CHAPTER 4

CYTOTOXIC ACTIVITY

4.1 General introduction

Persicaria chinensis var. *chinensis* is used traditionally to clear heat and eliminate toxins as well as to treat dysentery, inflammatory skin disease, eczema and corneal nebula (Wan *et al*., 2009; Xie *et al*., 2007; Yang *et al*., 2009). Clinical and pharmacognostic studies show that, this plant can be used to treat inflammation, injury such as contusion, sprain and flu infection (Lin *et al*., 2001; Lin *et al*., 2006).

Polygonum chinense also shows *in vitro* antibacterial properties against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Li *et al*., 2004). Research from China demonstrated the *in vitro* antitumour activity against human hepatocellular carcinoma cell line, Bel-7404, of *Polygonum chinense* using MTT assay (Wei *et al*., 2003). However, the constituents of antitumour properties of *Polygonum chinense* still require further investigation and confirmation (Xie *et al*., 2007).

This project is undertaken to evaluate the cytotoxic activity of the crude methanol, dichloromethane and water extracts of the leaves, stems and whole plant of both the lowland cultivated and highland wild varieties of *P. chinensis* var. *chinensis* by an *in vitro* growth inhibition assay system against selected cancer cell lines of human epithelial carcinoma of cervix cell line (CaSki), human epithelial adenocarcinoma of ovary cell line (SKOV-3), human epithelial adenocarcinoma of colon cell line (HT-29), human epithelial carcinoma of lung cell line (A549), human epithelial adenocarcinoma of mammary gland (breast) cell line (MCF7) and normal human lung fibroblasts cell (MRC-5).

4.2 Materials and Methods

4.2.1 Samples of *Persicaria chinensis* var. *chinensis*

Approximately 2 kg each of the leaves, stem and the whole plant (leaves and stem) of lowland cultivated *Persicaria chinensis* var. *chinensis* were collected from the lowland study site at Sagil, Tangkak, Johor. The highland wild samples of *P. chinensis* var. *chinensis* were collected from Gunung Ulu Kali, Bentong, Pahang, Malaysia (Figure 4.1).

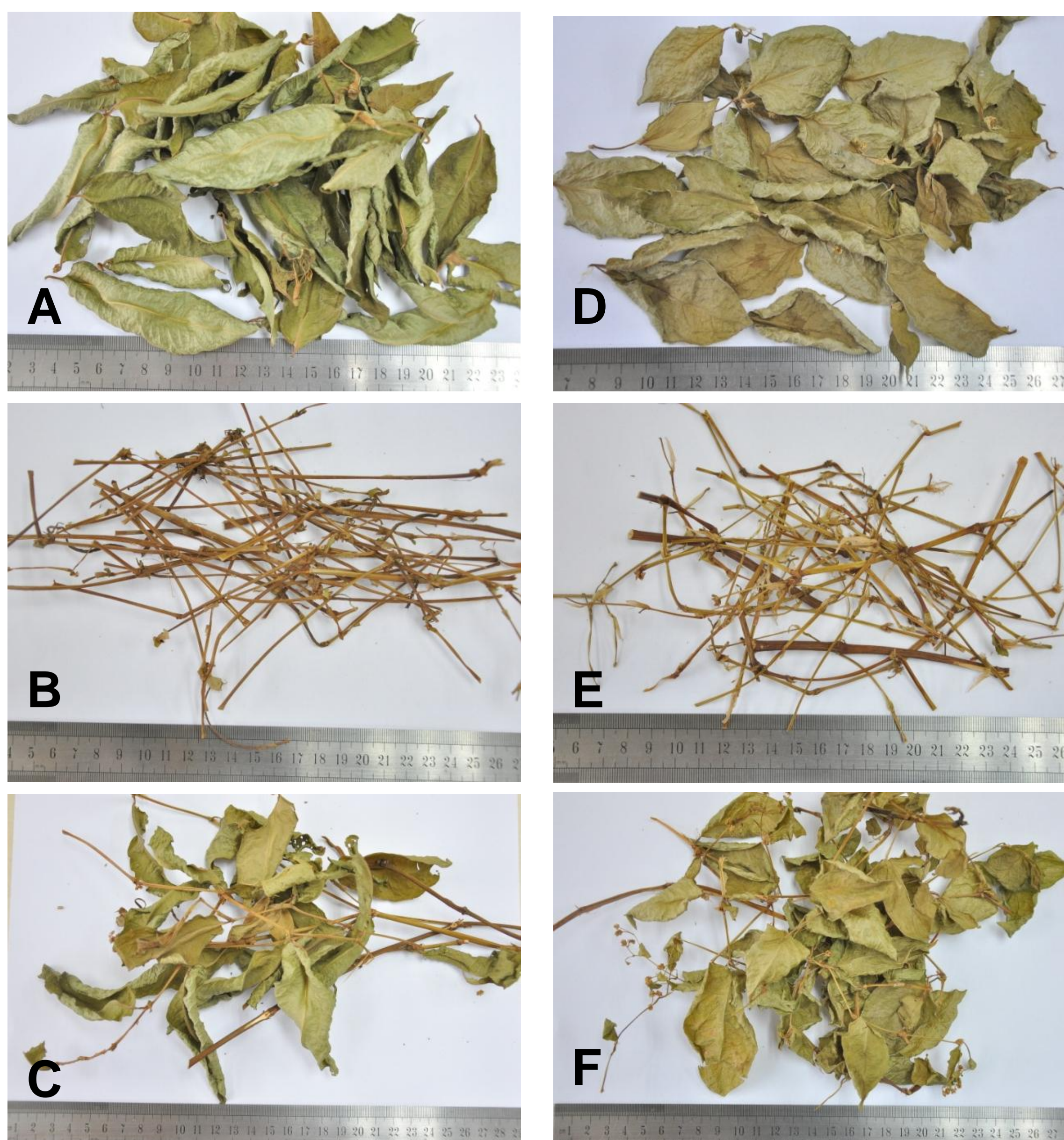

Figure 4.1: The samples of *P. chinensis* var. *chinensis* after drying. A–C: highland variety. D–F: lowland variety. A & D: leaves. B & E: stems. C & F: whole plants.

4.2.2 Preparation of crude extracts

The different plant parts from fresh samples collected were immediately separated from each other, i.e. whole plant, leaf and stem. They were subsequently washed with tap water, cleaned and dried with towels and tissue paper before being placed into an oven (Memmet) at 40 ℃. After 72 hours of oven-drying, the dried samples were grounded into fine powder in a blender. 40 g of the powder from each sample was successively extracted with 400ml of dichloromethane, 400 ml of methanol and 400 ml of distilled water for 3 days with constant shaking (200 rpm) on a Series 25D incubator shaker (New Brunswick Scientific) at room temperature (28 ℃).

Crude dichloromethane and methanol extracts were obtained after the solvent was removed using a rotary evaporator (Bruchi) whereas the crude water extract was obtained after freeze-drying. 20 mg crude dichloromethane and methanol extracts were then redissolved in 1 ml dimethylsulfoxide (DMSO) (Sigma) in a 1.5 ml provial to obtain a stock solution of 20 mg/ml. 20 mg crude water extract was redissolved in 1 ml sterile distilled water to obtain a stock solution of 20 mg/ml. The stocks were stored at -20 ℃ (Freezer, Acson) until use. The concentration of the samples was prepared according to the requirement for the neutral red cytotoxicity assay.

4.2.3 Glassware preparation and sterilization technique

Pipettes, beakers, bottles (Schott Duran), conical flasks and other required glasswares were soaked overnight in 7x detergent (Flowlab), and later washed with tap water. All the glasswares were rinsed with distilled water before drying in an oven (Memmert) at 60 ℃. Beakers and conical flasks were covered with aluminum foil; all the tips and provials were sealed in plastic bags.

Schott bottles with plastic caps were loosely screwed to allow penetration and escape of steam during autoclave sterilization. All glasswares, tips and provials were

autoclaved for 20 minutes at 120 ℃ and 1.1 kg/cm^2 (15 lb) pressure. After cooling, the loose caps were tightened immediately and the tips and provials were dried in an oven (Memmert) at 60 ℃. Pipettes were placed in an aluminum pipette canister with the tips at the closed end of the canister. The canister was closed tightly and heat sterilized at 180 ℃ for 2 hours in a dry heat sterilization oven (Memmert).

4.2.4 Cell Lines

Human epithelial adenocarcinoma of ovary cell line (SKOV-3) (ATCC No: HTB-77), human epithelial carcinoma of cervix cell line (CaSki) (ATCC No: CRL-1550), human epithelial adenocarcinoma of mammary gland (breast) cell line (MCF7) (ATCC No: HTB-22), human epithelial carcinoma of lung cell line (A549) (ATCC No: CCL-185), human epithelial adenocarcinoma of colon cell line (HT-29) (ATCC No: HTB-38) and normal human lung fibroblasts cell MRC-5 (ATCC No: CCL-171) were purchased from the American Tissue Culture Collection (ATCC, USA). The viability of the cells was checked before and after treatment by the tryphan blue exclusion dye method (section 4.3.2). Cell stocks were stored frozen in liquid nitrogen (-196 ℃) until use.

4.2.5 Revival of Cells

Prior to thawing of each frozen cell line, 20% of supplemented Dulbecco's Modified Eagle's Medium (Sigma) or RPMI 1640 medium (Sigma) was thawed at 37 ℃ (Appendix D). One millilitre of the culture medium was then pipetted into a sterile centrifuge tube ready to be used. Vials containing frozen cells were removed from the liquid nitrogen tank before plunging into a beaker of ice and then transferred to a 37 ℃ water bath (Grand Instruments) for quick thawing using gentle hand agitation. The cells in the cryovial were then transferred into a pre-prepared sterile centrifuge tube (Orange

Scientific) containing 1 ml of 20% culture medium and spun at 1000 rpm (Clements 2000) for 5 minutes at room temperature. The supernatant was discarded and the pellet resuspended and mixed in the 1 ml of 20% culture media. 9 ml of 20% culture medium was transferred into a 25 ml tissue culture flask (Nunc) and 1 ml of the resuspended cells were added into the culture flask containing 9 ml of culture medium. The suspended cells were observed for any contamination under the inverted microscope (Leica). The cells were subsequently incubated at 37 ℃ in a 5% CO_2 incubator (Jouan).

4.2.6 Maintenance of Cells

CaSki, MCF7, HT-29 and A549 cells were cultured and maintained in RPMI 1640, SKOV-3 cells in DMEM medium (Dulbecco's Modified Eagle's Medium) and MRC-5 in Eagle's Minimum Essential Medium (MEM) respectively, in 25 ml tissue culture flasks. These cultures were incubated in a humidified atmosphere at 37 ℃ in a 5% CO_2 incubator. These cultures were subcultured every 2 or 3 days and the 10% culture medium (Appendix D) was changed every 2 days or alternatively from time to time based on the colour changes. The cells were routinely checked under an inverted microscope (Leica) for any contamination. When the cells were fully confluent, they were subcultivated.

4.2.7 Subcultivation of Cells

When adherent cells attached to form a single layer in a tissue culture flask at the confluent stage, they were then ready for subcultivation. 10% culture medium was thawed in a 37 ℃ water bath and accutase (ICT) was also thawed at room temperature. Confluent cells were washed twice using phosphate buffer saline (PBS) at pH 7.2. The cells were first detached from the flask by incubating in 1 ml accutase and 3 ml PBS solution for 10 minutes at 37 ℃. The flask was gently tapped to help detach the cells

still attached to the bottom of the flask. The cell suspension was then transferred into a sterile centrifuge tube and centrifuged for 5 minutes at 1000 rpm at room temperature. The supernatant was discarded and 3 ml of 10% supplemented culture medium was added to resuspend the pellet. The cells were then distributed into 3 flasks (2 new flasks and 1 current flask) each containing 9 ml of culture medium. The flasks were then further incubated in a humidified atmosphere at 37 ℃ in a 5% CO_2 incubator.

4.2.8 Cryopreservation of cells

The monolayer confluent cells were fed with fresh 10% supplemented medium 24 hours before freezing. The medium in the tissue culture flask was discarded and the cells were washed with 10 ml of sterile PBS pH 7.2 (Appendix D). The cells were detached from the tissue culture flask as described in section 4.2.7. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 3 ml of freezing medium. The cell suspension was aliquoted in 1 ml amounts into 3 sterile cyro-freezing vials (Nalgene). The vials were left to stand in a polystyrene cup which was kept in the vapour phase in a liquid nitrogen tank at -70 ℃ overnight. The vials were then plunged into the liquid nitrogen tank and stored at -196 ℃.

4.3 Analysis for cytotoxic activity of *P. chinensis* var. *chinensis* extracts

The leaves, stems and whole plants crude extracts derived from the highland and lowland variety of *P. chinensis* were evaluated for their cytotoxic activities against selected cancer and normal cell lines using the neutral red assay.

4.3.1 Serial dilution of *P. chinensis* var. *chinensis* stock solutions

Aliquots of 10 μl from the plant crude methanol and dichloromethane extract stock solutions (20 mg/ml) were diluted in 90 μl of 10% DMSO to produce a concentration of 2000 μg/ml (tube A). It was then further diluted by adding 10 μl from tube A into 90 μl of 10% DMSO to provide a concentration of 200 μg/ml (tube B). 10 μl from tube B was added into another 90 μl of 10% DMSO to provide a final concentration of 20 μg/ml (tube C). (Same method was applied to plant crude water extracts but by replacing DMSO with sterile distilled water)

4.3.2 Incubation of cell lines with *P. chinensis* var. *chinensis* extracts

The confluent cells were detached from the tissue culture flask as described in section 4.2.7. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 ml of supplemented medium to produce a stock cell suspension. For cell enumeration, 100 μl of the stock cell suspension was transferred into a vial with 900 μl of 0.4% tryphan blue (Appendix D) and mixed. 20 μl of the suspension with dye was loaded at the two edges of the cover slip of a haemacytometer (Scherf). The haemacytometer was then examined under a microscope using objective lens 20x (Leica). The unstained viable cells were counted. The cell suspension at a concentration of 3×10^4 cells/ml was prepared according to the formula below:

$$P_1 \times 10^5 \times V_1 = P_2 \times V_2$$

P_1 : Average number of viable cells counted from the haemacytometer

10^5 : Counting chamber conversion factor & dilution factor with dye

V_1 : Volume of stock cell suspension needed

P_2 : The desired cell concentration in the cell suspension

V_2 : Volume of 10% supplemented medium used for seeding

200 µl of the cell suspension with known cell density was transferred into the wells of a 96-wells plate (Nunc) and incubated for at least 4 hours in a humidified atmosphere at 37 ℃ in a 5% CO_2 incubator to allow the cells to adhere and achieve 60–70% confluence before the test agents were added. The cells in the 96-wells plate were then treated with fresh plant stock solution serially diluted at varying concentrations (1, 10, 25, 50, 75 and 100 µg/ml) and subsequently incubated for 72 hours in a humidified atmosphere at 37 ℃ in a 5% CO_2 incubator. All tests were performed in triplicates and untreated cells with growth medium only served as negative controls.

4.3.3 Neutral Red Cytotoxicity Assay

The neutral red cytotoxicity assay was carried out based on the initial protocol described by Borenfreund and Puerner (1985). It quantifies the accumulation of the neutral red dye in the lysosomes of viable and uninjured cells.

At the end of the 72 hours incubation period, the medium in each well with or without the plant extracts was discarded followed by the addition with 200 µl of neutral red (NR) medium with the concentration of 50 µg/ml. The plate was further incubated in a 5% CO_2 incubator at 37 ℃ for another 3 hours to allow maximum uptake of the neutral red dye into the lysosomes of viable and uninjured cells. After the incubation

period, the medium was discarded and the cells in each well were washed with 200 μl of Neutral Red washing solution (Appendix D). The dye was eluted from the cells by incubation with 200 μl of Neutral Red resorb solution for 30 minutes at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). Dye absorbance was measured at 540 nm using ELISA reader (Titertek Multiskan MCC/340).

The average data from the triplicates for each plant extract concentration was expressed in terms of percentage of inhibition relative to the negative controls. The percentage of inhibition of each test sample was calculated according to the following formula:

$$\% \text{ of inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\ \%$$

Percentage of inhibition values were then used to plot dose-response curves. The IC_{50} is the concentration of a drug required for 50% inhibition *in vitro* (Cheng & Prusoff, 1973; Neubig *et al.*, 2003) and was estimated from the plotted dose-response graphs according to the following formula:

$$\frac{\%_b - 50\%}{[\ ]_b - X} = \frac{50\% - \%_a}{X - [\ ]_a}$$

$[\ ]_a$ = concentration A ; $\%_a$ = % of inhibition caused by $[\ ]_a$
$[\ ]_b$ = concentration b ; $\%_b$ = % of inhibition caused by $[\ ]_b$
X = concentration of the extract when 50% of inhibition occurred.

*From the calculated percentage of inhibition, we use mathematical calculation to estimate that the concentration that can inhibit 50% of cell death actually falls in between the $[\text{concentration}]_a$ with its % of inhibition and $[\text{concentration}]_b$ with its % of inhibition.

According to the criteria of the American National Cancer Institute, the extract that gave IC_{50} being lower than 30 μg/ml was considered a crude extract promising for further purification (Suffness & Pezzuto, 1990), or considered cytotoxically active.

4.4 Results

4.4.1 Yield of crude extracts

The leaves, stems and whole plants of highland and lowland varieties of *P. chinensis* var. *chinensis* were extracted using three solvents namely methanol, dichloromethane and water. The yield was calculated according to the formula below:

$$\text{Yield (\%)} = \frac{\text{Weight of concentrated extract}}{\text{Weight of sample (40g)}} \times 100\ \%$$

The yield calculated for all 18 extracts are shown in Table 4.1. Total yield percentages ranged from 9.08% to 23.48%. Overall, the lowland variety showed higher percentages of total yield as compared to the highland variety. Leaf extracts were found to yield higher percentages as compared to the stem and whole plant extracts. Generally, the yield percentages from the methanol extracts (ranged from 5.69% to 15.23%) were found to be higher as compared to the water (ranged from 2.73% to 6.20%) and dichloromethane (ranged from 0.62% to 2.05%) extracts.

