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„Extracts of anti-malarial and anti-inflammatory healing plants as oncolytic concept“

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ABBREVIATIONS

417  murine NPM-ALK positive ALCL cell line
ATP  adenosine triphosphate
ALL  acute lymphoblastic leukemia
AML  acute myeloid leukemia
ALCL anaplastic large cell lymphoma
ALK anaplastic lymphoma kinase
ASE accelerated solvent extraction
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
CAM cell adhesion molecules
Cdc2 cell division control protein 2
Cdk cyclin-dependent kinase protein
Cip/Kip cyclin dependent kinase inhibitor proteins
CLL chronic lymphoblastic leukemia
CML chronic myeloid leukemia
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DNase deoxyribonuclease
E2F a group of genes that codifies a family of trancription factors
ECL enhanced chemiluminescence
EDTA ethylenediaminetetraacetic acid
Erk extracellular –signal-regulated kinases
FACS fluorescence activated cell sorting
FCS fetal calf serum
FDA US Food and Drug Administration
G0, G1, G2 gap phases of the cell cycle
HL-60 human promyelocytic leukemia cell line
HLF human lung fibroblasts
INK4 inhibitor of cyclin-dependent kinase 4
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>M-phase</td>
<td>mitosis</td>
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<tr>
<td>NPM</td>
<td>nucleophosmin</td>
</tr>
<tr>
<td>p21</td>
<td>cyclin-depenedent kinase inhibitor 1</td>
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<td>p53</td>
<td>tumor suppressor protein</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose)polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PIC</td>
<td>protease inhibitor cocktail</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsufonyl fluoride</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>platelet-derived growth factor β</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RPMI</td>
<td>cell culture medium (Rosewell Park Memorial Institute)</td>
</tr>
<tr>
<td>S-phase</td>
<td>DNA synthesis during cell cycle</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SR-786</td>
<td>human NPM-ALK positive ALCL cell line</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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1 INTRODUCTION

Cancer is one of the leading causes of death worldwide. For 2010, the United States National Institute of Cancer estimates about 1.5 million new cases of cancer and more than 500 thousands of deaths from cancer in the USA (www.cancer.gov). Apart from avoidable risks such as smoking, the most important risk factor for cancer is supposed to be the increase in life expectancy since most cancers occur in people over the age of 65, however, also younger adults and children are diagnosed with cancer. Thus, there exists a constant need for the development of novel anti-neoplastic agents.

Natural products represent a vital resource for therapeutic principles as about 60 % of all anti-neoplastic drugs used in Western medicine originate from natural sources including plants, microorganisms and marine organisms (Cragg and Newman 2007). Moreover, approximately 80 % of the world’s population relies on medical plants for their primary health care, especially in less-developed countries. As a result, numerous traditional healing plants successfully passed hundreds and even thousands of years of application in a variety of diseases. Two popular examples of plant derived drugs are vincristine, a vinca alkaloid from Catharantus rosea (formerly known as Vinca roseus), and paclitaxel, which naturally occurs in the bark of Taxus brevifolia. Vincristine and paclitaxel, which are main agents applied in chemotherapy, demonstrate the pivotal role of plants in the discovery of new lead compounds. In both cases, interest in pharmacological research was triggered by the traditional uses of the plants as home remedy.

To discover new potential lead compounds against cancer, the present work focussed on the ethnomedical knowledge of the ancient civilization of the Central American Mayas. Their traditional medicine is based on the rich biodiversity of the rain forest, and is still practiced effectively to cure a variety of diseases. In this manner, we made use of the long-lasting medical experience of the Mayas in plant selection for detailed screening.

The first plant investigated, Critonia morifolia (Asteraceae), was selected based on its traditional anti-inflammatory uses, as similar signaling pathways are commonly upregulated both in inflammatory conditions and cancer. Despite of its curative uses as home remedy only limited phytochemical and no pharmacological research results were published. Neurolaena lobata (Asteraceae), the second plant, is described as effective home remedy to cure protozoal ailments and malaria, in particular. Its anti-protozoal activity is already scientifically
documented. Furthermore, first hints on potential cytotoxicity were published (François et al. 1996), however, more detailed investigations are still missing.

The potential anti-carcinogenic properties of these two ethno-pharmacological healing plants from Guatemala were tested in human HL-60 promyelocytic leukemia cells to assess their anti-proliferative and pro-apoptotic activity. For each plant, the most active extract out of five, obtained through serial extraction using solvents of increasing polarity, was studied in more detail. Western blots and FACS analyses were applied to gain further insights into the underlying mechanisms of growth inhibition and apoptotic trigger. Investigations on *C. morifolia* were limited to HL-60 cells only. In case of *N. lobata*, all experiments, apart from initial screening in HL-60 cells, were performed in either human and/or murine NPM-ALK positive ALCL (anaplastic large cell lymphoma) cell lines SR-786 and 417, respectively. Moreover, the *N. lobata* extract was tested in normal human lung fibroblasts (HLF) to rule out unspecific cytotoxicity.
2 LITERATURE SURVEY

2.1. Cell cycle and cancer

The cell cycle is an ordered series of events that is required for duplication of an eukaryotic cell and subsequent division into two identical daughter cells. Complex networks of regulatory factors influence whether a cell proliferates, stays in a quiescent state or dies. During cell cycle progression, cells go through numerous internal checkpoints to verify proper completion of the previous step prior proceeding to the next step. Disorders in cell cycle regulation are associated with a variety of diseases including cancer (Meeran and Katiyar 2008).

2.1.1. Basic regulation of the cell cycle

The duration of cell cycle of eukaryotic cells has been defined as the interval between the completion of mitosis by a cell and completion of mitosis by at least one of its daughter cells (Meeran and Katiyar 2008). Strict regulation of the cell cycle is essential to provide a correct duplication of genetic information as well as its correct segregation during mitosis.

The cell cycle of eukaryotic cells comprises four distinct phases (Figure 1):

G1 (Gap phase 1) cellular growth, preparing for DNA synthesis
S (S-phase) DNA synthesis and replication
G2 (Gap phase 2) preparation for mitosis
M (mitosis) cell division

G1-, S- and G2-phase together are also referred to as interphase. Additionally, cells in G1-phase may leave cell cycle and enter a temporarily or even permanently quiescent state termed G0 in dependence on environmental and developmental signals (van den Heuvel 2005).
Cell cycle phases are tightly regulated by cyclin-dependent kinases (Cdks). Even though Cdk protein levels are constant throughout the cell cycle, Cdk activity requires forming of complexes with accessory subunits known as cyclins (Murray 2004). Cell cycle related cyclins are synthesized and destroyed at specific times during the cell cycle, thereby modulating Cdks kinase activity (Figure 1).

Cdk/cyclin complexes include three interphase Cdks (Cdk2, Cdk4 and Cdk6), the mitotic Cdk1 (also known as cell division control protein 2 (cdc2)), and ten cyclins that belong to four distinct classes (A, B, D and E) (Malumbres and Barbacid 2009). However, only certain Cdk/cyclin complexes are supposed to control cell cycle progression. For example, D-type cyclins that bind preferably to Cdk4 and Cdk6 play an important role in the transition from G1 to S-phase upon mitogenic stimuli. The activation of these complexes allows the expression of E-type cyclins which bind to Cdk2. Cdk2/cyclin E further promotes G1/S transition and initiates DNA replication (Meeran and Katiyar 2008). Subsequently, Cdk2 is activated by association to cyclin A in the late stage of S-phase driving progression into G2-phase. At the end of interphase, Cdk1 associates with cyclin A to facilitate the onset of mitosis. Subsequently, cyclin A degradation enables the formation of Cdk1/cyclin B complexes which finally drives cells through mitosis (Malumbres and Barbacid 2009).
In addition to regulation by cyclins, Cdk activity is modulated by two classes of Cdk inhibitors. The first family is identified as the Cip/Kip family including p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}. These proteins are able to interact with multiple Cdk/cyclin complexes, thereby inhibiting Cdk activity throughout the cell cycle. The members of the INK4 family, composed of p16\textsuperscript{INK4B}, p16\textsuperscript{INK4A}, p18\textsuperscript{INK4C} and p19\textsuperscript{INK4D}, specifically block the association of Cdk4/6 and cyclin D, thereby inhibiting G1/S transition (Meeran and Katiyar 2008). Commonly, Cdk inhibitor proteins are upregulated in response to anti-proliferative signals.

In many cancers certain Cdk-cyclin complexes are deregulated, resulting in continued proliferation and unscheduled re-entry into the cell cycle.

2.1.2. DNA damage checkpoints

The cellular DNA integrity of every mammalian cell is constantly exposed to both intrinsic (e.g. byproducts from oxidative respiration) and external sources (e.g. chemicals, radiation, cigarette smoke) of DNA-damaging agents as well as occasional DNA mismatch (Jackson and Bartek 2009). Therefore, cells have evolved several mechanisms to cope with the constant attack on their DNA. Dependent on the type of DNA lesion, a variety of repair mechanisms exists. Cells response to DNA-damage ranges from direct repair to halting of the cell cycle and undergoing of programmed cell death (i.e. apoptosis).

Mammalian cells may only withdraw from the cell cycle when they experience growth-factor deprivation and/or inhibitory signals in early to mid G1-phase. If cells pass through the pRb (retinoblastoma protein)/E2F (transcription factor)-controlled restriction point, they are committed to complete the cell cycle and cell division. However, in response to genotoxic stress, checkpoint networks can delay cell cycle progression in G1, S or G2-phase (Kastan and Bartek 2004). These DNA damage checkpoints refer to signal transduction pathways induced by DNA damage that halt the cell cycle until DNA is repaired (Malumbres and Barbacid 2009). This mechanism is supposed to ensure the maintenance of genomic integrity and the prevention of cancer.

G1 and G1/S checkpoint

The acquisition of abnormalities during G1/S phase appears to be a crucial step in the development of cancer. Hence, the G1/S checkpoint prevents the replication of damaged DNA.

The most important checkpoint response to DNA damage in late G1 is mediated through the ATM/ATR-Chk1/Chk2-Cdc25A pathway. Depending on the type of DNA-damage, ATM/ATR
phosphorylate Chk2/Chk1 which in turn phosphorylate Cdc25A on its several serine residues, triggering its degradation. This results in the failure of Cdk2 activation, which is crucial for transition from G1 to S-phase, and consequently an accumulation of cells in G1 (Kastan and Bartek 2004).

Moreover, the cell cycle protein p53 is one of the most important regulatory proteins in G1 checkpoint. Phosphorylation of p53 at Ser15 by ATM/ATR and at Ser20 by Chk1/Chk2 leads to a maintained G1/S arrest. A key transcriptional target of p53 is the Cdk-inhibitor protein p21, which silences the G1/S-promoting Cdk2/cyclin E kinase, thereby causing G1 arrest (Kastan and Bartek 2004). Additionally, p21 binds to the Cdk4/cyclin D complex resulting in a hypophosphorylation of pRb, thereby suppressing the pRb/E2F pathway and causing cell cycle arrest (Meeran and Katiyar 2008). p21 can also be regulated via p53-independent pathways, e.g. c-Myc and BRCA1 (Abukhdeir and Park 2008).

Notably, the Chk1/Chk2-Cdc25A checkpoint is considered to be implemented more rapidly and independently of p53 whereas p53 and p21 appear to play a role in sustained G1 arrest (Kastan and Bartek 2004).

S-phase checkpoint

In general, the S-phase checkpoint network is activated to ensure appropriate DNA duplication in case of DNA damage. At least two parallel branches are involved in slowing down ongoing DNA synthesis to facilitate DNA repair prior to synthesis (Falck et al. 2002). An important mechanism includes the activation of the ATM/ATR-Chk1/Chk2-Cdc25A pathway after DNA damage. Both Chk1 and Chk2 are associated with inhibitory phosphorylation of its substrate Cdc25A, targeting it for degradation (Mailand et al. 2000; Madlener et al. 2009). In return, the downregulation of Cdc25A inhibits the further activation of the Cdk2/cyclin E complex which is needed to proceed in S-phase (Falck et al. 2002). In addition, the ATM-Nbs1-SMC1 pathway defines a separate branch of the intra-S-phase checkpoint (Yazdi et al. 2002).

Both branches of intra-S-checkpoint have to be interfered concomitantly to abrogate inhibition of DNA replication (Falck et al. 2002).
G2-M checkpoint

The G2-M checkpoint prevents cells from initiating mitosis when they either experience DNA damage during G2 and/or progressed into G2 without proper repair in previous phases or inappropriate replication of DNA in S-phases.

The essential mitosis-promoting activity of the Cdk1/cyclin B complex makes it the critical target of the G2 checkpoint. Upon various stresses, Cdk1/cyclin B activity is inhibited by ATM/ATR-Chk1/Chk2 and/or p38 via regulating degradation and inhibition of the Cdc25 phosphatase family that is normally responsible for Cdk1 activation (Kastan and Bartek 2004). p53 and BRCA1 are reported to play an important role in maintaining G2 arrest. By regulating transcriptional programs, p53 and BRCA1 lead to an upregulation of cell cycle inhibitors such as Cdk-inhibitor p21, GADD45a (growth arrest and DNA-damage-inducible 45 alpha) and 14-3-3 sigma proteins (Meeran and Katiyar 2008). Besides, these p53 downstream effectors can also be regulated in a p53-independent manner as tumor cells that are defective in p53 still tend to selectively accumulate in G2 phase after DNA damage. Moreover, other upstream regulators of Cdc25 phosphatases and/or Cdk1/cyclin B seem to be targeted by DNA-damage induced mechanisms (Kastan and Bartek 2004).

2.2. Cell death programs

Cell death is a pivotal process during development, immune regulation and homeostasis in multicellular organisms. Numerous human pathologies are associated with its dysregulation (Duprez et al. 2009). Due to morphological criteria, three main types of cell death are classified as apoptotic, necrotic, or to be associated with autophagy.

2.2.1. Apoptosis

Morphological features of apoptosis are cell shrinkage, chromatin condensation, and membrane blebbing. Apoptosis is referred to as intrinsic programmed cell death mechanism that results in controlled breakdown of the cell into apoptotic bodies, which are subsequently engulfed by surrounding cells and phagocytes (Duprez et al. 2009).

Two protein families are mainly involved in apoptosis, namely caspases (cysteinyl aspartate-specific proteases), which mediate the execution, and the Bcl-2 family, which control mitochondrial integrity. Caspases can be subdivided into initiator (caspases-2, -8, -9, and -10) and effector (caspases-3, -6, and -7) caspases. Initially, all of them are expressed in their inactive proenzyme form and require proteolytic cleavage to be activated. Cleavage may be
mediated by intrinsic and extrinsic pathways. Among others, the intrinsic pathway is regulated by antagonizing anti-apoptotic and pro-apoptotic (during cellular stress) members of the Bcl-2 family. Upon mitochondrial damage, cytochrome c is released from the mitochondria into the cytosol where it associates with Apaf (apoptotic protease activating factor) and ATP (adenosine triphosphate), activating procaspase-9. The extrinsic pathway of apoptosis is mainly mediated by stimulation of receptors of the TNFR (tumor necrosis factor receptors) family, such as Fas (Duprez et al. 2009).

It is generally believed that apoptosis does not induce immunological response and therefore does not provoke inflammation (Duprez et al. 2009).

### 2.2.2. Necrosis

Necrosis is morphologically characterized by cytoplasmatic and organelle swelling and the final loss of cell membrane integrity. Thus, cellular contents are released into the surrounding extracellular matrix provoking immunological response and inflammation (Duprez et al. 2009).

Typically, necrosis is characterized in negative terms by the absence of caspase activation, cytochrome c release and DNA oligonucleosomal fragmentation (Krysko et al. 2008). As necrotic cell death generally results from a severe physical damage, such as hyperthermia and ischemia, it has been described an uncontrolled cell death that lacks underlying signaling events. However, evidence emerged that in certain conditions, necrosis is guided by strictly regulated signaling pathways, which are initiated by diverse stimuli. Moreover, in some conditions when apoptosis is hampered, necrosis might acts as a kind of back-up for cell death (Duprez et al. 2009).

### 2.2.3. Autophagy

Cell death associated with autophagy represents a catabolic pathway that allows cells to degrade and recycle cellular components. Morphologically, autophagy is characterized by the presence of double-membrane vesicles, which contain sequestered proteins and organelles. Autophagy at basal levels helps cells to maintain intracellular homeostasis and serves as cell survival mechanism during nutrient deprivation (Jin and White 2007). However, massive autophagy is suggested to play a role in cell death, often associated with features of apoptotic or necrotic cell death (Duprez et al. 2009).
2.3. Carcinogenesis

The process of the transformation of normal cells into cancer cells is referred to as carcinogenesis. Usually, it takes years to decades from initial genomic changes within a “cancer cell” to the clinical outcome of cancer.

For a long time, a multistep model of cancer development has been accepted. By now, it is evident that this model does not meet the complexity of the process, however, it still has its eligibility. In the following section, two distinct ways of characterizing the development of cancer are described. The multistep model functionally groups carcinogenesis into three distinct phases, whereas the other way is by listing acquired features of mutant cells during carcinogenesis, referred to as “hallmarks of cancer” defined by Hanahan and Weinberg (2000).

In general, the two models do not exclude but can rather be considered as supplementing each other.

2.3.1. The multistep model

Traditionally, the development of cancer is operationally divided into three phases: initiation, promotion and progression (Figure 2).

Initiation is characterized by DNA mutations in a cell such as single nucleotide polymorphisms, gene depletion or amplification, and chromosomal translocations leading to irreversible genomic changes. Either extrinsic (e.g. chemicals, radiation, cigarette smoke) or intrinsic agents produced during normal physiological processes within a cell may cause initiation. Usually, multiple mutations must occur for a tumor cell to arise (Barrett 1993), which explains that an initiated cell may stay in a quiescent state for several years.

