universität wien

DISSERTATION

Bioassay guided fractionation of

Peucedanum ostruthium (L.) W.D.J. Koch

and *Melampyrum pratense* L.

focusing on anti-inflammatory activities

Verfasserin

Mag. Sylvia Vogl

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 091 438
Dissertationsgebiet lt. Studienblatt:	Dr.-Studium der Naturwissenschaften Botanik (Stzw)
Betreuerin / Betreuer:	Univ. Prof. Dr. Brigitte Kopp

Acknowledgements

In this place my sincerest thanks are given to …

… Univ. Prof. Mag. Dr. Dr. h.c. Brigitte Kopp for the possibility to work on such an interesting and important topic, as well as for her heartily guidance through it.

… Ao. Univ. Prof. Mag. Dr. Gottfried Reznicek for the constant help with the phytochemical work.

… Ao. Univ. Prof. Mag. Dr. Johannes Saukel for the backup at the plant gathering as well as for his advices concerning microscopy and statistics.

… Dr. Martin Zehl for the numerous LC-MS analyses and the essential support and corrections for my first publications.

… Univ. Prof. Dr. Verena Dirsch, Dr. Atanas Atanasov, Dr. Elke Heiß, Dr. Helge Joa and Nanang Fakhrudin, PhD. from the Department of Pharmacognosy, University of Vienna for the pharmacological testing.

… Dr. Valery Bochkov, Mag. Judit Mihaly, Mag. Markus Binder and Dr. Murty Bulusu for the cooperation at the Department of Vascular Biology and Thrombosis Research, Medical University of Vienna.

… Dr. Daniela Schuster and Dr. Gerhard Wolber from the Department of Pharmaceutical Chemistry, University of Innsbruck for the *in silico* screening.

… Ao. Univ.-Prof. Dr. Ernst Urban from the Department for Medicinal Chemistry, University of Vienna for the NMR- analyses.

… my colleagues from the Department of Pharmacognosy, especially Dr. Paolo Picker, Dr. Judith Singhuber, Dr. Kerstin Kainz and Mag. Oliver Donath, the best colleagues and friends one could ever imagine, who were always willing to help.

… my partner Mag. Michael Burgert for his endless love, support and good advices.

… and last but not least I thank my family, especially my mother Angela Vogl, for the constant encouragement and backup.

Abbreviations

ACE	acetylcholinesterase
ASR	anisaldehyde sulfuric acid reagent
CC	column chromatography
CD14	cluster of differentiation 14
COX-1, -2	cyclooxygenase-1, -2
cPLA2	cytosolic phospholipase A2
CXCR1/ CXCR2	CXC-motive-chemokine receptor 1/2, also known as Interleukin-8-receptor alpha/ beta
3D	three-dimensional
DAD	diode array detector
DCM	dichloromethane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary DNA
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ELSD(-LT)	evaporative light scattering detector(-low temperature)
ER	estrogen receptor
eNOS	endothelial nitric oxide synthase
EtOAc	ethyl acetate
FID	flame ionization detection
FXR	farnesoid X receptor
GC	gas chromatography
qPCR	quantitative polymerase chain reaction
GR	glucocorticoid receptor
HAc	acetic acid
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
HUVEC	human umbilical vein endothelial cells
H_2SO_4	sulfuric acid
ICAM-1	intercellular adhesion molecule-1

IFN-γ	interferon-γ
IκB	inhibitor of kappa-light-chain-enhancer of activated B cells
IKK	IκB kinase
IL	interleukin
5-LO	5-lipoxygenase
LPS	lipopolysaccharide
LXR	liver x receptor
MAPK	mitogen-activated protein kinase
MeCN	acetonitrile
MeOH	methanol
MW	molecular weight
mPGES-1	microsomal prostaglandin e synthase 1
mRNA	messenger RNA
MS	mass spectrometry
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	nuclear magnetic resonance
NP	natural product
NPPR	natural products-polyethylene glycol reagent
NSAIDs	nonsteroidal anti-inflammatory drugs
p. a.	pro analysis
PAF	platelet-activating factor
PCR	polymerase chain reaction
PPARs	peroxisome proliferators-activated receptors
Rf	retention factor
RNA	ribonucleic acid
RXR	retinoid X receptor
SPE	solid phase extraction
TLR4	toll-like receptor 4
TLC	thin layer chromatography
TNF-α	tumor necrosis factor α
UV	ultraviolet light
VCAM-1	vascular cell adhesion protein-1
VS	vanillin sulfuric acid reagent
VSCMs	vascular smooth muscle cells

Contents

1 Introduction 1

1.1 Peucedanum ostruthium 3

1.1.1 Botanical description 3

1.1.2 Traditional and pharmaceutical usage 4

1.1.3 Components 8

1.2 Melampyrum pratense 9

1.2.1 Botanical description 9

1.2.2 Traditional and pharmaceutical usage 11

1.2.3 Components 11

1.3 Targeting inflammation 13

1.3.1 Nuclear factor κB (NF-κB) 15

1.3.2 Pro-inflammatory mediators 17

1.3.3 Nuclear receptors 21

1.4 Objective 24

2 Materials and Methods 25

2.1 The VOLKSMED-DATABASE 25

2.2 Literature research 27

2.3 Plant material 27

2.4 Reagents and solvents 31

2.5 Reference compounds 31

2.6 Extraction 34

2.6.1 Accelerated Solvent Extractor (ASE) 35

2.6.2 Ultrasonic bath 36

2.6.3 Extraction under reflux 36

2.6.4 Chlorophyll separation 36

2.6.5 Tannin separation 37

2.7 Solid Phase Extraction (SPE) 38

2.8 Chromatographic methods 40
2.8.1 Thin Layer Chromatography (TLC) 40
2.8.2 Column chromatography (CC) 43
2.8.3 High Performance Liquid Chromatography (HPLC) 44
2.8.4 Gas Chromatography (GC) 47
2.9 Mass spectrometry (MS) 47
2.10 Nuclear Magnetic Resonance (NMR) Spectroscopy 47
2.11 Pharmacological test systems 48
2.11.1 Downregulation of LPS or TNF-α induced IL-8 and E-selectin 48
2.11.2 Luciferase cell based assays 51
2.12 In silico screening 51
3 Results 52
3.1 Extraction and purification 52
3.2 Giving scientific evidence for the traditional usage of several Austrian medicinal plants with potential anti-inflammatory properties (manuscript) ... 55
3.3 Plant extracts in cell based anti-inflammatory assays – Methods for the removement of activity masking bulk components (manuscript) 108
3.4 Bioactivity guided isolation of the active principles from Peucedanum ostruthium 129
3.4.1 Pharmacophoric profiling of *Peucedanum ostruthium* compounds against anti-inflammatory targets 143
3.5 Identification and quantification of coumarins in Peucedanum ostruthium (L.)Koch by HPLC-DAD and HPLC-DAD-MS 152
3.6 Identification of ostruthin from Peucedanum ostruthium rhizomes as an inhibitor of vascular smooth muscle cell proliferation 160
3.7 Bioactivity guided isolation of active compounds from Melampyrum pratense 165
3.7.1 TLC investigation of *Melampyrum pratense* extracts and fractions 169
3.7.2 Isolation and identification of bioactive compounds 171

3.7.3 Pharmacological results 180
3.7.4 Pharmacophoric profiling of *Melampyrum pratense* compounds against anti-inflammatory targets 185

4 Discussion 191

4.1 Peucedanum ostruthium 193

4.2 Melampyrum pratense 199

5 Summary 205

6 Zusammenfassung 207

7 References 209

***Curriculum vitae* 224**

1 Introduction

The present work was conducted in the frame of the NFN project "Drugs from Nature Targeting Inflammation" (DNTI). The aim of this project was to find new bioactive natural compounds of traditionally used plants and/ or new anti-inflammatory modes of action of those.

Secondary metabolites of plants are compounds with no apparent function in the primary metabolism of the organism; however, they represent an important tool for its defense and provide one means of competition in its environment. Mankind has been using such metabolites as therapeutic agents since ancient times (Adams *et al.*, 2009; Hostettmann *et al.*, 2007; Silva *et al.*, 1998). Analysis of drugs developed between 1981 and 2006 showed that natural products (NPs) or NP-derived compounds comprised 28% of all new chemical entities (NCEs). In addition, 24% of all NCEs were related to NPs (Fig. 1). Nowadays about 60% of the new pharmaceutics are based on molecular structures of natural resources (Kinghorn, 2008; Newman, 2008; Newman *et al.*, 2009; Newman *et al.*, 2007; Newman *et al.*, 2003; Potterat *et al.*, 2008).

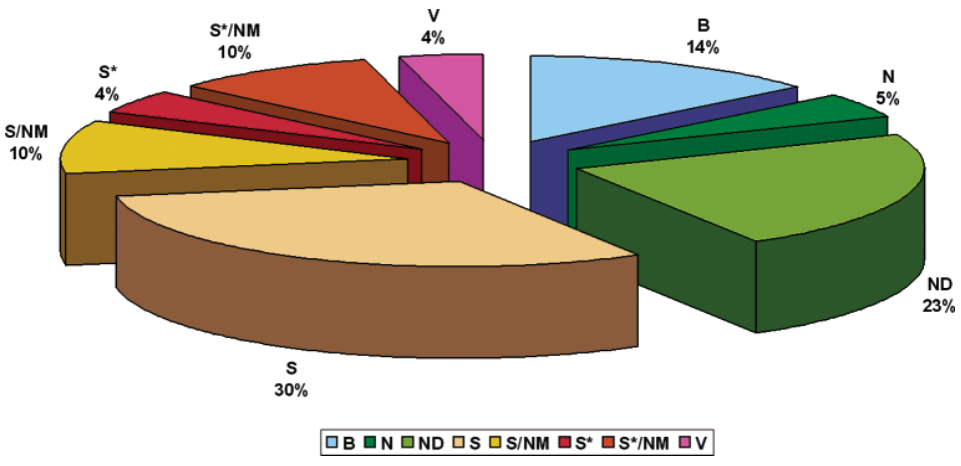


Fig. 1 All new chemical entities, approved as drugs between 01/1981–06/2006, by source (total number = 1184). B - biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host. N - natural product. ND - derived from a natural product and is usually a semisynthetic modification. S - totally synthetic drug, often found by random screening/modification of an existing agent. S* - made by total synthesis, but the pharmacophore is/was from a natural product. V - vaccine. NM - natural product mimic (Newman *et al.,* 2007).

Inflammation is a common denominator in a variety of diseases; therefore, natural compounds with anti-inflammatory action are an important group of therapeutics (Dvorak *et al.*, 2006).

Thus, Austrian medicinal plants with a potential anti-inflammatory activity, recorded in the VOLKSMED database, were chosen for further investigation in cell based *in vitro* assays, regarding their potency to inhibit NF-κB, activate PPAR-α or -γ and to reduce the production of interleukin 8 or E-selectin.

During this ethno-pharmacological screening two species were selected as interesting candidates for further bioassay guided fractionation, namely *Peucedanum ostruthium* (L.) W.D.J. Koch and *Melampyrum pratense* L..

Bioactivity guided isolation is a common approach in drug discovery from natural resources. In its course several cycles of fractionation are usually needed to obtain a pure compound which in the end, is expected to be an active principle and may serve as a lead structure for drug development (Fig. 2. Koehn *et al.,* 2005).

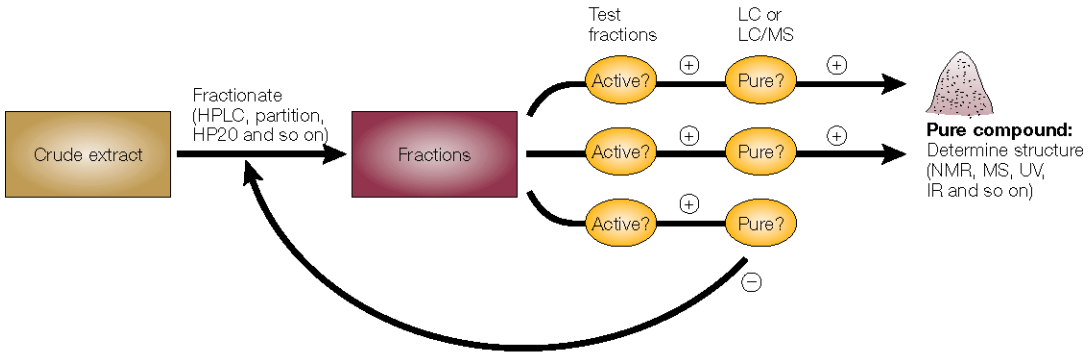


Fig. 2 Generic scheme for bioassay guided fractionation. "HP20" is a solid-phase adsorber; HPLC - high-performance liquid chromatography; IR - infrared spectroscopy; LC/MS - liquid chromatography/mass spectrometry; NMR - nuclear magnetic resonance; UV - ultraviolet (Koehn *et al.*, 2005).

1.1 *Peucedanum ostruthium*

1.1.1 Botanical description

The masterwort, *Peucedanum ostruthium* (L.) W.D.J. Koch, is a perennial Apiaceae shrub growing on nitrogen rich, humous, humid soil, preferable in subalpine tall forbs or alder meadows. It is wide spread in the alpine region and elsewhere in middle and northern Europe mostly of anthropogenic origin (Fig. 4, p. 4). This species of the family Apiaceae is characterized by hollow stems, white to light pink petals and three-part, trilobate leaves (Fig. 3). It can reproduce via generation of stolons and reaches heights between 30-100 cm (Fischer *et al.*, 2008).

2n = 22



Fig. 3 *Peucedanum ostruthium* (L.) W.D.J. Koch - Habitus.
Source: "Köhler's Medizinal-Pflanzen in naturgetreuen Abbildungen mit kurz erläuterndem Texte: Atlas zur Pharmacopoea germanica“ (Köhler, 1987).

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Rosidae*
Order	*Apiales*
Family	*Apiaceae* – Carrot family
Genus	*Peucedanum L.* – peucedanum
Species	*Peucedanum ostruthium* (L.) W.D.J. Koch – masterwort

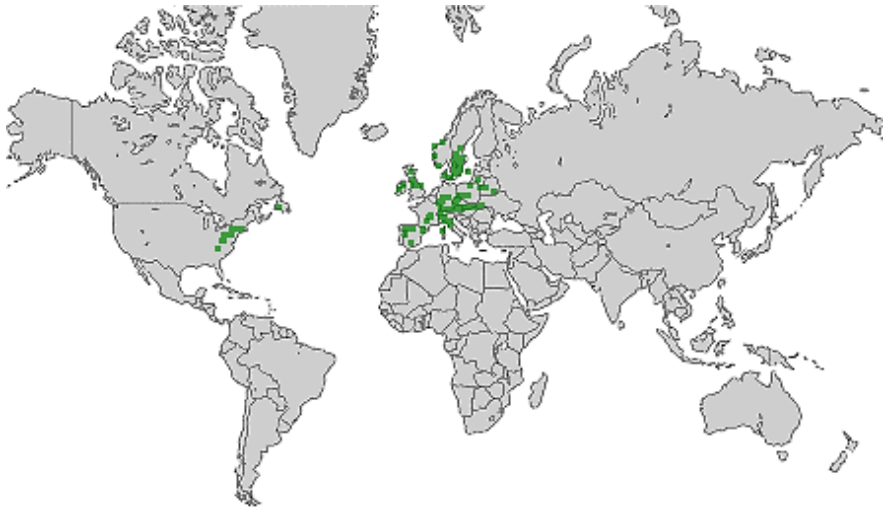


Fig. 4 Distribution of *Peucedanum ostruthium* (L.) W.D.J. Koch.
Source: http://www.wilde-planten.nl/meesterwortel.htm.

1.1.2 Traditional and pharmaceutical usage

Leaning on reports of the VOLKSMED database, in Austrian ethnomedicine the masterwort is mainly used in South Tyrol, Vorarlberg and Salzburg (Fig. 5, p. 5), obviously because it is preferably growing in the alpine region. It is also known as a traditional remedy in England (masterwort, pellitory of Spain), France (impératoire, benjoin des maléfices), the Netherlands (meesteewortel), Italy (imperatoria, erba rena, elafobosco erba rena), Denmark (Mesterrod), Sweden (Mästerrot), the Czech Republic (Všedobr horní), Hungary (Mestergyörkér), former Bohemia (vsčdobr), Croatia (pukavica) and Russia (carskij korenj).

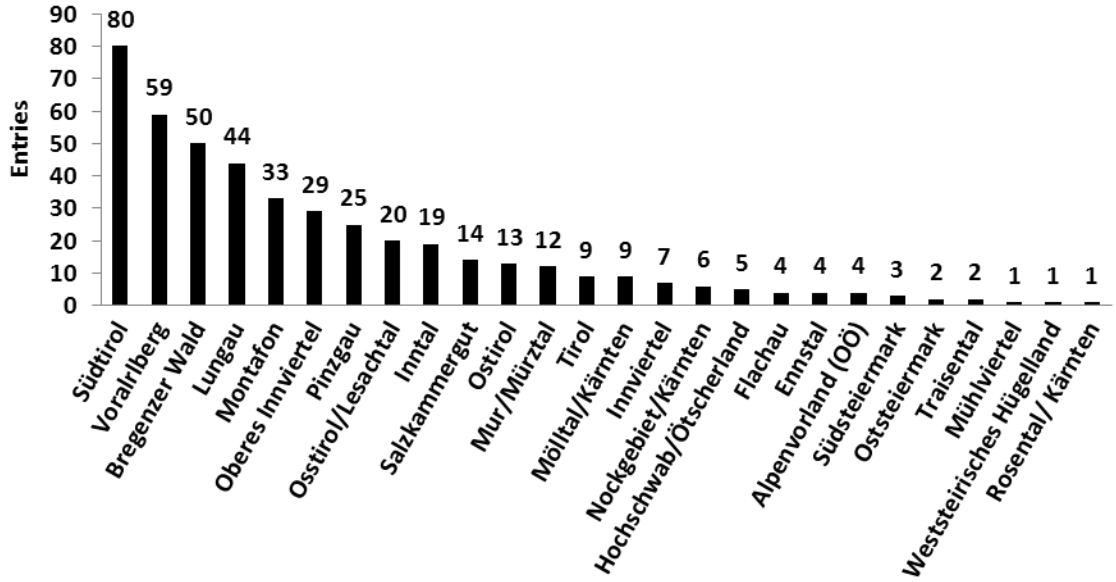


Fig. 5 Regions within Austria where *P. ostruthium* has been mainly used; modified from Gerlach (2008).

In Austrian traditional medicine several species of the genus *Peucedanum* have been used. The most common one is the masterwort *Peucedanum ostruthium*. Ointments and alcoholic extracts of the rhizomes have been externally applied for skin lesions, inflammation and rheumatism. Teas, liqueurs and wines have been prepared mainly for gastrointestinal diseases, for ailments of the cardiovascular system and the respiratory tract (Fig. 6) (Gerlach *et al.*, 2006).

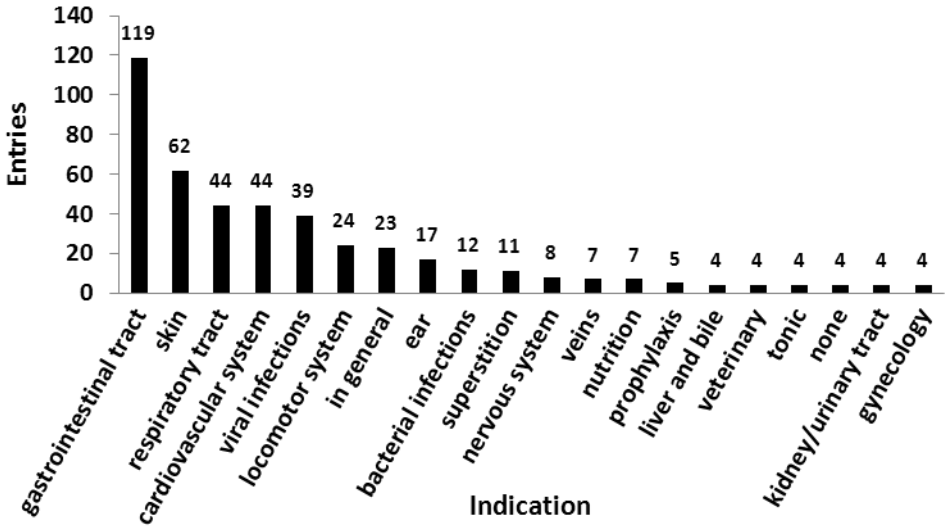


Fig. 6 Typical application fields of *P. ostruthium* in traditional Austrian medicine; modified from Gerlach (2008).

In a diploma thesis composed in the frame of the VOLKSMED database Kneip (1989) categorized the main fields of application of *Peucedanum ostruthium* into cardiotropic, antiphlogistic and for stomach and liver disorders. For ailments of the cardiovascular system wine made of *P. ostruthium* was traditionally applied if people suffered from circulatory disorders, calcification and varicose veins. Latter have also been treated with compresses of fresh leaves. Furthermore, masterwort root tea was ingested against stroke and alcoholic beverages produced from the herb were used for prevention and post-treatment of strokes. A tea of the roots should be helpful for purification of the blood and compresses of the leaves were prepared when people suffered from sepsis. Fresh roots were chewed whereas dried ones were fumigated against inflammatory diseases. Moreover, roots were taken into the mouth for ailments of the throat or after extraction of teeth. Wounds were healed with alcoholic beverages or compresses of leaves. Additionally, liqueurs and wines were applied to treat gastrointestinal diseases, either for the cattle (Kneip, 1989).

The masterwort was mentioned for the first time in the "Macer floridus" from the monk Odo von Meung (~ 840-1100) describing it as a universal remedy against liver dysfunctions, cough and breathing difficulties. In the 10th century the French physician Floridus recommended a plant called "struthion, ostruthium" against jaundice and other liver dysfunctions, internal stones, spitting of blood and leprosy (Madaus, 1976). However, the traditional usage of the masterwort was mainly documented in the herbal books of the 15th and 16th century.

Hildegard von Bingen recommended the plant if people suffered from fever, pneumonia and infectious diseases (Tschirch, 1917). In the middle age masterwort was used as remedy against infections of humans and animals, e.g. for foot-and-mouth disease. Paracelsus appreciated the masterwort for healing diverse ailments especially for liver dysfunctions like jaundice, but even as protection against pest, the Black Death. Wolff (Scrutinium Amuletorum medicum, 1690) recommended binding the cut drug to thumbs and toes against epilepsy. Leonhardt Fuchs wrote a whole chapter about the habitat, the time of harvest, the shape, the nature and the vigor of *P. ostruthium* in his book "The new herbal of 1543"(Kneip, 1989). Together with *Angelica* and *Levisticum* the masterwort was part of the „Spiritus carminativus Sylvii.

In the herbal books of the 20th century the usage is mainly described for gastrointestinal diseases (Hegi, 1906; Madaus, 1976), diarrhea and colic (Flamm *et al.*, 1940) but also against cold, fever, bronchial catarrh and asthma (Hegi, 1906; Madaus, 1976), and if women suffered from uteri spasm and dysmenorrhea (Flamm *et al.*, 1940).

Additionally, Madaus (1967) stated that, the masterwort should be helpful against urinary and kidney stones, atherosclerosis and to prevent strokes, to treat anti-inflammatory diseases like rheumatism or sciatica, if people suffered from diabetes and against pollinosis. According to Madaus (1976) ointments made from the roots or the leaves were used externally against tumors and to treat sepsis. Fresh leaves were applied on poorly healing sores. Far more abundant was the internal application of the underground parts of *P. ostruthium*. For this Madaus provided recipes concerning the preparation of the pharmaceutical drug, like maceration in liqueur and wine for bronchitis or teas prepared from powdered drug to heal gastrointestinal disorders.

Tschirch stated in his book „Handbuch der Pharmakognosie“(1917) that until 1882 the Pharmacopoeia germanica II as well as until 1893 the Pharmacopoeia helvetica III contained a monograph on the masterwort. The German Pharmacopeia from 1941 also contained a monograph on the masterwort in its 6th supplement.

Today, the pharmaceutical drug is described as grayish brown, annulated, longitudinal corrugated 4 to 8 cm long rhizomes. The elliptic to round cross section consists of a dark brown periderm and a white parenchyma with huge resin ducts (Kubelka *et al.*, 1984). The identity is checked via TLC (coumarin components show fluorescence under UV 365) (Hänsel *et al*., 2010). The essential oil content is in the range of 1.1-1.8 % (Cisowski *et al.*, 2001). The drug called “Radix Imperatoria” is about to be included in the Austrian Pharmacopeia again.

In Traditional Chinese Medicine (TCM) the hot and bitter drug belongs to the metal element associated with autumn, the lungs and large intestine, as well as to the fire element describing early summer, the heart, and small intestines (Bensky *et al.*, 2004).

In homeopathy remedies of masterwort are used against gastrointestinal and skin diseases.

1.1.3 Components

Various compounds have been identified in *P. ostruthium,* including flavonoids, phthalides and most notably are the high amounts of essential oil and coumarins (structures are shown in Tab. 21- Tab. 28, pp. 143- 150).

1.1.3.1 Essential oil

In the family of Apiaceae the essential oil components are one of the most investigated compound classes concerning the efficacy of medicinal drugs. Nevertheless, investigations concerning the volatile compounds of *P. ostruthium* are scarce. Cisowski *et al.* (2001) published an essential oil content of 0.9 % for the herb and 1.25 % for the rhizome (per dry weight basis). GC-MS and GC-FID analyses detected 39 compounds in the essential oil of the rhizomes, mainly comprised of sabinene (35.2 %) and 4-terpineol (26.6 %). In the herb 44 essential oil components were detected and 39 of them identified. The main compounds were β-caryophyllene (16.1 %) and α-humulene (15.8 %), followed by sabinene (4.7 %). The coumarin osthole was detected in both essential oils (5.5 % in the one of the herb and 5.1 % in the one of the rhizome).

1.1.3.2 Coumarins

Only few works have been published concerning the coumarin composition of *P. ostruthium*. 1932 Butenandt and Marten, described osthole, ostruthin and hydroxypeucedanin hydrate as constituents of the masterwort. Almost simultaneously Spath and Klager identified hydroxypeucedanin (1933) and ostruthin (1934). In 1996 the isolation and structure elucidation of two new coumarins, 6-(3-carboxybut-2-enyl)-7-hydroxycoumarin and the 3-acetate of oxypeucedanin hydrate, from the roots of *Peucedanum ostruthium* was published (Hiermann *et al.*, 1996). During a screening for new acetylcholinesterase (AChE) inhibitors the coumarins ostruthin, imperatorin, ostruthol, and oxypeucedanin hydrate and the chromone derivative peucenin were isolated from the DCM extract of *P. ostruthium* roots by bioassay guided fractionation (Urbain *et al.*, 2005). Reisch *et al.* (1975) separated the five nonglycosidic furanocoumarins oxypeucedanin, oxypeucedanin hydrate, tert-O-methyloxypeucedanin hydrate, isoxypeucedanin and a new furanocoumarin named pabulenone (Reisch *et al.*, 1975).

1.2 *Melampyrum pratense*

1.2.1 Botanical description

Melampyrum pratense L. is an annual herb that reaches up to 30 cm in height. It consists of narrow, lanceolate, decussate leaves, dentate hypsophylls and white flowers with a yellow pharynx, blooming between June and August (Fig. 7). This species preferably grows on acidic, meager grounds of woods, heaths and marshes. Although its name suggests that its growing on meadows it is one of the most abundant plants in Austrian forests, living mainly on spruces (Fischer *et al.*, 2008).



Fig. 7 Habitus of *Melampyrum pratense* L..
Original book source: Flora von Deutschland, Österreich und der Schweiz (Thomé, 1905).

Melampyrum pratense L. or common cow-wheat belongs to the family of Orobanchaceae. The genus *Melampyrum* includes about 10-20 species of herbaceous flowering plants, native to temperate regions of the Northern Hemisphere (Fig. 8). They grow as hemi parasites, which means that they are actually autotrophic plants but gain some mineral nutrients from other plants.

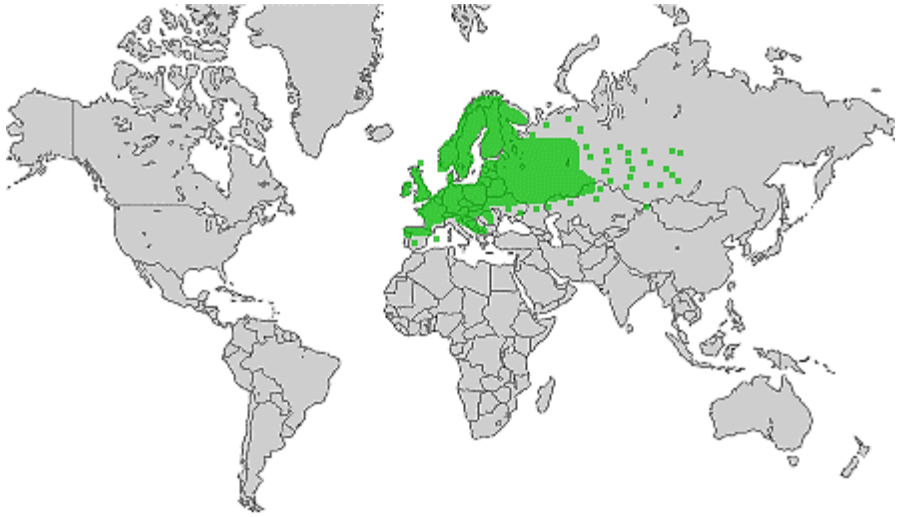


Fig. 8 Distribution of *Melampyrum pratense* L..
Source: http://www.wilde-planten.nl/meesterwortel.htm.

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Asterids*
Order	*Lamiales*
Family	*Orobanchaceae* – Broomrape family
Genus	*Melampyrum L*.
Species	*Melampyrum pratense* L. – Common cow- wheat

1.2.2 Traditional and pharmaceutical usage

In Cavan, England, the plant, also known as golden wheat, was recommended as decoction for kidney ailments (Maloney, 1972). The seeds "Semina Melampyri lutei" have been used as stimulating agent (Geiger, 1939). *Melampyrum* species are used in Romanian traditional medicine for rheumatic disorders and skin infections, suggesting an anti-inflammatory potential (Mogoşan *et al.*, 2008).

In Austria *Melampyrum* species have rarely been traditionally applied. This plant was chosen due to the very promising results of its relative genus *Euphrasia* (typical application fields see Fig. 9), which species are hard to define and laborious to gather. The high growing *M. pratense* on the other hand occurs in huge populations and is easy to collect. Additionally, after pharmacological testing, it turned out to be even more potent than *Euphrasia sp.*.

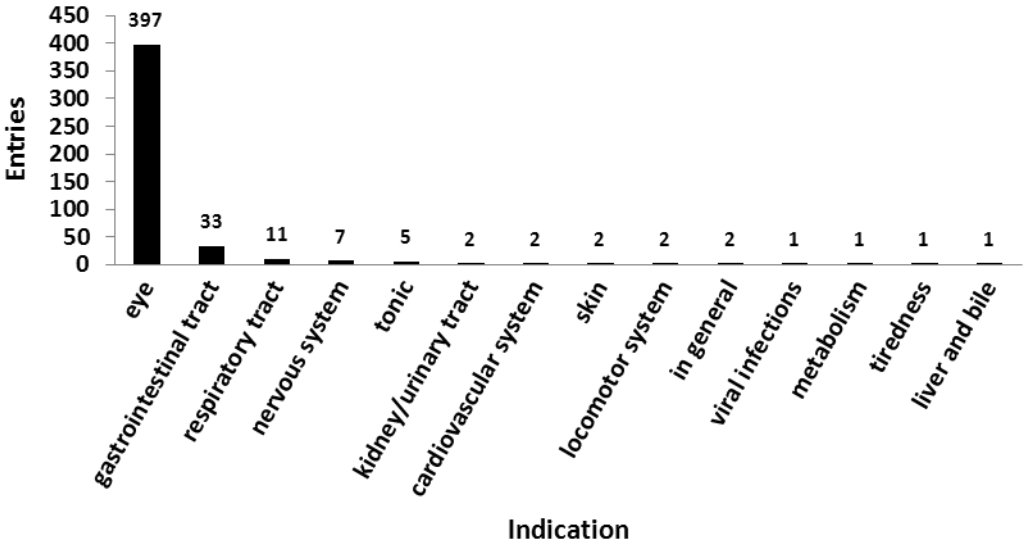


Fig. 9 Typical application fields of *Euphrasia sp.* in traditional Austrian medicine, modified from Gerlach (2008).

1.2.3 Components

There are some publications on the constituents of various *Melampyrum* species especially *M. arvense* but very little has been published concerning the compounds of *M. pratense* (chemical structures are given in Tab. 36- Tab. 40, pp. 186- 189).

Next to iridoids and flavonoids Kirmizibekmez *et al.* (2011) identified a lignan glycoside dehydrodiconiferyl alcohol 9-O-β-glucopyranoside, benzoic acid, β-sitosterol and a fatty acid mixture in *M. arvense*.

Flavonoids and phenolic acids isolated from the herbs of *M. pratense* and *M. nemorosum* growing in the Ural region have been examined by two-dimensional paper chromatography (Galishevskaya *et al.*, 2011). In this study 23 phenolic compounds including 17 flavonoids and 6 phenol carboxylic acids (e.g. chlorogenic, caffeic, and ferulic acid) have been identified in *M. pratense*.

Additionally, alkaloids, saponins and tannins have been found in *Melampyrum* species (Karimova *et al.*, 1966).

1.2.3.1 Iridoids

Kirmizibekmez *et al.* (2011) isolated aucubin, melampyroside, mussaenoside, mussaenosidic acid and 8-*epi*-loganin from the water subextract of *M. arvense*. The aucubin content was determined in *M. arvense* as 2.68 %, *M. nemorosum* as 0.43 % and *M. pratense* as 5.11 %, respectively (Broda *et al.*, 1969). Suomi *et al.* (2001) quantified iridoidglycosides in *M. pratense* and *M. sylvaticum* using optimal on-line combination of partial filling micellar electrokinetic chromatography and electrospray ionization mass spectrometry (ESI-MS). In both species traces of catalpol and aucubin (ca. 100 mg/L), were found but no ketologanin, verbenalin or 10-cinnamoyl catalpol, detected in other Orobanchaceae. Loganin was only identified in *M. sylvaticum* (ca. 40 mg/L) (Suomi *et al.*, 2001).

1.2.3.2 Flavonoids

Cinaroside, luteolin, quercitrin and hyperoside have been identified on TLC using reference compounds. The flavonoid content, measured by HPLC, in various organs varied within 0.08-3.17 % for *M. pratense* and 0.04-2.29 % for *M. nemorosum* with maximum content in the generative organs and minimum in the roots (Galishevskaya *et al.*, 2011). Apigenin and luteolin 7-O-β-glucopyranoside have been isolated from the water extract of *M. arvense* (Kirmizibekmez *et al.*, 2011).

1.3 Targeting inflammation

Inflammation is the body's reaction to exogenous stimuli, which can be physical e.g. mechanical lesions and irradiations, or chemical, caused by infectious agents and by malignant or altered normal cells. It includes cross-linking of diverse vascular and cellular reactions as well as antigen specific and unspecific immune defense. Cardinal symptoms are *calor* (heat), *tumor* (edema), *rubor* (redness), *dolor* (pain), and *functio laesa* (loss of function).

The inflammatory pathway consists of four components: inflammatory inducers like lipopolysaccharides (LPS) or tumor necrosis factor-α (TNF-α), the sensors, such as toll-like receptors (TLRs), detecting them, the inflammatory mediators induced by the sensors on mast cells, dendritic cells and macrophages, and the target tissues that are affected by the inflammatory mediators. The types of cells migrated to the site of inflammation and the pathway induced depends on the nature of the inflammatory trigger. Neutrophil infiltration is a hallmark of acute inflammation whereas the characteristic cell components of chronic inflammation are macrophages, lymphocytes and plasma cells. Bacterial pathogens are detected by receptors of the innate immune system, such as TLRs (Medzhitov, 2010).

The inflammatory process starts with an increase in vascular permeability and local vasodilation (acute phase). Subsequently, leukocytes from the blood infiltrate into the tissues (subacute phase). Finally, in the chronic proliferative phase, tissue degeneration and fibrosis take place. Each of these phases includes different mediators. Histamine, produced by mast cells, leads to an increase in diameter and permeability of blood vessels. Serotonin, released by thrombocytes, effects their aggregation and mediates hyperalgesia while the complement factors C3a and C5a evoke the release of histamine. The production of nitrogen monoxide (NO), bradykinin and prostaglandins also leads to vasodilation and hyperalgesia. Furthermore, leukotrienes as well as platelet-activating factors (PAFs) are generated by granulocytes, leucocytes and mast cells. Proteases and cytokines are released and reactive oxygen species (ROS) are exerted to kill bacteria.

Although there are various molecular mediators involved in the development of the inflammatory response, many pro-inflammatory pathways converge at the point of the activation of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB).
All these processes are essential for the survival of the organism as they include the fight against pathogens and restoring tissue homeostasis. Nevertheless, they can become harmful when the response is exaggerated and sustained without an apparent benefit (Barreiro *et al.*, 2010; Mutschler *et al.*, 2001).
Various pathological conditions like arthritis, atherosclerosis, the metabolic syndrome, sepsis, allergies and auto-immune diseases or cancer involve inflammation. For most of these conditions no satisfying treatment of the inflammatory part is available. General treatment relies on steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs). The latter lead to the inhibition of cyclooxygenase (COX) an enzyme involved in the formation of prostaglandin H_2. COX exists in two isoforms having different functions. COX-1 the constitutive expressed enzyme is responsible for the synthesis of prostaglandins in many cells and tissues e.g. in kidneys and thrombocytes. It is also expressed in gastric epithelial cells where it functions as the major source of the beneficial cytoprotective prostaglandin formation. Thus, inhibition of COX-1 in the gastrointestinal tract accounts for the gastric adverse effects of NSAIDs. COX-2 production is immediately induced by cytokines and other inflammatory mediators and therefore highly expressed in case of inflammation and pain reactions. COX-2 selective inhibitors lead to antiphlogistic, analgetic and antipyretic effects of NSAID. However, studies indicate that COX-2 is also constitutively produced in kidney, uteri and the spinal cord and inhibition of it appears to cause severe cardio-vascular side effects (Burke et al., 2006). Therefore, the search for new anti-inflammatory drugs still represents an important field in drug discovery.

In the present study a panel of functional cell models were used for the identification of active natural products that interfere with TNF-α or LPS-induced expression of adhesion molecules (E-selectin) or IL-8, inhibit the NF-κB pathway and/or activate nuclear receptors (PPARs, LXR, FXR).

1.3.1 Nuclear factor κB (NF-κB)

NF-κB is a transcriptional protein complex that is ubiquitously expressed and critically involved in the inflammatory and innate immune responses, but also in proliferation and cell survival/apoptosis (Hayden *et al.*, 2011; Lawrence, 2009; Sethi *et al.*, 2008). It regulates the expression of an array of inflammatory response genes such as cytokines (e.g. TNF, IL-1β, IL-6/10/12, IFN-γ), chemokines (e.g. monocyte chemotactic protein-1 - MCP-1), mediator producing enzymes (e.g. COX-2, arachidonate 5-lipoxygenase, inducible nitric oxide synthase), adhesion molecules (e.g. E-selectin, ICAM-1), enzymes for extracellular matrix degradation (e.g. matrix metallopeptidase 9), as well as regulators of apoptosis (e.g. inhibitors of apoptosis) or cell proliferation (e.g. cyclin D). Therefore, targeting different steps of NF-κB signaling cascade is considered to be a good approach to combat many different inflammatory conditions (Lawrence *et al.*, 2010).

In its inactive form NF-κB is sequestered in the cytosol by interactions with IκB (inhibitor of NF-κB) proteins. Stimulation of the cell with a NF-κB activator (e.g. pro-inflammatory cytokine such as IL-1 or TNF-α), initiates signal transduction cascades that ultimately result in phosphorylation and subsequent degradation of IκB, leading to release and nuclear translocation of NF-κB. Phosphorylation of IκB occurs by the IκB kinase (IKK) complex.

Fig. 10 shows the different pathways leading to NF-κB activation. The mammalian NF-κB family consists of p50 (also known as NF-κB1), p52 (also known as NF-κB2), p65 (also known as ReLA), ReL and ReLB. These five members exhibit unique functions which analysis is complicated as they form homo- and heterodimers masking their unique roles in knockout animals (Hayden *et al.*, 2008). The inflammatory response depends on the pro-inflammatory transcriptional programs regulated by NF-κB and their inducing stimulus. Therefore, induction by LPS can lead to the expression of genes different to those transcribed upon TNF-α induction (Ghosh *et al.*, 2008).

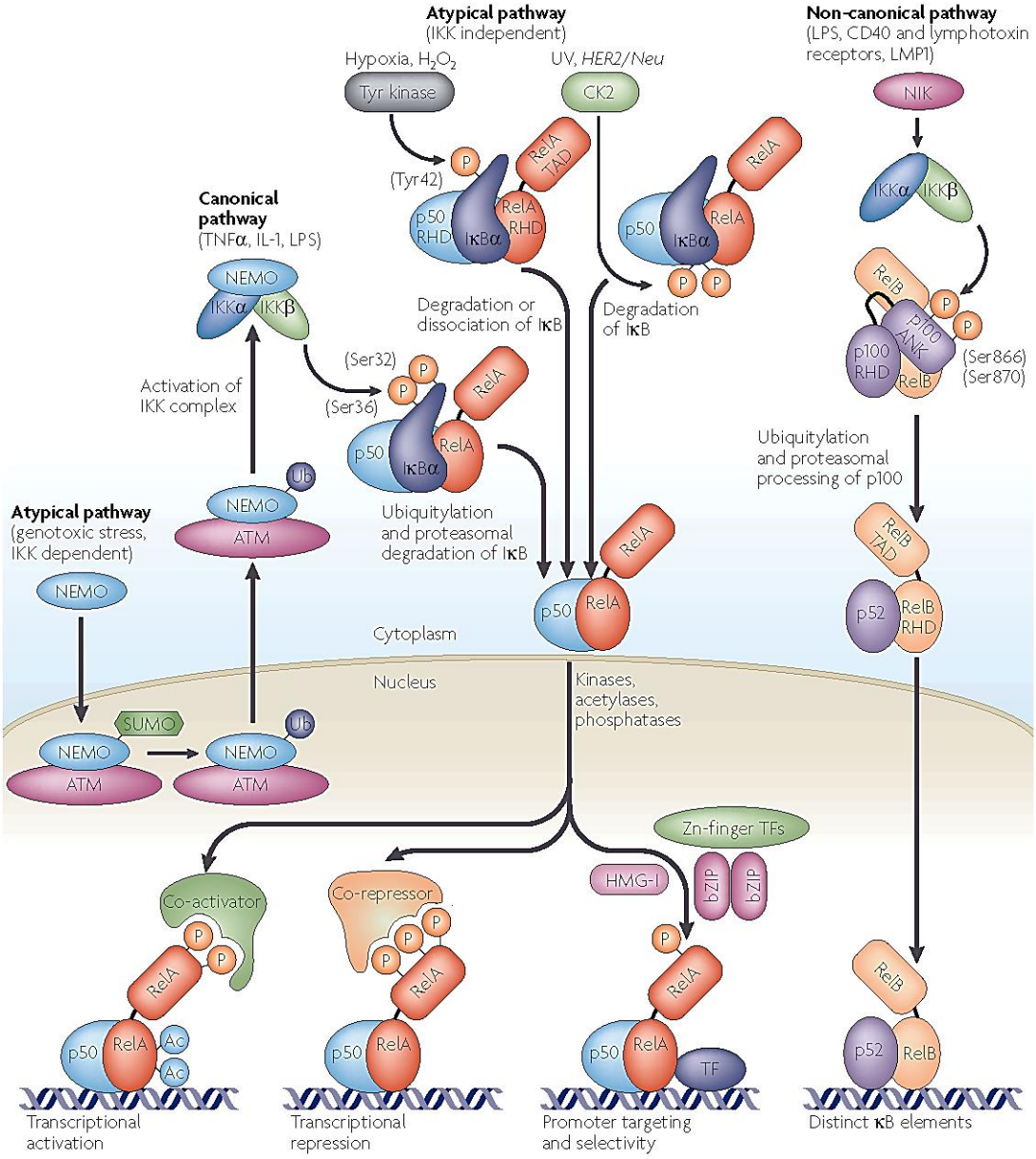


Fig. 10 Pathways leading to activation of NF-κB (Perkins, 2007).

The canonical pathway is induced by tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and many other stimuli. It is dependent on activation of IKKβ, resulting in the phosphorylation of IκBα which subsequently is degraded. Consequently, NF-κB complex is released and can relocate to the nucleus. The IKK-dependent activation may also be promoted via genotoxic stress. Here, NF-κB essential modifier (NEMO) migrates to the nucleus, where it is sumoylated and then ubiquitylated, transmigrates back to the cytoplasm together with ataxia telangiectasia mutated (ATM) checkpoint kinase, and activates IKKβ. The atypical IKK-independent pathways of NF-κB activation include casein kinase-II (CK2) and tyrosine-kinase-dependent pathways. The non-canonical pathway starts with activation of IKKα by the NF-κB-inducing kinase (NIK), followed by phosphorylation of the p100 NF-κB subunit. Consequently, p100 is processed to p52, which can lead to the activation of p52–RelB heterodimers that translocate to the nucleus and target distinct κB elements.

1.3.2 Pro-inflammatory mediators

Many of the inflammatory processes are mediated by the transcription factor NF-κB. Major target genes of NF-κB include those for transcription of adhesion molecules (e.g. E-selectin), cytokines (e.g. TNF-α), chemokines (e.g. IL-8), growth factors, and enzymes like COX-2 producing inflammatory mediators. Most prominent inducers of inflammation are cytokines such as TNF-α and IL-1, as well as components released by bacteria such as LPS. Fig. 11 gives an overview of the signaling cascade of the inflammatory process and the involved pro-inflammatory mediators.

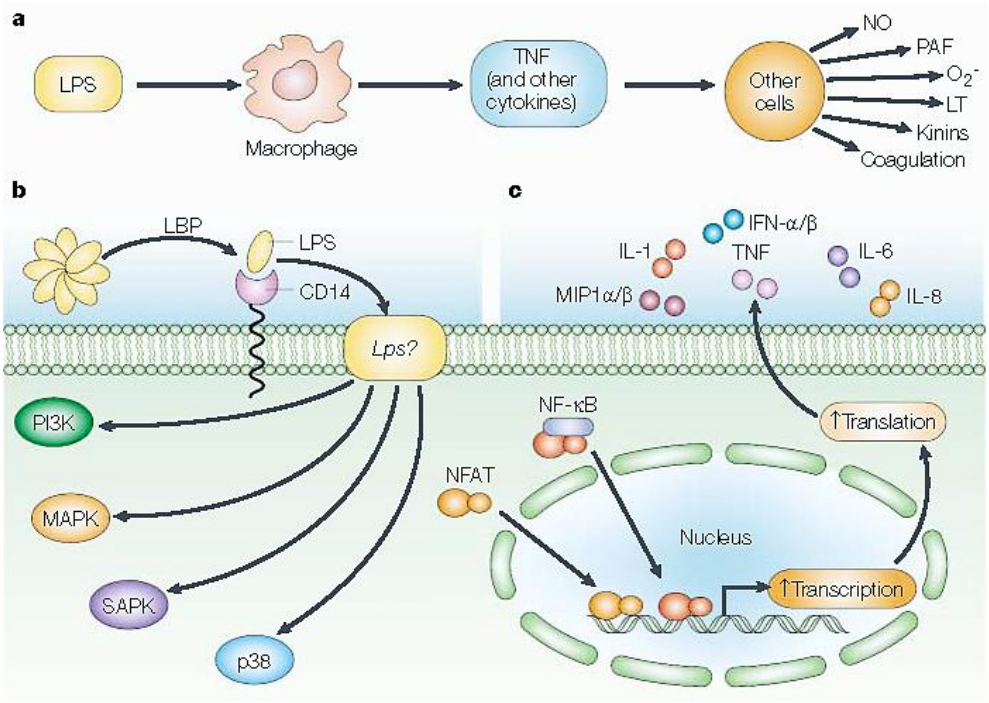


Fig. 11 Signaling cascade of the inflammatory process and its pro-inflammatory mediators. (Beutler *et al.*, 2003).The endotoxin lipopolysaccharide (LPS) is recognized by macrophages, leading to the release of tumor-necrosis factor (TNF), a soluble mediator of LPS toxicity **(a)**. LPS-binding protein (LBP), which binds plasma LPS and conveys it to the surface of cells in a bioactive form and CD14 are extracellular components of the LPS-sensing pathway. Additionally, LPS is responsible for the activation of several protein kinases (PI3K - phosphatidylinositol 3-kinase; MAPK - mitogen-activated protein kinase; SAPK - stress-activated protein kinase) **(b)**. The main transcription factor for induction of the gene encoding TNF is the nuclear factor-κB - NF-κB. It leads to the transcription of several pro-inflammatory cytokines (interferon - IFN α/γ, interleukin - IL-1 and 6) and chemokines (IL-8 and MIP1 - macrophage inflammatory protein 1) **(c)**. LT - lymphotoxin; NFAT - nuclear factor of activated T cells; NO - nitric oxide; PAF - platelet-activating factor.

1.3.2.1 Lipopolysaccharide (LPS)

Lipopolysaccharides are cell wall polymers of gram-negative bacteria released after their decomposition. In the host they act as endotoxins stimulating macrophages to release pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, TNF-α and platelet-activating factor (PAF).

LPS as a prototypical endotoxin binds the CD14/TLR4/MD2 receptor complex in various cell types. Several LPS receptors have been identified in different macrophages upon which CD14 is the most thoroughly studied. Bacterial LPS typically consist of a hydrophobic domain known as lipid A, or endotoxin, a nonrepeating "core" oligosaccharide, and a distal polysaccharide also known as O-antigen (Raetz *et al.*, 2002). The O-antigen is a repetitive glycan polymer comprising the outermost domain of the LPS molecule which varies from strain to strain. Besides oligosaccharides directly attached to lipid A the core domain usually contains sugars such as heptose and non-carbohydrate components e.g. phosphate or amino acids. The lipid A structure, a phosphorylated glucosamine disaccharide with multiple fatty acids, is known to be very variable, expressing properties ranging from LPS agonists, through weak endotoxicity to LPS antagonists (Stewart *et al.*, 2006).

1.3.2.2 Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α) is a key cytokine that has been shown to play important physiologic (e.g., inflammation) and pathophysiologic (e.g., various liver pathologies) roles. It was identified in 1975 as an endotoxin-induced glycoprotein, which caused haemorrhagic necrosis of sarcomas in mice (Carswell *et al.*, 1975). TNF-α induces three major cellular responses, namely the cell survival through NF-κB activation, apoptosis, and necrosis. It has been related to a diverse range of inflammatory, infectious and malignant conditions. The efficacy of anti-TNF-α antibodies or administration of soluble TNF-α receptors (TNFRs) in controlling disease e.g. rheumatoid arthritis and other inflammatory conditions indicates its importance in drug therapy (Bradley, 2008).

The two different receptors for TNF-α, TNF-R1 and TNF-R2 are differentially expressed on cells and tissues and initiate distinct as well as overlapping signal transduction pathways. TNF-R1 can be found in most tissues, and is activated by both the membrane-bound and the soluble trimeric forms of TNF-α. TNF-R2, on the other hand is only expressed in cells of the immune system, and responds to the membrane-bound form of the TNF-α homotrimer. Studies have shown that the pro-inflammatory and the programmed cell death pathways, activated by TNF-α and associated with tissue injury, are mediated through TNF-R1. The role of TNF-R2 signaling is less well characterized, but TNF-R2 has been shown to mediate signals promoting tissue repair and angiogenesis (Bradley, 2008).

The TNF-α receptor system has a prominent role in the pathogenesis of chronic inflammatory and autoimmune disorders. The central role of TNF-α in drug discovery has been demonstrated by the ability of agents that block the action of TNF-α to treat a variety of human diseases, including many inflammatory conditions like rheumatoid arthritis (Segal *et al.*, 2008), ankylosing spondylitis, inflammatory bowel disease and psoriasis (Bradley, 2008) as well as major depression (Dowlati *et al.*, 2010), Alzheimer's disease (Swardfager *et al.*, 2010), and cancer.

1.3.2.3 E-selectin

During inflammation, expression of E-selectin on endothelial cells is induced by the release of cytokines, like IL-1 and TNF-α by damaged cells.

E-selectin, like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) belongs to the endothelial adhesion molecules. It plays an important role in recruiting leukocytes to the site of injury as it recognizes and binds to sialylated carbohydrates present on their surface proteins. Additionally, E-selectin and P-selectin bind to their ligands on neutrophils inducing rolling behavior on the endothelial surface (Fig. 12, p. 20).

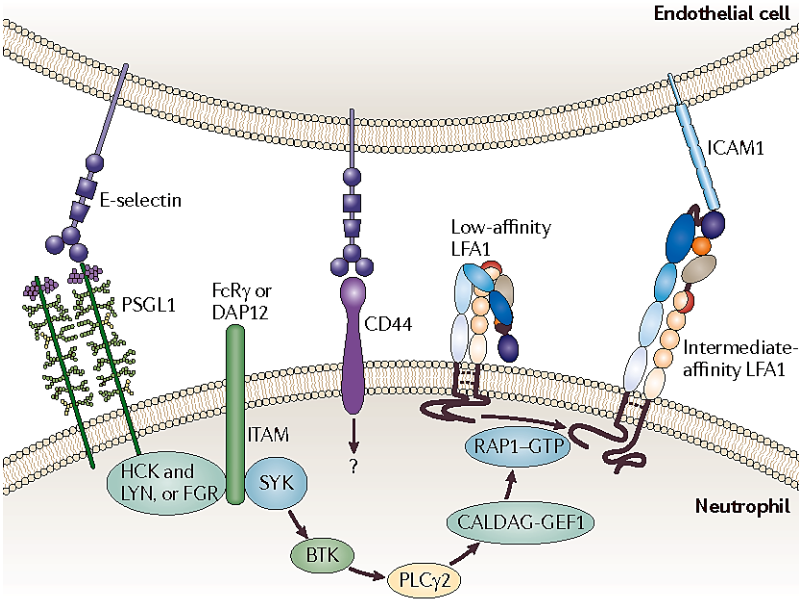


Fig. 12 E-selectin signaling cascade (Hogg *et al.*, 2011). Signaling from E-selectin to its ligands P-selectin glycoprotein ligand 1 (PSGL1) and CD44 on neutrophils gives rise to a defined pathway consisting of a SRC family tyrosine kinase (FGR, or HCK and LYN), an immunoreceptor tyrosine-based activation motif (ITAM)-containing protein (Fc receptor γ-chain (FcRγ) or DAP12) and spleen tyrosine kinase (SYK). Downstream of SYK lies the TEC family kinase Bruton's tyrosine kinase (BTK), which activates phospholipase Cγ2 (PLCγ2). Recent findings indicate CALDAG-GEF1 (Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor 1; also known as RASGRP2) and RAP1 playing a role downstream of SYK. ICAM1 - intercellular adhesion molecule 1; LFA1 - lymphocyte function-associated antigen 1 (also known as αLβ2 integrin).

1.3.2.4 Interleukine-8 (IL-8)

Interleukins belong to the cytokines, secreted proteins or signaling molecules. The majority of interleukins are synthesized by helper $CD4^+$ T lymphocytes, as well as through activated monocytes, endothelial cells, keratinocytes and fibroblasts.

Interleukin-8 (IL-8), a prototype of CXC chemokines, was discovered in 1987 (Baggiolini *et al.*, 1992). It is produced by stimulated monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, chondrocytes and a number of tumor cell lines. It mainly acts as a polynuclear neutrophil attractant on T-lymphocytes and basophils (chemotactic activity) and its production can be induced by TNF-α and IL-1 (Baggiolini *et al.*, 1995; Schroeder, 1992). Additional stimulants of the synthesis of IL-8 are bacterial lipopolysaccharides, concanavalin A, double-stranded RNA, phorbol esters and viruses.

From the several receptors capable to bind IL-8 the most frequently studied types are the G protein coupled serpentine receptors CXCR1 and CXCR2 (Mukaida *et al.*, 1998). The function of IL-8 is not species-specific as it acts in humans as well as in rodent and rabbit cells. Its activity on neutrophil granulocytes makes it different to all other cytokines. There it leads to an increase in cytosolic calcium levels and the release of enzymes. It promotes the production of reactive oxygen species, it is chemotactic for all known types of migratory immune cells e.g. granulocytes, endothelial cells, macrophages, mast cells and keratinocytes (Schroeder, 1992). Its anti-inflammatory activities are related to the fact that it is able to inhibit the adhesion of leukocytes to activated endothelial cells. It plays a key role on the pathogenesis and development of inflammation and cancer. It is present in a number of diseases with neutrophil infiltration, such as gingivitis, psoriasis or rheumatoid arthritis (Broaddus *et al.*, 1996).

1.3.3 Nuclear receptors

Important mediators inhibiting NF-κB responses are nuclear receptors, among those the glucocorticoid receptor, the peroxisome proliferator-activated receptors (PPARs), the farnesoid X receptor (FXR), the liver X receptor (LXR) and the orphan nuclear receptors class 4A (NR4A). Nuclear receptors are thought to exert their anti-inflammatory activities by repressing the activity of other transcription factors, such as STAT or AP1 proteins, both involved in the induction of a variety of pro-inflammatory genes (Bamborough *et al.*, 2009; Brasier, 2010; Glass *et al.*, 2006; Karin *et al.*, 2004).

1.3.3.1 Peroxisome proliferator-activated receptors (PPARs)

Next to NF-κB, another ubiquitously expressed class of transcription factors that are relevant regulators of the inflammatory response are the nuclear receptors (Glass *et al.*, 2006). Recently, much attention has been drown to the peroxisome-proliferator-activated receptors (PPARs), and liver X receptors (LXRs), controlling the expression of inflammatory and metabolic genes in macrophages and lymphocytes (Chawla, 2010; Huang *et al.*, 2010; Neve *et al.*, 2000).

PPARs are ligand-activated transcription factors exerting important functions in lipid and glucose and glucose metabolism, as well as in inflammation (

Fig. 13).

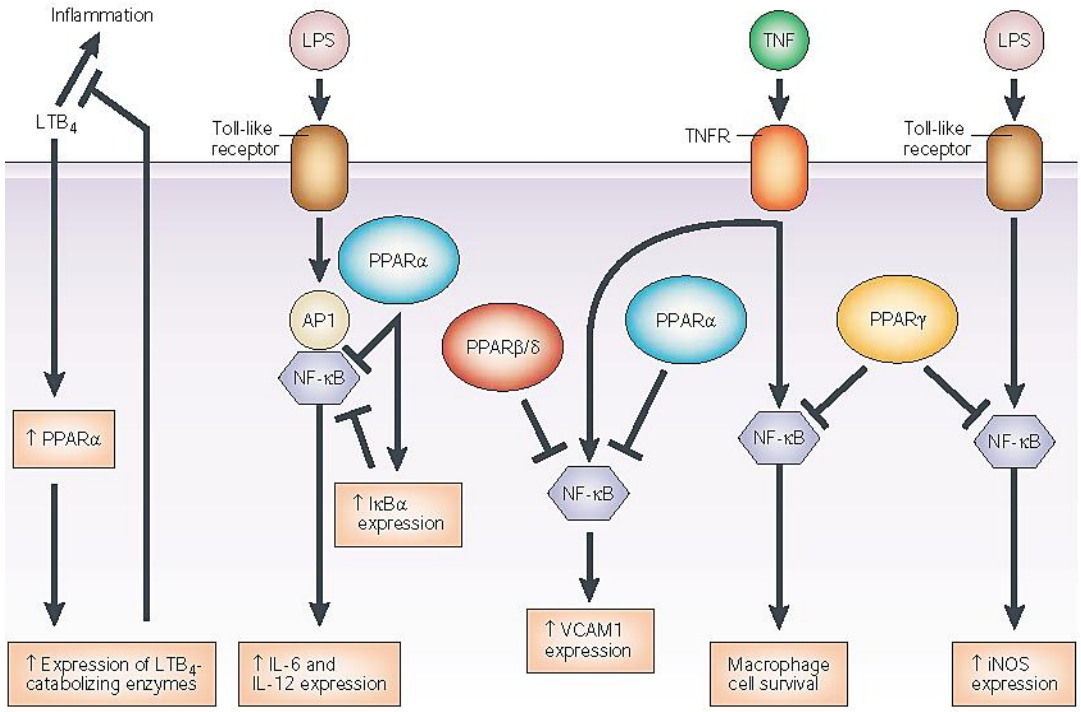


Fig. 13 Molecular mechanisms of PPAR-mediated regulation of inflammatory responses. (Daynes et al., 2002). The peroxisome proliferator-activated receptors (PPARs) regulate inflammatory responses via transactivation and transrepression. In most cases they exert their anti-inflammatory properties by antagonizing nuclear factor-κB (NF-κB) and AP1 signalling pathways. By inhibiting NF-κB and AP1, the PPARs repress the expression of several genes that are involved in the inflammatory response, including cytokines, cell-adhesion molecules and other pro-inflammatory signal mediators, such as inducible nitric oxide synthase (iNOS). PPARα has also been reported to control the duration and magnitude of the inflammatory response through its ability to induce expression of genes encoding proteins that are involved in the catabolism of pro-inflammatory lipid mediators. LPS - lipopolysaccharide; TNF - tumour-necrosis factor; TNFR - TNF receptor; IκBα - inhibitor of NF-κB, α; LTB4 - leukotriene B4; IL – interleukin; VCAM1 - vascular cell adhesion molecule 1.

There are three subtypes of this nuclear receptor known, namely PPAR-α, -β/δ, and -γ. PPAR-α, highly expressed in the vascular wall, skeletal muscle, heart, liver, and kidney is mainly involved in the regulation of lipid catabolism (Fruchart, 2009). Its activation was also shown to inhibit proliferation (Gizard *et al.*, 2008a; Gizard *et al.*, 2008b) and to modulate the inflammatory response of vascular smooth muscle cells (VSMCs).

PPAR-β/δ can be found in various tissues but mainly in brain, adipose tissue, and the skin. It is involved in the regulation of lipid metabolism and energy expenditure (Yumuk, 2006) as well as in inflammation (Chawla, 2010). Via alternative splicing after transcription, PPAR-γ exists in three isoforms. PPAR-γ1 is more or less abundant in all tissues, including heart, muscle, colon, kidney, pancreas, and spleen. The 30 amino acids longer PPAR-γ2 is mainly expressed in adipose tissue. PPAR-γ3 can be found in macrophages, large intestine and white adipose tissue. The activation of PPAR-γ increases insulin sensitivity and contributes to the regulation of genes involved in inflammation, hypertension, and atherosclerosis (Glass *et al.*, 2006; Glass *et al.*, 2010; Li *et al.*, 2004; Pascual *et al.*, 2007).

Once activated by their ligands, the PPARs translocate into the nucleus, form heterodimers with another member of the nuclear receptors super-family, the retinoid X receptor (RXR), and subsequently bind to PPAR response elements (PPREs) located in the promoter regions of PPAR-responsive target genes.

To avoid side effects like those of the common PPAR-γ agonist thiazolidinedione the development of new PPAR ligands is of high interest. Promising strategies to achieve that goal are the development of dual PPAR-α/-γ agonists, PPAR-α/-β/-γ pan-agonists, or partial agonists of PPAR-γ (Yumuk, 2006).

1.4 Objective

The aim of this study was to find active natural compounds of traditionally used plants from Austrian folk medicine exerting anti-inflammatory effects. The obtained results should contribute to a better understanding of the efficacy of some plant extracts used in Austrian ethnomedicine. In the course of a bioassay-guided fractionation the biological activity of extracts, fractions and compounds of highly promising plants identified should be determined. LC-MS, GC-MS, and different NMR-techniques were used for the chemical identification of the active or lead compounds. In particular, *Peucedanum ostruthium* (L.) Koch as well as *Melampyrum pratense* L. were investigated for their *in vitro* anti-inflammatory modes of action.

The evaluation of the pharmacological activity and the mechanism of action on the molecular level of selected bioactive constituents by multidisciplinary research activities (*in vitro* enzyme- or cell-based systems, *in vivo* models, virtual bioactivity profiling) should lead to the identification of new drug scaffolds. Moreover, natural products that are used for mechanistic studies could contribute to a better understanding of diverse signaling pathways involved in certain inflammatory disorders.

Chronic vascular inflammatory diseases are assumed to be the main cause of death and morbidity in the Western world and are expected to be the number one global killer by 2020 (Lopez Alan *et al.*, 2006). Therefore, inflammatory diseases impose an enormous burden on mankind. Although a multitude of inducers of inflammatory responses are known, there is much less information about inhibitors of this process. Several ethnopharmacological approaches claim success in treatment of inflammation, lacking however the objective scientific evaluation of their efficacy. Still, the variety of extracts of botanic origin used in traditional medicine makes this field attractive for research directed at identification of natural anti-inflammatory compounds with unknown mechanisms of action, which may find controlled therapeutic application. In the end we attempted to analyze natural plant materials starting from the complex raw extracts, following their activity changes during successive steps of separation right up to the isolation of single active compounds evaluating the multifaceted regulatory mechanisms reflecting the complexity of their composition.

2 Materials and Methods

2.1 The VOLKSMED-DATABASE

The starting point of our work was the so called VOLKSMED-database (Gerlach *et al.*, 2006) that has been completed up to now. This database (DB) was created by gathering information about traditionally used plants in Austria and the immediate vicinity (Fig. 14 and Tab. 1, p. 26). In this course 1857 persons were interviewed about their customs in the use of medicinal plants in a time frame from 1983 to 1995. The DB only refers to knowledge that had been passed on from generation to generation and does not lean on any existing literature. Besides an exact botanical identification, it contains information about plant parts used, preparations and application methods, internal or external use, indications, and the number of citations. Some examples for traditionally used plants listed in the VOLKSMED-database are listed below:

- Extracts of the leaf of *Plantago lanceolata*, for example, were traditionally recommended when people suffered from cold or chronic bronchitis. Fresh leaves of this plant species have also been used for wound healing and against diarrhea.
- Extracts from *Euphrasia officinalis* were applied externally for eye infections, like conjunctivitis and for skin inflammations but also internally to treat colds, flu, bronchitis or sore throat.
- People used the fruits of *Berberis* for lung, liver and bowl diseases.
- Extracts from *Symphytum officinale* roots and leaves were known to be effective against bone and joint conditions, inflammation, swelling, sprains and skin problems.

The most cited species in the course of inflammation were the leaves of *Salvia officinalis* (1100 citations) and the roots of *Symphytum officinale* (1010 citations). Related taxons/genera (e.g. *Epilobium montanum* to *E. parviflorum* and *E. angustifolium* or *Melampyrum* to *Euphrasia*), as well as various plant organs (e.g. also the leaves of *Geum urbanum)* were sometimes included, in order to understand relationship of different species and to eventually find a higher diversity of active compounds.

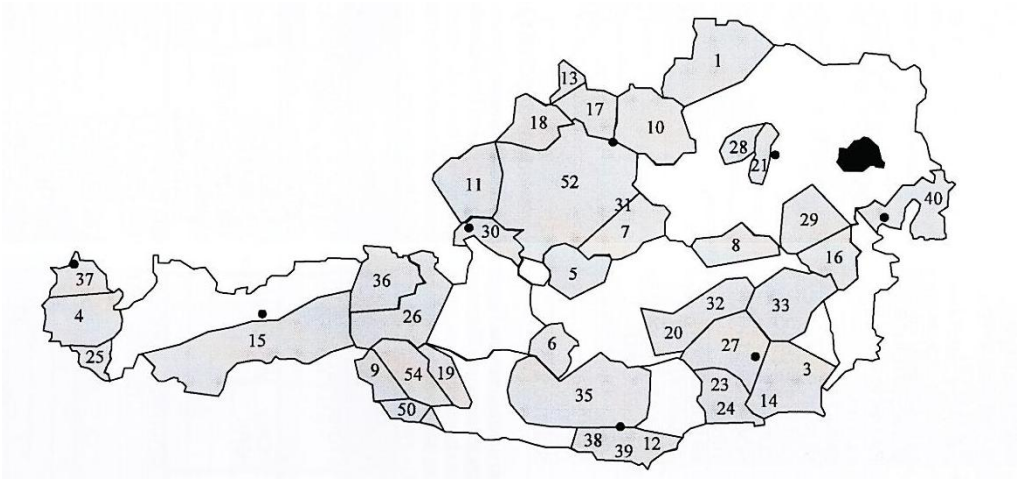


Fig. 14 Map of Austria showing locations where interviews concerning the traditional knowledge on Austrian ethnomedicine have been performed, numbers refer to regions indicted in Tab. 1.

thesis	region
1	Waldviertel
3	Südsteiermark
4	Vorarlberg
5	Salzkammergut
6	Lungau
7	Oberösterreichisches Alpenvorland
8	Hochschwab Ötscherland
9	Osttirol
10	Unteres Mühlviertel
11	Oberes Innviertel
12	Unterkärnten
13	Oberes Mühlviertel
14	Weststeirisches Hügelland
15	Inntal
16	Bucklige Welt
17	Mühlviertel
18	Innviertel
19	Mölltal/KTN
20	Mur/Mürztal
21	Traisental/NÖ
23	Südsteiermark

thesis	region
24	Südsteiermark
25	Montafon
26	Pinzgau
27	Zentrale Steiermark
28	Dunkelsteiner Wald
29	Rax/Schneeberg
30	Flachau
31	Ennstal
32	Mur/Mürztal
33	Oststeiermark
34	Südtirol
35	Nockgebiet/KTN
36	Tirol
37	Bregenzer Wald
38	Rosental/KTN
39	Südkärnten/Osten
40	Burgenland
50	Osttirol/ Lesachtal
52	Innviertel
54	Osttirol
55	Südtirol

Tab. 1 List of regions where interviews for the VOLKSMED DB were performed (Gerlach, 2008).
NÖ - Lower Austria, KTN - Carinthia.

2.2 Literature research

In-depth literature research concerning 226 drugs of 150 plant species which were reported in the VOLKSMED-database as traditionally used anti-inflammatory agents has been carried out. Therefore, the databases Chemical Abstracts (CAPLUS) and Medline were scanned via the user interface SciFinder Scholar. Search criteria were the name of the plant species or genera defined with terms concerning inflammation (antiphlogistic, anti-inflammatory, etc.) in order to avoid the reinvestigation of already well-known plants.

Permanent literature research was conducted to find suitable detection systems (HPLC, TLC, etc.) for the selected plants *M. pratense* and *P. ostruthium*, constituents already identified in those species and state of the art literature. Therefore further databases, e.g. ISI Web of knowledge, SCOPUS, BIOSIS and Google Scholar were searched.

2.3 Plant material

24 plant samples were field-collected and another 26 drugs were purchased from different companies in Austria and Germany (Tab. 2 - Tab. 4, pp. 28-30). The plant material was identified by macroscopic and microscopic methods. In close cooperation with a project partner from the Institute of Botany and Pharmacognosy, Veterinary University of Vienna, new methods like DNA fingerprinting were applied for the characterization of selected species (*Peucedanum ostruthium*, *Melampyrum pratense*). Voucher specimens of all species are deposited at the Department of Pharmacognosy, University of Vienna, Austria.

The field collected plant material was air-dried under light exclusion and at room temperature, to avoid the development of compound artifacts due to chemical reactions via enzymatic degradation.

Family	Species	Plant part	Sample date	Reference source/ sample location
Apiaceae	*Angelica archangelica*	root	31.03.2008	Kottas Pharma GmbH, Vienna, Austria
Apiaceae	*Angelica sylvestris*	root	09.07.2008	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Apiaceae	*Peucedanum ostruthium*	root	31.03.2008	Kottas Pharma GmbH
Apiaceae	*Peucedanum ostruthium*	leaf	19.08.2008	Hochwechsel/ Steyersberger Schwaig, Lower Austria (NÖ)
Apiaceae	*Pimpinella major*	root	07.08.2008	Ebenwald Kienstein, NÖ
Apiaceae	*Sanicula europaea*	root	29.05.2008	Jubiläumswarte, Vienna, Austria
Malvaceae	*Malva sp.*	leaf	31.03.2008	Kottas Pharma GmbH
Malvaceae	*Malva neglecta*	herb	12.08.2008	Nodendorf (Ernstbrunn), NÖ
Malvaceae	*Tilia sp.*	herb	31.03.2008	Kottas Pharma GmbH
Onograceae	*Circea lutetiana*	herb	11.08.2008	Sagberg, Lower Austria
Onograceae	*Epilobium angustifolium*	herb	31.03.2008	Kottas Pharma GmbH
Onograceae	*Epilobium montanum*	herb	10.09.2008	Hochwechsel/ Steyersberger Schwaig, NÖ
Onograceae	*Epilobium parviflorum*	herb	31.03.2008	Kottas Pharma GmbH
Orobancha-ceae	*Euphrasia officinalis*	herb	10.09.2008	Hochwechsel/ Steyersberger Schwaig, NÖ
Orobancha-ceae	*Euphrasia rostkoviana*	herb	31.07.2008	Murtal (Hintermuhr), Lungau, Salzburg, Austria
Orobancha-ceae	*Melampyrum pratense*	herb	16.07.2008	Neustift am Walde Nord, Vienna, Austria
Orobancha-ceae	*Melampyrum sylvaticum*	herb	July 2008	Laab im Walde, NÖ
Plantagina-ceae	*Plantago lanceolata*	leaf	31.03.2008	Kottas Pharma GmbH
Plantagina-ceae	*Veronica chamaedrys*	herb	07.05.2008	Neustift am Walde, Vienna, Austria

Tab. 2 List of the investigated plant drug samples.

Family	Species	Plant part	Sample date	Reference source/ sample location
Plantaginaceae	*Veronica officinalis*	herb	19.06.2008	Neustift am Walde, Vienna, Austria
Plantaginaceae	*Veronica sp.*	herb	31.03.2008	Kottas Pharma GmbH
Polygonaceae	*Rumex alpinus*	leaf	10.09.2008	Die Hohe Student, Styria, Austria
Polygonaceae	*Rumex alpinus*	root	10.09.2008	Die Hohe Student, Styria, Austria
Rosaceae	*Agrimonia sp.*	herb	31.03.2008	Kottas Pharma GmbH
Rosaceae	*Filipendula ulmaria*	flower	31.03.2008	Kottas Pharma GmbH
Rosaceae	*Filipendula ulmaria*	leaf	28.07.2008 31.07.2008	Weißpriachtal, Salzburg, Austria Gontal, Carinthia, Austria
Rosaceae	*Filipendula vulgaris*	flower	19.06.2008	Neustift am Walde, Vienna, Austria
Rosaceae	*Geum montanum*	root	July 2008	Schattneralm, Styria, Austria
Rosaceae	*Geum urbanum*	herb	31.07.2008	Alfred Galke GmbH, Gittelde, Germany
Rosaceae	*Geum urbanum*	root	22.09.2008	Alfred Galke GmbH
Rosaceae	*Potentilla anserina*	herb	31.03.2008	Kottas Pharma GmbH
Rosaceae	*Ribes nigrum*	fruit	09.07.2008	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Rosaceae	*Rosa canina*	fruit	31.03.2008	Kottas Pharma GmbH
Rosaceae	*Sorbus aucuparia*	fruit	31.03.2008	Kottas Pharma GmbH
Scrophulariaceae	*Verbascum sp.*	flower	09.07.2008	Alfred Richter GmbH. & CO.KG
Urticaceae	*Urtica dioica*	herb	09.07.2008	Alfred Richter GmbH. & CO.KG
Verbenaceae	*Verbena officinalis*	herb	31.03.2008	Kottas Pharma GmbH

Tab. 2 List of the investigated plant drug samples (continued).

Voucher no.	Species + used plant part	Reference source/ sample location	Sample date
Po001	*P. ostruthium* dried root material	Kottas Pharma GmbH, Vienna, Austria - Lot no.: 2634 wild collected in Italy	Nov. 2007
Po002	*P. ostruthium* dried root material	Kottas Pharma GmbH, Vienna, Austria - Lot no.: 9110258#2 wild collected in Italy	Dec. 2009
Po003	*P. ostruthium* dried root material	Kwizda Pharmahandel GmbH, Vienna, Austria - Lot no.: 9201025 wild collected in Italy	May 2009
Po004	*P. ostruthium* dried root material	Alfred Galke GmbH, Gittelde, Germany - Lot no.: 16000 wild collected in Italy	March 2010
Po005	*P. ostruthium* dried root material	Alfred Richter GmbH. & CO.KG, Kufstein, Austria - Lot no.: 156567 wild collected in Italy	April 2009
Po006	*P. ostruthium* dried root material	Caelo, Hilden, Germany - Lot no.: 09161905 wild collected in Italy	June 2009
Po007	*P. ostruthium* dried root material	wild collection from Gontal, Carinthia, Austria, 1800 m above sea level, 3 km west of Katschberg	July 2008
Po008	*P. ostruthium* dried root material	wild collection from the Weißpriachtal, Salzburg, Austria, 1200 m above sea level, 3 km north-western of Hinterweißpriach	July 2008

Tab. 3 List of additionally investigated *Peucedanum ostruthium* rhizome samples for the development of an HPLC-DAD quantification method for coumarins.

Voucher no.	Sample location/ date	Plant part used
S 1	Weißpriachtal, Salzburg, Austria, 1200 m above sea level (Salzburg), 2009	fresh rhizomes
S 2	Riedingtal, Salzburg, 2009	dried rhizomes
S 3	Gontal, Carinthia, Austria, 1800 m above sea level (Carinthia), 2007	fresh rhizomes
S 4	Königsalm, NÖ, 2008	fresh rhizomes

Tab. 4 List of self-made liqueurs prepared from *Peucedanum ostruthium* rhizomes.

2.4 Reagents and solvents

All solvents used for extraction, chromatography and other phytochemical work were from highest purity grade available and are listed below in Tab. 5.

Solvent	Purchased from
Acetonitrile (MeCN) HPLC-grade	VWR, BDH Prolabo, Darmstadt, Germany
Chloroform GPR Rectapur (p.a.)	VWR, BDH Prolabo, Darmstadt, Germany
Dichloromethane (DCM) AnalR Normapur (p.a.)	VWR, BDH Prolabo, Darmstadt, Germany
Diethyl ether GPR Rectapur (p.a.)	VWR, BDH Prolabo, Darmstadt, Germany
n-hexane HPLC-grade/ p.a.	VWR, BDH Prolabo, Darmstadt, Germany
Ethyl acetate (EtOAc) p.a.	VWR, BDH Prolabo, Darmstadt, Germany
Methanol (MeOH) HPLC-grade/ p.a.	VWR, BDH Prolabo, Darmstadt, Germany
Toluol p.a.	VWR, BDH Prolabo, Darmstadt, Germany
Ethanol 96 % p.a.	Carl Roth GmbH und CoKG, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO) p.a.	Carl Roth GmbH und CoKG, Karlsruhe, Germany
Acetic acid 100 % (HAc) p.a.	Carl Roth GmbH und CoKG, Karlsruhe, Germany
Formic acid > 98 % p.a.	Carl Roth GmbH und CoKG, Karlsruhe, Germany
Sulfuric acid conc. 95 % (H_2SO_4)	Gatt-Koller, Absam, Austria
Water (H_2O) p.a.	distilled water from the University of Vienna

Tab. 5 List of reagents and solvents used.

2.5 Reference compounds

Tab. 6, p. 32 shows a list of all reference components used for TLC, LC-MS, HPLC-DAD and HPLC-ELSD identification of coumarins in *Peucedanum ostruthium*. Tab. 7, p. 33 includes a list of fatty acid references used for GC-MS or TLC comparison. In Tab. 8, p. 33 and Tab. 9, p. 34 all reference compounds used for *Melampyrum pratense* are listed.

	CAS	MW	Company	Charge no.
Simple coumarins				
Auraptene	495-02-3	298.38	Chromadex, Irvine, USA	0001190
Osthole	484-12-8	244.29	Chromadex, Irvine, USA	00015420
Ostruthin	148-83-4	298.38	isolated, structure and purity proven by NMR (> 98 %)	
Scopoletin	92-61-5	192.17	Extrasynthese, Lyon, France	0528
Umbelliferon	93-35-6	162.14	Extrasynthese, Lyon, France	0529
Linear Furano-coumarins				
Bergapten	484-20-8	216.19	Extrasynthese, Lyon, France	0552S
Bergaptol	486-60-2	202.16	Extrasynthese, Lyon, France	0590S
Bergamottin	7380-40-7	338.40	Extrasynthese, Lyon, France	0550S
Heraclenin	2880-49-1	286.28	Chromadex, Irvine, USA	00008101
Imperatorin	482-44-0	270.29	Herboreal Ltd, Dalkeith, UK	B 705
Isoimperatorin	482-45-1	270.28	Herboreal Ltd, Dalkeith, UK	B 706B
Isopimpinellin	482-27-9	246.22	Extrasynthese, Lyon, France	0536S
Ostruthol	642-08-0	386.40	isolated, structure and purity proven by NMR (> 98 %)	
Oxypeucedanin	737-52-0	286.28	Herboreal Ltd, Dalkeith, UK	B0460
Oxypeucedanin hydrate	24724-52-5	304.30	Herboreal Ltd, Dalkeith, UK	B0576
Angular Furano-coumarins				
Isobergapten	482-48-4	216.19	Extrasynthese, Lyon, France	0538S
Pimpinellin	131-12-4	246.22	Herboreal Ltd, Dalkeith, UK	B 0637

Tab. 6 Reference coumarin compounds used for *P. ostruthium.*

	CAS	MW	Company	Charge no.
Fatty acids				
linoleic acid	60-33-3	280.45	Sigma-Aldrich, Vienna, Austria	92321
lineolenic acid	463-40-1	278.43	Sigma-Aldrich, Vienna, Austria	1344
oleic acid	112-80-1	282.46	Sigma-Aldrich, Vienna, Austria	75090
palmitic acid	57-10-3	256.428	Sigma-Aldrich, Vienna, Austria	P0500
stearic acid	57-11-4	284.48	Sigma-Aldrich, Vienna, Austria	85679

Tab. 7 Fatty acids used for identification in *P. ostruthium* and *M. pratense*.

	CAS	MW	Company	Charge no.
Flavones				
Apigenin	520-36-5	270.25	Extrasynthese, Lyon, France Phytolab, Vestenbergsgreuth (Vebg.), Germany	05081808 2968
Luteolin	491-70-3	286.25	Extrasynthese, Lyon, France Phytolab, Vebg., Germany	02071720 5864
Chrysoeriol/ 3‘-Me-Luteolin	491-71-4	300.29	Carl Roth GmbH und CoKG, Karlsruhe, Germany	47792261
Diosmetin / 4‘-Me-Luteolin	520-34-3	300.26	Carl Roth GmbH und CoKG, Karlsruhe, Germany	30680794
Flavonoles				
Kämpferol	520-18-3	286.24	Carl Roth GmbH und CoKG, Karlsruhe, Germany	3689559
Quercetin	117-39-5	302.24	Phytolab, Vebg., Germany	2926
Monoglycosides				
Luteolin-7-O-glucoside	5373-11-5	448.38	Extrasynthese, Lyon, France	04111604
Hyperoside	482-36-0	464.38	Extrasynthese, Lyon, France	03120118
Diglycosides				
Rutin	153-18-4	610.52	Carl Roth GmbH und CoKG, Karlsruhe, Germany	4480011

Tab. 8 Flavonoid references used for *M. pratense*: aglyca, mono- and diglycosides.

	CAS	MW	Company	Charge no.
Iridoid glycosides				
Aucubin	479-98-1	270.25	Phytolab, Vebg., Germany	5970
Catalpol	2415-24-9	286.25	Phytolab, Vebg., Germany	1344
Loganin	18524-94-2	390.39	Phytolab, Vebg., Germany	3518
Loganic acid	22255-40-9	376.36	Phytolab, Vebg., Germany	3495
Mussaenosidic acid	82451-22-7	376.36	AnalytiCon Discovery GmbH Potsdam, Germany	AD111810-01

Tab. 9 Iridoid Glycosides used as reference compounds for *M. pratense.*

2.6 Extraction

Prior to extraction the cut and dried plant material was grinded. The extraction itself was performed with the polar organic solvent methanol and the nonpolar one dichloromethane, as the active compounds may be of polar or nonpolar nature.

In order to find out the most efficient, as well as fastest method for an initial screening of several plant species two model plants, namely *Calluna vulgaris* and *Filipendula ulmaria* were extracted first with dichloromethane and then with methanol.

Five gram of the grinded plant material were extracted regarding following methods:

1. ASE extraction using three cycles at once with 30 mL solvent released into a single vial, i.e. during cycles part of the solvent was released from the extraction cell, containing the plant material, and fresh one was replenished so that all the extract is released into one vial.
2. ASE extraction with 3 x 15 mL solvent released into three individual vials, i.e. three separate consecutive extraction cycles were used whereby the whole solvent was released in a separate vial at the end of each cycle.
3. Extraction in the ultrasonic bath at room temperature with 3 x 15 min using 15 mL of solvent for each extraction cycle.

All extracts were then evaporated to dryness at 40 °C, under reduced pressure and weighed to evaluate the extract yields.

2.6.1 Accelerated Solvent Extractor (ASE)

Extraction by ASE was applied for the general screening of the 26 selected plant species, and scaled-up for the isolation of active compounds from *P. ostruthium* and *M. pratense*. A detailed description on the extraction parameters is given in chapter 3.2, pp. 55 ff.

The Accelerated Solvent Extractor (ASE 200 from Dionex Austria GmbH, Vienna, Austria) works under pressure (0.3 - 20 MPa) and with high temperature (up to 200 °C). It consists of a solvent supply with the possibility to use up to 4 different solvents at once, an instrument computer for the programming, an oven with trays for the steel extraction cells (1-100 mL) containing the (plant) material and trays for vials into which the extracted fluid is released (Fig. 15).

Compared to techniques like Soxhlet and sonication, ASE generates results in a fraction of the time. Additionally, the amount of extraction solvent needed is reduced. The increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent below its boiling point.

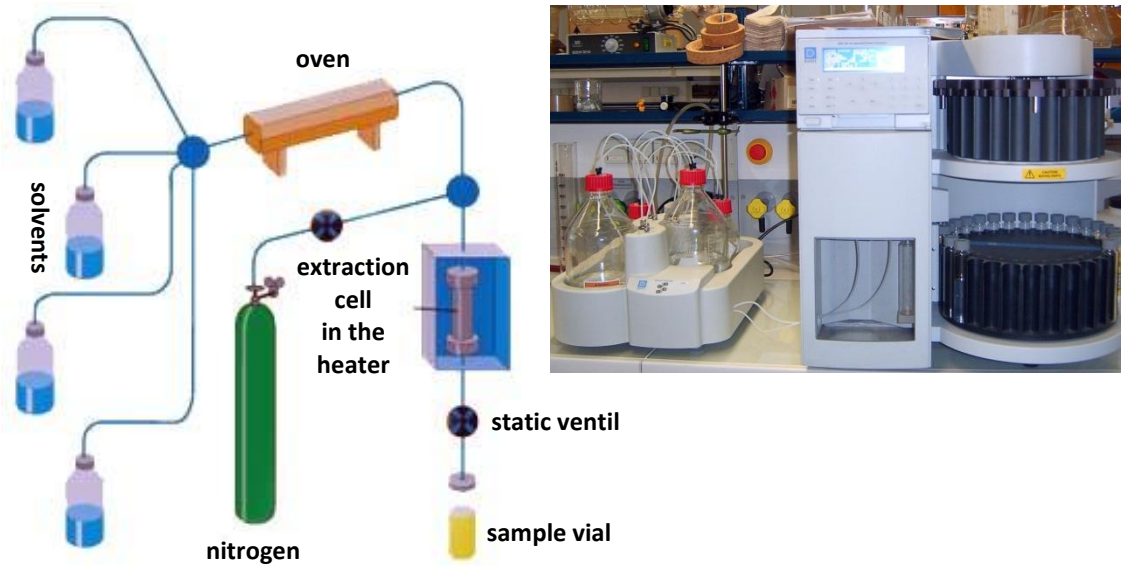


Fig. 15 Left: scheme of an ASE assembling (source: http://www.dionex.com**).**
Right: overview of the used instrument.

2.6.2 Ultrasonic bath

Ultrasonic bath extraction was performed for the preliminary tests to find the best extraction method for a screening of several plants (results see chapter 3.1, p. 52 ff.). Therefore 3 times an extraction for 15 min each was conducted. The plant material was transferred into a suitable flask with the selected solvent and put into a water bath transmitting the ultrasonic sound. With the ultrasonic bath it is possible to extract higher amounts of sample material. It is a fast and easy to handle method but has higher solvent consumption and the lower reproducibility than ASE.

2.6.3 Extraction under reflux

This method was chosen for the quantification of coumarins in *P. ostruthium* (chapter 3.5, p. 151 ff.) as well as for the preliminary tests described in chapter 2.6, p. 34.
This procedure works with a water bath, regulated temperature and a reflux cooler for constant circulation of the extraction solvent, reducing the solvent amount needed.

2.6.4 Chlorophyll separation

Chlorophyll, if present, has been removed from the dichloromethane extracts, using a liquid-liquid-partition in DCM and MeOH : H_2O (1 : 1). Briefly, 1 g extract was dissolved in 150 mL DCM, 150 mL MeOH : H_2O (1 : 1) were added and DCM was evaporated under reduced pressure (800-600 mbar) and at 40 °C. Consequently, insoluble chlorophyll precipitated in the methanol-water phase and could be filtered off.



Fig. 16 *M. pratense* after chlorophyll separation from the DCM extract.

2.6.5 Tannin separation

Tannins were separated from the methanolic extracts using a method published by Wall *et al.* (1996) shown in Fig. 17.

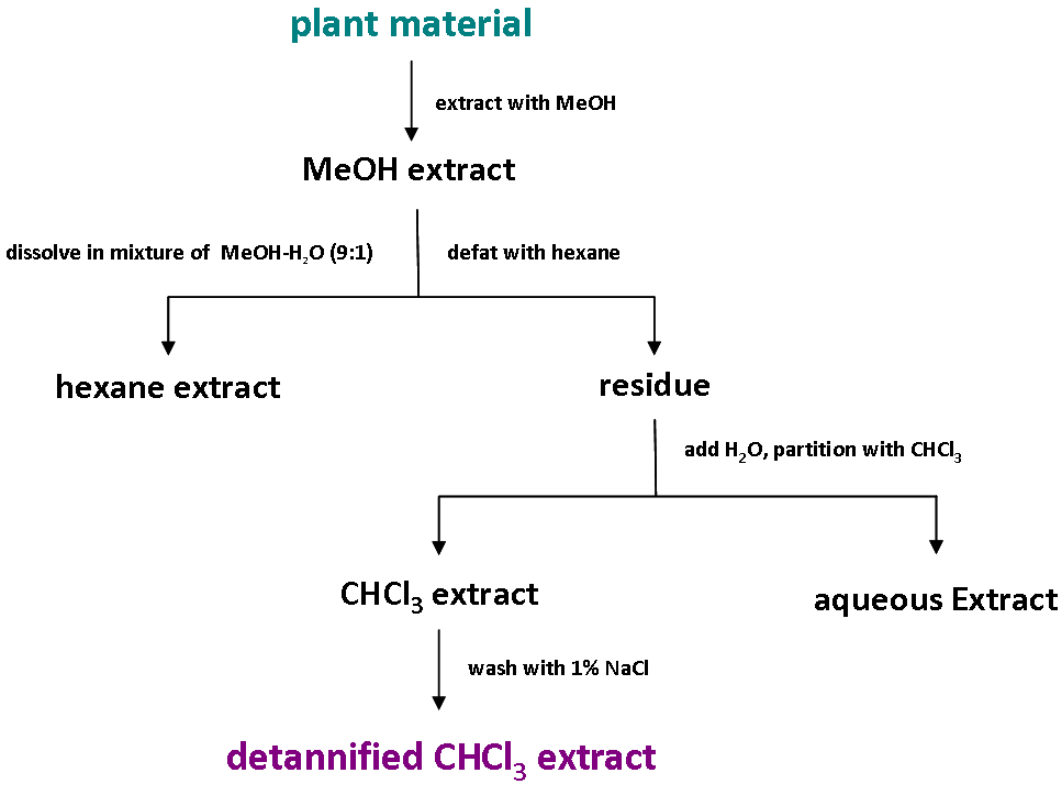


Fig. 17 Schematic description of the separation procedure of tannins (Wall *et al.*, 1996).

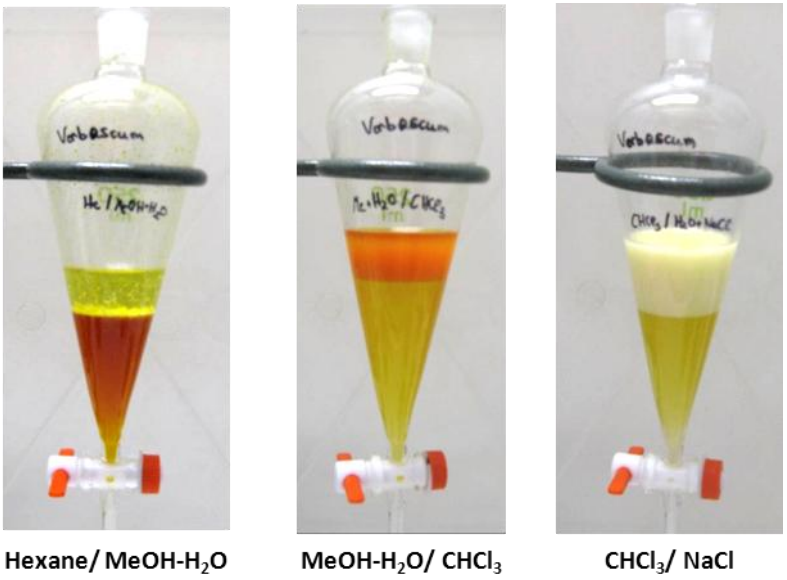


Fig. 18 Various separation steps of the tannin separation of *Verbascum sp.*.

2.7 Solid Phase Extraction (SPE)

Solid phase extraction is a fast and reproducible method for sample preparation and purification. This vacuum liquid chromatography works with the same sorbents (silica gel or RP18 material) like HPLC with particle sizes around 40 µM. SPE on 60cc Bond Elut C18 cartridges (Varian, Harbor City, CA, USA) was performed for a first chromatographic separation of the crude DCM and MeOH extracts. 20 cc Bond Elut Si cartridges (Varian, Harbor City, CA, USA) were utilized for further purification on silica gel. The cartridges were prewashed with H_2O and MeOH and conditioned with 30 % MeOH/H_2O for C18 as well as with EtOAc and n-hexane for the silica gel material. After the application of the extract, successive elution of solvents of different polarity was performed under reduced pressure (flow rate: ≈ 2 drops/sec). A typical assembling of an SPE apparatus consists of a glass chamber where reduced pressure is produced via a pump, leading to a faster elution. The polypropylene extraction cartridges with the sorbent are situated on the top of the chamber. Erlenmeyer flasks serve as collection vials for the different fractions (Fig. 19). All system used for fractionation and purification are listed in Tab. 10, p. 39.

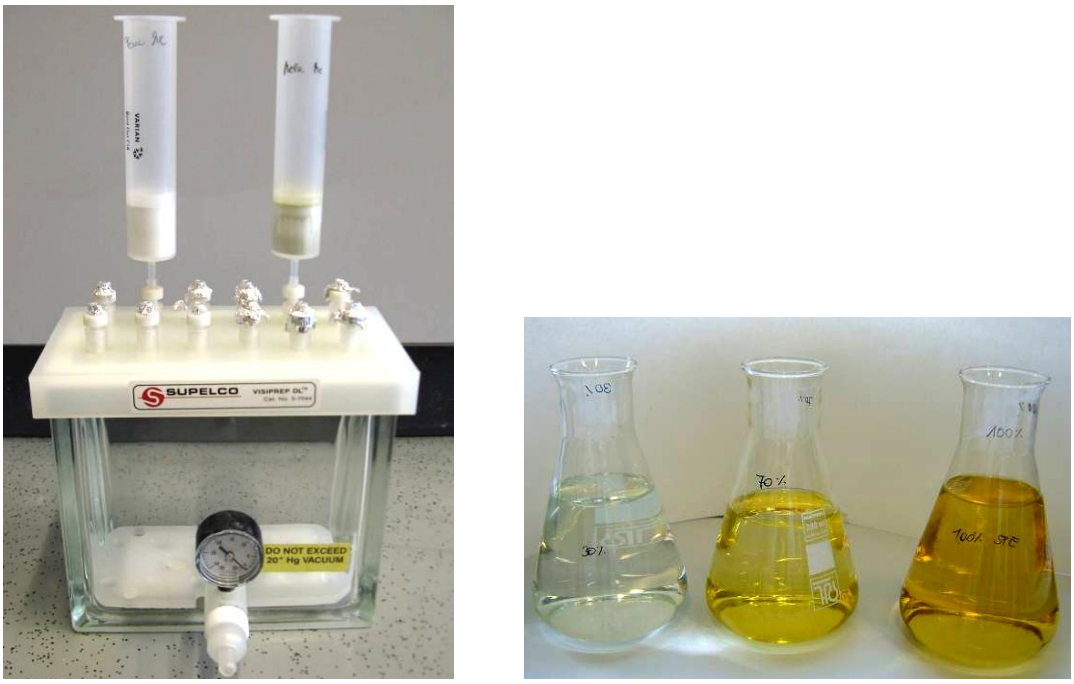


Fig. 19 Images of the SPE procedure of *M. pratense* and *P. ostruthium*: left hand side apparatus for SPE fractionation with cartridges and vacuum chamber; right hand side collected fractions of *P. ostruthium.*

SPE system 1	
Mobile phase:	MeOH : H_2O = 30 : 70 → 70 : 30 → 100 : 0
Stationary phase:	C18
Reservoir volume:	55 mL
Flow:	2 drops per second
Fraction Size:	collecting fractions of 5 reservoir volumes of each concentration
Plant used for:	*M. pratense, P. ostruthium* - fractionation of crude extracts

SPE system 2	
Mobile phase:	n-hexane : EtOAc = 90 : 10 → 85 : 15 → 80 : 20 → 75 : 25 → 70 : 30
Stationary phase:	silica gel
Reservoir volume:	15 mL
Flow:	2 drops per second
Fraction Size:	collecting fractions of 100 mL of one solvent system
Plant used for:	*P. ostruthium* - purification of ostruthin

SPE system 3	
Mobile phase:	n-hexane : EtOAc = 80 : 20 → 75 : 25
Stationary phase:	silica gel
Reservoir volume:	15 mL
Flow:	2 drops per second
Fraction Size:	collecting fractions of 100 mL of one solvent system
Plant used for:	*P. ostruthium* - purification of ostruthol and peucenin

SPE system 4	
Mobile phase:	MeOH : H_2O = 0 : 100 → 10 : 90 → 20 : 80
Stationary phase:	C18
Reservoir volume:	6 mL
Flow:	2 drops per second
Fraction Size:	collecting fractions of 30 mL of one solvent system
Plant used for:	*M. pratense*- purification of Melampyroside and Mussaenoside

Tab. 10 List of used SPE systems.

2.8 Chromatographic methods

These physical separation methods are based on interactions of components with a stationary and a mobile phase. Basically, they are important for a preparative separation of natural compounds and a first identification using reference compounds.

2.8.1 Thin Layer Chromatography (TLC)

For TLC silica gel 60 F_{254} plates (10 x 10 and 20 x 10 cm) with particle sizes 5-40 µM (classic TLC) or 2-10 µm (HPTLC) from Merck, Darmstadt, Germany were used. The separation capacity is better the smaller the particle size and the tighter the particles are arranged. Therefore, HPTLC usually leads to a better separation. The development of the plates was performed in suitable glass chambers equipped with filter paper for saturation of the gas phase. Samples were usually applied in bands in a concentration of 1 mg/mL solvent (5-20 µL) using 5 µL glass capillaries.

This simple and rapid chromatographic technique was used for analysis of fractions during the development of column chromatography or SPE in order to compare fractions and control the separation of the compounds. The used TLC systems are listed in Tab. 11, p. 40-42 .

TLC system for separation of coumarins	
Mobile phase 1: Mobile phase 2: Mobile phase 3:	toluene : ether = 1 : 1 saturated with 10 % acetic acid (Wagner & Bladt, 1996, pp. 140-144) toluene : EtOAc = 85 : 15 (Herde, 2005) n-hexane : EtOAc = 70 : 30
Detection:	UV_{366}, UV_{254}
Analyses of:	Under UV_{366} **coumarins** appear as bright blue bands, furano- and pyranocoumarins as yellow brownish zones. Under UV_{254} some coumarins show quenching effects (Wagner & Bladt, 1996, pp. 350-351).
Plant used for:	*P. ostruthium*

Tab. 11 Description of the different TLC systems used.

TLC system for identification of fatty acids	
Mobile phase:	EtOAc : MeOH : H_2O = 97 : 3 : 10
Detection:	anisaldehyde sulfuric acid reagent (ASR) UV_{366}, UV_{254}
Analyses of:	**Fatty acids** are detected as violet- grayish zones. Under UV_{366} coumarins appear as light blue or yellow brownish zones. Under UV_{254} some coumarins show quenching effects (Wagner *et al.*, 1996).
Plant used for:	*P. ostruthium*

TLC system for iridoids and flavonoids	
Mobile phase 1: Mobile phase 2:	EtOAc : MeOH : H_2O = 75 : 15 : 8 $CHCl_3$: MeOH : H_2O = 80 : 20 : 10
Detection:	vanillin sulfuric acid reagent (VSR), ASR UV_{366}, UV_{254}
Analyses of:	After spraying with VS or ASR diverse colored zones appear (Wagner & Bladt, 1996, pp. 84-85): → violet red zones of iridoid glycosides → blue zones secoiridoid glycosides → yellow zones flavonoid glycosides Under UV_{254} secoiridoid glycosides and xanthones give fluorescence quenching zones (Wagner & Bladt, 1996, pp. 86-87).
Plant used for:	*M. pratense*

TLC system for separation of essential oil components	
Mobile phase:	toluene : EtOAc = 97 : 3
Detection	VS UV_{366}, UV_{254}
Analyses of:	**Essential oil** components (terpenoids, phenylpropanoids) appear as red-violet to brown violet zones. (Wagner & Bladt, 1996, pp. 168-193)
Plant used for:	*M. pratense*

Tab. 11 Description of the different TLC systems used (continued)**.**

TLC system for iridoids	
Mobile phase:	$CHCl_3$: MeOH : H_2O = 60 : 40 : 4
Detection:	ASR, VS, UV_{366}
Analyses of:	Drugs with **iridoid glycosides** (e.g. *Euphrasia* - relative of *Melampyrum pratense*) from Wagner & Bladt, 1996; pp. 88-89. Under Vis iridoid glycosides react with ASR reagent as grey, blue or violet zones. Under UV_{366} brown to greenish yellow fluorescence can be observed.
Plant used for:	*M. pratense*

TLC system for flavonoids	
Mobile phase:	EtOAc : formic acid : HAc : H_2O = 100 : 11 : 11 : 26
Detection:	natural products-polyethylene glycol reagent (NPPR) UV_{366}
Analyses of:	Under UV_{366} **flavonoids** appear as yellow-orange zones and phenolic acids as blue bands (Wagner & Bladt, 1996, pp. 210-235).
Plant used for:	*M. pratense*

Tab. 11 Description of the different TLC systems used (continued).

Anisaldehyde sulfuric acid reagent (ASR), used for TLC of *M. pratense* and *M. sylvaticum* was composed as following: MeOH : HAc : H_2SO_4 conc. : anisaldehyde in a ratio of 85 : 10 : 5 : 0.5. First, 0.5 mL anisaldehyde were mixed with 5 mL HAc and 85 mL MeOH and then H_2SO_4 was added drop-wise. After spraying the TLC plate was developed at 10 °C for 5-10 min and evaluated under visible light as well as under UV-365 nm (Wagner & Bladt, 1996, p. 359).

For the vanillin sulfuric acid reagent (VSR) two solutions, namely 1 % ethanolic vanillin (solution 1) and 10 % sulfuric acid (solution 2) have been prepared. The TLC plate was sprayed first with solution 1 and immediately afterwards with solution 2. After development of 5-10 min at 110 °C the plate was detected under visible light (Wagner & Bladt, 1996, p. 364).

Natural products-polyethylene glycol reagent (NPPR) consisted of two separate reagents, a 1 % methanolic diphenylboric acid-β-thylamino ester and a 5 % polyethylene glycol-4000 (PEG). Again latter solution has to be sprayed after the first. After drying the plate was evaluated under UV-365 nm (Wagner & Bladt, 1996, p. 362).

2.8.2 Column chromatography (CC)

Common CC is a classical chromatographic method with relatively high particle sizes of 63-200µm. The systems used for separation and isolation of compounds from *P. ostruthium* and *M. pratense* are listed in Tab. 12.

The collected fractions were investigated using TLC (used methods see chapter 2.8.1, pp. 40-42). CC was used for the fractionation of 30 % and 70 % SPE subfraction of *Melampyrum pratense* DCM extract and purification of several compounds (Tab. 12).

CC system 1	
Mobile phase:	n-hexane : EtOAc = 95 : 5 → from fraction 95: n-hexane : EtOAc = 90 : 10
Stationary phase:	silica gel 60, Merck, particle size: 63-200 µM
Column diameter:	60 x 0.5 cm
Flow:	4 drops per minute
Fraction Size:	collecting fractions of 3 mL each 30 min
Plant used for:	*P. ostruthium* - purification of isoimperatorin and osthole

CC system 2	
Mobile phase:	EtOAc : MeOH : H_2O = 85 : 15 : 5 → from fraction 265: EtOAc : MeOH : H_2O = 85 : 20 : 5
Stationary phase:	silica gel
Column diameter:	90 x 2 cm
Flow:	4 drops per minute
Fraction Size:	collecting fractions of 3 mL each 30 min
Plant used for:	*M. pratense* 30 % MeOH SPE subfraction from the chlorophyll free DCM extract

CC system 3	
Mobile phase:	$CHCl_3$: MeOH : H_2O = 90 : 3.5 : 0.2 → from fraction 675 = 85 : 8 : 0.5
Stationary phase:	silica gel
Column diameter:	90 x 2 cm
Flow:	4 drops per minute
Fraction Size:	collecting fractions of 3 mL each 30 min
Plant used for:	*M. pratense* 70 % MeOH SPE subfraction from the chlorophyll free DCM extract

Tab. 12 Column chromatography parameters used for separation and purification of compounds.

2.8.3 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography was used to identify, quantify and purify individual components of a mixture. Generally, it is differentiated between analytical and preparative HPLC, latter is used for separation and purification of compounds. The HPLC systems applied in the present work are listed in Tab. 15.

The columns used can include polar stationary phases, like silica gel or most recently HILIC but preferably they run with apolar C18 material (reversed phase) which includes silica treated with RMe_2SiCl, where R is a straight chain alkyl group (columns used see Tab. 13). For detection diode array detectors (DAD) and low temperature light scattering detectors (ELSD-LT) were used. Additionally, an instrument coupled to an electrospray ionization ion trap mass spectrometer (ESI-IT-MS) was implemented. The HPLC devices used in the present work are listed in Tab. 14. For interpretation of analyses Shimadzu LC Solution Version 1.2 or 1.03 SP was used.

The solvents used were water and the organic solvents acetonitrile or methanol. Acid was often added to the respective solvent, so that the components were present in their protonated form.

Column 1:	**Luna C18 (2),** Phenomenex, Aschaffenburg, Germany
Length: Diameter: Particle size:	250 mm 4.6 mm 5 µM
Guard Column:	Hypersil ODS C18, 5 µm, 4 x 4 mm

Column 2:	**Dionex Acclaim 120 C 18,** Dionex, Germering, Germany
Length: Diameter: Particle size:	150 mm 2.1 mm 3 µm
Guard Column:	Acclaim 120 C18, 5 µm, 10 x 2.1 mm

Column 3:	**Nucleosil 100-7 C18,** Machery-Nagel, Düren, Germany
Length: Diameter: Particle size:	250 mm 21 mm 5 µm

Tab. 13 HPLC columns used for isolation and identification of various components of *P. ostruthium* and *M. pratense*.

Instrument 1:	**Shimadzu SCL-10A**, Shimadzu Österreich GmbH, Korneuburg, Austria
Pump:	LC-10AD
Degasser:	DGU-14A
Controller:	SCL-10A
Auto sampler:	SIL-10AD
Detector:	photodiode array detector (SPD-M10A)

Instrument 2:	**Shimadzu CBM-20A**
Pump:	LC-20AD
Degasser:	DGU-20A5
Controller:	CBM-20A
Auto sampler:	SIL-20AC HT
Detector:	photodiode array detector (SPD-M20A)
	low-temperature-light scattering-detector**:** ELSD-LT
Column oven:	CTO-20AC

Instrument 3:	**Shimadzu LC-8A preparative Liquid Chromatograph**
Pumps:	2x LC-8A
Controller:	CBM-20A
Auto sampler:	SIL-10AD
Fraction Coll.:	FRC-10A
Detector:	photodiode array detector (SPD-M20A9)

Instrument 4:	**Dionex UltiMate 3000 RS-LC**, Dionex Austria GmbH, Vienna, Austria
Ion source:	orthogonal electrospray ionization
Detector:	three-dimensional quadrupole ion trap mass spectrometer
	capillary voltage: -0,4 V; *nebulizer:* 20 psi (N_2); *collision gas:* helium

Tab. 14 List of HPLC devices used for separation and identification.

HPLC system 1	(Ganzera *et al.*, 1997)
Mobile phase:	**A:** H_2O + 0.01 % HAc, **B:** MeCN + 0.01 % HAc
Gradient:	15 % B (3 min), in 2 min to 20 % B, in 10 min to 30 % B, in 3 min to 45 % B, in 7 min to 50 % B, in 10 min to 70 % B, in 3 min to 15 % B and in 5 min to 95 % B
Stationary phase:	column 1
Temperature:	room temperature
Flow:	1 mL/min
Plant used for:	*P. ostruthium* - first HPLC investigations

Tab. 15 Conditions and parameters of the applied HPLC methods.

HPLC system 2	(Vogl *et al.*, 2011)
Mobile phase:	**A:** H_2O + 0.01 % HAc, **B:** MeCN + 0.01 % HAc
Gradient:	in 6 min 25-37 % B, in 8 min 37-45 % B, in 10 min 45-65 % B, in 1 min 65-95 % B, and for 5 min isocratic at 95 % B
Stationary phase:	column 2
Flow:	0.5 mL/min
Temperature:	38 °C
Plant used for:	*P. ostruthium* – identification and quantification of coumarins

HPLC system 3	preparative HPLC
Mobile phase:	**A:** H_2O, **B:** MeCN
Gradient	15 % B (for 3 min), in 2 min to 20 % B, in 10 min to 30 % B, in 3 min to 45 % B, in 7 min to 50 % B, in 10 min to 70 % B, in 3 min to 85 % B and in 5 min to 95 % B
Stationary phase:	column 3
Flow:	20.8 mL/min
Temperature:	room temperature
Plant used for:	*P. ostruthium* – fractionation of Peuc Det 70

HPLC system 4	developed by the author of the thesis
Mobile phase:	**A:** H_2O + formic acid (pH = 2.5), **B:** MeCN
Gradient:	98 % B (for 5 min), in 11 min to 90 % B, in 24 min to 80 % B, in 20 min to 50 % B
Stationary phase:	column 2
Flow:	0.4 mL/min
Temperature:	35 °C
Plant used for:	*M. pratense* - chromatography and LC-MS identification of iridoids in the crude MeOH extract

HPLC system 5	developed by the author of the thesis
Mobile phase:	**A:** H_2O + formic acid (pH = 2.5), **B:** MeCN
Gradient	98 % B (5 min), in 5 min to 85 % B, in 3 min to 79 % B, in 27 min to 65 % B, in 10 min to 15 % B
Stationary phase:	column 2
Flow:	0.4 mL/min
Temperature:	35 °C
Plant used for:	*M. pratense* - chromatography and LC-MS identification of flavonoids in Mela Det, OC as well as in the SPE subfractions thereof

Tab. 15 Conditions and parameters of the applied HPLC methods (continued).

2.8.4 Gas Chromatography (GC)

In order to identify volatile compounds GC-MS analysis was carried out. The analyses were performed on a Shimadzu (Kyoto, Japan) GC-2010 gas chromatograph coupled to a quadrupole mass selective detector (Shimadzu GCMS-QP2010). Helium was used as carrier gas at a constant flow mode of 1.9 mL/min. Electron ionization mass spectra were recorded in the range 40-700 m/z. Chromatographic separation was performed on a Phenomenex Zebron capillary column (60 m x 0.25 mm x 0.25 µm), containing silicon (methyl-silicon) as stationary phase modified with 5 % phenyl silicon to increase polarity. 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 min. LC solution software was applied for the analysis. All samples were dissolved to 0.1 mg/mL in DCM or MeOH and ultrasonicated for 10 minutes. 1 µL sample was injected, injector temperature was 270 °C and detector temperature was 250 °C (**method 1**).

