# Chapter 5

# Cytotoxic activity of *Pteleopsis myrtifolia* leaf extracts


## Abstract

There is considerable interest and need for the discovery of additional novel natural products and their semisynthetic analogs as potential cancer chemotherapeutic drugs. Since other researchers found that a methanolic extract of leaves of a Tanzanian *Pteleopsis myrtifolia* tree inhibited the growth of three human cancer cell lines, we investigated the cytotoxic activity of South African *P. myrtifolia* leaf extracts. Several leaf extracts (cold water, hot water, hot water without tannins (H-t), ethanol, methanol, methanol without tannins (M-t) and chloroform) of *P. myrtifolia* were tested against different human cell lines (MCF-12, MCF-7, H157, $WHCO_3$, HeLa). Standard cell culture techniques and crystal violet staining was used to measure cell growth spectrophotometrically. Graphs drawn from calculations made from spectrophotometer readings, indicated that for the non-cancerous human cell line, MCF-12A (breast), the growth was not inhibited at 10 μg/ml by all five extracts, and significantly inhibited at a concentration of 100 μg/ml by the hot water and methanol leaf extracts. The absence of extensive growth inhibition may indicate that the plant extracts are not toxic to the cell line at the concentrations used. The growth of the cancerous cell line $WHCO_3$ were inhibited by all plant extracts, (significant less growth inhibition occurred at 20 μg/ml than at 60 μg/ml), the growth of cell lines MCF-7 and H157 by all plant extracts except the M-t extract (with significant less growth inhibition at 20 μg/ml than at 60 μg/ml), and the growth of cell line HeLa by all plant extracts except the hot water and chloroform extracts ((with significant less growth inhibition at 20 μg/ml than at 60 μg/ml). When the hot water and methanol extracts were tested without tannins in a separate experiment, both the H-t and M-t extracts, inhibited growth of the H157 cell line more than the same extracts with tannin. These extracts without tannins inhibited growth less than

the extracts with tannins for the HeLa cell line. $GI_{50}$ values were reached for all extracts at 100 μg/ml for the MCF-7 and $WHCO_3$ cell lines. No extracts reached $GI_{50}$ values for the MCF-12A cell line, and only the hot water, methanol and chloroform extracts reached their $GI_{50}$ values for the H157 cell line. LC values were reached for the cold water, hot water, methanol and ethanol extracts for the MCF-7, $WHCO_3$ and HeLa cell lines. No extracts reached constant lethal concentration values for the cell lines H157 and MCF-12. The cell lines differed in their sensitivity to the plant extracts and were most sensitive to the hot water and methanol extracts.

## 5.1 Introduction

### 5.1.1 Secondary metabolites from plants in the treatment of cancers

Today, natural products still represent more than 80% of pharmacological lead compounds. They constitute a practically endless source of novel substances able to enrich therapeutics. In the field of chemotherapeutics several substances were isolated, amongst which the Madagascan periwinkle, *Catharanthus roseus* that were the source of, vincaleukoblastine and leurocristine, marketed under the names of Velbe ®, and Oncovin ®, respectively. These two drugs have now been used for over thirty years in the chemotherapy of cancers and leukaemia. In 1974 Dr Lang Lois and Dr Portier managed to synthesize vincristine and vinblastine, as well as another original substance, Navelbine ® which exhibited therapeutic properties superior to the previous drugs (Portier *et al*., 1996).

A number of other secondary metabolites and their derivatives of plant origin, as well as natural products of marine and microbial origin are currently in preclinical and clinical trials as potential anticancer agents. One such is combretastatin from the Combretaceae plant family. It was isolated from the bark of the South African *Combretum caffrum* by Dr. Gordon Gragg in the laboratories of Prof. Pettit at Arizona State University. It had a very high affinity for tubulin. Since the yield was very low – 26.4 mg was isolated from 77 kg dry stem bark – several forms

were synthesised and tested. Experiments examining of the effect of combretastatin A4 and combretastatin A4 phosphate on murine tumors demonstrated that combretastatin A4 phosphate caused selective extensive vascular shutdown of tumors. The vascular shutdown was followed by large-scale cell death and necrosis within 24 h after administration (Chaplin *et al*., 1999).

Wall and co-workers (Wani *et al*., 1971) discovered a substance in the trunk bark of the yam (*Taxus* sp), which he called taxol. This substance remained unexplored for years, probably due to the facts that taxol can only be extracted from multi-centuried trees, the trees are very slow growing, the yield is very low and taxol not easily soluble. Several years later, the biological mode of action of taxol was discovered by Susan Horwitz (Schiff *et al*., 1979). Taxol belongs to a group of substances called “spindle poisons” and inhibits the disappearance of spindle after cell division by preventing the depolymerization of microtubules and thus cell progression.

In Tanzania, 47 plants were evaluated for cytotoxic activity by using methanolic extracts and three human cancer cell lines. From the nine plants traditionally used to treat cancer, only two exhibited a cytotoxic effect. From the 38 plants that are used to treat non-cancer diseases, 14 exhibited a cytotoxic effect. *Pteleopsis myrtifolia* was one of the plants not traditionally used to treat cancers, which had cytotoxic effects. At 100 μg/ml, 75-100% inhibition of growth was obtained *in vitro* for the HT29 (colon adenocarcinoma) and A431 (skin carcinoma), and 25-50% for HeLa (cervical carcinoma) cancer cell lines (Kamuhabwa *et al*., 2000).

### 5.1.2 Incidence of cancer

Cancer is the second leading cause of death amongst Americans. One out of every four deaths in the U.S. is due to cancer. In the United States in 1999, over 1500 people were expected to die of cancer each day. On a worldwide basis, figures for death rates for all cancer sites

combined, increased from 1990 up to 1994, decreased from 1994 up to 1998 and stabilised from 1998 through 2000. Increases in breast cancer amongst woman and prostate cancer amongst men are masked by a decrease in all cancer sites combined statistics. The Centres for Disease Control and Prevention (CDC), National Cancer Institute (NCI) and North American Association of Central Cancer Registries (NAACCR) released a United States Cancer Report in November 2003 (to date the most up to date available) with cancer incidence data up to the year 2000. The data covers 84% of the United States (US) population, over 60 primary cancer sites for men and woman, and provide specific information with regard to geographic area, race, sex and age:

- The most common childhood cancers are leukaemia (incidence highest amongst 1- to 4 - year- olds), lymphomas (incidence highest amongst 15- to 19-year-olds), cancer of the central nervous system and reticuloendothelial neoplasms.
- White men and woman are more often affected by melanomas of the skin and cancer of the brain than are black or Asian men and woman.
- Black men and woman are more affected by multiple myelomas than are white or Asian men and woman.
- Asian men and woman have higher incidence rates of liver and intrahepatic bile duct cancer and stomach cancer than do white or black men and woman (Centres for Disease control and Prevention, 2004).

During the 20th century, the leading cause of death in the U.S. shifted from infectious to chronic diseases. Seven out of every 10 U.S. residents who die each year, do so as a result of chronic (e. g., cancer, diabetes) disease. Chronic diseases can be prevented or controlled by adopting behaviours (eating nutritious foods, being physically active) (Medical News Today, 2004)

### 5.1.3 Underlying cause in many types of cancer

The underlying cause in many types of cancer seem to be free radical damage to the DNA of cells, triggering their altered behaviour. Since reactive oxygen radicals play an important part in carcinogenesis, it would be important to develop a course of action to prevent these free radicals from causing damage. Intake of antioxidant nutrients and supplements provide protection against free radical activity, therefore antioxidants present in consumable fruits, vegetables, neutraceuticals and beverages have received considerable attention as cancer chemopreventative agents (Muktar *et al*., 1994). Diet plays the most important preventative role in protection from cancer and Holford (1997) states that the balance between one's intake of antioxidants and exposure to free radicals may literally be the balance between life and death. Fruit, vegetables and seeds are top of the anti-cancer foods and are rich in phytochemicals, vitamins A and C, selenium, vitamin E, calcium and zinc. Plants make protective compounds (phytochemicals) to protect themselves from radiation, insects and humans. Ingestion of a plant rich diet, confers the chemoprotective effect of phytochemicals to man.

