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1. AIMS OF THE WORK

Cancer is one of the biggest medicinal problems in the recent time, so that the research and development of new therapeutic strategies is very important for human healthcare. In many cases, the new concepts are derived from natural sources, especially plants.^{(1),(2)}

Plants have always been used in the traditional medicine for the treatment of various diseases, including cancer, so that a substance from natural origin or a derivate thereof is an interesting source for new anti-cancer drug concepts. Natural substances are known for a very specific interaction with their targets due to their long evolution over thousands of years and their high stereochemical complexity, leading to drugs with a high effectiveness and low adverse effects at the same time.^{(3),(4)}

The aims of this diploma thesis were to investigate purified dry extracts of five different Cameroonian plant species (*Dichrostachys glomerata*, *Lippia rugosa*, *Pittosporum mannii*, *Plectranthus glandulosus* and *Zornia glochidiata*) on their cytotoxic effects on HL-60 promyelocytic leukaemia cells by proliferation analysis, cell death analysis and western blotting, as well as their metabolism using RP-HPLC.

These in-vitro investigations are important in order to determine whether these purified plant extracts might be used as a potential source for new anti-cancer therapy agents in the future.

2. INTRODUCTION

2.1. Cancer

Cancer is currently one of the biggest burdens of mankind, causing 7 million deaths worldwide every year (statistics from the year 2000), that means more than the most prominent infectious diseases – malaria, HIV/AIDS and tuberculosis – combined. Statistics postulate even an enormous increase of new cases over the next years (up to 15 million new cases in 2020), especially in the developing countries (up to 70 %), where patients can not afford the high therapy costs due to their low income.^{(1),(2)} In Germany, up to 30 % of deaths are caused by cancer.⁽⁵⁾

Chemotherapy is a bigger part in the treatment of cancer, especially when metastases, relapses or disseminated tumors are existent and surgery or radiotherapy can not be used. Although some patients can be cured from cancer with chemotherapy (especially when they suffer from fast growing cancers such as leukaemia), in many cases the therapy is only palliative, that means it prolongs the lifetime and reduces the symptoms but can not prevent the progression and death.⁽⁵⁾

Additionally, today's cytostatic drugs are known for severe adverse effects such as bone marrow suppression, mucositis, stomatitis, alopecia, nausea and vomiting due to unspecific cell interactions, so that the actual scientific research tries to figure out the molecular "dysfunctions" of cancer cells in order to detect new targets for more specific drugs (higher success rate at lower adverse effects).⁽⁵⁾

Cancers are characterized by the so called "hallmarks of cancer", including cellular growth without stimulation, resistance to inhibitory signals, the suppression of apoptosis, the potential for infinite replication (due to the expression of telomerases), the induction of angiogenesis and the invasion in other tissues causing metastasis.⁽⁶⁾ In addition, also the deregulated cellular metabolism, the ability of avoiding the destruction via the immune system, tumor-promoting inflammation and gene instability causing mutations are counted to these main characteristics.⁽⁷⁾

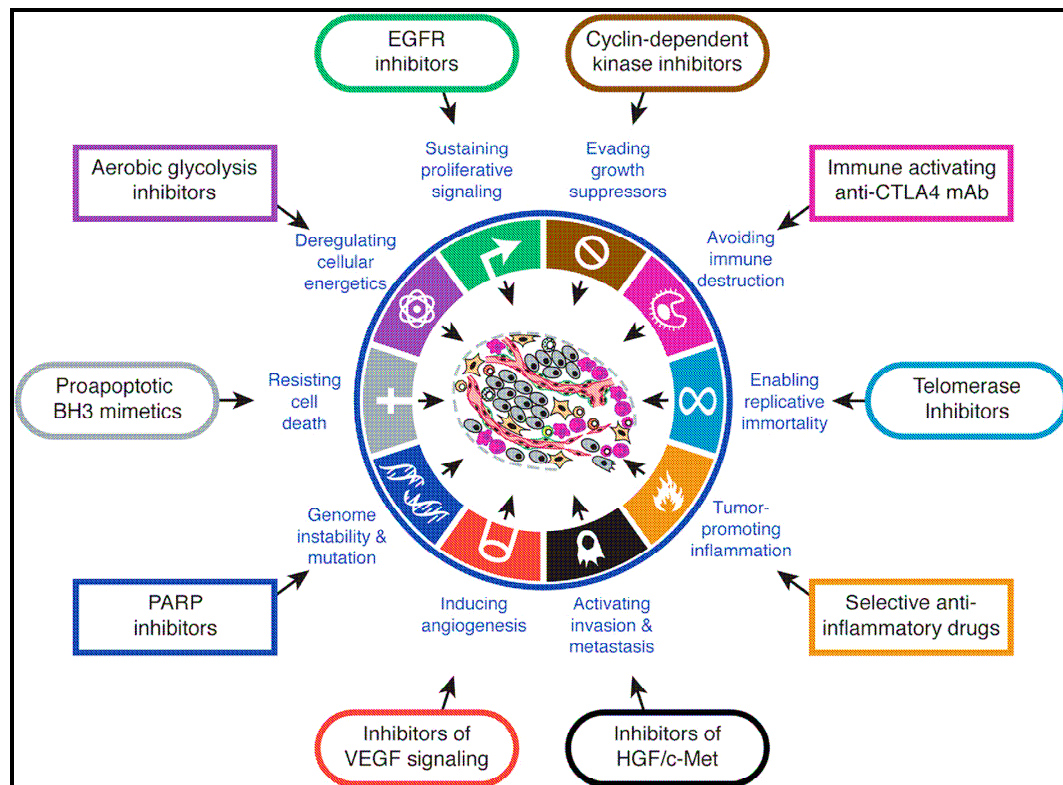


Figure 1: Cancer characteristics and therapeutic targeting options⁽⁷⁾

2.2. Cell cycle

Cellular growth is divided in five main phases, known as the phases of the cell cycle. In the G_0 -phase, the cells are resting, but have the ability to return in the active cycle by entering the G_1 -phase. In G_1 , the cells prepare themselves for the following S-phase (= synthesis), in which the DNA is replicated. Afterwards, in the G_2 -phase, the replication is controlled, and finally, the actual division is executed in the so-called M-phase (= mitosis).⁽⁵⁾ The whole cycle is controlled at several checkpoints, for example between the G_1 - and the S-phase, between the S- and the G_2 -phase and between the G_2 - and the M-phase.⁽⁸⁾ When DNA-damages are detected at one of these checkpoints, the cell cycle is stopped and reparation processes are activated. If the reparation attempts are not successful, the programmed cell death, apoptosis, is initiated.⁽⁵⁾ The checkpoints are controlled by extracellular growth signals, but also by signals from oncogene products, which stimulate the cell cycle progression. On the contrary, tumor-suppressing agents (such as p53) lead to an arrest of the cell cycle and to programmed cell death.⁽⁵⁾

When a dysregulation in this cycle occurs, for example due to a mutation in the p53 tumor suppressor gene, the formation of a tumor can be the result.⁽⁵⁾

2.3. Cell death

Generally, two different types of cell death are known – necrosis (or necrotic cell death) and apoptosis (also called programmed cell death).

Necrosis is characterized by a massive increase of the cell volume (oncosis), which is leading to a rupture of the plasma membrane, followed by a loss of intracellular contents.⁽⁹⁾ Due to the appearance of intracellular contents in the intercellular space, an inflammation in the surrounding tissue is initiated. Although necrosis was considered for a long time as “accidental” or “uncontrolled”, newer investigations showed a regulated progression of the necrotic cell death by various factors such as PARP-1.⁽¹⁰⁾

On the other hand, apoptosis describes a specific morphological aspect of cell death, where the cell volume is reduced (pyknosis) and chromatin condensation, nuclear fragmentation and membrane blebbing take place. Finally, the remaining fragments are removed by phagocytosis, so that no inflammatory process occurs.^{(5),(9)}

Apoptosis is induced either by an intrinsic or by an extrinsic pathway.⁽⁹⁾ Extrinsically, so called “death receptors”, like FAS (also known as CD 95) or the TNF-receptor are activated by their ligands (FASL, TNF), so that FADD (FAS associated death domain protein) can bind and activate procaspase 8.⁽⁵⁾

In the intrinsic way, p53 tumor suppressor protein is expressed after DNA damage, which stimulates the formation of bax. Bax leads to the liberation of cytochrome C from mitochondria, which activates the procaspase 9 together with APAF-1 (apoptosis activating factor) to its active form, caspase 9.⁽⁵⁾

Caspases are cysteine-proteases, which are activated in cascades and lead to the degradation of apoptosis inhibitors (positive feedback mechanism). Additionally, they catalyse the degradation of cytoskeleton proteins like actin or membrane proteins like lamin A. Furthermore, caspases cause the activation of various enzymes of the cell signalling system, whereof the CAD-nuclease (caspase activated DNase) is the most important one, because it causes the typical morphological signs of apoptosis.⁽⁵⁾

2.4. Leukaemia

Leukaemia is a summarizing term for various cancers of the blood forming system, which all show an abnormal increase of white blood cells due to an abnormal multiplying of their precursor cells. It can be caused by various triggers such as radiation, genetic and congenital factors, virus infections, chemicals and drugs. According to the type of affected cells, leukaemia can be classified as lymphocytic (= lymphoblastic) or myeloid (= myelogenous).⁽¹¹⁾ Furthermore, acute forms of leukaemia and chronic forms can be distinguished. Acute leukaemia forms are abruptly beginning, rapidly progressing diseases and known as highly malign types of cancer, leading to death in a very short time when not treated immediately.⁽⁵⁾ In this forms, immature (so-called “blastic”) cells are predominant. The onset of the disease is characterized by fever, bone pain and influenza-like symptoms. Besides, headache, loss of energy and appetite, anaemia, painful hepato- and splenomegaly and a depressed immune status are reported.⁽¹¹⁾

On the contrary, the chronic forms of leukaemia are characterized as slowly progressing diseases with a higher percentage of mature cells, which are however not functional. They develop slowly, in up to 25 % of the cases with no symptoms, so that they might be undetected for a long time. In the other cases, fever, loss of appetite and energy, sweating, enlarged lymph nodes anaemia and infections appear.⁽¹¹⁾

Leukaemia actually affects far more adults (90%) than children, although it accounts for nearly 30 % of all children’s cancerous diseases. About 60 % of the adult cases are acute forms of leukaemia, while even 95 % of childhood leukaemia cases are acute.⁽¹¹⁾

The survival rates of leukaemia have improved significantly in the past decades due to the development of newer therapy strategies including multiple drug therapies and bone marrow transplantation, but it is still a highly malign form of cancer, which is causing many cases of death. In the year 2000, about 256.000 new cases of leukaemia were reported worldwide by Globocan 2000, and approximately 209.000 patients died from it. The relative five year survival rate in the developing countries of the world is estimated – depending on the region – somewhere between 3,2 % (countrified China) and 26,4 % (India).^{(11),(12)}

2.5. HL-60 cells

The HL-60 cell line was derived by S. J. Collins et al., established in the year 1977 by leukopheresis from the peripheral blood leucocytes of S. G., a 36-year-old Caucasian female, who suffered from acute promyelocytic leukaemia and resulted in the development of a growth-factor-independent immortal cell line, which still provides a continuous source of human cells up today.^{(13),(14)}

As a characteristic of transformed cells, they are tumorigenic, which means that they produce subcutaneous myeloid tumors after injection in nude mice. The phenotype is known as stable through at least 85 passages and no DNA herpes virus or RNA retrovirus was isolated in the cells.⁽¹³⁾ They are graded as biosafety level 1⁽¹⁵⁾, meaning that they are unlikely to cause diseases in humans and during the work with this material, only general hygienic and appropriate safety procedures must be used.⁽¹⁶⁾

The cells grow in single-cell suspension without any tendency to clump or to adhere to plastic or glass.⁽¹³⁾ The predominant cell type (characterized by light and electron microscopy) is a neutrophilic abnormal promyelocyte with prominent nuclear/cytoplasmic asynchrony. Up to 10 % of the cultured cells differentiate spontaneously; the differentiation can also be stimulated by various substances.^{(13),(14)}

HL-60 cells are generally round or ovoid with a modal cell diameter of 13 µm (range from 9 µm to 25 µm). Larger cells are frequently binucleate. Typically, they contain large rounded nuclei with regular, distinct margins and 2 to 4 nucleoli. The cytoplasm is deeply basophilic with azurophilic granules.⁽¹³⁾

The cells express surface receptors for Fc fragments and for the complement system and show phagocytic activity and responsiveness to chemotactic stimuli. They also produce tumor necrosis factor (TNF) after stimulation with phorbol myristic acid.^{(13),(14)}

Cytogenetic analyses revealed aneuploidy and other karyotypic abnormalities, such as monosomy, trisomy, tetrasomy, and various chromosomal translocations.⁽¹⁴⁾ The most consistent karyotypic abnormalities were the deletion of the chromosomes 5, 8 or X. In early culture passages cells with 44 chromosomes predominated; however, clones with 45 or 46 chromosomes dominate after further passaging.^{(13),(14)}

The HL-60 cell genome contains a 4-fold to 30-fold amplified c-myc proto-oncogene. Additionally, it was found that the p53 gene has been deleted and one allele of the GM-CSF gene is rearranged and partly deleted.⁽¹⁴⁾

All these properties have made this special cell line an important model for studying the molecular events of (myeloid) cell differentiation and the effects of various elements such as pharmacological substances on the cellular growth process.⁽¹⁴⁾

2.6. Plant products as source for anti-cancer agents

Plants and other natural products have played a significant role in human healthcare for thousands of years - especially in the treatment of infectious diseases or cancer. Astonishingly, only 1 % of the approximately 300.000 higher plant species show a tradition of food use, whereas about 10 to 15 % are extensively reported for application in medicinal belongings. Most of the potential application possibilities were found by trial and error, based on practices in humans.⁽¹⁷⁾

A review by the FDA even showed that 62 % of the new and approved anti-cancer drugs are either natural products or derivates thereof, contain pharmacophores derived from natural sources or are (modified) natural products attached to targeting systems.^{(3),(4)}

The traditional use of plants and plant-products has been a major source for the discovery of novel anti-cancer agents, but still today, these products are important sources for active antineoplastic compounds, presumably due to their highly complex molecular structures containing several chiral centres compared to small molecule drugs, which might be the reason for their specific biological activities.^{(3),(4)}

The beginning of the scientific search for anti-cancer agents in plants started in the 1950s, when the alkaloids vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae), were discovered by chance, when during investigations on the hypoglycaemic effect of the plant extract a reduction in the white blood cell count was observed.^{(18),(19)} These substances, and their more recent semi-synthetic derivatives vindesine and vinorelbine, are still used (in combination with other

anti-cancer agents) for the treatment of different forms of cancer like leukaemia, lymphomas, breast and lung cancers or Kaposi's sarcoma.⁽¹⁸⁾

By and by, more plant-derived anti-cancer agents were searched and discovered, for example, derivatives of epipodophyllotoxine from *Podophyllum* sp. (Podophyllaceae), which had a long history of medicinal use in India and was already known for over 70 years as the main component of the extract, when in the 1950s the correct structure was firstly reported.⁽¹⁸⁾ Extensive research in this field led to etoposide and teniposide, semi-synthetic derivatives of epipodophyllotoxine, which are even more potent and show less adverse effects than epipodophyllotoxine itself. Etoposide and teniposide are used today in the treatment of lymphomas, bronchial and testicular cancers.^{(18),(19)}

Thereupon, the United States National Cancer Institute (NCI) started a plant collection and evaluation program in 1960, leading to numerous new chemotypes showing cytotoxic activities, such as the taxanes paclitaxel, which was isolated firstly from the bark of *Taxus brevifolia* Nutt. (Taxaceae), used in the traditional medicine of Native American tribes and the Ayurvedic medicine system, and its analogue docetaxel, which can be produced semi-synthetically via conversion of key precursors which are found in the leaves of different *Taxus species*, the so called baccatins.^{(18),(19)} These baccatins are a major and renewable source for the production of this important class of drugs. Paclitaxel is used for the treatment of ovarian, breast and non-small cell lung cancer, but also Kaposi's sarcoma, rheumatoid arthritis, psoriasis and multiple sclerosis, while docetaxel is mainly used for the treatment of breast and lung cancer. Additionally, there are several derivated taxane-analogues in preclinical development.⁽¹⁸⁾

Another very important class of anti-cancer drugs was derived from camptothecin, a component of *Camptotheca acuminata* Decne (Nyssaceae), which was tested by the NCI in the 1970s as anti-cancer agent, but dropped due to severe bladder toxicity. After years of intensive research, the more effective and less toxic derivatives topotecan, which is used for the treatment of ovarian and lung cancers, and irinotecan, used in colorectal cancers, were developed.⁽¹⁸⁾

Other agents in clinical use are harringtonine and homoharringtonine, isolated from *Cephalotaxus harringtonia* var. *drupacea* Sieb and Zucc. (Cephalotaxaceae), which are applied successfully as a racemic mixture for the treatment of acute and chronic myelogenous leukaemia in China. The mixture is even efficient in patients with forms of leukaemia, which are resistant to the standard treatment.⁽¹⁸⁾

The search for novel bioactive compounds from plants is still continued by collaboration among scientists in the whole world.^{(18),(19)}

2.7. Cameroonian flora and its use

One of the richest plant diversity of Tropical Africa is found in Cameroon (South West and North West provinces)⁽²⁰⁾, so that Cameroon is also known as a “refugium” for various threatened species or a “biodiversity hotspot” with a high rate of endemism.⁽²¹⁾ This high rate of biodiversity (rank 5th in Africa after the Republic of Congo, South Africa, Madagascar and Tanzania)⁽²²⁾ with about 8620 plant species (thereof 155 classified as threatened)⁽²³⁾ is caused by Cameroon’s numerous eco-systems including the Sahelian, mountains, humid tropical forest and coastal regions.⁽²²⁾

Although many plants are used in the Cameroonian folk medicine or as traditional spices, only 289 species belonging to 89 families are documented in their traditional use and even less have been investigated scientifically and studied on their molecular interaction mechanisms.⁽²³⁾ The main problem is that the knowledge of the preparation and their traditional use is only passed orally from generation to generation, risking the loss of this precious information, so that a scientific look into this subject and a documentation thereof is needed urgently.⁽²⁴⁾

So far, investigations already showed powerful antimalarial^{(25),(26),(27)}, antimicrobial^{(28),(29),(30),(31),(32)}, antioxidative⁽³³⁾, anxiolytic⁽³⁴⁾, sedative and anticonvulsant⁽³⁵⁾ properties of various Cameroonian plants.

