PHYTOCHEMICAL SCREENING AND NATURAL KILLER CELLS IMMUNOMODULATION EFFECTS OF *Pereskia bleo* LEAVES EXTRACT ON CERVICAL CANCER CELLS

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by

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LIST OF SYMBOLS

°C	Degree Celsius
ml	Milliliter
mm	Millimeter
g	Gram
g/mol	Gram/molecule
%	Percentage
µg/ml	Microgram/milliliter
mg/ml	Milligram/milliliter
pg/ml	Picogram/milliliter
µl	Microliter
nm	Nanometer
α	Alpha
β	Beta
γ	Gamma

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CDKs	Cyclin-dependent kinases
CINV	Chemotherapy-induced nausea and vomiting
CNS	Central nervous system
CIPN	Chemotherapy-induced peripheral neuropathy
CO_2	Carbon dioxide
CXCR4	C-X-C chemokine receptor type 4
DISC	Death-inducing signal complex
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FasL	Fas Ligand
FBS	Fetal bovine serum
FCS	Flow cytometry standard
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GC-MS	Gas Chromatography-Mass Spectrometry
GI	Gastrointestinal tract
GvHD	Graft versus host disease
HLA	Human leukocyte antigen
IC_{50}	Half maximal inhibitory concentration
IFN-α	Interferon alpha
IFN-γ	Interferon gamma

IgG	Immunoglobulin G
IL	Interleukin
KARs	Activating killer cell-immunoglobulin-like receptors
KIRs	Inhibitory receptors of NK cells include killer immunoglobulin-like receptors
LILRs	Leukocyte immunoglobulin-like receptors
LSM	Lymphocyte separation medium
MDR	Multidrug resistance
MHC-I	Major histocompatibility complex class 1
MOMP	Mitochondrial outer membrane permeabilization
MRSA	Methicillin Resistant *Staphylococcus aureus*
MTT	3-[4,5-dimethyl thiazol-2-yl] 2,5-diphenyl tetra-zolium bromide
NCI	National Cancer Institute
NIST	National Institute of Standards and Technology
NK	Natural Killer
NKG2A	Natural killer group 2 member A
NKG2D	Natural killer group 2 member D
NLRP3	Nod-like receptor family pyrin domain containing 3
PBEA	Ethyl acetate extract of *Pereskia bleo* leaves
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PI	Propidium Iodide
PS	Phosphotidylserine
OD	Optical density
RNA	Ribonucleic acid
SD	Standard deviation
TAMs	Tumor associated macrophages
TCR	T-cell receptor
Th1	T helper type 1
TILs	Tumor-infiltrating lymphocytes
TKIs	Tyrosine kinase inhibitors
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
Tregs	Regulatory T-cells
TRAIL	TNF-related apoptosis-inducing ligand

PENILAIAN FITOKIMIA DAN KESAN IMUNOMODULASI SEL PEMBUNUH SEMULA JADI OLEH EKSTRAK DAUN *PERESKIA BLEO* KE ATAS SEL KANSER SERVIKS

ABSTRAK

Pereskia bleo merupakan tumbuhan berdaun dan boleh dimakan. Ia dikenali sebagai Pokok Jarum Tujuh Bilah di kalangan penduduk setempat dan mempunyai sifat anti-kanser. Kajian ini bertujuan untuk menjelaskan mekanisme tindakan tumbuhan ini sebagai anti-kanser untuk mendorong kematian sel dan menilai kesan imunostimulasi ke atas sel pembunuh semulajadi (sel NK) sebagai potensi tambahan ke atas kesan anti-cancer. Dalam kajian ini, daun *P. bleo* diekstrak dengan menggunakan beberapa teknik dan pelarut organik dengan polariti yang berlainan yang kemudiannya dianalisis menggunakan GC-MS. Ekstrak tersebut juga diuji untuk kesan sitotoksiknya terhadap sel-sel HeLa, MDA-MB-231, SW480 dan NIH/3T3 menggunakan asai MTT. Ekstrak yang mempunyai kesan sitotoksik paling kuat dan sel kanser yang berkaitan seterusnya diuji sama ada mampu mengaruh kematian sel melalui perencatan kitaran sel, asai Annexin V / PI dan pengukuran protein apoptotik menggunakan sitometri aliran. Selain daripada itu, sel-sel NK didedahkan dengan pelbagai kepekatan ekstrak etil asetat daun *P. bleo* (PBEA) dan kadar perkembangannyaa ditentukan melalui asai MTT. Sel NK dari individu sihat dan pesakit kanser serviks kemudiannya dirawat dengan PBEA berkepekatan 14.4 µg/ml selama 24 jam untuk dinilai aktiviti sitotoksinya. Kematian sel-sel sasaran dikenalpasti melalui sitometri aliran sementara itu asai ELISA dilakukan untuk menentukan penghasilan perforin, granzim B, IFN-γ dan IL-2. Keputusan kajian menunjukkan kehadiran terpenoid, sterol, alkaloid, flavonoid, fenol, asid lemak dan vitamin E di

dalam daun *P. bleo* bersama-sama sebatian baru iaitu (-)-Loliolide, neophytadiene, β-tokoferol, γ-tokoferol, squalene, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, 4-vinyl-syringol, phenol,2-methoxy-4-(1-propenyl) and asid heksadekanoik. PBEA memperlihatkan nilai IC_{50} terendah (14.37 ± 8.40 μg/ml) menunjukkan kesan sitotoksik terkuat secara selektif ke atas sel HeLa. Analisis kitaran sel menunjukkan perencatan perkembangan sel HeLa yang dirawat dengan PBEA pada fasa G_0/G_1 dibuktikan dengan pengumpulan sel yang signifikan pada fasa ini (P<0.05). Pemeriksaan morfologi terhadap kematian sel HeLa menunjukkan kehadiran cebisan nukleus, kondensasi kromatin sementara kejadian apoptosis juga telah dikesan di dalam asai Annexin V/PI. Analisis protein apoptotik menunjukkan pertambahan protein pro-apoptotik (Bax, p53 dan caspase-3) dan perencatan protein anti-apoptotik Bcl-2 (P<0.05). Sementara itu, perkembangan sel NK selepas 24 jam dirawat dengan PBEA didapati meningkat secara signifikan berbanding 48 dan 72 jam (P<0.05). Sel HeLa mengalami peningkatan apoptosis yang ketara dan meningkatkan ekspresi granzim B serta IFN-γ selepas diinkubasi dengan sel NK pesakit kanser yang dirawat dengan PBEA. Oleh itu, penemuan kami menunjukkan PBEA menyebabkan kematian sel kanser serviks HeLA dan merangsang pengaktifan sel NK dari pesakit kanser yang meningkatkan kesan sitotoksiknya terhadap sel HeLa. Hasil kajian ini memberikan kefahaman tentang keberkesanan *P. bleo* sebagai ejen pencegahan kimoterapi dan seterusnya membuka ruang bagi kajian selanjutnya.

PHYTOCHEMICAL SCREENING AND NATURAL KILLER CELLS IMMUNOMODULATION EFFECTS OF *PERESKIA BLEO* LEAVES EXTRACT ON CERVICAL CANCER CELLS

ABSTRACT

Pereskia bleo is a leafy and edible plant, locally known as "Pokok Jarum Tujuh Bilah" which has anti-cancer properties. This study purposed to elucidate the underlying mechanism of this plant as anti-cancer in inducing cell death as well as to evaluate its immunostimulatory effects on Natural Killer cells (NK cells) as a potential additional anti-cancer effect. In this study, the leaves of *P. bleo* were extracted using different techniques and solvent polarities, and subsequently subjected to GC-MS analysis. The extracts were tested for its cytotoxic effects on HeLa, MDA-MB-231, SW480 and NIH/3T3 cell lines using MTT assay. The most cytotoxic extract and its corresponding cancer cell lines were investigated for their cell death induction through cell cycle arrest, Annexin V/PI assay and measurement of apoptotic proteins using flow cytometry. NK cells were exposed to different concentrations of ethyl acetate extract of *P. bleo* leaves (PBEA) and its proliferation rate was determined *via* MTT assay. NK cells from healthy individuals and cervical cancer patients were treated with 14.4 µg/ml of PBEA and co-cultured with target cells for 24 h to evaluate its cytotoxic activity. Target cells death was identified by flow cytometry while ELISA assay was performed to determine the production of perforin, granzyme B, IFN-γ and IL-2. Results showed the presence of terpenoids, sterols, alkaloids, flavonoids, phenols, fatty acids and vitamin E in the extracts of *P. bleo* leaves together with new compounds namely (-)-Loliolide, neophytadiene, β-tocopherol, γ-tocopherol, squalene, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, 4-vinyl-syringol, phenol,2-methoxy-4-(1-

propenyl) and hexadecanoic acid. PBEA exhibited the lowest IC_{50} value (14.37 ± 8.40 µg/ml) indicated the strongest cytotoxic effect selectively on cervical cancer cells (HeLa). The cell cycle analysis showed inhibition of cell proliferation at G_0/G_1 phase in PBEA treated HeLa cells as evidenced by a significant accumulation of the cells at this phase ($P<0.05$). Morphological examination on PBEA treated HeLa cell showed the presence of fragmented nuclei and condensation of chromatin while apoptosis was detected in the Annexin V/PI assay. Analysis of apoptotic proteins revealed a significant upregulation of pro-apoptotic proteins (Bax, p53 and caspase-3) while downregulation of anti-apoptotic protein Bcl-2 ($P<0.05$) in PBEA treated HeLa cells. Meanwhile, NK cells proliferation at 24 h was found significantly increased compared to 48 h and 72 h of PBEA treatment ($P<0.05$). Apoptosis of HeLa cells was markedly increased in PBEA treated NK cells from cancer patients. This extract also enhanced granzyme B and IFN-γ expression in NK cells from cancer patients. Thus our findings demonstrated that PBEA induced cell death in the cervical cancer cells (HeLa) and stimulate activation of NK cells from cervical cancer patients which enhanced cytotoxic effect against HeLa cells. These results provide some insight into the effectiveness of *P. bleo* as a potential chemopreventive agent which open up for further studies.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer is a leading cause of death worldwide thus making it a public health concern (Kooti *et al.*, 2017). It is known to cause alteration of cells' genes, disturbance of growth signaling receptor for activation of cancer cells, evasion from apoptosis and immune surveillance escape which make them resistant to cell death and sustain proliferation in the host's body (Hanahan and Weinberg, 2016).

Nowadays, chemotherapy remains the treatment option for various types of cancer coupled with either radiotherapy or surgery (Chen *et al.*, 2018). Chemotherapeutic drugs aim to eliminate proliferating cancer cells through the mechanism of apoptosis (Chen *et al.*, 2018; Liu *et al.*, 2015). However, the killing action of these drugs is non-specific which kill not only the malignant cells but also the normal cells thus prompting several adverse side effects such as cardiac dysfunction, bone marrow suppression and cognitive impairment (Demaria *et al.*, 2017; Dietrich and Kaiser, 2016; Norwood Toro *et al.*, 2019). Apart from targeting cancer cell death, enhancement of immune response is another approach to eliminate cancer cells as offer by modern modalities for instance hormonal therapy, immunotherapy and cell based therapy (Koury *et al.*, 2018). Nonetheless, these modalities also exhibit adverse side effects such as neurotoxicity (Naran *et al.*, 2018). Furthermore, the emerging of drug resistance to chemotherapeutic agents has become a major challenge in cancer treatment (Chen *et al.*, 2018). All these limitations have reduced the response rates of the patients leading to ineffectiveness of the treatment and diminishing their quality of life.

Nowadays, medicinal plants provide new approach in the development of therapeutic agents for cancer treatment with promising results without harming the normal cells (Zaid *et al.*, 2017). These plants consist of flavonoids, phenolic compounds and alkaloids that work synergistically thus responsible for their anti-cancer property as well as other pharmacological properties (Yuan *et al.*, 2017). Therefore, availability of plant-based compounds with minimal side effects and less toxic effects to the healthy cells is desired for prevention and treatment of cancer (Greenwell and Rahman, 2015). Several medicinal plants that have been reported useful in the prevention and cancer treatment such as *Annona muricata* for colon and breast cancer, *Abrus precatorius* for breast cancer and *Clinacanthus nutans* for cervical cancer (Kim *et al.*, 2018; Moghadamtousi *et al.*, 2015; Sofi *et al.*, 2018; Zakaria *et al.*, 2017). In addition, medicinal plants can modulate the immune response against cancer cells.

Pereskia bleo (*P. bleo*) is a well-known medicinal plant in Malaysia that possess various health benefits for instance muscle ache relieve, detoxification, hemorrhoid, hypertension as well as in cancer prevention and treatment (Malek *et al.*, 2009; Yen *et al.*, 2013). The leaves of this plant have been reported to show several biological activities including anti-cancer effects (Abdul-Wahab *et al.*, 2012; Sim *et al.*, 2010a; Sri Nurestri *et al.*, 2008; Wahab *et al.*, 2009).

Cytotoxic activity of this plant leaves has been demonstrated on several cancer cell lines: human colon carcinoma (HCT 116), nasopharyngeal epidermoid carcinoma (KB), human hormonal-dependent breast cancer (MCF-7) and human cervical cancer (CasKi) (Sri Nurestri *et al.*, 2008). *P. bleo* leaves methanol extract and ethyl acetate fraction along with α-tocopherol compound isolated from this plant were found highly

cytotoxic against KB cell line while no cytotoxic effects observed in normal human fibroblast cell line (MRC-5) (Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008). Furthermore, *P. bleo* leaves methanol extract promoted cell death in T47-D cell line (breast carcinoma) by apoptosis *via* c-myc pathway and caspase-3 activation (Tan *et al.*, 2005). Therefore, *P. bleo* serves a promising plant candidate for cancer therapy. However, deeper understanding and fundamental information needed to be gathered about this plant.

1.2 Rationale of study

Chemotherapy has been a mainstay of cancer treatment for decades. However, it causes cytotoxic effects to the normal cells which induce adverse effects in the patients due to the non-specific action of the chemotherapeutic agents. Apart from that, the emerging of chemotherapy resistance has become one of the obstacles to the effectiveness of cancer treatment.

Nowadays, many people are seeking for complementary alternative medicine that harmless to the normal cells and effective to eliminate cancer cells. Medicinal plants can serve as an alternative for the treatment of cancer with natural immunoadjuvant, negligible side effects and effective in killing cancer cells. Previous studies of *P. bleo* have shown the leaves of this plant comprised anti-cancer effects. However, this effect was only reported in studies where fraction extracts and single compound were used (Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008). In traditional practice, the plants usually taken in crude or raw form and often claimed more effective compared to purified single compounds due to benefit of synergistic action from the compounds mixture in the crude extract (Caesar and Cech, 2019). Besides, up until

now, there is no report on immunomodulatory effects of *P. bleo* leaves in eliminating cancer cells.

Thus, it is essential to explore the anti-cancer activity and immunostimulatory effects of *P. bleo* leaves crude extracts against cancer cell lines in order to provide better understanding on its ability as anti-cancer and stimulatory agents for cancer treatment.

1.3 Objectives of study

1.3.1 General objective

To study apoptosis induction and immunomodulatory activity of *P. bleo* leaves extracts on cancer cell line.

1.3.2 Specific objectives

1. To analyze phytochemical compounds of *P. bleo* leaves extracts *via* gas chromatography-mass spectrometry (GC-MS).
2. To determine the cytotoxic effects of *P. bleo* leaves extracts on selected cancer and normal cell lines for their potential as anti-cancer agents.
3. To investigate the mechanism of cell death induced by the selected *P. bleo* leaves extract that exert the most potent cytotoxic effects on its corresponding cancer cells.
4. To evaluate immunostimulatory effects induced by the selected *P. bleo* leaves extract on activation of Natural Killer (NK) cells against cervical cancer HeLa cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

When abnormal cells in the body begin to proliferate uncontrollably, this condition leads to the formation of a malignant tumor known as cancer (Abbas and Rehman, 2018). These cancer cells undergo mutation leading to metabolic transformations that inhibits tumor suppressor genes and activate oncogenes. As a result, this condition promotes cancer progression when glucose consumption is increased, mitochondrial respiration is reduced, reactive oxygen species generation rises and cell death is resisted (Ribas *et al.*, 2016).

In 2018, approximately 18.1 million of new cancer incidences worldwide were reported while 9.6 million of deaths were caused by this disease (Bray *et al.*, 2018). The most common cancer among men were lung, prostate and colorectal cancer while in female, breast cancer recorded the highest incidence followed by colorectal and lung cancer (Bray *et al.*, 2018). Overall, mortality according to cancer types are as follows: lung cancer (18.4 %) followed by breast cancer (11.6 %), prostate cancer (7.1 %) and colorectal cancer (6.1 %) (Bray *et al.*, 2018).

In Malaysia, the largest incidence of cancer among males was prostate, colorectal and bladder (Azizah *et al.*, 2019). Meanwhile, breast cancer, corpus uteri and thyroid were the most common cancer identified among females (Azizah *et al.*, 2019).

2.1.1 Hallmark of cancer

Tumorigenesis is a complex and multistep process. The mutation of oncogenes and tumor-suppressor genes take place in normal cells resulting in fast proliferation and resistance to cell death (Yuan *et al.*, 2016). Normal cells evolve into a malignant state when they acquire several biological capabilities during tumorigenesis. Once the biological capabilities are obtained, they become the hallmark of cancer which describes the concept of tumor development. Eight biological capabilities have been highlighted by Hanahan and Weinberg (2016) as the hallmark of cancer, each with their own functional role, as summarize below:

Hallmark 1: Sustaining proliferative signaling

Normal cells maintain their proliferation and tissue homeostasis through a growth signaling pathway in a cell cycle that are tightly regulated (Matson and Cook, 2017). In the context of cancer, sustaining proliferative signaling involves the mutation of genes in cancer cells known as oncogenes which eventually promotes the uncontrolled proliferation of daughter cells (Martincorena and Campbell, 2015). Cancer cells can sustain proliferative signaling *via* several mechanisms such as the production of their own growth factor ligands that stimulate proliferation or altering receptor signaling by producing more receptor proteins on cancer cells for their activation (Hanahan and Weinberg, 2016).

Hallmark 2: Evading growth suppressors

Cancer cells have the ability to escape the negative regulation process that is mostly governed by tumor suppressor genes. Tumor suppressors are essential to limit the growth and proliferation of the cell. Examples of genes that codes for tumor

suppressors include retinoblastoma-associated (RT) and TP53 that play a crucial role in cellular regulation whether to proliferate or undergo senescence or apoptosis.

Hallmark 3: Resisting cell death

The mechanism of triggering cell death includes apoptosis, necrosis and autophagy. Cancer cells have to evade these processes in order to continue their proliferation, expansion and progression to a higher state of malignancy. Apoptosis is the most prominent programmed cell death, where the cells are genetically programmed to die. The apoptotic program can be triggered *via* two mechanisms which are intrinsic and extrinsic pathways (Hanahan and Weinberg, 2016). Furthermore, apoptosis deregulation in cancer cells has led to the development of cancer treatment that uses apoptosis as a tool to inhibit their proliferation (Abraha and Ketema, 2016).

Hallmark 4: Enabling replicative immortality

There are two proliferation barriers in the cell growth and division cycle which are senescence (viable state) and crisis (leading to cell death) that normal cells can bypass. The telomerase functions as a protector in the cell that restricts them from unlimited replication (immortalization) (Hanahan and Weinberg, 2016). The presence of telomerase activity is almost absent in normal cells indicated by short telomeres which leads to the activation of either one of the proliferation barriers (Hanahan and Weinberg, 2016). In contrast, cancer cells exhibited high telomerase activity characterized by the increased telomere size which prevents them from triggering senescence or apoptosis and eventually lead to unlimited replication (Hanahan and Weinberg, 2016).

Hallmark 5: Inducing angiogenesis

Nourishment (nutrients and oxygen) and waste elimination (metabolic waste and carbon dioxide) are essential in both normal cells as well as cancerous ones (Fadaka *et al.*, 2017). This is made possible *via* angiogenesis, the formation neovasculature in tumours (Hanahan and Weinberg, 2016). It is a process where new blood vessel forms and activated by the cancer cells in order to sustain and expand neoplastic growth (Liao and Johnson, 2007).

Hallmark 6: Activating invasion and metastasis

Due to the aggressive nature of cancer cells, they attack neighbouring tissue and the circulatory systems including blood and lymphatic vessels. These vessels are used as pathways for the spread of cancer cells to nearby or distant organs. The tissue-draining of the lymphatic vessel enables the invasion of cancer cells to lymph nodes leading to metastasis. Invasion and migration are often associated with an advanced stage of cancer progression (Su *et al.*, 2017).

Hallmark 7: Deregulating cellular energetics and metabolism

The uptake of glucose as an energy source in the presence of oxygen are markedly higher for cancer cells in order to support their proliferation and sustainability in the tumor microenvironment (Warburg *et al.*, 1927). In addition, lactate, is a toxic waste from the cells that undergoing anaerobic and aerobic glycolysis has been recognized as tumor-promoters, serving as metabolic fueling for the cancer cells (Dhup *et al.*, 2012).

Hallmark 8: Avoiding immune destruction

Cancer cells continue grow in the body and exhibited 'cancer immunoediting' which enable them to escape from immune surveillance (Lussier and Schreiber, 2016). During this phase, cancer cells manage to evade from immune surveillance and control through several mechanisms including the absence of tumor-antigen recognition due to the tumor or effector cells modification, cell death resistance and immunological proofing *via* immunosuppressive factors secretion (Malmberg, 2004).

2.1.2 Treatment modalities for cancer

Cancer treatments consist of several common methods namely chemotherapy, surgery, radiotherapy, immunotherapy, targeted therapy and hormonal therapy (Chen *et al.*, 2018). In Malaysia, surgery, radiotherapy, chemotherapy as well as hormonal therapy are presently available for cancer treatment (Taib *et al.*, 2017; Tamin, 2017; Wong, 2014). The success of the treatment depends on the cancer type, tumour area and stage (Abbas and Rehman, 2018).

Surgery is a conventional treatment for benign and malignant solid tumors (Abbas and Rehman, 2018; Tohme *et al.*, 2017). This treatment involves the removal of solid tumors and becomes a preferred treatment option compare to radiotherapy and chemotherapy due to its minimal risk of surrounding tissues damage during the tumor removal (Abbas and Rehman, 2018). Radiotherapy utilizes electron beams, x-rays, or gamma rays to kill tumor cells (Terasawa *et al.*, 2009). This therapy often used in combination with chemotherapy, immunotherapy or surgery (Baskar *et al.*, 2012). Radiation therapy alone can be used in treatment of cancers such as lung carcinomas (non-small cell), prostate carcinomas, and cervix carcinomas (Baskar *et al.*, 2012). In

addition, radiotherapy in combination with other treatments are used to treat breast carcinoma, local advanced cervix carcinoma and local advanced lung carcinomas (Baskar *et al.*, 2012)

Chemotherapy uses cytotoxic mediators targeting cells that are rapidly dividing, interfere with the cell division process, stimulate the expression of pro-apoptotic proteins and suppression of anti-apoptotic proteins which ultimately leads to apoptosis in cancer cells (Hassan *et al.*, 2014; Jones and Ocen, 2020). This mechanism is shown in several chemotherapeutic agents such as taxane (e.g., paclitaxel and docetaxel) that slow down the mitosis of the cancer cells when they intervene with microtubule polymerization and prompt the cell death *via* apoptosis (Jordan *et al.*, 1996). 5-5-Fluorouracil (5-FU) is an FDA-approved breast and colorectal cancer treatment (Ajani, 2006; Ershler, 2006). This drug causes the disruption of nucleoside metabolism and alteration of the DNA and RNA thus causing cancer cell death (Longley *et al.*, 2003).

Apart from the conventional treatment methods, there are some of modern cancer treatments option such as immunotherapy and hormonal therapy. Cancer immunotherapy is designed to enhance immune response towards combating cancer cells (Sambi *et al.*, 2019). Example of immunotherapy including the administration of exogenous cytokines, therapeutic vaccines, cancer vaccines and cell-based therapies (Ventola, 2017b). Mechanism of the action exerted by immunotherapy is specific towards target cancer cells while sparing the normal cells (Imai and Takaoka, 2006). Treatment strategies involved the using of monoclonal antibodies, small molecule inhibitors and nano-particulate antibody conjugates (Lord and Ashworth, 2008; Padma, 2015; Sanna *et al.*, 2014). For instance, epidermal growth factor receptor (EGFR)

tyrosine kinase inhibitors (TKIs) is used in targeted treatment for EGFR-mutation-positive non-small cell lung cancer (Skinner *et al.*, 2018).

Hormonal therapy involves the administration of exogenous hormones in hormone-dependent cancer which modulate the endocrine system by reducing the production of the hormone or interfering with the activity of receptor (Fairchild *et al.*, 2015). This therapy is used for treatment in breast and prostate cancer (Awan and Esfahani, 2018; Brawer, 2006). For example, anti-hormonal agents that selectively regulate estrogen receptor (e.g., tamoxifen) and aromatase inhibitors are used for breast cancer treatment (Awan and Esfahani, 2018). In addition, certain hormones such as exogenous corticosteroids have general antineoplastic effects on cancer cells by causing apoptosis thus included in almost all chemotherapy protocols for lymphoid malignancy (Roth *et al.*, 2010; Schmidt *et al.*, 2004).

2.1.3 Limitations of current cancer drugs

Chemotherapy remains a mainstay for current cancer treatment which usually offer alongside other conventional treatment options like surgical intervention and radiotherapy (Senapati *et al.*, 2018; Singh *et al.*, 2019). In general, chemotherapeutic drugs work *via* different mechanisms that cause oxidative stress, DNA damage, cell cycle arrest or cytoskeleton damage, targeting both dividing cancer and dividing healthy cells (Basu and Krishnamurthy, 2010; Qi *et al.*, 2018; Trendowski, 2014; Yokoyama *et al.*, 2017).

Although the aim of chemotherapy is the eradication of cancer cells, it also attacks normal cells causing adverse effects in multiple organ systems (Mittra *et al.*, 2017). Such debilitating effects have cause a major clinical problem, leading to a low

survival rate in the majority of cancer patients (Nurgali *et al.*, 2018). In addition, chemotherapeutic drugs toxicity observed in patients contribute to inefficacy of these anticancer agents (Gewirtz *et al.*, 2010).

Chemotherapy-induced nausea and vomiting (CINV) are among the major concern in cancer patients during chemotherapy regimens. This symptom can be acute (occur less than 24 hours after treatment) or delay (occur after 24 hours and up to 8 days of treatment) (Roscoe *et al.*, 2004). CINV will become an anticipated response in the following chemotherapy cycles which involves nausea and vomiting (Roscoe *et al.*, 2011). Vomiting is an action that is prompted once the body recognizes the presence of harmful elements in the body. This reflex can damage cells of the stomach and intestines (Mustian *et al.*, 2011). When foreign substances are detected by the mucosa of the gastric or small intestine, it stimulates the vagal afferents' interaction with the hindbrain, a component of the central nervous system (CNS), contributing to an emetic response as an efferent vagal action (Mustian *et al.*, 2011). Once acute, delayed and anticipatory CINV turns severe, patients are less likely to comply with their chemotherapy regiment, while those who do will be susceptible to a compromised bodily function, anxiety, depression leading to a poorer quality of life (Rodríguez, 2013; Roscoe *et al.*, 2011).

Apart from CINV, most cancer patients undergoing cytotoxic therapy experienced fatigue. Fatigue is related to the activation of pro-inflammatory cytokines induced by chemotherapeutic agents or by the tumor itself (Bower and Lamkin, 2013). About 30% to 60% of cancer patients reported experience moderate to severe fatigue during chemotherapy, leading to discontinuation of treatment in some patients and cause significant impairment in the patients' quality of life (Bower, 2014).

Mucositis is another common side effects due to cancer chemotherapy. Mucositis-related chemotherapy causes mucosal injury in gastrointestinal (GI) tract which damage normal cells that are rapidly dividing (Cinausero *et al.*, 2017). Gastrointestinal mucositis may lead to local ulceration and pain, which in turn results in susceptibility to sepsis, anaemia, fatigue, anorexia, malabsorption and weight loss (Nurgali *et al.*, 2018). Due to this gastrointestinal side effects, susceptible patients become reluctant to adhere to their chemotherapy regiment followed by the discontinuation of the treatment altogether. This will subsequently reduce their quality of life and survival rate (Cinausero *et al.*, 2017).

Meanwhile, a lot of anti-cancer drugs such as angiogenesis inhibitors, platinum-based agents, taxanes, proteasome and vinca alkaloids causes chemotherapy-induced peripheral neuropathy (CIPN) (Nurgali *et al.*, 2018). The side effects of long-term CIPN results in ataxia, insomnia and depression, thus diminishing the ability to function and living quality in cancer survivors (Nurgali *et al.*, 2018).

Most cytotoxic drugs have immune suppressive side effects. They act by eliminating dividing haematopoietic cells that will be manifested as severe neutropenia and cytopenia (Hashiguchi *et al.*, 2015). Treatment-associated neutropenia remains dose-limiting toxicity of cancer chemotherapy due to adverse effects including susceptibility to life-threatening infection, elevated risk of bleeding, decreased immunity and fever (Dinan *et al.*, 2015; Fontanella *et al.*, 2014). Besides, neutropenia and its complications make it necessary for early termination of treatment, delays or dose reductions (Dinan *et al.*, 2015).

In addition, multidrug resistance (MDR) poses a challenge to the potency of cancer chemotherapy. Drug resistance in cancer occur during the invasion and

metastasis of cancers (Mansoori *et al.*, 2017). It is manifested in the form of reduced sensitivity towards drugs that are supposed to inhibit tumor growth by interfering with the membrane transport involving the P-glycoprotein product, modification of target enzyme, impairment in drug activation, suppression of apoptosis, promoting DNA repair and mutation in cell cycle proteins such as p53 (Krishna Vadlapatla *et al.*, 2013; Luqmani, 2005; Mansoori *et al.*, 2017). Nowadays, MDR has emerged as a major challenge in cancer chemotherapy leading to many treatment failures and severe adverse effects in patients (Ye *et al.*, 2019).

2.2 Apoptosis and cancer

2.2.1 Mechanism of apoptosis

Apoptosis is essential in the case of normal development and homeostasis. The normal cells usually undergoing apoptosis when they are damaged in various ways, mislocalized or inappropriately proliferating (Hanahan and Weinberg, 2016).

Apoptosis can be activated by two pathways which are extrinsic and intrinsic pathway. Extracellular ligands such as tumor necrosis factor (TNF), Fas Ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) affects apoptotic signaling through the extrinsic pathway. Apoptosis occurs when these receptors are activated through the formation of a death-inducing signal complex (DISC) after the caspases cascade is activated (Jan and Chaudhry, 2019). Apoptosis is triggered through the intrinsic pathway is controlled by the Bcl-2 family consisting of pro-apoptotic (Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL and Mcl1) proteins (Elmore, 2007; Llambi *et al.*, 2011). Upon activation by stimuli such as DNA damage, deprivation of cytokines and growth factors, cytochrome c is released into the cytosol and converge the

activation of caspase cascade of pro-apoptotic proteins leading to the mitochondrial outer membrane permeabilization (MOMP) causing cell death (Green and Llambi, 2015; Zaman *et al.*, 2014).

Caspase cascade signaling system is important in apoptosis as it is controlled by various proteins that either promote or inhibit apoptosis (Green and Llambi, 2015; Ng *et al.*, 2013). There are two types of caspases: the initiator (caspase-2, -8, -9, and -10) and the effector caspases (caspase-3, -6, and -7) (Parrish *et al.*, 2013). In the intrinsic pathway, the cell death is initiated by the release of cytochrome c which forms an apoptosome complex with Apaf-1 proteins and activates caspase-9 (initiator caspases). Meanwhile, the stimulation of death receptor (e.g. FasL) that binds to the intracellular domain receptor (e.g. FADD) which induces cell death through the extrinsic pathway is triggered by the activation of caspase-8 or -9 (initiator caspases) (Baliga and Kumar, 2003; Parrish *et al.*, 2013). The activation of initiator caspases either by extrinsic or intrinsic pathways eventually causes cell death via the activation of downstream effector caspases such as caspase-3 (Porter and Janicke, 1999). Figure 2.1 shows overview of extrinsic and intrinsic pathways involved in apoptosis.

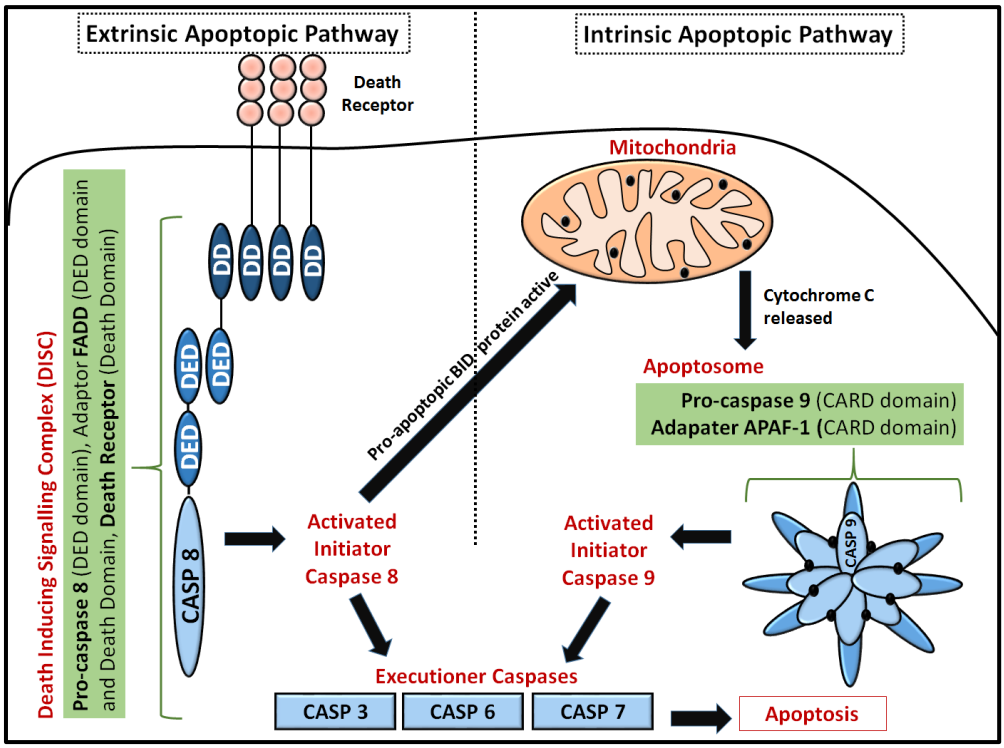


Figure 2.1 Mechanism of apoptosis *via* extrinsic and intrinsic pathway (Source: Google image free to use license)

2.2.2 Targeting apoptosis in cancer treatment

One way of treating cancer is to eliminate the uncontrolled proliferate cancer cells. Targeting apoptosis is an effective method in cancer treatment by modulating the cells' own mechanism of death in order to terminate them (Pfeffer and Singh, 2018). Common strategies of chemotherapeutic drugs target various stages of apoptosis pathways such as pro-apoptotic proteins stimulation and suppression of anti-apoptotic molecules (Hassan *et al.*, 2014).

Several agents act as apoptotic signals that have been discovered include inhibitors for Bcl-2, ligands for death-receptors, inhibition of X-linked inhibitor of apoptosis (XIAP) proteins and alkylphospholipid analogs (APL). Venetoclax is an example of Bcl-2 inhibitor that is used in leukemia treatment (Sharma and Pollyea,

2018). XIAP inhibitor such as antisense oligonucleotide AEG35156 was clinically tested to reduce the expression of XIAP and increase cytotoxic activity against solid tumors (Miura *et al.*, 2011).

2.3 Immune modulation for treatment of cancer

What begins as a mutation in the genetic material in normal cells and physiological alteration in cancer cells and defence mechanisms in the body, soon develops into a malignant form known as cancer (Furuta *et al.*, 2010). These cellular alterations lead to other problems such as the loss of function in tumor suppressor genes resulting in cellular immortality, proliferation and carcinogenesis (Wang *et al.*, 2018). This is where the role of the immune system comes in by preventing the spread of tumor and carcinogenesis (Koury *et al.*, 2018).

Immune modulation in cancer refers to treatments that modulate patients' immune response to control the growth and eliminate the tumor cells (Naidoo *et al.*, 2014). Immunotherapy is a modern strategy for cancer treatment which modulates the immune system of the patients to selectively kill target cancer cells (Koury *et al.*, 2018). This technique makes use of a process called immunoediting, also known as immune surveillance, where cells within the immune system suppress tumor growth and progression by identifying and dismissing malignant cells (Ribatti, 2017).

Cancer cells use several mechanisms to evade from host immune surveillance to reestablish their growth and continue to progress such as upregulation of checkpoint receptor ligands that essentially prevent tumor-infiltrating lymphocytes (TILs) from entering the tumor mass, upregulation of immune-suppressing cells including regulatory T-cells (Tregs), downregulating the antigen presentation system or induction

of the production of suppressive cytokines such as IL-10 and TGF-β (Park *et al.*, 2018; Reeves and James, 2017; Thepmalee *et al.*, 2018; Wan, 2010).

This section will provide the details regarding the roles of major immune cells involved in cancer progression and targeted therapy including macrophages, neutrophils, natural killer (NK) cells, T cells and B cells. Apart from that, strategies that have been developed to manipulate anti-tumor response and targets in modern immunotherapy will also be highlighted.

2.3.1 Immune cells in cancer

Various immune cell types infiltrate the tumor environment and interaction between tumor and immune cells give rise to production of cytokines and growth factors that facilitate tumor cells in sustaining survival and metastasis (Gun *et al.*, 2019). Interestingly, apart from pro-humoral role, these cells have potential as anti-cancer (Gun *et al.*, 2019). Despites multifunctional roles of these immune cells (such as macrophages, neutrophils, NK cells, T cells and B cells), understanding their roles contributes toward development of innovative anti-cancer strategies (Gun *et al.*, 2019).

Macrophages are immune cells that are essential for normal physiological processes such as fighting infections, wound healing as well as promoting diseases such as autoimmune disorders and tumorigenesis (Wynn *et al.*, 2013). Generally, macrophages can be activated by interferon gamma (IFN-γ) IL-4, IL-10 which in return they produce pro-inflammatory cytokines and nitric oxide against bacterial, virus infections as well as involved in wound healing (Wynn *et al.*, 2013). However, tumor associated macrophages (TAMs) induce cancer metastasis by promoting angiogenesis, inducing tumor growth and enhancing tumor-cell migration and invasion (Dandekar *et*

al., 2011). The presence of TAMs in the tumor microenvironment has been associated with poor prognosis for breast, prostate, ovarian, cervical, endometrial, esophageal and bladder cancers (Dandekar *et al.*, 2011).

