universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Mechanism of action of natural compounds enhancing macrophage cholesterol efflux“

verfasst von

Nicole Schilcher

angestrebter akademischer Grad

Magistra der Pharmazie (Mag.pharm.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 449
Studienrichtung lt. Studienblatt:	Pharmazie
Betreut von:	Univ. Prof. Dr. Verena Dirsch

Abstract

The life-threatening disease atherosclerosis results from atherosclerotic plaque formation in the arterial vessel wall. The critical steps in pathogenesis of atherosclerosis involve excessive cholesterol deposition in the atherosclerotic plaque and formation of cholesterol-enriched proatherogenic macrophage foam cells.

The reverse cholesterol transport (RCT) is a process which exerts an anti-atherosclerotic effect, where cholesterol is transported from the macrophage cell to the liver, then transformed into bile acids and finally fecal-excreted. The macrophage cholesterol efflux, is considered to be the initial step of RCT, therefore, enhancement of macrophage cholesterol transport can be used to prevent the development of atherosclerosis.

Based on our preliminary research there are three natural compounds known that dose-dependently activate the macrophage cholesterol efflux in THP-1 cells without any cytotoxicity (measured with Resazurin Assay). The first compound is Piperine, a pungent constituent isolated from fruits of black pepper and long pepper. The second compound is Leoligin, the major lignan from *Leontopodium alpinum*. The third is Evodiamine, a bioactive compound of the traditional Chinese herbal drug Fructus Evodiae.

To find the mechanism of action of these natural compounds enhancing the macrophage cholesterol efflux, Western blotting was performed to detect the expression levels of proteins involved in the macrophage cholesterol efflux, particularly the transmembrane proteins from ABC (Adenosin triphosphate-binding cassette) transporters superfamily type A1 and G1 (ABCA1 and ABCG1), the bidirectional transmembrane protein SR-BI (Scavenger receptor class B type I), and the nuclear receptors PPARγ (Peroxisome proliferator-activated receptor γ) and RXRα (Retinoid X receptor α). With this method, increased protein expression of the transmembrane protein ABCA1 was identified. Quantitative real time PCR was further performed to evaluate the ability of the natural compounds to modify ABCA1 mRNA levels. The obtained result show that the active compounds can increase the ABCA1 mRNA level significantly, but the effect of up-regulation of protein expression obtained from results of western blotting is stronger. Therefore it is suggested a complex mechanism of action by which Piperine, Leoligin and Evodiamine are enhancing macrophage cholesterol efflux.

Zusammenfassung

Die lebensbedrohliche Erkrankung Atherosklerose entsteht durch Bildung von atherosklerotischem Plaque in der arteriellen Gefäßwand. Entscheidende Schritte der Pathogenese der Atherosklerose umfassen übermäßige Einlagerung von Cholesterol in den atherosklerotischen Plaque und die Bildung von mit Cholesterol angereicherten pro-atherogenetischen Makrophagen-Schaumzellen.

Der Reverse Cholesterol Transport (RCT) ist ein Prozess mit einem anti-atherosklerotischen Effekt, in welchem Cholesterol von den Makrophagen zur Leber transportiert, weiters in Gallensäuren umgewandelt und anschließend fäkal ausgeschieden wird. Der Cholesterol Efflux durch Makrophagen wird für den initialen Schritt des RCT gehalten, weshalb die Verbesserung des Makrophagen Cholesterol Transportes zur Verhinderung der Entstehung von Atherosklerose genutzt werden kann.

Aufgrund vorläufiger Untersuchungen sind drei natürliche Verbindungen bekannt, welche Dosis-abhängig den Makrophagen Cholesterol Efflux in THP-1 Zellen aktivieren können, ohne Zytotoxizität aufzuweisen (gemessen mit Resazurin Assay). Die erste Verbindung ist Piperin, ein Scharfstoff der aus den Früchten des Schwarzen Pfeffers und Langen Pfeffers isoliert wird. Die zweite Verbindung ist Leoligin, das Haupt-Lignan von *Leontopodium alpinum.* Drittens Evodiamin, eine Verbindung aus der traditionellen chinesischen Droge Fructus Evodiae.

Um den Wirkmechanismus dieser natürlichen Verbindungen, welche den Makrophagen Cholesterol Efflux verbessern, zu finden, wurde die Methode des Western Blottings angewandt, um den Expressionslevel von Proteinen die am Makrophagen Cholesterol Efflux beteiligt sind, insbesondere die transmembranären Proteine der ABC (Adenosin triphosphate-binding cassette) Transporter Familie vom Typ A1 und vom Typ G1 (ABCA1 und ABCG1), das bidirektionale Protein SR-BI (Scavenger receptor class B type I) und die nuklearen Rezeptoren PPARγ (Peroxisome proliferator-activated receptor γ) und RXRα (Retinoid X receptor α), zu ermitteln. Anhand dieser Methode wurde eine gesteigerte Expression des transmembranären Proteins ABCA1 identifiziert. Um die Fähigkeit der Modifikation des ABCA1 mRNA Levels der natürlichen Verbindungen zu evaluieren, wurde im nächsten Schritt die Methode der quantitativen real time PCR angewandt. Die erhaltenen Ergebnisse zeigen, dass die aktiven Verbindungen zwar den ABCA1 mRNA Level signifikant

erhöhen können, jedoch der Effekt der Hochregulierung der Protein Expression, erhalten durch die Ergebnisse des Western Blottings, stärker ist. Deshalb wird ein komplexerer Wirkmechanismus erwartet, durch welchen Piperin, Leoligin und Evodiamin den Makorphagen Cholesterol Efflux verbessern können.

Table of content

1. Introduction

1.1 Atherosclerosis

As a disease of the large arteries, atherosclerosis, which is commonly also designated as hardening of arteries, is well known as the primary cause of heart disease and stroke, causing about 50 % of all deaths in westernized societies (1). And according to the World Health Organization (WHO) cardiovascular disease (CVD), whose main cause is atherosclerosis along with hypertension, accounted in 2005 for 30 % of the deaths globally (2).

Because formation of atherosclerosis can occur in any artery of the body, we know a lot of atherosclerosis-related disorders, for example coronary heart disease, carotid artery disease, peripheral artery disease, chronic kidney disease (3).

There are not only genetic but also environmental factors related to the formation of this dangerous disease. Genetic factors are for example hyperlipidemia (elevated levels of LDL/VLDL (low density lipoprotein/very low density lipoprotein), lipoprotein(a)), elevated levels of homocysteine, reduced levels of HDL (high density lipoprotein), hypertension, diabetes and obesity and systemic inflammations. Environmental factors include for example a high-fat diet, smoking, low antioxidant levels and lack of exercise (1,4).

Hallmark of atherosclerosis are atherosclerotic plaques, which are composed of various lipids and different cell types, that narrow the arterial lumen, leading to high blood pressure and other diseases. Some of the most important components of the atherosclerotic plaques are cholesterol, fatty acids, lipid-laden macrophages, necrotic cellular debris and fibromuscular-connective tissue (1,5).

1.1.1 Atherosclerotic plaque

Atherosclerotic plaque formation results in a high number of circulating monocytes, which migrate into the developing plaque. After that, the differentiation into macrophages with a pro-inflammatory phenotype takes place. Due to the pro-inflammatory environment, the macrophages can accumulate cholesterol and thus „macrophage foam cells“ are developed. As a consequence of “macrophage foam cell“ production, a life-threatening acute myocardial infarction and stroke can occur when the plaque brakes, narrowing the vessel lumen and decreasing blood-flow (6). In summary for the development of atherosclerosis,

the accumulation of cholesterol in atherosclerotic plaque and the formation of foam cells are considered to be a very important step (7,6).

Regarding to the ratio of cholesterol esters to total cholesterol, two different kinds of cells can be differentiated. Lipid-loaded cells are cells with a ratio less than 50 % and foam cells are cells that have a ratio more than 50 % (8).

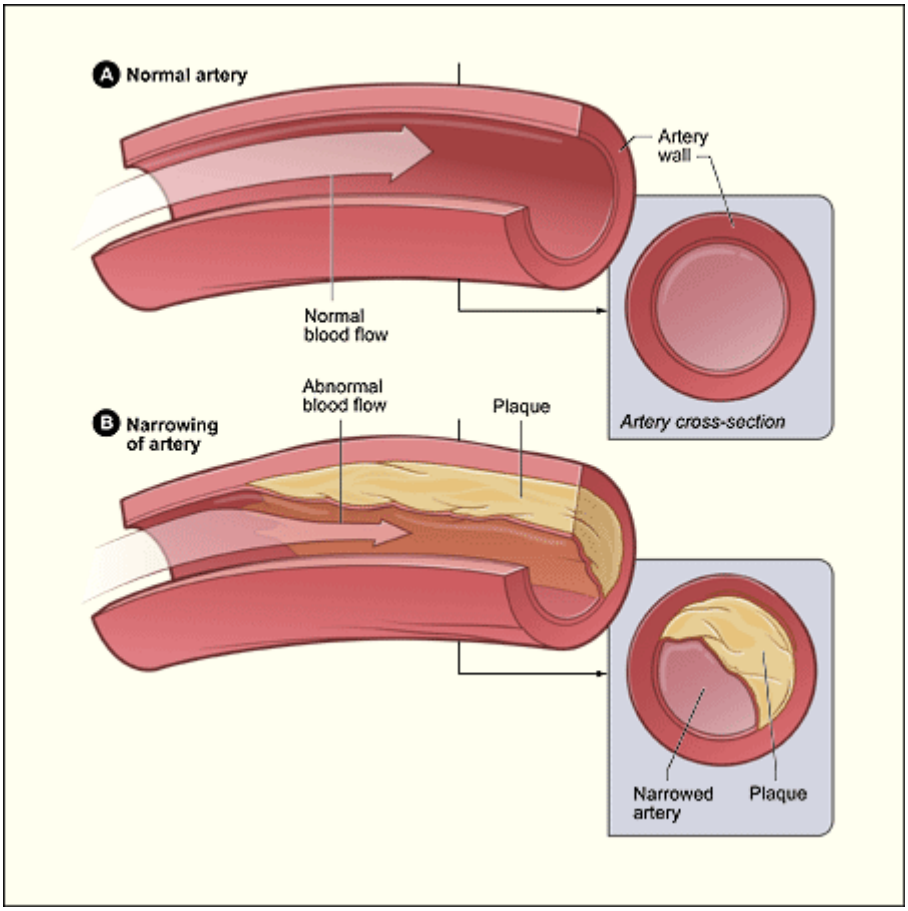


Figure 1: Atherosclerotic plaque formation.

This figure shows a normal artery and an atherosclerotic plaque-formation narrowed artery. Plaque is composed of cholesterol, fatty acids, lipid-laden macrophages, necrotic cellular debris and fibromuscular-connective tissue. By narrowing of the artery, blood flow decreases and in further consequence life-threatening heart attack and stroke can occur.

(Figure adapted from (3))

1.2 Reverse cholesterol transport

The high level of cholesterol in blood can cause the development of atherosclerosis and this level can be regulated by several mechanisms, for example by cholesterol uptake, biosyntheses, transportation, metabolism and secretion (9,10,11).
Regulating cholesterol blood levels might prevent the formation of atherosclerotic plaques and in further consequence to prevent the occurrence of heart attack and stroke. The RCT (reverse cholesterol transport) is a process, which contributes to the regulation of the cholesterol level in the blood. It transports cholesterol from the macrophage "foam" cells to the liver. In the liver the cholesterol is bound to bile acids and transformed to bile salts and in the end it is eliminated by fecal excretion. So the RCT has the potential to prevent atherosclerosis by decreasing the amount of cholesterol in blood plasma (8).

To give an overview of the process of RCT in more detail, it should be noticed that the RCT starts with the process of cholesterol efflux where FC (free cholesterol) is effluxed via transmembrane transporters from peripheral cells, which are not only macrophages but also peripheral non-macrophage cells, to acceptors in the extracellular environment (process of macrophage cholesterol efflux explained in detail in 1.2.1). These extracellular acceptors mainly include apoA-I (apolipoprotein A-I) and HDL. After that, within the HDL the free unesterified cholesterol is converted to cholesteryl esters through the enzyme lecithin cholesterol acyltransferase (LCAT) and in further consequence a mature HDL particle arises which is transported to the liver. In the liver the HDL cholesterol can be taken up selectively through the transport protein SR-BI (scavenger receptor class B type I) and subsequently follows the transformation to bile salts. These built bile salts are secreted into the bile and further excreted via the feces (12).

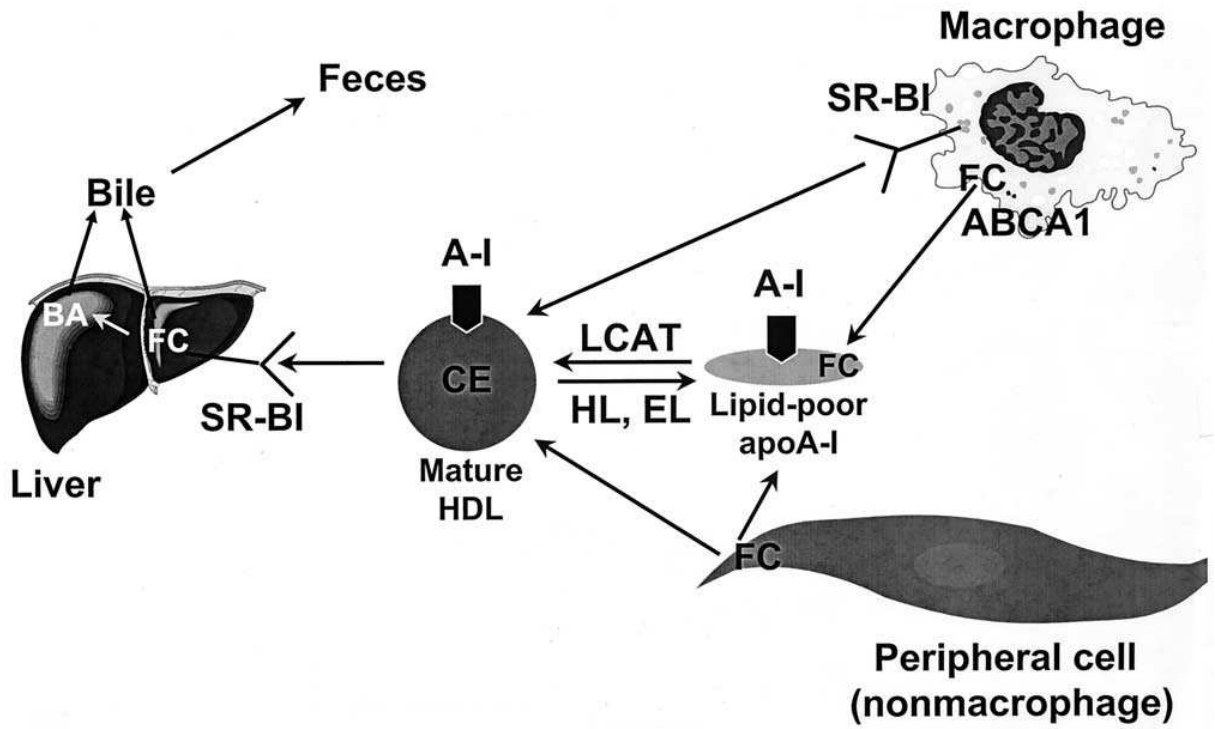


Figure 2: Reverse cholesterol transport (RCT)

During the initial step of RCT, named cholesterol efflux, free cholesterol (FC) is effluxed out of macrophage and nonmacrophage cells to acceptors in the extracellular environment. Therefore, the lipid-poor apoA-I accepts FC via the tansmembrane transport protein ABCA1 or mature HDL accepts FC via another tranmembrane transport protein, called scavenger receptor class B1 (SR-BI). After that, FC is converted into CE (cholesteryl ester) by the LCAT (enzyme lecithin cholesterol acyltransferase). HDL is taken up in the liver via SR-BI, in the liver FC is converted into BA (bile acids) and subsequently secreted into bile and finally fecal excreted.