Several of these plant species are also used to treat cancer in the traditional medicine, but are not listed in the Cameroonian pharmacopoeia. In order to obtain effective therapy strategies for cancer, new drugs, particularly gained from these plants used in the traditional medicine, should be investigated.⁽²⁾

The aim of this work was – as already mentioned – to explore the anti-cancer effects of purified dry extracts from five different Cameroonian plants. In the following sections, the habiti and the traditional uses of these plants are presented:

2.7.1. *Dichrostachys glomerata*



Figure 2: Morphology of *Dichrostachys glomerata*⁽³⁶⁾

Dichrostachys glomerata Chiov. (Fabaceae-Mimosoideae), synonym of *Mimosa glomerata* Forssk. or *Dichrostachys cinera* subsp. *cinera* Wight & Arn.⁽³⁷⁾, is a shrub or small tree (1 to 12 m height) with a rough bark⁽³⁸⁾; very common in tropical Africa, in parts of South Africa but also in Arabia, tropical Asia and Australia.⁽³⁹⁾ It is usually forming thickets on disturbed scrublands in the savannah.⁽⁴⁰⁾

It is armed with spines. The bole is short, sometimes twisted and mostly irregular. The leaves have 4 to 41 pairs of pinnae and show a length of up to 20 cm; the leaflets are linear to oblong. The inflorescences are yellow in the upper bisexual part; mauve, pink or white in the lower neuter part.^{(38),(41)}

The fruits of *Dichrostachys glomerata* are used as a traditional spice in the west region of Cameroon, but several studies also showed powerful medicinal properties of this fruits. Various fruit extracts turned out to be powerful antioxidants⁽⁴²⁾, show in vitro antibacterial activities (even in multi-drug resis-

tant strains)⁽⁴³⁾ and reduce cardiovascular risk factors in vivo, such as blood cholesterol, triglycerides, glucose and HbA_{1c}, blood pressure or body fat.⁽⁴⁴⁾ In addition, a macerate of *Dichrostachys glomerata* bark can be used for furuncle or blennorrhoea treatment and as preservative in the food industry.⁽⁴⁵⁾

A methanolic fruit extract also showed in vitro a moderate inhibitory effect on different cancer cell-lines (MiaPaCa-2, CEM/ADR5000 and CCRF-CEM).⁽⁴⁶⁾ In the folk medicine, parts of the whole plant are used as antidotes (treating stings, bites, etc.), painkillers, for the cure of arthritis or rheumatism, swellings, oedema and venereal diseases.⁽⁴¹⁾ Additionally, the stem bark is used for the preparation of adstringents, emetics, laxatives, abortifacients or repellents and for the treatment of diarrhoea and other stomach troubles. The roots are used as diuretics or laxatives, for pulmonary troubles, insanity and leprosy. Last but not least, the leaves are used as molluscicides or for the treatment of naso-pharyngeal affections.⁽⁴¹⁾

2.7.2. *Lippia rugosa*



Figure 3: Morphology of *Lippia multiflora*⁽⁴⁷⁾

Lippia rugosa A. Chev. (Verbenaceae) is found from Guinea to West Cameroon. Traditionally, it is used on the Vogel Peak Massif for anchoring the

roof of round-huts. Its habitus is similar to *Lippia mulitflora* Moldenke (see Figure 3), which is a woody pubescent herby shrub, up to 3 m high. The leaves are bluish-green, the fragrant flowers are white or lilac. Typically, it is growing on waste ground or in the savannah.⁽⁴⁸⁾

The leaves are used in the form of a decoction in the traditional Cameroonian medicine to treat typhoid fever and rheumatism. In vitro studies also showed that preparations of *Lippia rugosa* might be potentially used as anti-fungal agent against *Aspergillus* species, which are known as dangerous food poisons due to Aflatoxin production.^{(49),(50)}

2.7.3. *Pittosporum mannii*



Figure 4: Morphology of *Pittosporum mannii*⁽⁵¹⁾

Pittosporum mannii Hook f. (Pittosporaceae), synonym of *P. viridiflorum* Sims, also known as Bourangal in Bororo language, is widely spread in the savannah zone of Adamawa, in the highlands of West Cameroon and mount Mandara.⁽⁵²⁾

It is a very polymorphic shrub or small tree up to 12 m height with rough grey bark. The leaves are either obovate or broadly oblanceolate and up to 17,5 cm long. The leaf colour is deep green and sometimes glossy on the above side; paler and often yellowish-green beneath. The inflorescences are

mostly many-flowered terminal racemose panicles with strongly sweet-scented, white, cream, yellowish or greenish flowers.^{(53),(54)}

Pittosporum mannii is restricted to Fernando Po, Cameroon's mountains, the Bamenda Highlands and the Bambutos. It grows at and above 2000 m.⁽⁵⁴⁾ Infusions of the stem bark are used to treat intestinal and infectious diseases such as fever, malaria, liver disorders or stomach ache; additionally it is used as antidote for snakebites or insect bites. The dried internal part of the bark is also used to cure worm infections in infants.⁽⁵²⁾ For chest pain, cough or amenorrhoea, infusions of equal parts of *Pittosporum mannii* and *Bridelia micrantha* Baill. (Euphorbiaceae) adding honey are taken orally.⁽⁵⁵⁾ Additionally, it can be used as repellent against *Anopheles arabiensis*.⁽⁵⁶⁾

In vitro tests also showed significant antioxidant properties, which might explain (partly) the use of *Pittosporum mannii* for the treatment of infections and inflammations in the traditional Cameroonian medicine.⁽⁵²⁾ In studies, methanol and water extracts were toxic against Vero E6 cells⁽⁵⁷⁾ and A2780 human ovarian cancer cells⁽⁵⁸⁾. This fact might refer to pittoviridoside, a cytotoxic triterpenoid saponin, which was found in *Pittosporum mannii*.⁽⁵⁸⁾

According to personal information from Dr. Momeni, University of Ngaoundere, some kind of toxicity was also observed in humans: Test persons, which were consuming larger amounts or concentrated infusions of the stem bark vomited after the intake.

2.7.4. *Plectranthus glandulosus*



Figure 5: Morphology of *Plectranthus* sp.⁽⁵⁹⁾

Plectranthus glandulosus Hook f. (Lamiaceae) is a coarse, scrambling to erect, glandular, strongly aromatic herb and up to 3 m high⁽⁶⁰⁾. The leaves are long petioled or cordate-ovate and glabrous, up to 12 cm long and nearly as long as broad. The copious violet inflorescences are ample, decompound, ordered in terminal panicles with slender, glandular-pubescent branchlets.⁽⁶¹⁾

It is found in openings in the montane forest and amongst scrubs⁽⁶⁰⁾ at about 2100 m above sea level in the Cameroonian Mountains⁽⁶¹⁾, from Mali to Fernando Po.⁽⁶²⁾

It is cultivated and used as a traditional aromatic spice in the Adamawa region of Cameroon, e.g. for sauces.⁽⁶²⁾ In addition, macerations of the leaves, taken orally, are used to treat malaria, as mosquito repellents and for the cure of internal or lower abdominal inflammation.⁽⁵⁵⁾ *Plectranthus glandulosus* is also known as powerful antifungal⁽⁵⁰⁾ and antibacterial⁽⁶³⁾ agent in different in-vitro assays.

2.7.5. *Zornia glochidiata*



Figure 6: Morphology of *Zornia glochidiata*⁽⁶⁴⁾

Zornia glochidiata Reichb. ex. DC. (Fabaceae-Papilionoideae), also known as *Zornia diphylla* Braun & Massey, is an annual (rarely perennial) herb, which is widespread starting from Senegal and Eritrea, throughout tropical

Africa, reaching Cape Province, south-west Africa and also Madagascar.^{(65),(66),(67)}

The stems are erect or decumbent, 10 to 70 cm tall, sometimes slightly woody at the base and usually not surviving the dry season. Its upper leaflets are lanceolate to ovate-lanceolate and rounded at the base, while the lower leaflets are broader. The Flowers are very inconspicuous, usually yellow, but also white or orange, with or without reddish-purple veins.^{(65),(66)}

It grows in an altitude range from 0 to 1800 m⁽⁶⁵⁾ on grassland, roadsides, in open woodland, waste places or riverbanks; mostly on sandy soil, in rocky places or on sand dunes, but also in semi-evergreen forest.⁽⁶⁶⁾

Decoctions of the roots are used to treat paralysis, epilepsy, convulsions and spasms, yellow fever, malaria and as a repellent.⁽⁶⁸⁾ Various compositions containing *Zornia glochidiata* are also used for the treatment of common cold⁽⁶⁹⁾, oedema⁽⁷⁰⁾, bronchitis, complicated with cor pulmonale⁽⁷¹⁾, or even gastric cancer⁽⁷²⁾.

Its use for the treatment of gastrointestinal disorders is supported by a study which showed spasmolytic and antimicrobial activities of *Zornia glochidiata*.⁽⁷³⁾ A n-hexane extract also showed a promising anticancer activity by inducing apoptosis in-vitro (DLA-cells) and in-vivo (mice), whereas no acute toxicity could be detected, which also explains the traditional use for the treatment of gastric cancer.⁽⁷⁴⁾

3. METHODS

If not mentioned otherwise, all chemicals were purchased from Sigma (Munich, Germany).

3.1. Extraction methods used to gain the tested extracts

All plants were extracted, purified and evaporated by the team of Dr. Jean Momeni, department of chemistry, university of Ngaoundere in Cameroon. The gained purified dry extracts were sent to Austria by mail.

Table 1: Information on the used plant sources and the purification methods

Plant species	Used parts and quantity	Collection area and date	Purification method(s)
<i>Dichrostachys glomerata</i>	fruits (800 g)	local market of Ngaoundere (December 2010)	CC
<i>Lippia rugosa</i>	leaves	University Campus of Ngaoundere (August 2010)	CC & TLC
<i>Pittosporum mannii</i>	stem bark (1500 g)	Babouantou, upper Nkam division of western Cameroon (August 2009)	CC & TLC
<i>Plectranthus glandulosus</i>	leaves (1300 g)	“Champs de Prière” in Ngaoundere (Sept. 2010)	CC
<i>Zornia glochidiata</i>	whole plants	Dang, near the Campus of the University of Ngaoundere (July 2009)	CC

In order to get the extracts, all extraction steps were performed three times and followed by concentration. The selected parts of the plants – as listed in Table 1 – were powdered and then firstly extracted with hexane, the remaining residual powder was extracted with ethyl acetate and finally, the newly gained residual powder was extracted with ethyl alcohol.

For the experiments, the ethyl alcohol extracts of *Dichrostachys glomerata*, *Lippia rugosa*, *Pittosporum mannii* and *Zornia glochidiata* were purified and evaporated; the tested dry extract of *Plectranthus glandulosus* was gained

by mixing the hexane extract with the ethyl acetate extract, followed by purification and evaporation.

The purification was performed either by using column chromatography (CC) on silica gel, followed by preparative thin layer chromatography (TLC) using hexane/ethylacetate/methanol in increasing polarity as eluent or by using column chromatography on silica gel with hexane/ethylacetate in increasing polarity as eluent only (see Table 1).

3.2. Cell culture – general methodology

HL-60 promyelocytic leukaemia cells (purchased from ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium supplemented with 1 % L-glutamine, 1 % penicillin-streptomycin-mixture and 10 % heat inactivated foetal calf serum (FCS)⁽⁷⁵⁾. The culture conditions were set to 37 °C in a humidified atmosphere (95 % humidity) containing 5 % CO₂, as recommended by ATCC⁽¹⁵⁾. All media and supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

In order to keep the cells in an exponential growth phase over the whole research period, cells were always seeded at a concentration of approximately $1,0 \cdot 10^5$ cells/ml into T-25 Nunc tissue culture flasks or T-75 Nunc tissue culture flasks and – according to their doubling time of about 16 hours – subcultured every other day or every third day, that means every time when a number of $1,0$ to $2,0 \cdot 10^6$ cells/ml was reached, which could be detected roughly due to the change of the media colour from purple-red to yellow. Exact concentrations were determined every time using a CASY[®] TTC cell counter (Roche, Basel, Switzerland).

The CASY[®]-Technology of Schärfe System is a German development which combines the Resistance Measurement Principle (a particle measurement technique), with Pulse area analysis, a modern method of signal evaluation.

The measurement is performed by suspending the cells in CASY[®] ton buffer and aspirating them at a constant flow speed through a precision measuring pore of defined geometry. It is important that the cells are passing through the measuring pore one after another.⁽⁷⁶⁾

During the measurement process, via two platinum electrodes a pulsed low voltage field (1 MHz) is applied to the measuring pore. The electrolyte-filled

pore represents a defined electrical resistance, so that the level of resistance increases when a cell displaces a quantity of electrolyte corresponding to its volume, and this increase can be measured. Due to the high frequency signal scanning, a large measuring range from very small debris particles to very large cell aggregates is provided at maximum resolution and reproducibility.⁽⁷⁶⁾

CASY[®] does not only count the cells but also measures their size (volume), which makes viability checks possible.⁽⁷⁶⁾

For the experiments, the critical particle size was set to 9,6 μm , so that only cells larger than this size were counted as viable cells and smaller particles or cell fragments did not falsify the results.

3.3. Proliferation inhibition assay

2,0 ml of HL-60-cell-suspension at a concentration of approximately $1,0 \cdot 10^5$ cells/ml were given into Nunc 6-well plates. In order to get this concentration, HL-60-cells were seeded in T-25 Nunc tissue culture flasks, incubated and counted using the CASY[®] TTC cell counter. Finally, the stock-suspension was diluted with fresh and warmed-up media to the target concentration.

5,0 mg of each purified dry extract were resolved in 100,0 μl dimethylsulfoxide (DMSO) by vortexing in order to get a stock-solution, which was again diluted 1:10 and 1:100 to gain lower concentrated stocks. This was performed in order to achieve the same DMSO concentration in every sample-well (0,2 %), so that differences in the proliferation level could not be caused by the cytotoxic effect of DMSO, but only by the tested plant extract. The concentration of 0,2 % DMSO was aimed in order not to influence the cells in their natural growth rate, which could be observed at a concentration of about 0,5 % or higher. All solutions were frozen and stored at -20 $^{\circ}\text{C}$ until they were used.

