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# DIPLOMARBEIT

Titel der Diplomarbeit

Discovery of novel anti-inflammatory compounds  
targeting NF- $\kappa$ B

Verfasserin

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## Abstract

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Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is the generic name for a family of pro-inflammatory transcription factors, which are activated by numerous stimuli and which themselves lead to the transcription of various target genes like those encoding for inflammatory cytokines, adhesion molecules, enzymes and others. NF- $\kappa$ B is crucial for the proper immune response, since it plays an important role in innate and adaptive immunity. In case it is deregulated it can also be a major contributor to inflammation and linked diseases like cancer. These facts make NF- $\kappa$ B a promising target in drug discovery.

Since the plant kingdom provides a huge number of still unidentified plants and constituents with potential pharmacological effects, it was the aim of this work to discover new anti-inflammatory agents from plant sources which target the NF- $\kappa$ B signaling pathway. Mostly less scientifically examined plants were selected through ethnopharmacological or computer-aided approaches.

A cell-based NF- $\kappa$ B luciferase reporter gene assay was used to identify active extracts and compounds.

In this work a lot of active plant extracts and active compounds derived from plant material have been identified. For the most active substances the IC<sub>50</sub> was determined. Plumericin from the Amazonian plant *Himatanthus sucuuba* was the most effective compound identified in this work. This compound was shown to inhibit the NF- $\kappa$ B pathway upstream from I $\kappa$ B- $\alpha$ , but the target still remains unknown.

As further interesting outcome it has been found that extracts from *Morus alba* have a direct inhibiting effect on the luciferase enzyme, showing one limitation of this popular screening assay.

Additionally extracts from *Notopterygium* sp. have been found not only to be very active as NF- $\kappa$ B inhibitors but also to have unspecific inhibitory or possibly toxic effects.

In summary new compounds which target the NF- $\kappa$ B signaling pathway and which therefore represent potential leads for the development of novel anti-inflammatory drugs, have been identified in this work. Furthermore, a number of active extracts have been identified which could be further fractionated to gain even more active compounds that could be useful to combat diseases related to inflammation.



# Zusammenfassung

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Nuclear factor  $\kappa$ B (NF- $\kappa$ B) ist die gängige Bezeichnung einer Familie proinflammatorischer Transkriptionsfaktoren, die durch zahlreiche Stimuli aktiviert werden und selbst die Transkription vieler Zielgene herbeiführen. So führen sie unter anderem zur Transkription derjenigen Gene, die für inflammatorische Cytokine, Adhäsionsmoleküle und Enzyme kodieren. NF- $\kappa$ B ist essentiell für eine funktionierende Immunantwort, da er eine wichtige Rolle in der unspezifischen und spezifischen Immunabwehr übernimmt. Im Falle einer Deregulierung der NF- $\kappa$ B Signalkaskade, wird diesem Transkriptionsfaktor aber auch eine bedeutende Rolle im Entzündungsgeschehen und in den damit verbundenen Krankheiten, wie z.B. Krebs, zugeschrieben. Diese Tatsache führt dazu, dass NF- $\kappa$ B ein vielversprechendes Ziel bei der Erforschung neuer Wirkstoffe darstellt.

Das Pflanzenreich enthält viele noch unerforschten Pflanzen und Inhaltsstoffe mit möglichen pharmakologischen Effekten. So war es das Ziel dieser Arbeit neue antientzündliche Wirkstoffe pflanzlicher Herkunft zu finden, die eine Hemmung der NF- $\kappa$ B Signaltransduktion bewirken. Hauptsächlich wurden hierfür wissenschaftlich wenig untersuchte Pflanzen über eine computergestützte oder ethnopharmakologische Herangehensweise ausgewählt

Zur Identifizierung aktiver Extrakte und Inhaltsstoffe wurde ein zellbasierter *luciferase reporter gene assay* verwendet.

In dieser Arbeit wurden viele wirksame Pflanzenextrakte und aktive Inhaltsstoffe aus pflanzlichem Material identifiziert. Für die aktivsten Substanzen wurden die IC<sub>50</sub> Werte bestimmt. Plumericin aus der Amazonas-Pflanze *Himatanthus siccuba*, war die wirkungsvollste Substanz, die in dieser Arbeit gefunden wurde. Es konnte gezeigt werden, dass Plumericin den NF- $\kappa$ B Signalweg oberhalb von I $\kappa$ B- $\alpha$  hemmt. Jedoch muss der Angriffspunkt noch bestimmt werden.

Als weiteres interessantes Ergebnis wurde entdeckt, dass Extrakte aus der Pflanze *Morus alba* eine direkte Hemmwirkung auf das Enzym Luciferase besitzen. Dieses Resultat zeigt eine Schwäche dieses populären Screeningassays.

Zusätzlich wiesen die Extrakte aus der Pflanze *Notopterygium* sp. nicht nur NF- $\kappa$ B hemmende Wirkungen auf, sondern zeigten auch unspezifische inhibitorische oder möglicherweise sogar toxische Wirkungen.

Zusammenfassend wurden in dieser Arbeit neue Substanzen mit hemmender Wirkung auf den NF- $\kappa$ B Signaltransduktionsweg identifiziert, die als mögliche Leitstrukturen für die Entwicklung neuer antientzündlicher Arzneistoffe herangezogen werden können. Des Weiteren wurden aktive Extrakte gefunden, die zusätzlich fraktioniert werden könnten, um weitere aktive Inhaltsstoffe zu gewinnen. Diese könnten sich als nützlich im Kampf gegen Krankheiten, die mit Entzündungen einhergehen, erweisen.

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## **B. Introduction**

## B. Introduction

### 1. Aim of the work

Before the first synthetic drug phenazone was introduced to the market in 1884, with its trade name antipyrine<sup>1</sup>, mainly herbs and plant products and fewer also minerals and animal products were used for thousands of years to treat all kind of ailments. While the development of synthetic drugs flourished since their first introduction at the end of the 19th century, the use of herbal drugs diminished in the Western world. Lately however, the interest and research in plant derived drugs started to increase again. A lot of plants and their extracts have been examined since then and the traditional use could often be confirmed by scientific research. Various herbal products like *Arnicae flos*, *Calendulae flos*, *Matricariae flos* and *Salicis cortex* are known for hundreds of years for their anti-inflammatory or anti-rheumatic action and their effects have been scientifically proven.<sup>2</sup> But still a lot of potentially interesting and effective plants remain that are not well scientifically investigated.

The nuclear factor  $\kappa$ B (NF- $\kappa$ B) is an important transcriptional factor involved in physiological, inflammatory, and immune responses, but in case it is deregulated it develops to a major contributor in pathogenic inflammation and chronic inflammatory diseases as asthma and rheumatoid arthritis, as well as in inflammation-linked diseases like cancer.<sup>3-5</sup>

Known potent anti-inflammatory drugs are amongst others glucocorticoids. One mechanism of action to obtain their effective anti-inflammatory property is the inhibition of NF- $\kappa$ B activity.<sup>6</sup> The mechanism of action of these potent drugs shows that NF- $\kappa$ B is a good choice as a possible target for the discovery of effective anti-inflammatory drugs.

Since the plant kingdom still represents a little investigated but huge source for prospective drugs, a main goal of this work was to find novel NF- $\kappa$ B inhibitors from plant sources, which show a high inhibitory potential and therefore could possibly show superior attributes compared to the currently available anti-inflammatory drugs. As part of the DNTI project part 04 “from cell based assays to molecular mechanisms” the work was focused on the identification of new natural products with potential anti-inflammatory activities in cell based models relevant for inflammation.

## **2. DNTI**

This thesis is part of the NFN (Nationales Forschungsnetzwerk) project “Drugs from nature targeting inflammation” (DNTI). This project represents an interdisciplinary collaboration between different project parts (PP) at different universities in Austria with the goal to discover new drugs from plant sources to combat inflammation. DNTI is divided in 8 collaborating groups as listed below.

### **2.1. PP01: Co-ordination**

Director of this organizational part is Univ.-Prof. Dr. Hermann Stuppner from the Institute of Pharmacy at the University of Innsbruck. This project part is mainly responsible for the documentation, management, and controlling of the research process and all administrative and financial tasks that come along with this duty.

### **2.2. PP02: Identification of bioactive natural products by virtual screening**

Supervisor of this group is Dr. Daniela Schuster (Institute of Pharmacy, University of Innsbruck). The aim of this project part is the implementation of computer-aided methods for the identification of bio-active substances out of plant sources.

For the achievement of this goal four different methods are being used or are under development. These are: pharmacophore modeling, database mining, *in silico* counter screening based on parallel pharmacophore virtual screening, and last the methods of docking and scoring.

### **2.3. PP03: From *in silico* plant selection to bioactive natural products**

Head of this project part is Univ.-Prof. Dr. Hermann Stuppner (Institute of Pharmacy, University of Innsbruck). The aim is to discover bioactive constituents from natural materials in a target orientated manner thereby applying integrated computational tools into the field of pharmacognosy. This group is responsible for the evaluation of hit candidates and the forwarding of those ones to PP02 in order to upgrade the

database. Virtual hits with a high ranking should then be isolated from natural sources.

### **2.4. PP04: From cell-based assays to molecular mechanisms**

Univ.-Prof. Dr. Verena M. Dirsch (Department of Pharmacognosy, University of Vienna) is the leader of this project part. The main goal of the group is to provide the DNTI project with appropriate cellular models for screening and bio-guidance of plant extracts for the identification of novel NF- $\kappa$ B inhibitors and PPAR $\alpha$ , PPAR $\gamma$ , and PPAR $\beta/\delta$  agonists. A further important task is the mechanistic characterization of the molecular mode of action of the identified compounds.

### **2.5. PP05: From ethnomedicine to bioactive natural products via bioassay-guided isolation**

Under the supervision of Univ.-Prof. Dr. Rudolf Bauer (Institute of Pharmaceutical Sciences, University of Graz) this project part works on the isolation of active compounds from selected plants used in traditional Chinese medicine. The plant material is previously identified and phytochemically characterized. Additional pharmacological characterization is done by cellular and enzymatic *in vitro* assays.

### **2.6. PP06: Reemerging medicinal plants: bioactivity and biotechnology**

The head of this project part is Univ. Prof. Mag. Dr. Dr .h. c. Brigitte Kopp (Department of Pharmacognosy, University of Vienna). The VOLKSMED database was scanned for plants with documented anti-inflammatory abilities but with a yet not characterized mode of action. The now ongoing work is the identification and phytochemical characterization of the used plant material and thereupon the isolation of the active compounds.

### 2.6.1. VOLKSMED database

The VOLKSMED database is a collection based on the knowledge of traditional medicine collected in various diploma theses in the past. Students therefore interviewed people, who still preserved this traditional knowledge, all over Austria and South-Tirol. Special attention was paid to differentiate between real traditional knowledge and new acquirements from the modern medicine. Therefore especially old people were interrogated who gained the ancient herbal medicine knowledge from their ancestors. Originating from the collected information there is now a database including a lot of plants with ethnopharmacological use. Information about the utilized part of the plant for the needed indication, and the kind of preparation, as well as the application is recorded for each plant.

### 2.7. PP07: Medical plants: molecular characterization and domestication

Univ.-Prof. Dipl. Ing. Dr. Chlodwig Franz and his group (Institute for Applied Botany and Pharmacognosy, University of Veterinary Medicine Vienna) work on the aim to deliver plant material of defined quality. The identification and characterization of plant material is completed with DNA sequence data and molecular markers analysis. This group deals also with plant cultivation, aiming to deliver defined plant materials, in order to be investigated by the other groups.

### 2.8. PP08: *In vitro* and *in vivo* models for inflammation

Compounds which were previously identified and analyzed by other project partners are evaluated by the group of Dr. Valery Bochkov (Department of Vascular Biology and Thrombosis Research Medical University of Vienna) using *in vitro*, *ex vivo* and *in vivo* models relevant for inflammation.

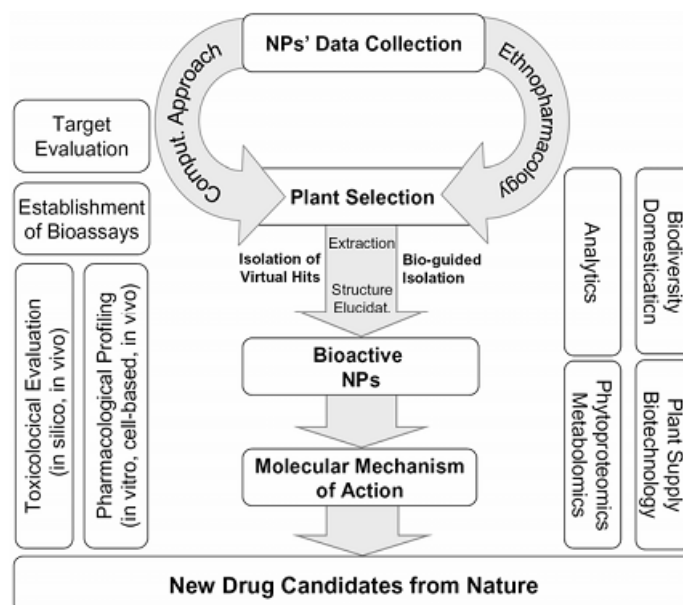


**Figure 1: Overview over the workflow of the DNTI project. Taken from the NFN-application DNTI.**

### **3. Nuclear factor $\kappa$ B: pathways and associated molecules**

#### **3.1. Nuclear factor $\kappa$ B and its various stimuli and target genes**

The nuclear factor  $\kappa$ B (NF- $\kappa$ B) was identified 25 years ago as the nuclear factor that binds the  $\kappa$  light chain enhancer in B cells and therefore was named NF- $\kappa$ B.<sup>7</sup> Since then it has been a major target of scientific investigations.

NF- $\kappa$ B is regulated by hundreds of stimuli and activates thereupon hundreds of target genes. The different activators can be amongst others arranged into the following groups: bacteria and bacterial products (e.g. LPS), viruses and viral products, stress, cytokines (e.g. IL-1/2/12/15/17 and 18, TNF- $\alpha$ ), mitogens, growth factors (e.g. PDGF), hormones (e.g. FSH), and other physiological and apoptotic mediators. While the various cellular targets can be ordered into the following main groups: chemokines and cytokines (e.g. TNF- $\alpha$ , IL-6/8/9/11/12/15), immune receptors, adhesion molecules (e.g. ICAM-1, VCAM-1), acute phase proteins, inflammatory enzymes (COX-2, 5-LOX), cell surface receptors, regulators of apoptosis, and growth factors (e.g. PDGF, GM-CSF).<sup>8</sup>

#### **3.2. Nuclear factor $\kappa$ B and its role in immunity, inflammation, and cancer**

The classical NF- $\kappa$ B pathway is essential for the proper working of innate immunity. As stated above, NF- $\kappa$ B activation causes transcription of genes encoding amongst others adhesion molecules like intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial-leukocyte adhesion molecule 1 (ELAM-1), as well as enzymes which produce secondary inflammatory mediators like COX-2, which leads to the production of mostly pro-inflammatory prostaglandins. The mentioned molecules are important mediators in innate immune response since they are required for the migration of inflammatory and phagocytic cells to the place of inflammation in order to respond to infection and injury. These mediators also lead to the activation of the recruited cells when they reach the place of inflammation. For example macrophages produce inflammatory mediators like chemokines, TNF- $\alpha$ , and others upon NF- $\kappa$ B activation as a result to bacteria and bacterial product stimulation. Production and secretion of cytokines like IL1- $\beta$  and TNF- $\alpha$  upon NF- $\kappa$ B binding to  $\kappa$ B

sites in the promoters of these target genes result in an amplifying loop of activation since these molecules are NF- $\kappa$ B pathway activators as well. These examples show the importance of precise regulation of the NF- $\kappa$ B activity.

The alternative NF- $\kappa$ B pathway has been shown to play an important role in adaptive immunity as it is crucial for spleen microarchitecture development and the proper maturation of B-cells.<sup>9</sup>

Uncontrolled inflammation was found to increase the risk of autoimmune diseases and cancer. NF- $\kappa$ B shows diverse tumor promoting functions. Activation of different target genes by NF- $\kappa$ B can increase tumor growth and progression. Binding of TNF- $\alpha$  or IL-1 to their receptors on cells that are fated to go through malignant conversion, causes the expression of anti-apoptotic genes which lead to the survival of these cells. Classical pathway activation in inflammatory cells, which were recruited to tumorigenic cells, promotes tumor development as well, since it leads to the expression of genes that encode secreted cytokines, growth factors, and survival factors which thereupon act on the malignant cells.<sup>3</sup>

### 3.3. The five members of the mammalian NF- $\kappa$ B family

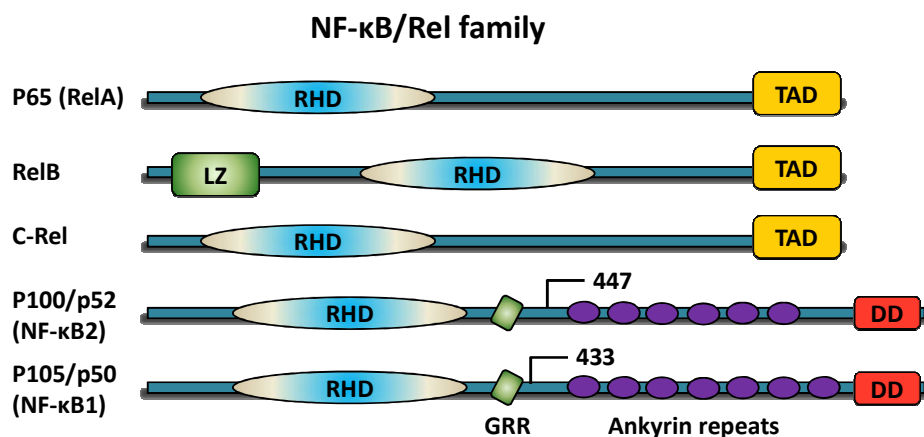


**Figure 2: Schematic illustration of the mammalian NF- $\kappa$ B family members.** P100 and p105 are processed to p52 and p50 respectively. Numbers indicate the assumed sites of cleavage at amino acid 447 for p100 and amino acid 433 for p105. For details see text. DD: death domain; GRR: glycine rich region; LZ: leucin zipper domain; RHD: Rel homology domain; TAD: transactivation domain. Adapted from Hayden and Ghosh.<sup>10,11</sup>

NF- $\kappa$ B is the term of use for a family of proteins which consists of the five following members in mammals: RelA (p65), RelB, c-Rel, NF- $\kappa$ B1 (meaning p50 and its

precursor p105) and NF- $\kappa$ B2 (meaning p52 and its precursor p100). The mostly referred to molecule when talking of NF- $\kappa$ B is the p65/p50 dimer.

Common feature of the NF- $\kappa$ B family members is the shared highly conserved 300 amino acid Rel homology domain (RHD). It enables the molecule to dimerize, to interact with the inhibitor of  $\kappa$ B (I $\kappa$ B), and to translocate to the nucleus and bind to DNA.

RelA, RelB, and c-Rel share C-terminal transactivation domains (TAD), while NF- $\kappa$ B1/2 both have a long C-terminal domain with multiple copies of ankyrin repeats, which inhibit their activation (**Figure 2**).<sup>12</sup> As the TAD is needed for the regulation of transcription and p50 and p52 lack TADs, they have three possible ways of acting. Since NF- $\kappa$ B proteins bind as dimers to  $\kappa$ B sites in promoters or enhancers of target genes, p50 and p52 can form heterodimers with TAD-containing family members and thus can regulate transcription. Other possibilities are that p50 and p52 directly recruit other TAD-containing proteins to the  $\kappa$ B-sites, or that they repress transcription by binding to  $\kappa$ B sites as homodimers.<sup>10</sup>

While all family members are able to form hetero- and homodimers, RelB cannot form homodimers and it does not heterodimerize with c-Rel or p65.<sup>11</sup>

The family members appear in unstimulated cells as homo- or heterodimers bound to proteins of the I $\kappa$ B family, which prevent their translocation to the nucleus and subsequent transcription of target genes.<sup>11,12</sup>

### **3.4. Inhibitor of $\kappa$ B (I $\kappa$ B) family members**

Inactive NF- $\kappa$ B molecules are bound to inhibitor of  $\kappa$ B (I $\kappa$ B) proteins. These proteins all share the multiple ankyrin repeat region which is needed for protein to protein interactions like the retention of NF- $\kappa$ B molecules in the cytoplasm.<sup>13</sup> The different family members can be ordered into the following groups.

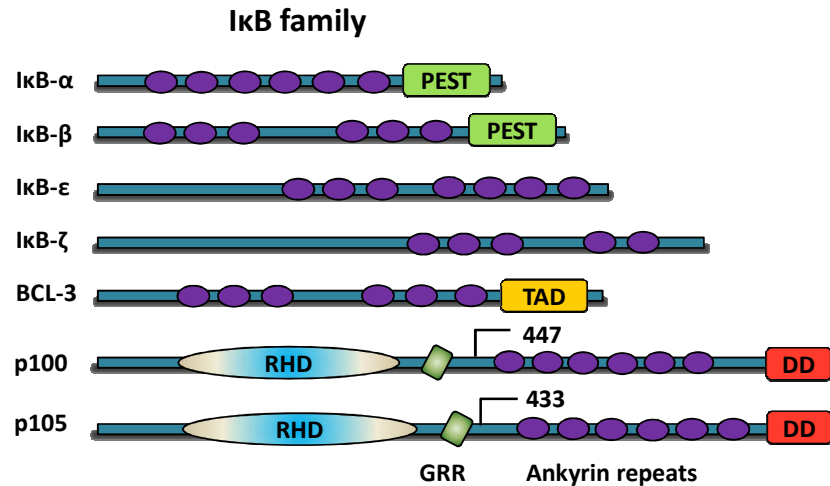


**Figure 3: Schematic illustration of the IκB family members.** P100 and 105 are precursor proteins of p52 and p50 respectively. Numbers indicate the assumed sites of cleavage at amino acid 447 for p100 and amino acid 433 for p105. For details see text. DD: death domain; GRR: glycine rich region; PEST: proline, glutamic acid, serine, and threonine rich region; RHD: Rel homology domain; TAD: transactivation domain. Adapted from Ghosh and Hayden.<sup>10,11</sup>

### 3.4.1. Typical family members

The “typical” family members are IκB-α, IκB-β, and IκB-ε. IκB-α is the prototypical and best studied member of the IκB family. These three family members operate as the classical inhibitors by retaining NF-κB dimers in the cytosol, hence they inhibit the transcription of target genes. IκB-α and IκB-ε are degraded upon IKK phosphorylation and their expression is upregulated by NF-κB. The only difference between those two lies in the delayed degradation and resynthesis of IκB-ε.<sup>14</sup>

### 3.4.2. Precursor proteins

There are the two “precursor” IκB molecules p100 and p105. Contrary to other IκB members these molecules are generally only partly degraded and therefore do still bind to the NF-κB molecule after degradation. However, it depends on the serine residues on which p105 is being phosphorylated whether it is completely degraded like other IκBs or just its C-terminal domain to release p50. P100 is processed to p65.<sup>14</sup> Both “precursor” IκB molecules contain a C-terminal death domain (DD). The DD of p100 has been described to function as a processing inhibitory domain (PID).<sup>11</sup> This PID inhibits basal processing to p52 in order to ensure selective processing

regulated in response to receptor binding and following signaling.<sup>15</sup> P100 and p105 also contain a glycine rich region (GRR) which prevents the total proteolysis of these proteins as it serves as a stop signal.<sup>11</sup>

### 3.4.3. The atypical family members

Last there are the “atypical” I $\kappa$ Bs, I $\kappa$ B- $\zeta$  and Bcl-3. They seem to regulate NF- $\kappa$ B signaling in a different way compared to other I $\kappa$ Bs. Bcl-3 contains a TAD analogous to the NF- $\kappa$ B family members and functions in the nucleus instead of the cytoplasm. I $\kappa$ B- $\zeta$  is rather unsimilar to the other I $\kappa$ Bs and is not expressed continuously.<sup>14</sup>

## 3.5. Inhibitor of $\kappa$ B kinases (IKKs)

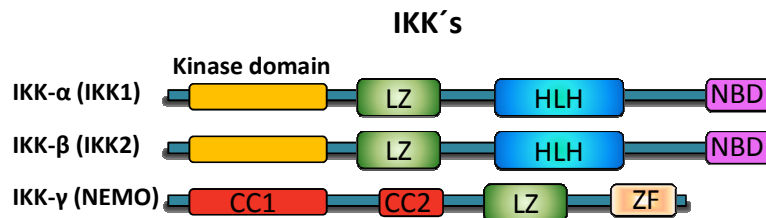


**Figure 4: Schematic illustration of the inhibitors of  $\kappa$ B kinases.** For details, see text. CC: coiled-coil domain; HLH: helix-loop-helix domain; LZ: leucine zipper domain; NBD: NEMO-binding domain; ZF: zinc finger domain. Adapted from Ghosh and Hayden.<sup>10</sup>

There are three major inhibitors of  $\kappa$ B kinases (IKKs, **Figure 4**). IKK- $\alpha$ , also referred to as IKK-1, IKK- $\beta$  also known as IKK-2, and the NF- $\kappa$ B essential modulator (NEMO) also called IKK- $\gamma$ . All members contain a leucine zipper domain (LZ) which in the case of IKK- $\alpha/\beta$  is needed for their dimerization and the kinase activity. Those two kinases are mostly known to form heterodimers. This dimer then binds NEMO through the NEMO-binding domain (NBD) present on both IKK- $\alpha$  and IKK- $\beta$ . Hence the core complex consists of the two catalytic kinases IKK- $\alpha$  and IKK- $\beta$  and the regulatory non catalytic subunit NEMO.<sup>14</sup>

NEMO contains a coiled-coil motif in its N-terminal half, responsible for interactions with the IKKs. Another coiled-coil motif in its c-terminal half, a leucine zipper, and a zinc finger motif are involved in oligomerization and interaction with the upstream molecules.<sup>12</sup>

### 3.6. NF- $\kappa$ B pathways

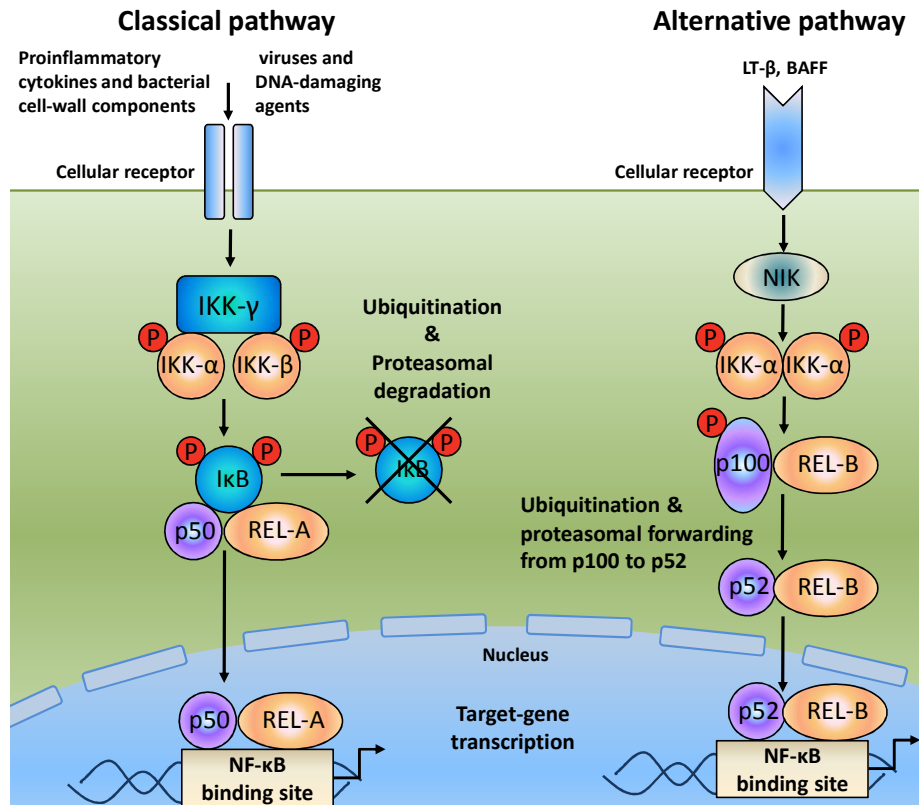


**Figure 5: Schematic simplified illustration of the two main signaling pathways leading to NF- $\kappa$ B activation.** Demonstrated are on the left side the classical pathway and on the right side the alternative pathway. The main differences can be found in the composition of the IKK complex and NF- $\kappa$ B dimers. For details see section 3.6.2 and 3.6.1. BAFF: B-cell activating factor; LT- $\beta$ : Lymphotoxin- $\beta$ ; P: phosphate.

