# PHYTOCHEMICAL CHARACTERIZATION, INDUCTION OF APOPTOSIS AND ACTIVATION OF NATURAL KILLER (NK) CELLS BY *Abrus precatorius* LEAVES EXTRACT ON HUMAN BREAST CANCER CELL LINE

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*Faliq Adeeba*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | Degree Celsius |
| µg | Microgram |
| ABP | Name of a peptide from *Abrus* lectin |
| AD | Alzheimer's disease |
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| AGP | Name of a peptide from *Abrus* lectin |
| AKT | Protein kinase B |
| ALS | Amyotrophic lateral sclerosis |
| ANOVA | Analysis of variance |
| APME | *Abrus precatorius* methanol leaves extract |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| BALB/c | Albino laboratory-bred strain mice |
| Bax | Bcl-associated X |
| BC | Before Christ |
| Bcl-2 | B-cell Lymphoma 2 |
| BSA | Bovine serum albumin |
| CAM | Complementary and alternative medicine |
| Caspase | Cysteine-aspartic proteases |
| CD | Cluster of differentiation |
| CO2 | Carbon dioxide |
| COMT | catechol-O-methyl-transferase |
| DL | Dalton's Lymphoma |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic |
| ELISA | Enzyme-linked immunosorbent assay |
| FA | Fatty acids |
| FADD | Fas dissociated death domain |
| FasL | Fas ligand |
| FITC | Fluorescein isothiocyanate |
| FLIP | Fas-associated death domain-like interleukin-1-β-converting enzyme-inhibitory protein |

| | |
|---|---|
| FSC | Forward scatter |
| GCMS | Gas chromatography Mass spectrometry |
| GzmB | Granzyme B |
| h | hour |
| HRP | Horseradish peroxidase |
| IC50 | Inhibitory concentration at 50% |
| IFN-γ | Interferon gamma |
| IL | Interleukin |
| kDa | Kilodalton |
| KIRs | Killer-cell immunoglobulin-like receptors |
| MAG | Monoacylglycerol |
| MAPK | Mitogen Activated Protein Kinase |
| MCG | Microglial cells |
| MHC 1 | Major histocompatibility complex 1 |
| ml | Millilitre |
| MMP | Matrix metalloproteinases |
| MOMP | Mitochondrial Outer Membrane Protein |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| NCI | National Cancer Institute |
| NCR | Natural cytotoxic receptor |
| NF-κB | nuclear factor kappa b |
| NK | Natural killer |
| nm | Nanometre |
| NO | nitric oxide |
| OD | Optical density |
| PBMC | Peripheral blood mononuclear cells |
| PE | Phycoerythrin |
| PI | Propidium iodide |
| PPARγ | Peroxisome Proliferator-Activated Receptor gamma |
| PRF1 | Perforin |
| RT | Room temperature |
| SSC | Side scatter |
| STAT3 | Signal Transducer and Activator of Transcription 3 |

| | |
|---|---|
| TNF-α | Tumor Necrosis Factor alpha |
| TRAIL | TNF Releated Apoptosis Inducing Ligand |
| TSG | Tumour Suppressor Gene |
| USA | United State of America |
| USD | United State Dollar |
| USM | Universiti Sains Malaysia |
| WHO | World Heatlth Organization |
| XIAP | X-linked inhibitor of apoptosis |

**PENCIRIAN FITOKIMIA, PENGARUHAN APOPTOSIS DAN PENGAKTIFAN SEL PEMBUNUH SEMULAJADI (NK) OLEH EKSTRAK DAUN *Abrus precatorius* KE ATAS TITISAN SEL KANSER PAYUDARA MANUSIA**

## ABSTRAK

Kanser masih merupakan salah satu masalah global yang mengancam populasi dunia secara amnya. Pencarian penawar untuk kanser masih lagi dijalankan dengan pesat. Walaupun perubatan konvensional masih merupakan pilihan nombor satu dalam rawatan kanser, perubatan secara tradisi tetap juga menjadi pilihan oleh pesakit kanser. Pendekatan rawatan tradisional yang menggunakan tumbuhan ubatan masih lagi diamalkan secara meluas sejak berpuluh dan ratusan tahun yang lampau. Kebolehan tumbuhan berubat untuk menghalang pembiakan sel kanser berserta kemampuannya untuk mengaktifkan sistem imun tubuh badan merupakan salah satu strategi yang paling ideal untuk melawan kanser. Oleh itu, pemahaman dan pembuktian secara saintifik berkenaan mekanisma keupayaan tumbuhan berubat untuk melawan kanser akan mengurangkan jurang ilmu pengetahuan yang belum lagi diterokai berkenaan tumbuhan tersebut. Dalam kajian ini, satu tumbuhan berubat yang dikenali sebagai *Abrus precatorius* atau pokok saga, diselidiki. Daun tumbuhan ini digunakan secara tradisional untuk merawat pelbagai penyakit termasuk kanser. Daun tumbuhan ini dipilih dan diekstrak menggunakan beberapa teknik pengekstrakan dengan pelarut yang berbeza. Analisis fitokimia dilakukan menggunakan GC-MS. Keupayaan ekstrak tumbuhan ini untuk menghalang pembiakan sel kanser dianalisis menggunakan asai MTT. Ekstrak terbaik yang menunjukkan $IC_{50}$ paling rendah ke atas sel kanser terpilih seterusnya dipilih untuk analisis selanjutnya untuk melihat mekanisma induksi

kematian sel. Ini diukur dengan analisis penghentian kitaran sel, pewarnaan apoptosis dengan AnnexinV/PI, dan akhir sekali dengan mengukur ekspresi protein p53, Bax, Bcl-2 dan Caspase-3. Keupayaan ekstrak ini untuk merangsang tindakbalas sistem imun dengan mengaktifkan sel pembunuh semula jadi (NK) dinilai melalui uji kaji yang melibatkan proses pengkulturan sel NK bersama dengan sel kanser sasaran, MDA-MB-231. Ini diamati dengan analisis kematian sel sasaran dan kuantifikasi rembesan sitokin interleukin-2 (IL-2) dan interferon gamma (IFN-γ) berserta perforin (PRF-1) dan granzyme B (GzmB). Hasil kajian mendapati, ekstrak yang diperoleh melalui kaedah Soxhlet menggunakan pelarut etil asetat dan metanol mempunyai sebatian finolik dan terpenoid yang paling tinggi berbanding ekstrak yang lain. Ekstrak metanol yang diperolehi secara kaedah Soxhlet (APME) menunjukkan $IC_{50}$ paling rendah ke atas sel kanser MDA-MB-231. Analisis selanjutnya menggunakan sitometri aliran, menunjukkan kemampuan APME untuk mengaruh kematian sel secara apoptosis melalui perencatan DNA di fasa G0/G1 dalam kitaran sel, peningkatan ekspresi protein p53, Bax dan Caspase-3; dan diregulasi protein Bcl-2. APME juga mampu mengaktifkan sel NK (daripada penderma sihat) untuk menjadi sitotoksik dan mengakibatkan apoptosis ke atas sel kanser. Peningkatan rembesan IFN-γ dan PRF-1 dapat dilihat dari eksperimen ko-kultur ini. Penemuan ini menunjukkan keupayaan *A. precatorius* untuk bertindak sebagai agen anti proliferasi ke atas sel kanser dan perangsang ke atas sel NK dari penderma yang sihat. Ini mungkin diakibatkan oleh kehadiran pelbagai sebatian kimia dalam profil tumbuhan tersebut yang bertindak secara sinergistik.

**PHYTOCHEMICAL CHARACTERIZATION, INDUCTION OF APOPTOSIS AND ACTIVATION OF NATURAL KILLER (NK) CELLS BY *Abrus precatorius* LEAVES EXTRACT ON HUMAN BREAST CANCER CELL**

## ABSTRACT

Cancer is still one of the global menace and poses a threat to the general world population. The search for cancer cure is also still on the race. Although conventional medicine remains the number one choice in cancer treatment, traditional approach is also still one of the favourable choices made by cancer patients to deal with this horrible disease. Traditional approaches, mainly by utilising medicinal plants are widely sought after in many countries since centuries ago. The ability of medicinal plants to exhibit their anti-proliferative activity, together with the ability to activate immune responses would be the ideal strategy to beat the disease. Therefore, understanding the mechanisms of medicinal plants displaying their anticancer properties scientifically would fill the gap of unknown knowledge about them. A medicinal plant known as *Abrus precatorius* or 'saga' were used in this study. This plant has been utilised traditionally to cure various ailments including cancer. The leaves of *A. precatorius* were selected to be extracted by different extraction techniques which employed different types of solvent. GC-MS was employed to provide the phytochemical analysis of the extracts. The ability of those extract to inhibit proliferation in cancer cells were measured using MTT assay. The best extract exhibiting the lowest inhibitory concentration ($IC_{50}$) on the selected cancer cell, was selected to determine the mechanisms of action in inducing the cell death. Cell cycle arrest analysis, apoptosis staining with AnnexinV/PI and quantification of the expression of p53, Bac, Bcl-2 and Caspase-3 proteins were used to determine the

mechanism of cell deaths. Finally, the ability of the extract to induce immune response by activating NK cells was determined in a co-culture experiment of the NK cells with the target cell, MDA-MB-231 cells. This was observed by the analysis of target cell deaths and quantification of the secretion of cytokines, interleukin-2 (IL-2) and interferon gamma (IFN-γ); and the degranulation of the cytotoxic granules by quantifying the perforin (PRF-1) and granzyme B (GzmB). The results showed that the ethyl acetate and methanol extracts prepared using Soxhlet contained the highest phenolic and terpenoid compounds comparing to the other extracts. The methanol extract obtained by Soxhlet, APME (*A. precatorius* methanol extract), exhibited the lowest $IC_{50}$ value on MDA-MB-231 cells. Further analysis by flow cytometry revealed APME induced cell death on MDA-MB-231 cells *via* apoptosis, through DNA arrest at G0/G1 cycle, coupled with an increase of p53, Bax and Caspase-3 expression and decrease of Bcl-2 expression. APME was also found to activate NK cells (from healthy donor) by causing NK cell cytotoxic activity *via* apoptosis in the target cells. Increased levels of INF-γ and PRF-1 were also observed in this co-culture experiment. These findings reflect the ability of *A. precatorius* leaves extract to exhibit the anti-proliferative effect on cancer cells and stimulatory effect on NK cells from the healthy donors. This might be due to the presence of various phytochemical compounds in the extract that might act synergistically.

# CHAPTER 1

# GENERAL INTRODUCTION

Cancer is one of the leading causes of death worldwide. Choices of cancer treatments are bone marrow transplantation, chemotherapy, hormone therapy, immunotherapy, radiotherapy and surgery. Cancer chemotherapy employing cytotoxic drugs targeting the apoptosis pathway still remains as the main choice to eradicate cancer cells in medical oncology. However, the non-specificity of the drugs might also cause toxicity to the neighbouring normal healthy cells. Prolong exposure to these drugs lead to severe toxicity effects including drug resistance, infertility and carcinogenicity. This limitation makes the search for alternative anticancer agents that are not toxic to normal and healthy cells, in demand. Therefore, using the guide from folklore medicinal practices (Ulung and Studi, 2014), a medicinal plant, *Abrus precatorius,* was chosen for this current study. This medicinal plant works wonder in traditional medicinal practices where it is widely used for many ailments such as coughs, diarrhoea, wound healing and even including as anticancer and anti virus (Ulung and Studi, 2014). The investigation started with the phytochemical analysis of the plant extract, ability of the plant extract to promote cell death, and finally the ability of the extract to activate the natural killer (NK) cells activity.

## 1.1 Medicinal plants as potential anticancer agent

Herbal medicine or medicinal plants or also referred as botanical medicine or phytomedicine, is broadly defined as the use of part or whole plants for illness prevention or treatment. Traditionaly, plants have always been the main source of continuous remedies for mankind since thousand years ago. Their therapeutic potentials were based on the observations and findings they made throughout those years. This knowledge of practising traditional medicinal plant is known as wisdom.

It led to the continuous use of the medicinal plants, and has become the basis of many studies that led to the discovery of drugs for many diseases including cancer.

Most of the drugs available currently, are obtained from medicinal plants, such as reserpine from *Rauvolfia sepentina,* atropine from *Atropa belladonna* and morphine, obtained from the unripe seedpods of *Papaver somniferum* dried latex (Mondal *et al.*, 2016). Aspirin, the analgesic drug is also one of the important example of drug discoveries from willow bark tree (Gonzalez and Morer, 2017). Different plant extracts have been proven to exert different biological activities among others include anticancer. Medicinal plants known to exhibit anticancer properties are generally comprised of a large collection of phenolic compounds. These compounds are found to inhibit carcinogenesis by interfering at the specific stages of the event (David *et al.*, 2016). Therefore identification of the composition of phytochemicals in those plants helped us to understand and discover more about the medicinal properties of those plants, besides laying out the information needed to support the traditional wisdom of the use of medicinal plants.

### 1.2 Anti-proliferative Activity and Apoptosis effect of Medicinal Plants

Many studies have been carried out to investigate the effectiveness of a medicinal plant extracts to induce apoptosis in cancer cells. The effectiveness of the plant extracts to exhibit the anti-proliferative activity on cancer cells was firstly investigated, then 'how' or the mechanism of the cell death was determined. This is often involved the determination of the $IC_{50}$ of the extract, followed by analysis of the apoptosis proteins expression. $IC_{50}$ is the half maximal of the inhibitory concentration to evaluate the performance of a test substance or drug (Sebaugh, 2011). Apoptosis is an orchestrated programmed cell death that is characterized by specific biochemical and morphological properties.

Apoptosis is a regulated and controlled pathway of multicellular organisms to eliminate unwanted cells. Failure of apoptosis leads to uncontrolled cell proliferation that may lead to cancer. One of the ways for cancer treatment is targeting the apoptosis pathway, either stimulating the pro-apoptotic proteins or inhibiting anti-apoptotic proteins. These apoptotic proteins are known to regulate the event of the cell death. Among the widely investigated proteins are p53, Bcl-2 family proteins and the caspases (Roy *et al.*, 2018). These proteins create a network of communication among each other as a response to the stimuli of initiating cell deaths. The stimuli includes DNA damage or stressed cells due to heat, radiation or cytotoxic exposure. Medicinal plants have been promoted as potential chemoprevention agents due to the human consumption as dietary supplement and health maintenance purposes since decades ago. Many scientific evidence have demonstrated that medicinal plants can inhibit the carcinogenesis process effectively (Singh *et al.*, 2019). As an alternative therapy, medicinal plants were also administered to cancer patients to prevent and treat cancer in recent years (Gezici and Şekeroğlu, 2019). Therefore, the understanding of a medicinal plant extract ability to cause cell death through apoptosis will open up to possibilities of new cancer therapy or chemoprevention.

### 1.3 Medicinal plants and Natural Killer Cells

Another area of interest in the medicinal plants research is the study on immune response towards the introduction of the plant extract. Furthermore, this information would answer if certain medicinal plants would stimulate the immune response in order to promote cancer cell deaths. The immune system consists of cells that prevent, detect and eliminate pathogens and unwanted cells in the body. Natural killer (NK) cells are unique innate immune cells that are important in cancer immune surveillance. NK cells kill target cells by recognition of the target cells surface proteins mainly the

lacking of MHC class I protein. However, cancer cells have their own mechanisms to evade this immune - surveillance. Some natural compounds have demonstrated the ability to act as NK cells stimulator. Vitamins are known to be useful for our body, and vitamins such as vitamin A, B, C, D and E also have been found to help the stimulation of NK cells. As reviewed by Grudzien and Rapak (2018), phytochemicals that were found to act as NK cells stimulator are genistein, curcumin, ginseng extract, garlic extract, resveratrol, poison gooseberry extract, kumquat pericarp extract, prostratin, lectin and polysaccharides.

### 1.4 *Abrus precatorius* as potential anticancer agent

*A. precatorius* is native to India and mostly grows in tropical and subtropical areas of the world. In traditional Hindu medicine, it has been used since ancient times where in some regions the leaves were chewed to treat mouth ulcer. These similar practices were also found in other ancient cultures including China. The leaves are also used as nerve tonic and are useful for its anti-inflammatory properties to treat wounds and swellings. Oil extracted from the *A. precatorius* seeds are used to promote hair growth while the roots are used for jaundice, gonorrhoea and haemoglobinuria (Samy *et al.*, 2008).

Traditionally in Malaysia, the leaves of *Abrus precatorius* are used to treat ailments such as fever, ulcer and mouth cancer (Ulung and Studi, 2014). These traditional practices however have never been documented and the usage of the plant is only based on popular folklore among the local people. Decoction of the leaves is widely practised as the treatment for cold, coughs and colic. Juice from the leaves is applied to swellings by mixing with oil (Bamola *et al.*, 2018). Mixture of rice starch and the leaf paste are consumed orally for anthrax treatment (Pokharkar *et al.*, 2011).

Powdered leaves paste is used for conjunctivitis and convulsion in children (Joshi and Tyagi, 2011).

*Abrus precatorius* is one of the medicinal plants listed to exhibit many types of biological activities including anticancer (Ghosh *et al.*, 2017; Gul *et al.*, 2018; Lebri *et al.*, 2015; Oladimeji *et al.*, 2016; Sofi *et al.*, 2018). M. Gul et al., (2013, 2018) reported anti-proliferative activities of *A. precatorius* against human acute monocytic leukemia cell line (THP-1), while Sofi et al., (2013, 2018) reported the anti-proliferative activities on MDA-MB-231. However, Sofi et al., (2013, 2018) used the aqueous extract and fractions prepared from gradient elution of ethyl acetate extract. Therefore, *A. precatorius* serves as a promising plant as the possible candidate for cancer therapy. However, deeper understanding and fundamental information needed to be gathered about this medicinal plant.

## 1.5 Rationale & Objectives of this study

Many people nowadays are looking for an alternative or complementary treatment to chemotherapy that is not only effective to eradicate cancer cells but also harmless to other healthy and normal functioning cells and tissues. Previous studies of *A. precatorius* have shown the ability of the plant to exhibit anticancer properties and it has been used in traditional settings since many years ago. However, most of these studies are from India and Africa. Less is known about the ability of our home grown species. In traditional setting, medicinal plants are mostly taken raw in crude extract form. Synergistic actions among phytocompounds in the crude extract might contribute to the medicinal properties of these plants (Ma *et al.*, 2009), furthermore, few studies have suggested that the crude extract usages were more effective compared to using isolated single compound (Aiyelaagbe *et al.*, 2011; Rasoanaivo *et al.*, 2011).

A deeper understanding of the ability of *A.precatorius* to induce cytotoxicity and promote immune stimulation were investigated, in order to provide beneficial fundamental pharmacological information on this medicinal plant. Therefore, the objectives of this study are as follows:

**General Objective:**

To study the ability of *Abrus precatorius* leaves as an anticancer agent through its ability to induce apoptosis and to promote the activation of natural killer cell.

**Specific Objectives:**

1. To employ different extraction strategies on *A. precatorius* leaves employing different extraction processes and solvents and analyse the presence of the phytochemical compounds by gas chromatography mass spectometry (GC-MS)

2. To determine the effects of the extracts as anti-proliferative agents on the selected normal and cancer cell lines and to investigate the mechanism of cell death imposed by the selected extract on the corresponding cancer cell.

3. To observe the ability of the selected extract to induce Natural Killer (NK) cells activation in co-culture experiment with the selected cancer cells, using NK cells isolated from healthy and cancer donors.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 CANCER

The word ‘cancer’ is originated from the word ‘carcinoma’ from Latin that means, crab. Cancer is the most feared disease and it refers to the malignant tumours resulted from abnormal cell growth. Cancer is one of the leading causes of death globally with 9.6 millions mortalities in 2018 (World Health Organization, 2018). The prevalence is increasing in both men and women where one in every six deaths is due to cancer. Among the top leading cancer fatalities are colorectal, stomach, lung and breast. In Malaysia, out of 43, 837 new cases reported in 2018, 7593 of them were breast cancer cases (World Health Organization, 2019a). Prevalence of cancer cases reported in Malaysia is presented in Figure 2.1. Most of the cancers affect the age group of 50 – 60, however the incident of the disease is not affected by sex. Conversely, the site of growth differs between men and women, which cause men to be associated with intestine, prostate and lung cancer, while women are mostly affected by breast, uterus, gall-bladder and thyroid cancer.

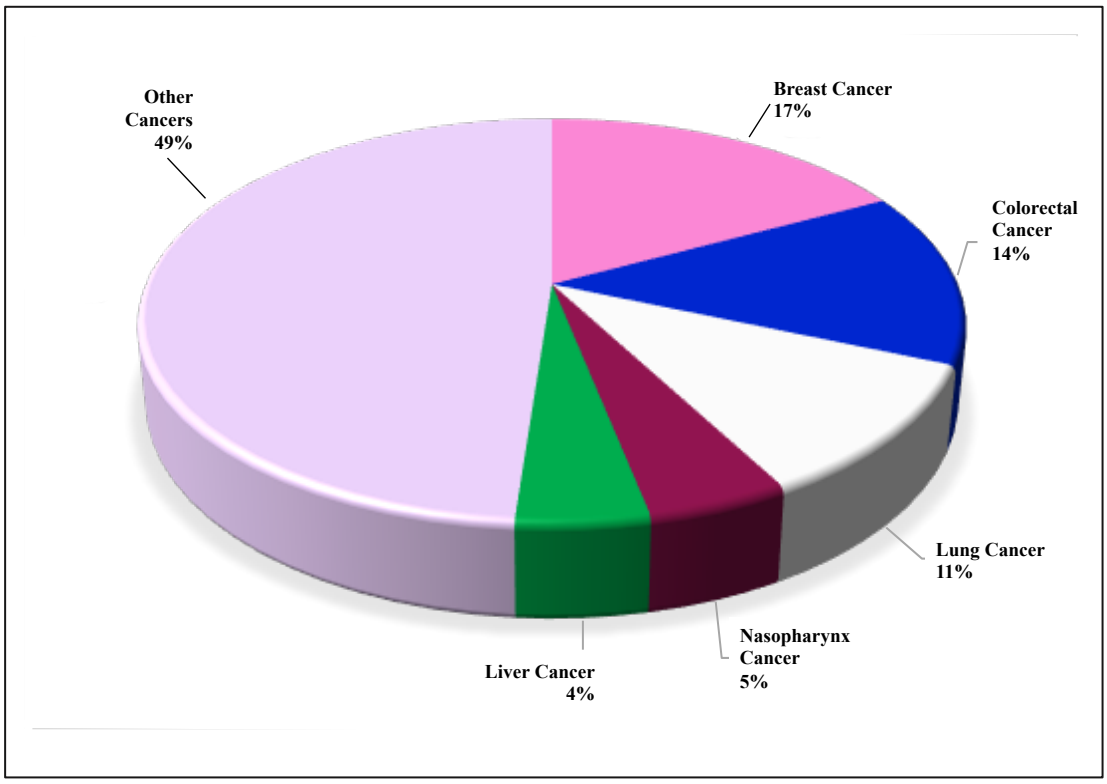



Figure 2.1: **Cancer cases reported in Malaysia in the year 2018**
The chart is recreated from the data published by
(World Health Organization, 2019a)

### 2.1.1 Hallmark of Cancer

Normal cells have many factors controlling their growth and proliferation. Their growth are normally regulated by growth factors. If the cells are damaged, there will be another regulatory mechanisms that will stop their growth and division until they are repaired. If the damage is irreparable, the cells will "self destruct". Therefore, in order for cancer cells to survive, they have to overcome these regulatory factors controlling the normal cells mechanisms. Hanahan and Weinberg (2016) has outlined eight hallmark capabilities of most forms of cancers. Each capability has a different functional role. The hallmark of cancers are as follow:

1. “Sustaining proliferating signalling”.

Generally, one of the known criteria of cancer is the uncontrollable cell proliferation. The inappropriate cell proliferation is resulted from disrupt cellular regulatory network. Induction and repressive signals control cell proliferation. The inductive signals are chronically sustained, causing inappropriate stimuli for cell proliferation. This often involves gene mutations that drive the cancer cell proliferation. These mutated genes are known as oncogenes.

2. “Evading growth suppressors”

Cancer occurs when tumour suppressor genes (TSGs) failed to stop initiation of cancer cell-division process. In the cells internal system, p53, one of the TSGs, mediates the cells regulation to ensure they only proceed to their growth and division cycle after appropriate state of cell physiological is achieved. In a stressful event in the cell, p53 will be activated and induce programmed cell death, thus stopping cell proliferation. However, mutation or defect in p53 pathway were identified in majority of human cancers that allows continuous cancer cell proliferation.

3. “Resisting cell death”

Normal and healthy cells have the ability to “kill themselves” in an orchestrated cell death program known as apoptosis. Besides apoptosis, cell death also occurs by autophagy, and necroptosis. Cancer cells lose this ability to self-destruct thus promoting continous proliferation. Proper signalling to induce cell death is disrupted causing cancer cells to resist cell death.

4. “Enabling Replicative Immortality”

Normal cells are able to die after several cell division processes however cancer cells are able to escape this and become immortal, where they can not divide (senescence)

or die. This is due to the length of the telomeres in cancer cells DNA that has been manipulated to increase at each division time, therrefre avoiding senescence.

5. “Inducing angiogenesis”

Angiogenesis is a process that demonstrates the formation of new blood vessels. Cancer cells are able to initiate angiogenesis to ensure that they receive continuous oxygen and other nutrients supply. Cancer cells need to activate their “angiogenic switch” which reduce the factors inhibiting the formation of new blood vessels and increase the factors promoting formation of new blood vessels.

6. “Activating invasion and metastasis”

Established cancer cells can become invasive and migratory. Cancer cells are able to invade neighbouring tissues including the blood and lymphatic vessel that provide a pathway for the cells to disseminate to other anatomical sites. This is where the tumour will be categorized as being benign or malignant.

7. “Deregulating Cellular Energetics and Metabolism”

Cancer cells utilize the abnormal metabolic pathway to create energy to support their proliferation and survival. This is a concept introduced almost a century ago by Otto Warburg where the cancer cells uptook glucose and demonstrated glycolysis, even in the presence of oxygen. This aerobic glycolysis produces building blocks and ATP required for cell growth and division.

8. “Avoiding Immune Destruction”

Cancer cells must find ways to  avoid the immune surveillance. They are able to avoid the immune surveillance because of most of the antigens expressed on cancer cells are most likely shared by their normal cell-of-origin. Antigens on the cells are being ignored by the immune system and reflecting immune self-tolerance of the cancer cells. Some of the cancer cells are also able to express antigens that are not tolerateable

by the immune system, such as new antigens produced due to the genome mutation and embryonic antigens.

### 2.1.2 Cancer therapy

Upon diagnosis with cancer, patients will be subjected to different types of treatments based on the type of cancer, locality, and stage. Cancer treatments available today are surgery, chemotherapy, radiotherapy, immunotherapy, vaccination, photodynamic therapy, stem cell transformation or any combination of the aforementioned treatments. These treatments are normally accompanied by side effects including toxicity, non-specificity, restriction in metastasis and fast clearance (Mukherjee and Patra, 2016; Patra *et al.*, 2014). A lot of efforts were made to reduce the side effects of cancer therapy such as preventing damage of the chemotherapy drugs on neighbouring cells, aggregate drug accumulation and lesion efficiency, acquiring novel drug delivery and targeting system (Vinogradov and Wei, 2012).

Chemotherapeutic agents work on different molecular targets, such as:

1) topoisomerase inhibitors such as irinotecan and doxorubicin
2) alkylating agents such as oxaliplatin, melphalan, carboplatin, and cisplatin
3) microtubule acting agents such as vincristine, vinblastine, paclitaxel and docetaxel

These drugs give side effects such as neutropenia, diarrhoea, cardiotoxicity, nephrotoxicity, gastrointestinal toxicity, hematologitoxicity and many more (Caruso *et al.*, 2000; Iqbal *et al.*, 2017; Weaver, 2014). The aforementioned drugs are very effective on a broad range of cancers however, their limitations are not disregardable, among others include the high price-tag, not eco-friendly, besides having the side

effects and toxicity. Cancer cells might develop drug resistant as they progress through mutation. For example, MCF-7, breast cancer cells exhibit over-expressed drug resistant genes (*ABCA12* and *ABCA4*) when docetaxel was applied. However, downregulation of those genes was observed when application of docetaxel was applied alongside with curcumin, a phytocompound found in tumeric (Aung *et al.*, 2017). Therefore, applying single-target anticancer agent is not the only option for efficacy of cancer treatment. Thus, employing phytochemicals and their analogues serve as alternative promising options for cancer treatment for better and lesser toxicity treatment (Singh *et al.*, 2016).

## 2.2 COMPLEMENTARY AND ALTERNATIVE MEDICINE

A huge reservoir of bioactive compounds exists in over 400 000 species of plants on Earth, but only a small percentage have been examined in research studies. Plants have been and continue to be an important source for therapeutic uses. In many developed countries, plant products use in complementary and alternative medicine (CAM) are popular. Approximately, more than 80% of the population worldwide depend on the traditional medicine or folk medicine as their primary healthcare needs as reported by WHO (Qi, 2013).

Herbal medicine usages in Asia embodies the history of the interaction between human and the environment. In Africa, the ratio of traditional healers to population is 1:500, however, the ratio of medical doctors to the population is broader at 1:40 000. This might be due to the locality of majority of the African population that lives in the rural areas. On that note, even in well-developed countries equipped with advanced conventional healthcare system like Singapore and Korea, 76-86% of their respective population still relies on traditional medicine (Qi, 2013). About 62.9% of cancer

patients in non-Asian countries reported to have used CAM (Saghatchian *et al*., 2014). Approximately 40% of the cancer patients in Australia, New Zealand, Europe, Canada and the United States were reported to use CAM (Horneber *et al*., 2012).

Findings from the 2015 National Health Morbidity Survey showed that 29.95% of Malaysian used CAM with consultation in their lifetime (World Health Organization, 2019b). Report from WHO (2019) also stated that 9 million users of CAM were reported among the Malaysian estimated population of 30 million. In Malaysia, the reported use of CAM was USD 500 million, annually, comparing to about USD 300 million spent on the use of conventional medicines (World Health Organization, 2002). Among CAM practices available in Malaysia and recognized by the Traditional and Complementary Medicine (Recognized Practice Areas) Order 2017, are traditional Malay medicine, traditional Indian medicine, traditional Chinese medicine, Islamic medical practice, homeopathy, chiropractic and osteopathy (World Health Organization, 2019b).

CAM users among cancer patients in Asian countries were reported as follows: 97.2% - China (Chen *et al.*, 2008), 79.3% - Taiwan (Ku and Koo, 2012), 60.9% - Thailand (Puataweepong *et al.*, 2012), 55.0% - Singapore (Chow *et al.*, 2010) and 57.4%-Korea (Kang *et al.*, 2012). According to the study by Siti *et al.* (2009), the usage of CAM among Malaysian's adult was estimated about 67.6-71.2% during their lifetime. They also highlighted the main CAM used were biological-based therapies (88.9%), manipulative and body-based therapies (27.0%), mind-body medicine (11.1%) and traditional medicine (1.9%) (Siti *et al.*, 2009). The usage of CAM among Malaysian breast cancer patients were 64.0% (Shaharudin *et al.*, 2011) and 88.3% (Gopal *et al.*, 2013), while the prevalence of CAM usage among breast cancer

survivors in Peninsular Malaysia was 51.0% (Saibul *et al.*, 2012). And in recent studies, CAM users among Malaysian breast cancer patients was 70.7% (Chui *et al.*, 2018) and 34.8% (Zulkipli *et al.*, 2018). Dietary supplementation was reported as the most frequent use of CAM.

High demand on CAM usages indicates that more information is needed to be explored and disseminate to the mass especially on the efficacy of the utilization of medicinal plants as well as the toxicity dosage of the plant. Most CAM practices are based on cultural and historical influences and this knowledge was passed on from one generation to another generation, however, scientific evidence supporting their usages are lacking. Malaysia has been actively regulating the traditional medicine practices in order to control the usages and practices in this country. Efforts were made in order to document all information as a reference for practitioners and consumers. Traditional medicine units were also being set up in 15 hospitals around Malaysia. Integrative traditional medicine and practices are practised in addition to the conventional allopathic medicine and many patients have benefited from this integrative programme since its introduction in 2007 (Meow, 2018).

## 2.3 MEDICINAL PLANTS AND CANCER

Medicinal plants have always centred around the traditional medicine practices. These plants have also continuously providing resources for mankind in search of remedies to various diseases and ailments. Historically, the initial usage of medicinal plants originated from China in 5000 BC. Tyler (1999) reported that natural medicines were widely used up until the first half of twentieth century, when after that synthetic medicine took the front seat. Natural products such as vegetables, fruits, tea, grains, spices, nuts, herbs, and medicinal plants are rich in phenolic, flavonoids, alkaloids, carotenoids, vitamins, minerals and other organic materials. Therapeutics

capabilities of these plants, especially the medicinal plants include antiviral, antitumour, antimalarial, and anti-inflammatory activity.

One of strategy to combat cancer is through chemoprevention using natural product to suppress, prevent and reverse pre-malignancy before the cancer become aggressive. Scientific interest towards medicinal plants to combat cancer has recently gained popularity. 35 000 plant species were screened by The National Cancer Institute, USA (NCI) for the anticancer activities and among that about 3000 plants were able to demonstrate reproducible anticancer activity (Desai *et al.*, 2008; Roy *et al.*, 2018).

Anticancer medicinal plants are known to contain a huge reservoir of polyphenolic components (David *et al.*, 2016) and other phytocompounds that are able to inhibit progression and development of cancer (Aung *et al.*, 2017). Table 2.1 listed some medicinal plants with reported anticancer activity in the year 2018 and 2019.