For the analyses of fatty acids samples were preliminary derivatized with trimethylsulfoniumhydroxide (TMSH). For this all samples were dissolved to 5 mg/mL in DCM or MeOH and ultrasonicated for 10 min. 200 µL of the dissolved sample was mixed with 50 µL of TMSH (Fluka, Steinheim, Germany), resulting in a concentration of 4 mg/mL. The mixture was shaken for 30 sec and transferred to the GC-MS. The method used worked with a temperature gradient from 120 to 320 °C in 15 min with a 5 min hold on 320 °C (**method 2**).

2.9 Mass spectrometry (MS)

Using MS allows the identification of a substance due to its molecular weight and fragmentation scheme of specific structures. MS was conducted as described in chapter 3.5, p. 151 ff.

2.10 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR was used for the identification of purified compounds, which structures could not be clarified solely with MS. NMR was conducted as described in chapter 3.5, p. 151 ff.

2.11 Pharmacological test systems

The crude and purified extracts were tested *in vitro* regarding their anti-inflammatory activity by two project partners from the Department of Pharmacognosy, University of Vienna and the Department of Vascular Biology and Thrombosis Research from the Medical University of Vienna. For all bioassays the samples were further diluted in dimethyl sulfoxide (DMSO) and this stock solution was further mixed with buffer. Detailed description on the methods is given in chapter 3.2, pp. 55 ff.

All extracts, subfractions and pure compounds have been investigated by three closely collaborating groups:

→ The cooperation partner from the Department of Vascular Biology and Thrombosis Research from the Medical University of Vienna investigated them for their potency of a downregulation of tumor necrosis factor-α (TNF-α) or Lipopolysaccharide (LPS) stimulated E-selectin and interleukin-8 (IL-8) mRNA or protein.

→ The project partner from the Department of Pharmacognosy, University of Vienna tested all extracts, derived subfractions and pure compounds PPAR-α and -γ activation, as well as on the inhibition of NF-κB in cell-based luciferase reporter gene assays.

2.11.1 Downregulation of LPS or TNF-α induced IL-8 and E-selectin

2.11.1.1 mRNA level

A general anti-inflammatory activity was measured by the quantification of expression of the pro-inflammatory genes, IL-8 and E-selectin. The major readouts were the mRNA levels normalized to a housekeeping gene β2-microglobulin.

The inflammatory process was induced by activation of endothelial cells either with the inflammatory cytokine TNF-α, or the bacterial product LPS. These compounds are known to stimulate distinct but partially overlapping signaling pathways with documented role in acute and chronic inflammation. TLR 4 and TNF-α receptor play a key role in the activation of other cell types, e.g. leukocytes.

In human umbilical vein endothelial cells (HUVEC), selected for our investigations, the expression of E-selectin on the transcriptional level has maximal levels within 4 hours after stimulation at the cell surface (Kosonen *et al.*, 2000).

RT-qPCR was selected for quantification of the two selected inflammatory genes IL-8 and E-selectin, allowing for the assessment of the regulation of other genes from stored cDNA when considered necessary later on (i.e. for identification of mechanism). Detailed description of the method is given in chapter 3.2, p. 55 ff.

This screening started with pooled crude extracts of 10 plant species at concentrations of 100 µg/mL. Further on, the single extracts of the active pools only were tested.

2.11.1.2 Protein level - ELISA

Further on, a protein ELISA for IL-8 and E-selectin stimulated by LPS/ TNF-α was developed by the cooperation partner from the Department of Vascular Biology and Thrombosis Research from the Medical University of Vienna (see chapter 3.2, p. 55 ff.). The detailed protocol and the working steps of ELISA performed by the author of this thesis are given in Tab. 16.

ELISA (Enzyme-linked immunosorbent assay) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. For the readout a Synergy H4 Hybrid Reader (BioTek, Winooski, USA) with the Software, Gene 5 (Version 1.10) was used. It was performed at 450 nm with reference wavelength of 620 nm. Samples (SPL) were measured in triplicates next to each other; blanks (BLK) were included in triplicates as well. A typical plate layout is shown in Fig. 20.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SPL1	SPL1	SPL1	SPL9	SPL9	SPL9	SPL16	SPL16	SPL16	SPL24	SPL24	SPL24
B	SPL2	SPL2	SPL2	SPL10	SPL10	SPL10	SPL17	SPL17	SPL17	SPL25	SPL25	SPL25
C	SPL3	SPL3	SPL3	SPL11	SPL11	SPL11	SPL18	SPL18	SPL18	SPL26	SPL26	SPL26
D	SPL4	SPL4	SPL4	SPL12	SPL12	SPL12	SPL19	SPL19	SPL19	SPL27	SPL27	SPL27
E	SPL5	SPL5	SPL5	SPL13	SPL13	SPL13	SPL20	SPL20	SPL20	SPL28	SPL28	SPL28
F	SPL6	SPL6	SPL6	SPL14	SPL14	SPL14	SPL21	SPL21	SPL21	SPL29	SPL29	SPL29
G	SPL7	SPL7	SPL7	BLK	BLK	BLK	SPL22	SPL22	SPL22	BLK	BLK	BLK
H	SPL8	SPL8	SPL8	SPL15	SPL15	SPL15	SPL23	SPL23	SPL23	SPL30	SPL30	SPL30

Fig. 20 Plate Layout for ELISA readout using a Synergy Plate Reader. Controls: blank (BLK): buffer plus the monoclonal antibody; background includes buffer, monoclonal antibody and the enzyme; negative control has the whole system but unstimulated sample and the positive control includes only agonist.

Working step	Reagents	Effect
coating	4 µg/mL PBS 100 µL o/n 4 °C	monoclonal antibody specific for IL-8 binds to surface
wash	PBST 0.05 % 3 x 300 µL/well	in order to wash off the excessive/ non-specifically bound antibody (Ab)
blocking	1 % BSA/PBS 300 µL/well 1 h rest	Bovine Serum Albumin (BSA) → for coating the surface in between Abs
wash	PBST 0.05 % 5 x 300 µL/well	PBS (Phosphate Puffer)
standard, sample dilution	0.1 % BSA + 0.05 % PBST 100 µL/well o/n 4 °C	→ IL-8 (antigen) binds to Antibody
wash	PBST 0.05 % 5 x 300 µL/well	
detection Ab (antibody)	20 ng/mL 0.1 % BSA + 0.05 % PBST 100 µL/well 2 h rest	→ Ab binds to IL-8 12000 µL buffer + 66,7 µL Ab 2 x 5000 + 2 x 1000 µL – 60 + 6,7 µL
wash	PBST 0.05 % 5 x 300 µL/well	
streptavidin	0.1 % BSA + 0.05 % PBST 1/200 100 µL/well 20 min rest	→ enzyme binds to Ab 12000 µL buffer + 60 µL enzyme
wash	PBST 0.05 % 5 x 300 µL/well	
substrate	TMB 100 µL/well	peroxidase cuts substrate → blue mix 2 part substrate 1 : 1 6000 µL A + 6000 µL B
STOP	2N H_2SO_4 50 µL/well	acetic milieu stops reaction → yellow
read	450/620 nm	using ELISA Synergy H4 Hybrid Reader

Tab. 16 Description of the working steps for the ELISA of various extracts subfractions and compounds from *P. ostruthium* and *M. pratense.* PBS (Phosphate Buffered Saline) - routine buffer used in antigen/antibody reactions ensuring that the pH and osmotic conditions are optimal for the antigen/antibody reaction to take place. PBST (phosphate buffered saline with Tween 20)**.** PBST has the detergent Tween added and was used to wash off non-specifically bound material from the first and secondary antibodies during the assay. TMB (3,3',5,5'-Tetramethylbenzidine) for detection of horseradish peroxidase activity, yielding a blue color (Amax = 370 nm and 652 nm) that changes to yellow (Amax = 450 nm) upon addition of a sulfuric or phosphoric acid stop solution. Bovine Serum Albumin (BSA).

2.11.2 Luciferase cell based assays

2.11.2.1 Activation of PPARs

The luciferase cell based measurements of activation of PPAR-α or -γ were performed by the project partner from the Department of Pharmacognosy, University of Vienna, as described in chapter 3.2, p. 55 ff.

2.11.2.2 Inhibition of NF-κB

The luciferase cell based inhibition of NF-κB was measured by the project partner from the Department of Pharmacognosy, University of Vienna, as described in chapter 3.2, p. 55 ff.

2.12 *In silico* screening

The project partner form the University of Innsbruck provided a pharmacophore model collection for anti-inflammatory pharmacological targets from the arachidonic acid cascade (COX-1, COX-2, 5-LO, cPLA2α, and mPGES-1), the nuclear receptor family (FXR, LXR, PPAR, and GR), and the NF-κB signaling pathway (IKK-2 and the NF-κB DNA binding domain). The pharmacophore was applied to virtually screen known natural compounds in order to predict promising products for isolation and provide suggestions for anti-inflammatory mechanisms of these compounds. A pharmacophore model is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response (IUPAC definition). They consist of chemical features which represent physiochemical parameters such as hydrophobic, aromatic, hydrogen bond donor or acceptor, cationic, or anionic. The positions of these features can be directly derived from a 3D structure of a bioactive molecule bound to its binding site on a macromolecule (structure-based model) or from a 3D alignment and elucidation of common chemical functionalities of active ligands (ligand-based model). Pharmacophore models are well-established tools for virtual screening. The screening procedure is further described in chapter 3.2, p. 55 ff.

3 Results

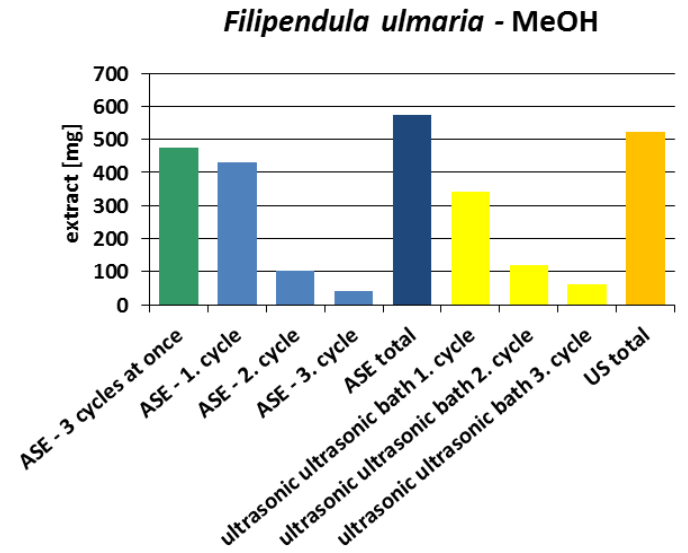
In the following part the results will be displayed in form of four different manuscripts. Further work and more detailed information on the phytochemical part of this work will be presented in separate chapters before the respective manuscripts.

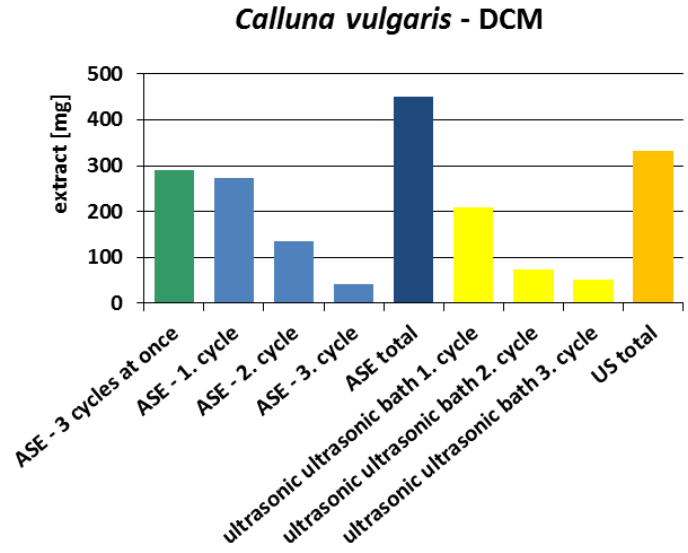
The first two publications deal with the general screening of various traditional Austrian healing plants in anti-inflammatory test systems. Manuscript 1 (chapter 3.2, pp. 55 ff.) investigates various traditional plant species from Austria for their anti-inflammatory potency in different *in vitro* cell based assays and computer aided *in silico* models. Second manuscript (chapter 3.3, pp. 108 ff.) deals with the influence of bulk components like chlorophyll or tannins on initial screening of crude plant extracts. The third publication (chapter 3.5, pp. 151 ff.) describes the identification, and quantification of coumarins in *Peucedanum ostruthium* (L.) Koch by HPLC-DAD and HPLC-DAD-MS. Last manuscript (chapter 3.6, pp. 160 ff.) deals with the identification of ostruthin from *Peucedanum ostruthium* rhizomes as an inhibitor of vascular smooth muscle cell proliferation.

3.1 Extraction and purification

Before extraction of the selected plant species, preliminary tests were performed to evaluate the efficiency of different extraction methods. Therefore, the two model plants *Calluna vulgaris* and *Filipendula ulmaria* were extracted as described in chapter 2.6, p. 34.

Fig. 21 (pp. 53-54) shows that the second method (extraction with ASE in 3 concecutive cycles) provided the best results regarding extract yields both, for the two model plants as well as for the different solvents tested. Additionally, this method is easy to handle, fast and well reproducible. Therefore, running three times one extraction cycle with the ASE was chosen as the best and most efficient extraction method for the screening.





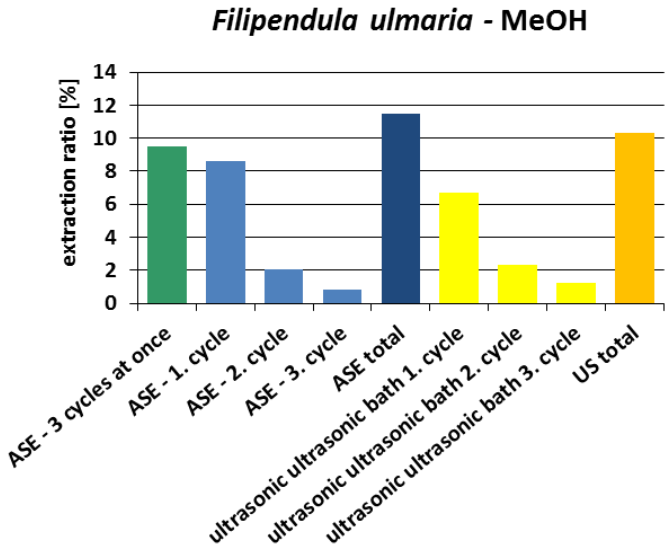


Fig. 21 Continues on next page

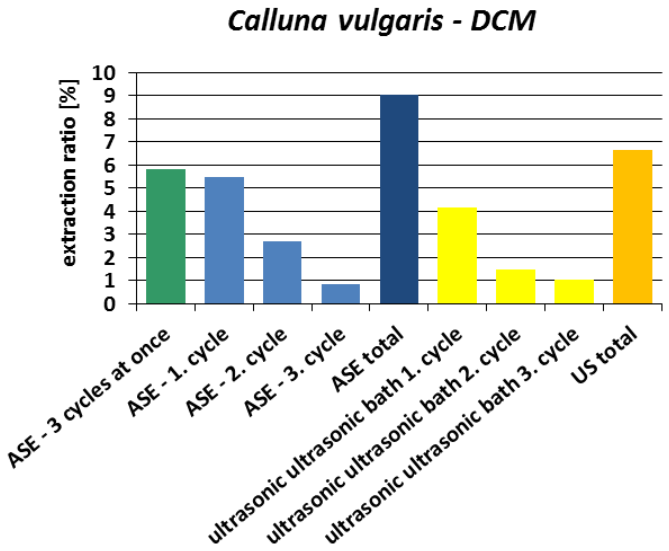


Fig. 21 Comparison of extraction yields (upper two graphs) and the extraction ratio [% w extract/w drug] (below) of various extraction techniques: green bar represents the extraction with 3 cycles at once on the ASE. Blue bars show the results of the 3 single cycles run with ASE (light blue) and the total of them (dark blue). Finally, the amount of extracts from the ultrasonic extraction is expressed in the yellow bars, once again the 3 individual extracts (light yellow) and the combined one (dark yellow).

For the chlorophyll separation different methods were evaluated as well. To investigate the influence of different purification methods and the influence of chlorophyll or polyphenols on the used test systems, comparative studies were carried out within project partners from the University of Vienna, the Medical University of Vienna, the University of Innsbruck, and the University of Graz (see chapter 3.3, pp. 108 ff.).

From all tested methods liquid-liquid-repartition between DCM and a mixture of MeOH : H_2O = 1 : 1 turned out to be most suitable regarding effectiveness and handling (Fig. 22).

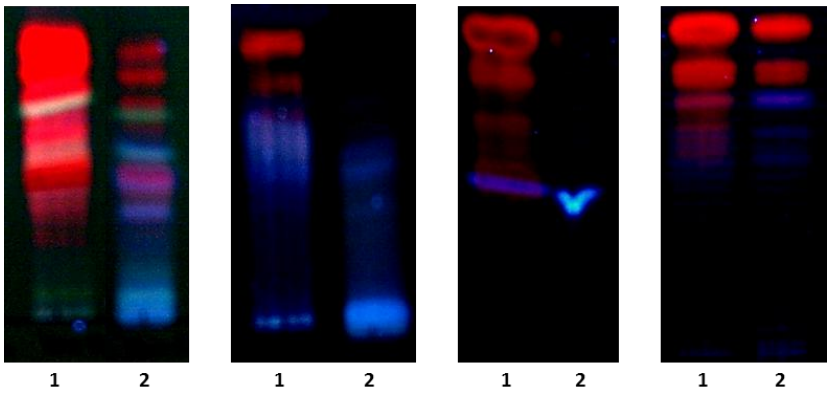


Fig. 22 DCM extracts before (1) and after (2) chlorophyll separation: from left to right: *Veronica chamedris, Verbena officinalis, Malva sp., Epilobium parviflorum*.
TLC was performed on silica gel using toluol : formylacetat : formic acid = 10 : 8 : 2, detection under UV_{254}. Chlorophyll is represented as red fluorescending bands.

3.2 Giving scientific evidence for the traditional usage of several Austrian medicinal plants with potential anti-inflammatory properties (manuscript)

This manuscript was prepared for submission. Plant collection was done together with Dott. Paolo Picker and ao. Univ. Prof. Dr. Johannes Saukel (Dept. of Pharmacognosy, University of Vienna). All of the phytochemical work was performed by the author of this thesis together with Dott. Paolo Picker from the Dept. of Pharmacognosy, University of Vienna. Pharmacological testing has been conducted by colleagues from the Institute for Vascular Biology and Thrombosis Research of the Medical University from Vienna (downregulation of IL-8 and E-selectin) and the Department of Pharmacognosy of the University of Vienna (activation of PPARs and inhibition of NF-κB). The *in silico* screening of known compounds was performed by co-workers from the Department of Pharmaceutical Chemistry of the University of Innsbruck. Literature research and creation of the mol-files was done by the author of this thesis.

Giving scientific evidence for the traditional usage of several Austrian medicinal plants with potential anti-inflammatory properties

Sylvia Vogl[a†], Paolo Picker[a†], Judit Mihaly-Bison[b], Nanang Fakhrudin[a‡], Daniela Schuster[c], Gerhard Wolber[c], Atanas Atanasov[a], Christoph Wawrosch[a], Gottfried Reznicek[a], Verena Maria Dirsch[a], Johannes Saukel[a], Brigitte 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 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:** phone +43-1-4277-55273, fax +43-1-4277-9552, e-mail johannes.saukel@univie.ac.at

Keywords:
Traditional remedies
Austrian herbals
Inflammation
In silico screening
Cell based *in vitro* models

Abstract

Ethnopharmacological relevance

In Austria, like in most Western countries, traditional knowledge about medicinal plants growing in the surrounding area became scarce. Nevertheless, via the so called VOLKSMED database it was possible to gather the remaining lore concerning Austria´s ethnomedicine. Hence, a further step was to relate this basic information to scientific results.

Aim of the study

The present study aims to give scientific evidence for an age-long traditional usage of plants naturally growing in Austria and the immediate vicinity, focusing on their anti-inflammatory properties.

Material and methods

Extracts of different polarity were produced from 75 drugs of 63 plant species. In a further step, the bulk constituents chlorophylls and tannins were depleted. Crude and purified extracts of all species were then evaluated for their *in vitro* anti-inflammatory activity, by assessment of their effect on gene expression (E-selectin, IL-8), induced by inflammatory stimuli (TNF-α, LPS) in endothelial cells, as well as their potential to activate PPAR-α and -γ and to inhibit the TNF-α-induced NF-κB activation. Additionally, computer-aided *in silico* screening of known compounds of the selected species was performed.

Results

Extracts from 71 of 75 drugs were active in at least one of the pharmacological *in vitro* test systems. The *in silico* hits comprised components mainly belonging to the group of fatty acids, triterpenes and phenylpropanderivates present in 40 of the investigated species.

Conclusion

Our research supports the efficacy of plants reported in Austrian folk medicine for diseases associated with inflammatory processes.

1. Introduction

The search for bioactive compounds is one of the central subjects of industrial and academic natural products chemistry. Nowadays about 60 % of the new pharmaceutics are based on molecular structures of natural resources (Newman and Cragg, 2007).
In contrast to Traditional Chinese Medicine (TCM), Traditional European Medicine (TEM), including Austria's ethnomedicine, has been barely scientifically explored (Adams et al., 2009). Although the advance of Western medicine led to repression of traditional preparations, Austria and its adjacent regions have a rich history in traditional folk medicine. In order to conserve this knowledge, the so called VOLKSMED database was generated (Gerlach et al., 2006). This database was created by gathering information about traditionally used plants in Austria and the immediate vicinity. It only refers to knowledge that had been passed on from generation to generation and does not lean on any existing literature. Besides an exact botanical identification, it contains information about plant parts used, preparation and application methods, internal or external use, indications and the number of citations. This database was the basis for the selection of plant drugs to be evaluated on various anti-inflammatory targets, using both *in silico* and *in vitro* assays.
Inflammation is a part of various pathological conditions e.g. arthritis, atherosclerosis, the metabolic syndrome, allergies and other auto-immune diseases, even cancer involves inflammation. For most of these conditions no satisfying treatment of the inflammatory part is available. General treatment relies on steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) which have several adverse effects (Burke et al., 2006). Therefore, the search for new anti-inflammatory drugs still represents an important field in drug discovery.
Many of the inflammatory processes are mediated by the transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB). Major target genes of NF-κB include those for transcription of adhesion molecules (e.g. E-selectin), cytokines (e.g. tumor necrosis factor (TNF)-α), growth factors and enzymes (e.g. Cyclooxygenase- (COX)-2) producing inflammatory mediators. Most prominent inducers of inflammation are cytokines such as TNF-α and interleukin (IL)-1, as well as components released by bacteria such as lipopolysaccharide (LPS). Important mediators inhibiting NF-κB responses are nuclear receptors, like the glucocorticoid receptor, the peroxisome

proliferator-activated receptors (PPARs), the farnesoid X receptor (FXR), the liver X receptor (LXR) and the orphan nuclear receptors class 4A (NR4A). Nuclear receptors are thought to exert their anti-inflammatory activities by repressing the activity of other transcription factors, such as STAT or AP1 proteins, both involved in the induction of a variety of pro-inflammatory genes (Bamborough et al., 2009; Brasier, 2010; Glass and Ogawa, 2006; Karin et al., 2004).

In the present study, a panel of functional cell models together with computer aided pharmacophores of possible inflammatory targets were used for the identification of active natural products. Thereby, interference with TNF-α or LPS induced expression of the adhesion molecule E-selectin or the chemokine IL-8, inhibition of the NF-κB pathway and activation of nuclear receptors (PPAR-α and -γ) were investigated, in order to get insight in various steps of the complex inflammatory process.

2. Materials and methods

2.1. Literature Research

In a first step, the databases Chemical Abstracts (CAPLUS) and Medline were scanned via the user interface SciFinder Scholar concerning 226 plant species which were reported in the VOLKSMED database as traditionally used anti-inflammatory agents (e.g. for colds, rheumatism, gastro-intestinal diseases). The search was limited to terms concerning inflammation (antiphlogistic, anti-inflammatory, etc.) in order to avoid re-investigations.

Due to the results of this literature research and due to the availability of the drugs, 63 plant species were selected for further investigations. Hence, compounds already known to be present in each of these plant species were extracted from the existing literature and mol-files thereof were created for the in silico screening.

2.2. Plant material

Part of the plant material could be purchased from various suppliers (Kottas Pharma GmbH, Vienna, Austria; Alfred Richter GmbH. & CO.KG, Kufstein, Austria and Alfred Galke GmbH, Gittelde, Germany). Several species have been collected in Austria and air dried at room temperature (Table 1, pp. 65-70). The plant material was authenticated

by Prof. Johannes Saukel. Voucher specimens are deposited in the Herbarium of the Institute of Pharmacognosy, University of Vienna.

2.3 Reagents and Chemicals.

Methanol (MeOH), chloroform, hexane and dichloromethane (DCM) were of p.a. grade (VWR, Vienna, Austria). DMSO p.a. for *in vitro* tests was purchased from Carl Roth GmbH und Co. KG, Karlsruhe, Germany. Water was distilled by an automatic distillation apparatus (IKA-Dest M3000, IKA®-Werke GmbH & Co. KG, Staufen, Germany).

2.4 Extraction and removal of bulk constituents

The dried and powdered plant material was first processed with the nonpolar dichloromethane (DCM) and subsequently with the polar solvent methanol (MeOH) using an accelerated solvent extractor ASE200 (Dionex Austria GmbH, Vienna, Austria). The instrument was equipped with 22 mL stainless steel extraction cells and 60 mL glass collection bottles. The extraction was performed at 40 °C and 150 bar using 3 extraction cycles, 5 min heat-up time, 2 min static time, 10 % flush volume and 60 sec nitrogen purge.

Subsequently, tannins and chlorophyll were excluded from polar and nonpolar extracts, respectively, in order to avoid possible interferences with the assays and to increase the relative amount of the active compounds. The tannins removal method was based on liquid-liquid partitions between $CHCl_3$ and mixtures of MeOH/H_2O (Wall et al., 1996). For the exclusion of the chlorophyll from the green plant material, a method based on a liquid-liquid partition between dichloromethane and a mixture of MeOH/H_2O was used. Shortly, 1 g of dichloromethane extract was dissolved in 150 mL dichloromethane. Further, the same amount of MeOH/H_2O (1:1) was added. Finally, the dichloromethane was removed under reduced pressure leading to precipitation of the chlorophyll, which could easily be filtered. This process leads to a loss of about 50-90 % of yield of crude extract. Figure 1 gives an overview of extraction and purification processes, as well as pharmacological evaluation steps.

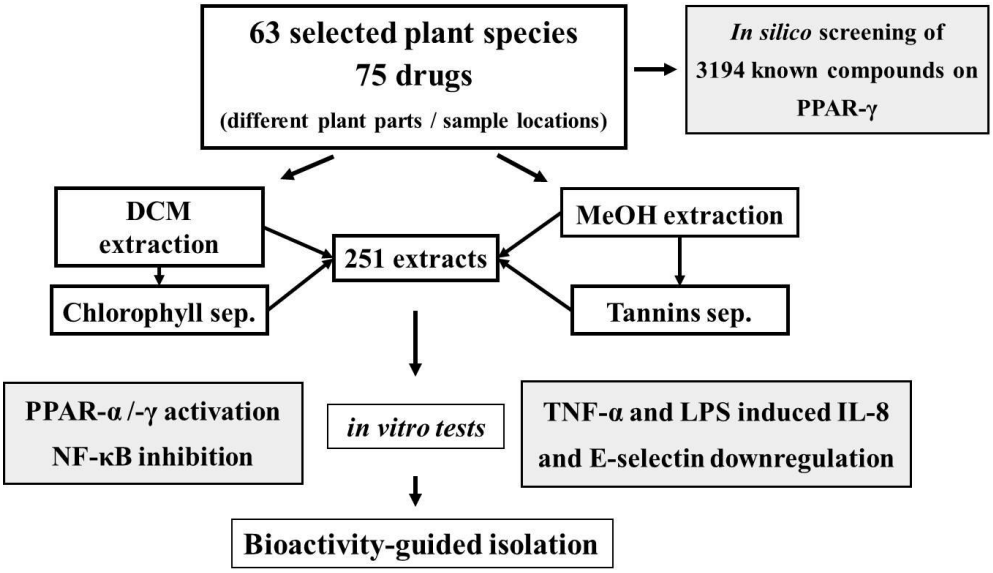


Figure 1 Overview of the plant processing and used test systems, DCM dichloromethane, MeOH methanol.

2.5. Luciferase based cell assays

2.5.1. PPAR transactivation assay

6.106 HEK293T cells were seeded in 10 cm dishes overnight. On the next day the cells were transfected by the calcium phosphate precipitation method with a total of 10 µg DNA including PPAR luciferase reporter construct (PPRE-tk3x-Luc), expression plasmid for the corresponding PPAR receptor protein and pEGFP-C1 (Clontech Laboratories, Inc. Mountain View, USA) as internal control. The ratio of the DNA of PPRE:PPAR:EGFP was kept 2:2:1. Six hours later the cells were transferred into 96 well plates and the medium was exchanged with a Dulbecco's Modified Eagle Medium (DMEM) supplemented containing 5 % charcoal stripped fetal bovine serum. One hour later the indicated treatments were performed and the cells were incubated in cell culture incubator for 18 h. Cells were then washed with phosphate buffered saline (PBS) and lysed. The luminescence of the firefly luciferase as well as the fluorescence of enhanced green fluorescent protein (EGFP) were quantified on a GeniosPro plate reader (Tecan, Grödig, Austria). The luciferase signal was normalized with the EGFP derived fluorescence to account for differences in the cell number and/or transfection efficiency.

2.5.2. NF-κB transactivation assay

Human embryonic kidney 293 cells stably transfected with the pNFκB-luc (293/NFκB-luc cells, Panomics, RC0014) were seeded in 10 cm dishes and transfected with 5 µg

pEGFP-C1 (Clontech Laboratories, Inc. Mountain View, USA). Six hours later the cells were transferred into 96 well plates containing a serum-free Dulbecco's Modified Eagle Medium (DMEM) and incubated in the cell culture incubator overnight. On the next day, cells were pretreated with the indicated extracts or compounds for one hour. For the assay cells were stimulated with 2 ng/mL human recombinant TNF-α for 6h, the medium was then removed and the cells were lysed with luciferase lysis buffer (Promega, Ludwigshafen/ Mannheim, Germany). Plant extracts and reference pure compounds were tested at a concentration of 10 µg/mL in at least three independent experiments. Parthenolide was used as positive control at a concentration of 5 µM. The luminescence of the firefly luciferase and the fluorescence of EGFP were quantified on a GeniosPro (Tecan, Grödig, Austria) plate reader. The luciferase signal derived from the NF-κB reporter was normalized with the EGFP derived fluorescence to account for differences in the cell number and/or transfection efficiency.

2.6. Assessment of general anti-inflammatory properties

A general anti-inflammatory activity was measured by the quantification of expression of the pro-inflammatory genes IL-8 and E-selectin. The major readouts were the mRNA levels of those inflammatory adhesion molecules normalized to a housekeeping gene β2-microglobulin. The inflammatory process was induced by activation of endothelial cells either with the inflammatory cytokine TNF-α or the bacterial product LPS. These compounds are known to stimulate distinct but partially overlapping signaling pathways with documented role in acute and chronic inflammation. RT-qPCR was selected for quantification of the two selected inflammatory genes IL-8 and E-selectin, allowing for the assessment of the regulation of other genes from stored cDNA when considered necessary later on (i.e. for identification of mechanism).

This screening started with pooled crude extracts of 10 plant species at concentrations of 100 µg/mL. Further on, the single extracts of the active pools (10µg/mL) only were tested.

2.6.1. Cells and cell culture

TERT technology (hTERT) immortalized human vascular endothelial cells (HUVEC-tert) (Chang et al., 2005) were grown in M199 medium (Sigma-Aldrich, St. Luis, USA) supplemented with 20 % fetal bovine serum (Sigma, Taufkirchen, Germany),

endothelial cell growPh supplement (Technoclone, Austria) and antibiotics. Experiments were performed in 12 well plates (NUNC, Roskilde, Denmark) in M199 medium containing 1 % bovine serum albumin (Applichem, Darmstadt, Germany) and 3 % fetal bovine serum.

2.6.2. E-selectin and IL-8 assay

Monolayers of subconfluent quiescent HUVECtert cells were pre-treated for 10 minutes with the plant material or inhibitor as indicated, followed by stimulation with 100 ng/mL of TNF-α (PeproTech, Rocky Hill, USA) or LPS (Sigma-Aldrich, St. Luis, USA) for 30 minutes or 4 hours, respectively. RNA was extracted from the cells using QIAzol lysis reagent (Qiagen, Hilden, Germany), 900 ng of RNA was reverse transcribed with MulV-RT using Oligo d(T) primers (Applied Biosystems, Carlsbad, USA). Relative expression of the genes of interest was determined by Q-PCR (Roche, Basel, Switzerland). Primers were designed with PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, USA) using the reference mRNA sequences of respective genes from the GeneBank (URL: http://www.ncbi.nlm.nih.gov).

For inteleukin-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 calculated by normalization to a housekeeping gene β2-microglobulin using the mathematical model by Pfaffl (Kadl et al., 2002), and presented as fold variation over the control.

2.7. Statistical analysis

Statistical analysis of the data was performed with the Prism 4.03 software (GraphPad Software Inc., La Jolla, USA). Values were calculated as means ± S.E.M. of at least two independent experiments.

2.8. *In silico* screening

Mol-files from 3194 known structures published for the respective taxa were created for the *in silico* screening. Pharmacophore models for the anti-inflammatory pharmacological target from the nuclear receptor family PPAR-γ were applied to virtually screen these natural compounds. Therefore, the plant extract constituents were submitted to

conformer generation using Accelrys Discovery Studio 2.1 (DS 2.1, Accelrys Software Inc., San Diego, USA) with 255 conformers / molecule, fast algorithm. The resulting multi-conformational database was submitted to the ligand profiler module of DS 2.1 using the flexible fitting algorithm. Detailed information regarding the models and the screening procedure can be found in (Markt et al., 2008; Markt et al., 2007). For the screening, all available models were used.

3. Results and discussion

As the survey on the VOLKSMED database for indications related to inflammation revealed the huge number of 9190 citations, the most promising 226 drugs were chosen. After a literature research concerning the investigation status with special emphasis on the planned *in vitro* test systems 63 plant species from 26 families, respectively were selected for phytochemical and pharmacological processing.

Table 1 (pp. 65-70) gives information about the investigated plant species, namely the scientific name, the botanical family, the plant part used, its indication and application methods, as well as sample date and location or purchase date and company.

With 9 selected species, the Lamiaceae was the most frequent family investigated in this study, what may be due to the essential oil components, rendering them interesting healing plants for traditional choice. Most common application forms were teas prepared from the herb.

Rosaceae with 8 and Apiaceae with 5 investigated species were also highly represented. Tannins, known for their antiphlogistic activity, are an important compound class in the family of Rosaceae. From members of this plant family the fruits were mainly used prepared as jellies and syrups.

The family of Apiaceae is characterized by its high amounts of essential oil and coumarins, which are also interesting in course of traditional application. From Apiaceae drugs people traditionally preferred the roots, probably because of their size and flavors. Here typical beverages are liqueurs and wines, besides the tea.

PLANT	PLANT-PART	FAMILY	SAMPLE DATE	APPLICATION	INDICATION	SAMPLE LOCATION/ SOURCE
Agrimonia sp. Agrimoniae Herba (Ph. Eur.)	herb	Rosaceae	31.03.2008	internal as tea	liver and bile, gastrointestinal, respiratory tract	Kottas Pharma GmbH, Vienna, Austria
Agropyron repens (L.) P. Beauv.	rhizome	Poaceae	16.09.2008	drug internal and external, internal as tea, syrup or cold maceration in water	fever	Kottas Pharma GmbH, Vienna, Austria
Ajuga reptans L.	herb	Lamiaceae	04.2008	internal as tea	respiratory tract	Laab im Walde, Lower Austria
Ajuga genevensis L.	herb	Lamiaceae	04.2008	internal as tea	respiratory tract	Laab im Walde, Lower Austria
Alnus viridis (Chaix) DC.	leaf	Betulaceae	07. 2008	drug internal and external	fever, infections	Rüßgütl bei Konigsch, Lungau, Salzburg, Austria
Angelica archangelica L.	root	Apiaceae	31.03.2008	internal as tea or tincture	gastrointestinal tract, respiratory tract, nervous system, fever, infections, flu	Kottas Pharma GmbH, Vienna, Austria
Angelica sylvestris L.	root	Apiaceae	09.07.2008	internal as tea or tincture	gastrointestinal tract, respiratory tract, nervous system, fever, infections, flu	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Bellis perennis L.	flower	Asteraceae	31.07.2008	internal as tea, leaves as salad	respiratory tract, gastrointestinal	Alfred Galke GmbH, Gittelde, Germany
Berberis vulgaris L.	fruit	Berberidaceae	31.03.2008	internal as tea, jelly or syrup	fever, respiratory tract, infections, colds, flu	Kottas Pharma GmbH, Vienna, Austria
Beta vulgaris L.	beet	Chenopodiaceae	31.07.2008	internal drug or juice, external compresses of drug	respiratory tract, fever, infections	Alfred Galke GmbH, Gittelde, Germany
Betonica officinalis L.	herb	Lamiaceae	12.06.2008	internal as tea, external as compress or bath	gastrointestinal tract, respiratory tract, nervous system, skin, gynecology	Neustift am Walde, Vienna Austria
Calluna vulgaris (L.) Hull	herb	Ericaceae	31.03.2008	internal as tea	kidney and urinary tract	Kottas Pharma GmbH, Vienna, Austria
Capsella bursa-pastoris (L.) Medik.	herb	Brassicaceae	16.09.2008	internal as tea or tincture, external as tincture, tea, ointment, bath or powder	locomotor system, gynecology, cardiovascular system, hemostasis, skin	Kottas Pharma GmbH, Vienna, Austria
Circea lutetiana L.	herb	Onograceae	11.08.2008	external as cold maceration in ethanol, internal as tea	rheumatism and gout, infections, fever	Sagberg, Lower Austria

PLANT	PLANT-PART	FAMILY	SAMPLE DATE	APPLICATION	INDICATION	SAMPLE LOCATION/ SOURCE
Epilobium parviflorum Schreb.	herb	Onograceae	31.03.2008	internal as tea	prostate, kidney and urinary tract	Kottas Pharma GmbH, Vienna, Austria
Epilobium angustifolium L.	herb	Onograceae	31.03.2008	internal as tea	prostate, kidney and urinary tract	Kottas Pharma GmbH, Vienna, Austria
Epilobium montanum L.	herb	Onograceae	10.09.2008	internal as tea	prostate, kidney and urinary tract	Hochwechsel/ Steyersberger Schwaig, Lower Austria
Equisetum arvense L.	herb	Equisetaceae	09.07.2008	internal as tea, external as bath and compress	kidney and urinary tract, locomotor system, rheumatism and gout, skin	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Equisetum palustre L.	herb	Equisetaceae	08.06.2008	internal as tea, external as bath and compress	intervertebral disks, lung cancer	Neusiedlersee- region, Bgld., Austria
Euphrasia rostkoviana Hayne	herb	Orobanchaceae	31.07.2008	internal as tea, external as compress	eyes, gastrointestinal tract	Murtal (Rotgüldensee) Lungau, Salzburg, Austria
Euphrasia officinalis L.	herb	Orobanchaceae	31.03.2008	internal as tea, external as compress	eyes, gastrointestinal tract	Kottas Pharma GmbH, Vienna, Austria
Filipendula ulmaria (L.) Maxim.	flower	Rosaceae	31.03.2008	internal as tea	rheumatism and gout, infections, fever	Kottas Pharma GmbH, Vienna, Austria
Filipendula ulmaria (L.) Maxim.	leaf	Rosaceae	28.07.2008 31.07.2008	internal as tea	rheumatism and gout, infections, fever	Weißpriachtal, Salzburg, Austria Gontal, Carinthia, Austria
Filipendula vulgaris Moench	flower	Rosaceae	19.06.2008	internal as tea	rheumatism and gout, infections, fever	Neustift am Walde, Vienna Austria
Gentiana punctata L.	leaf	Gentianaceae	24.07.2008	internal and external as liqueur and tea	gastrointestinal tract, skin, locomotor system, liver and bile, pediatrics, fever, flu, rheumatism and gout	Pöllatal (Lisertal), Carinthia, Austria
Gentiana punctata L.	root	Gentianaceae	24.07.2008	internal and external as liqueur and tea	gastrointestinal tract, skin, locomotor system, liver and bile, pediatrics, fever, flu, rheumatism and gout	Pöllatal (Lisertal), Carinthia, Austria
Glechoma hederacea L.	herb	Lamiaceae	31.03.2008	internal as salad and tea	liver and bile, gastrointestinal tract, respiratory tract, food, kidney and urinary tract, fever, flu	Kottas Pharma GmbH, Vienna, Austria

PLANT	PLANT-PART	FAMILY	SAMPLE DATE	APPLICATION	INDICATION	SAMPLE LOCATION/ SOURCE
Geum montanum L.	root	Rosaceae	07.2008	internal as tea	rheumatism and gout, infections, fever	Schattneralm, Styria, Austria
Geum urbanum L.	herb	Rosaceae	31.07.2008	internal as tea	rheumatism and gout, infections, fever	Alfred Galke GmbH, Gittelde, Germany
Geum urbanum L.	root	Rosaceae	22.09.2008	internal as tea	rheumatism and gout, infections, fever	Alfred Galke GmbH, Gittelde, Germany
Hippophae rhamnoides L.	fruit	Elaeagnaceae	31.07.2008	internal as tea, juice or syrup	infections, colds, flu	Alfred Galke GmbH, Gittelde, Germany
Hypericum maculatum Crantz	herb	Hypericaceae	07. 2008	external as oil, ointment or cold maceration in ethanol, internal as oil and tea	skin, locomotor system, nervous system, gastrointestinal tract, respiratory tract, kidney and urinary tract, cardiovascular system, infections, rheumatism and gout	Gontal, Carinthia, Austria
Linum usitatissimum L.	seeds	Linaceae	09.07.2008	internal soaked or as tea, external as compress or oil	respiratory tract, eyes, infections, colds, flu, fever, rheumatism and gout	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Lycopodium L.	herb	Lycopodiaceae	31.03.2008	external as compress, internal as tea	locomotor system, skin, liver and bile, kidney and urinary tract, infections, rheumatism and gout	Kottas Pharma GmbH, Vienna, Austria, Saukel
Majorana hortensis Moench	herb	Lamiaceae	09.07.2008	internal as herb	gastrointestinal tract, infections	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Malva L. *Malvae Folium* (PH. Eur.)	leaf	Malvaceae	31.03.2008	external as bath, internal as tea	skin, gastrointestinal tract, respiratory tract	Kottas Pharma GmbH, Vienna, Austria
Malva neglecta	herb	Malvaceae	12.08.2008	external as bath, internal as tea	skin, gastrointestinal tract, respiratory tract	Nodendorf (Ernstbrunn), Lower Austria
Melampyrum pratense L.	herb	Onograceae	16.07.2008	-	-	Neustift am Walde, Vienna Austria
Melampyrum sylvaticum L.	herb	Onograceae	07. 2008	-	-	Laab im Walde, Lower Austria
Melissa officinalis L.	leaf	Lamiaceae	31.07.2008	internal as tea, external essential oil	gastrointestinal tract, nervous system, liver and bile, food	Alfred Galke GmbH, Gittelde, Germany

PLANT	PLANT-PART	FAMILY	SAMPLE DATE	APPLICATION	INDICATION	SAMPLE LOCATION/ SOURCE
Origanum vulgare L.	herb	Lamiaceae	09.07.2008	internal as tea, external as ointment	gastrointestinal tract, respiratory tract, nervous system	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Petasites hybridus (L.) P. Gaertn., B. Mey. & Scherb.	leaf	Asteraceae	10.09.2008	internal as tea or cold maceration in ethanol, external as compress or maceration in vinegar	infections, fever, flu, colds	Die Hohe Student, Styria, Austria
Peucedanum ostruthium (L.) W.D.J. Koch	root	Apiaceae	31.03.2008	internal as tea, liqueur and wine, external as fumigation, tincture and incense	gastrointestinal tract, skin, respiratory tract, cardiovascular system, viral infects, infections, fever, flu, colds	Kottas Pharma GmbH, Vienna, Austria
Peucedanum ostruhtium (L.) W.D.J. Koch	leaf	Apiaceae	19.08.2008	internal as tea and liqueur, external as fumigation, tincture and incense	gastrointestinal tract, skin, respiratory tract, cardiovascular system, viral infects, infections, fever, flu, colds	Hochwechsel/ Steyersberger Schwaig, Lower Austria
Picea abies (L.) H. Karst.	shoot tip	Piceaceae	10.06.2008	external as resin, bath, inhalation, ointment, tea, internal as syrup, tea	respiratory tract, skin, locomotor system, viral infects, gastrointestinal tract	Wechsel, Mariensee, Lower Austria
Pimpinella major (L.) Huds.	root	Apiaceae	07.08.2008	internal as drug, tea, in milk, liqueur	respiratory tract, fever, infections, colds, flu	Ebenwald Kienstein, Lower Austria
Plantago lanceolata L.	leaf	Plantaginaceae	31.03.2008	internal as syrup or tea, external as fresh stumped leaves	respiratory tract, skin, insect bites, viral infects	Kottas Pharma GmbH, Vienna, Austria
Potentilla anserina L.	herb	Rosaceae	31.03.2008	internal as tea	gastrointestinal tract, gynecology, spasm	Kottas Pharma GmbH, Vienna, Austria
Prunella vulgaris L.	herb	Lamiaceae	19.06.2008	internal as tea	respiratory tract, infections	Neustift am Walde, Vienna, Austria
Ribes nigrum L.	fruit	Rosaceae	09.07.2008	internal as food or syrup	gastrointestinal tract, viral infects, locomotor system, respiratory tract, cardiovascular system	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Rosa canina L.	fruit	Rosaceae	31.03.2008	internal as tea	kidney and urinary tract, viral infects, food	Kottas Pharma GmbH, Vienna, Austria

PLANT	PLANT-PART	FAMILY	SAMPLE DATE	APPLICATION	INDICATION	SAMPLE LOCATION/ SOURCE
Rumex alpinus L.	leaf	Polygonaceae	10.09.2008	internal as drug	viral infects	Die Hohe Student, Styria, Austria
Rumex alpinus L.	root	Polygonaceae	10.09.2008	internal as drug	viral infects	Die Hohe Student, Styria, Austria
Salvia officinalis L.	herb	Lamiaceae	09.07.2008	internal as tea or chewed	respiratory tract, mouth, gastrointestinal tract, skin	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Sambucus ebulus L.	fruit	Adoxaceae	09. 2008		fever, respiratory tract	Laab im Walde, Lower Austria
Sambucus nigra L.	fruit	Adoxaceae	31.03.2008	internal as tea, jelly, juice or syrup	viral infects, fever, flu, colds, respiratory tract, mouth, gastrointestinal tract, skin	Kottas Pharma GmbH, Vienna, Austria
Sambucus nigra L.	flowers	Adoxaceae	05. 2011	internal as tea or syrup	viral infects, fever, flu, colds, respiratory tract, mouth, gastrointestinal tract, skin	Sagberg, Lower Austria
Sanicula europaea L.	root	Apiaceae	29.05.2008	internal as tea, external as ointment	skin, respiratory tract, locomotor system, gastrointestinal tract, infections	Jubiläumswarte, Vienna, Austria
Sorbus aucuparia L.	fruit	Rosaceae	31.03.2008	internal as tea, syrup, jelly or liqueur	respiratory tract, fever, infections, colds, flu, rheumatism and gout	Kottas Pharma GmbH, Vienna, Austria
Symphytum officinale L.	root	Boraginaceae	31.03.2008	external as ointment, compress or alcoholic digestion, internal as tea or tincture	locomotor system, gastrointestinal tract	Kottas Pharma GmbH, Vienna, Austria
Symphytum officinale L.	leaf stem	Boraginaceae	08.06.2008	external as ointment, compress, or alcoholic digestion, internal as tea or tincture	locomotor system, gastrointestinal tract, rheumatism and gout	Neusiedlersee- region, Bgld., Austria
Tilia L.- *Tilliae flos* (Ph. Eur.)	flowers	Malvaceae	31.03.2008	internal as tea	fever, flu, viral infects, respiratory tract	Kottas Pharma GmbH, Vienna, Austria
Tussilago farfara L.	leaf	Asteraceae	09.07.2008	internal as tea or syrup, external direct or stumped	respiratory tract, skin, locomotor system, viral infects, flu, colds, fever, rheumatism and gout	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Urtica dioica L.	herb	Urticaceae	09.07.2008	internal as tea or fresh leaves	kidney and urinary tract, gastrointestinal tract, locomotor system, skin, hemorrhage, cardiovascular system, rheumatism and gout, flu	Alfred Richter GmbH. & CO.KG, Kufstein, Austria

PLANT	PLANT -PART	FAMILY	SAMPLE DATE	APPLICATION	INDICATION	SAMPLE LOCATION/ SOURCE
Vaccinium myrtillus L.	fruit	Ericaceae	09.07.2008	internal as fresh food, liqueur or tea	gastrointestinal tract, diabetes	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Vaccinium vitis-idaea L.	fruit	Ericaceae	19.08.2008	internal as jelly or syrup	fever, gastrointestinal tract, kidney and urinary tract, food	Hochwechsel/ Steyersberger Schwaig, Lower Austria
Verbascum sp. Verbasci flos (Ph. Eur.)	flower	Scrophulariaceae	09.07.2008	internal as tea, external as ointment, tea, bath or compress	respiratory tract, skin, veins, gastro-intestinal tract, locomotor system	Alfred Richter GmbH. & CO.KG, Kufstein, Austria
Verbena officinalis L.	herb	Verbenaceae	31.03.2008	internal as tea or liqueur	fever, infections	Kottas Pharma GmbH, Vienna, Austria
Veronica chamaedrys L.	herb	Plantaginaceae	07.05.2008	internal as tea	nervous system, respiratory tract, cardiovascular system, metabolism	Neustift am Walde Nord, Vienna, Austria
Veronica officinalis L.	herb	Plantaginaceae	19.06.2008	internal as tea	nervous system, respiratory tract, cardiovascular system, metabolism	Neustift am Walde, Vienna, Austria
Veronica L.	herb	Plantaginaceae	31.03.2008	internal as tea	nervous system, respiratory tract, cardiovascular system, metabolism	Kottas Pharma GmbH, Vienna, Austria

Table 1 List of investigated plant species, including their families, the used drugs, sample date and location, as well as information about their traditional usage as documented in the VOLKSMED database.

3.1. Cell based *in vitro* anti-inflammatory activity

In table 2 (pp. 87-92) all results of the *in vitro* cell based assays are presented, regarding PPAR-α and -γ activation, NF-κB inhibition and downregulation of TNF-α- or LPS induced expression of IL-8 or E-selectin mRNA. For latter some results are missing as here pools of 10 plants were screened initially and only the plants from the most active pools were individually tested. The results are shown together with the species name, the plant part used and the extracts thereof, prepared for scientific investigations.

Extracts from 71 of 75 drugs were active in at least one of the pharmacological test systems. 30 drugs showed an activity in the general anti-inflammatory assay. Extracts from 28 drugs showed a strong activation of PPAR-α and/or -γ (activation of more than 2 fold at a concentration of 10 µg/mL). Strong inhibition of NF-κB (inhibition of more than 80 % at 10 µg/mL) was detected in extracts from 21 drugs (see table 2, pp. 87-92).

This striking hit rate of 95 % supports the ethnopharmacological screening approach compared to random screening with an average hit rate of 10 % (Harvey, 2002). Moreover, it finally provides some scientific evidence for the traditional usage of several Austrian healing plants.

When searching literature for the investigated plant species no relevant studies in the concept of inflammation have been found concerning *Agropyron repens, Ajuga genevensis, Angelica sylvestris, Bellis perennis, Capsella bursa-pastoris, Circea lutetiana, Epilobium montanum, Equisetum palustre, Filipendula vulgaris, Gentiana punctate, Geum montanum, Hypericum maculatum, Lycopodium sp., Majorana hortensis, Melampyrum pratense, Pimpinella major, Rumex alpinus* and *Sorbus aucuparia.*

In the context of inflammation ***Agrimonia eupatoria*** herb was found to suppress lipopolysaccharide (LPS) induced nitric oxide production in BV2 microglial cells as well as the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin 1 beta (IL-1β) and IL-6 in a dose-dependent manner (Lee et al., 2005). Moreover, significant scavenging capacity of reactive oxygen species (ROS) by its polyphenols (Correia et al., 2007), anti-oxidant capacities (Correia et al., 2006) as well as a reduction of nitric oxide (NO) in LPS activated RAW 264.7 macrophage cells ($p < 0.05$) and suppression of nitric oxide synthase (iNOS) expression (Jung Chang et al., 2010) have been reported for this species. Nevertheless, no publications could be found concerning a NF-κB inhibition, detected in our study, which may be evoked by the fla-

van-3-ols, flavonols, flavones, procyanidines and phenolic acids identified for *Agrimonia eupatoria* (Correia et al., 2007).

An ***Ajuga reptans*** extract standardized for 50 % phenylpropanoid content was already tested for anti-inflammatory and antioxidant activity in mice (Marzani et al., 2009). Furthermore, the phenylpropanoid glycoside, teupolioside was shown to reduce pro-inflammatory cytokines release, the appearance of nitrotyrosine and poly (ADP-ribose) polymerase immunoreactivity in the colon of rats, besides an up-regulation of ICAM-1 as well as the expression of P-selectin. Teupolioside also decreased proMMP-9 Matrix (metallopeptidase 9) and -2 activity, induced in the colon of rats by dinitrobenzene sulfonic acid administration (Di Paola et al., 2009). Korkina et al. (2007) reported a significantly accelerated wound healing and anti-inflammatory action in the excision wound model, an inhibition of ROS release from the whole blood leukocytes and a ferrous ion chelating capacity for this compound. Moreover, teupolioside inhibited chemokine and growth factor expression by cultured human keratinocytes treated with pro-inflammatory cytokines, TNF-α and interferon-gamma (IFN-γ) (Korkina et al., 2007). In this study, a downregulation of pro-inflammatory cytokine mRNA of E-selectin and IL-8 was affected by the extracts of *A. reptans* herb as well as by those of *Ajuga genevensis*, what correlates with the reported activities.

Concerning ***Alnus viridis***, nothing has been published implicating a PPAR activating or NF-κB inhibiting activity as observed in our assays. Only oregonin, mainly found in *Alnus* species was reported to display anti-inflammatory and anti-oxidative activities (Kuo et al., 2008).

Coumarins isolated form ***Angelica archangelica*** roots have already been investigated for anti-inflammatory activity in terms of *in vitro* cyclooxygenase-1 (COX-1) and 5-lipoxygenase (5-LOX) inhibition. No activity was found in the COX-1 assay but osthole and oxypeucedanin hydrate isovalerate could inhibit 5-LOX (Roos et al., 1997). In our case, a moderate NF-κB inhibiting activity was observed by *A. archangelica*, while a strong one as well as a strong PPARs activation was demonstrated for the tannin free extract of *Angelica sylvestris*.

Concerning ***Berberis vulgaris***, most studies deal with the alkaloid berberine, isolated from its roots. Cui et al (2009) demonstrated that this compound was able to decrease Th17 and Th1 cytokine secretion and lowered the activity of STAT1 and STAT4 through the suppression of p38 MAPK and JNK activation in type 1 diabetic non-obese

diabetic mice (Cui et al., 2009). An anti-inflammatory property of another alkaloid from this species, berbamine, was also indicated via a selective down-regulation of STAT4 as well as by an inhibition of IFN-γ in experimental autoimmune encephalomyelitis (Ren et al., 2008). Moreover, cannabisin G and (±)-lyoniresinol isolated form the root bark of *B. vulgaris* exhibited antioxidant activity with IC_{50} values of 2.7 and 1.4 μg/mL in a hydroxyl radical scavenging assay (Tomosaka et al., 2007). In the present study, only the detannified extract of this plant species showed moderate activity on PPAR-α.

An antioxidant activity was observed by the total flavonoids from ***Betonica officinalis*** leaves and roots (Hajdari et al., 2010). In the concept of inflammation rosmarinic acid was identified in the aerial parts of *B. officinalis* (Czigle et al., 2007). Nonetheless, nothing is known about an activity on the suppression of the chemokine IL-8 or the pro-inflammatory cell adhesion molecule E-selectin.

Extracts from the aerial parts of ***Calluna vulgaris*** were investigated *in vivo* in mice models for their anti-inflammatory and anti-nociceptive activities and the flavonol kaempferol-3-O-β-D-galactoside was isolated as the active principle (Orhan et al., 2007). Ursolic acid, identified in heather flowers, was found to inhibit LOX and COX and to block macrophage arachidonic acid metabolism in mouse peritoneal macrophages (Najid et al., 1992). In a screening of Swedish traditional remedies *C. vulgaris* and *Geum urbanum* were reported to inhibit prostaglandin biosynthesis and platelet activating factor (PAF)-induced exocytosis *in vitro*. Moreover, *C. vulgaris* was identified as one of the most potent COX inhibitors (Tunon et al., 1995). In the present study, an inhibition of NF-κB was described for the first time for this plant species.

Anti-inflammatory and anti-oxidant activities of ***Epilobium angustifolium*** and ***Epilobium parviflorum*** have been related to their content of oenothein B, quercetin-3-O-glucuronide and myricetin-3-O-rhamnoside. Extracts from the herb of these two *Epilobium* species suppressed the activity of hyaluronidase and lipoxygenase with IC_{50}s around 5 μg/mL and 25 μg/mL. This inhibition was related to the high contents of oenothein B, revealing an IC_{50} of 1.1 μM. Moreover, the extracts as well as the pure compound oenothein B (IC_{50} = 15.4 μM) were able to decrease the release of myeloperoxidase and reactive oxygen species (ROS) from stimulated neutrophils (Kiss et al., 2011). The flavonoid myricetin 3-O-β-D-glucuronide, isolated from the leaves of *E. angustifolium*, exhibited anti-inflammatory activity on carrageenan-induced edema in the rat hind paw (>10 fold more effective than indomethacin) and an inhibitory effect on prosta-

glandin biosynthesis (as effective as indomethacin) (Hiermann et al., 1991). Juan et al. (1988) showed that the aqueous extracts from both *E. angustifolium* and *E. parviflorum* (5 times less active) reduced the *in vitro* release of 6-keto-prostaglandin F1α, PG(prostaglandin)E2, and PGD2 from rabbit ear in a concentration dependent manner (Juan et al., 1988). The ethanolic extract of *E. parviflorum* herb revealed hydroxyl radical scavenging activity, inhibited COX-1 and -2 catalyzed prostaglandin biosynthesis and the growth of *Escherichia coli* (Steenkamp et al., 2006). Results of the present screening revealed only a moderate potency to inhibit NF-κB concerning *E. angustifolium* and *E. parviflorum*. However, the less investigated species *E. montanum* did moreover activate PPARs and reduce the release of IL-8 and E-selectin mRNA after stimulation with TNF-α and LPS.

Equisetum arvense herb was reported to suppress the production of ROS, superoxide anions and hydroxyl radicals generated in cell-free systems and human neutrophils. Moreover, extracts from the aerial parts of *E. arvense* inhibited carrageenan induced paw edema in rats (Oka et al., 2007). We however, found an activity of the apolar extracts from *E. arvense* on NF-κB and PPARs. The extracts from its related species *Equisetum palustre* were more effective on the downregulation of the pro-inflammatory mediators IL-8 and E-selectin.

Concerning species from the genus *Euphrasia*, ***Euphrasia officinalis*** just inhibited NF-κB; whereas, ***Euphrasia rostkoviana*** was additionally active on PPARs in our screening. Xu et al. (2008) detected highest antioxidant (ROS) activity in a phenolic acid fraction of *E. officinalis* herb, followed by its flavonoids, and lowest activity from its iridoid glycosides. Yet, the flavonoid fraction was the most potent one with regard to inhibition of NO production LPS stimulated Raw 264.7 macrophages (Xu et al., 2008).

The flowers of ***Filipendula ulmaria*** were reported to have complement inhibitory activity (Halkes et al., 1997), while an anti-inflammatory effect was shown by a decrease in the synthesis of IL-2 by splenocytes and by suppression of pro-inflammatory cytokines production in delayed-type hypersensitivity reaction (Churin et al., 2008). We could detect an activation of PPARs by *F. ulmaria* leaves and an inhibition of NF-κB by the herb. The flowers of *Filipendula vulgaris* on the other hand, were able to reduce IL-8 and E-selectin production.

Recently, rosmarinic acid analogs obtained from the herb of ***Glechoma hederacea*** var. *longituba* were shown to inhibit NF-κB production (Luciferase assay) and the induction

of COX-2 and iNOS mRNA in HepG2 cells (Kim et al., 2011). In the present study, extracts from *Glechoma hederacea* were not just able to inhibit NF-κB but did also exert strong activation of PPARs. Moreover, this plant species was reported to inhibit IFN-γ and LPS induced production of NO in a dose-dependent manner, as well as the production of pro-inflammatory cytokines, IL-12p70 and TNF-α. However, it increased IFN-γ/ LPS induced IL-12p40 production and did not affect the IL-6 production (An et al., 2006).

Hippophae rhamnoides L., commonly known as sea buckthorn, is also used in Indian and Chinese traditional medicine. Polyphenols, polyphenolcarboxylic acids, flavones and flavone derivatives as well as carotene derivatives, isolated form the berries, have been related to its antioxidant free radical scavenging activities (Popescu et al., 2011). Kwon (2011) isolated casuarinin from the leaves of *H. rhamnoides* and studied its effect on the TNF-α-induced ICAM-1 expression in a human keratinocytes cell line HaCaT. Pre-treatment with casuarinin inhibited TNF-α-induced protein and mRNA expression of ICAM-1 as well as TNF-α-induced NF-κB activation. Furthermore, the activation of ERK and p38 MAPK was suppressed in a dose-dependent manner and TNF-α-induced pro-inflammatory mediators, such as IL-1β, IL-6, IL-8, and MCP-1 could be decreased after pre-treatment with casuarinin (Kwon et al., 2011). The phenolic compounds (+)-catechin, (+)-gallocatechin, and (-)-epigallocatechin and the tritepenoid ursolic acid were isolated from a 70% ethanol extract of the branches from sea buckthorn and evaluated for their inhibitory effects on 12-O- tetradecanoylphorbol-13-acetate (TPA) induced inflammation (1 μg/ear) in mice. (-)-epi-gallocatechin and ursolic acid showed an effect with a 50 % ID of 1.7 and 0.2 μmol/ear (Yasukawa et al., 2009). However, in our study no activity was observed by *H. rhamnoides* fruit extracts concerning NF-κB inhibition, PPAR activation or downregulation of IL-8 and E-selectin.

The fixed oil obtained from the seeds of linseed (***Linum usitatissimum***), containing alpha-linolenic acid, was screened for an anti-inflammatory activity *in vivo* using carrageenan, leukotriene and arachidonic acid induced paw edema models in rats. Significant inhibition of paw edema was observed in the highest dose (3 mL/kg) in all the models (Singh et al., 2008). In the present study, no *in vitro* activity was observed by the different linseed extracts on NF-κB, PPAR, IL-8 and E-selectin.

Leaves from ***Malva sylvestris*** showed very strong antioxidant properties, including radical-scavenging activity (EC_{50} = 0.43 mg/mL), reducing lipid peroxidation in liposomes

(0.04 mg/mL) and brain cells homogenates (0.09 mg/mL). Responsible for this effect could be the high contents of antioxidants (phenols, flavonoids, carotenoids, tocopherols), unsaturated fatty acids (α-linolenic acid) and minerals (Barros et al., 2010). Pirbalouti (2010) evaluated the wound healing activity of diethyl ether extracts from *M. sylvestris* flowers at 200 mg/kg/day dose in alloxan-induced diabetic rats. The extract-treated diabetic rats showed significant reduction in the wound area compared to the control. Moreover, tissue samples obtained from the 9^{th} and 18^{th} day after treatment revealed increased well organized bands of collagen, more fibroblasts and few inflammatory cells (Pirbalouti Abdollah et al., 2010). In our study, extracts from ***Malva neglecta*** and *Malva sp.* (***Malvae Folium*** according to Ph. Eur.) were effective modulators of PPARs, NF-κB, E-selectin and IL-8.

Two sulfated triterpenes, two ionol derivatives, caffeic and rosmarinic acid isolated from the fresh leaves and stems of ***Melissa officinalis*** neither affected cell viability nor caused the release of pro-inflammatory mediators or a decrease of trans-epithelial electrical resistance in the reconstituted human epidermis (Mencherini et al., 2009). Ethanolic extracts from the leaves of *M. officinalis* dose-dependently inhibited acetic acid-induced visceral pain, with ID_{50} value of 241.9 mg/kg as well as early (neurogenic pain) and the late (inflammatory pain) phases of formalin-induced licking and glutamate-induced pain (ID_{50} = 198.5 mg/kg, rosmarinic acid ID_{50} = 2.64 mg/kg) (Guginski et al., 2009). Antioxidant capacities (Trolox equivalent antioxidant capacity = 4.06±0.31 mM) of this plant species have been linked to its content of phenolics (calculated as quercetin equivalent 1370.09±41.38 μM) (Ivanova et al., 2005). Furthermore, rosmarinic acid from *M. officinalis* was shown to inhibit several complement-dependent inflammatory processes on both the classical pathway C3-convertase and on the cobra venom factor-induced, alternative pathway convertase *in vitro* (max. inhibition of classical pathway lysis observed at 2.6 mM). Moreover, significant inhibition of lysis of pre-formed EA43b cells by dilutions of human or rabbit serum in the presence of rosmarinic acid (1 mM) together with an inhibition of C5a generation was reported (Peake et al., 1991). In the present study, an *in vitro* inhibition of NF-κB and an activation of PPARs by the apolar extracts of *M. officinalis* leaves were observed.

Origanum vulgare has been reported to possess anti-inflammatory activities *in vitro* and *in vivo.* The anti-inflammatory constituents were identified as rosmarinic acid, oleanolic acid and ursolic acid (Shen et al., 2010). Supercritical fluid extracts of the

herb from oregano with the main compounds, trans-sabinene hydrate, thymol and carvacrol were able to decrease pro-inflammatory TNF-α, IL-1β and IL-6 cytokines synthesis and to increase the production of anti-inflammatory cytokine IL-10 in oxidized-LDLs activated THP-1 macrophages (Ocana-Fuentes et al., 2010). Carvacrol, a monoterpenic phenol isomeric with thymol, highly present in oregano essential oils, was reported to be responsible for several biological activities of oregano such as antimicrobial, anti-tumor, anti-mutagenic, anti-genotoxic, analgesic, anti-spasmodic, anti-inflammatory, angiogenic, anti-parasitic, anti-platelet, AChe inhibitory, anti-elastase, insecticidal, anti-hepatotoxic and hepatoprotective activities (Baser, 2008). During a screening of 34 dietary plants on their ability to induce basal NF-κB activity or inhibit LPS induced NF-κB activity (monocytes stably transfected with a NF-κB-luciferase reporter construct) apigenin (present in oregano and onion), carnosol (rosemary, thyme) and sulforaphane (cruciferous vegetables) were identified as the most potent NF-κB inhibitors. The spices, oregano ($p < 0.001$), thyme ($p < 0.001$), clove ($p < 0.001$) and turmeric ($p < 0.001$) exhibited strong inhibition of LPS induced NF-κB activity (less than 15 % compared to control); inhibition of TNF-α induced activation was found to be even stronger (Paur et al., 2008). Ivanova et al. (2005) described antioxidant capacities (Trolox equivalent antioxidant capacity = 5.87±0.2 mM) of this plant species linked to its content of phenolics (calculated as quercetin equivalent 1653.61±11.52 μM). In the present study, a moderate activation of PPAR-α was reached by the detannified methanolic extract while the NF-κB activation was inhibited by the DCM extract.

Petasites hybridus is mainly associated with toxicity. However, recent studies indicated that there were no signs of hepatocellular toxicity at estimated therapeutic C_{max} levels of 60 ng/mL. Nonetheless, when investigating liver function *in vitro* at > 170-fold of therapeutic C_{max} levels, including cytotoxicity (lactate dehydrogenase, MTT and ATP), transaminase activities (alanine aminotransferase and aspartate aminotransferase), albumin synthesis, urea and testosterone metabolism to assay for cytochrome P 450 monooxygenase activity only rhizome extracts rich in petasin (37 % petasin) evoked liver toxicity (Anderson et al., 2009). Lipophilic extracts of *P. hybridus* rhizomes with different levels of content of petasin and isopetasin were investigated for an inhibition of COX-1 and -2 isoenzymes and inhibition of the expression of COX-2 and p42/44 MAP kinase in rat primary microglial cells were tested. All extracts were weak direct inhibitors of COX-1 (IC_{50} > 400 μg/mL) but strong inhibitors of the inducible isoform

COX-2 (IC_{50} = 20.0 µg/mL - 60.6 µg/mL). As pure petasin and isopetasin neither inhibited COX-1 nor COX-2, this activity was not correlated to the content of these compounds. Moreover, the extracts inhibited LPS induced and thus COX-2-mediated PGE2 release in primary rat microglial cells (IC_{50} = 2.4 µg/mL - 5.8 µg/mL) (Fiebich et al., 2005). *In vitro* studies suggested that an extract of *P. hybridus* (Ze339), with petasins considered being the pharmacologically active fraction, blocks leukotriene synthesis in monocytes and granulocytes (Thomet et al., 2001). Furthermore, this extract was reported to inhibit the allergic response via reduction of airway hyperresponsiveness and eosinophil recruitment into the bronchoalveolar lavage fluid upon allergen challenge in mice. These effects were associated with reduced IL-4, IL-5 and RANTES (chemokine (C-C motif) ligand 5) production in the bronchoalveolar lavage (Brattstrom et al., 2010). However, these findings could not be approved in a clinical trial with humans (Jackson Catherine et al., 2004). The present study also suggests an influence on pro-inflammatory mediators *in vitro*, as IL-8 and E-selectin could be suppressed by *P. hybridus* leaf extracts.

Several compounds derivatised from osthole, isolated from ***Peucedanum ostruthium*** roots, showed moderate inhibitory activity in the humoral immune response to sheep erythrocytes in mice *in vitro*, as well as on Con A- and pokeweed mitogen-induced mouse splenocyte proliferation. Moreover, three compounds inhibited TNF-α in rat and human blood cells, whereas one derivative stimulated the production of both cytokines. Stimulation of IL-6 as well as a suppression of the carrageenan induced inflammation in mice (56.5 % and 68.3 % inhibition, resp.) was observed by two substances (Zimecki et al., 2009). Extracts from the roots of *P. ostruthium* were investigated *in vivo* for their antiphlogistic and antipyretic effects as well as for their influence on arachidonic acid metabolism. Thereby, oral administration of 10 % ethanolic extract led to a marked inhibition of carrageenan induced rat paw edema. Here, 6-(3-carboxybut-2-enyl)-7-hydroxycoumarin was determined to be the anti-inflammatory principle of the plant (inhibition of ≤50 % at 30 µg/kg). Furthermore, the extract and the coumarin were characterized as dual inhibitors of COX and 5-LOX activities (Hiermann and Schantl, 1998). In our study, the detannified MeOH extracts of roots and leaves were potent inhibitors of NF-κB and showed a strong activation of PPARs. The dichloromethane extract on the other hand, was active on IL-8, E-selectin and NF-κB.

The lignan 7-hydroxymatairesinol extracted from the heartwood of ***Picea abies*** was reported to reduce LPS stimulated TNF-α secretion in THP-1 cells in a dose dependent manner as well as TNF-α mRNA. In human polymorphonuclear leukocytes this compound concentration dependently reduced ROS and IL-8 production (Cosentino et al., 2010). On the other hand, Maeaettae et al. (2006) showed that hardwood and softwood dusts induced TNF-α, CCL2, CCL3, CCL4 and CXCL2/3 chemokines expression and inhibited IL-1β and CCL24expression in RAW 264.7 cells (Maeaettae et al., 2006). However, we found only moderate inhibition of NF-κB and weak downregulation of TNF-α induced production of E-selectin mRNA evoked by *P. abies* shoot tip extracts.

COX-1 inhibitory activity of ***Plantago lanceolata*** and ***Plantago major*** methanolic leaf extracts with an IC_{50} of 2.00 and 0.65 mg/mL, respectively as well as an inhibition of 12-LOX (IC50 = 0.75 and 1.73 mg/mL) was demonstrated (Beara et al., 2010). The herbal phenylethanoid acteoside isolated from *P. lanceolata* L. leaves was reported to have anti-oxidative properties and to significantly ameliorate colitis induced by dextran sulfate sodium in Balb/c in mice. T cells isolated from mesenteric lymph nodes were stimulated with anti-CD3 antibody in the presence of IL-2; after incubation for 24 h, IL-1β, IL-6, IL-12, TNF-α and IFN-γ levels in supernatants were analyzed and a significant down-regulation of IFN-γ secretion (195 pg/mL with 600 µg acteoside vs. 612 pg/mL with PBS, $P < 0.02$) was observed (Hausmann et al., 2007). In the modified hen's egg chorioallantoic membrane test *P. lanceolata* fluid extracts were comparable to hydrocortisone, phenylbutazone, and diclofenac-Na regarding their anti-inflammatory effects (Marchesan et al., 1998). Phenylethanoids, acteoside and plantamajoside isolated from the herb of *P. lanceolata* showed inhibitory effects on arachidonic acid-induced mouse ear oedema (Murai et al., 1995). Moreover, a hydroalcoholic extract from this plant species was reported to suppress 5-LOX and COX-2 in cell-free systems (Herold et al., 2003). In the present analyses extracts from plantain were able to inhibit NF-κB and to activate PPARs.

A methanolic extract of from the herb of ***Potentilla anserina*** was claimed to inhibit TNF-α-induced cell damage of L-929 cells in MTT assay (Muraoka et al., 2011). However, nothing is known about an activity of *P. anserina* extracts on NF-κB or PPARs, as observed in our study.

Ethanolic extracts from ***Prunella vulgaris*** herb were shown to significantly inhibit LPS stimulated prostaglandin E2 (PGE2) and nitric oxide production in RAW 264.7 mouse

macrophages at 30 µg/mL without affecting cell viability. This effect could only be partly explained by an effect of rosmarinic acid. LPS induced COX-2 and iNOS protein expression were reduced by *P. vulgaris* extract whereas rosmarinic acid inhibited only COX-2 expression (Huang et al., 2009). Moreover, *P. vulgaris* extracts as well as rosmarinic acid reduced ROS production, intracellular glutathione depletion as well as lipid peroxidation in LPS treated cells. Furthermore, LPS induced downregulation of IL-1β, IL-6 and TNF-α and suppression of iNOS was observed (Zdarilova et al., 2009). Extracts of this plant species were reported to suppress phorbol 12-myristate 13-acetate and calcium ionophore A23187-stimulated TNF-α, IL-6 and IL-8 secretion in human mast cells and inhibited A23187-induced NF-κB/DNA binding activity as well as NF-κB-dependent gene reporter assay (Kim et al., 2007). In the present study, we also observed a strong inhibition of NF-κB besides activation of PPAR and downregulation of E-selectin.

Several *in vitro* and *in vivo* studies indicate that anthocyanins and other polyphenols in berries have a range of potential health benefits including anti-oxidant, anti-inflammatory, cytoprotective and neurological effects. In a study conducted by Hurst et al. (2010), the proanthocyanin-enriched blackcurrant (***Ribes nigrum***) fruit extract in contrast to the anthocyanin-enriched one suppressed both IL-4- and IL-13-stimulated CCL26 secretion in a dose dependent manner. IL-4-stimulated CCL26 secretion was also decreased by epigallocatechin and to a lesser extent by epicatechin, metabolites identified in the proanthocyanidin extract. Moreover, epigallocatechin was able to reduce cellular phosphorylated STAT-6/STAT-6 ratio and like its original extract it potentiated the ability of IFN-γ to suppress IL-4-stimulated CCL26 secretion (Hurst et al., 2010). Furthermore, black current fruit extracts more than doubled basal NF-κB activity compared to controls in a screening of dietary plants on monocytes stably transfected with a NF-κB-luciferase reporter construct (Paur et al., 2008). Karlsen (2007) investigated the effect of anthocyanins isolated from bilberries and black currants in cultured monocytes. Here, these compounds were found to efficiently suppress LPS induced activation of NF-κB. In a clinical trial differences were observed in several NF-κB related inflammatory mediators, namely the pro-inflammatory chemokines IL-8, RANTES and IFNα (an inducer of NF-κB activation): changes in the anthocyan supplementation group (45, 15, and 40 % decreases from baseline, resp.) differed from those in the placebo group (20, 0, and 15 % decreases from baseline, resp.). Similarly,

changes in IL-4 and IL-13, two cytokines that mediate pro-inflammatory responses and induce NF-κB activation, in the anthocyan supplementation group (60 and 38 % decreases from baseline, resp.) tended to differ from those in the placebo group (4 and 6 % decreases) (Karlsen et al., 2007). Proanthocyanidins isolated from *R. nigrum* leaves were reported to inhibit carrageen in-induced pleurisy in rats by reducing pleural exudate formation and polymorphonuclear neutrophils infiltration. Moreover, immunohistochemical investigations on lung sections showed a reduced production of endothelial cell adhesion molecules. *In vitro* this was validated by an observed inhibition of ICAM-1 but not IL-8 and VEGF165 mRNA expression (Garbacki et al., 2005). Prodelphinidins, from *R. nigrum* leaves, decreased prostaglandin E2 and selectivity COX-2 of the COX enzymes *in vitro*. In the whole blood assay though, no effects on COX activity could be found (Garbacki et al., 2002). In our study, however, we could not observe an effect of black current fruit extracts on NF-κB but activation of PPARs and reduction in stimulated IL-8 and E-selectin mRNA production was detected.

Rosa canina fruits (rose hip) were reported to contain antioxidant nutrients and an anti-inflammatory galactolipid (Kirkeskov et al., 2011). Dichloromethane extracts from rose hip powder and the triterpene acids oleanolic acid and ursolic acid (IC_{50} 21 ± 6 μM) isolated thereof significantly and concentration dependently inhibited LPS induced IL-6 release from Mono Mac 6 cells (Saaby et al., 2011). Rose hip powder extracts of different polarity were tested for inhibition of cyclooxygenase (COX-1, COX-2) and of 5-LOX-mediated leukotriene B4 (LTB4) formation as well as for DPPH-radical-scavenging capacity. In contrast to ineffective water and methanol extracts, n-hexane and dichloromethane extracts were potent inhibitors of COX-1, COX-2 and 5-LOX-mediated leukotriene B4. Triterpene acids ursolic acid, oleanolic acid and betulinic acid, as well as the fatty acids oleic, linoleic and α-linolenic acid were identified in the active extracts. The methanolic extract on the other hand, was the most potent DPPH radical scavenger well correlated with its total phenolic content (Wenzig et al., 2008). *In vivo* *R. canina* fruit extract inhibited the development of carrageenan induced edema similar to indomethacin (Lattanzio et al., 2011). We detected a moderate activation of PPARs in the detannified MeOH extract of *R. canina* fruits, which has not been investigated so far.

Sage (***Salvia officinalis***) was reported to have similar anti-oxidant potentials as synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxylanisole, cur-

rently used as food additives (Krishnaiah et al., 2011). Moreover, extracts from sage were able to decrease pro-inflammatory IL-6 and TNF-α release. Simultaneously, an increase of the anti-inflammatory IL-10 and reduction of COX-2 or iNOS expression have been observed (Mueller et al., 2010). The phenolic diterpenes carnosic acid and carnosol, present in several labiate herbs, like *Rosmarinus officinalis* (Rosemary) and *S. officinalis* were found to activate PPAR-γ and to inhibit the formation of pro-inflammatory leukotrienes in intact human polymorphonuclear leukocytes (IC_{50} = 15-20 μM and 7 μM, resp.) as well as purified recombinant 5-LOX (IC_{50} = 1 μM and 0.1 μM, resp.) (Poeckel et al., 2008). Moreover, hexane and ethylacetate fractions of sage were shown to inhibit the protein and mRNA expression of TNF-α and IL-6 (100 μg/mL) as well as nitrite accumulation and the LPS/ IFN-γ induced iNOS protein (50 μg/mL) in LPS stimulated RAW 264.7 cells (Hyun et al., 2004). *In vivo* aqueous and butanol leaf extracts caused analgesic effect in the hot-plate latency assay as well as in early and late phases of formalin-induced paw licking in rats (Qnais Esam et al., 2010). The chloroform extract and its main component ursolic acid (ID_{50}=0.14 μM/cm^2 - 2 fold more potent than indomethacin) were investigated for topical anti-inflammatory properties (Baricevic et al., 2001). In the present study, PPAR-γ activation by *S. officinalis* leaf extracts could not be detected, however, most extracts were strong inhibitors of NF-κB.

Extracts from the leaves of ***Sambucus ebulus*** and ursolic acid isolated thereof reduced TNF-α induced expression of VCAM-1 (IC_{50} 6.25 μM), ursolic acid additionally inhibited ICAM-1 (IC_{50} = 3.13-6.25 μM) (Schwaiger et al., 2011). Aqueous extracts from *S. ebulus* fruit revealed NO scavenging activity (Ebrahimzadeh et al., 2010). In a review of Shokrzadeh et al. (2010) anti-inflammatory, anti-nociceptive, anti-cancer, anti-angiogenic and anti-oxidative activities were described for this plant species and ebulitin, ebulin 1, flavonoids, athocyanins among other components have been identified as active ingredients (Shokrzadeh and Saeedi Saravi, 2010). Anti-inflammatory *in vivo* activity was claimed for the models carrageenan and serotonin induced hind paw edema in mice, and adjuvant-induced chronic arthritis in rats and associated to the content of chlorogenic acid (Yesilada, 1998). In our study, *S. ebulus* fruit extracts were able to reduce the production of either IL-8 or E-selectin after stimulation with TNF-α and LPS.

Detannified MeOH extracts of ***Sambucus nigra*** flowers and fruits on the other hand, were strong activators of PPARs and potent inhibitors of NF-κB. The standardized

black elderberry fruit extract, Sambucol, was shown to be effective *in vitro* against 10 strains of influenza virus. Moreover, the production of the inflammatory cytokines IL-1β, TNF-α, IL-6 and IL-8 was significantly increased by this extract (2-45 fold), compared to LPS, in human monocytes (Barak et al., 2001). The elder flower extract on the other hand was found to potently inhibit cytokine production, integrin activation, or induction of the oxidative burst via inhibition of NF-κB and phosphatidylinositol 3-kinase (Harokopakis et al., 2006).

Concerning ***Sanicula europaea***, only an *in vivo* study of its saponins injected into rats showed reduction of the inflammatory responses after injections of nucleic acid Na salt or ovalbumin into the hindpaw (Jacker and Hiller, 1976). The present study, however, indicates an *in vitro* anti-inflammatory activity of *S. europaea* roots due to a decreased production of IL-8 and E-selectin after stimulation with TNF-α and LPS.

A glycopeptide isolated from the aqueous extract of ***Symphytum officinale*** roots exerted dose dependent antiphlogistic effect on carrageenan induced rat paw edema with an ED_{50} of 61 µg/kg p.o. (control indomethacin 10 mg/kg). Further investigations on the release of arachidonic acid, COX and LOX metabolites as well as on arachidonic acid induced platelet aggregation indicate that the isolated glycopeptide inhibits the release of prostaglandins and leukotrienes via decreasing the expression of phospholipase A2 (Hiermann and Writzel, 1998). The present analyses revealed a potency of the leave and root extracts to activate PPARs and to downregulate IL-8 and E-selectin mRNA.

The flavonoids present in the flowers of ***Tilia × viridis*** have been shown to exert antioxidant properties, acting as ROS scavengers, principally on hydrogen peroxide and the superoxide anion (Marrassini et al., 2011). Activation of PPARs by the dichloromethane extract and moderate inhibition of NF-κB by the detannified MeOH extract of *Tilia sp.* (*Tilliae flos* in accordance with the Ph. Eur.) was detected in the present study.

Ethyl acetate extracts of ***Tussilago farfara*** leaves inhibited the production of NO, TNF-α, and IL-6 tested by Griess assay and ELISA, and LPS induced phosphorylation of NF-κB P-65, NF-κB binding protein, tested by Western blot in LPS stimulated RAW 264.7 cells (Xu et al., 2011). Moreover, tussilagone, a sesquiterpenoid isolated from the flower buds of *T. farfara*, inhibited the production of NO, TNF-α, and prostaglandin E2 (PGE2) as well as iNOS and COX-2 expression in LPS stimulated RAW264.7 cells and murine peritoneal macrophages (Hwangbo et al., 2009). Furthermore, antioxidant capacities were shown by a decreased lipid peroxidation initiated by Fe^{2+}/ascorbic acid in

rat brain homogenates, and DPPH radicals scavenging properties (Cho et al., 2005). We could evaluate an activation of PPARs and an inhibition of NF-κB by *T. farfara* leave extracts of different polarity.

Extracts from ***Urtica dioica*** leaves have revealed antagonist activity against the histamine-1 receptor (IC_{50} = 193 (±71) $\mu g/mL^{-1}$) as well as reduction of prostaglandin formation through inhibition of COX-1 (IC_{50} = 1160 (±47) $\mu g/mL^{-1}$), COX-2 (IC_{50} = 275 (±9) $\mu g/mL^{-1}$) and hematopoietic prostaglandin D2 synthase (295 (±51) $\mu g/mL^{-1}$), central enzymes in pro-inflammatory pathways (Roschek et al., 2009). Due to a recent review several clinical and experimental studies suggest that nettle herb has some anti-inflammatory properties (Chrubasik et al., 2007). A polysaccharide fraction isolated from a water extract of the nettle roots showed activity in the carrageenan rat paw edema model and lymphocyte transformation test (Wagner et al., 1989). Nettle leaf extracts potently inhibited NF-κB activation in various cell types and in response to several stimuli. This effect was not mediated by a direct modification of DNA binding, but rather by preventing degradation of its inhibitory subunit IκB-α (Riehemann et al., 1999). These findings are supported by an inhibition of NF-κB in the present study together with an activation of PPARs.

Treatment with extracts from ***Vaccinium myrtillus*** fruits were reported to ameliorate the 2,4,6-trinitro-1-chlorobenzene induced scratching behavior, and ear swelling in a mouse model of chronic allergic contact dermatitis (Yamaura et al., 2011). Moreover, this plant was able to reduce ROS level in HT-29 (250 μg/mL; 24 h, $p<0.05$) and Caco-2 (50 μg/mL; 1 h, $p<0.05$) cells (Kraus et al., 2010). In a clinical trial supplementation with *V. myrtillus* juice resulted in significant decreases in plasma concentrations of C-reactive protein (CRP), IL-6, IL-15 and monokine induced by INF-γ (MIG) whereas, an increase in TNF-α was observed. Furthermore, the polyphenols quercetin, epicatechin, and resveratrol from bilberries inhibited LPS induced NF-κB activation in a monocytic cell line (Karlsen et al., 2010). The anthocyanidins delphinidin 3-O-glucoside and delphinidin 3-O-galactoside were found to be the most effective soybean LOX-1 (IC_{50} values of 0.43 and 0.49 μM) and 5-LOX (IC_{50} values of 2.15 and 6.9 μM) inhibitors (Knaup et al., 2009). In cultured monocytes, anthocyanins isolated from bilberries and black currants efficiently suppressed LPS induced activation of NF-κB. In a clinical trial, changes in the NF-κB-controlled pro-inflammatory chemokines IL-8, RANTES and IFNα in the anthocyanin supplementation group (45, 15, and 40 % decreases from

baseline, resp.) differed from those in the placebo group (20, 0, and 15 % decreases from baseline, resp.) ($P < 0.050$). Similarly, changes in IL-4 and IL-13 in the anthocyanin supplementation group (60 and 38 % decreases from baseline, resp.) varied compared to those in the placebo group (4 and 6 % decreases) ($P = 0.056$ and, $P = 0.089$, resp.) (Karlsen et al., 2007).

Phenolics from ***Vaccinium vitis-idaea*** fruits were shown to suppress IL-6 and TNF-α production at a concentration of 100 µg/mL but had no significant effect on IL-1β production (Kylli et al., 2011). Moreover, extracts from *V. vitis-idaea* berries inhibited platelet activating factor induced exocytosis *in vitro* (Tunon et al., 1995). The present investigations showed that extracts from *V. vitis-idaea* and *V. myrtillus* fruits revealed a potency to inhibit NF-κB, activate PPAR and suppress the production of IL-8 and E-selectin mRNA.

Verbascum mucronatum flower extracts displayed anti-inflammatory, antinociceptive and wound healing activities on the carrageenan induced hind paw edema model in mice and the incision and excision models in mice and rats. Within this study four iridoid glucosides, ajugol, aucubin, lasianthoside I, catalpol, two saponins, ilwensisaponin A and C and a phenylethanoid glycoside, verbascoside were isolated of which only verbascoside revealed significant wound healing activity as well as antinociceptive and anti-inflammatory potentials (Akdemir et al., 2011). Antioxidant activities were shown for ethanolic extracts of the aerial parts of *Verbascum xanthophoeniceum*, collected phenylethanoid glycosides fractions and forsythoside B, verbascoside and leucosceptoside B in 2,2'-diphenyl-1-picrylhydrazyl (DPPH),, oxygen radical absorbance capacity, hydroxyl radical averting capacity, ferric-reducing antioxidant power, and superoxide anion radical scavenging assays (Georgiev et al., 2011). The recent study revealed a new activity of the dichloromethane extract of *Verbasci flos* (in accordance with the Ph. Eur.) on PPARs.

Verbena officinalis whole plant showed moderate inhibition (50 %) of COX-1 (Lin et al., 2008). *In vivo* an anti-inflammatory activity using carrageenan paw edema model was shown for extracts of different polarity, however the chloroform extract showed highest activity. Hereof β-sitosterol, ursolic acid, oleanolic acid, 3-epiursolic acid, 3-epioleanolic acid and minor triterpenoids of derivatives of ursolic acid and oleanolic acids were isolated. From the MeOH extract 2 iridoid glucosides, verbenalin and hastatoside, a phenylpropanoid glycoside, verbascoside and β-sitosterol-D-glucoside were

purified (Deepak and Handa, 2000). However, nothing is known about an inhibition of NF-κB or suppression of IL-8 and E-selectin mRNA, detected in the present investigations.

The last genera investigated were *Veronica* species. Here, extracts of ***Veronica chamedrys*** and ***Veronica officinalis*** revealed only an activity on PPARs and on pro-inflammatory mediators, IL-8 and E-selectin. *Veronica sp.* however, was also active on NF-κB. Nothing has been found about the two *Veronica* species investigated in the concept of inflammation. However, *in vivo* anti-inflammatory and antinociceptive investigations of *Veronica anagallis-aquatica* L. aerial parts showed an inhibitory activity against carrageenan induced hind paw edema and of p-benzoquinone-induced writhing in mice with the methanolic extract. Bioactivity guided isolation led to the identification of aquaticoside A, aquaticoside B, aquaticoside C, veronicoside, catalposide, verproside, verminoside and martynoside of which verproside and catalposide were found to possess potent antinociceptive and anti-inflammatory activities, per os without inducing any apparent acute toxicity as well as gastric damage (Kuepeli et al., 2005). Methanolic extracts of *V. cymbalaria, V. hederifolia, V. pectinata var. glandulosa, V. persica* and *V. polita* showed inhibitory activity of NO release in LPS stimulated macrophages and cytotoxic activity against KB epidermoid carcinoma and B16 melanoma. After partition between water and chloroform, water fractions significantly reduced NO production without any cytotoxicity, while chloroform fractions showed cytotoxicity. Moreover, the water fractions of the five *Veronica* species revealed radical scavenging activity in the DPPH assay (Harput et al., 2002).

Sample			Results						
SPECIES	PLANT PART	EXTRACT	PPAR-α	PPAR-γ	NF-κB	TNF-α INDUCED		LPS INDUCED	
						E-selectin	IL-8	E-selectin	IL-8
Agrimonia sp.	herb	DCM	no	no	moderate				
		wCh	no	no	strong				
		MeOH	no	no	no				
		Det	no	no	no				
Agropyron repens	rhizomes	DCM	strong	strong	no	strong	moderate	moderate	strong
		MeOH	no	no	no	moderate	moderate	no	moderate
		Det	no	moderate	no				
Ajuga genevensis	herb	DCM	no	no	no	strong	moderate	no	moderate
		OC	no	no	no				
		MeOH	no	no	no	strong	no	strong	no
		Det	no	no	no				
Ajuga reptans	herb	DCM	no	no	no	no	moderate	no	moderate
		OC	no	no	no				
		MeOH	no	no	no	strong	no	strong	no
		Det	no	no	no				
Alnus viridis	leaves	DCM	no	no	moderate				
		OC	no	no	strong				
		MeOH	no	no	no				
		Det	moderate	moderate	strong				
Angelica archangelica	roots	DCM	no	no	moderate				
		MeOH	no	no	no				
		Det	no	no	no				
Angelica sylvestris	roots	DCM	no	no	no	strong	moderate	moderate	strong
		MeOH	no	no	no	moderate	no	no	strong
		Det	strong	moderate	strong				
Bellis perennis	flowers	DCM	strong	moderate	moderate				
		MeOH	no	no	no				
		Det	moderate	moderate	no				
Berberis vulgaris	fruits	DCM	no	no	no				
		MeOH	no	no	no				
		Det	moderate	no	no				
Beta vulgaris	roots	DCM	no	no	no				
		MeOH	no	no	no				
		Det	no	no	no				
Betonica officinalis	herb	DCM	no	no	no	strong	strong	little	no
		OC	no	no	no	strong	strong	strong	strong
		MeOH	no	no	no	moderate	strong	moderate	strong
		Det	no	no	no	strong	strong	little	strong
Calluna vulgaris	herb	DCM	no	no	moderate				
		OC	no	no	moderate				
		MeOH	no	no	no				
		Det	no	no	no				
Capsella bursa-pastoris	herb	DCM	strong	no	strong	strong	little	moderate	strong
		OC	strong	moderate	no				
		MeOH	no	no	no	moderate	moderate	no	strong
		Det	strong	strong	strong				

Sample			Results						
SPECIES	PLANT PART	EXTRACT	PPAR-α	PPAR-γ	NF-κB	TNF-α INDUCED		LPS INDUCED	
						E-selectin	IL-8	E-selectin	IL-8
Circea lutetiana	herb	DCM	no	no	no				
		OC	moderate	no	no				
		MeOH	no	no	no				
		Det	no	no	no				
Epilobium angustifolium	herb	DCM	no	no	no				
		OC	no	no	moderate				
		MeOH	no	no	no				
		Det	no	no	no				
Epilobium montanum	herb	DCM	no	no	no	moderate	no	moderate	strong
		OC	moderate	no	moderate				
		MeOH	no	no	no	no	no	no	no
		Det	strong	moderate	moderate				
Epilobium parviflorum	herb	DCM	no	no	moderate				
		OC	no	no	no				
		MeOH	no	no	no				
		Det	no	no	no				
Equisetum arvense	herb	DCM	no	no	strong				
		OC	moderate	moderate	moderate				
		MeOH	no	no	no				
		Det	no	no	no				
Equisetum palustre	herb	DCM	no	no	no	no	no	no	moderate
		OC	no	no	moderate				
		MeOH	no	no	no	strong	no	moderate	no
		Det	no	no	no				
Euphrasia officinalis	herb	DCM	no	no	strong				
		OC	no	no	no				
		MeOH	no	no	no				
		Det	no	no	strong				
Euphrasia rostkoviana	herb	DCM	no	no	no				
		OC	strong	no	moderate				
		MeOH	no	no	no				
		Det	strong	moderate	no				
Filipendula ulmaria	leaves	DCM	no	no	no				
		OC	moderate	moderate	no				
		MeOH	no	no	no				
		Det	no	no	no				
Filipendula ulmaria	herb	DCM	no	no	moderate				
		OC	no	no	moderate				
		MeOH	no	no	no				
		Det	no	no	strong				
Filipendula vulgaris	flowers	DCM	no	no	no	strong	moderate	no	moderate
		MeOH	no	no	no	strong	moderate	strong	no
		Det	no	no	no				
Gentiana punctata	leaves	DCM	no	no	no				
		OC	no	no	no				
		MeOH	no	no	no				
		Det	no	no	no				

Sample			Results						
SPECIES	PLANT PART	EXTRACT	PPAR-α	PPAR-γ	NF-κB	TNF-α INDUCED		LPS INDUCED	
						E-selectin	IL-8	E-selectin	IL-8
Gentiana punctata	roots	DCM	no	no	no				
		MeOH	no	no	no				
		Det	moderate	no	no				
Geum montanum	roots	DCM	no	no	no	strong	strong	little	no
		MeOH	no	no	no	no	strong	no	no
		Det	moderate	moderate	no				
Geum urbanum	herb	DCM	moderate	moderate	strong				
		OC	strong	strong	moderate				
		MeOH	no	no	no				
		Det	no	no	moderate				
Geum urbanum	roots	DCM	moderate	no	moderate	no	strong	moderate	no
		MeOH	no	no	no	no	strong	no	no
		Det	strong	moderate	moderate				
Glechoma hederacea	herb	DCM	no	no	no				
		OC	no	moderate	moderate				
		MeOH	no	no	no				
		Det	strong	strong	strong	strong	strong	no	no
Hippophae rhamnoides	fruits	DCM	no	no	no				
		MeOH	no	no	no				
		Det	no	no	no				
Hypericum maculatum	herb	DCM	no	moderate	moderate				
		OC	moderate	no	no				
		MeOH	no	no	no				
		Det	moderate	no	no				
Linum usitatissimum	seeds	DCM	no	no	no				
		MeOH	no	no	no				
		Det	no	no	no				
Lycopodium sp.	herb	DCM	no	no	strong				
		OC	no	no	strong				
		MeOH	no	no	no				
		Det	moderate	moderate	no				
Majorana hortensis	herb	DCM	no	no	no				
		OC	no	no	strong				
		MeOH	no	no	no				
		Det	strong	moderate	moderate				
Malva neglecta	herb	DCM	no	no	no	strong	no	little	strong
		OC	strong	moderate	no				
		MeOH	no	no	no	no	no	no	strong
		Det	moderate	no	moderate				
Malva sp.	leaves	DCM	moderate	no	strong				
		OC	strong	strong	no	strong	moderate	no	no
		MeOH	no	no	no				
		Det	no	strong	moderate				
Melampyrum pratense	herb	DCM	no	no	no	strong	little	moderate	strong
		OC	no	no	no	moderate	strong	moderate	strong
		MeOH	no	no	no	strong	strong	strong	strong
		Det	no	no	strong	strong	strong	moderate	strong

Sample			Results						
						TNF-α INDUCED		LPS INDUCED	
SPECIES	PLANT PART	EXTRACT	PPAR-α	PPAR-γ	NF-κB	E-selectin	IL-8	E-selectin	IL-8
Melissa officinalis	leaves	DCM	no	no	moderate				
		OC	strong	strong	moderate				
		MeOH	no	no	no				
		Det	no	no	strong				
Origanum vulgare	herb	DCM	no	no	moderate				
		OC	no	no	no				
		MeOH	no	no	no				
		Det	moderate	no	no				
Petasites hybridus	leaves	DCM	no	no	no	strong	moderate	moderate	moderate
		OC	no	no	no				
		MeOH	no	no	no	no	strong	no	moderate
		Det	no	no	moderate				
Peucedanum ostruthium	leaves	DCM	no	no	no	strong	strong	no	no
		OC	no	no	no				
		MeOH	no	no	no				
		Det	moderate	strong	moderate	no	little	no	strong
Peucedanum ostruthium	roots	DCM	no	no	strong				
		MeOH	no	no	no				
		Det	strong	moderate	strong				
Picea abies	shoot tip	DCM	no	no	moderate	no	no	no	no
		OC	no	no	no				
		MeOH	no	no	no	little	no	no	no
		Det	no	no	no				
Pimpinella major	roots	DCM	no	no	moderate	no	strong	no	no
		MeOH	no	no	no	no	moderate	no	no
		Det	no	no	moderate				
Plantago lanceolata	leaves	DCM	no	no	strong				
		OC	no	moderate	strong				
		MeOH	no	no	no				
		Det	strong	moderate	moderate				
Potentilla anserina	herb	DCM	no	no	strong				
		OC	no	no	no				
		MeOH	no	no	no				
		Det	no	moderate	no				
Prunella vulgaris	herb	DCM	no	no	no	strong	no	no	no
		OC	moderate	moderate	strong				
		MeOH	no	no	no	strong	no	moderate	no
		Det	no	no	no				
Ribes nigrum	fruits	DCM	moderate	moderate	no	moderate	strong	no	no
		MeOH	no	no	no				
		Det	strong	moderate	no	no	strong	no	moderate
Rosa canina	fruits	DCM	no	no	no				
		MeOH	no	no	no				
		Det	moderate	moderate	no				

Sample			Results						
SPECIES	PLANT PART	EXTRACT	PPAR-α	PPAR-γ	NF-κB	TNF-α INDUCED E-selectin	TNF-α INDUCED IL-8	LPS INDUCED E-selectin	LPS INDUCED IL-8
Rumex alpinus	leaves	DCM	no	no	no	strong	little	moderate	no
		OC	no	no	no				
		MeOH	no	no	no	no	strong	no	moderate
		Det	no	no	no				
Rumex alpinus	roots	DCM	no	no	no	no	strong	no	no
		MeOH	no	no	no	no	strong	no	strong
		Det	no	no	no				
Salvia officinalis	leaves	DCM	no	no	strong				
		OC	no	no	strong				
		MeOH	no	no	no				
		Det	no	no	strong				
Sambucus ebulus	fruits	DCM	no	no	no	no	little	moderate	no
		MeOH	no	no	no	strong	strong	little	moderate
		Det	no	no	no				
Sambucus nigra	flowers	DCM	no	no	no				
		MeOH	no	no	no				
		Det	strong	moderate	no				
Sambucus nigra	fruits	DCM	no	no	no				
		MeOH	no	no	no				
		Det	strong	strong	strong				
Sanicula europaea	roots	DCM	no	no	no	moderate	moderate	strong	strong
		MeOH	no	no	no	strong	moderate	no	strong
		Det	no	no	no				
Sorbus aucuparia	fruits	DCM	no	no	no				
		MeOH	no	no	no				
		Det	strong	strong	no				
Symphytum officinale	stems	DCM	no	no	no	moderate	no	strong	strong
		OC	strong	moderate	no				
		MeOH	no	no	no	no	no	no	no
		Det	strong	moderate	no				
Symphytum officinale	leaves	DCM	no	no	no	moderate	no	strong	strong
		OC	moderate	no	no				
		MeOH	no	no	no	strong	no	no	strong
		Det	no	no	no				
Symphytum officinale	roots	DCM	moderate	moderate	no				
		MeOH	no	no	no				
		Det	no	no	no				
Tilia sp.	flowers	DCM	moderate	moderate	no				
		MeOH	no	no	no				
		Det	no	no	moderate				
Tussilago farfara	leaves	DCM	moderate	no	moderate				
		OC	strong	moderate	moderate				
		MeOH	no	no	no				
		Det	moderate	moderate	no				

Sample			Results						
SPECIES	PLANT PART	EXTRACT	PPAR-α	PPAR-γ	NF-κB	TNF-α INDUCED		LPS INDUCED	
						E-selectin	IL-8	E-selectin	IL-8
Urtica dioica	leaves	DCM	strong	no	no				
		OC	strong	strong	moderate				
		MeOH	no	no	no				
		Det	no	moderate	moderate				
Vaccinium myrtillus	fruits	DCM	moderate	moderate	no	no	no	no	moderate
		MeOH	no	no	no	strong	little	strong	little
		Det	moderate	moderate	no				
Vaccinium vitis-idaea	fruits	DCM	no	no	no	no	no	no	little
		MeOH	no	no	no				
		Det	moderate	strong	moderate	no	strong	moderate	no
Verbascum sp.	flowers	DCM	strong	moderate	no				
		MeOH	no	no	no	no	no	no	no
		Det	no	no	no				
Verbena officinalis	herb	DCM	no	no	strong	strong	no	no	moderate
		OC	no	no	no				
		MeOH	no	no	no				
		Det	no	no	strong				
Veronica chamaedrys	herb	DCM	no	no	no				
		OC	strong	moderate	no	moderate	moderate	moderate	strong
		MeOH	no	no	no	strong	no	no	strong
		Det	no	no	no				
Veronica officinalis	herb	DCM	no	no	no	strong	little	moderate	strong
		OC	strong	moderate	no				
		MeOH	no	no	no	strong	no	no	no
		Det	no	no	no				
Veronica sp.	herb	DCM	no	no	strong				
		OC	moderate	strong	moderate				
		MeOH	no	strong	no				
		Det	no	no	no				

Table 1 Results of the *in vitro* screening of different extracts produced from 75 traditionally used Austrian drugs on various anti-inflammatory targets. Extracts were tested in triplicates at a concentration of 10 µg/mL on PPARs activation (activ.) as well as on NF-κB inhibition (inhib.) and at 100 µg/mL concerning LPS or TNF-α induced downregulation of interleukine-8 (IL-8) and E-selectin (E-sel.) mRNA; < 25 % inhibition/ activation is considered as no activity (no), 25-50 % inhibition/ activation is interpreted as a low activity (little), 50-75 % inhibition/ activation is considered as a moderate activity (moderate) and high activity is defined as > 75-100 % inhibition/ activation (strong). DCM - dichloromethane extract, MeOH - methanol extract, OC - dichloromethane extract without chlorophyll, Det – methanol extract without polyphenols.

3.2. Computer aided in silico binding studies

In the *in silico* screening of 3194 known compounds, PPAR-γ binding facility of 82 active compounds, of which 45 components belonged to the group of fatty acids, 20 triterpenes and 6 phenylpropanderivates, from 40 plant species was predicted (see table 3, pp. 94-97). These results should help to predict promising products for isolation and provide suggestions for their anti-inflammatory mechanism of action.

Fatty acids are present in most plant drugs and their PPAR binding affinity is already known (Cury-Boaventura et al., 2006; Forman et al., 1996; Hihi et al., 2002).

From all the (furano)coumarins screened only those with a geranyl side chain, namely ostruthin and aurapten *in silico* binding affinity to PPAR-γ.

Triterpenes revealed a high binding affinity to PPAR-γ. PPAR activation by triterpenes was published for both *in silico* (Correia et al., 2006) and *in vitro* (Bae et al., 2010; Correia et al., 2007) screenings of natural compounds.