Adapted from Karin and Greten<sup>3</sup> and additionally modified from Hayden and Ghosh.<sup>11</sup>

To generally understand the signaling leading to NF- $\kappa$ B activation, a small simplified pathway summary is given here. For details see the sections below.

First membrane receptor activation is mediated through numerous stimuli and leads to a downstream signal transduction (**Figure 5**). This signal transduction results in the activation of the IKK complex which phosphorylates I $\kappa$ B family members followed by ubiquitination and degradation of the latter. Hence the NF- $\kappa$ B dimer is set free and can translocate to the nucleus where it leads to target gene transcription. So far three specific pathways leading to NF- $\kappa$ B activation are known.<sup>12</sup>

### 3.6.1. The classical pathway with a focus on TNF-R1 signaling

The classical (canonical) pathway is the best studied pathway and it is known to be the pathway which is activated by the majority of stimuli.

Since the agent used to stimulate the NF- $\kappa$ B pathway in this work is the cytokine TNF- $\alpha$ , the canonical pathway will be described with a focus on TNF-R stimulation with TNF-R1 as a general representative.

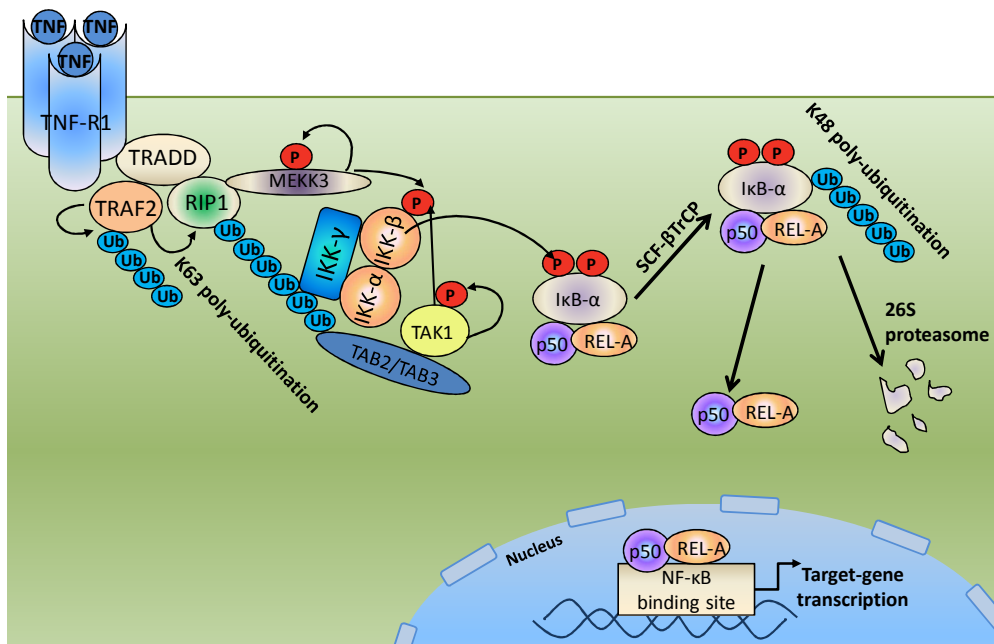


**Figure 6: Schematic illustration of the TNF-R1 stimulated pathway signaling to NF- $\kappa$ B.** For explanation and further abbreviations see section 3.6.1. P: phosphate; Ub: ubiquitin.

Adapted from Verstrepen L. et al.<sup>12</sup>

Given that TNF receptors do not have an intrinsic enzymatic activity they signal by recruiting intracellular adaptors. Binding of trimeric TNF leads to receptor aggregation and dissociation of the endogenous inhibitor of TNF receptor activity called silencer of death domain (SODD).<sup>11</sup>

The following described pathway is illustrated in **Figure 6** and is also presented in a shortened and simplified version in **Figure 5**.

The intracellular part of TNF-R1 contains a death domain (DD) which so far was blocked by SODD and now serves as a docking site through which the adaptor protein TNF-R1 associated death domain protein (TRADD), which also contains a DD, can interact throughout DD interactions. TNF receptor-associated factor 2 (TRAF2) and the serine/threonine kinase named receptor interacting protein 1 (RIP1)

are subsequently recruited by TRADD. TRAF2 then undergoes auto-poly-ubiquitination and afterwards poly-ubiquitinates RIP1 at Lys 377 using K63-linked poly-ubiquitin chains. This ubiquitination leads to the recruitment of transforming growth factor  $\beta$ -activated kinase 3 (TAB3) and TAB2. TAB2 then recruits TAK1.

The IKK complex is recruited by the poly-ubiquitination of RIP1 as well, and binds via NEMO to the K63 poly-ubiquitin chain of RIP1. The co-recruitment of the IKK complex and TAK1 enables TAK1 to phosphorylate IKK- $\beta$  which leads to the activation of the IKK complex.

In addition to those mentioned factors RIP1 also recruits the mitogen-activated protein kinase kinase kinase 3 (MEKK3) upon TNF- $\alpha$  stimulation. MEKK3 phosphorylates IKK- $\beta$  as well, hence leads to the activation of the IKK complex.

Activation of the IKK complex causes I $\kappa$ B- $\alpha$  phosphorylation by IKK- $\beta$  at the two serine residues Ser32 and Ser36. The SCF- $\beta$ TrCP molecule recognizes phosphorylated I $\kappa$ B- $\alpha$  via its  $\beta$ TrCP1 and  $\beta$ TrCP2 components while the SCF element binds Ubc4/5 which K48-poly-ubiquitinates I $\kappa$ B- $\alpha$  at Lys21 and Lys22.

This causes the recognition and degradation by the 26S proteasome which results in the release of the NF- $\kappa$ B dimer and its translocation to the nucleus where it can bind to the NF- $\kappa$ B binding sites and thus lead to the transcription of target genes as listed in **section 3.1**.<sup>12</sup>

### 3.6.2. The alternative pathway

The main known stimuli for this pathway are Lymphotoxin- $\beta$  (LT $\beta$ ) and B-cell activating factor (BAFF). In general receptor occupation leads to the activation of the NF- $\kappa$ B inducing kinase (NIK). Contrary to the classical pathway the alternative, also called non-canonical pathway, depends on IKK- $\alpha$  alone and does not require IKK- $\beta$  or IKK- $\gamma$ . Hence in this pathway the IKK complex consist only of an IKK- $\alpha$  homodimer. NIK seems to directly phosphorylate IKK- $\alpha$  and thereupon IKK- $\alpha$  phosphorylates p100, which dimerizes with RelB. Hence after phosphorylation and partial degradation of the C-terminal section of p100 the p52/RelB dimer is generated and subsequently translocates to the nucleus (**Figure 5**).<sup>9,15</sup>

### 3.6.3. The atypical pathway

There exists a third pathway which is the least examined so far. It has been documented that agents that cause lesions in DNA, like UV or doxorubicin, might lead to IKK-independent degradation of I $\kappa$ B, which then mostly phosphorylates the p50/p65 dimer. Since this pathway has been mainly described in response to DNA damage, it is hypothesized that the IKK-independent pathway might have developed to obtain NF- $\kappa$ B activity in DNA damaged cells.<sup>15</sup>

## 4. Firefly luciferase in reporter gene assays

### 4.1. Luciferase converts luciferin to oxyluciferin and light

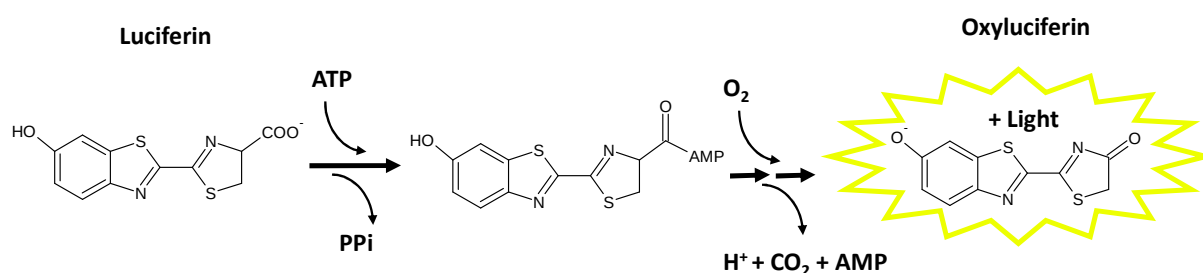


**Figure 7: Shortened chemical reaction of luciferin to oxyluciferin catalyzed by luciferase.** PPi: inorganic pyrophosphate; AMP: adenosine mono phosphate.

Adapted from Fan and Wood.<sup>16</sup>

The main assay used in this work utilizes luciferase from the firefly *Photinus pyralis*. It is an enzyme that does not need posttranslational modification to express activity and therefore is highly eligible for transfection, so as to use it in screening assays. In order that the enzymatic reaction takes place, ATP and luciferin are needed as substrates. The luciferase assembles luciferin with ATP to the enzyme-bound intermediate luciferyl-AMP and thereby releases pyrophosphate. Through some intermediated steps O<sub>2</sub> reacts with luciferyl-AMP thus creates oxyluciferin, which is a tautomer of its keto and enol form, in a high energy state. Oxyluciferin then returns to the ground state and thereby emits visible yellow-green light with a wavelength maximum of 562-570 nm (**Figure 7**). Luciferin can also be oxidized to oxyluciferin without enzymatic help. But this reaction takes place without luminescence.<sup>16,17</sup>

### 4.2. Firefly luciferase expressed in HEK-293/NFκB-luc cell line

In the assay that was mainly utilized in this work the HEK-293/NFκB-luc cell line was used, which is stably transfected with a luciferase reporter construct having 6 copies of the NF-κB response element (see also **D Methods section 1.1**). Cell stimulation with TNF-α leads to receptor binding and subsequent activation of the canonical pathway which ends in the activation of NF-κB, as described above (**section 3.6.1**).

Thereupon NF-κB molecules can bind to their response elements leading to the transcription of the luciferase gene. In case that inhibition of NF-κB activation is mediated somewhere in the signaling pathway by a test sample, no NF-κB molecules are set free from IκB binding and can therefore not bind to the target promoter sequences. Thus no luciferase transcription takes place and the substrate luciferin is not oxidized by the missing luciferase, hence no light emission can be measured.

## 5. EGFP in cellular assays

### 5.1. GFP from *Aequorea victoria*

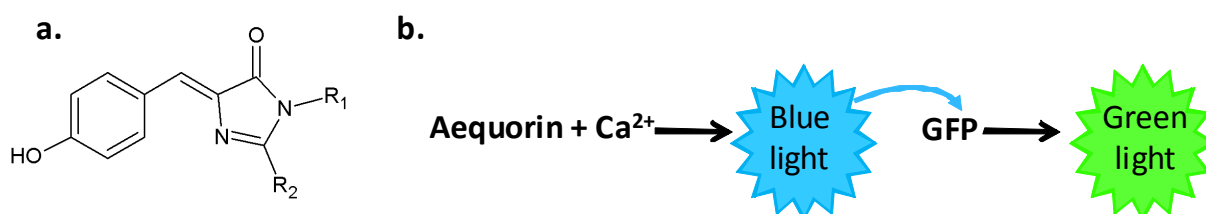


**Figure 8: Structure of the *Aequorea* GFP chromophore (a.) and schematic reaction of light transfer in *Aequorea* (b.).** Aequorin emits blue light upon calcium binding. The blue light is absorbed by GFP which thereupon emits green light. Adapted from Zimmer.<sup>18</sup>

The green fluorescent protein (GFP) was isolated from the jellyfish *Aequorea victoria*. This jellyfish contains two bioluminescent proteins which were both extracted and described one after another by Osamu Shimomura who, amongst others, won the Nobel Prize in 2008 for the discovery and development of the green fluorescent protein.

The first described protein was aequorin, while GFP was described in 1974<sup>19</sup> as the second one since it was harder to isolate. Upon calcium binding aequorin emits blue light ( $\lambda_{\text{max}} = 470 \text{ nm}$ ) which is absorbed by the GFP and thereupon it fluoresces green

( $\lambda_{\text{max}} = 509 \text{ nm}$ ). For this reason *Aequorea victoria* appears green although it contains the blue light emitting protein aequorin.<sup>18</sup>

## 5.2. EGFP as internal control in cell-based assays

The protein that was used in this work is a mutant version called enhanced GFP (EGFP). Wild-type GFP has two excitation peaks and shows a sub-optimal expression in mammalian cells as well as low brightness. EGFP is more adequate for being used in molecular assays since it is optimized for higher expression in mammalian cells, shows only one excitation peak, a six-fold increased brightness, and a four-fold increased rate of oxidation of chromophore.<sup>18</sup>

Since the tested plant extracts may as well contain toxic compounds or compounds with unspecific inhibitory action, no or low oxyluciferin luminescence could not only indicate inhibition of NF- $\kappa$ B activity, but can also be caused by these mentioned reasons or by low cell density in the well caused by poor cell distribution. To differentiate real active samples from ones with unspecific inhibitory action, and to normalize the luminescence to the cell number, the cells were additionally transfected with a pEGFP-N1 plasmid as internal control. Measured luminescence was normalized to fluorescence in order to account for unspecific inhibitory action of the test samples or differences in the cell number.



## C. Materials

## C. Materials

### 1. Samples

#### 1.1. Samples obtained from collaboration partners in Vienna

Phytochemical work was done by the group of Prof. Brigitte Kopp and Prof. Gottfried Reznicek at the Department of Pharmacognosy, University of Vienna.

##### 1.1.1. Plants derived from the VOLKSMED database.

| Plant name                   | Family           | Organ used |
|------------------------------|------------------|------------|
| <i>Betonica officinalis</i>  | Lamiaceae        | Herb       |
| <i>Dryopteris filix-mas</i>  | Aspidiaceae      | Herb       |
| <i>Glechoma hederacea</i>    | Lamiaceae        | Herb       |
| <i>Malva</i> sp.             | Malvaceae        | Leaves     |
| <i>Melampyrum</i> sp.        | Scrophulariaceae | Herb       |
| <i>Peucedanum ostruthium</i> | Apiaceae         | Rhizome    |

**Table 1: List of plants from Vienna which are derived from the VOLKSMED database.**

##### 1.1.1.1. *Betonica officinalis*

*Betonica officinalis* herb was collected at Neustift am Walde. A dichloromethane (DCM) extract was obtained (sample code 1091). Fraction 1092 was gained through solid phase extraction (SPE) with 30% methanol (MeOH).

##### 1.1.1.2. *Dryopteris filix-mas*

The investigation of this plant was not part of the NFN project.

All fern samples were collected at the same location (Ebenwaldhöhe, Niederösterreich, near Kleinzell) in the same period (October) in different years. The plants used to obtain extract 997 were collected in 2006, extract 998 in 2007, and 999 as well as 1000 in 2009. The only difference between the latter two was that the plants used to obtain sample 999 like those for samples 997 and 998 were dried, while plants for sample 1000 were freshly frozen. Out of these four collections chlorophyll-depleted DCM extracts were made. Another DCM extract from the dried samples collected in 2009 was made but without chlorophyll separation (1001).

Out of each DCM extract a SPE with decreasing MeOH concentrations was made (100%; 70%; 30%).

|                                                |                    |                    |
|------------------------------------------------|--------------------|--------------------|
| 997: DCM 2006, dried, chlorophyll removed      |                    |                    |
| 1010: SPE 100% MeOH                            | 1011: SPE 70% MeOH | 1012: SPE 30% MeOH |
| 998: DCM 2007, dried, chlorophyll removed      |                    |                    |
| 1013: SPE 100% MeOH                            | 1014: SPE 70% MeOH | 1015: SPE 30% MeOH |
| 999: DCM 2009, dried, chlorophyll removed      |                    |                    |
| 1016: SPE 100% MeOH                            | 1017: SPE 70% MeOH | 1018: SPE 30% MeOH |
| 1000: DCM 2009, frozen, chlorophyll removed    |                    |                    |
| 1019: SPE 100% MeOH                            | 1020: SPE 70% MeOH | 1021: SPE 30% MeOH |
| 1001: DCM 2009, dried, chlorophyll not removed |                    |                    |

**Table 2: Fractions from *Dryopteris filix-mas*. Three subfractions were obtained from each DCM extract except from the one with sample code 1001.**

#### 1.1.1.3. *Glechoma hederacea*

*Glechoma hederacea* herb was used for extraction. It was provided by Kottas Pharma GmbH. Four initial MeOH extracts were generated. Extract 168 and 713-715 were depleted from polyphenols using different separating methods which will not be described in detail here, whereas extract 25 was not depleted from polyphenols.

Out of extract 168 a SPE with 100% MeOH (786) and with 70% MeOH (785) was made. These two fractions were further divided using a preparative high performance liquid chromatography (HPLC) thereby collecting subfractions 1057-1064 out of fraction 786 and subfractions 1104-1110 out of fraction 785 (**Figure 9**).

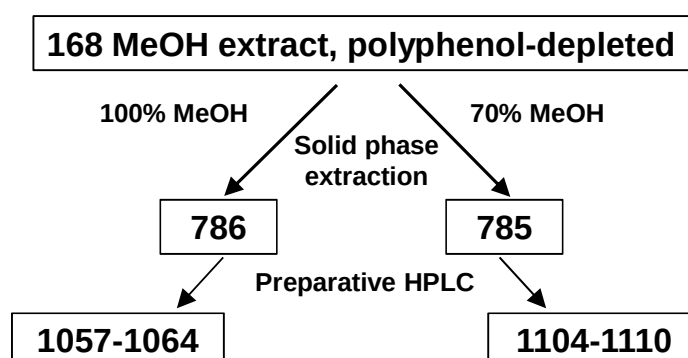


**Figure 9: Overview over the fractionation scheme of the *Glechoma hederacea* MeOH extract 168.**

1.1.1.4. *Malva* sp.

*Malva* sp. (Ph.Eur.) leaves were provided by Kottas Pharma GmbH. A crude DCM extract with the sample code 28 was made out of these leaves. Three further DCM extracts 716-718 were made but additionally depleted from chlorophyll using different methods which will not be described in detail here.

1.1.1.5. *Melampyrum* sp.

*Melampyrum* sp. was collected at Neustift am Walde. Two extracts were made out of the herb (**Figure 10**). Extract 277 was extracted with DCM and chlorophyll was removed. 279 was extracted with MeOH and depleted from tannins.

Extract 277 was further fractionated using SPE. The column was first eluted with 30% MeOH giving fraction 840, second with 70% MeOH making fraction 841, and the last fraction 842 was eluted with 100% MeOH. These three fractions were not tested in this work. But pure compounds (1329-1330) were gained out of sample 840 using silica gel column chromatography (SGCC) with EtOAc:MeOH:H<sub>2</sub>O (17:3:3) as solvent.

Sample 841 was fractionated using the same method but CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (9:1:1) as solvent. Again two pure substances were gained (1331; 1332) and 4 subfractions (1333-1336) which contained at least 3 substances each.

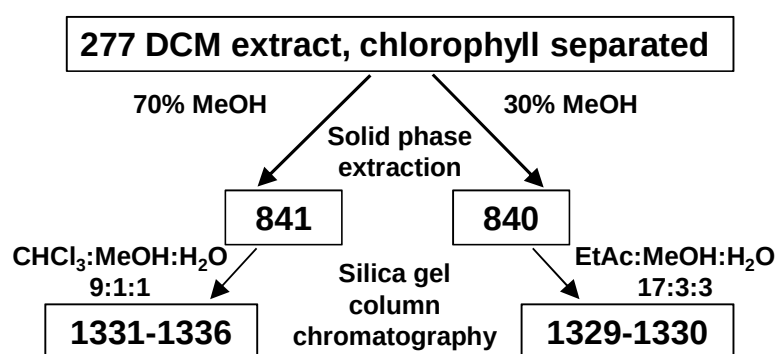


**Figure 10: Overview over the fractionation scheme of the *Melampyrum* sp. DCM extract 277.**

1.1.1.6. *Peucedanum ostruthium*

In order to obtain a crude extract, *Peucedanum ostruthium* rhizomes were extracted with DCM (sample code 31). Another extract was gained through extraction with MeOH and following removal of tannins. Out of this tannin-depleted MeOH extract three SPE fractions were generated utilizing 30%, 70%, and 100% MeOH as solvent. Fraction 797 gained by eluting with 70% MeOH was further fractionated with

preparative HPLC using a phthalate free solvent. Out of this fractionation the tested subfractions 1307-1317 were gained.

#### 1.1.2. Plants with use in Traditional Chinese Medicine (TCM)

TCM plants were provided by Plantasia GmbH.

| Plant name                 | Drug name | Family   | Organ used |
|----------------------------|-----------|----------|------------|
| <i>Albizia julibrissin</i> | Hehuanpi  | Fabaceae | Bark       |
| <i>Pinellia</i> sp.        | Banxia    | Araceae  | Rhizome    |

**Table 3: Listing of plants from Vienna which are used in TCM.**

##### 1.1.2.1. *Albizia julibrissin*

A crude DCM extract was made out of the bark. Out of this extract the subfractions 1156 and 1157 were generated through several column chromatographic (CC) steps. Fractions 1361-1373 were gained from a tannin-depleted MeOH extract.

##### 1.1.2.2. *Pinellia* sp.

Four extracts were made out of *Pinellia* sp. herb. A DCM extract and a MeOH extract were both generated from raw herb material gaining samples 993 and 994, respectively. Other extracts were made out of the processed drug according to TCM processing. Sample 995 was gained with DCM and sample 996 with MeOH extraction.

## 1.2. Samples obtained from collaboration partners in Graz (TCM plants)

The phytochemical work related to these samples was done by the group of Prof. Rudolf Bauer at the Department of Pharmacognosy, University of Graz.

| Plant name                   | Drug name   | Family         | Organ used         |
|------------------------------|-------------|----------------|--------------------|
| <i>Clematis armandii</i>     | Chuanmutong | Ranunculaceae  | Caulis             |
| <i>Lonicera japonica</i>     | Rendongteng | Caprifoliaceae | Caulis             |
| <i>Notopterygium incisum</i> | Qianghuo    | Apiaceae       | Underground organs |
| <i>Sophora flavescens</i>    | Kushen      | Fabaceae       | Root               |
| <i>Thlaspi arvense</i>       | Baijiangcao | Brassicaceae   | Herb               |
| <i>Typhonium giganteum</i>   | Baifuzi     | Araceae        | Rhizome            |

**Table 4: Listing of plants from Graz with use in TCM.**

### 1.2.1. *Clematis armandii*

Oleanolic acid found in the caulis of *Clematis armandii* was tested in this work.

### 1.2.2. *Lonicera japonica*

*Lonicera japonica* caulis was extracted with DCM gaining sample 1209. This extract was fractionated with a liquid-liquid partitioning using MeOH and n-hexan, generating fraction 1210 out of the MeOH phase. Sample 1210 was further partitioned on a sephadex column whereby subfractions 1345-1349 were gained.

### 1.2.3. *Notopterygium* sp.

*Notopterygium* sp. underground parts were extracted with DCM. This extract was further fractionated yielding the three samples 970-972. Out of fraction 970 the two subfractions 975 and 976 were generated.

Another crude DCM extract out of *Notopterygium incisum* underground parts (1243) was made. This extract was then partitioned into 10 different fractions 1244-1253.

### 1.2.4. *Sophora flavescens*

11 flavonoids and the chromanocoumaran maackiain which are known to be ingredients of *Sophora flavescens* root<sup>20-22</sup> were purified by the group of Prof. Bauer and tested in this work.

### 1.2.5. *Thlaspi arvense*

*Thlaspi arvense* herb was extracted with MeOH gaining sample 984. This extract was fractionated into samples 985-991.

### 1.2.6. *Typhonium giganteum*

The crude DCM extract 1254 was gained from *Typhonium giganteum* rhizomes.

## 1.3. Samples obtained from collaboration partners in Innsbruck

### 1.3.1. Predicted p50 binders

A virtual screening of the Specs database to identify substances predicted to interfere with the interfacial NF- $\kappa$ B/DNA binding was performed by the research group of Dr. Daniela Schuster at the Institute of Pharmacy, University of Innsbruck. 25 substances were predicted and tested in this work.

### 1.3.2. Ethnopharmacological approach

Phytochemical work as well as isolation and identification of pure compounds were done by the research group of Prof. Judith Rollinger and Prof. Hermann Stuppner at the Institute of Pharmacy, University of Innsbruck.

| Plant name                    | Family      | Organ used |
|-------------------------------|-------------|------------|
| <i>Garcinia nigrolineata</i>  | Guttiferae  | Leaves     |
| <i>Himatanthus sucuuba</i>    | Apocynaceae | Bark       |
| <i>Morus alba</i>             | Moraceae    | Root bark  |
| <i>Sideritis hyssopifolia</i> | Lamiaceae   | Herb       |

**Table 5: Listing of plants from Innsbruck found by an ethnopharmacological approach.**

### 1.3.3. *Garcinia nigrolineata*

An ethanol (EtOH) extract out of the leaves was gained. In a further step a liquid-liquid partitioning was done gaining sample 1093 from the ethyl acetate (EtOAc) phase.

