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DISSERTATION

Bioactivity guided fractionation of

Betonica officinalis and *Glechoma hederacea*

focusing on anti-inflammatory activities

Verfasser

Paolo Picker

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“This is the true joy in life, the being used for a purpose recognized by yourself as a mighty one,
the being thoroughly worn out before you are thrown on the scrap heap,
the being a force of Nature instead of a feverish selfish little clod of ailments and grievances
complaining that the world will not devote itself to making you happy.”

George Bernard Shaw

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Contents

1 Introduction

1.1 Folk Medicine

Austrian traditional folk medicine represents a valuable source of information in the finding of new active principles. Medicinal plants are used since decades for the treatment of every form of illness, but in most cases their active constituents and the relative mode of action are still unknown. As nowadays about 60% of the new pharmaceutics derive from molecular structures of natural origin, natural products in general play a dominant role in the development of drugs for the treatment of human diseases (Newman and Cragg, 2007).

Folk medicine exists since ever, as humans always tried to find ways of lessening pain and to remedy any form of mental or physical problems. In 18th and 19th century medical services were too expensive or too far away for most people, that's why they usually relied on the knowledge of non-professional practitioners to treat their illnesses.

Since the starting point of this study was represented by the popular medicine, only traditionally used plants were selected on the basis of information that had been passed on from generation to generation in Austria. These type of data are collected in the "Volksmed-Database", which was created by means of interviews with 1857 persons from 1983 to 1995. They were questioned about their customs in the use of medicinal plants, in order to collect the current traditional knowledge.

The database includes exact botanical descriptions, information about the part of the plant used, indications, preparation and application method, as well as number of citations. Of about 100 plant species traditionally used in Austria against several diseases, where *Hypericum perforatum* L. resulted as the most often used, the therapeutic properties of the most part have still to be scientifically proven (Gerlach et al, 2006; Benedek, 2007).

An ointment from *Symphytum officinale* roots, for example, whose anti-inflammatory properties are described in the Austrian popular medicine, was found to be at least so active as a Diclofenac ointment in the treatment of acute unilateral ankle sprain (Predel et al, 2005).

The antibacterial activity of *Leontopodium alpinum*, which is traditionally used as a tea or cooked in milk to treat dysentery, was confirmed in vitro against various strains of *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas aeruginosa*,

Staphylococcus aureus, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Dobner et al, 2003).

1.2 Inflammation

Inflammation (from the Latin - *inflammare:* to set on fire) is a common denominator of a variety of diseases including arthritis, atherosclerosis, allergies and cancer. Therefore, natural compounds with anti-inflammatory properties represent an important group of therapeutics (Dvorak et al, 2006). Whereas inflammation pathways and relative inducers are mostly known, there is less information about the inhibitors of these processes. The main goal of this thesis was the identification of substances from Austrian medicinal plants, possibly with new anti-inflammatory mechanisms of action, which could potentially find therapeutic application.

The inflammatory response is characterized by coordinate activation of various signaling pathways, that regulate expression of both pro- and anti-inflammatory mediators in resident tissue cells and leukocytes recruited from the blood (Lawrence, 2009).

In general, inflammation represents a protective response of the organism against harmful physical, chemical or biological stimuli, aimed to the elimination of the initial cause of cellular and tissue damage. It consists of a sequence of events, which determine an intense vascular reaction characterized by the following five cardinal signs: *calor* (heat), *tumor* (edema), *rubor* (redness), *dolor* (pain) and *functio laesa* (loss of function). The first four signs were described for the first time over 2000 years ago by the Roman encyclopedist and healer Aulus Cornelius Celsus, while the sign *functio laesa* was added later by Galen (Sobolewski et al, 2010). Heat is intended as an increased tissue temperature, consequence of the vasodilation; edema is caused by migration of blood cells to the damaged tissue; redness is due to increased vascular activity in the involved area; pain is caused by stimulation of peripheral nociceptors by mediators such as bradykinin, while the loss of function is the possible consequence of the previous events.

A controlled inflammatory process is beneficial, as it provides protection and repair in case of several threats, but it can also become detrimental in case of dysregulation causing, for instance, septic shock. The elimination of the infectious agents, followed by a resolution and repair phase (mediated by tissue-resident and

recruited macrophages), represents the goal of the successful acute inflammatory response. Of relevant importance in the transition from inflammation to resolution is the switch from pro-inflammatory prostaglandins to anti-inflammatory lipoxins, which promote the recruitment of monocytes, responsible for dead cells removing and tissue remodelling, instead of neutrophils (Medzhitov, 2008).

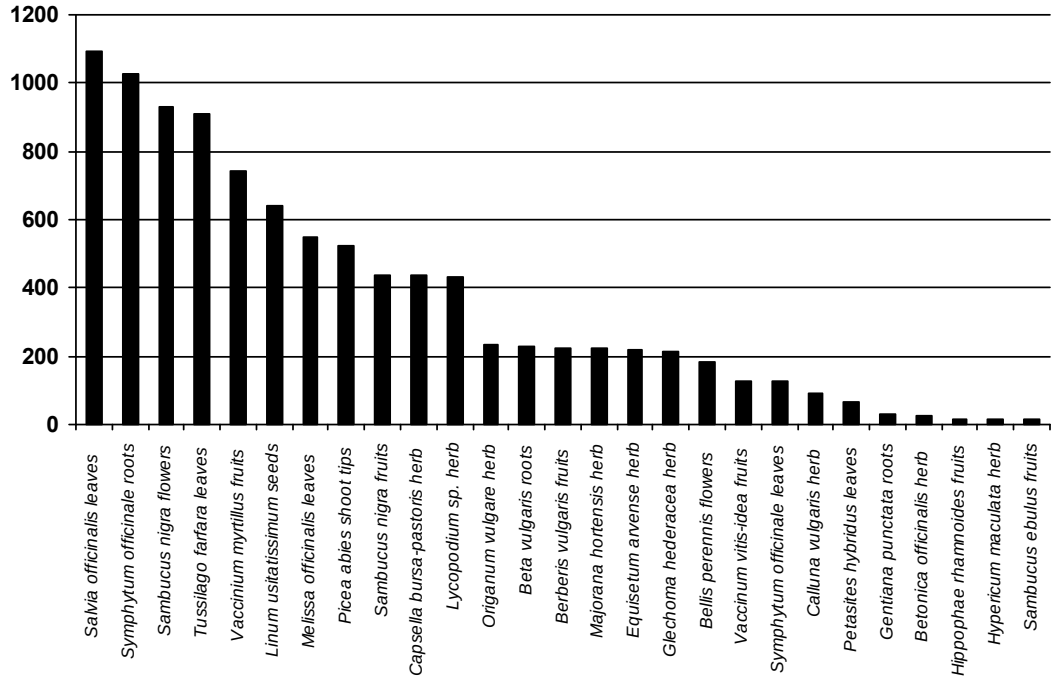
At a basic level, the process can be classified in acute and chronic. Acute inflammation is the fast response to injurious agents, which consists in the delivery of leukocytes and plasma proteins to the damaged site. The transmigration is consented by vasodilatation and consequent increased permeability, and promoted by cell adhesion molecules like E-selectin, P-selectin, ICAM (intracellular adhesion molecule) and VCAM (vascular cell adhesion molecule). Subsequently, the pathogens are engulfed and digested by macrophages (phagocytosis), which also stimulate lymphocytes to play their role. Chronic inflammation can be represented by persistent acute inflammation or a type of autoimmune reaction. In this case, permanent inflammation and tissue healing take place simultaneously. Examples of chronic inflammation are asthma, rheumatoid arthritis and multiple sclerosis.

In summary, inflammation is a complex phenomenon which involves several cell types in different pathways. On the one hand the acute inflammatory response represents a vital defense system of the organism, on the other hand chronic inflammation can lead to pathologies such as cancer, diabetes, Alzheimer's disease and neuropathic pain states (Sobolewski et al, 2010).

1.3 Selection of the Drugs

Since inflammation represents the fulcrum of this study, all indications that can be related to it were used as filter options in the Volksmed-Database. From the numerous hits (9190 citations) the most promising 226 drugs were chosen due to number of citations. After a literature research, 35 drugs of 31 plant species from 17 families were selected to be investigated, as they were not or poorly studied so far. As *Sambucus nigra* (flowers and fruits) resulted the most cited species, it has been considered interesting to also include the related taxon *Sambucus ebulus*, with the aim of getting a comprehensive picture of their relationship with each other. An overview of the plant species with more than 10 citations in the Volksmed-Database, intended as the number of mentions occurred in the interviews, is shown in Fig. 1.

Figure 1: Plant species with more than 10 citations in the Volksmed-Database



In a first step, polar and non polar extracts of all selected drugs were generated and pharmacological screened in vitro on different targets focusing on anti-inflammatory activities.

In a general ranking (see Table 6, pag. 30), the herbs of the Lamiaceae *Betonica officinalis* and *Glechoma hederacea* were determined as promising candidates and selected to be subjected to a bioactivity guided fractionation, in order to identify and pharmacologically evaluate their active constituents.

1.4 *Betonica officinalis* L.

Betonica officinalis L. (syn: *Stachys officinalis* L.), commonly known as Wood betony, is a perennial herb found growing wild in Europe, North Africa and western Asia (Fig. 2). The stems, slender and square, have a height from 15 to 40 cm, while the stalked basal leaves are oval. The dense and tubular reddish-purple magenta flowers have five lobes, from which the lower three ones are bent back. A persistent smooth five-toothed calyx contains the fruit, which consists of four small nutlets (Tobyn et al, 2010).

Wood betony was once the sovereign remedy for all maladies of the head (Grieve, 1971). The old italian proverb "sell your coat and buy betony" indicates the versatility of this plant as a remedy for several diseases. In Austrian folk medicine, the aerial parts of the plant are mainly prescribed as an aqueous infusion against inflammatory disorders of the upper respiratory system and as an ointment for the treatment of skin lesions (Gerlach et al, 2006).

Iridoid glycosides, flavonoids and phenylethanoid glycosides (Jeker et al, 1989; Kobzar and Nikonov, 1986; Miyase et al, 1996) were already identified in the aerial parts of the plant. They were also found to contain 0.5% of essential oil, with a mixture of isocaryophyllene and β-caryophyllene (22.9%) as its principal component (Chalchat et al, 2001).

Glycosides from *Betonica officinalis* were found to possess hypotensive activity (Zinchenko et al, 1962), while antioxidant activity was observed by total flavonoids contained in the leaves, more than in the roots (Hajdari et al, 2010). The plant was also found to possess strong antioxidant activity in phosphomolybdenum and lipid peroxidation assays (Matkowski and Piotrowska, 2006), as well as in DPPH and FRAP experiments (Hajdari et al, 2010).

Potent in vivo anti-inflammatory activity was determined in the related species *Stachys inflata* using carrageenan-induced paw edema and formalin tests (Maleki et al, 2001).

The aerial parts of the plant at flowering stage (5 kg) were field-collected in June 2008 in Neustift am Walde (Vienna, Austria), and dried at room temperature. Voucher specimens (Bet-hb-08_1) are deposited at the Department of Pharmacognosy, University of Vienna.

Figure 2: *Betonica officinalis* L.
(source: Botanical.com)

1.5 *Glechoma hederacea* L.

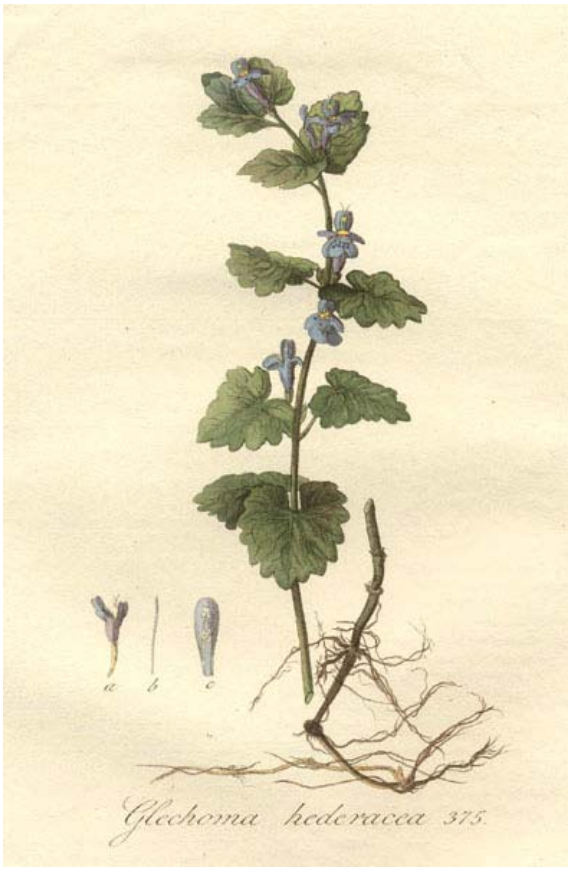
Commonly known as Ground ivy, *Glechoma hederacea* L. (Fig. 3) is a perennial hairy herb with unbranched square stems, which bear numerous, kidney-shaped dark green leaves, stalked and opposite to one another, and characterized by rounded indentations on the margins. Purplish blue flowers with small white spots are placed in the axils of the upper leaves (Grieve, 1971).

Common to Europe and the United States, the plant is found growing in shady places, waste grounds, dry ditches and on the sides of moist meadows (Felter and Lloyd, 2003). According to Green (1832), the Ground ivy expels the plants which grow near it, impoverishing pastures. In Austria, the aerial parts of the plant are traditionally used mainly in the form of tea, against cold and influenza, gastrointestinal disorders, respiratory and urinary tract inflammations. The most recurrent indication, however, is for the treatment of liver and gall bladder diseases (Gerlach et al, 2006).

Flavonoids, triterpenoids, sesquiterpenoids, alkaloids, glycosides and hydroxycinnamic acids have already been isolated from the aerial parts of the plant (Zieba, 1973; Milovanovic et al, 1995; Kumarasamy et al, 2003; Kikuchi et al, 2008; Yamauchi et al, 2007; Vavilova et al, 1988; Stahl et al, 1972). An aqueous extract was found to inhibit the production of nitric oxide in IFN-γ- and LPS-stimulated mouse peritoneal macrophages, through inhibition of iNOS expression (An et al, 2006), while anti-hypertensive activity, determined by higher sodium excretion rate, was observed in spontaneously hypertensive rats (Watanabe et al, 2007).

The dried aerial parts of the plant (2 kg) were obtained from the drug store Kottas Pharma GmbH (Vienna, Austria; Batch Nr.: KLA70586). A second sample of the plant, field-collected in Laab im Walde (Austria), was chromatographically and pharmacologically compared with the first one. Voucher specimens of the two plant samples (Gle-hb-08_1 and Gle-hb-08_2, respectively) are deposited at the Department of Pharmacognosy, University of Vienna.

Figure 3: *Glechoma hederacea* L.
(source: Flora batava by Jan Kops, Herman Christiaan, et al.)

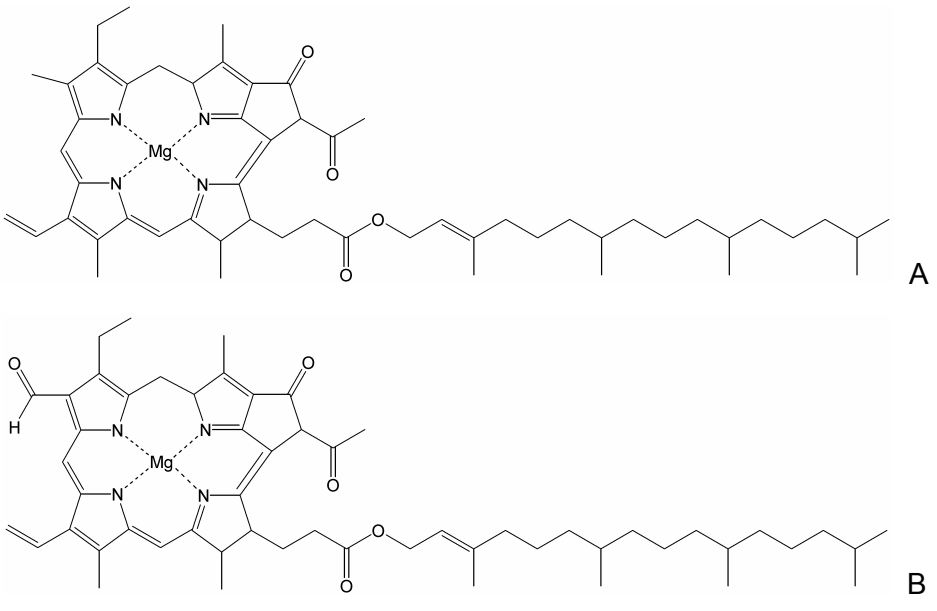


1.6 Chlorophyll and Polyphenols in Plant Extracts

Chlorophyll can represent the major part of nonpolar plant extracts. Besides the possible interference in the in vitro test systems, its removal results in a significantly increased relative concentration of the active compounds. Polyphenols, on the other hand, can form tight complexes with metal ions, proteins and polysaccharides (Potterat and Hamburger, 2006), leading to false positive or false negative results in cell-based assays. Their removal, furthermore, should result in the enrichment of the compounds of interest as well. Thus, nonpolar and polar extracts were purified from chlorophyll and polyphenols, respectively, with the removal techniques described in chapters 2.6 and 2.7.

A comparison between three different chlorophyll and polyphenols removal methods was also carried out, in order to find the most effective way of purification and to examine whether such ubiquitous plant constituents lead to any problems in the used cell-based anti-inflammatory tests. Different methodologies aimed to their removal were applied, and their selectivity for the target molecules was evaluated by chromatographic techniques. Extracts from the herbs of *Malva sp*. and *Glechoma hederacea* were used for this purpose, and the possible influence of the pure compounds chlorophyll A, chlorophyll B, tannic acid, epicatechin gallate, and rosmarinic acid (Fig. 4), which is known to be present in *Glechoma hederacea* (Okuda et al, 1986), was additionally evaluated in the same in vitro assays.

Figure 4: Chemical formulae of the tested ubiquitous plant components (A: chlorophyll A; B: chlorophyll B; C: tannic acid; D: epicatechin gallate; E: rosmarinic acid)



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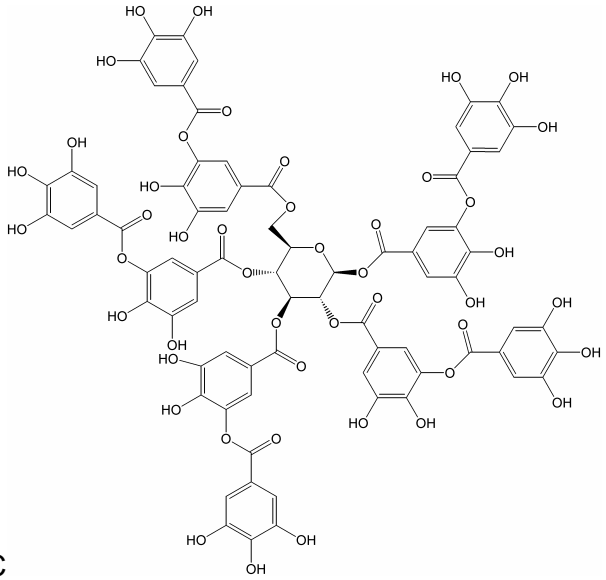

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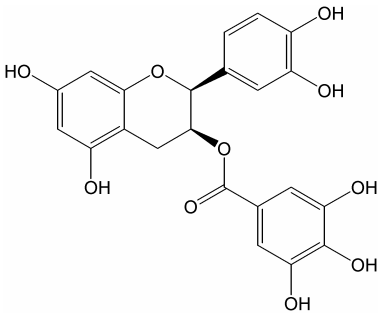

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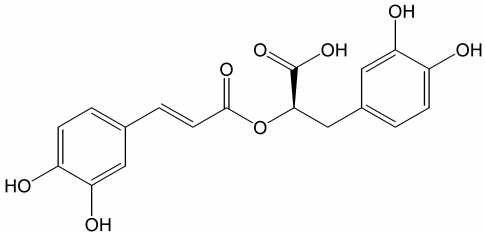


The phytochemical work of these specific investigations was performed together with ao. Univ.-Prof. Dr. Judith M. Rollinger, Institute of Pharmacy, University of Innsbruck; Univ.-Prof. Dr. Rudolf Bauer, Institute of Pharmaceutical Sciences, University of Graz and Mag. Sylvia Vogl, Department of Pharmacognosy, University of Vienna. The pharmacological tests were carried out in cooperation with Judit Mihaly-Bison and Priv.-Doz. Mag. Dr. Valery Bochkov, Department of Vascular Biology and Thrombosis Research, Medical University of Vienna and with Dr. Atanas G. Atanasov, Department of Pharmacognosy, University of Vienna.

1.7 Biological Targets

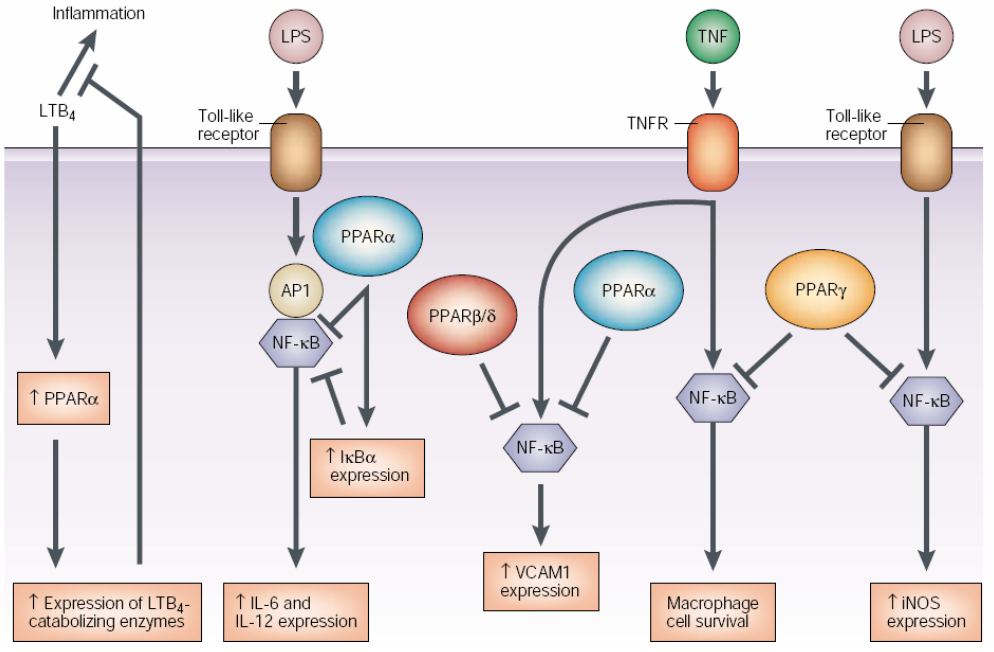
The cell-based evaluation of compounds focusing on anti-inflammatory activities can be carried out on several biological targets. Our cooperation with the Medical University of Vienna, as well as with the pharmacological group “Molecular Targets” of the Department of Pharmacognosy, University of Vienna, gave us the opportunity to perform the bioactivity guided fractionation using five different in vitro assays. The ability of the candidates to activate the peroxisome proliferator-activated receptors –α and –γ, to inhibit the TNF-α-induced NF-κB activation, as well as the TNF-α- and LPS-induced E-selectin and IL-8 expression should result in an overview of their potential as anti-inflammatory agents.

1.7.1 Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are members of the nuclear-hormone-receptor superfamily and are able to transduce a wide variety of signals, including inflammatory events, into a defined and ordered set of cellular responses at the level of gene transcription. So far, three PPAR isoforms – PPAR-α, PPAR-β/δ and PPAR-γ – have been identified and cloned (Daynes and Jones, 2002). The receptors PPAR-α and –γ, in particular, are also expressed in endothelial cells and vascular smooth muscle cells, where they are considered to play an important role in the regulation of inflammatory responses (Blaschke et al, 2006).

The ability of the PPARs to regulate inflammatory responses depends on their transactivation and transrepression capacities. Most of their anti-inflammatory properties arise through their ability to antagonize the nuclear factor κB (NF-κB) and AP1 signaling pathways (Fig. 5). In this way, the PPARs repress the expression of several genes that are involved in the inflammatory response, such as cytokines, cell-adhesion molecules and other pro-inflammatory signal mediators. PPAR-α has also been reported to control duration and magnitude of the inflammatory response, through the expression of genes encoding proteins that are involved in the catabolism of pro-inflammatory lipid mediators (Daynes and Jones, 2002). The aim was therefore to discover potent activators of PPAR-α and -γ by utilizing HEK293 cell-based luciferase reporter gene assays.

Figure 5: Role of PPARs in regulation of inflammatory responses
(source: Daynes and Jones, 2002)



1.7.2 Nuclear Factor κB (NF-κB)

The one of the nuclear factor κB has long been considered a prototypical proinflammatory signaling pathway, as it results in the expression of proinflammatory genes including cytokines, chemokines, and adhesion molecules (Lawrence, 2009). Therefore, much attention has focused on the development of anti-inflammatory drugs targeting on it (Karin et al, 2004).

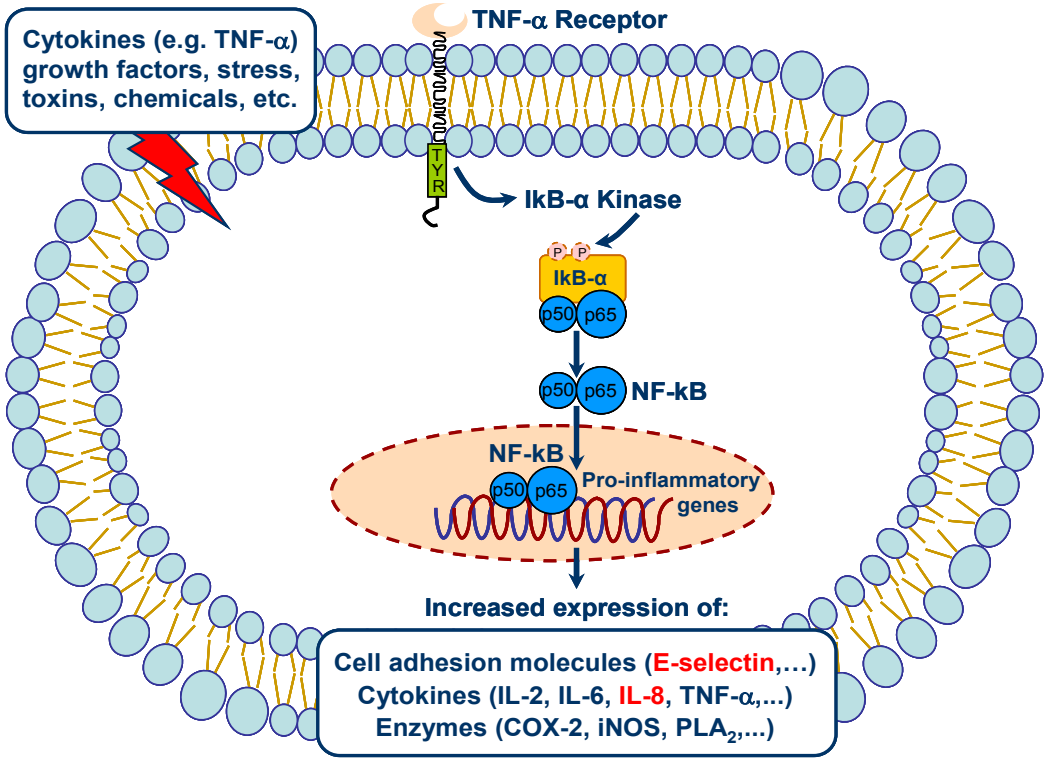
In physiological conditions, the heterodimer NF-κB (p50/p65) is found in the cytoplasm of endothelial cells, inactivated by the protein IκB-α. The local release of cytokines like TNF-α by damaged cells activates the IκB-α kinase, which phosphorylates IκB-α. This results in the release of NF-κB, which translocates to the nucleus, where the subunit p65 activates the transcription of pro-inflammatory genes. Among others, the transcription of the cell adhesion molecule E-selectin and the chemokine IL-8 is activated (Fig. 6).

Pharmaceuticals such as aspirin or sodium salicylate are able to inhibit the NF-κB activation blocking IκB-α phosphorylation and degradation (Pierce et al, 1996).

Lawrence et al. (2001) demonstrated the involvement of NF-κB also in the resolution of acute inflammation using pharmacological inhibitors. They confirmed the expected role of NF-κB in pro-inflammatory gene induction, but they also showed its role in the expression of anti-inflammatory genes and induction of leukocyte apoptosis during the resolution of inflammation. Inhibition of NF-κB during the resolution phase prolonged inflammatory response and inhibited apoptosis, in conflict with the generally accepted view that NF-κB was anti-apoptotic in inflammatory cells.

As the NF-κB pathway represents a valuable target for testing anti-inflammatory candidates, a HEK293 cell-based luciferase reporter gene assay (specific for NF-κB) was also selected for the investigations of this thesis.

Figure 6: Role of NF-kB in the inflammation process
(source: Mutschler et al, *Arzneimittelwirkungen*)



1.7.3 E-selectin and Interleukin-8 (IL-8)

The downregulation of stimulated E-selectin and IL-8 in endothelial cells represents also an important tool in the field of inflammation, particularly as their expression can be correlated with the NF-κB activation. Moreover, the possibility to stimulate the endothelium with two inflammatory stimuli (TNF-α and LPS), having different chemical nature and interacting with different receptors, enhances the reliance of the test system. These compounds are known to play a role in distinct but partially overlapping signaling pathways in the inflammation process.

In human umbilical vein endothelial cells (HUVEC), which were selected for our investigations, the expression of E-selectin can be induced, for instance, by lipopolysaccharide (LPS) on the transcriptional level and its maximal levels are expressed within 4 hours after stimulation at the cell surface (Kosonen et al, 2000; Bevilacqua et al, 1987; Vestweber and Blanks, 1999).

Previously known as endothelial leukocyte adhesion molecule-1 (ELAM-1), E-selectin is expressed exclusively in endothelial cells as consequence of IL-1, TNF-α, or LPS stimulation (De Rose et al, 1998; Bevilacqua et al, 1989). Precisely, it promotes the “rolling step”, namely the reversible adhesion of leukocytes to the endothelium, which then fix themselves to the surface of the vessel in the “sticking step”. Subsequently, the leukocytes migrate through the vessel to the site of injury or infection (diapedesis) via a gradient of chemotactic factors (Frenette and Wagner, 1997; Verbeuren et al, 2009).

Interleukin-8 is a potent chemotactic factor with a key role in host defense mechanisms, which release is consequence of inflammatory signals from a variety of cells such as neutrophils, smooth muscle cells and endothelial cells (Yuan et al, 2009; Mukaida et al, 1998). In the inflammation process, this chemokine is responsible for the leukocyte recruitment to the endothelium. Secreted as consequence of monocytes and macrophages activation, IL-8 determines the directional migration of neutrophils, basophils and T lymphocytes (Brat et al, 2005; Baggiolini et al, 1989; Rossi and Zlotnik, 2000).

1.8 Objective

As the pharmacologically active principles of most medicinal plants are still unknown, the aim of this thesis was to find new anti-inflammatory modes of action and / or new active natural compounds in Austrian traditionally used plant species. Considering that medicinal plants are often prescribed in the form of tea, their active principles represent only a minor part of an aqueous extract. Therefore, it would be of great interest to isolate and identify these constituents, in order to evaluate their biological properties in the form of pure compounds.

The possible influence of ubiquitous plant constituents such as chlorophyll and phenolic compounds in the employed in vitro assays, as well as the choice of adequate techniques to remove them, represented also an important point of this thesis.

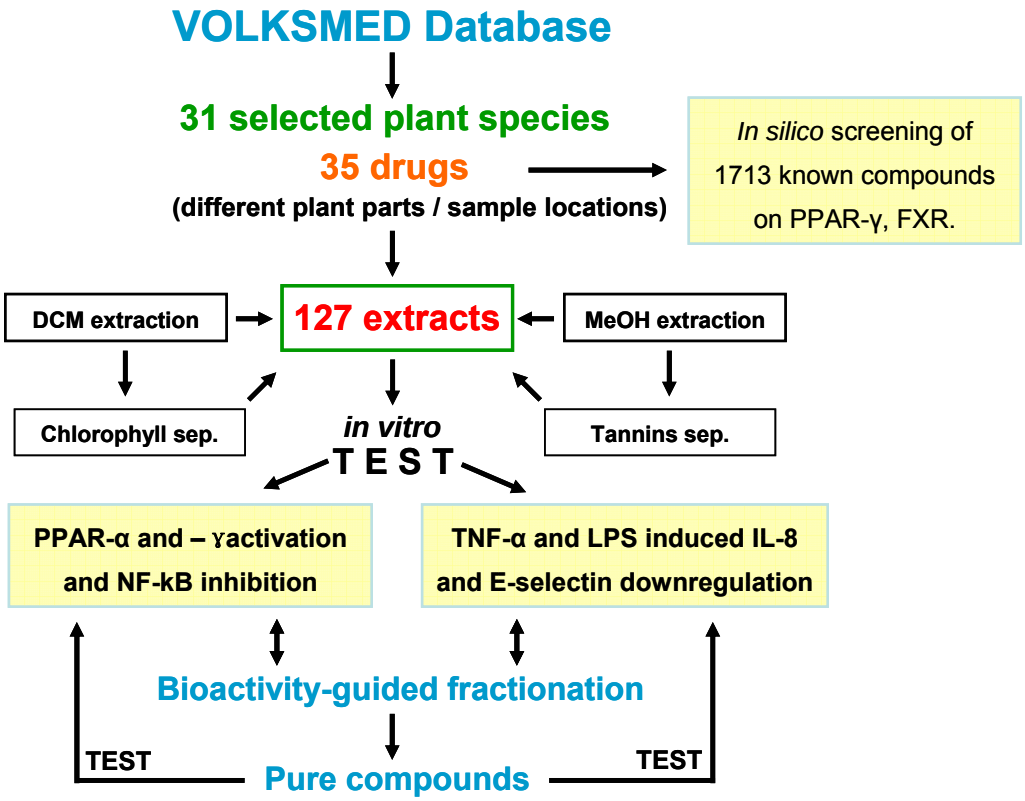
The bio-evaluation of plant materials used in traditional medicine is widely attempted. Furthermore, most studies focus solely on the identification of constituents or assessment of single biologic activities of the identified compounds,

eventually overlooking multifaceted regulatory mechanisms reflecting the complexity of their composition.