Compound	Comp. class	Plants published for	PPAR-γ
112-54-9 lauraldehyde	**aldehyde**	***Capsella bursa-pastoris, Tilia sp, Vaccinium myrtillus, Hippophae rhamnoides***	**x**
2922-51-2 2-heptadecanone	**ketone**	***Bellis perennis, Sambucus ebulus***	**x**
221001-23-6 (-)-tejedine	**alkaloid**	***Berberis vulgaris***	**x**
148-83-4 ostruthin	**coumarin**	***Peucedanum ostruthium***	**x**
495-02-3 aurapten	**coumarin**	***Peucedanum ostruthium***	**x**
1002-84-2 pentadecanoic acid	**fatty acid**	***Bellis perennis, Filipendula ulmaria/ vulgaris, Sambucus ebulus, Tussilago farfara, Urtica dioica***	**x**
10233-13-3 isopropyl laurate	**fatty acid**	***Tilia sp.***	**x**
110-27-0 isopropyl myristate	**fatty acid**	***Tilia sp.***	**x**
111-61-5 ethyl stearate	**fatty acid**	***Potentilla anserina***	**x**
111-62-6 ethyl oleate	**fatty acid**	***Potentilla anserina, Hippophae rhamnoides***	**x**
1115-01-1 methyl 9,10-dihydroxystearate	**fatty acid**	***Sambucus nigra***	**x**
112-39-0 methyl palmiate	**fatty acid**	***Bellis perennis, Betonica officinalis, Equisetum arvense, Hippophae rhamnoides, Origanum vulgare, Tussilago farfara, Vaccinium myrtillus***	**x**
112-63-0 methyl linoleate	**fatty acid**	***Bellis perennis, Betonica officinalis, Equisetum arvense, Origanum vulgare, Sambucus ebulus, Verbascum sp.***	**x**
112-80-1 oleic acid	**fatty acid**	***Agropyron repens, Euphrasia sp, Euphrasia rostkoviana, Hippophae rhamnoides, Peucedanum ostruthium, Potentilla anserina, Prunella vulgaris, Ribes nigrum, Rosa canina, Sambucus nigra, Sambucus ebulus, Sambucus racemosa, Urtica dioica***	**x**
112-85-6 docosanoic acid	**fatty acid**	***Agropyron repens, Capsella bursa-pastoris, Prunella vulgaris, Sambucus nigra***	**x**
124-06-1 ethyl myristate	**fatty acid**	***Potentilla anserina, Hippophae rhamnoides***	**x**
142-91-6 isopropyl palmiate	**fatty acid**	***Bellis perennis, Origanum vulgare***	**x**
15514-85-9 9-hydroxy-10,12-octadecadienoic acid	**fatty acid**	***Glechoma hederacea***	**x**
18104-44-4 β-dimorphecolic acid	**fatty acid**	***Glechoma hederacea***	**x**
2197-37-7 linoleic acid	**fatty acid**	***Filipendula ulmaria/ vulgaris***	**x**
2197-51-5 6,9-octadecadienoic acid	**fatty acid**	***Prunella vulgaris***	**x**
24163-97-1 all-cis-farnesyl acetate	**fatty acid**	***Tilia sp.***	**x**

Compound	Comp. class	Plants published for	PPAR-γ
273944-60-8 3,6,17-eicosatrienoic acid	**fatty acid**	***Prunella vulgaris***	**x**
28290-77-9 6,9-linoleic acid	**fatty acid**	***Agropyron repens, Malva sp.***	**x**
301-00-8 methyl linolenate	**fatty acid**	***Bellis perennis, Origanum vulgare, Tussilago farfara, Verbena officinalis***	**x**
373-49-9 palmitoleic acid	**fatty acid**	***Bellis perennis, Hippophae rhamnoides, Linum usitatissimum, Sambucus ebulus, Urtica dioica***	**x**
463-40-1 linolenic acid	**fatty acid**	***Agropyron repens, Bellis perennis, Equisetum arvense, Euphrasia sp., Filipendula ulmaria-vulgaris, Hippophae rhamnoides, Linum usitatissimum, Malva sp, Peucedanum ostruthium, Potentilla anserina, Ribes nigrum, Rosa canina, Sambucus nigra, Sambucus ebulus, Symphytum officinale, Urtica dioica, Vaccinium vitis ideae***	**x**
503-05-9 malvalic acid	**fatty acid**	***Malva sp.***	
506-12-7 margaric acid	**fatty acid**	***Bellis perennis, Filipendula ulmaria-vulgaris, Hippophae rhamnoides, Malva sp.***	**x**
506-26-3 gamma-linolenic acid	**fatty acid**	***Hippophae rhamnoides, Malva sp, Ribes nigrum, Symphytum officinale***	**x**
506-30-9 arachidic acid	**fatty acid**	***Agropyron repens, Capsella bursa-pastoris, Hippophae rhamnoides, Potentilla anserina, Prunella vulgaris, Sambucus nigra, Urtica dioica***	**x**
506-32-1 arachidonic acid	**fatty acid**	***Epilobium angustifolium, Ribes nigrum, Urtica dioica***	**x**
506-37-6 nervonic acid	**fatty acid**	***Sambucus nigra***	**x**
506-46-7 hexacosanoic acid	**fatty acid**	***Lycopodium sp, Sambucus nigra***	**x**
544-63-8 myristic acid	**fatty acid**	***Agropyron repens, Bellis perennis, Calluna vulgaris, Capsella bursa-pastoris, Filipendula ulmaria-vulgaris, Geum montanum, Geum urbanum, Hippophae rhamnoides, Linum usitatissimum, Malva sp, Plantago lanceolata, Potentilla anserina, Sambucus ebulus, Tussilago farfara, Urtica dioica***	**x**
54665-32-6 9-oxo-10,12-octa-decadienoic acid	**fatty acid**	***Glechoma hederacea***	**x**
557-59-5 lignoceric acid	**fatty acid**	***Agropyron repens, Malva sp, Sambucus nigra***	**x**
57-10-3 palmitic acid	**fatty acid**	***Agrimonia sp Agropyron repens, Angelica archangelica, Bellis perennis, Calluna vulgaris, Capsella bursa-pastoris, Equisetum arvense, Equisetum palustre, Euphrasia sp, Filipendula ulmaria/vulgaris, Geum montanum, Geum urbanum, Hippophae rhamnoides, Linum usitatissimum, Lycopodium sp, Origanum vulgare, Peucedanum ostruthium, Potentilla anserina, Prunella vulgaris, Ribes nigrum, Rosa canina, Sambucus nigra, Sambucus ebulus, Tilia sp, Tussilago farfara, Urtica dioica, Vaccinium vitis-idaea, Verbena officinalis***	**x**
57-11-4 stearic acid	**fatty acid**	***Agropyron repens, Angelica archangelica, Bellis perennis, Calluna vulgaris, Capsella bursa-pastoris, Euphrasia sp, Filipendula ulmaria/vulgaris, Hippophae rhamnoides, Linum usitatissimum, Origanum vulgare, Peucedanum ostruthium, Potentilla anserina, Prunella vulgaris, Ribes nigrum, Sambucus nigra, Sambucus ebulus, Sambucus racemosa, Urtica dioica***	**x**

Compound	Comp. class	Plants published for	PPAR-γ
60-33-3 linoleic acid	**fatty acid**	***Angelica archangelica, Bellis perennis, Equisetum arvense, Euphrasia sp, Hippophae rhamnoides, Linum usitatissimum, Peucedanum ostruthium, Potentilla anserina, Ribes nigrum, Rosa canina, Sambucus nigra, Sambucus ebulus, Sambucus racemosa, Tilia sp, Tussilago farfara, Urtica dioica***	**x**
628-97-7 ethyl palmitate	**fatty acid**	***Betonica officinalis, Filipendula ulmaria-vulgaris, Hippophae rhamnoides, Prunella vulgaris, Sambucus ebulus, Vaccinium myrtillus***	**x**
638-53-9 tridecanoic acid	**fatty acid**	***Bellis perennis***	**x**
638-59-5 myristyl acetate	**fatty acid**	***Angelica archangelica, Lycopodium sp.***	**x**
64265-94-7 9-oxo-10,12,15-octadecatrienoic acid	**fatty acid**	***Glechoma hederacea***	**x**
646-30-0 nonadecanoic acid	**fatty acid**	***Malva sp***	**x**
738-87-4 sterculic acid	**fatty acid**	***Malva sp.***	**x**
822-32-2 cetyl alcohol acetate	**fatty acid**	***Euphrasia sp, Euphrasia rostkoviana***	**x**
89886-42-0 9-hydroxy-10,12,15-octadecatrienoic acid	**fatty acid**	***Glechoma hederacea***	**x**
19103-54-9 salvigenin	**flavonoid**	***Salvia officinalis, Veronica sp.***	
24163-97-1 all-cis-farnesyl acetate	**isoprenoid**	***Tilia sp.***	**x**
40266-29-3 cis-2-trans-6-farnesyl acetate	**isoprenoid**	***Origanum vulgare, Tilia sp.***	**x**
4128-17-0 all-trans-farnesyl acetate	**isoprenoid**	***Origanum vulgare, Tilia sp.***	**x**
84254-88-6 geranyl angelate	**isoprenoid**	***Bellis perennis***	
93-28-7 acetyleugenol	**phenylpropane**	***Melissa officinalis***	**x**
99353-00-1 methyl rosmarinate	**phenylpropane**	***Melissa officinalis, Prunella vulgaris***	**x**
48228-96-2 stigmastan-3-ol, acetate (6CI,9CI)	**steroid**	***Potentilla anserina***	**x**
915-05-9 sitosteryl acetate	**steroid**	***Equisetum arvense, Euphrasia sp, Euphrasia rostkoviana, Potentilla anserina***	**x**

Compound	Comp. class	Plants published for	PPAR-γ
108908-93-6 Ursa-12,20(30)-dien-28-oic acid, 2,3-dihydroxy-, methyl ester, (2α,3α)- (9CI)	**triterpene acid**	**Prunella vulgaris**	**x**
26563-64-4 Methyl 3-epimaslinate	**triterpene acid**	**Prunella vulgaris**	**x**
26563-68-8 3-epi-maslinic acid	**triterpene acid**	**Prunella vulgaris**	**x**
485-18-7 Methyl 2α,3α-diacetoxyurs-12-en-28-oate	**triterpene acid**	**Berberis vulgaris**	**x**
500574-38-9 simulanol	**triterpene acid**	**Berberis vulgaris**	**x**
52213-28-2 3-Epicorosolic acid methyl ester	**triterpene acid**	**Prunella vulgaris**	**x**
989-30-0 α-ursolic acid	**triterpene acid**	**Glechoma hederacea**	**x**
102519-34-6 Olean-12-en-28-oic acid, 2,3,23-trihydroxy-, (2α,3α,4α)-	**triterpene**	***Prunella vulgaris***	**x**
1035139-04-8 Ursane-3,23-diol, (3β,4α)-	**triterpene**	***Prunella vulgaris***	**x**
1035139-07-1 Ursane-2,3,19-triol, (2α,3β)-	**triterpene**	***Prunella vulgaris***	**x**
1035139-09-3 Ursane-2,3-diol, (2α,3β)-	**triterpene**	***Prunella vulgaris***	**x**
103974-74-9 Urs-12-en-28-oic acid, 2,3,23-trihydroxy-, (2α,3α,4α)-	**triterpene**	***Prunella vulgaris***	**x**
108908-94-7 Oleana-11,13(18)-dien-28-oic acid, 2,3,23-trihydroxy-, methyl ester, (2α,3α,4β)- (9CI)	**triterpene**	***Prunella vulgaris***	**x**
108925-78-6 Oleana-11,13(18)-dien-28-oic acid, 2,3,23-trihydroxy-, (2α,3α,4β)- (9CI)	**triterpene**	***Prunella vulgaris***	**x**
117589-42-1 13,27-Cycloolean-11-en-28-oic acid, 2,3,23-trihydroxy-, methyl ester, (2α,3α,4β)- (9CI)	**triterpene**	***Prunella vulgaris***	**x**
117589-43-2 13,27-Cycloolean-11-en-28-oic acid, 2,3,23-trihydroxy-, (2α,3α,4β)- (9CI)	**triterpene**	***Prunella vulgaris***	**x**
143839-01-4 Ursa-12,20(30)-dien-28-oic acid, 2,3,23-trihydroxy-, (2α,3α,4α)-	**triterpene**	***Prunella vulgaris***	**x**
52213-27-1 Urs-12-en-28-oic acid, 2,3-dihydroxy-, (2α,3α)-	**triterpene**	***Prunella vulgaris***	**x**
89786-73-2 Urs-12-en-28-oic acid, 2,3,23-trihydroxy-, methyl ester, (2α,3α,4β)- (9CI)	**triterpene**	***Prunella vulgaris***	**x**
89786-83-4 Urs-12-en-28-oic acid, 2,3,23-trihydroxy-, (2α,3α,4β)-	**triterpene**	***Prunella vulgaris***	**x**

Table 2 List of the compounds evaluated as binding to the *in silico* target PPAR-γ. An x indicates a hit.

4. Conclusion

Despite the once wide use of traditional European herbals, modern science has barely started to scientifically explore this treasure (Adams et al., 2009). When entering the term "Traditional European Medicine (TEM)" in the database of SciFinder only 11 citations are found. The concept "Traditional Chinese Medicine (TCM)" on the other hand reveals 25947 and "Ayurveda" 970 references. Two years ago Adams et al. (2009) stated that they found 7 hits for TEM, 4264 for TCM and 550 concerning Ayurveda. This shows a strong increase of interest in TCM but nearly non regarding European ethnomedicine. A reason for this phenomenon is of course the avoidance of traditional ethnomedicine in Europe during the advance of Western medicine, whereas TCM is and was an integral part of the Chinese health system until now. Actually, in Europe nowadays TCM seems even more popular than its own traditional remedies.

The present study provides a collection of several antiphlogistic plant species traditionally used in Austria and the immediate vicinity, which have been barely scientifically investigated until now. Finally, multifunctional *in vitro* anti-inflammatory activities of extracts prepared from the medicinal herbs were compatible with their usage as traditional remedies against diverse inflammatory diseases. 95 % of the screened extracts had high potency to activate PPARs, inhibit NF-κB activation or downregulate the important mediators of inflammation, IL-8 and E-selectin *in vitro*. Moreover, an *in silico* screening of already known compounds, identified and published for the investigated species, provided several possible scaffold candidates that may contribute to the effectiveness of the plant extracts.

In conclusion, the present study of several traditional Austrian healing plants provides some evidence for the efficacy of an ethnopharmacological screening approach, on the one hand. However, foremost it supports the traditional application of several medicinal plant drugs from Austria on a scientific basis.

5. References

Adams, M., Berset, C., Kessler, M., Hamburger, M., 2009. Medicinal herbs for the treatment of rheumatic disorders--a survey of European herbals from the 16th and 17th century. Journal of Ethnopharmacology 121, 343-359.

Akdemir, Z., Kahraman, C., Tatli, I.I., Kuepeli Akkol, E., Suentar, I., Keles, H., 2011. Bioassay-guided isolation of anti-inflammatory, antinociceptive and wound healer glycosides from the flowers of *Verbascum mucronatum* Lam. Journal of Ethnopharmacology 136, 436-443.

An, H.-J., Jeong, H.-J., Um, J.-Y., Kim, H.-M., Hong, S.-H., 2006. *Glechoma hederacea* inhibits inflammatory mediator release in IFN-gamma and LPS-stimulated mouse peritoneal macrophages. Journal of Ethnopharmacology 106, 418-424.

Anderson, N., Meier, T., Borlak, J., 2009. Toxicogenomics applied to cultures of human hepatocytes enabled an identification of novel *Petasites hybridus* Extracts for the treatment of migraine with improved hepatobiliary safety. Toxicological Sciences 112, 507-520.

Bae, H., Kim, H.-J., Shin, M., Lee, H., Yin, C.S., Ra, J., Kim, J., 2010. Inhibitory effect of *Agrimoniae* Herba on lipopolysaccharide-induced nitric oxide and proinflammatory cytokine production in BV2 microglial cells. Neurological Research 32, S53-S57.

Bamborough, P., Callahan James, F., Christopher John, A., Kerns Jeffrey, K., Liddle, J., Miller David, D., Morse Mary, A., Rumsey William, L., Williamson, R., 2009. Progress towards the development of anti-inflammatory inhibitors of IKKbeta. Current Topics in Medicinal Chemistry 9, 623-639.

Barak, V., Halperin, T., Kalickman, I., 2001. The effect of sambucol, a black elderberry-based, natural product, on the production of human cytokines: I. Inflammatory cytokines. European Cytokine Network 12, 290-296.

Baricevic, D., Sosa, S., Della Loggia, R., Tubaro, A., Simonovska, B., Krasna, A., Zupancic, A., 2001. Topical anti-inflammatory activity of *Salvia officinalis* L. leaves: the relevance of ursolic acid. Journal of Ethnopharmacology 75, 125-132.

Barros, L., Carvalho, A.M., Ferreira, I.C.F.R., 2010. Leaves, flowers, immature fruits and leafy flowered stems of *Malva sylvestris*: A comparative study of the nutraceutical potential and composition. Food and Chemical Toxicology 48, 1466-1472.

Baser, K.H.C., 2008. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Current Pharmaceutical Design 14, 3106-3119.

Beara, I.N., Orcic, D.Z., Lesjak, M.M., Mimica-Dukic, N.M., Pekovic, B.A., Popovic, M.R., 2010. Liquid chromatography/tandem mass spectrometry study of anti-inflammatory activity of Plantain (*Plantago* L.) species. Journal of Pharmaceutical and Biomedical Analysis 52, 701-706.

Brasier, A.R., 2010. The nuclear factor-κB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovascular Research 86, 211-218.

Brattstrom, A., Schapowal, A., Maillet, I., Schnyder, B., Ryffel, B., Moser, R., 2010. *Petasites* extract Ze 339 (PET) inhibits allergen-induced Th2 responses, airway inflammation and airway hyperreactivity in mice. Phytotherapy research : PTR 24, 680-685.

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. Experimental Cell Research 309, 121-136.

Cho, J., Kim, H.M., Ryu, J.-H., Jeong, Y.S., Lee, Y.S., Jin, C., 2005. Neuroprotective and antioxidant effects of the ethyl acetate fraction prepared from *Tussilago farfara* L. Biological & Pharmaceutical Bulletin 28, 455-460.

Chrubasik, J.E., Roufogalis, B.D., Wagner, H., Chrubasik, S.A., 2007. A comprehensive review on nettle effect and efficacy profiles, part I: herba urticae. Phytomedicine 14, 423-435.

Churin, A.A., Masnaia, N.V., Sherstoboev, E.I., Shilova, I.V., 2008. Effect of *Filipendula ulmaria* extract on immune system of CBA/CaLac and C57Bl/6 mice. Eksperimental'naia i klinicheskaia farmakologiia 71, 32-36.

Correia, H., Gonzalez-Paramas, A., Amaral, M.T., Santos-Buelga, C., Batista, M.T., 2006. Polyphenolic profile characterization of *Agrimonia eupatoria* L. by HPLC with different detection devices. Biomedical Chromatography 20, 88-94.

Correia, H.S., Batista, M.T., Dinis, T.C.P., 2007. The activity of an extract and fraction of Agrimonia eupatoria L. against reactive species. BioFactors 29, 91-104.

Cosentino, M., Marino, F., Maio, R.C., Delle Canne, M.G., Luzzani, M., Paracchini, S., Lecchini, S., 2010. Immunomodulatory activity of the lignan 7-hydroxymatairesinol potassium acetate (HMR/lignan) extracted from the heartwood of Norway spruce (*Picea abies*). International Immunopharmacology 10, 339-343.

Cui, G., Qin, X., Zhang, Y., Gong, Z., Ge, B., Zang, Y.Q., 2009. Berberine Differentially Modulates the Activities of ERK, p38 MAPK, and JNK to Suppress Th17 and Th1 T Cell Differentiation in Type 1 Diabetic Mice. Journal of Biological Chemistry 284, 28420-28429.

Cury-Boaventura, M.F., Kanunfre, C.C., Gorjao, R., de Lima, T.M., Curi, R., 2006. Mechanisms involved in Jurkat cell death induced by oleic and linoleic acids. Clinical Nutrition 25, 1004-1014.

Czigle, S., Haznagy-Radnai, E., Toth, J., Mathe, I., Grancai, D., 2007. Rosmarinic acid in the genus *Stachys* L. Revista de Medicina si Farmacie 53, 114-117.

Deepak, M., Handa, S.S., 2000. Antiinflammatory activity and chemical composition of extracts of *Verbena officinalis*. Phytotherapy Research 14, 463-465.

Di Paola, R., Esposito, E., Mazzon, E., Riccardi, L., Caminiti, R., Dal Toso, R., Pressi, G., Cuzzocrea, S., 2009. Teupolioside, a phenylpropanoid glycosides of *Ajuga reptans*, biotechnologically produced by IRBN22 plant cell line, exerts beneficial effects on a rodent model of colitis. Biochemical Pharmacology 77, 845-857.

Ebrahimzadeh, M.A., Nabavi, S.F., Nabavi, S.M., Pourmorad, F., 2010. Nitric oxide radical scavenging potential of some Elburz medicinal plants. African Journal of Biotechnology 9, 5212-5217.

Fiebich, B.L., Grozdeva, M., Hess, S., Huell, M., Danesch, U., Bodensieck, A., Bauer, R., 2005. *Petasites hybridus* extracts in vitro inhibit COX-2 and PGE2 release by direct interaction with the enzyme and by preventing p42/44 MAP kinase activation in rat primary microglial cells. Planta Medica 71, 12-19.

Forman, B.M., Chen, J., Evans, R.M., 1996. The peroxisome proliferator-activated receptors: ligands and activators. Annals of the New York Academy of Sciences 804, 266-275.

Garbacki, N., Angenot, L., Bassleer, C., Damas, J., Tits, M., 2002. Effects of prodelphinidins isolated from *Ribes nigrum* on chondrocyte metabolism and COX activity. Naunyn-Schmiedeberg's Archives of Pharmacology 365, 434-441.

Garbacki, N., Kinet, M., Nusgens, B., Desmecht, D., Damas, J., 2005. Proanthocyanidins, from *Ribes nigrum* leaves, reduce endothelial adhesion molecules ICAM-1 and VCAM-1. Journal of Inflammation (London, United Kingdom) 2, online Journal.

Georgiev, M., Alipieva, K., Orhan, I., Abrashev, R., Denev, P., Angelova, M., 2011. Antioxidant and cholinesterases inhibitory activities of *Verbascum xanthophoeniceum* Griseb. and its phenylethanoid glycosides. Food Chemistry 128, 100-105.

Gerlach, S., Saukel, J., Kubelka, W., 2006. Pflanzen in der österreichischen Volksmedizin. Die „VOLKSMED-Datenbank“. Sci Pharm. 74(Suppl 1), 36.

Glass, C.K., Ogawa, S., 2006. Combinatorial roles of nuclear receptors in inflammation and immunity. Nature Reviews Immunology 6, 44-55.

Guginski, G., Luiz, A.P., Silva, M.D., Massaro, M., Martins, D.F., Chaves, J., Mattos, R.W., Silveira, D., Ferreira, V.M.M., Calixto, J.B., Santos, A.R.S., 2009. Mechanisms involved in the antinociception caused by ethanolic extract obtained from the leaves of *Melissa officinalis* (lemon balm) in mice. Pharmacology, Biochemistry and Behavior 93, 10-16.

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

Halkes, S.B.A., Beukelman, C.J., Kroes, B.H., Van den Berg, A.J.J., Van Dijk, H., Labadie, R.P., 1997. A strong complement inhibitor from the flowers of *Filipendula ulmaria*. Pharmaceutical and Pharmacological Letters 7, 79-82.

Harokopakis, E., Albzreh Mohamad, H., Haase Elaine, M., Scannapieco Frank, A., Hajishengallis, G., 2006. Inhibition of proinflammatory activities of major periodontal pathogens by aqueous extracts from elder flower (*Sambucus nigra*). Journal of Periodontology 77, 271-279.

Harput, U.S., Saracoglu, I., Inoue, M., Ogihara, Y., 2002. Anti-inflammatory and cytotoxic activities of five *Veronica* species. Biological & Pharmaceutical Bulletin 25, 483-486.

Harvey, A.L., 2002. Natural products for high-throughput screening. Advances in Phytomedicine 1, 39-44.

Hausmann, M., Obermeier, F., Paper, D.H., Balan, K., Dunger, N., Menzel, K., Falk, W., Schoelmerich, J., Herfarth, H., Rogler, G., 2007. *In vivo* treatment with the herbal phenylethanoid acteoside ameliorates intestinal inflammation in dextran sulphate sodium-induced colitis. Clinical and Experimental Immunology 148, 373-381.

Herold, A., Cremer, L., Calugaru, A., Tamas, V., Ionescu, F., Manea, S., Szegli, G., 2003. Hydroalcoholic plant extracts with anti-inflammatory activity. Roumanian archives of microbiology and immunology 62, 117-129.

Hiermann, A., Reidlinger, M., Juan, H., Sametz, W., 1991. Isolation of the antiphlogistic principle from *Epilobium angustifolium*. Planta Medica 57, 357-360.

Hiermann, A., Schantl, D., 1998. Antiphlogistic and antipyretic activity of *Peucedanum ostruthium*. Planta Medica 64, 400-403.

Hiermann, A., Writzel, M., 1998. Antiphlogistic glycopeptide from the roots of *Symphytum officinale*. Pharmaceutical and Pharmacological Letters 8, 154-157.

Hihi, A.K., Michalik, L., Wahli, W., 2002. PPARs: transcriptional effectors of fatty acids and their derivatives. Cellular and Molecular Life Sciences 59, 790-798.

Huang, N., Hauck, C., Yum, M.-Y., Rizshsky, L., Widrlechner, M.P., McCoy, J.-A., Murphy, P.A., Dixon, P.M., Nikolau, B.J., Birt, D.F., 2009. Rosmarinic Acid in *Prunella vulgaris* Ethanol Extract Inhibits Lipopolysaccharide-Induced Prostaglandin E2 and Nitric Oxide in RAW 264.7 Mouse Macrophages. Journal of Agricultural and Food Chemistry 57, 10579-10589.

Hurst, S.M., McGhie, T.K., Cooney, J.M., Jensen, D.J., Gould, E.M., Lyall, K.A., Hurst, R.D., 2010. Blackcurrant proanthocyanidins augment IFN-γ-induced suppression of IL-4 stimulated CCL26 secretion in alveolar epithelial cells. Molecular Nutrition & Food Research 54, S159-S170.

Hwangbo, C., Lee, H.S., Park, J., Choe, J., Lee, J.-H., 2009. The anti-inflammatory effect of tussilagone, from *Tussilago farfara*, is mediated by the induction of heme oxygenase-1 in murine macrophages. International Immunopharmacology 9, 1578-1584.

Hyun, E.-A., Lee, H.-J., Yoon, W.-J., Park, S.-Y., Kang, H.-K., Kim, S.-J., Yoo, E.-S., 2004. Inhibitory effect of *Salvia officinalis* on the inflammatory cytokines and inducible nitric oxide synthesis in murine macrophage RAW264.7. Yakhak Hoechi 48, 159-164.

Ivanova, D., Gerova, D., Chervenkov, T., Yankova, T., 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. Journal of Ethnopharmacology 96, 145-150.

Jacker, H.J., Hiller, K., 1976. Antiexudative efficiency of the saponins from *Eryngium planum* L. and *Sanicula europaea* L. Pharmazie 31, 747-748.

Jackson Catherine, M., Lee Daniel, K.C., Lipworth Brian, J., 2004. The effects of butterbur on the histamine and allergen cutaneous response. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology 92, 250-254.

Juan, H., Sametz, W., Hiermann, A., 1988. Anti-inflammatory effects of a substance extracted from *Epilobium angustifolium*. Agents and Actions 23, 106-107.

Jung Chang, H., Kim, J.-H., Park, S., Kweon, D.-H., Kim, S.-H., Ko, S.-G., 2010. Inhibitory effect of *Agrimonia pilosa* Ledeb. on inflammation by suppression of iNOS and ROS production. Immunological investigations 39, 159-170.

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

Karlsen, A., Paur, I., Boehn, S.K., Sakhi, A.K., Borge, G.I., Serafini, M., Erlund, I., Laake, P., Tonstad, S., Blomhoff, R., 2010. Bilberry juice modulates plasma concentration of NF-κB related inflammatory markers in subjects at increased risk of CVD. European Journal of Nutrition 49, 345-355.

Karlsen, A., Retterstoel, L., Laake, P., Paur, I., Kjoelsrud-Boehn, S., Sandvik, L., Blomhoff, R., 2007. Anthocyanins inhibit nuclear factor-κB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. Journal of Nutrition 137, 1951-1954.

Kim, J.P., Song, S.B., Lee, I.S., Kim, Y.H., Yoo, I.D., Ryoo, I.J., Bae, K.H., 2011. Anti-inflammatory activity of constituents from *Glechoma hederacea* var. longituba. Bioorganic & Medicinal Chemistry Letters 21, 3483-3487.

Kim, S.-Y., Kim, S.-H., Shin, H.-Y., Lim, J.-P., Chae, B.-S., Park, J.-S., Hong, S.-G., Kim, M.-S., Jo, D.-G., Park, W.-H., Shin, T.-Y., 2007. Effects of *Prunella vulgaris* on mast cell-mediated allergic reaction and inflammatory cytokine production. Experimental Biology and Medicine (Maywood, NJ, United States) 232, 921-926.

Kirkeskov, B., Christensen, R., Buegel, S., Bliddal, H., Danneskiold-Samsoe, B., Christensen, L.P., Andersen, J.R., 2011. The effects of rose hip (*Rosa canina*) on plasma antioxidative activity and C-reactive protein in patients with rheumatoid arthritis and normal controls: A prospective cohort study. Phytomedicine 18, 953-958.

Kiss, A.K., Bazylko, A., Filipek, A., Granica, S., Jaszewska, E., Kiarszys, U., Kosmider, A., Piwowarski, J., 2011. Oenothein B's contribution to the anti-inflammatory and antioxidant activity of *Epilobium sp.* Phytomedicine 18, 557-560.

Knaup, B., Oehme, A., Valotis, A., Schreier, P., 2009. Anthocyanins as lipoxygenase inhibitors. Molecular Nutrition & Food Research 53, 617-624.

Korkina, L.G., Mikhal'chik, E.V., Suprun, M.V., Pastore, S., Dal Toso, R., 2007. Molecular mechanisms underlying wound healing and anti-inflammatory properties of naturally occurring biotechnologically produced phenylpropanoid glycosides. Cellular and Molecular Biology (Sarreguemines, France, Online) 53, 84-91.

Kraus, M., Kahle, K., Ridder, F., Schantz, M., Scheppach, W., Schreier, P., Richling, E., 2010. Colonic availability of bilberry anthocyanins in humans. ACS Symposium Series 1035, 159-176.

Krishnaiah, D., Sarbatly, R., Nithyanandam, R., 2011. A review of the antioxidant potential of medicinal plant species. Food and Bioproducts Processing 89, 217-233.

Kuepeli, E., Harput, U.S., Varel, M., Yesilada, E., Saracoglu, I., 2005. Bioassay-guided isolation of iridoid glucosides with antinociceptive and anti-inflammatory activities from *Veronica anagallis-aquatica* L. Journal of Ethnopharmacology 102, 170-176.

Kuo, C.-H., Lee, C.-W., Lai, Y.-C., Lee, S.-S., 2008. Determination of oregonin in *Alnus* plants and biological samples by capillary electrophoresis. Journal of Pharmaceutical and Biomedical Analysis 47, 195-200.

Kwon, D.-J., Bae, Y.-S., Ju, S.M., Goh, A.R., Choi, S.Y., Park, J., 2011. Casuarinin suppresses TNF-α-induced ICAM-1 expression via blockade of NF-κB activation in HaCaT cells. Biochemical and Biophysical Research Communications 409, 780-785.

Kylli, P., Nohynek, L., Puupponen-Pimia, R., Westerlund-Wikstrom, B., Leppanen, T., Welling, J., Moilanen, E., Heinonen, M., 2011. Lingonberry (*Vaccinium vitis-idaea*) and European Cranberry (*Vaccinium microcarpon*) Proanthocyanidins: Isolation, Identification, and Bioactivities. Journal of Agricultural and Food Chemistry 59, 3373-3384.

Lattanzio, F., Greco, E., Carretta, D., Cervellati, R., Govoni, P., Speroni, E., 2011. *In vivo* anti-inflammatory effect of *Rosa canina* L. extract. Journal of Ethnopharmacology 137, 880-885.

Lee, H.J., Kim, J.S., Jin, C., Ryu, J.-H., 2005. Inhibitory activity of medicinal herbs on nitric oxide synthesis in activated macrophages. Natural Product Sciences 11, 16-21.

Lin, G.D., Li, R.W., Myers, S.P., Leach, D.N., 2008. A method of selecting plants with anti-inflammatory potential for pharmacological study. Natural Product Communications 3, 71-76.

Maeaettae, J., Luukkonen, R., Husgafvel-Pursiainen, K., Alenius, H., Savolainen, K., 2006. Comparison of hardwood and softwood dust-induced expression of cytokines and chemokines in mouse macrophage RAW 264.7 cells. Toxicology 218, 13-21.

Marchesan, M., Paper, D.H., Hose, S., Franz, G., 1998. Investigation of the antiinflammatory activity of liquid extracts of *Plantago lanceolata* L. Phytotherapy Research 12, S33-S34.

Markt, P., Petersen, R.K., Flindt, E.N., Kristiansen, K., Kirchmair, J., Spitzer, G., Distinto, S., Schuster, D., Wolber, G., Laggner, C., Langer, T., 2008. Discovery of Novel PPAR Ligands by a Virtual Screening Approach Based on Pharmacophore Modeling, 3D Shape, and Electrostatic Similarity Screening. Journal of Medicinal Chemistry 51, 6303-6317.

Markt, P., Schuster, D., Kirchmair, J., Laggner, C., Langer, T., 2007. Pharmacophore modeling and parallel screening for PPAR ligands. Journal of Computer-Aided Molecular Design 21, 575-590.

Marrassini, C., Anesini, C., Ferraro, G., 2011. HPLC Fingerprint of a flower extract of *Tilia* × *viridis* and correlation with antiproliferative and antioxidant activity. Phytotherapy Research 25, 1466-1471.

Marzani, B., Benedusi, A., Giuliani, G., Buonocore, D., Marzatico, F., 2009. *Ajuga reptans*: biological activity of a dry phenylpropanoid-standardized extract. Cosmetic Technology (Milano, Italy) 12, 19-23.

Mencherini, T., Picerno, P., Russo, P., Meloni, M., Aquino, R., 2009. Composition of the fresh leaves and stems of *Melissa officinalis* and evaluation of skin irritation in a reconstituted human epidermis model. Journal of Natural Products 72, 1512-1515.

Mueller, M., Hobiger, S., Jungbauer, A., 2010. Anti-inflammatory activity of extracts from fruits, herbs and spices. Food Chemistry 122, 987-996.

Murai, M., Tamayama, Y., Nishibe, S., 1995. Phenylethanoids in the herb of *Plantago lanceolata* and inhibitory effect on arachidonic acid-induced mouse ear edema. Planta Medica 61, 479-480.

Muraoka, O., Yoshikawa, M., Morikawa, T., Ninomiya, K., 2011. Anti-TNF-α agents and hepatoprotective agents derived from *Potentilla anserina*, pharmaceuticals containing theagents, and new saponin and polyphenol compounds. (Kinki University, Japan; DiabetymCo., Ltd.). Application: JP, p. 42pp.

Najid, A., Simon, A., Cook, J., Chable-Rabinovitch, H., Delage, C., Chulia, A.J., Rigaud, M., 1992. Characterization of ursolic acid as a lipoxygenase and cyclooxygenase inhibitor using macrophages, platelets and differentiated HL60 leukemic cells. FEBS Letters 299, 213-217.

Newman, D.J., Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years. Journal of Natural Products 70, 461-477.

Ocana-Fuentes, A., Arranz-Gutierrez, E., Senorans, F.J., Reglero, G., 2010. Supercritical fluid extraction of oregano (*Origanum vulgare*) essential oils: Anti-inflammatory properties based on cytokine response on THP-1 macrophages. Food and Chemical Toxicology 48, 1568-1575.

Oka, M., Tachibana, M., Noda, K., Inoue, N., Tanaka, M., Kuwabara, K., 2007. Relevance of anti-reactive oxygen species activity to anti-inflammatory activity of components of Eviprostat, a phytotherapeutic agent for benign prostatic hyperplasia. Phytomedicine 14, 465-472.

Orhan, I., Kuepeli, E., Terzioglu, S., Yesilada, E., 2007. Bioassay-guided isolation of kaempferol-3-O-β-D-galactoside with anti-inflammatory and antinociceptive activity from the aerial part of *Calluna vulgaris* L. Journal of Ethnopharmacology 114, 32-37.

Paur, I., Austenaa, L.M., Blomhoff, R., 2008. Extracts of dietary plants are efficient modulators of nuclear factor kappa B. Food and Chemical Toxicology 46, 1288-1297.

Peake, P.W., Pussell, B.A., Martyn, P., Timmermans, V., Charlesworth, J.A., 1991. The inhibitory effect of rosmarinic acid on complement involves the C5 convertase. International Journal of Immunopharmacology 13, 853-857.

Pirbalouti Abdollah, G., Azizi, S., Koohpayeh, A., Hamedi, B., 2010. Wound healing activity of *Malva sylvestris* and *Punica granatum* in alloxan-induced diabetic rats. Acta poloniae pharmaceutica 67, 511-516.

Poeckel, D., Greiner, C., Verhoff, M., Rau, O., Tausch, L., Hoernig, C., Steinhilber, D., Schubert-Zsilavecz, M., Werz, O., 2008. Carnosic acid and carnosol potently inhibit human 5-lipoxygenase and suppress pro-inflammatory responses of stimulated human polymorphonuclear leukocytes. Biochemical Pharmacology 76, 91-97.

Popescu, M., Danciu, T., Danciu, E., Ivopol, G., Manea, S., 2011. Natural antioxidants, free-radical-scavengers and minerals, in fresh juices and vegetables. Revista de Chimie (Bucharest, Romania) 62, 761-765.

Qnais Esam, Y., Abu-Dieyeh, M., Abdulla Fuad, A., Abdalla Shtaywy, S., 2010. The antinociceptive and anti-inflammatory effects of *Salvia officinalis* leaf aqueous and butanol extracts. Pharmaceutical biology 48, 1149-1156.

Ren, Y., Lu, L., Guo, T.B., Qiu, J., Yang, Y., Liu, A., Zhang, J.Z., 2008. Novel Immunomodulatory properties of berbamine through selective down-regulation of STAT4 and action of IFN-γ in experimental autoimmune encephalomyelitis. Journal of Immunology 181, 1491-1498.

Riehemann, K., Behnke, B., Schulze-Osthoff, K., 1999. Plant extracts from stinging nettle (*Urtica dioica*), an antirheumatic remedy, inhibit the proinflammatory transcription factor NF-κB. FEBS Letters 442, 89-94.

Roos, G., Waiblinger, J., Zschocke, S., Liu, J.H., Klaiber, I., Kraus, W., Bauer, R., 1997. Isolation, identification, and screening for COX-1- and 5-LO-inhibition of coumarins from *Angelica archangelica*. Pharmaceutical and Pharmacological Letters 7, 157-160.

Roschek, B., Jr., Fink, R.C., McMichael, M., Alberte, R.S., 2009. Nettle extract (*Urtica dioica*) affects key receptors and enzymes associated with allergic rhinitis. Phytotherapy Research 23, 920-926.

Saaby, L., Jager, A.K., Moesby, L., Hansen, E.W., Christensen, S.B., 2011. Isolation of immunomodulatory triterpene acids from a standardized rose hip powder (*Rosa canina* L.). Phytotherapy Research 25, 195-201.

Schwaiger, S., Zeller, I., Poelzelbauer, P., Frotschnig, S., Laufer, G., Messner, B., Pieri, V., Stuppner, H., Bernhard, D., 2011. Identification and pharmacological characterization of the anti-inflammatory

principal of the leaves of dwarf elder (*Sambucus ebulus* L.). Journal of Ethnopharmacology 133, 704-709.

Shen, D., Pan, M.-H., Wu, Q.-L., Park, C.-H., Juliani, H.R., Ho, C.-T., Simon, J.E., 2010. LC-MS method for the simultaneous quantitation of the anti-inflammatory constituents in oregano (*Origanum* Species). Journal of Agricultural and Food Chemistry 58, 7119-7125.

Shokrzadeh, M., Saeedi Saravi, S.S., 2010. The chemistry, pharmacology and clinical properties of *Sambucus ebulus*: A review. Journal of Medicinal Plants Research 4, 095-103.

Singh, S., Nair, V., Jain, S., Gupta, Y.K., 2008. Evaluation of anti-inflammatory activity of plant lipids containing alpha-linolenic acid. Indian journal of experimental biology 46, 453-456.

Steenkamp, V., Gouws, M.C., Gulumian, M., Elgorashi, E.E., van Staden, J., 2006. Studies on antibacterial, anti-inflammatory and antioxidant activity of herbal remedies used in the treatment of benign prostatic hyperplasia and prostatitis. Journal of Ethnopharmacology 103, 71-75.

Thomet, O.A.R., Wiesmann, U.N., Schapowal, A., Bizer, C., Simon, H.U., 2001. Role of petasin in the potential anti-inflammatory activity of a plant extract of *Petasites hybridus*. Biochemical Pharmacology 61, 1041-1047.

Tomosaka, H., Chai, H., Chin, Y.-W., Salim, A.A., Keller, W.J., Kinghorn, A.D., 2007. Antioxidants from *Berberis vulgaris* (Barberry). Abstracts of Papers, 234th ACS National Meeting, Boston, MA, United States, August 19-23, 2007, AGFD-132.

Tunon, H., Olavsdotter, C., Bohlin, L., 1995. Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. Journal of Ethnopharmacology 48, 61-76.

Wagner, H., Willer, F., Kreher, B., 1989. Biologically active compounds from the aqueous extract of *Urtica dioica*. Planta Medica 55, 452-454.

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.

Wenzig, E.M., Widowitz, U., Kunert, O., Chrubasik, S., Bucar, F., Knauder, E., Bauer, R., 2008. Phytochemical composition and in vitro pharmacological activity of two rose hip (*Rosa canina* L.) preparations. Phytomedicine 15, 826-835.

Xu, L., Li, C., Zhang, M., Dai, Y., Huang, F., 2011. Effect of the ethyl acetate extract of Farfarae Flos on inflammatory factors. Zhongguo Yaoke Daxue Xuebao 42, 64-67.

Xu, Y., Wu, Q., Pan, M.-H., Ho, C.-T., Simon, J.E., 2008. Phytochemical screening and bioactive properties of the medicinal plant eyebright (*Euphrasia officinalis*). Abstracts of Papers, 236th ACS National Meeting, Philadelphia, PA, United States, August 17-21, 2008, AGFD-143.

Yamaura, K., Shimada, M., Ueno, K., 2011. Anthocyanins from bilberry (*Vaccinium myrtillus* L.) alleviate pruritus in a mouse model of chronic allergic contact dermatitiss. Pharmacognosy Research 3, 173-177.

Yasukawa, K., Kitanaka, S., Kawata, K., Goto, K., 2009. Anti-tumor promoters phenolics and triterpenoid from *Hippophae rhamnoides*. Fitoterapia 80, 164-167.

Yesilada, E., 1998. Evaluation of the anti-inflammatory activity of the Turkish medicinal plant *Sambucus ebulus*. Chemistry of Natural Compounds 33, 539-540.

Zdarilova, A., Svobodova, A., Simanek, V., Ulrichova, J., 2009. *Prunella vulgaris* extract and rosmarinic acid suppress lipopolysaccharide-induced alteration in human gingival fibroblasts. Toxicology in Vitro 23, 386-392.

Zimecki, M., Artym, J., Cisowski, W., Mazol, I., Wlodarczyk, M., Glensk, M., 2009. Immunomodulatory and anti-Inflammatory activity of selected osthole derivatives. Zeitschrift fuer Naturforschung Section C 64, 361-368.

3.3 Plant extracts in cell based anti-inflammatory assays – Methods for the removement of activity masking bulk components (manuscript)

This manuscript was prepared for submission and was modified from a former version included in the PhD thesis of Dott. Paolo Picker (Picker, 2011). Phytochemical work, except MS analyses (Dr. Martin Zehl, Dept. of Pharmacognosy, University of Vienna) was performed by the author of this thesis and Dott. Paolo Picker from the Dept. of Pharmacognosy, University of Vienna. Co-workers from the University of Graz and Innsbruck each performed two different methods for the removal of bulk components CR2 and PR2 and CR3 and PR3, respectively. Pharmacological testing concerning the downregulation of IL-8 after stimulation with TNF-α or LPS, apart from ELISA of IL-8, has been performed by colleagues from the Institute for Vascular Biology and Thrombosis Research of the Medical University from Vienna. Cell based luciferase assays, for activation of PPARs and inhibition of NF-κB was conducted by co-workers from the Department of Pharmacognosy, University of Vienna.

Plant extracts in cell based anti-inflammatory assays – Methods for the removement of activity masking bulk components

Sylvia Vogl[a†], Paolo Picker[a†], Judit Mihaly-Bison[b], Markus Binder[b], Nanang Fakhrudin[a‡], Atanas G. Atanasov[a], Elke H. Heiss[a], Martin Zehl[a], Johannes Saukel[a], Christoph Wawrosch[a], Andreas Schinkovitz[c], Rudolf Bauer[c], Judith M. Rollinger[d], Hermann Stuppner[d], Verena M. Dirsch[a], Valery Bochkov[b], Gottfried Reznicek[a]*, Brigitte 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:** phone: +43-1-4277-55210, fax: +43-1-4277-9552, email: gottfried.reznicek@univie.ac.at

Keywords:
chlorophyll, tannins, inflammation, cell based assays, plant extracts

Abstract

Ethnopharmacological relevance

During an *in vitro* screening of 75 traditionally used Austrian medicinal plant drugs in the prominent cell-based anti-inflammatory assays, inhibition of activation of the nuclear factor-κB (NF-κB) and reduction of the expression of pro-inflammatory interleukin-8 (IL-8), the question arose if bulk constituents, such as chlorophylls and tannins, present in plants in high amounts, should be removed before testing. It was previously shown that these ubiquitous plant constituents can interfere with some biological *in vitro* assays. However, a possible influence on the intended targets has never been investigated before.

Aim of the study

Moreover, chlorophylls and tannins represent a significant portion of plant extracts, decreasing the relative amount of other bioactive components. Therefore, the aim of this study was to examine (i) whether chlorophyll and phenolic compounds from plant extracts may lead to false positive or false negative results in three selected cell-based anti-inflammatory test systems never investigated for such an interference before, (ii) whether their elimination markedly alters the bioactivity of extracts, and (iii) whether there are differences between distinct clearance procedures.

Material and methods

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 IL-8, induced by the pro-inflammatory stimuli tumour 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.

Results

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.

Conclusion

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; Kinghorn et al., 2011; Newman, 2008; Newman and Cragg, 2007). To identify new potential bioactive compounds, biological *in vitro* assays are used (Swinney and Anthony, 2011).

Therefore, in a first step traditionally used medicinal plants from Austria and the immediate vicinity were gathered in the so called VOLKSMED database (Gerlach et al., 2006). In a second step, plants of this database associated with a usage against inflammatory diseases (rheumatism, wound healing, bronchitis, colds, etc.) were chosen for a screening in different *in vitro* cell based assays indicating an inflammatory action, i.e. inhibition of the nuclear factor kappa B (NF-κB) and downregulation of lipopolysaccharide (LPS) or tumor necrosis factor (TNF-α) stimulated expression of interleukin (IL-8).

When testing plant extracts in biological *in vitro* assays, a potential interference of ubiquitous substances, such as chlorophyll and polyphenols (e.g. tannins) 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). Therefore, cell-based *in vitro* assays are highly at risk to be influenced by these bulk components.

Nevertheless, to the best of our knowledge until now, an influence was only shown for tannins in cell-based *in vitro* assays regarding anti-bacterial, anti-fungal, anti-viral and anti-parasitic potential (Cos et al., 2006; Tan et al., 1991; Wall et al., 1996). Additionally, antioxidant capacities of tannins have been previously explored (Wei et al., 2011; Zhang et al., 2010), while Feldman et al. (2002) reported an inhibition of TNF-α secretion from LPS- stimulated human peripheral blood mononuclear cells by designed dimeric gallotannin analogues (Feldman Ken et al., 2002). Concerning chlorophylls, studies only indicate an anti-oxidative capacity (Cho et al., 2000; Park et al., 2007).

So far, investigations of chlorophylls, tannins and rosmarinic acid on the prominent pro-inflammatory mediators NF-κB, LPS, TNF-α and interleukins are rare. However, plant extracts as well as plant-derived polyphenols have been repeatedly shown to be efficient modulators of these inflammatory mediators (Cho et al., 1998; Dvorak et al., 2006; Geraets et al., 2009; Gutierrez-Venegas et al., 2006; Hamalainen et al., 2007; Jeong et al., 2002; Liao et al., 2010; Nam, 2006; Paur et al., 2008). Therefore, it is important to evaluate possible false positive screening results evoked by these compounds.

Relative to tannins, most non-polyphenolic compounds appear in much lower amounts in polar plant extracts (Silva et al., 1998). Chlorophyll typically represents more than a half of crude nonpolar extracts, displaying a possible masking effect of active components. We therefore hypothesized that the elimination of bulk constituents, such as tannins, rosmarinic acid and chlorophylls, may result in a higher specific bioactivity of plant extracts, which consequently should be purified prior to pharmacological evaluation *in vitro*.

2. Materials and Methods

2.1. Plant material

The dried leaves of *Malva sp. (Malvae Folium* according to the European Pharmacopoeia, Ph. Eur.) and the dried aerial parts of *Glechoma hederacea* L. (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 of p.a. grade for extraction and HPLC grade for HPLC analyses (VWR, Vienna, Austria). Formic acid p.a. 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 (Vienna, Austria), while rosmarinic acid (98 % purity) was obtained from Extrasynthese (Genay, France).

2.3. Extraction

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 Austria GmbH, Vienna, Austria) 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.

Subsequently, DCM extracts from *Malva sp.* were used for the chlorophyll elimination comparison; while MeOH extracts from *Glechoma hederacea* were subjected to the different tannin elimination procedures (extraction yields see Table 1, p. 128).

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 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 MeOH/H_2O, was filtered.

For the second method (CR2), preparative TLC plates (20x20 cm, silica gel 60, Merck, Darmstadt, Germany) were pre-developed in DCM until the solvent front reached the end of the plate, in order to remove impurities. Next, the extract 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 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 tannins

Three methods to eliminate tannins (or rosmarinic acid, which is known to be present in *Glechoma hederacea* (Okuda et al., 1986)) 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 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 tannin-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. 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 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 of sample solution was 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 Laboratories, Inc. Mountain View, USA). Six hours later cells were transferred to 96 well plates and incubated at 5 % CO_2 and 37 °C overnight. The medium was exchanged with a serum-free

Dulbecco's modified eagle medium (DMEM) and the indicated treatments were performed on the next day. 30 min after treatment cells were stimulated with 2 ng/mL recombinant human TNF-α for 4 h, the medium was removed and the cells were lysed. Plant extracts as well as reference pure compounds (10 µg/mL) were tested in at least three independent experiments with four replicates each; parthenolide (5 µM) was used as positive control. The luminescence of the firefly luciferase and the fluorescence of EGFP were quantified on a GeniosPro plate reader (Tecan, Grödig, Austria). Consequently, the luciferase signal 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, USA), cell growth supplement (Promocell, Germany) and antibiotics. At least three independent experiments with six replicates each were performed in 96 well plates in M199 medium containing 1 % bovine serum albumin (BSA) (Applichem, Darmstadt, Germany) and 3 % serum.

Plant extracts were tested at a concentration of 10 µg/mL, reference pure compounds at 10 µM; BAY 11-7082 was used as positive control (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, USA) or 300 ng/mL of LPS (Sigma-Aldrich, St. Louis, USA) 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, USA). Supernatants were transferred into 96 well plates (NALGE-NUNC Int., Rochester, USA) 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, USA), while the optical density (OD) was measured with a SynergyHT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, USA) 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 of all, 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 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, 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) were evaluated.

Fig.1 (p. 123) 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). Moreover, these compounds did not activate NF-κB or induce IL-8 expression, when applied to unstimulated cells (data not shown).

- *Figure 1* (p. 123) **-**

Chlorophyll-depleted extracts, tested at 10 µg/mL in TNF-α- or LPS-stimulated cells, differently influenced IL-8 expression or NF-κB activity, depending on the used elimination method. The crude DCM extract from *Malva sp.* significantly inhibited NF-κB

activation and IL-8 expression in TNF-α- and LPS-stimulated cells. However, all chlorophyll depletion methods resulted in a clear loss of activity (Fig. 2, p. 124).
- *Figure 2* (p. 124) **-**

The differences between the depletion methods were even more pronounced for the tannin eliminations. Method PR1 led to a strong NF-κB and IL-8 inhibitory activity of the *Glechoma hederacea* methanol extract (Figs. 3a, 3b and 3c, p. 125). In contrast, methods PR2 and PR3 resulted in extracts revealing the same inactivity as the crude methanol extract (Figs. 3a and 3b, p. 125) or led to a loss of activity compared to the crude methanol extract (Fig. 3c, p. 125).
- *Figure 3* (p. 125) **-**

To explain these differences, the chlorophyll- and tannin-depleted extracts were analytically evaluated. The yields of the purified extracts after the different purification procedures (w/w, relative to the crude extracts), as well as those of the extractions (drug extract ratio, DER), are listed in Table 1 (p. 128) .
- *Table 1* (p. 128) **-**

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 sp.* (Fig. 4, p. 126), was quantitatively removed by all three methods. Additionally, this purification process resulted in the enrichment of other more polar constituents (methods CR1 and CR3). However, the ELSD detection (Fig. 5, p. 126-127) 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 visualized in the HPLC-UV chromatogram due to the lack of chromophores.
- *Figures 4, 5* (p. 126-127) **-**

HPLC analyses of *Glechoma hederacea* extracts (Fig. 6, p. 127) showed the successful clearance of rosmarinic acid, 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, may be responsible for the activity of the relative rosmarinic acid free extract.
- *Figure 6* (p. 127) **-**

These results 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. This was shown during the screening of the 75 plant drugs, where a (higher) activity was usually reached by the chlorophyll or tannin/ rosmarinic acid depleted extracts compared to the original crude dichloromethane or methanolic extracts.
On the other hand, depletions are not specific and may lead to the loss of bioactive constituents. Comparing the advances of the used depletion methods, the partition procedure for chlorophyll as well as that for polyphenols is easy to handle and does not lead to any loss of compounds in total on stationary phases. The separation of chlorophyll on TLC, on the other hand is quite laborious and not exhaustive. Separation with classical column chromatography on the other hand, is more specific and leads to a first fractionation of compounds.

In conclusion, our analyses 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 testing of the crude extracts should always be included in order to evaluate a possible loss of active compounds. Additionally, 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

This work is funded by the Austrian Science Fund, NFN: S107-B03.

References

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

Cho, J.Y., Lee, J., Park, J., Park, M.H., 1998. Isolation of tumor necrosis factor-alpha inhibitory components from *Angelica koreana.* Yakhak Hoechi 42, 125-131.

Cho, K.J., Han, S.H., Kim, B.Y., Hwang, S.G., Park, K.K., Yang, K.H., Chung, A.S., 2000. Chlorophyllin suppression of lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells. Toxicology and applied pharmacology 166, 120-127.

Cos, P., Vlietinck, A.J., Vanden Berghe, D., Maes, L., 2006. Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. Journal of Ethnopharmacology 106, 290-302.

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

Dvorak, Z., Vrzal, R., Maurel, P., Ulrichova, 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, 117-128.

Feldman Ken, S., Wilson Sarah, L., Lawlor Michael, D., Lang Charles, H., Scheuchenzuber William, J., 2002. *In vitro* and *in vivo* inhibition of LPS-induced tumor necrosis factor-alpha production by dimeric gallotannin analogues. Bioorganic & Medicinal Chemistry 10, 47-55.

Foster, B.C., Arnason, J.T., Briggs, C.J., 2005. Natural health products and drug disposition. Annual Review of Pharmacology and Toxicology 45, 203-226.

Geraets, L., Haegens, A., Brauers, K., Haydock, J.A., Vernooy, J.H.J., Wouters, E.F.M., Bast, A., Hageman, G.J., 2009. Inhibition of LPS-induced pulmonary inflammation by specific flavonoids. Biochemical and Biophysical Research Communications 382, 598-603.

Gerlach, S., Saukel, J., Kubelka, W., 2006. Pflanzen in der österreichischen Volksmedizin. Die „VOLKSMED-Datenbank“. Scientia Pharmaceutica 74(1), 36.

Gutierrez-Venegas, G., Kawasaki-Cardenas, P., Rita Arroyo-Cruz, S., Maldonado-Frias, S., 2006. Luteolin inhibits lipopolysaccharide actions on human gingival fibroblasts. European Journal of Pharmacology 541, 95-105.

Hamalainen, M., Nieminen, R., Vuorela, P., 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 (E-journal).

Harvey, A.L., 2007. Natural products as a screening resource. Current Opinion in Chemical Biology 11, 480-484.

Jeong, H.-J., Koo, H.-N., Na, H.-J., Kim, M.-S., Hong, S.-H., Eom, J.-W., Kim, K.-S., Shin, T.-Y., Kim, H.-M., 2002. Inhibition of TNF α and IL 6 production by aucubin through blockade of NF-κB activation in RBL-2H3 mast cells. Cytokine 18, 252-259.

Kinghorn, A.D., Pan, L., Fletcher, J.N., Chai, H., 2011. The Relevance of Higher Plants in Lead Compound Discovery Programs. Journal of Natural Products 74, 1539-1555.

Liao, P.-C., Chien, S.-C., Ho, C.-L., Wang, E.I.C., Lee, S.-C., Kuo, Y.-H., Jeyashoke, N., Chen, J., Dong, W.-C., Chao, L.K., Hua, K.-F., 2010. Osthole Regulates Inflammatory Mediator Expression through Modulating NF-κB, Mitogen-Activated Protein Kinases, Protein Kinase C, and Reactive Oxygen Species. Journal of Agricultural and Food Chemistry, 58(19), 10445-10451..

Nam, N.-H., 2006. Naturally occurring NF-κB inhibitors. Mini-Reviews in Medicinal Chemistry 6, 945-951.

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

Newman, D.J., Cragg, G.M., 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, 1108-1111.

Park, J.-Y., Park, C.-M., Kim, J.-J., Noh, K.-H., Cho, C.-W., Song, Y.-S., 2007. The protective effect of chlorophyll a against oxidative stress and inflammatory processes in LPS-stimulated macrophages. Food Science and Biotechnology 16, 205-211.

Paur, I., Austenaa, L.M., Blomhoff, R., 2008. Extracts of dietary plants are efficient modulators of nuclear factor kappa B. Food and Chemical Toxicology 46, 1288-1297.

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

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

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

Tan, G.T., Pezzuoto, J.M., Kinghorn, A.D., Hughes, S.H., 1991. Evaluation of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Journal of Natural Products 54, 143-154.

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.

Wei, S.-D., Chen, R.-Y., Liao, M.-M., Tu, N.-W., Zhou, H.-C., Lin, Y.-M., 2011. Antioxidant condensed tannins from *Machilus pauhoi* leaves. Journal of Medicinal Plants Research 5, 796-804.

Zhang, L.-L., Lin, Y.-M., Zhou, H.-C., Wei, S.-D., Chen, J.-H., 2010. Condensed tannins from mangrove species *Kandelia candel* and *Rhizophora mangle* and their antioxidant activity. Molecules 15, 420-431.

Figures and Tables

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-α (TNF): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

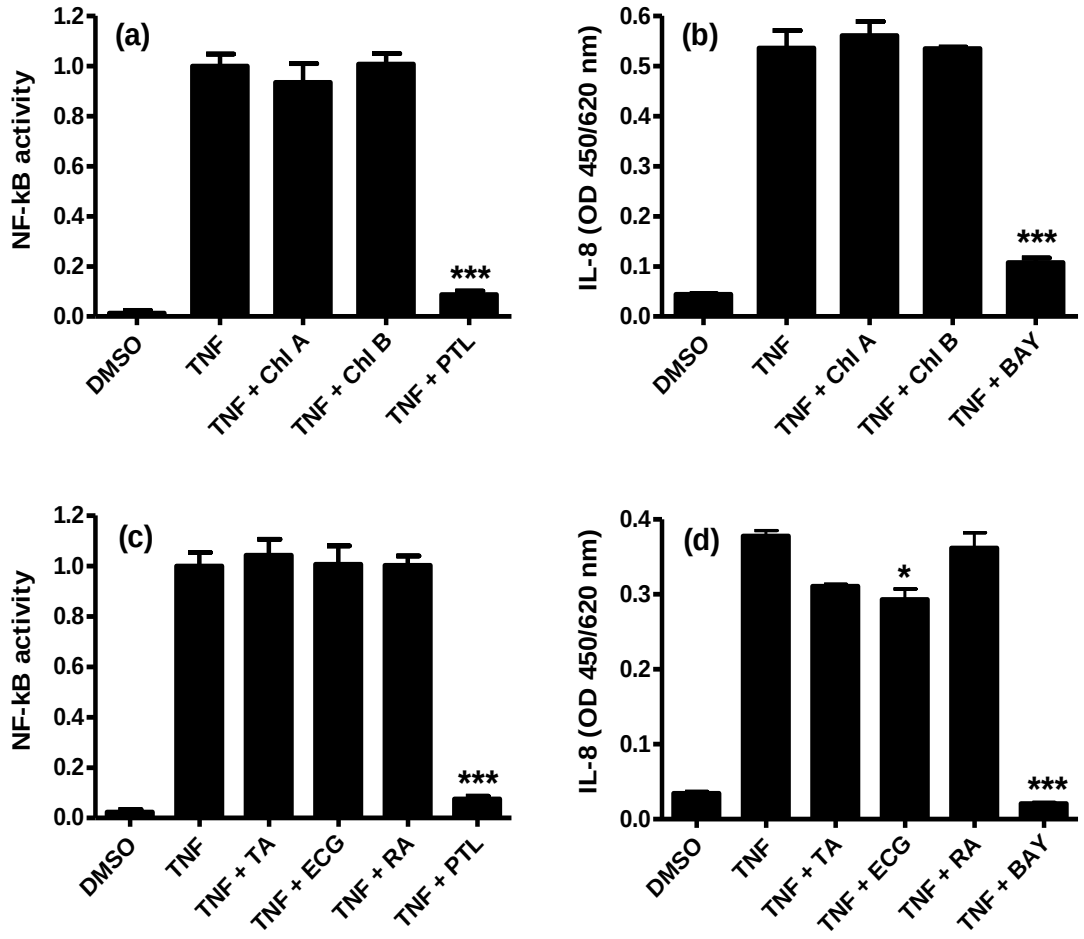


Figure 2: Effects of *Malva* DCM extract (Mal DCM) and the chlorophyll free subextracts CR1, CR2 and CR3 on NF-κB activity in TNF-α-stimulated cells (a) and IL-8 expression in TNF-α- (b) or LPS- (c) stimulated cells at 10 µg/mL. Positive control (5 µM): parthenolide (PTL) or BAY 11-7082 (BAY). Bars represent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

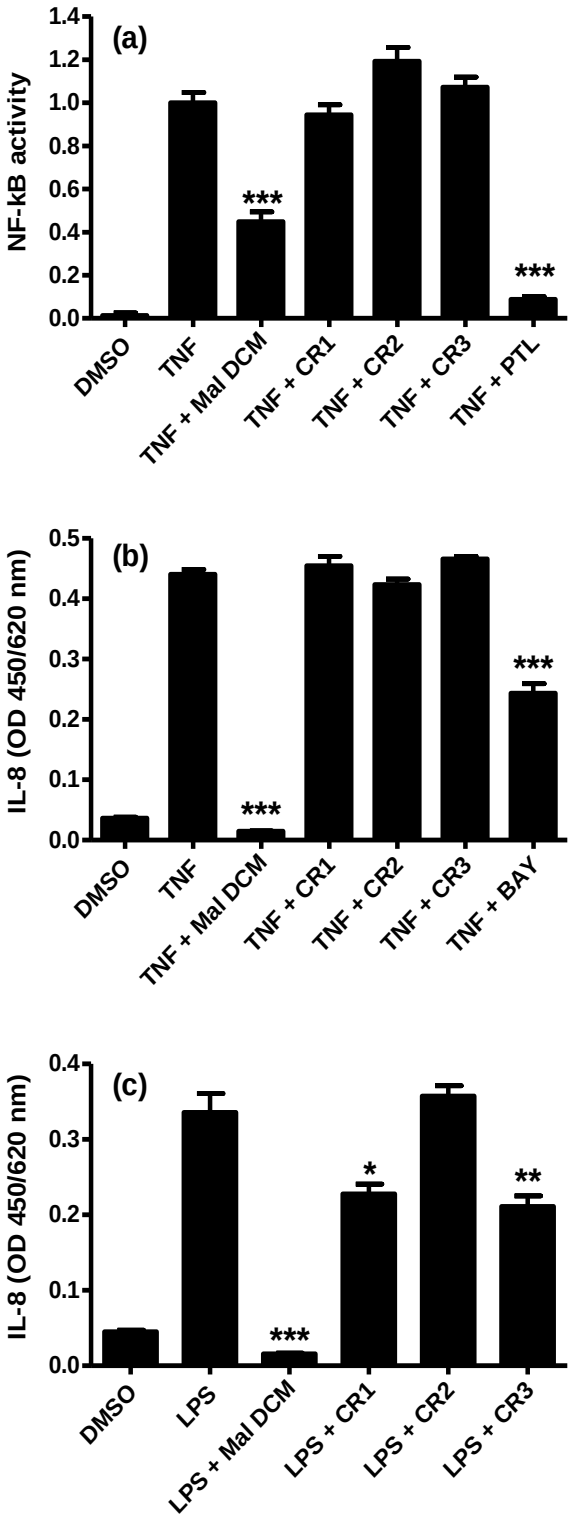


Figure 3: Effects of *Glechoma hederacea* MeOH extract (Gle MeOH) and its tannin depleted subextracts (PR1, PR2 and PR3) on NF-κB activity in TNF-α-stimulated cells (a) and IL-8 expression in TNF-α- (b) and LPS- (c) stimulated cells at 10 µg/mL. Positive control (5 µM): parthenolide (PTL) or BAY 11-7082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

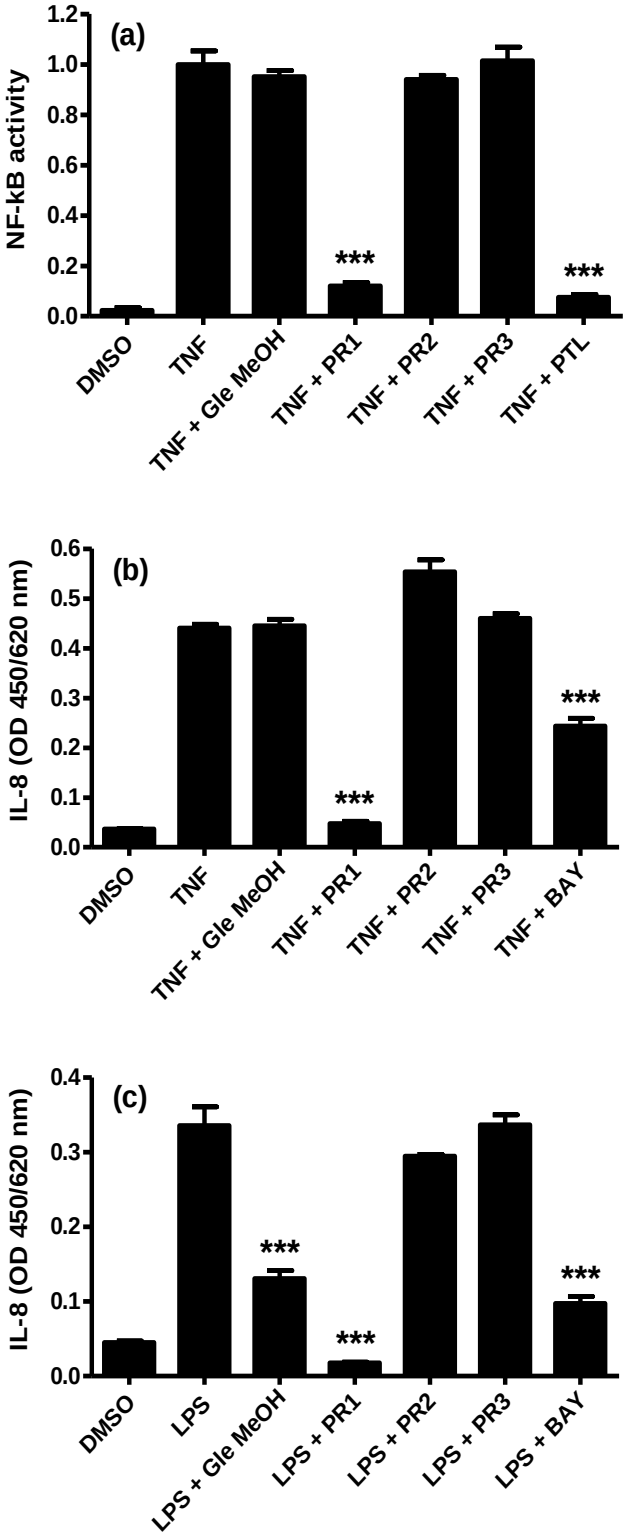


Figure 4: Comparison of the crude dichloromethane extract of *Malva ssp.* (DCM) with the chlorophyll free subextracts (CR1, CR2, CR3) using HPLC-UV detection at 254 nm. Analyses were performed on an Atlantis T3 (Waters, Milford, MA, USA) analytical column (150 x 3 mm, 3 µm) at an oven temperature of 25 °C. The flow rate was 0.7 mL/min and the injection volume was 20 µL of each sample. The mobile phase consisted of H_2O adjusted to pH 2.8 with formic acid (solvent A) and MeCN (solvent B) with the following gradient: 25-55 % of B in in 60 min, 55-85 % of B in 40 min. peak 4: The retention times of the peaks were as follows: chlorophyll, Rt: 97 min.

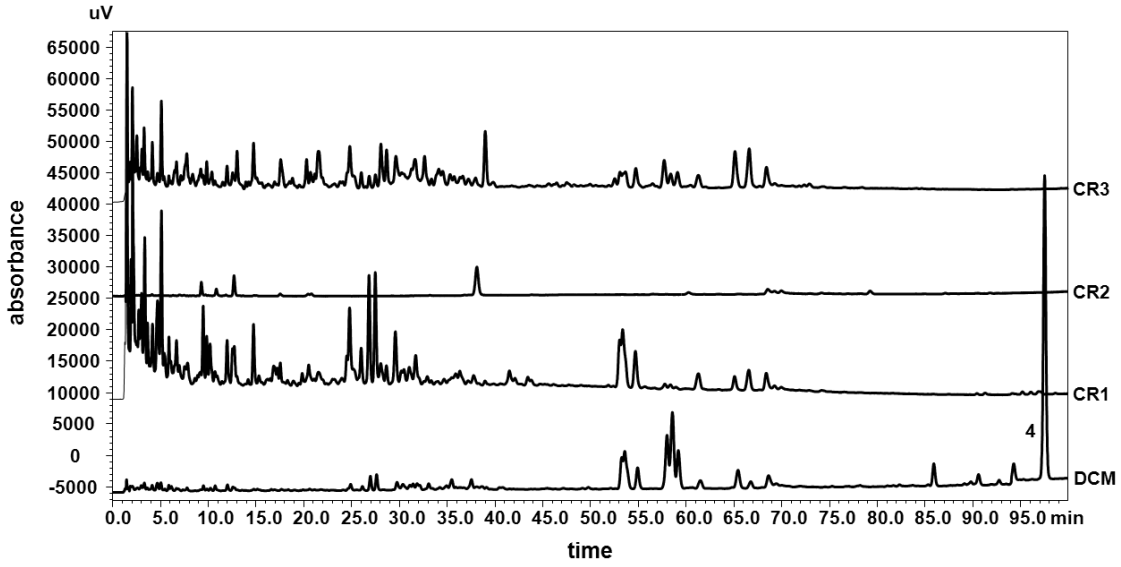


Figure 5: Comparison of the crude dichloromethane extract of *Malva ssp.* (DCM) with the chlorophyll free subextracts (CR1, CR2, CR3) using HPLC-ELSD. Analyses were performed on an Atlantis T3 (Waters, Milford, MA, USA) analytical column (150 x 3 mm, 3 µm) at an oven temperature of 25 °C. The flow rate was 0.7 mL/min and the injection volume was 20 µL of each sample. The mobile phase consisted of H_2O adjusted to pH 2.8 with formic acid (solvent A) and MeCN (solvent B) with the following gradient: 25-55 % of B in 60 min, 55-85 % of B in 40 min. The retention times of the peaks were as follows: peak 1: linolenic acid, Rt: 83.5 min; peak 2: linoleic acid, Rt: 90.5 min; peak 3: oleic acid, Rt: 96.5 min; peak 4: chlorophyll, Rt: 97 min.

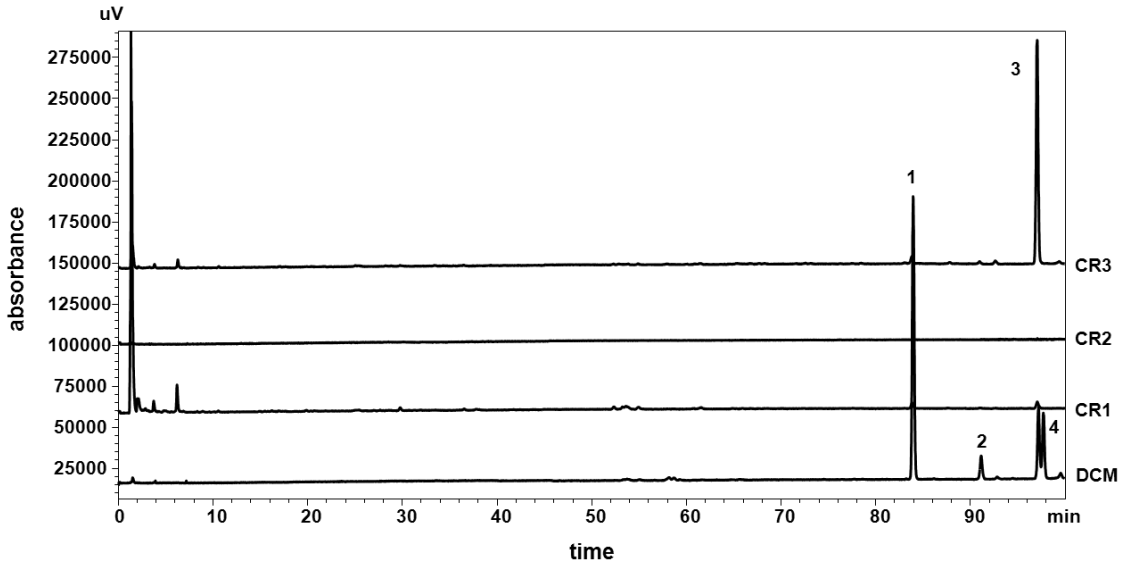


Figure 6: Comparison of the crude methanolic extract (MeOH) of *Glechoma hederacea* with the extracts after different methods of tannin removal (PR1, PR2, PR3) via HPLC-UV (254 nm). Analyses were performed on an Atlantis T3 (Waters, Milford, MA, USA) analytical column (150 x 3 mm, 3 µm) at an oven temperature of 25 °C. The flow rate was 0.7 mL/min and the injection volume was 20 µL of each sample. The mobile phase consisted of H_2O adjusted to pH 2.8 with formic acid (solvent A) and MeCN (solvent B) with the following gradient: 2-32 % of B in 75 min. The retention times of the peaks were as follows: peak 5: cyclic hydroxamic acid 2-benzoxazolinone, Rt: 31.8 min; peak 6: rosmarinic acid, Rt: 52.5 min.

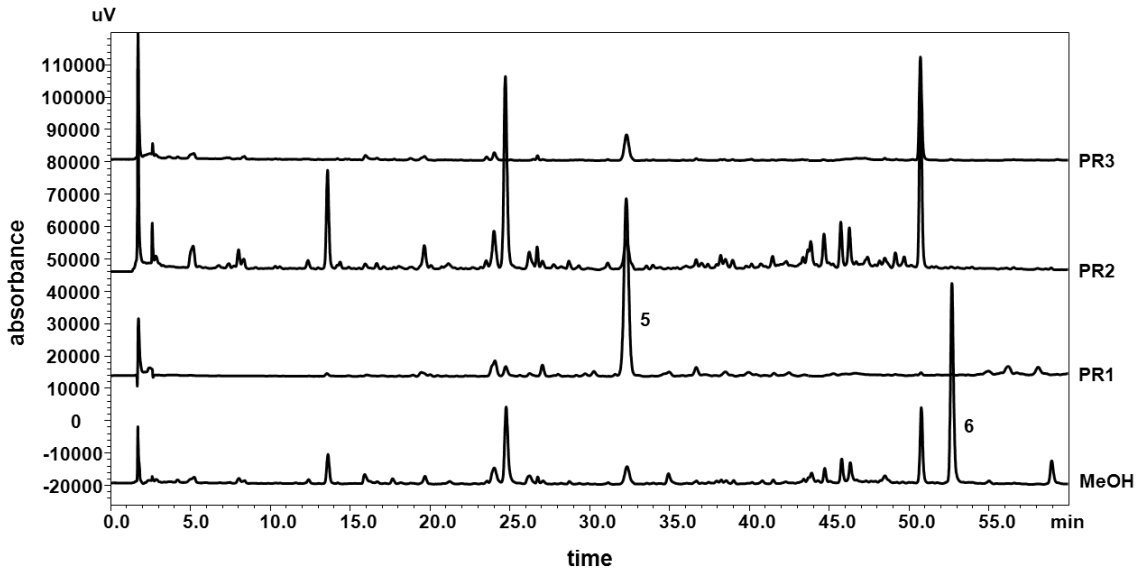


Table 1: Extraction yields: drug extract ratio (DER- values), yield of purified extracts after removal of bulk components [%, w/w crude extract].

	Malva sp.	***Glechoma hederacea***
Initial weight of crude drug	**3 g**	**3 g**
Drug extract ratio (DER)	**DCM extract: 8 : 1**	**MeOH extract: 5 : 1**
Percentage on the crude extract after removal of bulk components	CR1: 11.0 %	PR1: 8.0 %
	CR2: 40.0 %	PR2: 72.0 %
	CR3: 34.6 %	PR3: 48.6 %

3.4 Bioactivity guided isolation of the active principles from *Peucedanum ostruthium*

After the initial extract screening highest anti-inflammatory activity was found in the detannified MeOH extract of *P. ostruthium* (Tab. 18, p. 131) with a 2.5 and 3 fold activation of PPAR-α and PPAR-γ, respectively (Fig. 23, p. 130). NF-κB was even down-regulated to the base level by the DCM as well as by the detannified MeOH extracts (Fig. 24, p. 130). Therefore, a further fractionation of the detannified MeOH extract with SPE (**SPE system 1**, Tab. 10, p. 39) was performed, yielding 3 subfractions of different polarity. Extraction yields of ASE extraction and subsequent fractionation via SPE are given in Tab. 17.

Thereby, high PPARs activation and strong NF-κB inhibition was reached by the 70 % and 100 % MeOH fractions (Tab. 18, p. 131). Strongest activation of PPARs was found in the 70 % MeOH fraction (2 fold activation of PPAR-α and 1.8 fold of PPAR-γ), followed by the 100 % MeOH fraction (1.5 fold activation of PPARs), and almost no activation was observed in the 30 % MeOH fraction (Fig. 25, p. 131). Concerning NF-κB inhibition, weak inhibition was detected in the 30 % MeOH fraction, in contrast the 70 % and 100 % MeOH fraction showed a strong inhibition of 100 % (Fig. 25, p. 131).

ASE extraction			
crude drug [g]	**367.5**		
sample ID	**extract**	**% of starting material**	**% of crude drug**
Peu DCM	37.8 g	**10.3**	**10.3**
Peu MeOH	81.2 g	**22.1**	**22.1**
tannin separation			
starting MeOH extract [g]	**51.9**		
Peuc Det	2.3 g	**4.4**	**0.62**
SPE			
starting MeOH extract [mg]	**2263**		
Peu Det 30	181 mg	**8.0**	**0.05**
Peu Det 70	925 mg	**40.9**	**0.25**
Peu Det 100	716 mg	**31.6**	**0.19**

Tab. 17 Extraction yields of the ASE extraction of the crude plant material, tannin separation and solid phase extraction (SPE) of *P. ostruthium* rhizomes (Peu). DCM - dichloromethane extract, MeOH - methanol extract, Det - detannified methanol extract, 30 - SPE with 30 % MeOH, 70 - SPE with 70 % MeOH, 100 - SPE with 100 % MeOH.

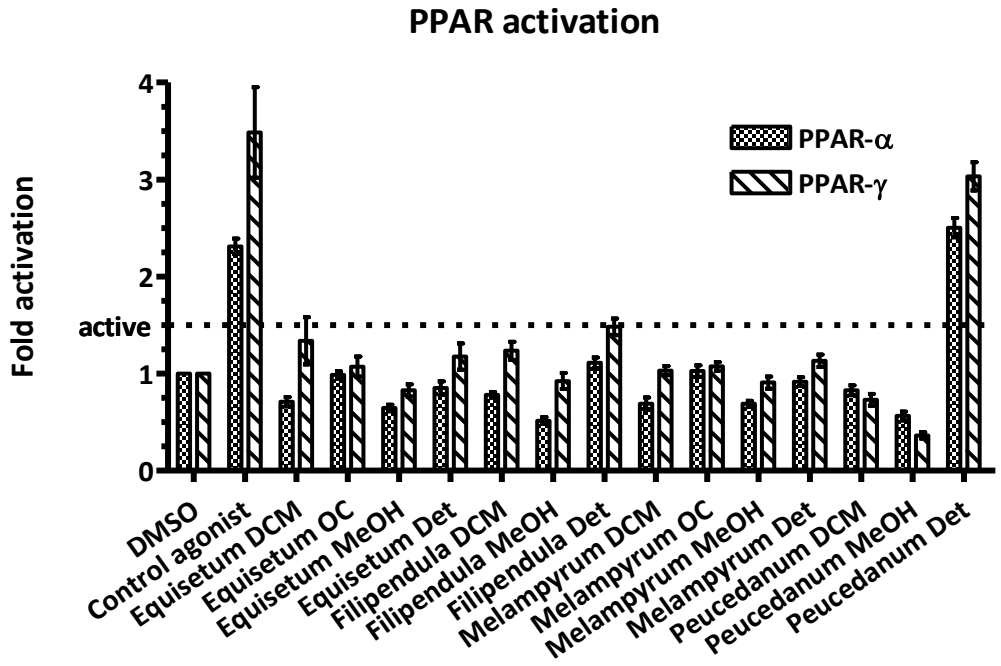


Fig. 23 Results of the initial screening of selected plant species on PPARs activation. DCM – dichloromethane extract, OC - DCM extract without chlorophyll, MeOH - methanolic extract, Det - detannified MeOH extract. Extracts were tested in triplicates at a concentration of 10 µg/mL on PPAR-α and -γ activation. PPAR-α control agonist: GW7647 (50 nM), PPAR-γ agonist: Troglitazone (5 µM). Bars represent mean values ± SEM. Dotted line set at 50 % activation, representing promising activity.

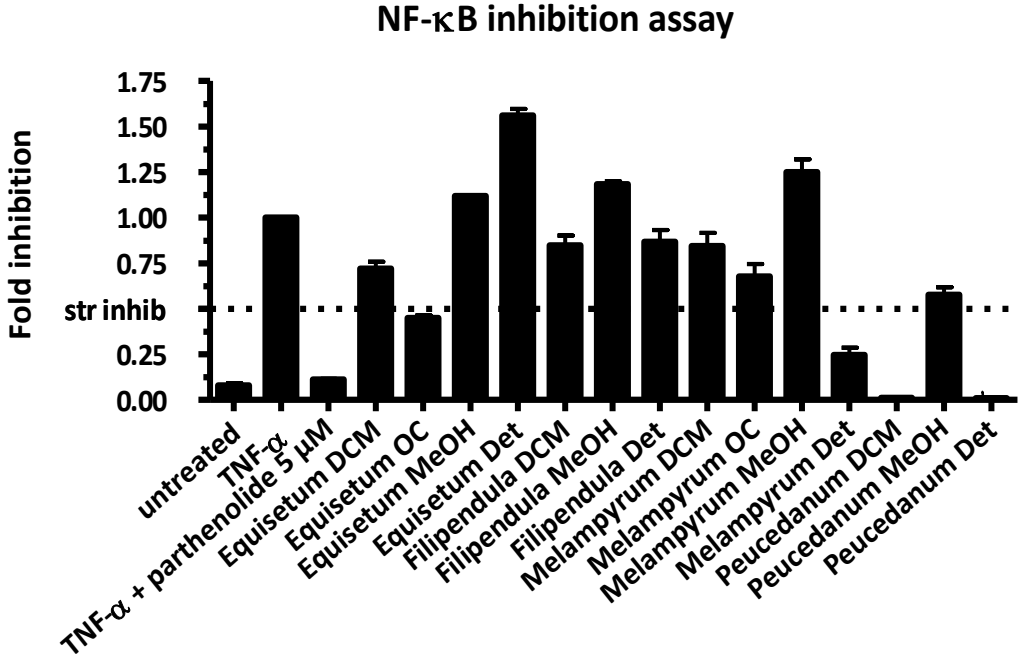


Fig. 24 Results of the initial screening of selected plant species on NF-κB inhibition. DCM – dichloromethane extract, OC - DCM extract without chlorophyll, MeOH - methanolic extract, Det - detannified MeOH extract. Extracts were tested in triplicates at a concentration of 10 µg/mL Bars represent mean values, error bars SEM. Dotted line set at inhibition of 50 %, representing strong inhibitors (str inhib).

Plant material	Sample ID	Preparation	PPAR-α activ.	PPAR-γ activ.	NF-κB inhib.	E-sel. +TNF	IL-8 +TNF	E-sel. +LPS	IL-8 +LPS
P. ostruthium root	Peuc DCM	DCM extract	-	-	+++	+++	+++	-	-
	Peuc MeOH	MeOH extract	-	-	-	-	+	-	-
	Peuc Det	detannified MeOH extract	+++	+++	+++	+++	+	+++	+++

Initial extract	Sample ID	Preparation	PPAR-α activ.	PPAR-γ activ.	NF-κB inhib.	E-sel. +TNF	IL-8 +TNF	E-sel. +LPS	IL-8 +LPS
detannified MeOH extract	Peuc Det 30	SPE with 30 % MeOH	-	+	+	+++	++	++	++
	Peuc Det 70	SPE with 70 % MeOH	+++	++	+++	+++	-	+	+
	Peuc Det 100	SPE with 100 % MeOH	++	++	+++	+++	+	+++	+++

Tab. 18 Results of the initial screening of *P. ostruthium* crude extracts on various anti-inflammatory targets (above). Results of the SPE subfractions of *P. ostruthium* detannified MeOH extract (below). Extracts and fractions were tested in triplicates at a concentration of 10 µg/mL on PPARs activation (activ.) as well as on NF-κB inhibition (inhib.) and LPS or TNF-α induced downregulation of interleukine-8 (IL-8) and E-selectin (E-sel.) mRNA; <25 % inhibition/ activation is considered as no activity (-), 25-50 % inhibition/ activation is interpreted as low activity (+), 50-75 % inhibition/ activation is considered as moderate activity (++) and high effect is defined as > 75-100 % inhibition/ activation (+++). Extracts chosen for further bioassay guided isolation are highlighted in grey.

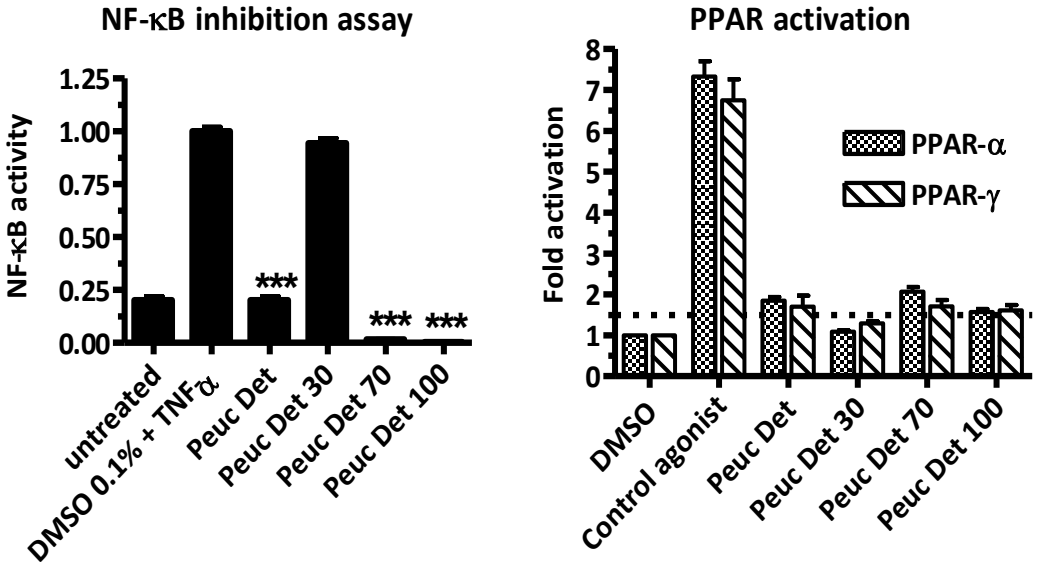


Fig. 25 PPAR activation assay (right) and NF-κB inhibition assay (left) of the detannified *P. ostruthium* extract and its SPE subfractions (eluted with 30 % MeOH = Peuc Det 30, with 70 % MeOH = Peuc Det 70 and with 100 % MeOH = Peuc Det 100). All subfractions were tested in quadruples at a concentration of 10 µg/mL. PPAR-α control agonist: GW7647 (50 nM), PPAR-γ agonist: Troglitazone (5 µM). Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF-α: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dotted line is set for significant actives.

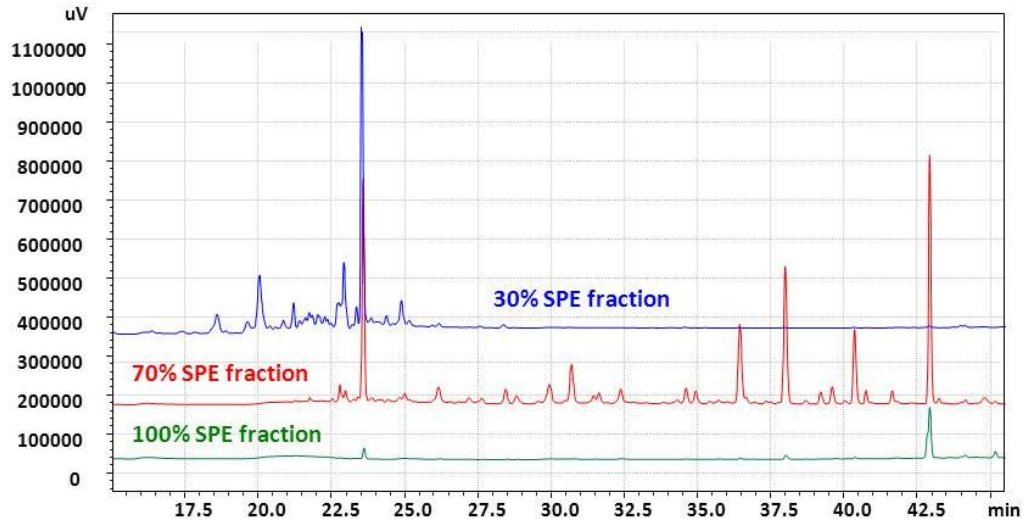


Fig. 26 HPLC-DAD comparison (310 nm) of *P. ostruthium* detannified extract SPE subfractions (eluted with 30 %, 70 % and 100 % MeOH, see SPE system 1, Tab. 10, p. 39). HPLC analyses were performed using column 1, instrument 1 and HPLC system 1 (described in Tab. 13-Tab. 15, pp. 44-46)

As the 30 % and 100 % methanolic subfractions contained compounds also present in the 70 % fraction (Fig. 26), further fractionation and isolation was conducted with the latter one. Therefore, fractionation on preparative HPLC (Fig. 27) was performed using instrument 3, column 3 and HPLC system 1 (Tab. 13 - Tab. 15, pp. 44-46), application of 6 x 100 mg in 1 mL MeCN each and injection volume of 700 µL. The following peak-based unification yielded 9 fractions in total (amounts are given in Tab. 19, p. 133), whereby all fractions displayed activity in some assays (see Tab. 20, p. 133).

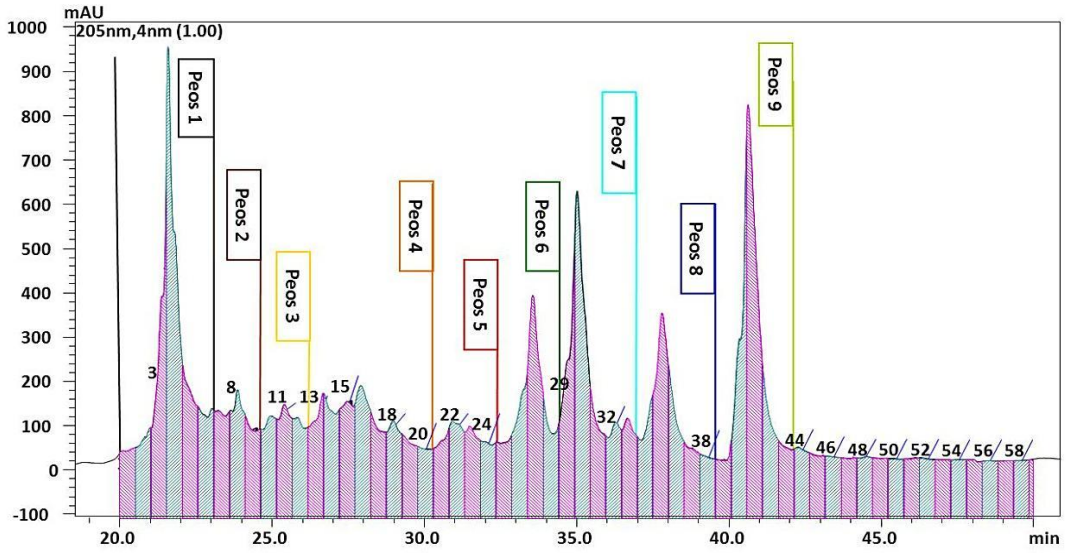


Fig. 27 HPLC-UV Chromatogram of preparative HPLC of Peuc Det 70 illustrating the peak based unification to subfractions Peos 1-9. Measurements were performed on instrument 3 with column 3 and HPLC system 1 (described in Tab. 13 - Tab. 15, pp. 44-46).

preparative HPLC			
application amount 6 x 70 mg = 420 mg			
sample ID	**extract [mg]**	**% of starting material**	**% of crude drug**
Po V1	21.9	**5.2**	**0.003**
Peos 1 oxypeucedanin hydrate	78.7	**18.7**	**0.010**
Peos 2 mix bergaptol, xanthotoxin and others	39.2	**9.3**	**0.005**
Peos 3 acetyl- oxypeucedanin hydrate and others	23.0	**5.5**	**0.003**
Peos 4 oxypeucedanin methanolate, bergapten, isopimpinellin and oxypeucedanin	53.8	**12.8**	**0.007**
Peos 5 oxypeucedanin	19.7	**4.7**	**0.003**
Peos 6 ostruthin + peucenin	36.7	**8.7**	**0.005**
Peos 7 imperatorin	46.8	**11.1**	**0.006**
Peos 8 isoimperatorin + osthole	28.7	**6.8**	**0.004**
Peos 9 ostruthin	30.1	**7.2**	**0.004**
Po N9	18.8	**4.5**	**0.002**

Tab. 19 Amounts of the fractions from preparative HPLC of *P. ostruthium* 70 % SPE subfraction of de-tannified MeOH extract (Peos 1-9), PV1 - forerunnings, Po N9 - tailings. Portions on the starting material and the initial crude drug are marked blue.

Mother sample	Sample ID	Preparation	PPAR-α activ.	PPAR-γ activ.	NF-κB inhib.	E-sel. +TNF	IL-8 +TNF	E-sel. +LPS	IL-8 +LPS
SPE 70 % MeOH	Peos 1	prep HPLC	-	-	-	+	+	-	+++
	Peos 2	prep HPLC	-	-	+	+	-	+	+
	Peos 3	prep HPLC	-	-	++	++	+	+	++
	Peos 4	prep HPLC	-	-	+++	+	-	+	+
	Peos 5	prep HPLC	+	++	++	-	-	++	-
	Peos 6	prep HPLC	-	-	+++	-	-	+	+
	Peos 7	prep HPLC	-	-	+	-	+	+	+
	Peos 8	prep HPLC	+	-	+++	-	-	+	++
	Peos 9	prep HPLC	++	++	+++	-	-	+	++

Tab. 20 Results of the preparative HPLC subfractions of *P. ostruthium* 70 % SPE fraction of MeOH Det extract. All subfractions were tested in at least triplicates at a concentration of 10 µg/mL on PPARs activation (activ.) as well as on NF-κB inhibition (inhib.) and LPS or TNF-α induced downregulation of interleukine-8 (IL-8) and E-selectin (E-sel.) mRNA**;** < 25 % inhibition/ activation is considered as no activity (-), 25-50 % inhibition/ activation is interpreted as low activity (+), 50-75 % inhibition/ activation is considered as moderate activity (++) and high effect is defined as > 75-100 % inhibition/ activation (+++).

Subfraction 1 (Peos 1) mainly consisted of oxypeucedanin hydrate, identified with a reference compound via HPLC-DAD and LC-MS (Fig. 28, p. 134).

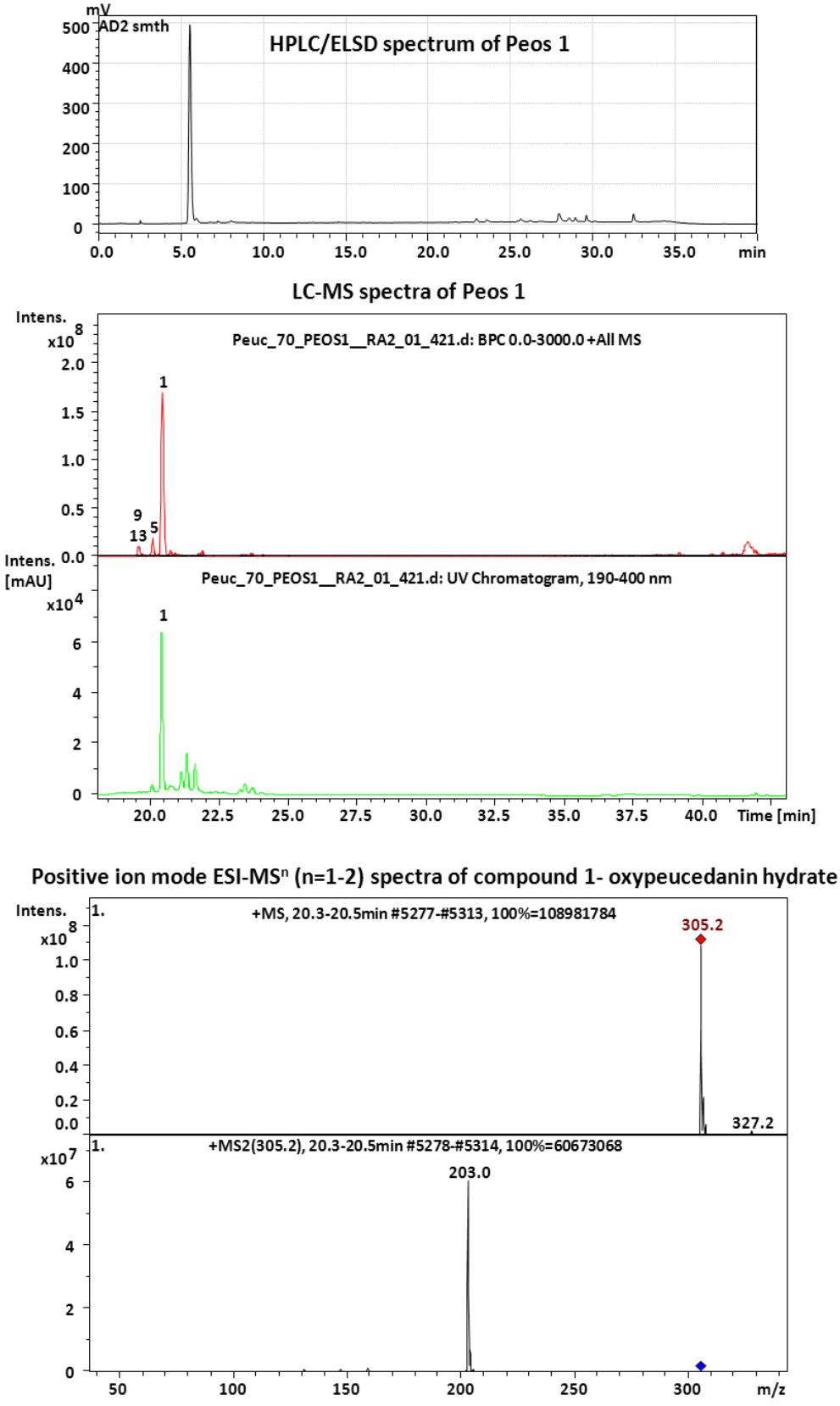


Fig. 28 HPLC-DAD/ELSD-MS investigations of Peos 1: first HPLC-ELDS-LT chromatogram, generated on HPLC instrument 2, using column 2 and HPLC system 2, LC-MS measurements below were performed on instrument 4, with column 1 and HPLC system 1 (Tab. 13-Tab. 15, pp. 44-46). **1** - oxypeucedanin hydrate, **5** - 2”- or 3”-acetyloxypeucedanin or 2”- or 3”-acetylheraclenin, **9** - xanthotoxol, **13** - oxypeucedanin.

Subfraction 2 (Peos 2) contained several compounds including bergaptol and xanthotoxin. In combined subfraction 3 (Peos 3) acetyloxypeucedanin hydrate was present among several others. Peos 4 was a mixture of several coumarins, of which oxypeucedanin methanolate, bergapten, isopimpinellin and oxypeucedanin were identified by HPLC-DAD and LC-MS, using reference compounds for comparison of UV-spectra and fragmentation pattern. In combined fraction Peos 5 HPLC-DAD-MS analyses showed oxypeucedanin as main component.

Peos 6 mainly contained ostruthol and some peucenin (Fig. 29 and Fig. 30, pp. 135-136). The main component identified in Peos 7 was imperatorin and little amounts of osthole were found (Fig. 31, p. 137). Osthole was also present in traces in Peos 8 together with the main compound isoimperatorin (Fig. 32, p. 138). From combined fraction Peos 9 ostruthin was isolated and identified using LC-MS and NMR spectroscopy.

The 9 HPLC subfractions were tested in all anti-inflammatory *in vitro* assays to evaluate fractions for further isolation (results see Fig. 34 and Fig. 35, p. 140).

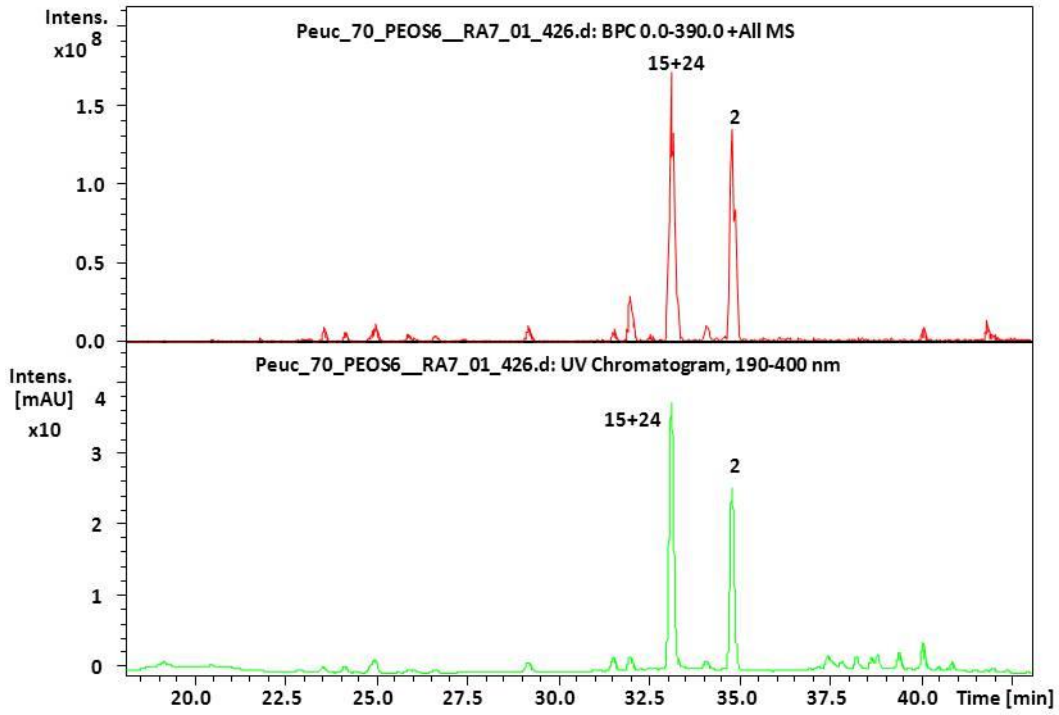


Fig. 29 LC-MS investigations of Peos 6: LC-MS measurements were performed on instrument 4, with column 1 and HPLC system 1 (Tab. 13-Tab. 15, pp. 44-46); **15** - ostruthol, **24** - peucenin, **2** - imperatorin.

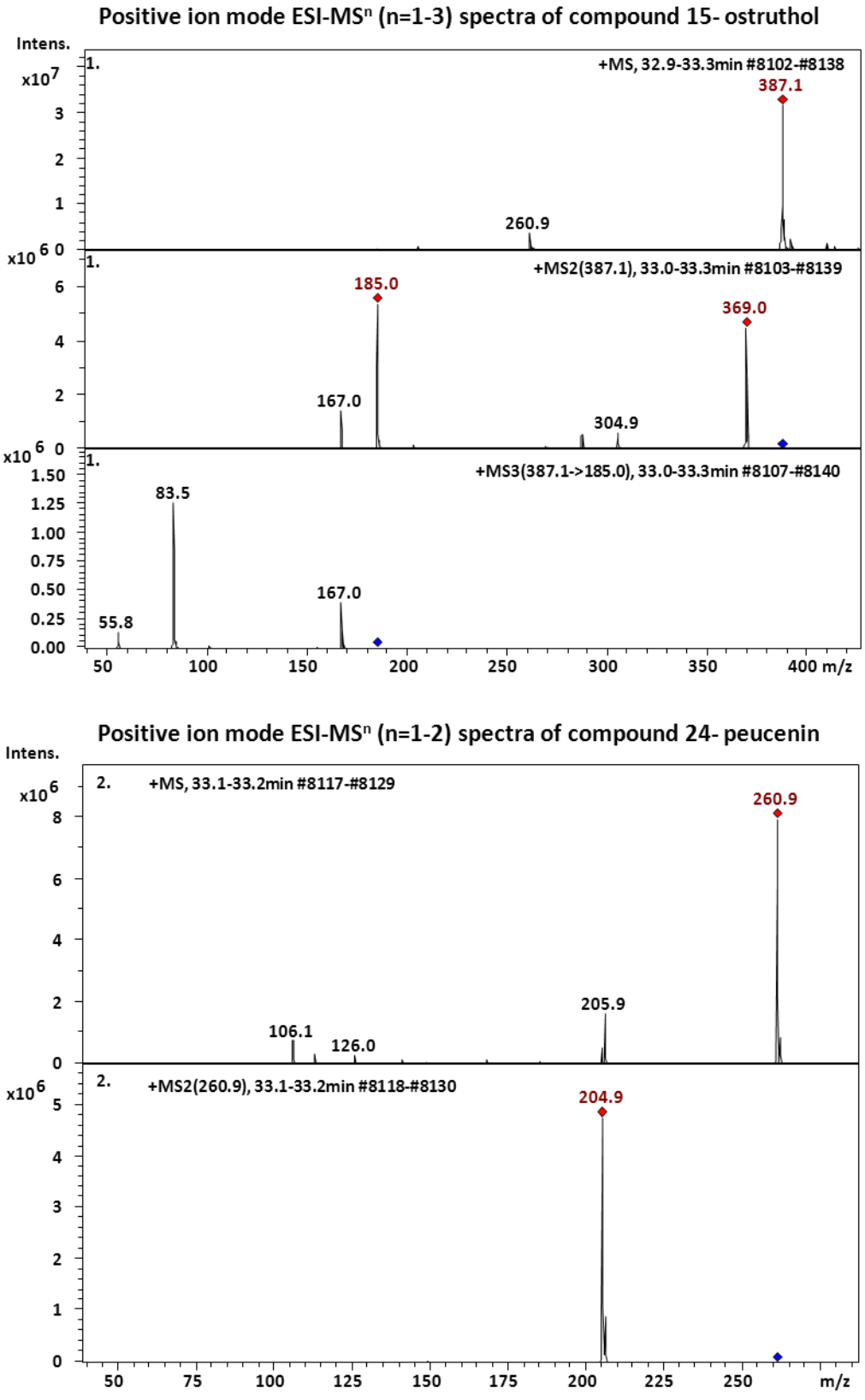


Fig. 30 ESI-MS chromatograms of ostruthol and peucenin, the main compounds of Peos 6.
LC-MS analyses were performed on instrument 4, with column 1 and HPLC system 1.

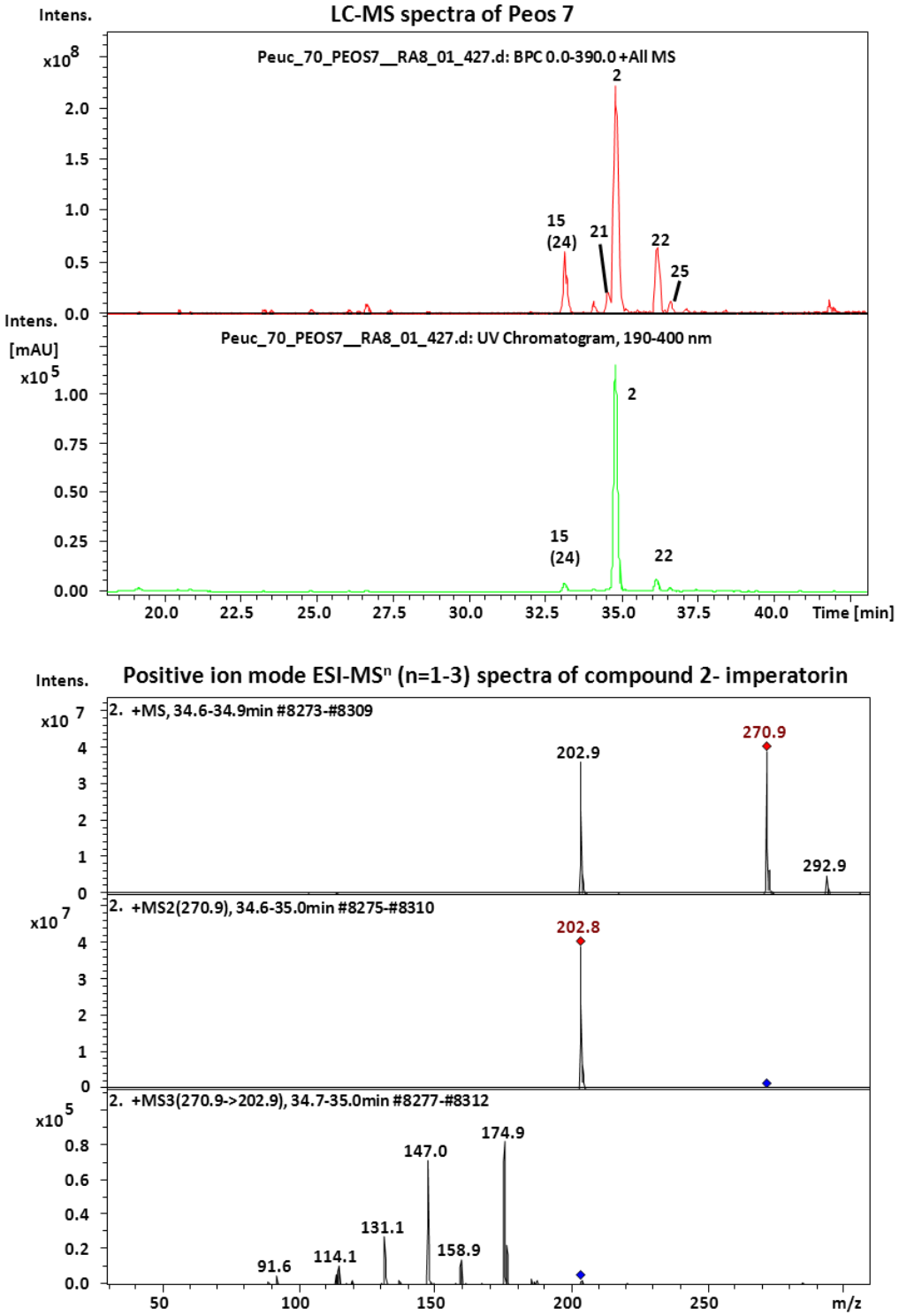


Fig. 31 LC-MS investigations of Peos 7 (above), ESI-MS chromatograms of imperatorin identified in Peos 7 (below): LC-MS measurements were performed on instrument 4, using column 1 and HPLC system 1. **15** - ostruthol, **21** - maybe 3'-O-Acetylhamaudol, **22** - maybe phellopterin, **24** - peucenin, **2** - imperatorin, **25** - osthole.

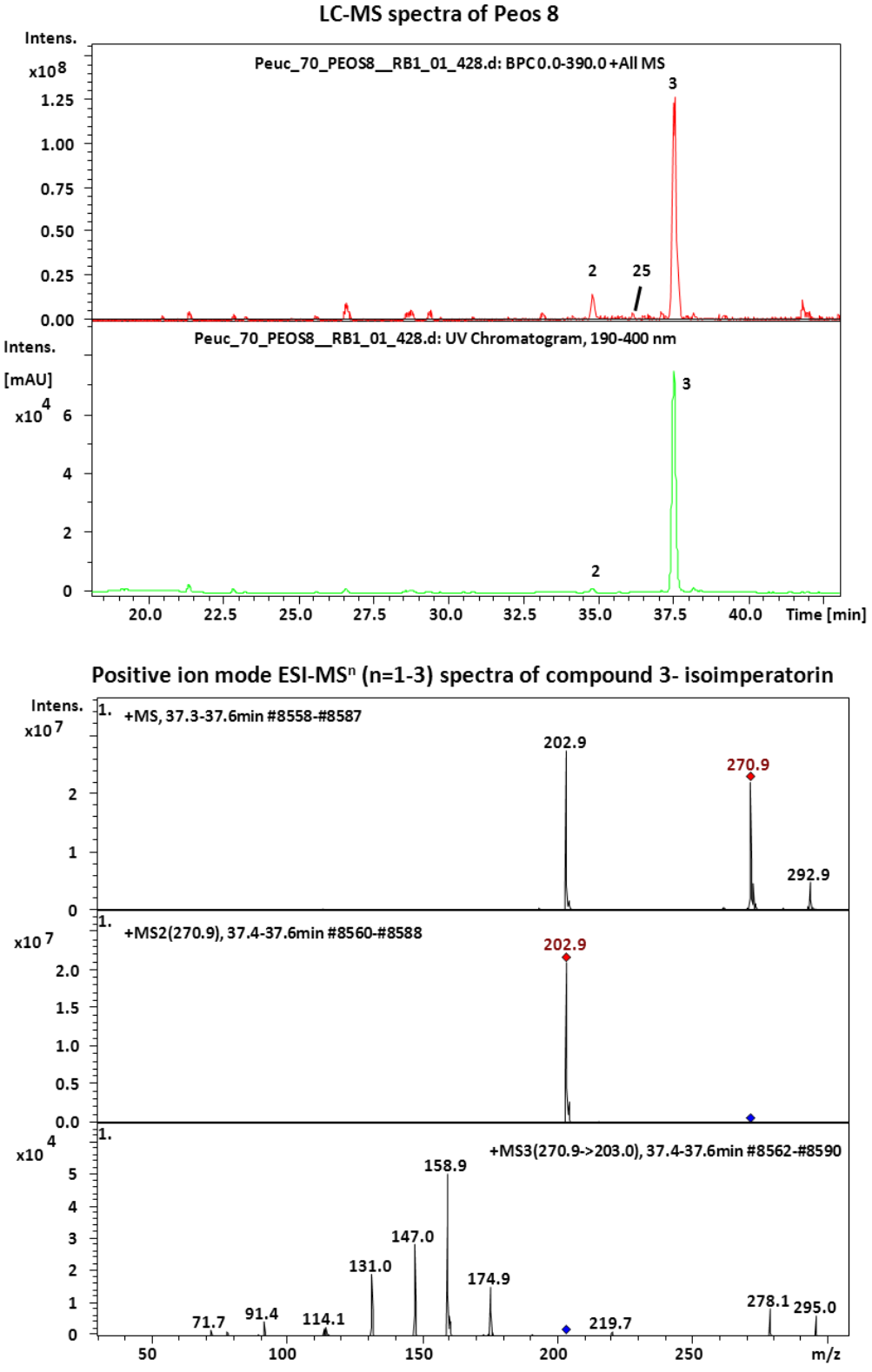


Fig. 32 LC-MS investigations of Peos 8 (above), ESI-MS chromatograms of isoimperatorin, the main compound of Peos 8 (below) LC-MS measurements were performed on instrument 4, with column 1 and HPLC system 1. **2** - imperatorin, **3** - isoimperatorin, **25** - osthole.

