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The enrichment of *Pluchea odorata* anti-neoplastic activities
and the analysis of underlying mechanisms

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ABBREVIATION INDEX

ADP/ATP	Adenosine diphosphate/adenosine triphosphate
AIF	Apoptosis inducing factor
ASE	Accelerated solvent extraction
ASR	Anisaldehyde sulphuric acid reagent
ATCC	American type culture collection
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia mutated and Rad3-related
Bcl-2	B-cell lymphoma 2
CAM	Cell adhesion molecules
CC	Column chromatography
Cdc	Cell division control
Cdk	Cyclin dependent kinase
CH ₂ Cl ₂	Dichloromethane
CHCl ₃	Chloroform
Chk2	Check point kinase 2
CIP/KIP	cell cycle proteins
CKI	Cyclin dependent kinase inhibitor
DAPI	4',6-Diamidin-2-phenylindol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FCS	Fetal calf serum
G0, G1, G2	Gap phases of the cell cycle
GF	Growth factor
GS	Growth signals
HL60	Promyeloic leukaemia cells
HPLC	High performance liquid chromatography
IC50	Concentration causing 50% inhibition
INK4	Tumor suppressor protein
mA	Milliampere
MCF7	Breast cancer cells
MeOH	Methanol
mM	Millimol
M-phase	Mitosis
p21	CDK inhibitor 1
p38	Mitogen-activated protein kinase
p53	Tumor suppressor protein
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly[ADP-ribose]polymerase
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline/tween
PE	Petroleumether

PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulfonyl fluoride
pRp	Retinoblastoma protein
PVDF	Membranes for Western Transfer and Sequencing
rpm	Revolutions per minute
RPMI	Cell culture medium (Roswell park memorial institute)
RP-18 SPE	Reversed phase-18 solid phase extraction
RV	Reservoir volume
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SPE	Solid phase extraction
S-phase	DNA synthesis
TBS	Tris buffered saline
TBS/T	Tris buffered saline/tween
TLC	Thin layer chromatography
TNF α	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)-aminomethan
UV	Ultraviolet
VLC	Vacuum liquid chromatography

1 INTRODUCTION

A high percentage of medicinal drugs used in western medicine are derived from natural products and therefore ethno-pharmacological remedies of exotic civilisations can be a rich source for the development of novel drugs [CRAGG et al., 2006]. Particularly the rainforests possess an incredible potential concerning medicinal genetic resources, some of which may yield drugs to treat numerous diseases [CSEKE et al., 2006]. The majority of drugs used in western medicine are derivatives of naturally occurring substances (metabolites of plant or microbial origin) [CRAGG et al., 2006].

A success story in natural product drug discovery is paclitaxel (Taxol ®) from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae). The antitumor activity of Taxol is based on its ability to stabilize microtubules in tumor cells, promoting mitotic arrest and cell death. Several Native American tribes have used *Taxus* species for the treatment of non-cancerous diseases [CRAGG and NEWMAN, 2005].

There are innumerable higher plants, which have not been explored phytochemically yet. But it is anticipated that many of them contain bioactive compounds for the development of drugs to combat cancer diseases. Thus, we concentrate on the medicine of the Maya, who developed an advanced pharmaceutical science because of their habitat, the world's third richest in numbers of vascular plant species [BORCHARDT, 2004].

For the first time the anti-cancer activity of extracts of the ethnopharmacological Mayan healing plant, *Pluchea odorata* (Asteraceae), was described by Gridling et al. The most active dichloromethane extract was specially investigated, whereby it showed distinct anti-proliferative effects. Its pro-apoptotic properties were massive. Conclusively substantiated the dichloromethane extract of *P. odorata* may contain several pro-apoptotic principles. Hence, the effectiveness of the extract may be based on the synergism of these principles [GRIDLING et al., 2009].

The aim of recent investigations was separating these principles with strong anti-neoplastic activity derived from the aerial parts of *P. odorata* (L.) Cass., which are used by the Maya to

treat severe inflammatory conditions. Hence, bioassay-guided fractionation was carried out to enrich the active compounds. The plant material was subsequently extracted with different solvents: petroleum ether and dichloromethane. The dichloromethane extract by ASE showed the highest anti-proliferative activity on HL60 cell line. Thus, it was re-chromatographed by Vacuum Liquid Chromatography (methanol-water in different ratios), normal Column Chromatography (chloroform-methanol-water gradient) on silica gel KG60, and RP-18 Solid Phase Extraction (methanol-water).

The obtained fractions were tested in human HL60 promyelocytic leukaemia cells regarding their anti-cancerogenic properties. Furthermore, the most potent isolated fractions were investigated on their apoptosis-inducing potential. Western blotting displayed data on the underlying mechanisms. The bioassay-guided fractionation indicated that the anti-proliferative activity of *P. odorata* was due to a fraction containing highly apolar constituents. Qualitative investigations on other *Pluchea* species reported sesquiterpenes and flavonoids as major compounds [FATOPE et al., 2004; VERA et al., 2008]. Positive reactions with spraying reagent anisaldehyde sulphuric acid on TLC propose the presence of sesquiterpenes in the active fractions.

2 LITERATURE SURVEY

2.1 CANCER

2.1.1 Cell cycle and cancer

The mammalian cell cycle consists of four phases, G1, S phase, G2 and M phase, each of which should be completed successfully before the next begins. Through major cell cycle transitions the progression of eukaryotic cells is mediated. Thereby, the sequential assembly and activation of cyclin dependent kinases (CDK) is important [VIALARD et al., 2001]. The aberrant regulation of the cell cycle can lead to carcinogenesis. Mutations in genes of the molecular components of the growth factor signaling pathway and in genes that code for regulation of the cell cycle can lead to growth signal autonomy, one of the six hallmarks of cancer.

Cell cycle progression, as an ordered and tightly-regulated process, involves multiple checkpoints that assess extracellular growth signals, cell size and DNA integrity. For the induction of cell cycle progression cyclin dependent kinases (CDKs) and their cyclin partners are positive regulators and cyclin dependent kinase inhibitors (CKIs) are negative regulators. There are two types of CKIs, the INK4 family and the CIP/KIP family, of which, p21, directly inducible by p53, is an example. The abnormal expression or activation of positive regulators and suppression of negative regulators can be one origin of cancer [PARK and LEE, 2003].

2.1.2 What is Cancer?

Cancer is broadly defined as group of diseases, in which cells in a part of the body begin to grow out of control. More than 100 cancer types have been classified. The tissues of origin consist of epithelial cells (carcinomas), mesoderm cells (sarcomas) or glandular tissue (adenocarcinomas), such as breast tissue. Cancers differ in characteristics, therefore treatment must be adapted [PECORINO, 2008].

2.1.3 The six hallmarks of Cancer

A goal of cancer research is to find opportunities to counteract mechanisms of malignant tumors and thereby reduce mortality rate with cancer as the underlying cause of death. Considering the numerous and complex properties of cancers, the six essential alterations in cell physiology that collectively dictate malignant growth, were experimentally discovered by Hanahan and Weinberg [2000]. These alterations are essential for carcinogenesis and thereby they are also potential targets for the design of new therapeutics [PECORINO, 2008].

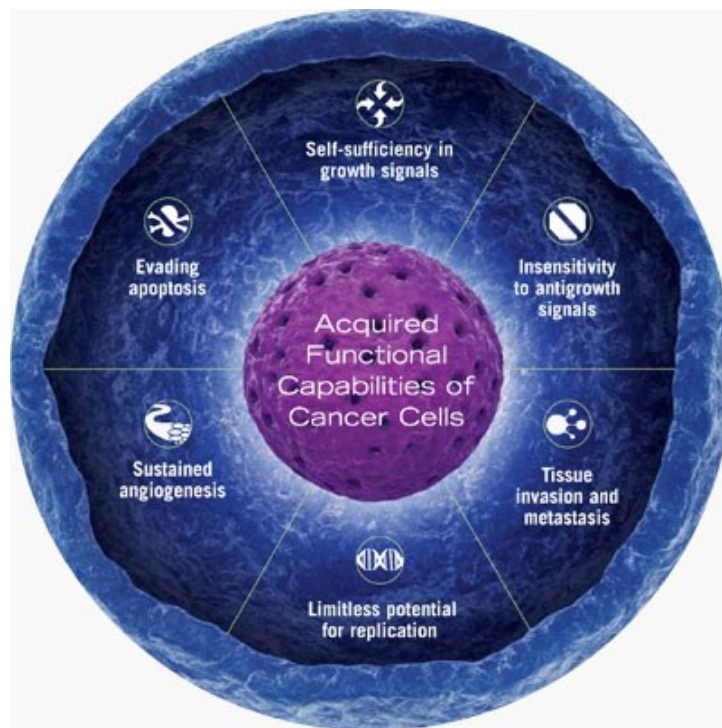


Figure 1 Six essential alterations in cell physiology [HANAHAN and WEINBERG, 2000]

2.1.3.1 Acquired functional capabilities of cancer cells

- Self-sufficiency in growth signals
- Insensitivity to antigrowth signals
- Evading apoptosis
- Limitless potential for replication
- Sustained angiogenesis
- Tissue invasion and metastasis

The defined hallmarks can be found in most types of human cancer. Cancer is characterized by unregulated cell growth and most carcinogens are agents that cause alterations to the DNA sequence or mutations, therefore cancer results from alterations in DNA [PECORINO, 2008].

2.1.3.1.1 Self-sufficiency in growth signals

Cancer cells are able to proliferate by producing their own mitogenic growth signals, independent on normal growth factor signaling. Thereby, tumor cells are reducing their dependence on stimulation from their microenvironment, hence a critically important homeostatic mechanism for ensuring a proper behaviour of cell types is disrupted. In contrast the division of normal cells needs external signals from growth factors. Growth factor pathways that are affected by acquired mutations lead to unregulated growth.

There are different molecular strategies for achieving growth signal autonomy, involving alteration of extracellular growth signals, of transcellular transducers of those signals, or of intracellular circuits that translate those signals into action [HANAHAN and WEINBERG, 2000]. Many cancer cells acquire the ability to create a positive feedback signaling loop often termed autocrine stimulation, whereby they obviate dependence on GFs from other cells [FEDI et al., 1997].

During tumor pathogenesis cell surface receptors, that transduce growth-stimulatory signals are deregulated. The overexpression of GF receptors, occurring in many cancers, may enable the cancer cell to become hyper-responsive to ambient levels of growth factors.

Ligand-independent signaling, elicited by gross overexpression of GF receptors, can also be the result of structural alteration of receptors [HANAHAN and WEINBERG, 2000]. Moreover cancer cells are able to switch the types of expressed extracellular matrix receptors (integrins), favoring receptors which transmit pro-growth signals. These cell surface receptors link cells to the extracellular matrix (ECM), whereby the integrin receptors gain ability to transduce signals that provoke various cell behaviours, ranging from quiescence to motility, resistance to apoptosis and entrance into the cell cycle. The failure of binding can impair cell motility, induce apoptosis, or cause cell cycle arrest [GIANCOTTI and RUOSLAHTI, 1999].

In the majority of human tumors cell-to-cell growth signaling operates, because the several distinct cell types appear to communicate via heterotypic signaling. And tumor cells are able to co-opt their normal neighbours by inducing them to release abundant fluxes of growth-stimulation signals [SKOBE and FUSENIG, 1998].

2.1.3.1.2 Insensitivity to antigrowth signals

To maintain homeostasis cells respond to inhibitory signals, but cancer cells do not, because acquired mutations interfere with the inhibitory pathways [PECORINO, 2008]. There are multiple antiproliferative signals within normal tissues to maintain homeostasis; these are soluble growth inhibitors and immobilized inhibitors in the extracellular matrix.

Antigrowth signals are able to block proliferation, whereby they operate with two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent (G_0) state, and when extracellular signals permit, cells may reemerge. Otherwise, cells may be induced to enter into post-mitotic states and therefore permanently relinquish their proliferative potential [HANAHAN and WEINBERG, 2000].

To prosper incipient cancer cells have to evade these antiproliferative signals. Perhaps all proliferative signals are funneled through the retinoblastoma protein (pRb), that blocks proliferation, when in a hypophosphorylated state [WEINBERG, 1995]. Cancer cells have the opportunity to disrupt the pRb pathway, whereby E2Fs is liberated and thus allows cell proliferation. The pRb signaling circuit can be disrupted in a variety of ways.

Cancer cells are also able to turn off expression of integrins or other cell adhesion molecules. Thereby antigrowth signals are blocked, but those that convey progrowth signals are favoured. Cell proliferation is not only depending on mere avoidance of cytostatic antigrowth signals, but i.e on constraining cell multiplication by instructing cells to enter irreversibly into post-mitotic states by using diverse mechanisms that are incompletely understood. It is apparent that tumor cells use various strategies to avoid terminal differentiation [HANAHAN and WEINBERG, 2000].

2.1.3.1.3 Evading apoptosis (programmed cell death)

Cancer cells are able to evade apoptotic signals, but normal cells are removed by apoptosis. Expansion of cell number is determined by the rate of cell proliferation, but also by the rate of programmed cell death (apoptosis). One hallmark of most and perhaps all types of cancer may be the acquired resistance toward apoptosis. The apoptotic program may be present in all cell types throughout the body, whereby this program is triggered by various signals and it unfolds in a series of steps, until the shriveled cell corpse is engulfed by nearby cells and disappears. There are two classes of components of the apoptotic machinery, sensors and effectors. Sensors are monitoring the extracellular and intracellular environment and they regulate the second class of components, the effectors of apoptotic death.

Cytochrome C, a potent catalyst of apoptosis, is released by mitochondria. Many of the signals, that elicit apoptosis, converge on mitochondria [GREEN and REED, 1998]. The various proteins of the Bcl-2 family, which have either proapoptotic or antiapoptotic function, are able to act through cytochrome C release. Cytochrome C released from mitochondria can activate intracellular proteases, termed caspase 8 and caspase 9, which are able to activate more effector caspases, that execute the death program through destruction of the genome

[HANAHAH and WEINBERG, 2000]. The investigations of apoptosis in cancer at the molecular level were opened up by the discovery of the upregulation of bcl-2 oncogen via chromosomal translocation in follicular lymphoma and its recognition as having antiapoptotic activity [VAUX et al., 1988]. Existing data indicate that the presence of overexpressed oncogenes can trigger the apoptotic program.

2.1.3.1.4 Limitless Replicative Potential

Cancer cells are able to maintain the length of their telomeres that results in unlimited replicative potential. Within normal cells the chromosomal ends, telomeres, are shortened during every round of DNA replication. That is an autonomous counting device to define a finite number of cell doublings [PECORINO, 2008].

Not only growth signal autonomy, insensitivity to antigrowth signals and resistance to apoptosis does ensure expansive tumor growth, but also failures in an intrinsic, cell-autonomous program that otherwise limits the multiplication of perhaps all types of mammalian cells. When this program is disrupted a clone of cells expands to a size that constitutes a macroscopic, life-threatening tumor.

Cells in culture have a finite replicative potential, after a certain number of doublings cells stop growing – a process termed senescence. The circumvention of senescence is possible by disabling their pRb and p53 tumor suppressor proteins. Thereby, cells are able to continue multiplying for additional generations, until they enter into the crisis state. This state is characterized by massive cell death, but also the occasional emergence of a variant cell that has acquired the ability of immortalization [WRIGHT et al., 1989].

The counting devices for cell generations are the ends of chromosomes. These telomeres are consisting of several thousand repeats of short 6 bp sequence element. During each cell cycle telomeric DNA loses approximately 100 bp, the replicative cell generations lose their ability to protect the ends of chromosomal DNA, that results in the death of the affected cells [COUNTER et al., 1992].

In virtually all types of malignant cells telomerase maintenance is evident and clearly a key component of the capability for unlimited replication [SHAY and BACCHETTI, 1997]. There are two mechanisms to maintain telomeres. On the one hand, malignant cells do so by upregulating expression of the telomerase enzyme. The enzyme adds hexanucleotide repeats onto the ends of telomeric DNA [BRYAN and CECH, 1999]. On the other hand, cells activate a mechanism, termed ALT. Thereby the maintenance of telomeres is through recombination-based interchromosomal exchanges of sequence information [BRYAN et al., 1995].

2.1.3.1.5 Sustained angiogenesis (formation of new blood vessels)

Blood vessels are responsible for supplying normal cells with oxygen and nutrients. Cancer cells are able to induce angiogenesis, the growth of new blood vessels. That is necessary for survival and expansion of tumors. The imbalance between angiogenic inducers and inhibitors can activate the angiogenic switch [PECORINO, 2008].

During organogenesis growth is coordinated and once a tissue is formed, the process of angiogenesis is carefully regulated. The evidence is, that cells within aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. Incipient neoplasias have to develop angiogenic ability to progress a larger size [HANAHAN and FOLKMAN, 1996].

Angiogenesis is encouraged or blocked by counterbalancing positive and negative signals. There are angiogenesis-initiating signals like vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2). One angiogenesis inhibitor is thrombospondin-1. Tumors seem to gain the ability to induce and sustain angiogenesis during development, via an „angiogenic switch“. Tumor angiogenesis offers an attractive therapeutic target [HANAHAN and WEINBERG, 2000].

2.1.3.1.6 Tissue Invasion and Metastasis

During the development of most types of human cancer, pioneer cells are spawned that travel to distant sites, where they found new colonies. These metastases are the cause of most human

cancer deaths. Invasion and metastasis are very complex processes, but also allied processes and utilizing similar operational strategies. In cells possessing invasive or metastatic capabilities, several classes of proteins are altered. The affected proteins include cell-cell adhesion molecules (CAMs), such as E-cadherin, and integrins, which link cells to extracellular matrix substrates, whereby all of these „adherence“ interactions convey regulatory signals to the cell (APLIN et al., 1998). E-cadherin acts as a suppressor of invasion and metastasis by epithelial cancers.

Changes in expression of CAMs and integrin appear to play critical roles in the processes of invasion and metastasis. Also extracellular proteases are involved in invasive and metastatic capability, whereby protease genes are upregulated, protease inhibitor genes are downregulated, and inactive forms of proteases are converted into active enzymes. Clearly central to the acquisition of invasiveness and metastatic ability are the activation of extracellular proteases and the altered binding specificities of cadherins, CAMs, and integrins [HANAHAH and WEINBERG, 2000].

2.1.4 Cancer and Inflammation

Many types of cancer are caused by inflammation and specific infectious agents, through several mechanisms, which are involved in the process of cancer initiation and promotion. Especially long-term exposure to infectious agents and chronic inflammation can lead to cancer, but these causes of human cancer can be prevented.

There are various viruses and bacteria, acting as infectious agents involved in carcinogenesis. Some viral and bacterial infections are able to induce a chronic inflammatory response that contributes to carcinogenesis. Under normal conditions inflammation is a defense mechanism against infectious agents, which is highly regulated. But around a tumor the body creates an inflammatory microenvironment characterized by inflammatory cells, growth factors, and reactive oxygen/nitrogen species [PECORINO, 2008].

The linkage between inflammation and cancer is part of an paradigm of carcinogenesis, because smoldering, nonresolving inflammation sets the stage for mutagenesis, angiogenesis

and metastasis [BALKWILL and MANTOVANI, 2010]. The possible mechanisms of this linkage includes induction of genomic instability, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation, resistance to apoptosis and tumor neovascularization [KUNDU and SURH, 2008].

Approximately 20 % of all malignancies are initiated or exacerbated by inflammation, whereby the aggravation of inflammation is effected by increased production of cytokines, proteolytic enzymes, lipid mediators and prostaglandins. Also the infiltration of macrophages is a common feature of inflammation and angiogenesis. Therefore inflammatory and angiogenic responses can be targets for development of anticancer therapeutic drugs [ONO, 2008].

2.1.5 Apoptosis

The highly regulated process of cell death controls cell numbers and gets rid of cells, that have extensive DNA damage, it therefore plays an important role in tumor suppression. The aberrant regulation of cell growth, differentiation and apoptosis can give rise to tumors.

Apoptotic cells are characterized by shrinkage, membrane blebbing, chromatin condensation and precise fragmentation. And they are swept clean during phagocytosis by macrophages. This contrasts with the process of necrosis. Cells swell, membranes become leaky and cells spill out their contents into the surrounding tissue and cause inflammation [PECORINO, 2008].

2.1.6 Cell death programs

The cells of metazoans have developed complex self-destruction mechanisms, collectively called programmed cell death, which includes the invention of intrinsic and extrinsic trigger factors. In multicellular organisms homeostasis is maintained by the balance between cell proliferation and cell death. There are several morphologically discernible forms of cell death.

The type I cell death is divided into caspase-dependent apoptosis and caspase-independent apoptosis. Caspase-dependent apoptosis is a process of cell suicide eliminating unwanted

cells. Apoptosis involves the action of catabolic enzymes (proteases like caspases, nucleases) and stepwise DNA degradation. The remaining material is phagocytosed by unaffected neighboring cells.

In a caspase-independent fashion the apoptotic chromatin degradation can be achieved also by AIF (apoptosis inducing factor). The two mechanisms of apoptosis execution exist together and may cooperate.

The type II cell death involves lysosomes for the cell death by autophagy, whereby parts of membranes of subcellular organelles such as Golgi assemble to autophagosomes. The difference to apoptosis is the remaining intact cytoskeleton. Type I and type II cell death can occur in mixed form within the same tissue or cell type and even within the same cell. Apoptosis is required for homeostasis, hence the dis-regulation of cell death programs, more specifically, an abnormal resistance to apoptosis induction causes inter alia cancer due to the persistence of mutated cells.