Of the several possible in vitro anti-inflammatory targets, the peroxisome proliferator-activated receptors α and γ, the nuclear factor κB, the cell adhesion molecule E-selectin and the cytokine interleukin-8 were chosen in this study for the investigation of the selected plants.

Leitmotif of the thesis was definitely a bioactivity guided fractionation, which led from the selected plant materials to the isolation and testing of promising anti-inflammatory substances (Fig. 7).

Figure 7: Overview of the investigation process from the selection of the plant species to the testing of pure compounds isolated thereof



2 Material and Methods

2.1 Plant Material

15 plant samples were field-collected from 2008 to 2009 together with Mag. Sylvia Vogl in different locations in Austria, while further 20 drugs were obtained from drug stores. The plant material was authenticated by Prof. Johannes Saukel and dried at room temperature. Voucher specimens thereof are deposited at the Department of Pharmacognosy, University of Vienna.

As the extractions should be followed by several purification and fractionation steps, it is of relevant importance to dispose of large amounts of homogenous plant material. Therefore, the finding of adequate quantities of wild plant samples and their identification represented a crucial initial phase of this study. At least 2 kg of each plant species were collected from the wild or purchased from the companies Alfred Richter GmbH, Kottas Pharma GmbH and Alfred Galke GmbH.

After the authentication, the plant material was dried at room temperature and finely grinded (particle size: 0.75 mm) before the extraction. The investigated plant species ordered by family are listed in Table 1.

Table 1: Investigated drugs and their sources

Family	Species	Plant Part	Plant Source
Adoxaceae	*Sambucus ebulus*	Fruits	Laab im Walde / Wolfsgraben
Adoxaceae	*Sambucus nigra*	Flowers	Alfred Richter GmbH
Adoxaceae	*Sambucus nigra*	Fruits	Kottas Pharma GmbH
Asteraceae	*Bellis perennis*	Flowers	Alfred Galke GmbH
Asteraceae	*Petasites hybridus*	Leaves	Hoher Student
Asteraceae	*Tussilago farfara*	Leaves	Alfred Richter GmbH
Berberidaceae	*Berberis vulgaris*	Fruits	Kottas Pharma GmbH
Betulaceae	*Alnus viridis*	Leaves	Lungau
Boraginaceae	*Symphytum officinale*	Stem	Neusiedlersee region
Boraginaceae	*Symphytum officinale*	Leaves	Neusiedlersee region
Boraginaceae	*Symphytum officinale*	Roots	Kottas Pharma GmbH
Brassicaceae	*Capsella bursa-pastoris*	Herb	Kottas Pharma GmbH
Chenopodiaceae	*Beta vulgaris*	Roots	Alfred Galke GmbH

Elaeagnaceae	*Hippophae rhamnoides*	Fruits	Alfred Galke GmbH
Equisetaceae	*Equisetum arvense*	Herb	Alfred Richter GmbH
Equisetaceae	*Equisetum palustre*	Herb	Neusiedlersee region
Ericaceae	*Calluna vulgaris*	Herb	Kottas Pharma GmbH
Ericaceae	*Vaccinium myrtillus*	Fruits	Alfred Richter GmbH
Ericaceae	*Vaccinum vitis-idea*	Fruits	Hochwechsel
Gentianaceae	*Gentiana punctata*	Leaves	Pollertal / Kärnten
Gentianaceae	*Gentiana punctata*	Roots	Pollertal / Kärnten
Hypericaceae	*Hypericum maculata*	Herb	Katschberg
Lamiaceae	*Ajuga genevensis*	Herb	Weinviertel / Lahner
Lamiaceae	*Ajuga reptans*	Herb	Laab im Walde / Wolfsgraben
Lamiaceae	*Betonica officinalis*	Herb	Neustift am Walde
Lamiaceae	*Glechoma hederacea*	Herb	Kottas Pharma GmbH
Lamiaceae	*Majorana hortensis*	Herb	Alfred Richter GmbH
Lamiaceae	*Melissa officinalis*	Leaves	Alfred Galke GmbH
Lamiaceae	*Origanum vulgare*	Herb	Alfred Richter GmbH
Lamiaceae	*Prunella vulgaris*	Herb	Neustift am Walde
Lamiaceae	*Salvia officinalis*	Leaves	Alfred Richter GmbH
Linum	*Linum usitatissimum*	Seeds	Alfred Richter GmbH
Lycopodiaceae	*Lycopodium sp.*	Herb	Kottas Pharma GmbH
Poaceae	*Agropyron repens*	Rhizomes	Kottas Pharma GmbH
Piceaceae	*Picea abies*	Shoot tips	Wechsel / Mariensee

2.2 Reference Compounds

The identification of the isolated substances was performed also by comparison with reference pure compounds, which sources and purity factors are listed in Table 2.

Table 2: Purity factors and origin of the reference compounds

Reference compound	Purity	Company
Acacetin	98%	Phytolab, Hamburg, Germany
Apigenin	98%	Phytolab, Hamburg, Germany
2-benzoxazolinone	98%	Sigma Aldrich, Steinheim, Germany
Chlorophyll A	95%	Fluka Chemical Corp., Ronkonkoma, NY, USA
Chlorophyll B	95%	Fluka Chemical Corp., Ronkonkoma, NY, USA

Epicatechin gallate	98%	Sigma Aldrich, Steinheim, Germany
Eupatorin	97%	ABCR, Karlsruhe, Germany
Harpagide	95%	Phytolab, Hamburg, Germany
Harpagoside	95%	Phytolab, Hamburg, Germany
Rosmarinic acid	98%	Extrasynthese, Genay, France
Tannic acid	-	Fluka Chemical Corp., Ronkonkoma, NY, USA

2.3 In Silico Screening

Virtual screening is a well-established tool for predicting biological activities of small organic molecules and selecting promising compounds for biological testing (Reddy et al, 2007; Rester, 2008; Kirchmair et al, 2008; Schneider, 2010; Markt et al, 2011). An extensive literature survey resulted in a list of compounds, known to be present in the selected plant species, which were converted into mol-files for further processing in the in silico screening on two different molecular targets. In order to predict their biological effects, the huge number of 1713 structures was virtual screened for their potential to activate the PPAR-γ as well as the Farnesoid X receptor (FXR), whose signaling mechanisms are known to be involved in anti-inflammatory responses. FXR is also a nuclear receptor, particularly expressed in the liver, which plays a role in inflammation as a negative modulator in the NF-κB pathway. Its activation, followed by the translocation into the nucleus, was found to inhibit the expression of inflammatory mediators in response to the hepatic NF-κB activation in vitro (Wang et al, 2008).

Compounds which were identified in the selected plants *Betonica officinalis* and *Glechoma hederacea* (see chapter 3.2.2) were additionally tested in a second time for their ability to bind the enzymes 5-lipoxygenase (5-LOX) and IkappaB kinase-2 (IKK-2), which are responsible for the synthesis of proinflammatory leukotrienes and for the NF-κB activation, respectively.

All molecules were submitted to conformational model generation using DiscoveryStudio 2.5. A maximum of 250 conformers with an energy maximum of 20 kcal above the minimum was calculated in “fast” mode. The parallel profiling was performed using the “rigid fitting” option.

These experiments were carried out in cooperation with Dr. Daniela Schuster, Institute of Pharmacy, Computer-Aided Molecular Design Group, University of Innsbruck.

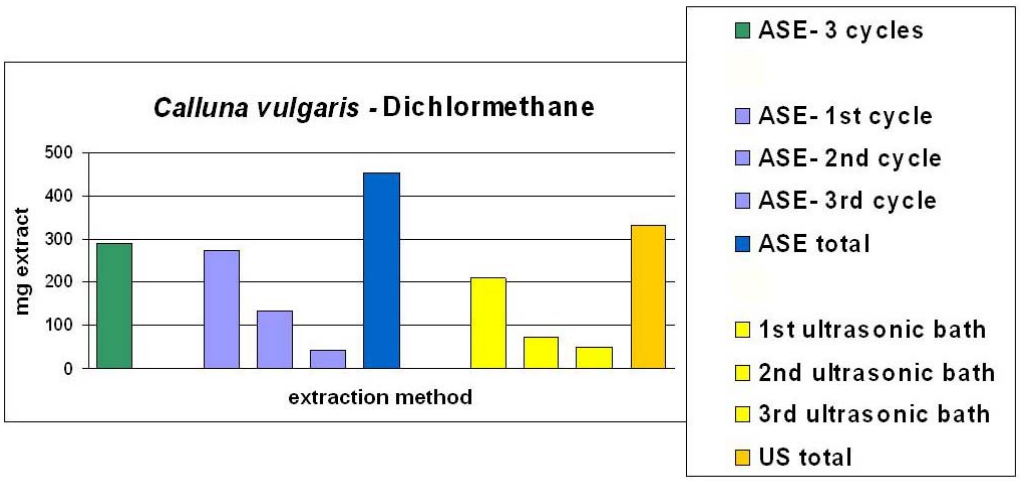
2.4 Extraction Techniques Comparison

Before starting the experimental work, some preliminary tests were performed, in order to compare the efficiency of different extraction methods, using the herb of *Calluna vulgaris* as model drug. The plant material (5.0 g) was first extracted three times with dichloromethane as solvent in an ultrasonic bath, for 15 minutes at room temperature. The yield was then compared with two extraction methods using an Accelerated Solvent Extractor ASE200 (Dionex Corp., Sunnyvale, CA, USA). Compared with other extraction techniques, the ASE generates results in a fraction of the time. Increased temperature accelerates the extraction kinetics, while elevated pressure by means of nitrogen keeps the solvent below its boiling point. In addition to speed, ASE offers a lower cost per sample by reducing solvent consumption up to 90%.

This automatized extractor allows basically to perform extractions in one or more cycles, intended as the number of times to perform the static heating and flushing steps. When more than one cycle is programmed, the flush volume is divided among the cycles.

The first method with the ASE consisted in an extraction with 3 cycles, while in the second one the plant material was extracted three times using one cycle. The results showed that the method where the plant material was extracted three times with one cycle was the most efficient (Fig. 8). Therefore, this method was applied for the extraction of all other drugs.

Figure 8: Extraction techniques comparison



2.5 Extraction

In a first time, amounts between 4 and 8 g of each drug were extracted, depending on the bulkiness of the plant material and on the necessity to mix it with diatomaceous earth, which is essential in the case of the fruits, acting as a dispersant and drying agent. As the used 33 ml extraction cells did not allow the processing of larger drug amounts, a second extraction was necessary in some cases.

In order to cover a wide range of polarity, the same plant material was extracted first with dichloromethane (DCM) and, after drying via nitrogen, with methanol. Both solvents were of analytical grade and purchased from Merck (Darmstadt, Germany).

The employed Dionex ASE200, which was equipped with 33 ml stainless steel extraction cells and 60 ml glass collection bottles, was programmed with the following conditions: 3 extraction cycles (3 times 1 cycle), 5 min heat-up time, 2 min static time, 10% flush volume, 60 sec nitrogen purge, 40 °C oven temperature and 150 bar pressure. The extracts were taken to dryness under reduced pressure, weighed and prepared for further processing.

The extraction yields (w/w) of the selected 35 drugs are listed in Table 3.

Table 3: Extraction yields (w/w) of the 35 investigated drugs

Plant species	Plant part	DCM extract (Yield %)	MeOH extract (Yield %)
Agropyron repens	Rhizomes	1.3	27.0
Ajuga genevensis	Herb	3.3	24.8
Ajuga reptans	Herb	2.7	22.9
Alnus viridis	Leaves	9.4	28.0
Bellis perennis	Flowers	4.1	15.8
Berberis vulgaris	Fruits	0.2	44.8
Beta vulgaris	Roots	0.5	14.6
Betonica officinalis	Herb	1.6	15.4
Calluna vulgaris	Herb	8.7	27.4
Capsella bursa-pastoris	Herb	1.8	17.8
Equisetum arvense	Herb	6.5	14.6
Equisetum palustre	Herb	2.6	6.8
Gentiana punctata	Leaves	5.7	46.5
Gentiana punctata	Roots	4.2	44.2

Glechoma hederacea	Herb	2.5	7.7
Hippophae rhamnoides	Fruits	18.3	23.4
Hypericum maculata	Herb	27.0	34.6
Linum usitatissimum	Seeds	75.7	11.2
Lycopodium sp.	Herb	1.7	6.6
Majorana hortensis	Herb	11.6	30.5
Melissa officinalis	Leaves	4.6	6.6
Origanum vulgare	Herb	10.1	27.9
Petasites hybridus	Leaves	4.0	17.6
Picea abies	Shoot tips	4.0	37.6
Prunella vulgaris	Herb	2.5	14.3
Salvia officinalis	Leaves	24.3	9.2
Sambucus ebulus	Fruits	1.3	84.8
Sambucus nigra	Flowers	5.9	18.9
Sambucus nigra	Fruits	0.7	16.3
Symphytum officinale	Leaves	3.2	10.8
Symphytum officinale	Roots	0.7	5.9
Symphytum officinale	Stems	4.2	14.4
Tussilago farfara	Leaves	9.1	18.6
Vaccinium myrtillus	Fruits	10.7	84.3
Vaccinum vitis-idea	Fruits	8.4	57.9

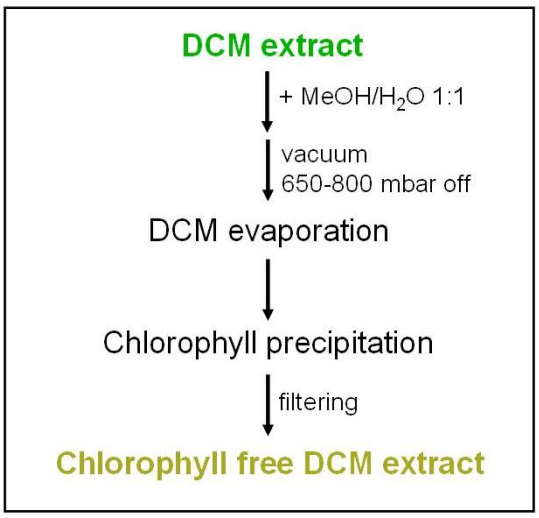
2.6 Chlorophyll Removal

All nonpolar extracts with a presumable significant content of chlorophyll (herbs and leaves) were subjected to a purification process, which was based on a liquid-liquid partition between DCM and a mixture of MeOH/H_2O (Fig. 9).

Dry extracts were redissolved in a defined volume of DCM (6.67 mg/ml), the same amount of MeOH/H_2O 1:1 was added and the two obtained phases were shortly shaked. As the DCM phase was completely removed under reduced pressure, the most nonpolar constituents, mainly chlorophyll, precipitated in the MeOH/H_2O phase and could be filtered off.

The aqueous phase was finally taken to dryness under reduced pressure, yielding the chlorophyll free DCM extract (wCh).

Figure 9: Scheme of the chlorophyll removal process

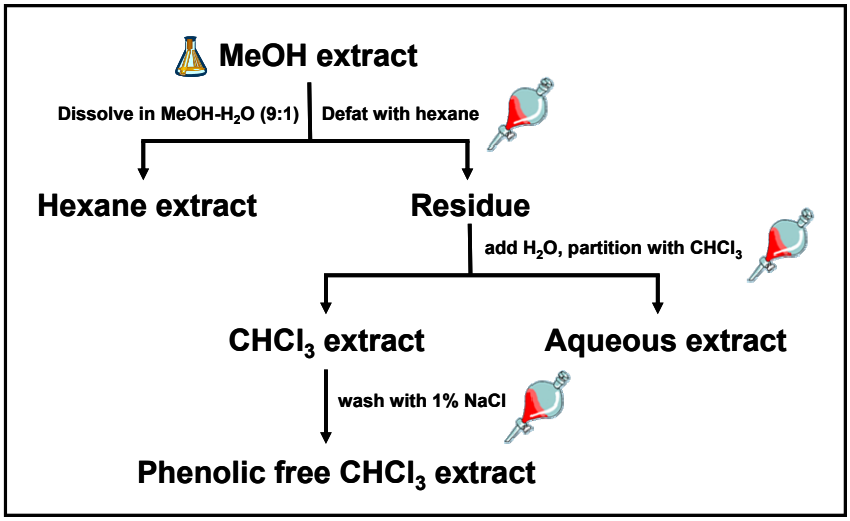


2.7 Polyphenols Removal

Polyphenols were removed from the polar extracts, according to Wall et al. (1996). The purification process is based on liquid-liquid partitions between chloroform and mixtures of MeOH/H_2O (Fig. 10).

Dry MeOH extracts were first redissolved in a mixture of MeOH/H_2O (9:1) and defatted by partition with hexane. Subsequently, the aqueous phase whose polarity was increased by addition of water (MeOH/H_2O 3:1), was partitioned with chloroform and the consequent chloroform extract was further washed with a 1% NaCl solution, generating the phenolic free $CHCl_3$ extract (wP).

Figure 10: Scheme of the polyphenols removal process



2.8 Solid Phase Extraction (SPE)

Despite the extraction with two different solvents and the described purification processes, crude extracts might contain a large number of constituents. Therefore, a solid phase extraction represented the first fractionation step of the selected drugs *Betonica officinalis* and *Glechoma hederacea*, with the aim of separating constituents of different polarities, simplifying moreover their subsequent isolation. On the basis of their positive results in the pharmacological screening, the chlorophyll free dichloromethane extract from *Betonica officinalis* and the phenol-free methanol extracts from both plants were subjected to this type of fractionation.

Bond Elut C18 (10 g) cartridges (Varian, Harbor City, CA, USA) with a reservoir volume (RV) of 60 ml were applied on a vacuum box, whose pressure was set at 5.0 mmHg off. The stationary phase was washed with 5 RV of distilled water and methanol, and conditioned with 2 RV of the initial elution concentration of 30% MeOH prior to the fractionation.

Extract amounts between 200 and 350 mg could be applied on the cartridges, depending on their solubility in the smallest possible volume of DCM or MeOH (approx. 2.0 ml), which has to be evaporated by the vacuum once the extract is adsorbed on the stationary phase. After the application of the extracts, the cartridges were eluted with a flow rate of about 2 drops per second with aqueous solutions of 30%, 70% and 100% MeOH (each 5 RV) in succession, obtaining three fractions (A, B and C, respectively) of decreasing polarity. These were taken to dryness under reduced pressure and prepared to be subjected to the successive chromatografic analyses.

2.9 Chromatographic Methods

2.9.1 Gas Chromatography – Mass Spectrometry (GC-MS)

In order to identify the volatile constituents of the mentioned SPE-fractions, GC-MS analyses were carried out using a Shimadzu (Kyoto, Japan) GC-2010 gas chromatograph equipped with a Phenomenex Zebron ZB-5 capillary column (thickness 0.25 µm, length 60 m, diameter 0.25 mm) and coupled to a quadrupole mass selective detector Shimadzu GCMS-QP2010. Data were acquired using a Shimadzu GCMSsolution software ver.2.50.

Samples were dissolved in methanol or dichloromethane at the concentration of 10 mg/ml. Injection volume was one microliter, injector temperature was 270 °C and detector temperature was 250 °C. Oven temperature program consisted of an initial temperature of 50 °C, increased to 270 °C at 3 °C/min and maintained at this level for 15 minutes. Carrier gas (Helium 5.0) was used at constant flow mode at 1.9 ml/min. Electron ionization mass spectra were recorded in the range 40-700 m/z.

2.9.2 High Pressure Liquid Chromatography (HPLC)

A Shimadzu (Kyoto, Japan) HPLC system consisting of a system controller (CBM-20A), a membrane degasser (DGU-20A5), a solvent delivery unit (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC), a photodiode array detector (SPD-M20A) and a low temperature light scattering detector (ELSD-LT, 40 °C) was used for all measurements. Data were acquired using a Shimadzu LCsolution software ver.1.25. Methanol and acetonitrile (chromatographic grade) were purchased from Merck (Darmstadt, Germany). Water was distilled by an IKA-Dest M3000 automatic water distillation apparatus (IKA, Staufen, Germany) and adjusted to pH 3.0 with concentrated formic acid (Carl Roth, Karlsruhe, Germany) in all measurements.

Phytochemical samples were dissolved in MeOH at the concentration of 5 mg/ml when analyzed with analytical columns, or at the concentration of 25 mg/ml if semipreparative columns were used.

In Table 4 are listed the fractions from *Betonica officinalis* and *Glechoma hederacea* selected to be fractionated by HPLC, as consequence of their activity in the pharmacological tests. The experimental parameters used for each fraction are listed in the following Table 5. The sample B2C (isolated peak from B2) was subjected to a further purification, as it resulted to be a mixture of two compounds. Each HPLC run was preceded by an equilibration time of 10 minutes with the initial mobile phase composition at the defined temperature, and followed by 10 minutes purge time with an elution of 100% MeOH or 95% MeCN.

Table 4: Samples fractionated by HPLC

Plant species	Sample	Preparation
Betonica officinalis	**B1**	DCM extract - 30% SPE fraction
	B2	DCM extract - 70% SPE fraction
	B2C	Fraction from B2
Glechoma hederacea	**G1**	MeOH extract - 30% SPE fraction
	G2	MeOH extract - 70% SPE fraction
	G3	MeOH extract - 100% SPE fraction
	G4	MeOH extract – polyphenols removed (Kottas Pharma)
	G5	MeOH extract - polyphenols removed (Laab im Walde)
	G2D	Fraction from G2
	G2E	Fraction from G2

Table 5: HPLC parameters

Sample	Stationary Phase	Mobile Phase (v/v)	Flow Rate (ml/min)	Injection Volume (µl)	Oven Temperature (°C)
B1	LiChrospher 100 RP-18, 250 x 4, 5 µm	Water (A) and Methanol (B), 5-100% of B in 60 min	1.0	10	25
B2	Aquasil C18 250 x 4.6, 5 µm	Water (A) and Acetonitrile (B), 40-48% of B in 25 min, 48-95% of B in 10 min	1.0	10	15
B2C	Luna C18 250 x 4, 5 µm	Water (A) and Acetonitrile (B), 44-46% of B in 15 min	1.0	10	16
G1	LiChrospher 100 RP-18, 250 x 4, 5 µm	Water (A) and Methanol (B), 5-100% of B in 60 min	1.0	10	25
G2	LiChroCART RP-18e 250 x 10, 5 µm	Water (A) and Methanol (B), 50-100% of B in 90 min	3.0	100	25
G3	LiChrospher RP-18 250 x 4, 5 µm	Water (A) and Methanol (B), 85-100% of B in 45 min	1.0	10	15
G4	Atlantis T3 150 x 3, 3 µm	Water (A) and Acetonitrile (B), 2-32% of B in 75 min	0.7	10	25
G5					
G2D	Atlantis T3 150 x 3, 3 µm	Water (A) and Acetonitrile (B), 40-55% of B in 45 min	0.5	10	25
G2E	Atlantis T3 150 x 3, 3 µm	Water (A) and Acetonitrile (B), 50-65% of B in 60 min	0.5	10	25

2.9.3 High Pressure Liquid Chromatography – Mass Spectrometry (HPLC-MS)

HPLC-MS measurements were carried out in cooperation with Dr. Martin Zehl, Department of Pharmacognosy, University of Vienna. The analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal electrospray ionization (ESI) source (HCT, Bruker Daltonics, Bremen, Germany). The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage: 4.0 or 3.7 kV, nebulizer 30 psi (N_2), dry gas flow 8 L/min (N_2) and dry temperature 340 °C or 350 °C. Positive and negative ion mode multistage mass spectra (at least MS^3) were obtained in automated data-dependent acquisition (DDA) mode. Helium was used as collision gas, the isolation window was 4 Th, and the fragmentation amplitude was set to 1.0 V. All measurements were performed with the conditions (mobile/stationary phase, gradient elution, flow rate, injection volume, oven temperature) already listed in Table 5.

2.10 High Resolution Mass Spectrometry (HRMS)

HRMS measurements were performed by Dr. Martin Zehl, in cooperation with Jürgen König and Martina Köberl, Department of Nutritional Sciences, University of Vienna. Spectra of isolated compounds were recorded on an ESI-Qq-TOF mass spectrometer (micrOTOF-Q II, Bruker Daltonics, Bremen, Germany) in negative ion mode. The sum formula was determined using the SmartFormula algorithm based on the mass accuracy and True Isotope Pattern analysis. Additional off-line negative ion mode ESI-MS^n spectra of these compounds were obtained on the HCT instrument using direct infusion.

2.11 NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer using $CDCl_3$ [$\delta(^1H)$ = 7.26 ppm and $\delta(^{13}C)$ = 77.00 ppm] or CD_3OD [$\delta(^1H)$ = 3.31 ppm and $\delta(^{13}C)$ = 49.00 ppm] as solvent. The experiments were performed in cooperation with ao. Univ.-Prof. Mag. Dr. Ernst Urban, Department of Medicinal Chemistry, University of Vienna.

2.12 Biological Testing

2.12.1 PPAR-α / -γ Activation and NF-κB Inhibition

PPARs and NF-κB assays were carried out in cooperation with Dr. Atanas G. Atanasov, Department of Pharmacognosy, University of Vienna. In both cases, phytochemical samples were tested in at least three independent experiments.

Human Embryonic Kidney, HEK293 cells were seeded in 10 cm dishes. After 24 hours, the cells were transiently transfected with the calcium phosphate precipitation method with 10 µg DNA including PPAR-α or -γ expression plasmids, PPAR luciferase reporter construct pPPRE-tk3x-Luc and green fluorescent protein plasmid. After 6 hours, the cells were transferred in 96 well plates and the medium was replaced with a DMEM supplemented with 5% charcoal stripped FBS. Cells were subsequently incubated for 18 hours with the indicated concentration of extracts / compounds. GW7647 at 50 nM and troglitazone at 5 µM were used as positive controls for PPAR-α and –γ, respectively.

In the NF-κB inhibition assay, the same type of cells, stably transfected with the pNFκB-luc (293/NFκB-luc cells, Panomics, RC0014) and seeded in 10 cm dishes, were transiently transfected with green fluorescent protein plasmid. Six hours later, cells were transferred in 96 well plates and incubated with serum-free DMEM. After 24 hours, cells were treated with the indicated concentration of extracts / compounds and stimulated with 2 ng/ml human recombinant TNF-α for 6 hours. The known NF-κB inhibitor parthenolide (PTL) was used as positive control at the concentration of 5 µM.

In both PPARs and NF-κB assays, cells were finally lysed and the luciferase activity was quantified on a GeniosPro plate reader (Tecan, Austria) and normalized with the green fluorescence level to account for differences in the cell number and / or transfection efficiency.

2.12.2 TNF-α / LPS-induced E-selectin and IL-8 Downregulation

The experiments on E-selectin and IL-8 downregulation were performed together with Judit Mihaly-Bison and Priv.-Doz. Mag. Dr. Valery Bochkov, Department of Vascular Biology and Thrombosis Research, Medical University of Vienna.

Plant components were tested in a characteristic inflammatory reaction, where the endothelium is activated by inflammatory cytokine (TNF-α) or bacterial product (LPS). In a first phase, the downregulation of E-selectin and IL-8 was evaluated at the mRNA level, through isolation of RNA from the treated cells and quantification of the genes of interest by normalization to a housekeeping gene β_2-microglobulin.

In a second time, ELISA experiments were additionally performed to assess the effect of the candidates at the protein level. This further step was carried out in order to confirm at the post-transcriptional level the activities observed through the RNA isolation. This confers more reliability to the study, as the expression of a certain protein is not always closely related to the relative mRNA transcript level.

2.12.2.1 E-selectin and IL-8 mRNA

For the first phase, TERT technology (hTERT) immortalized human vascular endothelial cells (HUVECtert) (Chang et al, 2005) were grown in M199 medium (Sigma-Aldrich, St. Luis, MO) containing 20% fetal bovine serum (Sigma, Taufkirchen, Germany), endothelial cell growth supplement (Technoclone, Austria) and antibiotics. Experiments were performed in triplicates using 12 well plates (NUNC, Roskilde, Denmark) in M199 medium supplemented with 3% fetal bovine serum and 1% bovine serum albumin (Applichem, Darmstadt, Germany).

Monolayers of subconfluent quiescent cells were treated for 10 minutes with the indicated concentration of extracts / compounds and stimulated with 100 ng/ml of TNF-α (PeproTech, Rocky Hill, NJ) or LPS (Sigma-Aldrich, St. Luis MO) for 30 minutes or 4 hours, respectively. RNA was extracted from the cells using QIAzol lysis reagent (Qiagen, Hilden, Germany) and 900 ng thereof were reverse transcribed with MuIV-RT using Oligo d(T) primers (Applied Biosystems, Carlsbad, CA). The relative expression of the genes of interest was determined by Q-PCR (Roche, Basel, Switzerland).

Primers were designed with a PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA) using the reference mRNA sequences of respective genes from the GeneBank (http://www.ncbi.nlm.nih.gov). For IL-8 primers 5'-ctcttggcagccttcctgatt-3' (forward) and 5'-tatgcactgacatctaagttctttagca-3' (reverse), for E-selectin 5'-ggtttggtgaggtctgctc-3' (forward) and 5'-tgatctgtcccggaactgc-3' (reverse) were used. Relative quantification of the investigated genes was performed by normalization to a housekeeping gene β_2-microglobulin using the

mathematical model by Pfaffl (Kadl et al, 2002) and presented as fold variation over the control.

Due to the high number of extracts, the first screening experiments were conducted on pools of 10 crude plant extracts at the concentration of 100 µg/ml (each extract 10 µg/ml, results not shown). Only extracts composing active pools were individually evaluated in a second screening phase, at the concentration of 10 µg/ml.

2.12.2.2 E-selectin and IL-8 ELISA

Immortalized human umbilical vein endothelial cells (HUVECtert) (Chang et al, 2005) were grown in M199 containing 20% FBS, 1% PSF (penicillin, streptomycin, fungicide), 1% glutamine and 0,4 % ECGS/H (Endothelial Cell Growth Supplement / Heparin; PromoCell, Cat. No. C-30140). Confluent HUVEC-Tert cells were seeded into 96 well plates and incubated overnight for attachment of cells (~ 16 hours).

Phytochemical samples were diluted to their appropriate end concentration in M199 containing 1% PSF and 2% FBS. TNF-α (PeproTech, Cat. No. 300-01A) and LPS (Sigma Aldrich, Cat. No. L2880-25mg) were directly diluted with the samples, to a concentration of 200 ng/ml, which is twice the desired end concentration of agonists. BAY 11-7082 was used as positive control at the concentration of 5 µM. Experiments were performed in six replicates, using 6 adjacent horizontal wells on the 96-well plate. The medium was removed from each replicate row and 50 µl of diluted samples were added in their appropriate end concentration.

Samples were incubated for 30 minutes, allowing them to take effect on their cellular targets. 50 µl of 2-fold concentrations of agonists (diluted in media containing a certain sample, as described) were added to the replicate rows containing the same sample in which also the agonist was diluted. Cells were then incubated for 6 hours.

Detection of secreted IL-8 directly from the medium was performed using the human CXCL8/IL-8 ELISA DuoSet ELISA Development kit (R&D Systems, Cat. No. DY208) and the TMB 2-Component Microwell Peroxidase Substrate Kit (VWR International, Cat. No. 50-76-00). ELISA was performed in 96 well NUNC plates for immune reactions (NUNC; F8 MAXISORP, Cat. No. 468667).

After treatment of the NUNC plates with coating antibody, the culture medium of the stimulated cells was applied on the NUNC ELISA plates in a manner that within one replicate, 50 µl of two wells were pooled to one well of the NUNC ELISA plate,

meaning that six identical treated biological replicates became three analytical replicates. The medium containing secreted IL-8 from stimulated cells was incubated overnight with the capturing antibody and then removed. Washing steps were carried out five times with 100 µl of PBST (0,05% Tween 20). Detection antibody and streptavidin (coupled to HRP) were subsequently applied.

Concerning the E-selectin experiments, the medium was thoroughly removed from the stimulated cells. The cells were fixed with freshly diluted 0.1% glutaraldehyde (Merck, New Jersey, US; # 1.04239.0250) for 15 minutes at 4 °C and then washed with 100 µl of PBST (0,05% Tween 20). Washing steps were carried out 2 times. In all steps liquids were removed using a vacuum pump with 8 tips. The dish was blocked by incubation with PBS containing 1% BSA (Albumin – Fraktion V; AppliChem, Darmstadt, D) for one hour at 37 °C. All subsequent dilutions were carried out in PBS containing 0.1% BSA (same supplier). Human E-selectin/CD62E MAb (Clone BBIG-E4), Mouse IgG1 (R&D Systems, Minneapolis, US; # BBA16) was diluted to a concentration of 0.3 µg/ml and used as primary antibody to detect membrane – bound E-selectin. The cells were incubated with primary antibody for one hour at 37 °C and washed twice. HRP-conjugated sheep anti-mouse IgG polyclonal antibody (CE-Healthcare, Little Chalfont Buckinghamshire, UK; # NA931V) was diluted 1:1500 and used as secondary antibody. Cells were incubated with secondary antibody at 37 °C for one hour and washed twice.

In both IL-8 and E-selectin experiments, turnover of substrate for HRP was performed according to the instructions manual of the TMB 2-Component Microwell Peroxidase Substrate Kit (VWR International). Quantification of IL-8 and E-selectin was carried out measuring the optical density (OD) using a SynergyHT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) at 450 nm, using 620 nm wavelenght as reference.

2.12.3 Statistical analysis

Statistical analysis of the data was performed with the Prism 4.03 software (GraphPad Software Inc., La Jolla, CA). The experimental data are presented as means ± standard error of the mean (SEM) from at least three independent experiments.

Statistical significance was determined by ANOVA using Bonferroni post hoc test. P values < 0.05 were considered significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

3 Results

3.1 Biological Screening

The results of the screening performed on the selected 35 drugs are listed in Table 6 (legend see Table 7). The phenol-free MeOH extract from *G. hederacea*, originally excluded from E-selectin / IL-8 testing as the crude extract composed an inactive pool, was added later due to the strong activity exhibited on PPARs and NF-κB. That extract was selected for further investigations together with the extracts from *Betonica officinalis*, which were able to strongly downregulate E-selectin and IL-8.

Table 6: Pharmacological screening of crude and purified extracts. All samples were tested at 10 µg/ml. Extracts with no E-selectin / IL-8 available results are intended as not active, as part of inactive pools.

