IN VITRO SCREENING OF CYTOTOXIC EFFECT AND ANTIOXIDANT ACTIVITY OF *Pereskia bleo* AND C*entella asiatica* CRUDE EXTRACTS

By

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ABSTRACT

IN VITRO SCREENING OF CYTOTOXIC EFFECT AND ANTIOXIDANT ACTIVITY OF *Pereskia bleo* AND C*entella asiatica* CRUDE EXTRACTS

Vimalan Rengganaten

Pereskia bleo from the family of Cactaceae and *Centella asiatica* from the family of Umbelliferae are two well known medicinal plants. These plants are often used traditionally as anti-inflammatory, antioxidant and anti-rheumatic. In this study, the cytotoxic effect and antioxidant activity of these plants were evaluated. Possible synergistic interaction between these two plants was studied at ratio 1:1, 3:7 and 7:3. The whole plants were extracted using cold solvent extraction to produce ethanolic and hexane crude extracts. The cytotoxic effect of the crude extracts was measured using MTT assay. The ethanolic and hexane crude extracts of *Pereskia bleo* and *Centella asiatica* possessed high cytotoxic effect against K-562 cells upon 48 hours of treatment. The *Pereskia bleo* crude yielded IC_{50} values of 21.5 µg/mL and 32.5 µg/mL, and the *Centella asiatica* crude yielded IC_{50} values of 30.0 µg/mL and 32.0 µg/mL for ethanolic and hexane extracts, respectively. The highest cytotoxic effect was observed in the mixture ratio of 3:7 of hexane crude extracts with IC_{50} value of 21.0 µg/mL. This suggests that possible synergism activity may exist at this ratio between these two plant samples. The antioxidant activity was measured using DPPH assay. The highest antioxidant activity was

observed in the ethanolic crude extract of *Pereskia bleo*, with IC_{50} value of 475 µg/mL. Lower antioxidant activity was yielded among the mixture ratio of crude extracts, suggesting a possible antagonism interaction. The crude extracts of *Pereskia bleo* and *Centella asiatica* could be potential cytotoxic and antioxidant agents. The possible synergistic interaction could exist between the plant samples. Further studies should investigate the synergistic interaction using purified compounds from *Pereskia bleo* and *Centella asiatica*.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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VIMALAN RENGGANATEN

APPROVAL SHEET

This project report entitled "***IN VITRO* SCREENING OF CYTOTOXIC EFFECT AND ANTIOXIDANT ACTIVITY OF *Pereskia bleo* AND *Centella asiatica* CRUDE EXTRACTS**" was prepared by VIMALAN RENGGANATEN and submitted as partial fulfillment of the requirements for degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **VIMALAN RENGGANATEN** (ID No: **09ADB07208**) has completed this final year project entitled **"*IN VITRO* SCREENING OF CYTOTOXIC EFFECT AND ANTIOXIDANT ACTIVITY OF *Pereskia bleo* AND *Centella asiatica* CRUDE EXTRACTS"** supervised by Ms. Sangeetha Arullappan from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

Yours truly,

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(VIMALAN RENGGANATEN)

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
ATCC	American Tissue Culture Collection
CML	Chronic Myeloid Leukemia
DMSO	Dimethyl sulfoxide
DPPH assay	2,2-diphenyl-1-picrylhydrazyl
FBS	Fetal bovine serum
IC_{50}	Concentration causing 50% inhibition of the desired activity
MTT assay	3- [4, 5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide
NCI	National Cancer Institute
PBS	Phosphate buffered saline
rpm	revolution per minute
WHO	World Health Organization
K-562 cells	Human myeloblastic leukemia cells
RPMI	Roswell Park Memorial Institute

CHAPTER 1

INTRODUCTION

Throughout the ages, humans have relied on nature, especially plants for their basic needs such as food source, clothing and most importantly medicines (Gurib-Fakim 2006). The utilization of natural plant for disease treatment begins from ancient civilization of Chinese and Indian (Phillipson 2001). The current focus and interest on producing medicinal agent has shifted towards the field of phytochemistry. Different plants have been studied, analyzed and characterized for its medicinal value based on their main biological compounds available (Briskin 2000).

According to Gurib-Fakim (2006), there are approximately 250 000 species of plants available globally, however only 1% of tropical species have been studied for their medicinal properties. As reported by Adenan (as cited in Aziz et al., 2003), Malaysia is the 12^{th} most biodiverse nation in the world, which makes up 15 000 species of flowering plants with over 3000 species of medicinal plant. However only 50 of these medicinal plants are being used or researched scientifically for its medicinal properties.

Besides exploring each plant for its own medicinal property, another novel concept that has been recently introduced in the field of antimicrobial drug research is the effect of synergism. Most of the natural extracts have one or more active compounds that give the plant its medicinal value. In synergism,

the active compounds from two or more plants are mixed in a proportion to produce an interaction that might be heightened due to the combination via agonism, or in reduce the pharmacological effect via antagonism (Ulrich-Merzenich et al., 2010).

In current drug therapy, to obtain the desirable therapeutic effect, two or more synthetic drugs are often mandatorily administered. These interactions of drugs should be studied as it opens wide opportunity to produce an effective drug. The activity of synergism using medicinal plants has shown to have substantial amount of pharmacology properties (Adwan et al., 2009). Hence, this novel concept should be heavily adopted especially in the phytochemistry field.

Pereskia bleo and *Centella asiatica* are two known medicinal plants in Malaysia, which have been analyzed for it's their secondary metabolites and biological activities (Pittella et al., 200l; Malek et al., 2009). These two plants were used to explore the novel concept of synergism in this study.

The death tolls by cancer increase every year. Within 3 years period of 2004 to 2007, more than 213 500 primary brain and nervous system tumors were diagnosed (Kohler et al., 2011). However, till the date few drugs have been synthesized which are capable to slow down the proliferation of the cancer cells (Kostova 2005). Therefore, the quest to find an effective anticancer is still ongoing. Hence, the objectives of this study are as follows:

1. To isolate the crude extracts from *Pereskia bleo* and *Centella asiatica* using ethanol and hexane via cold extraction method,
2. To determine the concentration of the crude extracts that will decrease the K-562 cell viability by 50% (IC_{50}) using MTT assay,
3. To determine the percentage of radical scavenging activities of the crude extracts using DPPH assay,
4. To determine the possible synergistic cytotoxic effect and antioxidant activity between *Pereskia bleo* and *Centella asiatica* crude extracts at different mixture ratios (1:1, 3:7 and 7:3).

CHAPTER 2

LITERATURE REVIEW

2.1 *Pereskia bleo*

2.1.1 General Description

Pereskia bleo is locally known as Jarum tujuh bilah among the Malay community and as Seven star needle (qi xing zhen) among the Chinese community. The taxonomical classification of the *Pereskia bleo* is shown in Table 2.1. *Pereskia bleo* is a member of the cactus family, Cactaceae with a subfamily of Pereskioideae (Malek et al., 2009; Butterworth and Wallace 2005).

Pereskia bleo has been traditionally used in Malaysia by various ethnic groups in battling cancer, diabetes, hypertension and diseases associated with rheumatism and inflammation (Malek et al., 2009). The morphology of this plant is rather unique as compared to its other family members. *Pereskia bleo* has thorny spines covered around its stem in a group of seven on each areole, hence the name "Seven star needle", where the thorns act as a natural defense for the plant (Lee et al., 2009). *Pereskia bleo* is widely distributed around the world as there are evidences stating that *Pereskia bleo* is being used in countries ranging from Panama to Malaysia (Sim et al., 2010). Figure 2.1 shows the image of *Pereskia bleo*.

Table 2.1: Taxonomical classification of *Pereskia bleo* (Butterworth and Wallace 2005).

Division	**Class**
Kingdom	Plantae
Family	Cactaceae
Subfamily	Pereskioideae
Genus	*Pereskia*
Species	*Pereskia bleo*

Figure 2.1: Image of *Pereskia bleo* @ Vimalan Rengganaten

2.1.2 Bioactive Compounds

According to Doestch et al. (as cited in Malek et al., 2009), there are four alkaloids extracted and identified from *Pereskia bleo*. These include 3,4-dimethyoxy-β-phenethylamine, 3-methyoxytyramine, mescaline and tyramine. Using active ethyl acetate fractions, four compounds were isolated, namely dihydroactinidiolide, sterols, α-tocopherol and phytol (Malek et al., 2009). The function of each of the compounds studied by various researches is shown in Table 2.2. It shows that *Pereskia bleo* has a wide range of antioxidants, providing the basic understanding that it could be an essential component in battling cancer.

Table 2.2: Function of the bioactive compounds found in *Pereskia bleo* (Roeder 2005).

Compounds	**Functions**
3,4-dimethyoxy-β-phenethylamine	Neuromodulator (Roeder 2005)
Mescaline	Hallucinogen (Bunzow et al., 2001)
3-methoxytyramine	Neuromodulator (Netscher 2007)
Tyramine	A type of adrenergic transmitter (Sotnikova et al., 2010)
Dihydroactinidiolide	Flavoring in tea and tobacco (Sotnikova et al., 2010)
Sterols	Anti-atherosclerosis, antibacterial, anti-inflammation, antioxidant (Malek et al., 2009)
α-tocopherol	Dietary antioxidant (Malek et al., 2009)
Phytol	Precursor of vitamin E synthesis (Bruhn et al., 2002)

2.1.3 Previous Investigations

2.1.3.1 Antioxidant Properties

Sim et al. (2010) stated that hexane crude extracts of *Pereskia bleo* showed remarkable antioxidant activity with EC_{50} of 210 µg/mL, followed by ethyl acetate extracts with EC_{50} (median effective concentration) of 225 µg/mL using DDPH assay. In a different study by(Lee et al., 2009., *t*-butanol extracts of the stems of *Pereskia bleo* showed higher antioxidant activity as compared to methanol, ethyl acetate and water extracts. By using ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assay, *t*-butanol extracts of stem showed a scavenging activity of 450 µmole TE/g dry weight with respect to Trolox Equivalent.

Meanwhile, Hassanbaglou et al. (2012) presented a study using the leaves of *Pereskia bleo* on its antioxidant activity by experimenting using different solvent extracts and antioxidant assays. The assays include DPPH assay, ferric reducing antioxidant power (FRAP assay) and β-carotene-linoleic acid. As overall, ethyl acetate extracts showed higher scavenging activity using all the three assays, with an IC_{50} of 168.35 ± 6.5 µg/ml, as compared to methanol, hexane and ethanol extract of the leaves of *Pereskia bleo.*

2.1.3.2 Cytotoxic Properties

According to Malek et al. (2009), methanolic extracts, hexane and ethyl acetate fractions showed toxic effect against KB cell line (human nasopharyngeal epidermoid carcinoma cell line), CaSki cell line (human cervical carcinoma cell line), HCT 116 cell line (human colon carcinoma cell line) and MCF-7 cell line (hormone-dependent breast carcinoma cell line). The highest antiproliferative effect was observed using methanol crude extracts and ethyl acetate fractions against KB cells with IC_{50} between 6.5 and 4.5 µg/mL. None of the extracts showed any toxic effect against the normal cell line, MRC-5 (non-cancerous human fibroblast cell line).

Lee et al. (2009) reported that all methanol, ethyl acetate, butanol and aqueous extracts of the stem of *Pereskia bleo* did not exhibit antiproliferative effect on normal mouse fibroblast (NIH/3T3), further proving that *Pereskia bleo* may not be toxic to non cancerous cells with a selective inhibitory property. *In vivo* testing of oral administration of methanolic crude extracts showed no acute toxicity among the mice subjects (Sim et al., 2010).

Meanwhile, Tan et al. (2005) reported that methanolic extracts of *Pereskia bleo* against human breast carcinoma cell line (T-47D) produced a remarkable EC_{50} of 2.0 µg/mL. Using ultrastructural analysis, the mechanism of action of the compounds from methanolic crude extracts was determined, where *Pereskia bleo* crude is believed to induce DNA fragmentation which results in apoptosis.

2.2 *Centella asiatica*

2.2.1 General Description

Centella asiatica is commonly known as “pegaga” by the Malays, the Indian pennywort in English and “valarai” in Tamil (Globinmed 2010). *Centella asiatica* belongs to the Umbelliferae family (Pittella et al., 2003). The taxonomical classification is shown in Table 2.3.

In Ayurvedic medication, *Centella asiatica* is used extensively in treating several disorders, such as insanity, asthma, leprosy, ulcers, eczema and improving the wound healing process (Pittella et al., 2009). Traditionally it is believed to enchance memory and improve nerve function. It is also a common spice used in Asian cookings (Soumyanath et al., 2012). Besides that, this plant is also believed to have anti-inflammatory, antiproliferative and collagen synthesizing activities (Jayashree et al., 2003).

Centella asiatica has a pantropical distribution including Southeast Asia, extending even to some subtropical regions. *Centella asiatica* have a smooth, rossette form leaves, with greenish long petals (Globinmed 2010). It is a small perennial herb with long stolons and nodes at the rooting system. It often grows in a damp, slightly shaded, fertile soils, normally along stream river banks (Globinmed 2010). Figure 2.2 shows the image of *Centella asiatica*.

Table 2.3: Taxonomical classification of *Centella asiatica* (Jayashree et al., 2003; Pittella et al., 2009).

Division	**Class**
Kingdom	Plantae
Family	Umbelliferae
Subfamily	Apiaceae
Genus	*Centella*
Species	*Centella asiatica*

Figure 2.2: Image of *Centella asiatica* @ Vimalan Rengganaten.

2.2.2 Bioactive Compounds

The major group of phytochemical available in *Centella asiatica* is triterpene glycosides with asiaticoside as the main bioactive compound. It also has other compounds such as centellasaponin, asiaticoside, madecassoside, sceffoleoside and asiatic acid (Pittella et al., 2009). The function of each of these compounds is shown in Table 2.4.

Table 2.4: Function of bioactive compounds found in *Centella asiatica.* (Won et al., 2010).

Compounds	**Functions**
Centellasponin	Collagen I synthesis (Won et al., 2010)
Asiaticoside	Anti-inflammatory (Zheng and Qin 2007)
Madecassoside	Anti-inflammatory (Zheng and Qin 2007)
Sceffoleoside	Anti-inflammatory (Zheng and Qin 2007)
Asiatic acid	Anti-proliferative (Tang et al., 2009)

2.2.3 Previous Investigations

2.2.3.1 Antioxidant Properties

In a study by Zainol et al. (2003), different parts of *Centella asiatica* showed different level of antioxidant activity using ferric thiocyanate and thiobarbituric acid assay. It was reported that, the antioxidant activity from the leaves and roots of *Centella asiatica* were almost as high as the α-tocopherol, which is a well known antioxidant agent with total phenolic content between 3.23 g/100 g to 11.7 g/100 g.

Besides that, after 14 days of oral administration of methanolic crude extracts of *Centella asiatica* in a lymphoma-bearing mice, Jayashree et al. (2003) reported there was a significant increment of endogenous antioxidant enzymes such as superoxide dismutase, catalase and glutathione. Lymphoma progresses with the increased action of free radicals such as reactive oxygen species. The endogenous antioxidant neutralizes these radicals, which in return slows the progression of the lymphoma (Jayashree et al., 2003).

2.2.3.2 Cytotoxic Properties

Aqueous extracts of *Centella asiatica* inhibited the proliferation of keratinocyte, which is a hyperproliferative skin disorder known as psoriasis, with IC_{50} of 209.9 ± 9.8 mg/mL (Sampson et al., 2001). In a different study, *Centella asiatica* restrained the formation of lesion caused by ethanol. The oral administration of the crude extracts prior to the consumption of ethanol showed promising results in battling gastric lesion. It is reported that *Centella asiatica* may strengthen the mucosal barrier and the antioxidant activity produced by it reduces the radicals (Cheng and Koo 2000).