The process of promotion refers to the influence of non-carcinogenic substances on the clonal expansion of initiated cells and is supposed to be substance as well as tissue specific. Effects of promoting agents seem to be reversible, which suggests an epigenetic mechanism (Hennings et al. 1993). The end product of promotion is commonly a benign foci of pre-neoplastic cells (Barrett 1993).

These pre-neoplastic cells must undergo an additional step to convert to malignant neoplasms. This transformation from benign lesions to malignant cancers is termed progression. Malignant neoplasms are distinct from benign tumors regarding their cellular morphology, growth, differentiation, and invasiveness. Moreover, neoplasms differ in their responsiveness to certain chemical treatments (Barrett 1993).
2.3.2. The Hallmarks of Cancer

By now, more than hundred distinct types of cancer, and subtypes of tumors can be found within specific organs. Although specific characteristics among cancer cells may vary substantially, Hanahan and Weinberg (2000) defined six essential alterations in cell physiology that are collectively responsible for the malignant growth: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. These capabilities are acquired during tumor development and represent a successful breaching of the anti-cancer mechanisms hardwired into cells.
Figure 3. Manifestation of six essential alterations in cell physiology collectively dictating malignant growth (Hanahan and Weinberg 2000).

**Self-sufficiency in growth signals**

Normal cells remain in a quiescent state unless they are stimulated by mitogenic growth stimuli. These exogenous signals are transmitted into the cell by transmembrane receptors and induce proliferation. Three distinct mechanisms leading to growth signaling autonomy are described below.

Most importantly, many cancer cells reduce their dependence on exogenously derived signals by generating their own growth signals, creating a positive feedback loop. This property inhibits homeostatic regulation that normally ensures a proper behavior of the cells in a tissue.

An overexpression of growth factor receptors, often carrying tyrosine kinase activity, is also found in several cancers. Receptor overexpression may increase responsiveness to growth signals enabling cells to proliferate also at ambient levels of growth factors that normally would not trigger cell cycle.
A third mechanism by which cells become less dependent on growth stimuli is a modulation in the expression levels of extracellular matrix receptors (integrins), favoring those which act pro-mitotic. Integrins physically link cells to the extracellular matrix and transduce signals into the cytoplasm which influence cell behavior, ranging from quiescence, activating proliferation and resistance to apoptosis.

Abandoning the reductionist type of view focusing solely on cancer cells, heterotypic signaling between the diverse cell types within a tumor is proven to contribute to unscheduled tumor cell proliferation. Within normal tissue, cells are known to largely influence their neighbors to grow. Cancer cells may acquire the ability to co-opt their normal nearby cells by inducing them to release growth signals. These complex interactions need to be considered when trying to understand the development of cancer.

**Insensitivity to anti-growth signals**

Anti-growth signals include both soluble and immobilized inhibitors embedded in the surfaces of neighbor cells and in the extracellular matrix. These signals contribute to the quiescent state of cell and tissue homeostasis by two distinct mechanisms. First, cells can be forced into a resting phase (G0) in which they may remain for a long period of time unless inhibitory signals vanish or growth signals induce proliferation. Alternatively, cells may be induced to permanently stop proliferation activity, usually associated with specific differentiation. Both strategies are described in further detail below.

During G1 phase of cell cycle, the so-called growth phase, normal cells monitor their external environment. Based on the sensed signals, cells decide whether to proceed to S-phase and therefore proliferate or to enter a quiescent state. Thus, it is essential for cancer cells to evade these anti-growth signals. Evidence is mounting that many if not all growth-inhibiting signals are funneled by retinoblastoma protein (pRb) and its relatives, p107 and p130. Hypophosphorylation of pRb blocks proliferation by targeting E2F transcription factors that modulate the expression of numerous genes crucial for progression from G1 into S-phase. In the absence of pRb or disruption of the pRb pathway, pro-mitotic E2Fs are liberated and facilitate cell proliferation. Among other anti-growth factors, effects of TGFβ (transforming growth factor β) on the pRb pathway are the best documented. Preventing the phosphorylation that inactivates pRb, TGFβ blocks the transition into S-phase. A variety of strategies evading the pRb circuit are found in tumor tissues e.g. down-regulation of TGFβ receptors, displaying mutant receptors or mutation of downstream signaling proteins.
Additionally, cancer cells may also reduce expression of integrins and other cell adhesion molecules that send growth-inhibitory signals, favoring those that transmit growth stimuli.

Apart from temporarily halted cell cycle mediated by the pRb pathway, normal tissues indefinitely constrain cell multiplication by promoting cell differentiation. Apparently, cancer cells use various strategies to avoid terminal differentiation. One of them involves the c-Myc oncogene, over-expressed in many tumors that impairs differentiation and promotes growth.

**Evading apoptosis**

Within a normal tissue, cell number is determined not only by proliferation but also by the rate of cell death. Typically, programmed cell death in terms of apoptosis represents a major regulator of cell population. Thus, acquisition of resistance towards apoptosis is characteristic of most and perhaps all types of cancer.

Apoptosis can be roughly divided into two stages. In the first step, sensors permanently monitor the extracellular and intracellular environment for detecting abnormalities influencing the cells’ well-being. In response to detrimental conditions, these signals initiate the activation of components of the second level, which function as executioners of programmed cell death. Trigger of apoptosis include DNA damage, signaling imbalance provoked by oncogenes, survival factor insufficiency, and hypoxia.

Numerous signal cascades may lead to apoptosis by converging to the ultimate effectors of programmed cell death. Intracellular proteases termed caspases or cytochrome C released by mitochondria trigger downstream activation of more effector caspases that finally execute apoptosis (see also 2.2.1).

Circumvention of apoptosis may be acquired by a variety of strategies, including the loss of pro-apoptotic regulators (e.g. p53) and/or trigger of anti-apoptotic components of the signaling circuitry. Since most regulatory and effector components are present in redundancy, tumor cells that have lost a certain pro-apoptotic component are likely to still own intact components of similar apoptotic pathways. Thus, this fact has to be considered when trying to target a specific type of cancer by chemotherapy.

**Limitless replicative potential**

The three capabilities mentioned above, growth signal autonomy, insensitivity to growth-inhibitory signals and resistance to apoptosis, lead to independence of a cell’s proliferation behavior from signals in its environment. However, recent research indicates that
this acquired disruption in cell-to-cell signaling is not sufficient to ensure expansive tumor growth. Many and maybe all mammalian cells appear to possess an intrinsic program that limits their multiplication. This program is regarded as acting autonomously of cell-to-cell interactions.

Normal cells in culture are demonstrated to have a finite replicative potential whereas tumor cells appear to be immortalized, suggesting that the ability of unlimited multiplication was acquired during tumor progression \textit{in vivo}. Thus, the generational limit of normal somatic cells acts as a barrier to cancer.

This phenomenon is caused by the progressive erosion of telomeres during DNA replication cycles. Telomeres, the end of chromosomes, are composed of several thousand repeats of a 6 bp sequence element and function as stabilizers of chromosomal DNA. During each cell cycle, replicative generations show a loss of 50-100 bp of telomeric DNA from the ends of every chromosome. As a result, telomeres lose their ability to protect the chromosomal DNA through successive cycles of replication. The unprotected ends participate in chromosomal fusions, typically yielding in the death of the affected cell. Of malignant cells, 85-90\% succeed in maintaining telomeres during S-phase by upregulating expression of the telomerase enzyme, which adds 6 bp repeats onto the ends of telomeric DNA. The second mechanism helps to maintain telomeres through recombination of interchromosomal exchanges of sequence information. Normal tissue cells lack of telomere maintenance mechanisms. While the absence of telomerase is one cause for cellular aging on the one hand, it also acts as anti-cancer mechanism on the other. Hence, ensuring telomere length above critical threshold is a key component of the capability for unlimited replication.

\textit{Sustained angiogenesis}

The adequate supply with oxygen and nutrients is crucial for cell function and survival. Coordinated growth of blood vessels during organogenesis ensures that virtually all cells in a tissue reside within 100 μm of a capillary blood vessel. The formation of new blood vessels within a tissue, the process of angiogenesis, is carefully regulated. Proliferating cells do not possess an intrinsically angiogenic ability.

Angiogenesis is encouraged by the counterbalance of positive and negative signals. Like growth signals, these stimuli encompass soluble and immobilized factors. Important examples of angiogenesis-initiating signals are the vascular endothelial growth factor (VEGF) and
fibroblast growth factor (FGF 1/2), each binding to transmembrane tyrosine kinase receptors displayed by endothelial cells. Integrins are also part of the regulatory process.

Malignant cells appear to acquire the ability to induce angiogenesis in an early to midstage event during tumor development. Tumors evidence a changed balance of angiogenesis inducers and inhibitors. For example, many tumors reveal an increased expression of VEGF and/or FGFs. In others, expression of endogenous inhibitory factors is downregulated. Furthermore, proteases are emerging as another dimension of regulation by modulating pro- and anti-angiogenic molecules.

Due to high metabolic activity and expansive growth of tumor tissue, sufficient supply with oxygen and nutrients can only be guaranteed by angiogenesis. Thus, mechanisms of sustained angiogenesis are considered an attractive therapeutic target.

*Tissue invasion and metastasis*

An important property of tumor cells is their ability to invade adjacent tissues, which happens sooner or later during cancer development. This process of invasion and metastasis enables cells to escape from primary tumors and may colonize in distant settlements. Like in the host tumor tissue, successful growth depends upon the other five hallmarks of cancer. Although the complexity of invasion and metastasis remains incompletely understood, various contributing strategies are broadly identified.

Several classes of proteins involved in the adherence of cells to their surroundings in tissues are altered in invasive and metastatic cells. Notably, molecules of cell-to-cell adhesion (CAMs) and integrins, which link cells to the extracellular matrix, are affected. A well-documented example of alteration in cell-to-environment interactions involves the adhesion molecule E-cadherin, which is ubiquitously expressed on epithelial cells. Coupling between adjacent cells by E-cadherin transmits anti-growth signals. Apparently, the function of E-cadherin is lost in most epithelial cancers by mechanisms including mutational inactivation, transcriptional repression and increased proteolysis.

Another parameter influencing the invasive and metastatic potential of cancer cells are the alterations in extracellular proteases expression. In general, protease genes are upregulated whereas protease inhibitors are downregulated, not only in malignant cells but rather in conscripted stromal cells.
Nevertheless, further insights into the regulatory circuits and molecular mechanisms that facilitate tissue invasion and metastasis are required for the development of effective therapeutic strategies.

2.4. Leukemia

Leukemia is a cancer of the blood or bone marrow that is characterized by an abnormal increase in blood cells, typically leukocytes. The term leukemia encompasses a heterogeneous spectrum of hematopoietic malignancies. It is subdivided into four major groups, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoblastic leukemia (CLL), and chronic myeloid leukemia (CML). Radiation, genetic and congenital factors, chemicals, drugs and viruses are discussed as possible causes of leukemia (www.cancer.gov).

Leukemia is the most common cancer diagnosed in children aged younger than 15 in the United States. In this age group, acute lymphocytic leukemia comprises approximately 75 % of all cases. Conversely, AML represents only 15-20 % of all childhood leukemia diagnoses (Deschler and Lübbert 2006).

Symptoms of leukemia include infection, anemia, easy bleeding and bruising, shortness of breath, petechia, overall weakness and weight loss or loss of appetite. Leukemia is diagnosed by extensive examination of the blood and the bone marrow (www.cancer.gov).

In the present study, preliminary screenings were performed in acute promyelocytic HL-60 cells, presenting a subtype of AML.

Acute myeloid leukemia

AML represents a highly malignant neoplasm which constitutes the leading cause of death due to cancer in children and young adults (Deschler and Lübbert 2006). The disease is characterized by an increase in immature blood cells that fail to differentiate.

Acute promyelocytic leukemia (APL) accounts for approximately 10 % of all acute myeloid leukemias and is associated with blocked granulocytic differentiation. In over 98 % of APL, the retinoic acid receptor alpha (RARα) gene is fused to the promyelocytic leukemia (PML) gene via t(15;17)(q21;q22) translocation. This chromosomal rearrangement results in the oncogenic promyelocytic leukemia-retinoid acid receptor α (PML-RARα) fusion protein (Vitoux et al. 2007). Currently, all-trans retinoic acid (RA) and arsenic trioxide are applied in clinical practice to successfully treat the disease. Moreover, new compounds that appear to directly or indirectly target the PML-RARα function are in preclinical development (Vitoux et al. 2007).
2.5. Lymphoma

Lymphoma is a type of cancer beginning in the cells of the immune system, i.e. B- and T-lymphocytes, and natural killer cells. According to the histological morphology, lymphomas are classified into two main types: Hodgkin lymphoma (HL), which is characterized by the presence of malignant Reed-Sternberg cells, and non-Hodgkin (NHL) lymphomas. Several risk factors, including genetic disposition, have been linked to the development of lymphoma but exact etiology is still unknown (www.cancer.gov).

Symptoms caused by lymphomas are nonspecific and therefore can be caused by numerous conditions unrelated to cancer. Typical symptoms are enlarged lymph nodes, fever, chills, night sweats and fatigue as well as unexplained weight loss. Diagnosis is made by blood test, biopsy of a swollen lymph node and/or bone marrow and imaging studies like X-ray (www.cancer.gov).

For *N. lobata*, more detailed examinations on anti-neoplastic activity of the dichloromethane extract were performed in human (SR-786) and murine (417) anaplastic large cell lymphoma cell lines.

**Anaplastic large cell lymphoma**

Anaplastic large cell lymphoma (ALCL) accounts for less than 2% of all lymphomas, but represents about 10-15% of childhood lymphoma. ALCL was initially described by Stein et al. in 1985 and is classified as a unique entity among non-Hodgkin’s lymphoma arising from T/null cells. ALCL is morphologically characterized by large neoplastic lymphoid cells with high expression levels of the cytokine receptor CD30 (initially termed Ki-1 antigen) at a membrane and Golgi pattern (Stein et al. 1985). Malignant cells tend to grow cohesively and invade lymph node sinuses. However, also extranodal involvement is observed as ALCL often presents with infiltration of the bone, skin and lung.

The WHO lymphoma classification distinguishes systemic from cutaneous ALCL (Swerdlow et al. 2008). Two distinct entities are listed among systemic ALCL: the anaplastic lymphoma kinase ALK-positive (ALK+) ALCL comprising about 50-85% of all cases, and ALK-negative (ALK-) ALCL which resembles the ALK+ ALCL variant, but lacks the expression of ALK protein. The third type, a cutaneous ALCL (cALCL), is also ALK deficient (Stein et al. 2000). Predominance in ALK+ ALCL is observed in children, which accounts for approximately 90% of all ALCL cases.
The role of ALK in ALCL

The transmembrane receptor tyrosine kinase ALK was first described as NPM (nucleophosmin)-ALK fusion protein in ALCL in 1994. The hybrid protein NPM-ALK is caused by the chromosomal rearrangement t(2;5) of the NPM gene, located on 5q35 and the ALK gene, located on 2p23 (Morris et al. 1994). Investigations in vivo, using transgenic mouse models, established NPM-ALK as a causative protein in the development of ALCL (Chiarle et al. 2003). ALK fusion proteins in ALCL are not limited to NPM-ALK, but currently it is considered the most important and definitively best investigated (Palmer et al. 2010).

In systemic ALCL, the expression of ALK turned out to be an important prognostic factor. Thus, patients diagnosed with ALK+ ALCL are reported to have a more favorable clinical outcome in terms of 5-year survival rate compared to those diagnosed with ALK- ALCL. However, the frequency of ALK expression in ALCL varies among age groups with a higher prevalence in pediatric and adolescent patients and exhibiting predominance in male. As age-adjusted clinical outcome did not exhibit a superior prognosis in dependence of ALK expression, the favorable prognosis of ALK+ patients may be largely dictated by the younger age (Savage et al. 2008).

Currently, combinatorial chemotherapy (CHOP: cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), prednisone) is applied in the first treatment approach of ALCL patients, sometimes combined with radiotherapy. A lot of ongoing research tries to discover a treatment directly targeting ALK (Palmer et al. 2010).

Most ALK+ ALCL patients respond with complete remission upon first-line treatment. However, high relapse rates as well as long term effects of chemotherapy and radiation therapy have to be considered, particularly in pediatric and adolescent patients, as both treatments potentially damage normal cells which might turn into secondary malignancies within decades of life time (Meadows et al. 2009; Reiter 2009; Freed and Kelly 2010).

2.6. Natural products in drug discovery

Currently, the process of drug development from initial discovery of a potential therapeutic agent to subsequent market launch of a drug takes approximately ten years upwards and costs about one billion dollars (Balunas and Kinghorn 2005; Li and Vederas 2009).

Although 50% of the best-selling pharmaceuticals in use today are derived from natural sources (Schuster 2001), reduced emphasis in the pharmaceutical industry on the drug
discovery from natural products was observed during the past decade (Koehn and Carter 2005). This development can be attributed to several factors, whereby the introduction of high-throughput screening (HTS) against defined molecular targets probably represents the most important one. This trend is enhanced by advances in molecular biology, cellular biology and genomics, which lead to increased numbers of identified molecular targets.

In HTS, preferably synthetic chemical libraries are utilized which shortens drug discovery timelines and money, as HTS of natural sources faces a number of difficulties (Li and Vederas 2009). A major hurdle to overcome is that initial extracts of natural material consist of a complex mixture. This makes it difficult and time consuming to isolate the active principles and elucidate their structures. The key compound may be unstable, or the activity is based on two or more synergistic constituents that may disappear upon separation. Additionally, the screening of natural products encompasses the high probability of duplication, i.e. the isolated active compound might be already known and thus cannot be patented (Li and Vederas 2009).