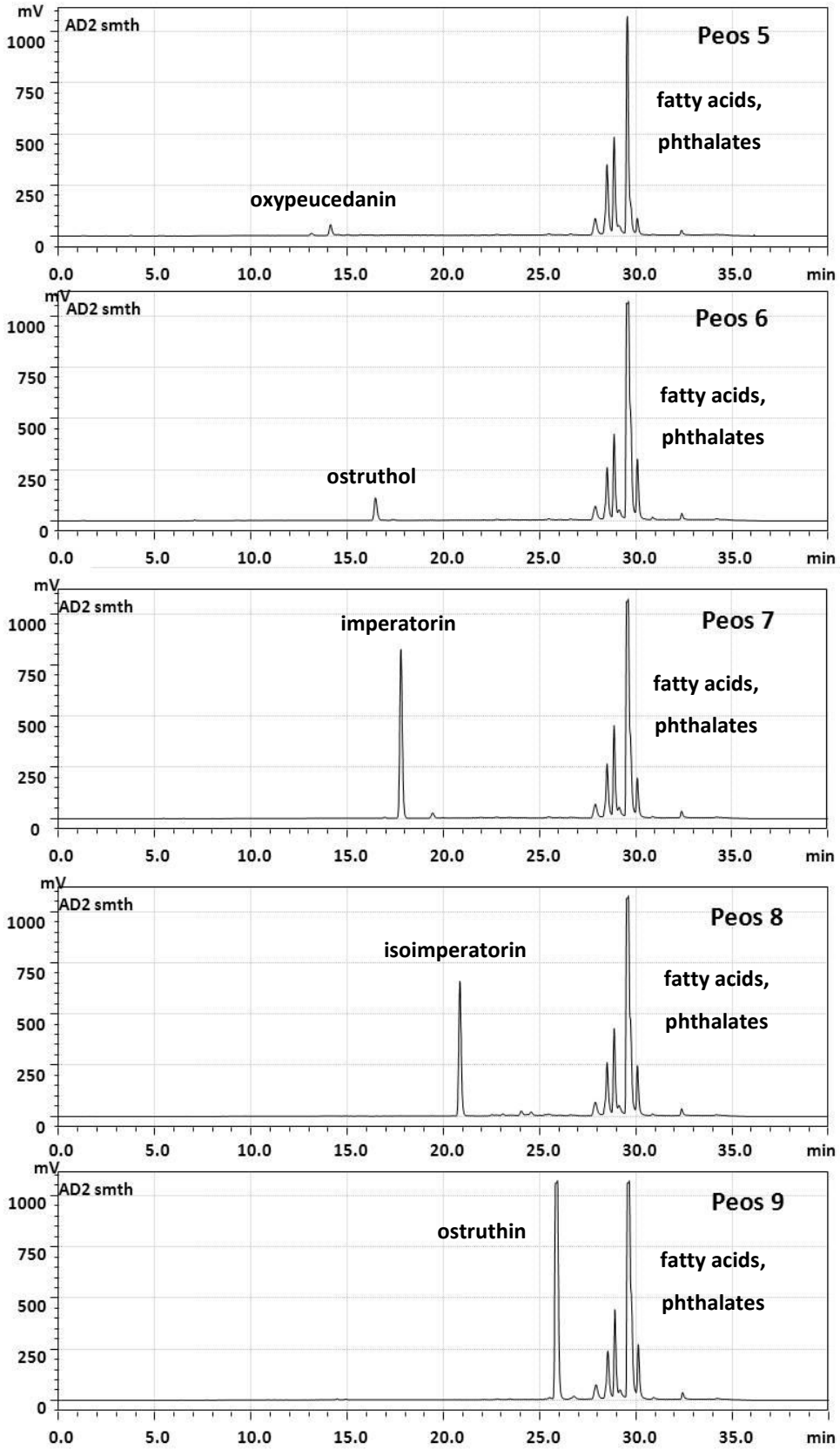


Fig. 33 HPLC-ELSD-LT analyses of *P. ostruthium* fractions of preparative HPLC. Meaurements were performed on HPLC instrument 2, using column 2 and HPLC system 2.

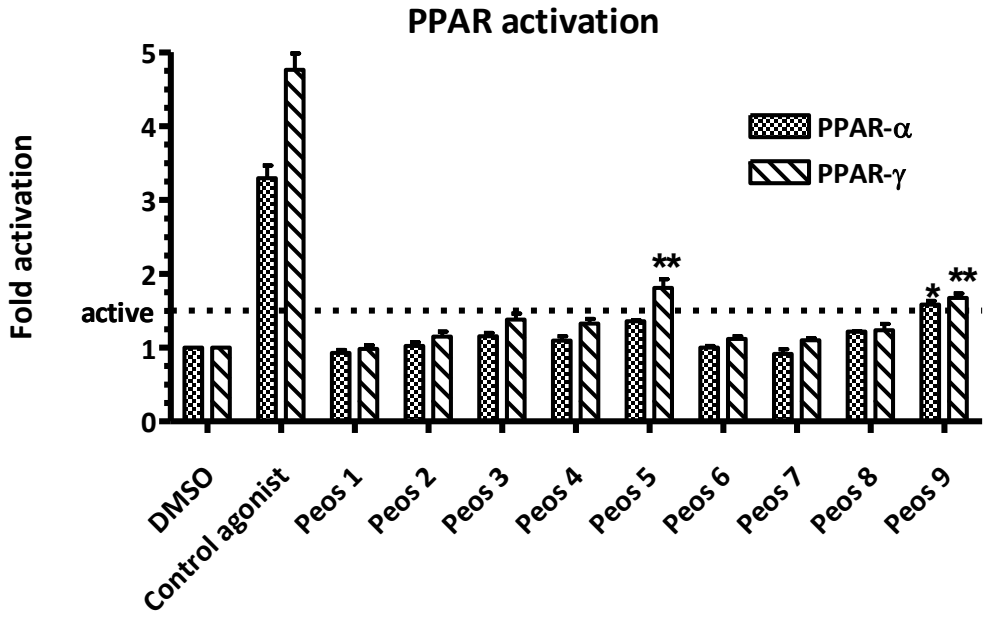


Fig. 34 PPAR activation assay of combined subfractions from preparative HPLC of *P. ostruthium* 70 % MeOH SPE fraction. All subfractions were tested in quadruples at a concentration of 10 µg/mL. PPAR-α control agonist: GW7647 (50 nM), PPAR-γ agonist: Troglitazone (5 µM). Bars represent mean values, error bars consider SEM, stars indicate significance compared to DMSO: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

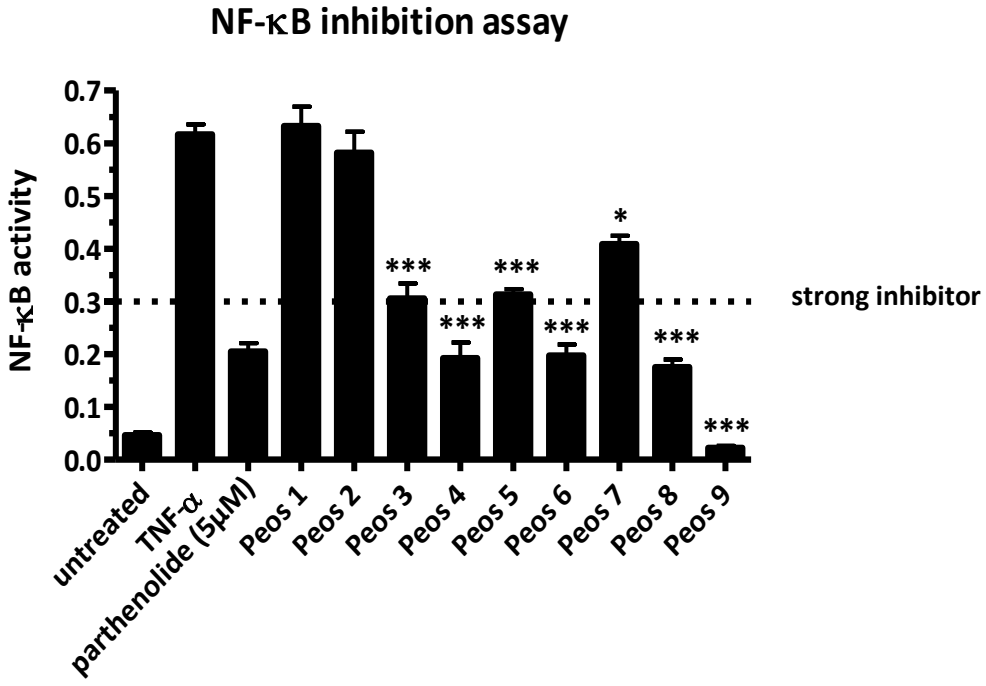


Fig. 35 NF-κB inhibition assay of combined subfractions from preparative HPLC of *P. ostruthium* 70 % MeOH SPE fraction. All subfractions were tested in quadruples at a concentration of 10 µg/mL bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF-α: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Subsequently, the compounds of these fractions were identified using TLC (Fig. 36), HPLC-DAD-MS for coumarins (see chapter 3.2.1, p. 151) and GC-MS for volatile compounds and fatty acids (results see annex I). Most compounds were common (furano)coumarins and fatty acids and could be purchased in order to perform HPLC-DAD-MS comparison and/ or pharmacological testing.

Two coumarins, three furanocoumarins and one chromone were further purified and isolated using **SPE system 2** (see chapter 2.7, p. 39) for ostruthin, **SPE system 3** (see chapter 2.7, p. 39) for separation of ostruthol and peucenin from imperatorin, as well as **CC system 1** (see chapter 2.8.2, p. 43) for separation of isoimperatorin and osthole. All compounds were tested in the respective anti-inflammatory test systems in several independent experiments at a concentration of 10 µM in the PPAR and NF-κB assay and 500 ng/mL to test for LPS or TNF-α induced IL-8 and E-selectin mRNA. Most of the coumarins had an effect in the general anti-inflammatory assay on the mRNA level, which could not be confirmed on protein level. Except of bergamottin, the coumarins did not show activation of PPARs (Fig. 38, p. 142), even those predicted by *in silico* screening (aurapten and ostruthin, see chapter 3.4.1, p. 143). Inhibition of NF-κB could only be achieved by ostruthin (Fig. 37, p. 142). That the isolated coumarins from the active fractions (Fig. 34, p. 140 and Fig. 35, p. 140) did barely result in active components might be explained by the other substances present in those fractions (Fig. 33, p. 139). On the one hand, natural occurring fatty acids and on the other hand, the solvent impurities phthalates were identified using GC-MS (see annex I) and LC-MS.

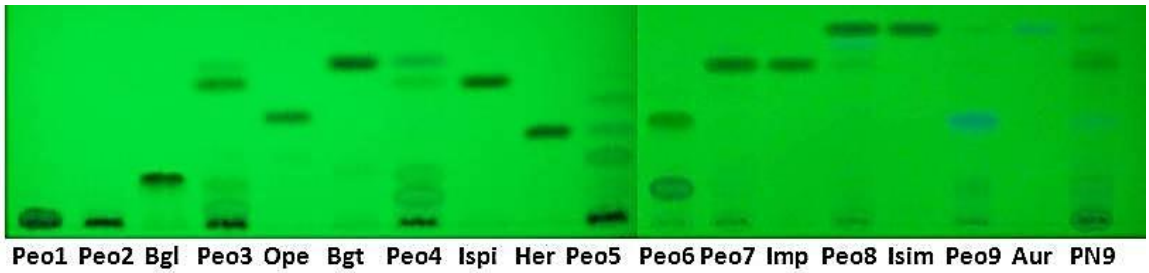


Fig. 36 TLC-comparison on silica plates of the collected subfractions of preparative HPLC (Peos 1-9). TLC was performed using the TLC system for coumarins with mobile phase 2, described in Tab. 11, p.40, detection was performed under UV_{254}; Bgl - bergaptol, Ope - oxypeucedanin, Bgt - bergapten, Ispi - isopimpinellin, Her - heraclenin, Imp - imperatorin, Isim - Isoimperatorin, Aur - aurapten.

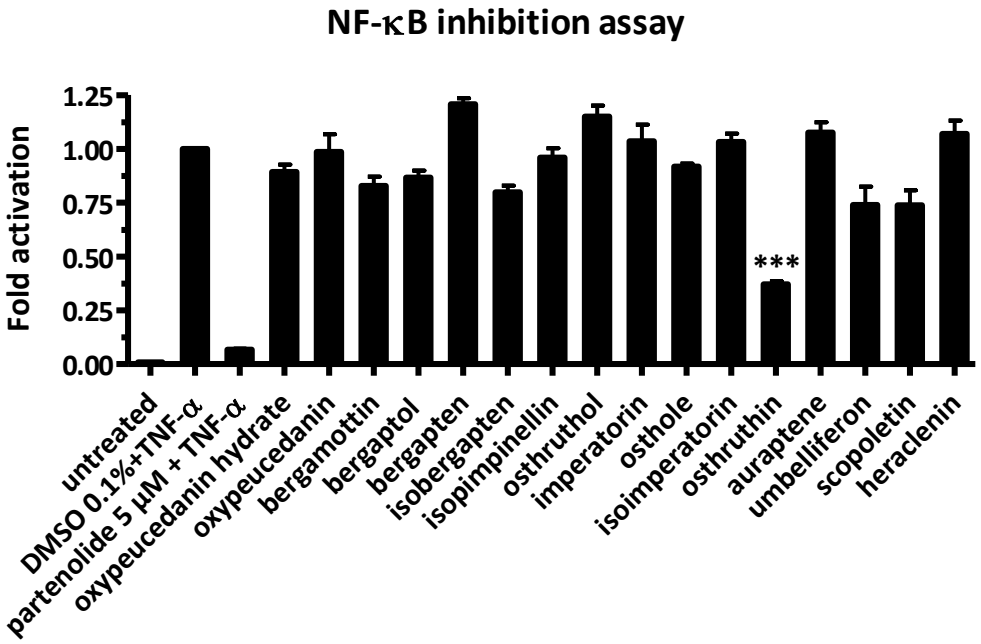


Fig. 37 NF-κB inhibition assay of compounds identified/ isolated from *P. ostruthium* roots. Last four are coumarins of other origin for structure related activity observation. Compounds were tested in quadruples at a concentration of 10 µM. Bars represent mean values, error bars consider SEM, stars indicate significance compared to DMSO: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

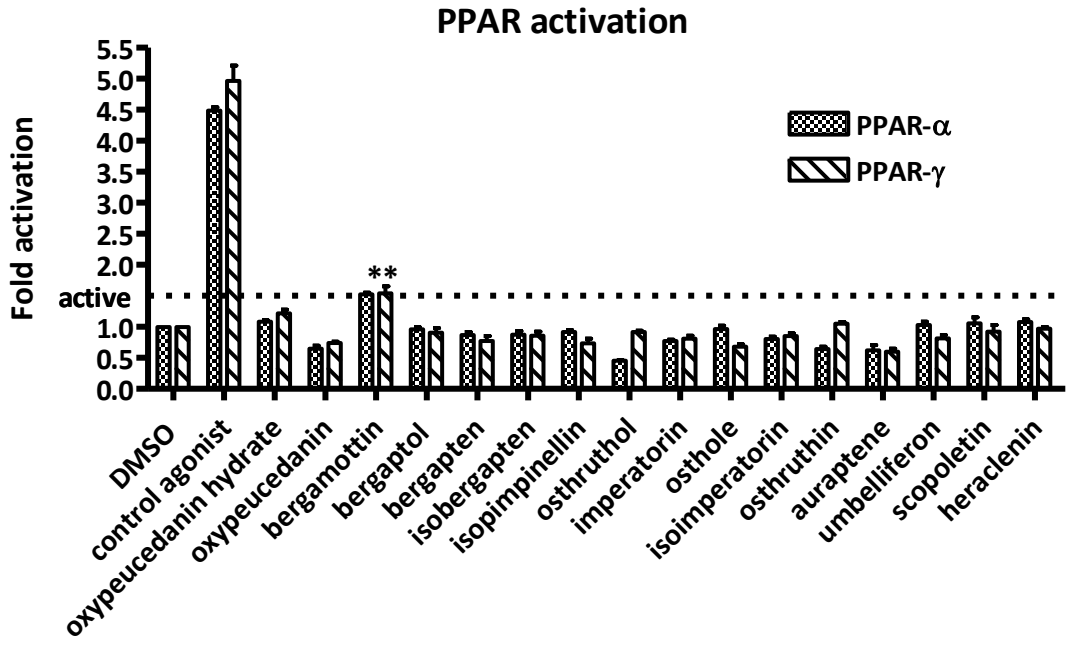
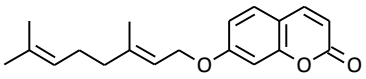
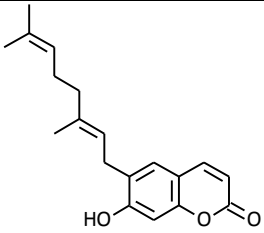
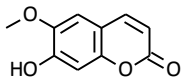
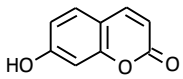
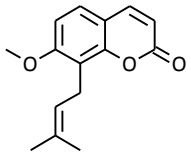
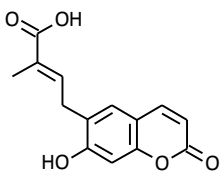


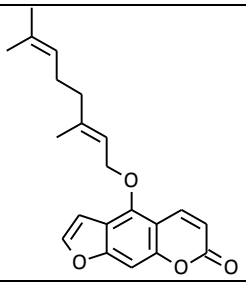
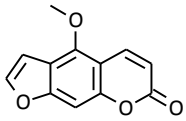
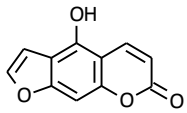
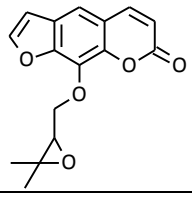
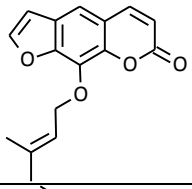
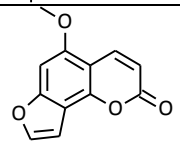
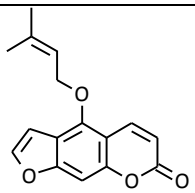
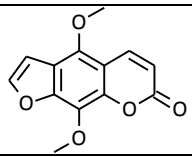
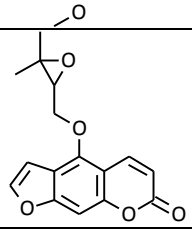
Fig. 38 PPAR activation assay of compounds identified/ isolated from *P. ostruthium* roots. Last four are coumarins of other origin for structure related activity observation. PPAR-α control agonist: GW7647 (50 nM), PPAR-γ agonist: Troglitazone (5 µM). Compounds were tested in quadruples at a concentration of 10 µM. Bars represent mean values, error bars consider SEM, stars indicate significance compared to DMSO: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4.1 Pharmacophoric profiling of *Peucedanum ostruthium* compounds against anti-inflammatory targets

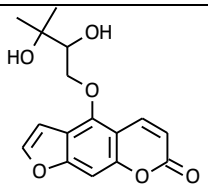
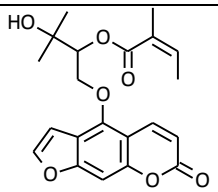
This work was done in cooperation with Dr. Daniela Schuster and Dr. Gerhard Wolber from the Department of pharmacological Chemistry of the University of Vienna. From the 71 structures published for *Peucedanum ostruthium* besides the ubiquitously fatty acids the (furano)coumarins revealed binding affinity to COX-2. Additionally, double prenylated coumarins as well as furanocoumarins did bind *in silico* to PPAR-γ. All published structures as well as the results of the screening are shown in Tab. 21 - Tab. 28.

Coumarins									
Substance	**Structure**	**COX-2**	**mPGES-1**	**cPLA2α**	**p38**	**IKK2**	**FXR**	**LXR**	**PPAR-γ**
Aurapten 495-02-3 $C_{19}H_{22}O_3$ 298.38		x							x
Ostruthin 148-83-4 $C_{19}H_{22}O_3$ 298.38		x							x
Scopoletin 92-61-5 $C_{10}H_8O_4$ 192.17		x							
Umbelliferon 93-35-6 $C_9H_6O_3$ 162.14		x							
Osthole 484-12-8 $C_{15}H_{16}O_3$ 244.29		x							
2-Butenoic acid, 4-(7-hydroxy-2-oxo-2H-1-benzopyran-6-yl)-2-methyl- 182692-57-5 $C_{14}H_{12}O_5$ 346.33		x							

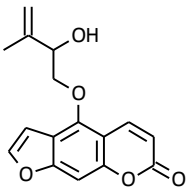
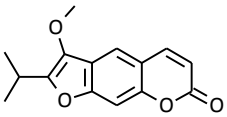
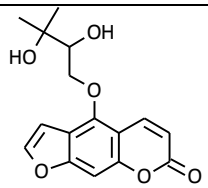
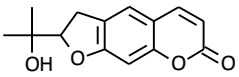
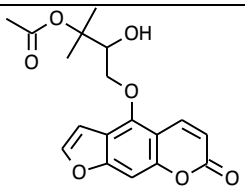
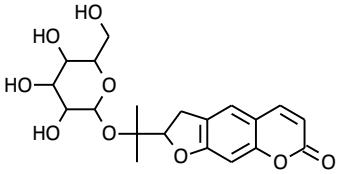
Tab. 21 *In silico* screening results of diverse coumarins found in *P. ostruthium.* A positive match in the pharmacophoric pocket is indicated with an x.

Furanocoumarins									
Substance	**Structure**	**COX-2**	**mPGES-1**	**cPLA2α**	**p38**	**IKK2**	**FXR**	**LXR**	**PPAR-γ**
Bergamottin 7380-40-7 $C_{21}H_{22}O_4$ 338.40		x					x		x
Bergapten 484-20-8 $C_{12}H_8O_4$ 216.19		x							
Bergaptol 486-60-2 $C_{11}H_6O_4$ 202.16		x							
Heraclenin 2880-49-1 $C_{16}H_{14}O_5$ 286.28		x							
Imperatorin 482-44-0 $C_{12}H_8O_4$ 270.28		x							
Isobergapten 482-48-4 $C_{12}H_8O_4$ 216.19		x							
Isoimperatorin 482-45-1 $C_{16}H_{14}O_4$ 270.28		x							
Isopimpinellin 482-27-9 $C_{13}H_{10}O_5$ 246.22		x							
Oxypeucedanin 737-52-0 26091-73-6 $C_{16}H_{14}O_5$ 286.28		x							

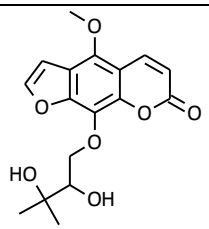
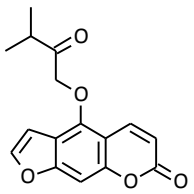
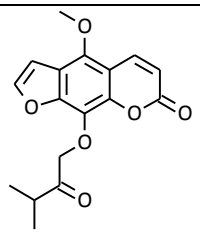
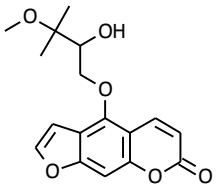
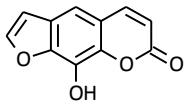
Tab. 22 continues on next page.

Substance	Structure	COX-2	mPGES-1	cPLA2α	p38	IKK2	FXR	LXR	PPAR-γ
Oxypeucedanin hydrate 2643-85-8 $C_{16}H_{16}O_6$ 304.29		x							
Ostruthol 642-08-0 $C_{21}H_{22}O_7$ 386.40		x							

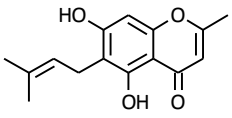
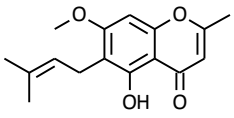
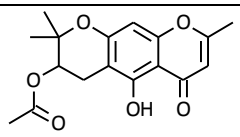
Tab. 23 *In silico* screening of the furanocoumarins published for *P. ostruthium.* A positive match in the pharmacophoric pocket is indicated with an x.

Substance	Structure	FXR	PPAR-γ
Pabulenol 33889-70-2 $C_{16}H_{14}O_5$ 286.28			
Peucedanin 133-26-6 $C_{15}H_{14}O_4$ 258.27			
Prangolarin hydrate 2643-85-8 $C_{16}H_{16}O_6$ 304.29			
Marmesin 13849-08-6 $C_{14}H_{14}O_4$ 246.26			
3'-acetate of oxypeucedanin hydrate 182692-58-6 $C_{18}H_{18}O_7$ 346.33			
(-)-Marmesinin; Ammijin 495-30-7 $C_{20}H_{24}O_9$ 408.40			

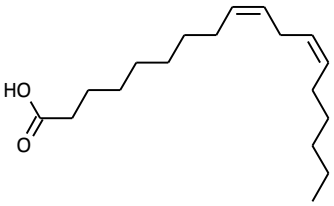
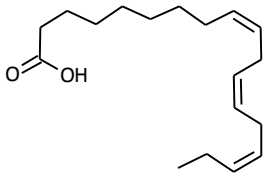
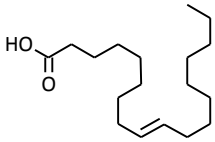
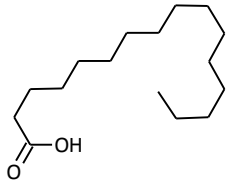
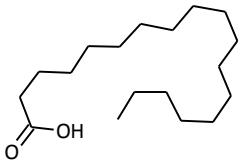
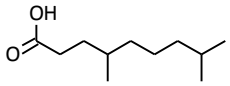
Tab. 23 continues on next page.

Substance	Structure	FXR	PPAR-γ
Byankagelicine 482-25-7 $C_{17}H_{18}O_7$ 334.32			
Isooxypeucedanin; Pabulenone 5058-15-1 $C_{16}H_{14}O_5$ 286.28			
Anhydroby-akangelicin; Isobyakangelicol 35214-81-4 $C_{17}H_{16}O_6$ 316.31			
Alatol; Oxypeucedanin methanolate; 52939-12-5 $C_{17}H_{18}O_6$ 318.32			
Xanthotoxol 2009-24-7 $C_{11}H_6O_4$ 202.16			

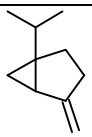
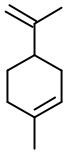
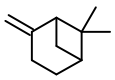
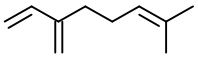
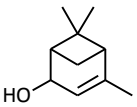
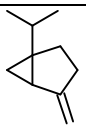
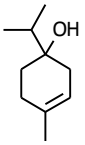
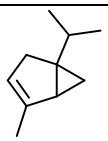
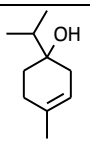
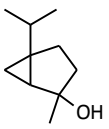
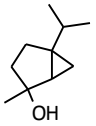
Tab. 24 Results of the molecular target screening on PPAR-γ and FXR of furanocoumarins identified in *P. ostruthium.* *In silico* binding in the respective target is indicated with an x.

Chromones			
Substance	**Structure**	**FXR**	**PPAR-γ**
Peucenin 578-72-3 $C_{15}H_{16}O_4$ 260.29			
Peucenin-7-methyl ether 13544-40-6 $C16H_{18}O_4$ 274.31			
Hamaudol-7-acetate 30358-88-4 $C_{17}H_{18}O_6$ 318.32			

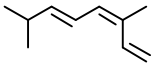
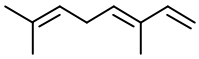
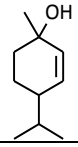
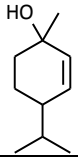
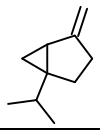
Tab. 25 *In silico* screening results on PPAR-γ and FXR of chromones found in *P. ostruthium.*

Fatty acids									
Substance	**Structure**	**COX-2**	**mPGES-1**	**cPLA2α**	**p38**	**IKK2**	**FXR**	**LXR**	**PPAR-γ**
Lineolic acid 60-33-3 $C_{18}H_{32}O_2$ 280.45		x					x	x	x
Lineolenic acid 463-40-1 $C_{18}H_{30}O_2$ 278.43		x						x	x
Oleic acid 112-80-1 $C_{18}H_{34}O_2$ 282.46		x					x	x	x
Palmitic acid 57-10-3 $C_{16}H_{32}O_2$ 256.42		x					x	x	x
Stearic acid 57-11-4 $C_{18}H_{36}O_2$ 284.48		x					x	x	x
4,6-Dimethylnonanoic acid 7540-70-7 $C_{11}H_{22}O_2$ 186.29									

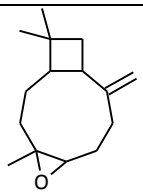
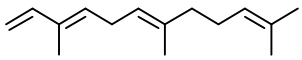
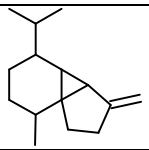
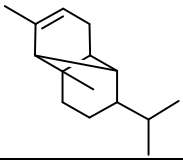
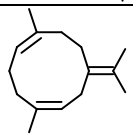
Tab. 26 *In silico* screening results of diverse fatty acids published for *P. ostruthium*. A positive match in the pharmacophoric pocket is indicated with an x.

Monoterpenes			
Substance	**Structure**	**FXR**	**PPAR-γ**
l-Sabinene 10408-16-9 $C_{10}H_{16}$ 136.23	 Rotation (-)		
α-Limonene 138-86-3 $C_{10}H_{16}$ 136.23			
ß-Pinene 127-91-3 $C_{10}H_{16}$ 136.23			
Myrcene 123-35-3 $C_{10}H_{16}$ 136.23			
trans-Verbenol 1820-09-3 $C_{10}H_{18}O$ 152.23	 		
d-Sabinene 2009-00-9 $C_{10}H_{16}$ 136.23	 Rotation (+)		
d-Terpin-1-en-4-ol 2438-10-0 $C_{10}H_{18}O$ 154.25	 		
α-Thujene 2867-05-2 $C_{10}H_{16}$ 136.23			
cis-Thujane-4-ol 20126-76-5 $C_{10}H_{18}O$ 154.25	 Rotation (-)		
trans-4-Thujanol 17699-16-0 $C_{10}H_{18}O$ 154.25	 		
l-Terpinen-4-ol 15537-55-0 $C_{10}H_{18}O$ 154.25	 		

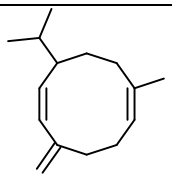
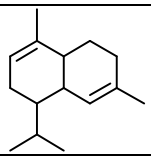
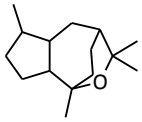
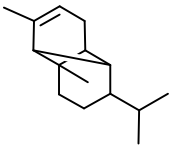
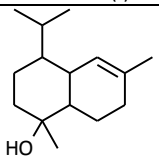
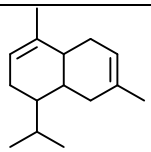
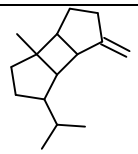
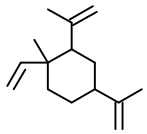
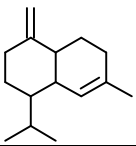
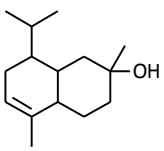
Tab. 26 continues on next page.

Substance	Structure	FXR	PPAR-γ
cis-Ocimene 27400-71-1 $C_{10}H_{18}$ 137.23			
trans-Ocimene 27400-72-2 $C_{10}H_{18}$ 137.23			
cis-p-Menth-2-en-1-ol 29803-81-4 $C_{10}H_{18}O$ 154.25			
trans-p-Menth-2-en-1-ol 29803-82-5 $C_{10}H_{18}O$ 154.25			
Sabinene 3387-41-5 $C_{10}H_{16}$ 136.23			

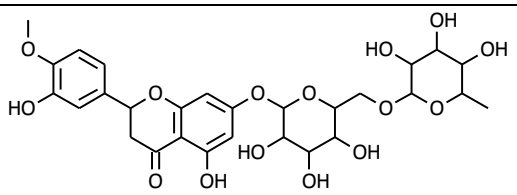
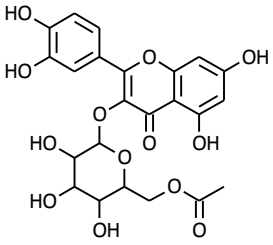
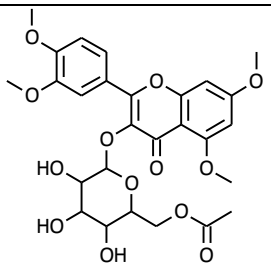
Tab. 27 Results of the molecular target screening on PPAR-γ and FXR of monoterpenes identified in *P. ostruthium*.

Sesquiterpenes			
Substance	**Structure**	**FXR**	**PPAR-γ**
β-Caryophyllene oxide 1139-30-6 $C_{15}H_{24}O_5$ 220.35			
α-Farnesene 502-61-4 $C_{15}H_{24}$ 204.35			
β-Cubebene 13744-15-5 $C_{15}H_{24}$ 204.35			
(+)-Ylangene 14912-44-8 $C_{15}H_{24}$ 204.35			
Germacrene B 15423-57-1 $C_{15}H_{24}$ 204.35			

Tab. 27 continues on next page.

Substance	Structure	FXR	PPAR-γ
Germacrene D 23986-74-5 C_{15} H_{24} 204.35			
(-)-α-Cadinene, 24406-05-1 C_{15} H_{24} 204.35			
(-)-Kessane 3321-66-2 C_{15} H_{26} O 222.37			
Aglaiene; α-Copaene 3856-25-5 C_{15} H_{24} 204.35	 Rotation (-)		
l-α-Cadinol 481-34-5 C_{15} H_{26} O 222.37			
(-)-β-Cadinene 523-47-7 C_{15} H_{24} 204.35			
β-Bourbonene 5208-59-3 C_{15} H_{24} 204.35	 Rotation (-)		
β-Elemene 33880-83-0 C_{15} H_{24} 204.35			
(±)-γ-Cadinene 39029-41-9 C_{15} H_{24} 204.35			
2-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro- 2,5-dimethyl-8-(1-methylethyl)- 50895-55-1 C_{15} H_{26} O 222.37			

Tab. 28 *In silico* screening results on PPAR-γ and FXR of sesquiterpenes found in *P. ostruthium*.

Flavonoids			
Substance	**Structure**	**FXR**	**PPAR-γ**
Hesperidine 520-26-3 C_{28} H_{34} O_{15} 610.56			
4H-1-Benzopyran-4-one, 3-[(6-O-acetyl-**β**-D-glucopyranosyl)oxy]-2-(3,4-dimethoxyphenyl)-5,7-dimethoxy- 54542-52-8 C_{27} H_{30} O_{13} 562.52			
Quercetin-3-O-(6-acetylglucoside) 54542-51-7 C_{23} H_{22} O_{13} 506.41			

Tab. 29 Results of the molecular target screening on PPAR-γ and FXR of flavonoids identified in *P. ostruthium.*

The coumarins ostruthin and osthole, the chromone peucenin as well as the furanocoumarins oxypeucedanin hydrate, oxypeucedanin, oxypeucedanin methanolate, bergamottin, bergapten, bergaptol, isopimpinellin, pabulenol, osthrutol, imperatorin, osthole, isoimperatorin and xanthotoxin have also been identified in this study.

As isolation and identification has only been performed for the active detannified MeOH extract, representing the apolar $CHCl_3$ portion of the MeOH extract, and the nonpolar DCM extract the polar flavonoids already described for *P. ostruthium* were not identified in this study. The greatest part of the volatile mono- and sesquiterpenoids, on the other hand have likely been lost during extraction and isolation. Therefore, only the sesqiterpene caryophyllene oxide was detected with GC-MS analyses. Some components that have only been described for the fruits of *P. ostruthium*, like the coumarins umbelliferone, aurapten and scopoletin, the furanocoumarins marmesin and angelicin derivatives or the chromones peucenin-7-methylether and hamaudol have also been included in the *in silico* screening.

3.5 Identification and quantification of coumarins in *Peucedanum ostruthium* (L.)Koch by HPLC-DAD and HPLC-DAD-MS

Sylvia Vogl,[†] Martin Zehl,*[,†] Paolo Picker,[†] Ernst Urban,[‡] Christoph Wawrosch,[†] Gottfried Reznicek,[†] Johannes Saukel,[†] and Brigitte Kopp[†]

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

[‡]Department for Medicinal Chemistry, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

This paper was published in the Journal of Agricultural and Food Chemistry, J. Agric. Food Chem. 2011, 59, 4371–4377 (dx.doi.org/10.1021/jf104772x). All of the phytochemical work, except NMR measurements (Prof. Ernst Urban, Dept. of Pharmaceutical Chemistry, University of Vienna), as well as MS analyses (Dr. Martin Zehl, Dept. of Pharmacognosy, University of Vienna) were done by the author of this thesis. Quantification of the coumarins as well as statistics for the method development were performed by the author of this thesis.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Identification and Quantification of Coumarins in *Peucedanum ostruthium* (L.) Koch by HPLC-DAD and HPLC-DAD-MS

Sylvia Vogl,[†] Martin Zehl,[*,†] Paolo Picker,[†] Ernst Urban,[‡] Christoph Wawrosch,[†] Gottfried Reznicek,[†] Johannes Saukel,[†] and Brigitte Kopp[†]

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

[‡]Department for Medicinal Chemistry, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

Supporting Information

ABSTRACT: The rhizomes of *Peucedanum ostruthium* (L.) Koch (masterwort) are traditionally used in the alpine region as ingredient of liqueurs and bitters, and as a herbal drug. A sensitive and specific high-performance liquid chromatography−diode-array detection−mass spectrometry (HPLC-DAD-MS) method has been developed for the simultaneous identification and quantification of its main coumarins, oxypeucedanin hydrate, oxypeucedanin, ostruthol, imperatorin, osthole, isoimperatorin, and ostruthin. Fast HPLC separation could be achieved on an Acclaim C18 column (150 mm × 2.1 mm i.d., 3 μm) using a mobile phase gradient of acetonitrile−water modified with 0.01% acetic acid. The quantification by HPLC-DAD was performed with imperatorin as external standard and validated to demonstrate selectivity, linearity, precision, and accuracy. The content of the main coumarins was quantitated in various batches of commercial and field-collected rhizomes of *Peucedanum ostruthium*, as well as in beverages prepared thereof.

KEYWORDS: *Peucedanum ostruthium*, HPLC-DAD-MS, validation, quantification, coumarins, oxypeucedanin hydrate, oxypeucedanin, osthole, imperatorin, isoimperatorin, ostruthol, ostruthin

INTRODUCTION

Comprising over 120 species, the genus *Peucedanum* is one of the most species-rich genera of the Apiaceae. While many members of the family Apiaceae are commonly used in cuisine, particularly as herbs and spices, this is rarely the case for the *Peucedanum* species. A notable exception is the masterwort (*Peucedanum ostruthium*). Its main distribution is in the alpine region, where the rhizomes have been traditionally used for the preparation of teas, liqueurs, and bitters.[1] Teas are typically prepared from dried and cut roots, which are commercially available in drugstores under the name "Radix Imperatoriae" or "Rhizoma Imperatoriae". Various tea recipes can be found, where masterwort is either the sole ingredient or is mixed with other herbal drugs. The masterwort roots are also popular for the preparation of liqueurs and bitters by means of cold soaking. Typically, the dried or fresh masterwort roots are cut, placed in a container, and covered with ethanol (38%). After an appropriate soaking time, usually several weeks, the preparation is filtered and diluted to reduce bitterness.

The usage of masterwort for the preparation of teas, liqueurs, and bitters is strongly linked to its important relevance in Austrian traditional medicine. These preparations are particularly recommended for gastro-intestinal diseases, but also for disorders of the cardiovascular system, the respiratory tract, and to treat tiredness.[1]

Coumarins represent a very interesting main compound class in *Peucedanum* species.[2] At least 1300 natural coumarins have been identified so far, mainly in the families of Rutaceae and Apiaceae.[3] Within the Apiaceae family, coumarin patterns can be used to distinguish between closely related species.[4] Members of this compound class are increasingly recognized as valuable health-promoting constituents of edible plants and herbal plant preparations due to their wide spectrum of biological activities. Recent studies on various coumarins have revealed, among others, antioxidant,[5] antimycobacterial,[6] anticoagulant, antitumoral, antiviral, antifungal, and antiinflammatory activities.[3,7−9] Coumarins from *Peucedanum ostruthium*, most notably ostruthol, have also been shown to act as inhibitors of acetylcholinesterase, which implicates a high potential for the treatment of Alzheimer's disease.[10]

However, coumarins and furanocoumarins have also been linked to phototoxic, mutagenic, carcinogenic, and hepatotoxic effects.[11−13] In 2004, the European Food Safety Authority (EFSA) established a tolerable daily intake of coumarin (1,2-benzopyrone) of 0.1 mg/kg body weight based on its hepatotoxicity.[14] Very high concentrations of coumarin have been found in cassia cinnamon, which lead to public discussions in Germany and Austria regarding the safety of cinnamon-containing foods.[11,15] Although relevant data on other coumarin derivatives are scarce, it is not unlikely that they are also toxicologically relevant or may interact with drugs such as warfarin.[16] Consequently, there is a need for the quantification of the content of coumarins in food and herbal remedies, like those containing *Peucedanum ostruthium*.

Received: December 13, 2010
Accepted: February 22, 2011
Revised: February 21, 2011
Published: March 22, 2011

ACS Publications © 2011 American Chemical Society

Up to now, there are no qualitative or quantitative standards for the quality control of the commercial drug "Radix/Rhizoma Imperatoriae". Drugstores and distributors of this herbal drug specify the content of essential oil as a quality feature, but the content of the coumarins is typically not determined. This potentially important information is thus not available to the consumers.

Several methods have been published for the quantification of coumarins in foods and herbal drugs. For instance, HPLC methods have been developed for the determination of coumarins in citrus products[17] and in *Angelicae dahuricae* Radix.[18,19] GC-MS has been applied for the quantification of coumarins in bergamot oil,[20] citrus fruits, and vegetables of the Apiaceae family.[21] However, to the best of our knowledge, there is no method available for the quantification of the main coumarins in masterwort roots. Therefore, the aim of this study was to establish a specific HPLC method for this purpose and to quantify the main coumarins in commercial and field-collected samples of *Peucedanum ostruthium* and preparations thereof. Furthermore, the method was validated according to the ICH guidelines[22] on the basis of specificity, linearity, precision, and accuracy.

MATERIALS AND METHODS

Reagents and Chemicals. Methanol (MeOH), dichloromethane, and acetonitrile (MeCN) were HPLC-grade (VWR, Vienna, Austria). Glacial acetic acid was purchased from Carl Roth (Karlsruhe, Germany). Water was distilled by an IKA-Dest M3000 automatic water distillation apparatus (IKA, Staufen, Germany). Imperatorin, isoimperatorin, oxypeucedanin, and oxypeucedanin hydrate were all of 99% purity and purchased from Herboreal Ltd. (Edinburgh, UK). We obtained osthole with a purity of 98.9% from Chromadex (Irvine, USA).

Ostruthol and ostruthin were isolated from the dichloromethane extract of *Peucedanum ostruthium* rhizomes using preparative HPLC. The purity was confirmed to be >98% by HPLC with DAD and ELSD detection as well as by NMR.

Samples and Preparations of *Peucedanum ostruthium* Rhizomes. Six samples of "Radix/Rhizoma Imperatoriae" were purchased from various companies in Austria and Germany (two batches from Kottas Pharma GmbH, Vienna, Austria; one each from KWIZDA, Vienna, Austria; Alfred Richter GmbH & CO KG, Kufstein, Austria; Alfred Galke GmbH, Gittelde, Germany, and Herba Chemosan, Vienna, Austria). Another two batches were field-collected and identified by Dr. Saukel (dried root material collected in 2008 at the Weisspriachtal, Salzburg, Austria, 1200 m above sea level, and dried root material collected in 2008 at Gontal, Carinthia, Austria, 1800 m above sea level). Voucher specimens are deposited at the Department of Pharmacognosy, University of Vienna, Austria.

For exhaustive extraction of the coumarins, 0.5 g dried and powdered material was processed with 20 mL of dichloromethane using reflux extraction for 15 min at 40 °C. Subsequently, the extracted solution was filtered, evaporated to dryness, and redissolved in 20 mL of MeOH.

In order to quantify the coumarin content in traditional formulations, water extract and liqueurs were prepared and analyzed. The water decoction was obtained by extraction of 0.5 g dried and powdered drug with 20 mL of water for 15 min in a water bath at 100 °C. This procedure was chosen for a direct comparison of the extraction efficiency of water with dichloromethane. In the case of a traditional masterwort tea, cut pieces would be used instead of the powder. The extracted solution was filtered, centrifuged, and used directly for HPLC measurements. Furthermore, we investigated four traditionally prepared liqueurs produced in different years from roots of various sample locations. The roots of the masterwort have been cut, put into a bottle, filled up with ethanol (38%), and kept for at least 4 weeks. For the analysis, the solution was filtered, centrifuged, and used directly for HPLC measurements. The liqueur obtained in the above-described way is typically diluted 1:10−1:15 with ethanol (38%) before consumption.

Identification and Quantification of the Coumarins by HPLC-MS and HPLC-DAD. An HPLC instrument from Shimadzu (Kyoto, Japan) consisting of a CBM-20A system controller, a DGU-20A5 membrane degasser, an LC-20AD solvent delivery unit, an SIL-20AC HT autosampler, a CTO-20AC column oven, and an SPD-M20A photodiode array detector was used for quantification. Data analysis was performed using the chromatography software LCsolution Ver.1.2 (Shimadzu). Chromatographic separation was achieved on a 150 mm × 2.1 mm i.d., 3 μm, Acclaim 120 C18 reversed-phase column, with a 10 mm × 2.1 mm i.d., 5 μm, Acclaim 120 C18 guard column from Dionex (Germering, Germany), at 38 °C and a flow rate of 0.5 mL/min. Water and MeCN, both modified with 0.01% acetic acid, were used as mobile phases A and B, respectively. Gradient elution was performed as follows: 25−37% of B in 6 min, 37−45% of B in 8 min, 45−65% of B in 10 min, 65−95% of B in 1 min, and isocratic at 95% of B for 5 min. The injection volume was 5 μL for all samples. The DAD collected data from 190 to 400 nm.

Tentative identification of the main coumarins was facilitated by HPLC-DAD-MS. These analyses were performed on an UltiMate 3000 RSLC-series system (Dionex) coupled to an HCT 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (Bruker Daltonics, Bremen, Germany). HPLC separation was carried out as described above. The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage, −4.0 kV; nebulizer, 20 psi (N_2); dry gas flow, 6 L/min (N_2); and dry temperature, 350 °C. The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode where each MS scan (m/z 100−700) was followed by MS^2 scans (m/z 40−700, fragmentation amplitude of 0.7 V) of the two most intense precursor ions, and MS^3 scans (m/z 40−700, fragmentation amplitude of 1.0 V) of the most intense fragment ion in each MS^2 scan. Helium was used as collision gas, the isolation window was set to 4 m/z, and three spectra were averaged per scan.

The identity of the main compounds was confirmed either by comparison with reference compounds (**1**, **2**, and **4**−**6**, see Figure 1) or by isolation and structural characterization by 1D and 2D NMR and MS (**3** and **7**).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) 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. ^1H and ^{13}C NMR data of **3** and **7** were in accordance with previously published data.[23−25]

Imperatorin (**4**) was chosen as an external standard for the quantification of the identified major coumarins by HPLC-DAD. Stock solutions of **4** in MeOH were prepared in a concentration of 2 mg/mL and further diluted in MeOH to obtain the appropriate working solutions for external calibration and validation. The detector signal at 310 nm was used for the quantification of the coumarins. As the structures and therefore the UV spectra and absorption coefficients at 310 nm differ between **4** and the six other coumarins, a UV-response factor was calculated for each substance. For that, **1**−**7** were separately weighed and dissolved in MeOH to make stock solutions of 0.25 mg/mL. Those stock solutions were mixed and further diluted with MeOH by a factor of 10. The purity of the coumarins, including the two isolated compounds **3** and **7**, was confirmed by HPLC analysis (UV detection at 205, 254, and 310 nm).

Validation of the Analytical Method. The specificity of the method was shown by testing the peak purity of the analytes in the samples on the basis of the online UV and MS spectra. The precision (i.e., repeatability of measurements) was determined by at least triplicate

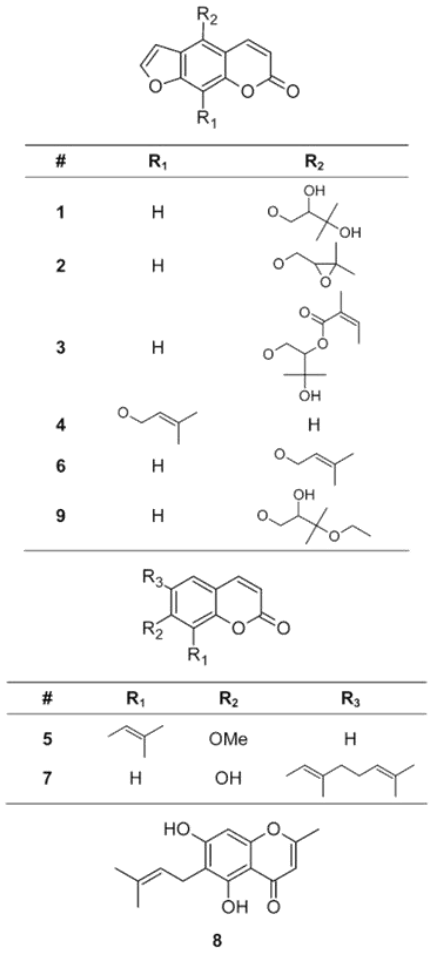


Figure 1. Structure of the main coumarins in *Peucedanum ostruthium*. Linear furanocoumarins: oxypeucedanin hydrate (**1**), oxypeucedanin (**2**), ostruthol (**3**), imperatorin (**4**), isoimperatorin (**6**), and oxypeucedanin ethanolate (**9**). Simple courmarins: osthole (**5**) and ostruthin (**7**). Chromones: peucenin (**8**).

analysis of sample R1 (Kottas Pharma GmbH, Vienna, Austria, 2008) on three different days, whereby the sample was independently prepared on each day. Intra- and interday variability was evaluated for all seven main coumarins and expressed by the relative standard deviations (RSD). The accuracy of the method was estimated by means of recovery experiments with all seven coumarins. Therefore, an aliquot of the dry sample drug (R1) was spiked with 2.08 mg of each compound (dissolved in MeOH) per g drug. After evaporation of the methanol, the sample was analyzed in pentuplicate. The sensitivity and linear range of the method were shown for the external standard imperatorin as well as for the mixture of the seven reference coumarins. The concentration range was evaluated for imperatorin starting at very high concentrations (2 and 1.5 mg/mL) and further stepwise dilution by a factor of 2 or 10 down to the limit of detection. The Pearson product−moment correlation coefficient (r) of the calibration curve was evaluated. The lower limit of detection (LLOD) and quantification (LLOQ) values were demonstrated as the concentration where the signal-to-noise ratio (S/N) had a defined value (for LLOD, $S/N \sim 3$, and for LLOQ $S/N \sim 10$, where N is the height of the noise measured at the retention time of the reference peak when the blank is analyzed, and S is the height of the signal of the reference).

RESULTS AND DISCUSSION

Optimization of the HPLC Method. In addition to the essential oil components, coumarins represent an interesting compound class within the Apiaceae family. Recent studies showed their broad spectrum of activities and their potency as new drug leads.[3,5−8,26−30]

Currently, there are no investigations on the coumarin content of *Peucedanum ostruthium*, but several HPLC methods for the analysis of coumarins were published. Different methods from literature using gradient as well as isocratic elution were tested for the analysis of the coumarins in *Peucedanum ostruthium*.[4,31] Unfortunately, even with simple adaptations, none of the tested methods provided sufficient peak purity for the accurate quantification of the main coumarins as determined by HPLC-MS. Therefore, a novel method was developed using a C18 column with smaller particle size and a higher temperature. Baseline separation (resolution >1.5) of all relevant components (Figure 1) in the apolar extract of the dried root material of *Peucedanum ostruthium* could be achieved on the Acclaim C18 column with a gradient of H_2O and MeCN containing 0.01% acetic acid in just 30 min (Figure 2).

Identification of the Main Coumarins in *Peucedanum ostruthium* Rhizomes. On the basis of the HPLC fingerprint, 7 major coumarin constituents (**1**−**7**) were selected for the quantitative analysis (Figures 1 and 2). Identification of these components was based on their characteristic MS^n spectra (Table 1). The $[M + H]^+$ ions of the linear hydroxyfuranocoumarin derivatives all showed neutral loss of their oxygen-bound side chain to yield the fragment ion at m/z 203 (protonated hydroxyfuranocoumarin). Further fragmentation of this ion in MS^3 allows the differentiation of 5- and 8-hydroxyfuranocoumarins via the ratio of the intensities of the fragment ions at m/z 175 and m/z 159 as described previously.[32] In the case of **3**, an intense fragment ion corresponding to the protonated (dehydrogenated) side chain is obtained at m/z 185. The fragmentation of **5** has also been described, and **7** shows the analogous neutral loss of the side chain followed by the loss of CO.[33] The identity of **1**, **2**, and **4**−**6** was further confirmed by comparison of their retention times and UV spectra with authentic references. In addition, **3** and **7** were isolated by semipreparative HPLC in order to facilitate comprehensive structure elucidation by NMR spectroscopy. Several less prominent compounds, such as the chromone **8**, were identified by LC-MS only (data not shown). Khaled et al.[24] also reported **1**−**8** as the main compounds of the dried roots but without any quantitative analyses. The coumarin glycosides reported in the study by Khaled et al.[24] were not detected in any of the investigated samples, including a methanolic extract of dried roots that was enriched in more polar compounds.

Quantification of the Main Coumarins in the *Peucedanum ostruthium* Rhizome. The content of the main coumarins was quantitated in various batches of commercial and field-collected rhizomes of *Peucedanum ostruthium* as well as in liqueurs and in a water decoction. For this, the HPLC-DAD method has been validated using the external standard imperatorin (**4**). With an injection volume of 5 μL, the linear range for **4** was determined to span concentrations from 61 ng/mL to 1.0 mg/mL with an linear regression equation of $y = 23850014x$ and an r of 0.99996. The calibration curves for the other coumarins were also linear over the investigated concentration range of approximately 0.2 μg/mL to 0.2 mg/mL (Table 2). Relative response factors to **4** were

Journal of Agricultural and Food Chemistry ARTICLE

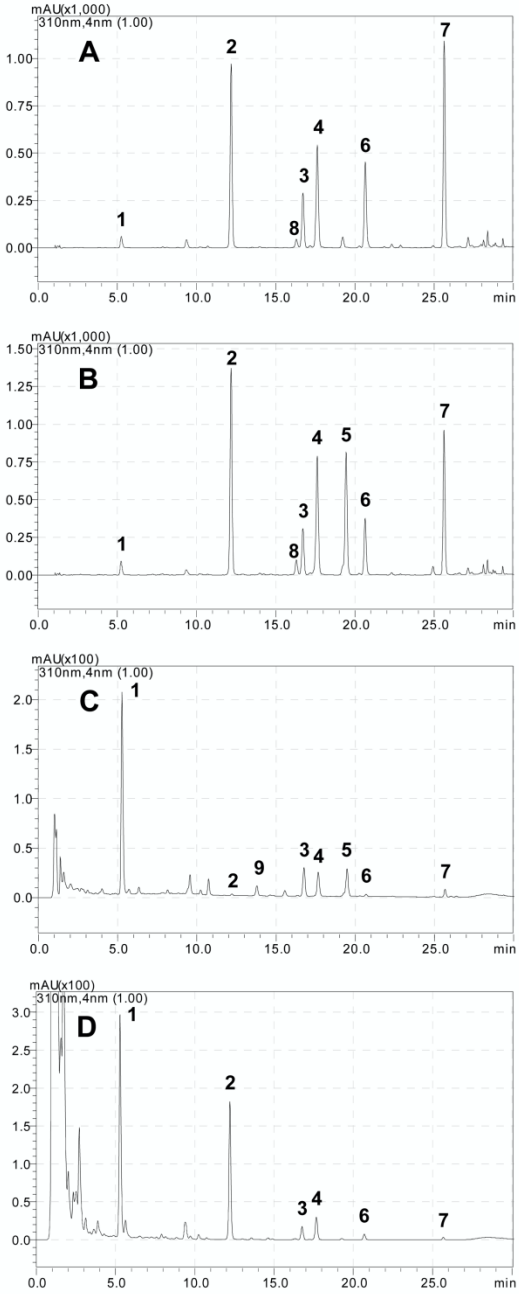


Figure 2. HPLC chromatograms (310 nm) of (A) the dichloromethane extract of commercial *Peucedanum ostruthium* rhizome (Kottas 2010), (B) the dichloromethane extract of the *Peucedanum ostruthium* rhizome from a wild collection at Weisspriachtal, Salzburg, (C) a liqueur of *Peucedanum ostruthium* rhizome, and (D) a water decoction of the *Peucedanum ostruthium* rhizome (prepared from sample Kottas 2010). Peak numbers refer to the compounds shown in Figure 1.

determined for **1**−**3** and **5**−**7** (Table 2). The LLOD of the external standard was 15 ng/mL (S/N = 3.6), whereas the LLOQ was found to be 61 ng/mL (S/N = 8.6). Data on the precision expressed as intra- and interday RSD were determined from

Table 1. MS^n Fragmentation Patterns of the Main Coumarins in *Peucedanum ostruthium* Rhizome Extracts and Preparations

#[a]	$[M + H]^+$	main fragment ions (>10% rel. int.)
1	305.0	MS^2 [305.0]: 202.8
		MS^3 [202.8]: 174.9, 173.8, 158.9, 146.9, 131.0
2	287.0	MS^2 [287.0]: 202.8
		MS^3 [202.8]: 174.8, 173.8, 172.8, 158.8, 146.9, 130.9, 114.1
3	387.1	MS^2 [387.1]: 369.1, 305.0, 184.9, 166.9
		MS^3 [369.1]: 287.0, 268.9, 202.8, 166.9
		MS^3 [184.9]: 166.9, 83.3
4	270.9	MS^2 [270.9]: 202.8
		MS^3 [202.8]: 174.8, 158.8, 146.9, 130.9
5	245.0	MS^2 [245.0]: 188.8
		MS^3 [188.8]: 160.9, 158.8, 130.9
6	270.9	MS^2 [270.9]: 202.8
		MS^3 [202.8]: 158.8, 146.9, 130.9
7	299.1	MS^2 [299.1]: 174.8
		MS^3 [174.8]: 146.9
8	261.0	MS^2 [261.0]: 204.8
9	333.1	MS^2 [333.1]: 202.8
		MS^3 [202.8]: 174.9, 158.9, 157.8, 146.9, 130.9

[a] Numbers refer to the compounds shown in Figure 1.

Table 2. Relative UV-Response Factors to Imperatorin (f_i), Linear Regression Equations, and Correlation Coefficients (r or Pearson Product-Moment) for the Seven Coumarins

#[a]	f_i [b]	linear regression equation[b]	r[b]
1	1.7	$y = 12295600x$	0.99112
2	0.8	$y = 26169348x$	0.99571
3	1.7	$y = 12558206x$	0.99760
4		$y = 23850014x$	0.99996
5	0.6	$y = 33689612x$	0.99997
6	0.8	$y = 28921459x$	0.99779
7	1.5	$y = 14131201x$	0.99304

[a] Numbers refer to the compounds shown in Figure 1. [b] The values were determined from 5 replicates performed in one day.

three independently prepared samples of one batch and are given in Table 3. The interval between the first two and the third analysis was around seven months, which also indicates the high stability of the coumarins upon storage of the dried root material. The higher RSD values for **5** can be explained by the presence of an only partly resolved impurity that requires peak splitting during data analysis. The accuracy and extraction efficiency were estimated by an analysis of a sample spiked with (relatively small) amounts of all seven coumarins (Table 4).

With one exception (collected sample from Weisspriachtal, Salzburg, 2008; R7), variations of the coumarin content between the drug samples were rather low for a biological material with relative standard deviations lower than ±30% (Table 5). In the dichloromethane extracts of these root samples (R1−R6 and R8), **7** was quantitated as the main coumarin (38−41% of the total coumarin content), followed by **2** (17−20%), **4** (12−15%), **3** (12−14%), **6** (9−10%), **1** (2−7%), and **5** composing just about 1%. The sample collected from Weisspriachtal in 2008 (R7) differed mainly by a significantly higher absolute and relative

Journal of Agricultural and Food Chemistry ARTICLE

content of **5** (8.8 mg/g and 11.1%, respectively) and a somewhat higher content of **2** and **4**. The total coumarin content (i.e., sum of the contents of the seven quantitated coumarins) in the dichloromethane extracts represented 68−78%, whereas the amount on gram dry weight of the drug made up 4−8%.

Analysis of Liqueurs and a Water Decoction Prepared from the *Peucedanum ostruthium* Rhizomes. In Austrian traditional medicine, particularly in the alpine region, the rhizomes of *Peucedanum ostruthium* have been and are still used for the preparation of teas, liqueurs, and bitters. To investigate whether significant amounts of the different coumarins can be extracted with the rather polar solvents used for the preparation of liqueurs (ethanol/water) and teas (hot water), we have analyzed four traditionally prepared liqueurs and a tea-like water decoction made of one of the commercial "Radix/Rhizoma Imperatoriae" samples. The analysis was performed with the same method optimized for the dichloromethane extracts, and no sample preparation except filtration and centrifugation was conducted. Interestingly, all seven coumarins could be detected and quantified in the four different liqueurs, although the absolute content differed very strongly (Table 6). In comparison to the other extracts, the preparation of the liqueurs was not standardized. Neither the amount of drug per bottle nor the "extraction time" was identical between the batches. Furthermore, the liqueurs were made with cut root material, which most likely has an influence on the extraction rates of the compounds. Still, the relative content of the different coumarins was surprisingly similar, with the most polar coumarin **1** found in the highest concentration (64−85%), followed by **3** (5−17%), **4** (5−9%), **5** (1−5%), **7** (1−4%), **6** (0.5−2%), and **2** (0.4−2%). In the water decoction, the most dominant coumarin was again **1**, comprising 64%, followed by **2** (21%), **3** (6%), **4** (6%), **7** (2%), and **6** (2%).

Obviously, the extraction efficiency for the different coumarins with hot water and 38% ethanol is strongly dependent on the polarity of the compounds. However, this alone does not explain the extremely high content of **1** and the very low content of **2** in the liqueurs. In a recent study, it was shown that **1** is formed by hydrolytic ring-opening of **2** during sample preparation.[34] In the case of the liqueurs, where the plant material is kept in the solvent for a long time (up to two years in our case), the majority of **1** is presumably a transformation product of **2**. This hypothesis is supported by the tentative identification of **9** in the liqueurs, which most likely results from ethanolytic epoxide ring-opening as a second transformation product of **2** (Figure 2C). Analogously, oxypeucedanin methanolate was observed in MeOH extracts of *Peucedanum ostruthium* rhizomes (data not shown). Transformation of **2** to the hydrate has most likely also occurred in water decoction, but the reaction is less complete due to the rather short extraction and processing time before analysis. Theoretically, **1** could also result from hydrolysis of the ester function of **3**, but no experimental evidence for this pathway was observed.

Table 3. Intra- and Interday Repeatability of the Method

#[a]	mean level ± SD [mg/g][b]	intraday repeatability RSD [%][c]	interday repeatability RSD [%][d]
1	3.48 ± 0.11	1.02	7.61
2	9.90 ± 0.06	0.94	0.96
3	7.59 ± 0.08	0.79	0.89
4	6.82 ± 0.09	0.89	1.15
5	0.25 ± 0.02	1.36	9.86
6	4.84 ± 0.22	1.00	4.78
7	22.15 ± 0.91	0.46	4.47

[a] Numbers refer to the compounds shown in Figure 1. [b] Coumarin content in a Radix Imperatoriae sample (R1, Kottas Pharma GmbH, Vienna, Austria, 2008) as quantitated from the dichloromethane extract ($n = 11$). [c] Average of the RSD values ($n = 3-5$, each) obtained on three different days. [d] RSD of the three average values ($n = 3-5$, each) obtained on three different days.

Table 4. Recovery of the Seven Coumarins

#[a]	R1 unspiked mean level ± SD [mg/g][b]	R1 spiked mean level ± SD [mg/g][c]	recovery [%]
1	3.23 ± 0.02	5.29 ± 0.08	99
2	9.82 ± 0.06	11.81 ± 0.10	95
3	7.63 ± 0.07	9.46 ± 0.07	87
4	6.88 ± 0.05	8.67 ± 0.09	86
5	0.23 ± 0.00	1.88 ± 0.02	79
6	4.60 ± 0.04	6.34 ± 0.11	84
7	21.21 ± 0.11	24.05 ± 0.28	136

[a] Numbers refer to the compounds shown in Figure 1. [b] Coumarin content in an unspiked Radix Imperatoriae sample (R1, Kottas Pharma GmbH, Vienna, Austria, 2008) as quantitated from the dichloromethane extract ($n = 5$). [c] Coumarin content in a spiked aliquot of sample R1 (2.08 mg each of **1**-**7** per g drug) as quantitated from the dichloromethane extract ($n = 5$).

Table 5. Coumarin Content in Different Samples of Radix Imperatoriae as Quantitated from the Dichloromethane Extracts

	roots (coumarin content in mg/g dry weight ± SD)[b]								
#[a]	R1	R2	R3	R4	R5	R6	R7	R8	average R1−R8
1	3.48 ± 0.11	1.58 ± 0.03	3.78 ± 0.04	2.19 ± 0.02	3.93 ± 0.07	3.44 ± 0.02	2.41 ± 0.01	3.08 ± 0.02	2.99 ± 0.83
2	9.90 ± 0.06	12.72 ± 0.63	9.96 ± 0.17	6.91 ± 0.05	9.80 ± 0.06	10.29 ± 0.01	17.21 ± 0.07	13.11 ± 0.06	11.24 ± 3.08
3	7.59 ± 0.08	8.73 ± 0.36	7.69 ± 0.16	5.28 ± 0.08	8.09 ± 0.08	7.39 ± 0.20	8.96 ± 0.04	9.22 ± 0.09	7.88 ± 1.24
4	6.82 ± 0.09	10.07 ± 0.41	7.45 ± 0.04	4.88 ± 0.06	7.04 ± 0.06	6.82 ± 0.12	14.27 ± 0.03	11.42 ± 0.25	8.60 ± 3.08
5	0.25 ± 0.02		0.51 ± 0.00	0.32 ± 0.00	0.41 ± 0.01	0.43 ± 0.02	8.81 ± 0.04	0.49 ± 0.01	1.60 ± 3.18
6	4.84 ± 0.22	6.50 ± 0.39	4.96 ± 0.09	3.96 ± 0.10	5.04 ± 0.04	5.02 ± 0.04	4.98 ± 0.05	6.99 ± 0.03	5.29 ± 0.97
7	22.15 ± 0.91	25.05 ± 0.11	22.83 ± 0.11	16.47 ± 0.13	23.74 ± 0.11	23.22 ± 0.09	21.08 ± 0.09	31.31 ± 0.21	23.23 ± 4.13

[a] Numbers refer to the compounds shown in Figure 1. [b] Samples: R1, Kottas Pharma GmbH, Vienna, Austria, 2008; R2, Kottas Pharma GmbH, Vienna, Austria, 2010; R3, Herba Chemosan, Vienna, Austria, 2009; R4, KWIZDA, Vienna, Austria, 2009; R5, Alfred Galke GmbH, Gittelde, Germany, 2009; R6, Alfred Richter GmbH & CO KG, Kufstein, Austria, 2009; R7, dried root material collected in 2008 at the Weisspriachtal in Salzburg, Austria; and R8, dried root material collected in 2008 at the Gontal in Carinthia, Austria; $n = 3$, except for R1 where $n = 11$.

dx.doi.org/10.1021/jf104772x |J. Agric. Food Chem. 2011, 59, 4371–4377

Table 6. Coumarin Content in Different Beverages Prepared from the *Peucedanum ostruthium* Rhizome

	liqueurs[b] mean level ± SD in [μg/mL]				water extract[c] mean level ± SD in [μg/mL]
#[a]	S1	S2	S3	S4	T
1	125.21 ± 0.31	511.49 ± 3.34	2574.75 ± 5.14	2574.04 ± 3.82	180.96 ± 2.68
2	0.71 ± 0.09	12.88 ± 1.54	34.36 ± 0.62	82.16 ± 3.38	60.40 ± 1.22
3	21.43 ± 0.38	133.58 ± 0.98	146.84 ± 2.17	268.79 ± 9.61	18.43 ± 0.86
4	11.69 ± 0.19	56.65 ± 0.56	164.30 ± 2.27	323.01 ± 2.36	15.94 ± 0.57
5	8.17 ± 0.10	35.47 ± 0.88	26.89 ± 0.29	66.53 ± 4.37	
6	0.93 ± 0.13	4.89 ± 0.66	22.11 ± 0.48	60.75 ± 2.67	4.90 ± 0.21
7	4.53 ± 0.29	35.15 ± 0.25	33.98 ± 0.43	126.70 ± 7.00	4.92 ± 0.16

[a] Numbers refer to the compounds shown in Figure 1. [b] Samples: S1, liqueur prepared from roots from Gontal in Carinthia, 2007; S2, liqueur prepared of roots from at the Königsalm in Lower Austria, Austria, 2008; S3, liqueur prepared of roots from Weisspriachtal in Salzburg, 2009; and S4, liqueur prepared of roots from the Riedingtal in Salzburg, Austria, 2009; $n = 3$. [c] T, water decoction of R1; $n = 3$.

Overall, the coumarin content of the liqueurs and the water decoction is potentially high enough to exert biological effects upon consumption. Considering that the liqueurs analyzed in this study are typically diluted 1:10 to 1:15 before consumption to reduce bitterness, low milligram quantities of coumarins, mostly oxypeucedanin hydrate (**1**), are taken up with a typically applied volume of 20−40 mL. The concentration of coumarins in the water decoction is most likely higher than in traditional masterwort teas due to differences in the way of preparation. Estimating that traditional tea preparations are less concentrated by a factor of about 10 compared to liqueurs, similar amounts of coumarins are ingested since much larger volumes of tea (approximately 250 mL) are consumed.

In conclusion, the above-described method is well suited for fast profiling and quantification of the main coumarins for quality control of the drug "Radix Imperatoriae" and various preparations thereof.

■ ASSOCIATED CONTENT

Supporting Information. Calibration function of **4**, ^{1}H, ^{13}C, and 2D NMR spectra of compounds **3** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +43-1-4277-55219. Fax: +43-1-4277-55256. E-mail: martin.zehl@univie.ac.at.

Funding Sources

This work is funded by the Austrian Science Fund (FWF): NFN-Project S10704-B037.

■ REFERENCES

(1) Gerlach, S.; Saukel, J.; Kubelka, W. Pflanzen in der österreichischen Volksmedizin. Die „VOLKSMED-Datenbank". *Sci. Pharm.* **2006**, *74*, S36.

(2) Hadacek, F.; Muller, C.; Werner, A.; Greger, H.; Proksch, P. Analysis, isolation and insecticidal activity of linear furanocoumarins and other coumarin derivatives from *Peucedanum* (Apiaceae: Apioideae). *J. Chem. Ecol.* **1994**, *20*, 2035–2054.

(3) Curini, M.; Cravotto, G.; Epifano, F.; Giannone, G. Chemistry and biological activity of natural and synthetic prenyloxycoumarins. *Curr. Med. Chem.* **2006**, *13*, 199–222.

(4) Ganzera, M.; Sturm, S.; Stuppner, H. HPLC-MS and MECC analysis of coumarins. *Chromatographia* **1997**, *46*, 197–203.

(5) Thuong, P. T.; Hung, T. M.; Ngoc, T. M.; Ha, D. T.; Min, B. S.; Kwack, S. J.; Kang, T. S.; Choi, J. S.; Bae, K. Antioxidant activities of coumarins from Korean medicinal plants and their structure-activity relationships. *Phytother. Res.* **2010**, *24*, 101–106.

(6) Schinkovitz, A.; Gibbons, S.; Stavri, M.; Cocksedge, M. J.; Bucar, F. Ostruthin: An antimycobacterial coumarin from the roots of *Peucedanum ostruthium*. *Planta Med.* **2003**, *69*, 369–371.

(7) Garcia-Argaez, A. N.; Ramirez Apan, T. O.; Parra Delgado, H.; Velazquez, G.; Martinez-Vazquez, M. Anti-inflammatory activity of coumarins from *Decatropis bicolor* on TPA ear mice model. *Planta Med.* **2000**, *66*, 279–281.

(8) Genovese, S.; Curini, M.; Epifano, F. Prenyloxyphenylpropanoids as a novel class of anti-inflammatory agents. *Antiinflamm. Antiallergy Agents Med. Chem.* **2010**, *9*, 158–165.

(9) Riveiro, M. E.; De Kimpe, N.; Moglioni, A.; Vazquez, R.; Monczor, F.; Shayo, C.; Davio, C. Coumarins: old compounds with novel promising therapeutic perspectives. *Curr. Med. Chem.* **2010**, *17*, 1325–1338.

(10) Urbain, A.; Marston, A.; Hostettmann, K. Coumarins from *Peucedanum ostruthium* as inhibitors of acetylcholinesterase. *Pharm. Biol.* **2005**, *43*, 647–650.

(11) Abraham, K.; Wohrlin, F.; Lindtner, O.; Heinemeyer, G.; Lampen, A. Toxicology and risk assessment of coumarin: Focus on human data. *Mol. Nutr. Food Res.* **2010**, *54*, 228–239.

(12) da Silva, V. B.; Kawano, D. F.; Carvalho, I.; da Conceicao, E. C.; de Freitas, O.; da Silva, C. H. T. P. Psoralen and bergapten: in silico metabolism and toxicophoric analysis of drugs used to treat vitiligo. *J. Pharm. Pharm. Sci.* **2009**, *12*, 378–387.

(13) McKenna, K. E. Iatrogenic skin cancer: induction by psoralen/ultraviolet A and immunosuppression of organ transplant recipients. *Photodermatol. Photoimmunol. Photomed.* **2004**, *20*, 289–296.

(14) Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contacts with Food (AFC) on a request from the Commission related to coumarin (question number EFSA-Q-2003-118). *EFSA J.* **2004**, *104*, 1−36 (available at http://www.efsa.europa.eu/en/efsajournal/pub/104.htm).

(15) Woehrlin, F.; Fry, H.; Abraham, K.; Preiss-Weigert, A. Quantification of flavoring constituents in cinnamon: high variation of coumarin in cassia bark from the German retail market and in authentic samples from Indonesia. *J. Agric. Food Chem.* **2010**, *58*, 10568–10575.

(16) Segal, R.; Pilote, L. Warfarin interaction with *Matricaria chamomilla*. *Can. Med. Assoc. J.* **2006**, *174*, 1281–1282.

(17) Dugo, P.; Piperno, A.; Romeo, R.; Cambria, M.; Russo, M.; Carnovale, C.; Mondello, L. Determination of oxygen heterocyclic components in citrus products by HPLC with UV detection. *J. Agric. Food Chem.* **2009**, *57*, 6543–6551.

(18) Park, A. Y.; Park, S. Y.; Lee, J.; Jung, M.; Kim, J.; Kang, S. S.; Youm, J. R.; Han, S. B. Simultaneous determination of five coumarins in *Angelicae dahuricae* Radix by HPLC/UV and LC-ESI-MS/MS. *Biomed. Chromatogr.* **2009**, *23*, 1034–1043.

(19) Zheng, X.; Zhang, X.; Sheng, X.; Yuan, Z.; Yang, W.; Wang, Q.; Zhang, L. Simultaneous characterization and quantitation of 11 coumarins in *Radix Angelicae Dahuricae* by high performance liquid chromatography with electrospray tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2010**, *51*, 599–605.

(20) Belsito, E. L.; Carbone, C.; Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M. C. Comparison of the volatile constituents in cold-pressed bergamot oil and a volatile oil isolated by vacuum distillation. *J. Agric. Food Chem.* **2007**, *55*, 7847–7851.

(21) Peroutka, R.; Schulzová, V.; Botek, P.; Hajšlová, J. Analysis of furanocoumarins in vegetables (*Apiaceae*) and citrus fruits (*Rutaceae*). *J. Sci. Food Agric.* **2007**, *87*, 2152–2163.

(22) International Conference on Harmonization (ICH). ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1). 1994, (available at http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html).

(23) Liu, R.; Sun, Q.; Shi, Y.; Kong, L. Isolation and purification of coumarin compounds from the root of *Peucedanum decursivum* (Miq.) Maxim by high-speed counter-current chromatography. *J. Chromatogr., A* **2005**, *1076*, 127–132.

(24) Khaled, S. A.; Szendrei, K.; Novak, I.; Reisch, J. Coumarin glycosides from *Peucedanum ostruthium*. *Phytochemistry* **1975**, *14*, 1461–1462.

(25) Perel'son, M. E.; Sheinker, Y. N.; Syrova, G. P. NMR spectra of natural coumarin derivatives II. Furocoumarins. *Chem. Nat. Compd.* **1971**, *7*, 557–562.

(26) Bartnik, M.; Glowniak, K.; Jakubowicz-Gil, J.; Pawlikowska-Pawlega, B.; Gawron, A. Effect of peucedanin and bergapten (5-MOP), furanocoumarins isolated from *Peucedanum tauricum* Bieb. (Apiaceae) fruits, on apoptosis induction and heat-shock protein expression in HeLa cells. *Herba Pol.* **2006**, *52*, 71–78.

(27) Epifano, F.; Genovese, S.; Menghini, L.; Curini, M. Chemistry and pharmacology of oxyprenylated secondary plant metabolites. *Phytochemistry* **2007**, *68*, 939–953.

(28) Ikeda, R.; Wada, M.; Nishigaki, T.; Nakashima, K. Quantification of coumarin derivatives in Noni (*Morinda citrifolia*) and their contribution of quenching effect on reactive oxygen species. *Food Chem.* **2009**, *113*, 1169–1172.

(29) Lee, S. O.; Choi, S. Z.; Lee, J. H.; Chung, S. H.; Park, S. H.; Kang, H. C.; Yang, E. Y.; Cho, H. J.; Lee, K. R. Antidiabetic coumarin and cyclitol compounds from *Peucedanum japonicum*. *Arch. Pharm. Res.* **2004**, *27*, 1207–1210.

(30) Liao, P. C.; Chien, S. C.; Ho, C. L.; Wang, E. I.; Lee, S. C.; Kuo, Y. H.; Jeyashoke, N.; Chen, J.; Dong, W. C.; Chao, L. K.; Hua, K. F. Osthole regulates inflammatory mediator expression through modulating NF-κB, mitogen-activated protein kinases, protein kinase C, and reactive oxygen species. *J. Agric. Food Chem.* **2010**, *58*, 10445–10451.

(31) Eeva, M.; Rauha, J.-P.; Vuorela, P.; Vuorela, H. Computer-assisted, high-performance liquid chromatography with mass spectrometric detection for the analysis of coumarins in *Peucedanum palustre* and *Angelica archangelica*. *Phytochem. Anal.* **2004**, *15*, 167–174.

(32) Zhang, H.; Gong, C.; Lv, L.; Xu, Y.; Zhao, L.; Zhu, Z.; Chai, Y.; Zhang, G. Rapid separation and identification of furocoumarins in *Angelica dahurica* by high-performance liquid chromatography with diode-array detection, time-of-flight mass spectrometry and quadrupole ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2167–2175.

(33) Chen, Y.; Fan, G.; Zhang, Q.; Wu, H.; Wu, Y. Fingerprint analysis of the fruits of *Cnidium monnieri* extract by high-performance liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *43*, 926–936.

(34) Gökay, O.; Kühner, D.; Los, M.; Götz, F.; Bertsche, U.; Albert, K. An efficient approach for the isolation, identification and evaluation of antimicrobial plant components on an analytical scale, demonstrated by the example of *Radix imperatoriae*. *Anal. Bioanal. Chem.* **2010**, *398*, 2039–2047.

3.6 Identification of ostruthin from *Peucedanum ostruthium* rhizomes as an inhibitor of vascular smooth muscle cell proliferation

Helge Joa,[†,§] Sylvia Vogl,[†,§] Atanas G. Atanasov,*[,†] Martin Zehl,[†] Thomas Nakel,[†] Nanang Fakhrudin,[†,⊥] Elke H. Heiss,[†] Paolo Picker,[†] Ernst Urban,[‡] Christoph Wawrosch,[†] Johannes Saukel,[†] Gottfried Reznicek,[†] Brigitte Kopp,[†] and Verena M. Dirsch[†]

[†] Department of Pharmacognosy, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

[‡] Department for Medicinal Chemistry, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

[§] These authors contributed equally to this work.

[⊥] Permanent address: Department of Pharmaceutical Biology, Gadjah Mada University, Sekip Utara, Yogyakarta, 55281, Indonesia

This manuscript was published in the Journal of Natural Products, 2011, 74(6), 1513-1516 (DOI: 10.1021/np200072a). All of the phytochemical work, except NMR measurements (Prof. Ernst Urban, Dept. of Pharmaceutical Chemistry, University of Vienna), as well as the MS analyses (Dr. Martin Zehl, Dept. of Pharmacognosy, University of Vienna) were done by the author of this thesis. All the pharmacological testing has been performed by the second first author Dr. Helge Joa from the Department of Pharmacognosy of the University of Vienna.

JOURNAL OF NATURAL PRODUCTS

NOTE

pubs.acs.org/jnp

Identification of Ostruthin from *Peucedanum ostruthium* Rhizomes as an Inhibitor of Vascular Smooth Muscle Cell Proliferation

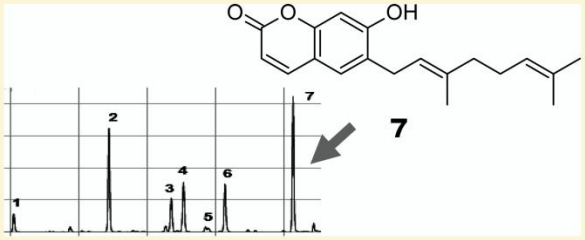
Helge Joa,[†,§] Sylvia Vogl,[†,§] Atanas G. Atanasov,[*,†] Martin Zehl,[†] Thomas Nakel,[†] Nanang Fakhrudin,[†,⊥] Elke H. Heiss,[†] Paolo Picker,[†] Ernst Urban,[‡] Christoph Wawrosch,[†] Johannes Saukel,[†] Gottfried Reznicek,[†] Brigitte Kopp,[†] and Verena M. Dirsch[†]

[†]Department of Pharmacognosy, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

[‡]Department for Medicinal Chemistry, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

Supporting Information

ABSTRACT: Inhibition of vascular smooth muscle cell (VSMC) proliferation is of substantial interest in combating cardiovascular disease. A dichloromethane extract from the rhizomes of *Peucedanum ostruthium*, a traditionally used Austrian medicinal plant with anti-inflammatory properties, was examined for a putative antiproliferative activity in rat aortic VSMC. This extract inhibited serum (10%)-induced VSMC proliferation concentration dependently. Further identification and biological testing of its major constituents revealed that the coumarin ostruthin (7) is the major antiproliferative substance. In summary, a new bioactivity of *P. ostruthium* rhizomes is described, and 7 has been identified as the responsible compound.



Coronary artery disease induced by atherosclerosis is one of the major causes of death in the Western world.[1] One major pathological change associated with atherosclerosis is thickening of the arterial wall and progressive narrowing of the arterial lumen, leading to insufficient blood flow. Proliferation of vascular smooth muscle cells (VSMC) in the intima layer of the arterial wall is one of the major components of the narrowing process, besides ongoing inflammatory processes.[2] There are already several pharmaceuticals used to combat pathological VSMC proliferation, but their action is not optimal due to long-term side effects. Therefore, there is still a need to identify new active compounds.[3,4]

Natural products represent an excellent resource for the identification of new lead structures.[5] Austria and its adjacent regions have a rich history in traditional folk medicine. In order to conserve this ethnopharmacological knowledge, the VOLKSMED database was created.[6] Since inflammation is associated with very diverse pathological conditions, including atherosclerosis, plants used traditionally to treat inflammatory conditions were retrieved from the VOLKSMED database. In an ongoing screening program, extracts of these plants were tested for their ability to inhibit VSMC proliferation. This bioactivity screening procedure revealed an activity of the dichloromethane (CH_2Cl_2) extract from *Peucedanum ostruthium* (L.) WDJ Koch (Apiaceae) rhizomes. *Peucedanum* species, especially the rhizomes of *P. ostruthium*, have a long history in Austrian traditional medicine for the treatment of inflammatory diseases. Furthermore, an anti-inflammatory action of a *P. ostruthium* root extract has been demonstrated in vivo on oral administration to rats with carrageenan-induced rat paw edema.[7]

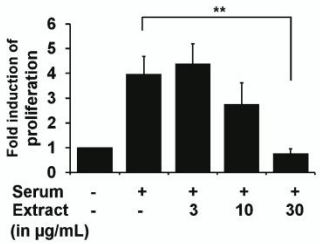


Figure 1. *Peucedanum ostruthium* rhizome CH_2Cl_2 extract inhibits the proliferation of VSMC. The extract was applied at 3, 10, and 30 μg/mL, and after 30 min the cells were stimulated with 10% serum for 48 h, followed by quantification of the proliferation by the resazurin conversion method. Final DMSO concentration in all samples was 0.1%. All values were normalized to the signal obtained from the unstimulated, vehicle (0.1% DMSO)-treated cells and therefore represent the fold activation of proliferation above the basal level. The graph is representative for three independent experiments with consistent results (mean ± SD), $^{**}p < 0.01$ (ANOVA/Tukey).

To test whether the CH_2Cl_2 extract of *P. ostruthium* rhizomes concentration dependently inhibits serum (10%)-induced proliferation of rat aortic VSMC, this extract was applied at concentrations of 3 to 30 μg/mL (Figure 1), and resazurin conversion after 48 h was measured.[8] Serum-stimulated VSMC were taken as positive control and serum-starved cells as negative control. Data quantification revealed that the CH_2Cl_2 extract of

Received: January 25, 2011
Published: May 31, 2011

ACS Publications Copyright © 2011 American Chemical Society and American Society of Pharmacognosy

Journal of Natural Products NOTE

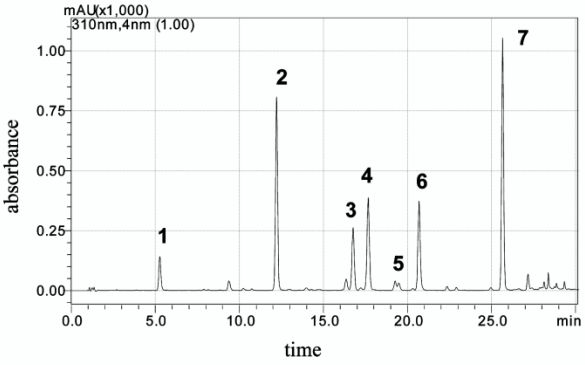
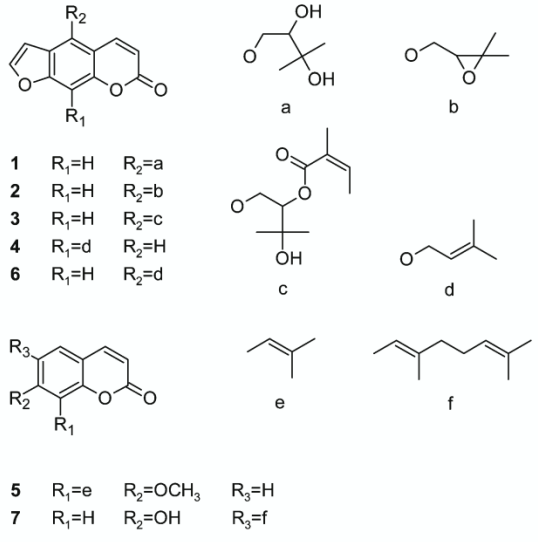


Figure 2. HPLC chromatogram (310 nm) of the CH_2Cl_2 extract of *Peucedanum ostruthium* rhizome. Peak identities: **1**, oxypeucedanin hydrate; **2**, oxypeucedanin; **3**, ostruthol; **4**, imperatorin; **5**, osthole; **6**, isoimperatorin; **7**, ostruthin.

P. ostruthium rhizomes inhibits the proliferation of VSMC with an IC_{50} value of 24 ± 14 μg/mL.

It has been reported previously that coumarins are major bioactive constituents of extracts from *P. ostruthium* rhizomes.[7,9] Furthermore, in a parallel study, we have performed a comprehensive qualitative and quantitative analysis of the main coumarins in extracts from *P. ostruthium* rhizomes.[10] High-performance liquid chromatography−diode-array detection−mass spectrometry (HPLC-DAD-MS) profiling of the CH_2Cl_2 extract investigated in this study (Figure 2) led to the identification of seven coumarins as major constituents (**1**−**7**). The total coumarin content in this CH_2Cl_2 extract was determined to be 78%, whereas the content per gram dry weight of plant material was 6%. Ostruthin (**7**) was quantified as the main coumarin (41% of the total coumarin content), followed by oxypeucedanin (**2**, 18%), ostruthol (**3**, 13%), imperatorin (**4**, 12%), isoimperatorin (**6**, 9%), oxypeucedanin hydrate (**1**, 6%), and osthole (**5**, 1%).



1 R_1=H R_2=a
2 R_1=H R_2=b
3 R_1=H R_2=c
4 R_1=d R_2=H
6 R_1=H R_2=d

5 R_1=e R_2=OCH_3 R_3=H
7 R_1=H R_2=OH R_3=f

After the identification of the seven major coumarin constituents of the CH_2Cl_2 extract of *P. ostruthium* rhizomes, their individual antiproliferative activities in serum-stimulated VSMC were investigated. Compounds **1**, **2**, and **4**−**6** were obtained from commercial sources. Ostruthol (**3**) and ostruthin (**7**) were isolated by preparative HPLC, the identity was confirmed by mass spectrometry and 1D and 2D NMR spectroscopy, and their purity was shown to be >98% by HPLC-DAD, HPLC-ELSD, and NMR. Compounds **1**−**7** were all applied at 30 μM, but only **7** exhibited significant antiproliferative activity (Figure 3). Since **7** was the main compound in the extract (Figure 2), as well as the most active among the seven major constituents (Figure 3), it is concluded that **7** is the major active compound in the CH_2Cl_2 extract of *P. ostruthium* rhizomes inhibiting VSMC proliferation. Furthermore, **7** constitutes around 32% of the total extract. The IC_{50} value determined for the CH_2Cl_2 extract, i.e., 24 μg/mL, corresponds to about 26 μM of **7**. Since **7** inhibited VSMC proliferation with an IC_{50} of 11 ± 2 μM, its activity can entirely explain the inhibitory properties of the plant crude extract.

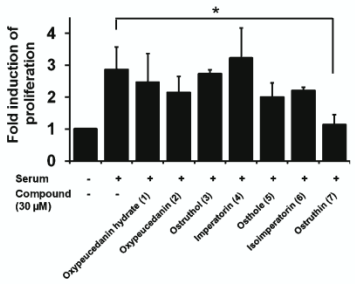


Figure 3. Ostruthin (**7**) is the active compound of the *Peucedanum ostruthium* CH_2Cl_2 extract. The major compounds of this extract, oxypeucedanin hydrate (**1**), oxypeucedanin (**2**), ostruthol (**3**), imperatorin (**4**), osthole (**5**), isoimperatorin (**6**), and ostruthin (**7**), were tested for an antiproliferative action on VSMC utilizing the resazurin conversion method. All compounds were tested in a concentration of 30 μM. Ostruthin (**7**) was the only compound significantly inhibiting serum-stimulated VSMC proliferation (mean ± SD, n = 3, $^*p < 0.05$; ANOVA/Tukey).

The resazurin assay method used is based on the metabolic conversion of resazurin to the fluorescent resorufin, which correlates to the cell number. To validate the potential of **7** to inhibit VSMC proliferation, the rate of de novo DNA synthesis via BrdU incorporation was measured (Figure 4). As expected, ostruthin concentration dependently inhibited the serum-induced DNA synthesis, with an IC_{50} of 17 ± 6 μM. Furthermore, the IC_{50} value at which the CH_2Cl_2 extract inhibited DNA synthesis was 18 ± 4 μg/mL. Since 18 μg/mL extract solution corresponds to 19 μM ostruthin, the DNA synthesis inhibitory properties of the plant crude extract can also be entirely explained by that single chemical constituent. No significant differences in the cell viability assessed by trypan blue exclusion test was detected in the presence of ostruthin in the used concentration range, with a cell viability of 98 ± 2% and 92 ± 6% for the untreated and ostruthin (30 μM)-treated cells, respectively (mean ± SD, n = 4, $p > 0.05$, Student's t test).

An antiproliferative action of **7** in VSMC has not been reported before. However, one study examined an antiproliferative action (IC_{50} of 13.6 ± 1.8 μM) of the structurally similar osthole (**5**) isolated from *Angelica pubescens* in the smooth muscle cell line A10.[11] In contrast, when applied at 30 μM we did not observe any significant inhibitory action by osthole in our model system utilizing primary rat aorta VSMC (Figure 3). Since the structurally similar linear furanocoumarins (Figure 3) also do not have an effect on the proliferation of VSMC, it was concluded

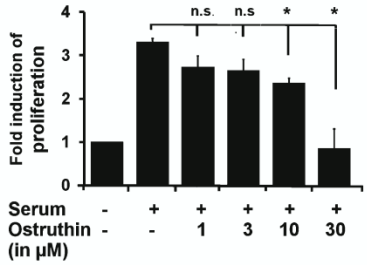


Figure 4. Ostruthin (7) inhibits serum-stimulated proliferation of VSMC in a concentration-dependent manner. Serum-starved VSMC were pretreated with the indicated concentrations of **7** or DMSO (0.1%) for 30 min, BrdU (10 μM) was added, and cells were stimulated with 10% serum for 20 h. Incorporation of BrdU was measured at the end of the stimulation period. All values were normalized to the signal obtained from the unstimulated, vehicle (0.1% DMSO)-treated cells and therefore represent the fold activation of proliferation above the basal level (SEM, $n = 3$, $^*p < 0.05$; ANOVA/Tukey).

that the geranyl group at the C-6 position may be necessary for the inhibitory effect. To validate this specific effect of the geranyl group, the inhibitory potential for several further coumarins and furanocoumarins was tested, namely, auraptene, bergamottin, bergapten, bergaptol, heraclenin, isobergapten, isopimpinellin, scopoletin, and umbelliferone (Figures S1 and S2, Supporting Information), which all share some structural similarity to **7**, but lack the C-6 geranyl group. Indeed, none of these compounds significantly inhibited VSMC proliferation at a concentration of 30 μM, underlining the specific action of **7**, among the compounds tested.

EXPERIMENTAL SECTION

Reagents. Cell culture reagents including Dulbecco's modified Eagle's medium (DMEM) without phenol red containing 4.5 g/L glucose, glutamine, calf serum, penicillin, and streptomycin were obtained from Lonza Group Ltd. (Basel, Switzerland). Trypsin was purchased from Invitrogen (Carlsbad, CA), and DMSO was from Fluka (Buchs, Switzerland). Acetonitrile, dichloromethane, ethyl acetate, hexane, and methanol (MeOH) were HPLC grade and purchased from VWR (Vienna, Austria). Glacial acetic acid was obtained from Carl Roth (Karlsruhe, Germany). Imperatorin (**4**), isoimperatorin (**6**), oxypeucedanin (**2**), and oxypeucedanin hydrate (**1**) were all of 99% purity and purchased from Herboreal Ltd. (Edinburgh, U.K.). Osthole (**5**) with a purity of 98.9% was from Chromadex (Irvine, CA). Water was distilled by an automatic water distillation apparatus (IKA Dest. M3000). All other reagents were from Carl Roth (Karlsruhe, Germany), if not stated otherwise.

Cell Culture. VSMC from thoracic aortas of Sprague−Dawley rats were kindly provided by Dr. Kathy K. Griendling (Emory University, Atlanta, GA). VSMC were grown and seeded at the indicated density in DMEM containing glutamine, penicillin/streptomycin, and 10% calf serum. Basic cell parameters were checked routinely using a cell viability analyzer (ViCell, Beckman Coulter, Brea, CA), and cell passages 5 to 15 were used for the experiments.

Plant Material. *P. ostruthium* rhizomes were purchased from Kottas Pharma GmbH, Vienna, Austria (collected in 2008, batch: KLA70807). The species identity of the plant material was authenticated macroscopically and microscopically by one of the authors (J.S.), and chemical fingerprinting was done by TLC and HPLC-DAD-MS. A voucher specimen is deposited at the Department of Pharmacognosy, University of Vienna, Austria (number: Po001).

Extraction and Isolation of Ostruthol (3) and Ostruthin (7). The dried and powdered roots of *P. ostruthium* (367.5 g) were extracted with CH_2Cl_2 and subsequently with MeOH using an ASE200 accelerated solvent extractor (Dionex Corp., Sunnyvale, CA). The instrument was equipped with 22 mL stainless steel extraction cells and 60 mL glass collection bottles. Three extraction cycles with 5 min heat-up time, 2 min static time, 10% flush volume, and 60 s nitrogen purge were used. Extraction was done at 40 °C and 150 bar. We obtained 37.8 g of CH_2Cl_2 extract and 81.2 g from the MeOH extract. From the MeOH extract, tannins were depleted using a method published by Wall et al.[12] A 2.3 g amount of the detannified MeOH extract was then further fractionated by solid-phase extraction on a 60 cm^3 Bond Elut C_{18} cartridge (Varian, Harbor City, CA) eluted with solutions of 30%, 70%, and 100% MeOH. A 181 mg amount of the 30% MeOH subfraction, 925 mg of the 70% MeOH subfraction, and 716 mg of the 100% MeOH SPE subfraction were obtained. A 420 mg aliquot of the 70% MeOH subfraction was separated by preparative HPLC using an instrument from Shimadzu (LC-8A) with an autosampler (SIL-10AP), a diode array detector (SPD-M20A9), and a fraction collector (FRC-10A). Chromatographic separation was obtained at room temperature on a VP 250/21 Nucleosil 100-7 C_{18} column at a flow rate of 20.8 mL/min. The solvent system consisted of H_2O (A) and acetonitrile (B) using gradient elution starting with 15% B for 3 min, in 2 min to 20% B, in 10 min to 30% B, in 3 min to 45% B, in 7 min to 50% B, in 10 min to 70% B, in 3 min to 85% B, and in 5 min to 95% B. Six runs with an average load of 70 mg of extract were performed, leading to a total of 420 mg. Fractions were collected every 30 s starting after 20 min, obtaining nine subfractions. Subfraction 6 (37 mg) contained mainly **3** and low amounts of peucenin. An 18 mg portion of this fraction was purified on silica gel SPE cartridges using hexane and ethyl acetate (8:2), resulting in 3.8 mg of peucenin. Elution with hexane and ethyl acetate (3:1) yielded 12 mg of **3** (purity >98%, confirmed by HPLC-ELSD and NMR). Subfraction 9 contained pure **7** (30 mg; purity >98%, confirmed by HPLC-ELSD and NMR). The identification of **3** and **7** was confirmed by 1D and 2D NMR spectroscopy, ESIMS, and comparison of the ^1H and ^{13}C NMR data with literature values.[10,13−15]

Identification and Quantification of Coumarins by High-Performance Liquid Chromatography−Diode Array Detector (HPLC-DAD) and HPLC-DAD−Mass Spectrometry. Comprehensive qualitative and quantitative analysis of the main coumarins in extracts from *P. ostruthium* rhizomes was recently described elsewhere.[10] Briefly, analytical HPLC separation was performed on an Acclaim C_{18} reversed-phase column from Dionex (2.1 × 150 mm, 3 μm) at 38 °C and a flow rate of 0.5 mL/min with a water (A) and acetonitrile (B) gradient containing 0.01% acetic acid. The gradient was 25−37% B in 6 min, 37−45% B in 8 min, 45−65% B in 10 min, 65−95% B in 1 min, and isocratic at 95% B for 5 min. Tentative identification of the main coumarins was facilitated by HPLC-DAD-MS in an automated data-dependent acquisition mode. These 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 ESI source (HCT, Bruker Daltonics, Bremen, Germany). The identity of **1**, **2**, and **4**−**6** was confirmed by comparison of their retention times and UV spectra with authentic references.[10] The main coumarins were quantified by a validated HPLC-DAD method at a detection wavelength of 310 nm with imperatorin as external standard. A Shimadzu Prominence HPLC system was used for these analyses.

Resazurin Conversion Assay. The assay is based on the metabolic conversion of resazurin to fluorescent resorufin, which correlates with cell number.[8] Cells were seeded in transparent 96-well plates (1×10^4 cells/well), serum-starved for 24 h, pretreated with the indicated concentrations of the respective samples or vehicle for 30 min, and subsequently stimulated for 48 h with calf serum (10%). Then, cells were washed with PBS and incubated in DMEM containing 10 μg/mL resazurin for 2 h. Samples were measured by monitoring the increase

in fluorescence at a wavelength of 580 nm using an excitation wavelength of 535 nm in a 96-well plate reader (Tecan GENios Pro; Tecan Group Ltd., Männedorf, Switzerland).

5-Bromo-2′-deoxyuridine Incorporation. As a measurement of DNA synthesis, 5-bromo-2′-deoxyuridine (BrdU) incorporation was determined using a cell proliferation kit (Roche Diagnostics) as previously described.[16] Briefly, VSMC were seeded into black (transparent flat bottom) 96-well plates (1×10^4 cells/well), serum-starved for 24 h, pretreated with test samples or vehicle (0.1% final concentration of DMSO in all samples) for 30 min, and subsequently stimulated with calf serum (10%). After 30 min, BrdU (10 μM) was added, and the cells were cultivated for another 20 h. Afterward, cells were fixed and stained with an enzyme-coupled anti-BrdU antibody according to the manufacturer's instructions. Relative light units were determined using a 96-well plate reader (Tecan GENios Pro; Tecan Group Ltd., Männedorf, Switzerland).

Trypan Blue Exclusion Test. The trypan blue viability test is based on the principle that living cells possess intact cell membranes that are not permeable to the trypan blue dye, whereas dead cells have disrupted membrane integrity and in the presence of the dye are colored blue. To assess the cell viability, VSMC were seeded in six-well plates (1×10^5 cells/well), serum starved for 24 h, pretreated with ostruthin for 30 min, and subsequently stimulated with calf serum (10%). The viability of the cells was assessed 24 h later by trypan blue exclusion utilizing an automated cell viability analyzer (ViCell, Beckman Coulter, Brea, CA).

Statistical Analysis. Data were evaluated as means ± standard deviation from at least three independent experiments. IC_{50} values were determined by nonlinear regression using Data Analysis Toolbox software (MDL Information Systems Inc., Nashville, TN). Statistical significance was determined by ANOVA using Tukey post hoc test, and the results with $p < 0.05$ were considered significant.

■ ASSOCIATED CONTENT

Supporting Information. Results regarding the antiproliferative activity of additional structurally related coumarins and furanocoumarins are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +43-1-4277-55226. Fax: +43-1-4277-9552. E-mail: atanas.atanasov@univie.ac.at.

Notes

[⊥]Permanent address: Department of Pharmaceutical Biology, Gadjah Mada University, Sekip Utara, Yogyakarta, 55281, Indonesia.

Author Contributions

[§]These authors contributed equally to this work.

■ ACKNOWLEDGMENT

We thank J. Benedics, H. Beres, and D. Schachner for their excellent technical assistance. This work was supported by the Austrian Science Fund (FWF) [Grants NFN S10704-B03, NFN S10706-B03, and NFN S10708-B03].

■ REFERENCES

(1) Rader, D. J.; Daugherty, A. *Nature* **2008**, *451*, 904–913.
(2) Ivey, M. E.; Osman, N.; Little, P. J. *Atherosclerosis* **2008**, *199*, 237–247.
(3) Dzau, V. J.; Braun-Dullaeus, R. C.; Sedding, D. G. *Nat. Med.* **2002**, *8*, 1249–1256.
(4) Raja, S. G. *Ann. Thorac. Surg.* **2006**, *81*, 1162–1171.
(5) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461–477.
(6) Saukel, J.; Kubelka, W. *Sci. Pharm.* **1993**, *62*, 100.
(7) Hiermann, A.; Schantl, D. *Planta Med.* **1998**, *64*, 400–403.
(8) Ahmed, S. A.; Gogal, R. M., Jr.; Walsh, J. E. *J. Immunol. Methods* **1994**, *170*, 211–224.
(9) Hiermann, A.; Schantl, D.; Schubert-Zsilavecz, M.; Reiner, J. *Phytochemistry* **1996**, *43*, 881–883.
(10) Vogl, S.; Zehl, M.; Picker, P.; Urban, E.; Wawrosch, C.; Reznicek, G.; Saukel, J.; Kopp, B. *J. Agric. Food. Chem.* **2011**, *59*, 4371–4377.
(11) Guh, J. H.; Yu, S. M.; Ko, F. N.; Wu, T. S.; Teng, C. M. *Eur. J. Pharmacol.* **1996**, *298*, 191–197.
(12) 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.; Farnsworth, N. R.; Cordell, G. A.; Kinghorn, A. D. *Phytomedicine* **1996**, *3*, 281–285.
(13) Liu, R.; Sun, Q.; Shi, Y.; Kong, L. *J. Chromatogr. A* **2005**, *1076*, 127–132.
(14) Khaled, S. A.; Szendrei, K.; Novak, I.; Reisch, J. *Phytochemistry* **1975**, *14*, 1461–1462.
(15) Perel'son, M.; Sheinker, Y.; Syrova, G. *Chem. Nat. Compd.* **1971**, *7*, 557–562.
(16) Schwaiberger, A. V.; Heiss, E. H.; Cabaravdic, M.; Oberan, T.; Zaujec, J.; Schachner, D.; Uhrin, P.; Atanasov, A. G.; Breuss, J. M.; Binder, B. R.; Dirsch, V. M. *Arterioscler. Thromb. Vasc. Biol.* **2010**, *30*, 2475–2481.

3.7 Bioactivity guided isolation of active compounds from *Melampyrum pratense*

Referring to the results of the group from the Medical University of Vienna the methanolic (MeOH) as well as the dichloromethane (DCM) extract of *Melampyrum pratense* showed strong downregulation of TNF-α and LPS induced production of E-selectin and IL-8 mRNA (Tab. 30).

Rank	Plant species	DCM extract				MeOH extract				Pool
		TNF induced		**LPS induced**		**TNF induced**		**LPS induced**		
		E-sel	**IL-8**	**E-sel**	**IL-8**	**E-sel**	**IL-8**	**E-sel**	**IL-8**	
1	*Sanicula europea*	++	++	+++	+++	+++	++	-	+++	6
2	***Melampyrum pratense***	**+++**	**+++**	**nd**	**++**	**+++**	**++**	**+++**	**+(+)**	**5**
3	*Agropyron repens*	+++	++	++	+++	++	+(+)	-	++	6
4	*Filipendula vulgaris*	+++	++	nd	++	+++	++	+++	-	5
5	*Veronica chamaedrys*	++	++	++	+++	++(+)	-	-	+++	6
6	*Angelica sylvestris*	+++	+(+)	++	+++	++	-	-	+++	6
7	*Capsella bursa pastoris*	+++	(+)	++	+++	+(+)	+	-	+++	6
8	*Symphytum officinalis*	++	-	+++	+++	++(+)	-	-	+++	6
9	*Ajuga genevensis*	+++	++	nd	++	+++	-	+++	-	5
10	*Petasites hybridus*	+++	++	+(+)	++	nd	++(+)	-	++	7
11	*Veronica officinalis*	+++	+	++	+++	+++	-	-	-	6
12	*Betonica officinalis*	+++	+++	nd	-	+++	-	+++	-	5
13	*Sambucus ebulus*	nd	+	+(+)	-	+++	+++	+	++	7
14	*Rumex alpinus*	++(+)	+	++	-	nd	+++	-	++	7
15	*Malva neglecta*	+++	-	+	+++	-	-	-	+++	6

Tab. 30 Ranking DCM and MeOH extracts of the 15 most promising plant species. Extracts were tested in triplicates at a concentration of 10 µg/mL concerning LPS or TNF-α induced downregulation of interleukine-8 (IL-8) and E-selectin (E-sel.) mRNA**;** < 25 % inhibition/ activation is considered as no activity (-), 25-50 % inhibition/ activation interpreted as low activity (+), 50-75 % inhibition/ activation is considered as moderate activity (++) and high effect is defined as > 75-100 % inhibition/ activation (+++), nd means not determined. Plant chosen for further bioassay guided isolation is highlighted in grey.

Further tests of the crude and the purified extracts on LPS or TNF-α induced down-regulation of IL-8 and E-selectin mRNA, as well as on PPARs activation and on NF-κB inhibition showed equal or even better results for chlorophyll freed and detannified crude extracts (Tab. 32, p. 167). Consequently, further fractionation of these extracts was performed using solid phase extraction (**SPE system 1**, chapter 2.7, p. 39). Extractions yields are represented below in Tab. 31.

crude drug [g]	**82.1**		
sample ID	**extract**	**% of starting material**	**% of crude drug**
Mela DCM	1.63 g	**2.0**	**2.0**
Mela MeOH	12.73 g	**15.5**	**15.5**
removal of chlorophylls			
starting DCM extract [mg]	**1500**		
Mela OC	263 mg	**17.5**	**0.3**
Mela Chloro	1203 mg	**80.2**	**1.5**
Recovery	**1466 mg**	**97.8**	
tannin separation			
starting MeOH extract [mg]	**12000**		
Mela Det	583 mg	**4.9**	**0.7**
Mela Hex	628 mg	**5.2**	**0.8**
Mela MeOH/H_2O	8850 mg	**73.8**	**10.8**
Mela Nacl	nd.	**nd.**	
Recovery	**10061 mg**	**83.8**	
SPE			
starting OC extract [mg]	**260**		
Mela OC 30	93 mg	**36.0**	**0.11**
Mela OC 70	86 mg	**33.0**	**0.10**
Mela OC 100	53 mg	**20.5**	**0.07**
Recovery	**232 mg**	**89.5**	**0.28**
starting MeOH extract [mg]	**291**		
Mela Det 30	31 mg	**10.8**	**0.04**
Mela Det 70	59 mg	**20.1**	**0.07**
Mela Det 100	186 mg	**64.0**	**0.23**
Recovery	**276 mg**	**94.9**	

Tab. 31 Extraction yields of the ASE extraction of the crude drug, chlorophyll removal, tannin separation and solid phase extraction (SPE) of *M. pratense* (Mela). DCM - dichloromethane extract, OC - DCM extract without chlorophyll, MeOH - methanol extract, Det - detannified MeOH extract , Hex – n-hexane phase, 30 - SPE with 30 % MeOH, 70 - SPE with 70 % MeOH, 100 - SPE with 100 % MeOH, nd. – not determined. Portions on the starting material and the initial crude drug are marked blue.

Plant material	Sample ID	Preparation	PPAR-α activ.	PPAR-γ activ.	NF-κB inhib.	E-sel. +TNF	IL-8 +TNF	E-sel. +LPS	IL-8 +LPS
Melampyrum pratense herb	Mela DCM	DCM extract	-	-	-	+++	+	++	+++
	Mela OC	chlorophyll freed DCM extract	-	-	-	++	+++	++	+++
	Mela MeOH	MeOH extract	-	-	-	+++	+++	+++	+++
	Mela Det	detannified MeOH extract	-	-	+++	+++	+++	++	+++

Tab. 32 Results of the initial screening of *M. pratense* extracts on various anti-inflammatory targets. Extracts were tested in triplicates at a concentration of 10 µg/mL on PPARs activation (activ.) as well as on NF-κB inhibition (inhib.) and LPS or TNF-α induced downregulation of interleukine-8 (IL-8) and E-selectin (E-sel.) mRNA**;** < 25 % inhibition/ activation is considered as no activity (-), 25-50 % inhibition/ activation is interpreted as low activity (+), 50-75 % inhibition/ activation is considered as moderate activity (++) and high effect is defined as > 75-100 % inhibition/ activation (+++).

The gained SPE subfractions were further investigated for their anti-inflammatory activity in the respective *in vitro* assays. Regarding LPS or TNF-α induced downregulation of IL-8 and E-selectin mRNA highest activities were found in the 70 % SPE fraction of the chlorophyll free DCM extract (Tab. 33, p. 168) as well as in the 30 % and 70 % SPE subfraction of the detannified MeOH extract (Tab. 33, p. 168).

Investigations on PPARs activation and NF-κB inhibition showed highest activities in the 30 % and 70 % SPE fractions of the DCM extract and in the 70 % SPE subfraction of the detannified MeOH extract (Tab. 33, p. 168). The promising results of the 100 % SPE fractions of the chlorophyll free DCM extract were expected to be induced by fatty acids concentrated in those fractions and identified by TLC using reference compounds (TLC system for identification of fatty acids, Tab. 11, p. 41).

TLC investigations, described in chapter 3.7.1, as well as HPLC-ELSD/DAD/MS analysis were done to give more information on the composition of the extracts and SPE subfractions.

Initial extract	Sample ID	Preparation	PPAR-α activ.	PPAR-γ activ.	NF-κB inhib.	E-sel. +TNF	IL-8 +TNF	E-sel. +LPS	IL-8 +LPS
chlorophyll freed DCM extract	Mela OC 30	SPE with 30 % MeOH	-	+	+	++	-	++	-
	Mela OC 70	SPE with 70 % MeOH	+++	+++	+++	+++	-	+++	+++
	Mela OC 100	SPE with 100 % MeOH	+++	+++	+++	-	-	++	++

Initial extract	Sample ID	Preparation	PPAR-α activ.	PPAR-γ activ.	NF-κB inhib.	E-sel. +TNF	IL-8 +TNF	E-sel. +LPS	IL-8 +LPS
detannified MeOH extract	Mela Det 30	SPE with 30 % MeOH	-	+	+++	++	-	++	+++
	Mela Det 70	SPE with 70 % MeOH	+++	+++	+++	+++	-	++	++
	Mela Det 100	SPE with 100 % MeOH	-	+	+	-	-	+++	+++

Tab. 33 Results of the SPE subfractions of *M. pratense* chlorophyll separated DCM extract (above) detannified MeOH extract (below).

Fractions were tested in triplicates at a concentration of 10 µg/mL on PPARs activation (activ.) as well as on NF-κB inhibition (inhib.) and LPS or TNF-α induced downregulation of interleukine-8 (IL-8) and E-selectin (E-sel.) mRNA; < 25 % inhibition/ activation is considered as no activity (-), 25-50 % inhibition/ activation is interpreted as low activity (+), 50-75 % inhibition/ activation is considered as moderate activity (++) and high effect is defined as > 75-100 % inhibition/ activation (+++).