Other than that, *Centella asiatica* also increases the production of IL-2 (interleukin 2) and TNF-α (tumor necrosis factor-α). It also showed that albino mice treated with *Centella asiatica* crude extracts yield higher response to both primary and secondary antibodies against BSA (bovine serum albumin), hence was concluded it may have chemo preventive or anticancer potential (Punturee et al., 2005).

2.3 Cancer

According to American Cancer Society (ACS) (2012), cancer is defined as a disease whereby the cells gain functionality due the abnormality in the cells which causes uncontrolled proliferation and gain the ability to invade other tissues. Through blood circulation system and lymphatic system, these cells are able to spread elsewhere in the body.

In 1998, lung, liver, breast, leukemia and stomach cancer were the top five cause of death among Malaysian cancer patient (Lim 2003). However, the top five frequent cancers in Peninsular Malaysia from 2003 to 2005 were breast, large bowel, lung, cervix uteri and leukemia. Large bowel cancer is the most common cancer among males of the major ethnic groups in Malaysia, meanwhile breast cancer among all females (Lim et al., 2008).

Chronic myeloid leukemia (CML) is one of the most heavily studied malignancies with regards with its association with the Philadelphia chromosome. CML occurs often due to abnormal chromosome translocations which involves the ABL proto-oncogene on chromosome 9 and the BCR gene on chromosome 22 (Deininger et al., 2000). Due to this translocation, the myeloid cells gain functionality by dividing and proliferating without control, and hence the leukemia.

K-562 cell or also known as human myeloid leukemia cell line is derived from culturing the leukemic cells from patients suffering CML. It is suspension in nature and presents a lymphoblastic morphology. K-562 cells are cultured in RPMI 1640 medium supplemented with 10% of FBS (Assef et al., 2003).

2.4 Cytotoxic Assays

A cell dies because it lose its membrane integrity permanently, and there are three types of cell death; apoptosis, necrosis and autophagic cell death (Golstein and Kroemer 2006). Cytotoxicity is one of the parameter that often associated with cell death and proliferation of the cell (Weyermann et al., 2005). Hence, cytotoxicity becomes a very valuable assay in determining the efficiency and efficacy of a natural derivative in battling cancer cells.

In general, there are a few methods available to analyze the cytotoxicity established by a compound. The most common technique to study the cytotoxicity *in vitro* is by using the trypan blue as the indicator. It is based on the intact membrane integrity of viable cells as compared to apoptotic cells where the cytoskeleton framework is destroyed. The absorption and staining of the dye is limited to only viable cell as the dead cells have compromised cell membrane (Riss and Moravec 2004).

MTT (3- [4, 5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide) assay is a sensitive technique that measures the cell proliferation, the apoptotic or necrosis metabolic events, leading to reduction of cell viability. The MTT compound is a yellowish water-soluble tetrazolium salt. The reduction of MTT compound into an insoluble purple formazan dye crystals, indicates that the cells in are viable, as the reduction is governed by succinate dehydrogenase which belongs to the active mitochondrial respiratory pathway (Fotakis and Timbrell 2006). Using a spectrophotometer, upon the addition of cosolvent to solubilize the formazan crystal, the colorimetric reading is taken at 570 nm. This directly rely information about the cell viability (Hamid et al., 2004).

Like MTT assay, XTT (2, 3-bis [2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide) is a tetrazolium salt which is reduced by succinate dehydrogenase via the mitochondrial respiratory pathway. The mechanism of action in identifying cytotoxicity is similar to MTT assay, the major difference is XTT is converted to water soluble formazan product which does not need any solubilization to read its colorimeter reading at 480 nm (Kuhn et al., 2003). Besides these, other assays include sulforhodamine B (SRB) assay, neutral red (NR) assay, lactate dehydrogenase (LDH) assay and clonogenic assay can be used to evaluate the cytotoxic effect of a compound (Riss and Moravec 2004).

2.5 Antioxidant Assays

Free radicals are often associated with numerous human diseases, and often antioxidant enzymes becomes a valuable resource in battling the formation of radicals (Temple 2000). Natural antioxidants, such as vitamins, phenolics and carotenoids are gaining attention among the scientific community as it stands a chance in lowering the risk of cancer and other radical associated diseases (Sharma and Bhat 2009).

DPPH, 2,2-diphenyl-1-picrylhydrazyl assay is one of the method to study the level of antioxidant from natural derivatives. DPPH is a stable free radical and in the presence of antioxidant, this radical will be reduced to DPPHH. The reduction of DPPH is observed by the change in color from purple to yellowish solution. The degree of discolorization stands as indicator for the antioxidant properties a compound contain (Satynarayana and Subhramanyam 2009).

There are several other assays available that could estimate the potential scavenging antioxidant activity of a compound. These includes, ferric reducing antioxidant power (FRAP), oxygen radical absorption capacity (ORAC) and 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) assay (Thaiponga et al., 2006).

FRAP assay is a colorimetric evaluation of the antioxidant capacity. It is based on the principle of reduction of ferric tripyridyltriazine complex to a blue colored compound called ferrous tripyridyltriazine at low pH in the presence of antioxidants. This reduction is measured at 593 nm (Guo et al., 2003; Kaushik et al., 2012).

2.6 Synergistic Interaction

Synergism is said to be present when the effect from a combined mixture is greater than the sum from their individual effects (Kepner 2004). Traditional medicinal systems such as Ayuverdic and ancient Chinese medicine have been using the concept of synergism, where often a mixture of plants was used instead of one species alone (Mukherjee et al., 2011).

In monodrug therapy, the chemical agent could only be directed against a single individual molecular target to exhibit its effect. Besides that, the buffering effect from drug-mitigating response of the body system against the monodrug therapy further reduces the efficacy of this therapy. Hence, the attention has shifted towards multidrug therapy (Zimmermann et al., 2007).

The synergistic multidrug therapy shows higher efficacy as compared to the monodrug therapy. Due to the multiple mode of actions from the multiple agents, the biological system is unable to develop adaptive resistant towards the drugs (Zimmermann et al., 2007). Hence, using the concept of synergism in the multidrug therapy, a higher desired therapeutic effect can be achieved

with reduction in the dosage usage and the undesired toxicity (Chou 2010). The exhibition of multiple modes of action in the synergistic multidrug therapy becomes an essential tool in battling multifactorial diseases such as cancer (Zimmermann et al., 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

Fresh whole plant of *Pereskia bleo* and *Centella asiatica* were bought from Taman Herba Batu Gajah and Kampar wet market, respectively on September 2012. These plants were authenticated by Dr. Goh Teik Khiang of Department of Agricultural and Food Science, Faculty of Science, Universiti Tunku Abdul Rahman, Malaysia.

3.1.2 Cancer Cell Line

The human cell line used was human myelogenous leukemia (K-562). It was purchased from American Tissue Culture Collection (ATCC). The K-562 was preserved at -80ºC.

3.1.3 Chemicals and Solvents

The type of chemicals and solvents used with respect to their brand and manufacturer is as shown in Table 3.1.

Table 3.1: The list of chemicals and solvents used.

Chemicals and Solvents	**Brand/ Manufacturer**
Ethanol	Irama Canggih Sdn Bhd (Malaysia)
Hexane	Irama Canggih Sdn Bhd (Malaysia)
DPPH reagent	Sigma-Aldrich®
L-Ascorbic acid powder	Sigma-Aldrich®
D-α-tocopherol	Sigma-Aldrich®
MTT reagent	Bio Basic Inc. (Canada)
Dimethly sulfoxide (DMSO)	System Chemar®
Doxorubicin hydrochloride	Fisher Scientific
5-flurouracil	Fisher Scientific
0.4% Trypan blue solution	Sigma-Aldrich®
D-Phosphate buffered saline (PBS)	Nacalai Tesque, Inc
US Origin Fetal bovine serum (FBS)	Jrscientific Inc.
RPMI 1640 medium	Nacalai Tesque, Inc
Penicilin-streptomycin solution	Embryomax®

3.1.4 Equipments and Apparatus

The type of equipments used with respect to their brand is as shown in Table 3.2.

Table 3.2: The list of equipments and apparatus used.

Equipment	**Brand**
Rotary Evaporator	BUCHI Rotavapor
Incubator	Memmert
Sonicator	Elmasonic S100H
5% CO_2 Incubator	BINDER
Centrifuge	HERAEUS MULTIFUGE 1S-R
Microplate reader	BIORAD Model 680 Microplate reader

3.2 Methodology

3.2.1 Preparation of Plant Materials

Roughly 3 kg of the *Pereskia bleo* and *Centella asiatica* were washed to remove the debris and soil. It was then left to dry for a week under room temperature. The wet and dry weights were measured. The dried plant samples were pulverized into powder using a laboratory blender.

3.2.2 Plant Extraction

Sequential extraction was carried out using ethanol and hexane solvent. The powdered samples of *Pereskia bleo* and *Centella asiatica* were separately soaked in 95% ethanol for one week at room temperature. The solvent-containing extracts were filtered using cotton and filter paper, and the solvents were evaporated at roughly 40ºC using rotary evaporator. The concentrated crude extracts were dried at 37ºC in incubator and its' final weight were recorded. The extraction was repeated thrice. The crudes were stored at 4ºC until further use. The procedure was repeated using hexane, where the powdered plant samples were dried and soaked with hexane (modified from Malek et al., 2009).

3.2.3 Preparation of Plant Stocks

The main stocks were prepared by diluting 100 mg of the crude extracts in 1 mL of DMSO to produce 100 mg/mL. The stocks were then filtered using nylon syringe filter (0.22 µm). The working stock of 1 mg/mL was prepared by diluting the main stock with basic RPMI 1640 medium. Five different ratios were prepared, 1:0, 0:1, 1:1, 3:7 and 7:3 from the working stock respect to *Pereskia bleo*: *Centella asiatica*. The preparation for the ratios is shown in Table 3.3. For each ratio, five different concentrations ranging from 20 µg/mL to 100 µg/mL were prepared. For antioxidant assay, working stock of 1 mg/mL was prepared, where 20 mg of the extract was dissolved in 20 mL of 95% ethanol. Using serial dilution, five dilution concentrations were prepared ranging from 200 µg/mL to 1000 µg/mL.

Table 3.3: Preparation of stock ratios.

Ratio	Working Stock of *Pereskia bleo* (µL)	Working Stock of *Centella asiatica* (µL)	Final volume (µL)
1:0	1000	0	1000
0:1	0	1000	1000
1:1	500	500	1000
3:7	300	700	1000
7:3	700	300	1000

3.2.4 Preparation of Complete Medium

The basic medium used was RPMI 1640 (Roswell Park Memorial Institute). It was supplemented with 10% FBS (fetal bovine serum) culturing the K-562 cells. The 10% FBS was heat-inactivated (56ºC for 30 minutes) prior to its usage. The complete medium was kept in 4ºC until further use.

3.2.5 Preparation of Positive and Negative Controls

Doxorubicin and 5-fluorourical were used as positive controls in the experiment. The working stock of 1 mg/mL was prepared for both these agents using sterile distilled water as a diluent, and dilutions ranging from 20 µg/mL to 100 µg/mL were prepared. The negative control in this assay was 1% DMSO. The 1% DMSO was diluted using basic RPMI 1640 medium from its 100% DMSO stock.

Ascorbic acid was used as positive control in DPPH assay. A concentration of 1 mg/mL of ascorbic acid using 95% ethanol as diluent was prepared. A set of five dilutions were prepared ranging from 200 µg/mL to 1000 µg/mL. The negative control used in this assay is 95% ethanol. The same concentration of ethanol was used as diluents in preparing the plant extract stock.

3.2.6 Preparation of Reagents for Bioassays

For cytotoxicity assay, a concentration of 5 mg/mL of the MTT solution was prepared using PBS and it was wrapped with aluminum foil. The solution was prepared freshly prior to use. For antioxidant assay, DPPH solution (0.1 mM) was prepared where 0.04 g of the DPPH powder was dissolved in 100 mL of 95% ethanol. It was wrapped with aluminum foil.

3.2.7 Preparation of Freezing Medium

The freezing medium was prepared by adding 7.5 mL of basic medium supplemented with 2 mL of 10% FBS and 0.5 mL of 100% DMSO.

3.2.8 Culture and Subculture of K-562 Cells

The cryopreserved cells were thawed at 37ºC before it was transferred into a culture flask containing 5 mL of complete RPMI 1640 medium. The cells were observed under inverted microscope before incubating it overnight in 5% CO_2 incubator. Upon reaching 70-80% confluency, the cell suspension was centrifuged at 1000 rpm for 10 minutes, and the supernatant was discarded in order to remove the presence of DMSO from the medium.

The pellet was washed using PBS and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded. Finally, the pellet was resuspended with 5 mL complete RPMI 1640 medium and later transferred to a new culture flask. The cells were incubated in 5% CO_2 incubator until further use. The cells were subcultured upon reaching a confluency of 70-80%. The cell suspensions were transferred to a 15 mL centrifuge tube. It was then centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended with 5 mL sterile PBS. It was centrifuged at 1000 rpm for 10 minutes and the supernatant was discarded. This step was repeated twice. Finally, the pellet was resuspended with 5 mL complete RPMI 1640 medium and later transferred to a new culture flask. The cells were incubated in 5% CO_2 incubator until further use.

3.2.9 Cryopreservation of K-562 Cells

The cell suspension was observed under inverted microscope to confirm its confluency and to observe any sign of contamination. The cell suspension was then centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended with 5 mL PBS. It was centrifuged at 1000 rpm for 10 minutes and the supernatant was discarded. This step was repeated twice.

The pellet was resuspended with 6 mL of freezing medium. A total of 1.5 mL of the suspension was transferred to three autoclaved cryovials, respectively. The cryovials were initially stored at -20ºC for a week before transferring them to -80ºC for long term storage (modified from Meng et al., 2004).

3.3 Cell Quantification

The concentration of the cells in the culture flask was determined using a hemocytometer. Into an eppendoff tube, 10 µL of cell suspension and 10 µL of trypan blue dye were added in 1:1 dilution ratio. It was left to incubate for one minute. Next, 10 µl of suspension from the eppendoff tube was transferred to each side of the hemocytometer grid. An inverted microscope was used to count the cells on all the four grids at 10X magnification. The K-562 cell viability was calculated using the formula shown in Appendix A.

3.4 Bioassays

3.4.1 MTT Assay

The cells were seeded at a concentration of 4000 cells per well. The designs of the 96 well plate are shown in Appendix B. In each well, 180 µL of cell suspension was added. Basic RPMI 1640 medium was added as a sterility control. The wells were observed under the inverted microscope to ensure equal amount of cells were transferred in to the wells. The plate was incubated for 2 hours before its treatment.

After the 2 hours of incubation, the dilutions from the plant stock (20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL) were added to the cells. From each concentration, 20 µL was transferred into their respective wells. As for the positive control, 20 µL of the different concentrations was added into its respective well. For the negative control meanwhile, 20 µL of 1% DMSO was added to its respective wells. The assay was duplicated for

each concentration, and the assay was triplicated for each plate. The assay was repeated using the different ratios. Upon treating the cells, the plates were left for 24 hours and 48 hours in 5% CO_2 incubator.

After the incubation, 20 µL of the MTT solution was added to all the treated wells and incubated at 5% CO_2 incubator for 4 hours. Then, the 96 well plates were centrifuged at 1000 rpm for 10 minutes. Next, 100 µL of the supernatant was discarded from each well and 100 µL of 100% DMSO solution was added. The DMSO was thoroughly mixed in each well before its absorbance at 560 nm was measured using a microplate reader. The absorbance readings were recorded and the percentage cell viability was calculated. The percentage cell viability was calculated using the formula shown in Appendix C. Graphs of percentage cell viability against the concentration of crude extracts were plotted to determine the IC_{50} value.