Respectively 4,0 μl of the main stock solution, both diluted stock solutions and pure DMSO were added to three wells of cells each, obtaining the following final concentrations: 0 μg purified dry extract/ml cell suspension (control), 1 μg purified dry extract/ml cell suspension, 10 μg purified dry extract/ml cell suspension and 100 μg purified dry extract/ml cell suspension.

After 24 hours and 48 hours exposure of the cells to the tested dry extracts, the samples were resuspended using a 1000 µl pipette. Cell counts (using again the CASY[®] TTC cell counter) and – if possible – IC₅₀ values were determined.

The percentages of the HL-60 cell proliferation in comparison to the untreated control sample were calculated using the following formula according to Strasser, Maier et al.^{(77),(78)}

$$proliferation_{(in \% of control)} = \frac{sample_{48h} - sample_{24h}}{control_{48h} - control_{24h}} \cdot 100$$

Sample_{48h} is the number of viable cells after 48 hours exposure to the tested dry extract concentration, sample_{24h} the number of viable cells after 24 hours extract treatment and control_{48h}/control_{24h} are the numbers of viable cells after 48 hours/24 hours without extract treatment (DMSO only).

3.4. Apoptosis/necrosis assay

Cell death was analysed using a Hoechst 33258/propidium iodide (HOPI) double staining, according to the method published by Grusch et al.^{(79),(80)}

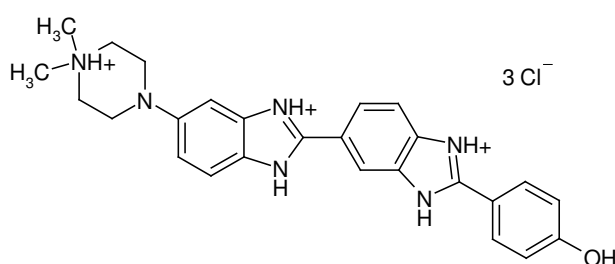


Figure 7: Hoechst 33258

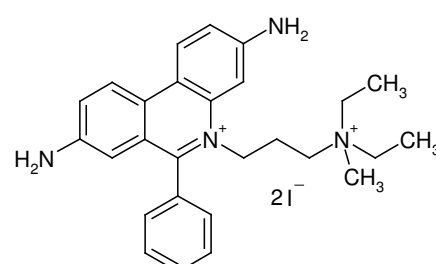


Figure 8: Propidium iodide

HL-60 cells were seeded in Nunc 6-well plates at a concentration of $1,0 \cdot 10^5$ cells/ml and exposed in triplicates to 0 µg/ml (control), 5 µg/ml, 10 µg/ml and 25 µg/ml purified *Pittosporum mannii* dry extract for 24 hours and 48 hours. Directly afterwards, the samples were resuspended, 100,0 µl of each sample were transferred in a Nunc 96-well plate and Hoechst 33258

and propidium iodide were added to the cells at a final concentration of 5 µg/ml and 2 µg/ml. Then, the samples were incubated at 37 °C for 60 minutes again, leaving enough time for the substances to stain and for the sedimentation of the cells, so that they remain in the same focus layer for the following examination with a Zeiss Axiovert 35 fluorescence microscope (Zeiss, Oberkochen, Germany), equipped with a DAPI filter. The cells were photographed with a Nikon D7000 DSLR camera (Nikon, Tokyo, Japan) and analysed by visual examination. Cells were judged by their morphology and the integrity of the cell membranes by the propidium iodide staining and counted manually.

Both stains are DNA intercalating fluorescent dyes and therefore known as mutagenic. Hoechst 33258, a bis-benzimide dye, shows an excitation wavelength of 352 nm and an emission of 461 nm, resulting in a cyan-blue staining of the DNA.⁽⁸¹⁾ It stains the nuclei of all cells (no matter whether the cell membrane is integer or not), allowing to monitor nuclear changes, such as chromatin condensation and nuclear fragmentation during apoptosis.

On the contrary, propidium iodide shows an emission maximum of 617 nm (bright red light), when it is excited with 535 nm.⁽⁸²⁾ However, when propidium iodide is excited with the wavelength of the DAPI filter (depending on the filter type between 340 nm and 380 nm, usually 365 nm)⁽⁸³⁾, a light pink emission can be observed. Propidium iodide is excluded from cells with an integer cell membrane (that means viable and early apoptotic cells); therefore, an uptake of propidium iodide in a cell indicates membrane destruction, which is characteristic for necrotic and late apoptotic cells.

So, both dyes combined allow due to their selective uptake to distinguish between viable cells (blue, see Figure 9), early apoptotic cells (bright blue “spots” due to chromatin condensation and nuclear fragmentation, see Figure 10), late apoptotic cells (bright pink “spots”, see Figure 11) and last but not least necrotic cells (huge and pink, see Figure 12).

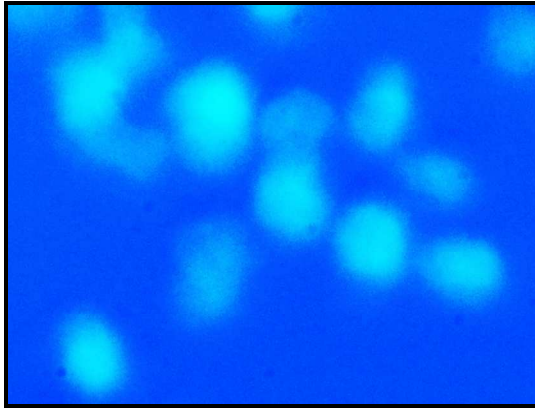


Figure 9: viable cells



Figure 10: early apoptotic cells

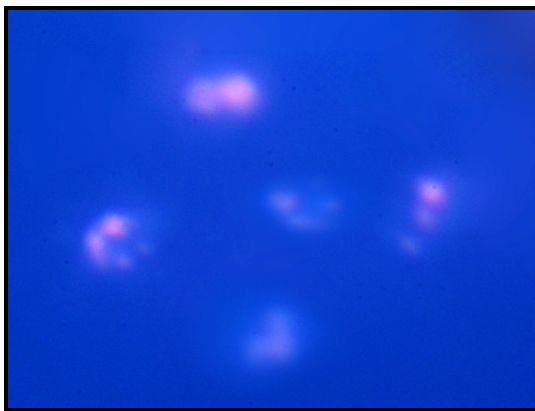


Figure 11: late apoptotic cells

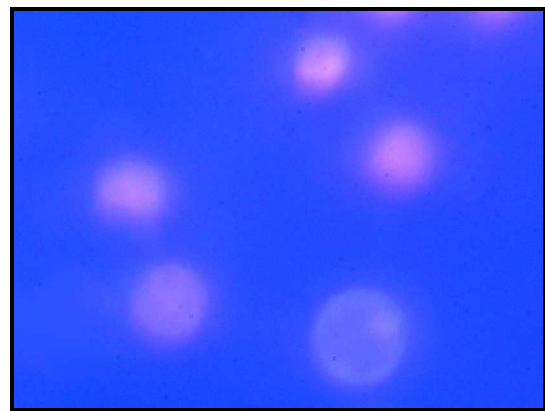


Figure 12: necrotic cells

3.5. Western Blotting

The tests were performed either as a dose response or as a time response analysis. For the dose response testing, HL-60 human promyelocytic leukaemia cells were seeded in five T-75 Nunc tissue culture flasks at a concentration of $1,0 \cdot 10^5$ cells/ml and treated with *Pittosporum mannii* dry extract in the following concentrations: 0 µg/ml (control), 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml for 8 hours. For the time response analysis, HL-60 cells were seeded in a T-225 Nunc tissue culture flask (at $1,0 \cdot 10^5$ cells/ml) and incubated with 25 µg/ml *Pittosporum mannii* dry extract. Samples were taken after 0 h, 2 h, 4 h, 8 h, 24 h and 48 h of incubation, respectively.

At every sampling time point, exactly $2,0 \cdot 10^6$ cells were harvested after being counted using the CASY TTC[®] cell counter, in order to monitor the cell growth process and to ensure that the exact amount of cells were taken from every sample for the further analysis. The samples were centrifuged

(1000 rpm, 5 min, 4 °C) and washed with cold PBS twice, then, the cells were lysed in a buffer containing 50 mM TRIS, 150 mM NaCl, 1 % Triton-X 100, 1 mM phenylmethylsulfonylfluoride (PMSF) and 2,5 % PIC. After the next centrifugation step (12000 rpm, 20 min, 4 °C) the supernatants were transferred in Eppendorf test tubes (Eppendorf AG, Hamburg, Germany) and stored at -20 °C until the further analysis was continued.

Equal amounts of the protein samples were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4 % stacking gel, a 10 % separating gel (discontinued electrophoresis) and a TRIS glycine electrophoresis buffer.

The samples as well as the PageRuler® Prestained Protein Ladder marker (Thermo Fisher Scientific Inc., Waltham, MA, USA) were loaded into the gel slots and separated using firstly 80 V, then 120 V. Afterwards, the samples were transferred onto Amersham Hybond-P PVDF membranes (GE Healthcare, Buckinghamshire, UK) using a TRIS glycine methanol transfer buffer at 4 °C and 100 V. Equal protein loading was controlled by staining with Ponceau S.

After washing with TRIS-buffered saline (TBS) pH 7,6, the membranes were blocked for approximately 1 hour in 5 % non-fat dry milk in TBS containing 0,1 % Tween-20 (blocking solution), so that unspecific binding of the antibody to free positions of the membrane could be prevented. Then, the membranes were incubated with the first antibody, diluted 1:500 – 1:1000 in blocking solution by gently rocking overnight at 4 °C and washed with TBS containing 0,1 % Tween-20 (TBS-Tween). Subsequently, the second antibody (peroxidase conjugated swine anti-rabbit IgG or rabbit anti-mouse IgG) was added in a dilution of 1:2000 – 1:5000 in blocking solution and incubated for 1 hour, followed again by washing with TBS-Tween.

The chemoluminescence was developed with the Amersham ECL-plus Western Blotting Detection System Kit (GE Healthcare) and detected after approximately 1 minute of incubation with a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland). After the detection, the membranes were washed and stored at 4 °C until further use with different antibodies.

The mouse monoclonal (ascites fluid) anti- β -actin clone AC-15 antibody, cat no. A5441, was purchased from Sigma and the mouse PARP-1 (F-2) monoclonal antibody, cat no. sc 8007, as well as the rabbit p21 (C-19) polyclonal affinity purified antibody, cat no. sc-397, were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). The rabbit phospho-p38 MAPK (Thr180/Tyr182) (12F8) monoclonal antibody, cat no. 4631, and the rabbit p38 MAPK polyclonal affinity purified antibody, cat no. 9212, were bought from Cell Signalling (Danvers, MA, USA). Both peroxidase conjugated antibodies (swine anti-rabbit and rabbit anti-mouse IgG) were from Dako (Glostrup, Denmark).

3.6. UV/vis-spectrum analysis

The analysis via UV/visible spectrophotometry was performed in order to identify relevant wavelengths (absorption maxima) for the detection of the extract components in the RP-HPLC (described in section 3.7.).

1,0 mg of the purified *Pittosporum mannii* dry extract was solved completely in 1,0 ml DMSO and filled into a quartz glass cuvette. Then, a wavelength scan from 190 nm to 1100 nm was performed with a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan).

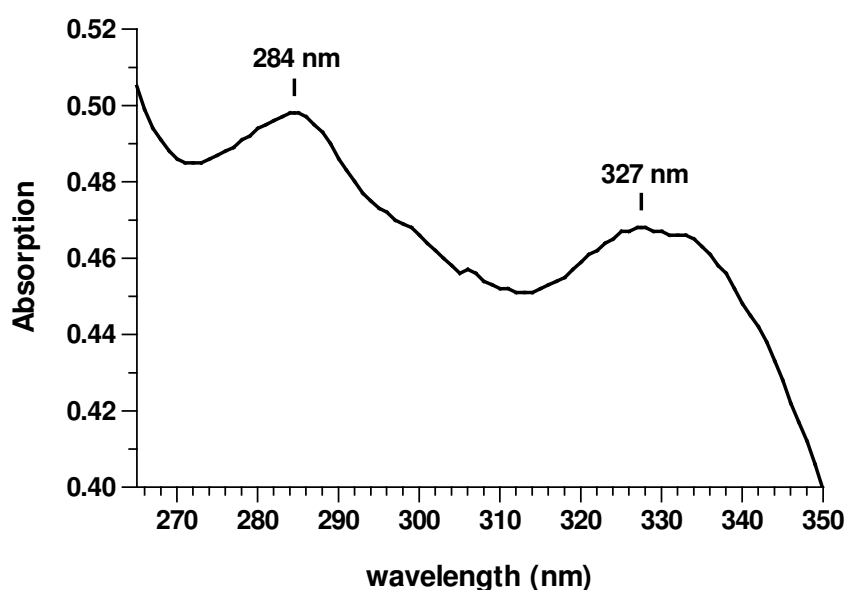


Figure 13: UV/Vis spectrum of purified *Pittosporum mannii* dry extract

Two relevant maxima were detected – one at 284 nm and one at 327 nm (as shown in Figure 13). Due to the fact that we do not know neither the qualitative nor the quantitative constitution of the extract, it was not possible to calculate the molar extinction coefficient.

3.7. Reversed phase HPLC

3.7.1. Sample preparation

For the sample containing purified *Pittosporum mannii* dry extract only, 1,1 mg dry extract were solved in 1,0 ml of a 1 + 9 mixture of DMSO and 10 mM ammonium acetate/acetic acid buffer pH 5,0 by vortexing.

In order to get the samples of *Pittosporum mannii* dry extract and its metabolites in the HL-60 culture media, cells were seeded in four T-75 Nunc tissue culture flasks at two different concentrations respectively: $1,0 \cdot 10^5$ cells/ml and $5,0 \cdot 10^5$ cells/ml. One sample of each concentration level was incubated with 20 µg *Pittosporum mannii* dry extract per ml, while the other one was only treated with DMSO (control).

Directly afterwards, 10 ml cell suspension were taken from every culture flask, transferred into 15 ml Falcon® conical tubes (BD Biosciences, Franklin Lakes, New Jersey, USA) and centrifuged at mild conditions (5 minutes, 900 rpm, 4 °C) in order to prevent the cells from being disrupted. The supernatant media were transferred in new Falcons and stored at -20 °C until further analysis.

The remaining culture flasks were incubated for 24 hours (conditions as mentioned above); then, the sampling procedure was repeated, so that we received four more samples: two new samples, representing the components of *Pittosporum mannii* dry extract in the medium after 24 hours of incubation at two different cell concentrations and a control sample of each concentration.

Shortly before the samples were about to be injected into the RP-HPLC system, they were defrosted and mixed with the same amount of ice cold ethanol in order to precipitate the bigger part of the proteins. Then, the samples were centrifuged (10 minutes, 12000 rpm), the supernatants were filled into

HPLC sample vials and placed in the HPLC auto sampling table. The injection volume was set to 40,0 µl per injection.

3.7.2. Conditions

The determination of the components of *Pittosporum mannii* dry extract was performed using a Merck Hitachi “LaChrom” HPLC system (Darmstadt, Germany), equipped with a L-7200 auto sampler injector placed on a Peltier sample cooling thermostat, a L-7100 pump module, a L-7300 column oven (set at 30 °C), a D-7000 interface and a L-7400 UV/vis-detector. The chromatographic separation was performed on a Hypersil BDS-C₁₈ column (5 µm, 250 x 4,6 mm), preceded by a Hypersil BDS-C₁₈ precolumn (5 µm, 10 x 4,6 mm), at a flow rate of 1,0 ml per minute.

The mobile phase was mixed from 10 mM ammonium acetate/acetic acid buffer pH 5.0 and methanol in form of a continuous gradient to elute the components according to their lipophilicity.

The gradient started from 5 % methanol and increased linearly to 90 % at 22,7 minutes (according to an increase of 3,75 % per minute), where it remained constant for five minutes. Then, the percentage of methanol was reduced to 5 % within 2 minutes and left at this level until minute 32 in order to equilibrate the column before the next sample was applied.