Table 4.1: Yield percentages from the highland and lowland variety of *P. chinensis* var. *chinensis*.

Plant	Part of the plant	Extraction solvent	Weight (g)	Yield (%)	Total yield (%)
Highland wild variety	Leaves	methanol	2.5819	6.45	11.70
		DCM	0.5874	1.47	
		water	1.5113	3.78	
	Stems	methanol	2.2779	5.69	9.08
		DCM	0.2638	0.66	
		water	1.0908	2.73	
	Whole plants	methanol	2.4079	6.02	10.86
		DCM	0.4422	1.11	
		water	1.4942	3.74	
Lowland cultivated variety	Leaves	methanol	6.0928	15.23	23.48
		DCM	0.8195	2.05	
		water	2.4799	6.20	
	Stems	methanol	4.8529	12.13	17.00
		DCM	0.2493	0.62	
		water	1.6985	4.25	
	Whole plants	methanol	4.3950	10.99	17.20
		DCM	0.6693	1.67	
		water	1.8174	4.54	

4.4.2 Cytotoxic activity of *P. chinensis* var. *chinensis*

The crude methanol, dichloromethane and water extracts of the leaves, stems and whole plants of the lowland and highland varieties of *P. chinensis* var. *chinensis* were evaluated for their cytotoxic potential against various human cancer cell lines and normal cell lines. The extracts were tested at varying concentrations of 1, 10, 25, 50, 75 and 100 μg/ml for 72 hours in a humidified atmosphere at 37 ℃ in a 5% CO_2 incubator. The negative control consisted of cells not treated with plant extracts. Figure 4.3 shows various untreated human cancer cell lines and normal cell lines. These controls exhibited normal proliferation rates and showed no signs of death after the incubation time of 72 hours.

The average percentage of triplicates and standard deviations of the 18 crude *P. chinensis* extracts against CasKi, SKOV-3, HT-29, A549, MCF7 and normal human lung fibroblasts cell, MRC-5 cells are shown in Appendix G. The cytotoxicity data obtained were expressed as inhibition percentage relative to negative controls and the IC_{50} values were estimated from the plotted dose-response graphs according to the formula mentioned in the materials and methods (graphs in Figures 4.4–4.6). IC_{50} value is the concentration of extract which results in 50% killing of the cells. Extracts with IC_{50} value $\leq$ 30 μg/ml are considered active for the cytotoxicity assay (Suffness & Pezzuto, 1990).

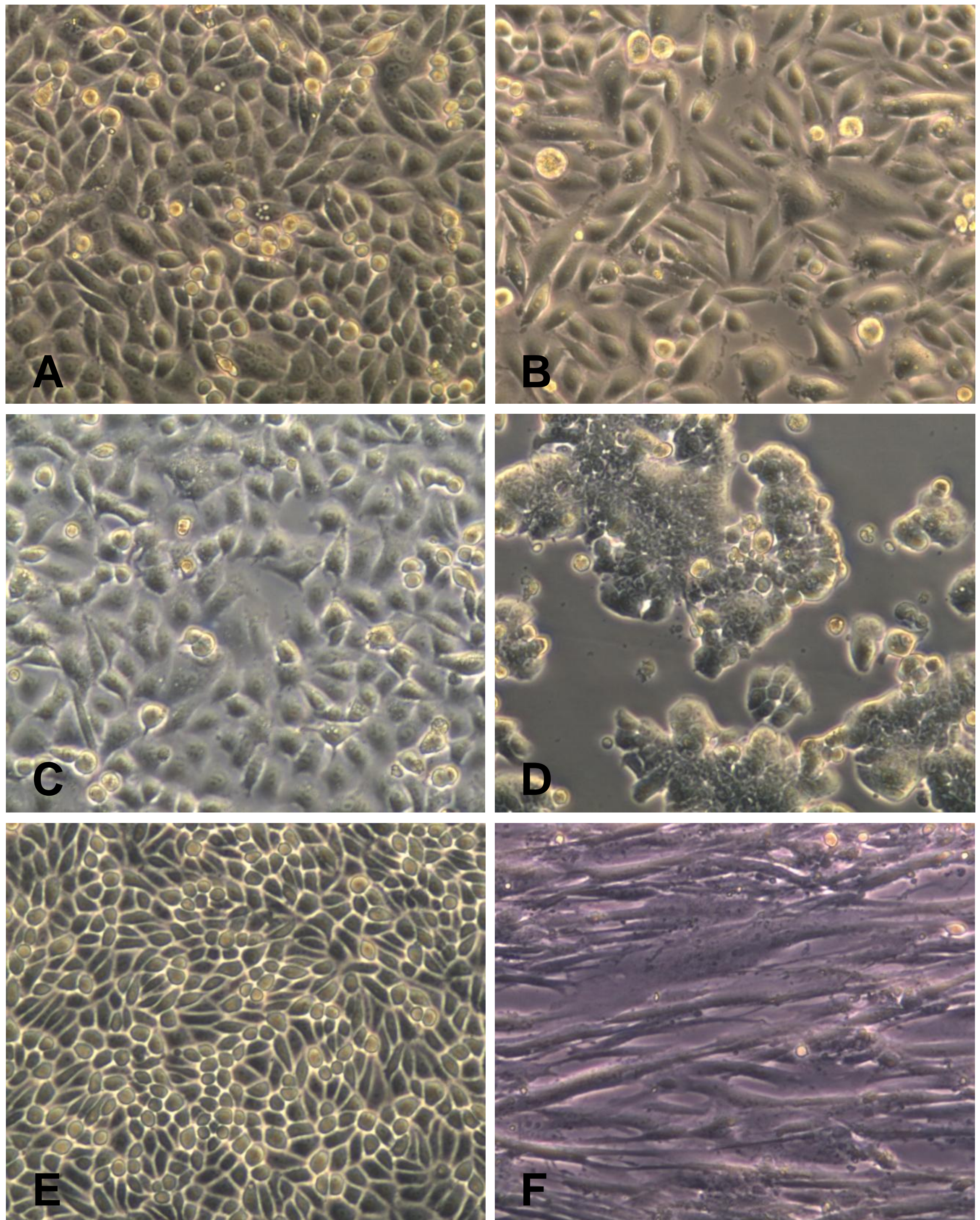

Figure 4.2: Photomicrographs (40× magnification) of various human cancer cell lines (A–E) and normal human cell lines (F). (A): CaSki cells incubated in RPMI 1640 medium. (B): MCF7 cells incubated in RPMI 1640 medium. (C): A549 cells incubated in RPMI 1640 medium. (D): HT-29 cells incubated in RPMI 1640 medium. (E): SKOV-3 cells incubated in DMEM medium. (F): normal human lung fibroblasts cell MRC-5 incubated in MEM medium.

4.4.2. (a) Cytotoxic activity of the methanol extracts of *P. chinensis* var. *chinensis*

As illustrated in Figures 4.4 A–L, all the cancer cell lines evaluated showed different cytotoxic profiles when treated with methanol crude extracts of different parts of the plant derived from lowland and highland varieties. The IC_{50} value for the methanol crude extract was determined and summarized in Tables 4.2 and 4.3.The increased inhibition activity against the cancer cells with increasing concentrations of the methanol extracts were observed after 72 hours of treatment. The results revealed that the leaf crude methanol extracts of both lowland and highland varieties of *P. chinensis* var. *chinensis* exhibited higher inhibition against all cancer cells tested as compared to dichloromethane and water extracts.

The results showed that the leaf crude methanol extracts of both the lowland and highland varieties of *P. chinensis* var. *chinensis* exhibited higher inhibition against all cancer cells tested as compared to the stem and whole plant methanol extracts. The IC_{50} values of the lowland and highland leaf crude methanol extracts ranged from 11 μg/ml–53 μg/ml and 19 μg/ml–62 μg/ml, respectively. The whole plant crude methanol extracts of the highland and lowland varieties exhibited higher inhibition than the stem crude methanol extracts. The IC_{50} values of the lowland and highland whole plant crude methanol extracts ranged from 46 μg/ml–63 μg/ml and 22 μg/ml–73 μg/ml, respectively.

i) Human epithelial carcinoma of cervix cell line, CaSki

Leaf methanol extract from the lowland variety inhibited the proliferation of CaSki with inhibition percentage of 49% at 10 μg/ml, 60% at 25 μg/ml and 86% at 100 μg/ml [Figure 4.4 (B)]. The resulting IC_{50} value of 11.37 μg/ml showed that the extract was actively cytotoxic against CaSki. However, the leaf methanol extracts from the highland variety did not show active cytotoxic activity against CaSki resulting IC_{50}

value of 53.45 μg/ml. Other parts of the plants (stem and whole plant) from the lowland and highland variety were also not active against CaSki and showed IC_{50} values of 54 μg/ml to 85 μg/ml (Tables 4.2 & 4.3).

i) Human epithelial adenocarcinoma of ovary cell line, SKOV-3

The leaf methanol crude extract of the highland variety showed inhibition to the proliferation of the SKOV-3 with the killing percentage of 34% at 10 μg/ml, 60% at 25 μg/ml and 91% at 100 μg/ml. The resulting IC_{50} value of 19.38 μg/ml indicated that the extract is actively cytotoxic against SKOV-3. The inhibition percentages against SKOV-3 cells by the whole plant methanol extract of the highland variety showed 35% at 10 μg/ml, 54% at 25 μg/ml and 90% at 100 μg/ml with a resulting IC_{50} value of 21.71 μg/ml indicated that the extract is actively cytotoxic against SKOV-3. All the other extracts derived from different plant parts of the highland and lowland varieties were not active against SKOV-3 with IC_{50} values ranging from 39 μg/ml to 69 μg/ml (Tables 4.2 & 4.3).

ii) Human epithelial adenocarcinoma of colon cell line, HT-29

All of the methanol crude extracts (leaf, stem and whole plant) derived from the lowland and highland varieties were not active against HT-29. The resulting IC_{50} values ranged from 40 μg/ml to 82 μg/ml (Tables 4.2 & 4.3).

iii) Human epithelial carcinoma of lung cell line, A549

Similar to the HT-29, all the methanol crude extracts from the leaf, stem and whole plant derived from the highland and lowland varieties were not actively cytotoxic against A549.The resulting IC_{50} values ranged from 48 μg/ml to 97 μg/ml (Tables 4.2 & 4.3).

iv) Human epithelial adenocarcinoma of mammary gland (breast) cell line, MCF7

The inhibition percentages of the MCF7 cells for the crude methanol extract of the leaves of the lowland variety were 46% at 25 μg/ml, 69% at 50 μg/ml and 77% at 100 μg/ml. This crude methanol extract of the leaf derived from the lowland variety attained IC_{50} values of 29.27 μg/ml indicated that the extract is actively cytotoxic against MCF7. Crude methanol extracts of the stem and whole plant of the lowland variety and the leaf, stem and whole plant were not actively cytotoxic against MCF7; showed IC_{50} values ranging from 49 μg/ml to 69 μg/ml (Tables 4.2 & 4.3).

v) Normal human lung fibroblasts cell, MRC-5

All of the methanol crude extracts (leaf, stem and whole plant) derived from the lowland and highland varieties were not actively cytotoxic against normal human lung fibroblasts cell, MRC-5. The IC_{50} values ranged from 85 μg/ml and above. This indicated that the methanol crude extracts did not inhibit the growth of normal human fibroblast, MRC-5 (Tablse 4.2 & 4.3).

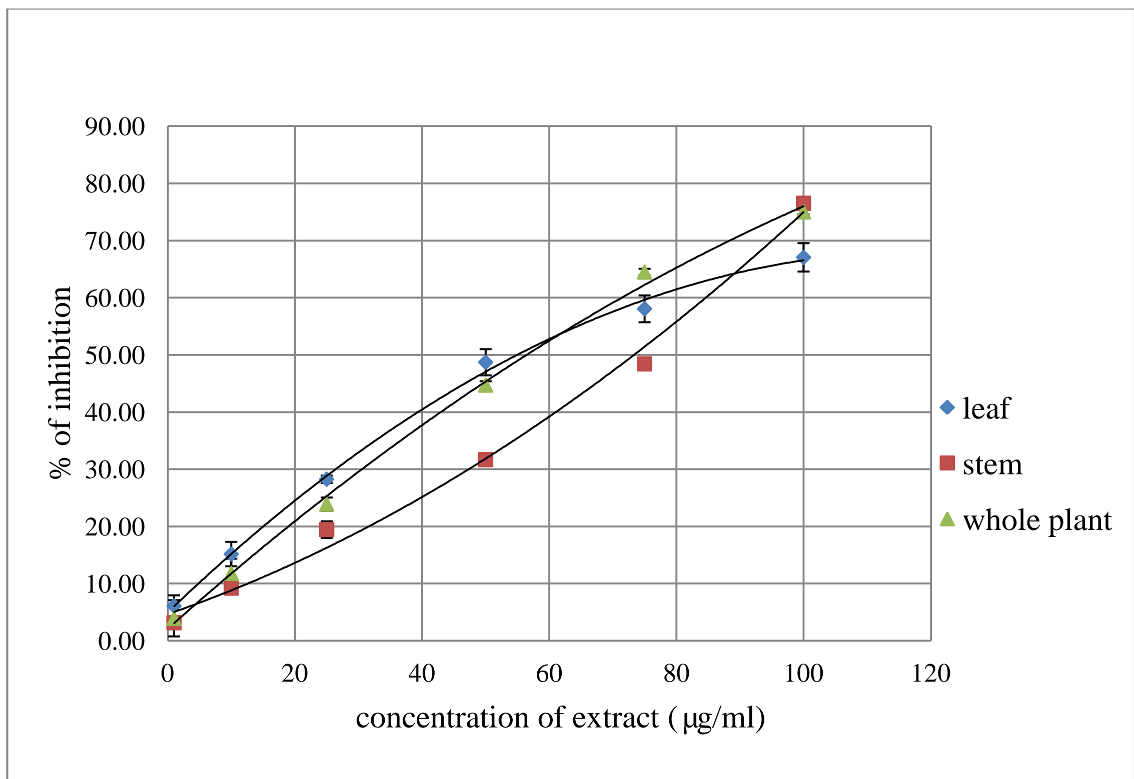

Figure 4.3 (A): The *in vitro* growth inhibition of CaSki cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

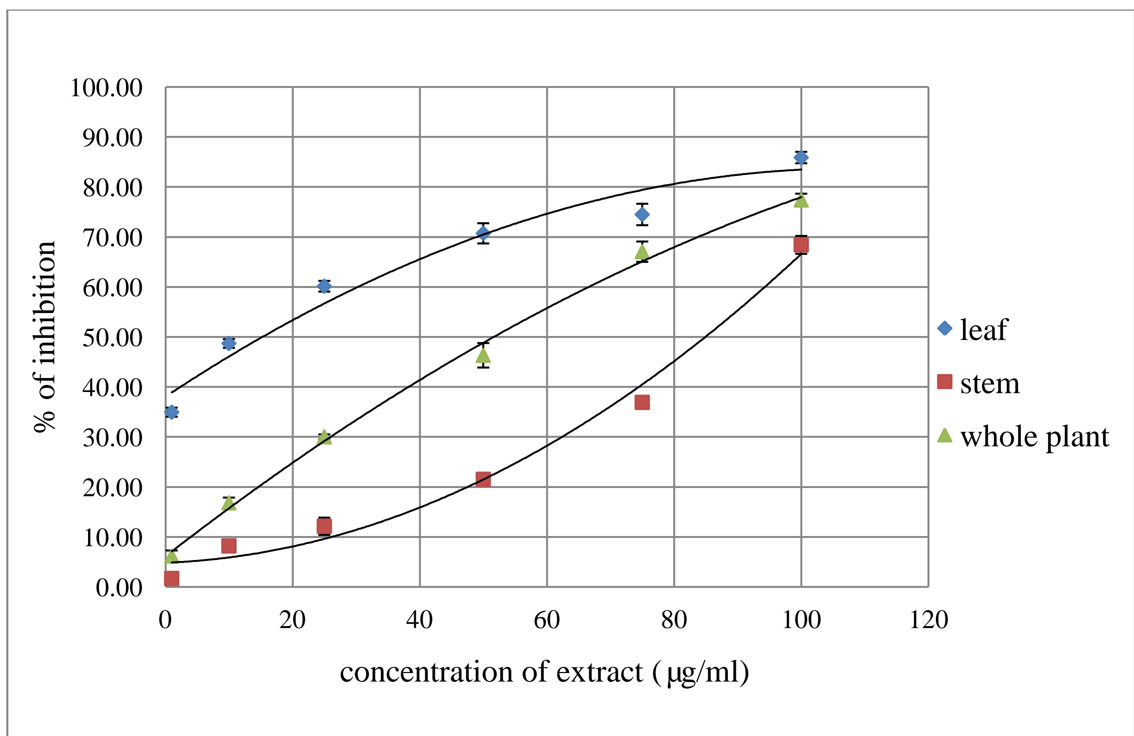

Figure 4.3 (B): The *in vitro* growth inhibition of CaSki cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

