universität wien

DIPLOMARBEIT

PPARα- AND PPARγ-LUCIFERASE REPORTER ASSAYS APPLIED FOR THE DISCOVERY OF NOVEL AGONISTS FROM NATURAL SOURCES

angestrebter akademischer Grad

Magister der Pharmazie (Mag.pharm.)

Verfasser:	Christian Maier
Matrikelnummer:	0106466
Studienrichtung/Studienzweig (lt. Studienblatt):	A449 Diplomstudium Pharmazie
Betreuerin:	Univ. Prof. Dr. Verena M. Dirsch

Wien, am 31.1.11

Abstract

The need for an effective therapy of diseases related to inflammation is still a challenge. In the modern western society pathologies such as the metabolic syndrome, cancer, allergies, and arthritis belong to the most frequent disorders. The role of inflammation for the development of these diseases has been well studied recently. Several triggers for the development of inflammatory processes could be identified although a lot is still unclear. The progress of that research field though gives hope for the establishment of innovative and more effective anti-inflammatory treatments.

The peroxisome proliferator-activated receptors (PPARs) represent ligand-activated transcription factors, which belong to the nuclear receptor family. Various studies have shown the anti-inflammatory potential of the PPARs, demonstrated by inhibition of proinflammatory cytokines and the antagonism with the "master regulator" of inflammation, NFκB (nuclear factor "kappa-light-chain-enhancer" of activated B cells), among other effects.

Primary aim of this work is the identification and characterization of PPARα and or PPARγ activating compounds, which derive from natural sources and are capable to antagonize inflammatory processes in the cardiovascular system.

This work is done as part of the DNTI (Drugs from Nature Targeting Inflammation) project of the NFN (Nationales Forschungsnetzwerk), an interdisciplinary network, which interlinks the knowledge and the skills of members of pharmacy, chemistry, veterinarian and human medicine from different universities in Austria.

The selection of natural products (NPs) is achieved in two different ways, by an ethnopharmacological, based on the VOLKSMED and TCM databases, and a computational approach, using 3-D multiconformational molecule databases. The ethnopharmacological approach starts with the knowledge of the traditional ways of usage, whereas the computational approach is achieved using ligand based pharmacophore models. A bioassay-guided isolation, respectively isolation of virtual hits will lead to promising bioactive NPs, which are then investigated on basis of cellular assay systems for the molecular mechanisms of their action.

The task of this work within the DNTI project was to apply a luciferase reporter assay to identify activators of PPARα and PPARγ *in vitro*. Active plant extracts which met these

properties were then further fractionated by the respective project partners to detect the active principles.

When pure active compounds are identified, the anti-inflammatory potential towards acute and chronic cardiovascular inflammatory processes will be tested *ex vivo* and *in vivo* by partners at the Medicinal University of Vienna.

Zusammenfassung

Die Entwicklung einer effektiven Therapie von Erkrankungen die mit Entzündungen verbunden sind, ist immer noch eine Herausforderung. In unserer modernen, westlichen Gesellschaft gehören Erkrankungen, wie das Metabolische Syndrom, Krebs, Allergien oder Arthritis zu den am häufigsten auftretenden physischen Fehlsteuerungen. Die Rolle von Entzündungen unter den genannten Erkrankungen wurde in letzter Zeit intensiv erforscht, wobei noch viele Fragen offen bleiben. Der Fortschritt auf diesem Forschungsfeld gibt allerdings Hoffnung, eine innovative und effektivere anti-inflammatorische Therapie etablieren zu können.

Die Peroxisom-Proliferator-aktivierten Rezeptoren (PPARs) sind Liganden gebundene Transkriptionsfaktoren, die zur Familie der nukleären Rezeptoren gehören. Verschiedene Studien haben das anti-inflammatorische Potential der PPARs belegt, das unter anderem durch die Hemmung von proinflammatorischen Cytokinen und dem „Hauptregulator“ von Entzündungen, NFκB, erreicht wird.

Primäres Ziel dieser Arbeit ist die Identifikation und Charakterisierung von PPARα und oder PPARγ aktivierenden und von natürlichen Quellen stammenden Substanzen, die inflammatorische Prozesse im kardiovaskulären System antagonisieren können. Diese Arbeit ist Teil des DNTI (Drugs from Nature Targeting Inflammation) Projekts des NFN (Nationales Forschungsnetzwerk), ein interdisziplinäres Netzwerk, das das Wissen und die Kompetenzen von Mitgliedern aus den Bereichen Pharmazie, Chemie, Veterinär- und Humanmedizin von verschiedenen Universitäten in Österreich verbindet.

Die Selektion von Naturstoffen (Engl.: natural products, NPs) wird auf zwei verschiedenen Wegen erreicht. Einerseits durch einen ethnopharmakologischen Ansatz, basierend auf den VOLKSMED und TCM Datenbanken und andererseits durch einen computergestützten Zugang auf der Basis von multikonformationalen 3-D Moleküldatenbanken. Der ethnopharmakologische Ansatz startet mit dem Wissen über traditionell verwendete Arzneipflanzen, während der computergestützte Zugang über auf Liganden basierende Pharmakophormodelle erreicht wird. Eine Bioassay geleitete Isolation, beziehungsweise Isolation von virtuellen Hits führt zu den entwicklungsfähigen bioaktiven NPs, die anschließend auf der Basis von zellulären Assay-Systemen auf den molekularen Wirkmechanismus untersucht werden.

Die Aufgabe dieser Arbeit innerhalb des DNTI Projekts war die Anwendung eines Luziferase Reportergen-Assays, um Aktivatoren von PPARα und PPARγ *in vitro* zu identifizieren. Aktive Pflanzenextrakte, die diese Eigenschaften erfüllten, wurden dann von den entsprechenden Projecktpartnern weiter fraktioniert, um die Wirkkomponenten festzustellen.

Im Fall der Detektierung von Reinsubstanzen, werden diese *ex vivo* und *in vivo* durch die Partner an der Medizinischen Universität Wien auf ihr antiinflammatorisches Potential gegenüber akuten und chronischen kardiovaskulären inflammatorischen Prozessen untersucht.

A TABLE OF CONTENTS

B Introduction

1. Aim of this work

The potential of drugs deriving from natural sources for the treatment of diseases is reflected by the dimension of all new chemical entities (NCEs) on the drug market. It has been shown, that between 1981 and 2006 among all NCEs the natural products, or compounds which derived from natural products, covered 28% of the newly introduced drugs.[1] This fact reveals that natural products (NPs) still represent an essential and significant source for the discovery of new leads and development of new drugs.

1.1. The role of inflammation

Diseases, which are connected with inflammation (e.g. cancer, auto-immune diseases, allergies, sepsis, the metabolic syndrome, arthritis and atherosclerosis) belong to the most frequently appearing physical disorders.[2] Inflammation arises in response to injury of the tissue caused e. g. by infectious or physical agents, as well as by malignant cells. This makes clear that within such pathological processes inflammation plays an important role.[3]

The effective treatment of inflammation is one of the biggest challenges of our century, especially as adverse effects of the established anti-inflammatory therapies reduce the therapeutical quality.[4]

A central role in inflammation plays the vasculature, as the process is started there by a cytokine-mediated activation, followed by adhesion and transmigration of leucocytes into the surrounding tissue.[5] Here, the nuclear transcription factor NFκB is responsible for a variety of proinflammatory responses, as known target genes of NFκB are adhesion molecules, cytokines, growth factors and enzymes like COX-2. It is known, that this nuclear transcription factor can be activated by proinflammatory cytokines, like TNF-α (tumor necrose factor α) and IL-1 (interleukin-1), as well as by LPS (lipopolysaccharids).[3] For the treatment of inflammation the inhibition of NFκB is of high interest because of the broad spectrum of proinflammatory effects associated with the

activation of this transcription factor.[6] Newer evidence suggest that also the nuclear receptors class 4, the liver X receptor and the PPARs (Peroxisome proliferators activated receptors) are potent inhibitory mediators.[7]

Inflammational response caused by tissue injury is mediated by a variety of transcription factors such as NFκB, activated protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and signal transducer and activator of transcription (STAT). As a consequence the proinflammatory cytokines, chemokines and adhesion molecules are regulated up. Here an activation of PPARγ leads to an efficient suppression of this inflammatory cascade, inhibiting the transcription factors (NFκB, AP-1, NFAT, STAT) and thereby reducing the expression of several cytokines, chemokines and adhesion molecules. Among the known cytokines, inhibited in this way are interleukin 1h (IL-1h), IL-2, IL-6, IL-10, IL-12, tumor necrosis factor α (TNF- α) and interferon-γ (IFN- γ). The monocyte chemoattractant protein-1 (MCP-1) belongs to the chemokines, the intercellular adhesion molecule-1 (ICAM-1), the vascular cell adhesion molecule-1 (VCAM-1) and the platelet/ endothelial cell adhesion molecule-1 (PECAM-1) are adhesion molecules. All these proteins can be inhibited as a consequence of the PPARγ activation. A PPARγ agonism moreover inhibits the expression of the inducible nitric oxid synthase (iNOS) and of the cyclooxygenase-2 (COX-2), two well known enzymes, involved in proinflammatory pathways.[8]

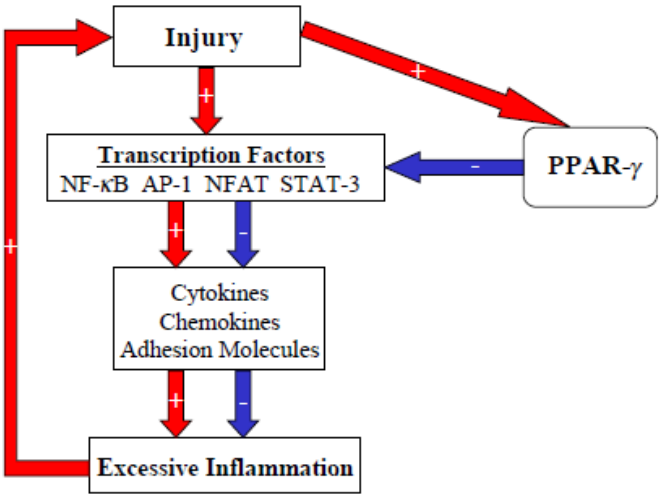


Figure 1: The effect of PPARγ activation on inflammatory pathways.

Picture taken from: Abdelrahman, M. & al. (2005).[8]

1.2. Organization of the DNTI

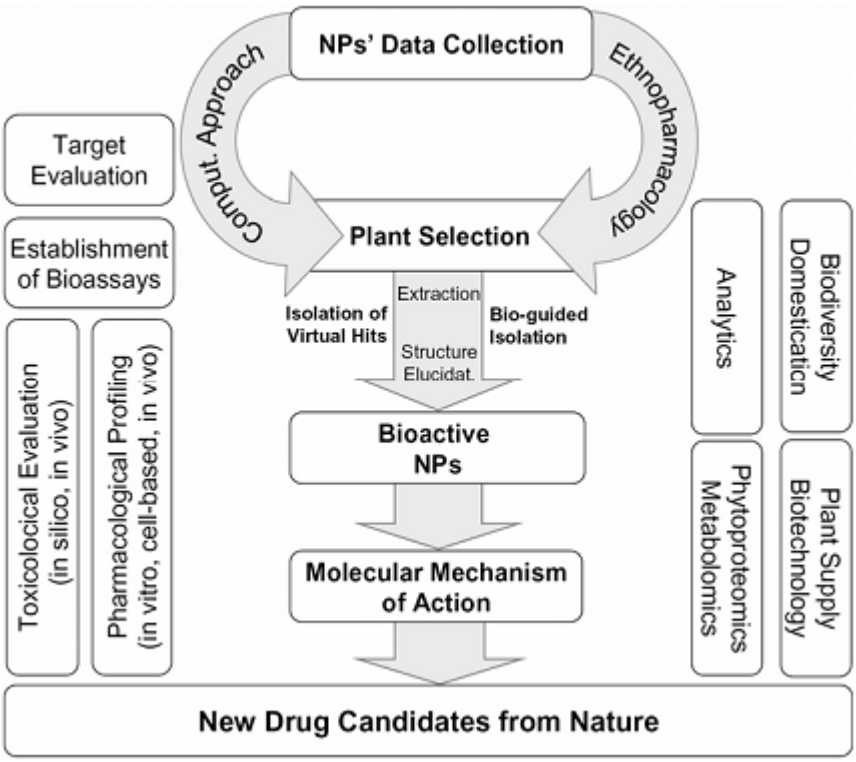


Figure 2: Scheme of the planned workflow of the NFN project.

Picture taken from the NFN-Application DNTI – Drugs from Nature Targeting Inflammation.[9]

This work represents a part of the DNTI (Drugs from Nature Targeting Inflammation) project, which has the primary aim to identify and characterize compounds deriving from natural sources, which are able to antagonize inflammatory processes in the cardiovascular system.

The DNTI project is funded by the Austrian Science Fund (FWF) as a part of the NFN (Nationales Forschungs Netzwerk) program. It combines the scientific knowledge and potential of consortium members with expertise in pharmacy, biology, chemistry, veterinarian and human medicine to discover new drug candidates from nature.

This introduction of the major aims and the collaboratory relations within the different project groups is meant to give a basic overview for the structure of DNTI. The detailed working steps for the investigation of promising new drug candidates from natural

sources are further discussed in chapter 3, where the different approaches to determine bioactive candidates for the PPAR testing are presented.

To identify promising new drug candidates DNTI utilizes two major approaches: one based on computational techniques, and one based on ethnopharmacological knowledge. The selection of promising natural products or plant material is achieved based on both the ethnopharmacological as well as the computational approaches.

The computational approach is mainly conducted by network partners from the University of Innsbruck, who develop 3-D pharmacophore models based upon proteins that are pharmacological targets in inflammation. The computational approach also comprises the creation of 3D multiconformational molecule databases with natural products, which was also realized by project partners from the University of Innsbruck. The created databases contain structures of natural products that are used for virtual screening against the developed pharmacophore models based on proteins relevant for the inflammatory process. Structures that are predicted hits (e.g. pharmacophores that fit with the active binding site of the model of the respective protein involved in inflammation) are then evaluated, as far as their chemical stability, toxicity, drug likeness is concerned.

The ethnopharmacological approach is achieved through knowledge of the traditional usage of medicinal plants out of the VOLKSMED database (focused on Austrian medicinal plants, described in chapter 3) and the Traditional Chinese Medicine (TCM). The isolation of active compounds from promising plants is realized by a bioassay-guided procedure.

Several plants derived from the TCM and from the VOLKSMED database were analyzed as a part of ongoing projects different than DNTI.

Plants deriving from the VOLKSMED and TCM databases are used in collaboration with project partners from the Department of Pharmacognosy at the University of Vienna.

The morphological, phytochemical and biomolecular identification and characterization of plants, containing novel natural products, as well as the development of strategies for the conservation of collected endangered species is done by collaboration partners from the Institute for Applied Botany and Pharmacognosy at the Veterinarian University

of Vienna. The same project partners are also responsible for the cultivation of plants, needed as starting material for further investigations.

After the selection of promising medicinal plants or natural products through the above described computational and ethnopharmacological approaches, the next step is to validate their pharmacological activity. This diploma work is done as a part of the DNTI dealing with the identification of plant extracts or natural products activating PPARα and PPARγ. Upon the identification of plant extract with promising activity bio guided fractionation is further done together with project partners providing expertise in phytochemistry to enable the chemical identification of the responsible bioactive compounds. Suitable cellular assay systems are next used to characterize in details the molecular mechanisms of action of the identified natural products. The most promising pure compounds will be finally forwarded to collaboration partners at the medical University of Vienna for testing of their anti-inflammatory potential *ex vivo* and *in vivo*. The *in vivo* tests models that are used in particular address the effectiveness of the identified compounds in acute and chronic cardiovascular inflammatory processes.[9]

2. The peroxisome proliferator-activated receptors (PPARs)

The PPARs are ligand-activated transcription factors, belonging to the nuclear receptor gene family.[10] Until now three isotypes of PPARs have been identified: PPARα (NR1c1), PPARβ/δ (NR1C2), and PPARγ (NR1C3).[11] These three isotypes show a differential tissue expression. Whereas PPARα is mainly expressed in the liver, kidney, heart, skeletal muscle and brown fat, which are tissues characterized by a high level of fatty acid metabolism, PPARβ/δ is expressed ubiquitously and represents an important mediator regarding the ß-oxidation of fatty acids, energy homeostasis and thermogenesis. PPARγ can be found primarily in the white and brown adipose tissue and limited amounts in the skeletal muscle and heart. Cells of the vascular wall such as endothelial cells and vascular smooth muscle cells, as well as invaded macrophages and foam cells show expression of PPARα and PPARγ.[12] Since the PPARs show a very broad expression pattern, they influence a large number of cell types, including immune cells, as shown in figure 3.[13]

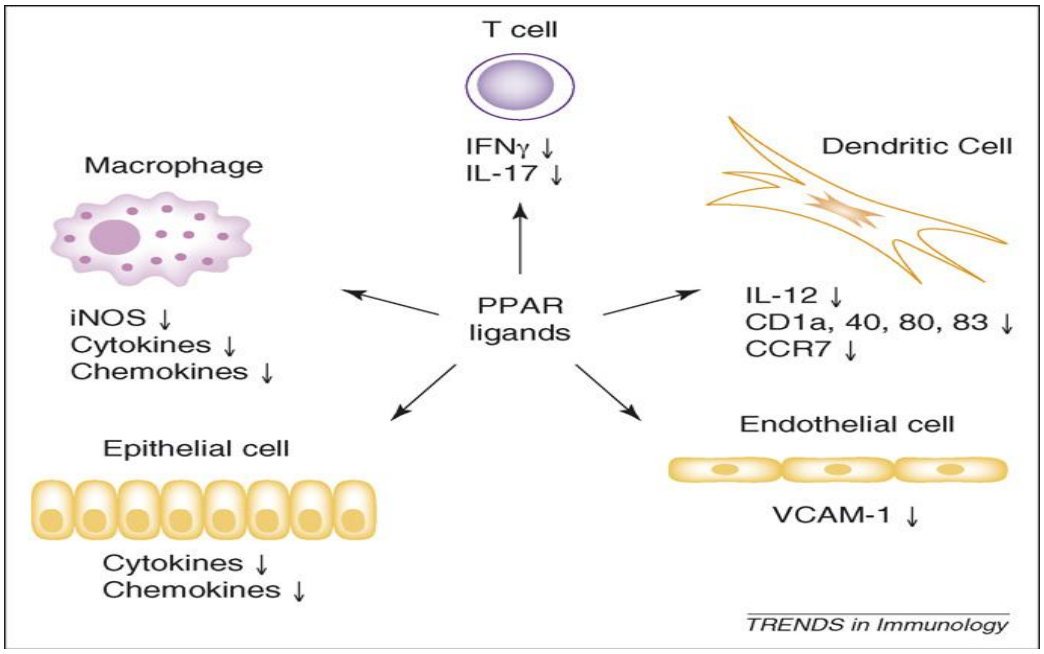


Figure 3: The multiple responses resulting of PPAR activation in different cell types.

Picture taken from: Straus, D. S., and Glass, C.K. (2007).[13]

Native and modified polyunsaturated fatty acids, as well as eicosanoids, are natural activators of PPARs, whereas several synthetical ligands have been also described which bind to the large binding pocket of the PPARs. Known activators of PPARα, PPAR β/δ and PPARγ are for example the fibrates, GW501516 and thiazolidinediones, respectively.[14]

Upon activation by the binding of a ligand, PPARs form a heterodimer with the retinoid-X-receptor (RXR). The heterodimer then binds to peroxisome proliferator-response element (PPRE), a specific DNA sequence located in the promoter of the regulated target genes, leading to stimulation of their transcription.[12]

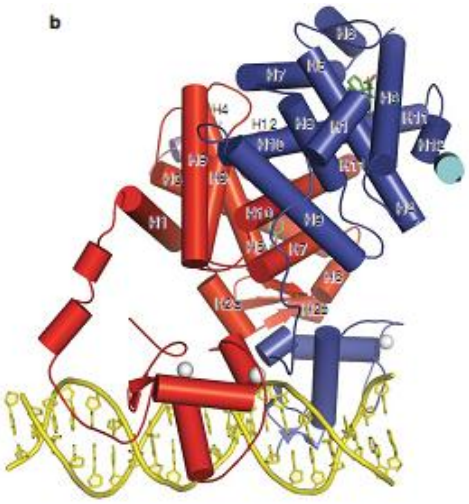


Figure 4: The structure of the PPARγ (red) – RXRα (blue) complex on PPRE.