Sample			Results						
Species	Plant Part	Extract	PPAR-α activation	PPAR-γ activation	NF-κB inhibition	TNF-α-induced		LPS-induced	
						E-selectin	IL-8	E-selectin	IL-8
Agropyron repens	Rhizomes	DCM	strong	strong	no	strong	moderate	moderate	strong
		MeOH	no	no	no	moderate	moderate	no	moderate
		wP	no	moderate	no	-	-	-	-
Ajuga genevensis	Herb	DCM	no	no	no	no	moderate	no	moderate
		wCh	no	no	no	-	-	-	-
		MeOH	no	no	no	strong	no	strong	no
		wP	no	no	no	-	-	-	-
Ajuga reptans	Herb	DCM	no	no	no	no	moderate	no	moderate
		wCh	no	no	no	-	-	-	-
		MeOH	no	no	no	strong	no	strong	no
		wP	no	no	no	-	-	-	-
Alnus viridis	Leaves	DCM	no	no	moderate	-	-	-	-
		wCh	no	no	strong	-	-	-	-
		MeOH	no	no	no	-	-	-	-
		wP	moderate	moderate	strong	-	-	-	-
Bellis perennis	Flowers	DCM	strong	moderate	moderate	-	-	-	-
		MeOH	no	no	no	-	-	-	-
		wP	moderate	moderate	no	-	-	-	-
Berberis vulgaris	Fruits	DCM	no	no	no	-	-	-	-
		MeOH	no	no	no	-	-	-	-
		wP	moderate	no	no	-	-	-	-
Beta vulgaris	Roots	DCM	no	no	no	-	-	-	-
		MeOH	no	no	no	-	-	-	-
		wP	no	no	no	-	-	-	-
Betonica officinalis	Herb	DCM	no	no	no	strong	strong	weak	strong
		wCh	no	no	no	strong	strong	strong	strong
		MeOH	no	no	no	moderate	strong	moderate	strong
		wP	no	no	no	strong	strong	weak	strong

Calluna vulgaris	Herb	DCM wCh MeOH wP	no no no no	no no no no	moderate moderate no no	- - - -	- - - -	- - - -	- - - -
Capsella bursa-pastoris	Herb	DCM wCh MeOH wP	strong strong no strong	no moderate no strong	strong no no strong	strong - moderate -	weak - moderate -	moderate - no -	strong - strong -
Equisetum arvense	Herb	DCM wCh MeOH wP	no moderate no no	no moderate no no	strong moderate no no	- - - -	- - - -	- - - -	- - - -
Equisetum palustre	Herb	DCM wCh MeOH wP	no no no no	no no no no	no moderate no no	no - strong -	no - no -	no - moderate -	moderate - no -
Gentiana punctata	Leaves	DCM wCh MeOH wP	no no no no	no no no no	no no no no	- - - -	- - - -	- - - -	- - - -
Gentiana punctata	Roots	DCM MeOH wP	no no moderate	no no no	no no no	- - -	- - -	- - -	- - -
Glechoma hederacea	Herb	DCM wCh MeOH wP	no no no strong	no moderate no strong	no moderate no strong	- - - strong	- - - strong	- - - no	- - - no
Hippophae rhamnoides	Fruits	DCM MeOH wP	no no no	no no no	no no no	- - -	- - -	- - -	- - -
Hypericum maculata	Herb	DCM wCh MeOH wP	no moderate no moderate	moderate no no no	moderate no no no	- - - -	- - - -	- - - -	- - - -
Linum usitatissimum	Seeds	DCM MeOH wP	no no no	no no no	no no no	- - -	- - -	- - -	- - -
Lycopodium sp.	Herb	DCM wCh MeOH wP	no no no moderate	no no no moderate	strong strong no no	- - - -	- - - -	- - - -	- - - -
Majorana hortensis	Herb	DCM wCh MeOH wP	no no no strong	no no no moderate	no strong no moderate	- - - -	- - - -	- - - -	- - - -
Melissa officinalis	Leaves	DCM wCh MeOH wP	no strong no no	no strong no no	moderate moderate no strong	- - - -	- - - -	- - - -	- - - -

Origanum vulgare	Herb	DCM wCh MeOH wP	no no no moderate	no no no no	moderate no no no	- - - -	- - - -	- - - -	- - - -
Petasites hybridus	Leaves	DCM wCh MeOH wP	no no no no	no no no no	no no no moderate	strong - no -	moderate - strong -	moderate - no -	moderate - moderate -
Picea abies	Shoot tips	DCM wCh MeOH wP	no no no no	no no no no	moderate no no no	no - weak -	no - no -	no - no -	no -- no -
Prunella vulgaris	Herb	DCM wCh MeOH wP	no moderate no no	no moderate no no	no strong no no	strong - strong -	no - no -	no - moderate -	no - no -
Salvia officinalis	Leaves	DCM wCh MeOH wP	no no no no	no no no no	strong strong no strong	- - - -	- - - -	- - - -	- - - -
Sambucus ebulus	Fruits	DCM MeOH wP	no no no	no no no	no no no	no strong -	weak strong -	moderate weak -	no moderate -
Sambucus nigra	Flowers	DCM MeOH wP	no no strong	no no moderate	no no no	- - -	- - -	- - -	- - -
Sambucus nigra	Fruits	DCM MeOH wP	no no strong	no no strong	no no strong	- - -	- - -	- - -	- - -
Symphytum officinale	Leaves	DCM wCh MeOH wP	no moderate no no	no no no no	no no no no	moderate - strong -	no - no -	strong - no -	strong - strong -
Symphytum officinale	Roots	DCM MeOH wP	moderate no no	moderate no no	no no no	- - -	- - -	- - -	- - -
Symphytum officinale	Stems	DCM wCh MeOH wP	no strong no strong	no moderate no moderate	no no no no	moderate - no -	no - no -	strong - no -	strong - no -
Tussilago farfara	Leaves	DCM wCh MeOH wP	moderate strong no moderate	no moderate no moderate	moderate moderate no no	- - - -	- - - -	- - - -	- - - -
Vaccinium myrtillus	Fruits	DCM MeOH wP	moderate no moderate	moderate no moderate	no no no	no strong -	no weak -	no strong -	moderate weak -
Vaccinum vitis-idea	Fruits	DCM MeOH wP	no no moderate	no no strong	no no moderate	no no -	no strong -	no moderate -	weak no -

Table 7: Results legend

Activity	PPAR-α and -γ	NF-κB	E-selectin / IL-8
strong	> 2 fold activation above the control	> 80% inhibition	> 75% inhibition
moderate	50-100% activation above the control	50-80% inhibition	50-75% inhibition
weak	-	-	25-50% inhibition
no	< 50% activation above the control	< 50% inhibition	< 25% inhibition

DCM	Crude dichloromethane extract
wCh	Dichloromethane extract without chlorophyll
MeOH	Crude methanol extract
wP	Methanol extract without polyphenols

3.2 Phytochemical Analyses

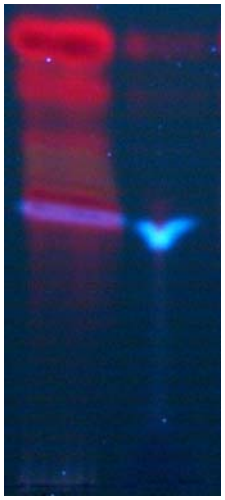
3.2.1 Purification and Solid Phase Extraction

Chlorophyll and polyphenols eliminations, performed respectively on crude DCM and MeOH extracts, resulted on average in low yields, as the removal of other constituents is also to be expected. On the other hand, considering only PPARs and NF-κB assays (where both crude and purified extract were screened, see Table 6), the activity of extracts without chlorophyll or polyphenols was higher in 57 cases, while only in 6 cases the purification processes resulted in a decreased biological activity.

The purification yields for the selected plants *Betonica officinalis* and *Glechoma hederacea*, relative to the crude extracts, are listed in Table 8.

Table 8: Chlorophyll and polyphenols removal yields (w/w)

Plant species	Plant part	After chlorophyll removal (yield %)	After polyphenols removal (yield %)
Betonica officinalis	Herb	15.9	3.5
Glechoma hederacea		12.5	8.1



The effectivity of the chlorophyll removal could be verified by thin layer chromatography, using Merck silica gel 60 F254 plates (8 cm development) as stationary phase and a mixture of toluol, ethylformiat and concentrated formic acid (5+4+1) as mobile phase. Detection was performed at 366 nm. The TLC chromatograms in Fig. 11 show the successful separation of the chlorophyll (red zone at front) from the DCM extract of *Folium Malvae.*

Figure 11: TLC chromatograms of *Malva* DCM extract before (Left) and after (Right) the chlorophyll removal

As purified extracts were more active than crude extracts, the successive solid phase extraction was performed with them. The results of this first fractionation step for the two selected plants are listed in Table 9 (wCh = without chlorophyll; wP = without polyphenols).

Table 9: Solid phase extraction yields (w/w)

Species	Extract	Fraction	Preparation	Yield %
Betonica officinalis	DCM wCh	**B1**	30% MeOH	47.8
		B2	70% MeOH	23.6
		B3	100% MeOH	17.1
	MeOH wP	**B4**	30% MeOH	12.4
		B5	70% MeOH	26.8
		B6	100% MeOH	51.9
Glechoma hederacea	MeOH wP	**G1**	30% MeOH	13.8
		G2	70% MeOH	21.1
		G3	100% MeOH	47.1

Once pharmacological evaluated (results in chapter 3.3), the above listed SPE fractions B1, B2, G1, G2 and G3 were selected to be further fractionated by HPLC, in order to identify and isolate their active constituents. Despite their activity, fractions B3, B5 and B6 were excluded from further investigations, as preliminary HPLC-MS analyses demonstrated their high relative content of DEHP (also found in G3, see pag. 42) or closely related structures.

3.2.2 Chromatographic Separation and Structure Elucidation

3.2.2.1 *Betonica officinalis*

The HPLC-ELSD chromatogram of the fraction **B1** (Fig. 12) showed the presence of a main component (B1A, Rt: 21.5), which was tentatively identified by LC-MS, yielding an $[M + Na]^+$ ion at *m/z* 429.1 in positive ion mode and an $[M - H]^-$ ion at *m/z* 405.1 in negative ion mode LC-MS (MW = 406).

The MS^n spectra indicated the presence of an acetyl- and a hexosyl-group, which pointed towards the known constituent 8-O-acetylharpagide (Kobzar, 1986; Jeker et al, 1989) (Fig. 13).

Its isolation by HPLC was carried out avoiding heat and acidified water, as these factors are known to cause iridoids degradation. Once isolated in sufficient amount (3.79 mg), the compound was subjected to 1D and 2D NMR analyses and its chemical structure could be confirmed.

Figure 12: HPLC-ELSD chromatogram of B1

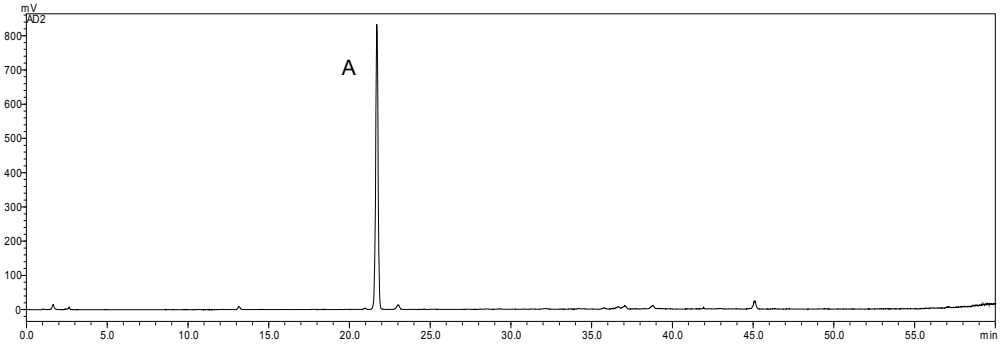
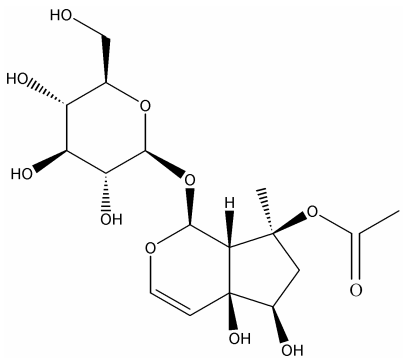
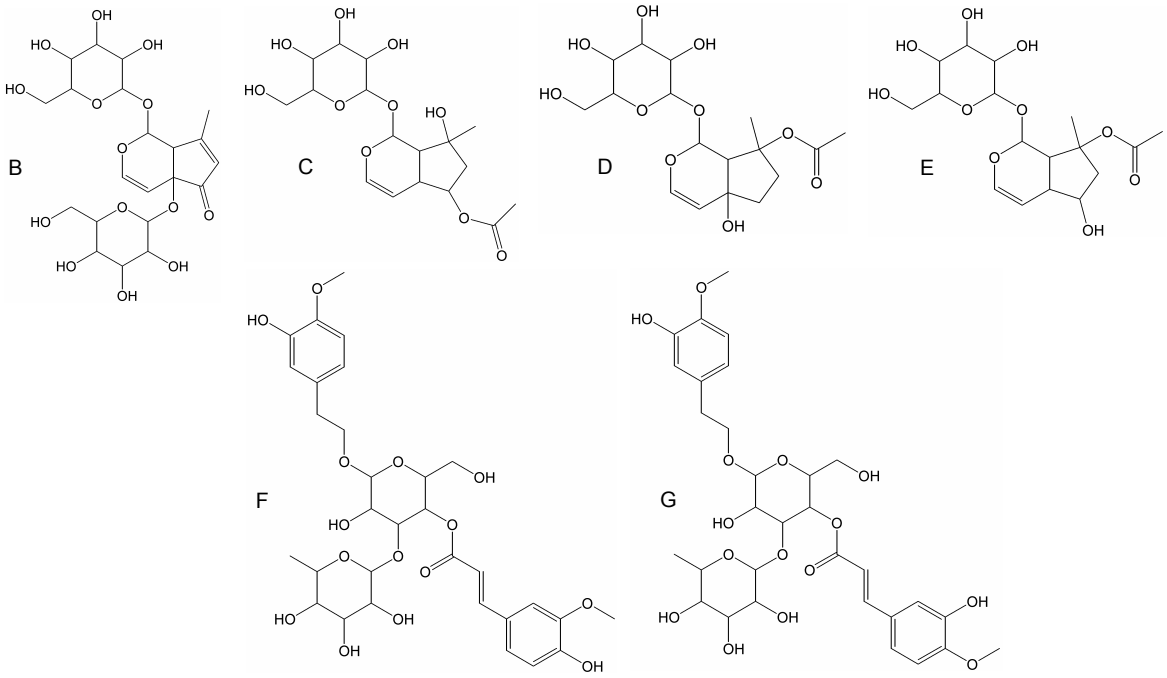


Figure 13: Chemical formula of 8-O-acetylharpagide (B1A)



Six other substances could also be detected by LC-MS as trace components of the fraction (Fig. 14), however they were not isolated and further processed due to their low relative amount. They were tentatively identified as the already known compounds allobetonicoside (B1B), 6-O-acetylmioporoside (B1C), reptoside (B1D), ajugoside (B1E), martynoside and one isomer of it (B1F and B1G).

Figure 14: Chemical formulae of the minor components identified in B1



The HPLC-ELSD fractionation of **B2** (Fig. 15) consented the isolation of four compounds (B2A, B2B, B2D, B2E) and one mixture of two substances (B2C). The further fractionation of B2C (Fig. 16) yielded the pure compound B2C1, which represented the major part of the mixture according to the ELSD detection. With regard to NMR structure elucidation and biological testing, amounts between 2.64 and 5.45 mg of the pure compounds were isolated by HPLC.

In the LC-MS analysis, compounds B2A and B2B were both detected as $[M - H]^-$ ions at *m/z* 347.1 and yielded nearly identical fragment ion spectra, dominated by consecutive neutral loss of two molecules of CO_2, which could, however, not be matched to known constituents of *Betonica officinalis*. HRMS of the isolated pure compounds showed the $[M - H]^-$ ions at *m/z* 347.1858 (B2A) and *m/z* 347.1844 (B2B), matching to a molecular formula of $C_{20}H_{28}O_5$ (calcd. *m/z* 347.1864 for $C_{20}H_{27}O_5^-$).

Compound B2C1 yielded an $[M + H]^+$ ion at *m/z* 345.1 in positive ion mode and an $[M - H]^-$ ion at *m/z* 343.1 in negative ion mode LC-MS. The on-line UV spectrum, with λ_{max} at 274 nm and 342 nm, and the fragmentation of the $[M - H]^-$ ion, which showed consecutive neutral loss of three methyl radicals ($CH_3^{\bullet}$), indicated that B2C1 is a trimethoxy-dihydroxyflavone. Compound B2C1 was finally identified as eupatorin, a known constituent of *Stachys swainsonii* (Skaltsa et al, 2007), by comparison of UV spectra (Fig. 17) and HPLC retention times with a commercial reference compound.

HRMS of the isolated pure compound B2D showed the $[M - H]^-$ ion at *m/z* 361.2029, matching to a molecular formula of $C_{21}H_{30}O_5$ (calcd. *m/z* 361.2020 for $C_{21}H_{29}O_5^-$). Fragmentation in MS^2 yielded, among others, the neutral loss of a methyl radical ($CH_3^{\bullet}$). Further fragmentation of the resulting fragment ion at *m/z* 346.1 gave an MS^3 spectrum that closely resembled the MS^2 spectra of compounds B2A and B2B, suggesting that compound B2D is an O-methylated derivative of compound B2A or B2B.

Compound B2E yielded an $[M + H]^+$ ion at *m/z* 329.1 in positive ion mode LC-MS. The fragmentation pattern and the on-line UV spectrum, which showed λ_{max} at 276 nm and 329 nm, indicated that B2E could be salvigenin, a known constituent of *Stachys swainsonii* and *Stachys ionica* (Skaltsa et al, 2007; Meremeti et al, 2004).

Figure 15: HPLC-ELSD chromatogram of B2

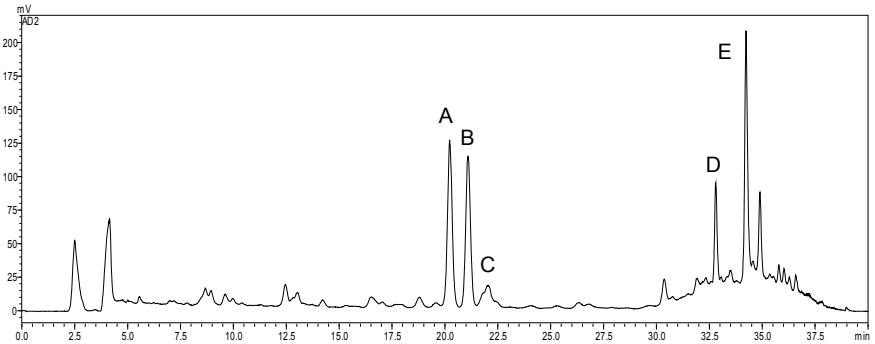


Figure 16: HPLC-UV (Left) and -ELSD (Right) chromatograms of B2C

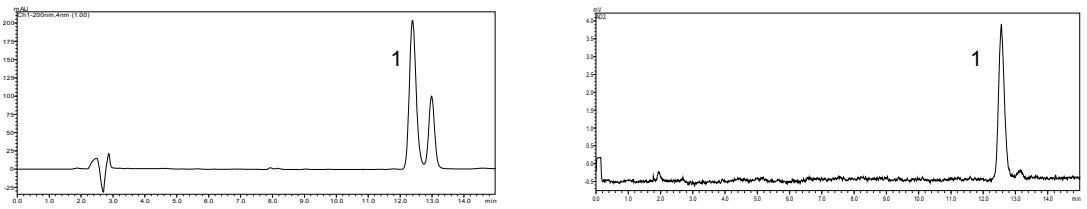
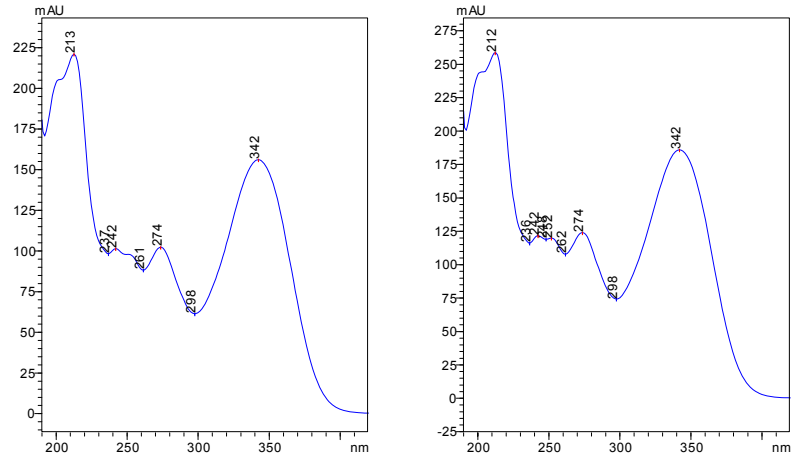
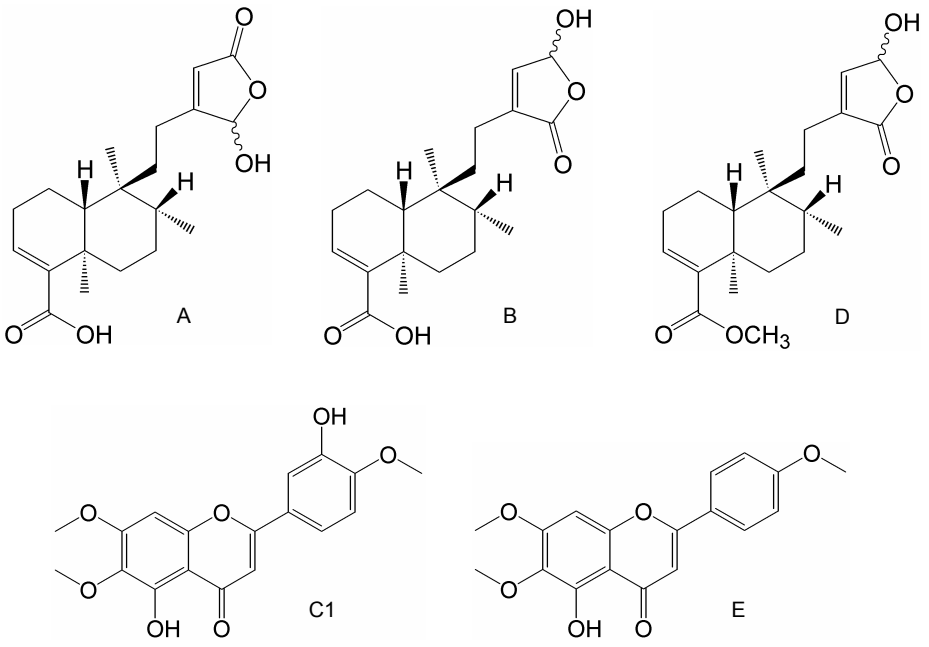


Figure 17: UV spectra of B2C1 (Left) and commercial eupatorin (Right)



Extensive 1D and 2D NMR experiments on the isolated pure compounds finally permitted to conclude the structure elucidation. Thus, the five structures from B2 were determined as follows (Fig. 18): 16-hydroxycleroda-3,13-dien-16,15-olide-18-oic acid (B2A); 15-hydroxycleroda-3,13-dien-16,15-olide-18-oic acid (B2B); eupatorin (B2C1); 15-hydroxycleroda-3,13-dien-16,15-olide-18-oic acid methyl ester (B2D); salvigenin (B2E).

Figure 18: Chemical formulae of the compounds isolated from B2



3.2.2.2 *Glechoma hederacea*

GC-MS and HPLC-MS analyses of **G1** permitted to identify for the first time in this plant the cyclic hydroxamic acid 2-benzoxazolinone (Fig. 19), as the main component of the fraction according to the ELSD detection.

First, its identification was consented by GC-MS analyses (Fig. 20) with a similarity index of 92% (G1A, R_t: 40.4 min). Additionally, 2(4H)-benzofuranone and eicosanoic acid methyl ester were found to be present in a much lower amount with a 91% similarity index. The HPLC-ELSD and -UV (270 nm) chromatograms of the same fraction (Fig. 21) proved that the presence of a main component (G1A, R_t: 23.5 min) was not limited to the volatile constituents. The UV spectrum of G1A and its HPLC retention time were found to match with the ones of the commercial reference compound 2-benzoxazolinone (Fig. 22). Subsequently, HPLC-MS analyses were performed using the same conditions as above, in order to gain a further confirmation of its identity. As expected, the obtained mass spectrum of G1A was found to be according to the one acquired by GC-MS (data not shown).

The isolation of the compound by HPLC (2,49 mg) was followed by extensive 1D and 2D NMR experiments, which confirmed its identity as 2-benzoxazolinone.

Figure 19: Chemical formula of 2-benzoxazolinone from G1

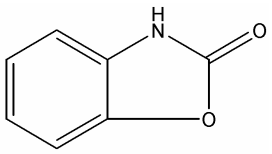


Figure 20: GC-MS chromatogram of G1

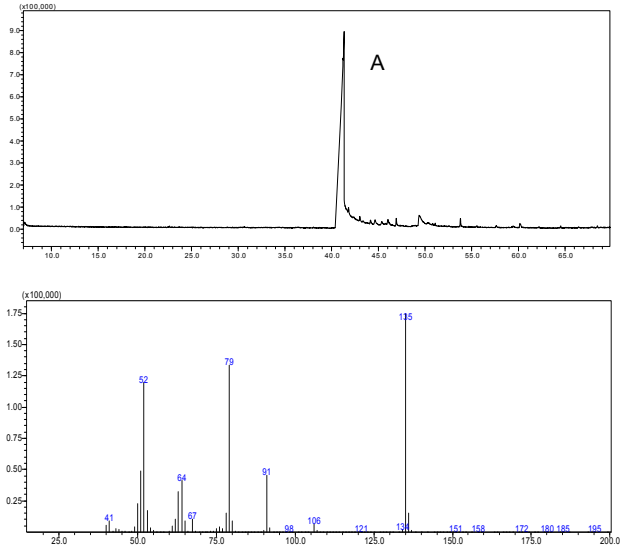


Figure 21: HPLC-UV (Left) and -ELSD (Right) chromatograms of G1

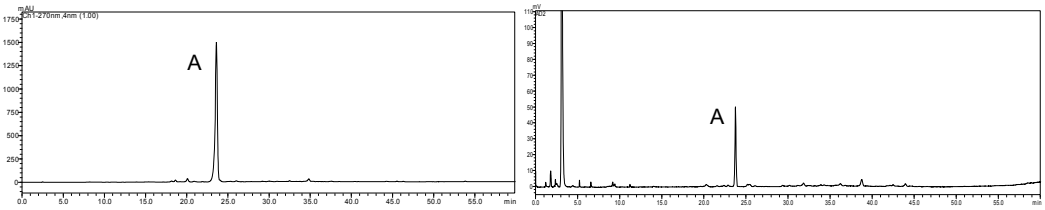
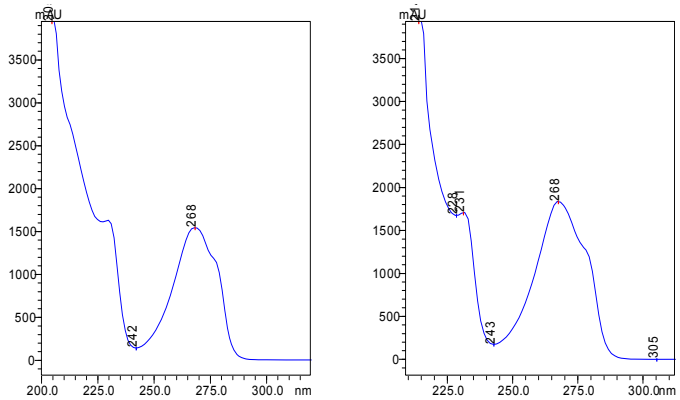


Figure 22: UV spectra of G1A (Left) and commercial 2-benzoxazolinone (Right)



The semipreparative HPLC fractionation of **G2** yielded seven subfractions, which were generated as shown in Fig. 23, as this complex mixture of compounds could not be analytically processed. The collected subfractions were pharmacologically evaluated and all of them were found to be active on the usual targets (results see Table 12, pag. 54). Several HPLC analyses followed by LC-MS measurements consented to identify the main component of **G2D** as the flavonoid acacetin (G2D1, Rt: 15.7), while the triterpene esculentic acid (G2E1, Rt: 10.9) was identified in **G2E** (Figs. 24 and 25). Their chemical formulae are shown in Fig. 26.

Figure 23: HPLC-ELSD chromatogram of G2

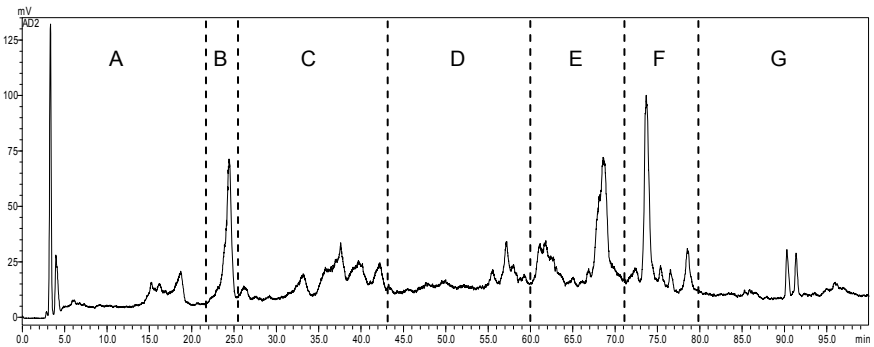


Figure 24: HPLC-UV (340 nm) chromatogram of G2D

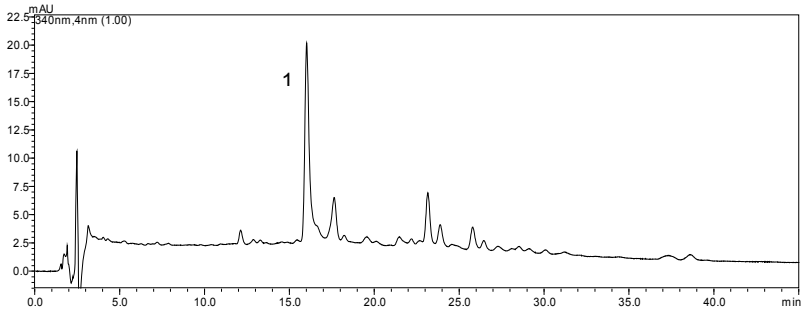


Figure 25: HPLC-ELSD chromatogram of G2E

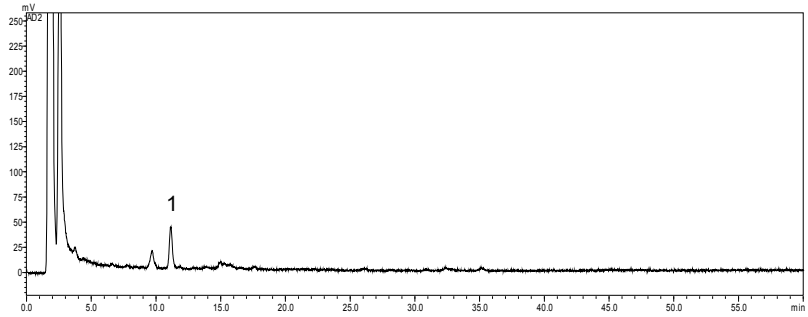
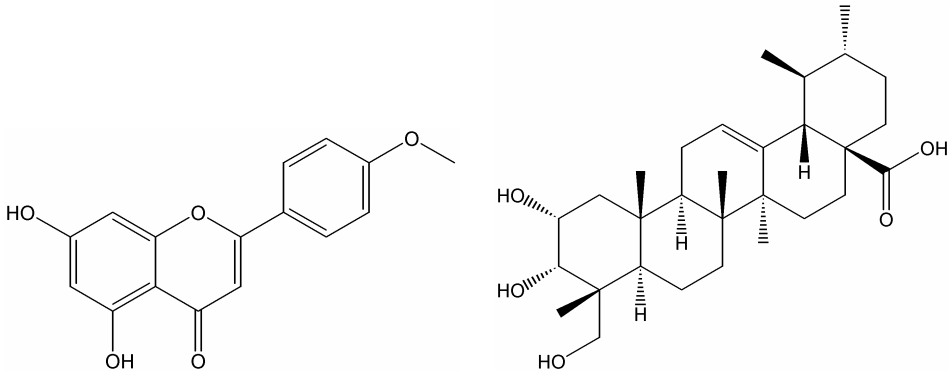


Figure 26: Chemical formulae of acacetin from G2D (Left) and esculentic acid (Right) from G2E



The HPLC-ELSD chromatogram of **G3** is shown in Fig. 27. This analytical fractionation consented to isolate and pharmacologically evaluate three fractions and five pure compounds, whose tentative structure elucidation was performed by HPLC-MS.

The major components of this fraction were not considered of particular interest, as they were identified as chlorophyll catabolites. Compound G3F was identified as

phaeophorbide A (Fig. 28), which derives from the chlorophyll degradation (via chlorophyllide) by the enzyme chlorophyllase (Schelbert et al, 2009), while compounds G3B, G3E, G3H and fraction G3G were found to be closely related structures.

Compound G3D was found to be diethylhexylphthalate (DEHP), which is a commonly used plastic softener and, therefore, does not belong to the plant. The presence of this compound in the fraction is due to contamination of the solvents used for the extraction, as extensive HPLC and GC-MS analyses demostrated (data not shown).

Figure 27: HPLC-ELSD chromatogram of G3

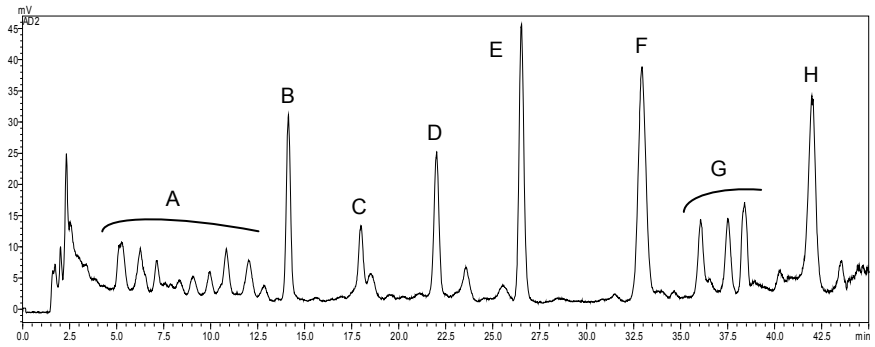
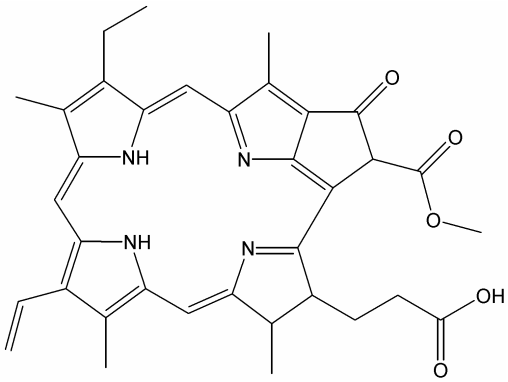


Figure 28: Chemical formula of phaeophorbide A from G3



The chromatographic comparison between the phenol-free MeOH extracts of *Glechoma hederacea* from two different locations (**G4** and **G5**) is shown in Fig. 29, through the overlapping of their HPLC-UV chromatograms.