### 1.3.4. *Himatanthus sucuuba*

*Himatanthus sucuuba* bark was extracted with MeOH delivering the crude extract 642. Out of this extract fractions were generated by extracting it with EtOAc (643) and n-butanol (644). After several rounds of fractionation five pure compounds have been isolated out of the bark of *Himatanthus sucuuba*: biochanin A, dihydrobiochanin A, plumericin, plumieridin, and one new substance.

### 1.3.5. *Morus alba*

The root was extracted with MeOH gaining samples 310 and 311.

### 1.3.6. *Sideritis hyssopifolia*

A crude MeOH extract was made out of the herb. After liquid-liquid partitioning subfractions were obtained, and from one of the active subfractions a diterpene isomer mixture was gained (sample code 1194).

## 2. Cell culture materials

Freshly made reagents and solutions were sterilized or sterile filtrated before use. After opening they were stored at 8°C and used not longer than 4 weeks. All cell culture reagents were provided by Lonza Group Ltd. (Basel, Switzerland) unless otherwise stated.

| Name                           | Components (provider)                           | Amount or concentration |
|--------------------------------|-------------------------------------------------|-------------------------|
| <b>Cell culture media</b>      |                                                 |                         |
| <i>HEK-293/NF-κB-luc media</i> |                                                 |                         |
| Growth medium                  | L-glutamine                                     | 2 mM                    |
|                                | Hygromycin B                                    | 100 µg/ml               |
|                                | (Hygrogold™ InvivoGen)                          |                         |
|                                | Penicillin potassium salt                       | 100 U/ml                |
|                                | Streptomycin sulfate                            | 100 µg/ml               |
|                                | Fetal bovine serum                              | 10%                     |
|                                | In DMEM                                         | 500 ml                  |
| Seeding medium                 | Same as growth medium but without hygromycin B. |                         |
| Experimental medium            | L-glutamine                                     | 2 mM                    |
|                                | Penicillin potassium salt                       | 100 U/ml                |
|                                | Streptomycin sulfate                            | 100 µg/ml               |
|                                | Fetal bovine serum                              | 0.1%                    |
|                                | In DMEM                                         | 500 ml                  |
| <i>HUVECtert medium</i>        |                                                 |                         |
| Growth medium                  | Hydrocortisone                                  | 4-15 pg/ml              |
|                                | Recombinant human epidermal growth factor       | 0.5 - 4 ng/ml           |
|                                | Amphotericin B                                  | 50 pg/ml                |
|                                | Benzylpenicillin                                | 100 U/ml                |
|                                | Gentamicin                                      | 50 ng/ml                |
|                                | Penicillin potassium salt                       | 100 U/ml                |
|                                | Streptomycin sulfate                            | 100 µg/ml               |
|                                | Bovine brain extract                            | 0.4%                    |
|                                | Fetal bovine serum superior (Biochrom)          | 10%                     |
|                                | In EBM                                          | 500 ml                  |

| Cell culture buffers                           |                                           |             |
|------------------------------------------------|-------------------------------------------|-------------|
| PBS pH 7.4                                     | NaCl                                      | 36.0 g      |
|                                                | Na <sub>2</sub> HPO <sub>4</sub>          | 7.4 g       |
|                                                | KH <sub>2</sub> PO <sub>4</sub>           | 2.15 g      |
|                                                | In ddH <sub>2</sub> O                     | 5000 ml     |
| NaH <sub>2</sub> PO <sub>4</sub> stock for HBS | NaH <sub>2</sub> PO <sub>4</sub>          | 5.25 g      |
|                                                | In ddH <sub>2</sub> O                     | 500 ml      |
| 2 x HBS pH 7.0                                 | NaCl                                      | 8.0 g       |
|                                                | NaH <sub>2</sub> PO <sub>4</sub> stock    | 10 ml       |
|                                                | HEPES (Fluka)                             | 6.5 g       |
|                                                | Adjust pH to 7.0 with diluted NaOH or HCl |             |
| Cell culture reagents and solutions            |                                           |             |
| Trypsin/EDTA in PBS                            | Trypsin (Invitrogen)                      | 0.05%       |
|                                                | EDTA                                      | 0.02%       |
|                                                | in PBS                                    |             |
| Gelatin solution 0.1%                          | Gelatin (Fluka)                           | 1 g         |
|                                                | ddH <sub>2</sub> O                        | Ad. 1000 ml |
| Poly-D-lysine stock                            | Poly-D-Lysine (Sigma)                     | 50 mg       |
|                                                | ddH <sub>2</sub> O                        | 50 ml       |
| Prior to use                                   | Poly-D-lysine stock                       | 250 µl      |
|                                                | PBS                                       | 10 ml       |
| Transfection solution                          | 2 x HBS                                   | 720 µl      |
|                                                | 2 M CaCl <sub>2</sub>                     | 94 µl       |
|                                                | pEGFP-N1 plasmid (Clontech)               | 4 µg        |
|                                                | ddH <sub>2</sub> O                        | Ad. 1500 µl |
| Miscellaneous                                  |                                           |             |
| Positive control                               | Parthenolide (Alexis)                     |             |
| Stimulating agent                              | Recombinant human TNF-α (Sigma)           |             |

**Table 6: Cell culture media, buffers, and reagents.**

### 3. Luciferase reporter gene assay

| Name                         | Components (provider)                     | Amount or concentration |
|------------------------------|-------------------------------------------|-------------------------|
| <b>Buffers and solutions</b> |                                           |                         |
| Luciferin solution           | D-Luciferin sodium salt (Synchem)         | 25 mg                   |
|                              | ddH <sub>2</sub> O                        | 8.27 ml                 |
| Luciferin buffer             | Luciferin solution                        | 1.1 ml                  |
|                              | 1 M Tricine buffer pH7.8                  | 220 µl                  |
|                              | ddH <sub>2</sub> O                        | 9.2 ml                  |
| 0.1 M ATP-solution pH 7.0    | ATP disodium salt (Sigma)                 | 1 g                     |
|                              | 1 N NaOH                                  | 3.3 ml                  |
|                              | ddH <sub>2</sub> O                        | 13.4 ml                 |
| ATP-Tricine-buffer           | 1 M Tricine pH 7.8                        | 220 µl                  |
|                              | 0.5 M MgCl <sub>2</sub>                   | 473 µl                  |
|                              | 0.1 M ATP-solution pH 7.0                 | 407 µl                  |
|                              | ddH <sub>2</sub> O                        | 9.9 ml                  |
| Lysis buffer (for one plate) | 5 x Reporter lysis buffer (Promega )      | 1.2 ml                  |
|                              | 270 mM Coenzyme A trilithium salt (Sigma) | 6 µl                    |
|                              | 1 mM DTT (Fluka)                          | 6 µl                    |
|                              | ddH <sub>2</sub> O                        | 4.8 ml                  |

*Table 7: Luciferase reporter gene assay buffers and solutions.*

### 4. Protein extraction and quantification

| Name                              | Components (provider)                                              | Amount or concentration |
|-----------------------------------|--------------------------------------------------------------------|-------------------------|
| <b>Protein extraction buffers</b> |                                                                    |                         |
| Lysis buffer (for 6 wells)        |                                                                    |                         |
| Stock solution pH 7.5             | HEPES                                                              | 50 mM                   |
|                                   | NaCl                                                               | 50 mM                   |
|                                   | NaF                                                                | 50 mM                   |
|                                   | Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub> x 10H <sub>2</sub> O | 10 mM                   |
|                                   | EDTA                                                               | 5 mM                    |
|                                   | Na <sub>3</sub> VO <sub>4</sub>                                    | 1 mM                    |
|                                   | In ddH <sub>2</sub> O                                              |                         |

|                               |                                                                        |         |
|-------------------------------|------------------------------------------------------------------------|---------|
| Prior to use                  | Stock solution pH 7.5                                                  | 645 µl  |
|                               | Complete® (Roche Diagnostics) tablet solved in 2 ml ddH <sub>2</sub> O | 30.3 µl |
|                               | 0.1 M PMSF (in isopropanol)                                            | 7.5 µl  |
|                               | Triton® X-100 (10% in ddH <sub>2</sub> O)                              | 75 µl   |
| <b>Protein quantification</b> | Roti®-Quant (Carl Roth)                                                |         |

**Table 8: Protein extraction and quantification.**

## 5. SDS- PAGE

| Name                       | Components (provider)                   | Amount or concentration |
|----------------------------|-----------------------------------------|-------------------------|
| <b>Buffers</b>             |                                         |                         |
| 3 x SDS sample buffer      | Bromophenol blue                        | 15.0 mg                 |
|                            | Glycerol                                | 30.0 ml                 |
|                            | SDS                                     | 6.0 g                   |
|                            | 0.5 M Tris-HCl (Fluka) solution, pH 6.8 | 37.5 ml                 |
|                            | ddH <sub>2</sub> O                      | Ad. 100 ml              |
| Prior to use               | 3 x SDS sample buffer                   | 85%                     |
|                            | β-Mercaptoethanol                       | 15%                     |
| Electrophoresis buffer 5 x | Glycine                                 | 72.0 g                  |
|                            | Tris-base (Carl Roth)                   | 15.0 g                  |
|                            | SDS                                     | 5.0 g                   |
|                            | ddH <sub>2</sub> O                      | Ad. 1000 ml             |
| <b>SDS-PAGE gels</b>       |                                         |                         |
| Resolving gel 10%          | ABA solution 30%                        | 2.5 ml                  |
|                            | SDS 10%                                 | 75 µl                   |
|                            | 1.5 M Tris-HCl (Fluka) pH 8.8           | 1.875 ml                |
|                            | ddH <sub>2</sub> O                      | 3.05 ml                 |
| Prior to use               | APS 10%                                 | 37.5 µl                 |
|                            | TEMED (Fluka)                           | 7.5 µl                  |

## C. Materials

|                         |                                            |         |
|-------------------------|--------------------------------------------|---------|
| Stacking gel            | ABA solution 30%                           | 640 µl  |
|                         | SDS 10%                                    | 37.5 µl |
|                         | 1.25 M Tris-HCl (Fluka)                    | 375 µl  |
|                         | pH 6.8                                     |         |
|                         | ddH <sub>2</sub> O                         | 2.62 ml |
| Prior to use            | APS 10%                                    | 37.5 µl |
|                         | TEMED (Fluka)                              | 7.5 µl  |
| Protein standard ladder | Precision Plus Protein™ Standard (BIO-RAD) |         |

**Table 9: SDS-PAGE buffers, gels, and protein standard.**

## 6. Western blotting and protein detection

| Name                         | Components (provider)             | Amount or concentration |
|------------------------------|-----------------------------------|-------------------------|
| <b>Buffers and solutions</b> |                                   |                         |
| Blotting buffer 5 x          | Glycine                           | 72.9 g                  |
|                              | Tris-base (Carl Roth)             | 15.17 g                 |
|                              | ddH <sub>2</sub> O                | Ad. 1000 ml             |
| Dilution prior to use        | Blotting buffer 5 x               | 100 ml                  |
|                              | Methanol                          | 100 ml                  |
|                              | ddH <sub>2</sub> O                | 300 ml                  |
| TBS-T 10 x pH 8.0            | NaCl                              | 11.1 g                  |
|                              | Tris-base (Carl Roth)             | 3.0 g                   |
|                              | Tween-20 (Sigma)                  | 1 ml                    |
|                              | ddH <sub>2</sub> O                | Ad. 1000 ml             |
| Dilution prior to use        | TBS-T 10 x pH 8.0                 | 100 ml                  |
|                              | ddH <sub>2</sub> O                | 900 ml                  |
| ECL- homemade                | 1 M Tris-base (Carl Roth)         | 500 µl                  |
|                              | pH 8.5                            |                         |
|                              | Luminol stock                     | 25 µl                   |
|                              | p-coumaric acid stock             | 11 µl                   |
|                              | ddH <sub>2</sub> O                | 4.5 ml                  |
|                              |                                   |                         |
| Prior to use                 | H <sub>2</sub> O <sub>2</sub> 30% | 3 µl                    |
| Luminol stock                | Luminol (Sigma)                   | 0.44 g                  |
|                              | DMSO (Fluka)                      | 10.0 ml                 |
| Coumaric acid stock          | p-coumaric acid (Sigma)           | 0.15 g                  |
|                              | DMSO (Fluka)                      | 10.0 ml                 |

|                   |                                             |             |
|-------------------|---------------------------------------------|-------------|
| APS 10%           | APS                                         | 1.0 g       |
|                   | ddH <sub>2</sub> O                          | Ad. 10.0 ml |
| Stripping agent   | NaOH                                        | 0.5 M       |
| Blotting membrane | Immuno-blot™ PVDF membrane 0.2 µm (BIO-RAD) |             |

**Table 10: Western blotting buffers and solutions.**

## 7. Antibodies

Anti-rabbit secondary antibody was diluted in 5% milk powder (Carl Roth) in TBS-T. All other antibodies were diluted in 5% BSA (Carl Roth) in TBS-T.

| Target                      | Dilution | Source | Provider                  |
|-----------------------------|----------|--------|---------------------------|
| <b>Primary antibodies</b>   |          |        |                           |
| IκB-α                       | 1:1000   | Rabbit | Cell Signaling Technology |
| α-tubulin                   | 1:500    | Mouse  | Santa Cruz                |
| <b>Secondary antibodies</b> |          |        |                           |
| HRP-linked anti-mouse IgG   | 1.6:1000 | Goat   | MP Biomedicals            |
| HRP-linked anti-rabbit IgG  | 1:2500   | Goat   | Cell Signaling Technology |

**Table 11: Antibodies.**

## 8. Technical equipment and scientific software

| <b>Technical equipment</b>               |                                         |
|------------------------------------------|-----------------------------------------|
| Heraeus® Multifuge® 1S-R Centrifuge      | Thermo Fisher Scientific Inc. (CA, USA) |
| Heraeus® Biofuge® fresco Microcentrifuge | Thermo Fisher Scientific Inc. (CA, USA) |
| HERAcell® 150 Incubator                  | Thermo Fisher Scientific Inc. (CA, USA) |
| HERAsafe® Laminar air flow workbench     | Thermo Fisher Scientific Inc. (CA, USA) |
| LAS-3000™ Luminescent Image Analyzer     | Fujifilm (Tokyo, JP)                    |
| Olympus BX51 Fluorescence Microscope     | Olympus Europa GmbH (Hamburg, DE)       |
| Olympus CKX31 Light Microspoe            | Olympus Europa GmbH (Hamburg, DE)       |
| PowerPac™ HC Power supply                | BIO-RAD Laboratoris (CA,USA)            |
| Tecan GENios™Pro, Tecan Sunrise™         | Tecan (Mannedorf, CH)                   |
| Vi-Cell™ XR Cell Viability Analyzer      | Beckman Coulter (CA, USA)               |
| <b>Scientific software</b>               |                                         |
| AIDA™ version 4.06                       | Raytest GmBH (Straubenhardt, DE)        |
| GraphPad PRISM™ version 5.01             | GraphPad Software, Inc. (CA, USA)       |
| Vi-Cell™ XR 2.03                         | Beckman Coulter (CA,USA)                |
| XFLUOR4 version 4.51                     | Tecan (Männedorf, Switzerland)          |
| XFLUOR4GENIOSPRO version 4.63            | Tecan (Männedorf, Switzerland)          |

**Table 12: Technical equipment and scientific software.**

## D. Methods

## D. Methods

### 1. Cell culture

In order not to stress the cells, all media and reagents were warmed for half an hour in a 37°C warm water bath before being used. Cell culture work was always done with sterile materials under sterile conditions in a HERAsafe® Laminar air flow workbench.

#### 1.1. HEK-293/NF-κB-luc cells

For NF-κB-dependent luciferase reporter gene assay the human embryonic kidney (HEK)-293/NFκB-luc cell line was used (Panomics Catalog# RC0014). This cell line is stable transfected with pNFκB-luc, a luciferase reporter construct regulated by 6 copies of the NF-κB response element and bearing a hygromycin resistance gene.

##### 1.1.1. Cultivation

HEK-293/NF-κB-luc cells were cultivated in HEK-293/NF-κB-luc growth medium (**Table 1**) in a 175 cm<sup>2</sup> flask in a humidified incubator at 37°C, 5% CO<sub>2</sub>. The cells were grown to near confluence and passaged twice a week or when needed for an experiment. Therefore the growth medium was discarded and the cell surface was washed with 10 ml PBS. Subsequently the cells were treated with 2 ml trypsin/EDTA for 2-3 min to detach them from the flask surface. Too long exposure was avoided in order not to stress the cells. Trypsin activity was stopped with 8 ml growth medium. Cell number and viability were determined using ViCell® cell viability analyzer, and 18 x 10<sup>6</sup> cells were seeded into a new 175 cm<sup>2</sup> flask containing 15-20 ml growth medium.

##### 1.1.2. Seeding for transfection

Cells were washed with 10 ml PBS and treated with 2 ml trypsin/EDTA for 2-3 min to detach them from the flask surface. After treatment with trypsin the cell suspension was transferred into a Falcon tube and centrifuged (1400 g, 20°C). The supernatant was discarded. The cell pellet was suspended by pipetting up and down several times in seeding medium (**Table 6**) to break cell clumps. Cells were counted via ViCell® and 8 x 10<sup>6</sup> cells were seeded into a 10 cm dish containing 10 ml seeding

medium and  $2 \times 10^6$  cells into a 6 cm dish containing 4 ml seeding medium. The cells were incubated over night at 37°C and 5% CO<sub>2</sub> before transfection.

#### 1.1.3. Transfection

Before further handling, all cells were transfected with 4 µg enhanced green fluorescent protein plasmid (pEGFP-N1) which was utilized as internal control. Cells were checked under the microscope next morning. The technique used to transfect the DNA into the cells was the calcium phosphate precipitation method.<sup>23</sup> Therefore 4 µg DNA was diluted in 750 µl 2 x HBS, 94 µl 2 M CaCl<sub>2</sub> solution and the mixture was filled up with ddH<sub>2</sub>O to a total volume of 1500 µl. The components were added in the following order into a 2 ml Eppendorf tube: 1) ddH<sub>2</sub>O; 2) DNA; 3) 2 x HBS; 4) CaCl<sub>2</sub> solution. The mixture was vortexed shortly and left for 20 min at room temperature to form a DNA complex. Next the solution was dropped equally onto the medium surface in the 10 cm dish grown with cells. To ensure the equal distribution the dish was shaken carefully. The cells growing in the 6 cm dish were left untransfected to be used as a background control later on. The plates were incubated for 6 h.

#### 1.1.4. Seeding for an experiment

After 6 h the transfection process was stopped by aspirating the medium and washing the cells with 10 ml PBS. PBS was removed and those cells in the 10 cm dish were treated with 2 ml trypsin/EDTA and those in the 6 cm dish with 1 ml trypsin/EDTA. After 2-3 min trypsin activity was stopped with 8 ml seeding medium for the bigger dish and 4 ml for the smaller one. The cell suspensions were transferred into Falcon tubes and centrifuged for 4 min (1400 g, 20°C). After centrifugation the supernatant was discarded and the cell pellet was suspended in experimental medium (**Table 6**) containing 0.1% serum. 500 µl cell suspension was transferred to the ViCell<sup>®</sup> counter.  $2 \times 10^4$  cells diluted in 100 µl seeding medium were seeded per well into a coated (see **1.1.5**) and transparent 96-well plate. 92 wells were seeded with transfected and 4 wells with untransfected cells, as shown in **Figure 11**. The plate was left in the incubator over night.

#### 1.1.5. Poly-D-lysine coating

To ensure that HEK-293/NF-κB-luc cells will not detach from the well surface during the experiment, the 96-well plate had to be coated with poly-D-lysine before seeding. For the coating of two plates 250 µl poly-D-lysine stock solution was diluted in 10 ml

PBS. 50  $\mu$ l diluted solution were applied per well and incubated for 10 min. Afterwards the solution was aspirated and the wells were washed 2 times with 100  $\mu$ l PBS per well. PBS was aspirated and the plate was used immediately or stored sterile at room temperature for the next experiment.

### 1.2. HUVECtert

For protein analysis experiments immortalized human umbilical vein endothelial cells (HUVEC) were used. HUVEC were immortalized by insertion of a human telomerase reverse transcriptase (hTERT)-cDNA.<sup>24</sup> To confirm the stability of HUVECtert compared to primary HUVEC those cells were tested on their ability to induce E-selectin, CD31, CD105, CD109, and von Willebrand factor, as well as on their ability to form vessel-like structures. The induction and the structures formation of HUVECtert were shown to be identical to that of low-passage primary HUVEC.<sup>24</sup>

#### 1.2.1. Cultivation

HUVECtert cell line was cultivated in HUVECtert growth medium (**Table 6**) in 10 cm dishes coated with gelatin. The cells were processed the same way as described above for HEK-293/NF- $\kappa$ B-luc cells but HUVECtert medium was used instead. Previous to an experiment  $2 \times 10^5$  cells diluted in 2 ml were seeded into each well of a 6-well plate and grown to near confluence.

#### 1.2.2. Gelatin coating

A 6-well plate was filled with 1 ml 0.1% gelatin solution per well. The plate was incubated for 10 min. The gelatin solution was aspirated and the plate was used directly. No washing step was done.

## 2. Luciferase reporter gene assay

Luciferase reporter gene assay experiments were done with HEK-293/NF- $\kappa$ B-luc cells. All solutions and media used on cells were sterile and warmed for half an hour in a 37°C warm water bath before treatment.

### 2.1. Sample dilution

After being seeded into a 96-well plate, cells were checked under the fluorescent microscope next morning to see if the transfection was efficient. Cells were treated

with different samples to find out their effect on NF- $\kappa$ B activation. Sample dilutions were always made freshly right before the experiment.

Sample stock solutions were stored in DMSO at -20°C. An aliquot was unfrozen and diluted in experimental medium to a four-fold higher concentration than the required final concentration in the well. If it was needed DMSO was added in order to always get a final concentration of 0.1% DMSO per well.

Parthenolide was used as a positive control in a final concentration of 5  $\mu$ M in experimental medium. This compound is a sesquiterpenelactone occurring in the aerial parts of *Tanacetum parthenium*<sup>2</sup>, and is a well known inhibitor of the NF- $\kappa$ B pathway.<sup>25</sup> Parthenolide mediates the inhibition of NF- $\kappa$ B DNA binding by inhibiting the phosphorylation of I $\kappa$ B- $\alpha$  and therefore its degradation.

A 0.1% DMSO control solution in experimental medium was prepared to serve as a negative control.

## 2.2. Cell treatment

The different wells of each 96-well plate were treated as shown in **Figure 11**. Cells were treated with 50  $\mu$ l sample dilution, parthenolide, or 0.1% DMSO dilution and left for half an hour pretreatment in the incubator. Untransfected cells were not treated and 4 wells with untreated but transfected cells were left as well. Treatment was always done in quadruplicate. After pretreatment the cells were stimulated with 50  $\mu$ l experimental medium containing TNF- $\alpha$  diluted to get a final concentration of 2 ng/ml. Untransfected cells and those 4 wells containing untreated cells for reference were not treated with TNF- $\alpha$ . Just additional 100  $\mu$ l experimental medium was added to those wells to ensure the same volume as the rest of the wells. The plate was incubated for 4 h. Shortly before stopping the treatment, cells were checked under the microscope to see if the treatment has affected the phenotype of the cells. Then the stimulation was stopped by aspirating the medium and the plate was immediately frozen at -80°C or measured instantly.

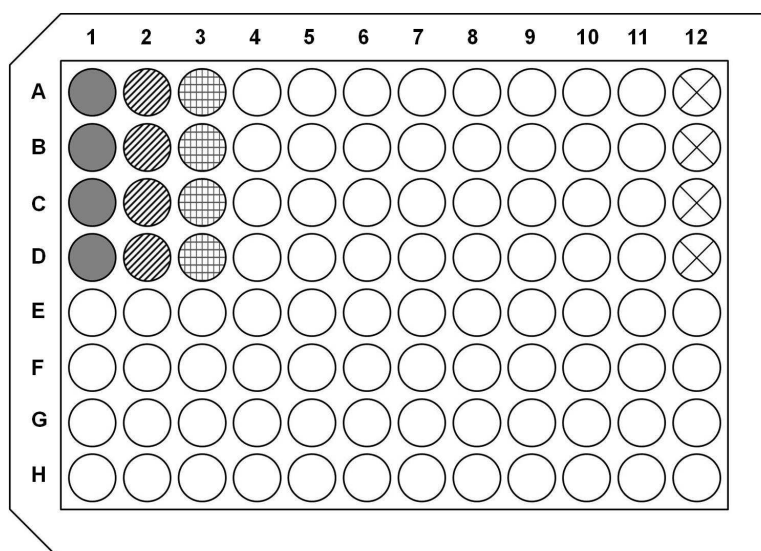


**Figure 11: The arrangement of different treatments for NF- $\kappa$ B luciferase reporter gene assay done in a 96-well plate.** Each sample and control was tested in quadruplicate. Crossed wells (A12 – D12) contained untransfected, untreated, and unstimulated cells for background correction. Grey group (A1 – D1): transfected but untreated and unstimulated cells. Hatched wells (A2 – D2): parthenolide control. Checkered wells (A3 – D3) contained stimulated cells treated only with 0.1% DMSO and were used as negative control for a maximal NF- $\kappa$ B activation. All other wells were used for the testing of 20 different samples in quadruplicate.

### 2.3. Cell lysates and measurement

The plate was taken out of the freezer and the cells were lysed immediately with 50  $\mu$ l luciferase lysis buffer. The plate was wrapped in aluminum foil to protect it from light and left for 10 min on a shaker. Afterwards 40  $\mu$ l cell lysate was transferred to a black 96-well plate. Measurement was done with Tecan GENios™Pro plate reader using auto injection for tricine-ATP-buffer and luciferin buffer (**Table 13** and **Table 14**). The luminescence signals obtained from each well were normalized to the EGFP-derived fluorescence to account for differences in the cell number.

| Measurement mode            | Fluorescence |
|-----------------------------|--------------|
| Excitation wavelength       | 485 nm       |
| Emission wavelength         | 520 nm       |
| Gain                        | Optimal      |
| Number of reads             | 1            |
| Integration time            | 1000 $\mu$ s |
| Lag time                    | 0 $\mu$ s    |
| Mirror selection            | Dichroic 3   |
| Time between move and flash | 40 ms        |

**Table 13: Parameters for fluorescence measurement using Tecan GENios™ Pro.**

| Measurement mode                  | Luminescence  |
|-----------------------------------|---------------|
| Integration time                  | 2000 ms       |
| Attenuation                       | None          |
| Time between move and integration | 50 ms         |
| Well kinetic number               | 1             |
| Well kinetic interval (minimal)   | 2020 ms       |
| Injector A/B volume               | 50 $\mu$ l    |
| Injector A/B speed                | 200 $\mu$ l/s |
| Injection mode                    | Standard      |

**Table 14: Parameters for luminescence measurement using Tecan GENios™ Pro.**

### 3. Protein analysis

Protein analysis experiments were done with the HUVECTert cell line. All solutions and media were sterile and warmed for half an hour in a 37°C warm water bath before being used on the cells.