Picture taken from: Chandra, V & al. (2008)[15]

The PPARs are known to play an essential role in metabolic pathways related to the control of the lipid and glucose homeostasis, and therefore represent highly promising targets for the therapy of metabolic diseases.[16] Newer evidence have shown that the PPARs are also critically involved in the regulation of inflammatory responses, as they control not just the transcription of metabolic but also inflammatory genes.[14]

2.1. PPARα

2.1.1. The metabolic role of PPARα

As the PPARα is highly expressed in tissues with metabolic functions, such as the liver, it plays a key role in the metabolism of fatty acids.[17] Endogenous (unsaturated fatty acids and eicosanoids) as well as synthetic substances as the fibrates are capable of activating the PPARα, which leads to a transcriptional activation of target genes involved in mitochondrial and peroxisomal fatty acid oxidation. By this mechanism, the capacity of the fat storage in the liver is decreased.[18] PPARα is known as the target of gemfibrozil, fenofibrate and clofibrate, a synthetic drug class with hypolidemic effects, used in the therapy of hypertriglyceridemia.[13]

PPARα is, together with the transcription factors sterol regulatory element-binding protein (SREBP-1c) and carbohydrate response element-binding protein (ChREBP), one of the main regulators of the adipogenesis in the liver. PPARα transcriptionally controls the expression of the primary transcription factors SREBP-1c and liver X receptor (LXR), which leads to transcriptional activation of the stearoyl CoA-desaturase, an important enzyme involved in glycolysis and lipogenesis. Therefore PPARα can be defined as balanced lipid sensor, mediating lipolytic as well as lipogenic effects.[19]

2.1.2. PPARα and inflammation

PPARα is not just involved in the regulation of metabolic pathways, but also inflammatory processes.[20] By inhibition of the expression of inflammatory genes, the activation of PPARα can reduce inflammation, provoked by proinflammatory cytokines. The immunosuppressive effects of the PPARα are overall a consequence of an inhibition of proinflammatory gene expression. In this way different proinflammatory transcription factors such as the signal transducer and activator of transcription (STAT), the activator protein-1 (AP-1), the COX-2 enzyme and the nuclear transcription factor NF-κB are inhibited.[18]

As molecular mechanism for the NFκB inhibition, a transcriptional upregulation of the IκBα protein is suggested. This protein is capable of keeping the NFκB in an inactivated

state and thus suppresses this inflammatory cascade by inhibition of the NFκB translocation to the nucleus. Furthermore the PPARα activation reduces the proinflammatory effects of the CAATT/ enhancer binding proteins, linking directly the coactivator glucocorticoid receptor interacting protein-1 (GRIP1) and the transcriptional intermediary factor-2 (TIF2), which represent further important transcription factors involved in inflammation. The IL-6 receptor downregulation and the IL-1 receptor antagonist (IL-1ra) upregulation by PPARα lead to an inhibition of effects induced by proinflammatory cytokines. The IL-1ra is produced endogenously by a variety of cell types, including immune cells.[18]

The multiple effects of PPARα on different inflammatory pathways underline the potential of PPARα agonists as a novel promising option for an anti-inflammatory therapy. Experimental evidences also demonstrate the important regulatory role of PPARα in the context of atherosclerosis development. Proinflammatory genes which play a central role in the pathogenesis of atherosclerosis such as the monocyte chemotactic protein-1 (MCP-1), TNFα, vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and interferone-γ, are suppressed upon activation of PPARα. As a result, especially the development of severe atherosclerotic lesions can be reduced significantly, whereas also proatherogenic effects were recognized, when the lesions were in an early stadium, or the inflammation process has not started yet. However the acute anti-atherosclerotic effects of PPARα activation are underlined by the decrease in the plasma concentrations of C-reactive protein (CRP) and serum amyloid A (SAA), which are acute phase proteins, playing direct as well as indirect roles in the development of atherosclerosis.[18]

The PPARα activation is also suggested to control the expression of genes, which are responsible for cholesterol transport and output of reactive oxygen, therefore playing a critical role in the development of macrophage foam cells relevant for the development of atherosclerotic lesions. The adhesion of leucocytes to activated endothelial cells of the arterial vasculature is also demonstrated to be inhibited by an activation of PPARα. Furthermore also immune cells like macrophages and neutrophils, playing an essential role in acute and chronic inflammation, are influenced by the activity of PPARα. The endogenous, chemotactic, inflammatory eicosanoid Leukotriene B_4 (LTB4) is also a direct ligand of PPARα and induces by activation of PPARα a feedback mechanism leading to its own degradation through enzymes envolved in the β and ω-oxidation.[19]

As a result of the above described effects, the pathogenesis of atherosclerosis is affected in an efficient way by PPARα activation at many different steps.[19]

2.2. PPARγ

2.2.1. The PPARγ agonists: thiazolidinediones and endogenous ligands

PPARγ is the molecular target of the thiazolidinediones (TZD, also known as glitazones), well known for their insulin-sensitizing and anti-diabetic effects, used in the treatment of diabetes mellitus type 2.[21] This class of synthetic drugs represents an efficient therapy option to reduce blood glucose levels particularly, when combined with diet, sulphonylureas, metformin or insulin. The first drug from the class of the glitazones, troglitazone, was approved by the FDA in the USA, but was withdrawn from the market due to dozens of deaths caused by its hepatotoxicity it however was withdrawn from the market. As the other glitazones like pioglitazone and rosiglitazone did not show a similar hepatotoxicity, it was suggested, that the toxicity was caused by the troglitazone-specific tocopherol side chain. Endogenous ligands such as unsaturated fatty acids and the 5-Hydroxyeicosatetraenoicacid (5-HETE) as well as other eicosanoids are also able to stimulate the PPARγ. PPARγ serves as a key regulator in adipogenesis and plays an essential role in insulin signaling and the pathophysiology of obesity.[22]

2.2.2. The role of PPARγ in metabolism

The two isoforms of PPARγ, namely PPARγ1 and PPARγ2, are expressed in the adipose tissue. PPARγ2 is even only expressed in this tissue. These two isoforms are the result of using alternative promoters and differential splicing, resulting in 30 additional amino acids at the N-terminus of PPARγ2. PPARγ plays an important role in the differentiation of adipocytes, since many genes which are involved in the lipid metabolism, such as fatty acid binding protein 4 (FABP4), phosphoenolpyruvat carboxykinase (PEPCK), acyl CoA synthase, lipoprotein lipase, and fatty acid transporters, have PPAR-response elements in their promotors. PPARγ activation in adipocytes leads to an increased uptake of free fatty acids (FFA), which can then be

catabolized or stored in the adipocytes. As a consequence, the circulating FFA in the blood are lowered, with the benefit that the muscle and liver steatosis, known triggers of insulin resistance, decrease. The insulin sensitizing mechanism in the liver leads to a suppression of glucose production, and the sensitizing effect in the muscle leads to an insulin-mediated uptake and storage of glucose in this tissue. The insulin sensitizing effects in liver and muscles are believed to be secondary effects, being a consequence of the PPARγ-induced adipocyte differentiation and gene regulation. Further reported molecular effects, caused by PPARγ activation are the enhancement of the tyrosyl phosphorylation of IRS (insulin receptor substrate) proteins and increased phosphatidyl inositol 3 kinase (PI 3 kinase) activity. The observed increase of the insulin sensitivity in the adipose tissue is a consequence of the PPARγ-mediated induction of the cCBL-associated protein (named after Casitas B-lineage Lymphoma) and IRS2, which are critically involved in the insulin signaling. The PPARγ activation also down regulates the transcription of 11b-hydroxysteroid-dehydrogenase 1 (11b-HSD-1) in the adipose tissue. This enzyme is responsible for the activation of cortison to the higher active cortisol and therefore regulates the glucocorticoid level. It is known, that the 11b-HSD-1 plays an important role in the development of insulin resistance, observed in patients with hypercorticosteroidism. Therefore, some of the insulin sensitizing effects may also be caused by this PPARγ-mediated decrease of 11b-HSD-1 expression. One major factor in the development of an insulin resistance is the proinflammatory cytokine TNF-α. This proinflammatory cytokine is, amongst others, expressed in the adipose tissue and antagonizes the insulin signal transduction, whereas PPARγ activation inhibits the effects of TNF-α (e.g. inhibits the TNF-α-induced downregulation of the expression of glucose transporters (GLUT)).[23]

2.2.3. PPARγ: bridging metabolism and inflammation

Today insulin resistance is not just seen as an isolated pathological process, it is associated with the cardiovascular risk factors obesity, dyslipidemia and hypertension, which are all known triggers for the development of atherosclerosis.[24]

The anti-inflammatory effects of the PPARγ activation are certainly linked to the anti-atherosclerotic action well evident in different animal models. The TZD drugs are known to reduce levels of proinflammatory factors such as the C-reactive protein and antagonize the effects caused by NFκB. In addition to that, adiponectin, another very

important protein with anti-inflammatory and anti-atherosclerotic properties seems to also be affected by the PPARγ. Adiponectin is expressed specifically in the liver and is able to lower glucose, triglycerides and FFA in the blood. It has been observed, that among patients with the metabolic syndrome, the adiponectin plasma levels are low. Adiponectin is in addition to its effect on the metabolism supposed to suppress vascular cell proliferation and has an effect on macrophage activity, processes which are known to be involved in the atherosclerotic pathogenesis. It was shown that the glitazones are able to increase the plasma levels of adiponectin in patients with NIDDM or obesity. Therefore the glitazones in particular, as well as PPARγ agonists in general, are besides their well known metabolic effects, good candidates to combat the pathogenesis of the metabolic syndrome and atherosclerosis.[23]

2.3. PPARβ/δ

Among the three known PPARs, the PPARβ/δ receptor is still the least well explored. Although this protein is found in numerous tissues, until now the specific endogenous ligands for the PPARβ/δ are not well elucidated. The existing experiments with knock out models of mice with PPARβ/δ deficiency however suggest, that this PPAR has a function in the wound healing process, lowers obesity, and similarly to the other two PPAR subtypes, works as a regulator of inflammatory processes.[18]

2.3.1. The metabolic effects of PPARβ/δ

PPARβ/δ activation results in an enhanced fatty acid oxidation in the adipose tissue and in the musculature of the heart. As a consequence, the mass of the adipose tissue lowers, and the contraction of the heart muscle is improved. The type 1 muscle fibers are also increased, which is an additional positive effect, resulting in improved heart persistency. The increased fatty acid oxidation in the adipose tissue and musculature is supposed to have a protective effect in the context of hepatic steatosis.[18]

2.3.2. The role of PPARβ/δ in inflammation

Whereas the anti-inflammatory potential of PPARα and PPARγ is intensively studied, much less is known about the effect of the PPARβ/δ in this context. It seems that this PPAR has dual properties, serving as either proinflammatory or anti-inflammatory transcription factor under different circumstances. In the non-ligand bound status, the PPARβ/δ forms a transcriptional complex with the retinoid-X-receptor α (RXRα) and the B-cell lymphoma-6 protein (BCL-6). The BCL-6 works as a suppressor protein of inflammation. If a ligand binds to the PPARβ/δ, the BCL-6 protein dissociates, and the activated complex starts the transcription of PPARβ/δ-dependent target genes. At the same time the released BCL-6 protein is known to affect macrophage functions by repressing the expression of proinflammatory genes. Further effects related with the pathogenesis of atherosclerosis can be observed in mice models upon activation of PPARβ/δ. Thus for example it has been demonstrated that by inhibition of the pro-atherosclerotic genes VCAM-1, MCP-1, and interferone-α (IFN-α), PPARβ/δ activation diminishes the development of atherosclerotic lesions. The effect on atherosclerotic progression is still controversial in human models, since there is evidence that PPARβ/δ activation in humans seems even to contribute to the atherosclerotic process, as it has been shown, that PPARβ/δ induces lipid accumulation in macrophages. These controversial experimental results underline the importance of further investigations in this topic.[18]

3. Samples

In this section the different approaches of the DNTI project to identify possible new PPARα and/or PPARγ agonists are explained in detail. Thereafter, plants which met these properties are introduced, focusing on known effects, working principles and ingredients.

3.1. Plants selected from the VOLKSMED database

The Department of Pharmacognosy, University of Vienna, collected information about plants, which were known to have an anti-inflammatory potential because of their traditional application, regarding pathological/physiological processes such as rheumatism, arthritis, fever, or pain, which are connected with inflammation. This information was retrieved using the VOLKSMED database, which contains information on traditionally used natural medicinal products (Plants, drugs) in Austria and South Tyrol. This database was created by the Department of Pharmacognosy, which assigned diploma theses with the purpose to collect data about natural medicinal products, used traditionally in rural and alpine regions of Austria and South Tyrol. The collected information was then transferred to the VOLKSMED database, in a continued process lasting over a period of twelve years. The database comprises essential characteristics of the used plants, such as an exact botanical description, the used part of the plant, indications, and preparation/application forms. As the specific mechanism of action is not yet known for the majority of the plants, the main task of our collaboration partners in house is to browse the VOLKSMED database for possible candidates and forward the information to our collaboration partners in Innsbruck for *in silico* screening. The crude extracts of the selected interesting plants are pharmacologically investigated using cell-based or enzymatic assays. After characterization of the activity of chosen plants, they are further fractionated using a bioassay-guided approach to identify the active compounds, which then can be tested in *in vivo* systems.[9]

The samples derived from the VOLKSMED database, which were tested for PPARα and PPARγ activation in the frame of this diploma work are summarized in table 1.

Table 1:

Internal identification number	Plant/Extract	Part used	Solvent
168	*Glechoma hederacea* extract (tannin separated)	herba	MeOH
784	*Glechoma hederacea* SPE fraction	herba	MeOH 30%
785	*Glechoma hederacea* SPE fraction	herba	MeOH 70%
786	*Glechoma hederacea* SPE fraction	herba	MeOH 100%
796	*Peucedanum ostruthium* SPE fraction (tannin separated)	radix	MeOH 30%
797	*Peucedanum ostruthium* SPE fraction (tannin separated)	radix	MeOH 70%
798	*Peucedanum ostruthium* SPE fraction (tannin separated)	radix	MeOH 100%
922-30	*Peucedanum ostruthium* SPE 70% MeOH fractions, HPLC subfractions	radix	Acetonitril/ H2O gradient

Table 1: Samples tested in this diploma work derived from plants chosen from the VOLKSMED database.

3.1.1. Promising plants chosen from the VOLKSMED database

Glechoma hedreacea Linn. (Lamiaceae)

Glechoma hederacea, also called “ground ivy” or “creeping Charlie” has been traditionally used in the folk medicine for centuries. It can be found on shady places, fences or hedges as well as at the margin of humid meadows in moderate climate areas of Asia, the USA and Europe.[25]

The traditional use of the herb is manifold, whereas abscess, arthritis, asthma, bronchitis, diabetes and inflammation are just a few of the pathological progresses for which the *Glechoma hederacea* herb is used. The ulcer-protective, antiviral and cytotoxic effects of *Glechoma hederacea* could be shown in different *in vitro* studies and there is newer evidence showing that the herb possesses also anti-inflammatory properties.[25] It was shown that *Glechoma hederacea* extract is able to inhibit the activation of the inducible NO synthase protein (iNOS) by interferon-γ or lipopolysaccharids in peritoneal macrophages of mice. In addition to that proinflammatory cytokines such as TNF-α were inhibited in this study, which suggests, that components of *Glechoma hederacea* are capable to counteract parameters related to inflammation.[26]

To the known compounds of *Glechoma hederacea* belong sesquiterpenoids, triterpenoids, flavonoid glycosides, rosmarinic acid[27] as well as the alkaloids hederacin A and B, cytotoxic alkaloids, which occur in the *Glechoma hederacea* in only low concentrations.[25]

Peucedanum ostruthium Linn. (Apiaceae)

The roots as well as the herb of *Peucedanum ostruthium* were already used in the 16th and 17th century in Europe for the therapy of disorders connected to inflammation such as rheumatism. The used parts of *Peucedanum ostruthium* were boiled in wine and drunk in order to ameliorate joint pains. They were also used external as schnaps that was applied on painful limbs.[30]

It was shown, that among the components of the 10% ethanolic extract of the root of *Peucedanum ostruthium*, the 6-(3-carboxybut-2-enyl)-7-hydroxycoumarin worked as an

inhibitor of cyclooxygenase and 5-lipoxygenase. This component was able to inhibit the formation of a carrageenan-induced rat paw edema significantly.[31]

Some osthole [7-methoxy-8-(3-methyl-but-2-enyl)-chromen-2-one] derivates, obtained by extraction of the roots, were also found to have an influence on the immune response, inhibiting the LPS induced TNF-α production.[32]

Some of the other known components of the rhizome are (+) sabinene and 4-terpineol as the quantitative most important compounds of the 44 identified.[33]

3.2. Plants selected based upon in silico tools

The main aim of this part of the NFN project (done by collaboration partners at the University of Innsbruck) is to identify bio-active compounds from natural sources, using computer based methods. The first approach to realize this aim is the pharmacophore modeling process. Here the binding of a compound to a biological target is virtually predicted by the application of three-dimensional space models using the structure-based or ligand-based pharmacophore modeling. For targets with known structure, such as the PPARs, ligands can be identified, which possess the appropriate chemical, functional groups for an efficient binding. If no 3-D structure of the target is available, special algorithms are used for a flexible ligand-based pharmacophore modeling. 3-D compound libraries of NP´s, which have been created in advance, are used for the database mining process. These libraries are virtually screened, using the generated pharmacophore models as tools for the identification of promising structures. The so received lists of interesting compounds are then computationally investigated regarding their chemical stability and the opportunity for chemical modifications. In order to assess for example undesired interactions with the cytochrome P450 enzyme system (CYPs 450), a parallel *in silico* pharmacophore-based counter screening is applied. This process is essential to refine the received hit lists. The final selection of virtually predicted hits is done by docking and scoring of the interesting structures with the 3D targets, whereas the interaction energy between the complex of the ligand-target is estimated. Three different 3D multiconformational molecule databases containing interesting structures for the virtual screening process are used: The NP-database, which consists of more than 110.000 NPs. The DIOS database with 9000 NPs is based

on ethnopharmacological knowledge, since it contains compounds present in the medicinal plants described in the famous Materia medica by Pedanius Dioscorides. The third multiconformational database derives from the TCM, with information for more than 10.000 metabolites. Besides the focus on the 3D databases, the collaboration partners at the University of Innsbruck evaluate the received virtual hits using literature-search and ranking of the hits according to their chemical stability, non toxicity, drug likeness and accessibility. For the isolation of predicted virtual hits from plant material, an emphasis is put on material which is known for its anti-inflammatory potential, as used empirically for inflammation-related diseases. The extraction and analytical investigation is done by different methods. With LC-MS (Liquid Chromatography-Mass Spectrometry) and GC-MS (Gas Chromatography-Mass Spectrometry) the bioactive compounds are identified. These compounds can then be isolated by preparative chromatographic systems and their structure is analyzed by MS and NMR (Nuclear Magnetic Resonance)-spectroscopy. The chosen compounds are finally forwarded for further enzymatic/cellular *in vitro* assay systems, respectively *in vivo* sytems.[9]

3.2.1. Pure compounds

Table 2:

Internal identification number	Plant/Extract	Part used	Molecular weight
793	*Krameria triandria*	Radix	264
794	*Krameria triandria*	Radix	250
795	*Krameria triandria*	Radix	266
913	*Doronicum austriacum*	Radix	180
914	*Doronicum austriacum*	Radix	338
915	*Doronicum austriacum*	Radix	320
916	*Doronicum austriacum*	Radix	218
917	*Citrus sinensis*	Pericarp	372
918	*Citrus sinensis*	Pericarp	402
919	*Citrus sinensis*	Pericarp	372
920	*Citrus sinensis*	Pericarp	342
921	*Citrus sinensis*	Pericarp	342

Table 2: Pure compounds tested in this diploma work, and the plants from which they originate.

3.2.2. Plant extracts containing predicted bioactive compounds

Table 3:

Internal identification number	**Plant/Extract**	**Part used**	**Solvent**
895-912	*Sideritis hyssopifolia*	herba	DMSO

Table 3: The Sideritis hyssopifolia fractions, containing virtually predicted bioactive compounds.

3.2.3. Promising plants chosen based upon computational methods

Sideritis hyssopifolia

The aerial parts of *Sideritis hyssopifolia* are used for the treatment of a variety of illnesses, because of its traditionally known anticatarrhal, anticephalgic, antidiarrhoeal, diuretic and hypotensive properties.[34] Flavones glycosides, such as hypolaetin-glycosides were isolated from the aerial parts. It is not known, whether this flavones are the active principles for the mentioned effects, of the plant.[35]

3.3. Plants tested for PPARα and PPARγ activity that are not part of the DNTI project

Several plants derived from the TCM and from the VOLKSMED database were analyzed as a part of ongoing projects different than DNTI. An essential step after fractionation of the initial extracts here again is the subsequent *in vitro* testing. In order to identify active compounds, the active extracts are further subject to activity guided isolation. The isolation and fractionation of the plant material is achieved via preparative thin layer chromatography, column chromatography, medium pressure liquid chromatography, preparative HPLC, and solvent partition chromatography.

3.3.1. Plants deriving from the TCM

The number of plants, used in the TCM for the therapy of inflammation-associated diseases is very big, and the TCM represents according to its 5000-year long history and vast empirical knowledge a very interesting and promising pool for drug discovery.[36]

Extracts and fractions from promising plants from the TCM with known anti-inflammatory effects are shown in table number four. They were provided by the working group of Prof. Kopp, Institute of Pharmacognosy, Vienna, for luciferase-based assay determination on their PPAR activity.

Table 4:

Internal identification number	**Plant/Extract**	**Part used**	**Solvent**
787	*Arisaema sp.*	Rhizoma praeparata	MeOH
788	*Arisaema* sp.	Rhizoma praeparata	MeOH (tannin separated)

789	*Arisaema sp.*	Rhizoma praeparata	Dichlormethane
790	*Pinellia* sp.	Rhizoma praeparata	MeOH (tannin separated)
791	*Pinellia* sp.	Rhizoma praeparata	Dichlormethane
792	*Pinellia* sp.	Rhizoma praeparata	Dichlormethane
809	*Albizzia julibrissin* SPE fraction	cortex	PE
810	*Albizzia julibrissin* SPE fraction	cortex	DC1
811	*Albizzia julibrissin* SPE fraction	cortex	DC2
812	*Albizzia julibrissin* SPE fraction	cortex	EtOAc

Table 4: Samples tested in this diploma work derived from plants chosen from TCM.

These samples do not belong to the DNTI project.

Arisaema species (Araceae)

Arisaema erubescens is known to have anti-cancer and anti-convulsant properties.[37] *In vitro* investigations of a lectin, received from the tubers of *Arisaema tortuosum*, have shown anti-proliferative activity in human cancer cell lines.[38] By isolation of the 2, 3-dihydroxypropyl 9Z, 12Z-octadecadienoate from *Arisaema amurense Max. var. serratum Nakai* one anti-inflammatory mechanism could be identified. This isolated compound has shown to be a strong inhibitor of the phospholipase A2 (PLA2), which is responsible for the biosynthesis of the proinflammatory prostaglandins, leucotriens and thromboxanes.[39] A common property of the *Arisaema sp*. is their toxicity, which is representative for this family (Araceae).[40]

Pinellia sp. (Araceae)

Pinellia ternata is used in combination with *Citrus reticulate* in the Korean traditional medicine for the treatment of inflammatory processes in the lung, such as asthma. It is known to ameliorate cough, is working antiemetic, antineoplastic and antifertilizing. Until now various components could be identified and it was shown, that the pinellic acid (9S, 12S, 13S-trihydroxy-10E-octadecenoid acid) has a modulatory effect on antigen-specific antibodies in the lung. Furthermore, pinellic acid is proposed as an efficient oral adjuvant for nasal influenza vaccine.[42] In a murine asthma model it was furthermore shown, that *Pinellia ternata* suppressed effectively the Th2 cytokines (IL-4, IL-5, IL-13), IgE and the expression of eosinophilic CCR3 in the lung. These findings suggest, that the compounds of *Pinellia ternata* have a immunmodulatory potential.[41]

Pinellia ternata is also used for the treatment of pathological processes related to obesity. It was shown that the water extract of *Pinellia ternata* is able to increase the expression of uncoupling proteins in the brown adipose tissue and of PPARα in the white visceral adipose tissue of obese Zucker rats, lowering FFA and triglicerides-blood levels consequentely.[43]

Albizzia julibrissin Durazz (Leguminosae)

The cortex of *Albizzia julibrissin* is known in the Chinese Pharmacopoeia for its sedative and anti-inflammatory activity and is in addition used to treat injuries from falls, carbuncles[28], confusion and many more.[29]

Until know saponins, lignans, phenolic glycosides, triterpenes and flavonoids could be isolated from *Albizzia julibrissin*. The methanolic extract shows radical scavenging characteristics on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, with 3´,4´,7-trihydroxyflavone, isookanin, luteolin, syringaresinol-glycoside, and the alibrissinosides A and B, two lately identified phenol-glycosides, being the main responsible compounds for this effect.[29]

An isolated julibroside j1 analogue showed cytotoxic effects against a specific cancer cell line (Bel-7402).[28]

3.3.2. Plants deriving from the VOLKSMED database

The plant fractions and subfractions of *Dryopteris filix-mas* were provided from Prof. Reznicek from the Department of Pharmacognosy in Vienna. The *Dryopteris filix-mas* fractions were the only investigated samples, chosen for determination of PPARα and PPARγ activity, as illustrated in table number five.