Whereas several peaks, even if in different relative amounts, were found to be common to both samples, the most relevant difference regarded the main components of the two extracts.

The already mentioned 2-benzoxazolinone, identified as compound G4B (Rt: 31.8), could not be detected in G5, where the main component was found to be compound G5A (Rt: 71.9). HPLC-MS analyses and the comparison with a commercial reference compound consented to identify G5A as the flavonoid apigenin (Fig. 30), which could be also detected in G4, although in a much lower amount.

Further investigations on a higher number of plant samples are required in order to explain the absence of 2-benzoxazolinone in G5.

Figure 29: HPLC-UV (254 nm) chromatograms of G4 and G5

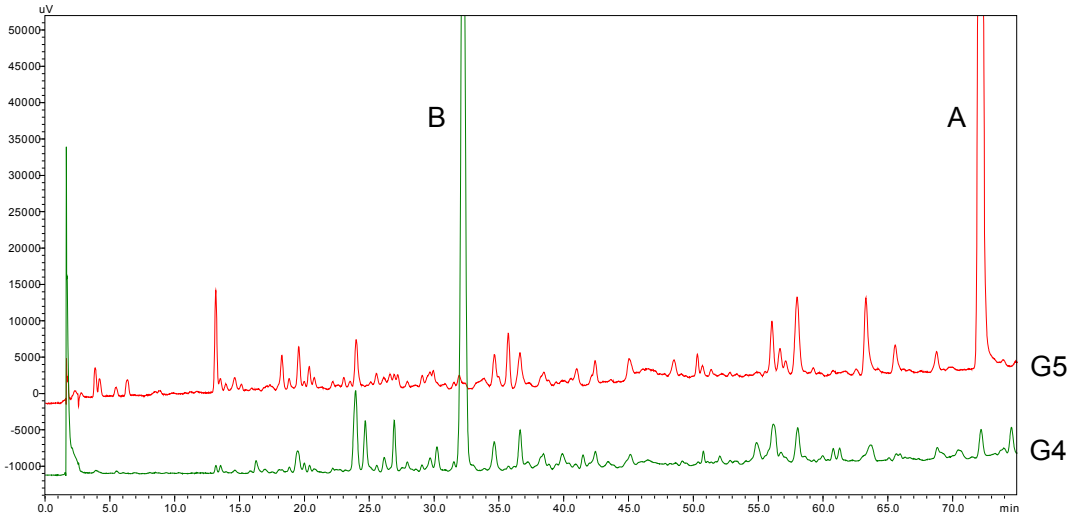
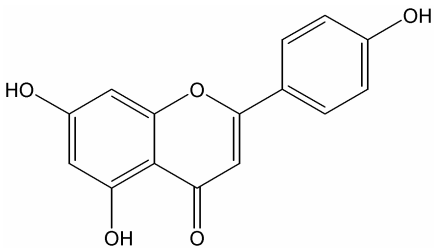


Figure 30: Chemical formula of apigenin from G5



3.2.3 HPLC Method Optimization

The HPLC fractionation of B2 from *Betonica officinalis* required an extensive method optimization, in order to achieve a satisfying separation of its main constituents. Several stationary phases were tested with different solvent systems at different oven temperatures, before the final method could be chosen. Flow rate and injection volume were always 1.0 ml/min and 10 µl, respectively. Representative of the optimization process are the four methods which follow.

In the first one a LiChrospher RP-18 column (250 x 4 mm, 5 µm) was used, with a linear gradient from 5% to 100% MeOH in 60 min (v/v) and the oven temperature set at 25 °C. The HPLC-ELSD chromatogram showed the presence of one main component (Fig. 31).

In the second method, where acetonitrile was used instead of methanol, the splitting of the main peak was observed, evidencing the presence of at least two main components (Fig. 32).

Figure 31: HPLC-ELSD chromatogram of B2 (LiChrospher RP-18, 5-100% MeOH in 60 min, 25°C)

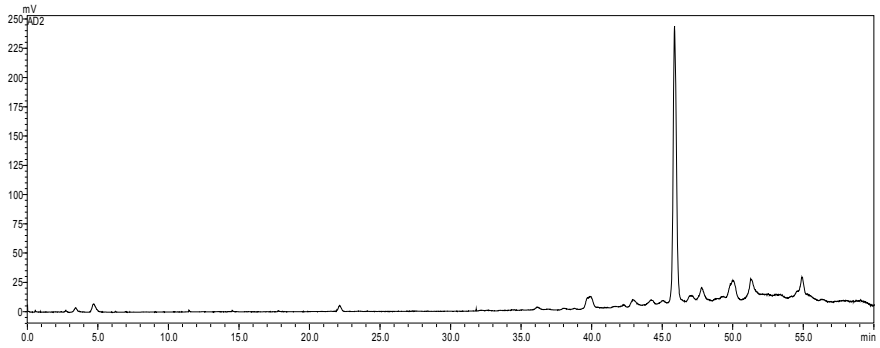
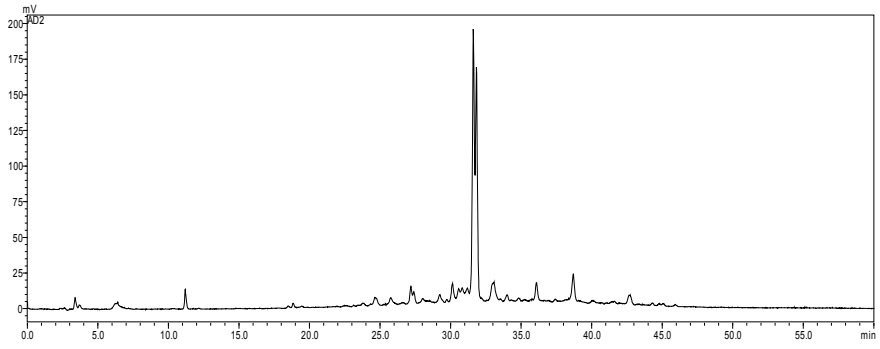
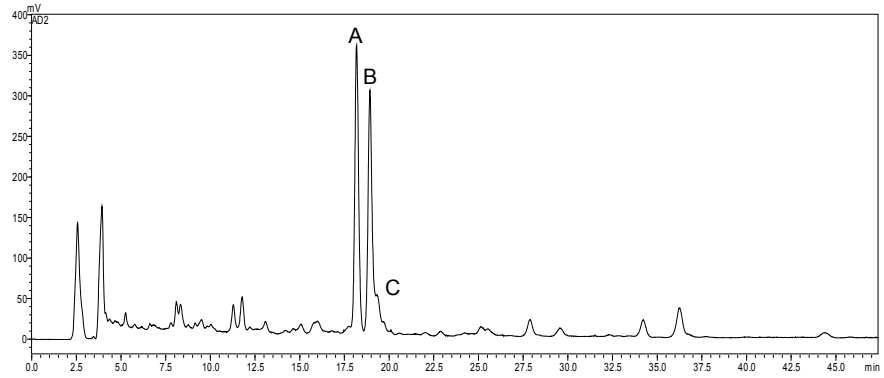


Figure 32: HPLC-ELSD chromatogram of B2 (LiChrospher RP-18, 5-100% MeCN in 60 min, 25°C)



The switch to an Aquasil C18 column (250 x 4.6 mm, 5 µm) in the third method, with a flat acetonitrile gradient (40-60% MeCN in 60 min, v/v), resulted in the separation of the two main peaks (A, B) but also in the co-elution of other constituents (C) with the second one (Fig. 33).

Figure 33: HPLC-ELSD chromatogram of B2 (Aquasil C18, 40-60% MeCN in 60 min, 25°C)



The decisive factor in the last method was the oven temperature set at 15 °C instead of 25 °C, as the initial acetonitrile gradient (40-48% MeCN in 25 min, v/v) was in line with the previous one.

Besides the good separation of the peaks A, B and C, two further prominent peaks (D, E) were observed in the purge step (48-95% MeCN in 10 min, 95% MeCN for 10 min, v/v) and could also be object of investigation (Fig. 34). The improvement in the last run can be distinctly appreciated with three different wavelenghts in Fig. 35.

Figure 34: HPLC-ELSD chromatogram of B2 (Aquasil C18, 40-48% MeCN in 25 min, 15°C)

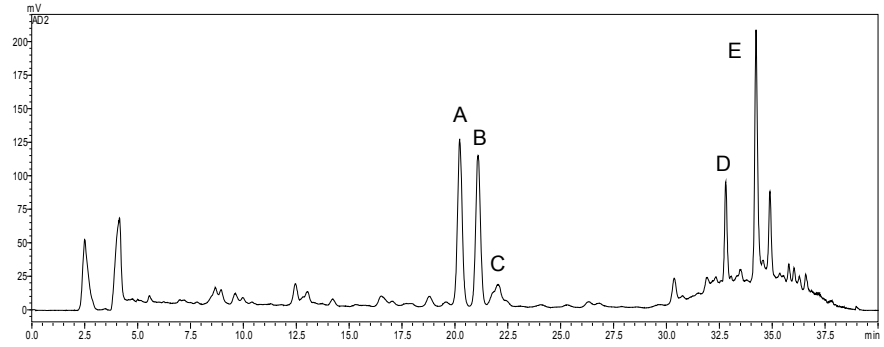
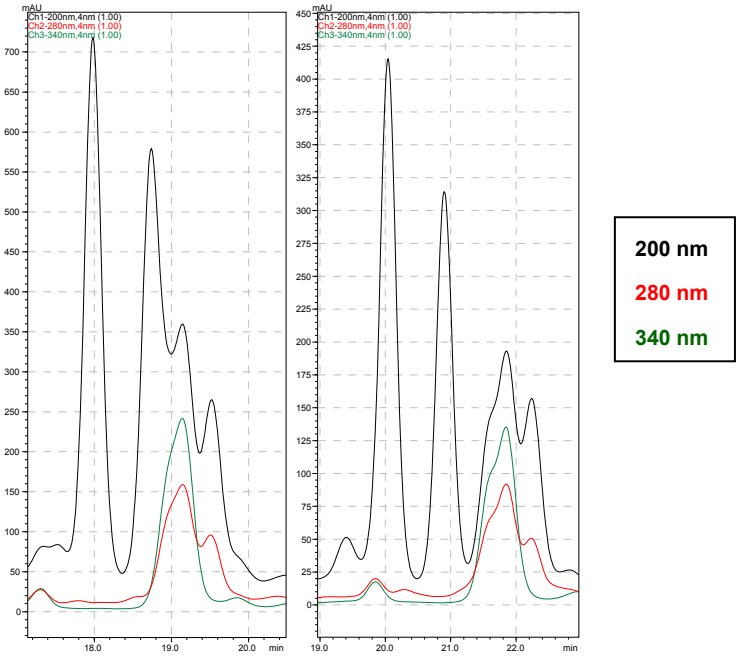


Figure 35: HPLC-UV chromatograms of B2
(Left: 40-60% MeCN in 60 min, 25 °C; Right: 40-48% MeCN in 25 min, 15 °C)



The separation of the two compounds composing B2C was also achieved after different trials. Different MeCN gradients (40-48% in 25 min; 41.5-43.5% in 15 min; 41.5-43.5% in 30 min, v/v) with a flow rate of 0.5 or 1.0 ml/min using a Luna C18 column (250 x 4 mm, 5 µm) at 15 °C resulted to be not suitable for the purpose.

A good separation was finally achieved using an extremely slow gradient (44-46% MeCN in 15 min, v/v) with a flow rate of 1.0 ml/min and the oven temperature set at 16 °C (Fig. 16, pag. 37).

3.2.4 Further GC-MS analyses

Besides of the described 2-benzoxazolinone, several other compounds (mainly fatty acids and essential oil components) were identified by GC-MS in the SPE fractions of *Betonica officinalis* and *Glechoma hederacea*.

MS spectra of the structures listed in Table 10 were found to match those stored in a library with a similarity index (SI) value of at least 90%. Fractions B4, B5, B6 and G2 were found to not contain significant amounts of volatile constituents.

Table 10: Compounds identified by GC-MS in fractions of *B. officinalis* and *G. hederacea*

Fraction	Compound
B1	Limonene dioxide
	4-(2,6,6-trimethyl-1-cyclohexen-1-yl)- 3-Buten-2-ol
	Loliolide
	cis-Z-α-Bisabolene epoxide
B2	5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone
	Caryophyllene oxide
	Oplopanone
	Thujopsan-2α-ol
B3	6,10,14-trimethyl-2-pentadecanone
	6,10-dimethyl-2-undecanone
	Palmitic acid
	Nonadecanol
	Eicosanoic acid, methyl ester
	Hexadecanoic acid
	Octadecanoic acid, methyl ester
	Hexanoic acid, 2-tetradecyl ester
	Stigmasta-4,7,22-trien-3β-ol
	Stigmasta-5,22-dien-3-ol, acetate
G1	2-benzoxazolinone
	3-hydroxymethyl-2-benzoxazolinone
	(1S,4R)-p-mentha-2,8-diene
	4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-ol
	4-tridecen-6-yne
	Eicosanoic acid, methyl ester
	Heptacosanoic acid, methyl ester
	Octadecanoic acid, methyl ester
G3	Tetradecanal
	Methyl dodecanoate
	6-octadecenoic acid, methyl ester
	5,8-octadecadienoic acid, methyl ester
	Methyl linoleate
	12-methyltetradecanoic acid, methyl ester

3.3 Biological Testing

The pharmacological results of the SPE fractions obtained from the extracts of *Betonica officinalis* and *Glechoma hederacea*, tested in the different assays at the concentration of 10 µg/ml, are listed in Table 11 (legend see Tab. 7, pag. 33).

In the following graphs, bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF or LPS (* P<0.05, ** P<0.01, *** P<0.001).

Table 11: Pharmacological results of the SPE fractions from *Betonica officinalis* and *Glechoma hederacea*, tested at 10 µg/ml (legend see Tab. 7)

Sample			Results						
Species	Extract	Fraction	PPAR-α	PPAR-γ	NF-κB	TNF-α-induced		LPS-induced	
						E-select.	IL-8	E-select.	IL-8
Betonica officinalis	DCM wCh	B1	-	-	-	moderate	moderate	weak	no
		B2	-	-	-	moderate	weak	strong	moder.
		B3	-	-	-	no	no	strong	strong
	MeOH wP	B4	-	-	-	no	no	moderate	no
		B5	-	-	-	no	no	strong	strong
		B6	-	-	-	moderate	no	strong	strong
Glechoma hederacea	MeOH wP	G1	no	no	no	strong	strong	no	no
		G2	strong	strong	strong	strong	strong	no	no
		G3	moderate	moderate	strong	strong	weak	no	no

3.3.1 *Betonica officinalis*

8-O-acetylharpagide (B1A), main component of **B1**, was pharmacologically evaluated at different concentrations showing significant activities on TNF-α-induced IL-8 and E-selectin at the mRNA level, similarly to the original fraction B1 at 10 µg/ml. B1A was found to strongly and dose-independently inhibit the TNF-α-induced IL-8 (Fig. 36), as well as TNF-α-induced E-selectin in a dose-dependent manner between 1.0 and 5.0 µg/ml (Fig. 37), while its activity at 10 µg/ml was lower in both cases probably due to a toxic effect.

In contrast, 8-O-acetylharpagide was inactive on these targets at the protein level. Furthermore, the closely related iridoids harpagide and harpagoside, even if not

identified in *Betonica officinalis*, were also tested for comparison reasons on the same targets with negative results (data not shown).

Figure 36: Effect of 8-O-acetylharpagide (B1A) on TNF-α-induced IL-8 mRNA

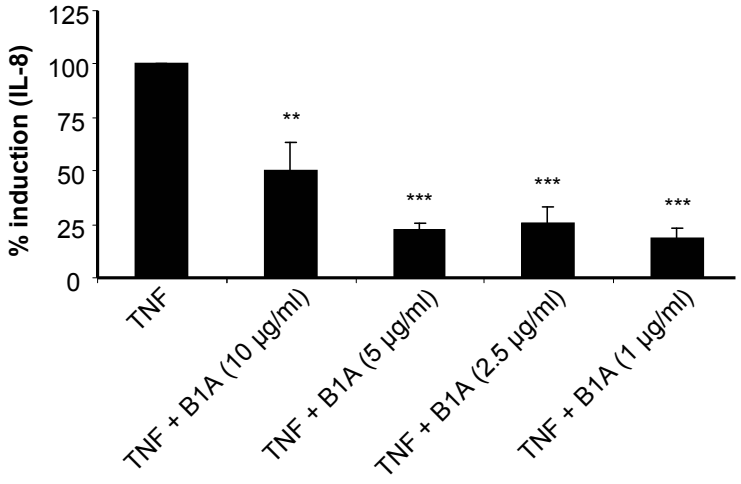
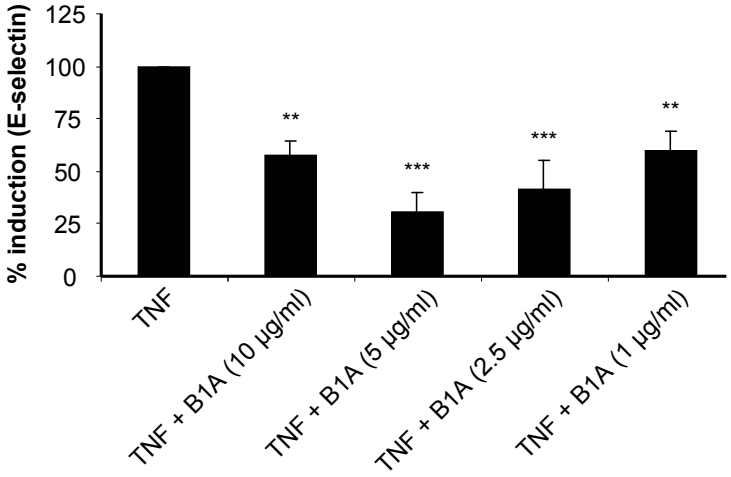


Figure 37: Effect of 8-O-acetylharpagide (B1A) on TNF-α-induced E-selectin mRNA



The testing of the fraction **B2** at the mRNA level revealed strong downregulations of IL-8 and E-selectin at the concentration of 10 µg/ml, particularly of the LPS-induced E-selectin, which was inhibited until the basal level.

Some of the substances isolated from this fraction exhibited significant activities on both targets at the concentration of 10 µg/ml (Figs. 38-41). Compound B2C1 (eupatorin) was able to strongly inhibit the LPS-induced expression of E-selectin and IL-8, while the close related flavonoid salvigenin (B2E) was only moderate active on LPS-induced E-selectin. Concerning the clerodane diterpenes (B2A, B2B and B2D), which were differently active, compound B2B showed the strongest activity on LPS-induced E-selectin, with a mean inhibition of 96.7%.

Figure 38: Effect of compounds from B2 at 10 µg/ml on TNF-α-induced IL-8 mRNA

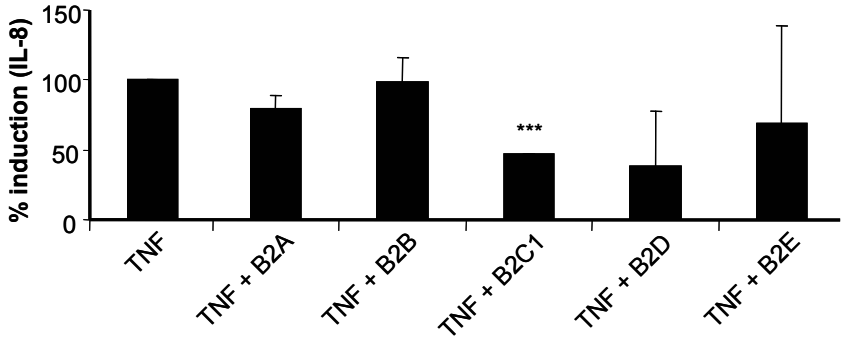


Figure 39: Effect of compounds from B2 at 10 µg/ml on LPS-induced IL-8 mRNA

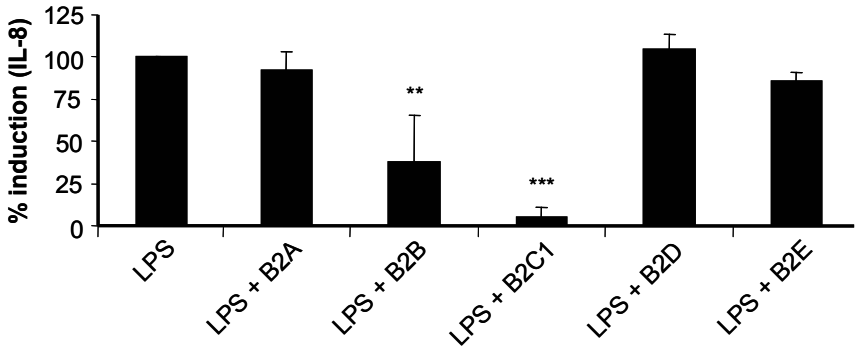


Figure 40: Effect of compounds from B2 at 10 µg/ml on TNF-α-induced E-selectin mRNA

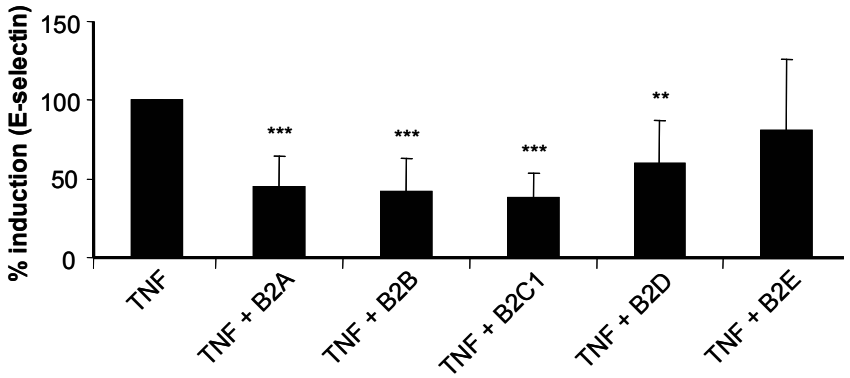
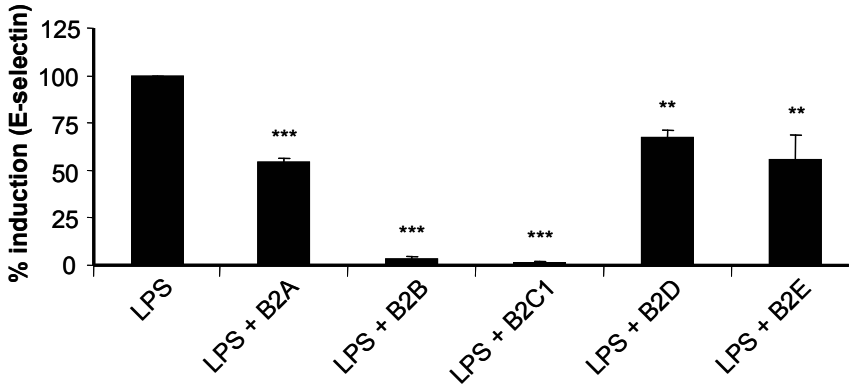


Figure 41: Effect of compounds from B2 at 10 µg/ml on LPS-induced E-selectin mRNA



The above described compounds were subsequently evaluated on the TNF-α- and LPS-induced IL-8 and E-selectin expression at the protein level by ELISA, with different results. Furthermore, the accomplished structure elucidation consented to obtain more reliable results through the testing at micromolar concentrations.

Whereas the original fraction B2, tested at 10 µg/ml, determined an almost complete inhibition of both targets (data not shown), the clerodane diterpenes B2A and B2B were found to be active at the concentration of 30 µM, particularly on the E-selectin expression (Figs. 44 and 45), while B2B could also significantly inhibit the TNF-α-induced IL-8 expression (Fig. 42). Much weaker but significant activities were observed for eupatorin (B2C1) and salvigenin (B2E), while the esterificated clerodane diterpene (B2D) was inactive (Figs. 42-45).

Figure 42: Effect of compounds from B2 at 30 µM on TNF-α-induced IL-8 ELISA

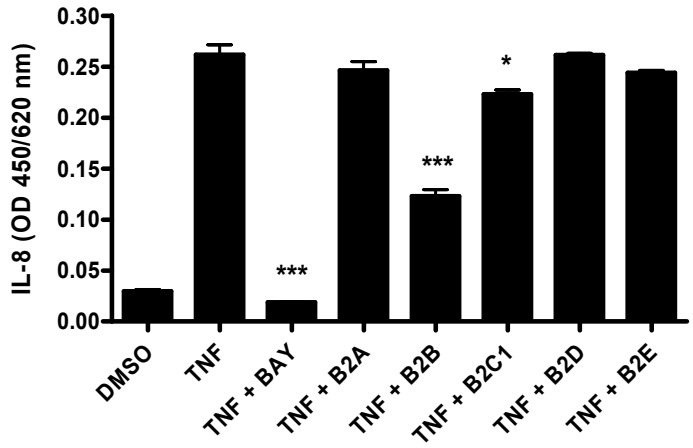


Figure 43: Effect of compounds from B2 at 30 µM on LPS-induced IL-8 ELISA

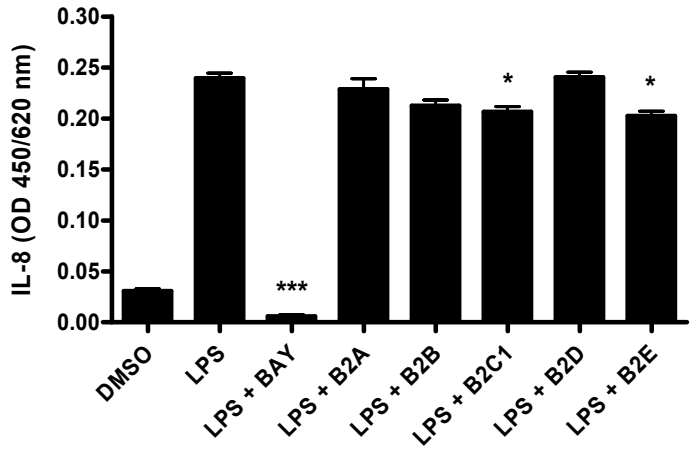


Figure 44: Effect of compounds from B2 at 30 µM on TNF-α-induced E-selectin ELISA

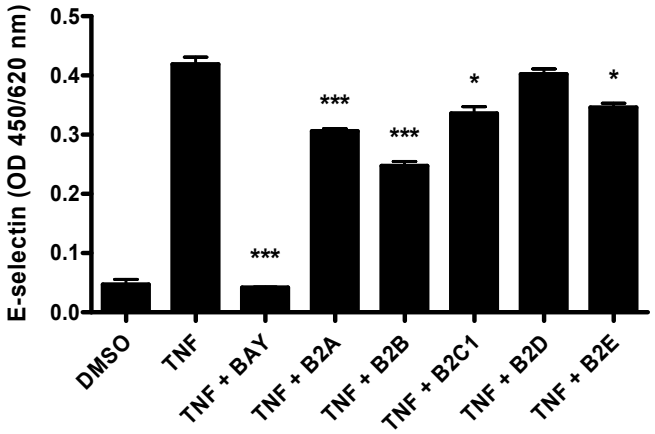
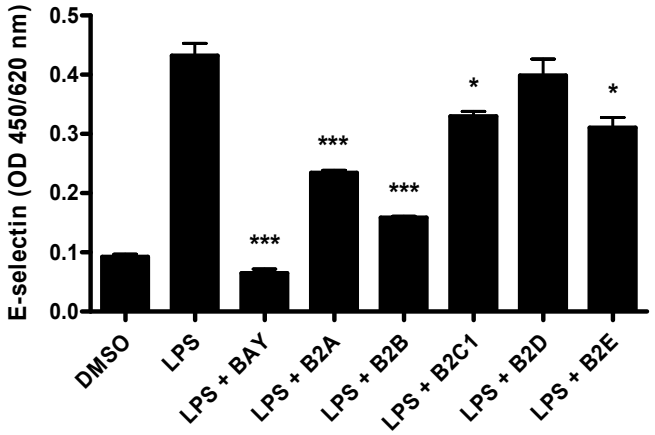
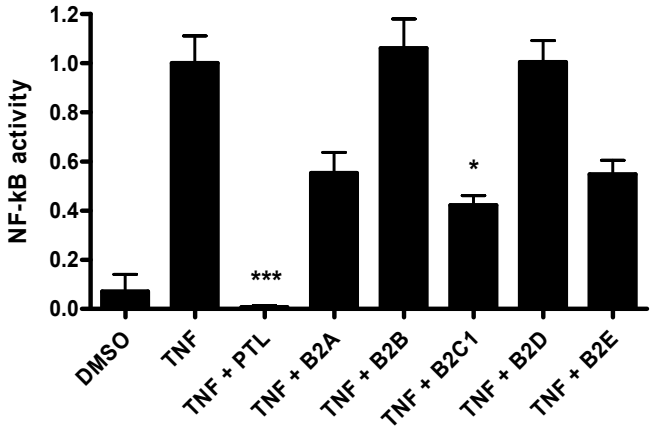


Figure 45: Effect of compounds from B2 at 30 µM on LPS-induced E-selectin ELISA



The subsequent testing of the above descibed compounds on the TNF-α-induced NF-κB activation, at the concentration of 30 µM, revealed a moderate inhibition by compounds B2A and B2E, while only the flavonoid eupatorin (B2C1) showed a significant inhibitory activity (Fig. 46).

Figure 46: Effect of compounds from B2 at 30 µM on the TNF-α-induced NF-κB activation



3.3.1.1 Structure-activity relationships

On the basis of these results, structure-activity relationships could be delineated between the clerodane diterpenes (B2A, B2B, B2D) and the flavonoids (B2C1, B2E). It seems to be that the lacton conformation of B2A decreases the activity, compared with the one of B2B. Further, the esterification of B2D determines a complete loss of activity in comparison with B2B. On the other hand, the alternative lacton conformation and the carboxylic function of B2B increase strongly the activity (Fig. 47).

Concerning the flavonoids B2C1 and B2E, which only differ for the hydroxyl group in the 3'-position of the first one, different activities were observed especially at the mRNA level in LPS-stimulated cells. That hydroxyl function seems to be critical for the strong activity of B2C1 (Fig. 48).

However, further investigations are necessary in order to confirm these assumptions and to identify the pharmacophore of these structures.

Figure 47: Structure-activity relationships between the clerodane diterpenes from B2

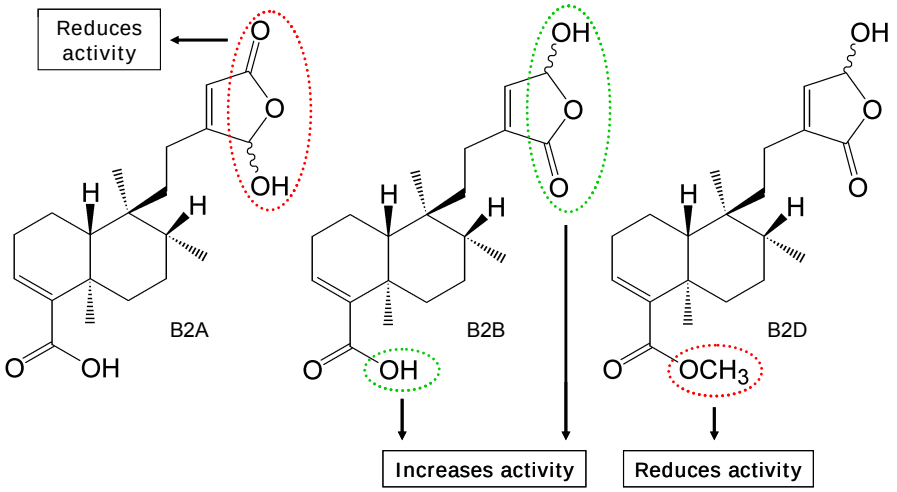
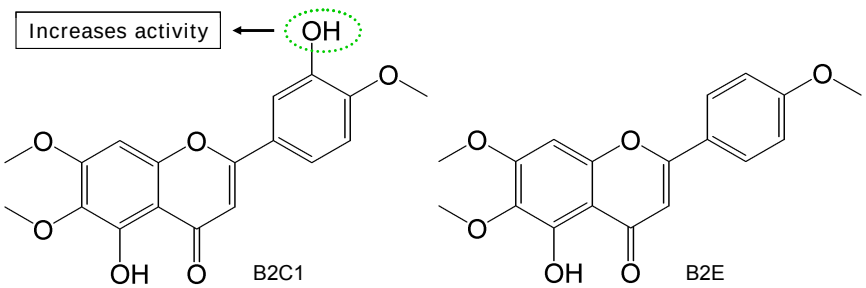


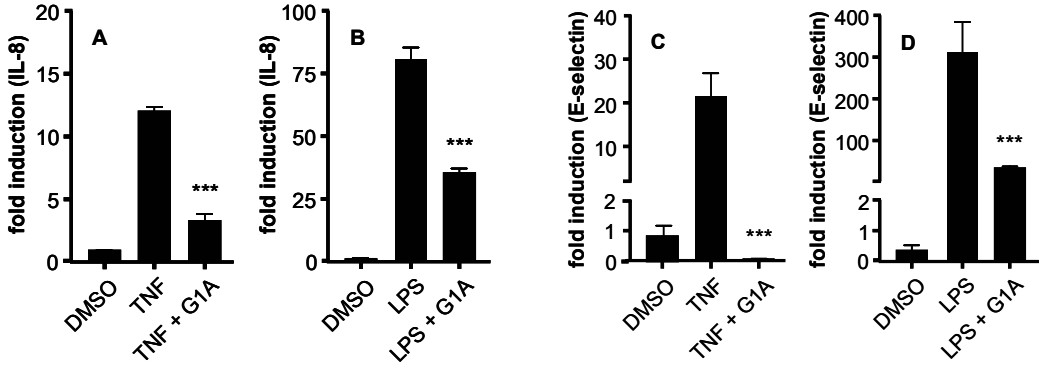
Figure 48: Structure-activity relationships between the flavonoids from B2



3.3.2 *Glechoma hederacea*

Tested at 10 µg/ml, the main component of **G1** 2-benzoxazolinone (G1A) was able to significantly inhibit the TNF-α- and LPS-induced expression of IL-8 and E-selectin (Fig. 49). However, this results were not reproducible at the protein level, where G1A was only weakly active on LPS-induced IL-8 (data not shown).

