PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATIONS ON CONSTITUENTS OF MEDICINAL PLANTS WITH POTENTIAL ANTI-CANCER ACTIVITY

Dissertation

Zur Erlangung des Doktorgrades an der Naturwissenschaftlichen Fakultät der Karl-Franzens-Universität Graz

Vorgelegt von

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Graz, März 2011
“Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt.”

(Albert Einstein)
ACKNOWLEDGEMENTS

I am very thankful to my adviser Prof. Dr. Rudolf Bauer who gave me the opportunity to perform my thesis at the Department of Pharmacognosy, Institute of Pharmaceutical Sciences, Karl-Franzens University of Graz, Austria. I thank him for the support, confidence and the freedom I had during my work.

I am also very thankful for the fruitful cooperation with the Center for Medical Research (ZMF), Medical University of Graz, Austria. Special thank to Dr. Beate Rinner (ZMF, Core facility flow cytometry) and her team members Heike Knausz and Karin Bürger for many experiments and stimulating discussions. Many thanks to Prof. Dr. Helmut Schaider (Cancer Biology Unit, Department of Dermatology and Centre for Medical Research) for support and providing the melanoma cell lines. Furthermore, I want to express my gratitude to Univ.-Ass. Dr. Birgit Lohberger (Department of Orthopedics and Orthopedic Surgery) and her coworkers for the time they invested in studying several pharmacological effects of some of my isolated substances. Last but not least, I want to thank Dr. Alexander Deutsch (Division of Hematology) for real-time PCR experiments and interesting discussions.

A special role was also occupied by Prof. Dr. Thomas Efferth and his coworkers who introduced me into cell culture techniques and different cell assays at the German Cancer Research Center (DKFZ), Heidelberg, Germany and provided several cancer cell lines. I also thank Thomas for several discussions, advices and encouragements.

This thesis would not have been possible without Dr. Herbert Böchzelt (Joanneum Research Forschungsgesellschaft mbH, Graz, Austria), Dr. Stefan Kahl and Prof. Xiao-Jiang Hao (Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China) who supplied me with the fundament of my thesis in form of a preliminary thesis and the plant material.

I am grateful to my colleagues Dr. Olaf Kunert and Dr. Martina Blunder for structure elucidation and Dr. Wolfgang Schühly for structure elucidation, support and providing me Helianthus angustifolius for activity-guided isolation. Moreover, I am thankful to Dr. Gerald Rechberger (Institute of Molecular Biosciences) for high-resolution mass analysis.
Great work was also performed by Prof. Dr. Günther Heubl (Department of Biology, Ludwigs-Maximilian University, Munich, Germany) concerning plant identification.

I thank Prof. Dr. Jose Rios (University of Valencia, Spain) and Assoc. Prof. Dr. Ioanna Chinou (University of Athens, Greece) for sending me several shikonin and alkannin derivatives.

Big thanks also to my office colleagues and friends Mag. Ute Widowitz and Mag. Elisabeth Feizlmayr for a great time, a lot of laughter, many discussions and support. I express also my gratitude to all other colleagues who supported me.

Sincere thanks to my parents and all good friends for permanently supporting me on my way and being there whenever I needed them!

Last but not least, I want to thank the Fonds zur Förderung der wissenschaftlichen Forschung (FWF) for financial support (Project number P 21114).
Ich versichere hiermit, dass die vorliegende Dissertation eine eigenständige Orginalarbeit darstellt, die von mir selbstständig durchgeführt, angeführt und abgefasst worden ist.

Jegliche Information, die ich aus bestehenden Arbeiten entnommen habe, wurde als Zitat erkenntlich gemacht.

Des Weiteren versichere ich, dass ich keine anderen als die angegebenen Hilfsmittel verwendet habe.

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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
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<tr>
<td>AP1</td>
<td>activator protein 1</td>
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<tr>
<td>ASE</td>
<td>accelerated solvent extraction</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>DAD</td>
<td>diode array detector</td>
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<td>DCM</td>
<td>dichloromethane</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>EtOAc</td>
<td>ethyl acetate</td>
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<td>FCPC</td>
<td>fast centrifugal partition chromatography</td>
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<td>FDA</td>
<td>US Food and Drug Administration</td>
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<tr>
<td>GI</td>
<td>growth inhibition</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
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<tr>
<td>MeOH</td>
<td>methanol</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NST/PEG</td>
<td>Naturstoff – polyethylene glycol</td>
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<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PE</td>
<td>petroleum ether</td>
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<tr>
<td>STS</td>
<td>soft tissue sarcoma</td>
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<tr>
<td>TCM</td>
<td>traditional Chinese medicine</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>XTT</td>
<td>sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate</td>
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1. INTRODUCTION

1.1. WHY WORKING ON ANTI-CANCER AGENTS?

Cancer is the second most common cause of death in most developed countries and an increasing cause of death in developing countries according to the World Health Organization (WHO). Already 50% of all cancer cases appear in developing countries in South America and Asia. A 45% increase of the number of global cancer deaths is expected by WHO from 2007 to 2030 since the global population increases and ages more and more. It is assumed that the world population increases by 80 million people each year and will be 7.5 billion in 2020 and 8.9 billion in 2050, whereby, the main growth will take place in developing countries. Life expectancy is rising from 41 years in 1950 to 64 years in 2000 and approximately 71 years in 2020. This will be also reflected in the world’s cancer incidence (Schwartsmann et al., 2002). WHO further estimates that 84 million people will die of cancer without intervention between 2005 and 2015.

In the last centuries, much research has been done on how people can fight cancer. The most common techniques comprise surgery, radiation therapy, chemotherapy, hormonal therapy and combinations of these. However, it must be remembered that especially medicinal treatments have still a limited effectiveness because of, for example, resistances of tumors and lethal side effects. All too often, the used anti-cancer drugs show no high specificity to cancer cells because cancer is in a sense a term for many diseases which all manifest in the excessive proliferation of cells of the body (Efferth et al., 2008). Therefore, the identification and development of new anti-cancer drugs are still important research objectives.

1.2. THE ROLE OF NATURAL PRODUCTS IN THE DEVELOPMENT OF NEW ANTI-CANCER AGENTS

To say it with the words of Tulp and Bohlin (2002): “In retrospect, the use of natural products has been the single most successful strategy in the discovery of novel medicines” and, furthermore, “for all important molecular targets in the human body, natural compounds exist in other species, and in more than one particular species.”
Natural products are compounds derived from plants, microorganisms, invertebrates or vertebrates. In plants, they typically comprise the secondary plant metabolites. In contrast to primary metabolites, secondary plant metabolites are not necessary for the plant’s primary or energy metabolism. At first, they were thought to be waste products. However, they are not waste but important for the ecological fitness of a plant. They help the plants protecting themselves from pathogens and herbivores such as insects, mammals, bacteria, fungi and viruses. Moreover, they play an important role in attracting animals responsible for pollination and seed dispersion and in competition with other plants (Wink, 1988).

In the search for new medicines, plants and their constituents have always played a crucial role, not only in the search for new anti-cancer drugs but also in the development of traditional medicine systems (Cragg et al., 2009). WHO estimates that about 65% of the world’s primary health care systems are based on traditional medicine which mostly relies on the usage of plants. Newman and Cragg (2007) have shown that out of 157 anti-cancer drugs approved by the FDA and 18 anti-cancer drugs reported in time-relevant textbooks, 113 are natural products, derived from natural products or inspired by natural products (figure 1). Twenty of all compounds available are biologicals or vaccines and counted neither for natural nor for synthetic drugs. Therefore, these 113 natural agents represent 72.9% of all available anti-cancer drugs and demonstrate the big influence of natural products in the development of new anti-cancer agents and the minor influence of combinatorial chemistry. Famous examples of natural products which are used today in cancer therapy are the vinca alkaloids (vinblastine, vincristine), taxanes (paclitaxel, docetaxel), podophyllotoxine (etoposide, teniposide), camptothecines (topotecan, irinotecan) and anthracyclines (doxorubicin, daunorubicin) (Effert et al., 2007a). The vinca alkaloids vinblastine and vincristine isolated from Catharanthus roseus G. were the first clinically used plant derived anti-cancer compounds (Cragg et al., 2009). Vinblastine was also used in this work to serve as positive control in several assays.

It is estimated that there are 300 000 to 500 000 higher plant species distributed all over the world but only 6 to 15% are phytochemically and pharmacologically investigated so far (Cragg et al., 2009). This further indicates that there is still a huge potential for the discovery of new plant derived compounds. Other sources of new bioactive metabolites comprise microorganisms or marine organisms. The chemotherapeutics daunomycin, doxorubicin, dactinomycin and mitomycin were isolated from Streptomyces species and are in clinical use now (Schwartzmann et al., 2002).
A classical method for finding new lead compounds is the extraction of plant material with solvents of different polarity and the subsequent activity-guided fractionation and isolation. There is a broad spectrum of in vitro assays using cancer cell lines to investigate the effects of plant extracts or natural compounds. Many are based on cytotoxicity such as the thymidine incorporation assay, ATP bioluminescence assay, LDH assay or assays based on tetrazolium salts (MTT, MTS or XTT). Others are for example biochemical- and genetics-based using transformed cells (Cragg et al., 2009, Hatok et al., 2009). In this thesis, the XTT viability assay should be mainly used to determine the cytotoxicity of extracts, subsequent produced fractions and finally isolated pure compounds. This assay is based on the cleavage of the tetrazolium salt XTT by mitochondrial dehydrogenases and provides an easy, safe and non-radioactive method to determine cell growth and viability (Scudiero et al., 1988). It is a rapid and reproducible method which reduces the amount of culture medium, cells and compounds required. Moreover, it allows a direct measurement of the samples in a microplate (Hatok et al., 2009). Since this assay gives no information about the molecular mechanism, further investigations of isolated compounds should reveal possible modes of action within the cells.

Figure 1. The origin of all available anti-cancer agents from the 1940s to June 2006 (Newman and Cragg, 2007). N: natural product, ND: derived from a natural product. Usually a semisynthetic modification, B: biological; usually a large (>45 residues) peptide or protein, S: totally synthetic drug, often found by random screening/modification of an existing agent, S*: made by total synthesis, but the pharmacophore is/was from a natural product, V: vaccine, subcategory NM: natural product mimic.
1.3. **The potential of traditional Chinese medicine in the discovery of new anti-cancer agents**

Traditional Chinese medicine (TCM) has developed over 5000 years and comprises a huge knowledge of plants, animals and minerals and their application as medicine. It is still an important part in the primary health care system in China especially in rural areas but also in urban and well-developed areas (Efferth et al., 2007a). In contrast to western allopathic medicine, TCM does not focus on single targets but considers the entire human body and designs individual therapies. These therapies do not only include herbal medicines but also acupuncture, massage and moxibustion (Konkimalla and Efferth, 2008). Since TCM comprises knowledge of millennia, it can be assumed that most inactive plants and recipes have disappeared over the years and that TCM is a treasure chest full of bioactive plants (Efferth et al., 2008). During the last centuries, TCM has also become very famous in Western countries and enormous effort has been made to investigate TCM on a scientific basis (Efferth et al., 2007a, Konkimalla and Efferth, 2008). In 2006, the export revenue of China was increased by 31% compared to 2005 and the tendency is still rising. 380 000 licenced TCM physicians are registered in China and, in 2005, 620 000 students went to 32 TCM colleges and universities (Konkimalla and Efferth, 2008).

Estimated 700 to 800 plants are used in TCM to treat cancer or related syndromes. Several tumor therapeutics have already been derived from or have a direct relationship to TCM and show a broad spectrum of actions within the cell (Efferth et al., 2007a). Some of them, for instance, disrupt the cell cycle. Vinca alkaloids inhibit the formation of microtubules by binding to tubulin. Microtubules are part of the mitotic spindle and are necessary during cell division. As a result of this inhibition, the cells are arrested in mitosis (Duflos et al., 2002). Paclitaxel, originally isolated from *Taxus brevifolia* but also present in *Taxus chinensis*, works the other way round by inhibiting depolymerisation of tubulin (Efferth et al., 2007a). Camptothecin was isolated from *Camptotheca acuminata* and inhibits the DNA ligation after topoisomerase I induced single-strand DNA breaks. These breaks are necessary for reducing the torsion tension of super-coiled DNA during transcription and replication. Although camptothecin could not be used as chemotherapeutic directly, it was the starting point of topotecan and irinotecan – two water soluble derivatives which are now used in cancer therapy (Pommier, 2006). During cell replication and division, topoisomerase II induces DNA double-strand breaks to reduce tension. It also repairs these breaks immediately. Through inhibition of this enzyme, these breaks exist longer which can lead to the induction of apoptosis (Giles...
and Sharma, 2005). Etoposide and teniposide, derivatives of podophyllotoxin which has been isolated from *Podophyllum peltatum*, are such inhibitors and established cancer therapeutics. Other targets of cancer agents comprise signal-transduction proteins such as kinases, angiogenesis and telomerases, enzymes involved in the immortalization of tumor cells. Compounds affecting these targets are emodin (from *Rheum palmatum*), capsaicin (from various *Capsicum* species) and verbascoside (from *Pedicularis striata*), respectively (Efferth et al., 2007a).

![Chemotherapeutics](image_url)

*Figure 2. Examples of plant-derived chemotherapeutics mentioned in the text.*

Except of *Helianthus angustifolius*, the plants investigated in this PhD thesis have been used in China. In a former PhD thesis (Kahl, 2005), a database including 561 species used to treat cancer or ulcers has been created. Immunostimulatory plants were not included. The Latin and Chinese name, Chinese characters, Pinyin transcription, the plant parts used and already performed studies can be found in this database. Sources consisted of the traditional Chinese medicine, the Chinese Materia Medica, which is a subcategory of TCM, the Chinese herbal folk medicine, which is not included in the Chinese pharmacopoeia, and some relevant publications (Kahl, 2005). Subsequently, 76 plants of this database were acquired or collected in China and subjected to initial screenings. Extracts reducing the cell growth of human CCRF-CEM leukemia cells below 20% of vehicle-treated control cells at a concentration of 10 µg/ml were regarded as active (Kahl, 2005, Efferth et al., 2008). Thereby, 23 out of 253 extracts showed high
activity (Efferth et al., 2008). In this work, some of these plants were further investigated, fractionated and the active compounds isolated. *Helianthus angustifolius* was found to exhibit high cytotoxicity in a screening of randomly collected plants from North America. Since almost nothing was known about this plant, it was included in this thesis and activity-guided fractionated using human CCRF-CEM leukemia cells as monitor.
2. GENERAL PART

2.1. WHAT´S WRONG WITH CANCER CELLS?

“Cancer is a group of diseases characterized by unregulated cell growth and the invasion and spread of cells from the site of origin, or primary site, to other sites in the body” (Pecorino, 2008). According to Hanahan and Weinberg (2000) cancer cells need to acquire autonomous growth, insensitivity to growth inhibitory signals, a way to evade programmed cell death (apoptosis), unlimited replication potential, sustained angiogenesis, tissue invasion and metastasis (figure 3). This process has several steps, involves several genetic alterations and is common to most, if not all, cancers. Especially the last point is characteristic for malignant tumors in contrast to benign tumors (Pecorino, 2008).

Since cancer development requires several steps and many cell defense mechanisms such as DNA repair and cell cycle checkpoints have to be overcome, it is comprehensible that there is an increased risk of cancer with age and that cancer is more prevalent in these days due to the fact that life expectancy was and is still increasing (Pecorino, 2008).

Cell proliferation, apoptosis and differentiation contribute to the overall cell number of an individuum. Changes or failures in these processes affect the balance and can lead to the development of cancer. Objectives in cancer therapy are the prevention of proliferation (cytostatic effects) and the killing of cancer cells (cytotoxic effects). Three main classes of cancer can be distinguished: Carcinomas which have their origin in epithelial cells, sarcomas which develop from mesoderm cells and adenocarcinoma which arise from glandular tissues. Up to now, over 100 types of cancer are described and classified (Pecorino, 2008).
2.2. Targets of Chemotherapy

“Conventional chemotherapy uses chemicals that target DNA, RNA, and protein to disrupt the cell cycle in rapidly dividing cancer cells and thus has broad specificity. The ultimate goal of cytotoxic chemotherapy is to cause severe DNA damage and to trigger apoptosis in the rapidly dividing cancer cells” (Pecorino, 2008).

Since cancer is characterized by abnormal cell proliferation, one target of chemotherapy is affecting the cell cycle itself. The cell cycle is a sequence of events a cell has to pass through between one cell division and the next. It is divided into four stages: G1/G0-, S-, G2- and M-phase (figure 4). In the so called S-phase, the DNA is replicated. In the M-phase, mitosis and cytokinesis take place, and at its end the two daughter cells divide. The two G-phases are called “gap”-phases in which the cell prepares all steps for the S- or M-phase. Most human cells are in the so called G0-phase. This is an inactive phase outside the cell cycle in which cells do not divide. Mitogens or growth factors are necessary to repatriate such cells into the cell cycle (Pecorino, 2008). In eukaryotic cells, control of the cell cycle is a basic process and several checkpoints make sure that all events of the actual phase are completed before the next phase can be entered. If anything is missing or defect, the cell pauses at a checkpoint and tries to complete the phase or repairs existing damages. If this is not possible, the cell undergoes apoptosis. There are at least three well-founded checkpoints: the G1 checkpoint, the S phase checkpoint, and the G2/M phase checkpoint. Central players in these checkpoints and cell cycle control are the so called cyclines and cyclin-dependent kinases (Cdns), checkpoint kinases (Chk) and dual specificity phosphatases (Newman et al., 2002). In human tumors, the cell cycle control is frequently defective. For instance, cyclines and Cdk genes are often overexpressed leading to growth signal autonomy. Flavopiridol a semi-synthetic flavonoid is a competitive inhibitor of all Cdns tested. It inhibits the expression of some cyclines and arrest cells in the G1/S- and G2/M-phase (Pecorino, 2008). Other important chemotherapeutic targets can be found during mitosis and S-phase. For example, tubulin interactive agents, actin inhibitors and kinesin inhibitors arrest cells in mitosis and prevent cell division. Topoisomerase II inhibitors such as doxorubicin disrupt DNA synthesis and arrest cells in the S-phase (Newman et al., 2002).
Figure 5. Overview of apoptosis regulation and signaling pathways (Cell Signaling Technology, 2008).
Apoptosis is a programmed cell death and cancer cells have found a way to escape it. It plays an essential role in the human morphogenesis, the control of the overall cell number and in eliminating cells with DNA damages and the potential to cause cancer. Therefore, induction of apoptosis is a main target for cancer chemotherapy. Apoptosis is a highly regulated process comprising cell shrinkage, membrane blebbing, chromatin condensation and precise fragmentation. Finally, macrophages and neighbored cells incorporate the remaining cell parts. In contrast to necrosis (uncontrolled cell death) where membranes lose their integrity, cells swell and spill out their contents into the surrounding tissue causing inflammations, apoptosis is not accompanied by any inflammation process (Pecorino, 2008, Bröker et al., 2005). In human cells, apoptosis can be induced by external “death factors” or internal signals such as DNA damage or oxidative stress. Therefore, two apoptotic ways can be distinguished: the extrinsic and intrinsic pathway. Central enzymes in both pathways are caspases which cleave proteins at aspartate residues. The name comes from cystein-rich aspartate proteases. 13 different caspases have been identified in mammals so far (Pecorino, 2008). They are synthesized as inactive procaspases consisting of a prodomain and p20 large and p17 small subunits. The activation needs a series of cleaving events: the small and large subunits are separated followed by the removal of the prodomain. Once activated, they possess the ability to activate the same and other caspases (Degterev et al., 2003).

An overview of the complexity of apoptosis is shown in figure 5. In the extrinsic pathway, death factors (for example Fas and TRAIL ligand) bind to death receptors (for example Fas and TRAIL receptor) and trigger the activation of the initiator caspase-8 through activation of death domains and adapter proteins. Caspase-8 then activates executioner caspases-3, -6 and -7 (Pecorino, 2008). In cancer chemotherapy, special attention has to be drawn at TRAIL ligand and receptor since it predominately kills cancer cells and spares normal cells as long as the tumor is not resistant to TRAIL (Fulda and Debatin, 2004).

The intrinsic pathway starts with the mitochondria. The B-cell lymphoma-2 (Bcl-2) protein family is located at the outer membrane of the mitochondria and comprises about 20 members and two groups. One group possesses anti-apoptotic properties the other one pro-apoptotic. The family is divided into three subfamilies which contain one to four Bcl-2 homology (BH) domains: the anti-apoptotic subfamily with four BH domains (bcl-2, bcl-xL, mcl1, bcl2a1, bcl-w and bcl-b), the pro-apoptotic bax-like subfamily lacking the BH4 domain (bax, bak and bok) and the structurally diverse BH3-only subfamily (bid, bad, bim, bik, bmf, noxa, puma and hrk) displaying homology only in the BH3 domain (Taylor et al., 2008, Pecorino, 2008). It is assumed that these proteins form or block channels in the outer membrane and, thus, regulate the release of apoptotic mediators
(for example apoptosis-inducing factor (AIF) and cytochrome c) from the intermembrane space of the mitochondria. The ratio of pro- and anti-apoptotic proteins determines the overall effect. By activation, bax or Bbk increase the permeability of the outer membrane with the help of bid and bim. Subsequently, cytochrome c and procaspase-9 are released into the cytosol and assemble into the so called apoptosome complex along with dATP and Apaf-1. This triggers the activation of the initiator caspase-9 which in turn activates the executioner caspases-3, -6 and -7. Both pathways are not separated from each other but cross-linked. For example, caspase-8 of the extrinsic pathway can activate bid. Bid activates the intrinsic pathway by activating bax and bak. Activation of the executioner caspases results in activation of DNase and the degradation of target proteins such as nuclear lamins, cytoskeletal proteins (actin and intermediate filaments) and kinases of cell signaling. This leads to cell shrinkage, membrane blebbing and DNA cutting into the typically in apoptosis occurring (multiples of) 180 bp (Pecorino, 2008).

Another form of cell death is mitotic catastrophe. It occurs during mitosis or because of mitotic failure. It probably results from defective cell cycle checkpoints and cellular damage and is supposed to prevent aneuploid cells. It happens close to or during metaphase or at the polyploidy checkpoint and is controlled by cell cycle specific kinases. Chromatin condensation, release of proapoptotic proteins, caspase activation and DNA degradation can be observed. Mitotic catastrophe can be induced by microtubule hyper- (taxanes, elutherobins) or depolymerizing agents (Vinca alkaloids, colchicines) (Castedo et al., 2004).

A non-apoptotic pathway beside necrosis is autophagy (self-digestion). Under physiological conditions, autophagy is important in cell housekeeping and recycling of whole organelles and macromolecules. Autophagosomes fuse with lysosomes to autolysosomes in which organelles or macromolecules are digested by hydrolases and released back to the cytosol. Increasing stress such as hypoxia increases autophagy activity, finally promoting the so called type II or autophagic cell death. Tamoxifen is one substance increasing the autophagy in breast cancer (Hoyer-Hansen and Jäättelä, 2008, Bröker et al., 2005). Another non-apoptotic death is the so called paraptosis. Thereby, mitochondria and endoplasmic reticulum swelling lead to cytoplasmic vacuolation. Mitogen-activated protein kinases, the receptor TAJ/TROY and insulin-like growth factor I receptor seems to be involved, instead of caspase activation or formation of apoptotic bodies. However, this kind of cell death is not yet fully understood (Bröker et al., 2005).

It is assumed that not only one death program can be activated at the same time and that cells can switch between different programs. The overall death program seems to be determined by the speed and effectiveness of its activation and execution (Bröker et al., 2005).
2.3. Investigated Plants

2.3.1. Bischofia javanica

Taxonomy
Class: Rosopsida
Subclass: Rosidae
Order: Euphorbiales
Family: Euphorbiaceae
Genus: Bischofia
Species: Bischofia javanica BLUME

The family of Euphorbiaceae comprises about 300 genera and 7500 species, occurs mainly in the tropics and shows a large variety from wide-leaved trees in tropical rainforests to succulents in arid climates. They are characterized by a superior, mostly three-locular ovary, which decompose very often in three parts when ripe. Flowers are inconspicuous and mostly dioecious (Frohne and Jensen, 1998).

Distribution and Plant. Bischofia javanica (figure 6) is an evergreen, dioecious tree, native to Okinawa, Japan, Southeast Asia and Australia (Wada and Tanaka, 2005; Misra and Tewari, 1971) and occurs in humid valley forests (Zheng et al., 2004). It reaches a height of 40 m and a diameter of 2.3 m. The trunk is short and erect; the bark is almost smooth, brown and contains a red milky sap. Leaves are trifoliate, rarely palmate. Petioles are 8 – 20 cm long. The male inflorescence is 8 – 13 cm long and pubescent to glabrous, the female is 15 – 17 cm and pendant. The berry-like fruits are brown and globular or subgloboar (Zheng et al., 2004).


Traditional Use. The juice of the leaves is used in India to treat wounds (Misra and Tewari, 1971). In TCM, the plant has a low significance in the treatment of cancer (Kahl,
2005). The leaves are applied to ulcers, the red sap of the stem to sores. The fruits are a tonic for babies and the roots administered as diuretic. Elsewhere, this species is used for burns, coughs, cracked feet, diarrhea, fever, gastritis, ophthalmia, sores, throat ailments, tonsillitis, toothache, urticaria and wounds (Duke and Ayensu, 1985a).

**Chemical composition and pharmacology.** *Bischofia javanica* was reported to possess antiulcer, antihelmintic, antidysenteric (Gupta et al., 1988) and antinematodal activities (Alen et al., 2000) as well as anti-microbial activities against a broad spectrum of microorganisms (Khan et al., 2001).

Concerning its anti-cancer activity, the pentacyclic triterpene betulinic acid and derivatives, isolated from the bark of *Bischofia javanica*, were reported to be potent DNA topoisomerase II inhibitors (Wada and Tanaka, 2005). Betulinic acid was also shown to be potently effective against a wide variety of cancer cells, whereas, healthy cells were not affected or much more resistant (Fulda, 2009, Mullauer et al., 2010, Zuco et al., 2002). It induced apoptosis via direct mitochondrial perturbations, caused loss of mitochondrial membrane potential and, therefore, led to the release of soluble factors such as cytochrome c, activation of caspase -3 and nuclear fragmentation. Moreover, it seems that betulinic acid has an influence on Bcl-2 family protein members. For example, upregulation of bax expression was observed, whereas, bak and bad expression levels were not influenced (Fulda, 2009). Additionally, betulinic acid was also able to suppress tumor growth in vivo. Intraperitoneal injection reduced the tumor development of established melanoma xenograft in nude mice (Pisha et al., 1995) and increased the survival time of mice in a xenograft mouse model of ovarian cancer (Zuco et al., 2002). Currently, betulinic acid is evaluated in a phase I/II clinical trial in the tropical treatment of dysplastic nevi (Fulda, 2009).

From wood and leaves of *Bischofia javanica*, β-sitosterin, β-(β-sitosteryl)-D-glucoside, stigmasterin and β-sitostenon were isolated (Gupta et al., 1988, Kahl, 2005, Misra and Tewari, 1971). Phytosterols like β-sitosterol are suggested to protect from the most common cancers in Western countries, such as colon, breast and prostate cancer (Awad and Fink, 2000). β-sitosterol inhibited the growth of COLO 320 DM colon cancer cells and exhibited chemopreventive effect in DMH-induced experimental carcinogenesis (Baskar et al., 2010). β-sitosterol glucoside decreased the growth and viability of HT-29 colon cancer cells, arrested the cells in the G2/M phase and led to apoptosis, whereas noncancerous COS-1 cells were hardly affected (Jayaprakasha et al., 2010).

Friedelin, friedelinol, friedelinolacetate, epifriedelinol, friedelan-3α-ylacetate and epifriedelanolacetate were isolated from leaves, stem, wood and bark of *Bischofia*
Bischofia javanica (Gupta et al., 1988, Kahl, 2005). Friedelin showed significant cytotoxic activity against HL-60 leukemia, SK-OV-3 ovarian, A549 lung adenocarcinoma and HT-29 colon cancer cells in vitro (Ding et al., 2010, Thao et al., 2010).

![Figure 7. Structures of compounds isolated from Bischofia javanica.](image)

β-Amyrin, ursolic acid and flavonoids like chrysoeriol, fisetin, quercetin, luteolin-7-O-glucoside and quercitrin were also isolated (Gupta et al., 1988, Misra and Tewari, 1971). Ursolic acid appeared to be protective against cancer, decreased the expression of apoptosis suppressor proteins in several cancer cell lines and was found to be a potent inhibitor of NF-κB (Aggarwal and Shishodia, 2006). Fisetin has been shown to have antiproliferative and apoptosis inducing effects in human prostate cancer cells (Haddad et al., 2010) and human pancreatic AsPC-1 cancer cells (Murtaza et al., 2009). It inhibited cyclin-dependent kinase activities in human HT-29 colon cancer cells resulting in cell cycle arrest (Lu et al., 2005). It also caused DNA condensation, cleavage of PARP and procaspases-9, -7, and -3, decrease of antiapoptotic bcl-xL and bcl-2 and increase in proapoptotic bak and bim levels in human HCT 116 colon cancer cells (Lim and Park, 2009). Quercetin has demonstrated strong growth inhibitory activity in breast, colon, lung and ovarian cancer cells (Jagtap et al., 2009). It induced apoptosis in HT-29 colon cancer cells, arrested the cells in the G1/G0 phase and upregulated proapoptotic proteins, such as AMPK, p53 and p21. In in vivo experiments, the tumor volume could be significantly reduced by quercetin over 6 weeks (Kim et al., 2010). In vivo and in vitro studies also revealed its chemopreventive effects against DNA damage and precancerous changes (Jagtap et al., 2009).

Bischofianin, a dimeric dehydroellagitannin consisting of galloylglucose and dehydroellagitannin moieties was isolated from the leaves of Bischofia javanica together with the tannins garaniin, corilagin, furosin, punicalagin and procyanidin B-1 (Tanaka et al., 1995).
**2.3.2. **Bryophyllum pinnatum

**Taxonomy**
- **Class:** Rosopsida
- **Subclass:** Rosidae
- **Order:** Saxifragales
- **Family:** Crassulaceae
- **Genus:** Bryophyllum
- **Species:** Bryophyllum pinnatum Kurz.

Crassulacea are succulent herbs with a typical five-fold florescence and a superior ovary and native to dry regions. The root tips are dark red because of anthocyanines. The Crassulacean acid metabolism, a special form of photosynthesis is named after them. This metabolism enables the plant to fix CO$_2$ during the night in form of malate. During the day, these C$_4$-acids are cleaved, CO$_2$ released and photosynthesis can take place without stomata opening and water loss (Frohne und Jensen, 1998).

**Distribution and Plant.** Bryophyllum pinnatum (figure 8) is native to Africa and cultivated and naturalized in the Chinese provinces Fujian, Guangdong, Guangxi, Taiwan and Yunnan. It is an herb with a size of 40-150 cm. The stems are usually branched; the leaves are pinnately with 3-5 leaflets. Flowers are pendulous; the calyx tubular. The corolla has a red to purple color and a sparsely ciliate base. It flowers from January until March (Zhengyi and Raven, 2001).

**Synonyms.** According to TROPICOS®, five synonyms are known: Bryophyllum calycinum Salisb., Cotyledon pinnata Lam., Kalanchoe pinnata (Lam.) Pers., Sedum madagascariense Clus., Verea pinnata (Lam.) Spreng.

**Traditional Use.** In China, Bryophyllum pinnatum is used as antiseptic, bactericidal, diuretic, emollient, hemostatic, soporific and vulnerary and to treat boils, bruises, bugbites, burns, cancer, cold, colic, congestion, corns, cough, dysmenorrhea, earache, enteritis, epilepsy, erysipelas, fever, gonorrhea, headache, hypertension, nephritis, neuralgia, ophthalmia, otalgia, palpitations, rheumatism, scabies, sores, spasms, sprains, swelling, tumors, ulcers, urethritis and wounds. Leaves are applied as
rubefacient for neuralgia, pain and rheumatism (Duke and Ayensu, 1985a). In Nigeria, it is used for earache, cough, diarrhea, ulcers, insect bites, heart troubles, dysmenorrheal, epilepsy, arthritis and abscesses (Akinsulire et al., 2007). In Guyana, the juice of fresh leaves is used as anti-inflammatory and antiseptic agent (Abdellaoui et al., 2010).

**Chemical Composition and Pharmacology.** Investigations of several traditionally prepared and methanolic leaves extracts of *Bryophyllum pinnatum* exhibited antimicrobial activity (Akinsulire et al., 2007). Phytochemical investigations revealed the presence of alkaloids, flavonoids, phenols, tannins, ascorbic acid, riboflavin, thiamine and niacin. Moreover, the leaves provide a good source for Ca, P, K, Mg, Na, Fe and Zn (Okwu and Josiah, 2006). In another study, quercetin-, kaempferol- and isorhamnetin-glucosides were isolated as active antimicrobial constituents, whereby, the viability of keratinocytes was decreased about 50% when treated with 50 µg/ml of the initial MeOH extract (Abdellaoui et al., 2010). The MeOH extract showed also cytotoxicity against several cancer cells lines. Bryophyllin A, a bufadienolide 1,3,5-orthoacetate and bersaldegenin-3-acetate were isolated (Yamagishi et al., 1988) and showed activity against several cancer cells lines (Yamagishi et al., 1989). Also Bryophyllin B (Yamagishi et al., 1989) and Bryophyllin C (Supratman et al., 2000) were identified, the last showing insecticidal activity.

![Figure 9. Structures of compounds isolated from Bryophyllum pinnatum.](image)

Bryophyllin A and C, daigremontianin, and bersaldegenin-3-acetate inhibited the activation of the Epstein-Barr virus early antigen by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate in Raji cells (Supratman et al., 2001). The Epstein-Barr virus has been regarded as a cause of some types of human cancers and, therefore, bufadienolides as chemopreventive agents. A kind of chemoprevention exhibited also the polar and non-polar lipid fraction of an EtOAc extract of *Bryophyllum pinnatum*. They
inhibited reversion mutations induced by ethyl methanesulfonate or 4-nitro-O-phenylenediamine and 2-aminofluorene in *Salmonella typhimurium* (Obaseiki-Ebor et al., 1993).

New triterpenoids and phenanthrenes were isolated from the leaves by Siddiqui et al. (1989) and named bryophyllol, bryophollone, bryophollenone and bryophynol. Additionally, 18α-oleanane, <i>W</i>-taraxasterol, α- and β-amyrin and its acetates and a new sterol were identified. Cao et al. (2005) identified a flavonoid LEF with a chemical structure of C<sub>26</sub>H<sub>28</sub>O<sub>15</sub> (quercetin3-α-L-rha-β-D-xyl) and a molecular weight of 580 g/mol.

### 2.3.3. *Caesalpinia sappan*

#### Taxonomy

Class: Rosopsida  
Subclass: Rosidae  
Order: Fabales  
Family: Caesalpiniaceae  
Genus: Caesalpinia  
Species: *Caesalpinia sappan* L.

*Caesalpinia sappan* (figure 10) is an up to 6 m tall tree. Leaves are 30-45 cm long, panicles terminal or axillary; petals yellow, broadly obovate and ca. 9 mm. Legumes are reddish brown, shiny, woody and contain 3 or 4 seeds. It flowers from May to October (Zhengyi and Raven, 2010). The drug Lignum Sappan is cylindrical or...
semicylindrical, 10-100 cm long and 3-12 cm in diameter. It is yellowish- to brownish-red with traces of knife cutting. The texture is hard. The drug is odorless and tastes slightly astringent (The State Pharmacopoeia Commission of the People’s Republic of China, 2005).

**Traditional use.** Traditionally used is the dried heart wood which is mostly collected in autumn. The wood is freed from the white sap and dried (The State Pharmacopoeia Commission of the People’s Republic of China, 2005). In TCM, it is used as analgesic and anti-inflammatory agent to treat traumatic diseases, menstrual disorders (Tang and Eisenbrand, 2011a), amenorrhea, abdominal pain, dysmenorrhea, blood stasis after delivery, traumatic swelling and pain (Wu, 2005, The State Pharmacopoeia Commission of the People’s Republic of China, 2005). Decoctions are taken internally for bruises and hemoptysis.