Furthermore, the problem of reliable access and supply, especially with respect to intellectuality property concerns of local governments and the United Nation (UN) Rio Convention on Biodiversity that redefined biodiversity ownership (Schuster 2001) makes drug development from natural sources less attractive to pharmaceutical industries. According to the convention, genetic resources have a potential value and thus belong to the country of origin.

However, the big advantage of natural products is that they comprise a vast diversity of complex structures and new chemical entities, whereas synthetic libraries typically show considerably less diversity (Koehn and Carter 2005). Therefore, natural products will remain an important source of new drugs in the long term.

### 2.6.1. Plants as source of anti-cancer agents

Of natural products, plants represent a particularly vital source of novel drugs, with a vast diversity of complex structures and new chemical entities. Typically, bioactive plant compounds provide an evolutionary advantage to the plant, as they play a role in defense mechanisms against e.g. bacteria, fungi, viruses and grazing. These plant constituents may offer the potential to cure a variety of conditions in humans.

In 1955, the National Institute of Cancer, through the Cancer Chemotherapy National Service Center, started an intensive plant screening project with the aim of identifying natural
products with anti-cancer activity. In course of the screening program more than 100,000 plant extracts were studied. A popular outcome of this program is paclitaxel (Taxol®), originally derived from the bark of *Taxus brevifolia* (Pacific yew tree), which is commonly used in the treatment of breast cancer (Cragg 1998).

Two main approaches can be applied in the discovery of new active plant compounds. On the one hand, plants can be randomly selected. The other one applies ethno-pharmacological knowledge in the selection. The latter offers the advantage that it usually saves from the high try-and-error rate revealed by broad spectrum screening. Moreover, side effects may be minimized according to the long history of successful usage as home remedy.

The schematic overview of drug development based on traditional uses of a plant in folk medicine is illustrated in Figure 4.

![Figure 4. Schematic overview of drug discovery from medical plants (Balunas and Kinghorn 2005).](image)

After a potential drug passed pre-clinical *in vitro* and *in vivo* bio-assays, the US Food and Drug Administration (FDA) for the USA and the European Medicines Agency (EMA) for Europe must approve the investigational application of a new drug before clinical trial can commence (Butler 2008). Once all clinical trials have been successfully completed, the FDA and EMA will approve the drugs market launch in the USA and Europe, respectively.
2.6.2. **Example: Vincristine**

Vincristine is a plant alkaloid derived from the leaves of *Catharantus rosea* (rosy prewinkle), formerly known as *Vinca roseus*, of the family *Apocynaceae*. Its medical properties were already described in the 17th century. Extracts from the plant, originating from Madagascar, were effectively used to treat conditions like hemorrhage, scurvy, toothache, wounds, diabetic ulcers and hyperglycemia (Gidding et al. 1999).

The indigenous reputation of the plant as an oral hypoglycemic agent motivated its phytochemical investigation. Although researchers failed to substantiate the hypoglycemic activity, certain extracts produced a prolongation of life in mice with a type of acute lymphocytic leukemia. Accordingly, these extracts were subjected to detailed fractionation. Numerous alkaloids were isolated of which vincristine and structurally similar vinblastine were identified the most effective anti-neoplastic agents (Noble 1990).

![Figure 5. Structural formula of vincristine (Gidding et al. 1999)](image)

The vinca alkaloid vincristine was shown to act as mitotic inhibitor. The main mechanism by which vincristine exerts its cytotoxicity is via interference with the microtubule formation and the mitotic spindle dynamics, thereby stopping cell division. Apoptosis is the ultimate result of this vincristine-mediated cellular disruption (Gidding et al. 1999).

Vincristine was approved by the US Food and Drug Administration (FDA) in 1963 as Oncovin (www.fda.gov). In chemotherapy, vincristine is administered in the form of reconstituted vincristine sulfate (molecular weight: 923.04), exclusively intravenous. The usage of vincristine is indicated in acute leukemia. Furthermore, it has been shown to be useful as component in
combinatorial chemotherapy in several types of cancer, including Hodgkin disease, non-Hodgkin lymphoma and neuroblastoma (Gidding et al. 1999).

The most frequent and clinical important side-effect of vincristine is neurotoxicity (Moore and Pinkerton 2009). By now, neuropathy represents the dose-limiting toxicity of vincristine, especially in children.

2.6.3. Potential anti-neoplastic activity of two ethnomedical plants from Guatemala

Like vincristine, more than 60 % of applied anti-cancer drugs are derived from natural sources, i.e. plants, microorganisms and marine organisms (Cragg and Newman, 2007). In less-developed countries, a majority of the population still relies heavily or even entirely on traditional healing plants in treating various conditions. Therefore, the applications in folk medicine are well delivered and may act as first hint in the new drug discovery process.

In the present work, two Guatemalan ethnomedical plants (Neurolaena lobata and Critonia morifolia) were selected for investigations of their oncolytic potential based on their long history of traditional uses.

**Critonia morifolia - Traditional uses**

Several traditional applications of *C. morifolia* are known with limitation to the usage of the plants leaves. The leaves are applied as steam bath in cases of swelling, retention of fluids, rheumatism, arthritis, paralysis and muscle spasms. Baths of boiled leaves alone or in combination with other healing plants are used to cure skin conditions, wounds, insomnia, flu and aches. Boils, cysts, pus-filled sores and even cancer are treated with leaves that were heated in oil prior to application directly on the swelling.

**Critonia morifolia - Research results**

Various sesquiterpene lactones and pyrrolizidine alkaloids, including a completely new one named morifoline (Wiedenfeld and Cetto 1998) were isolated of *C. morifolia* (Herz 2004). Despite of its manifold curative usage in folk medicine, only little pharmacological research is published on *Critonia morifolia*.

**Neurolaena lobata- Traditional uses**

*N. lobata* is widely used as home remedy to cure a variety of diseases, particularly malaria and amoebiasis. Traditionally, tea is taken to treat and prevent a variety of parasitic ailments such as malaria, fungus, ringworm, amoebas and intestinal parasites. It is prepared by boiling one
fresh leaf per cup for ten minutes. Up to three cups are consumed daily. Tea prepared of the leaves is also used to bathe wounds and infections, and applied to douche conditions of leukorrhea and vaginal itching. Wounds, skin conditions and sores may be treated by fresh juice from crushed leaves. The water from boiled leaves is also used as insecticide and fungicide in home and garden, and as hair wash to get rid of lice. In case of sores, fungus and infections, a powder of toasted leaves is directly applied. Also the roots are a respected remedy. The water of boiled roots is drunk as a blood cleanser (Arvigo and Balick 1998).

**Neurolaena lobata - Research results**

A variety of characteristic sesquiterpene lactones were isolated of *N. lobata*, among others neurolenin A, B, C, D, E and F and lobatin A, B and C (Passreiter et al. 1995). Another class of chemical found are pyrrolizidine alkaloids (Passreiter 1998).

Various studies approved the anti-parasitic properties of *N. lobata*. Extracts and pure sesquiterpene lactones of *N. lobata* were found to be active against *Plasmodium falciparum*, the clinically most important malaria pathogen, and *Plasmodium berghei* (François et al. 1996). The ethanol extract of *N. lobata* was reported to inhibit parasite growth of *Leishmania mexicana*, *Trypanosoma cruzi* and *Trichomonas vaginalis* (Berger et al. 2001).

Only few studies are available on cytotoxicity of *N. lobata*. François et al. (1996) discovered cytotoxic effects of sesquiterpene lactones in GLC4 and COLO 320 tumor cell lines. An assay performed on brine shrimp larvae *Artemia salina* exhibited only weak toxicity (Berger et al. 1998). In mice, oral and intraperitoneal administration of 500 mg/kg of the water, ethanol and dichloromethane extract every 48 hours for three weeks did not exhibit subacute toxicity and oral dosages up to 5 g/kg did not exhibit acute toxicity (Cáceres et al. 1998).

Furthermore, hypoglycemic activity of the ethanol extract was demonstrated *in vivo* (Gupta et al. 1984). A recent study reported an inhibitory effect of *Neurolaena lobata* extracts on the transfer of HIV from dendritic cells to lymphocytes *in vitro* (Bedoya et al. 2008).
3 MATERIAL AND METHODS

3.1. Plant Material

Plant material of Neurolaena lobata and Critonia morifolia was collected in Guatemala (Petén). Fresh material was stored at -80°C before it was lyophilized and ground. Freeze-drying was preferred over air-drying, as it is known to be more convenient in preserving volatile substances.

Of N. lobata, also air-dried material was available. Thus, its effectiveness was compared to that of freeze-dried plant material to consider the loss of potentially effective compounds at a higher dehydration temperature. Investigations on air-dried material were limited to the extract type of lyophilized plant that exhibited the strongest effects in previous cell culture assays.
3.1.1. *Critonia morifolia* (Petén, Guatemala)

![Critonia morifolia, Guatemala](image)

Figure 6 Critonia morifolia, Guatemala

<table>
<thead>
<tr>
<th>Kingdom:</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division:</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class:</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Order:</td>
<td>Asterales</td>
</tr>
<tr>
<td>Family:</td>
<td>Asteraceae</td>
</tr>
<tr>
<td>Genus:</td>
<td>Critonia</td>
</tr>
<tr>
<td>Species:</td>
<td><em>Critonia morifolia</em></td>
</tr>
</tbody>
</table>

*Critonia morifolia*, like *N. lobata*, belongs to the family *Asteraceae*. It was formerly known as *Eupatorium morifolia*. The genus *Critonia* comprises 43 species and is spread from Mexico to Argentina (Herz 2004).

The plant grows as herbaceous shrub up to four meters tall. The indigenous name *palo verde* (green stick) refers to its characteristically thick green, often woody, stems. The leaves are
deep green with toothed margins and vary from 10 to 40 cm in length. The colour of florescence ranges from greenish-yellow and turns into straw-brown when dry. *C. morifolia* is found in forests and at the edges of forests, riversides and roadsides.

3.1.2. *Neurolaena lobata* (Guatemala, Petén)

![Image of Neurolaena lobata](www.nybg.org/bsci/belize/Neurolaena_lobata.jpg)

![Image of Neurolaena lobata](www.phytobokaz.fr/images_galerie/799959083%20.jpg)

Kingdom: Plantae  
Division: Magnoliophyta  
Class: Magnoliopsida  
Order: Asterales  
Family: Asteraceae  
Subfamily: Asteroideae  
Genus: Neurolaena  
Species: Neurolaena lobata
Asteracea is the largest family of flowering plants and spread worldwide. It is divided into 11 subfamilies of which the subfamily Asteroideae comprises approximately 70% of specific diversity of the whole family. Since 2004, this subfamily is divided into three supertribes (Robinson 2004) of which Helianthodae comprises the species Neurolaena lobata. This plant is spread in Latin American countries and can be found especially in Guatemala and Costa Rica.

Neurolaena lobata is an herb growing from 1 to 4 meters tall. It has only a few main stems with numerous branches and yellow blooming florescence. The most common names are tres puntas, referring to the leaves shape with three distinctive points, and Jackass bitters, referring to the extremely bitter taste of the plants leaves. Its natural habitat in the rainforest encompasses clearings, roadsides, fields and pastures (Arvigo and Balick 1998).
3.2. Plant extraction

The plant powder was consecutively extracted with petroleum ether (PE), dichloromethane (CH₂Cl₂), ethyl acetate (EA), methanol (MeOH) and water (H₂O), leading to five extracts of distinct polarity. Starting with the least polar solvent, this method provides an initial separation of non-polar and polar bioactive plant constituents at minimal plant expense.

For further use in cell culture experiments only small amounts of the gained extracts were transferred into a 1.5 ml tube and dissolved in DMSO (dimethyl sulfoxide). DMSO dissolves both polar and non-polar compounds and is miscible with a wide range of solvents. To account for detrimental effects of DMSO on cell proliferation, apoptosis and cell cycle, controls were treated with the respective concentrations of DMSO used for sample treatment. Maximum concentration of DMSO was limited to 0.5 % to avoid cell damage due to toxicity of DMSO.

3.2.1. C. morifolia – Accelerated Solvent Extraction (ASE 2000)

Accelerated Solvent Extraction (ASE 2000, Dionex) is a fully automated technique to rapidly extract solid and semi-solid sample matrices with organic solvents as well as water. ASE allows extraction at temperatures above the solvents boiling point, using high pressure to keep the solvent liquid. Advantages to traditional extraction methods are the reduction of solvent needed, time savings and better solubility of constituents at elevated temperatures, though thermal degradation of some target analytes has to be considered.

Powdered leaves of C. morifolia were mixed 2:1 with diatomaceous earth, which acts as a dispersant and drying agent, prior to adding the sample to the extraction cells. Schedule (Table 1) was programmed and started. One by one, cells were filled with the solvent via HPLC-pump and heated followed by static extraction. Solvent containing the dissolved plant compounds was collected in vials. The solid plant material that remained in the cells was flushed with cold solvent (also collected in vials) and dried with nitrogen (N₂) automatically. For each solvent and cell, the cycle was repeated before moving to the next solvent. All vials containing the same solvent were transferred into a flask and subjected to rotary evaporation (Table 2).
Table 1. ASE conditions for extraction of *C. morifolia* and air-dried *N. lobata*.

<table>
<thead>
<tr>
<th></th>
<th>Preheat</th>
<th></th>
<th>Temperature</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heat</td>
<td>Static extraction</td>
<td>Pressure</td>
<td>150 bar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purge time</td>
<td>Cycles</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flush volume</td>
<td>Solvent</td>
<td></td>
</tr>
<tr>
<td>(of extraction volume)</td>
<td></td>
<td></td>
<td>(in order of application)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Petroleum ether</th>
<th>Dichloromethane</th>
<th>Ethyl acetate</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflux – water bath (°C)</td>
<td>60</td>
<td>40</td>
<td>92</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Rotary evaporator (mbar)</td>
<td>600</td>
<td>850</td>
<td>250</td>
<td>300</td>
<td>75</td>
</tr>
</tbody>
</table>

3.2.2. *N. lobata* – reflux-water bath extraction

First, plant powder consisting of lyophilized leaves, florescence and stipes of *N. lobata*, was mixed at a concentration of 1:10 with solvent in a flask, e.g. to 20 g plant material 200 ml solvent was added. In the next step the flask was sealed with Parafilm® and treated in an ultrasonic bath for ten minutes to burst the plant cells and therefore making greater amounts of compounds available. Then the flask was put on a reflux-water bath at a temperature depending on the solvents boiling point (Table 2) for one hour. Afterwards the content of the flask was filtered through a round filter with a pore diameter of 150 nm to separate solid and dissolved plant material. The solid plant residue was air-dried over night and reused for the following extraction step with the next, more polar solvent. The extracted material dissolved in the liquid phase was dried by rotary evaporation at a water bath temperature of 40°C and a pressure according to the solvents characteristics (Table 2) until complete dryness. Flasks containing the dried extracts were stored in a vacuum desiccator.

For air-dired *N. lobata*, the extraction was conducted as described for *C. morifolia* applying ASE 2000 (Table 1) but was limited to the first two solvents (petroleum ether and dichloromethane). Only the dichloromethane extract was used in cell culture experiments.

Table 2. Solvents (in order of extraction), corresponding temperatures for water bath extraction and pressure used for solvent evaporation.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Reflux – water bath (°C)</th>
<th>Rotary evaporator (mbar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>60</td>
<td>600</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>40</td>
<td>850</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>92</td>
<td>250</td>
</tr>
<tr>
<td>Methanol</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>Water</td>
<td>100</td>
<td>75</td>
</tr>
</tbody>
</table>
3.3.  Cell culture

HL-60, human promyelocytic leukemia cells, were purchased from ATCC (American Type Culture Collection). SR-786, NPM-ALK positive human ALCL (anaplastic large cell lymphoma) cells, and 417, NPM-ALK positive mouse ALCL cells, were kindly provided by Prof. Lukas Kenner. HL-60, SR-786 and 417 cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-Glutamine and 1% Penicillin/Streptomycin. Primary human lung fibroblasts (HLFs), were obtained from ATCC (designation LL 47 (MaDo)) and cultivated in DMEM medium supplemented with 10% FCS and 1% Penicillin/Streptomycin. Media and supplements were purchased from Life Technologies. All cells were maintained in humidified atmosphere containing 5% CO₂ at 37°C.

3.4.  Proliferation and cytotoxicity assays

Proliferation inhibition (HL-60)

Initially, HL-60 cells were treated with all of the plant extracts to determine their anti-proliferative effects and to figure out the most promising ones. Therefore, HL-60 cells were seeded in 24-well plates at a concentration of 1 x 10⁵ cells/ml allowing logarithmic growth within 72 hours. Afterwards cells were incubated with increasing concentrations of plant extracts (5 µg/ml, 15 µg/ml, 30 µg/ml, 60 µg/ml) for 72 hours. The dichloromethane extract of *N. lobata* was also applied in 2.5 and 10 µg/ml. After 24 hours and 72 hours cell number was determined using a KX-21 N microcell counter (Sysmex Coporation, Kobe, Japan). All experiments were carried out in triplicates. Anti-proliferative effects were calculated by applying the following formula:

\[
\frac{C_{72h+drug} - C_{24h+drug}}{C_{72h- drug} - C_{24h - drug}} \times 100 = \% \text{ of cell division}
\]

- \( C_{72h+drug} \) cell number after 72 h of drug treatment
- \( C_{24h+drug} \) cell number after 24 h of drug treatment
- \( C_{72h-drug} \) cell number after 72 h without drug treatment
- \( C_{24h-drug} \) cell number after 24 h without drug treatment

Only the extract of *Neurolaena lobata* showing the best anti-proliferative effect, the dichloromethane extract, was tested on other cell systems including SR-786, 417 and HLF, respectively. Experiments with extracts of Critonia morifolia were restricted to HL-60 cells only.
*Proliferation inhibition (SR-786, 417)*

Effects of the dichloromethane extract of N. lobata were evaluated in the cell lines SR-786 (human) and 417 (mouse) using a Casy® cell counter (Invitrogen, Life Technologies Inc.). SR-786 cells were seeded in a 48-well plate at a concentration of $2 \times 10^5$ cells/ml. Then, the cells were incubated with 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml of the extract for 48 hours. 417 cells were seeded in 48-well plates at a concentration of $1 \times 10^6$ cells/ml and incubated with 5 µg/ml, 10 µg/ml and 15 µg/ml extract for 72 hours. The same formula as for HL-60 cells was used for calculations. In case of SR-786 cells, cell number after 48 hours was used instead of 72 hours.