Table 2.1 : Medicinal plants with anticancer activities reported in the year 2018 & 2019

| Plant Scientific Name | Common Name | Reported Activity | Reference |
|---|---|---|---|
| ***Abrus precatorius*** | Pokok Saga | Induction of apoptosis and anti-proliferative activities against breast cancer cell, monocytic leukemia (THP-1), and chemopreventive effect in mice model experiment | Sofi *et al.* (2018), Gul *et al.* (2018), Wan-Ibrahim *et al.* (2019) |
| *Alangium salviifolium* | Sage | Induction of apoptosis and anti-proliferative activity against melanoma and non-melanoma cancer cells | Dhruve *et al.* (2019) |
| *Allium cepa* | Onion | Cytotoxic effect on colon cancer cells (WiDr) | Fadholly *et al.* (2019) |
| *Allium sativum* | Garlic | Anticancer effect on MKN74 cell line | Korga *et al.* (2019) |
| *Alpinia galanga* | Galangal | Induced cytotoxicity and apoptosis in human lung cancer cells and murine lymphoma<br>Anticancer effect in T47D cells | Lakshmi *et al.* (2019),<br>Dai *et al.* (2018), |
| *Annona muricata* | Soursop | Induced apoptosis in breast cancer cells | Kim *et al.* (2018a), Arif *et al.* (2018) |
| *Bacopa monnieri* | Indian pennywort | Inhibited growth of colon cancer cells by inducing cell cycle arrest and apoptosis | Smith *et al.* (2018) |
| *Brassica oleoracea* | Cabbage | Anticancer effect of ethanol extract on hepatotocellular carcinoma | Vanitha *et al.* (2018) |

Table 2.1 Continued

| Plant Scientific Name | Common Name | Reported Activity | Reference |
|---|---|---|---|
| *Caralluma retrospiciens* | Bitter cress | Apoptosis in breast cancer cell lines | Alallah *et al.* (2018) |
| *Carica papaya* L. | Papaya | *In vitro* and *in vivo* protective effect against oxidizing agent in cancer experimental models | Siddique *et al.* (2018) |
| *Coriandrum sativum* | Coriander | Anticancer effects on prostate cancer cell lines | Elmas *et al.* (2019) |
| *Crinum amobile* | Spider lily | Anticancer activity of chloroform leaves extract on MCF-7, MDAMB-231, HCT-116 and HT-29 cells | Lim *et al.* (2019) |
| *Curcuma longa* | Turmeric | Anti-proliferative activity in cancer cells | Sheikh *et al.* (2018) |
| *Cymbopogon citratus* | Lemongrass | Decreases prostate cancer and glioblastoma cell survival | Bayala *et al.* (2018) |
| *Eurycoma longifolia* | 'Tongkat Ali' | Anticancer efficacy against lung carcinoma (A-549 cells) and breast cancer (MCF-7 cells), through upregulation of p53 and Bax, down regulation Bcl-2 | Thu *et al.* (2018) |
| *Diosphyros kaki* L. | Persimmon | Inhibited liver tumour growth *in vivo via* enhancement of immune function in mice | Chen *et al.* (2018) |
| *Ficus deltoidea* | Mistletoe fig / 'Mas cotek' | Ethyl acetate extract demonstrated anti-proliferative activity on MCF-7, MDA-MB-231, HCT 116 | Abolmaesoomi *et al.* (2019) |

Table 2.1 Continued

| Plant Scientific Name | Common Name | Reported Activity | Reference |
|---|---|---|---|
| *Garcinia mangostana* | Mangosteen | Cytotoxic activity on HeLa cells | Muchtaridi *et al.* (2018) |
| *Glycine max* | Soybean | Downregulation of histone demethylase JMJD5 prevent the progression of breast cancer cells | Wang *et al.* (2018b) |
| *Lawsonia inermis* | Henna tree | Branch methanolic extract inhibited the invasion of HT1080 cells strongly | Nakashima *et al.* (2018) |
| *Moringa oleifera* | Moringa | Induction of apoptosis and downregulation of AKT pathway in human prostate cancer | Ju *et al.* (2018) |
| *Murraya koenigii* | Curry tree | Exhibited anticancer activity on various cancer cell lines | Samanta *et al.* (2018) |
| *Nigella sativa* | Black seed | Inhibited proliferation and angiogenesis, induced apoptosis in Hela and HepG2 | (Maqbool *et al.*, 2019) |
| *Ocimum tenuiflorum* | Holy basil | Anticancer activity of methanol leaves extract on MCF-7 cells | Lam *et al.* (2018) |
| *Orthosiphon stamineus* | Java Tea / 'Misai Kucing | Inhibit proliferation and induced apoptosis in uterine fibroid cells | (Pauzi *et al.*, 2018) |
| *Perekia bleo* | Rose cactus /'Duri 7' | Induced cell death by cell cycle arrest and apoptosis in HeLa | Mohd-Salleh *et al.* (2019) |
| *Syzgium polyanthum* | Bay leaf | Low cytotoxic effect against breast cancer cells MCF-7 | Nordin *et al.* (2019) |

### 2.3.1 Phytochemicals

Medicinal plants are generally known because of the medicinal properties that they exhibited through their biological activity. Active compounds or substances refers to the constituents produced or stored in the plants that have physiological effects on living organisms (Rafieian-Kopaei, 2012). Most medicinal plants used for treatment contain properties including compounds that give synergistic actions. These compounds are beneficial as a source of drugs discoveries (Rasool Hassan, 2012). Different parts of the plants are utilized for the medicinal purposes including root, seed, leaves, flowers, stem, bark, fruits, or even the whole plant. Active compounds from these organs may have indirect or direct therapeutic effect that make them suitable as medicinal agents.

Phytochemicals are any of biologically active compounds found naturally occurring in plants. The term 'bioactive compound' is defined by the ability of the compound to interact with one or more component of a living tissue to generate probable effects (Guaadaoui *et al.*, 2014). Some of these compounds interact with each other and gives synergistic actions and this interaction might be beneficial or harmful to either of the components that contribute to their biological activities. These compounds are also characterized by their ability to prevent certain disease development including cancer.

Plants contain thousands of phytochemicals that are generally classified into primary and secondary metabolite. Primary metabolites are compounds that are responsible for plant growth, development and reproduction. Secondary metabolites referes to compounds that do not involve in those processes (Singh, 2015). Some classified these secondary metabolites as non-nutrient compounds that have been found to exert biological activity in human and link with reductions of

noncommunicable chronic diseases (Liu, 2013). Out of that only a few belongs to the primary group while the rest are classified as secondary metabolites which are subdivided based on their chemical structures. Phytochemicals are also classified based on their biosynthesis pathways, botanical origins, or biological properties. Figure 2.2 exhibit the phytochemical classifications which consist of carbohydrate, lipids, terpenoids, phenolic acids and alkaloid or other nitrogen containing metabolites.

Phenolics are compounds with at least one aromatic ring containing hydroxyl group. This compound is easily found in vegetables, fruits, legumes, cereals, wine, chocolate, tea and coffee which contributed to more than 8000 of phenolic compounds have been isolated (Gao and Hu, 2010). Phenolics exhibited anti-proliferative effect on several cancer cells by inhibition of topoisomerase or phosphatidylinositol-3-kinase and also cell cycle arrest. Phenolic compounds can also accelerate oxidative damage either to the proteins, carbohydrates or to the DNA (Vaghora and Shukla, 2016). Another phytochemical group belongs to the phenolic is known as flavonoid. In a study using animal models, flavonoids were found to give protective effect against tumour initaiton and progression (Batra and Sharma, 2013). Alkaloids also have demonstrated the anti-proliferative effects on different types of cancers *in vivo* and *in vitro* (Lu et al., 2012). Some of the anticancer agents found from alkaloids groups are berberine, colchicine and morphine (Gach *et al.*, 2011; Sueoka *et al.*, 1996).

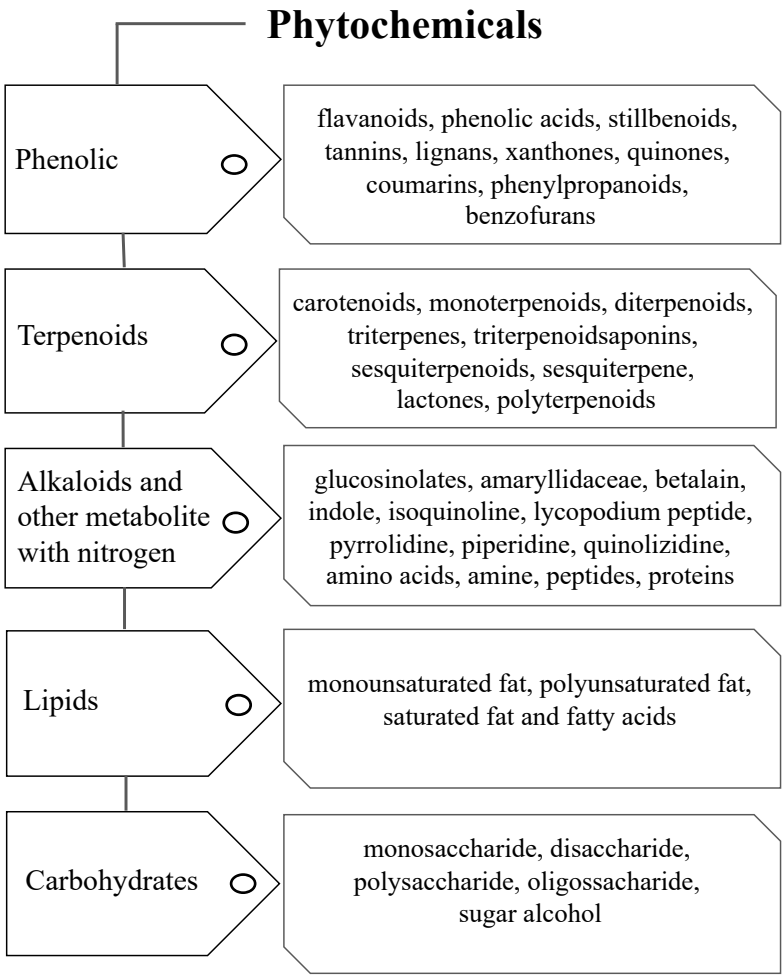



Figure 2.2: **Phytochemical classifications.**
Adapted from (Huang *et al.*, 2012; Mondal *et al.*, 2019; Nizami and Sayyed, 2018; Sharma *et al.*, 2017; Vermerris and Nicholson, 2007)

### 2.3.2 Plant extraction

Human depends on raw plant materials to access the medical needs in health maintenace and to cure diseases and ailment. Natural products extracted from medicinal plants either as crude extract or pure isolated compounds gives endless opportunity for novel drug discoveries since abundance of chemical diversity existed from plants on our planet. Historically, in most household, medicinal plants were used as a whole plant rather as a single pure compound (Cos *et al*., 2006). Definition of crude extract is the use of the substance obtained after the extraction process.

Extraction is a crucial process in analysis of medicinal plants to obtain bioactive compound from biomass materials. The extraction process is performed to increase the amount of target compound. This will increase the chance of obtaining maximum biological activities from the extract. This process starts with plant collection and identification, washing, drying, and grinding. Grinding to homogenize sample is important to increase surface contact area of the plant material with solvents. If the plant of interest is chosen based on traditional uses, therefore it is important that the preparation of the plant should be prepared as closely as possible mimicking the traditional preparation.

Maceration is a technique used widely in medicinal plant research and it is adapted from the art of wine making. This process involve soaking of the plant materials with a solvent in a closed container for a period of time, with frequent agitation (Chemat *et al.*, 2017). Basic principle of maceration is to soften the cell wall of the plant and then releasing the soluble phytochemicals. After the soaking period is over, the mixture is filtrated, and the flow-through is then collected and dried to obtain the extract. This process uses a large amount of solvent and a longer period of time.

Decoction is a method that uses similar principle as maceration except that the plant is boiled in a specific volume of water. This extraction process is normally done on hard plant materials such as root and barks and suitable for heat stable compound. Another extraction method is known as Soxhlet extraction. A porous bag known as "thimble" is used to place finely ground sample. Thimble is made of a strong cellulose or filter paper and it is placed in a thimble chamber of the Soxhlet apparatus (Figure 2.3). This is a hot continuous process. Solvent is placed in the bottom flask, boiled and the vapour arises will reach the sample thimble and condenses at the condensation chamber and finally drip back into the bottom flask. Contrary to maceration, Soxhlet

uses lesser quantity of solvent , however the solvent used must be high-purity and that could be costly (Azwanida, 2015). Figure 2.3 shows the conventional systems used for medicinal plants extraction.

Extraction efficacy is highly depending on the extraction method, temparature, time length and the solvent choice. Extraction yield obtained after an extraction process and its biologocial activity, does not only relies on the extraction technique but also the choice of the solvent used. In the same extraction condition, the most important paramenter that need to be consider is the solvent choice (Ngo *et al.*, 2017). Different types of solvents can be use in addition to water which include hexane, ethyl acetate, ethanol, methanol, chlorofom and many more. Choice of the solvent depends on each plant and the target compound (Ajanal *et al.*, 2012; Mahdi-Pour *et al.*, 2012).

Hexane is normally used to dissolve non-polar compounds such as wax, lipid, lignin and aglycon (Indarti *et al.*, 2019). Ethyl acetate is a semi polar solvent, used to dissolve semi and non polar compounds (Kasitowati *et al.*, 2019) such as sterol, alkaloid, terpenoid, and flavanoid. Methanol is able to extract polar compounds such as amino acids, sugar, glycoside, low and medium molecular weight phenolic compound, flavanoid, terpenoid, saponin, tannin and polyphenols (Solomon *et al.*, 2019; Wang *et al.*, 2019).

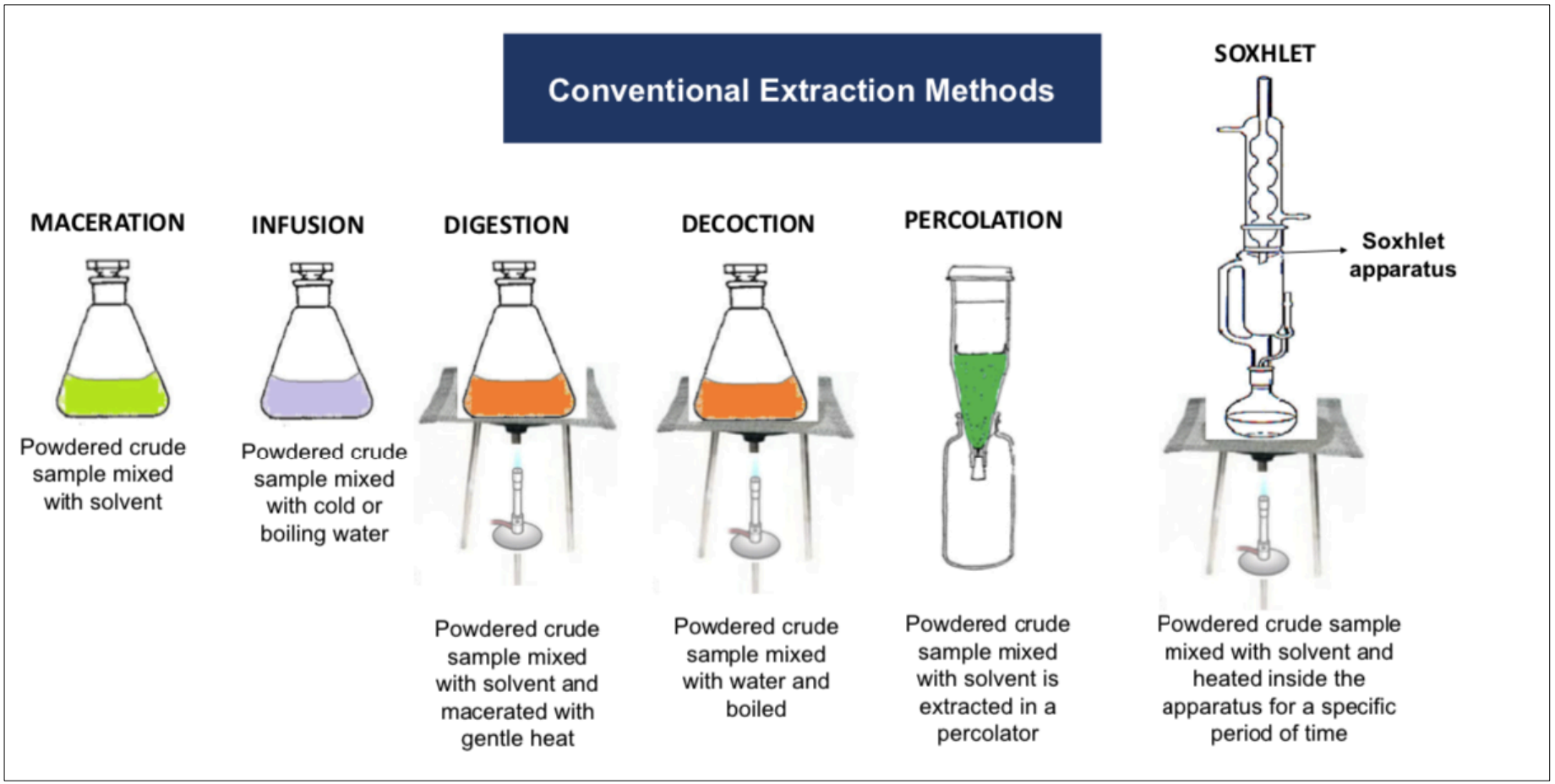



Figure 2.3: **The conventional extraction methods for medicinal plant extraction**. Modified from Belwal *et al.* (2018)

## 2.4 The medicinal plant: *Abrus precatorius*

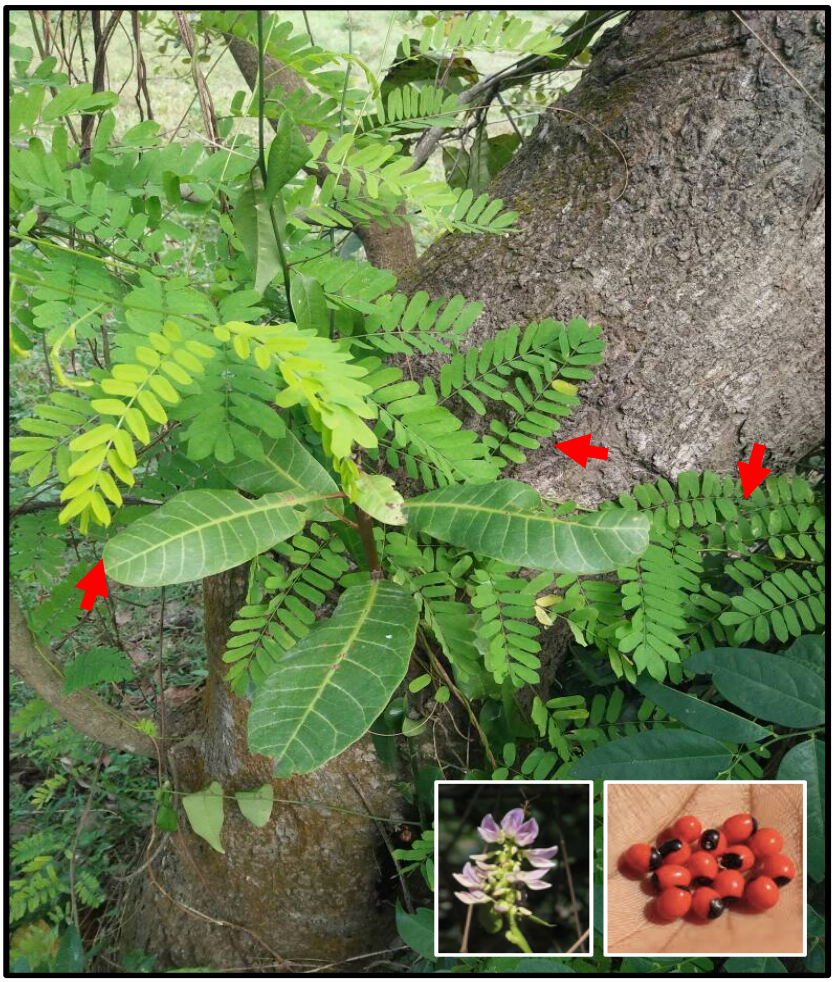


Figure 2.4: ***Abrus precatorius* plant twining around a tree.**
Red arrows pointed on the leaves of the plant used in this study.

*Abrus precatoriu,* as shown in Figure 2.4, is a flowering plant that belongs to legume family, Fabaceae. The common names of *A. precatorius* include: jequirity, Crab's eye, Rosary pea, precatory pea or bean, John crow bead, Indian licorice, Akar saga, and jumble bead. Phenotypically this plant is characterized as a slender, perennial climber that twines around trees, shrubs and hedges *Abrus* can be found in different parts of the world. There are different varieties of the *Abrus* as listed in Table 2.2.

Table 2.2: Different varieties of *Abrus* genus.

| ***Abrus* Varieties** | **Countries** | **Reference** |
|---|---|---|
| *Abrus aureus* | Madagascar | Solanki and Zaveri (2012) |
| *Abrus baladensis* | Somalia | Thulin (1994) |
| *Abrus bottae* | Saudi Arabia, Yemen | Al-Safadi (1994) |
| *Abrus canescens* | Africa | Agbagwa and Okoli (2005) |
| *Abrus cantoniensis* | China | Zhang *et al.* (2015) |
| *Abrus diversifoliatus* | Madagascar | Okhale and Nwanosike (2016) |
| *Abrus fruticulosus* | India, Brazil | de Vasconcelos *et al.* (2018) |
| *Abrus gawenensis* | Somalia | Thulin (1994) |
| *Abrus kaokensis* | Africa | Swanepoel and Kolberg (2011) |
| *Abrus laevigatus* | South Africa | Pandhure *et al.* (2010) |
| *Abrus longibracteatus* | Laos, Vietnam | Mondal and Parui (2014) |
| *Abrus madagascariensis* | Madagascar | Quattrocchi (2012) |
| *Abrus parvifolius* | Madagascar | Quattrocchi (2012) |
| ***Abrus precatorius*** | **India, Malaysia, Sri Lanka, Africa, Florida, Hawaii, South America, Australia, all tropical region** | (Pavithra *et al.*, 2019) |
| *Abrus pulchellus* | Africa, China | Zhang *et al.* (2015) |
| *Abrus sambiranensis* | Madagascar | Verdcourt (1970) |
| *Abrus schimperi* | Africa | Rahman *et al.* (2011) |
| *Abrus somalensis* | Somalia | Quattrocchi (2012) |

### 2.4.1 Traditional uses of *Abrus precatorius*

Aerial, leaves, stem and roots are all parts of *A. precatorius* used in the traditional system. Leaves are generally used to cure fever, stomach pain, headaches, asthma and skin diseases including itching and wound healing. Maceration of the leaves in warmed mustered oil is used to relieve pain of rheumatism affected area. Similarly, the juice of the leaves, blended in some oil is also used to relieve local pain (Joshi, 2019). In case of leukoderma and menorrhagia, powdered leaves mixed with sugar are consumed. Some practices take the leaves for managing diarrhoea, gastritis, insomnia, kidney diseases and cancer (Attal *et al.*, 2010).

Roots of *A. precatorius* is known to be emetic and alexiteric. Aqueous extract of the root is beneficial to relieve bad cough. It is used as substitute for liquorice and taken orally for sore-throat reliever. The roots also useful in treatment of gonorrhoea, jaundice and infections (Daniel, 2016). The seeds are used for nervous system remedy, and applied externally for skin diseases, ulcers and on hair. Seed paste can be applied for muscle stiffness, sciatica, shoulder pain, paralysis and other nervous disorder. For contraception, the seeds are taken orally to prevent conception by interfering the uterine function .

The traditional uses of *A. preactorius* also differ from one ethnic background to another. Not only differ on the choice of plant parts, but also methods in preparing them. Table 2.3 listed the traditional way of how *A. precatorius* is utilised in different parts of the world.

Table 2.3: Ethnomedicinal use of *A. precatorius* summarized from Ross (2003)

| Part of the plant | Preparation | Acclaimed medicinal use | Country |
|---|---|---|---|
| Leaves | Powder | Antidote | Brazil |
| | Decoction (dried) | Nerve tonic | |
| | Juice | Gonorrhoea, stomach-aches | East Africa |
| | Powder | Swellings, cuts | |
| | Decoction | Inflamed eyes, chest pain | |
| | Decoction | Coughs and flu | Haiti |
| | Decoction | Eye disease | India |
| | Decoction | Aphrodisiac, facilitate in childbirth | Ivory coast |
| | Juice | Coughs | Africa |
| | Paste | Antifertility | Sudan |
| | Juice | Antiinflammatory | Thailand |
| | Decoction | Coughs | Tanzania |
| Seeds | Powder | Aphrodisiac | Afghanistan |
| | Decoction | Malaria | Cambodia |
| | Powder | Intestinal worms, oral contraceptive | Africa |
| | Powder | Aphrodisiac | Egypt |
| | Decoction | Antifertility, contraceptive, for tuberculosis treatment and painful swelling | India |

Table 2.3 Continued

| Part of the plant | Preparation | Acclaimed medicinal use | Country |
|---|---|---|---|
| | Decoction | Aphrodisiac | Nepal |
| | Decoction | Aphrodisiac, abortion | Pakistan |
| | Decoction | Coughs | Virgin Island |
| | Decoction | Purgative | West Indies |
| Roots | Decoction | Asthma, aphrodisiac | Tanzania |
| | Decoction | Bronchitis, hepatitis | Taiwan |
| | Decoction | Antimalarial, anticonvulsant | Nigeria |
| | Decoction | Aphrodisiac | Mozambique |
| | Juice | Abortion | India |
| | Chewed | Aphrodisiac | Africa |
| | Chewed | Snake bite | Africa |
| | Decoction | Nerve tonic | Africa |

### 2.4.2 Phytochemistry of *Abrus precatorius*

Roots from *A. precatorius* have documented presence of compounds such as abrol, abrasine, pre-casine and precol. Meanwhile the mostly studied and potent bioactive compound from the seed is abrin. Abrin is toxic, containing poisonous protein, which could be fatal when ingested. However, the seed coat protects human from the toxin (Das *et al.*, 2016).

A review written by Ross (2003) listed all the compounds identified in the leaf of *A. precatorius* from the early years (1968 – 1981) include; arabinose, galactose, glycyrrhizin, hemiphloin, hypaphorine, inositol, montanyl alcohol, myricyl alcohol, pinitol, precatorine, trigonelline and xylose. Sweet tasting compounds were discovered in the leaves of *A. precatorius.* These compounds are asabrusoside, which is a triterpene glucoside; and glycyrrhizin. They are low in calorie but taste sweeter than normal sucrose. The level of sweetness is 30-100 times more than sucrose.

Compounds that have been discovered from the leaves are abrusoside- (A-E) (Choi *et al.*, 1989; Kinghorn and Soejarto, 2002), pterocarpan (Malele *et al.*, 2015); abrusprecatins A-C, medicarpin, maackiain and 4-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan (Li *et al.*, 2019); stigmasterol and β-monolinolein (Sofi *et al.*, 2018). Other compounds that were also discovered from the leaves are flavonoids (vitexin, liquirtiginin-7-mono- and diglycosided), triterpene (abrusgenic acid, abruslactone A, and mehyl abrusgenate) and toxifolin-3-glucosides (Attal *et al.*, 2010; Daniel, 2006).

### 2.4.3 Reported Pharmacological activities of *Abrus precatorius*

Anticancer activity of *A. precatorius* leaves has been previous mentioned at section 1.4. Here, the other pharmacological activities of *A. precatorius* in seeds, roots, aerial and leaves, are listed.

#### 2.4.3(a) Seeds

The microglial cells (MCG) activation from the Alzheimer's Disease (AD) patients' brain was able to be identified by *A. precatoirius* agglutinin. It was able to identify the MCG in the white matter of the cerebral from all AD patients studied. Identification of novel markers in MCG research is crucial in order to understand better the role of these cells in the metabolic/ dysmetabolic control of AD (Zambenedetti *et al.*, 1998). Anti-diabetic activity of the chloroform and methanol extract of *A. precatorius* was reported in diabetic rabbits (Monago and Alumanah, 2005). The chloroform and methanol seed extract also showed anti-fertility in rats (Bhutia *et al.*, 2009). Ethanolic seed extract was found to cause DNA damage in spermatozoa that lead to teratogenic effect which exhibit the antifertility activity of *A. precatorius* (Jahan et al., 2009). Anti-spermatogenic effect was shown by the *A. precatorius* hydraalcoholic extract (Soni *et al.*, 2015). Aborfacient and anti-implantation effect was also observed in pregnant rats (Solakhia *et al.*, 2019). *A. precatorius* seeds extract also showed potent antimicrobial activity (Bobbarala and Vadlapudi, 2009). The antibacterial and antifungal activity was because of the presence of certain compounds such as falvanoids, alkaloids and saponin (Vinayaka *et al.*, 2010). Cytotoxic effects on HepG2 cells was observed in the experiment using the hydroalcoholic seed extract (Gautam, 2017). Significant immunomodulating activity was observed on cellular immune response of the tumour bearing mice (Bhutia *et al.*, 2009).

### 2.4.3(b) Roots

Alcoholic extract and ethanol extract from fresh roots showed anti-convulsant activity and anti-depressant activity in mice, respectively (Moshi *et al.*, 2005; Okhale and Nwanosike, 2016).

### 2.4.3(c) Aerial and Leaves

Petroleum ether extract of the aerial part of the *A. precatorius* reported neuroprotective activity in hypoxic neurotoxicity rats (Premanand and Ganesh, 2010). The aqueous extract exhibited nephrotective effect on nephrotoxicity-induced HEK293 (Sohn *et al.*, 2009). Isoflavanquinone and abruquinone, isolated for the aerial part demonstrated antimalarial activity (Limmatvapirat *et al.*, 2004). Crude ethanol extract of the leaves exerted significant neuromascular effect by blocking the phrenic nerve diaphragm in the rat (Wambebe and Amosun, 1984). Antiepileptic activity was reported from the aqueous extract of the leaves (Moshi *et al.*, 2005). A recent study showed that the ethanolic extract exhibited analgesic and neuropharmacological activities that proposed the utilization of *A. precatorius* in pain management, psychiatric and neurological conditions (Mondal *et al.*, 2017). In another study, the methanolic leaves extract showed antidiabetic, antimicrobial and cytotoxic effect in brine shrimp lethality assay (Adekunle Alayande *et al.*, 2017).

## 2.5 APOPTOSIS

The first visualized human cell death was observed in a report on yellow fever in 1890 (Councilman, 1890), where he discovered vacuolated acidophilic bodies from hepatic tissue of yellow fever patients. This bodies were sometimes referred to as the Councilman bodies. Despite the early discovery of those bodies, it took about 70 years to reveal that the Councilman bodies were actually dying or dead cells with the help of the electron microscope (Bosurgi *et al.*, 2017; Kerr, 1969). It turned out that those

dying cells were not only unique in liver damage observation but also in other tissues and later, a detailed morphological and description of these cell deaths were published. This phenomenon was then termed as apoptosis (Kerr, 1971; Kerr *et al.*, 1972).

One of the causes of cancer is the failure of the cells to undergo apoptosis. Failure of apoptosis in cells lead to tumorigenesis by inhibition of the signals and activation of proteins involved in the apoptosis pathway. Apoptosis is process known for a programmed cell death or "cellular suicide". It is a key event in many biological processes, such as removal of the webbing between fingers and toes in embryos. This is known as programmed cell death because the death is specific and at the defined times during development (Hassan *et al.*, 2014). Another example of apoptosis is the resumption of the tadpoles' tails when they undergo metamorphosis. In human, apoptosis occurs continually. When cells are infected by pathogens or when the white blood cells reach the end of their lifespan, they are eliminated through apoptosis.

Apoptosis also occurs in a hormone-dependant physiologic involution such as the involution of endometrium lining during the menstrual cycle. Cell deletions in proliferation cell population such as in the intestinal crypt epithelium also demonstrate the event of apoptosis. Among pathological causes that lead to apoptosis are stimuli of mild injuries such as heat, radiation, or cytotoxic exposure that result in irreparable DNA damage, which ends with the suicidal pathway. The event of apoptosis is also beneficial in cell injuries cause by pathogens, besides it is mainly important in programming the cell death in tumours. Millions of cells die every minute in human body and being eliminated. However, mutations in some of the proteins that are involved in apoptosis pathway, may lead to tumour formation and cancer (Cao and Tait, 2018).

The first cell death programme was demonstrated in the nematode (*Caenorhabditis elegans*), where the key genes involved in apoptosis were identified (Malin and Shaham, 2015). Subsequent studies showed that many other organisms, including mammals, use similar proteins to commit cellular suicidal. Apoptosis is different from necrosis, another type of cell death. Necrosis involves swelling and rupture of the injured cells, while apoptosis involves a specific series of events that leads to cell death. Apoptosis is characterized by morphological changes including membrane blebbing, chromatin condensation, and nuclear fragmentation (Figure 2.6).

During the early stage of apoptosis in a cell, DNA segregates near the periphery of the nucleus thus the volume of the cytoplasm decreases. After that, cell begin to produce small bubble-like cytoplasmic extensions known as “blebs” and the nucleus and organelles begin to fragment into membrane-bound vesicles knows as apoptotic bodies. The apoptotic bodies are eliminated through phagocytosis by macrophages or degraded by lysosomes (Pfeffer and Singh, 2018).

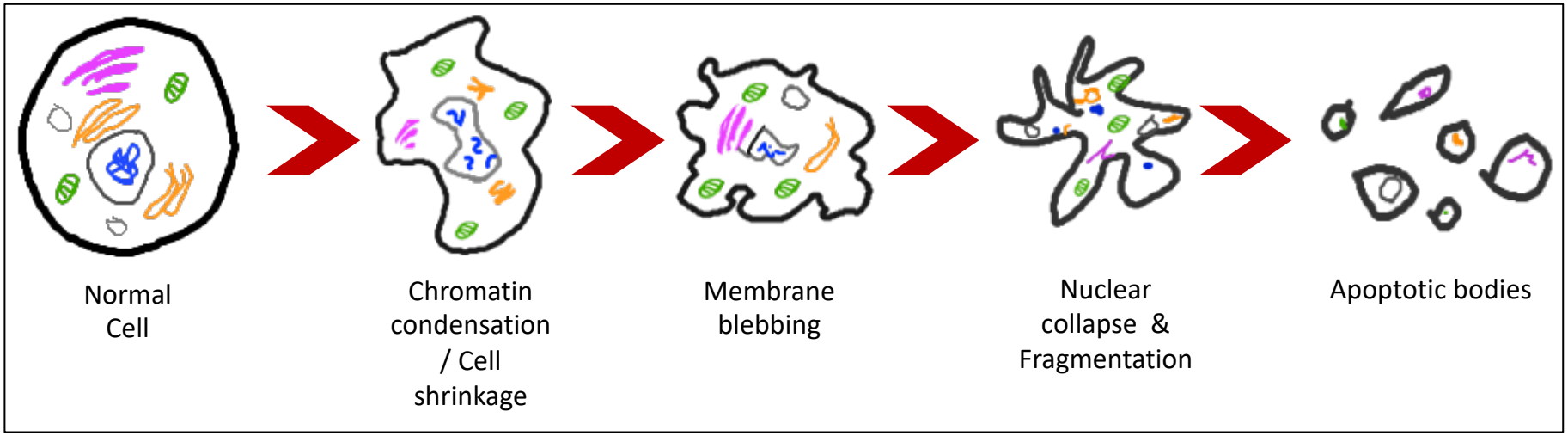



Figure 2.5: Drawn schematic diagram on morphological changes during apoptosis of a cell.

### 2.5.1 Apoptosis pathway

All of these morphological changes leading to apoptosis are the results of the activation of a cascade of intracellular factors known as caspases (cysteine aspartyl-specific proteases). It can occur by activation, up regulation and/or downregulation of many types of protein. There are two main routes where caspases can be activated and enters the apoptotic pathway.

The first pathway is known as the extrinsic pathway where cells receive death signals produced by cytotoxic-T cells in response of damaged or infected cells that initiate the apoptotic cascade (Zaman *et al.*, 2014). Two well-known death signals are received by the tumor necrosis factor (TNF) receptor and the Fas receptor or CD95. The CD95 ligand (Fas Ligand) will bind to the CD95/Fas receptors of the infected cell. This aggregation will result in the attachment of FADD (Fas associated death domain), an adaptor protein, to the cluster. This will activate the procaspase-8, a protease, to assemble at the site of the cluster and thus cleave into an active caspase-8 protein. Procaspase-8 also cleaved procaspase-3 into caspase-3 protein. Activation of caspase-8 and caspase-3 are important in order to activate subsequent steps in the apoptosis event such as the activation of Bid, a cell death protein, by interacting with the proteins at the surfaces of the mitochondria. This pathway may be inhibited by FLIP (FLICE-like inhibitory protein) protein that binds to procaspase-8, thus inhibit the proteins cleavage (Cao and Tait, 2018).