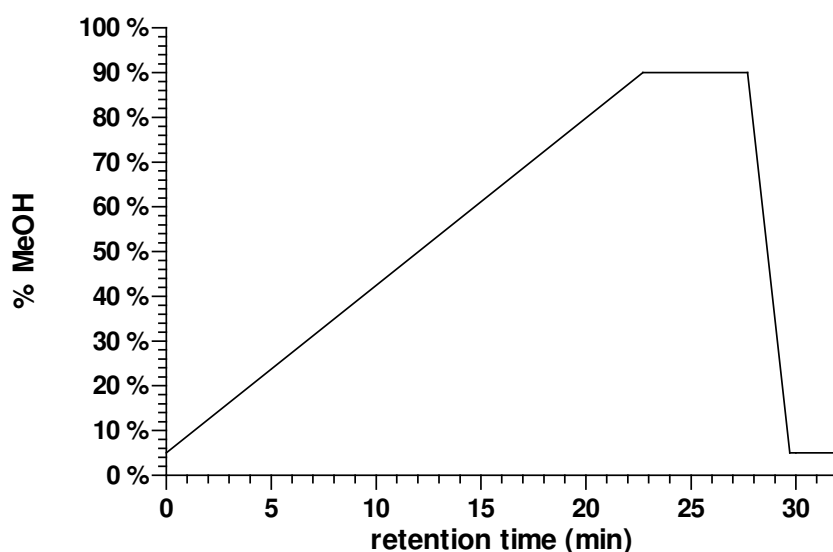


Figure 14: Used HPLC-gradient

Due to the fact that we could not determine any extinction coefficient, a quantification of the components could not be performed.

The UV-detection was performed firstly at both absorption maxima of *Pittonium sporum mannii* dry extract (284 nm and 327 nm), showing similar peaks but a better signal/noise-ratio of the baseline at 327 nm, so that the following analyses were performed at 327 nm only.

3.8. Statistical analyses and graphs

All proliferation and cell death experiments were performed in triplicates and analysed by t-test, using the Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). All calculations (mean, standard deviation) and all graphs were also created with this software. Asterisks indicate statistical significance at a 95 % confidence interval and the error bars represent $\pm$ standard deviation.

All chemical structures were drawn with MDL ISIS Draw 2.5 software (Symyx Technologies, Santa Clara, CA, USA).

4. RESULTS

4.1. Anti-proliferative activity

The purified dry extract of *Pittosporum mannii* showed a strong cell growth inhibition in our primary proliferation assay. In order to study its inhibitory properties more detailed, the proliferation inhibition assay was repeated using finer graduated concentrations of the dry extract at the same conditions as mentioned in section 3.3. The gained data was used to create cellular growth curves of the different samples (Figure 15).

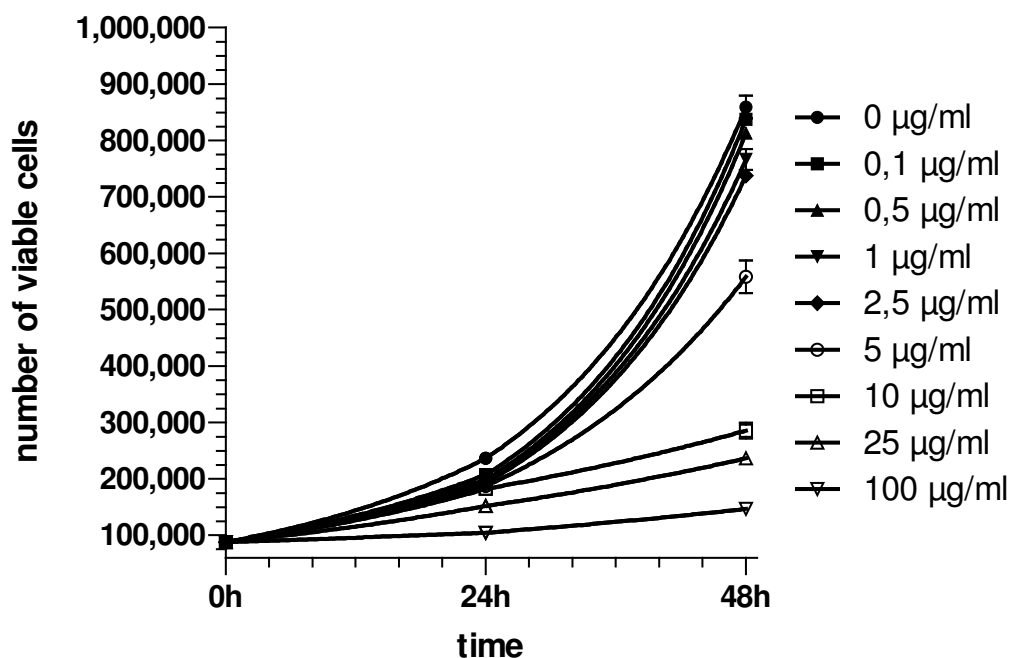


Figure 15: Cellular growth curves of HL-60 cells treated with different concentrations of purified *Pittosporum mannii* dry extract

In addition, the proliferation inhibition level was calculated with the formula mentioned in section 3.3. A dose dependent cell growth inhibition was observed (see Figure 16), ranging from a 98,6 % proliferation rate of the control samples, when treated with 0,5 µg/ml, to 59,5 % at 5 µg/ml and even up to only 6,8 % proliferation of the control samples in the highest concentration (100 µg/ml).

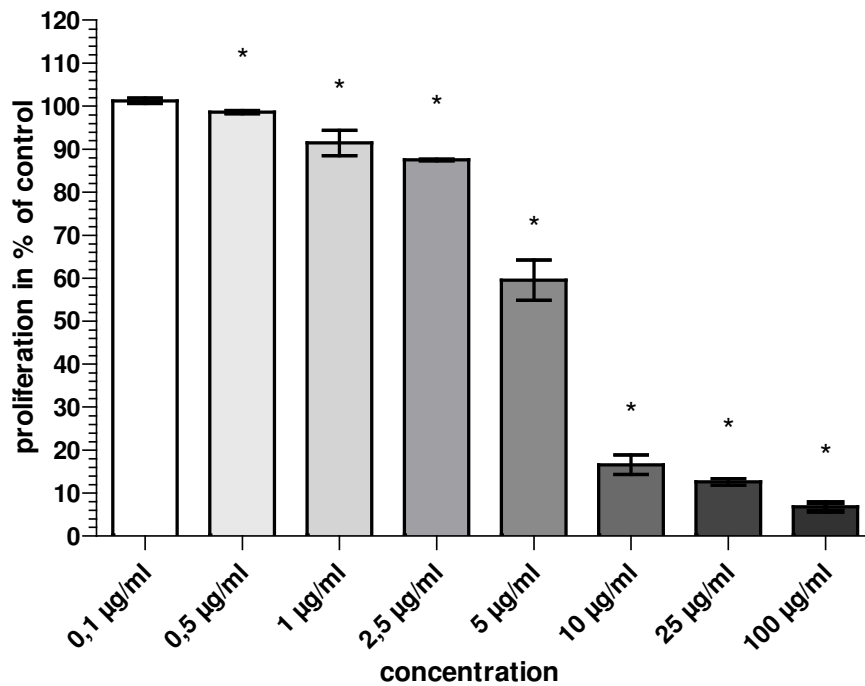


Figure 16: Proliferation inhibition of purified *Pittosporum mannii* dry extract in various concentrations

Using the gained proliferation inhibition data, it was possible to draw a dose-response curve and calculate the IC_{50} value. The IC_{50} of the purified *Pittosporum mannii* dry extract turned out to be $5,15 \mu\text{g/ml}$ ($\pm 0,18$), as shown in Figure 17.

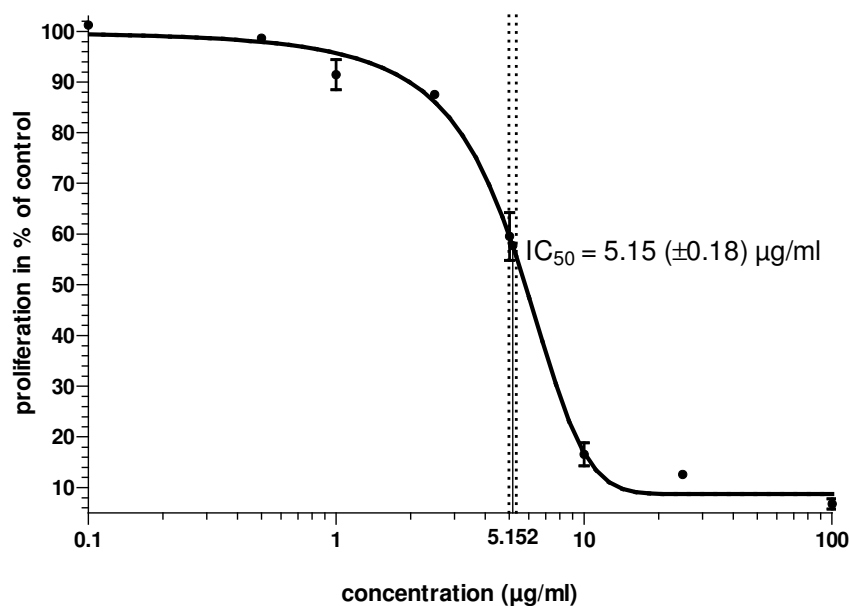


Figure 17: Dose response curve and IC_{50} of purified *Pittosporum mannii* dry extract

On the contrary, the purified dry extract gained from *Dichrostachys glomerata* did not show any HL-60 cell proliferation inhibition. In fact, it even raised the proliferation level of the cells slightly (about 105 % to 110 % of the control samples); however, this observed effect is not statistically significant.

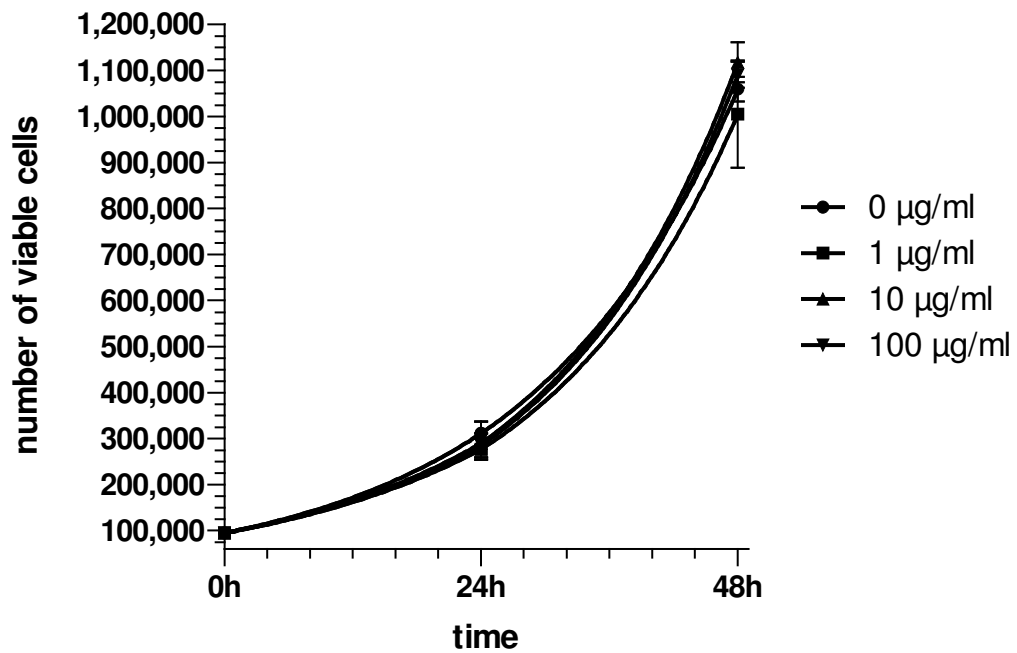


Figure 18: Cellular growth curves of HL-60 cells treated with different concentrations of purified *Dichrostachys glomerata* dry extract

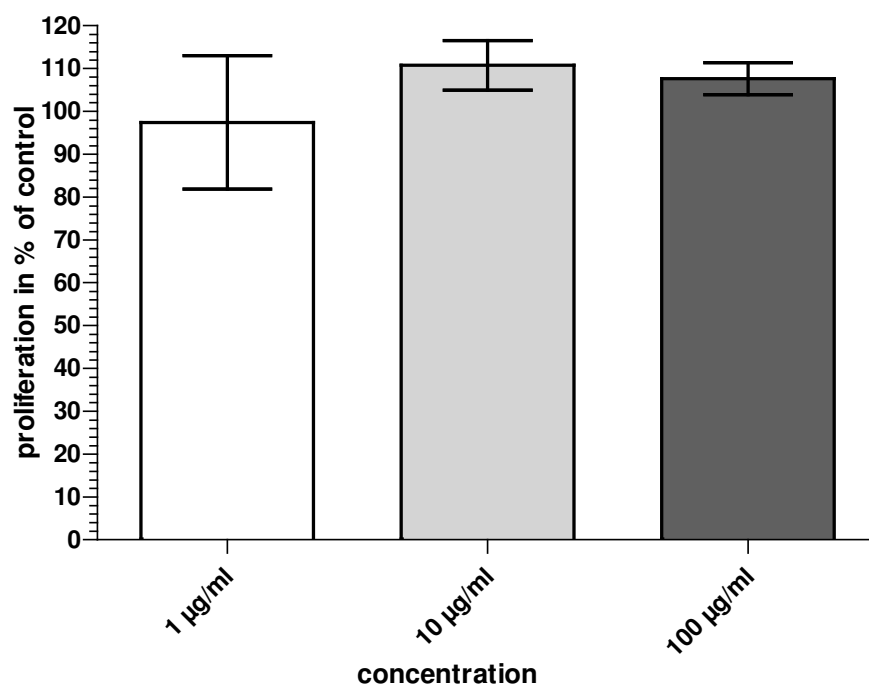


Figure 19: Proliferation inhibition of purified *Dichrostachys glomerata* dry extract

In the following figures, that show the cellular growth curves and the calculated proliferation inhibition levels of HL-60 cells treated with purified *Lippia rugosa* dry extract, it is visible that the extract did not inhibit the growth of the cells specifically.

Although there is a statistically significant reduction at 100 $\mu\text{g/ml}$ dry extract (86,8 % proliferation of the control), we assumed that the extract did not cause a specific cell death but only unspecific toxic effects due to the high extract concentration.