(Figure adapted from (12))

1.2.1 Cholesterol efflux

Cholesterol efflux, the initial step of the RCT, is a process by which cholesterol is transported from “macrophage foam cells” to the liver for final fecal excretion (8).

There are different known pathways of cholesterol efflux from macrophages to the HDL and these different pathways are for example the passive or diffusional efflux, the cholesterol efflux mediated by the bidirectional transmembrane protein SR-BI, the macrophage apoE (apolipoprotein E) secretion associated efflux and also the active cholesterol efflux mediated by the membrane transporters ABCA1 and ABCG1 (13).

There are different types of active and passive mechanisms described for macrophage cholesterol efflux. A passive process is called aqueous diffusion, where cholesterol from the cell surface is effluxed to various extracellular acceptors, driven by a concentration gradient between the cell membrane and the extracellular acceptor proteins. Other types of passive macrophage cholesterol efflux are the SR-BI mediated cholesterol efflux to HDL, where cholesterol can be effluxed by the bidirectional transmembrane transport protein SR-BI and finally the apoE secretion associated macrophage cholesterol efflux, where apolipoproteins are able to desorb phopholipids independently of transporters or receptors. Active cholesterol efflux is mediated by the transmembrane proteins ABCA1 to apoA-I and the ABCG1 to HDL using the energy of ATP (14).

But from all mentioned different cholesterol efflux pathways, the active cholesterol efflux mediated by ABCA1 and ABCG1 plays the most important role (13).

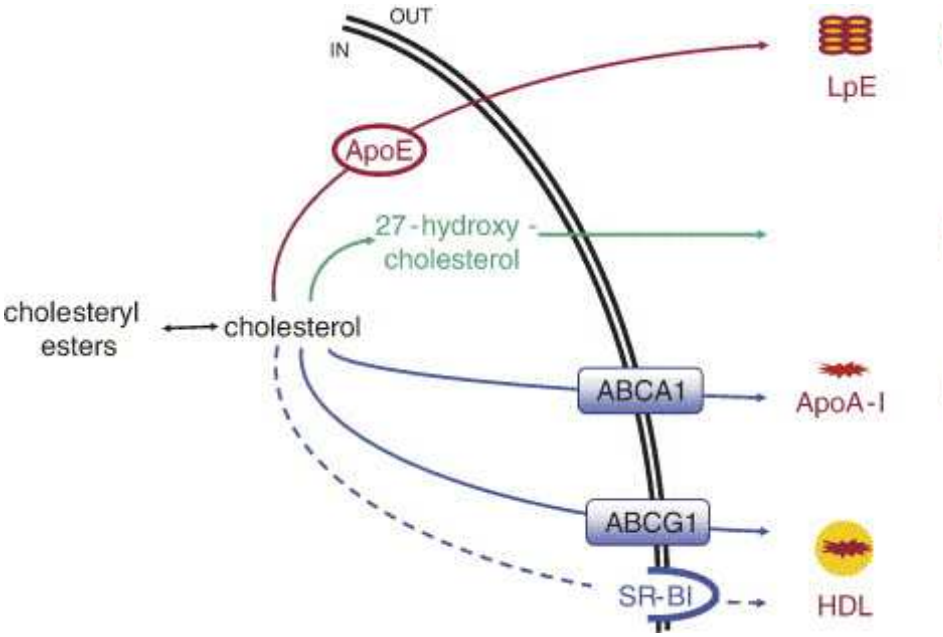


Figure 3: Different pathways of cholesterol efflux in macrophage cells

This figure shows the different pathways of macrophage cholesterol efflux: the passive efflux containing the aqueous efflux, efflux mediated by the bidirectional transmembrane protein SR-BI and efflux mediated by the secretion of lapidated apoE and the active efflux mediated by the transmembrane proteins ABCA1, ABCG1.

(Figure adapted from (14))

1.2.1.1 Transmembrane trafficking systems

Involved in the active macrophage cholesterol efflux are transmembrane trafficking systems, which mediate the transport of cholesterol from the macrophage foam cell to the acceptors in the extracellular environment.

1.2.1.1.1 Adenosin triphosphate-binding cassette (ABC) transporters

ABCA1 and ABCG1 are the two transporters from the large ABC superfamily that can cause cholesterol efflux, wherein the ABCA1 plays the most important role (8).

ABCA1

This unidirectional transporter uses the energy of adenosine triphosphate (ATP) in order to carry free unesterified cholesterol and phospholipids from intracellular side of the plasma membrane to extracellular apoA-I, which can be one way for the formation of HDL (15). So for the cholesterol efflux to the extracellular HDL, it can be mentioned that the interaction between ABCA1 and apoA-I is a very critical step (8).

Because of the role of ABCA1 in cholesterol efflux, it generates interest as a potential anti-atherogenic target. Therefore transgenic mice that over-express ABCA1 show increased plasma HDL levels, and also increased macrophage cholesterol efflux and a reduced diet-induced atherosclerosis level (16).

ABCG1

The ABCG1 transporter, in contrast to the ABCA1 receptor, which mediates the cholesterol efflux to the apoA-I (17,18), has a relative unspecific activity of cholesterol efflux. It mediates efflux on the one hand to HDL but on the other hand also to LDL and cyclodextrin (19).

1.2.1.1.2 Scavenger receptor class B type I (SR-BI)

This specific HDL receptor allows HDL, which can donate or accept cholesterol, to anchore to the plasma membrane and it is also responsible for the formation of hydrophobic channels that can transport cholesterol esters. So the SR-BI is a transporter that serves both the uptake of cholesterol esters and the cholesterol efflux (8).

This bidirectional transmembrane protein shows also a high expression in the liver and is therefore also a positive regulator of RCT from macrophages to the liver and in further consequence to the bile and feces (20).

1.2.1.2 Nuclear receptors

There are a lot of nuclear receptors, described so far, which exert a great significance for regulating the expression of RCT-related genes (8).

1.2.1.2.1 RXR (retinoid X receptor) and LXR (liver X receptor)

By forming a hetero-dimer, RXR and the LXR can promote the expression of RCT-related genes, such as ABCA1, ABCG1 and SR-BI (21,22).

1.2.1.2.2 PPAR (Peroxisome proliferator-activated receptor)

Among the PPAR family, there are three homeotypic isomers included (PPARγ, PPARβ/δ, and PPARα), which are involved in cholesterol efflux and their natural ligands are fatty acids and fatty acid derivatives (8).

PPAR forms a hetero-dimer with RXR, which induces the expression of for example SR-BI, ABCA1, ABCG1, and LXR (8).

To concentrate in detail on PPARγ, it is very important for the promotion of the adipocyte differentiation and of the formation of adipose tissue. Another important role of this nuclear factor is that it is able to up-regulate the expression of genes that are related to fatty acid transportation, -synthesis or esterification (8). PPARγ does not support the foam cell formation and in its activated state it inhibits the formation of macrophage-derived foam cells (23,24). It plays an important role for the enhancement of cholesterol efflux and for the attenuation of atherosclerosis, also because it induces caveolin-1 expression (25).

1.2.2 The PPARγ-LXRα-ABCA1/ABCG1 Pathway

Due to activation of this pathway, which consists of two nuclear receptors PPARγ and LXRα (binding as a heterodimer with RXRα) and the transmembrane proteins ABCA1 and ABCG1, macrophage cholesterol efflux can be enhanced (26,50).

After PPARγ is activated by agonists, a transcriptional cascade is started, where the LXRα (in dimer whit RXRα) gene is a direct target of PPARγ and these two nuclear factors start to cooperate to increase the expression of the transmembrane transporter proteins ABCA1 and ABCG1, which finally results in increase of the cholesterol effluxed out of macrophages (26,50).

1.2.3 Pioglitazone

The PPARγ agonist Pioglitazone is already known to be able to increase the expression of ABCA1 by activation of the PPARγ-LXRα-ABCA1 Pathway (27).

Basically the drug Pioglitazone belongs to the group of thiazolidinediones, which are used as oral antidiabetic drugs. The critical step in its mechanism of action is the activation of the nuclear receptor PPARγ, which can together with RXR increase protein expression of different target genes due to their influence on the DNA promotors. These expressed proteins can stimulate the differentiation of fat cells and also the glucose up-take. Consequently, the fasting blood sugar and the HbA_{1c} and also the concentration of triglycerides descend and the concentration of HDL increases (28).

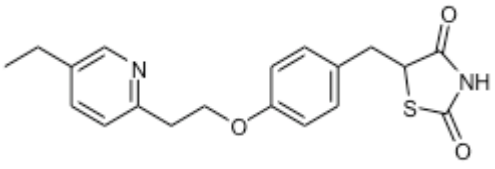


Figure 4: Chemical structure of Pioglitazone

(Picture adapted from (29))

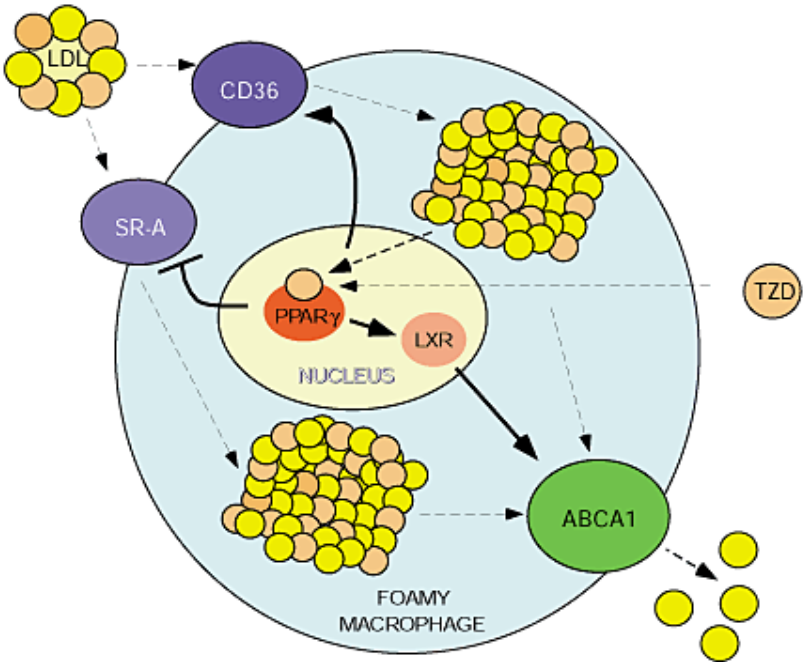


Figure 5: Enhancement of macrophage cholesterol efflux through the PPARγ-LXRα-ABCA1 pathway
This figure shows thiazolidinediones (TZD) which activate the nuclear factor PPARγ, leading to a increased expression of another nuclear factor LXRα, which induces the expression of the transmembrane transport protein ABCA1.
(Figure adapted from (30))

1.2.4 Active natural compounds for macrophage cholesterol efflux

In the following three active natural compounds known due to preliminary research (described in 1.3) to enhance macrophage cholesterol efflux are described.

1.2.4.1 Evodiamine

Evodiamine is the major bioactive compound of the traditional Chinese herbal drug Fructus Evodiae (Wuzhuyu, *Evodia rutaecarpa* (Juss.) Benth. (Rutaceae) fruits). In the traditional Chinese medicine (TCM) it is used for the treatment of inflammation-related disorders of the gastrointestinal tract, headache, menstrual complaints and mouth ulcers (31).
This compound was also reported to possess anti-obesity effects and to reduce body weight, adiposity, and serum levels of leptin and insulin significantly in mice fed a high-fat diet when it was applied orally as a food ingredient at 0.03 % for 2 months (32).

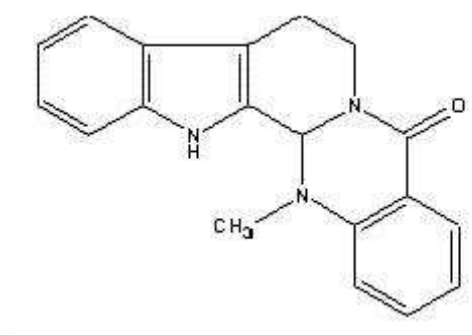


Figure 6: Chemical structure of Evodiamine
(Figure adapted from (33))

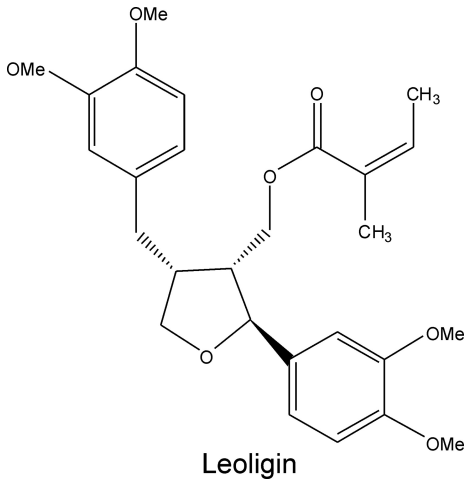
Figure 7: Picture of Evodia rutaecarpa
(Figure adapted from (34))

1.2.4.2 Leoligin

Leoligin is known as the major lignan from Edelweiss (*Leontopodium alpinum* Cass.).