The second pathway of apoptosis involves the mitochondria and it is also called as the intrinsic pathway. In this pathway, apoptosis occurs in absence of cell death signals. This pathway is triggered by signals including the deprivation of growth factor, DNA damage, and cytokine deprivation (Zaman *et al.*, 2014). At the mitochondria, the balance between death-promoting (proapoptotic) proteins and

death-preventing (antiapoptotic) proteins determine whether cell will undergo apoptosis. Among known proapoptotic proteins are Bax, Bid, Bak or Bad, while the antiapoptotic proteins are Bcl-2 and Bcl-x. In the case where a cell's DNA is injured by radiation, for example, apoptosis will be initiated by the activation of a protein knows as p53. The p53 protein will activate Bax, a proapoptotic protein. Mitochondrial permeability increases and cytochrome c molecules are released into the cytoplasm and recruits proteins such as Apaf-1, an apoptosis activating factor, and procaspase-9, which form a complex known as apoptosome. This assembly cleaved procaspase-9 to caspase-9 and activates the caspase-3, which then leads to the activation of caspases cascade. All of these events are summarised in Figure 2.7.

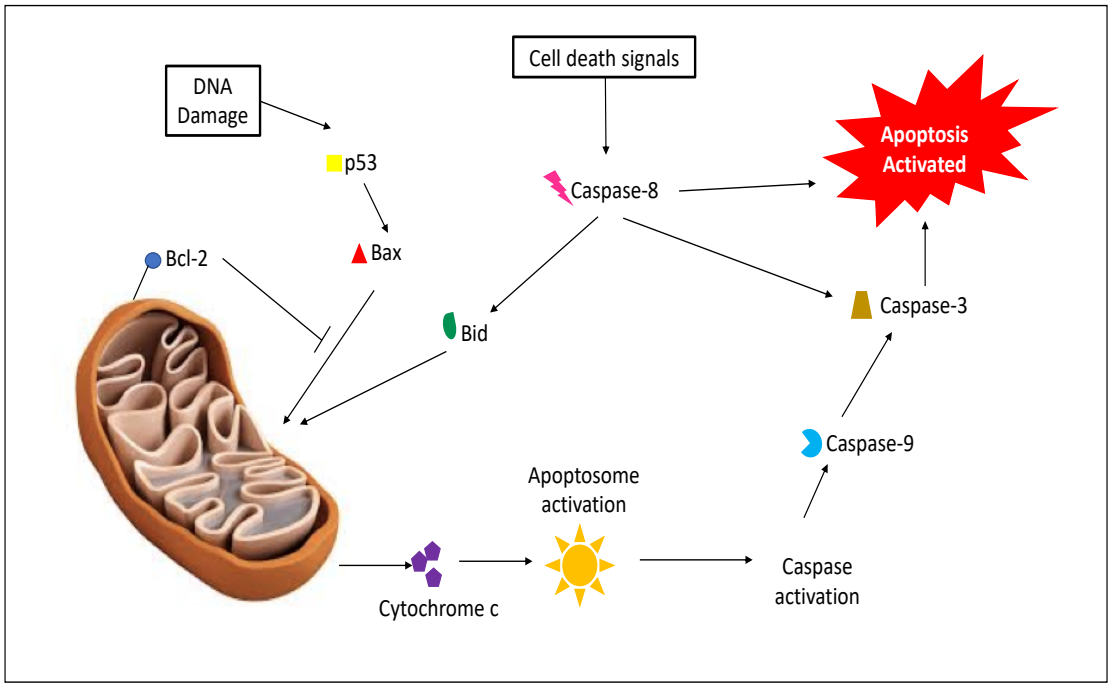



Figure 2.6: **Apoptosis pathway schematic diagram.**
Extrinsic pathway starts with external stimuli which induces the cell death signals; andintrinsic pathway starts with internal stimuli such as DNA damage. Adapted from the apoptosis event decription by Letai (2017)

### 2.5.2 Apoptosis proteins

#### 2.5.2(a) Tumour suppressor protein, p53

Tumour suppressor proteins are important in regulating cell division. Once damaged DNA is detected in a cell, these proteins will halt the cell proliferation until the damage is repaired. In some instances, specific tumour suppressor proteins will encourage damaged cell to cell death. However, when the genes encoding these proteins are malfunction, the cell with the damaged DNA will continue to proliferate which may cause further DNA damage or eventually leads to the formation of cancer.

Tumour suppressor protein p53 is encoded by *TP53* gene and approximately 50-55% of cancers loss this wild type activity of p53, making it the most silenced or mutated gene in cancer (Wang *et al.*, 2015). p53 activation causes responses such as apoptosis or cell cycle arrest thus preventing the development of tumour. Cell stresses such as DNA damage, hypoxia and oncogene activation increase p53 cellular level by phosphorylation and acetylation. However, in tumour bearing wild type p53, p53 function is downregulated thus inhibiting p53 responses, as shown in estrogen-receptor positive breast cancer, that directly interact with p53 and therefore prevent the p53-mediated apoptosis response (Bailey *et al.*, 2012b).

Scientists have demonstrated that p53 protein function also regulates metabolism and homeostasis in cells and tissues without causing cell cycle arrest or apoptosis (Schwartzenberg-Bar-Yoseph *et al.*, 2004; Wang *et al.*, 2015). A number of proteins are involved in regulation of p53 activity to sustain energy homeostasis and cell metabolism under normal and stresses physiological condition. p53 is an important protein engaging in controlling the changes in tumour cell metabolisms. Mutation in genes such as p53, leads to cancers in human. The p53 protein ensures response to

appropriate signals in order to decide the fate of the cell; therefore, known as “guardian of the genome” (Lane 1992), that plays a protective role in the whole body.

### 2.5.2(b) Bcl-2 Family Proteins

Bcl-2 family consist of 18 members which are categorised into three functional group based on their activities in apoptosis or their BH (Bcl-2 homology) domain. Anti-apoptotic Bcl-2 proteins are Bcl-2, Bcl-xL, Bcl-w, Mc11 and A1. These proteins contain four BH domains and prevent cell death by binding to the pro-apoptotic protein of the Bcl-2 family (Bax, Bak, or Bid) (Chipuk *et al.*, 2010). In order to maintain cell homeostasis or balance between pro- and anti- apoptotic proteins and control of the apoptosis, these proteins bind to each other and create a complex interaction network, therefore determine whether a cell lives or dies (Peña-Blanco and García-Sáez, 2018).

An example of a pro-apoptotic protein is Bid. Bid belongs to the Bcl-2 superfamily (Degli Esposti, 2002). Once in active form, Bid will initiate the action of mitochondria which will release the cytochrome c. Similar to the intrinsic pathway, Bax, another pro-apoptotic protein also belonging to the Bcl-2 superfamily also will initiate the action of mitochondria. Bax is activated by p53 protein and deactivated by Bcl-2 anti-apoptotic protein (McCurrach *et al.*, 1997; Peña-Blanco and García-Sáez, 2018).

### 2.5.2(c) Caspases

Caspase was first discovered as designated interleukin-1β converting enzyme and cytokine-processing enzyme (Cerretti *et al.*, 1992). Caspases belong to the cysteine proteases family and involved in protein hydrolysis (Lopez and Tait, 2015), which is the key event in apoptosis. Caspases are highly specific proteases synthesized as zymogens and activated by cleavage at aspartate, which generates large and small

units of mature enzyme (McIlwain *et al.*, 2015). Caspases are produced as inactive precursors known as procaspases. Once activated, caspases cleaved proteins within cells, resulting in precise and efficient killing of the cell in which they are activated.

Caspases are a group of proteins responsible for cleaving target proteins, especially in apoptosis. Two types of caspases classified based on their roles in apoptosis are known as the initiator caspases (caspase-2,-8,-9, and -10) and executioner caspases (caspase-3, -6 and -7) (Pfeffer and Singh, 2018). Caspase -8 and -9 are normally inactive pro-caspases and will be activated by dimerization instead of cleavage and this process is known as an "induced proximity model" (McIlwain *et al.*, 2015; Muzio *et al.*, 1998). In the model, the dimerization and activation of these caspases are reliable on the upstream signals. Activation of the executioner caspases are in controlled by their inactive pro-caspase dimers which needed the initiator caspases to cleave them. This mechanism prevents from inappropriate activation of the executioner caspases. Initiator caspases will cleave the executioner caspases between the large and small subunit that allows a conformational change on the active sites of the executioner caspase, and finally create a functional active mature caspase (McIlwain *et al.*, 2015). Once activated, caspase-8 will directly initiate apoptosis or cleave few other proteins including caspase-3, that eventually will initiate apoptosis after a series of proteins activation events occured.

### 2.5.3 Targeting apoptosis for Cancer treatment

A smart way discovered to treat cancer is to become the driver in controlling and possibly end the uncontrollable proliferating cancer cells. This can be achieved by using their own mechanism of programmed cell death, the apoptosis. Apoptosis plays a major role in cancer by inhibiting the tumour growth. Targeting apoptosis can be applied on various types of cancers as apoptosis evasion is one of the hallmarks of

cancer which is not specific to the type or the cause of cancer. Many anticancer drugs work by targeting different types of proteins or stages in either intrinsic pathway or the extrinsic pathway (Bao *et al.*, 2017; Liu and Zhu, 2017). The most common approaches reported are inhibition of anti-apoptotic proteins and stimulation of pro-apoptotic proteins (Hassan *et al.*, 2014). The research that has been done include Bcl-2 inhibitors (Zaman *et al.*, 2014), death-receptors ligands (Lopez and Tait, 2015), alkylphospholipid analogs (apoptosis signals) and inhibiton of XIAP (Hassan *et al.*, 2014). Though more researches are currently underway, there is no indication as of which target is the best. As we progress, more anticancer drugs inducing apoptosis will be determined.

## 2.6 CANCER AND IMMUNE RESPONSE

Cancer is often diagnosed long after it is initially formed. In 1909, Ehrlich (1909) believed that the immune system is able to protect host from cancer. However, his hypothesis was not experimentally proven because of the experimental tools and knowledge were inadequate at that time. He said,

> "in the enormously complicated course of fetal and post-fetal development, aberrant cells become unusually common. Fortunately, they remain completely latent thanks to the organism's positive mechanisms"
>
> (Ehrlich, 1909)

A resilient immune system is needed to eliminate or control the development of cancer. In other words, Burnet and Thomas postulated the immune surveillance hypothesis of cancer, which stated that sentinel thymus-dependent cells of the body constantly surveyed host tissues for emerging transformed cells. This hypothesis has

been supported by animal model experiment where mice with an immune-compromise status are susceptible to develop cancers compared to immune-competent mice (Burnet, 1957; Burnet, 2014; Mosier *et al.*, 1988; Thomas and Lawrence, 1959). Patients receiving organ transplants that were under treatment of prolong immunosuppressive drugs were developing tumours such as renal cancer, non-Hodgkin lymphoma, Kaposi sarcoma and other carcinomas (Burra and Rodriguez-Castro, 2015; Penn, 1993).

Over the years, treatments of cancers involved both ionizing radiation and chemotherapeutic agents were used to eradicate the mass of tumour. These treatments are beneficial and effective however there are cases of tumour relapse due to drug resistance of the tumour cells. Cancer immunotherapies are alternatives to the conventional treatments to enhance the ability of the immune system to kill-and-destroy cancer cell and control the advancement of tumour growth. Since the knowledge of the immune system is developing and slowly understood, various immune pathways are identified as candidates to potentiate anti-tumour responses in cancer patients. Several types of immunotherapy include monoclonal antibodies, non-specific immunotherapies, oncolytic virus therapy, T-cell therapy, cancer vaccines and other interventions (Hu *et al.*, 2019).

The immune system responses involved both adaptive long-lived responses and memory (T cells and B cells), and the innate cells that give short-lived responses, which involved the monocytes, macrophages, dendritic cells, and natural killer (NK) cells. The innate cells are the immediate responses that release the cytokines to lyse the abnormal cells or capturing debris from dead cells that resulted in specific foreign antigens peptide fragments, which are then identified by the adaptive T and B cells to generate antigen-specific responses. Major effector cells that target cancer cells

specifically are NK cells, dendritic cells, polymorphonuclear (PMN) leukocytes, macrophages, cytotoxic T lymphocytes (CTL) and mast cells (Yang *et al.*, 2019).

### 2.6.1 Natural Killer Cells

Natural Killer cells or also known as NK cells are a large granular lymphocyte (LGL) important to the innate immune system. NK cells was first discovered in 1970s (Pross and Jondal, 1975) through a series of experimentation on cancer patients with regards to cytotoxicity. NK cells are capable of killing virus-infected and cancer cells without initial activation thus the term "Natural Killer" cells were used. NK cells are originated from common lymphoid progenitor cells located in the bone marrow. NK cells live in the primary and secondary lymphoid tissue, including in the non-lymphoid tissue and also within the liver, lungs and peripheral blood (Freud *et al.*, 2014).

Ten to 15% of the circulating lymphocytes are the NK cells. NK cells are cytotoxic lymphocyte and can be characterised by the presence of surface marker protein expressing CD56 and CD16. They are also identified by $CD56^{+}/CD3^{-}$, where CD3 is specific for T-lymphocytes and is not expressed by NK cells (Grudzien and Rapak, 2018). Ten percent (10%) of the NK cells found in the peripheral blood are immature NK cells ($CD56^{bright}$ / $CD16^{dim}$) that produce cytokine which is important in the immunomodulation. $CD56^{bright}$ NK cells do not contain cytotoxic granules but are able to respond to target cells by producing the $T_H1$ cytokine IFNγ. While more than 90% of NK cells in peripheral blood are mature NK ($CD56^{dim}$/ $CD16^{bright}$), which is important in mediating immune response of NK cells (Daher and Rezvani, 2018). They carry cytotoxic granules and are effective killers. NK cells in the liver, lymph nodes and lung are less cytotoxic comparing to the blood NK. Although, NK cells from the uterus contain cytotoxic granules, they do not respond to target cells. They are mainly

functioning to produce angiogenic factors and mediating in placenta formation (Sojka *et al.*, 2019).

Lymphocytes activation, survival, proliferation and differentiation are regulated by cytokines. Interleukin in particular IL-2, IL-15, IL-12, IL-18 and IL-21 are known to promote NK cells proliferation and improve their anti-tumour ability (Floros and Tarhini, 2015; Hu *et al.*, 2019; Srivastava *et al.*, 2013). A 15 000-kDa α-helix cytokines known as IL-2 is produced predominant by activated $CD4^+$ and $CD8^+$ T cells. IL-2 is also produced by activated dendritic cells (DCs), NK cells, and NKT cells (Granucci *et al.*, 2001). IL-2 effects two important immune cell population, the T and NK cells. It is known that IL-2 plays a role in the these cells proliferation and not only that, it also enhances the NK cells cytotoxic ability aginst tumour cells (Floros and Tarhini, 2015; Srivastava *et al.*, 2013; Wang *et al.*, 1999). Recently, IL-2 is administered to induce NK proliferation in patient undergoing immunotherapy. However, IL-2 caused side effects including nausea, flu and dizziness, therefore limiting its application widely (Surayot and You, 2017). Furthermore, high dosage of IL-2 also can result in toxicity in patients (Suck and Koh, 2010).

NK cells have the ability to promote cytotoxic activity and cytokine production following immune system stimulation (Grossenbacher *et al.*, 2016). Recently, NK cells are classified as cytotoxic, interferon-γ (IFNγ)-producing innate lymphocytes (ILC) (Elemam *et al.*, 2017). IFNγ is responsible in modulating immune response in particular towards defence against intracellular pathogens and cancers. It is produced by NK cells, NKT cells, $CD8^+$ T cells and T-helper 1 ($T_H1$) $CD4^+$ cells (Schoenborn and Wilson, 2007). IFNγ supports $T_H1$ differentiation, enhancing the function of macrophages, encouraging leukocyte migration to the infection site, and exerting the

MHC expression for better T-cells recognition of infected or malignant cells (Farrar and Schreiber, 1993; Schroder *et al.*, 2004). Deficiency in IFNγ signalling in mice and humans contributes to the susceptibility to tumours and intracellular pathogens infections such as mycobacteria (Bustamante *et al.*, 2014; Ikeda *et al.*, 2002).

NK cells induce apoptosis in their targets by producing the TNF family molecules, cytokines and cytotoxic granules (Paul and Lal, 2017). NK cells respond to target cells, either virus infected or malignant cells that are lacking MHC class I molecules. NK cells activation is regulated by signals, either inhibitory or activating, from target cells in the pro- and anti- inflammatory microenvironment. Once activated, cytotoxic proteins are released from the pre-formed cytoplasmic granules of the NK cells by exocytosis at the NK-target cell interface (Lotzova and Herberman, 2019). This process is knows as degranulation which relase pore-forming protein, perforin and granzyme B.

NK cells directly kill their target cells by mediation of perforin/granzyme or by FasL mechanism (Lee *et al.*, 2018b). These cytotoxicity granules proteins critically drive direct cytotoxicity on target cells (Backes *et al.*, 2018). Once activated, NK cells release these proteins on the target cells. Perforin is the protein function to disrupt the cell membrane and granzymes are protein belonging to the serine proteases (Sabry and Lowdell, 2017). Perforin binds to the phospholipid on the components of the membrane lipid bilayer in a calcium dependent manner and finally, facilitate the entrance of the granzymes into the cytosol of the target cell. Granzymes will induce apoptosis once they are inside the target cell.

As mentioned earlier, NK cells also kill target cell by engagement of cell surface death receptors with the expression of Fas ligand (FasL) and TNF-related

apoptosis-inducing ligand (TRAIL). Once bound to the receptors on the target cells, these proteins will induce apoptosis. Another direct killing method of NK cells cytotoxicity involves antibody dependent cell mediated cytotoxicity (ADCC) and this is usually mediated by immunoglobulin G (IgG) in humans. NK cells also kill their target cells indirectly by producing cytokines and chemokines to destroy abnormal cells and restore the regulation of innate and acquired immune responses (Fauriat *et al.*, 2010).

### 2.6.2 Phytocompounds and Natural Killer cells

Medicinal plants contain phytocompounds that can stimulate NK cells effect. Vitamins are among the natural compound known to stimulate NK cells. Fat-soluble vitamin A such as retinol, retinal, retinoic acid (RA) and carotenoids were able to reduce tumour growth in several animal models. Administration of retinol in BALB/c mice (with breast cancer) resulted in increased splenic NK cells activity and the highest activity was recorded after 1 hour of the treatment (Fraker *et al.*, 1986). Vitamin B is important for cell metabolism and it a water soluble vitamins. Significant decrease of NK cells cytotoxicity was observed in the spleen of the mice with vitamin $B_{12}$ defeciency (Partearroyo *et al.*, 2013). The water soluble vitamin C also have demonstrated stimulatory NK cells activities. In an experiment conducted *in vivo*, blood from healthy individuals were collected before and after administration of vitamin C and aloe vera juice. NK cells cytotoxicity was significantly increased after the vitamin C supplementation (Toliopoulos *et al.*, 2012). Vitamin D is a insoluble vitamin belonging to the steroid group. In experiment comparing between control mice and high-fat diet mice showed that supplementation of vitamin D increased the NK cells actvity in lean mice but not in obese diet mice (Lee *et al.*, 2018a). Vitamin E is

another water insoluble vitamin that have demonstrated immunomodulatory effects in animals and human models (Mutalip, 2018).

Some other reported phytochemicals promoting NK cells acitivity are genistein, curcumin and ginseng. Genistein is an isoflavone compound identified in soybean. The investigation of genistein and NK cells activation started with the observation of lower incident rates of breast, colon and prostate cancer in countries with higher dietary consumption of soybean (Tse and Eslick, 2016). Genistein (0.1-0.5µmol/l) showed increased NK cells activity, however the activity declined with concentration more than 0.5µmol/l (Zhang *et al.*, 1999). Curcumin, form *Curcumin longa* showed that the increased dosage was able to increase NO production by NK cells therefore promoting apoptosis on the target cell (Bhaumik *et al.*, 2000). Ginseng plant was also able to significantly augment NK cells cytotoxicity (Takeda and Okumura, 2019) and treatment of polysaccharide from ginseng berry also showed NK cells cytotoxicity on cancer cells (Lee *et al.*, 2019a).

# CHAPTER 3

# GC-MS ANALYSIS OF PHYTOCHEMICAL COMPOUNDS of *Abrus precatorius* LEAVES EXTRACT

## 3.1 INTRODUCTION

Medicinal plants are gaining worldwide recognitions because of their diversity and have broad pharmacological activities from their therapeutic phytochemicals. Phytochemicals are extracted from different parts of plants like seeds, seed coats, barks, leaves, flowers, pulps, roots, and shoots. The extraction of the phytochemical compounds is significant in the exploration of new therapeutic biomolecules that are potential as medicinal agents. Plant contains a variety of chemical compounds that can be utilised to treat chronic and infectious diseases (Duraipandiyan *et al.*, 2006). Phenolic compounds and flavonoids, for instance, have a great impact on health and cancer prevention (Venugopal and Liu, 2012). To date, thousands of phytochemical compounds have been reported to have beneficial biological activities such as antimicrobial, antioxidant, anticancer, and many more.

The important part in studying the medicinal plants is the extraction procedure. Extraction is a standard procedure to separate phytochemical compounds using selective solvents. Decoction and maceration are commonly used in traditional practices while the Soxhlet extraction is more familiar in the industrial settings. Other modern extraction techniques include, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), which aim to increase yield with a minimal cost, while superficial fluid extraction (SFE) and accelerated solvent extraction are less preferred due to the high cost despite their efficiency (Azwanida, 2015).

Decoction is a method of extraction by means of boiling the plant materials to extract volatile compounds, oils, and various other phytochemical compounds. Maceration on the other hand is a technique adopted from wine making where plant materials are soaked in a closed container with a solvent for at least three days at room temperature (Handa, 2008). Soxhlet extraction or also known as continuous hot extraction uses the Universal Extraction System (Buchi), where heated solvent in a flask, vaporizes into the thimble containing grounded plant material, condenses in the condenser and drip back into the hot flask. This process is repeated until the colour of the solvents becomes clear. Decoction of the *Abrus precatorius* leaves is widely practised as the treatment for cold, coughs and colic. Juice from the leaves is applied to swellings by mixing with oil. Mixture of rice starch and the leaf paste are consumed orally for anthrax treatment (Pokharkar *et al.*, 2011). Powdered leaves paste are used for conjunctivitis and convulsion in children (Joshi and Tyagi, 2011).

Plant extracts encompass of numerous phytochemical compounds, which pose a challenge in order to separate and identify them due to their polarity. Chromatography is a process to separate any molecules based on their shape, size and charge. With the advancement of research and technologies, different separation techniques have been introduced to identify and isolate these compounds such as gas chromatography (GC), paper chromatography, thin layer chromatography (TLC), high-performance thin layer chromatography (HPTLC), column chromatography, overpressure layer chromatography (OPLC), and high-performance liquid chromatography (HPLC). GC is a technique used to separate volatile compounds, where the liquid phase is separated from the gas phase. It is one of the most important analytical methods in organic chemical analysis to determine individual substances in complex mixtures. Mass-spectrometry is an analytical method that measure masses within a sample by ionizing the chemical species and sort their ions based on the mass-

to-charge ration. This detection method provides meaningful data by determining the substance molecules or fragments directly. Therefore, the integration of gas-chromatography and mass-spectrometry into a single GC-MS system has been a great platform for many laboratories to run a quantified detection analysis due to its high selectivity and very high sensitivity (Belwal *et al.*, 2018).

Phytochemical analysis of the leaves and roots of *A. precatorius* demonstrated the presence of glycyrrhizin (Karwasara *et al.*, 2010), an important compound of liquorice (Killacky *et al.*, 1976), which is widely used in the food and pharmaceutical industry. A known triterpenoid and three novel triterpenoids were identified from the acid hydrolyzed methanol-soluble leaves extract (Kim *et al.*, 2002) of *A. precatorius*. From the *n*-butanol leaves extract of *A. precatorius,* other compounds identified were abrusoside A (Choi *et al.*, 1989), abrusosides B, C, D, plus three other sweet glycosides based on the novel cycloartane-type aglycone, abrusogenin (Kinghorn and Soejarto, 2002). This chapter described the phytochemicals identified in the leaves extracts of *A. precatorius*. Different extraction methods were performed and their phytochemicals were characterised in order to possibly identify all phytochemicals in the leaves of *A. precatorius*.

## 3.2 MATERIALS & METHODOLOGY

### 3.2.1 Plant collections

*A. precatorius* matured leaves (Figure 3.1) were collected from Kampung Sabak, Pengkalan Chepa, Kelantan, Malaysia, around the months of August till September. The plant was authenticated by Assoc. Prof. Dr. Rahmad Zakaria from the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia and the voucher specimen (USM 11730) was submitted for future references.

### 3.2.2 Preparation of leaves sample

The leaves of *A. precatorius* were collected, cleaned and oven-dried at 50ºC, and then later ground to fine powder with a mechanical grinder.

### 3.2.3 Aqueous decoction of *Abrus precatorius* leaves

Decoction method was used in traditional medicine and hence was applied in this experiment. For aqueous extract, 11g of dried fine powdered leaves were soaked in 450 ml water at 50°C until the water reduced to one-third of the initial volume (Wan-Ibrahim *et al.*, 2018). The extract was then freeze-dried for the subsequent analysis.

### 3.2.4 Maceration extraction of the leaves by hexane, ethyl acetate and methanol solvents

Three successive extractions of the *A. precatorius* leaves by maceration were performed following the method by Irshad *et al.* (2012) with modifications. Approximately 18g of dried powdered leaved were soaked in three different solvents successively for about one month each. Initially the leaves were macerated in 250ml of hexane (99%), followed by 250ml of ethyl acetate (99%) and lastly in 250ml of methanol (99%). During the maceration period, the mixture was manualy agitated in every other three days. After each solvent, the mixture of the leaves and solvent was filtered then the filtrate was left to dry under the fume hood to obtain the dried crude extract. The remaining leaves were also left under the fume hood to evaporate the remaining solvent before macerating with the following solvent.

### 3.2.5 Successive solvent Soxhlet Extraction

About 22g of ground *A. precatorius* was subjected to successive Soxhlet (Buchi) extraction with 250ml hexane, -ethyl acetate and -methanol, performed with

modification according to Kaneria and Chanda (2012) . Upon completion of the first extraction with hexane, the solution was dried using a rotary evaporator. The remaining powdered leaves in the thimble was left to dry overnight in fume hood to evaporate residual hexane. Then subsequent extraction with ethyl acetate was performed in the same manner and followed by methanol. All extracts were kept in -20°C until further used.

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

Hewlett Packard 6890 Gas Chromatograph with 5973N Mass Selective Detector was used to carry out the GC-MS. 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 flow rate at 1.0 ml/min with the oven temperature was programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 20°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively. One microliter sample was injected in splitless mode and was analysed in MS full scan mode (*m/z* 40-650). The electron ionisation was fixed at 70eV. Acquisition of data was performed using Chemsation software.

### 3.2.7 Identification of phytochemical compounds

The mass spectrum of the GC-MS was interpreted against the database of the National Institute of Standards and Technology (NIST02) and Wiley275 libraries with matches of ≥80 % to identify phytochemical compounds.

## 3.3 RESULTS

### 3.3.1 *Abrus precatorius* plant

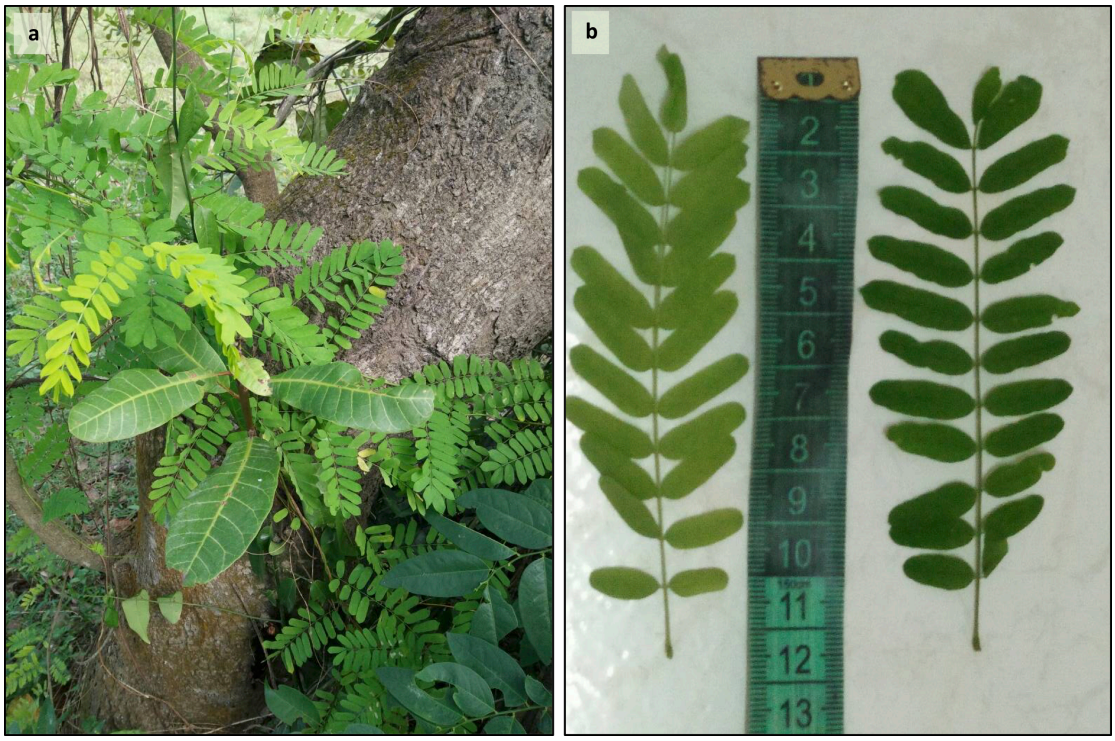



Figure 3.1: ***Abrus precatorius* leaves used in this study.**

#### 3.3.1(a) Yield of all extracts after each extraction

Figure 3.1 showed the leaves collected for this study. The yield obtained after each extractions of the *A. precatorius* leaves are as follows: aqueous extract (31.8%), hexane extract by maceration (5.55%), ethyl acetate extract by maceration (6.10%), methanol extract by maceration (8.67%), hexane extract by Soxhlet (4.67%), ethyl acetate extract by Soxhlet (4.76%), and methanol extract by Soxhlet (7.90%).

### 3.3.2 Aqueous Extract by decoction

The GC-MS analysis showed that the classes of compounds identified in aqueous extracts of *A. precatorius* leaves were phenolic compounds, terpenoids and steroids.. Seventeen chemical compounds were identified as shown in Table 3.1. The main class of compounds identified was phenolic compounds (2.82%). Four phenolic compounds were identified, and the major phenolic compound was 4-vinylphenol (1.17%). Other major compounds that found in *A. precatorius* leaves are methyl jasmonate (1.89%), decylenic alcohol (1.46%) and cis-11-Tetradecen-1-ol (1.41%). Methyl jasmonate is categorised as fragrance that belongs to structural group ketones cyclopentanones and cyclopentenones (Scognamiglio *et al.*, 2012). Decylenic alcohol is also belongs to the fragrance group and it is also known as Rosalva.

Table 3.1: Compounds Present in The Leaves Aqueous Extracts of *Abrus precatorius* Using GC-MS

| Retention time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Phenolic compound** | |
| 8.4 | 4-vinylphenol | 1.17 |
| 8.9 | p-Vinylguaiacol | 0.68 |
| 10.1 | β-Phenoxyethyl iso-butyrate | 0.47 |
| 11.2 | Cinnamaldehyde, β-hexyl- | 0.50 |
| | **Terpenoids** | |
| 9.9 | β-lonone | 0.17 |
| 12.6 | Phytol | 0.39 |
| | **Steroids** | |
| 16.5 | Stigmasterol | 0.31 |
| | **Others** | |
| 7.8 | 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- | 0.66 |
| 9.8 | 6-Tridecene | 0.13 |
| 10.7 | Methyl dihydrojasmonate | 1.89 |
| 10.9 | Propanoic acid,2-methyl-3-[4-t-butyl]phenyl- | 0.68 |
| 11.5 | Decylenic alcohol | 1.46 |
| 11.5 | 3-Decen-1-ol, (E)- | 1.07 |
| 11.6 | cis-11-Tetradecen-1-ol | 1.41 |
| 13.7 | Palmitic acid á-monoglyceride | 0.19 |
| 15.7 | 1-Heneicosanol | 0.72 |
| 16.6 | 1-Heptacosanol | 1.17 |

### 3.3.3 Maceration extraction of the leaves (Hexane)

36 compounds were identified in this extract. The main compounds in this hexane leaves extract by maceration are 1-Octscosanol (24.09%), 1-heptacosanol (21.80%) and oxirane heptadecyl- (20.85%). Twelve compounds were identified under the terpenoids group as listed in Table 3.2.

Table 3.2: Compounds Present in The Leaves Hexane Extracts (Maceration) of *Abrus Precatorius* Using GC-MS

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Terpenoids** | |
| 7.114 | Dihydromyrcenol | 0.01 |
| 7.387 | Linalool | 0.02 |
| 8.417 | Citronellol | 0.01 |
| 8.109 | m-methylacetophenone | 0.01 |
| 8.368 | β-cyclocitral | 0.01 |
| 8.627 | β -cyclohomocitral | 0.01 |
| 9.243 | Naphtalene,1,2,3,4-tetrahydro-1,1,6-trimethyl- | 0.02 |
| 9.733 | Geranyl acetone | 0.02 |
| 9.936 | β-lonone | 0.07 |
| 11.512 | Neophytadiene | 1.23 |
| 14.894 | Squalene | 0.91 |
| 14.957 | Geranylgeraniol | 0.13 |
| | **Others** | |
| 5.938 | 3-octanone | 0.01 |
| 10.195 | 2(4h)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7aa-trimethyl | 0.15 |
| 10.734 | Methyl dihydrojasmonate | 0.20 |
| 11.162 | Octanal, 2-(phenylmethylene)- | 0.08 |
| 13.339 | Ethyl eicosanoate | 0.18 |
| 13.451 | 4,8,12,16-tetramethylheptadecan-4-olide | 0.20 |
| 13.563 | Tetracosane | 1.00 |
| 13.815 | Butyl 9,12-octadecadienoate | 0.14 |
| 13.885 | Eicosane | 0.32 |
| 13.955 | 2- Monopalmitin | 0.66 |
| 14.151 | 4-methyl-1-anthracenamine | 0.08 |
| 14.305 | 16-heptadecenal | 0.16 |
| 14.495 | Heptadecane | 1.55 |
| 14.586 | Tetracosanoic acid, methyl ester | 0.80 |
| 15.076 | Octadecane, 1-chloro- | 1.98 |
| 15.363 | Tridecane | 0.86 |

Table 3.2 Continued

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| 15.531 | 1,19-eicosadiene | 7.98 |
| 15.839 | 1-heptacosanol | 21.80 |
| 15.923 | Octadecanal | 0.78 |
| 15.972 | 16-octadecenal | 1.07 |
| 16.441 | Oxirane heptadecyl- | 20.85 |
| 16.868 | 1-octacosanol | 24.09 |
| 17.582 | Dl-α-tocopherol | 0.62 |
| 19.585 | Cyclotriacontane | 0.20 |

### 3.3.4 Maceration extraction of the leaves (Ethyl acetate)

21 compounds were identified. The main compounds in this extract are 2-hexadecene,3,7,11,15-tetramethyl-(R-(R*,R*-E)- (16.02%), octacosyl acetate (8.67%) and phytol (7.6%).