*AlamarBlue® assay (417, HLF)*

Additionally to cell number determination of 417 cell line, the alamarBlue® assay (Invitrogen, Life Technologies) was applied to measure cytotoxicity. The active component of alamarBlue® is resazurin, which is a non-toxic and cell permeable compound. Resazurin acts as an indicator dye that is blue and non-fluorescent. Upon entering an active cell it is converted to bright red-fluorescent resuforin via reduction reactions of cell metabolism (Figure 9). The amount of fluorescence produced is proportional to the number of living cells and corresponds to the cells metabolic activity. Non-viable or damaged cells show a lower innate metabolic activity and thus generating a proportionally lower signal than healthy cells. Thus, this method allows both qualitative (relative) and a quantitative evaluation of cell viability and cytotoxicity.

![Figure 9](http://de.wikipedia.org/wiki/Resazurin)

Figure 9. Blue, non-fluorescent resazurin, indicator dye in alamarBlue®, is converted into red fluorecent resofurin via reduction reactions of metabolic active cells. [http://de.wikipedia.org/wiki/Resazurin](http://de.wikipedia.org/wiki/Resazurin)
HLF cells were seeded into 48-well plates at a concentration providing confluence within the wells. Together with 500 µl medium, HLF cells were grown for 24 hours. 417 cells were seeded into 48-well plates at a concentration of $1 \times 10^6$ cells/ml. Each well was filled with 500 µl cell suspension. Dichloromethane extract was added at concentrations of 2.5 µg/ml, 5 µg/ml and 10 µg/ml, HLFs were additionally treated with 15 µg/ml. An untreated control and pure medium were also assessed to determine differences in cell viability depending on drug treatment. After 24 hours, and 48 hours respectively, 50 µl alamarBlue® reagent was added to each well and incubated for approximately 90 minutes at 37°C until colour changed from blue to red. Afterwards the 48-well plate was placed into a multi-detection reader for fluorescence and absorbance (Synergy HT, Bio-Tek Instrument, Inc., Vermont, USA). Plate reader software KC-4 (Bio-Tek) was used to determine absorption at 570 nm. Experiments were done in triplicates. To calculate the differences in cell viability, mean blank value (only medium) was subtracted from all other measurement values to take fluorescence of the medium into account. Mean value of the control samples was considered as 100 % cell viability. The mean values of treated samples were described as percentage of control sample viability. Calculations were performed in Excel (Microsoft).

3.5. Apoptosis assay – Hoechst 33258 and propidium iodide double staining

Hoechst 33258 (HO) and propidium iodide (PI) double staining (both Sigma, St. Louis, MO) allows the determination of the type of death the cell is undergoing, i.e. apoptosis (early or late) or necrosis (Grusch et al. 2002). HL-60 cells were seeded in a 24-well plate at a concentration of $1 \times 10^5$ cells/ml and treated with increasing concentrations of the specified extracts (Table 3). After 24 hours of incubation, 100 µl cell suspension of each well was transferred into separate wells of a 96-well plate and Hoechst 33285 and propidium iodide were added at final concentrations of 5 µg/ml and 2 µg/ml, respectively. After one hour of incubation at 37°C, stained cells were examined and photographed on a fluorescence microscope (Axiovert, Zeiss) equipped with a DAPI filter. Type and number of cell deaths was evaluated by visual examination of the photographs according to the morphological characteristics revealed by HOPI staining. Experiments were performed in triplicates.
Table 3. Extracts and corresponding concentrations applied in apoptosis assay

<table>
<thead>
<tr>
<th>Neurolaena lobata</th>
<th>µg/ml</th>
<th>Critonia morifolia</th>
<th>µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE extract</td>
<td>5, 15, 30</td>
<td>PE extract</td>
<td>5, 15, 30</td>
</tr>
<tr>
<td>CH₂Cl₂ extract</td>
<td>5, 10, 15</td>
<td>CH₂Cl₂ extract</td>
<td>5, 15, 30</td>
</tr>
<tr>
<td>EA extract</td>
<td>5, 10, 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeOH extract</td>
<td>15, 30, 60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.6. Cell cycle distribution (FACS)

417 cells were seeded in a 6-well plate at a concentration of 1 x 10⁶ cells/ml. Then dichloromethane extract of *N. lobata* was added to a final concentration of 5 µg/ml, 10 µg/ml and 15 µg/ml. HL-60 cells were seeded in T-75 tissue culture flasks at a concentration of 2 x 10⁵ cells/ml and treated with 10 µg/ml and 15 µg/ml of the petroleum ether extract of *C. morifolia*. After 24 hours (for HL-60 cells also after 8 hours) of incubation at 37°C, cells were harvested, transferred into 15 ml tubes and centrifuged (4°C, 800 rpm, 5 min). The supernatant was discarded and the cell pellet was washed with cold PBS, centrifuged (4°C, 800 rpm, 5 min), resuspended in 1 ml cold ethanol (70%), and either fixed for 30 minutes at 4°C or stored at -20°C prior further handling. After two washing steps with cold PBS, the cell pellet was resuspended in 500 µl cold PBS and transferred into a 5 ml polystyrene round bottom tube. RNAsé A and propidium iodide were added to a final concentration of 50 µg/ml each and incubated for one hour at 4°C. The final cell number was adjusted between 0.5 and 1 x 10⁶ cells in 500 µl. Cells were analyzed by FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA). Cell cycle distribution was calculated with ModFid LT software (Verity Software House, Topsham, ME, USA).

3.7. Western blotting

*Lysate preparation*

417 cells were seeded in a 6-well plate at a concentration of 10⁶ cells/ml and treated with 10 µg/ml of the dichloromethane extract of *N. lobata*. After 0.5, 2, 4, 8 and 24 hours 4 x 10⁶ cells were harvested. Vincristine, a common plant based anti-cancer drug, was added at final concentrations of 1, 2.5 and 5 µM. Cells were harvested after 8 and 24 hours.

SR-786 cells were seeded in a T-75 tissue culture flask at a concentration of 2.5 x 10⁵ cells/ml and incubated with 15 µg/ml dichloromethane extract of *N. lobata*. 10⁶ cells were harvested.
after 4, 8 and 24 hours. Additionally, Proteasome Inhibitor IV (Cat.No. 539175, Merck) was added to a final concentration of 50 µM in a single experiment. In another experiment lysosome inhibitor ammonium chloride (NH₄Cl) was added at a concentration of 20 mM.

HLFs were grown in a 6-well plate to confluence and incubated with 10 µg/ml and 15 µg/ml dichloromethane extract of *N. lobata*. After 4, 8 and 24 hours, cells were harvested.

HL-60 were seeded in T-75 tissue culture flasks at a concentration of 1.8 x10⁵ cells/ml and incubated with 15 µg/ml petroleum ether extract of *C. morifolia*. Cells were harvested after 0.5, 2, 4, 8 and 24 hours.

All cells were washed with cold PBS twice and centrifuged at 1000 rpm for 5 min at 4°C. Cell pellet was lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1 % Triton-X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Protease Inhibitor Cocktail (PIC). The lysate was centrifuged at 12000 rpm for 20 min at 4°C. Supernatant was transferred into a 1.5 ml tube and stored at -20°C until further analysis.

**Gel electrophoresis (SDS-PAGE) and blotting**

Equal amounts of protein samples (lysate) were mixed with SDS (sodium dodecyl sulfate) sample buffer and loaded onto a 10 % polyacrylamide gel. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) at 120 Volt.

To make proteins accessible to antibody detection, they were electrotransferred from within the gel onto a PVDF (polyvinylidene difluoride) membrane (Hybond, Amersham, UK) at 95 Volt for 80 minutes. Membranes were allowed to dry for at least 30 minutes up to 2 hours to provide fixing of the proteins on the membrane. Methanol was used to remoisten the membranes. Equal sample loading was checked by staining the membrane with Ponceau S (Sigma-Aldrich).

**Protein detection**

After washing with PBS (Phosphate buffered saline, pH 7.2) or TBS (Tris buffered saline, pH 7.6), membranes were blocked in PBS- or TBS-milk (5 % non-fat dry milk in PBS containing 0.5 % Tween 20 or TBS containing 0.1 % Tween 20) for one hour. Then, membranes were washed with PBS/T (PBS containing 0.5 % Tween 20) or TBS/T (TBS containing 0.1 % Tween 20), changing the washing solution 4-5 times, for at least 20 min. Next, membranes were incubated with the primary antibody in blocking solution (according to the data sheet TBS-, PBS-milk or TBS-, PBS-BSA) diluted 1:500 – 1:1000, gently shaking at 4°C over night. Thereafter,
membranes were washed again with PBS/T or TBS/T and incubated with the secondary antibody (peroxidase conjugated anti-rabbit IgG or anti-mouse IgG) diluted 1:2000 in PBS- or TBS-milk at room temperature for one hour. Chemiluminescence was developed by ECL detection kit (Amersham, UK) and membranes were exposed to Amersham Hyperfilms.

**Antibodies**

CD246, ALK protein, monoclonal mouse, clone ALK1, code M7195 (DakoCytomation)

Nucleophosmin, monoclonal mouse, clone 376, code M7305 (DakoCytomation)

PDGF Receptor β (28E1) Rabbit mAB, #3169 (Cell Signaling)

Chk1 (2G1D5) Mouse mAb, #2360 (Cell Signaling)

Phospho-Chk1 (Ser345) Antibody, #2341 (Cell Signaling)

Chk2 Antibody, #2662 (Cell Signaling)

Phospho-Chk2 (Thr68) Antibody, #2661 (Cell Signaling)

Cleaved PARP (Asp214) Antibody (Mouse Specific), #9544 (Cell Signaling)

PARP-1 (F-2): sc-8007, mouse monoclonal (Santa Cruz Biotechnology, Inc.)

Cleaved Caspase3 (Asp175) Antibody, #9661 (Cell Signaling)

Caspase3 Antibody, #9662 (Cell Signaling)

PhosphoDetect Anti-H2AX (pSer139), DR 1017 (EMD4Biosciences)

Cdc25A (phospho S75) antibody, ab47279, rabbit polyclonal (abcam)

Phospho-Cdc25A-S177 Antibody, rabbit polyclonal, Cat. #AP3046a (Abgent)

Cdc25A (F-6): sc-7389, mouse monoclonal (Santa Cruz Biotechnology, Inc.)

Cdc25B (C-20): sc-326, rabbit polyclonal (Santa Cruz Biotechnology, Inc.)

Cdc25C (C-20): sc-327, rabbit polyclonal (Santa Cruz Biotechnology, Inc.)

Phospho-cdc2 (Tyr15)(10A11) Rabbit mAb, #4539 (Cell Signaling)

Cdc2 p34 (17): sc-54, mouse monoclonal (Santa Cruz Biotechnology, Inc.)

Phospho-p53 (Ser20) Antibody, #9287 (Cell Signaling)

p53, mouse monoclonal, Cat. No. 1767 (Immunotech, Coulter Company)

JunB (120): sc-73, rabbit polyclonal (Santa Cruz Biotechnology, Inc.)

c-Jun (H-79): sc-1694, rabbit polyclonal (Santa Cruz Biotechnology, Inc.)

Phospho-Akt (Ser473)(587F11) Mouse mAb, #4051 (Cell Signaling)

Akt Antibody, #9272 (Cell Signaling)

Cyclin D1 (M-20): sc-718, rabbit polyclonal (Santa Cruz Biotechnology, Inc.)

p21 (C-19): sc-397, rabbit polyclonal (Santa Cruz Biotechnology, Inc.)

c-myc Ab-2 (9E10.3), #MS-139-P1, mouse monoclonal (Thermo Fisher Scientific, Inc.)
3.8. Quantitative RT-PCR

RNA preparation
SR-786 cells were seeded in T-75 tissue culture flasks at a concentration of $2.5 \times 10^5$ cells/ml and incubated with 15 µg/ml dichloromethane extract of *N. lobata*. After 4 hours and 8 hours cells were harvested and homogenized using QIAshredder (50) (Cat. No. 79654, QIAGEN). The cells were further processed according to the instructions of RNeasy Mini Kit (50) (Cat. No. 74104, QIAGEN). Final RNA concentration was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Inc.). RNA was stored at -80°C until further progression.

cDNA synthesis
First-strand cDNA synthesis from 1000 ng RNA was performed using SuperScript®First-Strand Synthesis Systems for RT-PCR (Cat. No. 11904-018, Invitrogen). The enzyme reverse transcriptase enables the transcription of mRNA into cDNA. cDNA synthesis reaction was primed using the most nonspecific primers, random hexamers. With this method, all RNAs are templates for cDNA synthesis. Desired mRNA was obtained by choosing specific primers in the PCR (polymerase chain reaction). Finished cDNA was stored at -80°C.

cDNA synthesis

\[
\begin{align*}
8 \mu l & \text{ RNA (1 µg RNA + H}_2\text{O)} \\
1 \mu l & \text{ Random Hexamers} \\
1 \mu l & \text{ dNTPs}
\end{align*}
\]

\[
\begin{align*}
\text{add} \\
2 \mu l & \text{ 10x Buffer} \\
4 \mu l & \text{ MgCl}_2 \\
2 \mu l & \text{ DTT} \\
1 \mu l & \text{ RNase OUT}
\end{align*}
\]

\[
\begin{align*}
\text{add} & \text{ Superscript II RT} \\
1 \mu l & \text{ RNase H}
\end{align*}
\]

Figure 10. cDNA preparation from RNA samples using SuperScript®First-Strand Synthesis Systems for RT-PCR (Invitrogen).
Real time-PCR

The alteration in transcription levels of NPM-ALK in SR-786 cells after treatment with the dichloromethane extract of *N. lobata* was investigated by real-time PCR using TaqMan® detection system. The housekeeping-gene glyceraldehyde 3-phosphat dehydrogenase (GAPDH), which is stably and constitutively expressed at high levels in most tissues and cells, served as control gene. For each sample, 7 µl H₂O, 10 µl TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µl primer+probe and 2 µl cDNA, or 2 µl H₂O for negative controls, were filled into a tube of a 96-well optical reaction plate. In case of GAPDH, the primer and probe mixture from TaqMan® Gene Expression Kit (Applied Biosystems) was used. To detect NPM-ALK transcripts, forward primer (GTG GTC TTA AGG TTG AAG TGT GGT T) and reverse primer (GCT TCC GGC GGT ACA CTA CTA A) were mixed with the probe (TGC TGT CCA CTA ATA TGC ACT GGC CCT) prior adding to the rest. Final concentration of primers and probe in the sample mixtures were 0.5 µM and 0.25 µM, respectively. Cycle program (95°C for 10 min to activate polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) was started on Abi Prism 7000 Sequence Detection System (Applied Biosystems). Real time-PCR was performed in duplicates for each cDNA template and gene investigated. Negative controls, containing water instead of cDNA, confirmed the absence of RNA/DNA in all reagents applied in the assay.

Analysis of results

Comparative \( C_T (\Delta \Delta C_T) \) method (Livak and Schmittgen 2001) was used for calculating relative quantitation of gene expression. The \( C_T \) value is determined as the number of PCR cycles that is needed to reach a defined level of fluorescence and therefore newly synthesized DNA. The following formula was applied to quantify relative expression of the target gene NPM-ALK:

\[
\text{Ratio} = 2^{-\Delta \Delta C_T}
\]

\[
\Delta C_T = C_T \text{ target gene (NPM-ALK)} - C_T \text{ control gene (GAPDH)}
\]

\[
\Delta \Delta C_T = \Delta C_T \text{ drug treatment} - \Delta C_T \text{ control sample}
\]

3.9. Statistical analysis

Values were expressed as mean ± SD. Student’s t-test was applied to compare differences between control samples and treatment groups. Statistical significance level was set to \( p < 0.05 \).
4 RESULTS

4.1. Critonia morifolia

Lyophilized leaves of *Critonia morifolia* were subjected to sequential extraction with five solvents of increasing polarity applying ASE 2000 (Dionex). The obtained extracts were investigated for their anti-carcinogenic potential in HL-60 cells. Proliferation and apoptosis assays were performed to identify the most promising extract which effects were subsequently studied in more detail by western blot analysis.

4.1.1. Extract yields and stock calculation

The extract weights obtained from serial extraction of 22.52 g lyophilized leaves of *C. morifolia* with five solvents of increasing polarity are presented in Table 4.