#### 3.1. Cell treatment and protein extraction

After the cells were seeded into a 6-well plate they were left to grow until they almost reached confluence. 1 h before the experiment was started the medium was renewed by 1200  $\mu$ l fresh medium for one well containing unstimulated cells, and 1150  $\mu$ l fresh medium for all other wells. 3 wells were treated with 300  $\mu$ l sample dilution containing DMSO in a final concentration of 0.1%. Control cells were treated with parthenolide diluted to a final concentration of 10  $\mu$ M and 0.1% DMSO. Only 300  $\mu$ l medium

containing DMSO to yield a final concentration of 0.1% was filled into the other two control wells. Cells were pretreated with the samples for 30 min. Afterwards the cells were stimulated with 50  $\mu$ l medium containing TNF- $\alpha$  in a final concentration of 10 ng/ml. One control well containing only medium with 0.1% DMSO was left unstimulated. TNF- $\alpha$  treatment was 60, 30, 15, 10, and 5 min for time course experiment and 10 min for sample testing. The stimulation was stopped with cold PBS. The cells were washed twice with the cold PBS and the plate was put on ice. Cells were treated immediately with 100  $\mu$ l lysis buffer and scrapped after 5 min. The protein lysates were pipetted into Eppendorf tubes and centrifuged for 10 min (13.000 g/4°C). 5  $\mu$ l supernatant of each sample was diluted with 45  $\mu$ l ddH<sub>2</sub>O and used for Bradford protein quantification assay later on. The rest of the cell lysate was diluted in 3 x SDS sample buffer (1:3) and heated for 5 min at 95°C. This sample was used for SDS-PAGE.

### 3.2. Bradford protein quantification

To quantify the amount of protein in each lysate a Bradford assay was performed. The quantification is possible due to the shift in the absorbance maximum of Coomassie Brilliant Blue G-250 from 470 nm to 595 nm after binding to proteins.<sup>26</sup> A BSA standard curve in 7 increasing concentrations (50-500  $\mu$ g/ml) was prepared. The diluted (1:10) cell lysates and the different BSA solutions were pipetted in triplicate of 10  $\mu$ l into a 96-well plate. Bradford reagent was prepared by diluting Roti<sup>®</sup>-Quant with ddH<sub>2</sub>O (1:3). 190  $\mu$ l Bradford reagent was filled to each sample and standard protein. After 5 min incubation the absorbance was measured at 595 nm using a Tecan Sunrise<sup>™</sup> microplate reader.

### 3.3. SDS-polyacrylamide gel electrophoresis

To separate all proteins by size, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) according to Laemmli was performed.<sup>27</sup> Therefore a resolving gel was prepared and filled into a gel chamber. A small amount of isopropanol was poured on the gel to remove bubbles. The gel was allowed to polymerize for around 45 min. After that the isopropanol was removed and a freshly prepared stacking gel was poured onto the resolving gel. A 10-well comb was pulled into the still liquid gel. After the polymerization had finished an equal amount of 25  $\mu$ g of sample protein or 7  $\mu$ l Precision Plus Protein<sup>™</sup> Standard was loaded per lane. The gel chamber was put

into an electrophoresis chamber and filled with electrophoresis buffer. Electrophoresis was done for 21 min with 110 V following approximately 36 min with 200 V until the dye reached the anode.

### **3.4. Western blotting**

Now that the proteins were separated they had to be transferred to a membrane for detection. Before being used, the Immuno-blot™ PVDF membrane was incubated in methanol and blotting buffer. Membrane and gel were put together to a sandwich covered by a layer of filter paper and a fiber pad on each side. This sandwich was put into a cassette which itself was set into a cooled blotting chamber filled with blotting buffer. The blot was run for 90 min and constant voltage of 100 V.

### **3.5. Protein detection**

To detect the relevant proteins, primary antibodies with specific binding for the proteins of interest were used. Secondary antibodies, containing horse reddish peroxidase conjugate, had specific binding to the respective primary antibody.

After blotting the membrane was left for 1 h in 5% milk powder in TBS-T solution on a shaker, to block all the unspecific protein binding sites, followed by washing with TBS-T (3 x 10 min). Thereafter the membrane could be incubated with primary antibody solution over night at 4°C. Incubation was stopped next day with TBS-T washing (3 x 10 min) and the membrane was incubated with secondary antibody solution for 2 h at room temperature and washed again with TBS-T (3 x 10 min).

Detection was done with LAS-3000™ Luminescent Image Analyzer (Fujifilm, Tokyo, JP) using freshly made ECL solution. After measurement the antibodies were removed with 0.5 M NaOH solution, to enable incubation of the membrane with another antibody.

## 4. Statistics

For the calculation of the IC<sub>50</sub> value minimum three independent transfection experiments, done in quadruplicate, with minimum three different concentrations of the investigated compound, were used. Nonlinear regression with Data Analysis Toolbox software (MDL Information Systems Inc., Nashville, TN, USA) was utilized for the calculations. To examine the statistical significance of possible effects of samples on NF-κB activity in HEK-293/NF-κB-luc cells, one-way analysis of variance (ANOVA) was used followed by Bonferroni's test for comparison of more than two experimental conditions. Differences with  $p < 0.05$  were considered significant (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ). Presented bars in the figures represent means  $\pm$  standard deviation (SD). GraphPad Prism software (5.01) was used for the statistical analyses.

## **E. Results**

## E. Results

In this work extracts, fractions, and pure compounds obtained from 20 plants, known from traditional use in inflammatory diseases, have been tested on their potency to inhibit TNF- $\alpha$ -induced activation of the transcription factor NF- $\kappa$ B in 293/NF- $\kappa$ B-luc cells. Depending on the current stage of investigation, these were initial crude extracts or already processed subfractions from extracts that were tested positive before (see **C Materials section 1**). The extracts and fractions were mostly tested in a concentration of 10  $\mu$ g/ml. If some inhibitory or toxic effects were noticed, the concentration was lowered. 72 pure substances, isolated from active extracts or found by computational screening, were tested mostly at 30  $\mu$ M. If some borderline activity was detected, the test concentration was increased, whereas it was lowered if a strong activity or some unspecific inhibitory effects were observed. For the most active substances the IC<sub>50</sub> value was determined by dose-response experiments. All samples were initially tested in at least two independent experiments done in quadruplicate.

**Table 15** shows the summary of all experiments done in this work. Samples which showed a residual NF- $\kappa$ B activity lower than 50% compared to the negative vehicle control (experimental medium supplemented with 0.1% DMSO), meaning an inhibition higher than 50%, were considered active, hence are marked with a "+". Inactive samples are marked with a "-".

**Table 15: Overview of all tested plant extracts, fractions, and pure substances.** Samples were tested in the luciferase reporter gene assay for NF- $\kappa$ B inhibition. Cells were pretreated with the tested sample 30 min before NF- $\kappa$ B activation was stimulated with TNF- $\alpha$  (2 ng/ml, 4 h). Cells were lysed and luminescence and EGFP-derived fluorescence were measured. Samples, which showed an inhibition higher than 50% compared to 0.1% DMSO control were considered active (+) and those with a lower inhibition as inactive (-).

| <b>Plants and pure compounds derived from VOLKSMED database from collaboration partners in Vienna (Prof. Brigitte Kopp, Prof. Gottfried Reznicek)</b> |                                                       |             |                            |                       |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|-------------|----------------------------|-----------------------|
| Code                                                                                                                                                  | Plant species; substance name; annotations            | Plant organ | Solvent/method/annotations | NF- $\kappa$ B inhib. |
| 1091                                                                                                                                                  | <i>Betonica officinalis</i>                           | Hb          | DCM; pure compound         | -                     |
| 1092                                                                                                                                                  | <i>Betonica officinalis</i>                           | Hb          | 30% MeOH SPE fraction      | -                     |
| 493                                                                                                                                                   | <i>Dryopteris filix-mas</i><br>Collected 2007, dried  | Hb          | DCM                        | +                     |
| 997                                                                                                                                                   | <i>Dryopteris filix-mas</i><br>Collected 2006, dried  | Hb          | DCM, chlorophyll removed   | +                     |
| 1010                                                                                                                                                  | Fraction                                              |             | 100% MeOH SPE fraction     | -                     |
| 1011                                                                                                                                                  | Fraction                                              |             | 70% MeOH SPE fraction      | -                     |
| 1012                                                                                                                                                  | Fraction                                              |             | 30% MeOH SPE fraction      | -                     |
| 998                                                                                                                                                   | <i>Dryopteris filix-mas</i><br>Collected 2007, dried  | Hb          | DCM, chlorophyll removed   | +                     |
| 1013                                                                                                                                                  | Fraction                                              |             | 100% MeOH SPE fraction     | +                     |
| 1014                                                                                                                                                  | Fraction                                              |             | 70% MeOH SPE fraction      | +                     |
| 1015                                                                                                                                                  | Fraction                                              |             | 30% MeOH SPE fraction      | +                     |
| 999                                                                                                                                                   | <i>Dryopteris filix-mas</i><br>Collected 2009, dried  | Hb          | DCM chlorophyll removed    | +                     |
| 1016                                                                                                                                                  | Fraction                                              |             | 100% MeOH SPE fraction     | -                     |
| 1017                                                                                                                                                  | Fraction                                              |             | 70% MeOH SPE fraction      | +                     |
| 1018                                                                                                                                                  | Fraction                                              |             | 30% MeOH SPE fraction      | -                     |
| 1000                                                                                                                                                  | <i>Dryopteris filix-mas</i><br>Collected 2009, frozen | Hb          | DCM chlorophyll removed    | -                     |
| 1019                                                                                                                                                  | Fraction                                              |             | 100% MeOH SPE fraction     | -                     |
| 1020                                                                                                                                                  | Fraction                                              |             | 70% MeOH SPE fraction      | +                     |
| 1021                                                                                                                                                  | Fraction                                              |             | 30% MeOH SPE fraction      | -                     |
| 1001                                                                                                                                                  | <i>Dryopteris filix-mas</i><br>Collected 2009, dried  | Hb          | DCM containing chlorophyll | -                     |

## E. Results

|               |                                           |    |                                                                                           |             |
|---------------|-------------------------------------------|----|-------------------------------------------------------------------------------------------|-------------|
| 25            | <i>Glechoma hederacea</i>                 | Hb | MeOH                                                                                      | -           |
| 713           | <i>Glechoma hederacea</i>                 | Hb | MeOH, polyphenols removed                                                                 | +           |
| 714           | <i>Glechoma hederacea</i>                 | Hb | MeOH, polyphenols removed                                                                 | -           |
| 715           | <i>Glechoma hederacea</i>                 | Hb | MeOH, polyphenols removed                                                                 | -           |
| 168           | <i>Glechoma hederacea</i>                 | Hb | MeOH, polyphenols removed                                                                 | n.t.        |
| 786           | Fraction                                  |    | 100% MeOH SPE fraction                                                                    | +           |
| 1057-<br>1064 | Subfractions                              |    | HPLC<br>1060,1062<br>Remaining fractions                                                  | +<br>-<br>- |
| 785           | Fraction                                  | Hb | 70% MeOH SPE fraction (786)                                                               | +           |
| 1104-<br>1110 | Subfractions                              | Hb | HPLC                                                                                      | -           |
| 28            | <i>Malva</i> sp.                          | Fo | DCM                                                                                       | +           |
| 716           | <i>Malva</i> sp.                          | Fo | DCM, chlorophyll removed                                                                  | -           |
| 717           | <i>Malva</i> sp.                          | Fo | DCM, chlorophyll removed                                                                  | -           |
| 718           | <i>Malva</i> sp.                          | Fo | DCM, chlorophyll removed                                                                  | -           |
| 279           | <i>Melampyrum</i> sp.                     | Hb | MeOH, tannins removed                                                                     | +           |
| 277           | <i>Melampyrum</i> sp.                     | Hb | DCM, chlorophyll removed                                                                  | +           |
| 840           | Fraction                                  |    | 30% MeOH SPE fraction                                                                     | n.t.        |
| 1329-<br>1330 | Pure substances                           | Hb | From: EtOAc:MeOH:H <sub>2</sub> O=<br>17:3:3 SGCC subfraction                             | -           |
| 841           | Fraction                                  |    | 70% MeOH SPE fraction                                                                     | n.t.        |
| 1331-<br>1332 | Pure substances                           |    | From:CHCl <sub>3</sub> :MeOH:H <sub>2</sub> O=<br>9:1:1 SGCC subfraction                  | -           |
| 1333-<br>1336 | Subfractions                              |    | CHCl <sub>3</sub> :MeOH:H <sub>2</sub> O= 9:1:1<br>SGCC subfractions<br>1333<br>1334-1336 | +<br>-<br>- |
| 31            | <i>Peucedanum ostruthium</i>              | Rh | DCM                                                                                       | +           |
| 1072          | Ostruthin                                 |    |                                                                                           | +           |
| 1073          | Ostruthol                                 |    |                                                                                           | -           |
| 1399          | Osthole                                   |    |                                                                                           | -           |
| 1400          | Oxypeucedanin hydrate                     |    |                                                                                           | -           |
| 1307-<br>1317 | <i>Peucedanum ostruthium</i><br>Fractions | Rh | MeOH<br>Preparative HPLC with phthalate<br>free solvent                                   | -           |

| Plants and pure compounds derived from Traditional Chinese Medicine from Prof. Brigitte Kopp (Vienna) |                                            |             |                                                                                                     |              |
|-------------------------------------------------------------------------------------------------------|--------------------------------------------|-------------|-----------------------------------------------------------------------------------------------------|--------------|
| Code                                                                                                  | Plant species; substance name; annotations | Plant organ | Solvent/method/annotations                                                                          | NF-κB inhib. |
| 1156-1157                                                                                             | <i>Albizia julibrissin</i><br>Subfractions | Cx          | DCM<br>EtOAc<br>CHCl <sub>3</sub> :MeOH:H <sub>2</sub> O; CC                                        | -            |
| 1361-1373                                                                                             | <i>Albizia julibrissin</i><br>Subfractions | Cx          | MeOH tannins removed<br>CHCl <sub>3</sub> :MeOH:H <sub>2</sub> O; CC<br>1367<br>Remaining fractions | +<br>-       |
| 993                                                                                                   | <i>Pinellia</i> sp.                        | Hb          | DCM                                                                                                 | -            |
| 994                                                                                                   | <i>Pinellia</i> sp.                        | Hb          | MeOH                                                                                                | -            |
| 995                                                                                                   | <i>Pinellia</i> sp.                        | Hb p.       | DCM                                                                                                 | -            |
| 996                                                                                                   | <i>Pinellia</i> sp.                        | Hb p.       | MeOH                                                                                                | -            |
| Plant and compound gained through ethnopharmacological approach from Prof. Rudolf Bauer (Graz)        |                                            |             |                                                                                                     |              |
| Code                                                                                                  | Plant species; annotations                 | Plant organ | Solvent/method/annotations                                                                          | NF-κB inhib. |
| 1182                                                                                                  | <i>Avena sativa</i><br>Salcolin A/B        | Hb          |                                                                                                     | +            |
| Plants and pure compounds derived from Traditional Chinese Medicine from Prof. Rudolf Bauer (Graz)    |                                            |             |                                                                                                     |              |
|                                                                                                       | <i>Clematis armandii</i>                   | Cl          |                                                                                                     | n.t.         |
| 1350                                                                                                  | Oleanolic acid                             |             |                                                                                                     | +            |
| 1351                                                                                                  | Ursolic acid                               |             |                                                                                                     | +            |
| 1209                                                                                                  | <i>Lonicera japonica</i>                   | Cl          | DCM                                                                                                 | +            |
| 1210                                                                                                  | Fraction                                   |             | MeOH fraction                                                                                       | +            |
| 1345-1349                                                                                             | Subfraction                                |             | Sephadex fractions<br>1345-1347<br>1348-1349                                                        | -<br>+       |
| 971                                                                                                   | <i>Notopterygium</i> sp.                   | Rx&Rh       | DCM fraction                                                                                        | +            |
| 972                                                                                                   | <i>Notopterygium</i> sp.                   | Rx&Rh       | DCM fraction                                                                                        | +            |
| 970                                                                                                   | <i>Notopterygium</i> sp.                   | Rx&Rh       | DCM fraction                                                                                        | n.t.         |
| 975                                                                                                   | Subfraction                                |             |                                                                                                     | -            |
| 976                                                                                                   | Subfraction                                |             |                                                                                                     | + /t.        |

## E. Results

| 1243                                                                                                                                                                                                                     | <i>Notopterygium incisum</i>               | Rx&Rh       | DCM crude extract           | +               |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|-------------|-----------------------------|-----------------|
| 1244-<br>1253                                                                                                                                                                                                            | Fractions                                  |             | 1244,1253<br>1247<br>others | -<br>t.<br>+/t. |
|                                                                                                                                                                                                                          | <i>Sophora flavescens</i>                  |             |                             |                 |
| 1196                                                                                                                                                                                                                     | Kushenol D                                 |             |                             | -               |
| 1197                                                                                                                                                                                                                     | Sophoraflavanon B                          |             |                             | -               |
| 1198                                                                                                                                                                                                                     | Kushenol E                                 |             |                             | -               |
| 1199                                                                                                                                                                                                                     | Kushenol B                                 |             |                             | -               |
| 1200                                                                                                                                                                                                                     | Sophoraflavanon G                          |             |                             | -               |
| 1201                                                                                                                                                                                                                     | Kuraridin                                  |             |                             | +               |
| 1202                                                                                                                                                                                                                     | Isokuraridin                               |             |                             | +               |
| 1203                                                                                                                                                                                                                     | Norkurarinol                               |             |                             | -               |
| 1204                                                                                                                                                                                                                     | Kushenol P                                 |             |                             | -               |
| 1205                                                                                                                                                                                                                     | Kushenol I                                 |             |                             | -               |
| 1206                                                                                                                                                                                                                     | Kushenol M                                 |             |                             | -               |
| 1207                                                                                                                                                                                                                     | Biochanin A                                |             |                             | -               |
| 1208                                                                                                                                                                                                                     | Maackiain                                  |             |                             | -               |
| 984                                                                                                                                                                                                                      | <i>Thlaspi arvense</i>                     | Hb          | MeOH                        | -               |
| 985-<br>991                                                                                                                                                                                                              | Fractions                                  |             | MeOH                        | -               |
| 1254                                                                                                                                                                                                                     | <i>Typhonium giganteum</i>                 | Rh          | DCM crude extract           | -               |
| <b>Plants and pure compounds predicted as NF-κB inhibitors or found by ethnopharmacological approach from collaboration partners in Innsbruck (Prof. Judith Rollinger, Prof. Hermann Stuppner, Dr. Daniela Schuster)</b> |                                            |             |                             |                 |
| Code                                                                                                                                                                                                                     | Plant species; substance name; annotations | Plant organ | Solvent/method/annotations  | NF-κB inhib.    |
| 1084                                                                                                                                                                                                                     | Dihydrocapsaicin                           |             |                             | -               |
| 1085                                                                                                                                                                                                                     | Ergosterol                                 |             |                             | -               |
| 1086                                                                                                                                                                                                                     | Ganoderic acid B                           |             |                             | -               |
|                                                                                                                                                                                                                          | <i>Garcinia nigrolineata</i>               |             | EtOH                        |                 |
| 1093                                                                                                                                                                                                                     | Fraction                                   |             | EtOAc                       | -               |
| 310                                                                                                                                                                                                                      | <i>Morus alba</i>                          | CoRx        | MeOH                        | ?               |
| 311                                                                                                                                                                                                                      | <i>Morus alba</i>                          | CoRx        | MeOH                        | ?               |
| 1078                                                                                                                                                                                                                     | Pure compound                              |             |                             | -               |

|                                                                                              |                                                              |    |                        |   |
|----------------------------------------------------------------------------------------------|--------------------------------------------------------------|----|------------------------|---|
| 642                                                                                          | <i>Himatanthus sucuuba</i>                                   | Cx | MeOH                   | + |
| 643                                                                                          | Fraction                                                     |    | EtOAc                  | + |
| 644                                                                                          | Fraction                                                     |    | n-Butanol              | - |
| 1087                                                                                         | Plumericin                                                   |    |                        | + |
| 1088                                                                                         | Biochanin A                                                  |    |                        | + |
| 1089                                                                                         | Dihydrobiochanin A                                           |    |                        | - |
| 1090                                                                                         | Pure compound                                                |    |                        | - |
| 1359                                                                                         | Plumieridin                                                  |    |                        | + |
|                                                                                              | Lichen                                                       |    |                        |   |
| 1140                                                                                         | Compound CR1                                                 |    |                        | - |
| 1141                                                                                         | Methyl-beta-orcinolcarboxylat                                |    |                        | - |
| 1142                                                                                         | Atranorin                                                    |    |                        | + |
| 1143                                                                                         | Fumarprotocetraric Acid                                      |    |                        | - |
| 1144                                                                                         | Variolaric Acid                                              |    |                        | - |
| 1145                                                                                         | Olivetoric Acid                                              |    |                        | + |
| 1146                                                                                         | Salazinic Acid                                               |    |                        | - |
| 1147                                                                                         | Perlatolic Acid                                              |    |                        | + |
| 1148                                                                                         | Evernic Acid                                                 |    |                        | - |
| 1149                                                                                         | Physodic Acid                                                |    |                        | + |
| 1150                                                                                         | Scensidine                                                   |    |                        | - |
| 1151-1155                                                                                    | Pure compounds n.i.                                          |    |                        | - |
| 1194                                                                                         | <i>Sideritis hyssopifolia</i><br>Diterpene isomer<br>Mixture |    | MeOH                   | - |
| <b>Predicted p50 binders from collaboration partners in Innsbruck (Dr. Daniela Schuster)</b> |                                                              |    |                        |   |
| 1094-1103                                                                                    |                                                              |    | 1102<br>Remaining ones | + |
| 1213-1227                                                                                    |                                                              |    | 1215<br>Remaining ones | + |
|                                                                                              |                                                              |    |                        | - |

## E. Results

Table abbreviations

|       |                         |        |                                             |
|-------|-------------------------|--------|---------------------------------------------|
| +     | : Active                | Inhib. | : Inhibition                                |
| -     | : Inactive              | MeOH   | : Methanol                                  |
| ?     | : The assay failed      | n.i.   | : Not identified                            |
| CC    | : Column chromatography | n.t.   | : Not tested in this work                   |
| Cl    | : Caulis                | p.     | : processed drug. (TCM specific processing) |
| CoRx  | : Cortex radices        | Rh     | : Rhizoma                                   |
| Cx    | : Cortex                | Rx     | : Radix                                     |
| DCM   | : Dichloromethane       | SGCC   | : Silica gel column chromatography          |
| EtOAc | : Ethyl acetate         | SPE    | : Solid phase extraction                    |
| EtOH  | : Ethanol               | t.     | : Toxic                                     |
| Fo    | : Folium                |        |                                             |
| Hb    | : Herb                  |        |                                             |

Percentage inhibition mentioned in the following sections always refers to the mean inhibition of TNF- $\alpha$  (2 ng/ml) stimulated NF- $\kappa$ B activity compared to the vehicle control (experimental medium supplemented with 0.1% DMSO). Consequently the negative control always represents 0%, meaning that there is no inhibition, while 100% refers to complete inhibition of NF- $\kappa$ B to the background level. Plant extracts, fractions, and pure substances were considered to have a good inhibitory effect if the inhibition was higher than 50%.

## 1. Investigation of ubiquitous plant constituents

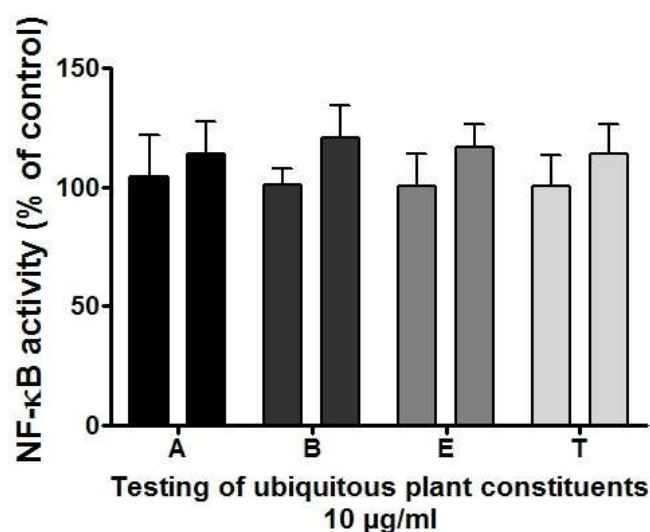


**Figure 12: Influence of tannic acid, chlorophylls, and epicatechin gallate on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with the samples (10 μg/ml) 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP- derived fluorescence. Two independent experiments were performed shown as the two bars grouped together. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate. All values are normalized to the vehicle control (experimental medium supplemented with 0.1% DMSO). A: chlorophyll A; B: chlorophyll B; E: epicatechin gallate; T: tannic acid.

Natural constituents like tannic acid, chlorophyll, and epicatechin gallate are very wide spread in plants. Hence, most extracts contain a high amount of these compounds, unless they are removed before. Therefore it is important to investigate if there is an inhibitory effect of these constituents on the utilized NF-κB activation assay.

**Figure 12** shows that no inhibitory effects on NF-κB were observed by chlorophyll A and B, epicatechin gallate, and tannic acid.