NK cells are innate cells with cytotoxic ability to eliminate tumor cells. These cells have lytic potential by releasing lytic granules or expressing death signals (Gun *et al.*, 2019). The mechanism of NK cells begins with the probing of other cells via activating or inhibitory receptors that will either allow or prevent the action of NK cells. Activating receptors recognizes foreign or stress-induced ligands while inhibitory receptors identify self-MHC-I molecules respectively (Gun *et al.*, 2019). For example, in an *in vitro* study, NK-mediated tumor lysis was activated when the NK cells recognized tumor antigen UL16-binding protein 2/5/6 on anaplastic thyroid carcinoma cells *via* natural killer group 2, member D receptor (NKG2D) (Wennerberg *et al.*, 2014). In a different study, it was reported that FasL-mediated malignant melanoma cells were eliminated upon the activation of NK cells once IL-18 was secreted as a response to the *in vivo* CXCR4 blockade on neutrophils or up-regulated NLRP3 inflammasome signalling in kupffer cells (Yang *et al.*, 2018).

In contrast, the adaptive immune system is slower in response to threats compared to cells of the innate immune system due to their antigen-specific action (Gun *et al.*, 2019). In the context of cancer, cytotoxic $CD8^+$ T cells from the T cells family have a significant role to play (Gun *et al.*, 2019). These cells are activated when the receptor on naïve T cells surface (TCR) engage with its specific antigenic peptide MHC-I on the tumor cells initiating target cell lysis upon the release of perforin and granzyme B which are cytotoxic molecules (Gálvez *et al.*, 2019; Gun *et al.*, 2019). The results of

this study highlight the potential of $CD8^+$ T cells in modulating antitumor immunity (Tsukumo and Yasutomo, 2018).

2.3.2 Types of immunotherapy

In recent years, a breakthrough in cancer treatment was achieved with the clinically approved immunotherapy modalities in patients (Ventola, 2017a). This new approach enhances the immune response in killing cancer cells including monoclonal antibodies, immune checkpoint inhibitors, cytokines, cancer vaccines and cell-based immunotherapy (Sambi *et al.*, 2019).

Where monoclonal antibodies are applied, they can either be conjugated or unconjugated with particular drugs to produce cytotoxic effects on cancer cells (Kimiz-Gebologlu *et al.*, 2018). These antibodies aim to block cell proliferation and some signaling pathways besides targeting a particular antigen on cancer cells (Papaioannou *et al.*, 2016). Rituxumab is an example of unconjugated monoclonal antibodies used to treat B-cell non-Hodgkin's lymphomas and it is the first monoclonal antibodies approved by FDA to be used in cancer treatment (Kimiz-Gebologlu *et al.*, 2018). Other example of monoclonal antibodies include Transtuzumab used in breast cancer treatment, Alemtuzumab for chronic lymphocytic leukemia and Panitumumab for metastatic colorectal cancer (van Krieken *et al.*, 2017; von Minckwitz *et al.*, 2017; Winqvist *et al.*, 2017).

Meanwhile, a different type of monoclonal antibody known as the immune checkpoint inhibitors enable T cells activation and tumor cells eradication by blocking immune checkpoint receptors (Sambi *et al.*, 2019). Two checkpoint inhibitors have been approved by FDA which are anti-programmed death ligand -1 (anti-PD-L1) and anti-

cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) (Ventola, 2017a). They have been successfully used in treating metastatic melanoma (Hugo *et al.*, 2016; Reddy *et al.*, 2017).

Infusion of specific cytokines is another approach of immunotherapy that can boost immune response to eliminate cancer cells (Klener *et al.*, 2015). Two cytokines have received FDA approval which are Interleukin -2 (IL-2) for hairy cell leukemia treatment and interferon-alpha (IFN-α) for advanced melanoma and metastatic renal cancer treatment (Berraondo *et al.*, 2019; Waldmann, 2018). Where IL-2 is used, it promotes T cells activity, especially tumor-infiltrating cells as well as increasing NK cells activity (Klener *et al.*, 2015). On the other hand, IFN-α enhances immune response by activating dendritic cells and promoting antigen presentation as well as enhances the T helper type 1 (Th1) cells response, cytotoxic T cells ($CD8^+$ T cells) activity and cytotoxic effects of NK cells (Alatrash *et al.*, 2013).

Vaccination for cancer is available for immunotherapeutic treatment that can induce immune response. Similar to the conventional vaccines, cancer vaccines consist of total or a portion of cancer cells or antigens (Sambi *et al.*, 2019). For instance, gp100 is used in the treatment of melanoma while E75 is useful for breast cancer treatment where these peptide-based vaccines respond to one tumor antigen in complex with its human leukocytes antigens (HLA) (Bianchi *et al.*, 2016; Clifton *et al.*, 2016). Apart from that, immune- or dendritic cell-based vaccines are produced by specifically extracting dendritic cells (DCs) and activating them with specific tumor antigen of interest and reintroduced into the patients to eliminate cancer cells of interest as illustrated in Figure 2.2 (Sambi *et al.*, 2019). Example of available cancer vaccines such as PA2024 for prostate cancer and TAA CA-125 for ovarian cancer (Sambi *et al.*, 2019).

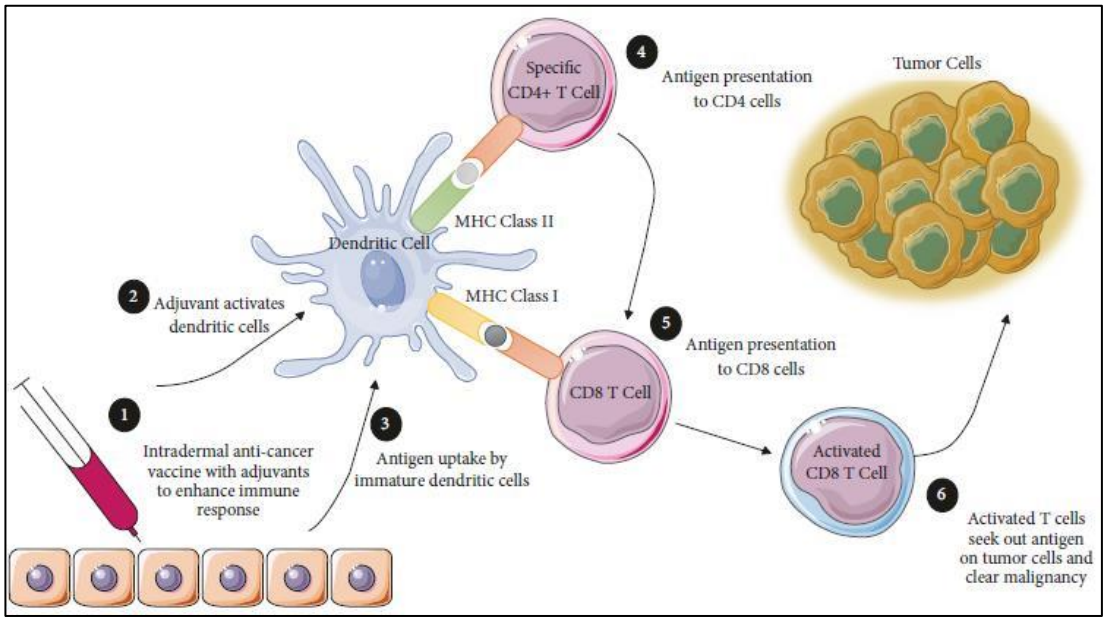


Figure 2.2 Mechanism of action of cancer vaccines. Cancer vaccines are administrated through intradermal injection (1) with adjuvants that activate dendritic cells (2). Immature dendritic cells take up the antigens; typically this antigen is uniquely expressed on tumor cells (3) and presents the antigen to CD4 cells (4) and CD8 cells (5). CD8 cells are then activated to seek out the antigen on the surface of tumor cells (6). Abbreviations: CD is cluster of differentiation and MHC is major histocompatibility complex (Sambi et al., 2019).

In cell-based immunotherapy, *ex vivo* cultured natural or genetically modified T cells are transferred into patients to eliminate cancer cells (Feng, 2013). Then, cytokines such as IL-2 are introduced along with the T cells for better efficacy (Alatrash *et al.*, 2013). Example of cell-based immunotherapies includes the infusion of autologous tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR)-transduced T cells and chimeric antigen receptor T cells (CAR T cells) (Koury *et al.*, 2018). It has been reported that the application of CAR T cells has been successful against acute and chronic B cell leukemia (Koury *et al.*, 2018). Table 2.1 presents some example of immunotherapy that is used in cancer treatment.

Table 2.1 Example of cancer immunotherapies with demonstrated efficacy in cancer treatment (Alatrash *et al.*, 2013; Weiner, 2015).

Immunotherapy	Type of cancer
Monoclonal antibodies	
Therapeutic monoclonal antibodies	Lymphomas, human epidermal growth factor receptor2-positive (HER-2+) breast cancer, colorectal cancer
Immune checkpoint blockers	Metastatic melanoma, renal cell carcinoma, non-small-cell lung cancer (NSCLC)
Cytokines	
High-dose recombinant interleukin-2	Metastatic melanoma, renal cell carcinoma
Interferon-alpha	
Vaccines	
Sipuleucel-T	Prostate cancer
gp100	Melanoma
Cell-based therapy	
Allogenic hematopietic stem cell transplant	Acute myeloid leukemia, hematologic malignancies
Autologous cell transfer	Metastatic melanoma
Genetically modified T-cell infusions	Leukemia, lymphomas

2.4 Natural Killer cells potential in cancer therapy

2.4.1 Natural Killer cells

NK cells are characterized by the expression of CD16 and CD56 and lacking of CD3 surface molecule (Chieregato *et al.*, 2017). These cells can be classified into $CD56^{bright}$ and $CD56^{dim}$. $CD56^{bright}$ NK cells is immature, present majority in lymph nodes and tonsils and have poor cytotoxic activity (de Jonge *et al.*, 2019). In contrast, $CD56^{dim}$ NK cells are mature and exert potent cytotoxic effects when attached to Fc receptors (CD16) on target cells which activates antibody-dependent cell-mediated

cytotoxicity (ADCC) (de Jonge *et al.*, 2019). They make up around 5 – 15 % of NK population in the peripheral blood (Mahapatra *et al.*, 2017).

Cytokines are not only responsible for the regulation of innate and adaptive immunity but also other biological processes in various cells such as growth, survival and proliferation of NK cells (Abel *et al.*, 2018). Interleukins (ILs) such as IL-2, IL-15, IL-21 are the keys to activate NK cells (Gasteiger *et al.*, 2013).

NK cells responses to transformed or virally-infected cells depend on interaction of signals received through their inhibitory and activating receptors (Tremblay-McLean *et al.*, 2019). Some of the activating receptors on NK cells include the natural cytotoxicity receptors (NKp30, NKp44, NKp46, NKp80), NKG2D, DNAX accessory molecule-1 (DNAM1), activating killer cell-immunoglobulin-like receptors (KARs) and others (Konjević *et al.*, 2017; López-Larrea *et al.*, 2008). Meanwhile, inhibitory receptors can identify MHC-I molecule. Examples of inhibitory receptors are NKG2A/CD94 (the c-type lectin), killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs) (Canossi *et al.*, 2016; Hatton *et al.*, 2016; Li *et al.*, 2011). The ligands for inhibitory receptors are mostly MHC-I molecules. NK cells do not attack healthy cells since they express MHC-I molecules (Isvoranu, 2017).

2.4.2 Natural Killer cells killing mechanism

NK cell-mediated cytotoxicity is controlled by inhibitory and activating receptors expressed on its surface. Ligation of these receptors with their corresponding ligands on target cells stimulates downstream signalling events and balance between inhibitory and activating signals subsequently leading to apoptosis (Ogbomo and Mody,

2016). NK cells cytotoxic activity can be mediated by lytic granules release pathway. In this pathway, a pore-forming molecules called perforin is released into the target cell membrane which allows delivery of granzyme B, thereby stimulate activation of caspases and induce target cell death (Leischner *et al.*, 2015).

Besides, the lytic granules pathway can be triggered in absent of caspases activation (Smyth *et al.*, 2005). Apart from lytic granules release pathway, NK cells cytotoxicity is modulated *via* activation of death receptor which involves ligation of NK cells death receptors such as Fas Ligand (FasL), tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL) to their related ligands on target cells (Smyth *et al.*, 2005). The activated receptor complex recruits the adaptor protein FADD and initiator caspases (caspase -8 or -10) leading to the formation of death-inducing signalling complex (DISC) which activate effector caspases and triggers cell death (Bratton and Cohen, 2001).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is another mechanism of NK cells cytotoxicity induce by binding of their CD16 receptor with Fc region of antibodies which together attach to the specific antigens present on the target cells (Wang *et al.*, 2015a). Activation of ADCC triggers the release of perforin, granzymes and cytokines finally induce target cells death (van der Haar Àvila *et al.*, 2019)

2.4.3 Natural Killer cells immunotherapy for cancer

As part of the innate immune system, NK cells possess the ability to eliminate tumours or infected cells in them without first being sensitized (Marcus *et al.*, 2014). They express inhibitory receptors (such as KIRs, NKG2A/CD94) that ligand with MHC-I molecules which are ubiquitously on normal cells and this activity block the

production of cytolytic granules and cytokine from NK cells thus preventing them from killing normal cells (Davis *et al.*, 2015). In contrast, abnormal cells such as cancer cells are lacking MHC-I molecules allowing NK cells to identify and eliminate them (Hu *et al.*, 2019). Therefore, NK cells have been targeted as a therapeutic option in treating cancer especially in adoptive immunotherapy (Ghaemdoust *et al.*, 2019).

In adoptive immunotherapy, NK cells are extracted from various sources and are cultured *in vitro*. After a special process, they will be injected to the patient's body and this therapy can be done *via* using autologous or allogenic NK cells (Ghaemdoust *et al.*, 2019). Autologous NK cells originate from the patient's own body whereas allogenic NK cells are obtained from various sources including hematopoietic stem cell transplantation or adoptive cell transfer models, NK cell lines and genetically modified NK cells (Geller and Miller, 2011; Ghaemdoust *et al.*, 2019).

Clinical trials have been carried out by using adoptive transfer of autologous NK and applied as a cancer treatment involving solid tumours including lymphoma, breast cancer, colon cancer, and lung cancer (Hu *et al.*, 2019). However, its efficacy is limited to only certain cancers (Burns *et al.*, 2003; Krause *et al.*, 2004). Allogenic NK cells products are used for the treatment of leukaemia, renal cell carcinoma, colorectal cancer and lymphoma (Harada *et al.*, 2017). A major concern with the application of allogenic NK cells is the start of graft versus host disease (GvHD) observed along with the use of immunosuppressive agents, injection of high-purity NK cells by CD3 depletion and selection of donors that is compatible with the host HLA (Iliopoulou *et al.*, 2010; Yoon *et al.*, 2010).

2.5 Medicinal plants as anti-cancer and immunostimulatory

Cancer is often a result of DNA aberrations in affected cells (Zaid *et al.*, 2017). Besides conventional cancer treatments like surgery, new approaches like cytotoxic regimes of compounds and radiation are gaining popularity which hinders the cellular replication system by primarily targeting rapidly dividing cells (Jones and Ocen, 2020). Nevertheless, these treatments are accompanied by side effects due to their low selectivity and rendered ineffective due to their increasing resistance (Zaid *et al.*, 2017).

The search for new modalities in the battle against cancer should focus on treatments that can overcome the resistance capability of cancer cells with almost insignificant side effects. Plant-derived drugs (particularly from medicinal plants) are desireable anti-cancer agents because they are natural, readily available and offer minimal side effects (Kooti *et al.*, 2017; Zaid *et al.*, 2017). Interestingly, almost 60% of drugs used in cancer treatment are derived from plant-based products (Kooti *et al.*, 2017). Examples of plant-based anti-cancer agents that have been clinically used are listed in Table 2.2.

Table 2.2 Plant-derived anti-cancer agents in clinical use

Drugs	Plant source	Uses for treatment	References
Vinca alkaloids			
Vincristine and vinblastine	*Catharanthus roseus* (Apocynaceae)	Breast cancer, Hodgkin's lymphoma, leukaemia, testicular cancer and lung cancer	(Cragg and Newman, 2005; Mann, 2002)
Taxanes			
Paclitaxel	*Taxus brevifolia*	Breast, ovarian, lung, head and neck, oesophageal, prostate and bladder cancers	(Kuruppu *et al.*, 2019)
Docetaxel	*Taxus baccata*	Breast, ovarian, head and neck, lung, gastric and bladder cancers	(Fu *et al.*, 2009)
Etoposide	*Podophyllum peltatum*	Hodgkin's and non-Hodgkin's lymphoma, lung, gastric, breast and testicular cancers	(Hande, 1998; Montecucco *et al.*, 2015)
Harringtonine and homoharringtonine	*Cephalotaxus harringtonia*	Acute and chronic myelogenous leukaemia	(Cragg and Newman, 2005; Moirangthem *et al.*, 2014)

2.5.1 Medicinal plants with anti-cancer effects

Plants produce a wide range of chemical compounds known as secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, phenolic compounds and others that responsible for their anticancer effects (Kooti *et al.*, 2017). These metabolites act by suppressing cancer's stimulating enzymes, repairing DNA, stimulating production of antitumor enzymes (caspase-3, -7, -8, -9, -10, -12) in cell and inducing antioxidant effects (Sakarkar and Deshmukh, 2011). Table 2.3 shows example of medicinal plants that effective in cancer treatment.

Table 2.3 Medicinal plants that possess anti-cancer effects

Plant name	Extracts used	Mechanism of action	References
Achillea wilhelmsii	Methanol extract of leaves	Induce apoptosis in colon, stomach, breast and human melanoma cells	(Azadbakht *et al.*, 2003; Dokhani *et al.*, 2005; Uddin *et al.*, 2011)
Ammi majus	Ethanol extract	Cytotoxic effects on human non-small cell lung carcinoma (H1299) cells line induced by apoptosis *via* inhibition of cytochrome p450 activity	(Shokoohinia *et al.*, 2014)
Annona muricata	Ethyl acetate extract of leaves	Induce G_1 cell cycle arrest and apoptosis *via* mitochondria-mediate pathway in human HCT-116 and HT-29 colon cells	(Moghadamtousi *et al.*, 2014)
Astrodaucus orientalis	Extract of root and above-ground part of plant	Anti-proliferative effects on breast cancer cells (T47D) *via* inhibition of cell cycle and induction of apoptosis	(Abdolmohammadi *et al.*, 2009)
Clinacanthus nutans	Methanol extract of leaves	Anti-tumor effects against 4 T1 tumor-bearing mice	(Rahman *et al.*, 2019)
Goniothalamus umbrosus	Ethyl acetate extract of leaves	Induce apoptosis in human breast cancer cells (MCF-7)	(Abdel-Wahab *et al.*, 2009)

2.5.2 Medicinal plants with immunostimulatory effects against cancer

Medicinal plants with immunomodulatory properties are a moderately concept in the phytomedicine (Hasson *et al.*, 2019). In addition to the enhancement of the humoral and cell-mediated immunity, immunomodulatory agents initiates the activation of the "non-specific" responses which the activation of the complement system, granulocytes, macrophages and natural killer cells (Hasson *et al.*, 2019). The activation of these essential immune cells initiates production of molecules such as cytokines that take part in the modulation and enhancement of the immune responses (Gummert *et al.*, 1999; Vigila and Baskaran, 2008). All these events produce alternatives to the current cancer chemotherapy. Immunomodulatory agents are non-specific compounds that work without antigenic specificity similar to the adjuvants that are associated with some vaccines (Gupta *et al.*, 2010; Liu *et al.*, 2016).

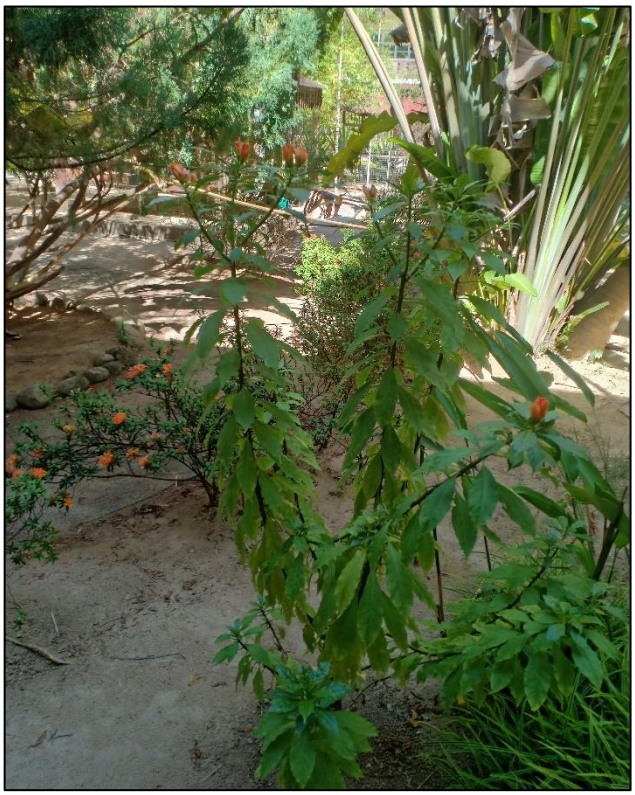
Several example of medicinal plants that demonstrated immunostimulatory effects in cancer such as *Tinospora cordifolia, Nitraria retusa* and *Solanum nigrum*. *Tinospora cordifolia* has showed immunomodulatory and anti-tumor activity when administered mice. Administration of methanol extract of this plant in mice has increased white blood cell count significantly, enhance macrophage activation and reduced solid tumor growth (Mathew and Kuttan, 1999). The chloroform extract of *Nitraria retusa* leaves that contain β-sitosterol and palmitic acid was reported for its anti-tumoral and immunostimulatory effects in mice bearing induced lung tumor by enhancing proliferation of cytotoxic T lymphocytes (CTLs) and splenocytes as well as reduction in metastatic tumor due to compound (Boubaker *et al.*, 2018). The enhance of immune response observed in breast tumor bearing-mice after treatment with polysaccharide fraction from *Solanum nigrum* (SN-ppF3) was indicated by increasing

of infiltrating-T cells, NK cells and macrophage in tumor tissues, higher apoptosis tumor cells and elevation of TNF-α, IFN-γ and IL-4 in treated mice (Razali *et al.*, 2016).

2.6 *Pereskia bleo*

Pereskia bleo (*P. bleo*) is an edible medicinal plant from the family of Cactaceae (Zareisedehizadeh *et al.*, 2014). It is originated from the western region of South America and commonly found in subtropical and tropical countries such as Malaysia, Indonesia, Singapore and India (Christophe, 2006). The scientific name of this plant is *Pereskia bleo* (Kunth) DC (Zareisedehizadeh *et al.*, 2014). This plant is famous among locals by the name of 'Pokok Jarum Tujuh Bilah' among Malay while "Cak Sing Cam' for the Chinese (Tan *et al.*, 2005; Wahab *et al.*, 2009).

P. bleo is a leafy and shrubby plant with a height between 0.8 to 8 m. The trunk of this plant is about 10 cm in diameter and comprises leafy branches bearing 5 to 7 black spines of 1 cm in length (Zareisedehizadeh *et al.*, 2014). This plant produces an orange-red rose-like flowers (Figure 2.3(i)) while the fruits are yellow in color, glossy, fleshy, thick walled and containing the brown or black seeds (Christophe, 2006; Sim *et al.*, 2010b). The leaves are glossy, thin, succulent and oblong in shape about 6 to 21 cm in length as presented in Figure 2.3(ii).



(i)

(ii)

Figure 2.3 *Pereskia bleo* used in this study produces an orange-red flower (i). The leaf of this plant is glossy approximately 20 cm in length (ii).

P. bleo is usually utilized for health and dietary purposes and in some places, it is popular as a food spice (Christophe, 2006; Malek *et al.*, 2009; Nugent, 1999). In Malaysia, the leaves of this plant are consumed raw as a salad or a drink in the form of tea from decoction of fresh leaves (Er *et al.*, 2007; Sri Nurestri *et al.*, 2008). In traditional medicine, this plant is useful in the treatment of ailments including hemorrhoid, diabetes, hypertension, headache, ulcer, infections, gastric pain, asthma and rheumatism (Er *et al.*, 2007; Malek *et al.*, 2009; Sim *et al.*, 2010b; Tan *et al.*, 2005). In addition, locals have claimed that the leaves of this plant are useful in the prevention and treatment of cancer (Yen *et al.*, 2013).

P. bleo leaves have been extensively studied as they are commonly utilized for the medical purposes in the traditional practice compared to the other parts. The earliest phytochemical study involving the leaves of *P. bleo* was done by Doetsch *et al.* (1980) that identified alkaloids like 3-methoxytyramine, tyramine and 3,4-dimethoxy-β-phenethylamine. In addition, the presence of β-sitosterol, phytol, vitamin E, 2,4-di-tert-butylphenol and α-tocopherol were reported in this plant's leaves (Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008). The leaves were also reported to contain high amount of phenolic compounds and phytochemicals including lactone, glycosides, fatty acids, alkaloids, sterols and terpenoids (Sim *et al.*, 2010a; Zareisedehizadeh *et al.*, 2014).

The leaves of *P. bleo* have shown various biological activities such as antioxidant, anti-microbial, anti-proliferative and cytotoxic effects. Its cytotoxic effects have been reported on several cancer cell lines. Crude, fraction and pure compounds have isolated from the leaves of this plant and evaluated for its cytotoxic effects on several cancer cell lines. For instance, methanol extract of *P. bleo* leaves was found to be cytotoxic against T-47D breast cancer cell line (EC_{50} value = 2.0 µg/ml) and induced apoptosis in this cells through c-myc pathway and caspase-3 activation (Tan *et al.*,

2005). Ethyl acetate fraction and crude methanol extract of this plant were demonstrated high cytotoxic effects against KB cancer cell line (human nasopharyngeal epidermoid carcinoma) at IC_{50} value of 4.5 and 6.5 µg/ml respectively (Sri Nurestri *et al.*, 2008). Several pure compounds isolated from this plant also showed significant cytotoxic effects against several cancer cell lines. For example, 2,4-di tert butylphenol exerted high cytotoxic effects on KB, MCF7 (human hormonal dependent breast cancer cells), CasKi (human cervical carcinoma) and A549 (human lung carcinoma) cell lines with IC_{50} value of 0.81, 5.75, 4.5 and 6.0 µg/ml respectively (Malek *et al.*, 2009). Apart from that, α-tocopherol was found highly cytotoxic against KB (IC_{50} value = 8.0 µg/ml), MCF7 (IC_{50} value = 7.5 µg/ml), CasKi (IC_{50} value = 6.0 µg/ml) and A549 (IC_{50} value = 6.0 µg/ml) (Malek *et al.*, 2009) cell lines.

As for its antioxidant activity, ethyl acetate and hexane extracts of *P. bleo* showed high antioxidant activity determined through assay such as β-carotene bleaching and scavenging effect on DPPH radicals (Hassanbaglou *et al.*, 2012; Sim *et al.*, 2010a; Wahab *et al.*, 2009). In antimicrobial activity, dichloromethane extract of *P. bleo* showed the strongest antimicrobial activity against Methicillin Resistant *Staphylococcus aureus* (MRSA) bacteria while hexane extract exhibited high antimicrobial activity and methanol extract showed moderate antimicrobial activity against both gram negative bacteria *Pseudomonas aeruginosa* and *Salmonella choleraesuis* (Wahab *et al.*, 2009).

Nowadays, modulation of the immune system to improve health status especially in the treatment of cancer has gained attention in the research field and continue to increase in demand due to limitations of the current cancer treatment. Therefore, numerous research involving medicinal plants are ongoing to explore for

their immunomodulatory effects on cancer cells. However, to our knowledge, study on immunomodulation of *P. bleo* leaves against cancer cells has never been done.

CHAPTER 3

PHYTOCHEMICAL SCREENING OF *Pereskia bleo* LEAVES EXTRACT *via* GC-MS

3.1 Introduction

Advancement and breakthroughs in the pharmaceutical industry are reliant upon traditional medicinal plants (Wright, 2005). This is mainly due to the fact that these plants contain metabolites be it in the form of crude or pure material that is used as a remedy that was passed down through generations for various ailments (Dekebo, 2019). Several example of modern drugs that are developed from plants such as Artemisinin isolated from *Artemisia annua* used for malaria treatment (Mander and Liu, 2010), Galantamine (Reminyl®) is used in treatment of Alzheimer's disease derived from *Galanthus woronowii* Losinsk. (Anand *et al.*, 2019; Kurz, 2002) and Vinblastine produced from *Catharanthus roseus* used as chemotherapy for Hodgkin's lymphoma (Kuruvilla, 2009; Seneca, 2007).

The bioactive compounds in plants can be classified into primary and secondary metabolites. Primary metabolites play a role in growth, development or reproduction via molecules like amino acids, carbohydrates and lipids (Dekebo, 2019). Meanwhile, secondary metabolites are divided into several classes such as terpenoids, phenolics, alkaloids, tannins and others that are used by plants in their defense mechanism against predators (Harborne, 1998). In addition, these compounds provide plants with their therapeutic properties such as antibiotic, anti-cancer, antifungal and antiviral that are beneficial to human health (Hussein and El-Anssary, 2018).

One of the commonly used medicinal plants is *Pereskia bleo* (*P. bleo*). The leaves of this plant are claimed to be effective in traditional cancer treatment when consumed raw or as tea (Abdul-Wahab *et al.*, 2012; Malek *et al.*, 2009; Yen *et al.*,

2013). Phytochemical studies on the leaves of *P. bleo* often uses fractions or pure compounds instead of crude extracts. In traditional medicine, a crude extract is preferred instead of an isolated single compound because the synergistic of all the compounds that are present in the plant offers greater effectiveness (Rasoanaivo *et al.*, 2011).

The first and crucial step in the isolation of bioactive compounds from medicinal plants is the extraction process. There are various extraction methods available to obtain the desired bioactive compounds from plants. The maceration, Soxhlet and decoction extraction techniques are some of the most used methods in medicinal plant studies. Maceration is a simple procedure that involves the soaking of plant materials in a solvent for a minimum of 3 days with frequent shaking at room temperature (Handa *et al.*, 2008). The low extraction temperature in this method can preserve some of the compounds from degradation (Wu *et al.*, 2015). Soxhlet extraction is a common practice for plant leaves extraction which result in good recovery of plant secondary metabolites (Dekebo, 2019) . This method usually requires the heating of plant samples at a high temperature which can increase the possibilities of losing thermolabile compounds (Zhang *et al.*, 2018). Decoction also involves soaking of the plant materials in boiled water. This method is suitable for extracting heat-stable compounds and hard plant structures such as barks and roots (Azwanida, 2015). Thus, different preparation methods will result in the isolation of different compounds since the heating process will cause some compounds to be degraded. This further emphasizes the importance of selecting the best extraction method in research.

Besides the methods of extraction, type of solvent for extraction also has a significant impact on the extraction of target compounds from medicinal plants that vary in terms of polarity. In the current study, four types of solvents were used for polarity

screening of *P. bleo* leaves. Hexane is used to extract nonpolar compounds, ethyl acetate for medium-polarity compounds, while methanol and aqueous solution for polar compounds. In addition to methanol and aqueous solution, ethanol is also useful for extracting compounds that are hydrophilic in nature (Bergs *et al.*, 2013; Sasidharan *et al.*, 2011). On the other hand, lipophilic extraction requires medium-polarity solvents such as ethyl acetate and dichloromethane (Bergs *et al.*, 2013; Sasidharan *et al.*, 2011). Meanwhile, solvents that are suitable for the extraction of non-polar compounds include petrol ether, chloroform and hexane (Bergs *et al.*, 2013).

Until now, gas chromatography-mass spectrometry (GC-MS) analysis remains a valuable method for the analysis of crude extracts of medicinal plants that contains volatile compounds. It is a rapid process with high sensitivity and selectivity, better resolution, high throughput and broad coverage (Deslauriers, 2002; Wang *et al.*, 2015b). Previous studies involving GC-MS on crude methanol extract of *P. bleo* leaves revealed the presence of β-sitosterol and stigmasterol while high amount of sugar and fatty acids were found in the aqueous extract (Sharif *et al.*, 2015). Despite the numerous studies involving this plant, the phytochemical investigation on its crude extract remains unexplored. In this study, we opted for the maceration, Soxhlet and aqueous decoction to determine the therapeutic potential of *P. bleo* leaves crude extracts while mimicking the traditional crude preparation at the same time. Thus, the present research was carried out to identify the phytochemicals present in the crude extracts of *P. bleo* leaves by using hexane, ethyl acetate, methanol and aqueous *via* GC-MS technique.

3.2 Materials and methods

3.2.1 Plant leaves collection and preparation

The leaves of *P. bleo* were collected from Kampung Cherang, Kota Bharu, Kelantan and verified by our botanist Dr. Rahmad Zakaria. A voucher specimen (Voucher No: 11575) was submitted to the herbarium at the School of Biology, Universiti Sains Malaysia (USM), Penang. The leaves were rinsed thoroughly with water before being subjected to oven-drying at 50°C and processed into powder form by grinding.

3.2.2 Maceration extraction of *P. bleo* leaves

A total of 10 g of *P. bleo* leaves powder were soaked in 500 ml of hexane, ethyl acetate and methanol successively for approximately 30 days at room temperature. Then, the extracts were filtered (Whatman paper no. 1) and concentrated by using a rotary evaporator.

3.2.3 Soxhlet extraction of *P. bleo* leaves

A total of 20 g of powdered leaves were extracted successively using hexane, ethyl acetate, and methanol solvent *via* Soxhlet apparatus. The leaves powder was loaded into ¾ of the thimble and placed in the chamber of the Soxhlet. Then, the Soxhlet extractor was placed in the flask containing hexane solvent and subjected to reflux heating. The solvent vapors travelled through the extractor and reached the thimble and returned to the flask. This process continued for 24 h until the solvent became clear. The thimble containing the plant leaves was dried under a fume hood for overnight and

this process was repeated for ethyl acetate and methanol. The extracts were then concentrated using the evaporator and stored at – 20 °C until use.

3.2.4 Aqueous decoction of *P. bleo* leaves

The aqueous extract was prepared by decoction method where 10 g of the *P. bleo* leaves sample were boiled in 450 ml water at 50°C until the water was reduced to one-third of its initial volume. Then, the sample was filtered with Whatman filter paper. Subsequently, the aqueous extract was frozen (-20˚C) overnight and dried using freeze dryer. The extract was stored at -20°C until further use.

3.2.5 Determination of extraction yield from *P. bleo* leaves

The extraction yield (%) for each solvent extract from the leaves of *P. bleo* obtained through maceration, decoction and Soxhlet extractions was calculated as follows:

$$\text{The yield of extract (\%)} = \frac{\text{Weight of the crude extract after evaporating process or freeze-drying}}{\text{Weight of dried leaves}} \times 100\ \%$$

3.2.6 Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

The GC-MS analysis of *P. bleo* leaves extracts were carried out by using Hewlett Packard 6890 Gas Chromatograph with 5973N Mass Selective Detector. The column was fused silica capillary, HP-5 column (30 m x 0.25 mm i.d x 0.25 µm film thickness) (Agilent Technologies, USA). The carrier gas was helium with a flow rate of 1.0 ml/min with the oven temperature programmed from 50°C (5 min) to 300°C (10

min) at a rate of 25°C/min. Both injection and interface temperatures were set at 280°C. One microliter sample was injected in splitless mode and analysed in MS full scan mode (m/z 40-650). The electron ionization was fixed at 70eV. Acquisition of data was performed using Chemsation software. Identification of phytochemical constituents was accomplished based on the similarity of more than 80 % between mass spectral with National Institute of Standards and Technology (NIST02) and Wiley 275 libraries.

3.3 Results

3.3.1 Yield of *Pereskia bleo* leaves extracts

Table 3.1 presented the percentage of extract yield from *P. bleo* leaves with different methods of extraction and solvents. In the maceration method, methanol produced the highest extraction yield (21.00 %), followed by ethyl acetate (12.00 %) and hexane (10.50 %). The same trend was also observed in Soxhlet extraction where methanol demonstrated the highest extract yield (28.00 %) followed by ethyl acetate (15.50 %) and hexane (12.50 %). The aqueous extract was obtained by using the decoction method which resulted in 25.30 % of extraction yield.

Table 3.1 Percentage of yield from *P. bleo* leaves extracts obtained *via* maceration, Soxhlet and decoction extraction.

Method of extraction	**Type of extract**	**Weight of dried leaves powder (g)**	**Weight of crude extract (g)**	**Yield (% w/w)**
Maceration	Hexane	10.00	1.05	10.50
	Ethyl acetate	10.00	1.20	12.00
	Methanol	10.00	2.10	21.00
Soxhlet	Hexane	20.00	2.50	12.50
	Ethyl acetate	20.00	3.10	15.50
	Methanol	20.00	5.60	28.00
Decoction	Aqueous	10.00	2.53	25.30

3.3.2 Identification of phytocompounds from the maceration of *P. bleo* leaves

In the present study, the leaves of *P. bleo* were extracted *via* maceration method using solvents of different polarity (hexane < ethyl acetate < methanol). The chemical

composition of each extract was analyzed using GC-MS. The identified compounds, their retention time (RT), percentage of composition (peak area), molecular weight and compound nature were detailed in Table 3.2, 3.3 and 3.4.

3.3.2(a) Hexane

Twenty-four compounds from sterols, terpenoids, fatty acids, phenolic compounds and others have been identified in the hexane extract of *P. bleo* leaves obtained *via* maceration as presented in Table 3.2. Sterols makes up the highest composition in the total compounds (23.35 %) in this extract where γ-sitosterol (17.53 %) was the prominent compound followed by campesterol (4.39 %), stigmasterol (1.13 %) and stigmast-7-en-3-ol, (3.beta.,5.alpha.)- (0.20 %). Terpenoids represent 12.53 % from the total compounds consisting of neophytadiene (5.84 %), phytol (5.22 %), squalene (1.05 %) and (-)-Loliolide (0.42 %). Phenolic compounds represent 9.32 % of the total compounds consisting of γ-tocopherol (6.13 %), 2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)- (2.15 %) and β-tocopherol (1.04 %). Other than that, vitamin E (13.31 %) and fatty acids such as 1-Heptacosanol (2.72 %), 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (2.69 %) as well as 9,12-octadecadienoic acid, methyl ester (2.55 %) were also identified in this study.