Currently the value of taking antioxidants during chemotherapy is researched. Cancer itself creates oxidative stress and impairs antioxidant status in the organism as a whole. Chemotherapy can overwhelm the antioxidant defence systems in the cell, which will lead to an increase in lipid peroxidation, which in turn leads to a decrease in cellular proliferation and therefore to a decrease in the effectiveness of chemotherapeutic agents. Patients with an impaired antioxidant status may become relatively resistant to chemotherapy, and there is evidence that antioxidants improve the antitumor response to antineoplastic agents. The value of antioxidants here is to overcome the growth inhibiting effects of oxidative stress and maintain responsiveness to chemotherapeutic agents (Drisko *et. al*., 2003)

### 5.1.4 Cytotoxic, antitumour, and antineoplastic activities

As mentioned in 1.1.9 of Chapter 1, the National Cancer Institute (NCI) has defined the following terms: 'cytotoxicity' refers to *in vitro* toxicity of tumour cells, while 'antineoplastic' and 'antitumour' should refer to *in vivo* activity in experimental systems (Ghisalberti, 1993). The NCI renamed the $IC_{50}$ value, the concentration that causes 50% growth inhibition, to the $GI_{50}$ value to emphasize the correction for the cell count at time zero (more detail in 5.2.6).

There is considerable interest in the discovery of additional novel natural products and their semisynthetic analogs as potential cancer chemotherapeutic drugs (Kinghorn *et al*., 1999). The aim of this research was to investigate leaf extracts from *P. myrtifolia* for their cytotoxic activity against one non-cancerous and four cancerous human cell lines.

## 5.2 Materials and Methods

### 5.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

### 5.2.2 Human cell lines

The following human cell lines of the American Tissue Culture Collection (ATCC) were chosen randomly from a standard battery of cell lines available at Department of Physiology. The ones chosen were a non-cancerous cell line - MCF-12A, and the cancerous cell lines MCF-7, H157, $WHCO_3$ and HeLa. They are described below in 5.2.2.1 and 5.2.2.2.

#### 5.2.2.1 Non-cancerous human cell line: (limited number of cell divisions).

##### 5.2.2.1.1 ATCC CRL- 10782 MCF–12A (mammary gland, human)

The MCF – 12A cell line was a gift from Professor Parker (Division of Medical Biochemistry, University of Cape Town). It is a non-tumourigenic epithelial cell line produced by long term

culture of non-cancerous mammary tissue in serum free medium with low $Ca^{++}$ concentration. MCF–12A was derived from adherent cells in the population; are positive for epithelial cytokeratins, exhibit three-dimensional growth in collagen and form domes in confluent cultures.

It's current medium for propagation: 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 µg/ml insulin, 500 ng/ml hydrocortisone and 5% foetal bovine serum. They have a population doubling time of 19 hours in the above medium.

#### 5.2.2.2 Cancerous human cell lines: (potential unlimited number of cell divisions)

##### 5.2.2.2.1 ATCC HBT- 22 MCF– 7 (human breast carcinoma cell line)

The MCF-7 cell line was supplied by Highveld Biological (Sandringham, SA). For the derivation of this line, cells from the pleural effusion were seeded in Eagle's minimum essential medium with non-essential amino acids, 20 µg/ ml insulin and 20% bovine calf serum. It has retained several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes.

Current medium for propagation: Eagle's minimum essential medium (MEM), at 37 °C in a humidified atmosphere containing 5% $CO_2$. Media were supplemented with 10% heat inactivated foetal calf serum, penicillin (100 µg/l), streptomycin (100 µg/l) and fungizone (250 µg/l).

##### 5.2.2.2.2 ATCC CRL- 5802 NCI-H157 (human squamous lung carcinoma)

The NCI – H157 is a non-small cell carcinoma cell line of the lung (NSCLC), originally derived by AF Gazdar, HK Oie, JD Minna and associates in 1979 from the pleural effusion of a 59-year-old male Caucasian with squamous cell carcinoma of the lung. It was given to Department of Physiology, University of Pretoria, as a gift from Dr. M de Kock (Dept. of Physiology, University

of the Western Cape). The cells express multiple markers of squamous differentiation. The line shows functional platelet-derived growth factor β (PDGF) receptors as well as mRNA expression of the 6-kb transcript for the PDGF β type receptor. The line expresses heterogeneous mRNA expression for PDGF A and B chain, transforming growth factor β and the epidermal growth factor.

NCI-H157 cells were grown as monolayers in the same medium as MCF-7.

#### 5.2.2.2.3 $WHCO_3$ (human oesophageal cancer cell line)

The $WHCO_3$ cell line was a gift from Professor Thornley (Department of Zoology, University of the Witwatersrand, Johannesburg, South Africa). $WHCO_3$ cells were obtained through biopsies from a patient with squamous oesophageal carcinoma. The cells were poorly differentiated non-keratinising squamous cell carcinoma.

The $WHCO_3$ cells were grown and maintained as monolayer cultures in minimum essential medium, containing 10% heat inactivated foetal calf serum and a 10% mixture of 10 µg/ml Penicillin, 10 µg/ml streptomycin and 25 µg/ml fungizone at 37 °C in a humidified atmosphere containing 5% $CO_2$.

#### 5.2.2.2.4 ATCC CCL-2 HeLa (human epitheloid carcinoma of cervix)

The HeLa cell line was purchased through Sterilab Services (Kempton Park, Johannesburg, South Africa) from the American Tissue Culture Collection (ATCC) (Maryland, USA). HeLa was the first aneuploid, epithelial-like cell line to be derived from human tissue and maintained continuously by serial cell culture. It was isolated by GO Gey, WD Coffman, and M.T. Kubicek in February 1951, from a carcinoma of the cervix of a 31-year-old Negro female (Cancer Res. 12: 264, 1952). In a recent re-examination of the original slides, Jones *et al*. (Obstet. Gynecol. 38: 945-949, 1971) diagnosed the tumour as an adenocarcinoma. Since its origin, it bas been

one of the most widely studied cell lines. HeLa cells have been reported to contain human papilloma virus 18 (HPV-18) sequences. It has to be handled as potentially biohazardous material under at least Biosafety Level 2 containment.

The current medium for propagation of HeLa cells is Eagle's MEM, in which they are grown as monolayers, at 37 °C in a humidified atmosphere containing 5% $CO_2$, with non-essential amino acids, 90% Earle's BSS, and 10% foetal bovine serum.

### 5.2.3 Plant extracts

The chloroform, ethanol, methanol and cold water extracts were prepared in the same way as described in 2.2.2 of Chapter 2. For the boiling water extract, dried, ground leaves of *P. myrtifolia* was boiled in distilled water (1:10) for 2 hours, cooled down and the supernatant centrifuged to separate any pieces of material from the extract. (For traditional use (venereal diseases), a concoction is made by boiling material for approximately 2 hours). Yield was determined and the dried extracts (from the extractants ethanol, methanol, cold water, and boiling water) were redissolved in distilled deionized water to a final concentration of 10 mg/ml and stored in a tightly sealed dark glass container at 5 °C. The dried extracts from chloroform were redissolved in dimethylsulfoxide (DMSO) to a final concentration of 1000 mg/ml (which served as a stock from which dilutions were made) and stored in a tightly sealed dark glass container at 5 °C. All final extracts were filter sterilised with 0.22 μm filters before use.

### 5.2.4 Statistical considerations

Statistical analysis of the data was conducted as discussed with Dr P. Becker of the unit for Biostatistics at the Medical Research Council (MRC). When exposing cells to the effects of plant extracts, biological variation was introduced within each cell line. The analytical variation

in the experimental procedures and biological variation within each cell line were analysed by determining ANOVA and students' t-test for paired samples, respectively. Quantitative experiments were repeated thrice and all experiments included an appropriate set of controls. $P < 0.05$ was considered significant.

Positive controls, such as vincristine or paclitaxel (taxol), were nor used due to budget constraints of the experiments. The NCI's values for vincristine or paclitaxel on human cell lines were compared to results of plant extracts (Table 5.2 in section 5.3).

