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Influence of medicinal plants on vascular smooth muscle cell proliferation

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Peucedanum Ostruthium

Picture taken from [1]

gewidmet meiner Familie

ABSTRACT

Atherosclerosis is the underlying cause of the diseases responsible for the most deaths worldwide, including ischemic heart disease and cerebrovascular disease. The accumulation of vascular smooth muscle cells (VSMC) within the intima of the arterial walls and the increase in extracellular matrix they produce, contributes to vessel narrowing in the pathogenesis of atherosclerosis and restenosis. One strategy for the treatment of vascular disease is to target cell cycle regulation of smooth muscle cells. The primary aim of this work is the identification and characterization of compounds which derive from natural sources and are capable to inhibit VSMC proliferation. This work was done as part of the national research network 'drugs from nature targeting inflammation (DNTI)', project 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 plants were selected either by a computational approach using pharmacophore models of relevant target proteins or an ethnopharmacological approach using traditional knowledge. The screening of about 150 different plant extracts via the resazurin conversion assay yielded several interesting candidate plant extracts, which inhibited the growth of VSMC, including *Sideritis hyssopifolia, Aristolochia debilis, Stephania tetrandra, Melia toosendan* and *Peucedanum ostruthium*. Furthermore, in a parallel study, a comprehensive qualitative and quantitative analysis of the main coumarins in extracts from *P. ostruthium* was performed by our collaboration partners at the University of Vienna and led to the identification of 7 major coumarins in those extracts. These coumarins were further forwarded to our group and testing via the resazurin conversion method revealed that the active principle inhibiting the growth of VSMC is ostruthin. The investigation of structurally similar linear furanocoumarins led to the conclusion that the geranyl group at the C-6 position is necessary for the inhibitory effect. Thus, we identified the anti-proliferative principle of the *Peucedanum ostruthium* extract and uncovered a novel bioactivity for ostruthin.

Zusammenfassung:

Arteriosklerose ist die zugrundeliegende Ursache für Erkrankungen, die weltweit die meisten Todesfälle verursachen. Zu diesen zählen koronare Herzkrankheiten und Schlaganfälle. Die Akkumulation und Einwanderung von vaskulären glatten Muskelzellen (VSMC) in die Intima, sowie deren starke Produktion an extrazellulärer Matrix trägt zum Prozess der Angiostenose (Gefäßverengung) bei. Eine Strategie zur Entwicklung neuer Medikamente und Therapien ist die Zellzyklusregulation von glatten Muskelzellen.

Das primäre Ziel dieser Arbeit war, die Identifizierung und Charakterisierung von Naturstoffen, die die Proliferation von VSMC inhibieren. Diese Arbeit wurde als Teil des nationalen Forschungsnetzwerkes „Drugs from Nature Targeting Inflammation“ (DNTI), welches vom FWF (Wissenschaftsfond) finanziert wird, durchgeführt. Dieses interdisziplinäre Netzwerk verbindet das Wissen und Können verschiedener Bereiche aus Pharmazie, Chemie, Veterinär- und Humanmedizin und verknüpft Universitäten in ganz Österreich. Im Zuge des Projektes wurden Pflanzen einerseits durch „Pharmacophore modelling“, also durch einen computergestützten Ansatz ausgewählt. Andererseits basierte die Auswahl auf einem ethnopharmakologischen Ansatz, welcher auf der Auswertung traditionellen Wissens beruht. Das Screening von 150 verschiedenen Pflanzenextrakten mittels ‚Resazurin Conversion assay‘ ergab einige interessante Ergebnisse. Folgende Pflanzenextrakte konnten das VSMC Wachstum hemmen; *Sideritis hyssopifolia, Aristolochia debilis, Stephania tetrandra, Melia toosendan* und *Peucedanum ostruthium.*

In einer parallelen Studie wurde von unseren Partnern eine quantitative und qualitative Analyse der Cumarine von *Peucedanum ostruthium* durchgeführt und die 7 bedeutendsten Cumarine identifiziert. Diese Cumarine wurden uns zur Verfügung gestellt und via Resazurin Assay überprüft. Dabei wurde Ostruthin als die aktive Substanz identifiziert. Die Untersuchung von strutkurell ähnlichen Furanocumarinen führte zum Schluss dass die Geranyl–Gruppe an der C-6 Position für den inhibierenden Effekt notwendig ist.

Somit konnten wir die antiproliferative Wirkung des *Peucedanum ostruthium* Extraktes identifizieren und eine neue Bioaktivität von Ostruthin enthüllen.

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B INTRODUCTION

1. Aim of this work

Atherosclerosis is the underlying cause of the top killing diseases worldwide, including ischemic heart disease and cerebrovascular disease [10]. Atherosclerosis involves multiple processes including endothelial dysfunction, matrix alterations, inflammation, and excessive vascular smooth muscle cell (VSMC) intimal migration and proliferation. An emerging strategy to develop means for the treatment of vascular disease is to target cell cycle regulation of smooth muscle cells [11-12]. Therefore, the identification of new compounds inhibiting VSMC proliferation is highly relevant, and in this work extracts from plants used in traditional medicine and known for their anti-inflammatory effects were examined for their putative antiproliferative action on VSMC.

2. Atherosclerosis

Progress in atherosclerosis research has been hindered due to the complexity of its genetic, biochemical, and environmental contribution factors. Nevertheless the development of new methods and techniques during the last three decades, including advances in cell culture, resulted in a better understanding of the underlying molecular mechanisms [6].

Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in the large arteries. The first observable change in the artery wall is the accumulation of lipoprotein particles and their aggregates in the intima layer. Subsequent recruitment of monocytes which migrate into the intima lead to the formation of sub-endothelial, macrophages called ‘foam cells’. The death of the ‘foam cells’ by apoptosis and necrosis contributes to the formation of a necrotic core of the atherosclerotic plaque [13].

Immune activation is ongoing in atherosclerotic lesions, triggering a fibroproliferative response mediated by intimal smooth muscle cells [13-15]. Over the years, this response may become so voluminous that vessel lumen is getting greatly diminished, blood flow is reduced, and ischemia sets in [13]. With disease progression, layers of fibrous connective tissue can replace the intima. The most advanced stages of atherosclerosis are characterized

with fissures, hematoma and thrombi, in the worst case resulting in stroke or myocardial infarction [16-17].

2.1. The structure of the arterial wall

The walls of blood vessels are mainly composed of endothelial cells, smooth muscle cells, and extracellular matrix (ECM), including elastin, collagen, and glycosaminoglycans. The vessel wall is comprised of three different layers called intima, media and adventitia (Figure B1), which are best distinguishable in arteries. The normal intima of large arteries has a continued endothelial monolayer seated on a basement membrane. The intima is separated from the media by the internal elastic lamina. The normal media mainly contains contractile VSMC surrounded by their own basement membrane [18]. These cells receive oxygen and nutrients, by direct diffusion from the vessel lumen, facilitated by holes in the internal elastic membrane [19].

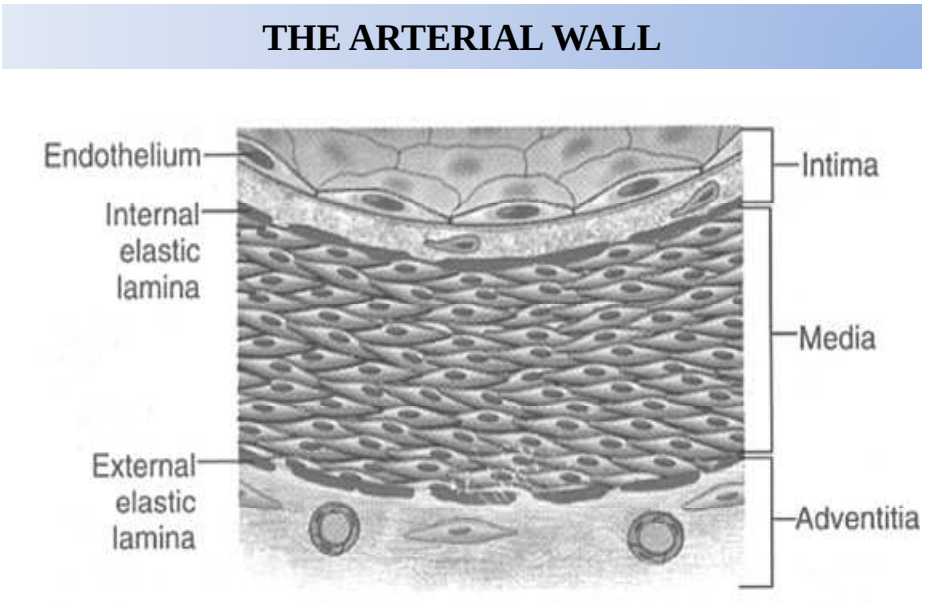


Figure B1 The arterial wall

Picture adapted from [4]

2.2. Triggers of atherosclerosis

Atherosclerotic lesions begin to develop under a damaged, dysfunctional endothelium, accompanied with changes in vasoconstriction, enhanced procoagulation, and enhanced leukocyte adhesion. The main triggers are a high saturated-fat diet, smoking, hypertension, obesity and insulin resistance [20-21]. Furthermore, a role for bacterial antigens and viral infection has been reported by various sources [22-25]. Additionally, a correlation between genetics and the severity of atherosclerotic lesions can be observed as well [26]. However, it is important to state that not one particular trigger alone but several events over a long time period, cause changes that can no longer be controlled nor repaired (see Figure B2).

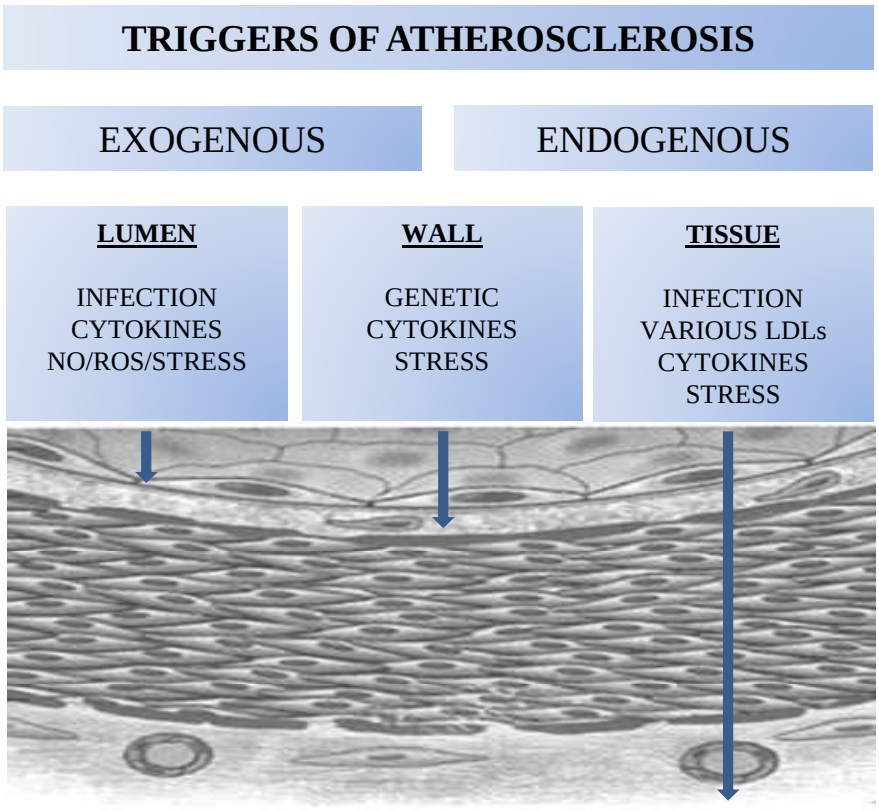


Figure B2: Triggers of Atherosclerosis

Adapted from [4], and [5]

2.3. Early phases of atherosclerosis

2.3.1. From lesion initiation to fatty streak formation

LDL enters the vessel wall by passive diffusion, and is retained in the vessel wall [27]. The endothelial cells in regions of arterial branching have polygonal shapes and no particular orientation. These latter areas show increased permeability to macromolecules such as low density lipoprotein (LDL) and are preferential sites for lesion formation [28]. Inside the intima the accumulation of lipids leads to the development of lipoprotein aggregates (see Figure B3) [6]. Furthermore, the modification of LDL contributes to the development of inflammation. These modifications could be mainly mediated by the uncoupled nitric oxide synthase (NOS), myeloperoxidase, xanthine oxidase, lipid oxygenase and NADPH oxidases [29-30]. Oxidative modifications seem to play a role, since oxidized low density lipoprotein (oxLDL) is not bound by the LDL receptor, but rather by so called scavenger receptors, expressed by macrophages and VSMC [31]. Raised oxLDL levels contribute to the expression of increased levels of vascular cell adhesion molecule 1 (VCAM-1), and other adhesion molecules, like E-selectin, P-selectin, and inter-cellular adhesion molecule 1 (ICAM-1) [9, 20]. Furthermore LDL regulates production of cytokines, such as interleukin 6 (IL-6) and growth factors, such as monocyte chemotactic protein MCP-1 [32-33]. As a consequence, patrolling monocytes enter the vessel wall by diapedesis between endothelial cells (EC) [34]. Subsequently, intimal monocytes mature into macrophages, express increased levels of scavenger receptors and engulf oxLD [20].

The incorporated cholesterol has a number of potential metabolic fates. Its esterification by acetyl-CoA acetyltransferase (ACAT-1) and the subsequent storage in lipid droplets leads to the formation of so called ‘foam cells’ (see Figure B3). The cells answer to excess intracellular cholesterol is the inhibition of the sterol regulatory element-binding protein (SREBP) transcription factors. These are required for cholesterol biosynthesis and LDL receptor expression [13, 35]. Unfortunately, cholesterol uptake via scavenger receptors is not altered. Therefore cholesterol efflux mechanisms are of great importance. The main ‘reverse cholesterol transport’ is likely to be via membrane transporters, with HDL serving as the

primary extracellular acceptor. Nevertheless, this so called 'HDL hypothesis' is still under debate [36].

Lipid-laden macrophages multiply and release several growth factors and cytokines, thus amplifying and sustaining inflammatory signals. VCAM-1, MCP-1, and macrophage colony-stimulating factor (M-CSF) appear to be the main mediators in the initiation and development of the initial lesion of atherosclerosis, the fatty streak [20].

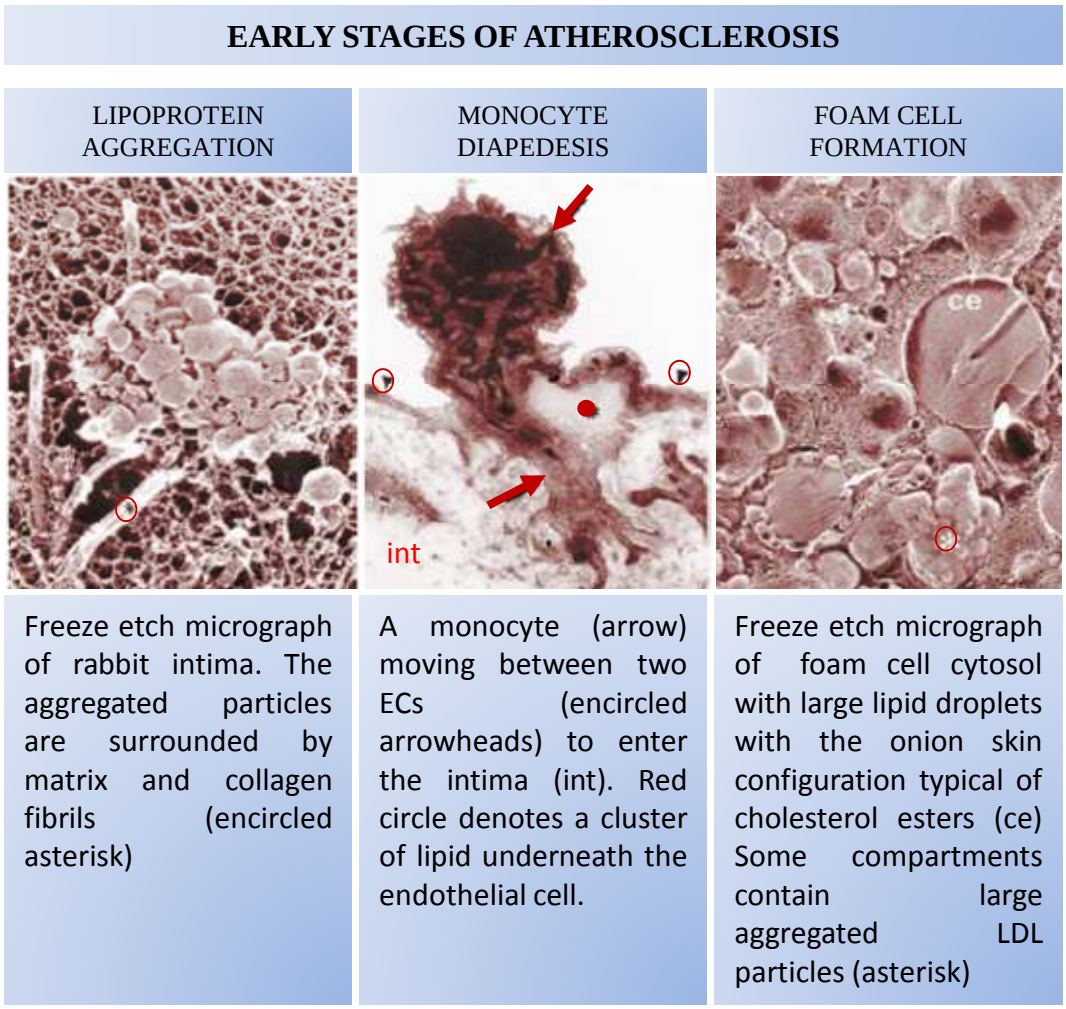


Figure B3: Early stages of atherosclerosis

Pictures and text adapted from Lusis [6]

2.4. Later phases of atherosclerosis

2.4.1. *Progression to complex plaque*

In disease progression, the inflammatory response is joined by a fibroproliferative response mediated by VSMC. These cells migrate from the medial layer of the artery wall, through the internal elastic lamina into the intima. They proliferate and synthesize excess ECM proteins. Furthermore they take up oxLDL, thereby contributing to foam cell formation [37]. In the center of an atheroma, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of smooth muscle cells and a collagen rich matrix [9]. Fibrous plaques are characterized by a growing mass of extracellular lipid, mostly cholesterol and by the accumulation of VSMC and extracellular matrix. Similarly, macrophages, EC, and VSMC appear to be activated based on their expression of numerous inflammatory products, such as tumor necrosis factor α (TNF-α), IL-6, and MCP-1. Thus, immune activation is ongoing in atherosclerotic lesions [9, 38]. During the progression of atherosclerosis, endothelial cells, foam cells, and smooth muscle cells die by apoptosis or necrosis. This leads to the formation of a lipid-rich core, totally devoid of supporting collagen, and a fragile and rupture prone fibrous cap [13].

2.4.2. *From fibrous lesion to plaque rupture and thrombosis*

High-risk plaques are characterized by angiogenesis [13]. This so called remodeling of the vessel may preserve the lumen and counteract intimal thickening. However, if the lesion grows faster than the remodeling process occurs, luminal stenosis develops [39]. The constant remodeling of the arterial wall is associated with the development of large plaques. Physical disruption of the atherosclerotic plaque, that ordinary protects the blood from the contact with the lipid core, is called plaque rupture [40]. Such events are particularly frequent in acute coronary syndromes. The rupture causes hemorrhage into the plaque, further luminal narrowing and the development of thrombi [41]. These may dispatch from the initial site of the lesion, flow with the blood stream and occlude downstream vessels with smaller diameter, leading to myocardial infarction (see Figure B4) [39]. Nevertheless

the disease does not always cause death. The development of so called 'buried plaques' involve again the accumulation of VSMC. On the one hand this leads to the prevention of the risk of plaque rupture, but on the other hand it contributes to stenosis and the development of angina [39].

