



universität
wien

DIPLOMARBEIT

Titel der Diplomarbeit

“Analysis of ethno-medical plants of the maya of
Central America for the development of new lead
compounds against cancer”

Verfasserin

Manuela Gridling

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.nat.)

Wien, am 01.10.2008

Studienkennzahl lt.: A474

Studienrichtung lt.: Ernährungswissenschaften

Betreuer: ao. Univ.-Prof. Dr. Karl-Heinz Wagner

TABLE OF CONTENTS

1.	INTRODUCTION	8
2.	LITERATURE SURVEY	10
2.1.	Cell-cycle	10
2.1.1.	Overview of cell cycle progression	10
2.1.2.	Cell-cycle control: checkpoints	13
2.2.	Cell death programs	17
2.2.1.	Apoptosis	18
2.2.2.	Autophagy	20
2.2.3.	Necrosis	20
2.2.4.	Apoptosis-necrosis: similar start-different finish	20
2.3.	Cancer	22
2.3.1.	Basics of cancer development	22
2.3.2.	The six hallmarks of Cancer	24
2.3.3.	Cancer and Inflammation	30
2.3.4.	Leukaemia	31
2.3.5.	Breast cancer	31
2.3.6.	Nutrition and Cancer	32
2.4.	Drugs	33
2.4.1.	Overview	33
2.4.2.	Development of drugs	34
3.	MATERIAL AND METHODS	36
3.1.	Plants	36
3.1.1.	Synonium podophyllum (Contra Hierba)	36
3.1.2.	Pluchea Odorata (Santa Maria)	37
3.1.3.	Lactuca sativa var. capitata (Lettuce - Green salad)	39
3.2.	Plant-extraction	40
3.3.	Cell Culture	41

3.4.	Proliferation-Assay	42
3.5.	Apopotose-Assay	42
3.6.	FACS-analysis	43
3.7.	Western blotting	43
3.8.	COX Inhibitor Screening Assay	46
3.9.	Total phenolics determination	47
4.	RESULTS	48
4.1.	Syngonium Podophyllum	48
4.1.1.	Stock calculation	48
4.1.1.1.	<i>Stipes and roots – lyophilized</i>	48
4.1.1.2.	<i>Stipes and roots – air dried</i>	50
4.1.2.	Antiproliferative activity of <i>S. podophyllum</i> extracts	52
4.1.3.	Anti-inflammatory activity evaluated as PGH ₂	54
4.2.	Pluchea odorata	54
4.2.1.	Stock calculation	54
4.2.1.1.	<i>Leaves – lyophilized</i>	54
4.2.1.2.	<i>Leaves, florescence and stipes - air dried</i>	56
4.2.2.	Anti-proliferative activity of <i>P. odorata</i> extracts	58
4.2.3.	Dichlormethan extract of <i>P. odorata</i> inhibits cell cycle in G2-M	61
4.2.4.	Inhibition of proliferation is preceded by the down-regulation of cell cycle protagonists	61
4.2.5.	The dichlormethan extract of <i>P. odorata</i> induces caspase 3 and apoptosis	63
4.3.	Lactuca sativa	67
4.3.1.	Stock calculation	67
4.3.2.	Anti-proliferative activity of <i>L. sativa</i> extracts	69
4.3.3.	Inhibition of proliferation by the water extract of <i>L. sativa</i> is preceded by the down-regulation of cell cycle protagonists	70
4.3.4.	The ethyl acetate and the water extract of <i>L. sativa</i> induce apoptosis	72

4.3.5.	Analysis of a potential KNO ₃ effect	75
4.3.6.	Total phenolic content of <i>L. sativa</i> ethyl acetate and water extract	76
5.	DISCUSSION	77
5.1.	<i>S. podophyllum</i>	77
5.2.	<i>P. odorata</i>	78
5.2.	<i>L. sativa</i>	82
6.	CONCLUSION	84
7.	SUMMARY	86
8.	ZUSAMMENFASSUNG	87
9.	REFERENCES	88
10.	DANKSAGUNG	94
11.	LEBENS LAUF	95
12.	PAPERS	96
13.	POSTERS	97

TABLE OF FIGURES

Figure 1	Cell Cycle	10
Figure 2	Mitosis	11
Figure 3	The G1/S checkpoint	14
Figure 4	G2 checkpoint response after genotoxic stress 1	16
Figure 5	G2 checkpoint response after genotoxic stress 2	17
Figure 6	A scheme of internalization mechanisms used by macrophages to engulf apoptotic and necrotic cells	18
Figure 7	Similar, distinct, and overlapping triggers of apoptosis I and apoptosis II	19
Figure 8	Apoptosis-necrosis: a similar start-different finish	21
Figure 9	Multistage carcinogenic model	23
Figure 10	Six essential alteration in cell physiology	24
Figure 11	Age standardist (world) incidence and mortality rates, female breast cancer in selectet countries	32
Figure 12	Development of new medical products	35
Figure 13	Syngonium podophyllum	36
Figure 14	Syngonium podophyllum	36
Figure 15	Pluchea odorata	37
Figure 16	Pluchea odorata	37
Figure 17	Lactuca sativa	39
Figure 18	Untreated HL-60 cells	41
Figure 19	Membran stained with Ponceau S	44
Figure 20	Anti-proliferative effects of air dried and freeze dried <i>S. podophyllum</i>	52
Figure 21	Anti-proliferative effects of <i>P. odorata</i> and <i>L. sativa</i> extracts	58
Figure 22	Cell cycle distribution of HL 60 cells upon treatment with <i>P. odorata</i> dichlormethane extract	61
Figure 23	Analysis of cell cycle-related protein and phospho-protein expression	62
Figure 24	Induction of apoptosis and necrosis by the dichloromethane extract of <i>P. odorata</i>	64
Figure 25	Western blot analysis of pro-apoptotic Caspase 3 and its target PARP	65
Figure 26	Western blot analysis of tubulin acetylation	66
Figure 27	Anti-proliferativ effects of <i>L. sativa</i> extracts	69
Figure 28	Analysis of cell-cycle related protein and phospho-protein expression	71
Figure 29	Induction of apoptosis and necrosis by <i>L. sativa</i>	72
Figure 30	Wester blot analysis of tubulin acetylation	74
Figure 31	Analysis of cell cycle related protein and phospho-protein expression	75

LIST OF TABLES

Table 1	Solvents and the specific temperature and pressure used for the evaporator	40
Table 2	Extract weights from <i>S. podophyllum</i> freeze dried	48
Table 3	Extract weights from <i>S. podophyllum</i> air dried	50
Table 4	IC 50 – values of <i>S. podophyllum</i>	53
Table 5	Extract weights from <i>P. odorata</i> freeze dried	55
Table 6	Extract weights form <i>P. odorata</i> air dired	56
Table 7	IC 50 – values of <i>P. odorata</i>	60
Table 8	Extract weights from <i>L. sativa</i>	67
Table 9	IC50 values of <i>L. sativa</i>	70
Table 10	Total Phenolics (Folin-Ciocalteu Method)	76

LIST OF ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
14-3-3 protein	a family of conserved regulatory molecules
ADP/ATP	adenosine diphosphate/adenosine triphosphate
AIF	apoptosis inducing factor
AKT	Serine/threonine-specific protein kinase PK3
ALL	acute lymphoblastic leukaemia
ALT	Alternative lengthening of telomeres
AML	acute myeloid leukaemia
ANT	mitochondrial adenine nucleotide translocase
Apaf1	Apoptotic peptidase activating factor 1
ATCC	american type culture collection
ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia mutated and Rad3-related
Bcl-2	B-cell lymphoma 2
BRCA1	breast cancer 1, early onset
CAD	Caspase Activated DNase
CAM	cell adhesion molecules
Cdc	cell division control
Cdk	cyclin dependent kinase
Cdkl	cyclin dependent kinase inhibitor
Cip/Kip	cyclin dependent kinase inhibitor proteins
CLL	chronic lymphoblastic leucaemia
CML	chronic myeloid leukaemia
COX	Cyclooxygenase
DMEM	Dulbecco's modified Eagl's medium
DNA	deoxy ribonucleic acid
DNA-PK	DNA protein kinase
DNase	deoxyribonuclease
dsRNA	Double-stranded RNA
DTT	dithiothreitol

E2F	a group of genes that codifies a family of transcription factors
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA and EIA	enzyme linked immunosorbent assay
Erk	MAP-Kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
G0, G1, G2	gap phases of the cell cycle
GADD45	Growth Arrest DNA Damage
GS	growth signals
HL-60	promyelocytic leukaemia cells
HPLC	high performance liquid chromatography
IC50	concentration causing 50% inhibition
Ig	Immunoglobulin
IFN- γ	Interferon-gamma
INK4	inhibitor of cyclin-dependent kinase 4
MCF-7	breast cancer cells
MCF-10A	human immortalized anomalous breast epithelial cell line
M-Phase	Mitosis
Mre11	Meiotic Recombination (double-strand break repair protein)
Nbs1	Nibrin (double-strand break repair protein)
P21	Cyclin-dependent kinase inhibitor 1A
P27	cyclin-dependent kinase inhibitors
P53	Tumor protein p53
PARP	poly[ADP-ribose]polymerase
PBS	phosphate –buffered saline
PG	Prostaglandin
PGF2 α	Dinoprost, Prostaglandin F _{2α}
PGHS	PGHS Prostaglandin G/H Synthase
PIC	Protease Inhibitor Cocktail
PI3	Peptidase inhibitor 3
PMSF	phenylmethylsulfonyl fluoride
pRb	retinoblastoma protein
PVDF	Membranes for Western Transfer and Sequencing
Rad11 Rad50	checkpoint Rad proteins
ROS	reactive oxygen species
RPMI	cell culture medium
S- Phase	DNA synthesis
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis
Ser	Serin
SMC1	ATPase
TBS	Tris buffered Saline
TNF α	tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
z-VAD-fmk	benzyloxycarbonyl Val-Ala-DL-Asp-fluoromethylketone

1. INTRODUCTION

Malign neoplasms are currently treated by surgical resection, radiation- and chemotherapy. More than 60% of the drugs used against cancer in western medicine are derivatives of naturally occurring substances (metabolites of plant or microbial origin) (Cragg and Newman 2006). Many traditional healing plants successfully passed several hundred years of empirical testing against specific diseases and thereby demonstrating that they are well tolerated in humans. Although quite a few ethno-pharmacological plants are applied against a variety of conditions there are still numerous plants that have not been cross-tested in diseases apart from the traditional applications.

The potential of plants of the tropical rain forest as a source for medicinally useful compounds is found in its high genetic diversity and its high concentration of active compounds. We have therefore focussed our interest on the knowledge of herbalists and *curanderos/curanderas* of very old cultures or civilizations living in high biodiversity regions still practising their traditional healing methods based on a rich botanical pharmacy. The long lasting medicinal experience of the Mayas of the tropical rain forest of Guatemala is a particularly precious knowledge, and moreover some Maya populations have been rather isolated for hundreds of years and still treat and heal diseases of many kinds using natural remedies from the neighbouring tropical rain forests, which are amongst the richest biodiversity areas in the world. Since the Maya medicine neither knew about leukaemia or cancer, nor remedies against it, our concept of plant selection was their traditional use (and they still are in use) against severe inflammations, because there are a variety of similar signalling pathways which are commonly up-regulated both in inflammatory conditions and in cancer.

Therefore, the aim of this project is the isolation of new lead substances with anti-neoplastic activity spectrum from scientifically as yet unexplored plants. It is intended to progress in a similar way as for the development of Taxol, which was already known as medicinal remedy (as metabolite in the bark of several

Taxus species) to the indigenous North American tribe of the Quinault who treat(ed) stomach and kidney complications. Two principle parameters guided the conception of this project:

- 1) the traditional use to treat inflammatory conditions and
- 2) the absence of records in western medicinal- and patent databases.

Here we describe for the first time the anti-cancer activity of extracts of two ethnopharmacological healing plants used against various inflammatory scenarios, *Pluchea odorata* (Asteracea) and *Syngonium podophyllum* (Araceae) in HL-60 promyelocytic leukaemia cells and MCF-7 breast cancer cells. The plants selected as such were extracted with solvents of increasing polarity (petroleum ether, dichlormethan, ethyl acetate, methanol and water). The extracts were tested in human HL-60 promyelocytic leukaemia and MCF-7 breast cancer cells regarding their anti-cancerogenic properties. The best extract types of the most bioactive plant parts were specifically investigated regarding their apoptosis-inducing potential. Western blot and FACS analyses aimed to obtain data on the underlying mechanisms.

As a negative control *L. sativa* was extracted and tested the same way as *P. odorata* and *S. podophyllum*.

2. LITERATURE SURVEY

2.1. Cell-cycle

The development of cancer is associated with disorders in the regulation of the cell cycle. Complex networks of regulatory factors dictate whether cells proliferate or die (Meeran and Katiyar 2008). Cell external signals decide until cells commit to go through the entire cycle, and cell intrinsic information control the progression through the cell cycle. Both together determine whether cells enter a division cycle. The basic components of this machinery are conserved in all eukaryotes (Heuvel 2005).

The DNA in every mammalian cell is under constant attack by agents that can either directly damage one of its three billion bases or break the phosphodiester backbone on which the bases reside, for example by solar radiation, polycyclic aromatic hydrocarbons and cigarette smoke, etc. Damage to cellular DNA can result in the development of cancer. Cells have developed several defense mechanisms to cope with this constant attack on their DNA, for example direct repair, halting cell-cycle progression or apoptosis (Meeran and Katiyar 2008).

2.1.1. Overview of cell cycle progression

The cell cycle is a recurring sequence of events that includes the duplication of cell contents and subsequent cell division. Traditionally the cell cycle *in* eukaryotic cell has been divided into four phases:

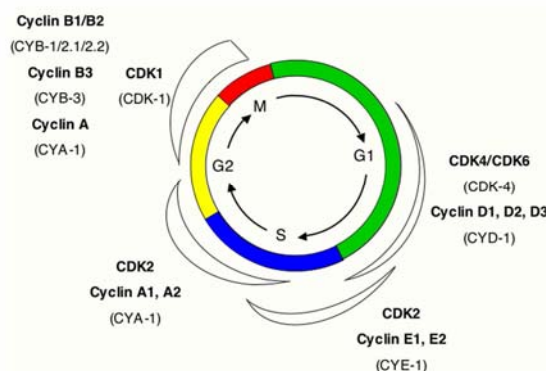


Figure 1: Cell Cycle (Heuvel 2005)

- (G1)** Gap phase 1 → connect the completion of M phase to initiation of S phase in the next cycle
- (S)** DNA synthesis → accurate duplication of the genome
- (G2)** Gap phase 2 → cells prepares itself for division
- (M)** Mitosis → chromosomes were separated and the cell divides
- (Meeran and Katiyar 2008)

Dependent on environmental and developmental signals, cells in G1 may temporarily or permanently leave the cell cycle and enter a quiescent or arrested phase know as G0 (Heuvel 2005).

The process of mitosis is divided into five scenes:

- Prophase: incipient chromosomal condensation
- Metaphase: chromosomal alignment
- Anaphase: segregation of sister chromosome
- Telophase: enclosure of the new chromosome in the new core
- Interphase: subsequent division of cellular material leading to the next interphase

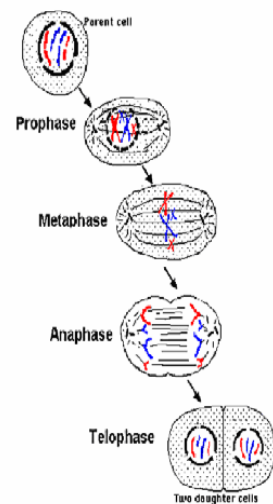


Figure 2: Mitosis

The Cell cycle is regulated by sequential activation and inactivation of many “check points”. They monitor the status of the cell as well as environmental cues. Check points are gene products or subset of gene products that, when mutated, confer independence on a cellular process that was previously dependent upon completion of another cellular process. In order to ensure proper cell cycle progression, the cells go through many internal checkpoints onto the next step (Meeran and Katiyar 2008).

Cyclins

Cyclins are able to form a complex with a CDK partner and thereby control various phases of the cell cycle (illustrated at figure 1). Their expression pattern dictates the point in the cell cycle at which they act. There are two types of cyclins:

- cell-cycle related cyclins (cyclin A, B, D and E) and
- non-cell cycle related cyclins (cyclin H and C)

Of the cell-cycle related cyclins, cyclin D and E play an important role in the transition from the G1 to S phase. Cyclin A has functions in both S phase and mitosis (Meeran and Katiyar 2008).

Cyclin dependent kinases (CDK)

Progression through the cell-division cycle is driven by activation and inactivation of cyclin-dependent kinases (CDKs). They trigger the transition to subsequent phases of cycle (Heuvel 2005). Different members of the CDK family, in association with different cyclins, switch throughout the cell cycle. Other family members regulate transcription, differentiation, and nutrient uptake, as well as other cellular functions. CDK protein levels are constant throughout the cell cycle; the CDKs are only functional during distinct intervals within the cell cycle. Notably, to enter the S phase all cells must activate cyclin-dependent kinases (Meeran and Katiyar 2008).

CDK inhibitors (CDKI)

There are two classes of Cdk inhibitors:

- the INK4 inhibitors, with p16^{INK4A}, p15^{INK4B}, p19^{INK4D} and p18^{INK4C},
- and the Cip/Kip inhibitors, with p21^{Waf1/Cip1}, p27^{Kip1} and p57^{Kip2}.

The INK4 family specifically inhibits the cell cycle, while the Cip/Kip family can inhibit Cdk activity during all phases of the cell cycle (Pietenpol and Stewart 2002). The relative concentrations of each of the two families of inhibitors determine their distribution among the various cyclin/CDK complexes and finally affect G1 progression. Cip1/p21 inhibits DNA replication and arrests growth in G1. Kip1/p27 also plays a role in the regulation of the restriction point. The

members of the INK4 family specifically block G1 progression by inhibiting the association of CDK4/6 with cyclin D. Impairment of a growth-stimulatory signaling pathway has been shown to stimulate the expression of CDKIs (Meeran and Katiyar 2008).

2.1.2. Cell-cycle control: checkpoints

Cells are constantly subject to mutation of their DNA, which is detrimental to the cells. But only rarely of that cells flourish as pathologic tumours. Cells respond to DNA damage by halting cell cycle progression or apoptosis. Failure of the quality control check points or a loss of balance of the regulatory molecules plays a major role in the development of cancer (Meeran and Katiyar 2008).

The choice of which repair system to use depends both on the type of lesion and on the cell-cycle phase of the cell. For example, a DNA double-strand break in S and G2 phases is readily repaired by homologous recombination fusion to it's intact sister chromatid. However, as cells progress into G2/M, the chromosomes are condensed in a highly ordered chromatin structure that makes homology search difficult (Branzei and Foiani 2008).

Checkpoints can either trigger arrest of cell cycle progression (G1, S or G2 phase) or trigger the induction of necessary repair genes. The cell cycle arrest allows the repair of genetic material thereby preventing secondary lesions and ensuring the appropriate progression into the next phase of the cycle (Meeran and Katiyar 2008). Checkpoint signaling may also result in activation of pathways leading to programmed cell death if cellular damage cannot be properly repaired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumour genesis (Pietenpol and Stewart 2002).

For example, after exposure of normal cells to genotoxic agents, p21^{Waf/Cip1} is induced by p53-dependent transactivation. The elevated p21 binds and inactivates cyclin D/Cdk4,6 and Cyclin D/Cdk2 complexes resulting in pRb hypophosphorylation and cell cycle arrest (figure 3) (Pietenpol and Stewart 2002).

Thus, the G1 checkpoint signals target two independent and critical tumor suppressor pathways, governed by p53 and pRb, which are most commonly deregulated in human cancers (Meeran and Katiyar 2008).

The S-phase checkpoint

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 regulated by two distinct pathways, named

- (1) ATM/ATR-Chk1-Cdc25A and
- (2) ATM-Nbs1-SMC1.

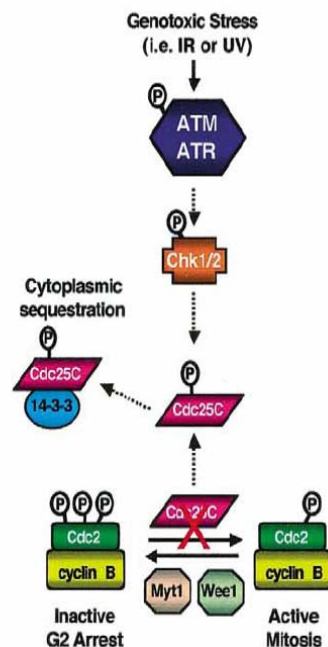
Ad 1) Depending on the type of DNA damage, ATM or ATR phosphorylates Chk1 which in turn phosphorylates Cdc25A. In response to genotoxic stress, the activity of Chk1 and Chk2 is enhanced. This leads to a down regulation of Cdc25A what in turn causes inactivation of Cyclin E-Cdk2.

Ad 2) This type of intra-S-Checkpoint reflects the impact of ATM-mediated phosphorylation of Nbs1 on several sides. It is required for activation of the Nbs1-Mre11-Rad11-Rad50 complex. Depending on the phosphorylation state of Nbs, the cohesin protein SMC1 is phosphorylated on Ser957 and Ser966 by ATM, which is required for the intra-S checkpoint.

These two kinds of intra-S-checkpoints have been documented in response to both ionizing radiation and UV radiation. Other proteins that are involved in the intra-S checkpoint too are for example 53BP1, BRCA1, FANCD2 and MDC1 (Meeran and Katiyar 2008).

The G2/M checkpoint

The G2/M checkpoint prevents cells from entering into mitosis when they experience DNA damage during G2 or when they carry unrepaired DNA from G1 or S to progress into G2 through inhibiting Cyclin B/Cdc2.



After DNA damage, members of the PI-3K family become activated and directly phosphorylate p53, including DNA-PK, ATM and ATR. ATM-dependent signaling induced by DNA damage also results in activation of Chk1 and Chk2 kinases, which mediate phosphorylation of p53. This is important for stabilization of the protein after DNA damage. After ATM-dependent activation, Chk1 and Chk2 also phosphorylate Cdc25C and Ser-216 and that furthermore generate a consensus binding site for 14-3-3 proteins. This inhibits Cdc2 activity (Pietenpol and Stewart 2002).