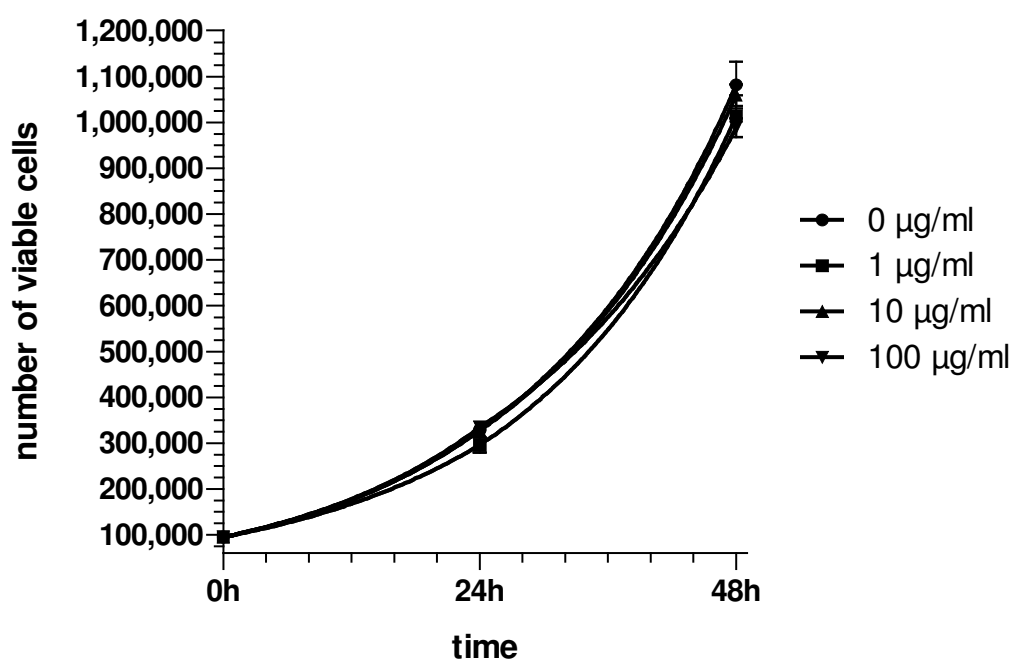


Figure 20: Cellular growth curves of HL-60 cells treated with different concentrations of purified *Lippia rugosa* dry extract

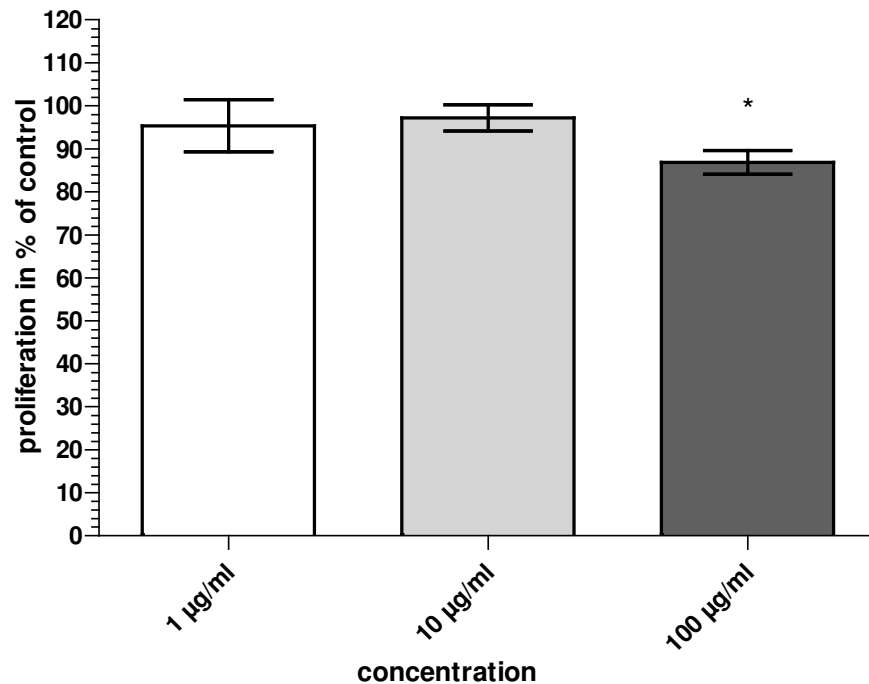


Figure 21: Proliferation inhibition of purified *Lippia rugosa* dry extract

The purified dry extract gained from *Plectranthus glandulosus* showed a minimal anti-proliferative effect; but only a very high concentration of the extract led to a statistically significant inhibition (77,8 % cellular growth of the control at 100 µg/ml; see Figure 23), which made us assume that further experiments with this extract are also gainless.

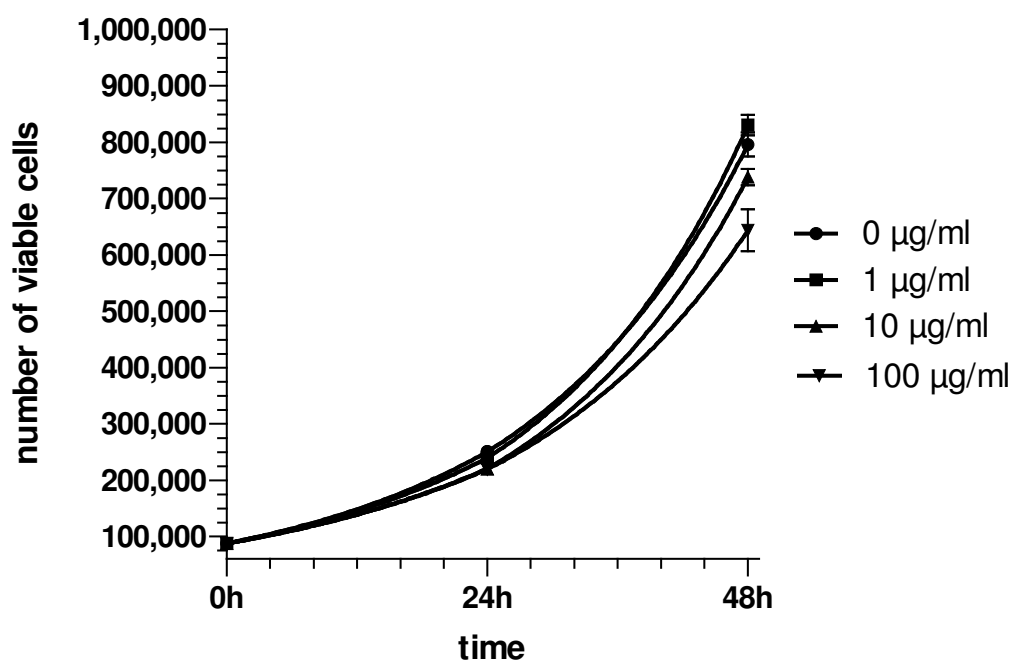


Figure 22: Cellular growth curves of HL-60 cells treated with different concentrations of purified *Plectranthus glandulosus* dry extract

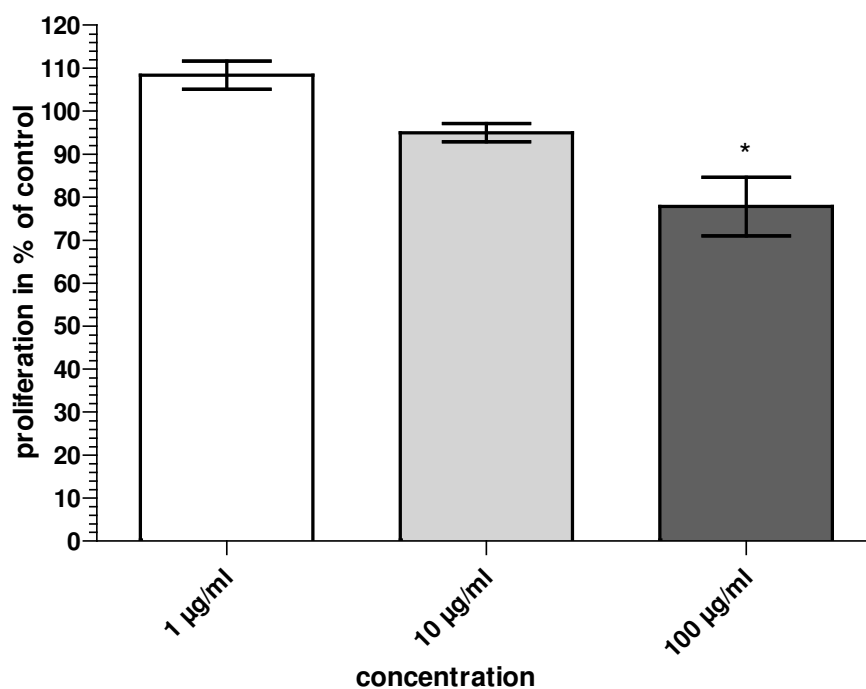


Figure 23: Proliferation inhibition of purified *Plectranthus glandulosus* dry extract

Furthermore, also the purified *Zornia glochidiata* dry extract did not show any statistically significant inhibition of the HL-60 cell proliferation, which is visible in Figure 24 and Figure 25.

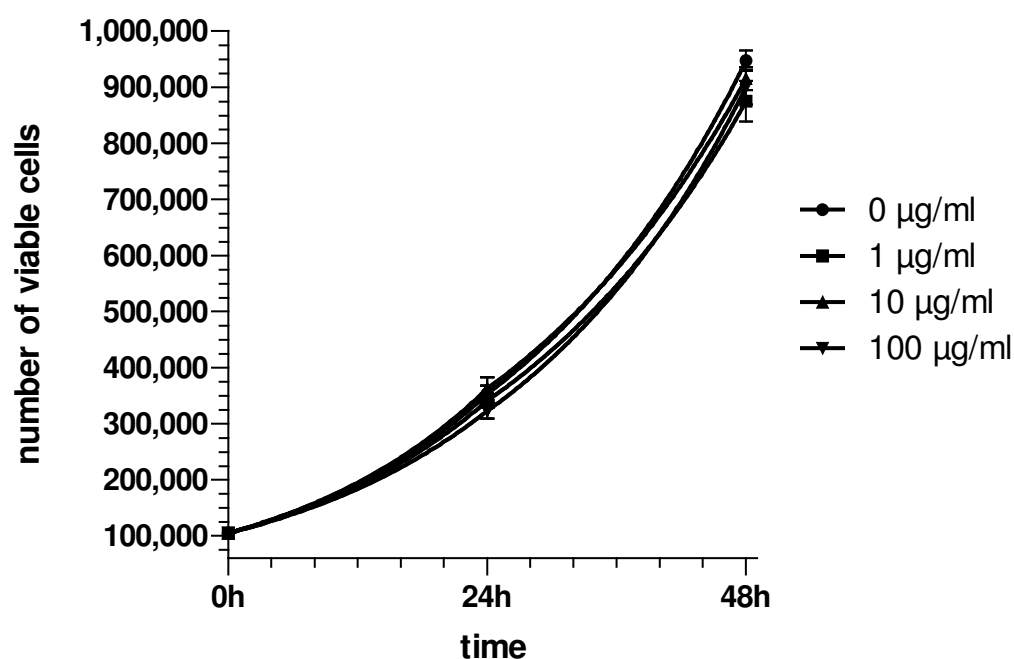


Figure 24: Cellular growth curves of HL-60 cells treated with different concentrations of purified *Zornia glochidiata* dry extract

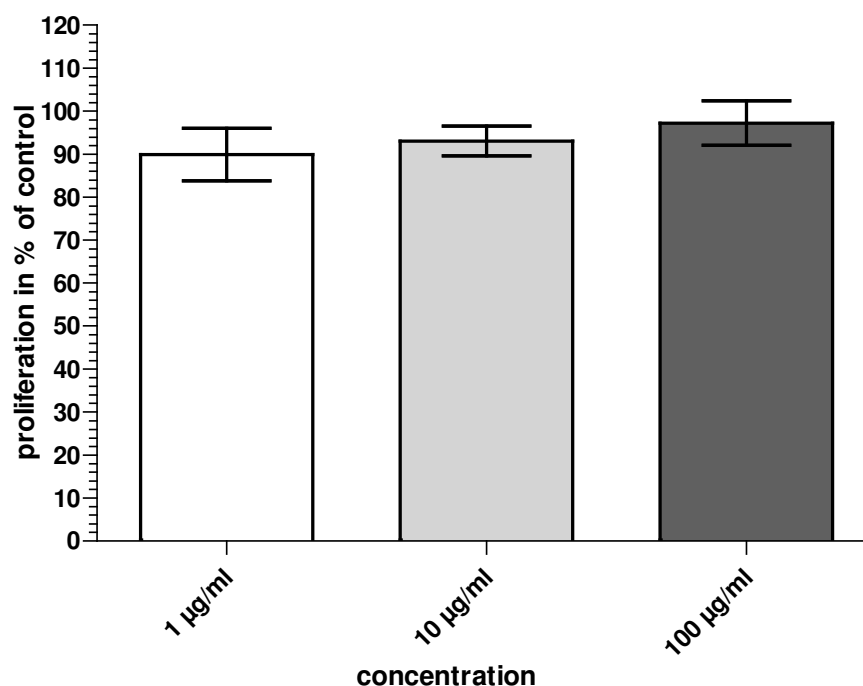


Figure 25: Proliferation inhibition of purified *Zornia glochidiata* dry extract

The fact that the purified dry extracts gained from *Dichrostachys glomerata*, *Lippia rugosa*, *Plectranthus glandulosus* and *Zornia glochidiata* did not show any significant inhibitory activity on the HL-60 cell proliferation – although some of them are already known as potential anti-cancer agents^{(46),(74)} – might belong to the performed purification of the extracts, so that the active components of these plants might have been removed during the processing.

Since no interesting effects of these extracts were observed, the further investigations of this thesis were only performed using the purified *Pittosporum mannii* dry extract.

4.2. Potential metabolism of *Pittosporum mannii* dry extract

Within the framework of the proliferation studies, not only different concentrations of the extract, but also different HL-60 cell concentrations were tested.

Interestingly, it was observed that variations of the cell number resulted in different cellular growth rates, although always the same concentration of the purified *Pittosporum mannii* dry extract (10 µg/ml) was used (see Figure 26 and Figure 27).

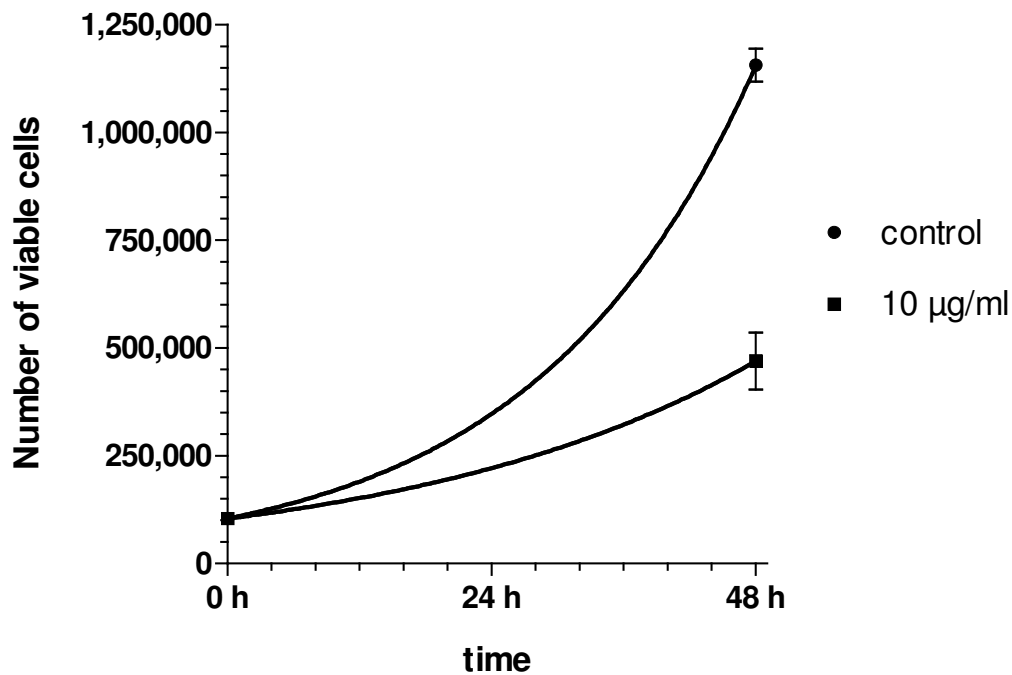


Figure 26: Effect of 10 µg/ml purified *Pittosporum mannii* dry extract on a low cell concentration ($1,0 \cdot 10^5$ cells/ml)

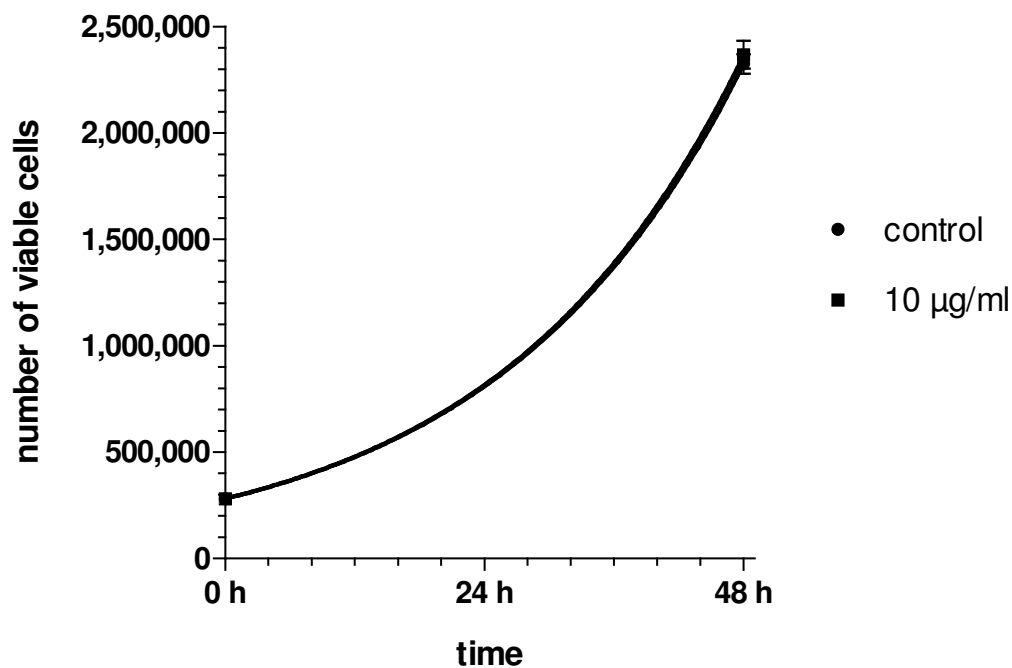


Figure 27: Effect of 10 µg/ml purified *Pittosporum mannii* dry extract on a high cell concentration ($5,0 \cdot 10^5$ cells/ml)

It was found out that higher cell numbers resulted in a lower anti-proliferative activity of the extract and vice versa. In Figure 28, the different proliferation inhibition levels – calculated with the formula mentioned above – are shown. While 10 µg/ml purified *Pittosporum mannii* dry extract led to a proliferation of only 34,8 % of the control sample in the samples containing $1,0 \cdot 10^5$ cells/ml, no anti-proliferative effect was observable at the higher cell concentration of $5,0 \cdot 10^5$ cells/ml anymore!