Inhibition of vascular intima hyperplasia and neointima formation in saphenous vein organ cultures as well as in an *in vivo* mouse model for venous bypass graft disease were shown by this compound (35).

The activation of cholesteryl ester transfer protein (CETP), an important mediator of lipoprotein metabolism and RCT representing a process of many steps finally resulting in cholesterol excretion out of the body, was shown by Leoligin (36).



Leoligin

Figure 8: Chemical structure of Leoligin

(Figure adapted from (37))

Figure 9: Picture of *Leontopodium alpinum*

(Figure is adapted from (38))

1.2.4.3 Piperine

Piperine is well known as the pungent constituent of the fruits of black pepper (*Piper nigrum* L., Piperaceae) and long pepper (*Piper longum* L., Piperaceae) (45,52).

Due to the reported immunomodulatory, anti-carcinogenic, antiasthmatic, hepatoprotective, anti-inflammatory (39), antimicrobial (40) and anti-ulcer activities (41) it is traditionally used for treatment of pain, chills, rheumatism, flu, muscular aches, colds, exhaustion, fevers, as a nerve tonic, to increase circulation of blood and to stimulate appetite and peristalsis (42,43,44).

The fruits of black pepper and its main bioactive compound piperine were also reported for reduction of cholesterol uptake and enhancement of translocation of cholesterol transporter proteins in Caco-2 cells (45).

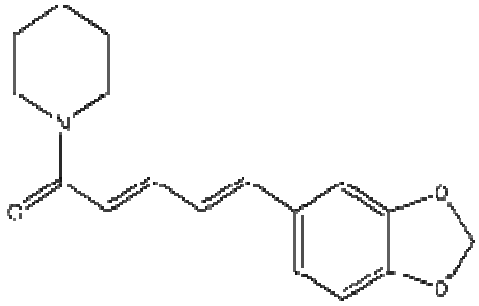


Figure 10: Chemical structure of Piperine
(Figure is adapted from (46))

Figure 11: Picture of black pepper

(Figure is adapted from (47))

1.3 Preliminary research

1.3.1 ApoA-I- mediated Cholesterol Efflux

Preliminary research conducted in the group has shown that Evodiamine, Leoligin and Piperine can dose dependently increase the ApoA-I-mediated cholesterol efflux.

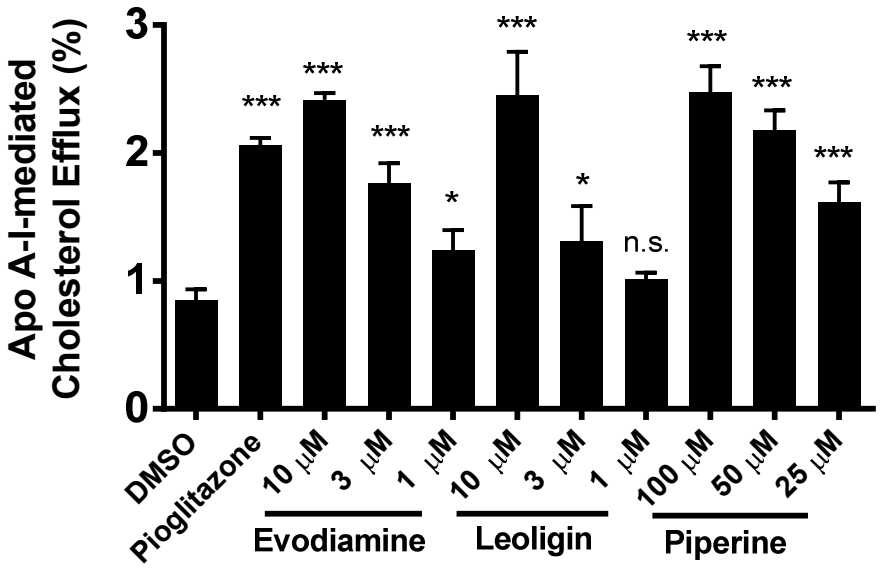


Figure 12: Dose dependent effect of the active compounds on apoA-I-mediated cholesterol efflux n=3 *p<0.05 **p<0.01 ***p<0.001 n.s. not significant, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post test; The data shown on this picture were generated by Limei Wang, a PhD student at the Department of Pharmacognosy, University of Vienna.

1.3.2 Lack of cytotoxicity of the active compounds

Based on own preliminary research, we also know that the active compounds do not show any cytotoxic effect determined by the resazurin assay, even if Evodiamine and Leoligin are applied at 40 µM, and Piperine is applied at 200 µM. They show the same effect as the control DMSO (0.1%), in contrast to the positive control Digitonin which can kill almost all of the cells at 50 µg/ml.

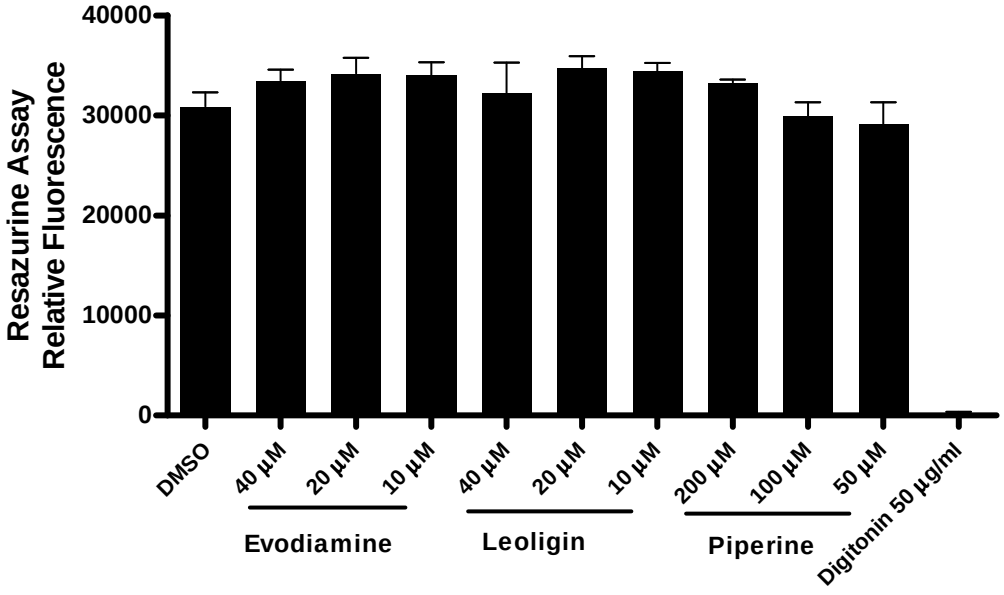


Figure 13: Cell viability in the presence of the active compounds, no significance compared to DMSO; n=3, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test; The data shown on this picture were generated by Limei Wang, a PhD student at Department of Pharmacognosy, University of Vienna.

2. Aim of the work

Preliminary research work has shown that three natural compounds, Piperine, Leoligin and Evodiamine can enhance the macrophage cholesterol efflux. Since the mechanism of action of the compounds remained unknown, in this work we focused on investigation of the expression level of proteins through which the excess cholesterol inside of the macrophages may be effluxed. Western blotting was used to detect the protein expression levels and qRT PCR to detect mRNA levels.

3. Materials and Methods

3.1 Cell culture

3.1.1 Cell line and medium

THP-1 (human monocytic leukaemia), a cell line derived from the peripheral blood of a one year old human male with acute monocytic leukaemia, was used for the experiments. The cells are large and round, and grow in suspension.

THP-1 cells were maintained in complete RPMI-1640 medium, containing 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % heat-inactivated fetal bovine serum (FBS). All of the supplements added to the RPMI-1640 medium, such as the L-glutamine, penicillin/streptomycin, and the calf serum should be sterile-filtrated first. The completed medium was stored at 4 °C and pre-warmed in a water bath at 37 °C before use. The medium was used up within one month after being prepared.

NAME	FINAL CONCENTRATION	CONTENT (STOCK)	PROVIDER
RPMI-1640 medium			Lonza
Penicillin/ Streptomycin Mixture	100 U/ml penicillin, 100 µg/ml streptomycin	10 000 U/ml potassium penicillin/10 000 mg/ml streptomycin sulphate	Lonza
L-glutamine	2 mmol/L L-glutamine	200 mM L-glutamine	Lonza
Heat-inactivated Fetal Bovine Serum	10 % heat-inactivated fetal bovine serum		Gibco

Table 1**:** The recipe of the complete RPMI-1640 medium

3.1.2 Subculturing

THP-1 cells were cultivated in cell culture incubator at 37 °C in an atmosphere containing 5 % CO_2. Since it is not recommended to let the cell concentration exceed 1 x 10^6 cells per ml,

the THP-1 cells were subcultured when the cell concentration reached 0.8 x 10^6 viable cells per ml (every two or three days).

For subculture, the whole cell suspension was taken out of the flask, and then centrifuged for four minutes at 150 x g. After centrifuging, the supernatant was thrown away and the cell pellet was resuspended in fresh complete medium. Then the cells were counted with Vi-Cell™ XR Cell Viability Analyzer (provider Beckmann Coulter) and finally seeded at 0.2 x 10^6 viable cells per ml into a new flask.

3.2 Western blotting

3.2.1 General information

Western blotting is used to identify the up-regulation or down-regulation of specific cellular proteins. A lot of proteins are involved in the cholesterol efflux, such as the transmembrane proteins, ABCA1, ABCG1, SR-B1, and the nuclear receptors, PPARγ, RXRα.

Generally, after THP1 cells were differentiated with PMA for 72 hours to become macrophages, the treatment with compounds was conducted twice for overall 30 hours. After that, the cells were lysed and the protein was extracted. Afterwards, the protein extracted from THP1 cells were separated with a gel, subsequently transferred to a PVDF membrane, probed with a protein-specific antibody, and detection was performed with LAS 3000^{TM} imager (provider Fujifilm) and the evaluation was done by AIDA® software (provider Raytest GmbH).

3.2.2 Cell preparation and protein extraction

3.2.2.1 Seeding cells

THP-1 cells were seeded in 6 well plates at a concentration of 0.2 x 10^6 viable cells per ml. Each well contained 4 ml of cell suspension with 0.8 x 10^6 viable cells and 200 nM phorbol-12-myristate-13-acetat (PMA). Then the cells were incubated for 72 hours to allow differentiation.

3.2.2.2 First treatment

After 72 hours of differentiation, the medium was removed, and the adherent cells were washed once with 4 ml phosphate buffered saline (PBS) per well. The needed compounds

(shown in Table 5) were dissolved in 2 ml serum-free medium with 0.1 % bovine serum albumin (BSA) together with 20 µg per ml unesterified cholesterol (UC). Then the cells were incubated for 24 hours.

NAME	FINAL CONCENTRATION	CONTENT (STOCK)	PROVIDER
RPMI-1640 medium			Lonza
Penicillin/ Streptomycin Mixture	100 U/ml penicillin, 100 µg/ml streptomycin	10 000 U/ml potassium penicillin/10 000 mg/ml streptomycin sulphate	Lonza
L-glutamine	2 mmol/L L-glutamine	200 mM L-glutamine	Lonza

Table 2: recipe of the serum free medium

NAME	CONTENT
PBS pH 7,4	123 mM NaCl 10 mM Na2HPO4 3 mM KH2PO4

Table 3: recipe of PBS

NAME	CONTENT	PROVIDER
Unesterified cholesterol (UC)	unesteriefied cholesterol delivered as a water-soluble complex with methyl-β-cyclodextrin	Sigma-Alrich

Table 4: information for the used unesterified cholesterol

3.2.2.3 Second treatment

The second treatment was performed due to preliminary experiments from Limei Wang, a PhD student at the Department of Pharmacognosy, University of Vienna, showed better results.

The same compounds used for the first treatment (shown in Table 5) were dissolved in serum-free medium.

After removing the medium from the first treatment, the cells were washed twice with 4 ml PBS per well and then treated for the second time with the same compounds. The cells were incubated for 6 hours.

3.2.2.4 Investigated compounds

The name, final concentration, and the provider of compounds used for first and second treatment of THP-1 cells are shown in Table 5.

NAME	FINAL CONCENTRATION	PROVIDER
Piperine	50 µM	Sigma
Leoligin	10 µM	Synthesized and provided from the group of Prof. Marko, Institute of Applied Synthetic Chemistry, Vienna University of Technology
Evodiamine	10 µM	Sigma
Pioglitazone	10 µM	Molekula

Table 5: Compounds for treatment of THP-1 cells

3.2.2.5 Lysing cells

The 6 well plates were placed on ice and further steps were also performed on ice to avoid protein degradation. The medium was aspirated with a Pasteur pipette, making sure that the cells are not scratched or destroyed. Then the cells were washed once with ice cold PBS and then the PBS was aspirated.

After that 100 µl of cold lysis buffer were added to each well. Two different lysis buffers were used, RIPA buffer for the nuclear proteins and NP40 buffer for the transmembrane proteins.

The cells lysed by RIPA buffer were incubated for 7 to 10 minutes on ice and then scratched vigorously by using a cell-scraper. The cell lysate was transferred to new eppendorf tubes. Sonification for 10 s was applied to release the proteins from the cells.

The cells lysed by NP40 buffer were incubated for 30 min on ice with gentle shaking. Afterwards the cells were scratched vigorously by using a cell-scraper, and tthe cell lysate was transferred into new eppendorf tubes.

All of the steps conducted above should be performed on ice. In order to remove the cell debris, the tubes containing cell lysate (lysed with RIPA buffer and NP40 buffer) were centrifuged at 4 °C at 16060 g for 10 minutes and then the supernatant containing proteins was transferred into new tubes.