3.4.2 DPPH Assay

In antioxidant assay, 1 mL of the each stock dilution was transferred into its respective test tubes. The test tubes were covered with aluminum foil. Into each test tube, 4 mL of the prepared DPPH solution was added. It was left to incubate for 30 minutes before reading its absorbance at 517 nm. The procedure was repeated with the positive and negative control. The percentage radical scavenging activity was calculated as shown in Appendix C. Graphs of percentage radical scavenging activity against the concentration of crude extracts were plotted to determine the IC_{50} value.

3.5 Data Analysis

The MTT and DDPH assay was repeated thrice. The mean value and standard deviation from the result was calculated by using Microsoft Office Excel 2007. Standard deviation showed how much variation there is from the mean value. A low standard deviation indicated that the data points are close to the mean value.

CHAPTER 4

RESULTS

4.1 Percentage Yield of *Pereskia bleo* and *Centella asiatica* Extracts

Table 4.1 shows the total percentage of water content of *Pereskia bleo* and *Centella asiatica*. *Centella asiatica* (89.6%) contained more water than *Pereskia bleo* (87.6%). Table 4.2 shows the percentage of crude yielded using ethanol and hexane for *Pereskia bleo* and *Centella asiatica*. Figure 4.1 shows the graphical representation of data from Table 4.2. The ethanolic crude extracts of *Centella asiatica* and *Pereskia bleo* recorded the highest percentage of yield, with 11.97% and 9.32%, respectively. The hexane extracts yielded lowest amount of crude, 0.49% for *Pereskia bleo* and 1.01% for *Centella asiatica*. This suggests that, the major compound in both the plant samples are polar compounds.

Table 4.1: Percentage of water content of plant samples.

Plant samples	Wet weight (g)	Dry weight (g)	Percentage of water content (%)
Pereskia bleo	3000	372	87.6
Centella asiatica	3000	309	89.7

Table 4.2: The percentage of crude yielded from ethanol and hexane solvent for *Pereskia bleo* and *Centella asiatica.*

Plant samples	Solvents	Dry Weight (g)	Crude Weight (g)	Percentage of yield (%)
Pereskia bleo	Ethanol	372	34.66	9.32
	Hexane	372	1.81	0.49
Centella asiatica	Ethanol	372	37.00	11.97
	Hexane	372	3.11	1.01

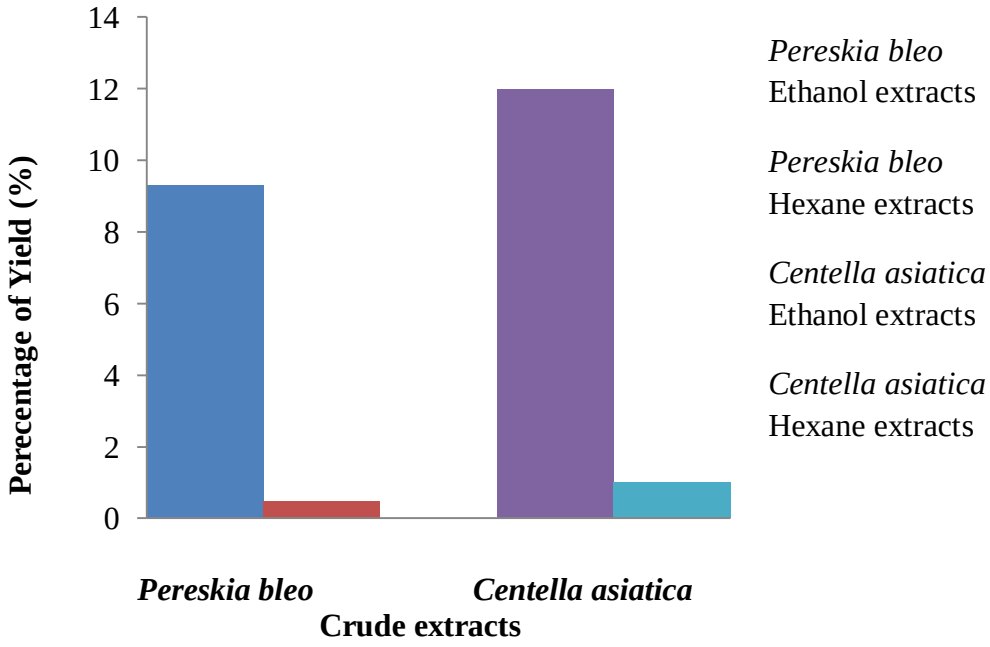

Figure 4.1: The percentage of yield of *Pereskia bleo* and *Centella asiatica* crude extracts using ethanol and hexane solvent.

4.2 MTT Assay

4.2.1 K-562 Cancer Cells

The K-562 cells are suspension cells that appeared to be floating and spherical in shape as shown in Figure 4.2. The cells were only used in the assay when it reaches confluency of 70% to 80%. The cells took around 72 hours to reach confluency. Every three days or once the cells reach confluency, they were subcultured.

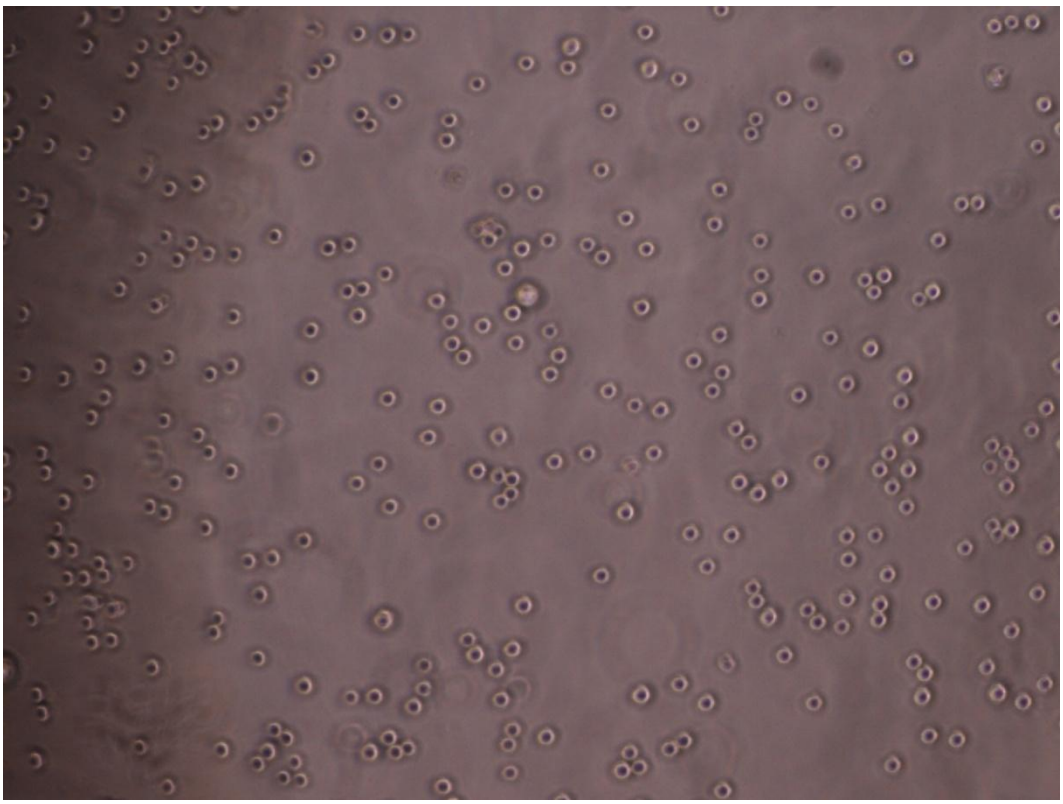

Figure 4.2: Morphology of K-562 cancer cells cultured in RPMI 1640 for 48 hours at 100X magnification.

The percentage of viability of K-562 cells after treatment with *Pereskia bleo* and *Centella asiatica* crude extracts is shown in Table 4.3. For *Pereskia bleo*'s ethanolic crude extracts, the highest cell viability was observed at 60.0 µg/mL (84.90 ± 0.153%) meanwhile the lowest cell viability was observed at 40.0 µg/mL (45.20 ± 0.040%). For hexane crude extracts, the highest cell viability was observed at 100.0 µg/mL (75.45 ± 0.109%), meanwhile the lowest at 20 µg/mL (42.56 ± 0.004%).

The ethanolic crude extracts of *Centella asiatica* showed the highest cell viability at 100.0 µg/mL (75.05 ± 0.037%) and the lowest at 40.0 µg/mL (45.69 ± 0.007%). The hexane crude extracts of *Centella asiatica* meanwhile showed the highest cell viability at 20.0 µg/mL (77.01 ± 0.027%) and the lowest at 80.0 µg/mL (46.38 ± 0.004%). For both *Pereskia bleo* and *Centella asiatica*, lower cell viability was observed upon 48 hours of treatment as compared to 24 hours.

Figure 4.3 to Figure 4.6 show the graphical representation of the cell viability of the K-562 cells upon treatment with *Pereskia bleo* and *Centella asiatica* crude extracts, and the positive controls, 5- fluorouracil and doxorubicin. The graph shows a fluctuating trend of cell viability. There is an inconsistency in the changes of the cell viability as the concentration increases.

The IC_{50} values obtained from graphical interpolation upon treatment with *Pereskia bleo* and *Centella asiatica* crude extracts were tabulated in Table 4.6. The lowest IC_{50} value was yielded from ethanolic crude extracts of *Pereskia bleo* and *Centella asiatica*, 21.5 µg/mL and 30.0 µg/mL, respectively. Lower IC_{50} values were obtained at 48 hours of treatments. Both the hexane crude extracts showed higher IC_{50} values compared to ethanolic crude extracts.

Table 4.3: The percentage of viability of K-562 cells treated with *Pereskia bleo* and *Centella asiatica* extracts after 24 and 48 hours treatment.

Plant sample	Crude Extracts	Concentration (µg/mL)	Percentage of viability (%)	
			24 hours	48 hours
Pereskia bleo	Ethanol	20.0	69.38 ± 0.176	50.42 ± 0.017
		40.0	75.96 ± 0.004	45.20 ± 0.040
		60.0	84.90 ± 0.153	47.04 ± 0.065
		80.0	75.67 ± 0.039	52.08 ± 0.014
		100.0	82.53 ± 0.109	53.72 ± 0.030
	Hexane	20.0	63.30 ± 0.017	42.56 ± 0.004
		40.0	72.86 ± 0.103	54.54 ± 0.063
		60.0	69.90 ± 0.045	49.82 ± 0.075
		80.0	72.09 ± 0.060	51.27 ± 0.027
		100.0	75.45 ± 0.109	49.35 ± 0.009
Centella asiatica	Ethanol	20.0	70.52 ± 0.029	54.30 ± 0.027
		40.0	64.67 ± 0.034	45.69 ± 0.007
		60.0	73.49 ± 0.071	45.57 ± 0.027
		80.0	72.64 ± 0.023	46.35 ± 0.021
		100.0	75.05 ± 0.037	51.90 ± 0.010
	Hexane	20.0	77.01 ± 0.027	55.67 ± 0.025
		40.0	57.44 ± 0.011	48.46 ± 0.043
		60.0	65.84 ± 0.009	47.95 ± 0.040
		80.0	67.48 ± 0.009	46.38 ± 0.004
		100.0	72.46 ± 0.030	47.41 ± 0.010

Results are expressed as mean ± standard deviation (n=3)

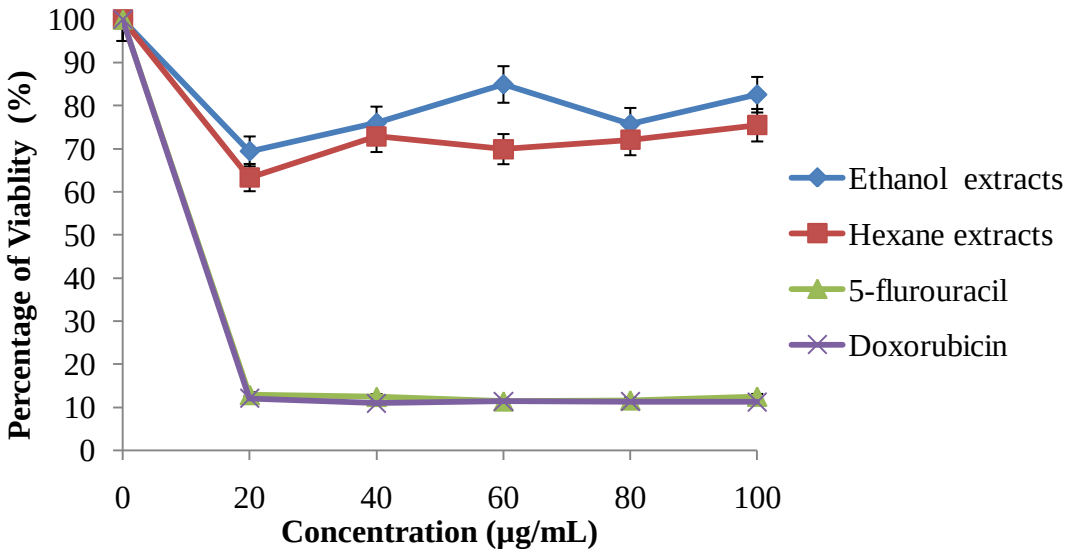

Figure 4.3: The percentage cell viability of K-562 cells after 24 hours treatment with various concentrations of extract of *Pereskia bleo* and the positive controls. The IC_{50} values were determined from graphical interpolation.

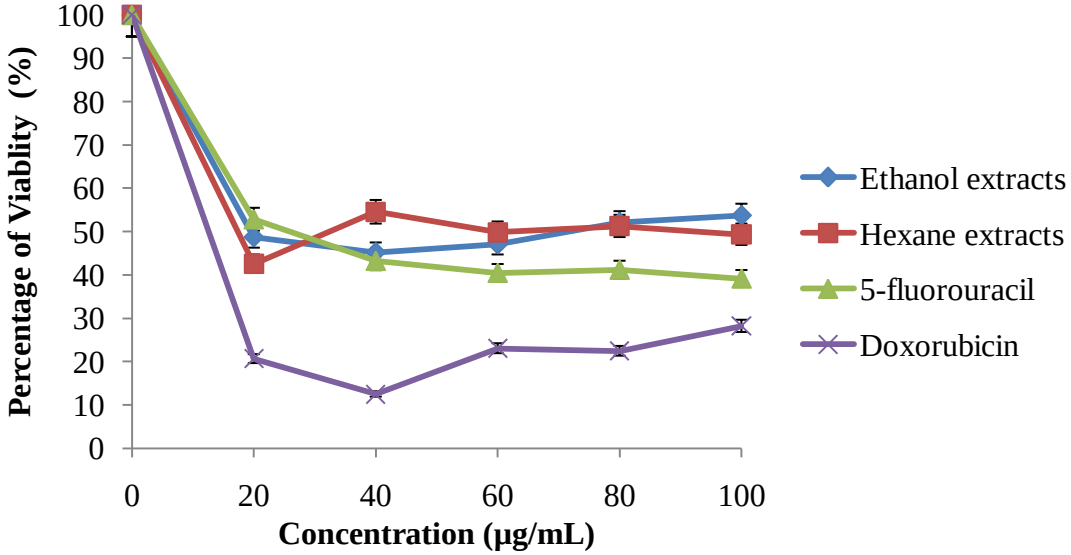

Figure 4.4: The percentage cell viability of K-562 cells after 48 hours treatment with various concentrations of extract of *Pereskia bleo* and the positive controls. The IC_{50} values were determined from graphical interpolation.