## 2. Discovery of plant extracts with an NF- $\kappa$ B inhibitory potency

### 2.1. Plants derived from the VOLKSMED database (samples delivered from collaboration partners in Vienna)

#### 2.1.1. *Dryopteris filix-mas* L., Aspidiaceae (not part of the NFN)

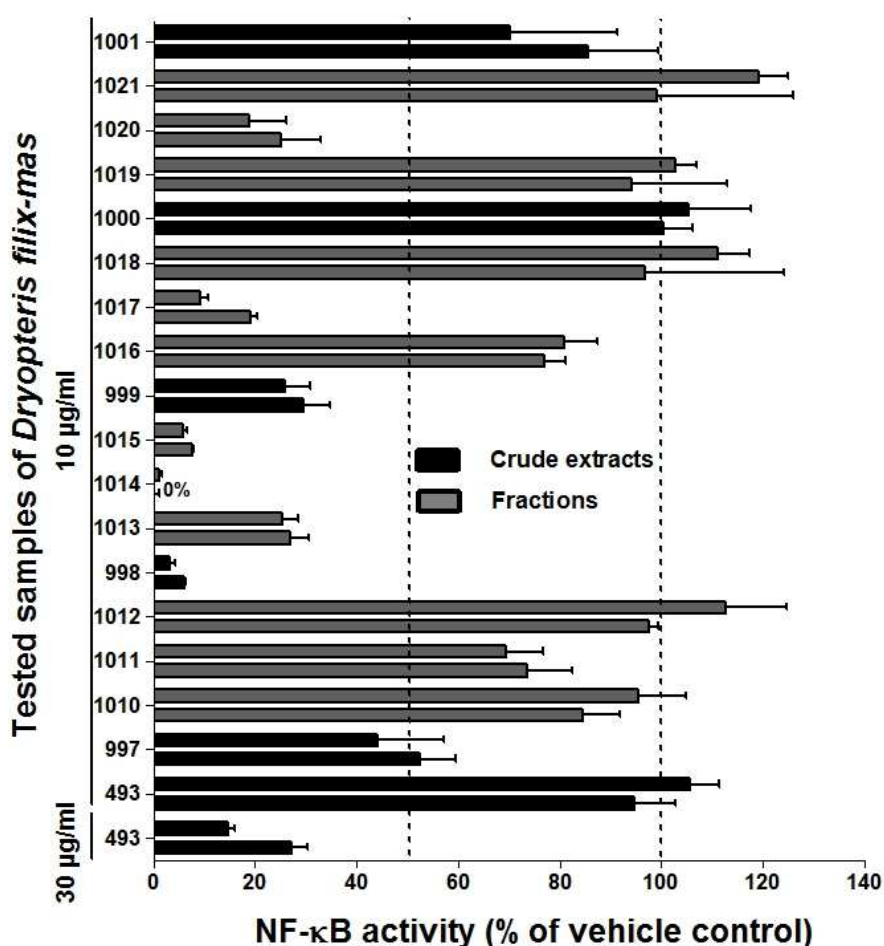


**Figure 13: Effects of *Dryopteris filix-mas* samples on NF- $\kappa$ B activity.** HEK 293/NF- $\kappa$ B-luc cells were treated with the samples (10 µg/ml or 30 µg/ml) 30 min prior to TNF- $\alpha$  stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Samples were tested in two independent experiments shown as two bars grouped together and assigned to the sample code. Each bar represents the mean  $\pm$  SD NF- $\kappa$ B activity of one experiment done in quadruplicate with the values expressed as % of vehicle control (experimental medium supplemented with 0.1% DMSO). Black bars represent the crude extracts and grey bars their fractions.

**Figure 13** shows the testing of different dichloromethane (DCM) chlorophyll depleted (except sample 493 and 1000 and its fractions) extracts and their fractions made from *Dryopteris filix-mas* herb. The samples differed in the years the plant was collected. All extracts and fractions were tested at 10 µg/ml, and extract 493 was tested a second time at 30 µg/ml.

Sample 997 (plant material collected in 2006) showed only a low mean activity with 48% and 56% inhibition compared to vehicle control. Its fractions 1010-1012 did not show an inhibition since their measured NF-κB activity was higher than 50%.

Extract 998 (plant material collected in 2007) was highly effective with 94% and 97% inhibition. All originated fractions show an inhibition higher than 50%. 1013 was moderately effective with 73% and 75% inhibition. Fractions 1014/15 seemed highly active with a mean inhibition >90%. Fraction 1015 inhibited NF-κB activity by 92% and 94%, while 1014 showed both times almost a total inhibition with a mean of 99% and 100%.

Extract 493 (plant material collected in 2007, chlorophyll not removed) did not show an effect at 10 µg/ml. Since the related but chlorophyll depleted extract 998 inhibited the NF-κB activity in this concentration, extract 493 was tested again at 30 µg/ml and was moderately active with 73% and 85% inhibition.

Extract 999 (plant material collected in 2009) showed a moderate activity with 70% and 74% inhibition compared to the control. Fractions 1016 and 1018 were not effective, while fraction 1017 was the most active of all three fractions with 81% and 91% inhibition.

Extract 1000 (plant material collected in 2009), being the same as 999 but with the plant material frozen freshly instead of dried, did not show any inhibitory effect. Its fractions 1021 and 1019 were inefficient as well. Fraction 1020 was the most active one with a NF-κB inhibition of 75% and 81%.

Extract 1001 was obtained from the same plant material as 999 but was not depleted from chlorophyll. In comparison to that one it was inefficient.

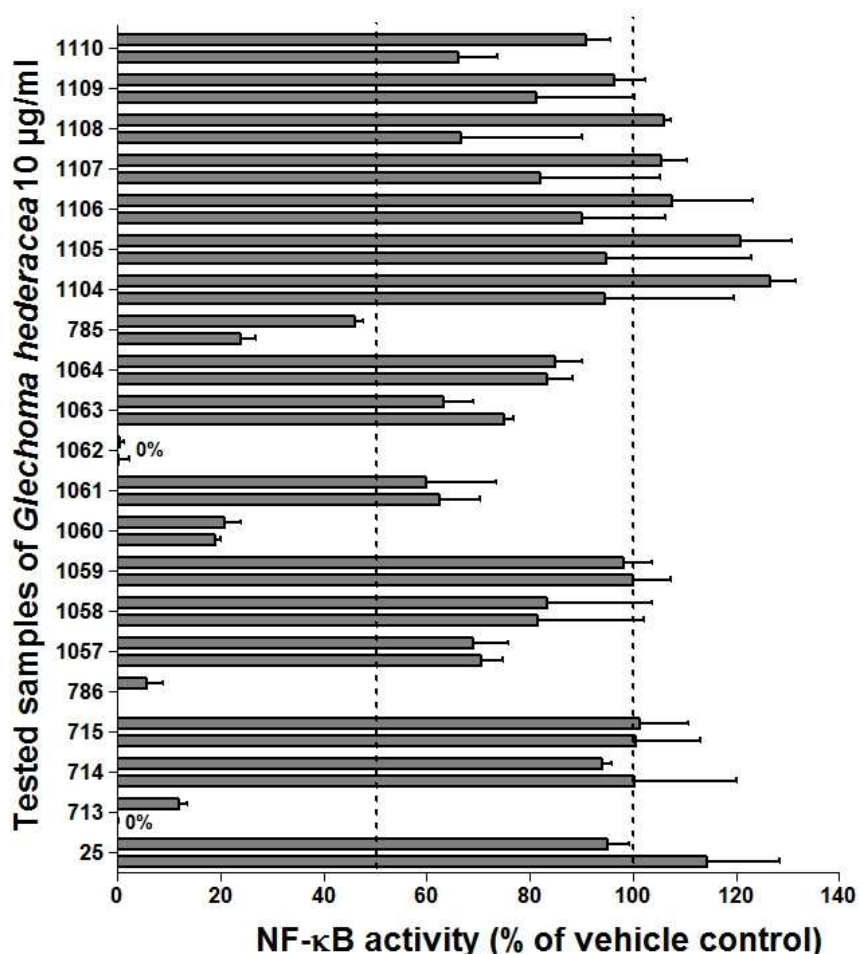
2.1.1.2. *Glechoma hederacea* L., Lamiaceae

**Figure 14: Effects of *Glechoma hederacea* samples on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with the samples (10 µg/ml) 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Samples were tested in two independent experiments shown as bars grouped together and assigned to the sample code. Sample 786 was tested only once just to confirm the previously observed inhibitory effect. Each bar represents the mean ± SD of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO).

Four different methanol (MeOH) extracts made from *Glechoma hederacea* herb have been tested. These are sample 25 and the tannic acid depleted extracts 713-715. Out of these four samples 25, 714, and 715 did not show any inhibitory effect. Sample 713 showed the highest activity with 88% and 100% NF-κB inhibition. Fraction 786 already showed positive effects in previous experiments. Hence in this experiment it was used just for confirmation while testing its subfractions 1057-1064. The repeated testing of 786 showed a high activity with 94% inhibition. Subfractions

1058, 1059, and 1064 were not active, and samples 1057 (29%, 31%), 1061 (38%, 40%), and 1063 (25%, 37%) showed some minor inhibition which however, remained smaller than 50%. Among the tested subfractions 1060 and 1062 were the most active. Subfraction 1060 inhibited NF- $\kappa$ B activity by 79% and 81% while 1062 showed both times a complete inhibition (100%).

Subfraction 785 already showed positive effects in previous experiments. In this work again an inhibition of 54% and 76% has been observed. Unfortunately none from its subfractions showed a relevant activity since no sample showed an inhibition higher than 50% as seen on **Figure 14**.

### 2.1.3. *Malva* sp., Malvaceae

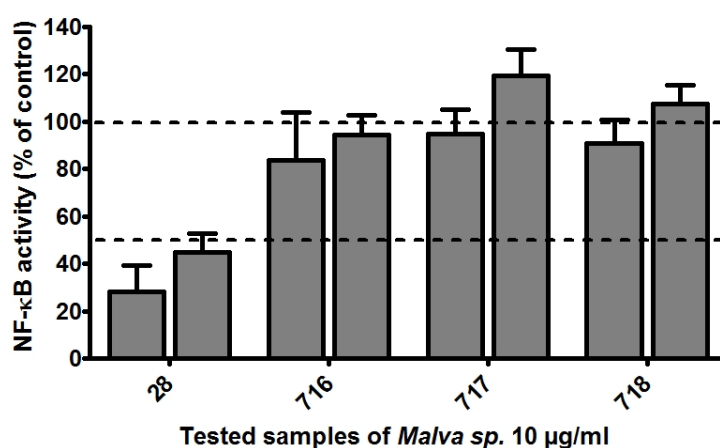


**Figure 15: Effects of tested samples of *Malva* sp. on NF- $\kappa$ B activity.** HEK 293/NF- $\kappa$ B-luc cells were treated with the samples (10  $\mu$ g/ml) 30 min prior to TNF- $\alpha$  stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Samples were tested in two independent experiments shown as two bars grouped together and assigned to the sample code. Each bar represents the mean  $\pm$  SD NF- $\kappa$ B activity of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO).

Four different DCM extracts out of *Malva* sp. leaf have been tested. Extracts 716-718 were inactive. Extract 28, being the only extract still containing chlorophyll, showed some activity with 55% and 72% NF- $\kappa$ B inhibition, respectively (**Figure 15**).

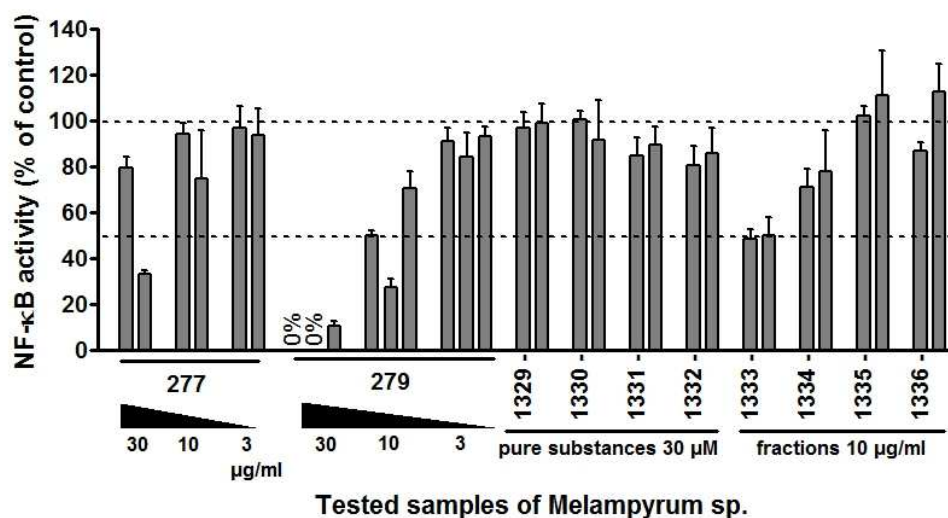
2.1.4. *Melampyrum* sp., Scrophulariaceae

**Figure 16: Effects of tested samples of *Melampyrum* sp. on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with the samples 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Samples 277 and 279 were tested at increasing concentrations (3, 10, 30 µg/ml), in two and three independent experiments, respectively. Pure compounds (1329-1332) were tested at 30 µM, and further fractions (1333-1336) at 10 µg/ml. Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Samples were tested in 2-3 independent experiments shown as bars grouped together and assigned to the sample code. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO).

**Figure 16** shows the results from the tested chlorophyll-depleted DCM extract (277) and from the tannin-depleted MeOH extract (279) of *Melampyrum* sp. herb at 3, 10, and 30 µg/ml. Pure substances (1329-1332) were tested at 30 µM and the fractions (1333-1336) were tested at 10 µg/ml.

Extract 277 did not show any inhibitory effect at 3 and 10 µg/ml. At 30 µg/ml in one of the experiments it showed 21% inhibition and in the other experiment the effect was stronger (67% inhibition).

Extract 279 was tested three times and was very active at 30 µg/ml. At this concentration it once showed 89% inhibition and twice a total inhibition of NF-κB activity (100%). The low EGFP-derived fluorescence (data not shown) indicated some possible toxic effects or unspecific inhibitory action at this concentration. The measurements at 10 µg/ml only showed a moderate activity with 30%, 50%, and 72% inhibition, while the concentration of 3 µg/ml was too low to show any activity.

The four pure compounds (1329-1332) were not active.

From the four subfractions 1333-1336 only 1333 showed a weak effect with 50% and 51% inhibition.

#### 2.1.5. *Peucedanum ostruthium* L., Apiaceae

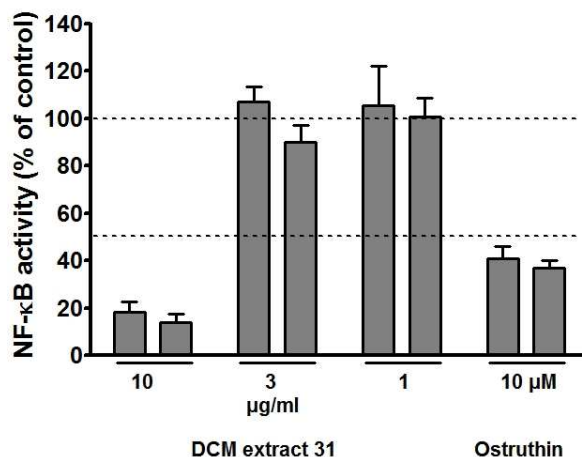


**Figure 17: Effects of *Peucedanum ostruthium* DCM extract and ostruthin on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with 3 or 10 μg/ml extract or 10 μM ostruthin 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Samples were tested in two independent experiments shown as two bars grouped together and assigned to the sample code. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO).

The crude DCM extract 31 from *Peucedanum ostruthium* rhizomes has been tested at 10 μg/ml and a strong mean inhibition of 82% and 86% has been observed as demonstrated in **Figure 17**. This extract was not active in lower concentrations (1 and 3 μg/ml). Out of the pure compounds ostruthin was the only active one with 59% and 63% inhibition at 10 μM. Other pure compounds and fractions originating from the crude extract were tested but found to be inactive (**Table 15**).

## 2.2. Plants used in the traditional Chinese medicine (TCM) (samples delivered from collaboration partners in Graz)

### 2.2.1. *Lonicera japonica* Thunb., Caprifoliaceae

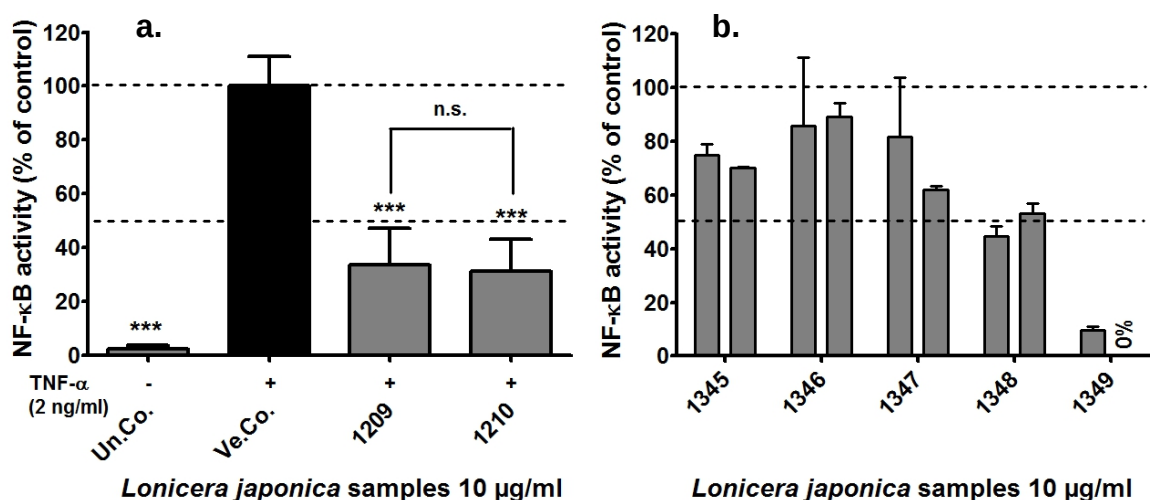


**Figure 18: Effects of *Lonicera japonica* extracts and fractions on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with the samples (10 µg/ml) 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. (a) Each bar represents the mean ± SD NF-κB activity of three independent experiments done in quadruplicate compared to the vehicle control (Ve.Co.: experimental medium supplemented with 0.1% DMSO). Un.Co.: untreated and unstimulated cells. \*\*\*: statistically significant with  $p < 0.001$  compared to the Ve.Co.; vehicle control; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test.). (b) Samples were tested in two independent experiments shown as two bars grouped together and assigned to the sample code. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate compared to vehicle control.

**Figure 18a** shows the results from the testing of *Lonicera japonica* caulis DCM extract (1209) and MeOH fraction (1210) on NF-κB activity at 10 µg/ml. Both samples showed significant ( $p < 0.001$ ,  $n = 3$ , ANOVA/Bonferroni) inhibition compared to the DMSO control with a mean inhibition of 66% for 1209 and 69% for 1210. The difference in inhibition was not significant between both samples. **Figure 18b** shows the results of the testing of fractions 1345-1349. 1345-1347 did not show considerable inhibition at 10 µg/ml since all NF-κB activities were higher than 50%. Fraction 1348 showed some borderline activity with 55% and 47% inhibition. Fraction 1349 was the most active one and induced highly efficient inhibition (90% and 100% in the two independent experiments).

### 3. Plant extracts showing NF- $\kappa$ B inhibition and potential toxic side-effects or unspecific inhibitory action

#### 3.1. Plants used in TCM (samples delivered from collaboration partners in Graz)

##### 3.1.1. *Notopterygium* sp. Apiaceae

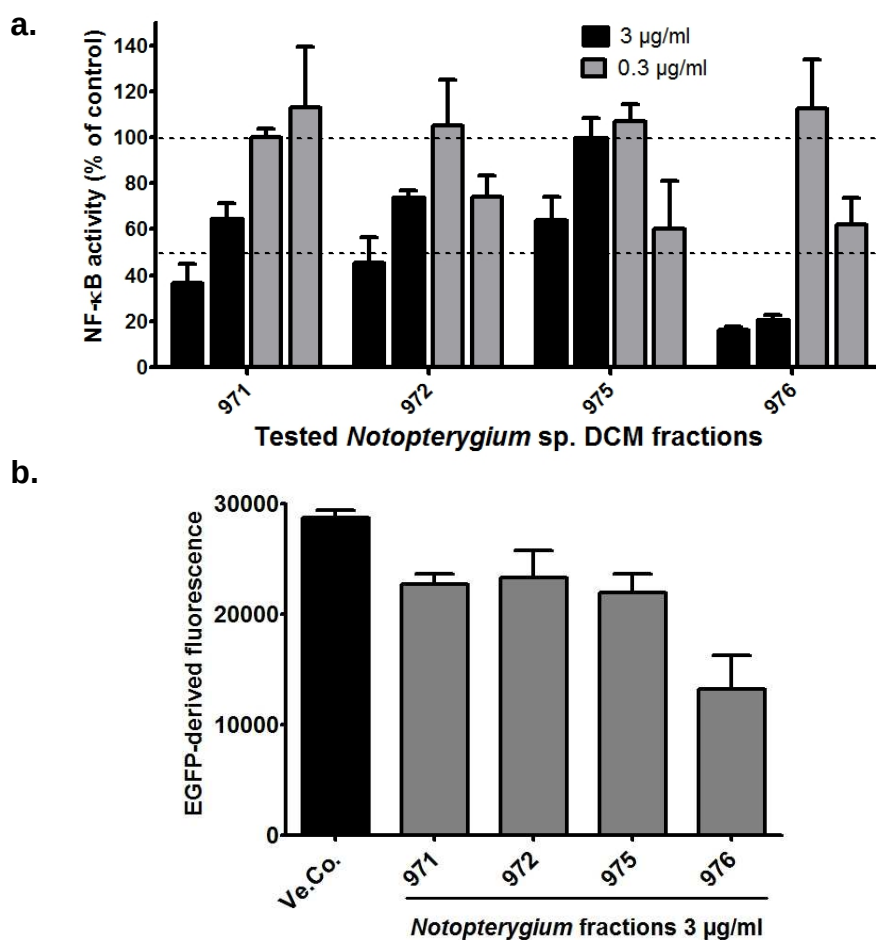
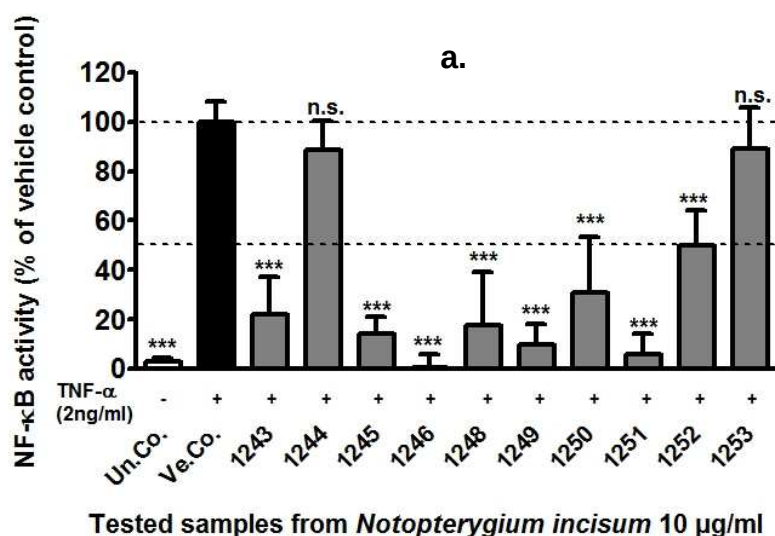


**Figure 19: Effects of *Notopterygium* sp. samples on NF- $\kappa$ B activity and EGFP-derived fluorescence.** HEK 293/NF- $\kappa$ B-luc cells were treated with the samples (0.3, 3  $\mu$ g/ml) 30 min prior to TNF- $\alpha$  stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. (a) Samples were tested in two independent experiments per concentration. These are shown as two bars grouped in terms of color and grouped together to four bars assigned to the sample code. The different concentrations are marked with grey (0.3  $\mu$ g/ml) and black (3  $\mu$ g/ml) color. Each bar represents the mean  $\pm$  SD NF- $\kappa$ B activity of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO). (b) EGFP-derived fluorescence. Each bar represents the mean  $\pm$  SD fluorescence of one representative experiment out of two done in quadruplicate. (Ve.Co.: vehicle control).

Several fractions out of a DCM extract of *Notopterygium* sp. underground parts have been tested. Previous experiments showed toxic or unspecific inhibitory effects of this plant extract at 10 µg/ml. This was the reason why the initial testing concentration was lowered to 3 µg/ml.

None of the samples inhibited NF-κB by more than 50% in a concentration of 0.3 µg/ml (**Figure 19a**). At 3 µg/ml 975 was still not active, and fractions 971 and 972 showed a moderate activity with 36% and 64% (971), and 26% and 55% (972) inhibition. 976 showed the highest potency with 80% and 84% inhibition at 3 µg/ml. A change in the cellular morphology has been observed through the microscope for both concentrations after 4 h treatment. Such morphological changes often indicate cellular stress or toxicity. The EGFP-derived fluorescence was lowered in concentrations of 3 µg/ml also indicating possible toxic or unspecific inhibitory effects. Especially strongly reduced was the fluorescence after treatment with fraction 976 with a mean of 13203 relative fluorescence units compared to the vehicle control with 28703.



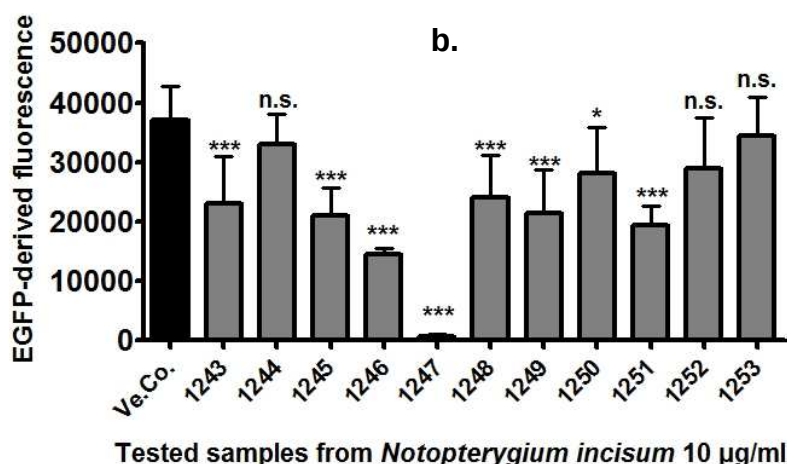


**Figure 20: Effects of *Notopterygium incisum* samples on NF- $\kappa$ B activity and EGFP-derived fluorescence.** HEK 293/NF- $\kappa$ B-luc cells were treated with the samples (10  $\mu$ g/ml) 30 min prior TNF- $\alpha$  stimulation (2 ng/m; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Bars represent the mean  $\pm$  SD (a) NF- $\kappa$ B activity or (b) EGFP- derived fluorescence alone, of three independent experiments each done in quadruplicate compared to the vehicle control (Ve.Co.: experimental medium supplemented with 0.1% DMSO). Un.Co.: untreated and unstimulated cells. \*\*\*:  $p < 0.001$ ; \*:  $p < 0.05$ ; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test).