When apoptotic execution pathways are blocked or as a result of a variety of stresses the necrotic (accidental) cell death can occur. Co-occurring are also self destruction programs, which stand in apparent contrast to necrosis. Apoptosis and other forms of active cell death are major factors in the evolution of multicellular organisms [HUETTENBRENNER et al., 2002].

2.2 CURRENT STATE OF RESEARCH – GENUS *PLUCHEA*

Gridling et al. undertook preceding investigations on *Pluchea odorata* (L.) Cass. (Santa Maria), a plant used by the Maya to treat inflammatory conditions. Novel therapeutic concepts were tested in the human promyelocytic leukaemia cell line HL60. The concept of plant selection was their traditional use against severe inflammations, because there are various similar signalling pathways both in inflammatory conditions and in cancer.

The most effective extract of *P. odorata* was the dichloromethane extract, suggesting that the active principle(s) is (are) non-polar compound(s), which inhibited inflammatory responses and exhibit anti-cancer activity [GRIDLING, 2008]. To isolate these compounds the bioassay-guided fractionation of the *P. odorata* CH₂Cl₂ extract was task of this diploma thesis and also current investigations.

The genus *Pluchea* (Asteraceae), comprising 80 species, contains various compounds. The leaves of *Pluchea carolinensis* (Jacq.) G. Don, a branched shrub from the Caribbean and surrounding regions, contains terpenoids, such as alpha-amyrin. Derivatives of cuauthemone 7 and 9 to 15, which are found in abundance in *Pluchea*, were found in aerial parts of *Pluchea carolinensis* [ROBINEAU, 1991].

Two sesquiterpenes, godotol A and godotol B, were isolated from *Pluchea arabica*. Previous investigations of *Pluchea* plants have shown the presence of eudesmane sesquiterpenes, monoterpenes, lignan glycosides, pentacyclic triterpenoids and flavanoids [FATOPE et al., 2004]. Also Marwah describes the wound-healing plant *Pluchea arabica*, traditionally used to treat sores and boils, containing the phytoconstituents sesquiterpenes, flavonoids, chromones and eudesmanes [MARWAH et al., 2007]. Decoctions of *Pluchea sagittalis* are used in traditional medicine and antioxidant and anti-inflammatory effects by CH₂Cl₂ extracts have been reported [PÉREZ-GARCÍA et al., 1996,2005].

The root extract of the Indian medicinal plant *Pluchea indica* (L.) Less.(Asteraceae) contains β-sitosterol and stigmasterol [GOMES et al., 2007] and eudesmane-type sesquiterpenes, plucheols A, B and plucheoside E. And the pure compound R/J/3 was isolated from *Pluchea indica* (L.) Less., a plant which is known for its anti-inflammatory activities and the isolated Tiophen showed the most pronounced anti-proliferative activity [BISWAS et al., 2005].

From the whole plant of *Pluchea arguta* a eudesmane sesquiterpene was isolated and identified as 4-epiplucheinol [AHMAD and FIZZA, 1986]. Plucheinol and (3αH)-plucheinol are eudesmane-type sesquiterpenes isolated from *Pluchea chingoyo* [ZABEL et al., 1982].

The genus *Pluchea* contains various flavonoids, the commonly known compounds isorhamnetin was isolated from *P. symphytifolia* W. T. Gillis and for the first time eupalitin has been isolated from *Pluchea* genera [CORDOVA et al., 2006].

From the leaves of *Pluchea odorata* (L.) Cass. four derivatives of a dehydroeudesman-8-one were obtained [ARRIAGA-GINER et al., 1983]. Two eudesmane derivatives, plucheinol and its monoester derivative 3 α (2',3'-dihydroxy-2'-methyl-buteryl oxy)-4 α ,11-dihydroxy-6,7-dehydroeudesman-8-one were isolated as minor constituents [ARRIAGA and BORGES-DEL-CASTILLO, 1985]. Investigations of the roots of *Pluchea odorata* Cass. yields a carvotageton derivative and the phthalic acid derivative, while the aerial parts contain besides the already known eudesmane derivative two further compounds of this type [BOHLMANN and ZDERO, 1975].

Whether the different *P. odorata* populations also exhibit different anti-cancer activity needs to be established and could elucidate which type(s) of the plant constituents may be responsible for the described anti-neoplastic effects.

3 MATERIAL AND METHODS

3.1 PLANT

Pluchea odorata (L.) Cass. (Santa Maria)



Figure 2 *Pluchea odorata*



Figure 3 *Pluchea odorata*: florescence

http://www.researchlearningcenter.org/bloom/species/Pluchea_odorata

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclassis:	Asteridae
Order:	Asterales
Family:	Asteraceae
Genus:	Pluchea
Species:	<i>Pluchea odorata</i>

The family of *Asteraceae* is probably the largest family of flowering plants. It includes more than 25,000 species world-wide, growing everywhere. The family *Asteraceae* shows remarkable variation in growth form and morphology because they occur in so many different localities and habitats.

Pluchea odorata (L.) Cass., by common name Saltmarsh Fleabane, Sweet-scent or in Central America known as Santa Maria, has pink to lavender coloured flowers. It is a woody shrub up to 3 metres high and with aromatic leaves. The distribution ranges from Northern America to the states of Southern America: Belize, Guatemala, Panama, Puerto Rico, Venezuela and Ecuador. It is used in traditional medicine as an antidote, astringent, diaphoretic and emmenagogue agent.

It is used as a treatment against common cold, hypertension, neuralgia and pneumonia [JOHNSON, 1999]. In traditional medicine it is prepared like tea. Therefore three leaves of *P. odorata* are boiled in three cups of water for two minutes followed by 15 minutes of steeping. This beverage is used against asthma attacks, coughs and colds, but also as ablutions against swellings, tumors and inflammations [ARVIGO and BALICK, 1994].

3.2 SAMPLE PREPARATION

The fresh plant material of *P. odorata* from Guatemala has been stored deep-frozen at -80°C . For further extraction procedures the aerial plant parts, leaves, caulis and florescence of *P. odorata* were lyophilized.

3.3 EXTRACTION WITH DICHLOROMETHANE (CH_2Cl_2)

First the freeze dried plant parts were milled (RETSCH ZM 100). Afterwards the obtained powder was extracted. To obtain the dichloromethane extract, 20 g milled plant was mixed with 200 ml (1:10) of petroleum ether (PE) and treated in an ultra sonic bath for 10 minutes to break the cell walls of the plant. Then it was placed to a reflux-water bath at 40°C (table 1).

After 1 hour the solvent was filtered through a round filter (Schleicher & Schuell, Microscience, 595, Ref Nr 10311612 – diameter 150 nm).

- The retained plant material (residue) was dried on a sheet of paper over night, before reuse for next extraction with dichloromethane.
- The liquid phase (solvent), which contained the dissolved material, was evaporated by a rotavapor (Heidolph WB 2001) and a water bath at 40°C until complete dryness.

The dried retained plant material was mixed with 200 ml (1:10) of CH_2Cl_2 and treated in an ultra sonic bath for 10 minutes. Then it was placed to a reflux-water bath at 50°C (table 1).

After 1 hour the solvent was filtered through a round filter. The liquid phase (solvent) which contained the dissolved material was evaporated under vacuum by a rotavapor until complete dryness.

3.4 EXTRACTION WITH CHLOROFORM (CHCl₃)

To obtain the chloroform extract, 20 g milled plant material was mixed with 200 ml (1:10) of methanol (MeOH) and treated in an ultra sonic bath for 10 minutes to break the cell walls of the plant. Then it was placed to a reflux-water bath at 79°C (table 1).

After 1 hour the solvent was filtered through a round filter. The supernatant liquid phase (solvent), which contained the dissolved material was evaporated under vacuum by a rotavapor until complete dryness.

The retained MeOH extract was dissolved in 100 ml MeOH:H₂O (9:1) and afterwards it was degreased with 100 ml petroleum ether. The mixture of immiscible liquids was put into a separating funnel, shaken and allowed to settle for three times.

The obtained PE extract was evaporated and stored. The aqueous MeOH extract was mixed with 100 ml H₂O (1:1). By using a separating funnel the partition with 200 ml CHCl₃ was carried out.

The aqueous extract was evaporated and stored. The chloroform extract was washed twice with 200 ml saline solution (NaCl 1%) and Na₂SO₄ (Natrium sulfuricum siccatum) was added to eliminate contained water out of the organic solvent. After filtering through a round filter the chloroform extract was evaporated.

solvent	°C of reflux water bath	evaporator mbar
petroleum ether	40	650
dichloromethane	50	650
methanol	79	300

Table 1 Solvents and their temperature and pressure for evaporation

3.5 EXTRACTION OF *P. ODORATA* WITH CH₂Cl₂ BY ASE 200 - accelerated solvent extractor (DIONEX)



Figure 4 ASE 200

http://www.dionex.com/enus/webdocs/4198_ASE_200_V10.pdf

The ASE 200 Accelerated Solvent Extractor executes extractions in shorter time and with less solvent than traditional extraction techniques. This technique uses conventional liquid solvents for extracting solid and semisolid samples. Due to elevated temperatures and pressures the efficiency of the extraction process is increased. The technology is completely automated.

3.6 CELL CULTURE

HL60 promyelocytic leukaemia cells and MCF7 breast cancer cells were purchased from ATCC. HL60 cells were grown in RPMI 1640 medium and MCF7 cells in Dulbecco's Modified Eagle Medium (DMEM; GIBCO). The media were supplemented with 10 % heat-inactivated fetal calf serum (FCS), 1% Glutamax and 1% Penicillin-Streptomycin. Cells were kept in humidified atmosphere containing 5% CO₂ at 37°C. All media and supplements were obtained from Life Technologies.

3.6.1 Proliferation Assay - Proliferation inhibition analysis

HL60 cells were seeded in T-25 tissue culture flasks or in 24-well plates at a concentration of 1×10^5 cells per ml cell culture medium and incubated with increasing concentrations of plant extracts or fractions. Cell counts and IC_{50} values were determined at 24 h and 72 h using a KX-21 N microcell counter (Sysmex Corporation). All experiments were done in triplicate.

The percent of cell division progression compared to the untreated control was calculated as follows:

$$[(C_{72\text{ h} + \text{drug}} - C_{24\text{ h} + \text{drug}}) / (C_{72\text{ h} - \text{drug}} - C_{24\text{ h} - \text{drug}})] \times 100 = \% \text{ cell division}$$

$C_{72\text{ h} + \text{drug}}$... cell number after 72 h of drug treatment

$C_{24\text{ h} + \text{drug}}$... cell number after 24 h of drug treatment

$C_{72\text{ h} - \text{drug}}$... cell number after 72 h without drug treatment

$C_{24\text{ h} - \text{drug}}$... cell number after 24 h without drug treatment

3.6.2 Apoptosis Assay - Determination of cell death by Hoechst 33258/propidium iodide (HOPI) double staining

To determine the type of cell death, HL60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells per ml cell culture medium and MCF7 cells were seeded in 96-well plates at a concentration of 1×10^4 per ml medium, grown for 24 hours. Then they were treated with increasing concentrations of extract or fractions (0.5 mg/ml, 1 mg/ml, 4 mg/ml, 20 mg/ml and 0.4 $\mu\text{g/ml}$, 0.8 $\mu\text{g/ml}$, 1.5 $\mu\text{g/ml}$, 3 $\mu\text{g/ml}$, respectively) for different periods of time (extract for 96 hours, fractions for 8 and 24 hours).

Hoechst 33258 and propidium iodide were added to the cells at final concentrations of 5 and 2 $\mu\text{g/ml}$, respectively. After 60 min of incubation at 37°C cells were examined on a Zeiss Axiovert fluorescence microscope equipped with a DAPI filter. Cells were photographed and analysed by visual examination to distinguish between apoptosis and necrosis [GRUSCH et al., 2002]. Cells were judged according to their morphology and the integrity of their cell membrane on the basis of propidium iodide exclusion. Those experiments were done in triplicate.

3.7 SEPARATION OF CHLOROPHYLL - Fractions F1/2 and F2/11,12,13,14,15,16

Fractions F1/2 (eluate of *P. odorata* ASE - CH₂Cl₂ extract), fractionated by Vacuum Liquid Chromatography, and F2/11, F2/12, F2/13, F2/14, F2/15 and F2/16 (eluates of fraction F1/3), fractionated by Column Chromatography, were dispensed in two liquid solvents of different polarity, in CH₂Cl₂ and MeOH-H₂O (1:1).

1 g of fraction was dissolved in 150 ml CH₂Cl₂ (fraction in mg x 0.15) and afterwards in 150 ml of methanol – water (1:1). The dichloromethane was evaporated at 40°C and 600-800 bar. Insoluble chlorophyll precipitated in the methanol–water phase and was eliminated by filtration.

3.8 CHROMATOGRAPHY

3.8.1 Thin Layer Chromatography (TLC)

Stationary phase: silica gel plates 60 F254 (Merck, Darmstadt, Germany)

Mobile phase:

solvent system	CHCl ₃ :MeOH:H ₂ O (ml)
1	95:1.5:0.1
2	90:3.5:0.2
3	85:8:0.5
4	80:10:1
5	70:22:3.5
6	60:40:10

Table 2 Composition of solvent system 1 – 6

Detection for solvent systems 1-6: After samples were separated, the TLC plates were analysed under UV₃₆₆, UV₂₅₄ and visible light, before and after spraying them with anisaldehyd sulphuric acid reagent (ASR). For visualisation of constituents, spraying the plates with ASR was followed by heating at 100°C for 5 min. The ASR compounds were 0.5 ml anisaldehyd, 10 ml glacial acetic acid, 85 ml methanol and 5 ml H₂SO₄ (sulfuric acid).

3.8.2 F1 Vacuum Liquid Chromatography (VLC)

4 g of the CH₂Cl₂ extract was dissolved in 15 ml CH₂Cl₂. The thus obtained solution was mixed with 6 g silica gel and evaporated to dryness and stored in a desiccator for further use. To obtain a homogenous powder it was refined in a mortar and placed on top of a column (31x 4 cm), filled with 100 g silica gel. In order not to loose the powdered sample, it was covered with sea sand as ballast.

1000 ml of each of the following mobile phases was used for fast elution with vacuum (table 3).

solvent	relation
petroleum ether	
CHCl ₃	
CHCl ₃ : MeOH	9:1
CHCl ₃ : MeOH	7:3
CHCl ₃ : MeOH	5:5
CHCl ₃ : MeOH	3:7
CHCl ₃ : MeOH	1:9
MeOH : H ₂ O	7:3
MeOH : H ₂ O	1:1
H ₂ O	

Table 3 Mobile phases of VLC F1 (detailed relation)

3.8.3 FX Column Chromatography (CC) - fractionation of F1/2

After chlorophyll was eliminated in the first step, the separation of fraction F1/2 was carried out on a 80x2 cm column, filled with 150 g silica gel. 410.6 mg of fraction F1/2 was dissolved in 6 ml chloroform and mixed with 8 g of silica gel. Afterwards the solvent was evaporated to dryness and the prepared fraction stored in a desiccator over night. After refining it in a mortar it was placed on top of the column and covered with sea sand. Screening was performed with solvent system 4 (80:10:1).

For the elution of compounds three different mobile phases were used:

430 ml CHCl_3 : MeOH : H_2O (100:10:1). Followed by 200 ml of CHCl_3 : MeOH : H_2O (90:10:0.1) and 286 ml of CHCl_3 : MeOH : H_2O (70:22:3.5).

3.8.4 F2 Column Chromatography (CC) - fractionation of F1/3

Chromatographic separation was performed on a 80x2 cm column, filled with 150 g silica gel. 1.46 g of fraction F1/3 (CHCl_3 :MeOH 9:1) was dissolved in 9 ml chloroform and mixed with 1.5 g silica gel. Then, the solvent was evaporated to dryness and stored in a desiccator over night. After refining it in a mortar it was placed on top of the column and covered with sea sand. A fluid reservoir for different solvents was fixed above the column and the following solvents, shown in table 4 below, were used for elution. The solvent MeOH : H_2O (95:5) was passed through the column by applying vacuum.

solvent	relation	amount (ml)
CHCl_3		500
CHCl_3 : MeOH : H_2O	95 : 1.5 : 0.1	600
CHCl_3 : MeOH : H_2O	90 : 3.5 : 0.2	562
CHCl_3 : MeOH : H_2O	85 : 8 : 0.5	2000
MeOH : H_2O	95 : 5	500

Table 4 Solvent systems of CC F2

3.8.5 F3 Column Chromatography (CC) - fractionation of F2/13

30 mg of fraction F2/13 (after separation of chlorophyll) were dissolved in 2 ml CHCl_3 . The thus obtained solution was mixed with 430 mg silica gel and evaporated to dryness and it was stored in a desiccator over night. For receiving a homogenous powder it was refined in a mortar and placed on top of a column (98x1cm), filled with 75 g silica gel. The powder was covered with sea sand.

The mobile phase was CHCl_3 :MeOH (95:5) for slower elution and therefore a more precise separation.

3.8.6 F4 Reversed Phase - Solid Phase Extraction (SPE) – fractionation of F3/3

The used stationary phase was a Bond Elut C18 cartridge (Column specification: 1210-2058; 1 ml/50 mg/40 μm), which is of extreme retentive nature for non polar compounds and a benefit when unknown compounds vary widely in structure.

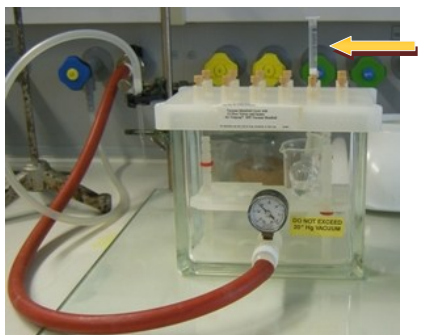


Figure 5 SPE equipment:
Bond Elut C18 cartridge on top (see the arrow),
glass tray connected with vacuum pump

First step was the conditioning of cartridge with MeOH. Then fraction F3/3 was dissolved in CHCl_3 and eluted by vacuum as fraction F4/1.

Afterwards the cartridge was eluted by vacuum with different solutions of MeOH and H_2O (90 %, 80 % and 70% MeOH) and CHCl_3 :MeOH (1:1), respectively. Thereby, fractions F4/2, F4/3, F4/4 and F4/5 were obtained. In between each elution the cartridge was dried by vacuum for 2 minutes.

3.8.7 F5 Reversed Phase - Solid Phase Extraction (SPE) – fractionation of F4/1

As stationary phase a Bond Elut C18 cartridge (Column specification: 1210-2058; 1 ml/50 mg/40 μm) was used.

First step was the conditioning of cartridge with MeOH. Then, fraction F4/1 was dissolved in CHCl_3 and eluted by vacuum as fraction F5/1.

Afterwards the cartridge was eluted by vacuum with two different solutions of MeOH and H_2O (98 % and 96 % MeOH). Thereby, fractions F5/2 and F5/3 were obtained. By elution of CHCl_3 :MeOH (1:1) fraction F5/4 was gained. In between each elution the cartridge was dried by vacuum for 2 minutes.

3.9 WESTERN BLOTTING

3.9.1 Preparation of lysates

HL60 cells were seeded in a tissue culture flask at a concentration of 1×10^6 cells per ml medium and incubated with 3 $\mu\text{g/ml}$ fraction (F2/11, F2/13 and F3/4, respectively) for 0.5 h, 2 h, 4h, 8h and 24 hours. At each time point, 2×10^6 cells were harvested, placed on ice and centrifuged (1000 rpm, 4°C, 4 min). Then, the supernatant (medium) was discarded and the pellet was washed twice with cold PBS (pH 7.2), and centrifuged (1000 rpm, 4 °C, 4 min).

The pellets lysed in 150 μl buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1 % Triton X-100, 2.5 % 0.5 mM PMSF and PIC (Sigma, Schnellendorf, Germany). Debris was removed by centrifugation (12,000 rpm, 4 °C, 20 min) and the supernatant was stored at -20°C until further analyses.

3.9.2 SDS-PAGE and electrotransfer

Equal amounts of lysate (protein samples) were loaded onto 10 % polyacrylamide gels. Proteins were electrophoresed (PAGE) at 120 volt (50 mA) for approximately 2 hours and then electroblotted onto PVDF membranes (Hybond P, Amersham, Buckinghamshire, UK) at 100 Volt and 4°C for 1 hour. To confirm equal sample loading, membranes were stained with Ponceau S.

3.9.3 Immuno reaction

After washing with TBS or PBS, the membranes were blocked for 1 hour in blocking solution containing 5 % skimmed milk in TBS and PBS with 0.5 % Tween 20, respectively, washed for 30 min (changing the washing solution 5 times) in TBS/T or PBS/T, and incubated by gentle rocking with primary antibodies in blocking solution (dilution 1:500) at 4 °C overnight.

Then, the membranes were washed in TBS/T or PBS/T (4 x for 10 min) and further incubated with the second antibody (peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, dilution 1:2000), for 1 h at room temperature. The membranes were washed with TBS/T or PBS/T (4 x for 10 min) and the chemoluminescence (ECL detection kit, Amersham, Buckinghamshire, UK) was detected by exposure of the membranes to Amersham Hyperfilm™ ECL.

The antibody against Phospho-Cdc25A (S75) was from Abcam (Cambridge, MA, USA) and against Phospho-Cdc25A (S177) was from Abgent (San Diego, CA, USA). Anti-H2AX (pSer139) was from Calbiochem (San Diego, CA, USA) and the antibodies against Cleaved Caspase-3 (Asp175), Chk2, Phospho-Chk2 and Phospho-cdc2 (Tyr15) were from Cell Signaling (Danvers, MA, USA).