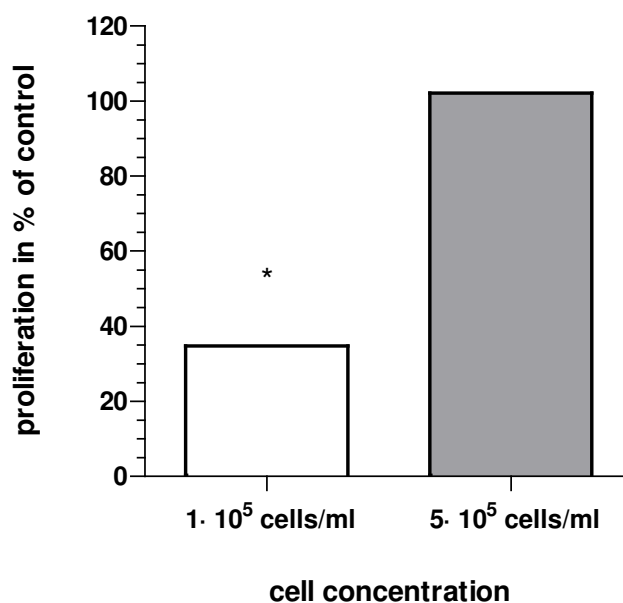


Figure 28: Proliferation inhibition of 10 µg/ml purified *Pittosporum mannii* dry extract on different HL-60 cell concentrations

We postulated that the reduction of the anti-proliferative effect of the purified *Pittosporum mannii* dry extract might belong to an extensive metabolism of the components by the HL-60 cells. It is known that HL-60 cells show a highly expressed enzyme equipment, such as cytochrome-P450-isoenzymes, for various metabolising activities.⁽⁸⁴⁾

In order to verify this hypothesis, the media of cultures containing different HL-60 cell concentrations, treated always with the same amount of purified *Pittosporum mannii* dry extract, were analysed by RP-HPLC using the UV-absorption at 327 nm for detection.

4.3. RP-HPLC results

4.3.1. *Pure purified Pittosporum mannii* dry extract

At first, the pure purified *Pittosporum mannii* dry extract was analysed in order to examine the relevant retention times of the extract components.

After it was evident that the used solvent and buffer did not show any significant peaks at the wavelength of 327 nm (see Figure 29), the dry extract sample was injected.

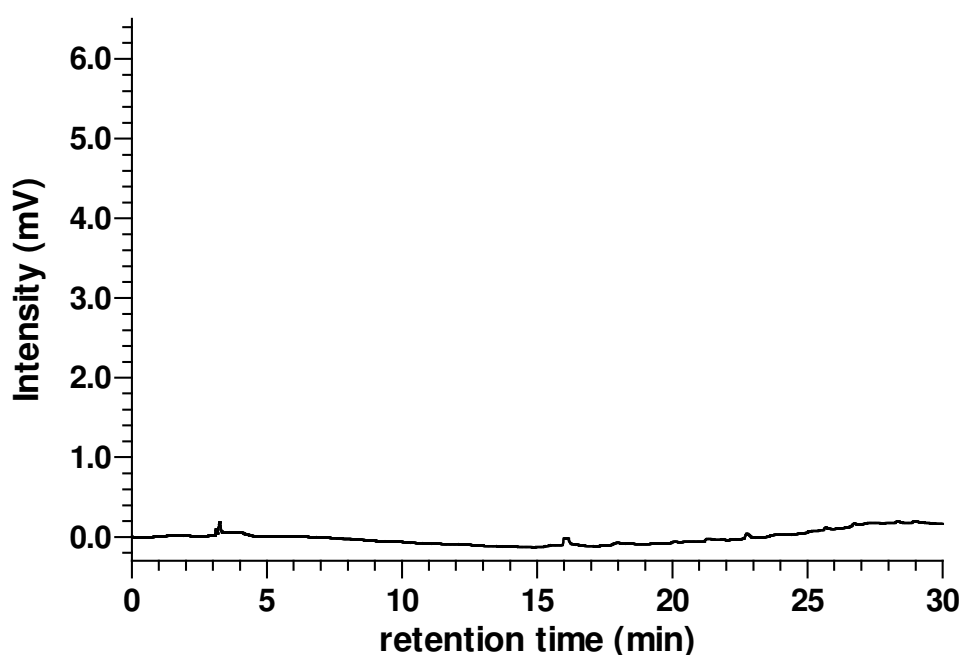


Figure 29: HPLC baseline graph (DMSO + ammonium acetate buffer pH 5,0 1:10 only)

The graph of the analysed sample (Figure 30) showed a lot of peaks between 17 and 23 minutes, so it was proved that the extract contains many different compounds – at least five main components between 19,00 and 21,00 minutes (retention times: 19,55 min; 19,99 min; 20,47 min; 20,61 min and 21,00 min) and several dozens components of lesser intensity were detected.

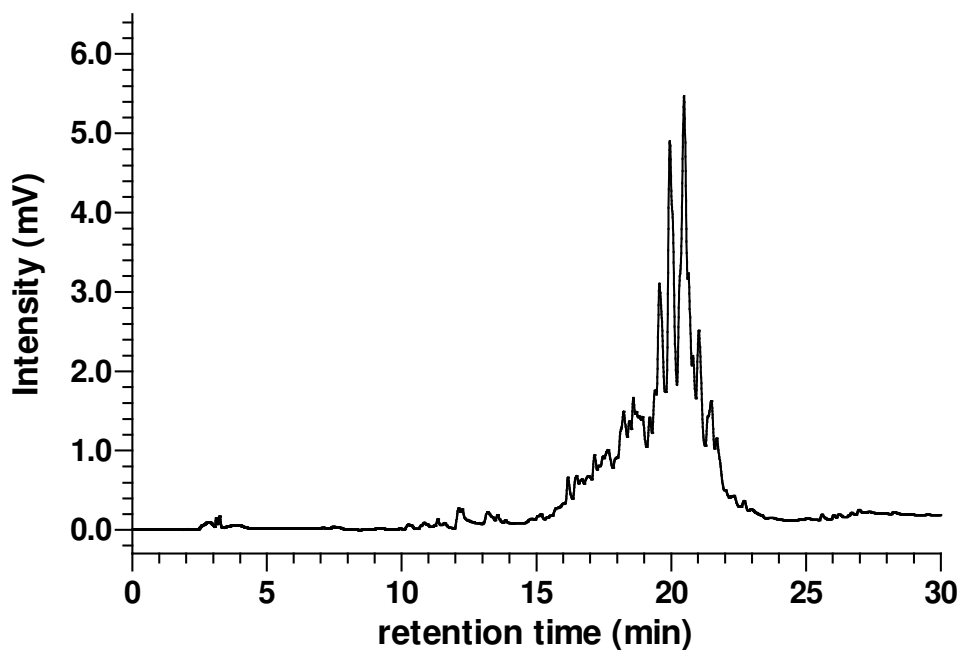


Figure 30: HPLC graph of pure purified *Pittosporum mannii* dry extract

4.3.2. Cell media treated with *Pittosporum mannii* dry extract

For the next step, the HL-60 cell medium of an untreated culture was analysed to assure that no culture ingredients show an absorption at the relevant retention times.

As shown in Figure 31, no relevant peaks between 17 and 23 minutes were visible in the used HPLC system, making a selective detection of the components of the purified *Pittosporum mannii* dry extract in the HL-60 culture medium possible.

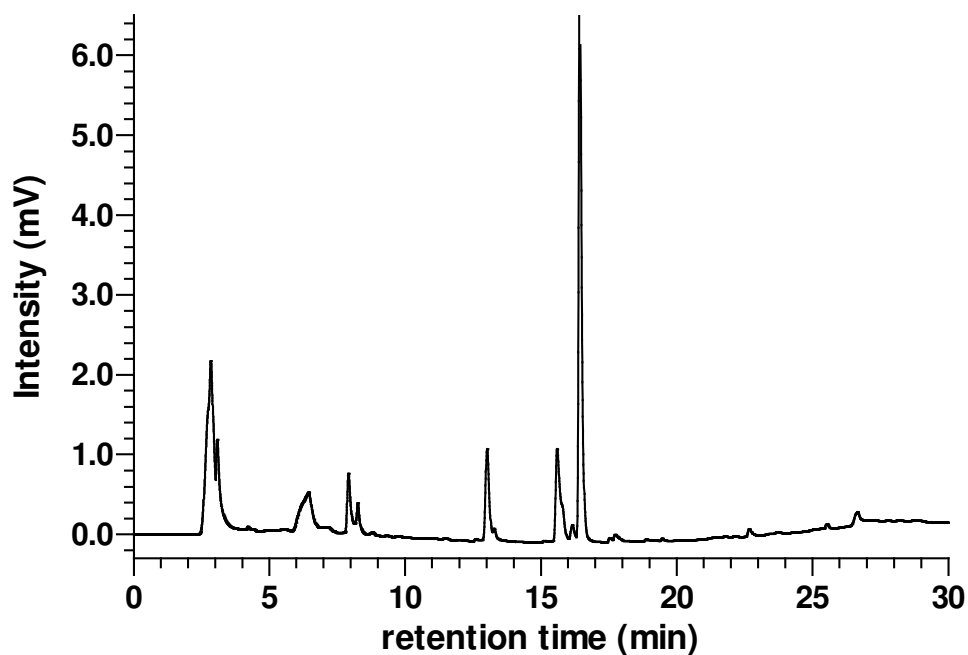


Figure 31: HPLC graph of the medium from an untreated HL-60 culture

In order to monitor the potential transformation of the *Pittosporum mannii* dry extract components, samples taken immediately after the addition of the extract (Figure 32 & Figure 33) and after 24 hours incubation (Figure 34 & Figure 35) were analysed.

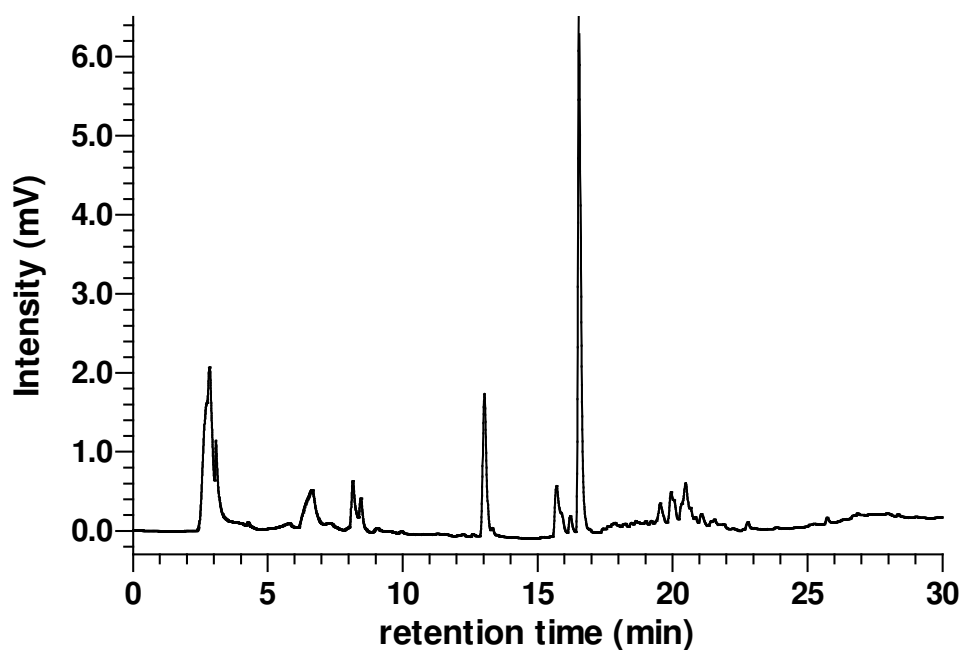


Figure 32: HPLC graph of the medium from a culture of $1,0 \cdot 10^5$ HL-60 cells/ml immediately after adding 30 µg/ml purified *Pittosporum mannii* dry extract

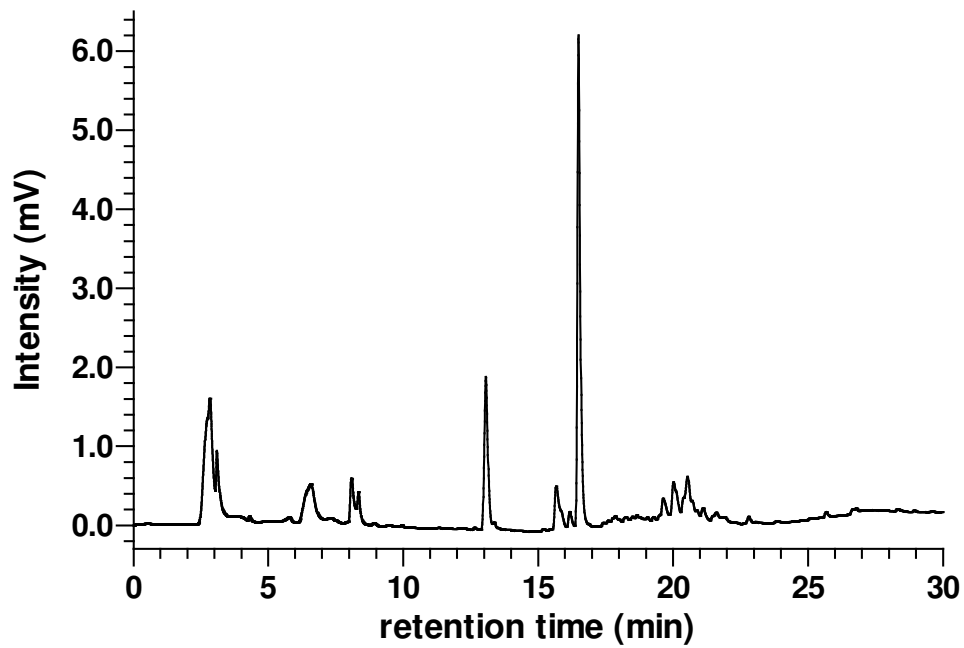


Figure 33: HPLC graph of the medium from a culture of $5,0 \cdot 10^5$ HL-60 cells/ml immediately after adding 30 µg/ml purified *Pittosporum mannii* dry extract