Table 3.2 Phytochemical compounds identified in the hexane extract of *P. bleo* leaves

Compounds name	**Retention time**	**Peak area (%)**	**Molecular weight (g/mol)**	**Nature of compounds**
2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	10.195	0.22	180	Others
Cyclopentaneacetic acid,3-oxo-2-pentyl-, methyl ester	10.657	0.13	226	Fatty acid
(-)-Loliolide	11.280	0.42	196	Terpenoids
3-Eicosyne	11.610	0.37	279	Others
Hexadecanoic acid, methyl ester	11.855	1.90	270	Fatty acid
9,12-Octadecadienoic acid, methyl ester	12.513	2.55	294	Fatty acid
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	12.541	2.69	292	Fatty acid
Phytol	12.583	5.22	297	Terpenoids
Octadecanoic acid, methyl ester	12.625	0.67	294	Fatty acid
4,8,12,16-tetramethylheptadecan-4-olide	13.444	0.48	325	Alkene hydrocarbon
5,9,13-Pentadecatrien-2-one,6,10,14-trimethyl-	13.535	0.19	262	Others
Tetracosanoic acid, methyl ester	14.578	0.46	383	Others
Squalene	14.879	1.05	411	Terpenoids
2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-	15.244	2.15	403	Phenol
β-tocopherol	15.538	1.04	417	Phenol
γ-tocopherol	15.594	6.13	417	Phenol

1-Heptacosanol	15.706	2.72	397	Fatty acid
Vitamin E	15.902	13.31	431	Vitamin E
Campesterol	16.364	4.39	401	Sterols
Stigmasterol	16.511	1.13	413	Sterols
n-Tetracosanol-1	16.560	1.20	355	Fatty acid
γ-sitosterol	16.819	17.53	415	Sterols
Stigmast-7-en-3-ol, (3.beta.,5.alpha.)-	17.106	0.20	415	Sterols
Neophytadiene	18.121	5.84	279	Terpenoids

3.3.2(b) Ethyl acetate

The GC-MS analysis of ethyl acetate extract from the leaves of *P. bleo* obtained *via* maceration has identified 23 individual compounds including sterols, terpenoids, fatty acids, phenolic compounds and others as listed in Table 3.3. Majority of the compounds were represented by terpenoids (23.94 %) consisting of phytol (16.09 %), neophytadiene (5.12 %) and (-)-Loliolide (2.73 %). In addition, sterols recorded 7.78 % from the total compounds in this extract that was made up of γ-sitosterol (9.63 %), campesterol (3.23 %), stigmastan-3,5-diene (1.31 %) and stigmasterol (0.82 %). Phenolic compounds represented 4.34 % of the total compounds composition with the presence of γ-tocopherol (2.42 %), 2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)- (1.82 %) and 4-vinyl-2-methoxy-phenol (0.10 %). Vitamin E was presented at 3.49 % of the compounds presented in the ethyl acetate extract of *P. bleo* leaves while fatty acids such as nonanoic acid,9-(3-hexenylidenecyclopropylidene)-2-hydroxy-1-(hydroxymethyl) (6.04 %), 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (5.90 %) and hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester (2.54 %) were also identified.

Table 3.3 Phytochemical compounds identified in the ethyl acetate extract of *P. bleo* leaves

Compounds name	**Retention time**	**Peak area (%)**	**Molecular weight (g/mol)**	**Nature of compounds**
4-vinyl-2-methoxy-phenol	8.977	0.10	150	Phenol
1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	9.467	0.08	206	Others
(-)Loliolide	11.287	2.73	196	Terpenoids
Neophytadiene	11.504	5.12	279	Terpenoids
Hexadecanoic acid, ethyl ester	12.121	0.16	284	Fatty acid
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	12.541	5.90	292	Fatty acid
Phytol	12.590	16.09	297	Terpenoids
9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	12.786	1.18	306	Fatty acid
9-Octadecenamide, (Z)-	12.849	0.55	281	Fatty acid
3,4-Dimethyl-3-cyclohexene-1-carbaldehyde	13.332	1.74	138	Others
4,8,12,16-Tetramethylheptadecan-4-olide	13.444	0.51	323	Alkene hydrocarbon
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	13.941	2.54	331	Fatty acid
Nonanoic acid,9-(3-hexenylidenecyclopropylidene)-2-hydroxy-1-(hydroxymethyl)	14.522	6.04	353	Fatty acid
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	14.795	1.99	278	Fatty acid
2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-	15.243	1.82	403	Phenol
γ-tocopherol	15.594	2.42	417	Phenol
1-Heptacosanol	15.706	2.39	397	Fatty acid

Stigmastan-3,5-diene	15.818	1.31	397	Sterols
Vitamin E	15.895	3.49	431	Vitamin E
Campesterol	16.364	3.23	401	Sterols
Stigmasterol	16.511	0.82	413	Sterols
Octacosyl acetate	16.56	1.13	453	Fatty acid
γ-sitosterol	16.812	9.63	415	Sterols

3.3.2(c) Methanol

The methanol extract of *P. bleo* from the maceration method has identified thirty-six phytocompounds *via* GC-MS. Table 3.4 showed the list of compounds consisting of sterols, terpenoids, fatty acids, phenolic compounds, alkaloids among others. Terpenoids recorded the highest composition (5.61 %) of the total compounds which contained (-)-Loliolide (2.39 %), phytol (1.75 %), neophytadiene (1.22 %) and 4,8,12,16-Tetramethylheptadecan-4-olide (0.25 %). Phenolic compounds represented 4.25 % of the total compounds composition that was made up of 4-vinyl-phenol (2.18 %) and 2-Methoxy-4-vinylphenol (2.07 %). Meanwhile sterols exhibited 1.16 % with the presence of β-sitosterol (0.93 %), campesterol (0.13 %) and stigmasterol (0.10 %). Interestingly, indole (0.18 %) from the alkaloids group was discovered for the first tim in the leaves extract of this plant. Fatty acids such as hexadecanoic acid, 2,3-dihydroxypropyl ester (3.52 %) and 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (2.13 %) as well as vitamin E (0.23 %) were also present in the extract.

Table 3.4 Phytochemical compounds identified in the methanol extract of *P. bleo* leaves

Compounds name	**Retention time**	**Peak area (%)**	**Molecular weight (g/mol)**	**Nature of compounds**
Butyrolactone	4.096	0.18	86	Others
Pyridine,2,4,6-trimethyl-	6.085	0.06	121	Others
2-Pyrrolidinone	7.268	0.69	85	Others
Methyl salicylate	8.186	5.66	152	Others
4-vinyl-phenol	8.403	2.18	120	Phenol
1H-Pyrrole-2,5-dione,3-ethyl-4-methyl-	8.473	1.16	139	Others
Indole	8.865	0.18	117	Alkaloids
2-Methoxy-4-vinylphenol	8.977	2.07	150	Phenol
Cyclopropane, octyl-	9.292	0.25	154	Others
1-Hexadecanol	9.831	0.56	242	Fatty acid
2-Naphtalenamine	10.251	0.54	143	Aromatic amine
3-Methyl-4-phenylpyrrole	10.405	0.45	157	Others
4-(1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	11.189	0.92	180	Others
(-)-Loliolide	11.308	2.39	196	Terpenoids
Neophytadiene	11.512	1.22	279	Terpenoids
2-Pentadecanone,6,10,14-trimethyl-	11.533	0.64	268	Others
Hexadecanoic acid, methyl ester	11.855	1.70	270	Fatty acid
Cyclotetradecane	12.415	0.87	196	Others
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	12.541	2.13	292	Fatty acid
Phytol	12.583	1.75	297	Terpenoids
Methyl 16-methyl-heptadecanoate	12.625	0.34	299	Others
Cyclohexyl-15-crown-5	12.695	0.24	302	Others

Bis(2-ethylhexyl) maleate	12.723	0.27	341	Others
Decyltetraglycol	12.786	0.84	335	Others
2-Butanedioic acid (E)-, bis(2-ethylhexyl) ester	12.996	0.98	341	Others
4,8,12,16-Tetramethylheptadecan-4-olide	13.437	0.25	325	Terpenoids
Hexagol	13.724	0.91	282	Others
Hexadecanoic acid, 2,3-dihydroxypropyl ester	13.941	3.52	331	Fatty acid
13-tetradecenal	14.501	1.44	210	Others
hexaethylene glycol monododecyl ether	15.062	0.89	451	Others
1-Octacosanol	15.699	0.38	411	Fatty acid
Vitamin E	15.895	0.23	431	Vitamin E
Campesterol	16.357	0.13	401	Sterols
Stigmasterol	16.504	0.10	413	Sterols
n-tetracosanol-1	16.553	0.28	355	Fatty acid
β-sitosterol	16.798	0.93	415	Sterols

3.3.3 Identification of phytocompounds from Soxhlet extraction of *P. bleo* leaves

Besides the maceration method, the current study also has successively extracted compounds from the leaves of *P. bleo* using Soxhlet extractor with solvents of different polarity (hexane < ethyl acetate < methanol). The extracts obtained were subjected to GC-MS analysis for identification of the presence phytoconstituents in each extract. The results were shown in Table 3.5, 3.6 and 3.7 which detailed the list of compounds, their retention time (RT), percentage of composition (peak area), molecular weight and compound nature.

3.3.3(a) Hexane

A totoal of 19 compounds have been identified from the hexane extract of *P. bleo* from the Soxhlet method as listed in Table 3.5. This extract consisted of terpenoids, sterols and others compounds. The most abundant compound was sterols representing 10.05 % of the total compound composition, indicating the presence of γ-sitosterol (8.68 %) and campesterol (1.37 %). Terpenoids represented 9.75 % of the total compounds in this extract and were made up of neophytadiene (5.15 %), (Z)-1,3-Phytadiene (2.55 %), (-)-Loliolide (1.04 %) and squalene (1.01 %). Other compounds were also found in this extract such as vitamin E (4.16 %) and fatty acids contained predominantly by hexadecanoic acid as well as methyl ester (6.29 %).

Table 3.5 Phytochemical compounds identified in the hexane extract of *P. bleo* leaves

Compounds name	Retention time	Peak area (%)	Molecular weight (g/mol)	Nature of compounds
Benzoic acid, methyl ester	8.202	1.67	136	Others
Octanoic acid, methyl ester	8.426	0.37	158	Fatty acid
Decanoic acid, methyl ester	9.749	0.24	186	Fatty acid
Dodecanoic acid, methyl ester	10.813	2.34	214	Fatty acid
2(4H)-Benzofuranone,5,6,7,7a-tetradhydro-4,4,7a-trimethyl-	10.981	0.54	180	Others
Methyl tetradecanoate	11.752	0.84	242	Fatty acid
(-)-Loliolide	12.06	1.04	196	Terpenoids
Neophytadiene	12.256	5.15	279	Terpenoids
(Z)-1,3-Phytadiene	12.431	2.55	279	Terpenoids
Hexadecanoic acid, methyl ester	12.599	6.29	271	Fatty acid
n-Hexadecanoic acid	12.739	11.03	256	Fatty acid
Heptadecanoic acid, methyl ester	12.991	0.56	285	Fatty acid
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	13.299	9.29	293	Fatty acid
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	13.446	35.12	265	Others
Hexadecanoic acid, 2,3-dihydroxypropyl ester-	14.692	0.68	331	Fatty acid
Squalene	15.743	1.01	411	Terpenoids
Vitamin E	17.262	4.16	431	Vitamin E
Campesterol	18.032	1.37	401	Sterols
γ-sitosterol	18.719	8.68	415	Sterols

3.3.3(b) Ethyl acetate

The GC-MS analysis of the ethyl acetate extract from *P. bleo* leaves showed the presence of twenty-two phytochemical compounds as presented in Table 3.6. Flavanoids were the highest compounds (20.81 %) identified in this extract with the predominate compound of 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl. Terpenoids were also identified at 9.38 % of the total compound composition comprising of neophytadiene (4.29 %), phytol (3.59 %) and (-)-Loliolide (1.50 %). Meanwhile, phenolic compounds represented 1.93 % of the total compound containing 4-vinyl-syringol (1.32 %), 4-vinylphenol (0.53 %) and γ-tocopherol (0.08 %). Sterols made up 1.76 % of the total extract compositions consisting of γ-sitosterol (1.56 %) and campesterol (0.20 %). Other compounds that were identified include vitamin E (0.36 %) and fatty acids such as linolenic acid (8.75 %).

Table 3.6 Phytochemical compounds identified in the ethyl acetate extract of *P.bleo* leaves

Compounds name	**Retention time**	**Peak area (%)**	**Molecular weight (g/mol)**	**Nature of compounds**
Protoanemonine	5.429	0.08	96	Others
Decane	7.186	0.28	142	Hydrocarbon
1,2,3-Propanetriol, monoacetate	8.257	11.03	134	Others
4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	8.608	20.81	144	Flavonoids
4-vinylphenol	9.105	0.53	120	Phenol
Dodecanoic acid, methyl ester	10.813	1.03	214	Fatty acid
Dodecanoic acid	10.981	1.26	200	Fatty acid
4-vinyl-syringol	11.065	1.32	180	Phenol
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	11.891	1.31	180	Others
(-)-Loliolide	12.074	1.50	196	Terpenoids
Neophytadiene	12.256	4.29	279	Terpenoids
Hexadecanoic acid, methyl ester	12.599	1.12	271	Fatty acid
Hexadecanoic acid	12.746	5.25	256	Fatty acid
Trans - sinapyl alcohol	12.928	0.78	210	Others
Phytol	13.341	3.59	297	Terpenoids
Linolenic acid	13.453	8.75	278	Fatty acid
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	14.699	3.42	331	Others
Ethyl linoleolate	15.995	0.86	306	Others
γ-tocopherol	16.793	0.08	417	Phenol
Vitamin E	17.262	0.36	431	Vitamin E
Campesterol	18.032	0.20	410	Sterols
γ-sitosterol	18.725	1.56	415	Sterols

3.3.3(c) Methanol

Twenty-eight compounds were identified in *P. bleo* methanol extract by GC-MS analysis as shown in Table 3.7. The prevailing compounds were from phenolic group (34.93 %) consisted of phenol, 2,6-dimethoxy- (11.64 %), 2-Methoxy-4-vinylphenol (8.30 %), 4-vinylphenol (5.78 %), phenol, 2-methoxy-4-(1-propenyl)- (3.90 %), 4-vinyl -syringol (3.72 %) and γ-tocopherol (1.59 %). On the other hand, 11.12 % of this extract was represented by terpenoids which consisted of neophytadiene (8.44 %), (Z)-1,3-Phytadiene (1.84 %), squalene (0.61 %) and olean-12-en-28-oic acid, 3-oxo-,methyl ester (0.23 %). Sterols comprising of γ-sitosterol (0.47 %), stigmasta-3,5-dien-7-one (0.21 %) and campesterol- (0.21 %) representing 0.89 % of the total compound composition of this extract. Meanwhile, vitamin E was found at 1.28 % of total compounds content as well as fatty acids such as 9,12,15-Octadecatrienoic acid, methyl ester (3.38 %) and methyl palmitate (3.32 %).

Table 3.7 Phytochemical compounds identified in the methanol extract of *P. bleo* leaves

Compounds name	**Retention time**	**Peak area (%)**	**Molecular weight (g/mol)**	**Nature of compounds**
2(3H)-Furanone, dihydro-	4.765	0.57	86	Others
Pyrazine, trimethyl-	6.364	0.31	122	Others
Pyrazine, 3-ethyl-2,5-dimethyl-	6.971	0.45	136	Others
Benzoic acid, methyl ester	7.374	1.06	136	Others
2-Isobutylthiazolidine	8.073	0.57	145	Others
4-vinylphenol	8.562	5.78	120	Phenol
2-Methoxy-4-vinylphenol	9.002	8.30	150	Phenol
Phenol, 2,6-dimethoxy-	9.236	11.64	154	Phenol
Phenol, 2-methoxy-4-(1-propenyl)-	9.735	3.90	164	Phenol
DL-Proline, 5-oxo-, methyl ester	9.9	3.75	143	Others
4-vinyl -syringol	10.327	3.72	180	Phenol
Neophytadiene	11.455	8.44	279	Terpenoids
(Z)-1,3-Phytadiene	11.632	1.84	279	Terpenoids
Methyl palmitate	11.811	3.32	271	Fatty acid
n-Hexadecanoic acid	12.381	2.49	256	Fatty acid
9,12,15-Octadecatrienoic acid, methyl ester	12.496	3.38	292	Fatty acid
Hexadecanamide	12.84	2.28	255	Fatty acid
Behenic alcohol	13.805	0.63	327	Others
Hexadecanoic acid, 2-hydroxy-1(hydroxymethyl)ethyl ester	13.923	2.78	331	Others
9,17-Octadecadienal, (Z)-	14.491	3.57	264	Others
13-Docosenamide	14.713	0.94	338	Others
Squalene	14.819	0.61	411	Terpenoids
γ-tocopherol	15.515	1.59	417	Phenol
Vitamin E	15.81	1.28	431	Vitamin E
Campesterol	16.264	0.21	410	Sterols

γ-sitosterol	16.688	0.47	415	Sterols
Stigmasta-3,5-dien-7-one	17.073	0.21	411	Sterols
Olean-12-en-28-oic acid, 3-oxo-,methyl ester	18.764	0.23	469	Terpenoids

3.3.4 Identification of phytocompounds from decoction of *P. bleo* leaves aqueous extract

In this study, the aqueous extract of *P. bleo* leaves was also obtained *via* decoction method. Identification of the phytocompounds was performed using GC-MS analysis. From the results, sixteen compounds have been found as listed in Table 3.8. The most abundant compound was from phenols (4.34 %) consisting γ-tocopherol (2.38 %) and 2-Methoxy-4-vinylphenol (1.96 %). Alkaloids represented 2.64 % from the total compound composition containing 1,2,3,4-Tetrahydro-cyclopenta(b)indole (1.80 %) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (0.84 %). Phytol, a predominant compound (1.31 %) from the terpenoid group was also identified in the aqueous extract of this plant. Sterols represented 0.91 % of the total compound composition comprising mainly of γ-sitosterol. Vitamin E was recorded at 0.52 % while fatty acids were also presented in the extract such as n-Tetracosanol-1 (1.56 %).

Table 3.8 Phytochemical compounds identified in the aqueous extract of *P. bleo* leaves

Compounds name	**Retention time**	**Peak area (%)**	**Molecular weight (g/mol)**	**Nature of compounds**
Pyrazine, trimethyl-	6.218	0.34	122	Others
Thiazolidine,2-isobutyl-	8.067	1.90	145	Others
Benzofuran,2,3-dihydro-	8.389	0.82	120	Others
2-Methoxy-4-vinylphenol	8.977	1.96	150	Phenol
4-methyl-2,5-dimethoxybenzaldehyde	10.314	0.76	198	Others
1,2,3,4-Tetrahydro-cyclopenta(b)indole	10.405	1.80	157	Alkaloids
Methyl dihydrojasmonate	10.65	2.30	226	Fatty acid
Octanal, 2-(phenylmethylene)-	11.162	1.28	216	Others
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	11.932	0.84	210	Alkaloids
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	12.534	0.38	292	Fatty acid
Phytol	12.583	1.31	296	Terpenoids
γ-tocopherol	15.587	2.38	416	Phenol
n-Tetracosanol-1	15.699	1.56	354	Fatty acid
Vitamin E	15.888	0.52	430	Vitamin E
1-Heptacosanol	16.553	0.91	396	Fatty acid
γ-sitosterol	16.791	2.98	414	Sterols

3.4 Discussion

Nowadays, medicinal plants have attracted the focus of researchers due to their health benefits and also a source of therapeutic compounds that lead to the discovery and development of new drugs. Medicinal plants possess extensive of secondary metabolites and phytochemicals which work synergistically contributing to its efficacy in the treatment of various health problems. In this regard, extraction and analysis of plant material are important in the development, upgrading, and quality control of herbal formulations (Sasidharan *et al.*, 2011).

Numerous studies have been done on this plant, nonetheless, its phytochemical studies remained unexplored. Most phytochemical studies involving this plant leaves usually use fractions instead of crude extracts. However, in traditional practice, the leaves are commonly prepared as crude. Thus, the present study was conducted to investigate the phytochemicals presented in the crude extracts of *P. bleo.* The leaves samples were extracted using solvents of different polarities through maceration, Soxhlet and decoction extraction methods. The crude extracts obtained were then subjected to GC-MS analysis for phytochemical compounds identification.

In the medicinal plant analysis, extraction is an important step to obtain phytochemical compounds of interest from the plant materials (Sasidharan *et al.*, 2011). The target compounds comprise of different structures that determine their solubility in the different polarity solvents that are used for extraction (Złotek *et al.*, 2016). Therefore, the efficacy of extraction largely depends on the type of solvent that significantly influence the recovery of extraction yield from the plant materials. According to the results, high extraction yield was produced by methanol solvent followed by ethyl acetate and hexane in both successive maceration and Soxhlet

extraction method. The aqueous solvent from decoction method also found producing high extraction yield. This was probably due to the presence of high level of polar compounds with high molecular weight in the plant materials that is soluble in high polarity solvents like methanol and aqueous. According to Truong *et al.* (2019), highly polar solvents enhanced the efficacy of the extraction process where they found that methanol extract of *Severinia buxifolia* produced the highest extraction yield compared to other tested solvents. In other study by Tambunan *et al.* (2017) demonstrated that the highest extract yield of *Ageratum conyoides* Linn. Leaves was obtained by aqueous extract (30.20 %) followed by ethanol extract (15.00 %). The current results indicated that the polarity of solvents influences the extraction yield.

As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. The current study used maceration, Soxhlet and decoction techniques to extract *P. bleo* leaves because they are commonly used in the extraction process of medicinal plants. In successive extraction method, the Soxhlet method outperformed the maceration method recording the best percentage of extract yield. The order of solvent effectiveness is as follows: methanol > ethyl acetate > hexane. Similar findings have been reported by Muthukumarasamy *et al.* (2018) where the Soxhlet extraction of *Osbeckia parvifolia* plant demonstrated excellent recovery over other extraction methods with the highest percentage of yield obtained in methanol followed by ethyl acetate, ethanol and hexane. There are several factors that can enhance the efficacy of extraction including a longer extraction period. However, this will not work once the equilibrium between solute and solid material has been achieved (Zhang *et al.*, 2018). In the current study, Soxhlet extraction recorded the best recovery percentage in the shortest time compared to maceration even though

maceration method required a longer extraction period about 30 days which may be caused by the equilibrium between the solvent and the plant materials.

The GC-MS analysis was conducted to identify the presence of phytocompounds in the leaves of *P. bleo* leaves extracts obtained by successive extraction with different solvents *via* maceration, Soxhlet and decoction extraction method. GC-MS is an ideal method for analysis of volatile components, terpenoids, lipids, fatty acids, phenolics, alkaloids and glycosides in the medicinal plants (McGhie and Rowan, 2012; Sixto *et al.*, 2019; Stashenko and Martínez, 2012). Our findings highlighted that all extracts from the leaves of *P. bleo* contain compounds like sterols, terpenoids, phenolic, flavonoids and alkaloids.

The crude hexane extract of *P. bleo* leaves showed the presence of terpenoids, sterols and phenolic compounds from maceration while a mixture of sterols and terpenoids were recorded from the Soxhlet extraction. Sterols were the highest phytocompound isolated in this extract obtained from both extraction methods which consist predominantly of γ-sitosterol. Terpenoids were the second-highest phytocompound isolated in this extract from both extractions with neophytadiene as the main compound. Hexane is effective for the extraction of non-polar compounds such as lipid, lignin, terpenoids and sterols (Cowan, 1999; Houghton and Raman, 2012). Phytochemical study of hexane extract from *Pluchea indicia Less* leaves reported similar findings where sterols were the highest compound found in this extract beside flavonoids, phenolic and alkaloid (Widyawati *et al.*, 2014). In addition, the current study identified low levels of phenolic compounds in the crude hexane extract from the maceration while there were no phenolic compounds detected from Soxhlet method. A study has reported the presence of phenolic content at a low level in *Terminalia*

ferdinandiana leaves together with flavonoids and tannins after being soaked in the hexane solvent at 4°C for 24 h (Courtney *et al.*, 2015). Solvents such as hexane, chloroform, propanol, ethyl acetate, ethanol, methanol and water are commonly used to extract phenolic compounds and the capacity of the compounds is affected by the polarity of the solvent (Aires, 2017). However, temperature also plays an important role in the recovery of phenolic compounds because some thermolabile phenolic compounds are vulnerable to high temperatures close to their boiling points (Seidel, 2012). Thus, this finding suggested that phenolic compounds might be degraded when extracted using Soxhlet method probably due to excess temperature during extraction that exceeds the boiling point of the hexane.

Terpenoids were found in abundance in the ethyl extract of *P. bleo* leaves from maceration followed by sterols and phenolic compounds. Phytol was the major content of terpenoids in this extract. Terpenoids and phenolic compounds were reported as the major compounds present in ethyl acetate extract of *Durio zibethinus* L. leaves prepared through maceration for 48 h at room temperature (Aruan *et al.*, 2019). While in the Soxhlet method, flavonoids were dominated by the compound 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl in the ethyl extract of this plant followed by terpenoids, phenols and sterols. The most common solvents for flavonoids extraction are methanol and ethanol using extraction methods such as percolation, dipping, continuous reflux, reflux and decoction (Chun, 1998; Feng *et al.*, 2017). For instance, in a report by Hossain and Rahman (2015) showed that high flavonoids were found in the methanolic extract of *Orthosiphon stamineus* leaves obtained using the Soxhlet method. However, in the current study, flavonoids in the leaves of *P. bleo* were best extracted using ethyl acetate obtained with the Soxhlet method. This finding suggested that ethyl acetate can be one of the best solvents for flavonoids extraction. Apart from

that, the current findings, showed that the presence of terpenoids was higher in maceration extraction compared to Soxhlet. Several studies have reported low recovery of terpenoids at high temperatures due to degradation and instability of the compounds with increasing temperature during extraction (McGraw *et al.*, 1999; Yang *et al.*, 2007). Thus, the recovery of terpenoids was excellent through maceration compared with Soxhlet extraction as these compounds are less stable and easily degraded with the presence of heat.

Methanol and aqueous are the polar solvents that are commonly used in extraction of medicinal plants. Methanol is effective for the extraction of polar compounds such as sugar, amino acid, glycoside compounds, phenols, tannin, saponin and terpenoids (Cowan, 1999; Houghton and Raman, 2012). The methanol extract of *P. bleo* leaves obtained through maceration showed the highest amount of terpenoids predominated by (-)-Loliolide followed by phenolic compounds, sterols and alkaloids. Meanwhile in Soxhlet extraction, phenolic compounds were detected the highest amount (predominant compounds namely 2-Methoxy-4-vinylphenol) followed by terpenoids and sterols. The aqueous solvent is effective to extract compounds including amino acid, sugar, glycoside and aglycon compounds (Houghton and Raman, 2012; Liu *et al.*, 2011). Phenolic compounds were the highest compounds present in the aqueous extract of *P. bleo* leaves obtained through decoction followed by alkaloids and sterols. These findings showed that phenols and sterols are thermolabile compounds as well as heat-stable as they have better recovery from Soxhlet and decoction which were carried out in the presence of heat. Meanwhile terpenoids are not heat stable compounds and can be isolated at low temperature.

In phytochemical research, it is important to choose proper methods of extraction for the isolation of target compounds as heat can degrade thermolabile compounds. In this study, various phytochemical compounds have been isolated from the leaves of *P. bleo* extracts. Table 3.9 summarizes similar and different compounds that have been isolated from the leaves of this plant between two methods of extraction maceration and Soxhlet. Previous studies on the phytochemical investigation involving this plant revealed the presence of dihydroactinidiolide, a mixture of sterols (campesterol, stigmasterol, β-sitosterol), 2,4,-di-tert-butylphenol, α-tocopherol, phytol, vitexin and vitamin E in the ethyl extract fraction of *P. bleo* leaves (Abdul-Wahab *et al.*, 2012; Malek *et al.*, 2009; Rahman, 2008). Meanwhile, β-carotene, α-tocopherol and a mixture of flavonoids (catechin, quercetin, epicatechin, myricetin) were found in dried leaves of *P. bleo* (Abdul-Wahab *et al.*, 2012). In addition, β-sitosterol was present in the hexane and ethyl acetate fraction of this plant while dichloromethane extract contained β-sitosterol glucoside (Abdul-Wahab *et al.*, 2012; Rahman, 2008).

As for the maceration method, the current findings showed the presence of several new compounds from the leaves of *P. bleo* such as β-tocopherol, 1-Heptacosanol, n-Tetracosanol-1 and indole together with previously reported compounds namely stigmasterol and β-sitosterol. Stigmasterol is one of the plant sterols that demonstrated anti-tumor activity and a popular antioxidant effect. Stigmasterol isolated from *Bacopa monnieri* Linn exerted anti-tumor effect on Ehrlich Ascites tumor in mice by reducing the proliferation and size of the tumor as well as improving other biochemical parameters (Ghosh *et al.*, 2011). In another study, mice with skin tumors showed a reduction in the size of tumor, papillomas cell numbers, level of lipid peroxidase and DNA damage after 16 weeks of treatment with stigmasterol isolated from *Azadirachta indica* plant (Ali *et al.*, 2015). Thus, the current findings suggested

that all the above mention compounds obtained *via* maceration method were heat-sensitive compounds as they are stable at room temperature extraction.

Meanwhile, in the Soxhlet method, the newly isolated compounds were 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, 4-vinyl-syringol, Phenol, 2,6-dimethoxy, phenol, 2-methoxy-4-(1-propenyl)-, Stigmasta-3,5-dien-7-one and hexadecanoic acid as shown in Table 3.9. 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (DDMP) is a flavanoid that exhibits anti-proliferative, pro-apoptotic effects, protective effects and antioxidant activity. DDMP isolated from onions induced cell death in colon cancer cells (HCT116 and SW620) through suppression of anti-apoptotic proteins Bcl-2 and the expression of pro-apoptotic proteins Bax (Ban *et al.*, 2007). DDMP isolated from *Plukenetia conophora* plant exhibited protective effects where it significantly reduced nitric acid and malondialdehyde levels in the reproductive toxicity of male rats induced by cadmium chloride (Olaniyan *et al.*, 2018). DDMP also demonstrated strong antioxidant activity in glucose-histidine Maillard reaction products (Yu *et al.*, 2013). Besides that, hexadecanoic acid, also known as n-hexadecanoic acid exhibited anticancer effects when it was isolated from the crude chloroform extract of *Kigelia pinnata* leaves showing the cytotoxic effects on human colorectal carcinoma (HCT-116) cells (Ravi and Krishnan, 2017). Other than that, it possesses antibacterial activity. This was evident in a study by Johannes and Litaay (2017) where n-hexadecanoic acid that was isolated from hydroid *Aglaophenia cupressina* Lamaoureoux at a concentration of 30 ppm inhibited the growth of *Salmonella typhi* after 48 hours incubation. Furthermore, anti-inflammatory effects have also been reported as a characteristic of n-hexadecanoic acid (Aparna *et al.*, 2012). Meanwhile, 4-vinyl-syringol and phenol, 2,6-dimethoxy are well known for their antioxidant activity (Adelakun *et al.*, 2012; Galano *et al.*, 2011; Nyanhongo *et al.*, 2013). Phenol,

2-methoxy-4-(1-propenyl) also known as isoeugenol has been reported to exhibit anti-microbial and cytotoxic effects (Atsumi *et al.*, 2000; Foo *et al.*, 2015). Thus, it can be suggested that these compounds are heat stable because they were not degraded at high temperature during Soxhlet extraction.

Apart from that, there were several similar new active compounds present in the leaves of *P. bleo* extract obtained with maceration and Soxhlet methods such as (-)-Loliolide, neophytadiene, γ-tocopherol, squalene, γ-sitosterol, 4-vinylphenol, 2-Methoxy-4-vinylphenol, 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z) and together with previously reported compounds such as phytol, campesterol and vitamin E. γ-sitosterol has been previously reported to influence cholesterol synthesis in liver and intestinal cell lines (Ho and Pal, 2005). It also acts as a cytotoxic sensitizing agent (Carter *et al.*, 2007). In addition, γ-sitosterol from *Strobilanthes crispus* leaves extract was found to be cytotoxic against colon and liver cancer cell lines (Endrini *et al.*, 2014). Besides that, neophytadiene is widely known for its antioxidant properties (Yamuna *et al.*, 2017). Meanwhile, γ-tocopherol is popular for its antioxidant properties and recent studies have also reviewed other benefits of these compounds such as anticancer, anti-inflammatory and cancer preventive effects (Cervinkova *et al.*, 2016; Constantinou *et al.*, 2019). Phytol is believed to have anticancer properties (Jeong, 2018). It has been reported to trigger apoptosis in liver and lung cancer cells activated via caspase 3 and 9 pathway (Kim *et al.*, 2015; Thakor *et al.*, 2017). In addition, 4-vinyl-2-methoxy-phenol or 2-methoxy-4-vinylphenol (2M4VP) showed anti-cancer effects on Panc-1 and SNU-213 pancreatic cell lines. Moreover, 2M4VP reduced the cells viability of Panc-1 cells by inhibiting the expression of the cells nuclear antigen (PCNA) proteins while supressing the migration of Panc-1 and SNU-213 cells (Kim *et al.*, 2019). In addition, 2M4VP possesses anti-inflammatory activity (Jeong *et al.*, 2011; Sridevi *et al.*, 2014).

It was observed in a study where 2M4VP extracted from pine needles was had strong anti-inflammatory effects in LPS-stimulated RAW264.7 cell line (Jeong *et al.*, 2011). On top of that, chemopreventive effects of squalene were evident while the formation of a preneoplastic lesion in the colonic was inhibited in rats after they were given a dietary containing 1% squalene, with no significant effect on serum cholesterol (Rao *et al.*, 1998). Other than that, squalene is also a potential anticancer agent in combination with other commercial anti-cancer agents such as adriamycin, bleomycin or cis-dichlorodiamminoplatinum which demonstrated cytotoxic effects against sarcoma cells (S180) (Nakagawa *et al.*, 1985). Thus, the current findings suggest that all the compounds mentioned above were stable at both low and high temperature since they present in maceration (at room temperature) and Soxhlet (high temperature) extraction methods.

There are limited studies on the chemical compounds from the aqueous extract of *P. bleo* leaves compared to other *Pereskia* species. Sim *et al.* (2010a) reported the presence of phenolic compounds in the aqueous extract of *P. bleo* leaves which are concomitant with the present finding except it only measured total phenolic content. Meanwhile, the present study has elucidated the compounds from the phenolic group namely 2-Methoxy-4-vinylphenol and γ-tocopherol. Sharif *et al.* (2015) reported that aqeous extract of *P. bleo* leaves contains fatty acids and high content of myo-inositol and sugars (galactose and phenanthrene). On the contrary, our GC-MS analysis revealed the presence of alkaloids, terpenoids, fatty acids and phenolic compounds. Phenolic compounds consisting of 2-Methoxy-4-vinylphenol and γ-tocopherol were the major compounds identified in the aqueous extract of *P. bleo* leaves. These differences might be due to the difference in temperature used during extraction. Sharif *et al.* (2015) carried out the extraction at 30^{o}C while the temperature used in this study was 50^{o}C.

Extraction at a higher temperature releases higher phenolic compound compared to a lower temperature (Prenesti *et al.*, 2007).

The current findings showed that there were various phytochemical compounds presented in the leaves of *P. bleo* identified *via* GC-MS analysis and the potential active compounds together with their biological activities were listed in Table 3.10. Thus, solvents such as hexane, ethyl acetate, methanol and aqueous solution were successful and suitable for the isolation of wide range of non-polar and polar compounds in the leaves of *P. bleo*. Methanol and aqueous solvents recorded the highest extraction yield obtained from maceration, Soxhlet and decoction extraction methods due to abundant of polar compounds with high molecular weight. Maceration method was the best technique for the isolation of terpenoids and phenolic compounds. Soxhlet method demonstrated the best recovery for flavonoids as well as phenolic compounds.

Table 3.9 Comparison and similar phytochemical compounds isolated from the leaves of *P. bleo* between maceration and Soxhlet

Maceration	Soxhlet
2H-1-Benzopyran-6-ol,3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl
4,8,12,16-tetramethylheptadecan-4-olide	(Z)-1,3-Phytadiene
1-Heptacosanol	Hexadecanoic acid
Stigmasterol	Dodecanoic acid, methyl ester
n-Tetracosanol-1	Pyrazine, trimethyl-
β-tocopherol	Benzoic acid, methyl ester
β-sitosterol	4-vinyl-syringol
Stigmast-7-en-3-ol, (3.beta.,5.alpha.)-	Phenol, 2,6-dimethoxy-
Stigmastan-3,5-diene	Phenol, 2-methoxy-4-(1-propenyl)-
Indole	Stigmasta-3,5-dien-7-one

Similar compounds

(-)-Loliolide
Hexadecanoic acid, methyl ester
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
9,12,15-Octadecatrienoic acid, methyl ester
Phytol
Squalene
γ-tocopherol
Vitamin E
Campesterol
γ-sitosterol
4-vinylphenol
2-Methoxy-4-vinylphenol
Neophytadiene
Hexadecanoic acid, 2,3-dihydroxypropyl ester

Table 3.10 Ten potential active compounds presented in the leaves of *P. bleo* identified *via* GC-MS and their biological activities.

Nature of compounds	Compounds name	Biological activities	References
Flavonoids	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	Anti-proliferative, pro-apoptotic effects	(Ban *et al.*, 2007)
		Protective effects	(Olaniyan *et al.*, 2018)
		Antioxidant	(Yu *et al.*, 2013)
Sterols	γ-sitosterol	Cytotoxic agent	(Carter *et al.*, 2007)
	Campesterol	Anti-angiogenic effects	(Choi *et al.*, 2007)
		Anti-cancer	(Awad *et al.*, 2003; Kpoviessi *et al.*, 2008)
		Anti-inflammatory	(Moreno-Anzúrez *et al.*, 2017)
Phenolic	γ-tocopherol	Anti-inflammatory	(Himmelfarb *et al.*, 2007; Wolf, 2006)
		Anti-cancer agent	(Wolf, 2006)
		Antioxidant	(Singh *et al.*, 2008)
	2-Methoxy-4-vinylphenol	Anti-microbial, antioxidant, analgesic	(Sridevi *et al.*, 2014)
		Anti-inflammatory	(Jeong *et al.*, 2011; Sridevi *et al.*, 2014)
		Anti-cancer effects	(Kim *et al.*, 2019)
Terpenoids	(-)-Loliolide	Antioxidant	(Yang *et al.*, 2011)
		Anti-cancer	(Machado *et al.*, 2012)
	Neophytadiene	Antioxidant	(Yamuna *et al.*, 2017)
	Phytol	Anti-cancer	(Jeong, 2018)
		Antioxidant	(Santos *et al.*, 2013)

		Antinociceptive	(Santos *et al.*, 2013)
		Anti-bacterial	(Pejin *et al.*, 2014)
Vitamin E	Vitamin E	Antioxidant	(Niki and Noguchi, 2004)
		Anticancer	(Yu *et al.*, 2009)
Fatty acid	Hexadecanoic acid	Anti-cancer	(Ravi and Krishnan, 2017)
		Anti-bacterial	(Johannes and Litaay, 2017)
		Anti-inflammatory	(Aparna *et al.*, 2012)

3.5 Conclusion

In conclusion, the recovery of extraction yield and compounds of interest from the plant materials are largely depend on the suitable extraction and solvent types. Our findings showed Soxhlet and decoction extractions were the ideal technique to extract phenolic and flavonoids compounds from the leaves of *P. bleo*. Maceration was best for extraction of terpenoids and phenolic compounds from this plant leaves. Terpenoids, sterols, alkaloids, fatty acids, flavonoids, vitamin E and phenolic compounds were identified from the crude extracts of *P. bleo* leaves. Furthermore, several new compounds identified from the leaves of *P. bleo* including (-)-Loliolide, neophytadiene, β-tocopherol, γ-tocopherol, squalene, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, 4-vinyl-syringol, phenol,2-methoxy-4-(1-propenyl) and hexadecanoic acid which possess anti-cancer and antioxidant properties that contribute to the therapeutic benefits of this plant.