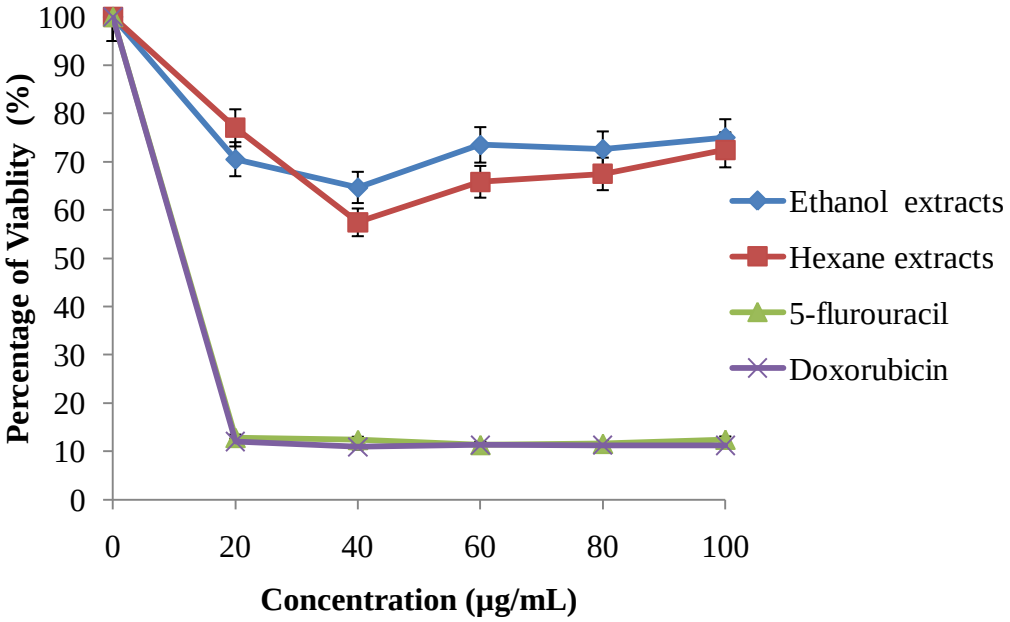

Figure 4.5: The percentage cell viability of K-562 cells after 24 hours treatment with various concentrations of extract of *Centella asiatica* and the positive controls. The IC_{50} values were determined from graphical interpolation.

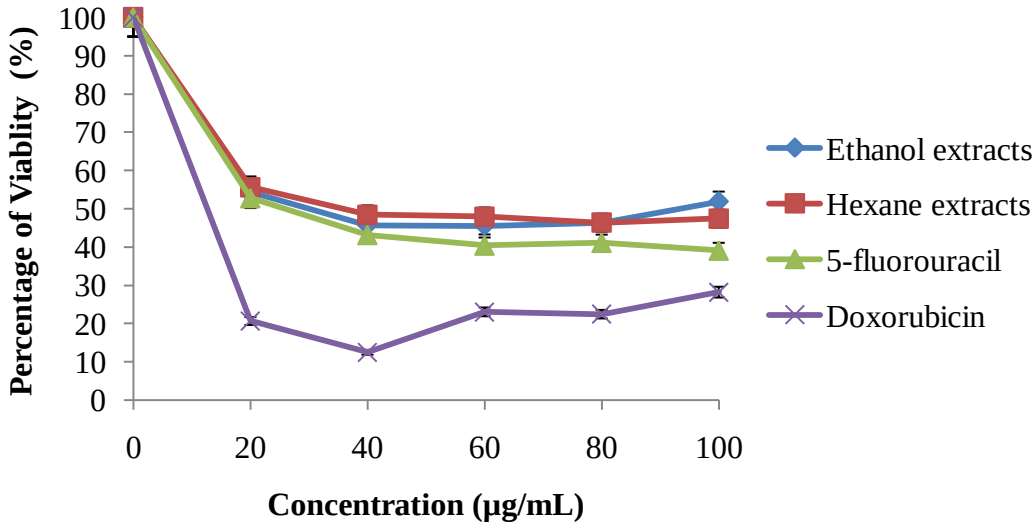

Figure 4.6: The percentage cell viability of K-562 cells after 48 hours treatment with various concentrations of extracts of *Centella asiatica* and the positive controls. The IC_{50} values were determined from graphical interpolation.

The percentage of viability of K-562 cells after treatment with various mixture ratios between *Pereskia bleo* and *Centella asiatica* crude extracts is shown in Table 4.4. At ratio 1:1, the highest cell viability for ethanolic crude extracts was at 40.0 µg/mL (89.41 ± 0.165%) and the lowest cell viability at 20.0 µg/mL (45.44 ± 0.388%). For hexane crude extracts meanwhile the highest cell viability was observed at 100.0 µg/mL (66.36 ± 0.053%) and the lowest cell viability at 20.0 µg/mL (43.42 ± 0.381%).

At ratio 3:7, the highest cell viability for ethanolic crude extracts was observed at 60.0 µg/mL (84.23 ± 0.088%) meanwhile the lowest cell viability at 100.0 µg/mL (58.10 ± 0.024%). For hexane crude extracts, the highest cell viability was observed at 100.0 µg/mL (84.87 ± 0.229%) meanwhile the lowest cell viability was at 20.0 µg/mL (67.31 ± 0.175%).

At ratio 7:3, the highest cell viability for ethanolic crude extracts was observed at 20.0 µg/mL (93.13 ± 0.116%) meanwhile the lowest cell viability at 80.0 µg/mL (46.38 ± 0.057%). For hexane crude extracts, the highest cell viability was observed at 80.0 µg/mL (99.80 ± 0.177%) meanwhile the lowest cell viability was at 60.0 µg/mL (49.52 ± 0.073%). Among all the ratios, the lowest cell viability was observed at ratio 1:1 hexane crude extracts.

Figure 4.7 to Figure 4.12 shows the graphical representation of the cell viability of the K-562 cells upon treatment with various mixture ratios of *Pereskia bleo* and *Centella asiatica*. A general trend of fluctuating was observed in all the graphs. However, lower cell viability was observed in 24 hours of treatment of the mixture ratio crude extracts rather 48 hours.

The IC_{50} values obtained from graphical interpolation were tabulated in Table 4.6. Lower IC_{50} values were obtained in 24 hours treatment of the mixture ratio crude extracts compared to 48 hours. Hexane crude extracts at ratio 3:7 showed the lowest IC_{50} value of 21.0 µg/mL.

Table 4.4: The percentage of viability of K-562 cells after treated with crude extracts at different ratio mixtures after 24 and 48 hours treatment.

Mixture Ratios	Crude Extracts	Concentration (µg/mL)	Percentage of Viability (%)	
			24 hours	48 hours
1:1	Ethanol	20.0	45.44 ± 0.388	56.74 ± 0.301
		40.0	73.98 ± 0.095	89.41 ± 0.165
		60.0	70.15 ± 0.115	40.35 ± 0.183
		80.0	61.17 ± 0.337	52.66 ± 0.161
		100.0	48.40 ± 0.322	73.60 ± 0.298
	Hexane	20.0	63.63 ± 0.207	43.42 ± 0.381
		40.0	61.86 ± 0.183	65.39 ± 0.115
		60.0	61.39 ± 0.066	52.42 ± 0.367
		80.0	53.17 ± 0.113	50.18 ± 0.242
		100.0	66.36 ± 0.053	50.49 ± 0.372
3:7	Ethanol	20.0	61.61 ± 0.030	67.32 ± 0.175
		40.0	58.27 ± 0.050	72.47 ± 0.129
		60.0	59.78 ± 0.043	84.23 ± 0.088
		80.0	56.83 ± 0.026	76.42 ± 0.215
		100.0	58.10 ± 0.024	76.73 ± 0.376
	Hexane	20.0	67.31 ± 0.175	72.35 ± 0.109
		40.0	72.47 ± 0.129	77.93 ± 0.060
		60.0	84.23 ± 0.088	73.93 ± 0.206
		80.0	76.42 ± 0.215	78.12 ± 0.286
		100.0	76.73 ± 0.376	84.87 ± 0.229
7:3	Ethanol	20.0	51.90 ± 0.013	93.13 ± 0.116
		40.0	53.49 ± 0.011	62.44 ± 0.244
		60.0	48.21 ± 0.062	89.21 ± 0.014
		80.0	46.38 ± 0.057	61.99 ± 0.089
		100.0	49.24 ± 0.034	65.82 ± 0.220
	Hexane	20.0	56.96 ± 0.082	96.01 ± 0.302
		40.0	50.87 ± 0.073	64.62 ± 0.211
		60.0	49.52 ± 0.128	82.90 ± 0.187
		80.0	54.65 ± 0.068	99.80 ± 0.177
		100.0	51.20 ± 0.133	71.90 ± 0.342

Results are expressed as mean ± standard deviation (n=3)

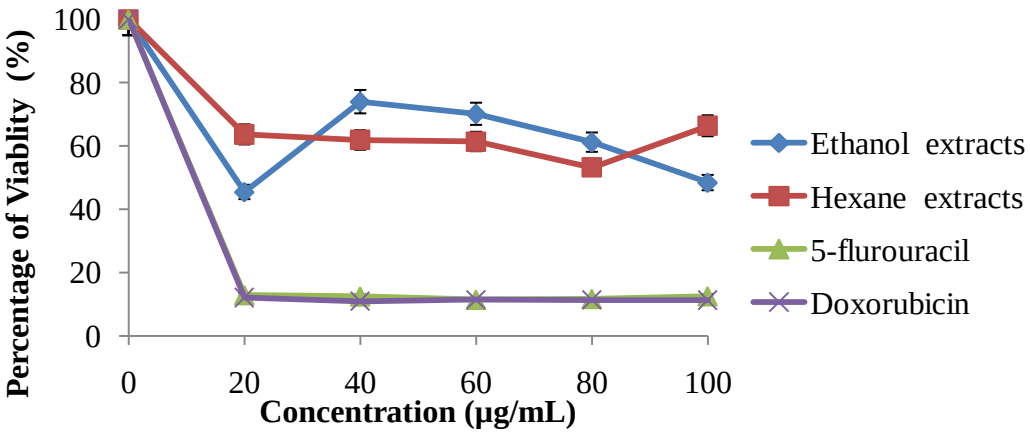

Figure 4.7: The percentage cell viability of K-562 cells after 24 hours treatment with various concentrations of extracts of *Pereskia bleo* and *Centella asiatica* at ratio of 1:1, and the positive controls. The IC_{50} values were determined from graphical interpolation.

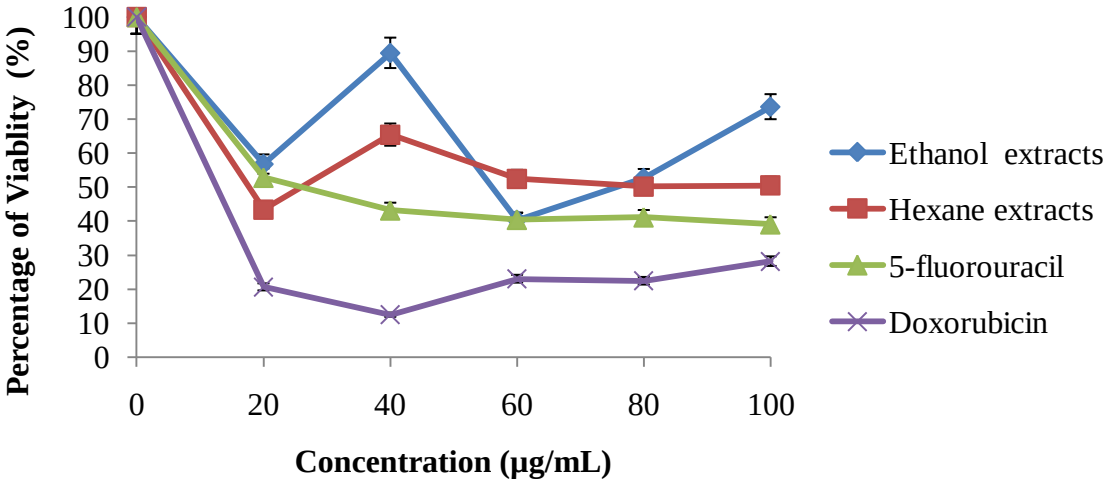

Figure 4.8: The percentage cell viability of K-562 cells after 48 hours treatment with various concentrations of extracts of *Pereskia bleo* and *Centella asiatica* at ratio of 1:1, and the positive controls. The IC_{50} values were determined from graphical interpolation.

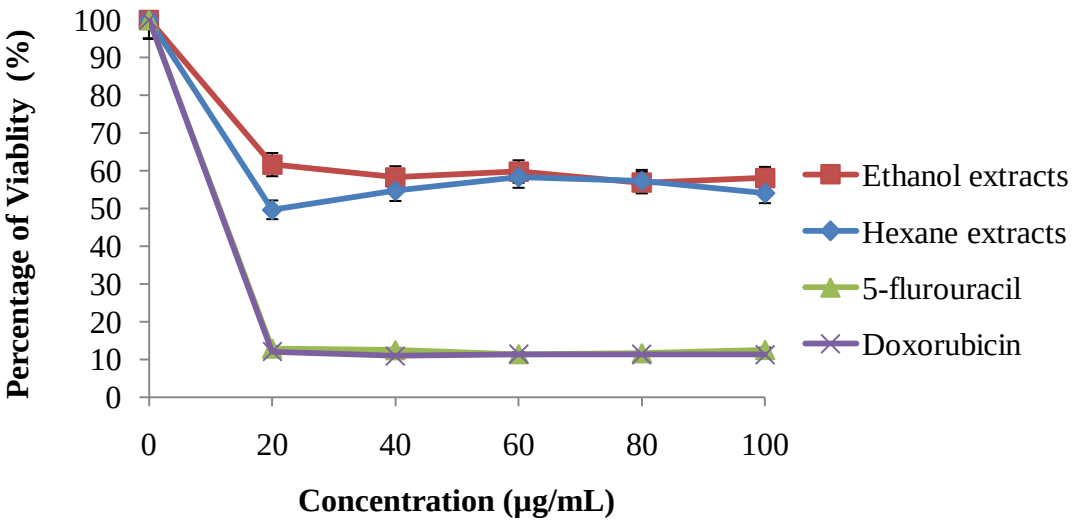

Figure 4.9: The percentage cell viability of K-562 cells after 24 hours treatment with various concentrations of extracts of *Pereskia bleo* and *Centella asiatica* at ratio of 3:7, and the positive controls. The IC_{50} values were determined from graphical interpolation.

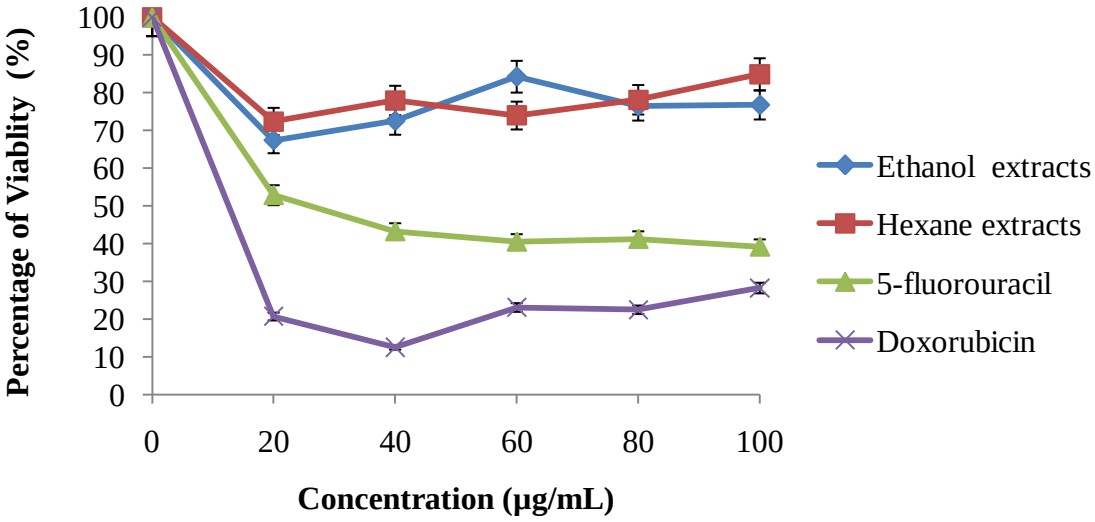

Figure 4.10: The percentage cell viability of K-562 cells after 48 hours treatment with various concentrations of extracts of *Pereskia bleo* and *Centella asiatica* at ratio of 3:7, and the positive controls. The IC_{50} values were determined from graphical interpolation.