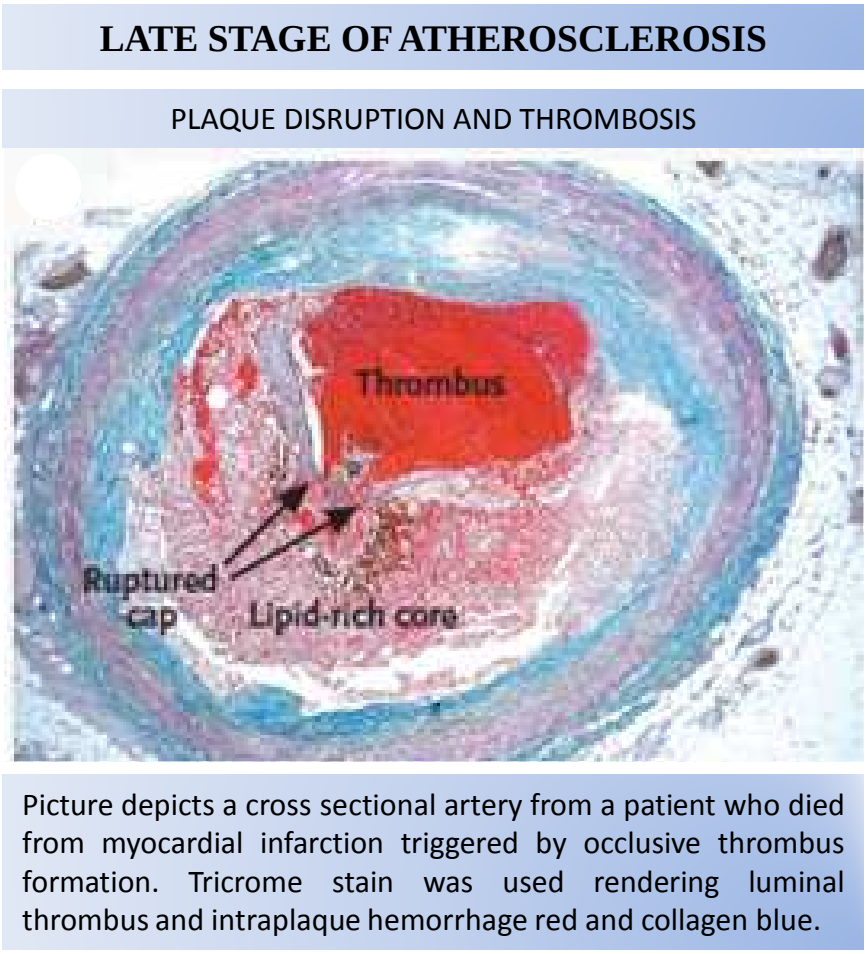


Figure B4: Late stage of atherosclerosis

Picture adapted from Hansson [9]

3. The role of VSMC on the pathogenesis of atherosclerosis

3.1. VSMC phenotype switching and motility

Medial VSMC are responsible for vasoconstriction and dilation in response to normal stimuli. Therefore the main part of the medial VSMC exhibit a contractile phenotype, producing low levels of ECM and increased intracellular myofilament [19]. Furthermore the normal adult artery shows very low apoptotic and mitotic indices.

Local factors, such as inflammatory cells, cytokines, oxLDL and systemic factors including hyperglycemia, blood pressure and flow, substantially alter the normal balance of proliferation [42]. Under pathological conditions, VSMC are able to undergo a reverse phenotypic shift [43]. The resulting VSMC exhibit a synthetic, proliferative state and are able to migrate from the media to intimal regions. Since not all fatty streaks progress to form complex atheroma, the arrival of VSMC and their production of ECM probably provide the transition towards a complex lesion [19].

In the native vessel, VSMC are surrounded by matrix molecules. These are important in maintaining tissue structure and play key roles in guiding cell functions [44]. Matrix metalloproteinases (MMP) are believed to play an important role to catalyze the removal of the basement membrane around VSMC and facilitate migration of the cells [45]. The migratory and proliferative activities of VSMC are regulated by growth promoters and inhibitors. Several growth factors and cytokines have been implicated in this regulation, including platelet derived growth factor (PDGF), transforming growth factor β (TGF-β), fibroblast growth factor (FGF), and proinflammatory cytokines such as TNF-α, IL-1, and IL-6 [5, 46].

The process of phenotype switching is very complex and involves the less investigated production of pro-proliferative enzymes and proteins necessary for cell migration [47].

The expression of specific cell-surface receptors and the binding to extracellular ligands stimulate VSMC migration and proliferation [48]. The movement of VSMC involves a plasma lamella which binds to extracellular substrates. Myosin contractions propel the cell forward in the direction of this anchoring leading edge. Furthermore the formation of a fingerlike

structure called podosome which consists mainly of actin filaments, is necessary for the remodeling of ECM [49]. The cells always move in the direction of a chemoattractant [50-51]. Diverse signal transduction systems, such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) pathways have been proposed to translate these mitogenic stimuli within VSMC [52-54].

3.2. Types of VSMC and VSMC growth

3.2.1. Heterogeneity of VSMC

It has become clear that VSMC in the vessel wall are phenotypically and functionally heterogeneous, influenced by both, developmental and environmental factors. At least four cell subpopulations differing in morphological appearance, expression pattern and growth capabilities were found investigating rat media [43]. Similar heterogeneity was observed for human VSMC [55]. As stated above two morphologic phenotypes are usually defined, namely the epithelioid (synthetic) and the spindle shaped cell (contractile) [43]. Contractile VSMC express high levels of contractile proteins, such as myosin, and do not proliferate. In contrast, synthetic VSMC produce high levels of α-Actin and extracellular matrix proteins, and are able to proliferate. In atherogenesis, intimal thickening is accompanied with the reexpression of fetal VSMC genes, suggesting that there is significant plasticity in VSMC function [56].

3.2.2. Hypertrophy and hyperplasia

Hypertrophy refers to an increase in smooth muscle cell size. It is a reversible mechanism when unaccompanied by endoreduplication. Increases in cell volume are a consequence of both, increased intracellular protein and intracellular water, which occur as a consequence of stimulation of protein synthesis and inhibition of protein degradation [56].

Hyperplasia refers to an increase in VSMC number. Hyperplasia is also a common component of hypertension, and is associated with many other vascular diseases like atherosclerosis, restenosis, and the response to vascular injury [56].

3.3. Activation of VSMC

The activation of VSMC is dependent on a complex signaling network of different growth factors and include tyrosine kinase receptors and secreted factors like PDGF, secreted factors coupled to G-protein receptors like angiotensin II , and other secreted molecules like transforming growth factor β (TGF β) [56].

Furthermore it includes the response to arterial injury and oxidative stress.

3.3.1. Platelet derived growth factor (PDGF)

Platelet derived growth factor (PDGF) was first isolated from serum and it was proofed to stimulate the proliferation of VSMC [57]. It plays a pivotal role in the complex pattern of growth factor signaling and probably is the most important chemoattractant and mitogen for VSMC [56-57].

It exists as a dimer composed of two of four homologous but distinct peptides termed PDGF-A, PDGF-B, PDGF-C and PDGF-D. [58-59]. The latter proteins bind to two related PDGF receptors, PDGFR-α and PDGFR-β. The most important roles for VSMC activation are thought to be via the PDGF-B and PDGFR-β. The activation of the receptor leads to the induction of cellular proliferation, chemotaxis and actin reorganization [59].

In the healthy media, contractile VSMC produce nearly undetectable amounts of PDGF and PDGFR [56-57]. Nevertheless recent studies showed that intimal VSMC may exhibit a different expression with high levels of PDGF-B [57, 60].Additionally, the pharmacological blockage of PDGF-B resulted in a diminished growth response of VSMC in diseased vessels [59]. In summary, PDGF is a critical growth factor for VSMC mediating hyperplasia, hypertrophy, and remodeling [56].

3.3.2. *ROS*

Oxidative stress in cardiovascular biology was once considered only in terms of injury, damage, and dysfunction [61]. However, it became clear that ROS are playing an important role in the increase of cell proliferation [62-63].

The predominant enzyme sytems that generate ROS in VSMC, are the NADPH oxidases (Nox) and the inducible nitrite oxidase (iNOS) [64]. VSMC contain numerous sources of ROS with the major forms being superoxide anion ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2).

Low levels of ROS lead to increased expression and secretion of growth factor. ROS have many different effects depending on ease of transit across cell membranes, local concentration, and duration of exposure.

In conclusion, ROS are powerful intra and extracellular mediators of VSMC growth in response to a variety of growth factors [63].

4. Cell cycle control in atherosclerosis

Mitogenic growth factor signaling ultimately induces regulatory mechanisms that are of relevance for cell division and modulate the cell cycle [11]. After differentiation, VSMC enter and stay in a growth arrested state named G_0 (gap 0) phase of the cell cycle. As already stated above, VSMC are able to dedifferentiate and re-enter the cell cycle [65]. During gap period 1 (G_1), necessary factors for proliferation are assembled. By passing the G_1/S checkpoint the cell enters the synthetic (S) phase. As soon as DNA replication is completed, the cell enters gap phase 2 (G_2). By passing G_2/M checkpoint, the cell enters mitotic phase (M phase). Finally the cell divides. Central role in the cell cycle control system plays a family of protein kinases known as cyclin-dependent kinases (CDK). The activity of these kinases rises and falls as the cell progresses through the cycle. Cyclical changes in CDK activity are controlled by a complex array of enzymes and other proteins. CDK, as their name implies, are inactive unless they are tightly bound to proteins called cyclins [66]. Further regulation is achieved by phosphorylation/dephosphorylation cycles and so called CDK inhibitory proteins (CKI). Phase specific cyclin-CDK complexes confer specificity and orderly progression through

the cell cycle. The CKI are divided into two distinct families. The CDK interacting protein/kinase inactivating protein (Cip/Kip) family inhibits a wide spectrum of CDK/cyclin complexes including p21 and p27 [12, 67].

In addition, cell cycle progression is modulated by transcription factors that transactivate CDKs and CKIs like the E2F family of transcription factors and p53 [68].

Since proliferation of VSMC is an essential hallmark in the development of atherosclerosis, investigation of proteins associated with cell cycle control and further pharmacological studies to develop new cytostatic drugs are warranted, and hopefully will lead to improved treatment options in the foreseeable future.

4.1. Role of selected VSMC cycle regulators in the pathogenesis of atherosclerosis

4.1.1. Cyclin D1

Cyclin D1 serves as a key sensor and integrator of extracellular signals of cells in early to mid-G_1 phase [69]. The binding to CDK 4 and 6 forms a kinase complex which is able to phosphorylate Rb [70] thereby ending the active repression of E2F transcriptional activity and promoting the transition from the G_1 to the S-phase of the cell cycle [69].

4.1.2. Retinoblastoma protein (Rb)

Hypophosphorylated Rb induces cell cycle arrest in G_1 by active repression of transcription through the formation of the Rb-E2F complex. In proliferating cells, CDK/cyclin activation causes the accumulation of hyperphosphorylated Rb protein, and thus the release of E2F. This leads to the transactivation of various target genes necessary for cell cycle progression, such as those encoding cyclin E, cyclin A and CDK1. Loss of function studies revealed that Rb deficiency triggers p53-dependent apoptosis and results in embryonic lethality [71]. Furthermore Rb modulates VSMC proliferation senescence and apoptosis [72].

4.1.3. *p27*

P27 contributes to growth arrest through the inhibition of cyclin-CDK complexes. It is a tumour suppressor, whose inactivation in mice led to the development of larger lesions with increased VSMC and macrophage proliferation. Furthermore its inactivation led to the upregulation of proinflammatory cytokines underlining its potential atheroprotective role [73].

4.1.4. *p53*

The tumor suppressor p53 is a central regulator of the cell cycle and influences the transcription of over 150 genes [74]. P53 becomes activated by stress signals including oxidative stress and mechanical injury. Activation of p53 may either lead to reversible cell cycle arrest, during the G_1- or G_2-phase of the cell cycle or induce apoptosis [75]. Furthermore recent studies connect its activity to the formation of the podosome necessary for VSMC migration [49].

The role of P53 in atherogenesis was first demonstrated by its inactivation in apoE-null mice. The latter study demonstrated that accelerated atheroma development, coincided with increased cell proliferation [12, 76]. However, transfection of an extra allele of p53 didn't show any effect on native atherosclerosis, but it reduced neointimal thickening induced by mechanical injury [77]. Furthermore, effects on VSMC apoptosis were not observed considering several studies, using such mouse models [78-80].

4.1.5. *P21*

P21 is a major downstream target of p53 and mediates cycle arrest in response to DNA damage [81]. Nevertheless, a certain amount of p21 is required for normal cell cycle progression. It stabilizes and promotes active cyclin-CDK complex formation in a dose-

dependent manner [3]. However binding of high amounts of p21 to cyclin-Cdk complexes inhibits RB phosphorylation and induces cell cycle arrest in G_1 phase [82].

Furthermore p21 is able to block the ability of proliferating cell nuclear antigen (PCNA) to bind polymerase δ (polδ) by competing on the same binding site, leading to a DNA replication block and an intra-S-phase arrest [3]. This suggests that p53 and p21 could play an important role in restenosis after stenting [83].

5. New perspectives on atherosclerosis and restenosis treatment

Nowadays, the standard blood flow restoring strategies include percutaneous transluminal coronary angioplasty (PTCA), intracoronary stent introduction, and coronary artery bypass surgery [84]. Restenosis is arbitrarily defined as a greater than 50% narrowing of vessel diameter after successful surgical treatment and remains a significant problem in interventional cardiology [85]. Restenosis is mostly caused by two major processes: intimal hyperplasia and constrictive remodeling of the arterial wall. These processes are probably due to an over exaggerated healing response, triggered by the surgical procedure. The development of drug eluting stents (DES) reduced the rates of restenosis and target lesion revascularisation [86]. However, in stent restenosis after DES placement still exists as a problem and current DES have failed to demonstrate superiority over bare metal stents for the femoropopliteal segment. [87] Furthermore, the promising initial results led to the rapid adoption and wide spread use of DES even in more complex patients and lesions [88-89]. However, following studies revealed restenosis rates re-increasing to the double-digit level [86]. The precise reason for the late increase in neointimal hyperplasia in DES is still unclear, but it may be related to a delayed healing response, persistent biological reaction caused by the drug after implantation, or a hypersensitivity reaction to the durable polymer used in DES [86].

5.1. Standard procedures of interventional cardiology

5.1.1. PTCA & stenting

Angioplasty is performed to open a narrowed artery, in which a balloon-tipped catheter is inserted into an artery and threaded to the affected part. A stent is stainless tube with slots. It is mounted on a balloon catheter in a collapsed state. When the balloon is inflated, the stent opens up and pushes itself against the inner wall of the artery. This holds the artery open when the balloon is deflated and removed (see Figure B5). Nowadays a broad variety of balloons and stents are used ranging from the simple bare metal stent to complex DES or biodegradable stents [90-91].

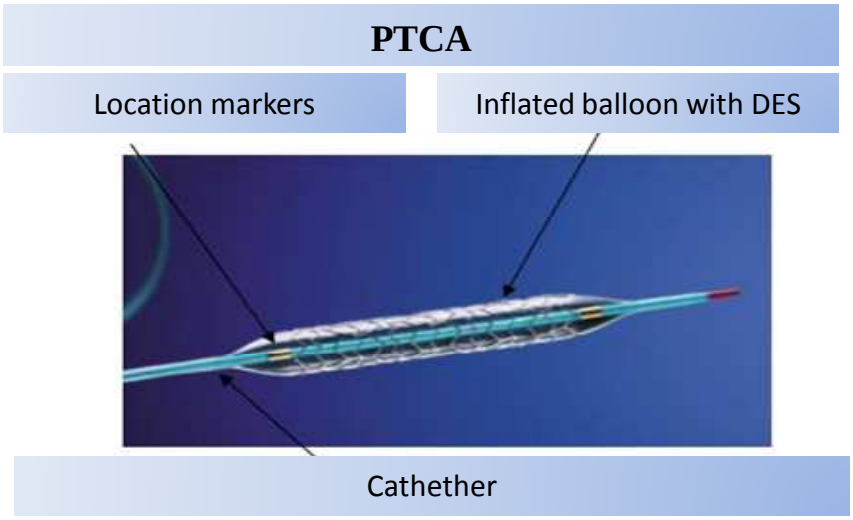


Figure B7: PTCA

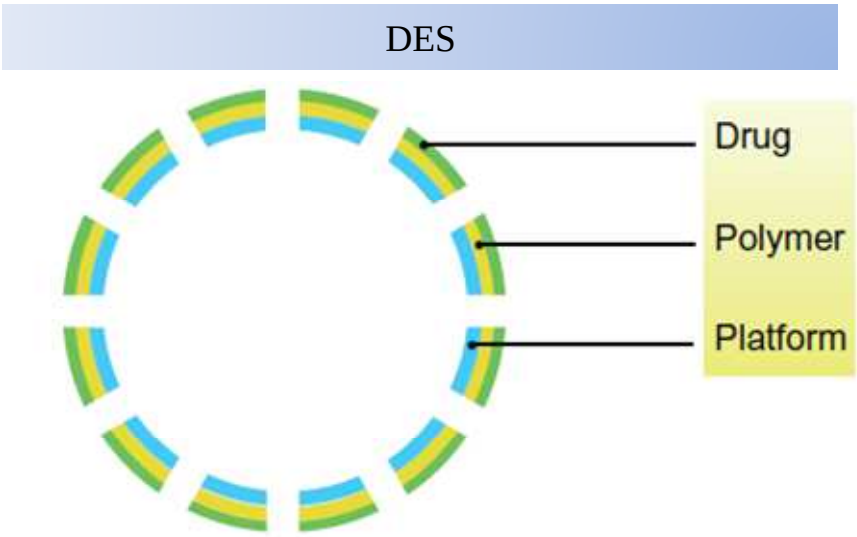
TAXUS Express Paclitaxel-Eluting Coronary Stent System, Taken from [2]

5.1.2. Drug eluting stents

A drug eluting stent is a peripheral or coronary stent placed into narrowed, diseased arteries that slowly releases a drug with growth inhibitory or cytotoxic properties. Ideally a smooth, thin layer of endothelial cells grows over the stent and the device is incorporated into the

artery, reducing the tendency for clotting [92-93]. Standard DES consist of three different elements, namely the drug, the polymer and the stent platform (see Figure B5) [7]. The most used drugs nowadays include sirolimus and paclitaxel.

Sirolimus and its analogs (e.g. zotarolimus, everolimus, biolimus) have a cytostatic effect. They inhibit mammalian target of rapamycin (mTor) and thereby suppress VSMC migration and proliferation by arresting the cell cycle in G_1-phase [94]. Paclitaxel, another drug used in DES, has a cytotoxic effect. It inhibits mitosis by preventing microtubule depolymerisation and thereby induces apoptosis [94].



Schematic representation of the structure of a DES

Picture adapted from [7]

6. Samples

This section describes the origin of the tested plant extracts, most of which were selected within the DNTI (drugs from nature targeting inflammation) project. Full list of all samples tested in this work is presented in Figure C3.

6.1. Plants selected within the DNTI project

6.1.1. Plants derived from TCM

The group of Prof. Bauer at the TCM Research Center Graz selected about 100 plant species, which have been used in TCM for anti-inflammatory purposes. These were selected based on the information in text books and databases. A part of the plant material was also selected by existing information on constituents available from a search in Chemical Abstracts and Medline. Plants without known active compounds were preferred. Plant material was either provided from reliable commercial suppliers in Europe (Plantasia; Chinamed; Sinomed; Herbasin; Complemedis) or through collaborative partners in China (Institute of Botany, Kunming; Institute of Medicinal Plant Development, Beijing; Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing; Shanghai Research Center for Modernization of Traditional Chinese Medicine, Shanghai). Some of the plant material was also available from a collaborative project on Chinese medicinal plants between the group of Prof. Bauer and Joanneum Research, Graz [95].

6.1.2. Plants derived from the VOLKSMED database

The group of Prof. Brigitte Kopp at the Department of Pharmacognosy, University of Vienna, selected plants from the VOLKSMED database. This database, founded twelve years ago was created to collect data about natural medicinal products, used traditionally in rural and alpine regions of Austria and South Tyrol. In this way a great deal of data was compiled containing the exact botanical description of the plants and the corresponding indication and preparation forms. Possible candidate plants retrieved from this database were extracted with different solvents and forwarded to different DNTI collaboration groups for pharmacological investigation utilizing cell-based assays. Afterwards active plant extracts are further fractionated using a bioassay-guided approach to identify the active compounds [3].

6.1.3. *Plants derived from in silico methods*

The groups of Dr. Schuster and Prof. Stuppner at the University of Innsbruck (Institute of Pharmacy) are dedicated to the target oriented search of bioactive plant constituents using modern in silico tools (pharmacophore based virtual screening) and supplied us with part of the tested plant samples (Figure C3) [96]. The first step in pharmacophore modeling is to predict the binding of a compound to a biological target, which is achieved by the application of three-dimensional space models using the state-of-the-art software Ligand Scout [97]. If no experimental 3D structures are available, special algorithms are used for a flexible ligand-based pharmacophore modeling. 3D compound libraries are used for the data mining process. Three different 3D molecule databases are used: The database DIOS consists of 2752 compounds from phytochemical reports of anti-inflammatory medicinal plants described by the ethnopharmacological source '*De material medica*' of Pedanius Dioscorides. NPD contains almost 80 000 compounds gathered arbitrarily from natural sources [98]. The third multiconformational database derives from the TCM, with information of more than 10.000 metabolites [99]. These libraries are virtually screened using the generated pharmacophore models as tools for the identification of promising structures. Afterwards the compounds of interest are computationally investigated regarding their chemical stability and the opportunity of chemical modifications. Subsequently, refinement procedures such as parallel in silico pharmacophore-based counter-screening are used to predict unwanted secondary effects [100]. The final selection of hits was done estimating the interaction energies of the target-ligand complexes. Finally the compounds are ranked using criteria like, chemical stability, non toxicity, drug likeness, accessibility, and known anti-inflammatory action. Subsequently extracts are prepared from medicinal plants that are known to contain the compounds of interest and these extracts are forwarded to different DNTI collaboration groups for pharmacological evaluation.