Table 3.3: Compounds Present in The Leaves Ethyl acetate Extracts (Maceration) of *Abrus precatorius* Using GC-MS

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Phenolic compounds** | |
| 8.984 | 2-Methoxy-4-vinylphenol | 0.72 |
| | **Terpenoids** | |
| 12.59 | Phytol | 7.60 |
| 14.886 | Squalene | 0.73 |
| | **Steroids** | |
| 16.28 | 7-ergosterol | 2.06 |
| 16.805 | β-Sitosterol | 1.78 |
| | **Others** | |
| 7.121 | 1-Ethyl-2-pyrrolidinone | 0.01 |
| 8.41 | Coumaran | 0.12 |
| 9.229 | Naphthalene,1,2-dihydro-2,5,8-trimethyl- | 0.09 |
| 9.474 | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone | 0.06 |
| 11.519 | 2-hexadecene,3,7,11,15-tetramethyl-(R-(R*,R*-E)- | 16.02 |
| 11.855 | Methyl hexadecanoate | 1.70 |
| 12.786 | Ethyl linolenate | 0.18 |
| 13.941 | 2-Palmitoglycerol | 1.88 |
| 14.515 | Nonanoic acid,9-(3-hexenylidenecyclopropylidene)-,2-hydroxy-1-(hydroxymethyl) | 5.03 |
| 15.055 | Cyclotriacontane | 2.52 |
| 15.244 | D-δ-tocopherol | 2.54 |
| 15.902 | Vitamin E | 2.05 |
| 16.133 | Z-14-Nonacosane | 2.48 |
| 16.574 | Octacosyl acetate | 8.67 |
| 17.141 | Triacontyl acetate | 2.44 |
| 17.764 | Cyclotriacontane | 1.08 |

### 3.3.5 Maceration extraction of the leaves (Methanol)

21 compounds were identified. The main compounds group in this extract are the phenolic compounds (3.44%), consisted of phenol, 4-Vinylguaiacol, syringol, methylparaben, and 4-methyl-2,5-dimethoxybenzaldehyde. Cyclotetracosane was the most abundant compound in this extract (3.00%).

Table 3.4: Compounds Present in The Leaves Methanol Extracts (Maceration) of *Abrus precatorius* Using GC-MS

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Phenolic compounds** | |
| 6.246 | Phenol | 0.22 |
| 8.984 | 4-Vinylguaiacol | 0.72 |
| 9.201 | Syringol | 0.95 |
| 9.831 | Methylparaben | 0.34 |
| 10.328 | 4-methyl-2,5-dimethoxybenzaldehyde | 1.21 |
| | **Steroids** | |
| 16.805 | β-Sitosterol | 0.48 |
| | **Terpenoids** | |
| 8.872 | Indolizine | 0.25 |
| | **Others** | |
| 7.681 | Butanedioic acid, hydroxy-,dimethyl ester | 0.60 |
| 8.185 | Methyl salicylate | 0.10 |
| 8.396 | Coumaran | 2.35 |
| 9.467 | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-y)ethanone | 0.28 |
| 10.734 | Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester | 0.91 |
| 11.210 | 2-Propenoic acid,3-(4-hydroxyphenyl)-, methyl ester | 2.58 |
| 11.862 | Hexadecanoic acid, methyl ester | 2.08 |
| 13.948 | 2-Monopalmitin | 2.82 |
| 15.054 | Cyclotetracosane | 3.00 |
| 15.601 | γ-Tocopherol | 0.80 |
| 15.706 | Cyclooctacosane | 1.12 |
| 15.902 | Vitamin E | 0.55 |
| 16.574 | Triacontyl acetate | 2.01 |
| 17.764 | 1-Octacosanol | 0.07 |

### 3.3.6 Soxhlet Extraction of the leaves (Hexane)

22 compounds were identified from this extract. Main compound identified from this extract is oxirane, hexadecyl- at 15.72%, followed by 1-eicosanol at 10.53%.

Table 3.5: Compounds identified in the leaves of *A. precatorius* hexane extracts (Soxhlet)

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Phenolic compounds** | |
| 11.870 | Phenol, 3-isopropoxy-5-methyl | 0.05 |
| | **Terpenoids** | |
| 10.722 | Dihydroactinidiolide | 0.12 |
| 12.003 | Neophytadiene | 1.54 |
| 11.814 | (-)-Loliolide | 0.35 |
| 18.151 | Alnulin | 0.23 |
| | **Steroids** | |
| 17.437 | Campesterol | 0.33 |
| | **Others** | |
| 2.621 | Octane | 0.10 |
| 4.924 | Nonane | 1.00 |
| 10.57 | Dodecanoic acid | 0.05 |
| 10.834 | 3-Mercapto-2(1H)-pyridinone | 0.04 |
| 11.660 | Myristic acid | 0.08 |
| 11.682 | 1-Methylbicyclo(6.3.0)undec-5-en-9-one | 0.09 |
| 12.185 | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | 0.57 |
| 12.354 | Hexadecanoic acid, methyl ester | 0.05 |
| 12.564 | Hexadecanoic acid | 5.63 |
| 13.031 | 2-Pentadecanone,6,10,14-trimethyl- | 0.11 |
| 13.047 | 9,12,15-Octadecatrienoic acid, methyl ester | 0.18 |
| 13.096 | Octadecanoic acid | 5.24 |
| 14.477 | 2-Monopalmitin | 0.23 |
| 15.035 | 9,12,15-Octadecatrienoic acid | 0.18 |
| 15.077 | Octadecanoic acid,2-hydroxy-1-( hydroxymethyl)ethyl ester | 0.35 |
| 15.378 | Cyclooctacosane | 0.13 |
| 15.792 | Bicyclo (10.8.0)eicosane, (E)- | 0.37 |
| 16.415 | Stigmasta-5,22-dien-3-ol, acetate, (3.beta.,22Z)- | 0.46 |
| 16.541 | 1-Eicosanol | 10.53 |
| 16.744 | Vitamin E | 0.37 |
| 18.067 | (23S)-ethylcholest-5-en-3.beta.-ol | 0.49 |
| 18.403 | 1-Tetracosanol | 0.69 |
| 18.788 | Oxirane, hexadecyl- | 15.72 |
| 19.152 | Stigmast-4-en-3-one | 0.08 |

### 3.3.7 Soxhlet Extraction of the leaves (Ethyl acetate)

Neophytadiene (32.56%) was the main compound idetified in this extract. 37 compounds were identified from this extract.

Table 3.6: Compounds identified in the leaves of *A. precatorius* ethyl acetate extracts (Soxhlet)

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Phenolic compounds** | |
| 8.531 | Benzoic acid | 0.30 |
| 8.860 | 4-vinyl-phenol | 3.26 |
| 9.834 | 2-Methoxy-4-vinylphenol | 0.99 |
| 15.75 | Naringenin | 0.91 |
| 16.688 | 3-Methoxy-4,5,7-trihydroxyflavone | 0.43 |
| 17.479 | Cirsimaritin | 4.48 |
| | **Terpenoids** | |
| 10.722 | Dihydroactinidiolide | 0.54 |
| 11.822 | (-)-Loliolide | 2.16 |
| 12.011 | Neophytadiene | 32.56 |
| 13.089 | Phytol | 1.71 |
| | **Steroids** | |
| 17.99 | β-Sitosterol | 0.64 |
| | **Others** | |
| 2.705 | 2-Propenoic acid, 2-methyl-,methyl ester | 0.41 |
| 3.657 | 2-Pentanone,4-hydroxy-4-methyl- | 0.35 |
| 8.089 | 2-Pentene,(Z)- | 0.46 |
| 8.713 | Benzoic acid, 2-hydroxy-, methyl ester | 0.12 |
| 8.958 | 1H-Pyrrole-2,5-dione,3-ethyl-4-methyl- | 0.25 |
| 9.378 | Benzonitrile, 2-methyl- | 0.19 |
| 9.735 | Naphtalene,1,2-dihydro-1,1,6-trimethyl- | 0.26 |
| 9.973 | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopentan(c)pyran-1-yl)ethanone | 0.22 |
| 10.561 | 2,5-Cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethyl)- | 0.57 |
| 10.750 | Dodecanoic acid | 0.57 |
| 10.820 | 2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone | 1.16 |
| 11.660 | Myristic acid | 2.30 |
| 11.569 | 1,2-Benzenediol,3,5-bis(1,1-dimethylethyl)- | 0.40 |
| 11.709 | 1-Methylbicyclo(6.3.0)undec-5-en-9-one | 1.05 |
| 12.508 | Hexadecanoic acid | 4.76 |
| 12.620 | Ethyl palmitate | 0.22 |
| 12.872 | Margaric acid | 0.19 |
| 13.201 | Linolenic acid | 5.59 |

Table 3.6 Continued

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| 13.264 | Stearic acid | 2.97 |
| 13.362 | Ethyl stearate | 0.25 |
| 14.363 | Heptacosanol | 0.41 |
| 15.028 | Nonanoic Acid,9-(3-Hexenylidenecyclopropylidene)- | 1.71 |
| 15.351 | 4,5'-Dihydroxy-7-methoxyflavanone | 0.44 |
| 15.841 | Stigmastan-6,22-dien,3,5-dehydro- | 0.29 |
| 16.408 | Stigmastan-3,5,22-trien | 0.90 |
| 16.744 | Vitamin E | 0.76 |

### 3.3.8 Soxhlet Extraction of the leaves (Methanol)

A total of 29 compounds found in this extract. 4-vinylphenol and neophytadiene were the two main compounds indentified.

Table 3.7: Compounds identified in the leaves of *A. precatorius* methanol extracts (Soxhlet)

| Retention Time (min) | Name of Compound | Area (%) |
|---|---|---|
| | **Phenolic compounds** | |
| 6.787 | Phenol | 0.09 |
| 8.867 | 4-vinylphenol | 12.18 |
| 9.483 | 2-Methoxy-4-vinylphenol | 0.71 |
| 10.820 | 4-vinyl-syringol | 0.37 |
| 17.472 | Cirsimaritin | 0.53 |
| | **Terpenoids** | |
| 11.822 | (-)-Loliolide | 1.59 |
| 11.941 | Neophytadiene | 12.18 |
| 13.089 | Phytol | 0.31 |
| | **Steroids** | |
| 17.598 | Stigmasterol | 0.54 |
| | **Others** | |
| 2.545 | 2-Furancarboxaldehyde | 0.22 |
| 5.555 | Butyrolactone | 0.08 |
| 5.856 | 2-Hydroxy-2-cyclopenten-1-one | 0.80 |
| 7.928 | Benzoic acid, methyl ester | 0.56 |
| 8.159 | Octanoic acid, methyl ester | 0.13 |
| 8.783 | Benzoic acid,2-methyl-, methyl ester | 0.17 |
| 9.735 | Capric acid | 0.29 |
| 10.568 | Lauric acid, methyl ester | 0.22 |
| 10.764 | Dodecanoic acid | 1.16 |
| 11.506 | Tetradecanoic acid, methyl ester | 3.02 |
| 11.660 | Myristic acid | 2.77 |
| 12.032 | 2-Pentadecanone | 1.32 |
| 13.040 | Methyl,8,11,14-heptadecatrienoate | 0.51 |
| 13.187 | Linolenic acid | 0.55 |
| 13.25 | Stearic acid | 0.56 |
| 13.53 | Methanone, (4-chlorophenyl)(4-hydroxyphenyl)- | 0.11 |
| 14.440 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | 0.44 |
| 15.07 | Octadecanoic acid,2-hydroxy-1-(hydroxymethyl)ethyl ester | 0.37 |
| 15.652 | 4-amino-5-tert-butyl-4'-(dimethylamino)biphenyl-3-carbonitrile | 0.24 |
| 15.813 | Glycerol tricaprylate | 0.43 |

### 3.3.9 Comparison of the obtained between Maceration and Soxhlet by each solvent

#### 3.3.9(a) Hexane

Comparison of the compounds present in from both henaxe- Soxhlet and maceration techniques are as shown in Table 3.9. Both extracts shared eight same compounds. 1-Octacosanol appeared the highest with 24.31% (Soxhlet) and 24.09% (Maceration).

Table 3.9: Comparison of phytocompounds in *A. precatorius* leaves extracted with hexane by Soxhlet and maceration.

| | Soxhlet | Maceration | |
|---|---|---|---|
| 24.31% | 1-Octacosanol | | 24.09% |
| 0.24% | 1-Heptacosanol | | 21.80% |
| 2.17% | 16-Octadecenal | | 1.07% |
| 1.54% | Neophytadiene | | 1.23% |
| 0.02% | α-inone | | 0.02% |
| 0.31% | Octadecanal | | 0.78% |
| 0.23% | 2-Monopalmitin | | 0.66% |
| 0.49% | Eicosane | | 0.32% |
| 15.72% | Oxirane, hexadecyl- | Oxirane heptadecyl- | 20.85% |
| 10.53% | 1-Eicosanol | 1,19-Eicosadiene | 7.98% |
| 5.63% | Hexadecanoic acid | Octadecane,1-chloro- | 1.98% |
| 5.24% | Octadecanoic acid | Heptadecane | 1.55% |
| 1.01% | Celidoniol, deoxy- | Tetracosane | 1.00% |
| 1.00% | Nonane | Squalene | 0.91% |
| 0.69% | (23S)-ethylcholest-5-en-3.beta.-ol | Tridecane | 0.86% |
| 0.57% | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | Tetracosanoic acid, methyl ester | 0.80% |
| 0.49% | 1-Tetracosanol | dl-.α.-Tocopherol | 0.62% |
| 0.46% | Stigmasta-5,22-dien-3-ol, acetate, (3.beta.,22Z)- | Digiprolactone | 0.28% |
| 0.37% | Vitamin E | Cyclopentaneacetic acid, 3-oxo-2-pentyl-,methyl ester | 0.20% |
| 0.37% | Bicyclo (10.8.0)eicosane, (E)- | Cyclotriacontane | 0.20% |

Table 3.9 Continued

| Soxhlet | | Maceration | |
|---|---|---|---|
| 0.35% | (-)-Loliolide | 4,8,12,16-Tetramethylheptadecan-4-olide | 0.20% |
| 0.35% | Octadecanoic acid,2-hydroxy-1-(hydroxymethyl)ethyl ester | Eicosanoic acid, ethyl ester | 0.18% |
| 0.33% | Campesterol | 16-Heptadecenal | 0.16% |
| 0.23% | Alnulin | 2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl- | 0.15% |
| 0.18% | 9,12,15-Octadecatrienoic acid, methyl ester | Butyl 9,12-octadecadienoate | 0.14% |
| 0.18% | 9,12,15-Octadecatrienoic acid | Geranylgeraniol | 0.13% |
| 0.13% | Cyclooctacosane | Octanal, 2-(phenylmethylene)- | 0.08% |
| 0.12% | Dihydroactinidiolide | 4-Methyl-1-anthracenamine | 0.08% |
| 0.11% | 2-Pentadecanone,6,10,14-trimethyl- | β-lonone | 0.07% |
| 0.10% | Octane | Geranyl acetone | 0.02% |
| 0.09% | 1-Methylbicyclo(6.3.0)undec-5-en-9-one | Linalool | 0.02% |
| 0.08% | Stigmast-4-en-3-one | 3-Octanone | 0.01% |
| 0.08% | Myristic acid | Dihydromyrcenol | 0.01% |
| 0.05% | Dodecanoic acid | Ethanone, 1-(3-methylphenyl)- | 0.01% |
| 0.05% | Phenol, 3-isopropoxy-5-methyl- | β-cyclocitral | 0.01% |
| 0.05% | Hexadecanoic acid, methyl ester | Citronellol | 0.01% |
| 0.04% | 3-Mercapto-2(1H)-pyridinone | β -cyclohomocitral | 0.01% |

### 3.3.9(b) Ethyl acetate

Comparison of the compounds present in from both Ethyl acetate- Soxhlet and maceration techniques are as shown in Table 3.10. Both extracts shared seven similar compounds. Phytol appeared the highest with 1.71% (Soxhlet) and 7.60% (Maceration). Neophytadiene was the highest compound in the extract by Soxhlet and it was not identified in the extract by maceration.

Table 3.10: Comparison of phytocompounds in *A. precatorius* leaves extracted with ethyl acetate by Soxhlet and maceration.

| | **Soxhlet** | **Maceration** | |
|---|---|---|---|
| 1.71% | Phytol | | 7.60% |
| 0.31% | Nonanoic Acid,9-(3-Hexenylidenecyclopropylidene)- | | 5.03% |
| 0.41% | Heptacosanol | | 4.45% |
| 0.76% | Vitamin E | | 2.05% |
| 0.64% | β-Sitosterol | | 1.78% |
| 0.99% | 2-Methoxy-4-vinylphenol | | 0.13% |
| 0.26% | Naphtalene,1,2-dihydro-1,1,6-trimethyl- | | 0.09% |
| 32.56% | Neophytadiene | 2-hexadecene,3,7,11,15-tetramethyl-(R-(R*,R*-E)- | 16.02% |
| 5.59% | Linolenic acid | Octacosyl acetate | 8.67% |
| 4.76% | Hexadecanoic acid | Cyclotriacontane | 2.52% |
| 4.48% | Cirsimaritin | D-δ-tocopherol | 2.54% |
| 3.26% | 4-vinyl-phenol | Z-14-Nonacosane | 2.48% |
| 2.97% | Stearic acid | Triacontyl acetate | 2.44% |
| 2.30% | Myristic acid | 7-Ergostenol | 2.06% |
| 2.16% | (-)-Loliolide | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester | 1.88% |
| 1.16% | 2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone | Hexadecanoic acid, methyl ester | 1.70% |
| 1.05% | 1-Methylbicyclo(6.3.0)undec-5-en-9-one | Cyclotriacontane | 1.08% |
| 0.91% | Naringenin | Squalene | 0.73% |
| 0.90% | Stigmastan-3,5,22-trien | 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)- | 0.18% |

Table 3.10 Continued

| Soxhlet | | Maceration | |
|---|---|---|---|
| 0.57% | 2,5-Cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethyl)- | Coumaran | 0.12% |
| 0.57% | Dodecanoic acid | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-y)ethanone | 0.06% |
| 0.54% | Dihydroactinidiolide | 1-Ethyl-2-pyrrolidinone | 0.01% |
| 0.46% | 2-Pentene,(Z)- | | |
| 0.44% | 4,5'-Dihydroxy-7-methoxyflavanone | | |
| 0.43% | 3-Methoxy-4,5,7-trihydroxyflavone | | |
| 0.41% | 2-Propenoic acid, 2-methyl-,methyl ester | | |
| 0.40% | 1,2-Benzenediol,3,5-bis(1,1-dimethylethyl)- | | |
| 0.35% | 2-Pentanone,4-hydroxy-4-methyl- | | |
| 0.30% | Benzoic acid | | |
| 0.29% | Stigmastan-6,22-dien,3,5-dehydro- | | |
| 0.25% | 1H-Pyrrole-2,5-dione,3-ethyl-4-methyl- | | |
| 0.25% | Ethyl stearate | | |
| 0.22% | Ethyl palmitate | | |
| 0.22% | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopentan(c)pyran-1-yl)ethanone | | |
| 0.19% | Benzonitrile, 2-methyl- | | |
| 0.19% | Margaric acid | | |
| 0.12% | Benzoic acid, 2-hydroxy-, methyl ester | | |

### 3.3.9(c) Methanol

Comparison of the compounds present in from both methanol- Soxhlet and maceration techniques are as shown in Table 3.11. Both extracts shared only four similar compounds. They are hexadecenoic acid, methyl ester; 2-methoxy-4-vinylphenol, hexadecanoic acid, 2-monopalmitin and phenol. Hexadecanoic acid, methyl ester appeared the highest with 13.09% (Soxhlet) and 2.08% (Maceration).

Table 3.11: Comparison of phytocompounds in *A. precatorius* leaves extracted with methanol by Soxhlet and maceration.

| | **Soxhlet** | **Maceration** | |
|---|---|---|---|
| 13.09% | Hexadecanoic acid, methyl ester | | 2.08% |
| 0.71% | 2-Methoxy-4-vinylphenol | | 0.72% |
| 0.44% | 2-Monopalmitin | | 2.82% |
| 0.09% | Phenol | | 0.22% |
| 12.18% | Neophytadiene | 2-Propenoic acid,3-(4-hydroxyphenyl)-, methyl ester | 2.58% |
| 3.02% | Tetradecanoic acid, methyl ester | Coumaran | 2.35% |
| 2.77% | Myristic acid | Triacontyl acetate | 2.01% |
| 1.71% | 4-vinylphenol | Cyclotetracosane | 3.00% |
| 1.59% | (-)-Loliolide | 4-methyl-2,5-dimethoxybenzaldehyde | 1.21% |
| 1.32% | 2-Pentadecanone | Cyclooctacosane | 2.12% |
| 1.16% | Dodecanoic acid | Syringol | 0.95% |
| 0.80% | 2-Hydroxy-2-cyclopenten-1-one | Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester | 0.91% |
| 0.56% | Benzoic acid, methyl ester | γ-Tocopherol | 0.80% |
| 0.56% | Stearic acid | Butanedioic acid, hydroxy-,dimethyl ester | 0.60% |
| 0.55% | Linolenic acid | Vitamin E | 0.55% |
| 0.54% | Stigmasterol | β-Sitosterol | 0.48% |
| 0.53% | Cirsimaritin | Methylparaben | 0.34% |
| 0.51% | Methyl,8,11,14-heptadecatrienoate | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-y)ethanone | 0.28% |
| 0.43% | Glycerol tricaprylate | Indolizine | 0.25% |
| 0.37% | 4-vinyl-syringol | Methyl salicylate | 0.10% |

Table 3.11 Continued

| | Soxhlet | Maceration | |
|---|---|---|---|
| 0.37% | Octadecanoic acid,2-hydroxy-1-(hydroxymethyl)ethyl ester | 1-Octacosanol | 0.07% |
| 0.31% | Phytol | | |
| 0.29% | Capric acid | | |
| 0.24% | 4-amino-5-tert-butyl-4'-(dimethylamino)biphenyl-3-carbonitrile | | |
| 0.22% | Lauric acid, methyl ester | | |
| 0.22% | 2-Furancarboxaldehyde | | |
| 0.17% | Benzoic acid,2-methyl-, methyl ester | | |
| 0.13% | Octanoic acid, methyl ester | | |
| 0.11% | Methanone, (4-chlorophenyl)(4-hydroxyphenyl)- | | |
| 0.08% | Butyrolactone | | |

### 3.3.10 Compounds with reported biological activity

Known anticancer compounds identified from each *A. precatorius* extracts are listed in Table 3.12. The extracts are aqueous (AQ), hexane by maceration (HM), ethyl acetate by maceration (EAM), methanol by maceration (MM), hexane by Soxhlet (HS), ethyl acetate by Soxhlet (EAS), and methanol by Soxhlet (MS).

Table 3.12: Compunds identified in GCMS analysis with reported biological activity.

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| 4-vinylphenol | AQ, MS | *In vivo* and *in vitro* antiangiogenic activities, reducing the blood vessel number and tumour size | Yue *et al.* (2015) |
| p-Vinylguaiacol | AQ | Antioxidant and antibacterial | Ao *et al.* (2009) |
| 2-Methoxy-4-vinylphenol | EAS, MS, EAM | Anti-inflammatory Effect of 2-Methoxy-4-Vinylphenol via the Suppression of NF-κB and MAPK Activation, and Acetylation of Histone H3 | Jeong *et al.* (2011) |
| Phytol | AQ, EAM, EAS, MS | Cytotixicity and antitumour activity | de Alencar *et al.* (2019) |
| Citronellol | HM | Anti-inflammatory, analgesic | Santos *et al.* (2018) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Neophytadiene | HM, HS, EAS, MS | A good analgesic, antipyretic, anti-inflammatory, antimicrobial, antioxidant and anticancer compound | Kumbum and Sivarao (2012) |
| Squalene | HM, EAM | Cardioprotector, antioxidant, antibacterial and antifungal, anticancer, detoxifying agent. | Lozano-Grande *et al.* (2018) |
| Dihydroactinidiolide | HS, EAS | Antioxidative and anti aggregation agent, Inhibit acetylcholinesterase, demostrated anticancer activity | Das *et al.* (2018) |
| Geranylgeraniol | HM | Anti-inflammatory, anti-tumorigenic, neroprotective, enhanced testoteron and progesteron level | Ho *et al.* (2018) |
| | | Synergistic effect with d-δ-tocotrienol on prostate carninoma cells by cell cycle arrest at G1 | Yeganehjoo (2015) |
| β-Sitosterol | EAM, EAS, MM | Anti-proliferative effects and induced apoptosis in MCF7, HVT116 and HeLa | Alvarez-Sala *et al.* (2019) |
| | | Antidiabetic | Zeb *et al.* (2017) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Stigmasterol | AQ, MS | Membrane stabilizing activity in human red blood cell, axhibitng anti-inflammatory effect | Zeb *et al.* (2017) |
| β-lonone | AQ, HM | Anribacteria and anti-inflammatory | Kurashov *et al.* (2016) |
| Tetracosane | HM | Cytotoxic against MDA-MB-231, HT-29, and NIH(3T3). Induced apoptosis in AGS cells | Uddin *et al.* (2012) |
| 3-octanone | HM | Role as a human urinary metabolite, an insect attractant, a fungal metabolite, an antifeedant, a plant metabolite and a biomarker. | Berendsen *et al.* (2013) |
| Vitamin E | HS, EAS, EAM, MM | Antioxidant | Mutalip (2018) |
| D-δ-tocopherol | EAM | Anticancer | Duke (1992-2019) |
| Dl-α-tocopherol | HM | Antioxidant | Bharati (2019) |
| γ-Tocopherol | MM | Protect cells from NO damage<br>Potential biomarker in response to pathological condition, as general marker for mortality risk in populations | Sjöholm *et al.* (2000)<br>Chai *et al.* (2019) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Linolenic | EAS, MS | Antibacterial activity | Chandrasekaran *et al.* (2008) |
| Myristic acid | HS, EAS, MS | Enhanced plasma activity, increased immunemodulating activity | Tan *et al.* (2017) |
| (-)-loliolide | HS, EAS, MS | Inhibit cellular senecence in human dermal fibroblast (HDFs) | Yang *et al.* (2015) |
| | | Antibacterial, antitumour, anticancer | Grabarczyk *et al.* (2015) |
| 2-pentadecanone | MS | Wound healing and antibacterial acitivity, collagen depostion, fibroblast proliferation | Siyumbwa *et al.* (2019) |
| 2(4h)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7aa-trimethyl | HM | Antifungal, antialgal, antioxidant, anbacterial activity | Akhbari *et al.* (2012) |
| 9,12,15-Octadecatrienoic acid, methyl ester | HS | Anticancer, Antimicrobial, Antioxidant and Hyperchloesteralemic | Kumar *et al.* (2010) |
| Eicosane | HM, HS | Antifungal, antibacterial, antitumor and cytotoxic effects | Hsouna *et al.* (2011) |
| Hexadecanoic acid | HS | Cytotoxic to human leukemic cells | Lai *et al.* (2010) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Hexadecanoic acid, methyl ester | HS, EAM, MM, MS | Antifungal, Antioxidant, hypocholesterolemic nematicide, pesticide, antiandrogenic flavour, haemolytic, 5-Alpha reductase inhibitor, potent antimicrobial activity. | Hema *et al.* (2011) |
| Naringenin | EAS | Cytotoxic effect on colon carninoma<br>Inihibit migration of lung cancer (A549) cells by inhibing Akt activities and reduction of MMP-2 and MMP-9 activities | Song *et al.* (2016)<br>Chang *et al.* (2017) |
| Linalool | HM | Antifungal, anti-inflammatory | Dias *et al.* (2018) |
| Geranyl acetone | HM | Antimicrobial activity | Bonikowski *et al.* (2015) |
| Methyl dihydrojasmonate | AQ, HM | Antitumour drug compound | da Silva *et al.* (2015) |
| 4,8,12,16-tetramethylheptadecan-4-olide | HM | Against for PPARγ | Perumal *et al.* (2014) |
| 2-Monopalmitin | HS, MM, MS | Members of MAG. MAG is important critical nodein lipid signaling pathways. MAG lipase inhibitor controls FA release of the sysnthesis protumorigenic sigaling lipids. | Mulvihill and Nomura (2013) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Tetradecanoic acid, methyl ester | MS | Inhibit production of uric acid | Duke (1992-2019) |
| Tetracosanoic acid, methyl ester | HM | Inhibit production of uric acid | Duke (1992-2019) |
| Octadecanal | HM | Main compound found in crude extract of *Nostoc linckia*, algae, exhibited antimicrobial activity against *S, aureus* and *S. sonnei* | Al-Wathnani *et al.* (2012) |
| Octadecanoic acid | HS | Antioxidant, hypoglycemic and thyroid inhibitng properties | Duke (1992-2019) |
| 1-Octacosanol | HM, HS, MM | 90% of antioxidant activity from rice with high level of octacosanol | Cho *et al.* (2017) |
| Coumaran | EAM, MM | Antitubercular, anti-HIV, anticancer, cytotoxic, antiprotozoal | Ilya *et al.* (2018) |
| Methyl hexadecanoate | EAM | COMT inhibitors – decrease off time in Parkinson's desease patients | Duke (1992-2019) |
| Indolizine | MM | Medicinal moeity, anticancer | Sharma and Kumar (2014) |
| Butanedioic acid, hydroxy-,dimethyl ester | MM | Testosterone-hydroxylase-inducer<br>Inhibit production of uric acid | Duke (1992-2019) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Methyl salicylate | MM | Anti-inflammatory, analgesic, antipyretic and antithrombotic | Mao *et al.* (2014) |
| Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester | MM | COMT inhibitors – decrease off time in Parkinson's desease patients<br>Antioxidant activity through methyl guanidine inhibition | Duke (1992-2019) |
| 2-Propenoic acid,3-(4-hydroxyphenyl)-, methyl ester | MM | Antioxidant | Duke (1992-2019) |
| Cirsimaritin | EAS, MS | Inhibited NO production<br>Anti-inflammatory activity by inhibtion of c-fos and STAT3 phosphorylation in RAW264.7 cells | Shin *et al.* (2017a) |
| 3-Methoxy-4,5,7-trihydroxyflavone | EAS | Antioxidant, anti-inflammatory activities<br>Cytotoxic effect in human lung carcinoma | Limboonreung *et al.* (2019)<br>Wei *et al.* (2019) |
| 4-vinyl-syringol | MS | Inhibit gastric tumours initiation | Cao *et al.* (2015) |

Table 3.12 Continued

| **Compound Name** | ***A. precatorius* Extract** | **Reported Activity** | **Reference** |
|---|---|---|---|
| Octanoic acid, methyl ester | MS | COMT inhibitors – decrease off time in Parkinson's desease patients<br>Antioxidant activity through methyl guanidine inhibition | Duke (1992-2019) |
| Capric acid | MS | Bactericidal, anti-inflammatory activity | Huang *et al.* (2014) |
| Dodecanoic acid | MS, EAS | Bactericidal, anti-inflammatory activity | Huang *et al.* (2014) |
| Linolenic acid | EAS, MS | Anti-inflammatory activity on human corneal epithelial cells | Erdinest *et al.* (2012) |
| Stearic acid | EAS, MS | Lowering plasma cholesterol level | Bonanome and Grundy (1988) |
| 4-amino-5-tert-butyl-4'-(dimethylamino)biphenyl-3-0.24 carbonitrile | MS | Anti-inflammatory activity | Duke (1992-2019) |

Legends:

(HM) -hexane maceration; (HS) – hexane Soxhlet; (EAM) – ethyl acetate maceration; (EAS)- ethyl acetate Soxhlet; (MM) – methanol maceration; (MS) – methanol Soxhlet; (AQ) – aqueous extract; (MMP) – matrix metalloproteinases; (PPARγ) – peroxisome proliferator-activated receptor gamma; (NF-κB) – nuclear factor kappa β; (MAPK) – mitogen activated protein kinase; (MAG)- monoacylglycerol; (FA)-Fatty acids; (NO)-nitric oxide; (COMT) – catechol-O-methyl-transferase; (STAT3) – signal transducer and activator of transcription 3; (c-fos) – proto-oncogene;

## 3.4 DISCUSSION

Medicinal plants contain organic compounds, which include phenolic compounds, alkaloids, tannins, carbohydrates, flavonoids, terpenoids, and steroids. These compounds existed as a result from being synthesized by the primary or secondary metabolism of living thing.

Phytochemical studies of aqueous leaves extract of *A. precatorius* are still limited as it only identifies the polar compounds. The composition of the compounds identified in aqueous extracts of *A. precatorius* leaves is a complex mixture of several classes of components, mainly phenolic compounds, terpenoids and steroids. Phenolic compounds are identified by the presence of a phenol structure such as an aromatic benzene ring with at least a hydroxyl substituent (Patra and Saxena, 2009; Robbins, 2003; Vermerris and Nicholson, 2006). Study by Hussain and Kumaresan (2014) showed that only phenolic compounds and steroids were present in aqueous leaves extract of *A. precatorius*. The aqueous extract was obtained *via* Soxhlet extraction method, hence the prolonged heat exposure may resulted in the loss of terpenoids compounds in their extract.

4-vinylphenol is the main phenolic compound identified in aqueous, ethyl acetate (maceration) and methanol (Soxhket) extracts of *A. precatorius* leaves. Recent study of 4-vinylphenol showed that this compound has anti-angiogenic activities (Yue *et al.*, 2015). Other identified compounds that have some therapeutic activities were β-lonone, phytol and stigmasterol. β-lonone was found to have anti-proliferative (Faezizadeh *et al.*, 2016), antibacterial (Kubo *et al.*, 1993; Patra *et al.*, 2015) and antitumor activities (Cho *et al.*, 2016; Liu *et al.*, 2008; Sharma *et al.*, 2013; Yu *et al.*, 1995) besides it also can be used as fungicide, pesticide, and trichomonicide. Phytol

has antimicrobial (Pejin *et al.*, 2014), anticancer (Song and Cho, 2015) and anti-inflammatory activities (Silva *et al.*, 2014). Stigmasterol is listed as antihepatotoxic (El-Domiaty *et al.*, 2009), anti-inflammatory (Gabay *et al.*, 2010), antinociceptive (Kamurthy *et al.*, 2013), antiophidic, antiviral, cancer-preventive agent (Ali *et al.*, 2015; Kasahara *et al.*, 1994), hypocholesterolemic agent (Barriuso *et al.*, 2015), ovulant agent (Zaman *et al.*, 2015) and sedative agent (Habib *et al.*, 2007).