Figure 4: G2 checkpoint response after genotoxic stress (Pietenpol and Stewart 2002)

The maintenance phase of G2/M partially relies on the transcriptional programs regulated by BRCA1 and p53. p53-dependent mechanisms are also important for the maintenance of G2 arrest following DNA damage.

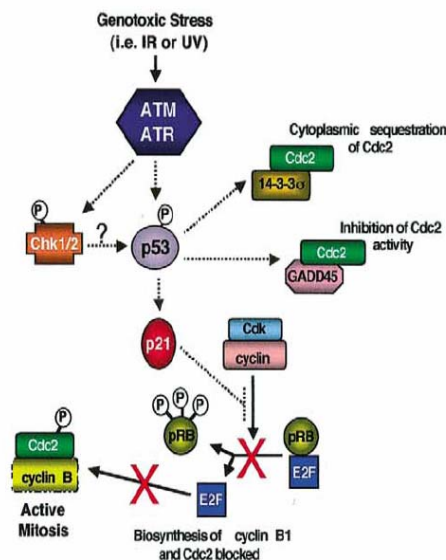


Figure 5: G2 checkpoint response after genotoxic stress (Pietenpol and Steward 2002)

p53 at G2/M phase works in three ways:

- (1) Through p21, GADD45, which causes the dissociation of the Cdc2 and cyclin B/Cdc2 complex
- (2) Through 14-3-3 sigma, which sequesters the cyclin B/Cdc2 complex in the cytoplasm and
- (3) Through repression of the transcription of Cdc2 and Cyclin B

(Meeran and Katiyar 2008)

2.2. Cell death programs

Homeostasis is maintained in multicellular organisms by a balance between cell proliferation and cell death. There are three major morphologies of cell death:

- apoptosis (type I)
- cell death associated with autophagy (type II)
- necrosis (type III).

Almost every dying or dead cell have the same final fate, namely engulfment by phagocytes. This clearance process (figure 6) is very important for the development and homeostasis. A defective or inefficient clearance may contribute to several human pathologies, for example systemic lupus

erythematosus, cystic fibrosis and chronic obstructive pulmonary disease (Krysko et al. 2007).

An important feature of currently used anti-cancer drugs is their potential to trigger programmed cell death (apoptosis) and to inhibit cell cycle progression. This, it is relevant to investigate whether cell cycle inhibition and apoptosis-induction becomes initiated by novel anti-cancer drugs, because these mechanisms are required to regulate tissue homeostasis, but frequently escape proper function in cancer cells.

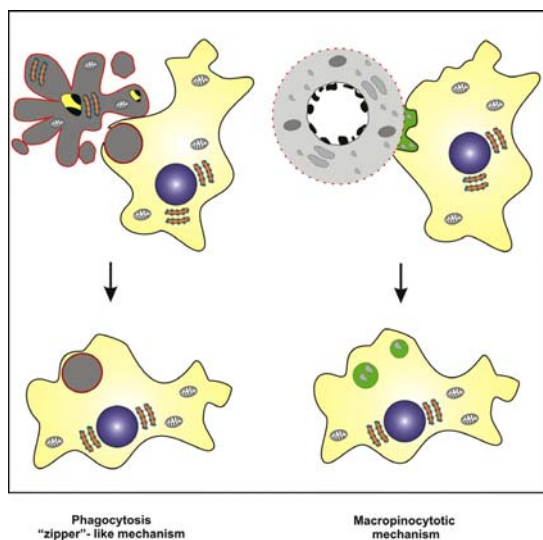


Fig. 6: A scheme of internalization mechanisms used by macrophages to engulf apoptotic and necrotic cells. (Krysko et al. 2007)

2.2.1. Apoptosis

Cells undergoing apoptosis show typical, well-defined morphological changes, like plasma membrane blebbing, chromatin condensation with margination of chromatin to the nuclear membrane, karyorrhexis (nuclear fragmentation), and formation of apoptotic bodies (Krysko et al. 2007).

There are two different types of apoptosis:

- 1) Caspase-dependent apoptosis
- 2) Caspase-independent apoptosis

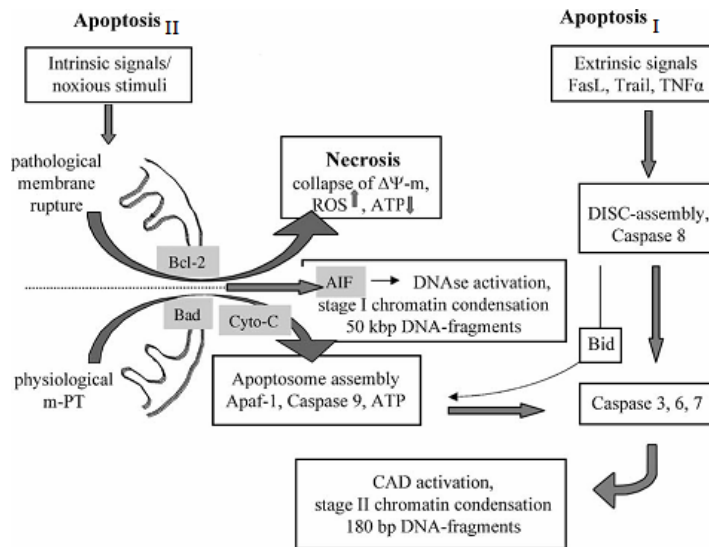


Fig.7: Similar, distinct, and overlapping triggers of apoptosis I and apoptosis II (Huettenbrenner et al. 2003)

Ad 1) Apoptotic cell death involves the orchestrated action of catabolic enzymes, like proteases and nucleases, within the limits of intact plasma membranes. It is accompanied by a characteristic change of nuclear morphology and chromatin biochemistry (stepwise DNA degradation). Caspases cleave their targets after aspartic acid residues and catalyze a highly selective pattern of protein degradation. Moreover, cellular organelles remain morphologically intact (only subtle changes of like partial protein degradation become manifest), whereas cells shrink and reduce intracellular potassium level.

Ad2) Execution of apoptotic chromatin degradation is not only achieved by executioner caspases and their effector CAD but also by AIF in a caspase-independent fashion. AIF activates a nuclear DNase which cuts genomic DNA into 50 kb fragments giving rise to a distinct nucleomorphological phenotype of chromatin condensation, the so-called stage I. Whereas CAD generates the phenotype of stage II chromatin condensation.

The two mechanisms of apoptosis execution exist together and may cooperate. It needs to be determined whether either of the pathways can be triggered

separately depending on the cell type, the context, or the stimulus (Huettnerbrenner et al. 2003).

2.2.2. Autophagy

Cells undergoing death associated with autophagy are characterized by the presence of double membrane autophagic vacuoles. Cells deprived of nutrient or obligate growth factors foremost activate autophagy. When cellular stress continuous, cell death may continue by autophagy alone. Alternatively it often comes associated with features of apoptotic or necrotic cell death (Krysko et al. 2007).

2.2.3. Necrosis

Necrosis is characterized by rapid cytoplasmic swelling and in the end a rupture of the plasma membrane and organelle breakdown. Necrosis has long been described as a consequence of extreme physicochemical stress, such as heat, somatic shock, mechanical stress, freeze thawing and high concentration of hydrogen peroxide. Because of the quick death due to the prior conditions, necrosis has been described as accidental and uncontrolled. However, many different cellular stimuli (TNF on certain cell lines, dsRNA, IFN- γ , ischemia) have been shown to induce necrosis. This induced necrotic cell death results from extensive cross-talk between several biochemical and molecular events at different cellular levels and it is as controlled and programmed as apoptosis. It is important to distinguish necrosis from other forms of cell death, because it is often associated with an unwarranted loss of cells in human pathologies. Furthermore it can also lead to local inflammation because of the release of intracellular factors from death cells, the so-called damage associated molecular pattern that alert the innate immune system (Krysko 2008).

2.2.4. Apoptosis-necrosis: similar start-different finish

Multiple stresses or physiological induction causes ion reflux of the mitochondrial pore transition (PT). Thereby the mitochondrial membrane potential $\Delta\Psi_m$ disturbs and in consequence an interference with mitochondrial

oxidative phosphorylation resulting in the decrease of ATP takes place. Due to the osmotic pressure, H₂O and Ca²⁺ ions cause swelling of the cytoplasm and organelles. Finally the cells burst and spill the fluid into the pericellular space. This phenomenon describes a classic necrotic phenotype. Another consequence of a disrupted $\Delta\Psi_m$ is the uncoupling of the respiratory chain. This lead to the hyperproduction of ROS, affection of the plasma potential, and subsequent irreversible oxidation of thiols and membrane lipids, as well as the depletion of glutathione.

Cytochrome C together with Apaf1 (both are released through the PT-pore complex) induces caspase 9, which directly translocates into the nucleus to induce caspase-independent stage I apoptosis.

Very likely, AIF exerts its activity during apoptosis and necrosis. Therefore, the extent of $\Delta\Psi_m$ disturbance may determine between life and deaths as a point of no return.

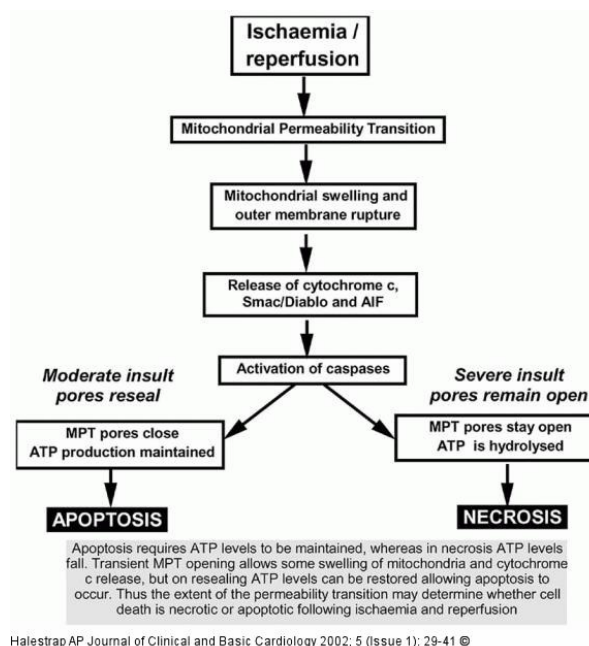


Figure 8: Apoptosis-necrosis: similar start-different finish (Halestrap 2002)

The PT-pore complex is controlled by Bcl-2 and Bcl-K_L and upon extopic overexpression. Bcl-2 inhibits PT and prevents both apoptosis and necrosis. Thus, early apoptotic onset and necrosis start with similar events such as PT.

Bcl-2 regulate another component of the PT-pore complex, called ANT. ANT exchanges mitochondrial matrix ATP for cellular ADP. ATP:ADP ratio determines whether a cell will vanish by necrosis or apoptosis. For example, noxious stimuli like stress or toxic compounds cause an ATP depletion and therefore necrosis. Exhausted ATP in consequence of excessive trauma blocks the apoptotic execution machinery downstream of PT and necrosis prevails.

However, the necrosis-apoptosis decision is not only taken by ATP. An example is a specific inhibition of caspases by z-VAD-fmk. This renders cells to exit by a necrotic phenotype although identical stimuli induce apoptosis when z-VAD-fmk is omitted.

Therefore, the status of $\Delta\Psi_m$ which depends on stress type and impact, not only switches between life and death, but gives also major directives for death modes such as necrosis, type II apoptosis, and caspase-independent apoptosis by AIF (Huettenbrenner et al. 2003).

2.3. Cancer

Tumours are complex tissues composed of ever-evolving neoplastic cells, matrix proteins that provide structural support and sequester biologically active molecules, and a cellular stromal component. Reciprocal interactions between neoplastic cells, activated host cells and the dynamic micro-environment in which they live enable tumour growth and dissemination (Van Kempen et al. 2006).

2.3.1. Basics of cancer development

Central to the development of cancer are genetic changes that endow these “cancer cells” with many of the hallmarks of cancer, such as self-sufficient growth and resistance to anti-growth and pro-death signals. However, while the genetic changes that occur within cancer cells themselves, such as activated oncogenes or dysfunctional tumour suppressors, are responsible for many

aspects of cancer development, they are not sufficient. Tumour promotion and progression are dependent on ancillary themselves.

Cancer results from the outgrowth of a clonal population of cells from tissue. The development of cancer, referred to as carcinogenesis, can be modelled and characterized in a number of ways. One way to describe this process is to illustrate the essential features of both cancer cells and tumours: the “hallmarks” of cancer (next chapter).

Cancer can also be considered with regard to a step-wise development functionally grouped into three phases: initiation, promotion and progression.

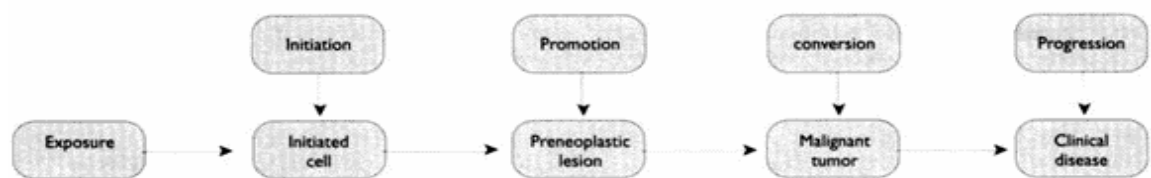


Figure 9: Multistage carcinogenic model (Harris 1991)

Initiation is characterized by genomic changes within the “cancer cell” such as point mutations, gene depletion and amplification, and chromosomal rearrangements leading to irreversible cellular changes. Tumour development is promoted by the survival and clonal expansion of these “initiated” cells. Progression encompasses a substantial growth in tumor size and either growth-related or mutually exclusive metastasis (Rakoff-Nahoum 2006).

The development of cancer in humans involves a complex succession of events that usually occur over many decades. During this multistep process, the genomes of incipient cancer cells acquire mutant alleles of proto-oncogenes, tumor-suppressor genes, and other genes that control, directly or indirectly, cell proliferation. Different combinations of these mutant alleles are found in the genomes of many distinct types of human cancer as well as in different cancers from the same tissue. An ever-increasing number of these genes have been shown to make contributions to the distinct steps involved in neoplastic transformation. The complexity of these observations provokes the question of

whether these genes and the more than 100 distinct types of human cancer can ever be rationalized in terms of a small number of underlying biologic and biochemical principles. Recent successes in the experimental transformation of human cells indicate that the disruption of a limited number of cellular regulatory pathways is sufficient to impart a tumourgenic phenotype to a wide variety of normal cells. These results, in turn, suggest a series of genetic and cellular principles that may govern the formation of most, if not all, types of human cancers (Hahn and Weinberg 2008).

2.3.2. The six hallmarks of Cancer

In the past decades researches revealed a small number of molecular, biochemical and cellular traits, the so called acquired capabilities. There are six essential alterations in cell physiology that collectively dictate malignant growth:

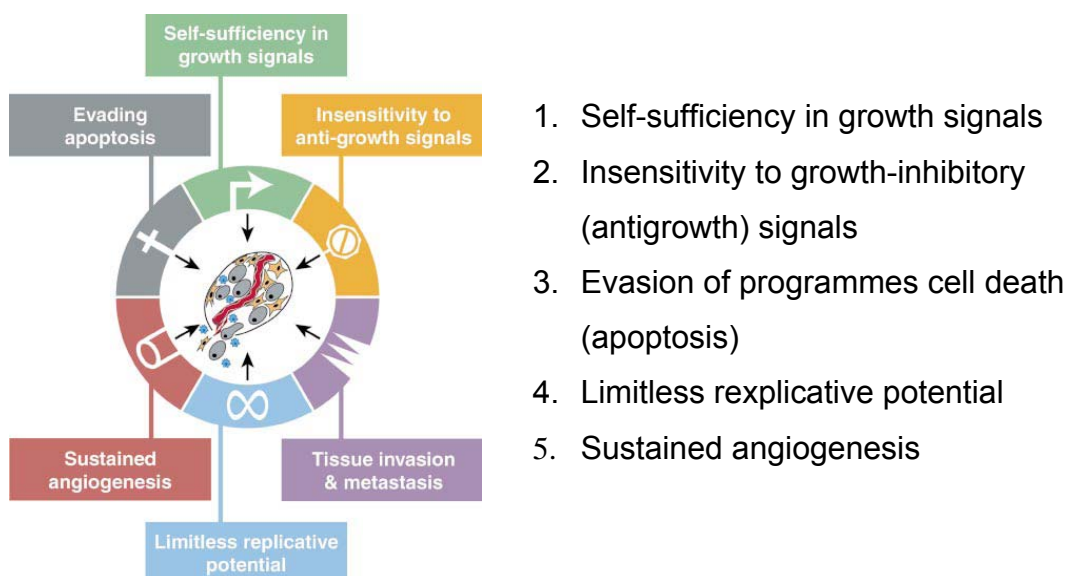


Figure 10: Six essential alterations in cell physiology (Hanahan and Weinberg 2000)

These traits can be found in most or perhaps all types of human cancer, because virtually all mammalian cells carry similar machinery, which regulates their proliferation, differentiation and death.

Genetic alterations are responsible for the transformation of normal cells into highly malignant derivatives. These cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis.

Self-Sufficiency in Growth Signals

Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. Many of the oncogenes act by mimicking these normal growth signals.

All tumour cells are able to synthesize their own growth signals hence they show a greatly reduced dependence on exogenous growth stimulation, thereby reducing their microenvironment. They may create a positive feedback signalling loop that is often termed autocrine stimulation.

But there are some other ways to lose dependency. For example receptor overexpression may enable the cancer cell to become hyper responsive to ambient levels of growth factors that normally do not trigger proliferation. Furthermore the ability of cancer cells to switch the types of extracellular matrix receptors which they express, favouring ones that transmit pro-growth signals, is another alternative too.

Moreover integrin receptor can bind to specific moieties of the extracellular matrix. This enables the transduction of signals into the cytoplasm and this influences cell behaviour, ranging from quiescence in normal tissue to motility, resistance to apoptosis and entrance into the active cell cycle.

Finally tumour cells are able to co-opt their normal neighbours by inducing them to release abundant fluxes of growth-stimulation signals either through direct contact or through secreted signal-molecules.

Insensitivity to Antigrowth Signals

Within a normal tissue, multiple antiproliferative signals are situated which maintain cellular quiescence and tissue homeostasis. Such signals are soluble

growth inhibitors and immobilized inhibitors. There are two distinct mechanisms whereby antigrowth signals can block proliferation:

1. Cells may be forced out of the active cycle into the quiescent (G0) state. Not until extra cellular signals permit, they may remerge in the future.
2. Cells have the ability to relinquish their proliferative potential by being induced to enter into post mitotic states. This was usually associated with acquisition of specific differentiation-associated traits.

Incipient cancer cells must evade these antiproliferative signals if they are to prosper.

One opportunity is to disrupt the pRb pathway, through that many and perhaps all antiproliferative signals are funnelled. That will lead to liberation of E2Fs and thus allows cell proliferation, rendering cells insensitive to antigrowth factors. These factors normally operate along this pathway to block advance through the G 1 phase of the cell cycle.

In addition, cancer cells are able to turn of expression of integrins and other cell adhesion molecules that send antigrowth signals, particularly those that convey progrowth signals.

Cell proliferation depends on more than an avoidance of cytostatic antigrowth signals. Our tissue also constrains cell multiplication. It may instruct cells to enter irreversibly into post mitotic, differentiated states by using diverse mechanisms that are incompletely understood. However it's apparent that tumour cells use various strategies to avoid this terminal differentiation.

Evading Apoptosis

The ability of tumour cell populations to expand in number is determed by rate of cell proliferation and by the rate of cell attrition, for what apoptosis represents a major source. Therefore resistance toward apoptosis is a hallmark of most and perhaps all types of cancer.

The discovery of the bcl-2 oncogene by its upregulation via chromosomal translocation in follicular lymphoma and its recognition as having antiapoptotic activity opened up the investigation of apoptosis in cancer at the molecular level. A cell's apoptotic program can be triggered by an overexpression of oncogene. Indeed, elimination of cells bearing activated oncogenes by apoptosis may represent the primary means by which such mutant cells are continually culled from the body's tissues. Collectively, observations argue that altering components of the apoptotic machinery can dramatically affect the dynamics of tumour progression, providing a rationale for the inactivation of this machinery during tumour development.

Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. The most commonly occurring loss of a proapoptotic regulator through mutation involves the p53 tumor suppressor gene. The resulting functional inactivation of its product, the p53 protein, is seen in greater than 50% of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade. Signals evoked by other abnormalities, including hypoxia and oncogene hyper expression, are also funnelled in part via p53 to the apoptotic machinery; these too are impaired at eliciting apoptosis when p53 function is lost. Additionally, the PI3 kinase AKT/PKB pathway, which transmits antiapoptotic survival signals, is likely involved in tumours.

We expect that virtually all cancer cells harbour alterations that enable evasion of apoptosis.

Limitless Replicative Potential

Many or perhaps all types of mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. A disruption of this program causes expansion of cell to a size that constitutes a macroscopic, life threatening tumour.

Cells in culture have a finite replicative potential. This can be avoided by disabling their pRb and p53 tumor suppressor protein. This follows massive cell

death, caryotypic disarray associated with end-to-end fusion of chromosomes and even the occasional emergence of variant ($1 \text{ in } 10^7$) cells that has acquired the ability to multiply without limit.

The counting device for cell generation, the so-called telomeres, is composed of several thousand repeats of a short 6 bp sequence element. During each cell cycle telomeric DNA loses 50-100 bp. Therefore cells lose their ability to protect the ends of chromosomal DNA and finally the affected cell dies. Hence, telomere maintenance is evident in virtually all types of malignant cells. There are two different possibilities how cells can protect their telomere:

1. Upregulation of expression of the telomerase enzyme, by adding hexanucleotide repeats onto the ends of telomeric DNA
2. Activating a mechanism – termed ALT – which appears to maintain telomeres through recombinant-based interchromosomal exchanges of sequence information

Therefore telomere maintenance is a clearly key component of the capability for unlimited replication, but another one, the circumvention of cellular senescence is undetermined.