6.2. Plants which were not part of the DNTI project

Furthermore several plants derived from the TCM and from the VOLKSMED database were analyzed as a part of ongoing projects outside of the DNTI.

6.2.1. *Plants deriving from the TCM*

Extracts and fractions from *Arisaema, Albizia* and *Pinellia* are known for their anti-inflammatory effects and were provided by the working group of Prof. Kopp, Department of Pharmacognosy, University of Vienna (Figure C3).

6.2.2. *Plants deriving from the VOLKSMED database*

6.2.2.1. *Dryopteris filix-mas*

Male Fern (*Dryopteris filix-mas*) is indicated as one of the plants with highest scores of usage as anti-inflammatory remedy in the VOLKSMED database, however, there are no scientific records explaining such usage. Furthermore, very promising preliminary results indicating an anti-inflammatory activity of *Dryopteris filix-mas* extracts in vivo as well as in vitro were obtained, validating the traditional ethnopharmacological usage. Therefore plant extracts provided by the working group of Prof. Reznicek, Department of Pharmacognosy, University of Vienna were also investigated in this work.

6.2.3. *Coffea arabica*

Coffee bean (*Coffea arabica*) was selected based on literature reports and extracts were provided by Prof. Reznicek, Department of Pharmacognosy, University of Vienna. The unique taste and smell of coffee derives from the interplay of over a thousand different compounds. Interesting compounds possibly targeting CVD are the caffeine, the diterpene alcohols cafestol and kahweol, chlorogenic acid, and other polyphenols [101]. The most promising substances with health promoting action in coffee are the polyphenols which can bind to

lower density lipoproteins, protect them from oxidation and thus be heart protective antioxidants [102]. The major polyphenol in coffee is chlorogenic acid and it is found to be the most powerful antioxidant of coffee [103]. In cellular studies, it was found that caffeine metabolites are inhibitors of the enzyme poly (ADP ribose) polymerase-1 in hydrogen peroxide-treated epithelial cells at physiological concentrations [104]. This might represent an anti-inflammatory action of caffeine. However, a meta analysis of well-conducted clinical trials of coffee or caffeine concluded increase in blood presure [105]. From the data presented, it can be concluded that only heavy consumption of coffee is harmful to the heart. Overall epidemiological studies show that the moderate consumption of coffee appears to confer cardiovascular benefit, with polyphenols present in filtered as well as unfiltered coffee having potential cardiovascular benefit [101].

C RESULTS

1. Screening plant extracts for inhibition of VSMC proliferation

1.1. Phenotypic determination of VSMC

VSMC from thoracic aorta Fischer rats, provided by Nanang Fakhrudin and Helge Joa (Department of Pharmacognosy, University of Vienna), were used for the main screen. Procedures were conducted as described in the materials and methods section. To avoid false data due to contamination the cells were consistently checked under the light microscope. Furthermore, identity of the cells was confirmed by fluorescence microscopy utilizing staining with smooth muscle α-actin antibody (Figure C1). No contaminations by other cell types or bacteria could be detected.

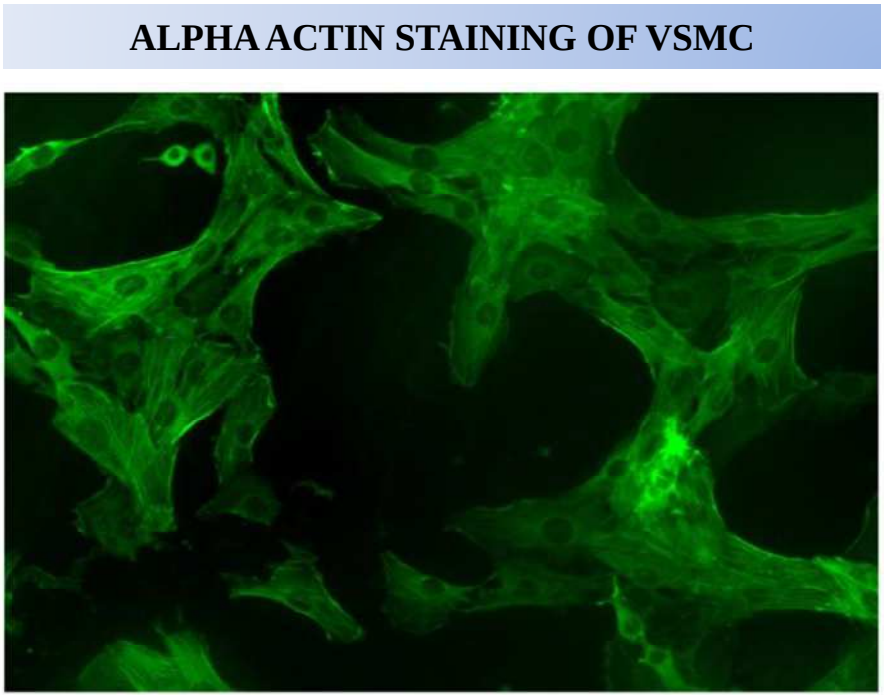


Figure C1: Alpha actin staining of VSMC

Photograph of rat VSMC stained with smoth muscle alpha-actin antibody. Data kindly provided by Helge Joa [3]

1.2. Resazurin conversion assay set up

The Resazurin conversion assay was performed in a 96 well plate set up. Figure C2 illustrates the pipetting scheme used for screening. All plant extracts were tested in a final concentration of 10 µg/ml. Within each 96-well plate sixteen samples were tested in quadruplicates. The stock solutions of all tested samples were prepared in DMSO. Therefore, to exclude adverse or unexpected effects of the solvent an extra DMSO control was always utilized (Figure C2: Yellow wells). Empty wells were also measured and the resulting background signal was subtracted from all other values. Unless otherwise stated, experiments were repeated twice.

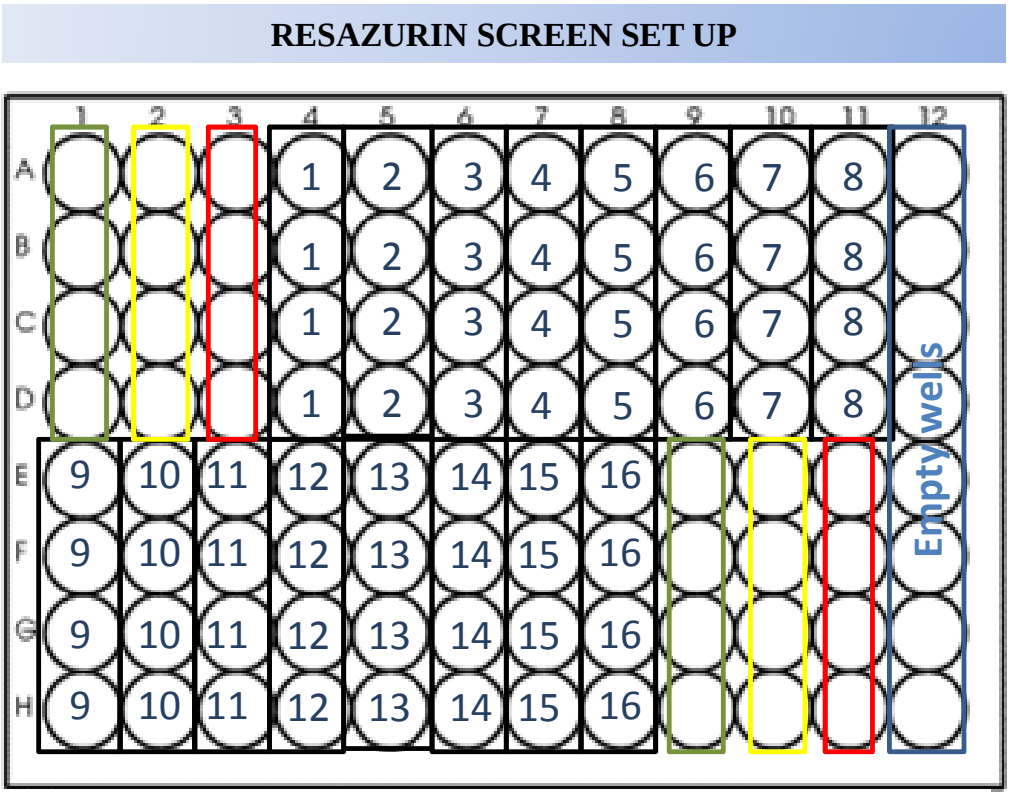


Figure C2: Resazurin Screen Set up

Green wells:	starved control	Yellow wells:	starved DMSO control
Red wells:	CS treated control	Blue wells:	empty control
Black wells:	CS treated samples 1-16		

1.3. Screening results

Figure C3 and C4 A-P show the list of the screened samples and the obtained experimental results. Each bar on Figure C4 represents one independent experiment performed in quadruplicate. 100% relative proliferation represents the relative fluorescence units (RFU) of the control treated with 10% calf serum, while 0% proliferation stands for RFU levels of the DMSO control. Except otherwise stated, all plant extracts were used in a final concentration of 10 µg/ml. Samples that induced more than 50% inhibition in the first two experiments were considered as antiproliferative. Samples which yielded RFU below the level of the DMSO control were considered as cytotoxic.

S Nr.	Plant/Extract	Solvent	Part used	Source	ACTIVITY on VSMC proliferation
56	*Poria cocos*	DCM	Radix	Prof. Bauer*	none
57	*Polygonum multiflorum*	DCM	Radix	Prof. Bauer	none
58	*Ophipogonis japonicus*	DCM	Radix	Prof. Bauer	none
59	*Pladticodon gradiflorum*	DCM	Radix	Prof. Bauer	none
60	*Bupleurum species*	DCM	Radix	Prof. Bauer	none
61	*Angelica sinensis*	DCM	Radix	Prof. Bauer	none
62	*Achyranthis bidentata*	DCM	Radix	Prof. Bauer	none
63	*Santalum album*	DCM	Lignum	Prof. Bauer	none
64	*Spatholobus suberrectus*	DCM	Caulis	Prof. Bauer	none
66	***Acorus tatarinowii***	**DCM**	**Rhizome**	**Prof. Bauer**	**increase**
68	*Gastrodia elata*	DCM	Rhizome	Prof. Bauer	none
69	*Prunus species*	DCM	Semen	Prof. Bauer	none
70	*Rhemania glutinosa*	DCM	Radix	Prof. Bauer	none
72	*Carthamus tinctorius*	DCM	Flos	Prof. Bauer	none
73	*Codonopsis species*	DCM	Radix	Prof. Bauer	none
74	*Polygonatum odoratum*	DCM	Radix	Prof. Bauer	none
75	*Ligusticum chuanxiong*	DCM	Rhizome	Prof. Bauer	none
76	*Crataegus pinnatifida*	DCM	Fructus	Prof. Bauer	none
77	*Astralagus mebranaceus*	DCM	Radix	Prof. Bauer	none
78	*Zingiberis officinalis*	DCM	Radix	Prof. Bauer	none
79	*Salvia militiorrhiza*	DCM	Radix	Prof. Bauer	none
80	*Paeonoa rubra*	DCM	Radix	Prof. Bauer	none
81	***Stephania tetrandra***	**DCM**	**Radix**	**Prof. Bauer**	**cytotoxic**
82	*Aconitum carmichaelii*	DCM	Radix	Prof. Bauer	none
84	*Curcurma species*	DCM	Radix	Prof. Bauer	none

85	*Corydalis yanhusuo*	DCM	Rhizome	Prof. Bauer	none
86	***Asari species***	**DCM**	**Radix + Rhizome**	**Prof. Bauer**	**minor decrease**
88	*Allium cacrostemon*	DCM	Bulbus	Prof. Bauer	none
89	*Trichosanthis species*	DCM	Radix	Prof. Bauer	none
90	*Notopterygii species*	DCM	Radix	Prof. Bauer	none
91	*Scutellaria baicalensis*	DCM	Radix	Prof. Bauer	none
92	*Ziziphus spinosa*	DCM	Semen	Prof. Bauer	none
94	*Schisandra chinesis*	DCM	Fructus	Prof. Bauer	none
98	*Panax notoginseng*	MeOH	Radix	Prof. Bauer	none
99	*Poria cocos*	MeOH	Radix	Prof. Bauer	none
100	*Polygonum multiflorum*	MeOH	Radix	Prof. Bauer	none
101	*Ophipogonis japonicus*	MeOH	Radix	Prof. Bauer	none
102	*Pladticodon gradiflorum*	MeOH	Radix	Prof. Bauer	none
103	***Bupleurum species***	**MeOH**	**Radix**	**Prof. Bauer**	**cytotoxic**
104	*Angelica sinensis*	MeOH	Radix	Prof. Bauer	none
105	*Achyranthis bidentata*	MeOH	Radix	Prof. Bauer	none
106	*Santalum album*	MeOH	Lignum	Prof. Bauer	none
107	*Spatholobus suberrectus*	MeOH	Caulis	Prof. Bauer	none
108	*Gardenia jasminoides*	MeOH	Fructus	Prof. Bauer	none
109	*Acorus tatarinowii*	MeOH	Rhizome	Prof. Bauer	none
110	*Leonurus japonicus*	MeOH	Fructus	Prof. Bauer	none
111	*Gastrodia elata*	MeOH	Rhizome	Prof. Bauer	none
112	***Prunus species***	**MeOH**	**Semen**	**Prof. Bauer**	**increase**
113	*Rhemania glutinosa*	MeOH	Radix	Prof. Bauer	none
114	*Forsythia suspensa*	MeOH	Fructus	Prof. Bauer	none
115	*Carthamus tinctorius*	MeOH	Flos	Prof. Bauer	none
116	*Codonopsis species*	MeOH	Radix	Prof. Bauer	none
117	*Polygonatum odoratum*	MeOH	Radix	Prof. Bauer	none

118	*Ligusticum chuanxiong*	MeOH	Rhizome	Prof. Bauer	none
119	*Crataegus pinnatifida*	MeOH	Fructus	Prof. Bauer	none
120	*Astralagus mebranaceus*	MeOH	Radix	Prof. Bauer	none
121	*Zingiberis officinalis*	MeOH	Radix	Prof. Bauer	none
122	*Salvia militiorrhiza*	MeOH	Radix	Prof. Bauer	none
123	*Paeonia rubra*	MeOH	Radix	Prof. Bauer	none
124	***Stephania tetrandra***	**MeOH**	**Radix**	**Prof. Bauer**	**inhibition**
125	*Aconitum carmichaelii*	MeOH	Radix	Prof. Bauer	none
126	*Akebia species*	MeOH	Caulis	Prof. Bauer	none
127	*Curcurma species*	MeOH	Radix	Prof. Bauer	none
128	*Corydalis yanhusuo*	MeOH	Rhizome	Prof. Bauer	none
129	*Asari spezies*	MeOH	Radix + Rhizome	Prof. Bauer	none
130	*Sargentodoxa cuneata*	MeOH	Caulis	Prof. Bauer	none
131	*Alllium macrostemon*	MeOH	Bulbus	Prof. Bauer	none
132	*Trichosanthis species*	MeOH	Radix	Prof. Bauer	none
133	*Notopterygii species*	MeOH	Radix + Rhizome	Prof. Bauer	none
134	*Scutellaria baicalensis*	MeOH	Radix	Prof. Bauer	none
135	*Ziziphus spinosa*	MeOH	Semen	Prof. Bauer	none
136	*Lonicera japonica*	MeOH	Caulis	Prof. Bauer	none
137	*Schisandra chinesis*	MeOH	Fructus	Prof. Bauer	none
138	*Artemisia scoparia*	MeOH	Herba	Prof. Bauer	none
139	*Buddleja officinalis*	MeOH	Flos	Prof. Bauer	none
581	*Chaenomelis specicosa*	DCM	Fructus	Prof. Bauer	none
582	*Chaenomelis specicosa*	MeOH	Fructus	Prof. Bauer	none
583	*Typhonium giganteum*	DCM	Rhizome	Prof. Bauer	none
584	*Typhonium giganteum*	MeOH	Rhizome	Prof. Bauer	none
585	***Melia toosendan***	**DCM**	**Fructus**	**Prof. Bauer**	**inhibition**

586	*Melia toosendan*	MeOH	Fructus	Prof. Bauer	none
587	*Xanthium sibiricum*	DCM	Fructus	Prof. Bauer	none
588	*Xanthium sibiricum*	MeOH	Fructus	Prof. Bauer	none
589	*Zanthoxylum species*	DCM	Pericarp	Prof. Bauer	none
590	*Zanthoxylum species*	MeOH	Pericarp	Prof. Bauer	none
591	*Vaccaria segetalis*	DCM	Semen	Prof. Bauer	none
592	*Vaccaria segetalis*	MeOH	Semen	Prof. Bauer	none
593	*Cassia species*	DCM	Semen	Prof. Bauer	none
594	*Cassia species*	MeOH	Semen	Prof. Bauer	none
595	*Victis trifolia*	DCM	Fructus	Prof. Bauer	none
596	*Victis trifolia*	MeOH	Fructus	Prof. Bauer	none
597	*Gardenia jasminoides*	DCM	Fructus	Prof. Bauer	none
645	*Typha spec.*	DCM	Pollen	Prof. Bauer	none
646	*Paeonia lactiflora*	DCM	Radix	Prof. Bauer	none
647	*Choerospondias axillaris*	DCM	Fructus	Prof. Bauer	none
648	*Ilex pubescens*	DCM	Cortex	Prof. Bauer	none
649	*Magnolia biondii*	DCM	Flos	Prof. Bauer	none
650	***Aristolochia debilis***	**DCM**	**Fructus**	**Prof. Bauer**	**inhibition**
651	*Prunella vulgaris*	DCM	Flos	Prof. Bauer	none
652	*Callicarpa bodinieri*	DCM	Herba	Prof. Bauer	none
653	*Aquilaria sinensis*	DCM	Resinatum	Prof. Bauer	none
654	*Broussonetia papyrifera*	DCM	Fructus	Prof. Bauer	none
655	*Lysimachia christinae*	DCM	Herba	Prof. Bauer	none
656	*Belamcanda chinensis*	DCM	Rhizome	Prof. Bauer	none
657	*Gentiana macrophylla*	DCM	Radix	Prof. Bauer	none
658	*Benincasa spec.*	DCM	Semen	Prof. Bauer	none
659	*Isatis indigotica*	DCM	Radix	Prof. Bauer	none
660	*Chrysanthemum indicum*	DCM	Flos	Prof. Bauer	none
661	***Evodia rutaecarpa***	DCM	Fructus	Prof. Bauer	**minor**