CHAPTER 4

CYTOTOXICITY, CELL CYCLE ARREST AND APOPTOSIS INDUCTION OF *Pereskia bleo* LEAVES EXTRACTS AGAINST SELECTED CANCER CELL

4.1 Introduction

To date, cancer remains the primary cause of death in humans worldwide in spite of the advancement in tools for its diagnosis, treatment and prevention (Razali *et al.*, 2016). It remains a global public health issue as its mortality and morbidity rate are predicted to increase every year (Pumiputavon *et al.*, 2017). Effective cancer treatment is important and urgently needed for future management of the disease. Conventional cancer treatments such as surgery, chemotherapy and radiotherapy, even though proven to be effective, remains unsatisfactory in some types of cancer due to their detrimental side effects, cancer prevalence and decline of general health in cancer patients (Schloss *et al.*, 2017). Thus, the discovery of novel potent anti-cancer agents for cancer treatment will always be in demand.

Elimination of cells from our body involves a number of mechanism including necrosis and apoptosis. Necrosis is a non-specific form of cell death indicated by disruption of cell membrane which prompt a localized inflammatory response and injury of the surrounding tissue as well as cells (Yang *et al.*, 2015). On the contrary, apoptosis involves an instant engulfment and removal of apoptotic bodies by phagocytosis without damage the surrounding cells and tissue (Wong, 2011). Besides, it is a natural cell death consequence from activation of a tightly regulated events that are frequently modified in cancer cells (Elmore, 2007; Hanahan and Weinberg, 2016). The mechanism of apoptosis is divided into two categories which are extrinsic (death receptor) and the intrinsic (mitochondrial) pathways. Therefore, apoptosis activation

has been pointed out as the main strategy in inhibiting the proliferation of cancer cells and in searching for anti-cancer drugs (Chen *et al.*, 2008).

The typical morphology that defining the apoptotic cells include cell shrinkage, fragmented nucleus, membrane disruption and blebbing as well as apoptotic body formation (Kumar et al., 2017; Saraste and Pulkki, 2000). Inverted or fluorescent microscopes are commonly used to detect the alterations in the cell structures due to apoptosis while Hoechst staining is the best method to identify condensed chromatins and fragmented nucleus of the apoptotic cells (Rahman *et al.*, 2013). Besides that, various assays have been developed for apoptosis detection such as DNA fragmentation, cytotoxicity and cell proliferation, flow cytometry apoptosis Annexin V/PI staining and apoptotic proteins expression (Banfalvi, 2017; Martinez *et al.*, 2010).

The application of natural products including medicinal plants as an alternative treatment for cancer has caught the attention of oncologists. Both plants and their products including crude extracts and pure compounds are recognized as pro-apoptotic agents due to the variation in their mechanism of actions and as well as their minimal side effects (Rasul and Ma, 2012; Wu *et al.*, 2002). Nevertheless, in traditional practice, plants are often consumed raw or in the form of crude extracts for medicinal purposes (Chai, 2011). In addition, studies have reported better efficacy of plant crude extracts compared to single compounds in cancer research (Aiyelaagbe *et al.*, 2011; Rasoanaivo *et al.*, 2011). Drugs that are derived from plants are often accessible, inexpensive and safe without significant detrimental effects towards consumers (Yadav and Agarwala, 2011). Furthermore, synergistic actions from various phytochemicals in the crude extracts may be the reason behind the healing potential of the plants (Ma *et al.*, 2009).

A number of popular medicinal plants have been studied for their apoptosis induction for example *Clinacanthus nutans*. Hexane extract of this plant was reported to induce apoptosis in HepG2 (liver), A549 (lung) and CNE1 (nasopharygeal) cancer cells modulated by oxidative stress and upregulation of caspases (-3, -8, -9) level (Ng *et al.*, 2017). Another study by Wan-Ibrahim *et al.* (2019) found methanol extract of *Abrus precatorius* promotes apoptosis in MDA-MB-231 breast cancer cells *via* inhibition of cell progression at G_0/G_1 phase and upregulation of Bax level. Aqueous extract of *Annona muricata* leaf exerted cytotoxic effects and triggered apoptosis in MDA-MB-231 cells through intrinsic pathway by producing reactive oxygen species (ROS) and cell cycle arrest at G_0/G_1 phase (Kim *et al.*, 2018). Ethanolic extract of *Moringa oleifera* leave induced cell death in liver HepG2, breast MCF7 and colon HCT 116 and Caco-2 cancer cells indicated by high number of apoptotic cells after treatment with this extract (Abd-Rabou *et al.*, 2017).

In this study, medicinal plant in focus is *Pereskia bleo* (*P. bleo*). The locals believe it can be used for cancer prevention and treatment (Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008; Yen *et al.*, 2013). In addition, there are many biological activities of *P. bleo* that have been reported including anti-cancer and anti-tumor, (Sri Nurestri *et al.*, 2008; Wahab *et al.*, 2009). Several *in-vitro* studies have reported the cytotoxic effects of fractionated extracts (hexane, ethyl acetate and water) and pure compounds in ethyl acetate extract from the leaves of *P. bleo* against various cancer cell lines such as human hormone-dependent breast carcinoma cell line (MCF7), human lung carcinoma cell line (A549), human colon carcinoma cell line (HCT 116), human nasopharyngeal epidermoid carcinoma cell line (KB) and human cervical carcinoma cell line (CasKi) (Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008). Meanwhile, the crude methanol extract from a mixture of the leaves and stems of *P. bleo* was found to be

cytotoxic towards human breast carcinoma cell line (T-47D) and caused apoptosis *via* the stimulation of caspase-3 and c-myc proteins (Tan *et al.*, 2005). However, there is a lack of information regarding its effects on other common cancer cell lines. Therefore, the current study aims to explore the cytotoxic response of crude extracts from *P. bleo* leaves on HeLa, MDA-MB-231 and SW480 cell lines. In order to understand the mechanism of cell death, the most active cytotoxic extract with its corresponding cancer cell was further investigated on apoptosis induction.

4.2 Materials and methods

4.2.1 Sample collection

Refer section 3.2.1 (page 39).

4.2.2 Maceration, Soxhlet and decoction extraction of *P. bleo* leaves

Refer section 3.2.2 for maceration (page 39), section 3.2.3 for Soxhlet (page 40) and section 3.2.4 for decoction (page 40).

4.2.3 Cell lines and cell culture

Cancer cell lines namely cervical (HeLa), breast (MDA-MB-231), colon (SW480) and normal mouse fibroblast cell line (NIH 3T3) were purchased from ATCC. A complete medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco) was used in the culture of the cell lines with 5% CO_2 at 37°C.

4.2.4 Anti-proliferative activity of *P. bleo* leaves extracts

An assay using 3-[4,5-dimethyl thiazol-2-yl] 2,5-diphenyl tetra-zolium bromide (MTT; Merck) was carried out to measure the cytotoxicity activity of *P. bleo* extracts. The cells were seeded in 96-well flat bottomed plates (Eppendorf) at a density of 5×10^4 cells per well in a final volume of 100µl/well. After 24 h incubation at 37°C with 5% CO_2, the cells were treated with 2 µl of a serial dilution of hexane, ethyl acetate, methanol and aqueous extracts of *P. bleo* leaves at concentrations starting from 0.3

µg/ml to 99 µg/ml (concentration of extract in the well). The serial concentrations of *P. bleo* leaves extracts were added into the wells shown as in Table 4.1. In addition, the cells were treated with a positive control tamoxifen (concentration of 0.3 µg/ml to 99 µg/ml) and a negative control DMSO (concentration < 1%). After 72 h of incubation period, 50µl MTT solution (2 mg/ml) were added to each well and further incubated for 4 h at 37°C. Then, 200µl of DMSO was added to each well to dissolve the MTT crystals and their absorbance was recorded at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. Each experiment was performed in triplicates. The percentage of cell viability was determined using the following formula:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance of treated cells (extracts/tamoxifen)}}{\text{Absorbance of DMSO}} \times 100\%$$

The IC_{50} values of the treated cancer and normal cells were determined by plotting a graph of cell's viability versus extract concentration. The extract with the lowest IC_{50} value and its corresponding cancer cell were used for further analysis in this study.

Table 4.1 Serial concentration of *P. bleo* leaves extracts

Stock extract concentration (mg/ml)	Concentration of extract in the well (mg/ml)	Concentration of extract in the well (µg/ml)
10.000	0.0990	99.00
5.000	0.0495	49.50
2.500	0.0248	24.80
1.250	0.0124	12.40
0.625	0.0062	6.20
0.313	0.0031	3.10
0.156	0.00155	1.55
0.078	0.00077	0.77
0.039	0.00039	0.39

4.2.5 Morphological assessment of apoptotic HeLa cells induced by PBEA

4.2.5(a) Bright field inverted microscopy

A total of 5×10^4 cells/ml of HeLa cells were seeded in a six-well plate and treated with IC_{50} value of ethyl acetate extract of *P. bleo* leaves (PBEA) and tamoxifen (positive control) at a concentration of 14.37 µg/ml and 2.71 µg/ml respectively and incubated for 24 h, 48 h and 72 h time points. The negative control used in this study was untreated cells. The cells were observed under an inverted microscope (OLYMPUS CK40) at 20× magnification to observe their morphological changes and evaluate the effect on HeLa cells after treatment with PBEA. The cells images were captured by using a camera (NIKON D5600).

4.2.5(b) Fluorescence microscopy

Hoechst 33258 staining was performed to determine the morphological changes in the nuclei of HeLa cells that were treated with PBEA. The cells were grown on glass

slides that were placed in a tissue culture dish and treated with PBEA (IC_{50} value =14.37 µg/ml) for 24 h, 48 h and 72 h. After each incubation time, the slides were washed twice with 1× PBS and fixed with 4% paraformaldehyde for 30 minutes at 4°C. Subsequently, the slides were incubated with 30 µg/ml of Hoechst 33258 solution for 30 minutes in the dark at room temperature. The slides were dried and mounted with DPX mounting medium and viewed under a fluorescence microscope (Imaging Source Europe GmBH, Bremen, Germany) at 40× magnification (excited at 350nm and emitted at 461nm). A brighter blue fluorescence nucleus is an indication of the apoptotic cells.

4.2.6 Induction of cell death in HeLa cancer cells induced by PBEA

4.2.6(a) Cell cycle assay

Flow cytometry was used to evaluate the changes in cell cycle distribution of HeLa cells induced by PBEA. The cells were treated with PBEA at a concentration of IC_{50} and incubated with 5% CO_2 at 37°C for several time intervals (24 h, 48 h and 72 h). Untreated cells were used as the control group in this study. After that, the samples were processed using BD Cycletest™ Plus DNA Kit (BD Bioscience) as per the manufacturer's protocol. The cells were resuspended in a buffer solution at concentration of 5×10^5 cells per sample. Then, the cells were incubated with trypsin buffer (10 min, room temperature), trypsin inhibitor and RNase buffer (10 min, room temperature) and lastly cold PI stain solution (10 min, 2°C – 8°C) on ice in the dark. After incubation, the samples were analyzed using FACSCANTO II (BD Bioscience). A minimum of 10 000 events was acquired per sample and the data were analyzed by using ModFiT LT 5.0 software.

4.2.6(b) Annexin V-FITC assay

Annexin V-FITC Detection Kit I (BD Bioscience) was used to identify the distribution of early or late apoptosis induced by PBEA towards HeLa cells. The assay was conducted according to the manufacturer's protocol. The cells were later incubated with the IC_{50} concentration of PBEA for 24 h, 48 h and 72 h with 5% CO_2 at 37ºC. Untreated cells were used as the control group. After that, the cells were washed twice with cold PBS and stained with 5µl FITC Annexin V and 5µl propium iodide (PI) for 15 min at room temperature in the dark. Results for the stained cells were obtained (10 000 events per sample) through FACSCANTO II (BD Bioscience). The data acquired were analyzed by using FlowJo_V10 software. The test was repeated thrice independently.

4.2.6(c) Apoptotic proteins expression

The expression of apoptosis proteins such as Bax, Bcl-2, p53 and caspase-3 in HeLa cells following treatment with PBEA were determined by the means of flow cytometry. Cells were incubated with the IC_{50} concentration of PBEA for 24 h, 48 h and 72 h with 5% CO_2 at 37ºC. Untreated cells were used as the control group. The proteins were stained with antibody conjugate according to the manufacturer's protocol. The cells were later harvested and washed with PBS. After that, the cells (1×10^6 cells) were fixed with ice-cold 70% ethanol for 1 hour at 4ºC. The cells were washed twice after fixation and blocked in 2% bovine serum albumin for 10 min at room temperature. After the blocking process, 100 µl of cells (1×10^5 cells) were transferred into flow tubes and stained with antibodies: Bax-PE (Santa Cruz), Bcl-2-Alexa Fluor 647 (Santa Cruz), p53-Alexa Fluor 480 (Santa Cruz) and caspase 3-Alexa Fluor 480 (Santa Cruz). Results for 10, 000 events per sample were recorded with FACSCANTO II (BD Bioscience).

The data were then analysed using FlowJo_V10 software. The experiment was carried out thrice independently.

4.2.7 Statistical analysis

Each data was presented as mean ± standard deviation (SD). The results were analyzed using repeated measure one-way ANOVA ($P<0.05$) and Tukey's multiple comparison test was done to determine the significance between groups. The normality was checked by Shapiro-Wilk test. All statistical analysis was performed with GraphPad Prism 7 software.

4.3 Results

4.3.1 Anti-proliferative activity of *P. bleo* leaves extracts

The anti-proliferative assay was done to evaluate the cytotoxic effects of *P. bleo* leaves extracts on the survival and growth of selected normal and cancer cells. The cell viability measured using MTT assay after 72 h treatment with each extract. The anti-proliferative effects can be determined with the IC_{50} concentration of the extracts where at this concentration has caused the inhibition of 50 % of the cell proliferation. At their IC_{50} concentrations, the plant extracts are considered active and demonstrate cytotoxic activity (Boik, 2001).

The cytotoxic activity of extract can be classified as the following: very active ($IC_{50} \leq 20$ µg/mL), moderately active ($IC_{50} > 20$ - 100 µg/mL), weakly active ($IC_{50} >$ 100 - 1000 µg/mL and inactive ($IC_{50} > 1000$ µg/mL) (Atjanasuppat *et al.*, 2009; Baharum *et al.*, 2014). In addition, the American National Cancer Institute (NCI) has established the cut off IC_{50} value less than 20 µg/ml for the most active extract or pure compound to avoid any undesired adverse effects (Srisawat *et al.*, 2013).

4.3.1(a) Anti-proliferative activity of *P. bleo* leaves extracts obtained *via* maceration extraction against selected cancer and normal cell lines

The MTT assay was carried out to determine the effect of *P. bleo* leaves extracts obtained *via* maceration on HeLa, MDA-MB-231, SW480 and NIH/3T3 cell lines. The maximum concentration of the extracts used were 99 µg/ml as stated in Table 4.1. However, there was no IC_{50} recorded at this maximum concentration, thus the maximum concentration of the extracts was increased to 990 µg/ml.

The results showed high percent of viability values (86 – 118%) in all cell types at the lowest concentration (4 µg/ml) of each *P. bleo* extract (hexane, ethyl acetate and methanol) after 72 h incubation period. The value of cell viability was low (5 – 19%) at the highest concentration (990 µg/ml) of *P. bleo* extracts in all cell types after 72 h of treatment except for methanol extract in SW480 cells which showed high percentage (56%). These results indicated that all extracts of *P. bleo* leaves have significantly reduced cell viability in HeLa (Figure 4.1), MDA-MB-231 (Figure 4.2), SW480 (Figure 4.3) and NIH/3T3 (Figure 4.4) after 72 h treatment ($P<0.05$). Tamoxifen was used as the positive control in this study (Figure 4.5).

Table 4.2 presented IC_{50} values of all extracts from maceration extraction towards all tested cell lines. Hexane and methanol extracts showed weak cytotoxic effect on normal cells and all cancer cells with IC_{50} values above 100 µg/ml. Ethyl acetate extract demonstrated very active cytotoxic effect on HeLa and MDA-MB-231 with IC_{50} values of 17.51 ± 8.6 µg/ml and 19.39 ± 1.26 µg/ml respectively. Meanwhile, SW480 showed moderate cytotoxic effect (IC_{50} value = 31.80 ± 16.1 µg/ml) and low cytotoxic effects at IC_{50} value of 182.0 ± 23.0 µg/ml on NIH/3T3 cells. The current findings showed that ethyl acetate extract obtained *via* maceration method possessed the potent cytotoxic effects against HeLa and MDA-MB-231.

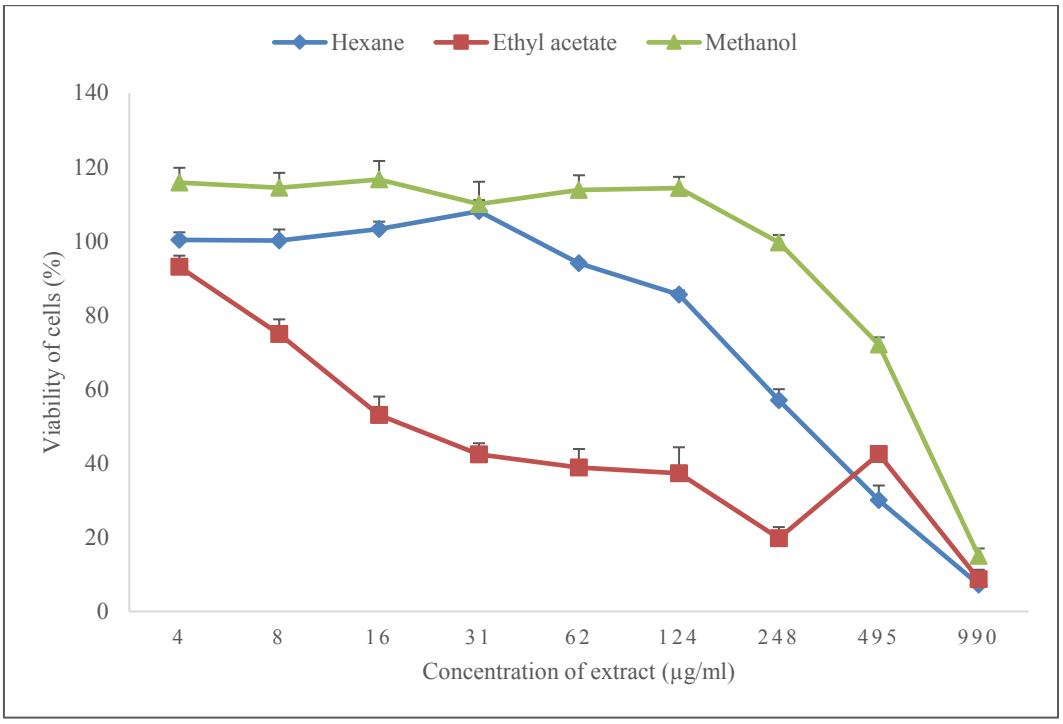


Figure 4.1 Representative graphs of the percentage of cell viability for HeLa after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (maceration extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

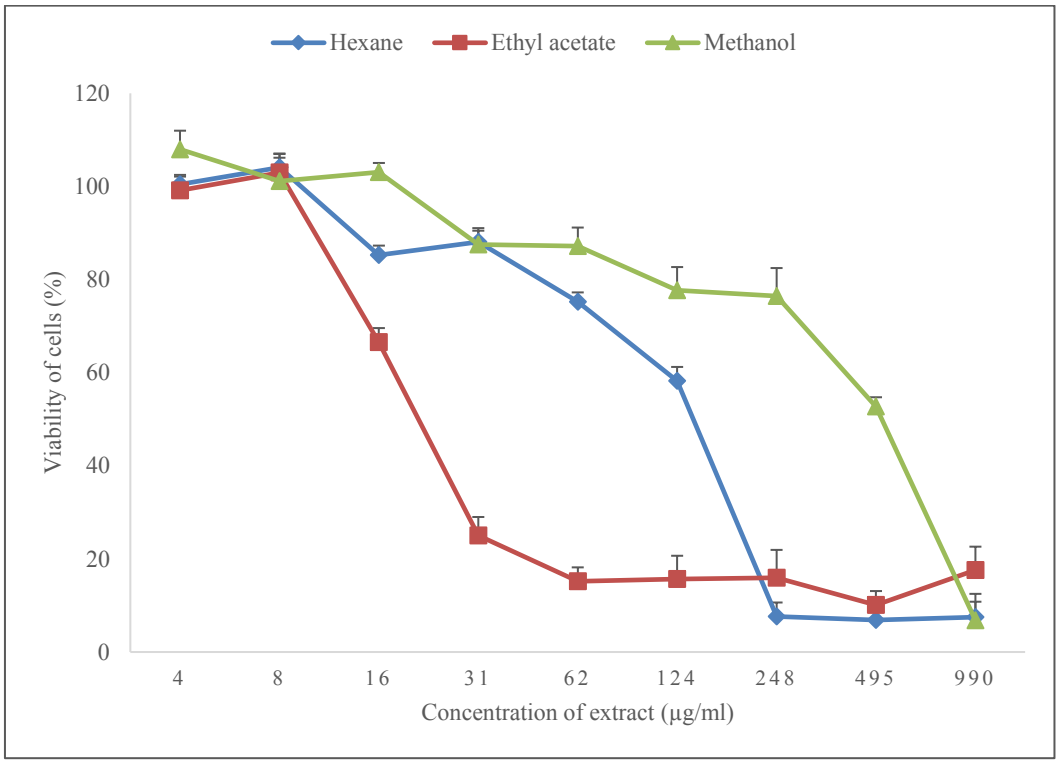


Figure 4.2 Representative graphs of the percentage of cell viability for MDA-MB-231 after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (maceration extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

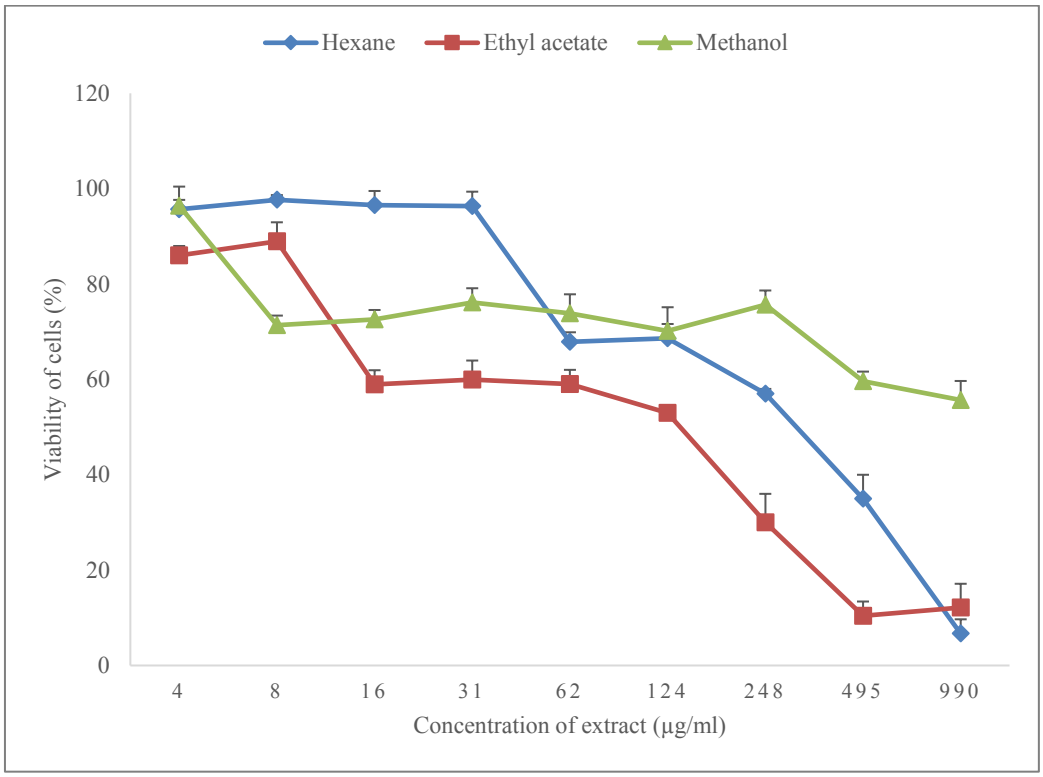


Figure 4.3 Representative graphs of the percentage of cell viability for SW480 after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (maceration extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

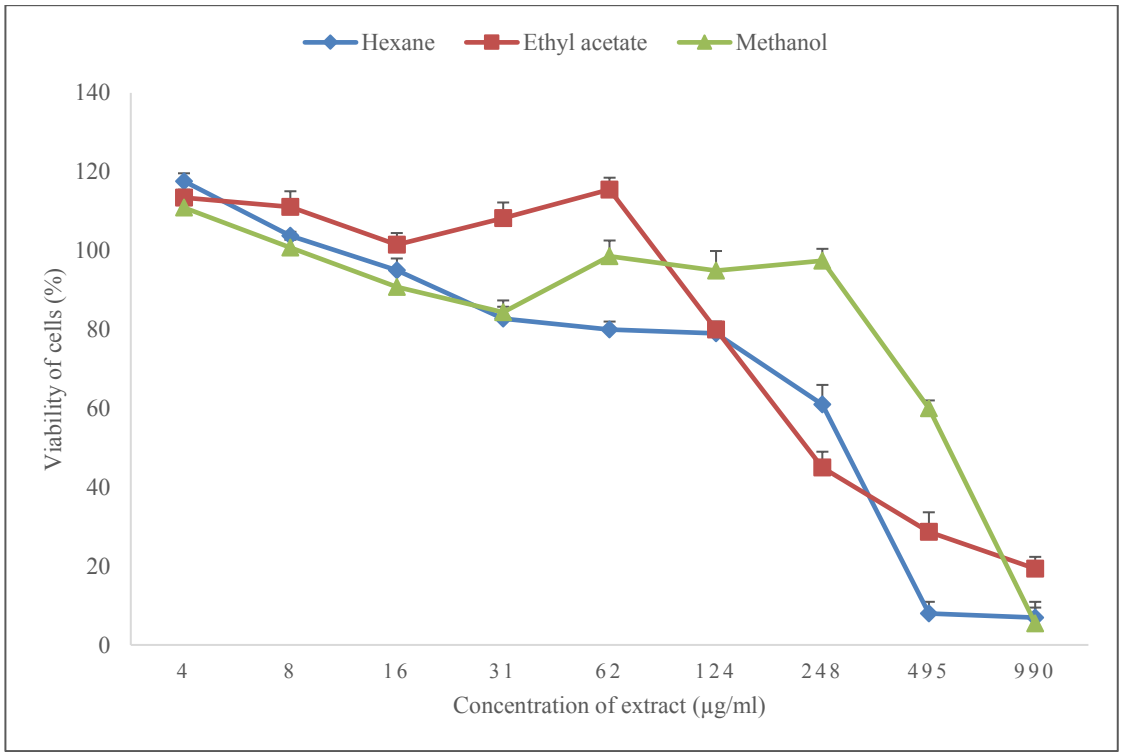


Figure 4.4 Representative graphs of the percentage of cell viability for NIH/3T3 after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (maceration extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

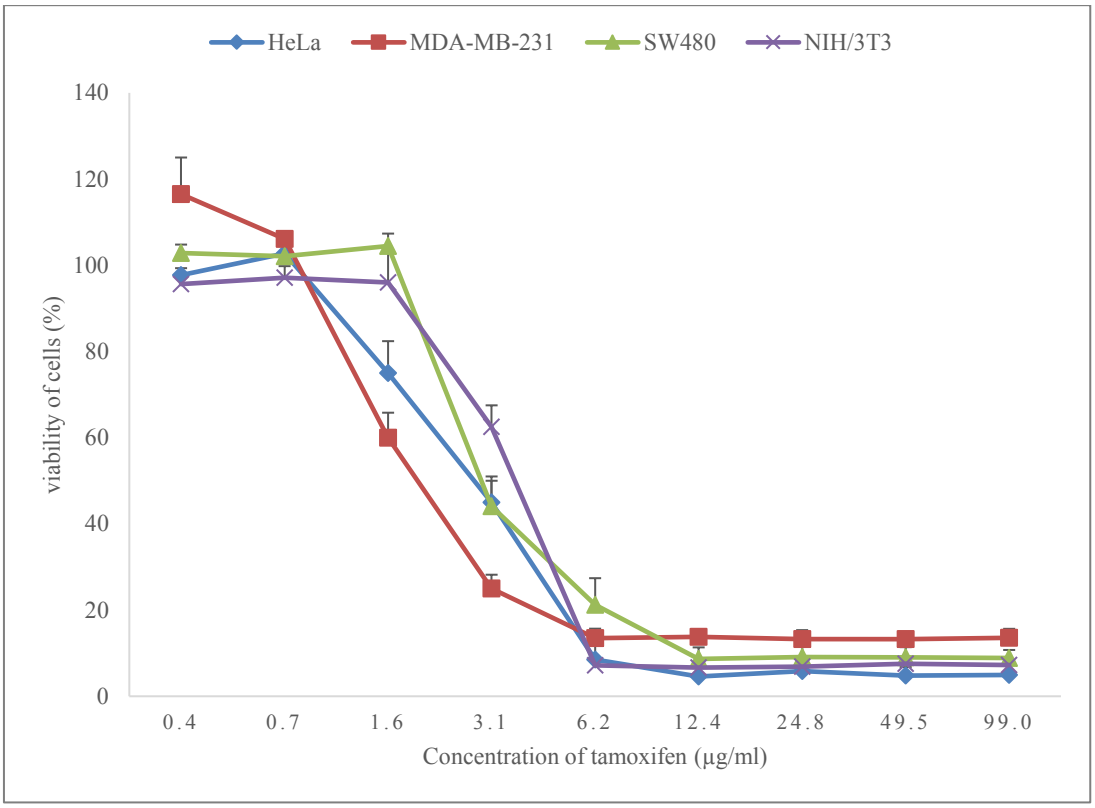


Figure 4.5 Representative graphs of the percentage of cancer and normal cells viability treated with various concentration of tamoxifen (positive control) for 72 h. Data were represented as mean ± SD from three independent experiments.

4.3.1(b) Anti-proliferative activity of *P. bleo* leaves extracts obtained *via* Soxhlet extraction against selected cancer and normal cell lines

Cell viability after 72 h treatment with all extracts obtained *via* Soxhlet method was measured using the MTT assay to explore the effect of the extracts on the selected normal and cancer cells. High percentage values in cell viability (92 – 129%) was observed in HeLa cells (Figure 4.6), MDA-MB-231 cells (Figure 4.7), SW480 (Figure 4.8) and NIH/3T3 (Figure 4.9) after 72 h treatment at the lowest concentration (0.4 µg/ml) of hexane, ethyl acetate and methanol extract. Treatment with hexane and ethyl acetate extracts at the highest concentration (99 µg/ml) showed low percent of viability (13 – 28 %) in HeLa and MDA-MB-231 cells (Figure 4.6 and 4.7). However, high percentage of cell viability (80 – 121%) was observed in SW480 and NIH/3T3 after 72 h incubation with the highest concentration of these extracts. Treatment with methanol extract at the highest concentration showed high values of cell viability (67 – 111%) in all cell types.

The results showed hexane and ethyl acetate extracts have significantly reduced the viability of HeLa and MDA-MB-231 cell after 72 h of treatment (P<0.05). Treatment with hexane and ethyl extract also showed reduction of cell viability in SW480 and NIH/3T3 cells (Figure 4.8 and 4.9) with no statistical differences. Methanol extract slightly reduced the viability in all cell types with no statistical differences. All cells were treated with tamoxifen as the positive control (Figure 4.10).

The summary IC_{50} values of all extracts and tamoxifen were shown in Table 4.2. Ethyl acetate showed very active cytotoxic effect on HeLa cells (IC_{50} value =

14.37±8.40 µg/ml) while moderate cytotoxicity was observed in MDA-MB-231 cells (IC_{50} value = 41.60±35.68 µg/ml). Hexane extract demonstrated moderate cytotoxic effects on HeLa cells (IC_{50} value = 20.24±11.69 µg/ml) and MDA-MB-231 (IC_{50} value = 43.76±36.86 µg/ml). On the other hand, both extracts showed weak cytotoxic effects on SW480 cells. Methanol extract also showed weak cytotoxic effects on all cancer cell lines. In addition, all extracts exerted low cytotoxic effects on normal cells NIH/3T3. Thus, our findings suggested that ethyl acetate extract from Soxhlet extraction exhibited the potent cytotoxic effects on HeLa cells.

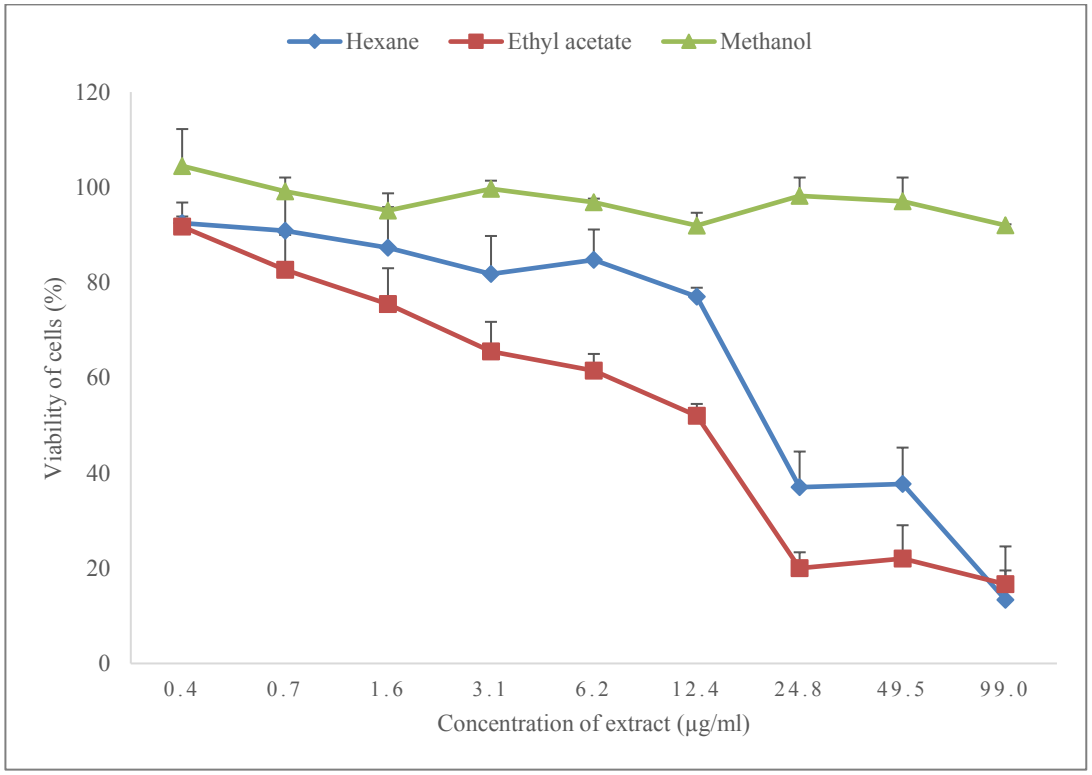


Figure 4.6 Representative graphs of the percentage of cell viability for HeLa after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (Soxhlet extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

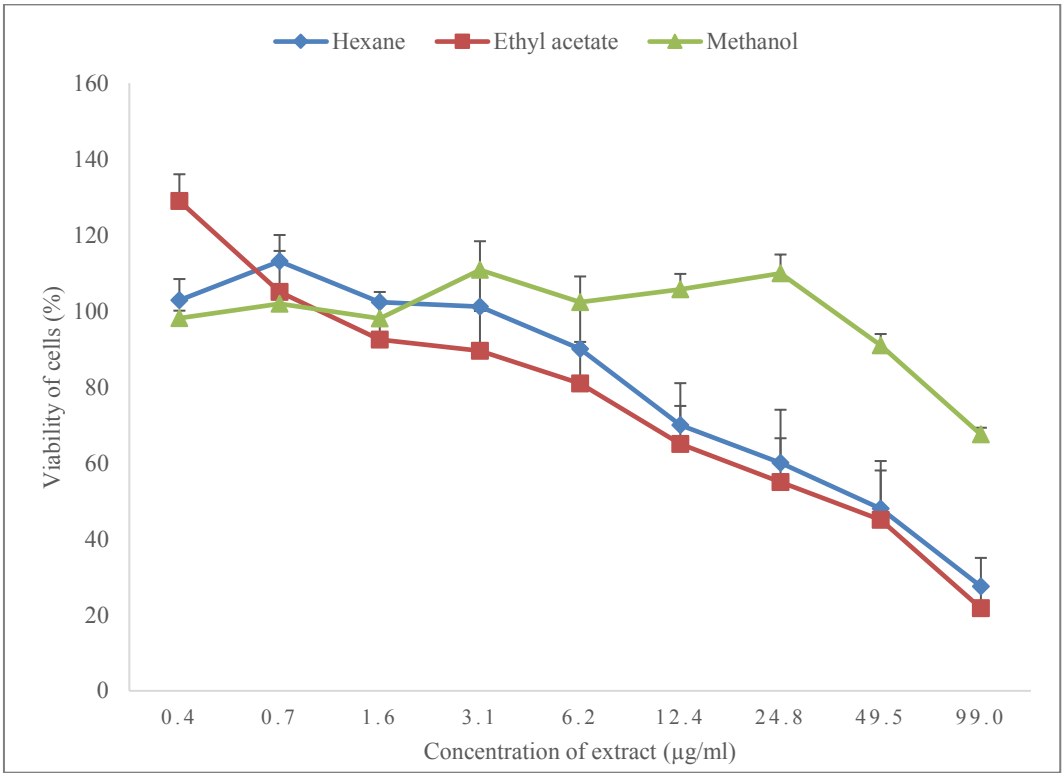


Figure 4.7 Representative graphs of the percentage of cell viability for MDA-MB-231 after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (Soxhlet extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

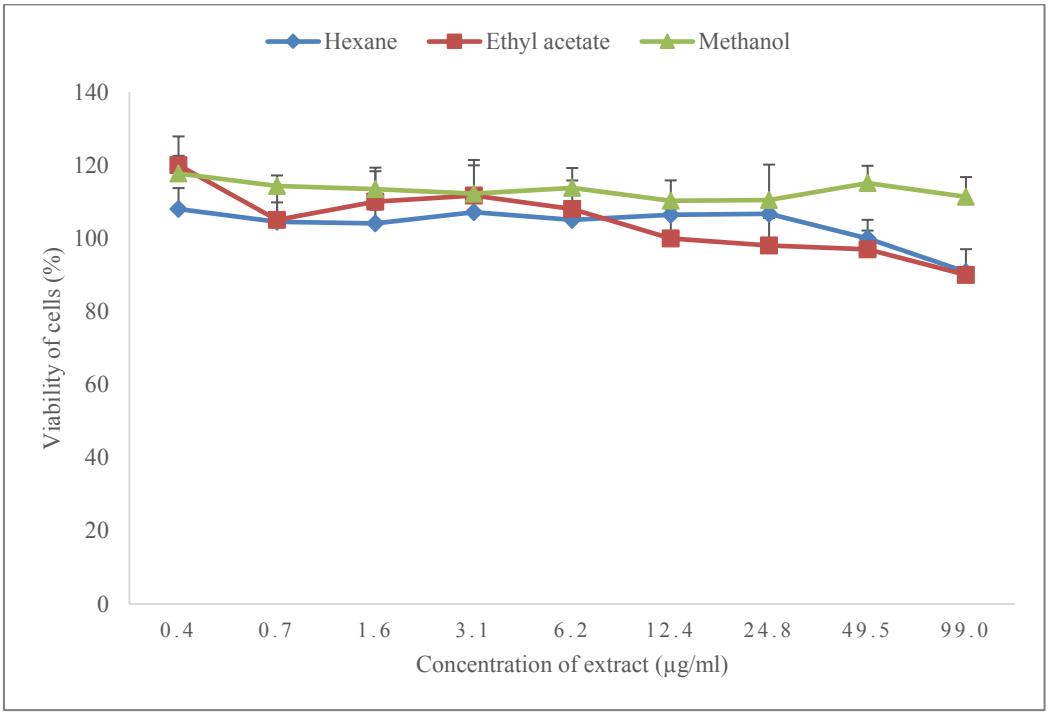


Figure 4.8 Representative graphs of the percentage of cell viability for SW480 after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (Soxhlet extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

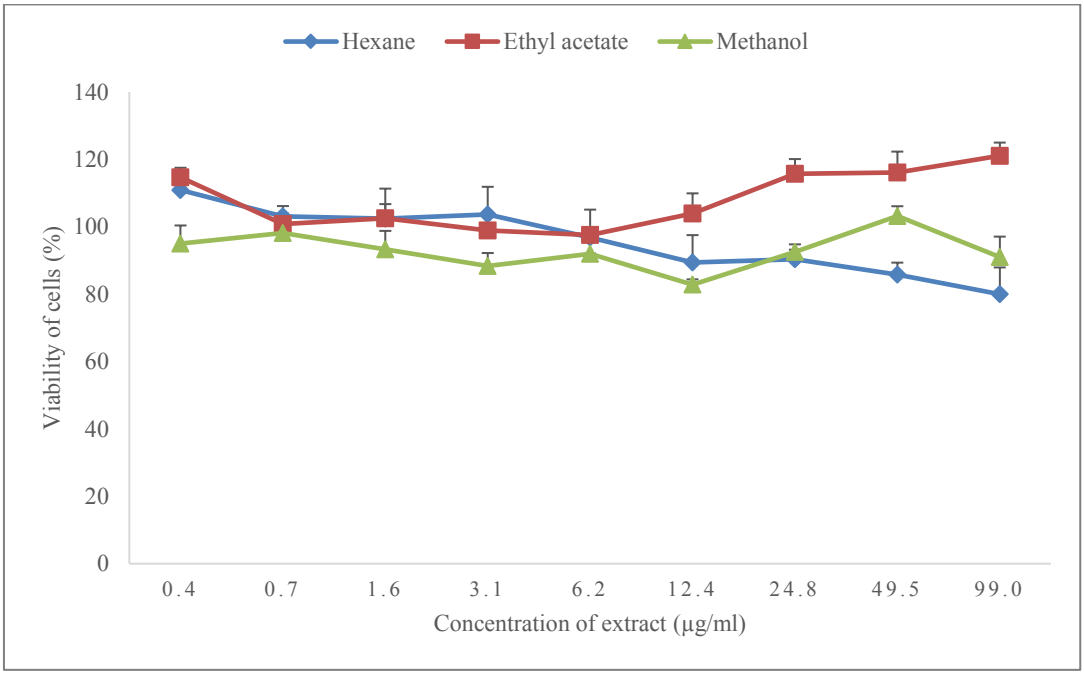


Figure 4.9 Representative graphs of the percentage of cell viability for NIH/3T3 after 72 h treatment with hexane, ethyl acetate and methanol extracts of *P. bleo* leaves (Soxhlet extraction) at various concentration. Data were represented as mean ± SD from three independent experiments.