Sustained Angiogenesis

The vasculature is crucial for the supply of oxygen und nutrients. All cells in a tissue reside within 100 μm of a capillary blood vessel. A coordinated growth of vessels and parenchyma ensured this closeness during organogenesis. Because cells within aberrant proliferative lesions initially lack angiogenesis ability, incipient neoplasias must develop angiogenic ability.

Counterbalancing positive and negative signals encourage, like endothelial growth factor (VEGF) and acidic or basic fibroblast growth factors (FGF1/2), or block, like thrombospondin-1, angiogenesis. Tumour may activate the angiogenic switch by changing the balance of these angiogenesis inducers and countervailing inhibitors, for example through altered gene transcription.

Integrin signaling contributes to this regulatory balance too, while quiescent vessel expresses one class of integrins and sprouting capillaries express another. Interference between these classes of integrins can inhibit angiogenesis.

Another way to regulate angiogenesis is proteases, which can control the bioavailability of angiogenic activators and inhibitors.

Tissue Invasion and Metastasis

Primary tumour masses of most types of human cancer spawn pioneer cells that move out and invade adjacent tissue. Then they travel to distant sites where they may succeed in founding new colonies. In effect these distant settlements of tumour cells, called metastasis, are the cause of 90 % of human cancer deaths. Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood.

There are several classes of proteins that possess invasive or metastatic capabilities. These proteins are involved in tethering of cells to their surroundings in a tissue are altered in cells. The affected proteins include cell-cell adhesion molecules (CAMs), for example E-cadherin expressed on epithelial cells, and integrins, which link cells to extra cellular matrix substrates. All of these “adherence” interactions convey regulatory signals to the cell. Changes in expression of CAMs and integrins play a critical role in the processes of invasion and metastasis, such as forced expression of integrin subunits in cultured cells can induce or inhibit invasive and metastatic behaviour.

The second general parameter of the invasive and metastatic capability involves extra cellular proteases. Some proteases are characteristically associated with the cell surface. Thereby they facilitate invasion by cancer cells

into nearby stoma, across blood vessel walls and through normal epithelial cell layers.

The activation of extra cellular proteases and the altered binding specificities of cadherin, CAMs and integrins are clearly central to the acquisition of invasiveness and metastatic ability. But the regulatory circuits and molecular mechanisms that govern these shifts remain elusive and, at present, seem to differ from one tissue environment to another (Hanahan and Weinberg 2000).

2.3.3. Cancer and Inflammation

An association between the development of cancer and inflammation has long been appreciated (Rakoff-Nahoum 2006). Recent data have expanded the concept that inflammation is a critical component of tumour progression. Many cancers arise from sites of infection, chronic irritation and inflammation (Coussens and Werb 2002).

Epidemiologic studies estimate that nearly 15 percent of the worldwide cancer incidence is associated with microbial infections. For example, chronic infection in immunocompetent hosts such as human papilloma virus or hepatitis B and C virus infection leads to cervical and hepatocellular carcinoma. Or conditions associated with chronic irritation and subsequent inflammation predispose to cancer, such as the long-term exposure to cigarette smoke, asbestos and silica (Rakoff-Nahoum 2006).

It has become evident that early and persistent inflammatory responses observed in or around developing neoplasm's regulates many aspects of tumour development, like matrix remodelling, angiogenesis and malignant potential, by providing diverse mediators implicated in maintaining tissue homeostasis, e.g., soluble growth and survival factors, matrix remodelling enzymes, reactive oxygen species and other bioactive molecules (Van Kempen et al. 2006).

In addition, tumour cells have co-opted some of the signalling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis. These insights are fostering new anti-inflammatory therapeutic approaches to cancer development (Coussens and Werb 2002).

2.3.4. Leukaemia

The first cell line which was used to perform the experiments was HL-60 promyelocytic leukaemia cells. Leukaemia is a blood or bone marrow cancer and is characterized by an abnormal proliferation of blood cells, mostly leukocytes. Leukaemias are a heterogeneous group of haematopoietic malignancies that include many diverse and biologically distinct subgroups. The four major subtypes of leukaemia described by most cancer registries include acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphoblastic leukaemia (CLL), and chronic myeloid leukaemia (CML) (Yang et al. 2003). The leukaemia show clear geographic, racial, ethnic, age, and gender variation in both incidence and mortality. The patterns of occurrence differ among subtypes. Known risk factors are ionizing, radiation, certain chemotherapeutic agents, and specific occupational chemical exposures. Some risk factors like infections agents, electromagnetic fields, cigarette smoking, other chemotherapeutic agents, and additional occupational chemical exposure are suspected in leukemogenesis (Groves et al. 1994).

2.3.5. Breast cancer

The second used cell line was MCF-7 breast cancer cells, which are frequently used in pharmacological studies. Cancer of the breast is the most common cancer in women worldwide. Around 1.15 million cases were recorded in 2002, accounting for around 23 per cent of all cancers in women (11 per cent overall). Observed rates of this cancer increase with industrialisation and urbanisation, and also with facilities for early detection. It remains much more common in high-income countries (figure11) but is now increasing rapidly in middle- and low-income countries, including Africa, much of Asia, and Latin America.

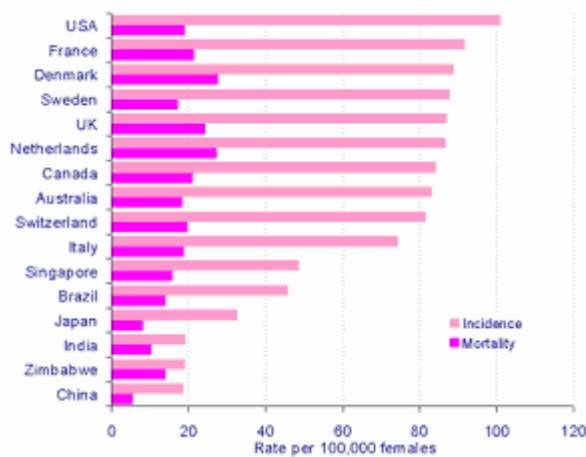


Figure 11: Age standardist (world) incidence and mortality rates, female breast cancer in selectet countries, 2002 estimates; UK Breast Cancer incidence statistics

Breast cancer is fatal in under half of all cases and is the leading cause of death from cancer in women (fifth overall), accounting for 14 per cent of all cancer deaths worldwide. Breast cancer is hormone related, and the factors that modify the risk of this cancer when diagnosed premenopausally and when diagnosed (much more commonly) postmenopausally are not the same. There is evidence showing the importance of early life events, including food and nutrition, as well as factors that affect hormone status, in modification of the risk of breast cancer (World Cancer Research Fund 2007).

2.3.6. Nutrition and Cancer

Food and nutrition modify the risk of cancers at a large number of sites. Vegetables, fruits, pulses (legumes), nuts, and seeds are sources of a wide variety of micronutrients and other bioactive compounds. Foods containing several of these constituents have been identified as being associated with cancer risk. However, it is not possible to ascribe the association between these foods and lower cancer risk to a causal effect of specific compounds with confidence, because each food contains a complex mixture of different constituents, all of which might also contribute to any effect (World Cancer Research Fund 2007).

Food components relevant to cancer development can be divided into macro- and micro components. The former tend to act indirectly. The latter usually have a clearly defined action, for example as genotoxic agents. Food can have both positive (carcinogenic) and negative (preventive) effects (Sugimura 2002).

For example, Quercetin is a flavonoid, which is a type of polyphenol. It is not an essential dietary component. Many studies in cultured cells and animals suggest that quercetin has antioxidant activity, which could lead to a range of biological activities, including reducing inflammation. Quercetin is found in apples, green and black tea, onions, raspberries, red wine, red grapes, citrus fruits, cherries, elderberries, broccoli, blueberries, cranberries, bilberries, and leafy, green vegetables, such as *L. sativa* (World Cancer Research Fund 2007).

2.4. Drugs

2.4.1. Overview

Many chemotherapeutic strategies interfere with important steps in cell cycle progression such as DNA replication and repair. Induction of apoptosis is another indicator for drug activity in cancer cells. However, the key drawback is that these drugs often cause severe side effects including immunotoxicity, neurotoxicity and nephrotoxicity.

Human tumour implanted animal models are often used for selecting anti-cancer drugs in drug discovery. The problems of in vivo models are, on the one hand, the long experimental duration, high costs and on the other hand, the limited predictive values because of species variations and other factors. That's why alternative methods such as in vitro cell-based assays, genomics and computational assays have gained more attention. Cultured human tumour cells, in our case HL 60 leukaemia cells and MCF-7 breast cancer cells, have been used as in vitro screening tools for identifying cancer drugs. Although activity oriented assays have been the major approaches of finding target-specific cancer drugs, minimizing toxic effects is also crucial to the development

of successful cancer drugs. It is recognized that understanding of toxicological properties in early phase will help to realise the bottlenecks and accelerate drugs discovery (W. Li et al. 2006).

In our particular case, the feature of nonspecific toxicity and undesired side effects is minimized because the tested plants have a long history of being used and well tolerated in humans.

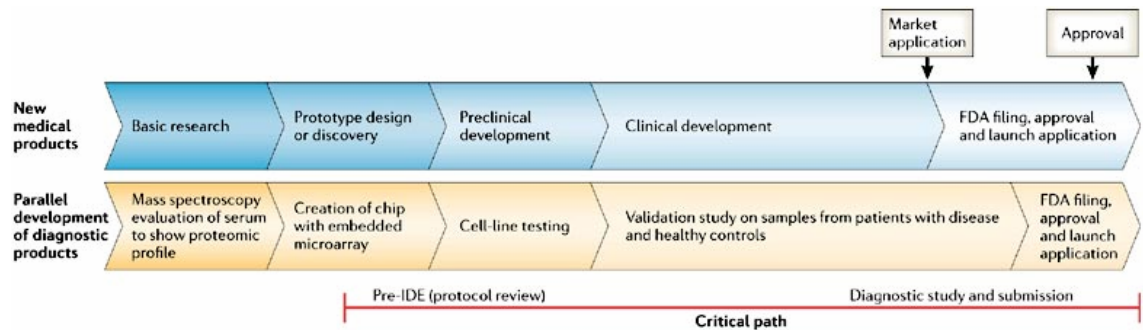
2.4.2. Development of drugs

The development of new medical products usually begins with basic research not knowing about any toxic side effects. Hence we selected two healing plants, which are still used by the Maya of the Guatemala/Belize lowland rain forest thereby eliminating the feature of non-specific toxicity. These plants are known to be successful in healing chronic inflammations and to be well tolerated by humans (in terms of side effects) and are unknown in the western medicinal and experimental literature (i.e. listed in PubMed)

The next step is the preclinical development. My part of this project was to study the possible in vitro anticancer activity of two plants which are used in traditional medicine to treat inflammations, because inflammation involves the activation of enzymes and intracellular pathways which may be also upregulated in tumour cells. In the traditional application the plants are air-dried which is a time-consuming process in the humid climate of the tropics and this may cause the degradation of active metabolites. From previous investigations with *A. alopecuroides* we know that freeze-drying of plants is mandatory for the preservation of their anti-cancer activity. Therefore, we compared the effectivity of the traditional- versus a modern preparation methods (freeze-drying of freshly harvested plants).

Further investigation would imply more preclinical test, the clinical development and end with a potential approval (in Europe through the „Richtlinie 2001/83/EG des Europäischen Parlaments und des Rates vom 6. November 2001 zur Schaffung eines Gemeinschaftskodexes für Humanarzneimittel“, in the USA

from the US Food and Drug Administration (FDA). Finally the product could be launched.



Copyright © 2006 Nature Publishing Group
Nature Reviews | Cancer

Figure 12: Development of new medical products (DE, investigational device exemption) (Gutman et al. 2006)

3. MATERIAL AND METHODS

3.1. Plants

3.1.1. *Syngonium podophyllum* (Contra Hierba)



Figure 13 : *Syngonium podophyllum*



Figure 14 : *Syngonium podophyllum*

(<http://sweetgum.nybg.org/common/imagedisplay.php?irn=181951>)

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopyda
Order:	Alismatales
Family:	Araceae
Genus:	<i>Syngonium</i>
Species:	<i>Syngonium podophyllum</i>

Syngonium is a genus of 33 species in the family Araceae. To this family belong 107 genres and over 3700 species. The parasitic vine grows on trees in forest clearings from Mexico to Panama. It can reach up to 5 m in length. In the adult form the large leaves are deeply lobed. The fruits are red. When a stem or a leaf breaks it exudes a sticky latex.

Traditional Uses:

A warm wash with leaves from *S. podophyllum* is used against sore, dry skin, fungus, itching, rashes and bruises. For rheumatism, arthritis, swellings and general pains a tincture of leaves soaked in alcohol is rubbed on painful areas. The important compounds are calcium oxalate and oxalic acid (Arvigo and Balick 1998).

3.1.2. *Pluchea Odorata* (Santa Maria)



Figure 15 *Pluchea odorata*
(www.fp.sfasu.edu)



Figure 16: *Pluchea odorata*
(www.delawarewildflowers.org)

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

Asteracea is the largest family of the order of the asterales. To this family belong 1528 genuses and 22750 species and it grows worldwide in every clime. The habitat of *P. odorata* are clearings, old fields and the edges of forests. It is a woody shrub up to 3 m of height with many branches. Like most species of this family the plant has aromatic leaves and brownish green flowers in large heads, turning to orange.

Traditional Uses:

A tea, made by boiling 3 *P. odorata* leaves in 3 cups of water for 2 minutes followed by 15 minutes of steeping, is used as a treatment against asthma attacks, coughs, colds and flu. A poultice with leaves warmed in oil should help against sore muscles, rheumatic pains, neuritis and arthritic joints. Furthermore *P. odorata* is used against infections and excessive bleedings after childbirth. Therefore the women sit over a steaming pot of herbs. Swelling, tumors, inflammations and bruises are also bathed in this herbal decoction (Arvigo and Balick 1998). The important compound of this plant is plucheinol.

3.1.3. *Lactuca sativa* var. *capitata* (Lettuce - Green salad)



Figure 17. *L. sativa*
(<http://en.wikipedia.org/wiki/>)

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Asterales
Family:	Asteraceae
Subfamily:	Cichorioideae
Genus:	<i>Lactuca</i>
Species:	<i>Lactuca sativa</i>
Form:	<i>Lactuca sativa</i> var. <i>capitata</i>

Lettuce is a cultivated form of *L. sativa*, which originally comes from neaples. The leaves are very compact and even after preparation very firm. Raw lettuce is used for salad and hamburgers, tacos and other dishes. In some countries lettuce is eaten cooked too.

3.2. Plant-extraction

The plants *P. odorata* and *S. podophyllum* were brought from Guatemala (San José/Petén). From these two plants I had two different materials:

Syngonium podophyllum

- air dried stipes and roots
- lyophilized stipes and roots from fresh plants

Pluchea odorata

- air dried leaves, florescence and stipes
- lyophilized leaves from fresh material

L. sativa was bought at a market in Vienna and afterwards lyophilized too.

The extractions were done in Prof. Dr. Brigitte Kopp's laboratory. (Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna)

First, the plant parts were milled (RETSCH ZM 100, sieve Nr. 05) and the obtained powder was weight out. Afterwards solvent was added with a concentration of 1:10 (to 50 g dried plant material 500 ml of e.g. petroleum ether was added). The solvent treated plant material (starting with the molst apolar solvent, PE) was dried for subsequent extractions with increasingly polar solvents. This methodical procedure is state of the art because it provides an initial seperation of apolar to polar bioactive plant constituents thereby providing maximal information by minimum plant expense.

Solvent	Reflux - water bath (°C)	Rotary evaporator (mbar)
Petroleum ether	40	650
Dichlormethan	50	650
Ethyl acetate	91	200
Methanol	79	300
Water	111	65

Table 1: solvents and the specific temperature and pressure used for the evaporator

The plant powder together with solvent was treated in an ultra sonic bath for 10 minutes to burst the cells of the plant. Then it was situated on a reflux – water bath. The temperature depends on which solvent was taken (shown in table 1).

After 60 minutes the solvent was filtered through a round filter (Schleicher & Schuell, Microscience, 595, RefNr. 10311612 – diameter 150 nm).

- The remaining plant material (residue) was dried on a sheet of paper to complete dryness before the next extraction with a more polar solvent followed (from petroleum ether to water).
- The material which was dissolved in the liquid phase (solvent) was gained by evaporating the solvent until complete dryness using a rotavapor (Heidolph WB 2001) with a temperature of the water bath (Heidolf VV 2011) of 40°C. The pressure is shown in table 1.

So step by step five plant extracts (PE extract, CH₂Cl₂ extract, EA extract, MeOH extract and water extract) were obtained. Finally the dried extracts were dissolved in ethanol for further use in cell culture experiments.

3.3. Cell Culture

HL-60 promyelocytic leukaemia cells, MCF-7 breast cancer cells and MCF-10A immortalized non-tumorigenic fibrocystic mammary gland epithelial cells were purchased from ATCC. HL-60 cells were grown in logarithmical growth phase in RPMI 1640 medium, MCF-7 and MCF-10A in DMEM medium, all of them supplemented with 10% heat inactivated fetal calf serum, 1% L-Glutamine and 1% Penicillin/Streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. All media and supplements were obtained from Life Technologies. All cell types were kept in humidified atmosphere containing 5 % CO₂ at 37°C.

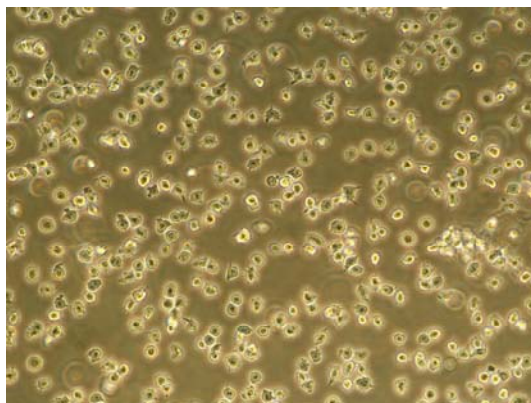


Figure 18: untreated HL-60 cells

3.4. Proliferation-Assay

Three different cell types were used: HL-60, MCF-7 and MCF-10A. To determine the antiproliferative effect of the plant extracts HL-60 cells were seeded in T-25 tissue culture flasks at a concentration of 0.1×10^6 cells per ml. MCF-7 and MCF-10A were seeded at a concentration of 0.01×10^6 cells per ml in 24-well plates and grown for 24 h. Then they were incubated with increasing concentration of plant extracts (500 µg/ml, 1 mg/ml, 4 mg/ml, 20 mg/ml) for 72 h. After 24 h and 72 h cells were counted and IC_{50} values for plant extracts were determined with a microcellcounter (Sysmex Corp., Japan). MCF-7 and MCF-10A had to be washed with PBS and trypsinised before. Experiments were done in triplicate.

Calculation to determine the percent of cell division:

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

$C_{72h + drug} \rightarrow$ cell number after 72 hours of drug treatment

(specific for the respective extract concentrations)

$C_{24h + drug} \rightarrow$ cell number after 24 hours of drug treatment

(specific for the respective extract concentrations)

$C_{72h - drug} \rightarrow$ cell number after 72 hours without drug treatment

$C_{24h - drug} \rightarrow$ cell number after 24 hours without drug treatment

3.5. Apoptose Assay – Hoechst 33258/propidium iodide (HOPI) double staining

To determine the type of cell death, HL-60 cells were seeded in T-25 tissue culture flasks (0.1×10^6 cells per ml) and MCF-7 were seeded in 24-well plates (0.01×10^6 cells per ml), grown for 24 h and treated with increasing concentrations (500 µg/ml, 1 mg/ml, 4 mg/ml, 20 mg/ml) of extract for 96 h. Afterwards MCF-7 cells were washed with PBS and trypsinised. Then Hoechst and propidium iodide were added directly to the cells at a final concentration of 5 µg/ml and 2 µg/ml for 1 hour at 37°C and then the stained cells were

photographed on Kodak Ektachrome P1600 film, analysed and counted manually. Experiments were done in triplicate.

3.6. FACS-analysis

HL-60 cells were seeded in T-25 tissue culture flasks with a concentration of 1×10^6 cells per ml and incubated with 500 µg/ml and 1 mg/ml *Pluchea odorata* dichloromethane extract at 37°C under cell culture conditions for 24 hours. Then the cells were washed with cold PBS, centrifuged (900 rpm, 5 min), resuspended and fixed in EtOH (70%) for 30 min at 4°C. After two further washing steps with cold PBS, RNase A and propidium iodide were added to a final concentration of 50 µg/ml each, incubated at 4°C over night and determined by FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Cell distribution was calculated with ModFid LT software (Verity Software House, Topsham, ME, USA)

3.7. Western blotting

Preparation of lysates

HL 60 cells were seeded in T-25 tissue culture flasks at a concentration of 1×10^6 cells per ml and incubated with 4 mg/ml extract (*P. odorata* and *L. sativa*) for 0.5, 1, 4, 8 and 24 hours. At every time point, 2×10^6 cells were harvested, washed twice with cold PBS, centrifuged at 1000 rpm for 5 minutes and lysed in 100 µl buffer (containing 150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton-X-100), 5 µl 1 mM phenylmethylsulfonylfluoride (PMSF) and 5 µl Protease Inhibitor Cocktail (PIC; from a 100 x stock). Afterwards the lysate was centrifuged for 20 min at 12000 rpm at 4°C, and the supernatant was stored at -20°C until further analyses.

SDS-PAGE and electrotransfer

Equal amounts of lysate (protein samples) were loaded onto a polyarylamide gels. Proteins were electrophoresed (PAGE) and then electro-blotted onto PVDF-membranes (Hybond- Amersham) at 20 V 4°C overnight or at 90 V for an hour (both at 4°C). To confirm equal sample loading, membranes were stained with Ponceau S.