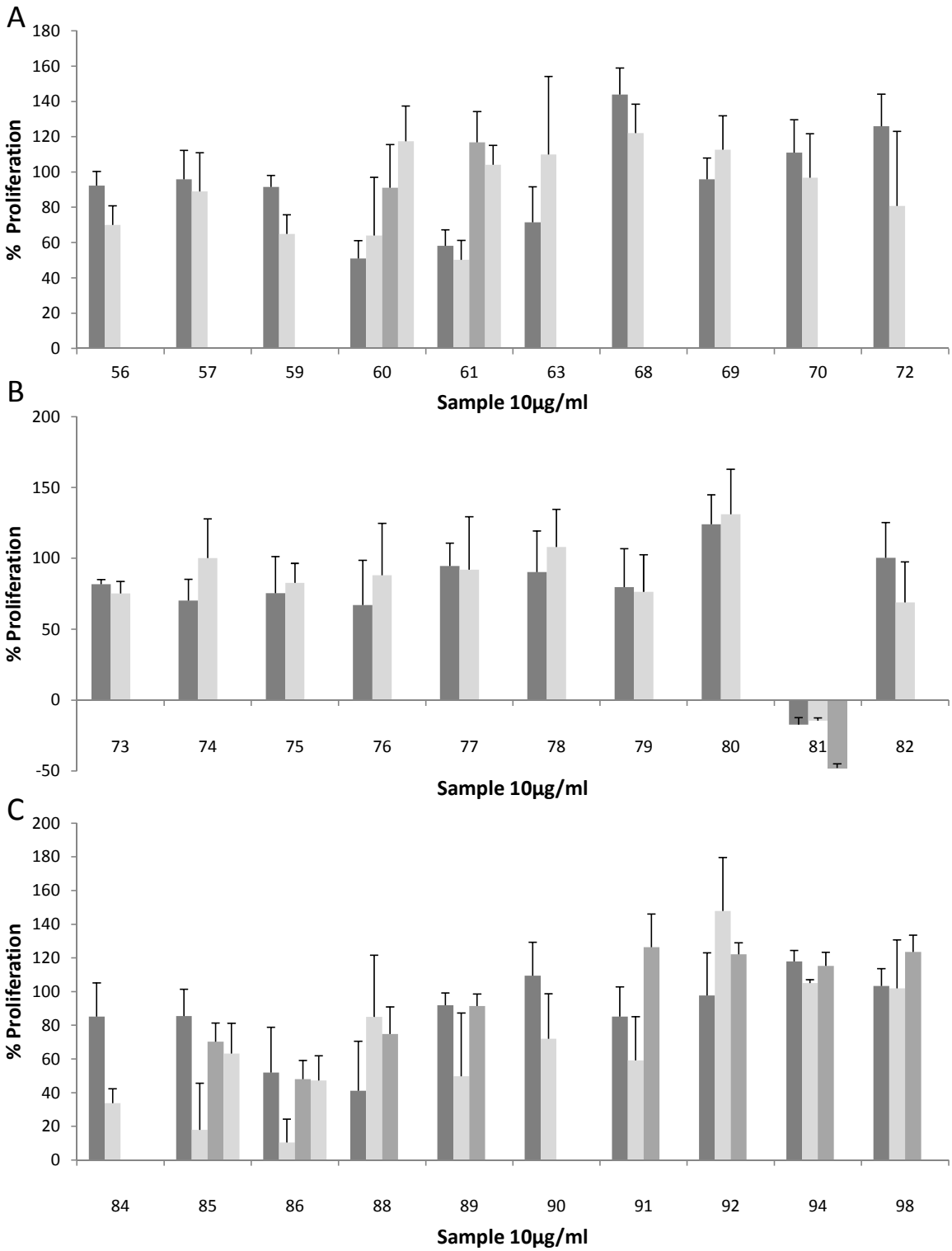
					decrease
662	*Cynanchum paniculatum*	DCM	Radix	Prof. Bauer	none
663	*Lindera aggregata*	DCM	Radix	Prof. Bauer	none
664	*Saposhnikovia divaricata*	DCM	Radix	Prof. Bauer	none
665	*Sophora japonica*	DCM	Flos	Prof. Bauer	none
666	***Piper kadsura***	DCM	Caulis	Prof. Bauer	**minor decrease**
667	*Lycium barbarum*	DCM	Fructus	Prof. Bauer	none
668	*Cimicifuga species*	DCM	Rhizome	Prof. Bauer	none
669	*Typha species*	MeOH	Pollen	Prof. Bauer	none
670	*Paeonia lactiflora*	MeOH	Radix	Prof. Bauer	none
671	*Choerospondias axillaris*	MeOH	Fructus	Prof. Bauer	none
672	*Ilex pubescens*	MeOH	Caulis	Prof. Bauer	none
673	*Magnolia biondii*	MeOH	Flos	Prof. Bauer	none
674	***Aristolochia debilis***	**MeOH**	**Fructus**	**Prof. Bauer**	**inhibition**
675	*Prunella vulgaris*	MeOH	Flos	Prof. Bauer	none
676	*Callicarpa bodinieri*	MeOH	Herba	Prof. Bauer	none
677	*Aquilaria sinensis*	MeOH	Resinatum	Prof. Bauer	none
678	*Broussonetia papyrifera*	MeOH	Fructus	Prof. Bauer	none
679	*Lysimachia christinae*	MeOH	Herba	Prof. Bauer	none
680	*Belamcanda chinensis*	MeOH	Rhizome	Prof. Bauer	none
681	*Gentiana macrophylla*	MeOH	Radix	Prof. Bauer	none
682	*Benincasa species*	MeOH	Semen	Prof. Bauer	none
683	*Isatis indigotica*	MeOH	Radix	Prof. Bauer	none
684	*Chrysanthemum indicum*	MeOH	Flos	Prof. Bauer	none
685	*Evodia rutaecarpa*	MeOH	Fructus	Prof. Bauer	none
686	*Cynanchum paniculatum*	MeOH	Radix	Prof. Bauer	none
687	*Lindera aggregata*	MeOH	Radix	Prof. Bauer	none
688	*Saposhnikovia divaricata*	MeOH	Radix	Prof. Bauer	none

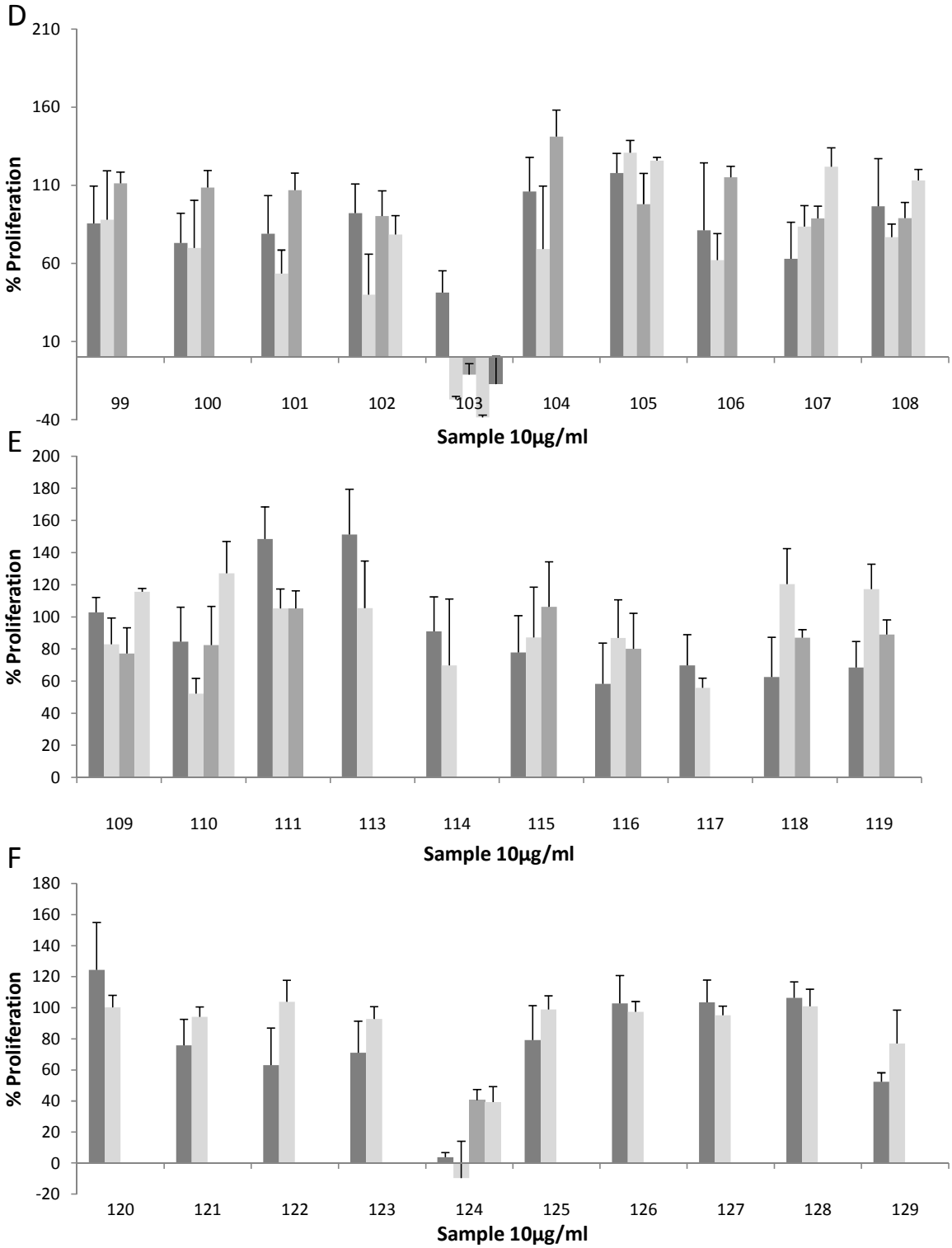
689	*Sophora japonica*	MeOH	Flos	Prof. Bauer	none
690	*Piper kadsura*	MeOH	Caulis	Prof. Bauer	none
691	*Lycium barbarum*	MeOH	Fructus	Prof. Bauer	none
692	*Cimicifuga species*	MeOH	Rhizome	Prof. Bauer	none
171	*Peucedanum ostruthium*	MeOH	Radix	Prof. Kopp**	none
168	*Glechoma hedera*	MeOH	Herba	Prof. Kopp	none
277	*Melampyrum species*	DCM	Herba	Prof. Kopp	none
279	*Melampyrum species*	MeOH	Herba	Prof. Kopp	none
487	*Dryopterix filix-mas*	EtAc	Fronds	Prof. Reznicek ***	none
492	*Dryopterix filix-mas*	DCM	Fronds	Prof. Reznicek	none
734	*Coffea arabica*	DCM	Semen	Prof. Reznicek	none
738	*Coffea arabica*	EtAc	Semen	Prof. Reznicek	none
742	*Coffea arabica*	PE	Semen	Prof. Reznicek	none
746	*Coffea arabica*	dH2O	Semen	Prof. Reznicek	none
811	*Albicia julibrissin*	DCM	Cortex	Prof. Kopp	none
812	*Albicia julibrissin*	EtAc	Cortex	Prof. Kopp	none
788	*Arisaema species*	DCM and MeOH	Rhizome + Pericarp	Prof. Kopp	none
724	***Sideritis hyssopifolia***	**MeOH**	**Herba**	**Prof. Stuppner**	**inhibition**
165	*Doronicum austriacum*	DCM	Radix	Prof. Stuppner ****	none
158	*Krameria triandra*	DCM	Radix	Prof.	none

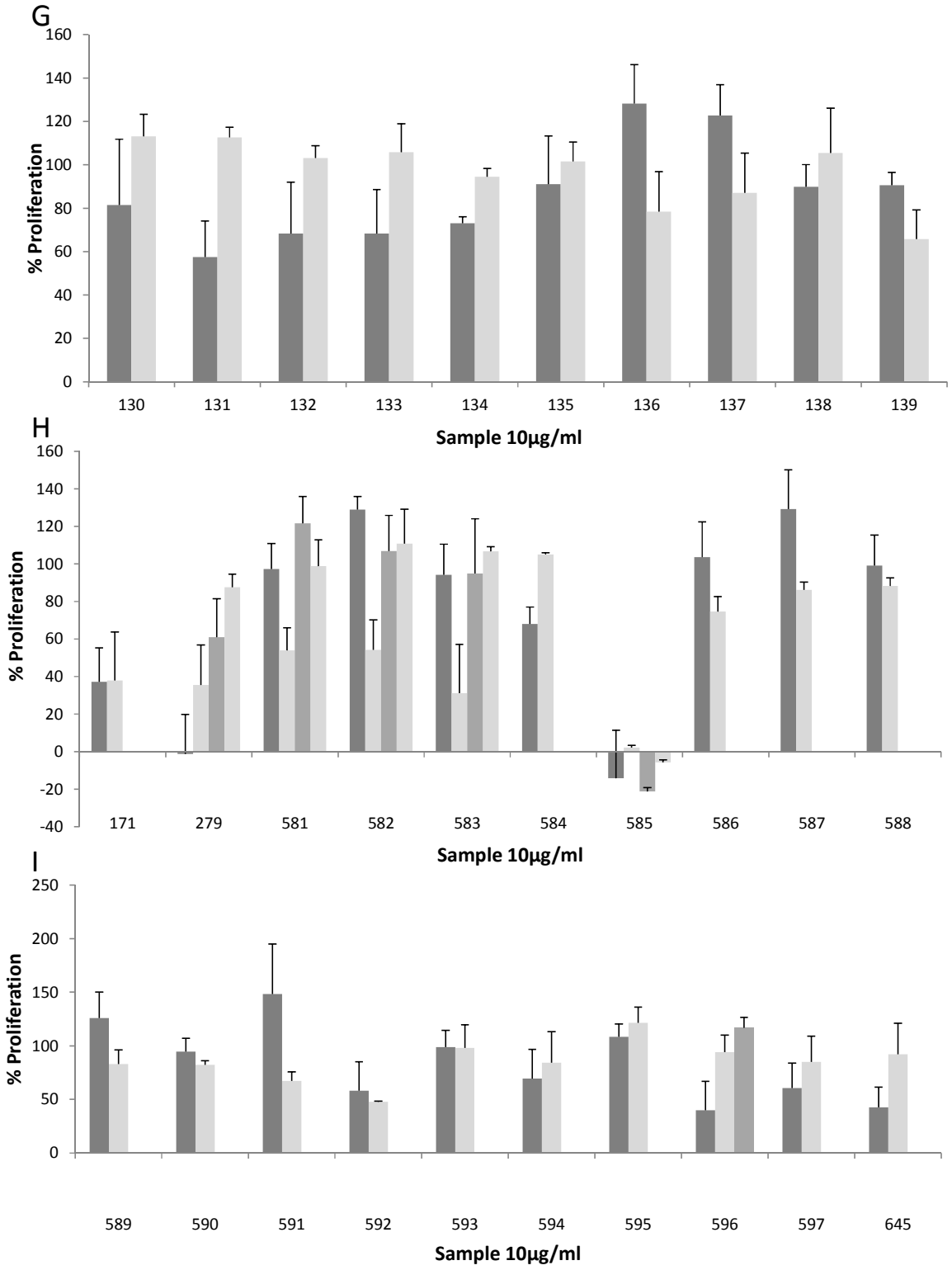
				Stuppner	
984	*Thlaspi arvense*	MeoH	Herba	Prof. Bauer	none
993	*Pinellia ternata (raw)*	DCM	Radix	Prof. Kopp	none
994	*Pinellia ternata (raw)*	MeOH	Rhizome	Prof. Kopp	none
995	*Pinellia ternata (processed)*	DCM	Rhizome	Prof. Kopp	none
996	*Pinellia ternata (processed)*	MeOH	Rhizome	Prof. Kopp	none

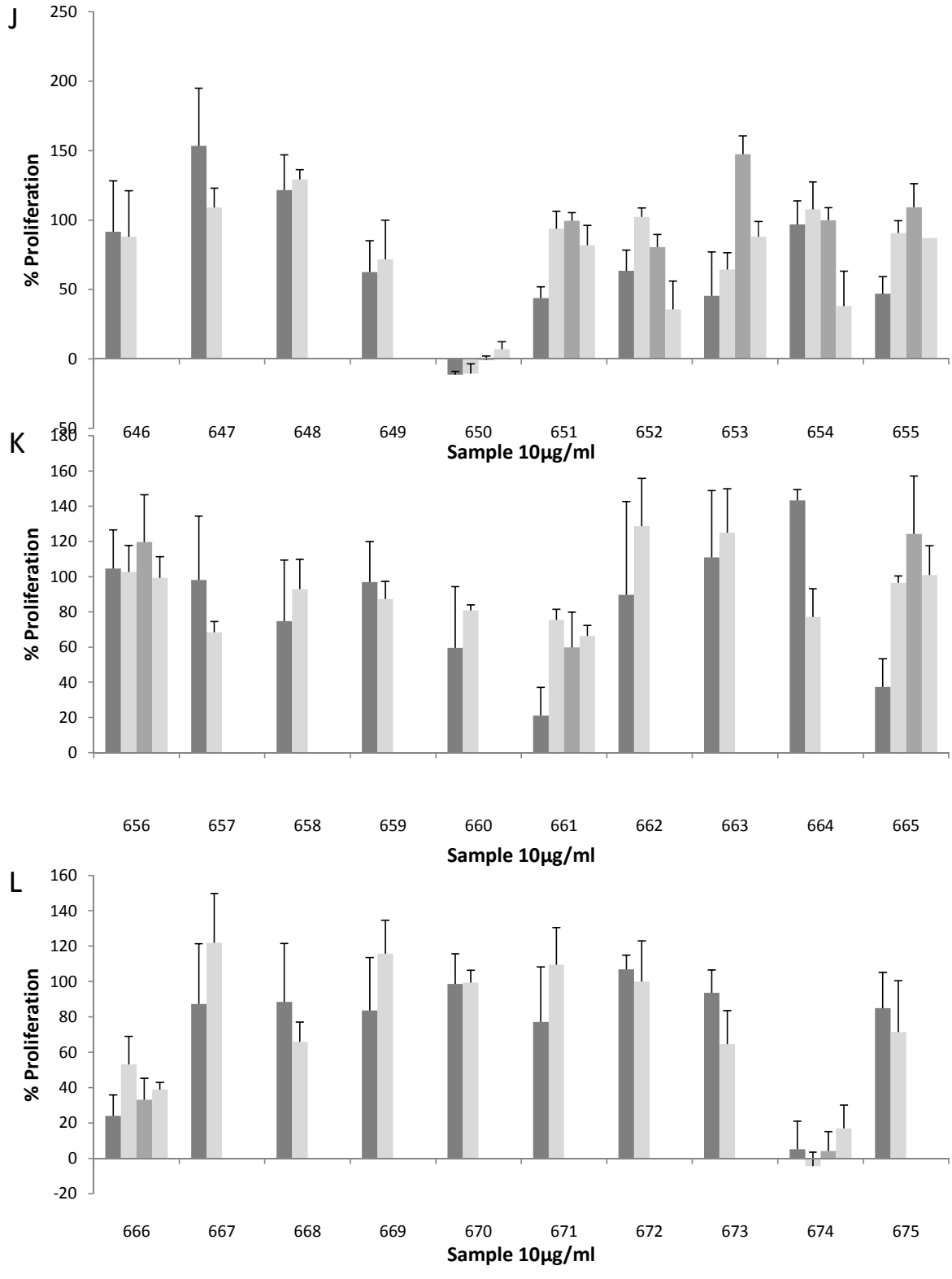
Figure C3: Complete list of plant extracts used for screening

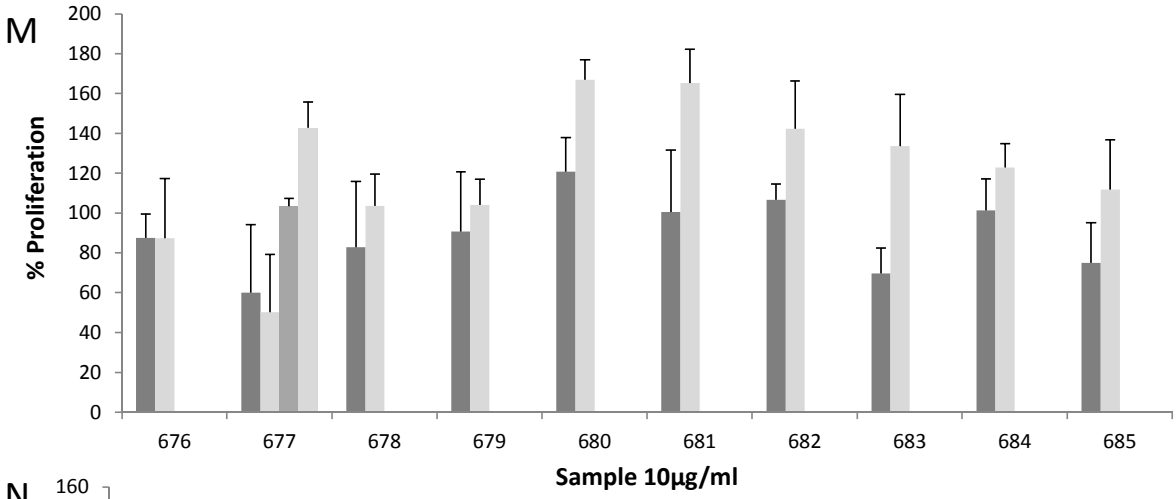
S Nr = Sample number; * Prof. Rudolf Bauer, Institute of Pharmaceutical Sciences, University of Graz; ** Prof. Brigitte Kopp, Department of Pharmacognosy, University of Vienna; *** Prof. Gottfried Reznicek, Department of Pharmacognosy, University of Vienna; **** Prof. Hermann Stuppner, Institute of Pharmacy, University of Innsbruck.