Figure 49: Effect of G1A at 10 µg/ml on TNF-α- and LPS-induced IL-8 (A-B) and E-selectin (C-D) mRNA



The results of the seven fractions from **G2**, isolated by semipreparative HPLC and tested at the concentration of 10 µg/ml, are listed in Table 12 (legend see Tab. 7). All fractions showed strong activity on TNF-α- and LPS-induced E-selectin at the mRNA level, five of them also on PPARs activation.

Similar results were obtained with the fractions generated from **G3** (Tab. 13). Diethylhexylphthalate (G3D) and phaeophorbide A (G3F) were also able to almost completely inhibit the TNF-α-induced NF-κB activation at 10 µg/ml. Phaeophorbide A was already found in *Isatis tinctoria* (Mohn et al, 2009) and in *Solanum dislorum*, where it was also active on NF-κB in PMA-induced HeLa cells (Heinrich, 2003).

Table 12: Pharmacological results of the fractions from G2 tested at 10 µg/ml

Fraction	PPAR-α	PPAR-γ	NF-κB	TNF-α-induced		LPS-induced	
				E-selectin	IL-8	E-selectin	IL-8
G2A	no	no	no	strong	moderate	strong	moderate
G2B	no	no	no	strong	moderate	strong	weak
G2C	strong	strong	no	strong	moderate	strong	weak
G2D	strong	strong	no	strong	moderate	strong	weak
G2E	strong	strong	no	strong	weak	strong	weak
G2F	strong	strong	no	strong	weak	strong	weak
G2G	strong	strong	no	strong	weak	strong	weak

Table 13: Pharmacological results of the fractions from G3 tested at 10 µg/ml

Fraction	PPAR-α	PPAR-γ	NF-κB	TNF-α-induced		LPS-induced	
				E-selectin	IL-8	E-selectin	IL-8
G3A	moderate	moderate	no	strong	weak	strong	weak
G3B	moderate	moderate	no	strong	weak	strong	moderate
G3C	moderate	moderate	no	strong	moderate	strong	weak
G3D	no	no	strong	strong	moderate	strong	moderate
G3E	moderate	no	no	strong	weak	strong	moderate
G3F	no	no	strong	strong	weak	strong	strong
G3G	no	no	no	strong	weak	strong	strong
G3H	no	no	no	strong	weak	strong	moderate

As the phenol-free MeOH extract **G5** (field-collected sample from Laab im Walde) was able to downregulate the IL-8 expression, its main component **apigenin** (G5A, see pag. 43) was also object of investigation, as well as the closely related flavonoid **acacetin** (G2D1), identified in the active fraction **G2D**.

The two flavonoids were tested at different concentrations on TNF-α- and LPS-induced IL-8 and E-selectin at the protein level, showing inhibitory activity in a dose dependent manner (Figs. 50-57). Subsequently, they were evaluated on TNF-α-induced NF-κB at the concentration of 10 µg/ml and found to significantly decrease its activation until the basal level (Fig. 58).

Figure 50: Effect of G5A (apigenin) on TNF-α-induced IL-8 ELISA

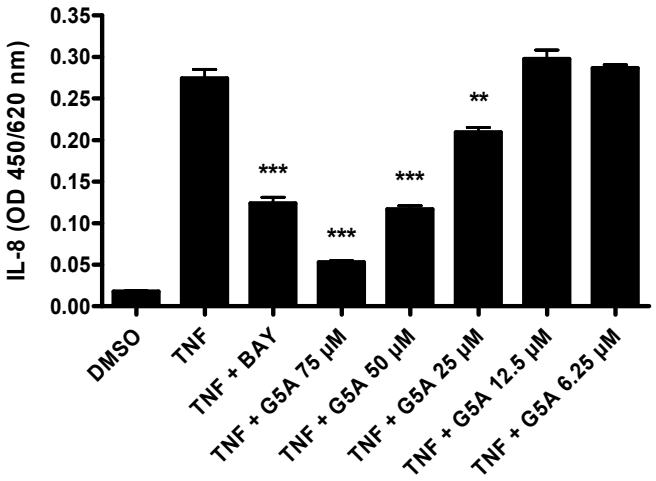


Figure 51: Effect of G5A (apigenin) on TNF-α-induced E-selectin ELISA

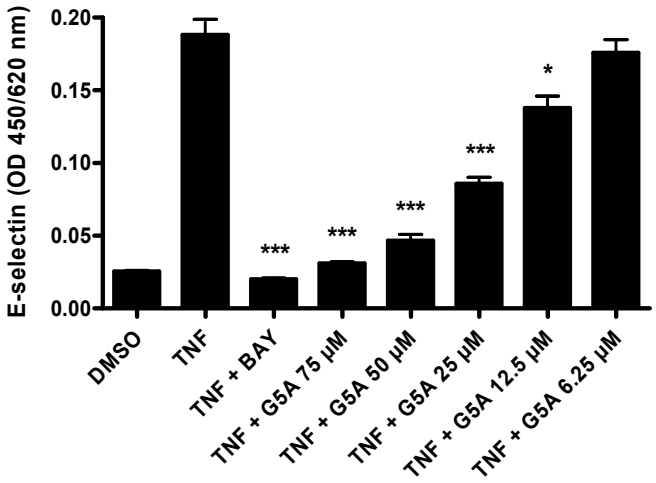


Figure 52: Effect of G5A (apigenin) on LPS-induced IL-8 ELISA

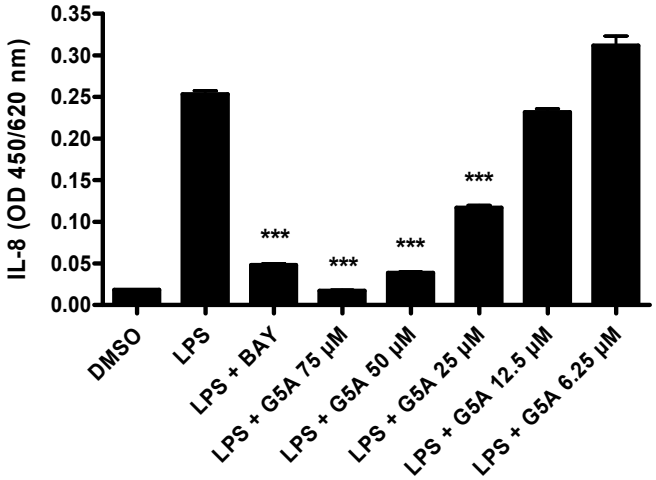


Figure 53: Effect of G5A (apigenin) on LPS-induced E-selectin ELISA

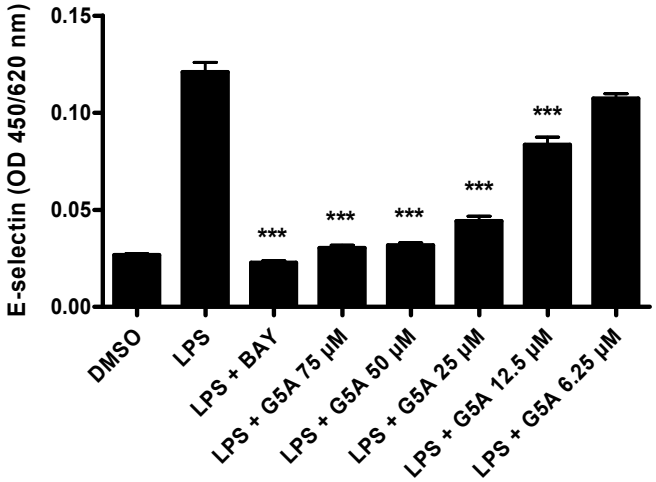


Figure 54: Effect of G2D1 (acacetin) on TNF-α-induced IL-8 ELISA

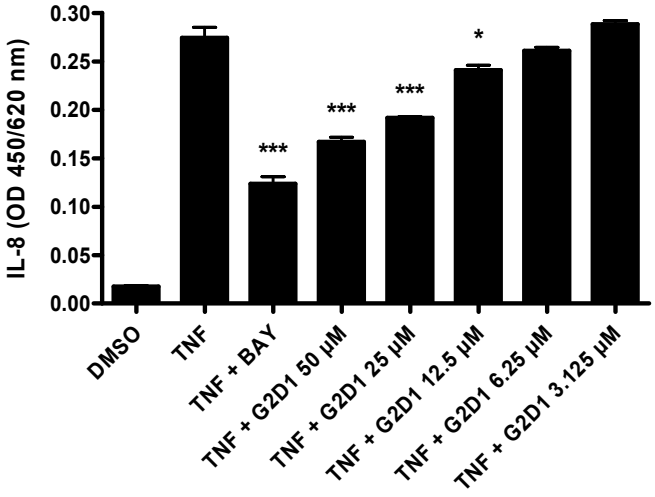


Figure 55: Effect of G2D1 (acacetin) on TNF-α-induced E-selectin ELISA

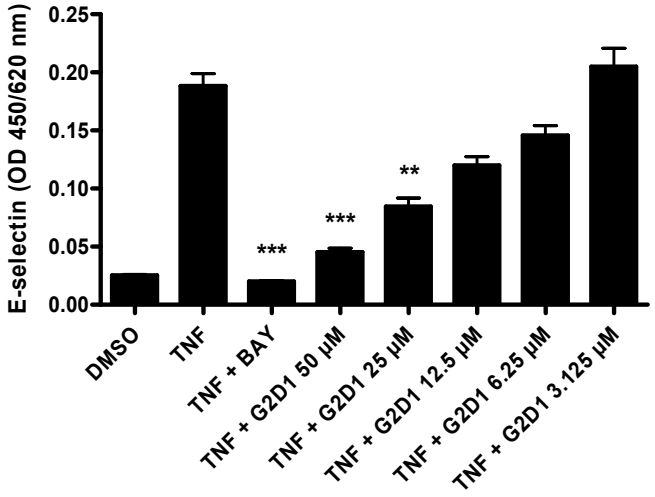


Figure 56: Effect of G2D1 (acacetin) on LPS-induced IL-8 ELISA

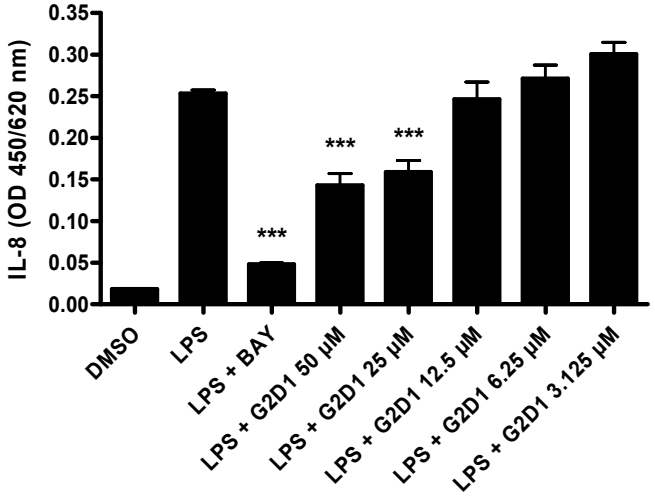


Figure 57: Effect of G2D1 (acacetin) on LPS-induced E-selectin ELISA

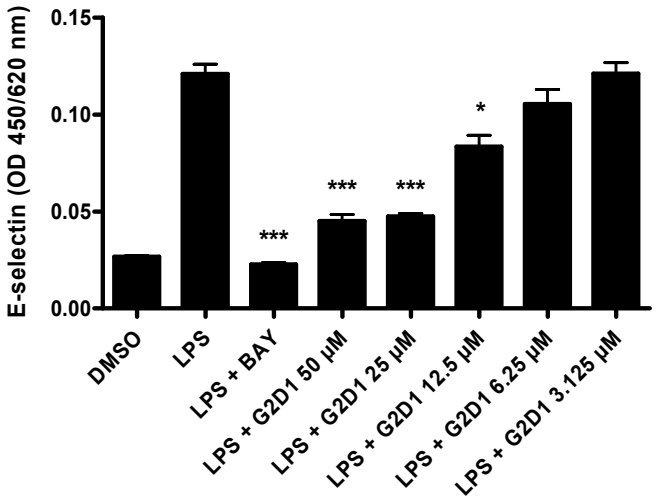
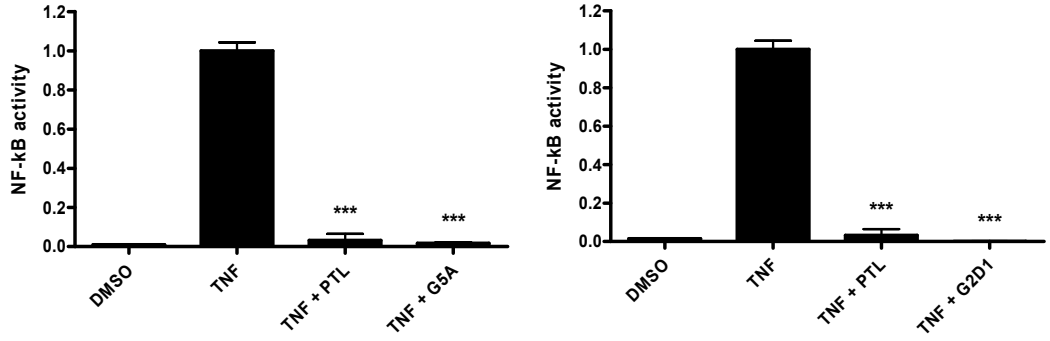


Figure 58: Effect of G5A (Left) and G2D1 (Right) at 10 µg/ml on TNF-α-induced NF-κB activation



EC_{50} were calculated with the inhibition values of apigenin and acacetin on TNF-α- and LPS-induced IL-8 and E-selectin (Tab. 14).

Table 14: EC_{50} of apigenin (G5A) and acacetin (G2D1) on TNF-α- and LPS-stimulated IL-8 and E-selectin

Compound	Target	Stimulation	EC_{50} (nM)	EC_{50} (95% confidence interval)
G5A	IL-8	TNF	31744	27767 to 36291
		LPS	18963	16399 to 21928
	E-selectin	TNF	17708	14871 to 21086
		LPS	13581	12243 to 15066
G2D1	IL-8	TNF	13854	11724 to 16372
		LPS	14177	10631 to 18906
	E-selectin	TNF	10968	8902 to 13513
		LPS	11689	9938 to 13748

3.4 In Silico Screening

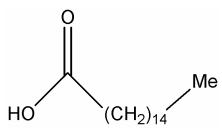
83 compounds from 26 plant species (33 fatty acids, 21 triterpenes, 11 triterpene acids, 5 alkaloids, 4 phenylpropanes, 3 isoprenoids, 3 alcohols, 1 aldehyde, 1 flavonoid and 1 steroid) were found to be active in the one and / or in the other in silico model (Tab. 15).

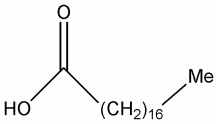
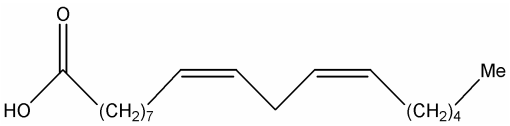
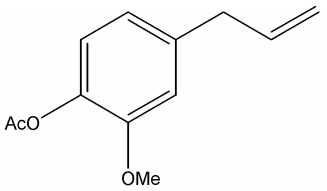
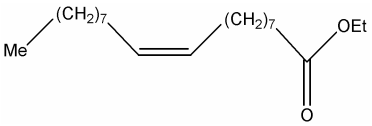
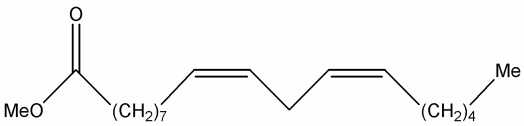
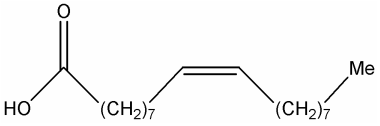
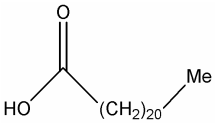
64 structures thereof were predicted to activate the PPAR-γ receptor, 52 the Farnesoid X receptor, while 29 were positive in both models. Therefore, these compounds can be supposed to possess anti-inflammatory properties.

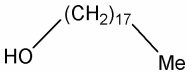
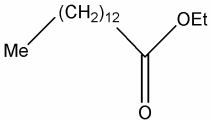
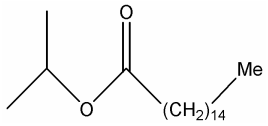
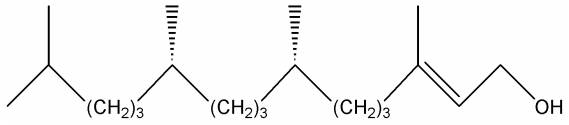
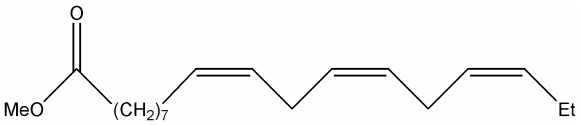
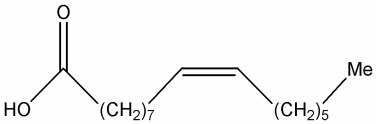
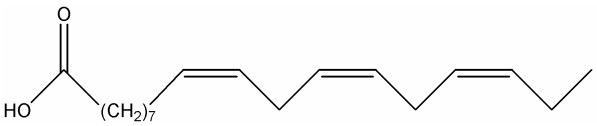
Concerning the screened known compounds from *Betonica officinalis* and *Glechoma hederacea*, 11 structures were active including 7 fatty acids (-esters) and the triterpene 3-epi-ursolic acid, which already showed anti-inflammatory activity in vivo (Pastorello et al, 2007).

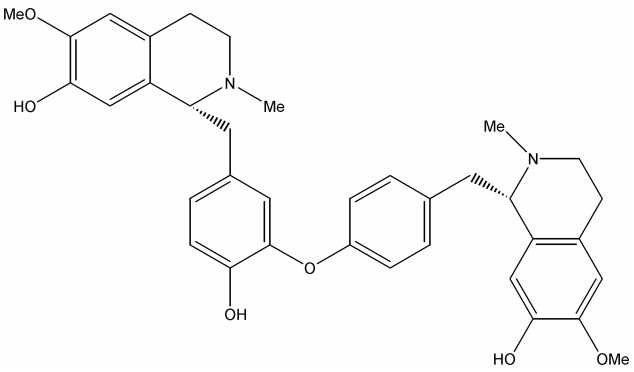
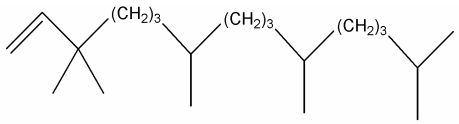
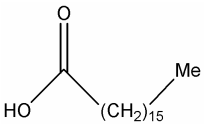
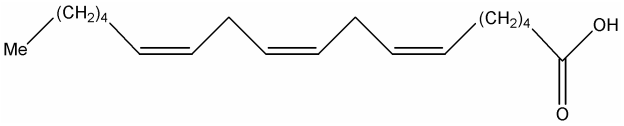
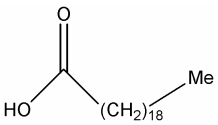
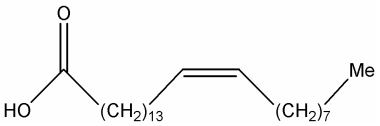
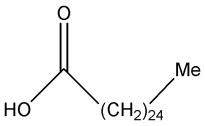
From the compounds investigated in vitro in this work, only the flavonoids eupatorin and salvigenin, identified in *Betonica officinalis*, were found to be active in the 5-LOX model, while the second one (already positive on FXR) was also active in the IKK-2 model.

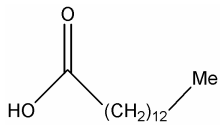
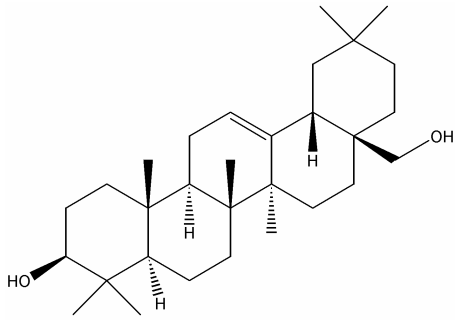
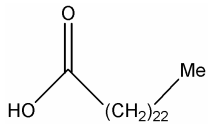
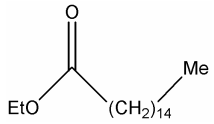
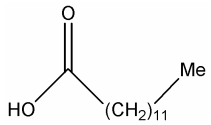
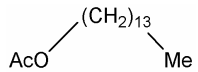
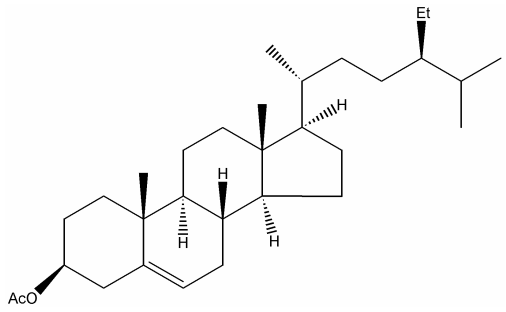
Table 15: In silico Hits

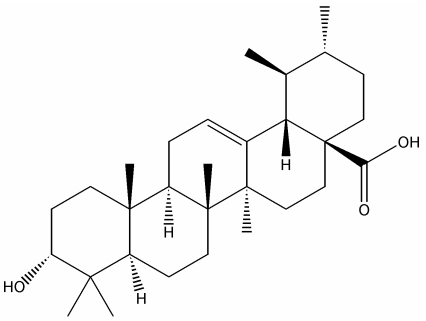
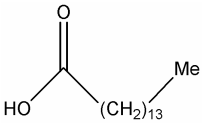
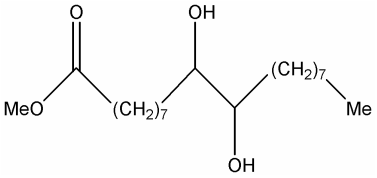
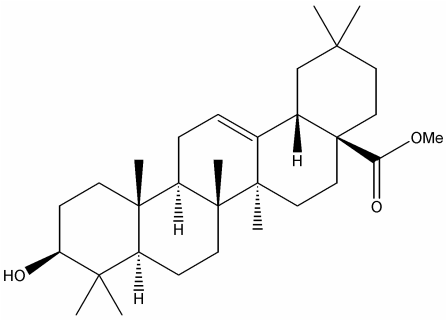
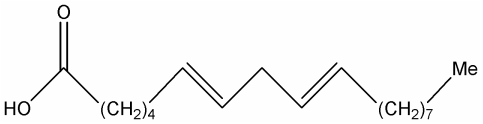
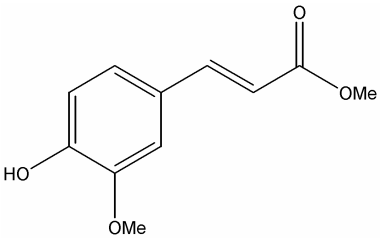
Structure	Plant species	PPAR-γ	FXR
 Hexadecanoic acid	*Agropyron repens* *Bellis perennis* *Calluna vulgaris* *Capsella bursa-p.* *Equisetum arvense* *Equisetum palustre* *Hippophae rhamn.* *Linum usitatissim.* *Lycopodium sp.* *Origanum vulgare* *Prunella vulgaris* *Sambucus nigra* *Sambucus ebulus* *Tussilago farfara* *Vaccinium vitis-id.*	+	+

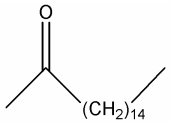
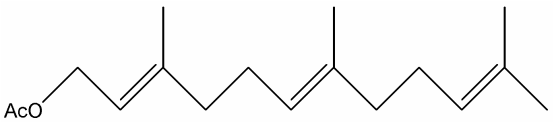
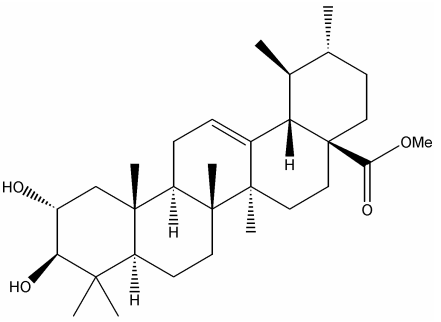
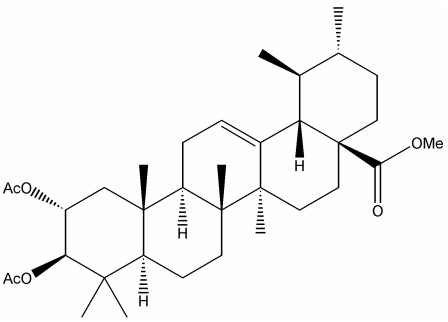
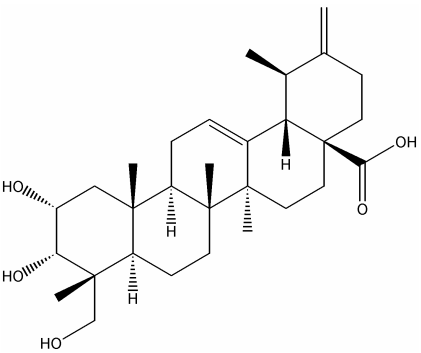
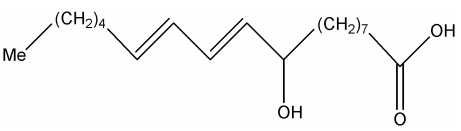
Structure	Plant species	PPAR-γ	FXR
 Octadecanoic acid	*Agropyron repens* *Bellis perennis* *Calluna vulgaris* *Capsella bursa-p.* *Hippophae rhamn.* *Linum usitatissim.* *Origanum vulgare* *Prunella vulgaris* *Sambucus nigra* *Sambucus ebulus*	+	+
 9,12-Octadecadienoic acid (9Z,12Z)	*Bellis perennis* *Equisetum arvense* *Hippophae rhamn.* *Linum usitatissim.* *Sambucus nigra* *Sambucus ebulus* *Tussilago farfara*	+	+
 Acetyleugenol	*Melissa officinalis*	+	-
 9-Octadecenoic acid (9Z)- ethyl ester	*Hippophae rhamn.*	+	+
 9,12-Octadecanoic acid (9Z,12Z)- methyl ester	*Bellis perennis* ***Betonica officinalis*** *Equisetum arvense* *Origanum vulgare* *Sambucus ebulus*	+	+
 9-Octadecenoic acid (9Z)	*Agropyron repens* *Hippophae rhamn.* *Prunella vulgaris* *Sambucus nigra* *Sambucus ebulus*	+	+
 Docosanoic acid	*Agropyron repens* *Capsella bursa-p.* *Prunella vulgaris* *Sambucus nigra*	+	+

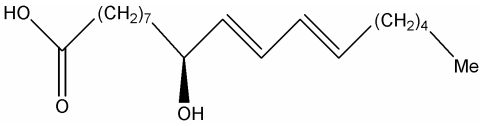
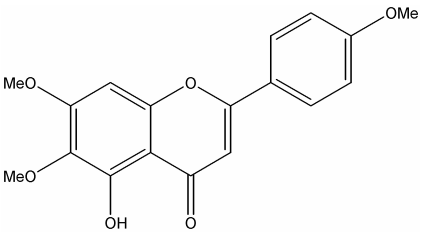
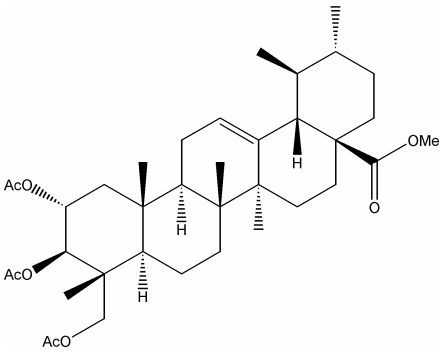
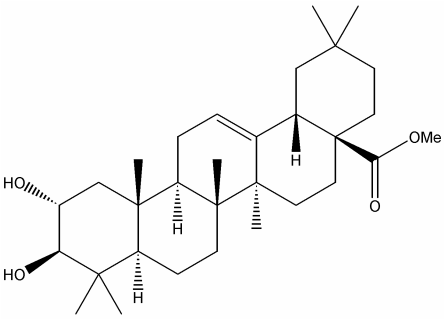
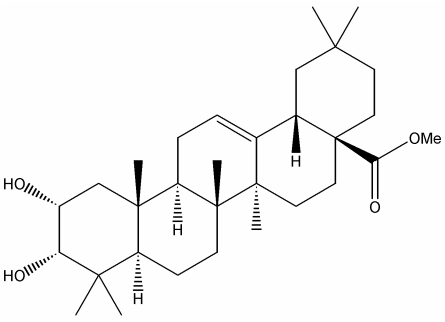
Structure	Plant species	PPAR-γ	FXR
 1-Octadecanol	***Betonica officinalis***	-	+
 Tetradecanoic acid ethyl ester	*Hippophae rhamn.*	+	+
 Hexadecanoic acid 1-methylethyl ester	*Bellis perennis* *Origanum vulgare*	+	+
 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl- (2E,7R,11R)	*Bellis perennis* *Equisetum arvense* ***Glechoma hed.*** *Majorana hortensis* *Origanum vulgare* *Picea abies* *Tussilago farfara*	-	+
 9,12,15-Octadecatrienoic acid methyl ester (9Z,12Z,15Z)	*Bellis perennis* *Origanum vulgare* *Tussilago farfara*	+	+
 9-Hexadecenoic acid (9Z)	*Bellis perennis* *Hippophae rhamn.* *Linum usitatissim.* *Sambucus ebulus*	+	+
 9,12,15-Octadecatrieonic acid (9Z,12Z,15Z)	*Agropyron repens* *Bellis perennis* *Equisetum arvense* *Hippophae rhamn.* *Linum usitatissim.* *Sambucus nigra* *Sambucus ebulus* *Symphytum off.* *Vaccinium vitis-id.*	+	-

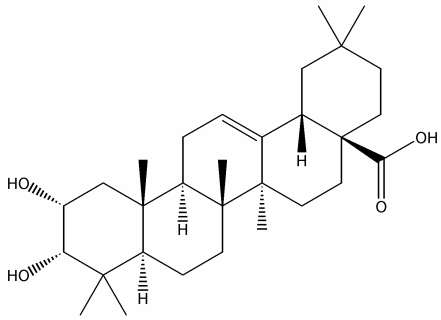
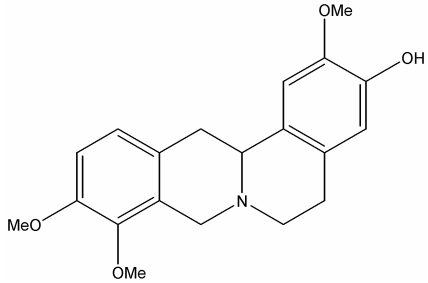
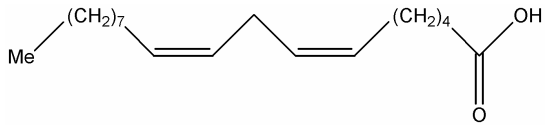
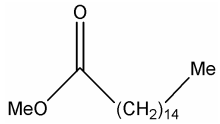
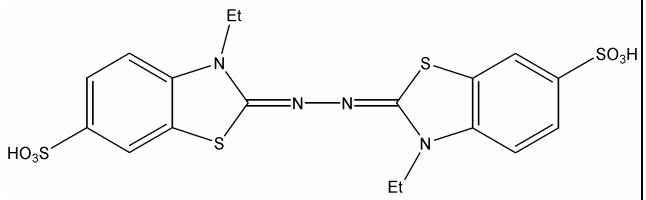
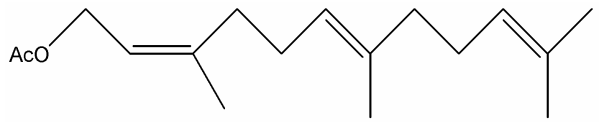
Structure	Plant species	PPAR-γ	FXR
 (+)-Berbamunine	*Berberis vulgaris*	+	-
 1-Hexadecen-3-ol, 3,7,11,15-tetramethyl	***Glechoma hed.***	-	+
 Heptadecanoic acid	*Bellis perennis* *Hippophae rhamn.*	+	+
 6,9,12-Octadecatrienoic acid (6Z,9Z,12Z)	*Hippophae rhamn.* *Symphytum off.*	+	+
 Eicosanoic acid	*Agropyron repens* *Capsella bursa-p.* *Hippophae rhamn.* *Prunella vulgaris* *Sambucus nigra*	+	-
 15-Tetracosenoic acid (15Z)	*Sambucus nigra*	+	-
 Hexacosanoic acid	*Lycopodium sp.* *Sambucus nigra*	+	-