Table 5:

Internal identification number	*Plant/Extract*	Part used	Solvent
870	*Dryopteris filix-mas* 100% MeOH fraction	herba	MeOH 100%
871-76	*Dryopteris filix-mas* subfractions from 870	herba	MeOH gradient
878	*Dryopteris filix-mas* 70% MeOH fraction	herba	MeOH70%
879-86	*Dryopteris filix-mas* subfractions from 878	herba	MeOH gradient

Table 5: Samples tested in this diploma work derived from plants chosen from the VOLKSMED database.

These samples do not belong to the DNTI project.

Dryopteris filix-mas Linn. (Dryopteridaceae)

The rhizome of the male fern is known to be effective in the therapy of tapeworms and influenza. Newer data suggests that the male fern also shows anti-cancer effects because of its kaempferol glycosides, which are known to affect the DNA polymerase. Furthermore the fatty acid synthase, which can be detected in high concentrations in malignant tissues, is inhibited by the alcoholic extract of the Male fern rhizome. By a

down-regulation mechanism of the PI3K/AKT and JNK pathways, this extract is able to inhibit the growth of cancer cells and even to induce apoptosis. On the contrary there are no data regarding the safety available.[44]

From the methanolic extracts of the rhizome of *Dryopteris crassirhizoma* two phloroglucinol components, flavaspidic acids PB and AB, could be identified. These components showed an efficient antimicrobial effect against Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus*, *Streptococcus mutans* and *Bacillus subtilis*.[45]

C) Materials and methods

1. DNA-plasmid preparation

1.1. DNA plasmids

A plasmid is defined as “linear or circular double-stranded DNA that is capable of replicating independently of the chromosomal DNA”. In bacteria, plasmids for example code for enzymes such as ß–lactamase, which confer antibiotic resistance. Plasmids are above all a prokaryotic characteristic, but can also be found in eukaryotic systems such as *Saccharomyces cerevisiae.* (http://www.biology-online.org/dictionary/Plasmid)

In the field of molecular biology, plasmids are used as vectors, “which are vehicles transferring genetic material from the donor organism to the target cell of the recipient organism”. (http://www.biology-online.org/dictionary/Vector)

Reporter genes encode a product, such as GFP or Luciferase, which can be investigated by fluorescence and luminescence measurement and are coupled to a promoter that contains upstream sequences such as the PPRE. Factors which are capable of activating the response element can so be investigated indirectly, as the expression of the reporter gene stands for the activation of the gene of interest. (http://www.biology-online.org/dictionary/Reporter_gene)

Another type of plasmids we used in this diploma work are expression vectors. Expression vectors are a type of plasmid containing the required regulatory sequences specifically used for the expression of a particular gene into proteins within the target cell. (http://www.biology-online.org/dictionary/Expression_vector)

The PPARα and PPARγ plasmids we used for the transfection of the HEK_293 cells are mammalian expression vectors and therefore produce eukaryotic proteins. (http://www.biology-online.org/dictionary/Mammalian_expression_vector)

These plasmids bear enhancer and promoter regions as regulatory sequences and use the transcription and translation machinery of the HEK_293 cell to express the receptor protein. (http://www.biology-online.org/dictionary/Expression_vector)

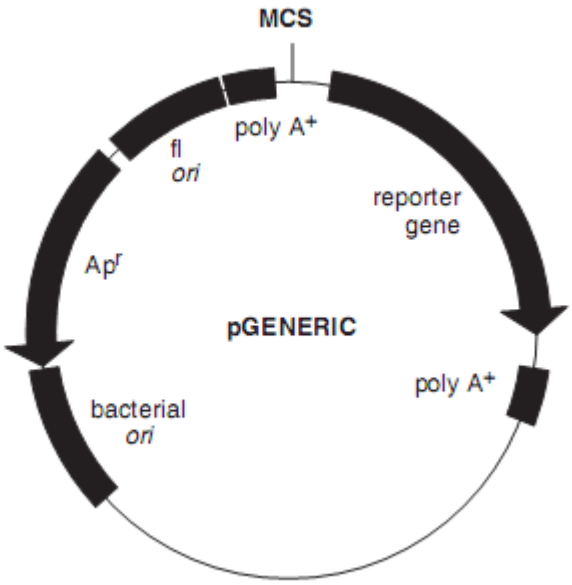


Figure 5: The principal organization of a typical DNA-plasmid, used in reporter gene assays.

Picture taken from: Wiley, J & al.[46]

The multiple cloning site (MCS) is used for the insertion of foreign DNA and therefore contains five to seven unique restriction endonuclease sites. For the PPAR-agonist screening, the plasmid that we used had an inserted PPAR response element (PPRE), ahead of the luciferase reporter gene. The backbone of the vectors usually also contains a bacterial origin of replication (bacterial ori) which is essential for the propagation of the vector in *E.coli*. The backbone of some vectors also contains a bacteriophage origin of replication (f1 ori) which is used for the production of single stranded DNA. The Ampicillin resistance gen (Ap^r), which encodes for β-lactamase, is essential for the selection of *E.coli* cells, which were able to take up the genes of interest (PPARα, PPARγ, Luciferin) as described in chapter 1.3. The plasmid encoding for EGFP that we used in this reporter assay additionally bears resistance for Kanamycin. The reporter genes (in the plasmids that we used firefly luciferase, or

EGFP) are usually flanked by polyadenylation signals, which are important for a proper and efficient processing of the reporter transcript in the mammalian cells.[46]

1.2. Preparation of competent E.coli (Escherichia coli) cells

Through the following process, the *E.coli* cells were rendered competent, which means capable of taking up DNA, in order to amplify the DNA plasmids, which were needed for the transfection of the HEK-293 cells, as described later on.

Introduction

In a first step, the *E.coli* cells were grown to the log phase (the phase of exponential growth) and then concentrated by centrifugation. The resuspension of the *E.coli* cell pellets in a $CaCl_2$ solution and the following heat shocking led to the desired DNA uptake by the cells. Afterwards the cells were grown at first in a nonselective medium, so that the cells were allowed to produce the plasmid-encoded antibiotic resistance proteins. Then we plated the cells on a medium which contains the selection antibiotics (Ampicillin and Kanamycin), and selected the colonies containing the plasmids that encode for PPARα, PPARγ, Luciferin and GFP in this way.

Table 6: The reagents were filtered sterile or autoclaved and stored at room temperature.

Materials and solutions (have to be sterile)	
One single colony of *E. coli* cells	
Beckman JS-5.2 rotor	
50 ml cooled polypropylene tubes	
$CaCl_2$ Solution:	60 mM $CaCl_2$
	15% glycerol
	10 mM PIPES, pH7

Additional reagents and equipment for the growth of bacteria in liquid media	
LB medium (500 ml):	5 g bacto tryptone 2.5 g bacto yeast 5 g NaCl Diluted with water up to 500 ml and autoclaved

.Table 6: Materials and reagents for the preparation of competent E. coli.

Practical part

At the first day we picked a single colony of *E. coli* and transferred it into 50 ml LB medium, for growing overnight at 37 °C while shaking moderately at 250 rpm. The next day, 4 ml of the culture were transferred into a sterile two liter flask, containing 400 ml of the LB medium, again grown at 37°C and shaken at 250 rpm. We controlled, that the OD_{590} (optical density at 590 nm, a measurement for the bacterial growth) did not exceed a value of 0.375, as an overgrowth of culture decreases the efficiency of transformation. Then we divided the culture into eight sterile, 50 ml polypropylene tubes, which were put on ice five to ten minutes, before centrifuging seven minutes at 1600 x g (3000 rpm in JS-5.2 rotor). After discarding the supernatant, we resuspended the cell pellets in 10 ml ice cold $CaCl_2$ solution before centrifuging at 1100 x g (2500 rpm) for further five minutes at 4 °C. This process was repeated without centrifuging and the cells kept on ice for 30 minutes. We received the final pellet after centrifuging again and resuspended it in 2 ml ice cold $CaCl_2$ solution, so that the cells could be dispensed into 250 µl aliquots in pre-chilled, sterile polypropylene tubes, and stored at -80 °C.

1.3. Obtaining single transformed E.coli colonies

Theoretical part

We used the competent *E. coli* cells, prepared as described in chapter 1.2. for plasmid uptake during transformation. The purpose was to receive single bacterial colonies, transformed with the plasmid bearing the appropriate antibiotic resistance gen.

Time consideration

After the transformation, the agar petry dishes need to be incubated overnight at 37°C, thus we plated them in the late afternoon.

Sterility consideration

Although strict sterility is not absolutely essential because the agar plates contain antibiotics, it is recommended to work as clean as possible. The important handling steps were therefore done in the range of the gas fire. The LB medium bottles were shortly boiled in the microwave after opening.

Table 7:

Materials	
LB medium	
Agar plates, containing the selection-antibiotics	
SOC medium (1000ml):	20 g Tryptone 5 g Yeast Extract 0.5 g NaCL 10 ml 250 mM NaCl Adjust pH 7 with 5 N NaOH Adjust volume to 1 L with deionized H_2O Autoclave for 20 min. Cool medium to 60°C, add 18 ml 20% filter-sterilized Glucose solution Add 5 ml of a sterile solution of 2 M $MgCl_2$

Table 7: Materials for the E. coli transformation.

Practical part

At first, we transferred the 40 µl aliquots of the competent *E. coli* bacteria, which we stored in the -80 °C freezer, into 1.5 ml tubes and put them on ice. One aliquot was prepared for each desired plasmid. An additional untransformed aliquot was prepared, which we used as a negative control and which was treated in the same way. 50 ng of the respective DNA plasmids (PPARα, PPARγ, PPRE, or GFP) were then transferred into the 1.5 ml tubes, containing the competent bacteria. During the following incubation on ice for 30 minutes, the bacteria were taking up the plasmid DNA. Meanwhile we set a heating block at 42 °C and melted the SOC medium, which was kept at -20 °C. After the end of the incubation step, the tubes containing the mixture of the competent bacteria with DNA, were put on the 42 °C heating block for exactly 45

seconds, and then immediately transferred on ice for one minute. 450 µl of the SOC medium were added, and the tubes incubated at 37 °C for one hour. During this time the bacteria had the possibility to express the resistance-conveying protein, which was encoded by the respective plasmid. After incubation, we centrifuged the tubes one minute and discarded the supernatant before resuspending the cell pellets in 50 µl of LB, containing the respective selection-antibiotic at the same concentration as in the agar plates. We transferred the bacterial suspension in one corner of the agar plate, containing the respective antibiotic, and carried out a fractionated streak. Before we incubated the plates at 37 °C overnight, we wrapped them with parafilm. Finally we put the plates in the refrigerator (4 °C), where they could be stored for several days safely, or handled them immediately for further extraction and purification.

1.4. DNA-midipreps

Theoretical part

DNA purification was done using the Pure Yield® Plasmid Midiprep System (Promega).This system represents a method to gain efficiently high quality and quantity plasmid-DNA out of transformed bacteria. The isolated DNA can be used further for transfection of the HEK-293 cells, as explained in the chapter 2.2.4. One advantage of the described midipreps isolation system is that as much as 100-200 µg of plasmid DNA from transformed *E.coli* cells could be obtained in just about 30 minutes. Furthermore, not just the quantity, also the quality of the obtained DNA is high.

By the following method the plasmids containing PPRE, PPARα, PPARγ and GFP, which have been amplified by the *E.coli* cells, were purified. The whole process was carried out at room temperature.

Table 8:

Materials
LB medium and selection antibiotics
Vacuum pump with vacuum manifold
Pure Yield™ Clearing column/ Binding column, Eluator™ Vacuum Elution device (Promega)

Solutions
Cell Resuspension/Lysis/Neutralization Solution™ (Promega)
Endotoxin Removal Wash Solution, ™ completed with the indicated amount of isopropanol (Promega)
Column Wash Solution™, completed by adding the respective amount of 95% EtOH (Promega)

Table 8: Materials and Solutions for the DNA-midipreps.

Before starting with the procedure we checked the clarity of the *Cell Lysis Solution*™, as an exposure to low temperature leads to SDS precipitate, resulting in poor lysis.

Practical part

In the morning we started a bacterial pre-culture and transferred 1-2 ml of the pre-culture into 150 ml LB medium at the late afternoon to start the main bacterial culture, which we incubated overnight. On the next day in the morning the DNA isolation was started, whereas 6 preps could be conveniently done at the same time. We centrifuged the flasks containing the main culture for 10 minutes at 5000 x g and discarded the supernatant. The cell pellets were then resuspended in 6 ml of the *Cell Resuspension*

Solution™ per flask. Next we lysed the cells by adding *Cell Lysis Solution*™ and rolling the flasks gently. The lysed cells were treated with *Neutralization Solution*™ afterwards for 2-3 minutes and a white precipitate was formed. We centrifuged the precipitated cells at room temperature for 15 minutes at 15000x g. The bulk of the cellular debris was pelleted in this way, so that after centrifugation we could separate the debris from the desired DNA using the *Pure Yield Clearing Columns*™ in the following way. First, the *Clearing Column*™ was mounted on the top of the *Binding Column*™ which was placed onto the vacuum manifold. We then poured the lysate into the *Clearing Column*™ and applied a vacuum. The lysate passed into the *Binding Column*™, where a special binding membrane retained the DNA. We continued the vacuum until all the liquid has passed through both columns, and then discarded the *Clearing Column*™ and added 5 ml of the *Endotoxine Removal Wash*™ to the *Binding Column*™. By applying the vacuum again the solution was sucked through the membrane and washing out contaminations such as endotoxines, proteins, RNA, and endonucleases. The membrane with the attached DNA was washed additionally with a *Column Wash Solution*™ and then dried by applying a vacuum for 30 seconds until the membrane appeared to be dry and we could not recognize an ethanol odor. Afterwards we removed the *Binding Column*™ from the vacuum manifold and put the tip of the column on a paper towel to remove the rest of ethanol in it. Afterwards, we collected the DNA in 1.5 ml microcentrifuge tubes, which were placed in the base of the *Eluator Vacuum Elution Device*™. The *Elution Device*™ was placed on the vacuum manifold and the *Binding Column*™ assembled on top of it, so that through adding 800 µl of nuclease free water, and applying a vacuum for one minute, the liquid containing the DNA could be collected in the tube. Finally we centrifuged the eluate in the microcentrifuge tube 10 minutes at maximum speed and transferred the supernatant into new tubes.[47]

2. Luciferase-based PPAR agonist screening

2.1. Theoretical part

2.1.1. Reporter genes used for the transfection of HEK-293 cells

The use of reorter genes, for transfection of HEK-293 cells as an example, is widely established as a reproducible procedure to analyze gen-expression and to measure the promoter activity, respectively. The transfection with fusion genes represents a temporary assay system, as the gene expression can be measured after introduction of the DNA for a definite time. The vector, by which the DNA can be introduced into the HEK-293 cells, contains the promoter and the enhancer sequence, which are fused to a reporter gene that encodes a reporter protein. The reporter molecules of interest in this assay, as described in the chapter 2.1.2, are the firefly luciferase and the EGFP. In case of PPRE, the sequence is inserted in the MCS of a promoter, which controls and promotes the expression of the luciferase reporter protein. The amount of luciferase, which is expressed, represents the ability of the inserted sequence to direct and promote the transcription.[46] The GFP reporter plasmid had a strong constitutively active promoter, and it was used as an internal control to account for transfection efficiency or unspecific effects of the investigated samples.

2.1.2. The reporter molecules: luciferase and GFP (Green Fluorescent Protein)

The firefly luciferase

The luciferase is a protein, isolated from *Photinus pyralis*. The cloning of the luc-gene marks the beginning of the first nonisotopic genetic reporter assays, with the advantage of a higher sensitivity and the independence from radioactivity. The luciferase catalyzes the bioluminescent reaction of the substrate luciferin, with ATP, Mg^{2+}, and molecular oxygen.[46] The luciferase catalyzed oxidation of luciferin is a two step process, as shown in figure six.

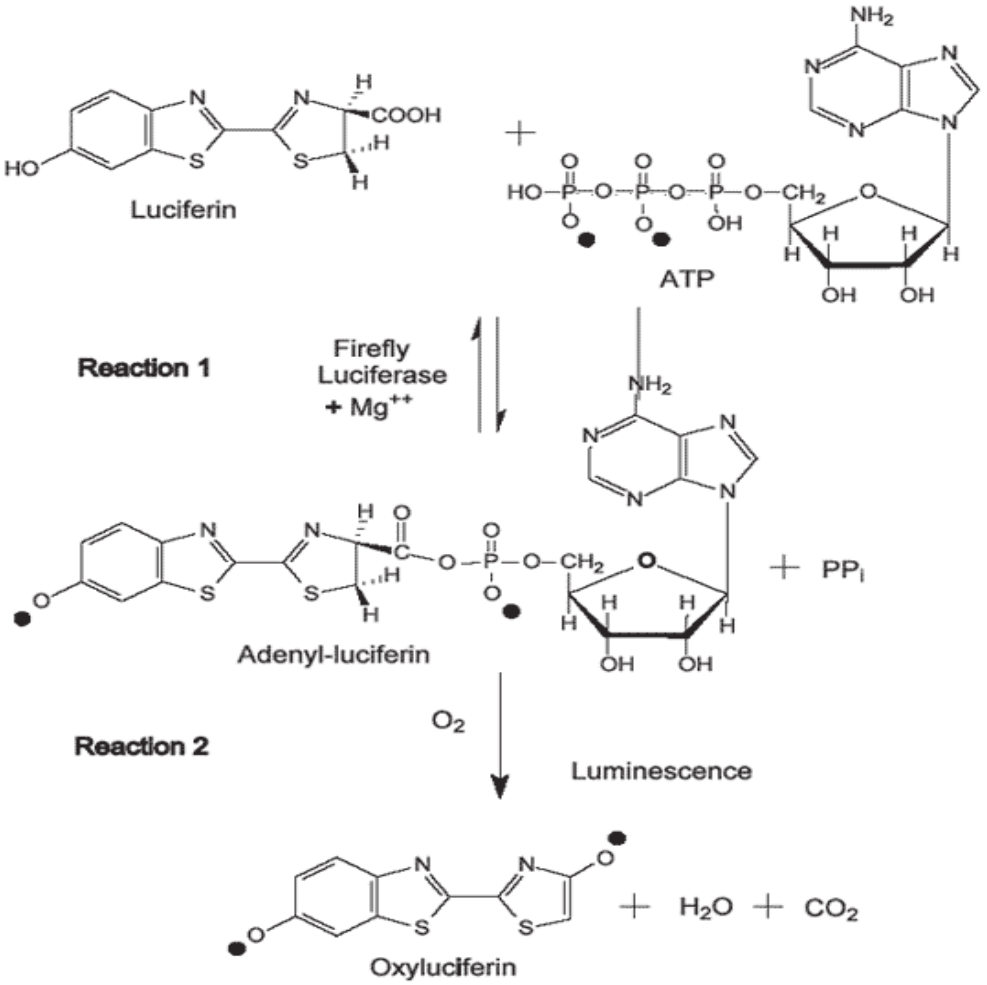


Figure 6: The luciferase catalyzed reaction is an ATP and Mg^{2+} dependent oxidation in two steps, with oxyluciferin, H_2O and CO_2 as products.

Picture taken from: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/luciferase.html.[48]

In a first step, the luciferin, which contains a heterocyclic system is adenylated and afterwards decarboxylized oxidatively. During this reaction, AMP, CO_2 and immediately formed light from the activated luciferin is produced [49]. By lysing the luciferase-containing cells, after transcription of the correspondent luc gen in the cell, and mixing the cell lysate with the corresponding reagents, a rapidly (less than one second) decaying flash of light is emitted. The luciferase activity of the according cell lysate corresponds to the total light emission and can be measured using a luminometer. Therefore it is possible to make an indirect estimation of the luciferase expression. As this reporter enzyme is very sensitive and has a high turnover, it is suitable for assay-

systems that are inducible by agents above basal expression levels of the cell. One disadvantage of the primly developed luciferase assay is the expensive performance and the unsatisfying reproducibility in the results, as the photon emission is very rapid. By adding Coenzyme A to the mixture of reagents, it is possible to receive a prolonged light emission and thus, the assay is more reproducible. The luciferase shows a preferential reaction with the luciferyl-Coenzyme A and therefore the extended light emission leads to an improvement, as far as the sensitivity is concerned.[46]

The green fluorescent protein: GFP

Another reporter protein that we used is the green fluorescent protein[50], cloned from *Aequorea victoria*. It is a widely used complete proteinogenous fluorophore that meets the requirements of an ideal fluorescent tag. The GFP can be detected in any organism, if a vector system is available, without causing harm to the functions of the cell. By using the GFP, it is also possible to target nearly any subcellular region, which is interesting for the observation of phenomena regarding the cell, *in vivo.*[46]

It is known already for several decades, that the jellyfish *Aequorea victoria* is able to emit green light, although the luminescence protein aequorin in it, emits blue light by binding Ca^{2+} ions. The reason for this phenomenon is that the blue light with a shorter wavelength is absorbed by the GFP, which consequently emits green light with a longer wavelength. After isolating and sequencing the protein it was used in 1994 for the first time for expression in *E. coli* and *Caenorhabditis elegans* by Chalfie et al.[51] It was possible to show, that the excitation of the fluorophore of the GFP does not need any further reagents or co-factors, so that the green light emission can be caused by excitation with long wave ultraviolet or blue light. The maximum excitation of the fluorophore is at a wavelength of 395 nm, resulting in an emission spectrum with one single peak at 509 nm. The GFP expression can be observed, using a fluorescence microscope, for a first, quick estimation of the cell transfection efficiency, as described in chapter 2.2.8. For the transfection of mammalian cells, such as HEK-293 utilized in this diploma work, various vectors for the GFP uptake are available. The problems with the wild type GFP, such as low fluorescence capacity, delayed chromophore formation and poor expression are mostly dissolved, using mutants of GFP, such as the enhanced GFP (EGFP), which we used in our experiments.[52]

Simultaneous quantification of the two used reporter molecules (luciferase and EGFP) provides information about the stimulation of the PPRE mediated transcription by PPAR agonists, the viability of the cells, as well as the transfection efficiency.