### 5.2.5 Growing cell lines at required density in multiwell plates

A primary culture was grown to confluency in a 25 $cm^2$ tissue culture flask containing 5 ml tissue culture medium. Before the start of the experiments, all medium was removed from the culture. The adhering cell monolayer was washed once or twice with a small volume of 37 °C PBS to remove any residual foetal bovine serum (FBS) that may inhibit the action of trypsin. Enough 37 °C trypsin/EDTA solution was added to the culture to cover the adhering cell layer. The plate was placed in the incubator for 1 to 2 min. The bottom of the plate was tapped lightly on the countertop to dislodge cells. The culture was checked with an inverted microscope to make sure that cells were rounded up and detached from the surface. If cells were not sufficiently detached, the plate was returned to the incubator for an additional minute or two. As soon as cells were detached, medium-containing serum (to inhibit further trypsin activity that might damage cells) was added. The cell suspension was transferred to a tube or flask. Cells were counted using a haemocytometer and diluted to the desired density. Tryphan blue (0.1%) exclusion staining was used to test for cell viability. 5 ml fresh medium was added to the original flask and returned to the incubator.

Aseptic techniques were used throughout all cellular experiments and preparation of multiwell

plates were carried out in a laminar flow cabinet.

Cell cultures were seeded (at the correct density - 5000 cells/ well (in 96 wells)) in 200 µl and incubated at 37 °C for 48 hours (2 days) after which they were inspected to see of they were growing and adhering to the bottom of the MPW. The outside wells of the 96-MWP were not used for experimental readings, because the loss of moisture from them over the 5 day duration of the experiment, might have given incorrect values. They were filled with 200 µl of 0.2% Triton X-100. When cell cultures grew and adhered to the bottom of the MWP, the plant extracts were added to the wells containing cells and the MWPs were incubated for another 3 days.

Initially each cell line was tested at 10 and 100 µg/ml of plant extract (Figure 5.1).

Three days after the plant extracts were added, the medium was discarded from and 100 µl 1% gluteraldehyde added to the wells and incubated at room temperature for 15 min. After 15 min, the gluteraldehyde was discarded and 100 µl of 0.1% crystal violet stain added and left at room temperature for 30 min. After 30 min, the plates were immersed in running tap water for 15 min and then dried thoroughly. 200 µl of 0.2% Triton X-100 were added to each well to solubilize the crystal violet stain and the plates incubated at room temperature for 30 min. After 30 min 100 µl of the solution was transferred to a clean micrometer plate and the absorbance read at 570 nm. Optical density is linearly related to the number of adhering cells with a sensitivity of ca.500 cells; therefore, the technique is applicable to study agents that affect cell proliferation (Gillies *et al*. 1986). This method makes use of a technique allowing rapid and reproducible quantification of cell number in cultures grown in 96 microtitre plates. Quantification is possible by solubilizing the adsorbed dye into a solution of Triton X-100 and determining optical density

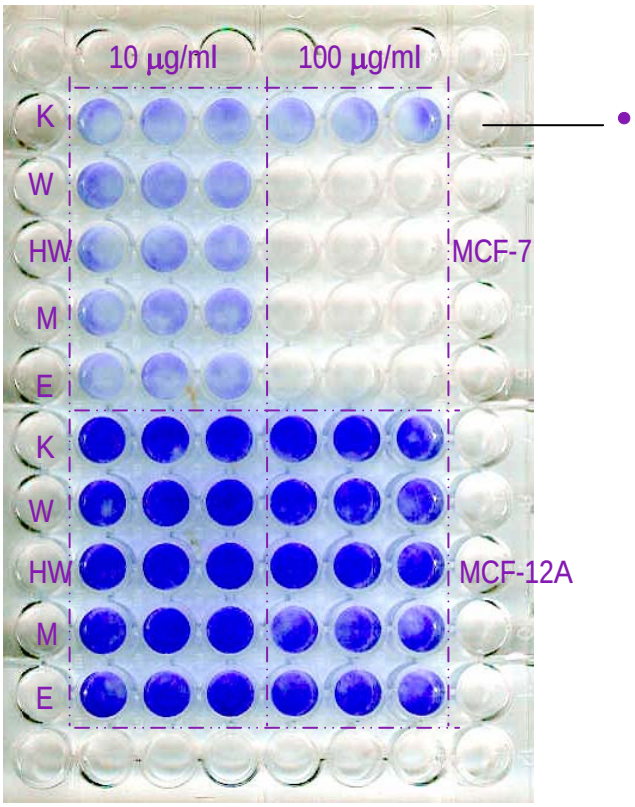


Figure 5.1. Scan of a 96-multiwell plate (MWP) with the human cell lines MCF-7 (top half) and MCF-12A (bottom half) at 10 (left) and 100(right) µg/ml of each of the plant extracts (rows marked: cold water (W), hot water (HW), methanol (M) and ethanol (E)). • The wells bordering the MWP contained only Triton X-100.

using a spectrophotometer. From the spectrophotometer readings, calculations were made and the graphs were drawn from the calculations to present the data in an easy to interpret format.

All plant extracts were also tested at 20, 40, 60 and 80 µg/ml. Figure 5.2 show the MCF-7 cell line at those concentrations.

The two plant extracts that inhibited the growth of the human cancer cell lines most were also tested without tannins. The tannins were removed from these extracts with the use of polyvinylpolypyrrolidone PVPP, described by Houghton & Raman (1998) and which is, according to these authors, useful for small-scale work.

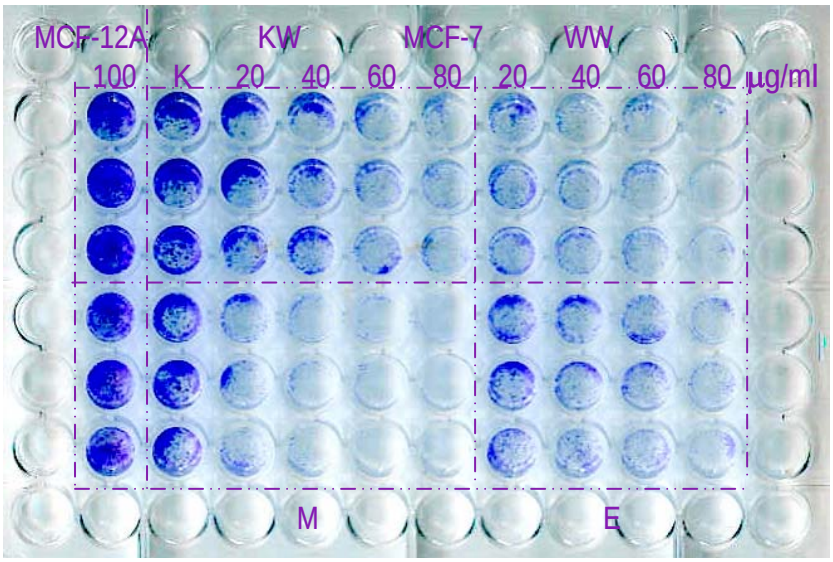


Figure 5.2. 96-MWP with the MCF-7 human cancer cell line and different plant extracts (KW = cold water, WW = warm water, M = methanol, E = ethanol) (columns) at concentrations 0, (0 = control = K), 20, 40, 60 and 80 µg/ml. The right column is the MCF-12A cell line at 100 µg/ml.

Time zero observations were made at the moment the plant extracts were added. The reason being that at this time the cancer cell cultures have already grown for 72 h. If this zero value is calculated, one is able to represent or calculate results in terms of growth inhibition by 50% ($GI_{50}$) and lethal concentration (LD) – and in addition, able to compare with other extracts tested by other researchers according to standards used by National Cancer Institute (NCI). To obtain this value a second set of controls was grown and at the moment the plant extracts were added to the first set, the second set of controls was fixated and stained. The value obtained here is subtracted from all wells. The control values (without plant extract) were taken as 100% cancer cell growth – and the different concentrations of plant extracts calculated as percentage cell growth inhibition.