NAME	CONTENT
RIPA buffer (stock solution)	50mM Tris/HCl pH 7.4 500mM NaCl 1% Nonidet P40 (repleaced by CA-630) 0.5% Na-Doc 0.1% SDS 0.05% NaN3 ddH2O

Table 6: recipe of stock solution of RIPA lysis buffer

NAME	CONTENT
RIPA buffer 1000 µl	RIPA 940 µl Complete® 25x 40 µl PMSF 0.1 M 10 µl NaF 1 M 5 µl Na_3VO_4 100 mM 5 µl

Table 7: recipe of RIPA lysis buffer

NAME	CONTENT
NP40 buffer (stock solution)	150 mM NaCl 50 mM HEPES pH 7.4 1% NP40

Table 8: recipe of stock solution of NP40 lysis buffer

NAME	CONTENT
NP40 buffer 1000 µl	NP40 940 µl Complete® 25x 40 µl PMSF 0.1 M 10 µl NaF 1 M 5 µl Na_3VO_4 100 mM 5 µl

Table 9: recipe of NP40 lysis buffer

3.2.3 Bradford assay

Bradford assay is a method which can be used to determine protein concentration.

Materials needed for this assay included: 96-well transparent plate, 1:10 protein sample dilutions which will be determined, albumin serial dilutions which will be used as standard, 1:10 dilution of complete lysis buffer which will be used to adjust the difference between sample dilutions and albumin dilutions and freshly prepared reagent solution (dd H2O and Roti®-Quant in ratio 1 : 3.75).

For each well, first 190 µl of reagent solution was added. Then for the well that was used for standard curve, 5 µl diluted lysis buffer and 10 µl albumin were added. For the well used for protein concentration determination, 5 µl of diluted sample and 10 µl dd H_20 were added. In the end the total volume in each well should be 205 µl. All of the samples were measured in triplicate. The plate was measured in a Sunrise™ plate reader (provider Tecan) at 595 nm. From the values that were obtained from albumin serial dilutions, a calibration curve was created which was used to determine the protein concentration.

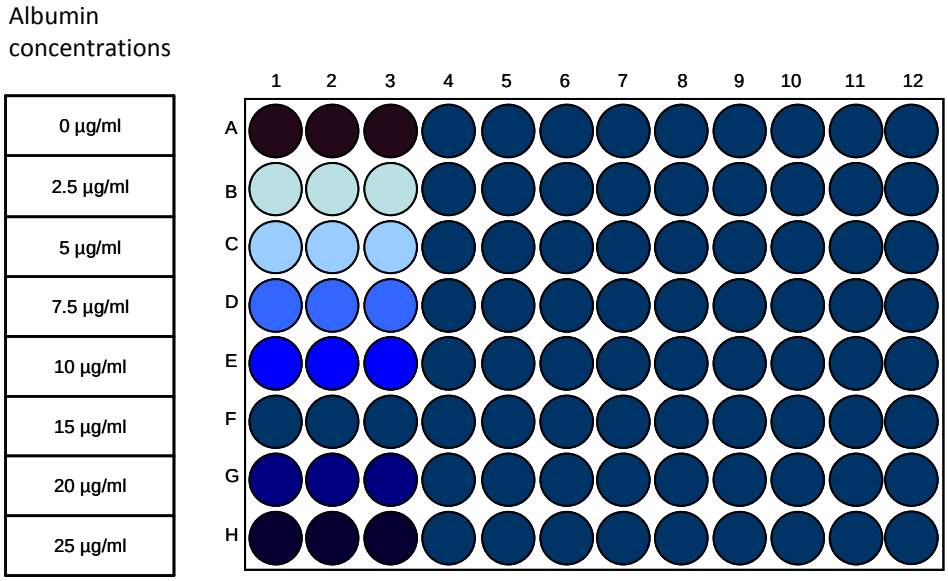


Figure 14: Schematic sketch of Bradford assay

All samples are measured in triplicate. First the different albumin concentrations to create a calibration curve and then the samples whose protein concentration should be determined, were measured.

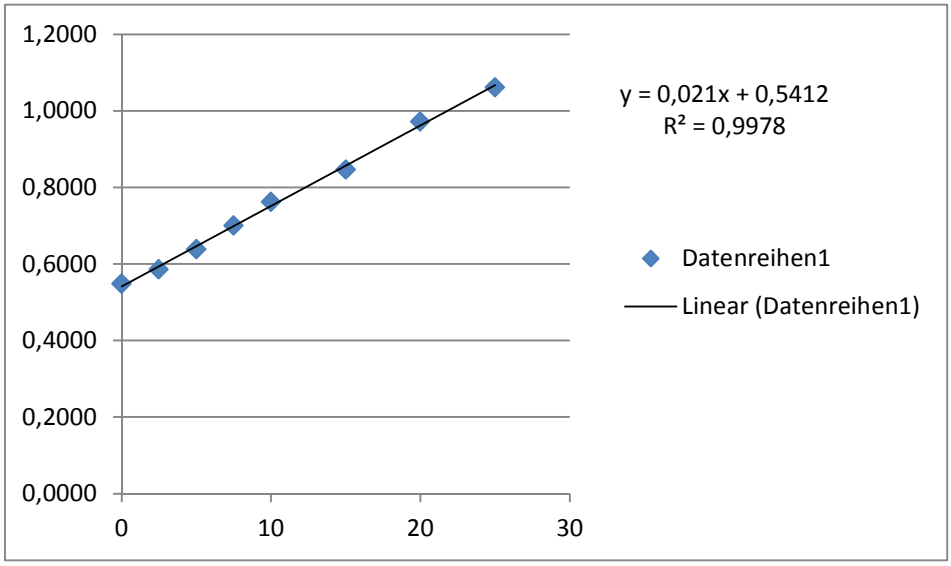


Figure 15: Calibration curve for Bradford assay

To determine the concentration of our samples a calibration curve is created by the absorption at 595 nm of the different albumin concentrations measured.

3.2.4 Protein denaturation and preparation of the samples for SDS-gel electrophoresis

For each protein sample, Sample buffer was added in a volume that corresponded to 1/3 of the sample volume. Then the protein samples together with Sample buffer were incubated at 95 °C for 8 to 10 minutes for protein denaturation. After cooled down to room temperature, they were ready to be used for SDS-gel electrophoresis.

NAME	CONTENT
Sample buffer 300 µl	3x SDS-Sample buffer 255 µl β-Mercaptoethanol 45 µl
3 x SDS Sample Buffer	0,5 M Tris-HCl pH 6,8 37,5 ml SDS 6 g Gycerol 30 ml Bromphenolblau 15 mg dd H_2O ad 100 ml

Table 10: Sample buffer contents

3.2.5 SDS-gel Electrophoresis

In order to separate the proteins by their molecular weight, SDS-gel electrophoresis was performed. 7.5 % SDS-gel was used based on the size of the proteins which will be detected. For each lane on the gels, 20 µg protein calculated with Bradford assay was loaded. The Precision Plus Protein All Blue standard (provider Bio-Rad) was used as molecular weight marker. The electrophoresis was performed at 25 mA per gel until the protein front had reached the bottom of the gel in the Epho-buffer.

NAME	AMOUNT
30 % PAA	1.875 ml
1,5 M Tris-HCl pH 8,8	1.875 ml
10 % SDS	75 µl
H_2O bidest.	3.675 mL
TEMED	7.5 µL
APS (10%)	37.5 µL

Table 11: Separating-gel

NAME	AMOUNT
30 % PAA	640 µl
1,25 M Tris-HCl pH 6,8	375 µl
10 % SDS	37.5 µL
H_2O bidest.	2.62 mL
TEMED	7.5 µL
APS (10%)	37.5 µL

Table 12: Collecting-gel

NAME	CONTENT
Epho-buffer 1000 ml	10 x Epho-buffer 100 ml Aqua bidest. 900 ml
10 x Epho-buffer	Tris-Base 30 g Glycine 144 g SDS 10 g Aqua bidest. ad 1000 ml

Table 13: Epho-buffer contents

3.2.6 Western blotting

The separated proteins were transferred from the gel to a polyvinylidenfluoride (PVDF) membrane which was activated in methanol and washed in blotting buffer. The following utensils were overlapped in order to make a “sandwich”: a filter paper, the gel, the membrane and a filter paper.

It was blotted at 100 V for 100 minutes in Blotting-buffer.

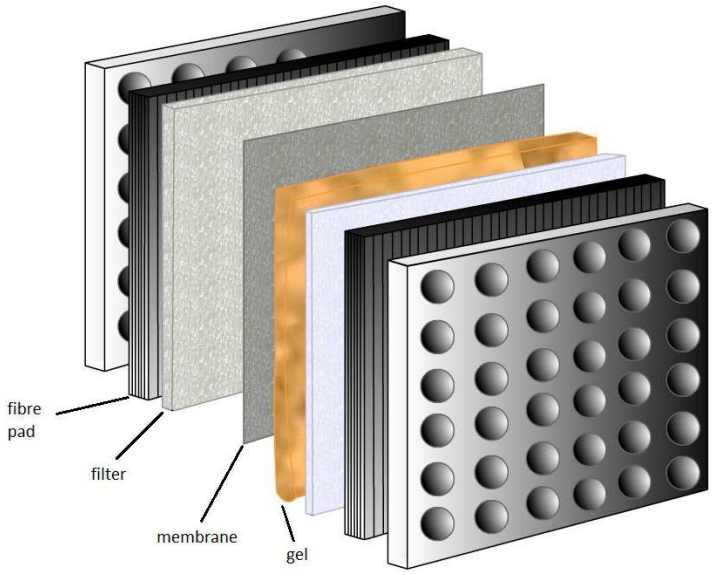


Figure 16: Schematic sketch of protein blotting

(Figure adapted from (48))

NAME	CONTENT
Blotting-buffer 500 ml	5 x Blotting-buffer 100 ml Methanol 100 ml Aqua bidest. 300 ml
5 x Blotting-buffer	Tris-Base 15,169 g Glycine 72,9 g Aqua bidest. ad 1000 ml

Table 14: Blotting-buffer contents

After the blotting, the membrane was blocked for one hour in 5 % milk powder in Tris-buffered-saline with Tween-20 (TBS-T) at room temperature, aiming to saturate the unspecific protein binding capacity of the membrane, to prevent the unspecific binding of the antibodies later on.

Then the membrane was washed with TBS-T three times 10 minutes each. Subsequently the first antibody was added and the membrane was incubated over night at 4 °C.

After the incubation the membrane was again washed 3 times (each time 10 minutes) with TBS-T and then the secondary antibody was added. Incubation time for the secondary antibody was 1 hour at room temperature. Then the membrane was washed again (3 times 10 minutes each) and afterwards it was detected with chemiluminescent reagent.

By using this method, also called immunodetection, we can detect our proteins of interest based on the antigen-antibody reaction. Therefore the first antibody binds to our protein we want to detect, then the second antibody coupled to a horse radish peroxidise binds on our first antibody and a antigen-antibody complex is built. Due to adding the chemiluminescent reagent our antigen-antibody complex can be visualized.

NAME	CONTENT
TBS-T pH 8,0	10 x TBS-T pH 8,0 100 ml Aqua bidest. 900 ml
10 x TBS-T pH 8,0	Tris-Base 30 g NaCl 111 g Tween 20 10 ml Aqua bidest. ad 1000 ml

Table 15: TBS-T pH 8,0 content

NAME	CONTENT
Chemiluminescent reagent	Aqua bidest. 4,5 ml 1 M Tris-Base pH 8,5 500 µl Luminol (stock) 22,5 µl p-Coumaric acid (stock) 11,2 µl H_2O_2 30 % 1,5 µl
Luminol stock solution	0,44 g Luminol 10 mL DMSO
p-Coumaric acid stock solution	0.15 g p-Coumaric Acid 10 mL DMSO

Table 16: Chemiluminescent reagent content

After the detection, the membrane was washed again the same way with TBS-T and then the first anti-mouse actin antibody was added and incubated over night at 4 °C.

Then the membrane was again washed with TBS-T and afterwards the secondary anti-mouse antibody was added to incubate it for 1 hour at room temperature. After the membrane was washed 3 times for 15 minutes with TBS-T, the actin was detected with chemiluminescent reagent. The detection of actin served as a control for equal protein loading.

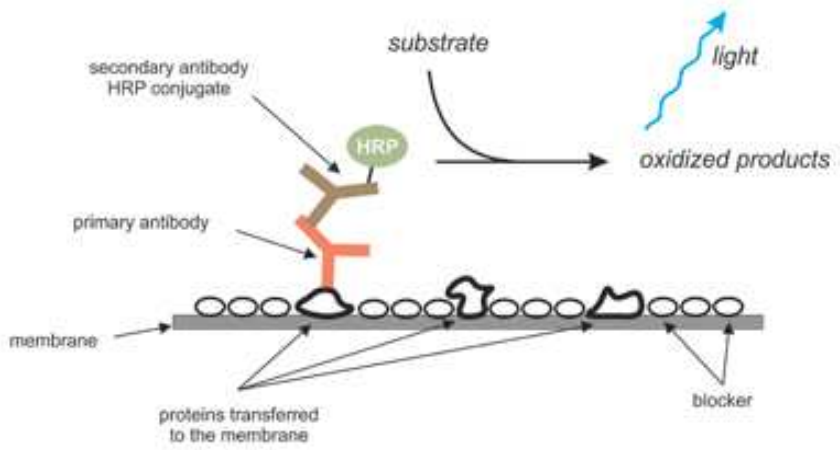


Figure 17: Detection with chemiluminescent reagent

The antigen-antibody complex can be visualised by immunodetection. The chemiluminescent reagent (content shown in Table 16) is added as substrate for the horseradish peroxidase (HRP), which results in oxidized products and light, which can be detected.

(Figure adapted from (49))

PROTEIN	MOLECULAR WEIGHT	MANUFACTURER	SPECIES	DILUTION
ABCA1	220 kD	Novus Biologicals	rabbit	1:500 in 5 ml 3 % milk in TBS-T
ABCG1	75,6 kD	Novus Biologicals	rabbit	1:500 in 5 ml 1 % milk in TBS-T
PPARγ	53,57 kD	Cell signalling	rabbit	1:1000 in 5 ml 5 % milk in TBS-T
RXRα	50-54 kD	Santa Cruz	rabbit	1:500 in 5 ml TBS-T
SR-BI	82 kD	Novus Biologicals	rabbit	1:1000 in 5 ml TBS-T
IgG horseradish peroxidase (HRP)-linked Antibody	various	Cell signalling	rabbit	1:1000
Actin	42 kD	mpbio	mouse	1:500 in 5 ml TBS-T
IgG HRP-linked Antibody	various	mpbio	mouse	1:500 in 5 ml TBS-T

Table 17: Antibodies

3.3 Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was performed to allow the quantitative determination of the expression of genes on the messenger ribonucleic acid (mRNA) level in THP-1 cells treated with the active compounds.