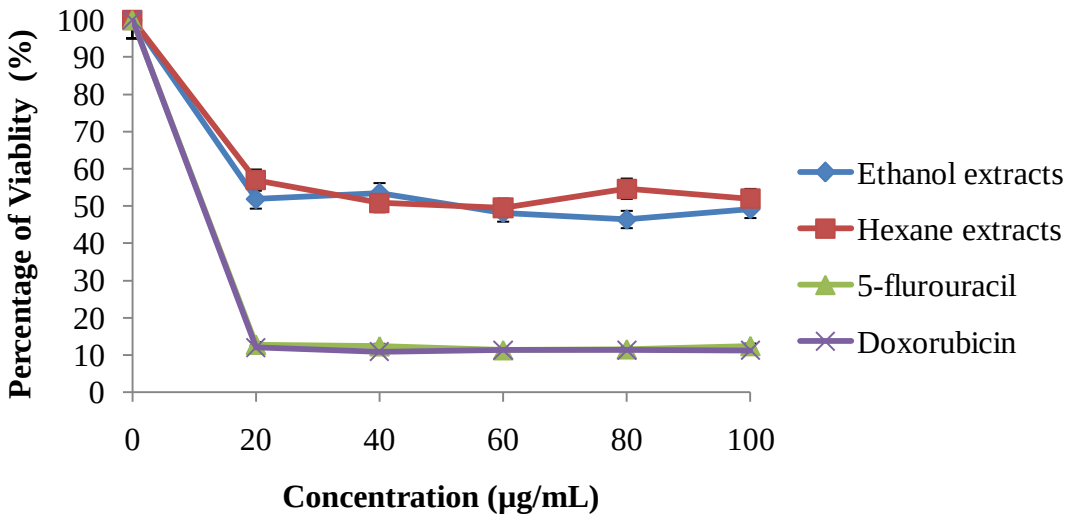

Figure 4.11: The percentage cell viability of K-562 cells after 24 hours treatment with various concentrations of extracts of *Pereskia bleo* and *Centella asiatica* at ratio of 7:3, and the positive controls. The IC_{50} values were determined from graphical interpolation.

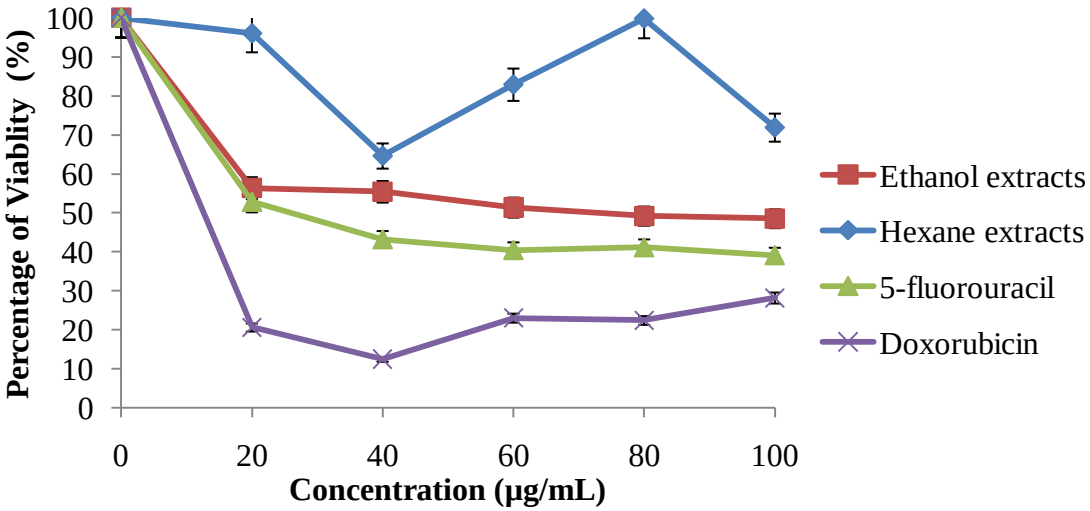

Figure 4.12: The percentage cell viability of K-562 cells after 48 hours treatment with various concentrations of extracts of *Pereskia bleo* and *Centella asiatica* at ratio of 7:3, and the positive controls. The IC_{50} values were determined from graphical interpolation.

4.2.2 Positive Controls

Commercialized anticancer drugs, 5-fluorouracil and doxorubicin were used as positive controls in treating K-562 cells. The percentage cell viability of K-562 cells treated with different concentration of the positive controls is shown in Table 4.5. The lowest percentage viability of 5-fluorouracil and doxorubicin was 11.36 ± 0.0012% and 10.93 ± 0.002%, respectively. The highest percentage viability of 5-fluorouracil and doxorubicin was 52.52 ± 0.040% and 28.31 ± 0.065%, respectively. Both 5-fluorouracil and doxorubicin yielded lower IC_{50} value at 24 hours treatment as referring to Table 4.6. Doxorubicin showed the lowest IC_{50} value of 11.0 µg/mL at 24 hours of treatment.

Table 4.5: Percentage of viability of K-562 cells treated with various concentrations of 5-fluorouracil and doxorubicin at 24 and 48 hours treatment.

Positive control	Concentration (µg/mL)	Percentage of viability (%)	
		24 hours	48 hours
5-fluorouracil	20.0	12.84 ± 0.023	52.52 ± 0.040
	40.0	12.41 ± 0.015	43.22 ± 0.043
	60.0	11.36 ± 0.012	40.46 ± 0.007
	80.0	11.61 ± 0.018	41.18 ± 0.134
	100.0	12.47 ± 0.013	39.14 ± 0.165
Doxorubicin	20.0	12.04 ± 0.050	20.65 ± 0.032
	40.0	10.93 ± 0.002	12.49 ± 0.025
	60.0	11.36 ± 0.011	23.05 ± 0.010
	80.0	11.30 ± 0.006	22.45 ± 0.033
	100.0	11.24 ± 0.005	28.31 ± 0.065

Results are expressed as mean ± standard deviation (n=3)

Table 4.6: The IC_{50} values of *Pereskia bleo* and *Centella asiatica* crude extracts, different ratio mixture and the positive controls against K-562 cells.

Plant sample/Ratio	Crude Extracts	IC_{50} value (µg/mL)	
		24 hours	48 hours
Pereskia bleo	Ethanol	>100	21.5
	Hexane	>100	32.5
Centella asiatica	Ethanol	>100	30.0
	Hexane	>100	32.0
1:1	Ethanol	23.5	57.0
	Hexane	>100	26.5
3:7	Ethanol	>100	>100
	Hexane	21.0	>100
7:3	Ethanol	52.5	>100
	Hexane	50.0	>100
5-fluorouracil	-	11.5	36.5
Doxorubicin	-	11.0	12.5

Results are expressed as mean ± standard deviation (n=3)

4.3 DPPH Assay

Table 4.7 shows the percentage radical scavenging activity of *Pereskia bleo* and *Centella asiatica* crude extracts after 30 minutes of incubation. For *Pereskia bleo*, the highest radical scavenging activity for ethanolic crude extracts was observed at 800 µg/mL (80.02 ± 0.003%) meanwhile the lowest radical scavenging activity at 200.0 µg/mL (30.36 ± 0.078%). For hexane crude extracts meanwhile, the highest radical scavenging activity was observed at 1000.0 µg/mL (46.13 ± 0.182%) and the lowest scavenging activity at 200.0 µg/mL (14.72 ± 0.023%).

The ethanolic crude extracts of *Centella asiatica* yielded the highest scavenging activity 800.0 µg/mL (44.59 ± 0.013%) meanwhile the lowest radical scavenging activity at 200.0 µg/mL (12.29 ± 0.065%). For hexane crude extracts meanwhile, the highest radical scavenging activity was observed at 800.0 µg/mL (14.02 ± 0.072%) and the lowest scavenging activity at 1000.0 µg/mL (1.12 ± 0.072%).

Figure 4.13 and Figure 4.14 show the graphical representation of the percentage radical scavenging activity of *Pereskia bleo* and *Centella asiatica* crude extracts. The radical scavenging activity increases as the concentration of the crude extracts increases, following a dose-dependent manner.

Table 4.10 shows the IC_{50} values of the scavenging activity of *Pereskia bleo* and *Centella asiatica* crude extracts. The lowest IC_{50} value shown was 475 µg/mL of *Pereskia bleo* hexane crude. Ethanolic crude extract of *Pereskia bleo* All *Centella asiatica* crude extracts yielded IC_{50} value of more than 1000 µg/mL.

Table 4.7: Percentage radical scavenging activity of *Pereskia bleo* and *Centella asiatica* crude extracts after 30 minutes of incubation.

Crude extracts	Concentration (µg/mL)	Radical scavenging activity (%)	
		Ethanol	Hexane
Pereskia bleo	200.0	30.36 ± 0.078	14.72 ± 0.023
	400.0	48.44 ± 0.055	19.67 ± 0.006
	600.0	70.03 ± 0.035	36.60 ± 0.115
	800.0	80.02 ± 0.003	44.99 ± 0.080
	1000.0	73.16 ± 0.062	46.13 ± 0.182
Centella asiatica	200.0	12.29 ± 0.065	4.91 ± 0.032
	400.0	21.76 ± 0.036	3.19 ± 0.024
	600.0	34.23 ± 0.007	7.36 ± 0.077
	800.0	44.59 ± 0.013	14.02 ± 0.072
	1000.0	42.30 ± 0.153	1.12 ± 0.072

Results are expressed as mean ± standard deviation (n=3)

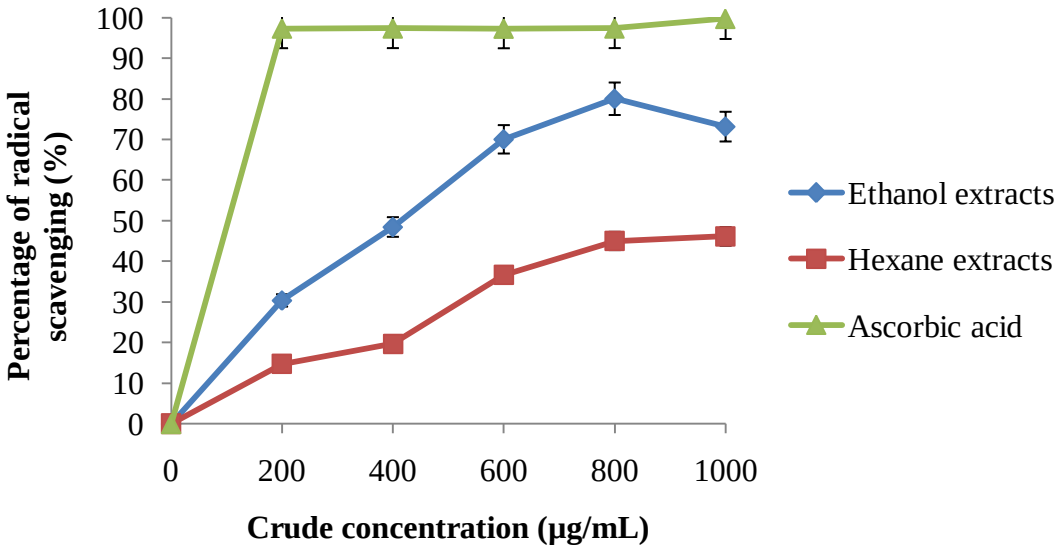

Figure 4.13: The percentage scavenging activity of *Pereskia bleo* crude extracts and ascorbic acid with different concentrations. The IC_{50} values were determined from graphical interpolation.

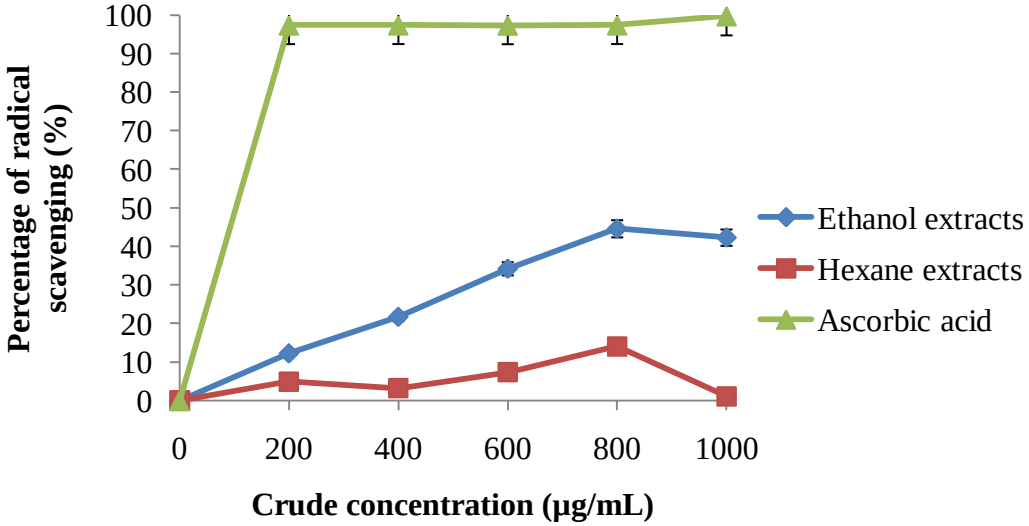

Figure 4.14: The percentage scavenging activity of *Centella asiatica* crude extracts and ascorbic acid with different concentrations. The IC_{50} values were determined from graphical interpolation.

Table 4.8 shows the percentage radical scavenging activity of different mixture ratio *Pereskia bleo* and *Centella asiatica* crude extracts after 30 minutes of incubation. For ratio 1:1, the highest scavenging activity using ethanolic crude extracts was observed at 1000.0 µg/mL (27.00 ± 0.366%) and lowest at 200.0 µg/mL (6.98 ± 0.027%). Meanwhile, the hexane crude extracts yielded the highest scavenging activity at 1000.0 µg/mL (56.40 ± 0.045%) and the lowest at 200.0 µg/mL (7.91 ± 0.023%).

For ratio 3:7, the highest scavenging activity using ethanolic crude extracts was observed at 1000.0 µg/mL (56.96 ± 0.045%) and lowest at 200.0 µg/mL (6.38 ± 0.023%). Meanwhile, the hexane crude extracts yielded the highest scavenging activity at 1000.0 µg/mL (17.78 ± 0.050%) and the lowest at 200.0 µg/mL (0.96 ± 0.011%).

For ratio 7:3, the highest scavenging activity using ethanolic crude extracts was observed at 1000.0 µg/mL (69.03 ± 0.185%) and lowest at 200.0 µg/mL (11.14 ± 0.110%). Meanwhile, the hexane crude extracts yielded the highest scavenging activity at 1000.0 µg/mL (34.84 ± 0.009%) and the lowest at 200.0 µg/mL (0.31 ± 0.190%). Overall, ethanolic crude extracts at ratio 7:3 yielded the highest radical scavenging activity when compared among all the other ratios.

Figure 4.15 to Figure 4.17 show the graphical representation of the percentage radical scavenging activity of the mixture crude extracts at ratio 1:1, 3:7 and 7:3. The graphs follow dose dependent manner trend, where as the concentration of the crude increases, the percentage radical scavenging activity increases accordingly.