2.2. Practical part: cell culture

2.2.1. Materials used in the cell culture

Table 9:

Name	**Ingredients**	**Amount**
Cell culture medium	DMEM with or without phenol red (Lonza)	500 ml
	Foetal bovine serum (FBS) (Gibco)	50 ml
	Penicilline (Lonza)	10.000 U/ml
	Streptomycine (Lonza)	10.000 U/ml
	L-Glutamine (Lonza)	200 mM

Table 9: The cell culture medium and ingredients.

This cell culture medium was used for all steps, in which the propagation of the HEK-293 cells was desired. The phenol red represents a good indicator, whether the pH of the medium is in the optimal range.

Medium without phenol red, containing stripped FBS instead of the normal FBS, was used for stopping the trypsinisation after harvest of the transfected cells. Furthermore, this so called “stripped medium” was used for the resuspension of the cell pellet after this process, before counting the number of viable cells.

The stripped medium does not contain any lipids, which could interact with the PPAR receptors and subsequently with the response element in this assay.

Table 10:

Name	Ingredients	Amount
2xHBS ph 7.5	NaCl	280 mM
	KCl	10 mM
	Na_2HPO_4. $2H_2O$	1.5 mM
	Dextrose	12 mM
	HEPES (Fluka)	50 mM

Name	Ingredients	Amount
PBS ph 7.4 (autoclaved)	NaCl	8 g
	KCl	200 mg
	Na_2HPO_4	1.44 g
	KH_2PO_4	240 mg
	ddH_2O	ad 800 ml
Trypsin/EDTA in PBS	Trypsin	0.05%
(Invitrogen)	EDTA (Fluka)	0.02%

Table 10: Solutions and buffers needed for cell maintenance (PBS, Trypsin) and transfection (HBS).

The HEK-293 cell line

The HEK-293 cell line represents a commonly used expression system for recombinant proteins. This cell line was generated over 30 years ago by the transformation of human embryonic kidney (HEK) cells, which were exposed to sheared fragments of human adenovirus type 5 (Ad5) DNA. By incorporation of the Ad 5 into the genome of the HEK-293 cells, the permanently transformed HEK-293 cell line was created.[53] The source of the now available HEK-293 cell line derives from the original transformation, done by Graham, Smiley, Russel and Nairn in 1977.[54]

The synthetic protein machinery of the HEK-293 cell line is able to express gene products, such as EGFP and luciferase, after introduction of the plasmid vectors into

the cells. The requirement for an efficient expression is that the plasmid vector has a strong promoter for mammalian expression, as is the case in the pc-DNA.

One advantage of the HEK-293 cells is, that also post-translational modifications are done, which are required to generate functional proteins of mammalian origin. The popularity of this cell line is also due to its fast and easy maintenance and the easily achieved high transfection efficiency. The above mentioned qualities make the HEK-293 cell line suitable to explore the effects of new drugs on specifically expressed drug targets, such as PPARα and PPARγ used in this diploma work.[53]

2.2.2. The maintenance of HEK-293 cells

Sterile work and arrangement

Before starting the work in the laminar air flow station, we sprayed EtOH 70% solution on the surface of the working space and sterilized the gloves in order to avoid contamination. All the materials that are needed for the work in the laminar flow station had to be treated in the same way with EtOH 70%. For the cell transfection, two dishes (10 cm) with six million and one dish (30 mm) with two million cells were needed.

Therefore the cells were seeded in flasks, containing the cell culture medium (Dulbecco´s modified Eagle´s medium, DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine, two days in advance. One flask (175 mm^2) was prepared with five million HEK-293 cells and 20 ml of medium, and one flask (75 mm^2) with 2 million HEK-293 cells and 10 ml of the medium.

Cell counting, using the Beckman Coulter VI-CELL®

In order to be able to seed the right amount of cells the Vi-Cell® was used for counting the cells and checking the cell viability.

The Beckman Coulter Vi-Cell® represents a cell viability analyzing device, which uses the" trypan blue dye exclusion method". Through this method it is possible to determine the number of viable cells exactly. The trypan blue dye can only be taken up by dead cells, as their membranes become permeable, therefore they can be distinguished from the viable cells because of their color.[55]

For checking the viability of the HEK-293 cells, an aliquot (e.g. 1000 µl) of the resuspended (in 10 ml DMEM) cell pellet was mixed with the trypan blue and transfered into a flow cell for further analyzes. The cell pellet was received following the washing and trypsinisation and resuspending steps, as presented in chapter 2.2.3. The definition of parameters for counting with the Vi-Cell® had to be done in advance.

The device next automatically makes up to 100 images and calculates the viability, using a proprietary algorithm. The amount of viable cells per ml could then be determined and therefore the necessary amount of cell suspension for further procedures calculated.

After the necessary amount of the HEK-293 cells was resuspended in the DMEM, the flask had to be shaken carefully, in order to achieve an optimal distribution, and incubated for two days. It was important to check the cells every day under the microscope to control their morphological shape.

2.2.3. Preparing the cells for transfection

Before the work was started, all the used solutions for the cell treatment had to be put at least for 15 minutes in a 37 °C water bath. After the pre-warmed cell culture media (DMEM with and without phenol red/ stripped serum), PBS, Trypsin, and the working materials as well as the surface were sterilized with alcohol (EtOH 70%). The flasks containing the HEK-293 cells were taken out of the incubator (5% CO_2, 37 °C) and checked under the microscope.

On the first day (at 16:00-17:00 h) we seeded six million HEK-293 cells in a 10 cm dish, which contained 15 ml of the culture medium and two million HEK-293 cells in a 30 mm dish containing 4 ml of the same medium.

Before the cells can be seeded in the dishes they had to be treated in the following way:

At first we aspirated the old medium out of the flasks, and washed the cells with 10 ml of PBS in a next step for one minute. After the PBS had been aspirated completely, we treated the cells with trypsin and incubated them for two minutes. This step was essential to be able to suspend the cells, which had been attached to the bottom of the flask. We also needed to make sure, that the trypsinisation did not take too long, as a longer trypsinisation leads to a decrease in cell viability. The flasks with the suspended cells were then taken out of the incubator and controlled under the microscope. During trypsinisation the cells were detached from the bottom of the vial, so that “floating globes” could be observed under the microscope. In order to stop the trypsinisation, we next added 8 ml of the culture medium into the flask. By further resuspending we made sure, that the trypsinisation is stopped fully. The cell suspension then was transferred into a falcon, (20 ml, respectively 10 ml) using cell strainers, (100 µm pore size) to remove cell conglomerates. To remove the medium containing the inactivated trypsin, we centrifuged the suspension at 1100 g, for five minutes at room temperature. After the centrifugation has been stopped, the cells were seen as a pellet on the bottom of the falcon and the medium could be aspirated with a pipette. We resuspended the pellet using 10 ml of the cell culture medium. A short vortexing (in medium speed for three seconds) ensured that the cells were distributed homogenously in the medium, so that the cell counting process could be started.

The cell counting, using the Vi-Cell®, as discussed in chapter 2.2.2.

For the cell transfection one dish (10 cm) with six million cells was needed and one dish (30 mm) with two million cells. The dishes had to be shortly shaken and the distribution of the cells checked under the microscope followed by incubation for 16 hours.

2.2.4. Transfection of HEK-293 cells

On the next day we transfected the cells, using the $CaCl_2$ method. The transfection of HEK-293 cells was done 16 hours after seeding.

Table 11:

Solutions
2 M $CaCl_2$ filter sterilized
2x HBS
H_2O sterile (dd H_2O)
DNA-plasmids
PPRE
PPARα, PPARγ
GFP

Table 11: Solutions and DNA-plasmids used for transfection of the HEK-293 cells.

Four eppendorf tubes were prepared for the transfection. In the first two tubes 720 µl of HBS is pipetted, in the second two tubes H2O, DNA (PPRE and GFP and either PPARα or PPARγ) and $CaCl_2$ are mixed. The amount of DNA used was 8 µg for PPRE and PPARα/PPARγ, and 4 µg for GFP. Since the stock concentrations of the DNA were different, we always needed to calculate the necessary amount of the plasmids to be pipetted. First the calculated amount of water was pipetted in the eppendorf tube, followed by the PPRE, GFP and in one of the tubes just PPARα, whereas in the other one just PPARγ instead. In the end the 44 µl of the $CaCl_2$ was added to the tubes and a short vortexing was done. The mixture was added drop wise to the first two tubes (with the HBS) and vortexed again. After 20 minutes of incubation of the mix it was vortexed one more time and then the two prepared 10 cm dishes containing six million HEK-293 cells could be transfected by adding the mixture drop wise. One dish was treated with the solution containing the PPARα plasmid and one with the solution containing the PPARγ plasmid. The complex was distributed drop wise on the dishes. After one hour

of incubation we viewed the cells under the microscope where the calcium phosphate-DNA complex could be seen attached to the surface of the cells. Five hours of incubation allowed the transfection to take place. To avoid possible toxic effects of the precipitate, we replaced the medium with fresh one.

2.2.5. Preparation of the samples

In the meantime the test samples, the agonists (GW 7647 as a potent PPARα and troglitazone as a potent PPARγ agonist) and DMSO as solvent control were prepared. First we thawed the stock solutions. It was important to keep in mind, that the components might be heat sensitive and so a longer exposition in the 37 °C warm water bath could lead to a decrease of effective components. Subsequently, the treatments were put into the laminar air flow, after one minute pre-thawing in the water bath for further thawing. After thawing of the stock solutions, the dilution of the tested samples and reference compounds were started.

2.2.6. Harvesting and reseeding of transfected HEK-293 cells

For the cell treatment in the 96 well-plate the following materials were needed:

Table 12:

Materials
Eppendorf® 8-channel pipettors (10-100µl and 30-300µl)
Two boxes of sterile tips for Eppendorf® pipettes (2-200µl)
One box of sterile tips for Eppendorf® pipettes (50-1000µl)
One Eppendorf® automatic pipetting-aid
Sterile graduated pipettes (5ml and 10ml)
Sterile reservoirs (a small reservoir for the treatments and 3 big reservoirs for cell seeding)
One 96 well plate
The test samples, the positive controls (GW 7647, troglitazone) and negative controls (DMSO) diluted in the DMEM medium
DMEM that contains 5% stripped FBS, Penicillin-Streptomycin and glutamine.

Table 12: Materials and solutions for harvesting, reseeding and cell treatment of the transfected cells.

The harvesting and reseeding process was done six hours after transfection.

After we placed the needed material into the laminar air flow station, we took the transfected cells out of the incubator (5 % CO_2, 37 °C) and viewed them under the microscope.
The calcium-phosphate-DNA complex could then be observed as small dark crystals next to the cells, which was already an indication whether the transfection procedure was successiful. In a first step the 2 big dishes (10 cm), that contained cells transfected with PPARα or PPARγ, PPRE, GFP, as well as the small dish (30 mm) containing

untransfected cells were processed by aspirating the medium containing the calcium phosphate precipitate. We then washed the cells with 10 ml of PBS and after the PBS has been discarded, detached them with trypsin (2 ml for the big dishes and 1 ml for the small dish, for two minutes at 37 °C). The trypsinisation was stopped by adding medium with stripped serum (8 ml for the big dishes, 4 ml for the small dish) and the obtained cell suspensions were transferred into three falcons for centrifugation at 1100 g, five minutes. We discarded the supernatant and resuspended the cells with DMEM supplemented with 5% stripped FBS, penicillin-streptomycin and glutamine (10 ml for the transfected cells, 5 ml for the untransfected). After we distributed the cells in the medium, the cell number could be determined using the Vi-Cell® as already discussed. 50 000 cells per well of the 96 well plate had to be seeded in 150 µl DMEM (5% stripped FBS, penicillin-streptomycin, glutamine). For a quick and equal distribution into the 96 well plate we used the Eppendorf® eight-channel pipette, and observed the cells under the microscope again, to check the accuracy of the pipetting process. The 96 well plate was placed into the incubator until 16:00, so that the cells had the possibility to attach to the bottom of the wells.

2.2.7. Cell treatment

One hour after harvesting and reseeding the cells, the diluted samples, the agonists, and the DMSO solvent control, which were kept at the refrigerator until this time, were added to the 96 well plate. This process was done using the multichannel pipette and reservoirs, following the shown plate design, whereas we divided the plate in two halves. Into the upper half of the plate the PPARα transfected cells, into the other half the PPARγ transfected cells were seeded.

No samples	GW	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	GW	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	GW	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	GW	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	Tro-glitazon	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	Tro-glitazon	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	Tro-glitazon	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected
No samples	Tro-glitazon	DMSO	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Samples	Un-transfected

*Figure 7: The plate design of a PPARα/*PPARγ *experiment.*

"No samples" designates transfected, but not treated cells, "GW" GW 7647 as PPARα and "Tro" Troglitazone as PPARγ agonist," DMSO "serves as a solvent control, "samples" tested fractions or compounds, "untransfected" cells as a control for transfection.

50 µl of the medium containing the agonists (GW 7647, troglitazone), of the tested compounds and the DMSO were added gently per well, in order to avoid damaging or flushing away the cells. The prepared solutions had to be mixed by vortexing shortly, before they could be added. After we added the compounds, the 96 well-plate was rocked back and forth gently and returned into the incubator for 18 hours (5 % CO_2, 37 °C).

Thereafter we aspirated the medium with a Pasteur pipette, starting with the untransfected cells, then the DMSO and finally the treatments. The plate then was measured immediately or put into the freezer for later processing.[47]

2.2.8. Fluorescence microscopy of transfected cells

In a first step it was essential to check the efficiency of the transfection process, by observing the 96 well-plate under the fluorescence microscope. If the transfection had worked properly, the GFP expression could be observed under the fluorescence microscope.

Principle and construction of the fluorescence microscope

The fluorescence microscopy is based on the fact, that a fluorochrome is able to emit detectable light after being irradiated with a light of certain wavelength. Through an excitation filter in the microscope the desired wavelength can be sorted out, that is able to excite the electrons in the GFP to a higher energy level. A second filter is needed to make the light emission visible, when the electrons turn to a lower energy level. According to the Stoke‘s Law the emitted light has a lower wavelength, so that the weaker emission light can be separated from the light, that has been used for exciting the electrons. A mercury lamp generates the exciting light.[56]

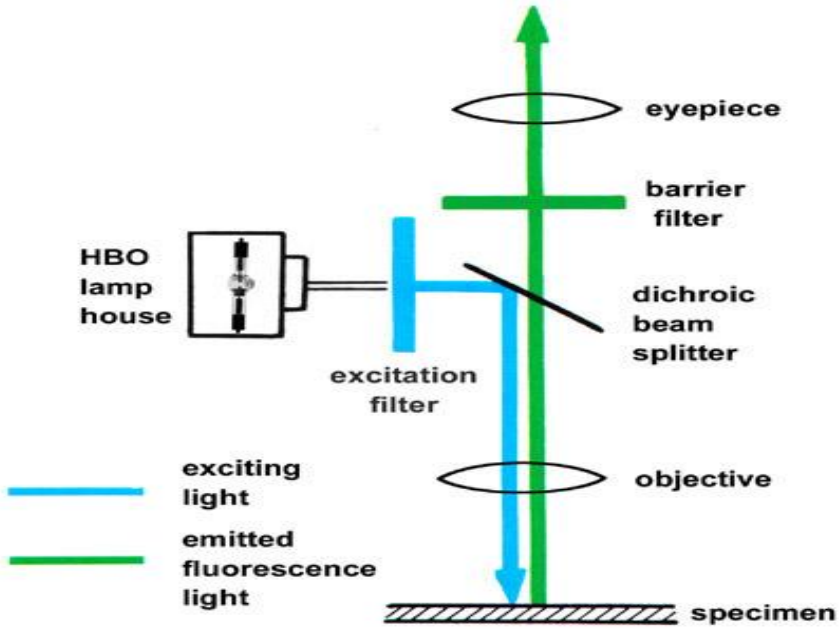


Figure 8: The principal of a fluorescence microscope.

Picture taken from: http://nobelprize.org/educational_games/physics/microscopes/fluorescence/.[56]

2.2.9. Fluorescence and luminescence measurement using the TECAN Genios PRO

Table 13:

Materials
One Eppendorf® 8-channel pipettor (10-100 µl)
Eppendorf® pipettors (10-100µl and 100-1000µl)
Sterile tips for Eppendorf® pipettes (2-200µl and 50-1000µl)
One Eppendorf® automatic pipetting-aid
One sterile graduated pipette (20ml)
One reservoir for the Lysis Buffer
Tecan Genios Pro fluorescence/luminescence analyzing device

Solutions and Buffers	**Ingredients**	**Amount**
Luciferin solution	Luciferin (Invitrogen)	25 mg
	dd H_2O	8.27 ml
ATP solution (ph 7.0)	ATP (Sigma)	1 g
	1 N NaOH	3.3 ml
	dd H_2O	13.4 ml
Luciferase buffer	Luciferin solution	1.1 ml
	1M Tricine buffer ph 7.8	220 µl
	dd H_2O	9.2 ml
Tricine- ATP-buffer	1 M Tricine	220 µl
	0.5 M $MgCl_2$	473 µl
	0.1 M ATP	407 µl
	0.27 M Coenzyme A	10 µl
	dd H_2O	9.9 ml
	DTT	11 µl
Lysis buffer	Lysis 5x Buffer (Promega)	2.4 ml
	dd H_2O	9.6 ml
	DTT	12 µl

Table 13: Materials, solutions and buffers needed for the luciferase assay.

The materials, solutions and buffers needed for the luciferase reporter assay are illustrated in table number 13.

Practical part

While the Luciferin solution and the ATP solution were thawed in a beaker with cold water for 30 minutes, the lysis buffer was prepared. It is important to keep the Luciferin solution always in the dark, as it is light sensitive.

Preparation of the Lysis buffer

2.4 ml Lysis 5x Buffer (Promega), 9.6 ml dd H2O and 12 µl of DTT were mixed in a glass and transferred into a plastic reservoir for multichannel pipetting. The 96 well plate containing the treated cells was taken out of the freezer. 50 µl of the Lysis Buffer was added per well, using the eight channel pipette, whereas the bottom of the plate must not be touched with the tips. We then covered the plate with aluminium foil and vortexed for ten minutes. In the meantime the TECAN Genios Pro (Serial number: 505000010; Firmware: V 3.20 03/05 Genios Pro, Version: V 4.63) was prepared for measurement.

The settings and parameters for the luminescence and fluorescence measurement with the Tecan Genios Pro are shown in table number 14 and 15.

Table 14:

Measurement mode	**Luminescence**
Integration time	2000 ms
Attenuation	none
Time between move and integration	1
Well kinetic number	2020 ms
Well kinetic interval	-250 ms
Injector A delay	50 µl
Injector A volume	200 µl/s
Injector A speed	-250 ms
Injector B delay	50µl
Injector B volume	200 µl/s
Injector B speed	standard

Table 14: Settings and parameters for the luminescence measurement with the Tecan Genios Pro.

Table 15:

Measurement Mode	**Fluorescence Top**
Excitation wavelength	485 nm
Emission wavelength	520 nm
Gain (optimal)	65
Number of reads	1
Integration time	1000 µs
Lag time	0 µs
Mirror selection	Dichroic 3
Time between move and flash	40 ms

Table 15: Settings and parameters for the fluorescence measurement with the Tecan Genios Pro.

We mounted the injector and started the washing process first. By selecting the injectors A+B and choosing to wash, prime and backflush, the tubes were filled with water. The ATP solution containing coenzyme A, and the Luciferin solution, which have been thawed in the meantime were attached to the TECAN. Through choosing the "prime" option, the tubes were filled with the reagents, and the TECAN reader was ready for measurement. For measuring one plate, 5 ml of ATP/Luciferin were needed, plus one ml for priming the tubes. After the plate was shaken for ten minutes, we transferred 40 µl out of each well into a black 96 well plate, which was used for the luminescence and fluorescence measurement. A one minute long shaking of the plate guaranteed an equal distribution of the lysed cells. Then we transferred the plate into the TECAN and the measurement of Luminescence and Fluorescence was started after the sledge with the injector was put in the injection position again. The results were saved and the TECAN cleaned by backflushing the reagents into the falcons. The remaining reagents were re-frozen at -80 °C. Finally we washed the injectors with EtOH 70% and water several times and backflushed the water into a waste falcon, so that the tubes were clean and empty for the next user.[47]

The calculation procedures

With the luminometer, it is possible to measure the emitted light in light units (LU). To account for a possible background luminescence the values obtained from the negative control (untransfected cells) were substracted from the results obtained with the test samples. From other side the untreated cells were transfected, but not treated with any samples, agonists or DMSO, and served as an internal control of the transfection efficiency.

The expression of the luciferase and therefore the ability to stimulate the promoter by the PPRE is certainly affected by the number of transfected cells. Here the measurement of the GFP served as an internal control, so that the relative light units (RLU) were calculated by dividing the values of the luciferase measurement by the values obtained from the GFP measurement. In this way the results obtained from the luciferase measurement are corrected taking into account the number of viable transfected cells.

In order to assure reproducibility of the received data, several (n= 2-4) independent experiments were carried out with all samples. For the statistical analyzes a two-tailed

paired Student t-test was done. The experimental results with $p \leq 0.05$ were considered significant.

The reference treatments

To assure a proper performance of the method, transfected cells treated with known agonists were used on each 96 well plate as positive control (GW 7647, troglitazone), whereas transfected cells treated with DMSO were always used as negative control. DMSO was used as solvent agent for all the tested samples. For all the tested samples the DMSO concentration was 0.1%. Therefore it is essential to know, whether the measured effect is derived from the sample or the DMSO. The cells that were treated with DMSO served as baseline value and the RLU of the DMSO control is defined as 1 fold activation of PPARα or PPARγ, respectively.