**Chemical composition and pharmacology.** A chloroform extract of *Caesalpinia sappan* led to an increase of cells in the SubG1 region and condensation and shrinkage of nuclei in HNSCC4 head and HNSCC31 neck cancer cells. Also the expression levels of p53 and p21\(^{WAF1/CIP1}\) were increased (Kim et al., 2005). A methanol, methanol-water (1:1) and water extract of this plant exhibited *in vitro* cytotoxicity against human HT-1080 fibrosarcoma, Hela cervix adenocarcinoma, A549 lung adenocarcinoma, murine 26-L5 colon cancer, murine LLC Lewis lung cancer and murine B16-BL6 cancer cells (Ueda et al., 2002).

The wood contains several phenolic pigments, whereby, brazilen is one of the major ones (Tang and Eisenbrand, 2011a). This substance has been reported to possess antibacterial (Xu and Lee, 2004) and DNA strand-scission activity in a cell free assay (Mar et al., 2003). A similar compound, brazilein, has been shown to downregulate protein and mRNA expression of survivin, a member of the inhibitor of apoptosis protein (IAP) family. It led to the activation of caspases-9 and -3 and PARP cleavage in HepG2 hepatocellular carcinoma cells (Zhong et al., 2009). A derivative of brazilen, tetracetyl-brazilen was isolated from the stem as well as protosappanin A (Xu et al., 1994).

A precursor of brazilen seems to be sappanchalcone, a chalcone methyl ether (Nagai et al., 1984). This compound exhibited cytoprotective activity as well as 4-O-methylepisappanol (Jeong et al., 2009). Another chalcone, named methoxychalcone, has been reported to induce cell cycle arrest and apoptosis in human A375 melanoma cells (Henmi et al., 2009) and human prostate cancer cells (Sun et al., 2010a, Szliszka et
3-deoxysappanchalcone has also been isolated together with 3,8-dihydroxy-4,10-dimethoxy-7-oxo-[2] benzopyranol[4,3-β] benzopyran, 3-deoxysappanone B, rhamnetin, protosappanin C, 3,7-dihydroxy-chroman-4-one, dimethyl adipate and daucosterin (Shu et al., 2008). Rhamnetin showed cytotoxicity against human Hela carcinoma cells (Sha et al., 2009). The 3-benzylchroman derivatives, protosappanin A, B, D, and E were also isolated in a search for new anti-inflammatory compounds (Washiyama et al., 2009).

Isoliquiritigenin 2'-methyl ether induced expression of heme oxygenase-1, an enzyme induced during oxidative stress, and led to apoptosis in primary and metastatic oral cancer cell lines (Lee et al., 2010b). Antioxidant activity relying on scavenging superoxide anion and hydroxyl radicals was reported from 1',4'-dihydrospiro[benzofuran-3(2H),3'-[3H-2]benzopyran]-1',6',6',7'-tetrol and 3-[[4,5-dihydroxy-2(hydroxymethyl) phenyl]-methyl]-2,3-dihydro-3,6-benzofurandiol (Safitri et al., 2003).

![Structures of compounds isolated from Caesalpinia sappan](image.png)

**Figure 11. Structures of compounds isolated from Caesalpinia sappan.**

Other compounds isolated from *Caesalpinia sappan* comprise the 3-benzylchroman derivative 3'-deoxy-4-O-methylepisappanol (Fu et al., 2008, Moon et al., 2010), the diterpenoids phanginin A-K (Yodsaoue et al., 2008), the homoisoflavan 7,3',4'-trihydroxy-3-benzyl-2H-chromene (Zhao et al., 2008), the flavonoid 3,8-dihydroxy 4,10-dimethoxy-7-oxo-[2] benzopyranol[4,3-β] benzopyran (Shu et al., 2008), Caesalpin J and P (Shimokawa et al., 1985, Miyahara et al., 1986), 4-O-methylsappanol, pulchoic acid (Jeong et al., 2009) and several other compounds related to brazilin (Saitoh et al., 1986, Fuke et al., 1985) such as sappanone A and B, sappanol and 3-hydroxysappanone B.
2.3.4. *Cinnamomum cassia*

**Taxonomy**

Class: Magnoliopsida  
Subclass: Magnoliidae  
Order: Laurales  
Family: Lauraceae  
Genus: Cinnamomum  
Species: *Cinnamomum cassia* Blume

![Figure 12. Cinnamomum cassia (Chinese Pharmacopoeia Commission, 2010).](image)

This family is spread in the tropics and subtropics and comprises about 3000 species. The only European species is *Laurus nobilis*. The typical flower is small, inconspicuous, green to creamy colored and actinomorphic. Potatoes berries or stone fruits contain one seed with a large embryo (Frohne and Jensen, 1998).

**Distribution.** *Cinnamomum cassia* (figure 12) occurs in the Chinese provinces Guangxi, Guangdong, Yunnan, Guizhou, Hainan, Hong Kong and Fujian in tropical to subtropical areas. It is also cultivated in India, Indonesia, Laos, Malaysia, Thailand and Vietnam (Zhongzhen, 2004, Zhengyi and Raven, 2008).

**Plant and Crude Drug.** *Cinnamomum cassia* is a medium-sized tree up to 10 m. The bark is gray-brown and up to 13 mm thick. Branches of the current year (yellow-brown) can be distinguished from older branches (dark brown) by their color. The leaves are alternate or subopposite; the petioles are robust. Leaf blades are leathery and elliptic to sub lanceolate, triplinerved, abaxially greenish opaque and, on the other site, green and shiny. Flowers are white and built in June to August. The perianth is yellowish brown
Cortex Cinnamomi is channeled or quilled, about 30-40 cm long and 3-10 cm wide/in diameter. The outer surface is grayish-brown and rough and has irregular fine wrinkles. The inner surface is reddish-brown and more even. It brakes easily. The odor is strongly aromatic; the taste sweet and pungent (The State Pharmacopoeia Commission of the People’s Republic of China, 2005).

**Traditional use.** In China, the stem bark of *Cinnamomum cassia* is considered as analgesic, antipyretic, astringent, diaphoretic, stimulant and tonic. In form of decoctions, it is used for amenorrhea, arthritis, cancer, chills, cold, colic, cough, diabetes, diarrhea, dizziness, dysmenorrhea, dyspepsia, dysuria, fever, headache, lumbago, rheumatism and stomachache (Duke and Ayensu, 1985b). In the Chinese pharmacopoeia, indications comprise furthermore: impotence, frigidity, inflammations of the eyes, sore throats, vomiting and neurosis (The State Pharmacopoeia Commission of the People’s Republic of China, 2005). The bark is mostly collected in autumn and dried in the shade (The State Pharmacopoeia Commission of the People’s Republic of China, 2005). The essential oil has cardiovascular, hypotensive and viricidal effects (Duke and Ayensu, 1985b) and is an analgesic and stomachic (Tang and Eisenbrand, 2011a). Additionally, young branches are in use as analgesic and antipyretic (Tang and Eisenbrand, 2011a). In Iran, tea of the bark is used for excessive salivation (Duke and Ayensu, 1985b).

**Chemical composition and pharmacology.** In a screening of 22 crude drugs, *Cinnamomum cassia* reduced mitochondrial membrane potential and activated caspases-3 in human HL-60 leukemia cells (Nishida et al., 2003). An aqueous extract of *Cinnamomum cassia* inhibited mouse melanoma progression and angiogenesis *in vivo* and potentiated the activity of CD8+ T cells in mice (Kwon et al., 2009). Moreover, the same extract reduced levels and activity of NF-κB and AP1 and their target genes and induced apoptosis (Kwon et al., 2010). Another aqueous extract was investigated against human cervical SiHa cancer cell lines. It inhibited proliferation of these cells, decreased cell migration by reducing MMP-2 expression and downregulated expression of the Her-2 oncoprotein. It also induced apoptosis by increasing intracellular calcium levels and causing loss of mitochondrial membrane potential (Koppikar et al., 2010). HPTLC analysis revealed that this extract was composed of several polyphenols and cinnamaldehyde as the major bioactive substance. This substance has been reported to inhibit proliferation and induce apoptosis by downregulation of bcl-xL, upregulation of p53 and bax proteins, cleavage of PARP as well as activation of caspases-3 in human PLC/PRF/5 hepatoma cells (Wu and Ng, 2007). Similar results were obtained using the...
human HepG2 hepatoma cell line (Ng and Wu, 2009). In HL-60 leukemia cells, apoptosis was initiated by ROS-mediated mitochondrial permeability transition and subsequent release of cytochrome c (Ka et al., 2003). Cinnamaldehyde together with 2-methoxycinnamaldehyde was also demonstrated to inhibit LPS-induced NF-κB activity in RAW 264.7 mouse macrophages (Reddy et al., 2004).

![Figure 13. Structures of compounds isolated from Cinnamomum cassia.](image)

2′-hydroxycinnamaldehyde inhibited the growth of 29 kinds of human cancer cells in vitro and SW-620 human tumor xenografts in nude mice (Lee et al., 1999). Further investigations showed that 2′-hydroxycinnamaldehyde inhibited AP-1 and bcl-2 activity, led to the activation of caspases-3 and, therefore, to apoptosis in SW-620 cells (Lee et al., 2007). In vivo investigations also revealed that 2′-hydroxycinnamaldehyde delayed hepatic tumor development probably by immunostimulating effects on T cells (Moon et al., 2006).

Also other major components of Cinnamomum cassia such as cinnamic acid and cinnamyl alcohol inhibited the growth of cancer cells (Ng and Wu, 2009). In melanoma cells, cinnamic acid led to cell differentiation, increased melanin production and decreased invasive capacity by modulating genes involved in tumor metastasis (Liu et al., 1995). In Caco-2 colon cancer cells, cinnamic acid inhibited DNA synthesis in growing cells and prolonged reduplication time – probably by interfering with the cAMP signaling pathway (Ekmekcioglu et al., 1998). An interesting observation was done by Brozic et al. (2009). They found that derivatives of cinnamic acid inhibit 17β-hydroxysteroid dehydrogenase type 1 which converts estrone to estradiol and displays an important target for the treatment of estrogen-dependent diseases, for example, breast cancer.

From aqueous extracts were further isolated: coumarin, β-sitosterol, choline, protocatechuic acid, vanillic acid and syringic acid. Also several diterpenes named cinn cassiol A, B, C1, C2, C3, D1, D2, D3, D4, and E and several glucosides of them, cinnzeylanol, cinnzeylanin, anhydrocinnzeylanol and anhydrocinnzeylanin were gained by fractionating extracts of the bark. Moreover, aromatic compounds – for example lyoniresinol 2α-O-β-D-glucopyranoside and syringaresinol – epicatechins, cinnamic
aldehyde cyclic glycerol 1,3-acetals and flavan-3-ol derivatives including procyanidin A₂, B₁, B₂, B₅ and B₇ have been reported (Tang and Eisenbrand, 2011a).

A GC-MS method for the simultaneous determination of cinnamaldehyde, copaene, cinnamic acid, coumarins, 2-methoxycinnamaldehyde, 2-methoxycinnamic acid and safrole was published by Lv et al. (2010).

### 2.3.5. Cocculus trilobus

**Taxonomy**

Class: Magnoliopsida  
Subclass: Ranunculidae  
Order: Ranunculales  
Family: Menispermaceae  
Genus: Cocculus  
Species: Cocculus trilobus (Thunb.) DC.

The family Menispermaceae contains about 450 species with many tropical lianes characterized by abnormal secondary growth. Flowers are diecious and small, fruits apocarpous. Seeds build massive cotyledons (Frohne and Jensen, 1998).

**Distribution and Crude Drug.** The plant (figure 14) is distributed in China, mainly in Shaanxi province and in southwestern, central and eastern regions (Zhongzhen, 2004). Moreover, it is produced in the Chinese provinces Anhui, Hunan, Hubei and Zhejiang (Hou and Jin, 2005). The roots are cylindrical or twisted, slightly torose, 10-20 cm in length and 1-2.5 cm in diameter. The outer surface is marked with curved grooves and sparse scars of branch roots. It is dull dark brown. The odour is faint, the taste slightly bitter (Zhongzhen, 2004).

![Figure 14. Cocculus trilobus (Zhang, 1994).](image)

**Traditional use.** In China, the drug is called mufângji. This description is also used for roots of Stephania tetrandra and Aristolochia fangchi which have similar indications but
differ in the chemical composition. Special attention has to be drawn at *A. fangchi* because it contains aristolochic acid, while the others do not (Wu et al., 2007). The roots of *Cocculus trilobus* are harvested during spring and autumn, washed, sliced and dried under the sun (Zhongzhen, 2004, Hou and Jin, 2005). They are used for arthritis, boils, paralysis, rheumatism, snakebites, sores, tumors, urethritis, arthralgia, edema, neurosis and neuralgia (Duke and Ayensu, 1985b, Zhongzhen, 2004, Hou and Jin, 2005). *Cocculus trilobus* is also used in treating cancer-related pain mostly along with other herbs (Hou and Jin, 2005). Decoctions of the stem are prepared to treat asthma, beriberi, bronchitis, fever, gonorrhea and paralysis. The whole plant is utilized for dropsy, fever and gonorrhea (Duke and Ayensu, 1985b).

**Chemical Composition and Pharmacology.** An ethyl acetate extract of *Cocculus trilobus* has been reported to inhibit the activity of sortase. Sortase is an enzyme, universal in gram-positive bacteria and anchors various surface proteins to the cell walls. Surface proteins are believed to be essential for pathogenesis of bacterial diseases and, therefore, provide a useful target in therapy (Kim et al., 2002).

The most important bioactive compounds in *Cocculus trilobus* are alkaloids. First isolated were the biscoclaurine alkaloids trilobine, homotrilobin and isotrilobine (Kondo and Nakazato, 1924, 1926a, 1926b). Trilobine and isotrilobine were shown to reverse doxorubicin resistance in human breast cancer cells, whereby, isotrilobine exhibited the same activity as verapamil (Hall and Chang, 1997). Trilobine also inhibited ADP-induced platelet aggregation *in vitro* and *in vivo* (Tan et al., 1991). From the roots, isotrilobine-N-2-oxide and nortrilobine were isolated as well (Chen et al., 1991). Structures of trilobamine and normenisarine were described in 1931 and 1955 (Inubushi, 1955, Nakano 1956). Magnoflorine was first isolated from the rhizome and vine in 1956 (Nakano, 1956) and exhibited growth inhibitory activity against HepG2 hepatocellular and U251 glioblastoma cell lines *in vitro*. However, it was not active against Hela cervical cancer cells (Mohamed et al., 2009). Moreover, magnoflorine and cocculine (also: sinomenine), another alkaloid isolated from *Cocculus trilobus* (McPhail et al., 1976, Juchi et al., 1978), were not cytotoxic to A549 non small cell lung adenocarcinoma, SK-OV-3 ovarian, SK-MEL-2 skin melanoma, XF498 CNS and HCT15 colon cancer cell lines (Min et al., 2006).

From a methanolic extract of the leaves, cocculolidine and isoboldine were isolated (Wada and Munakata, 1967). Cocculolidine showed insecticidal activity against leaf hoppers and Azuki-been weevils, whereby, the action is probably related to that of curare (Wada and Munakata, 1967). Another new alkaloid was isolated in 1969 by Ito et al. and named coclobine but no bioactivity has been reported so far. In 1977,
dihydroerysovine was firstly described in *Cocculus trilobus* (Ju-Ichi, et al., 1977). In 1978, coccutrine was isolated along with dihydroerysovine, cocculine and cocculolidine (Ju-Ichi et al., 1978).

![Figure 15. Structures of compounds isolated from *Cocculus trilobus*.](image)

In a search for new antitumor substances, a methanolic extract of *Cocculus trilobus* was activity-guided fractionated, yielding the alkaloid sinococuline as active principle against Sarcoma 180 ascites in mice (Itokawa et al., 1987a). It also exhibited cytotoxicity against human Raji Burkitt lymphoma, rat RN6 neurinoma, J774A1 BALB/c macrophages, mouse S180 sarcoma, rat E367 euroblastoma, human HepG2 hepatocellular cancer, B16 mouse melanoma and macrophages *in vitro*. It has been shown that it strongly decreased DNA synthesis, interfered with the cell metabolism of Raji, J774A1 and B16 cells and induced apoptosis in HL-60 leukemia cells. Moreover, it suppressed TNF and RNI production of endotoxin-stimulated macrophages (Liu et al., 1996). Another investigation of a methanolic extract led to the isolation of isosinococuline which showed activity against P-388 leukemia in mice *in vivo* (Itokawa et al., 1995).

### 2.3.6. Curcuma longa

**Taxonomy**
- Class: Liliopsida
- Subclass: Commelinidae
- Order: Zingiberales
- Family: Zingiberaceae
- Genus: Curcuma
- Species: *Curcuma longa* Linn.

![Figure 16. Curcuma longa](image)

*(Chinese Pharmacopoeia Commission, 2010).*
The family comprises about 1500 species and is specifically characterized by excretion cells which contain essential oil and non-volatile compounds, such as resins and dyes. They are tropical to sub-tropical plants with an herbaceous stem, distichously or convoluted leaves and conspicuous colored, large flowers (Frohne and Jensen, 1998).

**Distribution.** The native origin of *Curcuma longa* is unknown. It is cultivated throughout tropical areas of Asia, for example, in Fujian, Guangdong, Guangxi, Sichuan, Taiwan, Xizang and Yunnan (Zhengyi and Raven, 2000).

**Plant and Crude Drug.** *Curcuma longa* (figure 16) is about 1 m tall. Leaves are blade green and oblong. Petioles are 20-45 cm long. Inflorescences are on pseudostems. The calyx is white; the corolla pale yellow. It flowers in August. Rhizomes are many times branched, orange or bright yellow, cylindrical and aromatic (Zhengyi and Raven, 2000). The texture is hard and not easy to brake. The odor is characteristic and aromatic; the taste bitter and pungent (The State Pharmacopoeia Commission of the People’s Republic of China, 2005).

**Synonyms.** According to TROPICOS®, three synonyms for this plant are known: *Amomum curcuma* Jacq., *Curcuma domestica* Valeton and *Stissera curcuma* Raeusch.

**Traditional Use.** In China and India, the rhizome of *Curcuma longa* has a very long tradition as spice, medicine, cosmetic and dye (Hatcher et al., 2008). In TCM, roots of *Curcuma longa* are dug out in winter when the leaves whiter, washed and boiled or steamed thoroughly. Afterwards, the fibrous roots are removed. The root is sliced and dried in sunlight (Wu, 2005, The State Pharmacopoeia Commission of the People’s Republic of China, 2005). It is used to treat abdominal pain, chest pains, rheumatic pain of shoulders and arms, traumatic swelling, bruises, colic, coma, dysmenorrhea, fever, hepatitis, amenorrhea, sores and toothache. The roots are also applied externally in the case of inflammations and indolent ulcers and as decoction for ophthalmia (Duke and Ayensu, 1985b, The State Pharmacopoeia Commission of the People’s Republic of China, 2005).

**Chemical Constituents and Pharmacology.** In former times, turmeric was especially known for the coloring, flavoring and digestive properties. Three main phenolic pigments were isolated from the roots: curcumin, bisdemethoxy-curcumin and demethoxy-curcumin – also known as curcuminoids (Tang and Eisenbrand, 2011a, Araújo and Leon,
GENERAL PART

2001). Other pigments were dihydrocurcumin which is a keto-enol tautomer of curcumin (Tang and Eisenbrand, 2011a) and cyclocurcumin (Araújo and Leon, 2001). Curcumin I to III were also identified as C9-chain homologs of curcumin (Tang and Eisenbrand, 2011a). Curcumin has a broad spectrum of biological effects such as anti-inflammatory, anti-oxidant, anti/protozoal, anti-viral, chemopreventive and chemotherapeutic (Hatcher et al., 2008, Araújo and Leon, 2001). Thus, it has also several molecular targets. It binds physically to 33 different proteins including COX-2, protein kinase C, 5-lipoxygenase, tubulin, several transcription and growth factors and genes affecting proliferation, apoptosis, cytoprotection, metastasis and angiogenesis (Hatcher et al., 2008, Ravindran et al., 2009). Many cancer cells lines were inhibited in their growth by curcumin. Thereby, it is supposed that curcumin induces apoptosis on the one and inhibits proliferation on the other hand and that more than 40 biomolecules are involved. Curcumin activated both the intrinsic and extrinsic apoptotic pathway, led to DNA damage and endoplasmic reticulum stress. The expression of several pro-apoptotic genes were induced by curcumin, while anti-apoptotic genes were down-regulated. It also affected the cell cycle by arresting the cells in the G1 and G2/M phase, inhibited COX-2, 5-lipoxygenase, ornithine decarboxylase, phospholipase D and NF-κB. (Hatcher et al., 2008, Reuter et al., 2008, Ravindran et al., 2009). For myeloma, several cancers and others, clinical studies are currently in progress (Hatcher et al., 2008). However, it has been shown that curcumin orally administered to rats was excreted in the faeces to 75%, hardly absorbed and fast metabolized in the liver (Araújo and Leon, 2001). Nevertheless, curcumin showed significant effects distal the gastrointestinal tract in human clinical trials such as immunomodulation and contraction of the gall bladder (Hatcher et al., 2008).

![curcumin](image1) ![ar-tumerone](image2) ![α-tumerone](image3)

*Figure 17. Structures of compounds isolated from Curcuma longa.*

Sesquiterpene ketones have been shown to be the main constituents of the essential oil of the rhizome: ar-tumerone, α-tumerone, β-tumerone and curnole (Tang and Eisenbrand, 2011a). Ar-tumerone was shown to inhibit the proliferation of K562, L1210, U937, RBL-2H3, Molt 4B and HL-60 cancer cell lines *in vitro* through induction of apoptosis (Ji et al., 2004, Aratanechemuge et al., 2002). α-tumerone also reduced the...
growth of cancer cells (HepG2, MCF-7 and MDA-MB-231) and induced apoptosis in MDA-MB-231 cells. Furthermore, both substances exhibited immunomodulatory activities in human peripheral blood mononuclear cells (Yue et al., 2010). Several terpene compounds were found in the essential oil as well, such as α- and β-pinene, camphene, limonene, terpinene, caryophyllene, linalool, borneol, isoborneol, camphor, eugenol, cineole, curdione, curzerenone and curcumenes (Tang and Eisenbrand, 2011a).

*In vivo* toxicity studies with rodents and nonrodents revealed that *Curcuma longa* was not toxic even at high doses (Tang and Eisenbrand, 2011a).

### 2.3.7. *Helianthus angustifolius*

**Taxonomy**

Class: Rosopsida  
Subclass: Asteridae  
Order: Asterales  
Family: Asteraceae  
Genus: Helianthus  
Species: *Helianthus angustifolius* L.

![Figure 18. Helianthus angustifolius](picture taken by Aaron Sallegger, Okt. 2008, South Mississippi)

The family of Asteraceae comprises more than 25000 species, which are distributed worldwide and native to many different habitats. Aquatic plants and succulents occur inside the family, as well as small herbs and up to 20 m high tropical trees. Characteristic is an inferior ovary with only one ovule, achenes, a reduced calyx and the aggregation of the single flowers to a capitulum (Frohne and Jensen, 1998).

**Distribution.** The genus Helianthus consists of about 50 herbs and is native to North America (Weakley, 2010). The species *H. angustifolius* (figure 18) is native to savannahs, the Pine Barrens, ditches, marshes and wet meadows throughout the coastal plain of North America (Delaware, Florida, Georgia, North Carolina, South Carolina, Virginia) and lower Piedmont. Moreover, it is rare to upper Piedmont and the
mountain site in Georgia, North Carolina, South Carolina, Virginia and West Virginia (Radford et al., 1964, Weakley, 2010).

**PLANT.** The name Helianthus is derived from Greek *helios* (sun) and *anthos* (flower) and refers to the huge yellow flowers which are arranged sun-like and turn toward the sun (Hiller and Melzig, 2003). *Helianthus angustifolius* is a perennial herb with a fibrous root system and few, very short rhizomes. It is erect with one or several stems, 1-2 m in height and pubescent, at least below. Leaves are dark green, mostly alternate, below opposite, linear, 6-20 cm long and 3-15 cm wide. They are very scabrous above, densely pubescent beneath and contain often resin-dots. Phyllaries are lanceolate, slightly acuminate and usually shorter than the disk. Ray flowers are 2-4 cm long and yellow. Disk flowers are about 1 cm and purplish red. The nutlets are characterized by a dark brown color, a round or truncate shape and are glabrous or pubescent at apex (Radford et al., 1964, Correll and Johnston, 1996). It flowers from (July-) September until October (-frost) (Weakly, 2010) and produces dozens of flowers.

**SYNONYMS.** According to TROPICOS®, only *Helianthus angustifolius* var. *planifolius* Fernald is known as synonym.

**TRADITIONAL USE.** No traditional use was found for this particular species. However, some related Helianthus species are used by Native Americans. *Helianthus annuus* L. is a snakebite remedy, used as analgesic for chest pains, as aid for pulmonary troubles, cuts, diminishing thirst, sores and swellings as well as antirheumatic, anthelmintic, disinfectant and stimulant in form of cakes. It is also part of ceremonial medicines to lubricate or paint the face or body and in sun sand-painting ceremonies. It plays an important role in daily life since it is used as food in form of breads, cakes, gruel, porridge, eaten roasted or used as chewing gum. The flowers are used for decoration in dances and the stalks to make bird snares. The stems serve also as Holyway Prayer sticks (Moerman, 1998). Flowers are also folksy used for fever, especially in case of malaria. Oil of the fruits is taken in case of constipation, for preparation of ointments and creams and as cooking oil (Hiller and Melzig, 2003). Other Helianthus species, such as *H. anomalus* are dermatologicals, fodders and decorations. *H. cusickii* is a heart medicine, analgesic, carminative, dermatological or disinfectant, and *H. petiolaris* a dermatological, hunting medicine for good luck or ceremonial and/or decorative item, just to mention a few (Moerman, 1998).
CHEMICAL COMPOSITION AND PHARMACOLOGY. One of the most important compound classes in this family are sesquiterpene lactones. Over 3000 compounds have been isolated and identified so far from members of this family. Most of them were isolated from leaves or flowering heads. Thereby, their amount can reach up to 5% of dry weight (Zhang et al., 2005). Sesquiterpene lactones are mostly characterized by a $\alpha$-methylene-$\gamma$-lactone group. Many of them possess also $\alpha,\beta$-unsaturated carbonyl- or epoxy-groups. All these groups provide binding sites for biological nucleophiles such as thioles or amino groups. This in turn leads to a broad spectrum of various biological and pharmacological effects (Hänsel and Sticher, 2007). It has been shown that these compounds possess anti-microbial, anti-viral, anti-inflammatory and also anti-tumor properties. Their effect is mainly based on the covalent binding to free sulfhydryl groups in proteins which lead to a dysfunction of these proteins and several biological processes including cell signaling, proliferation, death and respiration (Zhang et al., 2005). The tribe of Heliantheae includes some important medicinal plant genera such as *Echinacea* and is known to be a rich source of secondary compounds of several compound classes. The tribe stands out due to the appearance of C-8$\beta$-substituted germacranolides, benzofuranes, benzopyranes and thymol derivatives. Also kauranes and deviated substances are characteristic (Frohne and Jensen, 1998).

2.3.8. *Hydnocarpus anthelmintica*

**TAXONOMY**

Class: Rosopsida  
Subclass: Rosidae  
Order: Violales  
Family: Flacourtiaceae  
Genus: Hydnocarpus  
Species: *Hydnocarpus anthelmintica* Pier.

*Figure 19. Hydnocarpus anthelmintica (Zhang, 1994).*

The Flacourtiaceae contain about 1300 species and are tropical lumbers. Flowers are partial spiral and have numerous stamina (Frohne and Jensen, 1998).

**DISTRIBUTION AND PLANT.** *Hydnocarpus anthelmintica* (figure 19) is a monoecious tree, 10 m or more high with a whitish grey bark. Leaves are alternate, shining green and
entirely pink when young. The flowers are unisexual or polygamous. Fruits are large and globose, seeds numerous and angular (Prajapati et al., 2006). It occurs abundantly in South East Asia (Jaroszewski et al., 1987).

**Traditional use.** In TCM, seeds of *Hydnocarpus anthelmintica* are a disinfectant in elephantiasis, gout, leprosy, rheumatism, scabies, syphilis and skin ailments. The oil is used to treat cancer and leprosy; the bark to treat fever (Duke and Ayensu, 1985a). It is mostly used in combination with other herbs. In the meantime however, it is rarely used internally because of its toxicity (Chen and Chen, 2004). The seed oil is also used in Thai herbal medicine to treat cancer, tuberculosis, leprosy and other dermatitis related diseases (Prachya et al., 2007).

**Chemical composition and pharmacology.** *Hydnocarpus anthelmintica* exhibited cytotoxicity against mouse leukemia, colon adenocarcinoma, osteosarcoma and cervix adenocarcinoma cells (de Padua et al., 1999). Several fungal endophytes were isolated from the branches. One of them, *Phomopsis sp.* was examined to produce mycoepoxydiene and the derivatives deacetylmycoepoxydiene and 2,3-dihydromycoepoxydiene (Prachya et al., 2007). Especially mycoepoxydiene exhibited strong cytotoxicity against several cancer cell lines (HepG2, A549, HCC-S102, HuCCA-1, KB, Hela, MDA-MB-231, T47D, HL-60 and P388).

![Figure 20. Structures of compounds isolated from Hydnocarpus anthelmintica.](image)

Combined chromatographic analysis of the seed oil revealed that it is mainly composed of 13-cyclopent-2-enyltridec-4-enoic acid. Other identified compounds were cis-4-hexadecenoic acid, C16 and C18 cyclopentyl fatty acids, and iso- and anteiso-methylbranched fatty acids (Christie et al., 1989). Hydnocarpic acid is demonstrable effective against leprosy (Duke and Ayensu, 1985a).

From leaves, epivolkkenin, a cyclopentoid cyanohydrin glucoside, was isolated, however, without any reported bioactivity (Jaroszewski et al., 1987).
2.3.9. *Lonicera japonica*

**TAXONOMY**
Class: Rosopsida
Subclass: Asteridae
Order: Dipsacales
Family: Caprifoliaceae
Genus: Lonicera
Species: *Lonicera japonica* Thunb. ex Murray

![Figure 21. Lonicera japonica (Chinese Pharmacopoeia Commission, 2010).](image)

About 500 members can be found in the family of Caprifoliaceae. Most of them are woody plants with opposite, single or pinnate leaves and pseudanthium-like inflorescences. Flowers are zygomorphic and normally sympetalous. Capsules, stone fruits or berries appear as fruits (Frohne and Jensen, 1998).

**DISTRIBUTION AND CRUDE DRUG.** Caulis Lonicerae is produced in the Chinese provinces of Zhejiang, Sichuan, Jiangsu and Henan (Wu, 2005). Moreover, it is distributed in Shangdong, Guangxi and Hong Kong (Zhongzhen, 2004). The drug is brownish-red or dull red on the outer surface and has fine longitudinal striae. The bark peels off and breaks easily. The odor is faint; the taste is slightly bitter but young branches are mild (Zhongzhen, 2004).

**TRADITIONAL USE.** Caulis and Flos Lonicerae (figure 21) are officially listed in the Chinese pharmacopoeia. Caulis Lonicerae is collected in autumn and winter. The branches are bundled and dried in the sun (Zhongzhen, 2004). Flos Lonicerae are the flower buds
which are collected in early summer and dried at cool and well-ventilated places (The State Pharmacopoeia Commission of the People’s Republic of China, 2005, Zhongzhen, 2004). Caulis Lonicerae is used as antiphlogistic and bacteriostatic in the case of fever, dysentery, abscesses and rheumatic swelling (Tang and Eisenbrand, 2011b), for carbuncles, sores and arthritis (Zhongzhen, 2004). The flowers are used in infusions for cutaneous infections, scabies, as antiseptic and diuretic. In form of a decoction, it is used for bacterial dysentery, cold, enteritis, infected boils, laryngitis, lymphadenitis, rheumatism, and sores and reported to be effective against flu and other infectious diseases as well as cancer – especially mammary carcinoma (Duke and Ayensu, 1985a). The plant is antidiarrhetic, antiphlogistic, diuretic and refrigerant. It is said to increase longevity and vitality when taken prolonged. In Japan and Vietnam, flowers and whole plant are used as diuretic and to wash tumors (Duke and Ayensu, 1985a).

**Chemical composition and pharmacology.** When apoptosis was induced by photodynamic therapy in CH27 lung carcinoma cells, alcoholic extracts of *Lonicera japonica* have been shown to be photosensitizing. Thereby, apoptosis was caspases-independently activated via AIF (Leung et al., 2008). A boiled aqueous extract of the flowers has been reported to inhibit COX-2, an enzyme involved in inflammation, by direct, transcriptional and post-transcriptional inhibition. Simultaneously, the viability of human A549 lung adenocarcinoma cells was not affected up to 100 mg/ml (Xu et al., 2007). One known compound of *Lonicera japonica* inhibiting COX-2, 5-lipoxygenase (Son et al., 2006) and, moreover, LPS-induced iNOS expression and NO production (Suh et al., 2006) is the biflavonoid ochnaflavone. Anti-inflammatory activity exhibited also loniceroside A and C in the croton oil-induced ear edema assay (Kwak et al., 2003).

From an ethanolic extract of the stem, protocatechuic acid, caffeic acid, macranthoin, esculetin, luteolin, quercetin, apigenin, luteolin-7-O-β-D-glucopyranoside, isorhamnetin-7-O-β-D-glucopyranoside, diosmetin-7-O-β-glucopyranoside, rhoifolin, lonicerin and hydnocarpin D were isolated (Zhang et al., 2009a). Luteolin was shown to inhibit phorbol 12-myristate 13-acetate (PMA) plus A23187-induced HMC-1 mast cell activation by blocking NF-κB activation, IκB degradation and inhibition of TNF-α, IL-8, IL-6 and COX-2 expression (Kang et al., 2010). It was also able to induce apoptosis in several cancer cells, for example, human HL-60 leukemia cells (Ko et al., 2002), A549 (Zhao et al., 2011) and CH27 lung carcinoma cells (Leung et al., 2005 and 2006). In these cases, the pro-apoptotic activity was based on an antioxidative effect (Leung et al., 2006), ATP-dependent processes and chaperone involvement (Lee et al., 2010a). It was also shown that luteolin arrests cells in the G1 phase and prevents DNA-synthesis (Ong
et al., 2010, Zhao et al., 2011). Moreover, luteolin as well as protocatechuic acid and chlorogenic acid was shown to decrease the viability of HepG2 hepatocellular carcinoma cells. Thereby, protocatechuic acid as well as the initial aqueous extract was able to induce cell death in a c-Jun N-terminal kinase-dependent manner (Yip et al., 2006). In human MCF-7 breast, A549 lung, HepG2 hepatocellular, Hela cervix and LNCaP prostate cancer cells, protocatechuic acid increased LDH leakage, DNA fragmentation, caspases-3 and -8 activity and decreased mitochondrial membrane potential and Na⁺/K⁺-ATPase activity. It was assumed that it causes not only apoptosis but also delays the invasion and metastasis (Yin et al., 2009). In fact, an inhibition of metastasis was observed in vivo in B16/F10 melanoma cells in mice: cell migration and invasion to the liver was inhibited at non-cytotoxic concentrations (Lin et al., 2011). Chlorogenic acid shows a wide spectrum of activities such as anti-HIV, anti-oxidant, anti-carcinogenic, anti-allergic and apoptosis-inducing activity. It has been assumed to upregulate death receptors and disrupt mitochondrial membrane potential which leads to release of cytochrome c, activation of caspases and, finally, to apoptosis. (Rakshit et al., 2010).