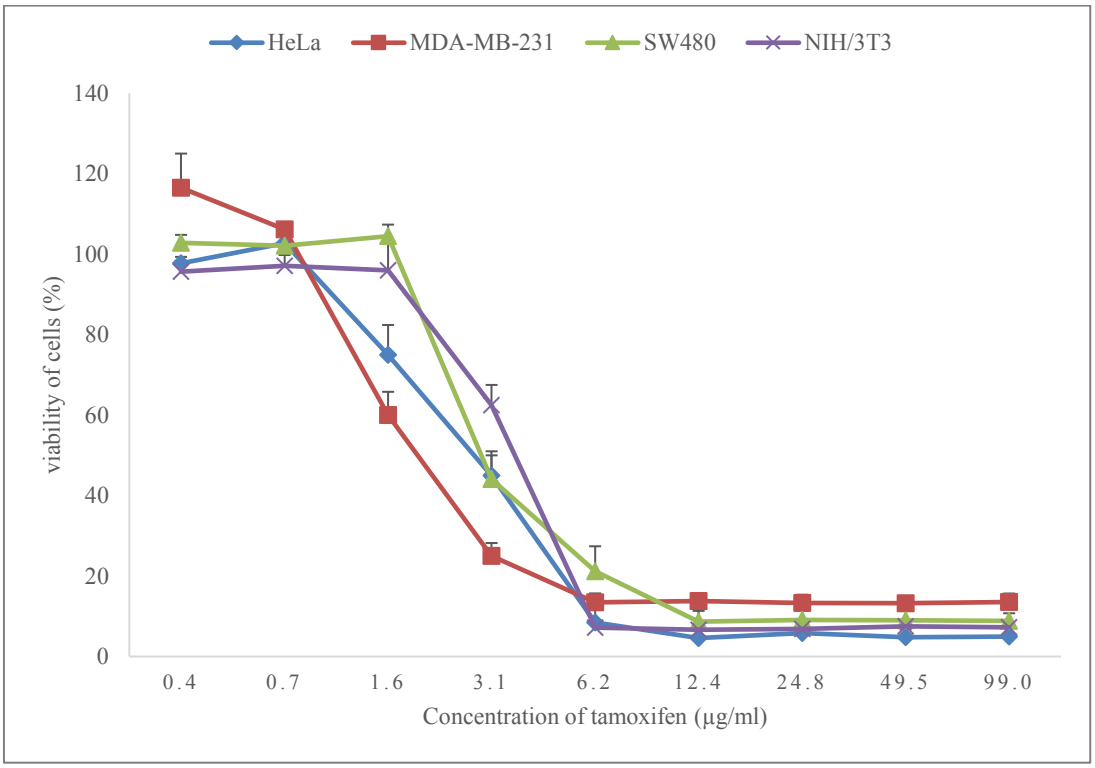


Figure 4.10 Representative graphs of the percentage of cell viability for cancer and normal cells viability treated with various concentration of tamoxifen (positive control) for 72 h. Data were represented as mean ± SD from three independent experiments.

4.3.1(c) Anti-proliferative activity of *P. bleo* leaves' aqueous extract obtained *via* decoction extraction against selected cancer and normal cell lines

There was no IC_{50} value detected for the treatment with the aqueous extract of *P. bleo* leaves within the range of tested concentration, which was more than 99 µg/ml**.** Therefore, the concentration was increased to the maximum 990 µg/ml**.** Treatment at the lowest concentration of aqueous extract (4 µg/ml) showed high percent values of cell viability (94 – 118%) in all cell types meanwhile low percent of cell viability (8 – 15%) observed in all cell types at the highest concentration of extract (990 µg/ml) after 72 h incubation.

The results showed the cell viability of cancer (HeLa, MDA-MB-231, SW480) and normal NIH/3T3 cells were significantly reduced after 72 h treatment with this extract as illustrated in Figure 4.11 ($P<0.05$). However, this extract exerted weak cytotoxic effects towards normal and all cancer cells with IC_{50} values of more than 100 µg/ml (Table 4.2).

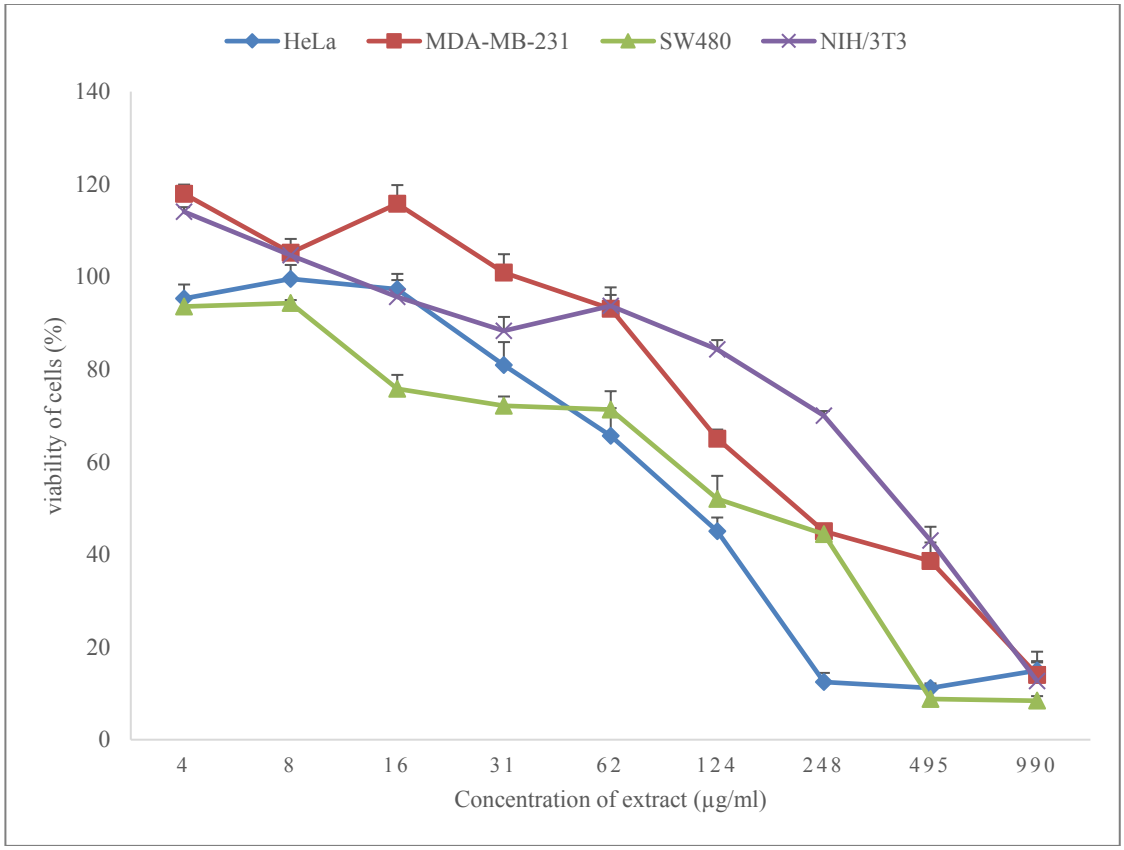


Figure 4.11 Percentage of cancer (HeLa, MDA-MB-231 and SW480) and normal cells NIH/3T3 after treatment with aqueous extract of *P. bleo* leaves at various concentration. Data were represented as mean ± SD from three independent experiments.

Table 4.2 IC_{50} values of cytotoxic effects from *P. bleo* leaves crude extracts against cancer (HeLa, MDA-MB-231, SW480) and NIH/3T3 normal cell lines.

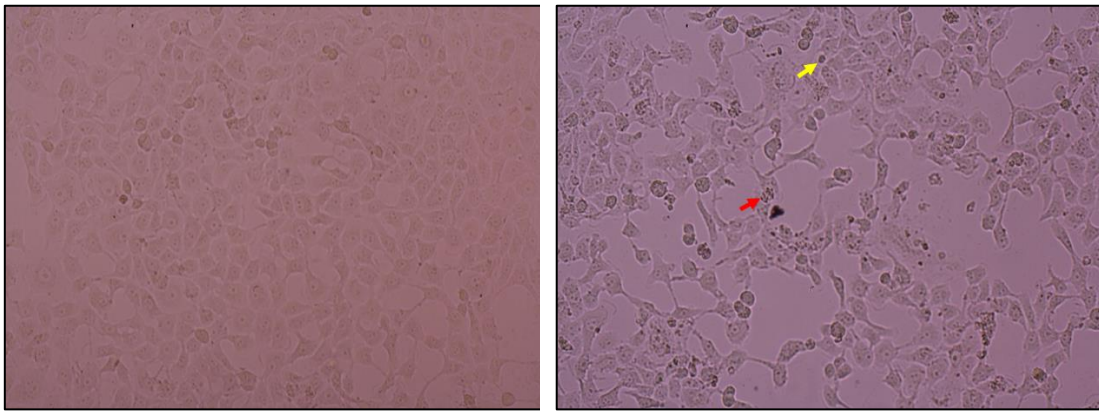
Type of extract	**IC_{50} values (µg/ml)**			
	HeLa	**MDA-MB-231**	**SW480**	**NIH/3T3**
Maceration				
Hexane	278.01 ± 12.8	95.75 ± 27.9	154.0 ± 2.0	275.0 ± 16.0
Ethyl acetate	17.51 ± 8.6	19.39 ± 1.26	31.80 ± 16.1	182.0 ± 23.0
Methanol	683.47 ± 15.7	213.23 ± 27.7	> 990	631.0 ± 22.0
Soxhlet				
Hexane	20.24±11.69	43.76±36.86	>99	>99
Ethyl acetate	14.37±8.40	41.60±35.68	>99	>99
Methanol	>99	>99	>99	>99
Decoction				
Aqueous	100.40 ± 2.3	224.31 ± 25.6	128.2 ± 7.5	359.5 ± 27.5
Tamoxifen	2.71 ± 0.88	2.24 ± 0.95	2.66 ± 0.22	3.78 ± 1.46

4.3.2 Morphological assessment of apoptotic HeLa cells induced by PBEA

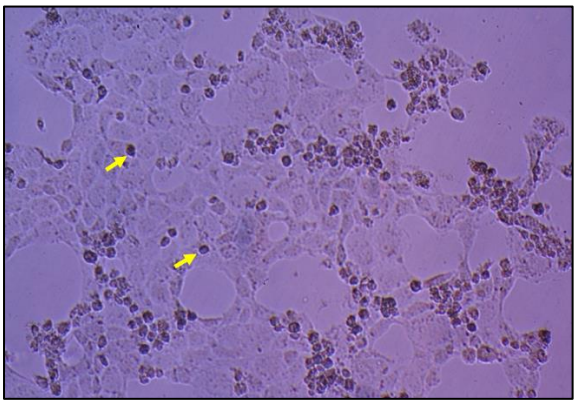
4.3.2(a) Bright field inverted microscopy

Apoptotic cells display characteristics such as membrane blebbing, cell shrinkage, condensation of chromatin and fragmented nuclei. The changes on cell morphology were visualized using a bright field microscope to determine the effect of PBEA on HeLa cells.

In comparison to control cells (untreated), morphological alterations in HeLa cells were observed after 24 h, 48 h and 72 h treatment with PBEA as shown in Figure 4.12, Figure 4.13 and Figure 4.14 respectively. Untreated cells maintained their morphology with rounded nuclei and attached to the surface of the tissue culture flask. In contrast, cells treated with PBEA showed typical apoptotic features such as nuclei fragmentation, condensed chromatin and shrinkage of cells. In addition, the viability of cells was significantly reduced after 72 h incubation with PBEA along with the appearance of rounding and losing contact with adjacent cells. Thus, our findings suggested HeLa cells underwent apoptosis after they were treated with PBEA.

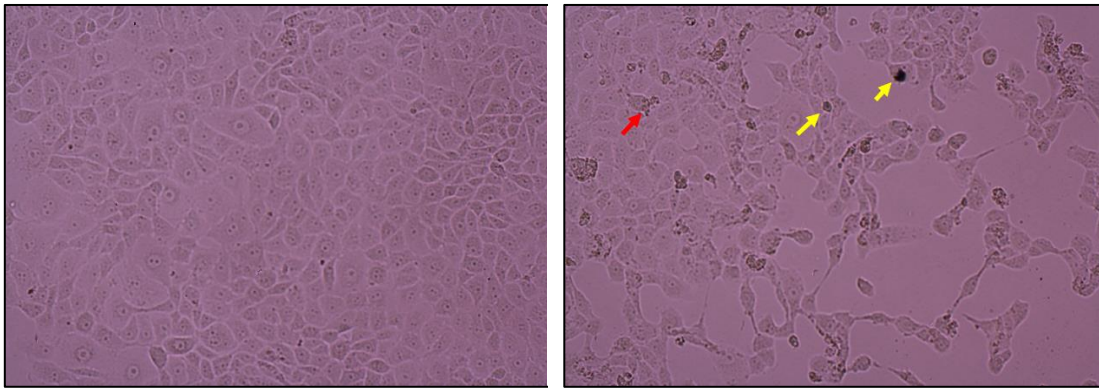


(i) (ii)

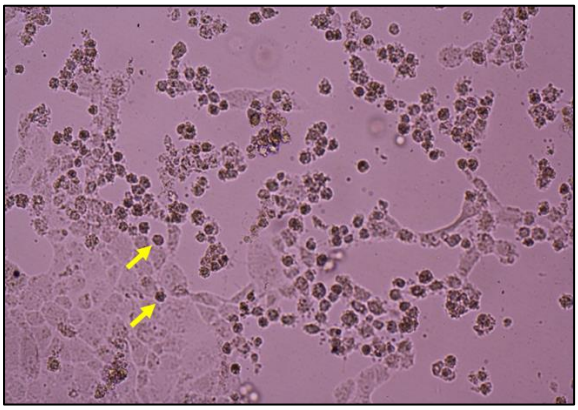


(iii)

Figure 4.12 Morphology of HeLa cells. (i) untreated 24 h, for 24 h treatment with PBEA (ii) and tamoxifen (iii). Apoptotic HeLa cells were indicated by condensation of chromatin (yellow arrow) and fragmented nuclei (red arrow). Bar scales represent 50 μm at 40 × magnification.



(i) (ii)



(iii)

Figure 4.13 Morphology of HeLa cells. (i) untreated 48 h, for 48 h treatment with PBEA (ii) and tamoxifen (iii). Apoptotic HeLa cells were indicated by condensation of chromatin (yellow arrow) and fragmented nuclei (red arrow). Bar scales represent 50 μm at 40 × magnification.

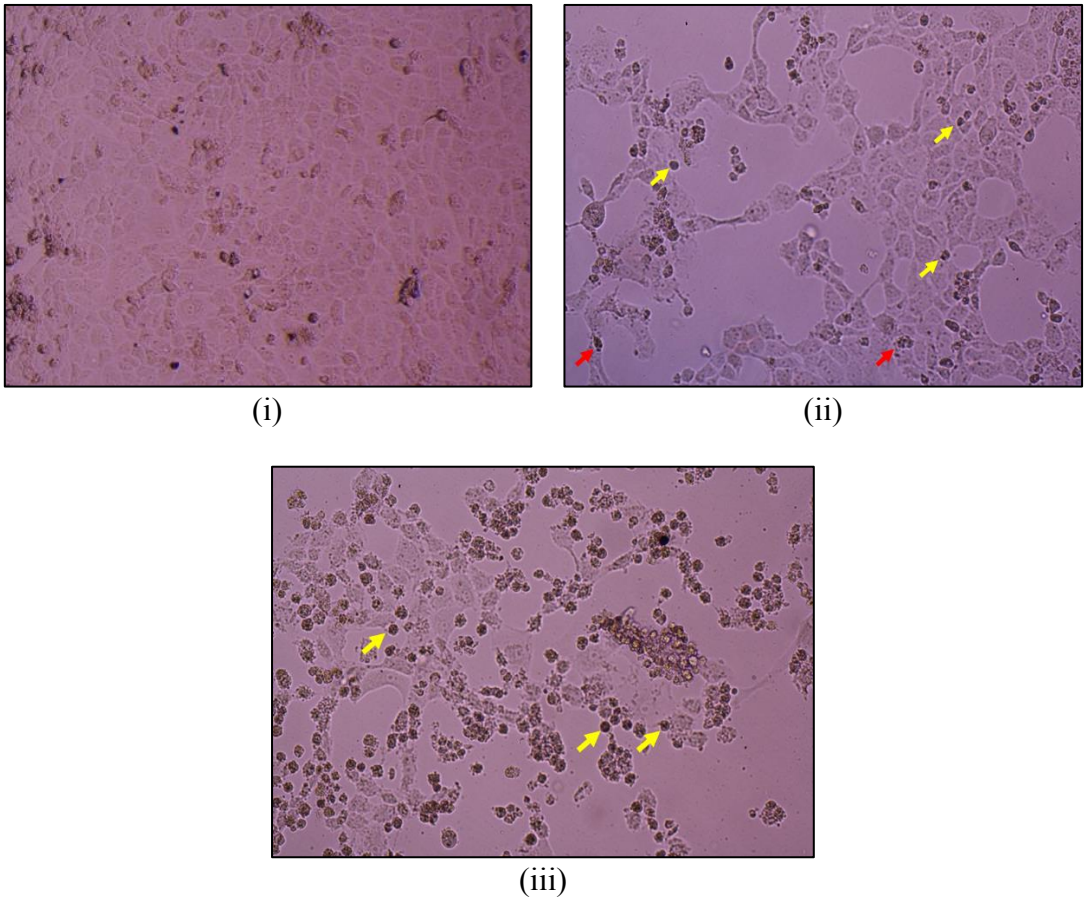


Figure 4.14 Morphology of HeLa cells. (i) untreated 72 h, for 72 h treatment with PBEA (ii) and tamoxifen (iii). Apoptotic HeLa cells were indicated by condensation of chromatin (yellow arrow) and fragmented nuclei (red arrow). Bar scales represent 50 μm at 40 × magnification.

4.3.2(b) Fluorescence microscopy

Apart from the bright field microscopy, apoptotic features can be observed *via* fluorescence microscopic examination using Hoechst 33258 staining. Our findings showed that PBEA treated HeLa cells demonstrated typical features of apoptosis such as condensed chromatin and fragmented nuclei when compared to control cells as shown in Figure 4.15. Control cells that were untreated exhibited rounded and intact nuclei with lighter blue staining as presented in Figure 4.15(i). In addition, the cells were attached to the tissue culture flask and in contact with the adjacent cells. On the other hand, the nuclei of apoptotic cells after treatment with PBEA were highly condensed, fragmented and in the form of crescents indicated by brighter blue fluorescence staining (Figure 4.15ii). After 48 h and 72 h incubation, PBEA treated HeLa cells started to lose their shape and lost in contact with the adjacent cells (Figure 4.15iii and Figure 4.15iv). Observation of cell death using Hoechst staining showed that PBEA induced apoptosis of HeLa cells at a concentration of 14.37 µg/ml.

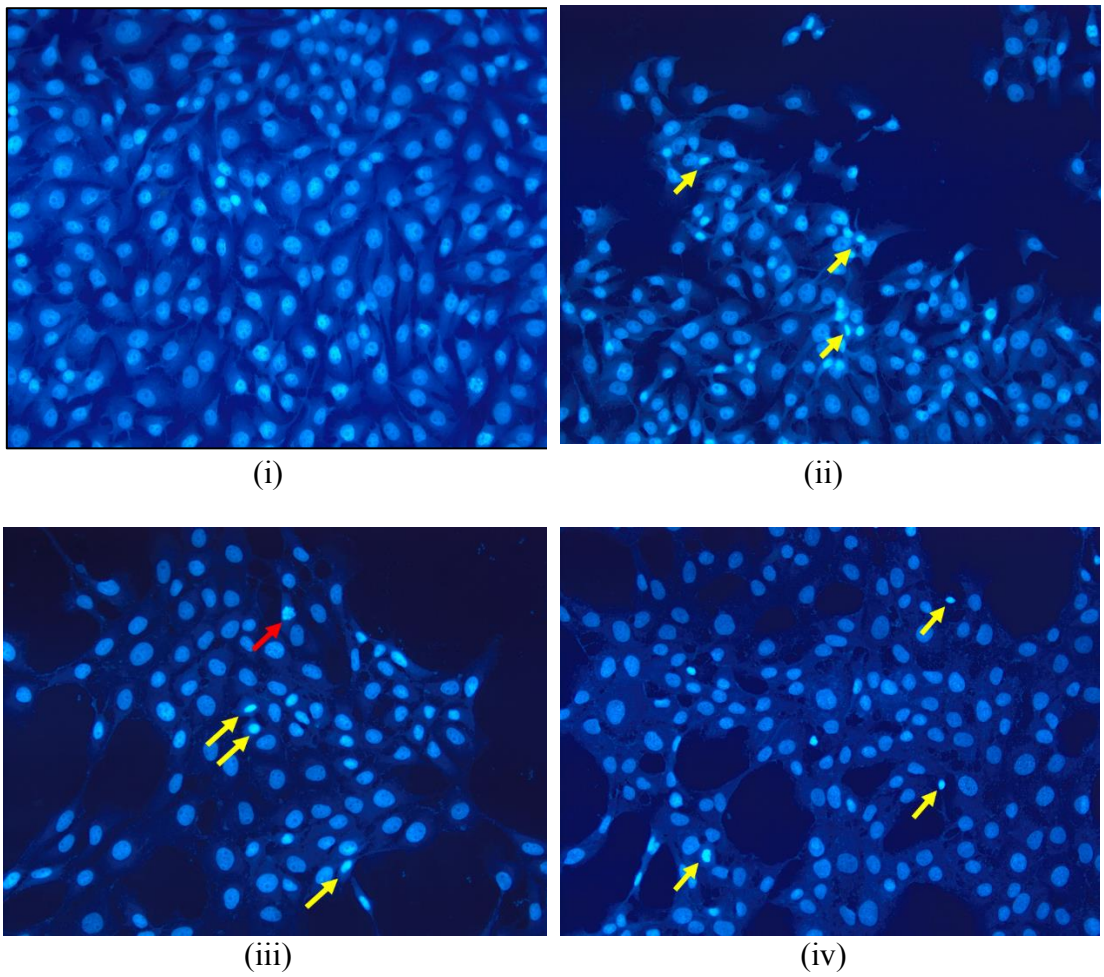


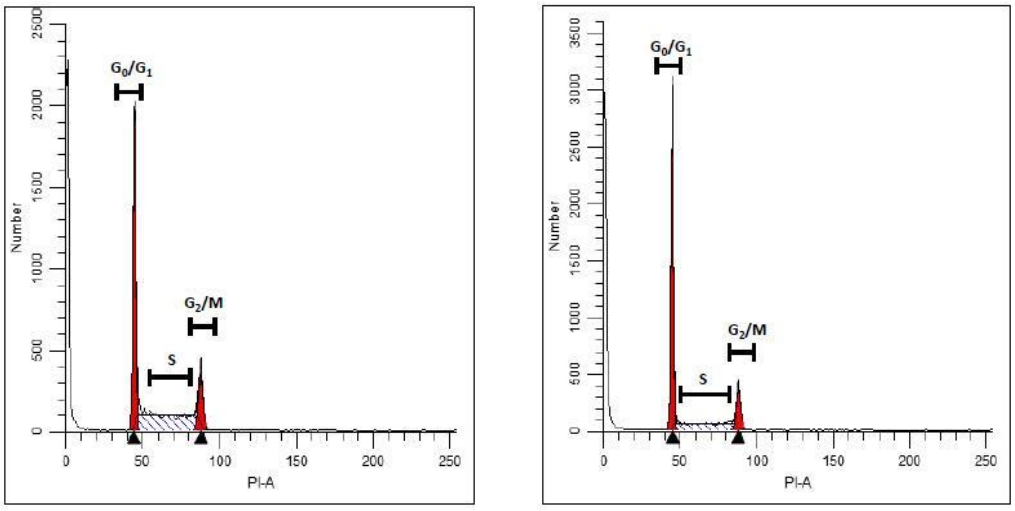
Figure 4.15 Morphology of HeLa cells stained with Hoechst. (i) control cells (ii) 24 h treatment with PBEA, (iii) 48 h treatment with PBEA and (iv) 72 h treatment with PBEA. Apoptotic HeLa cells were indicated by condensation of chromatin (yellow arrow) and fragmented nuclei (red arrow). Bar scales represent 50 μm at 40 × magnification.

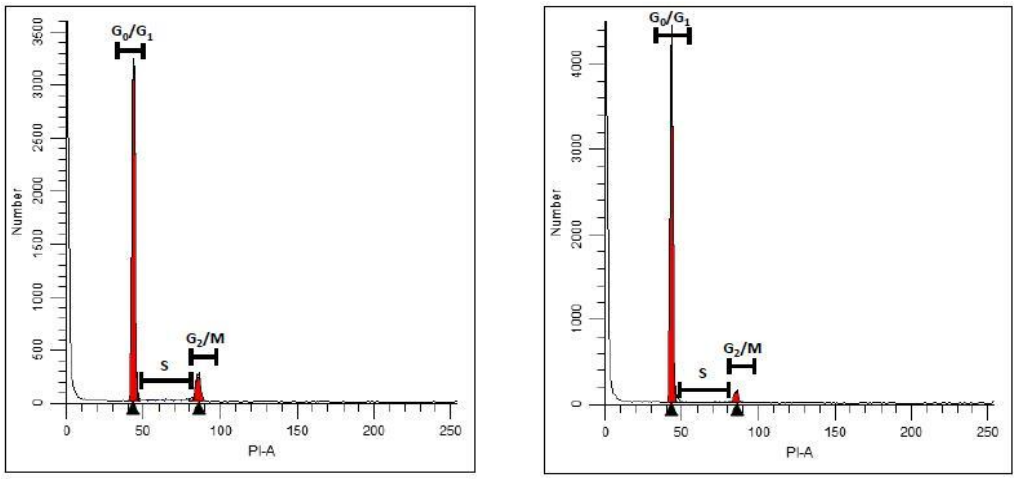
4.3.3 Cell death in HeLa cells induced by PBEA

4.3.3(a) Cell cycle assay

The effects of PBEA on cell cycle distribution in HeLa cells were evaluated by the analysis of cell cycle arrest using flow cytometry method. Cell cycle arrest analysis is based on the measurement of DNA content in the cells following PI staining (Wang *et al.*, 2011). The inhibition of cell proliferation (arrest of cells) is indicated by the accumulation of cells stained with PI at certain phases in the cell cycle (Cecchini *et al.*, 2012). Cells treated with the IC_{50} value of PBEA for 24 h, 48 h and 72 h were analyzed for the distribution in G_0/G_1, S and G_2/M phases of the cell cycle (Figure 4.16(i)).

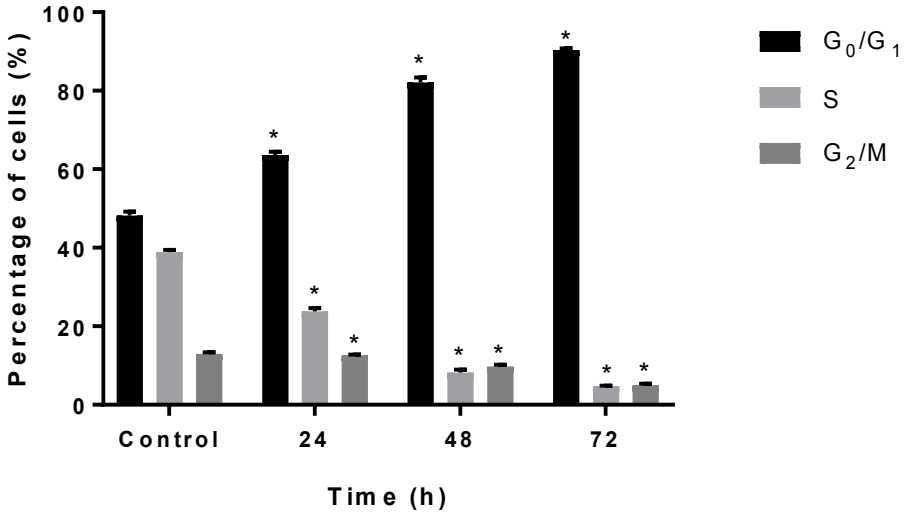
Figure 4.16(ii) shows the number of HeLa cells in G_0/G_1 phase were increased by 90 ± 0.38% after 72 h of treatment with PBEA, compared with control cells (P<0.05). The number of cells in S and G_2/M phases after 72 h of treatment with PBEA decreased to 5 ± 0.85% and 5 ± 1.03%, respectively, compared with control cells (P<0.05). Accumulation of cells at G_0/G_1 phase indicated the cell proliferation was halted at this phase. This showed that PBEA induced changes in the cell cycle progression of HeLa cells by causing the arrest of G_0/G_1 phase.





(i)

Figure 4.16 The effects of PBEA on the cell cycle of HeLa cells. (i) The flow cytometry histogram shows the DNA content and the corresponding percentage of cell distribution in the control and treated HeLa cells after 24 h, 48 h and 72 h. The distribution of cell cycle showed the accumulation of treated cells in the G_0/G_1 phase. (ii) The bar columns wer represented as mean±SD from three independent experiments. *$P<0.05$ when compared with the control group.



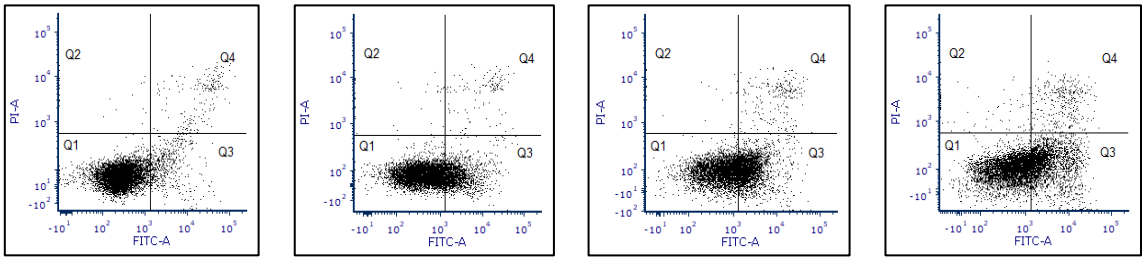
(ii)

Figure 4.16 Continue

4.3.3(b) Annexin V-FITC assay

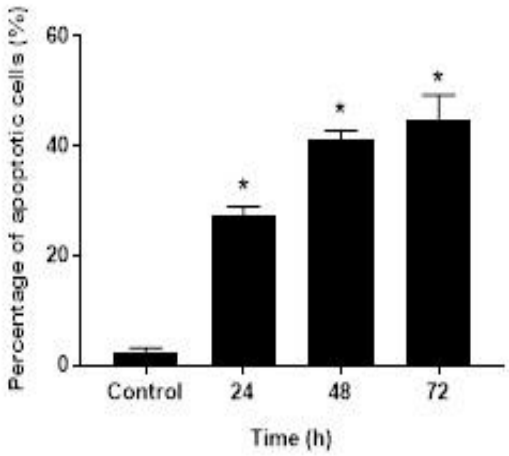
In order to identify whether or not the cells underwent apoptosis, control and treated HeLa cells with PBEA were stained with Annexin V (FITC) and PI to measure the presence of apoptotic cells. A negative result for both Annexin V and PI negative indicates viable cells. Meanwhile, V positive and PI negative is a sign of cells in the early apoptotic stage while cells that Annexin V and PI positive are in the late stage of apoptosis. Annexin V negative and PI positive shows necrotic cells. Apoptosis occurred in HeLa cells within 72 h upon treatment with PBEA as per flow cytometric analysis result as shown in Figure 4.17. The percentage of apoptotic cells measured after 24 h treatment with PBEA was 27.39±1.62 % while 40.96±1.8 % and 44.82±4.44 % after 48 h and 72 h of treatments respectively (Figure 4.17(ii)). The percentage of apoptotic HeLa cells increased after the treatment with PBEA. The apoptosis rate was

significantly different for every incubation period in treated groups compared to the control group (P<0.05).