Table 4. Extract weights corresponding to lyophilized plant material of *C. morifolia*.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Extract weight (g) corresponding to 22.52 g dried plant</th>
<th>Extract weight (µg) corresponding to 1 mg dried plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>1.3481</td>
<td>59.86</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.6347</td>
<td>28.18</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.3223</td>
<td>14.31</td>
</tr>
<tr>
<td>Methanol</td>
<td>3.1708</td>
<td>140.80</td>
</tr>
<tr>
<td>Water</td>
<td>4.1483</td>
<td>184.20</td>
</tr>
</tbody>
</table>

To determine anti-proliferative effects in HL-60 cells, extracts were applied at increasing concentrations (5 µg/ml, 15 µg/ml, 30 µg/ml, 60 µg/ml). Concentrated stock solutions of all extracts were prepared in DMSO and stored at -20°C. Control samples were treated with the amount of DMSO used in the highest concentration to take effects of DMSO into consideration. For all other samples, the DMSO concentration was adjusted accordingly (max. 0.5 % DMSO final concentration).
Table 5. Extract weights of *C. moriolia* applied in HL-60 proliferation assays.

<table>
<thead>
<tr>
<th>Extract type</th>
<th>Extract stock concentration (µg/µl DMSO)</th>
<th>Extract final concentration (µg/ml medium)</th>
<th>corresponding dried plant weight (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>12.07</td>
<td>5</td>
<td>83.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>250.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>501.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>1002.34</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>12.00</td>
<td>5</td>
<td>177.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>532.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>1064.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>2128.85</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>12.15</td>
<td>5</td>
<td>349.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>1048.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>2096.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>4193.02</td>
</tr>
<tr>
<td>Methanol</td>
<td>15.55</td>
<td>5</td>
<td>35.51</td>
</tr>
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<td></td>
<td></td>
<td>15</td>
<td>106.54</td>
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<td>30</td>
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<td></td>
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<td>60</td>
<td>426.14</td>
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<td>Water</td>
<td>43.80</td>
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<td>15</td>
<td>81.43</td>
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<td></td>
<td></td>
<td>30</td>
<td>162.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>325.73</td>
</tr>
</tbody>
</table>

4.1.2. *Anti-proliferative activity of C. morifolia extracts in HL-60 cells*

The extracts of *C. morifolia* were tested in logarithmically growing HL-60 cells at increasing concentrations. Proliferation inhibition of extract treated samples compared to solvent adjusted controls is illustrated in Figure 11. The petroleum ether extract exhibited a growth inhibition of more than 50 % at 5 µg/ml and 100 % at 15 µg/ml. Further, the dichloromethane and the ethyl acetate extract showed a dose-dependent response. When taking the corresponding dried plant weight into account (Table 5), the petroleum ether extract and the dichloromethane extract turned out to be the most effective. Therefore, an apoptosis analysis was conducted for these two extracts, but not for ethyl acetate extract. The methanol and the water extract were not taken into consideration for further experiments since the effects on proliferation in HL-60 cells were mostly insignificant.
Figure 11. Effects of different extracts of *C. morifolia* on the proliferation of HL-60 cells. Logarithmically growing cells were seeded at a concentration of $10^5$ cells/ml and treated with increasing concentrations (5, 15, 30 and 60 µg/ml) of extracts (petroleum ether, dichloromethane, ethyl acetate, methanol, water). Experiments were done in triplicate. Error bars indicate ±SD. Asterisks indicate significant alterations in proliferation compared to control ($p < 0.05$).
4.1.3. *Induction of apoptosis in HL-60 cells by extracts of C. morifolia*

Based on the results of the proliferation assay, the petroleum ether extract and the dichloromethane extract were investigated in more detail regarding their apoptotic potential. HL-60 cells were subjected to increasing concentrations of the specified extracts (Figure 12) for 24 hours. Afterwards, propidium iodide and Hoechst 33258 were added to the cells facilitating the distinction between viable, apoptotic and necrotic cells via visual examination.

![Graphs showing induction of apoptosis in HL-60 by petroleum ether and dichloromethane extracts of C. morifolia.](image)

**Figure 12.** Induction of apoptosis in HL-60 by the petroleum ether and the dichloromethane extract of *C. morifolia*. HL-60 cells were seeded at $10^5$ cells/ml and incubated with indicated concentrations of extracts (petroleum ether and dichloromethane) for 24 h. Afterwards cells were stained with propidium iodide (2 µg/ml) and Hoechst 33258 (5 µg/ml) and incubated for 1 h. Viable and apoptotic cells were photographed on a fluorescence microscope equipped with a DAPI filter and examined visually. Experiments were performed in triplicate. Error bars indicate ±SD. Asterisks indicate significant alterations in number of apoptotic cells compared to control ($p < 0.05$).

After 24 hours of incubation, the petroleum ether extract already exhibited a slight induction of apoptosis in HL-60 at 5 µg/ml. At 15 µg/ml, the petroleum ether extract induced cell death in almost 100% of the cells. Comparatively, 30 µg/ml of the dichloromethane extract were necessary to achieve similar results. Taking the corresponding plant weight into account, 250.58 µg/ml dried plant was needed to induce apoptosis in 100% of the cells in case of the petroleum ether extract, whereas 1064.42 µg/ml were required in case of the dichloromethane extract.

Thus, the petroleum ether extract of *C. morifolia* was identified the most active one. Its effects in HL-60 cells were studied in further detail by FACS and western blot analysis.
4.1.4. The petroleum ether extract represses c-Myc and cyclin D1 expression in HL-60 cells

Cyclin-dependent kinase (Cdk) inhibitor p21, is considered a tumor suppressor protein which acts as inhibitor of cell cycle progression in G1/S. One important inducer of p21 transcription is p53. As HL-60 cells are proven to be p53 deficient (Wolf and Rotter 1985), this pathway is excluded. Despite, p21 can be regulated by p53-independent pathways (Abukhdeir and Park 2008). At transcriptional level, for example, it can be regulated by the oncogene c-Myc (Coller et al. 2000).

c-Myc has a profound impact on cell proliferation, differentiation and apoptosis (Dominguez-Sola et al. 2007). Upregulation of c-Myc levels is common in many tumor types contributing to an abnormal proliferation rate.

Cyclin D1 promotes transition from G1 to S-phase in cell cycle in dependence on Cdk. In addition, cyclin D1 regulates and associates with transcription factors in a Cdk-independent manner. As the protein has impact on cell growth, metabolism, and cellular differentiation, overexpression of cyclin D1 plays a key role in the development of several cancers (Fu et al. 2004), and is classified, such as c-Myc, as a proto-oncogene.

Incubation of HL-60 cells with 15 µg/ml petroleum ether extract of C. morifolia caused a simultaneous repression of c-Myc and cyclin D1 after 30 minutes which intensified during the time course. However, HL-60 cells remained deficient in p21 expression during 24 hours of extract treatment.
Figure 13. Effects of the petroleum ether extract on the proto-oncogenes c-Myc and cyclin D1 and tumor suppressor protein p21 in HL-60 cells. Cells (1.8 x 10^5 cells/ml) were incubated with 15 µg/ml petroleum ether extract of C. morifolia and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and obtained proteins samples subjected to SDS-PAGE followed by western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

4.1.5. After a transient G2-M cell cycle inhibition, the petroleum ether extract of C. morifolia induces S-phase arrest in HL-60 cells after 24 hours of incubation

HL-60 cells were incubated with 10 µg/ml and 15 µg/ml petroleum ether extract of C. morifolia for 8 hours and 24 hours, respectively. Afterwards, cells were subjected to fluorescence activated cell sorting (FACS). Interestingly, the extract exhibited distinct effects depending on the duration of treatment.

After 8 hours, the cell population in G2-M phase increased from 15.9 % to 32.3 % (10 µg/ml) and up to 61.3 % (15 µg/ml; Figure 14a) upon extract treatment. The dose-dependent accumulation of HL-60 cells in G2-M was caused at the expense of both G0-G1 cells (from 32.0 % to 24.2 % and 10.0 %) and S-phase cells (from 52.1 % to 43.5 % and 28.4 %).

By contrast, FACS analysis after 24 hours of incubation with the petroleum ether extract revealed a cell cycle inhibition in S-phase at 15 µg/ml (Figure 14b). The treatment at this concentration resulted in a substantial increase of cells in S-phase from 38.8 % to 62.4 %, mainly at the expense of G0-G1 phase cells, which was reduced from 42.6 % to 23.3 %. The incubation of HL-60 cells with 10 µg/ml extract exhibited only a slight shift of cell population from G0-G1 to G2-M whereas S-phase remained unchanged. Therefore, the higher
concentration (15 µg/ml) of the petroleum ether extract was applied in the following experiments.

Figure 14. Analysis of cell cycle distribution in HL-60 cells after (a) 8 h and (b) 24 h of incubation with the petroleum ether extract of *C. morifolia*. HL-60 cells (1.8 x 10^5 cells/ml) were incubated with 10 µg/ml and 15 µg/ml of the petroleum ether extract of *C. morifolia*. After 8 h and 24 h cells were harvested and subjected to FACS analysis. Error bars indicate ±SD. Asterisks indicate significant alterations of cell population in cell cycle phase compared to control (p < 0.05). Experiments were performed in triplicate.

The expression of cell regulatory proteins was analyzed to investigate the mechanism of cell cycle arrest in HL-60 cells upon petroleum ether extract treatment.
4.1.6. **Modulations of cell regulatory proteins induced upon extract treatment**

The transient G2-M cell arrest after 8 hours and the final arrest in S-phase caused by the petroleum ether extract of *C. morifolia* were studied in further detail by investigating modulations of key proteins of the cell cycle. For this reason, HL-60 cells were treated with 15 µg/ml extract, harvested after 0.5, 2, 4, 8 and 24 hours and subjected to western blot analysis.

![Figure 15. Alteration in the expression of cell cycle regulatory proteins in HL-60 cells. HL-60 cells (1.8 x 10^5 cells/ml) were treated with 15 µg/ml petroleum ether extract of *C. morifolia*. After 0.5, 2, 4, 8 and 24 h, cells were harvested and lysed. Isolated protein samples were subjected to western blot analysis investigating the protein expression levels of the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.](image)

The entry of eukaryotic cells into mitosis is regulated by cyclin-dependant kinase 1 (Cdk1). Phosphorylation of Cdk1 at tyrosine 15 leads to an inactivation of Cdk1 (Norbury et al. 1991). The Cdc25 phosphatases, including Cdc25A, B and C, are pivotal triggers of the cell cycle by
activation of cyclin-dependent kinases (CdkS) via dephosphorylation (Hunter 1995). Despite the presence of all Cdc25s throughout the cell cycle, Cdc25A predominantly controls transition into S-phase via activation of Cdk2 (Hoffmann et al., 1994), but is also able to regulate G2-M progression (Kiyokawa and Ray 2008). On the other hand, Cdc25B and Cdc25C play a major role in G2-M transition via activation of the Cdk1/cyclin B complex (Hunter 1995). However, recent studies suggest an undefined level of functional redundancy among Cdc25 phosphatases (Kiyokawa and Ray 2008).

Antagonizing the Cdc25 dephosphorylation activity, Wee1 inhibits entry into mitosis by the phosphorylation of Cdk1 at the tyrosine 15 site (McGowan and Russel 1993). Wee1 phosphorylation at serine 642 leads to the nuclear-cytoplasmatic translocation. Although Wee1 activity is not altered by phosphorylation, p(642)Wee1 delocalization into cytoplasm prevents contact to its nuclear substrate Cdk1. Therefore, phosphorylation of Wee1 at Ser642 may promote the G2-M progression (Katayama et al. 2005).

The phosphatases Cdc25A and Cdc25B were upregulated between 2 and 8 hours of incubation with the petroleum ether extract prior to substantial downregulation thereafter. In contrast, Cdc25C levels continuously decreased during the time course, which correlated with the G2-M arrest revealed by FACS analysis after 8 hours.

Upon extract treatment, Cdk1 was partly dephosphorylated at Tyr15 after 8 hours, and completely after 24 hours which is indicative for its activation. As all three Cdc25 phosphatases were also downregulated within 24 hours, the decrease in kinase Wee1 was supposed to be responsible for Cdk1 activation that may have helped to overcome the initial G2-M arrest. Thus, the downregulation of Cdc25A and Cdc25B was most likely responsible for S-phase arrest after 24 hours.

These temporally separated and phase specific inhibitory properties on cell cycle arrest are probably due to distinct anti-neoplastic compounds that are contained in the petroleum ether extract of C. morifolia.
4.1.7  The petroleum ether extract induces apoptosis in HL-60 cells mediated by caspase-3

Caspase-3, a critical executioner of apoptosis, plays a major role in proteolytic cleavage of key proteins (Fernandes-Alnemri et al. 1994). Activation of caspase-3 requires cleavage into its 17 kD fragment.

One of the main targets of proteolytic activity of cleaved caspase-3 is the poly (ADP-ribose) polymerase (PARP). PARP, a 116 kD nuclear enzyme, appears to be involved in DNA repair in response to environmental stress (Satoh and Lindahl 1992). Full length PARP supports cells in maintaining viability whereas cleaved PARP (85 kD) facilitates cellular degradation (Beneke et al. 2000). Thus, cleavage of PARP serves as a marker of cells undergoing apoptosis.

An important marker of genotoxicity is the phosphorylated form of H2AX at serine 139, named γ-H2AX. Within minutes following DNA double-strand breaks, H2AX undergoes this specific phosphorylation by ATM (Rogakou et al. 1998) and localizes to sites of damage at subnuclear foci (Rogakou et al. 1999).

HL-60 cells treated with 15 µg/ml petroleum ether extract of C. morifolia exhibited an initial cleavage of caspase-3 within 2 hours which intensified during the time course. The cleavage of PARP after 4 hours correlates with the caspase-3 activation. In response to DNA double strand breaks initiated by apoptosis, H2AX was phosphorylated at Ser139 after 4 hours.

Additionally, Akt is known to play a critical role in apoptosis. This protein kinase represents an important downstream effector of PI3K (phosphatidylinositol 3-kinase) which promotes cell survival and prevents apoptosis when activated by phosphorylation at the serine 473 residue (Franke et al. 1997). As illustrated in Figure 16, untreated HL-60 did not express p(Ser473)Akt, neither it was induced by extract treatment whereas Akt was downregulated within 24 hours.

These findings suggest an induction of apoptosis by the petroleum ether extract of C. morifolia mediated by caspase-3. The later onset of H2AX phosphorylation, compared to cleavage of caspase-3, suggests that DNA double strand breaks in extract treated HL-60 cells are not a cause of the extract’s genotoxicity but a result of induced apoptosis. Therefore, the activation of downstream effectors of DNA damage, checkpoint kinases Chk1 and Chk2, were investigated.
Figure 16. Induction of pro-apoptotic key proteins in HL-60 cells after incubation with the petroleum ether extract of *C. morifolia*. HL-60 cells (1.8 x 10⁵ cells/ml) were incubated with 15 µg/ml extract and harvested after 0.5, 2, 4, 8 and 24 h, lysed and isolated protein samples were subjected to western blot analysis using the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

4.1.8. *Early onset of apoptosis, not genotoxicity, leads to checkpoint kinase activation in extract treated HL-60 cells*

To preserve a cell’s DNA integrity, a network of proteins communicates throughout the cell cycle with each other. In response to DNA damage, checkpoint kinases 1 and 2 (Chk1 and Chk2) are main mediators of cell cycle arrest to allow DNA repair prior to mitosis, or apoptosis in case of an irreparable damage (Zhou and Elledge 2000). Total expression level of Chk1 and Chk2 remained unchanged during 24 hours of extract treatment. The slight activation of Chk1 by phosphorylation at serine345 after 24 hours did not correlate with the observed S-phase arrest as Chk1 typically induces G2-M arrest (Liu et al. 2000). Chk2 was phosphorylated at the activating threonine 68 site within 4 hours of extract incubation (Figure 17). In general, this activation is caused by the ATM protein kinase after DNA damage (Ahn et al., 2000). The correlation of Chk2 activation, which was strongest after 24 hours, and the S-phase arrest observed at that timepoint is in line with previous findings (Koczor et al. 2009; Mazzacurati et al. 2004). Activated Chk2 was reported to phosphorylate the Cdc25A phosphatase on the serine 177 residue, targeting it for proteasomal degradation (Madlener et al., 2009). This
finding may explain the downregulation of Cdc25A after 24 hours of drug treatment, since the levels of p(S177)Cdc25A were increased (Figure 15).

Chk2 activation was probably due to DNA cleavage in the course of apoptosis, because caspase-3 activation occurred before Chk2 phosphorylation. Therefore, C. morifolia petroleum ether extract is not considered to damage DNA.

Figure 17. Activation of checkpoint kinases 1 and 2 after incubation of the petroleum ether extract of C. morifolia. HL-60 cells (1.8 x 10^5 cells/ml) were treated with 15 µg/ml of the petroleum ether extract of C. morifolia. After 0.5, 2, 4, 8 and 24 h, cells were harvested and lysed. Isolated protein samples were subjected to western blot analysis investigating the protein expression levels of the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.
4.2. Neurolaena lobata

Lyophilized aerial plant parts (leaves, florescence and stipes) of *N. lobata* were sequentially extracted by reflux-water bath extraction using five solvents of increasing polarity. The obtained extracts were tested for their anti-carcinogenic activity *in vitro*. Proliferation and apoptosis assays were performed to identify the extract containing the most active constituents.