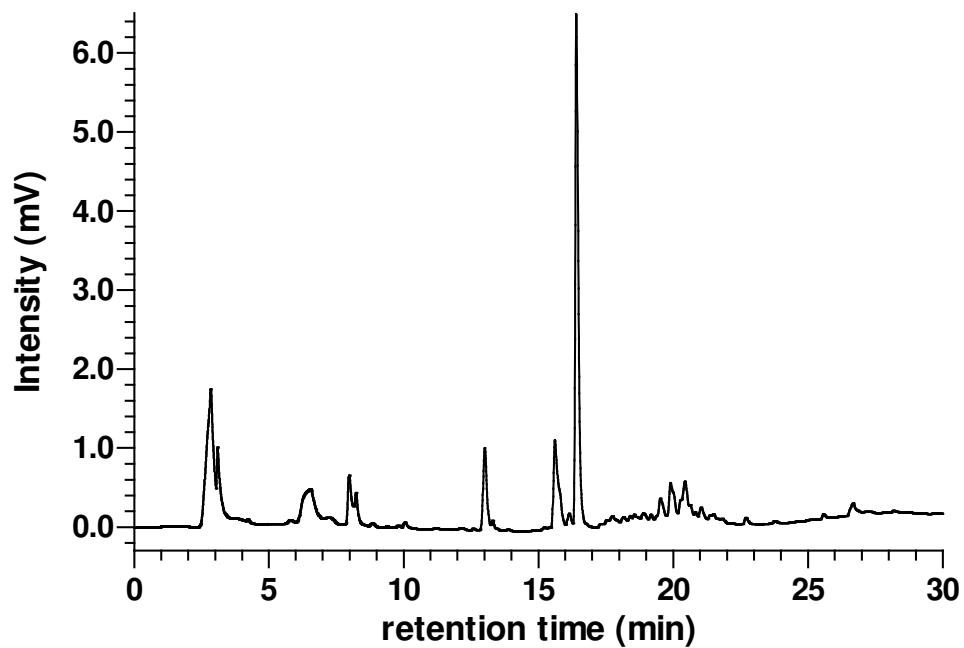


Figure 34: HPLC graph of the medium from a culture of $1,0 \cdot 10^5$ HL-60 cells/ml after the incubation with 30 µg/ml purified *Pittosporum mannii* dry extract for 24 hours

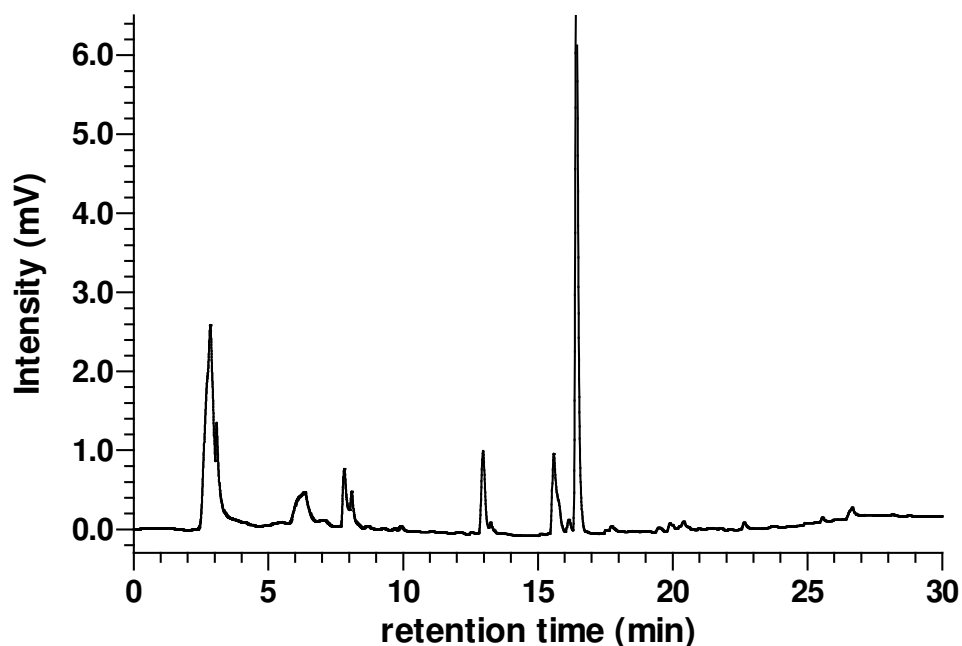


Figure 35: HPLC graph of the medium from a culture of $5,0 \cdot 10^5$ HL-60 cells/ml after the incubation with 30 $\mu\text{g/ml}$ purified *Pittosporum mannii* dry extract for 24 hours

It was found that the samples of the cultures with the higher cell concentration showed a very strong decrease of the main peaks between 19 and 21 minutes, while the peaks in the samples of the lower cell concentration did not show any significant variation at all. This confirms the hypothesis that the components of the purified *Pittosporum mannii* dry extract are extensively metabolised by a higher number of HL-60 cells.

We assume that the anti-proliferative activity of the extract somehow is a kind of on/off-effect, that means a low number of HL-60 cells is not capable of metabolising the components of the *Pittosporum mannii* dry extract in such a short time that they can survive the treatment, while a higher number of cells shows the ability of this required fast and almost quantitative metabolism.

Since the metabolites of almost any known substance are more hydrophilic than the substance itself (for example due to phase-I-reactions like oxidation, reduction or hydrolysis, often followed by phase-II-reactions like glucuronidation or sulfation)⁽⁵⁾, the resulting metabolites from the purified *Pittosporum mannii* dry extract should also be more hydrophilic, which would make them appear in a reversed phase HPLC at lower retention times.

In fact, no new peaks at lower retention times are visible in the analysed graphs, which is a potential indication for a destruction of the chromophoric system of the extract components during the metabolism, so that it is not possible to detect the metabolites at 327 nm anymore.

4.4. Cell death analysis

The Hoechst 33258 / propidium iodide double staining, followed by visual examination of the samples, showed a significant induction of necrosis in HL-60 cells treated with purified *Pittosporum mannii* dry extract in various concentrations. While 5 µg/ml led to 13,8 % and 38,6 % necrosis after 24 and 48 hours respectively, the highest concentration (25 µg/ml) even resulted in 27,6 % necrotic cells after 24 h and 73,2 % after 48 h (see Figure 36). Due to these results, the induction of necrosis by the extract is proved to be dose-dependent and time-dependent; the latter is represented by the massive increase of necrotic cells between 24 hours and 48 hours of incubation with the purified *Pittosporum mannii* dry extract.

Further investigations also showed significant necrosis already 4 hours after the dry extract was added.

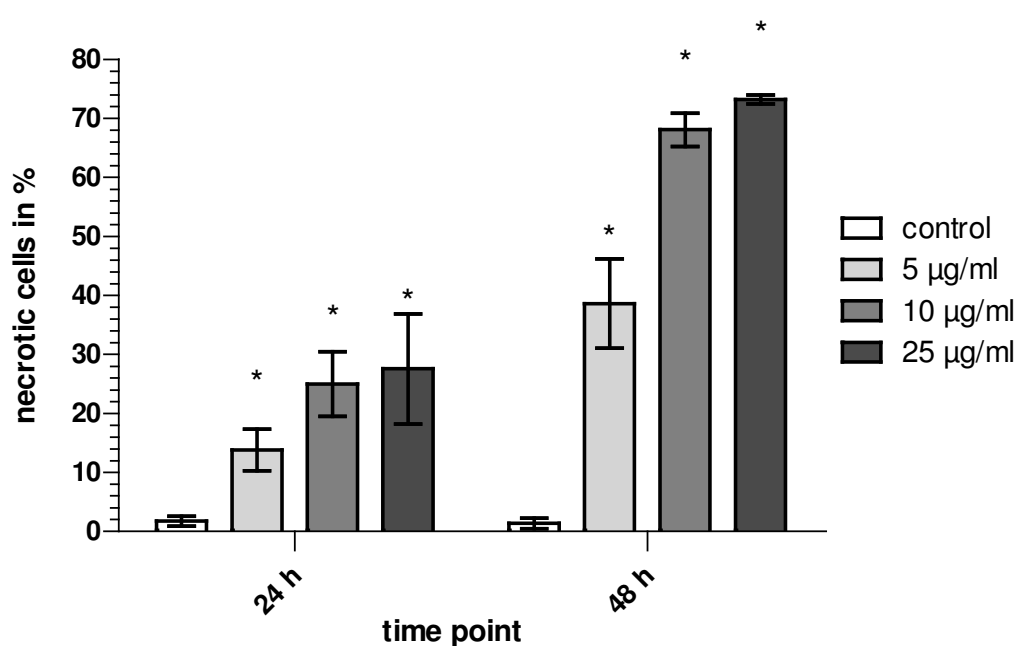


Figure 36: Necrotic cells after 24 h and 48 h incubation with different concentrations of purified *Pittosporum mannii* dry extract

Therefore, no important number of apoptotic cells could be observed in the prepared samples, which is also visible in Figure 37.

The increase after 24 hours is very small and only in the samples treated with higher concentrations of the *Pittosporum mannii* dry extract statistically significant. The number of apoptotic cells ranges from 3,5 % when treated with 5 µg/ml to 8,6 % at 25 µg/ml; furthermore, there is no additional raise in the amount of apoptotic cells between 24 hours and 48 hours, for example in the 25 µg/ml samples, the observed apoptotic level was about 8,6 % at both time points.

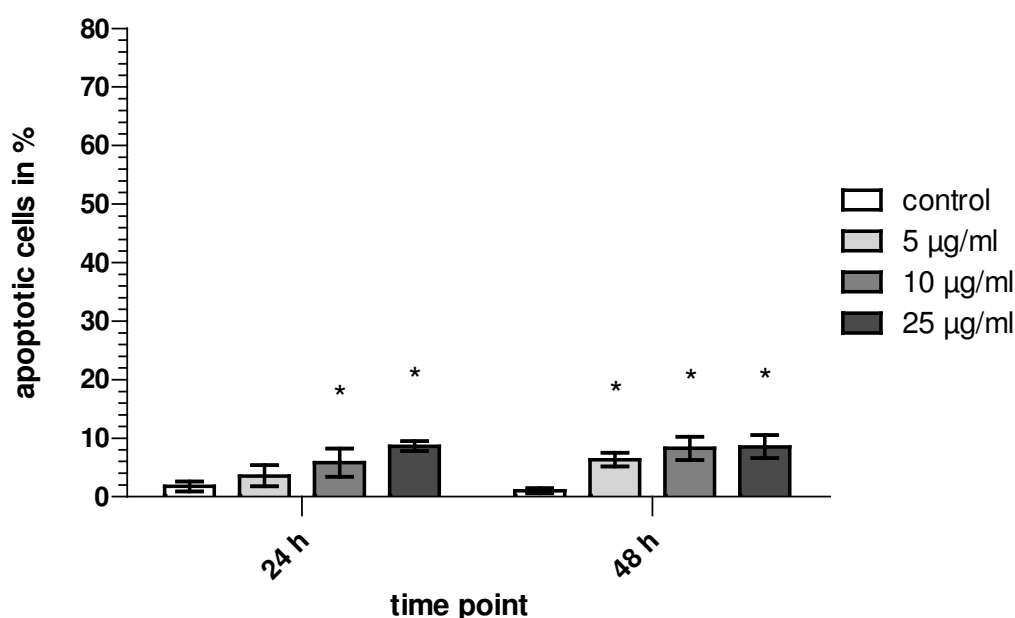


Figure 37: Apoptotic cells after 24 h and 48 h incubation with different concentrations of purified *Pittosporum mannii* dry extract

4.5. Western blots

4.5.1. Dose response analysis

The induction of necrosis is also visible in the result of the performed dose response Western blot analysis.

HL-60 cells were incubated with different concentrations of purified *Pittosporum mannii* dry extract and the β -actin-levels in the different samples after 8 hours of incubation were determined.

Usually, β -actin is expressed in an equal amount in all cells. Due to the fact that the induction of necrosis in the cells led to a massive destruction of the cell structures, which was also clearly observable visually by microscopy, also the β -actin concentration in the samples decreased the more purified *Pittosporum mannii* dry extract was added to the cell cultures (see Figure 38).

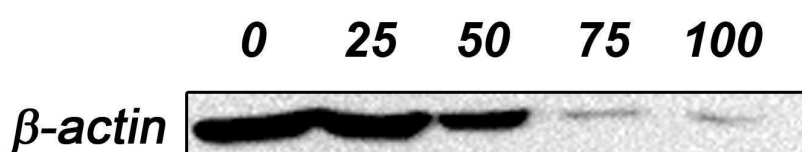


Figure 38: Western blot analysis of β -actin in HL-60 cells treated with 0 $\mu\text{g/ml}$ (control), 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 75 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ *Pittosporum mannii* dry extract for 8 hours

4.5.2. Time response analyses of some MAPK-pathway proteins

In order to find out more about the mechanism of the induction of cell death in the HL-60 cells by the *Pittosporum mannii* dry extract, the expression profiles of some mitogen-activated protein kinase (MAPK) pathway proteins, which are known as cell cycle regulators, were investigated.

Firstly, the expression of p21, a powerful and universal inhibitor of cyclin-dependent kinases⁽⁸⁵⁾, was examined. Cyclin dependent kinases are responsible for the progression of the cellular growth cycle because they are the key enzymes for the transition between the cell cycle phases at the checkpoints. Many cancer cells show a lack of p21, so that the typical uncontrolled cell proliferation is possible. On the contrary, overexpression of p21 inhibits the cell proliferation.⁽⁸⁵⁾

It was found out that the purified *Pittosporum mannii* dry extract increased the p21 expression level in the HL-60 cells significantly already within 2 hours (Figure 41).

Usually, p21 is induced by the p53 tumor suppressor gene, but due to the fact that the HL-60 cells are lacking the p53 tumor suppressing protein⁽⁸⁶⁾, another mechanism must have led to the upregulation of p21 in this experiment.

For example, also the activated p38 MAPKs are known for a direct activation and stabilisation of p21⁽⁸⁷⁾, so we decided to analyse the p38 MAPKs expression level in the treated HL-60 cells.

The group of p38 MAPKs can be subdivided in the four isoenzymes α , β , γ and δ .⁽⁸⁸⁾ They are member of a cell-signalling cascade, which is activated in response to DNA damage (double strand breaks), osmotic shock⁽⁸⁹⁾, lipopolysaccharides (LPS)⁽⁹⁰⁾, inflammatory cytokines (such as TNF) and cellular stress^{(91),(92)}, which is known as a prominent inducer of cell cycle arrest.

The p38 MAPKs are activated by phosphorylation at Thr₁₈₀ and Tyr₁₈₂^{(91),(92)} and lead on the other hand to phosphorylation of various transcription factors (for example Fos, Jun, Myc or Max)⁽⁹³⁾, so that they modulate the whole protein expression in the cells.

In addition, the p38 MAPKs play a central role in the regulation of the cell cycle checkpoints, such as the G₁/S- or the G₂/M-checkpoint.

At the G₁/S-checkpoint, they lead – as already mentioned – to an activation of p21 (directly or indirectly via p53), which inactivates the cyclin-E/CDK-2 complex.⁽⁸⁸⁾ In addition, p38 MAPKs inactivate Cdc25A, which usually stimulates the cyclin-D/CDK-4/6 and cyclin-E/CDK-2 complexes, so that these complexes are no longer present in an active condition.⁽⁸⁸⁾ The absence of these complexes leads to an inhibition of the cell cycle (see Figure 39).

At the G₂/M-checkpoint, they lead to an activation of p21 and of GADD45 α (growth arrest and DNA damage) inducible proteins, which both inactivate the cyclin-B/CDK-1 complex.⁽⁸⁸⁾ In addition, p38 MAPKs inactivate Cdc25B directly or indirectly via the activation of MK-2 (MAP-kinase activated protein kinase 2). Cdc25B is usually responsible for the activation of the cyclin-B/CDK-1 complex.⁽⁸⁸⁾ The absence of the active cyclin-B/CDK-1 complex leads again to an arrest of the cell cycle (see Figure 40).