Control **24 h** **48 h** **72 h**

(i)

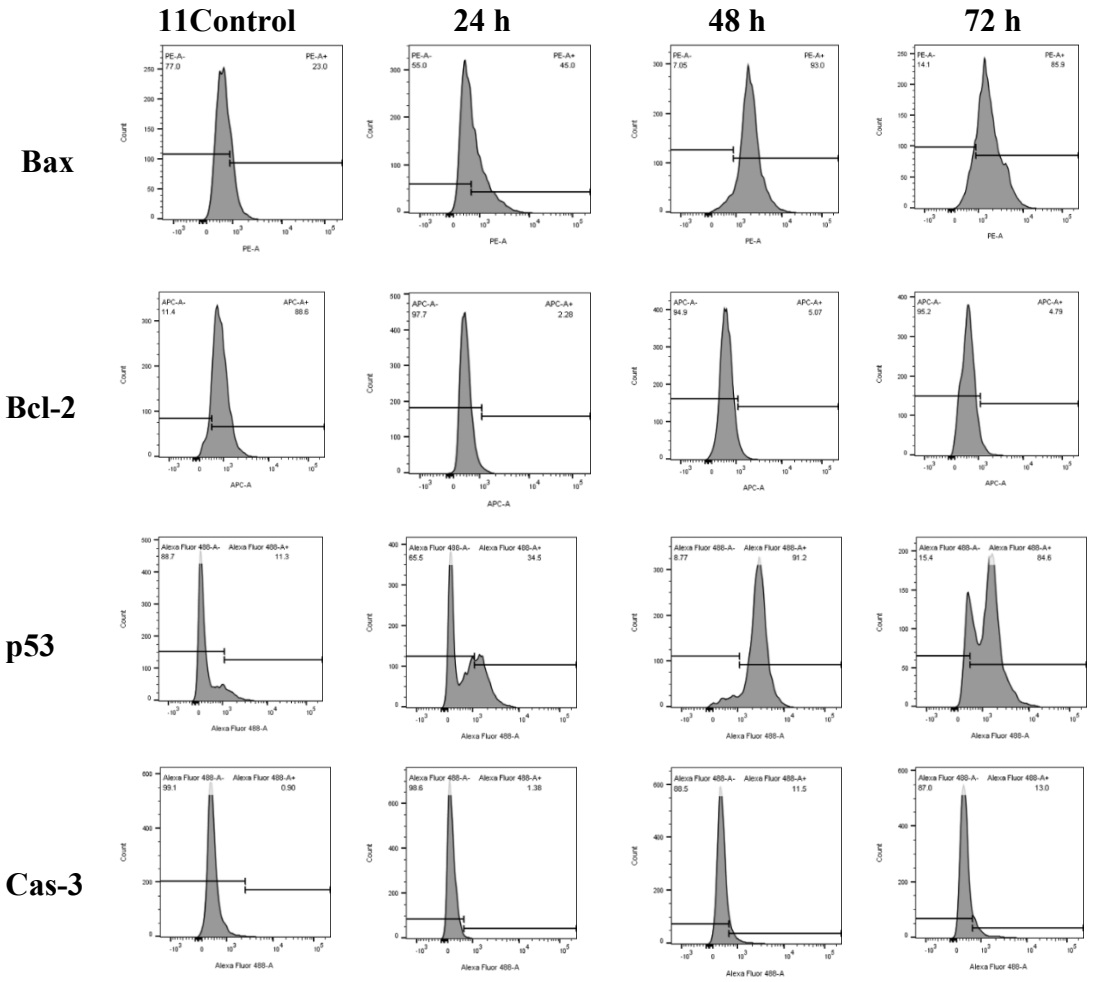


(ii)

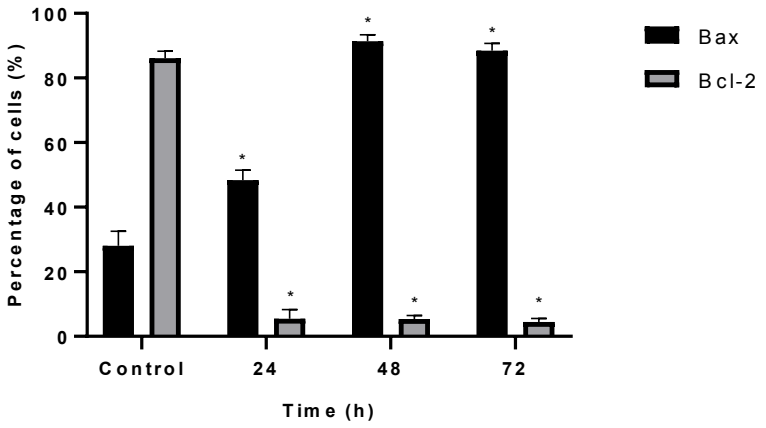
Figure 4.17 (i) Distribution of Annexin V-FITC staining dot plots in control and PBEA treated HeLa cells detected by flow cytometry after 24 h, 48 h and 72 h incubation. The four quadrants represent viable cells (Q1), necrotic cells (Q2), early apoptotic cells (Q3) and late apoptotic cells (Q4). (ii) The bar column illustrated the percentage of HeLa cells undergoing apoptosis. The data represent the mean±SD of three independent experiments. *P<0.05 when compared with the control group.

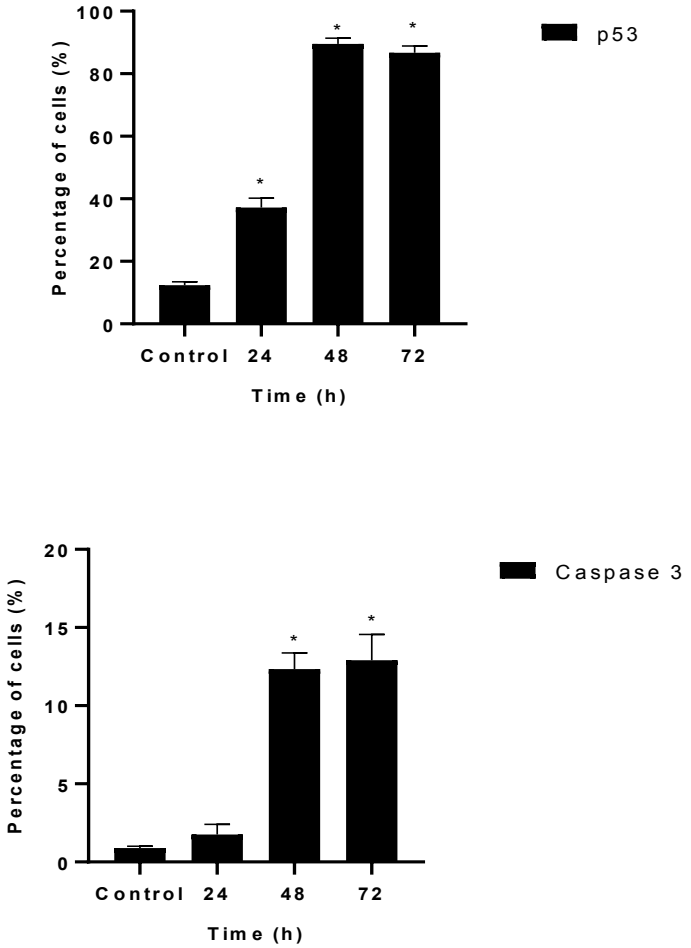
4.3.3(c) Apoptotic proteins expression

Apoptotic proteins (Bax, Bcl-2, p53 and caspase-3) expression levels in HeLa cells following treatment with 14.37±8.40μg/ml of PBEA were assessed by flow cytometry analysis (Figure 4.18). Figure 4.18(ii) shows the expression level of apoptotic protein Bax and p53 was upregulated to 65 % and 75 % respectively following the 72 h treatment period. Meanwhile, the level of anti-apoptotic protein Bcl-2 was downregulated (5 %) (Figure 4.18(ii)). There were significant differences observed for the expression levels of Bax, p53 and Bcl-2 treated and the control cells (P<0.05). Even though there was only a slight upregulation in the expression of caspase-3 (10 %), the results remain significantly different between treated and the control cells (P<0.05).


11Control
24 h
48 h
72 h
Bax
Bcl-2
p53
Cas-3

(i)


Percentage of cells (%)
100
80
60
40
20
0
Control
24
48
72
Time (h)
Bax
Bcl-2



(ii)

Figure 4.18 Flow cytometry analysis of apoptosis proteins expression in HeLa cells induced by PBEA. (i) Histograms of apoptosis protein Bax, Bcl-2, p53 and caspase-3 (cas-3) expression level measured in HeLa cells for control and treated with PBEA after 24 h, 48 h and 72 h incubation. (ii) The bar columns showed the percentage of apoptosis proteins expression in control and HeLa cells treated with PBEA. PBEA significantly upregulated expression of Bax, p53 and caspase-3, whereas Bcl-2 was downregulated. The data was shown as mean ± SD which represent three independent experiments. **P*<0.05 when compared with the control group.

4.4 Discussion

The search for anticancer agents from botanical sources has been gaining popularity worldwide due to their potential in arresting cancer cell proliferation with minimal side effects and easily accessible. At present, a large number of active compounds from natural sources such as herbs and medicinal plants have been isolated and developed as new drugs for cancer treatment (Wang *et al.*, 2013). *P. bleo* leaves is one of the important sources of medicine and traditionally used to treat various ailments such as haemorrhoid, hypertension, cancer, diabetes, infections, headaches, rheumatism, asthma as well as a health supplement (Er *et al.*, 2007; Malek *et al.*, 2009; Sim *et al.*, 2010b; Sri Nurestri *et al.*, 2008). Previous studies commonly used methanol crude extract and fractions for its anti-cancer study (Malek *et al.*, 2009; Tan *et al.*, 2005). In this study, a few solvents were utilized besides methanol which includes hexane, ethyl acetate and the aqueous solution obtained *via* conventional extraction methods to determine the cytotoxic effects of *P. bleo* leaves crude extracts on several common cancer cell lines such as HeLa, MDA-MB-231 and SW480 that have not been reported.

Two conventional successive extraction methods known as Soxhlet and maceration were used in this study. The different between these two methods are the presence of heat and time. Soxhlet method utilizes the heating during extraction with a shorter extraction time while maceration involves prolong soaking of the plant materials in the solvents at room temperature.

Pharmacology screening of plants is essential in the search for novel and effective drugs with minimal side effects (Kuete et al., 2013). National Cancer Institute (NCI), USA has established IC_{50} value below than 20µg/mL for *in vitro* cytotoxic activity of the crude extract which considered highly cytotoxic (Srisawat *et al.*, 2013).

According to Fatemeh and Khosro (2013), the IC_{50} value of a good anti-cancer agents should be as low as possible to avoid undesirable effects to the patients.

This study revealed that crude ethyl acetate extract (PBEA) obtained *via* Soxhlet method exhibited the strongest cytotoxic effects (IC_{50} value = 14.37± 8.40 µg/ml) among all the extracts on its corresponding cancer cell: HeLa cervical cancer cells. In addition, no cytotoxicity was observed in the normal cells (NIH/3T3) treated with PBEA at concentration of 14.37 µg/ml. The ability of the extract to distinguish between the normal and malignant cells is a crucial aspect of the development of an anti-cancer agent (Badmus *et al.*, 2015). This characteristic was evident for PBEA in this study hence making it a high potential option as an anti-cancer agent for the cervical cancer.

Apart from that, the effectiveness of PBEA towards HeLa cells possibly due to present of phytochemicals that can inhibit the cell's proliferation. PBEA is known to contain several useful phytochemicals such as terpenoids, and phenolic compounds (Abdul-Wahab *et al.*, 2012; Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008). Various studies have reported that terpenoids and phenols exhibited cytotoxic activity against several cancer cells like colon and liver (Endrini *et al.*, 2014; Sharma *et al.*, 2017). In addition, the current study has identified new flavonoid compound namely 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl as described in Chapter 3. These compound were found predominantly in PBEA obtained *via* Soxhlet method and have been reported for its anti-proliferative activity and pro-apoptotic effects (Ban *et al.*, 2007).

Previous studies on the cytotoxic effects of *P. bleo* leaves also found ethyl acetate (EA) fraction and α-tocopherol compound isolated from EA fraction exerted high cytotoxic effects against human nasopharyngeal cancer cells (KB) with IC_{50} values of 4.5 and 0.81 µg/ml respectively (Malek *et al.*, 2009; Sri Nurestri *et al.*, 2008). In

contrast, the current study found the crude PBEA was highly cytotoxic towards HeLa cells. However, in the traditional setting, the plant extract in the crude form is preferred compared to the pure compounds due to the synergistic actions from all compounds in the crude extract which contribute to the effectiveness of the plants (Chai, 2011; Ma *et al.*, 2009). Furthermore, studies have suggested that the crude extract usage offers better effectiveness compared to the pure compounds as all the compounds presented in the crude extract (Aiyelaagbe *et al.*, 2011; Rasoanaivo *et al.*, 2011).

Apart from the high cytotoxic effects against HeLa cells, PBEA from Soxhlet method showed moderate cytotoxic activity on breast cancer cells (MDA-MB-231). HeLa is a hormone-dependent cervical tumor cells while MDA-MB-231 is a hormone-independent breast tumor cells (Rampogu *et al.*, 2018; Robinson *et al.*, 1990). The variation in the effectiveness of PBEA towards these two types of cancer cells was possibly due to the presence of phytochemicals in this extract that enable it to inhibit the activity of the hormones involved. Al-Shehri and Moustafa (2019) reported a methanol extract of *Aerva javanica* (AJME) showed significant cytotoxic effects on hormone-dependent cancer cell lines namely prostate (PC3) and breast (MCF7) cells. They suggested that the activity of the implicated hormones might be suppressed by various effective phytochemicals in AJME. Thus, the presence of these phytochemicals including the new compound might contribute to the most potent cytotoxic effect of PBEA extracted *via* Soxhlet method on HeLa cancer cells.

Even though the methanol extract of *P. bleo* leaves obtained *via* both successive extractions gave the highest extraction yield in this study (as described in Chapter 3), there were no cytotoxic effects exhibited by these extracts on all cancer cell lines. Our results was in agreement with the findings from Er *et al.* (2007) that the methanol extract

of this plant showed no anti-proliferative effects on a mouse mammary cancer cells (4T1) as well as to the normal cells NIH/3T3 (IC_{50} values more than 200 µg/ml). In contrast, Tan *et al.* (2005) reported high cytotoxic activity exhibited by the methanol extract of this plant on T47-D breast cancer cells. The variation in the results obtained from the aforementioned studies and the current study might be due to the different cancer cells used and lower amount of effective phytochemicals isolated that are responsible to the cytotoxic activity of this plant extracts.

In this study, *P. bleo* leaves aqueous extract showed IC_{50} values above 100 µg/ml which indicates weak cytotoxic effects on all cell lines. A study by Er *et al.* (2007) also reported similar findings with the current study where no anti-proliferative activity demonstrated by the aqueous extract of this plant against 4T1 and NIH/3T3 cells. Another study found hot water extract of this plant possessed moderate cytotoxic effects towards a human colon cancer cells (HT29) which contrary to our findings (Lum *et al.*, 2013). In this study, the aqueous extract was not able to show cytotoxic effects possibly due to low amount of the effective compounds such as phytol and sterols (in Chapter 3) which may be responsible to the cytotoxic activity of this extract.

Since PBEA obtained *via* Soxhlet extraction was found to show the potent cytotoxic effects towards HeLa cells, only PBEA and HeLa cells were used for further analysis. In order to explore the cytotoxic effect of PBEA on HeLa cells, the changes in their structure were observed after 72 h treatment with PBEA. In this study, the microscopic examination of PBEA-treated HeLa cells found the presence of apoptotic cells indicated by fragmented nuclei and condensed chromatin. As the time of incubation period with PBEA increased to 72 h, HeLa cells underwent apoptosis when they appeared round, shrunken with condensed chromatin and lost their original

morphology as well as contact with the adjacent cells beside detaching from the tissue culture flask. The apoptotic cells were floated in the culture medium upon the loss of cellular adhesion to the substrate (Rahman *et al.*, 2013). Once the apoptotic monolayer adherent cells experience early detachment from their basal membrane, they are known as anoikis (Thuret *et al.*, 2003).

In the present study, apoptosis also was assessed using Hoechst 33258 staining. This method makes use of a permeable blue fluorescent nucleic acid dye called a Hoechst stain to detect the condensation of chromatin and the fragmented nuclei of cells that had undergone apoptosis (Rahman *et al.*, 2013). Our findings showed that HeLa cells that were treated with PBEA exhibited features of apoptosis such as cell shrinkage, highly condensed chromatin, nuclei fragmentation and decreased number of cells. In addition, crescent-shaped nuclei were also observed in PBEA treated HeLa cells. The appearance of crescent-shaped nucleus that picked up the brighter blue dye indicated the onset of early apoptosis event (Brady, 2004). Apart from that, cells in their later stage of apoptosis can be identified through their detachment from adjacent cells with convoluted outline as well as cell shrinkage (Saraste and Pulkki, 2000). Thus, our findings suggested that PBEA is effective in inhibiting the growth of HeLa cells and exerted cytotoxic effect on this cancer cells.

Cell cycle arrest and induction of apoptosis were tested to determine the mechanism involved in the inhibition of cell growth by PBEA leading to the cytotoxicity in HeLa cells. The ability of cells to sustain proliferation is a key factor in tumor progression and development. This is evident through the dysregulation of the cell cycle when the expression or activity of the related proteins are altered (Feitelson *et al.*, 2015). The current findings showed that PBEA significantly inhibits the

proliferation of HeLa cells. A defining characteristic of an anti-cancer agent is their ability to interfere with the cell cycle progression thus suppressing cancer cells growth (Bailon-Moscoso *et al.*, 2017). The cell cycle analysis demonstrated that PBEA arrested HeLa cells at G_0/G_1 phase in a time-dependent manner as the accumulation of the cells were noticeable at this stage after 72 h. This finding indicates that PBEA may inhibit the progression of proliferation in HeLa cells at the G_0/G_1 phase. Cell proliferation is controlled by the cyclin-dependent kinases (CDKs) such as CDK4 and CDK6 at each checkpoints and upon DNA damage, it can be inhibited by activation of the checkpoints (Otto and Sicinski, 2017). During cell division, the checkpoints that exist at each phase of the cell cycle will identify the potential DNA impairment which allows for cell repair to take place (Wiman and Zhivotovsky, 2017). Arresting DNA replication at the G_0/G_1 phase can direct the cells either to be repaired or to undergo apoptosis (Chen, 2016; Mantena *et al.*, 2006; Visconti *et al.*, 2016). In addition, numerous studies reported the ability of natural compounds from various plants in modulating the cell cycle arrest in the tumor cells. For instance, ethyl acetate extract of *Opuntia humifusa* was found to significantly inhibited the proliferation of HeLa cells by arresting the cell progression at G_1 phase associated cyclin D1, cyclin-dependent kinase 4 (CDK4) and phosphorylated retinoblastoma proteins (Hahm *et al.*, 2015). In another study, ethyl acetate extract of *Glycosmis parva* leaf reduced the viability of colorectal cancer cells HT-29 by inhibiting the cell proliferation through arresting the cell cycle at G_0/G_1 phase related with the suppression of cyclin A, cyclin B, COX-2 and BCL-2 expression which subsequently leads to apoptosis (Buranabunwong *et al.*, 2015). Thus, our findings suggested that PBEA was able to suppress the proliferation of HeLa cells by blocking the cell cycle progression at the G_0/G_1 phase. This should be looked into in future

studies where the blocking activity may be associated with the CDK mechanism or other pathways.

In order to confirm the onset of apoptosis following cell cycle arrest, HeLa cells treated with PBEA were stained with Annexin V-FITC and analyzed using flow cytometry. Apoptosis begins with the alteration of the plasma membrane which exposing phosphotidylserine (Segawa and Nagata, 2015). Annexin V is conjugated to the phosphotidylserine along with PI which binds to cells at different stages and distinguishes apoptosis from necrotic cells (Hingorani *et al.*, 2011). In the current study, apoptosis activation in a time-dependent manner was observed due to the accumulation of apoptotic cells in both early and late stage of apoptosis after 72 h of treatment with PBEA. These findings were in consistent with the morphological changes observed on PBEA-treated HeLa cells such as nuclei fragmentation, condensed chromatin, cells shrinkage and finally detachment of the apoptotic cells from the tissue culture flask. Thus, our results confirmed that HeLa cells had undergone apoptosis as a result of the cytotoxic effects induced by PBEA.

Exploitation of the apoptotic signaling pathways to induce the cancer cell death is the main strategy of most chemotherapeutic drugs currently used in the clinical oncology (Pistritto *et al.*, 2016). Apoptosis can occur either through the extrinsic pathway (death receptor) or the intrinsic pathway (mitochondrial). Upon stimulation by DNA damage for example triggering the activation of p53 (a tumor suppressor gene) which cause cell cycle arrest or apoptosis in the cells (Chen, 2016). Activated p53 causes alteration in the ratio of Bax/Bcl-2 apoptotic proteins which results in permeability of the mitochondrial outer layer (MOMP) subsequently activates caspases cascade leading to apoptosis (Aubrey *et al.*, 2018; Marchenko and Moll, 2014).

p53 plays a vital role in the tumor suppression. p53 loss its functions when in mutation form which associated with the majority of cancer and high level of mutant p53 proteins detected in the tumors promotes the tumorigenesis (Yue *et al.*, 2017). Our findings showed the increment of p53 level in HeLa cells after treatment with PBEA in a time-dependent manner. Increase in the expression level of p53 suggesting that the arrestment of DNA in the HeLa cells occurred. This is indicated by the inhibition of cell cycle progression at the G_0/G_1 phase leading to apoptosis as evidenced by the detection of apoptotic HeLa cells after 72 h of treatment with PBEA. A study by Saio *et al.* (2017) demonstrated similar findings where the aqueous-methanol extract of *Rhododendron arboretum* promoted apoptosis and cell cycle arrest in HeLa cells *via* upregulation of p53. Another study reported that p53 mediated apoptosis and cell cycle arrest in HeLa cells induced by the methanol extract of *Polyalthia longifolia* leaf (Vijayarathna *et al.*, 2017).

In healthy cells, the tumor suppressor p53 remained at low concentration. p53 protein level will elevate due to various stimuli and regulate the apoptotic gene expression besides arresting the cell cycle (Giono and Manfredi, 2007). It induces apoptosis by suppressing anti-apoptotic proteins such as survivin and Bcl-2. At the same time, pro-apoptotic protein such as Bax is activated thus initiating the caspase cascade (Amaral *et al.*, 2010; Wu *et al.*, 2013). Activation of pro-apoptotic proteins promoted mitochondrial outer membrane permeabilization (MOMP) and triggering the release of cytochrome c that will bind with Apaf-1 producing apoptosome which stimulates the occurrence of apoptotic (Kavitha *et al.*, 2017). The current results suggested that PBEA acted as an apoptosis inducer, suppressing Bcl-2 expression through the increment of p53 as well as Bax expression level in the HeLa cells. This observation is in line with a study by Wang *et al.* (2016) who reported that barberin hydrochloride acted as an

efficient apoptotic inducer in HeLa cells *via* upregulation of p53 and downregulation of Bcl-2 as well as cox-2 expression level. Other than that, a study by Cheng *et al.* (2017) recorded ampelopsin (AMP) identified from ethyl acetate extract of *Ampelopsis megalophylla* induces apoptosis in HeLa cells validated through downregulation of Bcl-2 expression and upregulation of Bax, cytochrome c, caspase 3 and caspase 9 suggesting apoptosis mediated *via* mitochondrial pathway.

Activation of caspases can be triggered through the extrinsic or intrinsic pathways and are crucial mediators in apoptosis with the activation of caspase 3 (downstream effector caspase). Our results showed that caspase-3 expression level was slightly increased in the HeLa cells upon treatment with PBEA. The current findings seem to support the previous study by Tan *et al.* (2005) where they discovered the methanol extract of *P. bleo* leaves induced apoptosis in the breast carcinoma cell line (T47-D) *via* activation of caspase-3 and c-myc. Thus, PBEA treatment induced the activation of caspase-3 through extrinsic or intrinsic pathway in HeLa cells that initiated the action of other proteins which leads to apoptosis.

According to the rise of cancer incidence globally associated to its morbidity, mortality and high cost of treatment, the important strategy to control this incidence is an early prevention of the disease. In regards to cancer, chemoprevention is one the potential approach which involves the usage of a natural, synthetic or biological agent to reduce the risk or delay the occurrence of malignancy (Steward and Brown, 2013). Understanding on how carcinogenesis works biologically and identification of potential molecular targets to disturb this process are important elements in chemoprevention (Steward and Brown, 2013). The success of chemoprevention has been demonstrated in cancer including prostate, colon and breast (Wu *et al.*, 2011). Meanwhile, when the

patient is diagnosed with cancer, treatment is an important opt to consider. The main goal of cancer treatment is to achieve a cure (Khan *et al.*, 2005). However, when cure is not possible due to advanced state of disease, treatment of cancer will focus on palliation aim for prolongation of patient's life span and reduce the sufferings (Khan *et al.*, 2005).

In addition, standardization of the plant and crude extract is very much essential to establish the correct identity and maintain their quality for future purposes. The process of standardization usually involves pharmacognostic techniques (morphological, anatomical and biochemical characteristics) and phytochemical studies (Calixto, 2000; Organization, 1998). As for standardization of *P. bleo* plant for future purposes, macro and microscopical examinations by botanist should be done for accurate identity of the plant. Environmental factor such as climate, altitude and place of origin can affect the content of active ingredients in a plant (Kunle *et al.*, 2012). Hence, it is important to ensure the same location as well as right plant part are taken to maintain the content of active ingredients for optimum activity of the plant used for instance in the future research. Phytochemical studies involving extraction with suitable solvents, purification and characterization of the active phytoconstituents in this plant should be done to assess the purity of the plant for future research or purposes. It is also important to perform toxicological studies to standardize this plant for future use as an herbal formulation (Kunle *et al.*, 2012).

The current findings showed the morphological alterations in HeLa cells treated PBEA with the presence of apoptotic features such as cell shrinkage, condensation of chromatin, fragmentation of nuclei and a significant reduction in the HeLa cell number. The morphological changes observed were in line with the detection of apoptotic event

from Annexin V staining. Thus, the results of this study clearly demonstrated that PBEA induced apoptosis in cervical cancer (HeLa cells) through Bax/Bcl-2 signalling pathway with the involvement of caspase-3, while inducing G_0/G_1 phase cycle arrest *via* the p53-mediated mechanism.

4.5 Conclusion

In conclusion, our findings demonstrated that PBEA at concentration of 14.37 µg/ml induced cell death in the cervical cancer HeLa cells indicated by the presence of apoptotic cells observed microscopically. This is confirmed by the evaluation of Bax/Bcl-2, caspase-3 and p53 expression level. PBEA also induced the G_0/G_1 phase cycle arrest *via* p53-mediated mechanism. Therefore, it can be concluded that *P. bleo* has the potential to become a chemopreventive agent. Further research, particularly *in vivo* study, should be carried out to provide further evidence of the anti-cancer properties of this plant.

CHAPTER 5

ACTIVATION AND CYTOTOXIC ACTIVITY OF NATURAL KILLER CELLS TOWARDS CERVICAL CANCER CELLS HeLa INDUCED BY *Pereskia bleo* LEAVES EXTRACT

5.1 Introduction

Immune system plays a role in protection, contributing to the homeostasis by removal of detrimental stimuli, invading pathogens as well as tumour cells from the body (Comi and Tondo, 2017). Immunosuppression increases susceptible to infections and malignancies (Tinguely, 2013). Immunomodulation is a process of modification of the immune response to increase the efficiency against infectious agents and tumors (Saroj *et al.*, 2012). Thus, immunostimulant is an immunomodulatory substance that is used to increase the immune response (Avorn, 2011). In recent years, immunostimulants have emerged as a new approach for the treatment of ailments such as cancer, immunodeficiency diseases and infectious diseases (Fraser and Poole, 2019; García-Martínez *et al.*, 2018; Pranchevicius and Vieira, 2013).

NK cells mediate cytotoxicity against tumor cells without prior sensitization upon activation (Marcus *et al.*, 2014). Due to that special ability, modulation of NK cells has been widely studied to develop an alternative method for the treatment of cancer besides the conventional chemotherapy (Kwon *et al.*, 2017).

Several studies have reported of substances that can stimulate NK cells activity for instance IFN-γ and IL-2 which are used as adoptive immunotherapy for cancer treatment (Garris *et al.*, 2018; Jiang *et al.*, 2016). However, cancer immunotherapy has its limitations, for instance, the administration of IL-2 can cause detrimental side effect and toxicity to the patients (Sharma *et al.*, 2017; Suck and Koh, 2010; Surayot and You, 2017).

Medicinal plants are well known for their benefits in the prevention and treatment of many diseases with minimal side effects. Medicinal plants used in traditional therapy are able to enhance immune response of patients with malignant disease especially when the host defence mechanism is suppressed due to chemotherapy (Singh *et al.*, 2016). Several medicinal plants as well as its isolated compounds from the natural products have been reported for their immunomodulatory effects on the activation of NK cells particularly. For instance, the extract of *Nigella sativum* was reported to promote activation and increase the cytotoxic activity of NK cells against human leukaemia cells (K562) by the production of IFN-γ and TNF-α and granzyme A release (Shabsoug *et al.*, 2008). A study revealed the increase in NK cells cytotoxic activity against a lymphoma (YAC-1) and K562 after treatment with an acetone extract from *Kumquat pericarp* (Nagahama *et al.*, 2015). In other study, a fermented extract of *Triticum aestivum* was found able to stimulate the NK cells activation and enhanced their killing activity in immunocompetent BALB/c mice through increased degranulation and high production of IFN-γ (Barisone *et al.*, 2018). In addition, a phenolic compound namely resveratrol was reported in promoting the cytotoxic effects of NK cells towards human A549 (lung), HepG2 (liver) and K562 (leukaemia) cancer cells by increased perforin release, suppression of NKG2D expression as well as increased kinase activity of JNK and ERK1/2 MAP (Lu and Chen, 2010).

As for *Pereskia bleo* (*P. bleo*), this edible medicinal plant is well known for its anti-cancer properties. Various reports regarding this plant indicated their anti-proliferative effect, anti-cancer effect and cytotoxic activity towards numerous cancer cell lines (Er *et al.*, 2007; Malek *et al.*, 2009; Tan *et al.*, 2005; Yen *et al.*, 2013). However, there is no report available on its immunomodulatory activity on NK cells.

In this study, we demonstrated that ethyl acetate extract of *Pereskia bleo* leaves (PBEA), significantly induces apoptosis in cervical cancer cells HeLa (Chapter 4). This have led us to evaluate the activation and cytotoxic activity of this plant extract on freshly isolated human NK cells from healthy and cervical cancer patients against HeLa cervical cancer cells *in vitro*.

5.2 Materials and methods

5.2.1 Preparation of extract

20 g of the powdered leaves was successively extracted with ethyl acetate solvent using the Soxhlet system as described in Chapter 3 (section 3.2.3, page 40). Then the extract was concentrated with rotary evaporator and kept at -20°C until use.

5.2.2 Human subjects

Human primary NK cells used in this study were obtained from blood samples collected from three healthy donors and three cervical cancer patients. All healthy donors were female aged between 18 to 45 years old with no acute or chronic disease, not consuming any immunosuppressive drugs, non-smokers and not pregnant. For cancer patients, the donors were female diagnosed with cervical cancer, aged between 45 to 60 years old, have not received any treatment, free of acute or chronic diseases, non-smokers and not pregnant. All donors have been informed and consented at the beginning of this study. This study protocol was reviewed and approved by the Human Research Ethics Committee of Universiti Sains Malaysia (JEPeM-USM) (JEPeM USM Code: USM/JEPeM/17100566).

5.2.3 Isolation of human NK cells

A total of 5 ml of fresh whole blood from each donor was collected in EDTA blood collection tubes (BD Vacutainer®). The blood was diluted at a ratio of 1:2 with 1x PBS. The diluted blood was layered on an equal volume of lymphocyte separation medium (LSM, Cornell) and separated by density gravity centrifugation at 400 x g

without brake for 30 minutes at room temperature. The layers formed after centrifugation were plasma, buffy coat containing peripheral blood mononuclear cells (PBMCs), LSM and erythrocytes as illustrated in Figure 5.1.

Buffy coat was instantly aspirated with caution and collected in 15 mL conical tube. The buffy coat was washed twice with sterile PBS and centrifuge at 300 x g for 10 minute at room temperature. The pellet (PBMCs) was resuspended in a complete RPMI medium (Gibco) which consisted of 10% FBS (Gibco) and 1% penicillin streptomycin (Gibco). The PBMCs were incubated for 24 h at 37 °C with 5% CO_2.

After 24 h incubation, monocytes become attached to the bottom of the T75 tissue culture flask (Eppendorf) while lymphocytes were suspended in the culture medium. The lymphocytes were collected in 15 mL conical tube. They were washed twice with 1x PBS centrifuged at 300 x g for 10 minutes at 4 °C and ready for the NK cells isolation process.

The isolation of NK cells from healthy donors and cervical cancer patients was performed using human NK Cell Isolation Kit (Miltenyi Biotec) by negative selection with LS column as per kit's instructions. Then cells (lymphocytes) were counted using automated cell counter (Invitrogen). After that, the cell pellet was resuspended in 40 µl of NK buffer (up to 10^7 of total cells). A total of 10 µl of NK Cell Biotin-Antibody Cocktail was added, mixed and incubated at 2 – 8 °C for 5 minutes. Another 30 µl of NK buffer was added followed by 20 µl of NK Cell MicroBead Cocktail. The mixture was mixed thoroughly and incubated at 2 – 8 °C. After 10 minutes, 500 µl of NK buffer was added to the cell suspension. Then, the cells suspension was placed in the LS column. Earlier the LS column was rinsed with 3 mL of NK buffer and placed in the magnetic field of MACS Separator. The flow-through cells (unlabeled cells) were

collected in 15 mL tube. The obtained unlabeled cells contained the enriched NK cells. Another 3 mL of NK buffer were applied onto the LS column and the pass-through cells were collected and combined with the previous unlabeled cells.

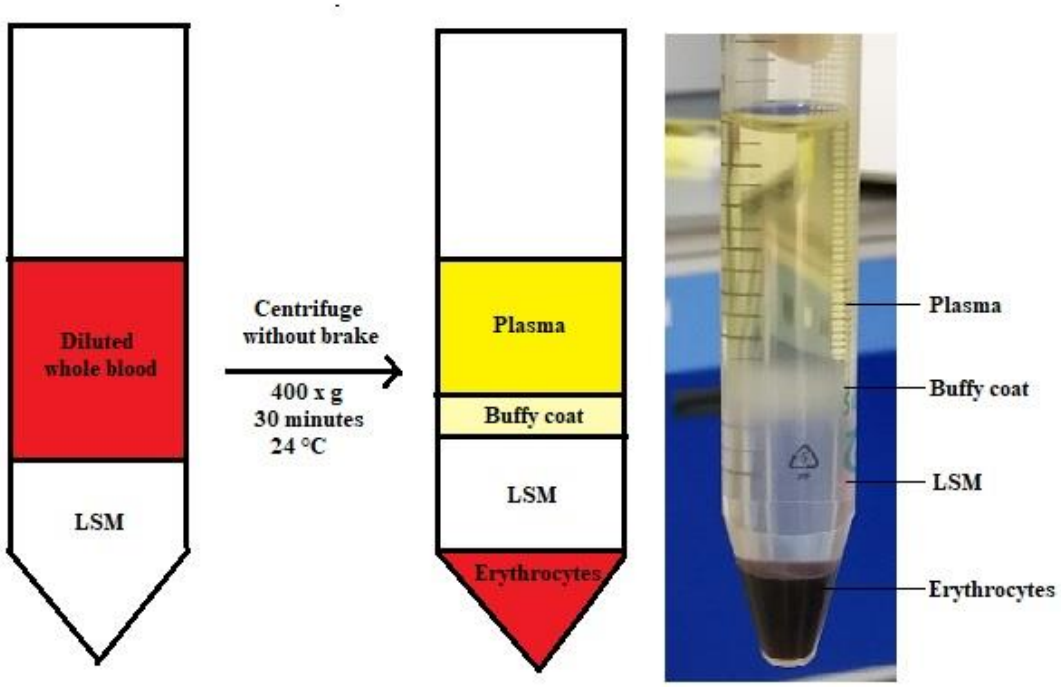


Figure 5.1 Separation of whole blood sample *via* a density gravity centrifugation.

5.2.4 Identification of human NK cells

To access the purity of the isolated NK cells, the unlabelled cells (enriched NK cells) were resuspended in PBS with a final concentration of 10^7 cells/ml. A total of 500 µl of blocking reagent bovine serum albumin (BSA, Miltenyi) was added to the cell suspension and incubated for 10 minutes at 4 °C. After that, 100 µl of the cell suspension was transferred into new labelled tubes and stained with 5 µl of anti-CD3-FITC (Santa Cruz) and 5 µl of anti-CD56-PE (Santa Cruz) for 30 minutes on ice under dark condition. 2 ml of PBS was added to the tubes and centrifuged for 5 minutes at 400 x g. Then, the supernatant was carefully aspirated and the pellet was resuspended in 500 µl of 1x PBS. The purity of the NK cells was assessed by flow cytometry through FACSCANTO (BD Bioscience) for minimum of 10,000 events per sample. The data was analysed using FCS Express De Novo software.

5.2.5 Proliferation assay of human NK cells

The cells from healthy individuals were subjected to a proliferation assay at several different incubation time (24 h, 48 h and 72 h) to determine the optimum period for NK cells proliferation. A volume of 100 µl of NK cells (5 x 10^3 cell/ml) were seeded from the complete medium DMEM into each well of flat bottom 96-well plates (Eppendorf) and incubated for 4 hours with 5% CO_2 at 37 °C. PBEA containing IC50 (14.37 ± 8.40 µg/ml) that has been subjected to serial dilution from 1 µg/ml until 200 µg/ml were used to treat the seeded NK cells at several incubation periods: 24 h, 48 h and 72 h in a humidified atmosphere (5% CO_2, 37 °C). The negative control of this experiment is DMSO at a concentration of less than 1%. After each incubation time, 20 µl of MTT reagent (5 mg/ml) was added into each well and further incubated for

4h at 37 °C. A volume of 100 µl of DMSO was added into each well and their optical density (OD) was measured using ELISA microplate reader at 570 nm. The experiment was done in triplicates and repeated 3 times using different donors. The percentage of NK cells proliferation was determined as following formula:

NK proliferation (%) = (Absorbance of treatment/absorbance of control) × 100 %

5.2.6 Co- culture of human NK cells with cervical cancer cells HeLa

Based on the results of the potent cytotoxic activity of PBEA obtained *via* Soxhlet method on HeLa cells (Chapter 4) as well as the NK cells proliferation, the co-culture experiments for NK cells stimulatory and cytotoxic activity on HeLa cells induced by PBEA was carried out at 24 h incubation. The overview of this experiment is illustrated in Figure 5.2.

5.2.6(a) Natural Killer cells

NK cells were isolated from 5 ml of fresh whole blood from donors and purified as described in section 5.2.3.

5.2.6(b) Target cells

Human cervical cancer cell line (HeLa) was purchased from ATCC (US). HeLa cells were grown in a complete media DMEM supplemented with 10% FBS (Gibco) and 1% penicillin streptomycin (Gibco).

5.2.6(c) Killing assay of human NK cells against HeLa cells induced by PBEA

Killing assays of human NK cells were performed by determining the percentage of target cell death after 24 h of PBEA treatment. Effector cells (NK cells of healthy or cervical cancer donors) were incubated with the target cells (HeLa) at specified ratio of effector to target (E: T = 25:1). Prior to the incubation with HeLa cells, NK cells were treated with PBEA (14.37 ± 8.40 μg/ml) for 4 hours with 5% CO_2 atmosphere at 37 °C. After activation, NK cells were transferred into a plate containing HeLa cells and incubated for additional 20 hours. NK cells without treatment with PBEA were used as a control in this study. After 24 h incubation, HeLa cells were collected and centrifuged at 300×g for 5 minutes. The supernatants were aspirated and kept to evaluate their level of cytokines as well as degranulation produced while the pellet was washed with PBS prior the staining process. Next, the cells were stained with Annexin V-FITC and PI for 15 minutes (in the dark). The target cell death was quantified using FACSCANTO (BD Bioscience) at 10,000 events per sample. The data analysis was performed using FCS Express 7 De Novo software. The experiment was conducted in independent triplicate.