Known agonists of PPARα and PPARγ: GW 7647 and troglitazone

GW 7647:

The GW 7647 is a selective and potent agonist of human PPARα. This synthetic drug is able to activate PPARα, PPARγ and PPARδ with EC_{50} values of 0.006, 1.1 and 6.2 µM. It is a very effective PPARα agonist with a high intrinsic activity. It was used in this assay in concentration of 50 nM as a positive control, to assure that the stimulation of the PPARα worked out properly and the transfection efficiency was adequate.[57]

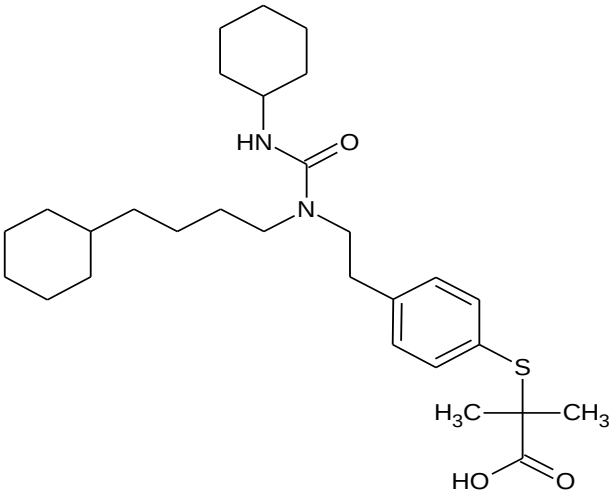


Figure 9: Chemical structure of GW 7647: 2-[[4-[2-[[(Cyclohexylamino)carbonyl](4-cyclohexylbutyl)amino]ethyl]phenyl]thio]-2-methylpropanoic acid.

Figure adapted from: http://www.caymanchem.com/app/template/Product.vm/catalog/10008613 [57]

Troglitazone

Troglitazone is a thiazolidinedione and was introduced as first synthetic PPARγ agonist for the treatment of insulin resistance and hyperglycemia in diabetes type 2 in the USA. As under the treatment of troglitazone death cases due to its hepatotoxicity were recognized, it was withdrawn from the market.

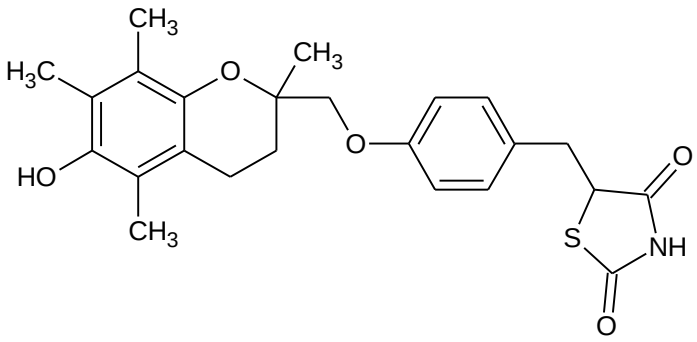


Figure 10: Chemical structure of troglitazone: (RS)-5-(4-[(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methoxy]benzyl)thiazolidine-2,4-dione.

Figure adapted from: http://www.caymanchem.com/app/template/Product.vm/catalog/71750/a/z. [58]

These adverse drug effects were caused because Troglitazone is metabolized to a structure with Vitamin E similarity. As it is a highly active and selective PPARγ agonist, with EC_{50} Values of 0.55 µM for human PPARγ, we used it as a positive control in a concentration of 10 µM for PPARγ activation. For troglitazone, no activation of PPARα/δ at this concentration was observed.[58]

The PPAR agonists were used as a positive control. The activation obtained was usually 3-5 fold above the level of the DMSO solvent control. This positive control served as a marker, whether the transfection efficiency was satisfying and the expressed receptors were stimulated by the known agonists.

D) Results

The results, received by the luciferase assay application are described in the following chapters (1.-3.). The tested extracts and fractions or pure compounds are classified according to their origin. Thus, the PPARα and PPARγ agonistic effects of plants deriving from the VOLKSMED database, the TCM database, and the chosen pure compounds and plants based upon a computational approach are listed separately.

The samples of greatest interest that exhibited a strong agonism on the PPARα and/or PPARγ are discussed separately. The plant extracts or subfractions were tested at 10 μg/ml, and the pure compounds at 30 μM concentration. Active samples were labeled with a "++" (activation more than 100% above the DMSO solvent control), moderately active samples were labeled with a "+" (activation between 50% and 100% above the DMSO solvent control), and the samples with no effect or effect lower than 50% induction with a "-" in the following chapters (1.-3.).

The internal identification number was used to easy identify the extracts/fractions in the laboratory during the screening process. All samples were tested two or more times until the reproducible results were obtained,

1. Plants deriving from the VOLKSMED database

Table number 16 illustrates the effects of the plants, deriving from the VOLKSMED database, on PPARα and PPARγ.

Table 16:

Internal identification number	**Plant/Extract**	**Part used**	**Solvent**	**Effect on PPARα**	**Effect on PPARγ**
784	*Glechoma hederacea* SPE fraction	herba	MeOH 30%	-	-

785	*Glechoma hederacea* SPE fraction	herba	MeOH 70%	++	++
786	*Glechoma hederacea* SPE fraction	herba	MeOH 100%	-	-
168	*Glechoma hederacea* extract (tannin separated)	herba	MeOH	+	+
796	*Peucedanum ostruthium* SPE fraction (tannin separated)	radix	MeOH 30%	-	-
797	*Peucedanum ostruthium* SPE fraction (tannin separated)	radix	MeOH 70%	+	-
798	*Peucedanum ostruthium* SPE fraction (tannin separated)	radix	MeOH 100%	-	-
922-923	*Peucedanum ostruthium* SPE 70% MeOH fraction, HPLC subfractions 1-2	radix	Acetonitril/ H_2O gradient	-	-
924	*Peucedanum ostruthium* SPE 70% MeOH fraction, HPLC subfraction 3	radix	Acetonitril/ H_2O gradient	-	+
925-926	*Peucedanum ostruthium* SPE 70% MeOH fraction, HPLC subfractions 4-5	radix	Acetonitril/ H_2O gradient	-	++
927-30	*Peucedanum ostruthium* SPE 70% MeOH fraction, HPLC subfractions 6-9	radix	Acetonitril/ H_2O gradient	-	+

Table 16: Samples deriving from the VOLKSMED database and their activity on PPARα or PPARγ .

The results are obtained from two or more independent experiments. All extracts or fractions were tested at concentration of 10 μg/ml. ("-" designates no activation, "+" activation of 50%-100%, and "++" a strong higher than 100% activation above the DMSO solvent control)

1.1 Highly active extracts/fractions

PPARα or PPARγ agonism with activation of more than 100% above the DMSO solvent control (two fold activation) was considered as highly effective. The samples meeting these properties are presented in chapter 1.1.1-1.1.2.

1.1.1. *Glechoma hederacea* Linn. (Lamiacaeae)

The fractions, received after Solid Phase Extraction (SPE) of the regarding herbal extract showed different activity on PPARα and PPARγ. The initial extract (168) as well as the methanolic sub fractions (784-86) did not contain tannins. The fraction which was eluted with 70% MeOH showed the greatest potential (785) (figure 11). The methanolic fraction 785 activated PPARα 2.75 fold, the PPARγ was activated 2.58 fold in comparison to the DMSO (one fold activation). The effect of the initial *Glechoma hederacea* fraction 168 was in comparison to sample 785 moderate, activating PPARα 1.9 fold and PPARγ 1.77 fold.

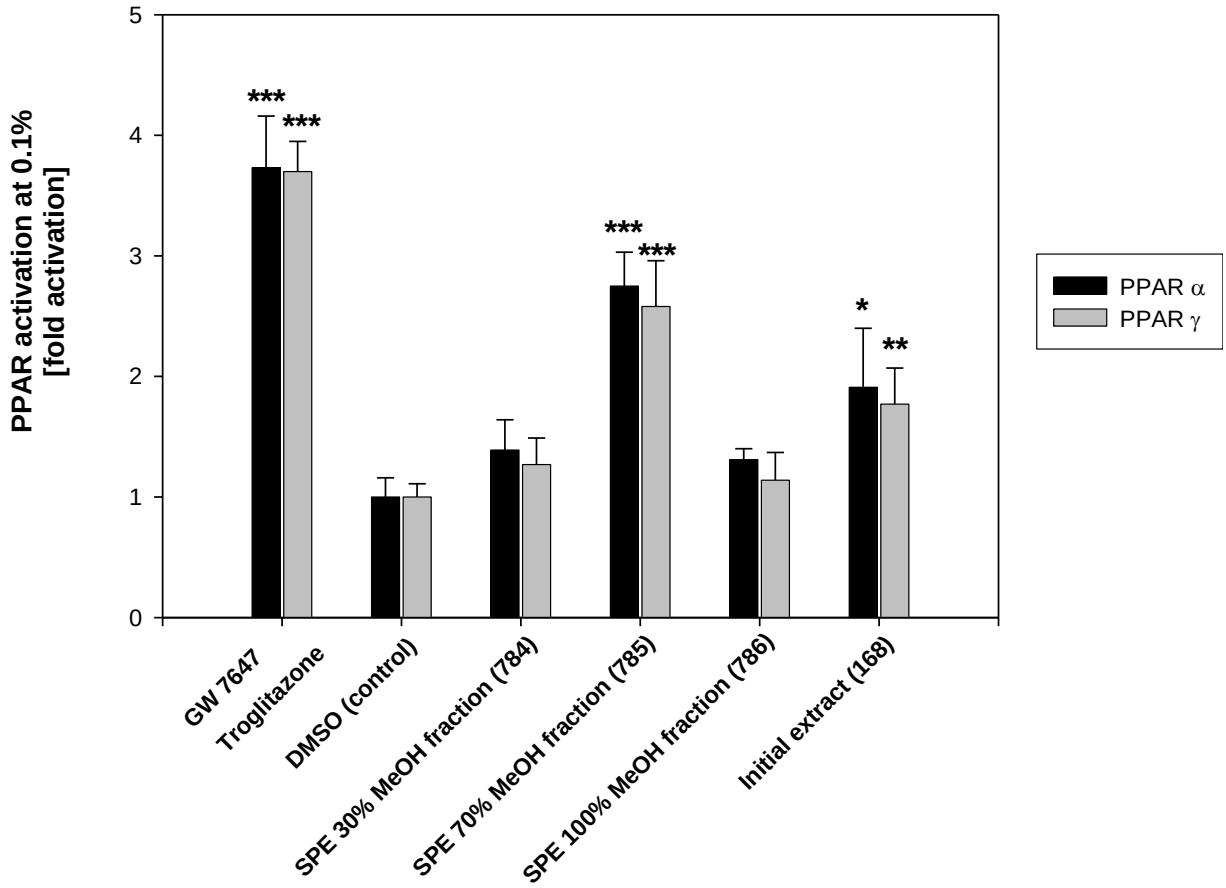


Figure 11: The Effects of Glechoma hederacea fractions on PPARα and PPARγ, in comparison to the known agonists of PPARα (black) and PPARγ (grey), GW 7647 and troglitazone, respectively.

The figure shows a representative graph out of two independent experiments performed in quadruplicate.

*Figure 11: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

1.1.2. *Peucedanum ostruthium* Linn. (Apiaceae)

Highly effective SPE fractions of the 70% methanolic *Peucedanum ostruthium* radix extract on PPARγ were the subfractions four and five, respectively sample number 925 and 926. All subfractions were obtained from HPLC through elution with Acetonitril and H_2O gradient. Sample number 925 and 926 exhibited properties of specific PPARγ agonists, not affecting the PPARα. For the subfraction four a 2.28 fold activation, for the subfraction number five a 2.35 fold activation of PPARγ could be determined.

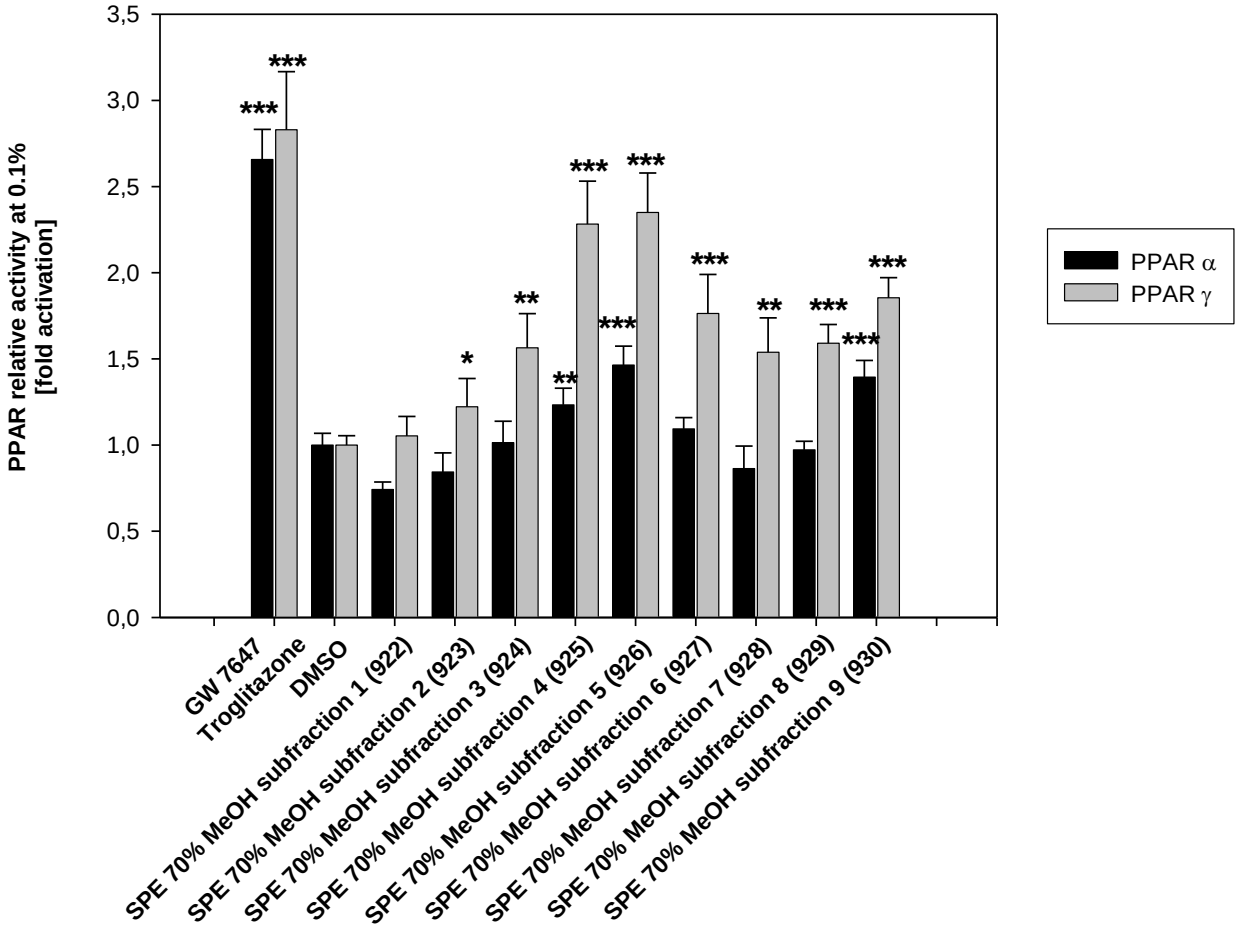


Figure 12: The effects of the Peucedanum ostruthium subfractions 922 to 930 on PPARα/γ compared with the agonists, GW 7647 and Troglitazone.

The figure shows a representative graph out of three independent experiments performed in quadruplet.

*Figure 12: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

1.2. Extracts and fractions, showing a moderate effect on PPARα and PPARγ

The samples, which were able to stimulate the PPARα and PPARγ less than 100%, but more than 50%, are presented in this chapter.

1.2.1. *Glechoma hederacea* Linn. (Lamiaceae)

The *Glechoma hederacea* fraction 168 is the initial fraction; by generating the sub fractions through SPE it was possible to receive sub fractions with different activity. The activity of this initial extract was in comparison to the SPE 70% MeOH fraction (sample 785) moderate, activating PPARα 1.9 and 1.6 fold and PPARγ 1.8 fold twice (Figure 11).

1.2.2. *Peucedanum ostruthium* Durazz. (Apiaceae)

The *Peucedanum ostruthium* 70% MeOH subfractions six to nine (927-930) showed a moderate effect on PPARγ. The subfraction seven and eight were able to activate the PPARγ 1.54 and 1.59 fold, the subfraction six 1.76 fold and the subfraction nine 1.87 fold, showing the highest activation within this group.

2. Compounds and plants selected based upon *in silico* tools

2.1. Virtually predicted pure compounds

Table number 17 illustrates the effects of pure compounds, selected based upon in silico tools, on PPARα and PPARγ.

Table 17:

Internal identification number	**Plant**	**Part used**	**Molecular weight**	**Effect on PPARα**	**Effect on PPARγ**
793	*Krameria triandria*	Radix	264	-	-
794	*Krameria triandria*	Radix	250	-	-
795	*Krameria triandria*	Radix	266	-	-
913	*Doronicum austriacum*	Radix	180	-	-
914	*Doronicum austriacum*	Radix	338	-	-
915	*Doronicum austriacum*	Radix	320	-	-
916	*Doronicum austriacum*	Radix	218	-	-
917	*Citrus sinensis*	Pericarp	372	-	-
918	*Citrus sinensis*	Pericarp	402	-	-
919	*Citrus sinensis*	Pericarp	372	-	-
920	*Citrus sinensis*	Pericarp	342	-	-
921	*Citrus sinensis*	Pericarp	342	-	-

Table 17: The virtually predicted pure compounds.

The results are obtained from three independent experiments. All compounds were tested at concentration of 30 μM. ("-" designates no activation)

2.2. Plants selected based on a computational approach

Table number 18 illustrates the effects of plants, selected based upon a computational approach, on PPARα and PPARγ.

The subfractions of *Sideritis hyssopifolia* A1-A11 were received by separation of a *Sideritis hyssopifolia* parent extract, which showed a highly effective PPARα activation. The samples 906-912 were prepared by separation of a *Sideritis hyssopifolia* parent extract, which was effective on both PPARs (Sideritis hyssopifolia B1-B7).

Table 18:

Internal identification number	**Plant/Extract**	**Part used**	**Solvent**	**Effect on PPARα**	**Effect on PPARγ**
895-896	*Sideritis hyssopifolia* A1-A2	herba	DMSO	-	-
897-899	*Sideritis hyssopifolia* A3-A5	herba	DMSO	++	++
900-901	*Sideritis hyssopifolia* A6-A7	herba	DMSO	-	+
902-903	*Sideritis hyssopifolia* A8-A9	herba	DMSO	++	++
904-905	*Sideritis hyssopifolia* A10-A11	herba	DMSO	++	+
906	*Sideritis* hyssopifolia B1	herba	DMSO	+	-
907-912	Sideritis hyssopifolia B2-B7	herba	DMSO	-	-

Table 18: Plant selection based upon a computational approach.

The results are obtained from three independent experiments. All extracts or fractions were tested at concentration of 10 μg/ml. ("-" designates no activation, "+" activation of 50%-100%, and "++" a strong higher than 100% above the DMSO solvent control).

2.2.1. Highly effective *Sideritis hyssopifolia* fractions

The tested *Sideritis hyssopifolia* subfractions A3-A5 (897-899) and A8-A9 (902-903) are characterized by a strong PPARα and PPARγ activation. The subfractions A10-A11 (904-905) showed high PPARα and moderate PPARγ activities. In detail, the subfractions A3-A5 activated the PPARα 3.44, 3.51, and 3.72 fold, the PPARγ was activated 3.02 2.00, and 2.60 fold. The highly effective subfractions A8-A9 activated the PPARα 3.30 and 3.75 fold, the PPARγ activation was 3.15 and 2.40 fold. The fractions A10 and A11 were highly effective on PPARγ with 2.28 and 2.38 fold activation, whereas the effect on PPARα was moderate with 1.64 and 1.86 fold activation.

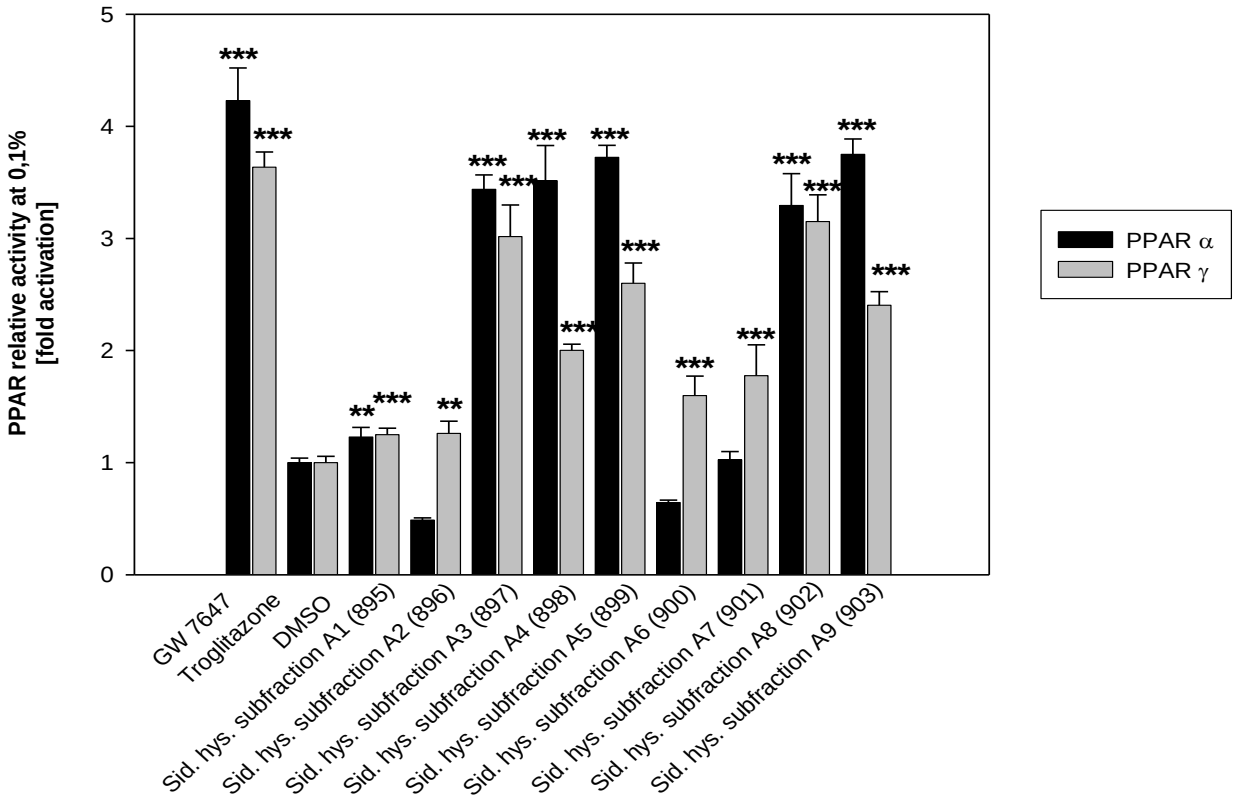


Figure 13: The Sideritis hyssopifolia subfractions (895-903).