The therapeutic activity of the aqueous leaves extract from *A. precatorius,* which in traditional practices obtained by decoction, might be due to the presence of phytol, stigmasterol and β-lonone. Individually, these phytochemical compounds have exhibited their bioactivities, however, it does not answer how it works as a whole crude extract as being practiced traditionally. Other studies indicated that other solvent extracts from *A. precatorius* leaves are rich in alkaloids, carbohydrates, steroids, phenolic compounds and terpenoids (Gul *et al.*, 2013; Hussain and Kumaresan, 2014; Yonemoto *et al.*, 2014).

In this present study, two different methods were applied for the successive solvent extraction, which were Soxhlet and maceration. Soxhlet extraction applied heat in a shorter time while maceration involved prolonged soaking without heat. Successive solvent extraction means the leaves were extracted first using hexane, ethyl acetate and lastly with methanol, in the manner of increasing polarity index (P'). Burdick and Jackson have arranged and listed solvents in order of increasing P' (Barwick, 1997). Hexane has a P' of 0.1, P' for ethyl acetate is 4.4 and P' for methanol is 5.1. Meanwhile aqueous has the highest P' at 10.2. Low P' solvent will extract higher volatile compound while the higher the P', less volatile compound will be extracted.

1-octacosanol was the main compound identified in both extraction by hexane Soxhlet or hexane maceration. This compound is a fatty acid alcohol mostly found in waxes of leaves and it is chemically similar vitamin E. To be beneficially for health, this compound must be taken as supplement because only a small amount of it can be ingested in the diet (Taylor *et al.*, 2003). Red-coloured rice of the Korean rice genotype presented highest antioxidant activity and contained the highest level of octacosanol (Cho *et al.*, 2017). Though not scientifically proven, this compound is used as supplement for many things including Parkinson disease, managing high cholesterol and atherosclerosis, improving athletic performances and also for amyotrophic lateral sclerosis (ALS). A study recently published showed that combined supplement with addition of 1-octacosanol can boost the fitness of drug detection dogs physically (Menchetti *et al.*, 2019). Another fatty alcohol highly presented in hexane maceration extract is heptacosanol (21.80%) however, only 0.24% was identified in the hexane Soxhlet extract. No health benefits or bioactivity has been associated with this compound. 1-eicosanol is another fatty alcohol highly presented in hexane Soxhlet extract which is mainly used as emollient in the cosmetics industry [PubChem CID=12404] (Kim *et al.*, 2018b).

Neophytadiene was the main compound found in the ethyl acetate Soxhlet extraction. It is an antioxidant compound known for its biological activity as anti-inflammaroty, antipyretic, good analgesic and antimicrobial (Swamy *et al.*, 2017). Neophytadiene belongs to a group of compounds known as sesquiterpenoids, which consist of terpenes containing three consecutive isoprene units. This compound was not found in the ethyl acetate maceration extract. This might be because the maceration technique did not use any heat compared to the Soxhlet extraction method. Neophytadiene is possibly occurred by dehydration of phytol (Changi *et al.*, 2012).

This also explained the reason that this compound could only be identified in extracts obtained via Soxhlet. The main compound found in the ethyl acetate maceration extract was 2-hexadecene,3,7,11,15-tetramethyl-(R-(R*,R*-E)-. Neophytadiene presence was very little with peak area of 1.54% in hexane Soxhlet extract, and 1.23% in hexane maceration extract. This compound was also one of the main compound identified in the methanol Soxhlet extract besides hexadecenoic acid, methyl ester.

Hexadecanoic acid, methyl ester is also known as methyl palmitate belongs to a group of fatty acid methyl ester [PubChem, CID=8181]. This compound has been reported to significantly induce dilation in aorta (Wang *et al.*, 2018a), reduce the levels of tumour necrosis factor-alpha (TNF-α), interleukin-10 (IL-10) and prostaglandin E2 (PGE2) without jeopardising the levels of ATP in cells. Besides that methyl palmitate is also reported to inhibit nitric oxide production and phagocytic activity of certain cells (Sarkar *et al.*, 2006; Wang *et al.*, 2018a; Wang *et al.*, 2010b). Methyl palmitate is also known as vasodilator which enhance blood flow in cerebral and promote neuronal cell survival after cardiac arrest. This therapeutic potential of methyl palmitate would lead to improvement of functional learning and memory subsequent of cardiac arrest-induced brain injury (Lee *et al.*, 2019b).

In this current study, extract from the ethyl acetate and methanol obtained by Soxhlet exhibited the highest phenolic and terpenoid compounds. 4-vinylphenol was the highest phenolic compound identified in the methanol extract (Soxhlet) and neophytadiene was the highest terpenoid compound identified in that extract. Cirsimaritin is another phenolic compound identified in both ethyl acetate and methanol (Soxhlet) extracts. This compound inhibited nitric oxide production and exhibited the anti-inflammatory activity (Shin *et al.*, 2017a). Another compound

found in the ethyl acetate (Soxhlet) profile is 3-Methoxy-4,5,7-trihydroxyflavone, also known as chrysoeriol. This compound is derived from luteolin, another phytocompound widely studied in medicinal plants. Recently it is found that this compound exhibited anti-inflammatory (Limboonreung *et al.*, 2019) and anticancer activity (Wei *et al.*, 2019).

Phytochemicals found in plants are generally known as primary and secondary compounds. Primary compounds are generally present as the building blocks of plants which includes sugars, proteins, and chlorophyll. Secondary compounds include phenolic compounds, alkaloids, terpenoids, steroids and many more (Wadood *et al.*, 2013). The biggest group of phytochemicals is the phenolic compound and most of these compounds are found in plant-based foods mainly fruits and vegetables such as cherries, grapes, citruses, tomatoes, apples, peaches and berries (Basli *et al.*, 2017). Phenolic compounds are widely studied for its health benefits especially the ability to exhibit as an anti-cancer agent. This ability might be attributed to the antioxidant activity poses by phenolic compounds. Oxidative stress is one of the causes in cancer occurrences. Phenolic compounds chemopreventive structure are able to induce cell cycle arrest thus inhibiting DNA binding and proliferation, and regulate the expression of ontogenesis and carcinogen metabolism (Huang *et al.*, 2009). Naringenin, a phenolic compound identified in the ethyl acetate extract (Soxhlet), exhibited cytotoxic effect on colon carninoma. In this particular study, naringenin was isolated from the citrus (Song *et al.*, 2016).

Terpenoids is also another compound of interest which have been identified to demonstrate the anti-proliferative activity on cancer cells. Subclasses of terpenoids are believed to contribute as anti-cancer agents include monoterpenoid, diterpenoid, triterpenoid and sesquiterpenoid (Huang *et al.*, 2012). In this current study, *A.*

*precatorius* leaves extract from ethyl acetate Soxhlet extraction presented the highest terpenoids at 36.97%. While the methanol Soxhlet extraction showed the highest phenolic compounds presented at 13.88% and terpenoids at 14.08%. Although there are a lot of therapeutic potential of the compounds identified in all extracts, our study are focusing on compounds promoting anticancer activity. Phenolic compounds found in all *A. preactorius* extracts were 4-methyl-2,5-dimethoxybenzaldehyde, cirsimaritin, and 4-vinyl-phenol. While compounds belong in the terpenoids group identified in all extracts include neophytadiene, phytol, squalene, and (-)-Loliolide. Neophytadiene presented in all extracts except ethyl acetate maceration and methanol maceration extracts.

Previous study of *A. precatorius* leaves showed that their biological activities are also related with their active compounds such as terpenoids and phenolics compounds. Yonemoto et al. (2014) found that terpenoids isolated from *A. precatorius* leaves had α-amylase inhibitory effect; one of the therapeutic approaches for preventing diabetes mellitus. Phenolic compounds such as flavanoids and phenolic acids are well known to have antioxidant and anti-proliferative activities (Gul *et al.*, 2013). A recent study from Iraq, using 80% aqueous ethanol solvents for *A. precatorius* leaves extraction by Soxhlet revealed the presence of several compounds including alkaloids, flavonoids, phytosterols and terpenoids. Three fractions were obtained from the crude extract that showed the presence of alkaloids (fraction 1), flavonoids (fraction 2) and steroids (fraction 3) (Khadem and Zahra'a, 2018).

## 3.5 CONCLUSION

Presence of phenolic compounds and terpenoids in most of the extracts showed that *A. preactorius* leaves might have effects on cancer cell proliferation and mortality. Major compound found consistently in all extract is neophytadiene, a terpenoids. However, little is known as to whether these identified compounds work individually or as a result of a synergistic effect. Therefore, in this study, we were exploring on the usage of *A. precatorius* leaves extracts in crude form rather than as isolated compounds. The ability of *A. precatorius* leaves extracts to possibly exert the anti-proliferative activity against selected normal and cancer cell line and its ability to induce apoptosis were further investigated and detailed in Chapter 4.

# CHAPTER 4

# ANTI-PROLIFERATIVE ACTIVITY AND APOPTOSIS INDUCTION of *Abrus precatorius* LEAVES EXTRACT ON CANCER CELLS

## 4.1 INTRODUCTION

Apoptosis is known as programmed cell death and it is the most studied and characterized form of cell death. Dying cells are packaged into fragments which later are consumed and eliminated by phagocytes. This process needs to occur without disturbing the function of surrounding healthy tissues (Green, 2011). Equilibrium between cell proliferation and cell death is important to ensure the cellular balance in functioning tissues and to avoid cell disruption. Many clinical diseases including cancer are developed because of this apoptosis imbalance either deficient or excessive (Cao and Tait, 2018). Initiation of apoptosis can occur either by the extrinsic or intrinsic pathway. The intrinsic pathway is also known as the mitochondrial pathway and the extrinsic pathway is also known as the death receptor pathway. Apoptosis is as important tool in cancer management used as a target by potent chemical or biological apoptosis-inducing agents (Sreelatha *et al.*, 2011).

Advances in the medical field have proven that allopathy treatment to be the preferable choice to combat cancer. However, these advances are used with concern due to their side effects and limitations. This scenario has generated an impact on the increase demand of traditional medicines through utilizing medicinal plants either as complementary to the allopathy treatments or as a complete alternative. Medicinal plants are widely sought as an alternative in various treatment in traditional medicine including cancer. Many issues were raised with this development especially concerning the toxicity and efficacy of the medicinal plants extract. Therefore, many studies have been conducted to fill in the knowledge gap in order to provide better

understanding of the biological activities and mechanism underlying those activities of the medicinal plants.

Among the other popular medicinal plants that is highly studied is the *Anonna muricata*. Ethanol extract of this plant was found to reduce viability and trigger apoptosis in liver cancer cell, HepG2 (Liu *et al.*, 2016). The apoptosis was triggered through activation of multiple proteins involved in the endoplasmic reticulum stress pathway. Another study by Smith *et al.* (2018), reported that bacopaside II, isolated from the plant *Bacopa monnieri* significantly reduced cell viability in colon cancer cells, SW 480, SW 620, Ht-29 and HCT 116. *Ixeris dentata* induced apoptosis by inhibiting p-Akt and p-NF- κB signaling pathway in MDA-MB-231 cells (Shin *et al.*, 2017b). Root extract from *Dillenia suffruticosa* also induced apoptosis, arrest DNA at G2/M cell cyle and activation of proapoptotic JNK1 and down regulation of ERK1 proteins (Foo *et al.*, 2016). Medicinal plants in focus of this thesis is *Abrus precatorius.* As mentioned earlier, many biological activities of *A. precatorius* have been reported including anticancer. However, pharmacological properties of *A. precatorius* collected from Malaysia need to be further explored.

Therefore, this chapter reported on the anti-proliferative activity of *A. precatorius* leaves extracts on selected cancer and normal cells. The extract with the lowest $IC_{50}$ with its corresponding cell was selected for further analysis to elucidate the involvement of apoptosis induction in promoting the cell deaths.

## 4.2 MATERIALS & METHODOLOGY

### 4.2.1 Cell culture

Human breast cancer cell lines, MDA-MB-231 and MCF-7; human colon cancer cell line, SW480; human cervical cancer cell line, HeLa; human normal breast cell, MCF-10A and mouse normal fibroblast cell, NIH(3T3); were obtained from American Type Cell Culture Collection (ATCC), Maryland, USA. Cells were seeded in 25cm$^2$ tissue culture flasks and grown at 37˚C under humidified 5% $CO_2$ in DMEM medium supplemented with 5% of FBS and 1% of penicillin-streptomycin. Confluent cells were harvested by trypsinization with trypsin-EDTA (0.25%) for about five minutes. Trypsinization was stopped with the addition of 1ml of complete medium. All selected cancer and normal cells used DMEM media except for MCF10-A. The ingredients for MCF10-A media is as listed in Appendix A.

### 4.2.2 Anti-proliferative activity assay of *A. precatorius* leaves extracts

Screening of anti-proliferative activity of *A. precatorius* leaves extract was performed on the selected cancer and normal cells. Extract that exhibited the lowest $IC_{50}$ value and its corresponding cell, was used for subsequent analysis in this study. Cells were cultured in 25cm$^2$ tissue culture flasks, trypsinized with trypsin-EDTA (0.25%) and seeded into the flat bottom 96-well plate. Cells were seeded at the centre of the plate (60 wells) with the concentration of 5 x $10^4$ cells/ml per well. Number of cells were determined by trpan blue exclusion assay and counted using hemocytometer (Appendix D). Wells at the edges of the plate were filled up with water to prevent the plate from drying during the incubation. The next day medium was discarded, then 200µL of fresh medium was added into the wells. Extracts of *A. precatorius* leaves were added following a serial dilution starting from 99µg/ml until 0.39µg/ml in each well. The diluted *A. precatorius* leaves extracts were added into the wells as according

to Table 4.1. Each concentration was added into three wells (triplicates) and each extract was tested for three times (n=3).

Table 4.1: Serial dilution calculation of *A. precatorius* extracts

| Well number | Initial Extract concentration (mg/ml) | Final Concentration of extract in well (mg/ml) | Final Concentration of extract in well (µg/ml) | Log 10 final concentrations |
|---|---|---|---|---|
| 1 | 10 | 0.099 | 99 | -1.00 |
| 2 | 5 | 0.0495 | 49.5 | -1.31 |
| 3 | 2.5 | 0.0248 | 24.8 | -1.61 |
| 4 | 1.25 | 0.0124 | 12.4 | -1.91 |
| 5 | 0.625 | 0.00618 | 6.18 | -2.21 |
| 6 | 0.3125 | 0.00309 | 3.09 | -2.51 |
| 7 | 0.156 | 0.00155 | 1.55 | -2.81 |
| 8 | 0.078 | 0.00077 | 0.77 | -3.11 |
| 9 | 0.039 | 0.00039 | 0.39 | -3.41 |

Anti-proliferative activity of *A. precatorius* leaves extracts was measured by the MTT (3-[4,5-dimethyl thiazol-2-yl] 2,5-diphenyl tetrazolium bromide) assay, which was performed after a 72h incubation post treatment with the extracts, tamoxifen (positive control) and DMSO (negative control). Absorbance was read at OD of 570nm. The absorption value at this wavelength directly represents the relative cell numbers with comparison to the control group (Igarashi and Miyazawa, 2001). The percentage of cell viability was determined according to the following equation.

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

The $IC_{50}$ values of all extracts on each cancer/normal cells were determined by plotting a graph of '$Log_{10}$ of the final extracts concentration' (as depicted in Table 4.1)

against 'Cell Viability'. The extract that induced lowest $IC_{50}$ values with its corresponding cell, was selected for the subsequent analysis of this study.

### 4.2.3 Morphology of cell death

From anti-proliferative assays, it was shown that *A. precatorius* methanolic leaves extract (Soxhlet) showed the lowest $IC_{50}$ value at 26.4µg/ml on MDA-MB-231 cells (Table 4.2). This extract was designated as APME for the rest of the report in this thesis. In order to visualize the morphological effects of APME on MDA-MD-231 cells, the APME-treated cells were observed under light microscope and fluorescent microscope.

#### 4.2.3(a) Bright field microscopy

Light microscope was used to view morphological effect of APME-treated MDA-MB-231 cells and images were recorded with Dino-Eye Eyepiece Camera. Cells were seeded in a six-well plate at $5x10^4$ cells/well. Cells were culturre using DMEM with 5% of FBS and 1% of penicillin-streptomycin. Cells were incubated in a humidified, 5% $CO_2$ incubator at 37°C, overnight. After cells were confluence, cells were treated with APME and further incubated for 24h, 48h and 72h. Untreated cells (contains <1% DMSO) at each time point were also observed alongside with Tamoxifen-treated cells.

#### 4.2.3(b) Fluorescent microscopy (Hoechst Staining)

Hoechst 33258 was used to visualize nuclear changes in apoptotic cells. The dye binds to the DNA in the cells, producing fluorescence blue color under fluorescence microscope. Cells were firstly seeded in a petri dish containing a sterile microscope slide at $5x10^4$ cells. They were incubated in a humidified, 5% $CO_2$

incubator at 37°C, overnight. The next day, cells were treated with APME for 24, 48h and 72h. After the incubation times, the slides were washed three times with PBS and dried. Then they were fixed with 4% paraformaldehyde (30 minutes) at 4°C. All slides were then incubated with 30 µg/mL Hoeshct 33258 (Invitrogen, USA) at room temperature in a dark condition for 30 minutes. Nuclear morphology of each different treatment time was examined under fluorescent microscope at 40X magnification (Imaging Source Europe GmbH, Bremen, Germany). For long-term storage, the slides were wrap with aluminum foil and kept in 4°C.

### 4.2.4 Cell Cycle Assay

MDA-MB-231 cells were treated with $IC_{50}$ of the APME and incubated for 24, 48 and 72h. Cells were harvested by trypsinization and the cell cycle assay was performed according to the manufacturer protocol, BD Cycletest™. All samples readings were acquired with FACSCANTO II (BD Bioscience). Data obtained was analysed with ModFit LT 5.0 software (Becton Dickinson, Franklin Lakes, NJ, USA).

### 4.2.5 Apoptosis Assays

#### 4.2.5(a) AnnexinV and PI staining

MDA-MB-231 cells were treated with $IC_{50}$ of the APME and incubated for 24h, 48h and 72h. Cells were harvested by trypsinization and the apoptosis assay was performed according to the manufacturer protocol, AnnexinV-FITC detection kit I (BD Bioscience). All samples readings were acquired with FACSCANTO II (BD Bioscience). Data obtained was analysed with FlowJo software.

#### 4.2.5(b) Bax, Bcl-2, Caspase-3 and p53 activity

MDA-MB-231 cells were treated with $IC_{50}$ of the APME and incubated for 24h, 48h and 72h. Cells were harvested by trypsinisation following each incubation time and washed twice with PBS. Ethanol (70%) was used to fix the cells at 4°C for an hour. Cells were washed twice with PBS and then blocked with 2% BSA for 10 minutes at room temperature. Another cell wash was performed, and cells were resuspended in PBS.

About 100µl of the cell suspension ($1x10^6$ cells) were mixed independently in different tubes, with antibodies (SantaCruz); Bax-PE (sc-7480), Bcl-2 – Alexa Fluor 647 (sc-7382), p53 – Alexa Fluor 488 (sc-126) and Caspase-3 – Alexa Fluor 488 (sc-7272). These cells-antibodies mixtures were incubated for 20 minutes at room temperature, then washed once and resuspended in 500µl PBS. All samples readings were acquired with FACSCANTO II (BD Bioscience). Data obtained was analysed with FlowJo software.

### 4.2.6 Statistical Analysis

The data were expressed as mean ±SD of three repeated experiments. The level of statistical significance was tested using repeated measure one-way ANOVA, followed by Dunnett's multiple comparison test. The difference was considered significant if $P<0.05$. Analyses were all done using GraphPad Prism7.

## 4.3 RESULTS

### 4.3.1 Anti-proliferative activity of *A. precatrius* leaves extracts

Anti-proliferative activity of *A. precatorius* was determined on human breast cancer cell lines, MDA-MB-231 and MCF-7; human liver cancer cell lines, SW480; human cervical cancer cell line, HeLa; human normal breast cell, MCF-10a and mouse normal fibroblast cell, NIH (3T3). The potential anti-proliferative activity of *A. precatorius* extracts was investigated by MTT assay. $IC_{50}$ values were determined to demonstrate the anti-proliferative effects of these extracts, with lower $IC_{50}$ values indicating higher anti-proliferative activity. The US National Cancer Institute and Geran Protocol (Geran *et al.*, 1972), listed out the criteria used to categorize cytotoxicity of plant extracts against cancer cell line as follows: highly cytotoxic ($IC_{50} \leq 20\mu g/ml$), moderately cytotoxic ($21 \leq IC_{50} \leq 200\mu g/ml$), weakly cytotoxic ($201 \leq IC_{50} \leq 500\mu g/ml$), and, no cytotoxicity ($IC_{50} \geq 501\mu g/ml$). $IC_{50}$ of all extracts were determined by plotting the graph of concentration of the extract(s) or tamoxifen vs percentage of cell viability.

### 4.3.2 Determination of the anti-proliferative activity of *A. precatorius* aqueous extract (decoction) on selected normal and cancer cells

*A. precatorius* aqueous leaves extract was prepared by decoction at 50°C. Initially the cells were treated with concentration as listed in Table 4.1 (page 88). However, no $IC_{50}$ value was obtained even the cells were treated at the maximum concentration of 99µg/ml. Therefore, the aqueous extract concentration was increased to 990µg/ml. It was performed in the same manner as explained in section 4.2.2. A log concentration vs cell viability was also plotted. Figure 4.1 showed the representative points of each concentration, presented in means with ±SD of three independent experiments. *A. precatorius* aqueous extract demonstrated a null toxicity against all cells, according to the standard criteria used by The US National Cancer Institute and Geran Protocol.

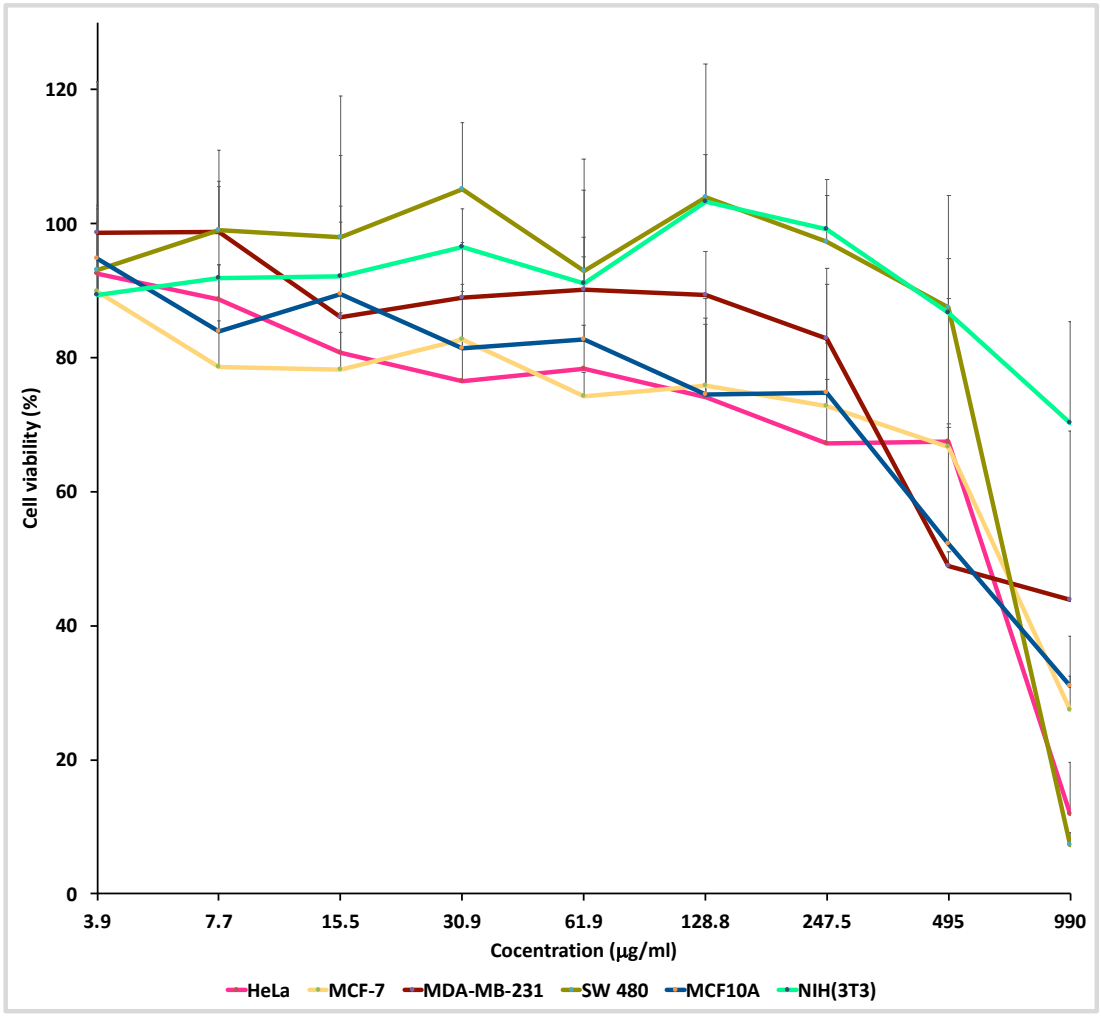



Figure 4.1: **Anti-proliferative activity of *A. precatorius* aqueous leaves extracts on selected cancer and normal cells.**

The results were expressed as mean, ±SD of three independent experiments with three replicates

### 4.3.3 Determination of the anti-proliferative activity of *A. precatorius* solvents extract (Soxhlet) on selected normal and cancer cells

There were two types of extraction methods used for *A. precatorius* leaves. Both methods applied solvents in a successive manner. Anti-proliferative activities of *A. precatotius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts are shown in Figure 4.2 -4.7.

#### 4.3.3(a) HeLa

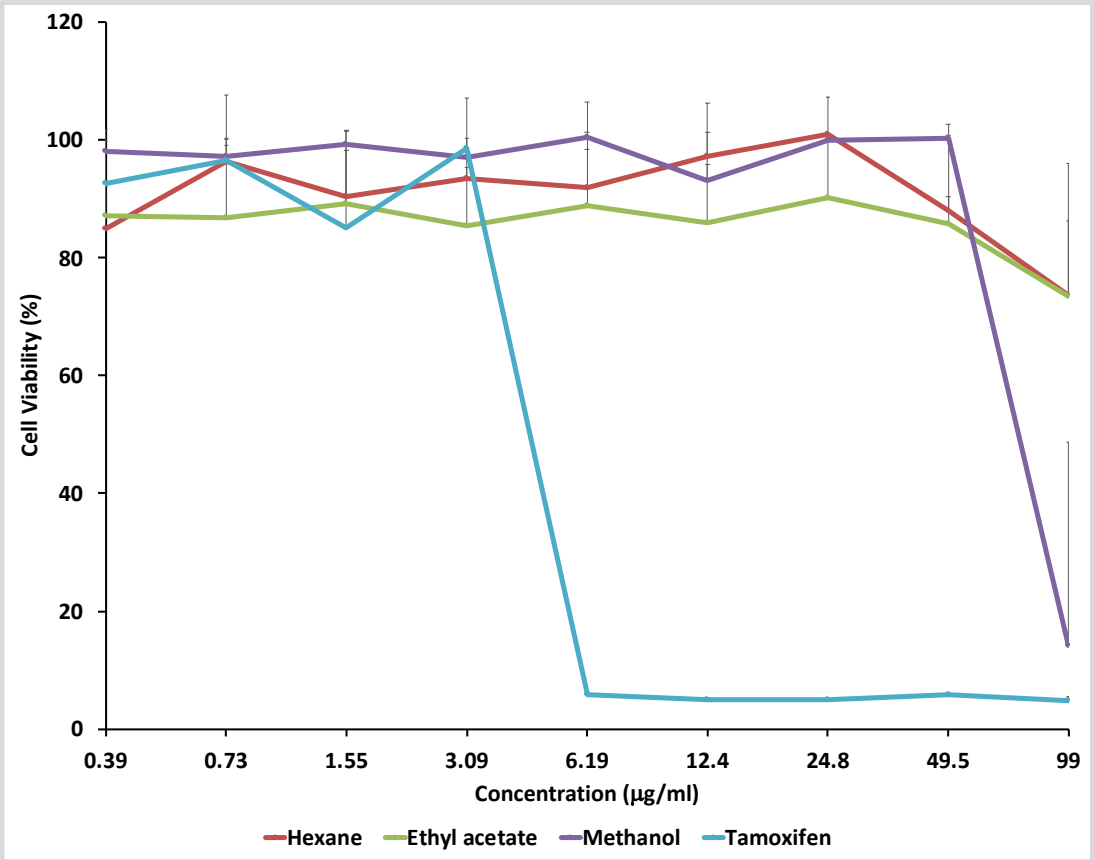



Figure 4.2: **Anti-proliferative activity of *A. precatorius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts on HeLa cells.**

The only $IC_{50}$ values obtained were for methanol extract was 73.6µg/ml and 4.32 µg/ml for Tamoxifen, while the rest of the extracts has the $IC_{50}$ values of >99 µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.3(b) MCF7

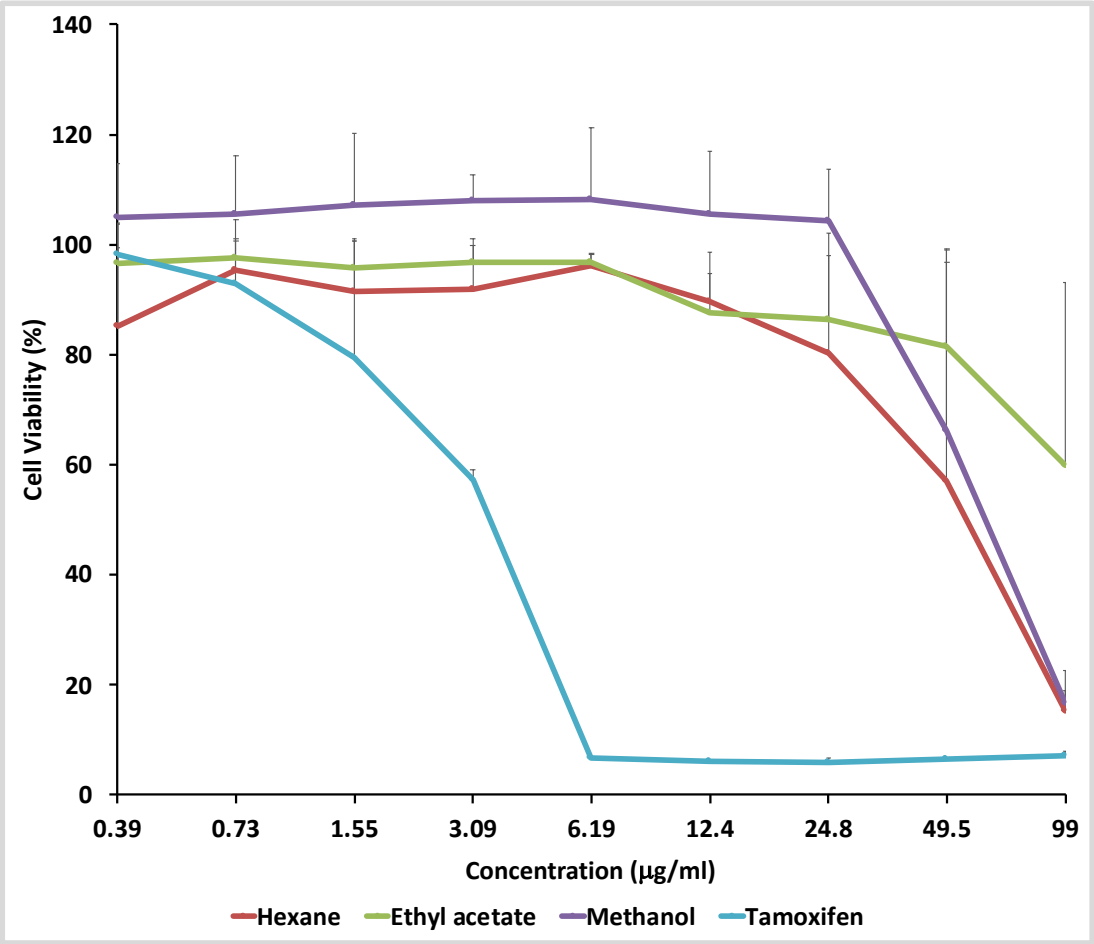



Figure 4.3: **Anti-proliferative activity of *A. precatorius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts on MCF7 cells.**

The $IC_{50}$ values obtained for hexane extract was 52.65µg/ml, ethyl acetate extract was 99µg/ml and methanol extract was 59.03µg/ml and 1.81µg/ml for Tamoxifen. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.3(c) MDA-MB-231

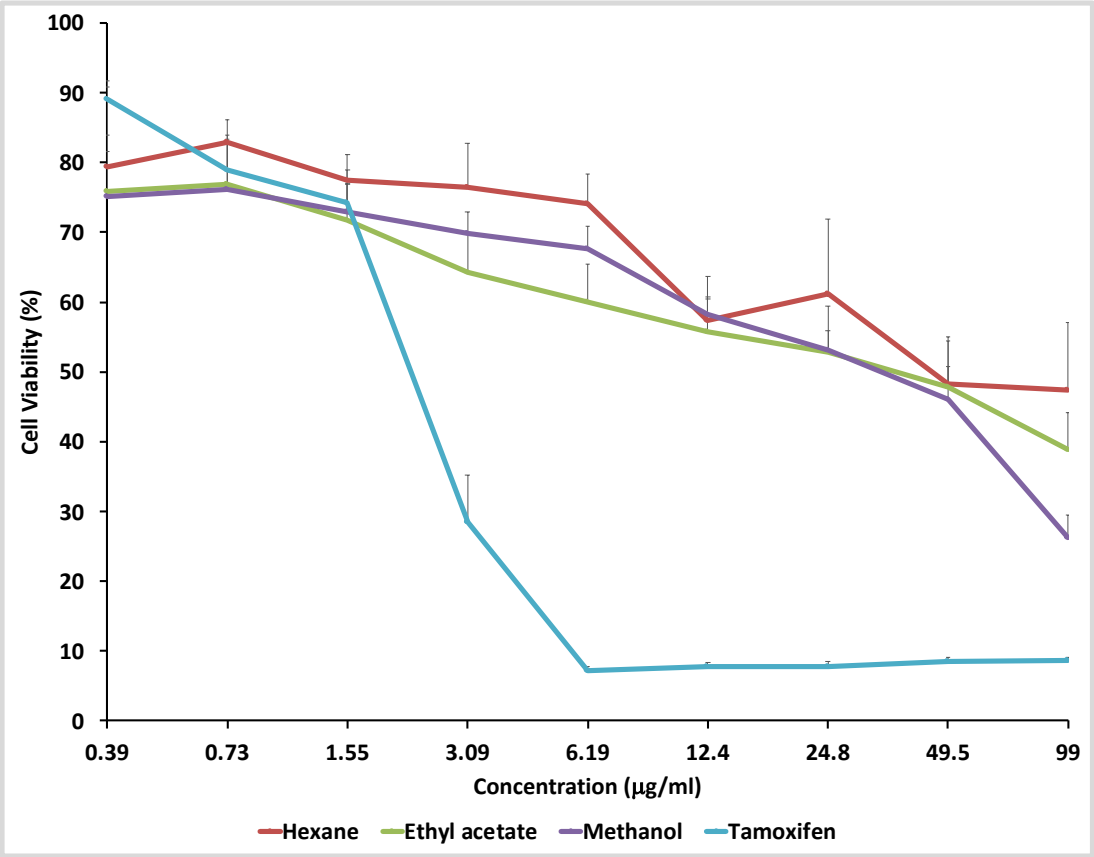



Figure 4.4: **Anti-proliferative activity of *A. precatorius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts on MDA MB-231 cells.**