Table 4.10 shows the IC_{50} values of the scavenging activity of the mixture crude extracts. All ethanolic crude extracts yielded lower IC_{50} value compared to the hexane crude extracts. The lowest IC_{50} value was observed using ethanolic crude extract at ratio 7:3 with IC_{50} of 730 µg/mL.

Table 4.8: Percentage radical scavenging activity of *Pereskia bleo* and *Centella asiatica* mixture crude extracts at different ratios after 30 minutes of incubation.

Ratios of Mixture	Concentration (µg/mL)	Radical scavenging activity (%)	
		Ethanol	Hexane
1:1	200.0	6.98 ± 0.027	7.91 ± 0.023
	400.0	17.89 ± 0.218	22.13 ± 0.033
	600.0	20.03 ± 0.101	34.67 ± 0.081
	800.0	21.16 ± 0.227	45.78 ± 0.037
	1000.0	27.00 ± 0.366	56.40 ± 0.045
3:7	200.0	6.38 ± 0.023	0.96 ± 0.011
	400.0	17.78 ± 0.033	1.47 ± 0.245
	600.0	39.92 ± 0.081	14.36 ± 0.110
	800.0	39.83 ± 0.038	14.58 ± 0.176
	1000.0	56.96 ± 0.045	17.78 ± 0.050
7:3	200.0	11.14 ± 0.110	0.31 ± 0.190
	400.0	29.91 ± 0.020	0.50 ± 0.085
	600.0	40.28 ± 0.134	15.96 ± 0.388
	800.0	54.14 ± 0.175	16.58 ± 0.089
	1000.0	69.03 ± 0.185	34.84 ± 0.009

Results are expressed as mean ± standard deviation (n=3)

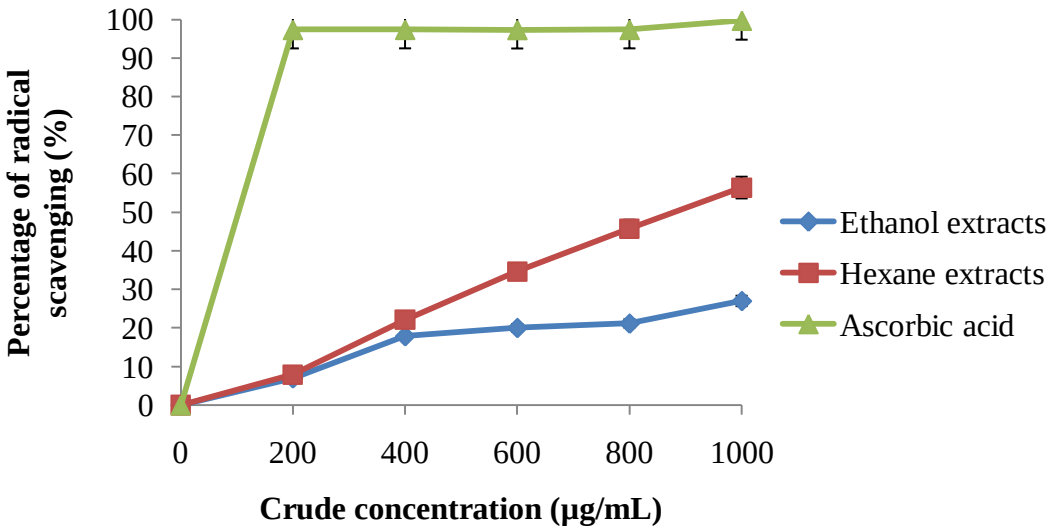

Figure 4.15: The percentage scavenging activity of *Centella asiatica* and *Pereskia bleo* crude extracts at ratio of 1:1, and ascorbic acid with different concentrations. The IC_{50} values were determined from graphical interpolation.

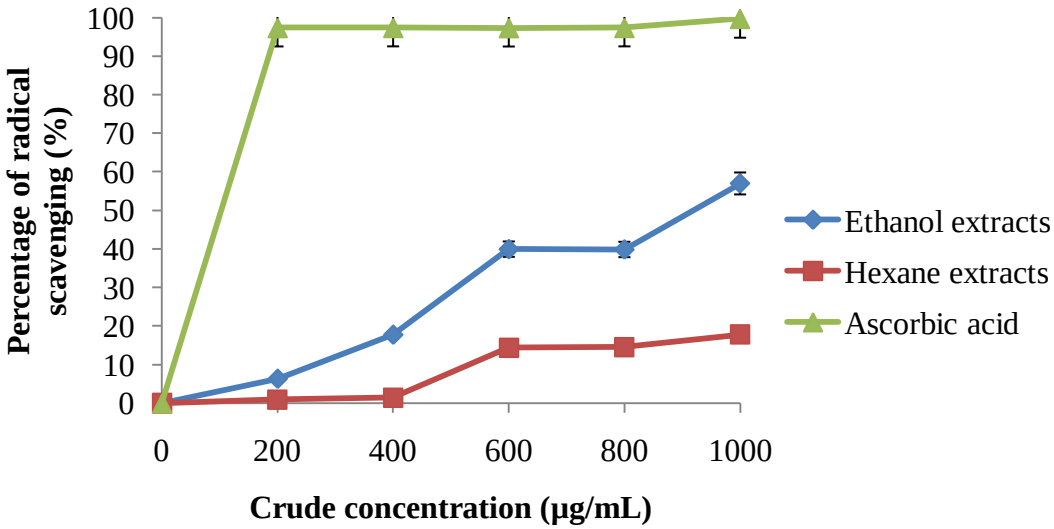

Figure 4.16: The percentage scavenging activity of *Centella asiatica* and *Pereskia bleo* crude extracts at ratio of 3:7, and ascorbic acid with different concentrations. The IC_{50} values were determined from graphical interpolation.

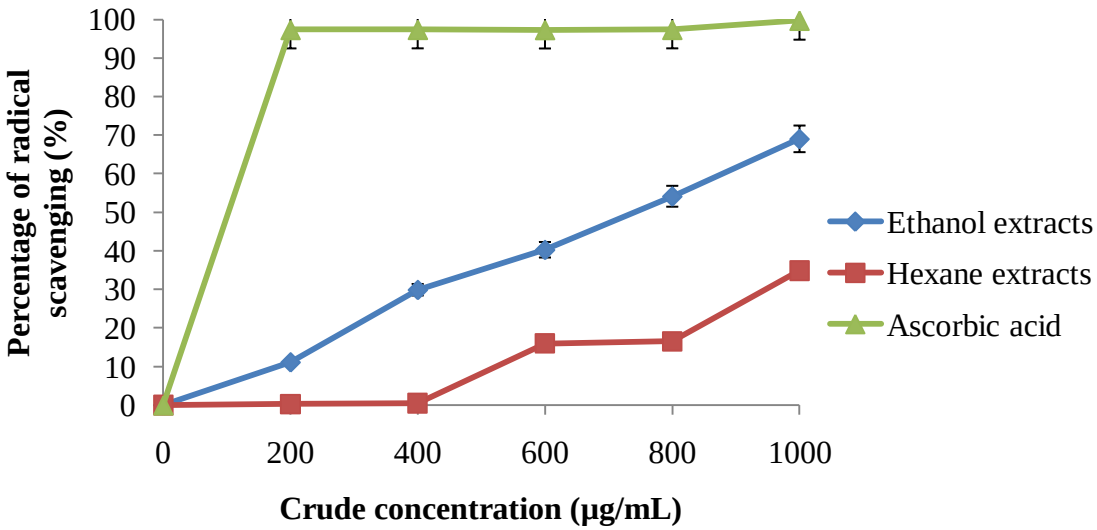

Figure 4.17: The percentage scavenging activity of *Centella asiatica* and *Pereskia bleo* crude extracts at ratio of 7:3, and ascorbic acid with different concentrations. The IC_{50} values were determined from graphical interpolation.

4.3.1 Positive Control

Table 4.9 shows the percentage radical scavenging activity of ascorbic acid. The highest radical scavenging activity was observed at 1000.0 µg/mL (99.73 ± 0.005%). The radical scavenging activity increases as the higher concentration of the ascorbic acid used. Table 4.10 shows the IC_{50} value of the scavenging activity of the ascorbic acid. Ascorbic acid yielded the lowest IC_{50} value as compared to the rest of the crude extracts, with an IC_{50} value of 105 µg/mL.

Table 4.9: Percentage radical scavenging activity of different concentrations of ascorbic acid.

Concentration (µg/mL)	Radical scavenging activity (%)
200	97.35 ± 0.001
400	97.37 ± 0.037
600	97.37 ± 0.005
800	97.37 ± 0.006
1000	99.73 ± 0.005

Results are expressed as mean ± standard deviation (n=3)

Table 4.10: The IC_{50} values of *Pereskia bleo* and *Centella asiatica* crude extracts, positive control and different ratio mixture in DPPH assay.

Plant sample/Ratio	Crude extracts	IC_{50} value (µg/mL)
Pereskia bleo	Ethanol	475
	Hexane	>1000
Centella asiatica	Ethanol	>1000
	Hexane	>1000
1:1	Ethanol	880
	Hexane	>1000
3:7	Ethanol	920
	Hexane	>1000
7:3	Ethanol	730
	Hexane	>1000
Positive control	Ascorbic acid	105

> Indicating possible antioxidant activity using higher crude extracts concentration.

CHAPTER 5

DISCUSSION

5.1 Plant Extraction

Plants have primary and secondary metabolites which make up their general organic composition. The primary metabolites such as phytosterols, lipids and amino acids, are involved directly in the growth and development of the plant. Most of these compounds are readily available in other further complex organisms such as human (Buchanan et al., 2000). However the secondary metabolites such as terpenoids, alkaloids and phenolic have the main focus in the extraction process (Sasidharan et al., 2011; Buchanan et al., 2000). Despite the functionality of most of the secondary metabolites in the plant remains unknown, these chemical compounds often posses pharmacological and biological activities (Visht and Chaturverdi 2012).

In order to yield maximum amount the crude extracts from the plant samples, fresh plant samples were filtered and bacterial and/or fungal infected samples were discharged. It was then washed to remove all the soil debris which can possibly affect the extraction process. Since hexane, a water insoluble solvent was used as part of the extraction solvent; the plant samples were dried to remove the water.

Two common methods of drying are air-drying and freeze-drying, however from previous investigation showed that *Pereskia bleo* and *Centella asiatica* are commonly dried via air drying, which was adapted into this study (Lee et al., 2009; Mohandas et al., 2006). The drying temperature was kept between 30ºC and 50ºC, as plants may have thermolabile compounds which can be easily destroyed in high temperature (de Paiva et al., 2004). Prior to extraction, the plant samples were authenticated by botanist to validate the genus and species of plant samples.

The plant samples were ground into powdered form in order to enhance the extraction kinetic via increasing the total surface are of the plant sample exposed to the extraction solvent and to create a homogenous mixture (Sasidharan et al., 2011). This will increase the effectiveness of the extraction process which may result in collection of more bioactive compounds such as phytols and sterols.

The extraction process can be carried out using different methods such as sonification, maceration and soxhlet extractions, microwave assisted extraction, solid-phase extraction and supercritical-fluid extraction (Sasidharan et al., 2011). This study adapted the most common, rapid and inexpensive extraction technique. Maceration or also known as cold solvent extraction, involve soaking the plant sample in selected solvents for a period time (Sarket et al., 2006). The longer the soaking period, the more compounds could be extracted out from the plant which posses the medicinal properties.

Upon soaking, the solvent was drained off from the plant before it was evaporated using rotary evaporator.

A successful extraction indicates variety of compounds have been isolated from the plant sample. This is highly dependent on the type of solvent used in the extraction process. The parameters that need to be looked into while choosing the type of extraction solvent are the nature of the targeted compound in the plant, the toxicity and melting point of the solvent which is preferably low and the solvent should not alter the structure and function of the extracted compounds (Ncube et al., 2008). Table 5.1 shows the different compounds extracted by using different solvent, in the line with the "like dissolves like" principle (Schmid 2001).

In this study, *Pereskia bleo* and *Centella asiatica* were extracted using ethanol and hexane. Malek et al. (2009) and Pittella et al. (2009) reported that *Pereskia bleo* and *Centella asiatica,* respectively, contain both polar and non-polar components, hence the choice of extraction solvent must include solvents that could extract out the polar and non-polar components. A polar solvent such as ethanol is commonly used to extract out polar compounds such as flavonoids (antioxidant) whereas non-polar solvents such as hexane extract out non-polar compounds such as terpenoids (antioxidant) (Visht and Chaturvedi 2012).

Table 5.1: Examples of bioactive compounds extracted by different solvents (Tiwari et al., 2011).

Water	Ethanol	Chloroform	Ether
Tannins	Tannins	Terpenoids	Alkaloids
Saponins	Polyphenols	Flavanoids	Terpenoids
Terpenoids	Terpenoids		Coumarins
Lectins	Alkaloids		

The crude extracts of the plant samples were dissolved in DMSO. As a cosolvent, DMSO improves the solubility of the plant bioactive compounds. Since crude extract contains diverse compounds, hence a universal solvent is needed to dissolve all the compounds to produce a homogenized solution (Violante et al., 2002). DMSO has been recorded to be toxic to cells at high concentration as it damages the integrity of the cell membrane, however studies have shown less than 5% DMSO is safe to be used in cytotoxic assay as it do not disrupt the integrity of the cells (Violante et al., 2002). Hence, 1% DMSO was used in this study.

The ethanolic extracts yielded the highest amount of crude as compared to the non-polar hexane extract for both the plant samples. The percentage yield of ethanolic crude extract of *Pereskia bleo* and *Centella asiatica* is high as 9.32% and 11.97%, respectively. The high amount of ethanol extract provides an insight that most components are at polar in nature for both these plant samples such as flavanoids and alkaloids.

5.2 Bioassay Results

5.2.1 Cytotoxic Assay

In vitro cytotoxicity has been a valuable assay for many pharmaceutical companies and cancer study researches, where the chemical cytotoxicity screening is analyzed due to its simplicity in the conduction of the assay and its high correlation to the *in vivo* data (Riss and Moravec 2004). Hence, this *in vitro* study helps in the identification and evaluation of the new therapeutic agents, either synthetic or natural product, on its effectiveness against cancer cells (Mirzayans et al., 2007). Using the *in vitro* cellular-based cytotoxicity bioassay, a compound is said cytotoxic agent when the compound exhibits its toxicity to the tumor cells, and is said to have anti-tumor properties when this toxicity is transferred to tumor cells *in vivo* experimentation (Itharat and Ooraikul 2007).

The IC_{50} value that is the concentration of drug required to inhibit the growth and proliferation of the cells by 50% was used an indicator in measuring the toxicity exhibited by the plant crude extracts (Fellows and O'Donovan 2007). By the standard set by National Cancer Institute, a crude extract is said have anti-tumor properties when the IC_{50} value is less than 50 µg/ml (Mans et al., 2000).

To study the cytotoxic effect of these plant extracts, MTT assay was used to evaluate the effectiveness of toxicity. MTT assay has been widely used, especially in studies involving chemosensitivity, radio sensitivity and immunological studies. MTT assay was used in this study due to its simplicity in the assay conduction, high reliability and sensitivity (Verma et al., 2010).

The growth curve of a cell includes the lag phase, the log phase and the stationary phase. The lag phase begins immediately after reseeding which could last up to 12 to 24 hours. In this phase, the cells recover themselves from the action of trypsin, remodel their cytoskeleton and prepare the necessary components for cell cycle. Then they enter the log phase where the population of the cells doubles. Due to the crowded environment and depleting nutrients, the cells eventually stop dividing and exit the cell cycle, which is known as the stationary phase (Freshney 2005). The K-562 cells were observed to reach 70% to 80% confluency in 72 hours of culturing, indicating the cells enter the stationary phase at 72 hours.