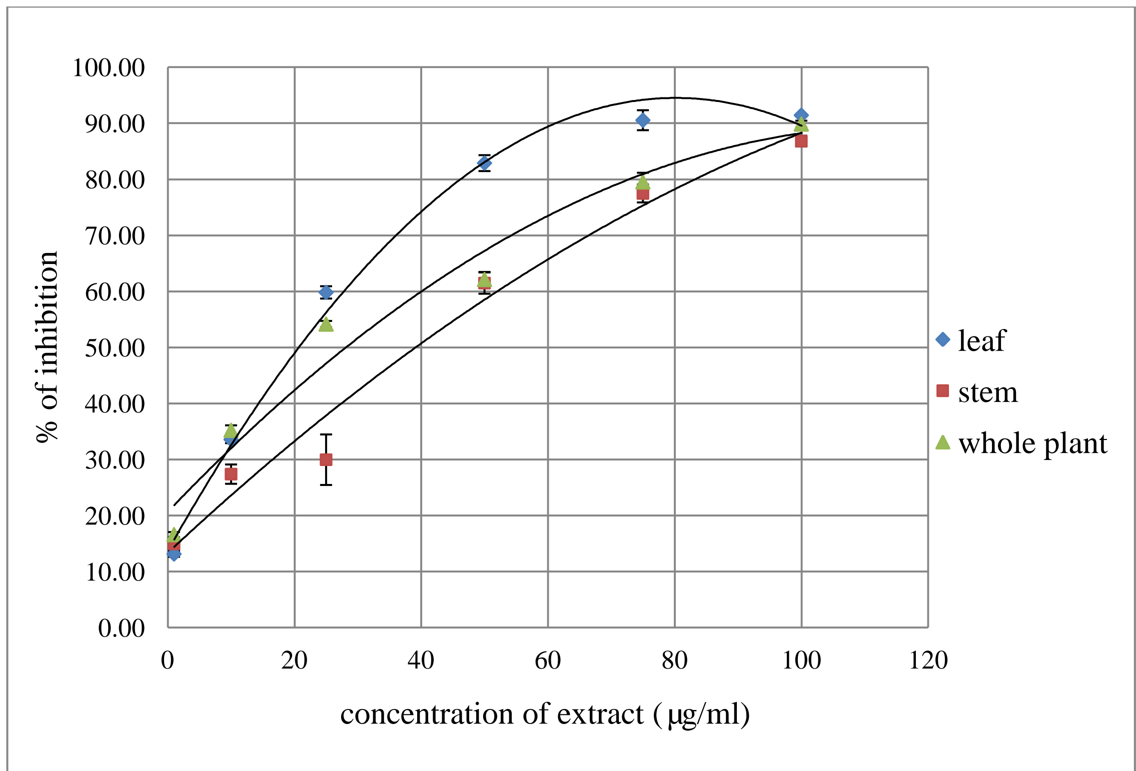

Figure 4.3 (C): The *in vitro* growth inhibition of SKOV-3 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

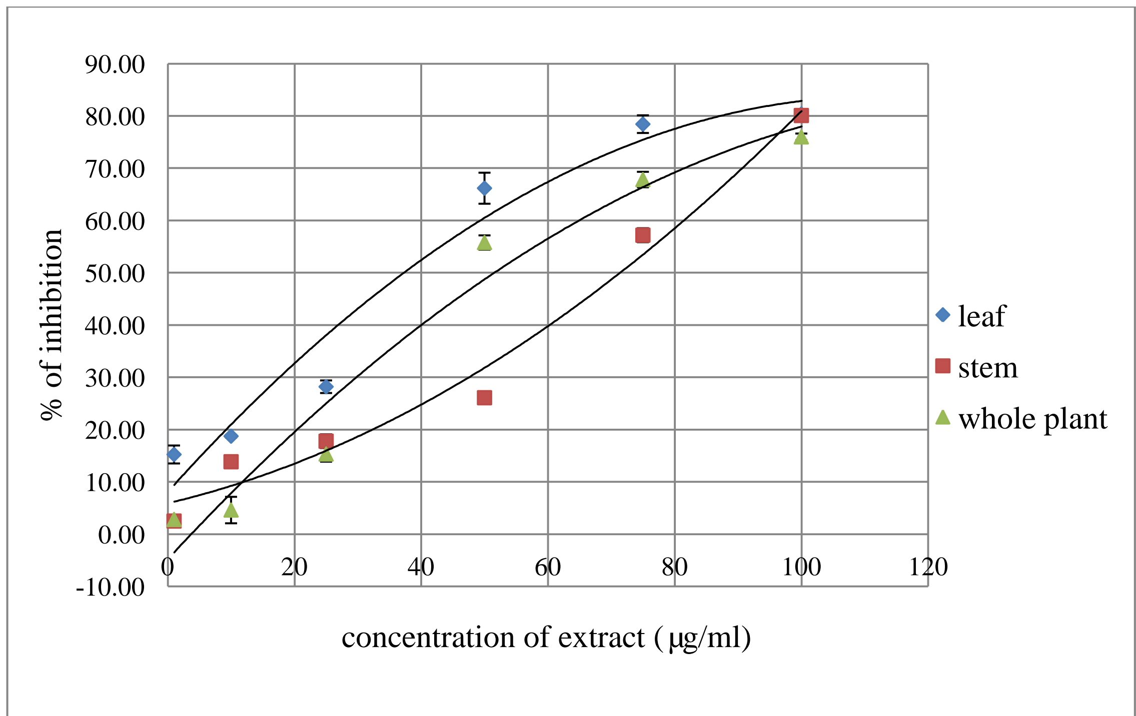

Figure 4.3 (D): The *in vitro* growth inhibition of SKOV-3 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

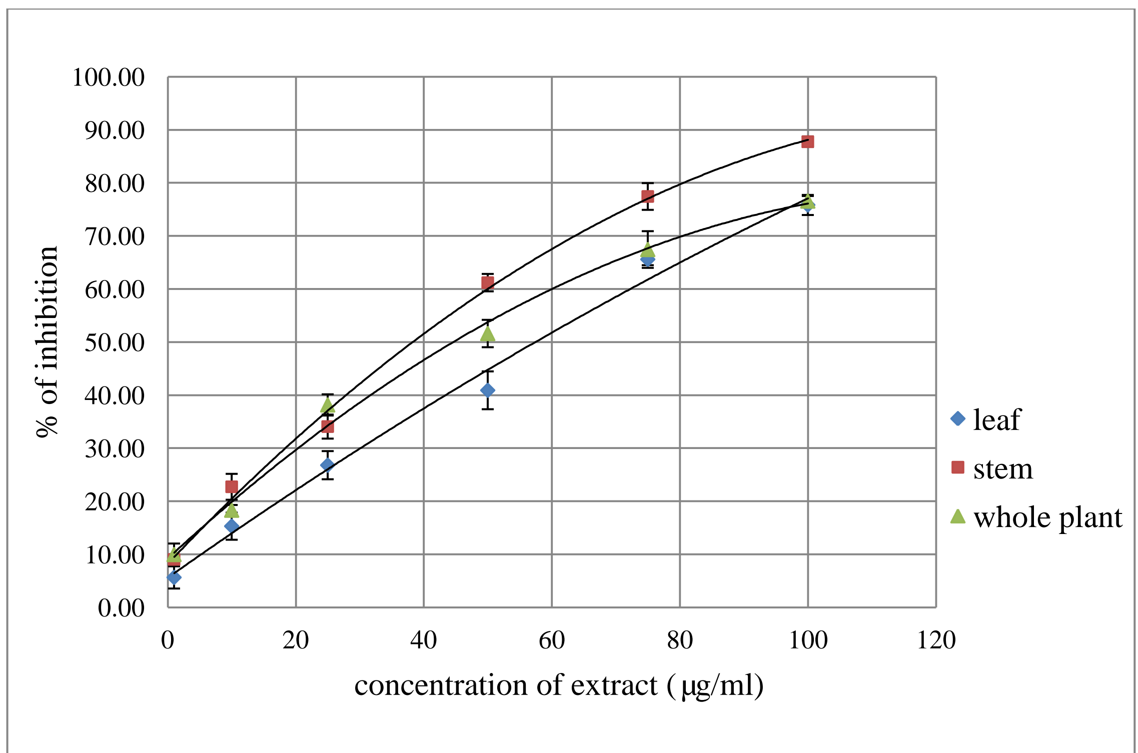

Figure 4.3 (E): The *in vitro* growth inhibition of HT-29 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

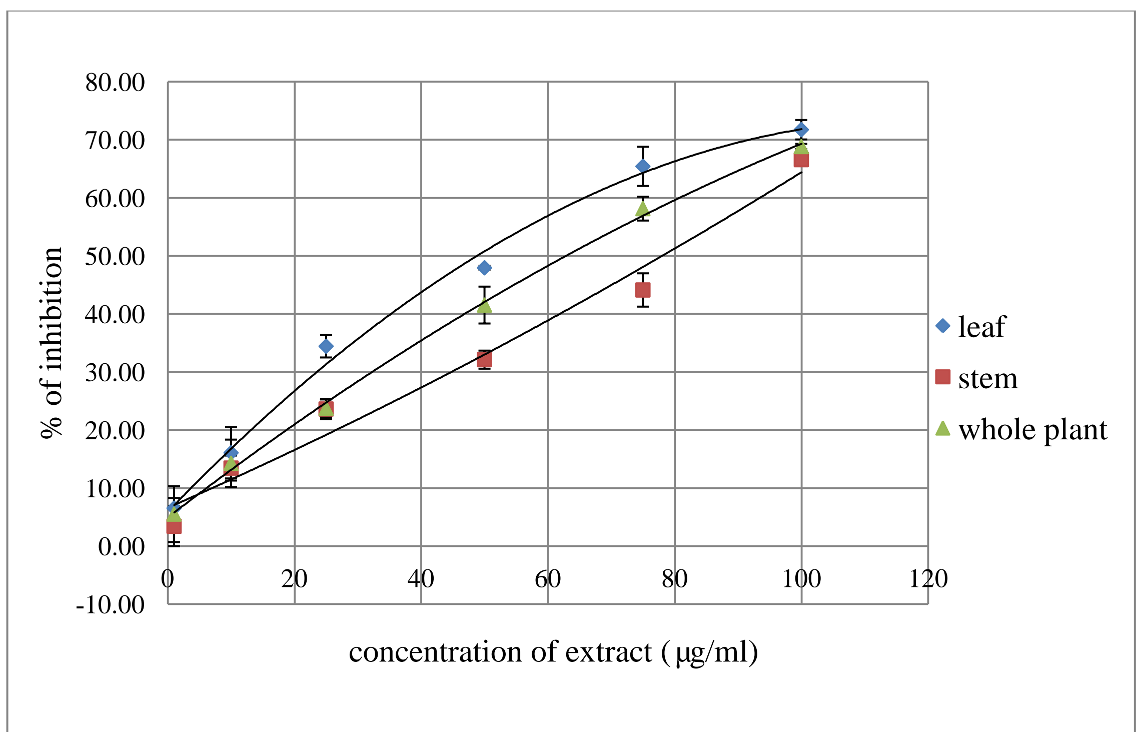

Figure 4.3 (F): The *in vitro* growth inhibition of HT-29 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

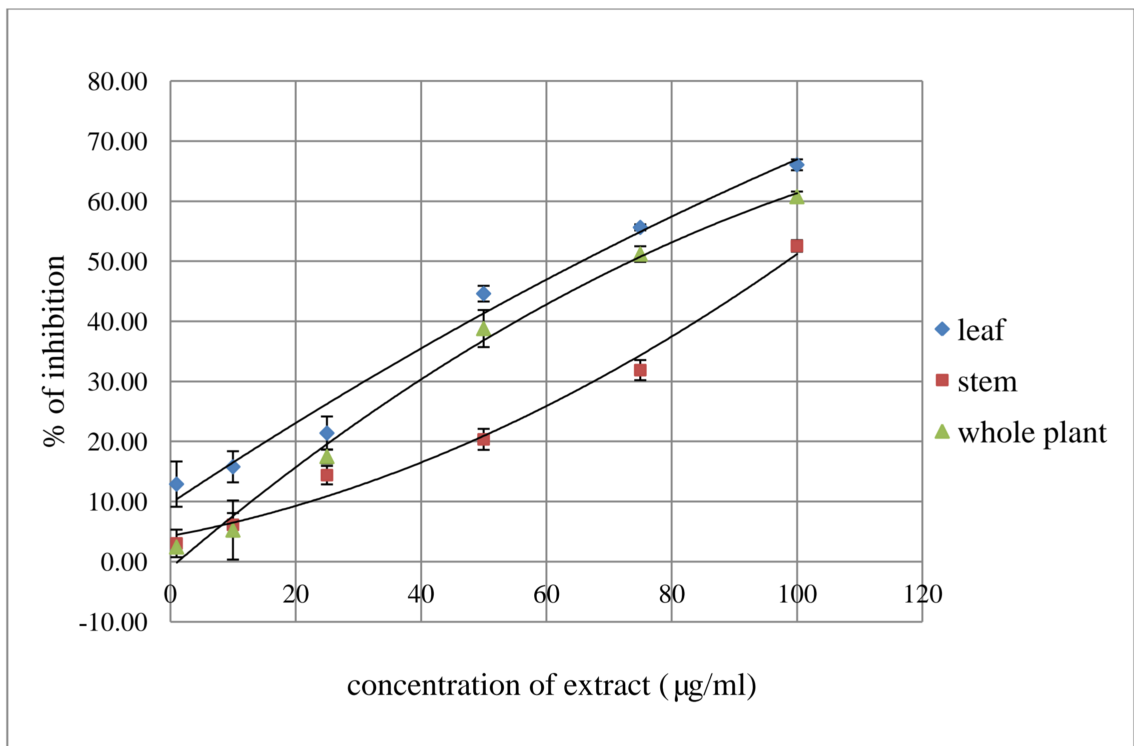

Figure 4.3 (G): The *in vitro* growth inhibition of A549 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

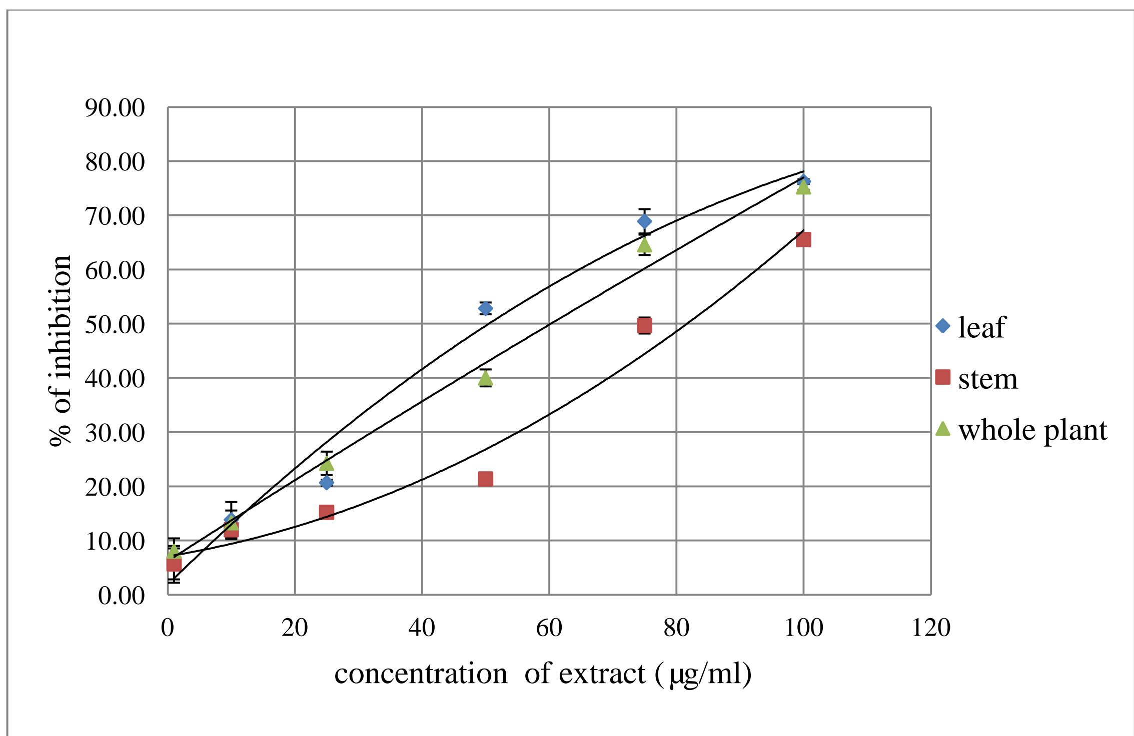

Figure 4.3 (H): The *in vitro* growth inhibition of A549 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

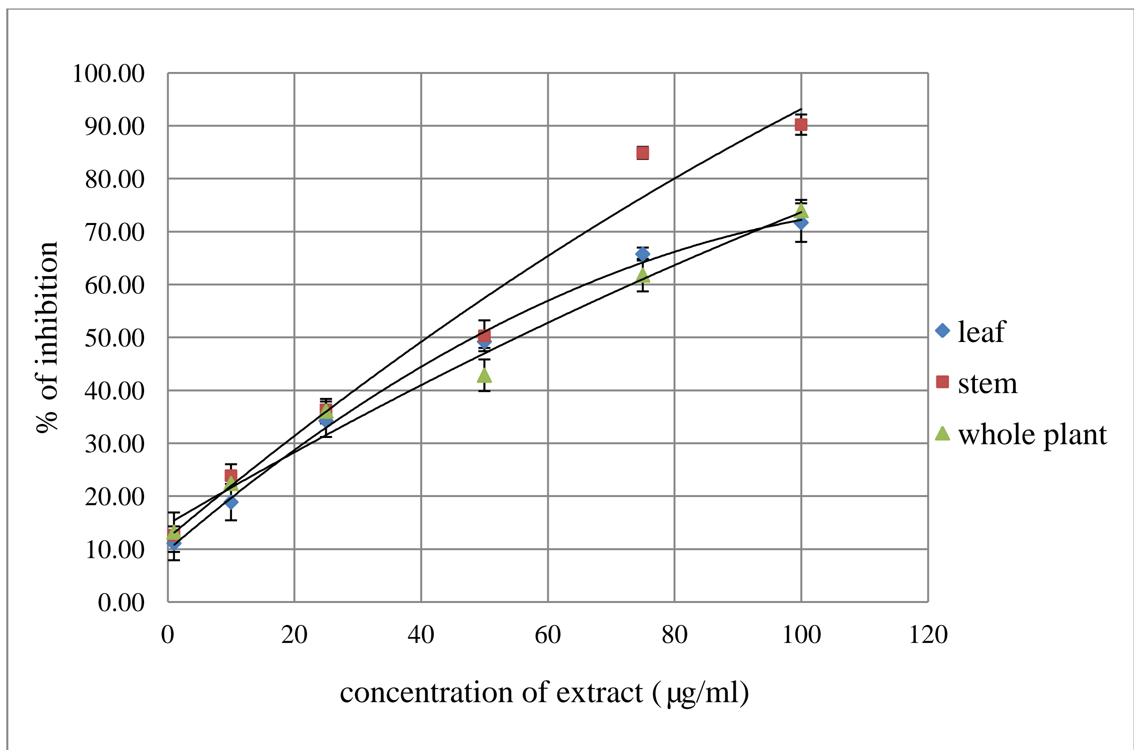

Figure 4.3 (I): The *in vitro* growth inhibition of MCF7 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