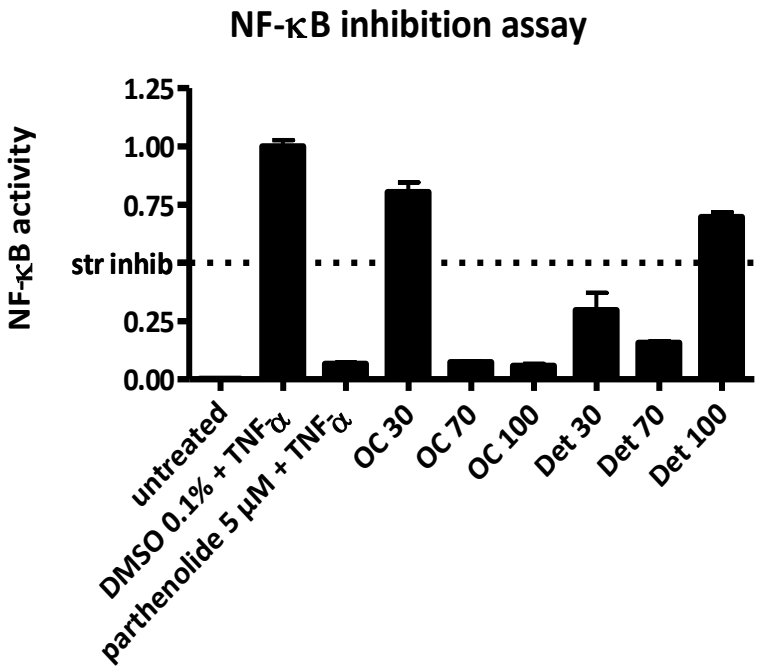


Fig. 39 NF-κB inhibition assay with SPE subfractions of *M. pratense*. Extracts were tested in triplicates at a concentration of 10 µg/mL. Bars represent mean values, error bars consider SEM, extracts below the dotted line are considered as strong inhibitors (str inhib).

3.7.1 TLC investigation of *Melampyrum pratense* extracts and fractions

In order to get a first impression of the composition of the extracts and derived SPE subfractions, different TLC systems, described in chapter 2.8.1, pp. 41-42, were carried out.

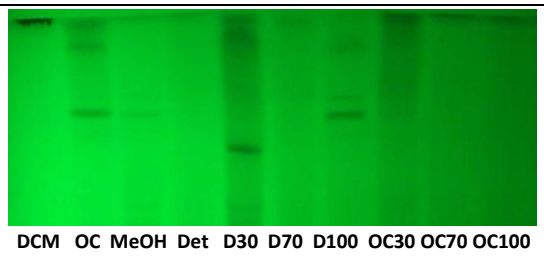
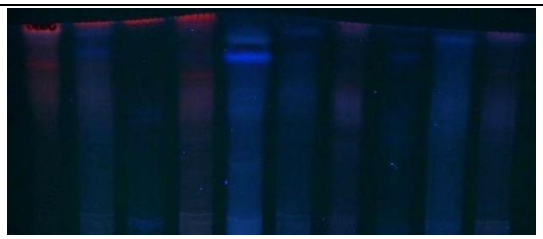
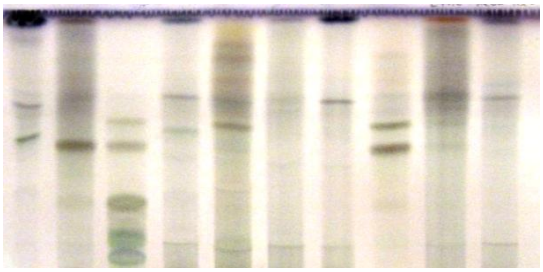
Both mobile phases of the **TLC system for iridoids and flavonoids** turned out to be suitable for comparison of all extracts and SPE fractions (Fig. 40, p. 170).

Mobile phase 1 was better suited for the methanolic fractions, whereas the more apolar mobile phase 2 gave better separation of the crude DCM extract and its SPE subfractions.

The **TLC system for separation of essential oil components** (TLC not shown) was too apolar for the investigated extracts and fractions.

The **TLC System for iridoids** on the other hand turned out to be too polar for the DCM extract without chlorophyll but was equally suitable for the methanolic fractions as TLC system 1 and mobile phase 1. Nonetheless, after spraying with AS some iridoid glycosides could be detected as grey, blue or violet zones in the upper front (TLC not shown).

In the methanolic crude extract, especially in the detannified fraction and its SPE subfractions the **TLC system for flavonoids** revealed the presence of flavonoids as yellow fluorescent bands after spraying with NPPR under UV_{366}.

Detection	***mobile Phase 1***
UV_{254}	
UV_{366}	
VIS - VSR	

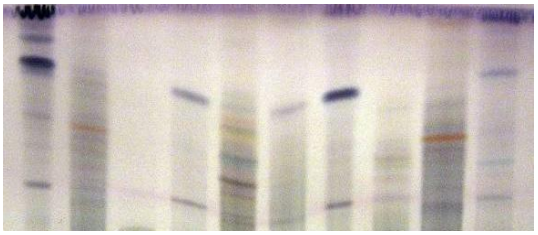
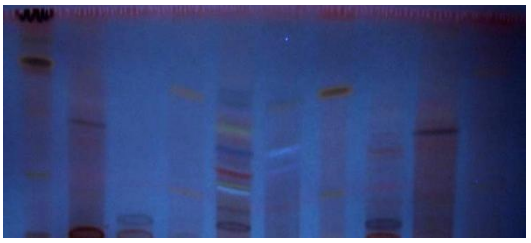
	mobile Phase 2
VIS - ASR	
UV_{366} - ASR	

Fig. 40 TLC systems used for *M. pratense* crude extracts and their SPE subfractions. VSR - vanillin sulfuric acid reagent, ASR - anisaldehyde sulfuric acid reagent, DCM - dichloromethane extract, OC - dichloromethane extract without chlorophyll, MeOH - methanol extract, Det/D - detannified methanol extract, D30 - SPE with 30 % MeOH, D70 - SPE with 70 % MeoH, D100 - SPE with 100 % MeoH

3.7.2 Isolation and identification of bioactive compounds

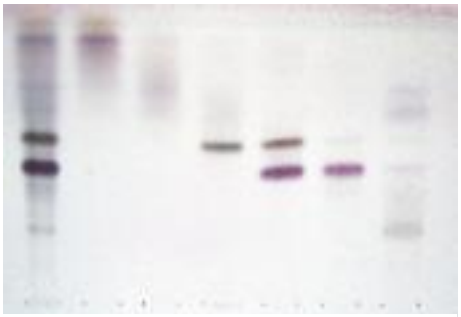
As TLC investigations showed mainly flavonoids in the methanolic fractions, isolation of active compounds was performed with the SPE subfractions of chlorophyll freed DCM extract. Mobile phase 1 of the TLC system for iridoids and flavonoids (Tab. 11, p. 42) was the basis for further separation of the more polar 30 % MeOH/H_2O SPE fraction of the chlorophyll separated DCM extract. Therefore, it was subjected to column chromatography on silica gel using **CC system 2** as described in Tab. 12, p. 43. Fractions were investigated using the initial TLC system with mobile phase 1 (Fig. 41, p. 172) and combined to 6 main fractions (amounts see Tab. 34). Combined fractions 51-75 (30/1) mainly contained a violet iridoid band, later identified with HPLC-DAD/-MS as aucubin, fractions 76-93 (30/2) showed impurities. Combined fractions 94-124 (30/3) revealed a prominent greyish green band later determined as melampyroside. Fractions 125-134 (30/4) contained melampyroside and a violet pinkish zone which was shown to be mussaenoside. This compound was enriched in fractions 135-184 (30/5). In the last combined fractions 185-240 (30/6) no compounds could be detected. Melampyroside and mussaenoside enriched in fraction 30/3 and 30/5, respectively were further purified by SPE using **SPE system 4** described in chapter 2.7, p. 39 and their structure was validated by with LC-MS and NMR (annex III).

Mela OC 30. silica gel column, EtOAC : MeOH : H_2O = 85 : 15 : 5			
crude drug [g]	**182.9**		
starting SPE extract [mg]	**178**		
sample ID	**extract [mg]**	**% of starting material**	**% of crude drug**
Mela 30/1	12.7	**7.0**	**0.007**
Mela 30/2	4.9	**3.0**	**0.003**
Mela 30/3 -Melampyroside	19.1	**10.7**	**0.010**
Mela 30/4 mix of Mussaenoside + Melampyroside	26.0	**14.6**	**0.014**
Mela 30/5 Mussaenoside	49.1	**27.5**	**0.027**
Mela 30/6	20.5	**11.5**	**0.011**
Mela wash	21.7	**12.2**	**0.012**
Recovery	**158.5**	**86.6**	

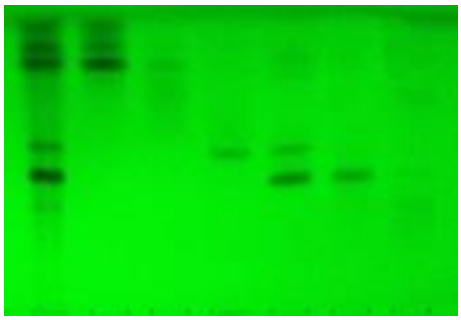
Tab. 34 Amounts of the respective column chromatography fractions from CC of *M. pratense* (Mela) 30 % SPE subfraction of chlorophyll free dichloromethane extract. Abbreviations see Fig. 41, p. 172. Portions on the starting material and the initial crude drug are marked blue.

TLC system for flavonoids and iridoids, mobile phase 1

Vanillin sulfuric acid reagent (VSR)



UV_{254}



Abbreviations

OC 30	30 % MeOH SPE fracdtion of DCM extract without chlorophyll
30/1	Combined fractions 51-75 of CC 2
30/2	Combined fractions 76-93 of CC 2
30/3	Combined fractions 94-124 of CC 2
30/4	Combined fractions 125-134 of CC 2
30/5	Combined fractions 135-184 of CC 2
30/6	Combined fractions 185-240 of CC 2

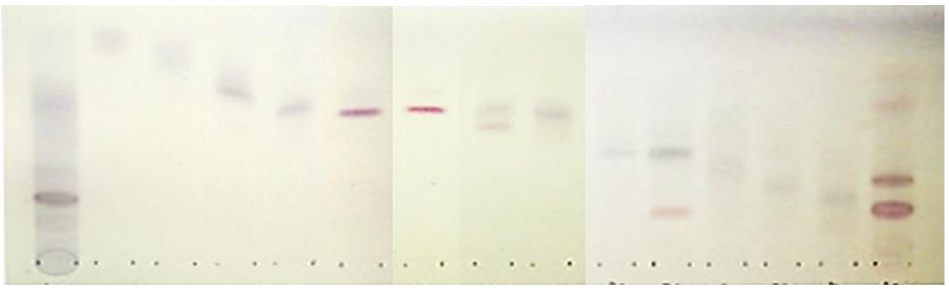
Fig. 41 TLC results of combined fractions from column chromatography of *M. pratense* 30 % MeOH SPE fraction of crude DCM extract without chlorophyll.

Mobile phase 2 of TLC system for iridoids and flavonoids (Tab. 11, p. 42) was successfully transferred to CC separation on silica gel of the 70 % MeOH/H_2O SPE fraction of the chlorophyll separated DCM extract using **CC system 3** as described in Tab. 12, p. 43. Fractions were investigated using the initial TLC system (Fig. 42, p. 173) with mobile phase 2 and combined to 13 main fractions (amounts see Tab. 35, p. 174). Combined fractions 90-130 (70/1), 131-155 (70/2) and 156-180 (70/3) showed light violet zones in the upper front of TLC system 3. In fractions 181-215 (70/4) a violet pinkish zone was detected after spraying with VS. After HPLC-ELSD/-DAD investigation this compound was identified as lunularin. Combined fractions 216-285 (70/5) as well as 286-320 (70/6) mainly consisted of a pinkish zone of lunularin (structure clarified via LC-MS and NMR, see annex III). Fractions 321-420 (70/7) and 421-675 (70/8) contained lunularin and another violet zone. Combined fractions 676-735 (70/9) and 736-865 (70/10) showed a weak zone of melampyroside.

The last three fractions combined fractions 866-1010 (70/11), 1011-1240 (70/12) and 1241-1440 (70/13) did not yield any detectable substances anymore.

TLC system for flavonoids and iridoids, mobile phase 2

Vanillin sulfuric acid reagent (VSR)



Abbreviations

OC 70	70 % MeOH SPE fracdtion of DCM extract without chlorophyll
70/1	Combined fractions 90-130 of CC 2
70/2	Combined fractions 131-155 of CC 2
70/3	Combined fractions 156-180 of CC 2 → after HPLC → impure Lunularin
70/4	Combined fractions 181-215 of CC 2 → after HPLC → impure Lunularin
70/5	Combined fractions 216-285 of CC 2 → Lunularin
70/6	Combined fractions 286-320 of CC 2 → Lunularin
70/7	Combined fractions 321-420 of CC 2
70/8	Combined fractions 421-675 of CC 2
70/9	Combined fractions 676-735 of CC 2
70/10	Combined fractions 736-865 of CC 2
70/11	Combined fractions 866-1010 of CC 2
70/12	Combined fractions 1011-1240 of CC 2
70/13	Combined fractions 1241-1440 of CC 2

Fig. 42 TLC results of combined fractions from column chromatography of *M. pratense* 70 % MeOH SPE fraction of crude DCM extract without chlorophyll.

Mela OC 70, silica gel column, $CHCl_3$: MeOH : H_2O = 90 : 3.5 : 0.2			
crude drug [g]	**24.38**		
starting SPE extract [mg]	**239**		
sample ID	**extract [mg]**	**% of strating material**	**% of crude drug**
90-130 Mela 70/1	1.9	**0.8**	**0.0008**
130-155 Mela 70/2	1.4	**0.6**	**0.0006**
156-180 Mela 70/3 impure Lunularin	3.9	**1.6**	**0.0016**
181-215 Mela 70/4 impure Lunularin	2.5	**1.1**	**0.0010**
216-285 Mela 70/5 pure Lunularin	23.4	**9.8**	**0.0096**
268-320 Mela 70/6 pure Lunularin	9.5	**4.0**	**0.0039**
321-420 Mela 70/7 Lunularin + impurities	27.0	**11.3**	**0.0111**
421-675 Mela 70/8	9.5	**4.0**	**0.0039**
676-735 Mela 70/9	17.4	**7.3**	**0.0071**
736-865 Mela 70/10	37.4	**15.7**	**0.0154**
866-1010 Mela 70/11	27.8	**11.6**	**0.0114**
1011-1240 Mela 70/12	16.3	**6.8**	**0.0067**
1241-1440 Mela 70/13 wash	21.1	**8.8**	**0.0087**
Recovery	199.2	**83.3**	

Tab. 35 Amounts of the respective column chromatography fractions from CC of *M. pratense* 70 % SPE subfraction of chlorophyll free dichloromethane extract. Abbreviations see **Fig. 42**. Portions on the starting material and the initial crude drug are marked blue.

After isolation of the main compounds an HPLC-DAD/ELSD system was developed for the identification of compounds in the crude extracts and SPE subfractions of *M. pratense* (Fig. 43 - Fig. 46, pp. 175-178).

Fig. 43, p. 175 shows that the chlorophyll freed DCM extract mainly contained mussaenoside, whereas mussaenoside and melampyroside were concentrated in the 30 % SPE subfraction. The main component of the 70 % SPE subfraction was lunularin. The identification was validated via comparison of retention times as well as UV chromatograms of the purified reference compounds (structure confirmed by NMR and LC-MS).

Besides mussaenoside and melampyroside, the methanolic crude extract contained several other iridoid glycosides, identified as aucubin, catalpol (shoulder of 2nd peak), loganin and mussaenosidic acid (Fig. 44, p. 175). Identification of those four compounds was done with commercial reference compounds via comparison of the UV-spectra (Fig. 45, pp. 160-177) and retention time as well as by LC-MS coupled detection.

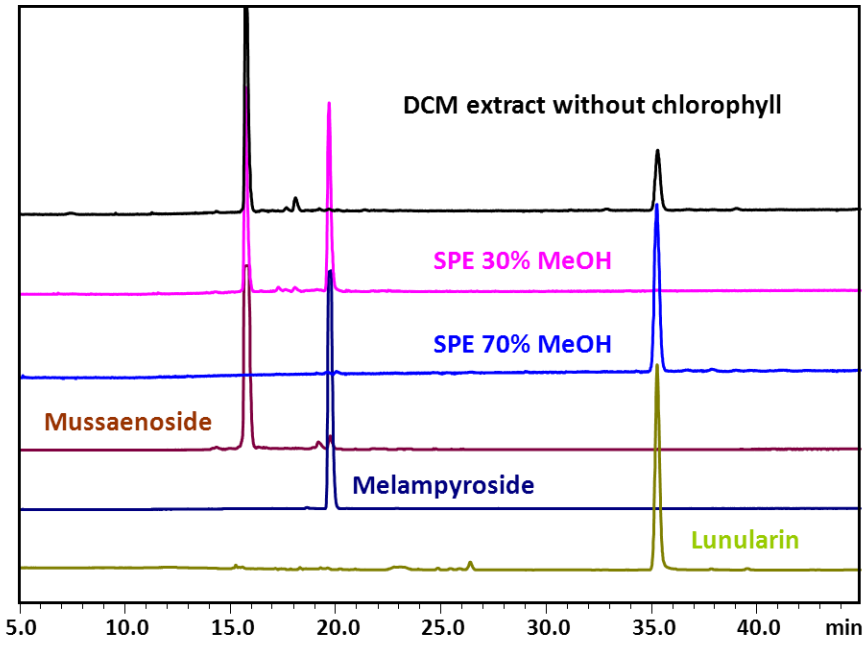


Fig. 43 HPLC-ELDS-LT chromatogram of the chlorophyll freed dichloromethane (DCM) extract and SPE subfractions (30 % MeOH and 70 % MeOH) of *M. pratense* in comparison with isolated reference compounds. Analyses were performed on HPLC instrument 2, using column 2 and HPLC system 5.

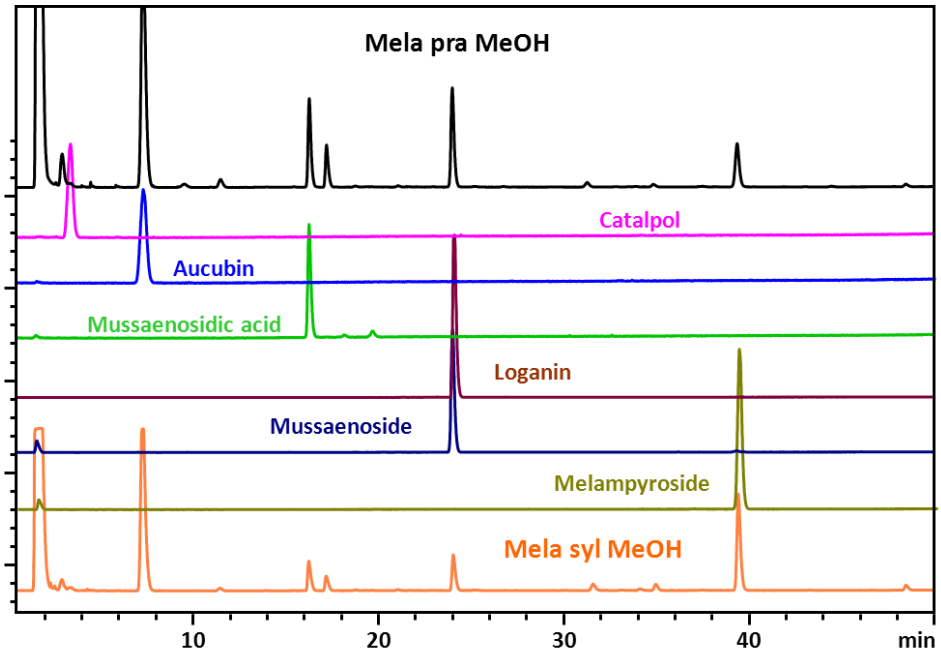


Fig. 44 HPLC-ELDS-LT chromatogram of the methanolic extract of *Melampyrum pratense* (Mela pra MeOH) and *Melampyrum sylvaticum* (Mela syl MeOH) in comparison with reference compounds. Analyses were performed on HPLC instrument 2, using column 2 and HPLC system 4.

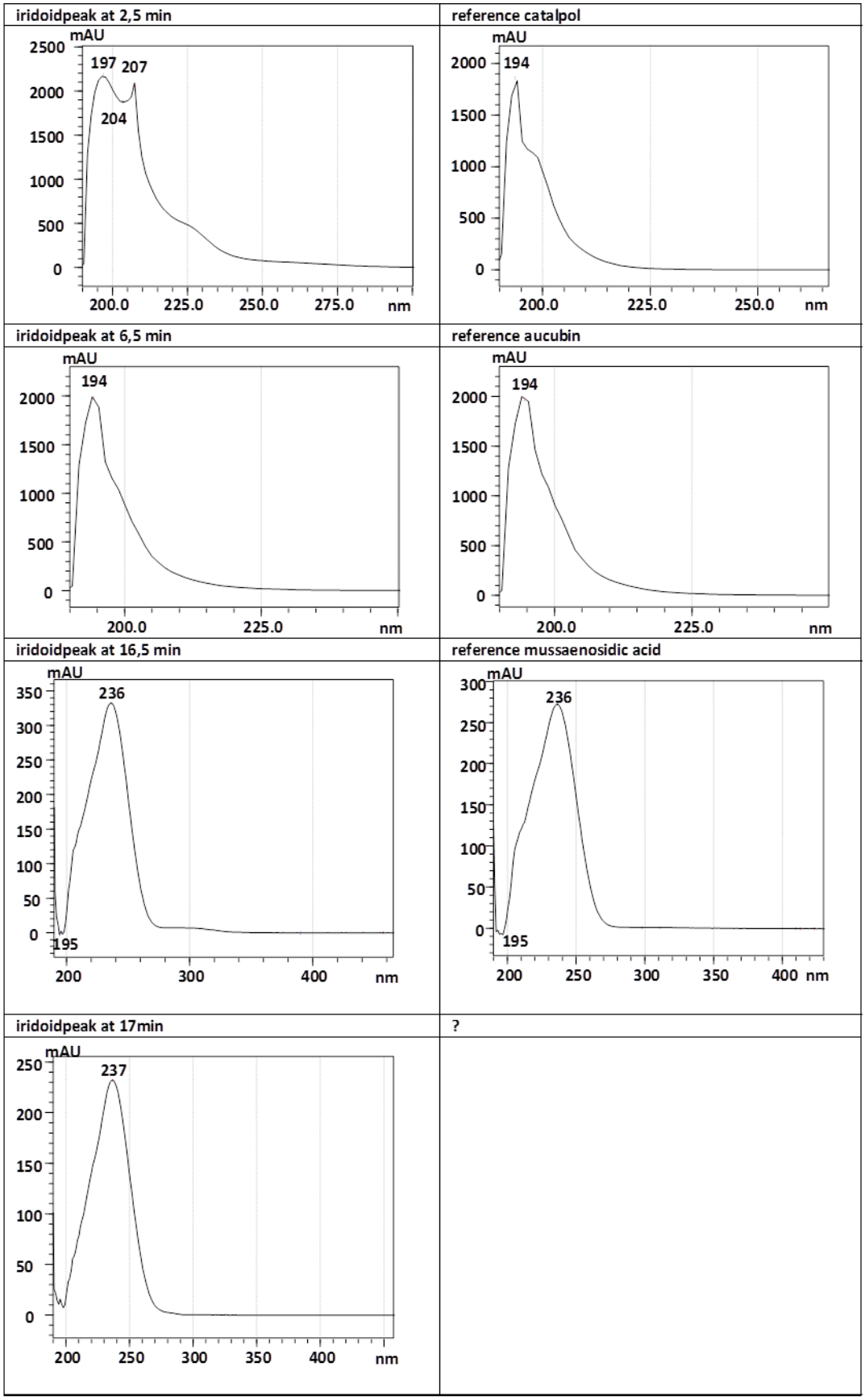


Fig. 45 continues on next page.

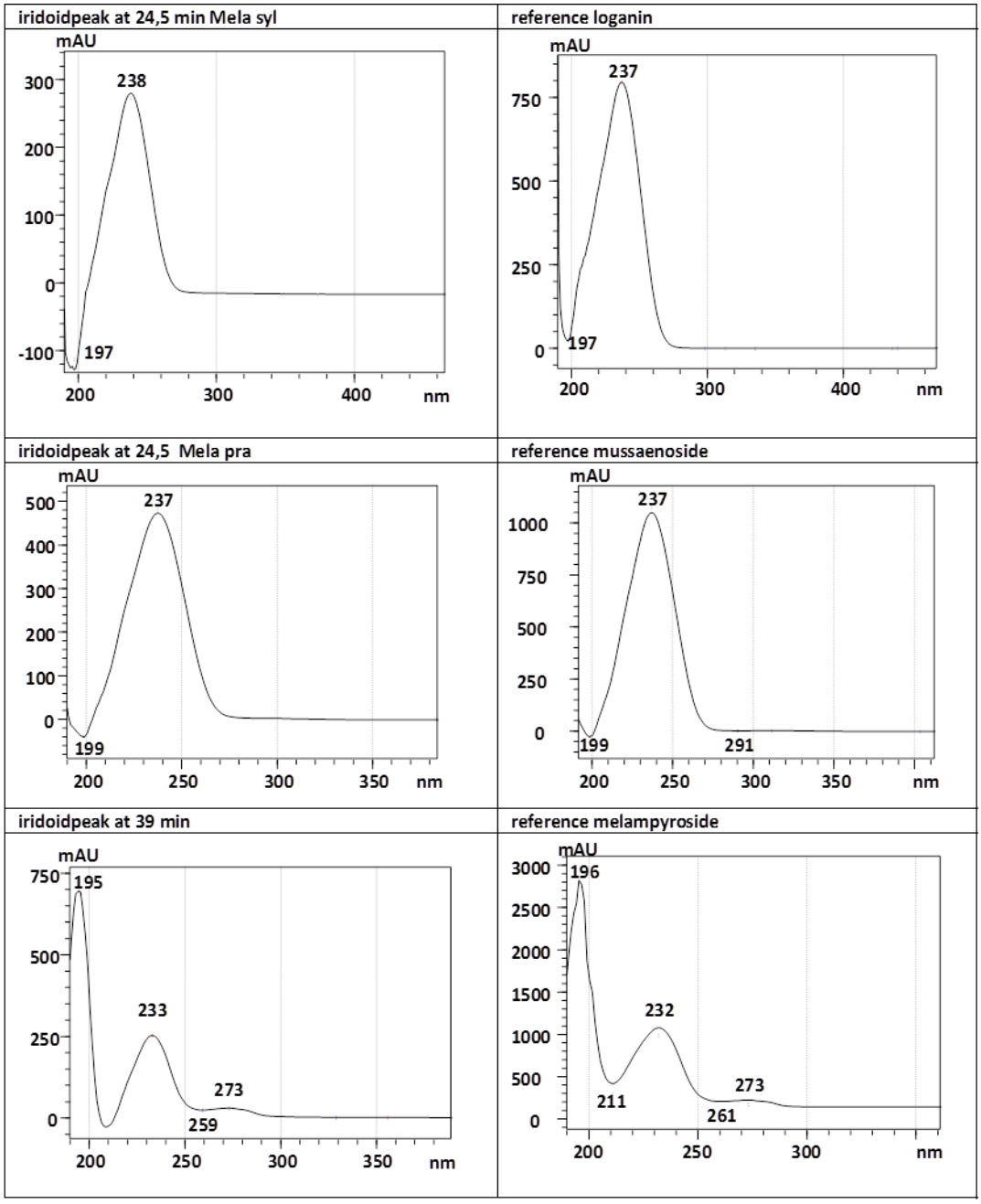


Fig. 45 UV spectra of the main peaks in the HPLC-ELSD chromatogram of *Melampyrum pratense/ sylvaticum* MeOH extract (Fig. 44) in comparison with the UV-spectra of the respective reference compounds.

The detannified MeOH extract on the other hand, mainly contained flavonoids of which luteolin, apigenin, chrysoeriol and traces of diosmetin could be identified using commercial reference compounds (Fig. 46). Luteolin was the main flavonoid in the detannified MeOH extract. Again, a comparison of the UV-spectra (Fig. 47, p. 179) and retention time was performed as well as a comparison with a MS database for flavonoids (Burgert, 2011).

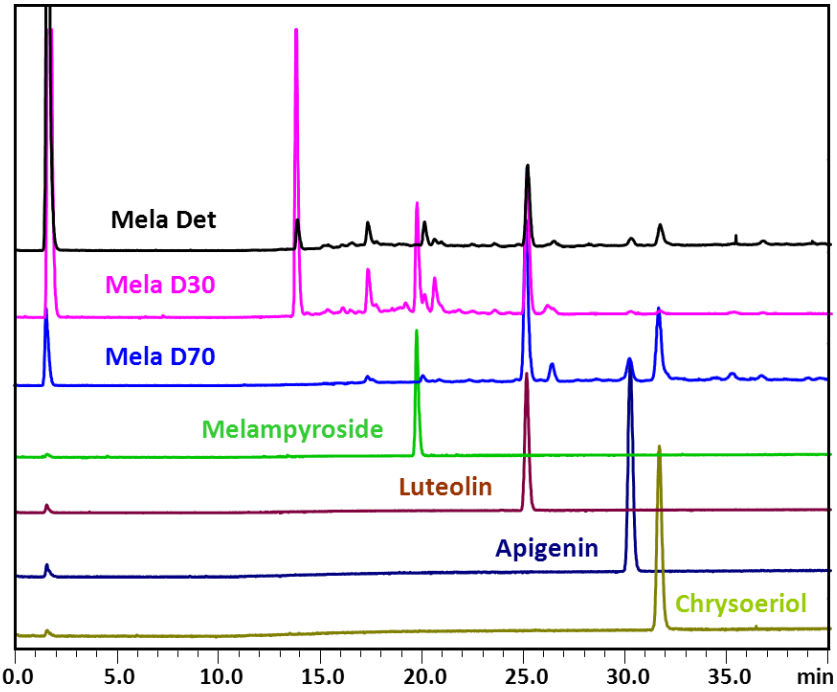


Fig. 46 HPLC-ELDS-LT chromatogram of the detannified MeOH extract (Mela Det) and SPE subfractions (30 % MeOH = Mela D30, and 70 % MeOH = Mela D70) of *M. pratense* in comparison with reference compounds. Analyses were performed on HPLC instrument 2, using column 2 and HPLC system 5.

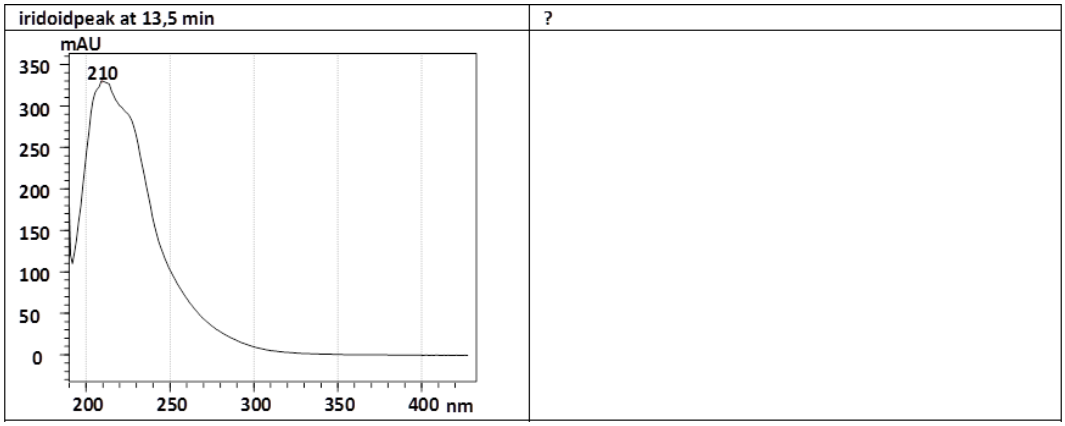


Fig. 47 UV spectra of the main peaks in the HPLC-ELSD chromatogram of *Melampyrum pratense/ sylvaticum* detannified MeOH extract (Fig. 42) in comparison with the UV-spectra of the respective reference compounds. Graph continues on the next page.

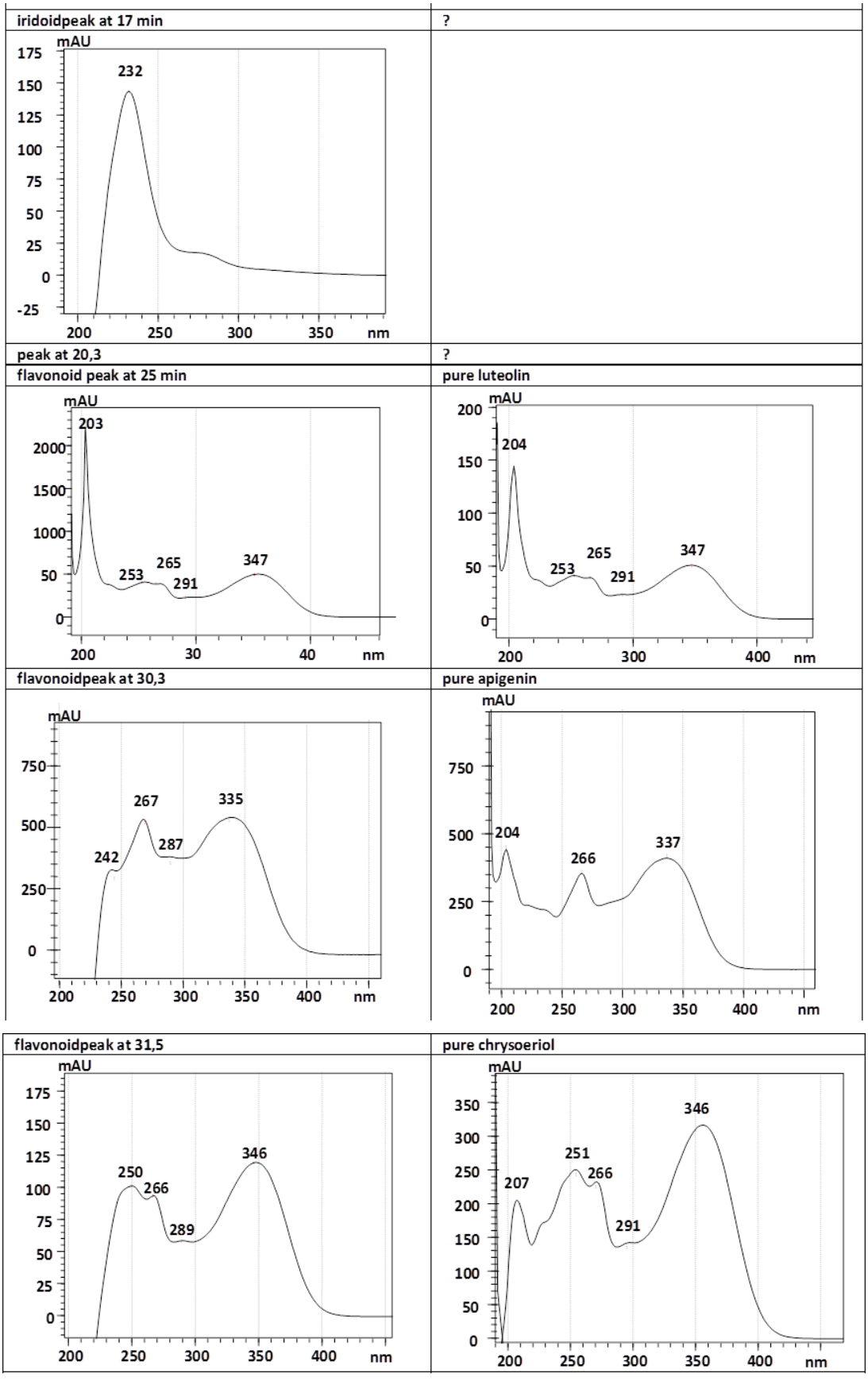


Fig. 47 continued.

3.7.3 Pharmacological results

The main compounds from *M. pratense* were then tested together with their initial extract in the respective anti-inflammatory test systems in several independent experiments, regarding PPARs activation, NF-κB inhibition and the downregulation of LPS or TNF-α induced production of IL-8 and E-selectin protein.

The iridoid glycosides mussaenosidic acid, melampyroside and mussaenoside displayed activity in the general anti-inflammatory assay on the protein level of E-selectin either after TNF-α or LPS stimulation (Fig. 48 and Fig. 49, p. 181). Inhibition was higher after stimulation with LPS, here aucubin and catalpol gave positive test results either. The potency of iridoidglycosides was specific for the downregulation of E-selectin as they did not downregulate IL-8 protein (Fig. 50, p. 181 and Fig. 51, p. 182), not activate PPARs and neither inhibit NF-κB (Fig. 52, p. 182). The flavonoids, apigenin and luteolin, on the other hand showed strong inhibition of NF-κB (Fig. 52, p. 182) as well as strong downregulation of both IL-8 and E-selectin protein (Fig. 48 - Fig. 51, pp. 180-182). The stilbene like compound lunularin, isolated only had an effect on the downregulation of E-selectin and IL-8 after stimulation with LPS but not with TNF-α.

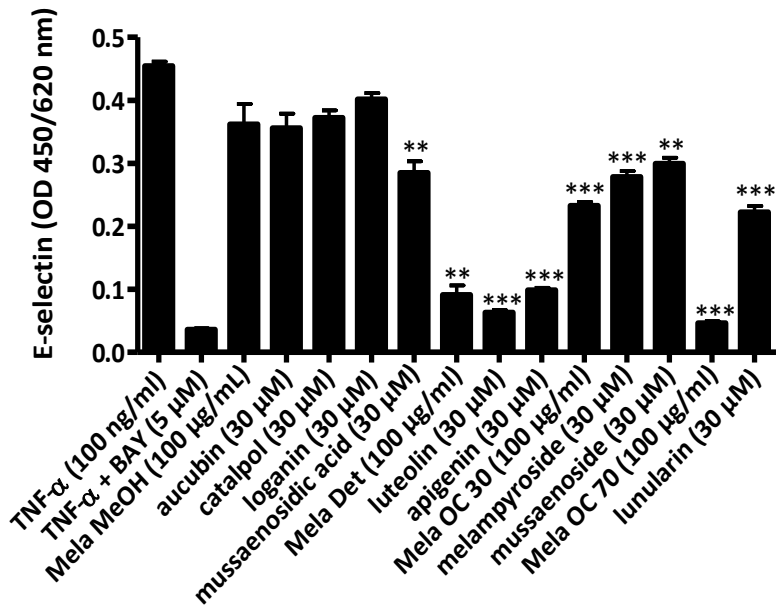


Fig. 48 Results of compounds identified/ isolated from *M. pratense* on the downregulation of E-selectin protein after stimulation with TNF-α. Compounds were tested 30 µM, fractions 100 µg/mL in triplicates in 2 independent experiments. Bay acts as positive control (5 µM). Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF-α: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

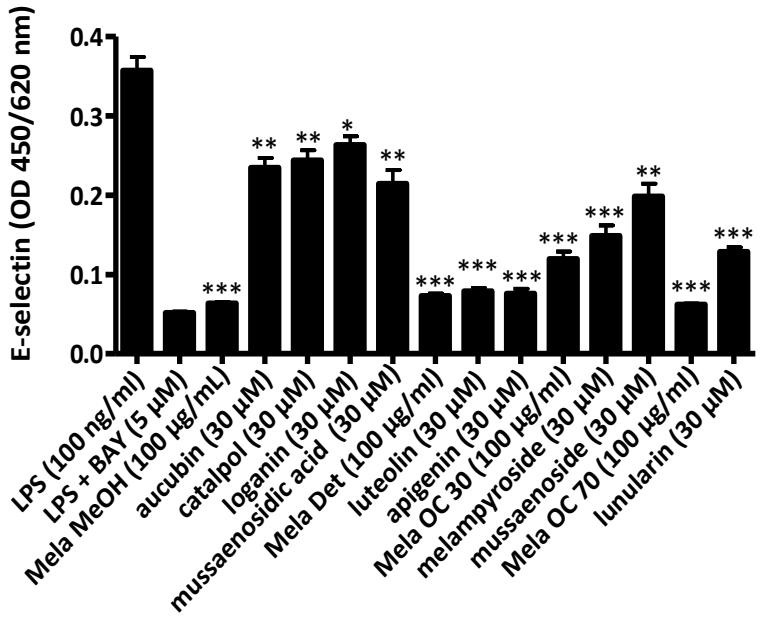


Fig. 49 Results of compounds identified/ isolated from *M. pratense* on the downregulation of E-selectin protein after stimulation with LPS. Compounds were tested 30 µM, fractions 100 µg/mL in triplicates in 2 independent experiments. Bay acts as positive control (5 µM). Bars represent mean values, error bars consider SEM, stars indicate significance compared to LPS: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

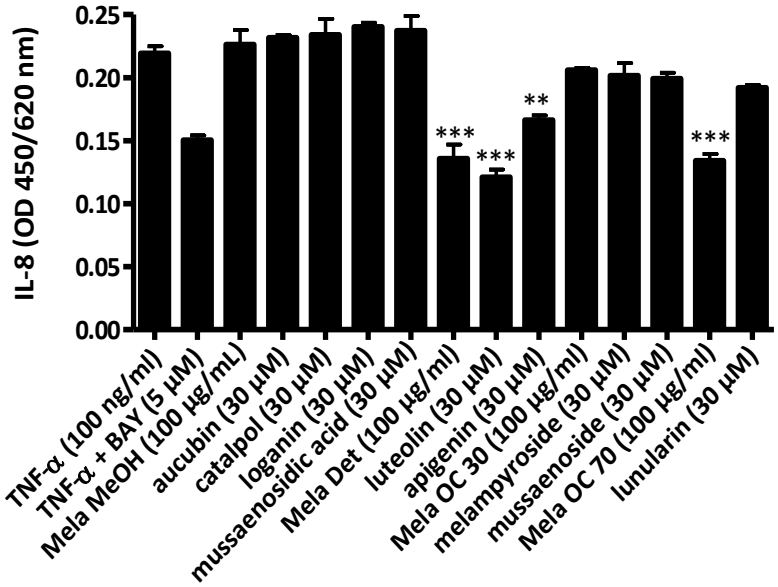


Fig. 50 Results of compounds identified/ isolated from *M. pratense* on the downregulation of IL-8 protein after stimulation with TNF-α. Compounds were tested 30 µM, fractions 100 µg/mL in triplicates in 2 independent experiments. Bay acts as positive control (5 µM). Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF-α: * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

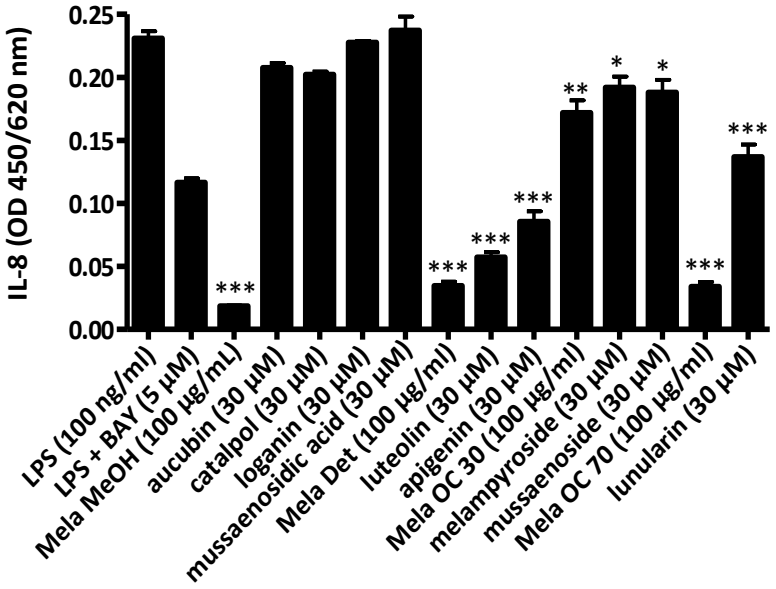


Fig. 51 Results of compounds identified/ isolated from *M. pratense* on the downregulation of IL-8 protein after stimulation with LPS. Compounds were tested 30 µM, fractions 100 µg/mL in triplicates in 2 independent experiments. Bay acts as positive control (5 µM). Bars represent mean values, error bars consider SEM, stars indicate significance compared to LPS: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

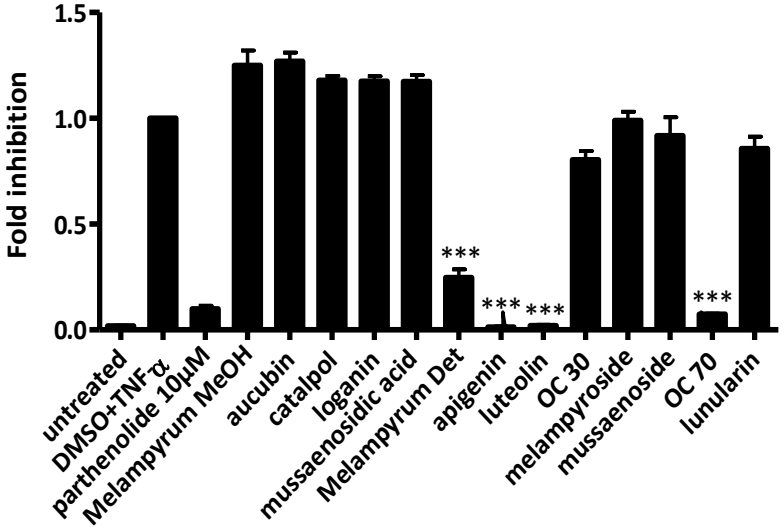


Fig. 52 Results of compounds identified/ isolated from *M. pratense* on NF-κB inhibition. Compounds were tested 30 µM with TNF-α cotreatment, fractions 100 µg/mL in triplicates in 2 independent experiments. Parthenolide (10 µM) acts as positive control. Bars represent mean values, error bars consider SEM, stars indicate significance compared to TNF-α induction: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Since luteolin and apigenin displayed highest activity on all anti-inflammatory targets dose response curves were evaluated (Fig. 53 - Fig. 54, pp. 183-184).

Luteolin was more effective on the downregulation of E-selectin with an EC_{50} of 5.67 µM after stimulation with TNF-α and 5.10 µM after stimulation with LPS. IL-8, on the other hand was downregulated with EC_{50s} of 32.09 µM and 27.06 µM, respectively. Apigenin showed good results concerning both E-selectin and IL-8 with EC_{50s} of 31.7 µM after stimulation with TNF-α and 18.9 µM after stimulation with LPS concerning IL-8. E-selectin was downregulated with EC_{50s} of 17.7 µM after stimulation with TNF-α and 13.6 µM after stimulation with LPS.

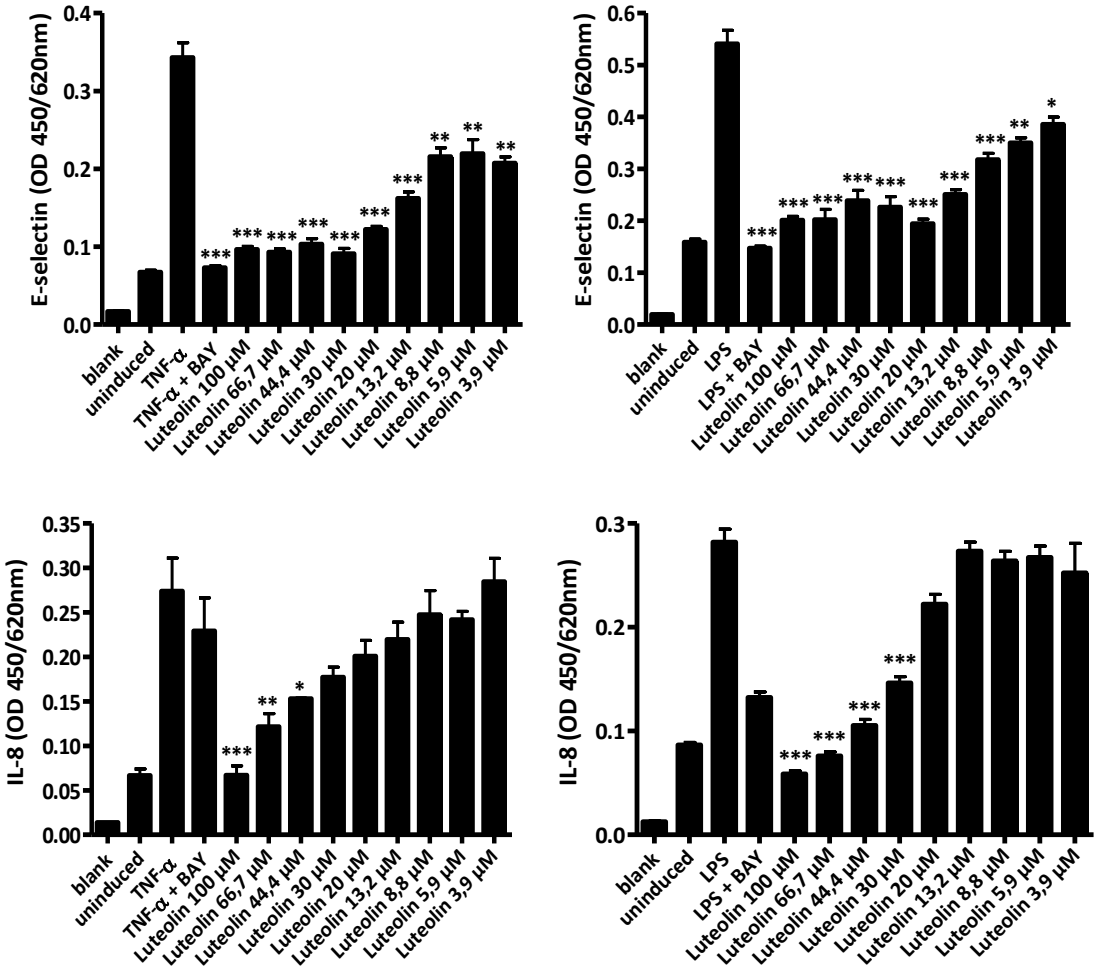


Fig. 53 Dose response of the flavonoid luteolin, the main compound of *M. pratense* and *M. sylvaticum* detannified extract and SPE subfractions on TNF-α (left) or LPS (right) induced production of E-selectin (above) and IL-8 (below): extracts were tested in triplicates in 2 independent experiments concentrations rising from 3.9 µM to 100 µM. Bars represent mean values, error bars consider SEM, stars indicate significance compared to E-selectin/ IL-8 amount after TNF-α or LPS stimulation: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

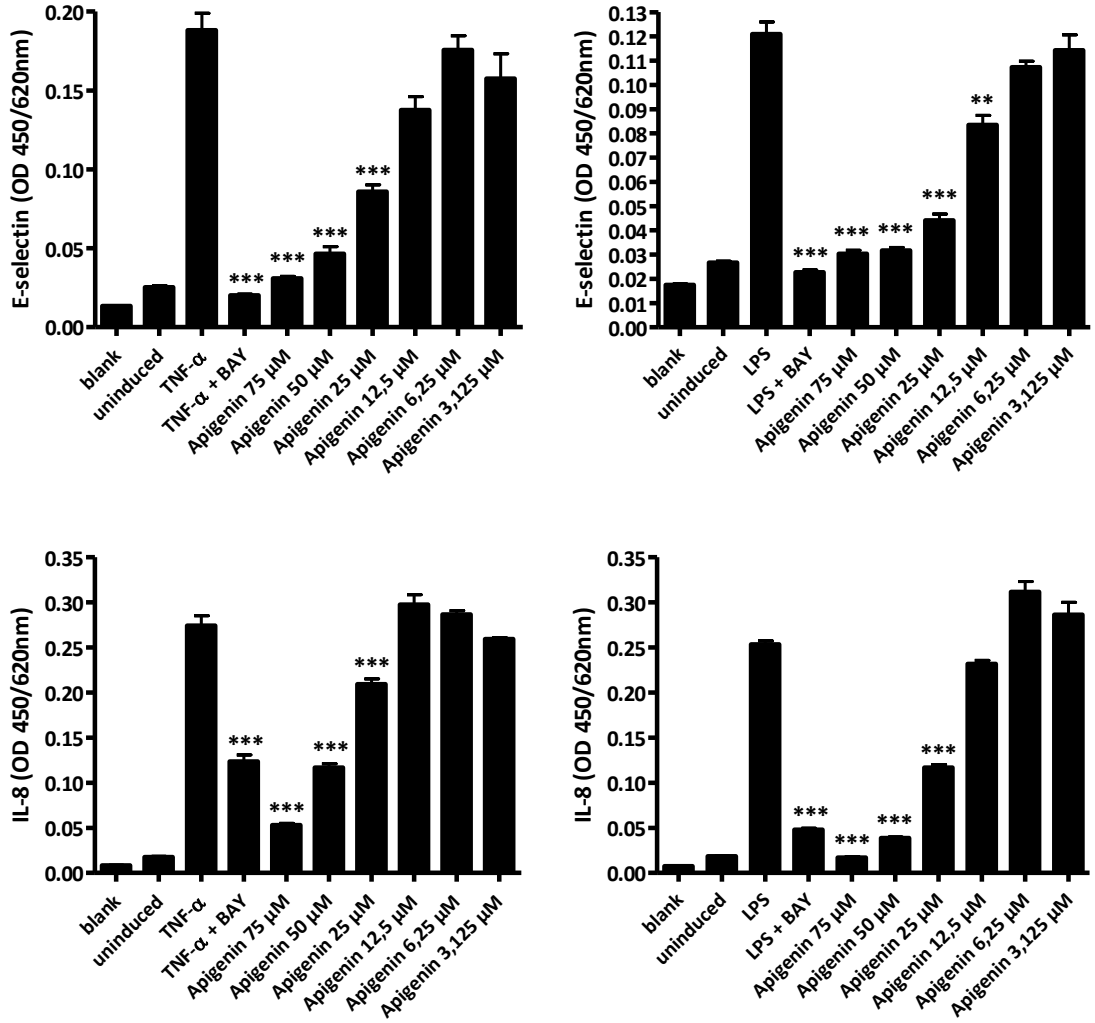
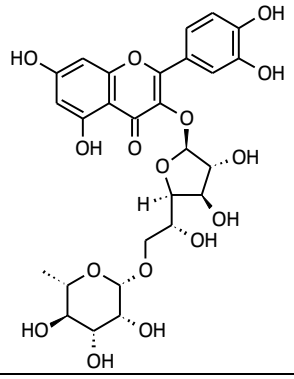
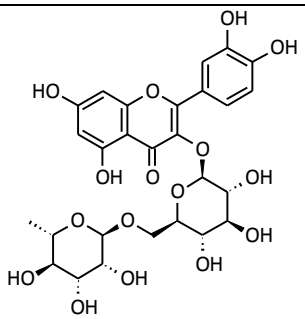
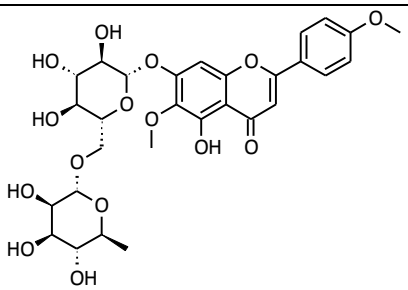
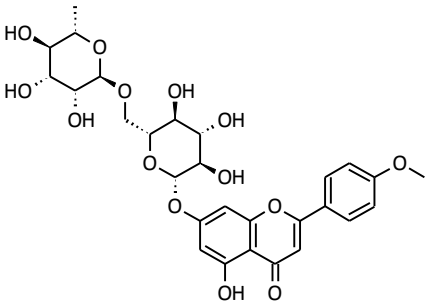


Fig. 54 Dose response of the flavonoid apigenin contained in *M. pratense* and *M. sylvaticum* detannified extract and SPE subfractions on TNF-α (left) or LPS (right) induced production of E-selectin (above) and IL-8 (below): extracts were tested in triplicates in 2 independent experiments concentrations rising from 3.125 µM to 75 µM. Bars represent mean values, error bars consider SEM, stars indicate significance compared to the amount of E-selectin/ IL-8 protein after TNF-α or LPS stimulation: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

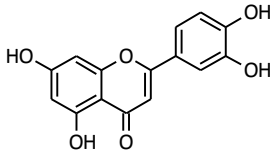
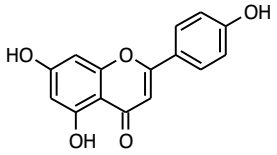
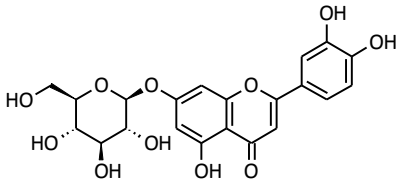
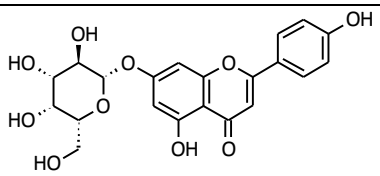
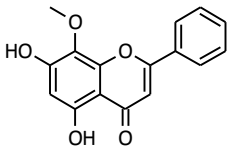
In conclusion, the flavonoids, apigenin and luteolin are likely to be the active compounds responsible for the positive results of the detannified methanolic extract of *M. pratense*. The high activity of the 70 % SPE fraction of the chlorophyll free extract can also be explained by its single main compound lunularin as the test concentration of the fraction (100 µg/mL) was much higher than that of the purified compound (30 nM). The iridoid glycosides mussaenosidic acid, melampyroside and mussaenoside displayed activity only on E-selectin just like the 30 % SPE fraction, where they were isolated from.

3.7.4 Pharmacophoric profiling of *Melampyrum pratense* compounds against anti-inflammatory targets

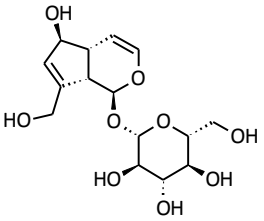
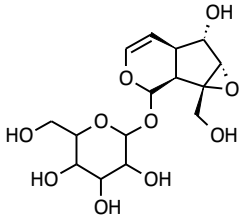
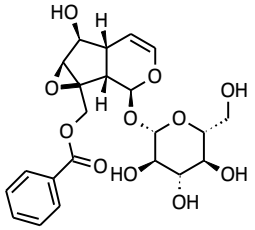
Results of the *in silico* screening of 32 structures published for *Melampyrum sp.* are shown in Tab. 36 - Tab. 40. Next to the ubiquitous fatty acids lineolic, oleic, palmitic and stearic acid only one of the components the phenolic lunularin provided a suitable pharmacophore for the PPAR-γ (Tab. 39, p. 188) and the ER-α binding pocket (Fig. 55).

Flavonoids			
Substance	**Structure**	**FXR**	**PPAR-γ**
Quercetin-3**β**-D-glucofuranosyl-6**β**-L-rhamnopyranoside 20364-84-5 $C_{27}H_{30}O_{16}$ 610.52			
Rutin; Quercetin-3-O-Rutinoside 153-18-4 $C_{27}H_{30}O_{16}$ 610.52			
Pectolinarin; Pectolinaroside 28978-02-1 $C_{29}H_{34}O_{15}$ 622.57			
Linarin; Acaciin 480-36-4 $C_{28}H_{32}O_{14}$ 592.55			

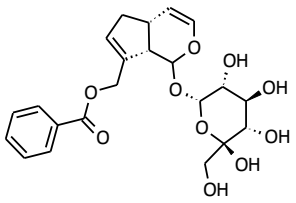
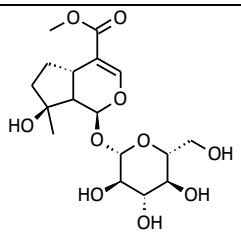
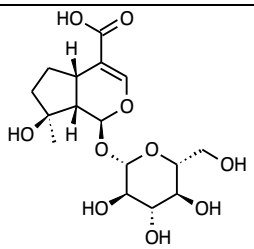
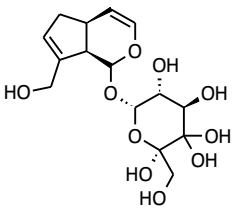
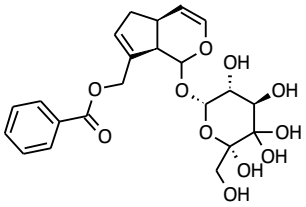
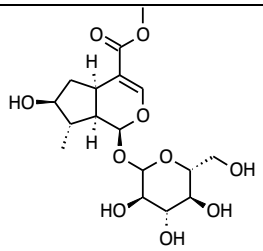
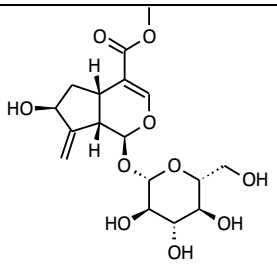
Tab. 35 continues on next page.

Substance	Structure	FXR	PPAR-γ
Luteolin 491-70-3 C_{15} H_{10} O_6 286.24			
Apigenin 520-36-5 C_{15} H_{10} O_5 270.24			
Cynaroside; Luteolin-7-D-glucopyranoside 5373-11-5 C_{21} H_{20} O_{11} 448.38			
Apigenin-7-β-D-glucopyranoside; Cosmetin 578-74-5 C_{21} H_{20} O_{10} 432.38			
Wogonin; 5,7-Dihydroxy-8-methoxyflavone 632-85-9 C_{16} H_{12} O_5 284.26			

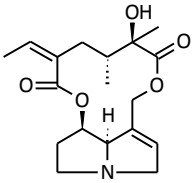
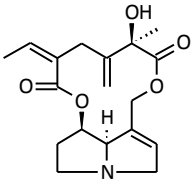
Tab. 36 *In silico* screening results of flavonoids found in *Melampyrum sp..*

Iridoids			
Substance	**Structure**	**FXR**	**PPAR-γ**
Aucubin 479-98-1 C_{15} H_{22} O_9 346.33			
Catalpol 2415-24-9 C_{15} H_{22} O_{10} 362.33			
10-Benzoylcatalpol; 10-O-Benzoylcatalpol 146714-07-0 C_{22} H_{26} O_{11} 466.44			

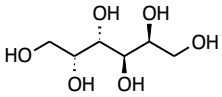
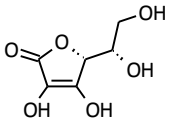
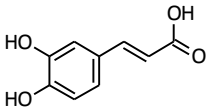
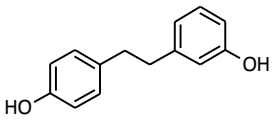
Tab. 36 continues on next page.

Substance	Structure	FXR	PPAR-γ
Melampyroside 55785-60-9 $C_{22}H_{26}O_{10}$ 450.44			
Mussaenoside 64421-27-8 $C_{17}H_{26}O_{10}$ 390.38			
Mussaenosidic acid 82451-22-7 $C_{16}H_{24}O_{10}$ 376.36			
Monomelittoside 20633-72-1 $C_{15}H_{22}O_{10}$ 362.33			
Globularifolin 73248-91-6 $C_{22}H_{26}O_{11}$ 466.44			
8-Epiloganin 79172-04-6 $C_{17}H_{26}O_{10}$ 390.38			
Gardoside methyl ester 93930-20-2 $C_{17}H_{24}O_{10}$ 358.38			

Tab. 37 Results of the computer aided pharmacophore modeling of iridoids found in *Melampyrum sp*..

Alkaloids			
Substance	**Structure**	**FXR**	**PPAR-γ**
Senecionine; Aureine; Senecionin 130-01-8 $C_{18}H_{25}NO_5$ 335.40			
Seneciphylline; α-Longilobine 480-81-9 $C_{18}H_{23}NO_5$ 333.38			

Tab. 38 Results of alkaloids from *Melampyrum sp.* screened anti-inflammatory on *in silico* targets.

Sugars, phenols and carboxylic acids			
Substance	**Structure**	**FXR**	**PPAR-γ**
Dulcite; Dulcitol; Melampyrit; 608-66-2 $C_6H_{14}O_6$ 182.17			
L-Ascorbic acid 50-81-7 $C_6H_8O_6$ 176.12			
Caffeic acid 331-39-5 $C_9H_8O_4$ 180.16			
Lunularin 37116-80-6 $C_{14}H_{14}O_2$ 214.26			x

Tab. 39 *In silico* screening results of compounds published for / isolated from (lunularin) *Melampyrum sp.*. A positive match in the pharmacophoric pocket is indicated by an x.

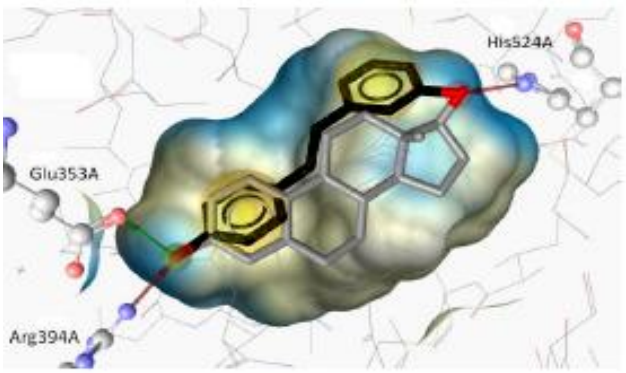
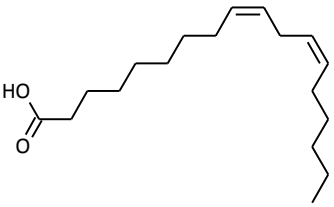
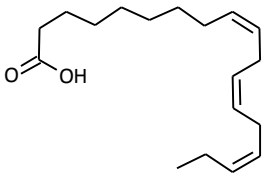
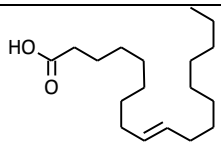
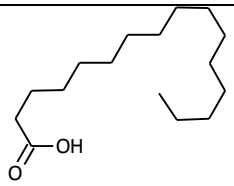
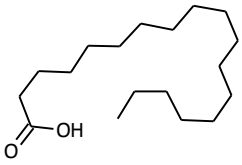
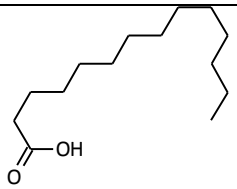
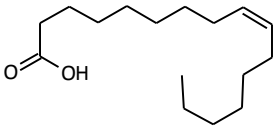
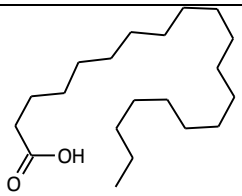


Fig. 55 Lunularin fitted into the ER-α binding pocket, yellow – hydrophobic features, red arrow – hydrogen bond acceptor, green arrow – hydrogen bond donor.

Fatty acids									
Substance	**Structure**	**COX-2**	**mPGES-1**	**cPLA2α**	**p38**	**IKK2**	**FXR**	**LXR**	**PPAR-γ**
Lineolic acid 60-33-3 $C_{18}H_{32}O_2$ 280.45		x					x	x	x
Lineolenic acid 463-40-1 $C_{18}H_{30}O_2$ 278.43		x						x	x
Oleic acid 112-80-1 $C_{18}H_{34}O_2$ 282.46		x					x	x	x
Palmitic acid 57-10-3 $C_{16}H_{32}O_2$ 256.42		x					x	x	x
Stearic acid 57-11-4 $C_{18}H_{36}O_2$ 284.48		x					x	x	x
Myristic acid 544-63-8 $C_{14}H_{28}O_2$ 228.37		x						x	x
Palmitoleic acid 373-49-9 $C_{16}H_{30}O_2$ 254.41		x					x	x	x
Eicosanoic acid 506-30-9 $C_{20}H_{40}O_2$ 312.53		x						x	x

Tab. 40 Computer aided pharmacophore results of fatty acids identified in *Melampyrum sp.*. A positive match in the pharmacophoric pocket is indicated by an x.

In the present work, the iridoid glycosides, melampyroside, mussaenoside and mussaenosidic acid were isolated and identified from the species *Melampyrum pratense* for the first time, the same counts for lunularin. Aucubin and loganin have already been descriebed for the investigated taxa and could be identified again using HPLC-DAD/-MS and reference compounds.

Additionally, the flavonoids apigenin, luteolin and chrysoeriol, already described in literature for other *Melapyrum* species were identified in this species for the first time. The fatty acids lineolic, oleic, palmitic and stearic acid were also identified in this study, for *Melampyrum pratense*, using TLC and reference compounds.

4 Discussion

In the present work a target-oriented approach (e.g. activation of PPAR-α and -γ or inhibition of NF-κB) as well as functional cellular models (e.g. inhibition of adhesion molecule surface expression in endothelial cells, VSMC proliferation and migration) were used to identify bioactive natural products as well as plant extracts and fractions, representing promising sources of bioactive compounds, that interfere with inflammatory processes.

The indispensible basis for the search of plant material was the long neglected but precious store of knowledge of Austrian traditional medicine. Despite the once wide use of traditional European herbals, modern science has barely started to scientifically explore this treasure (Adams *et al.*, 2009). When entering the term "Traditional European Medicine (TEM)" in the database of SciFinder only 11 citations are found. The concept "Traditional Chinese Medicine (TCM)" on the other hand reveals 25947 and "Ayurveda" 970 references. Two years ago Adams *et al.* (2009) stated that he found 7 hits for TEM, 4264 for TCM and 550 concerning Ayurveda. This shows a strong increase of interest in TCM but nearly non regarding European ethnomedicine. A reason for this phenomenon is of course the avoidance of traditional ethnomedicine in Europe during the advance of Western medicine, whereas TCM is and was an integral part of the Chinese health system until now. Actually, in Europe nowadays TCM seems even more popular than its own traditional remedies.

The written records concerning traditional European medicine started with Hippokrates (460-370 BC) – "De natura hominis", Diokles v. Karystos (ca. 360-260 BC) – "Rhizotomikon", Theophrastus of Eresos (371-287 BC) – "Enquiry into Plants" or "On the Causes of Plants" and Krateuas (100 BC) "Rhizotomikon". In the first century (AD) Pedanius Dioskurides wrote the "De materia medica" which was the most important work on herbal drugs for the following 1500 years. During the 15th and 16th century numerous eminent works were written like the herbal books of Hieronymus Bock/ Tragus (1498-1554), Leonhart Fuchs (1501-1566), Adam Lonicer (1528-1586) and Paracelsus (1493-1541).

A recent attempt to preserve the remains of knowledge about traditional Austrian herbal medicine is the creation of the VOLKSMED database at the University of Vienna. In its course several diploma thesis comprise interviews with people all around Austria and the immediate vicinity about traditional plants used, their indications, applications and preparations.

For the present work several plants with conveyed anti-inflammatory indications have been chosen out of this DB for an ethnopharmacological screening on the activation of the nuclear receptors PPAR-α and -γ, the inhibition of the transcription factor NF-κB and the downregulation of the adhesion molecule E-selectin and the chemokine IL-8, important mediators of the inflammatory response.

Finally, over 90 % of the investigated drugs revealed positive results in at least one of the engaged *in vitro* assay. Compared to an average hit rate of 10 % or below in random screenings (Cox, 1994; Harvey, 2002) this is a striking hit rate providing first evidence for the traditional application of several herbs on the one hand and the usefulness of an ethnopharmacological screening approach on the other hand. Based on the strength of the activity and due to a literature research on the existing scientific investigations of the active plants, the two promising plants *Peucedanum ostruthium* and *Melampyrum pratense* have been chosen for bioactivity guided fractionation and isolation of the components responsible for the observed anti-inflammatory activity of extracts and fractions.

Plant extracts are complex mixtures of numerous, highly diverse components. In times of High Throughput Screening (HTS) a purification of such mixtures as well as bioassay guided fractionation is generally regarded as too time consuming. Controversially, the complex chemistry provided by natural products represents an important pool for the discovery of new lead structures. Recent approaches make use of solid phase chromatography to enrich and purify extracts which can be easily automated (Balakin *et al.*, 2006; Eldridge *et al.*, 2002; Exarchou *et al.*, 2005; Harvey *et al.*, 2010; Lambert *et al.*, 2007; Schmid *et al.*, 1999). Together with technical advances in analytical techniques (e.g. microcoil NMR and coupled LC-MS-NMR) to rapidly determine the structures of isolated natural products and to avoid reisolation of already known compounds many difficulties of the use of natural products in HTS could be overcome (Cremin *et al.*,

2002; Wolfender *et al.*, 2010; Wolfender *et al.*, 2000). Another advantage of these modern methods is the decreased quantity of material needed for the determination of a structure (Hu *et al.*, 2005; Wolfender *et al.*, 2005).

For the present investigations an Accelerated Solvent Extractor (ASE) was chosen for first extraction of several plant species. This method guaranteed a fast, automated and reproducible extraction for the initial screening. A first and fast purification of the crude extracts was conducted via liquid/ liquid partitioning to avoid possible interference and/ or masking effects of bulk components, present in high amounts, such as chlorophylls and tannins. Further fractionation of the most promising extracts was conducted by SPE which works under reduced pressure and with reduced particle sizes rendering it much faster than common column chromatography (CC). Furthermore, the high standard of modern preparative and semi preparative HPLC columns often allows a separation of single compounds in a fraction of time compared to classical CC methods.

Finally, the structures of natural products can provide a basis for the design and synthesis of analogues that have improved pharmacological properties. Chemical reactions can implement additional drug-like features to natural products (Lopez *et al.*, 2007). Such combinatorial approaches have been successfully applied to a wide range of naturally derived chemical scaffolds, including steroids, terpenoids, flavonoids and alkaloids (Harvey, 2007).

4.1 *Peucedanum ostruthium*

This plant was chosen due to the high activity of its crude extracts in the PPAR activation and NF-κB inhibition assay. In the course of a bioactivity guided isolation the detannified MeOH extract was further fractionated by SPE and HPLC, leading to the isolation of six coumarins and one chromone. Additionally, several other coumarins were identified in the DCM as well as in the MeOH extract.

The initial crude detannified MeOH extract was able to activate PPAR-α 2.5 fold, even better than the positive control GW7647, and PPAR-γ 3 fold, comparable to troglitazone. The subsequent fractionation with SPE diminished the activity. Here only the 70 % methanolic subfraction showed good results with 2 and 1.8 fold activation of PPAR-α and PPAR-γ, respectively.

NF-κB was even downregulated to the base level by the DCM as well as by the detannified MeOH extracts, showing higher activity than the control agonist parthenolide. After fractionation with SPE the same effect was reached by the 70 % and 100 % methanolic subfraction. The following fractionation on preparative HPLC led to an additional loss of activity on PPARs as well as on NF-κB. From the coumarins isolated only bergamottin was able to activate PPARs (1.5 fold) and ostruthin could inhibit NF-κB (65 % inhibition). The antiphlogistic potency of ostruthin and its initial ethanolic extract of the roots of *Peucedanum ostruthium* have already been shown *in vivo* in a carrageenan induced rat paw edema test system (30 µg/kg of this compound inhibited the formation of edema to ≤ 50 %)(Hiermann *et al.*, 1998).

However, the positive results on PPARs and the strong inhibition of NF-κB could not be related to a single coumarin, tested at concentrations of 10 µM. Thus, it may need the composition of the coumarins, as in the whole extract, for an effect. A possibility to test this theory would have been to coapply the coumarins present in the active extract and to see if the effect compared to the results of the single tested coumarins was only additive or even synergistic. Unfortunately, this was not possible in the time frame of the project. Synergistic effects of coumarins with other components could also contribute to the higher activity of the initial extracts. Such an effect was shown in several publications (Kaneko *et al.*, 2003; Liao *et al.*, 2010b; Tirillini, 2000; Vera *et al.*, 2006).

Moreover, minor compounds, which were not isolated and tested but have high activity, might contribute to the overall activity of the extract as well. Additionally, volatile components might have been lost during the isolation process. Due to the fractionation on semipreparative HPLC conducted with water/ acetonitrile and subsequent evaporation of the fractions, a loss of volatile components would be inevitable.

Apiaceae are known to be rich in essential oil and components thereof were already linked to anti-inflammatory activity e.g. inhibitory activity against NF-κB mediated transcription in SW1353 cells (Tabanca *et al.*, 2007). Here, five pure compounds, 4-(2-propenyl)phenylangelate, 4-(3-methyloxiranyl)phenyltiglate, 4-methoxy-2-(3-methyloxiranyl)phenyl isobutyrate, 4-methoxy-2-(3-methyloxiranyl) phenylangelate and epoxy pseudoisoeugenol-2-methylbutyrate inhibited NF-κB mediated transcription with IC_{50} values of 5.5, 1.2, 0.01, 3.6 and 11 µ/mL. Measurements of the essential oil in the frame of a diploma thesis at the Department of Pharmacognosy (Waldbauer, 2011) exhibited a content of 0.4-0.7 % in different samples of *P. ostruthium* rhizomes. GC-MS analysis showed that the oil from the rhizome of *P. ostruthium* contains 39 compounds of which 29 were identified. Compounds present in largest quantities were sabinene (35.2 %) of which (+)-sabinene made up 96.54 % and 4-terpineol (26.6 %) of which (+)-4-terpineol accounted for 65.8 % (Cisowski *et al.*, 2001). Pharmacies and distributers of this herbal drug specify the content of essential oil as a quality characteristic, but it is neither sure that its proposed pharmaceutical properties are soley due to the essential oil, nor if there are still significant amounts of essential oil components present in the traditional preparations like a tea.

Furthermore, GC-MS measurements with the extracts, the SPE subfractions and the subfractions of preparative HPLC of 70 % SPE fraction of *Peucedanum ostruthium* revealed a high concentration of fatty acids in the PPAR active extracts and fractions and phthalides in the NF-κB actives. Fatty acids are already known as PPAR agonists (Cury-Boaventura *et al.*, 2006; Forman *et al.*, 1996; Hihi *et al.*, 2002). Gijbels *et al.* (1984) also found phthalides in the roots of *Peucedanum ostruthium*. Phthalide lactones from *Ligusticum* were identified as inhibitors of LPS-induced TNF-α production in monocytes and exhibited significant suppressive effects on TNF-α mediated NF-κB activation (Liu *et al.*, 2005b). Thus, it is not surprising that in our assays phthalides were also determined as potent inhibitors of NF-κB (100 % inhibition at 10 µM).

Nevertheless, coumarins are a very interesting main compound class in *Peucedanum* species (Hadacek *et al.*, 1994). Up to now, more than 1300 natural coumarins have been identified, mainly in the families of Rutaceae and Apiaceae (Curini *et al.*, 2006). For the plant itself they play an important role as phytoalexins (Glowniak *et al.*, 1994)

and insecticidal components (Hadacek *et al.*, 1994). Coumarins have been reported to act as analgesic, sedative, choleretic, antioxidant (Thuong *et al.*, 2010), immune stimulating, anti-inflammatory (Garcia-Argaez *et al.*, 2000; Genovese *et al.*, 2010), antifungal, anti-mycobacterial (Schinkovitz *et al.*, 2003), edema blocking, spasmolytic and photosensitizing compounds (Hänsel *et al*., 2010). Due to their wide spectrum of biologically activities, members of this compound class are increasingly recognized as valuable health-promoting constituents in various herbal drugs (Hoult *et al.*, 1996). Antibiotics like Novobiocin und Coumermycin A1 are derived from natural coumarins.

Their lipophilic characteristics make them resorbable into the gastrointestinal tract and the nervous system, where they may act as narcotics. Nevertheless, metabolic studies as well as reports on pharmacokinetics of other compounds than coumarin itself are rare.

The coumarins found in *Peucedanum ostruthium* have been reported to affect various targets linked to inflammation. Bergapten, xanthotoxin, imperatorin, isoimperatorin and isopimpinellin were already screened for COX-2 inhibition and bergapten, imperatorin and isoimperatorin had an effect revealing IC_{50} values of 10.7 µM and 24 µM (Hong *et al.*, 2008; Moon *et al.*, 2008; Yoo *et al.*, 2002). Osthole, oxypeucedanin and isoimperatorin showed significant inhibitory activities on TNF-α production. as 54.3±2.1 %, 33.6±1.0 % and 17.7±3.0 %, respectively, at 12.5 µg/mL (Cho *et al.*, 1998). Additionally, imperatorin and isoimperatorin antagonized IL-1β induced proliferation (Ji *et al.*, 1994). Oxypeucedanin hydrate, as well as oxypeucedanin were determined as potent inhibitors of NO production with IC_{50s} of 16.8 and 15.8 µg/mL (Ryu *et al.*, 2001; Wang *et al.*, 2000) and osthole was found to inhibit 5-LO and LPS induced prostaglandin E2 (Roos *et al.*, 1997). Osthole exhibited various pharmacological activities, including immunity-strengthening, anti-tumor, anti-hepatitis and anti-osteoporosis (You *et al.*, 2009; Zhou *et al.*, 2006). The anti-inflammatory action of osthole has already been explored on different targets (Liao *et al.*, 2010a; Liu *et al.*, 2005a; Zhou *et al.*, 2006; Zimecki *et al.*, 2009). It suppresses inflammatory response on NO levels (Liu *et al.*, 2005a). It was reported to be effective mainly as interleukin-1 blocker, but not as arachidonate blocker rendering it a potent anti-inflammatory agent even more potent than the prototype corticosteroid, prednisolone (Liu *et al.*, 1996).

Recently, it was published that it regulates inflammatory mediator expression through modulating NF-κB, mitogen-activated protein kinases, protein kinase C, and reactive oxygen species (Liao *et al.*, 2010a). It regulates enzyme protein expression of cholesterol-7-alpha-hydroxylase and diacylglycerol-O-acyltransferase-2 via activation of PPAR-α/ -γ in fat milk induced fatty liver rats (Zhang *et al.*, 2008) and was shown to inhibit alcohol induced fatty liver in mice (Sun *et al.*, 2009). It causes hypotension *in vivo*, and inhibits platelet aggregation and smooth muscle contraction *in vitro*. It may interfere with calcium influx and with cyclic nucleotide phosphodiesterases (Hoult *et al.*, 1996). Osthole was found to block the reduction of nerve growth factor (NGF) and suppressed interferon-γ (IFN-γ) increase in experimental autoimmune encephalomyelitis mice, what suggests it as a new pharmacological approach to treat multiple sclerosis (Chen *et al.*, 2010). Osthole, imperatorin and isoimperatorin showed a stronger vaso-relaxation effect than that of tetramethylpyrazine (Li *et al.*, 2009).

Additionally, the coumarins from *Peucedanum ostruthium*, ostruthin, imperatorin, oxypeucedanin hydrate and ostruthol in particular were found to inhibit acetylcholinesterase (0.001-1 µg/mL minimum inhibitory quantity), what might be useful in the treatment of Alzheimer`s disease (Hostettmann *et al.*, 2007; Urbain *et al.*, 2005). Antitumor activity was reported for isopimpinellin and imperatorin as they could significantly reduce overall binding of 7,12-Dimethylbenz[a]anthracen to epidermal DNA by 67 % and 52 %, respectively, when applied at doses of 400 nM (Cai *et al.*, 1997). This might render them as effective inhibitors of skin tumor initiation. Furthermore, oxypeucedanin hydrate, bergapten, imperatorin, isoimperatorin, isopimpinellin, osthole, ostruthol and oxypeucedanin, isolated from the chloroform extract of *Angelica archangelica* exhibited calcium agonist activity, which may be useful in the treatment of cancer (Harmala *et al.*, 1992). The antibacterial activity of *Peucedanum ostruthium* was linked to oxypeucedanin hydrate (Goekay *et al.*, 2010). Ostruthol and imperatorin isolated form *P. ostruthium* rhizomes were shown to slightly inhibit norepinephrine-induced contractions (Rauwald *et al.*, 1991). Isoimperatorin, imperatorin, oxypeucedanin and oxypeucedanin hydrate exhibited a significant inhibition on cell proliferation with ED_{50} values between 3.4 and 30 µg/mL depending on the cell line and compound (Kim *et al.*, 2007).

Imperatorin was reported to inhibit T-cell proliferation by targeting the transcription factor NFAT inhibitory with an $IC_{50} \sim 37$ µM (Marquez *et al.*, 2004).

Next to their interesting pharmacological activities coumarins are better known for their toxic effects. Depending on their structural features, coumarins show diverse activities which are of interest for pharmaceutical applications on the one hand, but also for their toxicity on the other hand. Hepatotoxic and carcinogenic aflatoxins for example have a coumarin moiety. In animal experiments hepatoxicity was found for coumarin at doses >> 100 mg/kg. Therefore, in 2004 the European Food Safety Authority (EFSA) derived a tolerable daily intake (TDI) for humans of 0.1 mg/kg body weight (Abraham *et al.*, 2010). Nevertheless, in a review from Fentem *et al.* (1993) concerning coumarin metabolism and hepatotoxicity species differences in coumarin hepatotoxicity appeared to be metabolism-mediated. In rats, where it is markedly hepatotoxic, coumarin is primarily metabolized via 3-hydroxylation and cleavage of the heterocyclic ring. Coumarin was shown to be less toxic in the baboon, gerbil and certain strains of mice, which resemble man in their extensive formation of the 7-hydroxy metabolite. Moreover, Fentem *et al*. (1993) states that liver toxicity in patients receiving relatively high daily doses of coumarin is very rare (Fentem *et al.*, 1993).

Furthermore, coumarins are known to display anticoagulant properties by antagonising Vitamin K. However, this activity possessed by some natural coumarins is not universal to this compound class. Essential for the anticoagulant activity, is a nonpolar carbon substituent at the position 3 of 4-hydroxycoumarin. Dicoumarol provided the basic structure of many anticoagulants (Hänsel *et al.*, 2010). It is a vitamin K antagonist, formed by the action of molds on coumarin in sweet clover. Nowadays, the commonly used clinical anticoagulant drugs are all fully synthetic compounds (e.g. acenocoumarol, phenprocoumon, warfarin).

Additionally, they are deposited in the skin, what is important in terms of the photosensitizing action of linear furanocoumarins, the psoralens (Satsyperova, 1968). The most prominent one is xanthotoxin (8-Methoxypsoralen, 8-MOP), which is even clinically applied. Photosensitizing substances make the skin more sensitive to sunlight as they absorb UV light in the range of 320-400 nm leading to radicals or pyrimidine dimers.

Ruta graveolens L., *Heracleum* species, *Pastinaca sativa* L., *Angelica archangelica* L., *Levisticum officinale* L. and the leaves of *Ficus carica* L. have been reported to lead to dermatitis after skin contact. Linear furanocoumarins, particularly psoralen, xanthotoxin (8-Methoxypsoralen, Ammoidin) and bergapten are said to be responsible for this action. On the other hand, the photosensitizing ability of furocoumarins provided successful clinical treatments against different diseases, from skin disorders like psoriasis or vitiligo to promising anti-carcinogenic uses (Berger *et al.*, 2010; Berger *et al.*, 2011; Berger *et al.*, 2008; Bethea *et al.*, 1999; Gasparro, 1996; Gasparro, 2000). Finally, 2004 the EFSA concluded that *in vivo*, coumarin does not bind in a covalent manner to the DNA as described (Cimino *et al.*, 1985; Murray, 2002) excluding a genotoxic mechanism by covalent cross-binding.

4.2 *Melampyrum pratense*

All crude extracts of *M. pratense* revealed a high activity in the general anti-inflammatory test systems i.e. downregulation of E-selectin and IL-8. In this assay an activation of the endothelium was achieved by two diverse stimuli, the inflammatory cytokine (TNF-α) or the bacterial product (LPS). These compounds stimulate distinct but partially overlapping signaling pathways implemented in acute and chronic inflammation. The appliance of two inflammatory agonists that reveal a distinct chemical nature and interact with different receptors should enhance the reliance of the screen. Toll-like receptor 4 and tumor necrosis factor receptor play a key role in the activation of other cell types, e.g. leukocytes. Therefore, the results of the screen for inhibitory plant compounds performed on endothelial cells are likely to be relevant to other cell types. The major readouts of the assay were mRNA levels of the inflammatory adhesion molecule E-selectin and the cytokine IL-8 normalized to a housekeeping gene β2-microglobulin. Finally, with this approach it was aimed to identify and quantify corresponding microRNAs, binding to complementary sequences on target mRNA transcripts. These post-transcriptional regulators are short non-coding RNAs that inhibit translation of target genes by binding to their mRNAs.

Recently, microRNAs are increasingly recognized as modulators of inflammation (Bhaumik *et al.*, 2009; Chen *et al.*, 2009; Dai *et al.*, 2011; Davidson-Moncada *et al.*, 2010).

Hence, all crude extracts were phytochemically investigated fort heir active compounds. With the concept of bioactivity guided fractionation and HPLC-ELSD/-DAD/-MS structure elucidations several iridoids and flavonoids were isolated and identified as potential active principles. Using HPLC-ELSD/-DAD/-MS analyses the iridoids mussaenoside, melampyroside, aucubin, catalpol, loganin and mussaenosidic acid were detected in the polar MeOH extract and the flavonoids luteolin, apigenin and chrysoeriol in its nonpolar detannified subfraction. The polar chlorophyll free part of the DCM extract mainly contained melampyroside, mussaenoside and lunularin, compounds that have been identified in *M. pratense* for the first time.

While IL-8 and E-selectin expressions were initially measured at the mRNA level, for the pure compounds newly established assays quantifying at the protein level were implemented. These experiments using ELISA readout were faster and gave more homogenous and stable results. Therefore, they may be considered as more reliable than those conducted at the mRNA level. Moreover, the post-transcriptional inflammatory effects are mediated by the protein expression, which can be distantly related to the mRNA transcript level.

As a result, the isolated iridoid glycosides were able to downregulate E-selectin, especially after stimulation with LPS, suggesting them to act on this specific pathway. The flavonoids, apigenin and luteolin, on the other hand showed strong inhibition of NF-κB as well as strong downregulation of both IL-8 and E-selectin protein. The stilbene like compound lunularin only had an effect on the downregulation of E-selectin and IL-8 after stimulation with LPS not with TNF-α. Additionally, this compound did bind *in silico* to the glucocorticoid receptor as well as to PPAR-γ. Latter result was confirmed *in vitro* using the PPARs activation assay.

The iridoids catalpol and aucubin can be found in many plant families and have already been described for *M. pratense* and other *Melampyrum* species (Suomi *et al.*, 2002; Suomi *et al.*, 2001).

Jeong *et al.* (2002) showed that aucubin inhibited the expression of TNF-α and IL-6 in Ag-stimulated rat basophilic leukemia (RBL)-2H3 mast cells in a dose-dependent manner with an IC_{50} of 0.101 and 0.19 µg/mL, respectively. In this study aucubin was also determined to inhibit Ag-induced nuclear translocation of the p65 subunit of NF-κB. Since activator protein-1 binding activity was not affected, their results suggested that aucubin is a specific inhibitor of NF-κB activation in mast cells, which might explain its beneficial effect in the treatment of chronic inflammatory diseases (Jeong *et al.*, 2002). However, during our investigations aucubin did not inhibit NF-κB at 30 µM.

The content of flavonoids (0.18-0.64 %) and iridoids (1.35-3.09 %) in the herb of *Melampyrum pratense* has been studied by Petrichenko *et al.* (2005). Additionally, the anticonvulsive activity has been determined *in vivo* and highest activity in the dose of 100 and 200 mg/kg has been found in the dry aqueous extract which was explained by the combined action of biologically active substances (Petrichenko *et al.*, 2005). The amount of flavonoids contained in *Melampyrum pratense* has also been evaluated by differential spectrophotometry. Thereby, the content was calculated with reference to the state standard sample of cinaroside, and was in the range of 0.1-0.7 %. (Petrichenko *et al.*, 2004).

Flavonoids are the most common group of plant polyphenols and are present in a wide range of fruits and vegetables. From the 5000 different flavonoids identified until now, in plants, most are conjugated with sugars (glycosides). There is an enormous interest in the possible health benefits of flavonoids, especially due to their potent antioxidant and free-radical scavenging activities observed *in vitro* (Ross *et al.*, 2002). They have been reported to possess anti-viral (Liu *et al.*, 2010; Orhan *et al.*, 2010; Savi *et al.*, 2010), anti-microbial (Doss *et al.*, 2011; Ozcelik *et al.*, 2011), anti-inflammatory (Chirumbolo, 2010; Figueirinha *et al.*, 2010; Serafini *et al.*, 2010) and anti-allergic potential expressed on different cell types, both from animals and humans. Inhibitory effects on numerous molecular targets in the micromolar concentration range, either by downregulating or suppressing various inflammatory pathways and functions, have been reported. Depending on little changes in the flavone-backbone flavonoids can play a modulating, biphasic and regulatory action on immunity and inflammation (Chirumbolo, 2010).

Intercellular adhesion molecule-1 (ICAM-1) has been implicated in the processes of inflammation and carcinogenesis. Investigations of the effects of flavonols (kaempferol, quercetin, and myricetin) and flavones (flavone, chrysin, apigenin, luteolin, baicalein, and baicalin) on the TNF-α stimulated ICAM-1 expression revealed kaempferol, chrysin, apigenin, and luteolin as active inhibitors of ICAM-1 expression. Additionally, apigenin and luteolin were shown to inhibit IKK activity, the IκBα degradation, NF-κB DNA-protein binding, and the NF-κB luciferase activity. The 3-mitogen-activated protein kinase (MAPK), activator protein-1 (AP-1), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activities were inhibited by apigenin and luteolin as well. Therefore, structure-activity relationships suggested the important role of OH- groups at positions 5 and 7 in the A ring and at position 4 in the B ring (Chen *et al.*, 2004). Numerous studies have already been conducted investigating the structure activity relationship of flavonoids in anti-inflammatory assays. In a comparative study on 37 flavonoids regarding their suppressive effect on TNF-α stimulated E-selectin expression in HUVECs fisetin, luteolin and apigenin (flavones), kaempferol and quercetin (flavonols), eriodictyol (flavanones), genistein (isoflavones) and butein (chalcones) exhibited inhibitory effects. Subsequent structure activity relations suggested that the C2-C3 double bond of the C-ring and an oxo-group in position 4 are essential for their inhibitory activities (Takano-Ishikawa *et al.*, 2003). Several flavonoids were investigated for the inhibitory effect on LPS-induced TNF-α production from macrophages. Positive results were provided for the flavones luteolin, apigenin and chrysin, the flavonols quercetin and myricetin, the flavanonol taxifolin and the anthocyanidin cyanidin chloride *in vitro*. Nevertheless *in vivo* serum TNF-α production was inhibited only by luteolin or apigenin, and only luteolin or quercetin inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear edema. These results suggest that the structure of luteolin is most suitable for the oral anti-inflammatory activity and that existence or disappearance of a hydroxy group may cause a loss of efficiency (Ueda *et al.*, 2004). Moreover, luteolin was shown to display excellent radical scavenging and cytoprotective properties, especially when tested in complex biological systems where it can interact with other antioxidants like vitamins. Nevertheless, its anti-inflammatory effects at micromolar concentrations can only be partly explained by its

antioxidant capacities. They include activation of antioxidative enzymes, suppression of the NF-κB pathway and inhibition of proinflammatory substances.

The glycosidic form of luteolin often present in plants is cleaved *in vivo* and the aglycones are conjugated and metabolized after nutritional uptake which has to be considered when evaluating *in vitro* studies (Seelinger *et al.*, 2008). Besides the inhibition of the induction of inflammatory cytokines such as TNF-α, IL-8 , IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) luteolin was found to attenuated cyclooxygenase (COX)-2 expression and intracellular Ca^{2+} levels (Kang *et al.*, 2010).

Metabolic studies concerning flavonoids showed deglycosylation, glucuronidation, sulfation and methylation steps occurring at various points during passage through the wall of the small intestine into the circulatory system and subsequent transport to the liver in the portal vein. Compounds that are not absorbed in the small intestine pass into the large intestine where they are degraded by the colonic microflora to phenolic acids, which can be absorbed into the circulatory system and subjected to phase II metabolites prior to excretion. Initially, the protective effect of flavonoids was thought to be due to their antioxidant properties by lowering the levels of free radicals within the body. However, recent studies indicate that the metabolites of those dietary phenolics exert modulatory effects in cells through selective actions on different components of the intracellular signalling cascades. Moreover, the intracellular concentrations required to affect cell signalling pathways are considerably lower than those necessary for the impact on antioxidant capacity (Crozier *et al.*, 2009).

The stilbene lunularin is mainly known from liverworts (Lu *et al.*, 2005; Ludwiczuk *et al.*, 2009). Besides, in higher plants it was found in celery, *Apium graveolens* (Zhou *et al.*, 2009) and *Morus* species (Syah *et al.*, 2004). Activity studies have only been conducted concerning antibacterial/ - fungal and antioxidant characteristics (Ramos *et al.*, 2006) but nothing has been found concerning antiphlogistic targets. Therefore, the recent study gives first insight in a possible anti-inflammatory action of this compound as it was able to downregulate E-selectin and IL-8 after stimulation with LPS and did bind *in silico* as well as *in vitro* to PPAR-γ.

In conclusion, I can say that the complexity of plant extracts means inexhaustible investigations of their various components as well as the interactions of all these compounds and their metabolites when considering *in vivo* effects. However, it is exactly this complexity making them so interesting and effective in and for a living organism. In this study the whole extracts generally revealed higher activity as the isolated "active principles", suggesting a higher value of extracts compared to single compounds. Moreover, the investigations of the present thesis provide some scientific evidence for the traditional usage of plant drugs. The ethnopharmacological screening approach was shown to be an efficient tool for the discovery of new scaffolds for drug development, which then could effectively be used in clinic.

5 Summary

In contrast to Traditional Chinese Medicine (TCM), Traditional European Medicine (TEM), including Austria`s ethnomedicine, got more and more lost during the advance of modern Western medicine. The generation of the so called VOLKSMED-database is an attempt to gather and conserve the remaining knowledge about the traditionally used medicinal plant species/ genera of Austria, its indications as well as the application and methods of preparation. Out of this database 37 drugs of 33 different species and 10 families respectively (Apiaceae, Malvaceae, Onograceae, Orobanchaceae, Plantaginaceae, Polygonaceae, Rosaceae, Scrophulariaceae, Urticaceae and Verbenaceae) were selected in order to substantiate their reported anti-inflammatory activities (gastrointestinal diseases, rheumatism, wound healing…). Selection was based on this anti-inflammatory action as well as on a literature research to evaluate the current state of investigation on the diverse plant species. Moreover, an intensive literature survey concerning the selected plants resulted in a list of known NPs, which were converted into mol-files for further processing in the *in silico* screening on different anti-inflammatory molecular targets (e.g. PPAR-γ or FXR).

Finally, 130 extracts of different polarity were prepared from these drugs and tested *in vitro* on various anti-inflammatory targets (activation of PPAR-α and -γ, inhibition of NF-κB, downregulation of IL-8 and E-selectin). This pharmacological screening resulted in 90 % actives of wich two very promising plants (*Melampyrum pratense* and *Peucedanum ostruthium*) were selected for further bioassay guided fractionation. For the isolation of active compounds various chromatographic techniques like CC (Column Chromatography), SPE (Solid Phase Extraction) and (preparative) HPLC (High-performance liquid chromatography) were used. The structure elucidation of the pure active constituents was performed by NMR (Nuclear Magnetic Resonance) and MS (Mass Spectrometry).

Concerning *Peucedanum ostruthium* the detannified MeOH extract showed highest activity. Therefore, further fractionated by solid phase extraction (SPE) on C18 cartriges was performed. The most active 70 % methanolic SPE subfraction therof was then separated via preparative HPLC. With further purification steps on silica gel six

coumarins and one chromone have been isolated. Several more coumarins have been identified using HPLC-DAD/-MS. However, the activity of the single compounds was much lower than that of the initial extracts. This effect might be due to the known PPAR ligands fatty acids and to the NF-κB inhibiting phthalates also identified in the active extracts and fractions via GC-MS. Another explanation would be synergistic effects of the single coumarins among themselves or together with other compound classes.

Concerning *Melampyrum pratense* high activity on the downregulation of IL-8 und E-selectin was found in all initial extracts. In the methanolic extract polar iridoid glycosides like aucubin were identified. In its apolar tannin free subextract flavonoids, especially apigenin und luteolin, were determined to be responsible for the activity of this extract. Moreover, the iridoid glycosides, melampyroside and mussaenoside, and the phenol lunularin were isolated from the chlorophyll depleted dichloromethan extract for the first time for this *Melampyrum* species. Again, these purified substances were found to account for the activity of their mother extract.

Finally, a first scientific indication for the traditional usage of several Austrian medicinal plants was provided. Moreover, some of the potential active principles in terms of an anti-inflammatory activity of *Peucedanum ostruthium* and *Melampyrum pratense* could be isolated and identified.

6 Zusammenfassung

Im Gegensatz zur Traditionell Chinesischen Medizin (TCM), ist die Traditionelle Europäische Medizin (TEM) und damit auch jene Österreichs, mit dem Fortschritt der westlichen Medizin immer mehr in Vergessenheit geraten. Die Entwicklung der sogenannten VOLKSMED Datenbank ist ein Versuch, das noch vorhandene Wissen über traditionell verwendete Medizinpflanzen aus Österreich, ihre Anwendungsgebiete, die Art der Anwendung und deren Zubereitungen zusammenzutragen und zu bewahren. Aus dieser Datenbank wurden 37 verschiedene Drogen von insgesamt 33 Pflanzenarten und 10 Familien (Apiaceae, Malvaceae, Onograceae, Orobanchaceae, Plantaginaceae, Polygonaceae, Rosaceae, Scrophulariaceae, Urticaceae and Verbenaceae) ausgewählt, um ihre überlieferte entzündungshemmende Wirkung (z.B. gegen Rheuma, zur Wundheilung, gegen Magenleiden usw.) auf wissenschaftlicher Basis zu untersuchen. Die Auswahl basierte zum einen auf eben dieser entzündungshemmenden Wirkung als auch auf einer Literatursuche zum aktuellen Stand der Wissenschaft zu den verschiedensten Pflanzenarten. Außerdem wurden im Zuge einer intensiven Literatursuche zu den ausgewählten Pflanzen mol-files der bereits bekannten Substanzen generiert, um diese *in silico* ebenfalls auf antiphlogistische Targets (e.g. PPAR-γ oder FXR) zu screenen.

Schließlich wurden von diesen Drogen 130 Extrakte unterschiedlicher Polarität hergestellt und *in vitro* auf verschiedenste antiphlogistische Zielstrukturen (Aktivierung von PPAR-α bzw. -γ, Inhibierung von NF-κB, und Herunterregulierung von IL-8 und E-selectin) getestet. Dieses pharmakologische Screening ergab insgesamt 90 % Treffer, aus denen zwei besonders wirksame Pflanzen für eine bioaktivitätsgeleitete Fraktionierung ausgewählt wurden, nämlich *Peucedanum ostruthium* und *Melampyrum pratense*. Für die Isolierung aktiver Einzelsubstanzen wurden verschiedenste chromatographische Techniken CC (Säulen Chromatographie), SPE (Festphasenextraktion) und (preparative) HPLC (Hochleistungsflüssigkeitschromatographie) angewendet. Die Strukturaufklärung der aufgereinigten Substanzen erfolgte mittels NMR Spektroskopie (Kernspinresonanzspektroskopie) und MS (Massen Spektrometire).

Bei *Peucedanum ostruthium* war der von Tanninen befreite MeOH Extrakt besonders wirksam. Daher wurde dieser in einem ersten Schritt mittles Festphasenextraktion in

C18-Kartuschen fraktioniert. Die aktivste 70 % methanolische Subfraktion hiervon wurde weiters mittels preparativer HPLC aufgetrennt. Nach weiteren Aufreinigungsschritten auf Silicagel wurden sechs Cumarine und ein Chromon isoliert. Einige weitere Cumarine konnten mittels HPLC-DAD/-MS identifiziert werden.

Die Aktivität der Einzelsubstanzen war allerdings nicht so hoch wie die der Ausgangsextrakte. Verantwortlich dafür könnten sowohl PPAR aktive Fettsäuren als auch NF-κB inhibierende Phthalate sein, die ebenfalls in größeren Mengen in den aktiven Extrakten/ Fraktionen mittels GC-MS nachgewiesen wurden. Eine andere Möglichkeit wäre ein synergistischer Effekt der einzelnen Cumarinen miteinander oder mit weiteren Inhaltsstoffen.

Alle Ausgangsextrakte von *Melampyrum pratense* zeigten eine starke Herunterregulierung von IL-8 und E-selectin. Im methanolischen Extrakt wurden polare Iridoidglykoside, wie z.B. Aucubin nachgewiesen. Im apolaren Tannin freien Anteil fand man hauptsächlich Flavonoide, insbesondere Apigenin und Luteolin, die sich für die Aktivität dieses Extrakts verantwortlich zeigten. Weiters wurde aus dem von Chlorophyll befreiten Dichlormethan Extrakt die beiden Iridoidglykoside Melampyrosid und Mussaenosid, sowie eine Stilben artige Substanz namens Lunularin zum ersten Mal für diese *Melampyrum* Art isoliert. Diese Reinsubstanzen trugen ebenfalls zur Wirkung der Ausgangsextrakte bei.

Letztendlich wurde mit der vorliegenden Arbeit versucht, erste wissenschaftliche Hintergründe für die traditionelle Anwendung zahlreicher österreichischer Medizinpflanzen zu liefern. Weiters konnten einige an der anti-inflammatorischen Wirkung von *Peucedanum ostruthium* und *Melampyrum pratense* mitbeteiligte Substanzen isoliert und identifiziert werden.

7 References

COPE Cytokines & Cells Online Pathfinder Encyclopedia

http://www.copewithcytokines.de 29.03.2011 19:30

Abraham K, Woehrlin F, Lindtner O, Heinemeyer G, Lampen A (2010). Toxicology and risk assessment of coumarin: Focus on human data. *Mol. Nutr. Food Res.* **54**(2)**:** 228-239.

Adams M, Berset C, Kessler M, Hamburger M (2009). Medicinal herbs for the treatment of rheumatic disorders--a survey of European herbals from the 16th and 17th century. *J Ethnopharmacol* **121**(3)**:** 343-359.

Baggiolini M, Clark-Lewis I (1992). Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* **307**(1)**:** 97-101.

Baggiolini M, Uguccioni M, D'Apuzzo M (1995). Interleukin-8 and other chemokines: New perspectives in inflammation. *Falk Symp.* **78**(Cytokines and the Liver)**:** 5-13.

Balakin KV, Savchuk NP (2006). Computational methods for analysis of high-throughput screening data. *Curr. Comput.-Aided Drug Des.* **2**(1)**:** 1-19.

Bamborough P, Callahan JF, Christopher JA, Kerns JK, Liddle J, Miller DD*, et al.* (2009). Progress towards the development of anti-inflammatory inhibitors of IKKbeta. *Curr Top Med Chem* **9**(7)**:** 623-639.

Barreiro O, Martin P, Gonzalez-Amaro R, Sanchez-Madrid F (2010). Molecular cues guiding inflammatory responses. *Cardiovasc. Res.* **86**(2)**:** 174-182.

Bensky D, Clavey S, Stöger E (2004). *Materia Medica: Chinese Herbal Medicine*. 3rd edition. Eastland Press.

Berger C, Hoffmann K, Vasquez JG, Mane S, Lewis J, Filler R*, et al.* (2010). Rapid generation of maturationally synchronized human dendritic cells: contribution to the clinical efficacy of extracorporeal photochemotherapy. *Blood* **116**(23)**:** 4838-4847.

Berger C, Lewis J, Girardi M, Tigelaar R, Edelson R (2011). Validity of evidence demonstrating efficacy of extracorporeal photochemotherapy. Reply to comments. *Blood* **117**(1)**:** 367.

Berger CL, Edelson R (2008). Cancer immunotherapy: from promise to practice. *Drugs Future* **33**(5)**:** 411-423.

Bethea D, Fullmer B, Syed S, Seltzer G, Tiano J, Rischko C*, et al.* (1999). Psoralen photobiology and photochemotherapy: 50 years of science and medicine. *J. Dermatol. Sci.* **19**(2)**:** 78-88.

Beutler B, Rietschel Ernst T (2003). Innate immune sensing and its roots: the story of endotoxin. *Nature reviews. Immunology* **3**(2)**:** 169-176.

Bhaumik D, Patil CK, Campisi J (2009). MicroRNAs: an important player in maintaining a balance between inflammation and tumor suppression. *Cell Cycle* **8**(12)**:** 1822.

Bradley JR (2008). TNF-mediated inflammatory disease. *J Pathol* **214**(2)**:** 149-160.

Brasier AR (2010). The nuclear factor-κB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc. Res.* **86**(2)**:** 211-218.

Broaddus VC, Hebert CA (1996). The role of interleukin-8 in inflammatory diseases. *Chemoattractant Ligands Their Recept.* **:** 1-28.

Broda B, Swiatek L, Druzynski J (1969). Aucubin determination in certain Scrophulariaceae species. *Acta Poloniae Pharmaceutica* **26**(3)**:** 255-258.

Burgert M (2011). Aufbau einer MS^n Datenbank mittels ESI-Ionenfallen-Massen-spektrometrie. Diploma Thesis, University of Vienna, Vienna.

Butenandt A, Marten G (1932). Vegetable, fish and insect poisons. V. Constituents of masterwort (*Imperatoria ostruthium*). *Justus Liebigs Ann. Chem.* **495:** 187-210.

Cai Y, Kleiner H, Johnston D, Dubowski A, Bostic S, Ivie W*, et al.* (1997). Effect of naturally occurring coumarins on the formation of epidermal DNA adducts and skin tumors induced by benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene in SENCAR mice. *Carcinogenesis* **18**(8)**:** 1521-1527.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A FIELD Full Journal Title:Proceedings of the National Academy of Sciences of the United States of America* **72**(9)**:** 3666-3670.

Chang MW-F, Grillari J, Mayrhofer C, Fortschegger K, Allmaier G, Marzban G*, et al.* (2005). Comparison of early passage, senescent and hTERT immortalized endothelial cells. *Exp. Cell Res.* **309**(1)**:** 121-136.

Chawla A (2010). Control of Macrophage Activation and Function by PPARs. *Circ. Res.* **106**(10)**:** 1559-1569.

Chen C-C, Chow M-P, Huang W-C, Lin Y-C, Chang Y-J (2004). Flavonoids inhibit tumor necrosis factor-α-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1)

in respiratory epithelial cells through activator protein-1 and nuclear factor-κ: Structure-activity relationships. *Mol. Pharmacol.* **66**(3)**:** 683-693.

Chen T, Huang Z, Wang L, Wang Y, Wu F, Meng S*, et al.* (2009). MicroRNA-125a-5p partly regulates the inflammatory response, lipid uptake, and ORP9 expression in oxLDL-stimulated monocyte/macrophages. *Cardiovasc. Res.* **83**(1)**:** 131-139.

Chen X, Pi R, Zou Y, Liu M, Ma X, Jiang Y*, et al.* (2010). Attenuation of experimental autoimmune encephalomyelitis in C57 BL/6 mice by osthole, a natural coumarin. *Eur. J. Pharmacol.* **629**(1-3)**:** 40-46.

Chirumbolo S (2010). The role of quercetin, flavonols and flavones in modulating inflammatory cell function. *Inflammation Allergy: Drug Targets* **9**(4)**:** 263-285.

Cho JY, Lee J, Park J, Park MH (1998). Isolation of tumor necrosis factor-alpha inhibitory components from *Angelica koreana*. *Yakhak Hoechi* **42**(2)**:** 125-131.

Cimino GD, Gamper HB, Isaacs ST, Hearst JE (1985). Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry. *Annu. Rev. Biochem.* **54:** 1151-1193.

Cisowski W, Sawicka U, Mardarowicz M, Asztemborska M, Luczkiewicz M (2001). Essential oil from herb and rhizome of *Peucedanum ostruthium* (L. Koch.) ex DC. *Z. Naturforsch., C: J. Biosci.* **56**(11/12)**:** 930-932.

Cox PA (1994). The ethnobotanical approach to drug discovery: strengths and limitations. *Ciba Found. Symp.* **185:** 25-41.

Cremin PA, Zeng L (2002). High-throughput analysis of natural product compound libraries by parallel LC-MS evaporative light scattering detection. *Anal. Chem.* **74**(21)**:** 5492-5500.

Crozier A, Jaganath IB, Clifford MN (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **26**(8)**:** 1001-1043.

Curini M, Cravotto G, Epifano F, Giannone G (2006). Chemistry and biological activity of natural and synthetic prenyloxycoumarins. *Curr. Med. Chem.* **13**(2)**:** 199-222.

Cury-Boaventura MF, Kanunfre CC, Gorjao R, de Lima TM, Curi R (2006). Mechanisms involved in Jurkat cell death induced by oleic and linoleic acids. *Clin. Nutr.* **25**(6)**:** 1004-1014.

Dai R, Ahmed SA (2011). MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. *Translational Research* **157**(4)**:** 163-179.

Davidson-Moncada J, Papavasiliou FN, Tam W (2010). MicroRNAs of the immune system: roles in inflammation and cancer. *Annals of the New York Academy of Sciences* **1183**(Year in Immunology 2)**:** 183-194.

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

Doss A, Vijayasanthi M, Parivuguna V, Venkataswamy R (2011). Antimicrobial effects of the flavonoid fractions of *Mimosa pudica* L. leaves. *J. Pharm. Res.* **4**(5)**:** 1438-1439.

Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK*, et al.* (2010). A meta-analysis of cytokines in major depression. *Biological psychiatry* **67**(5)**:** 446-457.

Dvorak Z, Vrzal R, Maurel P, Ulrichova J (2006). Differential effects of selected natural compounds with anti-inflammatory activity on the glucocorticoid receptor and NF-κB in HeLa cells. *Chem.-Biol. Interact.* **159**(2)**:** 117-128.

Eldridge GR, Vervoort HC, Lee CM, Cremin PA, Williams CT, Hart SM*, et al.* (2002). High-throughput method for the production and analysis of large natural product libraries for drug discovery. *Anal. Chem.* **74**(16)**:** 3963-3971.

Exarchou V, Krucker M, van Beek TA, Vervoort J, Gerothanassis IP, Albert K (2005). LC-NMR coupling technology: Recent advancements and applications in natural products analysis. *Magn. Reson. Chem.* **43**(9)**:** 681-687.

Fentem JH, Fry JR (1993). Species differences in the metabolism and hepatotoxicity of coumarin. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* **104**(1)**:** 1-8.

Figueirinha A, Cruz MT, Francisco V, Lopes MC, Batista MT (2010). Anti-inflammatory activity of *Cymbopogon citratus* leaf infusion in lipopolysaccharide-stimulated dendritic cells: Contribution of the polyphenols. *J. Med. Food* **13**(3)**:** 681-690.

Fischer M, Adler W, Oswald K (2008). *Exkursionsflora von Österreich, Liechtenstein und Südtirol*. Vol. 3. Ulmer Verlag, Stuttgart und Wien.

Flamm S, Kroeber L, Seel H (1940). *Pharmakodynamik deutscher Heilpflanzen*. Hippokrates-Verlag Marquardt & Cie, Stuttgart: Stuttgart. 179 ff.

Forman BM, Chen J, Evans RM (1996). The peroxisome proliferator-activated receptors: ligands and activators. *Ann N Y Acad Sci* **804:** 266-275.

Fruchart J-C (2009). Peroxisome proliferator-activated receptor-alpha (PPARα): At the crossroads of obesity, diabetes and cardiovascular disease. *Atherosclerosis* **205**(1)**:** 1-8.

Galishevskaya EE, Petrichenko VM (2011). Phenolic compounds from two *Melampyrum* species. *Pharmaceutical Chemistry Journal* **44**(9)**:** 497-500.

Ganzera M, Sturm S, Stuppner H (1997). HPLC-MS and MECC analysis of coumarins. *Chromatographia* **46**(3/4)**:** 197-203.

Garcia-Argaez AN, Ramirez Apan TO, Parra Delgado H, Velazquez G, Martinez-Vazquez M (2000). Anti-inflammatory activity of coumarins from *Decatropis bicolor* on TPA ear mice model. *Planta Med* **66**(3)**:** 279-281.

Gasparro FP (1996). Psoralen photobiology: recent advances. *Photochem. Photobiol.* **63**(5)**:** 553-557.

Gasparro FP (2000). The role of PUVA in the treatment of psoriasis. Photobiology issues related to skin cancer incidence. *Am J Clin Dermatol* **1**(6)**:** 337-348.

Geiger PL (1939). *Handbuch der Pharmazie zum Gebrauche bei Vorlesungen und zum Selbstunterrichte für Ärzte Apotheker und Droguisten*. Vol. 2: C. F. Winter. C. Gerold. 1843, Heidelberg und Wien.

Genovese S, Curini M, Epifano F (2010). Prenyloxyphenylpropanoids as a novel class of anti-inflammatory agents. *Anti-Inflammatory Anti-Allergy Agents Med. Chem.* **9**(2)**:** 158-165.

Gerlach S (2008) Wissenschaftliche Reflexionen über traditionelle Arzneipflanzen in Österreich. Doctor Thesis, University of Vienna, Vienna.

Gerlach S, Saukel J, Kubelka W (2006). Pflanzen in der österreichischen Volksmedizin. Die „VOLKSMED-Datenbank“. *Sci Pharm.* **74(Suppl 1):** 36.

Ghosh S, Hayden MS (2008). New regulators of NF-κB in inflammation. *Nat. Rev. Immunol.* **8**(11)**:** 837-848.

Gijbels MJM, Fischer FC, Scheffer JJC, Svendsen AB (1984). Phthalides in roots of *Capnophyllum peregrinum* and *Peucedanum ostruthium*. *Planta Med.* **50**(1)**:** 110.

Gizard F, Bruemmer D (2008a). Transcriptional control of vascular smooth muscle cell proliferation by peroxisome proliferator-activated receptor-γ: therapeutic implications for cardiovascular diseases. *PPAR Res.***:** online File 2295-2231-2290.

Gizard F, Nomiyama T, Zhao Y, Findeisen HM, Heywood EB, Jones KL*, et al.* (2008b). The PPARα/p16INK4a pathway inhibits vascular smooth muscle cell proliferation by repressing cell cycle-dependent telomerase activation. *Circ. Res.* **103**(10)**:** 1155-1163.

Glass CK, Ogawa S (2006). Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat. Rev. Immunol.* **6**(1)**:** 44-55.

Glass CK, Saijo K (2010). Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat. Rev. Immunol.* **10**(5)**:** 365-376.

Glowniak K, Dragan T, Zgorka G, Gawron A (1994). Phenolic and some other furanocoumarins in *Angelica* roots treated with fungi. *Acta Hortic.* **381**(International Symposium on Natural Phenols in Plant Resistance, Vol. 2)**:** 600-604.

Goekay O, Kuehner D, Los M, Goetz F, Bertsche U, Albert K (2010). An efficient approach for the isolation, identification and evaluation of antimicrobial plant components on an analytical scale, demonstrated by the example of Radix Imperatoriae. *Analytical and Bioanalytical Chemistry* **398**(5)**:** 2039-2047.

Hadacek F, Mueller C, Werner A, Greger H, Proksch P (1994). Analysis, isolation and insecticidal activity of linear furanocoumarins and other coumarin derivatives from *Peucedanum* (Apiaceae: Apioideae). *J. Chem. Ecol.* **20**(8)**:** 2035-2054.

Hänsel R, Sticher O (2010). *Pharmakognosie – Phytopharmazie*. 9th edn. Springer Medizin Verlag Heidelberg.

Harmala P, Vuorela H, Hiltunen R, Nyiredy S, Sticher O, Tornquist K*, et al.* (1992). Strategy for the isolation and identification of coumarins with calcium antagonistic properties from the roots of *Angelica archangelica*. *Phytochem. Anal.* **3**(1)**:** 42-48.

Harvey AL (2007). Natural products as a screening resource. *Curr. Opin. Chem. Biol.* **11**(5)**:** 480-484.

Harvey AL (2002). Natural products for high-throughput screening. *Adv. Phytomed.* **1**(Ethnomedicine and Drug Discovery)**:** 39-44.

Harvey AL, Clark RL, Mackay SP, Johnston BF (2010). Current strategies for drug discovery through natural products. *Expert Opin. Drug Discovery* **5**(6)**:** 559-568.

Hayden MS, Ghosh S (2011). NF-κB in immunobiology. *Cell Res.* **21**(2)**:** 223-244.

Hayden MS, Ghosh S (2008). Shared principles in NF-κB signaling. *Cell (Cambridge, MA, U. S.)* **132**(3)**:** 344-362.

Hegi G (1906). *Illustrierte Flora von Mitteleuropa*. 5/2. A. Pichler`s Witwe & Sohn: Vienna. 1396 ff.

Herde A Untersuchung der Cumarinmuster in Früchten ausgewählter Apiaceae. Doctor Thesis, University of Hamburg, Hamburg, 2005.

Hiermann A, Schantl D (1998). Antiphlogistic and antipyretic activity of *Peucedanum ostruthium*. *Planta Med.* **64**(5)**:** 400-403.

Hiermann A, Schantl D, Schubert-Zsilavecz M, Reiner J (1996). Coumarins from *Peucedanum ostruthium*. *Phytochemistry* **43**(4)**:** 881-883.

Hihi AK, Michalik L, Wahli W (2002). PPARs: transcriptional effectors of fatty acids and their derivatives. *Cell. Mol. Life Sci.* **59**(5)**:** 790-798.

Hogg N, Patzak I, Willenbrock F (2011). The insider's guide to leukocyte integrin signalling and function. *Nature Reviews Immunology* **11**(6)**:** 416-426.

Hong J, Shin KH, Lim SS, Kwak JH, Zee O, Ishihara K*, et al.* (2008). Lead compounds for anti-inflammatory drugs isolated from the plants of the traditional oriental medicine in Korea. *Inflammation Allergy: Drug Targets* **7**(3)**:** 195-202.

Hostettmann K, Marston A (2007). The search for new drugs from higher plants. *Chimia* **61**(6)**:** 322-326.

Hoult JR, Paya M (1996). Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *Gen Pharmacol* **27**(4)**:** 713-722.

Hu J-F, Garo E, Yoo H-D, Cremin PA, Zeng L, Goering MG*, et al.* (2005). Application of capillary-scale NMR for the structure determination of phytochemicals. *Phytochem. Anal.* **16**(2)**:** 127-133.

Huang W, Glass CK (2010). Nuclear receptors and inflammation control: Molecular mechanisms and pathophysiological relevance. *Arterioscler., Thromb., Vasc. Biol.* **30**(8)**:** 1542-1549.

Jeong H-J, Koo H-N, Na H-J, Kim M-S, Hong S-H, Eom J-W*, et al.* (2002). Inhibition of TNF α and IL 6 production by aucubin through blockade of NF-κB activation in RBL-2H3 mast cells. *Cytokine* **18**(5)**:** 252-259.

Ji Y, Zeng G, Rui Y (1994). Interleukin-1 beta induced proliferation of bovine cerebromicrovascular smooth muscle cells and antagonism of drugs. *Zhongguo Yaolixue Tongbao* **10**(4)**:** 271-273.

Joa H, Vogl S, Atanasov AG, Zehl M, Nakel T, Fakhrudin N*, et al.* (2011). Identification of ostruthin from *Peucedanum ostruthium* rhizomes as an inhibitor of vascular smooth muscle cell proliferation. *J. Nat. Prod.* **74**(6)**:** 1513-1516.

Kaneko T, Baba N, Matsuo M (2003). Protection of coumarins against linoleic acid hydroperoxide-induced cytotoxicity. *Chem.-Biol. Interact.* **142**(3)**:** 239-254.

Kang O-H, Choi J-G, Lee J-H, Kwon D-Y (2010). Luteolin isolated from the flowers of *Lonicera japonica* suppresses inflammatory mediator release by blocking NF-κB and MAPKs activation pathways in HMC-1 cells. *Molecules* **15:** 385-398.

Karimova SG, Smirnova SG, Nasyrov KM (1966). Chemical composition and pharmacology of the Scrophulariaceae. *Dtsch. Brau.***:** 153-155.

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.

Kim Y-K, Kim YS, Ryu SY (2007). Antiproliferative effect of furanocoumarins from the root of *Angelica dahurica* on cultured human tumor cell lines. *Phytother. Res.* **21**(3)**:** 288-290.

Kinghorn AD (2008). Drug discovery from natural products. *Foye's Princ. Med. Chem. (6th Ed.)***:** 10-25.

Kirmizibekmez H, Atay I, Kaiser M, Brun R, Cartagena MM, Carballeira NM*, et al.* (2011). Antiprotozoal activity of *Melampyrum arvense* and its metabolites. *Phytotherapy research : PTR* **25**(1)**:** 142-146.

Kneip M (1989). Volksmedizinische Anwendung von Heilpflanzen in Österreich. Diploma Thesis, University of Vienna, Vienna.

Koehn FE, Carter GT (2005). The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery* **4**(3)**:** 206-220.

Köhler FE (1987). *Köhlers Medizinal-Pflanzen in naturgetreuen Abbildungen und kurz erläuterndem Texte: Atlas zur Pharmacopoea germanica*. Band 2. Botanischer Verlag Franz Eugen Köhler [1883-1914], Gera-Untermhaus.

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. *Eur. J. Pharmacol.* **394**(1), 149-156.

Kubelka W, Schlederer H, Holzschuh L (1984). *Prüfkartei für Apotheken*. Österreichische Apotheker- Verlagsges.: Wien. II/152 ff.

Lambert M, Wolfender J-L, Strk D, Christensen SB, Hostettmann K, Jaroszewski JW (2007). Identification of natural products using HPLC-SPE combined with CapNMR. *Anal. Chem.* **79**(2)**:** 727-735.

Lawrence T (2009). The Nuclear Factor NF-kB Pathway in Inflammation. *Cold Spring Harbor Perspect. Biol.* **1**(6)**:** No pp given.

Lawrence T, Fong C (2010). The resolution of inflammation: Anti-inflammatory roles for NF-κB. *Int. J. Biochem. Cell Biol.* **42**(4)**:** 519-523.

Li AC, Glass CK (2004). PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. *J. Lipid Res.* **45**(12)**:** 2161-2173.

Li Z, Liu M, Yang H (2009). The study of vasoactive effect of pungent herbs' chemical constituents in Umbelliferae. *Zhongyao Yaoli Yu Linchuang* **25**(1)**:** 38-40.

Liao P-C, Chien S-C, Ho C-L, Wang EIC, Lee S-C, Kuo Y-H*, et al.* (2010a). Osthole regulates inflammatory mediator expression through modulating NF-κB, mitogen-activated protein kinases, protein kinase C, and reactive oxygen species. *Journal of Agricultural and Food Chemistry* **58**(19)**:** 10445-10451.

Liao Z-G, Liang X-L, Zhu J-Y, Zhao G-W, Yang M, Wang G-F*, et al.* (2010b). Correlation between synergistic action of Radix *Angelica dahurica* extracts on analgesic effects of *Corydalis* alkaloid and plasma concentration of dl-THP. *J. Ethnopharmacol.* **129**(1)**:** 115-120.

Liu J, Zhang W, Zhou L, Wang X, Lian Q (2005a). Anti-inflammatory effect and mechanism of osthole in rats. *Zhong Yao Cai* **28**(11)**:** 1002-1006.

Liu L, Ning Z-Q, Shan S, Zhang K, Deng T, Lu X-P*, et al.* (2005b). Phthalide lactones from *Ligusticum chuanxiong* inhibit lipopolysaccharide-induced TNF-α production and TNF-α-mediated NF-κB activation. *Planta Med.* **71**(9)**:** 808-813.

Liu SXL, Chiou GCY (1996). Effects of Chinese herbal products on mammalian retinal functions. *J. Ocul. Pharmacol. Ther.* **12**(3)**:** 377-386.

Liu T, Zhao J, Li H, Ma L (2010). Evaluation on anti-hepatitis viral activity of *Vitis vinifer* L. *Molecules* **15:** 7415-7422.

Lopez Alan D, Mathers Colin D, Ezzati M, Jamison Dean T, Murray Christopher JL (2006). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* **367**(9524)**:** 1747-1757.

Lopez SN, Ramallo IA, Sierra MG, Zacchino SA, Furlan RLE (2007). Chemically engineered extracts as an alternative source of bioactive natural product-like compounds. *Proc. Natl. Acad. Sci. U. S. A.* **104**(2)**:** 441-444.

Lu Z-Q, Fan P-H, Ji M, Lou H-X (2005). Terpenoids and bisbibenzyls from Chinese liverworts *Conocephalum conicum* and *Dumortiera hirsuta*. *J. Asian Nat. Prod. Res.* **8**(1-2)**:** 187-192.

Ludwiczuk A, Komala I, Pham A, Bianchini J-P, Raharivelomanana P, Asakawa Y (2009). Volatile components from selected Tahitian liverworts. *Nat. Prod. Commun.* **4**(10)**:** 1387-1392.

Madaus G (1976). *Lehrbuch der Biologischen Heilmittel Band II, Abteilung Heilpflanzen, Nachdruck 1976*. Band II. Olms Verlag, Auflage: (Nachdr. d. Ausg. Leipzig 1938) New York. 757.

Maloney B (1972). *Traditional Herbal Cures in Country Cavan. Part 1*. Vol. 1. Ulster Folklife, Northern Irland.

Marquez N, Sancho R, Ballero M, Bremner P, Appendino G, Fiebich BL*, et al.* (2004). Imperatorin inhibits T-cell proliferation by targeting the transcription factor NFAT. *Planta Med.* **70**(11)**:** 1016-1021.

Medzhitov R (2010). Inflammation 2010: New adventures of an old flame. *Cell* **140**(6)**:** 771-776.

Mogoşan C, Munteanu MF (2008). A comparative study on antiinflammatory effect of the tinctures from *Melampyrum bihariense* KERN and *Melampyrum cristatum* L. (Scrophulariaceae). *Farmacia* **LVI** (4)**:** 389-392.

Moon TC, Jin M, Son JK, Chang HW (2008). The effects of isoimperatorin isolated from *Angelica dahurica* on cyclooxygenase-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells. *Arch. Pharmacal Res.* **31**(2)**:** 210-215.

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 Rev. FIELD Full Journal Title:Cytokine & Growth Factor Reviews* **9**(1)**:** 9-23.

Murray RDH (2002). The naturally occurring coumarins. *Prog. Chem. Org. Nat. Prod.* **83:** 1-673.

Mutschler E, Geisslinger G, Kroemer HK, Ruth P, Schäfer-Korting M (2001). *Mutschler Arzneimittelwirkungen: Lehrbuch der Pharmakologie und Toxikologie*. 7th edition. Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart.

Neve BP, Fruchart JC, Staels B (2000). Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis. *Biochem. Pharmacol.* **60**(8)**:** 1245-1250.

Newman DJ (2008). Natural products as leads to potential drugs: an old process or the new hope for drug discovery? *J. Med. Chem.* **51**(9)**:** 2589-2599.

Newman DJ, Cragg GM (2009). Natural product scaffolds as leads to drugs. *Future Med. Chem.* **1**(8)**:** 1415-1427.

Newman DJ, Cragg GM (2007). Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **70**(3)**:** 461-477.

Newman DJ, Cragg GM, Snader KM (2003). Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* **66**(7)**:** 1022-1037.

Orhan DD, Oezcelik B, Oezgen S, Ergun F (2010). Antibacterial, antifungal, and antiviral activities of some flavonoids. *Microbiol. Res.* **165**(6)**:** 496-504.

Ozcelik B, Kartal M, Orhan I (2011). Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharm. Biol. (London, U. K.)* **49**(4)**:** 396-402.

Pascual G, Sullivan AL, Ogawa S, Gamliel A, Perissi V, Rosenfeld MG*, et al.* (2007). Anti-inflammatory and antidiabetic roles of PPAR-γ. *Novartis Found. Symp.* **286**(Fatty Acids and Lipotoxicity in Obesity and Diabetes)**:** 183-199.

Perkins ND (2007). Integrating cell-signalling pathways with NF-κB and IKK function. *Nat. Rev. Mol. Cell Biol.* **8**(1)**:** 49-62.

Petrichenko VM, Galishevskaya EE, Danilova VK, Shramm NI, Babiyan LK (2005). Standardisation and pharmaco-tecnological properties of the herb *Melampyrum pratense*. *Khimiko-Farmatsevticheskii Zhurnal* **39**(4)**:** 38-42.

Petrichenko VM, Galishevskaya YY (2004). Measurement of the amount of flavonoids in meadow cow-wheat (*Melampyrum pratensis*). *Farmatsiya (Moscow)*(1)**:** 24-25.

Picker P (2011) Bioactivity guided fractionation of *Betonica officinalis* and *Glechoma hederacea* focusing on anti-inflammatory activities. Doctor Thesis, University of Vienna, Vienna.

Potterat O, Hamburger M (2008). Drug discovery and development with plant-derived compounds. *Prog. Drug Res.* **65**(Natural Compounds as Drugs, Volume 1)**:** 45, 47-118.

Raetz CRH, Whitfield C (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71:** 635-700.

Ramos FA, Takaishi Y, Shirotori M, Kawaguchi Y, Tsuchiya K, Shibata H*, et al.* (2006). Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (*Allium cepa*) Skin. *Journal of Agricultural and Food Chemistry* **54**(10)**:** 3551-3557.

Rauwald HW, Brehm O, Odenthal KP (1991). Evaluation of the calcium antagonistic activity of *Peucedanum ostruthium* and *Olea europaea* constituents. *Pharm. Pharmacol. Lett.* **1**(2)**:** 78-81.

Reisch J, Khaled SA, Szendrei K, Novak I (1975). Natural product chemistry. 51. 5-Alkoxy-furanocoumarins from *Peucedanum ostruthium*. *Phytochemistry* **14**(8)**:** 1889-1890.

Roos G, Waiblinger J, Zschocke S, Liu JH, Klaiber I, Kraus W*, et al.* (1997). Isolation, identification, and screening for COX-1- and 5-LOX-inhibition of coumarins from *Angelica archangelica*. *Pharm. Pharmacol. Lett.* **7**(4)**:** 157-160.

Ross JA, Kasum CM (2002). Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **22:** 19-34.

Ryu SY, Kou NY, Choi HS, Ryu H, Kim TS, Kim KM (2001). Cnidicin, a coumarin, from the root of *Angelica koreana*, inhibits the degranulation of mast cell and the NO generation in RAW 264.7 cells. *Planta Med.* **67**(2)**:** 172-174.

Satsyperova IF (1968). Photosensitizing activity of furocoumarin-containing plants. *Rast. Resur.* **4**(4)**:** 570-579.

Savi LA, Caon T, de Oliveira AP, Sobottka AM, Werner W, Reginatto FH*, et al.* (2010). Evaluation of antirotavirus activity of flavonoids. *Fitoterapia* **81**(8)**:** 1142-1146.

Schinkovitz A, Gibbons S, Stavri M, Cocksedge MJ, Bucar F (2003). Ostruthin: an antimycobacterial coumarin from the roots of *Peucedanum ostruthium*. *Planta Med* **69**(4)**:** 369-371.

Schmid I, Sattler I, Grabley S, Thiericke R (1999). Natural products in high throughput screening: automated high-quality sample preparation. *J. Biomol. Screening* **4**(1)**:** 15-25.

Schroeder J-M (1992). Interleukin 8. *Adv. Neuroimmunol.* **2**(2)**:** 109-124.

Seelinger G, Merfort I, Schempp CM (2008). Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. *Planta Med.* **74**(14)**:** 1667-1677.

Segal B, Rhodus Nelson L, Patel K (2008). Tumor necrosis factor (TNF) inhibitor therapy for rheumatoid arthritis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **106**(6)**:** 778-787.

Serafini M, Peluso I, Raguzzini A (2010). Flavonoids as anti-inflammatory agents. *Proc. Nutr. Soc.* **69**(3)**:** 273-278.

Sethi G, Sung B, Aggarwal BB (2008). Nuclear factor-κ activation: from bench to bedside. *Exp. Biol. Med. (Maywood, NJ, U. S.)* **233**(1)**:** 21-31.

Silva GL, Lee I-S, Kinghorn AD (1998). Special problems with the extraction of plants. *Methods Biotechnol.* **4**(Natural Products Isolation)**:** 343-363.

Spath E, Klager K (1934). Natural coumarins. XII. Constitution of ostruthin (from *Imperatoria ostruthium*). *Ber. Dtsch. Chem. Ges. B* **67B:** 859-868.

Spath E, Klager K (1933). Plant fish poisons IV. Constitution of hydroxypeucedanin (from *Imperatoria ostruthium*). *Ber. Dtsch. Chem. Ges. B* **66B:** 914-922.

Stewart I, Schluter Philip J, Shaw Glen R (2006). Cyanobacterial lipopolysaccharides and human health - a review. *Environmental health : a global access science source* **5:** 7.

Sun F, Xie ML, Zhu LJ, Xue J, Gu ZL (2009). Inhibitory effect of osthole on alcohol-induced fatty liver in mice. *Dig Liver Dis* **41**(2)**:** 127-133.

Suomi J, Wiedmer SK, Jussila M, Riekkola M-L (2002). Analysis of eleven iridoid glycosides by micellar electrokinetic capillary chromatography (MECC) and screening of plant samples by partial filling (MECC): Electrospray ionisation mass spectrometry. *Journal of Chromatography A* **970**(1-2)**:** 287-296.

Suomi J, Wiedmer SK, Jussila M, Riekkola M-L (2001). Determination of iridoid glycosides by micellar electrokinetic capillary chromatography-mass spectrometry with use of the partial filling technique. *Electrophoresis* **22**(12)**:** 2580-2587.

Swardfager W, Lanctôt K, Rothenburg L, Wong A, Cappell J, Herrmann N (2010). A Meta-analysis of cytokines in Alzheimer's disease. *Biological psychiatry* **68**(10)**:** 930-941.

Syah YM, Achmad SA, Ghisalberti EL, Hakim EH, Makmur L, Soekamto NH (2004). A stilbene dimer, andalasin B, from the root trunk of *Morus macroura*. *J. Chem. Res.*(5)**:** 339-340.

Tabanca N, Ma G, Pasco DS, Bedir E, Kirimer N, Husnu K*, et al.* (2007). Effect of essential oils and isolated compounds from *Pimpinella* species on NF-kB: a target for antiinflammatory therapy. *Phytother. Res.* **21**(8)**:** 741-745.

Takano-Ishikawa Y, Goto M, Yamaki K (2003). Inhibitory effects of several flavonoids on E-selectin expression on human umbilical vein endothelial cells stimulated by tumor necrosis factor-α. *Phytother. Res.* **17**(10)**:** 1224-1227.

Thomé OW (1905). Flora von Deutschland Österreich und der Schweiz Vol. 4, 2nd edn, Friedrich von Zezschwitz, Verlag Flora von Deutschland, Gera, p 509.

Thuong PT, Hung TM, Ngoc TM, Ha DT, Min BS, Kwack SJ*, et al.* (2010). Antioxidant activities of coumarins from Korean medicinal plants and their structure-activity relationships. *Phytother. Res.* **24**(1)**:** 101-106.

Tirillini B (2000). Grapefruit: the last decade acquisitions. *Fitoterapia* **71**(Suppl. 1)**:** S29-S37.

Tschirch A (1917). *Handbuch der Pharmakognosie*. Verlag Chr. Herm. Tauchnitz, 1908, Leipzig. 904 ff.

Ueda H, Yamazaki C, Yamazaki M (2004). A hydroxyl group of flavonoids affects oral anti-inflammatory activity and inhibition of systemic tumor necrosis factor-α production. *Biosci., Biotechnol., Biochem.* **68**(1)**:** 119-125.

Urbain A, Marston A, Hostettmann K (2005). Coumarins from *Peucedanum ostruthium* as inhibitors of acetylcholinesterase. *Pharm. Biol. (Philadelphia, PA, U. S.)* **43**(8)**:** 647-650.

Vera N, Popich S, Luna L, Cravero R, Sierra MG, Bardon A (2006). Toxicity and synergism in the feeding deterrence of some coumarins on *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae). *Chem. Biodiversity* **3**(1)**:** 21-26.

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

Wagner H, Bladt S (1996). *Plant Drug Analysis*. 2nd edn. Springer: Berlin.

Waldbauer K (2011). Ergänzung und Erstellung der Monographien „Safran“ und „Meisterwurz“ für das Österreichische Arzneibuch. Diploma Thesis, University of Vienna, Vienna.

Wall ME, Wani MC, Brown DM, Fullas F, Olwald JB, Josephson FF*, et al.* (1996). Effect of tannins on screening of plant extracts for enzyme inhibitory activity and techniques for their removal. *Phytomedicine* **3**(3)**:** 281-285.

Wang CC, Lai JE, Chen LG, Yen KY, Yang LL (2000). Inducible nitric oxide synthase inhibitors of Chinese herbs. Part 2: Naturally occurring furanocoumarins. *Bioorg. Med. Chem.* **8**(12)**:** 2701-2707.

Wolfender J-L, Marti G, Queiroz EF (2010). Advances in techniques for profiling crude extracts and for the rapid identification of natural products: dereplication, quality control and metabolomics. *Curr. Org. Chem.* **14**(16)**:** 1808-1832.

Wolfender J-L, Queiroz EF, Hostettmann K (2005). Phytochemistry in the microgram domain - a LC-NMR perspective. *Magn. Reson. Chem.* **43**(9)**:** 697-709.

Wolfender J-L, Terreaux C, Hostettmann K (2000). The importance of LC-MS and LC-NMR in the discovery of new lead compounds from plants. *Pharm. Biol. (Lisse, Neth.)* **38**(Suppl.)**:** 41-54.

Yoo SW, Kim JS, Kang SS, Son KH, Chang HW, Kim HP*, et al.* (2002). Constituents of the fruits and leaves of *Euodia daniellii*. *Arch. Pharmacal Res.* **25**(6)**:** 824-830.

You L, Feng S, An R, Wang X (2009). Osthole: a promising lead compound for drug discovery from a Traditional Chinese Medicine (TCM). *Nat. Prod. Commun.* **4**(2)**:** 297-302.

Yumuk VD (2006). Targeting components of the stress system as potential therapies for the metabolic syndrome. The peroxisome-proliferator-activated receptors. *Annals of the New York Academy of Sciences* **1083**(Stress, Obesity, and Metabolic Syndrome)**:** 306-318.

Zhang Y, Xie M-L, Xue J, Gu Z-L (2008). Osthole regulates enzyme protein expression of CYP7A1 and DGAT2 via activation of PPARα/γ in fat milk-induced fatty liver rats. *J. Asian Nat. Prod. Res.* **10**(8)**:** 797-802.

Zhou K, Wu B, Zhuang Y, Ding L, Liu Z, Qiu F (2009). Chemical constituents of fresh celery. *Zhongguo Zhongyao Zazhi* **34**(12)**:** 1512-1515.

Zhou Z-w, Shen X (2006). Pharmacological activities of osthole. *Zhongguo Xinyao Zazhi* **15**(20)**:** 1726-1730.

Zimecki M, Artym J, Cisowski W, Mazol I, Wlodarczyk M, Glensk M (2009). Immunomodulatory and anti-inflammatory activity of selected osthole derivatives. *Z. fuer Naturforsch. C* **64**(5-6)**:** 361-368.

Curriculum vitae



Mag. Sylvia Vogl

address Hernalser Hauptsstrasse 79/1/24
A-1170 Wien

phone +43699/11686270

e-mail sylvia.vogl@univie.ac.at or sylvia_vog@yahoo.de

date of birth 1982-02-09

nationality Austria

parents Angela Maria Vogl, nee Marchart - employee in a flower shop
Dkfm. Dr. Horstmar Gynt Vogl - retiree

languages German (mother tongue)
English (fluent)
Spanish (fluent)
French (3 years grammer school)
Indonesian (basics)

Education

1988-1992: elementary school in A-7052 Müllendorf, Austria

1992-2000: secondary school Bundesgymnasium Eisenstadt in A-7000 Eisenstadt, Austria

2000-2007: diploma study in Biology/ Botany at the University of Vienna, A-1090 Vienna, Austria; Diploma Thesis at the Department for Ecophysiology and Functional Anatomy of Plants; Promoter: Prof. Dr. Harald Bolhàr-Nordenkampf; Topic: Changes in structural plant traits of soybean leaves as modified by CO_2, O_3 and $CO_2 + O_3$

2003-2007: studies in Social and Cultural Anthropology at the University of Vienna, Austria

Since 01/10/2007: PhD student at the Department of Pharmacognosy, University of Vienna, in frame of a NFN Project called “Drugs from nature targeting inflammation”; Promoter: Prof. Dr. Brigitte Kopp; Topic: Bioassay guided fractionation of *Peucedanum ostruthium*(L.) W.D.J. Koch and *Melampyrum pratense* L. focusing on anti-inflammatory activities.

Practical skills

HPLC, GC-MS, ELISA, Microsoft Office, Endnote, Statistics (Graph Pad Prism)

Further employments and activities

August 2000: employee at the public hospital “öffentliches Krankenhaus der Barmherzigen Brüder” in A-7000 Eisenstadt, Austria

2005/ 2006: Tutor at the Department of Ecophysiology and Functional Anatomy of Plants (University of Vienna)

2009: Assistant and administration of the TCM Summerschool in Chengdu, China (organized by the University of Vienna and the University of Innsbruck)

2009-2011: Teaching assistant at the course “methods for identification and characterization of pharmaceutically relevant organisms”

2010: Teaching assistant at the course “preparation and analysis of medicinal products of biogenous origin”

Aug. - Sep. 2003: eco-physiological - botanical excursion to Australia and New Zealand

July, August 2004: ethno-botanical excursion to Guatemala

July 2005: collaboration at the 17th International Botanical Congress at the Austria Center Vienna, A-1220 Wien

July, August 2005: collaboration at the SoyFACE project at the University of Illinois at Urbana-Champaign

Poster presentations

Vogl S, Castro JC, Meister M, Long S, Bolhàr-Nordenkampf H. The influence of elevated CO_2 and ozone concentration on leaf surface characteristics of soybean. ***16th conference of the Austrian Society of Plant Biology*** (ATSPB), Vienna, Austria, June 2006.

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 International Congress & Annual Meeting of the Society for Medicinal Plant and Natural Product Research – GA.*** Geneva, Switzerland, Aug. 16 - 20, 2009

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 NF-κB inhibition. ***57th International Congress & Annual Meeting of the Society for Medicinal Plant and Natural Product Research - GA***, Geneva, Switzerland, Aug. 16 - 20, 2009

Vogl S, Zehl M, Picker P, Urban E, Reznicek G, Saukel J, Wawrosch C, Kopp B. Rapid separation, identification, and quantification of coumarins in *Peucedanum ostruthium* by high-performance liquid chromatography with diode-array detection and mass spectrometry. ***58th International Congress & Annual Meeting of the Society for Medicinal Plant and Natural Product Research - GA***, Berlin, Germany, Aug. 29 - Sep. 2, 2010

Picker P, Mihaly-Bison J, Vogl S, Zehl M, Urban E, Reznicek G, Saukel J, Wawrosch C, Binder B, Kopp B. Bioactivity-guided fractionation of potential anti-inflammatory constituents from *Betonica officinalis*. ***58th International Congress & Annual Meeting of the Society for Medicinal Plant and Natural Product Research – GA.*** Berlin, Germany, Aug. 29 - Sep. 2, 2010

Publications

Joa H, Vogl S, Atanasov AG, Zehl M, Nakel T, Fakhrudin N, et al. (2011). Identification of Ostruthin from *Peucedanum ostruthium* Rhizomes as an Inhibitor of Vascular Smooth Muscle Cell Proliferation. J. Nat. Prod. 74(6): 1513-1516.

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

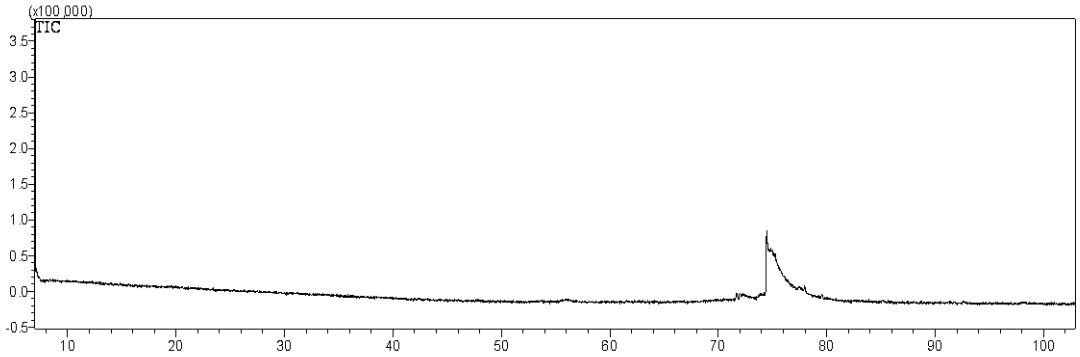
Annex I:

GC-MS analyses of *Peucedanum ostruthium*

extracts and subfractions

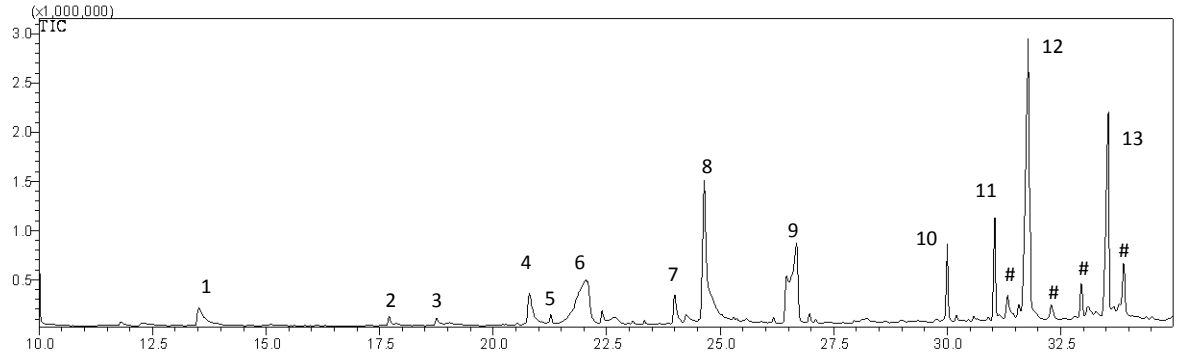
Peos 1

Method 1, 0.2 mg in 0.2mL MeOH



→ GC stationary phase

Method 2



column/ solvent residues

peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	94
2	GLOBULOL	17.5	$C_{15}H_{26}O$	85
	isopathulenol		$C_{15}H_{24}O$	84
	Kauran-18-al, 17-(acetyloxy)-, (4.beta.)-		$C_{22}H_{34}O_3$	83
3	SPATHULENOL	18.75	$C_{15}H_{24}O$	83
4	**Carboxylic acid**	20.8	$C_{12}H_{14}O_4$	81
	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-,methyl ester			
5	**Fatty acid**	21.2		
	Pentadecanoic acid, 14-methyl-, methyl ester		$C_{17}H_{34}O_2$	83
	METHYL HEXADECANOATE DB5-2592 (=METHYL PALMITATE)		$C_{17}H_{34}O_2$	83
	Dodecanoic acid, methyl ester / Methyl laurate		$C_{13}H_{26}O_2$	83

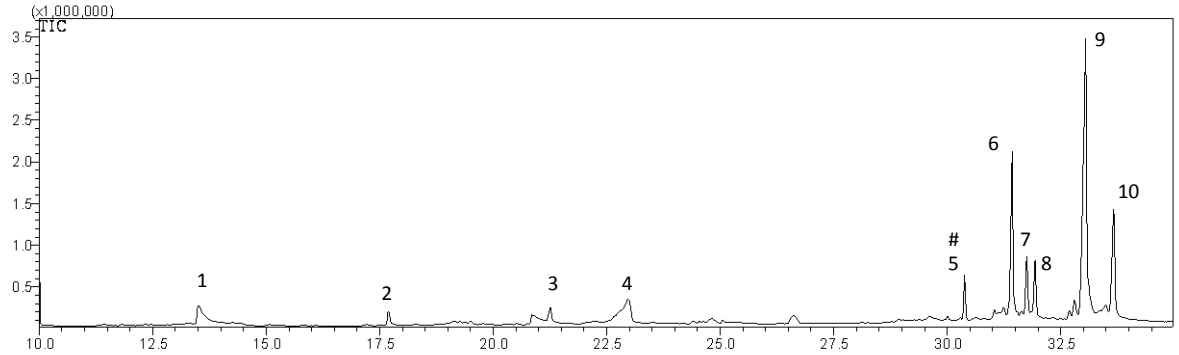
6	**Coumarin/ sesquiterpene**	22		
	delta.9-Tetrahydrocannabivarin		$C_{19}H_{26}O_2$	59
	3-PROPYL-.DELTA.9-TETRAHYDROCANNABINOL		$C_{19}H_{26}O_2$	59
	51FARNESOL		$C_{15}H_{26}O$	51
	BISABOLENAL <BETA-> DB5-2251		$C_{15}H_{22}O$	51
	OSTHOLE		$C_{15}H_{16}O_3$	51
7	**Coumarin**	24		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergapten		$C_{12}H_8O_4$	84
8	**Coumarin**	24.7		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergapten		$C_{12}H_8O_4$	96
9	Methyl 2-[2,4a-epidioxy-5,6,7,8-tetrahydro-5,5,8a-trimethyl-2H-	26.5	$C_{15}H_{22}O_5$	50
	17.beta.-Hydroxy-6-oxo-4,5-secoandrostan-4-oic acid methyl ester		$C_{20}H_{32}O_4$	49
	Androstan-3-one, 17-hydroxy-1-methyl-, (1.beta.,5.alpha.,17.beta.)-		$C_{20}H_{32}O_2$	49
10	**Coumarin**	30		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergapten		$C_{12}H_8O_4$	68
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	66
	Methoxsalen		$C_{12}H_8O_4$	64
11	**Coumarin**	31		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy-/ Bergaptan / Bergapten		$C_{12}H_8O_4$	70
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	68
	Methoxsalen		$C_{12}H_8O_4$	66
12	**Coumarin**	31.7		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy-/ Bergaptan / Bergapten		$C_{12}H_8O_4$	75
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	74
	Methoxsalen		$C_{12}H_8O_4$	71
13	**Coumarin**	33.5		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergapten		$C_{12}H_8O_4$	65
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	61
	Methoxsalen		$C_{12}H_8O_4$	60

Peos 2

Method 1, 0.2 mg in 0.2mL MeOH

→ no volatile substances detectable

Method 2



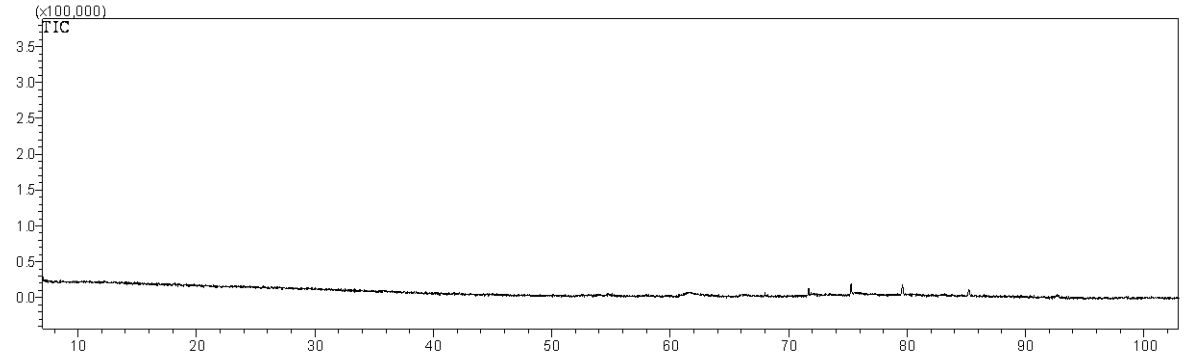
column/ solvent residues

peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	90
2	Globulol DB5-1841	17.7	$C_{15}H_{26}O$	86
	Acetoxyelemol <8-alpha-> DB5-2306 (=ACETATE II)		$C_{17}H_{28}O_3$	85
	Kauran-18-al, 17-(acetyloxy)-, (4.beta.)-		$C_{22}H_{34}O_3$	84
3	**Fatty acid**	21.3		
	Pentadecanoic acid, 14-methyl-, methyl ester		$C_{17}H_{34}O_2$	81
	Tetradecanoic acid, 12-methyl-, methyl ester		$C_{16}H_{32}O_2$	80
4	**Coumarin**	22.9		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergapten		$C_{12}H_8O_4$	72
	Methoxsalen		$C_{12}H_8O_4$	70
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	69
5	**Fatty acid residue**	30.4		
	5-Undecanol, 2-methyl-		$C_{12}H_{26}O$	70
	Silane			66
6	**Monoterpen/ fatty acid**	31.4		
	Linalool		$C_{10}H_{18}O$	62
	TRANS-2-PINANOL		$C_{12}H_{20}O$	62
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester / 2-Monopalmitin		$C_{19}H_{38}O_4$	62
7	**Fatty acid residue**	31.8		
	5-Undecanol, 2-methyl-		$C_{12}H_{26}O$	70

8	**Carboxylic acid**	32		
	2-Isopropyl-3-methoxycyclopropanecarboxylic acid		$C_8H_{14}O_3$	77
	1-Nonen-4-ol, 3,4-dimethyl-		$C_{11}H_{22}O$	76
	2,3-Octanediol		$C_8H_{18}O_2$	75
9	6-Nitro-cylohexadecane-1,3-dione	33	$C_{16}H_{27}NO_4$	71
	1,6-Octadiene, 3-ethoxy-3,7-dimethyl-		$C_{12}H_{22}O$	71
	1,6-Nonadien-3-ol, 3,7-dimethyl-		$C_{11}H_{20}O$	70
	Cyclohexan, 1,5-diisopropyl-2,3-dimethyl-		$C_{14}H_{28}$	69
10	**Carboxylic acid**	33.7		
	2-Isopropyl-3-methoxycyclopropanecarboxylic acid		$C_8H_{14}O_3$	75
	4-Heptanol, 4-propyl-		$C_{10}H_{22}O$	75
	5-Undecanol, 2-methyl-		$C_{12}H_{26}O$	75

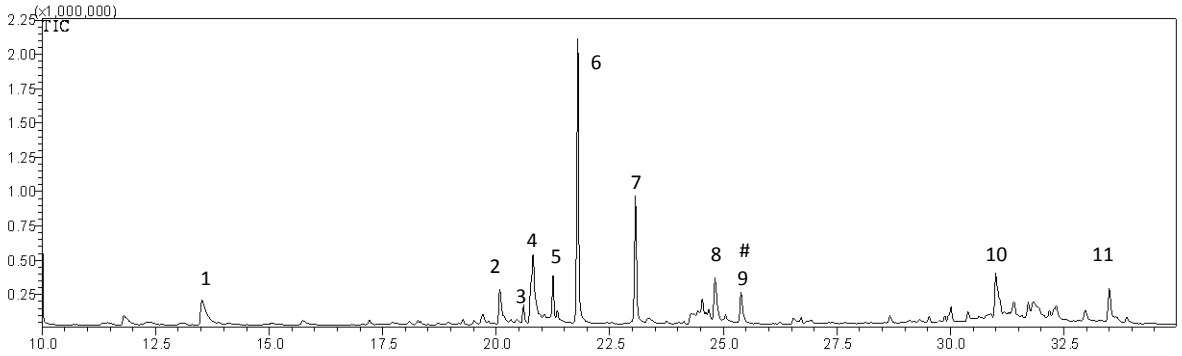
Peos 3

Method 1, 0.2 mg in 0.2mL MeOH



→ just GC stationary phase and septum bleed

Method 2



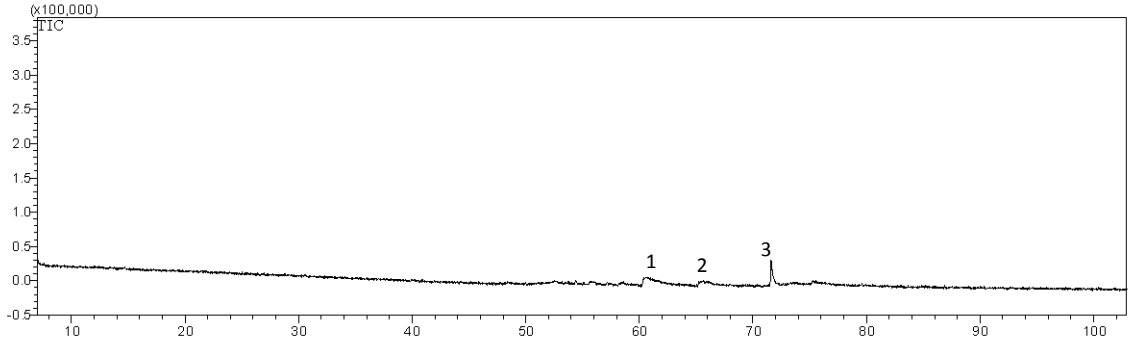
column/ solvent residues

peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	92
2	1,7-Dimethyl-1,8-naphthyridin-4-one-3-carboxhydrzide	20	$C_{11}H_{12}N_4O_2$	69
	1,5-Diazabicyclo[3.1.0]hexane, 6-(4-isopropylphenyl)- Pyridine,		$C_{13}H_{18}N_2$	68
	4-[4-hydroxy-5-methoxyphenyl]-		$C_{12}H_{11}NO_2$	68
3	**Sesquiterpene**	20.6		
	Caryophyllene oxide		$C_{15}H_{24}O$	84
4	**Carboxylic acid**	20.8		
	Buta-1,3-diyne, 1,4-bis(2-methoxycarbonylcyclopropyl)-		$C_{14}H_{14}O_4$	63
	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester		$C_{12}H_{14}O_4$	56
5	**Fatty acid**	21.2		
	Hexadecanoic acid, methyl ester / Methyl palmitate / Palmitic acid methyl ester		$C_{17}H_{34}O_2$	92
	Octadecanoic acid, methyl ester / Methyl stearate		$C_{19}H_{38}O_2$	87
	Pentadecanoic acid, 14-methyl-, methyl ester		$C_{17}H_{34}O_2$	87

6	**Coumarin**	21.8		
	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)-		$C_{15}H_{18}O_4$	73
	7H-Furo[3,2-g][1]benzopyran-7-one, 4,9-dimethoxy-		$C_{13}H_{10}O_5$	61
	4-Acetylisocoumarin		$C_{11}H_8NO_3$	61
7	**Carboxylic acid**	23		
	6-Methoxyquinoline-7-carboxylic acid, methyl ester		$C_{12}H_{11}O_3$	58
	2,6-di-n-propyl-4-(2-furyl)pyridine		$C_{15}H_{19}O$	57
	2-Naphthalenecarboxylic acid, 5,6,7-trimethoxy-, methyl ester		$C_{15}H_{16}O_5$	54
8	**Coumarin/ Chromone**	24.8		
	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)- 4-		$C_{15}H_{18}O_4$	69
	Acetylisocoumarin		$C_{11}H_8O_3$	61
	BERGAPTENE DB5-2830		$C_{12}H_8O_4$	61
9	**Carboxylic acid**	25.4		
	2',5'-Dimethoxy-2-biphenylcarboxylic acid		$C_{15}H_{14}O_4$	61
	Diisopropyl(1-naphthyloxy)silane		$C_{16}H_{22}OSi$	60
10	1,6-Octadiene, 3-ethoxy-3,7-dimethyl- / Ethyl linalool	31	$C_{12}H_{22}O$	74
	4-[3,4-Dimethoxycyclohexyl]-n-butanol		$C_{12}H_{24}O_3$	74
11	**Coumarin**	33.5		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten		$C_{12}H_8O_4$	70
	Isobergapten / 2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy-		$C_{12}H_8O_4$	67

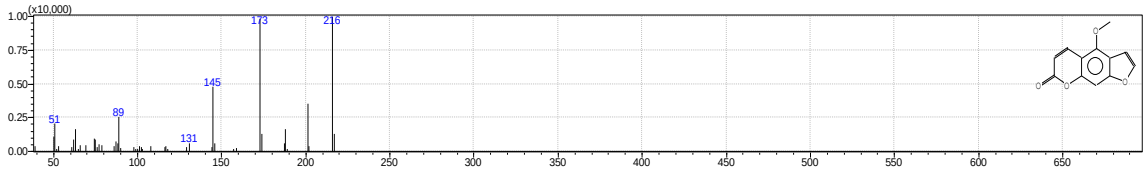
Peos 4

Method 1, 0.2 mg in 0.2mL MeOH



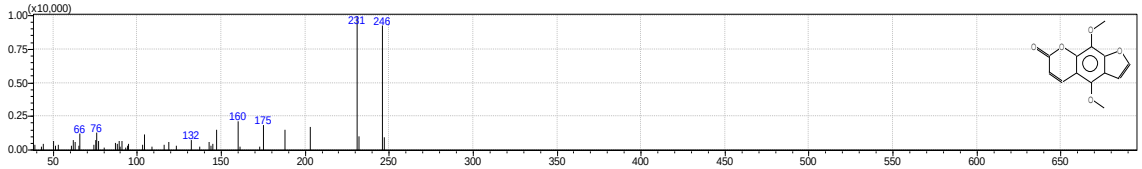
1. Peak → coumarin

88 %: 216: 7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- (Bergaptene)



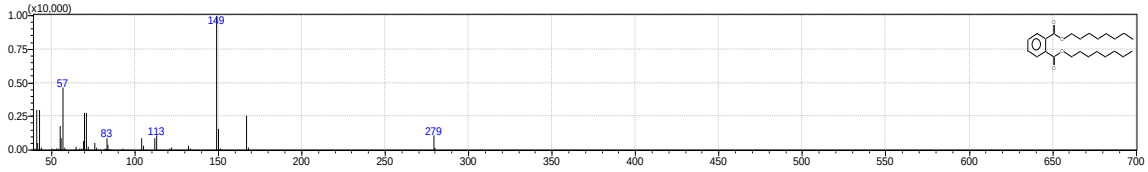
2. Peak → coumarin

84 %: 246: (iso)pimpinellin DB5-3162

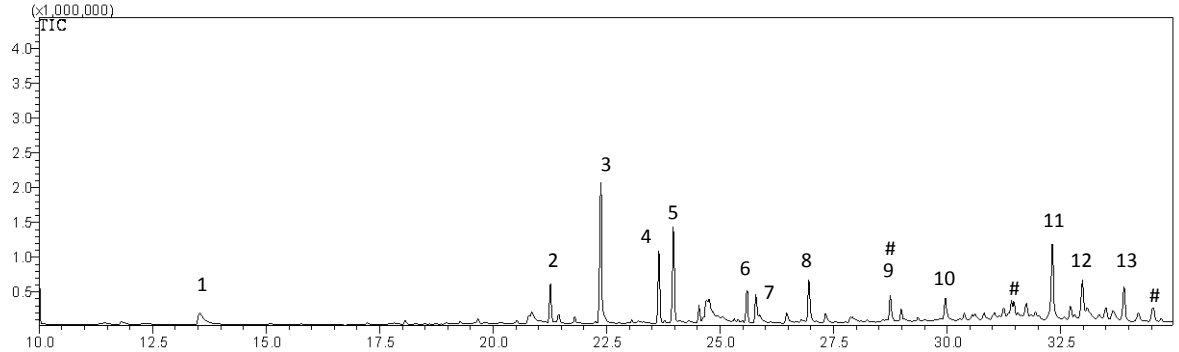


3. Peak → phthalate ester

94 %: 390: Di-n-octyl phthalate



Method 2



column/ solvent residues

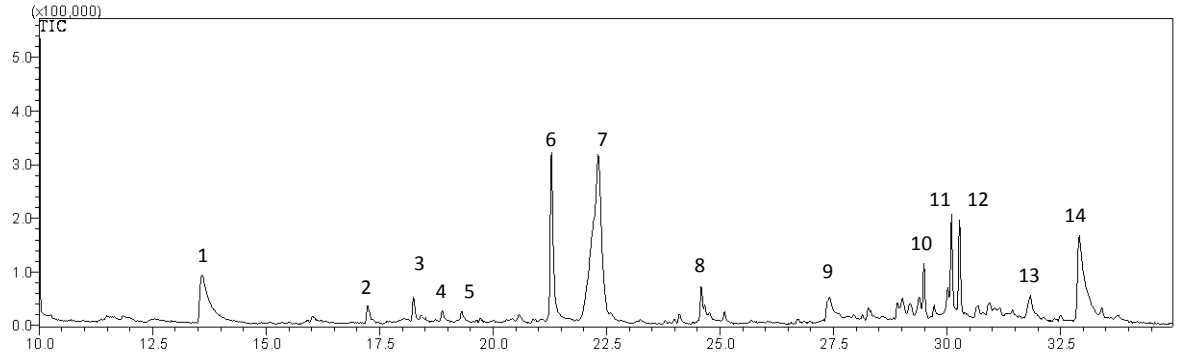
peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	92
2	**Fatty acid**	21.3		
	Hexadecanoic acid, methyl ester (CAS) Methyl palmitate Palmitic acid methyl ester		$C_{17}H_{34}O_2$	96
	Pentadecanoic acid, methyl ester (CAS) Methyl pentadecanoate		$C_{16}H_{32}O_2$	94
3	**Coumarin**	22.4		
	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)-		$C_{15}H_{18}O_4$	71
	Isopimpinellin, 7H-Furo[3,2-g][1]benzopyran-7-one, 4,9-dimethoxy-		$C_{13}H_{10}O_5$	66
4	**Coumarin**	23.5		
	Benzoic acid, 3-iodo-4-methoxy-, methyl ester		$C_9H_9IO_3$	59
	2-Cyclohexen-1-one, 3-methoxy-2-(2,4,5-trimethoxyphenyl)-		$C_{16}H_{20}O_5$	56
	8-Amino-6,7-dimethoxy-4-methylquinoline		$C_{12}H_{14}N_2O_2$	55
	ISOPIMPINELLIN DB5-3162		$C_{13}H_{10}O_5$	54
5	**Coumarin**	24		
	.alpha.-1-Naphthylisobutyric acid, 8-methoxy-, methyl ester		$C_{16}H_{17}FO_3$	57
	OSTHOL DB5-2999		$C_{15}H_{16}O_3$	52
6	**Coumarin**	25.6		
	2-ETHYL-5,6,7,8-TETRAHYDRO-8,9,9(5,9,9)TRIMETHYL-5,8-		$C_{14}H_{20}N_2$	52
	Xanthotoxin / 7H-Furo[3,2-g][1]benzopyran-7-one, 9-methoxy-		$C_{12}H_8O_4$	52
7	**Coumarin**	25.8		
	Osthole / 2H-1-Benzopyran-2-one, 7-methoxy-8-(3-methyl-2-butenyl)-		$C_{15}H_{16}O_3$	81
8	**Coumarin**	27		
	6-Acetyl-5-methoxy-2,7-dimethyl-1,4-naphthoguinone		$C_{15}H_{14}O_4$	69
	1,2,3-Trimethoxydibenzofuran		$C_{15}H_{14}O_4$	68
9	Spiro(pyrazole-3,2'-naphthalen-1'(2'H)-one, 2,4-dihydro-2-(2,4-dinitrophenyl)-5-methyl-5',7',8'-trimethoxy-	28.7	$C_{22}H_{20}N_4O_8$	58
	1,3-Dioxolo[4,5-g]furo[2,3-b]quinoline, 4,9-dimethoxy- / Flindersiamin		$C_{14}H_{11}NO_5$	57
	1,1'-Biphenyl, 4,2',3',4'-tetramethoxy-6-methyl-		$C_{17}H_{20}O_4$	56
10	**Coumarin**	30		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	72
11	INDENE, 1-(TRIMETHYLSILYL)-	32.3	$C_{12}H_{16}SI$	67
	1H-Indole-3-carboxaldehyde, 1-(trimethylsilyl)-Bicyclo[5.3.0]deca-		$C_{12}H_{15}NOSi$	67
	Hexanoic acid, 2-(methoxyimino)-, trimethylsilyl ester		$C_{10}H_{21}NO_3SI$	66
12	**Fatty acid**	33		
	1,6-Octadiene, 3-ethoxy-3,7-dimethyl- / Ethyl linalool		$C_{12}H_{22}O$	74
	7-Hexadecenoic acid, methyl ester,		$C_{17}H_{32}O_2$	74
13	1,3-Dimethoxy-2,3-dimethylbutane	33.8	$C_8H_{18}O_2$	75
	1-Trimethylsilyl-3-(dimethyl-n-pentylsilyl)propane		$C_{13}H_{32}Si_2$	71
	Butanoic acid, 3-(1-ethoxyethoxy)-2-methyl-, ethyl ester		$C_{11}H_{22}O_4$	71

Peos 5

Method 1, 0.2 mg in 0.2mL MeOH

→ no volatile substances

Method 2

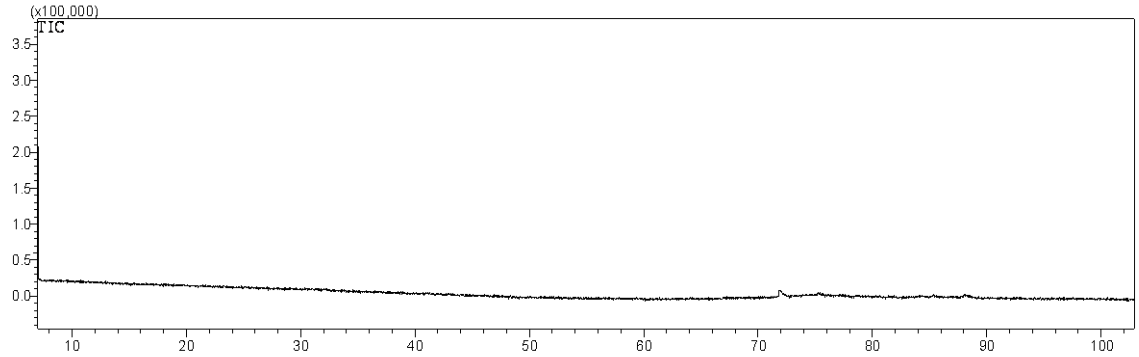


peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	92
	10-Undecenoic acid, methyl ester		$C_{12}H_{22}O_2$	85
2	**Fatty acid**	17.3		
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	90
	Octadecanoic acid, methyl ester / Stearic acid methyl ester		$C_{19}H_{38}O_2$	90
	Tridecanoic acid, methyl ester		$C_{14}H_{28}O_2$	90
	Hexadecanoic acid, 15-methyl-, methyl ester		$C_{18}H_{36}O_2$	90
	Nonadecanoic acid, methyl ester (CAS)		$C_{20}H_{40}O_2$	90
	Tetradecanoic acid, methyl ester (CAS) / Methyl myristate		$C_{15}H_{30}O_2$	90
3	**Sesquiterpene**	18.2		
	globulol DB5-1841		$C_{15}H_{26}O$	85
	Kauran-18-al, 17-(acetyloxy)-, (4.beta.)-		$C_{15}H_{24}O$	85
	17-ACETOXY-19-KAURANAL		$C_{22}H_{34}O_3$	83
	Perhydrocyclopropa[e]azulene-4,5,6-triol, 1,1,4,6-tetramethyl		$C_{15}H_{24}O$	83
	VERIDIFLOROL / VIRIDIFLOROL		$C_{15}H_{26}O_3$	82
	LONGIPINANOL DB5-1801 / TRANS-LONGIPINANOL		$C_{15}H_{26}O$	82
4	**Sesquiterpene**	18.9		
	LONGIPINANOL<EPI->DB5-1787/ CIS-LONGIPINANOL		$C_{15}H_{26}O$	80
	Diazoprogesterone		$C_{21}H_{30}N_4$	80
	CARYOPHYLLENE OXIDE DB5-1837		$C_{15}H_{24}O$	76
	Isoaromadendrene epoxide		$C_{15}H_{24}O$	76
	CEMBRENE DB5-2597		$C_{20}H_{32}$	75

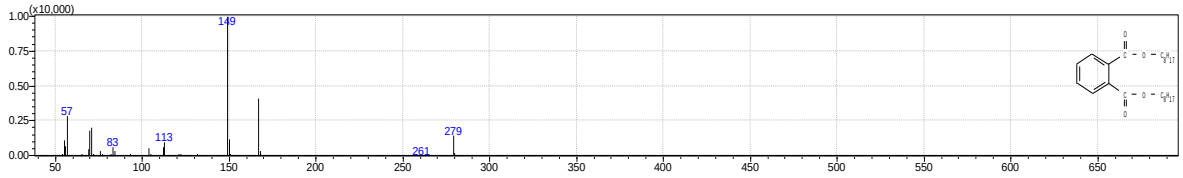
5	**Fatty acid**	19.3		
	Cyclopentanetridecanoic acid, methyl ester		$C_{19}H_{36}O_2$	88
	Pentadecanoic acid, 14-methyl-, methyl ester		$C_{17}H_{34}O_2$	87
	Tetradecanoic acid, 12-methyl-, methyl ester		$C_{16}H_{32}O_2$	86
6	1st main peak → **fatty acid**	21.3		
	Hexadecanoic acid, methyl ester / Methyl palmitate / Palmitic acid methyl ester		$C_{17}H_{34}O_2$	94
	Pentadecanoic acid, methyl ester / Methyl pentadecanoate		$C_{16}H_{32}O_2$	94
	Eicosanoic acid, methyl ester		$C_{21}H_{42}O_2$	94
7	2ndmain peak → **fatty acid**	22.4		
	Tetracosanoic acid, methyl ester		$C_{25}H_{50}O_2$	94
	Triacontanoic acid, methyl ester (CAS)		$C_{31}H_{62}O_2$	92
8	**Fatty acid**	24.6		
	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester		$C_{22}H_{38}O_2$	93
	11,14-Eicosadienoic acid, methyl ester		$C_{21}H_{38}O_2$	93
	7,10-Hexadecadienoic acid, methyl ester		$C_{17}H_{30}O_2$	92
9	**Monoterpen/ sesquiterpene/ fatty acid**	27.5		
	VERBENOL <TRANS->		$C_{10}H_{16}O$	61
	13,16-Seco-D-nor-5.alpha.-androsta-13(18),15-diene		$C_{18}H_{28}$	61
	CEMBRENE		$C_{20}H_{32}$	60
	CARYOPHYLLENE <14-HYDROXY-9-EPI-(E)-		$C_{15}H_{24}O$	60
10	(5.beta.,13.alpha.) 3.alpha.-Methoxy-3.beta.,19-epoxyandrost-8-ene-7.alpha.,17.beta.-diol, 4,4-dimethyl-	29.5	$C_{22}H_{34}O_4$	51
	Benzo[a]decalin-6-one, 9,9-dimethyl-2-n-pentyl-		$C_{21}H_{30}O$	48
11	3(2H)-Phenanthrenone, 2-(acetyloxy)-7-[(acetyloxy)acetyl]-1,4,4a,4b,5,6,7,8,10,10a-decahydro-1,1,4a,7-tetramethyl-, [2S-(2.alpha.,4a.alpha.,4b.beta.,7.beta.,10a.beta	30	$C_{24}H_{34}O_6$	53
	O-Acetyldihydrocrinine		$C_{18}H_{21}NO_4$	53
	Estr-4-en-3-one-1-acetonitrile, 17-acetoxy-.alpha.-(trimethylsilyloxy)-		$C_{25}H_{37}NO_4Si$	52
	Oxycodone		$C_{18}H_{21}NO_4$	51
12	3(2H)-Phenanthrenone, 2-(acetyloxy)-7-[(acetyloxy)acetyl]-1,4,4a,4b,5,6,7,8,10,10a-decahydro-1,1,4a,7-tetramethyl-, [2S-(2.alpha.,4a.alpha.,4b.beta.,7.beta.,10a.beta.)]-	30.3	$C_{24}H_{34}O_6$	53
	Estrone methyl ether thiosemicarbazone		$C_{20}H_{27}N_3OS$	53
	EPOXYGEDUNIN		$C_{28}H_{34}O_8$	53
13	4-(1,1-Dimethylallyl)-9-methoxy-7H-furo[3,2-g][1]benzopyran-7-one	31.8	$C_{17}H_{16}O_4$	51
	1-Methyl-1-n-tridecyloxy-1-silacyclobutane		$C_{17}H_{36}OSi$	50
14	**Coumarin**	32.9		
	7H-Furo[3,2-g][1]benzopyran-7-one, 9-methoxy-4-(3-methyl-2-butenyl)- / Alloimperatorin methyl ether		$C_{17}H_{16}O_4$	64

Peos 6

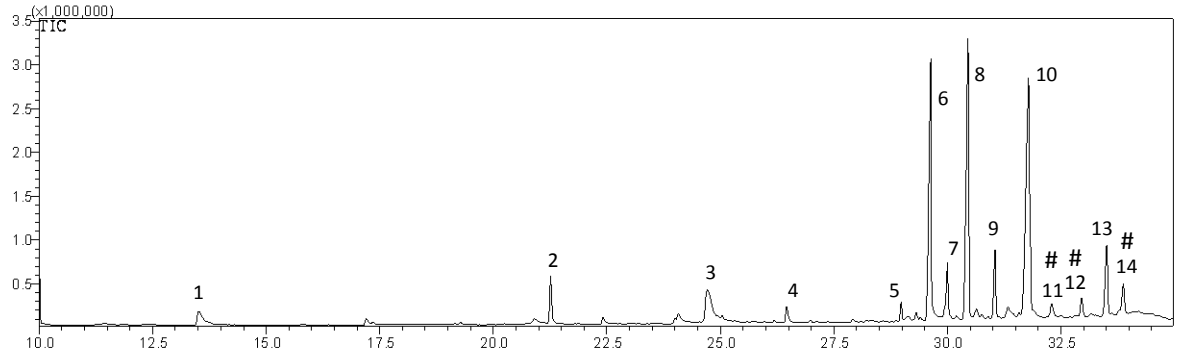
Method 1, 0.2 mg in 0.2mL MeOH



→ very low amounts of 1,2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate (72 %)



Method 2



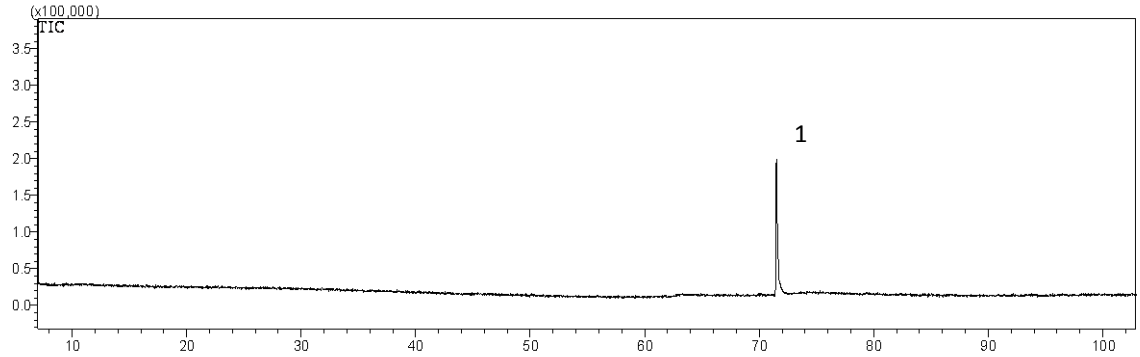
column/ solvent residues

peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	92
	10-Undecenoic acid, methyl ester		$C_{12}H_{22}O_2$	85
2	**Fatty acid**	21.3		
	Hexadecanoic acid, methyl ester / Methyl palmitate / Palmitic acid methyl ester		$C_{17}H_{34}O_2$	92
	Pentadecanoic acid, methyl ester / Methyl pentadecanoate		$C_{16}H_{32}O_2$	92
3	**Coumarin**	24.8		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten		$C_{12}H_8O_4$	91

4	**Coumarin**	26.5		
	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)-		$C_{15}H_{18}O_4$	71
	7H-Furo[3,2-g][1]benzopyran-7-one, 4,9-dimethoxy-		$C_{13}H_{10}O_5$	65
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten		$C_{12}H_8O_4$	63
5	3-Buten-2-ol, 2,3-dimethyl- (	29	$C_6H_{12}O$	67
	2-Propanol, 1-(1,3-dimethylbutoxy)-		$C_9H_{20}O_2$	65
6	1st main peak → **Chromone**	29.6		
	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-methyl-6-(3-methyl-2-butenyl)		$C_{16}H_{18}O_4$	75
7	**Coumarin**	30		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	59
8	2nd main peak → **Phenol / Chromone**	30.5		
	Phenol, 2,6-bis(1,1-dimethylpropyl)-4-methyl-		$C_{17}H_{28}O$	56
	3,3,6,6,9-Pentamethyl-3,4,5,6,7,9-hexahydro-2H-xanthene-1,8-dione		$C_{18}H_{24}O_3$	55
	4H-1-benzopyran-4-one, 2,3-dihydro-5-hydroxy-7-methoxy-2-methyl-6-(3-methyl-2-butenyl)- / DIHYDROPEUCENIN-7-METHYL ETHER		$C_{16}H_{20}O_4$	54
	Chromone, 5-hydroxy-8-isopentyl-7-methoxy-2-methyl- / DIHYDROHETEROPEUCENIN-7-METHYL ETHER		$C_{16}H_{20}O_4$	53
9	**Coumarin**	31		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	63
	3-Butyl-4,5-dimethyl-4-hydroxyhexahydropyrimidin-2-thione		$C_{10}H_{20}N_2OS$	62
10	3rd main peak → **Coumarin**	32		
	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten		$C_{12}H_8O_4$	74
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	73
	6-Methoxyfuro[2,3-h]chromen-2-one		$C_{12}H_8O_4$	71
	Methoxsalen		$C_{12}H_8O_4$	70
	Xanthotoxin / 7H-Furo[3,2-g][1]benzopyran-7-one, 9-methoxy-		$C_{12}H_8O_4$	69
11	2-ETHYLHEXANOIC ACID 1TMS	32.3	$C_{11}H_{24}O_2Si$	65
	3-Heptanol, 3-methyl-		$C_8H_{18}O$	65
	DIPROPYLACETIC ACID		$C_{11}H_{24}O_2Si$	65
12	1,1-Di(n-pentyloxy)-1-silacyclopentane	33	$C_{14}H_{30}O_2Si$	65
	1-Triisopropylsilyloxypentane		$C_{14}H_{32}OSi$	64
13	**Coumarin**	33.5		
	2H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten		$C_{12}H_8O_4$	70
	Methoxsalen		$C_{12}H_8O_4$	67
14	1,1-Di(n-pentyloxy)-1-silacyclopentane1-(n-Butoxy)-1-(n-hexyloxy)-1-	34	$C_{14}H_{30}O_2Si$	65

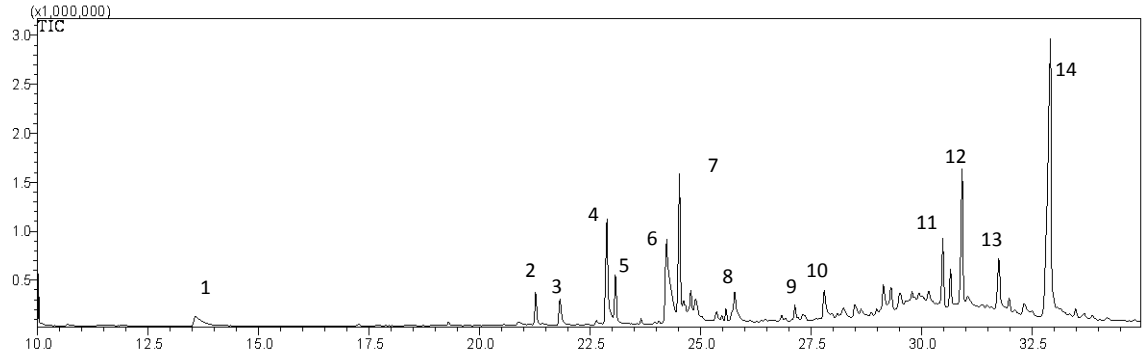
Peos 7

Method 1, 0.2 mg in 0.2mL MeOH



peak no.	main compounds	ret. time	formula	similarity [%]
1	Di-n-octyl phthalate	71.5	$C_{24}H_{38}O_4$	94
	1,2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate		$C_{24}H_{38}O_4$	94

Method 2

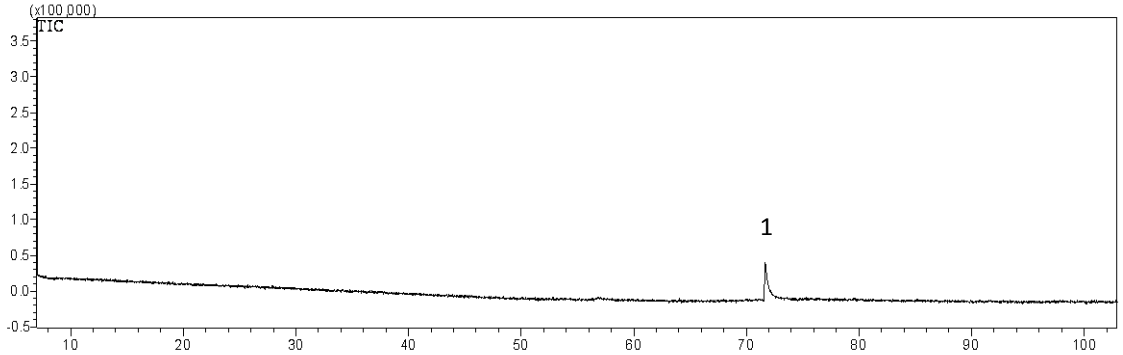


peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	91
	10-Undecenoic acid, methyl ester		$C_{12}H_{22}O_2$	84
2	**Fatty acid**	21.3		
	Hexadecanoic acid, methyl ester / Methyl palmitate / Palmitic acid methyl ester		$C_{17}H_{34}O_2$	95
	Pentadecanoic acid, methyl ester / Methyl pentadecanoate		$C_{16}H_{32}O_2$	93
3	**Coumarin**	21.7		
	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)-		$C_{15}H_{18}O_4$	73
	1,2,3,4-4H-Isoquinolin-1,3-dione, 4,4,5,6,8-pentamethyl-		$C_{14}H_{17}NO_2$	60
	4-Acetylisocoumarin		$C_{11}H_8O_3$	60

4	**Coumarin** 2H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten	22.8	$C_{12}H_8O_4$	69
5	6-Methoxyquinoline-7-carboxylic acid, methyl ester Isoxazol-5(4H)-one, 3-methyl-4-(2-methoxybenzylidene)- 2,6-di-n-propyl-4-(2-furyl)pyridine	23.1	$C_{12}H_{11}NO_3$ $C_{12}H_{11}NO_3$ $C_{15}H_{19}NO$	59 58 57
6	**Coumarin** 7H-Furo[3,2-g][1]benzopyran-7-one, 9-methoxy-/ Methoxsalen 2H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten	24.2	 $C_{12}H_8O_4$ $C_{12}H_8O_4$	 87 85
7	VETIVENENE <BETA-> DB5-1705 26-Amino-2,4-dimethyl-5-methoxyquinoline 2	24.6	$C_{15}H_{22}$ $C_{12}H_{14}N_2O$	57 56
8	**Coumarin** 2H-1-Benzopyran-2-one, 7-methoxy-8-(3-methyl-2-butenyl)- / Osthole	25.8	$C_{15}H_{16}O_3$	71
9	**Coumarin** 7H-Furo[3,2-g][1]benzopyran-7-one, 4,9-dimethoxy- / Isopimpinellin	27.2	$C_{13}H_{10}O_5$	79
10	2,2'-(2-Furylmethylene)bis(5-methylfuran) Furo[2,3-d]pyrimidin-4(5H)-one, 1,2-dihydro-2,2-dimethyl-3-phenyl	27.8	$C_{15}H_{14}O_3$ $C_{14}H_{14}N_2O_2$	53 53
11	1(2H)-Phenanthrenone, 3,4,9,10-tetrahydro-7-methoxy- 3(4H)-Dibenzofuranone, 4a,9b-dihydro-8,9b-dimethyl-	30.4	$C_{15}H_{16}O_2$ $C_{14}H_{14}O_2$	52 50
12	**Coumarin** 2H-1-Benzopyran-2-one, 7-methoxy-8-(3-methyl-2-butenyl)- / Osthole	31	$C_{15}H_{16}O_3$	57
13	5,10-Dihydroxy-2-methoxy-7-methyl-1,4-anthracenedione ALLOGIBBERINIC ACID	31.8	$C_{16}H_{12}O_5$ $C_{18}H_{20}O_3$	61 57
14	5,10-Dihydroxy-2-methoxy-7-methyl-1,4-anthracenedione Podocarpa-6,8,11,13-tetraen-12-ol, 13-isopropyl-, acetate	33	$C_{16}H_{12}O_5$ $C_{22}H_{30}O_2$	62 61

Peos 8

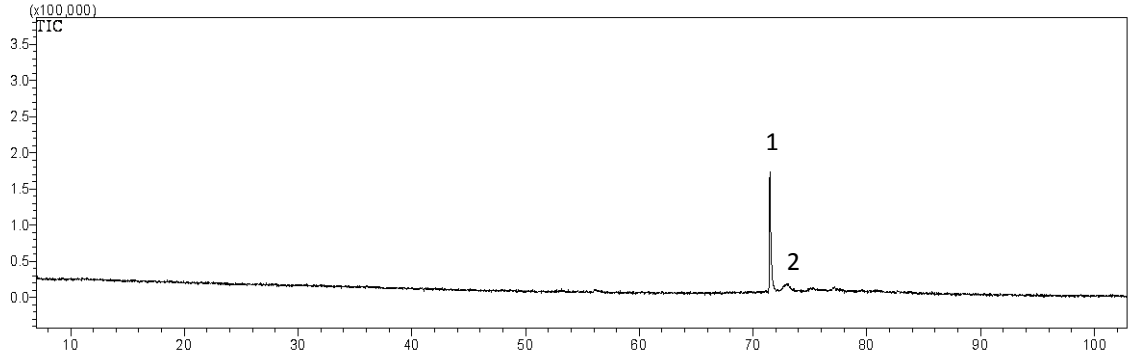
Method 1, 0.2 mg in 0.5mL MeOH



peak no.	main compounds	ret. time	formula	similarity [%]
1	Di-n-octyl phthalate 1,2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate	71.5	$C_{24}H_{38}O_4$ $C_{24}H_{38}O_4$	94 94

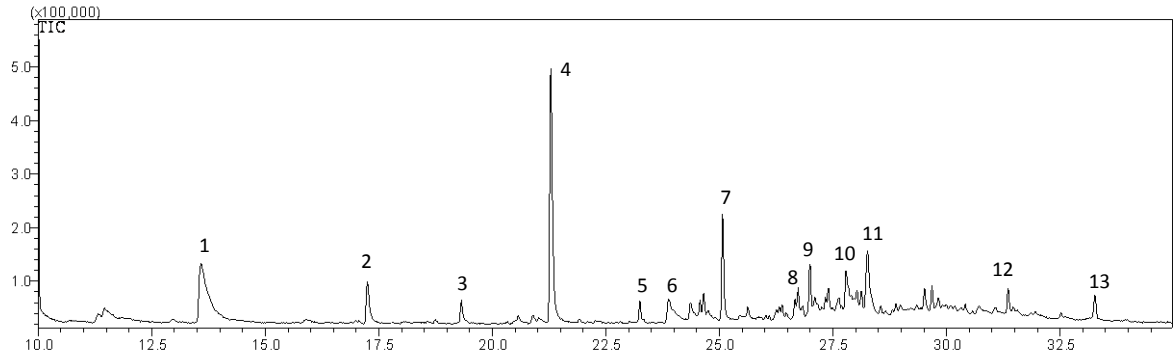
Peos 9

Method 1, 0.2 mg in 0.1mL MeOH



peak no.	main compounds	ret. time	formula	similarity [%]
1	Di-n-octyl phthalate	71.5	$C_{24}H_{38}O_4$	97
	1,2-Benzenedicarboxylic acid, dioctyl ester (CAS) Dioctyl phthalate		$C_{24}H_{38}O_4$	97
2	**monoterpene**	73		
	CARYOPHYLLENE <14-HYDROXY-9-EPI-(E)-		$C_{15}H_{24}O$	56

Method 2

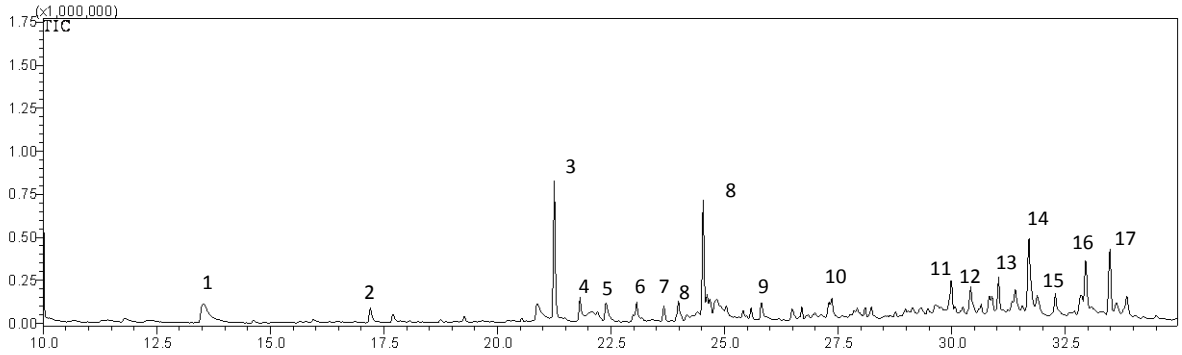


peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	91
	10-Undecenoic acid, methyl ester		$C_{12}H_{22}O_2$	84
2	**Fatty acid**	17.3		
	Pentadecanoic acid, methyl ester (CAS)		$C_{16}H_{32}O_2$	94
	Octadecanoic acid, methyl ester (CAS)/ Stearic acid methyl ester		$C_{19}H_{38}O_2$	93
	Tridecanoic acid, methyl ester		$C_{14}H_{28}O_2$	93
	Hexadecanoic acid, 15-methyl-, methyl ester		$C_{18}H_{36}O_2$	93
	Nonadecanoic acid, methyl ester		$C_{20}H_{40}O_2$	93

3	**Fatty acid**	19.4		
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	92
	Octadecanoic acid, methyl ester / Methyl stearate		$C_{19}H_{38}O_2$	91
	Eicosanoic acid, methyl ester / ARACHIDIC ACID METHYL ESTER		$C_{21}H_{42}O_2$	91
	Heneicosanoic acid, methyl ester		$C_{22}H_{44}O_2$	91
4	Main peak → **fatty acid**	21.3		
	Hexadecanoic acid, methyl ester / Palmitic acid, methyl ester		$C_{17}H_{34}O_2$	96
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	95
5	**Solvent/ column dirt**	23.2		
	1-Eicosanol		$C_{20}H_{42}O$	85
	METHYL 9,10-DIDEUTERO-9-OCTADECENOATE 1-Heptadecanol		$C_{19}H_{34}D_2O_2$	85
	1-Octadecanol (CAS) Stenol		$C_{17}H_{36}O$	83
	1-Hexadecene (CAS) Cetene		$C_{18}H_{38}O$	83
7	2-Pentenenitrile, 5-(p-anisyl)-3-methyl-	24	$C_{13}H_{15}NO$	85
	2-(4-Methoxyphenyl)ethanol		$C_9H_{12}O_2$	84
8	**Fatty acid**	25		
	Octadecanoic acid, methyl ester /Methyl stearate		$C_{19}H_{38}O_2$	94
	Docosanoic acid, methyl ester / Methyl behenate		$C_{23}H_{46}O_2$	93
9	Methyl (11R,12R,13S)-(Z)-12,13-epoxy-11-methoxy-9-octadecenoate	26.7	$C_{20}H_{36}O_4$	81
	1,6-Octadiene, 3-ethoxy-3,7-dimethyl- / Ethyl linalool		$C_{12}H_{22}O$	78
	Linalool / 1,6-Octadien-3-ol, 3,7-dimethyl-		$C_{10}H_{18}O$	75
10	1,6-Octadiene, 3-ethoxy-3,7-dimethyl- / Ethyl linalool	27	$C_{12}H_{22}O$	78
	9,12-Octadecadienal, dimethyl acetal		$C_{20}H_{38}O_2$	75
	NEOMENTHOL		$C_{10}H_{20}O$	74
11	**Fatty acid**	27.8		
	Hexadecadienoic acid, methyl ester		$C_{17}H_{30}O_2$	87
	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester		$C_{25}H_{42}O_2$	84
12	**Fatty acid**	28.3		
	7-Hexadecenoic acid, methyl ester, (Z)-		$C_{17}H_{32}O_2$	81
	Ethyl iso-allocholate		$C_{26}H_{44}O_5$	79
13	Octadecanoic acid, 2-oxo-, methyl ester	31.4	$C_{19}H_{36}O_3$	87
	Heptacosane, 1-chloro-		$C_{27}H_{55}Cl$	87
	Dodecane, 2,6,10-trimethyl- 212		$C_{15}H_{32}$	87
14	Heptacosane, 1-chloro-	33.3	$C_{27}H_{55}Cl$	88
	Octadecane, 1-chloro-		$C_{18}H_{37}Cl$	88
	Tetratriacontane		$C_{34}H_{70}$	88

Peuc 70

Method 2

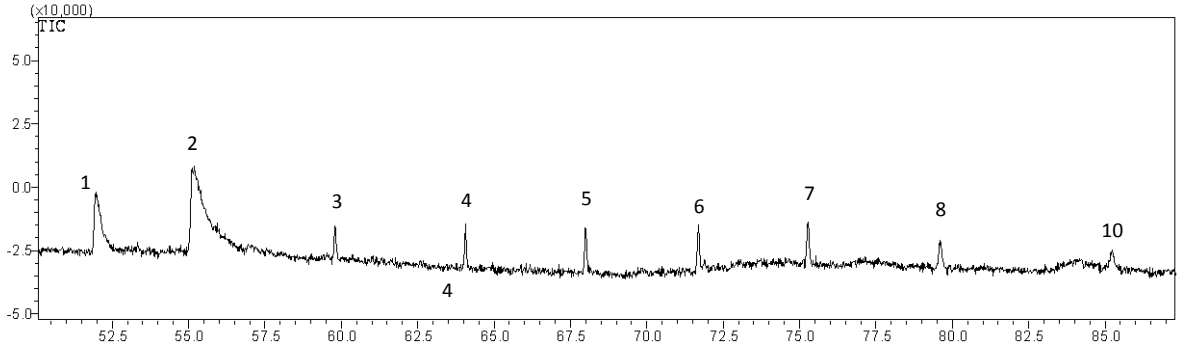


peak no.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	91
	10-Undecenoic acid, methyl ester		$C_{12}H_{22}O_2$	84
2	**Fatty acid**	17.3		
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	94
	Octadecanoic acid, methyl ester / Stearic acid methyl ester		$C_{19}H_{38}O_2$	93
	Tridecanoic acid, methyl ester		$C_{14}H_{28}O_2$	93
	Hexadecanoic acid, 15-methyl-, methyl ester		$C_{18}H_{36}O_2$	93
	Nonadecanoic acid, methyl ester		$C_{20}H_{40}O_2$	93
	Tetradecanoic acid, methyl ester / Methyl myristate		$C_{15}H_{30}O_2$	93
3	**Fatty acid**	21.3		
	Hexadecanoic acid, methyl ester / Palmitic acid, methyl ester		$C_{17}H_{34}O_2$	95
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	95
4	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)-	21.8	$C_{12}H_{12}O_2$	58
	5-Methyl-2-phenyl-2-hexenal		$C_{13}H_{16}O$	57
	2-Cyclopenten-1-one, 4-hydroxy-3-methyl-2-phenyl-		$C_{15}H_{18}O_4$	57
	1,3-Indandione, 2-ethyl-2-methyl-		$C_{12}H_{12}O_2$	57
5	**Coumarin**	22.4		
	3-Methoxycyclohex-2-en-1-one, 2-(2,5-dimethoxyphenyl)-		$C_{15}H_{18}O_4$	68
	2H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy- / Bergaptan / Bergapten		$C_{12}H_8O_4$	63
	7H-Furo[3,2-g][1]benzopyran-7-one, 9-methoxy-/ Methoxsalen / Xanthotoxin		$C_{12}H_8O_4$	61
6	Isoxazol-5(4H)-one, 3-methyl-4-(2-methoxybenzylidene)-	23	$C_{12}H_{11}NO_3$	55
	n-Octanoic acid, ethyldimethylsilyl ester		$C_{12}H_{26}O_2Si$	55
7	2-Cyclohexen-1-one, 3-methoxy-2-(2,4,5-trimethoxyphenyl)-	23.6	$C_{16}H_{20}O_5$	60
	Benzoic acid, 3-iodo-4-methoxy-, methyl ester		$C_9H_9IO_3$	53

8	1,3,5-Hexanetrione, 1-phenyl- Cyclopropane, 1-chloro-1-(3,3-diethoxy-1-propynyl)-2,2-dimethyl-		$C_{12}H_{12}O_3$ $C_{12}H_{19}ClO_2$	54 52
9	**Fatty acid** 9,12-Octadecadienoic acid, methyl ester, (E,E)- / Methyl linolelaidate / Linolelaidic acid, methyl ester 11,14-Eicosadienoic acid, methyl ester 7,10-Hexadecadienoic acid, methyl ester 9,12-Hexadecadienoic acid, methyl ester	24.5	 $C_{19}H_{34}O_2$ $C_{21}H_{38}O_2$ $C_{17}H_{30}O_2$ $C_{17}H_{30}O_2$	 87 86 85 85
10	**Coumarin** 2H-1-Benzopyran-2-one, 7-methoxy-8-(3-methyl-2-butenyl)- / Osthole	25.8	 $C_{15}H_{16}O_3$	 72
11	**Terpenoid** 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]- / d-Nerolidol DIHYDRO-CARVEOL	27.3	 $C_{15}H_2O$ $C_{10}H_{18}O$	 74 71
12	**Coumarin** 2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten	30	 $C_{12}H_8O_4$	 69
13	Quinoline, 6-methyl-2-phenyl- CANELLAL (=MUZIGADIAL) DB5-2807	30.5	$C_{15}H_{20}O_3$ $C_{16}H_{13}N$	55 55
14	**Coumarin** 2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten	31	 $C_{12}H_8O_4$	 67
15	**Coumarin** 2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten	31.7	 $C_{12}H_8O_4$	 69
16	**Coumarin** 2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten	32.3	 $C_{12}H_8O_4$	 65
17	Bicyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl- 1,3-Cyclohexadecanedione, 6-nitro-	33	$C_{10}H_{18}O_2$ $C_{16}H_{27}NO_4$	66 66
18	**Coumarin** 2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten	33.5	 $C_{12}H_8O_4$	 69

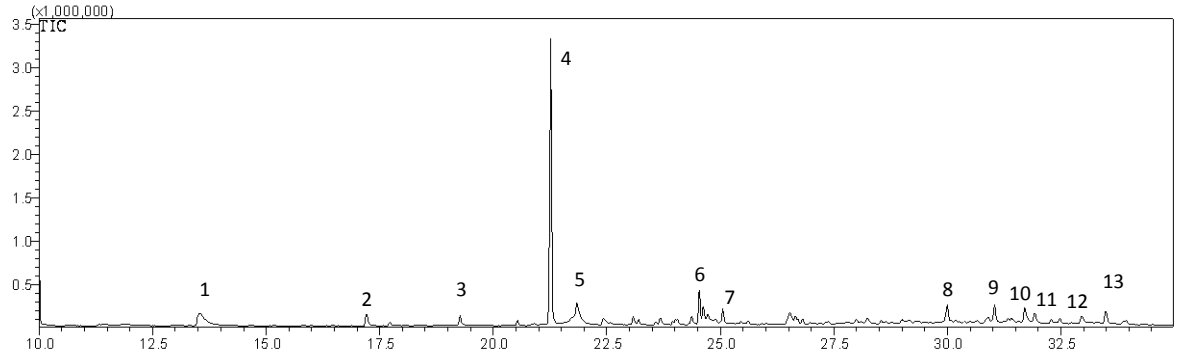
Peuc Det

Method 1, 1mg/ mL DCM



peak no.	main compounds	ret. time	formula	similarity [%]
1	**phthalate** 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (CAS) Isobutyl phthalate	52	$C_{16}H_{22}O_4$	93
2	**phthalate** 1,2-Benzenedicarboxylic acid, dibutyl ester (CAS) Dibutyl phthalate	55	$C_{16}H_{22}O_4$	94
3	**silikon from column** SILIKONFETT SE30 (GREVELS)	60		69
4	**silikon from column** Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- SILIKONFETT SE30 (GREVELS)	64		75 74
5	**silikon from column** EICOSAMETHYLCYCLODECASILOXANE SILIKONFETT SE30 (GREVELS)	68	$C_{20}H_{60}O_{10}SI_{10}$	82 77
6	**silikon from column** EICOSAMETHYLCYCLODECASILOXANE Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	71.5	$C_{20}H_{60}O_{10}SI_{10}$ $C_{16}H_{50}O_7Si_8$	80 79
7	**silikon from column** SILIKONFETT SE30 (GREVELS) EICOSAMETHYLCYCLODECASILOXANE	75.5	 $C_{20}H_{60}O_{10}SI_{10}$	 78 78
8	**silikon from column** SILIKONFETT SE30 (GREVELS)	79.5		77

Method 2



peak No.	main compounds	ret. time	formula	similarity [%]
1	**Fatty acid**	13.5		
	Nonanedioic acid, dimethyl ester		$C_{11}H_{20}O_4$	91
	10-Undecenoic acid, methyl ester		$C_{12}H_{22}O_2$	84
2	**Fatty acid**	17.3		
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	95
	Octadecanoic acid, methyl ester / Stearic acid methyl ester		$C_{19}H_{38}O_2$	94
	Tridecanoic acid, methyl ester		$C_{14}H_{28}O_2$	94
	Hexadecanoic acid, 15-methyl-, methyl ester		$C_{18}H_{36}O_2$	94
	Nonadecanoic acid, methyl ester		$C_{20}H_{40}O_2$	94
	Tetradecanoic acid, methyl ester / Methyl myristate		$C_{15}H_{30}O_2$	94
3	**Fatty acid**	19.4		
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	92
	Octadecanoic acid, methyl ester / Methyl stearate		$C_{19}H_{38}O_2$	91
	Eicosanoic acid, methyl ester / ARACHIDIC ACID METHYL ESTER		$C_{21}H_{42}O_2$	90
	Heneicosanoic acid, methyl ester		$C_{22}H_{44}O_2$	90
4	Main peak → **Fatty acid**	21.3		
	Hexadecanoic acid, methyl ester / Palmitic acid, methyl ester		$C_{17}H_{34}O_2$	97
	Pentadecanoic acid, methyl ester		$C_{16}H_{32}O_2$	94
5	Pheneacetic acid, 2-carboxy-3,5,6,.alpha.,.alpha.-pentamethyl-, methyl ester	21.8	$C_{15}H_{20}O_4$	56
	1,2,3,4-4H-Isoquinolin-1,3-dione, 4,4,5,6,8-pentamethyl-		$C_{14}H_{17}NO_2$	55
	Benzoic acid, 2-[2-isobutynitrilyl]-3,4,6-trimethyl-		$C_{14}H_{17}NO_2$	55
	2,3,5,6-Tetramethylterephthalaldehyde		$C_{12}H_{14}O_2$	55
	5-Methyl-2-phenyl-2-hexenal		$C_{13}H_{16}O$	55
	Artemisin		$C_{15}H_{18}O_4$	55
6	**Fatty acid**	24.5		
	9,12-Octadecadienoic acid, methyl ester, (E,E)- / Methyl linolelaidate / Linolelaidic acid, methyl ester 11,14-Eicosadienoic acid, methyl ester		$C_{19}H_{34}O_2$	96
			$C_{21}H_{38}O_2$	95

7	**Fatty acid**	25		
	Octadecanoic acid, methyl ester		$C_{19}H_{38}O_2$	87
	Eicosanoic acid, methyl ester		$C_{21}H_{42}O_2$	87
8	**Coumarin**	30		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	66
9	**Coumarin**	31		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	68
10	**Coumarin**	31.7		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	70
11	**Fatty acid**	32.3		
	Tetradecanoic acid, 12-methyl-, methyl ester		$C_{16}H_{32}O_2$	83
	Heptacosanoic acid, methyl ester		$C_{28}H_{56}O_2$	82
	Pentadecanoic acid, 14-methyl-, methyl ester		$C_{17}H_{34}O_2$	82
	Octadecanoic acid, methyl ester		$C_{19}H_{38}O_2$	82
12	6-Dimethylsilyloxytetradecane	33	$C_{16}H_{36}OSi$	66
13	**Coumarin**	33.5		
	2H-Furo[2,3-h]-1-benzopyran-2-one, 5-methoxy- / Isobergapten		$C_{12}H_8O_4$	69

Annex II:

Supporting information of the first publication

(Vogl *et al*., 2011)

Supporting Information

Identification and Quantification of Coumarins in *Peucedanum ostruthium* (L.) Koch by HPLC-DAD and HPLC-DAD-MS

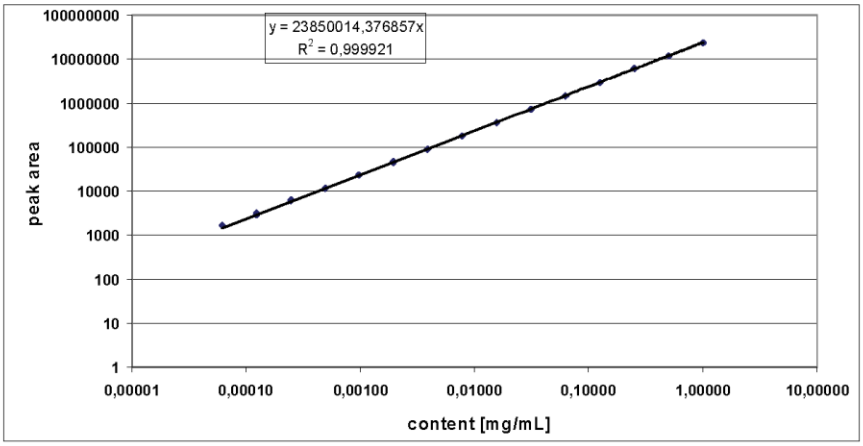
SYLVIA VOGL[†], MARTIN ZEHL[*,†], PAOLO PICKER[†], ERNST URBAN[‡], CHRISTOPH WAWROSCH[†], GOTTFRIED REZNICEK[†], JOHANNES SAUKEL[†], AND BRIGITTE KOPP[†]

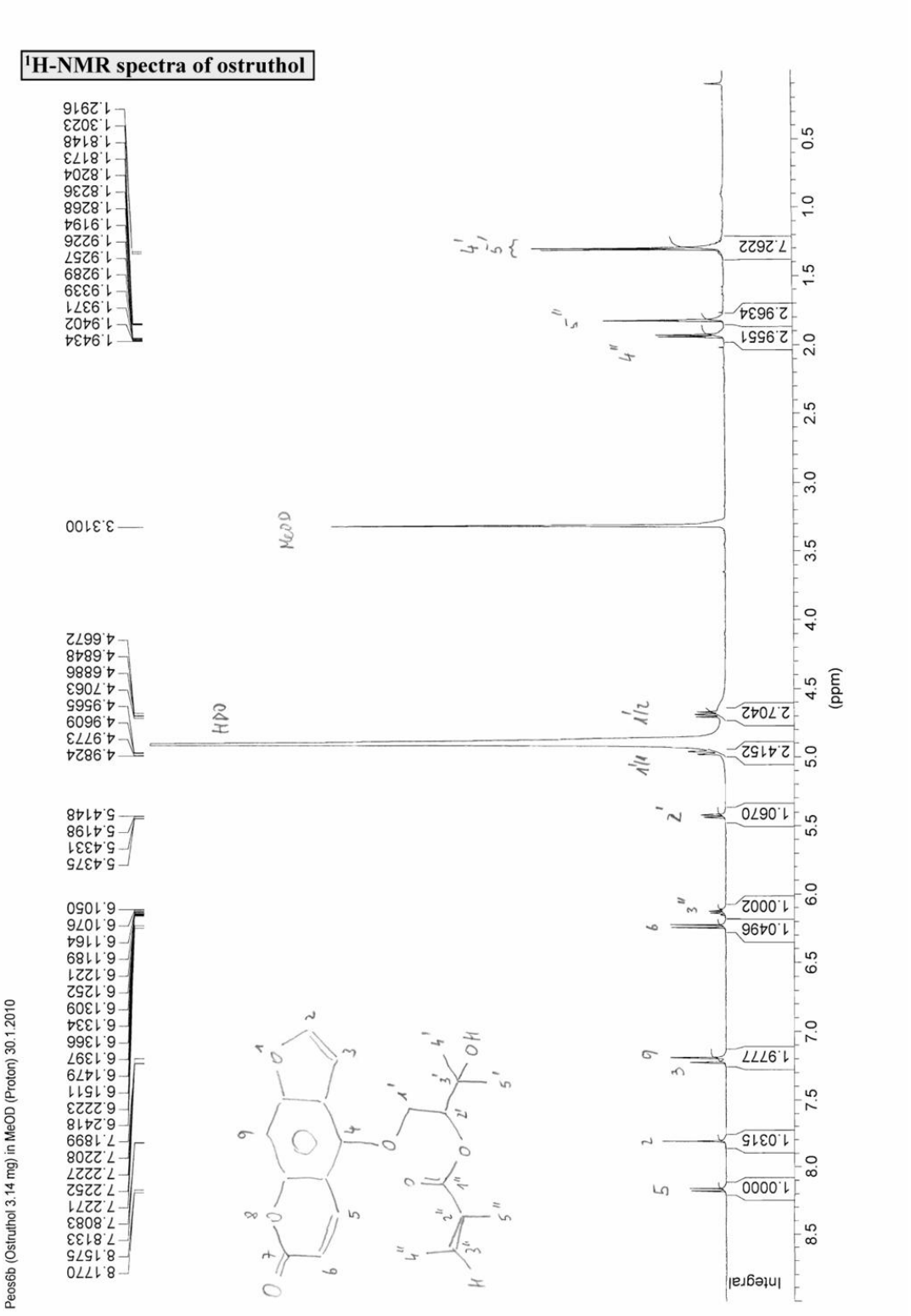
[†]Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

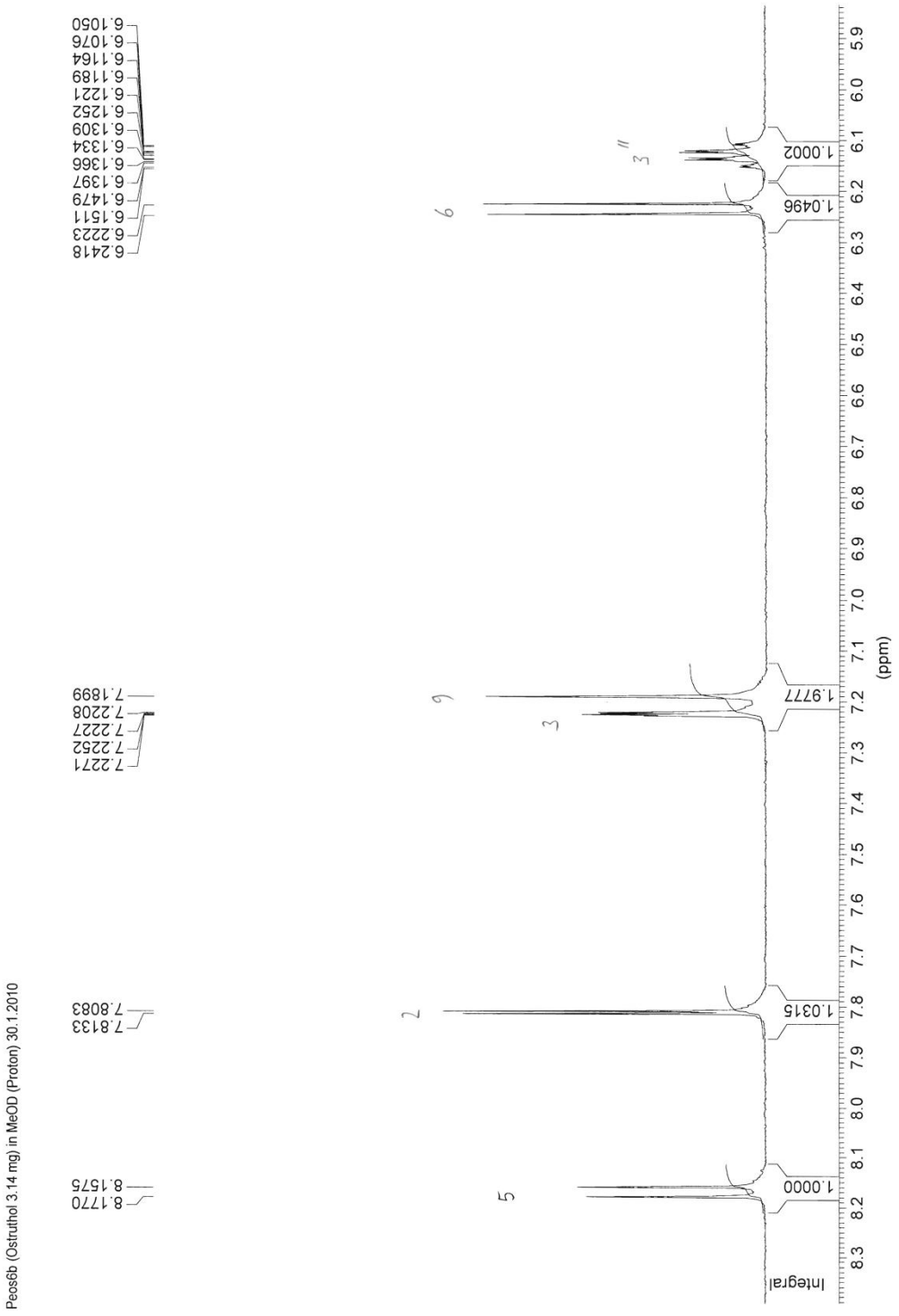
[‡]Department for Medicinal Chemistry, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

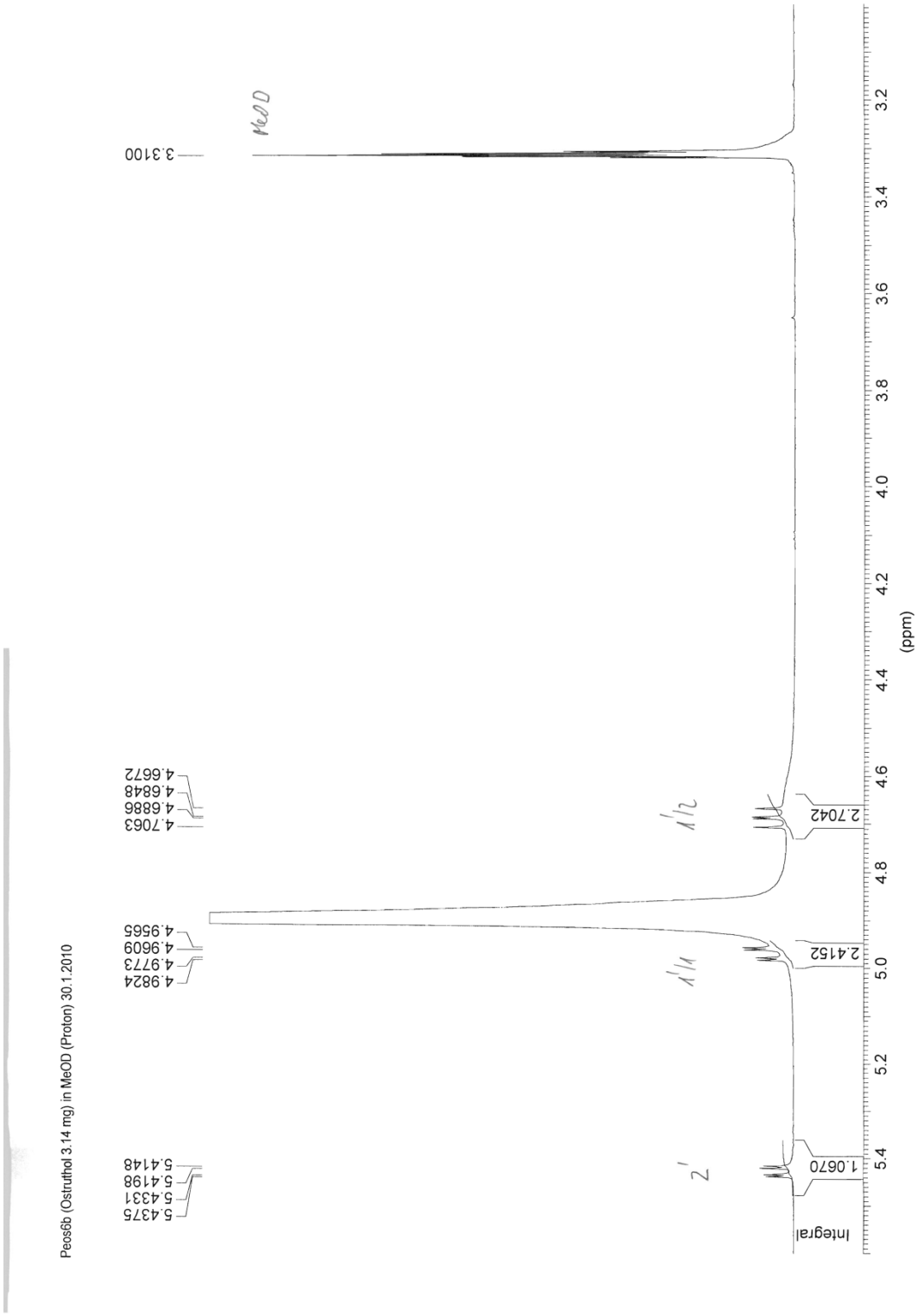
* To whom correspondence should be addressed. Phone: +43-1-4277-55219. Fax: +43-1-4277-55256. E-mail: martin.zehl@univie.ac.at

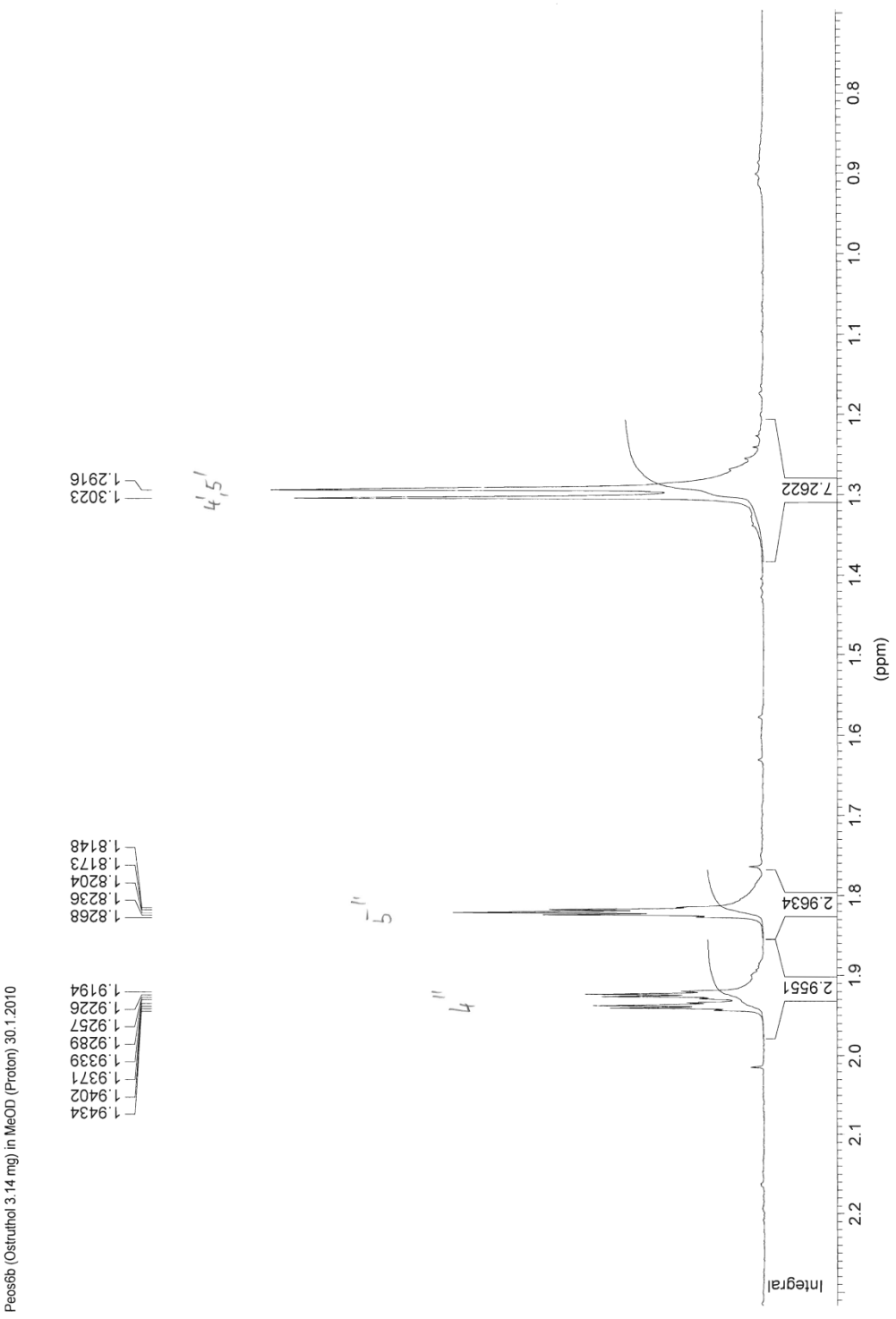
Figure S1. Calibration function (linear regression) for imperatorin.