From the results obtained, the individual crude extract of the plant samples showed possible cytotoxic activities. Overall, the ethanolic and hexane crude extracts of *Pereskia bleo* and *Centella asiatica* showed higher cytotoxic activities upon 48 hours treatment as compared to 24 hours treatment. This may due to the fact that some compounds are relatively slow in reacting. Hence, the compounds extracted from both the plant samples most probably required more time to absorbed by the cancer cells, activate its compounds and exhibit its cytotoxicity (Freshney 2005). Thus, in 24 hours of treatment, the

bioactive compounds in the plant crudes does not have enough time to exhibit its effect on the cells, which is represented by the high cell viability.

The percentage cell viability fluctuates between 40 to 70% throughout the various concentrations of the extracts, which results in non-linear graphs. This may be due to the usage of crude extract instead of purified compounds. Crude extracts contains various types of bioactive compounds which could interact among each other and yield different results (Buchanan et al., 2000).

Previous investigation on *Pereskia bleo* by Tan et al. (2005) is in agreement with the result obtained from this study. Tan et al. (2005) stated that the methanolic crude extract of *Pereskia bleo* yielded a promising anti-proliferative activity with IC_{50} value of 2.0 µg/mL against human mammary cell line, T47-D. They postulated that the crude extract induced apoptosis in T47-D cells via the activation of caspase 3 and c-myc pathways. Malek et al. (2009) meanwhile showed the methanolic crude extracts and ethyl acetate fractions of *Pereskia bleo* yielded IC_{50} values of 6.5 µg/mL and 4.5 µg/mL, respectively against KB (human nasopharyngeal epidermoid carcinoma) cell lines, meanwhile ethanolic and hexane crude extracts from this study yield IC_{50} value of 21.5 µg/mL and 32.5 µg/mL.

Factors such as difference in extraction solvent, type of cell line used or type of cytotoxic assay performed in evaluating the anti-proliferative properties of this plant maybe account for the variation in the IC_{50} values between these two studies. In term of extraction solvent, Tan et al. (2005) and Malek et al. (2009) used methanol as the solvent of extraction. Methanol has higher polarity as compared to ethanol, in return attracts more polar bioactive compounds from the plant. The higher number of bioactive constituents might give a higher cytotoxic activity (Slatnar et al., 2012). In the previous investigation, both the studies used monolayer cancer cells, as for this study, a suspension cancer cell line was used. Cancer cells differ morphologically; therefore it could be expected to observe variations in the interaction between the compound and the cell (Ariffin et al., 2012).

Centella asiatica on the other hand, also showed promising anti-proliferative activity using ethanol and hexane crude extracts, with IC_{50} values of 30.0 µg/mL and 32.0 µg/mL, respectively. Despite the low crude yield from the hexane extract as compared to the ethanol crude extracts (Table 4.2), there is less difference between the IC_{50} value of the ethanol and hexane crude extracts.

However, Pittella et al. (2009) reported that the aqueous extract of *Centella asiatica* does not show antiproliferative activity against mouse melanoma ($B_{16}F_1$), human breast cancer (MDA MB-231) and rat glioma (C_6) cell lines with IC_{50} value of 698.0 µg/mL, 648.0 µg/mL and 1000.0 µg/mL, respectively. This indicates that aqueous extraction may not be the ideal extraction solvent

as the ethanol and hexane solvent proved to be a better extraction solvent in this study. However, purified fractions of *Centella asiatica* showed antiproliferative effect with IC_{50} values of 17 µg/mL and 22 µg/mL against Ehrlich ascites tumor cells (Babu et al., 1995). This further proves that, *Centella asiatica* does contain potent anti-proliferative compounds and in the affirmative of the result obtained in this study.

Synergism occurs when the combination of the compounds significantly increases or reduces the effectiveness with respect to their effect on individual basis (Mukherjee et al., 2011). For decades, ethnopharmacology research has been focusing on the idea that one or a few active compounds in the plant are the key determinant of its therapeutic effects. However, ancient medicinal system such as Ayurvedic, believes that in order to yield maximum therapeutic effect, the synergistic interaction among all the ingredients of the plant is necessary. Besides that, the idea of focusing on a single active compound extracted from a natural product, which are normally low in availability may not be effective while dealing with multifactorial multitarget diseases such as cancer (Ulrich-Merzenich et al., 2010; Mukherjee et al., 2011).

A pharmacokinetic model, known as bioavailability model proposes that when two drugs are at synergistic, one of the drugs will interact with the other by increasing the drug's availability in the targeted region. The bioavailability is increased by the action of the one of the drugs which results in the enhancement of the second drug's entry into the cells or the delay in degradation of the second drug (Cokol et al., 2011). Hence, this model of

interaction is applicable for diseases such as cancer where this synergistic interaction could able to overcome the multitarget conditions.

The ratios tested were 3:7, 1:1 and 7:3, all with the respect of *Pereskia bleo*: *Centella asiatica*. Cytotoxic activities were observed in almost all the mixture ratios of both the hexane and ethanol crude extracts. The differences in the IC_{50} values yielded among the mixture ratios indicate the possible existence of synergistic interactions. Ethanolic and hexane crude extract at ratio 1:1 also showed high cytotoxic activities with IC_{50} value of 23.5 µg/mL and 26.5 µg/mL, respectively. The lowest IC_{50} value was shown by the hexane crude extracts ratio 3:7, that is 21.0 µg/mL. This indicates the interaction between the non-polar compounds from *Pereskia bleo* such as stigmasterol and campesterol, and non-polar compounds from *Centella asiatica* such as centellasaponin, might have produced an interaction that enhanced its cytotoxic activity (Malek et al., 2009; Pittella et al., 2009).

The ethanolic crude extracts at ratio 3:7 and hexane crude extracts at ratio 1:1 failed to exhibit any cytotoxic activities. A possible explanation is the interaction involved between the compounds in these two ratios was antagonism. Antagonism occurs when a mixture of compounds inhibit each other's activity which results in reduction of overall effect (Chou 2006). Hence, the reduction of the antiproliferative activity due to the antagonism interaction resulted in higher cell viability.

From the result obtained, higher cytotoxic activities were observed in the ratio mixtures crude extracts as compared to the individual crude extracts of the plant sample, thus indicating possible synergistic interaction. This shows that, lower amount of concentration of the crude extracts were needed to inhibit 50% proliferative effect of the cancerous cell when the crude extracts were mixed at ratio 1:1 and 3:7. A possible explanation might be the presence of more bioactive compounds in the mixture ratio created a broad spectrum cluster of compound that could have multiple mode of action in exhibiting its toxicity towards the cells (Ulrich-Merzenich et al., 2010).

A different trend of result was observed in the ratio mixture. Lower cell viability was shown in 24 hours treatment as compared to 48 hours treatment. Due to the unknown nature of the compounds available in the crude extracts and the type of synergistic interaction took place, not much could be deduced from this observation. However, this rationale should be kept in consideration, such that, some interactions yield short lived intermediates, which could exhibit their cytotoxicity in the beginning of the treatment, however the intermediates become less effective in longer incubation (Literak et al., 2003).

Doxorubicin and 5-flurouracil were used as positive controls in the study. These broad spectrum intercalating anticancer agents are commonly used in the cancer therapy (Sadeghi-aliabadi et al., 2010). Doxorubicin exhibits its toxicity on cancerous cells via topoisomerase II inhibition, DNA intercalation and free radical generation, in where any of this mechanism leads to cell death (Patel and Kaufman 2012). 5-flurouracil meanwhile exhibits its anticancer

activity either via its active metabolites which disrupt the RNA synthesis, inhibit the action of thymidylate synthase which is necessary for the DNA replication and repair, or intercalate into the DNA (Longley and Johnston 2007).

The IC_{50} values of these positive controls were lower as compared to all the crude extracts. However, the observed between IC_{50} values does not differ much to each other, indicating these plant extracts could be said as potential anti-proliferative agents. The general trend of all the crude extracts indicates inhibition of growth of the K-562 cell line as the concentration of the crude increases, hence suggesting a dose dependent inhibition.

5.2.2 DPPH Assay

Many human diseases, such as Alzheimer's disease and cancer, arise from oxidative stress, which is indicated by the presence of reactive oxygen species. Natural antagonists to this process are antioxidants (Hassanbaglau et al., 2012). Antioxidants have the capacity to neutralize or at times slow down the unnecessary oxidation process, by removing the free radicals, hence terminating the oxidative reaction (Temple 2000). Attempts to produce synthetic antioxidants have failed as studies show the synthetic compound may be carcinogenic. Hence, once again the attention has shifted towards natural product in producing powerful yet harmless antioxidant compounds in battling various diseases (Zainol et al., 2003).

Antioxidant compounds can be categorized into two groups, primary and secondary antioxidants (Maisuthisakul et al., 2007). This categorization is based on the mode of action against the oxidative stress. Primary antioxidant neutralizes the free radicals by donating hydrogen atoms or electrons which results in inhibition or delay in the oxidation process. The secondary antioxidants have various mode of action which includes binding of metal ions, scavenging oxygen, absorbing UV radiation and conversion of hydrogen peroxide to non-radical species (Maisuthisakul et al., 2007).

DPPH assay was used to measure the antioxidant properties of these plant samples. DPPH assay has been widely used in many experimentation due to its stability of the assay and the reproducibility of the result. Besides that, DPPH assay is inexpensive, straightforward and rapid in determining the scavenging activity of the compound (Thaipong et al., 2006; Yim et al., 2004).

Based on the results obtained from DPPH assay, the individual crude extracts of the plant samples, indicated *Pereskia bleo* and *Centella* asiatica have antioxidant activities. Ethanolic crude extracts of *Pereskia bleo* showed the highest percentage radical scavenging of 80.02 ± 0.003% at concentration 800 µg/mL, meanwhile hexane crude extract yielded the highest percentage radical scavenging at 1000 µg/mL with 46.13 ± 0.182%. This suggests that most of the bioactive compounds with antioxidant properties are at polar state, based on the percentage crude yield and the percentage radical scavenging. The polar compounds found in *Pereskia bleo* such as α-tocopherol has been proven to have high antioxidant capacity, hence explain the high percentage radical scavenging activities (Packer et al., 2001).

Although the percentage crude yield from hexane extracts of *Pereskia bleo* is the lowest, however the percentage radical scavenging was high in comparison with other crude extracts. The results obtained is in agreement with the study by Sim et al. (2010) that polar and non-polar solvent extracts of *Pereskia bleo* can be said as inhibitor of free radical. However, the result is not in agreement with Wahab et al. (2009) which stated that hexane extract yielded the highest percentage radical scavenging as compared to ethanol extract. This may due to

the difference in amount of dry plant sample used. The higher the amount of plant sample used in the extraction process, the more bioactive compound could be yielded, hence producing better scavenging activities.

The highest percentage radical scavenging for *Centella asiatica* was observed from ethanolic crude extracts with 44.59 ± 0.013% at concentration 800 µg/mL meanwhile low scavenging activity among the hexane crude extracts with 1.12 ± 0.072% at concentration 1000 µg/mL. The result indicated, as *Pereskia bleo*, most of the bioactive compounds with antioxidant activities were at polar state. Although the highest percentage crude yielded by ethanolic crude extracts of *Centella asiatica*, the amount of scavenging activity observed was moderate. The scavenging activity recorded might due to the presence of phenolic and flavonic constituents in the plant, which are ideal structures in the hydrogen-donation activity (Pittella et al., 2009).

Huang et al. (2005) suggested that the effectiveness of the antioxidant properties could be evaluated by the calculating the IC_{50} values. In this context, the IC_{50} refers to the concentration of an antioxidant at which 50% inhibition of free radical activity. Lower IC_{50} value indicates high scavenging activities as low amount of scavenger is required to achieve 50% scavenging reaction, hence making them an effective antioxidant. From the result, only ethanolic crude extracts of *Pereskia bleo* manage to yield low IC_{50} value of 475 µg/mL. Other crude extracts exhibited lower than 50% scavenging activity. Interestingly, a few ratio mixtures yielded moderate IC_{50} values. At ratio mixture of 1:1, 3:7 and 7:3 of the ethanolic crude mixture extract, the IC_{50}

values were 880 µg/mL, 920 µg/mL and 730 µg/mL, respectively. These IC_{50} values were higher compared to the IC_{50} value yield by ethanolic crude extract of *Pereskia bleo* alone, suggesting the interaction between these two plants might be antagonism, hence yielding less effective scavengers.

Ascorbic acid was used as the positive control in the DPPH assay. Often take as dietary antioxidant; ascorbic acid reduces the damage caused by the reactive species such as oxygen and nitrogen species. These radicals are known to cause lipid peroxidation, DNA and protein damage which is manifested in many clinical diseases (Naidu 2003). As antioxidant, ascorbic acid neutralizes the radical, thus preventing it from causing cellular damages. Ascorbic acid showed an IC_{50} value of 105 µg/mL, which was lower than the other crude extract mixtures. However, the value does not differ much and hence, these plant samples might have moderate antioxidant activities.

5.3 Study Limitations

The limitations of this study are more focused on the methodology of the experiment. Improper dissolution of the crude extracts in DMSO, especially the non-polar compounds may result in loss of some bioactive compounds during the filtration (Sasidharan et al., 2011). The lost of the bioactive compounds may result in inaccuracy of the result. Hence to avoid this, the working stock was sonicated to allow the DMSO dissolve the extracts.

Besides that, this study focuses more on the toxic effect on the cancerous cells. However, some phytochemical agents may not just exhibit its toxicity towards the targeted region; it causes unwanted side effects to untargeted regions (Sak 2012). Therefore, the full pharmacokinetic of the compounds extracted remained unknown. Other than that, the mechanism of action of these compounds is not studied.

5.4 Future Studies

In this study, compounds from plant crude extracts were investigated on its biological properties. It has become a common practice in isolating, identifying and elucidating the compounds extracted from the plant source. Further study show include bioactive compound separation techniques such as thin layer chromatography (TLC), purification techniques such as high performance liquid chromatography (HPLC) and elucidation techniques such as nuclear magnetic resonance (NMR) (Sasidharan et al., 2011). Upon isolation and structural elucidation, the resulting purified compound can be used in order to obtain a more accurate result.

Besides MTT assay, these plant samples should be tested using other *in vitro* assays. Numerous assays have been developed over the years to test the effectiveness of natural product compounds on their biological properties. Such assays include LDH release assay, neutral red assay and ATP content assay (Weyermann et al., 2005). Meanwhile, for the antioxidant assays, it is suggested to incorporate other *in vitro* antioxidant assays such as ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorption capacity) (Thaipong et al., 2006). By testing the cytotoxic activity and antioxidant properties using various assays, the comparative study could show the accuracy and reliability of the activities in the plant.

Since different compounds exhibit its toxicity by targeting at different molecular level. Some of the modes of actions used by phytochemical agent include modulation of gene expression, disruption in signal transduction pathway and interference with metabolic processes (D'Incalci et al., 2005). *Pereskia bleo* and *Centella asiatica* exhibited significant anti-proliferative activity. Hence, analysis on their mode of action could be useful information if these plants were to be commercialized as pharmaceutical products.

Cancer cells differ morphologically; therefore it could be expected to observe variations in the interaction between the compound and the cell. Hence, other type of cancer cells should be tested as well for the crude extracts effectiveness. Other than that, the toxicity on non-cancerous cell exerted by these compounds should be studied so that a safety guideline could be drawn.

Different combinations of ratio could be tested in order to find the best mixture ratio that yields the lowest IC_{50} value. Synergistic interaction might exist as the result shows, where it IC_{50} value obtained from the ratio mixture of the cytotoxicity assay is lower than the IC_{50} value of the individual plant. Also, the effect of mixture across the crude extract, that is the combination of polar and non-polar crude, should be tested for the interaction. This novel concept of synergism should be embedded in cytotoxicity and antioxidant study as it provides a prospective result.