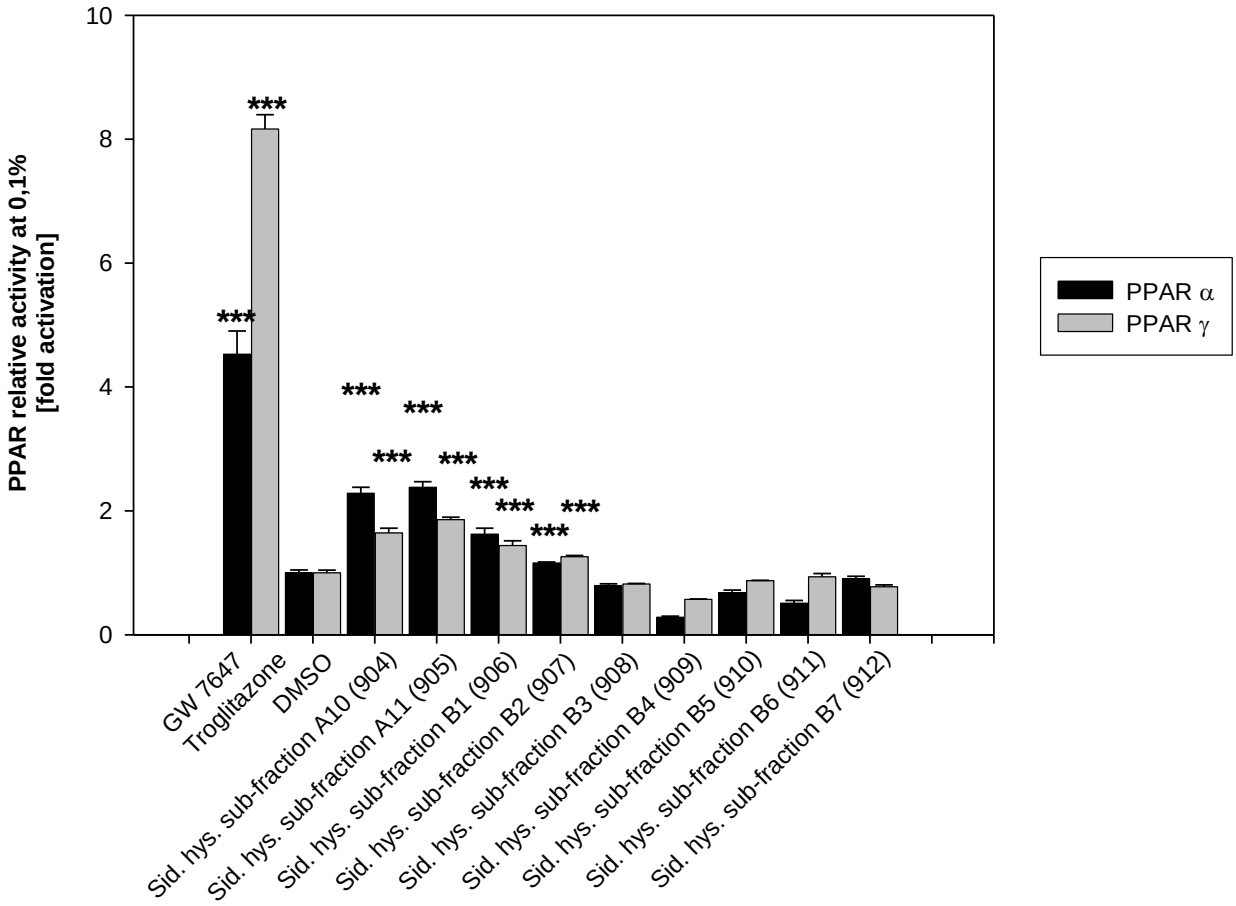


Figure 14: The Sideritis hyssopifolia subfractions (904-912).

The figures 13 and 14 show a representative graph out of three independent experiments performed in quadruplicate

*Figures 13 and 14: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

2.2.2. Moderately active *Sideritis hyssopifolia* subfractions

Although the initial extract of *Sideritis hyssopifolia* showed PPARα and PPARγ activation in previous experiments, the subfractions B1-B7 (906-912) did not exhibit the expected efficiency. Only the *Sideritis hyssopifolia* B1 subfraction (906) showed a moderate effect on PPARα, activating it 1.62 fold. The *Sideritis hyssopifolia* A6 and A7 subfractions (900 and 901) worked as moderately effective PPARγ agonists, activating it 1.60 and 1.78 fold.

3. Plants tested for PPARα and PPARγ activity that are not part of the DNTI project

3.1. Plants deriving from the TCM

Table number 19 illustrates the effects of plants deriving from the TCM database on PPARα and PPARγ.

Table 19:

Internal identification number	**Plant/Extract**	**Part used**	**Solvent**	**Effect on PPARα**	**Effect on PPARγ**
787	*Arisaema sp.*	Rhizoma praep.	MeOH	-	-
788	*Arisaema sp*.	Rhizoma praep.	MeOH (tannin separated)	++	++
789	*Arisaema sp*.	Rhizoma praep.	DC	+	+
790	*Pinellia sp*.	Rhizoma praep.	MeOH	-	-
791	*Pinellia sp*.	Rhizoma praep.	MeOH (tannin separated)	++	+
792	*Pinellia sp*.	Rhizoma praep.	DC	+	+

858-862	*Arisaema sp*. sub-subfraction 1-5 (from extract 789)	Rhizoma praep.	DC	-	-
863	*Arisaema sp*. sub-subfraction 6 (from extract 789)	Rhizoma praep.	DC	++	-
864	*Arisaema sp*. sub-subfraction 7 (from extract 789)	Rhizoma praep.	DC	++	-
865	*Arisaema sp*. sub-subfraction 8 (from extract 789)	Rhizoma praep.	DC	-	-
866	*Arisaema sp*. sub-subfraction 9 (from extract 789)	Rhizoma praep.	DC	+	-
867-869	*Arisaema sp*. sub-subfraction 10-12 (from extract 789)	Rhizoma praep.	DC	-	-
809	*Albizzia julibrissin* SPE fraction	cortex	PE	-	-
810	*Albizzia julibrissin* SPE fraction	cortex	DC1	-	-
811	*Albizzia julibrissin* SPE fraction	cortex	DC2	++	++
812	*Albizzia julibrissin* SPE fraction	cortex	EtOAc	++	++

Table 19: The TCM derived plants that are not part of the DNTI.

The results are obtained from three independent experiments. All extracts or fractions were tested at concentration of 10 μg/ml. ("-" designates no activation, "+" activation of 50%-100%, and "++" a strong activation higher than 100% above the DMSO solvent control)

3.1.1. Highly effective extracts and fractions

Arisaema species (Araceae)

Among the tested *Arisaema sp*. initial extracts, the methanolic with tannin separated fraction (788), did show a moderate effect on PPARα and a high effect on PPARγ, with a 1.91 and 2.23 fold activation within the performed experiment. The sample numbers 858-869 are subfractions from the *Arisaema sp*. Dichlormethane extract (789), won by SPE. Interestingly the subfractions six and seven (863 and 864) showed a strong effect on PPARα, activating it 2.10 and 2.21 fold, although the initial extract (789) did only show moderate effects, with a 1.69 fold activation for PPARα and a 1.59 fold activation for PPARγ.

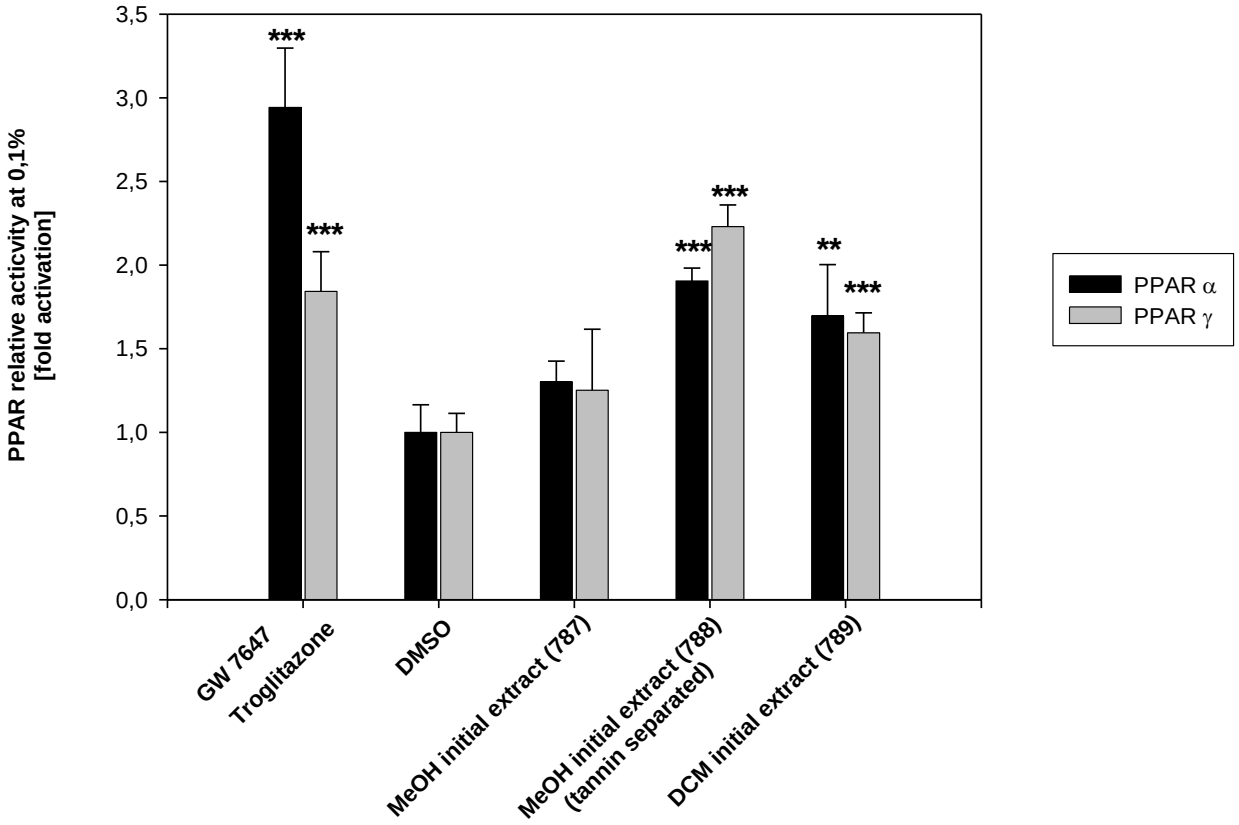


Figure 15: The effects of the Arisaema sp. initial extracts (787-89) on PPARα and PPARγ.

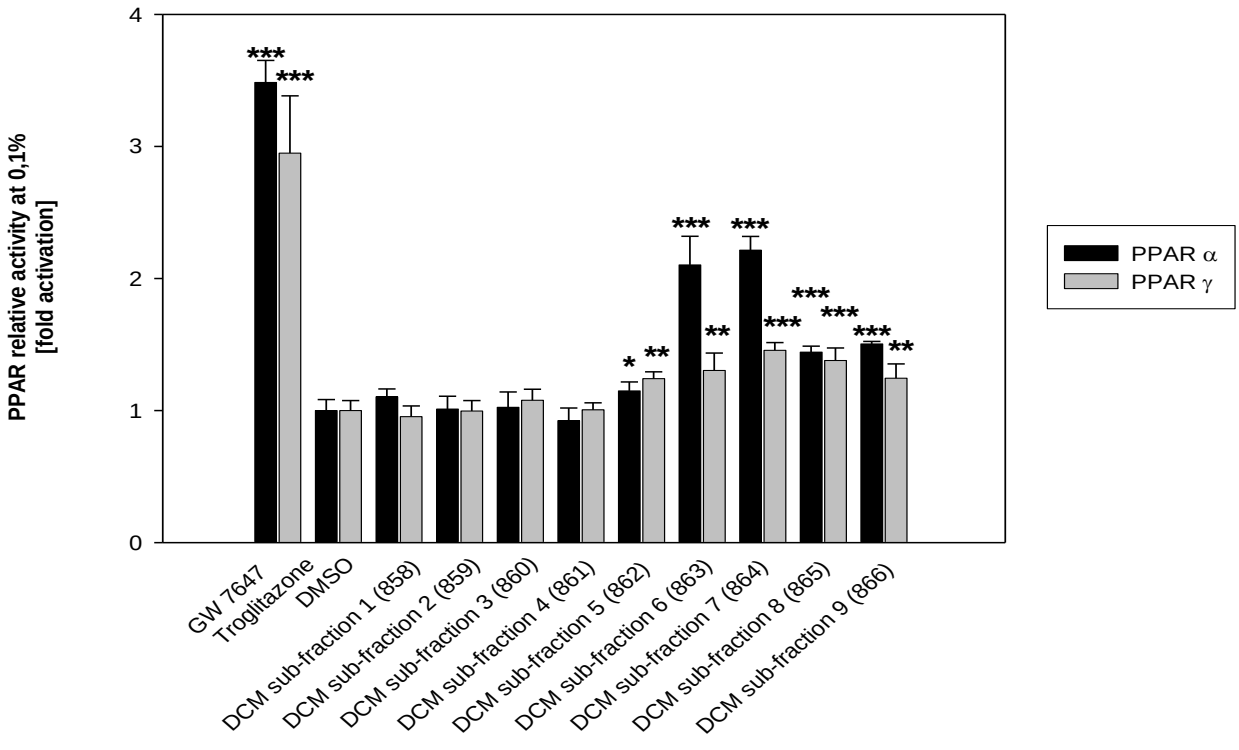


Figure 16: The effects of the Arisaema sp. DCM sub fractions (858-866) on PPARα and PPARγ.

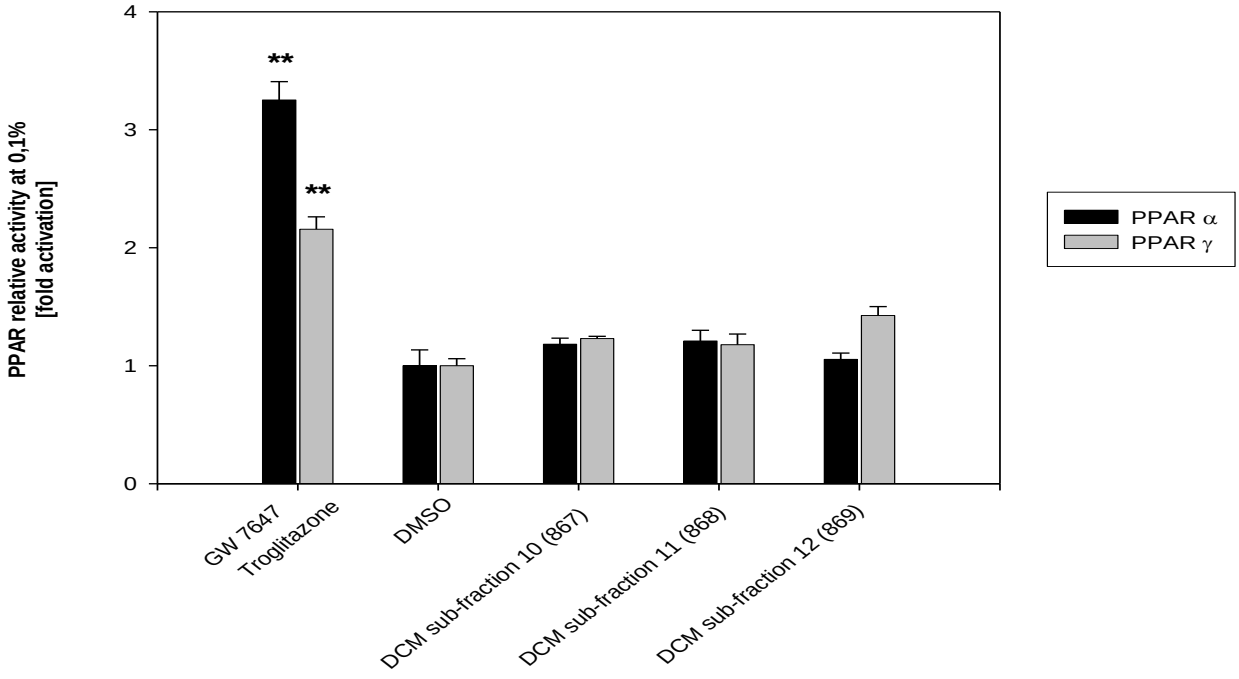


Figure 17: The effects of the Arisaema sp. DCM sub fractions (867-869) on PPARα and PPARγ.

The figures 15-17 show a representative graph out of three independent experiments performed in quadruplicate

*Figures 15-17: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

Pinellia sp. (Araceae)

The dichloromethane (792) extract of the rhizome of *Pinellia sp*. induced moderate activation of PPARα and a strong activation of PPARγ. The tannin separated methanolic (791) extract induced a strong activation of PPARα and a moderate of PPARγ. Sample number 791 and 792 activated the PPARα 2.07 and 1.83 fold, the PPARγ activation was 1.97 fold for fraction 791 and 2.11 fold for fraction 792.

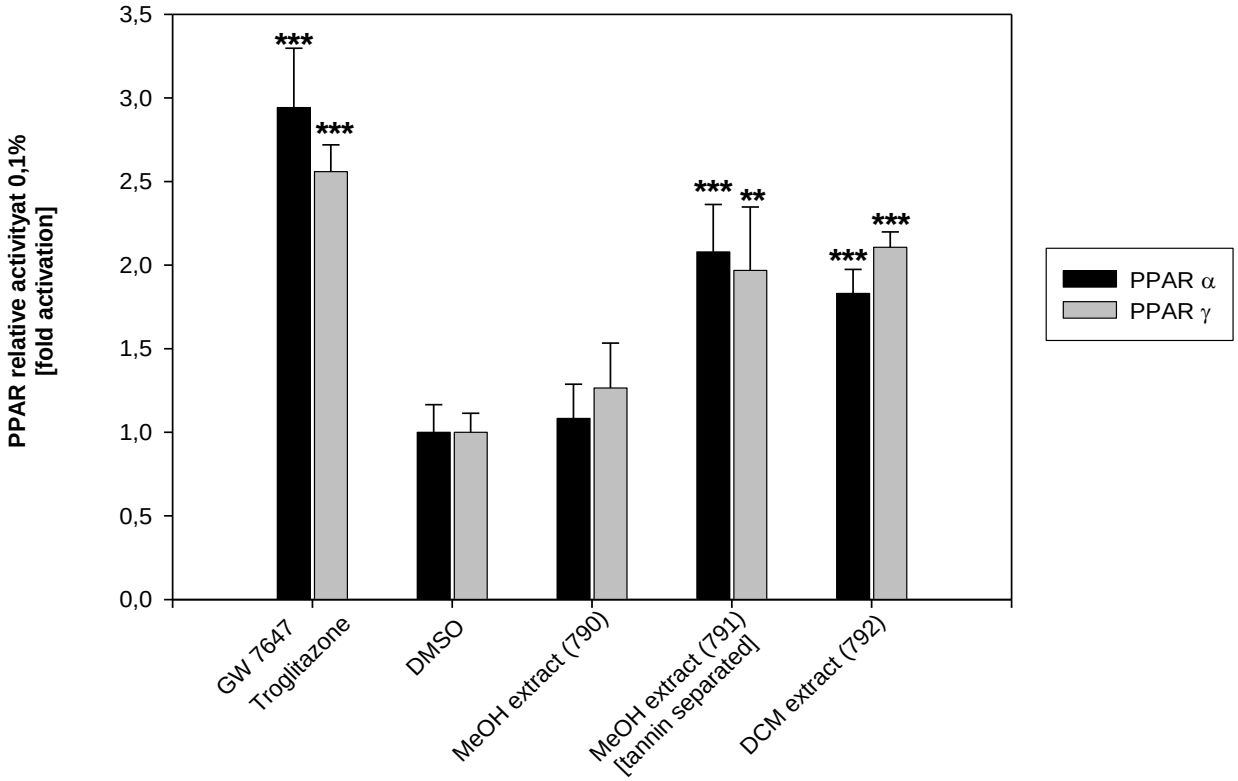


Figure 18: The effects of the Pinellia sp. DCM sub fractions (867-869) on PPARα and PPARγ.

The figure 18 shows a representative graph out of three independent experiments performed in quadruplicate

*Figure 18: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

Albizzia julibrissin Durazz (Leguminosae)

The fractions shown in figure 20 were eluted by SPE of the initial DCM extract using solvents with incrising polarity, whereas the fraction number 809 was received by elution with PE, the fractions number 810 and 811 with DC and the fraction number 812 with EtOAC. The initial extract was received from the cortex of *Albizzia julibrissin* and showed a high effect on PPARα and PPARγ. In the shown experiment (figure 12) on PPARα and PPARγ activation, fraction number 811 induced 2.4 fold activation of PPARα and 3.3 fold activation of PPARγ. Within this experiment the fraction number 812 induced a 2.69 and 2.65 fold activation of PPARα and PPARγ respectively.