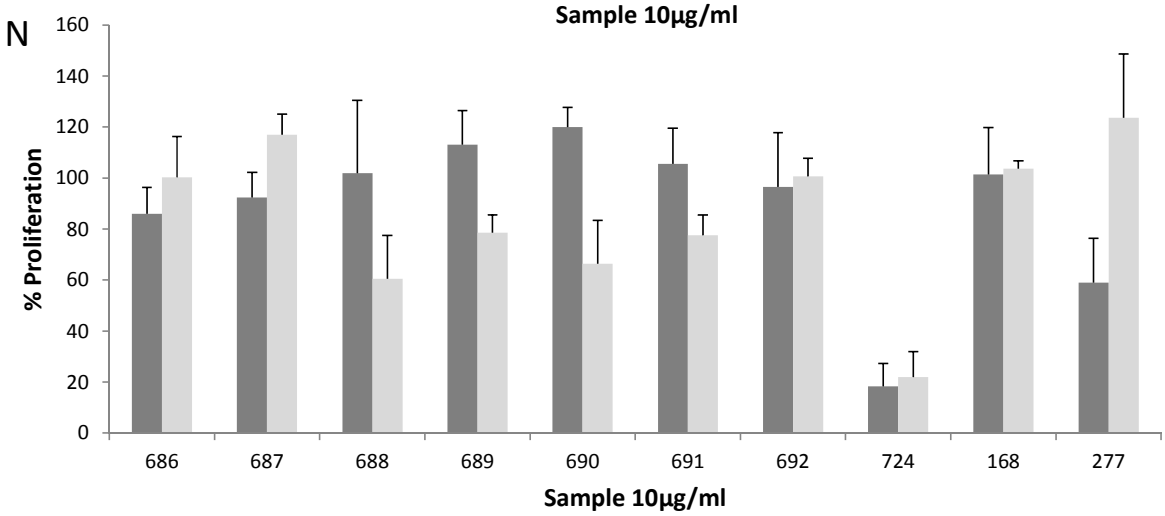

A
% Proliferation
180
160
140
120
100
80
60
40
20
0
56
57
59
60
61
63
68
69
70
72
Sample 10µg/ml
B
% Proliferation
200
150
100
50
0
-50
73
74
75
76
77
78
79
80
81
82
Sample 10µg/ml
C
% Proliferation
200
180
160
140
120
100
80
60
40
20
0
84
85
86
88
89
90
91
92
94
98
Sample 10µg/ml

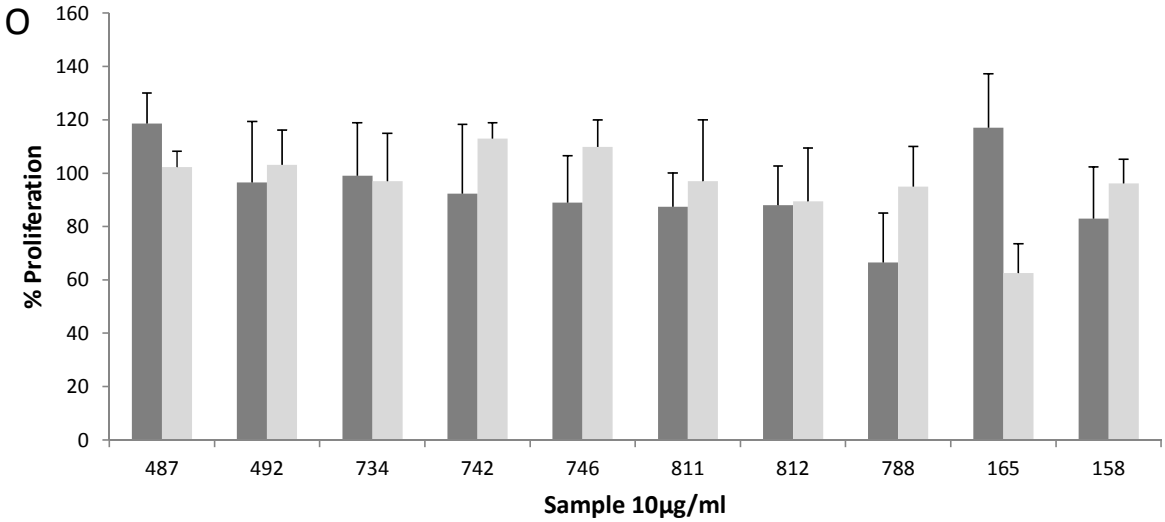

D
210
160
110
60
10
-40
% Proliferation
99
100
101
102
103
104
105
106
107
108
Sample 10µg/ml
E
200
180
160
140
120
100
80
60
40
20
0
% Proliferation
109
110
111
113
114
115
116
117
118
119
Sample 10µg/ml
F
180
160
140
120
100
80
60
40
20
0
-20
% Proliferation
120
121
122
123
124
125
126
127
128
129
Sample 10µg/ml


G
% Proliferation
0
20
40
60
80
100
120
140
160
130
131
132
133
134
135
136
137
138
139
Sample 10µg/ml
H
% Proliferation
-40
-20
0
20
40
60
80
100
120
140
160
171
279
581
582
583
584
585
586
587
588
Sample 10µg/ml
I
% Proliferation
0
50
100
150
200
250
589
590
591
592
593
594
595
596
597
645
Sample 10µg/ml


J
% Proliferation
250
200
150
100
50
0
-50
646
647
648
649
650
651
652
653
654
655
Sample 10µg/ml
K
% Proliferation
160
140
120
100
80
60
40
20
0
656
657
658
659
660
661
662
663
664
665
Sample 10µg/ml
L
% Proliferation
160
140
120
100
80
60
40
20
0
-20
666
667
668
669
670
671
672
673
674
675
Sample 10µg/ml


M
% Proliferation
200
180
160
140
120
100
80
60
40
20
0
676
677
678
679
680
681
682
683
684
685
Sample 10µg/ml


N
% Proliferation
160
140
120
100
80
60
40
20
0
686
687
688
689
690
691
692
724
168
277
Sample 10µg/ml


O
% Proliferation
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Sample 10µg/ml

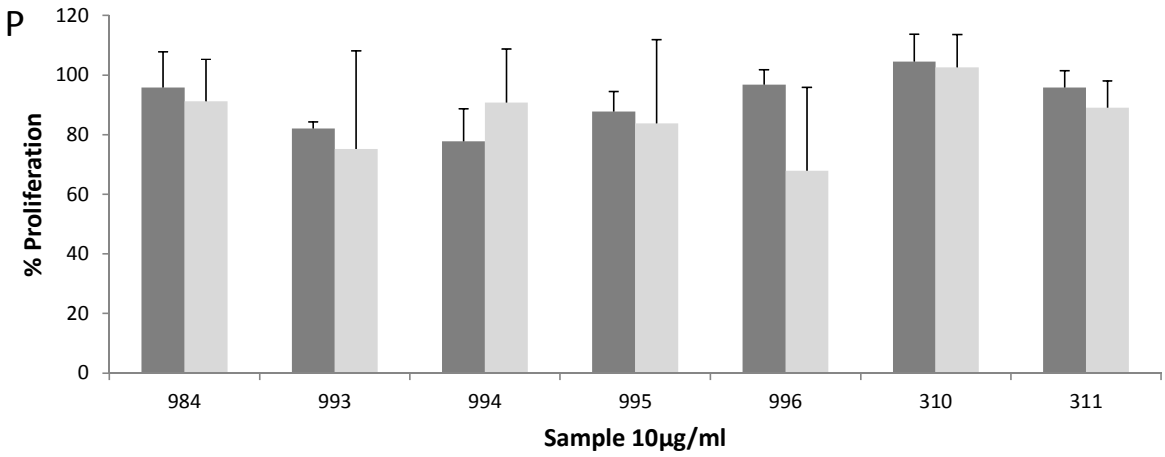


Figure C4 A-P: Screening results

Resazurin conversion assay: Serum starved VSMC were pretreated with plant extracts used in a final concentration of 10 µg/ml or vehicle (0,1% DMSO), and stimulated with serum for 48 h. Then the cells were incubated in DMEM containing 10 µg/ml resazurin and fluorescence was measured. Each bar represents one independent experiment performed in quadruplicate.

1.3.1. Plant extracts which showed contradictory results but proved to be non inhibitory

If plant extracts showed contradictory results in the first two performed experiments (e.g. in one of the experiments inhibiton over 50% and in the other no inhibition), they were subjected to a maximum of five independent experiments to verify their putative inhibitory potential. The cells were treated with special care and checked under the microscope to reveal possible cytotoxic action. Nevertheless, neither any visible effect could be detected, nor was any cytotoxicity revealed in repeated screening (Figure C5).

Sample number:					
60	61	85	88	89	90
91	92	94	98	99	100
104	118	119	101	115	116
105	106	107	108	109	110
111	101	102	581	582	583
595	596	651	652	653	654
655	656	665	677		

Figure C5:
Plant extracts which showed contradictory results in the first two tests and were further tested up to 5 times. Experimental results can be found in section 1.3. Figure C4.

1.4. Plant extracts which showed an increase in cell proliferation

Interestingly, the DCM extract of *Acorus tatarinowii* rhizomes (sample 66) and the MeOH extract of *Prunus species* semen seem to enhance proliferation (Figure C6). Due to time and material limitations, these plants were not subjected to further testing. Nevertheless this data might be valuable, considering the complexity of atherosclerosis. There is an upcoming interest in pharmaceuticals which prevent apoptosis in VSMC, in order to contribute to plaque stability. A further experimental approach would be the investigation of these

extracts without serum administration, and therefore to check whether these plant extracts are sufficient to induce cell proliferation. Furthermore putative antiapoptotic properties could be investigated.

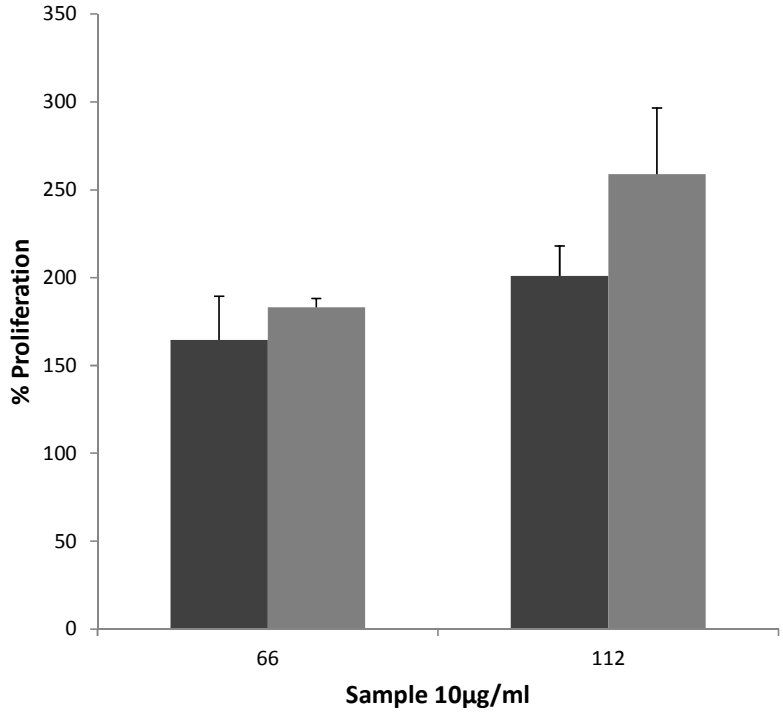


Figure C6: Resazurin conversion assay:

Serum starved VSMC were pretreated with plant extracts used in a final concentration of 10 µg/ml or vehicle (0.1% DMSO), and stimulated with serum for 48 h. Then samples were incubated in DMEM containing 10 µg/ml resazurin and fluorescence was measured. Each bar represents one independent experiment performed in quadruplicate

1.5. Cytotoxic plant extracts

The plant extracts shown in Figure C7 inhibit proliferation of VSMC. Nevertheless, light microscopy and the screening data suggest a strong cytotoxic activity on VSMC revealed by cellular defragmentation observed under the microscope, and reduction of the resazurine conversion far below the level of the DMSO treated control. Pharmacological application of toxic agents may result in the weakening of the vessel wall, thereby predisposing it to enlargement or aneurysm formation. Therefore, cytostatic agents would be preferred for potential pharmaceutical application. [11] Nevertheless, paclitaxel a drug found in the early sixties by a plant screening project, which exhibited cytotoxic features, is nowadays one of the most used drugs on DES. [106]

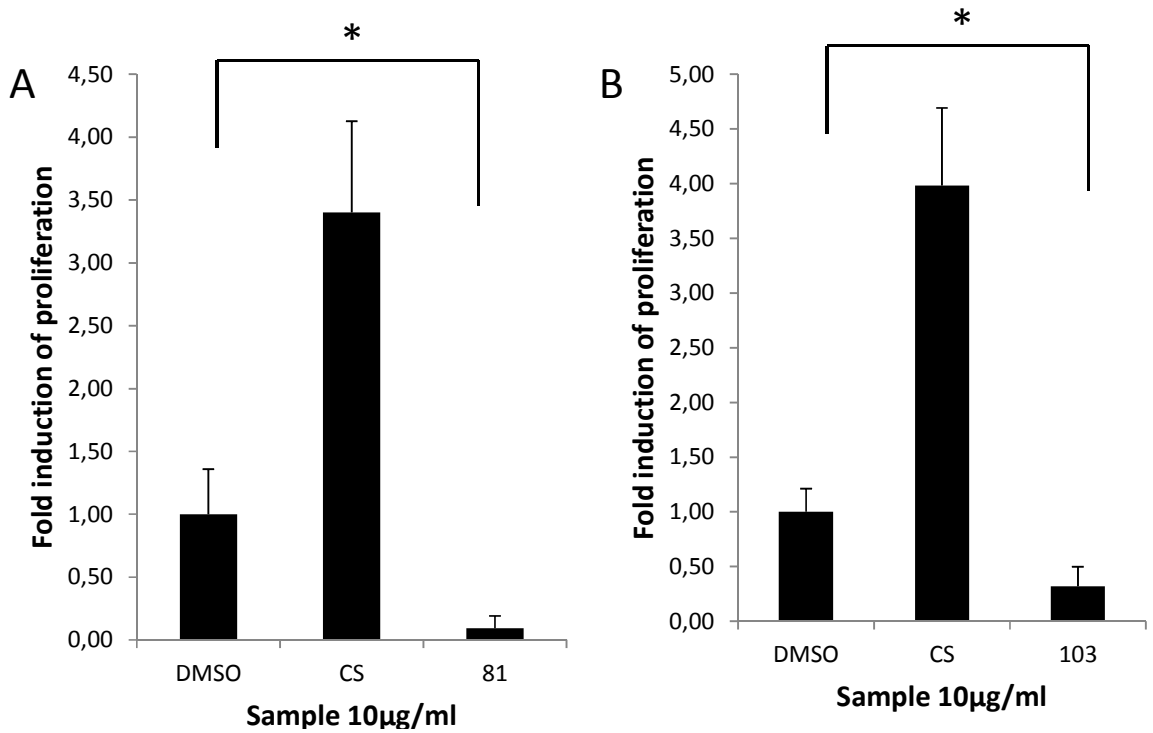


Figure C7: Cytotoxic action of the plant extracts 81 and 103

Resazurin conversion assay: Extracts were used in a final concentration of 10 µg/ml. The extracts were applied and after thirty minutes the cells were stimulated with 10% serum for 48 h. Final DMSO concentration in all samples was 0.1%. All values were normalized to the signal obtained from the unstimulated, vehicle (0.1% DMSO)-treated cells and therefore represent the fold activation of proliferation above the basal level. n=3, mean ± SD, * $p < 0.05$ (ANOVA/Tukey).

1.6. Plant extracts which showed inhibition of proliferation

1.6.1. Plant extracts which showed a slight reduction of proliferation

The DCM extract of *Asari species* radix and rhizome extract (Sample 86) and the DCM extract of *Piper kadsura* caulis (sample 666) showed slower and diminished growth compared to the calf serum control. Cytotoxicity could not be detected microscopically. Nevertheless since the effect was not strong these plant extracts were not subjected to further investigation in this work

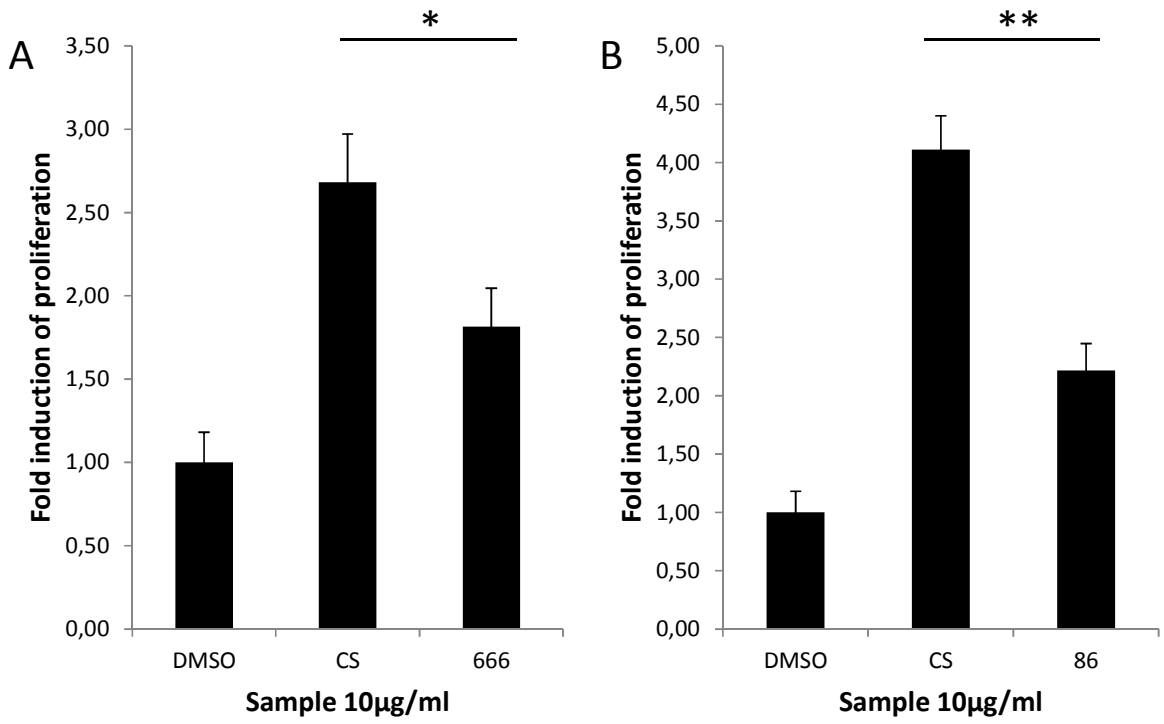
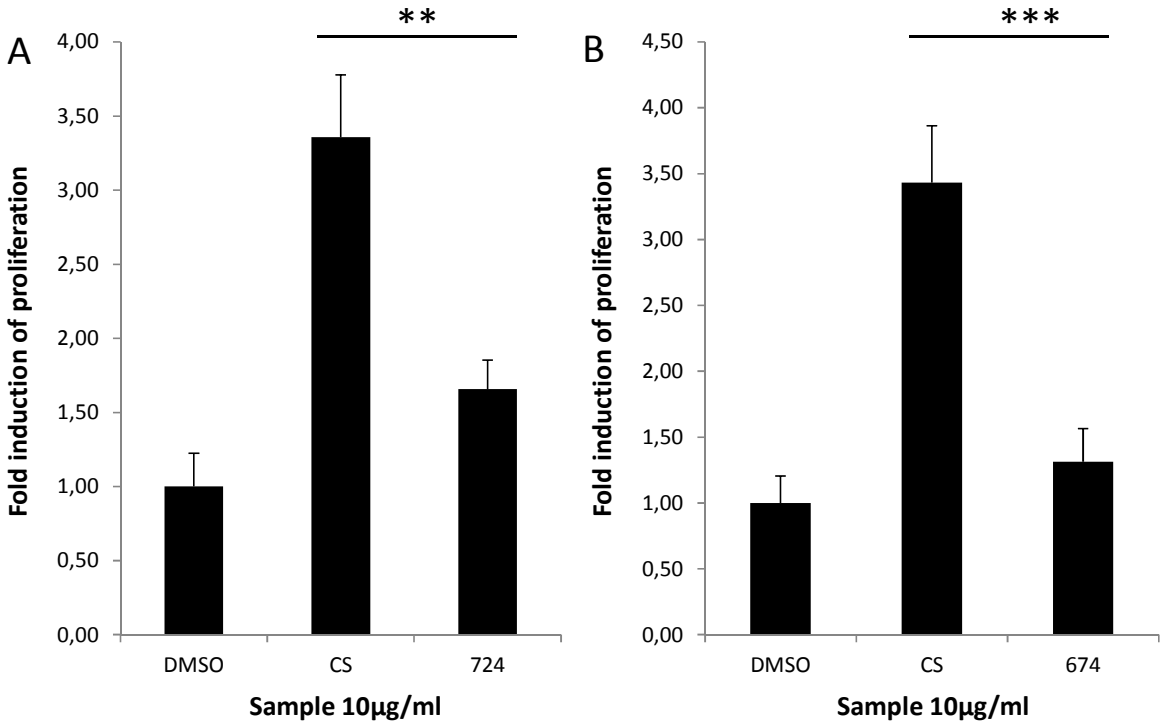


Figure C8: Inhibitory action of plant extracts 666 and 86

Resazurin conversion assay: Extracts were used in a final concentration of 10 µg/ml. The extracts were applied and after thirty minutes the cells were stimulated with 10% serum for 48 h. Final DMSO concentration in all samples was 0.1%. All values were normalized to the signal obtained from the unstimulated, vehicle (0.1% DMSO)-treated cells and therefore represent the fold activation of proliferation above the basal level. A) n=6, mean (±SD), * $p < 0.05$ (ANOVA/Tukey). B) n=4 , mean (±SD), ** $p < 0.01$ (ANOVA/Tukey)

1.6.2. Inhibitory plant extracts

The plant extracts shown in Figure C9 A – E exhibited strong inhibition without any visible proliferation under the microscope. Furthermore no cellular defragmentation could be detected microscopically. Therefore these plant extract represent the most interesting candidates for further studies apart from *Peucedanum ostruthium* which is presented in chapter three.