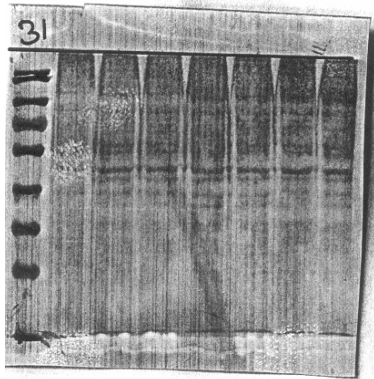


Figure 19: Membran stained with Ponceau S

Immuno-reaction

After washing with PBS/T (Phosphate Buffered Saline/0,5% Tween 20, pH: 7.2) or TBS/T (Tris Buffered Saline/0,1% Tween 20, pH: 7.6) the membranes were blocked in PBS- or TBS-Milk (5% non-fat dry milk in PBS containing 0.5% Tween or in TBS containing 0.1% Tween 20) for one hour and then washed with PBS/T or TBS/T for at least 30 min changing the washing solution 3-4 times. Afterwards the membranes were incubated with the first antibody (in blocking solution, dilution 1:500) by gently rocking at 4°C overnight. Then the membranes were washed with PBS/T or TBS/T for 30 min and incubated with the second antibody (anti-rabbit IgG or anti-mouse IgG, dilution 1:2000) for 1 hour at room temperature. After washing with PBS/T or TBS/T the chemo luminescence was detected by ECL detection kit (Amersham, UK) and the membranes were exposed to Amersham Hyper films.

Antibodies:

- Cyclin D1 (M-20) :sc-718, polyclonal (SANTA CRUZ BIOTECHNOLOGY; INC)

- P21 (C-19) :sc-397, polyclonal (SANTA CRUZ BIOTECHNOLOGY, INC)
- Phospho-p44/42 MAP Kinase (Thr202/Tyr204) E10, #9106, monoclonal (Cell Signaling)
- P44/42 MAP Kinase (137F5) Rabbit, #4695, monoclonal (Cell Signaling)
- Phospho-MEK 1/2 (Ser21/221), #9121, polyclonal (Cell Signaling)
- MEK 1/2 AB, #9122, polyclonal (Cell Signaling)
- Cleaved Caspase-3 (Asp175), #9661, polyclonal (Cell Signaling)
- PARP-1 (F-2): sc-8007, monoclonal (SANTA CRUZ BIOTECHNOLOGY, INC)
- MONOCLONAL ANTI-ACETYLATED TUBULIN, CLONE 6-11B-1, Mouse Ascited Fluid, Product No. T6793 (SIGMA)
- α Tubulin (DM1A): sc-32293, monoclonal (SANTA CRUZ BIOTECHNOLOGY, INC)
- β Tubulin (H-235): sc-9104, polyclonal (SANTA CRUZ BIOTECHNOLOGY, INC)
- Monoclonal Anti- β -Actin, Clone AC-15, mouse ascites fluid, Catalog Number A5441 (SIGMA)
- Cdc25A (phospho S17) antibody (ab18321), polyclonal (abcam)
- Phospho-cdc25a-S177 Antibody, polyclonal (ABGENT)
- Cdc25A (F-6): sc-7389, monoclonal (SANTA CRUZ BIOTECHNOLGOY, INC)
- Cdc25A (M-191): sc-7157, polyclonal (SANTA CRUZ BIOTECHNOLOGY, INC)
- Phospho-Chk2 (Thr68), #2661, polyclonal (Cell Signaling)
- Chk2 Antibody, #2662, polyclonal (Cell signaling)
- Phospho-cdc2 (Tyr15) (10A11) Rabbit mAb, #4539, monoclonal (Cell Signaling)
- Phospho-Chk1 (Ser345), #2341, polyclonal (Cell Signaling)
- Chk1, #234 (Cell Signaling)

Pierce EC Western Blotting Substrate Cat #32106 (Pierce)

Amersham Hyper films ECL – High performance chemiluminescence film (GE-Healthcare)

3.8. COX Inhibitor Screening Assay

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) enzymes catalyze the first step in the biosynthesis of prostaglandins (PG's), thromboxanes, and prostacylin; the conversion of arachidonic acid to PGH_2 .

COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs.

This assay measures $\text{PGF}_{2\alpha}$ produced by SnCl_2 reduction of COX-derived PGH_2 .

The COX screening assay was purchased from CAYMAN (Catalog No. 560131) and performed according to the manufacturer's instructions.

Performing COX-Reaction

Reagents: Reaction Buffer, COX-2 human recombinant, Heme, Arachidonic acid, Potassium hydroxide, Hydrochloric acid, Stannous Chloride

- Background tubes: Inactive COX-2 (COX-2 3 min in boiling water) was mixed with heme
- COX-2 100 % Initial activity tubes: COX-2 was mixed with heme
- COX-2 Inhibitor tubes: COX-2 was mixed with heme and afterwards extracts, derived from freeze dried stipes and roots from *Syngonium podophyllum*, were added in different concentrations (0,01 mg/ml; 0,1 mg/ml; 1 mg/ml; 4 mg/ml)

→ Incubation for 10 minutes at 37°C (on water bath)

COX-reaction was initiated by adding 10 μl of arachidonic acid to all test tubes following a further incubation for 2 min at 37°C water bath. Adding 1 M HCl stopped catalysis. To obtain a more stable prostaglandin saturated stannous chloride solution was added to each test tube (this reagent reduces PGH_2 to $\text{PGF}_{2\alpha}$).

Prostaglandins are quantified by EIA.

Performing the EIA

Regencies: EIA Buffer, Wash Buffer, Prostaglandin Standard, Prostaglandin Screening AchE Tracer, Prostaglandin Screening Antiserum

Each COX-reaction was assayed at two dilutions and each dilution was assayed in triplicate. Blanks, non-specific binding wells, maximum binding wells, total activity wells, Background samples (COX-2) and eight point standard curve (Prostaglandin Standard) were performed in duplicate. Afterwards the plate was covered with plastic film and incubated for 18 hours at room temperature.

Development and reading the plate

The plate was emptied and then washed five times. Ellmans's Reagents was added and the plate was covered with plastic film again. Now the plate was developed in the dark by using a shaker for 90 minutes and then was readed at a wavelength of 405 nm.

3.9. Total phenolics determination

Total phenolic content was determined using the Folin-Ciocalteu method, described by Singleton et al. and slightly modified according to Dewanto et al. 125 µl of suitable diluted extracts and 125 µl Folin-Ciocalteu reagent were merged. Afterwards 0.5 ml deionized water was added. The mixture was kept for 6 min, and then, 1.25 mL of a 7% aqueous Na_2CO_3 solution was added. The final volume was adjusted to 3 mL with water. After 90 min, the absorption was measured at 760 nm against water as a blank. The amount of total phenolics was expressed as gallic acid equivalents (GAE, mg gallic acid/100 g sample) through the calibration curve of gallic acid (Heimler et al. 2007).

4. RESULTS

4.1. *Syngonium Podophyllum*

The first tested plant was *S. Podophyllum*. Two different parts of this plant were analysed:

- stipes and roots – lyophilized
- stipes and roots – air dried

4.1.1. Stock calculation

4.1.1.1. *Stipes and roots – lyophilized*

Table 2a shows the extract weight in g derived from 51.6 g freeze dried plant of the five different solvent types (petroleum ether, dichlormethan, ethyl acetate, methanol, water) and the extract amount corresponding to 1 mg plant.

Four different concentrations were used to perform proliferation assays: 500 µg/ml, 1 mg/ml, 4 mg/ml and 20 mg/ml. The supplied extracts were added as shown in table 2b.

a)

Solvent	Extract weight (g) Derived from 51,6 g dried plant	Extract corresponding to 1mg plant
Petroleum Ether	0.44132	8.55 µg
Dichloromethane	0.13854	2.68 µg
Ethyl Acetate	0.23121	4.48 µg
Methanol	2.57482	48.90 µg
Water	2.14285	41.53 µg

b)

Extract Type	Concentration mg dried plant weight/ml medium	µg dried extract weight/ml medium
Petroleum Ether	0.5	4.28 µg
	1	8.55 µg
	4	34.20 µg
	20	171 µg
Dichlormethane	0.5	1.34 µg
	1	2.68 µg
	4	10.72 µg
	20	53.60 µg
Ethyl Acetate	0.5	2.24 µg
	1	4.48 µg
	4	17.92 µg
	20	89.60 µg
Methanol	0.5	24.45 µg
	1	48.90 µg
	4	195.60 µg
	20	978.00 µg
Water	0.5	20.77 µg
	1	41.53 µg
	4	166.12 µg
	20	830.60 µg

c)

PE	3 ml EtOH
CH ₂ Cl ₂	3 ml EtOH
EA	3 ml EtOH
MeOH	4 ml EtOH and 2 ml MeOH
H ₂ O	13 ml H ₂ O

Table 2: Extract weights from 51.6 g dried plant

The plant extracts were dissolved in different amounts of ethanol, methanol or water (see table 2 c). To avoid solvent bias, EtOH was added to the control and every other concentration to achieve similar solvent concentrations as in the highest extract concentration (20 mg/ml) e.g. for petroleum ether (in 5 ml Medium):

→ For control: ad 5.81 µl EtOH

→ For 500 µg/ml: ad 5.66 µl EtOH (5.81-0.15)

→ For 1 mg/ml: ad 5.52 µl EtOH (5.81-0.29)

→ For 4 mg/ml: ad 4.65 µl EtOH (5.81-1.16)

In general, this corresponded to an ethanol-concentration of 0.12% in every sample.

4.1.1.2. *Stipes and roots – air dried*

Table 3a shows the extract weight in g derived from 43.8 g air dried plant for the five different solvent types (petroleum ether, dichloromethane, ethyl acetate, methanol, water) and the extract amount corresponding to 1 mg plant.

Four different concentrations were used to perform proliferation assays: 500 µg/ml, 1 mg/ml, 4 mg/ml and 20 mg/ml. The supplied extracts were added as shown in table 3b.

a)

Solvent	Extract weight (g) Derived from 43.80 g dried plant	Extract corresponding to 1mg plant
Petroleum Ether	0.43452	9.92 µg
Dichloromethane	0.36114	8.25 µg
Ethyl Acetate	0.2309	5.27 µg
Methanol	1.51751	34.65 µg
Water	2.60450	59.46 µg

b)

Extract Type	Concentration mg dried plant weight/ml medium	µg dried extract weight/ml medium
Petroleum Ether	0.5	4.96 µg
	1	9.92 µg
	4	39.68 µg
	20	198.40 µg
Dichlormethane	0.5	4.13 µg
	1	8.25 µg
	4	33.00 µg
	20	165.00 µg
Ethyl Acetate	0.5	2.64 µg
	1	5.27 µg
	4	21.08 µg
	20	105.40 µg
Methanol	0.5	17.33 µg
	1	34.65 µg
	4	138.60 µg
	20	693.00 µg
Water	0.5	29.73 µg
	1	59.46 µg
	4	237.84 µg
	20	1189.20 µg

c)

PE	4 ml EtOH
CH ₂ Cl ₂	3 ml EtOH
EA	3 ml EtOH
MeOH	4 ml EtOH and 2 ml MeOH
H ₂ O	18 ml H ₂ O

Table3: Extract weights from 43.80 g dried plant

The plant extracts were dissolved in different amounts of ethanol, methanol or water (see table 3 c). To avoid solvent bias, EtOH was added to the control and every other concentration to achieve similar solvent concentrations as in the highest extract concentration (20 mg/ml) e.g. for dichlormethan (in 5 ml Medium):

→ For control: ad 6.85 µl EtOH

→ For 500 µg/ml: ad 6.68 µl EtOH (6.85-0.17)

→ For 1 mg/ml: ad 6.51 µl EtOH (6.85-0.34)

→ For 4 mg/ml: ad 5.48 µl EtOH (6.85-1.37)

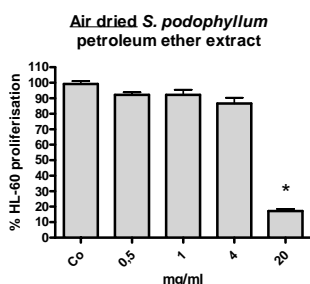
In general, this corresponded to an ethanol-concentration of 0.14% in every sample.

4.1.2. Antiproliferative activity of *S. podophyllum* extracts

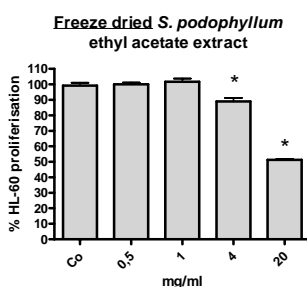
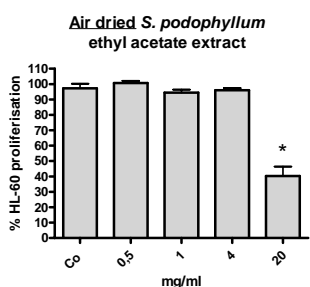
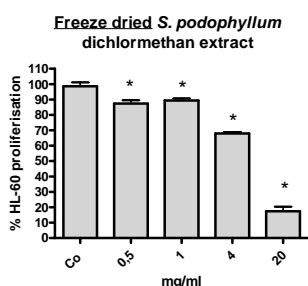
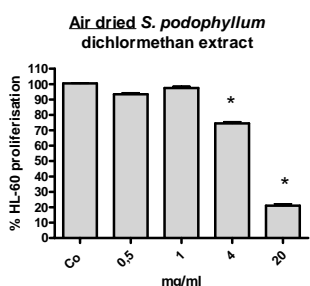
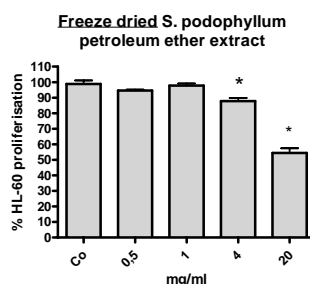
All extracts were tested in HL-60 cells. As illustrated in figure 20 all extracts showed an antiproliferative activity in HL-60 cells at a concentration of 20 mg/ml. The potential of the air dried material is rather similar to that of the freeze dried material regarding proliferation inhibitory effects. In both extract types methanol extract was the most effective with a growth inhibition of 80 % at a concentration of 4 mg/ml. Furthermore the dichlormethan extract has a comparatively high antiproliferative effect too with an inhibition of 30 % at a concentration of 4 mg/ml. All of the other extracts (petroleum ether, ethyl acetate and water) showed an effective growth inhibition up to nearly 90 % at a concentration of 20 mg/ml.

HL-60

air dried



freeze dried



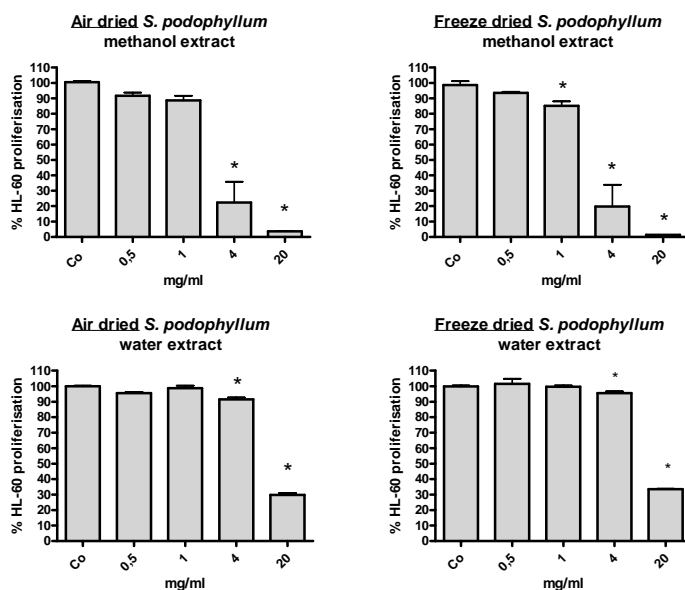


Figure 20 legend: Anti-proliferative effects of air dried and freeze dried *S. podophyllum* HL-60 cells were seeded into T-25 tissue culture flasks (1×10^5 cells / ml), grown for 24 hours to enter logarithmic growth phase, and incubated with 0.5, 1, 4, and 20 mg/ml of plant extracts (mg-amounts relate to freeze dried alternatively dried plant material before extractions). Controls received around 0.2% EtOH which was the ethanol concentration which cells had to experience together with the highest extract concentration. The other samples were also adjusted to equal ethanol concentrations to achieve similar solvent conditions. Cells were counted after 24, 48 and 72 hours of treatment and the percentage of proliferation between this time span was calculated in comparison to controls (controls were considered as 100 % proliferating cells and all other conditions were set in relation to this). Experiments were done in triplicate. Error bars indicate SEM, and asterisks significant proliferation inhibition compared to control (p<0.05).

Plant material			Solvent	IC 50
<i>S. podophyllum</i>	Stipes and roots	Lyophilized	Petroleum ether	n.r.
<i>S. podophyllum</i>	Stipes and roots	Lyophilized	Dichlormethan	9.5 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Lyophilized	Ethyl acetate	n.r.
<i>S. podophyllum</i>	Stipes and roots	Lyophilized	Methanol	2.6 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Lyophilized	Water	16 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Air dried	Petroleum ether	12.5 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Air dried	Dichlormethan	11.5 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Air dried	Ethyl acetate	17 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Air dried	Methanol	2.7 mg/ml
<i>S. podophyllum</i>	Stipes and roots	Air dried	Water	14.7 mg/ml

n.r. = not reached

Table 4: IC 50 - values of *S. podophyllum*

4.1.3. Anti-inflammatory activity evaluated as PGH₂

The extract with the highest antiproliferative activity of *S. podophyllum* was freeze dried methanol. Therefore this extract was used to perform this assay, but the results weren't significant. That's why they were not shown at this place.

Since the anti-proliferative activity was rather low and the anti-inflammatory activity didn't exist, we skipped further analyses with this plant.

4.2. *Pluchea odorata*

The more effective of the two tested plants was *P. odorata*. Two different parts of this plant were analysed:

- leaves, lyophilized
- leaves, florescence and stipes, air dried

4.2.1. Stock calculation

4.2.1.1. Leaves - lyophilized

Table 5a shows the extract weight in g derived from 35.8 g freeze dried plant for the five different solvent types (petroleum ether, dichlormethan, ethyl acetate, methanol, water) and the extract amount corresponding to 1 mg plant.

Four different concentrations were used to perform proliferation and apoptosis assays: 500 µg/ml, 1 mg/ml, 4 mg/ml and 20 mg/ml. The supplied extracts were added as shown in table 5b.

a)

Solvent	Extract weight (g) Derived from 35.8 g dried plant	Extract corresponding to 1mg plant
Petroleum Ether	0.97193	27.15 µg
Dichloromethane	0.9580	26.76 µg
Ethyl Acetate	0.46451	12.98 µg
Methanol	6.26163	174.91 µg
Water	3.9381	110.00 µg

b)

Extract Type	Concentration mg dried plant weight/ml medium	µg dried extract weight/ml medium
Petroleum Ether	0.5	13.76 µg
	1	27.15 µg
	4	108.60 µg
	20	543.00 µg
Dichlormethane	0.5	13.38 µg
	1	26.76 µg
	4	107.04 µg
	20	535.20 µg
Ethyl Acetate	0.5	6.49 µg
	1	12.98 µg
	4	51.92 µg
	20	259.60 µg
Methanol	0.5	87.46 µg
	1	174.91 µg
	4	699.64 µg
	20	3498.20 µg
Water	0.5	55.00 µg
	1	110.00 µg
	4	440.00 µg
	20	2200.00 µg

c)

PE	4 ml EtOH
CH ₂ Cl ₂	5 ml EtOH
EA	3 ml EtOH
MeOH	4 ml EtOH and 4 ml MeOH
H ₂ O	8 ml H ₂ O

Table 5: Extract weights from 35.80 g dried plant

The plant extracts were dissolved in different amounts of ethanol, methanol or water (see table 5 c). To avoid solvent bias, EtOH was added to the control and every other concentration to achieve similar solvent concentrations as in the highest extract concentration (20 mg/ml) e.g. for ethyl acetate (in 5 ml Medium):

- For control: ad 8.38 µl EtOH
- For 500 µg/ml: ad 8.17µl EtOH (8.38-0.21)
- For 1 mg/ml: ad 7.96 µl EtOH (8.38-0.42)
- For 4 mg/ml: ad 6.70 µl EtOH (8.38-1.68)

In general, this corresponded to an ethanol-concentration of 0.17% in every sample.

4.2.1.2. Leaves, florescence and stipes - air dried

Table 6a shows the extract weight in g derived from 38.75 g air dried plant for the five different solvent types (petroleum ether, dichlormethan, ethyl acetate, methanol, water) and the extract amount corresponding to 1 mg plant.

Four different concentrations were used to perform proliferation assays: 500 µg/ml, 1 mg/ml, 4 mg/ml and 20 mg/ml. The supplied extracts were added as shown in table 6b.

a)

Solvent	Extract weight (g) Derived from 38.75 g dried plant	Extract corresponding to 1mg plant
Petroleum Ether	0.6015	15.52 µg
Dichloromethane	0.27748	7.16 µg
Ethyl Acetate	0.10966	2.83 µg
Methanol	1.014	26.17 µg
Water	3.1978	82.52 µg

b)

Extract Type	Concentration mg dried plant weight/ml medium	µg dried extract weight/ml medium
Petroleum Ether	0.5	7.76 µg
	1	15.52 µg
	4	62.08 µg
	20	310.40 µg
Dichlormethane	0.5	3.58 µg
	1	7.16 µg
	4	28.64 µg
	20	134.20 µg
Ethyl Acetate	0.5	1.42 µg
	1	2.83 µg
	4	11.32 µg
	20	56.60 µg
Methanol	0.5	0.39 µg
	1	0.77 µg
	4	3.08 µg
	20	15.40 µg
Water	0.5	41.26 µg
	1	82.52 µg
	4	330.08 µg
	20	1650.40 µg

c)

PE	3 ml EtOH
CH ₂ Cl ₂	3 ml EtOH
EA	3 ml EtOH
MeOH	3 ml EtOH and 3 ml MeOH
H ₂ O	13 ml H ₂ O

Table 6: Extract weights from 38.75 g dried plant

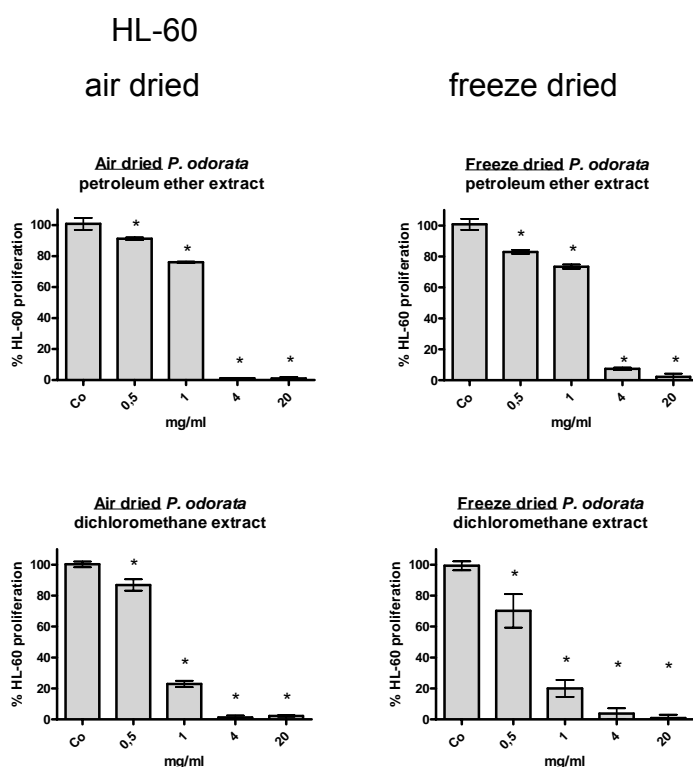
The plant extracts were dissolved in different amounts of ethanol, methanol or water (see table 6 c). To avoid solvent bias, EtOH was added to the control and every other concentration to achieve similar solvent concentrations as in the highest extract concentration (20 mg/ml) e.g. for petroleum ether (in 5 ml Medium):

- For control: ad 5.16 µl EtOH
- For 500 µg/ml: ad 5.03 µl EtOH (5.16-0.13)
- For 1 mg/ml: ad 4.90 µl EtOH (5.16-0.26)
- For 4 mg/ml: ad 4.13 µl EtOH (5.16-1.03)

In general, this corresponded to an ethanol-concentration of 0.1 % in every sample.