### 5.2.6 Calculation of growth inhibition, $GI_{50}$ and LC values

Graphs were drawn from the growth inhibitory effects of plant extracts for each cell line. For these graphs the control values were taken as 100% growth and the values obtained with plant

extracts, calculated as a percentage of the control. The 50% growth inhibition concentration ($GI_{50}$) as well as the lethal concentration (LC) formulas of NCI were applied and graphs drawn of resulting values. To calculate $GI_{50}$, the following formula was applied:

$$100 \times (T_{72}-T_0)/(K_{72}-T_0)$$

To calculate LC, the following formula was applied:

$$100 \times (T_{72}-T_0)/T_0$$

$T_{72}$ is the spectrophotometer reading of the specific cell line after 72 hours and crystal violet staining, $T_0$ is the spectrophotometer reading of the specific cell line at time zero, at the moment the plant extracts are added*, $K_{72}$ is the spectrophotometer reading of the control values (cells without any extract), of which an average is calculated and used. From each spectrophotometer reading, the average triton value is deducted. The graph values are the average of 3 repetitions.

* = A second set of controls (cells without any extract added) is used to obtain this value, since they are fixated and stained.

The TGI (total growth inhibition) is the concentration of plant extract or test drug where:

$(T_{72}-T_0)/(K_{72}-T_0) = 0$ and signifies a cytostatic effect.

LC50 is where $100 \times (T_{72}-T_0)/T_0 = -50$ and signifies a cytotoxic effect.

## 5.3. Results and discussion

After fixation and crystal violet staining, it was possible to see which plant extracts inhibited the cell lines extensively by the absence of colour in the wells of the MWP (Figure 5.3).

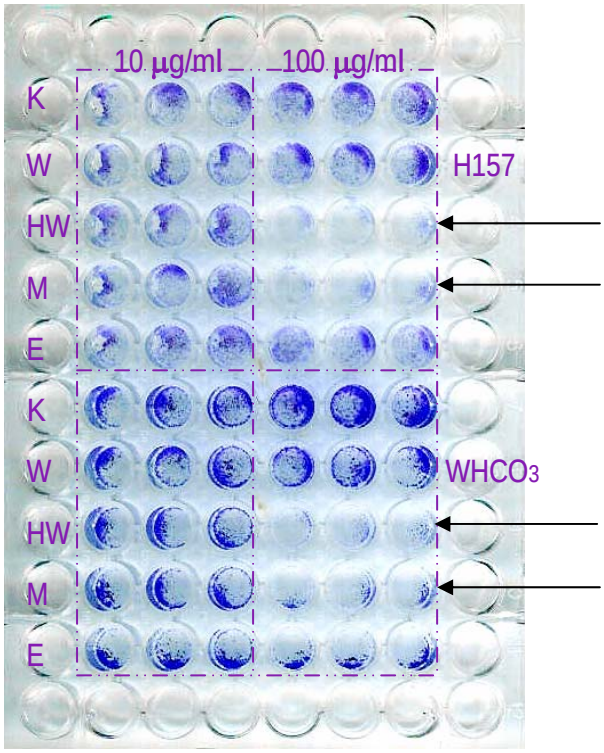


Figure 5.3. Scan of a MWP in which the inhibitory effects of the plant extracts hot water (HW) and methanol (M) against human cancer cell lines, (top half) H157 and (bottom half) $WHCO_3$, could be seen (indicated by arrows).

### 5.3.1 Non-cancerous human cell line MCF–12A

Graphs drawn from calculations made from spectrophotometer readings, indicated that for the non-cancerous human cell line MCF-12-A, the growth was not inhibited at 10 µg/ml for all extracts tested, and according to Figure 5.4a, slightly inhibited at a concentration of 100 µg/ml. Error bars, indicating standard deviation values, are present on all graphs. At 100 µg/ml, the MCF-12A cell's growth was inhibited significantly more than at 10 µg/ml only for the hot water and methanol extracts. Significant differences between concentration levels are indicated with stars at the higher concentration on graphs.

At the 100 µg/ml concentration, the cold water and methanol extracts' growth inhibition effects differed significantly. The same applied for the ethanol and methanol extracts. Extracts whose

growth inhibition effects were significantly different, are indicated with dots of the same colour in Table 5.1. For example, if the cold water and methanol extracts' growth inhibition effects differed significantly, each was given a dot of the same colour in Table 5.1. In addition, the ethanol and methanol extracts were given a dot of the same colour, a different colour than that of the cold water and methanol.

For the MCF-12A cell line the hot water and methanol extracts without tannins inhibited growth less (7% and 13%) than the extracts with tannins (27% and 38% respectively) (Figure 5.4b). This difference was not significant at a 5% confidence interval.

Formulas used by the National Cancer Institute for calculating 50% inhibitory concentration ($GI_{50}$) and lethal concentration (LC) were applied. The resulting values for the leaf extracts (cold and hot water, methanol, ethanol and chloroform and hot water and methanol extracts without tannins (H-t and M-t respectively)) were plotted; the 50% inhibitory concentration ($GI_{50}$) in Figure 5.4c, and the lethal concentration (LC) in Figure 5.4d. The $GI_{50}$-values (50% value of the control and y-axis at 50%) and the LC-values (no growth and y-axis below 0) were not reached.

The presence of growth inhibition only by the hot water and methanol extracts, may indicate that the plant extracts are not toxic to the cell line at the concentrations used. This is a very important result, since it indicates that the effects of the plant extracts are not due to general toxicity.

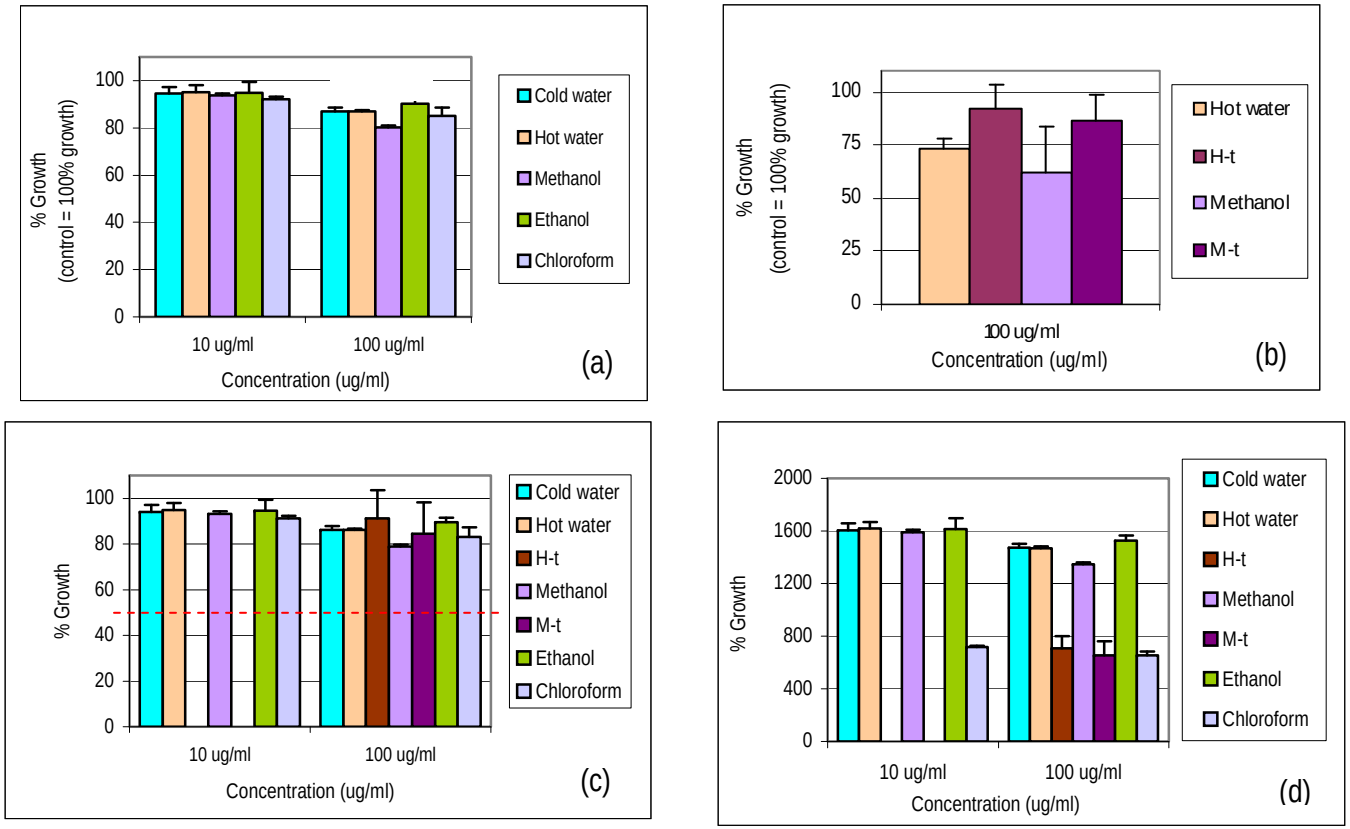


Figure 5.4. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the non-cancerous human cell line MCF-12A (breast), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values ($GI_{50}$) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