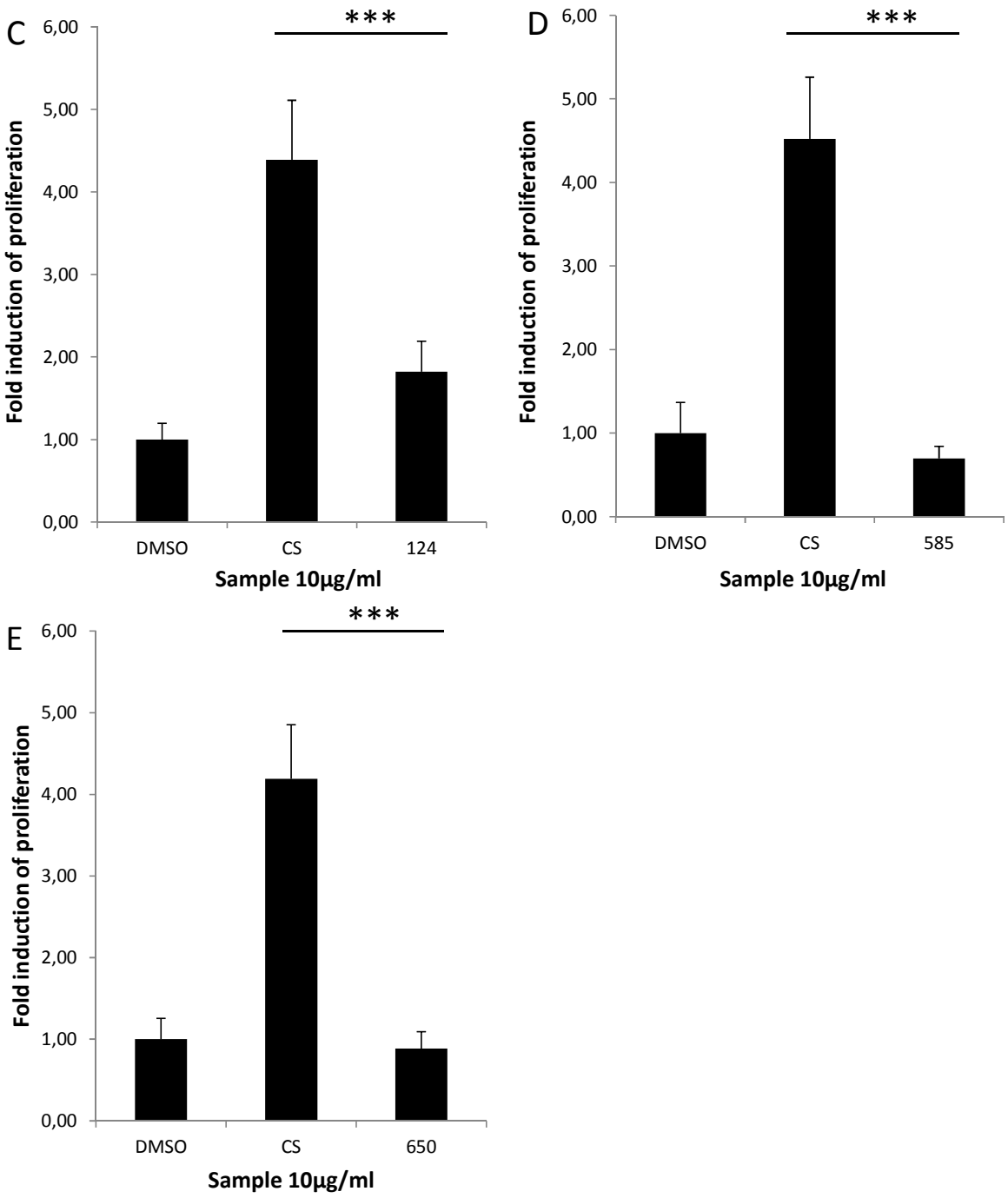


Figure C9 A-E: Plant extracts which induced inhibition of proliferation

Resazurin conversion assay performed as described in details in the methods section; A: 724 = *Sideritis hyssopifolia* MeOH extract n=3*;* B: 674 = *Aristolochia debilis* MeOH extract n=6 C: 124 = *Stephania tetranda* radix extracted with MeOH, n=4; D: 585 = *Melia toosendan* DCM extract, n=5; E: 650 = *Aristolochia debilis* DCM extract, n=6; mean (±SD), ** $p < 0.01$ *** $p < 0.001$ (ANOVA/Tukey).

2. Further investigation of active plant extracts

2.1. Inhibition of human umbilical vein endothelial cell proliferation

Plant extracts which actively inhibit the proliferation of VSMC without influencing endothelial proliferation would be of special interest for a potential use as stenting drugs. Therefore we have chosen the most promising candidates originating from the performed screening to investigate their effect on the proliferation of human umbilical vein endothelial cells (HUVEC). Tylophorine is known for its antiproliferative action in various cell types and was therefore chosen as a positive inhibition control. [3] Unfortunately the following investigated plant extracts, which proved to be active in VSMC, seem also to suppress the growth of HUVEC as well (Figure C10).

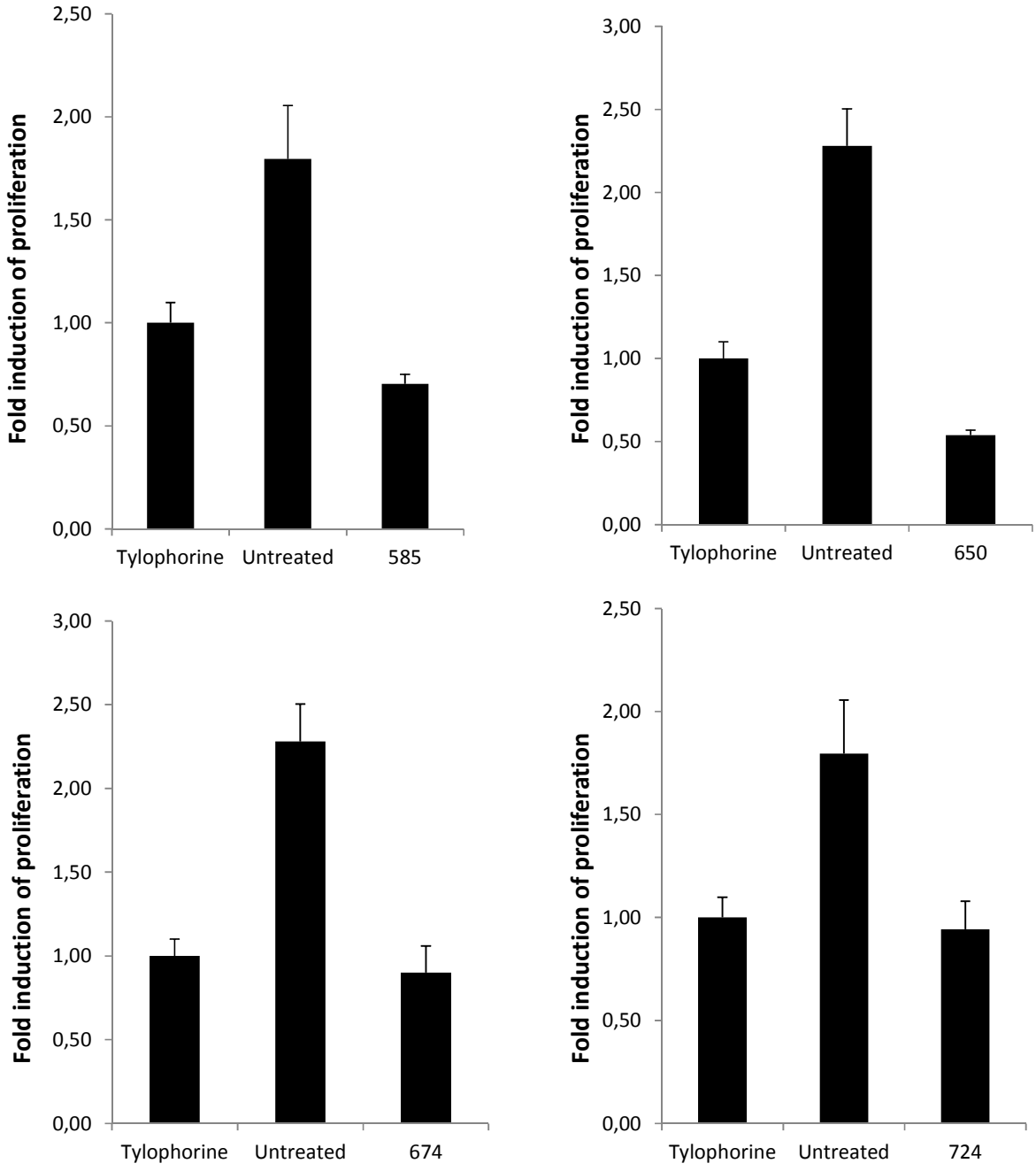


Fig C10: **Plant extracts tested for HUVEC proliferation inhibition**

Resazurin conversion assay: All plant extracts were used in a final concentration of 10 µg/ml. Tylophorine was used in a final concentration of 1 µM. Graphs represent one preliminary experiment and need further confirmation by additional independently performed experiments.

2.2. Determination of the type of cell cycle arrest induced by *Aristolochia debilis* extract

Due to the strong inhibition of proliferation by *A. debilis* extract in VSMC, we further analyzed its effect on the progression through the cell cycle phases. VSMC were starved and subsequently treated with *A. debilis* extract and calf serum (CS) for 20 h and then stained with probidium iodide (PI) to allow flow cytometric measurement of the DNA-content of single cells (Figure C11). Calf serum treatment leads to progression through the cell cycle as seen by higher percentages of cells in S-/G_2 phase compared to serum- starved cells being growth arrested in G_1-/G_0-phase of the cell cycle. Treatment of cells with both investigated *Aristolochia debilis* extracts (10 µg/ml) (sample 650 and 674) showed an accumulation of cells in G_1-phase compared to the serum treated control. A clear subG_1 peak indicative of cell death was not observed.

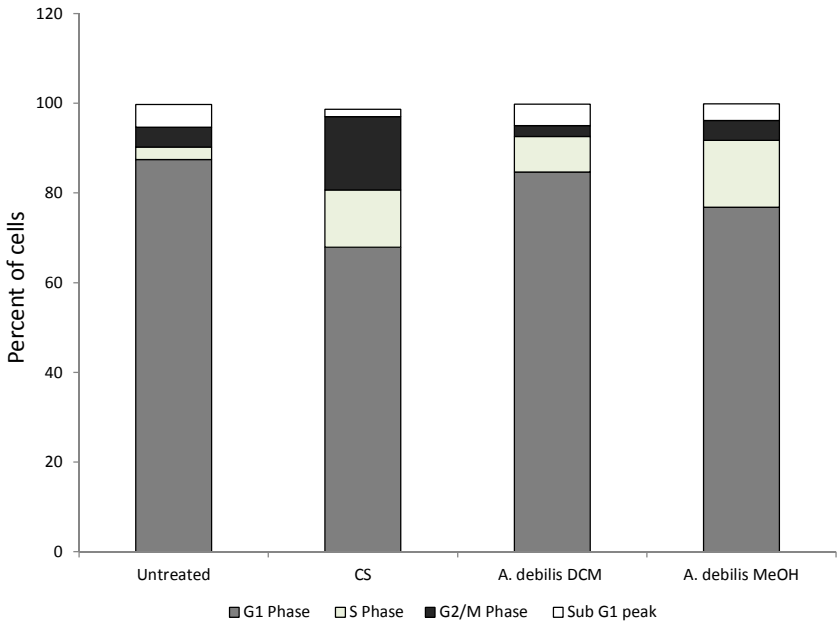


Fig C11: VSMC were synchronized by starvation, treated with 10 µg/ml *Aristolochia debilis* DCM and MeOH extract and then stimulated for 20 h with CS. Afterwards, VSMC were stained with propidium iodide. DNA contents were measured by flow cytometry. Graphs represent one preliminary experiment and need further confirmation by additional independently performed experiments.

3. Identification of ostruthin from *Peucedanum ostruthium* as antiproliferative agent

3.1. Resazurin convesion assay of *P. ostruthium* DCM extract and subfractions

Screening procedures revealed an activity of the DCM extract of *Peucedanum ostruthium* rhizomes on the proliferation of serum-stimulated rat aortic cells at 10 µg/ml (Figure C12 A). During the first step to downtrack the activity, we tested three different fractions obtained from the initial DCM extract. Solid phase extracts (SPE) with different concentrations of MeOH were provided by our collaboration partner Sylvia Vogl, Dept. Pharmacognosy.We found antiproliferative activity in subfraction 2 (Fig C12 C).

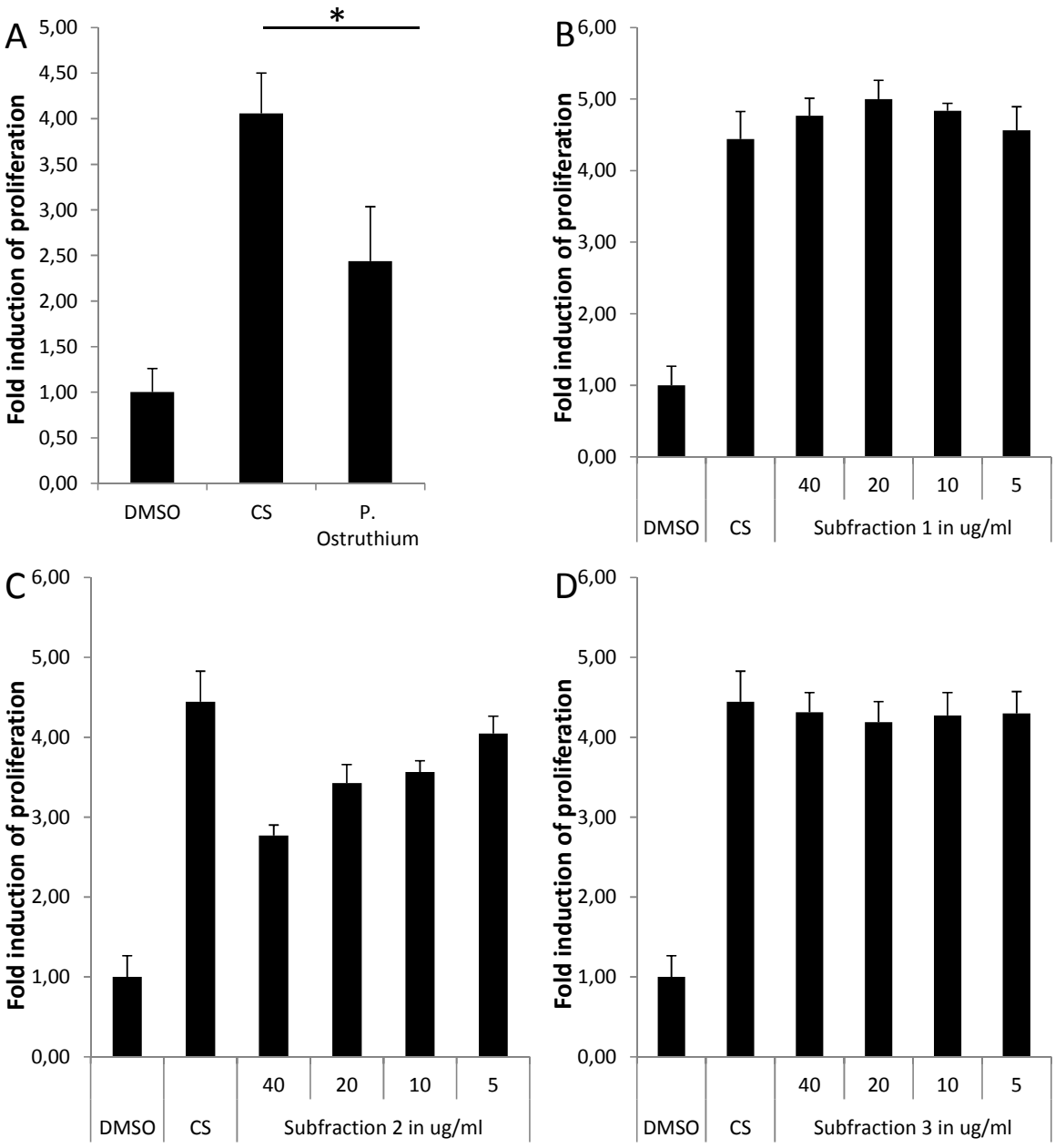


Figure C12: Antiproliferative action of *Peucedanum ostruthium*

Resazurin conversion assay was performed as described in details in the Methods section; A) Initial MeOH Extract of *Peucedanum ostruthium,* n=4, mean (±SD), * $p < 0.05$ (ANOVA/Tukey) B) SPE 30 % MeOH extract C) SPE 70 % MeOH extract D) SPE 100 % MeOH extract.

3.2. HPLC analysis of *P. ostruthium*

In a parallel study, a comprehensive qualitative and quantitative analysis of the main coumarins in extracts from *P. ostruthium* rhizomes was performed in the group of Prof. Kopp at the Department of Pharmacognosy, University of Vienna. As a result, the major constituents of the DCM extract were determined to be seven coumarins: oxypeucedanin hydrate, oxypeucedanin, ostruthol, imperatorin, osthole, isoimperatorin, and ostruthin (Figure C13) [8, 107].

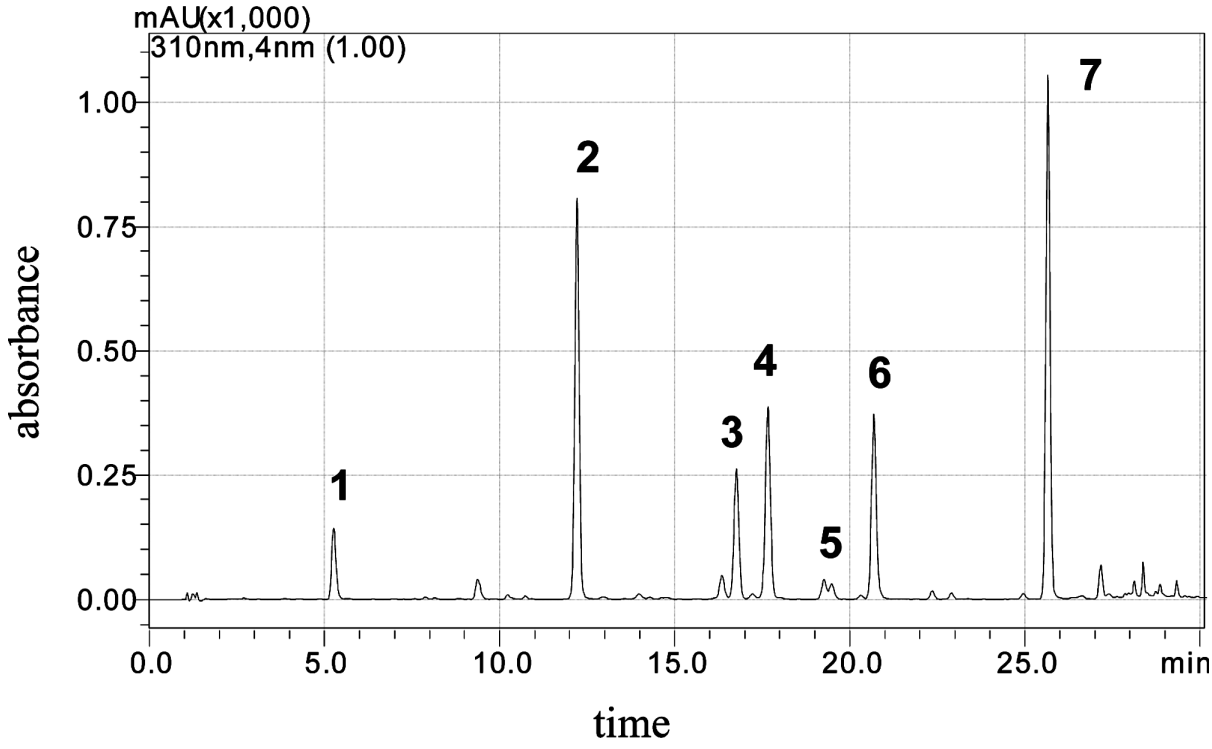


Figure C13: HPLC chromatogram UV detection at 310 nm of the CH_2Cl_2 extract of *Peucedanum ostruthium* rhizomes

Peak identities: 1, oxypeucedanin hydrate; 2, oxypeucedanin; 3, ostruthol; 4, imperatorin; 5, osthole; 6, isoimperatorin; 7, ostruthin.