The antibodies against Cdc2 p34 (17), Cdc25A (F-6), Cyclin D1 (M-20), PARP-1 (F-2) and α Tubulin (DM1A) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the antibodies against Anti- β -Actin and Anti-acetylated Tubulin were from Sigma (St. Louis, MO, USA).

3.9.3.1 Antibodies

- Phospho-Cdc25A (S75) antibody (ab47279), polyclonal (Abcam)
- Phospho-Cdc25A (S177) antibody, polyclonal (Agent)
- Anti-H2AX (pSer139), polyclonal, No. DR1017 (Calbiochem)
- Cleaved Caspase-3 (Asp175), #9661, polyclonal (Cell Signaling)
- Chk2, #2662, polyclonal (Cell Signaling)
- Phospho-Chk2 (Thr68), #2661, polyclonal (Cell Signaling)
- Phospho-cdc2 (Tyr15) (10A11), #4539, monoclonal, Rabbit mAb, (Cell Signaling)
- Cdc2 p34 (17): sc-54, monoclonal (Santa Cruz Biotechnology, Inc.)
- Cdc25A (F-6): sc-7389, monoclonal (Santa Cruz Biotechnology, Inc.)
- Cyclin D1 (M-20): sc-718, polyclonal (Santa Cruz Biotechnology, Inc.)
- PARP-1 (F-2): sc-8007, monoclonal (Santa Cruz Biotechnology, Inc.)

- α Tubulin (DM1A): sc-32293, monoclonal (Santa Cruz Biotechnology, Inc.)
- Anti- β -Actin, clone AC-15, mouse ascites fluid, No. A5441 (Sigma)
- Anti-acetylated Tubulin, clone 6-11B-1, mouse ascites fluid, No. T6793 (Sigma)

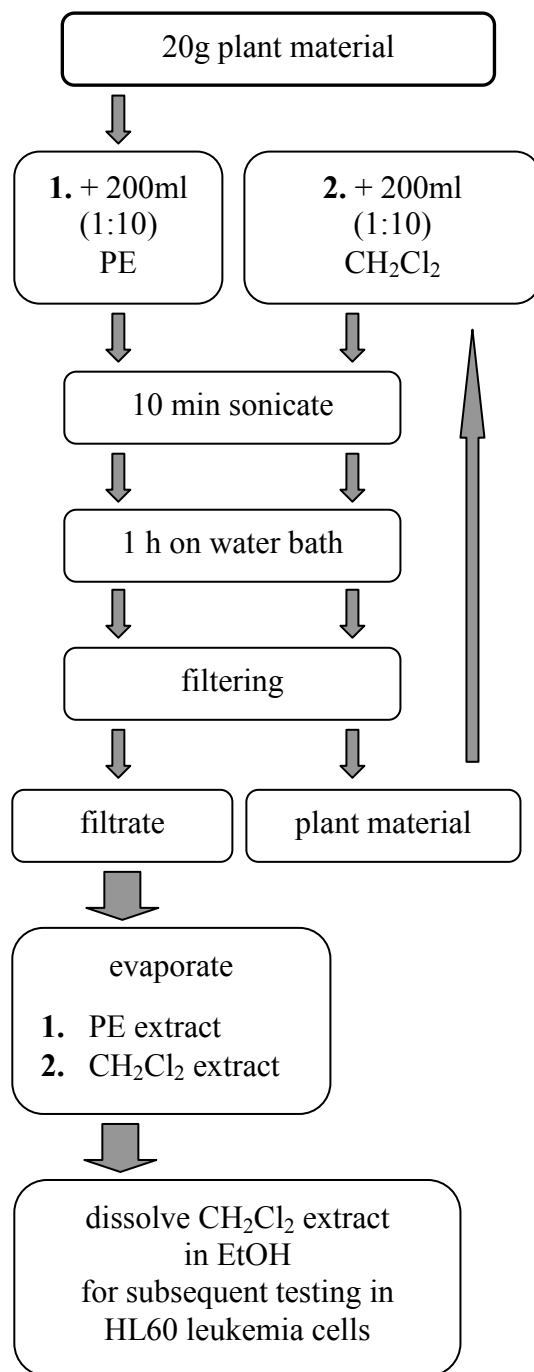
Pierce ECL Western Blotting Substrate Cat #32106 (Pierce) and Amersham Hyper films ECL – High performance chemiluminescence film (GE Healthcare) was used.

4 RESULTS AND DISCUSSION

4.1 EXTRACTION OF *PLUCHEA ODORATA*

Dried plant material of *Pluchea odorata* was extracted in two different ways (scheme 1: CH₂Cl₂ extraction; scheme 2: CHCl₃ extraction) to compare their anti-neoplastic activity in HL60 human leukemia cells. Subsequently, the more potent extract was compared with an accelerated extraction method (chapter 3.5, page 19). The following schemes (**scheme 1** and **scheme 2**) detail the workflow and give an overview of the methodology for a better attribution of results.

4.1.1 Extraction with dichloromethane (CH₂Cl₂)



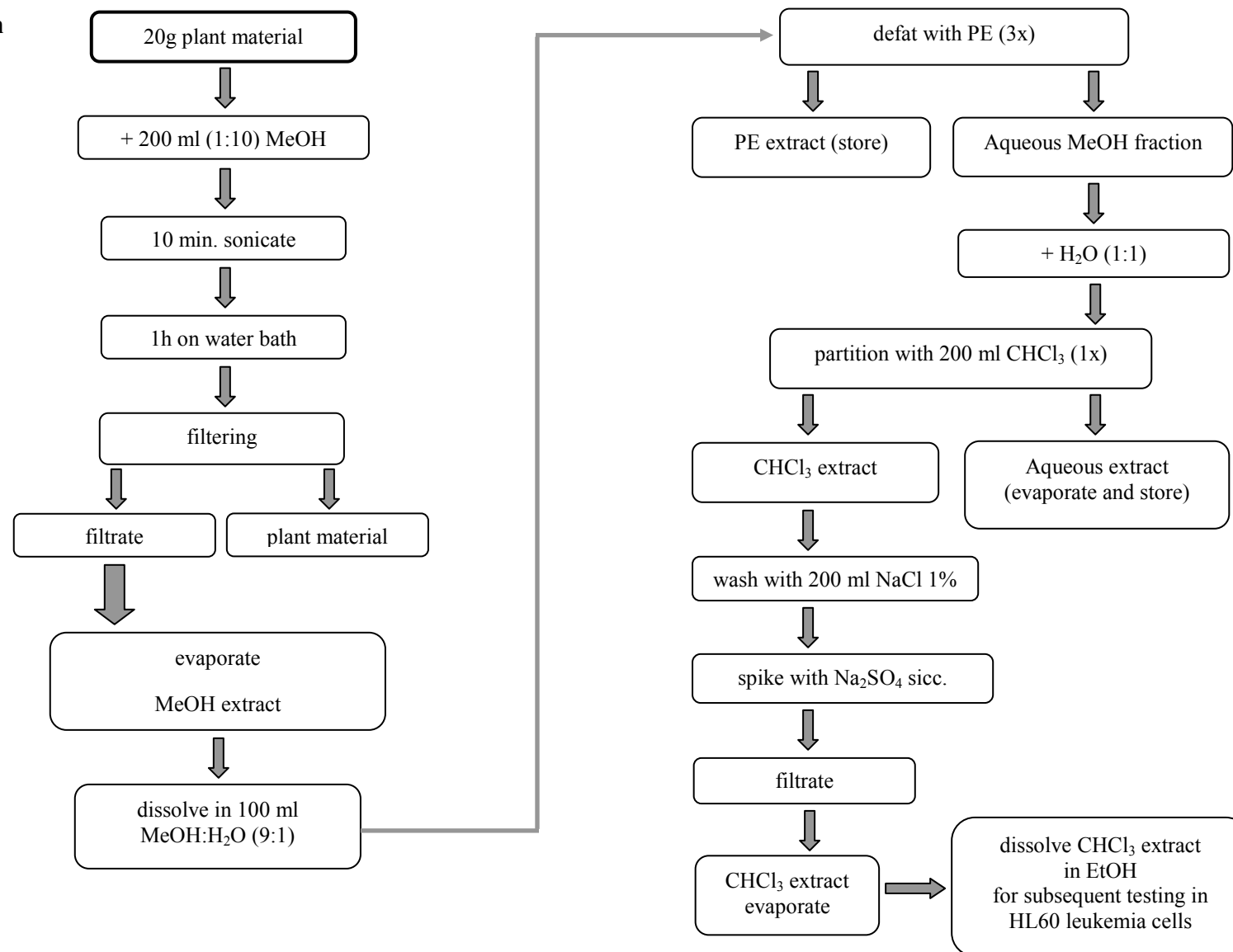
Scheme 1 Sequential extraction with petroleum ether and dichloromethane

20g of dried powdered plant material were dissolved in 200 ml PE (1:10), extracted by ultrasonication at room temperature for 10 min and at 40 °C on a reflux water bath for 1 hour. Then, the solvent was filtrated through a round filter and the obtained filtrate was evaporated for storing. Retained plant material was dried over night. Then it was mixed with 200 ml (1:10) of CH₂Cl₂ and extracted again by ultrasonication at room temperature for 10 min and at 50 °C on a reflux-water bath for 1 hour. The solvent was filtrated through a round filter and the obtained filtrate was evaporated by a rotavapor until complete dryness.

The dried CH₂Cl₂ extract of *P. odorata* (279.5 mg) was dissolved in 3 ml 96 % ethanol for subsequent testing.

In a subsequent Proliferation test the CH₂Cl₂ extract of *P. odorata* extract was compared with equal amounts CHCl₃ extract to evaluate the bioactive properties contained in the different extract types. Scheme 2 (page 32) shows the workflow of preparing the CHCl₃ extract of *P. odorata*.

4.1.2 Extraction with chloroform (CHCl₃)



Scheme 2 Extraction with CHCl₃

20g of dried powdered plant material were dissolved in 200 ml MeOH (1:10), extracted by ultrasonication at room temperature for 10 min and at 79 °C on a reflux water bath for 1 hour. Then, the solvent was filtrated through a round filter and the obtained filtrate was evaporated until dryness and redissolved in 100 ml MeOH:H₂O (9:1).

The retained MeOH extract was degreased with 100 ml PE. The mixture of immiscible liquids was put into a separating funnel, shaken and allowed to settle for three times. The obtained PE extract was evaporated and stored. The aqueous MeOH fraction was mixed with 100 ml H₂O (1:1). By using a separating funnel the partition with 200 ml CHCl₃ was carried out.

The aqueous extract was evaporated and stored. The CHCl₃ extract was washed twice with 200 ml saline solution (NaCl 1 %) and spiked with Na₂SO₄ (Natrium sulfuricum siccatum). After filtering through a round filter the chloroform extract was evaporated.

The dried CHCl₃ extract of *P. odorata* (260 mg) was dissolved in 1.5 ml 96 % ethanol for subsequent testing. The bioactivity of the CHCl₃- and CH₂Cl₂ extracts was compared by a Proliferation assay.

4.2 ANTI-PROLIFERATIVE ACTIVITY OF *P. ODORATA* EXTRACTS

To compare the anti-neoplastic activity of the CH₂Cl₂- and CHCl₃ extracts, the different extract types were tested in HL60 leukemia cells. Subsequent investigations were performed with the more potent extract type.

The cells were incubated with increasing concentrations (mg amounts of dried plant material before extraction) of the dissolved extract types. As shown in figure 6, both extracts indicated anti-proliferative effects. Whereas the stronger anti-proliferative activity was observed in the CH₂Cl₂ extract, which inhibited growth by ~ 75% at 0.5 mg plant material/ml, the inhibition of growth of CHCl₃ extract was weaker, approximately 60% at 0.5 mg plant material/ml after 72 hours of incubation.

Therefore, the CH₂Cl₂ extract was also tested by an apoptosis assay in MCF7 breast cancer cells, in which the cell death rate was more than 50% when treated with a concentration of 4 mg/ml after 96 hours (figure 7).

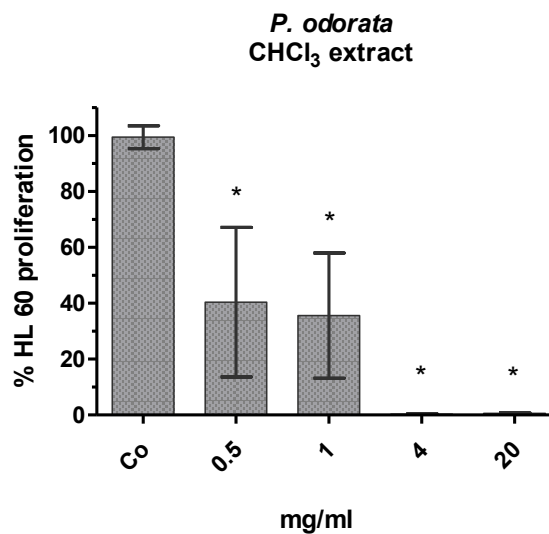
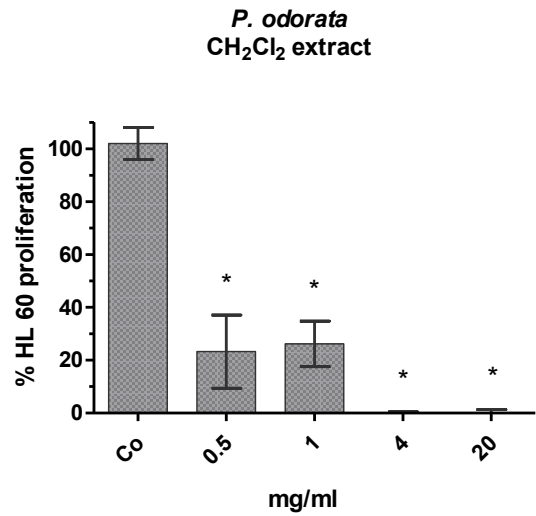


Figure 6 Anti-proliferative effect of CH₂Cl₂ - and CHCl₃ extract of *P. odorata*

HL60 cells were seeded into T-25 tissue culture flasks (1×10^5 cells/ml), incubated with 0.5, 1, 4 and 20 mg/ml of CH₂Cl₂ and CHCl₃ extract, respectively (mg-amounts relate to dried plant material before extractions) for 72 hours. Controls received 0.15 % to 0.30 % EtOH which was the highest EtOH concentration, cells had to experience in association with highest extract concentration. Equalization of EtOH was done in all samples to achieve same solvent conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

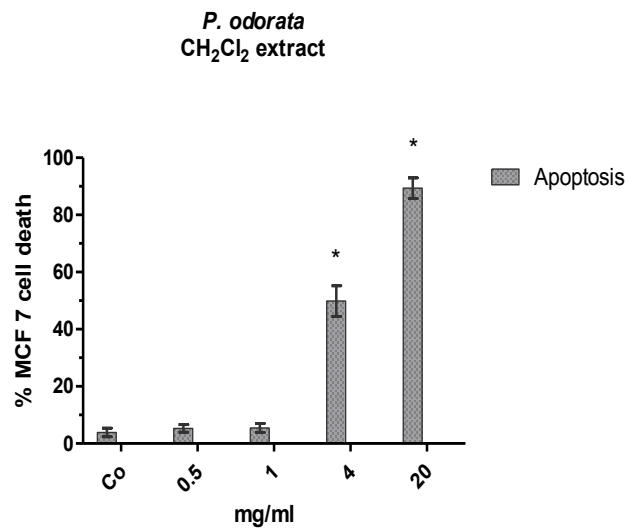


Figure 7 Induction of apoptosis by the CH₂Cl₂ extract of *P. odorata*

MCF7 cells were seeded in 96-well plates. Cells were allowed to attach over night and then they were incubated with 0.5, 1, 4 and 20 mg/ml of plant CH₂Cl₂ extract (mg-amounts relate to dried plant material before extractions) for 96 hours. Then, cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Nuclei with morphological changes which indicated apoptosis or necrosis were counted and percentages of vital, apoptotic and necrotic cells were calculated. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

4.2.1 Stock calculation – *Pluchea odorata*

Table 5 shows the extract weight in mg derived from 20 g freeze dried plant for the two different solvent types (dichloromethane, chloroform) and the extract amount in μg corresponding to 1 mg plant.

Four different concentrations were used to perform Proliferation assays: 0.5 mg/ml, 1 mg/ml, 4 mg/ml, 20 mg/ml (dried plant weight in mg/ml medium). These concentrations corresponded to different amounts of dried extract in $\mu\text{g/ml}$ medium.

The plant extracts were dissolved in different amounts of EtOH (3ml or 1.5ml, respectively).

solvent	extract weight (mg) derived from 20 g dried plant	extract (μg) corresponding to 1 mg plant
dichloromethane	279.5	13.98
chloroform	260.0	13.00
extract type	concentration mg dried plant weight/ml medium	μg dried extract/ml medium
dichloromethane	0.5	6.98
	1	13.97
	4	55.89
	20	279.48
chloroform	0.5	6.50
	1	13.00
	4	52.00
	20	260.00

Table 5 Stock calculation of *P. odorata* CH_2Cl_2 and CHCl_3 extract

Extract weight derived from 20 g dried plant material, extract amount corresponding to 1 mg plant and amounts of dried extract corresponding to different concentrations (dried plant weight/ml medium) of Proliferation assays.

4.3 EXTRACTION OF *P. ODORATA* with CH₂Cl₂ by ASE 200 – Comparison with common CH₂Cl₂ extraction

To compare the bio-activity of the conventional CH₂Cl₂ extract with the ASE - CH₂Cl₂ extract the lyophilised and milled plant material of *P. odorata*, 17.5 g plant powder, was extracted by ASE 200. The first cycle was performed with PE to eliminate chlorophyll and lipids, afterwards the same plant material was extracted three times with CH₂Cl₂. The supernatant was evaporated and the weight of the obtained dried extract (630 mg) was measured. The extraction was performed with a pressure of 150 bar and at 40°C.

For testing the ASE CH₂Cl₂ extract cells were incubated with increasing concentrations extract (shown in table 6 below). For further investigations an additional amount of 192 g lyophilised and milled plant material was extracted by ASE.

4.3.1 Stock calculation – *Pluchea odorata* ASE - CH₂Cl₂ extract

solvent	extract weight (mg) derived from 17.5g dried plant	extract (µg) corresponding to 1mg plant
dichloromethane	630	36
extract type	concentration mg dried plant weight/ml medium	µg dried extract/ml medium
dichloromethane	0.028	1
	0.139	5
	0.278	10
	0.417	15

Table 6 Stock calculation of *P. odorata* ASE - CH₂Cl₂ extract

Extract weight derived from 17.5 g dried plant material, extract amount corresponding to 1 mg plant and amounts of dried extract corresponding to different concentrations (dried plant weight/ml medium) of Proliferation assay.

The extraction by ASE 200 Accelerated Solvent Extractor was more efficient. In comparison with the conventional CH₂Cl₂ extraction the obtained extract amount was higher. The amounts are shown in table 7:

dried plant material in g	obtained dried extract in g	obtained dried extract in % of dried plant powder
extraction by ASE:		
192 g	4 g	2.08 %
17.5 g	0.630 g	3.6 %
conventional extraction:		
20 g	0.279 g	1.4 %

Table 7 Comparing obtained amount of dried extract by ASE and conventional extraction, respectively.

Less time for extraction was needed by ASE and the anti-proliferative activity of the conventional CH₂Cl₂ extract and the ASE - CH₂Cl₂ extract was similar (shown in figures 8 and 9). Because an extract concentration of 15 µg/ml or 13.97 µg/ml, respectively, inhibited HL60 cells proliferation by approximately 70 % after 72 hours. Therefore further extraction of *P. odorata* with dichloromethane was done by ASE.

4.3.2 Anti-proliferative activity of *P. odorata* ASE extract vs. conventional extract

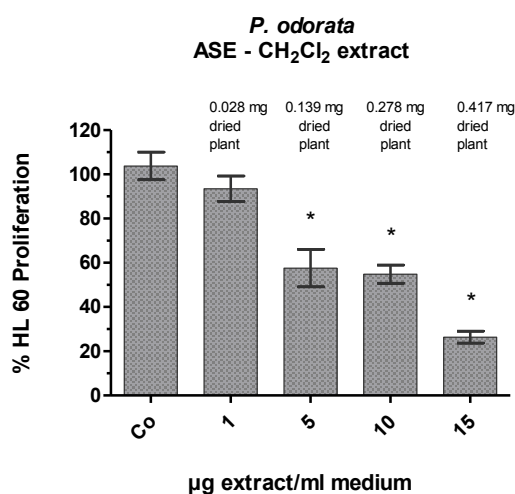


Figure 8 Anti-proliferative effect of ASE - CH₂Cl₂ extract of *P. odorata*

HL60 cells were seeded into T-25 tissue culture flasks (1×10^5 cells/ml), incubated with 1, 5, 10 and 15 µg/ml of ASE - CH₂Cl₂ extract for 72 hours. Control received 0.18 % EtOH which was the highest EtOH concentration, cells had to experience in association with highest extract concentration. Equalization of EtOH was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment. The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

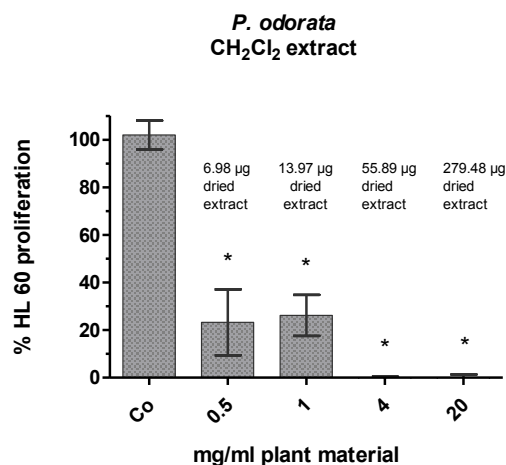


Figure 9 Anti-proliferative effect of CH₂Cl₂ extract of *P. odorata*

HL60 cells were seeded into T-25 tissue culture flasks (1×10^5 cells/ml), incubated with 0.5, 1, 4 and 20 mg/ml of CH₂Cl₂ extract (mg-amounts relate to dried plant material before extractions) for 72 hours. Control received 0.30 % EtOH which was the highest EtOH concentration, cells had to experience in association with highest extract concentration. Equalization of EtOH was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment. The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

4.3.3 Testing of different TLC systems – fingerprint of CH₂Cl₂ extract

To evaluate an adequate TLC system, 0.5 g of dried *P. odorata* CH₂Cl₂ extract was dissolved in 2 ml CH₂Cl₂. Spots of 20µl were applied on silica gel plates. To obtain the best separation 6 different solvent systems (less polar to polar) were tested (conditions see chapter 3.8.1, p21). Detection of each solvent system was performed under UV₂₅₄ with or without ASR.