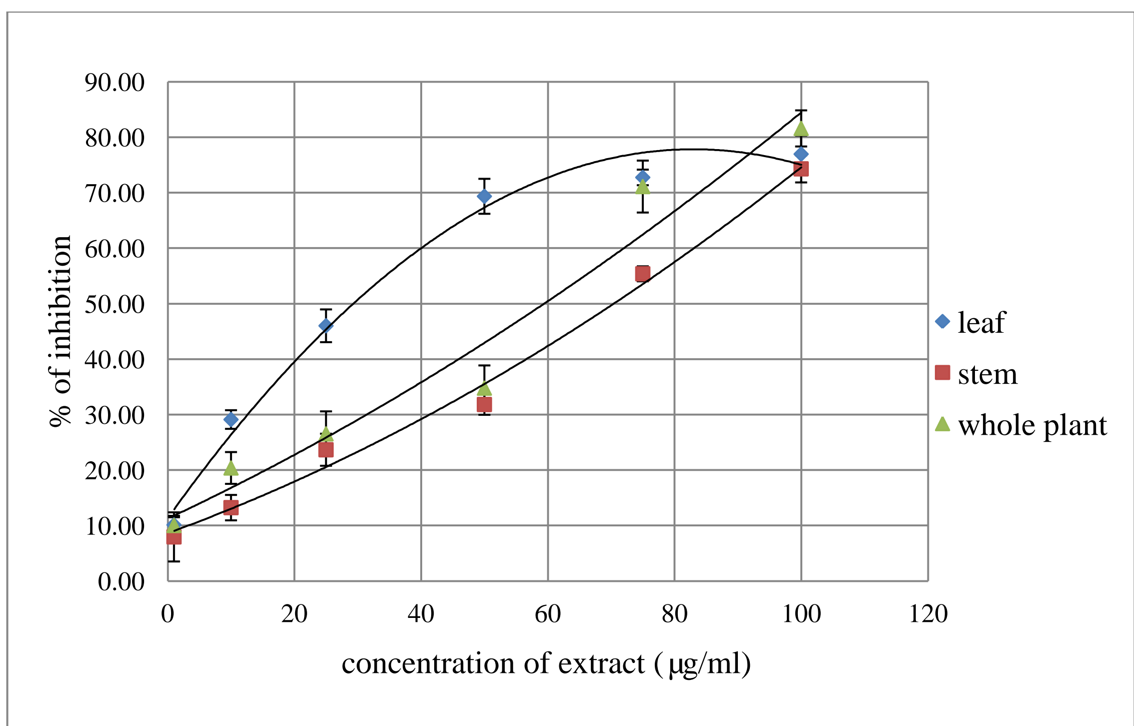

Figure 4.3 (J): The *in vitro* growth inhibition of MCF7 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

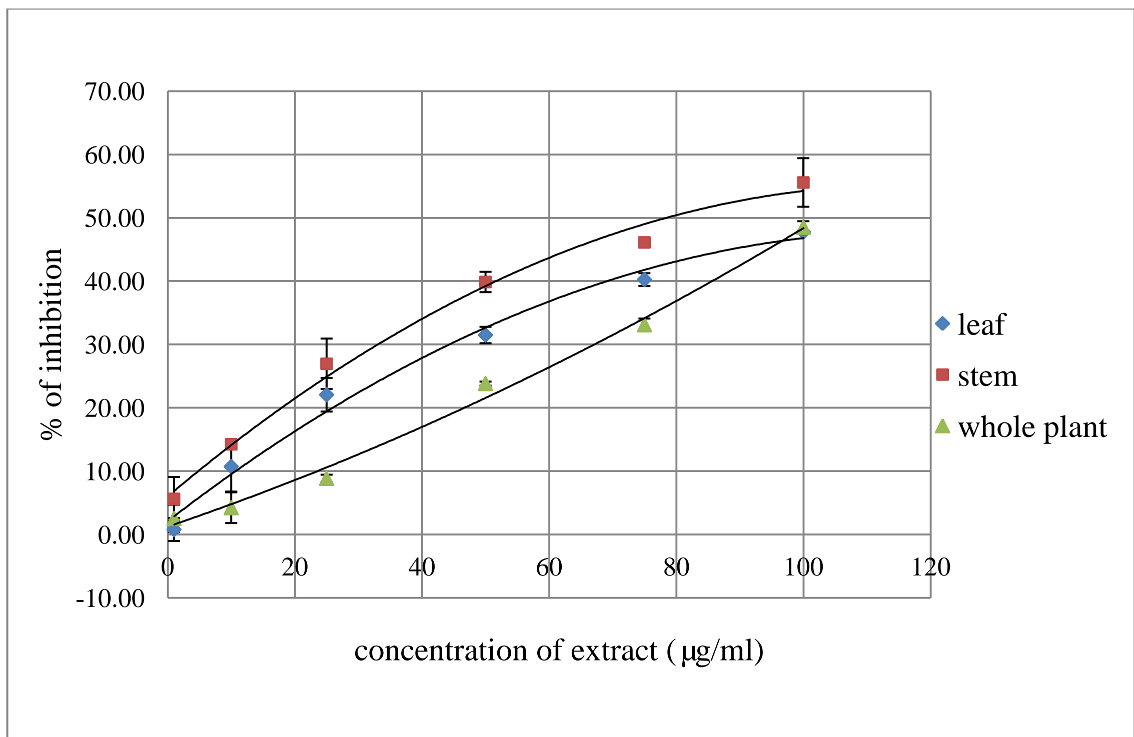

Figure 4.3 (K): The *in vitro* growth inhibition of MRC-5 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

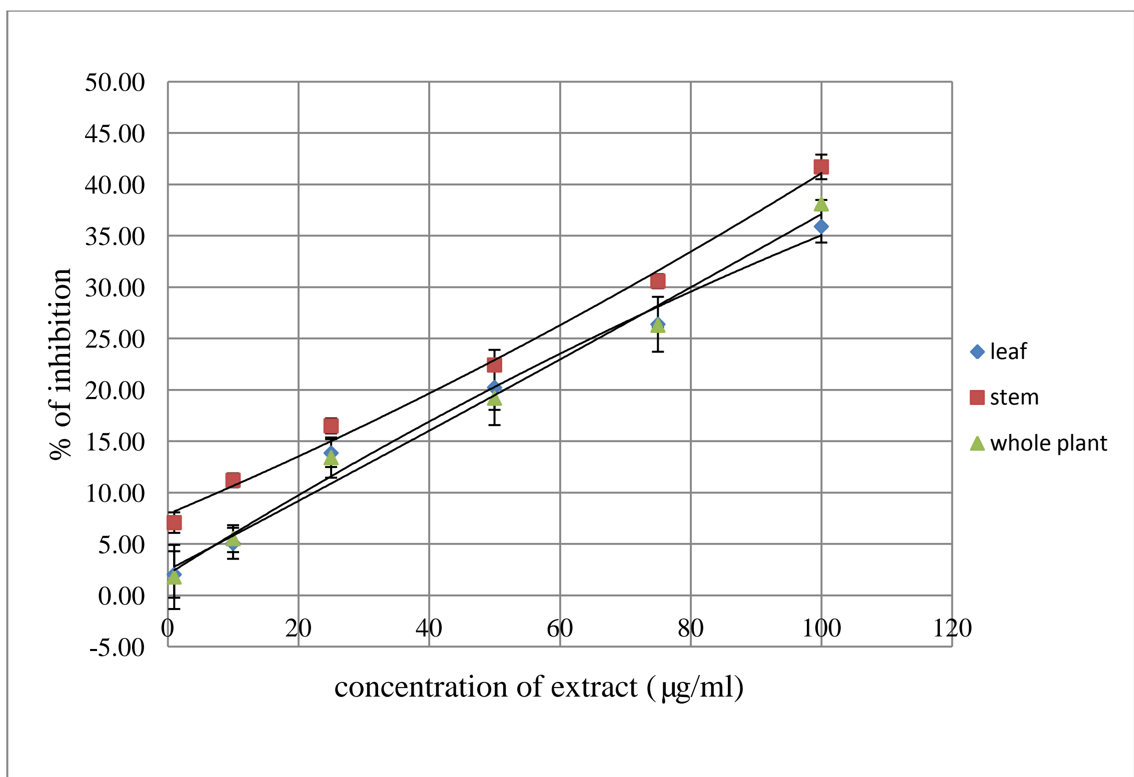

Figure 4.3 (L): The *in vitro* growth inhibition of MRC-5 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

4.4.2.(b) Cytotoxic activity of the dichloromethane extracts of *P. chinensis* var. *chinensis*

As illustrated from Figures 4.5 (A–L), all cancer cell lines evaluated showed different cytotoxic profiles when treated with dichloromethane crude extracts from different plant parts of the lowland and highland varieties. The IC_{50} values for the dichloromethane crude extract were determined and summarized in Tables 4.2 and 4.3.

The present study shows that most of the dichloromethane crude extracts from *P. chinensis* var. *chinensis* were not actively cytotoxic against all cancer cell lines tested with IC_{50} values of more than 30 μg/ml except the stem and whole plant crude dichloromethane extract of the highland variety when tested against HT-29 which showed IC_{50} value of 13.17 μg/ml and 21.86 μg/ml, respectively. All of the dichloromethane crude extracts from the lowland variety showed very low ability to retard the growth of MCF-7 with the IC_{50} values of more than 100 μg/ml.

i) Human epithelial carcinoma of cervix cell line, CaSki

The leaf, stem and whole plant crude dichloromethane extracts of the highland and lowland varieties showed IC_{50} values ranging from 55 μg/ml to more than 100 μg/ml (Tables 4.2 & 4.3).These results showed that all of the dichloromethane crude extracts were not actively cytotoxic against the CaSki cells.

ii) Human epithelial adenocarcinoma of ovary cell line, SKOV-3

The cytotoxic activity of all the crude dichloromethane extracts of the highland and lowland varieties were not actively cytotoxic against the SKOV-3 cells as the resulting IC50 values ranged from 77 μg/ml to 91 μg/ml (Tables 4.2 & 4.3).

iii) Human epithelial adenocarcinoma of colon cell line, HT-29

All the leaf, stem and whole plant crude dichloromethane extracts from the highland variety exhibited better growth inhibition compared to the lowland variety when tested against HT-29 cells, with IC_{50} values less than 56 μg/ml. The stem crude dichloromethane extracts of the highland variety against HT-29 exhibited an IC_{50} value of 13.17 μg/ml (Tables 4.2 and 4.3). The inhibition percentages against HT-29 cells by the stem dichloromethane extract of the highland variety were 35% at 10 μg/ml, 54% at 25 μg/ml and 90% at 100 μg/ml. The crude dichloromethane extract of the whole plant of the highland variety exhibited an IC_{50} value of 21.86 μg/ml against HT-29 cells. The growth inhibition percentages were 31% at 10 μg/ml, 55% at 25 μg/ml and 77% at 100 μg/ml. These stem and whole plant dichloromethane crude extracts were actively cytotoxic against HT-29 cell lines.

iv) Human epithelial carcinoma of lung cell line, A549

Similar to the SKOV-3 and CaSki cells, all the dichloromethane crude extracts from the leaf, stem and whole plant of the highland and lowland varieties were not actively cytotoxic against A549 cell lines. The resulting IC_{50} values ranged from 52 μg/ml to more than 100 μg/ml (Tables 4.2 & 4.3).

v) Human epithelial adenocarcinoma of mammary gland (breast) cell line, MCF7

Crude dichloromethane extracts of the leaf, stem and whole plant of the highland and lowland varieties were not actively cytotoxic against MCF7 with IC_{50} values ranging from 64 μg/ml to more than 100 μg/ml (Tables 4.2 & 4.3). All the dichloromethane crude extracts from the lowland variety showed very high IC_{50} values,

i.e. more than 100 μg/ml. These results showed a low ability to inhibit the growth of MCF-7 with the IC_{50} values of more than 100 μg/ml.

vi) Normal human lung fibroblasts cell, MRC-5

All of the dichloromethane crude extracts from the lowland and highland varieties were not actively cytotoxic against normal human lung fibroblasts cell, MRC-5 (Tables 4.2 and 4.3). The IC_{50} values ranged from 64 μg/ml to more than 100 μg/ml.

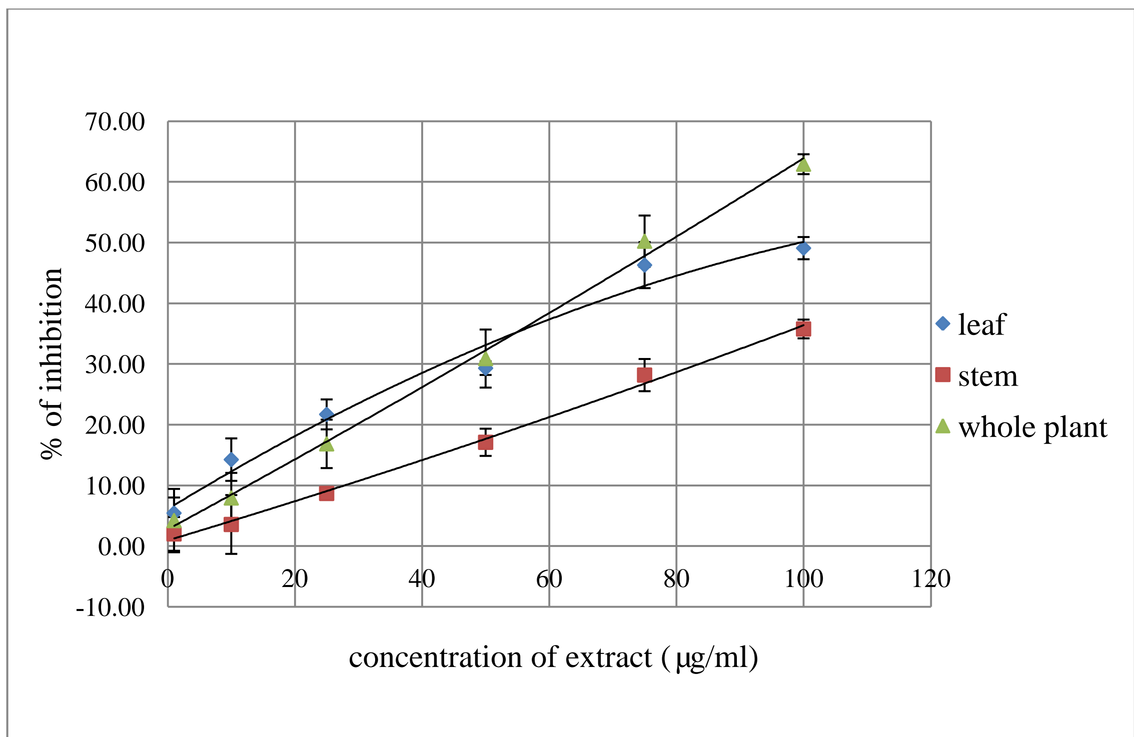

Figure 4.4 (A): The *in vitro* growth inhibition of CaSki cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

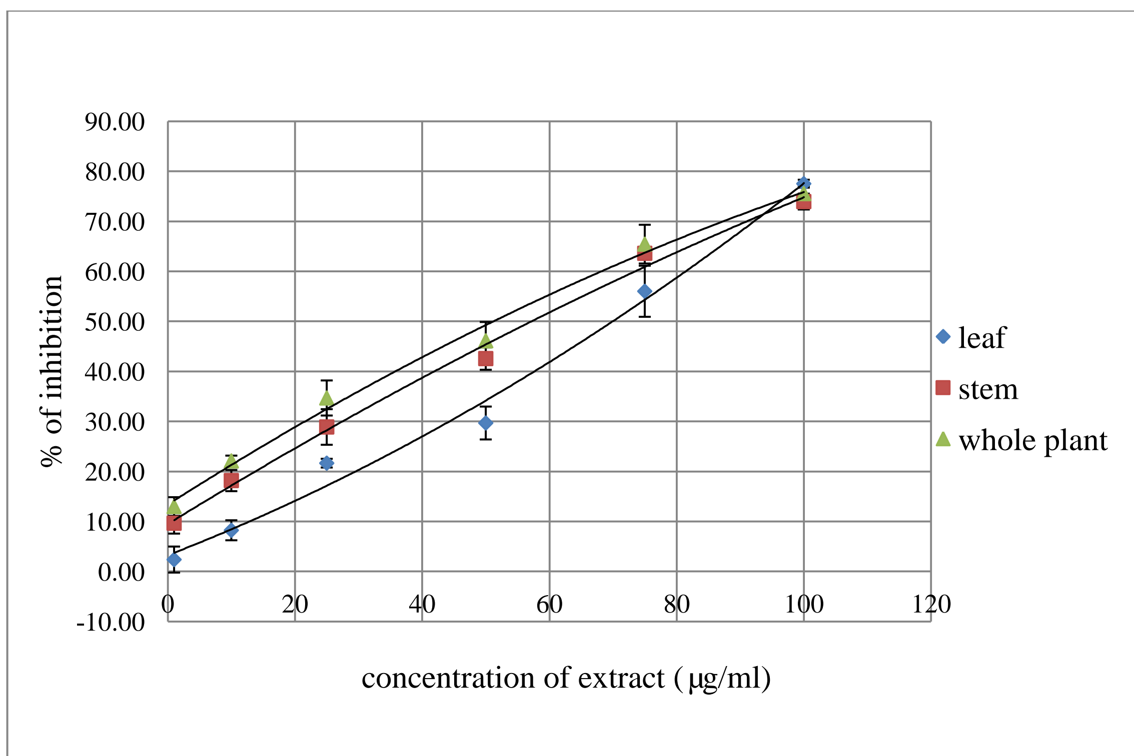

Figure 4.4 (B): The *in vitro* growth inhibition of CaSki cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