5.2.6(d) ELISA for degranulation (Perforin, Granzyme B) and cytokines (IFN-γ, IL-2) production

The expression of perforin, granzyme B, interferon gamma (IFN-γ) and interleukin 2 (IL-2) were determined using human PRF1 (Perforin 1), human GzmB (Granzyme B), human IFN-γ (Interferon gamma) and human IL-2 (Interleukin 2) kits from Elabscience as per manufacturer's protocol. A total of 100 μl of each sample [supernatant obtained from the NK cells co-culture experiment in section 5.2.6(c)] were added into each wells and incubated for 90 min at 37°C. Then, 100 μl of

biotinylated detection Ab working solution were added into each well after the removal of liquid and further incubated for 60 min at 37°C. Then, the plate was washed three times followed by addition of 100 µl of HRP conjugate working solution and incubated for 30 min at 37°C. After that, the plate was washed five times and 90 µl of substrate reagent were added into the wells followed by incubation for 15 min at 37°C (in the dark). The OD values of each well were measured with microplate ELISA reader at 450 nm. The results were expressed in pg/ml. Each experimental and control sample was assayed in triplicates.

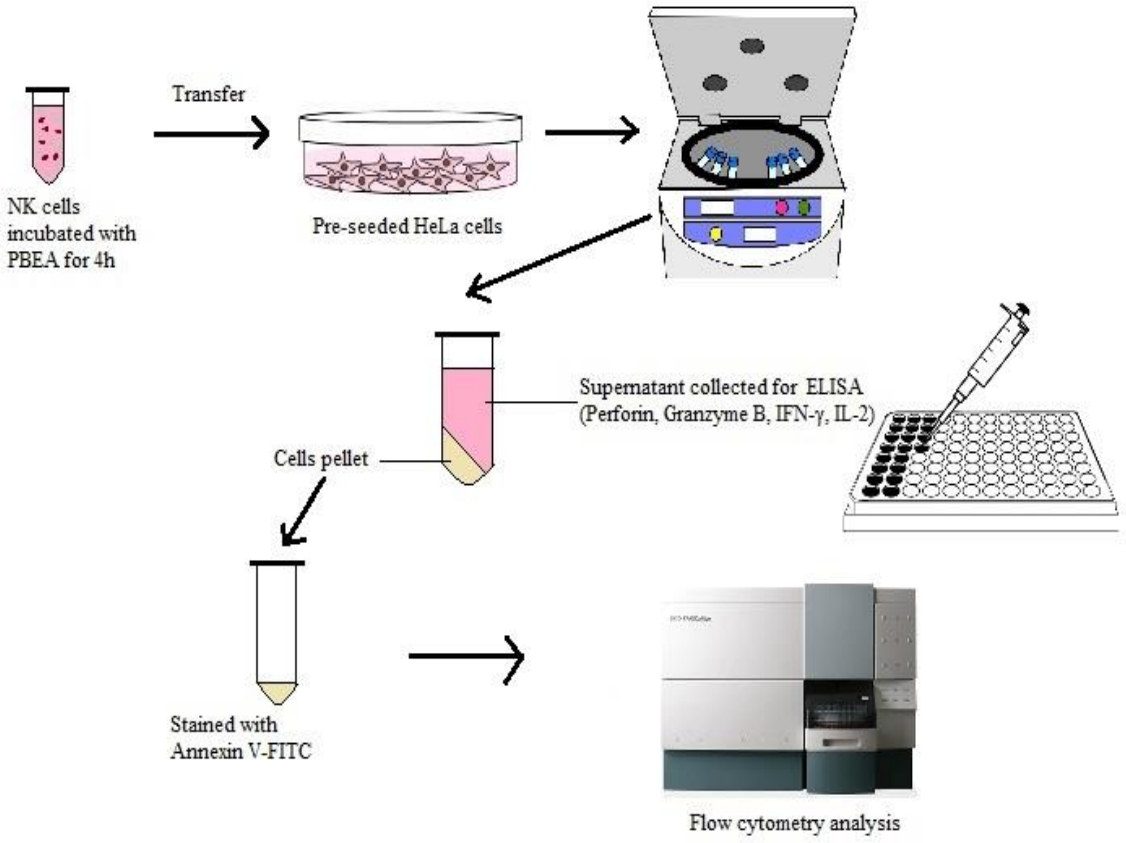


Figure 5.2 The overview of co-culture experiment of NK cells with target HeLa cells induced by PBEA

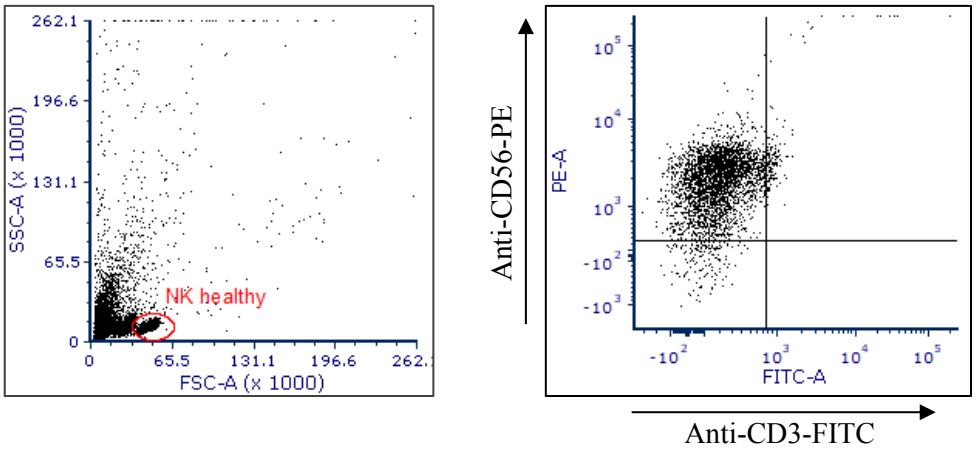
5.2.6(e) Statistical analysis

The data obtained were presented as mean ± SD. The results were analysed using independent t-test for the number of NK cells (healthy individuals and cervical cancer patients). One-way ANOVA test was conducted for the co-culture experiments of NK cells and Tukey's multiple comparison test was done to determine the significance between the groups. The significant value of the data was set at $P<0.05$. In addition, Shapiro-Wick test was performed for the data's normality. All statistical analysis was performed with GraphPad Prism 8 software.

5.3 Results

5.3.1 Purification of human NK cells

In order to evaluate the performance of Human NK cells Isolation kit (Miltenyi Biotech) with the condition of the present study, purification testing was carried out using three healthy donors. After the isolation, the expression of NK cells stained with anti-CD3-FITC and anti-CD56-PE were evaluated by flow cytometry analysis to confirm the presence of NK cells. Figure 5.3 showed the results of NK cells' purity from one of the healthy donors as a representative of the overall findings. The results were similar for all the three individual samples tested in this study. Our findings showed that about 85% of purified human NK cells ($CD3^{-}CD56^{+}$) was obtained through this kit. The purification testing was not carried out for the cervical cancer patient samples due to the limited number of NK cells.



(i) (ii)

Figure 5.3 Purification of human NK cells. (i) Representative dots plot for gating of NK cells population. (ii) Dots plot of purified NK cells indicated approximately 85% of $CD3^{-}CD56^{+}$ NK cells after isolation.

5.3.2 The number of NK cells from healthy individuals compared to cervical cancer patients

The isolation of human NK cells was performed using magnetic separation by negative selection. NK cells were obtained from three healthy and three cervical cancer donors. NK cells counts were determined using trypan blue exclusion method. In this study, NK cells from healthy individuals exhibited higher number of cells (7.8×10^5 cells/ml) compared to cervical cancer patients (3.15×10^5 cells/ml) as shown in Figure 5.4. NK cells count was significantly different *(P*<0.05) for both groups.

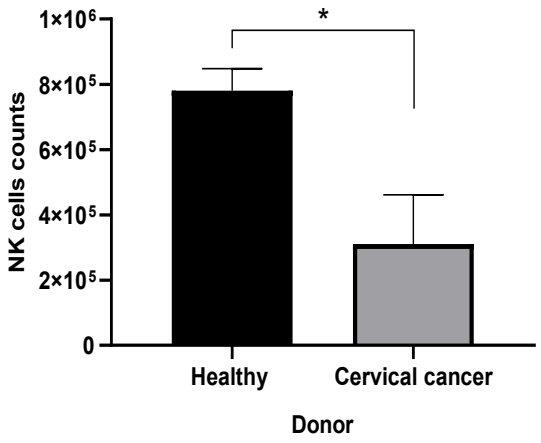


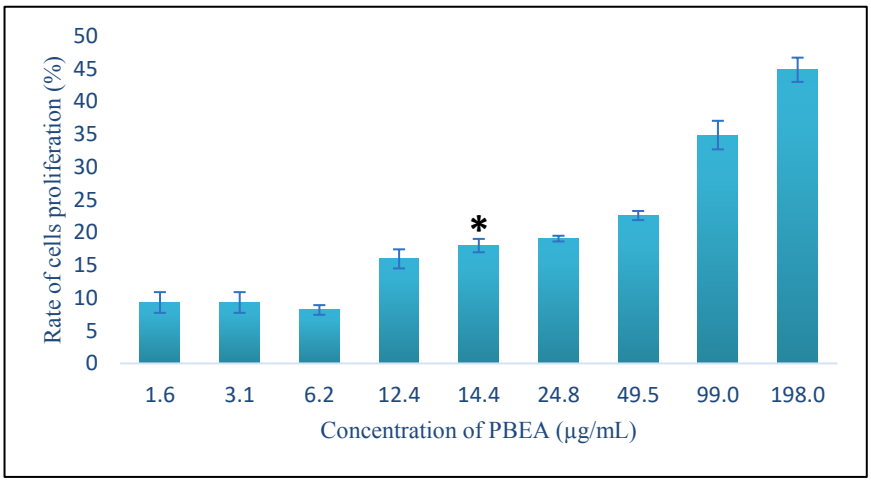
Figure 5.4 Number of human NK cells isolated from healthy and cervical cancer donors. Data are represented as mean ± SD from three independent experiments (n=3) with triplicates each. **P*<0.05 when compared to both groups.

5.3.3 PBEA enhanced the proliferation of human NK cells

In the current study, the freshly isolated human NK cells from healthy donors were incubated with PBEA at the various concentration (1 – 200 µg/mL) to determine their effect on the proliferation of NK cells after 24 h, 48 h, and 72 h. The IC_{50} value of PBEA (14.37± 8.40 µg/ml) from section 4.3.1(b) was also included in order to evaluate its proliferation effect on NK cells.

Figure 5.5(i) shows the increased of NK cells proliferation to 45 % with the increase in PBEA concentration after 24 h of treatment. Besides, the proliferation rate of PBEA treated-NK cells at concentration of 14.37 µg/ml (IC_{50} of PBEA) was significantly different in comparison to 48 h and 72 h treatment groups (P<0.05). The proliferation was not amplified after 48 h of treatment when the concentration of PBEA was less than 14.37 µg/ml (Figure 5.5 ii). However, when the concentration of PBEA was more than 14.37 µg/ml, the rate of NK proliferation was substantially increased to 35 % (Figure 5.5 ii).

At 72 h of treatment, the level of NK proliferation was observed to rise 25 % when treated with PBEA at concentration below 14.37 µg/ml (Figure 5.5 iii). The proliferation of NK cells was slightly decreased (10 %) at a concentration of 14.37 µg/ml and gradually increased to 35 % for concentrations of more than 50 µg/ml after 72 h of treatment (Figure 5.5 iii). This observation suggested that PBEA did not exert toxicity on human NK cells due to increase in their proliferation even at high concentration (200 µg/ml) treatment.



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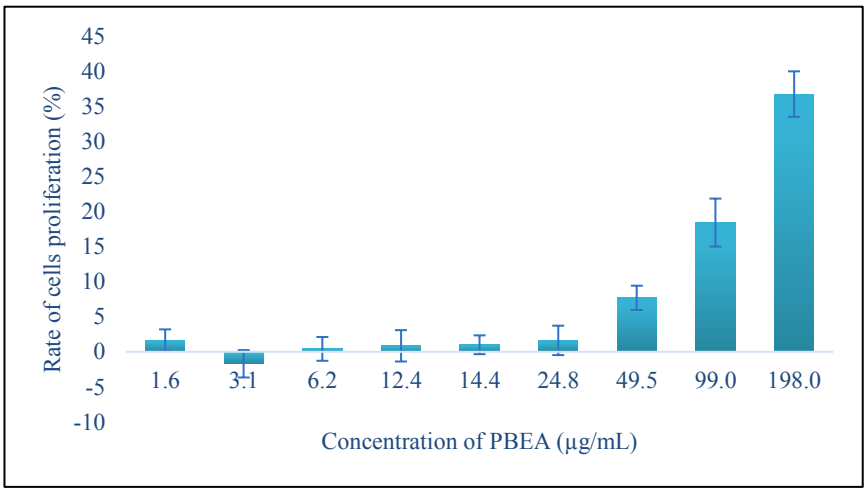

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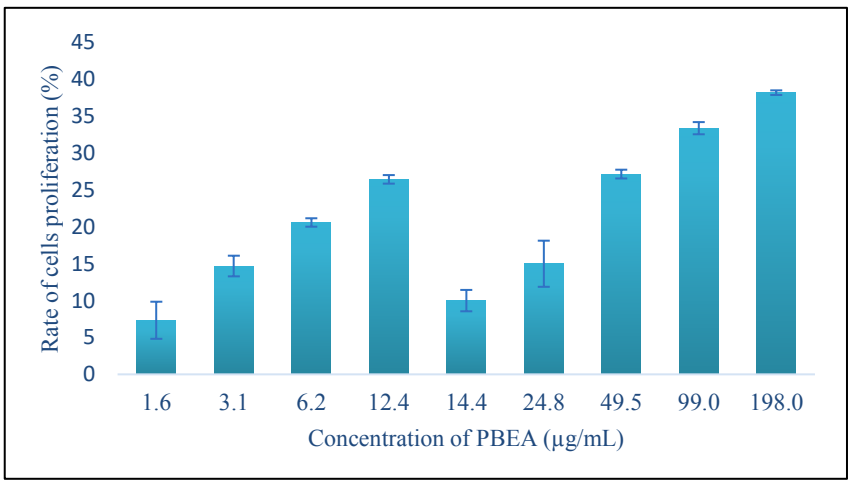


(iii)

Figure 5.5 Proliferation of NK cells after treatment with different concentration of PBEA (1 – 200 µg/mL) for (i) 24 h, (ii) 48 h and (iii) 72 h. *PBEA at concentration of 14.4 µg/ml is significant different when compare to 48 h and 72 h of PBEA treatment (P<0.05).

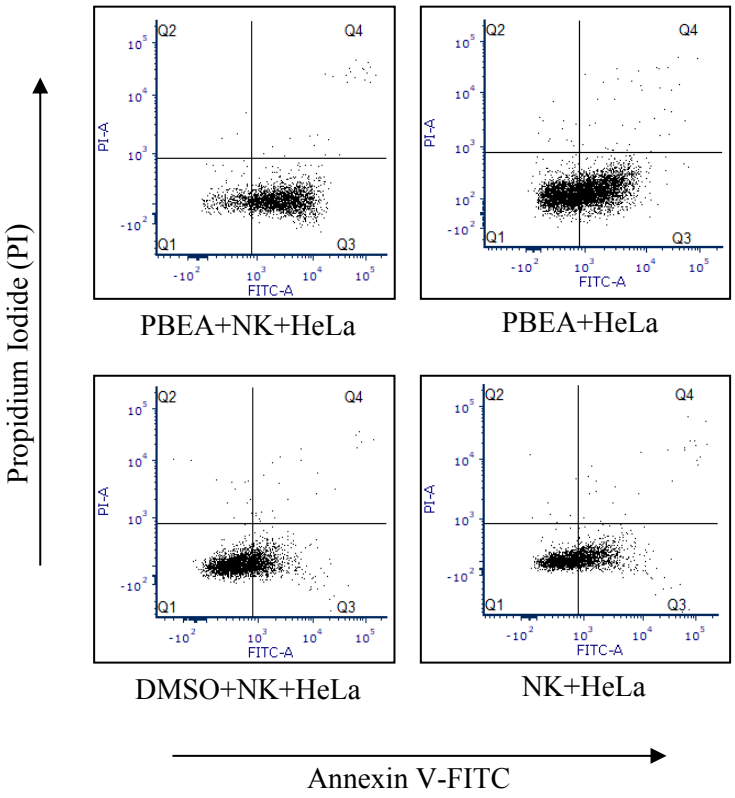
5.3.4 NK cells treated with PBEA enhanced the killing of target cells

Target cells killing assay of human NK cells activated by PBEA was assessed through flow cytometry. NK cells from healthy donors and the cervical cancer patients were treated with 14.37 μg/ml of PBEA prior to incubation with HeLa cells (target cells). As shown in Figure 5.6, the co-culture of HeLa cells with NK cells of healthy donors showed a substantial increase in the percentage of apoptotic target HeLa cells (88.65 ± 5.3 %) after treatment with PBEA (PBEA+NK+HeLa). A total of 53.73 ± 11.97 % of apoptotic cells were recorded in the treatment group without NK cells (PBEA+HeLa), 49.18 ± 16.01 % after treatment with DMSO (DMSO+NK+HeLa) and 64.62 ± 10.12 % without treatment with PBEA (NK+HeLa). The results demonstrated that the percentage of apoptotic cells in PBEA-treated group was significant different from the DMSO treated group and in the absence of NK cells (P<0.05).

Meanwhile in Figure 5.7, co-culture of HeLa cells with NK cells from cervical cancer patients showed high percentage of apoptotic target HeLa cells which was 74.62 ± 4.62 % after treatment with PBEA (PBEA+NK+HeLa). Other groups exhibited lower number of apoptotic cells which were 55.44 ± 3.55 % without NK cells (PBEA+HeLa), 60.00 ± 4.33 % with DMSO treatment (DMSO+NK+HeLa) and 57.81 ± 3.73 % without PBEA treatment (NK+HeLa). There were significant differences of apoptotic rate of HeLa cells in PBEA-treated NK cells from the groups that were not treated with PBEA as well as the groups that received DMSO treatment without NK cells (P<0.05).

Although the percentage of apoptotic HeLa cells was high in co-culture with NK cells from healthy donor compared to NK cells from cervical cancer donors, the results showed that PBEA has the ability to enhance the cytotoxic effects of NK cells

from cervical cancer donors to kill the target cells as indicated by high percentage of apoptotic HeLa cells in comparison to untreated control cells.



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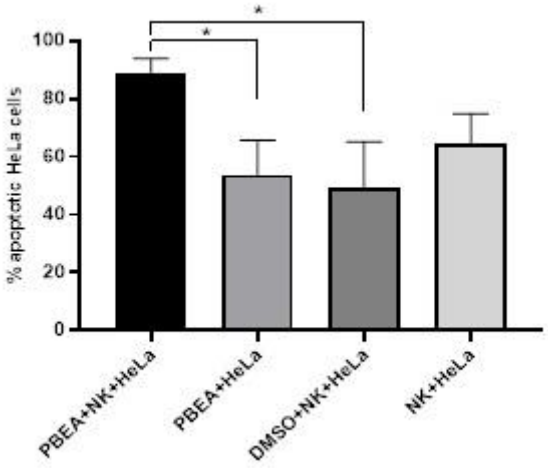
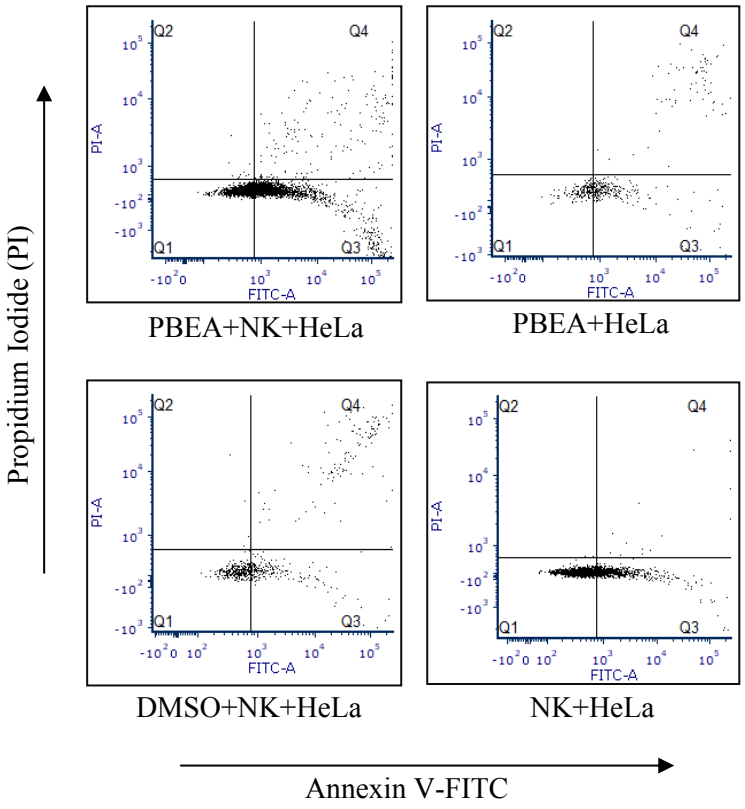


(ii)

Figure 5.6 Effect of NK cells from healthy donors on the target cells' death after stimulated with PBEA for 24 h . (i) The representative of dot plots distribution of HeLa cells co-culture with treated and untreated PBEA NK cells. The quadrants show the cells that are viable (Q1), necrotic (Q2), in early apoptosis (Q3) and late apoptosis (Q4). (ii) The bar graphs representing percentage of apoptotic HeLa cells after incubation with treated and untreated PBEA NK cells. Data are represented as mean ± SD from three independent experiments (n=3) with triplicates. *P<0.05 indicates significant different among the treatment groups.



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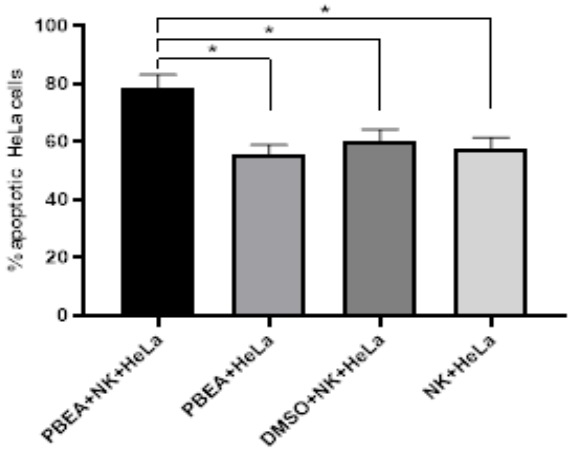

(ii)

Figure 5.7 Effect of NK cells from cervical cancer patients on the target cells death after stimulated with PBEA for 24 h. (i) The representative dot plots the distribution of HeLa cells co-culture with treated and untreated PBEA NK cells. The quadrants show the cells that are viable (Q1), necrotic (Q2), in early apoptosis (Q3) and late apoptosis (Q4). (ii) The bar graphs represent percentage of apoptotic HeLa cells after incubation with treated and untreated PBEA NK cells. Data are represented as mean ± SD from three independent experiments (n=3) with triplicates. **P*<0.05 indicates significant different among the treatment groups.

5.3.5 NK cells treated with PBEA enhanced cytokines (IFN-γ and IL-2) production

The production of NK cells granules (perforin and granzyme B) was measured to investigate the activation and cytotoxic activity of NK cells towards HeLa cells stimulated by PBEA. Based the results obtained, the expression of granzyme B was detected (9.39 ± 3.97 pg/ml) in PBEA-treated NK cells from cervical cancer patients. However, no expression of granzyme B measured in PBEA-treated NK cells from healthy donors. Perforin was also absent in all experiment conditions. In addition, the expression of IFN-γ was detectable in PBEA-treated NK cells from cervical cancer patients (19.82 ± 4.57 pg/ml) as well as in cells without PBEA treatment (19.60 ± 5.05 pg/ml), without statistical differences. On the other hand, the level of IL-2 expression was detectable in all treatment experiment conditions, without statistical differences. Table 5.1 summarized the results of perforin, granzyme B, IFN-γ and IL-2 released by PBEA-activated NK cells from healthy and cervical cancer donors that were incubated with HeLa cells.

Table 5.1 ELISA results of perforin, granzyme B, IFN-γ and IL-2 detection of human NK cells from healthy and cervical cancer donors stimulated by PBEA in co-culture with target HeLa cells.

	Concentration (pg/mL)			
	Perforin	**Granzyme B**	**IFN-γ**	**IL-2**
Healthy donors				
PBEA treated NK cells co-culture with HeLa cells	ND	ND	ND	9.86±0.94
NK cells co-culture with HeLa cells	ND	ND	ND	10.43±1.45
NK cells treated DMSO co-culture with HeLa	ND	ND	ND	9.34±1.75
Untreated NK cells	ND	ND	ND	8.76±0.22
Cervical cancer donors				
PBEA treated NK cells co-culture with HeLa cells	ND	9.39±3.97	19.82±4.57	10.60±1.81
NK cells co-culture with HeLa cells	ND	ND	19.60±5.05	13.20±3.78
NK cells treated DMSO co-culture with HeLa	ND	ND	ND	13.75±3.88
Untreated NK cells	ND	ND	ND	9.68±0.31

Values (n=3, 3 independent experiments with thrice replicates each) represent the means ± SD. ND indicates not detected.

5.4 Discussion

Innate immune response represents the first line defense mechanism in the body that provides a protective shield against infections and malignancies. Nowadays, the modulation of immune responses to improve health status especially in cancer has been extensively studied. Natural killer (NK) cells are one of the most important cells in the innate immune system and endowed with cytotoxic function (Habif *et al.*, 2019). NK cells are able to identify and kill tumor cells without prior activation and for this reason the cells have emerged as a therapeutic alternative for treatment of multiple malignancies (Nayyar *et al.*, 2019). Interestingly, numerous traditional medicinal plants have been reported which modulate immune responses against tumor cells. Several pure active compounds which have been isolated from plants such as resveratrol and lectins have been tested for its modulation effects on NK cells (Braedel-Ruoff, 2010; Lu and Chen, 2010; Rauf *et al.*, 2018). After demonstration of the potent cytotoxic activity of PBEA against HeLa cells (Chapter 4), we decided to evaluate if PBEA has additional immunomodulatory effects on NK cells that would potentiate its anti-tumor effects. In this regard, PBEA and HeLa cells were used to further the study and co-culture with NK cells. In this study, the immunomodulatory effect of PBEA was evaluated in a system composed of HeLa cells (target) and NK cells (effectors).

In the present study, NK cells were isolated from fresh whole blood samples of healthy and cervical cancer donors. The isolation of NK cells from both groups was performed using negative selection (Human NK Cell Isolation Kit) together with LS column. The isolation obtained $CD3^{-}CD56^{+}$ NK cells with purity of more than 85%. By using the same method and kit, Sugita *et al.* (2018) reported the similar purity of isolated NK cells from PBMCs (purity about 86%). In order to increase the purity of

NK cells, further purification with CD56 micromagnetic beads was suggested by Deng *et al.* (2014). The principle of CD56 micromagnetic beads purification was based on positive selection labelling of CD56 cells from PBMCs. Yi *et al.* (2018) demonstrated 70 % of initially enriched NK cells isolated using the same kit with slightly modifications. Then they further purified the NK cells using positive selection CD56 magnetic beads with more than 99 % of purity. Thus, according to the previous reports, a high degree of purity could be obtained with the inclusion of an additional final purification using CD56 micro magnetic beads.

In the present study, the results showed that the number of NK cells from healthy donors was higher compared to cervical cancer patients. About 10% of peripheral blood lymphocytes of healthy individuals contains NK cells, whereas, in cancer patients, the cells were recorded in low amounts (He *et al.*, 2016; Klingemann *et al.*, 2016; Levy *et al.*, 2011). Similarly, Levy *et al.* (2011) agreed that NK cells are not naturally present in the malignant tissue of advanced stage human neoplasm hence the low amount that was recorded. Studies have also reported the decrease number of NK cells observed in cervical cancer patients where these cells are often dysfunctional leading to defective NK cells cytotoxic activity against tumor cells (Arreygue-Garcia *et al.*, 2008; Garcia-Iglesias *et al.*, 2009). Other than that, Saito *et al.* (2013) reported a higher percentage of apoptotic cells (21.3 ± 11.6%) in gastric cancer patients compared to healthy individuals (11.2 ± 5.2%) which associated with the development of the cancer as well as low number of NK cells. Reduction of $CD56^{+}$ NK cells was also observed in advanced stage of colorectal and gastric cancer patients with liver metastasis (Gulubova *et al.*, 2009). The minimal presence of NK cells has been coined as the reason for metastasis due to the tumor cells escape from the liver immune control mechanism (Gulubova *et al.*, 2009). Furthermore, several studies also reported that

cancer patients with high progression of the disease exhibited a decrease in number of peripheral or tumor infiltrated NK cells which often have associated with low survival rate (Ishigami *et al.*, 2000; Li *et al.*, 2016; Peng *et al.*, 2017).

In pharmacological drug screening, a potential anti-cancer agent should exert greatest cytotoxic effect with almost negligible side effects. Based on the National Cancer Institute (NCI) guidelines, plant extracts with IC_{50} value less than 20 µg/mL are considered to have a potent cytotoxic effect (Srisawat *et al.*, 2013), which is in correspondence with the results of the cytotoxic effects of PBEA (Chapter 4). In the current study, the results showed the augmentation of NK cells proliferation as the concentration of PBEA increased to 200 µg/ml. This indicated that high concentration of PBEA was not toxic to the NK cells. This finding also was in agreement with the finding from the cytotoxic study of PBEA in Chapter 4 where concentration of PBEA more than 99 µg/ml was not toxic towards the normal cells. However, to avoid the direct killing of the target cells (HeLa), PBEA with concentration of 14.37 µg/ml was chosen to carry out the cytotoxic study of NK cells. The greatest proliferation was observed after 24 h of incubation with PBEA. The NK proliferation was not significant after 48 h and 72 h which could be influenced by the small sample size of the study (Angelo *et al.*, 2015). According to the results of the proliferation study, the evaluation of the effect of PBEA on the cytotoxic activity of NK cells was carried out using 24 h of PBEA treatment.

After activation, the cytotoxic activity of NK cells on target tumor cells can be determined based on the number of killed target cells (Li, 2010; Nishimura *et al.*, 2017). In the current study, target cells death was evaluated using Annexin V-FITC apoptosis assay. The results demonstrated that percentage of apoptotic HeLa cells was

markedly increased in the co-culture experiment of PBEA induced NK cells from cervical cancer patients when compared with the respective untreated control groups. According to this, it can be suggested that PBEA was able to promote the activation of NK cells to kill HeLa cells. A study by Surayot and You (2017) also demonstrate the usefulness of our experimental model, where they found that sulfated polysaccharides isolated from the seaweed *Codium fragile* enhanced NK cells cytolytic activity against HeLa cells. Yeap *et al.* (2013) reported the potential of a methanol extract from a traditional herb, *Rhaphidophora korthalsii* which increased the cytotoxic effect of NK cells against the leukemia cell line K562.

The killing pathway is used to evaluate the cytotoxic activity of NK cells by monitoring their response upon activation (Rudnicka *et al.*, 2015). The killing mechanism of NK cells is mediated through three pathways which are Perforin and Granzyme B-mediated pathway, Fas Ligand pathway and antibody-dependent cellular cytotoxicity (ADCC) pathway (Yoon *et al.*, 2015). Several parameters are used to measure activated NK cells response such as the number of apoptotic target cells, quantifying the expression of target markers expression (Fas Ligand, death receptors and caspases), degranulation release (granzyme A/B and perforin) or assessing cytokines secretion (IFN-γ, IL-12, IL-2) (Nishimura *et al.*, 2017; Rudnicka *et al.*, 2015; Yoon *et al.*, 2015).

The mechanism of action of NK cells begins by coming in tight contact with the target cells through the immune synapse then polarizing lytic granules as well as other organelles to the contact site (de Saint Basile *et al.*, 2010; Stinchcombe *et al.*, 2006). Perforin, a pore-forming protein that is contained within lytic granules will generates holes in the target cells membrane upon release followed by delivery of

granzyme to the cytosol of the target cells which initiates apoptosis (Backes *et al.*, 2018; Voskoboinik *et al.*, 2006). The current study showed perforin was not detected in all treatment groups (both from healthy and cancer donors). In contrast, the secretion of granzyme B was detected in the co-culture of NK cells from cancer patients treated with PBEA. This may be due to stimulation from PBEA which enhanced the activation of the NK cells' cytotoxicity towards the HeLa cells. This finding is in agreement with a previous report by Surayot and You (2017) which found that sulphated polysaccharides (SP) from *Codium fragile* enhances NK cells activation indicated by the release of granzyme B. In addition, absence of perforin and relatively low expression of granzyme B that were found in NK cells incubated with Hela cells in all treatment groups suggested that HeLa cells lack other activating signals for PBEA-activated NK cells from healthy and cancer patients. A study by Zhang *et al.* (2007) also reported no cytotoxic activity of NK cells against HeLa cells that was stimulated by recombinant membrane fractalkine (mhFKN) due to the absence of other activating signals for mhFKN-activated NK-92 cells on HeLa cells. The activation of NK cells is the result of the influence of the multitude of activating receptors such as the activating form of killer cell immunoglobulin-like receptor (KAR), NKG2D, NKp46, NKp44 and NKp30 which trigger the cytotoxic effects and cytokines release (Long *et al.*, 2013; Yoon *et al.*, 2015).

In addition to degranulation detection, cytokines expression such as IFN-γ was measured to evaluate NK cells cytotoxic activity upon stimulation with PBEA. IFN-γ is produced by NK cells after activation for the direct killing of the target tumor cells (Martín-Fontecha *et al.*, 2004; Schoenborn and Wilson, 2007). It was found that after 24 h incubation, the level of IFN-γ production was detected in co-culture of HeLa cells and NK cells from cancer patients in the groups of presence and absence of PBEA

treatment. According to these results, the expression of IFN-γ may be contributed by the stimulation effect from PBEA on NK cells. In addition, the increased production of IFN-γ also might be due to the influence by cytokines produced by other contaminating immune cells. A previous study showed that *Rhaphidophora khorthasii* modulated NK cells to produce IFN-γ due to IL-2 produced by the neighboring lymphocytes along with the stimulation by the extract (Yeap *et al.*, 2013).

In addition, the results obtained could be associated to the memory-like activity of NK cells from the patients, which could be pre-activated by stimulatory signals and tumor antigens (Pahl *et al.*, 2018). In this regard, the presence in HeLa cells of shared tumor antigens with the tumoral cells of the patients could induce the NK cells activation mediated by a recall response, evidenced by the production of granzyme B and IFN-γ, which was more potentiated in the presence of PBEA. This potential explanation is supported by the fact that these results were not observed in the healthy donors. Several studies indicated that NK cell activation by cytokines can induce the generation of NK cells with memory-like properties. The adoptive transfer of murine splenic NK cells pre-treated with IL-12, IL-15 and IL-18 into tumor-bearing mice effectively reduced tumor growth when compared to naïve nor IL-15- or IL-2-pretreated NK cells in combination with radiation therapy (Ni *et al.*, 2012). Similar to murine memory-like NK cells, human NK cells that were pre-activated with IL-12, IL-15 and IL-18 and subsequently rest for several days display increased IFN-γ production upon re-stimulation with cytokines or target cells compared with control NK cells (Ni *et al.*, 2012; Romee *et al.*, 2012). In addition, the memory-like NK cells demonstrated increase expression of activating receptors CD94, NKG2A, NKG2C and CD69 with lack of inhibiting receptors CD57 and KIR (Ni *et al.*, 2012; Romee *et al.*, 2012). Memory-like activation of NK cells also could be induced in response to

antigens. A study showed that NK cells of the mouse produced high level of IL-12, IFN-α and IFN-γ in response to re-exposure with the same hapten called dinitrofluorobenzene (DNFB) (Majewska-Szczepanik *et al.*, 2013).

According to the current findings, PBEA was found to be non-toxic to human NK cells, indicating that this extract can possibly represent a safe alternative to the existing therapies such as IFN-γ. A study conducted on the KHYG-1 NK cells line showed IFN-γ production and NK cell cytotoxic activity against K562 and YAC-2 cells, which were significantly higher after treatment with a *Fortunella crassifolia* fraction (Nagahama *et al.*, 2015). A similar mechanism could be operative with the use of PBEA, which should be explored in further studies.

Some studies have reported that activated NK cells produce IL-2, thus we decided to examine the presence of IL-2 in exploring the ability of PBEA in stimulating NK cells to produce IL-2. In this regard, the expression of IL-2 was detected in all treatment groups of NK cells from healthy and cancer patients. The detection of IL-2 expression may be explained by the synergistic effect from the contaminating cells and endogenous IL-2 production where activated NK cells particularly in cancer patients can produce IL-2 (Rangel-Corona *et al.*, 1998; Rimoldi *et al.*, 1993). IL-2 is well-known for its ability in stimulating proliferation of NK cells as well as enhances their cytotoxic activity against tumor cells (Floros and Tarhini, 2015; Srivastava *et al.*, 2013). Nowadays, IL-2 is used as immunotherapy to induce NK proliferation in cancer patients. However, the side effects of IL-2 such as nausea, flu-like symptoms and dizziness among others limit the wide application of IL-2 (Surayot and You, 2017). High dose consumption of IL-2 can also cause toxicity in patients (Suck and Koh, 2010).

Naturally, ADCC is one of the target cell death modes mediated by NK cells. It involves a process where antibodies such as Immunoglobulin G (IgG) become coated with the target cells and recruit effector cells (NK cells) to trigger target cell death *via* a lytic mechanism (Zahavi *et al.*, 2018). IgG facilitates the access of effector cells to the target cells with the Fab region of the IgG that binds to the target cells while the Fc portion associating with FcγR on NK cells that express CD16 (FcγRIII) (Fanger *et al.*, 1989; Prager *et al.*, 2019). Upon FcγR activation, NK cells release lytic granules to destroy antibody-sensitized target cells (de Saint Basile *et al.*, 2010). However, in our study, the target cells death observed in PBEA-induced NK cells from cancer patients probably was not related to ADCC killing mechanism of the target cells as the NK cells obtained with the exclusion of the plasma fraction of blood.