Solvent system 5 (CHCl₃:MeOH:H₂O; 70ml:22ml:3.5ml) showed a suitable separation of the rather apolar CH₂Cl₂ extract. Hence, this TLC solvent system was used for further TLC analysis (figure 10).

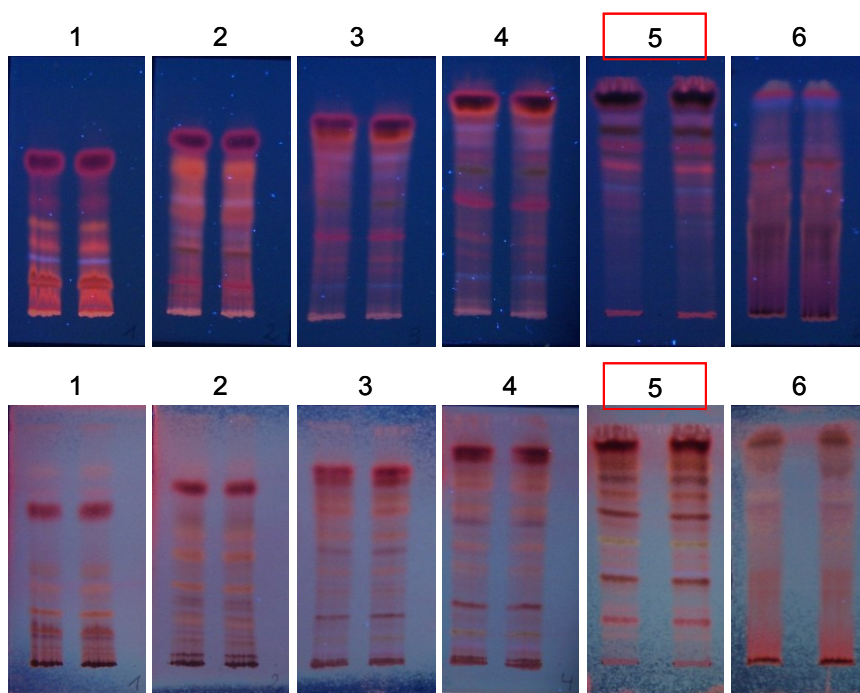


Figure 10 Above: *P. odorata* CH₂Cl₂ extract; solvent system 1-6; under UV₂₅₄ without reagent
Below: *P. odorata* CH₂Cl₂ extract; solvent system 1-6; under UV₂₅₄ with ASR

4.4 FRACTIONATION of ASE - CH₂Cl₂ EXTRACT of *P. ODORATA*

192 g dried and milled plant material of *P. odorata* was extracted with CH₂Cl₂ by ASE (see page 19). The obtained ASE - CH₂Cl₂ extract (4 g) was fractionated by VLC (F1, see page 22). The 10 resulting fractions (F1/1 – F1/10) were tested with regard to their anti-proliferative activity, whereby fraction F1/2 (apolar CHCl₃ – fraction) and fraction F1/3 (apolar CHCl₃:MeOH 9:1- fraction) showed significant effects.

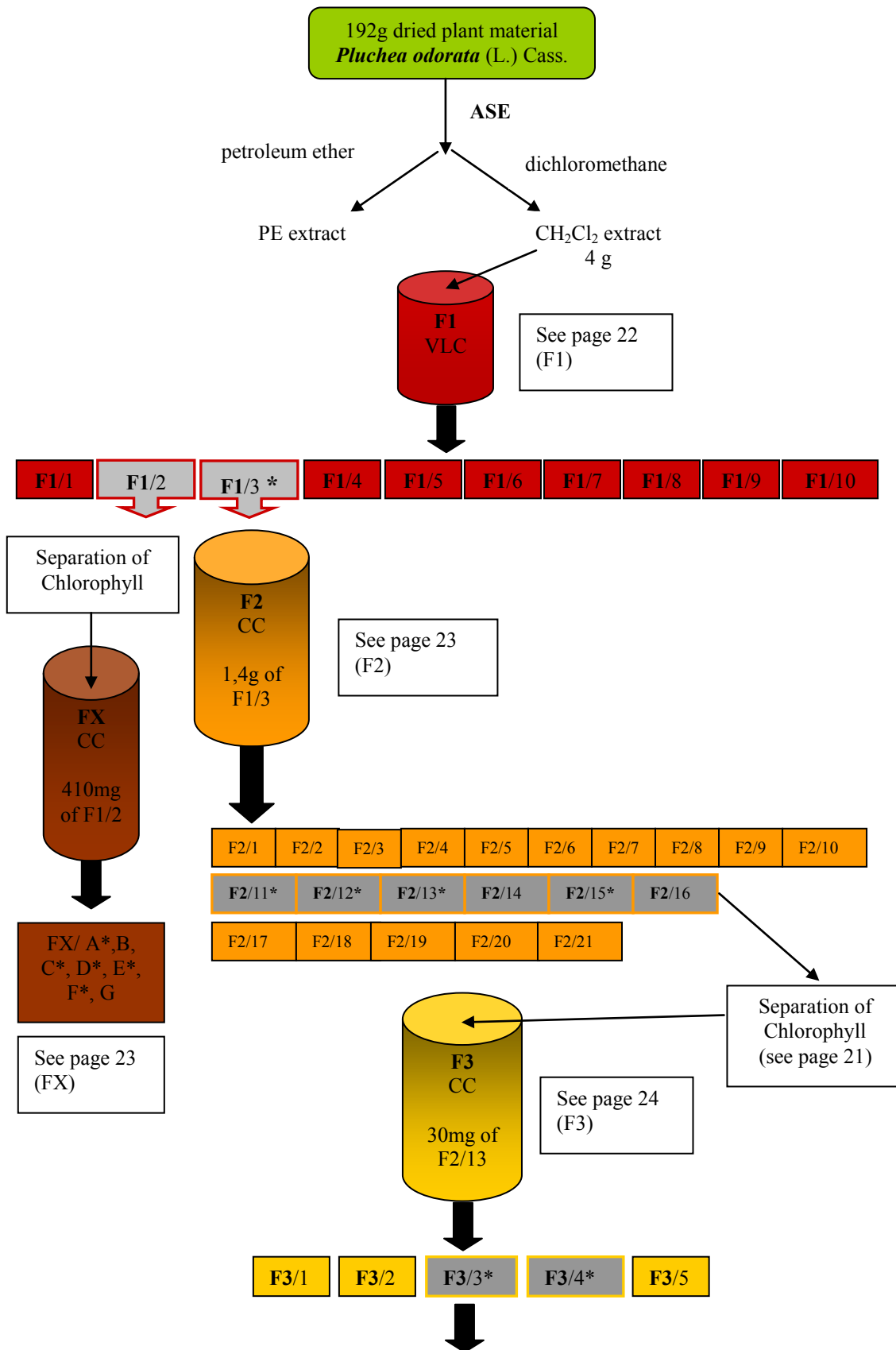
The fractionation of chlorophyll-free fraction F1/2 followed by CC (FX, see page 23), finally resulting in 7 fractions (potent fractions FX/A,C,D,E,F and inactive FX/B,FX/G). Investigations on fraction F1/2 stopped at this point, because it turned out that the presence or absence of chlorophyll did not make a difference regarding the anti-proliferative activity.

Fraction F1/3 was further fractionated by CC (F2, see page 23). Its 21 resulting fractions (F2/1 – F2/21) were tested concerning anti-proliferative activity, whereby fractions F2/11,12,13,14,15 and 16 (solvent relation CHCl₃:MeOH:H₂O 95:1.5:0.1 to 90:3.5:0.2, apolar range) showed significant growth inhibition.

Then, chlorophyll was separated from fractions F2/11 – F2/16, because it obscured the barmarks on TLC`s. From a quantitative point of view the biggest fraction, F2/13, was fractionated by CC (F3, see page 24) and the 5 obtained fractions (F3/1 – F3/5) were tested by Proliferation assay which showed the significant anti-proliferative activity of fractions F3/3 and F3/4.

Therefore fraction F3/3 was further fractionated by SPE (F4, see page 24), whereby 5 fractions were obtained. Fraction F4/1 and F4/2 were reunified and again fractionated by SPE (F5, see page 25). The steps of fractionation ended in two fractions, F5/1 and F5/2. The following scheme (see page 42f) overviews and details the workflow of fractionation steps, whereby the most active fractions are signed with an asterisk.

4.4.1 Fractionation – overview



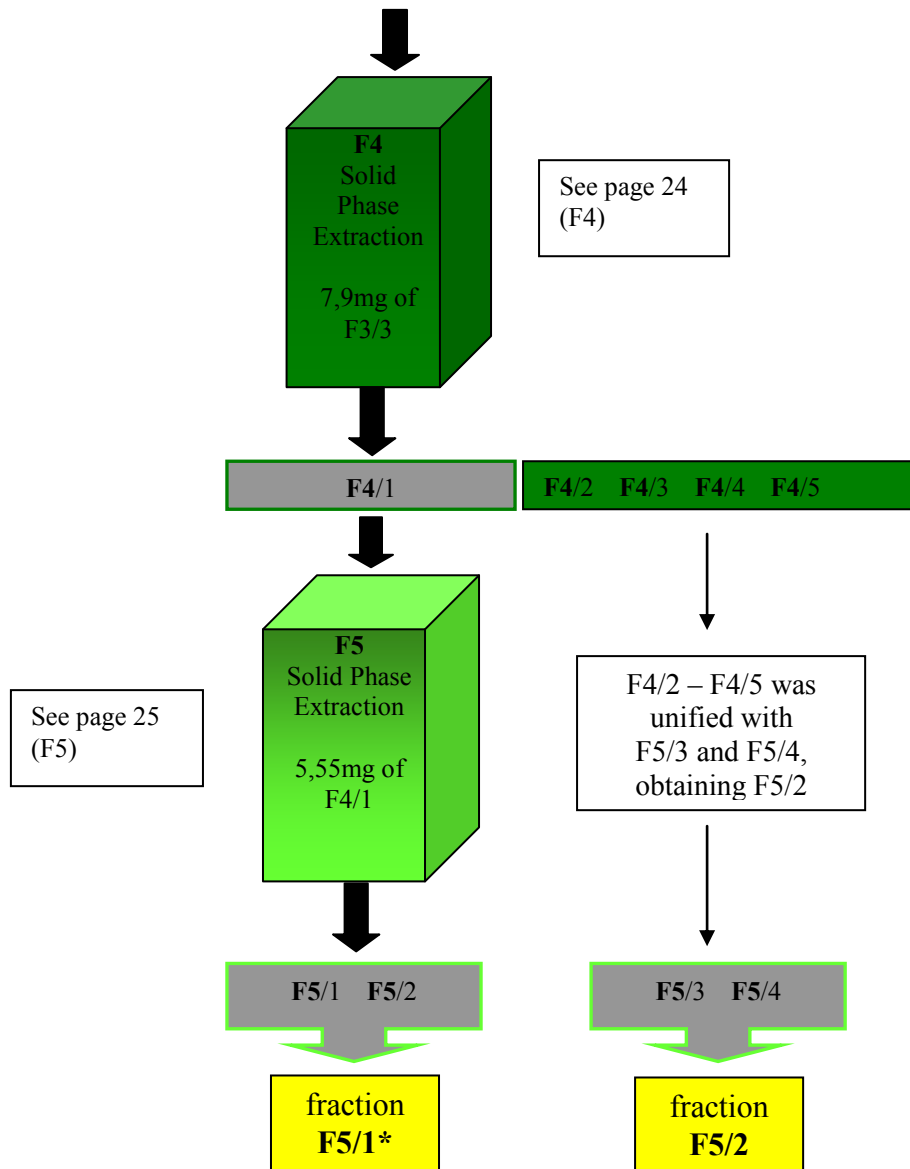


Figure 11 Fractionation steps - overview

4.4.2 F1 Vacuum Liquid Chromatography (VLC)

4 g of the CH₂Cl₂ extract of *P. odorata* was fractionated by VLC (see methods, p 22). 1000 ml of each of the mobile phases (solvents listed in table 8) was used for fast elution with vacuum. Solvent systems differed from TLC solvent systems due to different conditions between the separation techniques.

The 10 resulting fractions were analysed by TLC (figure 13). Ongoing they were evaporated to dryness and summed up, yielded a total weight of 3.95g (table 8), thus 98.8 % of starting material (4 g) was recovered. The biggest obtained fraction was the apolar CHCl₃ - fraction F1/2 (1.36 g) and the apolar (CHCl₃:MeOH 9:1) F1/3 (1.46 g). Resulting fractions (g) are also shown in a bar graph (figure 12).

fraction	solvent	relation	yield (g)
F1/1	PE		0.21
F1/2	CHCl ₃		1.36
F1/3	CHCl ₃ : MeOH	9:1	1.46
F1/4	CHCl ₃ : MeOH	7:3	0.45
F1/5	CHCl ₃ : MeOH	5:5	0.09
F1/6	CHCl ₃ : MeOH	3:7	0.14
F1/7	CHCl ₃ : MeOH	1:9	0.09
F1/8	MeOH : H ₂ O	7:3	0.08
F1/9	MeOH : H ₂ O	1:1	0.05
F1/10	H ₂ O		0.02
total			3.95

Table 8 Obtained fractions of VLC in gram and corresponding mobile phases

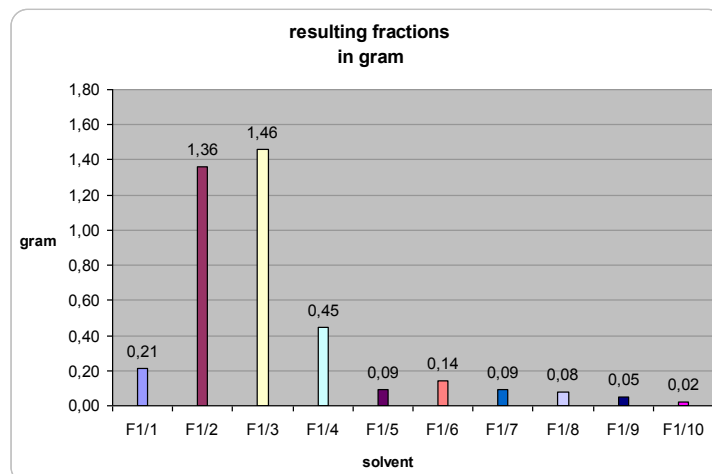
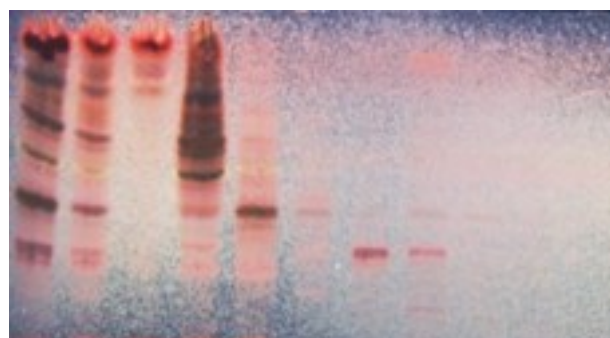


Figure 12 The type and amount of obtained VLC fractions (in gram) of the *P. odorata* ASE – CH₂Cl₂ extract

4.4.2.1 TLC of fractions F1/1 to F1/10

For comparison 20 µl of each fraction was applied on a silica gel plate.

Solvent system 5 (CHCl₃:MeOH:H₂O 70:22:3.5 ml) was used as mobile phase. After samples were separated, the TLC plate was sprayed with ASR, followed by heating at 100°C for 5 min to facilitate visualization, and analysed under UV₂₅₄ (see chapter 3.8.1, methods page 21).



P.o. F1/1 F1/2 F1/3 F1/4 F1/5 F1/6 F1/7 F1/8 F1/9 F1/10

Figure 13 TLC: *P. odorata* CH₂Cl₂ extract (P.o.); fractions F1/1 – F1/10; detection: ASR under UV₂₅₄; solvent system 5; by using the solvent systems PE and CHCl₃ all apolar components were eluted; by elution with CHCl₃:MeOH 9:1 most of components were obtained in apolar fraction F1/3; only few components seemed to be little more polar (see F1/5 to F1/10).

4.4.2.2 Anti-proliferative activity of fractions F1/1 to F1/10

To test the anti-proliferative activity of fractions F1/1 to F1/10 they were dissolved in DMSO and fraction F1/10 in DMSO, distilled water and EtOH (table 9). Fractions varied in solubility.

fraction	stock for proliferation assay
F1/1	1370µg/ 330µl DMSO
F1/2	1300µg/ 240µl DMSO
F1/3	1100µg/ 160µl DMSO
F1/4	1500µg/ 80µl DMSO
F1/5	1000µg/ 80µl DMSO
F1/6	1260µg/ 80µl DMSO
F1/7	1150µg/ 80µl DMSO
F1/8	1120µg/ 80µl DMSO
F1/9	1000µg/ 80µl DMSO
F1/10	1100µg/ 60µl DMSO + 100µl Aqua dest. + 30µl EtOH

Table 9 Stock solutions of F1/1 – F1/10 for Proliferation assay

HL60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells per ml, and incubated for 72 hours with 10 µg/ml of each fraction. Cells were counted after 24, 48 and 72 hours.

Fraction F1/3 (CHCl₃:MeOH 9:1) showed a significant growth inhibition up to 60 % at a concentration of 10µg/ml. The starting ASE – CH₂Cl₂ extract of *P. odorata* (10µg/ml) showed an effective growth inhibition around 40 % (see figure 8, p39). Thus, the bio-activity of F1/3 increased by 20 %. After 48 hours of incubation also fractions F1/2, F1/4 and F1/5 were active in comparison to control (figure 14; figure 15). Based on these results fractionation of F1/2 and F1/3 followed (see page 48-59).

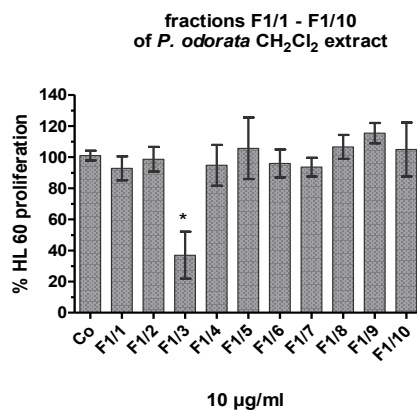


Figure 14 Anti-proliferative effect of fractions F1/1 to F1/10

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 10 µg/ml of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

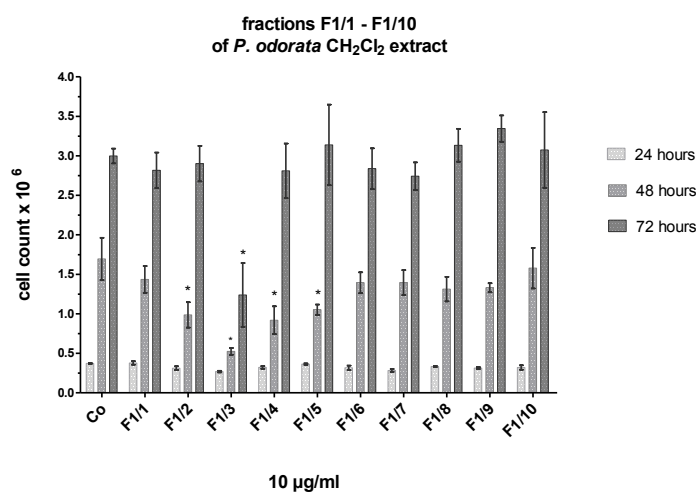


Figure 15 Anti-proliferative effect of fractions F1/1 to F1/10 (cell count)

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 10 µg/ml of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment and count of cells is shown as bar chart. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

4.4.3 FX Column Chromatography (CC) – fractionation of F1/2

4.4.3.1 Separation of chlorophyll of fraction F1/2

1.36 g of F1/2 was dissolved in 204 ml CH_2Cl_2 and then 204 ml of methanol-water (1:1) were added. The CH_2Cl_2 was evaporated at 40°C and 600-800 bar. The chlorophyll precipitated in the methanol-water phase and was filtered off under vacuum (see chapter 3.7, p21). The weight of the obtained dried fraction without chlorophyll was measured (410.6 mg). Therefore, 30.2 % of the initial fraction contained chlorophyll-free bioactitiy.



Figure 16 TLC: A: F1/2 **with chlorophyll**; detection: under UV_{366} ; solvent system 4
B: F1/2 **without chlorophyll**; detection: under UV_{366} ; solvent system 4

4.4.3.2 Fractionation of chlorophyll-free fraction F1/2 by CC

410.6 mg of fraction F1/2 were dissolved in 6 ml chloroform and mixed with 8 g of silica gel. Afterwards the solvent was evaporated to dryness and stored in a desiccator for further use. After refining it in a mortar it was placed on top of the 80x2 cm column, filled with 150 g silica gel, and covered with sea sand.