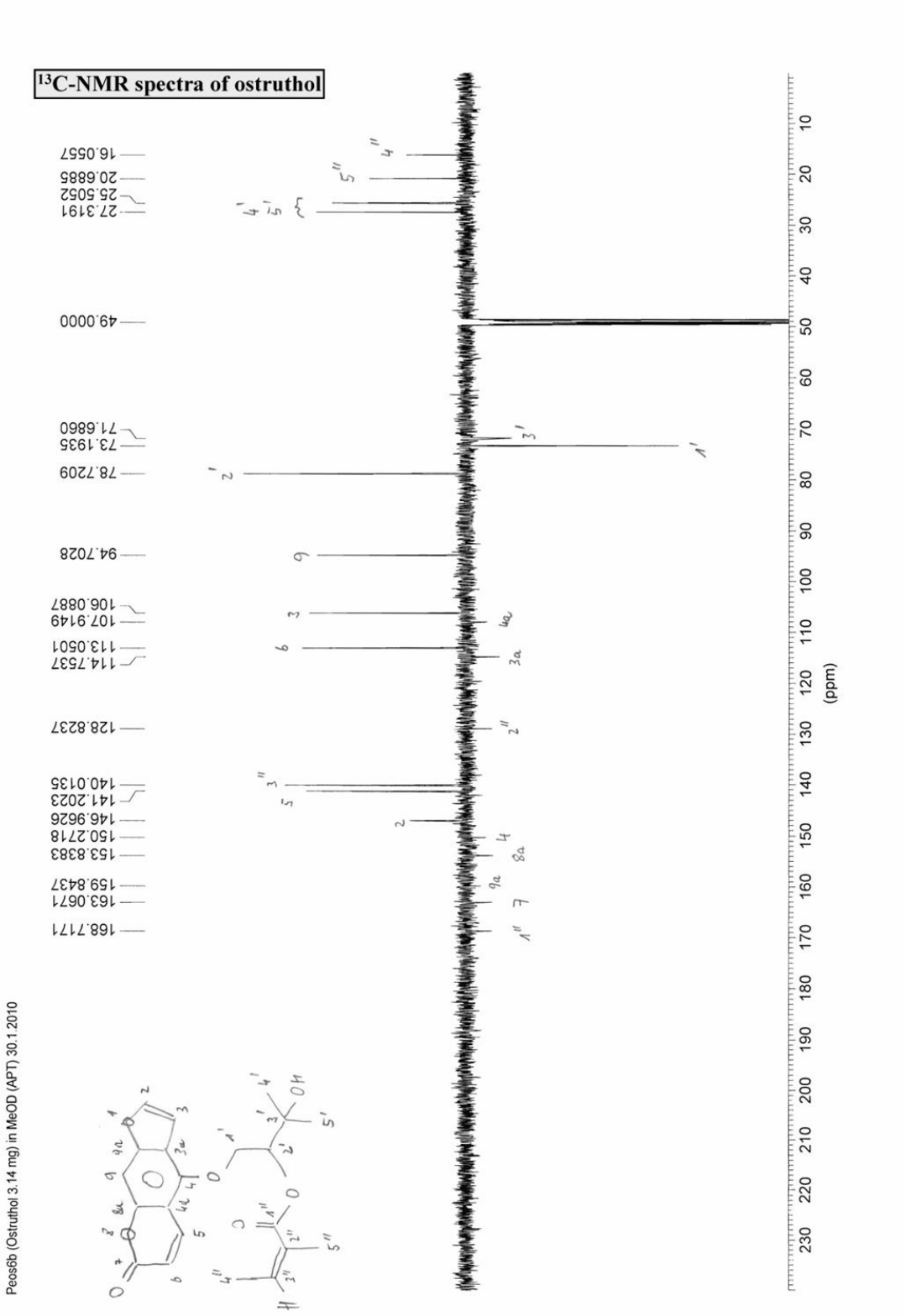


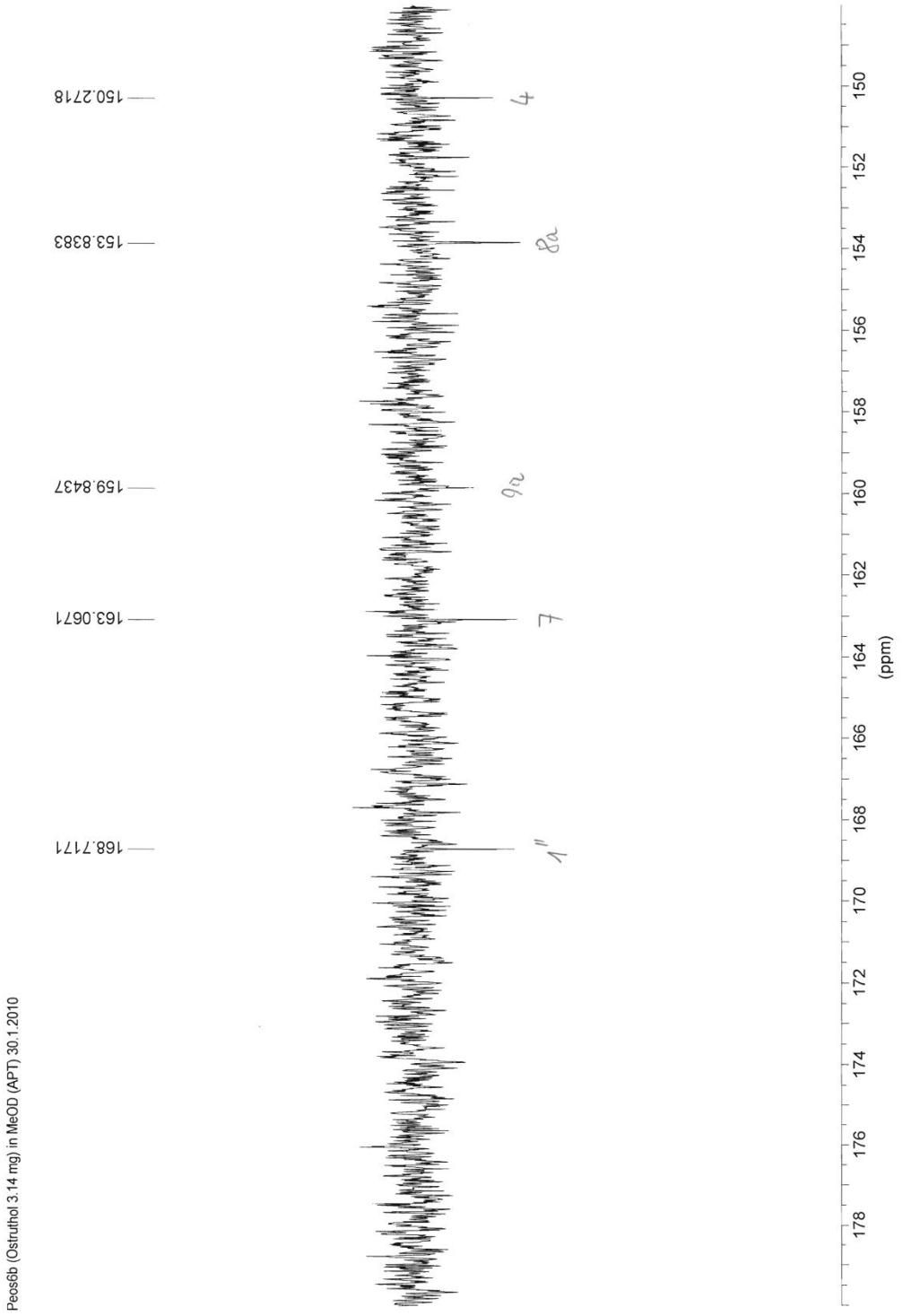

¹H-NMR spectra of ostruthol
Peos6b (Ostruthol 3.14 mg) in MeOD (Proton) 30.1.2010
MeOD
HDO
(ppm)
Integral

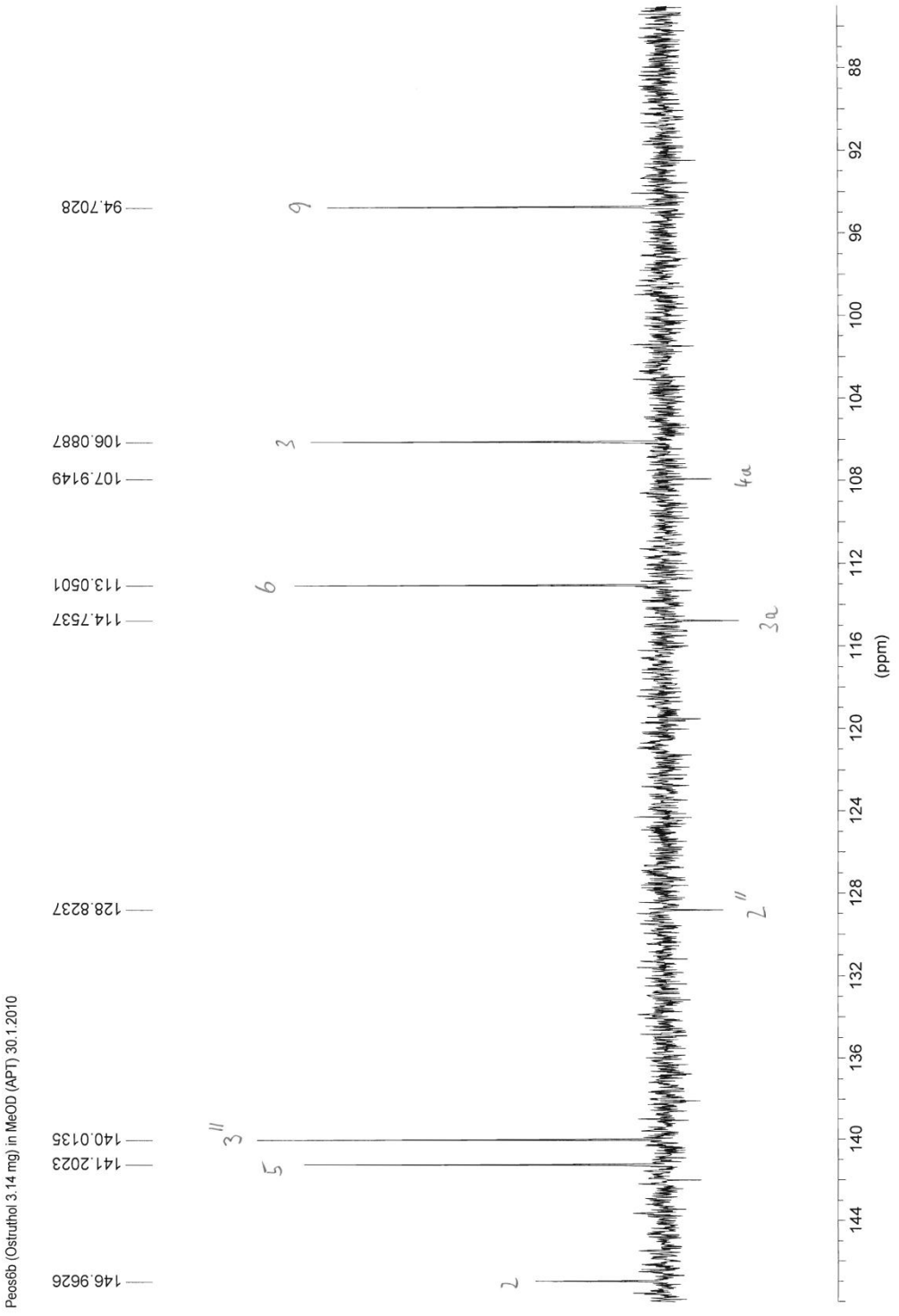

Peos6b (Ostruthol 3.14 mg) in MeOD (Proton) 30.1.2010
8.1770
8.1575
7.8133
7.8083
7.2271
7.2252
7.2227
7.2208
7.1899
6.2418
6.2223
6.1511
6.1479
6.1397
6.1366
6.1334
6.1309
6.1252
6.1221
6.1189
6.1164
6.1076
6.1050
Integral
1.0000
1.0315
1.9777
1.0496
1.0002
(ppm)

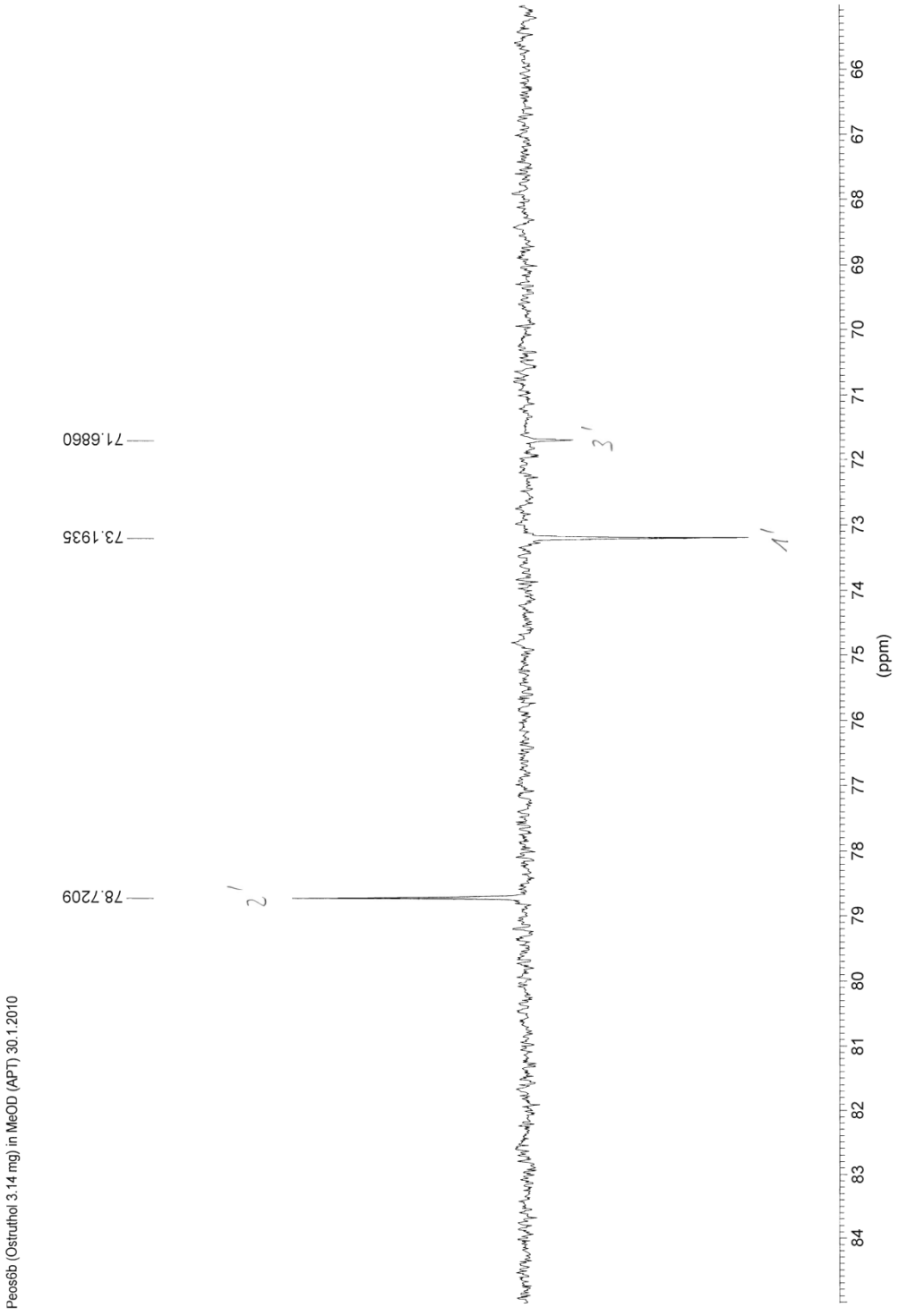

Peos6b (Ostruthol 3.14 mg) in MeOD (Proton) 30.1.2010
MeOD
3.3100
4.6672
4.6848
4.6886
4.7063
4.9565
4.9609
4.9773
4.9824
5.4148
5.4198
5.4331
5.4375
2.7042
2.4152
1.0670
Integral
(ppm)

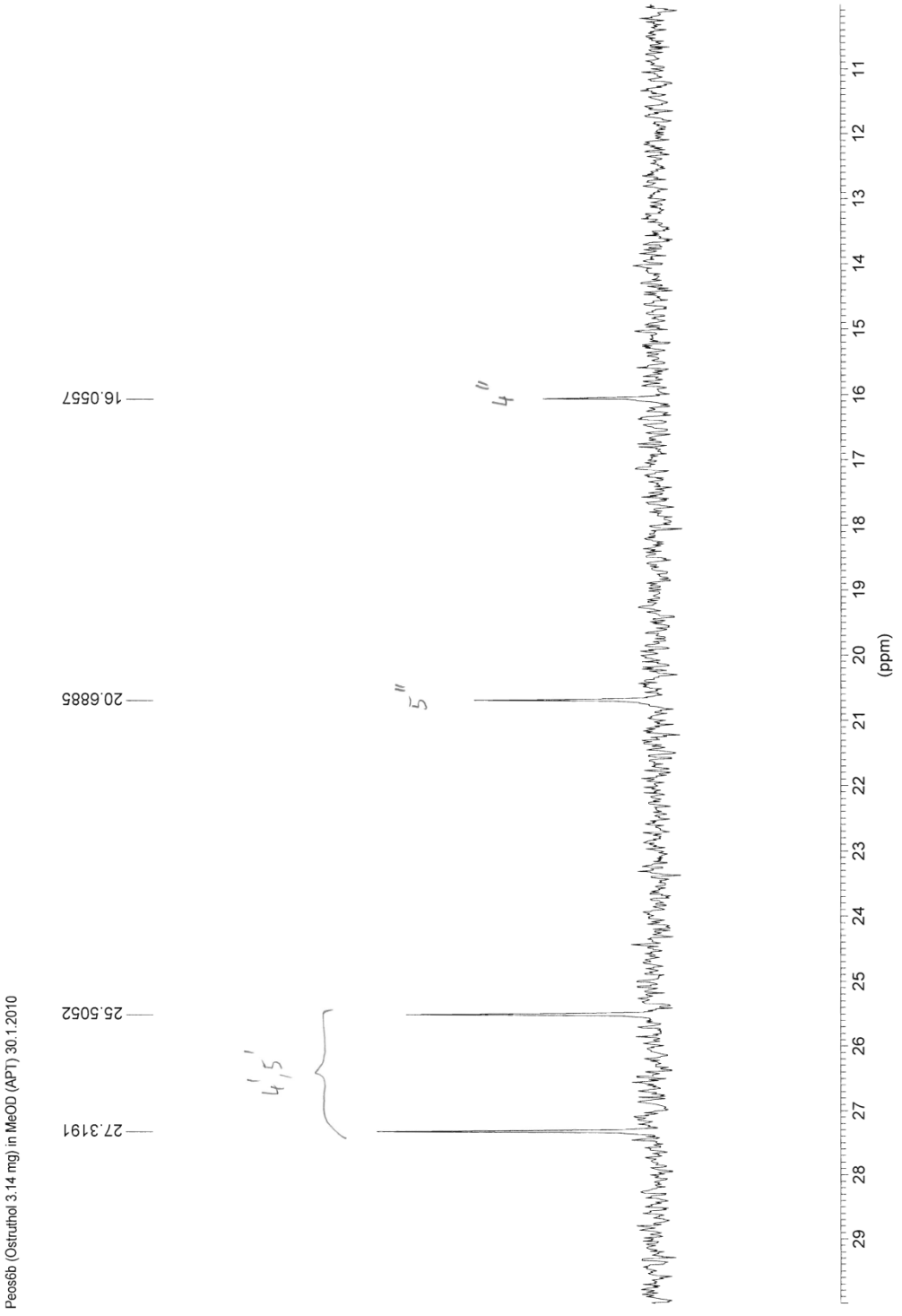

Peos6b (Ostruthol 3.14 mg) in MeOD (Proton) 30.1.2010
1.9434
1.9402
1.9371
1.9339
1.9289
1.9257
1.9226
1.9194
1.8268
1.8236
1.8204
1.8173
1.8148
1.3023
1.2916
4',5'
1''
5
4''
7.2622
2.9634
2.9551
Integral
(ppm)

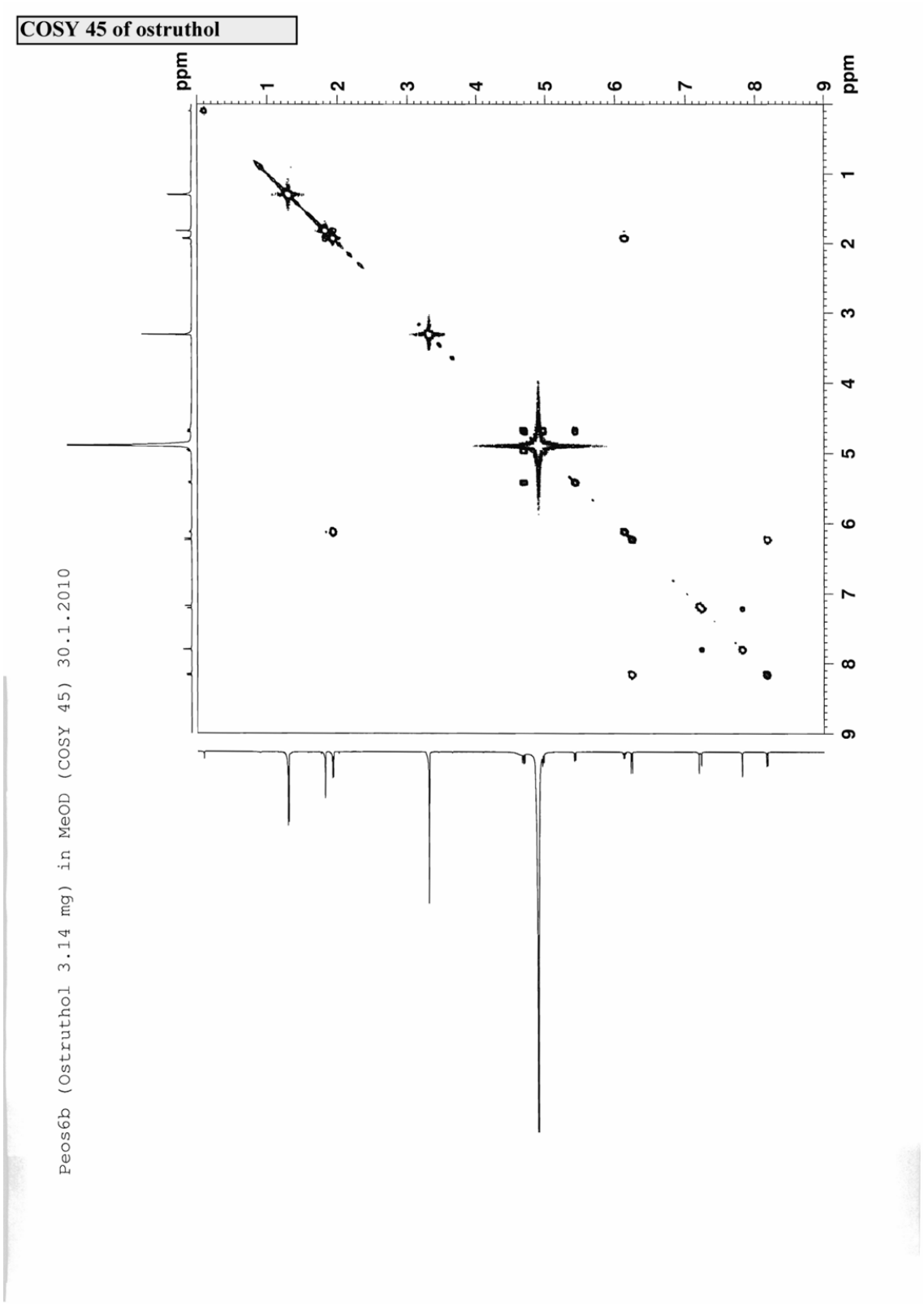

13C-NMR spectra of ostruthol
16.0557
20.6885
25.5052
27.3191
49.0000
71.6860
73.1935
78.7209
94.7028
106.0887
107.9149
113.0501
114.7537
128.8237
140.0135
141.2023
146.9626
150.2718
153.8383
159.8437
163.0671
168.7171
(ppm)
Peos6b (Ostruthol 3.14 mg) in MeOD (APT) 30.1.2010

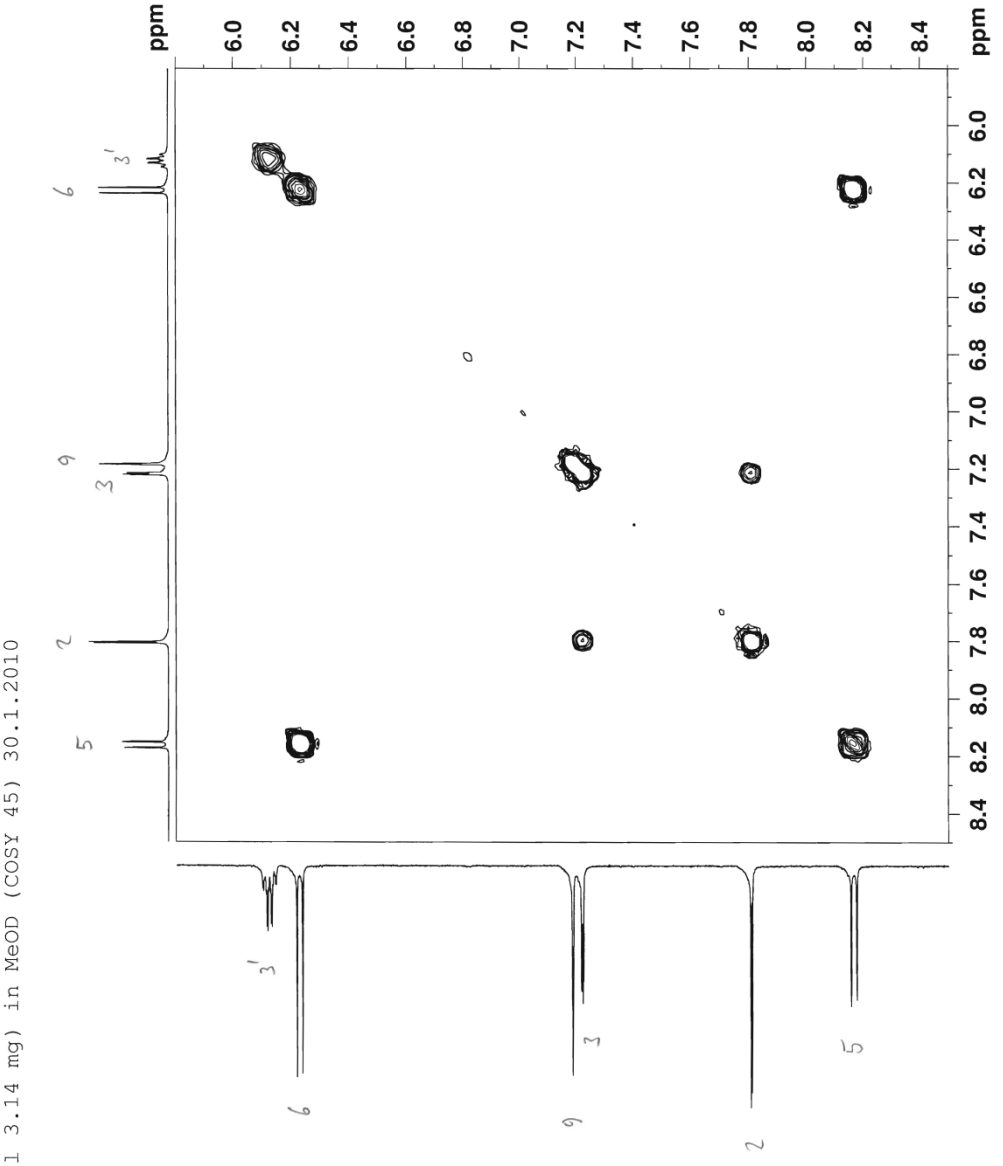

Peos6b (Ostruthol 3.14 mg) in MeOD (APT) 30.1.2010
150.2718
153.8383
159.8437
163.0671
168.7171
4
8a
9a
7
1''
150
152
154
156
158
160
162
164
166
168
170
172
174
176
178
(ppm)

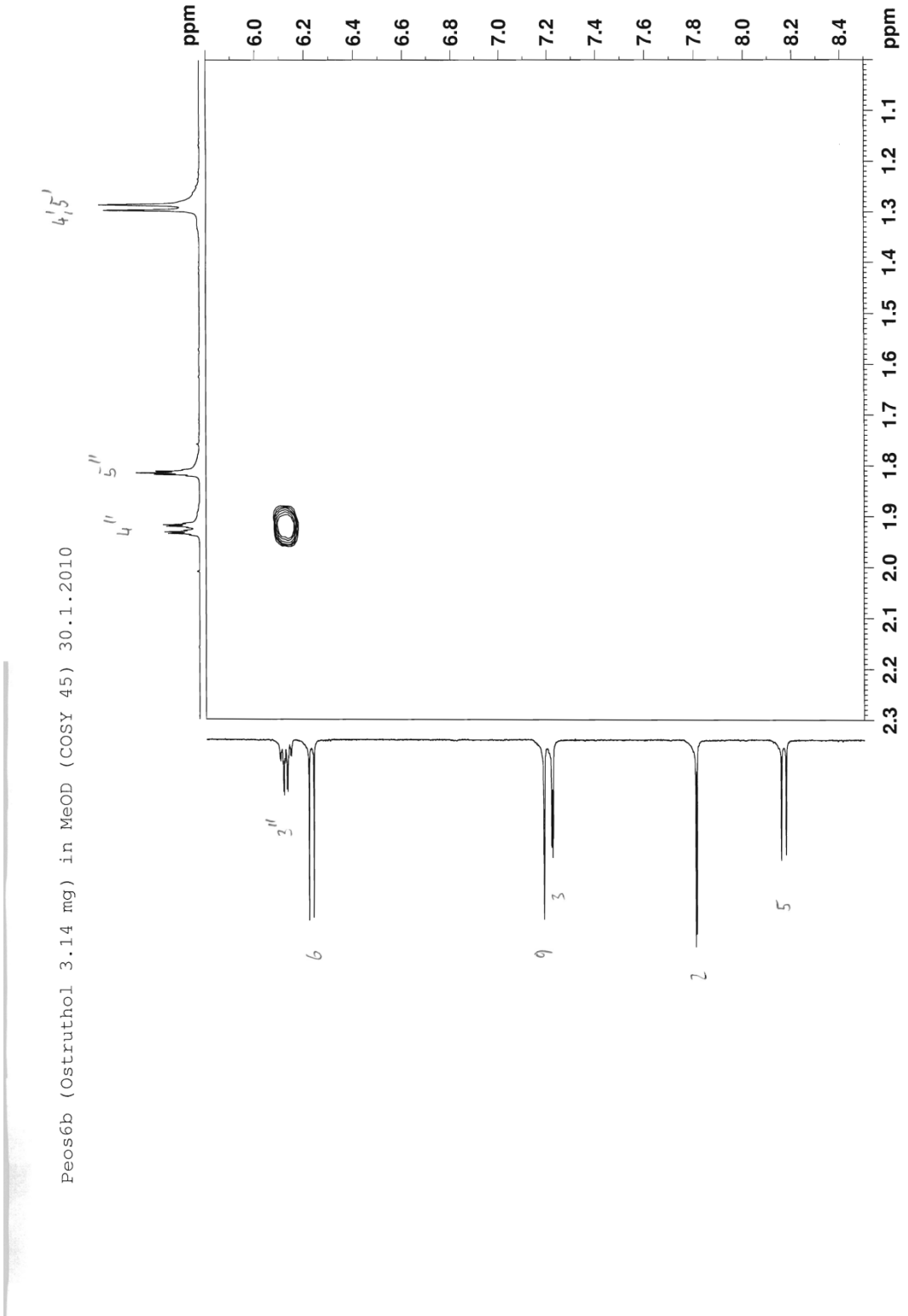

Peos6b (Ostruthol 3.14 mg) in MeOD (APT) 30.1.2010
94.7028
106.0887
107.9149
113.0501
114.7537
128.8237
140.0135
141.2023
146.9626
9
3
4a
6
3a
2''
3''
5
2
88
92
96
100
104
108
112
116
120
124
128
132
136
140
144
(ppm)

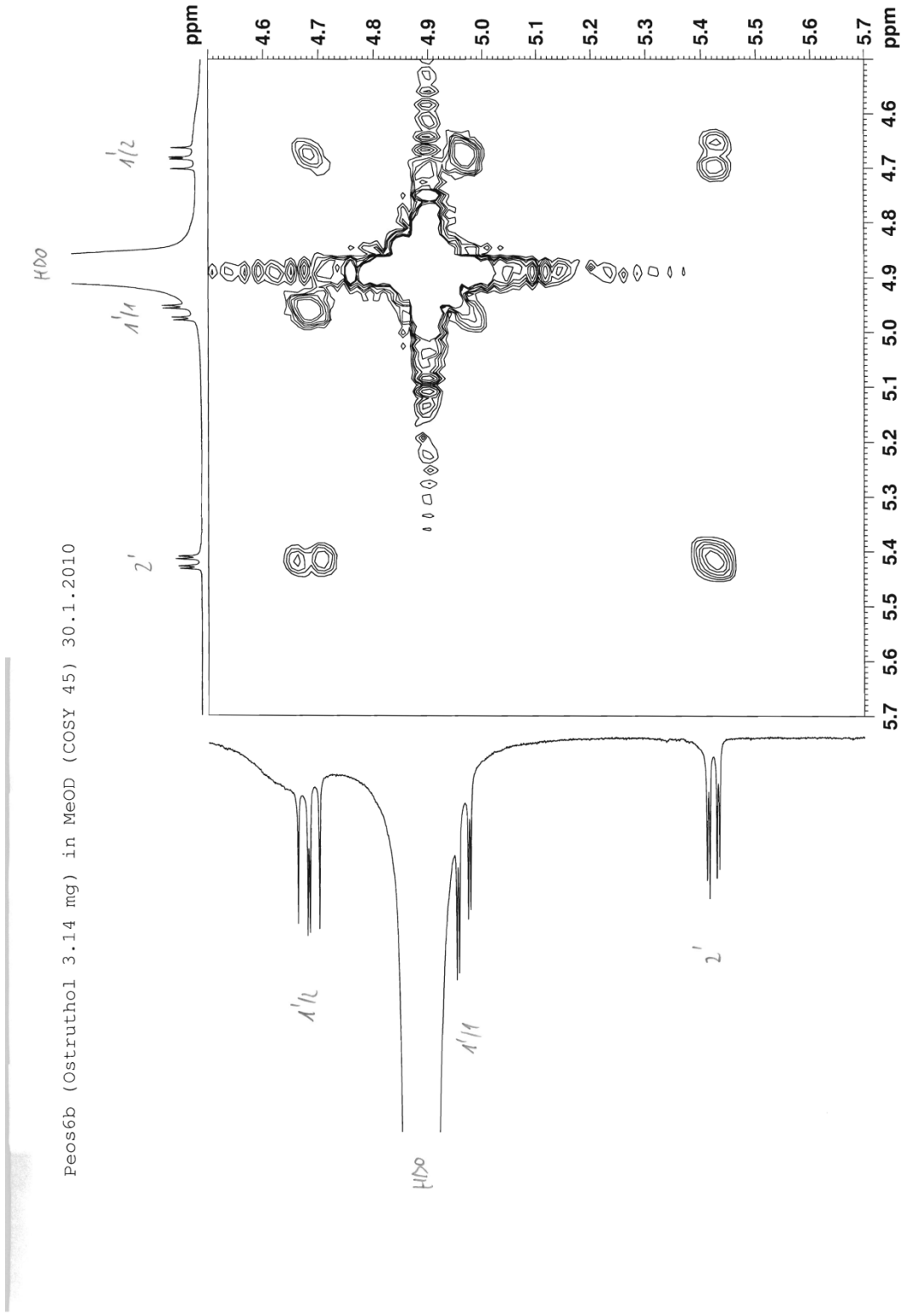

Peos6b (Ostruthol 3.14 mg) in MeOD (APT) 30.1.2010
78.7209
73.1935
71.6860
1'
2'
3'
84
83
82
81
80
79
78
77
76
75
74
73
72
71
70
69
68
67
66
(ppm)

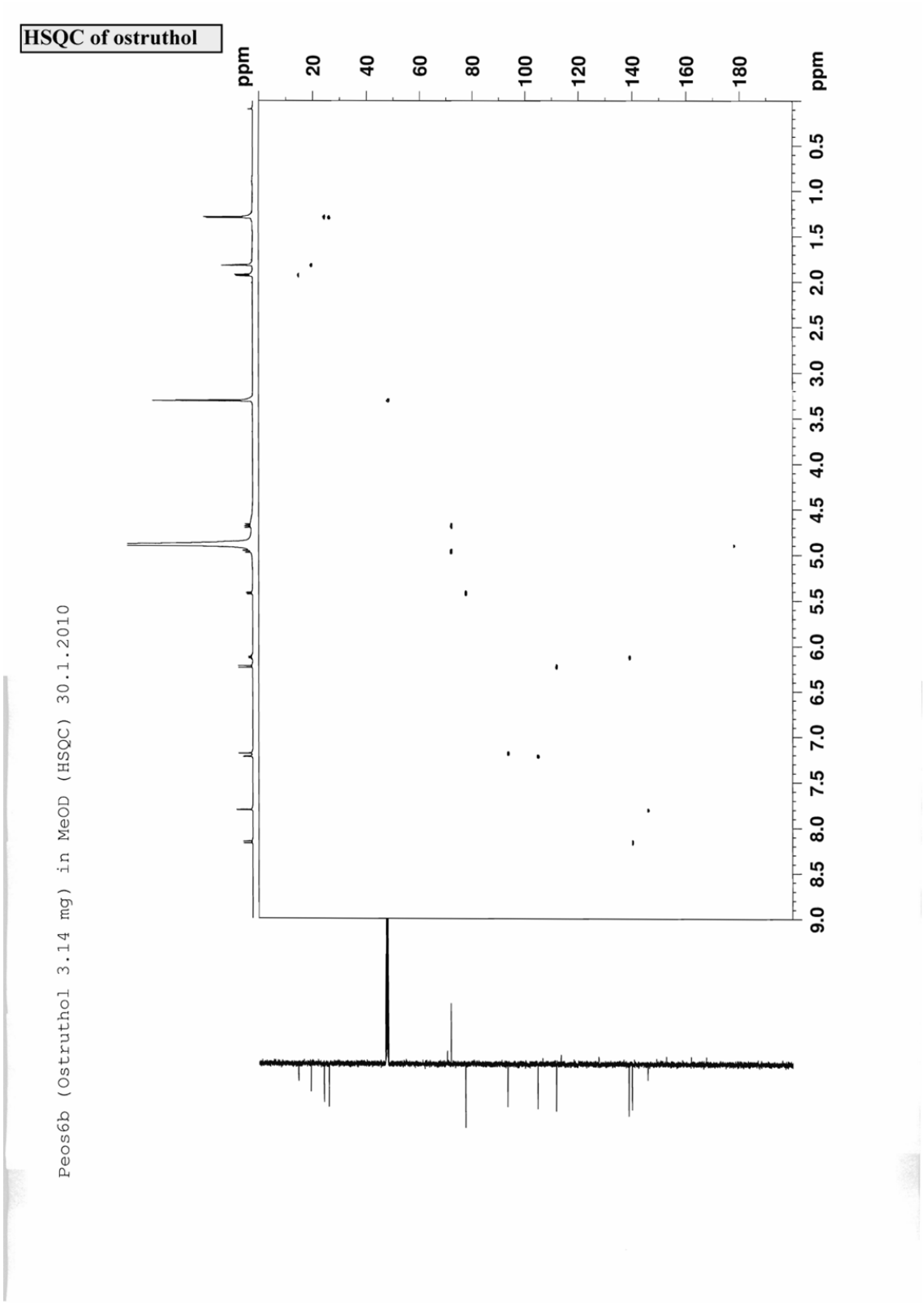

Peos6b (Ostruthol 3.14 mg) in MeOD (APT) 30.1.2010
16.0557
20.6885
25.5052
27.3191
4'
5'
5''
4''
(ppm)

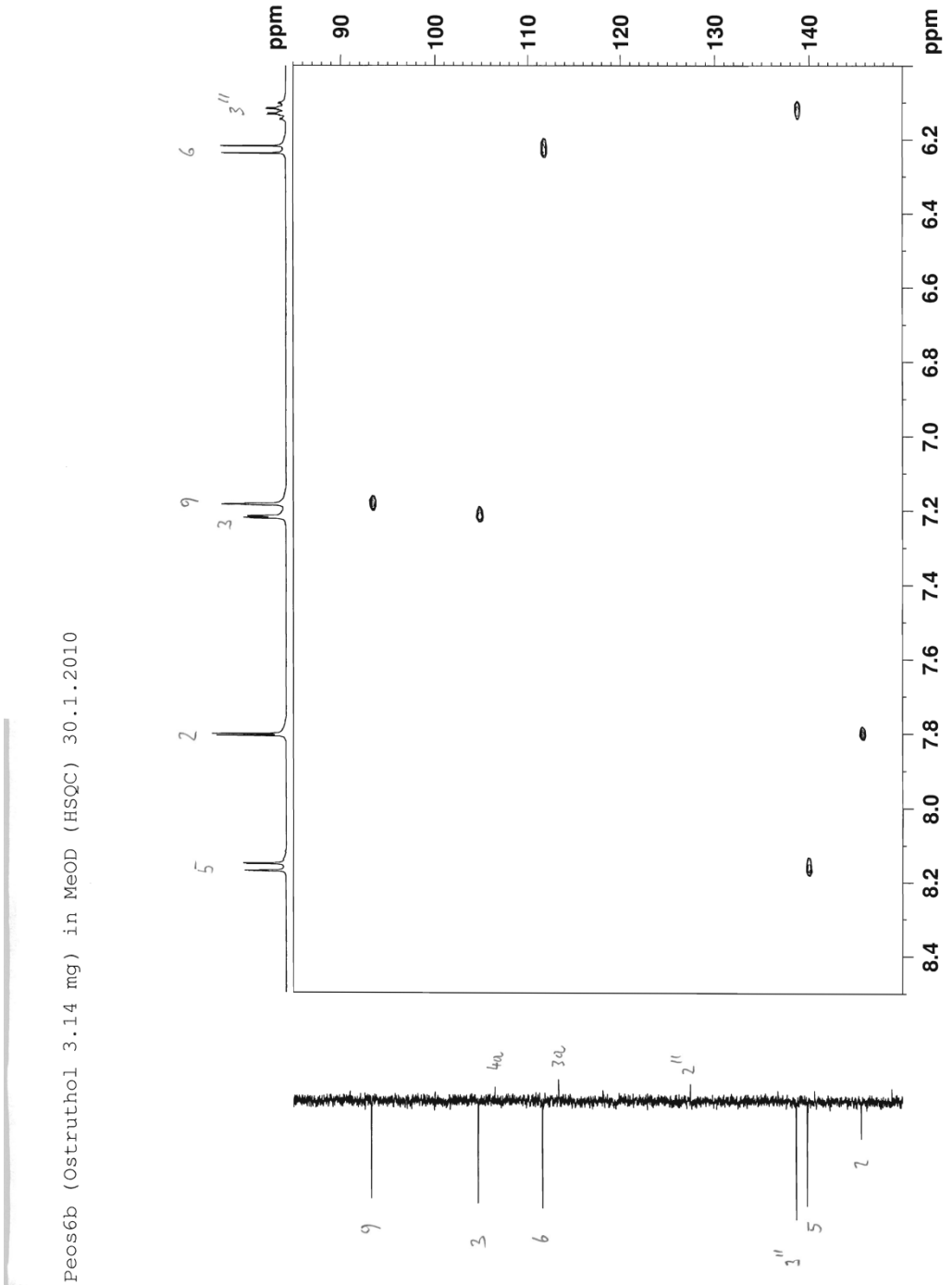

COSY 45 of ostruthol
ppm
1
2
3
4
5
6
7
8
9
Peos6b (Ostruthol 3.14 mg) in MeOD (COSY 45) 30.1.2010

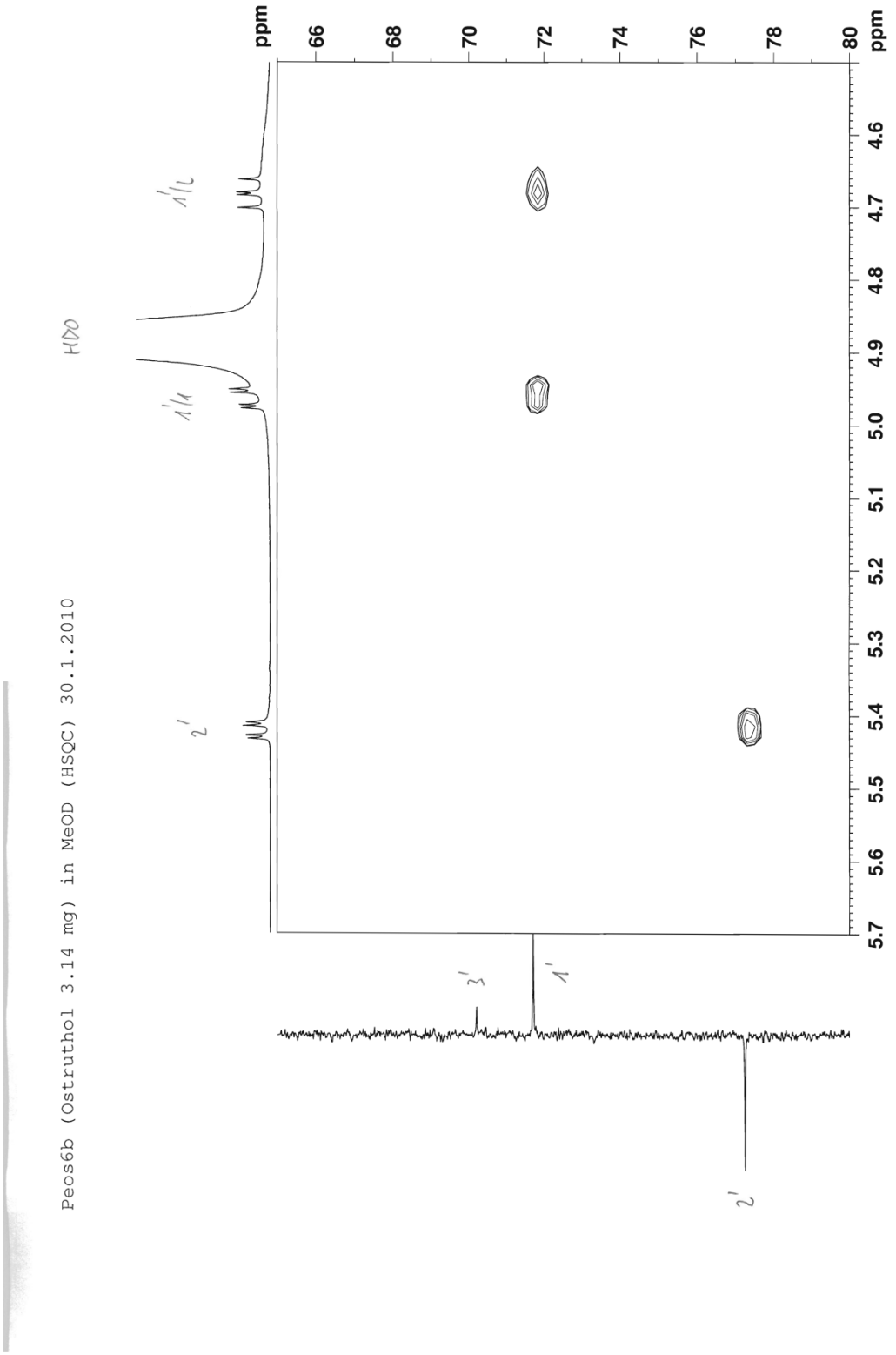

Peos6b (Ostruthol 3.14 mg) in MeOD (COSY 45) 30.1.2010
ppm
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
6
3'
9
3
2
5

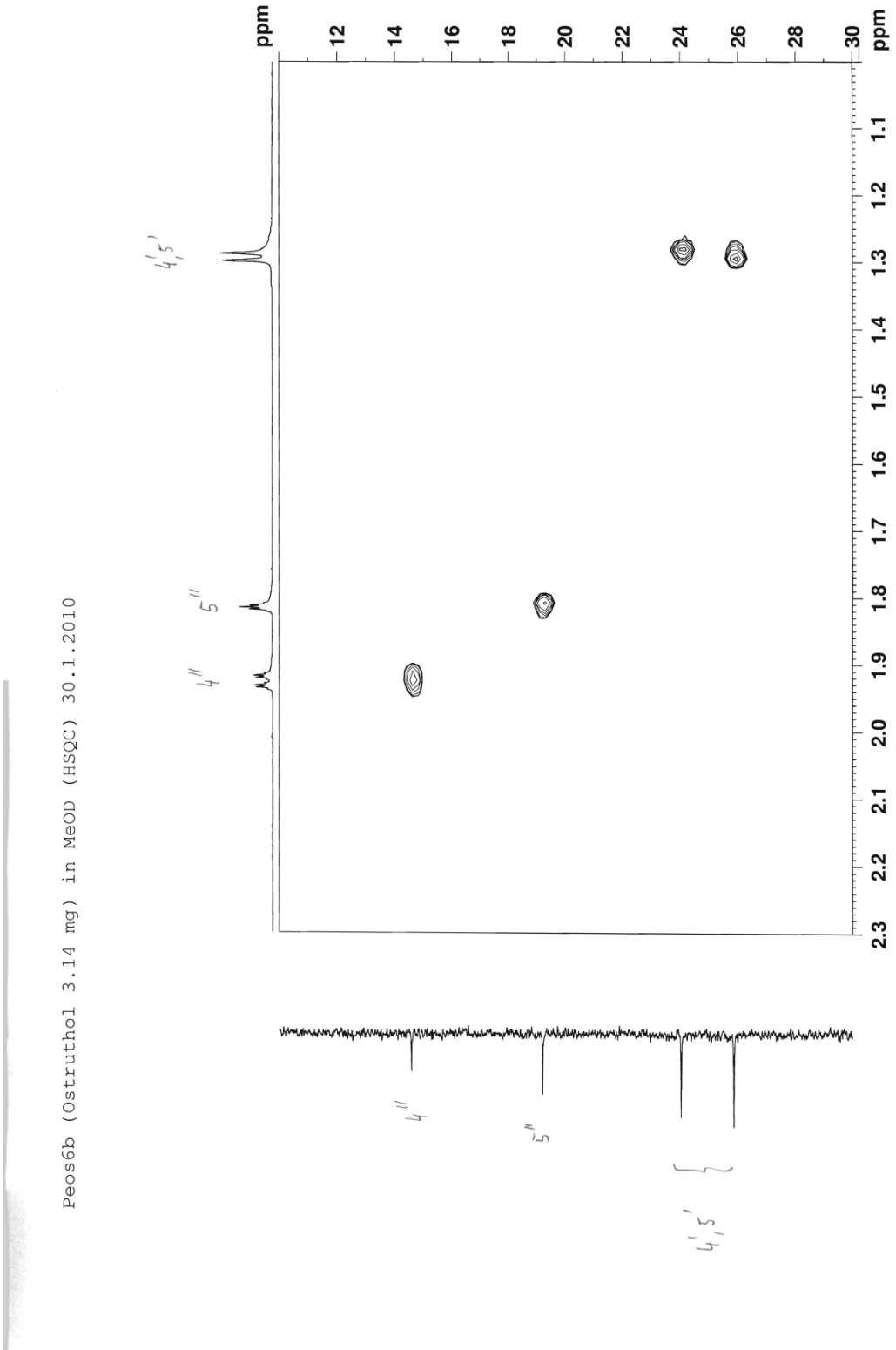

Peos6b (Ostruthol 3.14 mg) in MeOD (COSY 45) 30.1.2010
ppm
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
ppm
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
4',5'
5''
4''
2''
6
3
9
2
5

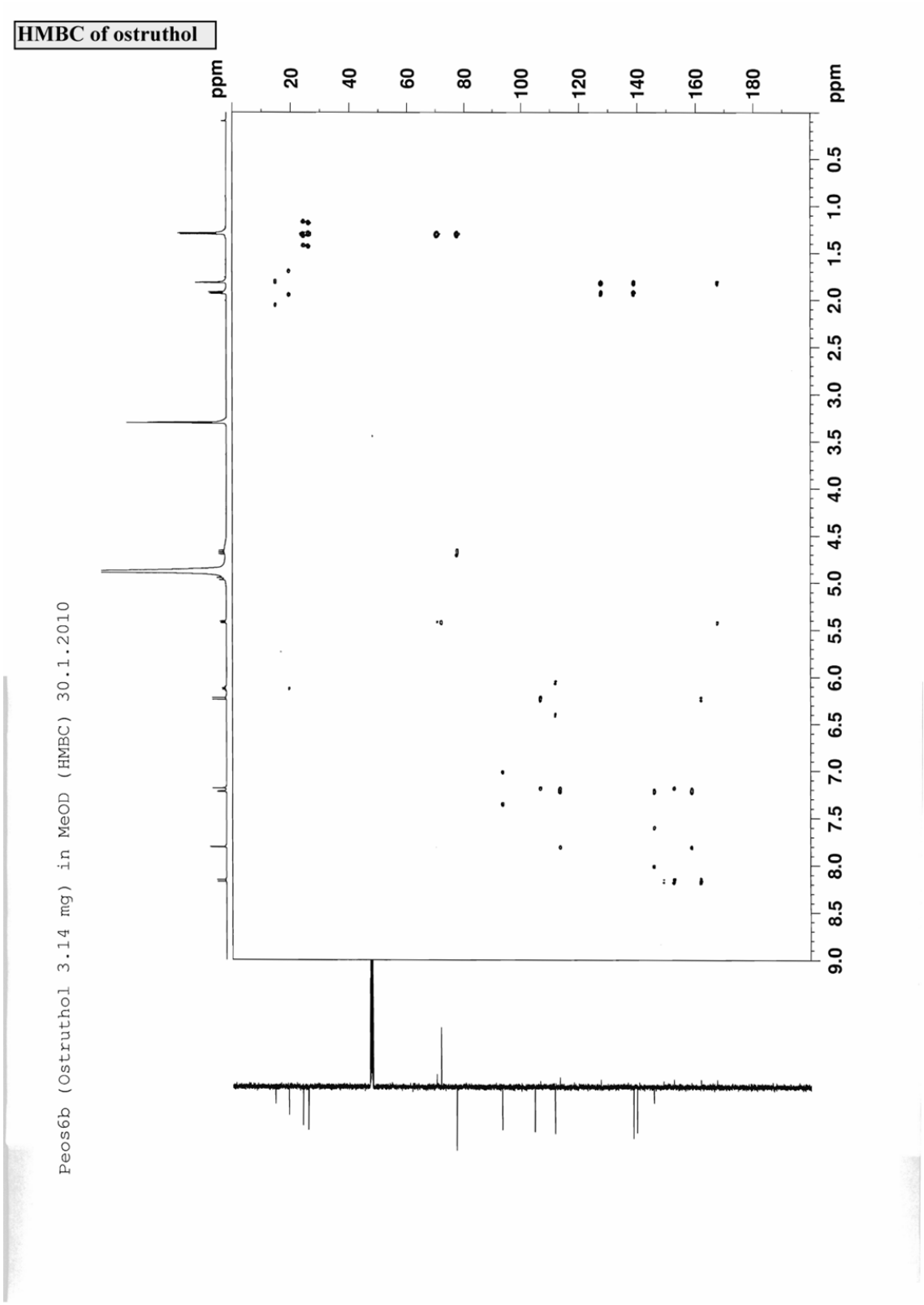

Peos6b (Ostruthol 3.14 mg) in MeOD (COSY 45) 30.1.2010
ppm
4.6
4.7
4.8
4.9
5.0
5.1
5.2
5.3
5.4
5.5
5.6
5.7
HDO
1'/2
1'/1
2'

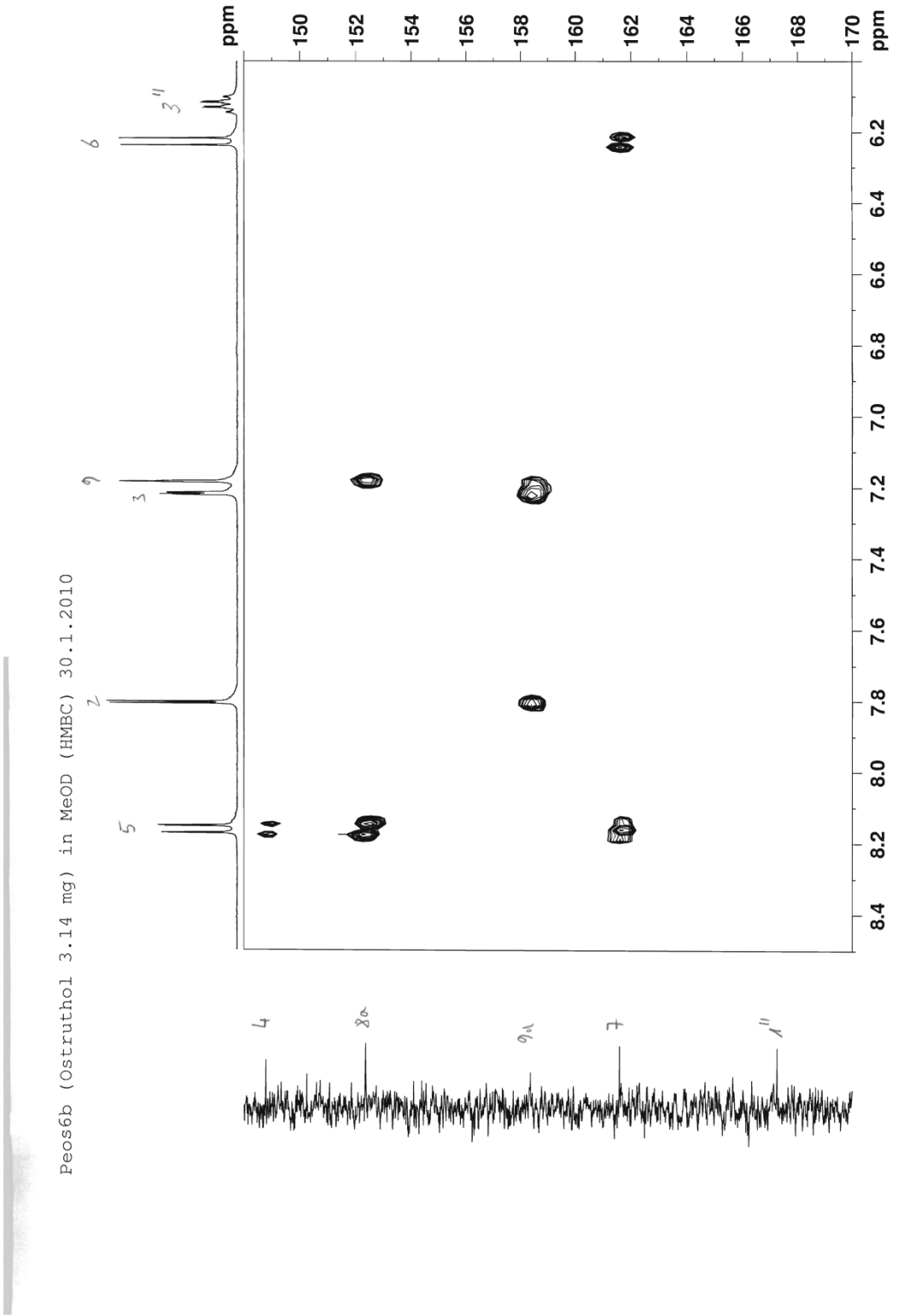

HSQC of ostruthol
ppm
20
40
60
80
100
120
140
160
180
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Peos6b (Ostruthol 3.14 mg) in MeOD (HSQC) 30.1.2010

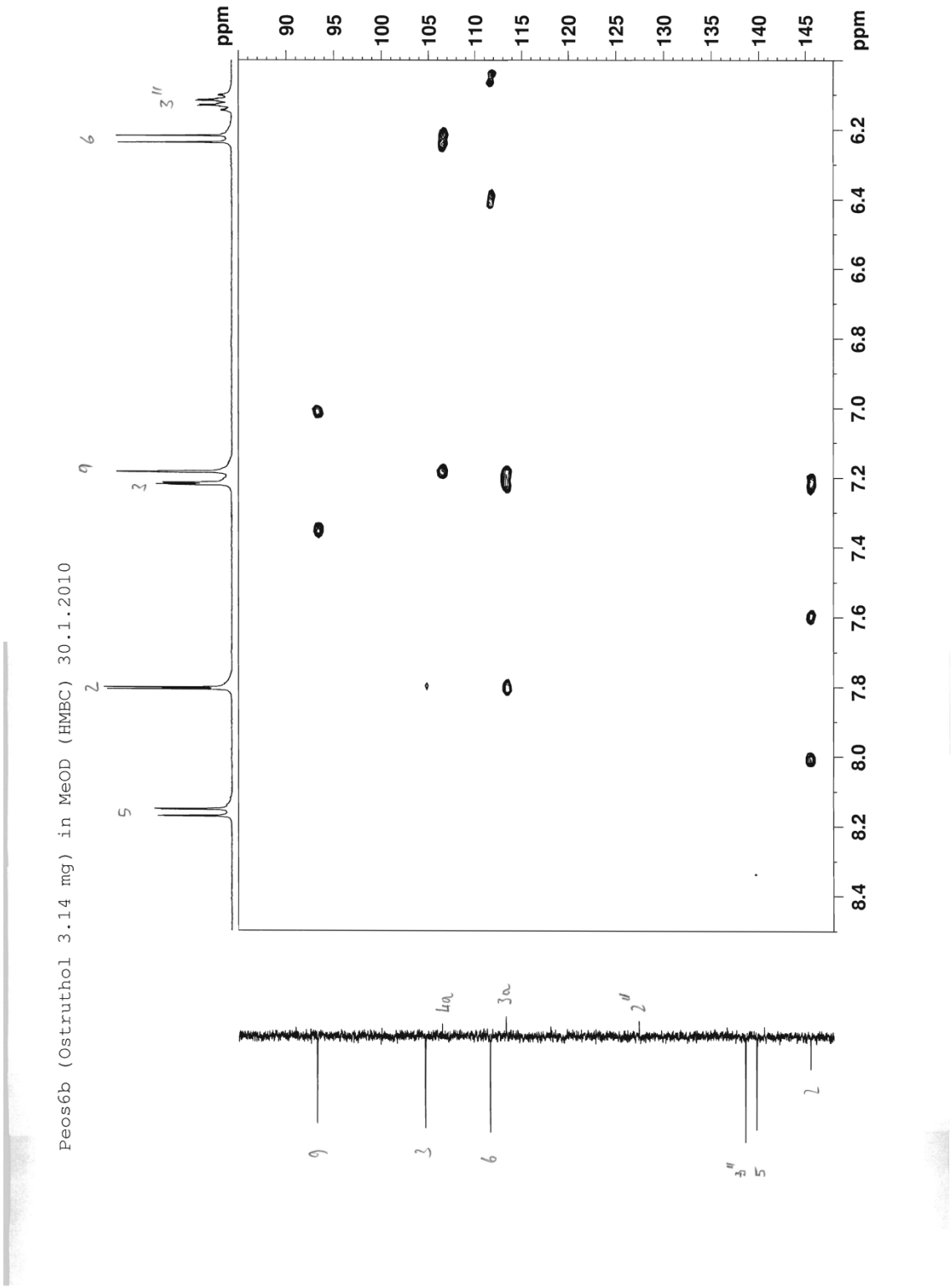

Peos6b (Ostruthol 3.14 mg) in MeOD (HSQC) 30.1.2010
ppm
90
100
110
120
130
140
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
6
3''
9
3
2
5
4a
3a
2''
9
3
6
3''
5
2

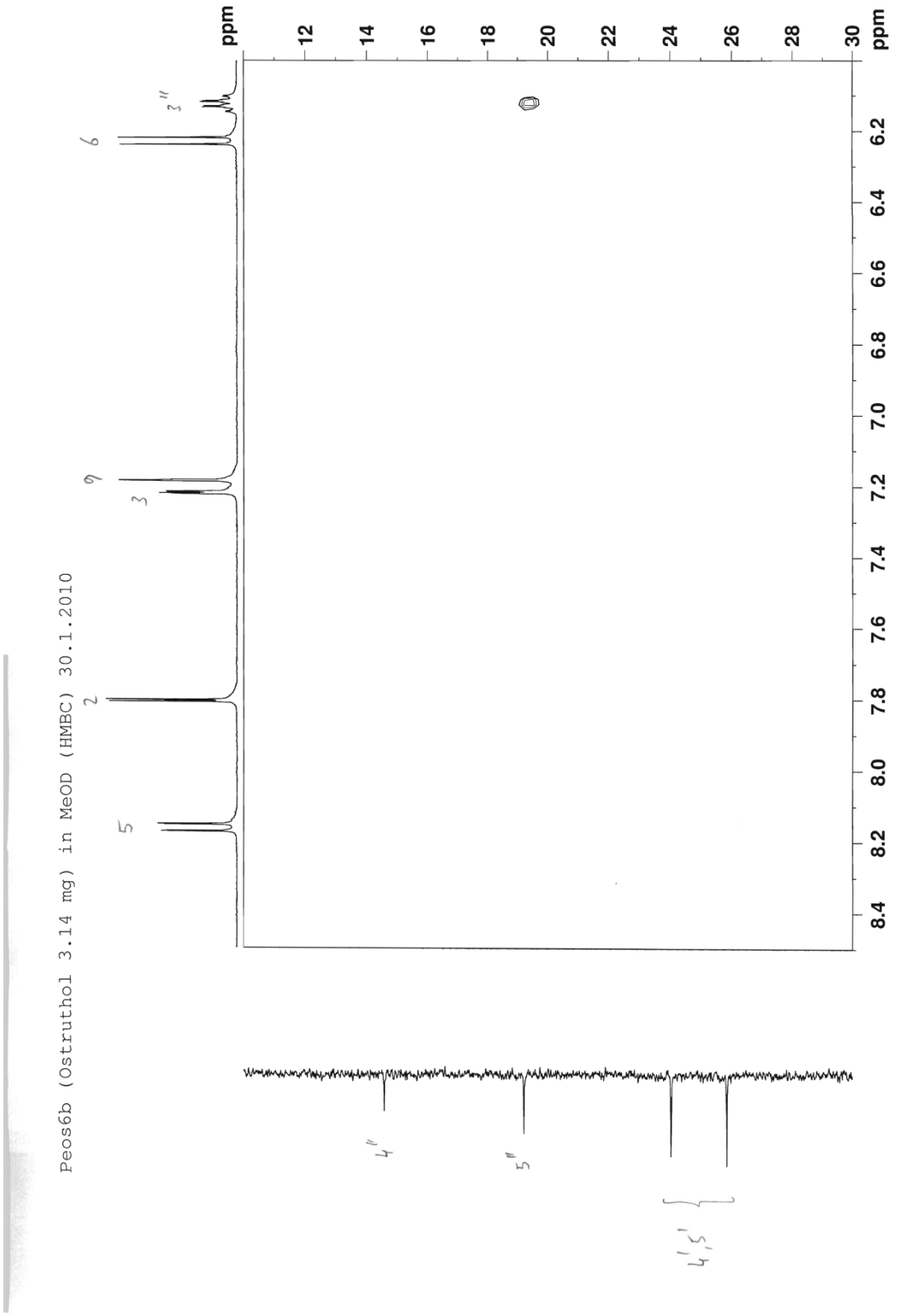

Peos6b (Ostruthol 3.14 mg) in MeOD (HSQC) 30.1.2010
HDO
1'
2'
3'
ppm
66
68
70
72
74
76
78
80
4.6
4.7
4.8
4.9
5.0
5.1
5.2
5.3
5.4
5.5
5.6
5.7
ppm

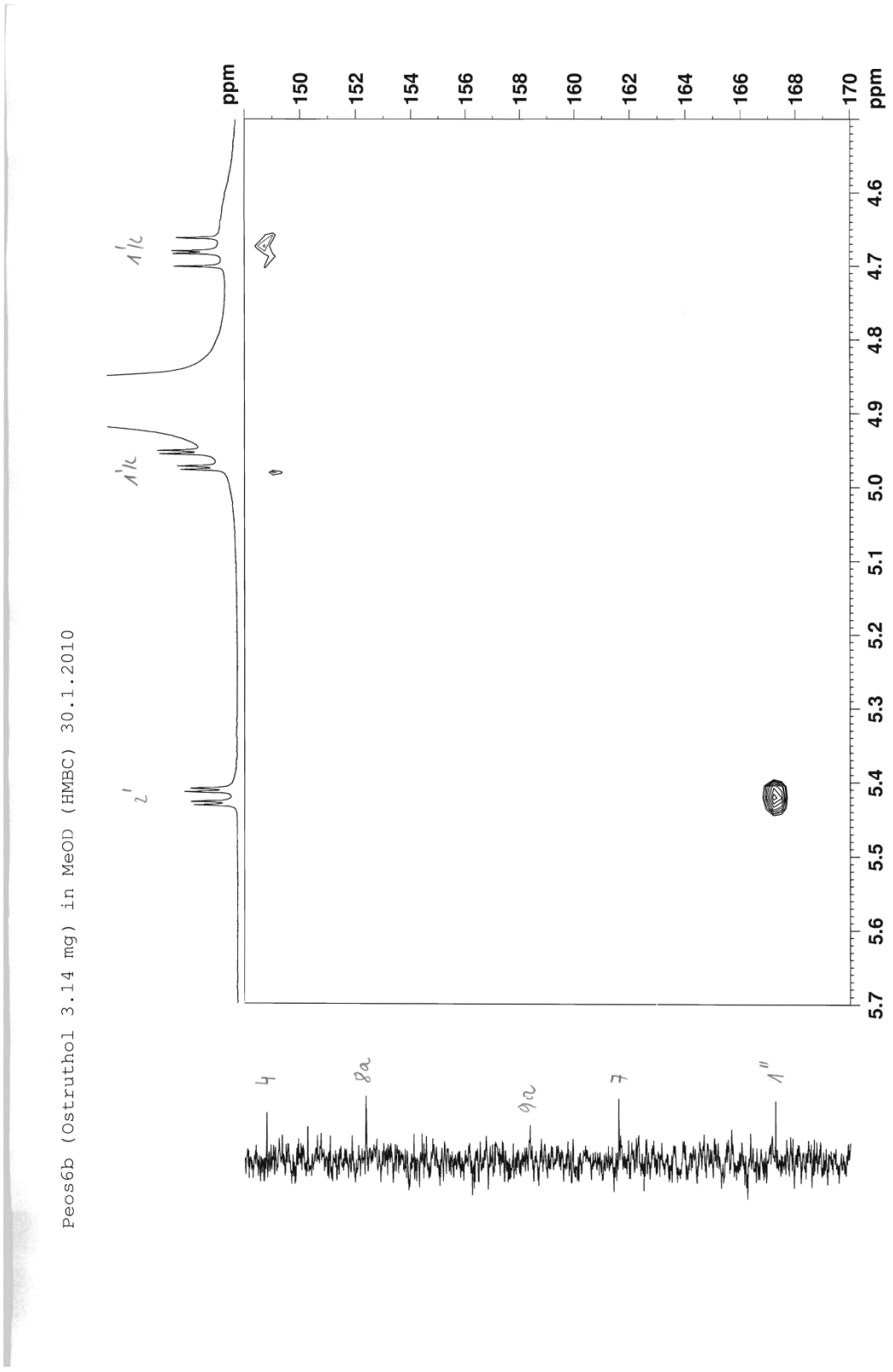

Peos6b (Ostruthol 3.14 mg) in MeOD (HSQC) 30.1.2010
ppm
12
14
16
18
20
22
24
26
28
30
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
4',5'
4''
5''

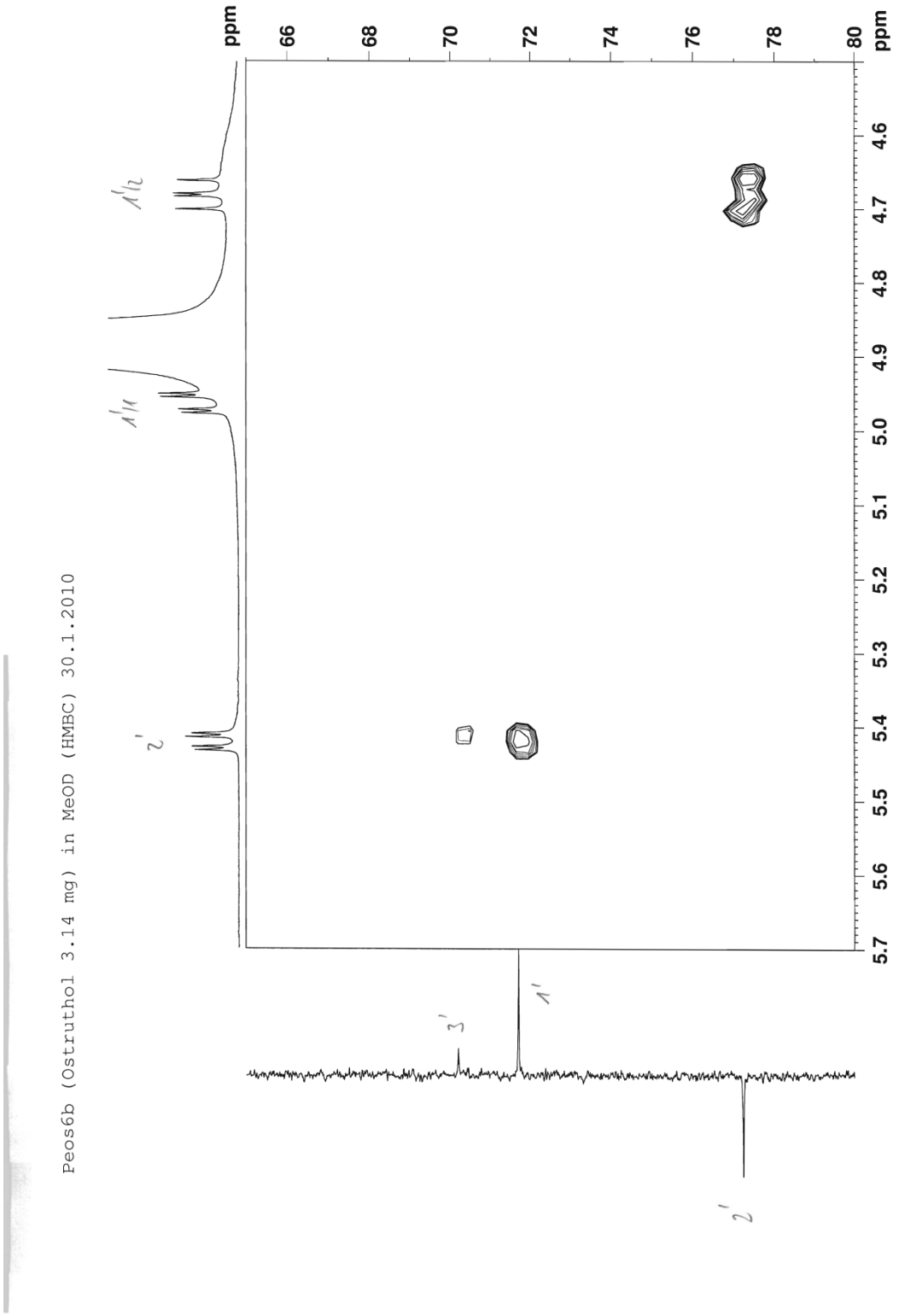

HMBC of ostruthol
ppm
20
40
60
80
100
120
140
160
180
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010

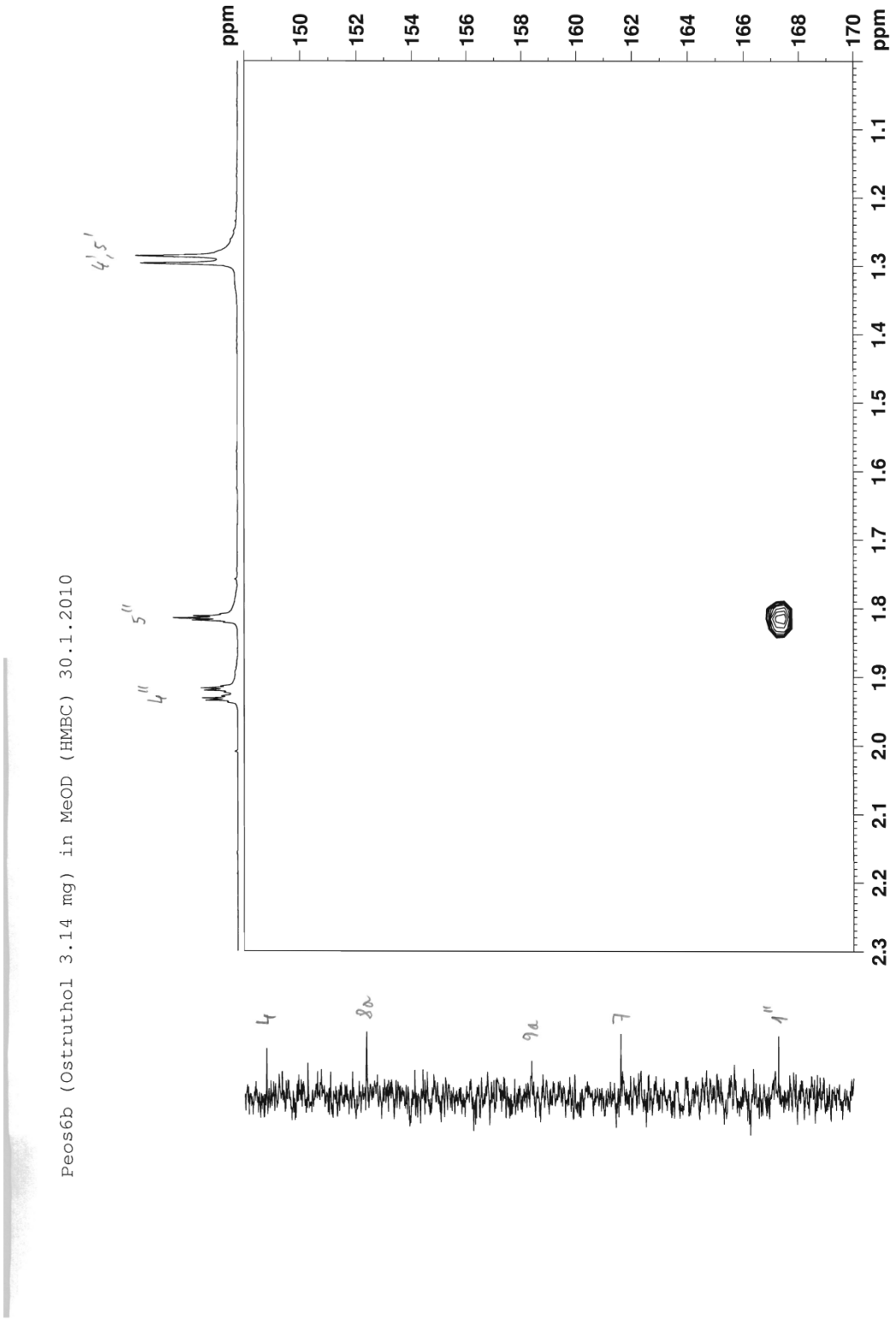

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
150
152
154
156
158
160
162
164
166
168
170
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
6
3''
9
3
2
5
4
8a
9a
7
1''

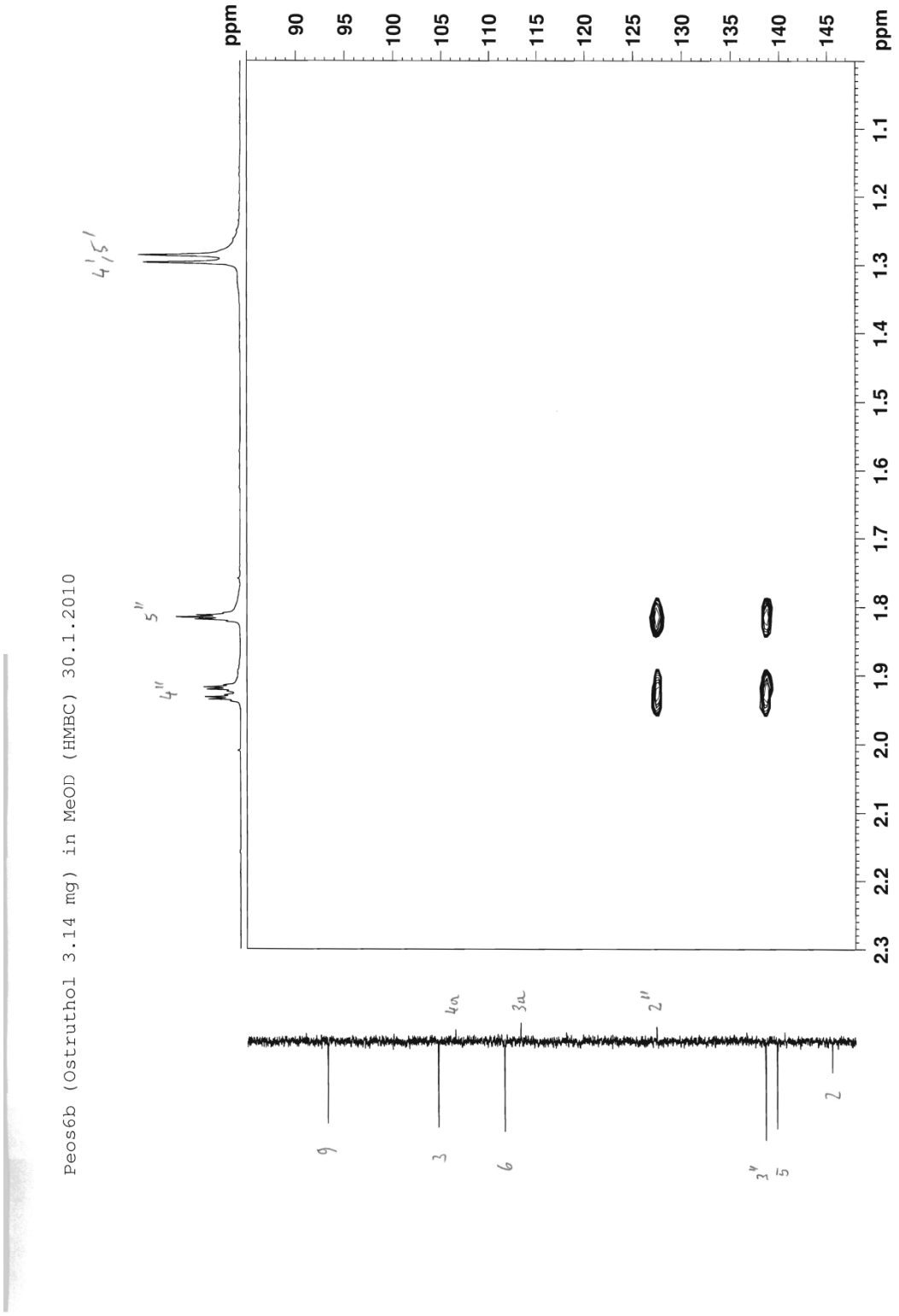

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
90
95
100
105
110
115
120
125
130
135
140
145
ppm
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
9
3
6
3''
5
2
4a
3a
2''
2

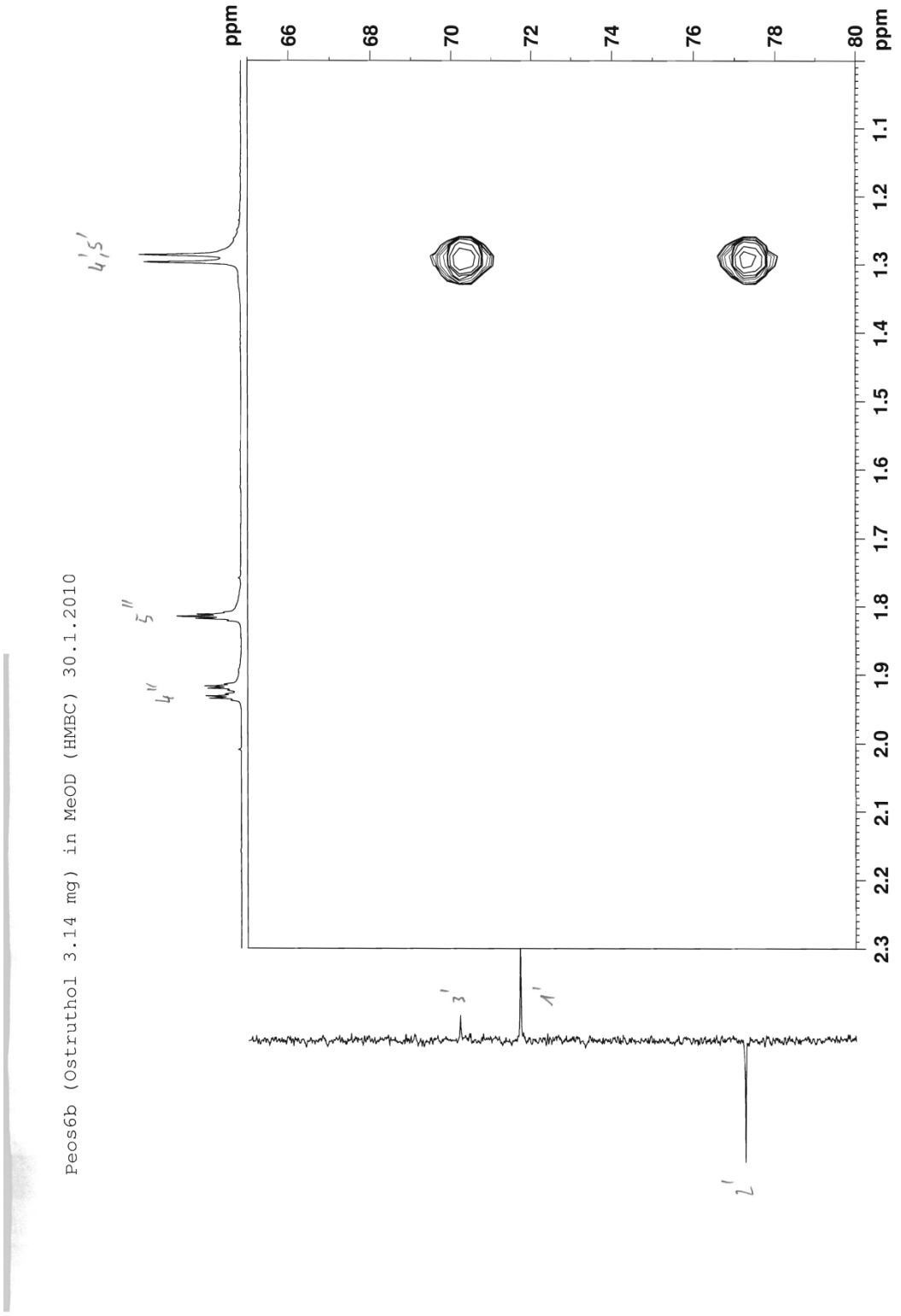

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
12
14
16
18
20
22
24
26
28
30
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
6
9
2
5
3
3''
4''
5''
4',5'

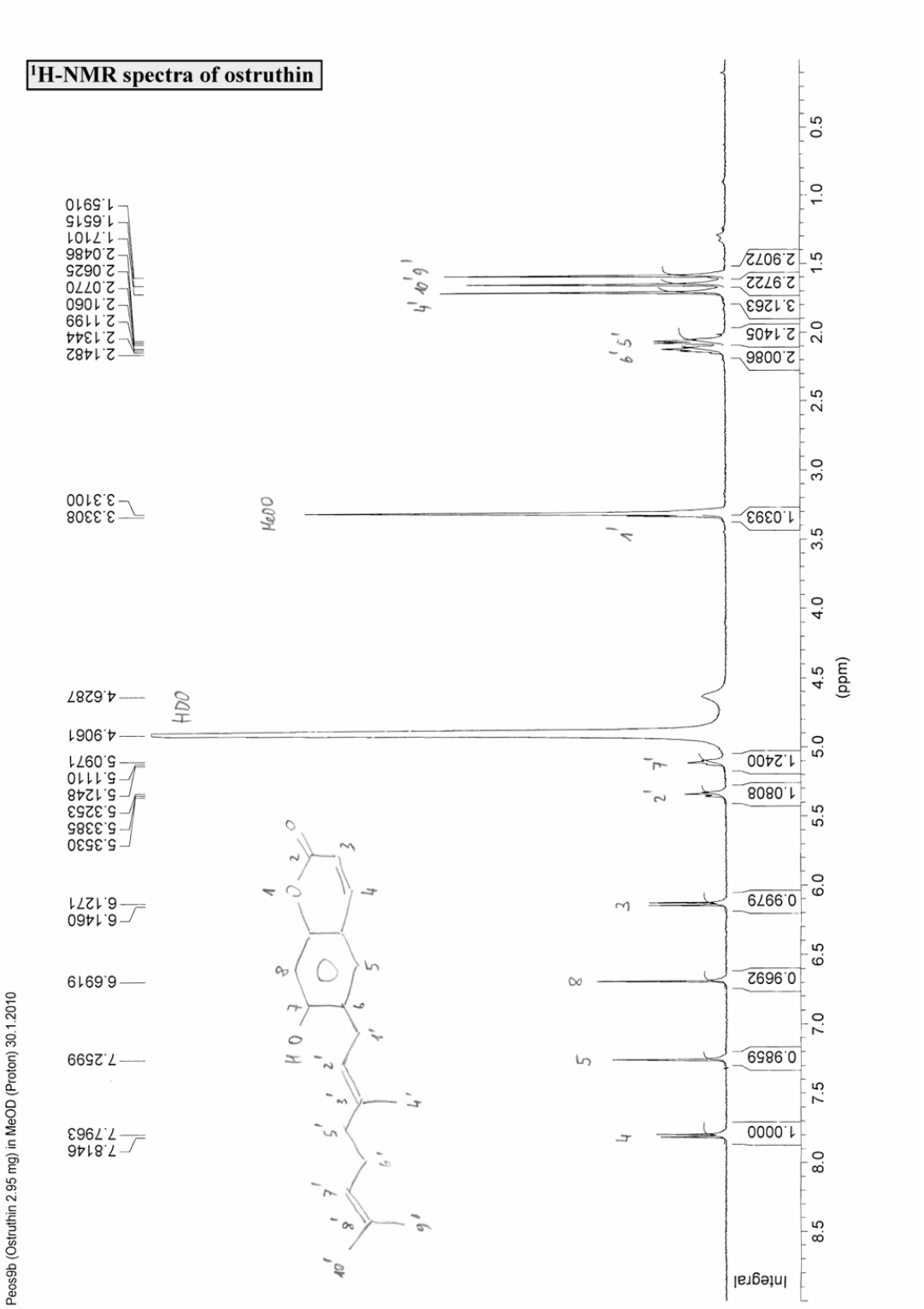

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
150
152
154
156
158
160
162
164
166
168
170
ppm
4.6
4.7
4.8
4.9
5.0
5.1
5.2
5.3
5.4
5.5
5.6
5.7
1'/1
1'/1
2'
4
8a
9a
7
1''

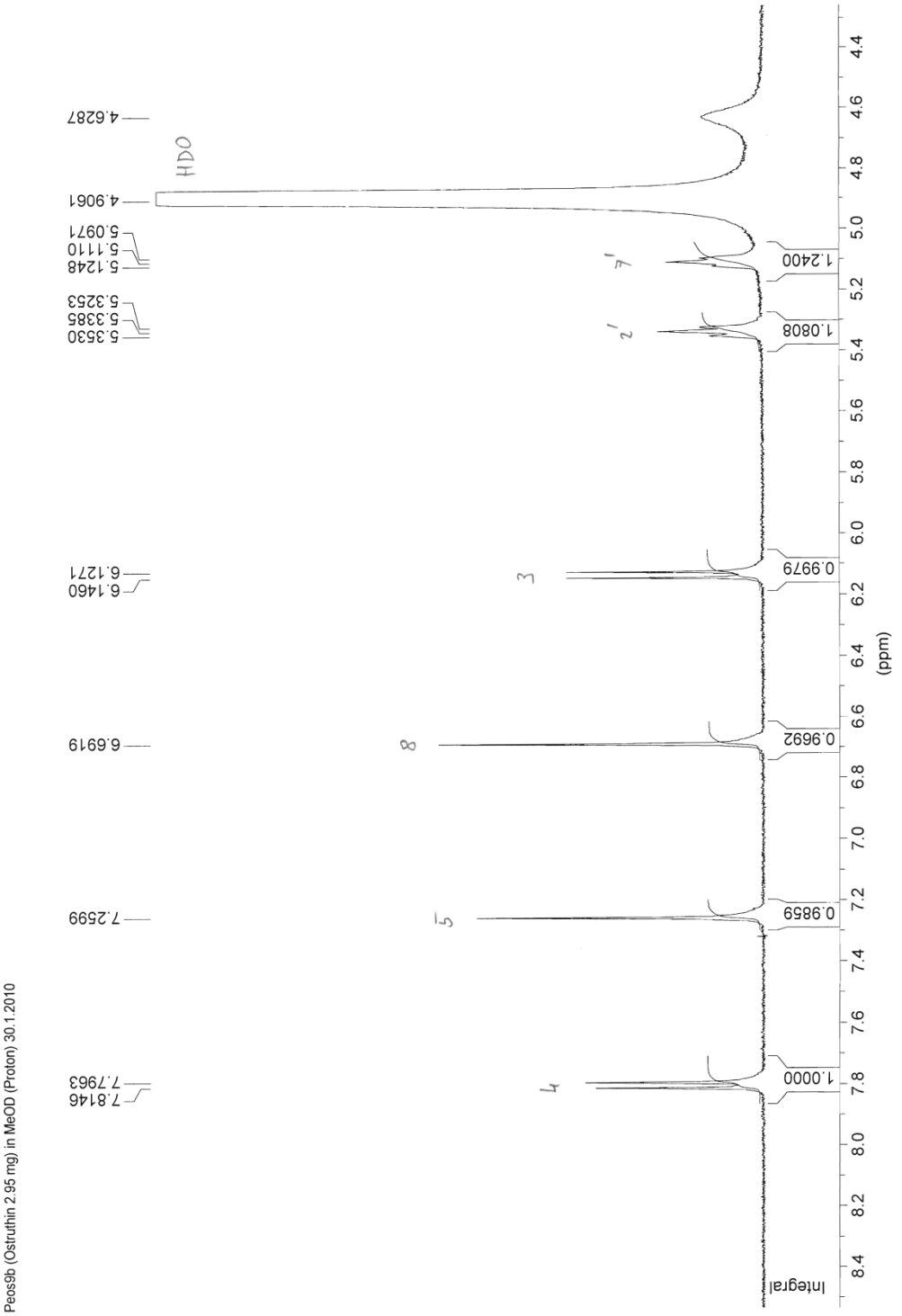

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
66
68
70
72
74
76
78
80
ppm
4.6
4.7
4.8
4.9
5.0
5.1
5.2
5.3
5.4
5.5
5.6
5.7
1'a
1'b
2'
3'
1'
2'

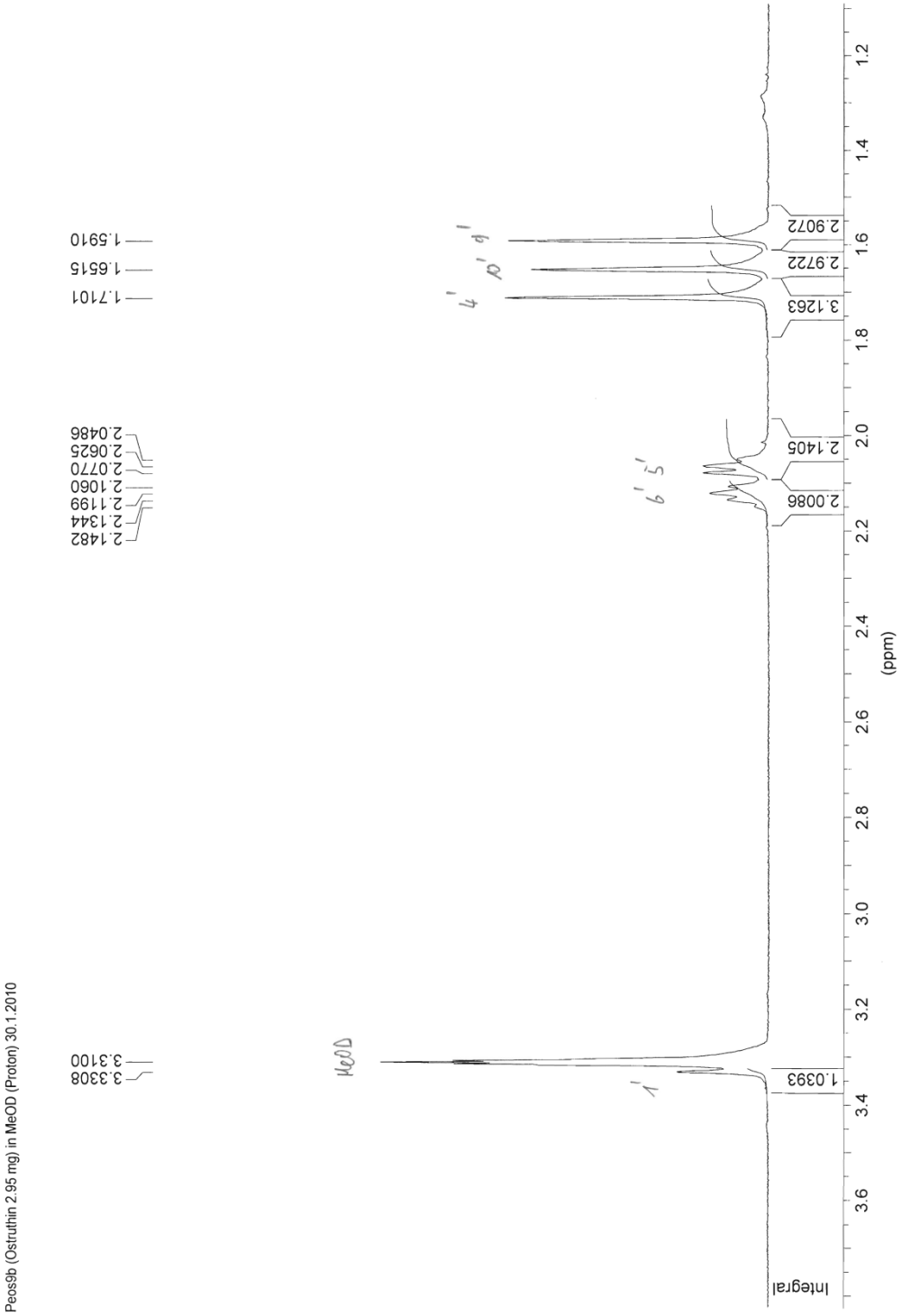

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
150
152
154
156
158
160
162
164
166
168
170
ppm
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
4',5'
5''
4''
4
8a
9a
7
1''

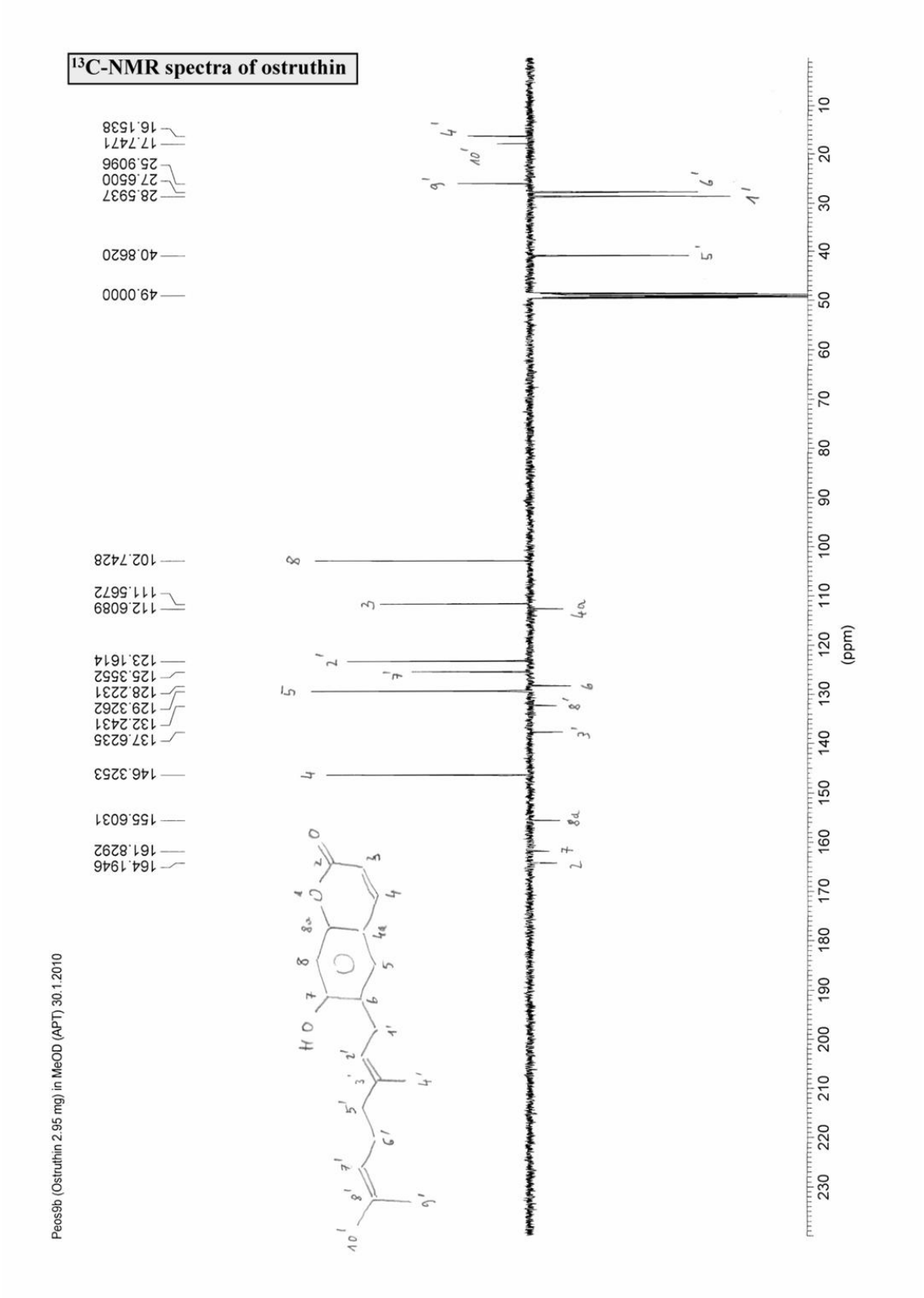

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
90
95
100
105
110
115
120
125
130
135
140
145
ppm
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
4',5'
5''
4''
4a
3a
2''
9
3
6
3''
5
2

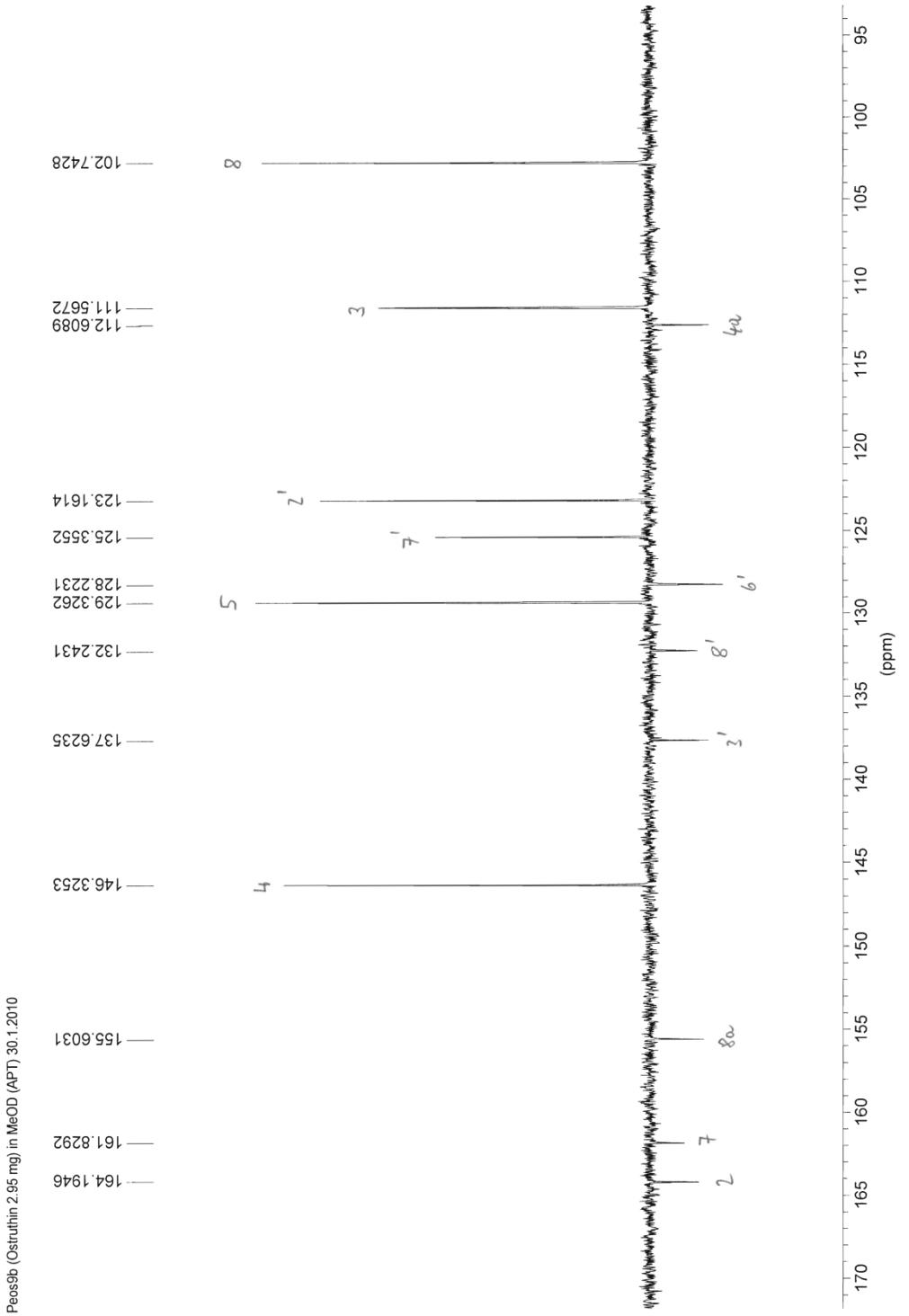

Peos6b (Ostruthol 3.14 mg) in MeOD (HMBC) 30.1.2010
ppm
66
68
70
72
74
76
78
80
ppm
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
4',5'
5''
4''
3'
1'
2'

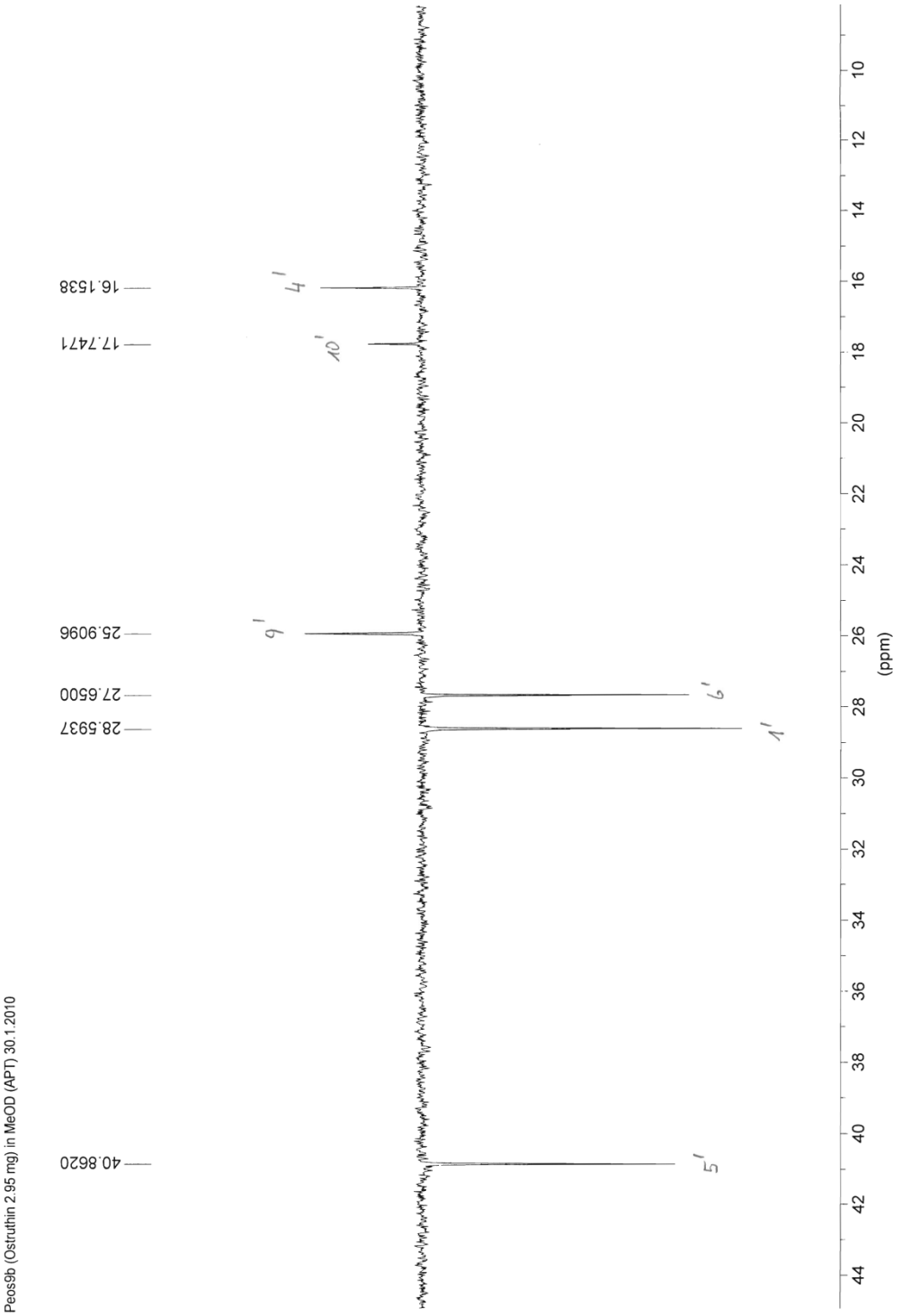

¹H-NMR spectra of ostruthin
Pecs9b (Ostruthin 2.95 mg) in MeOD (Proton) 30.1.2010
HDO
MeOD
Integral
(ppm)