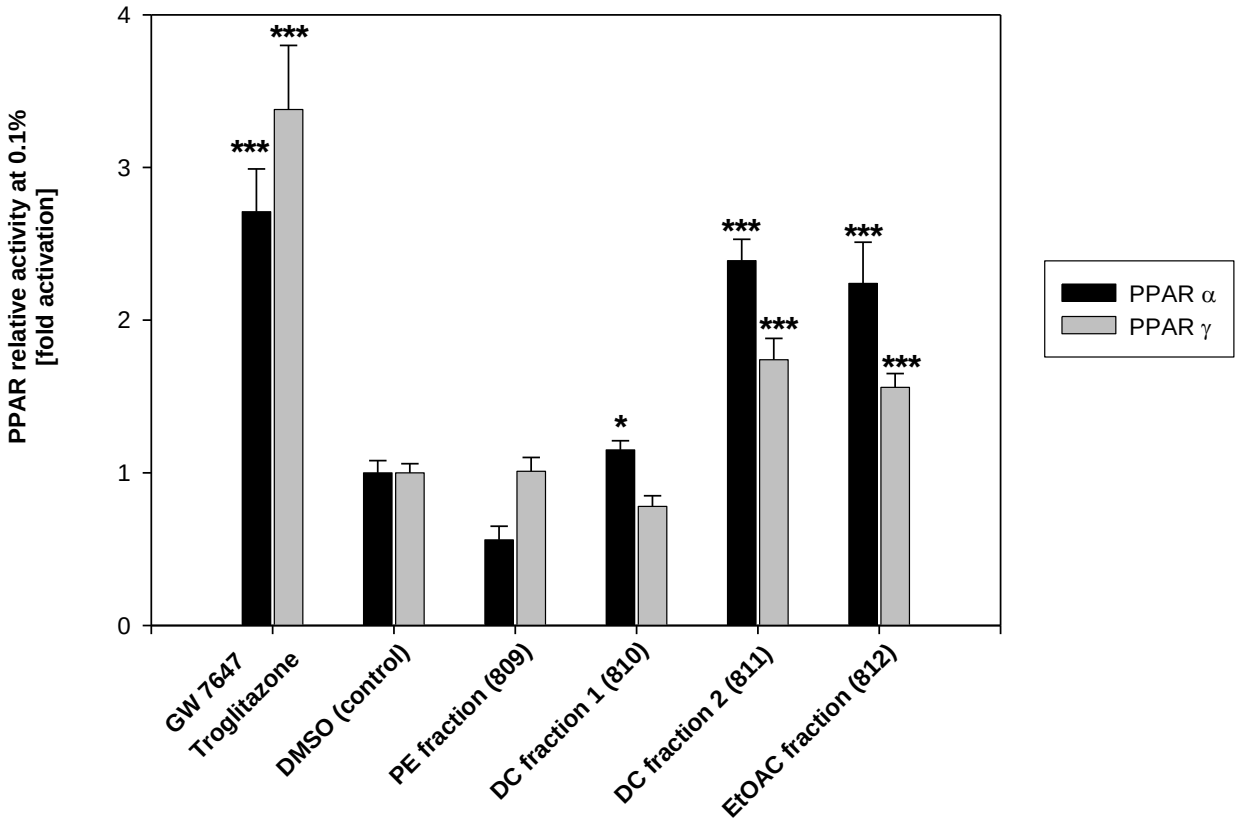


Figure 19: The biological activity of Albizzia julibrissin fractions on PPARα (black) and PPARγ (grey) activation, compared to the potent synthetic agonists GW 7647 and troglitazone.

The figure shows a representative graph out of three independent experiments performed in quadruplicate.

*Figure 19: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

3.2. Plants deriving from the VOLKSMED database

Table number 20 illustrates the effects of plants, deriving from the VOLKSMED database, on PPARα and PPARγ.

Table 20:

870	*Dryopteris filix-mas* 100% MeOH fraction	species	MeOH 100%	-	++
871-876	*Dryopteris filix-mas* subfraction 1-6 (from 870)	species	MeOH gradient	-	-
878	*Dryopteris filix-mas* 70% MeOH fraction	species	MeOH 70%	-	++
879-881	*Dryopteris filix-mas* subfraction 1-3 (from 878)	species	MeOH gradient	-	-
882	*Dryopteris filix-mas* subfraction 4 (from 878)	species	MeOH gradient	-	+
883-885	*Dryopteris filix-mas* subfraction 5-7 (from 878)	species	MeOH gradient	-	-
886	*Dryopteris filix-mas* subfraction 8 (from 878)	species	MeOH gradient	-	+

Table 20: Plants derived from the VOLKSMED database that are not part of the DNTI.

The results are obtained from three independent experiments. All extracts or fractions were tested at concentration of 10 μg/ml. ("-" designates no activation, "+" activation of 50%-100%, and "++" a strong activation higher than 100% above the DMSO solvent control)

3.2.1. Highly effective *Dryopteris filix-mas* fractions

From the investigated *Dryopteris filix mas* fractions, the 100% (870) and the 70% (878) methanolic initial fractions showed a high effect on PPARγ. For the 100% methanolic fraction no subfractions with relevant activity on PPARγ could be identified. As illustrated in figure 20 and 21 the sample number 870 was able to stimulate the PPARγ 2.07 fold; the sample number 878 showed an even stronger effect on PPARγ, activating it 3.79 fold.

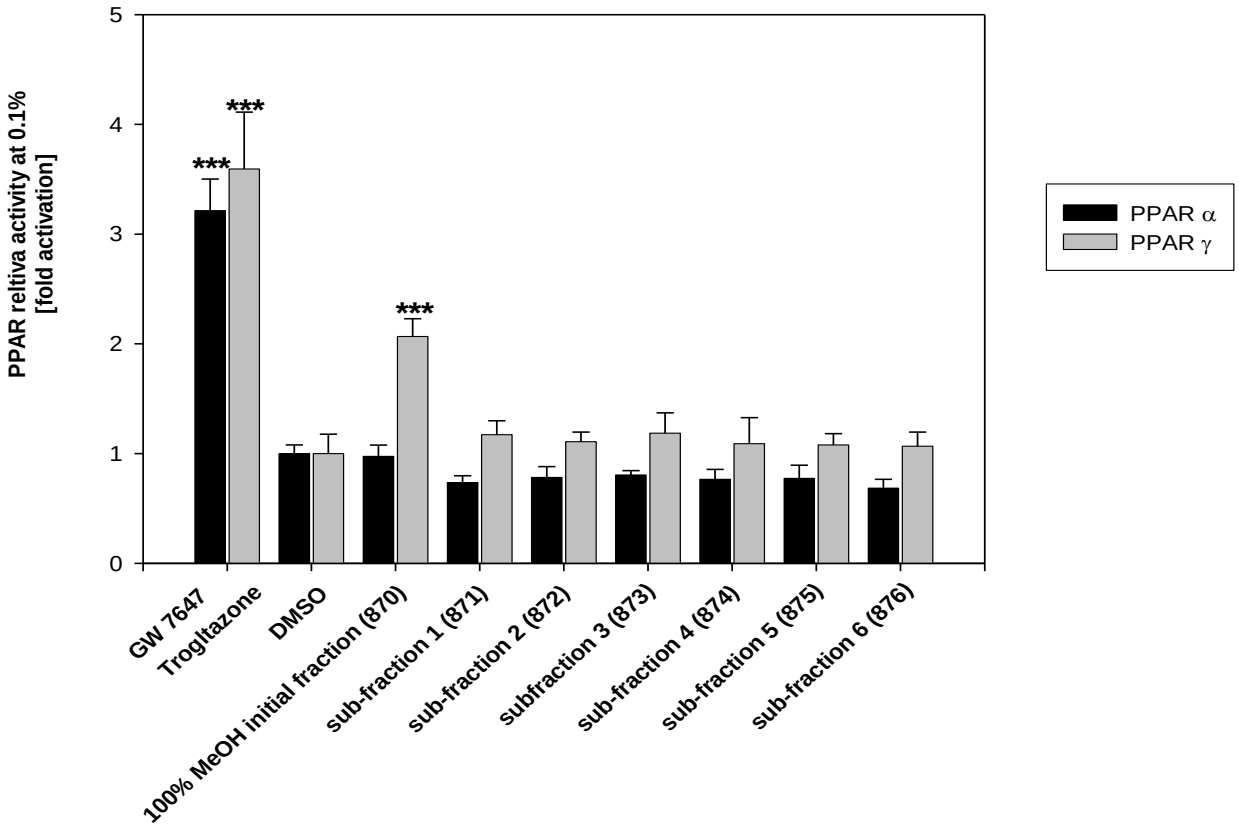


Figure 20: The Dryopteris filix mas initial fraction (870) and subfractions (871-76).

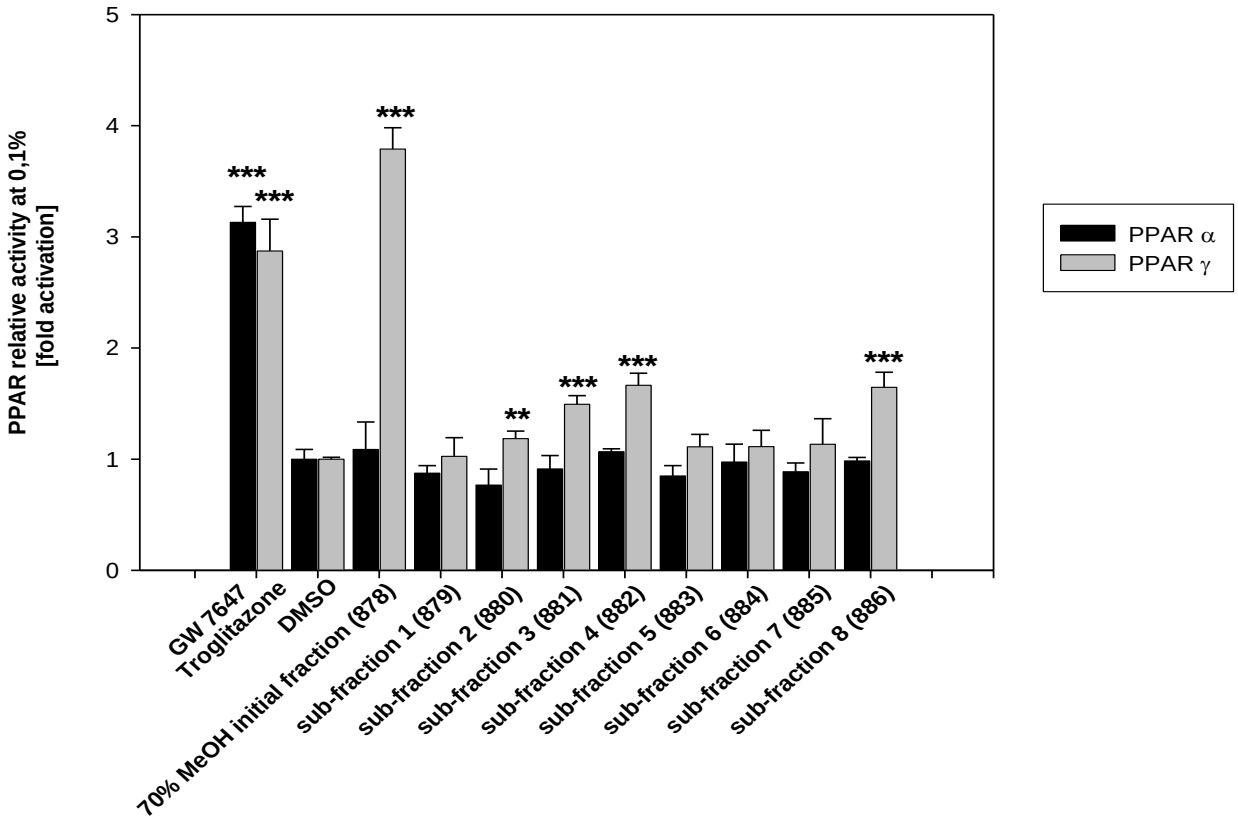


Figure 21: The Dryopteris filix mas initial fraction (878) and subfractions (879-86).

The figures 20 and 21 show a representative graph out of three independent experiments performed in quadruplicate.

*Figures 20 and 21: Unpaired two-tailed Student`s t-test: * : p ≤ 0.05, ** : p ≤ 0.01, *** : p ≤ 0.001: statistical significance.*

3.2.2. Moderately active *Dryopteris filix-mas* subfractions

The methanolic subfractions four (882) and eight (886) of *Dryopteris filix-mas*, originating from the 70% MeOH fraction (878), were able to stimulate the PPARγ 1.67 and 1.65 fold.

E) Discussion

1. Plants deriving from the VOLKSMED database (tested within the DNTI project)

The *Glechoma hederacea* fraction, which was eluted with MeOH 70%, was the most interesting fraction among the investigated fractions from this plant. As the fractions eluted with 30% and 100% MeOH did not show any effects, it can be concluded that the ratio between polarity/apolarity of the solvent achieved with 70% MeOH worked most successfully to isolate the bioactive compounds. Further fractionation and isolation processes should be carried out in order to detect the bioactive compounds. These compounds might be very interesting as they work on both PPARs with a similar activity, and there are indications from the literature that dual PPARα and PPARγ agonists are especially promising ligands. A new PPARα and PPARγ agonist, Aleglitazar, is currently being investigated within a phase II trial among patients with diabetes and coronary artery disease. Major cardiovascular endpoints (death, myocardial infarct, stroke) are reduced by this new dual working principle on PPARα and PPARγ.[59] If the *Glechoma hederacea* components can be identified, they may as well be interesting candidates for further lead development, as a dual activation of both investigated PPARs seems to be a benefit to co-morbidity patients, such as patients suffering from the metabolic syndrome.

The *Peucedanum ostruthium* subfractions four and five are the most promising ones for further investigations. Interestingly these subfractions seem to have compounds with a high affinity and selectivity to the PPARγ. The efficacy on PPARγ might be one reason why the roots of *Peucedanum ostruthium* are used for the therapy of rheumatism within Europe for already several centuries. It remains to be investigated whether the PPARγ activating principle is a new or known compound, such as ostholes and ostruthin.

2. Compounds and plants selected based upon *in silico* tools (tested within the DNTI project)

2.1. Virtually predicted pure compounds

2.1.1. *Krameria triandria* Ruiz. and Pav. (Krameriaceae)

The antioxidant and photoprotective characteristics of the *Krameria triandria* root extract could be proven in previously studies, using different cell models. The most important working principles for this effect could be identified, such as the 2-(2, 4-dihydroxyphenyl)-5-(E)-propenylbenzofuran.[60]

The polyphenolic constituents of the *Krameria trandria* roots are now known for several years, as they have been detected, using techniques such as the LC/MS (liquid chromatography/mass spectrometry) or FAB-MS (fast-atom bombardment mass spectrometry). One of the compounds, identified by this methods is the rhataniaphenol (2-(2-hydroxy-4-methoxyphenyl)-5-propenylbenzofuran), known for its radical scavenging activity.[61] The oligomeric proanthocyanidines of the Krameria triandria root extract are reported to have an antimicrobial effect.[62]

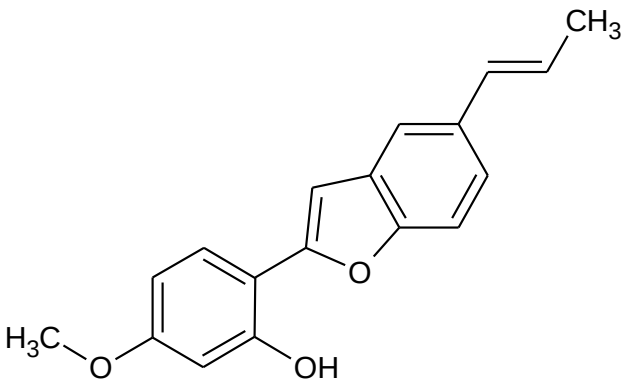


Figure 22: Structure of Rhataniaphenol: (2-(2-hydroxy-4-methoxyphenyl)-5-propenylbenzofuran).

Figure adapted from Facino, M. & al. (1997).[61]

The diseases aggravated by free radicals, such as atherosclerosis or coronary heart disease, could be affected positively by the *Krameria triandria* components consequently.[61]

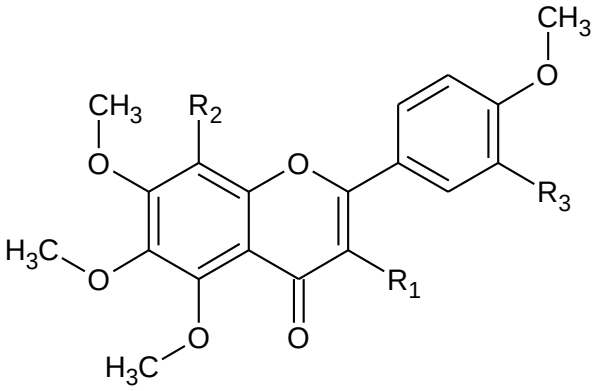
The *Krameria triandria* compounds, which we tested for PPARα and PPARγ- activity, did unfortunately not have the properties of a PPAR activating ligands.

2.1.2. *Citrus sinensis* Linn. (Rutaceae)

The pericarp of *Citrus sinensis* is known for its anti-inflammatory, antioxidant and hypolipidemic ingredients, the polymethoxylated flavones.[63] These flavones belong together with the O-glycosylated flavones, C-glycosylated flavones, O-glycosylated flavonols, O-glycosylated flavanones and phenolic acids and their ester derivatives to the main known flavonoid groups of the methanolic extract of the orange peel.[64]

The polymethoxylated flavones are also able to inhibit the growth of human breast cancer cells. For the apoptotic induction, a Ca^{2+}-dependent mechanism is reported. Especially the hydroxylated methoxyflavones, such as 5– hydroxy– 3, 6, 7, 8, 3´, 4´,- hexamethoxyflavone, lead to a Ca^{2+}-dependent activation of apoptotic caspases (caspase 12) in MCF–7 breast cancer cells.[65]

This fruit is used in combination with the *Phellodendron amurense* tree bark for the therapy of osteoarthritis, which represents the most frequently appearing form of arthritis. The polymethoxylated flavones are known to inhibit the TNF-α, IF-γ, IL-1 and IL-6 expression in adipocytes and therefore to target inflammation efficiently.[63]



A) R_1 = H; R_2 = OMe, R_3 = H

B) R_1 = OMe; R_2 = OMe; R_3 = OMe

C) R_1 = H; R_2= OMe; R_3 = OMe

D) R_1= H; R_2= H; R_3 = H

E) R_1= OMe; R_2= H; R_3 = OMe

F) R_1= H; R_2= H; R_3 = OMe

Figure 23: The chemical structure of some polymethoxylated flavones: A, tangeretin; B, heptamethoxyflavone; C, nobiletin; D, tetra-O-methylscutellarein; E, hexamethoxyflavone; F, sinensetin. Figure adapted from: Dugo, P & al (1996).[66]

The pure compounds of the pericarp of *Citrus sinensis* did not show any effects on the investigated PPARs. It seems that components of the *Citrus sinensis* pericarp are capable of ameliorating inflammatory processes[67], but the tested compounds in our assay did not indicate that such effects could be explained through PPARs activation. Further screening of 3D-multiconformational databases and docking/scoring processes might be interesting though, as the anti-inflammatory potential of this fruit has already been documented.[63]

2.2. Plants selected based upon a computational approach

Although the subfractions of the two *Sideritis hyssopofilia* extracts represent the only investigated samples of plants, selected based on a computational approach, the data of the PPAR activation experiments are very promising. Especially among the subfractions A1-A11, received by separation of the *Sideritis hyssopofilia* extract, which showed a strong PPARα activity in previous experiments, several samples could be identified as highly interesting. It is remarkable that within these eleven subfractions, five subfractions could be identified as highly effective extracts on both, PPARα and PPARγ and furthermore two fractions as highly effective on PPARα and moderately effective on PPARγ. These findings demonstrate, that the computational approach, from the selection of possible structures from multiconformational 3D-databases to pharmacophore modeling and docking and scoring, have a great potential in the development of new drugs. For the tested *Sideritis hyssopofilia* fractions A1-A11 it can be assumed with a high probability, that the separation of the PPARα and PPARγ active principles, will lead to one or more active compounds. Unfortunately the investigated *Sideritis hyssopofilia* B1-B7 did not confirm the high activation of PPARα and PPARγ as suggested in previous experiments. Only the first fraction, B1, was able to stimulate the PPARα moderately.

3. Plants testet for PPARα and PPARγ activity that are not part of the DNTI

3.1. Plants deriving from the TCM

Several independent publications show the therapeutic efficacy of the *Arisaema species*. The known effects differ from anti-proliferative to anti-inflammatory, underlining the importance of further investigations on the *Arisaema species*. The most effective fraction among the tested samples was definitely the tannin separated, methanolic extract (788). This result suggests that it would be essential to further process this extract, using the bio-assay guided fractionation principles, as done with the dichloromethane extract of *Arisaema sp*. (789), which showed only a moderate effect on both PPARs in our experiments. As mentioned in chapter 3.1.1., the sub fractions six and seven of the respective dichloromethane extract surprisingly induced a high effect on PPARα. It is possible that components, which interfered with the efficiency of the initial extract, were separated by the SPE, so that the respective sub fractions were activating the PPARα with a high selectivity and intrinsic activity. It would be furthermore important to characterize different species of *Arisaema* in order to address if the bioactivity could not be attributed just to a single species.

Previous studies have already revealed the potential of *Pinellia sp*. to improve diseases, related to the metabolic syndrome. It was found, that components of *Pinellia sp*. have an effect on essential enzymes in metabolic pathways, such as uncoupling proteins and PPARα, by an upregulation of the respective genes.[42] Our experiments were able to show furthermore that components of *Pinellia sp*., as in particular the tannin-separated, methanolic (791) and the dichloromethane (792) extract of the rhizome were found to have effects on both investigated PPARs. On the first look, the activity of these two fractions does not seem to be very high, but from other side it is interesting to mention the approximately equal activation of PPARα and PPARγ, underlining the importance of a further fractionation of these two extracts. As already discussed for *Arisaema*, it would be interesting as well for *Pinellia* to characterize different species.

The DC2 (811) and EtOAc (812) fractions, received by fractionation of the initial *Albizzia julibrissin* extract are as well interesting candidates for elucidation of bioactive compounds. Bio-assay guided fractionation to reveal the PPARα and PPARγ activating compounds is currently in progress with these two fractions. As the cortex of *Albizzia julibrissin* is known in the Chinese Pharmacopoeia for its anti-inflammatory properties, these traditional findings conform to the data collected in our experiments. The data from the tested *Albizzia julibrisin* extracts are comparable to the data received from the highly effective *Glechoma hederacea* extracts. If the PPARα and PPARγ agonism is achieved by a single molecule, it will as well be an interesting structure for further lead optimization.