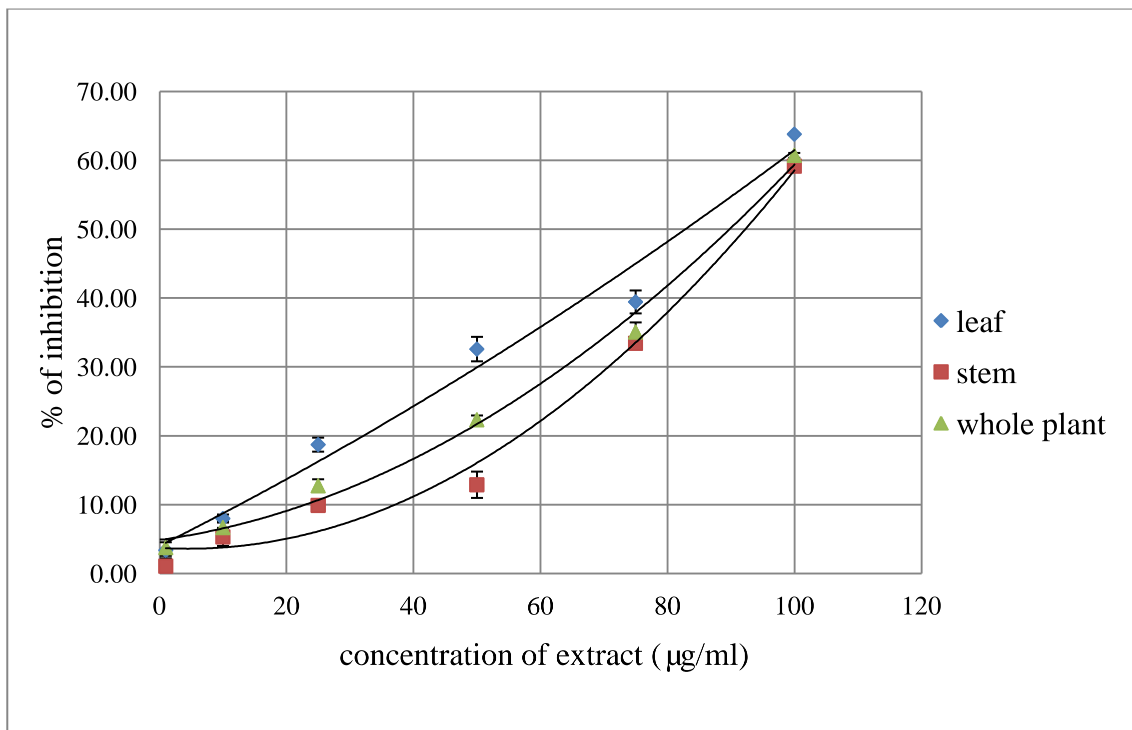

Figure 4.4 (C): The *in vitro* growth inhibition of SKOV-3 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

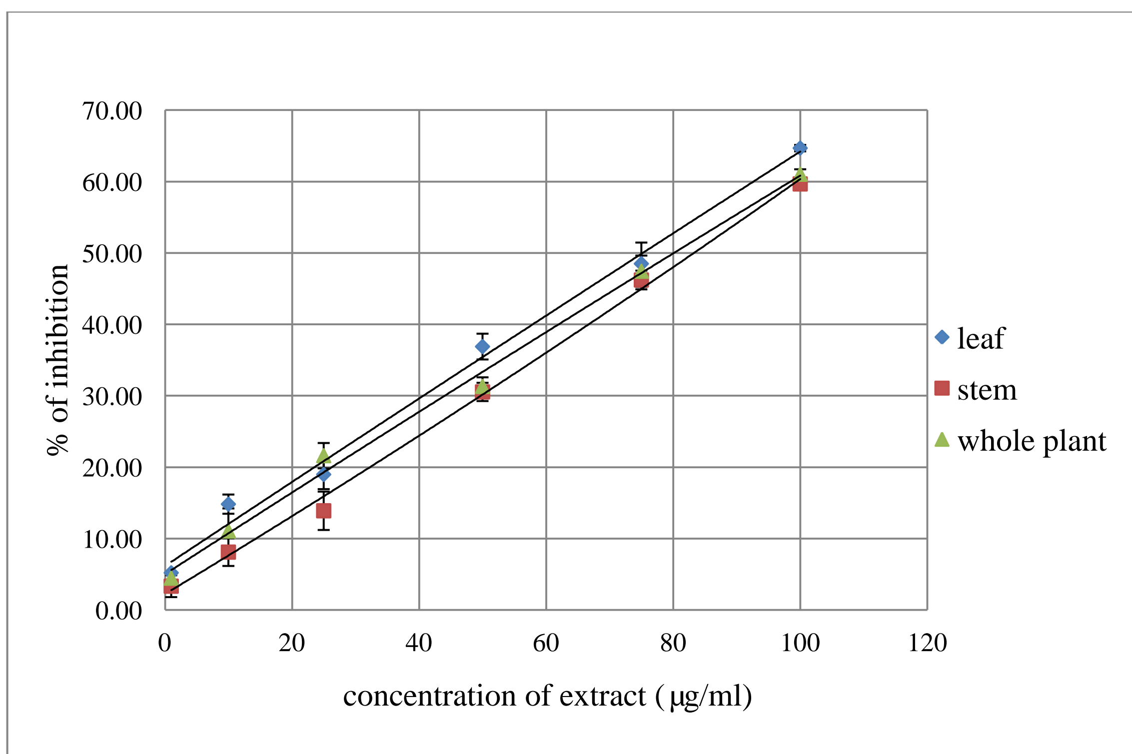

Figure 4.4 (D): The *in vitro* growth inhibition of SKOV-3 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

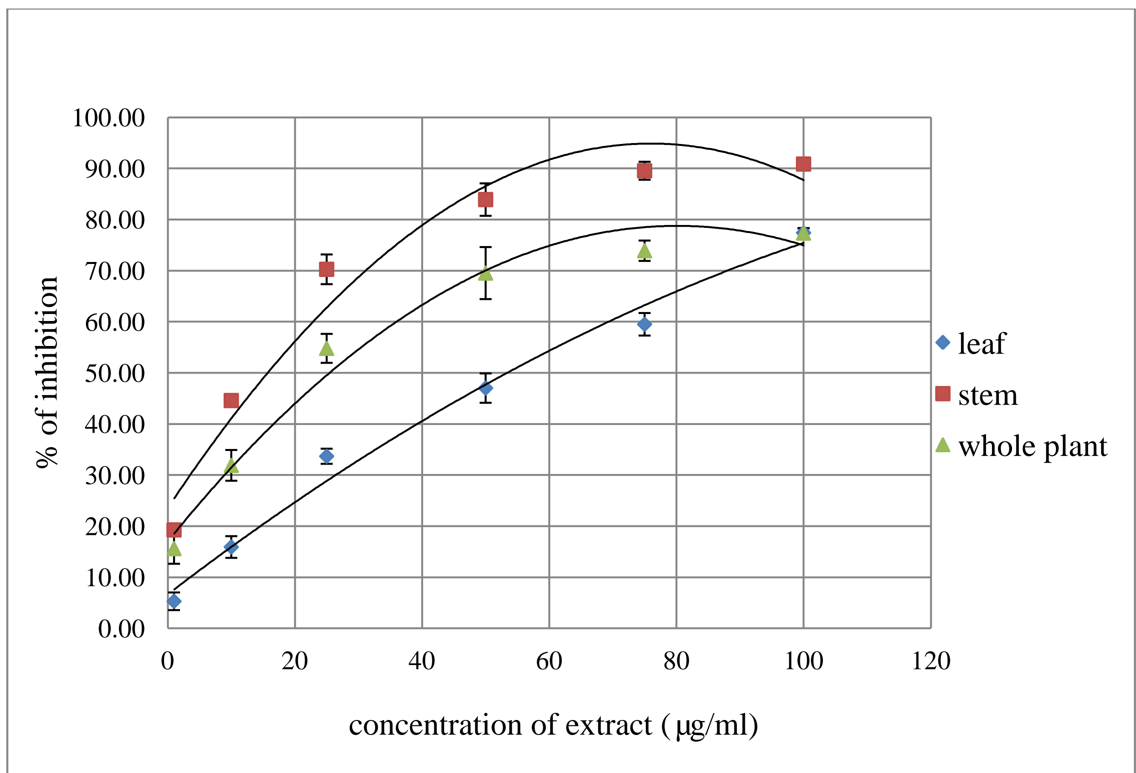

Figure 4.4 (E): The *in vitro* growth inhibition of HT-29 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

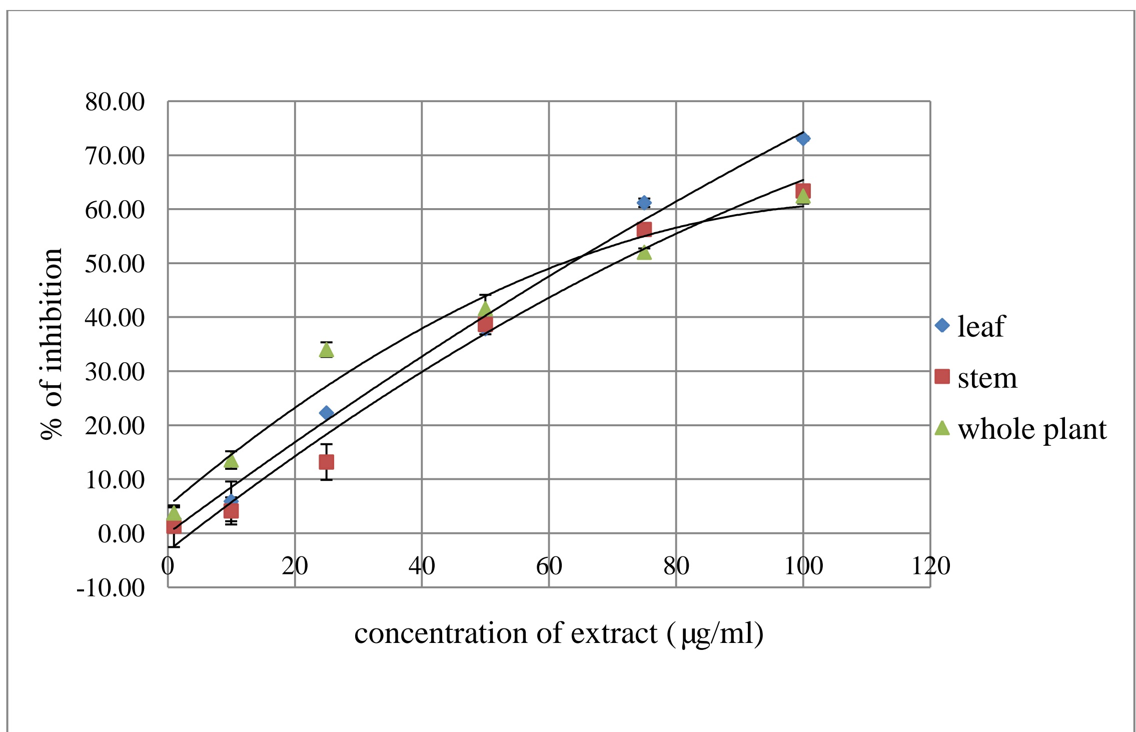

Figure 4.4 (F): The *in vitro* growth inhibition of HT-29 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

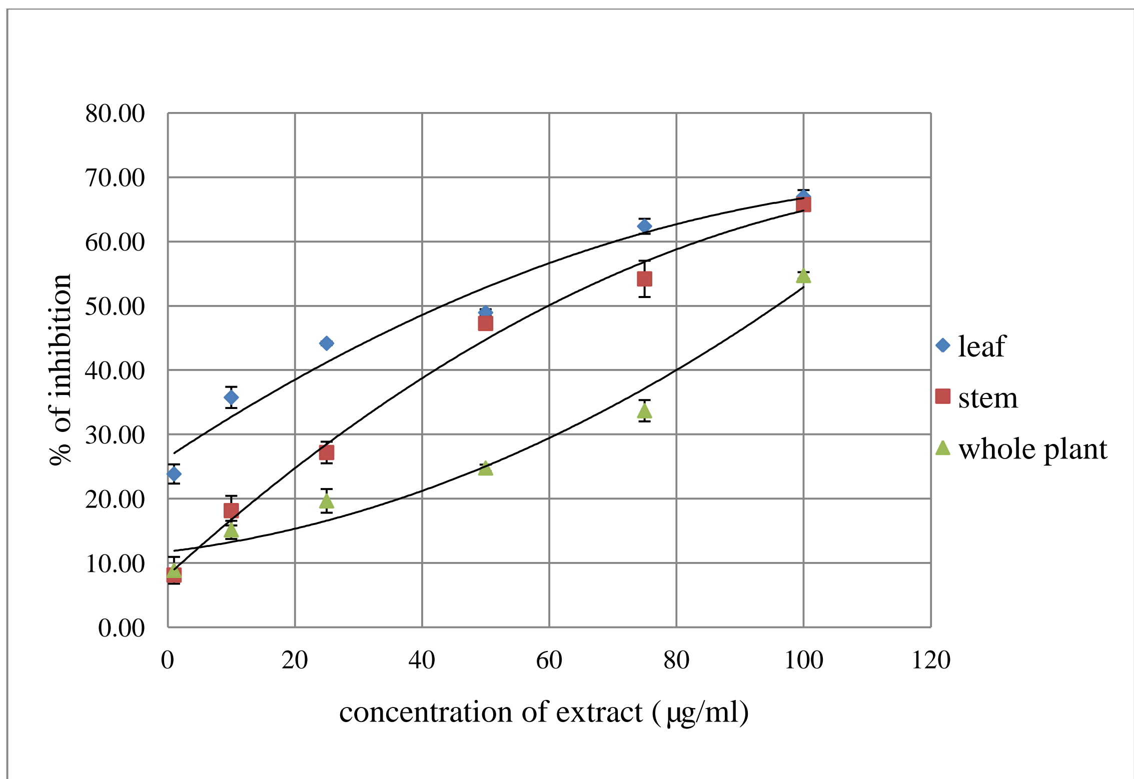

Figure 4.4 (G): The *in vitro* growth inhibition of A549 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

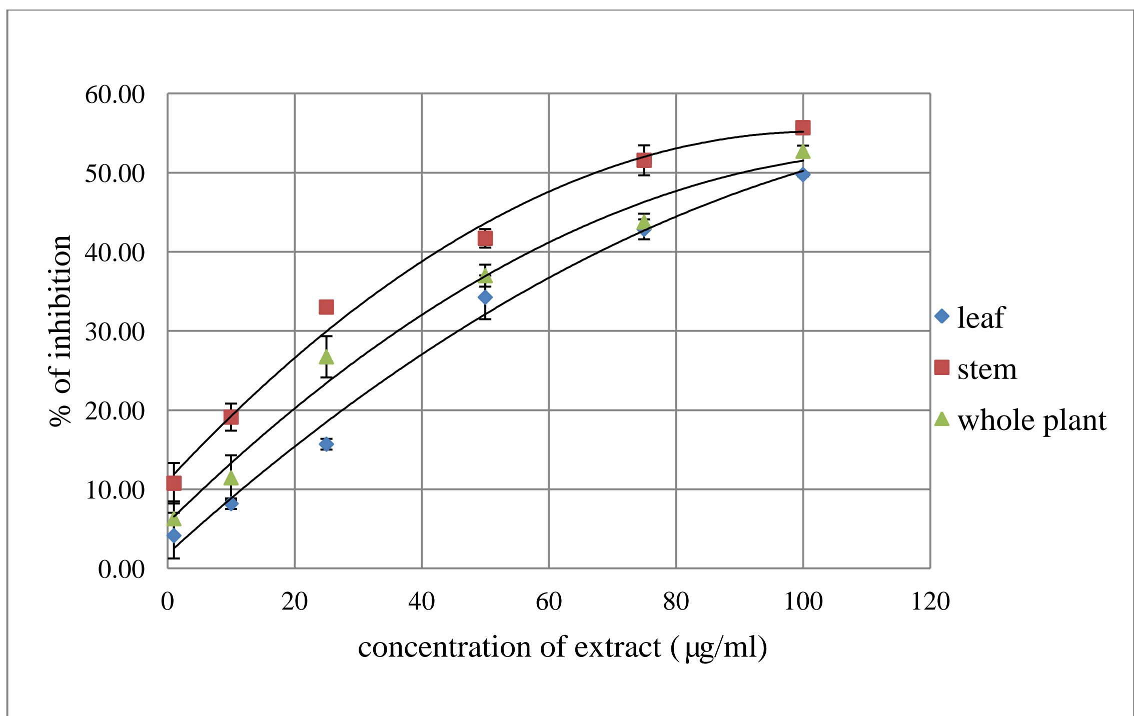

Figure 4.4 (H): The *in vitro* growth inhibition of A549 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