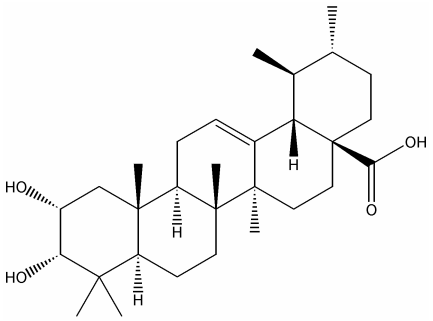
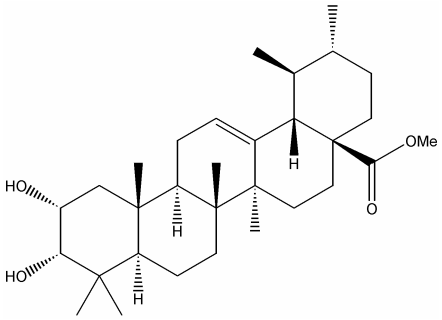
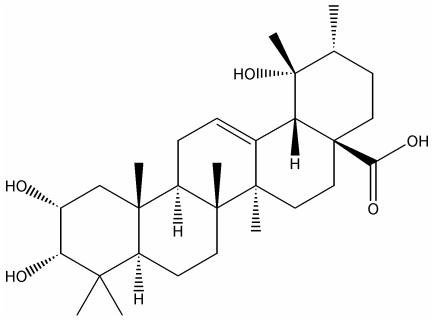
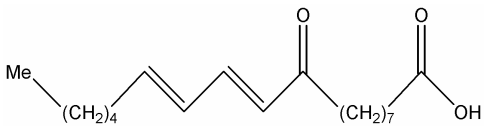
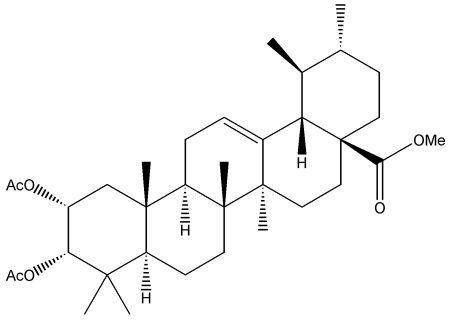
Structure	Plant species	PPAR-γ	FXR
 Tetradecanoic acid	*Agropyron repens* *Bellis perennis* *Calluna vulgaris* *Capsella bursa-p.* *Hippophae rhamn.* *Linum usitatissim.* *Sambucus ebulus* *Tussilago farfara*	+	-
 (+)-Erythrodiol	*Hippophae rhamn.* *Prunella vulgaris*	-	+
 Tetracosanoic acid	*Agropyron repens* *Sambucus nigra*	+	-
 Hexadecanoic acid ethyl ester	***Betonica officinalis*** *Hippophae rhamn.* *Prunella vulgaris* *Sambucus ebulus* *Vaccinium myrtillus*	+	+
 Tridecanoic acid	*Bellis perennis*	+	-
 1-Tetradecanol 1-acetate	*Lycopodium sp.*	+	+
 Sitosteryl acetate	*Equisetum arvense*	+	-

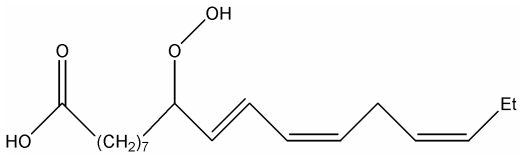
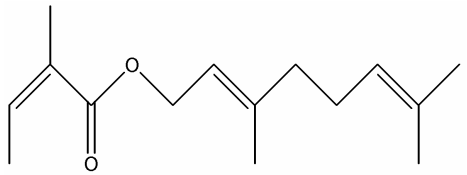
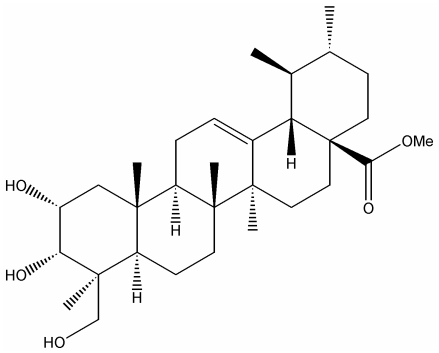
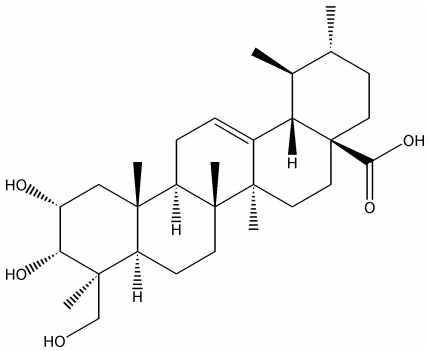
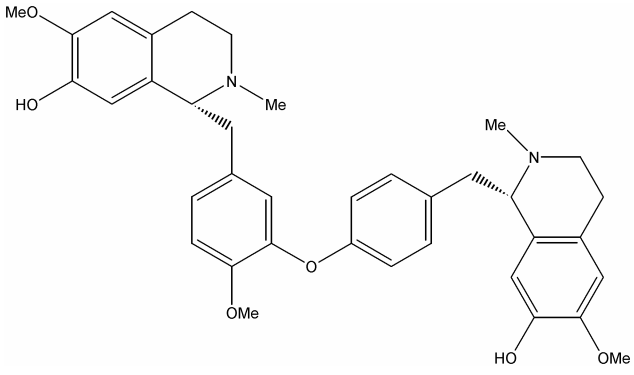
Structure	Plant species	PPAR-γ	FXR
 3-Epi-ursolic acid	***Glechoma hed.***	+	+
 Pentadecanoic acid	*Bellis perennis* *Sambucus ebulus* *Tussilago farfara*	+	+
 Octadecanoic acid 9,10-dihydroxy- methyl ester	*Sambucus nigra*	+	+
 Oleanolic acid methyl ester	*Prunella vulgaris*	-	+
 6,9-Octadecadienoic acid	*Prunella vulgaris*	+	+
 Ferulic acid methyl ester	*Lycopodium sp.*	+	-

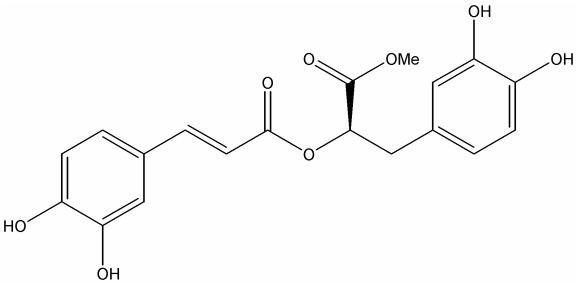
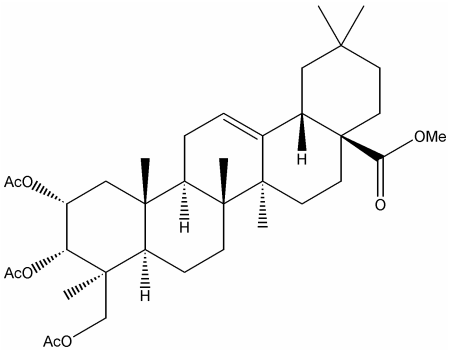
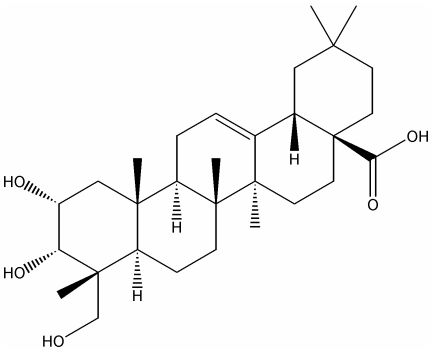
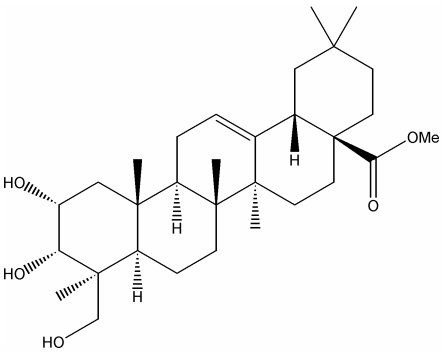
Structure	Plant species	PPAR-γ	FXR
 2-Heptadecanone	*Bellis perennis* *Sambucus ebulus*	+	+
 Acetic acid farnesyl ester	*Origanum vulgare*	+	-
 Corosolic acid methyl ester	*Prunella vulgaris* *Sorbus aucuparia*	-	+
 Corosolic acetate methyl ester	*Prunella vulgaris*	-	+
 Ursa-12,20(30)-dien-28-oic acid 2,3,23-trihydroxy-(2α,3α,4α)	*Prunella vulgaris*	+	-
 9-Hydroxy-10,12-octadecadienoic acid	***Glechoma hed.***	+	+

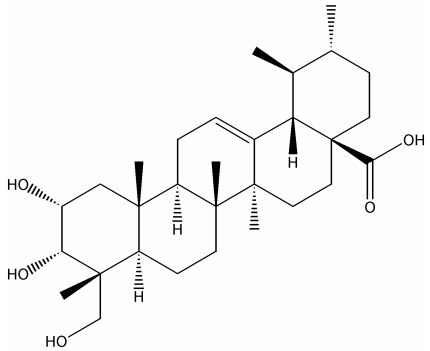
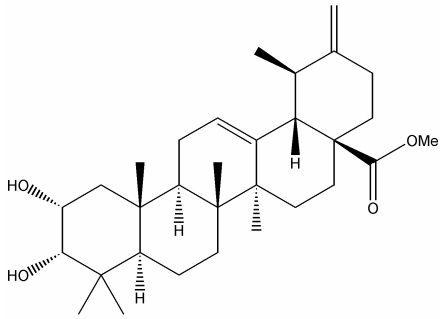
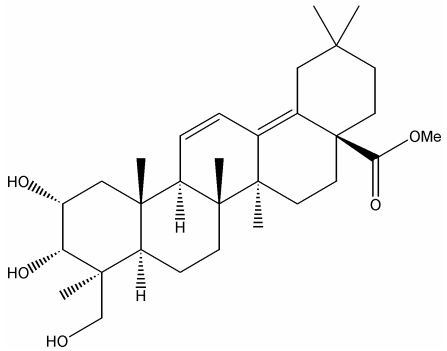
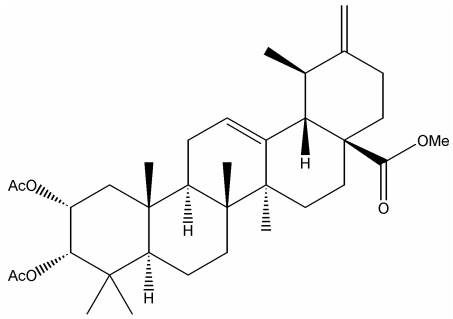
Structure	Plant species	PPAR-γ	FXR
 10,12-Octadecadienoic acid 9-hydroxy- (9S,10E,12E)	***Glechoma hed.***	+	+
 Salvigenin	*Salvia officinalis*	-	+
 Methyl 2α,3β,23-triacetoxyurs-12-en-28-oate	*Prunella vulgaris*	-	+
 Crategolic acid methyl ester	*Prunella vulgaris*	-	+
 Methyl 3-epimaslinate	*Prunella vulgaris*	+	+

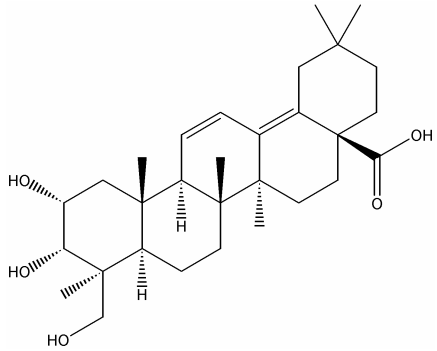
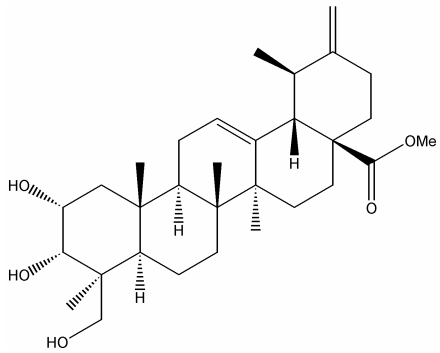
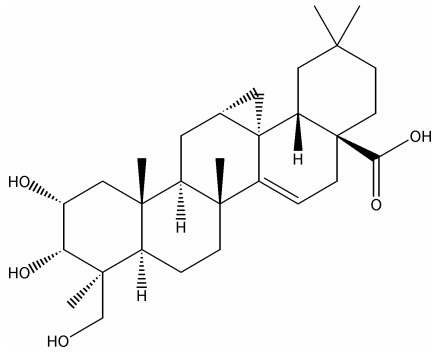
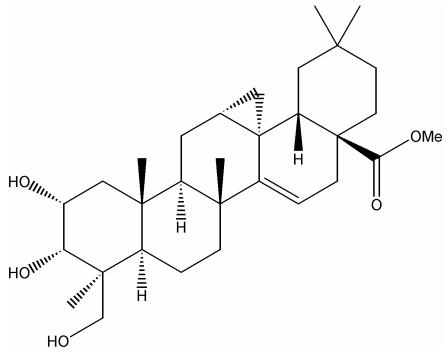
Structure	Plant species	PPAR-γ	FXR
 3-Epimaslinic acid	*Prunella vulgaris*	+	+
 (±)-Corypalmine	*Berberis vulgaris*	-	+
 (Z,Z)-6,9-Octadecadienoic acid	*Agropyron repens*	+	+
 Hexadecanoic acid, methyl ester	*Bellis perennis* ***Betonica officinalis*** *Equisetum arvense* *Hippophae rhamn.* *Origanum vulgare* *Tussilago farfara* *Vaccinium myrtillus*	+	+
 2,2'-(3-Ethyl-6-sulfobenzothiazolinone) azine	*Beta vulgaris* *Hippophae rhamn.* *Melissa officinalis*	-	+
 (2Z,6E)-Farnesyl acetate	*Origanum vulgare*	+	-

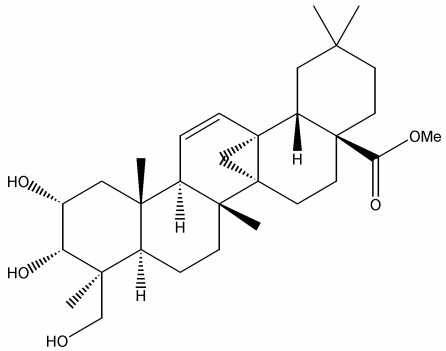
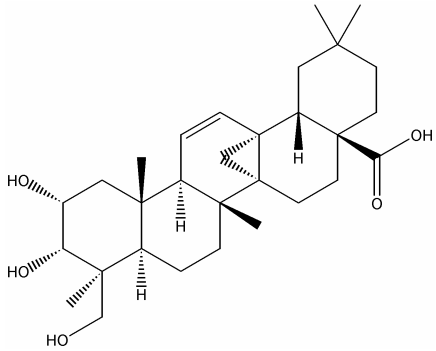
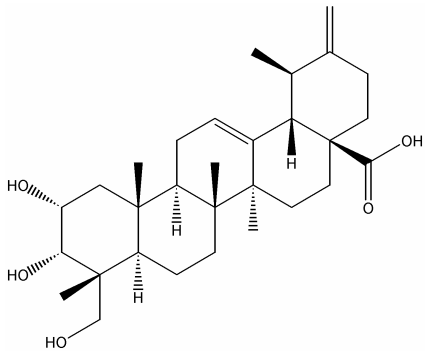
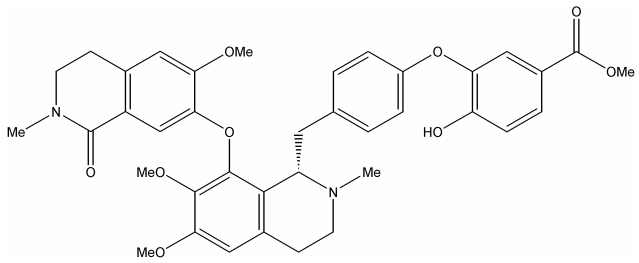
Structure	Plant species	PPAR-γ	FXR
 3-Epicorosolic acid	*Prunella vulgaris*	+	-
 3-Epicorosolic acid methyl ester	*Prunella vulgaris*	+	+
 Euscaphic acid	*Prunella vulgaris*	-	+
 9-Oxo-10,12-octadecadienoic acid	***Glechoma hed.***	+	+
 Methyl 2α,3α-diacetoxyurs-12-en-28-oate	*Prunella vulgaris*	-	+

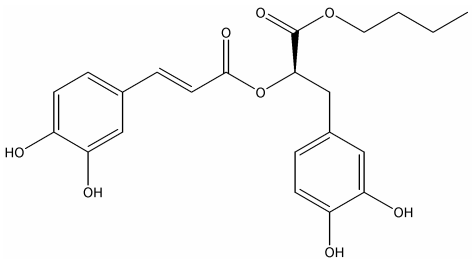
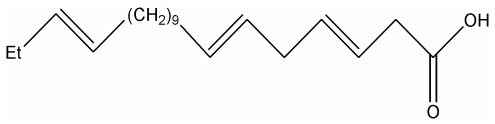
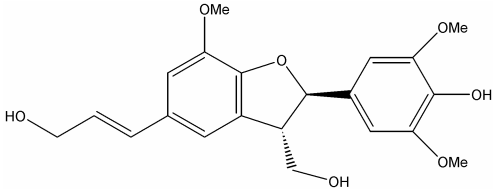
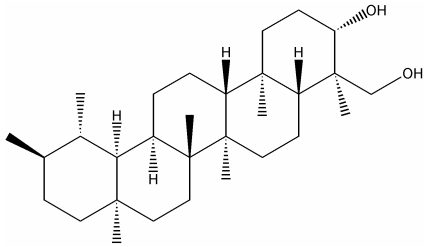
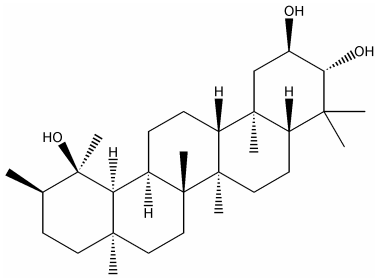
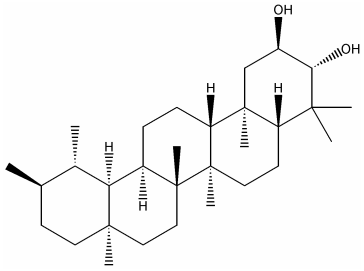
Structure	Plant species	PPAR-γ	FXR
 Linolenic acid 9-hydroperoxide	***Glechoma hed.***	+	+
 2-Butenoic acid 2-methyl-3,7-dimethyl-2,6-octadienyl ester (Z,E)	*Bellis perennis*	-	+
 Urs-12-en-28-oic acid 2,3,23-trihydroxy- methyl ester (2α,3α,4β)-(9CI)	*Prunella vulgaris*	+	-
 Urs-12-en-28-oic acid 2,3,23-trihydroxy (2α,3α,4β)	*Prunella vulgaris*	+	-
 (+)-Thaligrisine	*Berberis vulgaris*	-	+

Structure	Plant species	PPAR-γ	FXR
 Rosmarinic acid methyl ester	*Melissa officinalis* *Prunella vulgaris*	+	-
 Olean-12-en-28-oic acid 2,3,23-tris(acetyloxy)- methyl ester (2α,3α,4β)-(9CI)	*Prunella vulgaris*	-	+
 Isoarjunolic acid	*Prunella vulgaris*	+	-
 Olean-12-en-28-oic acid 2,3,23-trihydroxy- methyl ester (2α,3α,4β)-(9CI)	*Prunella vulgaris*	-	+

Structure	Plant species	PPAR-γ	FXR
 Esculentic acid	*Prunella vulgaris*	+	-
 Ursa-12,20(30)-dien-28-oic acid 2,3-dihydroxy- methyl ester (2α,3α)-(9CI)	*Prunella vulgaris*	+	+
 Oleana-11,13(18)-dien-28-oic acid 2,3,23-trihydroxy-methyl ester (2α,3α,4β)-(9CI)	*Prunella vulgaris*	+	-
 Ursa-12,20(30)-dien-28-oic acid 2,3-bis(acetyloxy)- methyl ester (2α,3α)-(9CI)	*Prunella vulgaris*	-	+

Structure	Plant species	PPAR-γ	FXR
 Oleana-11,13(18)-dien-28-oic acid 2,3,23-trihydroxy-(2α,3α,4β)-(9CI)	*Prunella vulgaris*	+	-
 Ursa-12,20(30)-dien-28-oic acid 2,3,23-trihydroxy- methyl ester (2α,3α,4β)-(9CI)	*Prunella vulgaris*	-	+
 (12R,13S)-2α,3α,24-Trihydroxy-12,13-cyclotaraxer-14-en-28-oic acid	*Prunella vulgaris*	+	-
 (12R,13S)-2α,3α,24-Trihydroxy-12,13-cyclotaraxer-14-en-28-oic acid methyl ester	*Prunella vulgaris*	+	-

Structure	Plant species	PPAR-γ	FXR
 13,27-Cycloolean-11-en-28-oic acid 2,3,23-trihydroxy-methyl ester (2α,3α,4β)-(9CI)	*Prunella vulgaris*	+	+
 13,27-Cycloolean-11-en-28-oic acid 2,3,23-trihydroxy-(2α,3α,4β)-(9CI)	*Prunella vulgaris*	+	-
 Ursa-12,20(30)-dien-28-oic acid 2,3,23-trihydroxy (2α,3α,4α)	*Prunella vulgaris*	+	-
 (-)-Tejedine	*Berberis vulgaris*	+	+

Structure	Plant species	PPAR-γ	FXR
 Butyl rosmarinate	*Prunella vulgaris*	+	-
 3,6,17-Eicosatrienoic acid	*Prunella vulgaris*	+	-
 (-)-Simulanol	*Berberis vulgaris*	+	-
 Ursane-3,23-diol (3β,4α)	*Prunella vulgaris*	+	-
 Ursane-2,3,19-triol (2α,3β)	*Prunella vulgaris*	+	-
 Ursane-2,3-diol (2α,3β)	*Prunella vulgaris*	+	-

3.5 Comparison of Methods for Removal of Bulk Constituents from Plant Extracts (Paper)

The following manuscript, entitled “Elimination of bulk polyphenols and chlorophyll from plant extracts influences their in vitro anti-inflamatory activity – the method matters”, concerns the chromatographic and pharmacological comparison of different extract purification methods. The author of this thesis, together with Mag. Sylvia Vogl, was responsible for the phytochemical work indicated with the codes CR1 and PR1, as well as for the chromatographic analyses. The manuscript has been submitted for publication.

Elimination of bulk polyphenols and chlorophyll from plant extracts influences their *in vitro* anti-inflammatory activity – the method matters

P. Picker[a†], S. Vogl[a†], J. Mihaly-Bison[b], M. Binder [b], N. Fakhrudin[a‡], A.G. Atanasov[a], A.M. Grzywacz[a], E.H. Heiss[a], M. Zehl[a], J. Saukel[a], C. Wawrosch[a], A. Schinkovitz[c], R. Bauer[c], J.M. Rollinger[d], H. Stuppner[d], V.M. Dirsch[a], V. Bochkov[b], G. Reznicek[a]*, B. Kopp[a].

[a]Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

[b]Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Schwarzspanierstrasse 17, A-1090 Vienna, Austria

[c]Institute of Pharmaceutical Sciences, Pharmacognosy, University of Graz, Universitätsplatz 4, A-8010 Graz, Austria

[d]Institute of Pharmacy/Pharmacognosy, University of Innsbruck, Innrain 52c, A-6020 Innsbruck, Austria

‡ Permanent address: Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, 55281, Yogyakarta, Indonesia

† These authors contributed equally to this work

* To whom correspondence should be addressed:

Prof. Dr. Gottfried Reznicek

Tel: +43-1-4277-55210

Fax: +43-1-4277-9552

E-mail: gottfried.reznicek@univie.ac.at

Abstract

Ubiquitous plant constituents, such as chlorophyll and phenolic compounds, can interfere with biological *in vitro* assays. Moreover, these substances represent a significant portion of plant extracts, decreasing the relative amount of other bioactive components. The aim of this study was to examine (i) whether chlorophylls and phenolic compounds from plant extracts may lead to false positive or false negative results in three selected cell-based anti-inflammatory test systems, (ii) whether their elimination markedly alters the bioactivity of extracts, and (iii) whether there are differences between distinct clearance procedures. Three commonly used methods to eliminate chlorophyll and phenolic compounds were applied; all of them efficiently removed bulk constituents as confirmed by HPLC and mass spectrometry. Plant extracts were evaluated before and after removal of chlorophyll and polyphenols for their potential to inhibit the activation of the transcription factor NF-κB and the expression of interleukin-8 (IL-8), induced by the pro-inflammatory stimuli tumor necrosis factor (TNF-α) or lipopolysaccharide (LPS) in human umbilical vein endothelial cells. Chlorophyll A and B, tannic acid, epicatechin gallate and rosmarinic acid (the latter three as representatives of common polyphenols) were also tested in these assays. Obtained results show that depending on the methods used, the activity of extracts can be strongly increased by purification, but also decreased due to loss of bioactive constituents. As none of the pure compounds showed an influence on the selected biological systems, except for a weak activity of epicatechin gallate on IL-8, the increase in activity observed after purification of extracts in some cases is likely due to an enrichment of the active compounds.

In summary, bulk compounds removal processes represent a valuable strategy for the biological evaluation of plant extracts, but their effectiveness has to be validated and a possible loss of active substances has to be considered.

1. Introduction

Plant-derived natural products represent an important source of new compounds effective against various diseases, including disorders associated with inflammation (Butler, 2008; Cragg and Newman, 2006; Foster et al., 2005; Harvey, 2007; Newman, 2008; Newman and Cragg, 2007). To identify new potential bioactive compounds, biological *in vitro* assays are used (Swinney and Anthony, 2011). When testing plant extracts in biological *in vitro* assays, a potential interference of ubiquitous substances, such as chlorophyll and polyphenols, must be considered since they may generate false positive or false negative results. Chlorophyll is known to interact with fatty acids, whereas tannins can form tight complexes with metal ions, proteins and polysaccharides (Potterat and Hamburger, 2006). Relative to tannins, most non-polyphenolic compounds appear in much lower amounts in polar plant extracts (Silva et al., 1998), while chlorophyll typically represents more than a half of crude nonpolar extracts. We therefore hypothesized that the elimination of bulk constituents, such as polyphenols and chlorophyll, may result in a higher specific bioactivity of plant extracts, which consequently should be purified prior to pharmacological evaluation *in vitro*.

Three common methods for the removal of chlorophyll and polyphenols, respectively, were applied in this study on extracts of two Austrian medicinal plants, *Folium Malvae* ÖAB (Malvaceae) and the aerial parts of *Glechoma hederacea* L. (Lamiaceae), respectively. The plant extracts were evaluated for their ability to inhibit TNF-α-induced NF-κB transactivation activity and the TNF-α- and LPS-induced interleukin-8 expression. Since the interference of chlorophyll and polyphenols with these specific assays was unclear, we tested effects of pure chlorophyll A, chlorophyll B, tannic acid, epicatechin gallate and also rosmarinic acid, which is known to be present in *Glechoma hederacea* (Okuda et al., 1986).

2. Materials and Methods

2.1. Plant material

The dried leaves of *Malva ssp. (Folium Malvae* according to the Austrian Pharmacopoeia, ÖAB) and the dried aerial parts of *Glechoma hederacea* (each 2 kg) were obtained from Kottas Pharma GmbH (Vienna, Austria), authenticated by Prof. Johannes Saukel and finely grinded before extraction. Voucher specimens (Mal-le-08_1 and Gle-hb-08_1, respectively) are deposited at the Department of Pharmacognosy, University of Vienna, Austria.

2.2. Reagents and Chemicals

Methanol, dichloromethane and acetonitrile were HPLC-grade (VWR, Vienna, Austria). Formic acid was purchased from Carl Roth (Karlsruhe, Germany). Chlorophyll A and B (both of 95% purity) and tannic acid were purchased from Fluka Chemical Corp. (Ronkonkoma, NY, USA). Epicatechin gallate (98% purity) was purchased from Sigma Aldrich (Steinheim, Germany), while rosmarinic acid (98% purity) was obtained from Extrasynthese (Genay, France).

2.3. Extraction

The extraction was performed using two different methods. Method A: 3.0 g grinded material of the two model plants were extracted first with DCM and after drying with MeOH, using an accelerated solvent extractor ASE200 (Dionex Corp., Sunnyvale, CA, USA) equipped with 22 mL stainless steel extraction cells and 60 mL glass collection bottles. The extraction conditions were the following: 3 extraction cycles, 5 min heat-up time, 2 min static time, 10% flush volume, 60 sec nitrogen purge, 40 °C oven temperature and 150 bar pressure.

Method B: 3.0 g plant material was extracted three times with 30 mL of DCM, respectively, in an ultrasonic bath for 15 min at room temperature; the solvent was evaporated under reduced pressure. The remaining plant material was extracted three times with MeOH using the same procedure.

Subsequently, DCM extracts from *Malva* were used for the chlorophyll elimination comparison, while MeOH extracts from *Glechoma hederacea* were subjected to the different polyphenols elimination procedures. The extraction yields are listed in Table 1.

2.4. Chlorophyll elimination

The first of three chlorophyll elimination methods (CR1), applied on the DCM extracts of *Malva*, was based on a liquid-liquid partition between DCM and a mixture of MeOH/H_2O. The extract (extraction method A) was dissolved in a defined volume of DCM (6.67 mg/mL) and the same amount of MeOH/H_2O 1:1 was added. Dichloromethane was removed under reduced pressure and chlorophyll, which precipitated in methanol/water, was filtered.

For the second method (CR2), preparative TLC plates (20x20 cm, silica gel 60, Merck) were pre-developed in DCM until the solvent front reached the end of the plate, in order to remove impurities. Next, the extract (extraction method A) was loaded onto the plate respecting a distance of 1 cm from the edges. Then the plate was developed in DCM until the solvent front progressed 16 cm from the starting line. Subsequently the plate was air dried and developed a second time utilizing a solvent mixture of 80% DCM and 20% EtOAc. The process was stopped once the solvent front had reached the upper border of the chlorophyll band minimizing the size of the latter. The entire silica gel, except areas containing chlorophyll and previously removed impurities (17-20 cm), was scratched from the plate and

sonicated in DCM and consecutively in MeOH. The obtained solution was finally filtered and dried under reduced pressure.

For the third method (CR3), the extract (extraction method B) was fractionated using column chromatography (Lichroprep RP18, 3.0 x 25 cm), and eluted with mixtures of MeOH and MeCN (300 + 0 mL, 150 + 50 mL, 50 + 50 mL, 25 + 75 mL, 0 + 150 mL) yielding two fractions: the MeOH eluate F1 (39.8 mg) and F2 (75.0 mg) by means of visual assessment and TLC monitoring (DCM - MeOH, 19:1; detection UV-VIS, vanillin-H_2SO_4). According to TLC, F1 was free from chlorophyll in contrast to the eluate F2.

2.5. Elimination of polyphenols

Three methods to eliminate polyphenols were applied on the MeOH extracts of *Glechoma hederacea*. Method PR1 was based on liquid-liquid partitions between $CHCl_3$ and mixtures of MeOH/H_2O (Wall et al., 1996). The extract (extraction method A) was first dissolved in MeOH/H_2O (9:1) and defatted by partition with hexane. H_2O was added to the aqueous phase generating a solution MeOH/H_2O 3:1, which was partitioned with $CHCl_3$. The organic phase was finally washed with 1% NaCl, yielding the phenol-free extract.

Method PR2: 5.0 g of Polyamide SC 6.6 (Macherey and Nagel, Duren, Germany) were mixed with distilled water. The suspension was incubated for 24 h before being transferred into a column. The column was rinsed with 150 mL MeOH and afterwards loaded with 92.4 mg of dissolved extract (extraction method A). Then the column was eluted with 250 mL MeOH and the obtained solution was finally dried under reduced pressure (Houghton and Raman, 1998).

Method PR3: The extract (extraction method B) was subjected to column chromatography (Polyamide 6S, Riedel DeHaen AG, Seelze, Germany) using a step gradient of H_2O and MeOH (60 + 0 mL, 30 + 30 mL, 15 + 45 mL, 10 + 50 mL, 0 +

350 mL) producing two fractions: circa 250 mL of eluate F1 (79.3 mg) and F2 (4.5 mg) by means of visual assessment and TLC monitoring (DCM – MeOH, 9:1; detection UV-VIS, vanillin-H_2SO_4). $FeCl_3$ solution (Ph.Eur.) was added to a sample of each fraction to assess the presence of phenolic compounds. Fraction F1 was considered free of phenolic compounds in the absence of a positive reaction.

2.6. High performance liquid chromatography (HPLC)

A Shimadzu (Kyoto, Japan) HPLC system consisting of a system controller (CBM-20A), a membrane degasser (DGU-20A5), a solvent delivery unit (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC), a photodiode array detector (SPD-M20A) and a low temperature light scattering detector (ELSD-LT, 40 °C) was used for the measurements. Chromatographic analyses of original and purified extracts were carried out on an Atlantis T3 (Waters, Milford, MA, USA) analytical column (150 x 3 mm, 3 µm) with an oven temperature of 25 °C. The flow rate was 0.7 mL/min and 20 µL sample were injected from a cooled (15 °C) autosampler tray. A gradient elution of H_2O (A, adjusted to pH 2.8 with formic acid) and MeCN (B) was used for *Malva* extracts (25-55% of B in 60 min, 55-85% of B in 40 min, v/v), while different conditions (2-32% of B in 75 min, v/v) were applied for *G. hederacea* extracts.

2.7. High performance liquid chromatography – mass spectrometry (HPLC-MS)

In order to identify the main constituents of the extracts and to prove the successful removal of the target substances, HPLC-MS analyses were performed using the same conditions as described above. The measurements were performed using an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal electrospray

ionization (ESI) source (HCT, Bruker Daltonics, Bremen, Germany). The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage: 4.0 or 3.7 kV, nebulizer: 30 psi (N_2), dry gas flow: 8 L/min (N_2), dry temperature: 340 °C or 350 °C. Positive and negative ion mode multistage mass spectra (at least MS^3) were obtained in automated data-dependent acquisition (DDA) mode. Helium was used as collision gas, the isolation window was 4 Th and the fragmentation amplitude was set to 1.0 V.

2.8. NF-κB transactivation activity

Human embryonic kidney 293 (HEK-293) cells stably transfected with an NF-κB-driven luciferase reporter gene (293/NFκB-luc cells, Panomics, RC0014) were seeded in 10 cm dishes and transfected with 5 µg pEGFP-C1 (Clontech). Six hours later cells were transferred to 96 well plates and incubated at 5% CO_2 and 37 °C overnight. On the next day the medium was exchanged with a serum-free DMEM and the indicated treatments were performed. After 30 min, cells were stimulated with 2 ng/mL recombinant human TNF-α for 4 h, then the medium was removed and the cells were lysed. Plant extracts as well as reference pure compounds were tested at the concentration of 10 µg/mL in at least three independent experiments with four replicates each, while parthenolide was used as positive control at the concentration of 5 µM. The luminescence of the firefly luciferase and the fluorescence of EGFP were quantified on a GeniosPro plate reader (Tecan). The luciferase signal derived from the NF-κB reporter was normalized by the EGFP derived fluorescence to account for differences in the cell number or transfection efficiency.