![Figure 22. Structure of compounds isolated from Lonicera japonica.](image)

From the flowers of *Lonicera japonica*, the following constituents were isolated: loniceroside A–E, loniceraflavone, chrysoeriol and derivatives, oleanolic acid derivatives, loganin, secoxyloganin, 7-epi-loganin, loganin acids, sweroside, vogeloside, epivogeloside, caffeic acid, p-hydroxybenzoic acid, β-sitosterol, daucosterol, rutin, hyperosides, macranthoidin A and B, chlorogenic and isochlorogenic acid, a methyl ester of it, tricin, apigenin and a methylether of it and quercetin together with a glucoside and a methylether of it (Tang and Eisenbrand, 2011b, Son et al., 1994, Li and Li, 2005, Phan et al., 2005, Liu and Yu, 2006, Choi et al., 2007, Lin et al., 2008, Qi et al., 2009). Moreover,
the cerebroside lonijaposide $A_1-A_4$, $B_1$ and $B_2$ (Kumar et al., 2006) and several derivatives of dicafeoylquinic acid were found (Peng et al., 2000, Qi et al., 2009). Also a cyclic peroxide, named shuangkangsu, has been isolated from the buds (Yu et al., 2008).

The caffeoylquinic acid derivatives 3,4-di-O-cafeoyl quinic acid methyl ester, 5-O-cafeoyl quinic acid methyl ester, 3,4-di-O-cafeoyl quinic acid, 1,3-di-O-cafeoyl quinic acid and chlorogenic acid were isolated from the leaves of *Lonicera japonica* (Ma et al., 2009). The biflavonoids 3’-O-methyl-loniflavone and loniflavone along with luteolin and chrysin were also isolated (Kumar et al., 2005). In another study, twelve triterpen saponins with oleanolic acid or hederagenin as aglycones were isolated and identified from the aerial parts of the plant (Kawai et al., 1988).

From the essential oil of the flowers, linalool, 2,6,6-trimethyl-2-vinyl-5-hydroxytetrahydropyran, pinene, hex-1-ene, hex-3-en-1-ol, *cis* and *trans*-2-methyl-2-vinyl-5-(α-hydroxyisopropyl)-tetrahydropuran, geraniol, α-terpineol, benzyl alcohol, β-phenylethyl alcohol, carvacrol and eugenol were isolated (Tang and Eisenbrand, 2011b).

To evaluate the quality of Flos Lonicerae, a new HPLC method based on their saponin content and using evaporative light scattering detection (ELSD) was published by Chai et al. (2005). Last year (2010), also a method for the differentiation of *Lonicera japonica* and close related species based on the PSBA-TRNH intergenic spacer as DNA bar code was published (Sun et al., 2011).

### 2.3.10. *Onosma paniculata*

**Taxonomy**

<table>
<thead>
<tr>
<th>Class: Rosopsida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subclass: Asterida</td>
</tr>
<tr>
<td>Order: Boraginales</td>
</tr>
<tr>
<td>Family: Boraginaceae</td>
</tr>
<tr>
<td>Genus: Onosma</td>
</tr>
<tr>
<td>Species: <em>Onosma paniculata</em> Bureau &amp; Franchet</td>
</tr>
</tbody>
</table>

![Figure 23. Onosma paniculata (Zhang, 1994).](image)

Boraginaceae are characterized through the strong development of kemps whose walls are hardened by SiO$_2$ and/or CaCO$_3$ inclusions. Flowers are zygomorphic, the ovary is
superior and develops four single-seeded achene-like nutlets. The family contains 146 genera and around 2000 species (Frohne and Jensen, 1998).

**Distribution.** *Onosma paniculata* (figure 23) appears on arid slopes and forest margins in heights of 2000-2300 m. It is native to West Guizhou, South-West and West Sichuan, central and North-West Yunnan (Zhengyi and Raven, 1995).

**Plant.** *Onosma paniculata* is a biennial herb, 40–80 cm tall and blackens after drying. Stems are unbranched and 5–10 cm in diameter at the base. The basal leaves are linear-lanceolate or oblanceolate, attenuate at base and acuminate at the apex. The upper and middle stem leaves are lanceolate or ovate-triangular, hastate at the base and acuminate at the apex. Inflorescences are terminal, bracts triangular. The calyx is 7–8 mm and enlarged in fruit. The corolla is blue-purple, tubular-campanulate and densely strigose outside. Nutlets are dark brown, not shiny and tuberculate. It flowers from June until September (Zhengyi and Raven, 1995).

**Synonyms.** According to TROPICOS®, two synonyms are known: *Onosma oblongifolium* W.W. Sm. & Jeffrey and *Onosma paniculatum* var. *hirsutistylum* Lingelsh. & Borza.

**Traditional Use.** In TCM, *Onosma paniculata* is used as Zicao. Zicao is actually a term for the roots of several plants of the Boraginaceae family containing naphthoquinone pigments as main constituents. It comprises several *Onosma*, *Arnebia* and *Lithospermum* species. Indications for Zicao are measles and other eruptive exanthema, skin infections, eczema, burns, scalds and constipation (Zhongzhen, 2004, The State Pharmacopoeia Commission of the People’s Republic of China, 2005). The roots are collected in spring and autumn, cleaned and dried under the sun (Zhongzhen, 2004). *Arnebia euchroma*, *Lithospermum erythrorhizon* and *Arnebia guttata* are officially listed in the Chinese pharmacopoeia (The State Pharmacopoeia Commission of the People’s Republic of China, 2005). *Lithospermum erythrorhizon* is also listed in the Japanese and Korean Pharmacopoeia and for five types of tumor in the Tibet-China pharmacopoeia, (Hu et al. 2006c, Duke and Ayensu, 1985a). In South-West China (Hu et al., 2006c) and other parts of the world (Duke and Ayensu, 1985a), several *Onosma* species are considered as carminative, cardiotonic, demulcent, diuretic, purgative, refrigerant, stimulant and tonic.

**History** (Papageorgiou et al., 1999). The history of Zicao and its main compounds shikonin and alkannin dates back to the time before Christ and took place in Asia and
Europe. In China, the plant *Lithospermum erythrorhizon* was first known for its silk coloring properties. Later, the surgeon Hua To (born ca. 136-141 AD) introduced it into TCM as medicine. In the 1596 written Pen Ts´ao Kang Mu, the roots of this plant are also listed. Even today, it is still listed in modern books of TCM – a fact displaying evidence for its medical properties. In Europe, the history is connected with the roots of *Alkanna tinctoria* and its main compound alkannin. It was also used for coloring textiles at times before Christ. The probably first documented medical usage of these roots is found in the work of Hippokrates (5.-4. century BC) and Theophrastus (4.-3. century BC). Also in the famous work *De Materia Medica* written by Dioscorides in 77 AD, the effects of these roots are extensively described. Since this book was the standard work for many centuries, the healing properties of *Alkanna tinctoria* were known in whole Europe. According to a recipe of William Salman (ca. 1710), the roots are boiled in olive oil together with cleaned earthworms and applied to deep wounds and punctures of the nerves. However, the use of this root seems to be fallen in oblivion since this time. Not before 1976, when Papageorgiou reported about the wound healing and antimicrobial properties of *Alkanna tinctoria*, the plant and its constituents gained new interest. Meanwhile several medicinal properties are reported. Today, shikonin and alkannin are not only used as medicine but also in cosmetics and food coloring in Japan, Europe and North America. In 1980, not less than 12 European countries approved these substances for food and wine coloring (Papageorgiou, 1980).

**CHEMICAL COMPOSITION AND PHARMACOLOGY.** *Onosma paniculata* and other Zicao plants are especially known for their naphthoquinone pigments. Thereby, the enantiomers shikonin and alkannin provide the basic structure and several derivatives of them are known (figure 24). Isohexenylnapthazarins are in the outer surface of the roots and can be found in at least 150 species of the genus *Lithospermum, Echium, Onosma, Anchusa* and *Cynoglossum* (Boraginaceae) (Papageorgiou, 1980). Shikonin was firstly isolated and identified by Majima and Kuroda in 1922; alkannin by Brockmann in 1935 (Papageorgiou, 1980). Several publications can be found describing the cytotoxic and anti-cancer activities of these compounds. In 1977, Sankawa et al. showed that shikonin completely reduced the growth of ascites cells of Sarcoma 180 in mice. Moreover, it induced apoptosis in several cancer cell lines such as HL-60 leukemia (Yoon et al., 1999), MCF-7 breast cancer (Hou et al., 2006), COLO 205 colon cancer (Hsu et al., 2004), A375-S2 melanoma (Wu et al., 2004), Tca-8113 oral squamous carcinoma (Min et al., 2008) and T24 bladder cancer cells (Yeh et al., 2007).

In COLO 205 cells, it was clearly shown that shikonin caused generation of reactive oxygen species, release of cytochrome c, activation of caspase-9 and -3, degradation of
PARP and DNA fragmentation while caspase-1 and -8 were not activated (Hsu et al., 2004). Shikonin also affected the cell cycle via cycline dependent kinases, cyclins and p21 (Yeh et al., 2007), increased p53 and bax protein levels and decreased bcl-2 protein levels and cyclin-dependent protein kinase 4 (Wu et al., 2004).

Another reported characteristic of these substances is the ability to induce a necroptotic death (Han et al., 2007, Xuan and Hu, 2009). Necroptosis was identified by Degterev et al. and is characterized by (a) necrotic cell morphology, (b) loss of plasma membrane integrity, (c) autophagy as downstream consequence, (d) rising of reactive
oxygen species in some cells, (e) loss of mitochondrial membrane potential and (f) the ability of necrostatin-1 to inhibit the process (Degterev et al., 2005). It is assumed to function as a backup when apoptosis is blocked (Han et al., 2009).

Plyta et al. (1998) reported on topoisomerase I inhibiting activities of several naphthoquinone derivatives which probably rely partially on the binding to a zinc finger domain of topoisomerase I. Fujii et al. (1992) referred to the inhibition of topoisomerase II by shikonin and Lu et al., (2002) had shown that shikonin and some derivatives inhibited telomerase, an enzyme essential in replication, cell cycle and senescence. Yang et al. (2009b) had demonstrated that shikonin also inhibited the proteasome in vitro and in vivo and, therefore, led to cell death and tumor growth inhibition. Also angiogenesis, a crucial step in tumor growth and metastasis, was inhibited by shikonin (Lee et al., 2008) and acetylshikonin (Pietrosiuk et al., 2004, Lee et al., 2008) in mice. The anti-cancer activity of shikonin was also investigated in a clinical study. A shikonin mixture significantly reduced tumor growth and improved the immune function and the life quality of late-stage lung cancer patients who were not treatable with operation, radiotherapy and/or chemotherapy (Guo et al., 1991).

Besides the anti-cancer activity, Zicao plants and their naphthoquinones are especially known for their wound healing activity. It has been shown that naphthoquinone derivatives induced reepithelialization, granulation tissue formation and, in contrast to the effects on tumors, angiogenesis (Lu et al., 2008, Papageorgiou et al., 1999). Recent experiments with beagel dogs showed that isoheaxenynaphthazarins decreased wound size significantly accompanied by similar effects (Karayannopoulou et al., 2010). Clinical studies confirmed the wound healing activities and the approval of some pharmaceutical formulations (for example HISTOPLASTIN RED® and HELIXDERM®) has been achieved. These formulations were successfully applied in the therapy of leprosy, decubitus and anal fissures (Papageorgiou et al., 2008, Papageorgiou et al., 1999). In cosmetics, root extracts of Lithospermum erythrorhizon have been shown to positively effect human skin hydration and transepidermal water loss (Chang et al., 2008). Furthermore, naphthoquinone derivatives have been reported to possess anti-inflammatory (Cheng et al., 2008, Tosun et al., 2008, Papageorgiou et al., 1999), antimicrobial (Shen et al., 2002), antiviral (Rajbhandari et al., 2007) and antifungal activities (Sasaki et al., 2002).

When considering these plants as medicine, another important class of natural products are pyrrolizidine alkaloids. This compound class is known for its hepatotoxic and carcinogenic effects. While Lithospermum erythrorhizon (Roeder, 2000) and Onosma species (Mroczek et al., 2004, Kretsi et al., 2003) contain high amounts of these
alkaloids, *Arnebia euchroma* contains only 10 ppm (Roeder, 2000). Therefore, a clear and unambiguous identification of the plant material is also of substantial interest.

Recently, new compounds have been isolated from the roots of *Arnebia euchroma*: octyl ferulate, a naphthoquinone dimer named arnebiabinone and the phenol ethyl 9-(2',5'-dihydroxyphenyl) nonanoate (Lui et al., 2010).

### 2.3.11. *Periploca sepium*

**Taxonomy**
- Class: Rosopsida
- Subclass: Asteridae
- Order: Gentianales
- Family: Asclepiadaceae
- Genus: Periploca
- Species: *Periploca sepium* Bunge

The family of Asclepiadaceae contains about 2000 species and has specialized flowers. Anthers and stigma are connected to a so called “gynostegium”. The pollen of one half of an anther are grouped into pollinia. Neighbored pollina are connected to form one pollination unit. They show a broad life-form spectrum, whereby, epiphytes and succulents are especially outstanding (Frohne and Jensen, 1998).

**Distribution.** *Periploca sepium* is mainly found in Chinese provinces such as Shanxi, Henan, Hebei, Shandong, Gansu and Hunan (Zhongzhen, 2004) and North and tropical Africa (Feng et al., 2008).

**Plant and crude drug.** *Periploca sepium* (figure 25) is a deciduous shrub up to 4 m high. Leaves are ovate-oblong and membranous. Pedicels are about 2 cm, sepals triangular-ovate and the corolla mostly purple and approximately 1.5 cm in diameter. Cymes are on lateral branchlets, often paired and sparsely-flowered. Seeds are oblong. It flowers from May to June (Zhengyi and Raven, 1995). The root is tubular or sulciform, 3-10 cm long, 1-2 cm in diameter and 2-4 mm thick. The outer surface is grayish-
yellowish-brown and the cork scales off easily. The inner surface is yellowish-white or pale reddish-brown and marked with fine longitudinal striae. It breaks easily. The odour is heavily aromatic, the taste bitter and slightly tongue-numbing (Zhongzhen, 2004).

**Traditional use.** The root bark is traditionally used in TCM to treat rheumatic arthritis with pain and weakness of the loins and knees, palpitation, dyspnoea and oedema of the lower extremities and has a strong insecticidal and cardiac action (Zhongzhen, 2004, The State Pharmacopoeia Commission of the People’s Republic of China, 2005, Bamba et al., 2007). However, it should not be taken for longer than one week because of its cardiac glycosides content (Hempen and Fischer, 2001). It is collected in spring and autumn, peeled off and dried in shadowy areas or under the sun (Zhongzhen, 2004). Additionally, *Periploca sepium* was utilized as firewood to light signal fires on the Great Wall of China (Bamba et al., 2007).

**Chemical composition and pharmacology.** In 1987 and 1988, Itokawa et al. published several papers about the investigations of an antitumor fraction from *Periploca sepium*. The MeOH extract of the root bark was activity-guided fractionated using Sarcoma 180 ascites in mice. This fractionation led to the isolation of pregnane glycosides, named periplocoside A, B, C, D, E, F, L, J, K, M and O and a steroidal compound S-20 (Itokawa et al., 1987b, 1988a, 1988b, 1988c). Periplocoside A showed significant antitumor activity against the Sarcoma 180 ascites (Itokawa, 1988a). Periplocoside A and E were shown to be immunosuppressive by direct inhibition of T cell activation in vitro and in vivo (Zhu et al., 2006, Zhang et al., 2009b). Periplocoside A, D, E, and F inhibited the proliferation of T cells in vitro without obvious cytotoxicity. Also other pregnane glycosides, such as Periperoxide A, B, C, D and E exhibited this proliferation inhibitory activity (Feng et al., 2008). Moreover, the methanolic root bark extract induced differentiation in mouse myeloid M1 leukemia cells. Activity-guided fractionation led to the isolation of several active pregnane glycosides, including plocoside A and B, and several active cardenolides (Umehara et al., 1995).

Periplocin, a cardenolid isolated from Cortex Periplocae inhibited the growth of human SW480 colon cancer cells via induction of apoptosis by targeting the beta-catenin/Tcf signaling pathway and down-regulation of survivin and c-myc (Zhao et al., 2010). The same compound activated caspases-dependent apoptosis in PC3 prostate cancer cells. Another cardenolid, periplocymarin sensitized U937 monocytic leukemia cells to TRAIL and led to caspases-dependent apoptosis in PC3 prostate cancer cells as well (Bloise et al., 2009).
Other compounds isolated from the root bark comprise the oligosaccharides perisesaccharides A, B, C, D and E (Wang et al., 2010b), the oligosaccharides D₂ and F₂, perisaccharide A, B and C, periplocogenin, pregnene-3β,20(S)-diol-3-O-[2-O-acetyl-β-D-digitopyranosyl-(1→4)-O-β-D-cymaropyranoside]-20-O-β-D-glucopyranosyl(1→6)-O-β-D-glucopyranosyl(1→2)-O-β-D-digitopyranoside, glycoside K, periplofenin, periforoside I, periforgenin A and pregn-5-ene-3β,16β,20(R)-triol-20-O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl(1→2)-O-β-D-digitopyranoside (Feng et al., 2008). Moreover, (24R)-9,19-cycloart-25-ene-3β,24-diol, (24S)-9,19-cycloart-25-ene-3β,24-diol, cycloeucalenol, β-amyrin acetate, α-amyrin, isovanillin and vanillin (Wang et al., 2007). Vanillin induced apoptosis and cell cycle arrest in human HT-29 colon cancer cells and enhanced TRAIL-induced apoptosis in human leukemia Hela cells (Ho et al., 2009, Lirdprapamongkol et al., 2010). Microarray analysis using hepatocarcinoma cells confirmed that vanillin affects clusters of genes involved in cell cycle and apoptosis and down-regulates cancer progression associated genes (Cheng et al., 2007).

GC-MS analysis of the volatile oil of the root bark showed that it is mainly composed of aromatic compounds, such as 4-methoxysalicylaldehyde (87.99%), eugenol (0.39%), cinnamaldehyde (0.32%), 2-hydroxy-4-methoxy-acetophenone (0.25%), anethole (0.22%) and p-anisaldehyde (0.17%) (Miyazawa et al., 2004). It has been reported that the volatile oil and 4-methoxysalicylaldehyde possess antimicrobial and antioxidant activities (Wang et al., 2010a). Investigations of the milky exudate revealed the presence of cis-polyisoprene (Bamba et al., 2007).
2.3.12. **Saussurea lappa**

**Taxonomy.**

Class: Rosopsida  
Subclass: Asteridae  
Order: Asterales  
Family: Asteraceae  
Genus: Saussurea  
Species: *Saussurea lappa* Clarke

The family of Asteraceae comprises more than 25000 species, which are distributed worldwide and native to many different habitats. There are aquatic plants, succulents, as well as small herbs and up to 20 m high tropical trees. Characteristic is an inferior ovary with only one ovule, achenes, a reduced calyx and the aggregation of the single flowers to a capitulum (Frohne and Jensen, 1998).

**Distribution.** *Saussurea lappa* (figure 27) is native to India and grows in sub alpine regions of Jammu and Kashmir, Himachal Pradesh and Uttarakhandal in height of 3200-3800 m (Pandey et al., 2007). It is also native to China – mainly to the provinces Yunnan, Sichuan and Tibet (Zhongzhen, 2004) – and to Pakistan. Due to increasing medicinal and commercial purposes, wildlife stocks decrease day by day. Therefore,
Saussurea lappa is also listed in Appendix I of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Kuniyal et al., 2005).

**Plant and Crude Drug.** *Saussurea lappa* is an erect, robust, pubescent, perennial herb and 1-2 m tall. The basal leaves are very large and have a long petiole. The upper leaves are smaller and have a short petiole. Flower heads are bluish-purple to almost black, stalkless, hard, rounded and 2-5 flowers are clustered together. Involucral bracts are ovate-lanceolate, purple, rigid and hairless. The corolla is about 2 cm long, tubular and also blue-purple to almost black in color. Anther tails are fimbriate, achenes curved, about 8 mm long and tip narrowed with one rib on each face (Pandey et al., 2007).

The root is cylindrical or semi-cylindrical, up to 40 cm long and 0.5-5 cm in diameter. The outer surface is yellowish- to dull grayish-brown and marked with conspicuous wrinkles, longitudinal grooves and scars of rootlets. It is hard, solid and does not brake easily. The odor is fragrant and distinctive, the taste slightly bitter (Zhongzhen, 2004).

**Synonyms.** According to TROPICOS®, six synonyms for this plant are known: *Saussurea costus* (Falc.) Lipschitz, *Saussurea lappa* (Decne.) Sch. Bip., *Aucklandia lappa* Decne., *Aucklandia costus* Falc., *Aplotaxis lappa* Decne., and *Theodorea costus* (Falc.) Kuntze.

**Traditional Use.** Dried roots of *Saussurea lappa* are traditionally used in different medicine systems. In China, it is known as Mu Xiang, which refers to “wood fragrance”, and used to alleviate abdominal distention, tenesmus, indigestion with anorexia, dysentery, nausea, vomiting (Pandey et al., 2007), asthma, cancer, cholera, rheumatism and cough (Duke and Ayensu, 1985a). Roots of two to three years old plants are collected in autumn and winter, cut into segments or sliced length-wise and dried under the sun or by wind. The cork is removed (Zhongzhen, 2004). In the Indian medical systems of Ayurveda, Siddha and Unani, Radix Saussureae is mainly used to treat asthma, cough, cholera, chronic skin diseases and rheumatism (Pandey et al., 2007). In Tibetan medicine, several traditional formulations containing these roots are found for the treatment of chronic inflammations of the lungs, cough and chest congestion. It is, for example, part of a remedy called “Padma 28” which is used to “support the body’s mechanisms and to help maintain good health” and was shown to possess anti-inflammatory activity (Moeslinger et al., 2000). In TCM and Ayurveda, decoctions of the root are prepared either as a single drug or in combination with other drugs. The oil of the root has a reputation for its fragrance and is, therefore, also used in perfumery (Sun
et al., 2003). The oil is obtained from the roots by steam distillation or solvent-extraction. Dried leaves are smoked as tobacco in northwestern Himalaya and used as fodder during fodder scarcity in winter (Kuniyal et al., 2005).

**Chemical composition and pharmacology.** Various extracts of *Saussurea lappa* and isolated natural products have been reported to possess anti-inflammatory, anti-cancer, hepato-protective, anti-ulcer, cholagogic, immunomodulatory, hypolipidaemic, hypoglycaemic, antispasmodic, antimicrobial, antiparasitic, antifeedant and CNS depressant activities (Pandey et al., 2007). In AGS gastric cancer cells, a methanolic extract of *Saussurea lappa* root led to caspase-3 activation, upregulation of p53, cell cycle arrest at the G2/M-phase, translocation of bax to the mitochondria and, therefore, to apoptosis (Ko et al., 2004 and 2005).

In *Saussurea lappa*, sesquiterpenes and sesquiterpene lactones are the most common constituents (Paul et al., 1960). Sesquiterpene lactones can be found in more than 100 plant families and display a huge and diverse group of natural products. Most of them exist within the Asteraceae family (Zhang et al., 2005). Costunolide and dehydrocostus lactone are the most famous and important representatives in *Saussurea lappa* (figure 28).

Regarding its anti-cancer activity, costunolide was reported to induce apoptosis in HL-60 leukemia cells by reactive-oxygen species-mediated mitochondrial membrane potential loss, followed by the release of cytochrome c and activation of caspase-9 and -3 (Lee et al., 2001). The involvement of ROS-species was also suggested by Park et al. (2001), since the level of the anti-apoptotic bcl-2 protein was decreased, whereas, PARP was activated. In NALM-6 cells, not only caspase-9 was activated and PARP cleaved, but also caspase-8 (Kanno et al., 2008). Choi and Lee (2009) could show that c-Jun N-terminal kinase activation (JNK) also contribute to the costunolide induced apoptosis in human leukemia cells. Another important target in cancer cells are telomerases which play an essential role in cell proliferation by adding TTAGGG repeats to telomeres. In the majority of cases they are activated in cancer cells (Counter et al., 1992). It was shown that costunolide inhibited telomerase activity in MCF-7 and MDA-MB-231 breast cancer and NALM-6 B-cell leukemia cells (Choi et al., 2005; Kanno et al., 2008) and, therefore, led to apoptosis. Another target in MCF-7 cells seems to be microtubules. Costunolide led to a massive polymerization of tubulin and, therefore, to a disturbed microtubule organization (Bocca et al., 2004). Also an antiangiogenic effect of costunolide by blocking the angiogenic factor signaling pathway was reported (Jeong et al., 2002). In Syrian golden hamsters and F344 rats, costunolide inhibited buccal pouch and intestinal carcinogenesis also in vivo (Ohnishi et al., 1997, Mori et al., 1994).
Dehydrocostus lactone induced apoptosis in DU145 prostate cancer cells (Kim et al., 2008). It caused activation of caspase-3, -7, -8 and -9, enhanced PARP cleavage, decreased anti-apoptotic proteins and increased pro-apoptotic. Similar effects were also observed in HepG2 and PLC/PRF/5 hepatocellular carcinoma cells (Hsu et al., 2009). It was shown that dehydrocostus lactone triggered endoplasmic reticulum stress (Hung et al., 2010, Kucharczak et al., 2003) and nuclear relocation of mitochondrial AIF and endonuclease G. NF-κB has been reported to counteract apoptosis induced by TNF-α, ionizing radiation and some anticancer drugs (Kucharczak et al., 2003). In HL-60 leukemia cells, dehydrocostus lactone inhibited NF-κB activation by preventing TNF-α-induced degradation and phosphorylation of I-κBα. Therefore, the activities of caspase-8 and -3 were increased and apoptosis triggered (Oh et al., 2004). Furthermore, an effect on cell cycle distribution was reported in ovarian SK-OV-3 cancer cells. The cells were arrested at the G2/M interface accompanied by a slight decrease in the expression of CDK4 and cyclin E and an increase in p21 (Choi and Ahn, 2009). Other cytotoxic sesquiterpene lactones from these roots comprise for example cynaropicrin, reynosid, dihydroreynosid, lappadilactone, lappalone, zaluzanin, arbusculin, santamarin and colartin (Pandey et al., 2007, Sun et al., 2003). Moreover, several germacrenes (de Kraker et al., 2001), sulfonated guaianolides (Wang et al., 2008), aplotaxene, β-sitosterol, linoleic and betulinic acid methyl esters (Choi et al., 2008) were isolated.

Costus root oil contains resinoids (6%), essential oil (~1.5%), inulin (18%), saussurine and other alkaloids (0.05%). It was shown to be hyposensitive, bronchodilatory and antiseptic against Streptococcus and Streptophylococcus (Kuniyal et al., 2005) and exhibited quite promising results as a non-specific agent for cancer immunotherapy (Takanami et al. 1987). Delactonized oil exhibited antispasmodic activity
in guinea pig ileum and dog and guinea pig trachea as well as hypotensive effects in anaesthetized dogs (Gupta and Ghatak, 1967).

### 2.3.13. **Zanthoxylum nitidum**

**Taxonomy**
- Class: Rosopsida
- Subclass: Rosidae
- Order: Rutales
- Family: Rutaceae
- Genus: Zanthoxylum
- Species: *Zanthoxylum nitidum* (Roxb.) DC

The Rutaceae contain about 1600 species, whereby, the genus Zanthoxylum is native to the tropics and shows some characteristics more typical to Magnoliidae, for example, unspecialized flower morphology. Rutaceae have actinomorphic flowers with five petals and a superior ovary with epitrop ovules (Frohne and Jensen, 1998).

**Distribution.** *Zanthoxylum nitidum* (figure 29) is found in Fujian, Hunan, Guangxi, Guangdong, Yunnan, Taiwan and Hong Kong (Zhongzhen, 2004) and occurs from India to northern Queensland, Australia (Deyun et al., 1996).

**Plant and Crude Drug.** *Zanthoxylum nitidum* is a morphologically variable species, found as liane in rainforests and as 1-3 m tall, climbing shrub in dryer habitats (Fang et al., 1993, Deyun et al., 1996). Radix Zanthoxyli is cylindrical, slightly curved, 0.7-5 cm or above in diameter, hard and dark brownish-yellow to light brown. When broken, surface shows thin cork. The odor is slightly fragrant, the taste pungent, tongue-numbing and bitter (Zhongzheng, 2004).

**Synonyms.** According to TROPICOS®, a synonym for this plant is *Fagara nitida* Roxb.

**Traditional Use.** In China, the roots are harvested throughout the year, cleaned, cut into slices and dried under the sun or used directly. Indications are trauma, rheumatic arthralgia, stomachache, toothache, burns, tetanus, bruises, rheumatism and scalds. It is
also used for venomous-snake bites (Duke and Ayensu, 1985b, Zhongzheng, 2004). The fruits are collected in autumn, cleaned and used unprepared. Best quality is obtained in Sichuan. They warm the spleen and stomach, alleviate pain and kill parasites. They are also used in combination with other plants. For example, along with Radix Ginseng and Rhizoma Zingiberis in “The Major Decoction for Rehabilitating the Spleen and Stomach” (Wu, 2005). Zanthoxylum nitidum is also part of a Chinese toothpaste and was shown to decrease the incidence of dental plaque and enhance gingival health (Wan et al., 2005).

**CHEMICAL COMPOSITION AND PHARMACOLOGY.** The most important bioactive substances found in Zanthoxylum nitidum belong to the alkaloids. Others are coumarins and lignins. Nitidine, the most famous alkaloid and mainly found in the roots (Zhang et al., 2001) has been reported to exhibit anti-cancer activity in B16 melanoma, MCF-7 and HS578T breast and DU145 and MPC3 prostate cancer cells (Del Poeta et al., 1999). As well as chelerythrine and isofagaridine, it inhibited topoisomerase I mediated DNA relaxation and, therefore, influenced important cellular processes such as DNA replication and transcription (Fang et al., 1993). Nitidine, along with dihydrochelerythrine, oxyavicine, 8-methoxychelerythrine and 8-hydroxydihydrochelerythrine also showed analgesic and anti-inflammatory activities in mice (Hu et al., 2006a).

Chelerythrine were shown to inhibit cell viability, increase DNA damage and induce the cyclin kinase inhibitors p21 and p27 in human LNCaP and DU145 prostate cancer cells (Malikova et al., 2006). In human HL-60 leukemia cells, they arrested the cells in the G1 phase, activated the mitochondrial apoptotic pathway and induced both, apoptosis and necrosis (Vrba et al., 2008). Moreover, chelerythrine induced apoptosis in osteosarcoma cells by activation of the RAF/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway (Yang et al., 2008b). 5,6-dihydro–6–methoxyxnitidine, skimmianine and 5–methoxydictamnine, alkaloids isolated from the roots, have been reported to be effective against hepatitis B virus. 5–methoxydictamnine and other alkaloids like dictamnine and gamma–fagarine were also isolated and shown to possess antimitotic and antifungal activities (Yang and Chen, 2008).

Skimmianine exhibited cytotoxicity against HL-60 leukemia, RAJI Burkitt lymphoma, Jurkat leukemia, MCF-7 breast cancer, KG-1a hematopoietic myeloid progenitor cells, Hep-2 larynx cancer (Varamini et al., 2009) and Hela cervical cancer cells (Jansen et al., 2006).

Other isolated compounds have been identified as: oxynitine, oxyavicine, oxychelerythrine, dihydrochelerythrine, 6–acetonyldihydrochelerythrine, norchelerythrine, decarine, arnottianamide, flindersine, 4–methoxy–1–methyl–2 quinolone, skimmianine,
gamma–fagarine, liriodenine, N–acetyldehydroanonaine, N–actetylanonaine, sesamin, episesamin, piperitol–3,3–dimethylallyl ether, xanthoxylol–3,3–dimethylallyl ether, savinin, 2,3–bis(3,4–methylenedioxybenzyl)but–2–en–4 olide, α–cadinol, anticopalol, spathulenol, aesculetin dimethyl ether, β–sitosterol and β–sitostenone. They were tested against MCF-7, NCI-H460 and SF-268 cancer cell lines, whereby, liriodenine was the most active compound (Yang et al., 2009a). Liriodenine also inhibited the growth of human A549 lung adenocarcinoma (Chang et al., 2004) and the hepatoma cell lines Hep G2 and SK-Hep-1 (Hsieh et al., 2005) by cell cycle arrest and, in lung cancer cells, activation of apoptosis.

Figure 30. Structures of compounds isolated from Zanthoxylum nitidum.

The alkaloids zanthomuurolanine, epi-zanthomuurolanine, zanthocadinanine A and B and epi-zanthocadinanine were isolated from the stem bark as well (Yang et al., 2008a). Moreover, β–amyrin, asirinin, dihydrogaagaridine, decarine acetate and hesperidin have been isolated from petrol, ethyl acetate and methanolic extracts (Deyun, 1996). Sesamin inhibited the growth of several cancer cells, such as leukemia, myeloma, colon, prostate, breast, pancreas and lung cancer and potentiated TNF-α–induced apoptosis (Harikumar et al., 2010). Hesperidin has been found in significant amounts (0.74%) in the roots by LC-ES-MS analysis (Lu et al., 2006). From an ethanolic extract of the roots, 2,4–dihydroxypyrimidine, syringic acid, 2,6–dimethoxy–1,4–benzoquinone, 4–hydroxybenzoic acid, ethylparaben, (Z)-3-(2,3,4-trimethoxyphenyl) acrylic acid, 5,6,7–trimethoxycoumarin, stigmast-9, stigmast-9-en-3-ol and daucosterol were isolated (Hu et al., 2006b).

Since alkaloids are the most important bioactive compound class found in Zanthoxylum nitidum, an HPLC-UV method has been developed to determine nine alkaloids simultaneously: berberrubine, coptisine, sanguinarine, nitidine, chelerythrine, liriodenine, 6,7,8-trimethoxy–2,3-methylendioxybenzophenantridine, oxyavicine and dihydrochelerythrine. Since variations in the chemical composition can lead to extreme changes in the therapeutic effects, this method was reckoned to provide a feasibility of quality control (Liang et al., 2006).
3. OWN INVESTIGATIONS AND DISCUSSION

3.1. PRELIMINARY PHARMACOLOGICAL INVESTIGATIONS

3.1.1. DETERMINATION OF CELL NUMBER AND VIABILITY

For the determination of cell number, a Neubauer hemocytometer or a CASY® counter was used. In the case of CCRF-CEM, MDA-MB-231, U251 and HCT 116 cells a new CASY® setup had to be developed. This was done with the help of CASY® excell and the CASY® blue Evaluation Programm. Three measurements with living and three measurements with dead cells were performed (3 x 400 µl each measurement). To get dead cell samples, cells were treated with 800 µl CASY® blue for 2 min. CASY® blue is an alcoholic ready-to-use reagent which creates standardized samples of dead cells. Afterwards, living and dead cell data were loaded into the CASY® blue Evaluation Programm and the settings of cursors used for determination of viability were calculated (see figure 31).
OWN INVESTIGATIONS AND DISCUSSION

### Measurement Parameters
- **Capillary**: 150 µm
- **Size Scale**: 40 µm
- **Sample Volume**: 3 x 400 µl
- **Dilution**: 200,00

### Results Cursor
- **Norm Cursor**: 7.88 µm, 0.8
- **Eval Cursor**: 12.94 µm, 1.1

### Assay Quality

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<td><strong>dead</strong></td>
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### Measurement Parameters
- **Capillary**: 150 µm
- **Size Scale**: 40 µm
- **Sample Volume**: 3 x 400 µl
- **Dilution**: 400,00

### Results Cursor
- **Norm Cursor**: 7.38 µm, 1.2
- **Eval Cursor**: 12.60 µm, 1.4

### Assay Quality

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3.1.2. **IC$_{50}$ Determination of Vinblastine**

Vinblastine is a known chemotherapeutic and served as positive control in the XTT viability assay. Therefore, first of all IC$_{50}$ values for each cell line were determined after 72 h.

**Figure 3.1**. Living (red line) and dead (blue line) cell distribution of CCRF-CEM, MDA-MB-231, U251 and HCT 116 cells. Vertical solid red and dotted blue lines: positions of the cursors used for determination of viability and cell number (CASY® blue Evaluation Programm).

**Figure 3.2**. Determination of IC$_{50}$ values of vinblastine on different cancer cell lines and a lung fibroblast cell line (MRC-5), n = 6, mean ± sem.
It could be shown that vinblastine reduced cell growth and proliferation dose-dependently. Moreover, these experiments have demonstrated the reproducibility of the assay (figure 32). For positive control purposes, vinblastine was used at a final concentration of 10 ng/ml (12.3 nM) in the case of cancer cells and 100 ng/ml (123 nM) in the case of MRC-5 lung fibroblasts.