Quantification measurements of the coumarin content in the DCM extract of *Peucedanum ostruthium* rhizomes resulted in ostruthin as the main coumarin (41% of the total coumarin content), followed by oxypeucedanin (18%), ostruthol (13%), imperatorin (12%), isoimperatorin (9%), oxypeucedanin hydrate (6%) and osthole composing just 1%. The total coumarin content in this extract made up 78%, whereas the amount on gram dry weight of drug made up only 6% (Figure C14) [8, 107].

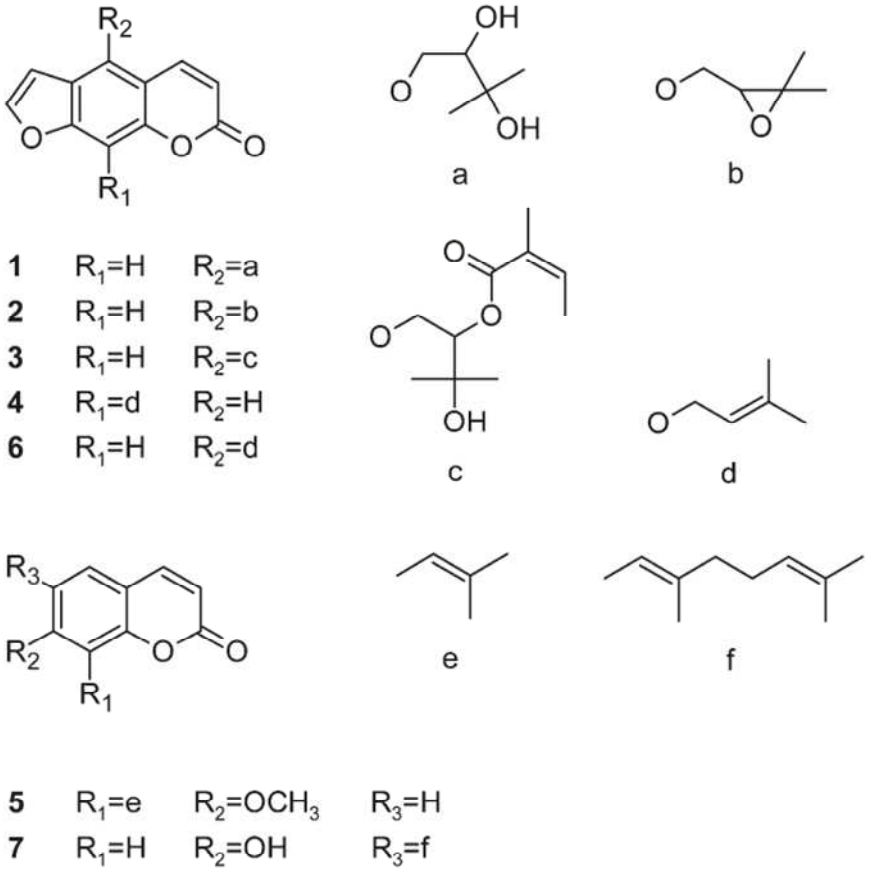


Figure C14: Structural formula of the seven main coumarins of *Peucedanum Ostruthium* 1, oxypeucedanin hydrate; 2, oxypeucedanin; 3, ostruthol; 4, imperatorin; 5, osthole; 6,isoimperatorin; 7, ostruthin. Figure adapted from [8].

3.3. Resazurin conversion assay with the main coumarins from the extract

In the first series of further experiments, the five most promising coumarins were chosen for further investigation (for osthole and oxypeucedanin hydrate see [3]). Ostruthin is the only compound of the tested coumarins which significantly inhibits serum-stimulated VSMC proliferation (Figure C15).

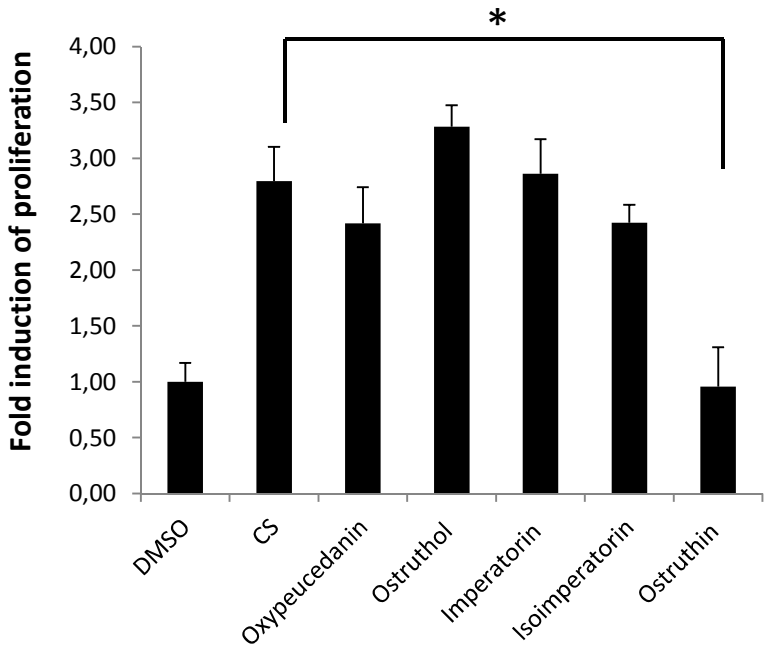


Figure C15: Antiproliferative action of Ostruthin

Resazurin conversion assay: All compounds were tested in a concentration of 30 µM. Ostruthin was the only compound significantly inhibiting serum-stimulated VSMC proliferation (mean (±SD), n = 3, *p < 0.05; ANOVA/Tukey)

3.3.1. Test of structurally related (furano)coumarins

Since the structurally similar linear furanocoumarins (Figure C15) did not exhibit an effect on the proliferation of VSMC, it was assumed that the geranyl group at the C-6 position may be necessary for the inhibitory effect. To validate this specific effect of the geranyl group, the inhibitory potential for several further coumarins and furanocoumarins was tested, namely, bergamottin, bergaptol, bergapten isobergapten, umbelliferone, scopoletin, auraptene, and heraclenin (Figure C16), which all share some structural similarity to Ostruthin, but lack the C-6 geranyl group. Indeed, none of these compounds significantly inhibited VSMC proliferation at a concentration of 30 µM, underlining the specific action of Ostruthin, among the compounds tested [8].

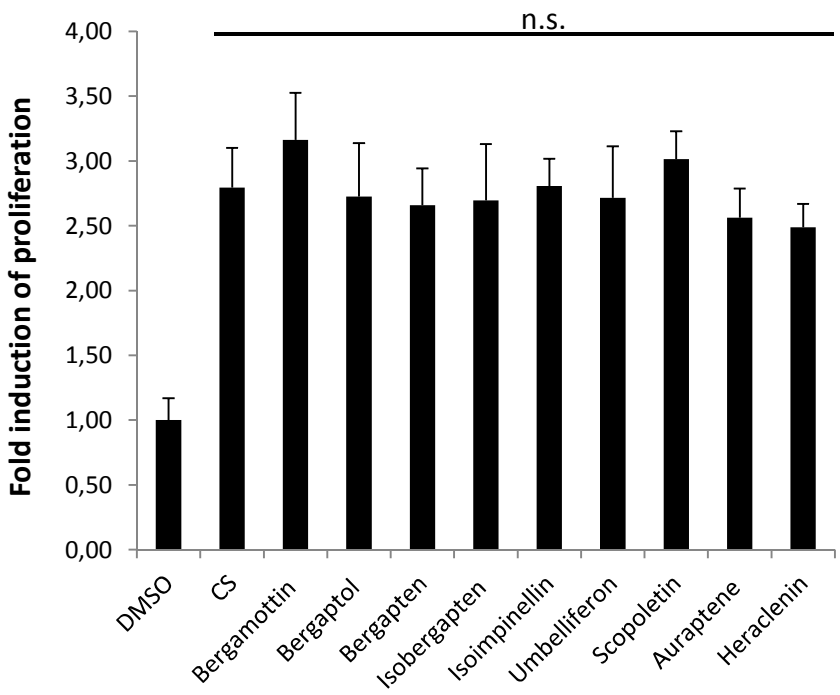


Figure C16: Action of related (furano)coumarins on VSMC proliferation

Resazurin conversion assay: All samples except DMSO control were treated with serum and used in a concentration of 30 µM (mean (±SD), n = 3, n.s. not significant, ANOVA/Tukey)

3.4. Ostruthin inhibits rat VSMC proliferation in a concentration-dependent manner

To check whether ostruthin inhibits the proliferation of VSMC in a concentration dependent manner, ostruthin was applied to VSMC in a concentration range of 10 – 30 µM and checked for inhibition of proliferation via the resazurin conversion method. Preliminary results obtained in this work (Figure C17) showed concentration-dependent inhibition of the proliferation of VSMC. This experimental setting was later used for further independent experiments performed by Helge Joa and it was finally established that ostruthin inhibits VSMC proliferation with an IC50 of 24 µM [8].

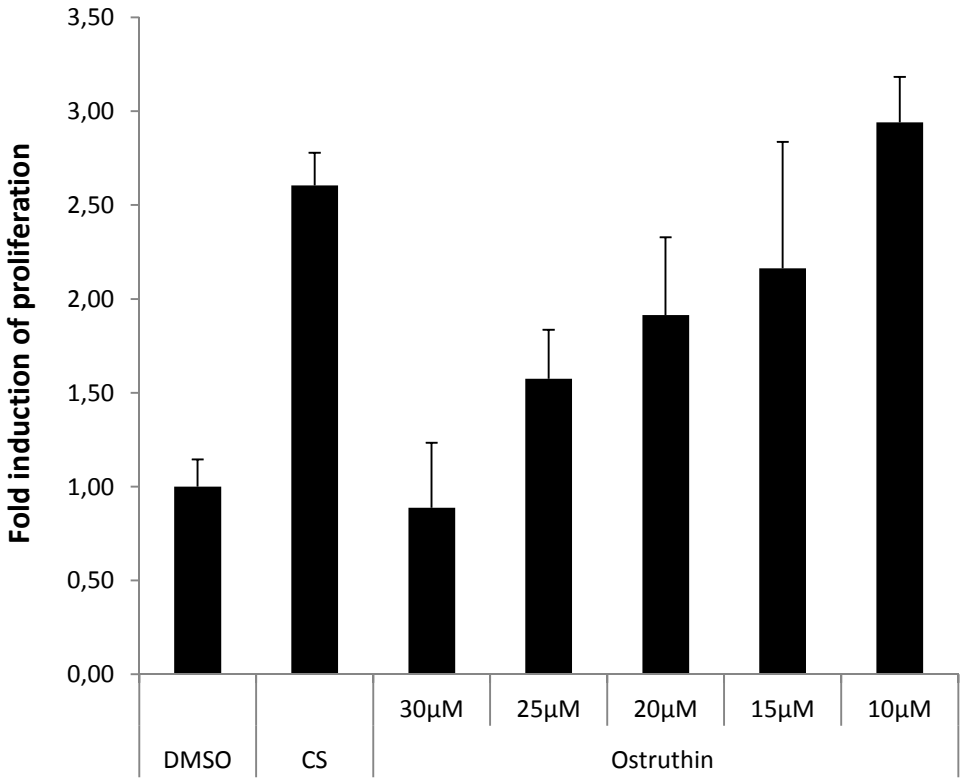


Figure C17: Concentration dependent inhibition of VSMC by ostruthin
Resazurin conversion assay: All samples except DMSO control were treated with serum. Ostruthin (10–30 µM) was applied. The graph represents two independently performed experiments in quadruplicate.

3.5. Ostruthin is less active in HUVEC

A very interesting finding was that the inhibitory potential of ostruthin in HUVEC was a bit smaller than in VSMC. We tested ostruthin up to a concentration of 90 µM and found an inhibitory action with an IC50 of 41 µM (Figure C18). However, since this experimental setting was performed just twice, this observation would need further confirmation by additional independently performed experiments.

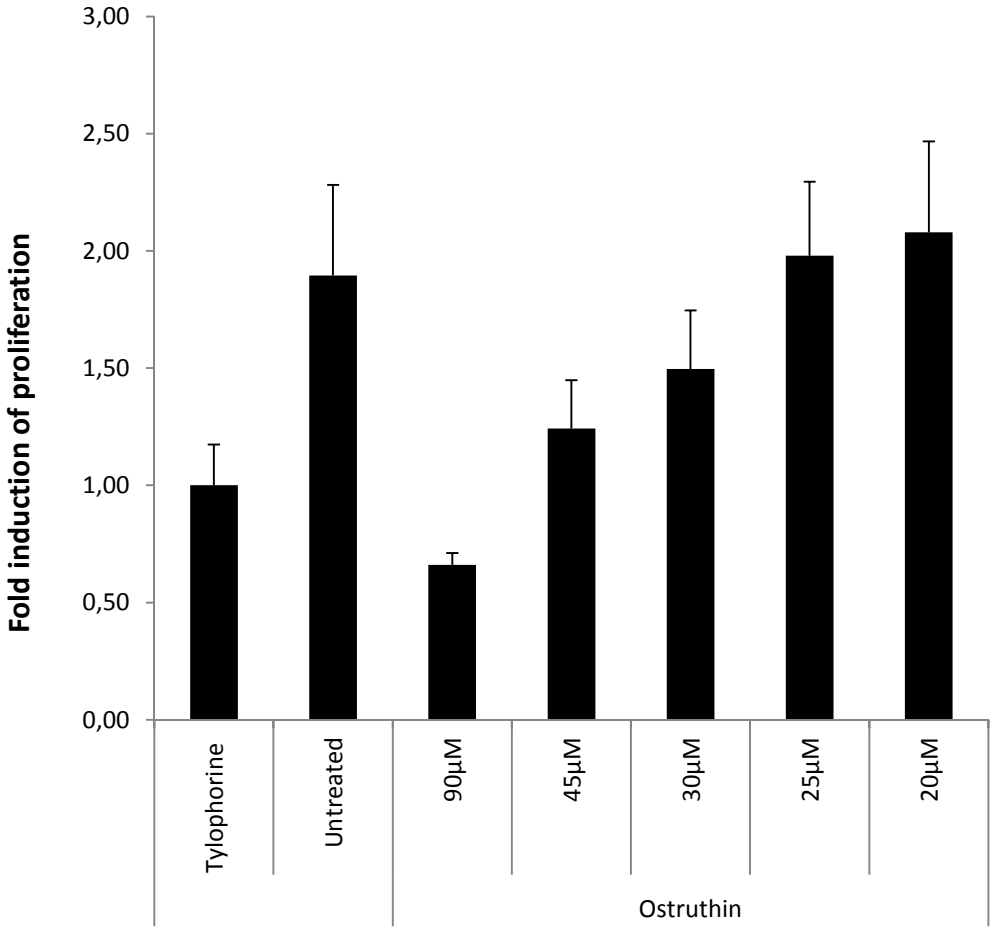


Figure C18: Concentration dependent inhibition of HUVEC by ostruthin

Resazurin conversion assay: All samples except DMSO control were treated with serum. Ostruthin (20–90 µM) was applied. The graph represents two independently performed experiments and needs further verification.

3.6. Ostruthin inhibits human VSMC (huVSMC) proliferation

The next step was to check whether the activity of ostruthin was restricted to rat VSMC. Therefore we have obtained human VSMC from the group of Dr. David Bernhard (AKH, Vienna). We optimized the resazurin conversion assay for this cell type and tested for ostruthin activity. Ostruthin used at 30 µM inhibits the proliferation of huVSMC as well (Figure C19).

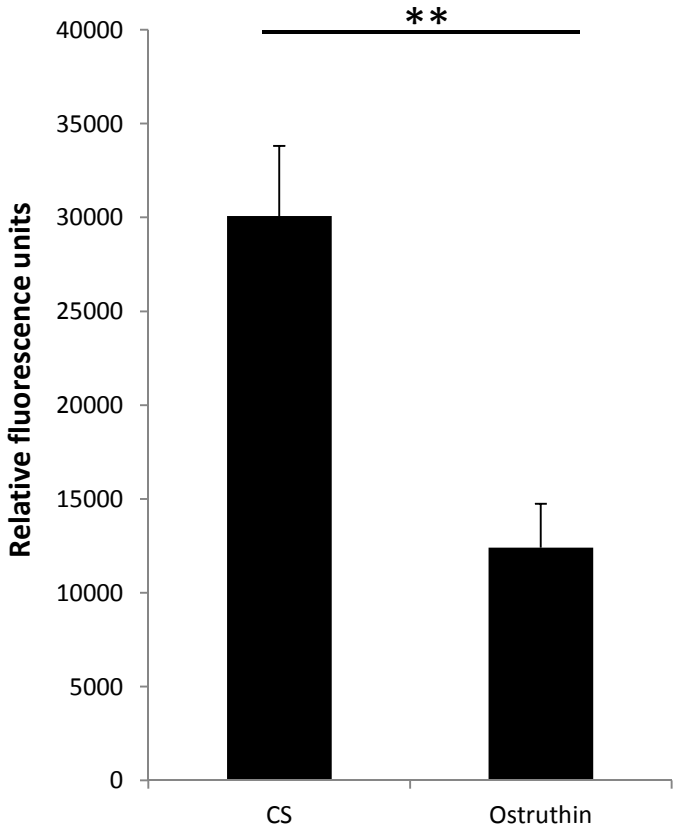


Figure C19: Ostruthin also inhibits huVSMC growth

Resazurin conversion assay in huVSMC: Ostruthin was used in a concentration of 30 µM; n=3; mean (±SD), ** $p < 0.01$, ANOVA/Tukey.

D DISCUSSION

During this diploma project, about 150 different plant extracts were tested for inhibition of VSMC proliferation. For several reasons we chose the resazurin conversion assay as the main screening method. It is a cheap and easy to handle application, which allows high throughput data generation in a 96-well plate set up and provides reliable results. Furthermore, a major advantage is the easy adaptation of this technique to different settings and cell types. Nevertheless, a drawback to this method is that it does not distinguish between inhibition of proliferation and cytotoxic or apoptotic effects.

1. Further considerations for targeting VSMC

Due to the complexity of atherosclerosis, there are different views considering the role of VSMC during the development and the progression of atherosclerosis. The large elastic arteries become dilated with advancing age and the intima thickens. The thickness of the arterial wall, as indexed by the thickness of the intimal and medial layers, increases in a linear fashion, nearly threefold, between the ages of 20 and 90 years, even in the absence of atherosclerotic plaques [108]. As a consequence, antiproliferative and anti-inflammatory treatment may slow down this thickening process and might play a major role in the prevention of atherosclerotic development. Therefore antiproliferative research may provide a theoretical basis for the development of a therapy.

Furthermore, the situation in late atherosclerosis is more complex, since VSMC produce connective tissue and are thought to contribute to plaque stability. Therefore Falk et al. even consider the recruitment, proliferation, and synthetic activities of VSMC as beneficial [13].
Further studies revealed that VSMC apoptosis is a critical process determining plaque stability which may in the worst case scenario result in plaque rupture [109].
In this late phase, substances which prevent VSMC apoptosis or which promote proliferation are of major interest. Figure C6 shows candidate plants for further pro-proliferative research.

A third application of antiproliferative research is the search for new stenting drugs. As stated above there are two different successful approaches dealing with restenosis. Firstly, there is the cytotoxic approach. Figure C7 shows such candidate plants.
Secondly, there is the cytostatic approach where drug eluting stents are used to inhibit VSMC proliferation (see chapter 5.1.2).
Nevertheless, the downfall of the cytotoxic approach is the high risk of thrombosis. Therefore it is absolutely clear that the second approach is the one we should follow. The screen yielded several such interesting plant extracts which will subsequently be discussed in more detail.