### 5.3.2 Cancerous human cell line MCF–7

The MCF-7 (breast) cancerous cells were inhibited by all plant extracts except the chloroform extract (Figure 5.5 top left). At 20 μg/ml, they were inhibited most by the H-t extract – 93% inhibition, and in decreasing order by the methanol, M-t, hot water, cold water, chloroform and ethanol extracts – 90%, 79%, 55%, 29%, 26% and 15% respectively. The effect in growth inhibition was significantly less at 10 μg/ml than 20 μg/ml for all extracts, less at 20 μg/ml than 40 μg/ml for all extracts except methanol, and less at 40 μg/ml than 60 μg/ml for all extracts except methanol and chloroform. In addition, the growth inhibition effect was significantly less at 60 μg/ml than 80 μg/ml only for the ethanol extract, and significantly less at 80 than 100 μg/ml only for the methanol extract. To simplify calculations and presentation of results, cell lines were analysed for significant differences of a specific extract's effect only for the 20, 60 and 100 μg/ml concentrations (at these concentrations the extracts without tannins were tested as well), and the difference in different extract's effect only at 100 μg/ml (where most cell lines had an effect). Growth inhibition was significantly less at the 20 μg/ml than the 60 μg/ml for the cold water, hot water, ethanol and chloroform extracts. So also, the growth inhibition was significantly less at 60 μg/ml than 100 μg/ml for the cold water, hot water, ethanol and methanol extracts. Significance is indicated by stars at the higher of the two concentrations at Figure 5.5a, as well as in Table 5.1.

At a concentration of 100 μg/ml, all leaf extracts except chloroform, inhibited growth by 90% or more. At 100 μg/ml, the difference in growth inhibition was significant between several extracts. These significant differences are indicated as dots of the same colour in Table 5.1. When the hot water and methanol extracts were tested without tannins in a separate experiment, the extracts without tannins inhibited MCF-7 cell's growth less (94.5% and 93.5%) than the extracts with tannins (98% and 93% respectively) (Figure 5b). This difference was significant for the hot

water and H-t extracts and is indicated with a star at the top of the extract without tannins in Figure 5b.

$GI_{50}$ and LC formulas of NCI were applied and graphs drawn of resulting values; the $GI_{50}$ values in Figure 5.5c, and the LC in Figure 5.5d. At 20 μg/ml the methanol, hot water and M-t were already below their $GI_{50}$ values. At 100 μg/ml all the plant extracts, except chloroform, were below the $GI_{50}$ values. The lethal concentrations were attained for all extract types except M-t.

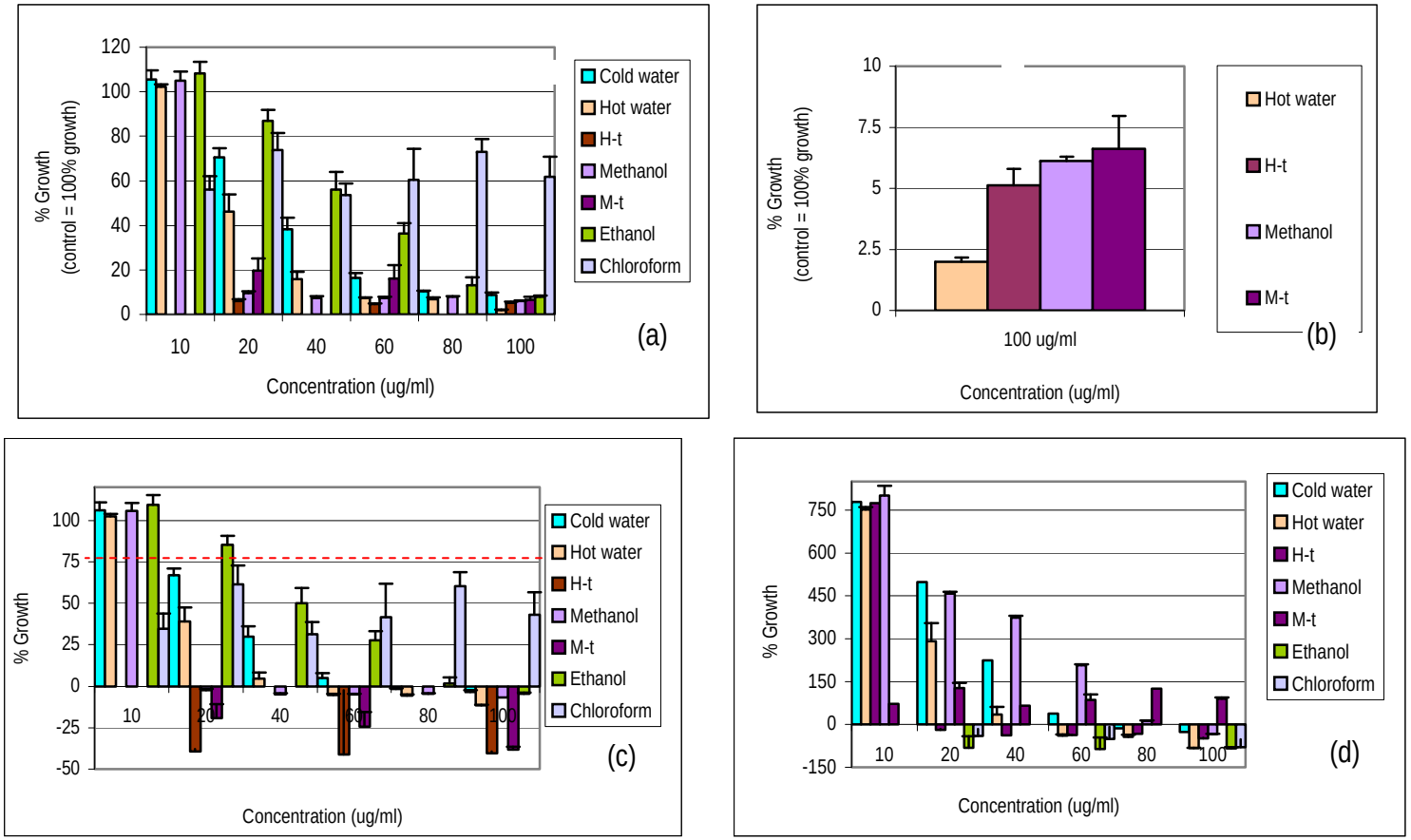


Figure 5.5. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the normal human cell line MCF-7 (breast), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values ($GI_{50}$) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

### 5.3.3 Cancerous human cell line H157

The H157 (lung) cells were least inhibited by the ethanol extract (Figure 5.6a). At concentrations of 100 μg/ml, growth was inhibited by 30% by the chloroform and 25% by the M-t extracts. The cold water and chloroform extracts inhibited growth significantly less at 20 μg/ml than 60 μg/ml, and significantly less at 60 μg/ml than 100 μg/ml. At 100 μg/ml, the difference in growth inhibition was significant for several plant extracts as indicated by dots of the same colour in Table 5.1. The hot water and methanol extracts without tannins inhibited growth more (8 and 30%) than the same extracts with tannins (no inhibition). This difference was significant for both extracts and is indicated with stars at the top of the extracts without tannins in Figure 6b.

The $GI_{50}$ and LC formulas of NCI were applied and graphs drawn of resulting values; the $GI_{50}$ values in Figure 5.6c, and the LC in Figure 5.6d. At 60 μg/ml, only the chloroform extract was below its $GI_{50}$ concentration. At 100 μg/ml all the plant extracts, except H-t, M-t and ethanol, were below the $GI_{50}$ values. The LC was not reached (to stay below zero) by any of the plant extracts in the 10 – 100 μg/ml concentration range.