### 3.2. Bischofia javanica

#### 3.2.1. Plant material, extraction procedure and TLC fingerprint

Leaves and branches were collected by S. Kahl in Xishuangbanna, China, in April 2003 and authenticated at the Kunming Institute of Botany, China (Kahl, 2005). In March 2008, fresh plant material was obtained from the Botanical Garden in Graz and dried at 30 °C. For screening purposes, 4.8 g of the material from China and 3.1 g of the new plant material was successively extracted with petroleum ether, ethyl acetate and methanol by ASE extraction. Extracts were evaporated to dryness under reduced pressure at 40 °C. The yields were: Chinese material: PE: 23.4 mg, EtOAc: 42.3 mg, MeOH: 309.5 mg; plant material from Graz: PE: 49.9 mg, EtOAc: 94.8 mg, MeOH: 374.8 mg. Furthermore, 4.4 g of the fresh material was subjected to maceration with MeOH for 40 h. The yield was 138 mg.

![Figure 33. TLC fingerprint of different extracts of Bischofia javanica sprayed with vanillin – sulfuric acid and photographed at vis.](image-url)
For TLC fingerprint analysis, 10 µl of a 5 mg/ml stock solution were applied to each of six TLC plates. Two plates respectively were developed with one of three solvent systems. Afterwards, one plate was sprayed with vanillin-sulfuric acid, the other one with NST/PEG – reagent. Best results are shown in figure 33 - 35.

Figure 34. TLC fingerprint of different extracts of Bischofia javanica photographed at 366 nm.

Figure 35. TLC fingerprint of different extracts of Bischofia javanica sprayed with Naturstoff – polyethylene glycole and photographed at 366 nm.
Comparison of the TLC fingerprints revealed some differences between the two plant materials used. At 366 nm, there was an additional blue zone in the Chinese ASE PE extract which was missing in the Austrian extract. Moreover, also in the ASE EtOAc and MeOH extract there was an additional blue zone in the Chinese material when sprayed with NST/PEG and viewed at 366 nm. Viewed under vis, the green and yellow zones of the ASE EtOAc extract were not yet extracted with PE in the Chinese plant material but in the material from Graz. However, there were also some additional red fluorescent zones in the plant material of Graz, which could not be found in the Chinese material. These red fluorescent zones are probably chlorophylls. The maceration of the fresh plant material seemed to be less effective than ASE extraction and revealed a similar zone pattern than the ASE EtOAc extract of the Graz material.

3.2.2. Pharmacological Investigations of Extracts

All extracts were subjected to the XTT viability assay. CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were treated with 10 µg/ml extract for 72 h. The MeOH macerate was also tested at 100 µg/ml.

![Graph showing metabolic active cells (% of control) for different extracts and concentrations](image_url)

*Figure 36. XTT viability assay of different extracts of Bischofia javanica. Incubation time: 72 h, concentration: 10 µg/ml. In the case of macerate (Graz plant material): 10 and 100 µg/ml. n = 6, mean ± sem.*
As shown in figure 36, neither the plant material from China nor the plant material of the Botanical garden in Graz showed strong growth inhibition of the cancer cells used. All extracts tested at 10 µg/ml were in the range of 85 – 110% of control and were not able to decrease the growth and viability of the cancer cells. Even at 100 µg/ml the macerate of *Bischofia javanica* showed only a slight inhibition of CCRF-CEM and MDA-MB-231 cells.

**3.2.3. DISCUSSION**

TLC investigation revealed some differences between the ASE extracts of the Chinese plant material and the material from Graz. Reasons for that could be age differences of the trees, different climate conditions (wild growing in China versus greenhouse in Graz), differences in soil properties and/or possible infestations by pests. Another explanation could also be that the natural product composition changed during storage of the Chinese plant material.

Some substances, isolated from *Bischofia javanica*, have been reported to inhibit the growth of cancer cells and to induce apoptosis. Betulinic acid in particular is relatively well investigated and even in a phase I/II clinical trial for topical treatment of dysplastic nevi (Fulda, 2009). It exhibited activity against a variety of cancer cells and induced apoptosis by a direct effect on mitochondrial membranes. This led to the release of soluble factors, such as cytochrome c, and, therefore, to the activation of different caspases. Also an inhibition of topoisomerase II by betulinic acid was described (Wada and Tanaka, 2005) and it showed some promising *in vivo* results as well (Pisha et al., 1995, Zuco et al., 2002). Worth mentioning is also that it hardly affected healthy cells (Fulda, 2009, Mullauer et al., 2010, Zuco et al., 2002). Other substances, isolated from different parts of *Bischofia javanica* which showed anticancer and apoptosis inducing activities were some phytosterols like β-sitosterin, triterpene such as friedelin and derivatives, and flavonoids such as fisetin, quercetin, and quercitrin. However, in our study, neither leaves collected in China nor plant material from the botanical garden in Graz could inhibit the growth of the cancer cells used (CCRF-CEM, MDA-MB-231, U251 and HCT 116) up to 10 or 100 µg/ml. Further investigations are necessary to determine whether the described active components are contained in the extracts prepared and whether the concentration was just too low.
3.3. **Bryophyllum pinnatum**

### 3.3.1 Plant Material, Extraction Procedure and TLC Fingerprint

*Bryophyllum pinnatum* (whole plant) was collected by S. Kahl in Xishuangbanna, China, in April 2003 and authenticated at the Kunming Institute of Botany, China (Kahl, 2005). 5.2 g freshly powdered plant material were subjected to ASE and successively extracted with PE and MeOH. Extracts were evaporated to dryness under reduced pressure at 40 °C. Yields were: 56.2 mg PE and 214.6 mg MeOH extract. For TLC fingerprint, a 5 mg/ml stock solution was prepared and 10 µl applied to each of six TLC plates. Respectively, two plates were developed with one of three solvent systems, analyzed at vis, 366 nm and 254 nm and sprayed with vanillin – sulfuric acid or NST/PEG reagent. Best results are shown in figure 37. Both extracts exhibited several red fluorescent zones, whereby, a few appeared in both, others only in one extract. The PE extract showed some additional blue to turquoise zones. When sprayed with vanillin – sulfuric acid several violet zones were observed.

![TLC fingerprint of different extracts of Bryophyllum pinnatum.](image)

### 3.3.2 Pharmacological Investigations of Extracts

Both extracts were subjected to the XTT viability and growth inhibition (GI) assay. CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were used for the XTT assay, CCRF-CEM for the GI assay. Extracts were tested at a final concentration of 10 µg/ml and incubated for 72 h (XTT) or 7 days (GI). As shown...
in figure 38, the PE extract had no effect on growth and viability of the cancer cell lines used. The MeOH extract inhibited proliferation of CCRF-CEM and HCT 116 cells to about 50% and 30% of control. MDA-MB-231 and U251 cells were not influenced. At the time of the GI assay, the extracts were ca. 10 month old. It could be shown that the growth inhibitory effect of the MeOH extract had almost disappeared after that time.

Figure 38. XTT viability (A) and GI (B) assay of different extracts of Bryophyllum pinnatum. A: XTT viability assay after 72 h, n = 6, mean ± sem, B: growth inhibition (GI) assay after 7 days, n = 3, mean ± S.D. Extract concentration: 10 µg/ml.

3.3.3. DISCUSSION

In this PhD thesis, the whole plant of *Bryophyllum pinnatum* was investigated regarding its cytotoxicity. A PE and MeOH extract were successively prepared by ASE and subjected to the XTT viability assay. After 72 h, CCRF-CEM leukemia and HCT 116 colon cancer cells showed a reduced proliferation and viability when treated with the MeOH extract, while MDA-MB-231 breast cancer and U251 glioblastoma cells were hardly affected in their growth. The PE extract showed no cytotoxicity against these cancer cell lines at a concentration of 10 µg/ml. MeOH extracts were also reported to show cytotoxicity against other cancer cells (Yamagishi et al., 1988, Abdellaoui et al., 2010). Nevertheless, up to now almost no death-inducing or proliferation inhibiting compounds were isolated whose mechanism of actions had been determined. Therefore, *Bryophyllum pinnatum* is a plant worth to be further investigated in this field. However, it was also shown that the activity of the MeOH extract was reduced after several months of storage indicating that the active compounds are not stable.
3.4. **CAESALPINIA SAPPAN**

### 3.4.1. PLANT MATERIAL, EXTRACTION PROCEDURE AND TLC FINGERPRINT

Leaves and branches of *Caesalpinia sappan* were collected by S. Kahl in Xishuangbanna, China in November 2002 and authenticated at the Kunming Institute of Botany, China (Kahl, 2005). Moreover, Lignum Sappan from December 2006 was obtained from Complemedis AG (Trimbach, Switzerland) in March 2008. Both (4.6 g and 4.5 g, respectively) were successively extracted with PE, EtOAc and MeOH by ASE extraction. Extracts were evaporated to dryness under reduced pressure at 40 °C. Yields were: Leaves and branches: PE: 74.7 mg, EtOAc: 118.8 mg, MeOH: 680.8 mg. Lignum: PE: 4.3 mg, EtOAc: 55.2 mg, MeOH: 314.8 mg. A 5 mg/ml stock solution of each extract was prepared and applied to each of six TLC plates. Respectively, two plates were developed with one of three solvent systems and, afterwards, viewed at vis, 366 nm and 254 nm and, finally, sprayed with vanillin-sulfuric acid or NST/PEG reagent. Best results are shown in figure 39.

![Figure 39. TLC fingerprint of different ASE extracts of Caesalpinia sappan leaves and branches or lignum.](image)
TLC fingerprint analysis of the extracts prepared revealed big differences between the zone pattern of leaves and branches and Lignum Sappan. While leaves and branches contain mostly red fluorescent substances – probably chlorophylls – when viewed at 366 nm, the wood displays mostly blue to turquoise zones. When sprayed with NST/PEG, Lignum Sappan exhibited a few additional zones, especially one red in the EtOAc and MeOH extract. In the zone pattern of leaves and branches was one additional, slightly yellow zone directly above the start. In the case of Sappan Lignum, extraction with PE was exhaustive, while the extraction with EtOAc was not exhaustive since the same zones appeared in the MeOH extract as well. In case of leaves and branches, extraction was not exhaustive in all three cases.

### 3.4.2. Pharmacological investigations of extracts

Extracts of *Caesalpinia sappan* were subjected to the XTT viability assay using CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells. Moreover, CCRF-CEM cells were incubated with the leaves/branches extracts for 7 days (GI assay). Extracts of the wood were tested in the XTT viability assay using the same cell lines as mentioned above. Results are shown in figure 40.

![Figure 40](image.png)

**Figure 40.** Effects of different ASE extracts of *Caesalpinia sappan*. On the one hand, leaves and branches were extracted, on the other hand, the wood (Lignum). A: XTT viability assay after 72 h, \( n = 6 \), mean ± sem, B: GI assay after 7 days, \( n = 3 \), mean ± S.D. Extract concentration: 10 µg/ml.
Extracts of leaves and branches showed no growth inhibitory or cytotoxic effects after 72 h as well as after 7 days. However, the EtOAc and MeOH extract of the wood decreased viability of CCRF-CEM leukemia cells significantly to about 15% of control cells and viability of the other cell lines to about 55 – 80% of control.

3.4.3. Discussion

Some extracts of this plant have been reported to exhibit anti-proliferative activity against several cancer cells lines (Kim et al., 2005, Ueda et al., 2002). In our study, it could be shown that some extracts of leaves and branches did not affect the growth and viability of several cancer cells lines after 72 h as well as after 7 days. However, an EtOAc and MeOH extract of the wood showed strong growth inhibitory activity against CCRF-CEM leukemia cells, whereby, breast, glioblastoma and colon cancer cells were less influenced. One of the major substance isolated from Lignum Sappan, brazilein, displayed DNA strand-scission activity in a cell free assay (Mar et al., 2003). This may contribute to the growth inhibitory activity observed. Another very similar compound, brazilein, activated several caspases and caused PARP cleavage in HepG2 cells (Zhong et al., 2009). Also several chalcones were reported to induce cell-cycle arrest and apoptosis in human cancer cell lines (Henmi et al., 2009, Sun et al., 2010a, Szliszka et al., 2010). This indicates that the growth inhibitory effects observed are not only based on cytotoxicity of the extracts. At the moment, the wood of *Caesalpinia sappan* is further investigated within another PhD thesis (Wagner, 2011). The active extracts will be activity-guided fractionated using the XTT viability assay and CCRF-CEM cells as monitor.

3.5. *Cinnamomum cassia*

3.5.1. Plant material, extraction procedure and TLC fingerprint

The bark of *Cinnamomum cassia* was obtained by S. Kahl at the medicinal plant market in Kunming, China, in November 2002 (Kahl, 2005). 5.1 g fresh ground plant material was successively extracted with PE and MeOH by ASE extraction. Extracts were evaporated to dryness under reduced pressure at 40 °C. 242.8 mg PE and 545.5 mg MeOH extract were gained. For TLC and HPLC fingerprint, a 5 mg/ml stock solution was prepared. 20 µl were applied to each of six TLC plates. Respectively, two were developed with one of the three solvent systems, analyzed at vis, 366 and 254 nm and
sprayed with vanillin – sulfuric acid or NST/PEG reagent. Best results are shown in figure 41. At 254 nm, a few fluorescence quenching substances could be found and several blue and red fluorescent substances at 366 nm. When sprayed with vanillin – sulfuric acid, several violet zones appeared in the PE extract and a few less in the MeOH extract. It can also be seen that the ASE extraction method used was not completely exhaustive.

![Figure 41. TLC fingerprint of different extracts of Cinnamomum cassia.](image)

**3.5.2. Pharmacological Investigations of Extracts and TLC Fingerprint of the PE Extract**

The two prepared extracts of *Cinnamomum cassia* were subjected to the XTT viability and GI assay. For the XTT assay, CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were used. For the GI assay, only CCRF-CEM cells were used. Extracts were tested at a final concentration of 10 µg/ml. As shown in figure 42, the PE extract showed a quite strong growth inhibitory activity on CCRF-CEM leukemia and HCT 116 colon cancer cells after 72 h (XTT assay). After 7 days, the growth inhibitory effect of the PE extract on CCRF-CEM leukemia cells was further enhanced. However, MDA-MB-231 breast cancer and U251 glioblastoma cells were not affected in their growth and viability after 72 h. On the other hand, the MeOH extract showed no growth inhibitory activity on the cancer cell lines used. A further TLC analysis of the PE extract was done in comparison with some pure compounds which have been isolated from the stem bark of *Cinnamomum cassia* and identified as main
constituents. As shown in figure 43, cinnamaldehyde, methoxy-cinnamaldehyde and cinnamic acid seem to be contained in this extract.

Figure 42. Effects of different ASE extracts of Cinnamomum cassia on CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells. A: XTT viability assay after 72 h, n = 6, mean ± sem, B: growth inhibition assay after 7 days, n = 3, mean ± S.D. Extract concentration: 10 µg/ml.

Figure 43. TLC fingerprint of the PE extract of Cinnamomum cassia and some pure compounds. A: 254 nm; B: 366 nm; C: sprayed with anisaldehyde – sulfuric acid. 1: PE extract; 2: cinnamaldehyde; 3: methoxy-cinnamaldehyde; 4: cinnamic acid.
Using the XTT viability and growth inhibition assay, it could be shown that a PE extract of *Cinnamomum cassia* strongly decreased the viability and proliferation of CCRF-CEM leukemia cells. TLC fingerprint revealed that cinnamaldehyde, methoxycinnamaldehyde and cinnamic acid are presumably contained in the active PE extract. Especially the blue fluorescence at 366 nm and the yellow color of methoxycinnamaldehyde after spraying with anisaldehyde – sulfuric acid reagent was also found in the PE extract. However, it seems that the main zone of the PE extract is not only composed of methoxycinnamaldehyde and, probably, cinnamaldehyde but also of at least a third substance which showed a violet color after spraying. Furthermore, it could be shown that the second main zone at 254 nm is none of the pure compounds investigated. All three compounds used for TLC have been reported to be effective against cancer cells. Cinnamaldehyde seems to induce apoptosis in several cancer cell lines via activating the intrinsic pathway (Koppikar et al., 2010, Wu and Ng, 2007, Ng and Wu, 2009, Ka et al., 2003). Moreover, it inhibited NF-κB activity which is mostly increased in cancer cells (Reddy et al., 2004). Cinnamic acid, on the other hand, seems to inhibit cancer progression by decreasing invasive capacity (Liu et al., 1995) and inhibiting DNA synthesis in colon cancer cells (Ekmekcioglu et al., 1998). This and the fact that the PE extract only inhibited the proliferation of two out of four cancer cells lines, makes cinnamon worth to be further investigated.

### 3.6. *Cocculus trilobus*

#### 3.6.1. Plant material, extraction procedure and TLC fingerprint

Dried roots of *Cocculus trilobus* were acquired by S. Kahl at the medicinal plant market in Kunming, China in November 2002 (Kahl, 2005). 5.2 g freshly ground plant material was successively extracted with PE and MeOH using ASE extraction and yielded 38 mg and 191 mg extract, respectively. For TLC fingerprint analysis, a 5 mg/ml stock solution was prepared and 20 µl applied to each of six TLC plates. Two plates were developed with one of three solvent systems, analyzed at vis, 366 nm and 254 nm and sprayed with vanillin-sulfuric acid or NST/PEG reagent. Best results are displayed in figure 44. When sprayed with vanillin – sulfuric acid, two main and some minor violet zones were found in the PE extract, whereby, the biggest one was also found in the MeOH extract in traces.
At 366 nm, some small blue fluorescent zones were found in the PE as well as in the MeOH extract.

![Figure 4. TLC fingerprints of different ASE extracts of Cocculus trilobus and aristolochic acid.](image)

Many blue fluorescent substances of the MeOH extract remained at the start and could not been separated with these solvent systems. Since *Cocculus trilobus* contains many alkaloids, these zones probably refer to the existence of alkaloids in the extracts. It can also be seen, that the ASE extraction method was not completely exhaustive since some zones occurred in the PE as well as in the MeOH extract. Moreover, it could be shown that aristolochic acid is contained neither in the PE nor in the MeOH extract.

### 3.6.2. Pharmacological Investigations of Extracts

All ASE extracts were subjected to the XTT viability and growth inhibition assay. For the XTT assay, CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were used. Cells were treated with the extracts (10 µg/ml) for 72 h and, afterwards, analyzed by a spectrophotometer. For the GI assay, CCRF-CEM cells were incubated for 7 days with the extract (10 µg/ml) and, subsequently, analyzed by a cell counter. Results are shown in figure 45. Growth and viability of breast, glioblastoma and colon cancer cells was hardly influenced by the PE extract and just slightly by the MeOH extract. Only CCRF-CEM cells exhibited a moderate decrease of cell viability and growth when treated with the MeOH extract. Longer incubation for 7 days hardly drew a distinction compared to the XTT assay.
3.6.3. Discussion

It is not 100% sure whether the plant material used is indeed *Cocculus trilobus* since there are also other plants sold as fängji (Kahl, 2005). Especially confusions with roots of *Stephania tetrandra* and *Aristolochia fangchi* are possible. Special attention has to be drawn at *Aristolochia fangchi* because of its aristolochic acid content. *Cocculus trilobus* and *Stephania tetrandra* contain no aristolochic acid (Wu et al., 2007). Using TLC, it could be shown that aristolochic acid was contained neither in the PE nor in the MeOH extract indicating that this plant material is not *A. fangchi*. Nevertheless, authentication based on DNA sequence analysis should be performed before further investigations are initiated.

In the XXT and growth inhibition assay, the MeOH extract led to a growth inhibition of about 50% of CCRF-CEM leukemia cells after 72 h as well as after 7 days. Also U251 and HCT 116 cells were slightly inhibited in their growth. Only the growth of MDA-MB-231 cells was not affected. The PE extract investigated showed no activity against the cell lines used. From *Cocculus trilobus*, several alkaloids were isolated and some of them, such as magnoflorin (Mohamed et al., 2009), sinococuline (Itokawa et al., 1987a, Liu et al., 1996) and isosinococuline (Itokawa et al., 1995) also showed anti-cancer activity.
Since all bioactive compounds isolated from *Cocculus trilobus* so far belong to the alkaloids, activity-guided isolation will presumably lead to compounds belonging to the alkaloids as well. The occurrence of many blue fluorescent zones in the TLC fingerprint also corroborates this assumption or at least the assumption of the appearance of several alkaloids in the MeOH extract. The results were also in accordance with the literature, since all alkaloids with anti-cancer activity reported so far were isolated from methanolic extracts (Itokawa et al., 1987a and 1995, Liu et al., 1996, Mohamed et al., 2009).

### 3.7. *Curcuma longa*

#### 3.7.1. Plant Material, Extraction Procedure and TLC Fingerprint

Rhizome of *Curcuma longa* was acquired by Prof. Dr. Thomas Efferth in Vietnam. 4.9 g fresh powdered plant material was successively extracted with *n*-hexane and MeOH and yielded 144.6 mg and 728.8 mg extract, respectively. 10 µl of a 5 mg/ml stock solution were applied to each of six TLC plates. Two were developed with one of three solvent systems, analyzed at vis, 254 nm and 366 nm and sprayed with vanillin – sulfuric acid or NST/PEG reagent. Best results are shown in figure 46. Both extracts exhibited yellow fluorescent zones at 366 nm and fluorescence quenching zones at 254 nm. When sprayed with vanillin – sulfuric acid, these zones became violet. It can also be seen that several zones appear in both extracts indicating that the extraction method was not completely exhaustive. The yellow fluorescent zones at 366 nm seem to be only a little extracted with hexane, but much more with MeOH.

![Figure 46. TLC fingerprint of different extracts of Curcuma longa (Rhizoma) and curcumin.](image-url)
3.7.2. Pharmacological investigations of extracts

The ASE extracts were subjected to the XTT viability and growth inhibition assay. CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were used for the XTT assay, CCRF-CEM cells for the GI assay. Extracts were tested at a final concentration of 10 µg/ml and incubated for 72 h (XTT) or 7 days (GI). As shown in figure 47, both extracts inhibited the growth of CCRF-CEM leukemia cells after 72 h as well as after 7 days. The proliferation of the other three cell lines used was not affected by the n-hexane extract, but by the MeOH extract. However, although MDA-MB-231 and HCT 116 cells were quite strongly inhibited in their growth, U251 cells showed only a slightly reduced viability.

Figure 47. A: XTT viability (n = 6, mean ± sem) and B: GI assay (n = 3, mean ± S.D.) of Curcuma longa extracts. Extract concentration: 10 µg/ml.

3.7.3. Discussion

In our investigations, a n-hexane and a MeOH extract of the rhizome of Curcuma longa were prepared and investigated regarding their growth inhibitory activity using several cancer cell lines. Both extracts strongly inhibited the proliferation of CCRF-CEM leukemia cells after 72 h as well as after 7 days. Comparing the effects on the other cancer cell lines, the MeOH extract seems to have a stronger inhibitory effect than the n-hexane extract. Analyzing the TLC fingerprint and literature data, this activity is probably based on the yellow fluorescent zones at 366 nm. TLC fingerprint further indicates that
the main zone is curcumin. Curcumin has been reported as one major compound of the rhizome being responsible for the coloring and flavoring properties and bioactivities of this plant. It exhibited anti-inflammatory, anti-oxidant, antiprotozoal, anti-viral, chemopreventive and chemotherapeutic effects (Hatcher et al., 2008, Araújo and Leon, 2001) and was shown to kill cancer cells by various mechanisms such as induction of apoptosis via the extrinsic and intrinsic pathway, affecting cell cycle and genes involved in proliferation, apoptosis, cytoprotection, metastasis and angiogenesis (Hatcher et al., 2008, Reuter et al., 2008, Ravindran et al., 2009).

### 3.8. **Helianthus angustifolius**

#### 3.8.1. Extraction and Activity–Guided Isolation of *Helianthus angustifolius*

*Figure 48. Isolation scheme of Helianthus angustifolius (flowers). DCM: dichloromethane, na: not active, ma: moderate active, active: less than 20% metabolically active cells compared to vehicle treated control cells.*

#### 3.8.1.1. Extraction and First Fractionation

Flowers of *Helianthus angustifolius* (225 g) were percolated with 4 x 1 l dichloromethane to yield 22 g extract. In a screening of several plants collected in North America using CCRF-CEM leukemia cells as monitor and the XTT viability assay, this extract exhibited...
OWN INVESTIGATIONS AND DISCUSSION

strong growth inhibitory activity: 0.2 ± 0.08% of control cells (10 µg/ml and 72 h incubation).

Figure 49. TLC fingerprint of fractions F3-12 of Helianthus angustifolius.

Figure 50. Activity of fractions F1-14 in the XTT viability assay. The fractions were obtained after fractionation of the dichloromethane extract of Helianthus angustifolius flowers. n = 3, mean ± sem, concentration: 10 µg/ml, incubation time: 72 h.
Therefore, this extract was subjected to activity-guided isolation using different chromatographic techniques. In a first approach, the extract was fractionated using open column chromatography. Silica gel served as stationary, several mixtures of n-hexane/EtOAc/MeOH as mobile phase. Fractions of 25 ml were collected and compared by their TLC fingerprint. Similar fractions were combined (figure 49). At the end, 14 fractions were obtained (F1-14), dried under reduced pressure at 40 °C and tested for their growth inhibitory activity in the XTT viability test. After 72 h, the growth of CCRF-CEM cells was strongly inhibited by fractions 11, 12 and 13. Fractions 1 to 9 had no effect on the growth and viability, fraction 10 and 14 only a very slight (figure 50).

3.8.1.2. **Fractionation of F12 with Solid Phase Extraction and Semi-preparative HPLC**

After column chromatography, the most active fraction (F12) was further separated by solid phase extraction. Thereby, two fractions (F12-1 and F12-2) were obtained. The first fraction showed again strong growth inhibitory activity (0.8 ± 0.2 up to 22.0 ± 0.1% of control cells), while the second fraction exhibited no activity (105.8 ± 0.7% of control). Fraction F12-1 was further fractionated using semi-preparative HPLC. 16 fractions were collected, while four of them appeared to be pure compounds (F12-1-H8, -H9, -H11 and -H12) after NMR measurements. All four sesquiterpene lactones isolated showed again high activity and IC\textsubscript{50} values were determined (figure 51 and 52).

![Figure 51. Comparison of IC\textsubscript{50} values of the isolated substances. n = 6, mean ± sem.](image-url)
Figure 52. IC₅₀ determination of A: F12-1-H8, B: F12-1-H9, C: F12-1-H11, D: F12-1-H12 on different cancer cell lines and MRC-5 lung fibroblasts. Incubation time: 72 h. n = 6, mean ± sem.

3.8.2. STRUCTURE ELUCIDATION OF ISOLATED SUBSTANCES

Structures of F12-1-H8, -H9, -H11 and -H12 were elucidated by analyzing 1D 1H- and 13C-NMR as well as 2D HSQC, HMBC and DQF-COSY spectra. Moreover, high resolution mass, optical rotation and UV spectra were recorded. NMR data indicated a 3,10-epoxydized and 8-esterified sesquiterpene lactone (furanogermacranolide) with a methacrylyl sidechain. The relative configuration of the sesquiterpene moiety was established by comparison with published NMR data (Gershenzon and Mabry, 1984).
Finally, F12-1-H8 was assigned as 8-methacrylyl-4,15-iso-atrIricolide. F12-1-H9, -H11 and -H12 showed a very similar proton resonance pattern indicating the presence of the same 4,15-iso-atrIricolide core sesquiterpene lacton. However, signals belonging to the esterified side chains were different. F12-1-H9 was esterified with an isobutyryl moiety, F12-1-H11 with a methylbutyryl and F12-1-H12 with an isovaleryl moiety. Therefore, F12-1-H9 was identified as 8-isobutyryl-4,15-iso-atrIricolide, F12-1-H11 as 8-(2-methylbutyryl)-4,15-iso-atrIricolide and F12-1-H12 as 8-isovaleryl-4,15-iso-atrIricolide (Kretschmer et al., 2011a). 1H and 13C-NMR data are given in table 1, structures of the isolated compounds in figure 53.

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Table 1. 1H and 13C NMR data of the isolated compounds.
3.8.3. Fractionation of F11 and F13 by FCPC

3.8.3.1. Fractionation and cytotoxicity of F11

600 mg of fraction 11 were dissolved in n-hexane/EtOAc (6:4) and subjected to FCPC. 192 fractions were obtained and analyzed by TLC (solvent system: n-hexane/EtOAc 6:4). Similar fractions were unified, finally, yielding twelve fractions (F11-1 to -12, figure 54). Fractions 2 to 12 were subjected to the XTT viability assay and tested at a final concentration of 10 µg/ml. CCRF-CEM leukemia cells were used (figure 56). The highest cytotoxic effect was found in fractions F11-3 to -6 and -11 to -12. Cells treated with these fractions for 72 h showed no metabolic activity at all. Fractions F11-7 and F11-8 also showed high proliferation inhibiting activity, whereas, fractions F11-9 and F11-10 inhibited the growth only to about 50 % of control.

3.8.3.2. Fractionation and cytotoxicity of F13

For FCPC, 1.7 g of fraction F13 were dissolved in n-hexane/EtOAc/MeOH/H2O (2:8:2:8). 199 fractions were collected and analyzed by TLC (solvent system: n-hexane/EtOAc 2:8). Similar fractions were unified and 8 fractions were finally obtained (F13-1 to -8, figure 55). Fractions F13-2 to F13-7 were subjected to the XTT viability assay using CCRF-CEM leukemia cells. Extracts were tested at 10 µg/ml and incubated for 72 h. While F13-2 and F13-3 showed strong growth inhibition, F13-4 to -7 showed almost no cytotoxicity (figure 56). However, reasons of time made it not possible to further
fractionate the active fractions of F11 and F13. However, because of TLC behavior and activity it can be assumed that similar compounds isolated from fraction 12 can also be found in these fractions.

![Figure 55. TLC fingerprint of fractions F13-1 to -8 at 366 nm (B) and sprayed with vanillin – sulfuric acid (A).](image)

![Figure 56. Cytotoxic effects of fractions F11-2 to 12 and F13-2 to 7 on CCRF-CEM cells after 72 h (XTT assay). Concentration: 10 µg/ml, n = 6, mean ± sem.](image)

**3.8.4. DISCUSSION**

The DCM extract of flowers of *Helianthus angustifolius* was subjected to activity-guided fractionation since it showed high activity in the XTT viability assay using CCRF-CEM leukemia cells. Using open column chromatography, SPE and semipreparative HPLC,
four pure compounds had been isolated. F12-1-H8 exhibited a molecular ion at m/z = 345.1361 [M + H]+ (calcd. for C_{19}H_{20}O_{6} + H: 345.1332) in the positive HR-TOF-ES-MS. The NMR data of F12-1-H8 were pointing to a 3,10-epoxydized and 8-esterified sesquiterpene lactone (furanogermacranolide) with a methacrylyl sidechain. Its constitution was established on the basis of 1D and 2D NMR experiments, the relative configuration of the sesquiterpene moiety by comparison with data given in Gershenzon and Mabry (1984). Therefore, F12-1-H8 was assigned as 8-methacrylyl-4,15-iso-atriplicolide. The other three compounds exhibited a very similar proton resonance pattern indicating the presence of the same 4,15-iso-atriplicolide core sesquiterpene lactone. However, signals attributable to the esterified side chains were different. F12-1-H9 was found to be esterified with an isobutyryl moiety, F12-1-H11 with a methylbutyryl and F12-1-H12 with an isovaleryl moiety. 8-methacrylyl-4,15-iso-atriplicolide, 8-isobutyryl-4,15-iso-atriplicolide and 8-(2-methylbutyryl)-4,15-iso-atriplicolide have been previously described from *Helianthus tuberosus* (Jerusalem artichoke) (Spring, 1991) but not from *Helianthus angustifolius*. 8-isovaleryl-4,15-iso-atriplicolide was isolated and described in this thesis for the first time.

Finally, all four compounds were subjected to the XTT viability assay using CCRF-CEM leukemia, MDA-MB-231 breast cancer, U251 glioblastoma, HCT 116 colon cancer and MRC-5 lung fibroblasts. All of them showed strong dose-dependent growth inhibitory activity and IC_{50} values were determined. IC_{50} values of CCRF-CEM cells were in the nano-molar range (300-500 nM). Compared to that, IC_{50} values against MRC-5 cells were found to be 12 to 16 fold higher. All compounds exhibited IC_{50} values between 1 and 2 μM against HCT 116 colon cancer cells and 3-7 μM against MDA-MB-231 breast cancer cells. IC_{50} values against the very aggressive U251 glioblastoma cells were in a range between 10-20 μM. Compared to the used non-cancer cells, HCT 116 exhibited a 3 to 4 fold higher sensitivity to the substances, while MDA-MB-231 showed about the same and U251 a lower sensitivity as expressed by their IC_{50} values (Kretschmer et al., 2011a).

It has been reported that an α-methylene-γ-lactone group is a structural prerequisite for the cytotoxicity of many sesquiterpene lactones. Especially if it is present in the form of an exo-methylene group, sesquiterpene lactones possess high cytotoxicity (Kupchan et al., 1971, Lee et al., 1971). Our data are in agreement with this assumption. Moreover, we can assume that the contribution of the ester moiety to cytotoxicity is generally rather marginal because of the small range of respective IC_{50} values. The exocyclic methylene group at position 15, which lacks heliangolides of the budleia A-type may further contribute to enhanced cytotoxicity of this series. Finally, the slightly higher cytotoxic effect of F12-1-H8, which bears an additional O=C-C=CH_{2} group in the ester
moiety, also fits into this picture. This may enhance the alkylating potency of the compound and, therefore, enhanced DNA-crosslinking that leads to higher cytotoxicity (Fischer et al., 1998, Schmidt, 1999a and b).

Fractions F11 and F13 were also subjected to activity-guided fractionation using FCPC. Eight new fractions of F11 and three fractions of F13 showed again high cytotoxicity and will be further fractionated in the future.

3.9. **Hydnocarpus anthelmintica**

3.9.1. **Plant Material, Extraction Procedure and TLC Fingerprint**

Fruits of *Hydnocarpus anthelmintica* were collected by S. Kahl in Xishuangbanna, China, in November 2002. They were opened and the white, soft tissue was removed. The seed was taken out and dried (Kahl, 2005). For extraction purposes, dried seeds were opened, the pit removed and the seed coat successively extracted with PE, EtOAc and MeOH by ASE. From 4.8 g plant material, 18.6 mg PE, 10.1 mg EtOAc and 87.8 mg MeOH extract were obtained. Furthermore, the seed was completely ground and extracted with PE, EtOAc and MeOH as well. The initial weight was 5.1 g and 749.5 mg PE, 23.8 mg EtOAc and 201.1 mg MeOH extract were gained. From each extract, a 5 mg/ml stock solution was prepared and applied to each of six TLC plates. Respectively, two plates were developed with one of three solvent systems, analyzed at vis, 366 nm and 254 nm and sprayed with vanillin – sulfuric acid or NST/PEG reagent. Best results are shown in figure 57. TLC fingerprints showed that the EtOAc and MeOH extracts of the complete seed and the seed coat had almost the same zone pattern. Only the PE extracts showed a slightly different pattern. This is probably due to the solvent PE which primarily degreases the sample. Since the seeds of *Hydnocarpus anthelmintica* contain a lot of fatty and oily substances, the compounds extracted from the seed coat and visible in TLC are probably overlaid by these fatty substances. Furthermore, the fingerprints showed again, that the three cycles during ASE extraction were not enough to extract the plant material exhaustively.
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3.9.2. Pharmacological Investigations of Extracts

All extracts were subjected to the XTT viability assay. Extracts of the seeds were tested against CCRF-CEM leukemia; extracts of the seed coat were tested on CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells. Moreover, the effects of all extracts were examined in the GI assay. In each case, a final concentration of 10 µg/ml was used (figure 58). After 72 h, only a very slight growth inhibitory effect was observed when CCRF-CEM and MDA-MB-231 cells were treated with the methanol extract of the seed coat. After 7 days, this effect was slightly enhanced and also observed in case of the EtOAc seed coat extract. Extracts of the whole semen had no cytotoxic effects on CCRF-CEM leukemia cells.
 OWN INVESTIGATIONS AND DISCUSSION

Figure 58. Effects of different ASE extracts of Hydnocarpus anthelmintica. On the one hand, only the seed coat (SC) was extracted, on the other hand, the whole seed (S). A: XTT viability assay after 72 h, n = 6, mean ± sem, B: GI assay after 7 days, n = 3, mean ± S.D. Extract concentration: 10 µg/ml.