4.2.1. Extract yields and stock calculation

Extract weights obtained from serial extraction of 20.04 g lyophilized plant material with five solvents of different polarity are shown in Table 6.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Extract weight (g) corresponding to 20.04 g dried plant</th>
<th>Extract weight (µg) corresponding to 1 mg dried plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>0.8863</td>
<td>44.23</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.5931</td>
<td>29.60</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.2935</td>
<td>14.65</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.0840</td>
<td>54.09</td>
</tr>
<tr>
<td>Water</td>
<td>2.4096</td>
<td>12.24</td>
</tr>
</tbody>
</table>

To investigate the anti-proliferative effects in HL-60 cells, extracts were applied at four increasing concentrations (5 µg/ml, 15 µg/ml, 30 µg/ml, 60 µg/ml). Concentrated stock solutions of all extracts were prepared in DMSO and stored at -20°C. Control samples were treated with the amount of DMSO used in the highest concentration to take effects of DMSO into consideration. For all other samples the DMSO concentration was adjusted accordingly (max. 0.5 % DMSO final concentration).
Table 7. Extract weights of *N. lobata* applied in HL-60 proliferation assays.

<table>
<thead>
<tr>
<th>Extract type</th>
<th>Extract stock concentration (µg/µl DMSO)</th>
<th>Extract final concentration (µg/ml medium)</th>
<th>corresponding dried plant weight (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>12.29</td>
<td>5</td>
<td>113.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>339.16</td>
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<tr>
<td></td>
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<td>30</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>1356.65</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>13.50</td>
<td>5</td>
<td>168.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>506.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>1013.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>2027.31</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>16.00</td>
<td>5</td>
<td>341.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
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<td></td>
<td></td>
<td>60</td>
<td>4096.76</td>
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<tr>
<td>Methanol</td>
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<td>Water</td>
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<td>30</td>
<td>249.50</td>
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<tr>
<td></td>
<td></td>
<td>60</td>
<td>499.00</td>
</tr>
</tbody>
</table>

4.2.2. **Anti-proliferative activity of *N. lobata* extracts in HL-60 cells**

All extracts of *N. lobata* were tested in logarithmically growing HL-60 cells at increasing concentrations. Figure 18 illustrates the proliferation inhibition of extract treated samples compared to the control samples only adjusted by the solvent (DMSO). The petroleum ether, dichloromethane and ethyl acetate extract showed a growth inhibition up to 100% even at low concentrations (5 µg/ml and 15 µg/ml). The methanol extract exhibited also a dose-dependent response, but at higher concentrations. Especially when taking the corresponding dried plant material into account (Table 7), the proliferation assay did not allow a clear decision on the most effective extract. Therefore, an apoptosis analysis was conducted for the four extracts exhibiting the strongest effects in the proliferation assay (see 4.2.3). Since the water extract seemed to enhance proliferation at lower concentrations before showing slightly inhibitory effects at higher concentrations, it was not taken into consideration for further experiments.
Figure 18. Effects of different extracts of *N. lobata* on the proliferation of HL-60 cells. Logarithmically growing cells were seeded at a concentration of $10^5$ cells/ml and treated with increasing concentrations (5, 15, 30 and 60 µg/ml) of extracts (petroleum ether, dichloromethane, ethyl acetate, methanol, water). Experiments were performed in triplicate. Error bars indicate ±SD. Asterisks indicate significant alteration in proliferation compared to control ($p < 0.05$).
4.2.3. *Induction of apoptosis in HL-60 cells by extracts of N. lobata*

Based on the results of the proliferation assay, four extracts (petroleum ether, dichloromethane, ethyl acetate, methanol) were further investigated regarding their apoptotic potential. HL-60 cells were subjected to increasing concentrations of the specified extracts (Figure 19). After 24 hours of incubation, propidium iodide and Hoechst 33258 were added to the cells to facilitate the distinction between viable, apoptotic and necrotic cells via visual examination.

![Graphs showing induction of apoptosis in HL-60 by extracts of N. lobata](image)

Figure 19. Induction of apoptosis in HL-60 by different extracts of *N. lobata*. HL-60 cells were seeded at $10^5$ cells/ml and incubated with indicated concentrations of extracts (petroleum ether, dichloromethane, ethyl acetate, methanol) for 24 h. Afterwards cells were stained with propidium iodide (2 µg/ml) and Hoechst 33258 (5 µg/ml) and incubated for 1 h. Viable and apoptotic cells were photographed on a fluorescence microscope equipped with a DAPI filter and examined visually. Experiments were performed in triplicate. Error bars indicate ±SD. Asterisks indicate significant alterations in number of apoptotic cells compared to control ($p < 0.05$).
Of the four extracts, the dichloromethane extract exhibited the highest induction of apoptosis at the lowest concentration of 5 µg/ml after 24 hours of incubation. To achieve a similar number of cell deaths, 15 µg/ml of the petroleum ether extract were necessary. The ethyl acetate extracts induced apoptosis in almost 100% of the cells at a concentration of 10 µg/ml, which was similar to the dichloromethane extract. The difference in effectiveness between the dichloromethane and the ethyl acetate extract at 5 µg/ml was more obvious when taking the corresponding dried plant weight into account (168.94 µg/ml for dichloromethane and 341.40 µg/ml for ethyl acetate, respectively).

Therefore, the dichloromethane extract of *N. lobata* was identified as the most effective one and was subjected to more detailed investigations. For all upcoming experiments a less concentrated stock of 3.7 mg extract per ml DMSO was used.

Due to the promising results of the proliferation assay and cell death analysis in HL-60 cells, the dichloromethane extract of *N. lobata* was chosen for further investigations in human (SR-786) and murine (417) NPM-ALK positive ALCL (anaplastic large cell lymphoma) cell lines as well as human lung fibrobalst (HLF).

4.2.4. **Anti-proliferative effects of *N. lobata* dichloromethane extract in human ALCL SR-786 cells with a NPM-ALK translocation**

SR-786 cells were seeded at 2 x 10^5 cells/ml and treated with increasing concentrations of *N. lobata* dichloromethane extract. Due to growth characteristics of this cell line, assessment of cell proliferation was limited to 48 hours instead of 72 hours. Therefore, proliferation was considered as the difference in cell number between 24 hours and 48 hours of incubation. Results, illustrated in Figure 20, indicated a similar activity of the extract in SR-786 cells as in HL-60 cells (Figure 29).
Figure 20. Anti-proliferative effects of the dichloromethane extract of *N. lobata* in SR-786 cells. Cells were seeded at 2 x 10^5 cells/ml and incubated with the dichloromethane extract of *N. lobata* at the indicated concentrations. Cell number was determined after 24 hours and 48 hours of treatment using a Casy cell counter (Inovartis). Proliferation of control samples was defined as 100% and treated samples were set in relation. Error bars indicate ±SD. Asterisks indicate significant alterations in proliferation compared to control (p < 0.05). Experiments were performed in triplicate.

As the proliferation assay indicated “negative” proliferation in SR-786 cells at higher concentrations of the *N. lobata* dichloromethane extract, the mechanism of cell death was investigated by western blot analysis.

### 4.2.5. *Inhibition of NPM-ALK, induction of apoptosis and caspase-3 in SR-786 cells*

First, one of the most critical executioners of programmed cell death, caspase-3, and its target PARP were investigated for their cleavage. The exposition to 15 µg/ml dichloromethane extract of *N. lobata* triggered apoptotic response in SR-786 cells, which was confirmed by cleavage of caspase-3 as well as cleavage of PARP after 24 hours of incubation (Figure 21).

To examine the genotoxic potential of the extract, the levels of γ-H2AX were analysed. Like caspase-3, H2AX was not phosphorylated at the serine 139 site until 24 hours of incubation (Figure 21).

Therefore, the dichloromethane extract induced apoptosis mediated by caspase-3 and is supposed not to induce genotoxicity in SR-786 cells.
Figure 21. Induction of pro-apoptotic key proteins in SR-786 cells after incubation with the dichloromethane extract of *N. lobata*. SR-786 cells (2.5 x 10^5 cells/ml) were incubated with 15 µg/ml extract, harvested after 4, 8 and 24 h, lysed and isolated protein samples were subjected to western blot analysis using the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

In most ALK-positive ALCL cells (like in SR-786 cells), the kinase domain of ALK is fused to NPM creating the constitutively expressed oncogenic fusion protein NPM-ALK (Morris et al., 1994). After 4 hours of drug treatment, the NPM-ALK protein was substantially downregulated.

Furthermore, both PI3K and Akt are reported to be permanently activated by NPM-ALK in NPM-ALK positive ALCL cells (Slupianek et al. 2001). Western blot analysis of untreated cells showed a low constitutive p(Ser473)Akt level, which further decreased upon treatment with the dichloromethane extract. The expression of Akt protein remained almost unchanged.
In the following investigations, the mechanism causing the reduction of the oncogenic NPM-ALK fusion protein by the dichloromethane extract was studied in more detail.

4.2.6. Reduction of NPM-ALK levels is a decisive property of the dichloromethane extract of N. lobata and is caused at transcriptional level

As illustrated in Figure 21, NPM-ALK was rigorously down regulated within 24 hours of treatment with the dichloromethane extract of N. lobata. To obtain further knowledge about the specificity of NPM-ALK degradation by the N. lobata extract, the most active extract of Critonia morifolia was tested as well. According to cytotoxicity indicated in HL-60 cells, SR-786 cells were incubated with the petroleum ether extract of C. morifolia at a concentration of 20 µg/ml. Subjecting protein samples to western blot analysis revealed that NPM-ALK levels were not affected upon treatment by this plant extract (Figure 22). Therefore, the effect of the N. lobata dichloromethane extract on NPM-ALK repression was considered a specific property.

![Figure 22. Effects of the petroleum ether extract of C. morifolia on NPM-ALK levels in SR-786 cells. SR-786 cells were incubated with 20 µg/ml petroleum ether extract of C. morifolia and harvested after 4, 8 and 24 h. Cells were lysed and isolated protein samples were subjected to SDS-PAGE followed by western blot analysis of NPM-ALK and NPM. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.](image)

Experiments applying a proteasome inhibitor and a lysosome inhibitor were performed to investigate whether the reduction in NPM-ALK protein level was due to increased protein degradation. For this reason, SR-786 cells (2.5 x 10^5 cells/ml) were incubated with 15 µg/ml of the extract and either Proteasome Inhibitor IV was added to a final concentration of 50 µM or ammonium chloride (NH₄Cl) at a final concentration of 20 mM (Cockle and Dean, 1982). After 8 hours cells were harvested, lysed and isolated protein was subjected to SDS-PAGE and western blot analysis for NPM-ALK. As illustrated in Figure 23, both inhibitors failed to prevent
the decrease in NPM-ALK protein levels in extract treated cells. The control samples solely exposed to either the proteasome inhibitor or lysosome inhibitor ensured that no toxicity was induced by the inhibitors themselves.

<table>
<thead>
<tr>
<th>(a)</th>
<th>SR-786</th>
<th>(b)</th>
<th>SR-786</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurolaena extr.</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lys. Inh.</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NPM/ALK</td>
<td>[Image]</td>
<td>[Image]</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>[Image]</td>
<td>[Image]</td>
<td></td>
</tr>
</tbody>
</table>

Figure 23. Investigation of NPM-ALK degradation mechanisms by the dichloromethane extract of *N. lobata*. SR-786 cells (2.5 x 10⁵ cells/ml) were incubated with 15 µg/ml dichloromethane extract of *N. lobata* together with either (a) 50 µM proteasome inhibitor (Proteasome Inhibitor IV, Merck) or (b) 20 mM lysosome inhibitor (ammonium chloride NH₄Cl). Potential toxicity of inhibitors was considered by treating control samples with the respective inhibitor only. After 8 h cells were harvested and lysed. Protein samples were subjected to SDS-PAGE followed by western blot analysis. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

In the next step, NPM-ALK expression was investigated at the transcriptional level. RNA samples of SR-786 cells were taken after 4 hours and 8 hours of incubation with 15 µg/ml extract. Although the same cell number was taken, the total amount of RNA obtained decreased from 545 ng/µl to 450 ng/µl after 4 hours and 420 ng/µl after 8 hours of treatment. Equal amounts of RNA samples were transcribed into cDNA and subjected to real-time PCR applying specific primers for the NPM-ALK transcript and GAPDH as internal control. The NPM-ALK transcriptional level was set into relation to the constitutively expressed GAPDH using the comparative Ct (ΔΔCt) method (Livak and Schmittgen 2001). NPM-ALK transcription was downregulated by 37 % after 4 hours and by 62 % after 8 hours of incubation. This result confirms the substantial reduction in NPM-ALK protein expression detected by western blot analysis after 24 hours of extract treatment and explains the mechanism by which NPM-ALK becomes repressed (Figure 24).
Figure 24. PCR analysis of alterations in the NPM-ALK gene expression level in dichloromethane extract treated SR-786 cells. Cells (2.5 x 10^5 cells/ml) were incubated with 15 µg/ml extract of *N. lobata* and harvested after 4 h and 8 h, respectively. RNA was isolated, transcribed into cDNA and subjected to real time-PCR using specific primers for NPM-ALK and GAPDH (as internal control). Relative quantitation was calculated by applying the comparative C\textsubscript{T} method. Experiments were performed in duplicate. Error bars indicate ±SD.

The NPM-ALK downregulation was considered an important property of the *N. lobata* dichloromethane extract. Since all tests so far were conducted with lyophilized plant material, the question of effectiveness of air-dried material emerged. Therefore, air-dried aerial parts of *N. lobata* were subjected to accelerated solvent extraction and cell culture assays (data not shown). Like when exposed to the extract of lyophilized plant, SR-786 cells exhibited a potent downregulation in NPM-ALK levels upon treatment with the dichloromethane extract of the air-dried material (Figure 25).
Figure 25. Downregulation of NPM-ALK in SR-786 cells upon treatment with the dichloromethane extract of air-dried N. lobata. SR-786 cells (2.5 x 10^5) were incubated at 10 µg/ml and 15 µg/ml dichloromethane extract of air-dried plant material. Cells were harvested after 4, 8 and 24 h, lysed and isolated protein samples were subjected to SDS-PAGE followed by western blotting for NPM-ALK. Equal sample loading was confirmed by Ponceau S staining and β-actin blot.

4.2.7. The dichloromethane extract induces G2-M arrest and activates Chk1 in SR-786 cells

The effects of two concentrations (10 µg/ml and 15 µg/ml) of the dichloromethane extract on the cell cycle were investigated in SR-786 cells (2.5 x 10^5 cells/ml) by FACS analysis after 24 hours.

The extract treatment caused a dose-dependent accumulation of SR-786 cells in G2-M at the expense of cells in S-phase. The cell population in G2-M phase increased from 4.3% to 31% (10 µg/ml) and up to 51.5% (15 µg/ml; Figure 26). Simultaneously, S-phase decreased to 26.8% and 21.8%, respectively. G0-G1 phase was elevated only at a lower extract concentration and reduced at 15 µg/ml when compared to control.
Figure 26. Analysis of cell cycle distribution in SR-786 cells after 24 h of incubation with the dichloromethane extract of *N. lobata*. SR-786 cells (2.5 x 10^5 cells/ml) were incubated with 10 µg/ml and 15 µg/ml of the dichloromethane extract of *N. lobata*. After 24 h cells were harvested and subjected to FACS analysis. Error bars indicate ±SD. Asterisks indicate significant alterations of cells in cell cycle phase compared to control (p < 0.05). Experiments were performed in triplicate.

To investigate which regulatory key proteins might have caused G2-M arrest, SR-786 cells were treated with 15 µg dichloromethane extract per ml medium for 4, 8 and 24 hours and subjected to western blot analysis.

Checkpoint kinase 1 (Chk1) is probably the most important regulatory cell cycle protein in G2-M phase arrest. In case of DNA damage, Chk1 is activated rapidly by the ATR pathway and mediates cell cycle arrest in G2-M phase to allow DNA repair prior to mitosis (Liu et al., 2000). The activating phosphorylation of Chk1 kinase occurs at the serine 345 residue (Shibata et al., 2010). Besides others, Chk1 has been identified to phosphorylate serine 75 of Cdc25A targeting it for degradation (Kiyokawa and Ray, 2008; Mailand, et al. 2000). In accordance with the G2-M arrest identified by FACS analysis, Chk1 was activated by phosphorylation at serine 345 residue, after 8 hours of incubation while total expression of Chk1 remained unchanged (Figure 27). Additionally, the activation level of Chk2 was examined and only a weak induction of p(Thr68)Chk2 was observed after 24 hours of extract treatment.
Confirming the failure of entry into mitosis by G2-M arrest, Cdk1 (Cdc2) showed increased phosphorylation at tyrosine 15 within 8 hours. However, substantial dephosphorylation was found after 24 hours. In general, activation (i.e. dephosphorylation at Tyr15) of Cdk1 can be attributed to the modulated expression of the Cdc25 phosphatases. As the level of Cdc25A was not altered upon drug treatment, Cdc25B and Cdc25C were analyzed. Although acting through distinct mechanisms, Cdc25B and Cdc25C primarily control entry into mitosis by targeting the Cdk1/cyclinB complex (Karlsson-Rosenthal and Millar, 2006). Western blot analysis of the proteins suggested that the down regulation of Cdc25C after 4 hours and 8 hours was compensated by increased levels of Cdc25B and vice versa after 24 hours of incubation (Figure 27). Thus, Cdc25 phosphatases were not supposed to be responsible for activation of Cdk1 after 24 hours, and a downregulation of the kinase Wee1 (not investigated), which is targeting Cdk1, seems to be more likely.
Figure 27. Alteration in the expression of cell cycle regulatory proteins. SR-786 cells (2.5 x 10^5 cells/ml) were treated with 15 µg/ml of the dichloromethane extract of *N. lobata*. After 4, 8 and 24 h, cells were harvested and lysed. Isolated protein samples were subjected to western blot analysis investigating expression levels of p(Ser345)Chk1, Chk1, p(Thr68)Chk2, Cdc25A, Cdc25B, Cdc25C, p(Tyr15)Cdk1 and Cdk1. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.
4.2.8. *N. lobata* dichloromethane extract modulates oncogenes and tumor suppressor protein expression

To obtain a better insight into the mechanism of growth arrest that was induced by the dichloromethane extract of *N. lobata*, the expression of relevant oncogenes was studied.