4.2.2. Anti-proliferative activity of *P. odorata* extracts

All extracts showed an antiproliferative effect in HL-60 cells (figure 21). A concentration of 4 mg/ml caused 95 to 100 % proliferation in every extract type. Unexpectedly, the way of plant drying did not affect the activity of the *P. odorata* dichloromethane extract, because air-dried and freeze-dried plant material arrested the cell cycle similarly and at low doses. The best result showed the dichloromethane extract with a growth inhibition of 80 % at a concentration of 1 mg/ml. Therefore a proliferation assay was made in MCF-7 breast cancer cells too. These cells are frequently used in pharmacological studies, because the vast majority of all agents, which exhibit activity in the NCI-60 cell line screen, are also active in MCF-7 breast cancer cells (Cragg 2000). Furthermore a proliferation assay in MCF-10A breast epithelial cells was also made.



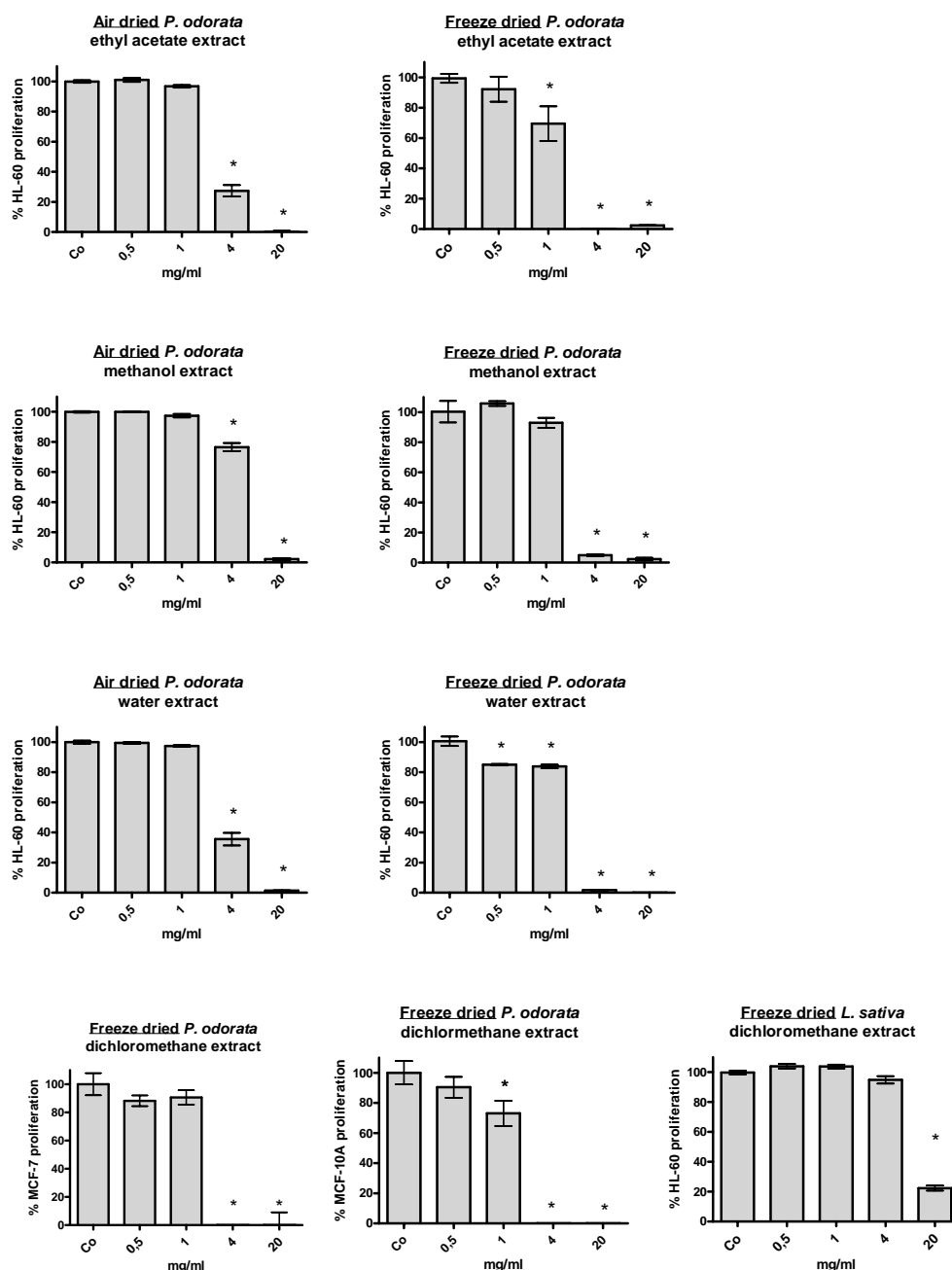


Figure 21 legend: Anti-proliferative effects of *P. odorata* and *L. sativa* extracts

HL-60 cells were seeded into T-25 tissue culture flasks (1×10^5 cells / ml), grown for 24 hours to enter logarithmic growth phase, and incubated with 0.5, 1, 4, and 20 mg/ml of plant extracts (mg-amounts relate to dried plant material before extractions). Controls received 0.10% to 0.45% EtOH which was the ethanol concentration which cells had to experience together with the highest extract concentration. The other samples were also adjusted to equal ethanol concentrations to achieve similar solvent conditions. Cells were counted after 24 and 48 hours of treatment and the percentage of proliferation between this time span was calculated in comparison to controls (controls were considered as 100 % proliferating cells and all other conditions were set in relation to this). MCF-7 and MCF-10A cells were seeded in 24 well plates, allowed to attach for 24 hours when extracts were applied as described above and cells counted after 24, 48, and 72 hours. Experiments were done in triplicate. Error bars indicate SEM, and asterisks significant proliferation inhibition compared to control ($p < 0.05$).

Both cells types caused a growth inhibition of 100 % at 4 mg/ml (figure 21). MCF-7 showed an antiproliferative activity of 10 %, MCF-10A of 25%. That's however less efficient than observed in HL-60 cells. As a negative control the dichlormethanextract of *L. sativa* was tested, because this is not a pharmacological plant that can be consumed in considerable amounts. 4 mg/ml dichlormethan extract of *L. sativa* was non-toxic. Unexpectedly, the 20 mg/ml concentration (corresponding to 320 µg dried extract/ml) exhibited significant anti-proliferative activity. This evidences that the anti-proliferative properties of *L. sativa* was not an effect of random plant toxicity or due to extremely high residue amounts in the *L. sativa* dichlormethan extract, but specific due to unknown apolar constituents.

Plant material			Solvent	IC 50
<i>P. odorata</i>	Leaves	Lyophilized	Petroleum ether	2 mg/ml
<i>P. odorata</i>	Leaves	Lyophilized	Dichlormethan	0.7 mg/ml
<i>P. odorata</i>	Leaves	Lyophilized	Ethyl acetate	1.8 mg/ml
<i>P. odorata</i>	Leaves	Lyophilized	Methanol	2.4 mg/ml
<i>P. odorata</i>	Leaves	Lyophilized	Water	2.2 mg/ml
<i>P. odorata</i>	Leaves, florescence and stipes	Air dried	Petroleum ether	2 mg/ml
<i>P. odorata</i>	Leaves, florescence and stipes	Air dried	Dichlormethan	0.8 mg/ml
<i>P. odorata</i>	Leaves, florescence and stipes	Air dried	Ethyl acetate	3 mg/ml
<i>P. odorata</i>	Leaves, florescence and stipes	Air dried	Methanol	9.6 mg/ml
<i>P. odorata</i>	Leaves, florescence and stipes	Air dried	Water	3.3 mg/ml

Table 7: IC 50 - values of *P. odorata*

4.2.3. Dichlormethan extract of *P. odorata* inhibits cell cycle in G2-M

HL-60 cells were seeded in T-25 tissue culture flasks with a concentration of 1×10^6 cells per ml and incubated with 500 µg/ml, and 1 mg/ml *P. odorata* dichlormethan extract at 37°C under cell culture conditions for 24 hours. Then the cells were stained and the respective cell cycle phase cell distribution was determined by FACS analysis.

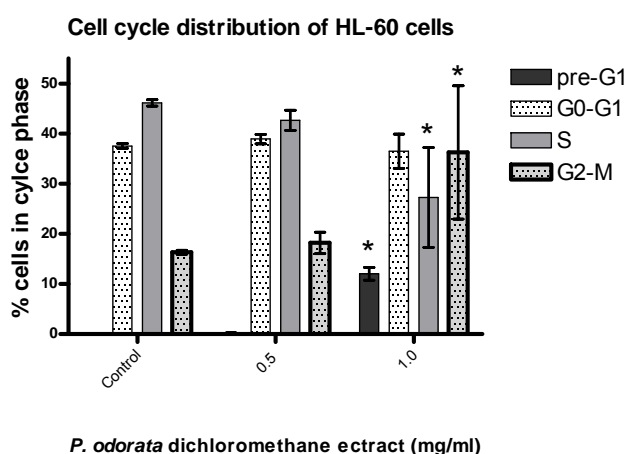


Figure 22: Cell cycle distribution of HL 60 cells upon treatment with of *P. odorata* dichloromethane extract

Logarithmically growing HL-60 cells were incubated with increasing concentrations of 0.5 and 1.0 mg/ml extract for 24 hours and then subjected to FACS analysis. Error bars indicate SEM and asterisks significance regarding the respective cell cycle phase of untreated controls ($p = 0.05$). Experiments were done in triplicate.

Figure 22 shows a significant increase in cells inhibited at the G2-M-Phase in the cell cycle progression with a higher number of cells in G2-M-Phase and lesser number of cells in S-Phase. Furthermore the quantity of pre-G1-Phase-cells growth up to 10 % at a concentration of 1 mg/ml.

4.2.4. Inhibition of proliferation is preceded by the down-regulation of cell cycle protagonists

Deregulated cell cycle progression is a hallmark of cancer. Cyclin-dependent kinases (CDK) play central roles in promoting cell cycle progression. Overexpression of CDK phosphatases often occurs in human cancer because uncontrolled activation of CDK's as well down-regulation of CDK's is the driving force of cancer cell proliferation. CDC 25 phosphatases are critical for timely

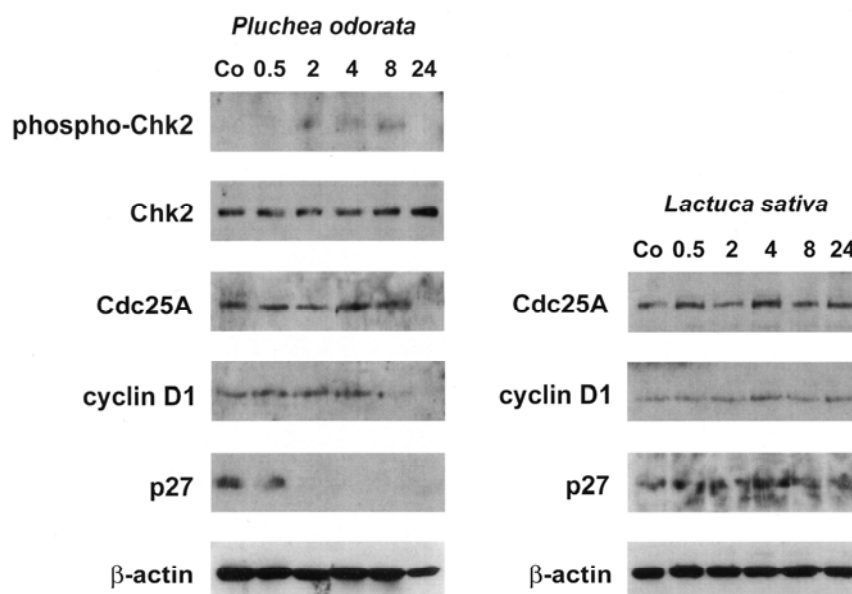
CDK activation in cell cycle progression. Studies using cultured cells suggest that CDC 25A regulates both G₁-S and G₂-M transition (Ray and Kiyokawa 2008) Chk2 was shown to target Cdc 25A to proteasomal degradation and therefore inhibit cell cycle.

4 mg/ml dichlormethane extract of *P. odorata* caused an activating phosphorylation of Chk 2 (figure 23a) after 2 hours followed by a decrease of Cdc 25A within 24 hours of incubation.

Cyclin D1 is an important regulator of G1 to S phase progression. Overexpression of cyclin D1 has been linked to the development and progression of cancer. Deregulated Cyclin D1 degradation appears to be responsible for the increased level of Cyclin D 1 in several cancers. Therefore Cyclin D1 is an attractive target for anti-cancer therapy (Alao 2007). Figure 23a shows that Cyclin D1 expression decreased after 8 hours too. *P. odorata* did not induce p21, a cell cycle inhibitor, but constitutive p27 expression became suppressed. This did not correlate with cell cycle inhibition.

On the other hand 4 mg/ml dichlormethanextract of *L. sativa* showed no effect in HL-60 proliferation, neither with cdc25 nor with Cyclin D1 or p27.

a)



b)

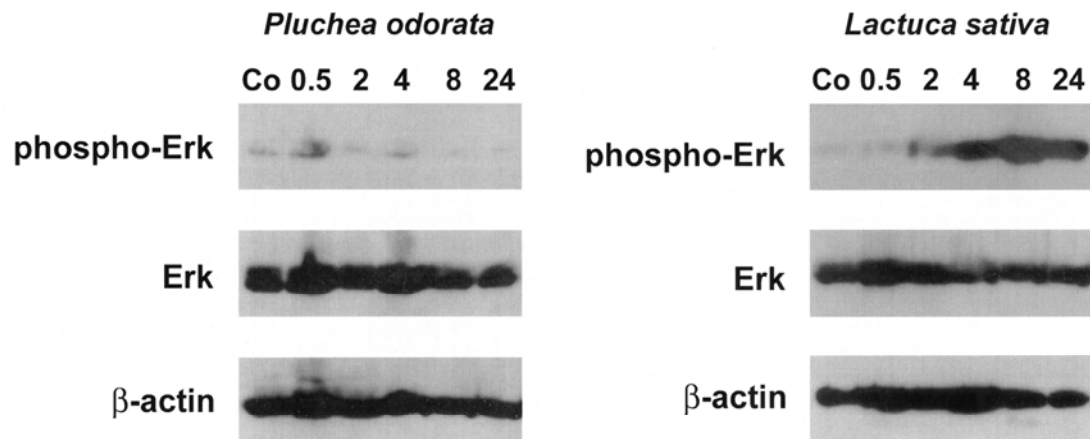


Figure 23 legend: Analysis of cell cycle-related protein and phospho-protein expression
 HL-60 cells (1×10^6 cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 hours when cells were incubated with 4 mg/ml dichloromethane extract of *P. odorata* and *L. sativa* for 0.5, 2, 4, 8, and 24 hours. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: (a) Cdc25A, phospho-Thr68-Chk2, Chk2, cyclin D1, p27; (b) phospho-Thr202/Tyr204-Erk, and Erk. Equal sample loading was controlled by Ponceau S staining and β-actin analysis.

Erk (MAP-Kinase) plays a critical role in the regulation of cell growth and differentiation. It is a major transducer of mitotic signalling. Figure 23b shows that Erk became dephosphorylated and therefore inactivated after 8 hours of treatment.

L. sativa dichloromethane extract showed an intense and long-lasting phosphorylation after 2 hours. In contrast, the prior presented Proliferation assay hasn't shown this effect. Therefore, the level of Erk did not correlate with the proliferation of HL-60 cells

4.2.5. The dichloromethane extract of *P. odorata* induces caspase 3 and apoptosis

The dichloromethane extract of *P. odorata* was further tested regarding pro-apoptotic properties in HL-60 and MCF-7 cells because a major of cytotoxic anticancer drugs is the potential to elicit cancer cell death. Both cell types induced apoptosis, in HL-60 earlier than in MCF-7. A concentration of 4 mg/ml was sufficient to eliminate 100 % of HL60-cells after 48 hours. MCF-7 cells caused a cell death rate of 85 % after 96 hours (figure 24).

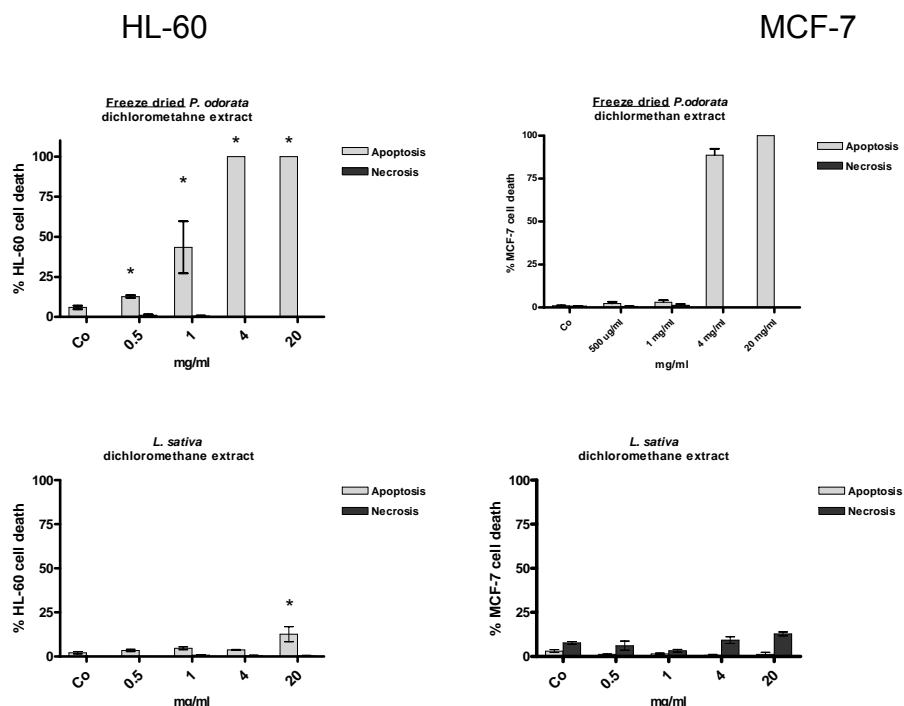


Figure 24 legend: Induction of apoptosis and necrosis by the dichloromethane extract of *P. odorata*

HL-60 cells were incubated for 48 hours and MCF-7 cells for 96 hours with increasing extract concentrations. 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 (see “Methods”) were counted and percentages of vital, apoptotic and necrotic cells were calculated. Experiments were done in triplicate. Error bars indicate SEM, and asterisks significant apoptosis induction compared to control (p<0.05).

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). In HL-60 cells Caspase 3 becomes activated by *P. odorata* extract. 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) (Fernandes-Alnemri et al. 1994).

As we have seen in prior figures, that dichloromethanextract of *P. odorata* starts to induces apoptosis at a concentration of 1 mg/ml in HL-60 cells. At a concentration of 4 mg/ml and 24 hour we see a distinctive cleavage of Caspase 3 to 19 kDa and 12 kDa, which is indicative for its activation. 4 mg/ml of the dichloromethan extract of *L. sativa* has no effect, Caspase 3 wasn't cleaved and

apoptosis was not induced. This antibody detects only the activated form at 19 kDa, but not full length caspase-3 or other cleaved caspases.

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. Figure 24 shows that PARP was cleaved after 24 hour *P. odorata* incubation, hence caspase 3 activation starts before PARP signature type cleavage, thereby evidencing that Caspase-3 was in fact functional. Therefore, it's safe to say that *P. odorata* triggers apoptotic cell death executed by Caspase-3. In contrast, the dichlormethanextract of *L. sativa* did not induce cell death in HL-60 or MCF-7 cells.