3.9.3. DISCUSSION

*Hydnocarpus anthelmintica* is a plant hardly investigated. A few publications can be found analyzing the oil of the seeds (Christie et al., 1989, Duke and Ayensu, 1985a). In 1999, de Padua et al. demonstrated that it was effective against several cancer cell lines *in vivo*. The only compounds isolated so far which exhibited cytotoxic activity were mycoepoxydiene and deacetylmycoepoxydiene (Prachya et al., 2007). However, these compounds were produced by an endophytic fungus and not by the plant itself. In this PhD thesis, several extracts of the seed coat and semen were investigated. Only the ethyl acetate and methanol extract of the seed coat exhibited a marginal proliferation inhibiting activity at 10 µg/ml. Higher concentrations should be tested to reveal if these extracts really have cytotoxic properties or not. If they have, this plant could be an interesting candidate to be further investigated.
3.10. *Lonicera japonica*

### 3.10.1. PLANT MATERIAL, EXTRACTION PROCEDURE AND TLC FINGERPRINT

Dried branches of *Lonicera japonica* cultivated in Yunnan were acquired by S. Kahl at the medicinal plant market in Kunming, China, in November 2002 (Kahl, 2005). Additionally, branches were purchased at the pharmacy Casa Medica (Austria) in November 2007 and from Complemedis AG (Switzerland) in October 2007.

![TLC fingerprints of different extracts of Lonicera japonica (branches)](image)

For successive ASE extraction with PE, EtOAc and MeOH, 5.2 g of the Chinese material, 5.1 g of the Austrian and 5.0 g of the material from Switzerland were used. The yields were: China: PE: 23 mg, EtOAc: 37 mg, MeOH: 259 mg; Austria: PE: 19 mg, EtOAc: 43 mg, MeOH: 178 mg; Switzerland: PE: 14 mg, EtOAc: 39 mg, MeOH: 409 mg. Moreover, from about 2 g of each material a decoction was prepared according to the
instructions of the Chinese pharmacopoeia. Decoctions yields were: China: 100 mg, Austria: 140 mg and Switzerland: 110 mg.

For TLC fingerprint analysis, only ASE extracts were used. A 5 mg/ml solution of each extract was prepared and 10 µl applied to each of six TLC plates. Two plates were developed with one of three solvent systems, analyzed at vis, 254 and 366 nm and sprayed with vanillin – sulfuric acid or NST/PEG reagent. Best results are shown in figure 59. No big differences between the three different plant materials could be observed by TLC analysis. They all exhibited a quite similar zone pattern. At 366 nm, a few zones of the PE extracts exhibited different fluorescence intensity, whereby, the strongest was found in the Chinese material. It can be seen that the ASE extraction method used was not completely exhaustive. However, it can be well observed that successive extraction with solvents of increasing polarities is an easy method to yield a wide spectrum of compounds on the one hand and allows a first fractionation on the other hand.

### 3.10.2. PHARMACOLOGICAL INVESTIGATIONS OF EXTRACTS

![Graph](image)

Figure 60. XTT viability assay of different extracts of Lonicera japonica acquired in China, Austria and Switzerland. Incubation time: 72 h, concentration: 10 µg/ml, n = 6, mean ± sem.
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All ASE extracts and decoctions of *Lonicera japonica* were subjected to the XTT viability assay. CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were treated with 10 µg/ml extract for 72 h. As shown in figure 60, neither the ASE extracts nor the decoctions showed high proliferation inhibitory activity. Only a few decreased the amount of metabolic active cells to about 70-80% of control. There was also no significant activity difference between the three plant materials used. Only the PE and EtOAc extract of the Austrian material exhibited about 20-30% more activity on CCRF-CEM cells than the extracts of the Chinese and Swiss material.

3.10.3. DISCUSSION

From the stem of *Lonicera japonica*, especially luteolin, protocatechuic acid and chlorogenic acid have been reported to possess anti-cancer activity (Kang et al., 2010, Yip et al., 2006, Ko et al., 2002, Zhao et al., 2011, Leung et al., 2005 and 2006, Lee et al., 2010a, Ong et al., 2010, Yin et al., 2009, Rakshit et al., 2010). Protocatechuic acid also inhibited metastasis of B16/F10 melanoma in mice (Lin et al., 2011). However, at a final concentration of 10 µg/ml, none of the extracts prepared in this thesis showed a strong growth inhibitory activity on leukemia, breast, glioblastoma and colon cancer cells in vitro. The maximal effect observed was in case of CCRF-CEM cells whose viability was about 70% compared to vehicle treated control cells. There was also no big difference between the three plant materials used regarding their cytotoxicity and TLC fingerprint. A non-cytotoxic effect was also reported for an aqueous extract which did not affect the viability of A549 lung cancer cells up to 100 mg/ml (Xu et al., 2007). This was also observed for the decoctions prepared. For further investigation, the extracts should be tested in higher concentrations. Since all three main substances mentioned above are commercial available, they can also be directly tested for their activity and their occurrence in these extracts.

3.11. *ONOSMA PANICULATA*

3.11.1. IDENTIFICATION OF PLANT MATERIAL

Roots of *Onosma paniculata* were purchased by S. Kahl at the medicinal plant market in Kunming, China, in October 2003. On the one hand, they were authenticated at the Kunming Institute of Botany, China. Using macroscopic characteristics, the plant material was first identified as *Arnebia euchroma*. To confirm or disprove this assumption, the
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Plant material was further identified using genomic analysis (Rinner et al., 2010). Thereby, the ITS2 region of nuclear DNA and trnL-F region of plastid DNA can be potentially used as a standard DNA barcode to identify medicinal plants and their closely related species (Chen et al., 2010). To estimate the reliability of species identification using a DNA barcoding technique, the Basic Local Alignment Search Tool (BLAST) can be used. It finds regions of local similarity between sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches. The obtained sequences were queried with the NCBI nucleotide database by a BLASTN 2.2.22 search (Zhang et al. 2000), which indicated a significant similarity to *Onosma paniculata* for both ITS (98% max identity to acc. no. EF199859) and trnL-F spacer (98% max identity to acc. no. EF199851; for the trnL-F 5'-intron 99% max identity to acc. no. EF199875). For *Arnebia euchroma*, the similarity values revealed were 84% for ITS and 96% for the trnL-F spacer, respectively.

**3.11.2. Extraction Procedure and TLC Fingerprint**

5.7 g fresh powdered plant material was subjected to ASE and successively extracted with PE and MeOH for screening purposes. Extracts were evaporated to dryness under reduced pressure at 40 °C. The yields were 0.05 g and 0.55 g respectively. 71.0 g fresh ground root material was extracted with PE by Soxhlet extraction for isolation purposes and 28.1 g were used for maceration with PE at room temperature. Yields were 1.07 g and 1.75 g, respectively. Moreover, for a decoction of the plant was prepared according to the guidelines of the Chinese Pharmacopoeia. For TLC fingerprint, 10 µl of a 5 mg/ml stock solution were applied to each of six TLC plates, developed with one of three different solvent systems and sprayed with vanillin – sulfuric acid or NST/PEG reagent. The best results are shown in figure 61 and 62.

Comparison of the ASE PE and MeOH extract revealed several red colored zones in the PE but not in the MeOH extract. This indicated the presence of naphthoquinone derivatives in the PE extract (figure 61). It also showed that the three cycles of ASE extraction were sufficient to prepare exhaustive extracts of the roots. The red zones also exhibited fluorescence at 366 nm and a blue-violet color when sprayed with vanillin-sulfuric acid. Moreover, two zones showed a turquoise-blue fluorescence at 366 nm and several additional bands could be observed after spraying with vanillin-\(\text{H}_2\text{SO}_4\). The MeOH extract contained substances which demonstrated a turquoise-blue fluorescence at 366 nm and a yellow and turquoise fluorescence after spraying with NST/PEG-reagent.
Comparison of differently prepared PE extracts (ASE, Soxhlet and maceration) demonstrated that there was no big difference between the extraction methods (figure 62). All extracts exhibited the same zone pattern. However, some red colored zones at vis seemed to be weaker in the macerate than in the other two extracts and, in the ASE extract, the lower blue fluorescent zone at 366 nm showed a lower fluorescence.
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3.11.3. PHARMACOLOGICAL INVESTIGATIONS OF EXTRACTS

3.11.3.1. XTT AND GI ASSAY

For screening purposes, a PE and MeOH extract of *Onosma paniculata* prepared by ASE were investigated regarding their growth inhibitory activity against CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells. Both extracts were tested at a concentration of 10 µg/ml and analyzed after 72 h (XTT assay) as well as after 7 days (growth inhibition assay).

![Graph A](image1.png)

![Graph B](image2.png)

*Figure 63. Activity of different extracts of Onosma paniculata. A: XTT viability assay after 72 h (mean ± sem, n = 6), B: growth inhibition assay (GI) after 7 days (mean ± S.D., n = 3), expressed as % of control. Extracts were tested at a final concentration of 10 µg/ml.*

In both cases, the PE extract strongly inhibited the growth and proliferation of the investigated cancer cells, while the MeOH extract had no effect. Subsequently, a Soxhlet PE extract, a PE macerate and a decoction were tested in the XTT assay. Both PE extracts again inhibited the growth of the cancer cells. This activity was comparable to that of the ASE PE extract (figure 63). Additionally, we also investigated the effect of a traditionally prepared decoction on CCRF-CEM cells since they were the most sensitive cells. However, this extract had no effect on the growth of the cancer cells. In the following, the Soxhlet PE extract was chosen for further pharmacological investigations and activity-guided isolation.
3.11.3.2. Morphological Changes

Morphological changes were studied in Hela, HT-29, THP-1, 769-P, SBc-L2, WM9, WM164 and WM35 cells. These cells were treated with different concentrations of the PE extract (0; 2.5; 5; 7.5 and 10 µg/ml) and grown at 37 °C in a humidified 5% CO₂ atmosphere for the adequate time period (24, 48 and 72 h). Afterwards, cells were gently rinsed with PBS and analyzed under a phase-contrast microscope for size, shape and integrity of cell membrane, cytoplasm and nuclei. First changes occurred already after 24 h and continued up to 72 h. The effect was not only time, but also dose dependent. Non-metastatic melanoma cells (SBc-L2) were influenced already after 24 h, whereas, metastatic melanoma cells (WM164) were not influenced before 48 h. After 72 h, WM35, WM9 and WM164 cells were much less dense than control cells. The other investigated cells also showed less density, cell shrinkage, detachment from the bottom and a significant changed morphology after 72 h (figure 64).

Figure 64. Morphological changes of different cancer cell lines under phase contrast microscopy (× 1000) after incubation with the PE extract for 72 h. First and third column: morphology of untreated cells (control). Second and fourth column: cells treated with 10 µg/ml Soxhlet PE extract.
3.11.3.3. **Cell Cycle**

Effects of the PE extract on cell cycle were studied in Hela, 769-P, HT-29, THP-1 and different melanoma cell lines from different tumor stages (WM35, WM9 and WM164). In Hela and THP-1 cells, the cell cycle distribution was changed only slightly. The G1-phase cells decreased, accompanied by a marginal increase of cells in the G2/M-phase (Hela) and cells in the S-phase (THP-1). In 769-P and HT-29 cells, the amount of cells in the G1-phase decreased to a greater extent than in Hela and THP-1 cells while cells in the S- and G2/M-phase increased. Thereby, the biggest increase was observed for 769-P cells. Cells in G2/M-phase increased about 11 fold. In the case of melanoma cell lines, the percentage of cells in the G1-phase decreased as well. In the case of WM9 and WM164 cells, about 3 times more cells were arrested in the S-phase compared to control cells. In WM35 cells, this phase increased only slightly. Cells in the G2/M-phase increased in all three melanoma cells, whereby, the largest shift was observed for WM9 cells. Representative data from two independent experiments are shown in figure 65.

**Figure 65.** Effects of the Soxhlet PE extract on cell cycle distribution. Shown are untreated control cells (Co) and cells treated with 10 µg/ml extract (OP) after 72 h. Y-axis denotes cell counts, x-axis represents DNA-content. Percentage of cells in the G1-, G2/M- and S-phase was calculated using FCS3-express.
3.11.3.4. **Activation of Caspase-3**

Activation of caspase-3 was investigated in Hela, THP-1, 769-P, HT-29, SBc-L2, WM35, WM9 and WM164 cells. In all of these cells lines, an activation of the enzyme could be observed. In the melanoma cells used (SBc-L2, WM35, WM9 and WM164), the activation seems to depend on the tumor stage (figure 66).

![Graphs showing caspase-3 activation](image)

*Figure 66. Investigations on caspase-3 activation. Y-axis denotes cell counts; x-axis represents fluorescence intensity of the activated caspase-3 antibody. The histograms were done by using FCS3-express. Green line represents untreated control cells, red line cells treated with 10 µg/ml Soxhlet PE extract for 72 h.*

3.11.4. **Activity-Guided Isolation of Active Constituents**

HPLC investigations of the Soxhlet PE extract revealed that it was composed of three main (retention times: 10.8 min, 22.7 min and 24.3 min, respectively) and some other compounds of less content (retention times: 8.5 min, 9.4 min and 19.1 min, respectively,
OWN INVESTIGATIONS AND DISCUSSION

figure 67). Since the substances could be separated by this method, the Soxhlet PE extract was fractionated using preparative HPLC. Out of 400 mg extract, the yields were:

- fraction 1 (retention time: 6.5 min): 1.70 mg
- fraction 2 (retention time: 8.5 min): 1.07 mg
- fraction 3 (retention time: 9.3 min): 0.79 mg
- fraction 4 (retention time: 10.8 min): 31.05 mg
- fraction 5 (retention time: 19.0 min): 6.13 mg
- fraction 6 (retention time: 22.7 min): 48.62 mg
- fraction 7 (retention time: 24.3 min): 46.34 mg

Figure 67. HPLC chromatogram of the Soxhlet PE extract and the UV spectra of the three main peaks (RP 18 column, 250 mm x 4 mm, 5 µm, mobile phase: H₂O and ACN).

NMR analysis revealed that the fractions of peak 2, peak 4, peak 6 and peak 7 (retention times: 8.5 min, 10.8 min, 22.7 min and 24.3 min, respectively, figure 67) were pure compounds, whereas, the other fractions were mixtures of several compounds. Fraction 3 was also a pure compound but too less to be unambiguous identified. To distinguish between the enantiomers alkannin and shikonin, CD measurements were performed. According to literature (Ikeda et al., 1991, Fukui et al., 1983), shikonin and its derivatives show a positive cotton effect, whereas, alkannin and derivatives have a negative one. The isolated substances showed a positive maximum at 356 – 358 nm and a negative at 305 – 315 nm and were, therefore, identified as shikonin derivatives. Along with with NMR data (table 2-4), fraction 2 could be identified as β-hydroxyisovalerylshikonin (Cui
et al., 2008), fraction 4 as acetylshikonin (Cui et al., 2008), fraction 6 as dimethylacrylshikonin (Han et al., 2008) and fraction 7 as epoxyshikonin (Cui et al., 2008) (figure 69). Epoxyshikonin was isolated from *Onosma paniculata* for the first time.

**Figure 68.** Extraction and isolation scheme of *Onosma paniculata* and activities in the XTT viability assay (active: less than 20% metabolic active cells after 72 h at a final extract/fraction concentration of 10 µg/ml).

**NMR and CD analysis:**

- **fraction 1**: mixture
- **fraction 2**: β-hydroxyisovalerylshikonin
- **fraction 3**: pure, but too less for NMR identification
- **fraction 4**: acetylshikonin
- **fraction 5**: mixture
- **fraction 6**: dimethylacrylshikonin
- **fraction 7**: epoxyshikonin

**Figure 69.** Structures of the isolated compounds.
Table 2. 1H and 13C NMR data of hydroxyisovalerylshikonin and acetylshikonin (measured in chloroform-d<sub>1</sub>).

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Table 2. 1H and 13C NMR data of hydroxyisovalerylshikonin and acetylshikonin (measured in chloroform-d<sub>1</sub>).
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Table 3. 1H NMR data of dimethylacylshikonin (measured in chloroform-d$_1$).

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Table 4. 1H NMR data of epoxyshikonin and acetylshikonin (measured in chloroform-d$_1$).
All fractions were tested in the XTT viability assay at 10 µg/ml. All of them decreased metabolic active cells after 72 h below 20% of control values (data not shown).

**Figure 70.** IC$_{50}$ determination of β-hydroxyisovalerylshikonin. XTT viability assay, 72 h. n = 6, mean ± sem.

**Figure 71.** IC$_{50}$ determination of acetylshikonin. XTT viability assay, 72 h. n = 6, mean ± sem.
Subsequently, IC<sub>50</sub> values of the four isolated and identified compounds were determined. Six different cancer cell lines (CCRF-CEM, MDA-MB-231, U251, HCT 116, SBC-L2, WM35, WM9 and WM164) and a lung fibroblast cell line (MRC-5) were used (figures 70 - 74).

Most IC<sub>50</sub> values were in the range between 0.6 and 31 µM. The highest were obtained in the case of the very aggressive melanoma cell line WM164 (acetylshikonin: 71.5 µM, epoxyshikonin: 52.3 µM). In contrast, CCRF-CEM leukemia cells reacted very sensitive
to the substances with IC\textsubscript{50} values ranging from 0.6 to 1.9 µM. IC\textsubscript{50} values of SBc-L2 and HCT 116 cells in the case of dimethylacrylshikonin and epoxyshikonin, respectively, were even lower than those of CCRF-CEM cells (1.1 µM and 1.3 µM).

The four melanoma cell lines used were from primary (SBc-L2, WM35) and metastatic (WM9, WM164) lesions and show an increasing aggressiveness. When treated with β-hydroxyisovaleryl- or dimethylacrylshikonin, IC\textsubscript{50} values increased consistently with their rising aggressiveness. Also when incubated with acetyl- or epoxyshikonin, the IC\textsubscript{50} values of WM164 cells were about 5.5 and 3.4 fold higher than those of SBc-L2 cells. However, in these cases the IC\textsubscript{50} values of WM35 were slightly higher than for WM9 cells. In general, dimethylacrylshikonin showed the lowest IC\textsubscript{50} on melanoma cells. MRC-5 lung fibroblast cells also showed very low IC\textsubscript{50} values of 2.4 to 3.9 µM which were lower than many of the respective concentrations of several cancer cells. However, CCRF-CEM leukemia cells were at least two fold more sensitive than MRC-5 cells.

Figure 7. Comparison of IC\textsubscript{50} values of β-hydroxyisovalerylshikonin, acetylshikonin, dimethylacrylshikonin and epoxyshikonin on different cancer and a lung fibroblast (MRC-5) cell line. XTT viability assay, 72 h, mean ± sem, n = 6.
3.11.5.2. **MORPHOLOGICAL CHANGES**

![Figure 75. Morphological changes of SBC-L2 cancer cells under phase contrast microscopy (× 1000) after incubation with the respective IC\(_{50}\) of dimethylacrylshikonin (1.1 µM) or epoxyshikonin (15.5 µM) for 24 or 48 h.](image)

![Figure 76. Morphological changes of WM35 cancer cells under phase contrast microscopy (× 1000) after incubation with the respective IC\(_{50}\) of dimethylacrylshikonin (2.3 µM) or epoxyshikonin (23.0 µM) for 24 or 48 h.](image)

Dimethylacryl- and epoxyshikonin were obtained as two main substances of the Soxhlet PE extract and further investigated. Since the melanoma cell lines used display different tumor stages within one kind of cancer, they were used for these investigations. SBC-L2, WM35, WM9 and WM164 cells were treated with their respective IC\(_{50}\) for 24 or 48 h.
Subsequently, cells were gently washed with PBS and analyzed with a phase contrast microscope regarding size, shape and integrity of cell membrane, cytoplasm and nuclei (figures 75 – 78). After 24 h as well as after 48 h, SBc-L2 cells showed a changed morphology. They were less dense than control cells, more round, smaller in shape and more cells were detached from the bottom. The same effects, but more intensified could be observed for the other three cell lines as well. Thereby, the effects were intensified after 48 h.

Figure 77. Morphological changes of WM9 cancer cells under phase contrast microscopy (× 1000) after incubation with the respective IC50 of dimethylacrylshikonin (2.7 µM) or epoxyshikonin (18.8 µM) for 24 or 48 h.

Figure 78. Morphological changes of WM164 cancer cells under phase contrast microscopy (× 1000) after incubation with the respective IC50 of dimethylacrylshikonin (8.3 µM) or epoxyshikonin (52.3 µM) for 24 or 48 h.
3.11.5.3. **Effects on Cell Cycle**

To investigate the effects of dimethylacyrlishikonin (figure 79) and epoxyshikonin (figure 80) on cell cycle distribution, melanoma cells were incubated with their respective IC\textsubscript{50} and double IC\textsubscript{50} for 24 h and 48 h. Vehicle treated cells served as control.

In case of SBc-L2 cells, dimethylacyrlishikonin increased the proportion of cells arrested in S- and G2/M-phase accompanied by a decrease of cells in the G1-phase. After 24 h as well as after 48 h, about half of all cells were arrested in the S-phase. Thereby, treatment with double IC\textsubscript{50} did not change the distribution significantly. Only a slight increase in S-phase cells was observed in comparison to the effect of the IC\textsubscript{50}. When SBc-L2 cells were treated with epoxyshikonin, a similar pattern was observed (decrease of cells in G1-phase and increase in S- and G2/M-phase cells). Treatment with 2 x IC\textsubscript{50} further increased cells in the G2/M-phase and slightly decreased cells in the S-phase. The effects after 24 h and 48 h were again comparable.
In the case of WM35 cells, dimethylacrylshikonin hardly affected the percentage of cells in the S-phase after 24 h as well as after 48 h, while epoxyshikonin increased S-phase cells from about 24% to 34-39%. In exchange, cells in G2/M-phase raised more when treated with dimethylacrylshikonin than with epoxyshikonin. In each case, the increases were again accompanied by a decrease of cells in the G1-phase.

In the case of WM9 cells, both substances changed the proportion of S-phase cells slightly, but increased cells in the G2/M-phase 3-5 fold. This effect was quite similar after 24 h and 48 h at both concentrations.

WM164 cells treated with dimethylacrylshikonin showed a different reaction. After 24 h as well as after 48 h, cells in the S-phase decreased. The amount of cells in the G1-phase increased slightly (IC_{50}) or remained unchanged (2 x IC_{50}) after 24 h but decreased dose-dependently after 48 h. Cells in the G2 phase increased in all cases. When treated with epoxyshikonin, the effect was comparable to the other cells lines. Cells in G1-phase decreased while S- and G2-phase cells increased. Only after 48 h and when treated with double IC_{50}, cells in the S-phase disappeared almost completely while about 60% were arrested in G2/M-phase.

Figure 8. Cell cycle distribution of different melanoma cell lines when treated with epoxyshikonin. A: Sbcl2-L2 cells, B: WM35 cells, C: WM9 cells, D: WM164 cells. Vehicle treated (control) and treated (respective IC_{50} and 2 x IC_{50}) cells were incubated for 24 h or 48 h and analyzed afterwards.
3.11.5.4. **Sub-G1 Peak**

Besides cell cycle distribution concerning G1-, S- and G2/M-phase, cells were also analyzed regarding the appearance of a Sub-G1 peak (figure 81). Once more, melanoma cells were incubated with the respective IC<sub>50</sub> and double IC<sub>50</sub> for 24 h and 48 h. Compared to control cells, the percentage of SBc-L2 cells showing a Sub-G1 peak increased 4 to 10 fold when treated with dimethylacrylshikonin and 12 to 16 fold in the presence of epoxyshikonin. In the case of WM35 cells, dimethylacrylshikonin increased the amount of Sub-G1 cells ca. 3 fold, epoxyshikonin only slightly after 24 h (IC<sub>50</sub>) and two-fold after 48 h. Also in the case of WM9 cells, epoxyshikonin hardly changed the percentage of Sub-G1 cells. In the presence of dimethylacrylshikonin, the percentage of Sub-G1 cells remained almost unchanged after 48 h, however, it was 2-4 fold increased after 24 h. In the case of WM164 cells, both substances led to the appearance of Sub-G1 peaks. In case of dimethylacrylshikonin, the percentage increased to same levels after 24 h and 48 h. In case of epoxyshikonin, the proportion of cells showing a Sub-G1 peak was further increased after 48 h compared to 24 h incubation.

![Figure 81. Percentage of Sub-G1 peaks of different melanoma cell lines. A: dimethylacrylshikonin, B: epoxyshikonin. Cells were treated with the respective IC<sub>50</sub> or double IC<sub>50</sub> for 24 or 48 h.](image)

3.11.5.5. **Activation of Caspase-3**

In case of SBc-L2, WM35 and WM164 cells, it was further investigated whether caspase-3 is cleaved and activated by dimethylacrylshikonin. In SBc-L2 cells, no activation of caspase-3 could be observed (figure 82). In case of WM35 (figure 83), cleavage of caspase-3 started after 10 h of incubation and continued to 72 h.
Figure 8.2. Analysis of caspase-3 cleavage in SBC-L2 cells. Y-axis denotes cell counts; x-axis represents fluorescence intensity of the activated caspase-3 antibody. Kontrolle: control cells, OP-P6-1: dimethylacrylshikonin, time specification depicts time of incubation. The table displays % of gated cells showing cleavage of caspase-3.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>SBC-L2 IC50 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.42</td>
</tr>
<tr>
<td>1h</td>
<td>2.38</td>
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<tr>
<td>3h</td>
<td>3.11</td>
</tr>
<tr>
<td>6h</td>
<td>1.79</td>
</tr>
<tr>
<td>12h</td>
<td>2.20</td>
</tr>
<tr>
<td>24h</td>
<td>3.12</td>
</tr>
<tr>
<td>48h</td>
<td>2.82</td>
</tr>
</tbody>
</table>

Figure 8.3. Analysis of caspase-3 cleavage in WM35 cells. Y-axis denotes cell counts; x-axis represents fluorescence intensity of the activated caspase-3 antibody. KTR: control cells, OP-P6-1: dimethylacrylshikonin, time specification depicts time of incubation. The table displays % of gated cells showing cleavage of caspase-3.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>WM35 IC50 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4.27</td>
</tr>
<tr>
<td>1h</td>
<td>3.82</td>
</tr>
<tr>
<td>3h</td>
<td>4.06</td>
</tr>
<tr>
<td>6h</td>
<td>5.20</td>
</tr>
<tr>
<td>12h</td>
<td>10.06</td>
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<tr>
<td>24h</td>
<td>27.39</td>
</tr>
<tr>
<td>48h</td>
<td>27.60</td>
</tr>
<tr>
<td>72h</td>
<td>36.57</td>
</tr>
</tbody>
</table>

However in WM164 cells, there was again no measurable activation of caspase-3 (figure 8.4). Altogether, it seems that dimethylacrylshikonin did not lead to the activation of caspase-3 in SBC-L2 and WM164 melanoma cell lines when they were treated with the
respective IC_{50}. In case of SBc-L2 and WM35 cells, activity of caspase-3 was further investigated using the CaspaseGlo® 3/7 assay (figure 85). While caspase-3 was activated in WM35 cells, it was only slightly activated in SBc-L2 cells.

![Graph showing caspase-3 cleavage](image1.png)

**Figure 84.** Analysis of caspase-3 cleavage in WM164 cells. Y-axis denotes cell counts; x-axis represents fluorescence intensity of the activated caspase-3 antibody. Kontrolle: control cells, OP-P6-1: dimethylacrylshikonin, time specification depicts time of incubation. The table displays % of gated cells showing cleavage of caspase-3.

<table>
<thead>
<tr>
<th>Time</th>
<th>WM164 IC_{50}</th>
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</thead>
<tbody>
<tr>
<td>1h</td>
<td>1.86</td>
</tr>
<tr>
<td>3h</td>
<td>1.47</td>
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<tr>
<td>6h</td>
<td>1.59</td>
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<td>12h</td>
<td>0.98</td>
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<tr>
<td>24h</td>
<td>3.67</td>
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<td>48h</td>
<td>3.04</td>
</tr>
<tr>
<td>72h</td>
<td>3.04</td>
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<th>Time</th>
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<td>3.04</td>
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<td>72h</td>
<td>3.04</td>
</tr>
</tbody>
</table>

**Figure 84.** Analysis of caspase-3 cleavage in WM164 cells. Y-axis denotes cell counts; x-axis represents fluorescence intensity of the activated caspase-3 antibody. Kontrolle: control cells, OP-P6-1: dimethylacrylshikonin, time specification depicts time of incubation. The table displays % of gated cells showing cleavage of caspase-3.

![Graph showing Caspase-3 activity](image2.png)

**Figure 85.** Caspase-3 activity after several incubation periods (CaspaseGlo® 3/7 assay); concentration: respective IC_{50}, n = 8, mean ± sem. DMSO-Co: vehicle treated control cells.
Final investigations comprised the relative mRNA expression levels of genes involved in apoptosis. Several genes of the extrinsic (TRAIL and its receptors DR4 and DR5) and the intrinsic (bid, bad, bik, bmf, and their antagonists bcl-2, bcl-x and mcl1) apoptotic pathway were chosen. Sbc-L2 and WM35 cells were treated with their respective IC_{50} of
dimethylacrylshikonin for several periods of time (figure 86). After 6h of treatment, a statistically significant up-regulation of bid, bik and bmf mRNA expression levels was found for WM35 cells. After 12h, also bad was statistically significant up-regulated in these cells. The highest expression levels of these genes were around seven fold induced for bmf and six fold for bad after 6 hours and 31 fold for bid after 48 h. In SBc-L2 cells however, no changed mRNA expression levels were measured. The mRNA expression levels of anti-apoptotic genes were not significantly changed in both cell lines. Investigations of TRAIL and its receptors DR4 and DR5 revealed that after 12h of treatment the mRNA expression levels of TRAIL were significantly higher in WM35 cells, while the expression levels of the receptors remained unchanged. In SBc-L2 cells, no alteration of the expression levels could be found.

### 3.11.5.7. **Comparison of Cytotoxicity of Different Shikonin and Alkannin Derivatives**

Several other shikonin and alkannin derivatives were provided by the University of Valencia, Spain (Prof. Dr. Jose Rios) and the University of Athens, Greece (Assoc. Prof. Dr. Ioanna Chinou). Shikonin, deoxyshikonin/alkannin, isobutyrylshikonin, isovalerylshikonin and (2-methyl-n-butyryl)shikonin were given by Prof. Rios, dimethylacrylalkannin, a mixture of shikonofuran C and D, β-hydroxyisovalerylalkannin, isobutyrylalkannin, α-methylbutyrylalkannin, teracrylalkannin, propionylalkannin, alkannin and acetylyalkannin were provided by Assoc. Prof. Chinou. IC$_{50}$ values of these substances were determined for CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells with the XTT viability assay after 72 h. Results are shown in figures 87 and 88.

Except of dimethylacrylalkannin and a mixture of shikonofuran C and D, all investigated shikonin and alkannin derivatives exhibited growth inhibitory activity against the cancer cell lines used. Dimethylacrylalkannin and shikonofuran C and D were not able to affect the cell growth up to 135 µM and 50 µg/ml, respectively.

Shikonin and its derivatives showed IC$_{50}$ values ranging from 0.15 µM to 12 µM. Most IC$_{50}$ values of CCRF-CEM leukemia cells were even in a nano-molar range. Also MDA-MB-231 breast and HCT 116 colon cancer cells revealed very low IC$_{50}$ values of about 1-3 µM. The highest concentrations for inhibiting growth and proliferation were needed in the case of U251 cells. Shikonin itself showed the highest activity of all tested shikonin derivatives.
Figure 87. **IC₅₀ values of different shikonin derivatives, determined by the XTT viability assay after 72 h.**

A: shikonin, B: deoxyshikonin/alkannin, C: isobutyrylshikonin, D: isovalerylshikonin, E: (2-methyl-n-butyryl)shikonin. n = 4, mean ± sem.
OWN INVESTIGATIONS AND DISCUSSION

**Concentration (µM)**

**Metabolic Active Cells (% of Control)**

**A**
- CCRF-CEM, IC<sub>50</sub>: 0.53 ± 0.02 µM
- MDA-MB-231, IC<sub>50</sub>: 5.60 ± 0.36 µM
- U251, IC<sub>50</sub>: 16.39 ± 0.45 µM
- HCT 116, IC<sub>50</sub>: 5.64 ± 0.14 µM

**B**
- CCRF-CEM, IC<sub>50</sub>: 0.39 ± 0.02 µM
- MDA-MB-231, IC<sub>50</sub>: 4.62 ± 0.14 µM
- U251, IC<sub>50</sub>: 3.17 ± 0.31 µM
- HCT 116, IC<sub>50</sub>: 1.75 ± 0.06 µM

**C**
- CCRF-CEM, IC<sub>50</sub>: 1.69 ± 0.15 µM
- MDA-MB-231, IC<sub>50</sub>: 12.21 ± 0.88 µM
- U251, IC<sub>50</sub>: 25.84 ± 0.66 µM
- HCT 116, IC<sub>50</sub>: 6.83 ± 0.85 µM

**D**
- CCRF-CEM, IC<sub>50</sub>: 1.22 ± 0.24 µM
- MDA-MB-231, IC<sub>50</sub>: 30.05 ± 5.92 µM
- U251, IC<sub>50</sub>: 41.70 ± 5.85 µM
- HCT 116, IC<sub>50</sub>: 7.97 ± 0.84 µM

**E**
- CCRF-CEM, IC<sub>50</sub>: 1.67 ± 0.38 µM
- MDA-MB-231, IC<sub>50</sub>: 27.59 ± 8.25 µM
- U251, IC<sub>50</sub>: 41.70 ± 5.85 µM
- HCT 116, IC<sub>50</sub>: 5.53 ± 0.87 µM

**F**
- CCRF-CEM, IC<sub>50</sub>: 0.98 ± 0.05 µM
- MDA-MB-231, IC<sub>50</sub>: 6.22 ± 0.46 µM
- U251, IC<sub>50</sub>: 19.17 ± 1.43 µM
- HCT 116, IC<sub>50</sub>: 1.52 ± 0.05 µM
IC$_{50}$ values of alkannin and its derivatives were in a range of 0.4 μM to 70 μM. Also in this case, CCRF-CEM leukemia cells displayed the highest sensitivity to the substances with IC$_{50}$ values at least two or three fold lower than those of the other cancer cell lines. U251 glioblastoma cells mostly exhibited the highest IC$_{50}$ values. In the case of β-hydroxyisovalerylalkannin this concentration was even six fold higher than those of MDA-MB-231 and HCT 116 cells. In general, HCT 116 cells reacted more sensitive to the treatment than MDA-MB-231 cells. Propionylalkannin was not able to reduce the growth of U251 cells up to 145 μM.