3.2. Plants deriving from the VOLKSMED database

Until now, there is little information on the components of the *Dryopteris filix-mas* rhizome. The results of our investigations support the idea to enforce investigations in this fern plant, as among the tested *Dryopteris filix-mas* extracts two fractions affected PPARγ significantly. Although the 100% MeOH initial fraction (878) was able to activate PPARγ over 100%, it was not possible to find any subfractions of this initial extract, with an enriched activity. Therefore it would be interesting to repeat the experiment at first with a dose-response experiment to confirm the effect of the initial extract. Afterwards the 100% MeOH initial fraction should be fractionated again and tested at a proper concentration for the PPAR activation in order to detect the effective subfractions.

For the 70% MeOH fraction, which had the highest efficacy on PPARγ among the tested *Dryopteris filix-mas* samples, it was possible to also detect bioactive subfractions. With a moderate effect on PPARγ, the subfractions four and eight did not meet the expectations, compared to the highly effective initial extract. One explanation might be that the components of these two subfractions might affect the PPARγ in a synergistic mode of action. This would explain why the initial extract has a significant higher effect on the PPARγ than the subfractions. In order to prove this hypothesis, it would be necessary to repeat the experiment, and if the results are confirmed to further investigate the working principle of these two subfractions by further extraction and isolation procedures.

F) References

1 Newman, D., J., Cragg, G., M. Natural Products as sources of New Drugs over the Last 25 Years. *Journal of Natural Products* **70** 461-477 (2007).

2 Pasternak, R-C & al. Atherosclerotic Vascular Disease Conference. *Circulation* **109**, 2605-2612 (2004).

3 Glass, Chrisopher, K., Ogawa, Sumito. Combinatorial roles of nuclear receptors in inflammation and immunity. *Nature Reviews Immunology* **6(1)** 44-55 (2006).

4 Solomon, D., H., Rassen, J., A., Glynn, R., J., Levin, R., Schneeweiss, S. The comparative safety of analgesics in older adults with arthritis. *Archives of Internal Medicine* **170 (22)** 1968-76 (2010).

5 De Martin, R., Hoeth, M., Hofer-Warbinek, R., Schmid J., A. The transcription factor NF-κB and the regulation of vascular cell function. *Atherosclerosis, Thrombosis, and Vascular Biology* **20** e83-e88 (2000).

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

7 Marx, N., Duez, H., Fruchart, J.-C., Staels, B. Peroxisome proliferators-activated receptors and atherogenesis. *Circulation Research* **94** 1168-1178 (2004).

8 Abdelrahman, M., Sivarajah, A., Thiemermann, C. Beneficial effects of PPAR-g ligands in ischemia-reperfusion injury, inflammation and shock*. Cardiovascular Research* **65**, 772-781 (2005).

9 in NFN-Application DNTI – Drugs from nature targeting inflammation.

10 Feige, J., N. & al. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Progress in Lipid Research* **45**, 120-159 (2006).

11 Escher, P., Wahli, W. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutuation Research* **448(2)** 121-38 (2000).

12 Cabrero, A., Laguna, J. C., Vázquez, M. Peroxisome proliferator-activated receptors and the control of inflammation. *Current drug targets. Inflammation and allergy* **3**, 243-8 (2002).

13 Straus, D. S., Glass, C.K. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. *Trends in Immunology* **12**, 551-8 (2007).

14 Hong, C. & Tontonoz, P. Coordination of inflammation and metabolism by PPAR and LXR. *Current Opinion in Genetics & Development* **18**, 461-467 (2008).

15 Chandra, V & al. Structure of the intact PPAR-γ-RXR-α nuclear receptor complex on DNA. *Nature*, Vol. **456** (2008).

16 Azhar, S. Peroxisome proliferator-activated receptors, metabolic syndrome and cardiovascular disease. *Future Cardiology* **6(5)** 675-91 (2010).

17 Yessoufou, A., Wahli, W. Multifaceted roles of peroxisome proliferatoractivated receptors (PPARs) at the cellular and whole organism levels. *Swiss Medical Weekly* **140**, w 13071 (2010).

18 Stienstra, R., Duval, C., Müller, M., Kersten, S. PPARs, Obesity, and Inflammation. *Hindawi Publishing Cooperation PPAR Research.* Volume 2007, Article ID 95974, 10 pages, doi: 10.1155/2007/95974.

19 Pyper, S. R., Viswakarma, N. Yu S. & Reddy, J. K. PPARα: energy combustion, hypolidemia, inflammation and cancer. *Nuclear Receptor Signaling* **8**, e002 (2010).

20 Crisafulli, C. & al. PPAR-alpha contributes to the anit-inflammatory activity of 17beta-estradiol. *Journal of Pharmacology and Experimental Therapeutics* **331(3)** 796-807 (2009).
21 Sugii, S. & al. PPAR gamma activation in adipocytes is sufficient for systemic insulin sensitization. *Proceedings of the National Academy* **106(52)** 22504-9 (2009).
22 Scheen, A. J. Thiazolidinediones and liver toxicity. *Diabetes & Metabolism* **3**, 305-13 (2001).
23 Moller, D. E., Berger, J.P. Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *International Journal of Obesity* **27**, 17-21 (2003).
24 Jomayvaz, F. R., Samuel, V. T., Shulman, G. I. The role of muscle insulin resistance in the pathogenesis of atherogenic dyslipidemia and nonalcoholic fatty liver disease associated with the metabolic syndrome. *Annual review of nutrition* **30**, 273-90 (2010).
25 Kumarasamy, Y., Cox, P. J., Jaspars, M., Nahar, L. & Sarker, S. D. Isolation, structure elucidation and biological activity of hederacine A and B, two unique alkaloids from Glechoma hederaceae. *Tetrahedron* **59**, 6403-6407 (2003).
26 An, H. J., Jeong, H. J., Um, J. Y., Kim, H. M. & Hong, S. H. Glechoma hederacea inhibits inflammatory mediator release in IFN-gamma and LPS-stimulated mouse peritoneal macrophages. *Journal of Ethnopharmacology* **106**, 418-424 (2006).
27 Matkowski, A. Antioxidant activity of extracts and different solvent fractions of Glechoma hederacea L. and Orthosiphon stamineus (Benth.) Kudo. *Advanced Clinical and Experimental Medicine* **17**, 615-624 (2008).
28 Zou, K., Zhao, Y. Y., Zhang, R. Y. A cytotoxic saponin from Albizia julibrissin. *Chemical & Pharmaceutical Bulletin* **54**, 1211-1212 (2006).
29 Jung, M. J., Kang, S. S., Jung, Y. J., Choi, J. S. Phenolic glycosides from the stem bark of Albizzia julibrissin. *Chemical & Pharmaceutical Bulletin* **52**, 1501-1503 (2004).
30 Adams, M., Berset, C., Kessler, M., Hamburger, M. Medicinal herbs for the treatment of rheumatic disorders-A survey of European herbals from the 16th and 17th century. *Journal of Ethnopharmacology* **121**, 343-359 (2009).
31 Hiermann, A., Schantl, D. Antiphlogistic and antipyretic activity of Peucedanum ostruthium. Planta Medica **64(5)**, 400-3 (1998).
32 Zimecki, M., Artym, J., Cisowski, W., Mazol, I., Włodarczyk, M., Gleńsk, M. Immunomodulatory and anti-inflammatory activity of selected osthole derivatives. *Journal of biosciences* **64**, 361-8 (2009).
33 Cisowski, W., Sawicka, U., Mardarowicz, M., Asztemborska, M., Luczkiewicz, M. Essential oil from herb and rhizome of Peucedanum ostruthium (L. Koch.) ex DC. *Journal of bioscience* **56**, 930-2 (2001).
34 Agelet, A., Vallés, J. Studies on pharmaceutical ethnobotany in the region of Pallars (Pyrenees, Catalonia, Iberian Peninsula). Part II. New or very rare uses of previously known medicinal plants. *Journal of Ethnopharmacology* **84**, 211-227 (2003).
35 Rodriguez-Lyon, M. L., Diaz-Lanza, A. M., Bernabé, M., Villaescusa-Castillo, L. Flavone glycosides containing acetylated sugars from Sideritis hyssopifolia. *Magnetic Resonance in Chemisry* **38**, 684-687 (2000).
36 But, P., P., H., Hu, S., Y., Kong, Y., C. Vascular plants used in Chinese medicine. *Fitoterapia* **3** 245-264 (1980).

37 Zhang, Y., Ke, W., Yang, J., Ma, A., Yu, Z. The toxic activities of Arisaema erubescens and Nerium indicum mixed with Streptomycete against snails. *Environmental Toxicology and Pharmacology* **27**, 283-286 (2009).
38 Dhuna, V., Bains, J. S., Kamboj, S. S., Singh, J., Saxena, S. & A. K. Purification and characterization of a lectin from Arisaema tortuosum Schott having in-vitro anticancer activity against human cancer cell lines. *Journal of Biochemistry and Molecular Biology* **38**, 526-532 (2005).
39 Chung, W., Goo, Y. M., Na, D. S., Kim, K. S. A PLA2 inhibitor from Arisaema amurense Max. var. serratum Nakai. *Archives of Pharmacal Research* **18**, 293-295 (1995).
40 Huai, H., Dong, Q., Liu, A. Ethnomedicinal analysis of toxic plants from five ethnic groups in China. *Ethnobotany Research & Applications* **8**, 169-179 (2010).
41 Ok, I. S., Kim, S.H., Kim, B.K., Lee, J.C., Lee, J.C. Pinellia ternate, Citrus reticulate, and their combinational prescription inhibit eosinophil infiltration and airway hyperresponsiveness by suppressing CCR3+ and Th2 Cytokines production in the ovalbumin-induced asthma model. *Mediators of inflammation* 413270. Epub (2009).
42 Nagai, T., Shimizu, Y., Shirahata, T., Sunazuka, T., Kiyohara, H., Omura, S., Yamada, H. Oral adjuvant activity for nasal influenza vaccines caused by combination of two trihydroxy fatty acid stereoisomers from the tuber of *Pinellia ternata. International Immunopharmacology* **10** 655-661 (2010).
43 Kim, Y.J., Shin, Y. O., Ha, Y. W., Lee, S., Oh, J. K. & Kim Y. S. Anti-obesity effect of Pinellia ternate extract in Zucker rats. *Biological & Pharmaceutical Bulletin* **29**, 1278-1281 (2006).
44 Mazzio, E. A., Soliman, K. F. A. In vitro screening for the tumoricidal properties of international medicinal herbs. *Phytotherapy Research* **23**, 385-398 (2009).
45 Lee, H. – B., Kim, J. – C., Lee, S. – M. Antibacterial activity of two phloroglucinols, flavaspidic acids AB and PB, from Dryopteris crassirhizoma. *Archives of Pharmacal Research* **32(5)** 655-9 (2009).
46 Wiley, J & al. Uses of Fusion Genes in Mammalian Transfection*. Current Protocols in Molecular Biology*, 9.6.1. – 9.6.12. (1996).
47 Promega, C. PureYield™ Plasmid Midiprep System. *Technical manual* **TM253**, 1-21 (2009).
48 in http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/luciferase.html.
49 in *Nonisotopic Assays for Reporter Gene Activity* (John Wiley & Sons, Inc., 2002).
50 Shariatmadari, R., Sipila, P. – P., Huhtaniemi, I. – T., Poutanen, M. Improved technique for detection of enhanced green fluorescent protein in transgenic mice. *Biotechniques* **30(6)** 1282-5 (2001).
51 Chalfie, M., Euskirchen, G., Ward, W., W., Prasher, D., C. Green fluorescent protein as a marker for gene expression. *Science* **263 (5148)**:802-5 (1994).
52 Wiley, J & al. Use of the A. Victoria Green Fluorescent Protein to Study Protein Dynamics in Vivo. *Current Protocols in Molecular Biology.* 9.7.22-9.7.28 (1996).
53 Thomas, P., Smart, T., G. HEK293 cell line: A vehicle for the expression of recombinant proteins. *Journal of Pharmacological and Toxicological Methods* **51**, 187-200 (2005).
54 Graham, F., L., Smiley, J., Russell, W., C., Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology* **36(1)** 59-74 (1977).
55 Wiley, J & Sons. *Current Protocols in Immunology*, A.3B.1 – A.3B.2 (1997).

56 in *Advanced Imaging Laboratory*. http://web.uvic.ca/ail/techniques/epi-fluorescence.html.
57 in http://www.caymanchem.com/app/template/Product.vm/catalog/10008613.
58 in http://www.caymanchem.com/app/template/Product.vm/catalog/71750/a/z.
59 Cavender, M. A., Lincoff, A. M. Therapeutic potential of Aleglitazar, a new dual PPAR alpha/gamma agonist: Implications for cardiovascular disease in patients with diabetes mellitus. *American Journal of Cardiovascular of Cardiovascular Drugs* **10**, 209-16 (2010).
60 Carini, M., Aldini, G., Orioli, M., Facino, R. M. Antioxidant and photoprotective activity of a lipophilic extract containing neolignans from Krameria triandria roots. *Planta Medicinica* **68**, 193-197 (2002).
61 Facino, M., Carini, M., Aldini, G., De Angelis, L. A rapid screening by liquid chromatoggraphy/mass spectrometry and fast-atom bombardment tandem mass spectrometry of phenolic constituents with radical scavenging activity, from Krameria triandria roots. *Rapid Communications in Mass Spectrometry* **11**, 1301-1303 (1997).
62 Scholz, E., Rimpler, H. Proanthocyanidins from Krameria triandria root. *Planta Medica* **55(4)** 379-84 (1989).
63 Oben, J., Enonchong, E., Kothari, S., Chambliss, W., Garrison, R., Dolnick, D. Phellodendron and Citrus extracts benefit joint health in osteoarthritis patients: a pilot, double-blind, placebo-controlled study. *Nutrition Journal* **8**, 1475-2891 (2009).
64 Kanaze, F., I., Termentzi, A., Gabrieli, C., Niopas, I., Georgarakis, M., Kokkalou, E. The phytochemical analysis and antioxidant activity assessment of orange peel (Citrus sinenesis) cultivated in Greece-Crete indicates a new commercial source of hesperidin. *Biomedical Chromatography* **23(3)** 239-49 (2009).
65 Sergeev, I., N., Li, S., Colby, J., Ho, C. – T., Dushenkov, S. polymethoxylated flavones induce Ca^{2+} - mediated apoptosis in breast cancer cells. *Life Sciences* **80** 245-253 (2006).
66 Dugo, P & al. Rapid Analyses of Polymethoxylated Flavones from Citrus Oils by Supercritical Fluid Chromatography. *Journal of Agricaltural and Food Chemistry* **44**, 3900-3905 (1996).
67 Ghanim, H., Upadhyay, M., Korzeniewski, K., Viswanathan, P., Abuaysheh, S., Mohanty, P., Dandona, P. Orange juice neutralizes the proinflammatory effect of a high-fat, high-carbohydrate meal and prevents endotoxin increase and Toll-like receptor expression. *The American Journal of Clinical Nutrition* **91(4)** 940-9 (2010).

G) Appendix

1. Abbreviations

A	
AMP	Adenosine 5´-monophosphate
AP-1	Activator protein-1
ATP	Adenosine 5´-triphosphate
Ad-5	Adenovirus type 5
B	
BCL-6	B-cell lymphoma-6 protein
C	
°C	degrees Centigrade
$CaCl_2$	Calcium Chloride
c-CBL	Casitas B-lineage lymphoma gen-c
C/EBPs	CCAAT/enhancer-binding proteins
CCR-3	Chemokine receptor type 3
ChREBP	Carbohydrate response element-binding protein
cm	centimeter
CO_2	Carbondioxide
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CYP-450	Cytochrome oxidase P-450 family
D	
DC	Dichloromethane
dd H_2O	distilled Water
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
DNTI	Drugs from Nature Targeting Inflammation
DPPH	1, 1-diphenyl-2-picrylhydrazyl radical

DTT Dithiothreitol, Dichlorodiphenyltrichloroethane

E

EC_{50} Effective concentration, defined as concentration of an agonist, which provokes half-maximal effects.

E.coli *Escherichia coli*

EDTA Ethylenediaminetetraacetic acid

EGFP Enhanced green fluorescent protein

11-β-HSD-1 11-β-hydroxysteroid-dehydrogenase-1

EtOAC Ethylacetate

F

FAB-MS Fast-atom bombardment mass spectrometry

FABP-4 Fatty acid binding protein-4

FBS Fetal bovine serum

FFA Free fatty acids

5-HETE 5-Hydroxyeicosatetraenicacid

G

g acceleration caused by gravity/ gramm

GC-MS Gaschromatography-Massspectrometry

GRIP-1 Coactivator glucocorticoid receptor interacting protein-1

GLUT Glucose transporter

H

HBS HBS medium is used for cell transfection

HEK-293 Human embryonal kidney cells-293

HPLC High Performance Liquid Chromatography

H_2O Water

I

ICAM 1 Intercellular adhesion molecule-1

IFN-α Interferone alpha

IκBα Inhibitor protein of NfκB

IL Interleukine

iNOS	inducible NO-synthase protein
IRS-2	Insulin receptor substrate-2
J	
JNK	C-Jun N-terminal kinase
K	
KCl	Kaliumchloride
KH_2PO_4	Kaliumdihydrogenphosphate
L	
LB medium	Lura-Bertani-Medium
LC-MS	Liquidchromatography-Massspectrometry
LPS	Lipopolysacharids
LU	Light-units
LTB-4	Leukotriene receptor B-4
LXR	Liver-X-receptor
M	
M	Molarity
MCP-1	Monocyte chemotactic protein-1
MCS	Multiple cloning site
Mg^{2+}	Ionized magnesium
MI	Myocardial infarct
µl	microliter
ml	milliliter
mm	milimeter
mM	milimolar
N	
N	Normality
NaCl	Natriumchloride
Na_2HPO_4	Dinatriumhydrogenphosphate
NCE´s	New Chemical Entities
MeOH	Methanol

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIDDM	Non insulin dependent diabetes mellitus
NFN	Nationales Forschungsnetzwerk
NMR	Nuclear magnetic resonance
NP´s	Natural products

O

OD_{590}	Optical density at 590 nm, a measurement for the bacterial growth.

P

pcDNA	Plasmid vector, for stable and transient expression in mammalian cells.
PBS	PBS medium, used in the cell culture for washing the cells.
PE	Petrolether
PEPCK	Phosphoenolpyruvat carboxykinase
PI3K/Akt	Phosphatidylinositol 3-kinases belong to a enzyme-family involved in cellular functions and activate the proteinkinase B, or also called Akt representing a signal pathway for cellular functions.
PIPES	Piperazine-N,N-bis(2-hydroxypropanesulfonic acid)
PLA-2	Phospholipase A-2
PMF´s	Polymethoxylated flavones
PPARα/γ	Peroxisome proliferator-activated receptors subtype alpha/gamma

S

SAA	Serum amyloid A, an acute phase protein.
SDS	Sodium dodecyl sulfate is a detergent, dissociating and unfolding proteins, used for SDS polyacrylamid gel electrophoresis.
SPE	Solid phase extraction
SOC	SOC medium is equivalent to the SOB (Super Optimal Broth) medium with 20mM glucose added and used for the creation of transformed competent bacteria.
SREBP-1c	Sterol regulatory element-binding protein-1c
STAT	Signal transducers and activators of transcription. The JAK (Janus-kinase)-STAT signalpathway contributes to the regulation of celluar functions, such as development and growth control.

T

TCM	Traditional Chinese Medicine
TIF-2	Nuclear receptor coactivator-2
TNF-α	Tumor necrose factor-α
TZD	Thiazolidinediones

U

U/ml	Units per milliliter

2. List of tables and figures

3. Curriculum Vitae

Personal Data

Name	Maier Christian
Date of Birth	5th of May, 1983
Place of Birth	Saalfelden am Steinernen Meer
Nationality	Austrian

Education

2001-2011	Studies of Pharmacy University of Vienna
1993-2001	Aufbaugymnasium Höhere Internatsschule des Bundes (H.I.B.), Saalfelden
1989-1993	Volksschule II Saalfelden

4. Acknowledgements

A study requires time, financial and personal efforts and above all the support and love of family, friends and colleagues. This part of my diploma thesis is dedicated to all people who accompanied me on my way.

I have always experienced myself as a happy and social person. Maybe I was already born with these attributes, but it were certainly the warming and loving surroundings of my family that influenced my personal development in this way. That is why I want to thank my parents, Waltraud and Alois Maier, for the best childhood a kid can dream of, for the efforts and patience you had to let me develop to who I am. Moreover I want to thank you for the personal and financial support during my study and the happy hours we spent together!

I had the big luck to grow up in a big family with one brother, Mr. Alexander Maier, and three sisters, Ms. Theresa Maier, Mrs. Sabina Brunner and Ms. Nicola Maier who always were, are and will be an essential part of my life. I hope I will be able to see you and your dear families as much as possible and to spend many happy days together. I also want to dedicate a special thank to my grandmas, Mrs. Marianne Maier, Mrs. Maria Bauer and Mrs. Elisabeth Eder (alias Mami) and especially to my godmother Mrs. Angelika Colombini (alias Angodika) with family for your generous and warm hearts towards poor students! Grazie per tutto e tanti auguri a voi!

If a boy becomes a man the best thing that can happen to him is to meet the woman for life. I had this great luck to meet and fall in love with the one girl already more than seven years, seven months and seven days ago. This part is dedicated to you, my best friend, companion and wife Ms. Marianne Eder, you make me complete.

Imagine the woods,
Without a tree,
Imagine the rivers,
Without the sea,
Imagine yourself,
Without me,
Imagine how lost,
I would be.

I want to thank you for your love and support, for your encouragement, for each moment we shared, if in happiness or tears; you made my life worth living! Without you I would not be where I am.

I also want to thank my parents-in-law, Mrs. Notburga Eder and Mr. Friedrich Eder for treating me like one of their children and the support during my studies. I hope that we will be able to spend many happy hours together and wish you health and a long life! Here I also want to dedicate a salute to my brother-in-law, Mr. Johannes Eder (alias Hannibunny), it is always awesome to spend some time with you.

What would life be without friends? I have the big luck to share some of my time with nice people, who I really appreciate. Thank you for being there! You know anyway who is meant!

Here I want to thank Mr. Prof. Christian Studenik for the support and nice conversations while enjoying a good cup of coffee!

I also want to thank Prof. Dirsch for the possibility to be part of the very interesting DNTI project, to expand my skills and learn new techniques. Here I also want to thank Dr. Atanas Atansov and Nanang Fakhrudin for the support and encouragement during my diploma thesis. Furthermore I want to thank my colleague Ms. Mag. pharm. Olivia Schrammel for the good cooperation and the nice time we spent besides working.