For the elution of all contained compounds a gradient from apolar to middle polar was chosen. Therefore three different mobile phases were used: The first phase was 430 ml of $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$ (100:10:1), whereby the elution rate was approximately 7 ml solvent per 30 minutes. This was followed by the second phase consisting of 200 ml $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$

(90:10:0.1) and finally 286 ml of the third middle polar phase consisting of CHCl_3 :MeOH:H₂O (70:22:3.5). The last two mobile phases were passed through the column by applying vacuum.

85 resulting fractions were collected and every fifth was tested by TLC (figure 17) to obtain a representative transect of fractions. Since distinct bar mark patterns occurred in the fractions FX/15 to FX/40 each single fraction was retested again by TLC using solvent system 4 (CHCl_3 :MeOH:H₂O 80:10:1) (figure 18). After comparing every fraction with each other, similar-looking fractions were pooled, finally resulting in 7 fractions (FX/A, B, C, D, E, F, G) (figure 19, 20, 21).

Screening was performed with solvent system 4 (80:10:1). For detection UV₃₆₆, UV₂₅₄ and visible light were used, before and after spraying the plate with anisaldehyd sulphuric acid reagent (ASR).

4.4.3.2.1 TLC of fractions FX/1 to FX/85

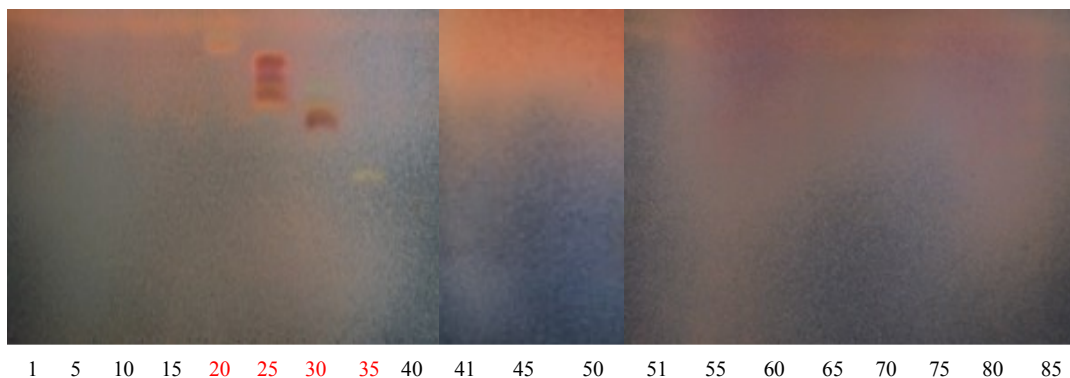


Figure 17 TLC: Fractions FX/1 – FX/85; detection: ASR under UV_{366} ; solvent system 4; every fifth fraction was tested. The apolar fractions 20, 25, 30 and 35 contained different compounds. No bar marks were detected after fraction FX/36 (see figure 18 below).

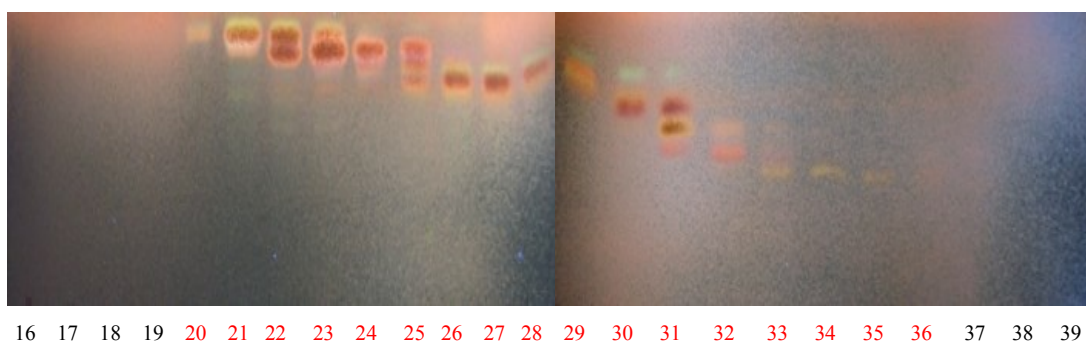


Figure 18 TLC: Apolar fractions FX/16 – FX/39 in detail; detection: ASR under UV_{366} ; solvent system 4; similarly looking fractions were pooled (containing one or more identical bar marks), finally resulting in 7 fractions, eluted by an apolar solvent system ($CHCl_3:MeOH:H_2O$ 100:10:1) (table 10 below).

Similarly looking fractions (based on similar bar mark patterns), that were obtained from CC-fractionation of F1/2, were recombined as follows:

obtained fractions	recombined as fraction:
1 - 21	FX/A
22 - 25	FX/B
26 - 29	FX/C
30 - 32	FX/D
33 - 50	FX/E
51 - 68	FX/F
69 - 85	FX/G

Table 10 Nomenclature of recombined fractions

4.4.3.2.2 TLC of fractions FX/A-G

The obtained 85 fractions, that were finally pooled in 7 fractions FX/A, B, C, D, E, F and G were reanalysed by TLC. For visualization, bar marks of each fraction were detected by different methods and wave lengths (see figures 19, 20, 21 below).

4.4.3.2.2.1 Fractions FX/A – G detected under UV₂₅₄, UV₃₆₆ and under UV₃₆₆ after spraying with ASR

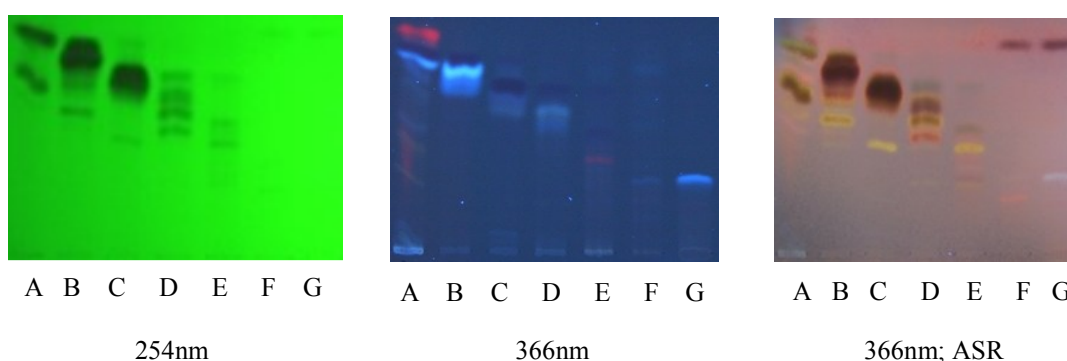


Figure 19 TLC: Fractions FX/A – G; detection: under UV₂₅₄; solvent system 4; using this detection method, bar marks of fraction A, B and C didn't appear separated. No bar marks were visible in fraction F and G.

Figure 20 TLC: Fractions FX/A – G; detection: under UV₃₆₆; solvent system 4 using this detection method, fraction A and B seemed to have an identical blue shining compound. Fraction C, D and E showed a blurred pattern. A luminous compound was visible in fraction G and the same ones, lower concentrated and therefore poor bright, in fraction F.

Figure 21 TLC: Fractions FX/A – G; detection: ASR under UV₃₆₆; solvent system 4 using this detection method, contained compounds of fractions are plainly visible.

The anti-proliferative activity of FX/A, FX/C, FX/D, FX/E and FX/F showed an inhibition of growth around 100 % at a concentration of 10µg/ml (figure 22, page 53). Fraction FX/B inhibited proliferation for around 30 %. The TLC pattern shows differences in main components of these fractions.

4.4.3.3 Anti-proliferative activity of fractions FX/A - FX/G

For testing the anti-proliferative activity of fractions FX/A to FX/G, they were first dissolved in CHCl₃ stock solution. Then the volume, which contained 1 mg fraction, was transferred into a reaction vial (table 11), dried under air stream and dissolved in 500 µl DMSO, resulting in working stocks of 2 µg/µl for each fraction.

fraction	yield (mg)/ CHCl₃ (µl)	CHCl₃ stock solution volume (µl) containing 1 mg fraction
FX/A	40.6/1500	37
FX/B	153.5/2000	13
FX/C	47.9/1200	25
FX/D	24.8/1500	60.5
FX/E	9.2/1000	109
FX/F	4.0/1000	250
FX/G	6.7/1000	150

Table 11 Prepared stock solutions of FX/A – FX/G for Proliferation assay

To determine the anti-proliferative effect of the fractions FX/A – FX/G, HL60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells per ml, and incubated for 72 hours with 10 µg/ml of each fraction. Cells were counted after 24, 48 and 72 hours.

Fractions FX/A, FX/C, FX/D, FX/E and FX/F inhibited HL60 growth up to nearly 100 % at a concentration of 10 µg/ml. In comparison, fraction FX/B inhibited proliferation only by 30 %. Due to changing affinity of potent principles to the stationary phase fraction FX/B was different and hereby less active (figure 22).

The starting material, the *P. odorata* ASE - CH₂Cl₂ extract, showed an inhibition of proliferation of 40 % at a concentration of 10 µg/ml (see figure 8, p39). After separation of the chlorophyll content, which was about 2/3 of this fraction (F1/2), the anti-proliferative activity was fully maintained and found in 5 different fractions (FX/A, FX/C – FX/F). Thus, most of the non-active content was eliminated and the active principle became increasingly purified.

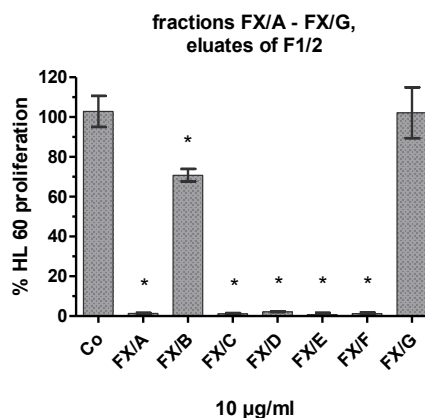


Figure 22 Anti-proliferative effect of fractions FX/A to FX/G

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 10 µg/ml of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

4.4.4 F2 Column Chromatography (CC) - fractionation of F1/3

In course of the first fractionation of *P. odorata* ASE – CH₂Cl₂ extract by VLC, 10 fractions (F1/1 – F1/10) were obtained and tested for anti-proliferative effect. Thereby fraction F1/3 was the most potent one after 72 hours of incubation (conc. 10 µg/ml) (figure 14, p 47) and contained the highest amount of obtained material (1.46 g).

Furthermore, treatment with fractions F1/2 and F1/4 showed decreased the cell numbers in comparison to control, but the obtained amount of material were less (1.36 and 0.45 g, respectively), than for F1/3. Consequently the fractionation of F1/3 (1.4 g) followed.

A fluid reservoir for different elution solvents was fixed above the fractionation column (table 12 below). The passage of the first four mobile phases was by gravity, and the fifth mobile phase MeOH:H₂O (95:5) was passed through the column by applying vacuum.

Some of the resulting 24 fractions (F2/19+20; F2/21+22+23) were pooled again, because they exhibited similar bar mark patterns. Then, these resulting 21 fractions were evaporated to dryness and the fraction weight was determined, which added up to 1.76 g (table 13, page 56). The fact that more material was regained after fractionation (1.76g) compared to the input (1.40g) is most likely due to minimal washout of column material.

mobile phase	relation	amount (ml)
CHCl ₃		500
CHCl ₃ : MeOH : H ₂ O	95 : 1.5 : 0.1	600
CHCl ₃ : MeOH : H ₂ O	90 : 3.5 : 0.2	562
CHCl ₃ : MeOH : H ₂ O	85 : 8 : 0.5	2000
MeOH : H ₂ O	95 : 5	500

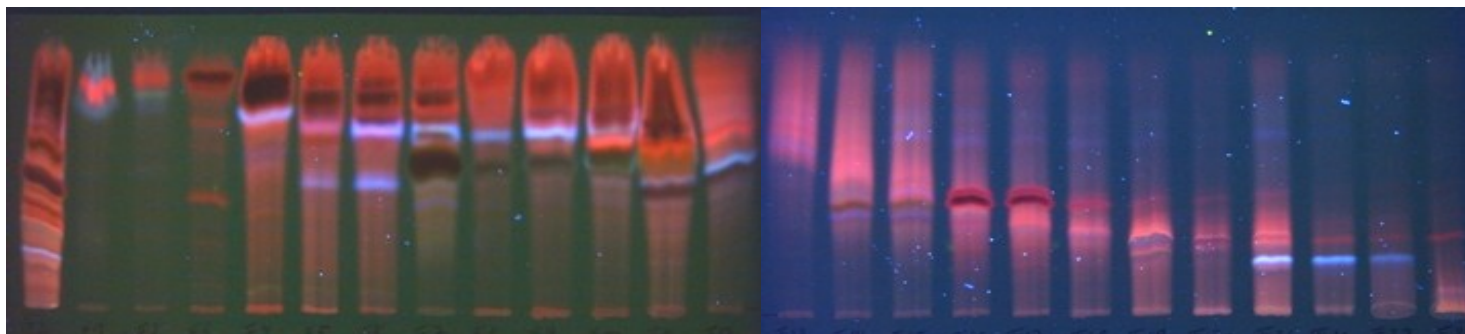
Table 12 Mobile phases of the fractionation of F1/3

Due to the apolar property of the starting material, fraction F1/3, the chosen gradient for mobile phases used for the subsequent fractionations (obtaining fractions F2/1 – 24) was also in an apolar range in the beginning to less polar MeOH:H₂O 95:5 at the end.

4.4.4.1 TLC of fractions F2/1- 24

The obtained 24 fractions, that were finally pooled in 21 fractions, were analysed by TLC. Screening was performed with solvent system 4 (80:10:1). For detection UV₃₆₆ was used, before and after spraying the plate with ASR (figures 23 and 24).

4.4.4.1.1 TLC of F2/1 – 24

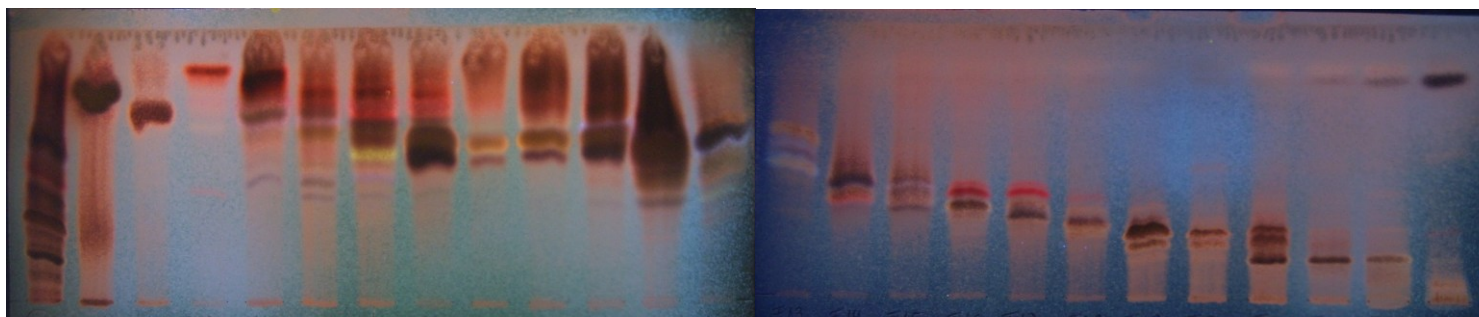


F1/3 F2/1 F2/2 F2/3 F2/4 F2/5 F2/6 F2/7 F2/8 F2/9 F2/10 F2/11 F2/12 F2/13 F2/14 F2/15 F2/16 F2/17 F2/18 F2/19 F2/20 F2/21 F2/22 F2/23 F2/24

Figure 23 Fractions F1/3; F2/1-24; solvent system 4; under UV₃₆₆ **without** ASR

Chlorophyll is luminous red, all fractions differs in main compounds and concentration, respectively. All obtained fractions followed an apolar gradient, except fraction F2/24 was eluted by the middle polar solvent system MeOH:H₂O 95:5.

Fractions F2/11 to F2/16 showed the highest and similar anti-proliferative activity, but TLC pattern shows differences in main compounds.



F1/3 F2/1 F2/2 F2/3 F2/4 F2/5 F2/6 F2/7 F2/8 F2/9 F2/10 F2/11 F2/12 F2/13 F2/14 F2/15 F2/16 F2/17 F2/18 F2/19 F2/20 F2/21 F2/22 F2/23 F2/24

Figure 24 Fractions F1/3, F2/1-24; solvent system 4; under UV₃₆₆ **with** ASR

Some bar marks are covered by chlorophyll, whereby visualization with ASR facilitates improved analysis.

4.4.4.2 Obtained fractions F2/1 to F2/21

fraction	solvent	relation	yield (mg)
F2/1	CHCl ₃		32.5
F2/2			30.5
F2/3			12.2
F2/4	▼		54.2
F2/5			17.8
F2/6			26.4
F2/7	CHCl ₃ :MeOH:H ₂ O	95 : 1.5 : 0.1	67.2
F2/8			24.8
F2/9	▼		48.5
F2/10			32.9
F2/11			113.2
F2/12	CHCl ₃ :MeOH:H ₂ O	90 : 3.5 : 0.2	90.7
F2/13			170.6
F2/14			88.6
F2/15	▼		26.3
F2/16			148.5
F2/17			44.1
F2/18	CHCl ₃ :MeOH:H ₂ O	85 : 8 : 0.5	64.9
F2/19			184.6
F2/20	▼		268.0
F2/21	MeOH:H ₂ O	95 : 5	213.0
total			1,760.0 mg

Table 13 Obtained 21 fractions in mg and correlated solvent systems

4.4.4.3 Anti-proliferative activity of fractions F2/1 to F2/21 (including chlorophyll)

For the preparation of the stock solutions to be tested in the Proliferation assay, fractions F2/1-21 were dissolved in DMSO and centrifuged at 12,000 rpm for 5 min. to precipitate insoluble debris. The fractions were divided into 5 groups (A, B, C, D, E) and tested in separate experiments to avoid biases and to assess the reproducibility of the fraction types and of the test system (table 14).

fraction	stock for proliferation assay
A1 = F2/1	1.06 mg/300µl DMSO
A2 = F2/6	1 mg/300µl DMSO
A3 = F2/11	1 mg/400µl DMSO
A4 = F2/16	1.21 mg/300µl DMSO
A5 = F2/21	1.09 mg/300µl DMSO
B1 = F2/2	0.98 mg/400µl DMSO
B2 = F2/7	1.18 mg/300µl DMSO
B3 = F2/12	1.15 mg/400µl DMSO
B4 = F2/17	1.16 mg/300µl DMSO
C1 = F2/3	0.76 mg/400µl DMSO
C2 = F2/8	0.60 mg/300µl DMSO
C3 = F2/13	1.10 mg/400µl DMSO
C4 = F2/18	1.25 mg/400µl DMSO
D1 = F2/4	1.17 mg/300µl DMSO
D2 = F2/9	1 mg/300µl DMSO
D3 = F2/14	1.14 mg/300µl DMSO
D4 = F2/19	1.07 mg/300µl DMSO
E1 = F2/5	1.11 mg/300µl DMSO
E2 = F2/10	0.64 mg/300µl DMSO
E3 = F2/15	0.99 mg/300µl DMSO
E4 = F2/20	1.61 mg/300µl DMSO

Table 14 Prepared stock of F2/1 to F2/21 for Proliferation assay

To determine the anti-proliferative effect of the fractions F2/1 to F2/21 HL60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells per ml. Cells were incubated for 72 hours with $10 \mu\text{g/ml}$ per fraction sample. The DMSO concentration was adjusted for every sample to 0.5 % (correlated with $10 \mu\text{g/ml}$).

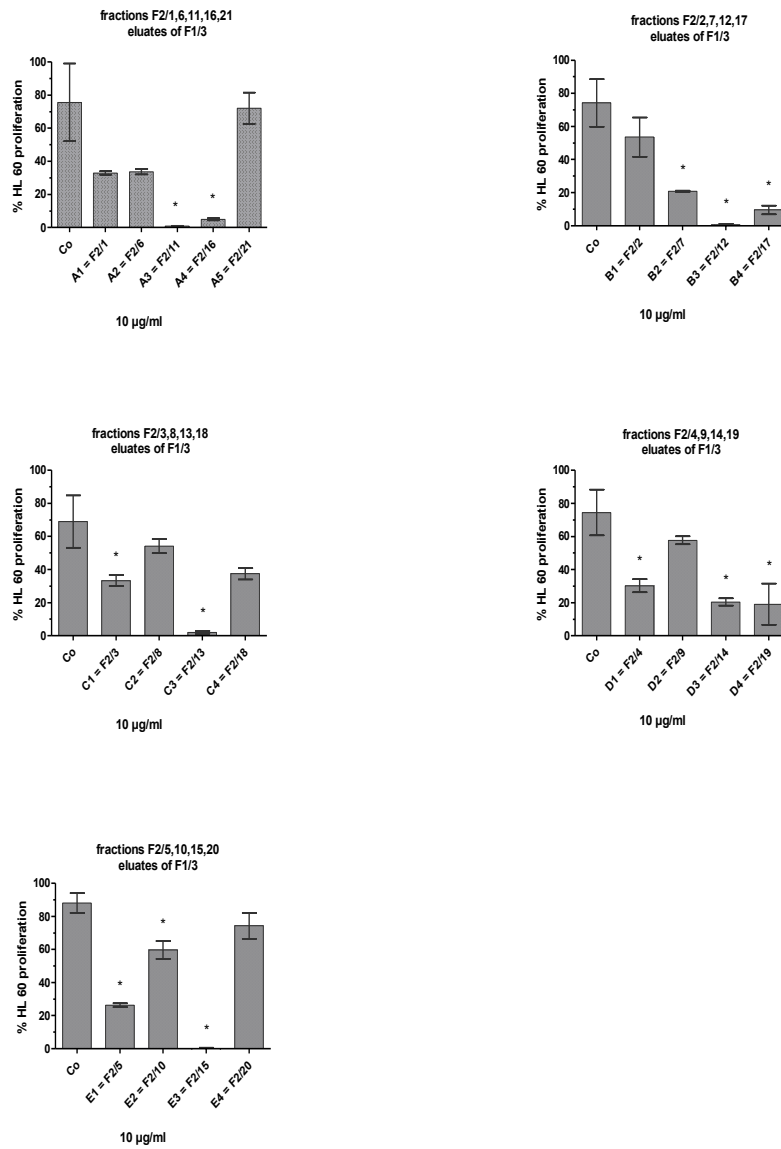


Figure 25 Anti-proliferative effect of fractions F2/1 to F2/21 (including chlorophyll)

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with $10 \mu\text{g/ml}$ of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

Fractions F2/11, F2/12, F2/13 and F2/15 showed an effective growth inhibition up to nearly 100 %, F2/16 up to 95 % and F2/14 up to 80 % at a concentration of 10 µg/ml (figure 25).