When the respective enantiomers were compared to each other, shikonin was more active than alkannin. However, acetylalkannin was more active than the shikonin enantiomere in case of MDA-MB-231 and HCT 116 cells. In the case of β-hydroxyisovalerlyshikonin and –alkannin, the shikonin derivative showed again higher activity than the alkannin derivative. The same behavior was observed for isobutyrylshikonin/–alkannin.
OWN INVESTIGATIONS AND DISCUSSION

3.11.6. DISCUSSION

3.11.6.1. PLANT IDENTIFICATION

Onosma paniculata was authenticated using its ITS2 region of nuclear DNA and trnL-F region of plastid DNA because of the macroscopic similarities between the different Zicao species. How similar these species can look like is also reflected by the fact that our plant material was first identified as Arnebia euchroma based on macroscopic characteristics. However, DNA analysis revealed that the highest similarity values obtained were found for Onosma paniculata (about 98%) and not Arnebia euchroma. An accurate identification of this plant was especially necessary, since the roots were obtained at the plant market in Kunming. On Chinese markets, Onosma paniculata and related species are often sold under the name Zicao not considering the exact plant species. However, it has been shown that shikonin containing plants vary in their content and composition of bioactive naphthoquinones which may be relevant to clinical applications (Hu et al., 2006c). Moreover, there are still concerns about safety and quality of TCM drugs since several plants are often used and sold under the same
Chinese drug name and the plant origin is not well documented (Bauer and Franz, 2010). This can lead to dangerous mistakes. The roots of *Stephania tetrandra* are officially listed in the Chinese Pharmacopoeia as analgesic and diuretic agent (Tang and Eisenbrand, 2011b). Inadvertently substitution of *Stephania tetrandra* with *Aristolochia fangchi* in weight-reducing pills led to progressive renal failure and ended in end-stage renal diseases and carcinoma in women. The mistake was then detected since the herb powder did not contain tetrandrine, which can be found in *Stephania tetrandra*, but aristolochic acids which are known for their carcinogenic activities in rats (Nortier et al., 2000). In case of Zicao species, it is known that some species contain not only shikonin or alkannin derivatives but also different amounts of pyrrolizidine alkaloids which are hepatotoxic and carcinogenic. For example, *Arnebia euchroma* contains only 10 ppm of these alkaloids and intoxications have not been reported so far. In contrast, *Lithospermum erythrorhizon* contains about 0.02% intermedine, myoscorpine and hydroxymyoscorpine in the dried root. Therefore, the internal use is not recommended (Roeder, 2000). Thirteen different pyrrolizidine alkaloids were also identified in *Onosma stellulatum* (Mroczek et al., 2004) and five in *Onosma leptantha* (Kretsi et al., 2003). This shows that a precise identification of Zicao plants is of substantial interest.

### 3.11.6.2. INVESTIGATIONS OF DIFFERENT ROOT EXTRACTS

In this PhD thesis, different extracts of the roots of *Onosma paniculata* were prepared using ASE, Soxhlet extraction, decoction and maceration and petroleum ether, methanol and/or water as solvents. Pharmacological investigations revealed that all PE extracts showed high cytotoxicity against CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells. In contrast, the MeOH extract and the decoction prepared did not show any cytotoxicity at 10 µg/ml. The facts that vegetable oil extractions of Zicao are recommended for external use (The State Pharmacopoeia Commission of the People’s Republic of China, 2005, Wu, 2005) and that a recipe of the 18th century describes the boiling of the roots of *Alkanna tinctoria* in olive oil for the application to deep wounds (Papageorgiou et al., 1999) allow to assume that these extracts may also be used for the treatment of cancer. When wound healing properties of Zicao roots were firstly reported evidence-based by Papageorgiou (1978), the active constituents were isolated from an unpolar hexane extract and identified as alkannin derivatives (Papageorgiou et al., 1999). Also other extracts of Zicao plants have been reported to inhibit the proliferation of several cancer cell lines (Lu and Liao, 1990, Wang and Li, 2003). The naphthoquinones pigment-LIII extract of *Arnebia euchroma* inhibited
the mitotic index and growth of normal human cells without inducing DNA damages (Lu and Liao, 1990). This indicates that side effects of the active constituents are acceptable. Although the Chinese pharmacopoeia of 2005 recommend only external use (The State Pharmacopoeia Commission of the People’s Republic of China, 2005), there are also citations for the internal use in form of root or whole plant decoctions (Wu, 2005, Duke and Ayensu, 1985a). However, the traditional prepared decoction showed no cytotoxicity toward CCRF-CEM leukemia cells indicating that these decoctions may not be able to fight cancer.

The active PE extract was further investigated regarding its effect on cell morphology, cell cycle and activation of caspase-3 (Rinner et al., 2010). CCRF-CEM, MDA-MB-231, U251, HCT 116, HT-29, Hela, 769-P, SBc-L2, WM35, WM9 and WM164 cells were used. In all cases, cell viability, growth and morphology were affected at the latest after 72 h of incubation. The four melanoma cells lines used were originally isolated from different stages of tumor progression. It could be observed that with increasing aggressiveness of the melanoma cells, the time to the first reactions of the cells also increased, indicating that the intensity of the effect depends on the tumor stage. Not only the morphology was influenced, but also the cell cycle. Cells were arrested in the S- and G2/M-phase accompanied by a decrease of cells in the G1-phase. Further investigations revealed that caspase-3, a key enzyme in the induction of apoptosis, was strongly activated in all cell lines. Shikonin was also shown to activate caspase-3 in leukemia, bladder and cervical cancer cells (Wu, 2005, Yeh et al., 2007, Yoon et al., 1999) and to inhibit topoisomerase II and NF-κB – potential targets in chemotherapy (Fuji et al., 1992, Min et al., 2008). Moreover, microarray experiments suggest that shikonin affects the DNA (Efferth et al., 2007a,b). The data of this thesis displayed that the PE extract of *Onosma paniculata* interfered with the cell cycle (especially at the G2/M-phase), led to the activation of caspase-3 and, finally, caused cancer cell death.

### 3.11.6.3. Activity-Guided Isolation and Pharmacological Investigations of Isolated Active Compounds

Many publications can be found describing the cytotoxic and anti-cancer activities of many different Zicao plants and shikonin or alkannin and derivatives thereof. However, *Onosma paniculata* is less investigated so far. HPLC fingerprint analysis revealed that the active PE extract was composed of three main and some minor compounds. Fractionation using preparative HPLC led to the isolation of four pure compounds: β-
hydroxyisovalerylshikonin, acetylshikonin, β-dimethylacrylshikonin and epoxyshikonin. The last two were isolated as main compounds of this extract. The structure and stereochemistry was analyzed using NMR and CD measurements and comparison of these data with literature data. Acetyl- and β-dimethylacrylshikonin were also isolated from a cyclohexane root extract of Onosma leptantha as anti-inflammatory and cytotoxic principles (Kundakovic et al., 2006).

Subsequently, IC50 values were determined after 72 h using the XTT viability assay. Eight different cancer cell lines (CCRF-CEM, MDA-MB-231, U251, HCT 116, SBC-L2, WM35, WM9 and WM164) and MRC-5 lung fibroblasts were used. Since it was shown that shikonin and derivatives polymerize and photooxidize in the presence of air, light, heat, acids and/or bases and loose activity (Papageorgiou, 1980, Papageorgiou et al., 1999), the substances were fumigated with nitrogen before storage, kept in dark at -20 °C and activity was regularly controlled by the XTT viability assay. For each assay, the substances were freshly dissolved in DMSO and used immediately. One benefit in this work was the one-step isolation of the substances from the PE extract since it has also been shown that biological activity can get lost during the isolation process due to polymerization of the compounds (Papageorgiou, 1980). All four isolated compounds decreased dose-dependently the viability and proliferation of all cancer cell lines and lung fibroblasts. Thereby, lung fibroblasts were inhibited in their growth in the same concentration range as many of the cancer cells. Only the leukemia cell line was about twice and six fold more sensitive to the treatment. However, in vivo experiments have shown that shikonin at a dose of 5-10 mg/kg/day did not exhibit cytotoxicity in mice while tumor growth was completely inhibited. Only higher doses (>15 mg/kg/day) led to the death of the experimental animals (Sankawa et al., 1977). In the case of the melanoma cell lines used, IC50 values increased with increasing aggressiveness. Altogether, dimethylacrylshikonin showed the highest activity against these cell lines. Further investigations of the two main compounds and their effects on the melanoma cells revealed that the morphology started to change already after 24 h and continued after 48 h. Also the cell cycle was already affected after 24 h. Yeh et al. (2007) had shown that shikonin influenced cyclin dependent kinase and cyclin activity by increasing p21 and downregulating cyclin E, CDK2 and CDK4 protein levels. In A375-S2 melanoma cells, cells were arrested in the G1 phase accompanied by an increase of p53 and bax protein levels and a decrease of CDK4 (Wu et al., 2004). However, CDK4 and p53 are mainly involved in the G1/S checkpoint and, thus, leading to an arrest in the G1 phase. Treatment with dimethylacryl- or epoxyshikonin mainly led to an arrest in the S- and/or G2/M-phase and was always accompanied by a decrease of cells in the G1 phase.
indicating other cellular targets. To verify this assumption, the cell cycle and enzymes/proteins involved have to be investigated in more detail in further experiments. In the case of dimethylacrylshikonin, which is less investigated compared to shikonin regarding its effects within the cell, it was also analyzed whether caspase-3 is activated in melanoma cells during the treatment. It could be shown that caspase-3 was cleaved and exhibited activity in WM35 cells, but not in SBc-L2 cells. Activation of the apoptotic machinery including several caspases has been reported for other cancer cell lines and shikonin as well (Hsu et al., 2004, Min et al., 2008, Wu et al., 2004, Yeh et al., 2007). Our results indicate that dimethylacrylshikonin leads to the activation of both pathways in WM35 cells since mRNA expression levels of bad, bid, bmf and TRAIL are significantly up-regulated. Nevertheless, it has also been shown that melanoma cells can undergo controlled cell death without activation of caspases and the release of cytochrome c. Oppermann et al. (2005) had shown that activation of the proapoptotic BH3-only protein natural born killer/Bcl-2 interacting killer (Nbk/Bik) led to typical phenomena of apoptosis such as DNA fragmentation and chromatin condensation without caspase activity. Hsu et al. (2004) have reported that in shikonin treated COLO 205 cells anti-apoptotic bcl-2 was down- and pro-apoptotic bad and bax expression was upregulated. Quite the same was also reported by Wu et al. (2004) in malignant melanoma cells. Expression levels of AIF were significantly increased long time before cytochrome c was released into the cytosol and caspases activated. It has been shown that AIF can directly and caspase-independently affect nuclei and cause DNA and chromatin fragmentation and that this process can precede the classical apoptotic pathway (Susin et al., 1999, Norberg et al., 2010). Thereby, bcl-2 acts as inhibitor of AIF release from the mitochondria (Susin et al., 1999). The importance of AIF-mediated cell death not only in human tumors but also neuronal cell death has been demonstrated several times. Moreover, AIF seems to have several physiological functions as redox-active protein and mitochondrial bioenergetics (Norberg et al., 2010). Although, we could not find an alteration in the mRNA expression levels of bcl-2 and bad in SBc-L2 cells, release of AIF would be worth to be further investigated. In other reports, shikonin and derivatives thereof were shown to induce necroptosis (Han et al., 2007, Xuan and Hu, 2009). The treatment was accompanied by necrotic morphology, loss of plasma and mitochondrial membrane potential and activation of autophagy without activation of caspases or translocation of AIF (Han et al., 2007). This cell death was identified by Degterev et al. (2005) and is characterized by (a) necrotic cell morphology, (b) loss of plasma membrane integrity, (c) autophagy as downstream consequence, (d) rising reactive oxygen species in some cells, (e) loss of mitochondrial membrane potential and (f) the ability of necrostatin-1 to inhibit the process. Necroptosis may function as a backup when apoptosis is blocked (Han et al.,
2009). Further investigations have shown that shikonin induced apoptosis or necrosis concentration dependently (Han et al., 2009). The present results from flow cytometric experiments revealed the appearance of a SubG1 peak when SBc-L2 cells were treated with dimethylacrylshikonin or epoxyshikonin indicating that DNA is fragmented. Also the cell shrinkage observed indicates cell degradation. Therefore, it is assumed that these cells undergo a rather controlled instead of uncontrolled cell death. A target of dimethylacrylshikonin could be AIF or other factors which lead to the release of AIF from the mitochondria – for example Ca^{2+} homeostasis or early lysosomal permeabilization (Norberg et al., 2010). Furthermore, another mechanism could be induction of a necroptotic cell death (Han et al., 2007). At the moment, the mitochondrial membrane potential is investigated using the JC-1 assay. If these results indicate loss of mitochondrial membrane integrity, a next step could be the treatment with necrostatin-1 which is an inhibitor of necroptosis (Han et al., 2007).

3.11.6.4. COMPARISON OF THE CYTOTOXICITY OF SEVERAL SHIKONIN AND ALKANNIN DERIVATIVES

It has been reported that the naphthaquinone moiety is necessary for anti-microbial activities since alkylation of the phenolic groups led to complete inactivity. The side chain is believed to serve as delivery system and to modify the overall activity (Papageorgiou, 1980). Also concerning topoisomerase I inhibition it was observed that substances lacking a phenolic hydroxyl group were not active in contrast to substances with one or two. Thereby, the side chain further affected the activity while acylated derivatives exhibited the highest activity (Plyta et al., 1998). Cui et al. (2008) suggested that the side chain and its substituent are not essential for the activity but the presence of a phenolic OH group. The chiral center was reported to have no effect on the cytotoxic activity (Lu et al., 2002).

IC_{50} values of shikonin, alkannin and seven derivatives respectively were determined after 72 h using CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells. Comparison of the activities have confirmed that the naphthaquinone moiety is more important for activity then the side chain. Also loss of the chiral center (deoxyshikonin/alkannin) had, in contrast to the report of Xuan and Hu (2009), no significant effect on the activity. The IC_{50} of this compound was in the same range than those of other derivatives. However, shikonin showed an 8 to 25 fold lower IC_{50} than alkannin. Similar to that also acetyl-, β-hydroxyisovaleryl- and isobutyrylshikonin exhibited lower IC_{50} than their respective
enantiomere indicating that the (R)-enantiomere possesses higher cytotoxicity than the (S)-enantiomere. The highest activity exhibited shikonin itself. Interestingly, dimethylacrylalkannin in contrast to dimethylacrylshikonin did not exhibit any cytotoxicity up to 100 µg/ml. Huang et al. (2004) also reported that this compound showed no significant cytotoxicity against human GLG-82 lung, nasopharyngeal CNE2, liver Bel-7402 and leukemia K-562 cancer cells while semisynthetic modification led to quite active derivatives thereof. However, acetylalkannin was also not active in that work while it showed high activity against cancer cell lines used in this thesis. Nevertheless, it has to be considered if there has been a stability problem during isolation, storage, shipment or testing. This may also explain why this substance was the only one showing only a slight red color in comparison to all other derivatives.

3.12. *Periploca sepium*

3.12.1. **PLANT MATERIAL, EXTRACTION PROCEDURE AND TLC FINGERPRINT**

Dried cortex of *Periploca sepium* was acquired by S. Kahl at the medicinal plant market in Kunming, China in November 2002 (Kahl, 2005). The plant material was not only authenticated by macroscopic analysis but also by DNA sequence analysis of the nuclear ribosomal internal transcribed spacer (ITS) (Wagner et al., 2010). 5.0 g freshly ground plant material was subjected to ASE extraction with PE and, successively, MeOH. After evaporation to dryness under reduced pressure, yields were 179.2 mg and 1524.4 mg, respectively. From each extract, a 5 mg/ml stock solution was prepared and applied to six TLC plates. Two plates were developed with one of three solvent systems respectively, analyzed at vis, 366 nm and 254 nm and, finally, sprayed with vanillin-sulfuric acid or NST/PEG. Best results are displayed in figure 90.

According to the TLC fingerprint, the ASE extraction method seems to be almost sufficient for an exhaustive extraction with petroleum ether and methanol. Spraying with vanillin-sulfuric acid revealed several violet zones in the PE extract, while most compounds of the MeOH extract seems to remain at the start. After spraying with NST/PEG reagent, a few blue fluorescent zones appeared in both extracts.
OWN INVESTIGATIONS AND DISCUSSION

3.12.2. PHARMACOLOGICAL INVESTIGATIONS OF EXTRACTS

After TLC fingerprint, both ASE extracts were subjected to the XTT viability and GI assay. Human CCRF-CEM leukemia, MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cell lines were used in the XTT assay, CCRF-CEM cells in the growth inhibition assay (figure 91).

![TLC fingerprint of a PE and MeOH extract of Periploca sepium prepared by ASE.](image)

Figure 90. TLC fingerprint of a PE and MeOH extract of Periploca sepium prepared by ASE.

Figure 91. Effects of different ASE extracts of Periploca sepium. A: XTT viability assay after 72 h, n = 6, mean ± sem, B: GI assay after 7 days, n = 3, mean ± S.D. Extract concentration: 10 µg/ml.
Extracts were tested at a final concentration of 10 µg/ml and effects analyzed after 72 h (XTT) as well as after 7 days (GI). While the PE extract had no effect on the growth and viability of the cancer cells used, the MeOH extract showed activity. However, it only affected CCRF-CEM and HCT 116 cells in their proliferation and viability but not MDA-MB-231 and U251 cells.

3.12.3. DISCUSSION

The MeOH extract investigated showed an interesting effect since it strongly inhibited the growth of CCRF-CEM leukemia and HCT 116 colon cancer cells, but not the proliferation of MDA-MB-231 breast and U251 glioblastoma cells. This may be a first hint that this extract shows not only cytotoxicity but rather some kind of a specific effect. However, further investigations are necessary to support this theory. In the literature, several compounds isolated from this plant have been reported to possess antiproliferative, apoptosis-inducing, cell cycle affecting or also immunomodulatory or rather immunosuppressive activities (Itokawa, 1988a, Zhu et al., 2006, Cheng et al., 2007, Feng et al., 2008, Ho et al., 2009, Zhang et al., 2009b, Lirdprapamongkol et al., 2010, Zhao et al., 2010). At the moment, *Periploca sepium* is being further investigated within another PhD thesis (Wagner, 2011). One part therein is the activity-guided fractionation using the XTT viability assay and CCRF-CEM leukemia cells as monitor to identify the active compounds in the MeOH extract.

3.13. SAUSSUREA LAPPA

3.13.1. PLANT MATERIAL, EXTRACTION PROCEDURE AND TLC FINGERPRINT

Roots were acquired by S. Kahl at the medicinal plant market in Kunming, China, in November 2002 and authenticated at the Kunming Institute of Botany, China (Kahl, 2005). For screening purposes, 5 g were successively extracted with PE and MeOH by ASE. All extracts were evaporated to dryness under reduced pressure at 40 °C. The yields were 0.20 g and 1.84 g, respectively. For isolation purposes, 55 g fresh powdered material was extracted with PE by Soxhlet extraction and more 36 g by maceration at room temperature. The yields were 1.74 g and 1.21 g, respectively. 20 µl of a 5 mg/ml stock solution were applied to six TLC plates, developed with one of three different solvent systems and sprayed with vanillin – sulfuric acid or NST/PEG reagent. The best result is shown in figure 92.
Figure 92. TLC fingerprint of extracts of *Saussurea lappa*, costunolide and dehydrocostus lactone.

All PE extracts exhibited the same TLC pattern after spraying with vanillin-$\text{H}_2\text{SO}_4$-reagent. They had two main zones of a violet-blue color and some other smaller zones. This showed that there was no big difference between the extraction processes used and that heat did not change the zone pattern. The TLC fingerprint also revealed that three cycles of ASE extraction were almost sufficient for an exhaustive extraction, since the zones of the PE extract were hardly visible in the MeOH extract. The two main compounds in the PE extracts were later identified as costunolide and dehydrocostus lactone, whereby, the upper band is dehydrocostus lactone, the lower costunolide.

### 3.13.2. Pharmacological Investigation of Extracts

For screening purposes, *Saussurea lappa* was successively extracted by ASE with PE and MeOH and both extracts were tested in the XTT viability and growth inhibition assay at a concentration of 10 µg/ml. Four different cancer cell lines were used (CCRF-CEM, MDA-MB-231, U251 and HCT 116). After 72 h, the PE extract showed strong growth inhibitory activity, while the MeOH extract had no effect on cell growth and viability. The same results were obtained after 7 days of incubation. For isolation purposes, more plant material was extracted by Soxhlet extraction and maceration. Both PE extracts showed again strong and comparable activity in the XTT viability assay after 72 h. This indicates that also the activity of the substances is not influenced by heat during the extraction process (figure 93).
Figure 93. Effects of different extracts of Saussurea lappa. A: XTT viability assay after 72 h (mean ± sem, n = 6), B: GI assay after 7 days (mean ± S.D., n = 3), expressed as % of control cells. Extracts were tested at a final concentration of 10 µg/ml.

### 3.13.3. ISOLATION AND IDENTIFICATION OF ACTIVE CONSTITUENTS

**Figure 94.** Extraction and isolation procedure of Saussurea lappa and activities in the XTT viability assay (active: less than 20% metabolic active cells compared to control cells after 72 h and at a final extract/fraction concentration of 10 µg/ml).
## OWN INVESTIGATIONS AND DISCUSSION

### measured data for costunolide

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Table 5. 1H and 13C NMR data of costunolide. 13C data were obtained from HSQC spectra. Therefore, data of quaternary C-atoms are not given in the table. Substances were measured in pyridin-d_5.

### measured data for dehydrocostus lactone

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Table 6. 1H and 13C NMR data of dehydrocostus lactone. 13C data were obtained from HSQC spectra. Therefore, data of quaternary C-atoms are not given in the table (n.d.). Substances were measured in pyridin-d_5.
HPLC and TLC analysis revealed that the active PE extract was composed of two main compounds (figure 95). Since these compounds could be separated by analytical HPLC, preparative HPLC was chosen for isolation. 200 mg extract were subjected to preparative HPLC which yielded 39.5 mg of a first and 67.7 mg of a second fraction. After analytical HPLC analysis, fraction one appeared as pure compound, while in the second fraction two peaks were observed. Therefore, fraction 2 underwent a second isolation process with the same parameters. Finally, 37.4 mg of fraction 2-1 were gained. Using NMR experiments (table 5 and 6) and literature data, fraction 1 was identified as costunolide (Jacobsson et al., 1995), fraction 2 as dehydrocostus lactone (Yuuya et al., 1999).

![HPLC chromatogram of the PE extract of Saussurea lappa. Stationary phase: RP18 column, mobile phase: H2O/ACN, detection at 220 nm.](image)

3.13.4. PHARMACOLOGICAL INVESTIGATIONS ON COSTUNOLIDE AND DEHYDROCOSTUS LACTONE

3.13.4.1. DETERMINATION OF IC50 VALUES USING THE XTT VIABILITY ASSAY

Costunolide and dehydrocostus lactone were tested in the XTT viability assay at a final concentration of 10 µg/ml. Since both showed strong growth inhibitory activity, IC50 values were determined. Six different cancer cell lines (CCRF-CEM, MDA-MB-231, U251, HCT 116, SBe-L2, WM35, WM9 and WM164) and a lung fibroblast cell line (MRC-5) were used.
In the case of costunolide, all IC₅₀ values were in the range of 9 to 20 µM, except of U251 cells with an IC₅₀ value over 100 µM. In general, dehydrocostus lactone showed a higher activity. The lowest IC₅₀ was only 2.5 µM and even in U251 cells, the IC₅₀ was only 30 µM. In the case of cancer cells, most IC₅₀ values were in the range of about 6 to 10 µM. For the MRC-5 lung fibroblasts, IC₅₀ values of 10.2 µM for costunolide and 13.3 µM for dehydrocostus lactone were examined. In case of costunolide, this concentration
was within the range of cancer cells or even a bit lower. In the case of dehydrocostus lactone, cancer cells reacted more sensitive to the treatment than lung fibroblasts – with exception of the very aggressive U251 cells.

![IC50 values comparison](image)

*Figure 98. Comparison of IC<sub>50</sub> values of costunolide and dehydrocostus lactone, determined by XTT viability assay after 72 h. n = 6, mean ± sem.*

### 3.13.4.2. **Effects of Costunolide and Dehydrocostus Lactone on Human Soft Tissue Sarcoma Cell Lines**

#### 3.13.4.2.1. **Effects on Cell Viability and Proliferation**

Human SW-872 liposarcoma, SW-982 synovial sarcoma and TE-671 rhabdomyosarcoma cells were incubated with different concentrations (ranging from 0.5 to 7.5 µg/ml) of costunolide or dehydrocostus lactone for 24 h, 48 h and 72 h. Figure 99 shows that both substances inhibited cell proliferation time- and dose-dependently. Moreover, dehydrocostus lactone showed again higher activity than costunolide.
Figure 99. Proliferation of human SW-872 (A, B), SW-982 (C, D) and TE-671 (E, F) sarcoma cell lines treated with several concentration of costunolide (left site) or dehydrocostus lactone (right site) for 24 h, 48 h and 72 h. ViaLight® assay, n = 8, mean ± sem. IC$_{50}$ values after 48 h are listed in the table below.
3.13.4.2.2. **Effects on Cell Cycle**

Human SW-872 liposarcoma, SW-982 synovial sarcoma and TE-671 rhabdomyosarcoma cells were incubated with the respective IC$_{50}$ of costunolide and dehydrocostus lactone for 24 h, 48 h and 72 h (figure 100). All three cancer cell lines showed S and G2/M phase arrest after 24 and 48 h when treated with costunolide. After 72 h however, the cell cycle returned to control levels, an effect which seemed to start already after 48 h and which occurred in all three cell lines. When treated with dehydrocostus lactone, cells were arrested in the S and G2/M phase. This was accompanied by a decrease of cells in the G1 phase. This effect was observed after all three incubation periods. Compared to costunolide, dehydrocostus lactone seems to have a stronger effect on the cell cycle.

![Figure 100](image-url)

*Figure 100. Influence of costunolide (A-C) and dehydrocostus lactone (D-E) on cell cycle distribution of soft tissue sarcoma cells. n = 3, mean ± S.D. A and D: SW-872 cells; B and E: SW-982 cells; C and F: TE-671 cells.*

3.13.4.2.3. **Effects on Cell Membrane**

The soft tissue sarcoma cell lines used were incubated with their respective IC$_{50}$ of costunolide and dehydrocostus lactone for 24 h, 48 h and 72 h (figure 101). Afterwards, they were stained with propidium iodide (PI) to detect membrane damages. For analysis, PI positive cells of untreated control cells were subtracted from PI positive cells of treated cells. In case of costunolide, the highest membrane damaging effect was observed after 24 h and was 10%. Longer incubation times resulted in less membrane damage. In the case of dehydrocostus lactone, the damaging effect was higher,
especially in SW-982 cells (24 h: about 26%, 48 and 72 h: about 10%). However, the membrane of TE-671 cells seems to be not damaged at all.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Figure 101.** Effects of costunolide (A) and dehydrocostus lactone (B) on cell membrane of different soft tissue sarcoma cell lines. Analysis was performed after incubation with the respective IC₅₀ for 24 h, 48 h or 72 h, cells were stained with PI and analyzed by flow cytometry. C: representative propidium iodide staining of SW-872 control and treated cells.

### 3.13.4.2.4. **Effects on apoptosis**

SW-872, SW-982 and TE-671 cells were incubated with their respective IC₅₀ of costunolide and dehydrocostus lactone for 24 h, 48 h and 72 h. Afterwards, cells were harvested, fixed and stained with DAPI, JC-1 or activated caspases-3 antibody. In all cases, no significant levels of cleaved caspases-3 or other apoptotic signs such as changes in the mitochondrial membrane potential could be detected (figure 102). Only when the cells were treated with the double IC₅₀ of dehydrocostus lactone, cleaved caspase-3 could be detected (figure 103).
Figure 102. Investigations on apoptosis. DAPI staining of A) untreated SW-982 cells, B) costunolide treated SW-982 cells (48 h) and C) dehydrocostus lactone treated SW-982 cells (48 h). D-G) Cells stained with JC-1 to analyze mitochondrial membrane integrity. Mitochondrial depolarization is indicated by decreasing red/green fluorescence intensity ratio. D) TE-671 control cells; E) CCCP (50 µM) pretreated positive TE-671 cells; F) dehydrocostus lactone treated TE-671 cells (48 h) and G) costunolide treated TE-671 cells (48 h). H) Cleaved caspases-3 levels in percent after treatment with costunolide or dehydrocostus lactone (IC₅₀). All experiments were done in triplicates and values are expressed in mean ± S.D. Cells were treated with their respective IC₅₀.
OWN INVESTIGATIONS AND DISCUSSION

Figure 103. Cleaved caspase-3 in A) SW-872, B) TE-671 and C) SW-982 cells when treated with the double IC$_{50}$ of dehydrocostus lactone. Cleaved caspase-3 was detected after 24 h and 48 h. Black line: untreated control cells; violet line: 24 h incubation; green line: 48 h incubation. D) Cleaved caspase-3 in SW-872 cells when treated with 2 x IC$_{50}$ of dehydrocostus lactone for 24 h, 48 h and 72 h. Apoblock was used as caspase inhibitor control. All experiments were performed in triplicates.

3.13.4.2.5. **Effects on the expression levels of ABC transporters**

Expression levels of three main drug transporters (ABCG2/BCRP1, ABCA2 and ABCB1/MDR1) were investigated by real-time PCR after soft tissue sarcoma cells were treated with the respective IC$_{50}$ of costunolide and dehydrocostus lactone (figure 104). While expression levels of ABCG2/BCRP1 and ABCA2 remained unchanged, the expression of ABCB1/MDR1 was significantly downregulated in dehydrocostus lactone treated SW-872 and costunolide and dehydrocostus lactone treated SW-982 cells. In TE-671 cells, the expression level of this transporter was upregulated.
Figure 104. Relative mRNA expression levels of ABCB1/MDR1 transporter. The expression was normalized (ΔCt) to the expression of β-actin, GAPDH and hprt-n housekeeping genes and compared to the respective ΔCt (ΔΔCt) of controls. * p < 0.05, n = 5, unpaired t-test.

3.13.4.2.6. Effects on MMP Expression Levels

SW-872, SW-982 and TE-671 cells were incubated with their respective IC₅₀ of costunolide and dehydrocostus lactone for 24 h, 48 h and 72 h. Afterwards, the expression levels of MMP-1, -2, -3 and -9 were analyzed using the MMP Fluorokine® MAP assay. As shown in table 7, the expression levels of MMP-2 and MMP-9 in TE-671 cells were significantly decreased, whereas, the expression of MMP-1 and -3 were not significantly changed when cells were treated with dehydrocostus lactone. Costunolide had no effect on the expression levels. In costunolide treated SW-982 cells, expression levels of all investigated MMPs were decreased at least after 24 h (MMP-1), whereas, dehydrocostus lactone only changed MMP-1 expression after 24 h. When SW-872 cells were treated with costunolide, MMP-3 and MMP-9 expression levels decreased. When treated with dehydrocostus lactone, the expression of MMP-1, -2 and -3 was lower.
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<td></td>
<td></td>
<td>MMP-2 expression [MFI]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>TE-671</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>33.5±3.8</td>
<td>38.3±10.2</td>
<td>39.8±4.9</td>
<td>24h</td>
<td>159.8±9.5</td>
<td>303.5±118.0</td>
</tr>
<tr>
<td>48h</td>
<td>82.5±13.7</td>
<td>74.2±7.7</td>
<td>85.5±4.1</td>
<td>48h</td>
<td>746.0±74.5</td>
<td>879.0±94.3</td>
</tr>
<tr>
<td>72h</td>
<td>90.0±13.5</td>
<td>103.1±21.0</td>
<td>123.8±8.4</td>
<td>72h</td>
<td>2286±385</td>
<td>2312±435</td>
</tr>
<tr>
<td>48h</td>
<td>24420±166</td>
<td>21375±962</td>
<td>26227±524</td>
<td>24420±166</td>
<td>21375±962</td>
<td>26227±524</td>
</tr>
<tr>
<td>72h</td>
<td>27209±729</td>
<td>26395±638</td>
<td>27751±585</td>
<td>27209±729</td>
<td>26395±638</td>
<td>27751±585</td>
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</tr>
<tr>
<td>SW-982</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>10749±412</td>
<td>7929±107</td>
<td>18823±1374</td>
<td>24h</td>
<td>2396±290</td>
<td>1190±207</td>
</tr>
<tr>
<td>48h</td>
<td>23809±151</td>
<td>22812±350</td>
<td>23901±113</td>
<td>48h</td>
<td>7215±727</td>
<td>3842±260</td>
</tr>
<tr>
<td>72h</td>
<td>25319±533</td>
<td>24892±447</td>
<td>25858±464</td>
<td>72h</td>
<td>17127±1353</td>
<td>8649±1384</td>
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<td>##</td>
<td>##</td>
<td>##</td>
<td>##</td>
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<tr>
<td>SW-872</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>56.8±24.0</td>
<td>50.0±23.0</td>
<td>46.9±24.0</td>
<td>24h</td>
<td>40.5±4.0</td>
<td>68.8±31.6</td>
</tr>
<tr>
<td>48h</td>
<td>54.5±12.0</td>
<td>56.37±26.0</td>
<td>45.15±16.0</td>
<td>48h</td>
<td>61.1±4.8</td>
<td>77.6±7.1</td>
</tr>
<tr>
<td>72h</td>
<td>49.4±22.0</td>
<td>46.7±12.0</td>
<td>37.3±14.0</td>
<td>72h</td>
<td>169.3±27.0</td>
<td>191.6±23.9</td>
</tr>
</tbody>
</table>
Table 7. MMP expression levels of soft tissue sarcoma cells after 24 h, 48 h and 72 h represented by mean fluorescence intensity (MFI). Untreated control cells in comparison with costunolide or dehydrocostus lactone treated cells (respective IC\textsubscript{50}). n = 5, mean ± sem. Student’s unpaired t-test was used to evaluate the differences between control cells and treated cells. p values: * p < 0.05, ** p < 0.01, *** p < 0.001.

### 3.13.5. Discussion

#### 3.13.5.1. Investigations on Extracts and Activity-Guided Isolation

Roots of *Saussurea lappa* were successively extracted with PE and MeOH and initially investigated regarding their effects on CCRF-CEM, MDA-MB-231, U251 and HCT 116 cancer cells. The plant is traditionally used in several systems of medicine such as traditional Chinese medicine, Indian Ayurveda, Siddha and Unani, and Tibetan medicine and used to treat several diseases including cancer, cholera, rheumatism and asthma (Pandey et al., 2007, Duke and Ayensu, 1985a). While the MeOH extract did not affect cell proliferation and viability, the PE extracts (ASE, Soxhlet and maceration) exhibited strong growth inhibition. It was also shown that different extraction methods did not change the TLC pattern and *in vitro* activity indicating heat-stability of the active compounds. Nevertheless, it has been shown that active compounds can also be extracted when using 80% MeOH only (Ko et al., 2004 and 2005). This methanolic extract induced apoptosis in AGS gastric cancer cells by affecting central steps of apoptosis and cell cycle.
Subsequently, the Soxhlet PE extract was subjected to activity-guided isolation. Since TLC and HPLC investigations revealed that this extract was composed of two main substances which could be separated by HPLC, preparative HPLC was chosen for fractionation. This led to the isolation of costunolide and dehydrocostus lactone as active principles. Both substances are sesquiterpene lactones which are primarily found in the Asteraceae family (Zhang et al., 2005). Determination of IC50 values using the XTT viability assay, the cell lines mentioned above, MRC-5 lung fibroblasts and several melanoma cell lines (SBc-L2, WM35, WM9 and WM164) revealed that both substances possess growth inhibitory activity. Thereby, dehydrocostus lactone exhibited lower IC50 values after 72 h than costunolide, in contrast to the reported effects on HepG2, Hela and OVCAR-3 cancer cells after 24 h of incubation (Sun et al., 2003). Interestingly, no big difference between the IC50 values in SBc-L2, WM35 and WM9 melanoma cells of different tumor stages, could be found. Also towards the most aggressive WM164 cells they had only slight higher IC50 values. However, lung fibroblasts were inhibited in their growth at similar concentrations. Nevertheless, it has been shown that costunolide reduced tumor burden and incidence also in vivo (Mori et al., 1994, Ohnishi et al., 1997). Both substances also exhibited in vivo gastroprotective activities in acidified ethanol-induced gastric mucosal lesions in Wistar rats (Matsuda et al., 2000). Moreover, an ethanolic extract at doses of 50-200 mg/kg significantly and dose-dependently reduced carrageenan induced paw edema in rats. The presence of sesquiterpene lactones in this extract is assumed (Gokhale et al., 2002).