The $IC_{50}$ obtained for hexane, ethyl acetate, methanol extract and tamoxifen were 45.60µg/ml, 54.5µg/ml, 26.4µg/ml and 2.27µg/ml, respectively. The results were expressed as mean, ±SD of three independent experiments with three replicates

### 4.3.3(d) SW 480

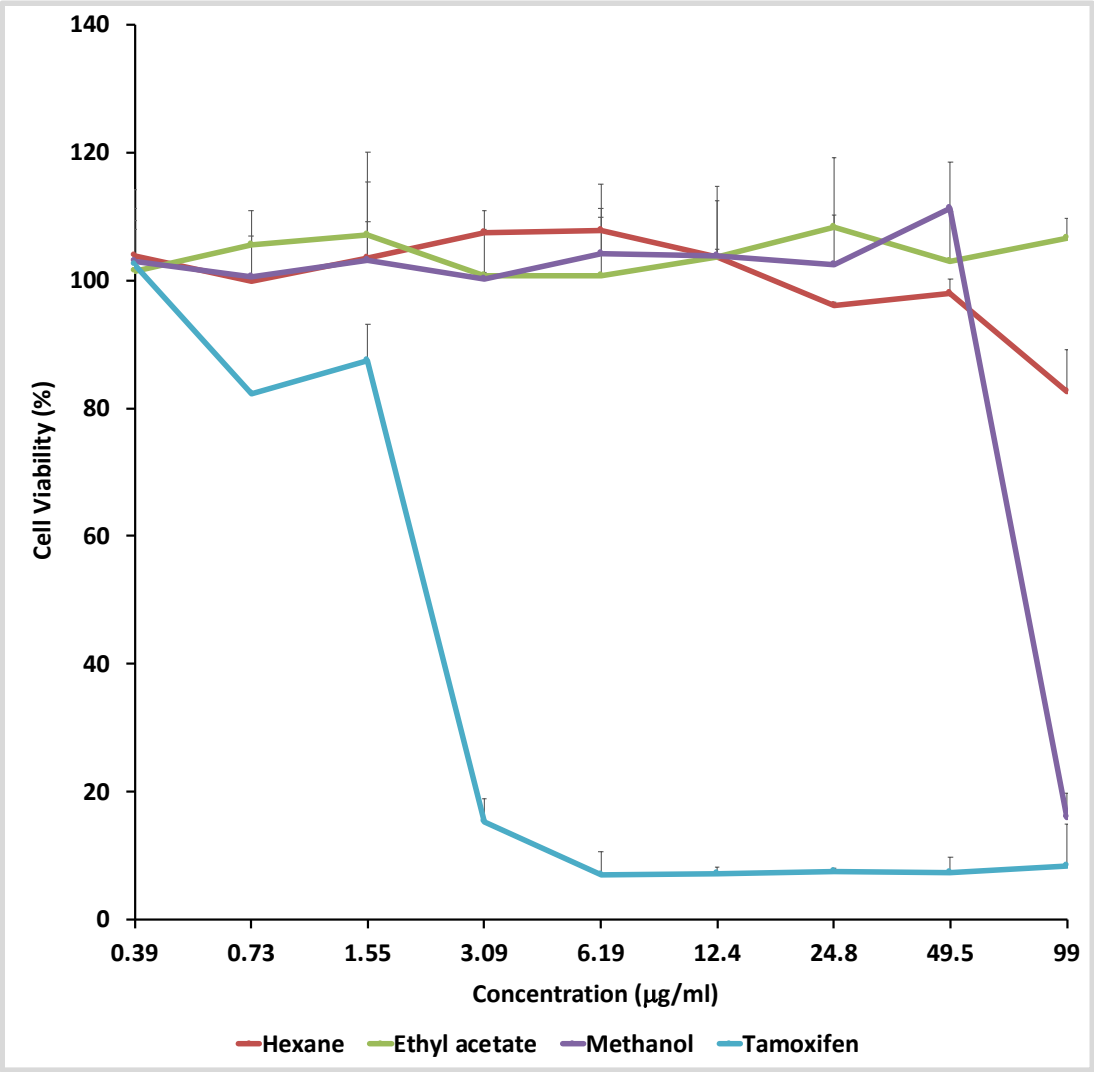



Figure 4.5: **Anti-proliferative activity of *A. precatorius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts on SW 480 cells.**

The $IC_{50}$ obtained for methanol extract is 77.23µg/ml and 2.31µg/ml for Tamoxifen, while the rest of the extracts demonstrated the $IC_{50}$ values of >99µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates

### 4.3.3(e) NIH(3T3)

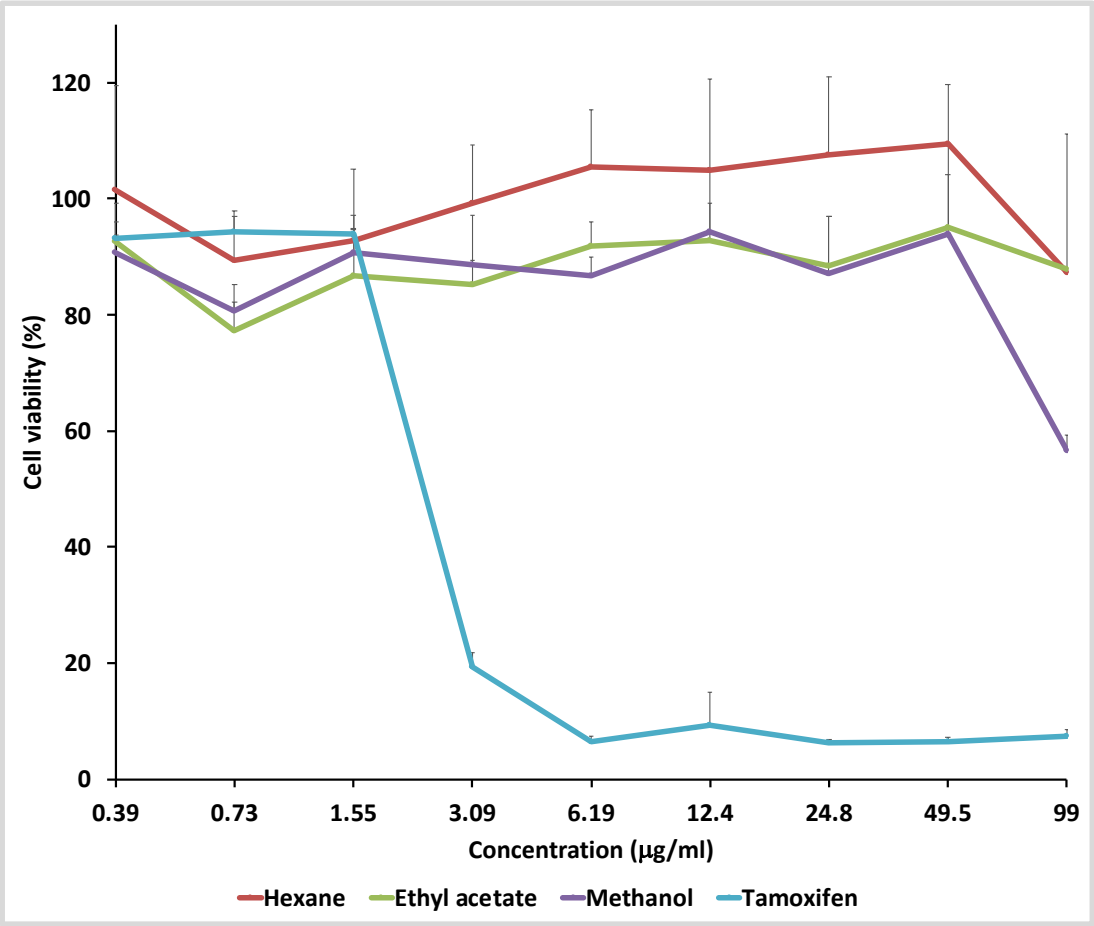



Figure 4.6: **Anti-proliferative activity of *A. precatorius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts on NIH(3T3) cells.**

The $IC_{50}$ obtained for Tamoxifen is 3.78µg/ml, while the rest of the extracts demonstrated the $IC_{50}$ values of >99µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates

### 4.3.3(f) MCF10A

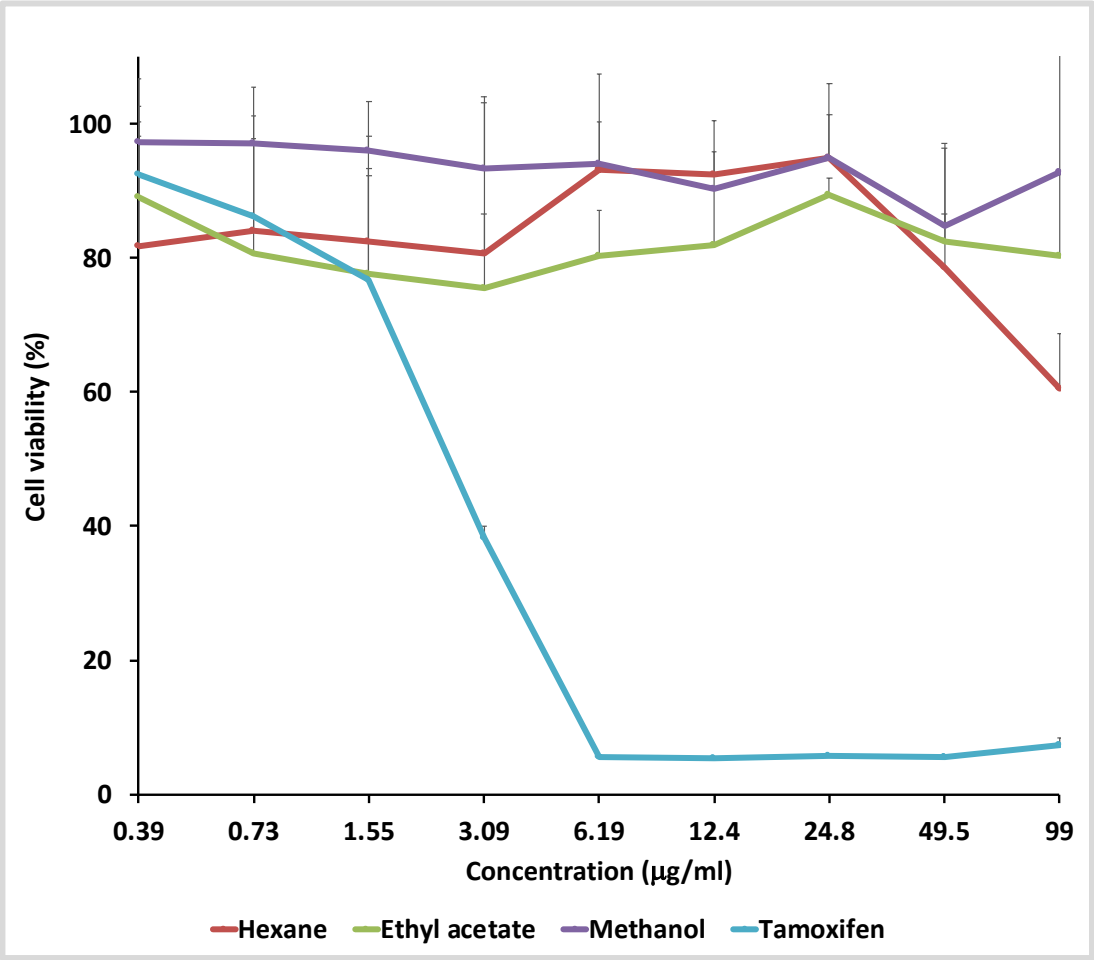



Figure 4.7: **Anti-proliferative activity of *A. precatorius* successive Soxhlet hexane-, ethyl acetate- and methanol- leaves extracts on MCF10A cells.**

The $IC_{50}$ obtained for Tamoxifen is 2.78µg/ml, while the rest of the extracts demonstrated the $IC_{50}$ values of >99 µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.4 Determination of the anti-proliferative activity of *A. precatorius* solvents extracts (Maceration) on selected normal and cancer cells

Another method used for *A. precatorius* leaves extraction was by maceration. The ground leaves were soaked with no heat successively with different solvents following their polarity. Initially the concentration of the extracts used were as stated in Table 4.1. However, no $IC_{50}$ was recorded in all extracts even at the maximum concentration used. Therefore, the extracts concentration was increased to the maximum of 495µg/ml. Anti-proliferative activities of *A. precatotius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts are shown in Figure 4.8 – 4.13.

### 4.3.4(a) HeLa

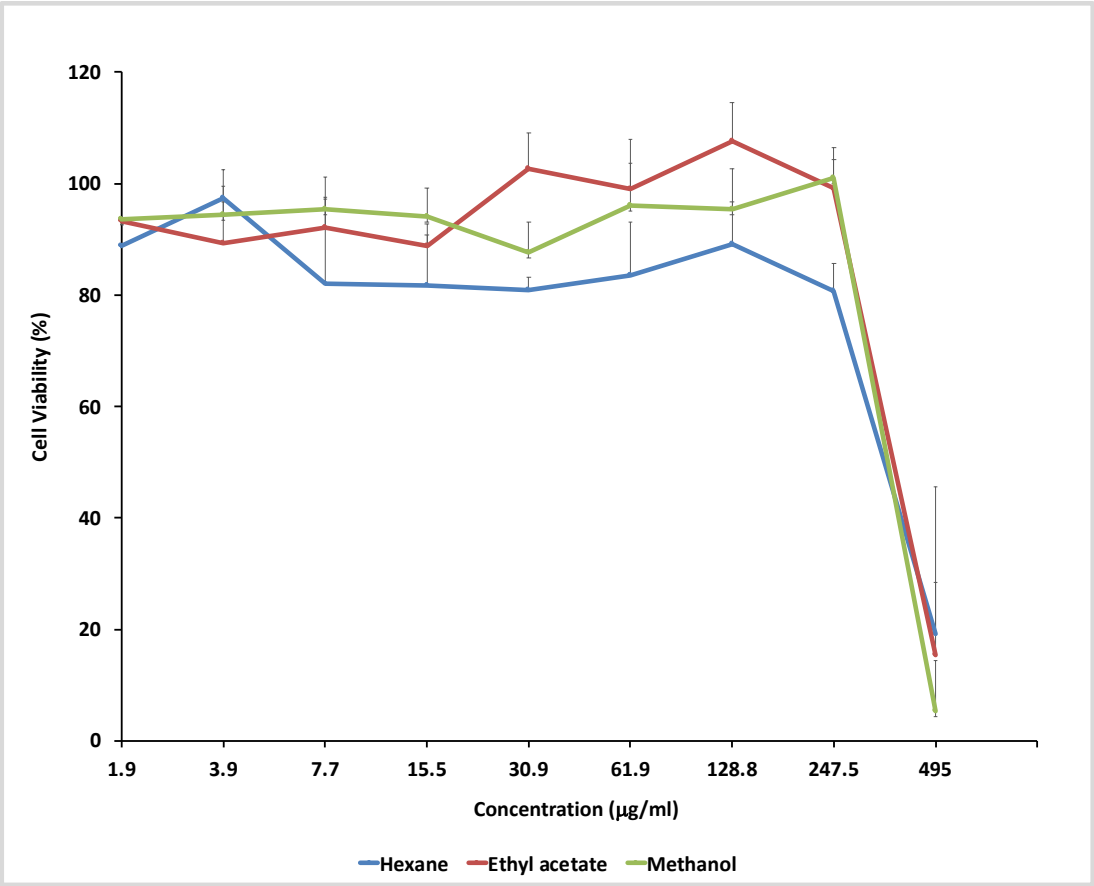



Figure 4.8: **Anti-proliferative activity of *A. precatorius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts on HeLa cells.**

The $IC_{50}$ obtained for hexane extract was 325µg/ml, ethyl acetate extract was 371µg/ml and methanol extract was 352 µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.4(b) MCF-7

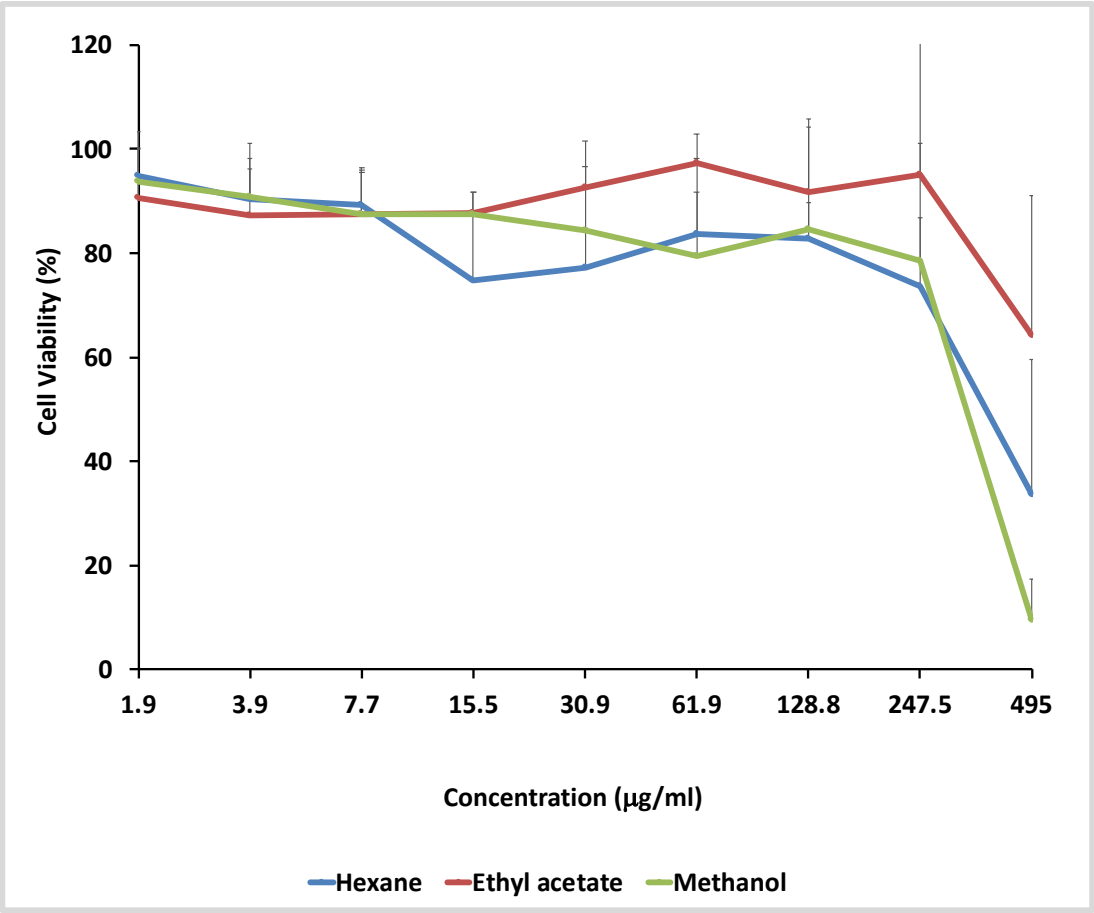



Figure 4.9: **Anti-proliferative activity of *A. precatorius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts on MCF-7 cells.**

The $IC_{50}$ obtained for hexane extract was 672µg/ml and methanol extract was 423µg/ml. While ethyl acetate extract was >495µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.4(c) MDA-MB-231

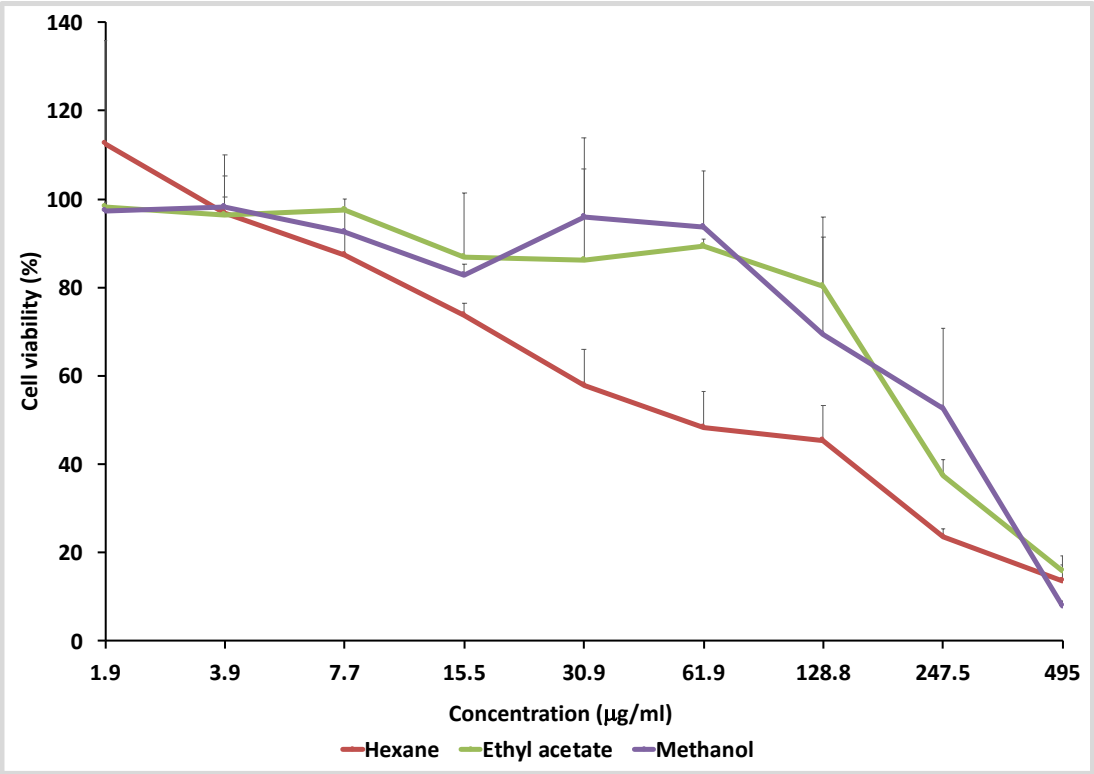



Figure 4.10: **Anti-proliferative activity of *A. precatorius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts on MDA-MB-231 cells.**

The $IC_{50}$ obtained for hexane extract was 80.75µg/ml, ethyl acetate extract was 207µg/ml and methanol was 255µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.4(d) SW480

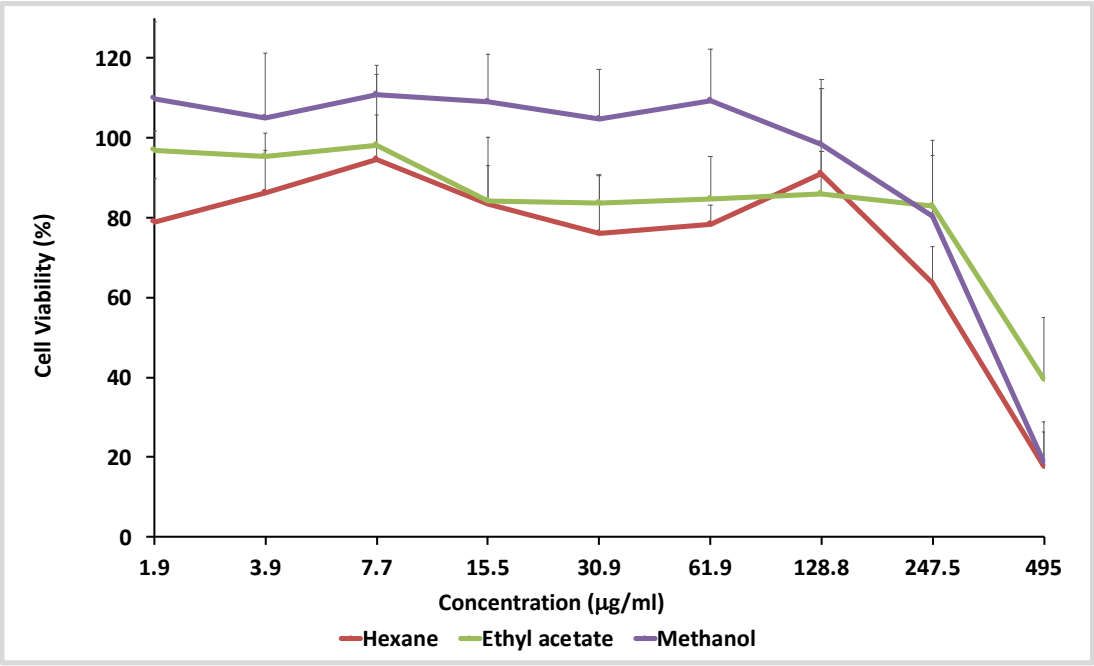



Figure 4.11: **Anti-proliferative activity of *A. precatorius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts on SW480 cells.**

The $IC_{50}$ obtained for hexane extract was 301.3µg/ml, ethyl acetate extract was 447.5µg/ml and methanol 350.3was µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.4(e) NIH (3T3)

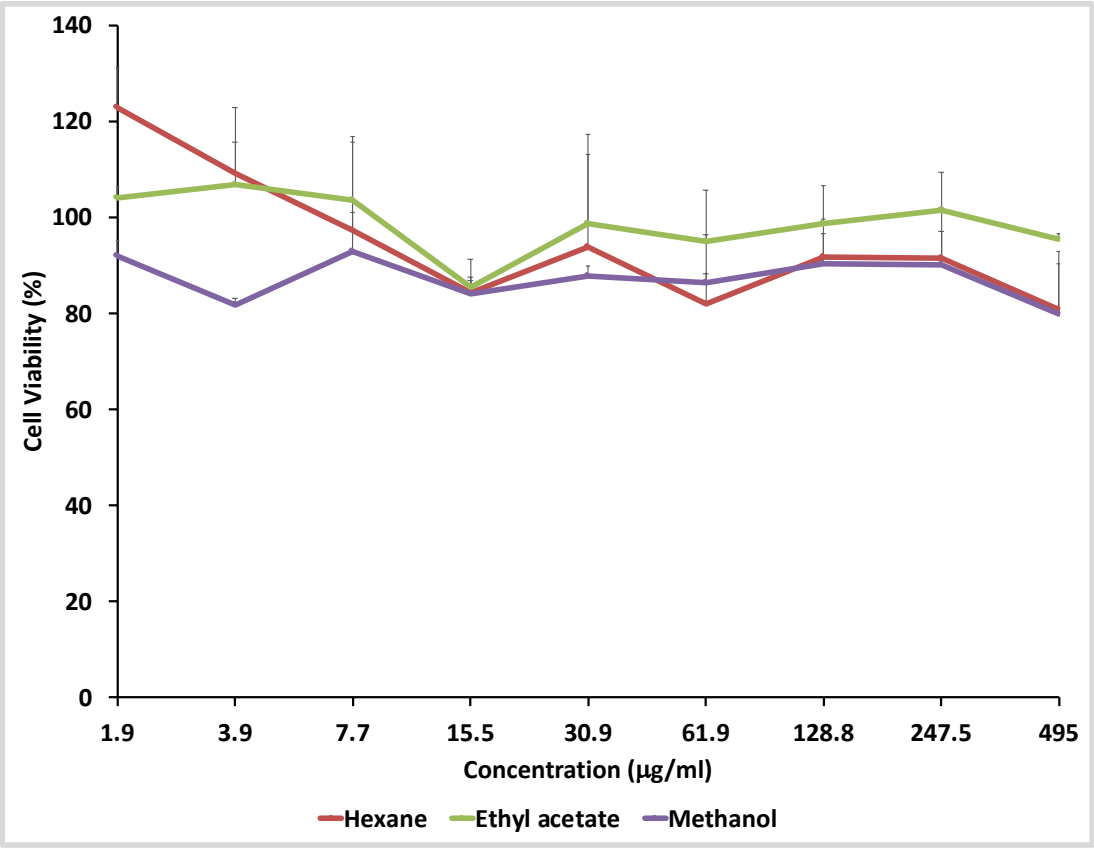



Figure 4.12: **Anti-proliferative activity of *A. precatorius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts on NIH(3T3) cells.**

No $IC_{50}$ was obtained even at the maximum concentration of 495µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.4(f) MCF10A

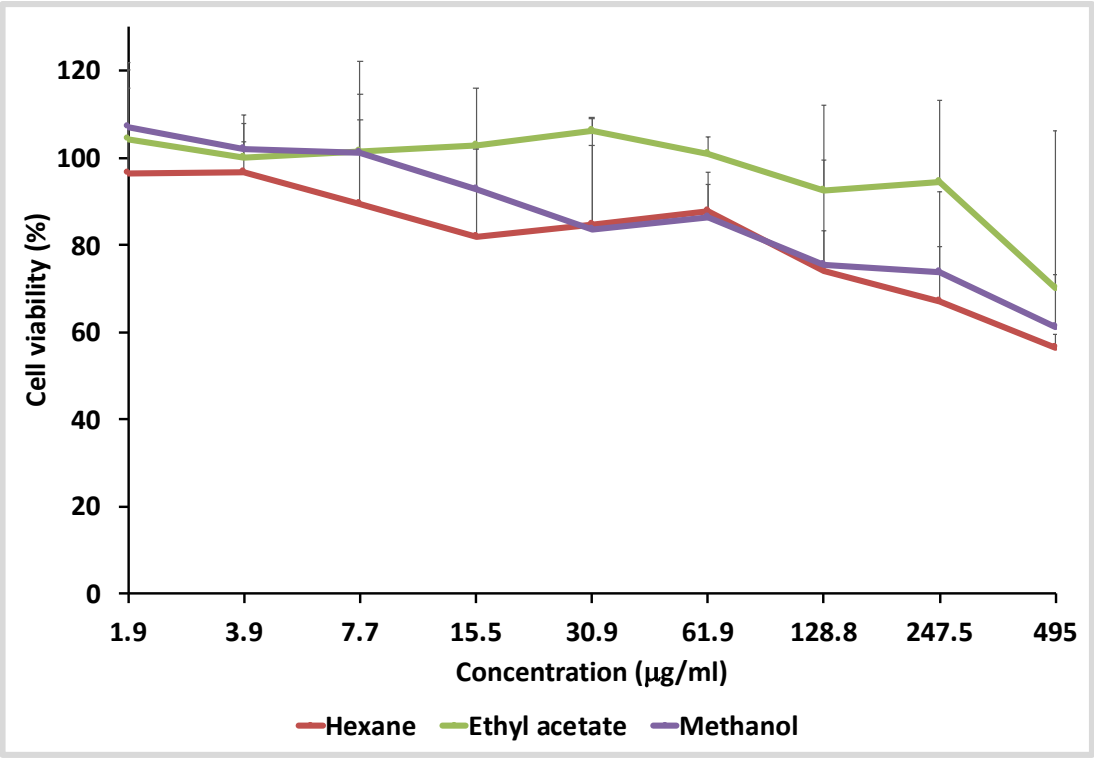



Figure 4.13: **Anti-proliferative activity of *A. precatorius* successive (maceration) hexane-, ethyl acetate- and methanol- leaves extracts on MCF10A cells.**

No $IC_{50}$ was obtained even at the maximum concentration of 495µg/ml. The results were expressed as mean, ±SD of three independent experiments with three replicates.

### 4.3.5 Summary of the $IC_{50}$ values of all *A. precatorius* leaves extracts

The $IC_{50}$ values of all extracts in inhibiting the cancer and the normal cells were summarized in Table 4.2. Methanol extract (Soxhlet) was identified to have the lowest $IC_{50}$ value among all extracts on MDA-MB-231 cells at 26.4±2.45μg/ml, which is categorized it to be moderately toxic. Methanol extract (Soxhlet) also exhibited moderate anti-proliferative activity on all cancer cells but not toxic on normal cells. Methanol extract (maceration) exhibited low cytotoxic activity on all cancer cells. Hexane extract (Soxhlet) exhibited moderate cytotoxicity on MCF-7 and MDA-MB-231 cells, while the maceration extract showed low anti-proliferative effect on HeLa, MCF-7, and SW 480. Ethyl acetate extract (Soxhlet) exhibited moderate anti-proliferative activity on MCF-7 and MDA-MB-231 cells, while low anti-proliferative activity on HeLa, MDA-MB-231, and SW 480 was exhibited by the ethyl acetate maceration extract. All extracts exhibited cytotoxic effects on MDA-MB-231 cells.

Table 4.2: $IC_{50}$ values of *A. precatorius* leave extracts against selected normal and cancer cell lines (µg/ml).

| Types of cancer cell lines | Hexane extract (Soxhlet) | Hexane extract (Maceration) | Ethyl acetate extract (Soxhlet) | Ethyl acetate extract (Maceration) | Methanol extract (Soxhlet) | Methanol extract (Maceration) | Aqueous Extract | Tamoxifen |
|---|---|---|---|---|---|---|---|---|
| HeLa (cervix) | >99 | 325±38.4 | >99 | 371±52.0 | 73.60±6.17 | 325±12.2 | 618±38.8 | 4.32±0.40 |
| MCF7 (breast) | 52.65±7.14 | 425.5±75.7 | 99.0±11.86 | >495 | 59.03±9.40 | 330±37.0 | 668±176 | 1.81±1.78 |
| MDA MB-231 (breast) | 45.60±11.60 | 80.75±64 | 54.50±9.05 | 206.5±9.2 | 26.40±5.40 | 254.5±57 | 537±32.5 | 2.27±0.38 |
| SW 480 (colon) | >99 | 301.3±39.1 | >99 | 447.5±31.8 | 77.23±6.39 | 350.3±28 | 785±131 | 2.31±0.59 |
| MCF-10a (normal breast) | >99 | >495 | >99 | >495 | >99 | >495 | 680±58.5 | 2.78±0.79 |
| NIH (normal fibroblast) | >99 | >495 | >99 | >495 | >99 | >495 | >990 | 3.78±1.78 |

### 4.3.6 Observation on morphological changes upon treatment with APME

Morphology of cell death can be observed microscopically. Two methods used in this study were direct observation via bright field microscopy and Hoechst staining via fluorescence microscopy. From previous anti-proliferative assays, *A. precatorius* methanolic leaves extract (Soxhlet) showed the lowest $IC_{50}$ value at 26.4µg/ml on MDA-MB-231 cells. Therefore, this extract (APME) was selected for further studies.

MDA-MB-231 cells were treated with APME for 24h, 48h, and 72h. The cells were viewed under inverted light microscope at 20X magnification and the images were captured with Dino-Eye Camera. The images are shown in Figure 4.14 (a) – (i). No significant change was observed for the APME-treated cells at 24h comparing to the untreated cells. At 48h and 72h, cells grew rapidly, and new cells formed layers, while older cells formed clumps on their surface. APME-treated cells were showing signs of apoptosis starting at 48h (Figure 4.14-e) post treatment. Longer time exposure showed more cells with membrane blebbing and ballooning. Cells were also unable to keep its spindle shaped cells. Rounder and shrunken cells signify dead cells and none newly formed cells were observed in Figures 4.14 (e), (f), (h) and (i). Tamoxifen-treated cells started to exhibit apoptotic-like structure at 24h post treatment. More cell deaths were observed in tamoxifen-treated cells at 48h and 72h post treatment.