A crude DCM extract from *Notopterygium incisum* (1243) showed upon testing a high activity with 78% inhibition at 10  $\mu$ g/ml. Its fractions (1244-1253) mostly showed a high activity as well (**Figure 20a**). Fractions 1244 and 1253 did not show a significant inhibition. Other fractions inhibited NF- $\kappa$ B activity significantly: 50% (1252), 69% (1250), 83% (1248), 86% (1245), 90% (1249), 94% (1251), and 100% (1246). NF- $\kappa$ B activity result of sample 1247 is not shown in **Figure 20a**, since the signal was very unspecific due to potential toxic effects. Cell morphology as well as EGFP-derived fluorescence (**Figure 20b**) indicated unspecific inhibitory or toxic action of most samples. The crude extract (1243) itself already showed a significant lower fluorescence compared to the control. Samples 1244, 1252, and 1253 did not show a significant decrease in fluorescence and they were the least NF- $\kappa$ B inhibiting samples as well. 1247 was very toxic with almost no living cells left in the well upon microscopy examination, and a very low fluorescence signal with a mean of 726 relative fluorescence units compared to 37029 of the vehicle control.

## 4. Pure compounds with an NF-κB inhibitory activity

### 4.1. Substances from plants used in TCM (samples delivered from collaboration partners in Graz)

#### 4.1.1. Oleanolic and ursolic acids from *Clematis armandii*, Franch. Ranunculaceae

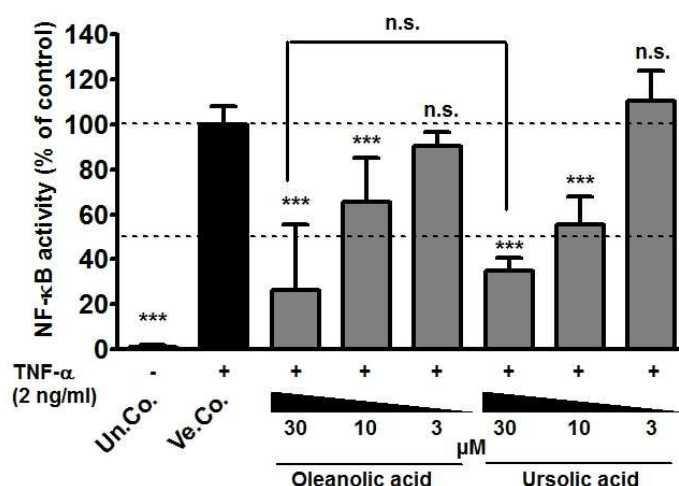


**Figure 21: Dose-dependent effects of oleanolic and ursolic acids on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with increasing concentrations of the two compounds (3, 10, 30 μM) 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. The figure shows the mean ± SD NF-κB activity of four independent experiments done in quadruplicate with all values normalized to the vehicle control (Ve.Co.: experimental medium supplemented with 0.1% DMSO). Un.Co.: untreated and unstimulated cells. \*\*\*:  $p < 0.001$ ; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test).

**Figure 21** shows the testing of oleanolic acid, a natural pentacyclic triterpenoid carboxylic acid found in *Clematis armandii*, and ursolic acid, a structurally similar substance. Both substances did not significantly inhibit NF-κB activity at 3 μM ( $p > 0.05$ ,  $n = 4$ , ANOVA/Bonferroni). Concentrations of 10 μM showed a significant effect ( $p < 0.001$ ) with a mean inhibition of 35% for oleanolic acid and 45% for ursolic acid. Both substances also showed a moderate activity at 30 μM. Oleanolic acid inhibited NF-κB activity by 74% and ursolic acid by 65%. There was no significant difference in the inhibitory potency of the two compounds at 30 μM.

#### 4.1.2. Kurarinone, isokurarinone, and other compounds from *Sophora flavescens* Ait., Fabaceae

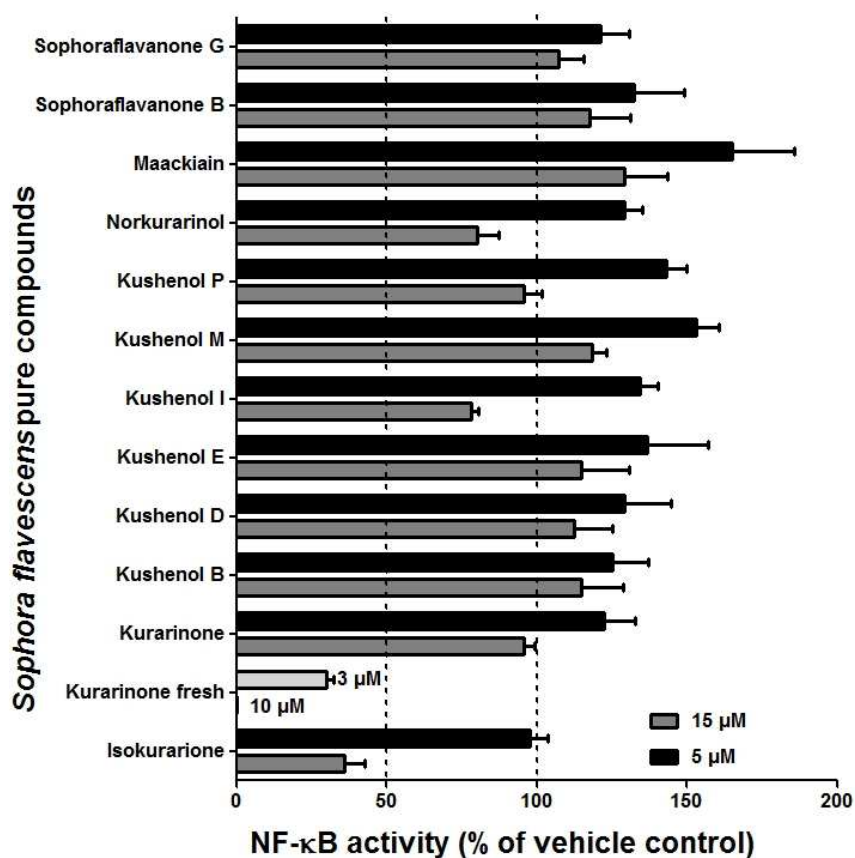


**Figure 22: Effects of pure compounds found in *Sophora flavescens* on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with the test samples 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. Tested concentrations were 5 and 15 μM, and additionally 3 and 10 μM for one different (fresh) batch of kurarinone. The different sample concentrations were tested in independent experiments shown as bars in different colors for the different concentrations and grouped together assigned to the compound name. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate compared to vehicle control (experimental medium supplemented with 0.1% DMSO).

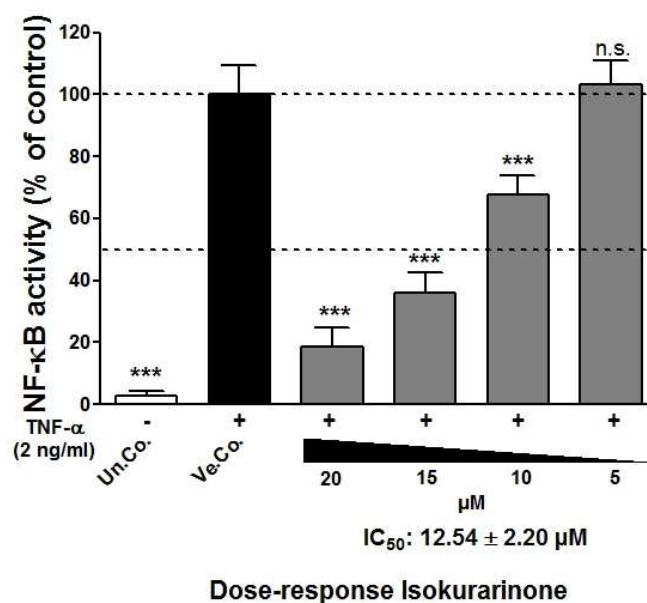


**Figure 23: Dose-response experiment with isokurarinone.** HEK 293/NF-κB-luc cells were treated with different concentrations of isokurarinone (5, 10, 15, 20 μM) or DMSO vehicle 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. The figure shows the mean ± SD NF-κB activity of three independent experiments done in quadruplicate compared to the vehicle control (Ve.Co.). Un.Co.: untreated and unstimulated cells. \*\*\*:  $p < 0.001$ ; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test).

11 flavonoids and the chromanocoumaran maackiain are known ingredients of the *Sophora flavescens* root.<sup>20-22</sup> The compounds have been tested first once in an initial concentration of 5 μM. After they showed no effect the concentration has been increased to 15 μM in the second experiment. Still most samples were inactive (**Figure 22**). Since the flavanon kurarinone was not active but is known to exhibit an anti-inflammatory activity from the literature<sup>28</sup> it was later tested again using the fresh sample. The fresh kurarinone sample was very active with 70% inhibition at 3 μM and 100% inhibition at 10 μM. Structurally similar isokurarinon (**Figure 24**) showed a moderate inhibiting potency at 15 μM in the first two experiments, and was tested in dose response experiments (**Figure 23**). The IC<sub>50</sub> was determined to be 12.54 ± 2.20 μM.

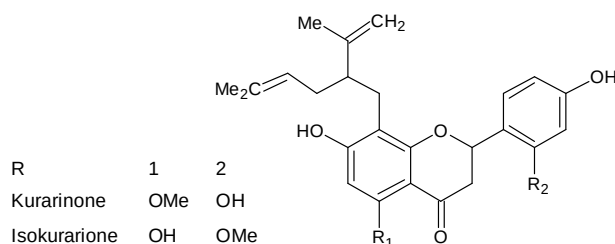


**Figure 24: Structure of the active substances kurarinone and isokurarinone.**

## 4.2. Ethnopharmacological approach (samples delivered from collaboration partners in Graz)

### 4.2.1. Salcolin A/B from *Avena sativa* L., Poaceae

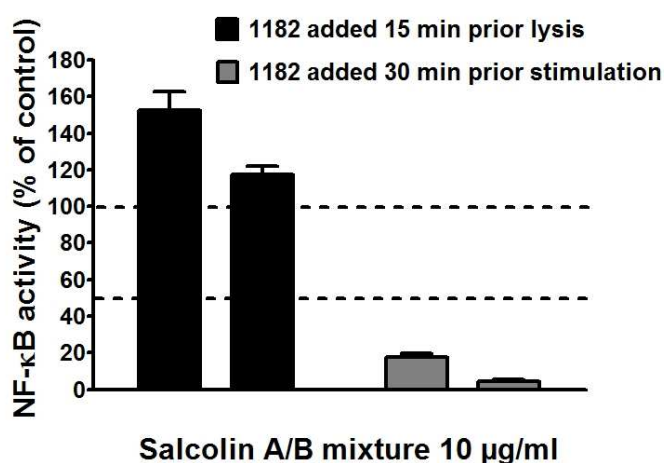


**Figure 25: Effects of salcolin A/B on NF-κB and luciferase activity.** HEK 293/NF-κB-luc cells were treated with sample 1182 (10 µg/ml) 30 min (grey bars) prior to TNF-α stimulation (2 ng/ml; 4 h) or 15 min prior to cell lysis (black bars) and luminescence as well as fluorescence were measured. Luciferase activity was normalized to EGFP-derived fluorescence. The two different treatment conditions were tested each in two independent experiments shown as bars in different colors and grouped together in the figure. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO).

A mixture of salcolin A and B has been tested. These are flavonolignans which were isolated from *Avena sativa*. This mixture showed a high NF-κB inhibiting potency of 83% and 95% in a concentration of 10 µg/ml (**Figure 25**). Since this mixture was very active, it was decided to verify that the observed effect is due to a real NF-κB inhibition, and is not an artifact due to direct inhibitory action of the luciferase enzyme itself. Hence a luciferase inhibiting test with this sample has been performed. The

salcolin mixture was thus added 15 min prior to cell lysis in two of the experiments, instead of 30 min prior to 4 h lasting TNF- $\alpha$  treatment. 15 min treatment is too short to influence the NF- $\kappa$ B-mediated luciferase gene expression, but would still be sufficient to directly inhibit the luciferase enzyme that has been already synthesized. The normalized luciferase activity for that treatment condition was even higher than vehicle control, showing that the sample did not inhibit the luciferase enzyme itself.

### 4.3. Ethnopharmacological approach (samples delivered from collaboration partners in Innsbruck)

#### 4.3.1. Secondary lichen metabolites

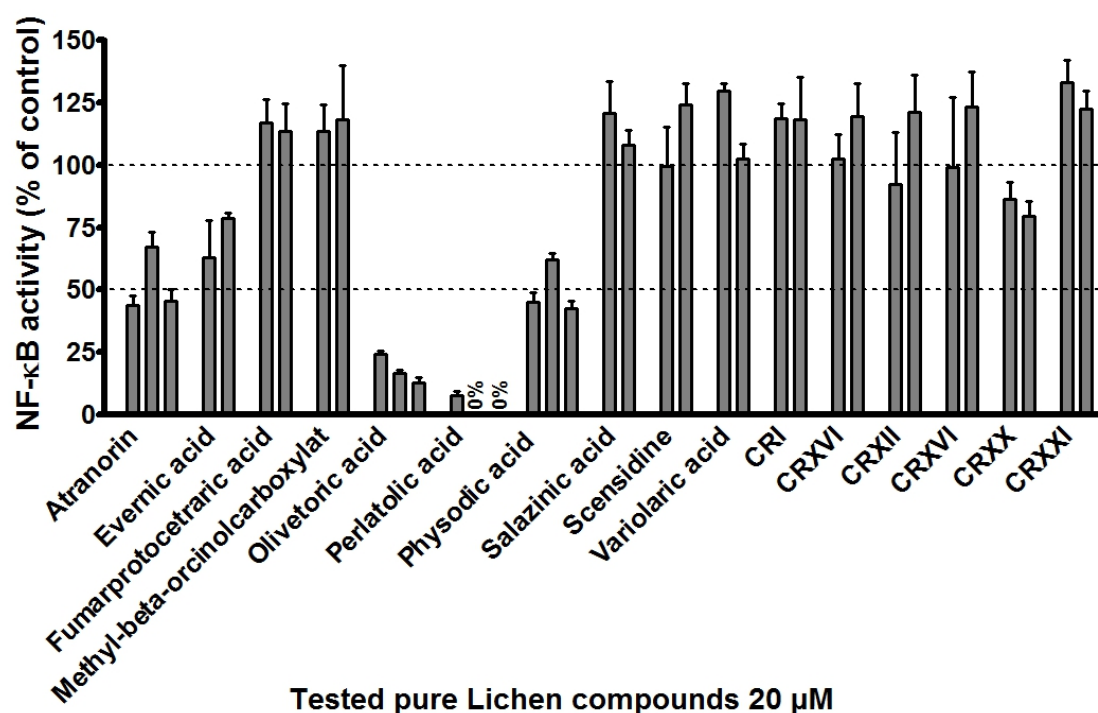


**Figure 26: Effects of pure substances found in lichens on NF- $\kappa$ B activity.** HEK 293/NF- $\kappa$ B-luc cells were treated with the samples (20  $\mu$ M) 30 min prior to TNF- $\alpha$  stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase signal was normalized to EGFP-derived fluorescence. Samples were tested in 2-3 independent experiments shown as bars grouped together assigned to the sample code. Each bar represents the mean  $\pm$  SD NF- $\kappa$ B activity of one experiment done in quadruplicate compared to the vehicle control (experimental medium supplemented with 0.1% DMSO).

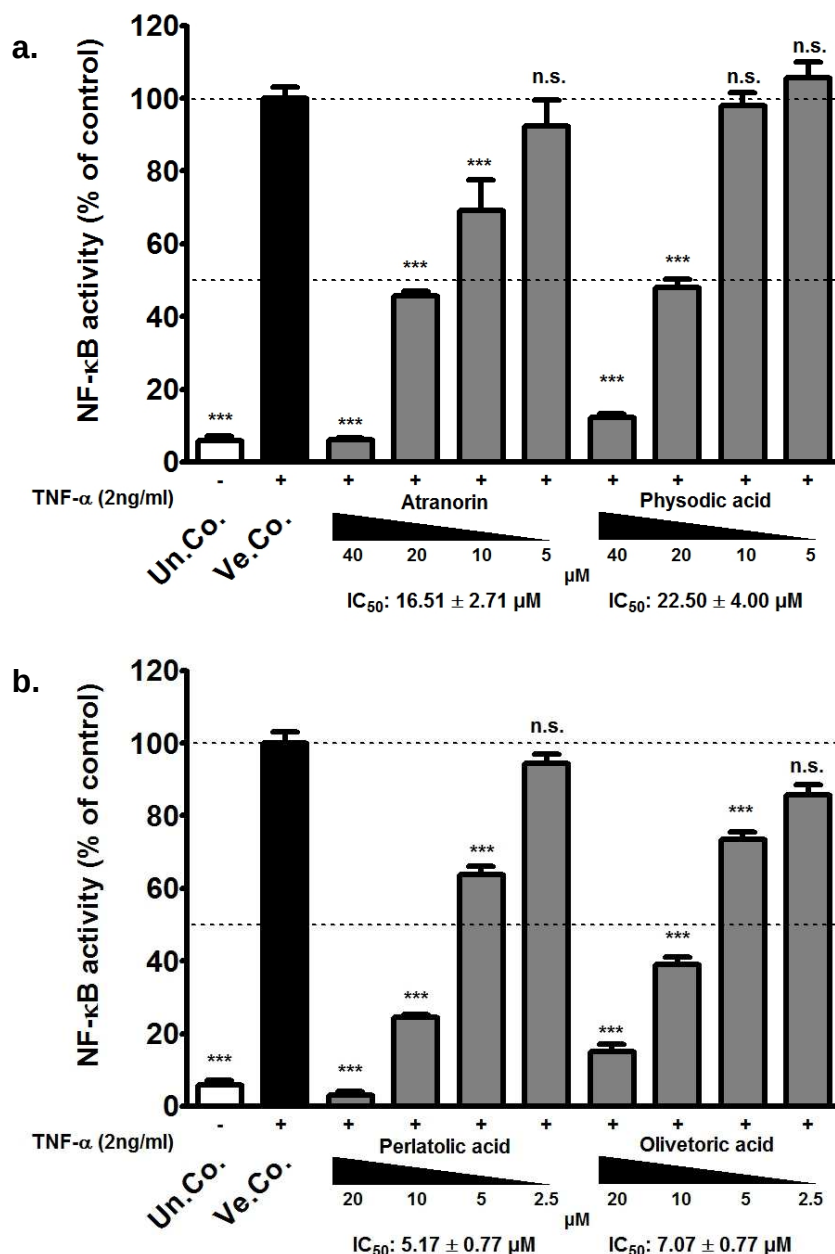


**Figure 27: Dose-response experiments with the active lichen pure substances atranorin, physodic acid, perlatic acid, and olivetoric acid.** HEK 293/NF-κB-luc cells were treated with different compound concentrations 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase signal was normalized to EGFP-derived fluorescence. Bars show the mean ± SD NF-κB activity of three independent experiments done in quadruplicate compared to the vehicle control (Ve.Co.: experimental medium supplemented with 0.1% DMSO). \*\*\*:  $p < 0.001$ ; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test). (a) Dose-response of the moderately active compounds atranorin and physodic acid tested at 40, 20, 10, and 5 μM. (b) Dose-response of the highly active compounds perlatic acid and olivetoric acid tested at 20, 10, 5, and 2.5 μM.

**Figure 26** shows the results of the testing of 16 pure compounds frequently found in lichens on NF- $\kappa$ B activity in a concentration of 20  $\mu$ M. 12 compounds did not show considerable activity at that concentration with the NF- $\kappa$ B activity in both experiments being above 50% compared to the DMSO control. These substances are: CRI (1140), CR XII (1152), CRXVI (1151), CRXV (1153), CRXX (1154), CRXXI (1155), evernic acid (1148), fumarprotocetraric acid (1143), methyl-beta-orcinolcarboxylat (1141), salazinic acid (1146), scensidine (1150), and variolaric acid (1144). Atranorin (1142) and physodic acid showed a moderate NF- $\kappa$ B inhibitory potency in the initial experiments at 20  $\mu$ M. Atranorin inhibited NF- $\kappa$ B activity by 33%, 55%, and 56% and physodic acid by 38%, 55%, and 57% at 20  $\mu$ M for all results. Perlatolic acid and olivetoric acid showed a higher inhibition at the same concentration (20  $\mu$ M). Olivetoric acid showed an inhibitory activity of 76%, 83%, and 87%, while perlatolic acid showed an even higher inhibition with 92% in one of the experiments and total inhibition (100%) in the other two tests. Consequently dose response experiments were done with all four active substances (**Figure 26**) to determine their  $IC_{50}$  (**Figure 27a and 27b**).  $IC_{50}$  was determined to be  $22.50 \pm 4.00$   $\mu$ M for physodic acid,  $16.51 \pm 2.71$   $\mu$ M for atranorin,  $7.07 \pm 0.77$   $\mu$ M for olivetoric acid, and the most potent substance was perlatolic acid with an  $IC_{50}$  of  $5.17 \pm 0.77$   $\mu$ M.

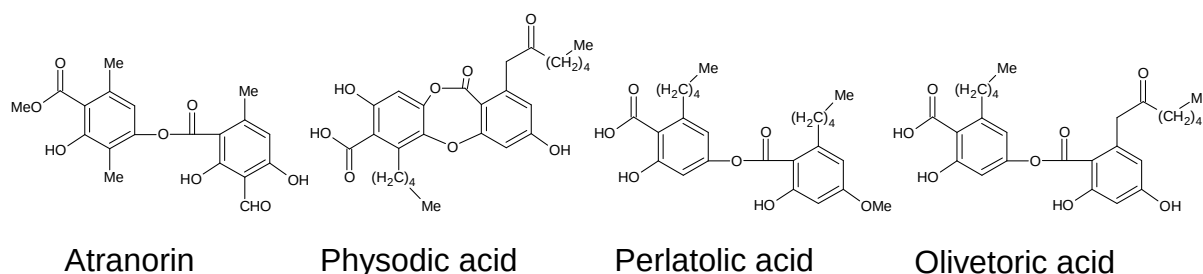


**Figure 28: Structures of the active lichen secondary metabolites.**

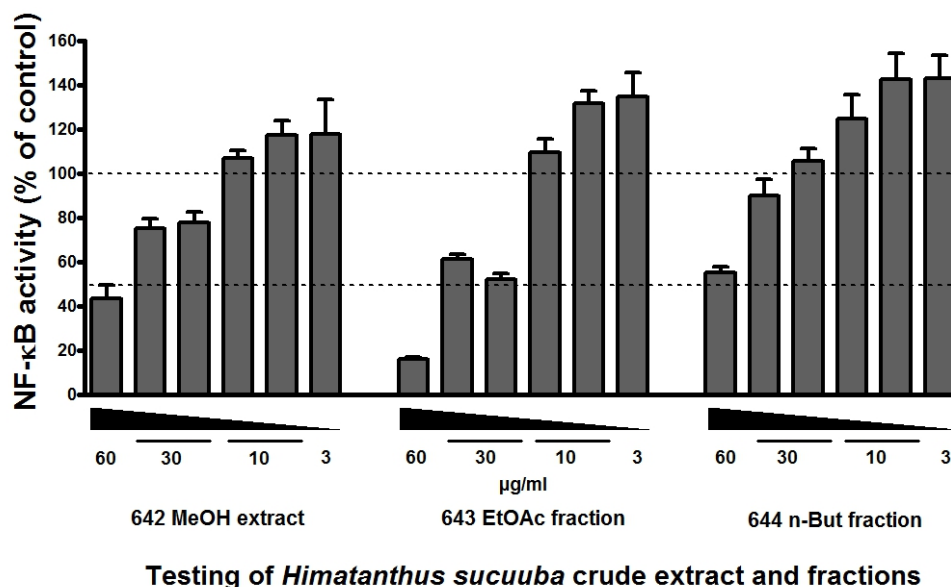
4.3.2. Plumericin from *Himatanthus sucuuba* M. Arg., Apocynaceae

**Figure 29: Effects of *Himatanthus sucuuba* cortex crude MeOH extract and its fractions on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with different sample concentrations for 30 min. Afterwards NF-κB activation was induced with TNF-α (2 ng/ml) for 4 h. Cells were lysed and luminescence and fluorescence were measured. Luciferase signal was normalized to the EGFP-derived fluorescence. 30 µg/ml and 10 µg/ml concentrations were tested twice while 60 µg/ml and 3 µg/ml were tested once. Each bar represents the mean ± SD of NF-κB activity in % of vehicle control of one independent experiment done in quadruplicate. EtOAc: ethyl acetate; n-But: n-butanol.

The crude MeOH extract of *Himatanthus sucuuba* cortex (642) and its EtOAc fraction (643), as well as the n-butanol fraction (644) have been tested for NF-κB inhibitory activity (**Figure 29**). NF-κB activity was not lower than control for the extract 642 at 3 and 10 µg/ml. At concentration of 30 µg/ml it only showed a low potency with 22% and 25% inhibition and there was 56% inhibition at 60 µg/ml.

Fraction 643 was very active at 60 µg/ml with 84% inhibition. 30 µg/ml inhibited the activity by 39% and 48%. Lower concentrations did not show any activity. 644 showed some considerable inhibition of 45% only at 60 µg/ml.