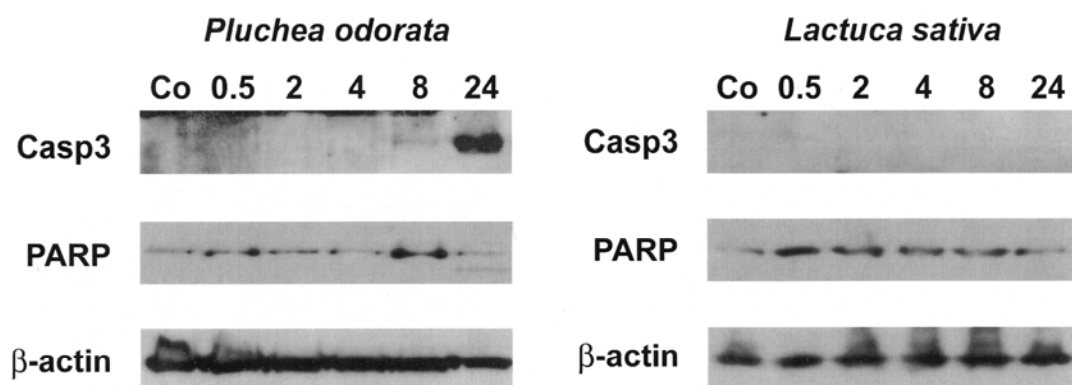


Figure 25 legend: Western blot analysis of pro-apoptotic Caspase 3 and its target PARP
HL-60 cells (1×10^6 cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 hours when cells were incubated with 4 mg/ml methanol extract derived from *P. odorata* and *L. sativa* for 0.5, 2, 4, 8, and 24 hours. Then isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: Caspase 3 which recognizes only the cleavage product indicating caspase activation, PARP which recognizes the full length form (116 kDa) and the signature-type cleaved product (85 kDa) which is generated by active Caspase 3. Equal sample loading was controlled by Poinceau S staining and β -actin analysis.

Tubulin is a major cytoskeleton component that has three distinct forms, α , β and γ -tubulin. α - and β -Tubulin compose a tubulin heterodimer, which multimerize to form a microtubule filament. They undergo a variety of post-translational modification, which may affect microtubule stability and protein interaction.

Microtubules are implicated in a variety of cellular functions including mitosis, intracellular transport, the maintenance of cell shape, and the formation of motile systems such as eukaryotic cilia and flagella (Piperno and Fuller 1985). The first tested antibody was directed against acetylated α -tubulin, because this protein could regulate the presence of microtubule in specific intracellular spaces by selective stabilization (Piperno et al. 1987). *P. odorata* dichlormethan extract (4 mg/ml) induced the acetylation of α -tubulin after 30 minutes, which intensified till 4 and remained for 24 hours.

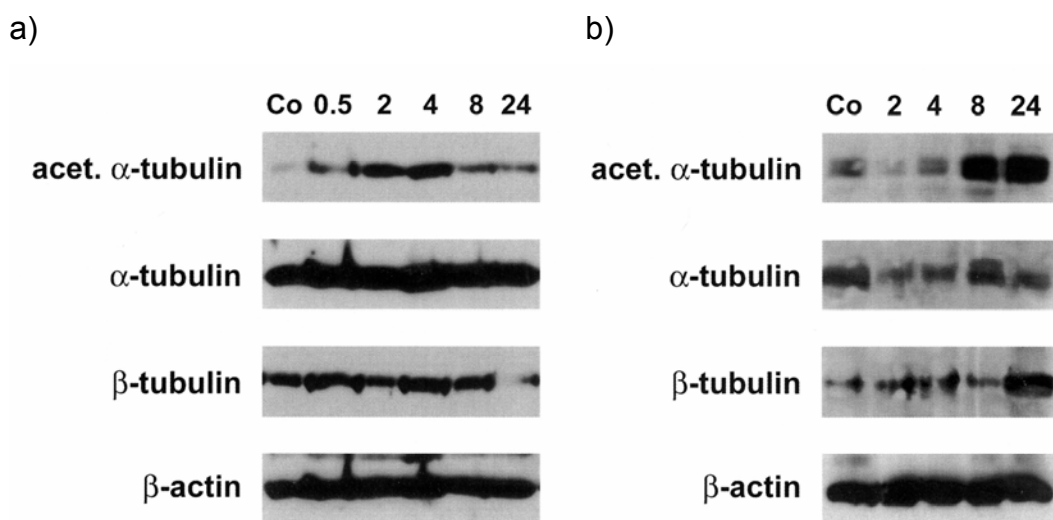


Figure 26 legend: Western blot analysis of tubulin acetylation
HL-60 cells (1×10^6 cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 hours when cells were incubated with 4 mg/ml methanol extract derived from (a) *P. odorata* and (b) 1 μ M paclitaxel for 2, 4, 8, and 24 hours. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: acetylated- α -tubulin, α -tubulin, and β -tubulin. Equal sample loading was controlled by Ponceau S staining and β -actin analysis.

Therefore, this extract type stabilises microtubule, such as *taxol*. *Taxol* is a major anti-cancer drug derived from the genus *Taxa* sp. The effectiveness of this drug is that it permits an increased acetylation, like *P. odorata* too, and this causes mitotic arrest and in consequence apoptosis.

4.3. *Lactuca sativa*

4.3.1. Stock calculation

Finally freeze dried *L. sativa* was analysed. Table 8a shows the extract weight in g derived from 18.8 g freeze dried plant for the five different solvent types (petroleum ether, dichloromethane, ethyl acetate, methanol, water) and the extract amount corresponding to 1 mg plant.

Four different concentrations were used to perform proliferation assays: 500 µg/ml, 1 mg/ml, 4 mg/ml and 20 mg/ml. The supplied extracts were added as shown in table 8b.

a)

Solvent	Extract weight (g)	
	Derived from 18.8 g dried plant	Extract corresponding to 1mg plant
Petroleum Ether	0.29398	15.64 µg
Dichloromethane	0.30170	16.05 µg
Ethyl Acetate	0.23782	12.65 µg
Methanol	3.23782	172.22 µg
Water	2.57090	136.75 µg

b)

Extract Type	Concentration mg dried plant weight/ml medium	µg dried extract weight/ml medium
Petroleum Ether	0.5	7.82 µg
	1	15.64 µg
	4	62.56 µg
	20	312.80 µg
Dichlormethane	0.5	8.03 µg
	1	16.05 µg
	4	64.20 µg
	20	321.00 µg
Ethyl Acetate	0.5	6.25 µg
	1	12.65 µg
	4	50.60 µg
	20	253.00 µg
Methanol	0.5	86.11 µg
	1	172.22 µg
	4	688.88 µg
	20	3444.40 µg
Water	0.5	68.38 µg
	1	136.75 µg
	4	547.00 µg
	20	2735.00 µg

c)

PE	1.5 ml EtOH
CH ₂ Cl ₂	1.5 ml EtOH
EA	1 ml EtOH
MeOH	1 ml EtOH and 1.5 ml MeOH
H ₂ O	8 ml H ₂ O

Table 8: Extract weights from 18.80 g dried plant

The plant extracts were dissolved in different amounts of ethanol, methanol or water (see table 8 c) To avoid solvent bias, EtOH was added to the control and every other concentrations to achieve similar solvent concentrations as in the highest extract concentration (20 mg/ml) e.g. for dichlormethan (in 5 ml Medium):

→ For control: ad 7.98 µl EtOH

→ For 500 µg/ml: ad 7.78 µl EtOH (7.98 -0.20)

→ For 1 mg/ml: ad 7.8 µl EtOH (7.98-0.40)

→ For 4 mg/ml: ad 6.38 µl EtOH (7.98-1.60)

In general, this corresponded to an ethanol-concentration of 0.16 % in every sample.

4.3.2. Anti-proliferative activity of *L. sativa* extracts

Overall it's evident that all extracts showed a growth inhibition at a concentration of 20 mg/ml (Figure 27). The antiproliferative effect in HL-60 cells was higher than in MCF-7 cells. The best result could be achieved in HL-60 as well as MCF-7 cells with the water extract. The inhibition was around 50 % at a concentration of 4 mg/ml.

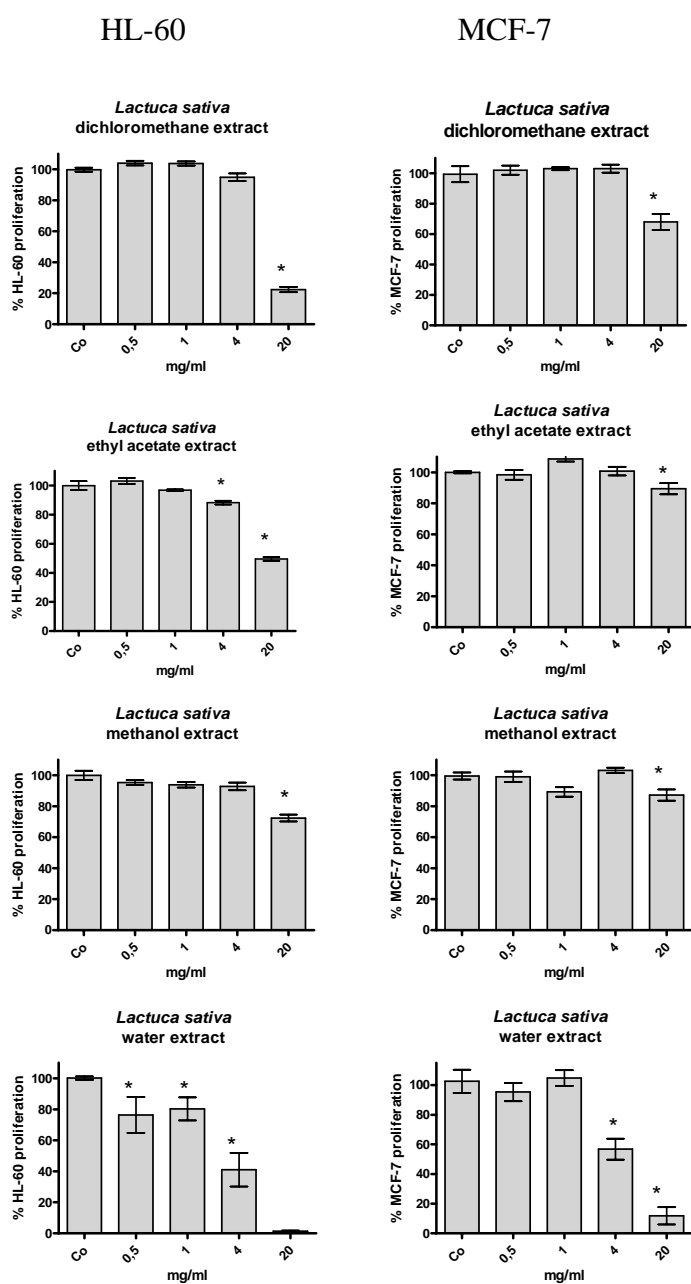


Figure 27 legend: Anti-proliferative effects of *L. sativa* extracts

HL-60 cells were seeded into T-25 tissue culture flasks (1×10^5 cells / ml), grown for 24 hours to enter logarithmic growth phase, and incubated with 0.5, 1, 4, and 20 mg/ml of plant extracts (mg-amounts relate to dried plant material before extractions). Controls received 0.1% to 0.3% EtOH which was the ethanol concentration which cells had to experience together with the highest extract concentration. The other samples were also adjusted to equal ethanol concentrations to achieve similar solvent conditions. Cells were counted after 24 and 48 hours of treatment and the percentage of proliferation between this time span was calculated in comparison to controls (controls were considered as 100 % proliferating cells and all other conditions were set in relation to this). MCF-7 and were seeded in 24 well plates, allowed to attach for 24 hours when extracts were applied as described above and cells counted after 24, 48, and 72 hours. Experiments were done in triplicate. Error bars indicate SEM, and asterisks significant proliferation inhibition compared to control ($p < 0.05$).

Plant material		Solvent	IC 50
<i>L. sativa</i>	Lyophilized	Petroleum ether	17 mg/ml
<i>L. sativa</i>	Lyophilized	Dichlormethan	14 mg/ml
<i>L. sativa</i>	Lyophilized	Ethyl acetate	20 mg/ml
<i>L. sativa</i>	Lyophilized	Methanol	n.r.
<i>L. sativa</i>	Lyophilized	Water	3.3 mg/ml

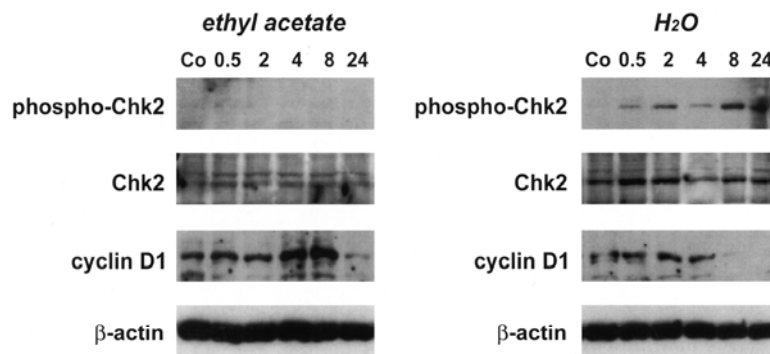
(n.r. = not reached)

Table 9: IC 50 - values of *L. sativa*

4.3.3. Inhibition of proliferation by the water extract of *L. sativa* is preceded by the down-regulation of cell cycle protagonists

4 mg/ml water extract of *L. sativa* caused phosphorylation and therefore an activation of Chk 2 after 30 minutes and intensified after 8 hours which was paralleled by a decrease of Cyclin D1 after 8 hours (figure 28a). This correlated with cell cycle inhibition of the water extract. On the other hand, ethyl acetate extract had no effect on Chk 2. After an incubation of 24 hours a decrease of Cyclin D1 was observed.

a)



b)

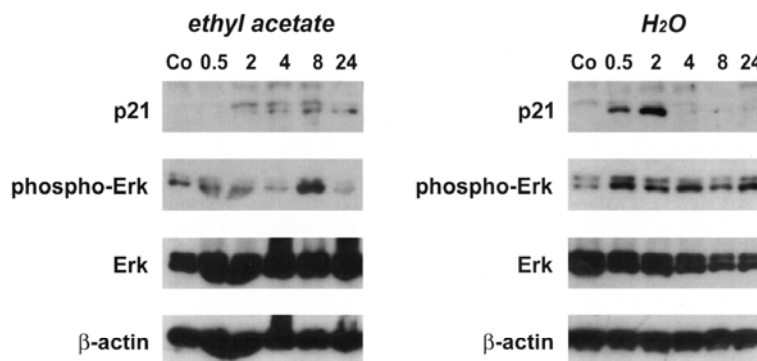


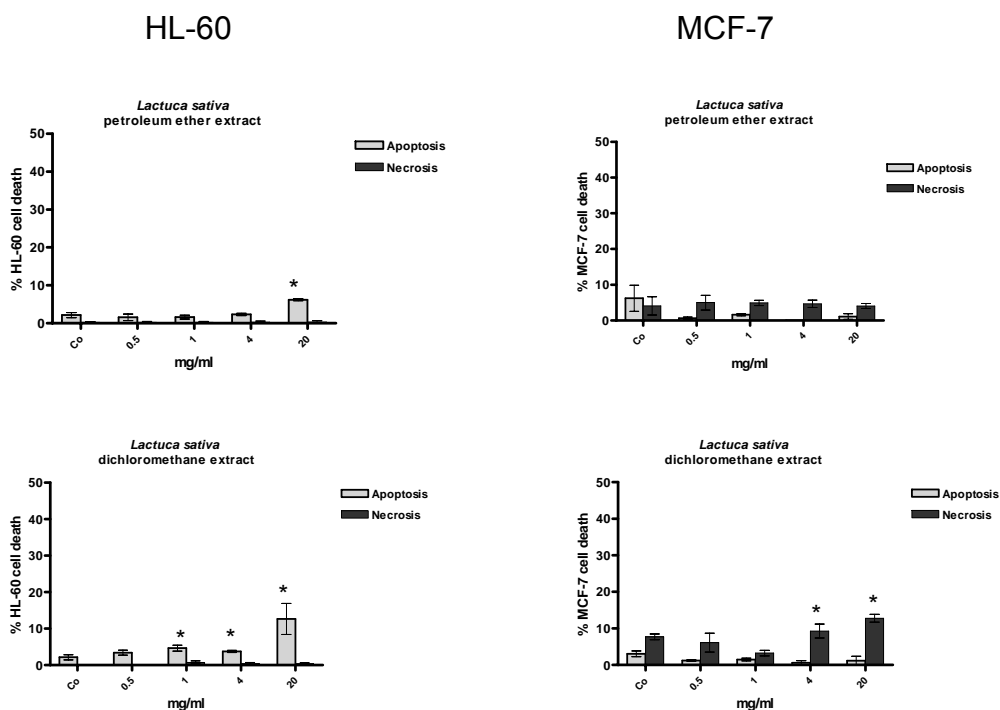
Figure 28 legend: Analysis of cell cycle-related protein and phospho-protein expression
 HL-60 cells (1×10^6 cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 hours when cells were incubated with 4 mg/ml *ethyl acetate* and water extract of *L. sativa* for 0.5, 2, 4, 8, and 24 hours. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: (a) phospho-Thr68-Chk2, Chk2, cyclin D1; (b) p21, phospho-Thr202/Tyr204-Erk, and Erk. Equal sample loading was controlled by Ponceau S staining and β-actin analysis.

Both extract types of *L. sativa* induced p21. Water extract showed a stronger effect than ethyl acetate but decreased after 4 hours while that of ethyl acetate persisted.

Erk showed late phosphorylation of MAP-Kinase with water extract. Ethyl acetate caused a phosphorylation of Erk after 8 hours with a following drop, simultaneously with that of Cyclin D1.

4.3.4. The ethyl acetate and the water extract of *L. sativa* induce apoptosis

First of all figure 29 shows that the extracts rather induced apoptosis in HL-60 while in MCF-7 slightly induced necrosis at the highest concentration (20 mg/ml) but this wasn't significant. The highest apoptosis induction could be achieved with 20 mg/ml water extract in HL-60 cells triggering around 60 % apoptotic cell death. The ethyl acetate extract showed a high apoptotic effect too with a rate of 30 %. To provide a possible explanation for the pro-apoptotic effect of EA- and in particular of the H₂O-extract, which could have based on a potential KNO₃ concentration, or on substantial amounts of phenolic components, respective tests were undertaken (see the following chapters). A non significant effect showed the extracts in MCF-7 cells. Only the water and methanol extract produces a minor necrotic effect of around 10 %.



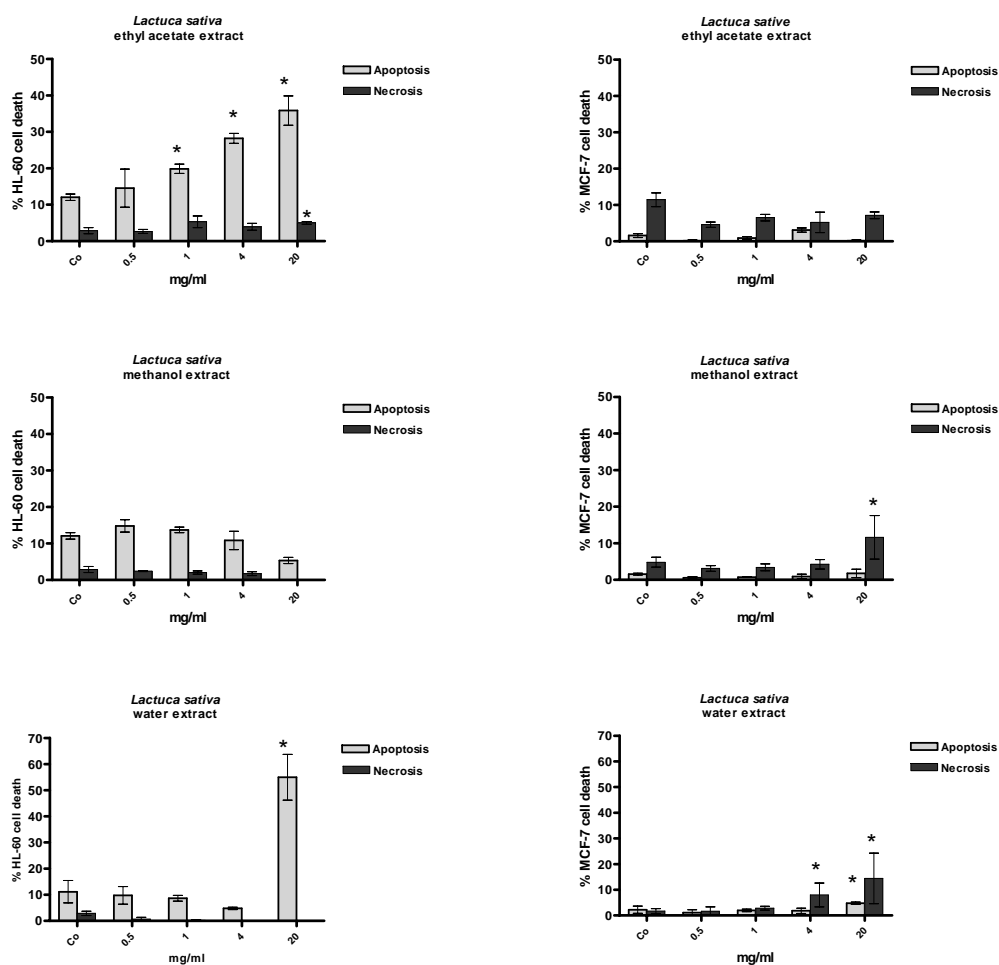


Figure 29 legend: Induction of apoptosis and necrosis by *L. sativa*

HL-60 cells were incubated for 48 hours and MCF-7 cells for 96 hours with increasing extract concentrations. 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 (see "Methods") were counted and percentages of vital, apoptotic and necrotic cells were calculated. Experiments were done in triplicate. Error bars indicate SEM, and asterisks significant apoptosis induction compared to control ($p < 0.05$).

As shown in figure 30, only the ethyl acetate extract induced acetylation of α -tubulin. This implicates that the ethyl acetate extract stabilizes microtubule and therefore might trigger mitotic arrest and apoptosis, which was in fact observed at a concentration of 4 mg/ml, whereas this concentration of water extract had no apoptotic effect.

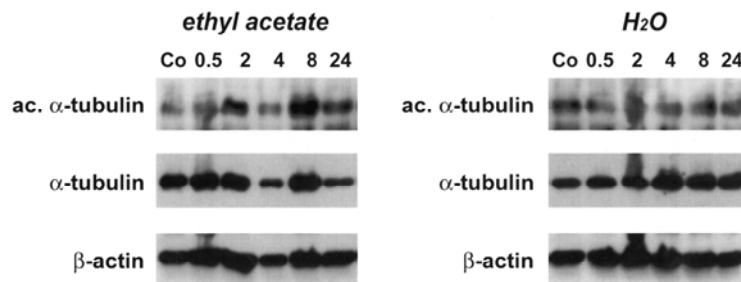


Figure 30 legend: Western blot analysis of tubulin acetylation

HL-60 cells (1×10^6 cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 hours when cells were incubated with 4 mg/ml methanol extract derived from ethyl acetate and water extract of *L. sativa* for 0.5, 2, 4, 8, and 24 hours. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: acetylated- α -tubulin, α -tubulin, and β -tubulin. Equal sample loading was controlled by Ponceau S staining and β -actin analysis.