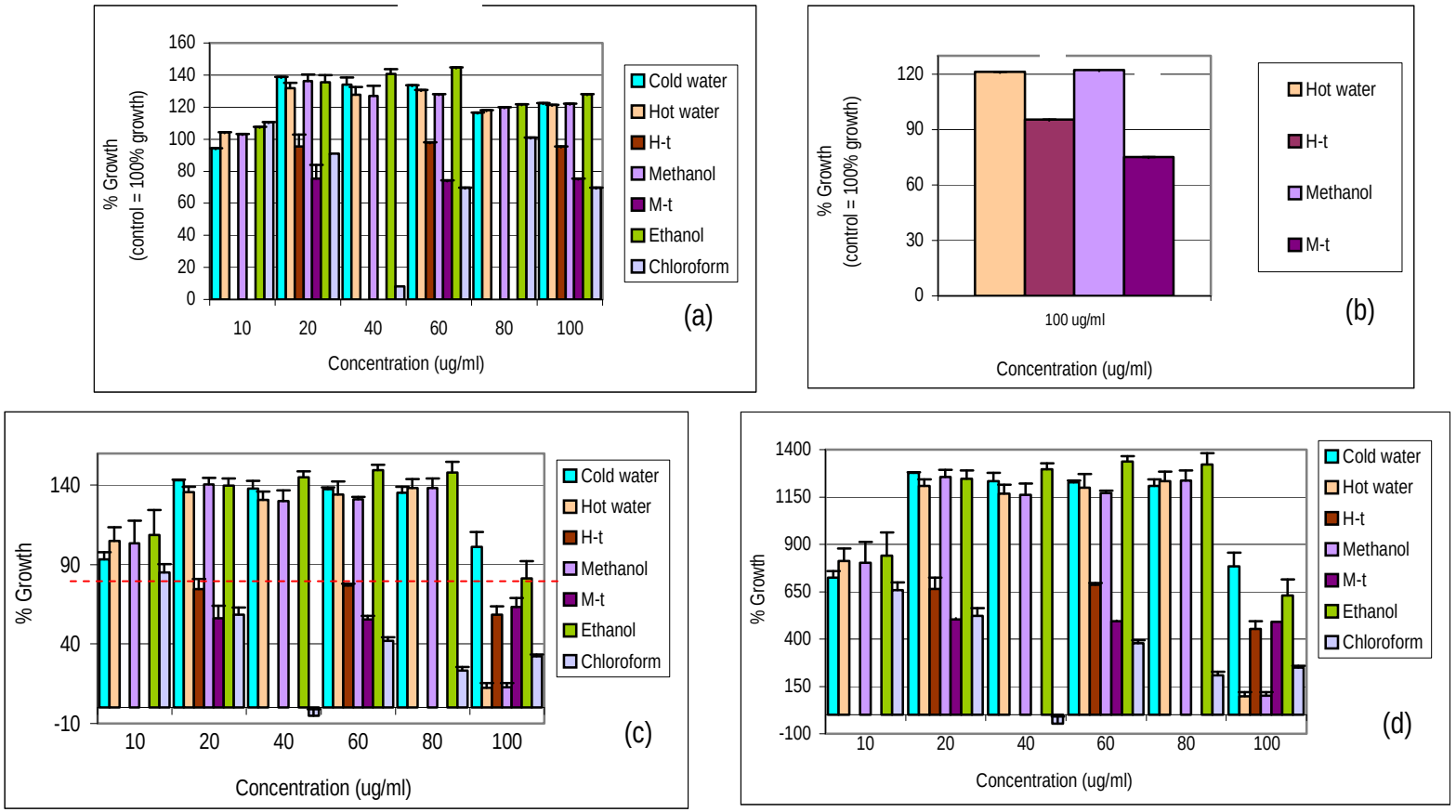


Figure 5.6. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the human cancer cell line H157 (lung), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values ($GI_{50}$) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

### 5.3.4 Cancerous human cell line $WHCO_3$

The cancerous cell line $WHCO_3$ (oesophagus), were inhibited by all plant extracts (Figure 5.7a and b). Growth inhibition was significantly less at the 20 μg/ml than at 60 μg/ml for all extracts. At 60 μg/ml, growth inhibition was significantly less than at 100 μg/ml for the cold water, hot water, methanol and chloroform extracts. Significance is indicated by stars at the higher of the two concentrations at Figure 5.7a, as well as in Table 5.1. At a concentration of 100 μg/ml, all leaf extracts except cold water, inhibited growth by 50% or more. At 100 μg/ml, the difference in growth inhibition was significant between several plant extracts as indicated by a dot of the same colour in Table 5.1. Although the hot water extract with tannins inhibited growth less (78%) than the same extract without tannins (84%) and the methanol extract inhibited growth more (75%) than the same extract without tannins (71%), this difference was not significant for a 5% confidence interval (Figure 5.7b).

$GI_{50}$ as well as LC formulas of NCI were applied and graphs drawn of resulting values; the $GI_{50}$ values in Figure 5.7c, and the LC in Figure 5.7d. At 20 μg/ml, the hot water, methanol, hot water and M-t extracts' growth inhibition were already below their $GI_{50}$ values. At 80 and 100 μg/ml all the plant extracts were below the $GI_{50}$ values. At 20 μg/ml, the hot water, methanol, and H-t have not yet attained their LC values. At 80 μg/ml all the plant extracts except methanol have not reached their LC values yet. Lethal concentrations were exhibited for all extract types except M-t at 100 μg/ml. Extracts that reach their $GI_{50}$ values but not their LC values at a specific concentration, have the desired effect. The desired effect is for plant extracts or compounds are to inhibit cell growth, but not be toxic (lethal).