Overexpression of certain cell growth and proliferation enhancing proteins are an important property of most cancer cells. So called oncogenes may prevent abnormal cells from cell death and promote uncontrolled growth when activated. On the other hand, proteins coded by tumor suppressor genes are able to repress cell cycle and/or facilitate apoptosis in abnormal cells. Both can be targets of anti-cancer drugs, namely suppressing oncogenes and promoting tumor suppressor genes.

One of the best investigated tumor suppressor genes is p53. Most cancer cells reveal a deregulation of p53 expression. In response to DNA damage p53 becomes phosphorylated at the serine 15 and serine 20 residues, typically phosphorylation at Ser20 is mediated by Chk1 and Chk2. This activation may lead to cell cycle arrest and DNA repair or apoptosis (Shieh et al., 1999). Upon treatment with the dichloromethane extract, p(Ser20)p53 levels increased only transiently within 4 hours whereas total p53 remained unchanged during the time course. Since activation of Chk1 happened after 8 hours (Figure 27), the activation of p53 can not be attributed to Chk1 kinase activity.

The transcription of Cdk-inhibitor p21 is known to be directly driven by p53. Despite, p21 can be regulated by many p53-independent pathways (Abukhdeir and Park 2008). At transcriptional level, for example, it can be regulated by the oncogene c-Myc (Coller et al. 2000). c-Myc is proven to have a profound impact on cell proliferation, differentiation and apoptosis. Upregulation of c-Myc expression is common in many tumor types. This contributes to an abnormal proliferation rate. Among others, c-Myc was discovered to downregulate p21 (Coller et al. 2000), and conversely c-Myc downregulation may result in p21 induction, as observed in SR-786 cells upon treatment with the *N. lobata* dichloromethane extract after 4 hours (Figure 28).
Figure 28. Effects of the dichloromethane extract on oncogenes and tumor suppressor proteins in SR-786 cells. SR-786 cells (2.5 x 10^5 cells/ml) were incubated with 15 µg/ml dichloromethane extract of N. lobata and harvested after 4, 8 and 24 h of treatment. Cells were lysed and obtained proteins samples subjected to SDS-PAGE and western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

The Jun family encompasses c-Jun, JunB and JunD. They are components of the transcriptional factor AP-1. Studies in knock-out mice have demonstrated roles for c-Jun in various tissues and developmental processes including T cell development (Riera-Sans and Behrens 2007). JunB and c-Jun are reported to be over-expressed in anaplastic large cell lymphoma favoring cancer progression (Mathas et al., 2002). Nevertheless, JunB and c-Jun are controversially discussed. Inconsistent reports in recent literature support the idea that both proteins may act as either an oncogene or a tumor suppressor in a cell-context-dependent manner (Shaulian 2010). The incubation of SR-786 cells with the extract transiently increased c-Jun expression whereas JunB was continuously downregulated (Figure 28).
Taking together, the activation of p53, induction of p21, and inhibition of c-Myc and JunB explain the growth arrest in SR-786 cells observed after incubation with the *N. lobata* dichloromethane extract.

4.2.9. **Proliferation inhibition in murine ALCL cells with a NPM-ALK translocation**

In preliminary studies, it was found that human ALCL cell lines SR-786 and Karpas 299 are deficient in membrane protein platelet-derived growth factor receptor β (PDGFR-β), although PDGFR-β is found in primary human ALCL specimens proven by immunohistochemical analysis (Laimer et al. 2010, in preparation). PDGF, and therefore also PDGFR-β, are recognized as important factors in cell proliferation, differentiation and growth (Gotzmann et al. 2006). This makes PDGF receptor an attractive target of anti-cancer drugs. To analyze potential effects of the extract on PDGFR-β, it was also tested in the murine ALCL cell line 417 which expresses high levels of PDGFR-β (Laimer et al. 2010, in preparation).

Two tests, on proliferation and cytotoxicity (see 4.2.11), were performed to investigate the effects of *N. lobata* dichloromethane extract in the NPM-ALK positive murine ALCL cell line 417. To measure proliferation, $10^6$ cells/ml were treated with increasing amounts of extract and incubated for 72 hours. 417 cells showed “negative” proliferation, i.e. the induction of cell death, at 10 µg/ml (Figure 29a) indicating a similar sensitivity to the drug in 417 compared to human HL-60 cells and SR-786 ALCL cells.
Figure 29. Anti-proliferative/apoptotic effects of dichloromethane extract of *N. lobata* in 417 cells. Cells (10⁶ cells/ml) were incubated at indicated concentrations of the dichloromethane extract for 72 h. Cell number was determined after 24 and 72 h using a Casy® cell counter. Proliferation of control samples was defined as 100 % and all other conditions were set in relation. Experiments were performed in triplicate. Error bars indicate ±SD. Asterisks indicate significant alterations in proliferation compared to control (p < 0.05).

Analysis of cell cycle distribution and protein expression patterns were conducted to determine at which stage and by which mechanisms cell proliferation was inhibited.

**4.2.10. **Cell cycle inhibitory effects in G2-M of the dichloromethane extract in 417 cells

Human NPM-ALK positive ALCL cell line SR-786 was proven to be arrested in G2-M phase upon treatment with the dichloromethane extract (Figure 26). Hence, murine 417 cells (10⁶ cells/ml) were incubated with 5 µg/ml, 10 µg/ml and 15 µg/ml of the dichloromethane extract for 24 hours and afterwards subjected to fluorescence activated cell sorting (FACS). Treatment of 417 cells with 5 µg/ml did not alter cell cycle distribution significantly. By contrast, application of 10 µg/ml resulted in an increase of 10 % in G0-G1 (from 46.1 % to 56.9 %) and from 7.7 % to 23 % in G2-M. Increases of cell population in G0-G1 and G2-M were at the expense of cells in S-phase which were reduced from 47.2 % to 20.1 % (Figure 30). This finding was consistent with the data obtained with SR-786 cells. At an extract concentration of 15 µg/ml, toxicity led to exorbitant cell death which did not allow FACS analysis for all three samples. Thus, this data was only assessed in duplicate not enabling for calculation of significance. Nevertheless, cells treated with 15 µg/ml extract followed the trend explored in cells at 10 µg/ml, namely an increase in cell population in G2-M phase at the expense of S-phase (Figure 30).
Cell cycle distribution of 417 cells after 24h of incubation with the dichloromethane extract of *N. lobata*

Figure 30. Cell cycle distribution of 417 cells upon treatment with the *N. lobata* dichloromethane extract. 417 (10⁶ cells/ml) were incubated at indicated concentrations of the dichloromethane extract of *N. lobata* for 24 h and then subjected to FACS analysis. Error bars indicate ±SD. Experiments were performed in triplicate, except for the concentration of 15 μg/ml, which was assessed in duplicate.

To investigate whether the same regulatory key proteins caused G2-M arrest in 417 cells like in SR-786 cells, 417 cells were incubated with 10 μg/ml of the dichloromethane extract and harvested after 0.5, 2, 4, 8 and 24 hours. Protein was isolated and subjected to western blot analysis (Figure 31).
Figure 31. Analysis of activation and deactivation of cell cycle regulatory proteins in 417 cells induced by the *N. lobata* dichloromethane extract. 417 cells (10⁶ cells/ml) were incubated at a concentration of 10 µg/ml dichloromethane extract and harvested at times indicated. Cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris-buffered saline (Tris pH 8), 1 % Triton X, 1 mM PMSF and 1 % PIC. The isolated protein was centrifuged and the supernatant was subjected to western blot analysis using the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

Like in SR-786 cells, the dichloromethane extract of *N. lobata* induced phosphorylation at Ser345 of Chk1 after 24 hours which confirms the G2-M arrest revealed by FACS analysis. In accordance, Cdc25A was degraded after 24 hours.

As shown in Figure 31, phosphorylation of Cdk1 was upregulated from 0.5 to 8 hours, thus blocking transition into mitosis. After 24 hours p(Tyr15)Cdk1 expression decreased, while total Cdk1 remained unaffected during 24 hours of treatment. In this case, the drug treatment decreased p(Tyr15)Cdk1 as well as Cdc25A in 417 cells after 24 hours. Thus, dephosphorylation was not attributable to increased phosphatase activity of Cdc25A but might was a result of decreased kinase activity of Wee1 (McGowan and Russel, 1993). Therefore, the effects observed in Cdk1 did not correlate with the growth phenotype.
In the next step, cytotoxic and apoptotic potential of the extract was investigated. Human lung fibroblasts were treated simultaneously to take possible cytotoxic effects in normal cells into account.

4.2.11. Induction of apoptosis is specific in 417 cells and mediated by caspase-3

Many anti-cancer drugs act by killing cells that divide rapidly, which is an important property of most cancer cells. However, those drugs are also affecting cells that proliferative rapidly under normal circumstances, i.e. cells in the digestive tract, bone marrow and hair follicle. Other cells in mammals replicate at a considerably lower rate which makes them less vulnerable to chemotherapy. Since the dichloromethane extract exhibited strong cytotoxic effects in HL-60, SR-786 and 417 cancer cells lines, the question of general toxicity in cells of mammalian origin emerged. To gain further insight into the specificity of cytotoxicity of the extract, normal human lung fibroblast (HLF) were subjected to extract treatment and analysis by alamarBlue® assay (Figure 32b). As 1 µg/ml extract did not exhibit a significant alteration in the proliferation of 417 cells, 2.5 µg/ml were used instead (Figure 32a). HLFs were seeded and allowed to attach for 24 hours prior treatment. At confluence, HLFs decelerate proliferation substantially while cells are still metabolically active. Thus, this system was used to simulate effects in normal cells (Li W et al. 2006). Additionally, apoptotic key proteins were investigated by western blot analysis in both 417 and HLFs.
Figure 32. AlamarBlue assay (a) in cell line 417 and (b) primary human lung fibroblasts after 48 h of incubation with the dichloromethane extract of *N. lobata* at indicated concentrations. 417 cells were seeded at $10^6$ cells/ml. HLFs were seeded at confluence and allowed to attach for 24 h prior treatment. After 48 h of incubation with the dichloromethane extract, 50 µl alamarBlue reagent (containing resazurin) was added to each well and cells were incubated for another 90 min. Metabolic active cells converted blue resazurin to red-fluorescent resofurin. Absorption was measured by a multi-detection reader at 570 nm. Calculated data based on the measurement (c) for 417 cells and (d) HLFs. Experiments were performed in triplicate. Error bars indicate ±SD. Asterisks indicate significant alterations in proliferation compared to control ($p < 0.5$).

By visual evaluation, HLFs seemed to be unaffected by the extract after 48 hours of incubation whereas 417 cells exhibited a substantial dose dependent response in metabolic activity indicated by the conversion of blue resazurin into red-fluorescent resofurin (Figure 32). This impression was validated by calculating differences in fluorescence based on the exact
measurement data. Thus, fluorescence produced by 417 cells reduced absorption by 10.3 % (not significant, p < 0.5), 26.7 % and 81.4 % with increasing concentrations of the dichloromethane extract (corresponding to 2.5 µg/ml, 5 µg/ml and 10 µg/ml, respectively; Figure 32c). In HLFs, the extract slightly attenuated the metabolism at 10 µg/ml and to 85.6 % at 15 µg/ml (both not significant, p < 0.05; Figure 32d). Western blot analysis of pro-apoptotic proteins was applied to confirm the findings stated above.

The dichloromethane extract of N. lobata exhibited strong apoptotic potential mediated by caspase-3 in SR-786 cells. The antibody used for detection of cleavage in 417 cells detects only activated large fragments (17/19 kD) but not full length caspase-3. Caspase-3 antibody detecting endogenous levels of full length caspase-3 (35 kD) as well as large fragments was applied for detection in HLF cells. The treatment of 417 cells with 10 µg/ml dichloromethane extract resulted in an activation of caspase-3 after 8 hours (Figure 33a) whereas caspase-3 remained full length (35 kD) in HLF cells exposed to 10 µg/ml and even 15 µg/ml extract after 24 hours (Figure 33b).

As target of activated caspase-3, cleaved PARP antibody, detecting the large fragment but not full length PARP, was used to indicate DNA damage in 417 cells. Cleavage of PARP already started after 4 hours of incubation with the extract and increased substantially within the following 20 hours (Figure 33a).

The dichloromethane extract of N. lobata induced phosphorylation of H2AX at Ser139 in 417 cells after 4 hours of incubation which intensified continuously until 24 hours. Like in human SR-786 cells, this correlated with caspase-3 activity and therefore, did not indicate genotoxicity.

In addition to the common pro-apoptotic proteins, two receptor tyrosine kinases (RTK) were investigated, the oncogenic NPM-ALK fusion protein and the cell growth stimulating protein PDGFR-β. The expression of both RTKs was considerably downregulated in 417 cells upon drug treatment, showing an earlier onset in NPM-ALK degradation (Figure 33a). In HLFs, the effect on PDGFR-β was less incisive and noticeable only after 24 hours of incubation. As proven by western blot analysis, HLFs are deficient in ALK expression. NPM expression was not affected by the extract in HLFs (Figure 33b).
Figure 33. Effects of the dichloromethane extract on pro-apoptotic proteins in cell line 417 and primary HLFs. 417 cells were seeded at $10^6$ cells/ml and incubated with 10 µg/ml of the dichloromethane extract of *N. lobata*. HLFs were seeded confluent and allowed to attach for 24 h prior incubation with 10 µg/ml and 15 µg/ml of the extract. At each time point indicated, cells were harvested and received protein samples (lysates) were subjected to western blot analysis to investigate cleavage of caspase-3 and PARP. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

So far, there exists no report about an anti-cancer drug that efficiently targets NPM-ALK. In the next step, the effect of the dichloromethane extract on NPM-ALK was compared to those of vincristine, a plant derived drug that is commonly used as part of the chemotherapy regimen in non-Hodgkin’s lymphoma such as ALCL.

**4.2.12. *N. lobata* dichloromethane extract, but not vincristine, substantially decreases levels of NPM-ALK in 417 cells**

As previously shown in this work, the NPM-ALK fusion protein was rigorously downregulated upon treatment with the dichloromethane of *N. lobata* but not the petroleum ether extract of *C. morifolia*. Therefore, this property was supposed to be characteristic for the *N. lobata* dichloromethane extract. To substantiate this hypothesis, its effects in 417 cells were compared to those of the chemotherapeutic agent vincristine.
During the dichloromethane extract treatment (10 µg/ml) decreased the expression of NPM-ALK in 417 cells within 4 hours and was continuously downregulated throughout the rest of the time course (Figure 33a). Vincristine, a plant derived alkaloid used in chemotherapy that acts as a mitotic inhibitor and leads to cell death, was used to treat 417 cells (10⁶ cells/ml) at increasing concentrations (1 µM, 2.5 µM and 5 µM). Protein samples were taken after 8 hours and 24 hours of incubation and subjected to western blot analysis. Compared to the dichloromethane extract of \( N. \) lobata, vincristine exhibited minimal cleavage of caspase-3 and no decrease in NPM-ALK expression after 8 hours. Prolonged exposure time to vincristine led to substantial activation of caspase-3 at all concentrations while protein levels of NPM-ALK were dose-independently reduced (Figure 34).

Furthermore, changes in the expression levels of PDGFR-β were assessed for both agents. The dichloromethane extract exhibited downregulating properties in PDGFR-β protein levels within 8 hours of incubation (Figure 33a). Vincristine treatment decreased PDGFR-β expression equally effective after 24 hours (Figure 34).

![Western blot analysis of effects of vincristine on NPM-ALK, PDGFR-β and caspase-3 levels in 417 cells. Cells (10⁶ cells/ml) were incubated with vincristine at concentrations of 1 µM, 2.5µM and 5 µM. Protein samples were taken after 8 h and 24 h. Isolated protein was subjected to western blot analysis applying antibodies against ALK (also detecting NPM-ALK fusion protein), PDGFR-β and caspase-3. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.](image)

**Figure 34.** Western blot analysis of effects of vincristine on NPM-ALK, PDGFR-β and caspase-3 levels in 417 cells. Cells (10⁶ cells/ml) were incubated with vincristine at concentrations of 1 µM, 2.5µM and 5 µM. Protein samples were taken after 8 h and 24 h. Isolated protein was subjected to western blot analysis applying antibodies against ALK (also detecting NPM-ALK fusion protein), PDGFR-β and caspase-3. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.
Direct comparison to vincristine leads to the assumption that the downregulation of NPM-ALK might be a decisive property of the *N. lobata* dichloromethane extract.
5 DISCUSSION

Based on the traditional uses in Mayan medicine for over thousand years, two ethnomedical plants from Central America, *C. morifolia* and *N. lobata*, were investigated for their potential anti-carcinogenic activity. Conditions treated by distinct preparations of aerial parts of these plants range from parasitic ailments to various states of inflammation and neoplasms (Arvigo and Balick 1998). Additionally, promising phytochemical and pharmacological research results have been published, particularly on *N. lobata*.

For investigational reasons, the collected fresh plant material from Guatemala was lyophilized to preserve also volatile compounds, and subjected to sequential extraction using five solvents of increasing polarity. This method facilitated an initial separation of plant constituents according to their polarity, and the obtained extracts were tested *in vitro* for their anti-neoplastic potential.