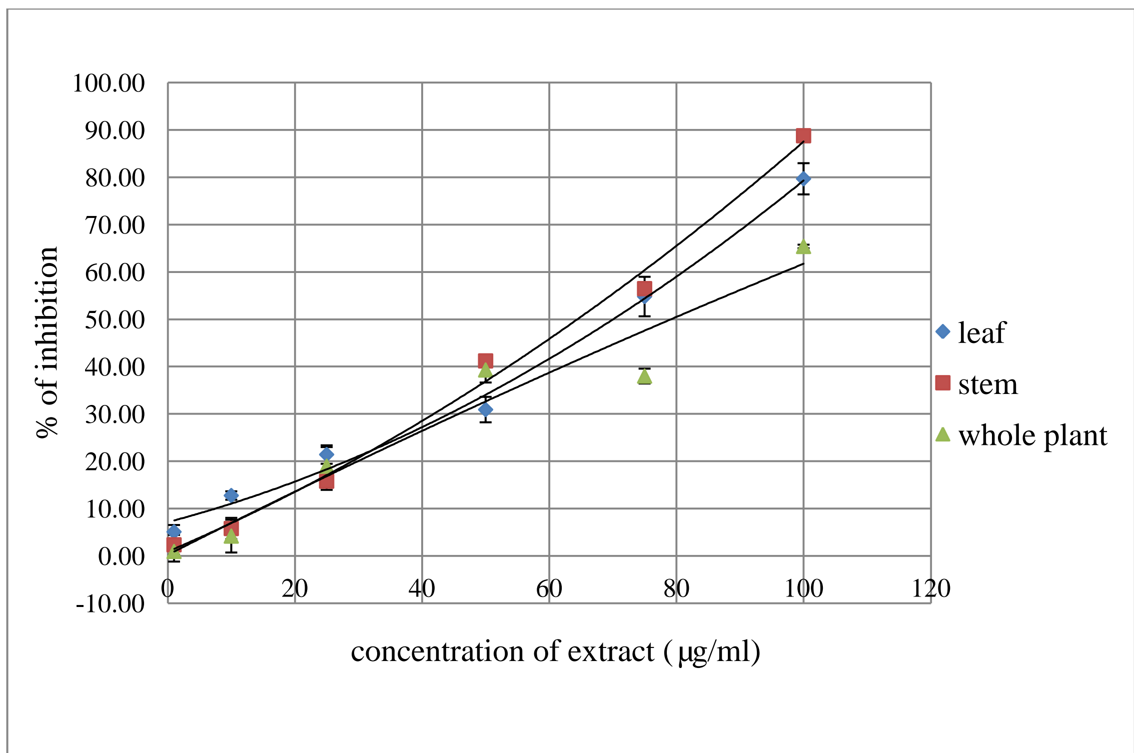

Figure 4.4 (I): The *in vitro* growth inhibition of MCF7 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

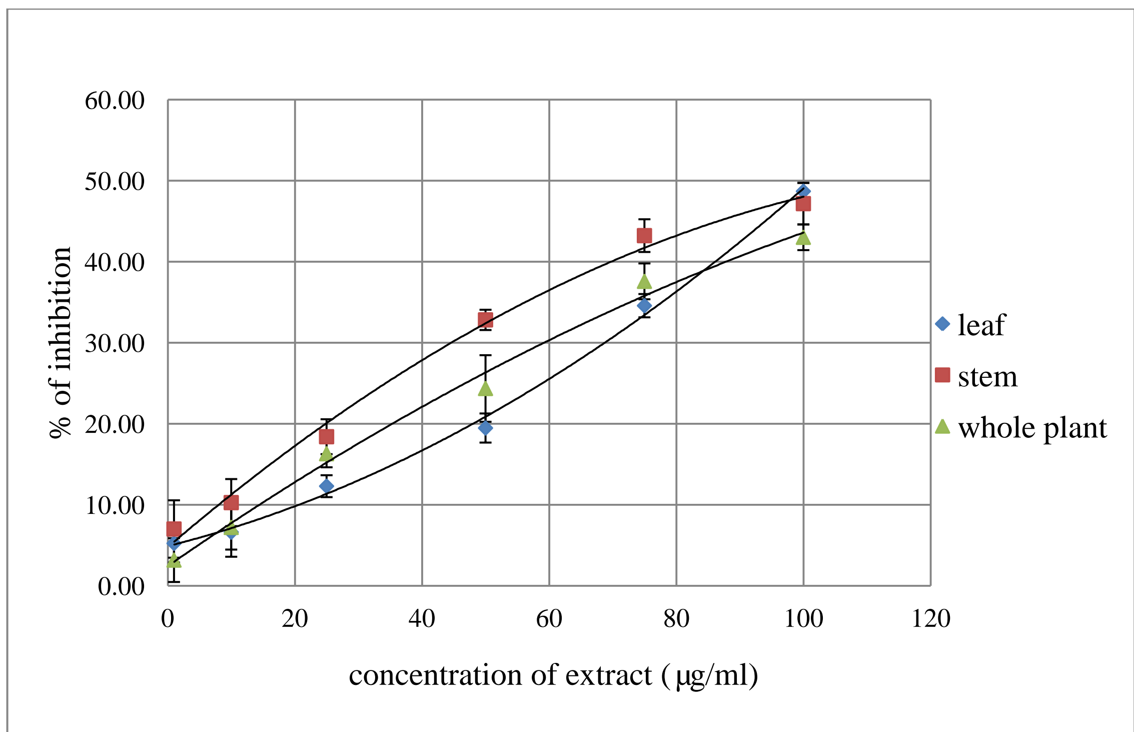

Figure 4.4 (J): The *in vitro* growth inhibition of MCF7 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

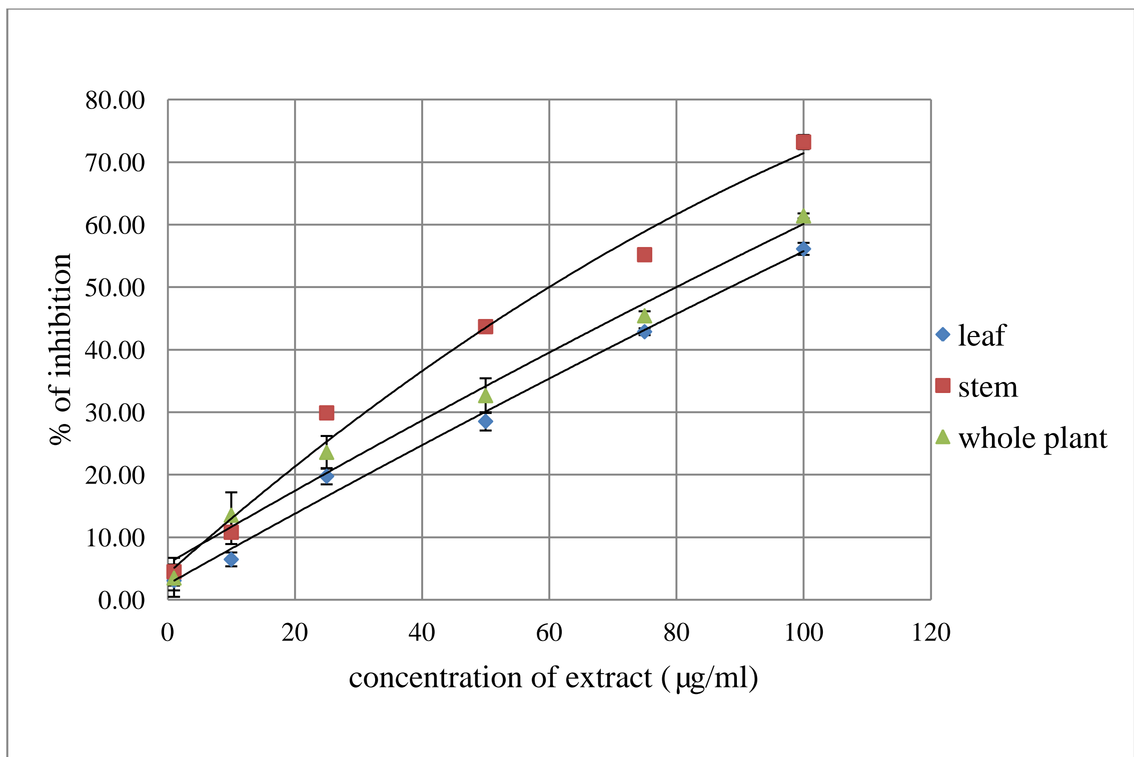

Figure 4.4 (K): The *in vitro* growth inhibition of MRC-5 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

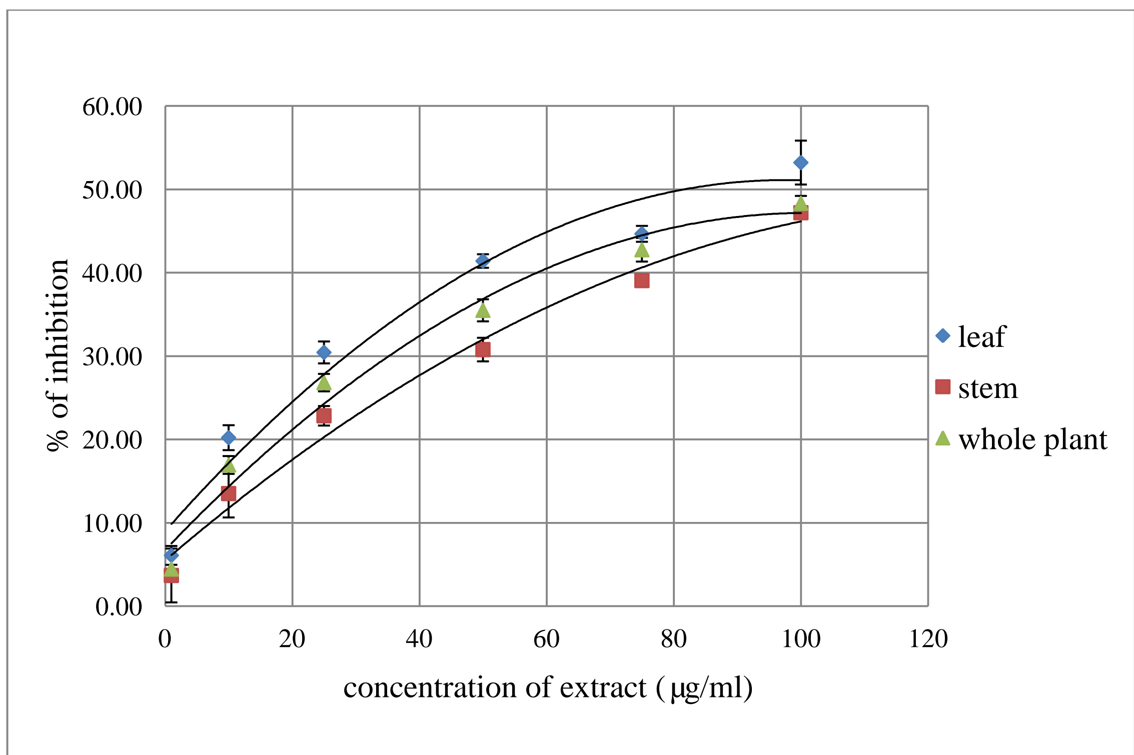

Figure 4.4 (L): The *in vitro* growth inhibition of MRC-5 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

4.4.2.(c) Cytotoxic activity of the water extracts of *P. chinensis* var. *chinensis*

Figures 4.6 (A–L) illustrates the growth inhibition percentages of all the cancer cell lines evaluated against water extracts of *P*. *chinensis* var. *chinensis*. Different cytotoxic profiles were observed when treated with water crude extracts derived from different plant parts of the lowland and highland varieties. The IC_{50} values for the water crude extract was determined and summarized in Tables 4.2 and 4.3.

i) CaSki, SKOV-3, HT-29, A549, MCF7 and MRC-5

Overall, the IC_{50} values of all the leaf, stem and whole plant crude water extracts derived from both the populations were high with the value of more than 60 μg/ml. The water extracts of *P*. *chinensis* var. *chinensis* are therefore considered as not actively cytotoxic against all of the cancer cell lines. All crude water extracts derived from both populations showed a low inhibition percentage of less than 40% and IC_{50} values of more than 100 μg/ml when tested on HT-29 and A549 cells at a high concentration of 100 μg/ml.

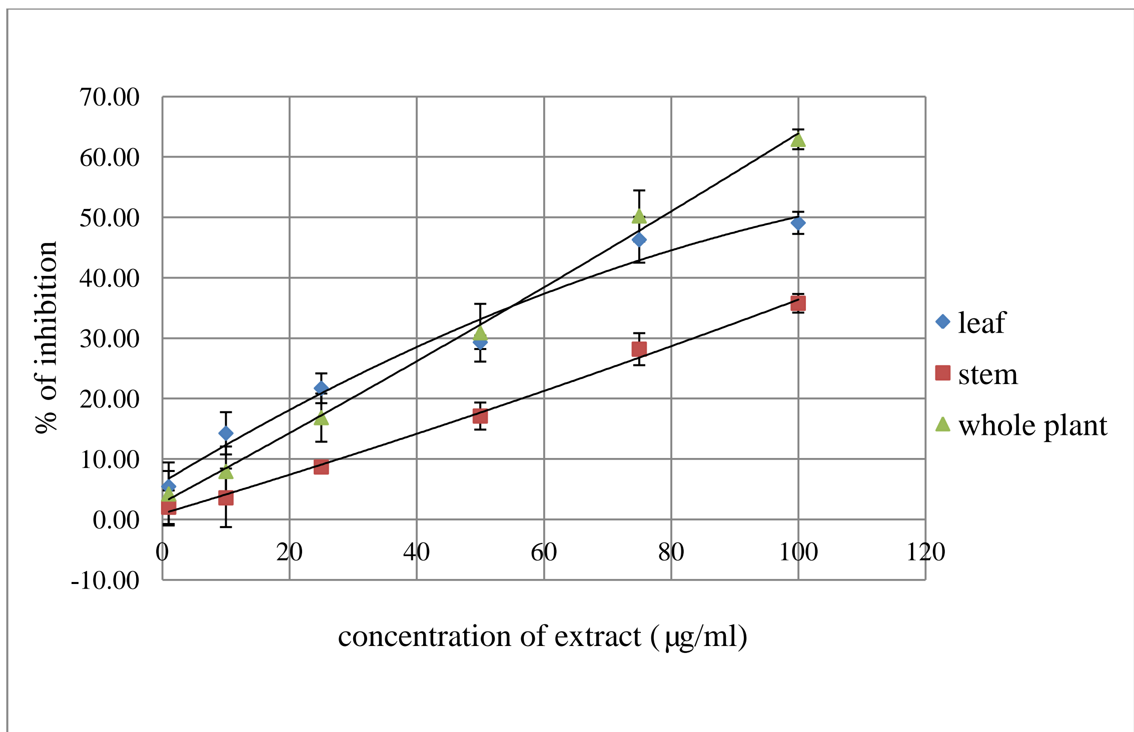

Figure 4.5 (A): The *in vitro* growth inhibition of CaSki cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

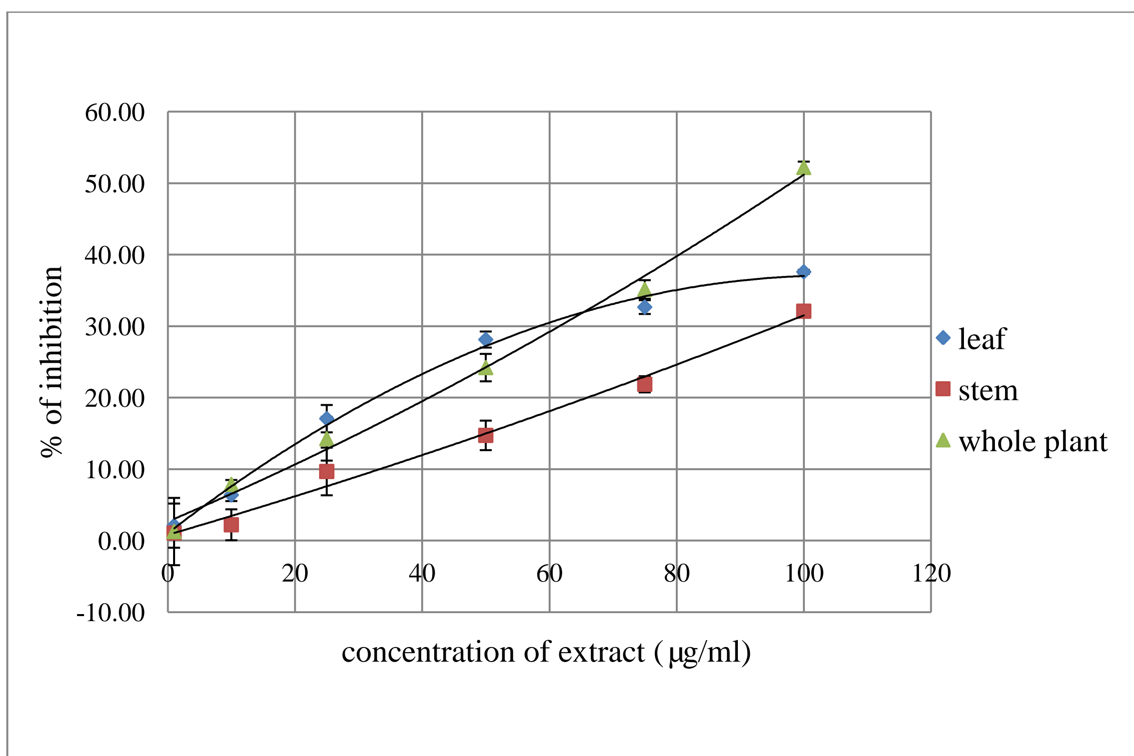

Figure 4.5 (B): The *in vitro* growth inhibition of CaSki cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