The overall starting extract, the *P. odorata* ASE – CH₂Cl₂ extract, inhibited HL60 cell proliferation up to 45 % after 72 hours of incubation with 10 µg extract/ ml medium. To recapitulate, fraction F1/2 showed a modification concerning count of cells after 48 h in comparison with control, but no significant anti-proliferative effect after 72 hours of incubation. But after separating chlorophyll and subsequent fractionation, yielding fractions of F1/2, fractions FX/A, C, D, E and F, inhibited HL60 growth up to nearly 100 % (conc. 10 µg/ml; incubation 72h)

Fraction F1/3 had an effective growth inhibition up to nearly 65 % at a concentration of 10 µg/ ml. After fractionation of F1/3, the resulting fractions F2/1 to F2/21 were tested. F2/11,12,13 and 15 showed an inhibition of growth up to 100%, therefore in the fractions F2/11 – 13 and F2/15 non-active compounds were not only eliminated, but the bioactive properties become further enriched.

Hence, a considerable enrichment of bioactive properties was attained, as the inhibition of growth by the starting ASE - CH₂Cl₂ extract was only 40 %, whereas fractions F2/11,12,13,15 and 16 and FX/A, C, D, E, F, respectively, inhibited cell proliferation by 100 %.

4.4.4.4 Separation of chlorophyll of fractions F2/11,12,13,14,15 and 16

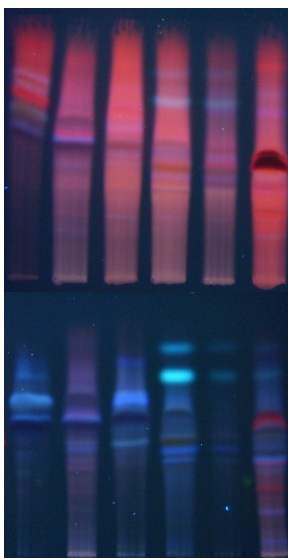
Only those fractions sharing highest anti-proliferative activity were selected to separate the chlorophyll. Without chlorophyll it is easier to identify each TLC - bar mark, to facilitate the separation of different compounds after visualization on TLC plates. Further investigations were only performed with fractions after the separation of chlorophyll.

Each fraction was mixed with adequate volume of CH₂Cl₂ and MeOH:H₂O (1:1) (table 15 below, methods p21). Then, CH₂Cl₂ was evaporated at 40°C and 600-800 bar. The chlorophyll precipitated in the methanol-water phase and was filtered off. The weight of the obtained dried fractions without chlorophyll was measured (table 15).

fraction with chlorophyll	CH₂Cl₂ (ml)	MeOH:H₂O (1:1) (ml)	yield of fraction without chlorophyll (in mg)	yield of fraction without chlorophyll (in %)
F2/11 (113.3 mg)	17	17	25.7	23
F2/12 (90.8 mg)	13.5	13.5	16.3	18
F2/13 (170.6 mg)	25.5	25.5	41.2	24
F2/14 (88.6 mg)	13.2	13.2	27.9	32
F2/15 (26.4 mg)	4	4	2.4	9
F2/16 (148.5 mg)	22	22	21.6	15

Table 15 Fractions F2/11-16 including chlorophyll (mg); adequate volume of CH₂Cl₂ and MeOH:H₂O for separating procedure; yield (mg) of fraction after separation; yield (%) of separated chlorophyll

4.4.4.4.1 TLC of fractions F2/11,12,13,14,15,16



F2/11 F2/12 F2/13 F2/14 F2/15 F2/16

Figure 26 Above: TLC of fractions F2/11,12,13,14,15,16 with chlorophyll (coloured red), solvent system 4; under UV_{366} without ASR

Below: TLC of fractions F2/11,12,13,14,15,16 after separation of chlorophyll, solvent system 4; under UV_{366} without ASR

Separation was not complete in F2/16, as it still shows red bar marks
Concerning the anti-proliferative effect no differences were observed between fractions with or without chlorophyll, therefore the chlorophyll is inactive.

4.4.4.2 Anti-proliferative activity of fractions F2/11, 12, 13, 14, 15 and 16 after separating chlorophyll

To determine the influence of chlorophyll on the anti-proliferative activity, fractions F2/11 – 16 were also tested after the separation of chlorophyll. HL60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells per ml. Then they were incubated for 72 hours with 10 $\mu\text{g/ml}$ of each fraction (dissolved in DMSO). No differences in effects were observed between chlorophyll-containing or chlorophyll-free fractions (figure 27 and 28 below).

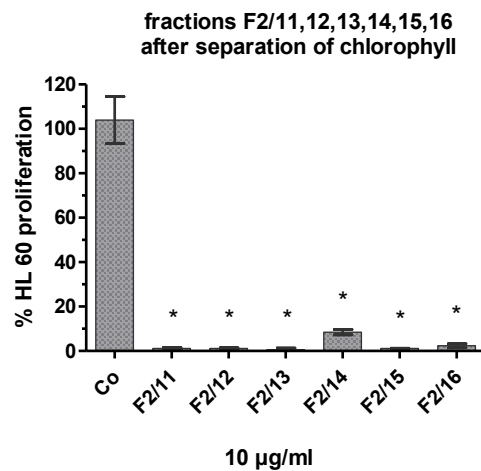


Figure 27 Anti-proliferative effect of fractions F2/11 to F2/16 without chlorophyll

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 10 $\mu\text{g/ml}$ of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

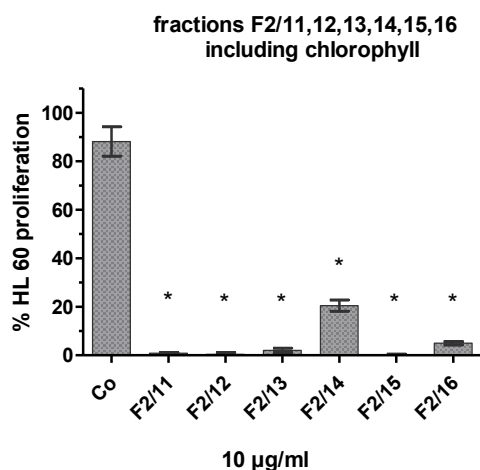


Figure 28 Anti-proliferative effect of fractions F2/11 to F2/16 including chlorophyll

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 10 µg/ml of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

After separation of chlorophyll fractions F2/11,12,13,15 and 16 showed an effective growth inhibition up to 100 % and fraction F2/14 up to 90 % at a concentration of 10µg/ml (figure 27).

4.4.4.4.3 Obtained fractions F2/11 to F2/16 after separating chlorophyll (in mg)

Based on initial weight of the fractions (including chlorophyll) from which the final fraction weight after separation of chlorophyll was subtracted, the calculated difference in weight was considered as the amount of chlorophyll. Possibly, also non-chlorophyll constituents were separated (figure 29).

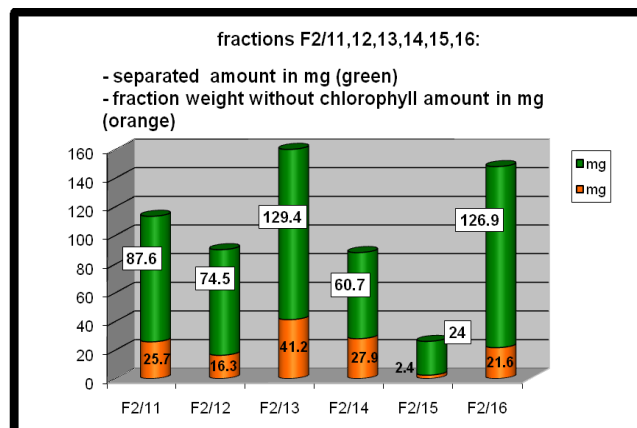


Figure 29 Histogram of fraction weight: chlorophyll included and separated, respectively

Fraction F2/11 contained 77 % chlorophyll, F2/12 contained 82 %, F2/13 76 %, F2/14 68 %, F2/15 91 % and F2/16 85 %. After separation of the chlorophyll content, which was about 68 % to 91 % of these fractions, the anti-proliferative activity was fully maintained and found in these 6 different fractions (F2/11 – F2/16).

About 23 % of the initial fraction F2/11 contained chlorophyll-free bio-activity, furthermore 18 % of F2/12, 24 % of F2/13, 32 % of F2/14, 9 % of F2/15 and about 15 % of F2/16.

Relating to fraction F1/2 30.2 % of the initial fraction contained chlorophyll-free bioactivity. After separation of the chlorophyll content, which was about 2/3 of this fraction (F1/2), the anti-proliferative activity was fully maintained and found in 5 different fractions (FX/A, FX/C – FX/F).

This means that non-active content was eliminated and the active principle became increasingly purified.

4.4.5 F3 Column Chromatography (CC) - fractionation of F2/13

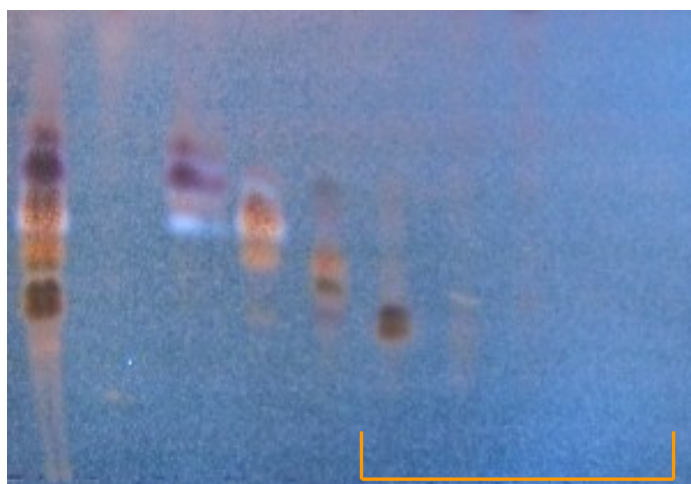
41.2 mg were obtained in fraction F2/13 after the separation of chlorophyll, which was the highest fraction amount. Therefore, 30 mg of F2/13 was dissolved in 2 ml CHCl_3 and used for further fractionation by Column Chromatography F3.

The CHCl_3 solution was mixed with 430 mg silica gel and evaporated to dryness. It was placed on top of a column (98x1 cm), filled with 75 g silica gel. The powdered sample was covered with sea sand. The mobile phase, CHCl_3 :MeOH (95:5; 700 ml), was of higher polarity than fraction F2/13, to achieve slower elution and therefore a more precise separation.

Approximately 2 ml fraction, that were obtained after elution, were collected in 318 tubes (altogether approximately 700 ml). Ongoing further 300 ml CHCl_3 :MeOH (95:5) and 300 ml CHCl_3 :MeOH (90:5) were passed through the column by applying vacuum, whereby the last two fractions were obtained.

To screen a representative transect of fractions, every tenth was tested by TLC (Mobile phase CHCl_3 :MeOH:H₂O: glacial acetic acid 90:10:0.1:0.1). For detection UV₃₆₆, UV₂₅₄ and visible light were used, before and after spraying the plate with anisaldehyd sulphuric acid reagent (ASR).

Fractions with similar looking bar mark pattern were pooled again. The 5 obtained fractions F3/1 – 5 were evaporated to dryness and had a total weight of 49.2 mg (table 16, figure 31). The fact, that more material was regained after fractionation (49.2 mg) compared to the input (30 mg), is most likely due to a washout of column material.



F2/13 F3/1 F3/2 F3/3 F3/4 pooled F3/5

combined fraction	yield (mg)
F3/1	3.4
F3/2	13.0
F3/3	7.9
F3/4	3.6
F3/5	21.3
	49.2

Figure 30 TLC of fractions F2/13, F3/1 – F3/5
solvent system CHCl_3 :MeOH:H₂O: glacial acetic acid
90:10:0.1:0.1; under UV₃₆₆ with ASR

Table 16 Fraction F3/1 - F3/5 in mg

Fractions F3/1 – F3/4 were obtained by the elution of column F3 with the mobile phase CHCl_3 :MeOH (95:5; 700 ml), fraction F3/5 resulted from elution with mobile phase CHCl_3 :MeOH (95:5; 300 ml) and mobile phase CHCl_3 :MeOH (90:5; 300 ml).

4.4.5.1 Obtained fractions F3/1 - 5 in mg

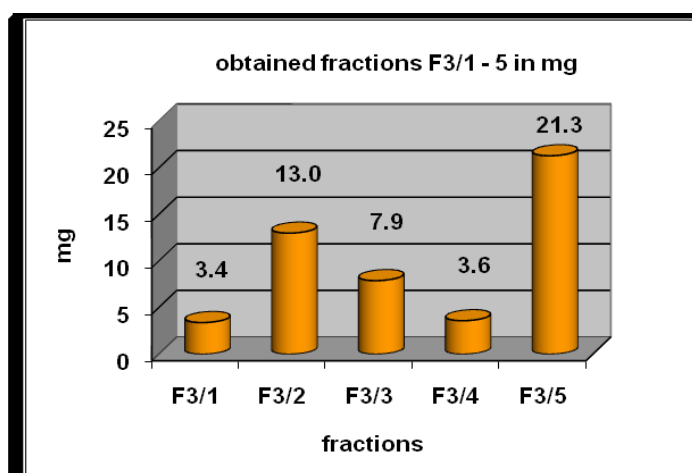


Figure 31 Fraction F3/1 to F3/5 in mg

4.4.5.2 Anti-proliferative activity of fractions F3/1 to F3/5

To determine the anti-proliferative effect of the fractions F3/1-5 HL60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells per ml. Then, they were incubated for 72 hours with $5 \mu\text{g/ml}$ of each fraction.

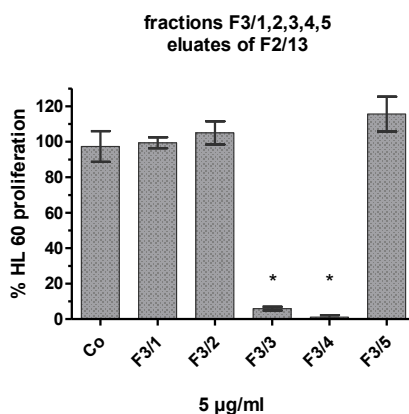


Figure 32 Anti-proliferative effect of fractions F3/1 to F3/5

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with $5 \mu\text{g/ml}$ of each fraction for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

Fractions F3/3 and F3/4 showed an effective growth inhibiting potential up to nearly 100 % at a concentration of $5 \mu\text{g/ml}$ after 72 hours (figure 32) and therefore, the bio-activity in F3/3 and F3/4 was approximately 200 % that of F2/13.

Fractions F3/4 and F3/3 were similar in anti-proliferative activity, but the obtained amount of F3/3 was higher (7.9 mg vs. 3.6 mg). Consequently the fractionation of F3/3 followed.

4.4.6 F4 Reversed Phase-Solid Phase Extraction (SPE) – fractionation of F3/3

For further accumulation of bio-active compounds, fraction F3/3 was fractionated by SPE. Firstly, the cartridge (Bond Elut C18; 1210–2058) was conditioned by washing the column with 2 RV (2 ml) MeOH and then dried by applying vacuum. After each step of fractionation with the different mobile phases the cartridge was dried for 2 min. before the next phase was applied.

Fraction F3/3 (7.9 mg) was dissolved in 500 μ l CHCl₃ and placed on top of the cartridge. By applying vacuum CHCl₃ passed through the column and fraction F4/1 was obtained. Additional fractions were collected by eluting the cartridge with 2 RV 90 % MeOH, further 2 RV 80 % MeOH, 2 RV 70 % MeOH and 4 ml CHCl₃:MeOH (1:1), getting fractions F4/2, F4/3, F4/4 and F4/5.

4.4.6.1 TLC of fractions F4/1 to F4/5

The obtained fractions were reanalysed by TLC, using the mobile phase CHCl₃:MeOH:H₂O:glacial acetic acid (90:10:0.1:0.1). For detection UV₃₆₆ was used after spraying the plate with ASR (figure 33 below).

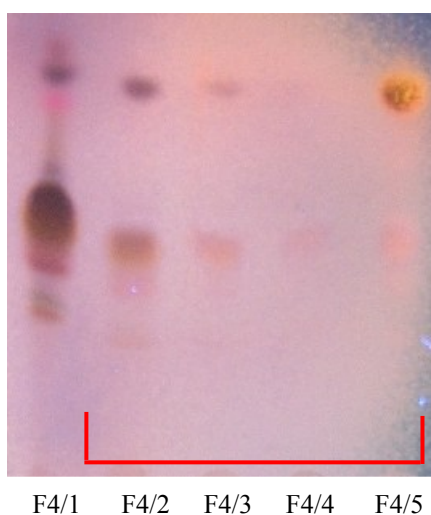


Figure 33 TLC of fractions F4/1 to F4/5; mobile phase CHCl₃:MeOH:H₂O:glacial acetic acid (90:10:0.1:0.1); detection under UV₃₆₆ with ASR

TLC showed poor separation of compounds, therefore similarly looking fractions F4/2, F4/3, F4/4 and F4/5 were pooled. The weight of obtained dried fractions was measured (F4/1: 5.55 mg; pooled fraction F4/2-5: 4 mg). A further separation step by Reversed Phase SPE (F5) with fraction F4/1 followed.

4.4.7 F5 Reversed Phase-Solid Phase Extraction (SPE) – fractionation of F4/1

Firstly the cartridge (Bond Elut C18; 1210–2058) was conditioned by washing the column with 2 RV (2 ml) MeOH and then dried by applying vacuum. After each step of fractionation with the different mobile phases the cartridge was dried for 2 min. before the next phase was applied.

Fraction F4/1 was dissolved in 80 μ l CHCl₃ and placed on top of the cartridge. By applying vacuum CHCl₃ passed through the column and fraction F5/1 was obtained.

Additional fractions were collected by eluting the cartridge with 2 RV 98 % MeOH, further 2 RV 96 % and 4 ml CHCl₃:MeOH (1:1), getting fractions F5/2, F5/3 and F5/4.

4.4.7.1 TLC of fractions F5/1 to F5/4

The obtained fractions were reanalysed by TLC, using the mobile phase CHCl_3 :MeOH:H₂O:glacial acetic acid (90:10:0.1:0.1). For detection UV₃₆₆ was used after spraying the plate with ASR (figure 34).

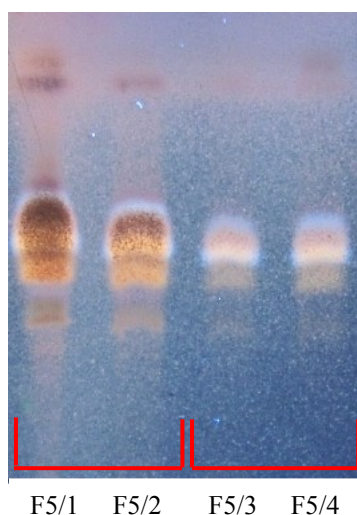


Figure 34 TLC of fractions F5/1 to F5/4; mobile phase CHCl_3 :MeOH:H₂O:glacial acetic acid (90:10:0.1:0.1); detection under UV₃₆₆ with ASR

Prepared TLC showed clear separation of F4/1, therefore F5/1 and F5/2 were pooled as one fraction. On the other hand F5/3 and F5/4 were pooled with fractions F4/2,3,4,5 of the F4 Reversed Phase SPE, because they showed similar bar mark pattern. Finally two fractions, named F5/1 and F5/2, were obtained and the anti-proliferative activity was tested (figure 35). All in all, there was not a significant further separation of compounds by applying SPE F5.

4.4.7.2 Anti-proliferative activity of fractions F5/1 and F5/2

To determine the anti-proliferative effect of through the last fractionation step obtained fractions F5/1 and F5/2 HL60 cells were seeded into 24-well plates at a concentration of 1×10^5 cells per ml. Cells were incubated for 72 hours with 10 $\mu\text{g/ml}$ per fraction sample. The DMSO concentration was adjusted for every sample to 0.5 % (correlated with 10 $\mu\text{g/ml}$).