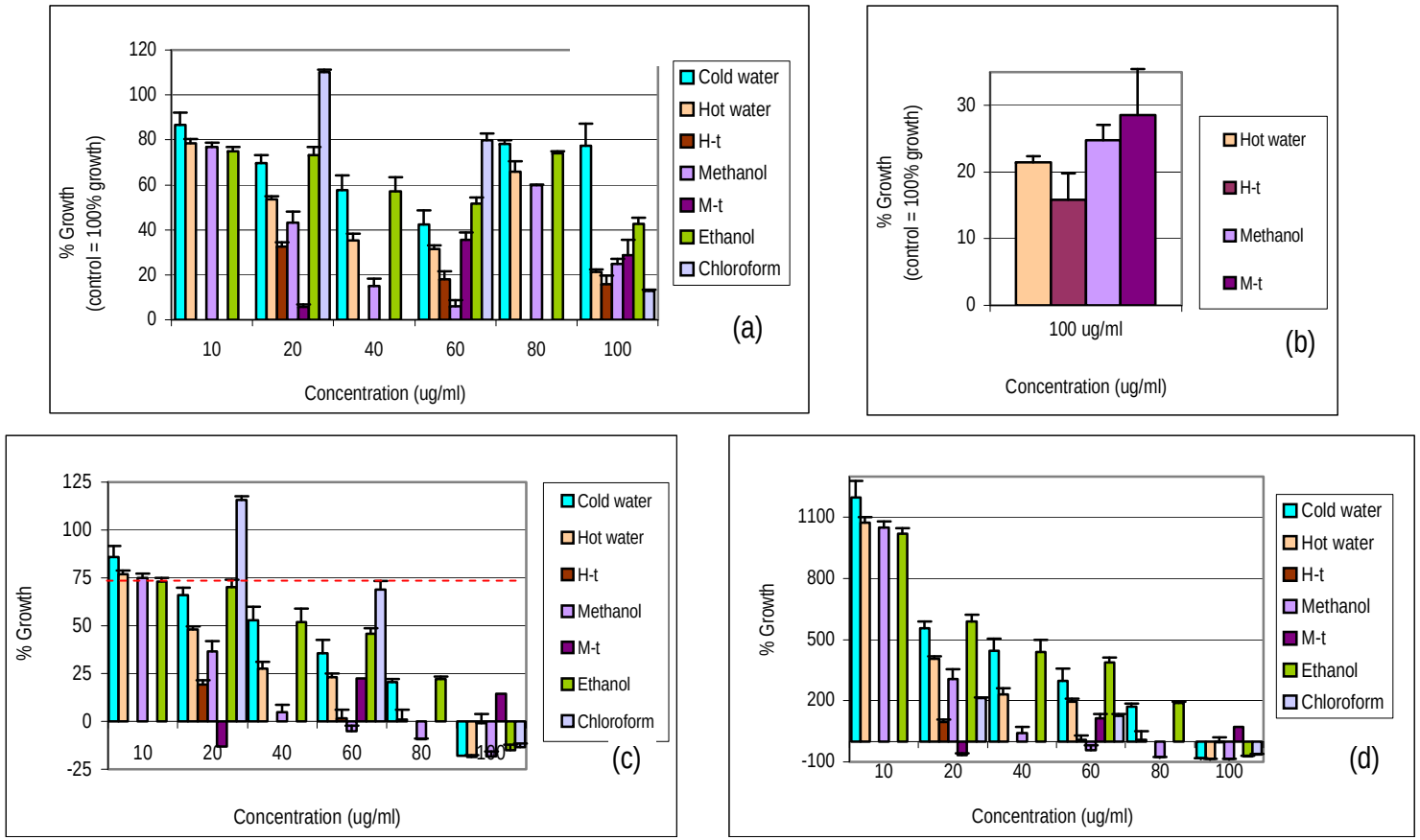


Figure 5.7. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the human cancer cell line $WHCO_3$ (oesophagus), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values ($GI_{50}$) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

### 5.3.5 Cancerous human cell line HeLa

The graphs of the HeLa (cervix) cell line indicated that these cancerous cells were inhibited by all extracts except chloroform (Figure 5.8a). At 60 μg/ml, all extracts except the chloroform extract, inhibited the HeLa cell line by 80% or more. All extracts, except the hot water and chloroform extracts, inhibited the HeLa cell line significantly less at 20 μg/ml than 60 μg/ml. Growth inhibition was significantly more at the 100 μg/ml than the 60 μg/ml only for the hot water and M-t extracts. Significance is indicated by stars at the higher of the two concentrations on Figure 5.8a, as well as in Table 5.1. At a concentration of 100 μg/ml, all leaf extracts except cold water, inhibited growth by 50% or more. At 100 μg/ml, the difference in growth inhibition was significant between several extracts as indicated by dots of the same colour in Table 5.1. The hot water and methanol extracts with tannins, inhibited growth more (95% and 89%) than the same extracts without tannins (84% and 41%) respectively (Figure 8b). This difference was significant for both extracts and is indicated with stars at the top of the extracts without tannins in Figure 5.8b.

The $GI_{50}$ and LC formulas of NCI were applied and graphs drawn of resulting values; the $GI_{50}$ values in Figure 5.8c, and the LC in Figure 5.8d. At 60 μg/ml the cold water, hot water, methanol, ethanol and H-t extracts were already below their $GI_{50}$ values. At 100 μg/ml all the plant extracts except chloroform were below the $GI_{50}$ values. The lethal concentrations were attained for all extract types except M-t and chloroform at 100 μg/ml.

The significant differences between the concentrations (20 and 60 μg/ml, as well as 60 and 100μg/ml) within one cell line as well as the between different plant extracts at 100 μg/ml for a specific cell line are listed in Table 5.1.

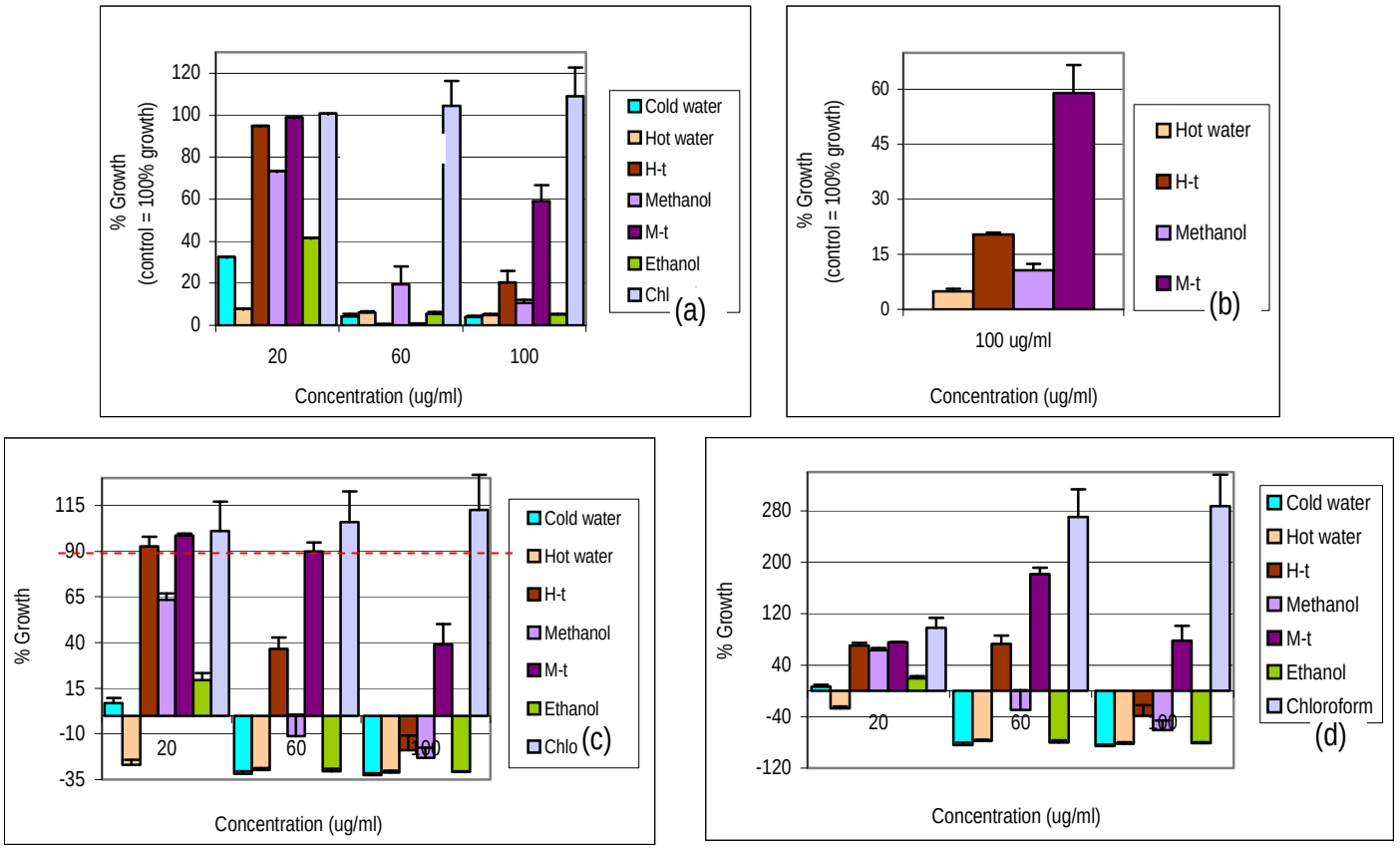


Figure 5.8. Effect of different *Pteleopsis myrtifolia* leaf extracts on growth of the human cancer cell line HeLa (cervix), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values ($GI_{50}$) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

Table 5.1. Significant differences between concentrations within one human cell line and between different leaf extracts of *Pteleopsis myrtifolia* at 100 µg/ml.