4.3.5. Analysis of a potential KNO_3 effect

To investigate whether the observed effects were due to KNO_3 , a fertilizer of which residual amounts are frequently found in *L. sativa*, HL-60 cells were incubated with 4 $\mu\text{g/ml}$ KNO_3 (4 μg KNO_3 /g plant weight is allowed by the legislation in the European Union) for 0.5, 2, 4, 8 and 24 hours. Figure 31 shows that KNO_3 had no effect, neither on Chk2 nor on p21, whereas Cyclin D1 expression was reduced after 8 hours, though not completely inhibited. Therefore the majority of the observed effects were not induced by fertilizer remnants.

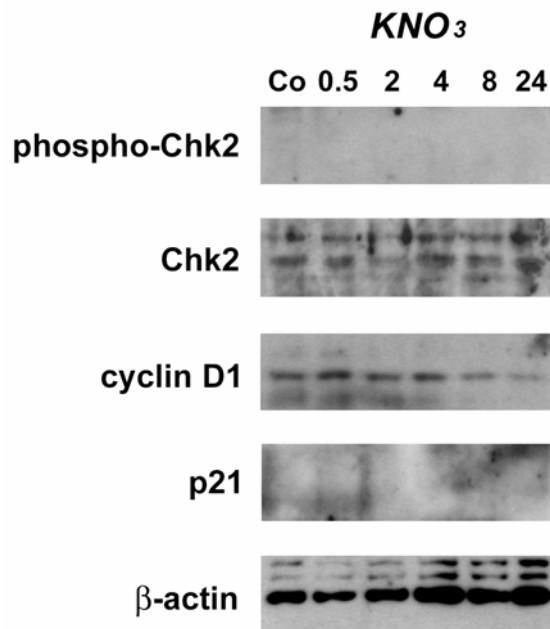


Figure 31 legend: Analysis of cell cycle-related protein and phospho-protein expression
HL-60 cells (1×10^6 cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 hours when cells were incubated with 4 $\mu\text{g/ml}$ KNO_3 for 0.5, 2, 4, 8, and 24 hours. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: phospho-Thr68-Chk2, Chk2, cyclin D1; p21. Equal sample loading was controlled by Ponceau S staining and β -actin analysis.

4.3.6. Total phenolic content of *L. sativa* ethylacetate- and water extract

The calculated total phenolic content (expressed as gallic acid and equivalents; table 10), was 3.18 mg/100 g in the ethyl acetate extract and 42.25 mg/100 g in water extract of *L. sativa*. Heimler et al. found higher polyphenol contents (about one - two orders of magnitude) in lettuce varieties. This discrepancy may be linked to the different extraction conditions. The low value in the ethyl acetate extract is based on the fact that phenolic compounds are not soluble in this solvent. Most phenolics, tannins, etc. are soluble in methanol and therefore remain in the methanol extract. That's why the total phenolic amount in the water extract was low too, because this extraction was subsequent to that with methanol. Therefore, the antiproliferative and antiapoptotic effect didn't result from phenolics whether in ethyl acetate nor in water extract.

<i>L. sativa</i>	µg gallic acid equivalent (after extraction)	g fresh plant	total phenolics (expressed as mg gallic acid/100g fresh plant)
Ethyl Acetate	22.91	0.72	3.18
Water	35.07	0.083	42.25

Table 10: Total phenolics (Folin-Ciocalteu Method)

5. DISCUSSION

To enable an initial and preliminary separation of plant components, the dried plant materials were serially/sequentially extracted with solvents of increasing polarity, which facilitated that different chemical substances from the plants were dissolved in the various extract types. Therefore, the five solvents exhibited distinct activities. In detail, the plant extracts were tested regarding their anti-leukaemic potentials such as the inhibition of proliferation and the induction of leukaemia cell death because these are hallmark for anti-neoplastic activities.

5.1. *S. podophyllum*

S. podophyllum can be found as a natural product against cancer and is recommended because of its anti-inflammatory-activity (Arvigo and Balick 1998). The Mayan use the plant against rheumatism, arthritis and swellings. However, the performed COX-Inhibitor Screening Assay showed that COX-2 isn't believed to be the target enzyme of the analysed methanol extract, and therefore PGH₂ is not the crucial metabolite. The anti-proliferative effects which are a hallmark for anti-tumor activity were only moderate in HL-60 cells upon *S. podophyllum* extract application. It can be speculated that the empirical anti-tumor effects observed by traditional healers were not due to cell cycle-inhibitory or pro-apoptotic properties (although we did not analyse the pro-apoptotic potential of *S. podophyllum*, because both mechanisms mostly appear together), but due to anti-angiogenic or anti-invasive properties, which have not been monitored by the here applied methods.

5.2. *P. odorata*

The anti-cancer effects of *P. odorata*, most and for all the pro-apoptotic properties were highly significant. The dichloromethane extract of *P. odorata* exhibited much higher activity. In the Mayan medicine decoctions of *P. odorata* are used to treat neuritis, rheumatic pain, and arthritis and early stage tumours. Patients are supposed to drink this decoction for several days in considerable quantities to treat cough and flu without side effects (Arvigo and Balick 1998).

P. odorata dose dependently arrested the cell cycle in G2-M phase. The interference with three independent mitotic mechanisms may have stalled the cells in this phase:

- 1) the de-phosphorylation of p44 and p42 mitogen activated protein (MAP) kinases (synonyms: Erk1 and Erk2), which function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation (Marshall 1995; Hunter 1995; Hill and Treisman 1995; Cowley et al. 1994), within 2 hours and therefore interruption of mitotic signaling. MAP kinases are activated by a wide variety of extracellular signals and transduce these mitotic signals through phosphorylation of threonine and tyrosine residues (Sturgill et al. 1983; Payn et al. 1991).
- 2) the *P. odorata* dichloromethane extract suppressed Cyclin D1 within 8 hours, which is required for the activation of Cdk4 and Cdk6 (Alao 2007; Lingfei et al. 1998), thereby affecting cell cycle progression. The D family of cyclins has been associated with a wide variety of proliferative diseases and cyclin D1 is classified as a proto-oncogene. 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; Inaba et al. 1992).
- 3) induction of checkpoint kinase 2 and consequently degradation of Cdc25A within 24 hours. Also Cdc25A is a proto-oncogene and coordinates the transit from G1 to S and also contributes to G2-M transit.

The cell cycle inhibitors p21 (which did not become upregulated; data not shown) and p27 (which became even downregulated) did not seem to play a role in this process.

P. odorata dichloromethane extract dose dependently triggered apoptosis which was preceded by an activation of Caspase-3 and degradation of its target PARP1. The proapoptotic potential was extraordinarily high, as 25 µg/ml dried extract remnant (corresponding to ~1 mg/ml dried plant weight) induced ~ 50 % cell death. The activity which inhibited 50 % proliferation were calculated 0.7 mg/ ml dried plant weight and it is very likely, that apoptosis had already a major contribution to this “anti-proliferative” activity. Both mechanisms were overlapping at the time points measured (and cannot be dissected by differently scheduled timing) and this allows the conclusion that the dichloromethane extract of *P. odorata* harbours an extraordinarily potent pro-apoptotic principle, which can be exploited for the development of a novel anti-cancer therapeutical approach. *Pluchea* species were shown to contain polyphenols, terpenoids and flavonoids (Alonso-Lopez et al. 1985; Wollenweber et al. 1985; Arriaga and Borges-del-Castillo 1985a; Arriaga and Borges-del-Castillo 1985b). When tested in HL-60- and other kinds of cancer cells, flavonoids and other (poly)phenolic compounds were shown to elicit apoptosis and inhibit the cell cycle. Frequently however, the proapoptotic concentrations had to be severalfold higher than those concentrations which inhibited proliferation (Bader et al. 2008; Madlener et al. 2007; Saiko et al. 2007; Horvath et al. 2006; Grusch et al. 2001; Rosenberger et al. 2000; Fritzer-Szekeres et al. 2000). This underscores the effectiveness of the *P. odorata* extract, which may be based on the synergistic activation of several pro-apoptotic principles.

Tubulins play a prevalent role in the differentiation of microtubule structure and function. Fine tuned orchestration of tubulin polymerization/de-polymerization events are required to enable mitotic spindle arrangements and cytokinesis. Therefore, the severe affection of tubulin dynamics upon stabilization is incompatible with functional cell division (Piperno and Fuller 1985) and causes mitotic catastrophe. Taxol exerts its anticancer effect by stabilizing/polymerizing

microtubules, which in turn triggers apoptosis due to mitotic arrest (Geney et al. 2005; Marcus et al. 2005). On the contrary, de-stabilization of tubulin such as by vincristine or colchicine, is cytotoxic (Marcus et al. 2005). Thus, tubulin-targeting drugs are validated anti-cancer therapeutics (Jordan and Wilson 2004). The polymerization of microtubules such as by taxol is associated with increased acetylation of α -tubulin, which is an established marker for microtubule stability (Piperno et al. 1987), because only polymerized but not non-polymerized microtubules become acetylated (Wilson and Forer 1997; Matsuyama et al. 2002). Histone deacetylase 6 (HDAC6) is to date the only known enzyme specifically de-acetylating α -tubulin (Hubbert et al. 2006) and hence, it controls the dynamics and stability of microtubules by promoting their disassembly (Matsuyama et al. 2002) and inhibition of HDAC6 results in α -tubulin acetylation on lysine 40 (Piperno et al. 1987). Also the inhibition of farnesyl transferase increases α -tubulin acetylation (Marcus et al. 2005) probably due to affection of the activity of the farnesyl-carrying mitotic microtubule-associated proteins CENP-E (Crespo et al. 2002).

Incubation with *P. odorata* dichloromethane extract rapidly (after 30 minutes) increased the acetylation of α -tubulin that still persisted after 24 hours of incubation. Therefore, this extract contained α -tubulin targeting activity, reminiscent of taxol, and arrested cells in G2-M causing mitotic catastrophe and cell death. The mechanism by which *P. odorata* extract interfered with microtubule dynamics will be addressed in future investigations.

To rule out the possibility, that the dichloromethane extracts generally contain non-specific toxic components we compared a dichloromethane extract prepared from *L. sativa* var. *capitata*, the green “iceberg” salad. Only the highest concentration tested (20 mg/ml) inhibited HL-60 cell proliferation but did not induce apoptosis and the dichloromethane rhizome extract of *S. podophyllum* was even less active. At the 4 mg/ml concentration, at which also the *P. odorata* extract was investigated by FACS- and by western blot analyses, *L. sativa* dichloromethane extract neither inhibited Cdc25A or cyclin D1 expression nor did caspase 3 become activated. Instead, it induced the

phosphorylation of Erk although we did not observe an acceleration of proliferation.

New agents with prospective anti-cancer activity are usually tested in a panel of 60 distinct cancer cell lines representing 9 distinct tumor entities (Weinstein 1997). However, the vast majority of all agents, which exhibit activity in the 60 cell line screen, are also active in MCF-7 breast cancer cells (Cragg 2000). Therefore, the dichloromethane extract of *P. odorata* was also tested in MCF-7 breast cancer cells and found to inhibit cell proliferation by ~ 90 % at a concentration of 4 mg/ml.

Upon testing whether *P. odorata* apolar dichloromethane-soluble constituents only target cancer cells we found that in the human immortalized anomalous breast epithelial cell line MCF-10A the antiproliferative effects were similar to those observed in MCF-7 cancer cells. This is most likely due to the fact that both cell lines duplicate similarly and also the vast majority of currently applied anti-cancer drugs combat cancer cells because they replicate faster than normal cells. Among polyphenols, flavonoids, and sesquiterpenes there are a plethora of bio-active compounds and studies more than 20 years ago analysed *P. odorata* regarding its constituents. It was shown that it contains flavonoids, triterpenes (Alonso-Lopez et al. 1985; Wollenweber et al. 1985), phytosterols (Dominguez and Zamudio 1972), and Plucheinol, a new eudesmane type sesquiterpen unique to *P. odorata* (Arriaga and Borges-del-Castillo 1985a; Arriaga and Borges-del-Castillo 1985b). A comparison between two *P. odorata* populations from Mexico and El Salvador showed that the Mexican population contained a greater number of triterpenes and flavonoids, whereas the population from El Salvador contained a greater number of sesquiterpenes (Wollenweber et al. 1985; Arriaga-Giner et al. 1983). 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 here described effects.

5.2. *L. sativa*

Unexpectedly, *L. sativa* water extract showed a distinct antiproliferative effect in HL-60 cells, so we decided to perform further analysis to clarify its effectiveness. In both cell types, HL-60 and MCF-7, a growth inhibition of 50 % at a concentration of 4 mg/ml could be achieved with the water extract. All the other extract types showed only a weak inhibition. In the cell death assay water and ethyl acetate extract triggered apoptosis. That's why these two extract types were used for western blot analysis and total phenolics determination.

The water extract was tested in a similar way as the *P. odorata* extracts shown before. Chk2 was activated after 30 minutes which was paralleled by a decrease of Cyclin D1 after 8 hours and therefore Cdk 4 and Cdk 6 were inactivated (Alao 2007; Lingfrei et al. 1998) resulting in an inhibition of the cell cycle progression. Furthermore, *L. sativa* upregulated p21, a specific inhibitor of Cdks, after 4 hours. This 21 kDa Cdk-interacting protein, p21, has been identified in cyclin A, cyclin D1 cyclin E and Cdk2 immunoprecipitates. The upregulation of p21 was independent of p53, which is the major regulator of p21, because HL-60 cells are p53 negative (Biroceccio et al. 1999). Additionally, the water extract induced long lasting phosphorylation of MAP-Kinase and therefore conflicting mitotic signaling. The ethyl acetate extract showed only a slight decrease of Cyclin D1 after an incubation of 24 hours and had no effect on Chk2. After 8 hours Erk was phosphorylated with a following drop, simultaneously with that of Cyclin D1.

In HL-60 cells *L. sativa* ethyl acetate and water extract triggered apoptosis at a concentration of 20 mg/ml. The proapoptotic potential, particularly of ethyl acetate, was relatively high. Even a concentration of 4 mg/ml causes apoptosis in 20 % of the cells. This correlates with previous investigations that *Lactuca* variants induce apoptosis in HL-60 cells (Chen et al. 2007). By contrast both extract types had no effect in MCF-7 cells. Only the water extract slightly induced necrosis at the highest concentration (20 mg/ml).

At a concentration of 4 mg/ml the ethyl acetate extract induced acetylation of α -tubulin after two hours and persisted after 24 hours of incubation. Therefore the ethyl acetate extract might trigger mitotic arrest and apoptosis, while the water did not show an influence.

To investigate whether the observed effects were due to KNO_3 , a fertilizer of which residual amounts are frequently found in *L. sativa*, HL-60 cells were treated with 4 $\mu\text{g/ml}$ KNO_3 (4 μg KNO_3/g fresh plant weight is allowed by the legislation in the European Union). The amount of nitrate markedly varies depending on the variety of vegetables, genetic factors, agricultural practices, climatic conditions, degree of maturity, and light. (Tosun and Ustun 2004). The conclusion was that KNO_3 had no effect on Chk2 or on p21. Only Cyclin D1 expression was slightly reduced after 8 hours. Therefore the antiproliferative and apoptotic effect of *L. sativa* were not induced by fertilizer remnants but by other mechanism(s).

A variety of polyphenolic compounds were investigated in different lettuce genotypes, for example quercetin, kaempferol, luteolin, apigenin, and crysoeriol derivatives (Heimler et al. 2007; Hohl et al. 2001). The total phenolic content in *L. sativa* expressed as gallic acid equivalents was 3.18 mg/100 g fresh weight in ethyl acetate extract and 42.25 mg/ 100 g fresh weight in water extract. This is 10-fold less than the total content of phenolic compounds found by Heimler et al. 2007. This discrepancy may be based on the fact that gallic acid is insoluble in ethyl acetate but soluble in methanol. That's why only small amounts of gallic acid remained in the extracts investigated (ethylacetate and water).

6. CONCLUSION

Leukaemias are characterized by an abnormal proliferation of blood cells, mostly leukocytes, but these diseases often escape after successful treatment. Therefore, we tested novel therapeutic concepts in the human promyelocytic leukaemia cell line HL-60 and chose a highly interdisciplinary approach that involved the long lasting empirical knowledge of the Maya, a more than thousand year old civilization with a highly developed plant-medicinal tradition. Maya medicine was unaware of leukaemia or cancer and therefore no remedies against these diseases were discovered. Our concept of plant selection is their traditional use against severe inflammations, because there are a variety of similar signalling pathways which are commonly up-regulated both in inflammatory conditions and in cancer. Here we compared the in vitro anticancer effects of extracts of two anti-inflammatory plants, *S. podophyllum* and *P. odorata*. As a negative control *L. sativa* was tested with the same methods like the other two plants.

The anti-proliferative effect of *S. podophyllum* was only moderate in HL-60 cells and the COX-Inhibitor Screening Assay showed no result. So we skipped further analysis with this plant.

On the other side, *P. odorata* extracts showed distinct antiproliferative effects. Particularly the proapoptotic properties were powerful. The dichloromethane extract of *P. odorata* contains apolar constituents, because the most effective was the dichlormethan extract, which inhibited inflammatory responses and exhibited anti-cancer activity, tested in HL-60, and MCF-7. *P. odorata* dose dependly arrested the cell cycle in G2-M phase based on the activation of checkpointkinase 2, and downregulation of Cdc25A and Cyclin D1 as well as inactivation of Erk1/2. The strong pro-apoptotic potential, through activation of Caspase 3 followed by PARP signature type cleavage, warrants further bioassay-guided fractionation to discover and investigate the active principle(s).

The hypothesis, that anti-inflammatory remedies might also be useful as anticancer agents in vitro was not valid for *S. podophyllum* but could be verified for *P. odorata*. This is a 50:50 situation and has therefore a much higher success rate regarding the identification of novel anti-cancer remedies compared to the rate observed when testing randomly collected (healing) plants.

L. sativa water extract showed an antiproliferative effect in HL-60 and MCF-7 cells through the activation of Chk2 and at the same time by a downregulation of Cyclin D, an upregulation of p21 and a phosphorylation of Erk1/2. Thereby the mitotic signaling was disrupted. Furthermore the water and the ethyl acetate extract triggered apoptosis in HL-60 cells, while the extracts had no effect in MCF-7 cells. These surprising findings open up the possibility for further research to detect the exact source, which is responsible for the distinct anti-cancerogenic effect of *L. sativa*.

7. SUMMARY

The aim of this diploma thesis was to investigate the anti-neoplastic potential of two healing plants, who were already used by the Maya of the Guatemala/Belize area against severe inflammatory conditions such as neuritis, rheumatism, arthritis, coughs, bruises and tumours.

S. podophyllum and *Pluchea odorata* were collected, dried, and extracted with five solvents of increasing polarity. To rule out the possibility, that the different solvent types contain non-specific toxic components we compared different extracts prepared from *L. sativa* var. *capitata*, the green “iceberg” salad. The inhibition of proliferation and the induction of cell death were investigated in HL-60 and MCF-7 cells, because these are endpoints to measure the efficiency of anti-cancer drugs. Western blot and FACS analyses aimed to obtain data on the underlying mechanisms.

While extracts of *S. podophyllum* showed only moderate anti-cancer activity and were therefore not further investigated, particularly the dichloromethane extract of *P. odorata* inhibited the cell cycle in G2-M which correlated with the activation of checkpoint kinase 2, and the down-regulation of Cdc25A and cyclin D1 as well as the inactivation of Erk1/2. This extract was an extraordinarily strong inducer of HL-60 and MCF-7 cell death activating caspase 3 followed by PARP signature type cleavage. The initiating death trigger was likely the stabilization of microtubules monitored by the rapid acetylation of α -tubulin, which was even more pronounced than that triggered by taxol.

L. sativa water extract showed a distinct antiproliferative effect in HL-60 and MCF-7 cells. It works in a similar way than *P. odorata*. Chk2 was activated which was paralleled by Cyclin D downregulation and p21 upregulation. Additionally, Erk1/2 was phosphorylated and therefore mitotic signaling was in conflict with p21 downregulation. Furthermore the water- and the ethyl acetates extract triggered apoptosis in HL-60 cells, while the extracts had no effect in MCF-7 cells.

8. ZUSAMMENFASSUNG

Das Ziel dieser Diplomarbeit war es das Potential von zwei Heilpflanzen zu untersuchen, die von den Mayas aus El Petén/Guatemala gegen Entzündungen, wie zum Beispiel gegen Neuritis, Rheuma, Arthritis, Erkältungen, Blutergüsse und Tumore, genutzt werden.

S. podophyllum und *P. odorata* wurden gesammelt, getrocknet und anschließend mit fünf Lösungsmitteln mit ansteigender Polarität extrahiert. Um die Möglichkeit auszuschließen, dass die unterschiedlich polaren Lösungsmittel Komponenten aus Pflanzen enthalten, die spezieübergreifend in verschiedensten Pflanzen enthalten sind, und in erhöhter Konzentration generell zell-toxisch wirken, verglichen wir sie mit verschiedenen Extrakte von *L. sativa* var. *capitata*, dem grünen „Eisberg“ Salat. Die Proliferationshemmung und die Induktion des Zelltodes wurden in HL-60 und MCF-7 Zellen ermittelt. Mit Hilfe von Western blot und FACS Analysen wurde versucht die grundlegenden Mechanismen zu ermitteln.

Während die Extrakte von *S. podophyllum* nur moderate anti-kanzerogene Aktivitäten zeigten und daher nicht weiter analysiert wurden, inhibierte vor allem das Dichlormethanextrakt von *P. odorata* den Zellzyklus in der G2-M Phase. Gleichzeitig wurde der Checkpoint Kinase 2, Cdc25A und Cyclin D1 herunterreguliert und Erk 1/2 inaktiviert. Dieses Extrakt induzierte außergewöhnlich stark den Zelltod in HL-60 und MCF-7 Zellen durch eine Caspase 3 Aktivierung mit darauffolgender PARP signature type cleavage. Der Auslöser des Zelltodes war wahrscheinlich die Stabilisierung der Mikrotubulie.