The data obtained by qRT-PCR can help us to assess the ability of the active compounds Evodiamine, Leoligin and Piperine to modify the levels of specific target mRNAs.

Total RNA of the treated cells was isolated by a total peqGOLD Total RNA Kit (provider Peqlab Biotechnologie).

Then a reverse transcription, a process in which single-stranded RNA is reversely transcribed into complementary DNA (cDNA), was performed. After that the process of qPCR was carried out with the cDNA.

3.3.1 Seeding of the cells

For seeding cells the same procedure described in 3.2.2.1 was performed.

3.3.2 Treatment of the cells

The treatment with the needed compounds was made the same as described in 3.2.2.2 and 3.2.2.3.

3.3.3 Extraction of the RNA

3.3.3.1 Homogenization and lysis

400 µl cell Lysis Buffer T were directly added to the cells for 5 minutes. Then the lysate was transferred into a DNA Removing Column, placed in a 2.0 ml Collection Tube and it was

centrifuged at 12.000 x g for 1 minute at room temperature. After that the flow-through lysate was transferred into a new 1.5 ml eppendorf tube.

3.3.3.2 Loading and binding

400 µl 70 % Ethanol was added to the lysate and mixed thoroughly. The PerfectBind RNA Column was placed in a new 2.0 Collection Tube and then the lysate was added directly to the membrane.
The assembly of PerfectBind RNA column and Collection Tube was centrifuged at 10.000 x g for 1 minute and after that the flow-through liquid and Collection Tube were discarded.

3.3.3.3 Washing 1

The PerfectBind RNA Column was placed in a fresh 2.0 ml collection tube and 500 µl RNA Wash Buffer 1 was added. Then it was centrifuged for 15 seconds at 10.000 x g. Subsequently the flow-trough liquid was again discarded and the collection tube was reused in the next step.

3.3.3.4 Washing 2

600 µl completed RNA Wash Buffer 2 was added to the PerfectBind RNA Column and it was again centrifuged for 15 seconds at 10.000 x g. The flow-through liquid was again discarded and the collection tube was reused. Then this step was repeated one more time.

3.3.3.5 Drying

In this very important step for removing ethanol from the column, the PerfectBind RNA Column was centrifuged for 1 minute at 10.000 x g to completely dry the column matrix.

3.3.3.6 Elution

The PerfectBind RNA Column was placed into a fresh 1.5 ml eppendorf tube and 60 µl sterile RNAse-free dH_2O was directly added to the binding matrix. After that it was centrifuged for 1 minute at 5.000 x g to elute RNA.

3.3.3.7 Quantitation and storage of RNA

For the quantification of RNA was used the "Nano drop 2000C Spectrophotometer" (provider Peqlab Biotechnologie) and the RNA was stored at -80 °C until further use.

3.3.4 Complementary desoxy-ribonucleic acid (cDNA) synthesis

The High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (provider Applied Biosystems™) components were placed and thawed on ice and then the 2 x RT master mix was also prepared and placed on ice.

COMPONENT	VOLUME (µL)/REACTION
10 x RT Buffer	2.0
25 x dNTP Mix (100 mM)	0.8
10 x RT Random Primers	2.0
MuliScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total per Reaction	10.0

Table 18: Contents of 2 x RT master mix

PCR stripes were used for synthesis of cDNA. 1 µg of RNA was added from each sample and filled up with water to a volume of 10 µl. Then 10 µl 2 x RT master mix were added to get a total volume of 20 µl. After the PCR stripes were closed tightly and the content was mixed,

they were put on the PCR machine (C1000^{TM} Thermal Cycler, provider: BIO-RAD), the cover was closed and the program was started.

	STEP 1	STEP 2	STEP 3	STEP 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 minutes	120 minutes	5 minutes	

Table 19: Thermal cycling conditions of RT-PCR

After the cDNA synthesis 30 µl of water were added to each sample to obtain a final volume of 50 µl and to get a concentration of 20 ng/µl cDNA (assuming that the reaction was complete). Then the cDNA was stored at -80 °C.

3.3.5 qPCR

First the reaction mix was prepared, and for calculating the volume it was taken care that each sample was measured in triplicate on a PCR white plate.

	For aimed gene (µl)	For control gene (µl)
LightCycler® 480 SYBR Green I Master Mix kit (provider Roche) 2 x Conc.	7,5	7,5
Primer solution 0.5 µM: HS_ABCA1_1_SG (provider Quanti Tect)	1,5	
Control gene primer solution 0.5 µM: 18S (provider Invitrogen)		1,5
PCR-grade water	4	4
Total volume	13	13

Table 20: Reaction mix contents

13 µl of reaction mix were added into each well of the PCR plate and subsequently 2 µl of the diluted cDNA were also added.

Then the PCR plate was sealed with a transparent foil and centrifuged for 3 minutes at 1000 x g.

Afterwards the plate was put in the PCR machine and the measurement was started.

4 Results and Discussion

It is well known that atherosclerosis leads, due to the formation of atherosclerotic plaques in the vessel wall, to decreased blood flow and in further consequence to heart attack and stroke and to death. Therefore it is important to find ways to avoid the atherosclerosis plaque formation, for example by increasing the RCT.

Macrophage cholesterol efflux, the initial step of RCT, plays an important role for prevention of the formation of atherosclerotic plaques.

Due to preliminary research, we know that three natural compounds Piperine, Leoligin, and Evodiamine can enhance the macrophage cholesterol efflux and therefore potentially also the RCT. Therefore we performed further experiments with these compounds to find their mechanism of action. The results obtained from the application of the different methods are shown below.

4.1 Western blotting

The aim of our work was to identify the mechanism of action of the active compounds which can enhance the macrophage cholesterol efflux. Therefore, we first tested the expression levels of proteins involved in the macrophage cholesterol efflux when cells were treated with these active compounds. Western blotting is a method which can be used to detect the changes of protein expression levels. Pioglitazone, which has been used clinically and reported to increase cholesterol efflux and some transporter protein expression, was used as positive control.

4.1.1 Expression level of nuclear receptors involved in cholesterol efflux

The obtained results of protein expression levels of the nuclear receptors PPARγ and RXRα, both of which were reported to participate in the macrophage cholesterol efflux, are presented in Figure 18 and Figure 19, respectively.

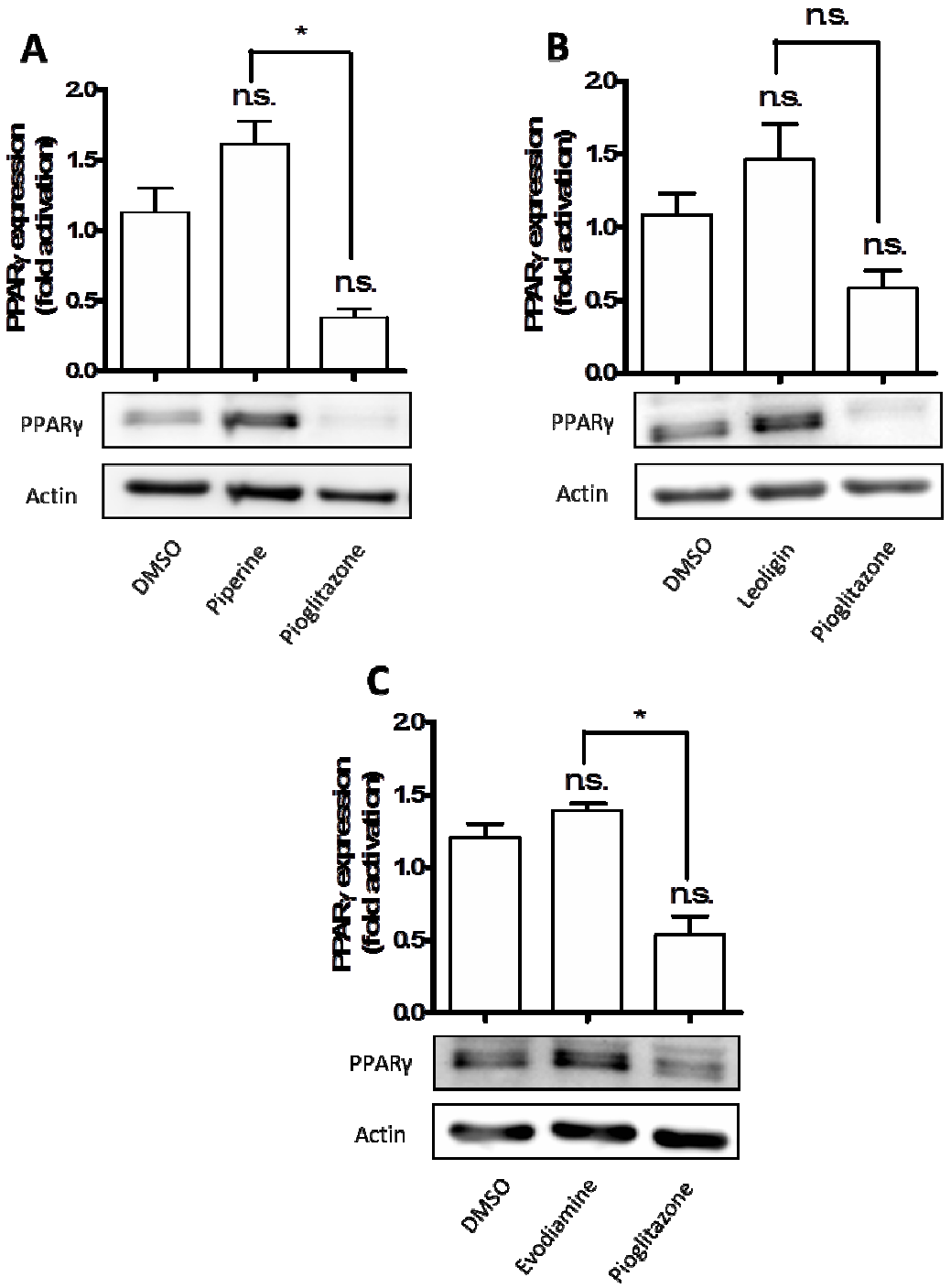


Figure 18: Results of PPARγ protein expression quantification after treatment with Piperine (A), Leoligin (B), or Evodiamine (C). THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine (A), 10 µM Leoligin (B), or 10 µM Evodiamine (C) for 24 hours. The results show tendency of a higher expression of PPARγ compared to DMSO (A), (B), (C). The PPARγ expression of 10 µM Pioglitazone shows a tendency of a lower expression compared to DMSO (A) (B), (C), a significantly lower expression compared to Piperine and Evodiamine(A), (C) and a tendency of lower expression compared to Leoligin (B); n=3 *p<0.05 n.s. not significant, normalized with Actin,

determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

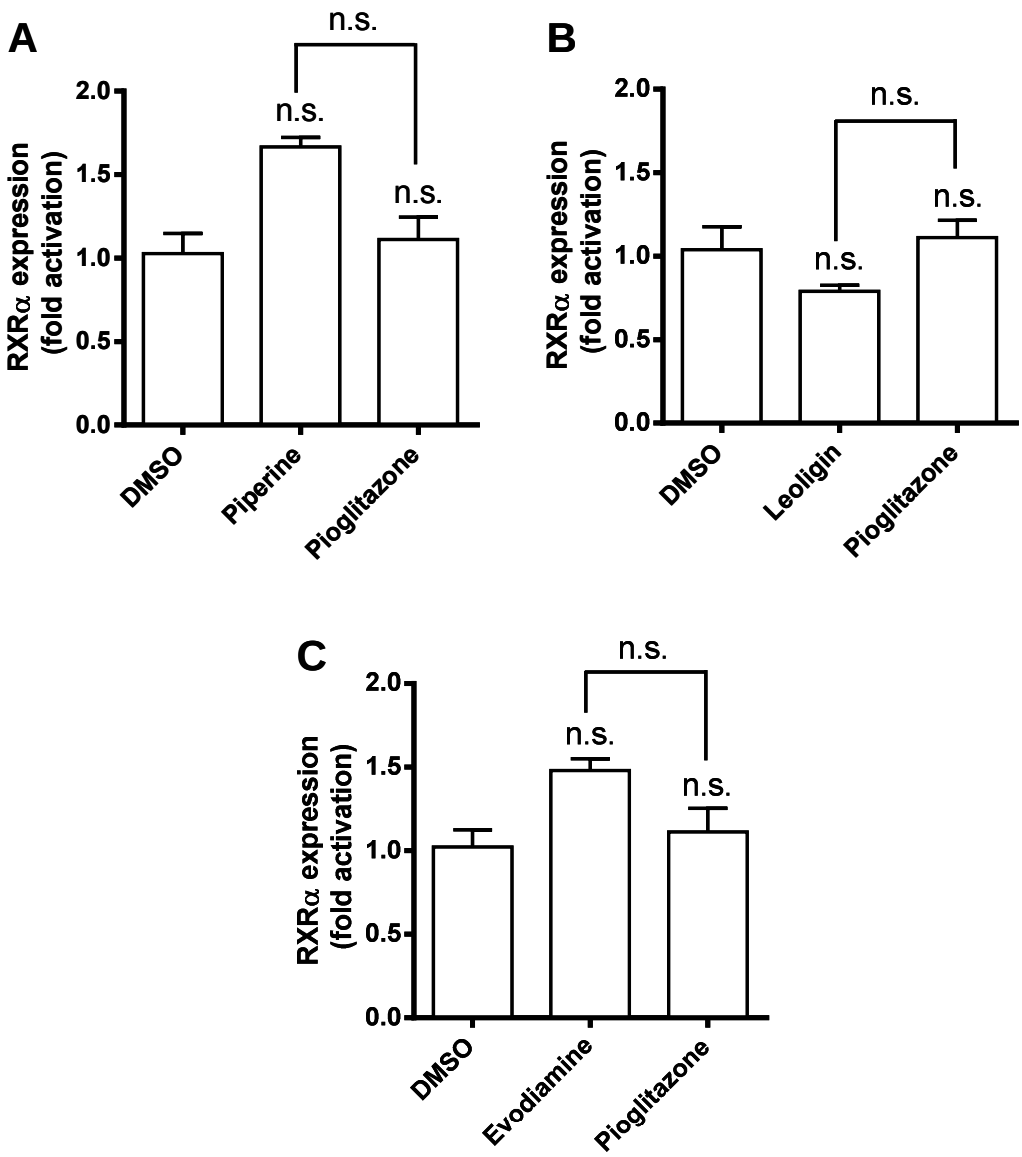


Figure 19: Results of RXRα protein expression quantification after treatment with Piperine (A), Leoligin (B), or Evodiamine (C).