| Cell line | Concentrations | | | Different plant extracts at 100 µg/ml | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| MCF-12A | 10 | 100 | | K | H | M | E | C | H-t | M-t |
| K | | | | ● | | ●● | ● | | | |
| H | | | | | | | | | | |
| M | | | | | | | | | | |
| E | | | | | | | | | | |
| C | | | | | | | | | | |
| H-t | | | | | | | | | | |
| M-t | | | | | | | | | | |
| MCF-7 | 60 < | 60 | 100 | K | H | M | E | C | H-t | M-t |
| K | | | | ●●● | ●●●●● | ●● | ●● | ●●●●● | ●●● | ●● |
| H | | | | | | | | | | |
| M | | | | | | | | | | |
| E | | | | | | | | | | |
| C | | | | | | | | | | |
| H-t | | | | | | | | | | |
| M-t | | | | | | | | | | |
| H157 | 20 | 60 | 100 | K | H | M | E | C | H-t | M-t |
| K | | | | ●●●● | ●●●● | ●● | ●●● | ●●●●● | ●●● | ●●● |
| H | | | | | | | | | | |
| M | | | | | | | | | | |
| E | | | | | | | | | | |
| C | | | | | | | | | | |
| H-t | | | | | | | | | | |
| M-t | | | | | | | | | | |
| $WHCO_3$ | 20 | 60 | 100 | K | H | M | E | C | H-t | M-t |
| K | | | | ●●●●● | ●● | ● | ●●● | ●●●● | ●● | ●●● |
| H | | | | | | | | | | |
| M | | | | | | | | | | |
| E | | | | | | | | | | |
| C | | | | | | | | | | |
| H-t | | | | | | | | | | |
| M-t | | | | | | | | | | |
| HeLa | 20 | 60 | 100 | K | H | M | E | C | H-t | M-t |
| K | | | | ●●● | ●●● | ● | ●● | ●●●● | ●●●● | ●●●●● |
| H | | | | | | | | | | |
| M | | | | | | | | | | |
| E | | | | | | | | | | |
| C | | | | | | | | | | |
| H-t | | | | | | | | | | |
| M-t | | | | | | | | | | |

K = cold water, H = hot water, M = methanol, E = ethanol, C = chloroform, H-t = hot water without tannins, M-t = methanol without tannins, ● indicate effects (growth inhibition at different concentrations) that differed significantly at a 5% confidence interval, ● indicate at which concentrations $GI_{50}$ values, but not LC values, (the desired effect) was reached.

The log values of (the $GI_{50}$ and LC growth inhibition values) the hot water and methanol extracts with and without tannins for the human cancer cell lines investigated, were compared with the log values of two extracts from the Combretaceae, as well two plant derived drugs, paclitaxel (taxol) and vincristine sulphate and human cell lines that were available from the NCI's trials (Table 5.2).

Table 5.2. Log values of (50% growth inhibition (GI50) and lethal concentration (LC) values) the hot water and methanol extracts with and without tannins and the human cancer cell lines MCF7, H157, $WHCO_3$ and HeLa, as well as the plant derived drugs paclitaxel (taxol) and vincristine sulphate and different human cell lines investigated by the National Cancer Institute*.

| Human cell lines | | MCF7 breast | H157 lung | $WHCO_3$ oesophagus | HeLa cervix |
|---|---|---|---|---|---|
| Extracts | | µg/ml | µg/ml | µg/ml | µg/ml |
| Hot | $GI_{50}$ | <1.30 | <2.00 | <1.30 | <1.30 |
| water | LC | <1.78 | - | <2.00 | <1.30 |
| Methanol | $GI_{50}$ | <1.30 | <2.00 | <1.30 | <1.30 |
| | LC | <1.30 | - | <1.78 | <1.78 |
| H-t | $GI_{50}$ | <1.30 | - | <1.30 | <1.78 |
| | LC | <1.30 | - | <2.00 | <2.00 |
| M-t | $GI_{50}$ | <1.30 | - | <1.30 | <2.00 |
| | LC | <1.30 | - | - | - |
| Human cell lines | | MCF7* breast | EKVX* non-small cell lung | K-562* leukemia | OVCAR-4* ovarian |
| Extracts | | µg/ml | µg/ml | µg/ml | µg/ml |
| N10109* | $GI_{50}$ | 2.00 | 0.4 | 1.4 | 1.5 |
| | LC | | | | |
| N10009* | $GI_{50}$ | 1.5 | - | 1.4 | 1.2 |
| | LC | | | | |
| Drugs | | µg/ml | µg/ml | µg/ml | µg/ml |
| Taxol* | $GI_{50}$ | -8.0 | -6.6 | -8.0 | -6.2 |
| | LC | | | | |
| Vincristine | $GI_{50}$ | -9.0 | -5.2 | -9.0 | -5.0 |
| sulphate* | LC | | | | |

* Cell lines, extracts (form Combretaceae) and drugs marked with an asterisk are from the National Cancer Institute's trials (Screening services, 1990).

## 5.4 Conclusions

The growth of the cancerous cell line $WHCO_3$ was inhibited by all plant extracts, (the 20 µg/ml

had significant less growth inhibition than 60 μg/ml), the growth of cell lines MCF-7 and H157 were inhibited by all plant extracts except the M-t extract (at 20 μg/ml there was significant less growth inhibition than at 100 μg/ml), and the growth of cell line HeLa by all plant extracts except the hot water and chloroform extracts (the 20 μg/ml significantly less than 100 μg/ml).

Although Kamuhabwa *et al*. (2000) found 25-50% inhibition of growth *in vitro* for the HeLa (cervical carcinoma) at 100 μg/ml of a methanol extract, results found in this study indicated 95% and 89% growth inhibition *in vitro* for the hot water and methanol extracts. This difference could be ascribed to differences in experimental procedure or composition of plant or cell line material.

Extracts with tannins inhibited the growth of the cancer cell lines MCF-7 and HeLa more than the same extracts without tannins. For the H157 cell line, extracts without tannins inhibited the growth more than extracts with tannins. Possible explanations could be that the H157 cells were more sensitive to tannins present than the other cell lines. After the tannins were removed, the active compound(s) were free to bring about more pronounced activity. This was not the case for the other cell lines, as they were less sensitive to tannins or responded to other compounds in the extract. Another explanation could be that the tannins bound to proteins concerned in the cell cycle and caused more inhibition of growth in the MCF-7 and HeLa cell lines.

$GI_{50}$ values were attained for all extracts at 100 μg/ml for the MCF-7 and $WHCO_3$ cell lines. All except the chloroform extract reached $GI_{50}$ values for the HeLa cell line. No extracts exhibited $GI_{50}$ values for the MCF-12A cell line, and only the hot water, methanol and chloroform extracts reached their $GI_{50}$ values for the H157 cell line. LC values were attained for the cold water, hot water, methanol and ethanol extracts for the MCF-7, $WHCO_3$ and HeLa cell lines. No extracts

reached constant lethal concentration values for the cell lines H157 and MCF-12. The cell lines differed in their sensitivity to the plant extracts, as they would as well to different chemotherapeutic drugs. They were most sensitive to the hot water and methanol (polar) extracts.

Often more differentiated cell lines (tumours) (like in this case $WHCO_3$, MCF-7 and HeLa) respond better to chemotherapy than less differentiated cell lines (like in this case H157). This was also seen in the response of these cell lines to plant extracts. The more differentiated cell lines showed more growth inhibition.

The result that the non-cancerous cell line MCF-12A is not extensively inhibited by the plant extracts, indicates that the growth inhibitory effects of cell lines are not the result of a general toxicity of leaf extracts. One can also argue that the non-cancerous cells grow slower than the cancerous ones and that the cancerous ones' cell cycle will therefore be more often affected by drugs than the non-cancerous cells. Within a 48 h test period – the time that the extracts were in contact with the cells (and is also the method the NCI uses for assays) the effect on cell cycle may not have been so pronounced as with longer periods. With most extracts, the $WHCO_3$ cell line reached $GI_{50}$ concentrations, but not LC at the 40, 60 and 80 μg/ml concentrations. Examples of other cell lines and plant extracts that reached $GI_{50}$ but not LC, were: the HeLa cell line and the hot water extract without tannins at 60 μg/ml as well as the methanol without tannins extract at 100 μg/ml, the MCF-7 cell line and the hot water extract at 20 μg/ml, as well as the cold and hot water extracts at 40 μg/ml, the H157 cell line and the chloroform extract at 60, 80 and 100 μg/ml, as well as the hot water and methanol extracts at 100 μg/ml (indicated by ~ at the concentrations that are in Table 5.1). In above-mentioned cases the desired effect has been reached; i.e. an effect that inhibits growth but are not lethal (toxic).

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