### 3.13.5.2. CONTINUATIVE PHARMACOLOGICAL INVESTIGATIONS OF COSTUNOLIDE AND DEHYDROCOSTUS LACTONE

The two isolated active compounds were further investigated regarding their effects in soft tissue sarcoma (STS) cell lines (SW-872, SW-982 and TE-671) (Kretschmer et al., 2011b). STS represent a rare group of malignant tumors of various histologies. They arise from a variety of connective tissues including blood vessels, muscles, fat, synovium and Schwann cells. In prognosis, histological subtype and grade are most important (Olstadt et al., 2003, Sessa et al., 2009). They frequently exhibit aggressive characteristics locally as well as in the formation of distant metastases. Moreover, only a few chemotherapeutics are available for systemic therapy (Chugh et al., 2005) since STS often show chemotherapy resistance. Therefore, novel strategies in the treatment of STS are needed and have to be tested.
Costunolide and dehydrocostus lactone dose- and time-dependently reduced the growth of all three STS cell lines, whereby, dehydrocostus lactone exhibited lower IC50 values than costunolide. Cell cycle investigations revealed that costunolide hardly affected the cell cycle distribution of SW-872 and SW-982 cells. In TE-671 cells, cells in the G1-phase were decreased accompanied by an increase of cells in the S- and G2/M-phase after 24 h and 48 h. However, after 72 h the cell cycle returned to control levels. Costunolide had been reported to activate caspase-3 by ROS-mediated mitochondrial membrane potential loss (Lee et al., 2001, Park et al., 2001). In soft tissue sarcoma cells however, no activation of caspase-3 could be detected when they were treated with IC50. Also by using DAPI and JC-1 staining, no apoptotic signals or disruption of mitochondrial membrane potential could be observed. Nevertheless, PI staining had shown that the cell membrane was hardly damaged by the treatment. The highest amount was 10% after 24 h which indicates that cell death was not relying on necrosis. However, the cellular target of costunolide has to be determined in further experiments.

In contrast, dehydrocostus lactone affected the cell cycle after 24 h, 48 h and 72 h. In each case G1-phase cells were diminished and cells in the G2/M phase elevated. The strongest effect was observed for TE-671 cells. The membrane damaging effect in SW-982 cells was higher than in costunolide treated cells. On the contrary, SW-872 cells’ membrane was hardly affected and the cell membrane of TE-671 cells only slightly. Several apoptosis assays revealed that also in the case of dehydrocostus lactone no apoptotic events could be measured when cells were treated with the respective IC50. Only when cells were treated with the double IC50, cleaved caspase-3 was detected and the percentage of activated caspase-3 could be reduced in the presence of ApoBlock (Kretschmer et al., 2011b).

Investigating the mRNA expression levels of transmembrane ATP binding cassette (ABC) transporter proteins ABCB1/MDR1, ABCG2/BCRP1 and ABCA2 revealed that costunolide significantly decreased the expression of ABCB1 transporter proteins in SW-982 cells. The other proteins were not significantly changed in their mRNA expression. Also dehydrocostus lactone reduced the expression of ABCB1 protein levels significantly in SW-872 and SW-982 cells. ABC transporters are named for their distinctive domains that bind ATP and are able to transport biological molecules across the cell membrane against a concentration gradient. The first identified ABC transporter was P-glycoprotein (P-gp) which is encoded by the MDR-1 gene and possesses a broad substrate recognition pattern (Litman et al., 2001). Amongst others, ABC transporters are important mediators of multiple drug resistance (Hrycyna et al., 1998, Allen et al., 1999, Litman et al., 2001). MDR1 and BCRP transporters have been shown to exhibit the most important factors for drug resistance in hepatocellular
carcinoma (Sun et al., 2010b). Inhibition of ABCG2 increased intracellular drug concentrations and the efficacy of chemotherapeutics in several tumors (Ebert et al., 2005). Therefore, dehydrocostus lactone and costunolide could provide candidates in the search for new cancer resistance circumventing drugs.

Finally, both substances were investigated regarding their effects on matrix metalloproteinases (MMP). The physiological role of MMPs is the degradation of extracellular matrix which plays an important role in wound healing, bone resorption and fetal development. Pathological activities are involved in arthritis, autoimmune diseases, heart failure and cancer (John and Tuszynski, 2001, Johnson et al., 1998). In tumors, they are frequently overexpressed and have been shown to play a role in tumor progression. The matrix degrading ability is probably used to spread to distant sites and they probably promote the growth of metastasized tumor cells (John and Tuszynski, 2001). Over 14 of these zinc-containing endopeptidases are known so far. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) possess a fibronectin-like domain responsible for collagen binding. Additionally, MMP-9 contains a collagen V-like sequence downstream the Zn$^{2+}$ binding site. MMP-1 (interstitial collagenase) plays a critical role in the angiogenic cascade and MMP-2 and -9 seem to cause tube formation (John and Tuszynski, 2001). MMP-3 is stromelysin-1 and has matrix components as substrate (Johnson et al., 1998). Inhibitors of these enzymes would have a huge potential in clinical applications. STS cells were treated with the respective IC$_{50}$ of costunolide or dehydrocostus lactone for 24 h, 48 h and 72 h. In summary, costunolide had the strongest inhibitory effect on MMP-3 and MMP-9 in SW-982 and SW-872 cells. Dehydrocostus lactone significantly inhibited especially MMP-1 and MMP-2 expression. The expression levels of MMPs in TE-671 cells were only affected by dehydrocostus lactone.

In summary, costunolide and dehydrocostus lactone have been identified as potential agents against STS cell lines although the detailed mechanism of action is not yet fully understood and has to be further elucidated.

3.14. **Zanthoxylum nitidum**

3.14.1. **Plant material, extraction procedure and TLC fingerprint**

Dried branches were acquired by S. Kahl at the medicinal plant market in Kunming, China, in November 2002 (Kahl, 2005). 5.1 g of freshly powdered plant material was subjected to successive ASE with PE, EtOAc and MeOH. After evaporation of the
OWN INVESTIGATIONS AND DISCUSSION

solvents under reduced pressure, the yields were: PE: 39.7 mg, EtOAc: 67.8 mg and MeOH: 240.5 mg. 10 µl of a 5 mg/ml stock solution were applied to each of six TLC plates. Two were developed with one of three TLC fingerprint solvent systems. Afterwards, they were examined at vis, 366 nm and 254 nm, and, finally, sprayed with vanillin–sulfuric acid or NST/PEG reagent. The best results are shown in figure 105. TLC comparison of the extracts revealed that the plant material was exhaustively or almost exhaustively extracted neither by PE nor by EtOAc. In the ethyl acetate as well as in the methanolic extracts many constituents of the precedent extract could be found. Even in the methanolic extract a few zones of the PE extract were still visible. Nevertheless, some compounds with blue fluorescence at 366 nm did not appear before extraction with MeOH. Some substances also showed fluorescence quenching at 254 nm, some of them appeared slightly blue. Moreover, many constituents showed fluorescence at 366 nm with or without NST/PEG reagent. After spraying with NST/PEG, only one band in the ethyl acetate and one in the methanolic extract appeared additionally.

Figure 105. TLC fingerprint of different ASE extracts of Zanthoxylum nitidum.

3.14.2. PHARMACOLOGICAL INVESTIGATION OF EXTRACTS

The extracts produced by ASE were subjected to the XTT viability and growth inhibition assay. All were tested at a final concentration of 10 µg/ml and investigated after 72 h or 7 days of incubation. Extracts were examined on CCRF-CEM leukemia after 72 h as well as after 7 days. Effects on MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells were studied after 72 h. As shown in figure 106, the methanolic extract reduced the growth of CCRF-CEM cells after 72 h to 39% of control values and after 7 days to 11% of control. Growth and viability of the other cancer cells lines used were hardly affected.
Figure 106. Effects of different ASE extracts of Zanthoxylum nitidum. A: XTT viability assay after 72 h, n = 6, mean ± sem, B: growth inhibition assay after 7 days, n = 3, mean ± S.D. Extract concentration: 10 µg/ml.

3.14.3. DISCUSSION

A petroleum ether, ethyl acetate and methanol extract of Zanthoxylum nitidum were investigated regarding their potential to inhibit the growth of different cancer cell lines. In the case of the GI assay, it could be shown that this potential increased with increasing extract polarity. Therefore, it is assumed that the activity is not correlated to the upper blue and yellow fluorescent compound in the TLC fingerprint, but rather to the yellow and blue fluorescent ones in the lower part of the TLC plate. Interestingly, using the XTT assay, CCRF-CEM leukemia cells were inhibited in their growth by the MeOH extract but not the other cancer cell lines used. This could be a first hint that this extract is not only cytotoxic but exhibits a kind of specific effect to several cancer cells. However, further investigations are necessary to support or contradict this theory. It is further assumed that the activity is probably correlated to alkaloids because of the fluorescent behavior of the extract in the TLC fingerprint and literature data. Several alkaloids such as nitidine (Zhang et al., 2001), chelerythrine (Fang et al., 1993, Malikova et al., 2006, Vrba et al., 2008, Yang et al., 2008b), and skimmianine have been reported to possess anti-cancer potential. They had an effect on topoisomerase I, DNA damage, cycline kinase inhibitors and/or activated the mitochondrial apoptotic pathway. All in all, Zanthoxylum nitidum is a plant worthy of further investigation to reveal the active principles and mechanisms of action.
The goal of this doctoral thesis was the phytochemical and pharmacological investigation of Chinese herbs with potential anti-cancer activity. In a preliminary doctoral thesis, a database including 561 species used to treat cancer or ulcers has been created. In the present PhD thesis, 12 of these plants were extracted and investigated regarding their cytotoxic effect on several cancer cell lines in vitro.

For screening purposes, the plant material was successively extracted with solvents of different polarity using accelerated solvent extraction and yielded 44 extracts. Additionally, traditional decoctions from two plants were prepared and three plants were extracted by maceration. For isolation purposes, Soxhlet extracts were prepared. Each extract was subjected to the XTT viability assay and growth inhibition assay to evaluate its cytotoxicity. CCRF-CEM leukemia, MDA-MB-231 breast cancer, U251 glioblastoma and HCT 116 colon cancer cells were used as standard cell lines. Six extracts showed high activity (below 20% of control) against one or several cell lines, whereby, the highest activity was found for Onosma paniculata and Saussurea lappa. Four extract exhibited moderate activity (between 20 and 60% of control).

Onosma paniculata was initially sold and identified as Arnebia euchroma based on morphological characteristics. In China, both plants are sold under the name Zicao, but differ in their chemical composition. By analyzing nuclear and plastid DNA, it clearly turned out to be Onosma paniculata. Four shikonin derivatives were identified as active principles. Their structures were elucidated by NMR and CD measurements. Epoxyskikonin was isolated from Onosma paniculata for the first time. IC$_{50}$ values were determined using eight different cancer cell lines and a lung fibroblast cell line. The active extract as well as the two main compounds were further investigated regarding their effects on several other cancer cells lines including melanoma cell lines from different tumor stages. The PE extract affected cell morphology and cell cycle (especially at the G2/M-phase) and led to cleavage of caspase-3. Concentrating on melanoma cell lines, dimethylacrylshikonin and epoxyskikonin were shown to affect cell morphology, cell cycle and the percentage of cells in the SubG1 region. Dimethylacrylshikonin also led to the cleavage and activation of caspase-3 in WM35 melanoma cells, but not in SBc-L2 cells. Dimethylacrylshikonin also changed the mRNA expression levels of
several pro-apoptotic genes in WM35 cells. Finally, the activities of the isolated compounds were compared to several other shikonin and alkannin derivatives.

In the case of Saussurea lappa, costunolide and dehydrocostus lactone were identified as active principles. Both structures were elucidated by NMR measurements. IC$_{50}$ values against several cancer cell lines and a lung fibroblast cell line were determined. In further investigations, the effects of these substances on three different soft tissue sarcoma cell lines were analyzed. Both influenced cell cycle distribution and the expression levels of several MMPs. The results indicate that costunolide is an inhibitor of MMP-3 and -9 and that dehydrocostus lactone inhibits MMP-2 and -9. Dehydrocostus lactone also affected the mRNA expression levels of the ABCB1 transporter. Apoptotic events such as disruption of the mitochondrial membrane potential or cleavage of caspase-3 were not found at IC$_{50}$. Cell membrane was only slightly damaged indicating no necrotic death.

Finally, in a screening of several plants collected in North America, a dichloromethane extract of the flowers of Helianthus angustifolius showed strong cytotoxicity. It was subjected to activity-guided isolation using several chromatographic techniques and CCRF-CEM cells as monitor. Four furanogermacranolides (8-methacrylyl-4,15-*iso*-atriplicolide, 8-isobutyryl-4,15-*iso*-atriplicolide, 8-(2-methylbutyryl)-4,15-*iso*-atriplicolide and 8-isovaleryl-4,15-*iso*-atriplicolide) were isolated as active principles and identified by several NMR experiments, optical rotation, UV spectra and high resolution mass spectrometry. All four exhibited strong cytotoxicity against the standard cancer and lung fibroblast cell lines. 8-Isovaleryl-4,15-*iso*-atriplicolide is reported in this PhD thesis for the first time. The other three compounds were isolated from this species for the first time.
Das Ziel dieser Doktorarbeit war die phytochemische und pharmakologische Untersuchung Chinesischer Heilkräuter bezüglich einer krebshemmenden Wirkung. In einer vorausgegangenen Arbeit wurde eine Datenbank erstellt, die 561 Pflanzen- und Tierarten umfasst, die in China für die Behandlung von Krebs oder Geschwüren verwendet werden. In der vorliegenden Doktorarbeit wurden zwölf dieser Pflanzen extrahiert und bezüglich ihrer zytotoxischen Wirkung auf verschiedene Krebszelllinien in vitro untersucht.


USAMMENFASSUNG


6. EXPERIMENTAL PART

6.1. PHYTOCHEMICAL INVESTIGATIONS

6.1.1. PLANT MATERIAL AND IDENTIFICATION

6.1.1.1. PLANT MATERIAL

_Curcuma longa_ was acquired by Prof. Dr. Thomas Efferth in Vietnam. _Caesalpinia sappan_, _Bischofia javanica_ (China), _Bryophyllum pinnatum_ and _Hydnocarpus anethelminthica_ were collected in Xishuangbanna, China, in 2002 or 2003 by Dr. Stefan Kahl. _Cinnamomum cassia_, _Cocculus trilobus_, _Lonicera japonica_ (China), _Onosma paniculata_, _Periploca sepium_, _Saussurea lappa_ and _Zanthoxylum nitidum_ were purchased at the medicinal plant market in Kunming, China, in November 2002 and Oktober 2003 by Dr. Stefan Kahl. A specimen copy of the plant material is deposited in the herbarium of the Institute for Plant Sciences, University Graz (Kahl, S., 2005). Moreover, _Bischofia javanica_ (Graz) was obtained from the Botanical Garden Graz (Austria), _Lonicera japonica_ (Switzerland) and _Caesalpinia sappan_ lignum from Complemedis AG (Trimbach, Switzerland) and _Lonicera japonica_ (Austria) from the pharmacy Casa Medica (Ragnitzerstr. 16, 8010 Graz, Austria). _Helianthus angustifolius_ was collected along highway 331 c. 2 Mi S of Tula in Lafayette Co., Mississippi, in October 2001 by Dr. Wolfgang Schuehly. A voucher of this plant (WS-62) is deposited at the Pullen Herbarium at the University of Mississippi, in Oxford, Mississippi.

6.1.1.2. PLANT AUTHENTICATION

Depending on the plant, identification was performed based on macroscopic characteristics at the Kunming Institute of Botany (Kahl, S., 2005) or genome analysis in collaboration with the Ludwig-Maximilian University of Munich, University of Veterinary Medicine of Vienna, or by the selling companies itself.

_Onosma paniculata Bur. & Franch._ was identified at the Kunming Institute of Botany and authenticated using genome analysis by. Prof. Dr. Guenther Heubl (Ludwig-Maximilian University of Munich). Total genomic DNA was extracted from the dried root using a modified CTAB (cetrimonium bromide) procedure of Doyle and Doyle (1990)
using 3% CTAB, 4% β-mercaptoethanol, 2 M NaCl and 5% PVP according to the protocol modified by Khan et al. (2007). For analysis, two noncoding regions, ITS (ITS1, 5.8S rDNA, ITS2) from nuclear DNA and trnL-F from chloroplast DNA, were chosen. The markers were amplified from total DNA via polymerase chain reaction (PCR) using Taq polymerase (Boehringer Ingelheim, Germany) and the primer pairs ITSleu1 (5‘-GTCCACTGAACCTTATCTATTAG-3’) and ITS4 (5‘-TCCGTAGGTGAACCTGCGG-3’) according to White et al. (1990). For amplification of the trnL-F spacer region the forward primer E (5‘-GGTTCAAGTCCCCTCTATCCC-3’) and reverse primer F (5‘-ATT'TGAAGTTGACACGAG-3’) according to Taberlet et al. (1991) were used. All PCR amplifications were carried out in a MWG thermocycler (Primus). The following program was chosen for ITS: (1) 94 °C for 2 min 30 sec, (2) 40 cycles at 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 1 min 15 sec and (3) a terminal extension phase at 72 °C for 10 min. For the trnL-F spacer each cycle consisted of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min 30 sec, the other steps remained unchanged. PCR products were purified with QuantumPrep Gel Slice Kit (Bio-Rad, Hercules, CA, USA). For sequencing an ABI 377 automated sequencer was used and performed as described elsewhere (Braeuchler et al., 2004). PCR products were purified and sequenced directly using the same primers as for amplification (Rinner et al., 2010).

**Periploca sepium Bunge** was identified at the University of Veterinary Medicine, Vienna in the course of another PhD thesis by analyzing the ITS1 region of the plant material (Wagner et al., 2011). Thereby, a modified CTAB-based DNA-extraction protocol was used for DNA isolation. For PCR reaction, the total reaction mixture of 45 μl containing 1.8 U of TaqPolymerase (HOT FIREPolRDNA Polymerase I, SolisBiodyne, Estonia), 600 nM of each primer ITS5 (5‘-GGAAGGAGAAGTCCCAACCC-3’) and ITS1 (5‘-GCTACGTCTTTCTCATGATGC-3’), 100 nM dNTPs, 2.5 mM MgCl₂, 1 x PCR-Buffer (80 mM Tris-HCL pH 9.4 – 9.5 at 25 °C; 20 mM (NH₄)₂SO₄; 0.02% w/v Tween-20) and 1 μl of genomic DNA. Amplification products were purified with a commercial kit (Invisorb SpinPCRapid Kit, Invitrek, Berlin, Germany) according to the manufacturer’s protocol. Sequence reaction was performed by IBL (Gerasdorf, Austria). The sequences were edited with the program Chromas (Technelysium, Tewantin, Australia). Alignments were constructed with the Clustal W algorithm of Mega 4.0 and the maximum composite likelihood model was chosen for the neighbor joining calculations. The BLAST search revealed 100% concordance with *Periploca sepium* (Wagner et al., 2010).
6.1.2. Extraction Methods

6.1.2.1. Preparation of Plant Material

Dried plant material was ground in a **ZM100 Retsch mill** (Retsch - Solutions in Milling and Sieving, Düsseldorf, Germany) or an **Analysenmühle IKA®**, Labortechnik A 10, Staufen, Germany. Afterwards, the powder was immediately extracted.

6.1.2.2. Accelerated Solvent Extraction (ASE)

ASE is an extraction method for solid and semisolid samples under elevated pressures (0.3 to 20 MPa) and temperatures (up to 200 °C). The samples are put into an extraction cell which is then filled up with solvent, closed and heated. When a certain pressure is reached, the cell is opened again and the extract is released in the collecting tray. Afterwards, cells are purged with fresh solvent. Remaining solvent is transferred to the collection tray with an inert gas (nitrogen) (Adam and Becker, 2000). According to Benthin et al. (1999) one to three extraction cycles of 5 to 6 min at temperatures somewhat above the boiling point are suitable for exhaustive or almost exhaustive extraction of medicinal plants. The instrument used was an **ASE 200 Accelerated Solvent Extraction System** from Dionex (Vienna, Austria).

Freshly powdered plant material was mixed with diatomaceous earth in a ratio of 4:1, filled in an extraction cell and extracted with the following ASE parameters:

<table>
<thead>
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<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
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<td>Heat</td>
<td>5 min</td>
</tr>
<tr>
<td>Static</td>
<td>5 min</td>
</tr>
<tr>
<td>Flush</td>
<td>40%</td>
</tr>
<tr>
<td>Purge</td>
<td>60 sec</td>
</tr>
<tr>
<td>Cycles</td>
<td>3</td>
</tr>
<tr>
<td>Pressure</td>
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</tr>
<tr>
<td>Temptures PE</td>
<td>50 °C</td>
</tr>
<tr>
<td>Temptures Hex</td>
<td>72 °C</td>
</tr>
<tr>
<td>Temptures EtOAc</td>
<td>81 °C</td>
</tr>
<tr>
<td>Temptures MeOH</td>
<td>68 °C</td>
</tr>
</tbody>
</table>

Plants were successively extracted with petroleum ether → ethyl acetate → methanol, petroleum ether → methanol or hexan → methanol. Afterwards, the solvent was evaporated under reduced pressure at 40 °C and dry extracts stored at -20 °C. The amount of plant material, solvents used and yields are listed in table 8.
<table>
<thead>
<tr>
<th>plant</th>
<th>used plant part</th>
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<th>solvent</th>
<th>yield (mg)</th>
</tr>
</thead>
<tbody>
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<td>leaves and caulìs</td>
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<td>PE</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
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</tr>
<tr>
<td><em>Bischofia javanica</em> (Graz)</td>
<td>leaves and caulìs</td>
<td>3.1</td>
<td>PE</td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>94.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>374.8</td>
</tr>
<tr>
<td><em>Bryophyllum pinnatum</em></td>
<td>whole plant</td>
<td>5.2</td>
<td>PE</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>214.6</td>
</tr>
<tr>
<td><em>Caesalpinia sappan</em></td>
<td>leaves and caulìs</td>
<td>4.6</td>
<td>PE</td>
<td>74.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>118.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>680.8</td>
</tr>
<tr>
<td><em>Caesalpinia sappan</em></td>
<td>lignum</td>
<td>4.5</td>
<td>PE</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>314.8</td>
</tr>
<tr>
<td><em>Cinnamomum cassia</em></td>
<td>cortex</td>
<td>5.1</td>
<td>PE</td>
<td>242.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>545.5</td>
</tr>
<tr>
<td><em>Cocculus trilobus</em></td>
<td>root</td>
<td>5.2</td>
<td>PE</td>
<td>38.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>191.2</td>
</tr>
<tr>
<td><em>Curcuma longa</em></td>
<td>rhizom</td>
<td>4.9</td>
<td>Hex</td>
<td>144.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>728.8</td>
</tr>
<tr>
<td><em>Hydnocarpus anthelmintica</em></td>
<td>seed coat</td>
<td>4.8</td>
<td>PE</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>87.8</td>
</tr>
<tr>
<td><em>Hydnocarpus anthelmintica</em></td>
<td>seed</td>
<td>5.1</td>
<td>PE</td>
<td>749.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>201.1</td>
</tr>
<tr>
<td><em>Lonicera japonica</em></td>
<td>caulìs</td>
<td>5.1</td>
<td>PE</td>
<td>19.5</td>
</tr>
<tr>
<td>(Austria)</td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>43.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>178.1</td>
</tr>
<tr>
<td><em>Lonicera japonica</em></td>
<td>caulìs</td>
<td>5.1</td>
<td>PE</td>
<td>23.9</td>
</tr>
<tr>
<td>(China)</td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>259.2</td>
</tr>
<tr>
<td><em>Lonicera japonica</em></td>
<td>caulìs</td>
<td>5.0</td>
<td>PE</td>
<td>14.3</td>
</tr>
<tr>
<td>(Switzerland)</td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>39.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>409.0</td>
</tr>
<tr>
<td><em>Onosma paniculata</em></td>
<td>root</td>
<td>5.7</td>
<td>PE</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>546.9</td>
</tr>
<tr>
<td><em>Periploca sepium</em></td>
<td>cortex</td>
<td>5.0</td>
<td>PE</td>
<td>179.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>1524.4</td>
</tr>
<tr>
<td><em>Saussurea lappa</em></td>
<td>root</td>
<td>5.0</td>
<td>PE</td>
<td>201.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>1836.3</td>
</tr>
<tr>
<td><em>Zanthoxylum nitidum</em></td>
<td>caulìs</td>
<td>5.1</td>
<td>PE</td>
<td>39.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtoAc</td>
<td>67.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>240.5</td>
</tr>
</tbody>
</table>

Table 8. Extracted plant material and yields of extracts.
6.1.2.3. Soxhlet Extraction

The principle of Soxhlet extraction is a continuous repeating percolation (Adam and Becker, 2000). It needs a cucurbit, a Soxhlet-extractor and a reflux condenser. The dry plant material was powdered and filled in the extraction thimble which was placed in the Soxhlet apparatus. The Soxhlet was twice filled with the adequate solvent until it overran and flowed in the round bottom flask. The reflux condenser was fixed in top of the Soxhlet, turned on and the solvent was heated until boiling. The extraction was stopped when the freshly extracted solution was no longer colored. The solvent was evaporated under reduced pressure at 40 °C. Dry extracts were stored at -20 °C.

Used plant material, solvent and yields:

<table>
<thead>
<tr>
<th>plant</th>
<th>used plant part</th>
<th>initial weight (g)</th>
<th>solvent</th>
<th>yields (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onosma paniculata</td>
<td>root</td>
<td>70.0</td>
<td>PE</td>
<td>1.1</td>
</tr>
<tr>
<td>Saussurea lappa</td>
<td>root</td>
<td>55.0</td>
<td>PE</td>
<td>1.7</td>
</tr>
</tbody>
</table>

6.1.2.4. Maceration

Maceration is the simplest form of extraction without elevated pressure or temperatures (Adam and Becker, 2000). Freshly pulverized plant material was mixed with 250 ml PE and stirred at room temperature for 24 h. The extract was filtered and the remaining plant material again extracted. Finally, both extracts were unified, filtered and evaporated to dryness under reduced pressure at 40 °C. Dry extracts were stored at -20 °C.

Used plant material, solvent and yields:

<table>
<thead>
<tr>
<th>plant</th>
<th>used plant part</th>
<th>initial weight (g)</th>
<th>solvent</th>
<th>yields (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bischofia javanica Graz</td>
<td>leaves and caulis</td>
<td>4.4</td>
<td>MeOH</td>
<td>0.14</td>
</tr>
<tr>
<td>Onosma paniculata</td>
<td>root</td>
<td>28.1</td>
<td>PE</td>
<td>1.7</td>
</tr>
<tr>
<td>Saussurea lappa</td>
<td>root</td>
<td>36.3</td>
<td>PE</td>
<td>1.2</td>
</tr>
</tbody>
</table>

6.1.2.5. Decoction

In traditional Chinese medicine, decoctions are oftentimes the typical form of application. In the case of Onosma paniculata and Lonicera japonica, a decoction was prepared according to the guidelines of the Chinese pharmacopoeia: crude crushed plant material was covered with water and cooked two times for 30 min each. Afterwards, the decoction
was filtered and lyophilized in a Virtis Sentry lyophilizer (The virtis company Gardine, New York, USA).

Used plants and yields:

<table>
<thead>
<tr>
<th>plant</th>
<th>used plant part</th>
<th>initial weight (g)</th>
<th>yields (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lonicera japonica Austria</td>
<td>caulis</td>
<td>1.9</td>
<td>0.14</td>
</tr>
<tr>
<td>Lonicera japonica China</td>
<td>caulis</td>
<td>1.8</td>
<td>0.10</td>
</tr>
<tr>
<td>Lonicera japonica Switzerland</td>
<td>caulis</td>
<td>1.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Onosma paniculata</td>
<td>root</td>
<td>20.0</td>
<td>1.43</td>
</tr>
</tbody>
</table>

6.1.2.6. **PERCOLATION**

Percolation is a continuous extraction in a cylindrical or conical tube performed at room temperature (Adam and Becker, 2000). Dried flowers of *Helianthus angustifolius* were four times extracted with 1l dichloromethane and dried under reduced pressure. The dried extract was stored at 4 °C.

<table>
<thead>
<tr>
<th>plant</th>
<th>used plant part</th>
<th>initial weight (g)</th>
<th>yields (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helianthus angustifolius</em></td>
<td>flower</td>
<td>225</td>
<td>22</td>
</tr>
</tbody>
</table>

6.1.3. **THIN LAYER CHROMATOGRAPHY**

6.1.3.1. **TLC FINGERPRINTS**

For TLC fingerprints, a 5 mg/ml solution of each extract or substance was prepared and 10 µl were applied to six TLC plates (three solvent systems, two spraying reagents). In the case of *Cinnamomum cassia*, *Cocculus trilobus* and *Saussurea lappa* 20 µl were applied. The layout of a TLC plate is shown in figure 107.

**TLC PLATES:** Silica gel 60 F<sub>254</sub>, 20 x 20 cm, Merck, Darmstadt, Germany. Plates were cut into 10 x 10 cm or 20 x 10 cm.
Three different solvent systems were used as mobile phases and freshly prepared before use:

<table>
<thead>
<tr>
<th></th>
<th>solvent system</th>
<th>methyl</th>
<th>ethyl</th>
<th>toluol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>chloroform</td>
<td>64</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>EtOAc</td>
<td></td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>toluol</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>36</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>EtOH</td>
<td>25</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>formic acid</td>
<td>8</td>
<td>formic acid</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>formic acid</td>
<td>8</td>
<td>formic acid</td>
<td>10</td>
</tr>
</tbody>
</table>

Plates were analyzed at vis, 366 nm and 254 nm. Afterwards, one plate was developed with vanillin-H$_2$SO$_4$ (Fluka, Steinheim, Germany), the other with Naturstoff – polyethylene glycol – reagent (Carl Roth GmbH & Co., Karlsruhe, Germany) and photographed at vis or 366nm, respectively.

Spraying reagents (Wagner et al., 1983):

- **Naturstoff-polyethylene glycol-reagent**: Consecutively, a 1% methanolic solution of Naturstoffreagenz A (β-aminodiethylester of diphenylboric acid, NST) and a 5% ethanolic polyethylene glycol 4000 solution (PEG) are sprayed on the plate and analyzed at 366 nm.

- **Vanillin-sulfuric acid (H$_2$SO$_4$)-reagent**: Successively, a 5% ethanolic vanillin solution and a 5% ethanolic sulfuric acid solution were sprayed on the plate. Afterwards, the plate was developed at 110 °C for 5 – 10 min and analyzed at vis.

All TLC plates were photographed with a Reprostar 3 (CAMAG Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Schweiz).

### 6.1.3.2. TLC fingerprint of Cinnamomum cassia

A second TLC fingerprint of the PE extract of *Cinnamomum cassia* was performed:

- **TLC plate**: Silica gel 60 F$_{254}$, 20 x 20 cm, Merck, Darmstadt, Germany.

- **Spraying reagent** (Wagner et al., 1983): Anisaldehyde-sulfuric acid-reagent: Consecutively, 0.5 ml anisaldehyde, 10 ml pure acetic acid, 85 ml MeOH and 5 ml sulfuric acid are mixed. The plate is sprayed, developed at 110 °C for 5 min and analyzed at vis.
• **COMPOUNDS USED:**
  - cinnamaldehyde: Fluka Chemie A.G., Buchs, Switzerland
  - methoxycinnamaldehyde: Sigma-Aldrich® Chemie GmbH, Steinheim, Germany
  - cinnamic acid: Schering-Kahlbaum A.G., Berlin, Germany

All compounds were dissolved in a concentration of 1 mg/ml. 10 µl were applied to the TLC plate. In the case of the PE extract, 15 µl of a 5 mg/ml solution were applied. Solvent system 3 served as mobile phase (see 6.1.3.1.)

### 6.1.4. ISOLATION METHODS

#### 6.1.4.1. Sesquiterpene lactones from *Helianthus angustifolius*

For the isolation of sesquiterpene lactones from *Helianthus angustifolius*, 25 g of silica gel (40 – 62 µm) were coated with 15.5 g of the dichloromethane extract. It was fractionated over 260 g silica gel (40 – 63 µm) using a mobile phase of *n*-hexane, EtOAc and MeOH and open column chromatography. The gradient used started with 100% *n*-hexane. The amount of EtOAc was increased 5% or 10% step wise and 2.5% MeOH was added to the mixture, when *n*-hexane/EtOAc ratio was 1:1. The MeOH portion was increased to 10% - 20% - 30% - 80% and 100%, while the *n*-hexane/EtOAc ratio remained the same. Each eluent mixture was 100 or 200 ml. Fractions of 25 ml were collected and compared by TLC fingerprint. Similar fractions were unified. Finally, 14 fractions (F1-14) were yielded (table 9).

<table>
<thead>
<tr>
<th>fraction</th>
<th>initial weight (g)</th>
<th>yield (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>15.5</td>
<td>0.25</td>
</tr>
<tr>
<td>F2</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>F3</td>
<td></td>
<td>1.79</td>
</tr>
<tr>
<td>F4</td>
<td></td>
<td>4.19</td>
</tr>
<tr>
<td>F5</td>
<td></td>
<td>2.32</td>
</tr>
<tr>
<td>F6</td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>F7</td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>F8</td>
<td></td>
<td>1.09</td>
</tr>
<tr>
<td>F9</td>
<td></td>
<td>0.27</td>
</tr>
<tr>
<td>F10</td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td>F11</td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>F12</td>
<td></td>
<td>1.39</td>
</tr>
<tr>
<td>F13</td>
<td></td>
<td>3.69</td>
</tr>
<tr>
<td>F14</td>
<td></td>
<td>0.23</td>
</tr>
</tbody>
</table>

*Table 9. Yields of fractions F1-14*
Fractionation of F12

For further investigations, fraction 12 was subjected to solid phase extraction (SPE). 810 mg were dissolved in MeOH and applied to solid-phase extraction (SPE) on cartridges (10 g of RP-18, 10 µm, International Sorbent Technology, Mid Glamorgan, UK). Collected fractions were unified according to their HPLC fingerprint and activity. One fraction (F12-1) was obtained by elution with 240 ml MeOH/H$_2$O (60:40) and a second (F12-2) by washing the column with 100% MeOH, acetone and dichloromethane, 30 ml each. Fraction F12-1 was finally fractionated by semi-preparative HPLC. It was dissolved in MeOH (40 mg/ml) and centrifuged for 10 min. This fractionation yielded four pure compounds (F12-1-H8, -H9, -H11 and -H12) whose structures have been identified by 1D and 2D NMR experiments. NMR data see table 1, page 74.

**SEMIPREPARATIVE HPLC OF F12-1:**

**INSTRUMENT:** L-6200A Intelligent Pump from Merck (Darmstadt, Germany)
L-4500 Diode Array Detector from Merck (Darmstadt, Germany)

**STATIONARY PHASE:** LiChroCART column (RP-18, 10 µm, 250 x 10 mm)

**MOBILE PHASE:** ACN / H$_2$O

**GRADIENT:**
0 – 45 min: 43% ACN
45 – 50 min: 43 - 100% ACN
50 – 60 min: 100% ACN

**FLOW RATE:** 2 ml/min

**DETECTION:** absorbance at 220 nm

**INJECTION VOLUME:** 100 - 200 µl

<table>
<thead>
<tr>
<th>fraction</th>
<th>initial weight (mg)</th>
<th>yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F12-1</td>
<td>810</td>
<td>206.4</td>
</tr>
<tr>
<td>F12-2</td>
<td></td>
<td>504.9</td>
</tr>
<tr>
<td>F12-1-H1</td>
<td></td>
<td>0.89</td>
</tr>
<tr>
<td>F12-1-H2</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>F12-1-H3</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>F12-1-H4</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>F12-1-H5</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>F12-1-H6</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>F12-1-H7</td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>F12-1-H8</td>
<td></td>
<td>2.08</td>
</tr>
<tr>
<td>F12-1-H9</td>
<td></td>
<td>14.38</td>
</tr>
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<td>F12-1-H9-2</td>
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<td>0.001</td>
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<td>50.57</td>
</tr>
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<td>F12-1-H11</td>
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<td>4.65</td>
</tr>
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<td>F12-1-H12</td>
<td></td>
<td>5.79</td>
</tr>
<tr>
<td>F12-1-H13</td>
<td></td>
<td>1.62</td>
</tr>
<tr>
<td>F12-1-H14</td>
<td></td>
<td>2.83</td>
</tr>
<tr>
<td>F12-1-H15</td>
<td></td>
<td>3.91</td>
</tr>
</tbody>
</table>

Table 10. Yields of fractions of F12 from Helianthus angustifolius.
**IDENTIFICATION OF ISOLATED COMPOUNDS:**

**8-METHACRYLYL-4,15-ISO-ATRIPICOLIDE** (F12-1-H8): light yellowish oil; \([\alpha]_D^{20} = -83.2\) (c 0.002, MeOH); UV (MeOH) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) = 211 (4.13), 280 (3.76); 1H and 13C NMR data, see table 1 (page 74); HR-MS TOF ES+: m/z = 345.1361 [M + H]+ (calcd. for \(\text{C}_{19}\text{H}_{20}\text{O}_{6}\) + H: 345.1332).