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine (A), 10 µM Leoligin (B), or 10 µM Evodiamine (C) for 24 hours. The results show a tendency of higher expression of RXRα compared to DMSO and also of higher RXRα expression compared to 10 µM Pioglitazone (A), (C). The result shows the tendency to have nearly the same expression level of RXRα compared to DMSO and compared to 10 µM Pioglitazone (B). The Pioglitazone treated group shows a trend to have the same expression compared to DMSO (A), (B), (C); n=3 n.s. not significant, normalized with Actin, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

Regarding the expression of PPARγ, Piperine, Leoligin and Evodiamine show all a tendency of up-regulation of protein expression compared to DMSO, but upon statistical evaluations these putative effects did not reach significance. If the active compounds were able to up-regulate the expression of the protein PPARγ, this could lead to enhancement of cholesterol efflux due to their influence on the PPARγ-LXRα-ABCA1/ABCG1 pathway, but further experiments are needed to conclusively confirm or exclude effects.

Piperine and Evodiamine show a slight tendency to up-regulate the RXRα protein expression level compared to DMSO and also compared to the positive control Pioglitazone. In contrast to that, Leoligin shows a trend to have nearly the same expression level of RXRα protein compared to DMSO and Pioglitazone. But based on statistical evaluation, the obtained results of RXRα protein expression of THP-1 cells treated with our active compounds did not reach significance. It is not completely excluded that Piperine and Evodiamine, but possibly not Leoligin, also able to increase the protein expression of RXRα and because this protein builds a dimer with LXRα, that they exert some influence on the PPARγ-LXRα-ABCA1/ABCG1 pathway. However, further experiments are needed to conclusively confirm or exclude effects.

It should be noticed that the obtained results of PPARγ expression in THP-1 cells treated with the positive control Pioglitazone show a significantly down-regulated effect of protein expression compared to Piperine and Evodiamine and compared to Leoligin the expression of PPARγ shows a tendency of down-regulation, but upon statistical evaluation the result obtained by Leoligin did not reach significance. The expression of PPARγ compared to DMSO also shows the trend of down-regulation (not significantly upon statistical tests).
The PPARγ agonist, Pioglitazone, has been reported to enhance cholesterol efflux from macrophages by increasing ABCA1/ABCG1 expression via the PPARγ-LXRα pathway (50).
Due to our results we could observe a trend of down-regulated expression of PPARγ in present of Pioglitazone, but this does not exclude that Pioglitazone acts as a ligand.
The ligand-activated transcription factor PPARγ, activated by its ligand Pioglitazone, shows degradation which is linked to its ligand-dependent activation (51).

So the agonist of PPARγ, Pioglitazone, enhances the macrophage cholesterol efflux by activating the PPARγ and in further consequence by activating the PPARγ-LXRα-ABCA1/ABCG1 pathway, but not by increasing the protein expression level of PPARγ. After binding to PPARγ it may induce the degradation of the protein.

4.1.2 Expression level of transmembrane transporter proteins involved in cholesterol efflux

The results of the measurements of the expression level of the transmembrane proteins ABCA1, ABCG1, and SR-B1, which are significantly involved in cholesterol efflux in macrophages, are shown in the following chapter.

4.1.2.1 ABCA1 and ABCG1

Figure 20 and Figure 21 show the expression levels of the transmembrane proteins ABCA1 and ABCG1. THP-1 cells were first differentiated with 200 nM for 72 hours to macrophage-like cells, then they were treated with the active compounds Piperine 50 µM, Leoligin 10 µM and Evodiamine 10 µM and compared to DMSO as control and Pioglitazone 10 µM as positive control.

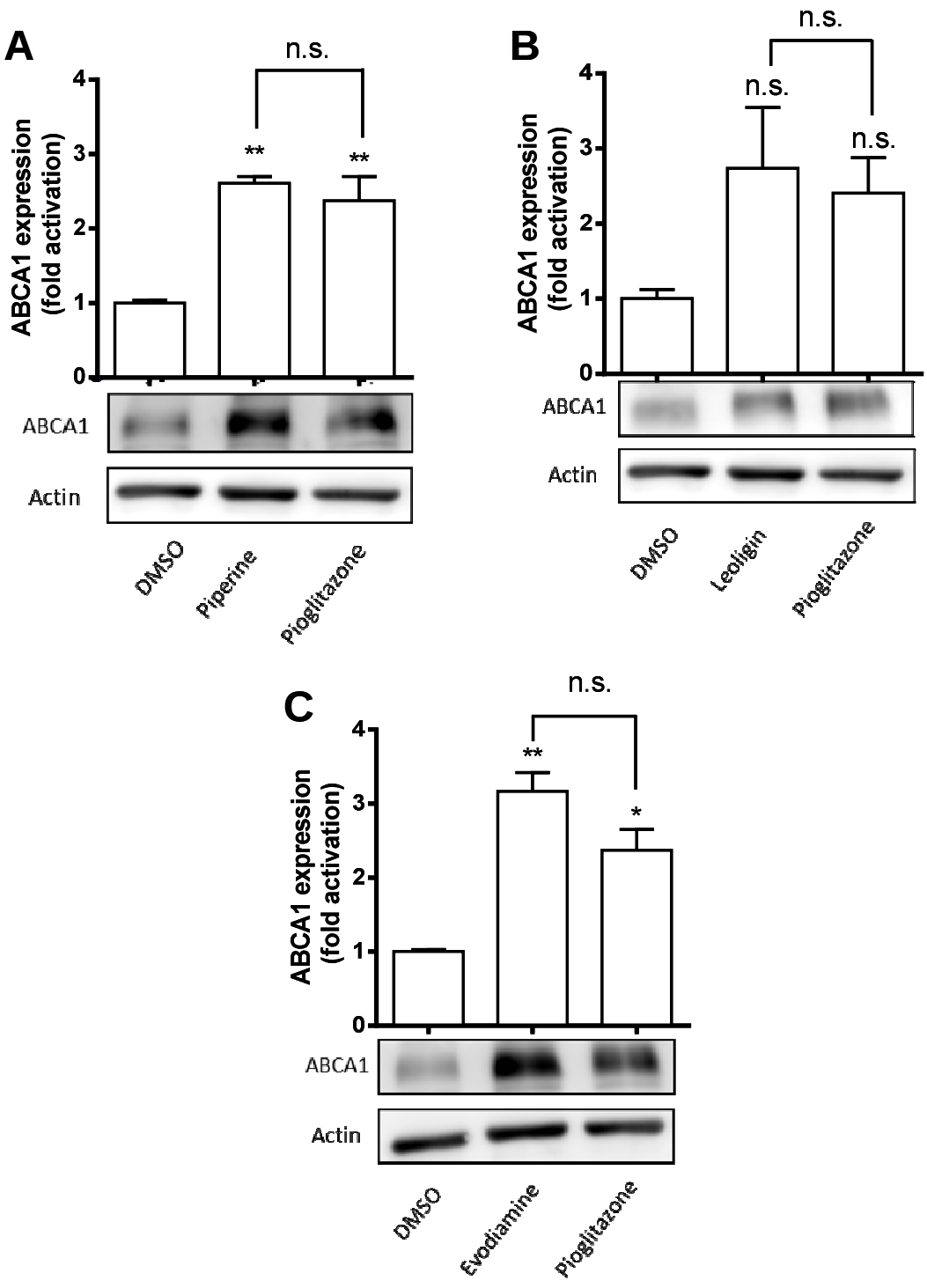


Figure 20: ABCA1 protein expression quantification after treatment with Piperine (A), Leoligin (B), or Evodiamine (C).

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine (A), 10 µM Leoligin (B), or 10 µM Evodiamine (C) for 24 hours. The figure shows a significantly higher expression of ABCA1 of cells treated with the active compound compared to DMSO (A), (C). The figure shows a tendency to up-regulate the ABCA1 protein expression compared to DMSO, but upon statistical evaluation it did not reach significance (B).The compounds show a

slight trend of up-regulation of ABCA1 expression compared to Pioglitazone (not significant) (A), (B) and also the tendency of up-regulation of expression level compared to Pioglitazone (not significantly) (C); n=3 *p<0.05 **p<0.01 n.s. not significant, normalized with Actin, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

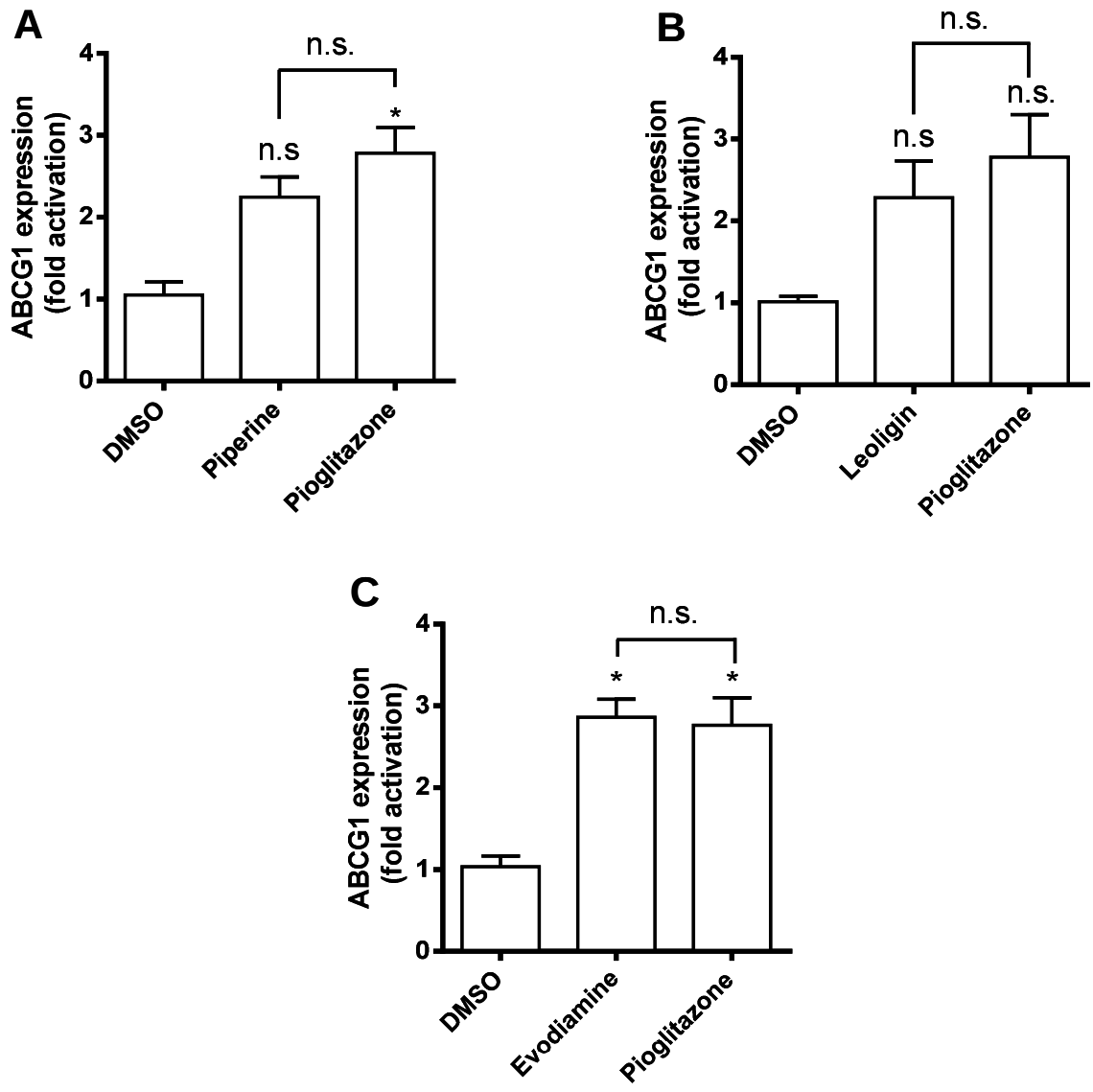


Figure 21: Results of quantification of ABCG1 protein expression upon treatment with Piperine (A), Leoligin (B), or Evodiamine (C).

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine (A), 10 µM Leoligin (B), or 10 µM Evodiamine (C) for 24 hours. The compounds show a tendency of up-regulation compared to DMSO (not significant) (A), (B) and an effect of up-regulation compared to DMSO (C). (A) and (B) show a tendency of down-regulation compared to Pioglitazone (not significant). The compound shows a tendency to have nearly the expression compared to Pioglitazone (not significant) (C). Pioglitazone shows an effect of up-regulation (A), (C) and a tendency of up-regulation (B) compared to DMSO ; n=3 *$p<0.05$ n.s. not significant, normalized with Actin, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

All of our active compounds show at least a tendency of up-regulation of expression level of both transmembrane proteins, ABCA1 and ABCG1, compared to the control DMSO.

In detail, for the ABCA1 protein, the compounds Piperine and Evodiamine show induced up-regulation of ABCA1 expression level compared to DMSO. In contrast to that, Leoligin just shows a tendency to up-regulate the expression level of ABCA1 in THP-1 cells, but upon statistical evaluation this putative effect of Leoligin did not reach significance.
Leoligin and Evodiamine indicate a tendency of a higher expression level of ABCA1 expression compared to Pioglitazone and the compound Piperine shows the trend to have nearly the same expression level as Pioglitazone, but based on statistical evaluation none of the compounds exhibited a significantly different effect compared to Pioglitazone.
Piperine and Evodiamine increased ABCA1 expression around 3-fold, therefore we can confirm that these two compounds have influence on the up-regulation of expression level of the transmembrane protein ABCA1.
Leoligin showed the tendency to increase the expression of ABCA1 around 3-fold. Further experiments are needed to confirm the putative influence of Leoligin on the ABCA1 protein expression.