MDA-MB-231 treated cells were also observed under fluorescent microscope to observe the nucleus changes. Hoechst 32588 was used to stain the cells as shown in Figure 4.15. This dye enters the cell and binds to the AT-rich segment of the DNA in the nucleus. This staining is important in order to demonstrate the DNA fragmentation as the hallmark of apoptosis. The density of the fluorescent was observed higher in

APME-treated cells at 48h and 72h which indicated that the DNA in those cells started to lose its integrity thus allowing more dye to bind to the exposed AT-regions.

### 4.3.6(a) Bright field microscopy

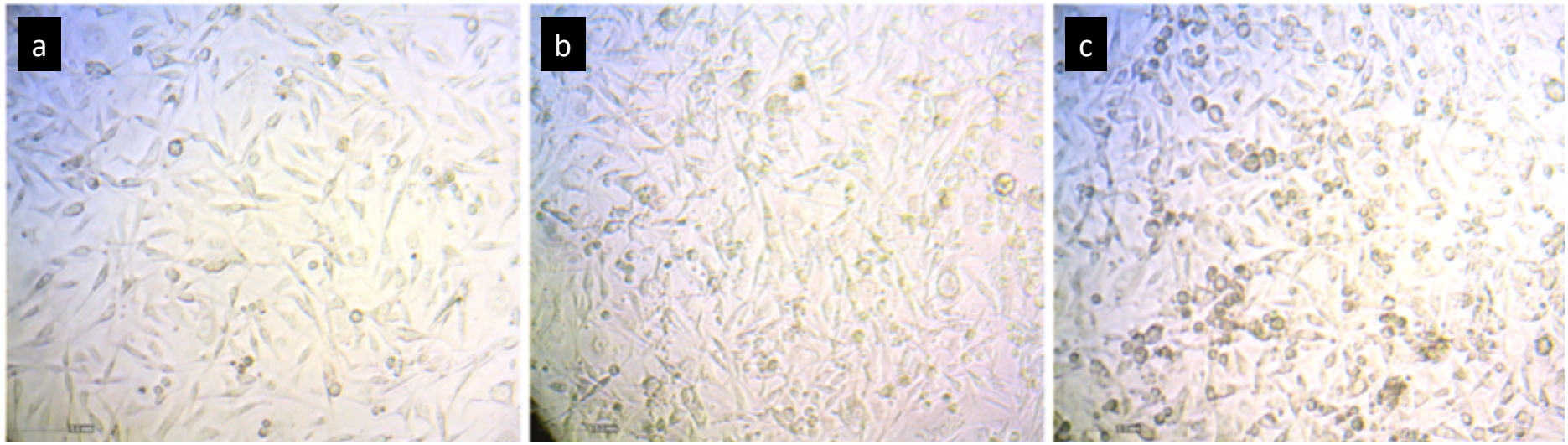



Figure 4.14 (a-c): **Bright field microscopy images of non-treated MDA-MB-231 at 24h, 48h and 72h.** Magnification: 20X..

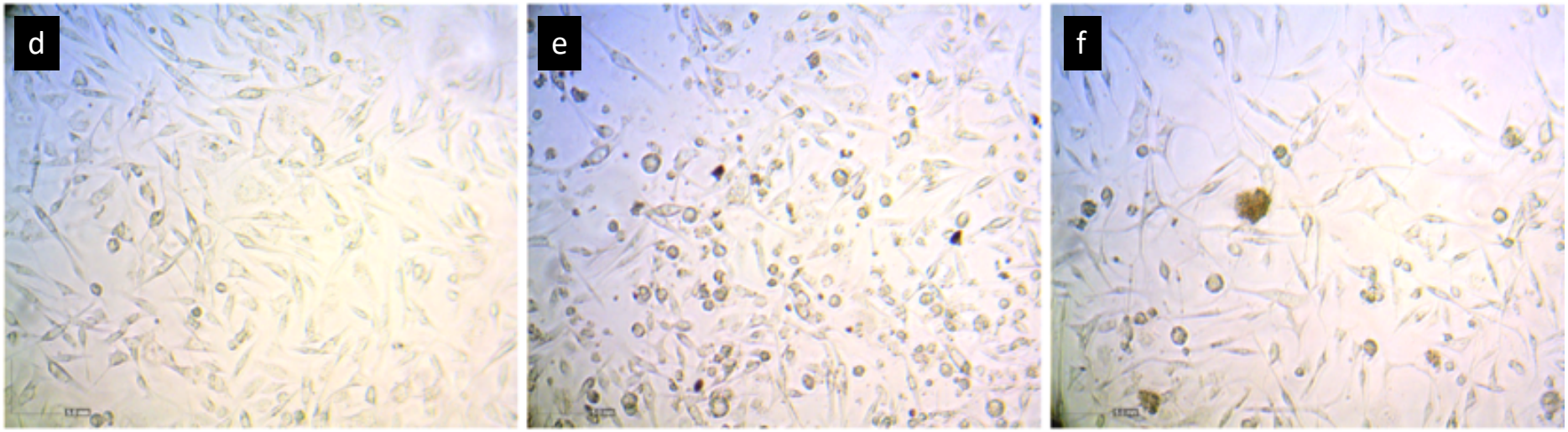



Figure 4.14 (d-f): **Bright field microscopy images of MDA-MB-231 cells treated with APME at 24h, 48h and 72h.** Magnification: 20X

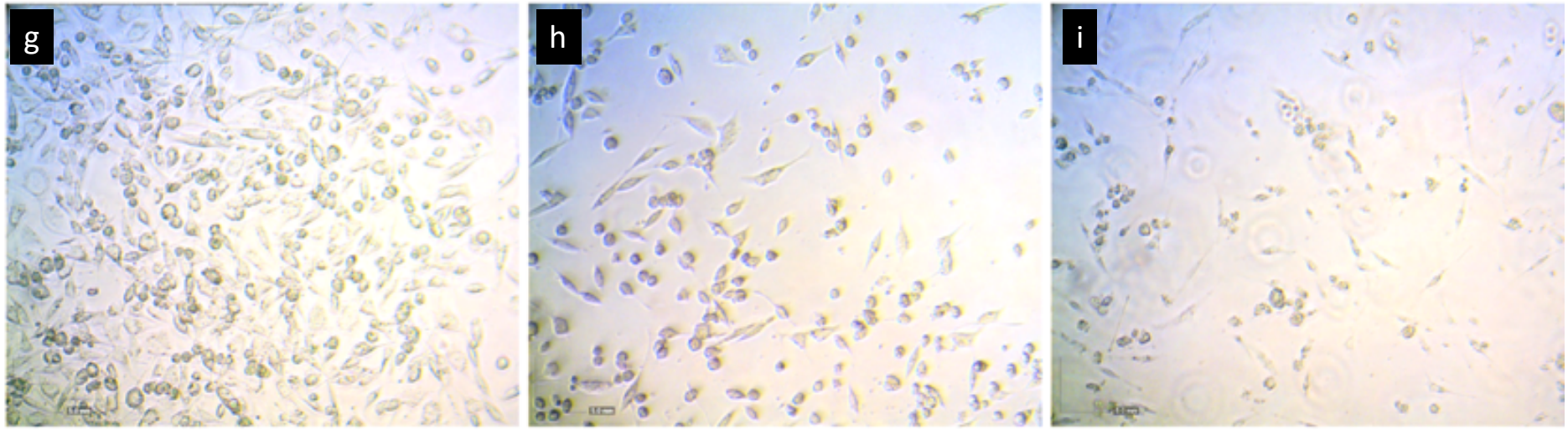



Figure 4.14 (g-i): **Bright field microscopy images of MDA-MB-231 cells treated with Tamoxifen at 24h, 48h and 72h.**
Magnification: 20X

### 4.3.6(b) Hoechst Staining

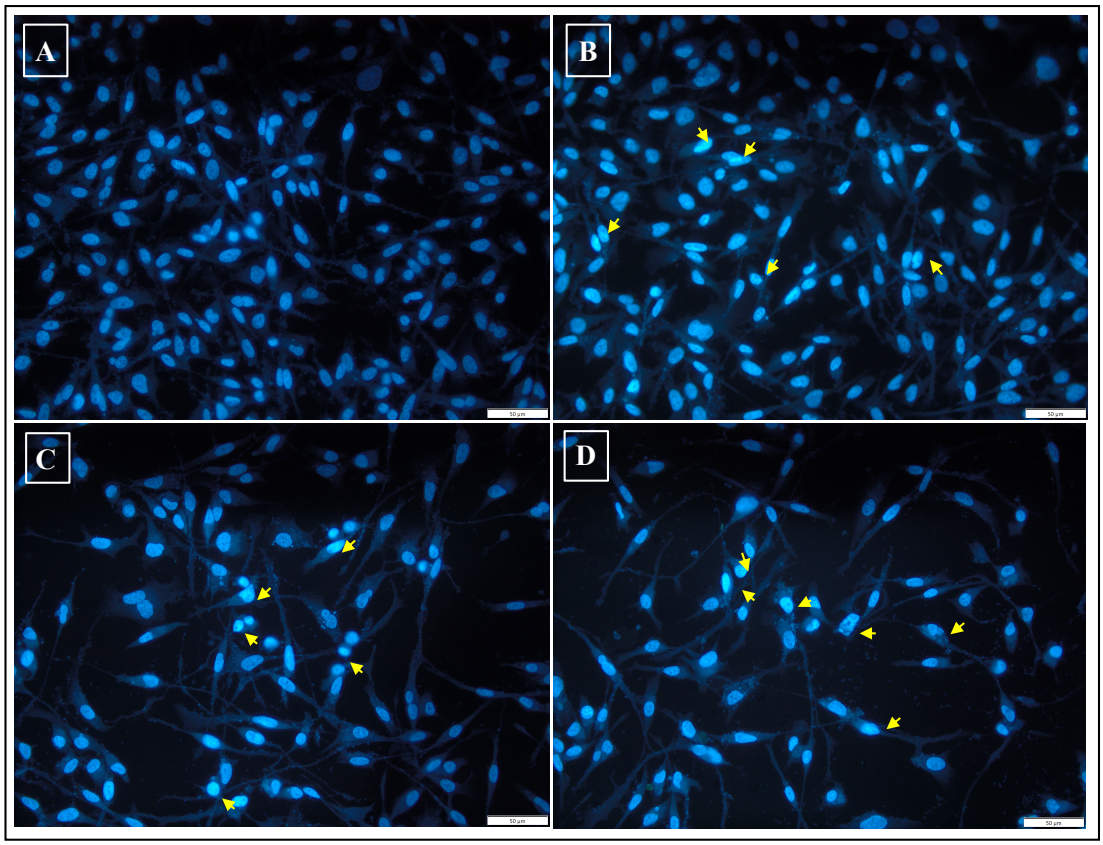



Figure 4.15 **Hoechst staining of MDA-MB-231 treated with APME.**

(A) Non-treated MDA-MB-231 cells; (B) MDA-MB-231 cells treated with APME at 24h; (C) MDA-MB-231 cells treated with APME at 48h (D) MDA-MB-231 cells treated with APME at 72hMagnification: 40X. Yellow arrows indicate cells containing fragmented DNA and condensed chromatin.

### 4.3.7 Cell Cycle Analysis

The ability of the APME to reduce cell viability could be due to cell death mediated by cell cycle arrest. Cell cycle arrest was investigated using flow cytometric analysis of propidium iodide stained DNA. Representative profiles of the cell cycle progression are presented in Figure 4.16 and percentage of each phase of cell cycle is presented in Figure 4.17. Significant increment of the arrest was seen at G0/G1 phase of the cell cycle, suggestive of cell death consistent with the reduction of cell growth as the percentage of cell population decreased in both S-phase and G2/M phase.

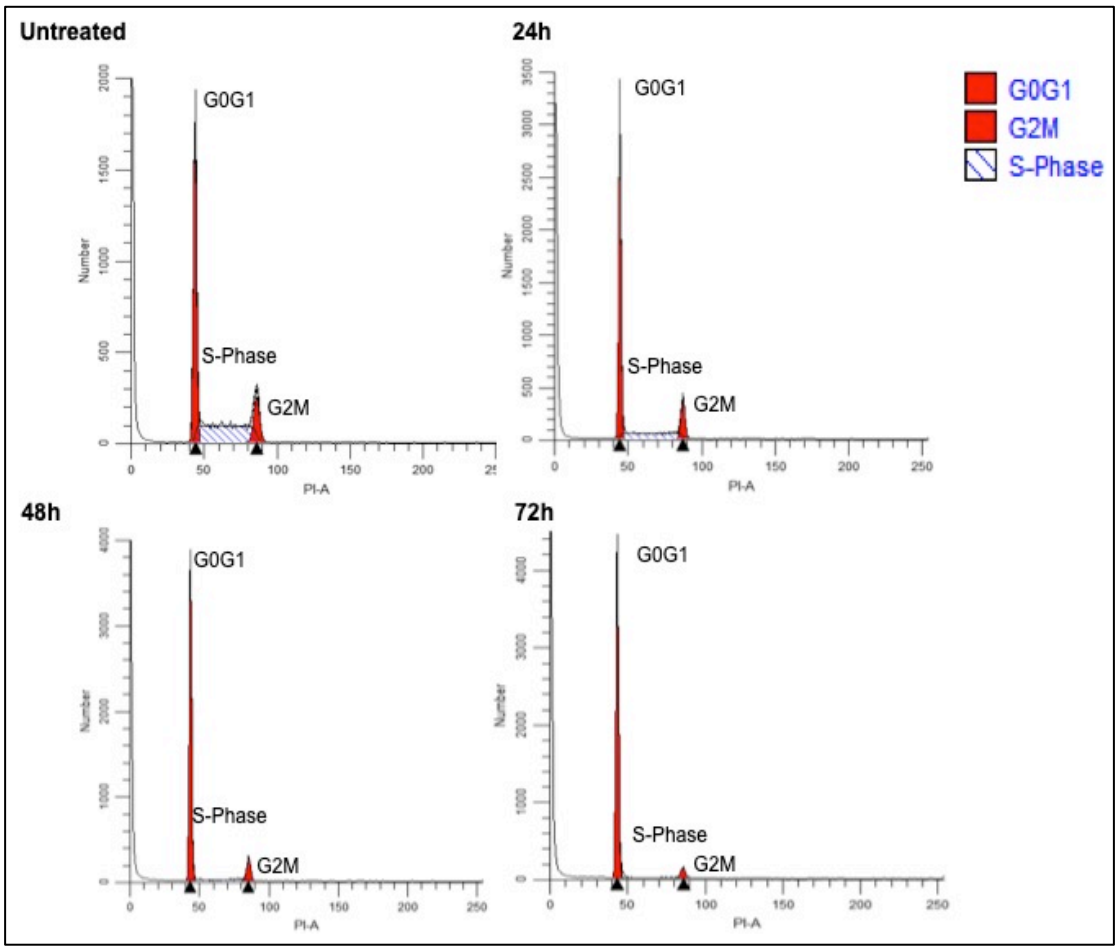



Figure 4.16: **Effects of APME on cell cycle progression in MDA-MB-231 cells.**

Histogram plot of cell cycle arrest analysis of MDA-MB-231 cells

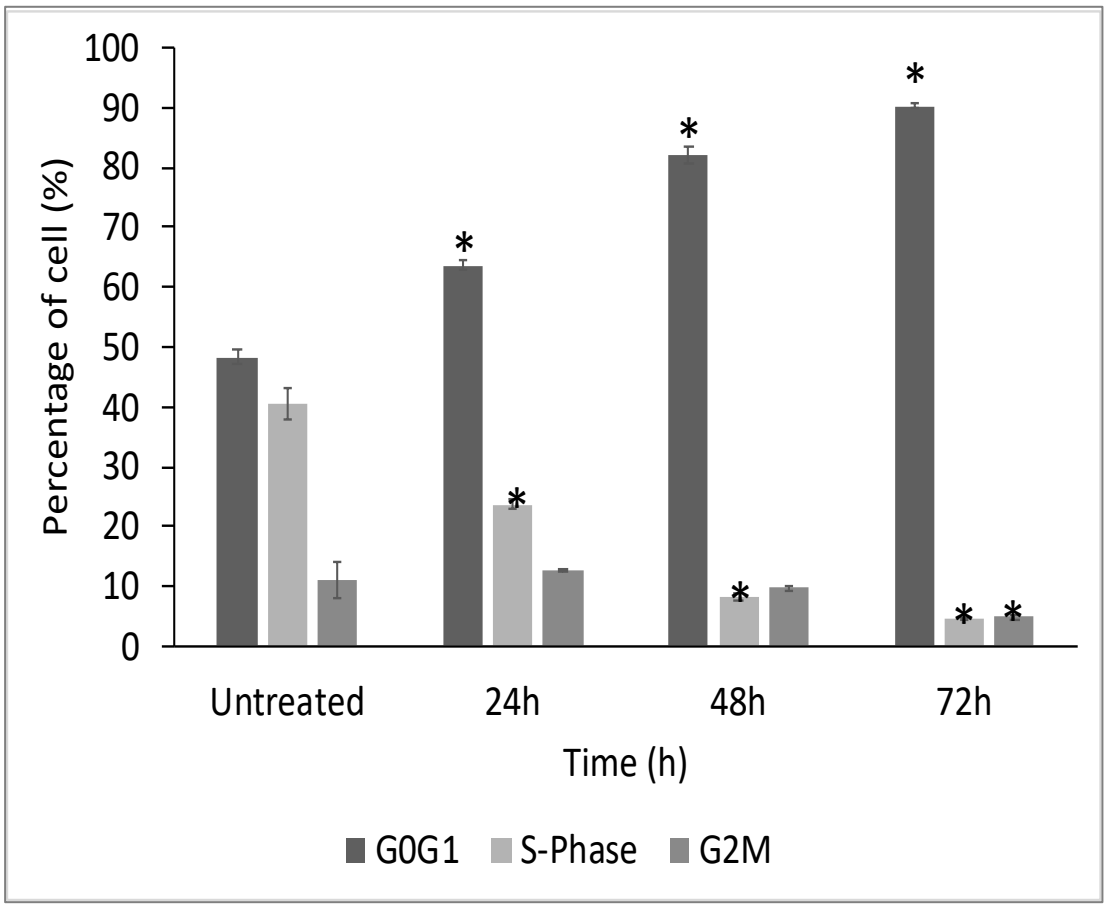



Figure 4.17: **Effects of APME on cell cycle progression in MDA-MB-231 cells.**

Graph represents the percentage of each phase of the cell cycle. Data are expressed as mean ±SD of three repeated experiments. $P<0.05$ is considered significance when comparing treated cells *vs* untreated cells.

### 4.3.8 Apoptosis Assay Analysis

#### 4.3.8(a) Annexin-V vs PI Staining

An apoptosis staining assay was performed using the AnnexinV-FITC detection kit I (BD Bioscience) to determine the ability of the APME to induce apoptosis. Representative profiles of the apoptosis assays are presented in Figure 4.18 and percentage of each phase of cell death progression is presented in Figure 4.19. Live cells did not uptake any stain and represented at Q3 (quadrant 3). AnnexinV bound to the phosphatidylserine of the plasma membrane which was exposed in early apoptosis (Q4; annexinV positive, PI negative). Late apoptotic cells lost their cell integrity thus allowing the penetration of PI (Q2; annexinV positive, PI positive), while necrotic cells were stained with PI only (Q1; PI positive). APME induced early apoptosis in MDA-MB-231 cells at 48h following treatment and late apoptosis at 72h after treatment.

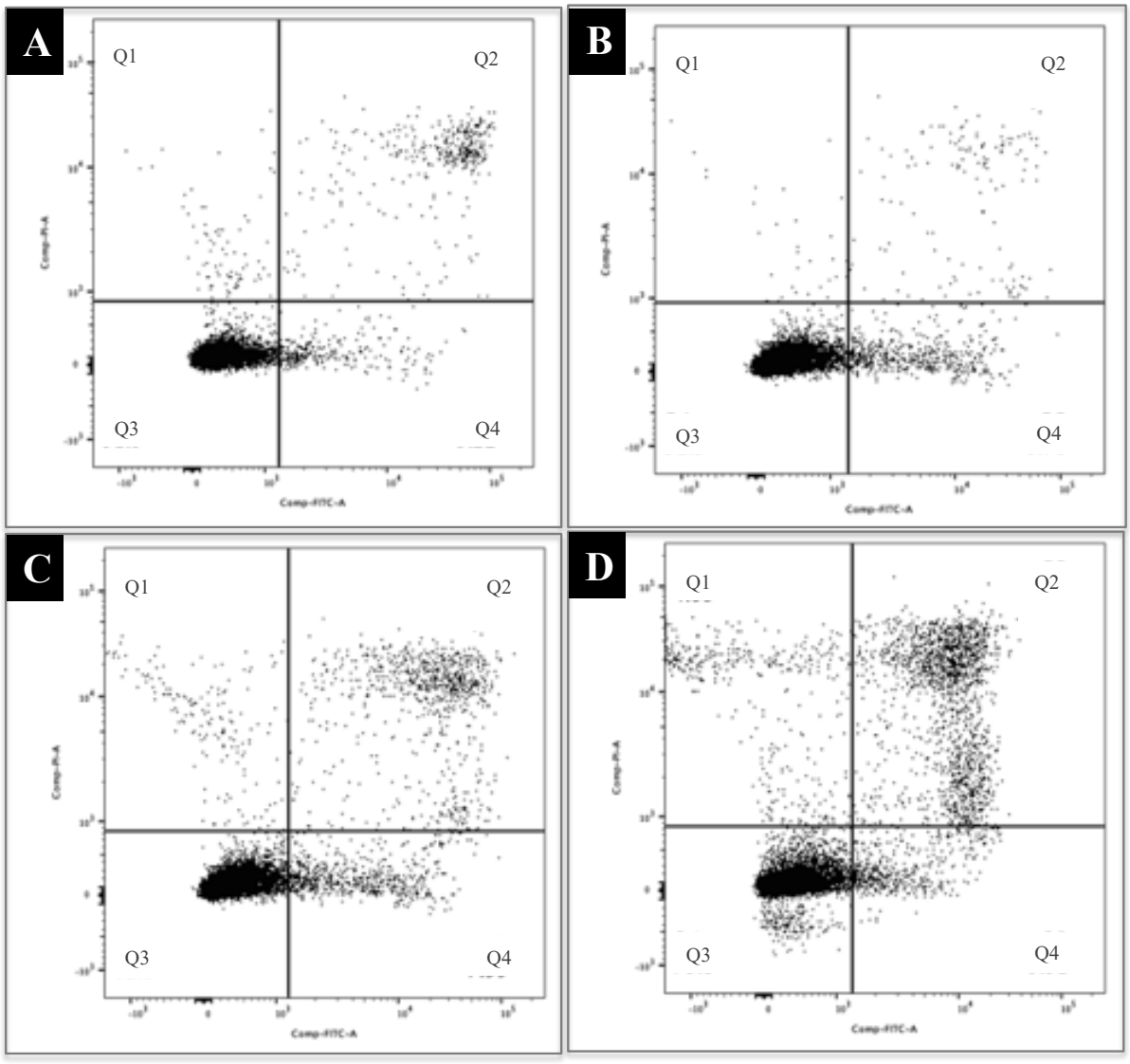



Figure 4.18: **The representative dot plot of the apoptosis assay in a time-dependent manner.**

(A) Non-treated MDA-MB-231 cells; (B) MDA-MB-231 cells treated with APME at 24h; (C) at 48h and (D) at 72h. Quadrants represent the percentage of cell populations; Q1- Necrosis, Q2- Late apoptosis, Q4- Early apoptosis, Q3-Live cells.

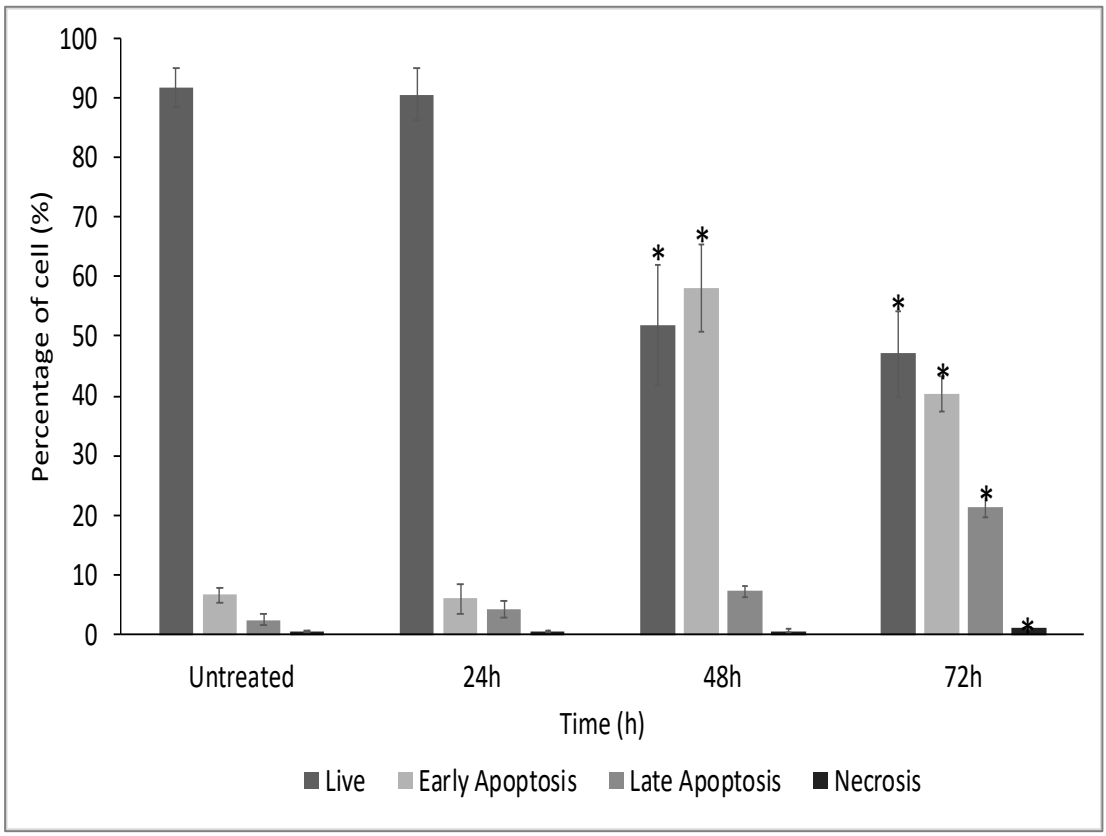



Figure 4.19: **Graph of the percentage of each phase of the MDA-MB-231 cell death following treatment with APME.**

Data are expressed as mean ±SD of three repeated experiments. $P<0.05$ is considered significance when comparing treated cells *vs* untreated cells.

### 4.3.8(b) p53, Bax, Bcl-2, and Caspase-3 protein expression

p53, Bax, Bcl-2 and Caspase-3 proteins expression levels in APME–induced apoptosis in MDA-MB-231 cells were measured by flow cytometry (Figure 4.20) at 24, 48, and 72h. Treatment of the MDA-MB-231 cells with the APME increased pro-apoptotic protein, Bax (Figure 4.22) and reduced anti-apoptotic protein, Bcl-2 (Figure 4.23) expressions in time dependant manner. No significant changes were observed on the p53 protein expressions (Figure 4.21). Caspase-3 protein expressions (Figure 4.24) were also increased in time dependant manner. These findings indicated that APME induced apoptosis in MDA-MB-231 cells by up regulating Bax protein and downregulating Bcl-2 protein. Increase in Caspase-3 expression also signifies the occurrence of apoptosis in MDA-MB-231 cells induced by APME. Combination of these findings might suggest that the apoptosis in MDA-MB-231 cells was triggered in the intrinsic pathway.

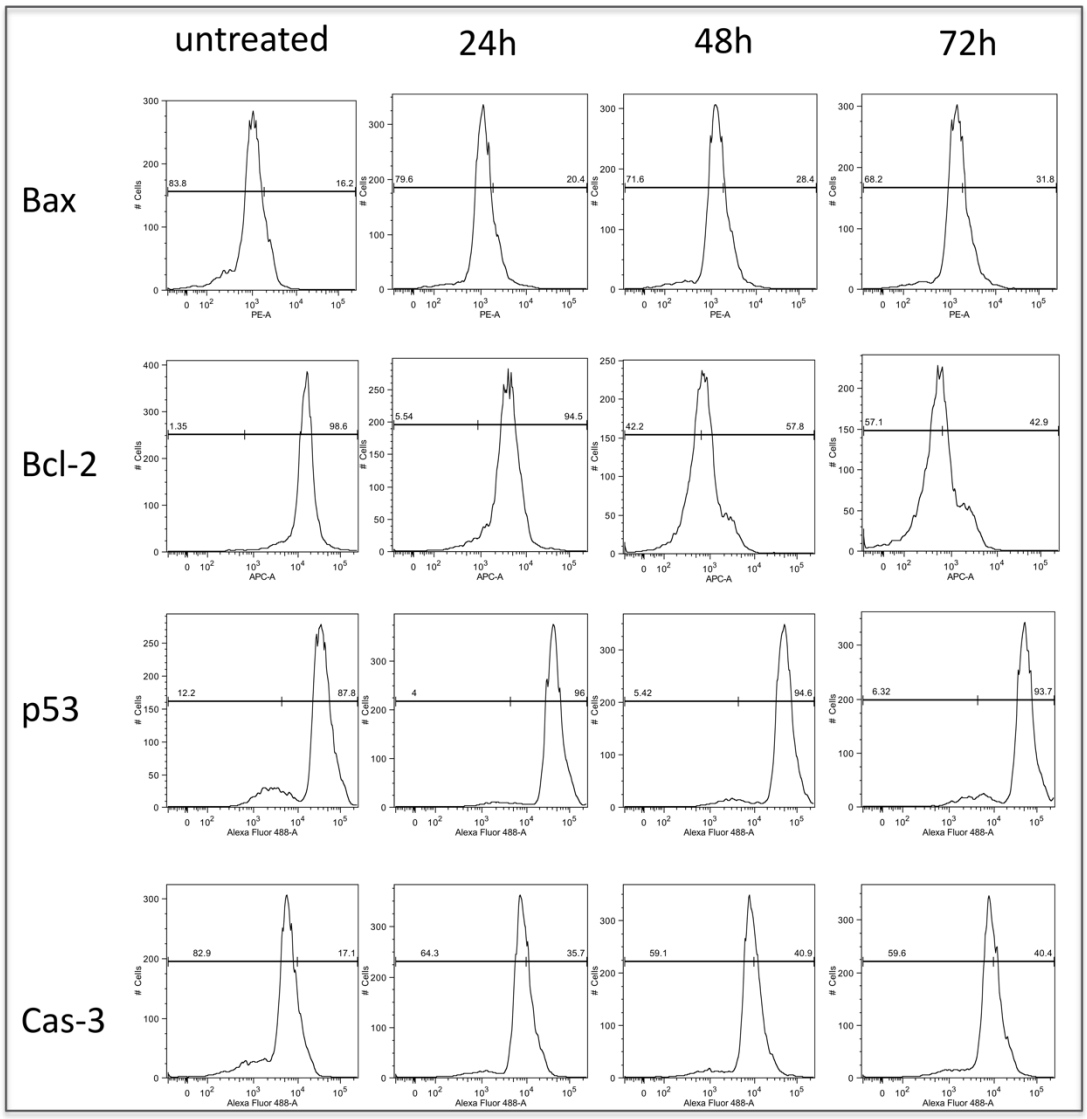



Figure 4.20: **Apoptosis proteins expression of Bax, Bcl-2, p53, and Caspase-3, following the treatment of MDA-MB-231 cell with APME by flow cytometry.**

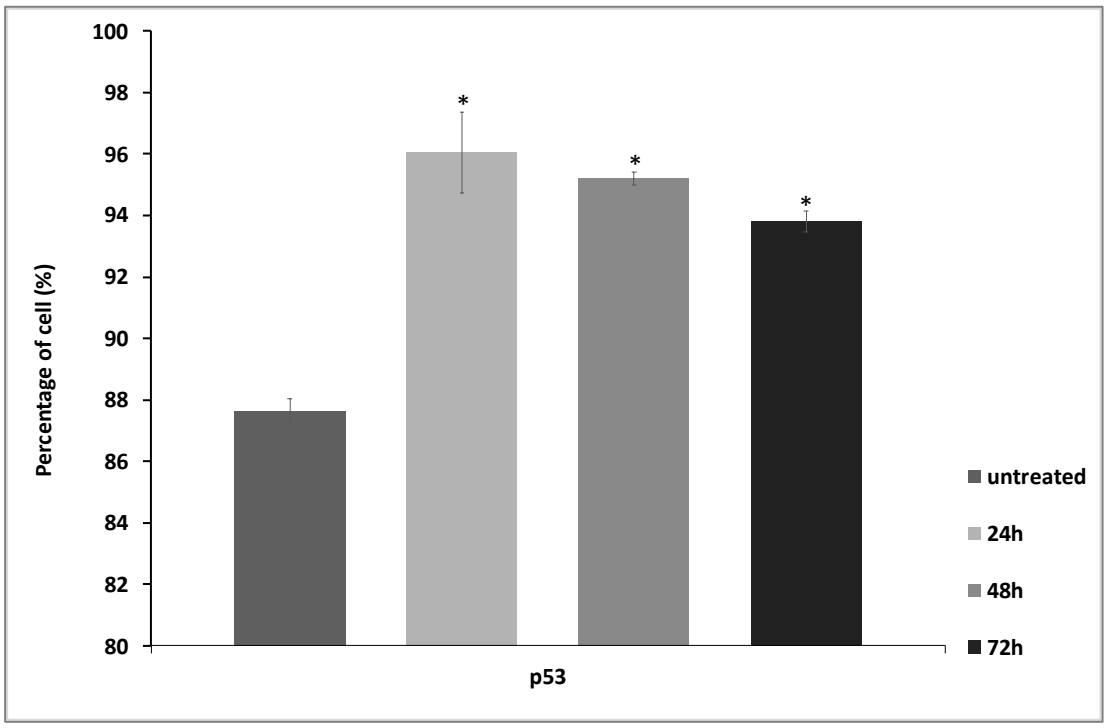



Figure 4.21: **Protein expressions of p53, following the treatment of MDA-MB-231 cell with APME by flow cytometry.**

Data are expressed as mean ±SD of three repeated experiments. $P<0.05$ is considered significance when comparing treated cells *vs* untreated cells.