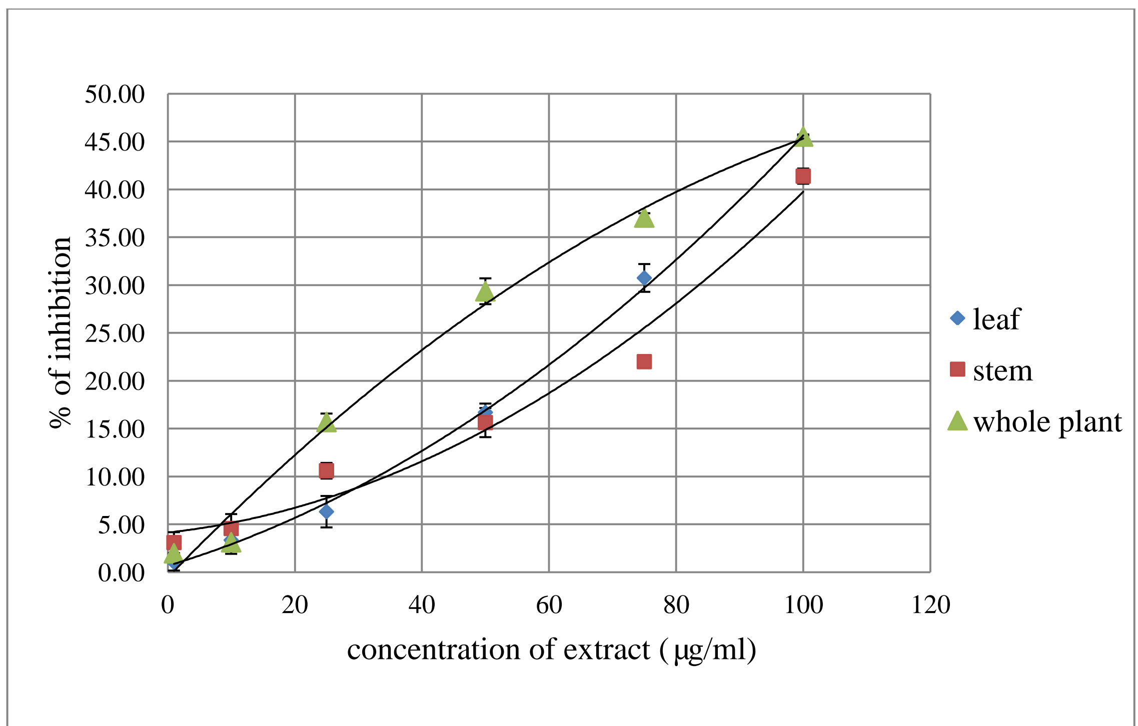

Figure 4.5 (C): The *in vitro* growth inhibition of SKOV-3 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

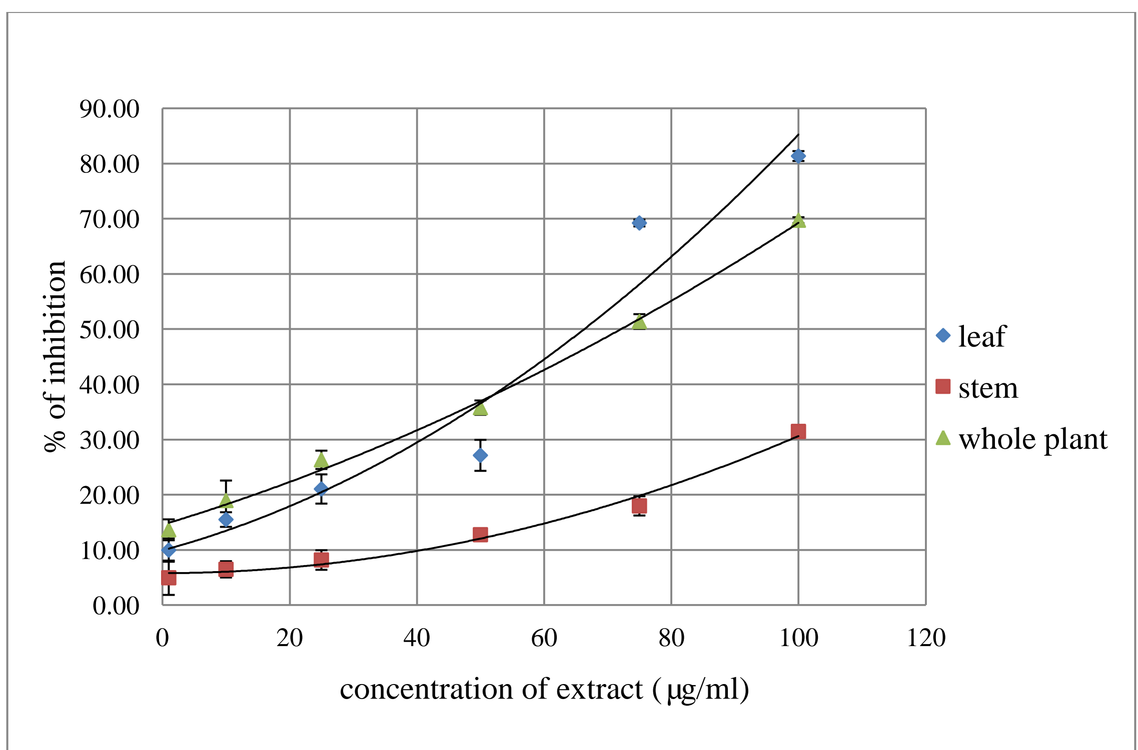

Figure 4.5 (D): The *in vitro* growth inhibition of SKOV-3 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

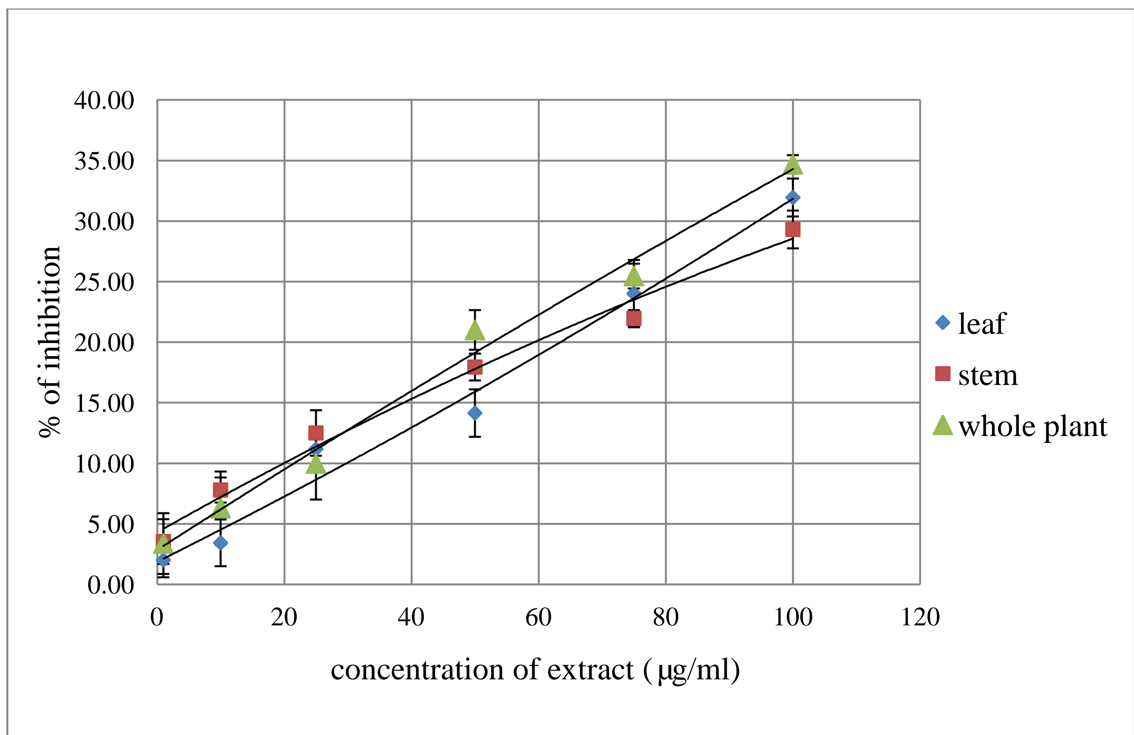

Figure 4.5 (E): The *in vitro* growth inhibition of HT-29 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

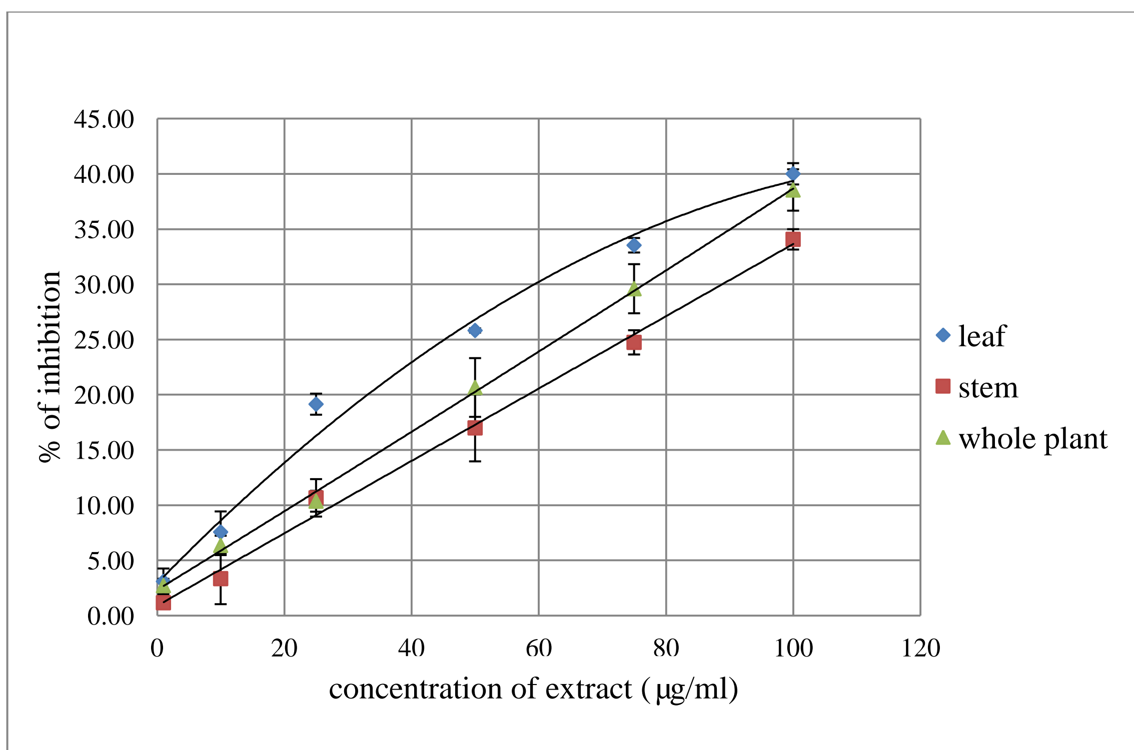

Figure 4.5 (F): The *in vitro* growth inhibition of HT-29 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

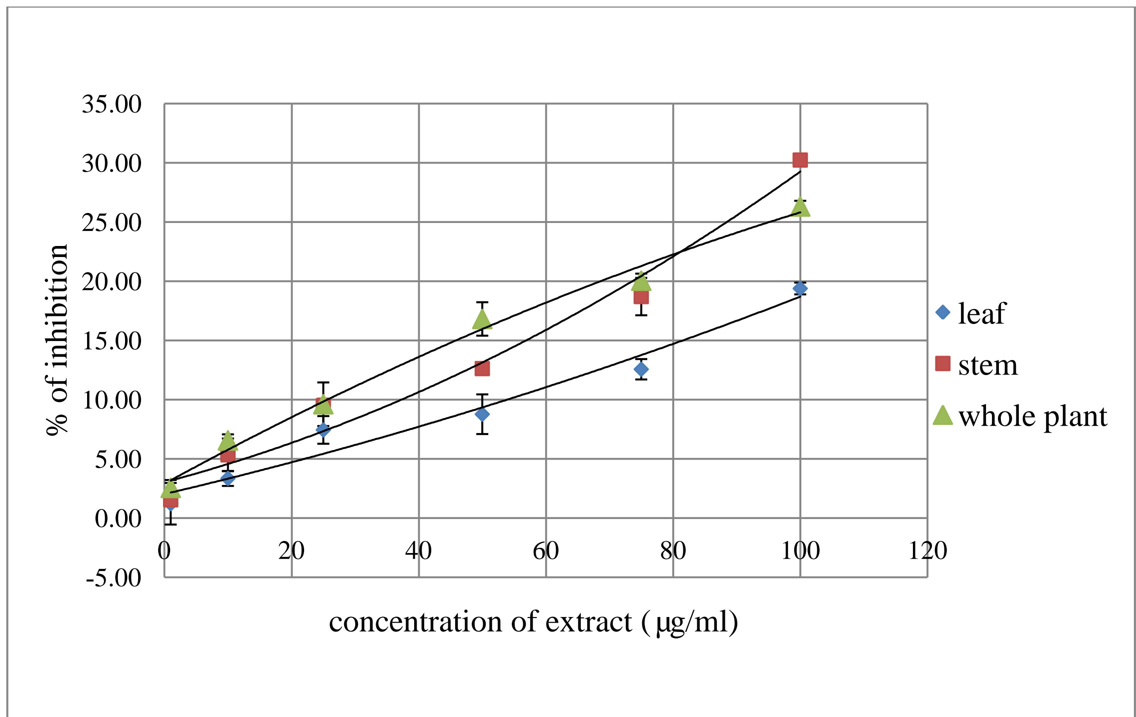

Figure 4.5 (G): The in vitro growth inhibition of A549 cells by crude water extracts of the highland variety of P. *chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

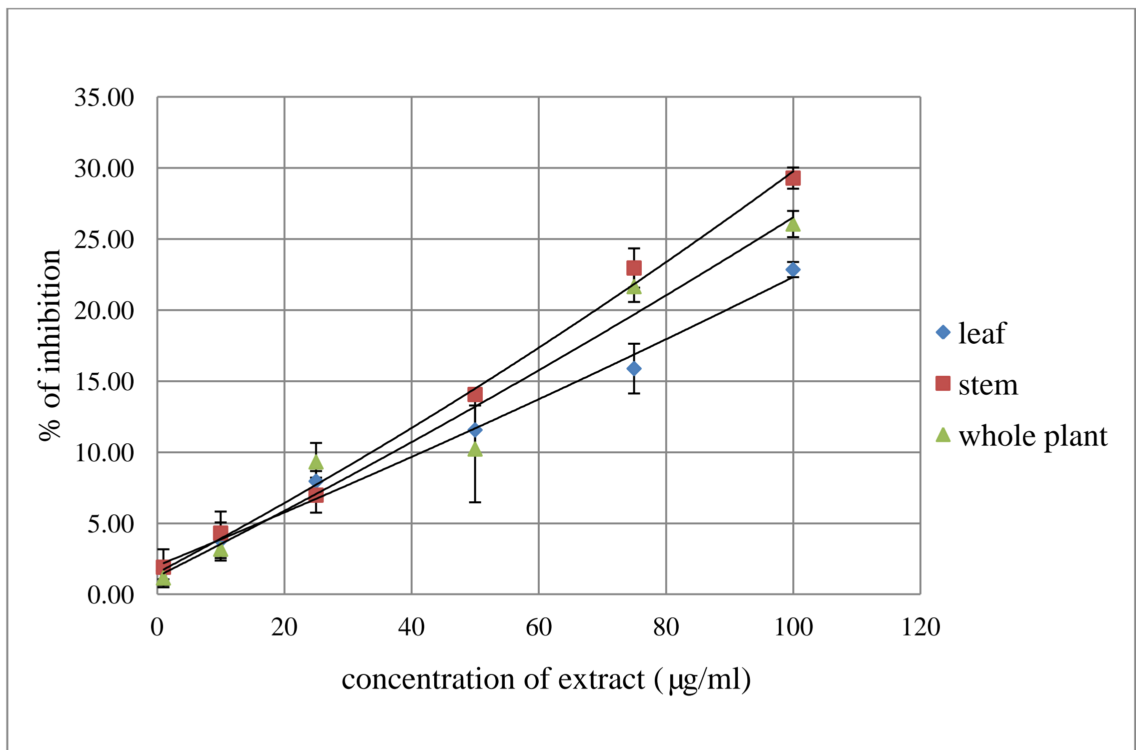

Figure 4.5 (H): The *in vitro* growth inhibition of A549 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

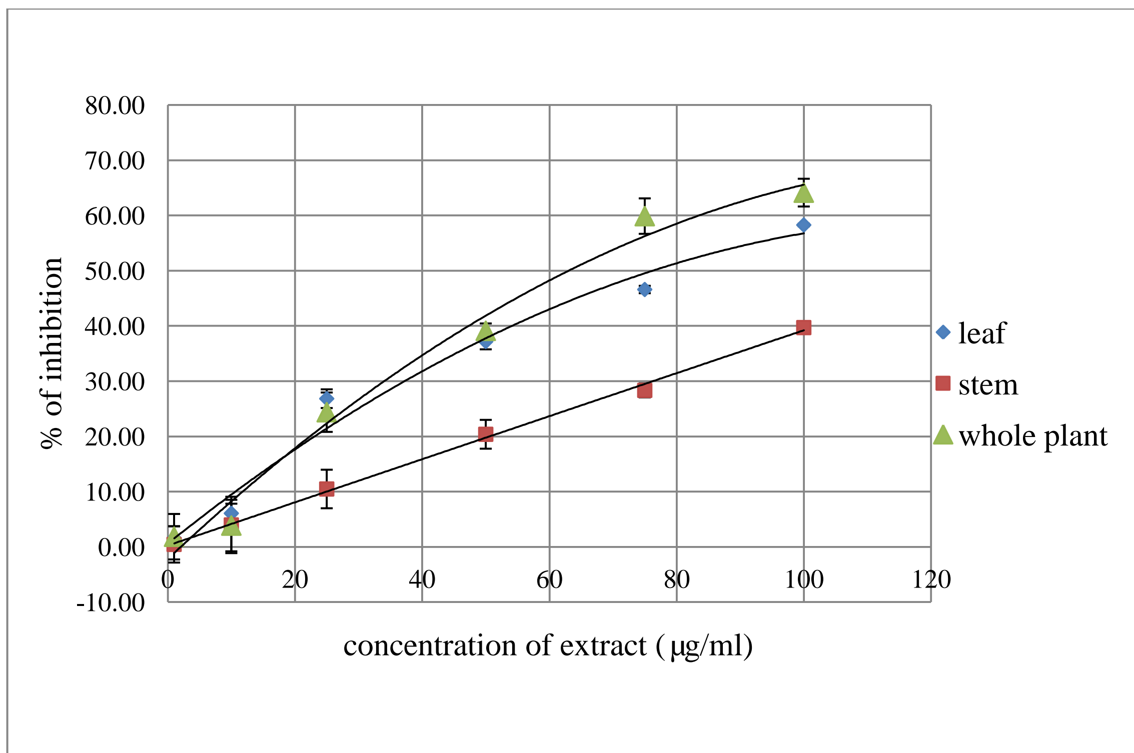

Figure 4.5 (I): The *in vitro* growth inhibition of MCF7 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