Das Wasser Extrakt von *L. sativa* zeigte einen distinktiven antiproliferativen Effekt in HL-60 und MCF-7 Zellen. Der Wirkungsmechanismus ist dem von *P. odorata* ähnlich: Chk2 wird aktiviert. Parallel dazu finden eine Herabregulation von Cyclin D und eine Aufregulation von p21 statt. Zusätzlich wird Erk1/2 phosphoryliert was gegensätzlich zur p21 Herabregulation steht. Außerdem induziert das Wasser- und das Ethylacetatextrakt apoptotischen Zelltod in HL-60 Zellen während sie keinen apoptotischen Einfluss auf MCF-7 Zellen hatten.

9. REFERENCES

Alao JP: The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Molecular Cancer*: 6-24, 2007.

Alonso-Lopez M, Arriaga-Giner FJ, Borges-del-Castillo J and Vasquez-Bueno P: N-alkanes and triterpenoids from some Salvadorian compositae. *Fitoterapia* 56(2): 123-125, 1985.

Arriaga FJ and Borges-del-Castillo J: A new eudesmane derivative from *Pluchea odorata*. *Planta Medica* 51(3): 290, 1985a.

Arriaga FJ and Borges-del-Castillo J: Carbon-13 NMR spectra of Plucheinol and its ester derivatives. *Magn. Res. Chem.* 23(6): 478-488, 1985b.

Arriaga-Giner FJ, Borges-del-Castillo J, Manresa-Ferrero MT, Vasquez-Bueno P, Rodriguez-Luis F and Valdes-Iraheta S: Eudesmane derivatives from *Pluchea odorata*. *Phytochemistry* 22(8): 1767-1769, 1983.

Arvigo R and Balick M: *Rainforest Remedies*, Lotus Press, Twin Lakes WI, 1998.

Bader Y, Madlener S, Strasser S, Maier S, Saiko P, Stark N, Popescu R, Huber D, Gollinger M, Erker T, Handler N, Szakmary A, Jäger W, Kopp B, Tentes I, Fritzer-Szekeres M, Krupitza G and Szekeres T: Stilbene analogues affect cell cycle progression and apoptosis independently of each other in an MCF-7 array of clones with distinct genetic and chemoresistant backgrounds. *Oncol Rep.* 19(3): 801-10, 2008.

Biroccio A, Del Bufalo D, Ricca A., D'Angelo C, D'Orazi G, Sacchi A, Soddu S and Zupi G: Increase of BCNU sensitivity by wt-p53 gene therapy in glioblastoma lines depends on the administration schedule. *Gene Therapy* 6: 1064-1072, 1999.

Branzei D and Foiani M: Regulation of DNA repair throughout the cell cycle. *Nature Reviews – Molecular cell biology* Volume 9: 297-308, 2008.

Chen YH, Chen HY, Hsu CL and Yen GC: Induction of Apoptosis by *Lactuca indica* L. in Human Leukemia Cell Line and its active Components. *J. Agric. Food Chem.* 55: 1743-1749, 2007.

Coussens LM and Werb Z: Inflammation and Cancer. *Nature* 420(6917): 860-7, 2002.

Cowley S, Paterson H, Kemp P and Marshall CJ: Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* 77: 841-852, 1994.

Cragg GM and Newman DJ: Antineoplastic agents from natural sources: achievements and future directions. *Expert Opin Investig Drugs* 9: 2783-2797, 2000.

Cragg GM, Newman DJ and Yang SS: Natural product extracts of plant and marine origin having antileukemia potential. The NCI experience (Review). *J Nat Prod*. 69(3): 488-98, 2006.

Crespo NC, Delarue F, Ohkanda J, Carrico D, Hamilton AD and Sebt SM: The farnesyl-transferase inhibitor, FTI-2153, inhibits bipolar spindle formation during mitosis independently of transformation and Ras and p53 mutation status. *Cell Death Differ*. 9(7): 702-9, 2002.

Newman DJ and Cragg GM: Natural Products as Sources of New Drugs over the last 25 years. *Journal of Natural Products*. Vol. 70, No. 3: 461-477, 2006.

Denecker G, Vercammen D, Declercq W and Vandenabeele P: Apoptotic and necrotic cell death induced by death domain receptors. *CMLS Cell. Mol. Life Sci*. Vol. 58: 356-370, 2001.

Dewanto V, Wu X, Adom KK and Liu RH: Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem*. 50: 3010-3014, 2002.

Dipankar R and Hiroaki K: Cdc25a Phosphatase: a Rate-limiting Oncogene that determines genomic stability. *Cancer Res*. 68 (5): 1251-1253, 2008.

Dmitri V, Krysko T, Berghe V, D'Herde K and Vandenabeele P: Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods* 44: 205-221, 2007.

Dolezal K, Popa I, Krystof V, Spíchal L, Fojtíková M, Holub J, Lenobel R, Schmölling T and Strnad M: Preparation and biological activity of 6-benzylaminopurine derivatives in plants and human cancer cells. *Bioorg Med Chem*. 14(3): 875-84, 2006.

Dominguez XA and Zamudio A: β -amyrin acetate and campestereol from *Pluchea odorata*. *Phytochemistry* 11(3): 1179, 1972.

Fernandes-Alnemri T, Litwack G and Alnemri ES: CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta- converting enzyme. *J. Biol. Chem*. 269: 30761-30764, 1994.

Fritzer-Szekeres M, Grusch M, Luxbacher C, Horvath S, Krupitza G, Elford HL and Szekeres T: Trimidox, an inhibitor of ribonucleotide reductase, induces apoptosis and activates caspases in HL-60 promyelocytic leukemia cells. *Exp Hematol*. 28(8): 924-30, 2000.

Geney R, Sun L, Pera P, Bernacki RJ, Xia S, Horwitz SB, Simmerling CL and Ojima I: Use of the tubulin bound paclitaxel conformation for structure-based rational drug design. *Chem Biol.* 12(3): 339-48, 2005.

Groves FD, Linet MS and Devesa SS: Epidemiology of human leukemia; *Curr Opin Hematol* 1(4): 321-6, 1994.

Grusch M, Fritzer-Szekeres M, Fuhrmann G, Rosenberger G, Luxbacher C, Elford HL, Smid K, Peters GJ, Szekeres T and Krupitza G: Activation of caspases and induction of apoptosis by novel ribonucleotide reductase inhibitors amidox and didox. *Exp Hematol.* 29(5): 623-32, 2001.

Grusch M, Polgar D, Gfatter S, Leuhuber K, Huettenbrenner S, Leisser C, Fuhrmann G, Kassie F, Steinkellner H, Smid K, Peters GJ, Jayaram HN, Klepal W, Szekeres T, Knasmüller S and Krupitza G: Maintenance of ATP favours apoptosis over necrosis triggered by benzamide riboside. *Cell Death Differ.* 9(2): 169-78, 2002.

Hanahan D and Weinberg RA: The Hallmarks of Cancer. *Cell.* Vol. 100: 57-70, 2000.

Heimler D, Isolani L, Vignolini P, Tombellin S and Romani A: Polyphenol Content and Antioxidative Activity in Some Species of Freshly Consumed Salads. *J. Agric. Food Chem.* 55: 1724-1729, 2007.

Hill CS and Treisman R: Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* 80: 199-211, 1995.

Hohl U., Neubert B., Pforte H., Schonhof I., Böhm H., (2001) Flavonoid concentrations in the inner leaves of head lettuce genotypes. *Eur Food Res Technol.* 213:205-211

Horvath Z, Murias M, Saiko P, Erker T, Handler N, Madlener S, Jaeger W, Grusch M, Fritzer-Szekeres M, Krupitza G and Szekeres T: Cytotoxic and biochemical effects of 3,3',4,4',5,5'-hexahydroxystilbene, a novel resveratrol analog in HL-60 human promyelocytic leukemia cells. *Exp Hematol.* 34(10): 1377-84, 2006.

<http://www.accessexcellence.org/RC/VL/GG/images/MITOSIS2.gif> (25.05.08)

UK Breast Cancer incidence statistics
<http://info.cancerresearchuk.org/cancerstats/types/breast/incidence/> (25.05.08)

Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF and Yao TP: HDAC6 is a microtubule-associated deacetylase. *Nature.* 23;417(6887): 455-8, 2002.

Huettenbrenner S, Maier S, Leisser C, Polgar D, Strasser S, Grusch M and Krupitza G: The evolution of cell death programs as prerequisites of multicellularity (Review). *Mutat Res.* 543(3): 235-49, 2003.

Hunter T: Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80: 225-236, 1995.

Inaba T, Matsushime J, Beach D and Ward DC: Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. *Genomics* 13: 565-574, 1992.

Lingfei K, Pingzhang Y, Zhengguo L, Jianhua G and Yaowu Z: A study on p16, pRb, cdk4 and cyclinD1 expression in non-small cell lung cancers. *Cancer Lett.* 130(1-2): 93-101, 1998.

Madlener S, Illmer C, Horvath Z, Saiko P, Losert A, Herbacek I, Grusch M, Elford HL, Krupitza G, Bernhaus A, Fritzer-Szekeres M and Szekeres T: Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukemia cells. *Cancer Lett.* 245(1-2): 156-62, 2007.

Marchart E, Krenn L and Kopp B: Quantification of the flavonoid glycosides in *Passiflora incarnata* by capillary electrophoresis. *Planta Med.* 69(5):452-6, 2003.

Marcus AI, Zhou J, O'Brate A, Hamel E, Wong J, Nivens M, El-Naggar A, Yao TP, Khuri FR and Giannakakou P: The synergistic combination of the farnesyl transferase inhibitor lonafarnib and paclitaxel enhances tubulin acetylation and requires a functional tubulin deacetylase. *Cancer Res.* 65(9): 3883-93, 2005.

Marshall CJ: Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80: 179-185, 1995.

Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, Seigneurin-Berny D, Osada H, Komatsu Y, Nishino N, Khochbin S, Horinouchi S and Yoshida M: In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J.* 21(24): 6820-31, 2002.

Meeran S.M., Kumar Katiyar S.K., (2008) Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Frontiers in Bioscience* 13. 2191-2202, January 1

Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ and Sturgill TW: Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.* 10: 885-892, 1991.

Pieper AA, Verma A, Zhang J and Snyder SH: Poly (ADP ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol Sci.* 20 (4):171-81, 1999.

Pietenpol JA and Stewart ZA: Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis. *Toxicology* 181-182: 475-481, 2002.

Piperno G and Fuller M: Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101: 2085-94, 1985.

Piperno G, LeDizet M and Chang XJ: Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. *J Cell Biol.* 104(2): 289-302, 1987.

Rakoff-Nahoum S: Why cancer and inflammation? (Review). *Yale J Biol Med.* 79(3-4): 123-30, 2006.

Ribeiro LR, Mantovani MS, Ribeiro DA and Salvadori DM: Brazilian natural dietary components (annatto, propolis and mushrooms) protecting against mutation and cancer (Review). *Hum Exp Toxicol.* 25(5): 267-72, 2006.

Rosenberger G, Fuhrmann G, Grusch M, Fassl S, Elford HL, Smid K, Peters GJ, Szekeres T and Krupitza G: The ribonucleotide reductase inhibitor trimidox induces c-myc and apoptosis of human ovarian carcinoma cells. *Life Sci.* 67(26): 3131-42, 2000.

Saiko P, Ozsvar-Kozma M, Bernhaus A, Jaschke M, Graser G, Lackner A, Grusch M, Horvath Z, Madlener S, Krupitza G, Handler N, Erker T, Jaeger W, Fritzer-Szekeres M and Szekeres T: N-hydroxy-N'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxy-benzamidine, a novel resveratrol analog, inhibits ribonucleotide reductase in HL-60 human promyelocytic leukemia cells: synergistic antitumor activity with arabinofuranosylcytosine. *Int J Oncol.* 31(5): 1261-6, 2007.

Sica A, Allavena P and Mantovani A: Cancer related inflammation: The macrophage connection. *Cancer Lett.* 267(2): 204-15, 2008.

Singleton VL, Orthofer R and Lamuela-Ravetos R: Analysis of total phenols and other oxidation substrates and antioxidants by means of the Folin-Ciocalteu reagent. *Methods Enzymol.* 299: 152-178, 1999.

Sturgill TW, Erikson LBRE and Maller JL: Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* 334: 715-718, 1988.

Sugimura T: Food and Cancer; *Toxicology* 181-182:17-21, 2002.

Tosun I and Ustun NS: Nitrate content of lettuce grown in the Greenhouse. *Bull. Environ. Contam. Toxicol.* 72:109-113, 2004.

Van der Heuvel S: Cell-cycle regulation. WormBook Sep21: 1-16, 2005.

Van Kempen LC, De Visser KE and Coussens LM: Inflammation, proteases and cancer. *European Journal of Cancer* 42 (6): 728-34, 2006.

Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace AJ Jr, Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, van Osdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE and Paull KD: An information-intensive approach to the molecular pharmacology of cancer. *Science* 275: 343-349, 1997.

Li W, Lam MS, Birkeland A, Riffel A, Montana L, Sullivan ME, Joseph M: Cell-based assays for profiling activity and safety properties of cancer drugs. *Journal of Pharmacological and Toxicological Methods* 54: 313-319, 2006.

Wilson PJ and Forer A: Effects of nanomolar taxol on crane-fly spermatocyte spindles indicate that acetylation of kinetochore microtubules can be used as a marker of poleward tubulin flux. *Cell Motil Cytoskeleton* 37(1): 20-32, 1997.

Wollenweber E, Mann K, Arriaga FJ and Yatskievych G: Flavonoids and terpenoids from the leaf resin of *Pluchea odorata*. *J. Biosciences* 40C(5-6): 321-324, 1985.

World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC: AICR, 2007.

Xiong Y, Menninger J, Beach D and Ward DC: Molecular cloning and chromosomal mapping of CCND genes encoding human D-type cyclins. *Genomics* 13: 575-584, 1992b.

Xiong Y, Zhang H and Beach D: D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* 71: 504-514, 1992a.

Yang X, Davies SM, Xiang Y, Robinson LL and Ross JA: Trends in Leukemia Incidence and Survival in the United States (1973-1998). *CANCER* Volume 97/Number 9: 2229-2235, 2003.

10. DANKSAGUNG

Ich bedanke mich bei meinem Betreuer Ao. Univ. Prof. Dr. Georg Krupitza für das Überlassen des Diplomarbeitsthemas, die ausgezeichnete Betreuung, seine Beratung in wissenschaftlichen und privaten Angelegenheiten und seine sehr hilfreiche Unterstützung bei Problemlösungen und spezifischen Fragestellungen. Ebenfalls bedanken möchte ich mich bei Ao. Univ. Prof. Dr. Karl-Heinz Wagner für die Betreuung und Unterstützung während meiner Diplomarbeit.

Univ. Prof. Mag. Dr. Brigitte Kopp und den Mitarbeitern des Departments für Pharmakognosie danke ich sehr für die kompetente Betreuung während der Extraktion des Pflanzenmaterials. Besonders bedanken möchte ich bei Univ.-Prof. Dr. Liselotte Krenn für die Durchführung der „Total phenolics determination“.

Meinen Dank gilt weiters meinen Kolleginnen Mag. Sibylle Madlener, Mag. Nicole Stark, Mag. Thanh-Phuong Vo Nha und Mag. Ruxandra Popescu für die sehr gute Zusammenarbeit in unserer Arbeitsgruppe und für die persönliche Unterstützung während der Erstellung der wissenschaftlichen Arbeit.

Ganz besonders bedanken möchte ich mich bei meinen Eltern Gabriela Bohle-Faast und Herbert Gridling die mir das Studium durch ihre großzügige finanzielle Unterstützung ermöglichten und mich meines gesamten Studiums immer hervorragend unterstützt haben.

Schließlich möchte ich mich noch bei meinem Freund Clemens und all meinen Freunden für die tolle private Atmosphäre während meines Studiums bedanken.

11. LEBENS LAUF

Name: Manuela Gridling

Geburtsdatum: 17. Februar 1981

Geburtsort: Dornbirn

Schulbildung:

1987 – 1991	Volksschule Dorf / Lauterach
1991 – 1996	Bundesgymnasium Blumenstraße / Bregenz
1996 – 1999	Handelsschule Bregenz
2000 – 2003	Matura an der Handelsakademie Bregenz (Aufbaulehrgang)

Berufserfahrung:

08/1999 – 08/2000 Angestellte

Landesregierung Vorarlberg

Praktikas

- ALMA Käsefabrikation & Export
reg.Gen.m.b.H./Bereich Qualitätskontrolle
- Landesregierung Vorarlberg-Umweltinstitut/Bereich
Trink- und Badewässerkontrolle
- Institut für Klinische Pathologie/Bereich
Tumorbiologie
- Hermann Pfanner Getränke Ges.m.b.H.
Bereich Qualitätskontrolle

div. Ferienjobs

ALMA Käsefabrikation & Export reg.Gen.m.b.H./Bereich
Produktion
POST AG – administrative Tätigkeiten

Studium:

ab Okt. 2003

Studium der Ernährungswissenschaften an der
Universität Wien

Jun. 2007 – Jun. 2008

Diplomarbeit am Institut für Klinische Pathologie,
Universität Wien,
Währinger Gürtel 18-20, 1090 Wien
Betreuer: Ao. Univ. Prof. Dr. Georg Krupitza
Thema: „Analysis of ethno-medical plants of the Maya of
Central America for the development of new lead
compounds against cancer”

12. PAPERS in preparation

In vitro anticancer activity of two ethno-pharmacological healing plants from Guatemala *Pluchea odorata* and *Phlebodium decumanum*

Gridling M., Stark N., Madlener S., Lackner A., Popescu R., Prinz S., Benedek B., Diaz R., Foster M., Thanh-Phuong Vo N., Huber D., Gollinger M., Saiko P., Mosgoeller W., de Martin R., Eytner R., Wagner K.H., Grusch M., Fritzer-Szekeres M., Szekeres T., Kopp B., Frisch R., Krupitza G.

Health beneficial in vitro effects of *Lactuca sativa*.

Gridling M., Popescu R., Kopp B., Wagner K.H., Krenn L., Krupitza G.

In vitro anticancer activity of two ethno-pharmacological healing plants from Guatemala *Anthurium schlechtendalii* and *Syngonium podophyllum*

Stark N., Gridling M., Madlener S., Herbacek I., Lackner A., Popescu R., Prinz S., Benedek B., Diaz R., Foster M., Thanh-Phuong Vo N., Huber D., Gollinger M., Saiko P., Mosgöller W., deMartin R., Eiter R., Grusch., Szekeres T., Fritzer-Szekeres M., Frisch M., Krupitza G.

In vitro anticancer activity of the ethnopharmacological healing plant *Hypericum adenotrichum* endemic to west Turkey

Özmen A., Gridling M., Madlener S., Thanh-Phuong Vo N., Stark N., Saiko P., Fritzer-Szekeres M., Szekeres T., Askin-Celik T., Krupitza G.

In vitro anticancer activity of *Scutellaria orientalis* ssp. *carica* endemic to western Turkey

Özmen A., Madlener S., Gridling M., Stark N., Saiko P., Michel B., Fritzer-Szekeres M., Szekeres T., Askin-Celik T., Krupitza G.

The role of piceatannol on primary transcription factor dependent steroid receptor function, the target gene expression of the transcription factors estrogen receptor and progesterone receptor

Thanh-Phuong Vo N., Madlener S., Gridling M., Horvath S., Probst P., Szekeres T., Fritzer-Szekeres M., Jäger W., Krupitza G.

Multifactorial anti-cancer effects of di-galloyl resveratrol encompass apoptosis, cell cycle arrest, and inhibition of lymphendothelial gap formation in vitro

Madlener S., Saiko P., Stark N., Popescu R., Gridling M., Phuong Tan N., Herbacek I., Davidovits A., Venkateswarly Somepalii, Geleff S., Grusch M., Trimurtulu G., Fritzer-Szekeres M., Szekeres T., Krupitza G.

Multifactorial anti-cancer effects of di-galloyl resveratrol encompass apoptosis, cell cycle arrest, and inhibition of lymphendothelial gap formation in vitro

Madlener S., Saiko P., Stark N., Popescu R., Gridling M., Thanh-Phuong Vo N., Herbacek I., Davidovits A., Venkateswarlu S., Geleff S., Grusch M., Kerjaschki D., Trimurtulu G., Fritzer-Szekeres M., Szekeres T., Krupitza G., *in preparation*

13. POSTERS

"In vitro anti-cancer activity of extracts derived from traditional healing plants"

Popescu R., Madlener S., Stark N., Gridling M., Davidovits A., Diaz R., Tut F., Herbacek I., Konkímalla V.B., Somepalii V., Saiko P., Horvath Z., Geleff S., Frisch R., Grusch M., Mosgöller W., Szekeres T., Efferth T., Trimurtulu G., Fritzer-Szekeres M., Kopp B., Krupitza G.

6th International Symposium on Ethnobotany Disciplines, Lima, Peru, September 2007

"Multifactorial anti-cancer effects of di-galloyl resveratrol encompass apoptosis, cell cycle arrest, and inhibition of lymphendothelial gap formation in vitro"

Madlener S., Saiko P., Stark N., Popescu R., Gridling M., Thanh-Phuong Vo N., Herbacek I., Davidovits A., Venkateswarlu S., Geleff S., Grusch M., Trimurtulu G., Fritzer-Szekeres M., Szekeres T., Krupitza G.

Jahrestagung der Deutschen Gesellschaft für Pathologie, Berlin, Germany, May 2008

"In vitro anti-cancer activity of extracts and isolated compounds derived from traditional healing plants of the Mediterranean and Central America"

Madlener S., Gridling M., Stark N., Popescu R., Konkimalla VB, Efferth T, Diaz R, Tut FM, Frisch R, Trimurtulu G, Lackner A, Herbacek I, Losert A, de Martin R, Eytner R, Vo NTP, Horvath Z, Saiko P, Fritzer-Szekeres M, Grusch M, Kopp B, Szekeres T, Krupitza G

13th World Congress on Advances in Oncology, 11th International Symposium on Molecular Medicine, Chersonissos, Crete, October 2008