For the second important transmembrane protein that belongs to the ABC superfamily, ABCG1, we could also observe a trend of up-regulation of protein expression level in THP-1 cells treated with Piperine and Leoligin, compared to the control DMSO (not significant). Evodiamine treated THP-1 cells could show an effect of up-regulation compared to the control DMSO. Compared to the positive control Pioglitazone, Piperine and Leoligin show the tendency to fail a higher expression level and Evodiamine shows the trend to have nearly the same expression level.
Because only Evodiamine could show an effect of up-regulation, further experiments are required to confirm or exclude effects on ABCG1 expression of Piperine and Leoligin treated THP-1 cells.

Both transmembrane proteins, ABCA1 and ABCG1, are involved in the active macrophage cholesterol efflux and therefore they are very important for the overall RCT rate. On the one hand the ABCA1 transfers the excess cholesterol specifically to the Apo-A-I, while the ABCG1

transfers cholesterol to HDL (8). The transcription of these two transmembrane proteins is controlled by the nuclear factors PPARγ and LXRα, forming the PPARγ-LXRα-ABCA1/ABCG1 pathway (26).

Because we could observe the effect of an increase of the protein expression of ABCA1 in THP-1 cells treated with Piperine and Evodiamine, and also because we could observe (presented in chapter 4.1.1) that these two active compounds show also the tendency to up-regulate the protein expression of nuclear receptors, we can suppose that the compounds are maybe able to enhance the macrophage cholesterol efflux due to their influence on the PPARγ-LXRα-ABCA1 pathway, but definitely, more detailed experiments are necessary to verify these hypothesis.

Also Leoligin is possibly able to enhance the macrophage cholesterol efflux by its influence on the PPARγ-LXRα-ABCA1 pathway, because due to our obtained results it shows a tendency to up-regulate both, the ABCA1 protein and the nuclear receptor PPARγ, but not the RXRα. However, since these putative effects did not reach statistical significance, clearly further experiments are needed to confirm or exclude them.

Furthermore, based on our results, that show effects of up-regulaton of ABCG1 expression in Evodiamine treated THP-1 cells and also because they showed tendency of up-regulation of the nuclear receptors, PPARγ and RXRα, we suppose that these compounds might have influence on the PPARγ-LXRα-ABCG1 pathway. But therefore, further experiments are necessary to verify this supposition.

Also Piperine and Leoligin treated THP-1 cells show tendency of up-regulation of ABCG1 protein, so we can not completely exclude that these active compounds might have influence on the PPARγ-LXRα-ABCG1 pathway. However, because we could not observe a statistically significant effect in THP-1 cells treated with Piperine and Leoligin, but just a tendency for increased ABCG1 expression, further research is required to confirm or completely exclude effect on the expression of this protein.

4.1.2.2 SR-BI

Figure 22 shows the protein expression level of the bidirectional transmembrane transporter protein SR-BI after THP-1 cells were differentiated for 72 hours with 200 nM PMA and treated with Piperine 50 µM, Leoligin 10 µM, or Evodiamine 10 µM. DMSO was used as control and as positive control was used Pioglitazone, 10 µM.

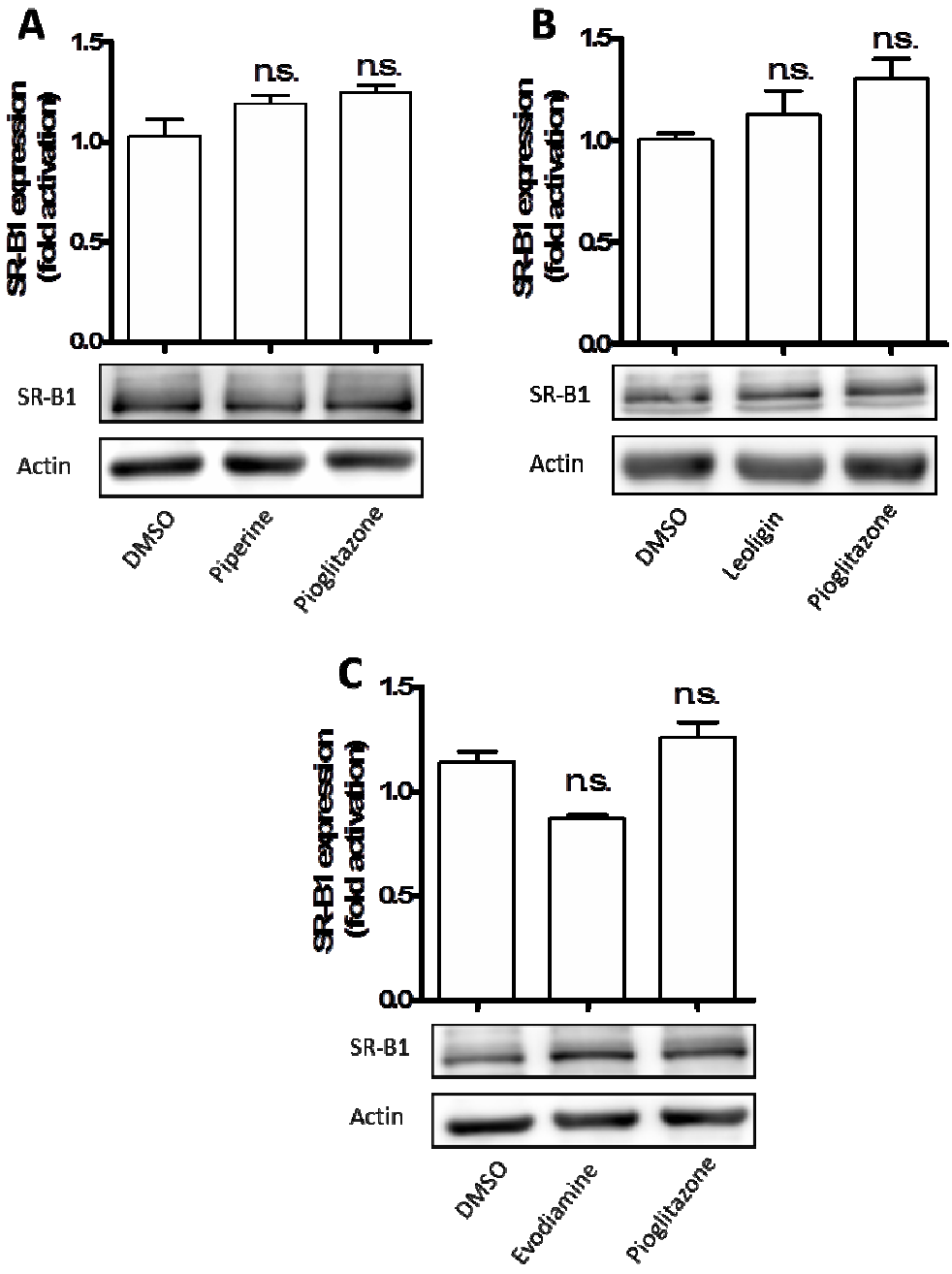


Figure 22: Results of quantification of SR-BI protein expression after treatment with Piperine (A), Leoligin (B), or Evodiamine (C).

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine (A), 10 µM Leoligin (B), or 10 µM Evodiamine (C) for 24 hours. No effect of up-regulation of the active compounds and Pioglitazone compared to DMSO was observed (A), (B), (C); n=3, normalized with Actin, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

There is no effect and no tendency of up-regulation of SR-BI expression in THP-1 in cells treated with Piperine, Leoligin and Evodiamine and also in THP-1 cells treated with Pioglitazone, compared to the DMSO treatment group.
Based on our results, the macrophage cholesterol efflux increasing activity of the investigated compounds is supposed not to be explained by their effect on SR-B1 expression level.

The bidirectional transmembrane protein SR-BI mediates both the cholesterol up-take and cholesterol efflux in macrophage cells, hepatocytes and other cells (8). Therefore, it can be suspected as a neutral characteristic of the compounds that they show no effects and no tendencies of enhancement of SR-BI expression level.

4.2 qRT PCR

4.2.1 mRNA expression level of the transmembrane protein ABCA1

Since the protein expression level of the transmembrane protein ABCA1 in THP-1 cells treated with Piperine and Evodiamine showed significant up-regulation and also treated with Leoligin showed the trend of up-regulation, we further investigated by qRT PCR the mRNA levels of ABCA1, to see if the increased protein amount is due to increased mRNA expression.

Figure 23, Figure 24 and Figure 25 show the ABCA1 mRNA levels in THP-1 cells differentiated with 200 nM PMA and treated with the active compounds Piperine 50 µM, Leoligin 10 µM, or Evodiamine 10 µM.

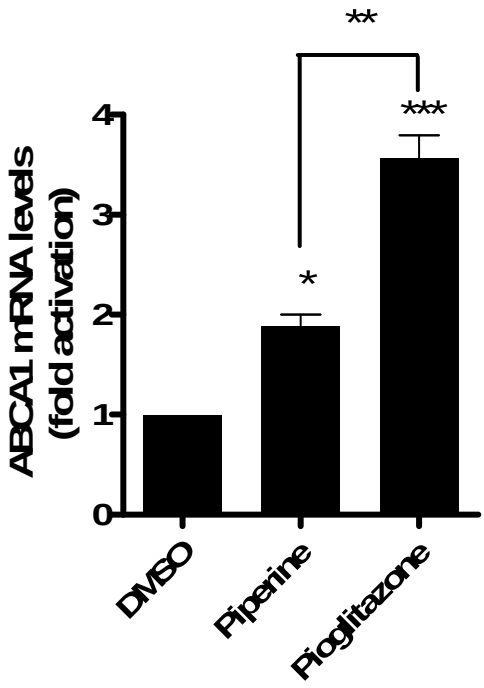


Figure 23: ABCA1 mRNA levels induced by Piperine

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine or 10 µM Pioglitazone for 24 hours. The data show us a higher expression of ABCA1 mRNA compared to DMSO (significant) and a lower ABCA1 mRNA expression compared to 10 µM Pioglitazone (significant); n=4 *p<0.5, **p<0.01, ***p<0.001, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

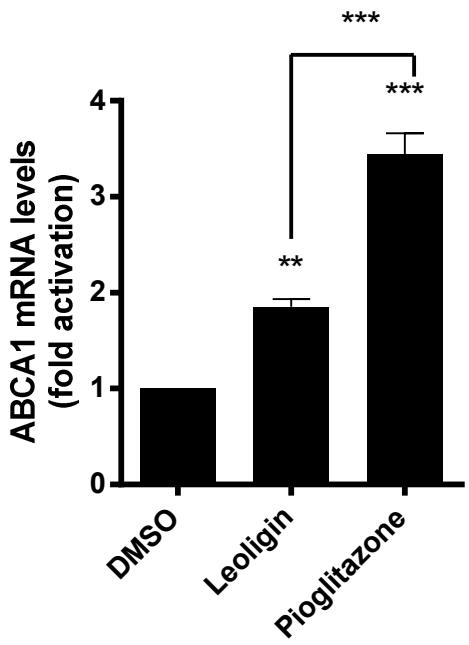


Figure 24: ABCA1 mRNA levels induced by Leoligin

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine or 10 µM Pioglitazone for 24 hours. The data show us a higher expression of ABCA1 mRNA compared to DMSO (significant) and a lower ABCA1 mRNA expression compared to 10 µM Pioglitazone (significant); n=4 **p<0.01, ***p<0.001, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

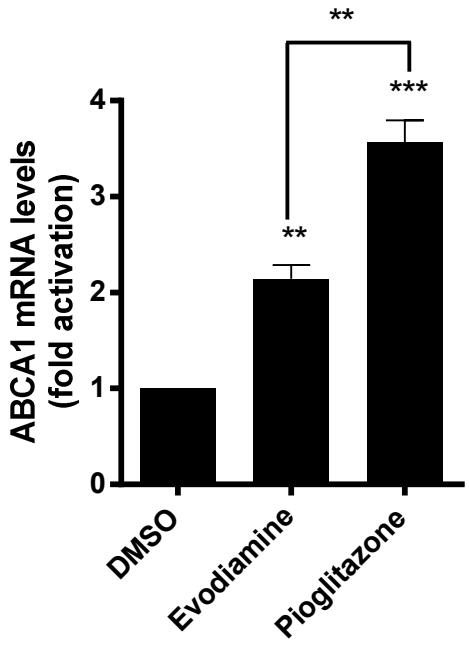


Figure 25: ABCA1 mRNA levels induced by Evodiamine

THP-1 cells first were differentiated with 200 nM PMA for 72 hours, then treated with 50 µM Piperine or 10 µM Pioglitazone for 24 hours. The data show us a higher expression of ABCA1 mRNA compared to DMSO (significant) and a lower ABCA1 mRNA expression compared to 10 µM Pioglitazone (significant); n=4 **p<0.01, ***p<0.001, determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni´s post hoc test.

All three tested compounds Piperine, Leoligin, and Evodiamine show a higher mRNA level of ABCA1 compared to the DMSO treatment group. But compared to the positive control, Pioglitazone, they show a lower mRNA level.

To compare all three active compounds, they all show nearly the same ABCA1 mRNA expression around 2-fold, therefore Piperine and Leoligin activated 1.9-fold and Evodiamine activated 2.2-fold.

Although the transmembrane protein ABCA1 shows a strong effect of up-regulaton in THP-1 cells when treated with the active compounds Piperine and Evodiamine, and a tendency of up-regulation of ABCA1 protein when THP-1 cells treated with Leoligin, the results obtained with the method of qRT PCR indicate that the mRNA level of ABCA1 is on the one hand

higher than the mRNA expression of DMSO but on the other hand also lower compared to the positive control Pioglitazone.

In contrast to the observed big increase of ABCA1 protein amount upon treatment with the investigated compounds Piperine and Evodiamine, and in contrast to the tendency of increased ABCA1 protein expression upon treatment with Leoligin, there is just a small increase in mRNA expression.

Therefore it is expected that aside of the mRNA levels increase other mechanisms of action of the investigated natural active compounds could also contribute for the observed ABCA1 protein expression.

4.3 Conclusion

In short, due to the obtained results, we can suppose more than one mechanism of action of the investigated natural compounds, Piperine, Leoligin and Evodiamine, to contribute to their macrophage cholesterol efflux enhancing properties.

There are lot of pathways described for macrophage cholesterol efflux, like the passive diffusion, the SR-BI mediated cholesterol efflux and the efflux mediated by the transmembrane proteins ABCA1 and ABCG1 (13).

We suppose that one mechanism of action of the investigated compounds is that they are probably able to enhance the macrophage cholesterol efflux because of their influence on the PPARγ-LXRα-ABCA1 pathway.