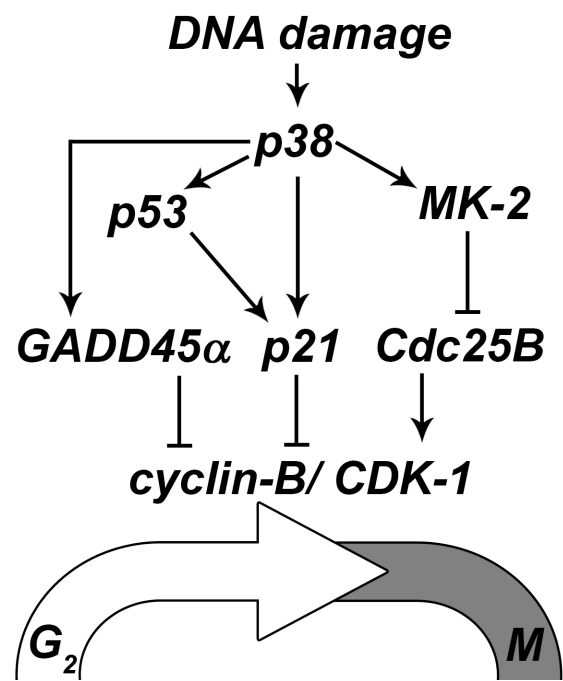
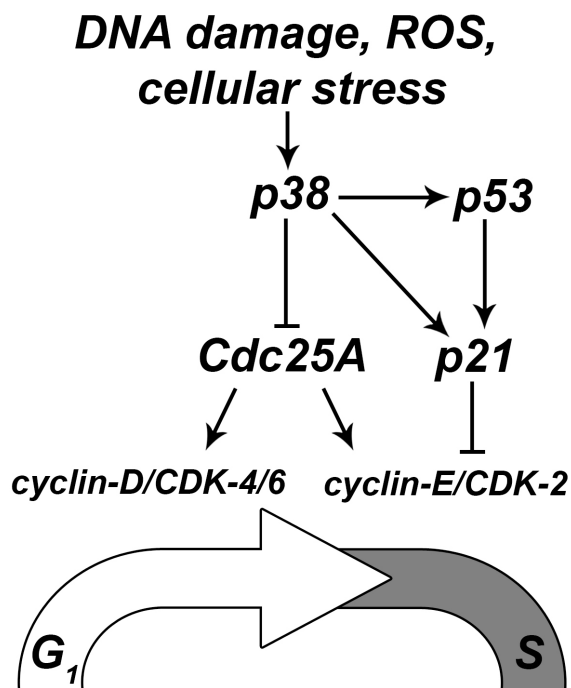


Figure 39: p38 MAPKs regulation mechanisms at the G_1/S -checkpoint⁽⁹⁴⁾

Figure 40: p38 MAPKs regulation mechanisms at the G_2/M -checkpoint⁽⁹⁴⁾

We monitored both states of the p38 MAPKs (the inactive, non-phosphorylated α -, β - and γ -isoforms and their active, phosphorylated pendants), so that a variation in the level of the active forms can be clearly explained by the activation process itself and not be falsified by a variation in the present protein concentration.

The purified *Pittosporum mannii* dry extract was able to induce the activation of p38 MAPKs after 4 hours (see Figure 41), which indicates that cellular stress might be also a possible reason for the arrest of the cell cycle.

On the other hand, the activation of p38 MAPKs appears later than the induction of p21 (see Figure 41), so that the upregulation of p21 in the treated HL-60 cells has to be independent from the p38 MAPKs and belong to a different, unknown trigger mechanism.

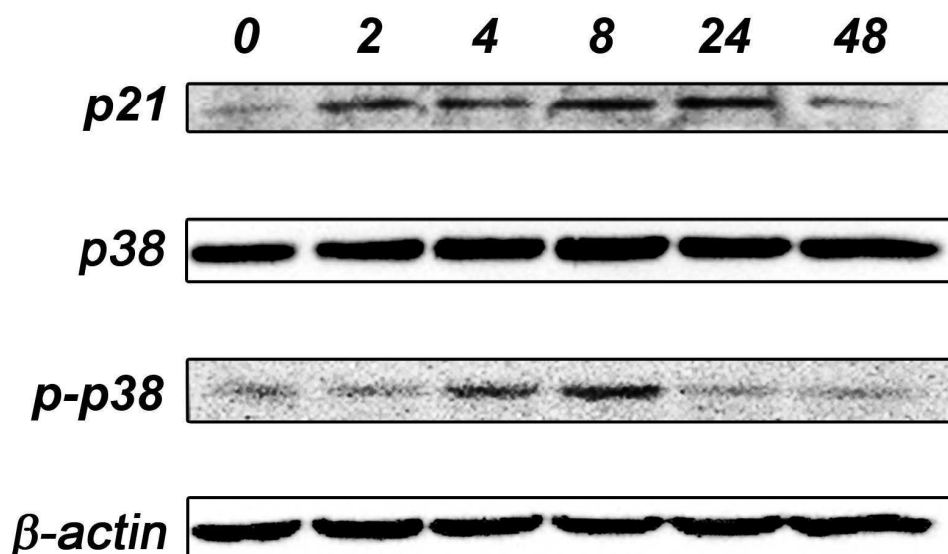


Figure 41: Western blot analyses of some MAPK-pathway proteins. HL-60 cells were treated with 25 μ g/ml *Pittosporum mannii* dry extract for 0 h, 2 h, 4 h, 8 h, 24 h, 48 h

4.5.3. Time response analysis of PARP-1 cleavage

Furthermore, we investigated the cleavage of the poly-(ADP-ribose)-polymerase (PARP-1) in the treated HL-60 cell cultures, which is known as a very meaningful marker for the detection of apoptosis or necrosis in cells.⁽⁹⁵⁾ PARP-1 is a DNA-repair associated nuclear enzyme⁽⁹⁶⁾, which is needed when DNA damages occur (single strand breaks). It mediates the binding of DNA-repair enzymes, such as DNA ligase III α , to the damaged site with its N-terminal zinc finger domain, so that the repair of the single strand break can be initiated.⁽⁹⁷⁾

During apoptosis, the 113 kDa PARP-1 protein is cleaved usually by caspase-3 in an 89 kDa C-terminal fragment and a 24 kDa N-terminal fragment.⁽⁹⁸⁾ Caspase-3 is a protease, which is important for the activation of the apoptotic cell death program.⁽⁹⁹⁾

On the other hand, during necrosis, smaller cleavage products at 50 kDa and between 40 and 35 kDa have been reported.⁽⁹⁶⁾ It is discussed that the necrotic pathway of PARP-1 cleavage is partly or totally mediated by

lysosomal proteases, which are released due to the cellular damage during necrosis.⁽⁹⁸⁾

The purified *Pittosporum mannii* dry extract did not show any cleavage of PARP-1 – neither the apoptotic nor the necrotic cleavage pattern – so that the induction of cell-death in the treated HL-60 cells, which was observed in the experiments mentioned above, has to be mediated by another mechanism than the activation of caspase-3 or lysosomal proteases. In addition, the expression level of PARP-1 did not vary in the monitored period of 48 hours either (see Figure 42).

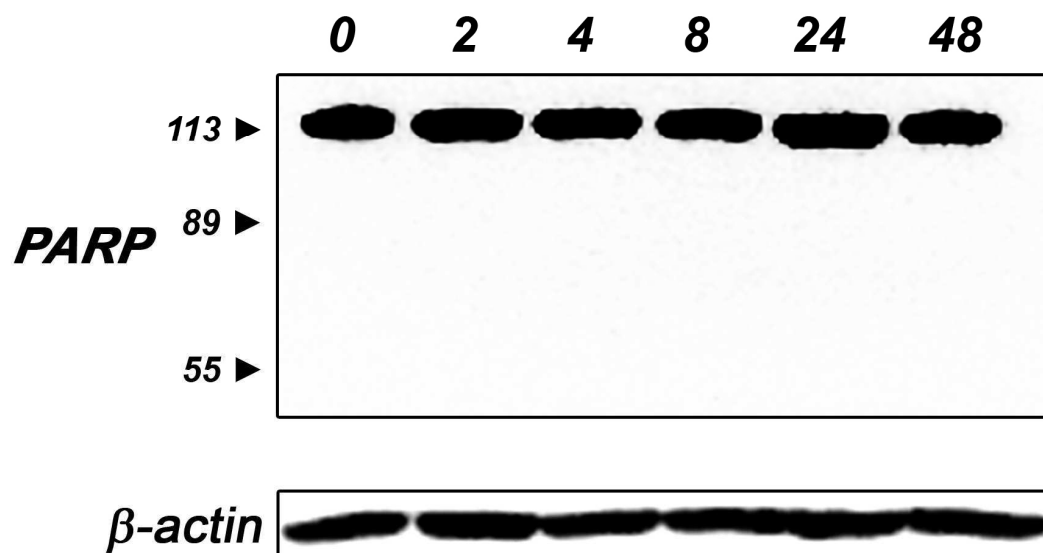


Figure 42: Western blot analysis of PARP-1 cleavage in HL-60 cells, treated with 25 μ g/ml *Pittosporum mannii* dry extract for 0 h, 2 h, 4 h, 8 h, 24 h, 48 h

5. DISCUSSION

The detection of new anti-cancer agents from natural sources is a highly significant area for scientific research.^{(4),(18)}

In this work, five purified plant extracts, isolated from different Cameroonian plant species (*Dichrostachys glomerata*, *Lippia rugosa*, *Pittosporum mannii*, *Plectranthus glandulosus* and *Zornia glochidiata*), which are used since ancient times in the traditional Cameroonian medicine or as spices, were investigated on their anti-cancer effects on HL-60 leukaemia cells. Since only the dry extract gained from *Pittosporum mannii* showed a significant proliferation inhibition in the assay, further experiments were performed only with this dry extract.

It is already known that *Pittosporum mannii* also showed cytotoxic effects on another cell line⁽⁵⁷⁾, so it would be interesting to find out, whether *Pittosporum mannii* influences the cellular growth rates of various cancer cell lines in the same amount as of the HL-60 cells or not.

In addition, it was found that the anti-proliferative components of this extract are extensively metabolised by HL-60 cells, which leads to the question if other cell lines are also capable to perform these transformation reactions.

Seo et al. discovered that the cytotoxic effect of *Pittosporum mannii* belongs to pittoviridoside, a monocatenar glycosylated triterpene saponin with an ursan-type aglycone structure.⁽⁵⁸⁾ However, pittoviridoside is not able to absorb UV-light due to its lack of a chromophoric structure. We assume that the purified *Pittosporum mannii* dry extract contained also (poly-)phenolic compounds because of its UV-absorption maxima at 286 nm and 327 nm, which is supported by Momeni et al., who detected and described these structure class in *Pittosporum mannii* too.⁽⁵²⁾ In turn, this fact leads to the question whether the observed cytotoxic effect of the present dry extract is caused by pittoviridoside only, or if other, still unknown substances are responsible for these effects too, which makes further investigations in this field necessary.

It was found that the purified *Pittosporum mannii* dry extract led to massive necrosis in the treated HL-60 cell cultures. Due to this unspecific and highly

toxic effect, its use in the anti-cancer therapy has to be discarded; but the very complex influence of its components on the cell-cycle makes other *Pittosporum mannii* extracts, differently gained than the present one, or pure isolated compounds and maybe even derivatives thereof, very interesting for further research works.

Since the plant is used in the traditional medicine on humans for a long time, the observed highly toxic properties of *Pittosporum mannii* are presumably also of toxicological interest. Known adverse effects of the medicinal use, such as nausea and vomiting (according to personal information from Dr. Momeni), can probably be explained by this toxic effect.

Besides, also differently purified extracts from the other investigated plant species, like *Dichrostachys glomerata* or *Zornia glochidiata*, which are already known as potential anti-cancer agents^{(46),(72),(74)}, might be of great interest and represent an important source for new anti-neoplastic agents.

We suggest the following preparation method, which provides a fast and efficient detection of active fractions, and after further purification maybe even active pure compounds.

Firstly, an extraction of a larger amount of the relevant plants should be performed in order to get higher yields of the total plant extracts. These total plant extracts should be investigated on their anti-proliferative and cytotoxic activity; then be fractionated (for example by column chromatography), followed by proliferation-inhibition analyses of these fractions. By using this preparation pathway, active fractions can be detected easily, and in addition, it allows to find out whether only one or several anti-proliferative agents are present in the examined plant. In the case of many active compounds, it can even be determined whether additive or synergistic effects of these substances are present.

In conclusion this work showed that substances or extracts from natural sources, especially gained from the African flora, are very fascinating and promising new therapy concepts for cancerous diseases, so that these sources should be further investigated.

6. ABSTRACT

Since cancer is still one of the biggest medicinal problems, new anti-cancer agents, especially from natural origin, are an important aim for scientific research work.

Five Cameroonian plant dry extracts (gained from *Dichrostachys glomerata*, *Lippia rugosa*, *Pittosporum mannii*, *Plectranthus glandulosus* and *Zornia glochidiata*), purified using chromatographic methods, were investigated on their cytotoxic effects on HL-60 leukaemia cells. Furthermore, the expression levels of cell-cycle regulating proteins and cell death mediators were determined by Western blot analyses.

Only the purified *Pittosporum mannii* dry extract led to a strong cell proliferation inhibition, so that further investigations were performed with this extract only. The cell proliferation compared with the control after 48 h ranged from 98,6 %, when treated with 0,5 µg/ml dry extract, up to only 6,8 %, when treated with 100 µg/ml. The IC₅₀ turned out to be 5,15 µg/ml (± 0,18). An extensive metabolism of the extract components by HL-60 cells was postulated due to the fact that in samples containing $1,0 \cdot 10^5$ cells/ml a proliferation of only 34,8 % of the control after 24 h was observed, while no anti-proliferative effect was detectable in samples with $5,0 \cdot 10^5$ cells/ml anymore, although all samples were treated equally with 10 µg/ml extract. This metabolism-thesis was proved using RP-HPLC, where a strong decrease of the components of the dry extract in HL-60 culture medium was monitored.

The *Pittosporum mannii* dry extract induced necrosis dose-dependent and time-dependent in a very strong degree; up to 27,6 % after 24 h and 73,2 % after 48 h, respectively. On the contrary, no important number of apoptotic cells (only 8,6 % at a concentration of 25 µg/ml after 48 h) and no time-dependent increase could be observed. It was found out that p21 and the p38 MAPKs were upregulated within 4 hours and 8 hours respectively, while no PARP-1 cleavage was observed.

All these results show that promising anti-neoplastic agents can be found in the African flora, which makes further investigations in this field very important.

7. ZUSAMMENFASSUNG

Da Krebs immer noch eines der größten Probleme der Menschheit darstellt, sind neue Wirkstoffe, insbesondere natürlichen Ursprungs, ein wichtiges Forschungsziel.

Im Rahmen der Arbeit wurden fünf chromatographisch gereinigte Pflanzentrockenextrakte aus dem Kamerun (gewonnen aus *Dichrostachys glomerata*, *Lippia rugosa*, *Pittosporum mannii*, *Plectranthus glandulosus* und *Zornia glochidiata*) auf ihre zytotoxischen Wirkungen auf HL-60-Leukämiezellen untersucht. Zusätzlich wurde die Expression von Zellzyklus-regulierenden Proteinen und Zelltod-Mediatoren durch Western-Blot-Analysen bestimmt.

Nur der gereinigte *Pittosporum mannii* Trockenextrakt führte zu einer starken Proliferationshemmung, sodass weitere Untersuchungen nur mit diesem Extrakt durchgeführt wurden. Die Zellproliferation, verglichen mit der Kontrolle nach 48 h, reichte von 98,6 % bei 0,5 µg/ml Extrakt bis hin zu nur 6,8 %, wenn die Zellen mit 100 µg/ml behandelt wurden. Die IC₅₀ beträgt 5,15 µg/ml (± 0,18). Ein extensiver Metabolismus der Extraktbestandteile durch HL-60-Zellen wurde postuliert, da in Proben mit $1,0 \cdot 10^5$ Zellen/ml eine Proliferation von nur 34,8 % der Kontrolle innerhalb von 24 h beobachtet wurde, während kein antiproliferativer Effekt in Proben mit $5,0 \cdot 10^5$ Zellen/ml mehr sichtbar war, obwohl alle gleichermaßen mit 10 µg/ml Extrakt behandelt wurden. Diese These wurde mittels RP-HPLC bewiesen, wo eine starke Abnahme der Extraktkomponenten im HL-60 Kulturmedium beobachtet wurde. Der *Pittosporum mannii* Trockenextrakt induzierte Nekrose dosisabhängig und zeitabhängig in einem starken Ausmaß; bis zu 27,6 % nach 24 h und 73,2 % nach 48 h. Im Gegensatz dazu konnte keine relevante Anzahl an apoptotischen Zellen (nur 8,6 % bei einer Konzentration von 25 µg/ml nach 48 h) und auch kein zeitabhängiger Anstieg beobachtet werden. Es stellte sich heraus, dass p21 und die p38 MAPKs innerhalb von 4 Stunden bzw. 8 Stunden hochreguliert wurden, während keine PARP-1-Spaltung erkennbar war. All diese Ergebnisse zeigen, dass vielversprechende antineoplastische Wirkstoffe in der afrikanischen Flora gefunden werden können und weitere Untersuchungen in diesem Forschungsgebiet sehr wichtig sind.

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