5.1. *C. morifolia*

Apart from inflammatory conditions, *C. morifolia* leaves are utilized as home remedy to cure boils, swellings and even “cancer” (severe skin damages with uncontrolled wound tissue growth: Arvigo and Balick 1998). Investigations in human promyelocytic leukemia HL-60 cells revealed the petroleum ether extract as the extract possessing the strongest anti-carcinogenic potential. Thus, the anti-proliferative and pro-apoptotic effects were due to the activity of apolar plant constituents. Besides, this finding is consistent with the traditional uses as the leaves are preheated in oil prior to application directly on the neoplastic tissue (Arvigo and Balick 1998). As it turned out during investigations, the activity of *C. morifolia* petroleum ether extract deteriorated when dissolved in DMSO and stored at -20°C within two weeks. Accordingly, fresh stock solutions were prepared regularly. Hence, active compounds contained in the extract are instable.

Like many human cancers, HL-60 show increased expression levels of the proto-oncogenes cyclin D1 and c-Myc, and lack of the tumor suppressor protein p53. These proteins are known to have impact on cell proliferation, differentiation and apoptosis, and thereby play an important role in the development of cancer (Fu et al. 2004; Dominguez-Sola et al. 2007). The *C. morifolia* petroleum ether extract simultaneously suppressed cyclin D1 and c-Myc within 30 minutes which intensified during the time course. In early G1 phase, D-cyclins bind to Cdk4
and Cdk6, thereby promoting the transition into S-phase (Pines 1991; Lingfei et al. 1998). Hence, the reduction in cyclin D1 would suggest a cell cycle inhibition in G1 phase, which contrasts both the G2-M and the S-phase arrest observed upon extract treatment after 8 hours and 24 hours, respectively. Therefore, the extract blocked also another cell cycle regulatory mechanism even before cyclin D1 suppression became effective. But as cyclin D1 also regulates and associates with transcription factors in a Cdk-independent manner (Fu et al. 2004), the influence of decreased cyclin D1 levels must have additively contributed to growth inhibition. HL-60 cells are p53 deficient (Wolf and Rotter 1985), nevertheless the expression of Cdk-inhibitor protein p21, which acts tumor suppressive and is (among others) induced by p53 and also Erk ½, or repressed at transcriptional level e.g. by c-Myc (Abukhdeir and Park 2008; Coller et al. 2000), was not affected by the extract.

The inhibition of uncontrolled cell proliferation is an essential property of anti-cancer drugs. Transiently, C. morifolia petroleum ether extract dose-dependently arrested the cell cycle in G2-M after 8 hours. This effect correlated with the continuous downregulation of phosphatase Cdc25C, which plays a major role in G2-M transition via activation of the Cdk1/cyclin B complex (Hunter 1995). The activation of Cdk1/cyclin B by dephosphorylation at Tyr15 is a crucial event to enable a cell to enter mitosis (Norbury et al. 1991). Notably, the initial G2-M arrest after 8 hours was shifted to inhibition of cell cycle progression in S-phase after 24 hours, which was confirmed by substantial dephosphorylation of Cdk1 from 8 to 24 hours, thus enabling cells to overcome the initial G2-M arrest. As Cdc25A, Cdc25B and Cdc25C levels were all downregulated at this time point, the activated state of Cdk1 was supposed to be attributable to the decrease in Wee1 levels as kinase Wee1 antagonizes the phosphatase activity of the Cdc25 family by phosphorylating Cdk1 at Tyr15 (Katayama et al. 2005). The S-phase arrest after 24 hours might have been due to the activation of Chk2, a downstream effector of ATM kinase in case of DNA damage (Ahn et al. 2000), which was strongest at that timepoint. This observation was paralleled by the phosphorylation of its substrate Cdc25A at the serine 177 residue. Phosphorylation of Cdc25A by a checkpoint kinase (Chk1, Chk2) targets it for rapid degradation (Mailand et al. 2000; Madlener et al. 2009). By dephosphorylating Cdk2, Cdc25A enables cells to transit from G1 to S-phase (Hoffmann et al. 2004) and therefore, the highest levels of Cdc25A and activated Cdk2 were reported at late S-phase (Molinari et al. 2000) indicating its need for proper DNA synthesis. Consequently, the degradation of Cdc25A might explain the arrest of cell population in S-phase as observed upon extract treatment after 24 hours. It remains to be established however, why cells did not arrest already in G1, since
cyclin D1 was completely downregulated at this time point, and it is likely that another cyclin may have substituted for cyclin D1 (i.e. cyclin D2 or D3).

The temporally separated cell cycle phase-specific inhibitory properties of the extract were probably due to distinct anti-neoplastic compounds that are contained therein.

Besides the inhibition of growth, another common feature of anti-cancer agents is their pro-apoptotic property like it was observed by the petroleum ether extract of *C. morifolia* and which was verified by the preceding activation of caspase-3 starting within 2 hours. Caspase-3 acts as critical executioner of apoptosis (Fernandes-Alnemri et al. 1994) and its main target PARP was signature-type cleaved after 4 hours, facilitating cellular degradation (Beneke et al. 2000). DNA double strand breaks were indicated by γ-H2AX after 4 hours. In general, phosphorylation of H2AX at Ser139 residue is considered a marker of genotoxicity (Rogakou et al. 1998). However, in this case, the later onset of H2AX phosphorylation compared to activation of caspase-3 suggested that DNA double strand breaks were not attributable to a genotoxic property of the extract but a result of the ongoing apoptotic degradation of the chromatin. Consistently, the activation of Chk2 occurred after the initiation of apoptosis as indicated by caspase-3 cleavage. This theory suggests an activation of Chk2 by ATM upon DNA cleavage initiated by the foregoing onset of apoptosis.

Thus, according to the early onset of apoptosis, the above discussed anti-proliferative effects of the *C. morifolia* petroleum ether extract found in HL-60 cells are probably attributable to the induction of cell death rather than growth inhibition, particularly for the 24 hours time point.

As a concluding remark, the *C. morifolia* petroleum ether extract has been demonstrated to exert strong anti-carcinogenic activity, especially in terms of pro-apoptotic effects. Nevertheless, the instability of active constituents and the difficult access to the plant may limit further investigations and applicability.
5.2. N. lobata

Aqueous preparations of *N. lobata* are widely used in folk medicine, especially to treat and prevent a variety of parasitic ailments such as malaria and amoebiasis (Arvigo and Balick 1998). In phytochemical research, a variety of characteristic sesquiterpene lactones were isolated of *N. lobata* including neurolenin A, B, C, D, E and F, and lobatin A, B, and C (Passreiter et al. 1995) as well as pyrrolizidine alkaloids (Passreiter 1998). Pharmacological studies approved anti-parasitic properties of distinct *N. lobata* extracts and plant constituents, i.e.: the dichloromethane extract and sesquiterpene lactones were reported to be active against the clinical most important malaria pathogen *Plasmodium falciparum* and *Plasmodium berghei* (François et al. 1996); and the ethanol extract was found to inhibit growth of *Leishmania mexicana*, *Trypanosoma cruzi* and *Trichomonas vaginalis* (Berger et al. 2001).

In addition, François et al. (1996) compared anti-plasmodial activities with cytotoxicity of sesquiterpene lactones isolated from the dichloromethane extract. Whereas neurolenin B exhibited the highest activity in *P. falciparum*, lobatin B was the compound with the strongest cytotoxic activity in GLC4 and COLO 320 tumor cell lines. The IC_{50} values against both tumor cell lines were much higher than those in the anti-plasmodial assay.

Consistent with previous investigations, the present study identified the dichloromethane extract of *N. lobata* to possess the highest anti-proliferative and pro-apoptotic potential in HL-60 cells. Therefore, this extract was chosen for further investigations in NPM-ALK positive anaplastic large cell lymphoma (ALCL) human cell line SR-786 and murine cell line 417. ALCL is a subtype of non-Hodgkin lymphoma classified among T/null cell lymphomas with aberrant cell surface expression of CD30. It is named after the typical morphology of the cells (Ambrogio et al. 2008). The majority of them carry chromosomal aberrations involving the anaplastic lymphoma kinase (ALK) of which the t(2;5)(p23;q35) translocation, which fuses the *ALK* gene to the nucleophosmin (*NPM*) gene, represents the most frequent one (Stein et al. 1985). The resulting NPM-ALK fusion protein with constitutive tyrosine kinase activity is considered to play an essential role in the pathogenesis of ALCL through its impact on proliferation, differentiation and apoptosis (Piva et al. 2006). Therefore, ALK is suggested as viable target for therapeutic intervention.
*N. lobata* dose-dependently arrested SR-786 cells in G2-M phase of the cell cycle, predominantly at the expense of S-phase cells. This effect was observed in both human SR-786 and murine 417 cells. Apart from the role of NPM-ALK, at least three independent mechanisms may have contributed to the accumulation of cells in this phase as revealed by western blot analyses:

(i) *N. lobata* extract induced checkpoint kinase 1 (Chk1), which is activated in response to DNA damage by ATR kinase (Liu et al. 2000) halting the cell cycle in G2-M phase to allow DNA repair prior to mitosis. A downstream target of activated Chk1 (and Chk2) is the inhibition of the Cdc25 phosphatase family. These proteins are required for cell cycle transit by activating cyclin-dependent kinases (Cdks) through dephosphorylation. Cdc25B and Cdc25C primarily control the entry into mitosis by activating the Cdk1/cyclin B complex (Karlsson and Millar 2006). The proteins seemed to compensate for each other as both proteins were inversely up- and downregulated during the time course of extract treatment. Thus, the alleged activation of Cdk1 after 24 hours of treatment was supposed to be the result of reduced kinase Wee1 activity targeting Cdk1 rather than increased Cdc25 phosphatase activity.

(ii) SR-786 cells exhibited dramatic alterations in their expression of oncogenic and tumor suppressor proteins upon incubation with the dichloromethane extract. The proto-oncogenic protein c-Myc, a main driver of unscheduled proliferation and overexpressed in many tumors, was completely downregulated within 4 hours. Moreover, the Cdk-inhibitor and tumor suppressor protein p21 was upregulated simultaneously. The main transcriptional regulator of p21 is p53 and in fact, p53 became activated within 4 hours of extract treatment. Whether this was the p21-inducing event remains obscure, since more than 50% of all cancers harbor a mutated and largely inactive p53 gene. As p21 was upregulated and temporally inverse to c-Myc suppression, this may have been the causal p21-regulatory mechanism (Coller et al. 2009).

(iii) Two major constituents of the transcription factor AP-1 (activating protein 1), JunB and c-Jun, were affected by the *N. lobata* extract. AP-1 is reported to play an essential role in certain cancers. In case of anaplastic large cell lymphoma, AP-1 is suggested to prevent cells from undergoing apoptosis, therefore promoting uncontrolled growth (Mathas et al. 2002). The dichloromethane extract induced a transient increase in c-Jun expression after 4 and 8 hours whereas JunB was continuously downregulated in
SR-786 cells. In NPM-ALK positive ALCL, especially the JunB level has been reported to be associated with carcinogenic transformation and may be regulated by NPM-ALK (Hsu et al. 2006), which is in line with the present findings as the decrease in NPM-ALK was paralleled by downregulation of JunB.

The major pathways engaged by NPM-ALK are the Jak/Stat, Ras/Mapk and PI3K pathways (Simonitsch et al. 2001; Polgar et al. 2005; Palmer et al. 2009). As proved by western blot analysis, treatment with the dichloromethane extract of *N. lobata* led to a substantial decrease in NPM-ALK protein expression that was caused at transcriptional level rather than protein degradation. The decrease in NPM-ALK protein was observed in both human SR-786 and murine 417 ALCL cell line. In contrast, the *C. morifolia* petroleum ether extract, which was tested in SR-786 cells, as well as the common plant derived chemotherapeutic agent vincristine used in ALCL therapy and tested in 417 cells, did not exhibit a downregulation of NPM-ALK levels upon treatment. Thus, the effect of *N. lobata* dichloromethane extract on NPM-ALK protein expression is proposed to be a specific property. However, because NPM-ALK plays a role in proliferation and apoptosis via several pathways its exact influence on proliferation and apoptosis is hard to determine.

PDGFR-β, like NPM-ALK, is a receptor tyrosine kinase playing an important role in proliferation, differentiation and growth (Gotzmann et al. 2006). Although primary human ALCL specimens express PDGFR-β as proven by immunohistochemical analysis, SR-786 and other human NPM-ALK positive ALCL cell lines are PDGFR-β deficient (Laimer et al. 2010, in preparation). Hence, murine cell line 417 was selected to examine the effects of *N. lobata* on this receptor tyrosine kinase. As for NPM-ALK, a substantial decrease in PDGFR-β protein expression was observed during the time course, leading to the assumption that *N. lobata* might contain an active principle which functions as a receptor tyrosine kinase inhibitor.

Apart from the downregulation of NPM-ALK, *N. lobata* dichloromethane extract triggered apoptosis in both SR-786 and 417 cell lines, which was associated with the cleavage of caspase-3, followed by phosphorylation of H2AX at Ser139 (γH2AX), as marker of double strand breaks caused by apoptosis. Cell death was induced at considerably low concentrations of 10 µg/ml extract (corresponding to 0.34 mg/ml dried plant weight) in murine and 15 µg/ml extract (corresponding to 0.5 mg/ml dried plant material) human ALCL cells, respectively.

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To gain further insight into the specificity of cytotoxicity and rule out overall toxicity, the extract was also tested in human lung fibroblasts (HLF). Prior treatment, HLFs were grown to confluence where they substantially decrease their proliferative activity while still being metabolically active simulating the homeostatic condition of normal cells, which generally proliferate at considerably lower rates than tumor cells. Indeed, the metabolism of HLFs was only insignificantly attenuated even at an extract concentration of 20 µg/ml. Furthermore, 15 µg/ml extract did not induce activation of caspase-3 in HLFs. According to these results, the *N. lobata* dichloromethane extract exhibited specificity towards cancer cells, which is confirmed by reports that only weak toxicity is observed in brine shrimp larvae *Artemis salina* and mice treated with a dichloromethane extract of *N. lobata* (Berger et al. 1998; Cáceres et al. 1998).

Whereas the *C. morifolia* extract is rather labile, the anti-neoplastic properties of the *N. lobata* are remarkably stable, which will facilitate further studies. Especially the contained sesquiterpene lactones could be of major interest since drugs derived from this substance class have already reached cancer clinical trials, e.g. artemisinin, thapsigargin, and parthenolide. The most promising feature of these drug candidates is their preferential selectivity toward tumor and cancer stem cells while sparing normal counterparts, which makes them superior to many conventional agents commonly used in chemotherapy (Ghantous et al. 2010; Zhang et al. 2005).

Concluding, there is sufficient evidence that *N. lobata* extract possesses specific anti-neoplastic properties downregulating oncogenes and oncogenic receptor-type tyrosine kinases with limited side toxicity and should therefore undergo testing *in vivo*. 
SUMMARY

Although many cancers can be treated efficiently in first line chemotherapy, relapse and refractory cancers require the discovery and development of new anti-cancer agents. The present work analyzed the potential anti-neoplastic activity of two rainforest species of the Asteraceae family, Critonia morifolia and Neurolaena lobata, that successfully passed hundreds of years of usage as home remedies in the long lasting medical tradition of the Central American Mayas. The plant material, collected in Guatemala, was lyophilized and sequentially extracted with five solvents of increasing polarity to provide an initial separation of plant constituents. For each plant, the extract exhibiting the most potent anti-proliferative and pro-apoptotic effects in HL-60 cells was subjected to more detailed investigations including western blot and FACS analyses.

The anti-proliferative properties of the C. morifolia petroleum ether extract turned out to be attributable to the induction of cell death as the apoptotic executioner protein caspase-3 was already activated within 2 hours of incubation. Changes in the levels of cell regulatory proteins were observed thereafter, in particular, Chk2 was activated upon DNA cleavage initiated by the foregoing onset of apoptosis, and this correlated with the S-phase cell arrest after 24 hours. The loss in bio-activity of this extract indicated that considerably unstable compounds triggered the anti-neoplastic effects, which may limit its potential use as anti-cancer remedy.

The other plant, N. lobata, tested in human SR-786 and murine 417 NPM-ALK positive ALC1 cell lines, was more promising. In both cell lines, 10-15 µg/ml of the dichloromethane extract inhibited the cell cycle in G2-M phase, which correlated with the activation of Chk1 and the depletion of the proto-oncogene c-Myc paralleled by the upregulation of the p21 tumor suppressor protein. The extract strongly triggered apoptosis, inducing caspase-3 followed by phosphorylation of H2AX. Overall toxicity was low since normal human lung fibroblasts remained viable even upon exposure to extract concentrations beyond 15 µg/ml. The extract led to a substantial decrease in oncogenic NPM-ALK transcript and protein expression, but not in that of nucleophosmin. This suggested that the extract affected a post-transcriptional mechanism, i.e. mRNA stability rather than mRNA transcription. The chemotherapeutic agent vincristine and the C. morifolia extract did not affect NPM-ALK expression. Apart from NPM-ALK, a second tyrosine kinase, namely PDGFR-β, was also downregulated by the N. lobata extract. Taken together, the N. lobata extract blocked the expression of several proto-oncogenes by a mechanism that needs to be determined in future investigations.

Die Proliferationshemmung des *C. morifolia* Petroleumetherextraktes beruhte vermutlich eher auf der frühen Induktion der Apoptose vermittelt durch aktive Caspase-3. Die Aktivierung des zellregulatorischen Proteins Chk2 und der damit korrelierende S-Phase Arrest nach 24 Stunden wäre demnach durch DNA-Schäden aufgrund des vorschreitenden Zelltod verursacht. Der rasche Verlust an Bioaktivität des Extraktes lässt auf in hohem Maße instabile Verbindungen schließen, die einen zukünftigen Einsatz als Chemotherapeutikum limitieren könnten.

REFERENCES


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