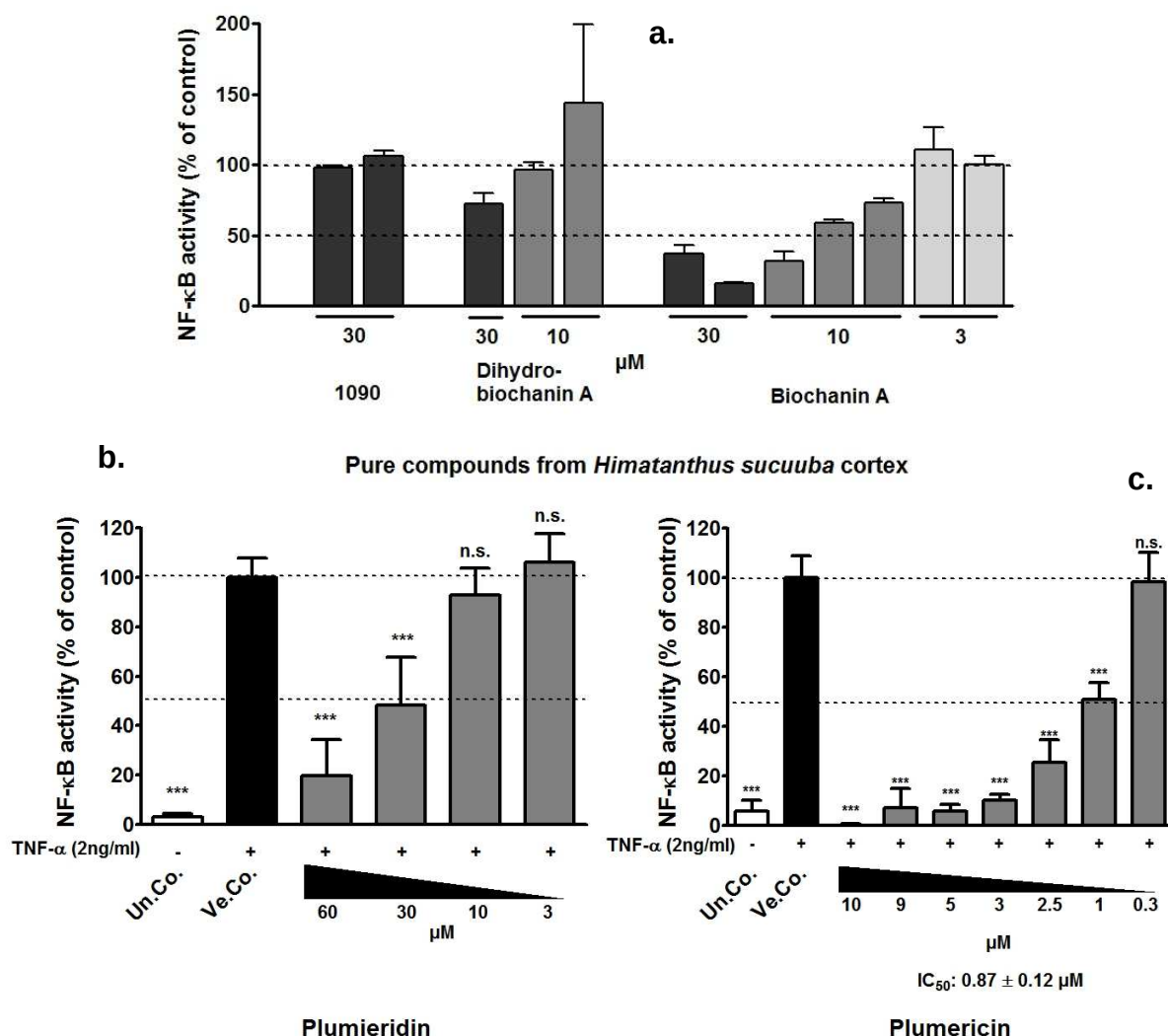


**Figure 30: Effects of *Himatanthus sucuuba* pure compounds on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with the test samples 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase signal was normalized to the EGFP-derived fluorescence. (a) Samples were tested in 2-3 independent experiments in different concentrations (3, 10 and/or 30 μM) represented by bars in different colors for the different concentration and grouped together assigned to the compound name or sample code. Each bar represents the mean ± SD NF-κB activity of one experiment done in quadruplicate. (b and c) Dose response experiments of the potent NF-κB inhibitor plumericin (c) and the structural related plumieridin (b). Bars show mean ± SD NF-κB activity in % of vehicle control of three (c) and five (b) independent experiments done in quadruplicate. \*\*\*:  $p < 0.001$ ; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test).

Several pure compounds (Figure 32) were isolated from the most active EtOAc fraction and have been tested for inhibition of TNF-α-induced NF-κB activity (Figure 30a). A previously unknown substance (1090) was isolated and tested twice, but it was inactive at 30 μM. Dihydrobiochanin, a flavonoid was tested twice at 10 μM and

once in a higher concentration (30  $\mu\text{M}$ ), but it did not show any significant activity as well. Biochanin A was tested in three concentrations (30, 10, 3  $\mu\text{M}$ ). It was not effective at 3  $\mu\text{M}$ , had moderate activity at 10  $\mu\text{M}$  (27%, 41%, 68%), and high to moderate activity at 30  $\mu\text{M}$  (63%, 84%). **Figure 30b** shows the outcome from the testing of plumieridin in dose-response experiments. Low (3, 10  $\mu\text{M}$ ) concentrations did not show a significant inhibition ( $p > 0.05$ ,  $n = 5$ , ANOVA/Bonferroni). However, when applied at 30  $\mu\text{M}$  and 60  $\mu\text{M}$  plumieridin showed a significant inhibition ( $p < 0.001$ ) compared to vehicle control, with 52% and 80% inhibition respectively.

The most potent substance found in the bark of *Himatanthus sucuuba* was plumericin. It was tested in dose-response experiments (**Figure 30c**). Plumericin showed no significant activity in a concentration of 0.3  $\mu\text{M}$ . But a concentration as small as 1  $\mu\text{M}$  already showed significant ( $p < 0.001$ ,  $n = 3$ , ANOVA/Bonferroni) inhibition with a mean of 49%, whereas 2.5  $\mu\text{M}$  plumericin inhibited NF- $\kappa\text{B}$  activity by 75%. Concentrations  $\geq 3 \mu\text{M}$  potently inhibited NF- $\kappa\text{B}$  activity to the background level. The  $\text{IC}_{50}$  of plumericin was determined to be  $0.87 \pm 0.12 \mu\text{M}$ .

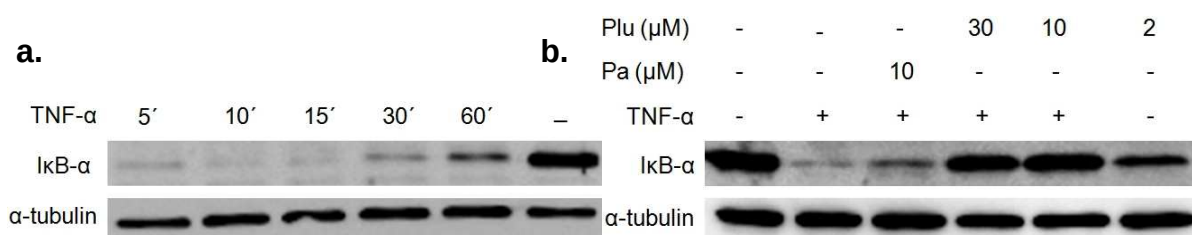


**Figure 31: Inhibitory effect of plumericin on TNF- $\alpha$ -induced I $\kappa$ B- $\alpha$  degradation.** (a) Time course experiment of I $\kappa$ B degradation after TNF- $\alpha$  stimulation. HUVEctert cells were treated with TNF- $\alpha$  (10 ng/ml) for the indicated time periods. Cell lysates were analyzed using western blot technique to detect I $\kappa$ B- $\alpha$  degradation and  $\alpha$ -tubulin. (b) Concentration-dependent inhibitory action of plumericin on TNF- $\alpha$ -induced I $\kappa$ B- $\alpha$  degradation. HUVEctert cells were treated with the indicated concentrations of plumericin or parthenolide (positive control, 10  $\mu\text{M}$ ) for 30 min prior to TNF- $\alpha$  treatment (10 ng/ml; 10 min). Cell lysates were analyzed using western blot technique to detect I $\kappa$ B- $\alpha$  degradation and  $\alpha$ -tubulin. Shown blots are representatives of (a) two and (b) four independent experiments. Pa: Parthenolide; Plu: Plumericin.

Since plumericin was the most potent NF- $\kappa\text{B}$  inhibitor found in this work further experiments have been performed, addressing the mechanism of action of this compound. To determine if plumericin inhibits the NF- $\kappa\text{B}$  signaling cascade upstream or downstream from the degradation of I $\kappa$ B- $\alpha$ , western blot experiments with HUVEctert cells were performed. To first determine the time point where I $\kappa$ B- $\alpha$  is

degraded the most after TNF- $\alpha$  treatment, a time course experiment has been performed (**Figure 31a**). Therefore the HUVECtert cells were stimulated with TNF- $\alpha$  for 5, 10, 15, 30, and 60 min. The highest state of degradation with almost no I $\kappa$ B- $\alpha$  left was seen after 10 min, and the later time points already displayed progressive I $\kappa$ B- $\alpha$  recovery. Consequently 10 min TNF- $\alpha$  treatment was used to investigate the effect of plumericin on I $\kappa$ B- $\alpha$  degradation. **Figure 31b** shows the strong inhibition of I $\kappa$ B- $\alpha$  degradation by plumericin at all tested concentrations (2, 10, 30  $\mu$ M) as compared to cells only treated with TNF- $\alpha$  showing almost full degradation.

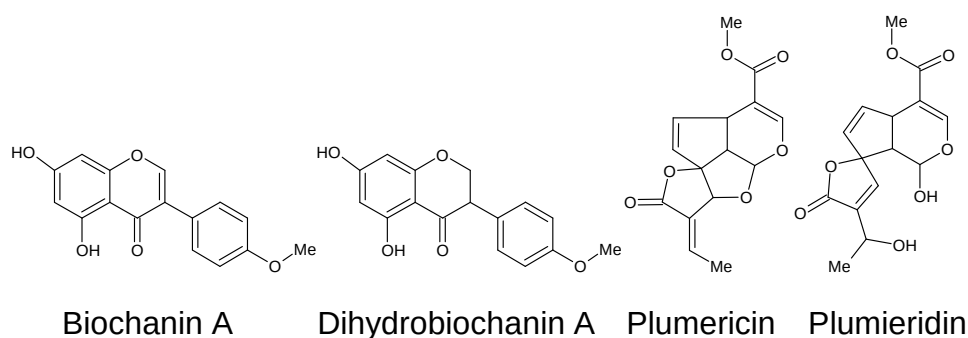


**Figure 32: Structures of the isolated substances from *Himatanthus sucuuba*.**

#### 4.4. Computational approach (samples delivered from collaboration partners in Innsbruck)

##### 4.4.1. Predicted p50 binders

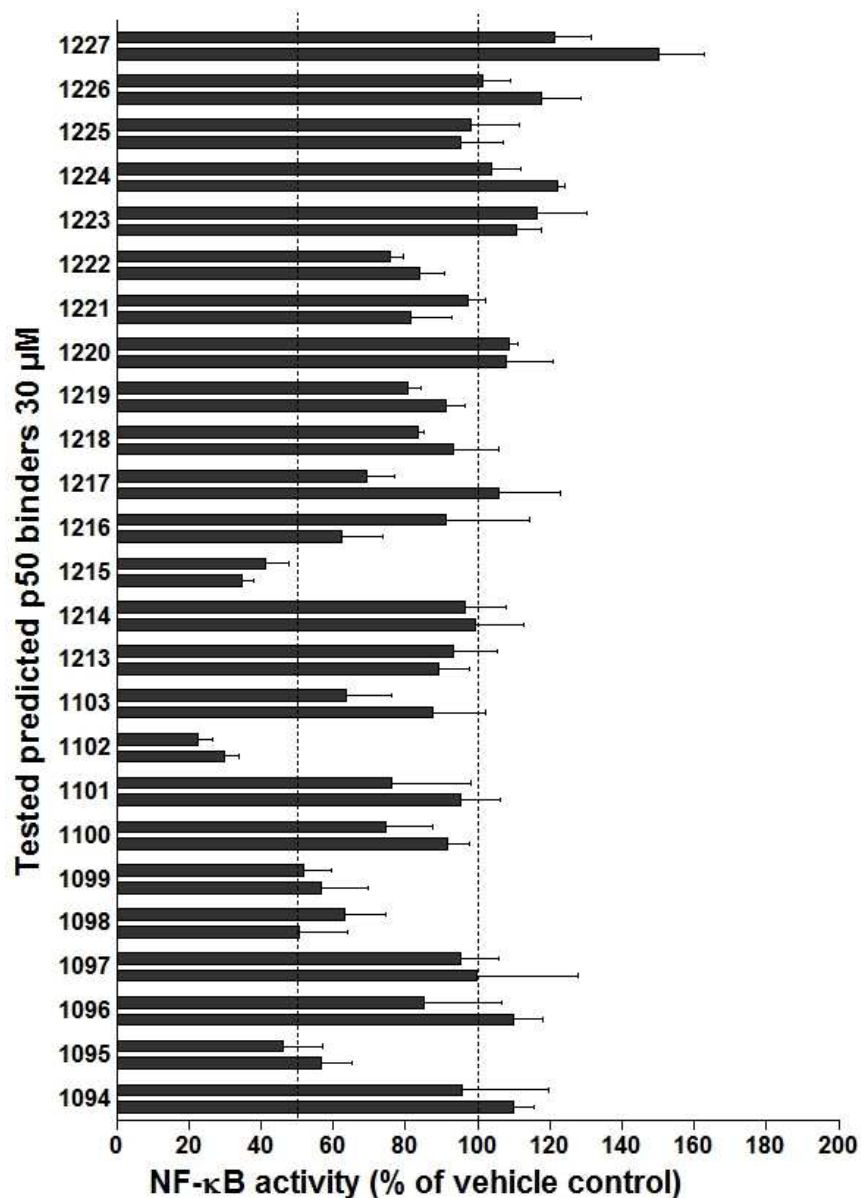


**Figure 33: Effects of all tested predicted p50 binders on NF- $\kappa$ B activation.** HEK 293/NF- $\kappa$ B-luc cells were treated with the samples (30  $\mu$ M) 30 min prior to TNF- $\alpha$  stimulation (2 ng/ml; 4 h). Cells were lysed and luminescence and fluorescence were measured. Luciferase activity was normalized to the EGFP-derived fluorescence. Samples were tested in two independent experiments shown as two bars grouped together and assigned to the sample code. Each bar represents the mean  $\pm$  SD NF- $\kappa$ B activity of one experiment done in quadruplicate in % of the vehicle control (experimental medium supplemented with 0.1 % DMSO).

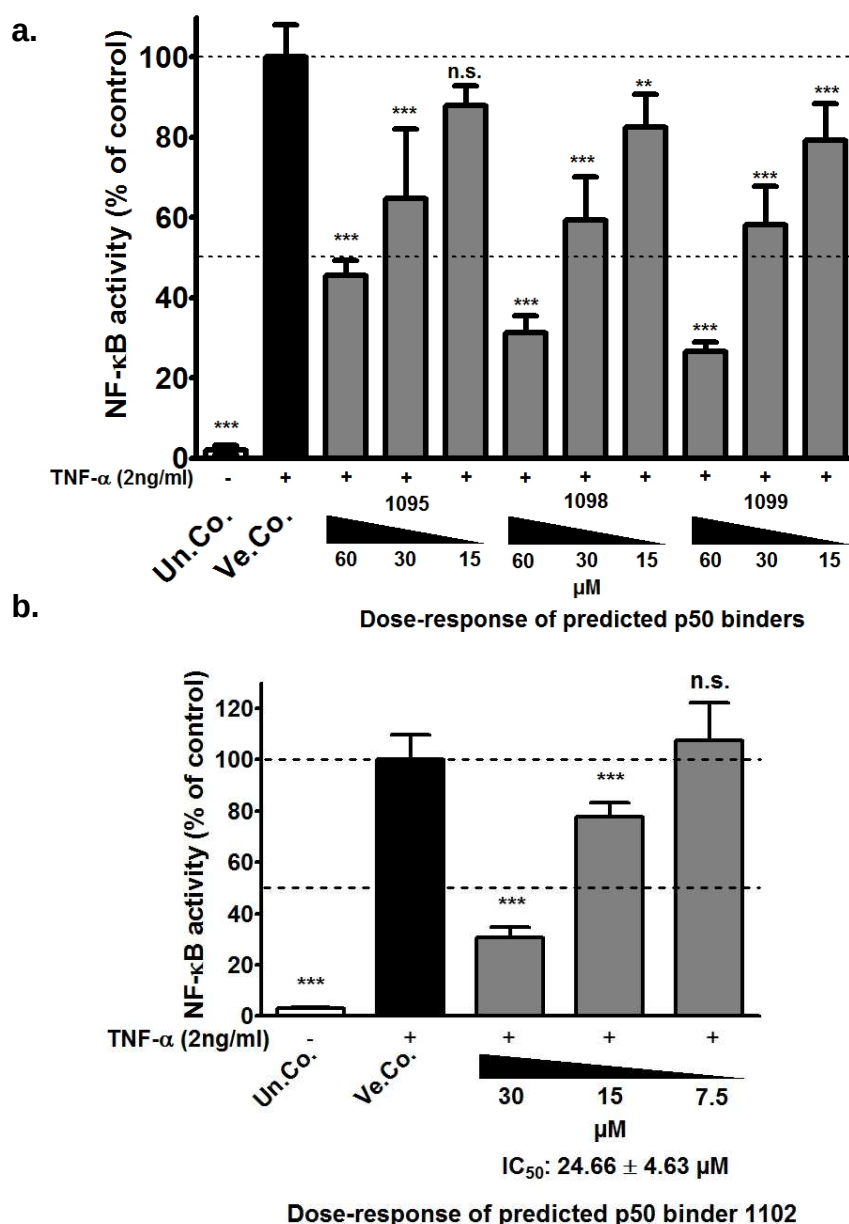


**Figure 34: Dose-response experiments with some of the predicted p50 binders on NF-κB activity.** HEK 293/NF-κB-luc cells were treated with increasing sample concentrations 30 min prior to TNF-α stimulation (2 ng/ml; 4 h). Luminescence and fluorescence were measured after cell lysis. Luciferase activity was normalized to EGFP-derived fluorescence. (a) Tested concentrations were 15, 30, and 60 μM. Bars show the mean ± SD NF-κB activity of four independent experiments done in quadruplicate. (b) Dose response experiment for IC<sub>50</sub> calculation of sample 1102. The figure shows means ± SD of the NF-κB activity obtained in three independent experiments done in quadruplicate. \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; n.s.: not significant (one-way ANOVA followed by Bonferroni's post-test).

Initially 25 predicted p50 binders found in a computational screening have been tested in two independent experiments done in quadruplicate at 30 μM (**Figure 33**). 20 of those substances did not inhibit NF-κB activity considerably. 3 compounds

showed border line activity with an inhibition close to 50% compared to the vehicle control (1095, 1098, 1099). Hence, they were further tested in dose response experiments to investigate the inhibitory effects more precisely (**Figure 34a**). Two further substances displayed even higher activity in the initial tests (**Figure 33**). 1215 showed 59% and 65% inhibition and 1102 inhibited NF- $\kappa$ B activity by 70% and 77%. In concentration-dependent experiments 1098 and 1099 showed a significant inhibition through all concentrations. 1095 was not as active and did not show a significant inhibition at 15  $\mu$ M. All samples showed a relevant inhibitory activity with over 50% inhibition only in a concentration of 60  $\mu$ M. 1095 showed a mean inhibition of 54%, 1098 of 69%, and 1099 of 73%. Thus the detected inhibition with these three compounds was not very high in spite of the high concentration applied. 1102 was the most potent p50 binder found in this work. It was tested in dose response experiments (**Figure 34b**). It showed a significant NF- $\kappa$ B inhibitory action in concentrations of 15  $\mu$ M (22%) and 30  $\mu$ M (69%). IC<sub>50</sub> was determined to be  $24.66 \pm 4.63$   $\mu$ M. **Figure 35** shows the structures of the virtually predicted p50 binders which were active (**Figure 35a**) compared to the structure of one which was not active (**Figure 35b**).

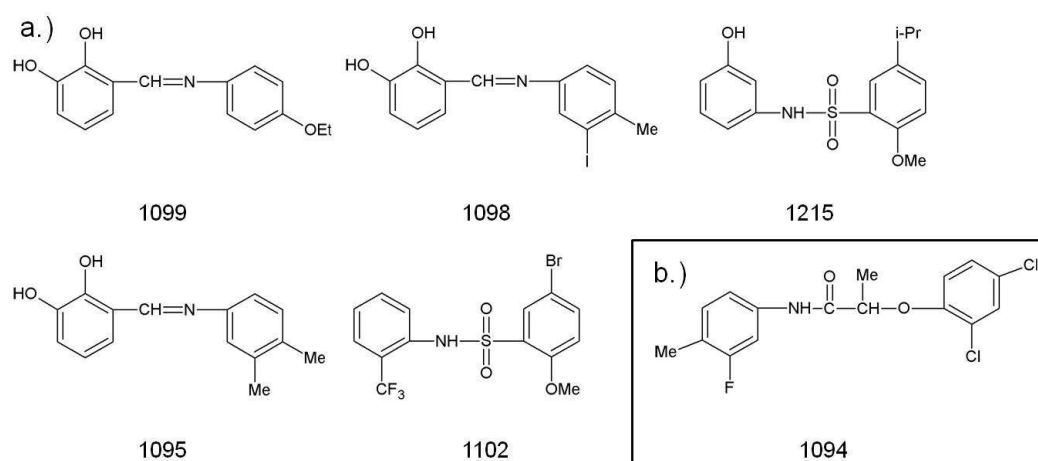


**Figure 35: Predicted p50 binders.** (a) The five predicted p50 binders which showed an NF- $\kappa$ B inhibiting action. (b) One example of a virtually predicted p50 binder that did not show any activity.

## 5. Plant extracts directly inhibiting the luciferase enzyme

### 5.1. Ethnopharmacological approach (samples delivered from collaboration partners in Innsbruck)

#### 5.1.1. *Morus alba* L., Moraceae

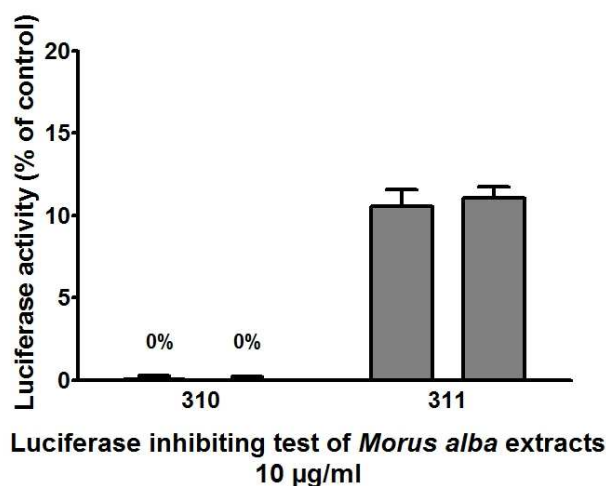


**Figure 36: Effects of *Morus alba* on luciferase activity.** NF- $\kappa$ B pathway in HEK 293/NF- $\kappa$ B-luc cells was stimulated with TNF- $\alpha$  (2 ng/ml) for 4 h. Samples (10 µg/ml) were added 15 min prior cell lysis. Cells were lysed and luminescence and fluorescence were measured. Luciferase signal was normalized to the EGFP-derived fluorescence. Each bar represents the mean  $\pm$  SD luciferase activity in % of vehicle control (experimental medium supplemented with 0.1% DMSO) of one independent experiment done in quadruplicate.

The effect of extracts 310 and 311 was already tested in previous works for NF- $\kappa$ B inhibition as well as for PPAR $\alpha$  and PPAR $\gamma$  activation. Both experimental methods were done with a luciferase reporter gene assay. Surprisingly the extracts induced low luciferase activity indicating antagonistic action on PPAR receptors and inhibiting effects on NF- $\kappa$ B at the same time. The outcomes of both tests taken together raised the question if these extracts are not simply inhibiting the luciferase enzyme itself rather than specifically inhibiting NF- $\kappa$ B and the PPARs.

**Figure 36** shows the results of the experiments performed to address direct luciferase inhibition by *Morus alba* extracts 310 and 311. HEK 293/NF- $\kappa$ B-luc cells were treated with TNF- $\alpha$  (2 ng/ml; 4 h). Cells were treated with the corresponding sample (10 µg/ml) 15 min prior to cell lysis. Such treatment time is too short to influence the NF- $\kappa$ B-mediated luciferase gene expression, but would be still sufficient to directly inhibit the luciferase enzyme that has been already synthesized. For

extract 310 no luminescence was measured indicating a total inhibition of the luciferase enzyme. Extract 311 showed a very high luciferase enzyme inhibitory potency as well, since both times only a mean of 11% luciferase activity compared to vehicle control was observed.



## **F. Discussion**

## F. Discussion

### 1. Investigation of ubiquitous plant constituents

Polyphenols, chlorophyll, and epicatechin gallate are natural ubiquitous constituents in plants. Extracts generated out of plant material consequently contain a huge amount of these substances and may therefore cause false results if any of these constituents interferes with the utilized bioassay. While tannins are known for their ability to form tight complexes with metal ions, polysaccharides, and proteins, chlorophyll may interact with fatty acids.<sup>29</sup> Hence it was important to investigate the effect of these plant constituents on the assay that has been used in this work.

Pure chlorophyll A/B, and the polyphenols epicatechin gallate, and tannic acid have been tested. No inhibition of NF- $\kappa$ B activity was observed (**Figure 12**). Consequently it can be assumed that no false positive results can be caused by these constituents in the utilized luciferase reporter gene assay. However, this experimental setting does not allow any conclusions on possible false negative results. Since the amount of the tested compounds is mostly many times higher than the amount of potentially active secondary metabolites, these results do not allow a conclusion on the possible concealing or diluting effect on active substances.

To additionally investigate any false negative results, extracts of two representative plants *Malva* sp. and *Glechoma hederacea* were made at our department.<sup>30</sup> Three different separating methods were used to remove chlorophyll and polyphenols, respectively. In this work some of these extracts were tested for their ability to inhibit NF- $\kappa$ B activation (see **2.1.2** and **2.1.3** in this chapter).

The results showed that depending on the used method and the constituent whose removal is desired, either a higher or a lower activity can be achieved after depleting. Active compounds can be also removed with the depleting steps, since the removing methods are not specific, showing the importance of the validation of the used depletion method. However, if only the desired ubiquitous constituents are removed, it is possible to enrich the extract with the active constituents leading to a higher activity.

Overall the performed study suggest that it is profitable to remove ubiquitous constituents in order to get an enriched extract while simultaneously using HPLC profiling to detect lost constituents and control the effectiveness of the depletion.<sup>30</sup>

## 2. Discovery of plant extracts with an NF- $\kappa$ B inhibitory action and effects of depletion from ubiquitous plant constituents

### 2.1. *Dryopteris filix-mas*

During the development of the VOLKSMED database plants with traditional use in rheumatic diseases, gout, as well as for all kind of aches affecting the musculoskeletal system were compiled, among others. The analysis of these plants showed that ferns were the most commonly used plants for external application in inflammation related complaints. In the traditional use there was no distinction between the different fern species.

*Dryopteris filix-mas* extracts from plant material collected in 2006 were already positively tested in an *in vivo* mouse-ear-edema-test, where the DCM extract and the 100% and 70% MeOH extracts showed positive effects compared to indomethacin.<sup>31,32</sup>

So far no other scientific investigations, concerning the anti-inflammatory effects of *Dryopteris filix-mas*, can be found.

In the experiments performed in this work (**Figure 13**) the SPE fractions from extract 998 (plant material collected in 2007) all seemed to contain some part of the potent constituents found in the extract, since they all showed an inhibition higher than 50%. From extracts 999 (plant material collected in 2009) and 1000 (collected also in 2009 but directly frozen without drying) only one fraction out of three per extract seemed to contain the active constituents, since only this fraction showed an activity whereas the other two did not. The highest amount of active constituents seemed to always be concentrated particularly in the 70% MeOH fractions, since out of each of the three SPE fractions these always were the most active ones. Therefore, it can be assumed that the active constituents have a polarity similar to that of the 70% MeOH solvent. Other conclusion concerning the active compounds can be drawn from the results obtained from the tests with samples 999 and 1000. Both were generated from the same plant material collection (in 2009) but one time the plant was dried (999) and one time freshly frozen (1000). 70% MeOH SPE fractions of both extracts showed similar activity, indicating that the active compounds do not metabolize through drying.