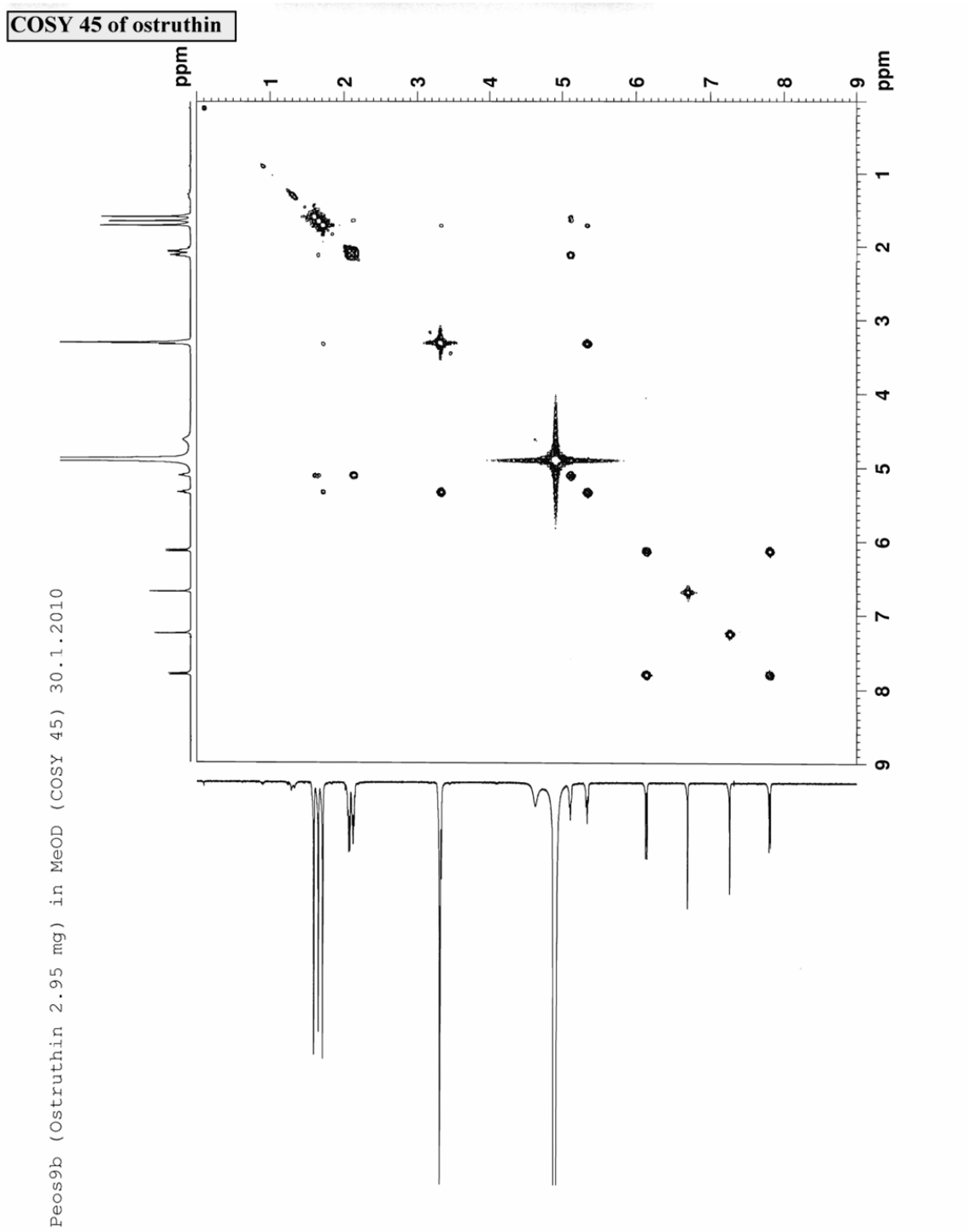

Peos9b (Ostruthin 2.95 mg) in MeOD (Proton) 30.1.2010
HDO
4.6287
4.9061
5.0971
5.1110
5.1248
5.3253
5.3385
5.3530
6.1271
6.1460
6.6919
7.2599
7.7963
7.8146
1.0000
0.9859
0.9892
0.9979
1.0808
1.2400
Integral
(ppm)

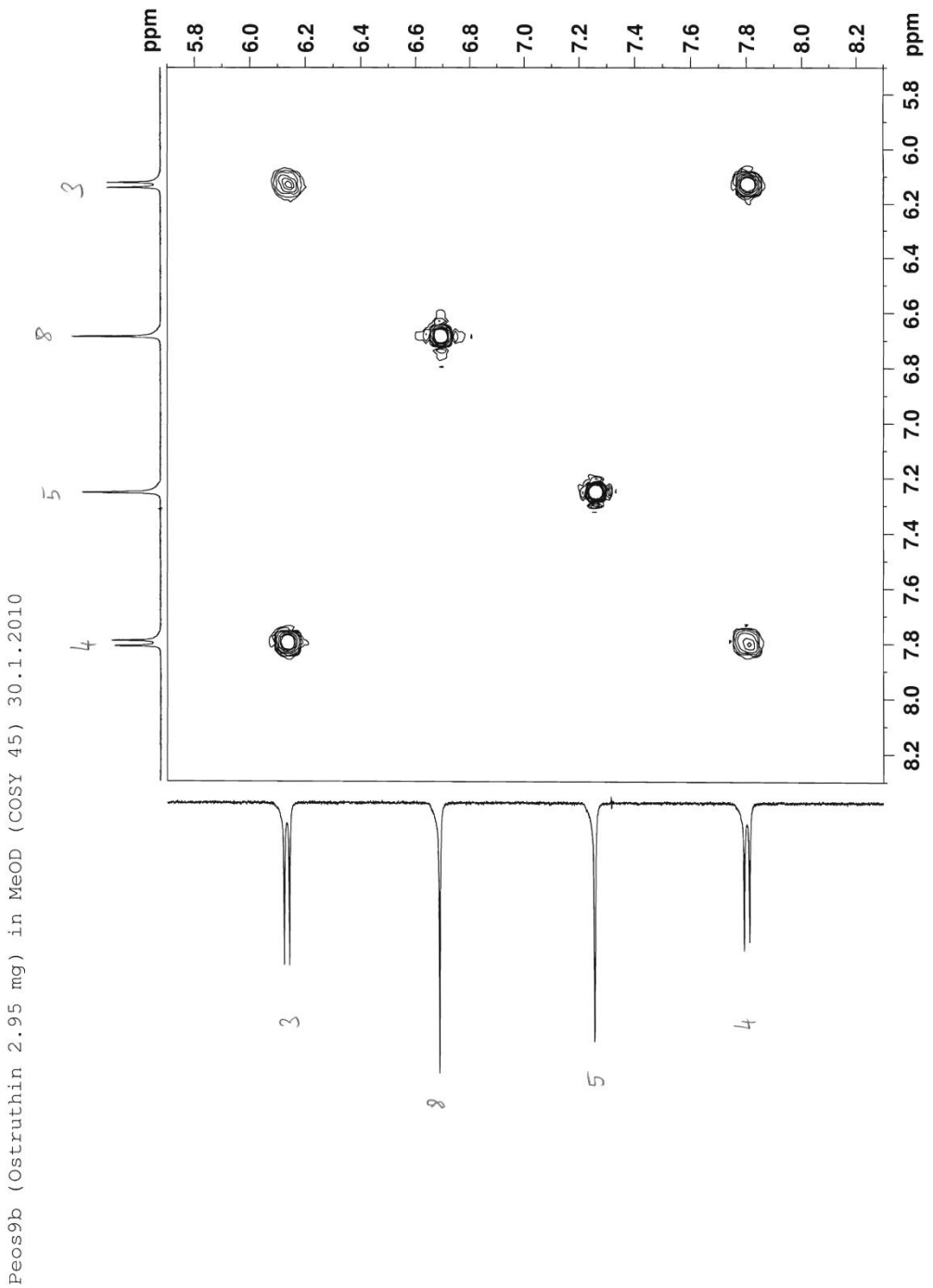

Peos9b (Ostruthin 2.95 mg) in MeOD (Proton) 30.1.2010
3.3308
3.3100
1.5910
1.6515
1.7101
2.0486
2.0625
2.0770
2.1060
2.1199
2.1344
2.1482
MeOD
1'
4'
5'
6'
1.0393
2.0086
2.1405
2.9072
2.9722
3.1263
Integral
(ppm)

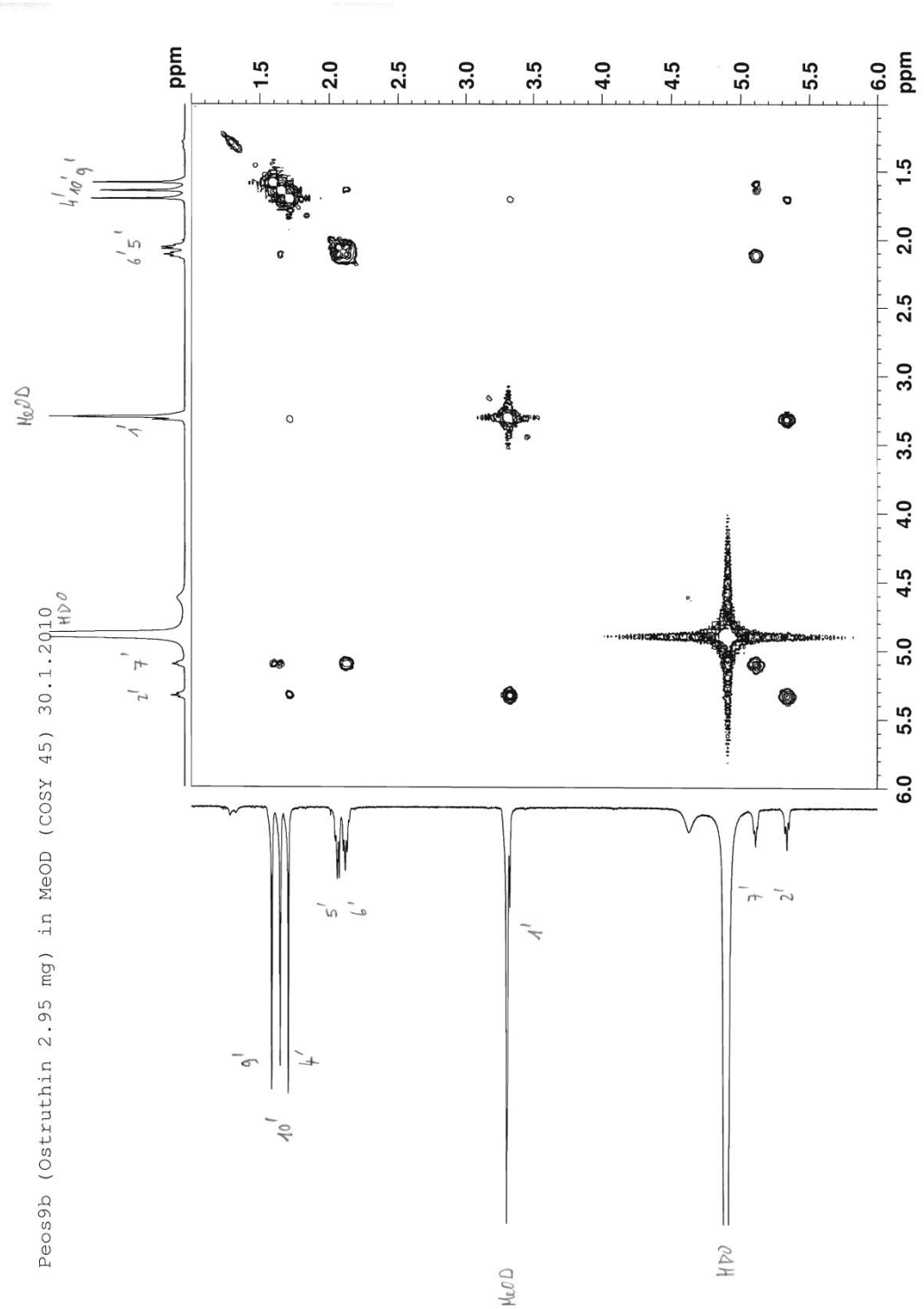

13C-NMR spectra of ostruthin
16.1538
17.7471
25.9096
27.6500
28.5937
40.8620
49.0000
102.7428
111.5672
112.6089
123.1614
125.3552
128.2231
129.3262
132.2431
137.6235
146.3253
155.6031
161.8292
164.1946
(ppm)
Peos9b (Ostruthin 2.95 mg) in MeOD (APT) 30.1.2010

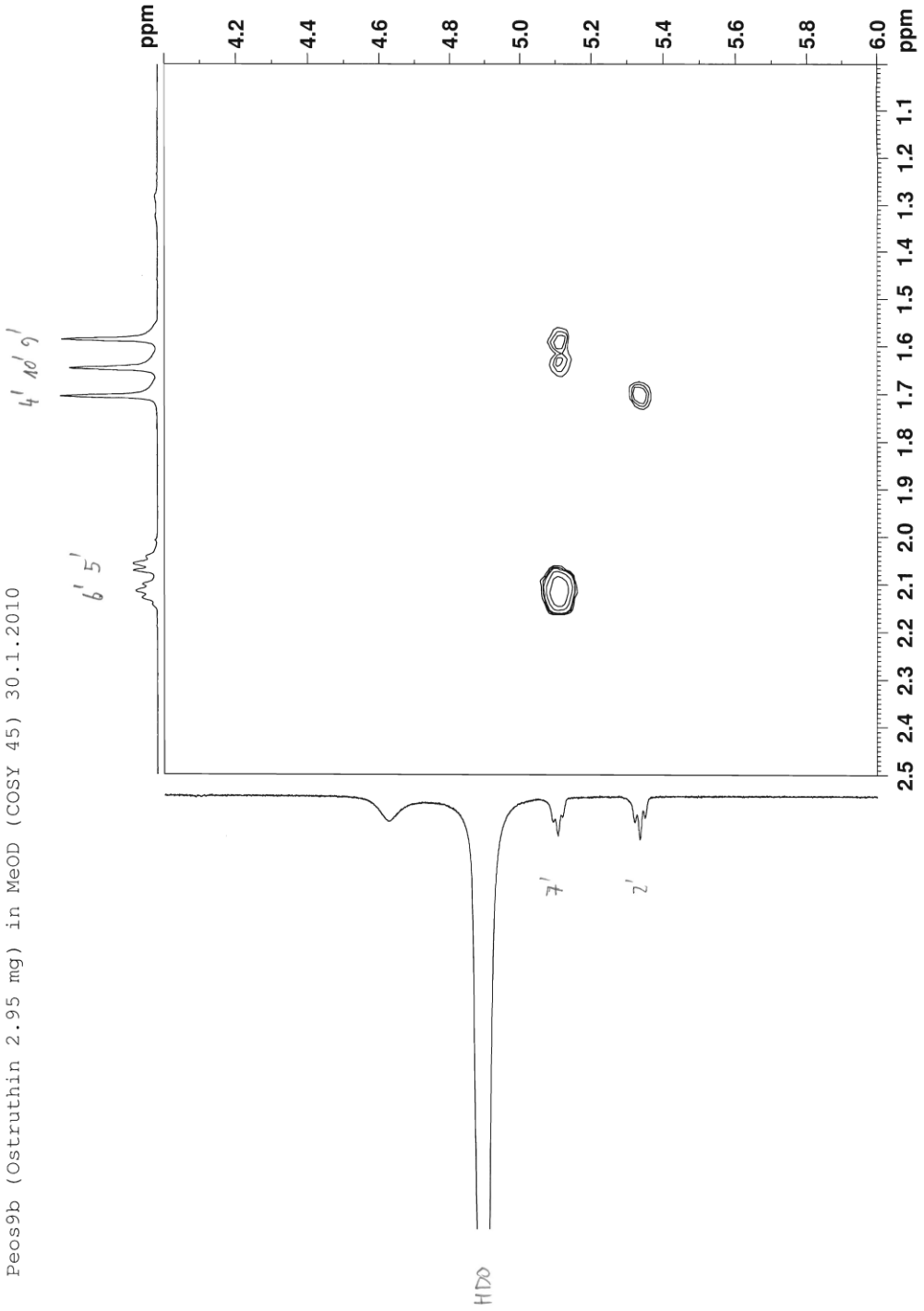

Peos9b (Ostruthin 2.95 mg) in MeOD (APT) 30.1.2010
102.7428
111.5672
112.6089
123.1614
125.3552
128.2231
129.3262
132.2431
137.6235
146.3253
155.6031
161.8292
164.1946
8
3
4a
2'
7'
6'
5
8'
3'
4
8a
7
2
95
100
105
110
115
120
125
130
135
140
145
150
155
160
165
170
(ppm)

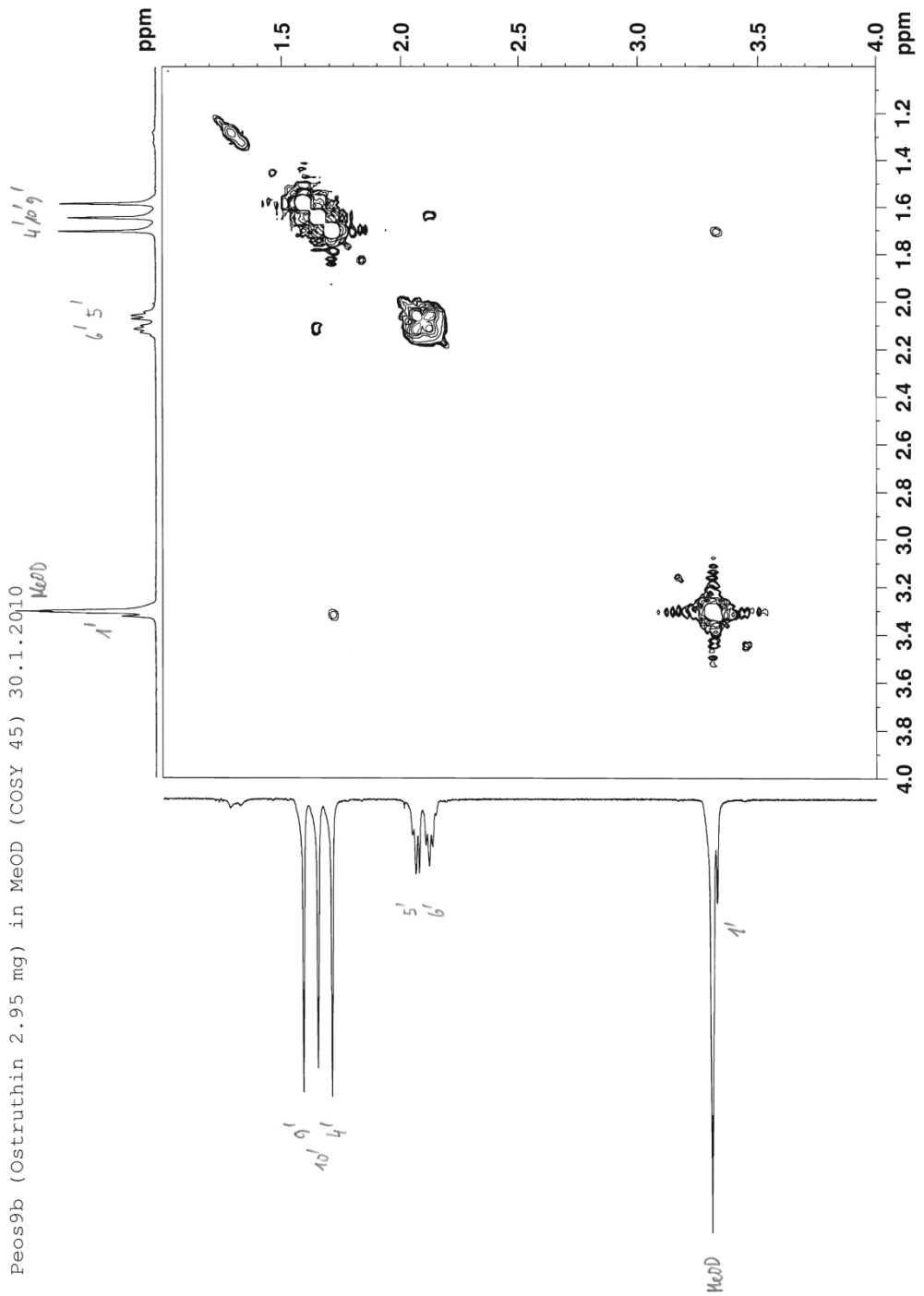

Peos9b (Ostruthin 2.95 mg) in MeOD (APT) 30.1.2010
16.1538
17.7471
25.9096
27.6500
28.5937
40.8620
4'
10'
9'
6'
1'
5'
(ppm)

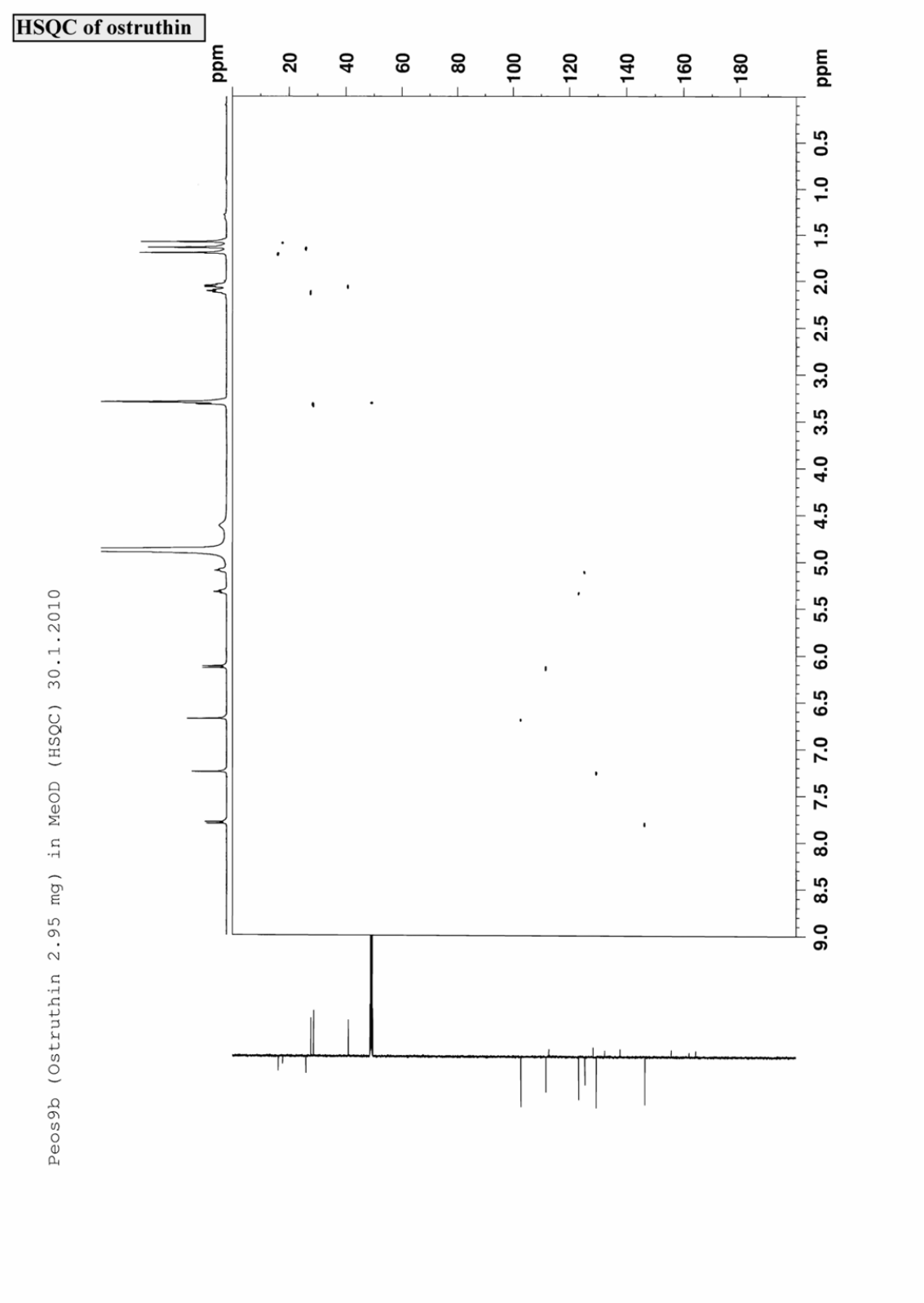

COSY 45 of ostruthin
ppm
1
2
3
4
5
6
7
8
9
ppm
Peos9b (Ostruthin 2.95 mg) in MeOD (COSY 45) 30.1.2010

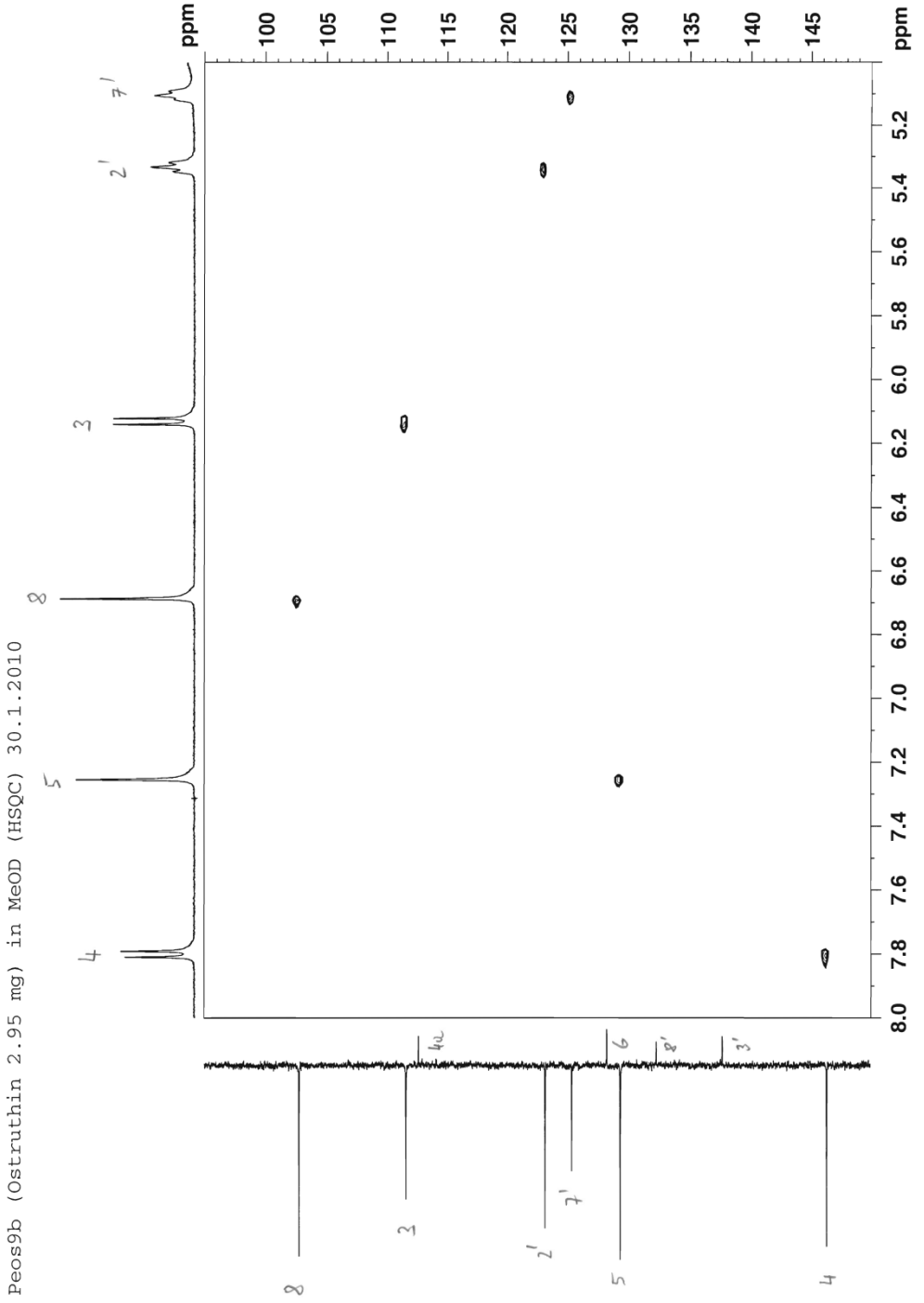

Peos9b (Ostruthin 2.95 mg) in MeOD (COSY 45) 30.1.2010
ppm
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
3
8
5
4

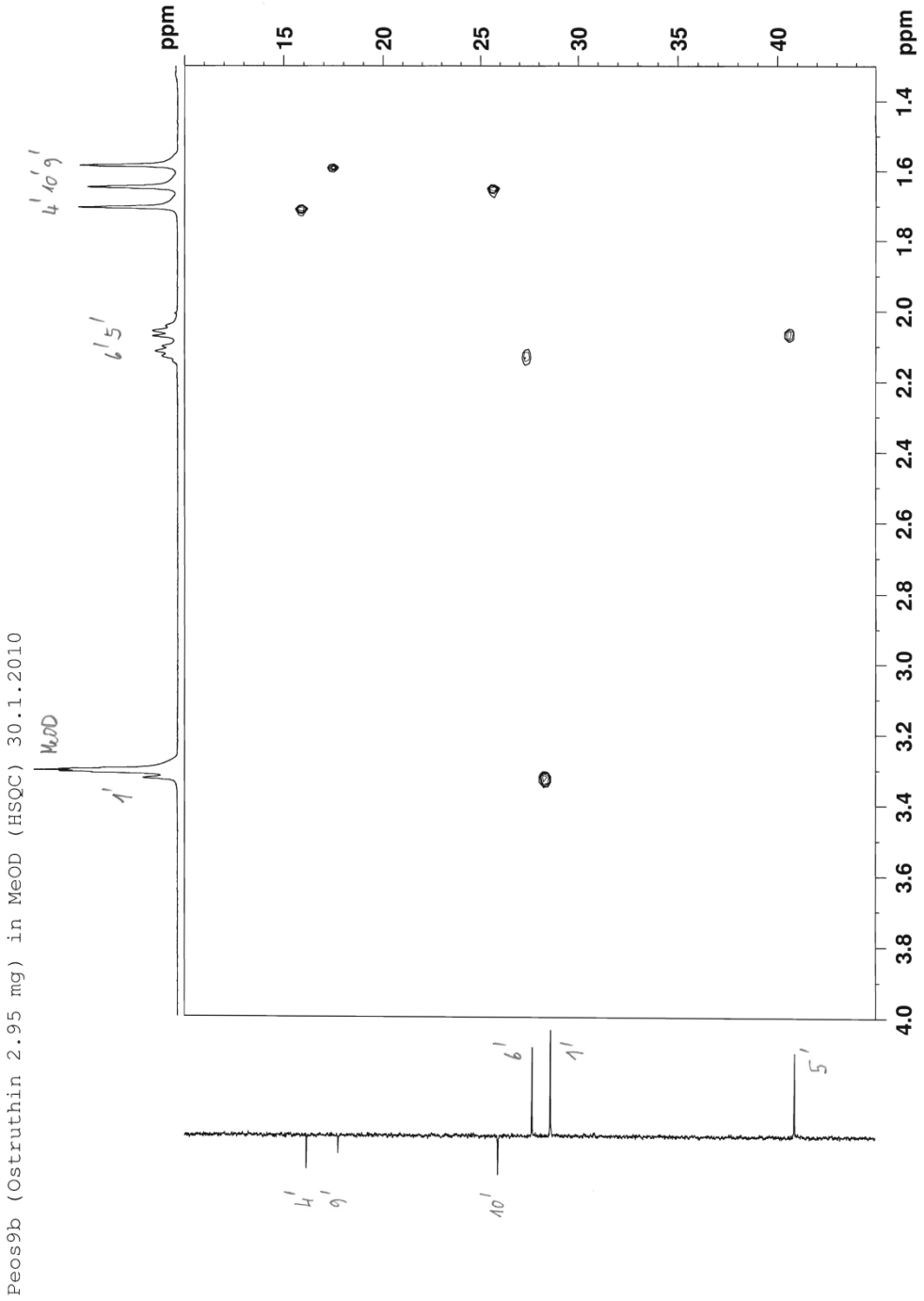

Peos9b (Ostruthin 2.95 mg) in MeOD (COSY 45) 30.1.2010
ppm
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
MeOD
HDO
1'
2' 7'
6' 5'
4' 10' 9'
9'
10'
4'
5'
6'
1'
7'
2'
MeOD
HDO

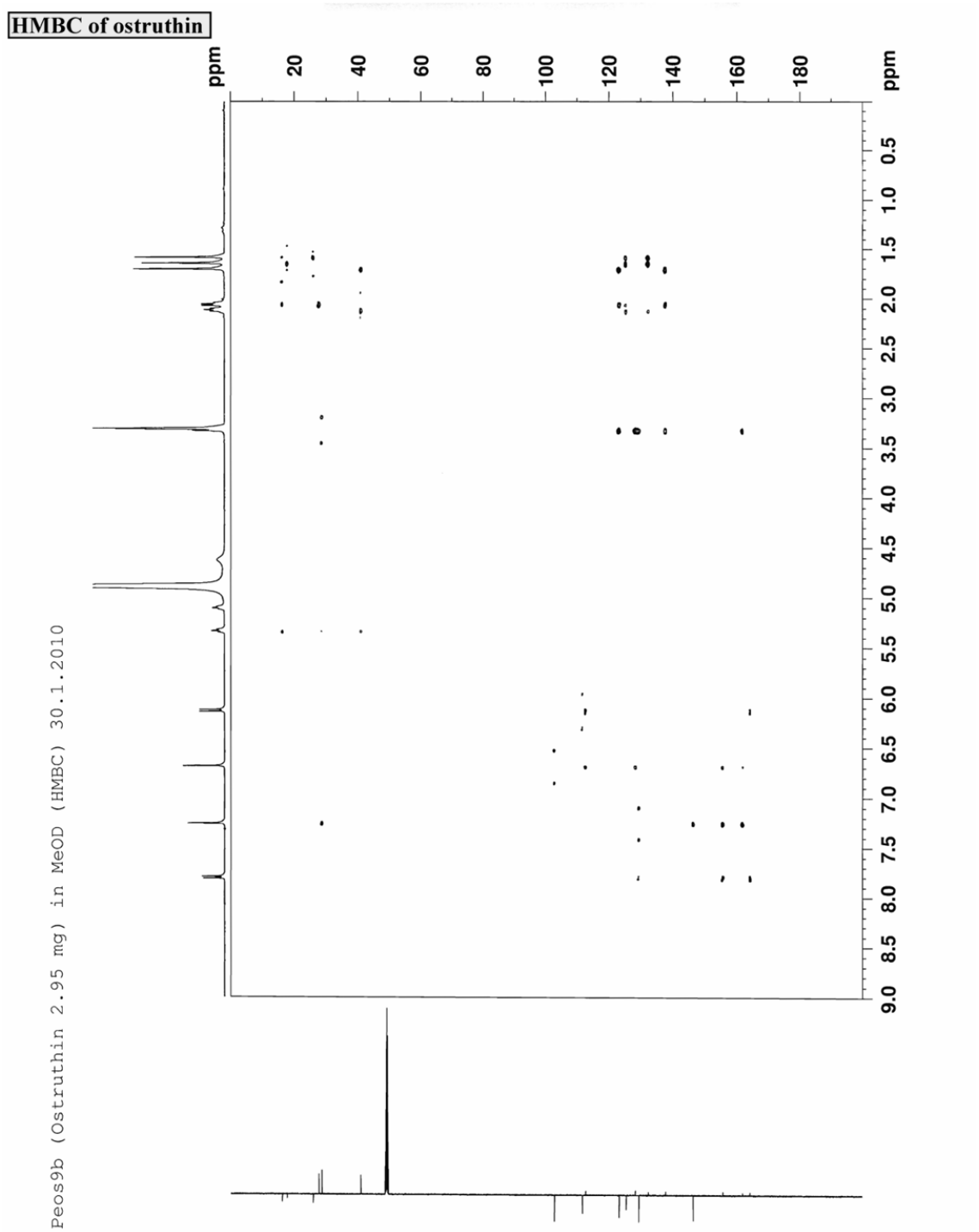

Peos9b (Ostruthin 2.95 mg) in MeOD (COSY 45) 30.1.2010
ppm
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
5.8
6.0
ppm
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
2.4
2.5
4' 10' 9'
6' 5'
7'
2'
HDO

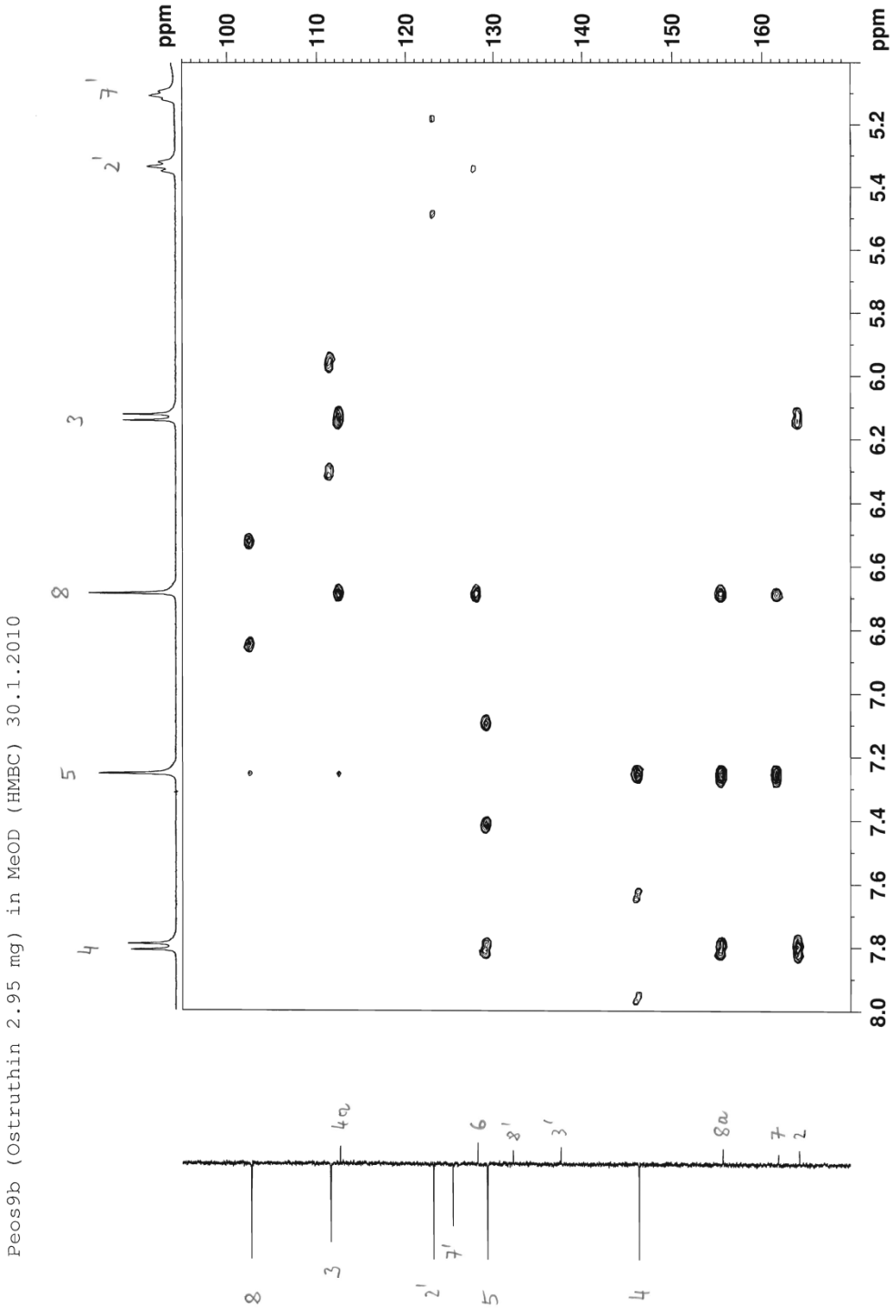

Peos9b (Ostruthin 2.95 mg) in MeOD (COSY 45) 30.1.2010
ppm
1.5
2.0
2.5
3.0
3.5
4.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
MeOD
1'
6' 5'
4' 9' 10'
5' 6'
1'
MeOD

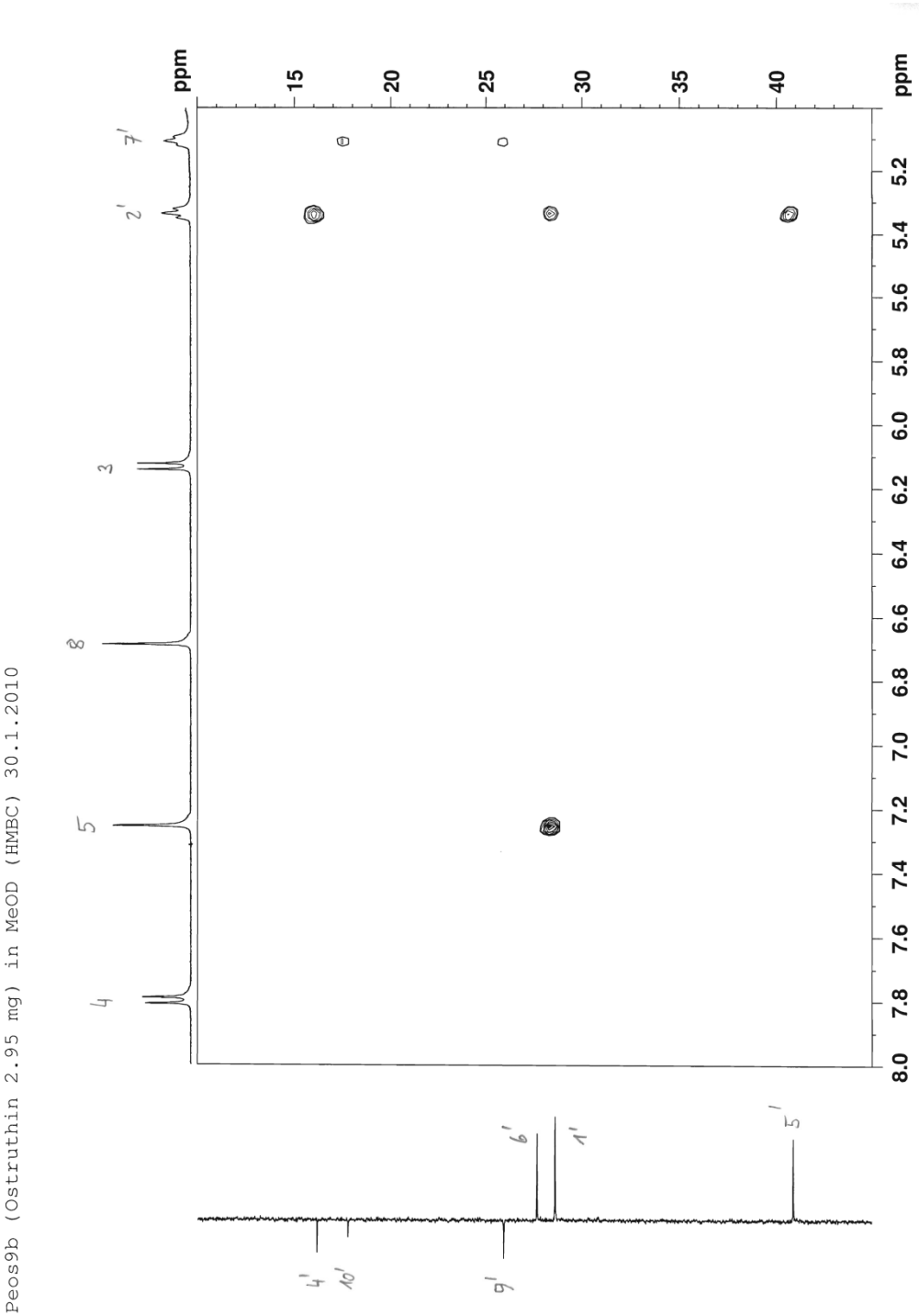

HSQC of ostruthin
ppm
20
40
60
80
100
120
140
160
180
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Peos9b (Ostruthin 2.95 mg) in MeOD (HSQC) 30.1.2010

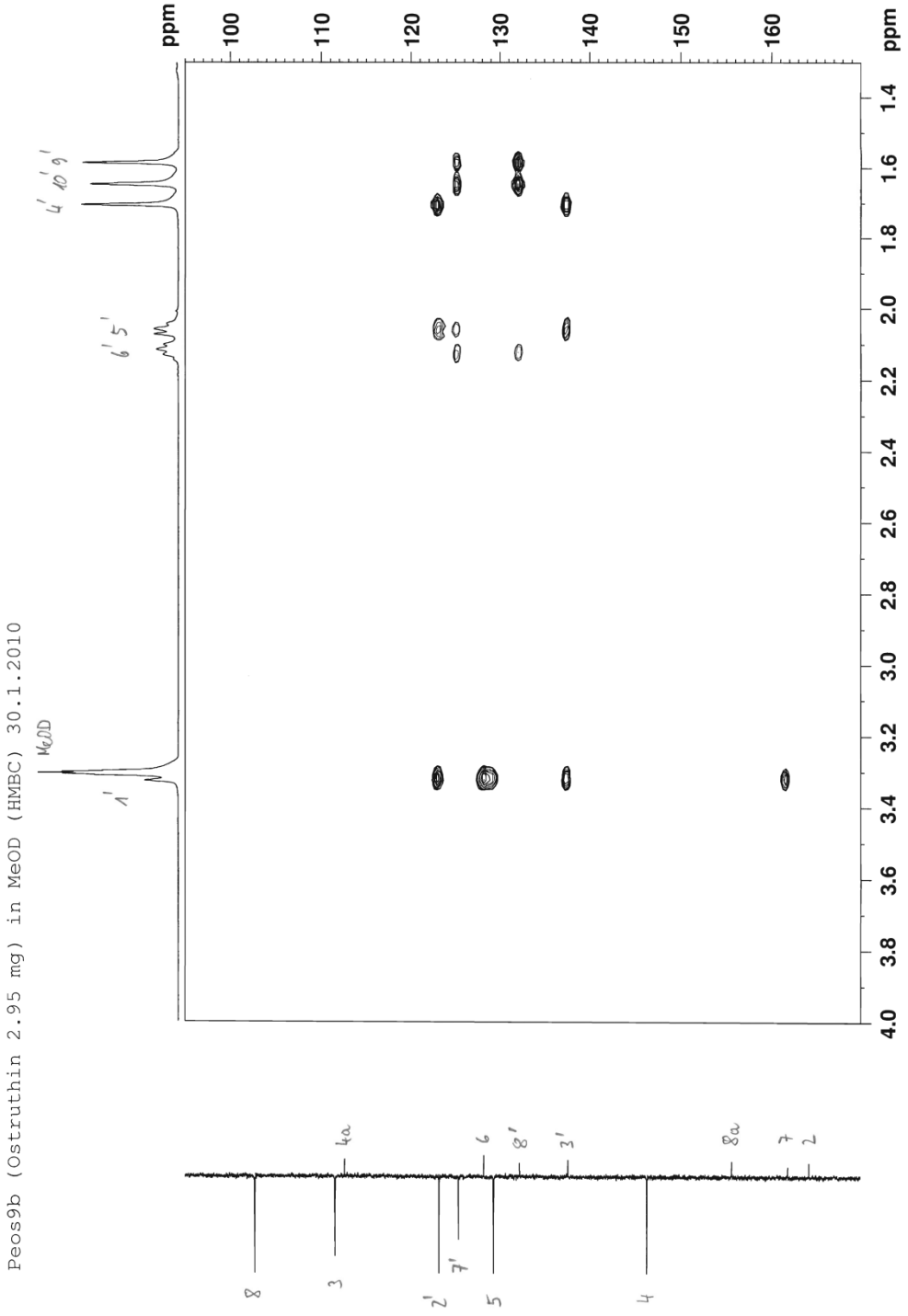

Peos9b (Ostruthin 2.95 mg) in MeOD (HSQC) 30.1.2010
ppm
100
105
110
115
120
125
130
135
140
145
5.2
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8
3
5
4
2'
7'
4a
6
8'
3'

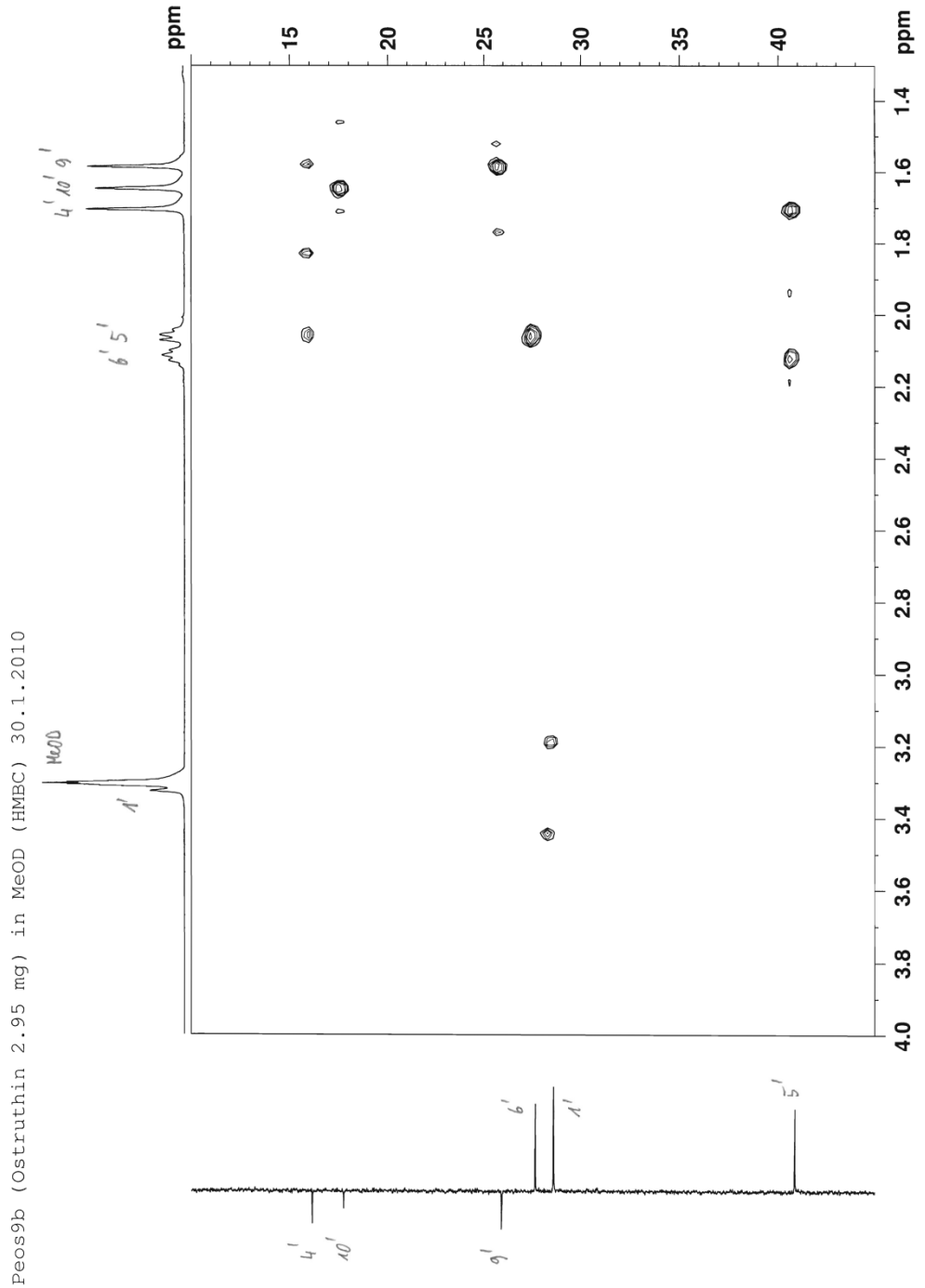

Peos9b (Ostruthin 2.95 mg) in MeOD (HSQC) 30.1.2010
MeOD
1'
6'
5'
4'
9'
10'
ppm
15
20
25
30
35
40
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0


HMBC of ostruthin
ppm
20
40
60
80
100
120
140
160
180
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Peos9b (Ostruthin 2.95 mg) in MeOD (HMBC) 30.1.2010


Peos9b (Ostruthin 2.95 mg) in MeOD (HMBC) 30.1.2010
ppm
100
110
120
130
140
150
160
ppm
5.2
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8
3
5
4
2'
7'
4a
6
8'
3'
8a
7
2


Peos9b (Ostruthin 2.95 mg) in MeOD (HMBC) 30.1.2010
ppm
15
20
25
30
35
40
ppm
5.2
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
7'
2'
3
8
5
4
6'
1'
5'
4'
10'
9'

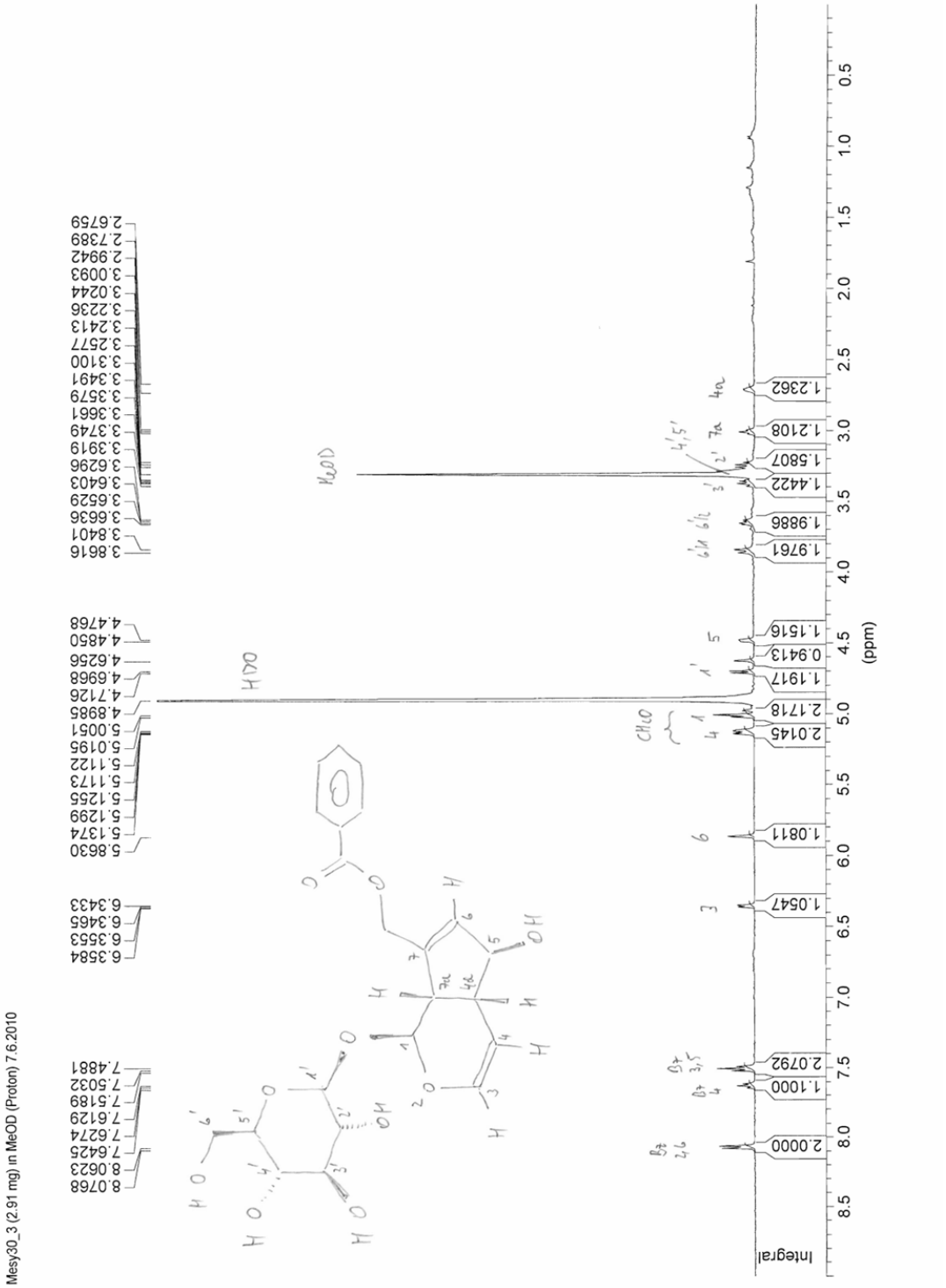

Peos9b (Ostruthin 2.95 mg) in MeOD (HMBC) 30.1.2010
MeOD
1'
6' 5'
4' 10' 9'
ppm
100
110
120
130
140
150
160
ppm
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
8
3
4a
2'
7'
5
6
8'
3'
4
8a
7
2

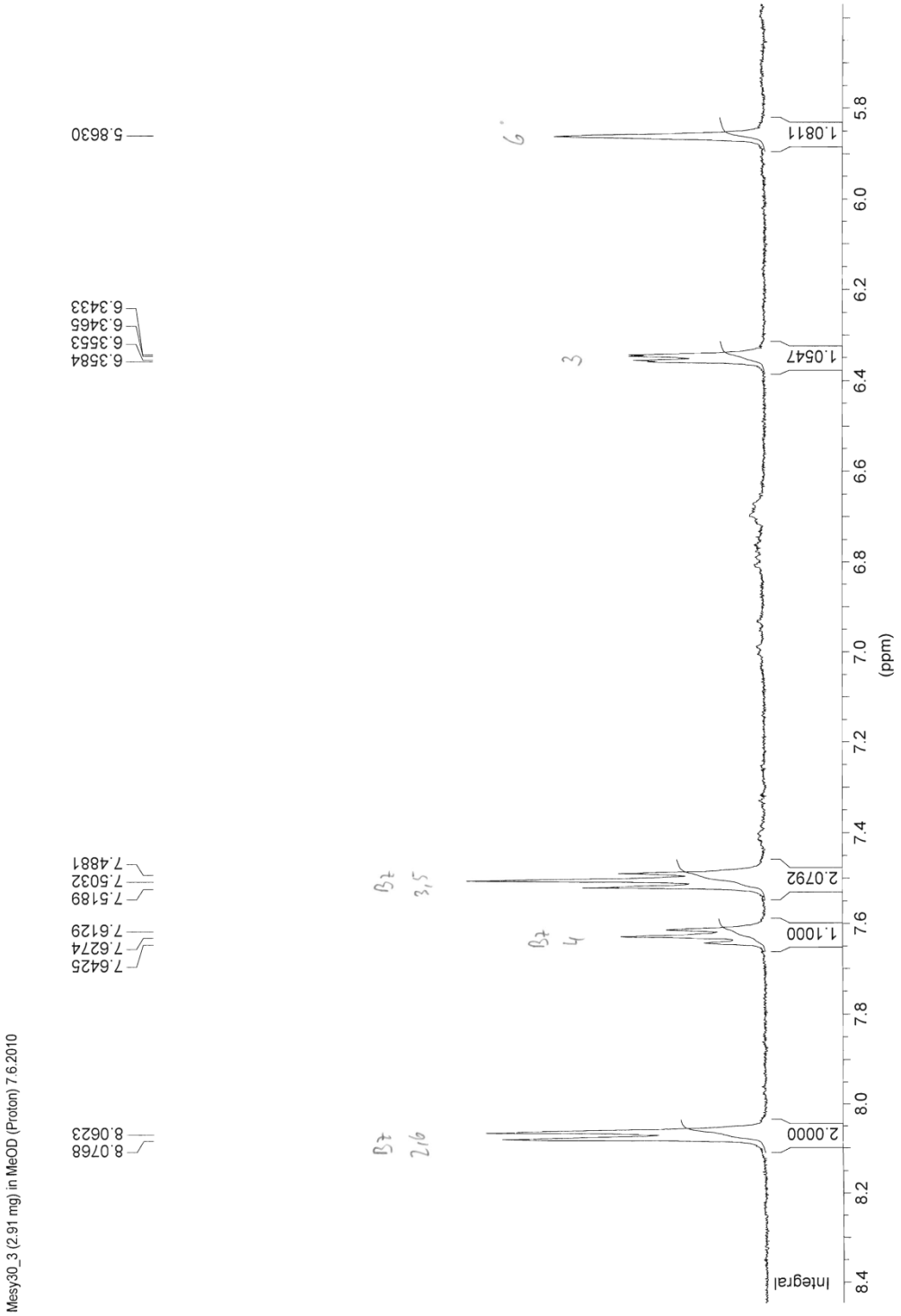

Peos9b (Ostruthin 2.95 mg) in MeOD (HMBC) 30.1.2010
MeOD
1'
6' 5'
4' 10' 9'
ppm
15
20
25
30
35
40
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
ppm
6'
1'
5'
4'
10'
9'

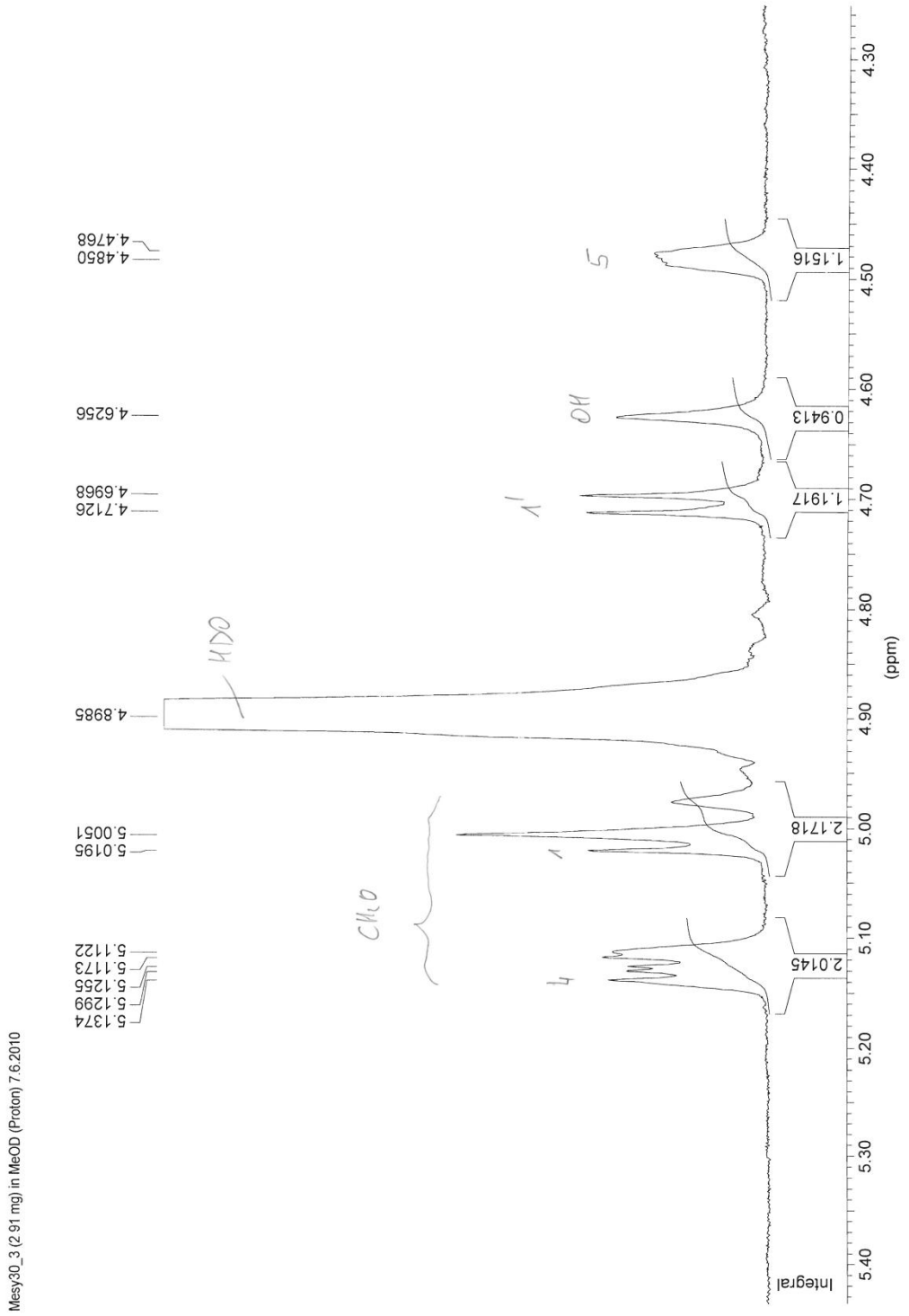
Annex III:

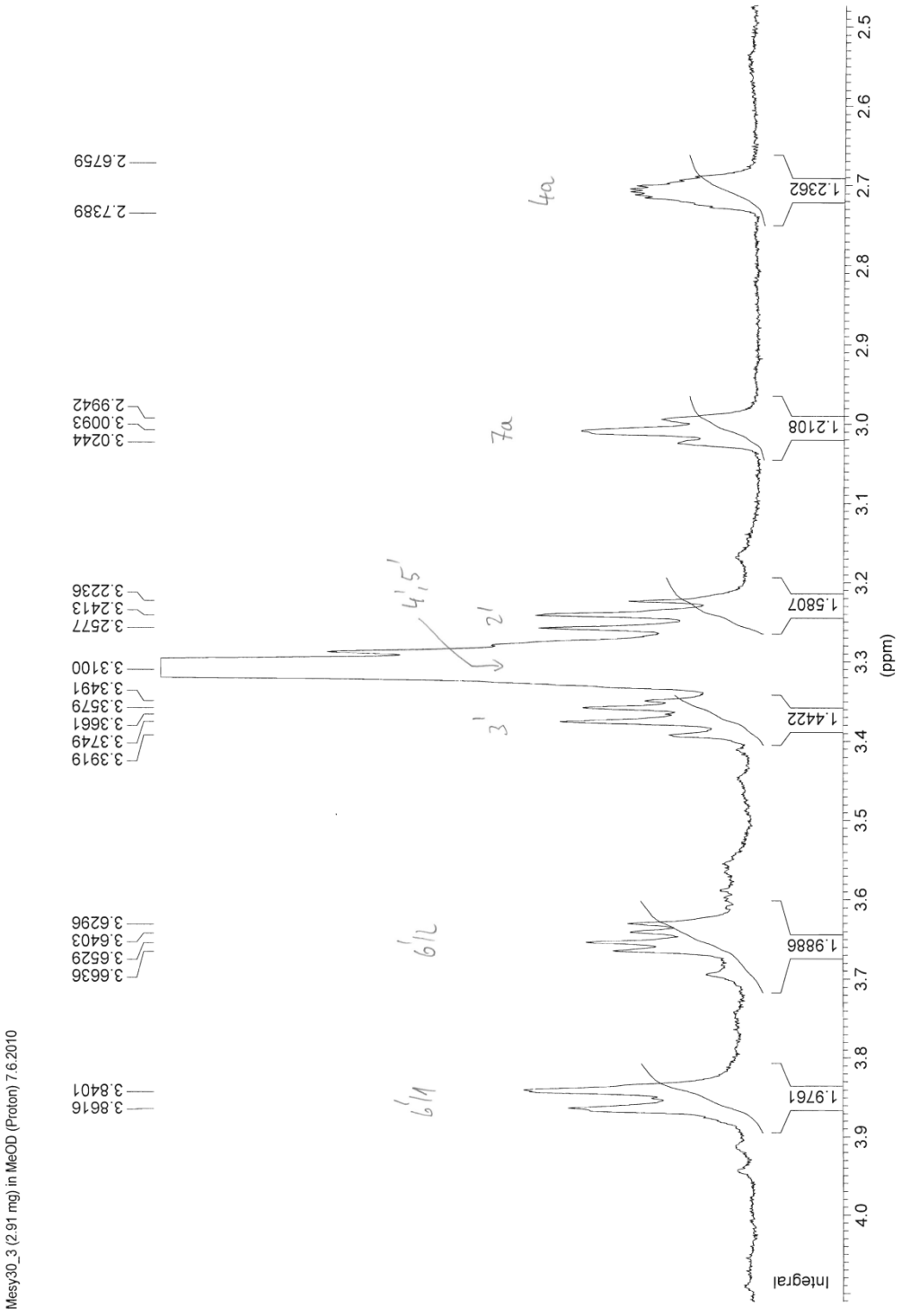
NMR spectra of melampyroside

^{1}H-NMR spectra of melampyroside



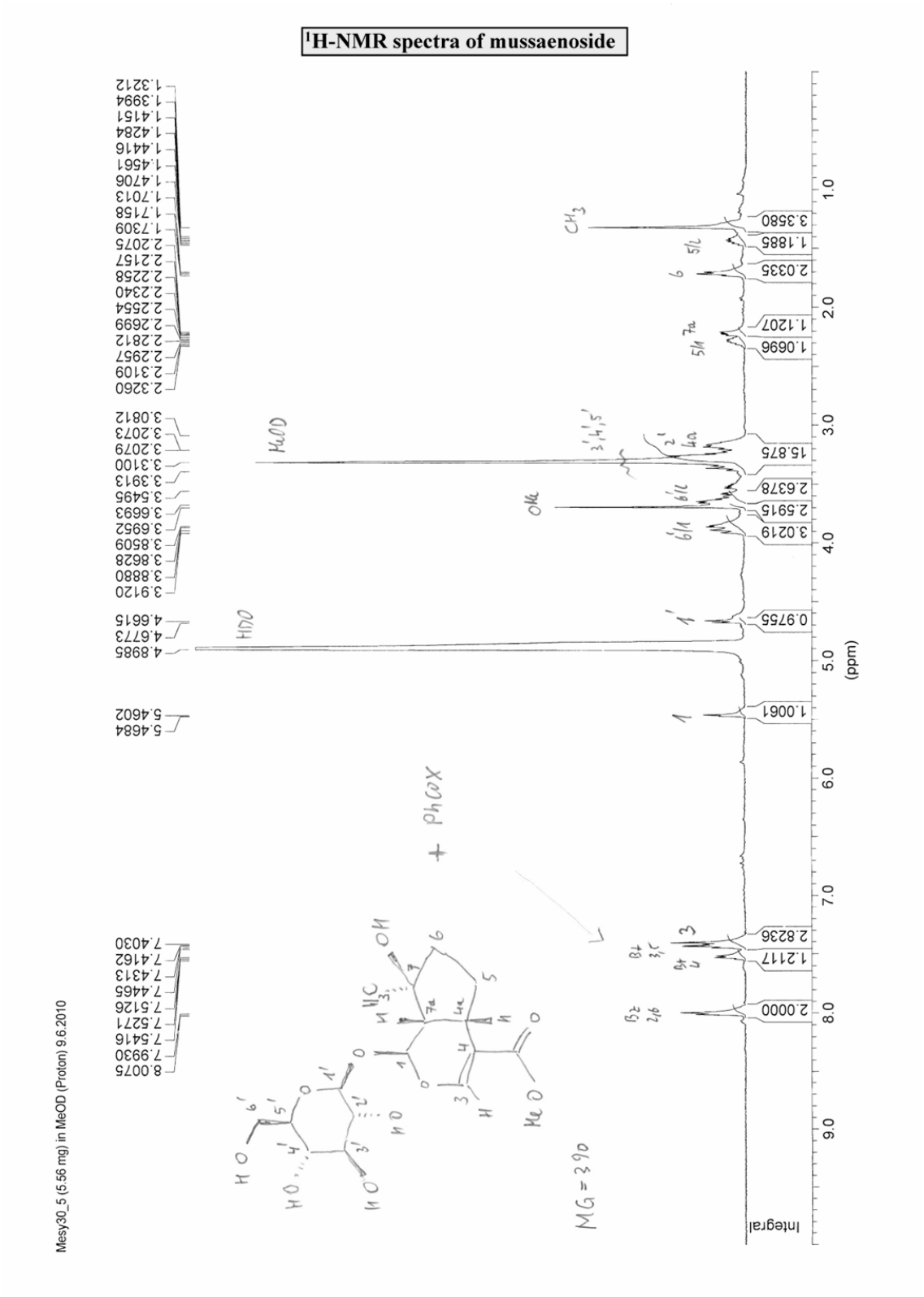

Mesy30_3 (2.91 mg) in MeOD (Proton) 7.6.2010
8.0768
8.0623
7.6425
7.6274
7.6129
7.5189
7.5032
7.4881
6.3584
6.3553
6.3465
6.3433
5.8630
Integral
2.0000
1.1000
2.0792
1.0547
1.0811
(ppm)


Mesy30_3 (2 91 mg) in MeOD (Proton) 7.6.2010
Integral
(ppm)
4.30
4.40
4.50
4.60
4.70
4.80
4.90
5.00
5.10
5.20
5.30
5.40
1.1516
0.9413
1.1917
2.1718
2.0145
4.8985
4.6256
4.7126
4.6968
4.4768
4.4850
5.0051
5.0195
5.1122
5.1173
5.1255
5.1299
5.1374
HDO
CH2O
OH
1
1'
4
5

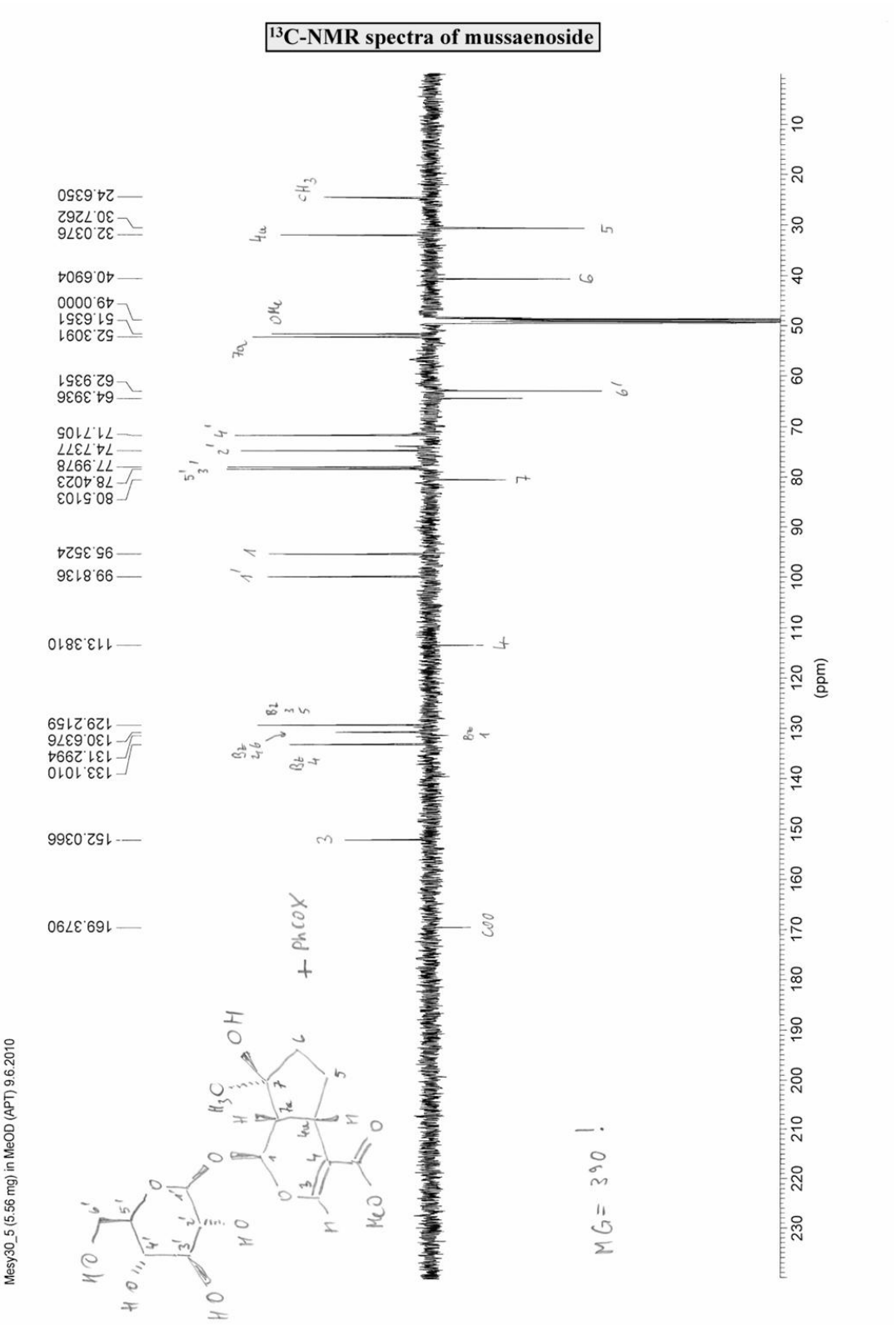

Mesy30_3 (2.91 mg) in MeOD (Proton) 7.6.2010
2.6759
2.7389
3.0244
3.0093
2.9942
3.2577
3.2413
3.2236
3.3100
3.3491
3.3579
3.3661
3.3749
3.3919
3.6296
3.6403
3.6529
3.6636
3.8401
3.8616
4a
7a
4',5'
2'
3'
6'2
6'1
1.2362
1.2108
1.5807
1.4422
1.9886
1.9761
Integral
(ppm)

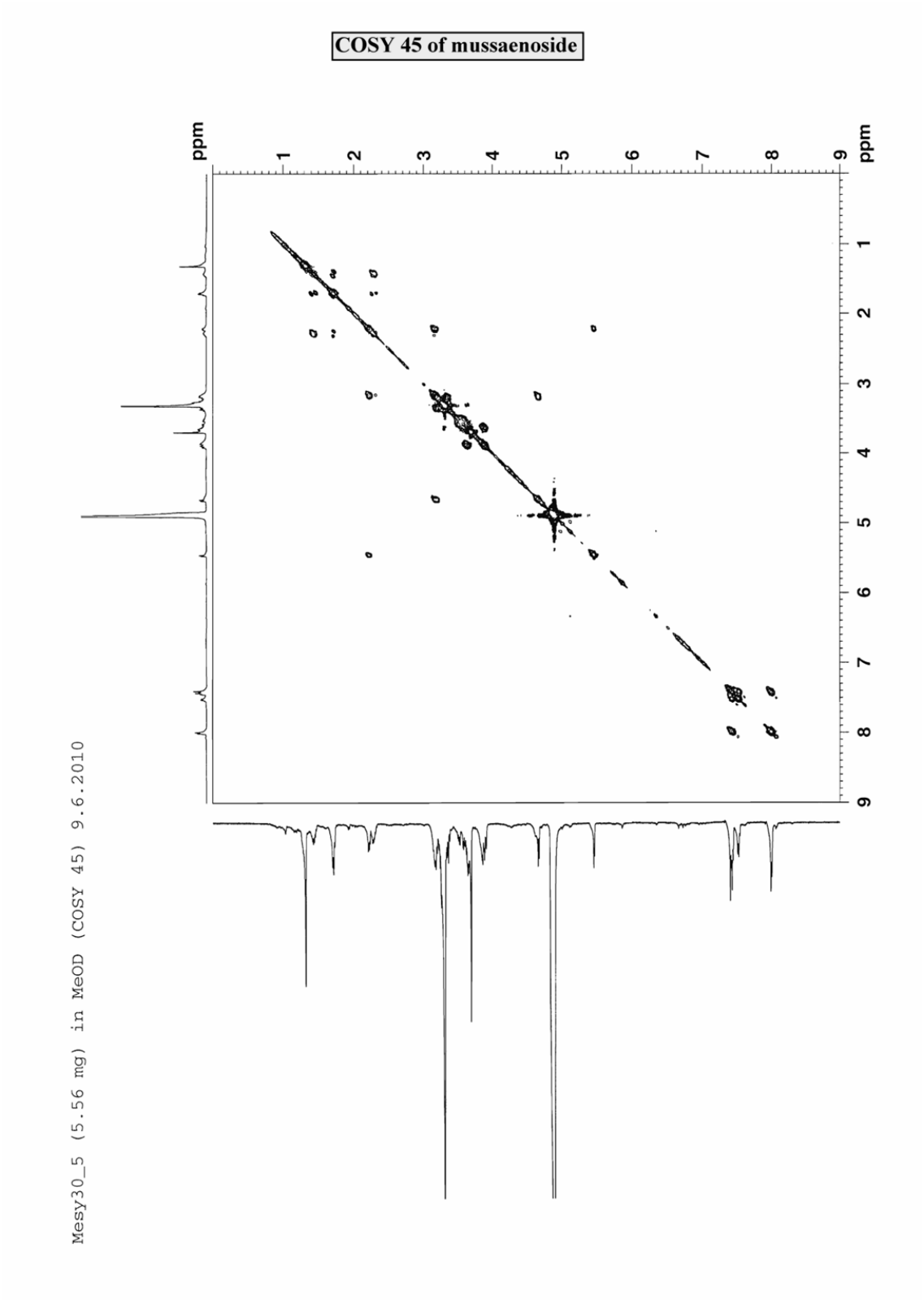
Annex IV:

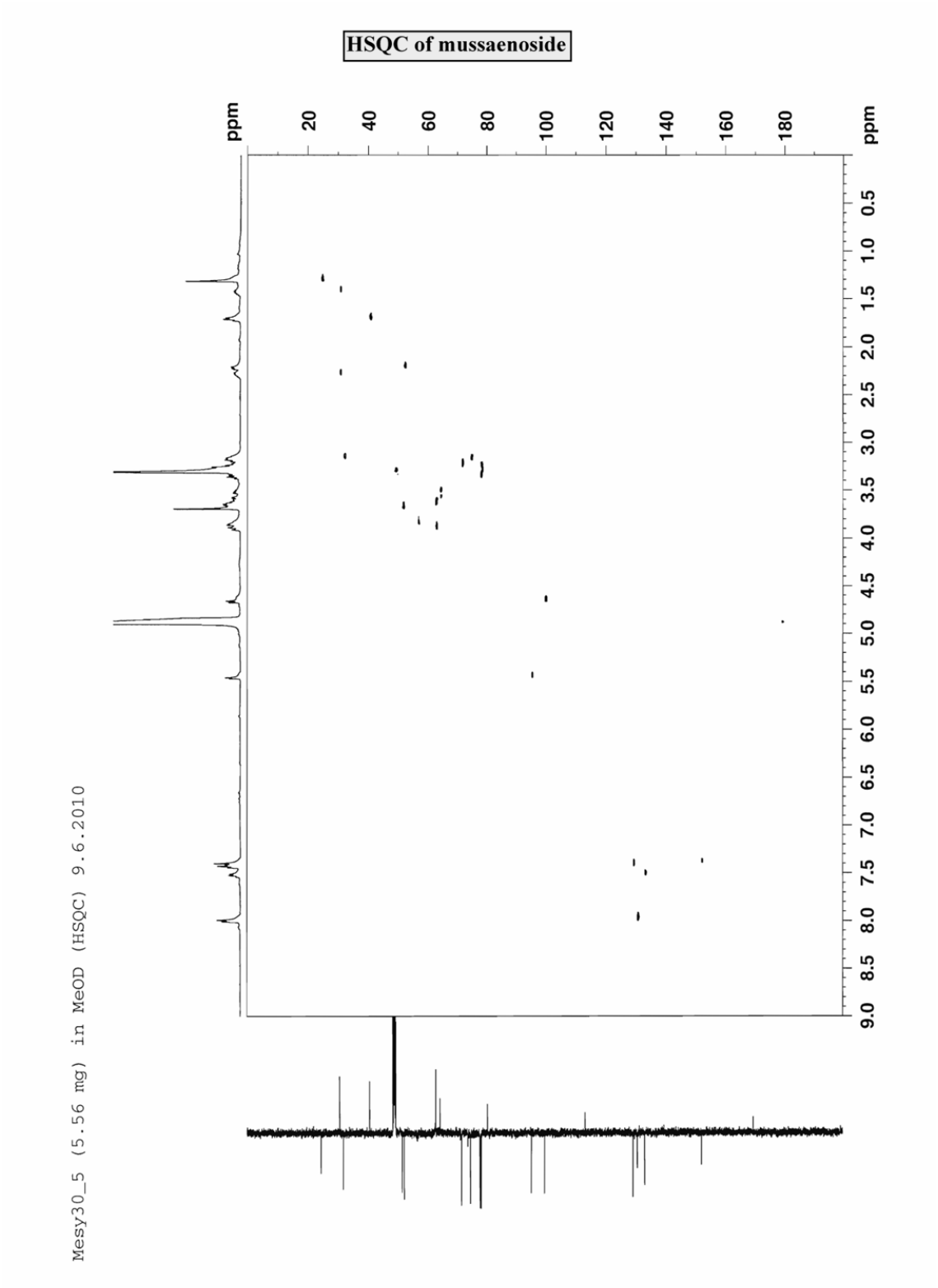
NMR spectra of mussaenoside

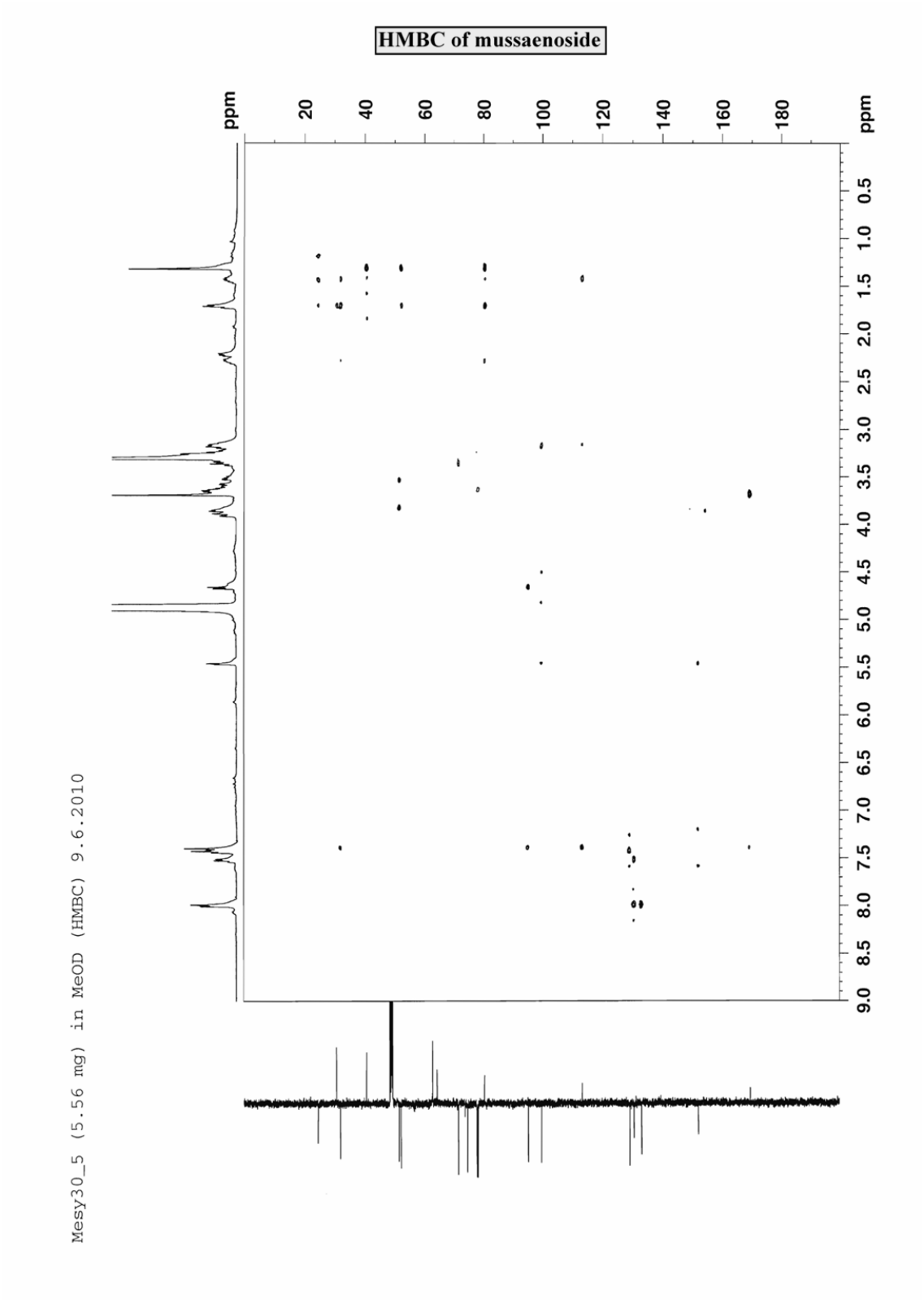

¹H-NMR spectra of mussaenoside
Mesy30_5 (5.56 mg) in MeOD (Proton) 9.6.2010
MG = 390
+ PhCOX
MeOD
OMe
CH3
Integral
(ppm)

^{13}C-NMR spectra of mussaenoside

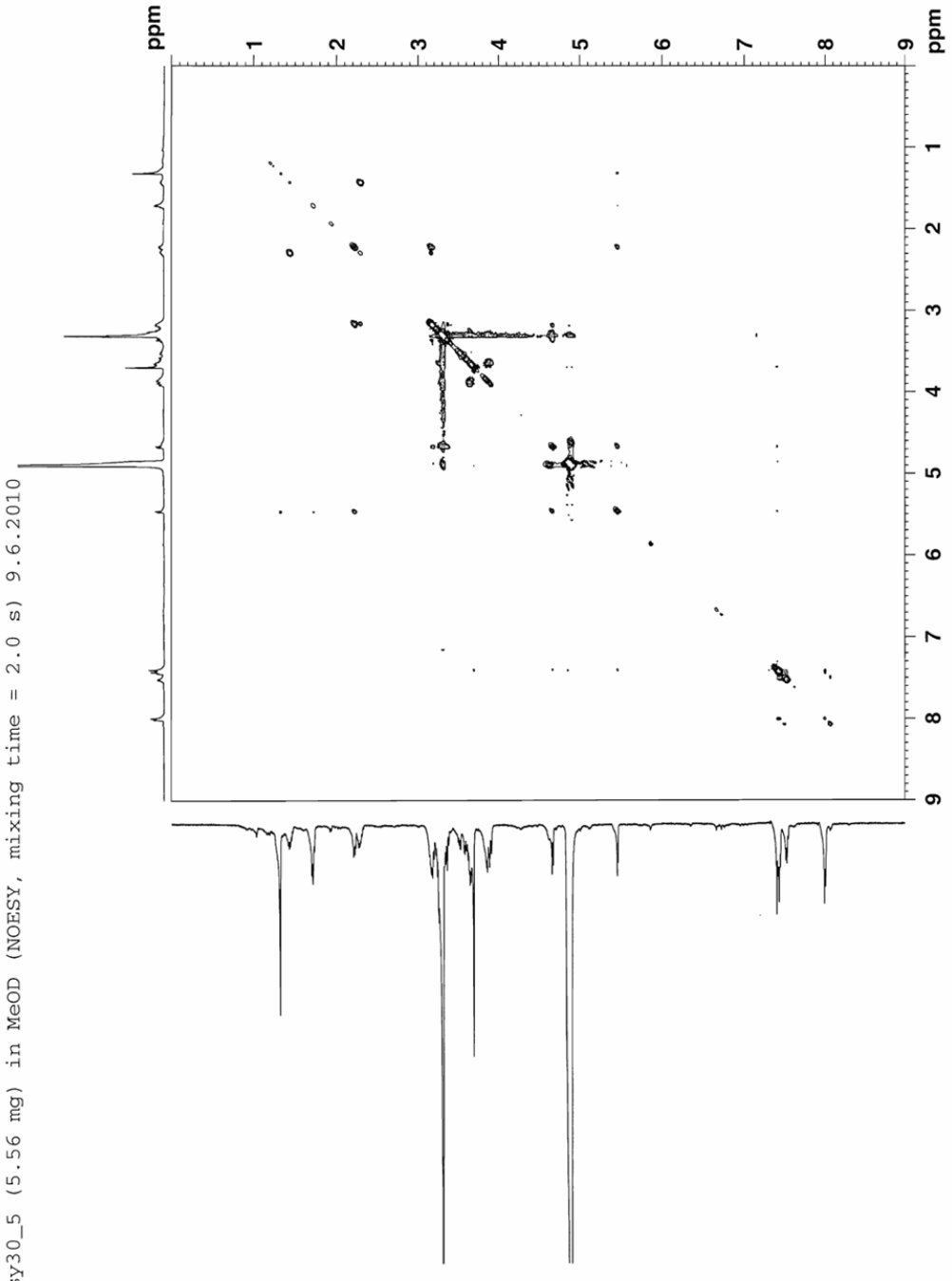



COSY 45 of mussaenoside
ppm
1
2
3
4
5
6
7
8
9
Mesy30_5 (5.56 mg) in MeOD (COSY 45) 9.6.2010


HSQC of mussaenoside
ppm
20
40
60
80
100
120
140
160
180
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Mesy30_5 (5.56 mg) in MeOD (HSQC) 9.6.2010


HMBC of mussaenoside
ppm
20
40
60
80
100
120
140
160
180
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Mesy30_5 (5.56 mg) in MeOD (HMBC) 9.6.2010

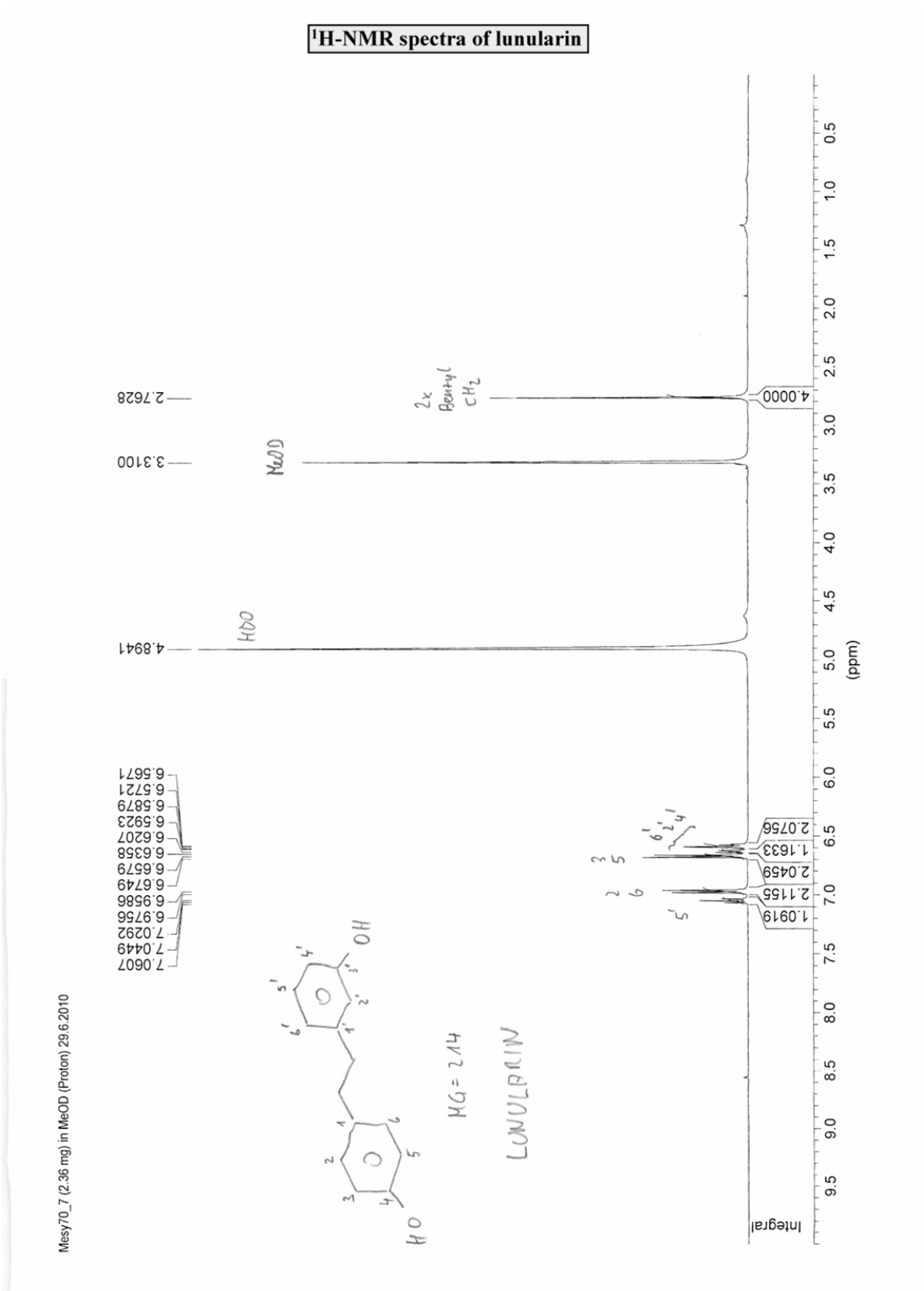
NOESY of mussaenoside

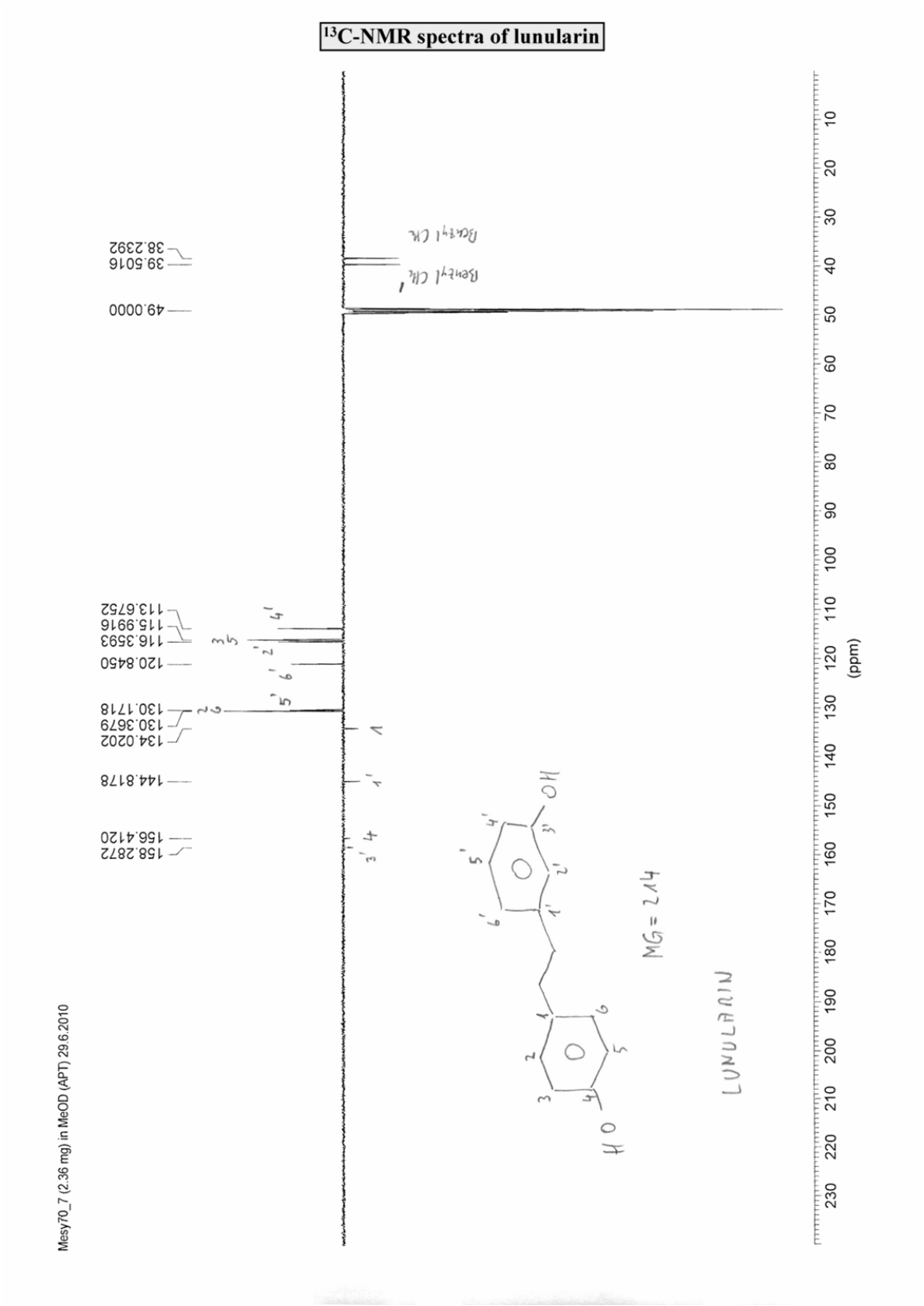


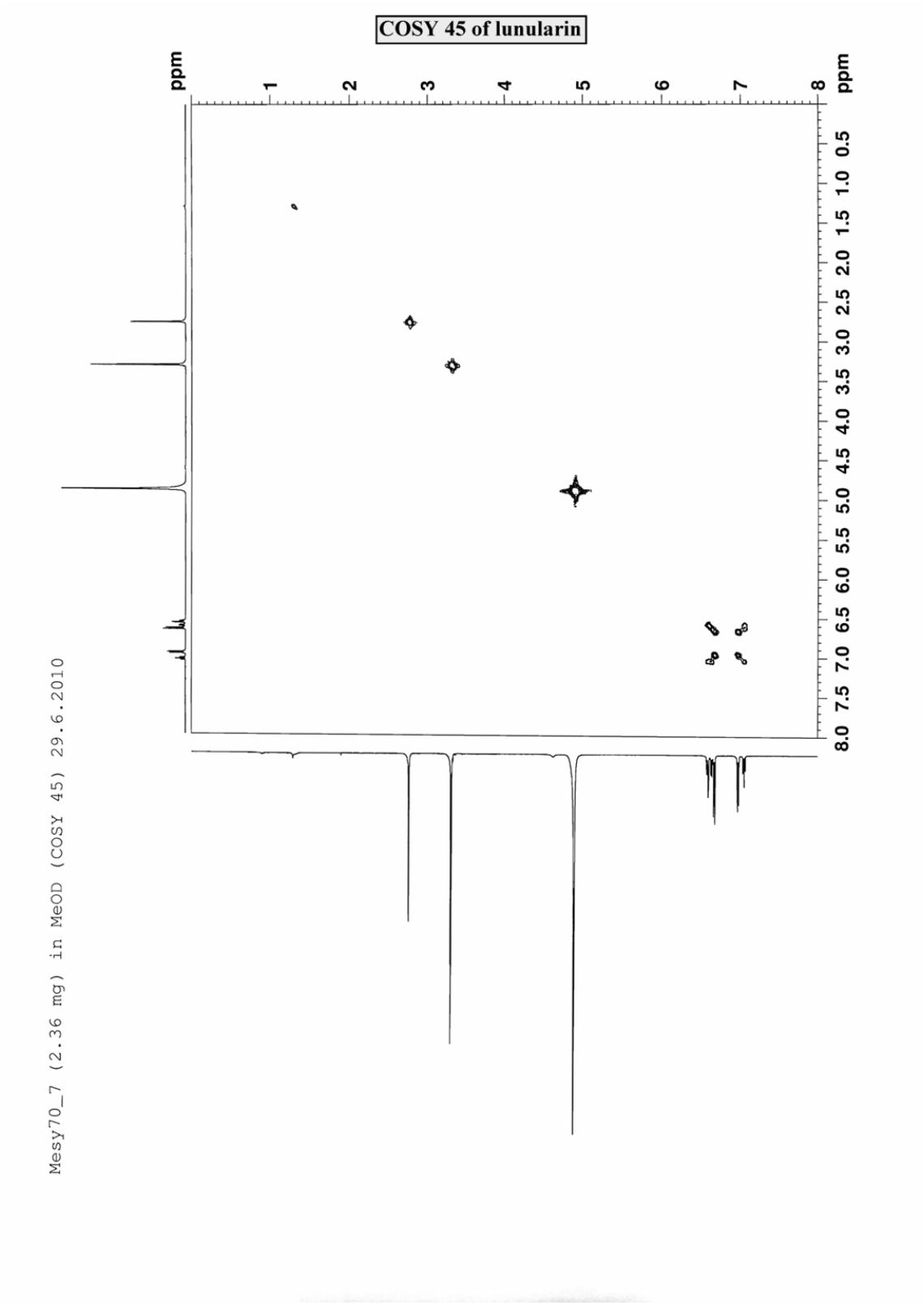
Annex V:

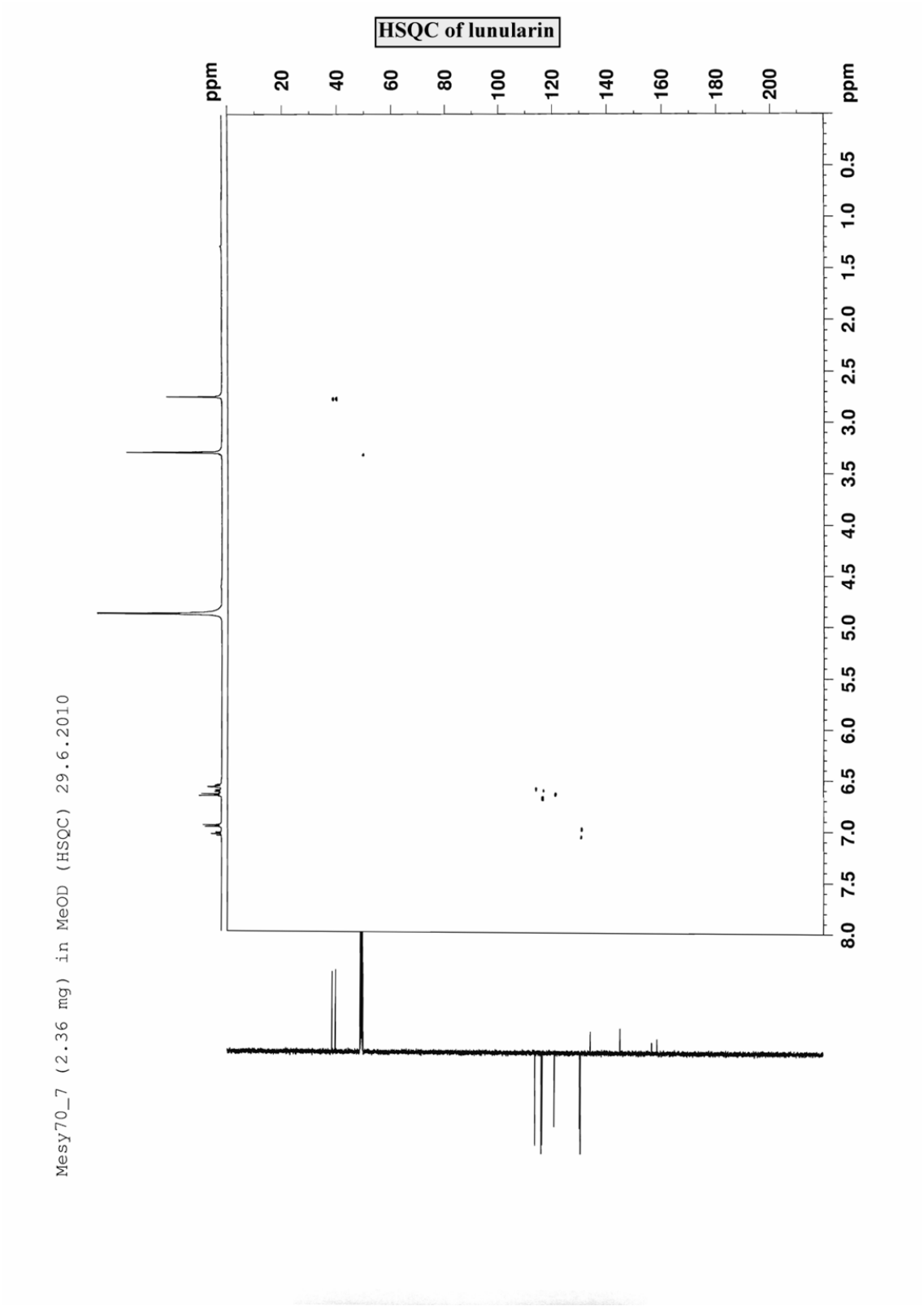
NMR spectra of lunularin

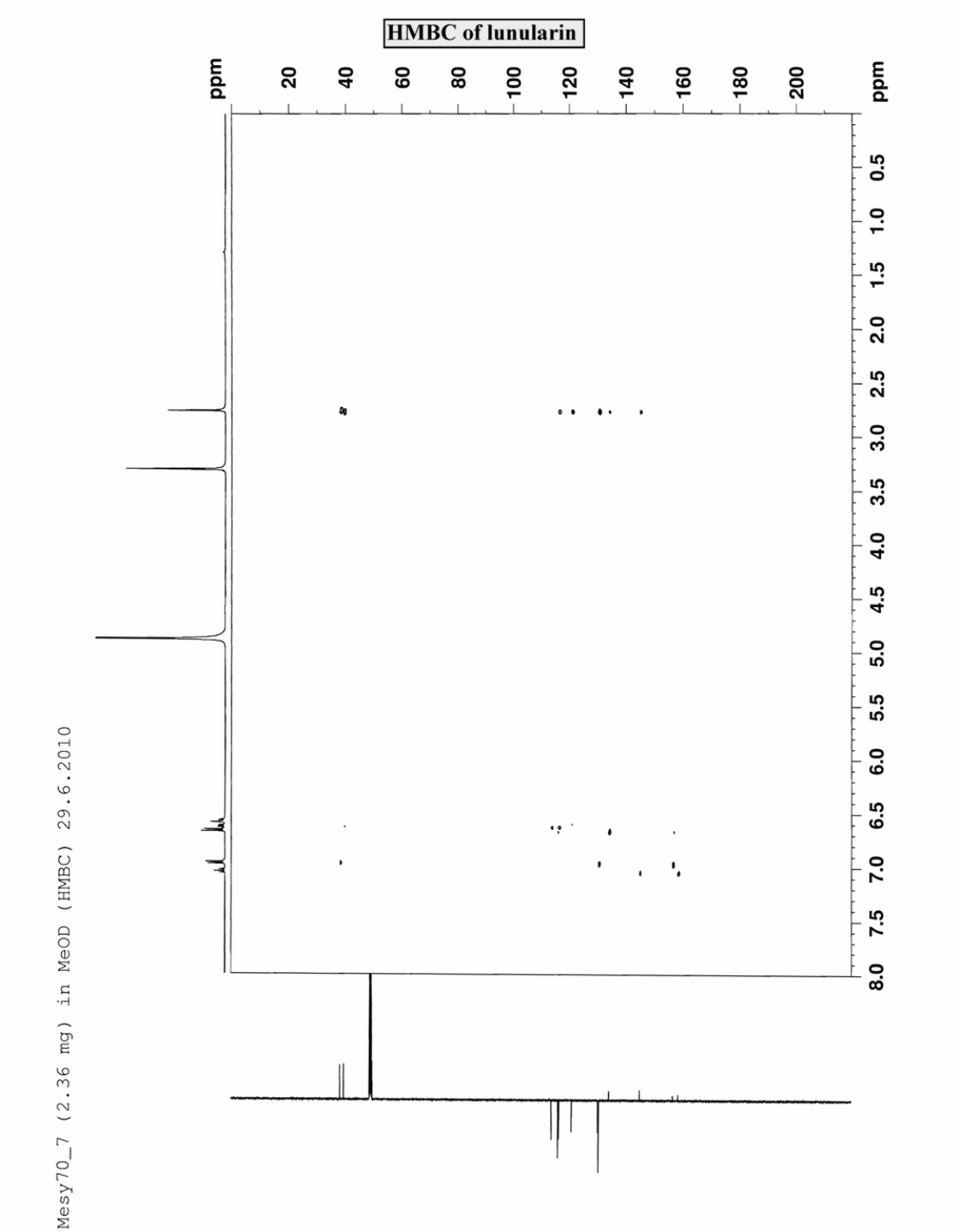
^{1}H-NMR spectra of lunularin




13C-NMR spectra of lunularin
Mesy70_7 (2.36 mg) in MeOD (APT) 29.6.2010
158.2872
156.4120
144.8178
134.0202
130.3679
130.1718
120.8450
116.3593
115.9916
113.6752
49.0000
39.5016
38.2392
(ppm)
MG = 214
LUNULARIN
HO
OH


COSY 45 of lunularin
ppm
1
2
3
4
5
6
7
8
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
Mesy70_7 (2.36 mg) in MeOD (COSY 45) 29.6.2010


HSQC of lunularin
ppm
20
40
60
80
100
120
140
160
180
200
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
Mesy70_7 (2.36 mg) in MeOD (HSQC) 29.6.2010


HMBC of lunularin
ppm
20
40
60
80
100
120
140
160
180
200
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
Mesy70_7 (2.36 mg) in MeOD (HMBC) 29.6.2010