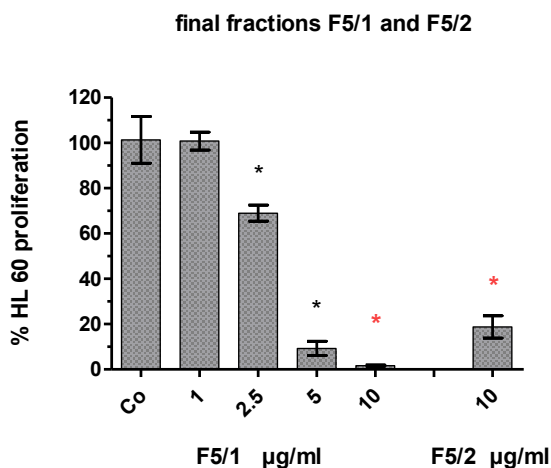


Figure 35 Anti-proliferative effect of fractions F5/1 and F5/2

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 1, 2.5, 5 and 10 $\mu\text{g/ml}$ of fraction F5/1 and 10 $\mu\text{g/ml}$ of fraction F5/2, respectively, for 72 hours. Control received 0.5 % DMSO which was the highest DMSO concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. All Asterisks indicate significant proliferation inhibition compared to control ($p < 0.05$). The red asterisks also mark the significance among the fractions F5/1 and F5/2 (conc. 10 $\mu\text{g/ml}$) and error bars SEM (see methods, page 20).

The initial fraction F3/3, before applying SPE F4 and SPE F5, showed an effective growth inhibiting potential up to nearly 100 % at a concentration of 5 $\mu\text{g/ml}$ after 72 hours (figure 32). Fraction F5/1 caused a cell growth inhibition around 100 % at a concentration of 10 $\mu\text{g/ml}$ (figure 35 above) and F5/2 showed 80 % inhibition.

Therefore no further enrichment of bioactive compounds in F5/1 and F5/2 was observed through the fractionation of F3/3 by SPE. But fraction F5/1 showed a significant bigger antiproliferative effect compared to F5/2.

Nevertheless, the advantage of this fractionation step was marginal and therefore the attempts to isolate the active principle(s) stopped at this point and mechanistic studies followed.

4.4.8 Final testing of potent fractions along the specific workflow

After finishing further fractionation steps Proliferation assays with potent fractions of workflow were done to directly compare the anti-proliferative effects at low fraction concentrations (3 $\mu\text{g/ml}$) and to demonstrate the enrichment of bio-activity.

Fractions F2/11, F2/13 and F3/4 showed the highest activities. Since, only fraction F2/11 and F2/13 are directly derived from the strong fraction F1/3 and F3/4 is directly derived from F2/13, we decided to further analyse fractions F2/11, F2/13 and F3/4. Therefore they were subsequently tested in Apoptosis assay and by Western blotting (see p76ff). Fractions FX/A to FX/G were eluates of F1/2, which was less potent than F1/3. FX/C and FX/E also showed significant activity and were set aside for future projects.

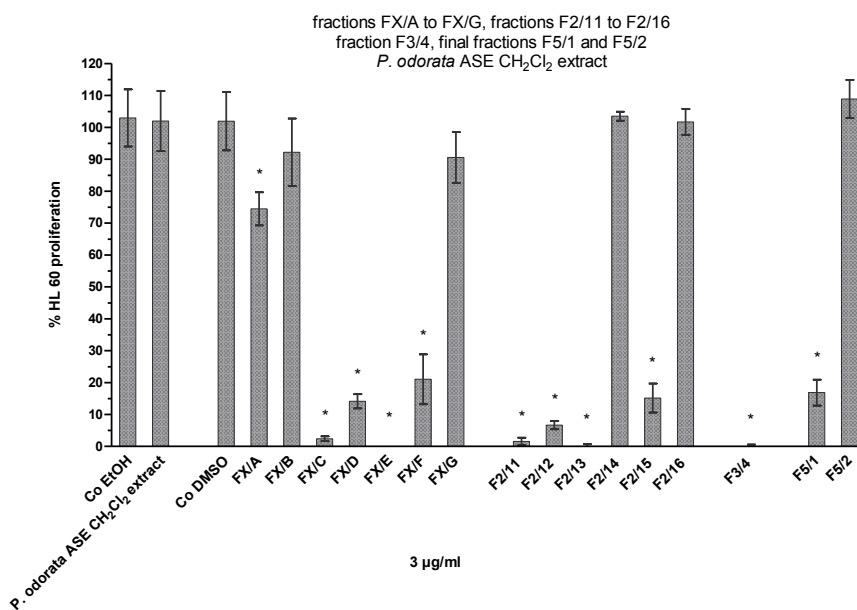


Figure 36 Anti-proliferative effect of *P. odorata* ASE CH_2Cl_2 extract and fractions FX/A to FX/G, F2/11 to F2/16, F3/4, F5/1 and F5/2

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 3 $\mu\text{g/ml}$ of each fraction for 72 hours. Control for all fractions received 0.5 % DMSO and control for *P. odorata* extract received 0.5 % EtOH (maximum), respectively, which was the highest concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO and EtOH, respectively, was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

To better illustrate and estimate the significant enrichment of potential active compounds during continuous fractionation, fractions of the successive workflow were tested by Proliferation assays, whereby HL60 cells were seeded into 24-well plates at a concentration of 1×10^5 cells per ml. Then they were incubated for 72 hours with $5 \mu\text{g/ml}$ and $10 \mu\text{g/ml}$, respectively, of fractions, which are shown in figure 37:

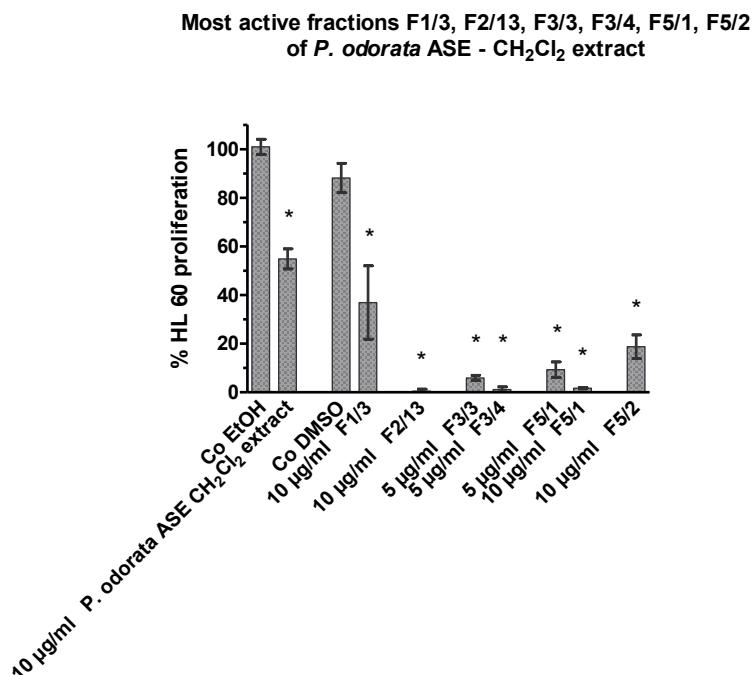


Figure 37 Anti-proliferative effect of fractions F1/3, F2/13, F3/3, F3/4, F5/1 and F5/2

HL60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 5 and $10 \mu\text{g/ml}$, respectively, of each fraction for 72 hours. Control for all fractions received 0.5 % DMSO and control for *P. odorata* ASE extract received 0.5 % EtOH (maximum), respectively, which was the highest concentration, cells had to experience in association with highest fraction/ extract concentration. Equalization of DMSO and EtOH, respectively, was done in all samples to achieve same conditions. Cells were counted after 24, 48 and 72 hours of treatment.

The percentage of proliferation between 24 and 72 hours was determined in comparison to control. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars SEM (see methods, page 20).

Fractions F3/4 and F3/3 were similar in anti-proliferative activity at a concentration of $5 \mu\text{g/ml}$, but the obtained amount of F3/3 was higher (7.9 mg vs. 3.6 mg). Consequently, the fractionation of F3/3 followed.

The obtained fractions F5/1 and F5/2 showed no significant level of activity, so evidently there was no further enrichment of active compounds after applying Solid Phase Extractions F4 and F5 (see page 68-73). Taken together the results shown in these graphs demonstrate that the enrichment of anti-proliferative activity from 10 µg/ml fraction F2/13 and F3/4 was at least more than 25 fold.

Fractions F2/11, F2/13 and F3/4 showed most significant anti-proliferative activity at a concentration of 3 µg/ml medium after 72 hours of incubation in HL60 cells. Therefore, they were subsequently tested by Western blot, analysing components of the cell cycle, because its deregulation is a hallmark of cancer and cell cycle protagonists such as Cyclin D1 and Cdc25A, oncogenes, which are upregulated in many cancer types (see page 77 ff).

4.5 ANALYSIS OF CELL CYCLE AND CHECKPOINT REGULATORS

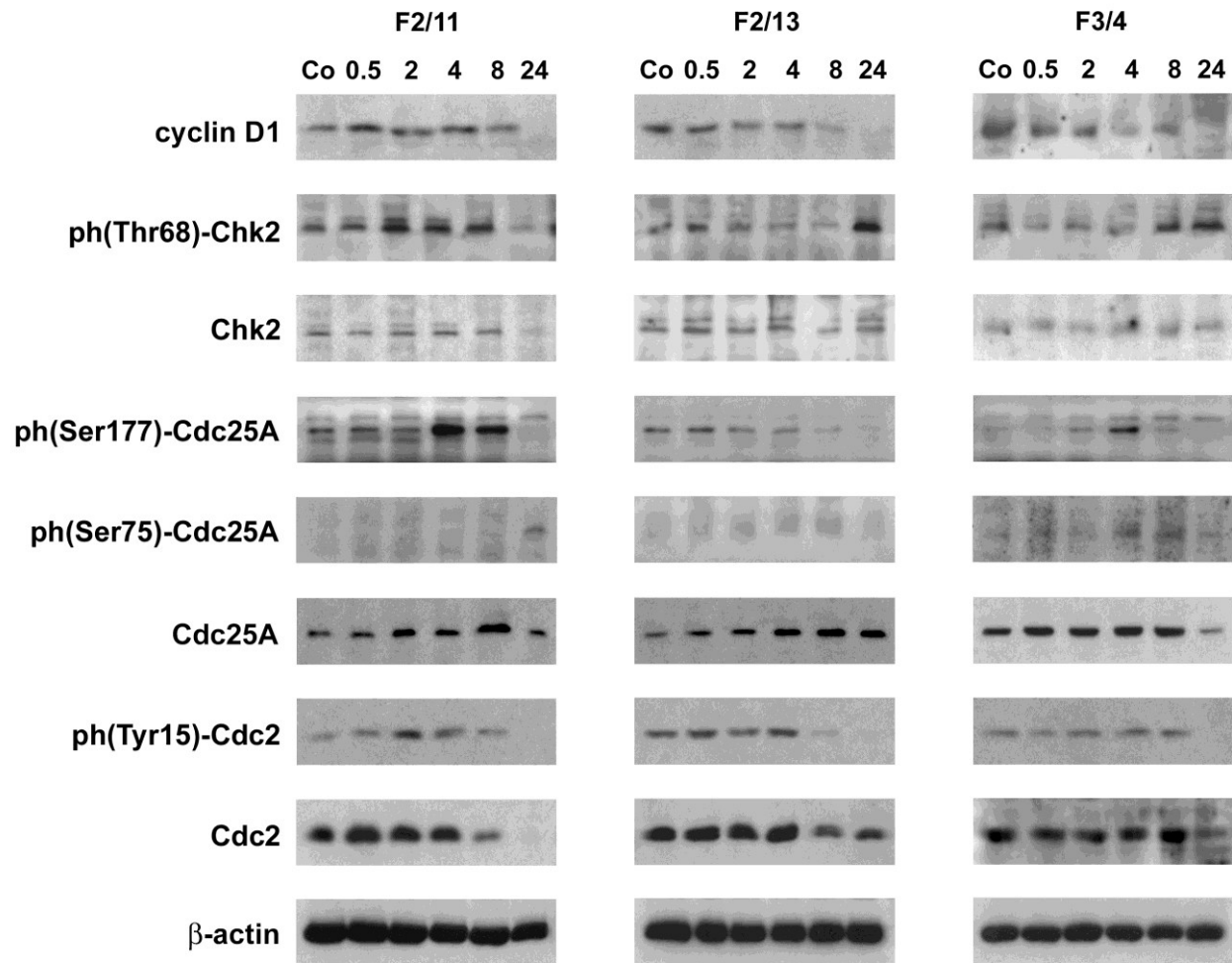


Figure 38 Analysis of cell cycle and checkpoint regulators

4.5.1 Cell cycle and checkpoint regulators

Each fraction, F2/11, F2/13 and F3/4, showed a significant anti-proliferative effect in HL60 cells (figure 36, p73), which was 100 % when cells were treated with 3 $\mu\text{g/ml}$ for 72 hours.

Exposure of HL60 cells to 3 $\mu\text{g/ml}$ fraction F2/11 completely blocked Cyclin D1 expression after 24 hours, whereas F2/13 reduced the Cyclin D1 level already after 8 hours and its consecutive fraction F3/4 down-regulated Cyclin D1 even after 30 minutes. This indicates, that F2/11 contains less potent bio-active principles targeting Cyclin D1 expression than F2/13. In F3/4, a direct fraction of F2/13, this activity was enriched, which constituted the aim of this work.

The proto-oncogene Cyclin D1 is an important regulator of G1 to S phase progression. It forms active complexes with its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6) that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein [ALAO, 2007]. The D family of cyclins is associated with various proliferative diseases. Temporally, the D cyclins appear in early G1, before the B cyclins, in the course of the cell cycle [XIONG et al. 1992a; XIONG et al., 1992b].

Depending on the type of DNA damage (genotoxic stress), ATM or ATR phosphorylates Chk2 or Chk1, which in turn phosphorylates Cdc25A. Thereby, Cdc25A becomes inactivated and causes the inactivation of Cyclin E-Cdk2.

The intra-S-phase checkpoint link functions to avoid the duplication of damaged or broken DNA which would eventually lead to genomic instability. This checkpoint is i.e. regulated by ATM/ATR-Chk2-Chk1-Cdc25A. Chk2 was phosphorylated at the activating Thr68 site upon treatment with all three fractions tested.

3 µg/ml F2/11 caused a rapid and transient phosphorylation of Chk2 within 2 hours of treatment and therefore its activation. In contrast, fraction F2/13 caused phosphorylation of Chk2 after 24 hours. Fraction F3/4 caused activation of Chk2 after 8 hours that intensified after 24 hours. Chk2 protein levels remained unchanged by incubation of HL60 cells with F2/13 and F3/4, but the level decreased upon incubation with F2/11 within 24 hours.

The analysis of Chk2 activity also supported the notion that different active principles are considered in F2/11 than in F2/13 and that the bioactive component(s) with higher activity becomes enriched in F3/4. Whereas F2/11 induces activation of Chk2 as the earliest of the events.

F2/13 and F3/4 induced Chk2 as the latest of the events analysed by Western blotting. This indicates that F2/11 contains a genotoxic component, which rapidly causes DNA damage and the subsequent gene expression alterations seem to be consequences of this genotoxicity.

In contrast the activation of Chk2 upon treatment with F2/13 and its derivative F3/4 seems to be the consequence of DNA damage caused by the comparatively late activation of Caspase 3 (see figure 41, page 83), which is activated earlier and stronger in F3/4 than in F2/13 and perfectly correlates with subsequent Chk2 activation.

Cdc25A is a direct target of Chk2 and Chk1 and activation of Chk2 can cause the phosphorylation of Ser177 of Cdc25A and Chk1 the phosphorylation of Ser75 of Cdc25A. Both phosphorylations inactivate Cdc25A.

F2/11 caused an intense phosphorylation of (Ser177)Cdc25A within 4 hours right after the activation of Chk2. Although, (Ser75)Cdc25A became phosphorylated, this was neither due to Chk1 nor to p38, because these kinases did not become activated (data not shown). In consequence, the phosphorylation level of Cdc2 increased because Cdc25A directly dephosphorylates this Cdc2 site, however when inactivated the kinase activity of Wee1 prevails, which genes rise to accumulating (Tyr15)Cdc2 phosphorylation.

In contrast, F2/13 caused a dephosphorylation of Cdc25A, which was shown to stabilise the protein [MADLENER et al., 2009] and also in this case the Cdc25A protein level increased due to reduced degradation rate and this was reflected by an increased dephosphorylation of (Tyr15)Cdc2 after 8 hours of treatment. Cdc2 (Cdk1) is mandatory for orchestrated G2-M transit. Cdc25A and Cdc25C dephosphorylate this protein, which activates its kinase domain. Cdc2 protein levels were much more stable in cells treated with F2/13 than with F2/11. This is consistent with the higher pro-apoptotic activity of F2/11 compared to F2/13 (page 83ff).

F3/4 caused a very transient phosphorylation of (Ser177)Cdc25A and a weak but more sustained phosphorylation of (Ser75)Cdc25A. This inactivation of Cdc25A may have caused the slight increase in (Tyr15)Cdc2 phosphorylation and the phosphorylation of (Ser75)Cdc25A seemed to be the trigger for its degradation after 24 hours.

4.6 INDUCTION OF APOPTOSIS UPON TREATMENT with *P. ODORATA* EXTRACT and FRACTIONS F2/11, F2/13, F2/14, F2/15 AND F3/4

In the second fractionation cohort, fractions F2/11 and F2/13 were most potent in the inhibition of proliferation. Fractions F2/14 and F2/15 were also tested in Apoptosis assay to monitor also their activity.

Fractions F3/4 and F3/3 were similar in anti-proliferative activity, but the obtained amount of F3/3 was higher (7.9 mg vs. 3.6 mg). Consequently, further fractionation was performed with F3/3, which consumed the entire material. Therefore, further investigations regarding apoptosis were done with fraction F3/4.

To determine the pro-apoptotic properties of the different fractions HL60 cells were seeded into 24-well plates at a concentration of 1×10^5 cells per ml. Cells were incubated for 24 hours with 3 $\mu\text{g/ml}$ per fraction sample and with 35 $\mu\text{g/ml}$ of *P. odorata* ASE - CH_2Cl_2 extract to demonstrate the enrichment of pro-apoptotic activities throughout the higher orders of fractionations. After incubation percentages of apoptotic cells were calculated.

Fraction F2/11 and F3/4 were the most active ones after 24 hours, because they induced apoptotic cell death in 100 % of HL60 cells. The higher concentrated *P. odorata* extract (35 $\mu\text{g/ml}$) was sufficient to eliminate around 65 % of HL60 cells, but the *P. odorata* extract at a low concentration of 3 $\mu\text{g/ml}$ was inactive (figure 39).

The most potent apoptotic fractions after 24 hours of incubation, F2/11 und F3/4, were further diluted (cells were incubated with 0.4 $\mu\text{g/ml}$, 0.8 $\mu\text{g/ml}$ and 1.5 $\mu\text{g/ml}$ of fractions). F2/11 caused 60 % cell death at a concentration of 1.5 $\mu\text{g/ml}$ medium, but fraction F3/4 caused 100% at the same concentration and even 60 % when applying the concentration of 0.8 $\mu\text{g/ml}$ medium and this result induced that the pro-apoptotic properties were enriched at least by 45 fold in fraction F3/4 (figure 39 and 40).

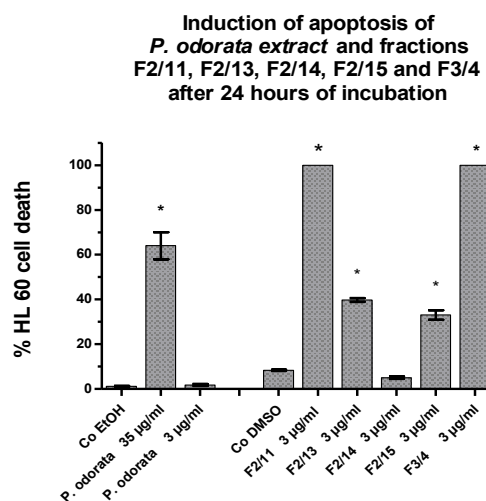


Figure 39 Induction of apoptosis by the *P. odorata* ASE - CH₂Cl₂ extract and proliferative active fractions F2/11, F2/13, F2/14, F2/15 and F3/4. HL60 cells were seeded into 24-well plates and incubated with *P. odorata* CH₂Cl₂ extract (conc. 35 µg/ml and 3 µg/ml) and with fractions F2/11, F2/13, F2/14, F2/15 and F3/4 (conc. 3 µg/ml for 24 hours). Controls and all fractions received 0.5 % DMSO and control for *P. odorata* ASE extract received 0.5 % EtOH, respectively, which was the highest concentration, cells had to experience in association with highest fraction/extract concentration. Then, cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Nuclei with morphological changes which indicated apoptosis or necrosis were counted and percentages of vital, apoptotic and necrotic cells were calculated. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control (p<0.05) and error bars SEM (see methods, page 20).

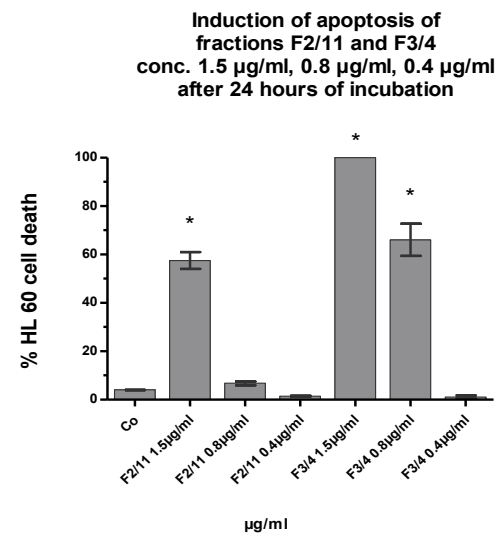


Figure 40 Induction of apoptosis by the active fractions F2/11 and F3/4. HL60 cells were seeded into 24-well plates and incubated with fractions F2/11 and F3/4 (conc. 1.5 µg/ml, 0.8 µg/ml, 0.4 µg/ml) for 24 hours. Control received 0.5 % DMSO, which was the highest concentration, cells had to experience in association with highest fraction concentration. Equalization of DMSO was done in all samples to achieve same conditions. Then, cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Nuclei with morphological changes which indicated apoptosis or necrosis were counted and percentages of vital, apoptotic and necrotic cells were calculated. Experiments were done in triplicate. Asterisks indicate significance compared to untreated control (p<0.05) and error bars SEM (see methods, page 20).