2.9. Interleukin-8 ELISA

TERT technology (hTERT) immortalized human umbilical vein endothelial cells (HUVECtert) (Chang et.al, 2005) were cultured in M199 medium supplemented with 20% fetal calf serum (both from Sigma-Aldrich, St Louis, MO), cell growth supplement (Promocell, Germany) and antibiotics. At least three independent experiments with six replicates each were performed in 96 well plates (Iwaki, Japan) in M199 medium containing 1% BSA (Applichem, Darmstadt, Germany) and 3% serum. Plant extracts were tested at the concentration of 10 µg/mL, reference pure compounds at 10 µM, while BAY 11-7082 was used as positive control at the concentration of 5 µM. Subconfluent HUVECtert cells were pre-treated for 30 min with the plant material or inhibitor as indicated, followed by stimulation with 100 ng/mL of TNF-α (PeproTech, Rocky Hill, NJ) or 300 ng/mL of LPS (Sigma-Aldrich, St. Louis, MO) for 6 h. Secreted IL-8 was determined by ELISA from the cell culture supernatants using the Quantikine® Human CXCL8/IL-8 Immunoassay Kit (R&D Systems, Minneapolis, MN). Supernatants were transferred into 96 well plates (NALGE-NUNC Int., Rochester, NY) coated with capturing antibody for IL-8 and developed with the respective detection antibody. Peroxidase activity was assessed with TMB 2-Component Microwell Peroxidase Substrate Kit (KPL, Gaithersburg, MD), while the optical density (OD) was measured with a SynergyHT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) at 450 nm using 620 nm wavelength as reference.

2.10. Statistical analysis

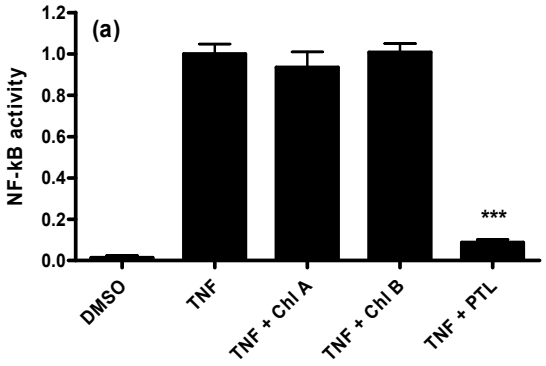
The experimental data are presented as means ± standard error of the mean (SEM) from three independent experiments. Statistical significance was determined by ANOVA using Bonferroni post hoc test. P values < 0.05 were considered significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

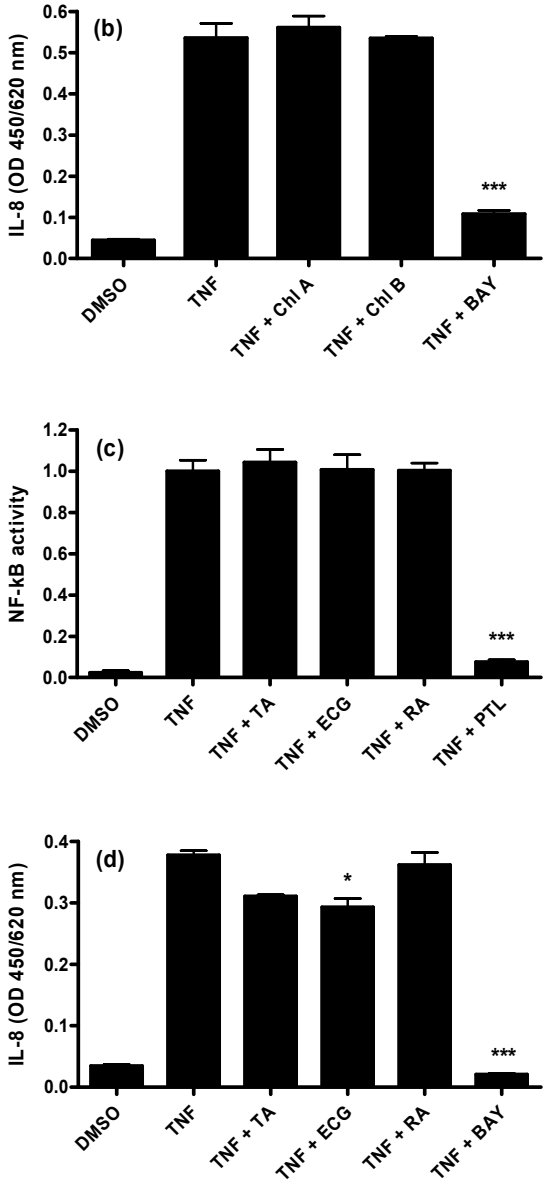
3. Results and Discussion

First, effective and fast methods for the elimination of polyphenols and chlorophyll from plant extracts were selected. To remove polyphenols, chromatography on polyamide using two different elution methods was chosen. Precipitation with gelatin/NaCl, polyvinylpyrrolidone (PVP) or caffeine was found to be inadequate, since these procedures unspecifically clear compounds with phenolic groups including flavonoids, whose removal is not desired. As a third method, we used liquid-liquid partition between $CHCl_3$ and mixtures of MeOH/H_2O published by Silva et al. (1998) and Wall et al. (1996), which is fast and easy to perform. To eliminate chlorophyll, we selected solvent partitioning between DCM and MeOH/H_2O (Silva et al., 1998), column chromatography (RP18), as well as preparative TLC (Sherma and Fried, 2004; Silva et al., 1998).

Fig.1 shows that neither chlorophyll A and B nor the polyphenols tannic acid, epicatechin gallate and rosmarinic acid influence the TNF-α-induced NF-κB activation at the concentration of 10 µg/mL (1a,1c), while only epicatechin gallate weakly inhibits the TNF-α-induced IL-8 expression at 10 µM (1d). Furthermore, they did not activate NF-κB or induce IL-8 expression, when applied to unstimulated cells (data not shown).

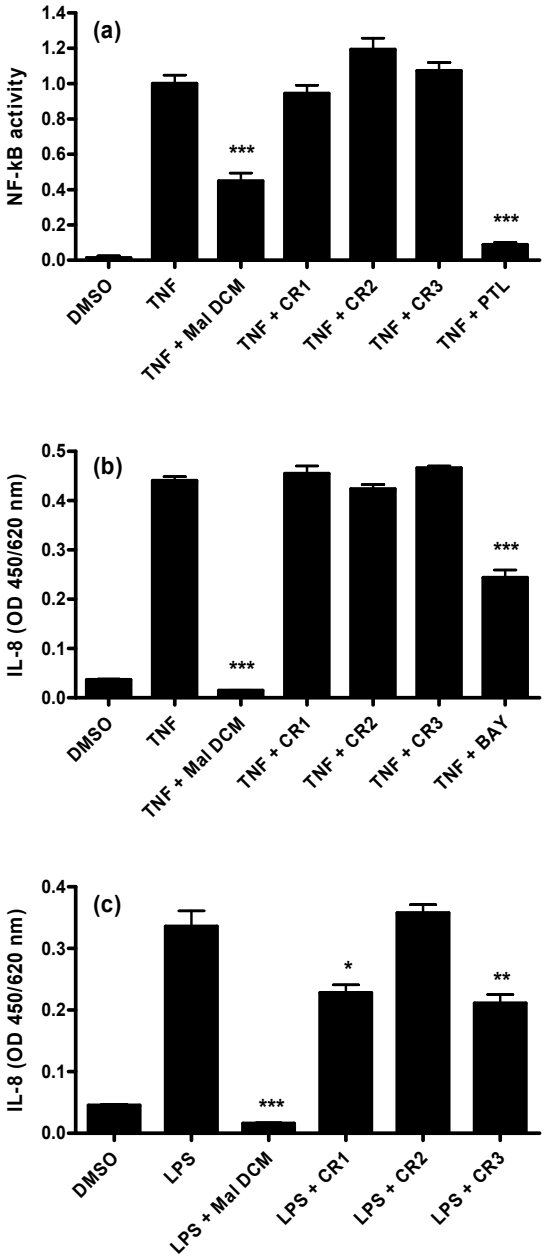
Figure 1: Chlorophyll A (Chl A) and B (Chl B), tannic acid (TA), epicatechin gallate (ECG) and rosmarinic acid (RA) do not inhibit NF-κB activation at 10 µg/mL (a,c) and do not interfere, except weakly ECG, with IL-8 expression at 10 µM (b,d) in TNF-α-stimulated cells. Positive control (5 µM): parthenolide (PTL) or BAY 11-7082 (BAY). Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF: * P<0.05, ** P<0.01, *** P<0.001.





Chlorophyll-depleted extracts, tested at 10 µg/mL in TNF-α- or LPS-stimulated cells, influenced IL-8 expression or NF-κB activity differently, depending on the used depletion method. The crude DCM extract from *Malva* significantly inhibited NF-κB activation and IL-8 expression in TNF-α- and LPS-stimulated cells. All chlorophyll clearance methods resulted in a clear loss of activity (Fig. 2).

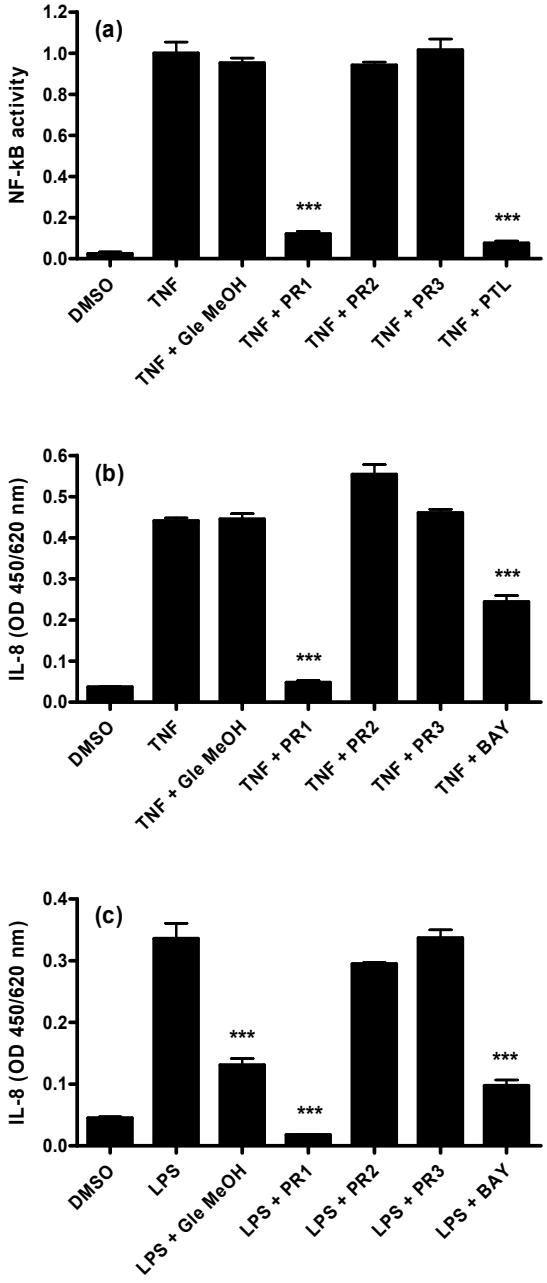
Figure 2: Effects of *Malva* DCM extracts purified from chlorophyll using methods CR1, CR2 and CR3 on NF-κB activity in TNF-α-stimulated cells (a) and IL-8 expression in TNF-α- (b) or LPS- (c) stimulated cells at 10 µg/mL. Positive control (5 µM): parthenolide (PTL) or BAY 11-7082 (BAY). Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF or LPS: * P<0.05, ** P<0.01, *** P<0.001.



Using different methods to clear extracts from polyphenols, the resulting differences are even more pronounced. Method PR1 led to disclosure of a strong NF-κB and IL-8 inhibitory activity of the *Glechoma hederacea* methanol extract (Figs. 3a, 3b and 3c). In contrast, methods PR2 and PR3 resulted in extracts that showed the same

inactivity as the crude methanol extract (Figs. 3a and 3b) or led to inactive extracts whereas the crude methanol extract showed inhibitory activity at 10 µg/mL (Fig. 3c).

Figure 3: Effects of *Glechoma hederacea* MeOH extracts purified from polyphenols using methods PR1, PR2 and PR3 on NF-κB activity in TNF-α-stimulated cells (a) and IL-8 expression in TNF-α- (b) and LPS- (c) stimulated cells at 10 µg/mL. Positive control (5 µM): parthenolide (PTL) or BAY 11-7082 (BAY). Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF or LPS: * P<0.05, ** P<0.01, *** P<0.001.



To explain these differences, the chlorophyll- and polyphenol-depleted extracts were analytically evaluated. The yields of the different purification procedures (w/w,

relative to the crude extracts), as well as those of the extractions (w/w, relative to the drug weight), are listed in Table 1.

Table 1: Extraction, chlorophyll and polyphenols removal yields (w/w)

Malva		***Glechoma hederacea***	
DCM extract (yield)	Chlorophyll removal (yield)	MeOH extract (yield)	Polyphenols removal (yield)
A (4.0%)	CR1 (11.0%)	A (8.5%)	PR1 (8.0%)
	CR2 (40.0%)		PR2 (72.0%)
B (3.8%)	CR3 (34.6%)	B (5.4%)	PR3 (48.6%)

HPLC analyses of the original and purified extracts were performed to verify the successful depletion of the target substances, to assess the enrichment of the other constituents as well as the unintended loss of non-target compounds. Comparison with pure reference compounds as well as LC-MS analyses permitted the identification of the substances of interest, demonstrating that chlorophyll and polyphenols were effectively removed by all three applied procedures.

Chlorophyll, identified as peak 4 (Rt: 97 min) in the DCM extract of *Malva* (Fig. 4), was quantitatively removed by all three methods, and this purification process resulted in the enrichment of other more polar constituents (methods CR1 and CR3). However, the ELSD detection (Fig. 5) demonstrates that peak 1 (Rt: 83.5 min), identified as linolenic acid and found to be the main component of the extract, was also removed, as was peak 2 (Rt: 90.5 min), identified as linoleic acid. In contrast, peak 3 (Rt: 96.5 min), which was found to be oleic acid, was saved and enriched only with method CR3. This last compound could not be observed in the HPLC-UV chromatogram due to the lack of chromophores.

Figure 4: HPLC-UV (254 nm) chromatogram of *Malva* DCM extracts (CR1, CR2, CR3: chlorophyll removal methods 1, 2, 3. DCM: crude dichloromethane extract).

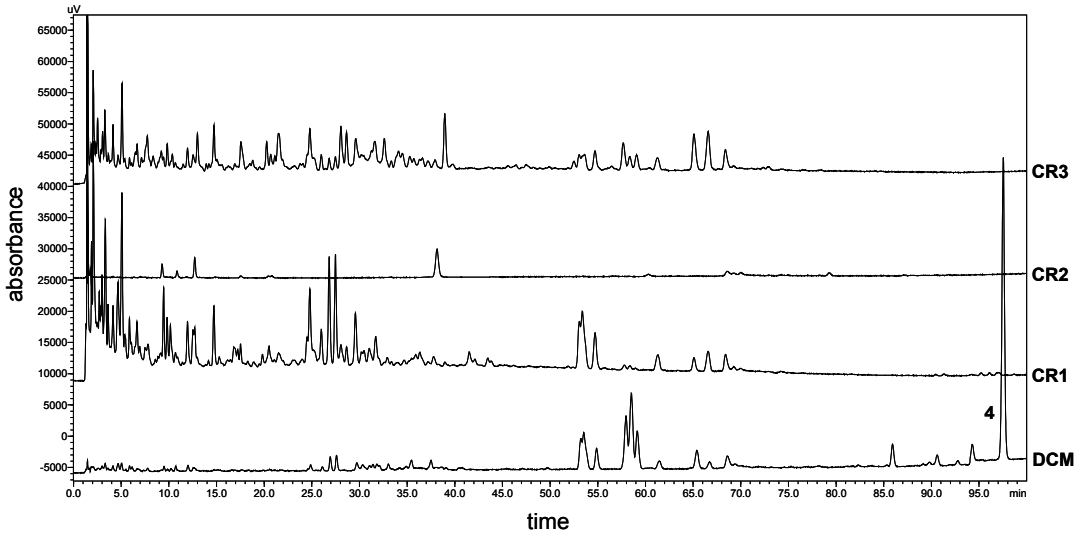
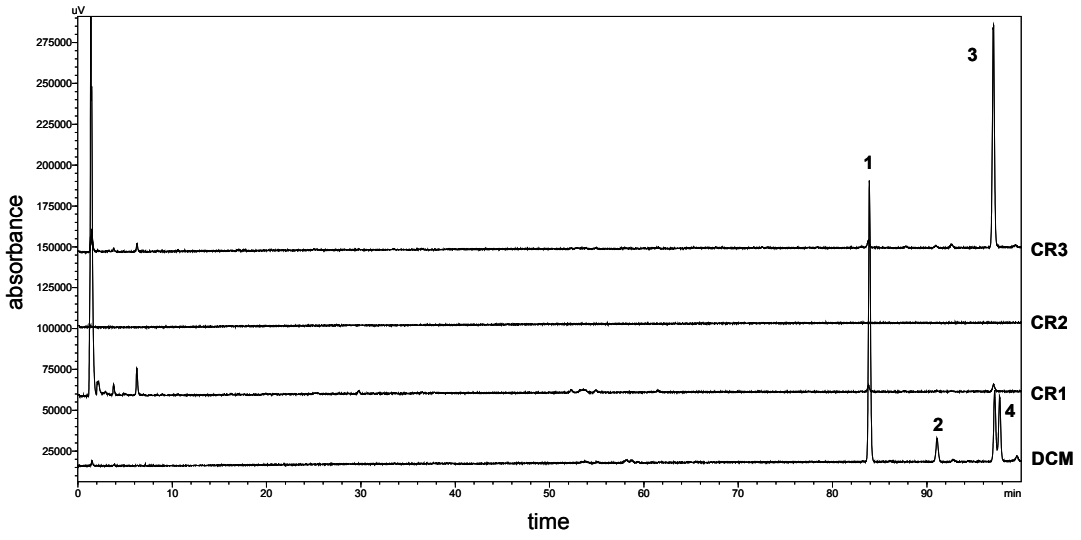
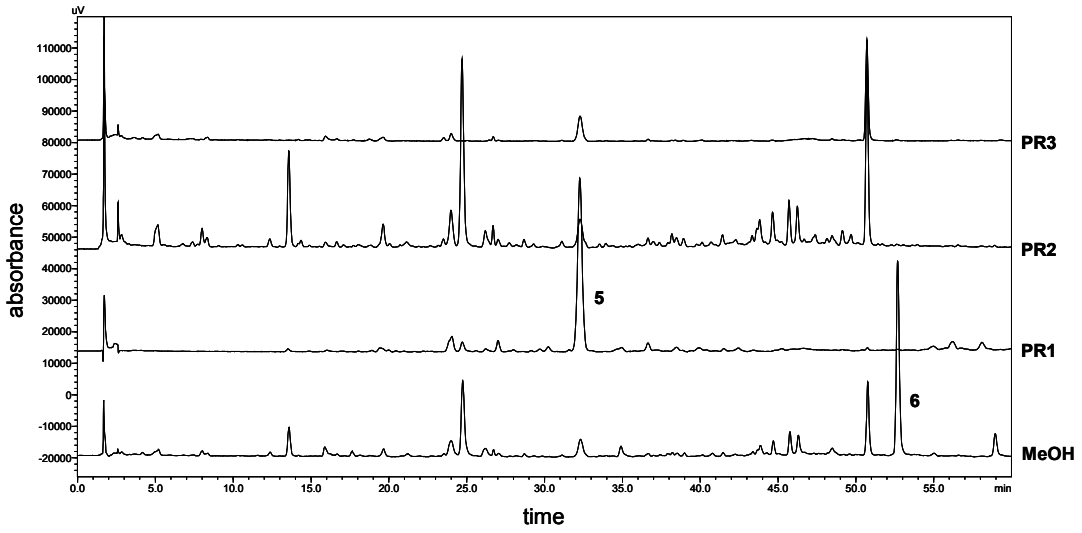


Figure 5: HPLC-ELSD chromatogram of *Malva* DCM extracts (CR1, CR2, CR3: chlorophyll removal methods 1, 2, 3. DCM: crude dichloromethane extract).



HPLC analyses of *Glechoma hederacea* extracts (Fig. 6) showed the successful clearance of rosmarinic acid, which was identified as peak 6 (Rt: 52.5 min), by all methods. Furthermore, method PR1 resulted in a strong enrichment of peak 5 (Rt: 31.8 min). This compound, identified by LC-MS as the cyclic hydroxamic acid 2-benzoxazolinone, can be supposed to be responsible for the activity of the relative phenol-free extract.

Figure 6: HPLC-UV (254 nm) chromatogram of *Glechoma hederacea* MeOH extracts (PR1, PR2, PR3: polyphenols removal methods 1, 2, 3. MeOH: crude methanol extract).



The data suggest that the method selected to remove bulk compounds has a dramatic influence on the *in vitro* anti-inflammatory activity of plant extracts. It can either enhance the specific activity due to enrichment of active components, or result in loss of general anti-inflammatory activity in cell-based *in vitro* assays due to unspecific co-depletion of active constituents.

Neither chlorophyll A and B nor the tested polyphenols, aside from a weak IL-8 inhibition by epicatechin gallate, interfered with the used cell-based anti-inflammatory assays. Nevertheless, high amounts of such bulk components in crude extracts can mask or dilute the effect of active compounds. On the other hand, depletions are not specific and may lead to the loss of bioactive constituents.

In conclusion, our data clearly show that the selection of a proper method to remove bulk constituents can be a valuable strategy to specifically enrich plant compounds influencing inflammatory parameters *in vitro*. However, a HPLC profiling before and after the clearance procedure is recommended, in order to ensure an effective elimination of the respective bulk compounds and to detect a possible loss of active constituents.

Acknowledgement

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References

Butler, M.S., 2008. Natural products to drugs: natural product-derived compounds in clinical trials. Nat. Prod. Rep. 25, 475-516.

Chang, M.W.-F., Grillari, J., Mayrhofer, C., Fortschegger, K., Allmaier, G., Marzban, G., Katinger, H., Voglauer, R., 2005. Comparison of early passage, senescent and hTERT immortalized endothelial cells. Exp. Cell Res. 309, 121-136.

Cragg, G.M., Newman, D.J., 2006. Natural product sources of drugs: plants, microbes, marine organisms, and animals. Compr. Med. Chem. II 1, 355-403.

Foster, B.C., Arnason, J.T., Briggs, C.J., 2005. Natural health products and drug disposition. Annu. Rev. Pharmacol. Toxicol. 45, 203-226.

Harvey, A.L., 2007. Natural products as a screening resource. Curr. Opin. Chem. Biol. 11, 480-484.

Newman, D.J., 2008. Natural Products as Leads to Potential Drugs: An Old Process or the New Hope for Drug Discovery? J. Med. Chem. 51, 2589-2599.

Newman, D.J., Cragg, G.M., 2007. Natural Products as Sources of New Drugs over the Last 25 Years. J. Nat. Prod. 70, 461-477.

Okuda, T., Hatano, T., Agata, I., Nishibe, S., 1986. The components of tannic activities in Labiatae plants. I. Rosmarinic acid from Labiatae plants in Japan. Yakuga. Zasshi. 106, 1108-1111.

Potterat, O., Hamburger, M., 2006. Natural products in drug discovery - concepts and approaches for tracking bioactivity. Curr. Org. Chem. 10, 899-920.

Sherma, J., Fried, B., 2004. Separation and determination of chloroplast pigments from spinach by thin-layer chromatography: A student laboratory experiment. J. Planar Chromatogr.-- Mod. TLC 17, 309.

Silva, G.L., Lee, I.-S., Kinghorn, A.D., 1998. Special problems with the extraction of plants. Methods Biotechnol. 4, 343-363.

Swinney, D.C., Anthony, J., 2011. How were new medicines discovered? Nat. Rev. Drug Discov. 10(7), 507-519.

Wall, M.E., Wani, M.C., Brown, D.M., Fullas, F., Olwald, J.B., Josephson, F.F., Thornton, N.M., Pezzuto, J.M., Beecher, C.W.W., et al., 1996. Effect of tannins on

screening of plant extracts for enzyme inhibitory activity and techniques for their removal. Phytomedicine. 3, 281-285.

4 Discussion

Folk medicine has always played an important role in the finding of new pharmaceutical strategies. As traditional knowledges often represented the starting point of the medical research, numerous natural products or structures derived threof are nowadays employed in the therapy of various diseases. Therefore, the scientific proof of claimed beneficial properties of plant extracts and moreover the identification of their active principles represent an important challenge.

The topic of this thesis was the investigation of Austrian medicinal plants traditionally used against inflammatory disorders, with the aim of confirming their beneficial properties on a scientific basis, revealing their active principles as well. For this purpose, a number of drugs were selected to be investigated, exclusively on the basis of their traditional anti-inflammatory applications. Besides the selection of the candidates, an important factor was the choice of an appropriate fractionation strategy, in the path from field-collected or purchased plant materials to the relative pure active compounds.

Different factors (e.g. synergism, potentiation, concentration), however, can differentiate the biological activity of crude extracts and pure compounds isolated thereof.

In several cases, we could observe stronger anti-inflammatory activities in extracts or fractions than in the main substances composing them, which were tested at different concentrations. Fraction B2 from *Betonica officinalis*, for instance, was able to strongly downregulate the IL-8 expression at the protein level. As all major components of this fractions were isolated and tested, two of them (the clerodane diterpenes B2A and B2B) showed a significant activity in the same assay, however much lower than that of the original fraction. This indicates that minor undetectable components or possibly a synergism between different constituents was responsible for part of the activity.

Moreover, the fact that no one of the isolated pure compounds showed significant activation of PPAR-α and -γ, despite even strong activities exhibited by the relative extracts or fractions, could be explained by such phenomena.

Furthermore, the possible influence of ubiquitous plant components such as chlorophyll and polyphenols, which often represent the main part of crude extracts, has to be considered. The testing of these substances demonstrated that none of them have pro- or anti-inflammatory activity in the applied in vitro assays. Therefore,

the higher activity of the purified extracts observed in most cases is due to enrichment of their active constituents.
In the screening phase of this study, for instance, the phenol-free MeOH extract of *Sambucus nigra* fruits showed strong activity in every used anti-inflammatory in vitro assay, in contrast with the original one, which was completely inactive. Similarly, the DCM extract of *Prunella vulgaris* herb acquired strong activity only after the chlorophyll removal.
On the other hand, chromatographic analyses indicated that the applied separation methods are not fully selective and they can also result in the loss of the active principles. The data suggest that selection of method for removal of bulk constituents can have dramatic influence on the anti-inflammatory activity of plant extracts. Thus, purification processes represent a valuable strategy for specific enrichment of plant compounds regulating inflammation, as first step of the isolation process, but their effectiveness should be always proved and the possible co-depletion of other substances has to be considered.
These investigations resulted in a relevant overview about pro and contra of commonly applied methods for the elimination of bulk components from plant extracts (submitted manuscript - chapter 3.5).

A bioactivity guided fractionation represented the ideal approach for the isolation of active compounds from the selected plant materials.
By this meaning, a step-by-step biological testing of extracts and derived fractions of different polarities could trace the right way of the research. In order to isolate pure compounds from the active fractions, different HPLC-based strategies were considered using semipreparative and analytical columns, depending on the complexity of the samples, as well as different detection systems. The employment of smaller particle size (3 µm) stationary phases resulted in excellent separation results, which were followed in some cases by satisfying LC-MS structure elucidations, performed also with the support of commercial reference compounds. On the other hand, these HPLC columns are characterized by a smaller loading capacity and therefore they are not suitable for the isolation of acceptable amounts of pure compounds. For this reason, columns with a higher particle size (5 µm) but at the same time a lower resolution were used for the isolation, even if this strategy required extensive method optimizations (see chapter 3.2.3). The achievement of pure compounds in sufficient amount was followed by the NMR structure elucidation, as well as by the biological testing.

The choice of appropriate molecular targets has also a crucial role in the finding of anti-inflammatory properties, as the possibilities are numerous. It is known that the nuclear factor κB plays an important role in inflammation, as its translocation into the nucleus of endothelial cells determines the transcription of pro-inflammatory genes. On the other hand, several factors can activate the NF-κB translocation and they represent even valuable targets. In addition to a cell-based NF-κB transactivation assay, where its inhibition by the candidates can be directly evaluated, the ability of extracts and pure compounds to activate the nuclear receptors PPAR-α and –γ, as well as to downregulate the TNF-α- or LPS-induced mediators E-selectin and IL-8 was also investigated both at the mRNA and at the protein level.
Hence, the activity of a candidate on the one and / or on the other molecular target can give an overview about its mechanism of action.

While IL-8 and E-selectin expression was initially measured at the mRNA level, we had in a second time the opportunity to perform these assays at the protein level. There is considerable uncertainty regarding the general, genome-wide correlation between levels of RNA and corresponding proteins (Gry et al, 2009). Comparing RNA and protein profiles of several gene products in different human cell lines, Gry et al. found significant correlations in only one third of the examined RNA species and corresponding proteins. As post-transcriptional effects are consequence of the protein expression, which can be distantly related to the mRNA transcript level, the experiments performed by ELISA concerning IL-8 and E-selectin downregulation should be considered more reliable than those conducted at the mRNA level.

The first relevant result about the general anti-inflammatory properties of the selected plants was achieved with the pharmacological screening, as only 4 of 35 investigated drugs (*Beta vulgaris* roots, *Gentiana punctata* leaves, *Hippophae rhamnoides* fruits, *Linum usitatissimum* seeds) did not show any activity, while several of the 31 other drugs showed strong activities in the applied assays.
Subsequently and after extensive literature surveys, the herbs of *Betonica officinalis* and *Glechoma hederacea* were chosen to be deeper investigated, as besides their promising results in the mentioned screening, they resulted to be less studied so far. Thus, the dried aerial parts of the two plants, which should be responsible for the therapeutic properties according to their traditional application, were re-extracted on a larger scale, depleted from bulk constituents and further processed.

Whereas the discussed purification processes were useful to enrich the relative concentration of the compounds of interest, the successive solid phase extraction turn out to be not less important, as it consented to work on more but simpler active fractions, instead of complex crude extracts. This first fractionation step permitted not only to exclude inactive fractions, but also to simplify the successive chromatographic isolation of pure compounds from the active ones.

As mentioned in chapter 3.2.2.1, some of the substances identified in *Betonica officinalis* (e.g. iridoids, flavonoids) are already known to be present in other *Stachys* species. On the other hand, three clerodane diterpenes were identified and isolated for the first time in this plant. Compound B2A was previously found in the leaves of *Echinodorus grandiflorus* (Costa et al, 1998), while compound B2B and its methyl ester B2D were identified in the aerial parts of *Grangea maderaspatana* (Krishna and Singh, 1999). However, no biological properties were reported for these compounds before. An in vivo strong anti-inflammatory activity was recently observed in some analogue clerodane diterpenoids from *Dodonaea polyandra*, which were found to be active in a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema model (Simpson et al, 2011). The similarity of the diterpenes from *B. officinalis* with these active structures suggests the possibility to evaluate them also in vivo.
Our investigations indicate the clerodane diterpenes 16-hydroxycleroda-3,13-dien-16,15-olide-18-oic acid (B2A) and 15-hydroxycleroda-3,13-dien-16,15-olide-18-oic acid (B2B), as well as the flavonoid eupatorin, as potential anti-inflammatory agents, being able to significantly inhibit of the TNF-α- and/or LPS-induced IL-8 and E-selectin expression.
The different activities shown by the compounds B2A and B2B, which differ only for the conformation of the lacton, consented to delineate structure-activity relationships between them, with a good correlation between mRNA and protein level regarding the downregulation of IL-8 and E-selectin. Nevertheless, their activity at the protein level was found to be weaker, with moderate but still significant anti-inflammatory properties.
So far, the results indicated the benefit of one lacton conformation than the other, as well as the negative effect of the esterification. In order to better delineate the pharmacophore of these compounds, further investigations will be performed in cooperation with the Department of Vascular Biology and Thrombosis Research, Medical University of Vienna. Similar structures should be synthesized starting from

commercial available compounds and their anti-inflammatory properties will be step-by-step evaluated and compared.

The iridoid 8-O-acetylharpagide, which was able to significantly inhibit TNF-α-induced E-selectin and IL-8 at the mRNA level, represents another interesting finding. Already known as constituent of *B. officinalis*, this compound was already found to possess antibacterial, antifungal, antispasmodic, cardiotonic, and also antipyretic activity when administered in higher doses (Shafi et al, 2004), while moderate anti-inflammatory activity was observed in vivo in a carrageenan induced paw edema model (Ahmed et al, 2003).

The bioactivity guided fractionation of *Glechoma hederacea* consented to identify, for the first time in this plant, the cyclic hydroxamic acid 2-benzoxazolinone, which showed anti-inflammatory properties on IL-8 and E-selectin at the mRNA level. Since the first report on it hypnotic properties (Lespagnol and Lefebvre, 1945), a number of derivatives of this compound have been tested for various biological activities (Çalış et al, 2001). This substance was previously isolated from *Acanthus ilicifolius* (Kapil et al, 1994) and *Calceolaria thyrsiflora* (Bravo et al, 2005), and found to possess leishmanicidal and antibacterial activity, respectively.
Interestingly, 2-benzoxazolinone could be detected in only one of two analyzed samples of *G. hederacea*, moreover as its main component. Although it has to be considered a natural compound, being already described to be present in other plant species, this finding could also indicate a possible contamination of the plant material obtained from Kottas Pharma GmbH. Besides the absence of 2-benzoxazolinone in the field-collected drug (Laab im Walde), it is even significant that this compound was never found in this plant species before. Analyses of further plant samples should clarify this inconsistency.

The flavonoid acacetin, known as a constituent of *Glechoma longituba* (Yang et al, 2006), was previously found to significantly inhibit protein and mRNA expression of iNOS and COX-2 in LPS-induced RAW 264.7 macrophages, without toxicity at concentrations between 5 and 40 µM (Pan et al, 2006). The same biological properties were observed in the close related compound apigenin, which also inhibited the NF-κB activation in an analogous test system (Liang et al, 1999). In contrast, both flavonoids were found to be inactive on the LPS-induced nitric oxide production in J774 macrophages, and toxic at the concentration of 100 µM

(Hamalainen et al, 2007). Finally, apigenin was found to inhibit the TNF-α-induced IL-8 production in human endothelial cells, but not the NF-κB activation (Gerritsen et al, 1995).
Besides the strong TNF-α-induced NF-kB inhibition, both flavonoids were able in our experiments to dose-dependently downregulate IL-8 and E-selectin at the protein level, both in TNF-α- and in LPS-stimulated cells. As the transcription of IL-8 and E-selectin is consequence of the NF-kB translocation into the nucleus, it might be assumed that the anti-inflammatory activity of the flavonoids is due to the significant limitation of that process.