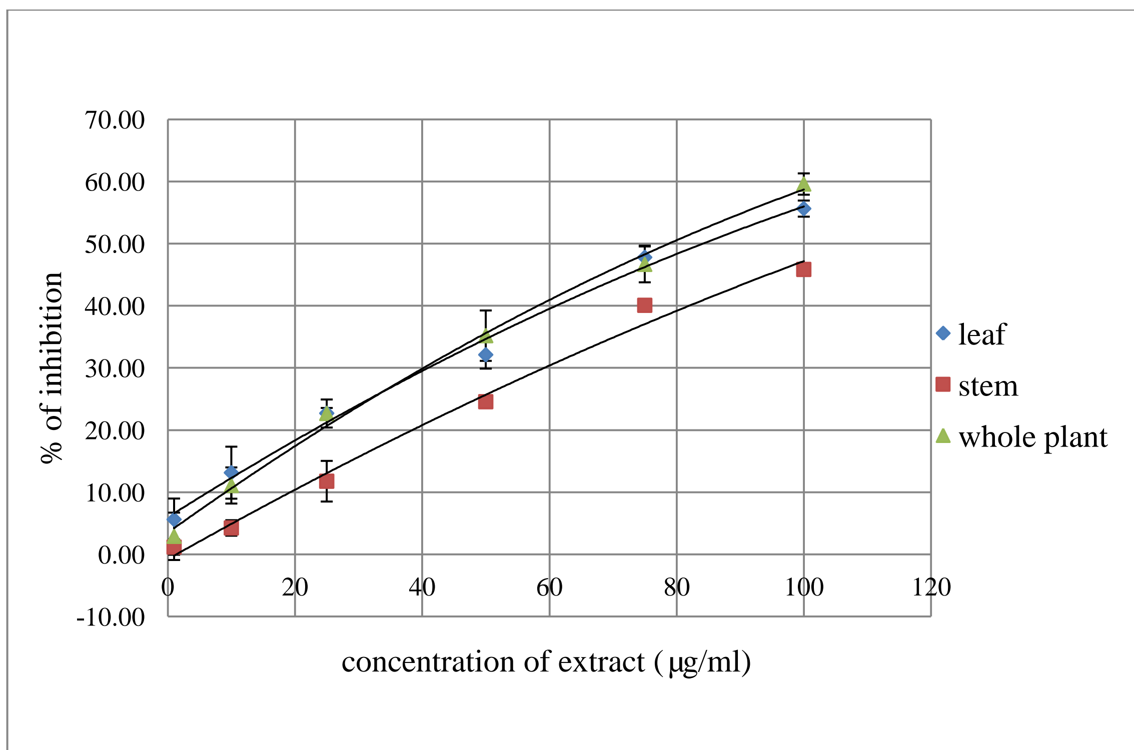

Figure 4.5 (J): The *in vitro* growth inhibition of MCF7 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

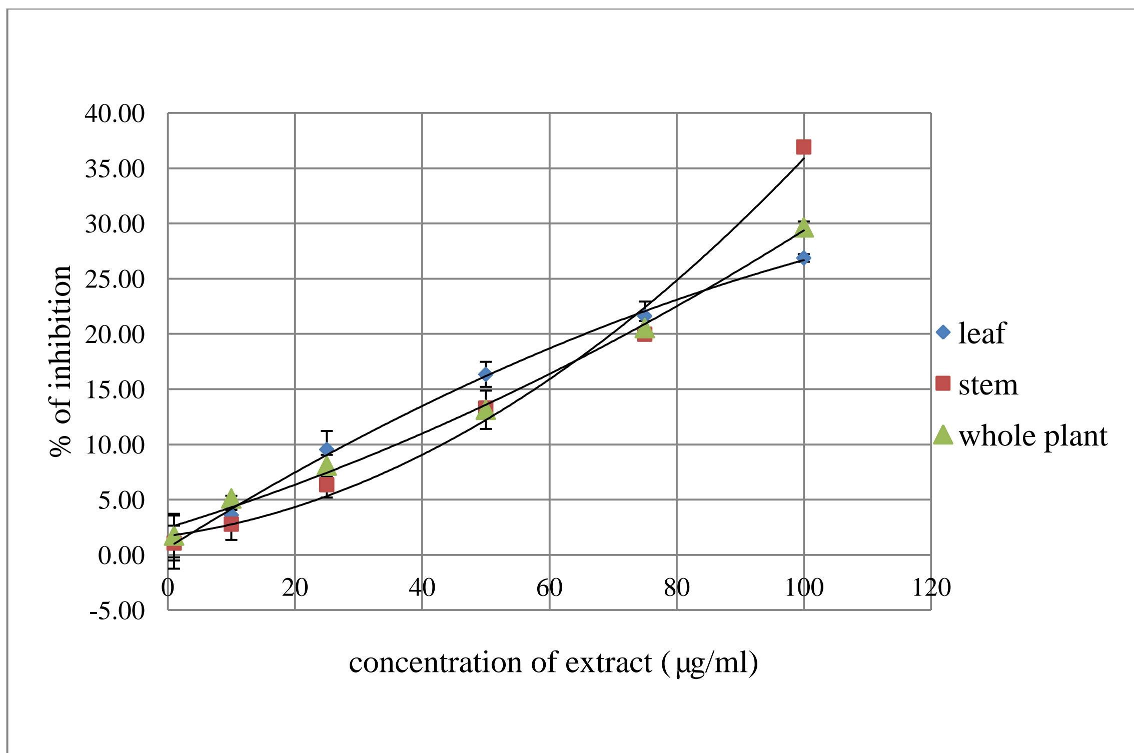

Figure 4.5 (K): The *in vitro* growth inhibition of MRC-5 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

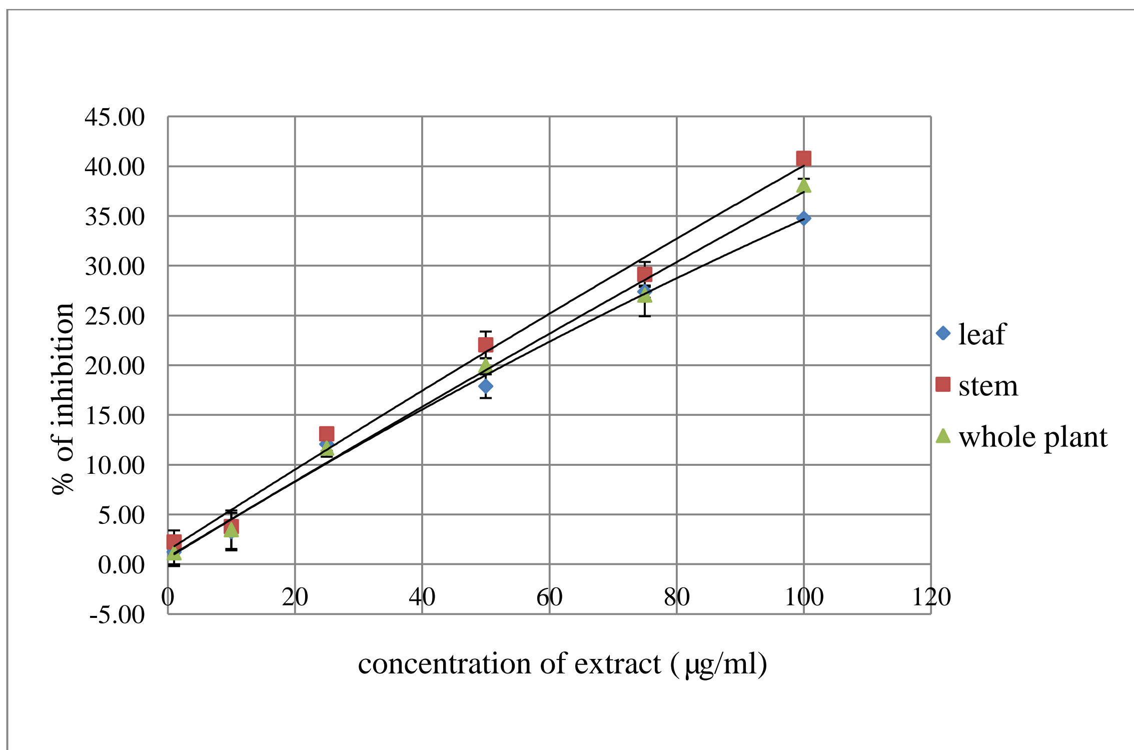

Figure 4.5 (L): The *in vitro* growth inhibition of MRC-5 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

Table 4.2: The IC_{50} values of crude methanol, dichloromethane and water extracts of the lowland cultivated variety of *P. chinensis* var. *chinensis* against different cell lines in the *in vitro* Neutral Red Cytotoxicity Assay.

Crude extracts of lowland cultivated variety of *P. chinensis* var. *chinensis*		IC_{50} value (µg/ml) against different cells					
		SKOV-3	CaSki	HT-29	A549	MCF7	MRC-5
Methanol	Leaf	39.34	**11.37**	52.95	47.78	**29.27**	>100
	Stem	69.20	85.37	81.54	75.49	69.29	>100
	Whole plant	46.41	54.40	62.73	60.15	60.48	>100
Dichloromethane	Leaf	77.29	69.29	63.04	>100	>100	90.61
	Stem	82.00	58.84	66.15	70.99	>100	>100
	Whole plant	79.67	54.99	70.23	92.37	>100	>100
Water	Leaf	63.57	>100	>100	>100	82.02	>100
	Stem	>100	>100	>100	>100	>100	>100
	Whole plant	72.80	96.78	>100	>100	81.44	>100

Table 4.3: The IC_{50} values of crude methanol, dichloromethane and water extracts of the highland wild variety of *P. chinensis* var. *chinensis* against different cell lines in the *in vitro* Neutral Red Cytotoxicity Assay.

Crude extracts of the highland wild variety of *P. chinensis* var. *chinensis*		IC_{50} value (µg/ml) against different cells					
		SKOV-3	CaSki	HT-29	A549	MCF7	MRC-5
Methanol	Leaf	**19.38**	53.45	59.21	62.22	51.21	>100
	Stem	40.89	76.40	39.68	96.87	49.47	85.36
	Whole plant	**21.71**	56.69	47.01	72.58	59.47	>100
Dichloromethane	Leaf	85.84	>100	55.98	51.97	69.98	88.43
	Stem	91.11	65.04	**13.17**	59.90	64.42	63.71
	Whole plant	89.56	82.90	**21.86**	94.35	85.93	82.15
Water	Leaf	>100	>100	>100	>100	82.28	>100
	Stem	>100	>100	>100	>100	>100	>100
	Whole plant	>100	72.45	>100	>100	63.10	>100

4.5 Discussion

Cytotoxicity assays are commonly used in preliminary *in vitro* antitumour screening tests. It is a rapid, inexpensive, standardized and sensitive method to measure drug-induced alterations in metabolic pathway or structural integrity which may or may not be related directly to cell death (Wilson, 1986). A number of methods have been employed for the detection of cytotoxicity or cell viability, i.e. the methyl tetrazolium assay (MTT), the ATP content of the treated cells, the lactate dehydrogenase leakage assay (LDH), a protein assay, and the neutral red uptake assay (NR). Weyermann *et al*. (2005) reported that, different cytotoxicity assays could give different results depending on the cell death mechanism induced. Fotakis & Timbrell (2006) reported that the MTT assay and the NR assay are the most sensitive cytotoxicity assays that show statistical significance between the treated cells and the controls.

In this study, NR assay was chosen instead of MTT assay to determine the cytotoxic effect of *P*. *chinensis* var. *chinensis* crude extracts against selected cancer cell lines. This selection was made due to several reasons. The MTT assay gives satisfactory responses using cell membrane damaging agents like triton X-100 and determines the activity of the mitochondria effectively, but this assay could be misleading if the toxic agent only influences intracellular activities, e.g. sodium azide which inhibits the respiratory chain. The MTT assay, which is dependent on enzymatic reactions, might also be influenced by enzyme inhibitors, for example chloroquine. NR assay on the other hand, is an inexpensive assay and is sufficient to measure the cell death (Weyermann *et al*., 2005). Moreover, NR assay requires fewer cells for analysis as a result of the optical density absorbance with the NR assay which is about twice than that obtained in the MTT assay, although both assay yield comparable ranking of cytotoxicity data (Borenfreund *et al*., 1988). In addition, the neutral red assay is also a useful tool to detect lysosomal damage when used in conjunction with other tests in

order to distinguish between cytotoxicity and organelle damage (Fotakis & Timbrell, 2006). However, in some cases, the NR assay is less sensitive (excitoxic model) and is not recommended for use in ion channel studies (Weyermann *et al*., 2005).

Previous studies have reported that, *P. chinensis* var. *chinensis* has produced certain chemical constituents which exhibited anti-inflammatory, anti-allergic properties (Tsai *et al*., 1998) as well as anti-fungal activity (Joshi *et al*., 1997).

Among the 18 tested extracts of *P. chinensis* var. *chinensis*, six extracts showed significant cytotoxic activity against CaSki, SKOV-3, MCF-7 and HT-29, i.e. the leaf methanol crude extract of the lowland and highland varieties, whole plant methanol crude extract of the highland variety, whole plant dichloromethane crude extract of the highland variety and also the stem dichloromethane crude extract of the highland variety. In contrast, none of the extracts assayed exhibited active cytotoxicity against A549 cell line. The presence of the apigenin, isorhamnetin, quercetin and luteolin in *P. chinensis* var. *chinensis* could explain the observed cytotoxic activity (Xie *et al*., 2007).

Apigenin is a flavonoid widely found in many fruits and vegetables. It has been shown to inhibit proteasome activity in breast cancer cells and breast cancer xenografts, induce apoptosis in leukemia cells and human breast cancer, exhibit anticancer activities (Chen *et al*., 2007) and inhibit UV-induced mouse skin tumourigenesis (Lepley *et al*., 1996). Apigenin can reduce the invasion of tumour cells, suppress tumour angiogenesis and therefore inhibit the growth of tumour cells and metastasis (Sun *et al*., 2004).

Luteolin is an important member of the flavonoid family. It has been reported that luteolin can inhibit the proliferation of serial tumour cells including solid tumours, human myeloid leukemia (Zhang *et al*., 2006) and may lead to growth inhibition of tumours and a reduced likelihood of cancer metastasis (Huang *et al*., 1999). Quercetin, a flavonoid, has been reported to have inhibitory effects on tumour cell properties and

proliferation (Huang *et al*., 1999; Wang *et al*., 2003), and also significantly inhibited the growth of the highly aggressive PC-3 prostate cancer cell line (Nair *et al*., 2004).

Isorhamnetin is a member of flavonoid components which has been used in the treatment of heart disease. The *in vitro* anticancer effect of isorhamnetin was observed on human esophageal squamous carcinoma cell line Eca-109 (Ma *et al*., 2007), It also exhibited noticeable anticancer effects on lung carcinoma cells (Yang *et al*., 2004; Zhu *et al*., 2005).

The other members in family Polygonaceae are well-known for their anticancer activities. For instance, *Fallopia japonica* (syn *Polygonum cuspidatum* and *Reynoutria japonica*) has well-documented anticancer activities *in vitro* and in bioassays based on animal models (Kintzios, 2006). The root extracts of *P*. *cuspidatum* contain the polyphenol resveratrol, (trans-3,4'5-trihydroxystilbene) and this compound has been shown to reduce, significantly, volume and weight of tumours as well as metastasis in highly metastatic Lewis lung carcinoma tumour-bearing mice (Kimura & Okuda, 2001). Emodin, another compound isolated from *P*. *cuspidatum*, has been reported to have anticancer properties against lymphocytic leukemia in mice (Hsu, 1989; Ogwuru & Adamczeski, 2000) and also acts as a strong inhibitor of a protein tyrosine kinase (Jayasuriya *et al*., 1992).

Recently, *Polygonum bistorta* was screened for its cytotoxicity against selected cancer cell lines and it has been reported to have moderate to very good activity, with the isolated compound, cycloartane-type triterpenoids, showing cytotoxic activities against P338 (Murine lymphocytic leukaemia), HepG2 (Hepatocellular carcinoma), J82 (Bladder transitional carcinoma), HL60 (Human leukaemia), MCF7 (Human breast cancer) and LL2 (Lewis lung carcinoma) cancer cell lines (Manoharan, 2006; Manoharan *et al*., 2007) .

The folk medicinal plant, *Polygonum hydropiper*, contains intense pungent substances in leaf and seed and is used against cancer (Hartwell, 1970). This plant has been reported to contain a potent cytotoxic warburganal, a drimane-type sesquiterpene dialdehyde (Fukuyama *et al*., 1980).

A common vegetable, *Polygonum odoratum*, is also reported to contain some flavonoids such as quercetin, rutin, catechin, isorhamnetin and kaempferol; the flavonoid, rutin, in combination with other flavonoids possess a strong anti-proliferative effect on breast cancer cells (Nanasombat & Teckchuen, 2009). *Polygonum hypoleucum*, a chinese medicinal plant, was found to have emodin and it has been shown to have successfully suppressed activities in various tumour cell proliferations (Kuo *et al*., 1997, 2001).

Techniques for cultivating medicinal plants always follow the same basic pattern but may differ in temperature requirements, pH preferences or the substrate on which these plants are grown. In this case, the highland and lowland varieties of *P. chinensis* var. *chinensis* are definitely grown in different environmental conditions, habitats, temperatures, rainfall, relative humidity, soil pH, soil type, the density of microbial life and so forth. As such in this study, the cytotoxic activities of the lowland and highland varieties of *P. chinensis* var. *chinensis* against different cell lines varied and these variations could be critically influenced by their physicochemical structure which may vary with habitats, cultivated conditions, isolation methods and other factors.

The lowland and the highland varieties of *P. chinensis* var. *chinensis* may produce different pure compounds resulting in different chemopreventive abilities to treat existing cancers. Therefore, these results strongly support further investigations of *P. chinensis* var. *chinensis* which involves looking into its possible antitumour and

anticancer properties; isolation of pure compounds, toxicity studies in order to determine its safety and selectivity index, *in vitro* pharmacological assays and so on.