**8-ISOBUTYRYL-4,15-ISO-ATRIPICOLIDE** (F12-1-H9): light yellowish oil; \([\alpha]_D^{20} = -89.9\) (c 0.01, MeOH); UV (MeOH) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) = 211 (4.89), 278 (4.66); 1H and 13C NMR data, see table 1 (page 74); HR-MS TOF ES+: m/z = 347.1436 [M + H]+ (calcd. for \(\text{C}_{19}\text{H}_{20}\text{O}_{6}\) + H: 347.1489).

**8-(2-METHYL-BUTYRYL)-4,15-ISO-ATRIPICOLIDE** (F12-1-H11): slightly yellow wax; \([\alpha]_D^{20} = +23.0\) (c 0.005, MeOH); UV (MeOH) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) = 211 (3.84), 274 (3.59); 1H and 13C NMR data, see table 1 (page 74); HR-MS TOF ES+: m/z = 361.1590 [M + H]+ (calcd. for \(\text{C}_{19}\text{H}_{22}\text{O}_{6}\) + H: 361.1646).

**8-ISOVALERYL-4,15-ISO-ATRIPICOLIDE** (F12-1-H12): slightly yellow wax; \([\alpha]_D^{20} = -16.0\) (c 0.001, MeOH); UV (MeOH) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) = 211 (3.88), 275 (3.60); 1H and 13C NMR data, see table 1 (page 74); HR-MS TOF ES+: m/z = 361.1647 [M + H]+ (calcd. for \(\text{C}_{19}\text{H}_{24}\text{O}_{6}\) + H: 361.1646).

**Fractionation of F 11 and 13**

Fraction 11 and 13 were further fractionated using Fast Centrifugal Partitioning Chromatography (FCPC). This technique is based on the distribution constant \(K\) of a substance between two immiscible phases. The big advantage of this method is that no compounds remain on the “column” (Friesen and Paul, 2005). Friesen and Pauli (2005) developed the HEMWat and ChMWat systems which are useful in many cases. These systems were tried in a preliminary test and the solvent combination with the best TLC results was finally used for FCPC.
**Instrument:**
FCPC-Kromaton-201, AlphaChrom OHG
ProStar 325 UV/VIS Detector, Varian
ProStar 210/215, PreStar 218 Solvent Delivery Module
(0.025 – 25 ml/min), Varian
ProStar 701/701A X-Y Fraction Collector, Varian

**Rotor:**
200 ml capacity, 1000 rpm

**Injection Loop:**
10 ml

**Solvent Systems:**
HEMWat -3: n-hexane/EtOAc/MeOH/H$_2$O (6:4:6:4) for F11
HEMWat +6: n-hexane/EtOAc/MeOH/H$_2$O (2:8:2:8) for F13

**Mode:**
ascending

**Sample Preparation:**

**F11:** 600 mg were dissolved in 7 ml mobile phase.

**F13:** 1.7 g was dissolved in 10 ml mobile and stationary phase.
Both were centrifuged for 20 min (2500 x g, Eppendorf Centrifuge 5810R, Hamburg, Germany) and the supernatant subjected to FCPC.

After 110 fractions (F11) and 121 fractions (F13), mobile phases were replaced by the stationary phases. In total, 192 fractions (3.75 ml) in the case of F11 and 199 fractions (3.75 ml) in the case of F13 were collected. All were analyzed by TLC (n-hexane/ EtOAc according to the HEMWat system) and similar fractions unified. Finally, from F11 twelve fractions (F11-1 to 12) and from F13 eight fractions (F13-1 to 8) were obtained (table 11).

<table>
<thead>
<tr>
<th>F11</th>
<th>F13</th>
</tr>
</thead>
<tbody>
<tr>
<td>fraction</td>
<td>initial weight (mg)</td>
</tr>
<tr>
<td>F11-1</td>
<td>0.62</td>
</tr>
<tr>
<td>F11-2</td>
<td>77.95</td>
</tr>
<tr>
<td>F11-3</td>
<td>36.92</td>
</tr>
<tr>
<td>F11-4</td>
<td>18.68</td>
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<td>F11-5</td>
<td>5.65</td>
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<td>F11-6</td>
<td>13.34</td>
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<tr>
<td>F11-7</td>
<td>8.01</td>
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<tr>
<td>F11-8</td>
<td>22.96</td>
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<td>F11-9</td>
<td>16.76</td>
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<td>F11-10</td>
<td>25.02</td>
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<td>F11-11</td>
<td>34.24</td>
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<td>F11-12</td>
<td>23.22</td>
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<tr>
<td>F13-8</td>
<td>0.59</td>
</tr>
<tr>
<td>F13-3</td>
<td>1700</td>
</tr>
</tbody>
</table>

**Table 11. Yields after fractionating F11 and F13 using FCPC**
The naphthoquinone derivatives investigated were isolated from *Onosma paniculata* using preparative HPLC. The active PE extract was dissolved in MeOH (40 mg/ml), 10 min centrifuged (2500 x g, Eppendorf Centrifuge 5810R, Hamburg, Germany) and the supernatant fractionated using the following instruments and parameters:

**INSTRUMENT:** Varian R PrepStar Model SD-1 (pump 1)
Dynamax Model SD-1 (pump 2)
Dynamax Absorbance detector Model UV-1

**STATIONARY PHASE:** VDSpher 100 C18 (250 x 25 mm, 10 µm)
(VDS Optilab Chromatographie Technik GmbH, Berlin, Germany)

**MOBILE PHASE:** ACN / H₂O

**GRADIENT:**
0 – 45 min: 70 – 100% ACN
45 – 60 min: 100% ACN

**FLOW RATE:** 20 ml/min

**DETECTION:** absorbance at 500 nm

**INJECTION VOLUME:** 800 – 900 µl

**IDENTIFICATION OF ISOLATED COMPOUNDS:**

**β-HYDROXYISOVALERYLSHIKONIN** (fraction 2): red powder; molecular formula: C_{21}H_{24}O_{7}; $\lambda_{max}$ = 215, 273, 503; 1H and 13C NMR data see table 2, page 92.

**ACETYLSHIKONIN** (fraction 4): red powder; molecular formula: C_{18}H_{18}O_{6}; $\lambda_{max}$ = 215, 273, 503; 1H and 13C NMR data see table 2, page 92.

**DIMETHYLACRYLSHIKONIN** (fraction 6): red powder; molecular formula: C_{21}H_{22}O_{6}; $\lambda_{max}$ = 215, 273, 503; 1H NMR data see table 3, page 93.

**EPOXYSHIKONIN** (fraction 7): red powder; molecular formula: C_{20}H_{20}O_{7}; $\lambda_{max}$ = 215, 273, 503; 1H NMR data see table 4, page 93.
**6.1.4.3. SESQUITERPENE LACTONES FROM SAUSSUREA LAPPA**

Costunolide and dehydrocostus lactone were isolated from *Saussurea lappa* using preparative HPLC. The active PE extract was dissolved in MeOH (40 mg/ml), 10 min centrifuged (2500 x g, Eppendorf Centrifuge 5810R, Hamburg, Germany) and the supernatant subjected to preparative HPLC:

**INSTRUMENT:** Varian R PrepStar Model SD-1 (pump 1)
Dynamax Model SD-1 (pump 2)
Dynamax Absorbance detector Model UV-1

**STATIONARY PHASE:** VDSpher 100 C18 (250 x 25 mm, 10 µm)
(VDS Optilab Chromatographie Technik GmbH, Berlin, Germany)

**MOBILE PHASE:** ACN / H$_2$O

**GRADIENT:**
- 0 – 10 min: 84% ACN
- 10 – 15 min: 84 - 100% ACN
- 15 – 20 min: 100% ACN

**FLOW RATE:** 20 ml/min

**DETECTION:** absorbance at 220 nm

**INJECTION VOLUME:** 500 – 1000 µl

**IDENTIFICATION OF ISOLATED COMPOUNDS:**

**COSTUNOLIDE** (fraction 1): white powder; molecular formula: C$_{15}$H$_{20}$O$_{2}$; $\lambda_{\text{max}}$ = 216; 1H and 13C NMR data see table 5, page 120.

**DEHYDROCOSTUS LACTONE** (fraction 2-1): white powder; molecular formula: C$_{15}$H$_{18}$O$_{2}$; $\lambda_{\text{max}}$ = 216; 1H and 13C NMR data see table 6, page 120.
6.1.5. SPECTROSCOPIC METHODS

6.1.5.1. Nuclear Magnetic Resonance Spectroscopy (NMR)

**INSTRUMENTS:**
- Varian UnityInova 600 MHz Spectrometer (599.84 MHz for $^1$H and 150.84 MHz for $^{13}$C)
- Varian UnityInova 400 MHz Spectrometer (399.98 MHz for $^1$H and 100.58 MHz for $^{13}$C)

**SOLVENTS:**
- chloroform-$d_1$ $\delta^1$H=7.27 $\delta^{13}$C=77.16 (Eurisotop)
- methanol-$d_4$ $\delta^1$H=3.31 $\delta^{13}$C=49.00 (Cambridge Isotope Laboratories)
- pyridine-$d_5$ $\delta^1$H=7.19, 7.55, 8.71 $\delta^{13}$C=123.5, 135.5, 149.9 (Sigma-Aldrich)

1D $^1$H- and $^{13}$C-NMR as well as 2D HSQC, HMBC and/or DQF-COSY spectra were measured. All measurements were recorded at 298 K using TMS as internal standard.

6.1.5.2. CD Measurements

CD measurements of shikonin derivatives were performed with a Jasco J-715 Spectropolarimeter. Samples were dissolved in chloroform (5 mM).

6.1.5.3. High Resolution Mass

High resolution mass was measured on a Synapt QTOF-HD-MS mass spectrometer (capillary voltage: 2.4 kV, source temp.: 100 °C, mass range: m/z 50-1000) (Waters, Manchester, UK).

6.1.5.4. Optical Rotation and UV Spectra

Optical rotations were measured on a Jasco P-2000 Polarimeter (Gross-Umstadt, Germany). UV spectra were recorded on a Specord 50 (190 – 700nm) (Analytik Jena, Jena, Germany). Samples were dissolved in MeOH.
6.2. Pharmacological investigations

6.2.1. Cell culture

Human CCRF-CEM and THP-1 leukemia and human MDA-MB-231 breast cancer cells were kept in RPMI 1640 medium (Gibco®, Invitrogen, Darmstadt, Germany) supplemented with 2 mM L-glutamine (Sigma-Aldrich®, MO, USA), 10% heat-inactivated fetal bovine serum (FBS) (PAA laboratories, Pasching, Austria), 100 units/ml penicillin and 100 µg/ml streptomycin (1% Pen/Strep) (PAA laboratories).

Human U251 glioblastoma, human HCT 116 colorectal carcinoma and human Hela cervix carcinoma cells were grown in high glucose Dulbecco’s modified Eagle medium (DMEM, Gibco®, Invitrogen), 4 mM L-glutamine, 10% FBS and 1% Pen/Strep.

Human HT-29 colon adenocarcinoma cells were grown in Ham’s F12 medium (Gibco®, Invitrogen) supplemented with 2 mM L-glutamine, 10% FBS and 1% Pen/Strep.

Human 769-P kidney carcinoma cells were cultured in RPMI 1640 medium, 10% FBS, 2 mM L-glutamine, 1% Pen/Strep and 1 mM sodiumpyruvate.

Human melanoma cells from primary (SBC-L2 and WM35) and metastatic (WM9 and WM164) lesions were kept in RPMI 1640 medium, 2% FBS, 2 mM L-glutamine and 1% Pen/Strep.

Human MRC-5 lung fibroblast cells were cultured in Minimum Essential Medium (MEM, Gibco®, Invitrogen) supplemented with 4 mM L-glutamine, 10% FBS and 1% Pen/Strep.

Human SW-872 liposarcoma, SW-982 synovial sarcoma and TE-671 rhabdomyosarcoma cells were grown in Dulbecco’s modified Eagle’s medium: Nutrient mixture F-12 (DMEM-F12, Gibco®, Invitrogen) supplemented with 5% FBS, 2 mM L-glutamine, 1% Pen/Strep and 0.25 µg Amphotericin B (PAA laboratories).

CCRF-CEM, MDA-MB-231, U251 and HCT 116 cells were a kind gift of Prof. Dr. Thomas Efferth (Cancer Research Center Germany, Heidelberg). THP-1, Hela, HT-29, 769-P and MRC-5 cells were provided by Dr. Beate Rinner (Center of Medical Research, Core Facility Flow Cytometry, Medical University of Graz, Austria). SBC-L2, WM9, WM35 and WM164 were given by Prof. Dr. Helmut Schaider (Cancer Biology Unit, Department of Dermatology and Centre for Medical Research, Medical University of Graz, Austria). SW-872, SW-982 and TE-671 cells were obtained from CLS cell line service (Eppelheim, Germany) and provided by Univ.-Ass. Dr. Birgit Lohberger (Department of Orthopaedic Surgery, Medical University of Graz, Austria).
Cells were kept in a humidified 5% CO$_2$ atmosphere at 37 °C in a Heraeus HERAcell 240 breeder (Thermo Fisher Scientific Inc., Vienna, Austria) or comparable. They were passaged at 90% confluence and medium replaced every 2-3 days if cells were not passaged. Adherent cells were harvested and trypsinated with 0.25% trypsin-EDTA-solution (Sigma-Aldrich®). The bench was a HERAsafe KS, Thermo Fisher Scientific Inc., Vienna, Austria or comparable.

**6.2.2. Determination of cell number**

Cell number was determined either with a Neubauer hemocytometer or a CASY® cell counter (Innovatis, Reutlingen, Germany). In the case of Neubauer hemocytometer, cells were 1:1 mixed with trypan blue and about 10 µl of this suspension was pipetted in the chamber. Only non-stained cells were taken for analysis. Cells were counted at hundredfold magnification with an Olympus CKX41 microscope (Hamburg, Germany) and four squares were analyzed. For cell counting using the CASY® counter, 25 µl or 50 µl of a cell suspension was mixed with 10ml CASY® ton solution and immediately analyzed with the respective setup. Only cells with a viability of more than 90% were used for assays.

**6.2.3. Viability assays**

**6.2.3.1. XTT viability assay**

The XTT viability assay is used to determine cellular proliferation and viability. It was first described in 1988 by Scudiero et al. and developed to improve already existing tetrazolium assays, especially the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Cell proliferation kit II (XTT) was obtained from Roche Diagnostics (Mannheim, Germany), Cat. No. 11 465 015 001. Aliquots (100 µl) of 5 x 10$^4$ cells/ml of adherent cell lines were seeded in 96-well microplates (flat bottom) and grown over night in a humidified 37 °C and 5% CO$_2$ atmosphere before extracts, fractions or pure compounds were added. This time allowed the cells to settle and attach. Aliquots (100 µl) of 1 x 10$^5$ cells/ml of suspension cells were treated immediately after seeding. To reduce evaporation effects and to measure the background absorbance of non-metabolized XTT solution, marginal wells were filled with medium only. Each sample was tested in two or
three independent wells per plate and minimum at two different cell passages. Since all test samples were dissolved in DMSO (dimethyl sulfoxide), control cells correspond to vehicle treated cells (0.5% DMSO final concentration). DMSO alone had no effect on cell growth and proliferation at that concentration. After adequate incubation time, 50 µl XTT solution was added. This solution consisted of a XTT labeling reagent and an electron-coupling reagent. Both were freshly mixed together in a ratio of 50:1 before adding to the wells. XTT is a yellow tetrazolium salt (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate) which is cleaved by mitochondrial dehydrogenases to form an orange formazan dye (figure 108). Since this conversion occurs only in metabolically active cells and the orange dye build is water soluble, the color change can be directly measured by a scanning multiwell spectrophotometer (Victor² 1420 Multilabel Counter, Wallac, PerkinElmer Life Sciences, Massachusetts, USA). Absorbance of each well was measured at 490 nm with a reference wavelength at 650 nm after 1.5 h (adherent cells) or 4 h (suspension cells) incubation with XTT at 37 °C and 5% CO₂. Viable cells were expressed as percentage of control and calculated with the following formula: (absorbance of treated cells/absorbance of untreated cells) × 100. Vinblastine (Vinblastine sulfate cryst.) served as positive control and was obtained from Sigma-Aldrich®.

Figure 108. Metabolization of XTT to a formazan salt.

6.2.3.2. ViaLight® ASSAY

The ViaLight® Plus Kit was obtained from Lonza (Lonza Rockland Inc., Rockland, USA). This assay measures cellular ATP levels using bioluminescence. ATP is indispensable in living cells to maintain viability and growth. In this assay, cellular ATP is used for the conversion of luciferin to oxyluciferin:

\[
\text{ATP + luciferin + O}_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin + AMP + P}_i + \text{CO}_2 + \text{LIGHT}
\]

The emitted light directly correlates with the ATP concentration and was measured with a LUMIstar luminometer (BMG Labtech., Offenburg, Germany).
Aliquots (100 µl) of 5 x 10^4 cells/ml were seeded in luminescence compatible 96 well microplates (Brand, Voerde-Friedrichsfeld, Germany) and grown over night before costunolide or dehydrocostus lactone was added. Cells were treated with 0 µg/ml, 2.5 µg/ml, 5 µg/ml and 7.5 µg/ml costunolide or 0 µg/ml, 0.5 µg/ml, 1 µg/ml, 1.5 Mg/ml, 2.0 µg/ml, 2.5 µg/ml, 5 µg/ml and 7.5 µg/ml dehydrocostus lactone for 24 h, 48 h and 72 h. Afterwards, the plates were removed from the incubator and cooled to room temperature. A one second integrated reading of each well was taken by the luminometer before 50 µl of cell lyses reagent was added to each well. After 10 min, 100 µl of AMR PLUS was added, incubated for 2 min at room temperature and plates analyzed with the luminometer. Results are represented as relative luminescence of control cells (100%).

**6.2.4. GROWTH INHIBITION ASSAY**

Aliquots (2ml) of 5 x 10^3 cells/ml in case of adherent cell lines were seeded in 6 well plates and grown over night before testing samples were added. Aliquots (1ml) of 5 x 10^4 cells/ml in case of suspension cells were pipetted in 24-well plates and immediately treated with the substances of interest. After 7 days of treatment, cells were harvested and counted. All experiments were done in triplicates. Vehicle treated control cells were incubated with 0.5% DMSO. Results are shown as vital cells (%).

**6.2.5. CELL MORPHOLOGY**

Cells at a density of 5 x 10^5 cells/well were seeded in 12 well plates and grown over night to settle and attach at the bottom completely. Subsequently, testing samples were added. Cells were kept at 37 °C and 5% CO₂ for the adequate time period. Afterwards, cells were gently rinsed with Dulbecco´s phosphate buffered saline (PBS, Gibco®, Invitrogen, Darmstadt, Germany) and photographed under a phase microscope (Olympus IX51, Japan).

**6.2.6. SUBG1 PEAK, CELL CYCLE AND PI STAINING**

The cell cycle is a sequence of events a cell passes between one cell division and the next. It is divided into four stages: G1/G0-, S-, G2- and M-phase. Since G1/G0-, S- and G2/M-phase differ in DNA content, they can be distinguished. In cells undergoing
apoptosis, nucleases are activated and cleave DNA in 180bp. These cells have a lower DNA content and staining with a DNA binding dye (propidium iodide) will reveal these cells in the so called sub G1 region (figure 109).

Adherent cells were grown over night before the substance of interest was added. Afterwards, cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere for the adequate time period. The final analysis was performed with a FACSCalibur flow cytometer (BD Biosiences, San Jose, USA) and the ModFit software.

Figure 109. Main phases of cell cycle (grey cycle) and an example of cell cycle distribution including a sub G1 peak (apoptosis).

**SubG1 peak and cell cycle:** Cells were harvested by trypsination in 10 ml media and centrifuged for 4 min at 4 °C and 200 xg. The pellet was resuspended with 5 ml PBS, centrifuged and resuspended with 5 ml fresh PBS. Cell number was then determined with a CASY® counter. 5 x 10⁵ cells/ml were again centrifuged and resuspended with 500 µl PBS. After adding 5 ml of 70% ice cold ethanol, cells were fixed for 10 min at 4 °C. For flow cytometric analysis, cells were again centrifuged (300 xg), two times washed with 5 ml PBS and, finally, resuspended with 250 µl PI-staining buffer (Beckman Coulter, Krefeld Germany) followed by incubation for 15 min at 37 °C.

**PI staining/membrane damage:** This method can also be used to examine if cell membrane was damaged by the substances. Cells were harvested and two times washed with PBS as described above. Afterwards, they were not fixed with ethanol, but stained immediately with the PI-staining buffer and analyzed by flow cytometry. In the case of membrane injury, PI can permeate into the cells. If no membrane damage
EXPERIMENTAL PART

occurred, cells are not stained and, therefore, can be distinguished. PI positive cells of untreated control cells were subtracted from PI positive cells of treated cells.

6.2.7. DAPI STAINING

DAPI (4′-6-diamidino-2-phenylindole) is a dye that forms fluorescent complexes with double-stranded DNA. Therefore, apoptotic cells can be distinguished from non-apoptotic cells by microscopic morphology/fluorescence analysis. 2 x 10⁵ cells were treated with the respective substance (costunolide and dehydrocostus lactone) for 24 h, 48 h and 72 h. Cells were harvested by trypsination, washed with PBS and centrifuged for 5 min at 228 x g. The cell pellet was resuspended in DAPI:MeOH 1:50 and incubated for 15 min at 37 °C in the dark. Untreated cells served as control. Finally, cells were analyzed under a fluorescence microscope.

6.2.8. ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL

5 x 10⁵ cells were harvested by trypsination, washed with PBS and incubated for 15 min at 37°C and dark with 1 µg/ml JC-1 (5,5′,6,6′-tetachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) fluorescent probe (MitoScreen Kit, BD Biosiences). JC-1 is a lipophilic fluorochrome used to analyze the mitochondrial membrane potential. In vital cells, it is uptaken in the mitochondria as monomers. This uptake is driven by the mitochondrial membrane potential. With increasing inner-mitochondrial JC-1 concentration, JC-1 starts to aggregate which results in a fluorescent shift from green to red. In cells with a disrupted mitochondrial membrane potential, JC-1 remains in the cytoplasm and in its monomeric form. Therefore, the ratio of red:green fluorescence intensity allows conclusions about the state of the mitochondrial membrane potential. Harvested and incubated cells were washed with PBS and analyzed by flow cytometry. As positive control, the mitochondrial membrane potential disrupter CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was used. Photomultiplier settings were arranged to detect JC-1 green fluorescence at 530 nm on FL1 and JC-1 red fluorescence at 590 nm on FL2. 20,000 events were analyzed. Finally, red:green ratio (aggregate:monomer) was calculated.
Caspases are proteases which play a central role in the regulation of apoptosis. Caspase-3 is activated by the extrinsic apoptotic pathway as well as the intrinsic and is, therefore, an important marker for the beginning of the apoptotic signaling pathway. Cells were incubated with the respective IC$_{50}$ and/or double IC$_{50}$ for different time periods. After trypsination, cells were centrifuged (300 xg, 4 min, 4 °C) and resuspended with 10 ml PBS. After being centrifuged again, 1 $\times$ 10$^5$ cells were resuspended in 0.5 ml PBS and 0.5 ml 4% formaldehyde. Cells were fixed for 10 min at 37 °C and stored at 4 °C. For permeabilization, 9 ml cold methanol was added and incubated for 30 min at 4 °C. For final staining, cells were again centrifuged (400 xg, 4 min, room temperature), the pellet resuspended in 2-3 ml incubation buffer and again centrifuged. Afterwards, the pellet was dissolved in 90 µl incubation buffer (100 ml PBS + 500 µl FBS) and incubated for 10 min at room temperature. 7 µl antibody solution was added and incubated for 30 – 60 min at room temperature in the dark. Before analyzing cells with the flow cytometer (FACSCalibur flow cytometer, BD Biosciences, San Jose, USA), 2-3 ml incubation buffer was added, centrifuged and the pellet resuspended with 300 µl PBS. Untreated cells were used as negative control. Analysis was performed with FACSDiva software (BD Biosciences); histograms were created with FCS express.

**ANTIBODIES:** FITC-conjugated monoclonal active caspase-3 antibody, Antibody-Alexa Fluor 488 conjugate (#9669, Cell Signaling Technology, Danvers, MA)

**USED FOR BLOCKING THE CASPASE ACTIVATION:** BD™ ApoBlock (BD Biosciences).

### 6.2.10. CASPASE-GLO® 3/7 ASSAY

To measure caspase-3 activity, the luminescent Caspase-Glo® 3/7 Assay (Promega, Mannheim, Germany) was used according to the manufacturer’s protocol. 10,000 cells/well (100 µl) were seeded into white-walled 96 well plates and treated with dimethylacylshikonin for 3, 6, 12, 24 and 48 h. For analysis, Caspase-Glo® 3/7 Reagent was added to each well resulting in cell lysis and the following reaction:
Thereby, luminescence directly correlates with the caspase activity and was measured 30 min after adding the Caspase-Glo® 3/7 Reagent. This reagent consisted of the Caspase-Glo® Substrate and Caspase-Glo® Buffer. Background luminescence was measured in wells containing Caspase-Glo® 3/7 Reagent, vehicle solution and cell culture medium. These values were subtracted from experimental values. Vehicle treated cells served as control.

### 6.2.11. ANALYSIS OF GENE EXPRESSION USING REAL-TIME PCR

Real-time PCR is a method to amplify and quantify targeted DNA. In contrast to standard PCR, the amplified DNA is not only detected at the end of the reaction, but in real time. It allows the quantification of the product by measuring the accumulation of the PCR product. More important than the amount of target DNA after a certain number of PCR cycles is the moment when the DNA product can be detected for the first time. The higher the starting copy number of target DNA is, the sooner an exponential increase of the fluorescence is observed and vice versa (Applied Biosystems, 2010).

### 6.2.11.1. RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was extracted using the Trizol® method (Invitrogen, Darmstadt, Germany) or the RNeasy Mini Kit (Qiagen, Hilden, Germany). Trizol® Reagent is a monophasic solution of phenol and guanidine isothiocyanate and provides a single-step RNA isolation method. It maintains the integrity of the RNA and lyses cells and cellular components. The RNeasy Mini Kit uses columns with silica membranes. Lyzed and homogenized samples diluted with EtOH are loaded on these columns. While RNA binds to them, all other compounds are washed away. Finally, RNA is gained by elution with water.

For subsequent cDNA synthesis, extracted RNA was treated with DNase I (10 units/µl, Roche Diagnostics, Mannheim, Germany) in the presence of RNase Inhibitor (40 units/µl, Roche Diagnostics, Mannheim, Germany) and 25 mM MgCl₂ for one hour at 37 °C. Some samples were tested for DNA contamination with a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Afterwards, total RNA was reversed transcribed into cDNA using 0.5 µg random hexamer primers and 200 U of reverse RevertAid™ H Minus M-MuLV RT (Fermentas, St. Leon-Rot, Germany) or the RevertAid cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Reversed transcription was performed according to the manufacturer’s protocol.
6.2.11.2. **Real-time PCR**

Real-time PCR was performed using an ABI Prism 7000 Detection system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Total reaction volume per well was 25 µl, containing 1 x SYBR® Green PCR Master Mix (Invitrogen, Darmstadt, Germany), 3 µl cDNA and forward and reverse primer, each at a concentration of 1 mM. The cycling protocol was as follows: one cycle at 50 °C for 2 min and 95 °C for 10 min followed by 50 cycles consisting of denaturation for 15 s at 95 °C, annealing of primers and elongation for 1 min at 60 °C. The specificity of all assays was already validated using genomic DNA and cDNA of appropriate positive and negative controls. GAPDH, β-actin, PPIA and/or hypoxanthine phosphoribosyltransferase (hrpt-n) served as housekeeping genes. All samples with a hrpt C_T value lower than 32 were included in the chemokine receptor expression profiling study. Peripheral mononuclear cells were isolated from blood of healthy donors and served as calibrator.

For quantification of gene transcription, the comparative threshold (C_T) was used. The C_T of the housekeeping gene was subtracted from the C_T of the target gene. This difference displayed the ΔC_T value. The ΔC_T of the calibrator was subtracted from the ΔC_T of the sample which is called ΔΔC_T. Results are expressed as relative units based on 2^ΔΔCT which gave the relative amount of target gene normalized to the endogenous control (geometric mean of all four housekeeping genes) and relative to the calibrator.

6.2.12. **Human MMP Fluorokine® MAP**

Soft tissue sarcoma cells were incubated the respective IC_{50} of costunolide or dehydrocostus lactone for 24 h, 48 h and 72 h. The supernatant was 5-fold diluted with the Calibrator Diluent. MMPs were analyzed using the MMP Fluorokine® MAP assay (R&D Systems, Wiesbaden, Germany) which was performed according to the manufacturer’s instruction in duplicates (R&D Systems, 2010). Analyte-specific antibodies are pre-coated onto color-coded microparticles. 50 µl diluted microparticles mixture and 50 µl standard or samples were added to each well of a pre-wet microplate. Plates were incubated at room temperature for 2 h using a microplate shaker (500 rpm). During this step, specific immobilized antibodies bind to MMP-1, MMP-2, MMP-3 or MMP-9. Afterwards, unbound substances were removed with Wash Buffer (3 x 100 µl) and 50 µl Biotin Antibody Cocktail was added. After 1 h of incubation, a further wash step removed unbound substances. Then, 50 µl diluted streptavidin-phycoerythrin conjugate (Streptavidin-PE) was added to the wells. This binds to biotinylated detection antibodies
and generates a signal. Microparticles were resuspended in 100 µl Wash Buffer and detected within 90 min by a Luminex analyzer (BioRad BioPlex™ dual laser).

### 6.2.13. Statistical Analysis

For IC\textsubscript{50} determination, the XTT viability or ViaLight™ assay was used as described above. At least four independent wells, two different cell passages and seven concentrations plus control cells (vehicle treated) were used. IC\textsubscript{50} values were calculated with **SigmaPlot 11.0** (Systat Software Inc., San Jose, CA, USA) and the four parameter logistic curve:

\[
y = \text{min} + \frac{(\text{max} - \text{min})}{1 + \left(\frac{x}{EC50}\right)^{-\text{Hillslope}}}
\]

Significant differences between control and treated cells were examined using Student’s unpaired t-test. Graphic data were drawn using SigmaPlot 11.0.

### 6.3. Collaborations

- Genome analysis for plant authentication was performed in collaboration with the Ludwig-Maximilian University of Munich (Prof. Dr. Günther Heubl) and the University of Veterinary Medicine of Vienna (Dr. Johannes Novak).
- Flow cytometric, real-time PCR and luminometric experiments were carried out at the Medical University of Graz, Austria (Dr. Beate Rinner, Dr. Alexander Deutsch).
- Cancer cell lines were provided by the German Cancer Research Center, Heidelberg, Germany (Prof. Dr. Thomas Efferth) and the Medical University of Graz, Graz, Austria (Dr. Beate Rinner, Univ.-Ass. Dr. Birgit Lohberger, Prof. Dr. Helmut Schaider).
- Plant material was obtained by Dr. Stefan Kahl in 2002 and 2003 (Kahl, 2005) in collaboration with the Joanneum Research Forschungsgesellschaft mbH, Graz, Austria, and by Dr. Wolfgang Schuehly, Karl-Franzens University, Graz, Austria.
- Shikonin derivatives were provided by Prof. Dr. José L. Rios, University of Valencia, Valencia, Spain.
- Alkannin derivatives were provided by Prof. Dr. Ioanna Chinou, University of Athens, Athens, Greece.
7. Literature


"Involvement of ROS in chlorogenic acid-induced apoptosis of Bcr-Abl(+) CML cells." Biochem. Pharmacol. 80(11): 1662 - 1675.


suppressing IL-17 production and inhibits differentiation of Th17 cells." Acta Pharmacol Sin. 30(8): 1144 - 1152.


CURRICULUM VITAE

Nadine Kretschmer, Dipl.-Biol.

PERSONAL DATA
Date of birth: 2nd June 1982
Place of birth: Ingolstadt, Germany
Nationality: German
Family status: Single
Language abilities: German (mother language), English

CAREER HISTORY
2009-present  Scientific project collaborator (FWF), Institute of Pharmaceutical Sciences, Dept. Pharmacognosy, University of Graz, Austria

2007-2008  Research Assistant, Institute of Pharmaceutical Sciences, Dept. Pharmacognosy, University of Graz, Austria

2007-2011  PhD thesis (Pharmacognosy), University of Graz, Austria
Topic of the thesis: “Phytochemical and pharmacological investigations on constituents of medicinal plants with potential anti-cancer activity” under the supervision of Prof. Dr. Rudolf Bauer

2001-2006  Studies of Biology, Technical University of Munich, Germany
Main subject: botany with emphasis on molecular biology, minor subjects: human biology, pharmacology and toxicology, limnology.
Diploma thesis: “Influence of Veratrum alkaloids on the catecholamine release at the ductus deferens and the adrenal medulla of the rat” under the supervision of Prof. Dr. Wolfgang Vierling
Overall assessment: passed with high distinction.

EDUCATION
1992-2001  Gnadenhal Gymnasium (Secondary School), Ingolstadt, Germany
1988-1992  Primary School, Großmehring, Germany

MAIN RESEARCH FOCUS
- Pharmacological assays and cell culture
- Extraction, fractionation and isolation of plant material
- Activity-guided isolation of active plant components
WORK EXPERIENCE

- **Student assistant** at the Institute of Pharmacology and Toxicology, Technical University of Munich, Germany, winter term 2006/2007
- **Student assistant** at the Department of Botany, Technical University of Munich, Germany, winter term 2005/2006

ADDITIONAL QUALIFICATIONS

- **Computer Know-how:** Microsoft Office (Word, Excel, PowerPoint, Outlook), SigmaPlot
- **Training Courses** at “Uni Graz for life”:
  - Basics of successful project management
  - Research project: From proposal to realization
  - Research projects, support programs, application
  - Scientific proposal and paper writing
  - Rhetoric and presentation
  - Speed reading
  - Humour at work
  - Competent handling of conflicts at work
- Other training courses:
  - QPCR Day Biomedica & Stratagene
  - Sample preparation and HPLC in pharmaceutical analysis (Sigma-Aldrich)
  - Seggauer Fortbildungstage 2009
  - Summer School of traditional Chinese medicine (attendance and organization)
  - English for academic purposes (University of Graz)

AWARDS:

- Best oral presentation at the Young Researcher Workshop of the 57th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Geneva, Switzerland, 2009
- Award “Best oral presentation” at the 26th Magnesium Symposium of the Society of Magnesium Research, Munich, Germany, 2006
PUBLICATIONS:


ORAL PRESENTATIONS:

- “Beeinflussung der Veratridin-induzierten Katecholamin-Freisetzung durch Magnesium am Samenstrang und am Nebennierenmark der Ratte“ at the 26. Magnesium-Symposium der Gesellschaft für Magnesium-Forschung e.V., Munich, Germany, October 2006

- “A petrol ether extract of *Onosma paniculatum* Bur. & Franch. shows strong anti-proliferative activity and induces apoptosis in human cancer cell lines” at the Young Researcher Workshop of the 57th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Geneva, Switzerland, August 2009

- “Pharmacological investigations on *Onosma paniculatum* and isolated shikonin derivatives” at the 1st Cell Culture Day of the Medical University of Graz, Graz, Austria, June 2010.