The study should also be extended using different solvent to extract the bioactive compounds present in the plant samples. The choice of extraction solvent used greatly affects the outcome of the study (Visht and Chaturvedi 2012). Hence using various solvent polarities, a broad spectrum of bioactive compounds could be extracted and tested for their activities.

Lastly, the accuracy of the *in vitro* data should be tested by conducting *in vivo* experimentation. Studies have showed that result from the *in vitro* at time is not represented when *in vivo* studies (Christou et al., 2001). This may be due to fact that *in vivo* study, the drug interaction involves the more complex system as compared to the *in vitro* studies.

CHAPTER 6

CONCLUSIONS

Data from the cytotoxicity and antioxidant assays suggested that both *Pereskia bleo* and *Centella asiatica* crude extracts have the potential as anticancer and antioxidant agents. The ethanolic and hexane crude extracts of *Pereskia bleo* yielded IC_{50} value of 21.5 µg/mL and 32.5 µg/mL, respectively. The ethanolic and hexane crude extracts of *Centella asiatica* yielded IC_{50} value of 30.0 µg/mL and 32.0 µg/mL, respectively. Possible synergism interaction may exist between these two plants as the lowest IC_{50} value was exhibited at ratio 3:7 using hexane crude extracts, with a remarkable IC_{50} of 21.0 µg/mL. Most of the ethanolic crude extracts showed high antioxidant activities. *Pereskia bleo* ethanolic crude extracts yielded the lowest IC_{50} value that is 475 µg/mL. The indication of the antioxidant can be related to the anticancer properties. Further analyses using purified compounds are necessary in order to confirm the result obtained.

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APPENDICES

Appendix A

Formula for Viable Cell Concentration Calculation

1. Average of viable cells

$$= \frac{(\text{Total cells in all grids})}{4}$$

2. Concentration of viable cells

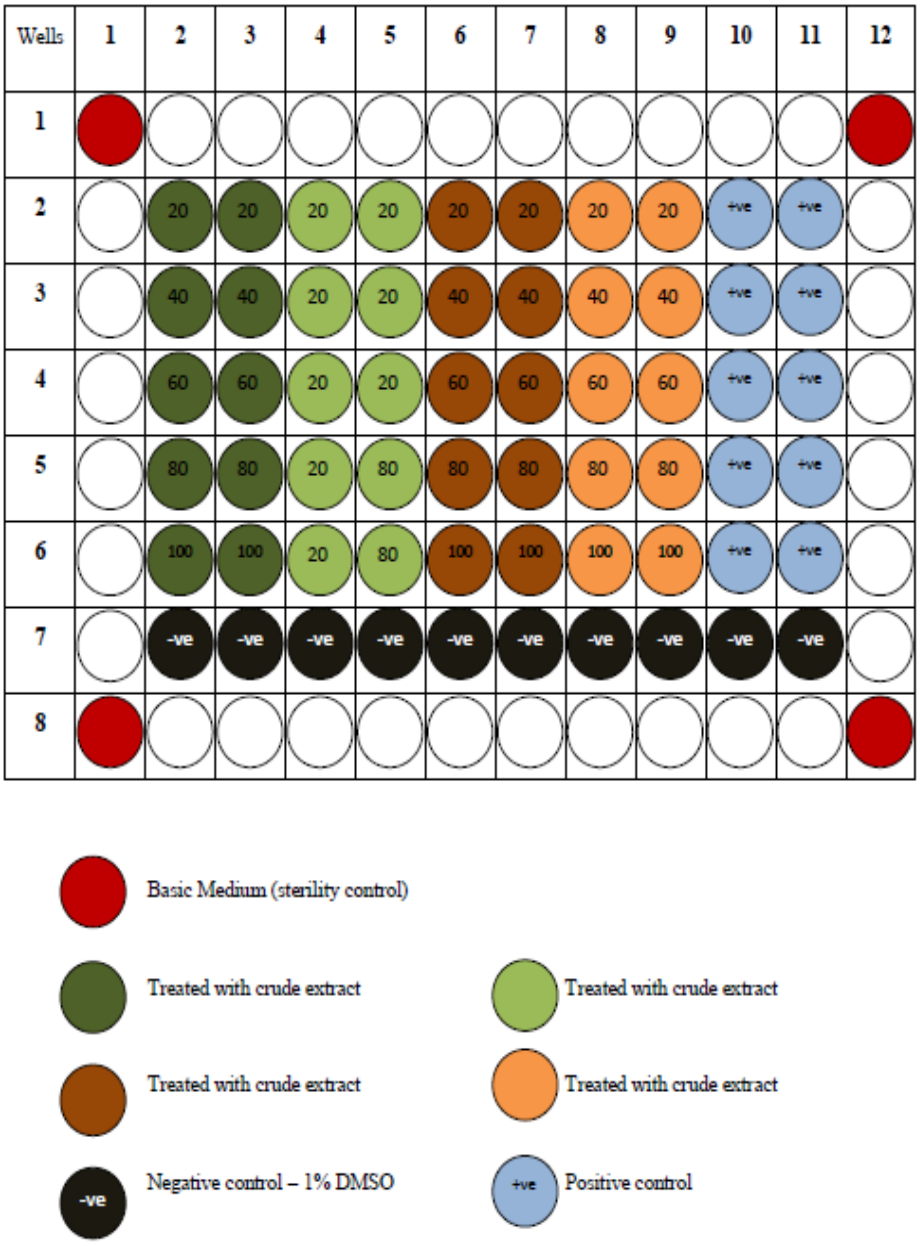
= Average number of viable cells X Dilution factor X 10^4 / mL

3. Total cell population in the original volume

= Concentration of viable cells X Volume of medium in the flask.

(Modified from Chen et al., 2009)

Appendix B



The basic 96-well plate design for MTT assay used in this study.

Appendix C

Formula for Percentage Cell Viability and Radical Scavenging Activity

1. Percentage cell viability

$$= \frac{Absorbance \ \ at \ 560 \ nm \ \ of \ \ treated \ \ well}{Absorbance \ \ at \ 560 \ nm \ \ of \ \ negative \ \ control} \times 100\%$$

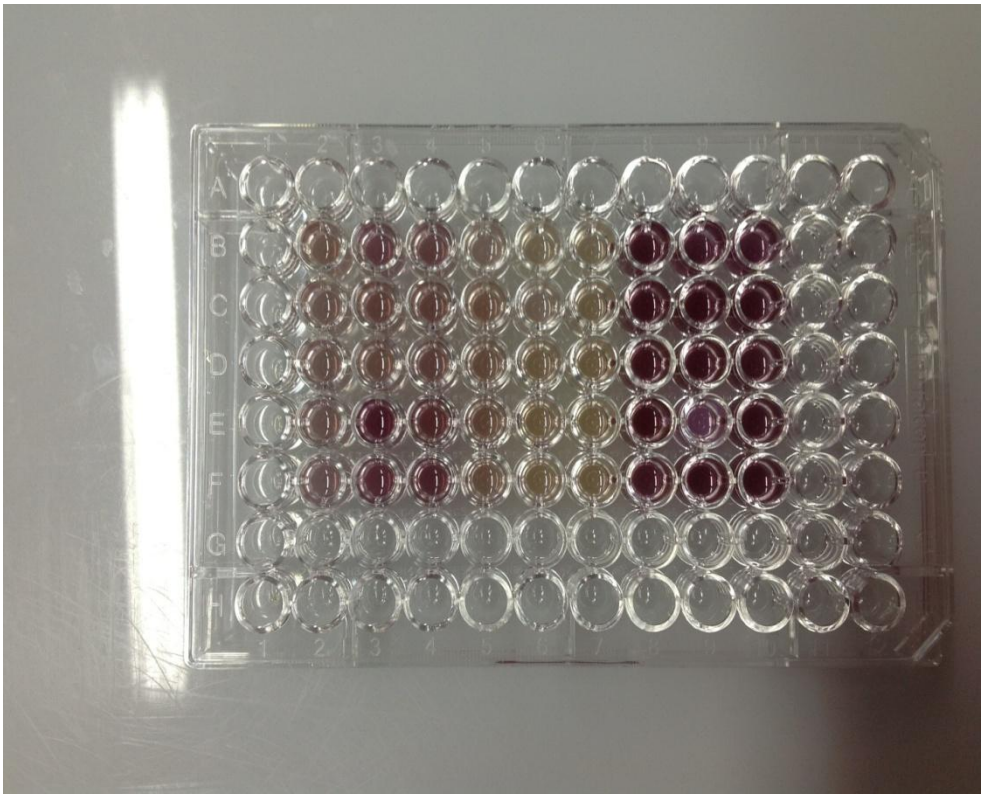
(Modified from Patel et al., 2009)

2. Percentage radical scavenging activity

$$= \frac{Abs.of \ \ negative \ \ control - Abs.of \ \ sample}{Abs.of \ \ negative \ \ control} \times 100\%$$

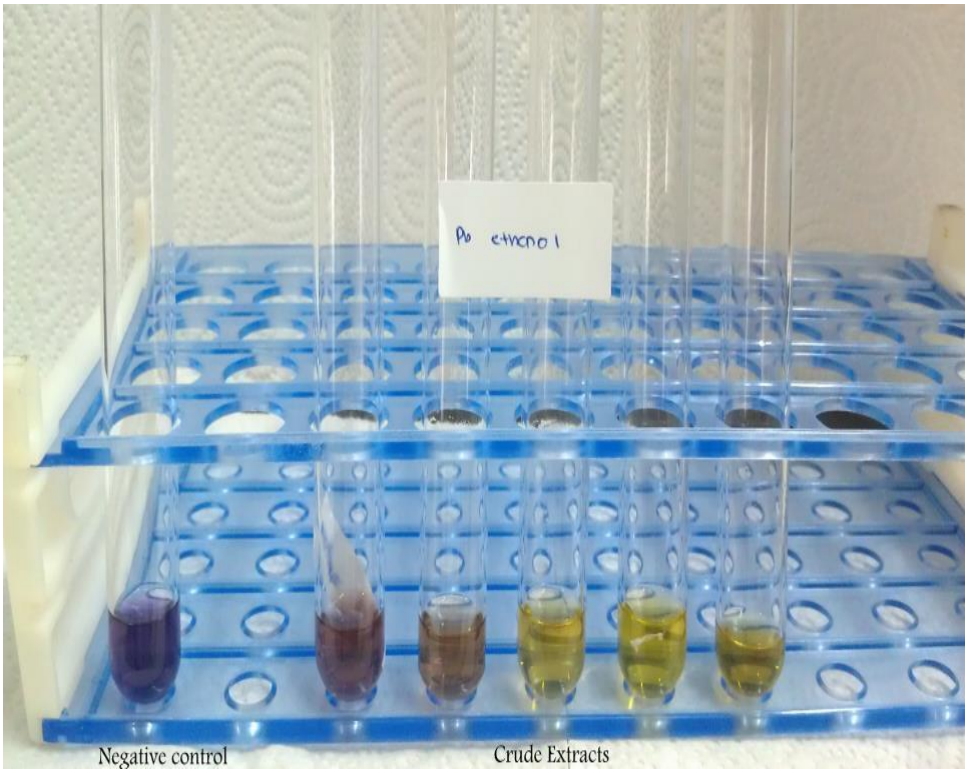
(Modified from Mandal et al., 2009)

Appendix D



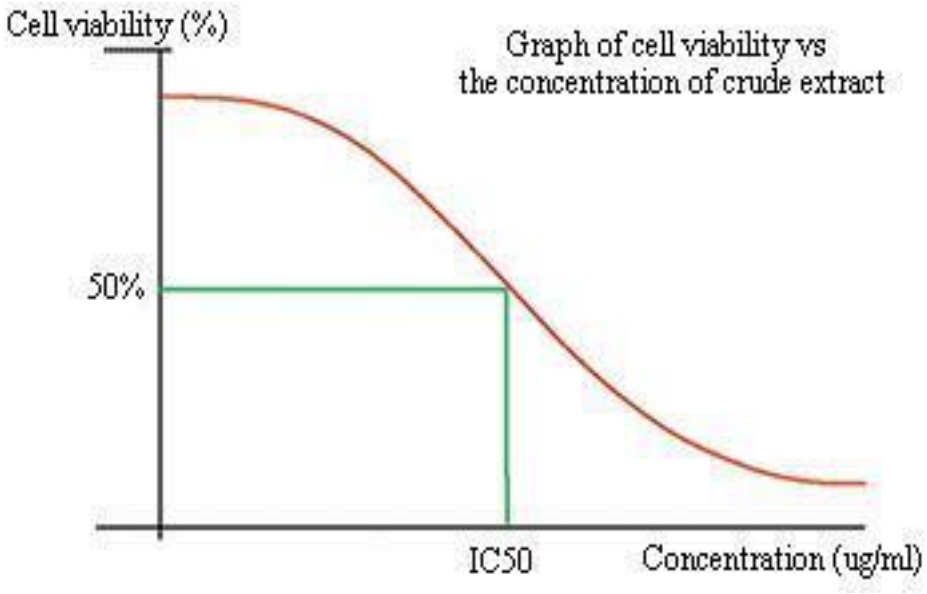
A 96-well plate showing the changes in the color upon treatment using MTT assay. The purple color indicate viable cells meanwhile the yellow color indicates non-viable cells.

Appendix E



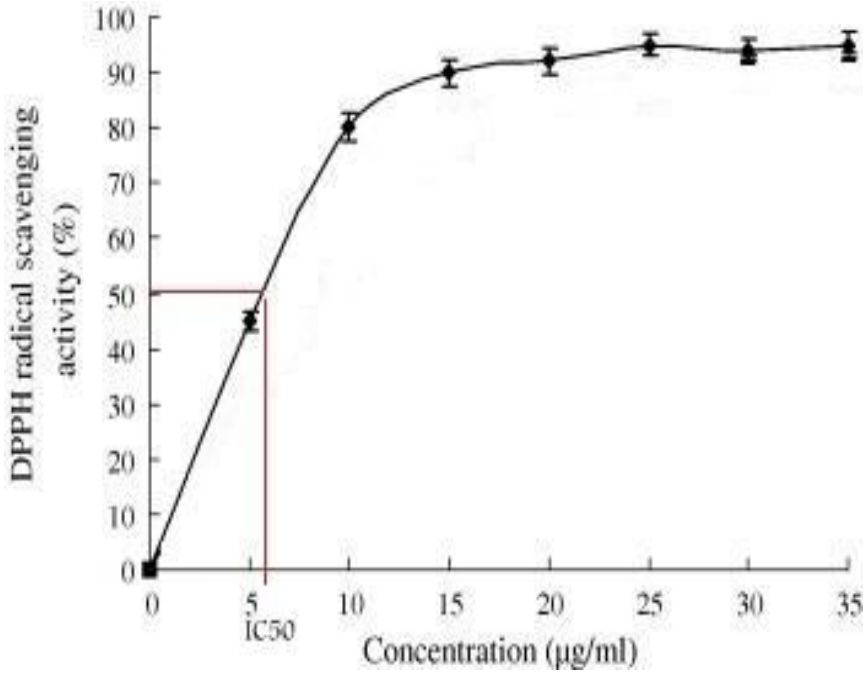
Several test tubes showing the changes upon treatment using DPPH assay. Violet color indicates high scavenging activity meanwhile purple color indicates low scavenging activity,

APPENDIX F



The graphical interpolation to determine the IC_{50} value from the graph of percentage of cell viability against the concentration of crude extracts.

APPENDIX G



The graphical interpolation to determine the IC_{50} value from the graph of percentage scavenging activity against the concentration of crude extracts.