2. Active plant extracts:

2.1. Melia toosendan

Almost every part of the genus *Melia* is used as traditional herbal medicine, such as for the treatment of leprosy, eczema, asthma, malaria, and fevers [110]. These plants are known as a rich source of bioactive limonoids. Furthermore, toosendanin, the main limonoid of *M. toosendan,* has been used as anthelmintic vermifuge against *Ascaris* for more than fifty years in China and recently its anti-proliferative and apoptotic action in various human cancer lines was described by Zhang et al [111]. Furthermore, Xu et al. report that treatment of PC12 cells with a crude extract of the fruits of *M. toosendan* reduces cell growth in a dose-dependent manner without detectable cytotoxicity. In accordance with the results of Xu et al., our screening data, as well as light microscopy, did not show any signs of cytotoxicity in our cells. In future it would be interesting to ascertain whether toosendanin is the main active substance.

2.2. *Sideritis hyssopifolia*

Sideritis hyssopifolia grows in mediterranean regions, the Balkans, the Iberian peninsula and can also be found in central Europe and temperate Asia [112]. Traditionally, it is used as a herb in the preparation of tea in case of digestive disorders and is believed to strengthen the immune system, suppress the common cold, influenza and other viruses [113]. Furthermore, essential oils obtained from *Sideritis hyssopifolia* have been shown to inhibit the growth of *Bacillus anthracis* and *S. aureus* [114]. In our experiments, the crude extract of *Sideritis hyssopifolia* inhibited VSMC and HUVEC proliferation. The findings of this diploma work present a novel action for *Sideritis hyssopifolia* and warrant further studies to identify the lead structures.

2.3. *Aristolochia debilis*

In traditional chinese medicine (TCM) *Aristolochia* species are used to treat snake and insect bites, to promote lactation or urination and reduce edema [115].

TCM is gaining popularity in Western countries but certain safety issues such as the deliberate use of high amounts of heavy metals and the presence of aristolochic acids (AA) in Chinese traditional herbal preparations of that plant, invariably require attention [116]. AA were shown to be nephrotoxic and carcinogenic in animal studies with rodents [116]. Aristolochic acid nephropathy (AAN), a progressive renal interstitial fibrosis frequently associated with urothelial malignancies, was initially reported in Belgium, in more than 100 patients after the intake of slimming pills containing the Chinese herb *Aristolochia fangchi* [117]. In our experiments no cytotoxicity could be detected even under microscopic investigation. Furthermore initial FACS experiments revealed a G_1 arrest for both the DCM and methanol extract of *A. debilis*. For future work with this plant it would be of tremendous importance to identify whether these effects are simply due to AA or if there are further bioactive compounds which are not toxic. Nevertheless, considering the fact that several sources report nephrotoxicity and cancerogenicity as a result of systemic long term intake or

the administration of high dosages of AA, local delivery on drug eluting stents or balloons may represent a novel use for *Aristolochia debilis* derived pharmaceuticals.

2.4. *Stephania tetrandra*

Stephania tetrandra is a Chinese medicinal herb which has been used traditionally as a remedy for neuralgia and arthritis in China [118]. During the nineties slimming pills erroneously contained powdered roots of plants - picked in China - belonging to the *Aristolochia* instead of *Stephania* family [119]. This is due to the fact that herbal ingredients are traded using their common Chinese Pin Yin name which can lead to confusion since 'Fang Ji' can be used to describe the roots of *Aristolochia fangchi*, *Stephania tetrandra* or *Cocculus species* [120]. *Stephania tetrandra* is not known to contain AA but the precautionary principle led the authorities to ban *Stephania tetrandra* and *Aristolochia fangchi* from the market permanently [119]. Nevertheless, recent studies revealed that treatment of human monocytes/macrophages with an extract of *Stephania tetrandra* lead to reduced IL-6 production without visible cytotoxic effects [118]. One main bioactive compound is tetrandine which has been used as an antihypertensive drug in China. We found an antiproliferative action for *S. tetrandra* extract in VSMC as well as in HUVEC without visible cytotoxicity.

2.5. *Peucedanum ostruthium*

The rhizomes of *Peucedanum ostruthium* are used in the Alpine region as an ingredient of liqueurs and bitters, and as a herbal drug against inflammation and for the treatment of cardiovascular disease [107, 121]. The DCM extract of *P. ostruthium* rhizomes proved to be active during the screening procedure. Therefore we decided to track the activity by bioassay-guided fractionation. The active subfraction 2 (Figure C12) was subjected to sensitive and specific high-performance liquid chromatography-diode-array detection-mass

spectrometry (HPLC-DAD-MS; developed and performed by Mag. Sylvia Vogl, Department of Pharmacognosy, University of Vienna). The isolation of all compounds was not possible. Nevertheless, this method led to the identification and quantification of 7 coumarins, which were the major constituents of the extract: oxypeucedanin hydrate, oxypeucedanin, ostruthol, imperatorin, osthole, isoimperatorin, and ostruthin [107]. Literature search also revealed that coumarins are the main bioactive compounds of *P. ostruthium* [122-123]. Among the isolated compounds just one was found to be active – the coumarin ostruthin (Figure C14) [8, 107]. A broad literature search revealed that ostruthin, imperatorin, ostruthol, and oxypeucedanin hydrate were found to inhibit acetylcholinesterase activity in a thinlayer bio autography assay [124] and that these compounds are thought to represent ideal candidates for drug development against Alzheimer's disease [125]. Furthermore a pronounced activity of ostruthin against several species of rapidly growing mycobacteria was observed by Schinkowitz et al [126]. An antiproliferative action for ostruthin in VSMC has never been observed before. In opposition to our findings, an antiproliferative action of osthole in the smooth muscle cell line A10 was reported by Guh et al [127].

In a following step, we tested related (furano)coumarins for their inhibitory action and compared their structural differences. Besides ostruthin none of the tested (furano)coumarins proved to inhibit VSMC proliferation. Therefore it can be concluded that the geranyl group in the C-6 position is necessary for the inhibitory effect [8]. Additionally, we tested the activity of ostruthin in huVSMC and found an activity similar to that in rat aortic VSMC. This points out that the activity of ostruthin is not restricted to rat cells. In the next logical step, HUVEC cells were tested with resazurin conversion assay. Initial experiments showed that ostruthin might be a bit less active in HUVEC cells compared to rat VSMC. Ostruthin represents an interesting target for further investigations, and may represent a promising lead compound with pharmacological potential.

E MATERIALS AND METHODS

1. Materials

All cell culture reagents and chemicals were obtained from Lonza Group Ltd. (Basel, Switzerland), Sigma Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) or Carl Roth (Karlsruhe, Germany), unless otherwise stated.

Tylophorine was initially provided by Prof. Proksch (University of Duesseldorf, Department of Pharmaceutical Biology), but for further studies bought from Alexis Biochemicals (Switzerland).

1.1. Cell culture media and reagents

1.1.1. Cell culture reagents

Name	Provider
Calf serum	Lonza Group Ltd. (Basel, Switzerland)
Complete protease inhibitor	Roche Diagnostics (Basel, Switzerland)
DMSO	Fluka (Buchs, Switzerland)
Dulbecco´s Modified Eagle Medium (DMEM) 4.5 g/L glucose, without L-glutamine, without phenol red	Lonza Group Ltd. (Basel, Switzerland)
Ham's F-12 medium	Invitrogen (Carlsbad, CA, USA)
M231	Invitrogen (Carlsbad, CA, USA)
L-glutamine 200 mM	Lonza Group Ltd. (Basel, Switzerland)
Penicillin/streptomycin mixture (10.000 U/ml potassium penicillin/10.000 µg/ml streptomycin sulphate)	Lonza Group Ltd. (Basel, Switzerland)
RPMI 1640	Lonza Group Ltd. (Basel, Switzerland)

Name	Provider
SMGS (smooth muscle growth supplement)	Lonza Group Ltd. (Basel, Switzerland)
Trypsin (1:250)	Invitrogen (Carlsbad, CA, USA)

Table D1:

Calf serum was heat inactivated (30 min, 56°C), aliquoted and stored at -20°C.

1.1.2. Cell culture media

Table D2: Cell culture media and buffers

Cell Culture solution	Volume	Used Reagents
Rat VSMC Growth Medium	10% 100 U/ml / 100□g/ml 2 mM final Volume 500 ml	Calf Serum Penicillin/Streptomycin L- Glutamine in DMEM
Rat VSMC Starvation Medium	0,1% 100 U/ml / 100□g/ml 2mM final Volume 500 ml	Calf Serum Penicillin/Streptomycin L- Glutamine in DMEM
Human VSMC Growth Medium	0,1% final Volume 500 ml	SMGS in M231
Human VSMC Stop Medium	10% 100 U/ml / 100□g/ml final Volume 500 ml	Calf Serum Penicillin/Streptomycin in RPMI 1640
Human VSMC Wash Medium	100 U/ml / 100□g/ml final Volume 500 ml	Penicillin/Streptomycin in RPMI 1640

Rat VSMC Starvation Medium	0,1%	Calf Serum
	100 U/ml / 100□g/ml	Penicillin/Streptomycin
	2 mM	L- Glutamine
	final Volume 500 ml	in DMEM
PBS	8.0 g	NaCl
	0.2 g	KCl
	1.15 g	Na_2HPO_4
	0.2 g	KH_2PO_4
	0.1 g	$MgCl_2 . 6H_2O$
	0.1 g	$CaCl_2 . 2H_2O$
	final volume 1000 ml	in Aqua dest.
Trypsin/EDTA in PBS	0.05%	Trypsin
	0.02%	EDTA

1.1. Growth factors

Table D3: Growth factors

Name	Provider
Recombinant PDGF-BB, human	Bachem (Weil am Rhein, Germany)

Lyophilized PDGF-BB was dissolved in sterile aqua bidest to a final concentration of 0.1 µg/µl and further diluted in sterile PBS + 0.2% BSA to a final concentration of 10 ng/µl. Aliquots with 20 µl were stored at -20°C and prior to experiments diluted with DMEM to a final concentration of 1 ng/µl.

1.2. Flow cytometry buffers

Table D7:

Flow cytometry solutions:	Volume	Used reagents
FACS buffer pH 7.37	8.12 g	NaCl
	0.26 g	KH_2PO_4
	2.35 g	Na_2HPO_4
	0.28 g	KCl
	0.43 g	LiCl
	0.2 g	Na_2N_3
	0.36 g	Na_2EDTA
	ad 1000 ml	Aqua dest.
HBSS buffer pH 7.4	8.18 mg	NaCl
	400 mg	KCl
	19.75 mg	Na_2HPO_4
	50 mg	KH_2PO_4
	180 mg	$CaCl_2 . 2H_2O$
	97.5 mg	$MgSO_4$
	1000 mg	Glucose
	4760 mg	HEPES
	ad 1000 ml	Aqua dest.

1.3. Other equipment

Table D8:

Miscellaneous material	
Name	Provider
96-well Microplates	Greiner bio-one (Frickenhausen, Germany)
Serological Pipettes	Sarstedt GmbH (Nümbrecht, Germany)
Tissue Culture Dishes (6 cm, 10 cm)	Greiner bio-one (Frickenhausen, Germany)
Tissue Culture Flask 75 cm2	Sarstedt GmbH (Nümbrecht, Germany)

Table D9:

Technical Equipment	
Name	Provider
Digital SLR Camera E-330	Olympus Europa GmbH (Hamburg, Germany)
FACSCalibur™	BD Biosciences Pharmingen (San Diego, CA, USA)
Fluorescence Microscope BX51	Olympus Europa GmbH (Hamburg, Germany)
Galaxy Mini Microcentrifuge	VWR International Inc. (West Chester, PA, USA)
HERAcell™ 150 Incubator	Thermo Fisher Scientific Inc. (Waltham, CA, USA)
Heraeus™ B15 Incubator	Thermo Fisher Scientific Inc. (Waltham, CA, USA)
Heraeus™ Fresco™ Centrifuge	Thermo Fisher Scientific Inc. (Waltham, CA, USA)
HERAsafe™ KS Workbench	Thermo Fisher Scientific Inc. (Waltham, CA, USA)
Light Microscope CKX31	Olympus Europa GmbH (Hamburg, Germany)
RCT basic Magnetic Stirrer IKA™	Laboratory equipment (Staufen, Germany)
Tecan GENios™ Pro Tecan Sunrise™	Tecan (Mannedorf, Switzerland)
Vibrax VXR basic Shaker IKA™	Laboratory equipment (Staufen, Germany)
Vi-Cell™ XR Cell Viability Analyzer	Beckman Coulter (Fullerton, USA)
Vortex Shaker	VWR International Inc. (West Chester, USA)

Table D10:

Name	Provider
GraphPad PRISM™ version 4.03	GraphPad Software, Inc. (San Diego, Ca, USA)
Vi-Cell™ XR 2.03	Beckman Coulter (Fullterton, Ca, USA)

2. Methods

2.1. Cell culture

2.1.1. Cell isolation

VSMC from thoracic aortas of Sprague-Dawley rats were kindly provided by Kathy K. Griendling (Emory University, Atlanta, USA). HVSMC were kindly provided by Prof. Bernhard (AKH).

2.1.2. Cell cultivation

Both human and rat VSMC were cultured in growth medium at 37°C and 5% CO_2. They were grown to 90% confluence and passaged twice per week. For passaging, cells were washed with PBS and trypsinized with Trypsin/EDTA for up to three minutes. Subsequently, growth medium was added and the cells were centrifuged for 4 min at 1000 rpm. The cell pellet was re-suspended in growth medium and the cells were counted with a trypan-blue staining based cell viability analyzer (VicellTM XR, Beckmann Coulter, Fullerton, CA, USA). Cells were seeded in flasks, in 6, 12, or 96-well plates or in 6 mm dishes and grown to designated density. Passages 6-15 were used for experiments. Dependent on experimental requirements, VSMC were precultured with starvation medium for 24 h.

2.1.3. Cell storage

Cells were trypsinized, centrifuged at 1250 rpm (333 g), and re-suspended in ice cold freezing medium Cryovials with $1x10^6$ cells were kept at 4°C for 20 min. Subsequently, they were frozen at -20°C for one day, at -80°C for at least two days and at last transferred into liquid nitrogen. For reuse, frozen cells were thawed, immediately resolved in growth media, and directly grown in a medium cell culture bottle to the desired confluency of 90%.

2.2. Proliferation assays

2.2.1. Resazurin conversion assay

The assay is based on the metabolic conversion of resazurin to fluorescent resorufin that correlates with the cell number. [128] Cells were seeded in transparent 96-well plates (1×10^4 cells/well), serum-starved for 24 h, pretreated with the indicated concentrations of the respective samples or vehicle for 30 min, and subsequently stimulated for 48 h with calf serum (10%). Then cells were washed with PBS and incubated in DMEM containing 10 µg/mL resazurin for 2 h. The formed resorufin was measured by monitoring the increase in fluorescence at a wavelength of 580 nm using an excitation wavelength of 535 nm in a 96-well plate reader (Tecan GENios Pro; Tecan Group Ltd., Männedorf, Switzerland).

2.3. Flow cytometry

Flow cytometry is based on the emission of optical signals by cells or cell particles when they pass a beam of laser light. It is a method used for cell cycle analysis, measurement of cell death, membrane potential and oxidative burst. Furthermore it is commonly used for detecting expression of fluorescently labelled proteins and surface antigens.

When a cell or a cell particle passes a laser beam it scatters the light in several ways. Hereby, the amount of scattered light correlates with the size and the complexity of the cell.

The Forward Scatter (FSC) detects the light that is bended by the cell along the beam, representing a measure for the Volume of the cell. The Sideward Scatter (SSC) detects the light fractions scattered at right angle to the laser beam, representing a measure for granularity and size of the nucleus of the cell. The gained signals are detected and processed by a computer system.

All flow cytometric investigations were performed on a FACSCalibur™ by illuminating the cells with an argon laser (488nm). PI was used as a fluorescence agent.

2.3.1. Propidium iodide staining

The cell cycle distribution of VSMC was determined by propidium iodide (PI) staining. [129] PI, is capable of labeling DNA by stoichiometrically binding to nucleic acids. Therefore, the fluorescence emission is proportional to the DNA content of a cell and can be detected by flow cytometry with 562-588 nm filters.

For the PI staining of CS-stimulated cell, VSMC were seeded in 6-well plates ($5x10^4$ cells/well) and remained in 10% CS-containing DMEM. After 24 h VSMC were treated with test compounds or vehicle (DMSO 0.1%) for the indicated time, and subsequently trypsinized, fixed with ice-cold hypotonic fluorochrome solution buffer containing 50 µg/ml PI, and incubated for 2 h at 4°C. Cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA) by using CellQuest™ Pro software.

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G APPENDIX

1. Abbreviations

A

AA	Aristolochic acid
AngII	Angiotensin II
ANP	Atrial natriuretic peptide
APS	Ammonium persulfate

B

bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin

C

c-Met	c-MET Receptor Tyrosine Kinase
CAK	CDK-activating kinase
CDKs	Cyclin-dependent kinase
cGMP	cyclic Guanosinmonophosphate
CNP1	C-Type natriuretic peptide
COX	Cyclooxygenase

D

DAG	Diacylglycerol
DNTI	Drugs from nature targeting inflammation
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPI	Diphenylene iodonium

E

EC	Endothelial cells

ECM	Extracellular matrix
EDTA	Ethylenediaminetetreacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
eNOS	endothelial Nitrite oxide synthase
ERK	Extracellular signal-regulated kinase

F

FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor

G

G_1	Gap phase 1
G_2	Gap phase 2
GAP	GTPase-activating protein
GSK-3	Glycogen synthase kinase-3

H

HBEGF	Heparin-binding EGF-like growth factor
HEPES	Hydroxyethylpiperazineethanesulfonic acid
HRP	Horseradish peroxidase
hypophosphorylated	phosphorylated to a less than normal extent, or less than fully
hyperphosphorylated	phosphorylated to a more than normal extent, or fully saturated with phosphate groups
HSP60	Heat shock protein 60
Huvec	Human umbellical vein cells
huVSMC	human Vascular smooth muscle cells

I

ICAM-1	Inter-Cellular Adhesion Molecule 1
IGF	Insulin growth factor 1
iNOS	inducible nitrit oxide synthase
IL-1	Interleukin 1
IL-6	Interleukin 6

J

JAK	Janus kinase
JNK	C-Jun N-terminal kinase

L

LDL	Low density lipoprotein
LPS	Lipopolysaccharide
lipoB	lipoprotein B
LO	Lipooxidase

M

MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MMP	Matrix metalloproteinase

N

NFN	Nationale Forschungsnetzwerke
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOX	NADPH oxidase

O

oxLDL	oxidized LDL

P

PAA	Polyacrylamide
PBS	Phosphate buffered saline
Pc	Polyclonal
PCNA	Proliferating Cell Nuclear Antigen
PCI	Percutaneous coronary intervention
PDGF	Platelet derived growth factor
PKC	Proteinkinase C
PLD	Phospholipase D
PMSF	Phenylmethanesulphonylfluoride
PTA	Percutaneous transluminal angioplasty
PTCA	Percutaneous transluminal coronary angioplasty

R

ROS	reactive oxygen species
RTK	Receptor tyrosine kinase

S

SDS	Sodium dodecyl sulfate
SPE	Solid phase extract

T

TBS-T	Tris-buffered saline/Tween 20 buffer
TEMED	Tetramethylethylenediamine
TCM	Traditional chinese medicine
TGF	Transforming growth factor
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor-α

TNF-β	Tumor necrosis factor-β
TRIS	Trihydroxymethylaminomethane

U

uPA	Urokinase plasminogen activator

V

VSMC	Vascular smooth muscle cells
VEGF	Vascular endothelial growth factor

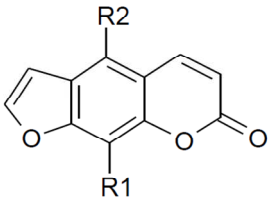
X

XO	Xanthine oxidase

2. Supplementary data

2.1 Chemical structure of coumarins

Linear furanocoumarins:



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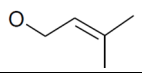
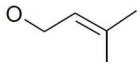
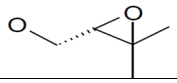
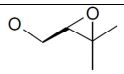
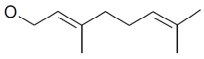
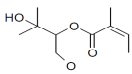
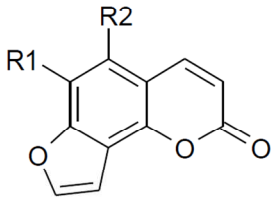
isoimperatorin	H	
imperatorin		H
heraclenin		H
oxypeucedanin	H	
bergamottin	H	
ostruthol	H	

Angular furanocoumarins:



	R1	R2
isobergapten	H	OMe

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