Samples 493 (plant material collected in 2007) and 1001 (plant material collected in 2009) represent DCM extracts still containing chlorophyll and each was compared to

the chlorophyll-depleted extract generated from the same plant collection. These extracts were 997 (2007) and 999 (2009), respectively. In both cases the chlorophyll-depleted extracts showed a higher activity. These experiments indicate that the active compounds might be enriched through the chlorophyll removing step, while the bulk amount of chlorophyll seems to conceal or just dilute the active constituents in extracts 493 and 1001.

Further activity-guided fractionation, especially of fractions gained from the dried fern collected in 2007, should be done, since they showed the highest activity.

### 2.2. *Glechoma hederaceae*

*Glechoma hederacea* is a widely spread plant in Europe and is used in the traditional Austrian medicine. In addition to a lot of other utilizations it is used against common cold, mucosa inflammations, sinusitis, and inflammatory diseases like gout and rheumatic arthritis.<sup>33</sup> *Glechoma hederacea* herb is known to contain 0.03-0.1% essential oil, tannins, flavonoids, triterpene carbonic acids, and contains traces of alkaloids like hederacin A and B.<sup>2</sup> Besides these components it was described to contain ursolic-acid related compounds.<sup>34</sup> Ursolic acid and the related compound oleanolic acid were found to be inhibitory in the utilized NF- $\kappa$ B assay (**Figure 21**).

*Glechoma hederacea* was previously described to have an anti-inflammatory action by inhibiting IFN- $\gamma$ -and LPS-stimulated production of the pro-inflammatory cytokines IL-12p70 and TNF- $\alpha$  in mouse peritoneal macrophages.<sup>35</sup> Rosmarinic acid analogues found in *Glechoma hederacea* were described to show inhibitory effects on TNF- $\alpha$ -induced NF- $\kappa$ B luciferase reporter activity in HepG2 cells.<sup>36</sup>

*Glechoma hederacea* seems to be a plant containing very potent constituents with NF- $\kappa$ B inhibitory action. Fractions 1062 and 1060 (**Figure 14**) seem to concentrate the active constituents since they showed the highest activity of all fractions. Further bioassay-guided fractionation should be done to characterize the active constituents. MeOH extracts 713-715 were gained from the same crude extract but were polyphenols-depleted using different methods. Rosmarinic acid was successfully removed from all three extracts. The results obtained upon testing of these samples show the importance of the use of a proper separation method since extract 713 was very active and extracts 714 and 715 were not active at all (see also section 1. in this chapter). The bulk polyphenols removal method used to gain extract 713 was the only of the three different methods which resulted in an enrichment of the substance

identified as cyclic hydroxamic acid 2 benzoxazolinone.<sup>30</sup> Since benzoxazolinone derivatives were reported to show an anti-inflammatory potency before<sup>37</sup> it can be assumed that it is one active constituent amongst others responsible for the observed activity. To confirm this hypothesis additional experiments with this substance should be done, as well as bioassay-guided fractionation of the extract could be further performed to investigate if there are any other active constituents. Also further experiments with fractions obtained from extracts not depleted from polyphenols should be done since rosmarinic acid analogues, which are polyphenols, could be removed with these methods as well, but may show an anti-inflammatory activity, as observed before.<sup>36</sup>

### **2.3. *Malva* sp.**

The term *Malvae folium* (Ph.Eur.) summarizes the dried leaves of the two species *Malva neglecta* Wallr. and *Malva sylvestris* L.. *Malvae folium* is known to cure ailments related to coughing.<sup>2</sup> In traditional Austrian medicine it is used to cure all kinds of inflammatory ailments.<sup>33</sup>

Out of the four different DCM extracts tested in this work only sample 28, being the single extract still containing chlorophyll, showed activity. Depleting steps for chlorophyll removal have been shown to remove other constituents as well.<sup>30</sup> Since the chlorophyll-depleted extracts do not show any activity compared to the activity of the crude DCM extract this might indicate that all separation methods led to a removal of active constituents. Further activity-guided experiments to characterize the active compounds should consequently be done with crude extracts still containing chlorophyll.

## **3. Discovery of plant extracts with an NF- $\kappa$ B inhibitory action**

### **3.1. *Lonicera japonica***

The TCM drug Rendongteng is the dry cane of *Lonicera japonica* Thunb. In TCM it is used as an antiphlogistic and bacteriostatic in the treatment of fever, dysentery, abscess, and rheumatic swelling.<sup>20</sup>

*Lonicera japonica* was found to have a phototoxic effect on human lung carcinoma cells.<sup>38</sup> The indicated antiphlogistic effects in TCM are supported by a publication in

which *Lonicera japonica* flower boiled aqueous extracts inhibited PGE<sub>2</sub> synthesis activity of COX-1 and COX-2 in Sf9 cells. However, the tested concentrations were quite high with an IC<sub>50</sub> value of 15 mg/ml for COX-2 and 56 mg/ml for COX-1, respectively.<sup>39</sup> Another anti-inflammatory effect of an aqueous extract of *Lonicera japonica* was shown by inhibition of trypsin-induced mice paw edema<sup>40</sup>, supporting application for treatment of rheumatic swelling.

Besides other constituents, luteolin is a prevalent compound in *Lonicera japonica* since it can be found in the flowers<sup>41</sup>, leaves<sup>42</sup>, and has also been isolated from a MeOH extract of the stems.<sup>43</sup> Luteolin was found to be a potent anti-inflammatory substance which was shown to inhibit I $\kappa$ B- $\alpha$  degradation and nuclear translocation of p65 NF- $\kappa$ B, as well as NF- $\kappa$ B-dependent reporter gene expression in phorbol 12-myristate 13-acetate- and A23187-stimulated HMC-1 cells.<sup>41</sup> LPS-induced DNA binding of NF- $\kappa$ B was also shown to be decreased by luteolin.<sup>44</sup>

It remains to be investigated if the main active component of the stem extracts examined in this work is luteolin as well. But since luteolin is soluble in organic solvents, and the tested crude extract was made with DCM, it seems likely that the used extract contains this potent flavonoid.

The sephadex fractions 1348 and 1349 generated out of the initial extract were the most active fractions found (**Figure 18**). It can be assumed that these fractions contain the greatest part of the active constituents and a further examination of these by bioassay-guided fractionation and isolation is recommended.

### 3.2. *Melampyrum* sp.

*Melampyrum arvense* was previously described to have an antiprotozoal activity<sup>45</sup> and an antioxidant capacity of *Melampyrum barbatum* flower and leaf was described as well.<sup>46</sup> But so far no other scientific investigations are known to back up the anti-inflammatory effects or way of action. Therefore it is of interest to further perform a broad investigation concerning this plant with potential interesting outcomes.

The MeOH extract 279 showed a strong inhibitory potency which indicates some very active constituents (**Figure 16**). Until now only fractions and pure compounds from the poorly active DCM extract 277 were tested and did not show much activity. Far more interesting would be the activity-guided fractioning of the active MeOH extract 279.

### 3.3. *Peucedanum ostruthium*

*Peucedanum ostruthium* rhizomes are specifically used in traditional Austrian medicine for cervical and pharyngeal inflammations as well as inflammation in general. Their use is also recommended for cardiovascular problems, gastrointestinal diseases, cough treatment, or improvement of wound healing.<sup>33</sup> The rhizomes are also used as an ingredient of teas, liqueurs, and bitters in the alpine region.<sup>47</sup>

Antimycobacterial activity of a DCM extract and the isolated compound ostruthin was described before<sup>48</sup> as well as an acetylcholinesterase inhibition caused by another DCM extract.<sup>49</sup> *Peucedanum ostruthium* rhizome extract and the isolated compound ostruthin were also found to be inhibitors of vascular smooth muscle cell proliferation at our department.<sup>50</sup> Antiphlogistic and antipyretic activities of an EtOH root extract were shown in a carrageenan-induced rat paw edema test.<sup>51</sup> In all cases coumarin derivatives were found to be the active constituents.

12 coumarins were isolated from *Peucedanum ostruthium* rhizomes at our department. 8 were tested previously and did not show any activity.<sup>52</sup> In this work so far only ostruthin out of the 4 tested coumarins was active (**Table 15, Figure 17**).

In conclusion in this work the DCM extract was found to have a good inhibitory action and a very high activity of the MeOH subfractions was also found previously.<sup>52</sup> But so far no highly active compound could be identified. Hence additional fractionation should be considered to gain the main active constituent or maybe several active compounds which could be collectively responsible for the potent NF- $\kappa$ B inhibition of *Peucedanum ostruthium* rhizomes besides ostruthin.

## 4. Plant extracts showing NF- $\kappa$ B inhibition and toxic side-effects

### 4.1. *Notopterygium* sp.

The root and rhizome of *Notopterygium incisum* (QiangHuo) are known in the TCM for their diaphoretic, antipyretic, and analgesic effects.<sup>53</sup> Thus, are used to cure rheumatism and to relieve headache appearing with a common cold.<sup>54</sup>

Bioassay-guided fractionation and isolation, which was done by collaboration partners from the University of Graz, and the corresponding experiments, which were performed at the Department of Pharmacognosy at the University of Vienna, led to

the discovery of six polyacetylenes as novel selective activators of PPAR $\gamma$ .<sup>55</sup> MeOH extracts of *Notopterygium incisum* underground parts have also been found to have anti-oxidative effects.<sup>56</sup> Constituents found in an n-hexane extract of *Notopterygium incisum* roots and rhizomes have already been shown to have an anti-inflammatory activity by inhibiting 5-LOX and COX *in vitro*. Phenethyl ferulate, faltarindiol, and (-)-bornyl ferulate were thereby identified as the main active constituents.<sup>57</sup> Another pharmacological study revealed analgesic effects, confirming the traditional use against pain. Thereby notopterol was identified as the active compound which also showed anti-inflammatory activity and a drug metabolism inhibitory effect in the liver<sup>58</sup> which was recently described to be caused by inhibition of CYP3A4.<sup>59</sup>

The DCM extracts and fractions tested in this work indicated cytotoxic effects which should be further investigated with proper methods. The underground parts of *Notopterygium* contain linear furocoumarins like notopterol which was mentioned before.<sup>54,58,60</sup> Furocoumarins, well known also as psoralenes, induce photochemical reactions with DNA.<sup>61</sup> Furocoumarins from *Notopterygium incisum* showed anti-proliferative, apoptotic, and cytotoxic activities against human and rat cancer cell lines. Amongst other linear furocoumarins, these effects were also caused by notopterol.<sup>54</sup> The described furocoumarins were gained out of a chloroform extract whereas DCM was the solvent used to obtain the extracts tested in this work. Since DCM and chloroform molecules only vary by one chlorine atom, the two solvents have a similar rank in the elutropic series. Hence it can be assumed that the extract used in this work contained furocoumarins which could be responsible for the observed toxic effects.

Most toxic compounds seem to be concentrated in fraction 1247 (**Figure 20b**) since there were almost no living cells left in the well after 4.5 h sample treatment.

In summary the performed experiments not only showed a potential toxicity or unspecific inhibitory effect of *Notopterygium incisum* root and rhizome extract but also a very high inhibitory action on NF- $\kappa$ B. Consequently, bioassay-guided fractioning should be done to identify all active and potentially toxic compounds. The above mentioned published results which reported an anti-inflammatory effect, were gained by more downstream experiments compared to the NF- $\kappa$ B luciferase reporter assay used in this work. Hence experiments with isolated compounds should be further performed to identify the more upstream targets and to get a better insight on the mode of action.

## 5. Discovery of pure compounds with NF- $\kappa$ B inhibitory activity

### 5.1. Oleanolic and ursolic acids from *Clematis armandii* caulis

Chuanmutong is the dry stem of *Clematis armandii* Franch or *Clematis montana* Buch.-Ham. and is used in TCM as an antipyretic and diuretic in the treatment of edema and rheumatism.<sup>20</sup>

Oleanolic acid is a natural pentacyclic triterpenoid carboxylic acid found in *Clematis armandii* caulis. Oleanolic acid was described to have antidiabetic effects in streptozotocin treated rats<sup>62</sup> and has been reported to have anti-inflammatory activities as it inhibited four different secretory phospholipases A2 gained from different sources.<sup>63</sup>

The structurally related compound ursolic acid was described to have an anti-inflammatory action in activated macrophages, which has been proposed to be caused due to an inhibition of NF- $\kappa$ B activation. On the other hand this compound showed a pro-inflammatory activity in unstimulated macrophages showing the dual effects of this constituent depending of the conditions.<sup>64</sup>

The oleanolic acid tested in this work was isolated by the group of Prof. Bauer out of one previously tested active *Clematis armandii* caulis DCM extract. The highest amount that was found in it was 0.714%. Corresponding to the tested concentration of the active extract, which was 10  $\mu$ g/ml, this would mean that oleanolic acid should show an inhibition on NF- $\kappa$ B activation in a concentration of 0.16  $\mu$ M, if it was the main active compound. However, the concentration in which oleanolic acid was experimentally observed to be active was >10  $\mu$ M (**Figure 21**). Consequently, the active concentration is too high to indicate that oleanolic acid is the main active agent in this extract. Therefore further investigations have to be done to identify the main active constituent, or possibly several active substances which collectively lead to the observed NF- $\kappa$ B inhibitory effect caused by the extract.

Ursolic acid is a very close related compound but is has not been described to occur in *Clematis armandii* caulis. It differs only by the positioning of one methyl group. Both substances showed a similar activity. Their structures could be further used as leads for the discovery of more active compounds.

## 5.2. Kurarinone and isokurarinone from *Sophora flavescens* root

*Sophora flavescens* root is used in TCM to treat fever, inflammatory disorders, ulcers, and skin burns.<sup>65</sup> It is also described to be used as an diuretic and for the treatment of diarrhea, gastrointestinal hemorrhage, and eczema.<sup>20</sup> The roots are known to contain flavonoids and alkaloids.<sup>20</sup> An anti-inflammatory effect of a flavonoid enriched fraction was shown through inhibition of LPS-induced PGE<sub>2</sub> production by direct inhibition of COX-2.<sup>65</sup> The compounds kurarinone and kuraridin were found to inhibit LPS-mediated NF-κB activation by blocking IκB-α degradation.<sup>28</sup>

Kurarinone was found to be an instable structure in this work, since the first testing showed no inhibition while the repeated experiment with a fresh sample showed a high activity (**Figure 22**). It was reported to be stable for temperatures below 25 °C and instable at 80 °C.<sup>66</sup> But it is not clear what may have caused the loss of activity during the storage in our laboratory.

For kurarinone, which seems to be more active than isokurarinone, additional dose-response experiments for the determination of IC<sub>50</sub> should be done. So far no anti-inflammatory effect was described for isokurarinon, making it a novel NF-κB inhibitor. Further investigation of both compounds should be made to determine their upstream targets and mode of action.

## 5.3. Lichen compounds atranorin, olivetoric, perlatolic, and physodic acids

Atranorin, olivetoric, perlatolic, and physodic acids are known lichen secondary metabolites.<sup>67-69</sup> Atranorin, olivetoric, and perlatolic acids have been reported to have an antimicrobial activity.<sup>68</sup> Atranorin was also shown to inhibit COX-1 in a dose-dependent manner and a dose-independent partial inhibition of COX-2 was also demonstrated.<sup>67</sup> But so far no other studies which would indicate an anti-inflammatory activity of those mentioned lichen compounds have been published. In this work the NF-κB inhibitory activity is demonstrated for the first time, thus four new potent substances are identified which are relevant for further investigation.

In summary two new moderately active compounds have been identified with an IC<sub>50</sub> of 22.50 ± 4.00 μM for physodic acid and 16.51 ± 2.71 μM for atranorin, as well as two novel highly effective compounds with an IC<sub>50</sub> of 7.07 ± 0.77 μM for olivetoric acid and 5.17 ± 0.77 μM for perlatolic acid (**Figure 26**).

#### 5.4. Discovery of plumericin as an NF- $\kappa$ B inhibitor

*Himatanthus sucuuba* is an Amazonian tree whose latex and stem bark are used in popular Amazonian medicine to cure a lot of ailments. The ethnopharmacological use of the bark differs by the regions it is used. In Peru it has been reported to be used to improve wound-healing, to treat tumors, boils and swellings, to have laxative and vermicide abilities, and to cause hallucinations. In Brazil it is said to have anti-ulcer and aphrodisiac effects. The Caboclos in the Amazon region use it as an analgesic and antitussive.<sup>70</sup>

Bioactivity-guided fractionation and isolation led to the discovery of five pure compounds, one new compound and four known structures (**Figure 32**). Among them was the highly active spirolactone iridoid plumericin. It has been previously described to have antimicrobial<sup>71</sup>, antileishmanial<sup>72</sup>, antifungal<sup>73</sup>, and also cytotoxic<sup>74</sup> activities. In this work a high NF- $\kappa$ B inhibitory potency has been detected for plumericin on TNF- $\alpha$ -stimulated HEK 293/NF- $\kappa$ B-luc cells, with an IC<sub>50</sub> of  $0.87 \pm 0.12$   $\mu$ M (**Figure 30**). The performed western blot experiments further indicated that the inhibitory target of plumericin can be found rather upstream in the NF- $\kappa$ B signaling cascade, as it inhibits I $\kappa$ B- $\alpha$  degradation in TNF- $\alpha$ -stimulated HUVECtert cells (**Figure 31**).

Since that result still leaves open a lot of possible ways of upstream action, the precise mechanism of inhibition should be further investigated using other experiments. First the effect on I $\kappa$ B kinase (IKK) which is responsible for the phosphorylation of I $\kappa$ B could be investigated. After phosphorylation by IKK, I $\kappa$ B is being ubiquitinated by ligases and conjugating enzymes and then degraded by the proteasome. Therefore effects on these machineries should also be considered. Depending on the outcome of these experiments even more upstream targets could be considered for investigation.

A proposed mechanism of action of some known NF- $\kappa$ B inhibitors is the reaction with cystein sulfhydryl groups of proteins by a Michael-type addition.<sup>75</sup> Plumericin and plumieridin both have two  $\alpha,\beta$ -unsaturated carboxyl groups each (compare **Figure 32**) which are potential Michael acceptors. Consequently this kind of reaction with proteins from the NF- $\kappa$ B signaling pathway should be considered as a possible way of inhibiting their action.

Plumieridin has a hemiacetal structure while plumericin forms its acetal, thereby gaining another cycle making plumieridin a three cyclic spirolactone iridoid and plumericin a four cyclic one. While the first stated structural similarities could be responsible for the NF- $\kappa$ B inhibitory potency observed for both structures, the last stated difference and particularly the exocyclic methylene group of plumericin seem to cause the higher effectiveness of that compound.

Additional experiments have been performed at the Department of Pharmacognosy at the University of Vienna, which showed that plumericin also inhibits surface expression of the adhesion molecules E-selectine, VCAM-1, and ECAM-1 in TNF- $\alpha$ -stimulated HUVEctert cells. Additionally the transcription factor Nrf2 which regulates various genes involved in the antioxidant response, prevention of cancer, vascular diseases, and inflammation was shown to be strongly activated.<sup>55</sup>

In summary plumericin was the most active compound found in this work. Further investigation on its mode of action e.g. to determine the direct upstream targets is of great interest. Additional investigations of the plant could be also considered since the traditional use shows a lot of interesting and different utilization. Therefore scientific evidence is needed to back up all the various traditional uses of *Himatanthus sucuuba*.

## 6. Plant extracts with a direct inhibitory activity on the luciferase enzyme

### 6.1. *Morus alba*

*Morus alba* is used especially in TCM and different parts of the plant are traditionally used to treat different ailments. Leaves are used as an antiphlogistic agent and root and bark are used as an anti-inflammatory remedy and as a diuretic. Additionally the dry young branches are used to treat arthritis and rheumatism and the fruits are used as a tonic and sedative.<sup>20</sup>

Antioxidant properties of extracts and compounds have been documented.<sup>76,77</sup> Published literature on inflammatory effects of *Morus alba* can be mostly found for leave extracts.<sup>78-80</sup>

An anti-inflammatory activity of the root bark has been reported by our collaboration partners in Innsbruck. The extract showed an inhibition of the enzymatic activity of COX-1 and COX-2.<sup>81</sup>

The flavonoid cudraflavone B was isolated from *Morus alba* root and showed promising anti-inflammatory properties *in vitro* by decreasing TNF- $\alpha$  transcription.<sup>82</sup>

*Morus alba* root extracts were previously described to show an NF- $\kappa$ B inhibitory potency in an NF- $\kappa$ B luciferase reporter gene assay in LPS/IFN- $\gamma$ -stimulated RAW 264.7 cells.<sup>83</sup>

However, the outcomes obtained in this work by testing *Morus alba* extracts for NF- $\kappa$ B inhibition showed that this extract rather contains constituents, which are potent inhibitors of the firefly luciferase enzyme, thus making the above stated published results questionable.

Still the obtained results do not allow conclusions on the activity of the tested samples, because neither positive nor negative effects on NF- $\kappa$ B activity of that sample can be monitored. With this obtained result it was illustrated that the utilized luciferase reporter gene assay has some limitations, making it unsuitable in a rare case like this.

The firefly luciferase inhibiting agent in the extracts remains to be characterized. So far a lot of different luciferase inhibitors like luciferin analogues, benzothiazoles, fatty acids, anesthetics, and alcohols, when used in higher concentrations, are known to inhibit the firefly luciferase.<sup>84</sup>

Since previous publications, as mentioned above, indicated an anti-inflammatory activity of *Morus alba*, the extracts and its fractions should be considered to be tested in other more suitable assays to clarify the possible effect on NF- $\kappa$ B signalling.



## **G. References**

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## **H. Appendix**

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### 1. Abbreviations

#### A

ABA : Acrylamide and bisacrylamide

ANOVA : Analysis of variance

APS : Ammonium persulfate

#### B

BAFF : B-cell activating factor

BSA : Bovine serum albumine

#### C

COX : Cyclooxygenase

#### D

DCM : Dichloromethane

DD : Death domain

DMEM : Dulbecco's modified Eagle's medium

DMSO : Dimethylsulfoxide

DTT : Dithiothreitol

#### E

EBM : Endothelial basal medium

ECL : Enhanced chemiluminescence

EDTA : Ethylenediaminetetraacetic acid

EtAc : Ethyl acetate

EtOH : Ethanol

#### G

GRR : Glycine rich region

#### H

HBS : HEPES buffered saline

HEK 293 : Human embryonic kidney cell line 293

HEPES : Hydroxyethylpiperazineethanesulfonic acid

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|             |                                                                                              |
|-------------|----------------------------------------------------------------------------------------------|
| HMC-1       | : Human mast cell line 1                                                                     |
| HPLC        | : High performance liquid chromatography                                                     |
| HUVEC(tert) | : Human umbilical vein endothelial cells (expressing human telomerase reverse transcriptase) |

**I**

|        |                                     |
|--------|-------------------------------------|
| ICAM-1 | : intercellular adhesion molecule 1 |
| IKK    | : Inhibitor of $\kappa$ B kinase    |

**L**

|             |                        |
|-------------|------------------------|
| LOX         | : Lipoxygenase         |
| LPS         | : Lipopolysaccharide   |
| LT- $\beta$ | : Lymphotoxin- $\beta$ |

**M**

|       |                                                    |
|-------|----------------------------------------------------|
| MEKK3 | : Mitogen-activated protein kinase kinase kinase 3 |
| MeOH  | : Methanol                                         |

**N**

|                |                                      |
|----------------|--------------------------------------|
| NBD            | : NEMO binding domain                |
| NEMO           | : NF- $\kappa$ B essential modulator |
| NF- $\kappa$ B | : Nuclear factor kappa B             |
| NIK            | : NF- $\kappa$ B inducing kinase     |

**P**

|      |                                      |
|------|--------------------------------------|
| PAGE | : Polyacrylamide gel electrophoresis |
| PBS  | : Phosphate buffered saline          |
| PGE  | : Prostaglandin E                    |
| PMSF | : Phenylmethanesulphonylfluoride     |

**R**

|      |                                  |
|------|----------------------------------|
| RHD  | : Rel homology domain            |
| RIP1 | : Receptor-interacting protein 1 |

### S

|      |                                    |
|------|------------------------------------|
| SD   | : Standard deviation               |
| SDS  | : Sodium dodecyl sulfate           |
| SGCC | : Silica gel column chromatography |
| SODD | : Silencer of death domain         |
| SPE  | : Solid phase extraction           |

### T

|               |                                                          |
|---------------|----------------------------------------------------------|
| TAB           | : TAK1- binding protein                                  |
| TAD           | : Transactivation domain                                 |
| TAK1          | : Transforming growth factor $\beta$ -activated kinase 1 |
| TBS-T         | : Tris buffered saline- Tween                            |
| TEMED         | : N,N,N',N'-tetramethylethylene diamine                  |
| TNF- $\alpha$ | : Tumor necrosis factor alpha                            |
| TRADD         | : TNF-R1 associated death domain protein                 |
| TRAF2         | : TNF receptor associated factor 2                       |
| TRIS          | : Tris(hydroxymethyl)aminomethane                        |

### V

|        |                                     |
|--------|-------------------------------------|
| VCAM-1 | : Vascular cell adhesion molecule-1 |
|--------|-------------------------------------|

## 2. Curriculum vitae

### Personal Data

|                          |                         |
|--------------------------|-------------------------|
| First and family name:   | Anna Maria Grzywacz     |
| Date and place of birth: | 29.04.1986, Łódź/Poland |
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### Educational background

|                 |                                                  |                    |
|-----------------|--------------------------------------------------|--------------------|
| 10/2005-09/2011 | Study of pharmacy, University of Vienna, Austria |                    |
| 09/2000-07/2005 | Solitude Gymnasium                               | Stuttgart, Germany |
| 09/1998-07/2000 | Herder Gymnasium                                 | Berlin, Germany    |
| 09/1993-07/1998 | Kath. Schule St. Paulus                          | Berlin, Germany    |
| 09/1992-07/1993 | Triftgrundschule                                 | Berlin, Germany    |

### Pharmaceutical work and training history

|                  |                                                                                                                                       |
|------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| 10/2010-06/2011  | Tutor in the laboratory exercises "Quantitative pharmazeutische Analytik", University of Vienna                                       |
| 02/2011          | Internship at the AGES PharmMed in the OMCL institute                                                                                 |
| 03/2010-07/2011  | Diploma thesis in the research laboratories of the "Molecular Targets" group at the Department of Pharmacognosy, University of Vienna |
| 07/2009; 08/2010 | Vacation job in the "Westend Apotheke" in Vienna                                                                                      |

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