Besides ADCC pathway and the release of perforin and granzyme, NK cells can induce apoptosis in the target cells through death receptors such as Fas Ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL). The activation of death receptors facilitates the formation of death-inducing signal complex that prompts the activation of caspase cascade (caspase 8 and 10) and ultimately result in apoptosis of the target cells (Strasser *et al.*, 2009; Wilson *et al.*, 2009). Although the exact mechanism of PBEA in activating immune cell cytotoxicity towards target HeLa cells is yet to be fully understood, it is predicted that FasL pathway is responsible for the activation of NK cells. HeLa cells are classified as Fas positive target cells in which the cells are highly sensitive to the apoptosis-inducing effects of Fas receptor stimulation (Cullen *et al.*, 2013). NK cells killing *via* Fas ligand-mediated pathway is induced through the formation of a death-inducing signaling complex which leads to the activation of caspases cascade and subsequently promotes apoptosis to the CD95-expressing target cells (Yoon *et al.*, 2015). In addition, NK cells serial killing

mechanism can switch from granzyme B-mediated cytotoxicity to death receptor pathway in a time dependent manner (Prager *et al.*, 2019). A study by Prager *et al.* (2019) demonstrated that the response of NK cells serial killing on target cells HeLa showed predominance of granzyme B activity at early time of incubation period (31 ± 16 min) and later at 45 ± 20 min, the cells develop a higher caspase 8 activity. In our study, PBEA-induced NK cells were incubated with the target cells for 24 hours with no perforin expression and low expression levels of granzyme B. These findings could be related to the shift of killing mode of NK cells from perforin granzyme pathway to death receptor pathway over time.

In addition, our findings showed that PBEA was capable of stimulating the activation NK cells of cancer patients by promoting cell death in the target cells. Despite the significant target cells death observed in PBEA-treated NK cells of cancer donors with no expression of perforin and slightly upregulated granzyme B and IFN-γ, it is possible that NK cells killing mechanism towards HeLa cells is also mediated by other pathways such as FasL. However, further investigations are required to elucidate NK cells killing mechanism enhanced by PBEA to provide scientific evidence for this assumption.

5.5 Conclusion

As a conclusion, our findings demonstrated that PBEA induced proliferation of NK cells in healthy donors and promoted cytotoxic activity of NK cells of cervical cancer patients by inducing cell death in HeLa cells. These results demonstrated the immunomodulatory effects of PBEA and support the future evaluation of this extract for further application as immunotherapy in the patients.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSION AND FUTURE RECOMMENDATIONS

6.1 General discussion

Over the last few decades, the search for the medicinal plants with anti-cancer properties and non-toxic to healthy cells remains relevant and desirable due to lack of tumor specificity and multidrug resistance of chemotherapy drugs (Greenwell and Rahman, 2015). *P. bleo*, an edible medicinal plant has been claimed by the locals for its effectiveness in curing cancer (Yen *et al.*, 2013). In order to explore its potential as a source for cancer treatment, it is important to study this plant for its anti-cancer properties to gather more information and understanding on its mechanism of action.

In cancer drug discovery, it is important to find cytotoxic agents that induce cell death with low or no toxicity toward normal cells. According to the NCI, anti-cancer agents with IC_{50} value below than 20 µg/ml is considered highly cytotoxic (Srisawat *et al.*, 2013). In this study, PBEA demonstrated high cytotoxic effects against cervical cancer cells (HeLa cells) while no cytotoxic effects were found on normal cell lines. These findings showed that PBEA has a potential as an anti-cancer agent that is non-toxic to the normal cells. Besides, an acute oral toxicity study found no toxicity in mice following administration of *P. bleo* crude extracts at the highest dose of 2500 mg/kg (Sim *et al.*, 2010b). As toxicity is a major concern with the use of medicinal plants in cancer treatment, hence, the findings of this study can provide a significant information in toxicity awareness for future *in vivo* study of *P. bleo*.

Apoptosis is a programmed cell death to remove damaged or aged cells from the body. One of the hallmarks of cancer is resistance of cancer cells to apoptosis that favor their survival which contribute to tumorigenesis (Hanahan and Weinberg, 2016).

Most of anti-cancer drugs are designed by targeting apoptosis induction to destroy the cancer cells (Hassan *et al.*, 2014). The current findings showed that *P. bleo* has an ability to trigger cell death in HeLa cells through stimulation of pro-apoptotic proteins that work in similar manner as chemotherapeutic drugs. This information provides on high value of *P. bleo* in the development of cancer treatment. However, this study is not intended to isolate the compounds from this plant but with the significant findings from this study, we want people to know about the goodness of this plant especially for health maintenance. In addition, the research on its compounds and therapeutic activities serve as a good platform to strengthen the proof of its anti-cancer properties claimed by people (Yen *et al*., 2013).

Studies have shown that the formation and progression of tumor are closely related to an individual's immune system (Wang *et al.*, 2020). Malignant cells can transform themselves to escape from immune surveillance leading to tumor progression and survival (Lussier and Schreiber, 2016). Anti-tumor immune response can be triggered through both innate and adaptive immunity with the involvement of dendritic cells, macrophages, NK cells, Tregs cells, B cells and $CD4^+$/$CD8^+$ T lymphocytes (Gun *et al.*, 2019; Sarvaria *et al.*, 2017). At present, cancer immunotherapy has gained more attention due to its efficacy in treating various cancer and safe (Wang *et al.*, 2020). The current study found that PBEA possess the ability to boost immune response to destroy the cancer cells by stimulating the activation of NK cells from cervical cancer patients towards the killing of HeLa cells. Furthermore, PBEA has promoted the proliferation of NK cells from healthy donors and this indicates that PBEA was safe and non-toxic to NK cells. Thus, this study has shown that PBEA could potentiate as immunotherapy for cervical cancer treatment in regard to its anti-cancer effects and safety.

On top of that, maintaining a healthy life by consuming plant-based foods is important to enhance the body's immunity against diseases especially cancer. High consumption of plant-based foods rich in antioxidant such as fruits, vegetables and legumes may prevent cancer or improve treatment has been supported by some studies. Results of these studies indicate that vitamins and some antioxidant compounds including carotenoids and flavonoids, are effective to inhibit growth of breast, stomach and human colorectal cancer cells (Martínez-Pérez *et al.*, 2016; Oliveira *et al.*, 2017; Wei *et al.*, 2019). A 6-months clinical trial involving consumption of plant-based diet consist of whole grain and vegetables showed a reduction in prostate-specific antigen (PSA) level among recurrent prostate cancer patients (Nguyen *et al.*, 2006). *P. bleo* contains high level of antioxidant as well other compounds that responsible for its anti-cancer properties which point out its benefit in prevention and cancer treatment. According to this, consumption of *P. bleo* is recommended as it may enhance the immune responses against cancer additionally the current findings supported the people's claim on the usefulness of this plant for cancer prevention and treatment.

This study showed that *P. bleo* has the potential to be an anti-cancer agent. Thus it is suggested to explore more on its anti-cancer immune response involving other types of cancer cells and other immune cells such as $CD4^+$ and $CD8^+$ T cells. Furthermore, a clinical trial as a plant-based diet among healthy people and cancer patients should be carried out to gather more information regarding its effectiveness in cancer prevention and treatment. This information is crucial to create as well as increase the awareness on usage of *P. bleo*.

Thus, our findings showed that PBEA promoted cell death in cervical cancer HeLa cells and possessed stimulatory effects on the activation of NK cells from

cervical cancer patients towards elimination of cancer cells reflecting its anti-cancer properties. Figure 6.1 illustrates the mechanism of action involved in the cytotoxic and immunostimulatory activity on cervical cancer HeLa cells induced by PBEA.

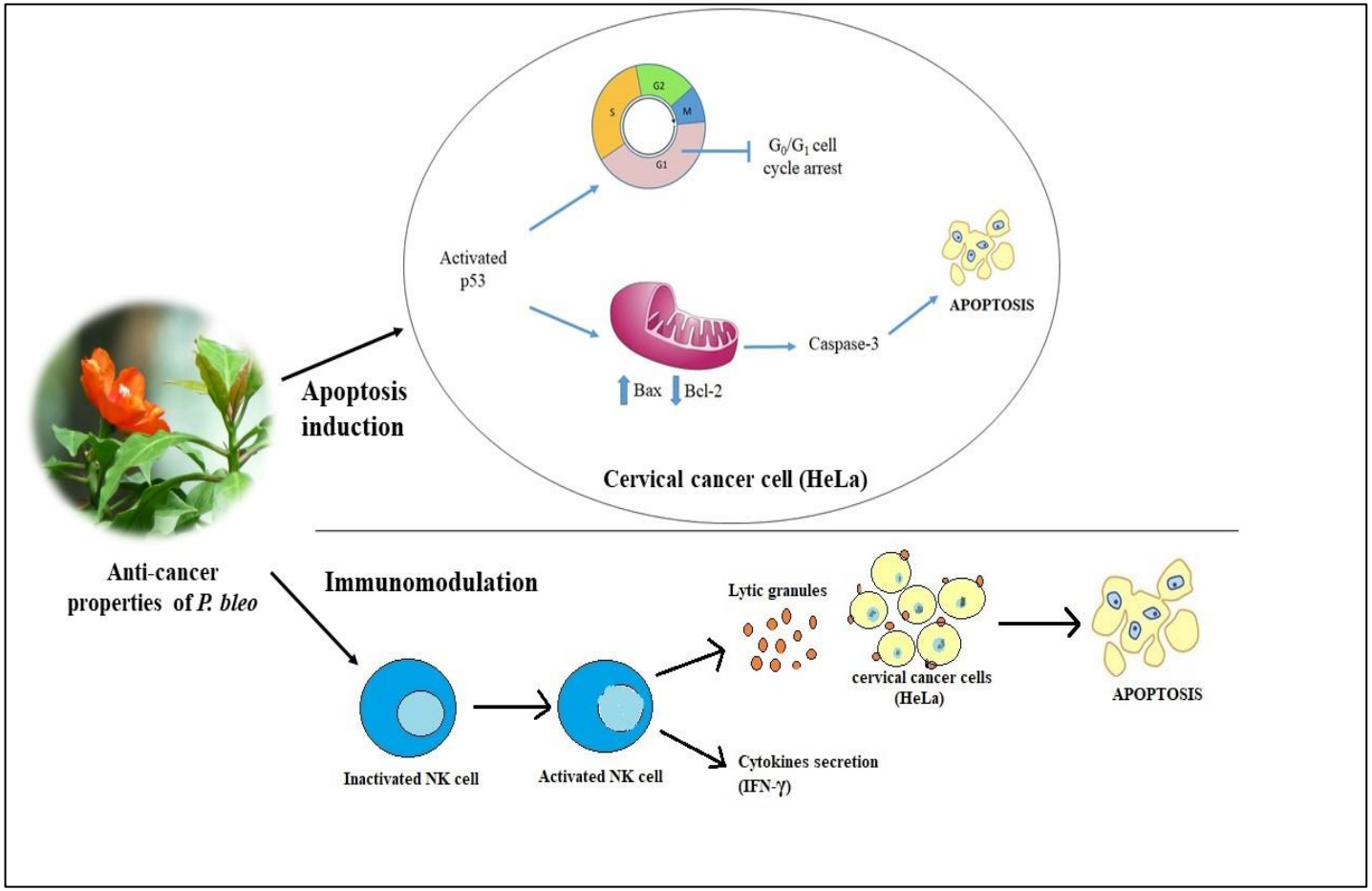

Figure 6.1 Mechanism of cytotoxic and stimulatory activity on cervical cancer HeLa cells induced by PBEA.

6.2 Conclusion

The phytochemicals screening on the leaves of *P. bleo* showed the presence of beneficial compounds that contribute to therapeutic activity of this plant. Ethyl acetate extract of *P. bleo* leaves (PBEA) demonstrated a potent cytotoxic effects against cervical cancer cell line (HeLa) associated with the synergistic effects of all compounds presented in the extract which need to further study in future. The results indicated that PBEA promoted cell death in HeLa *via* apoptosis and inhibition of cell cycle at G_o/G_1 phase mediated through p53 and caspase-3 activation. In addition, PBEA was found to enhance the activation of NK cells in cervical cancer patients by killing HeLa cells through the detection of granzyme B and IFN-γ as well as increased the percentage of apoptotic cells. Thus, it can be concluded that PBEA exerted cytotoxic effects and can potentially regulate NK cells in cervical cancer patients against cervical cells. These findings provided a better understanding of *P. bleo* as anti-cancer agents and potential as immunotherapy in cervical cancer treatment.

6.3 Recommendations for Future Research

The present study has many limitations due to the budget and time constraint. Thus, it is recommended for future studies to find a better approach in order to provide a better understanding on the anti-cancer activity of this plant. Suggestions for the future study on this plant anti-cancer activity are as following:

1. Measurement the effects of direct oral intake of *P. bleo* leaves among healthy individuals and cancer patients for its potential in chemoprevention and nutrition therapy.

2. Investigate the mechanism of cell death in cancer cells using other extracts of *P. bleo* leaves.
3. Study the mechanism of cell death using additional apoptosis markers such as Fas Ligand (FasL) receptor cell death, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), cytochrome-c and caspase-9.
4. Study on chemopreventive and anti-cancer effects of *P. bleo* leaves in animal models.
5. Increase the sample size of healthy individuals and cancer patients in NK cells activation study in order to provide better results and conclusion.
6. Study on the NK cells activation in promoting cancer cell death using other extracts of *P. bleo* leaves.
7. Additional of positive selection CD56 microbeads during purification to obtain maximum purity of NK cells.

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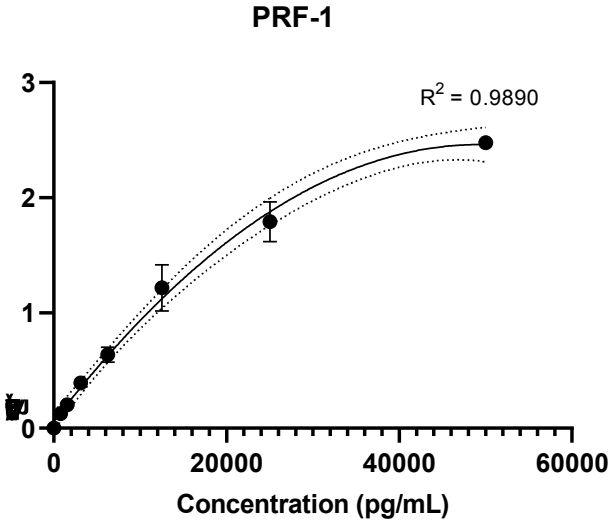
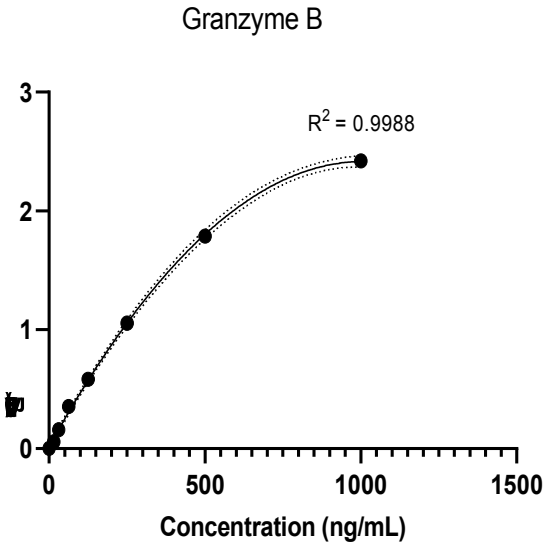
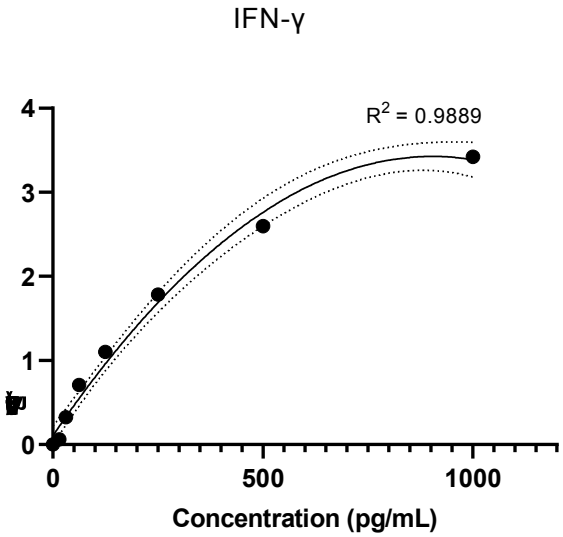
APPENDIX A: STANDARD CURVE OF PERFORIN FOR ELISA



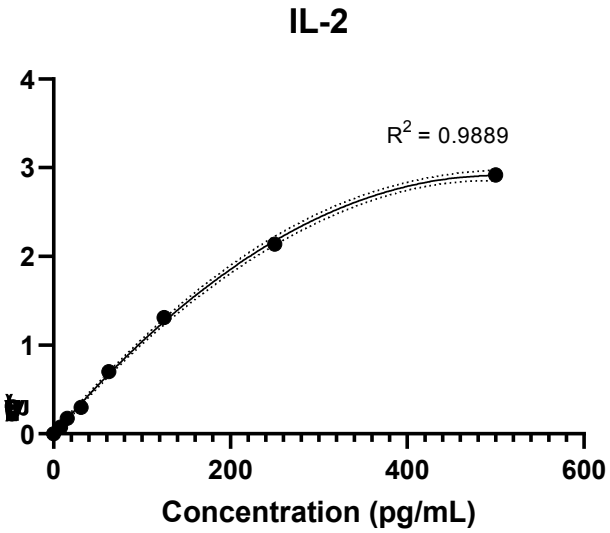
APPENDIX B: STANDARD CURVE OF GRANZYME B FOR ELISA



APPENDIX C: STANDARD CURVE OF IFN-γ FOR ELISA



APPENDIX D: STANDARD CURVE OF INTERLEUKIN-2 FOR ELISA



APPENDIX E: ETHICS APPROVAL FOR BLOOD SAMPLE COLLECTION

Jawatankuasa Etika
Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

7th July 2019

Dr. Norzila Ismail
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JEPeM USM Code: USM/JEPeM/17100566

Study Protocol Title: Understanding the Cytotoxic Effect of Natural Killer Cells towards Selective Cancer Cells Modulated by *Pereskia bleo, Abrus precatorius* and *Liposomes*.

Dear Dr.:

We wish to inform you that the Jawatankuasa Etika Penyelidikan Manusia, Universiti Sains Malaysia (JEPeM-USM) acknowledged receipt of Continuing Review Application dated 26th June 2019.

Upon review of JEPeM-USM Form 3(B) 2019: Continuing Review Application Form, the committee's decision for the **EXTENSION OF APPROVAL IS APPROVED (start from 7th July 2019 till 6th July 2020).** The report is noted and has been included in the protocol file.

JEPeM USM has noted that there is no research activity took place during the period of 13th June 2019 until 6th July 2019. The report is noted and has been included in the protocol file.

Principle Investigator (PI) should aware and concern about the ethical expiration of the study in the future.

Thank you for your continuing compliance with the requirements of the JEPeM-USM.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

(PROF. DR. HANS AMIN VAN ROSTENBERGHE)
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

c.c Secretary
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

JEPeM
JAWATANKUASA ETIKA
PENYELIDIKAN MANUSIA

JAWATANKUASA ETIKA PENYELIDIKAN (MANUSIA) – JEPeM USM
UNIVERSITI SAINS MALAYSIA

TEMPLATE BORANG MAKLUMAT DAN KEIZINAN PESERTA
TEMPLATE OF PARTICIPANT INFORMATION SHEET AND CONSENT FORM

(PROJEK PENYELIDIKAN)
(RESEARCH PROJECT)

Borang Maklumat dan Keizinan Peserta yang digunakan dalam Projek Penyelidikan mestilah mengikuti format maklumat berikut. Namun begitu pernyataan dan ayat yang digunakan hanyalah sebagai panduan sahaja.
The Participant Infomation and Consent Form *used in the Research Project must be according to these information formats. However, statements and phrases used only as a guide.*

- Tajuk Kajian / *Topic of the Research*
- Pengenalan / *Introduction*
- Tujuan Kajian / *Purpose of the Study*
- Kelayakan Penyertaan / *Participants Criteria*
- Prosedur-prosedur Kajian / *Study Procedures*
- Risiko / *Risks*
- Melaporkan Pengalaman Kesihatan / *Reporting Health Experiences*
- Penyertaan dalam Kajian / *Participation in the Study*
- Manfaat yang Mungkin Diperolehi / *Possible Benefits*
- Soalan / *Questions*
- Kerahsiaan / *Confidentiality*
- Tandatangan / *Signatures*

Sebagai **CONTOH**, sila rujuk Borang Maklumat dan Keizinan Peserta yang dilampirkan.
*As an **EXAMPLE**, please refer to the attached Participant Infomation Sheet and Consent Form.*

(Versi Bahasa Malaysia) / *(Bahasa Malaysia Version)*

1. **LAMPIRAN A**
   <Sila masukkan TAJUK KAJIAN>
2. **LAMPIRAN S (Borang Keizinan Peserta)**
3. **LAMPIRAN G (Borang Keizinan Peserta – *Sampel Genetik*)**
4. **LAMPIRAN P (Borang Keizinan Penerbitan Bahan yang Berkaitan dengan Peserta)**

(Versi Bahasa Inggeris) / *(English Version)*

1. **ATTACHMENT B**
   <Please add in the RESEARCH TITLE>
2. **ATTACHMENT S (Participant Information and Consent Form)**
3. **ATTACHMENT G (Participant Information and Consent Form – *Genetic Sample*)**
4. **ATTACHMENT P (Participant's Material Publication Consent Form)**

MAKLUMAT KAJIAN

Tajuk Kajian : Memahami kesan sitotoksik sel Pembunuh Semulajadi (Natural killer cell) ke atas sel kanser payudara dan serviks yang dimodulasi oleh ekstrak *Pereskia bleo, Abrus precatorius* and liposom.

Nama Penyelidik dan penyelidik bersama [sila sertakan no. Pendaftaran badan profesional (contoh MMC) sekiranya berkaitan :

Principal investigator:
Dr. Norzila Ismail (Pharmacology)
Co-Researchers:
1. Prof Dr Armando Acosta (INFORMM)
2. Dr Ramlah Kadir (Immunology)
3. Dr Rohimah Mohamud (Immunology)
4. Dr Wan Zainira Wan Zain (Surgery, No MMC 38667)
5. Dr Rahimah Rahim (O&G, No MMC 38338)
6. Puan Mazni Yusoff (Unit Perubatan Integratif)

PENGENALAN

Kajian ini adalah berkaitan dengan ujikaji kesan ekstrak Daun Saga (*Abrus precatorius*) dan ekstrak daun duri tujuh (*Pereskia bleo*) dan partikel nano semulajadi yang dikenali sebagai liposom, ke atas **sel darah putih** manusia iaitu sel NK (natural killer atau sel pembunuh semulajadi). **Kedua-dua jenis tumbuhan tersebut digunakan secara tradisional untuk merawat kanser oleh masyarakat tempatan**. **Kajian di peringkat sel dan molekul dalam makmal juga telah menunjukkan bahawa ia boleh membunuh sel kanser. Walaubagaimanapun, masih banyak peringkat kajian yang perlu dilakukan untuk memastikan ia benar-benar berkesan untuk membunuh kanser di dalam tubuh manusia**. **Manakala liposom pula adalah sejenis partikel nano-halus yang bersaiz 100 nanometer diameter yang diperolehi darpada bakteria yang tidak berbahaya. Kajian ke atas liposom adalah untuk menjadikannya sebagai molekul "pembawa" ubat terus ke lokasi kanser di dalam tubuh manusia bagi mengurangkan kesan sampingan ubat ke atas sel-sel yang sihat**.

Dalam kajian ini, kami akan melakukan ujian untuk mengetahui sama ada sel NK akan membunuh sel kanser dengan lebih baik atau tidak apabila dikulturkan bersama dengan ekstrak daun dua herba tersebut dan liposom. Sel NK manusia boleh diperolehi daripada darah manusia sebanyak 0.08-0.43%.

Adalah penting bagi anda membaca dan memahami maklumat kajian sebelum anda bersetuju untuk menyertai kajian penyelidikan ini dengan menderma sebanyak 10ml darah sahaja. **Walaupun sumbangan darah anda hari ini tidak memberi impak kepada tahap kanser anda, tetapi sumbangan anda hari ini mungkin akan memberi manfaat dalam penyelidikan kanser, yang seterusnya akan membuka kepada peluang kesembuhan kepada pesakit kanser pada masa akan datang.**

Penyertaan anda di dalam kajian ini dijangka **mengambil masa satu hingga dua jam** yang mana anda perlu menjawab beberapa soalan bagi memastikan anda memenuhi kriteria untuk pengambilan darah; dan juga proses pengambilan darah. Seramai 18 orang dijangka akan menyertai kajian ini.

TUJUAN KAJIAN

Kajian ini bertujuan untuk memahami mekansima ketoksikan sel NK ke atas sel kanser oleh ekstrak aktif daripada *Pereskia bleo* (duri tujuh) dan daun *Abrus precatorius* (saga) serta partikel nano liposom.

KELAYAKAN PENYERTAAN

Salah seorang kakitangan kajian akan membincangkan kelayakan untuk menyertai kajian ini. Adalah penting anda berterus terang kepada kakitangan tersebut termasuk sejarah kesihatan anda.

Kajian ini akan melibatkan individu yang mempunyai ciri-ciri berikut:

Bagi Peserta sihat:

Tiada penyakit berat
Tidak mengambil dadah immunosupresif
Umur 18-45 tahun
Tidak merokok
Tidak hamil

Bagi peserta pesakit kanser:

Disahkan mengidap kanser oleh doktor
Masih belum mendapat apa-apa rawatan
Umur 18-45 tahun
Tidak merokok
Tidak hamil

Kajian ini tidak akan melibatkan individu yang :

Bagi peserta sihat:

Mengambil dadah immunosupresif
Sedang mengalami demam
Enggan memberi kebenaran (consent)

Bagi peserta pesakit kanser:

Tidak didiagnos mempunyai kanser
Mengambil dadah immunosupresif
Mempunyai kanser dengan ko-morbiditi lain
Enggan memberi kebenaran (consent)

***Tidak mengambil dadah immunosupressif sekurang-kurangnya 3 bulan yang lepas.**

*** ko-morbiditi adalah kewujudan penyakit lain pada pesakit kanser, seperti tekanan darah tinggi, penyakit buah pinggang dan lain-lain.**

PROSEDUR-PROSEDUR KAJIAN

Peserta (yang boleh berbahasa melayu) hanya perlu mengisi borang maklumat yang diperlukan untuk mengetahui sama ada peserta termasuk dalam kriteria yang diperlukan. Jika peserta memenuhi kriteria, darah peserta akan diambil oleh individu terlatih sebanyak 10 ml. Setelah selesai, peserta akan diberi honorarium. **Sisa buangan saripada sampel kajian (sisa klinikal biohazard) akan dilupuskan dengan cara yang sistematik.**

RISIKO

Secara umumnya, pesakit tidak akan mengalami sebarang masalah atau risiko dalam proses pengambilan darah sebanyak 10 ml. Sila ikut arahan kakitangan yang mengambil darah dan hanya meninggalkan makmal selepas darah kering. Sila maklumkan kepada kakitangan kajian sekiranya anda menghadapi sebarang masalah atau mempunyai sebarang maklumat penting yang mungkin mengubah persetujuan anda untuk terus menyertai kajian ini.

MELAPORKAN PENGALAMAN KESIHATAN (Jika Kajian Melibatkan Kesihatan SAHAJA)

Sila hubungi kakitangan berikut pada bila-bila masa sekiranya anda mengalami sebarang masalah kesihatan, samada berkaitan atau tidak berkaitan dengan kajian ini.
Dr Wan Zainira Wan Zain **[No. Pendaftaran Penuh Majlis Perubatan Malaysia: 38667]** di talian 019-9216789 atau Dr Rahimah Rahim **[No. Pendaftaran Penuh Majlis Perubatan Malaysia: 38338]** di talian 012-8330274 secepat mungkin.

PENYERTAAN DALAM KAJIAN

Penyertaan anda dalam kajian ini adalah secara sukarela. Anda berhak menolak untuk menyertai kajian ini atau menamatkan penyertaan anda pada bila-bila masa, tanpa sebarang kehilangan manfaat yang sepatutnya anda perolehi. Hasil kajian ini tidak akan dimaklumkan kepada peserta kerana ia merupakan hasil dari kombinasi sel darah putih dari beberapa peserta dan bukan berorientasikan individu.

Penyertaan anda juga mungkin boleh diberhentikan oleh kakitangan kajian ini tanpa persetujuan anda sekiranya anda didapati tidak sesuai untuk meneruskan kajian ini berdasarkan protokol kajian. Kakitangan kajian akan memaklumkan anda sekiranya anda perlu diberhentikan dari menyertai kajian ini.

MANFAAT YANG MUNGKIN [Manfaat terhadap Individu, Masyarakat, Universiti]

Walaupun tiada manfaat secara langsung, walaubagaimanapun, secara tidak langsung hasil kajian ini akan dapat memberi manfaat kepada masyarakat umum untuk mendapat kesedaran (awareness) bahawa terdapat tumbuhan-tumbuhan yang bermanfaat untuk melawan kanser.

PERSOALAN

Sekiranya anda mempunyai sebarang soalan mengenai prosedur kajian ini atau hak-hak anda, sila hubungi;

Dr. Norzila Ismail
Jabatan Farmakologi
Pusat Pengajian Sains Perubatan
USM Kampus Kesihatan
09-7676135 / 010-2251980

Sekiranya anda mempunyai sebarang soalan berkaitan kelulusan Etika atau sebarang pertanyaan dan masalah berkaitan kajian ini, sila hubungi;

En. Mohd Bazlan Hafidz Mukrim
Setiausaha Jawatankuasa Etika Penyelidikan (Manusia) USM
Bahagian Penyelidikan dan Inovasi (P&I)
USM Kampus Kesihatan.
No. Tel: 09-767 2354 / 09-767 2362
Email : bazlan@usm.my or jepem@usm.my

KERAHSIAAN

Maklumat privasi seperti nama dan alamat serta identiti yang lain tidak akan diambil dalam kajian ini, melainkan status kesihatan serta soal selidik bagi memastikan pesakit memenuhi kriteria yang diperlukan dalam kajian. Oleh sebab itu, kerahsiaan peserta adalah selamat.

Data yang diperolehi dari kajian ini tidak akan mengenalpasti anda secara perseorangan. Hasil kajian tidak berkaitan dengan identiti individu yang terlibat dalam pendermaan darah dan mereka yang menderma tidak akan dihubungi semula. Walaubagaimanapun hasil kajian mungkin akan diterbitkan untuk tujuan perkongsian ilmu. Panel penilai JEPeM-USM dan ahli jawatankuasa terlibat boleh meneliti data kajian apabila diperlukan.

Bagi pesakit kanser, semua data dalam rekod perubatan anda mungkin akan disemak dan diuruskan oleh pakar klinikal yang terlibat dalam kajian ini, bagi tujuan memastikan anda memenuhi kriteria yang diperlukan dalam kajian. Kerahsiaan akan dijaga mengikut etika kerahsiaan pesakit. Darah yang diambil juga akan melalui proses kajian di dalam makmal tanpa mengenalpasti pemilik darah. **Setelah selesai kajian, sampel darah akan dilupuskan sebagaimana pelupusan sisa klinikal biohazard.**

Dengan menandatangani borang persetujuan ini, anda membenarkan penelitian rekod, penyimpanan maklumat dan pemprosesan data seperti yang dihuraikan di atas.

TANDATANGAN

Untuk dimasukkan ke dalam kajian ini, anda atau wakil sah anda mesti menandatangani serta mencatatkan tarikh halaman tandatangan (Lihat contoh Borang Keizinan Peserta di **LAMPIRAN S** atau **LAMPIRAN G (untuk sampel genetik)** atau **LAMPIRAN P**).

LAMPIRAN S

Borang Keizinan Peserta
(Halaman Tandatangan)

Tajuk Kajian: Memahami kesan sitotoksik sel Pembunuh Semulajadi (Natural killer cell) ke atas sel kanser payudara dan serviks yang dimodulasi oleh ekstrak *Pereskia bleo, Abrus precatorius* and liposom

Nama Penyelidik: **Dr. Norzila Ismail, Dr. Ramlah Kadir, Dr. Rohimah Mohamud, Prof Armando Acosta, Dr Wan Zainira Wan Zain (Surgery, No MMC 38667), Dr Rahimah Rahim (O&G, No MMC 38338),Puan Mazni Yusoff (Unit Perubatan Integratif)**

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan yang berikut:

- Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan Pesakit ini **termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian** dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.
- Semua soalan-soalan saya telah dijawab dengan memuaskan.
- Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.
- Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.
- Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Peserta untuk simpanan peribadi saya.

Nama Peserta

No. Kad Pengenalan Peserta

Tandatangan Peserta atau Wakil Sah

Tarikh (dd/MM/yy)
(Masa jika perlu)

Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan

Tarikh (dd/MM/yy)

Nama Saksi dan Tandatangan

Tarikh (dd/MM/yy)

Nota: i) Semua peserta yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran.

LAMPIRAN G

Borang Keizinan Peserta untuk Pengambilan *Sampel Genetik* (Halaman Tandatangan)

Tajuk Kajian: Memahami kesan sitotoksik sel Pembunuh Semulajadi (Natural killer cell) ke atas sel kanser payudara dan serviks yang dimodulasi oleh ekstrak *Pereskia bleo, Abrus precatorius* and liposom

Nama Penyelidik: **Dr. Norzila Ismail, Dr. Ramlah Kadir, Dr. Rohimah Mohamud, Prof Armando Acosta, Dr Wan Zainira Wan Zain (Surgery, No MMC 38667), Dr Rahimah Rahim (O&G, No MMC 38338),Puan Mazni Yusoff (Unit Perubatan Integratif)**

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan yang berikut:

- Saya telah membaca semua maklumat dalam Borang Maklumat dan Keizinan Pesakit ini **termasuk apa-apa maklumat berkaitan risiko yang ada dalam kajian** dan saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.
- Semua soalan-soalan saya telah dijawab dengan memuaskan.
- Saya, secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.
- Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.
- Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Peserta untuk simpanan peribadi saya.

Nama Peserta

No. Kad Pengenalan Peserta

Tandatangan Peserta atau Wakil Sah

Tarikh (dd/MM/yy)
Masa (jika perlu)

Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan

Tarikh (dd/MM/yy)

Nama Saksi dan Tandatangan

Tarikh (dd/MM/yy)

Nota: i) Lebihan sampel kajian ini akan dilupuskan dan tidak akan digunakan untuk tujuan lain kecuali setelah mendapat kebenaran daripada Jawatankuasa Etika Penyelidikan (Manusia), USM.
ii) Semua peserta yang mengambil bahagian dalam projek penyelidikan ini tidak dilindungi insuran.

LAMPIRAN P

Borang Keizinan bagi Penerbitan Bahan yang berkaitan dengan Peserta Kajian (Halaman Tandatangan)

Tajuk Kajian: Memahami kesan sitotoksik sel Pembunuh Semulajadi (Natural killer cell) ke atas sel kanser payudara dan serviks yang dimodulasi oleh ekstrak *Pereskia bleo, Abrus precatorius* and liposom

Nama Penyelidik: **Dr. Norzila Ismail, Dr. Ramlah Kadir, Dr. Rohimah Mohamud, Prof Armando Acosta, Dr Wan Zainira Wan Zain (Surgery, No MMC 38667), Dr Rahimah Rahim (O&G, No MMC 38338),Puan Mazni Yusoff (Unit Perubatan Integratif)**

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini.

Dengan menandatangani mukasurat ini, saya memahami yang berikut:

- Bahan yang akan diterbitkan tanpa dilampirkan dengan nama saya dan setiap percubaan yang akan dibuat untuk memastikan ketanpanamaan saya. Saya memahami, walaubagaimanapun, ketanpanamaan yang sempurna tidak dapat dijamin. Kemungkinan sesiapa yang menjaga saya di hospital atau saudara dapat mengenali saya.
- Bahan yang akan diterbitkan dalam penerbitan mingguan/bulanan/dwibulanan/suku tahunan/dwi tahunan merupakan satu penyebaran yang luas dan tersebar ke seluruh dunia. Kebanyakan penerbitan ini akan tersebar kepada doktor-doktor dan juga bukan doktor termasuk ahli sains dan ahli jurnal.
- Bahan tersebut juga akan dilampirkan pada laman web jurnal di seluruh dunia. Sesetengah laman web ini bebas dikunjungi oleh semua orang.
- Bahan tersebut juga akan digunakan sebagai penerbitan tempatan dan disampaikan oleh ramai doktor dan ahli sains di seluruh dunia.
- Bahan tersebut juga akan digunakan sebagai penerbitan buku oleh penerbit jurnal.
- Bahan tersebut tidak akan digunakan untuk pengiklanan ataupun bahan untuk membungkus.

Saya juga memberi keizinan bahawa bahan tersebut boleh digunakan sebagai penerbitan lain yang diminta oleh penerbit dengan kriteria berikut:

- Bahan tersebut tidak akan digunakan untuk pengiklanan atau bahan untuk membungkus.
- Bahan tersebut tidak akan digunakan di luar konteks – contohnya: Gambar tidak akan digunakan untuk menggambarkan sesuatu artikel yang tidak berkaitan dengan subjek dalam foto tersebut.

_____ _____

Nama Peserta

_____ _____ _____

No. Kad Pengenalan Peserta T/tangan Peserta **Tarikh** (dd/MM/yy)

_____ _____

Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan **Tarikh** (dd/MM/yy)

LIST OF PUBLICATIONS

1. **Mohd-Salleh, S. F.,** Wan-Ibrahim, W. S., & Ismail, N. (2019). *Pereskia bleo* Leaves Extract Induces Cell Death via Cell Cycle Arrest and Apoptosis in Cervical Cancer Cells HeLa. *Nutrition and cancer*, 1-9.

2. **Mohd-Salleh, S. F.**, Ismail, N., Wan-Ibrahim, W. S., & Tuan Ismail, T. N. N. (2020). Phytochemical Screening and Cytotoxic Effects of Crude Extracts of *Pereskia Bleo* Leaves. *Journal of Herbs, Spices & Medicinal Plants*, 1-12.

LIST OF PRESENTATIONS

Oct 2018 **Kelantan Research Day 2018**
(Oral)
GC-MS Analysis of *Pereskia bleo* Leaves Aqueous Extract
Siti Farhanah Mohd Salleh, Wan Suriyani Wan Ibrahim, Tuan Nadrah Naim Tuan Ismail and Norzila Ismail

Aug 2019 **3rd International Conference of Medical and Health Sciences – 24th National Conference of Medical and Health Sciences**
(Poster)
Crude Ethyl Acetate Extract of *Pereskia bleo* Promotes Cell Death in HeLa Cells *via* G_0/G_1 Cell Cycle Arrest and Apoptosis
Siti Farhanah Mohd Salleh, Norzila Ismail and Wan Suriyani Wan Ibrahim