4.7 COMPARISON of the ANTI-PROLIFERATIVE ACTIVITY with the PRO-APOPTOTIC ACTIVITY of FRACTIONS F2/11, F2/13 and F3/4

fraction	anti-proliferative activity [conc. 3µg/ml]	pro-apoptotic activity [conc. 3µg/ml] after 8 hours	pro-apoptotic activity [conc. 3µg/ml] after 24 hours
F2/13	100 %	5 %	40 %
F2/11	98 %	20 %	100 %
F3/4	100 %	40 %	100 %

Table 17 Comparison of F2/11, F2/13 and F3/4 in anti-proliferative with pro-apoptotic activity

Proliferation assays with potent fractions of workflow were done and fractions F2/11, F2/13 and F3/4 showed the highest anti-proliferative activity at low fraction concentrations (3 µg/ml). Fraction F3/4 was the most pro-apoptotic active ones after 8 hours of incubation. Fraction F2/11 and F3/4 were the most active ones after 24 hours, because they induced apoptotic cell death in 100 % of HL60 cells.

Fractions F2/13, F2/11 and F3/4 were further tested regarding cell death induction in HL60 cells because a major property of cytotoxic anticancer drugs is the potential to elicit apoptosis.

4.8 ANALYSIS OF APOPTOSIS RELATED PROTEINS

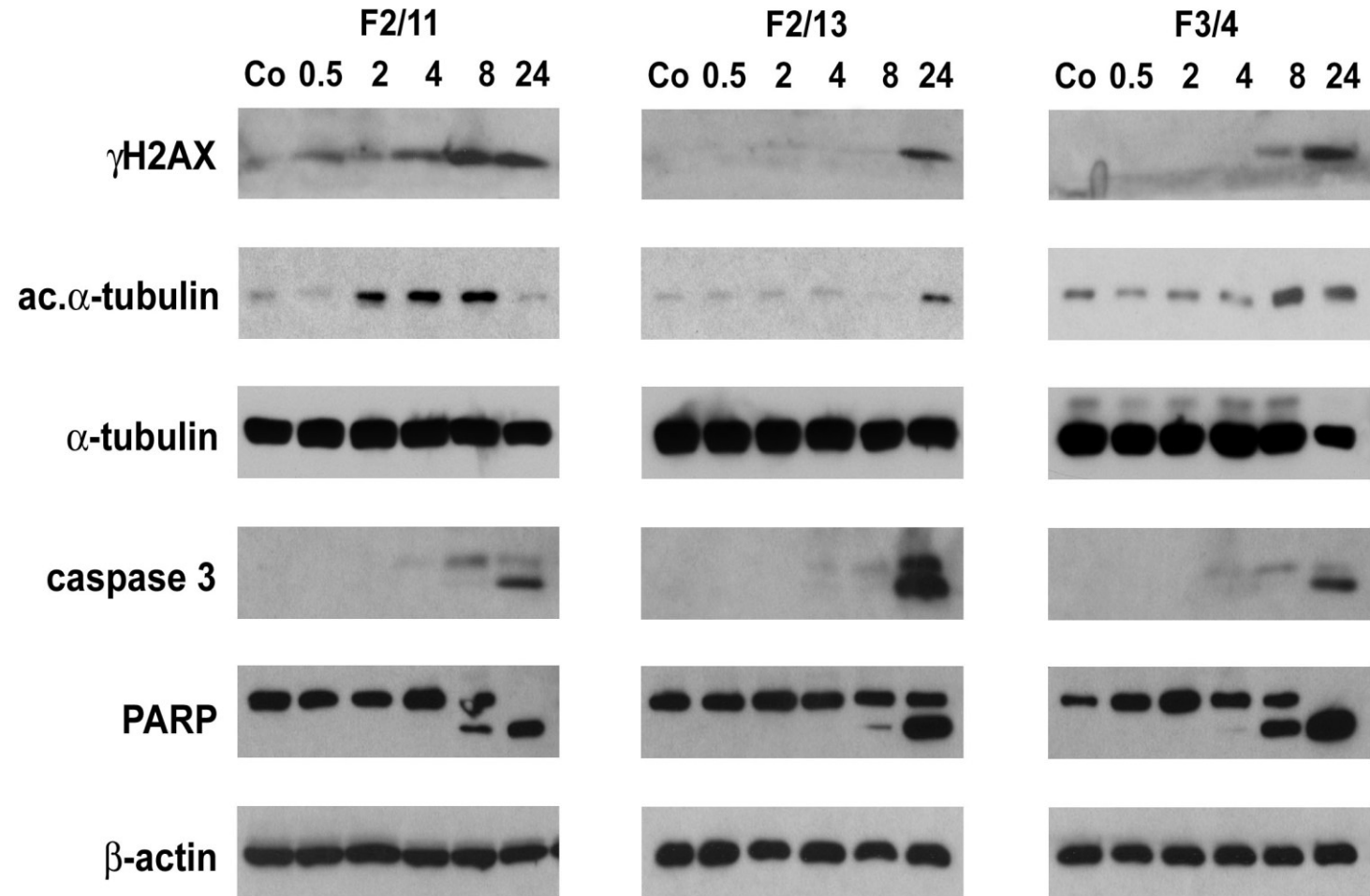


Figure 41 Analysis of apoptosis related proteins

We analysed apoptosis with a highly sensitive method that identifies very early hallmark phenotypes long before metabolism ceases and the cell actually dies, sometimes days later [FRITZER-SZEKERES et al., 2000; ROSENBERGER et al., 2000; GRUSCH et al., 2001]. Fraction F2/11 showed at a concentration of 1.5 µg/ml 60 % apoptosis after 24 hours. Fraction F3/4 caused 65 % apoptotic cells at a concentration of 0.8 µg/ml after 24 hours. At a concentration of 3 µg/ml and after 24 hours a distinctive cleavage of Caspase 3 to 19 kDa and 12 kDa was shown, which is indicative for its activation.

Western blotting aimed to elucidate different pro-apoptotic mechanism in the fractions F2/11, F2/13 and F3/4. Low concentrations of all fractions (3 µg/ml) were shown to induce H2AX phosphorylation (γ H2AX) in HL60 cells indicating genotoxicity. F2/11 caused phosphorylation of H2AX within 30 min, F2/13 within 24 hours and F3/4 after 8 hours. This is in agreement with the presence of a DNA damaging compound in F2/11, whereas induction of γ H2AX is the consequence of the activation of caspases by F2/13 and F3/4 as evidenced by PARP cleavage, in which this property was enriched. In F3/4 an additional execution caspase might have become activator to achieve this efficient signature type cleavage of PARP.

Caspases are the most important enzymes for apoptosis. Initiator caspases, like Caspase-8 and Caspase-9, cleave downstream pro-form caspases (Caspase 3/7/6). Caspase-3 plays a key role during apoptosis. It is totally or partially responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP). Also Caspase 6 and 7 cleave PARP and cooperate in this aspect [FERNANDES-ALNEMRI et al., 1994].

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that is activated by DNA strand breaks to participate in DNA repair [PIEPER et al., 1999]. Activated Caspase 3 cleaves the 116 kDa death substrate PARP into an 85 kDa fragment.

All fractions induced acetylation of α -tubulin. This implicates that they stabilize microtubule [PIPERNO et al., 1987], reminiscent of the mechanism of taxol and therefore, trigger mitotic arrest and apoptosis, which was in fact observed at a concentration of 3 µg/ml after 8 hours in F2/11 (20%) and F3/4 (40%), whereas this concentration of F2/13 had an apoptotic effect of

40 % after 24 hours. Tilting the fine-tuned equilibrium of polymerized/de-polymerized microtubule is incompatible with normal cell division and this causes not only cell cycle arrest, but also apoptosis.

Also, here F2/11 differed from F2/13 and F3/4, because in F2/11 tubulin acetylation was rapid and strong, whereas in fraction F2/13 it was observed after 24 hours only and less pronounced than in F2/11. F3/4 showed an enrichment in the tubulin acetylation property.

Therefore we could separate two very distinct anti-neoplastic properties in the fraction of the *P. odorata* ASE – CH₂Cl₂ extract. A genotoxic property which also causes strong tubulin polymerization inhibits cell growth and induces apoptosis in F2/11, and an even stronger pro-apoptotic property, which is not genotoxic but has a stronger impact on the expression of the oncogenes Cyclin D1 and Cdc25A as well as an activation of caspases.

This study evidences, that a traditional healing plant for the treatment of severe and chronic inflammations has the potential to be developed for an anti-neoplastic remedy. The discovery and the successful separation of a genotoxic property from an even more potent anticancer property that targets the expression of two potent oncogenes, Cyclin D1 and Cdc25A, makes further approaches to identify this highly pro-apoptotic component, a reasonable and fruitful undertaking.

5 CONCLUSION

Our investigations took aim at the anti-proliferative and pro-apoptotic active dichloromethane extract of the ethnopharmacological Mayan healing plant, *Pluchea odorata* (Asteracea) [GRIDLING et al., 2009]. To separate the principles from the aerial parts of *P. odorata* with anti-neoplastic activity bioassay-guided fractionation was carried out by (VLC) vacuum liquid chromatography (PE,CHCl₃-MeOH,MeOH-H₂O in different ratios), normal (CC) column chromatography (chloroform-methanol-water gradient) on silica gel KG60 and RP-18 (SPE) solid phase extraction (CHCl₃,MeOH,H₂O in different ratios). The bioassay-guided fractionation indicated that the anti-proliferative activity of *P. odorata* was due to fractions containing highly apolar constituents.

The most potent fractions that were separated throughout the workflow were tested by proliferation assay in HL60 cells to directly compare the anti-proliferative effects at low fraction concentration (3µg/ml) and to demonstrate the enrichment of bio-activity. The obtained results demonstrate that the enrichment of anti-proliferative activity was at least more than 25 fold.

Fractions F2/11, F2/13 and F3/4 showed most significant anti-proliferative activity at a concentration of 3 µg/ml medium after 72 hours of incubation in HL60 cells. Therefore they were subsequently tested by apoptosis assay and western blot, analysing components of the cell cycle and eliciting its deregulation, which is a hallmark of cancer.

Fractions showed the ability to down-regulate cell cycle protagonists such as Cyclin D1 and Cdc25A. These are oncogenes, which are upregulated in many cancer types. The Cyclin D1 expression differed in all three fractions, this indicates that the bio-active principles are different in each fraction. F2/13 contained more potent Cyclin D1 inhibitory activity than F2/11, which was further enriched in fraction F3/4.

F2/11 induces activation of Chk2 as the earliest of the events, whereas F2/13 and F3/4 as the latest. Chk2 protein levels decreased upon incubation with F2/11, but remained unchanged by incubation of HL60 cells with F2/13 and F3/4. This data attests the presence of different active principles contained in F2/11 and F2/13 and the enrichment of bioactive components in F3/4.

Fraction F2/11 obviously contains a genotoxic component which rapidly causes DNA damage and the subsequent gene expression alterations seem to be consequences of this genotoxicity. On the other side, the DNA damage upon treatment with F2/13 and its derivative F3/4 caused by the comparatively late activation of Caspase 3, which is activated earlier and stronger in F3/4 than in F2/13, elicits the activation of Chk2. (Ser177)Cdc25A is phosphorylated intensive by F2/11, in contrast, F2/13 caused a dephosphorylation of Cdc25A, which was shown to stabilise the protein.

Fraction F2/11 and F3/4 were the most active ones after 24 hours, because they induced apoptotic cell death in 100 % of HL60 cells after 24 hours, therefore they showed the higher pro-apoptotic activity compared to F2/13. The pro-apoptotic properties were enriched at least by 45 fold in fraction F3/4. All three fractions induced acetylation of α -tubulin and therefore, they trigger mitotic arrest and apoptosis. Two very distinct anti-neoplastic properties in the fraction of the *P. odorata* ASE – CH₂Cl₂ extract were separated, a genotoxic and an even stronger pro-apoptotic property.

6 ABSTRACT

The aim of this diploma theses was to enrich the anti-neoplastic activities of *Pluchea odorata* (L.) Cass., Asteraceae, and analyse the underlying mechanisms. In recent investigations a strong anti-neoplastic activity was found from aerial parts of *Pluchea odorata*, a plant used by the Maya to treat severe inflammatory conditions [GRIDLING et al., 2008].

Hence, bioassay-guided fractionation was carried out to enrich the active compound(s). Initially the plant material was extracted by (ASE) accelerated solvent extraction obtaining the bioactive *P. odorata* ASE - CH₂Cl₂ extract. It was re-chromatographed by (VLC) vacuum liquid chromatography (PE,CHCl₃-MeOH,MeOH-H₂O in different ratios), normal (CC) column chromatography (chloroform-methanol-water gradient) on silica gel KG60 and RP-18 (SPE) solid phase extraction (CHCl₃,MeOH,H₂O in different ratios). The bioassay-guided fractionation indicated that the anti-proliferative activity of *P. odorata* is due to a fraction containing highly apolar constituents.

As a result of five fractionation steps, an at least 25 fold enrichment of anti-proliferative activity was reached. To test the efficiency in anticancer effects the inhibition of proliferation and the induction of cell death were investigated in HL60 cells and MCF7 cells. The most potent fractions F2/11, F2/13 and F3/4 were tested by western blot to obtain evidence of underlying mechanisms. Differences between fractions in Cyclin D1 expression indicate, that F2/11 contains less potent principles than F2/13, but in F3/4 the activity was enriched.

Chk2 was phosphorylated at the activating Thr68 site upon treatment with all three fractions tested. F2/11 induced activation of Chk2 as the earliest of the events. Chk2 activity supported the notion that different active principles were contained in F2/11 and in F2/13, and that those components that more specifically interfered with cell cycle progression, became enriched in F3/4. Fraction F2/11 and F3/4 were the most pro-apoptotic active ones and the pro-apoptotic properties were enriched at least by 45 fold in fraction F3/4. Genotoxicity of fraction F2/11 was indicated by rapidly inducing phosphorylation of H2AX. Apparently, there is a DNA damaging compound in F2/11, whereas induction of γ H2AX is the consequence of the activation of caspases by F2/13 and F3/4 as evidenced by PARP cleavage, in which this

property was enriched. The acetylation of α -tubulin implicated that all three fractions stabilized microtubule, reminiscent of the mechanism of taxol and therefore, trigger mitotic arrest and apoptosis. F3/4 showed an enrichment in the tubulin acetylation property. Thus, we could separate two very distinct anti-neoplastic properties in the fraction of the *P. odorata* ASE – CH₂Cl₂ extract.

7 ZUSAMMENFASSUNG

Ziel dieser Diplomarbeit war die Anreicherung der anti-neoplastischen Aktivität der guatemalischen Pflanze *Pluchea odorata* (L.) Cass., Asteraceae, und die Untersuchung der zugrunde liegenden Mechanismen dieser Aktivität. Neueste Untersuchungsergebnisse zeigen die starke anti-neoplastische Aktivität der oberirdischen Pflanzenteile dieser traditionellen Heilpflanze, welche bei den Maya zur Behandlung schwerer Entzündungen eingesetzt wird [GRIDLING et al., 2008].

Auf Basis dieser Ergebnisse wurden zur Anreicherung aktiver Verbindungen, Bio-Assay unterstützte Fraktionierungen durchgeführt. Zu Beginn wurde das getrocknete, pulverisierte Pflanzenmaterial mit Hilfe der ASE (accelerated solvent extraction) extrahiert um das bioaktive *P. odorata* ASE - CH₂Cl₂ Extrakt zu erhalten. Darauf folgten die Fraktionierungsschritte durch (VLC) Vacuum liquid chromatography (PE,CHCl₃-MeOH,MeOH-H₂O in unterschiedlichem Mischungsverhältnis), durch (CC) Normal column chromatography (Chloroform-Methanol-Wasser Gradient) auf Silikagel KG60 und durch RP-18 (SPE) Solid phase extraction (CHCl₃,MeOH,H₂O in unterschiedlichem Mischungsverhältnis). Demzufolge ist die anti-proliferative Aktivität der *P. odorata* auf eine Fraktion zurückzuführen, welche hoch polare Bestandteile aufweist.

Durch fünf aufeinander folgende Fraktionierungsschritte konnte eine 25-fache Anreicherung der anti-proliferativen Aktivität erreicht werden. Zur laufenden Überprüfung der anti-karzinogenen Wirkung der erhaltenen Fraktionen wurde die Proliferationshemmung und die Induktion des Zelltodes in HL60 Zellen und MCF7 Zellen getestet. Die wirksamsten Fraktionen waren F2/11, F2/13 und F3/4, welche in der Folge mittels Western blot getestet wurden um zugrunde liegende Mechanismen aufzuzeigen. Die jeweils unterschiedliche Cyclin D1 Expression der einzelnen Fraktionen hat gezeigt, dass die Fraktion F2/11 schwächer wirksame Komponenten enthält als F2/13, und weist zugleich auf eine Aktivitätsanreicherung in Fraktion F3/4 hin.

Die Inkubation der Zellen mit den Fraktionen F2/11, F2/13 und F3/4 löste eine Phosphorylierung von Chk2 am aktivierenden Thr68 aus, wobei Fraktion F2/11 die Chk2 Aktivierung zum frühesten Zeitpunkt auslöste. Die beobachtete Chk2 Aktivität untermauerte den Hinweis darauf, dass sich die aktiven Verbindungen in F2/11 und F2/13 unterschieden. Gleichzeitig zeigte sie deutlich, dass diese Verbindungen, welche vornehmlich in die Zellzyklus-Progression eingegriffen haben, in F3/4 angereichert wurden.

Fraktion F2/11 und F3/4 wiesen die am stärksten pro-apoptotisch aktiven Verbindungen auf, wobei es durch die Fraktionierungsschritte zu einer 45-fachen Anreicherung in Fraktion F3/4 kam. Den Beweis der Genotoxizität der Fraktion F2/11 lieferte die rasch ausgelöste Phosphorylierung von H2AX. Offensichtlich ist in Fraktion F2/11 eine DNA-schädigende Komponente enthalten. Dahingegen fand die Induktion von γ H2AX durch F2/13 und F3/4 in Folge der Aktivierung von Caspasen statt, was durch die Aufspaltung von PARP belegt wurde.

Die Acetylierung des α -Tubulin hat gezeigt, dass die Mikrotubuli durch alle 3 Fraktionen stabilisiert wurden, und in der Folge mitotischen Arrest und Apoptose auslöste, was an den Mechanismus des Taxol erinnert. Fraktion F3/4 zeigte eine Steigerung der Tubulin-acetylierenden Wirkung. Somit haben wir die Auftrennung und Isolierung von zwei verschiedenen anti-neoplastisch wirksamen Komponenten oder Verbindungsgemischen aus dem *P. odorata* ASE – CH₂Cl₂ Extrakt erreicht.

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Working on my diploma research project
Cell culture (HL 60; MCF 7 cells), Apoptosis assay, Proliferation assay, Western blot

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Phytochemical research: Extraction of plants by ASE (Accelerated Solvent Extraction),
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02.07.2007 - 31.07.2007

*Sewage Treatment Plant Bregenz
Abwasserreinigungsanlage (ARA) Bregenz
Laboratory work: Testing of water- and sludge samples*

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*State Hospital Feldkirch
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Department of Pathology (Microbiological laboratory)
Preparing diagnosis data of urine samples*

General work experience

February 2002 - March 2008 (part-time employee)

„Personal Assistance“ of a handicapped person
„Persönliche Assistenz“

October 2005 - January 2006 (part-time employee)

BIZEPS - Zentrum für Selbstbestimmtes Leben, Vienna
Office assistant

January 2001 - August 2001

Fußach, Vorarlberg
Assistant to the mayor of Fußach: Office assignment

October 1999 - December 2000

Supermarket Spar Achsiedlung, Bregenz
Salesperson

Education

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University of Vienna
Nutritional sciences
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Commercial Highschool Bregenz
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11 PUBLICATIONS

Separation of anti-neoplastic activities by fractionation of a *Pluchea odorata* extract.

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Institute of Biology, Fen-Edebiyat Fakültesi, Adnan Menderes Universitesi, Aydin, Turkey.

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Institute of Biology, Fen-Edebiyat Fakültesi, Adnan Menderes Universitesi, Aydin, Turkey.

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Institute of Clinical Pathology, Medical University of Vienna, Vienna, Austria.

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Pro- and anti-carcinogenic mechanisms of piceatannol are activated dose-dependently in MCF-7 breast cancer cells.

Vo TP, Madlener S, Bago-Horvath Z, Herbacek I, Stark N, Gridling M, Probst P, Giessrigl B, Bauer S, Vonach C, Saiko P, Grusch M, Szekeres T, Fritzer-Szekeres M, Jäger W, Krupitza G, Soleiman A. Carcinogenesis. 2009 Aug 20.

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12 POSTER PRESENTATION

Ethnopharmacological investigations on *Pluchea odorata* (L.) Cass.

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