To describe this pathway, the activation of the nuclear receptor PPARγ by an agonist starts the cascade of the PPARγ-LXRα-ABCA1 pathway, where activation of PPARγ follows in an activation of another nuclear receptor, the LXRα. The activation of these nuclear factors leads to the increased expression of the transmembrane cholesterol transporter protein ABCA1 (26).

The investigated active compounds showed tendency to increase the expression of the nuclear receptor PPARγ (shown on Figure 18). Piperine and Evodiamine also showed tendency to increase the expression of the second nuclear receptor RXRα and Leoligin showed the tendency to have nearly the same expression compared to DMSO. Since these putative effects did not reach statistical significance, further experiments are needed to confirm or completely exclude them. If these putative effects confirm to be real, this could further lead to activation of LXRα, further regulating the expression of the transmembrane transporter protein ABCA1, which showed the effect of up-regulation in THP-1 cells treated with Piperine and Evodiamine, and trend of up-regulation in THP-1 cells treated with Leoligin (shown on Figure 20).

Therefore, it needs further research to study in more details the precise mechanisms by which the active compounds might enhance the macrophage cholesterol efflux through PPARγ-LXRα-ABCA1 pathway.

It is also not excluded that the active compounds might be able to enhance the macrophage cholesterol efflux by the PPARγ-LXRα-ABCG1 pathway, because we could observe tendencies of Piperine and Leoligin treated THP-1cells to up-regulate the ABCG1 expression level and because Evodiamine could even show an effect of up-regulation. The PPARγ-LXRα-ABCG1 pathway is a pathway, where similar to the PPARγ-LXRα-ABCA1 pathway the activation of PPARγ by an agonist and the followed activation of the other nuclear factor LXRα leads to the expression of the transmembrane protein ABCG1.
But the putative effects of Piperine and Leoligin are likely not a major part of the mechanism of action of our compounds, because our results indicate just a slight trend of change in the ABCG1 protein expression level upon treatment with Piperine and Leoligin (shown on Figure 21), in contrast to the increase of the expression level of ABCA1 in THP-1 cells treated with Piperine, and in contrast to the tendency of Leoligin to up-regulate the ABCA1 expressions level (shown on Figure 20). Except for THP-1 cells treated with Evodiamine, which showed nearly the same effect of up-regulation of ABCA1 and ABCG1 expression.

Definitely, the investigated compounds showed no significant effects on the expression level of the bidirectional membrane transporter SR-BI (shown on Figure 22), making this transporter an unlikely mediator of the efflux-enhancing action of the compounds. Furthermore, since the compounds are supposed not to enhance the SR-BI expression, they are probably also not expected to enhance the call ability for cholesterol import.

Since the compounds exerted an increasing effect on ABCA1 protein expression in THP-1 cells treated with Piperine and Evodiamine, and since they exerted a tendency of increase of ABCA1 protein expression in THP-1 cells treated with Leoligin, in the next step we thought that it was important to investigate the ABCA1 mRNA levels with the qRT PCR method, trying to correlate the increased ABCA1 protein expression with the mRNA levels of ABCA1.
But because we obtained from the qRT PCR measurements a lower increase of the mRNA expression level of ABCA1 than expected, we suppose that there are also other mechanisms of action of Piperine, Leoligin and Evodiamine that contribute to the ABCA1 protein increase. One possible such mechanism could be that the active compounds might also have influence on the stability of the transmembrane protein ABCA1. So we suppose that maybe treatment of the THP-1 cells with the active compounds could extend the half time life of the protein

ABCA1 and maybe that could be the reason why we could observe that big amount of expressed ABCA1 protein in Piperine and Evodiamine treated THP-1 cells and why we could observe a tendency of increased ABCA1 protein expression in Leoligin treated THP-1 cells, detected by western blotting. So this would also recommend further research.

The PPARγ agonist, Pioglitazone, which has been reported to enhance macrophage cholesterol efflux by activation of the PPARγ-LXRα-ABCA1/ABCG1 pathway (50), could not show enhancement of macrophage cholesterol efflux by increased PPARγ protein expression. In contrast to that, due to our obtained results we could observe that our natural active compounds showed the tendency to increase the expression level of PPARγ.
Since the western blotting results do not show the activation level of PPARγ, it still needs further research based on other methods to confirm whether our active compounds are able to act as ligands of PPARγ (and therefore able to activate the PPARγ) and in this way to enhance the macrophage cholesterol efflux by activating the PPARγ-LXRα-ABCA1/ABCG1 pathway.

Finally, on the one hand we could observe some effects, but on the other hand because a big part of the obtained results just showed trends and no significant effects upon statistical evaluation, and because we can reveal just a small part of mechanisms of action until now, it needs definitely further research to find all exact mechanisms by which the investigated natural compounds enhance the macrophage cholesterol efflux.

References

(1) Lusis AJ (2000), Atherosclerosis, Nature **407**(6801):233-41

(2) http://www.who.int/cardiovasular diseases/en

(3) www.nhlbi.nih.gov/health/health-topics/topics/atherosclerosis

(4) Pschryrembel (2004), Klinisches Wörterbuch, de Gruyter **260**:137

(5) McGill HC, McMahan CA, Herderick EE, Malcom GT, Tracy RE, Strong JP (2000), Origin of atherosclerosis in childhood and adolescence; The American journal of clinical nutrition **72**(5 Suppl):1307S-15S

(6) Li AC, Glass CK (2002), The macrophage foam cell as a target for therapeutic intervention, Nature medicine **8**(11):1235-42

(7) Marcel YL, Ouimet M, Wang MD (2008), Regulation of cholesterol efflux from magrophages, Current opinion in lipidology **19**(5):455-61

(8) Luo DX et al. (2010), A novel model of cholesterol efflux from lipid-loaded cells, Acta Pharmacologica Sinica **31**:1243-1257

(9) Ory DS (2004), Nuclear receptor signalling in the control of cholesterol homeostasis: have the orphans found a home?, Circ Res **95**:660-70

(10) Payne AH, Hales DB (2004), Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones; Endocr Rev **25**:947-70

(11) Von Bergmann K et al. (2005), Cholesterol and plant sterol absorption: recent insights, Am J Cardiol **96**:10D-14D

(12) Rader DJ (2002), High density lipoproteins and Atherosclerosis, The American Journal of Cardialogy **90**(8):62-70

(13) Tall AR et al. (2008), HDL, ABC Transporters, and Cholesterol Efflux: Implications for the Treatment of Atherosclerosis, Cell Metabolism **7**:365-375

(14) Cavelier C et al. (2006), Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1, Biochimica et Biophysica Acta **1761**:655–666

(15) Oram JF, Lawn RM (2001), The gatekeeper for eliminating excess tissue cholesterol, J Lipid Res **42**:1173-9

(16) Ohashi R. et al. (2005), Reverse cholesterol transport and cholesterol efflux in atherosclerosis, Q J Med **98**:845–856

(17) Vedhachalam C et al. (2007), ABCA1-induced cell surface binding sites for ApoA-I, Arterioscler Thromb Vasc Biol **27**:1603-9

(18) Vedhachalam C et al. (2007), Mechanism of ATP-binding cassette transporter A1-mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles, J Biol Chem **282**:25123-30

(19) Wang N et al. (2004), ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins, Proc Natl Acad Sci USA **101**:9774-9

(20) Yu XH et al. (2013), Foam cells in atherosclerosis, Clinica Chimica Acta **424**: 245–252

(21) Venkateswaran A et al. (2000), Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha, Proc Natl Acad Sci USA **97**:12097-102

(22) Repa JJ et al. (2000), Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers, Science **289**:1524-9

(23) Haraguchi G et al. (2003), PPAR(alpha) and PPAR(gamma) activators suppress the monocyte-macrophage apoB-48 receptor, J Lipid Res **44**:1224-31

(24) Ditiatkovski M et al. (2006), GM-CSF deficiency reduces macrophage PPAR-gamma expression and aggravates atherosclerosis in ApoE-deficient mice, Arterioscler Thromb Vasc Biol **26**:2337-44

(25) Hu Q et al. (2010), PPARgamma1-induced caveolin-1 enhances cholesterol efflux and attenuates atherosclerosis in apolipoprotein E-deficient mice, J Vasc Res **47**:69-79

(26) Chawla A et al. (2001), A PPARγ-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis, Molecular Cell **7**:161-171

(27) Ogata M et al. (2009), On the mechanism for PPAR agonists to enhance ABCA1 gene expression, Atherosclerosis **205**:413-419

(28) Mutschler E et al. (2008), Mutschler Arzneimittelwirkungen, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart **9**:419

(29) http://www.pharmawiki.ch/wiki/index.php?wiki=Pioglitazon

(30) Lazar MA (2001), Progress in cardiovascular biology: PPAR for the course, Nature Medicine **7**:23-24

(31) Adams M et al. (2004), Inhibition of leukotrine biosynthesis by quinolone alkaloids from the fruits of Evodia rutaecarpa, Planta media **70**(10):904-8

(32) Wang T et al. (2008), Evodiamine improves diet-induced obesity in a uncoupling protein-1-independent manner: involvement of antiadipogenic mechanism and

extracellularly regulated kinase/mitogen-activated protein kinase signalling, Endocrinology **149**(1):358-66

(33) http://www.pharma-chemical.com/detail_771.html

(34) http://www.agriculturesource.com/p-fructus-evodiae-p-e-30016.html

(35) Reisinger U et al. (2009), Leoligin, the major lignan from Edelweiss, inhibits intimal hyperplasia of venous bypass grafts, Cardiovascular research **82**(3):542-9

(36) Duwensee K et al. (2011), Leoligin, the major lignan from Edelweiss, activates cholesteryl ester transfer protein, Atherosclerosis **219**(1):109-15

(37) http://cardiovascres.oxfordjournals.org/content/early/2009/03/05/cvr.cvp059/F1.expansion

(38) *http://www.chirurgiemuw.at/abteilungen/surgical-research-laboratories/arbeitsgruppen-der-chirurgischen-forschung-nach-schwerpunkten/cardiac-surgery-research-laboratory/*

(39) Darshan and Doreswamy (2004), Patented antiinflammatory plant drug development from traditional medicine, Phytother Res **18**: 343–357

(40) Yang et al. (2002), A piperidine amide extracted from P longum L. fruit shows activity against aedes aegypti mosquito larvae, J Agr Food Chem **50**(13): 3765–3767

(41) Bai and Xu (2000), Protective action of piperine against experimental gastric ulcer, Acta Pharmacol Sinc **21**:357–359

(42) Pruthi JS 1(993), Major spices of india: crop management and post harvest technology, ICAR: New Delhi

(43) Ravindran PA (2000), Black pepper, piper nigrum. Medicinal and aromatic plants-industrial profiles, Phytochemistry **58**:827–829

(44) Sharon MH (2002), Herb-Drug Interaction Handbook, Church Street Books **12**:123-0527

(45) Acharaporn D et al. 2012, Black pepper and piperine reduce cholesterol uptake and enhance translocation of cholesterol transporter proteins, J Nat Med **67**:303–310

(46) http://foolsexperiments.wordpress.com/

(47) *http://www.21food.com/products/sarawak-faq-black-pepper-596558.html*

(48) http://www.chemgapedia.de/vsengine/vlu/vsc/de/ch/8/bc/vlu/proteinanalytik/elektrophorese.vlu/Page/vsc/de/ch/8/bc/proteinanalytik/methoden_protein/blot.vscml.html

(49) http://www.western-blot.us/index.php?page=detection

(50) Ozasa H et al. (2011), Pioglitazone enhances cholesterol efflux from macrophages by increasing ABCA1/ABCG1 expressions via PPARγ-LXRα pathway: Findings from in vitro and ex vivo studies, Atherosclerosis **219**(1): 141-50

(51) Hauser S et al. (2000), Degradation of the Peroxisome Proliferator activated Receptor γ is linked to the Ligand-dependent Activation, The Journal of biological chemistry **275**(24): 18527-18533

(52) Meghwal M et al. (2013), Piper nigrum and Piperine: An Update, Phytotherapie Research **27**(8):1121-30

Abbreviations

A	
ABC	Adenosine triphosphat-binding cassette protein
ABCA1	Adenosine triphosphat-binding cassette protein A1
ABCG1	Adenosine triphosphat-binding cassette protein G1
ApoA-I	Apolipoprotein A-I
ApoE	ApolipoproteinE

B	
BA	Bile acids
BSA	Bovine serum albumin

C	
cDNA	Complementary desoxyribonucleic acid
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease

D	
DMSO	Dimethylsulfoxid

F	
FBS	Fetal Bovine Serum
FC	Free cholesterol

H	
HDL	High density lipoprotein
HRP	Horseradish peroxidise-linked

I

IgG	Immunoglobulin G

L

LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LXR	Liver X receptor

M

mRNA	Messenger ribonucleic acid

P

PBS	Phosphate buffered saline
PMA	Phorbol-12-myristate-13-acetat
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
PVDF	Polyvinylidenfluoride

Q

qRT-PCR	Quantitative Real Time-Polymerase Chain Reaction

R

RCT	Reverse cholesterol transport
RPMI	Roswell Park Memorial Institute
RXR	Retinoid X receptor

S

SDS	Sodium dodecyl sulfate
SR-B1	Scavenger receptor class B type 1

T

TBS-T	Tris-buffered-saline with Tween

U

UC	Unesterified cholesterol

V

VLDL	Very low density lipoprotein

W

WHO	World Health Organization

Acknowledgements

First, I want to thank Univ. Prof. Dr. Verena Dirsch for giving me the opportunity to work in her research group the “Molecular Target Group”.

Particularly, I would also to thank my supervisor Limei Wang for her support and patience not only during practical work, but also during writing the diploma thesis.

Next I also want to thank the whole “Molecular Target Group” for their help and advices and also for the familiar atmosphere during the practical work.

Deepest thanks go also to my family, who always supported me not only during my study duration, but also in the rest of my life with great patience.

Curriculum Vitae

Personal Data

Name	Nicole Schilcher
Date of birth	08.02.1988
Place of birth	St. Pölten
Nationality	Austria

Educational Data

1994-1998	primary school
	Privatvolksschule der Englischen Fräulein St. Pölten
1998-2006	gymnasium
	Privatgymnasium der Englischen Fräulein St. Pölten

Study

10/2006-2013	Diploma study in Pharmacy
	University of Vienna
03/2013-06/2013	Diploma thesis (Department of Pharmacognosy)
	University of Vienna