Although the anti-inflammatory properties of the mentioned flavonoids are already described in the literature, they could explain the activity of the respective extracts / original fractions and, moreover, represent a scientific confirmation of the traditional applications of *Glechoma hederacea* against inflammatory diseases. Analogously, the bioactivity guided fractionation of *Betonica officinalis* resulted in the finding of active compounds and in the identification of three clerodanes diterpenes, which were never found in this plant species before.
However, further investigations including in vivo evaluations should be performed, in order to better understand the potential of these promising compounds as anti-inflammatory agents.

First and foremost, the pharmacological screening performed on 35 medicinal plants revealed to be highly significant, as the claimed anti-inflammatory properties of almost 90% of the selected drugs could be confirmed on a scientific basis.
This alone has to be considered a critical finding, as it shines a light on the Austrian traditional medicine and represents a step forward for the phytotherapy.

5 Summary

Austrian folk medicine represents an important starting point for the research of new active compounds in traditional used plants. Several drugs with claimed anti-inflammatory activity, in particular, were considered in this study, with the aim of identifying their active principles and giving a scientific evidence of their therapeutic properties. From the selected candidates, extracts covering a wide range of polarity were generated and subjected to depletion of their bulk constituents, i.e. chlorophyll and polyphenols from nonpolar and polar extracts, respectively, in order to enrich the concentration of the active compounds.

A large number of extracts with and without bulk constituents was screened using different cell-based anti-inflammatory assays. This resulted basically in two important results: besides the stronger activity observed in most cases in the purified extracts, almost 90% of the tested drugs showed activity in the one or in the other assay. Furthermore, a huge number of structures, known to be present in the selected plants, were screened in silico on different anti-inflammatory targets, revealing several interesting candidates.

Accomplished the screening, the herbs of *Betonica officinalis* and *Glechoma hederacea* were selected to be deeper investigated, through a bioactivity guided fractionation. Besides their positive results in the in vitro assays, both plants find application against inflammatory diseases in the Austrian folk medicine and, furthermore, they are less studied so far. The path between plant materials and pure compounds isolated thereof was covered through different phytochemical techniques, such as extraction, bulk constituents depletion, solid phase extraction, GC-MS, semipreparative and analytical HPLC analyses, while the conclusive structure elucidation was performed through NMR measurements.

The bioactivity guided fractionation of the two selected plants resulted in the identification by HPLC-MS and GC-MS of 46 compounds, 9 of which could be isolated and tested. Three clerodane diterpenes and the cyclic hydroxamic acid 2-benzoxazolinone were found for the first time in *Betonica officinalis* and *Glechoma hederacea*, respectively.

The pharmacological evaluation of the isolated pure compounds in different anti-inflammatory assays revealed the flavonoids apigenin and acacetin as the most active ones, as both were able to strongly downregulate IL-8 and E-selectin expression in a dose-dependent manner, as well as to inhibit the NF-κB activation. Furthermore, two of the clerodane diterpenes showed significant activities on IL-8

and E-selectin at the protein level. Other compounds, such as 2-benzoxazolinone, were active on IL-8 and E-selectin at the mRNA level. However, these activities were non reproducible at the protein level.

In conclusion, the present study confirmed the claimed anti-inflammatory properties of several Austrian medicinal plants, revealing moreover some of their active constituents.

6 Zusammenfassung

Die österreichische Volksmedizin repräsentiert eine wichtige Quelle für die Ermittlung neuer aktiver Substanzen aus traditionell angewandten Heilpflanzen. Verschiedene Drogen mit traditionell überlieferten anti-inflammatorischen Eigenschaften sind für diese Studie ausgewählt worden, um ihre Wirkprinzipien zu ergründen und einen wissenschaftlichen Beweis ihrer therapeutischen Wirksamkeit zu gewinnen.

Polare und unpolare Extrakte sind aus den ausgewählten Drogen hergestellt worden und beziehungsweise von Polyphenolen und Chlorophyll befreit worden, um die potentiell aktiven Inhaltstoffe anzureichen. Danach wurde eine große Anzahl von Extrakten mit und ohne Begleitsubstanzen in verschiedenen anti-inflammatorischen zellbasierten Assays gescreent. Dieses Screening ergab zwei Hauptresultate: neben der häufig stärkeren Aktivität der gereinigten Extrakte, zeigten nahezu 90% der getesteten Drogen Aktivität in dem einen oder anderen Assay. Darüber hinaus wurden zahlreiche bekannte Strukturen aus den ausgewählten Pflanzen in silico auf verschiedene entzündungshemmenden Targets gescreent, ebenfalls mit interessanten Resultaten.

Auf Grund der Screening-Ergebnissen wurden die oberirdische Pflanzenteile von *Betonica officinalis* und *Glechoma hederacea* ausgewählt und mit Hilfe einer bioaktivität-geleiteten Fraktionierung genauer untersucht. Neben ihren positiven Ergebnissen in den in vitro Assays, beide Pflanzen werden in der österreichischen Volksmedizin gegen Entzündung verwendet und sind aber bisher wenig untersucht worden.

Um aus dem pflanzlichen Ausgangsmaterial aktive Reinsubstanzen zu isolieren wurden verschiedenen phytochemischen Verfahren wie Extraktion, Abtrennung von Begleitsubstanzen, Festphasen-Extraktion, GC-MS, semipräparative und analytische HPLC Analysen durchgeführt. Die endgültige Strukturaufklärung erfolgte mittels NMR Spektroskopie.

46 Substanzen sind durch die bioaktivität-geleitete Fraktionierung der zwei ausgewählten Pflanzen identifiziert worden, 9 davon konnten isoliert und getestet werden. Drei Clerodan Diterpene und die zyklische Hydroxamsäure 2-benzoxazolinone sind zum ersten Mal in *Betonica officinalis* beziehungsweise in *Glechoma hederacea* nachgewiesen worden.

Die pharmakologische Evaluierung der isolierten Reinsubstanzen in verschiedenen Assays ermittelte die Flavonoide Apigenin und Acacetin als die aktivsten, da beide

eine relevante dosisabhängige Herunterregulation von IL-8 und E-selectin zeigten, sowie eine starke Inhibierung des Transkriptionsfaktors NF-κB. Darüber hinaus zeigten zwei der Clerodane Diterpene eine signifikante Aktivität auf IL-8 und E-selectin auf der mRNA-Ebene als auch auf Proteinebene, während die starke Wirkung von 2-benzoxazolinone auf mRNA-Ebene beschränkt war.

Zusammenfassend bestätigt die vorliegende Studie die behaupteten anti-inflammatorische Eigenschaften von mehreren österreichischen Heilpflanzen, und konnte zusätzlich einige ihrer aktiven Inhaltstoffe aufklären.

7 References

Ahmed B, Al-Rehaily AJ, Al-Howiriny TA, Abdelatee EK, Ahmad MS, 2003. Scropolioside-D2 and harpagoside-B: Two new iridoid glycosides from Scrophularia deserti and their antidiabetic and antiinflammatory activity. *Biological & Pharmaceutical Bulletin* **26**(4), 462-467.

An HJ, Jeong HJ, Um JY, Kim HM, Hong SH, 2006. *Glechoma hederacea* inhibits inflammatory mediator release in IFN-gamma and LPS-stimulated mouse peritoneal macrophages. *Journal of ethnopharmacology* **106**(3), 418-424.

Baggiolini M, Walz A, Kunkel SL, 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *Journal of Clinical Investigation* **84**(4), 1045-1049.

Benedek B, 2007. *Achillea millefolium* L. s.l. – Analysis of phenolic compounds and biological testing. PhD thesis, University of Vienna.

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr, 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proceedings of the National Academy of Sciences of the United States of America* **84**(24), 9238-9242.

Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B, 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* **243**(4895), 1160-1165.

Blaschke F, Takata Y, Caglayan E, Law RE, Hsueh WA, 2006. Obesity, peroxisome proliferator activated receptor, and atherosclerosis in type 2 diabetes. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**, 28-40.

Brat DJ, Bellail AC, Van Meir EG, 2005. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-Oncology* **7**(2), 122-133.

Bravo HR, Copaja SV, Figueroa-Duarte S, Lamborot M, San Martin J, 2005. 1,4-benzoxazin-3-one, 2-benzoxazolinone and gallic acid from *Calceolaria thyrsiflora* Graham and their antibacterial activity. *Zeitschrift für Naturforschung, C: Journal of Biosciences* **60**(5/6), 389-393.

Çalış Ü, Gökhan N, Erdogan H, 2001. Synthesis of some novel 3-methyl-6-(2-substituted propanoyl/propyl)-2-benzoxazolinone derivatives and anti-nociceptive activity. *Il Farmaco* **56**(9), 719-724.

Chalchat J, Petrovic SD, Maksimovic ZA, Gorunovic MS, 2001. Essential oil of *Stachys officinalis* (L.) Trevis., Lamiaceae from Montenegro. *Journal of Essential Oil Research* **13**(4), 286-287.

Chang M, Grillari J, Mayrhofer C, Fortschegger K, Allmaier G, Marzban G, Katinger H, Voglauer R, 2005. Comparison of early passage, senescent and hTERT immortalized endothelial cells. *Experimental cell research* **309**(1), 121-136.

Costa M, Tanaka CMA, Imamura PM, Marsaioli AJ, 1998. Isolation and synthesis of a new clerodane from *Echinodorus grandiflorus*. *Phytochemistry* Volume Date 1999, **50**(1), 117-122.

Daynes RA, Jones DC, 2002. Emerging roles of PPARs in inflammation and immunity. *Nature Reviews Immunology* **2**(10), 748-759.

De Rose V, Oliva A, Messore B, Grosso B, Mollar C, Pozzi E, 1998. Circulating adhesion molecules in cystic fibrosis. *American journal of respiratory and critical care medicine*, **157**(4 Pt 1), 1234-1239.

Dobner MJ, Schwaiger S, Jenewein IH, Stuppner H, 2003. Antibacterial activity of *Leontopodium alpinum* (Edelweiss). *Journal of ethnopharmacology* **89**(2-3), 301-303.

Dvorák Z, Vrzal R, Maurel P, Ulrichová J, 2006. Differential effects of selected natural compounds with anti-inflammatory activity on the glucocorticoid receptor and NF-κB in HeLa cells. *Chemico-Biological Interactions* **159**(2), 117-128.

Felter HW, Lloyd JU, 2003. Glechoma. *King's American Dispensatory*. Available online at: http://ibiblio.org/herbmed/eclectic/kings/glechoma.html.

Frenette PS, Wagner DD, 1997. Insights into selectin function from knockout mice. *Thrombosis and Haemostasis* **78**(1), 60-64.

Gerlach S, Saukel J, Kubelka W, 2006. Pflanzen in der österreichischen Volksmedizin - die „Volksmed-Datenbank". *Scientia Pharmaceutica* **74**, 36.

Gerritsen ME, Carley WW, Ranges GE, Shen CP, Phan SA, Ligon GF, Perry CA, 1995. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *American Journal of Pathology* **147**(2), 278-292.

Green T, 1832. The Universal Herbal or Botanical, Medical and Agricultural Dictionary.

Grieve M, 1971. A Modern Herbal: Volume II, **98**. New York: Dover Publications.

Gry M, Rimini R, Stromberg S, Asplund A, Ponten F, Uhlen M, Nilsson P. 2009. Correlations between RNA and protein expression profiles in 23 human cell lines. *BMC genomics* **10**, 365.

Hajdari A, Mustafa B, Franz Ch, Novak J, 2010. Total flavonoids, total phenolics and antioxidant activity of *Betonica officinalis* L. from Kosovo. *Acta Horticulturae* **860**, 75-80.

Hamalainen M, Nieminen R, Vuorela Pia, Heinonen M, Moilanen E, 2007. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediators of Inflammation*.

Heinrich M, 2003. Ethnobotany and natural products: the search for new molecules, new treatments of old diseases or a better understanding of indigenous cultures*? Current Topics in Medicinal Chemistry* **3**(2), 141-154.

Houghton PJ, Raman A, 1998. Laboratory handbook for the fractionation of natural extracts. *Science* **49**.

Jeker M, Sticher O, Calis I, Ruedi P, 1989. Allobetonicoside and 6-O-acetylmioporoside: two new iridoid glycosides from *Betonica officinalis* L. *Helvetica Chimica Acta* **72**(8), 1787-1791.

Kadl A, Huber J, Gruber F, Bochkov VN, Binder BR, Leitinger N, 2002. Analysis of inflammatory gene induction by oxidized phospholipids in vivo by quantitative real-time RT-PCR in comparison with effects of LPS. *Vascular Pharmacology* **38**(4), 219-227.

Kapil A, Sharma S, Wahidulla S, 1994. Leishmanicidal activity of 2-benzoxazolinone from *Acanthus ilicifolius* in vitro. *Planta Medica* **60**(2), 187-188.

Karin M, Yamamoto Y, Wang QM, 2004. The IKK NF-κB system: A treasure trove for drug development. *Nature Reviews Drug Discovery* **3**(1), 17-26.

Kikuchi M, Goto J, Noguchi S, Kakuda R, Yaoita Y, 2008. Glycosides from whole plants of *Glechoma hederacea* L. *Journal of Natural Medicines* **62**(4), 479-480.

Kirchmair J, Distinto S, Schuster D, Spitzer G, Langer T, Wolber G, 2008. Enhancing drug discovery through in silico screening: strategies to increase true positives retrieval rates. *Current Medicinal Chemistry* **15**(20), 2040-2053.

Kobzar AY, Nikonov GK, 1986. Flavonoids from the aerial parts of *Betonica officinalis*. *Khimiya Prirodnykh Soedinenii* (5), 636-637.

Kobzar AY, 1986. Phytochemical study of *Betonica officinalis*. I. Isolation of biologically active substances from the aerial parts of the plant. *Khimiya Prirodnykh Soedinenii* **2**, 239.

Kosonen O, Kankaanranta H, Uotila J, Moilanen E, 2000. Inhibition by nitric oxide-releasing compounds of E-selectin expression in and neutrophil adhesion to human endothelial cells. *European Journal of Pharmacology* **394**(1), 149-156.

Krishna V, Singh P, 1999. A clerodane derivative from *Grangea maderaspatana*. *Phytochemistry* **52**(7), 1341-1343.

Kumarasamy Y, Cox PJ, Jaspars M, Nahar L, Sarker SD, 2003. Isolation, structure elucidation and biological activity of hederacine A and B, two unique alkaloids from *Glechoma hederaceae*. *Tetrahedron* **59**(34), 6403-6407.

Lawrence T, 2009. The Nuclear Factor NF-kB Pathway in Inflammation. *Cold Spring Harbor Perspectives in Biology* **1**(6).

Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA, 2001. Possible new role for NF-κB in the resolution of inflammation. *Nature Medicine* **7**(12), 1291-1297.

Lespagnol A, Lefebvre M, 1945. Hypnotic effect of benzoxazolone substitutes. *Bulletin de la Societe Chimique de France* **12**, 386–388.

Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK, 1999. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis* **20**(10), 1945-1952.

Maleki N, Garjani A, Nazemiyeh H, Nilfouroushan N, Eftekhar Sadat AT, Allameh Z, Hasannia N, 2001. Potent anti-inflammatory activities of hydroalcoholic extract from aerial parts of *Stachys inflata* on rats. *Journal of Ethnopharmacology* **75**, 213-218.

Matkowski A, Piotrowska M, 2006. Antioxidant and free radical scavenging activities of some medicinal plants from the Lamiaceae. *Fitoterapia* **77**, 346-353.

Markt P, Schuster D, Langer T, 2011. Pharmacophore models for virtual screening. In Virtual Screening. Sotriffer C (Ed), Wiley-VCH, Weinheim.

Medzhitov R, 2008. Origin and physiological roles of inflammation. *Nature* **454**(7203), 428-435.

Meremeti A, Karioti A, Skaltsa H, Heilmann J, Sticher O, 2004. Secondary metabolites from *Stachys ionica*. *Biochemical Systematics and Ecology* **32**, 139-151.

Milovanovic M, Stefanovic M, Dermanovic V, 1995. Flavonoids from *Glechoma hederacea* L. *Journal of the Serbian Chemical Society* **60**(6), 467-469.

Miyase T, Yamamoto R, Ueno A, 1996. Phenylethanoid glycosides from *Stachys officinalis*. *Phytochemistry* **43**(2), 475-479.

Mohn T, Plitzko I, Hamburger M, 2009. A comprehensive metabolite profiling of *Isatis tinctoria* leaf extracts. *Phytochemistry* **70**(7), 924-934.

Mukaida N, Harada A, Matsushima K, 1998. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine & Growth Factor Reviews* **9**(1), 9-23.

Newman DJ, Cragg GM, 2007. Natural Products as Sources of New Drugs over the Last 25 Years. *Journal of Natural Products* **70**, 461-477.

Okuda T, Hatano T, Agata I, Nishibe S, 1986. The components of tannic activities in Labiatae plants. I. Rosmarinic acid from Labiatae plants in Japan. *Yakugaku Zasshi* **106**(12), 1108-1111.

Pan MH, Lai CS, Wang YJ, Ho CT, 2006. Acacetin suppressed LPS-induced up-expression of iNOS and COX-2 in murine macrophages and TPA-induced tumor promotion in mice. *Biochemical Pharmacology* **72**(10), 1293-1303.

Pastorello M, Ciangheroti CE, Colman TI, Amesty Á, Buitriago D, Israel A, 2007. Actividad antiinflamatoria del ácido 3-epi-ursólico y docking a la fosfolipasa A2. *Revista Facultad de Farmacia* **70**(1).

Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T, 1996. Salicylates inhibit IκB-α phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. *Journal of Immunology* **156**(10), 3961-3969.

Potterat O, Hamburger M, 2006. Natural products in drug discovery – concepts and approaches for tracking bioactivity. *Current Organic Chemistry* **10**, 899-920.

Predel HG, Giannetti B, Koll R, Bulitta M, Staiger C, 2005. Efficacy of a comfrey root extract ointment in comparison to a diclofenac gel in the treatment of ankle distortions: results of an observer-blind, randomized, multicenter study. *Phytomedicine: international journal of phytotherapy and phytopharmacology* **12**(10), 707-714.

Reddy AS, Pati SP, Kumar PP, Pradeep HN, Sastry GN, 2007. Virtual screening in drug discovery - a computational perspective. *Current Protein and Peptide Science* **8**(4), 329-351.

Rester U, 2008. From virtuality to reality – virtual screening in lead discovery and lead optimization: a medicinal chemistry perspective. *Current Opinion in Drug Discovery & Development* **11**(4), 559-568.

Rossi D, Zlotnik A, 2000. The biology of chemokines and their receptors. *Annual Review of Immunology* **18**, 217-243.

Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, Hortensteiner S, 2009. Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis. *The Plant Cell* **21**(3), 767-785.

Schneider G, 2010. Virtual screening: an endless staircase? *Nature Reviews Drug Discovery* **9**(4), 273-276.

Shafi N, Khan GA, Arfan M, Ahmad K, Gilani ND, 2004. Isolation and pharmacological screening of 8-O-acetylharpagide from *Ajuga bracteosa* Wall. *Pakistan Journal of Scientific and Industrial Research* **47**(3), 176-179.

Simpson BS, Claudie DJ, Gerber JP, Pyke SM, Wang J, McKinnon RA, Semple SJ, 2011. In Vivo Activity of Benzoyl Ester Clerodane Diterpenoid Derivatives from *Dodonaea polyandra*. *Journal of Natural Products* **74**(4), 650-657.

Skaltsa H, Georgakopoulos P, Lazari D, Karioti A, Heilmann J, Sticher O, Constantidinis Th, 2007. Flavonoids as chemotaxonomic markers in the polymorphic *Stachys swainsonii* (Lamiaceae). *Biochemical Systematics and Ecology* **35**, 317-320.

Sobolewski C, Legrand N, Morceau F, Diederich M, 2010. Inflammation: Novel arrows for an ancient target. *Biochemical Pharmacology* **80**(12), 1769-1770.

Stahl E, Datta SN, 1972. New sesquiterpenoids of the ground ivy (*Glechoma hederacea*). *Justus Liebigs Annalen der Chemie* **757**, 23-32.

Tobyn G, Denham A, Whitelegg M, 2010. The Western Herbal Tradition – 2000 years of medicinal plant knowledge (29), 307-316.

Vavilova NK, Fursa NS, Oshmarina VI, 1988. Hydroxycinnamic acids of *Glechoma hederacea*. *Khimiya Prirodnykh Soedinenii* **2**, 293-294.

Verbeuren T, Sansilvestri-morel P, Rupin A, Vallez, MO, Fratacci MD, Lerond L, Lavielle G, 2009. Association between an anti-atherothrombotic and an angiotensin-converting enzyme inhibitor. *Les Laboratoires Servier*. Patent number: US20090075976.

Vestweber D, Blanks JE, 1999. Mechanisms that regulate the function of the selectins and their ligands. *Physiological Reviews* **79**(1), 181-213.

Yamauchi H, Kakuda R, Yaoita Y, Machida K, Kikuchi M, 2007. Two new glycosides from the whole plants of *Glechoma hederacea* L. *Chemical & Pharmaceutical Bulletin* **55**(2), 346-347.

Yang NY, Duang JH, Li P, Qian SH, Hu WC, 2006. Chemical constituents of *Glechoma longituba*. *Zhongguo Tianran Yaowu* **4**(2), 98-100.

Yuan L, Nikolova-Krstevski V, Zhan Y, Kondo M, Bhasin M, Varghese L, Yano K, Carman C, Aird WC, Oettgen P, 2009. Antiinflammatory Effects of the ETS Factor ERG in Endothelial Cells Are Mediated Through Transcriptional Repression of the Interleukin-8 Gene. *Circulation Research* **104**(9), 1049-1057.

Wall ME, Wani MC, Brown DM, Fullas F, Olwald JB, Josephson FF, Thornton NM, Pezzuto JM, Beecher CWW, Farnsworth NR, Cordell GA, Kinghorn AD, 1996. Effect of tannins on screening of plant extracts for enzyme inhibitory activity and techniques for their removal. *Phytomedicine* **3**, 281-285.

Wang YD, Chen WD, Wang M, Yu D, Forman BM, Huang W, 2008. Farnesoid X receptor antagonizes NF-κB in hepatic inflammatory response. *Hepatology* **48**(5), 1632-1643.

Watanabe T, Kawata A, Inoue M, Ishihara S, Tsuji K, 2007. Antihypertensive effect of *Glechoma hederacea* extract in spontaneously hypertensive rats. *Nippon Shokuhin Kagaku Kogaku Kaishi* **54**(9), 415-418.

Zieba J, 1973. Isolation and identification of flavonoids from *Glechoma hederacea* L. *Polish journal of pharmacology and pharmacy* **25**(6), 593-597.

Zieba J, 1973. Isolation and identification of non-heteroside triterpenoids from *Glechoma hederacea* L. *Polish journal of pharmacology and pharmacy* **25**(6), 587-592.

Zinchenko TV, Fefer IM, 1962. Investigation of glycosides from *Betonica officinalis*. *Farmatsevtichnii Zhurnal* **17**(3), 35-38.

8 List of Abbreviations

AP1	Activator protein-1	IKK-2	IκB kinase-2
ASE	Accelerated solvent extraction	IL-8	Interleukin-8
BSA	Bovine serum albumin	iNOS	Inducible nitric oxide synthase
COX	Cyclooxygenase	LC-MS	Liquid chromatography mass spectrometry
DCM	Dichloromethane	5-LOX	5-lipoxygenase
DDA	Data-dependent acquisition	LPS	Lipopolysaccharide
DEHP	Diethylhexylphthalate	NF-κB	Nuclear factor κB
DPPH	2,2-diphenyl-1-picrylhydrazyl	NMR	Nuclear magnetic resonance
ECGS/H	Endothelial Cell Growth Supplement/Heparin	OD	Optical density
EGFP	Enhanced green fluorescent protein	PBS	Phosphate buffer solution
ELISA	Enzyme-linked immunosorbent assay	PBST	Phosphate-buffered saline Tween
ELSD	Evaporative light scattering detector	PMA	phorbol 12-myristate 13-acetate
ESI	Electrospray ionization	PPAR	Peroxisome proliferator activated receptor
FBS	Fetal bovine serum	PTL	Parthenolide
FRAP	Ferric reducing ability of plasma	PVP	Polyvinylpyrrolidone
FXR	Farnesoid X receptor	q-PCR	Quantitative polymerase chain reaction
GC-MS	Gas chromatography mass spectrometry	Rt	Retention time
HCT	High capacity ion trap	SEM	Standard error of the mean
HEK293	Human embryonic kidney 293	SI	Similarity index
HPLC	High performance liquid chromatography	SPE	Solid phase extraction
HRMS	High resolution mass spectrometry	TLC	Thin layer chromatography
HRP	Horseradish peroxidase	TMB	3,3,5,5-tetramethylbenzidine
HUVEC	Human umbilical vein endothelial cells	TNF	Tumor necrosis factor
ICAM	Intracellular adhesion molecule-1	ESI-TOF	Electrospray-Quadrupole-Time-of-Flight
IFN-γ	Interferon-γ	wCh	Without chlorophyll
IkB-α	Inhibitor κB-α	wP	Without polyphenols

9 Meeting Contributions

57th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research. August 16th – 20th, 2009 – Geneva, Switzerland.

Poster presentation

Screening of 35 plants used in Austrian folk medicine for PPAR-α and -γ activation and NFkB inhibition

Paolo Picker, Sylvia Vogl, Nanang Fakhrudin, Atanas Atanasov, Elke Heiß, Gottfried Reznicek, Johannes Saukel, Christoph Wawrosch, Verena M. Dirsch, Brigitte Kopp.

Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

Austria and its adjacent regions have a great history in traditional folk medicine. Folk-medicinal knowledge was collected over years and transferred to the VOLKSMED database [1] which contains an exact botanical description of each used plant. The aim of this study was to investigate the potential in vitro anti-inflammatory activity of plants selected from that database, using luciferase reporter gene assays.

Thirty five preselected plants were extracted with dichloromethane (DCM) and methanol (MeOH) using the Accelerated Solvent Extractor (Dionex ASE200). The chlorophyll, if present, was separated from the DCM extract, whereas the tannins were removed from the MeOH extract, in order to avoid possible interferences with the assay formats [2]. Crude and purified extracts were then examined for activation of PPAR-α and -γ and inhibition of NFκB using HEK293 cells transfected with green fluorescence protein plasmid (as internal control). The cells were also accordingly transfected with PPAR-α or -γ plasmids and reporter plasmid pPPRE-tk3x-Luc in the PPAR assay, while a pNFκB-luc transfection and a TNF-α stimulation were used in the NF-kB assay. Luciferase activity and fluorescence intensity were then measured using a GeniosPro plate reader.

The extracts of fifteen plants showed no activity in the applied assays, while the other twenty exhibited activity in one or more of the test systems. The three most active ones in both assays were the DCM extract with the chlorophyll separated of *Urtica dioica* leaves, the MeOH extract with the tannin separated of *Sambucus nigra* fruits and the DCM extract with the chlorophyll separated of *Prunella vulgaris* herb.

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References: 1.Saukel J (2006) Sci. Pharm. 74:36. 2.Potterat O and Hamburger M (2006) Curr. Org. Chem. 10:899-920

58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research. August 29th – September 2nd, 2010 – Berlin, Germany.

Short lecture

Bioactivity-guided isolation of potential anti-inflammatory constituents from *Betonica officinalis*

Picker P[1], Mihaly-Bison J[2], Vogl S[1], Zehl M[1], Urban E[3], Reznicek G[1], Saukel J[1], Wawrosch C[1], Binder BR[2], Kopp B[1]

[1]Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria
[2]Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Schwarzspanierstrasse 17, A-1090 Vienna, Austria
[3]Department for Medicinal Chemistry, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

Betonica officinalis (Lamiaceae) has been used in Austrian traditional medicine since ancient times against inflammatory disorders. The aim of this study was to investigate the anti-inflammatory properties of extracts, derived fractions, and isolated pure compounds of this plant by assessment of their effect on genes (E-selectin, IL-8) that are induced by inflammatory stimuli (TNF-α or LPS) in endothelial cells [1,2].

The plant material (herb) was extracted with dichloromethane (DCM) using an accelerated solvent extractor. Chlorophyll was separated by liquid-liquid-partition between DCM and a mixture of MeOH-H_2O 1:1, in order to increase the concentration of the active compounds. Since the purified DCM extract showed strong activity in the mentioned assay, a bioactivity-guided fractionation was carried out. Subfractions were obtained by solid-phase extraction using C18 cartridges eluted with 30%, 70%, and 100% MeOH. The 30% and the 70% subfractions, which showed highest activity, were further fractionated by HPLC in order to identify and investigate their active constituents, whose structures were elucidated by HPLC-MS, 1D, and 2D NMR spectroscopy.

Besides of some known polymethylated flavonoids (e.g. salvigenin), particularly the iridoid 8-O-acetylharpagide and two new diterpenoids were found to inhibit between 46% and 99% the LPS-stimulated induction of E-selectin at the concentration of 10 µg/ml, evidencing a considerable potential as new anti-inflammatory agents.

Acknowledgements: This work is funded by the Austrian Science Fund, NFN: S10704-B037

References: 1. Chang et al. (2005) Exp Cell Res. 309(1):121-36; 2. Kadl et al. (2002) Vascul Pharmacol. 38(4):219-27.

Curriculum Vitae

Personal details

Name:	Paolo Picker
Title:	Dott.
Date and place of birth:	7th June 1980, Sant'Agnello (Italy)
Nationality:	Italian
Family status:	Unmarried
Phone:	+43-(0)-680-2128862
Email:	paolo.picker@gmail.com
Address:	Eckpergasse 36/5 - 1180 Vienna, Austria

Professional experience

Since 04/2008:	Scientific co-worker at the Department of Pharmacognosy of the University of Vienna in the project "Drugs from Nature Targeting Inflammation" granted by the Austrian Science Fund
09/2006 - 07/2007:	Experimental diploma thesis in organic chemistry at the Department of Chemistry of Natural Substances, University of Naples, Italy. Topic: "Pepluane diterpenes with anti-inflammatory activity from *Euphorbia paralias* L.."
03/2005 - 09/2005:	Apprenticeship at the Pharmacy Bianchi in Meta, Italy

Education

Since 04/2008:	PhD studies at the Department of Pharmacognosy, University of Vienna
07/2007:	Diploma degree
10/1998 - 07/2007:	Study of Chemistry and Pharmaceutical Technology at the University of Naples
07/1998:	Graduation from grammar school
09/1993 - 07/1998:	Grammar school "Publio Virgilio Marone" in Meta

Languages

Italian:	Mother tongue

German:	Fluent in writing and speaking
English:	Fluent in writing and speaking

Teaching

10/2008 - 02/2011: Assistance in Phytochemistry courses ("Gewinnung und instrumentelle Analytik") at the Department of Pharmacognosy, University of Vienna

Publications

Picker P, Vogl S, Mihaly-Bison J, Binder M, Fakhrudin N, Atanasov AG, Grzywacz AM, Heiss EH, Zehl M, Saukel J, Wawrosch C, Schinkovitz A, Bauer R, Rollinger JM, Stuppner H, Dirsch VM, Bochkov V, Reznicek G, Kopp B. Elimination of bulk polyphenols and chlorophyll from plant extracts influences their in vitro anti-inflammatory activity – the method matters (submitted).

Joa H, Vogl S, Atanasov A, Zehl M, Nakel T, Fakhrudin N, Heiss E, Picker P, Urban E, Wawrosch Ch, Saukel J, Reznicek G, Kopp B, Dirsch V. Identification of ostruthin from *Peucedanum ostruthium* rhizomes as an inhibitor of vascular smooth muscle cell proliferation, *J.Nat.Prod*, 2011.

Vogl S, Zehl M, Picker P, Urban E, Wawrosch C, Reznicek G, Saukel J, Kopp B. Identification and Quantification of Coumarins in *Peucedanum ostruthium* (L.) Koch by HPLC-DAD and HPLC-DAD-MS. *J. Agricultural and Food Chemistry* 59(9): 4371-4377, 2011.

Short lectures

Picker P, Mihaly-Bison J, Vogl S, Zehl M, Urban E, Reznicek G, Saukel J, Wawrosch C, Binder BR, Kopp B. Bioactivity-guided isolation of potential anti-inflammatory constituents from *Betonica officinalis*. Young Researchers' Workshop, 58th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Berlin (Germany), 2010.

Poster presentations

Picker P, Mihaly-Bison J, Vogl S, Zehl M, Urban E, Reznicek G, Saukel J, Wawrosch C, Binder BR, Kopp B. Bioactivity-guided isolation of potential anti-inflammatory constituents

from *Betonica officinalis*. 58th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Berlin (Germany), 2010.

Vogl S, Zehl M, Picker P, Reznicek G, Saukel J, Wawrosch C, Urban E, Kopp B. Rapid separation, identification, and quantification of coumarins in *Peucedanum ostruthium*. 58th International Congress and Annual Meeting of the Society for Medicinal Plant Research, Berlin (Germany), 2010.

Picker P, Vogl S, Fakhrudin N, Atanasov A, Heiss E, Reznicek G, Saukel J, Wawrosch C, Dirsch VM, Kopp B. Screening of 35 plants used in Austrian folk medicine for PPAR-α and -γ activation and NFkB inhibition. 57th Annual Meeting and International Congress of the Society for Medicinal Plant Research, Geneva (Switzerland), 2009.

Vogl S, Picker P, Fakhrudin N, Atanasov A, Heiss E, Reznicek G, Saukel J, Wawrosch C, Dirsch VM, Kopp B. Influence of chlorophyll and tannins in plant extracts on cell-based luciferase reporter gene assays. 57th Annual Meeting and International Congress of the Society for Medicinal Plant Research, Geneva (Switzerland), 2009.

Fakhrudin N, Vogl S, Picker P, Heiss EH, Saukel J, Reznicek G, Kopp B, Atanasov A, Dirsch VM. Screening for discovery of novel peroxisome proliferator-activated receptor-alpha and -gamma agonists and nuclear factor-kB inhibitors by luciferase reporter gene assays. 21st Scientific Congress of the Austrian Pharmaceutical Society, Vienna (Austria), 2009