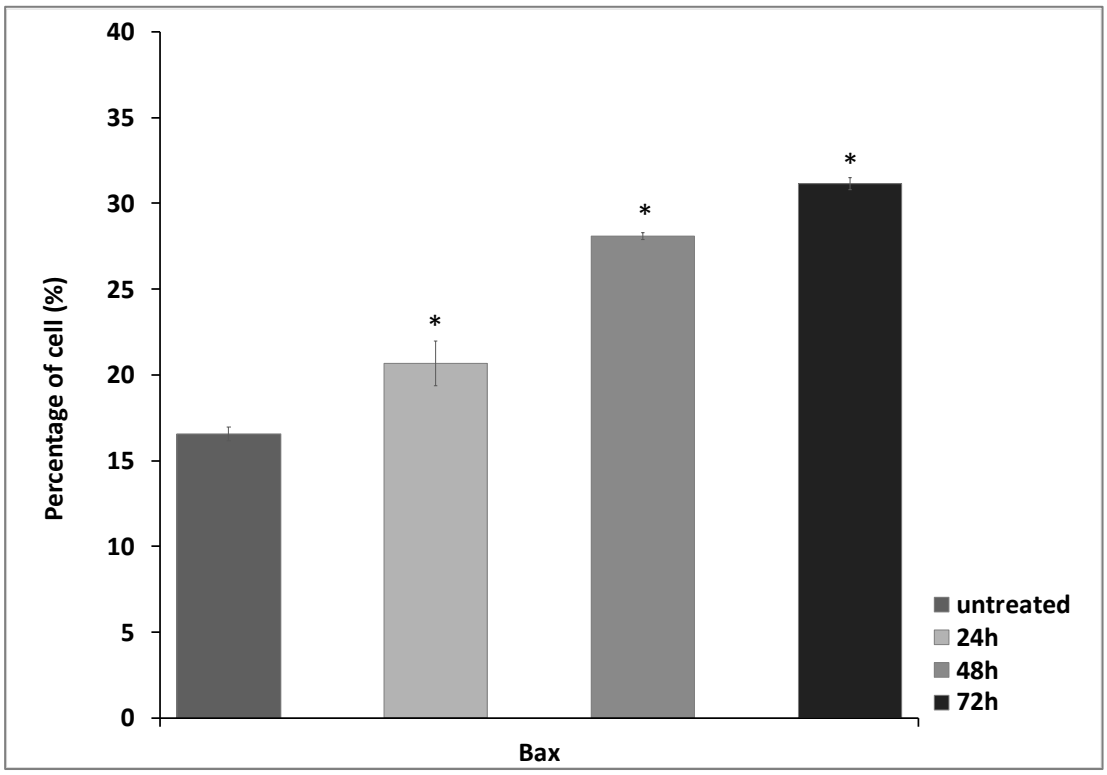



Figure 4.22: **Protein expressions of Bax, following the treatment of MDA-MB-231 cell with APME by flow cytometry.**

Data are expressed as mean ±SD of three repeated experiments. $P<0.05$ is considered significance when comparing treated cells *vs* untreated cells.

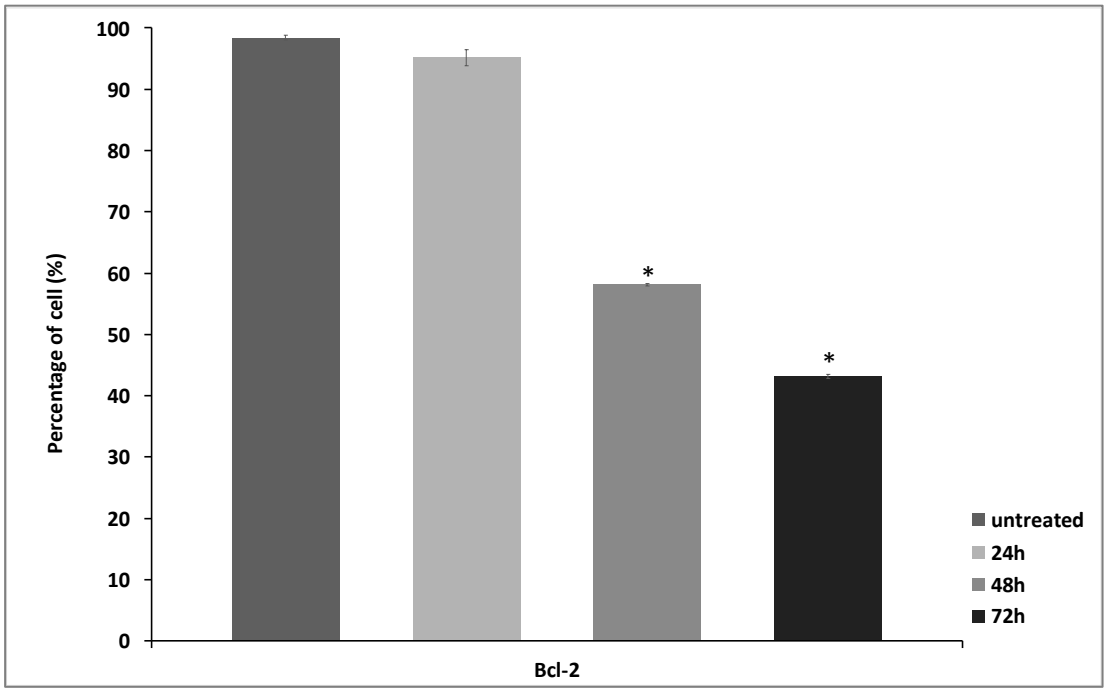



Figure 4.23: **Proteins expressions of Bcl-2, following the treatment of MDA-MB-231 cell with APME by flow cytometry.**

Data are expressed as mean ±SD of three repeated experiments. $P<0.05$ is considered significance when comparing treated cells *vs* untreated cells

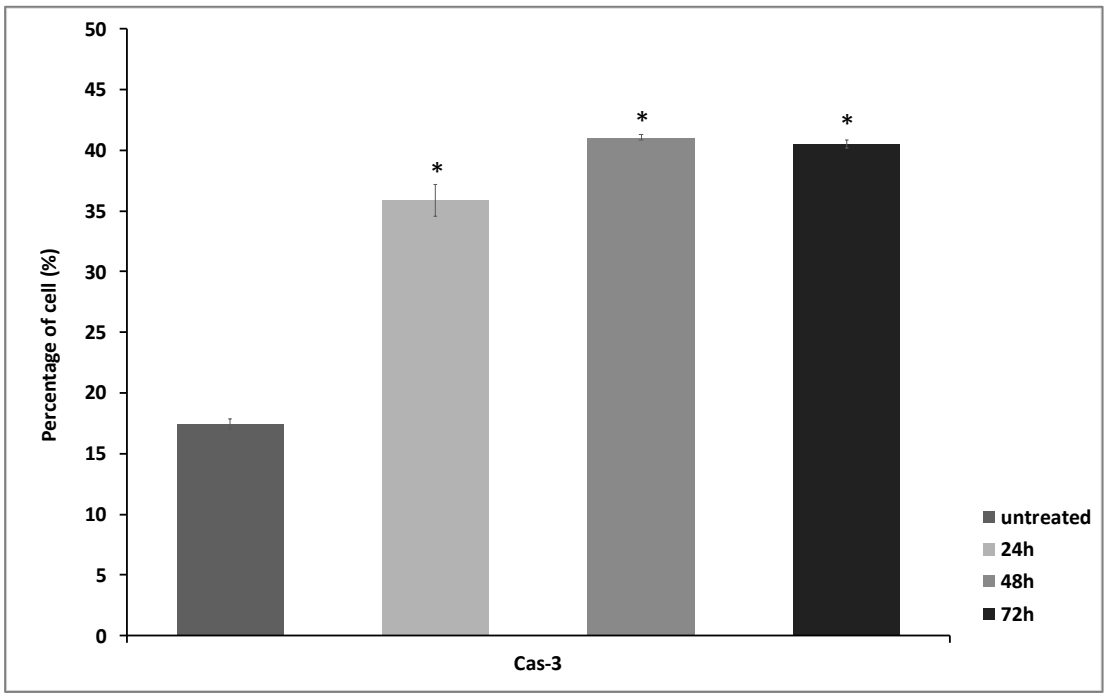



Figure 4.24: **Protein expressions of Caspase-3, following the treatment of MDA-MB-231 cell with APME by flow cytometry.**

Data are expressed as mean ±SD of three repeated experiments. $P<0.05$ is considered significance when comparing treated cells *vs* untreated cells.

## 4.4 DISCUSSIONS

A very large number of plant extracts have been screened for cytotoxic effects against cancer cell lines over the last 25 years and the traditional use of a considerable number of plants for cancer has been justified to some extent by the findings that have shown that their extracts are cytotoxic, especially if selectivity is demonstrated, either between different cancer cell lines or between cancer and non-cancer cell lines. Cytotoxicity is the common method to determine the anti-proliferative activity of an extract towards a cancer cell line. This method identifies the inhibitory effect of the extract on the cell that are actively undergoing mitotic division. The growth rate is indirectly measured by the formation of a colour, which also indicates the number of cells present. This is the basic principle applied in the MTT assay that was used in this study. In MTT, the mitochondria of the viable cells will reduce the tetrazolium salt into a coloured product called formazan. The instensity of this coloured product is measured spectrophotometrically with a plate reader. Such method can be used to determine whether the cytotoxicity of the cells is cytostatic or cytocidal (Houghton *et al.*, 2007). Thus, it will give an idea whether the extracts really affect the cells or merely just inhibit the growth.

This chapter reported on the potential of *A. precatorius* leaves to induce cell deaths in cancer cells. Previous chapter has highlighted the phytochemicals identified in *A. precatorius* leaves extracts and its possible compounds that contribute to the anti-proliferative activity in *A. precatorius* leaves. Previous study on various cancer cell lines showed that various phytocompound isolated from *A. precatorius* demonstrated inhibitory effects and these properties should be able to induce apoptosis on various types of cancers (Hickman, 1992). Extraction with water (aqueous) by decoction was performed to be as closely to the traditional practise as possible. Initially, the extracts

showed no anti-proliferative effect on any of the cells even at the highest concentration of 99µg/ml. Therefore, the extract was tested again on the cells with the maximum concentration at 990µg/ml. This was done to ensure whether *A. precatorius* aqueous leaves extract has any anti-proliferative effect because according to The US National Cancer Institute and Geran Protocol, plants extracts that are more than 501µg/ml is considered to not exhibit any cytotoxicity activity (Geran *et al.*, 1972).

*A. precatorius* aqueous leaves extracts could be categorized as non-toxic to cells. The lowest $IC_{50}$ values was 573µg/ml on MDA-MB-231 cells. This value is more than 501µg/ml, and this is definitely categorized as non-toxic. Lebri *et al.* (2015) performed a different aqueous extraction on *A. precatorius* leaves and discovered that it was able to inhibit 50% growth of murin mastocytoma cells (P815) at 200µg/ml. Sofi et al (2013) demonstrated anti-proliferative activity of the aqueous leaves extract of *Abrus precatorius* at 98µg/ml when tested against MDA-MB 231 cell line. Maximum inhibition of the cells at 75% was obtained at 600µg/ml after 48h of incubation (Sofi *et al.*, 2013). Their extract was obtained by longer maceration time (overnight) of sonicated powdered leaves in double distilled water, whereby in this study the aqueous extract was obtained by decoction at 50°C. In the GCMS report of *A. precatorius* aqueous leaves extract in Chapter 3, β-ionone was identified. This compound was reported to exhibit anti-proliferative effects on human leukemia cell line (Faezizadeh *et al.*, 2016). However, in this study, this extract was not able to show anti-proliferative effect below the standard range stipulated by The US National Cancer Institute and Geran Protocol, despite the presence of β-ionone in the extract.

Two different successive solvent extraction methods were applied this study. One was with the Soxhlet and the second one was by prolonged soaking or known as

maceration. The differences between these two methods are time and heat. Soxhlet took a shorter time with heat while maceration took a longer time without heat. Both started with hexane, followed by ethyl acetate and lastly with methanol. Extracts obtained by Soxhlet exhibited a better anti-proliferative activity against the selected cancer and normal cells compared to extracts obtained by maceration. The lowest $IC_{50}$ values was exhibited by the *A. precatorius* methanol leaves extract on MDA-MB-231 cells. In fact, this extract also has a moderate anti-proliferative effect on other cancer cells, HeLa, MCF7, and SW 480. On normal breast cell, MCF10A, and normal fibroblast cell, NIH(3T3), this extract failed to display any anti-proliferative activity at the maximum concentration of 99μg/ml. *A. precatorius* methanol leaves extract by maceration demonstrated weakly anti-proliferative effects on all cancer cells, and definitely exhibited non-cytotoxic on the normal breast cell, MCF10A and normal fibroblast cell, NIH(3T3). The presence of (-)-Loliolide may contribute to the better anti-proliferative effect of the *A. precatorius* methanol leaves extract by Soxhlet because of its anti-cancer ability (Samanta *et al.*, 2018). The GC-MS analysis also revealed that this extract contained high content of phenolic and terpenoid compounds. As discussed in Chapter 3, 4-vinylphenol exhibited antiangiogenesis and reduced the size of the tumour (Yue *et al.*, 2015), and this is the highest phenolic compound present in the extract. Both methanol extracts were able to display their anti-proliferatve activity on all cancer lines moderately by the Soxhlet extraction and lowly by the maceration extraction. These results indicate that *A. precatorius* methanol leaves extract both by Soxhlet extraction or maceration have the potential to induce cell deaths on those selected cancer cells but at the same time did not harm the normal cell lines.

*A. precatorius* ethyl acetate leaves extract by Soxhlet was able to moderately inhibit the growth of MDA-MB-231 cells at 54.50µg/ml and MCF7 at 99µg/ml. On the other hand, the maceration extracts exhibited weak cytotoxic on HeLa, MDA-MB-231 and SW 480. No activity was observed in MCF7, MCF10A and NIH(3T3) cells. Similar to methanol extract (Soxhlet), (-)-Loliolide was also identified in the ethyl acetate extract by Soxhlet. This compound was not identified in the maceration extracts. (-)-Loliolide was also presence in the *A. precatorius leaves* hexane extract (Soxhlet) and not detected in the maceration extract. Soxhlet *A. precatorius* leaves hexane extract exhibited moderate anti-proliferative activity on MDA-MB-231 cells at 45.60µg/ml and MCF7 cells at 52.65µg/ml. No cytotoxicity was observed on this extract on both MCF10A and NIH(3T3) cells at the maximum concentration of 99µg/ml. While the maceration extracts showed moderate anti-proliferative activity only on MDA-MB-231 cells at 80.75µg/ml, it also exhibited weak anti-proliferative activity on MCF7, HeLa, and SW480. Another study by Gul et al. (2013) claimed to have stronger anti-proliferative activity of the hexanol and ethanol leaves extracts of *Abrus precatorius* both in human colon adenocarcinoma cells (Colo-205) and human retinoblastoma cancer cells (Y79), while milder anti-proliferative activities were observed in human hepatocellular carcinoma cells (HepG2) and leukemia cells (SupT1). These two studies were different with the current study on the incubation time of the treated cells. They treated their cells for 48 h while ours was 72 h. From these findings, the Soxhlet *A. precatorius* methanol extract (APME) was identified as the most potent anti-proliferative extract on the MDA-MB-231 cells.

From this anti-proliferative experiment, both breast cancer cell lines showed high sensitivity towards all *A. precatorius* leaves extract, except for ethyl acetate extract by maceration. However, not all extracts were able to exclusively exhibit the

anti-proliferative activity in all cancer cell lines. All extracts were also unable to induce cytotoxicity on normal cells. This provide an inclination of the selectivity of *A. precatorius* leaves extracts which also conclude that MDA-MB-231 cells was the most sensitive cells towards those extracts.

Cytotoxicity activity alone could not conclude the anticancer properties of the extracts. Further studies are needed to determine the mechanism of the cell death. Some natural products are found to act by novel mechanism. For example, paclitaxel from species of yew (Taxus), inhibited mitosis by stabilizing microtubules and thus preventing the formation of tubulin, which in contrast to other anticancer agents that inhibit the formation microtubules since the beginning (da Rocha Dias and Rudd, 2001). APME was used to treat MDA-MB-231 cells in all subsequent assays to explore more on the activity of this extract. First, the APME-treated cells were observed for its morphological changes by light microscopy and fluorescence microscopy. APME-treated cells started to show signs of cell deaths starting at 48h. At 24h APME-treated cells did not exhibit significance difference with the untreated cells. Tamoxifen-treated cells started to demonstrated signs of apoptosis as early as 24h post treatment. Cells undergoing apoptosis were identified with the presence of membrane blebbing and ballooning, indicating that the plasma membrane started to lose its integrity. Apoptotic bodies were also observed in the APME-treated cells at 48h post treatment.

Further evaluation of the morphological changes of the apoptosis events, APME-treatted cells were also observed under fluorescence microscope following staining with Hoechst. This dye is generally used to observe nuclear changes in the cell. Besides Hoechst, another typical dye used for the same reason is 4',6-diamidino-2-phenylindole (DAPI). DNA becomes condensed and fragmented during apoptosis

and this event is the hallmark for cells undergoing apoptosis that distinguish them from necrosis cells and healthy cells. DNA fragmentation can be clearly observed in APME-treated cells after 72h post treatment.

For better understanding of the ability of APME to induce cell death, cell cycle analysis assay was performed. Cell growth and proliferation of mammalian cells occurs through cell cycle; thus, the inhibition of the cell cycle progression is the ideal target for anticancer agents (Kim *et al.*, 2008; Li and Blow, 2001). APME exhibited growth inhibitory effects on the MDA-MB-231 cells, inducing cell cycle arrest at G0/G1 phase. Increase percentage of cell population in G0/G1 phase and reduction of the population in S-phase, in time dependant manner proved this claim. On a contrary, a recent study showed that kaempferol, a flavonoid compound, induced cell cycle arrest at G2/M phase in MDA-MB-231 cells (Zhu and Xue, 2019). In S-phase, genetic information is transferred from one cell generation to another. Genome replication in S-phase is important to segregate two daughter cells during mitosis or the M-phase. Mitosis only occurs when S-phase is completed. Two gaps separate between M- and S-phase. Between M- and S-phase, there is the G1, and between S- and M-phase, there is G2. DNA damage activates these checkpoints. When growth arrest occurs at any checkpoints, cells will repair the damage. If the damage is repaired, cell progression will successfully resume, otherwise the cell will be eliminated through apoptosis (Li and Blow, 2001). DNA arrest occurred during G0/G1 phase in this current study, indicated that the cell proliferation was inhibited, thus showing reduction of the cell percentage in S-phase and G2/M phase. At this point, it is clear that cell proliferation was halted by DNA arrest at G0/G1 phase. Furthermore, it is important to establish if the cell inhibition was caused by apoptosis.

Apoptosis induction is regarded as the best strategies in cancer treatment. It is an important programmed cell death to eliminate unnecessary cells and thus became the common mode of action for most chemotherapeutic agents (Cao and Tait, 2018). Induction of apoptosis signifies the success of plant products as anticancer agents and it is the optimal way in cancer treatment. In order to confirm whether the inhibition of cell proliferation induced by APME is due to apoptosis, rather than necrosis, apoptosis assay using AnnexinV-FITC and PI staining was performed following the treatment with the extract. AnnexinV stains the phosphatidylserine of the inner cell membrane which is exposed during the early stage of apoptosis. Our results demonstrated that the APME promotes cell death via apoptosis. Early apoptosis occurred after 48h and eventually led to late apoptosis following 72h of treatment. These findings are in coherent with the morphological observation of the APME-treated MDA-MB-231 cells, where signs of cells undergoing apoptosis started to show at 48h post treatment. These signs explained the shrinkage of cells and eventually lead to rounded cells and detachment from the well surface.

Apoptosis occurs through a series of events, either by extrinsic pathway or intrinsic pathway, or both. One of the important proteins in inducing cell cycle arrest or apoptosis is the p53. This protein is encoded by the *TP53* gene. p53 is found mostly mutated or silence in cancer where about 50% to 55% of human cancers have loss of wild type p53 activity (Wang *et al.*, 2015). In normal condition, p53 is lowly expressed even undetectable, however, upon activation, p53 provides significance defence against tumour development by inducing cell cycle arrest or apoptosis. MDA-MB-231, a triple negative breast cancer (ER-) is known to express mutant p53 (Gartel *et al.*, 2003; Hui *et al.*, 2006), which enable the survival of the cells and contribute to suppressing apoptotic events. In ER+ breast cancer, though expressing wild type p53,

apoptosis responses by p53 are prevented because of the estrogen directly interacts with the wild type p53 (Bailey *et al.*, 2012a). p53 responses can be blocked in tumours with wild type p53 by downregulation of its activity or its protein effector activity. In our study, p53 expression was relatively high in MDA-MB-231 cells, however significant increment was observed at 24h post APME treatment. The level started to decrease insignificantly at 48h and significantly at 72h. Even though the level of p53 expression decreased at 72h, it was still significantly higher in comparison to the untreated cell. Thus, indicating that increased p53 levels contributed to the cell cycle arrest and/or apoptosis events in APME-treated MDA-MB-231 cells.

Decrease of mitochondrial outer membrane permeability (MOMP) indicated the irreversible events of early apoptosis. MOMP is highly regulated by anti-apoptotic and pro-apoptotic proteins (Czabotar *et al.*, 2014). Bax is a pro-apoptotic protein and Bcl-2 is an anti-apoptotic protein (Bai and Wang, 2014). Upon stimuli in the intrinsic pathway, Bax is activated which induce MOMP. In this pathway, Bcl-2 prevented MOMP by inhibiting the activity of the BH3-only proteins that are responsible to activate Bax. MOMP allows the release of intermembrane space proteins, such as cytochrome c and SMAC. Cytochrome c forms an apoptosome complex after binding to the APAF-1 protein. This complex is responsible to activate caspase-3 and caspase-7 which eventually lead to apoptosis.

Our results indicated that the expression of Bax proteins increased while Bcl-2 proteins decreased. Chien *et al.* (2009) demonstrated that quercetin induced apoptosis in MDA-MB-231 cells also by reducing Bcl-2 and increasing Bax protein levels. Overexpression of Bcl-2 like proteins such as Bcl-2, Bcl-xL and MCL-1 were detected in various cancers including lymphoma (Pepper *et al.*, 1998), lung cancer (Beroukhim *et al.*, 2010), neuroblastoma(Castle *et al.*, 1993) and breast cancer

(Olopade *et al.*, 1997). MOMP is an important event of apoptosis because once it is activated, cell will face death sentence regardless of caspase activation (Tait and Green, 2010).

Caspase-3 increased protein expression signified the apoptosis events in the MDA-MB-231 cells treated with the APME. Caspase-3 activation can occur both in the extrinsic or intrinsic pathway. Upon activated, caspase-3, which is also known as the executioner, cleaves hundreds of other proteins, that subsequently lead to the biochemical and morphological signals of apoptosis. These hallmark events include DNA fragmentation, plasma membrane blebbing and phosphatidylserine exposure (McIlwain *et al.*, 2015). As shown in the apoptosiss staining assay, exposure of phosphatidylserine was detected by AnnexinV-FITC and this indicates the early apoptosis event.

## 4.5 Conclusion

The results of this current study exhibited that APME inhibited MDA-MB-231 cell proliferation by causing DNA arrest at G0/G1 and inducing apoptosis cell death through upregulation of p53 and Bax, down regulation of Bcl-2 and increase expression of caspase-3 protein. These findings suggest that APME successfully promotes cell death in breast cancer cell, MDA-MB-231 *via* apoptosis possibly through mitochondria mediated pathway. Further analysis was perfomed to evaluate the ability of APME to promote the NK cells activation.

# CHAPTER 5

# INDUCTION of NATURAL KILLER (NK) CELL ACTIVITY ON MDA-MB-231 CELLS BY *Abrus precatorius* METHANOL LEAVES EXTRACT (APME)

## 5.1 INTRODUCTION

Elimination of cancer cells by the immune system is the desirable strategy to combat cancer naturally. This process utilises the highly specific coordinated immune system to remove cancerous cells from the host without interfering or damaging the surrounding cells. However, cancerous cells also have their own "mechanism" to delay or evade this immune surveillance and therefore remain active. Any anti-cancer agents that would augment the cytotoxicity of the immune cells in particular the Natural Killer cells (NK) is highly demanded and many researchers are currently looking into the insight of deeper understanding of these NK cells (Hu *et al.*, 2019). NK cells are innate immune cells which are able to eliminate various stressed or abnormal cells and even kill target cells without preliminary sensitization (Grossenbacher *et al.*, 2016; O'Sullivan *et al.*, 2015; Vivier *et al.*, 2011). NK cells are the preferred focus for the use in immunotherapy due to its independency on antigen specific T cells and its rapid proinflammatory cytokines secretion that initiate the adaptive immune response (Sconocchia *et al.*, 2014). NK cells release the cytolytic granules upon bridging immune synapses with target cells, including perforin and granzymes. These proteins function to induce target cell lysis and this process leading to their expressions is known as degranulation. NK cells degranulation is referred as indirect measurement of NK cells cytotoxicity (Topham and Hewitt, 2009)

Plant-derived medicines have an important role in influencing the effects on immune system. Discoveries of plant containing immunomodulatory properties is still relevant and in demand especially to enhance current practices in immunotherapy.

Several studies have been conducted to grasp the better understanding of how plant extracts or isolated phytocompounds affect the immunomodulation of NK cells. Studies on revasterol suggested that it promotes target cell apoptosis *via* caspase signalling pathway in high concentration ranges (Leischner *et al.*, 2015). Another study identified a fraction from *Caesalpinia spinosa,* rich with gallotannin as an anti-tumour agents in breast carcinoma and melanoma (Gomez-Cadena *et al.*, 2016). Lee and Cho (2018) demonstrated that curcumin augments the cytotoxicity effect of NK-92 on MDA-MB-231. For *Abrus precatorius,* to date most reported studies are on the seeds, particularly on lectin and abrin. NK cells activity in tumour-bearing mice was enhanced significantly by abrin (Ramnath *et al.*, 2006). *A. precatorius* seeds peptides AGP and ABP exerted immunostimulants response in Dalton's Lymphoma (DL) bearing mice (Bhutia *et al.*, 2009). Another study conducted on another species of *Abrus* known as *Abrus agglutinin* (seeds) also successfully demonstrated the ability of the seeds to stimulate spleen derived NK *in vitro* to exhibit cytotoxicity effect against Dalton's lymphoma ascites cells (DLAC) (Ghosh and Maiti, 2007).

Previous experiment has described the ability of *A. precatorius* methanol leaves extract (APME) to induce apoptosis in MDA-MB-231 cells. Therefore, the ability of APME to induce NK cells activity was investigated in this current Chapter. This was measured by the ability of APME-treated NK cells to promote cell death, measured by apoptosis staining of the target cell with AnnexinV/PI, and the production of interleukin-2 (IL-2), interferon gamma (IFN-γ), perforin (PRF-1) and granzyme B (GzmB) from the effector cell (NK cells).

## 5.2 MATERIALS & METHODS

### 5.2.1 Healthy and Cancer Donor Criteria

Three donors were chosen for both healthy and cancer individuals. 10ml of blood was obtained from each donor. All healthy donors were female with the age between 18 – 45 years old, no chronic disease, not consuming any immunosuppressive drugs, non-smokers, and not pregnant. For cancer donors, the selected donors were female between the age of 18 – 45 years old, diagnosed with breast cancer, not receiving any treatment yet including chemotherapy and radiotherapy or consuming any herbal and medicinal plants, non-smokers and not pregnant. Non-smokers were preferable because smoking impacts immune cells including the NK cells (Qiu *et al.*, 2017).

### 5.2.2 Isolation of human peripheral blood mononuclear cells (PBMC)

Whole blood sample from donors was collected in an EDTA vacutainer. The collected blood was diluted in PBS with ratio of 1:3 (v/v). The diluted blood was layered carefully on Lymphocyte Separation Media, LSM (Capricorn Scientific), and centrifuged at 400xg for 30 minutes at room temperature. After centrifugation, four layers were formed started with the blood plasma phase, interphase, LSM phase and erythrocyte phase (pellet), as illustrated in Figure 5.1. The interphase layer was collected carefully and transferred into a new 15ml tube. About 30ml of PBS was added to the tube and was centrifuged at 300xg for 10 minutes. This process was repeated twice. The pellet collected was the lymphocyte and it was resuspended in complete RPMI 1640 medium. The lymphocyte was transferred into the 75cm$^3$ flask and was incubated overnight in a humidified 5% $CO_2$ incubator at 37°C. This process was necessary to remove the monocyte by adherence to the flask plastic surface (Bose *et al.*, 2009). The next day, the supernatant was decanted into a new 15ml tube and

centrifuged at 400xg for 10 minutes. Washing with PBS was performed twice at 300xg for 10 minutes. The resulting pellet was resuspended in the RPMI media and the cell count was determined by the Trypan Blue exclusion assay.

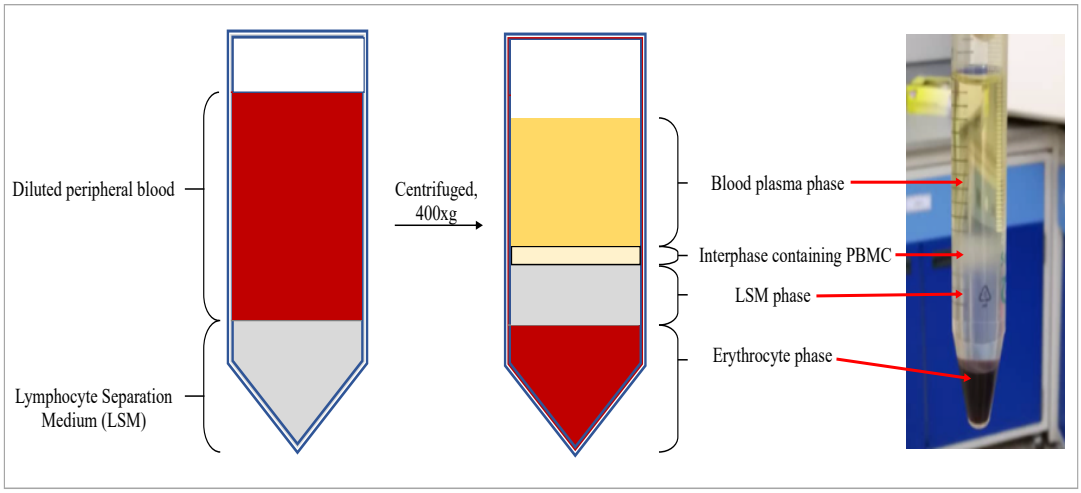



Figure 5.1: Isolation of peripheral blood mononuclear cells (PBMC)

### 5.2.3 NK Cell Isolation

Human NK Cell Isolation Kit (Miltenyi Biotec) was used to isolate the NK cells. The experiment was performed according to the manufacturer's protocol. This experiment was performed in cold and using pre-cooled solutions and buffers. After removal of monocyte by adherent to the plastic as described previously, the cell suspension was centrifuged to collect remaining lymphocytes. The cell pellet was resuspended in 40µl of buffer (refer Appendix B) for $10^7$ of the total cells. 10µl of NK Cell Biotin-Antibody Cocktail was added per $10^7$ of the total cells and mixed well. This mixture was incubated in the refrigerator (2-8°C) for 5 minutes. Then, 20µl of NK Cell Microbead Cocktail was added per $10^7$ of the total cells, mixed well and incubated in the refrigerator (2-8°C) for 10 minutes. 1000µl of buffer was added into

this suspension. Then, this suspension was proceeded to the subsequent magnetic cell separation.

First the column was placed onto the magnetic field of the MACS separator. The column was rinsed with 3ml of buffer then the suspension prepared earlier was applied into the column. The collected flow-through was the unlabelled cells which would represent the NK cells. The column was washed with the 3ml of buffer twice and was combined with the effluent collected earlier. This suspension was centrifuged at 300xg for 5 minutes and resuspended in 1ml PBS. The NK cells counts was determined using the Trypan Blue exclusion assay. Flow cytometry was used to verify the percentage of $CD56^{+}/CD3^{-}$ cells population after the separation.

### 5.2.4 NK Cell Proliferation Assay (MTT)

Isolated NK cells were seeded into the 96 wells plate. *A. precatorius* methanolic leaves extract (APME) was added in serial dilution with DMSO into each well starting from 200µg/ml until 1.56µg/ml. Untreated-NK cells and DMSO (<1%) were added into the wells as controls. The culture was incubated for 24h, 48h and 72h in a humidified 5% $CO_2$ incubator at 37°C. After each incubation point, 20µl of MTT (5mg/ml) was added into the well and incubated for 4h. After 4h, 100µl of DMSO was added into the wells to dissolve the formazan and the plate was read at absorbance 570nm. The absorption value at this wavelength directly represents the relative cell numbers with comparison to the control group (Igarashi and Miyazawa, 2001). The percentage of cell viability was determined by dividing the absorbance of the treated cells with the absorbance of the control and multiply by a hundred. Besides the diluted extract, the APME $IC_{50}$ (26.4µg/ml) obtained from previous experiment in Chapter 4 was also tested individually. This was done to ensure the effect of that concentration

on the NK cell proliferation. This part of the experiment in general was performed to determine the ideal concentration of APME needed to be used in the subsequent NK co-culture with MDA-MB-231 cells.

### 5.2.5 NK Cell Co-culture with MDA-MB-231

Graphical summary of this experiment is shown in Figure 5.2. This experiment was performed to evaluate the ability of APME in inducing NK cells activity on MDA-MB-231 cells.

#### 5.2.5(a) Cell Culture

MDA-MB-231 were seeded in 25cm$^2$ tissue culture flask and grown at 37°C under humidified 5% $CO^2$ in DMEM medium supplemented with 5% of FBS and 1% of penicillin-streptomycin. Confluent MDA-MB-231 cells were harvested by trypsinization.

#### 5.2.5(b) Co-cultutring of NK cells with MDA-MB-231 cells

This experiment was performed to evaluate the APME potential to induce anticancer immune response by activating the NK cells. Freshly isolated NK cells (effector) from each donor were pre-incubated for four hours according to the experimental design as described in Table 5.1, before being added into the pre-seeded (overnight) MDA-MB-231 (target) cell culture. The ratio of effector cells to target cells was set at 20:1 following the methods detailed in Ismail *et al.* (2012) and Nishimura *et al.* (2017) with given consideration of NK cells co-cultured with adherent cells (Surayot and You, 2017) and specifically with MDA-MB-231 cells (Fu *et al.*, 2015). Following the incubation, the medium was collected into a fresh tube and the adhered MDA-MB-231 cells were harvested by trypsinization. The trypsinized cells were combined with the collected medium and centrifuged at 300xg to separate the

cell pellet from the supernatant. The medium (supernatant) was transferred into a fresh 1.5ml tubes and kept at -80°C for further analysis. The collected pellet containing both NK cells and MDA-MB-231 cells were resuspended with RPMI 1640 complete medium and divided into two fresh tubes for further analysis.

### 5.2.5(c) NK Cell Staining

The number of NK cells in the culture was determined by staining the cells with PE-conjugated anti-CD56 (Santa Cruz) antibody and acquired by flow cytometry. The collected cells (100µl) following the co-culture experiment, was washed twice with 1XPBS. One mililitre of Bovine Serum Albumin (BSA) was added to the cells and incubated for 10 minutes at room temperature. Five microlitre (5µl) of the PE-conjugated anti-CD56 and FITC-conjugated anti-CD3 antibodies were added and mixed well. This suspension was incubated for about 30 minutes on ice. Then, the cells were washed with PBS (centrifuged at 300xg) twice to remove excess antibodies. The resulted pellet was resuspended with 500µl PBS and the number of NK cells was determined by flow cytometry FACSCANTO II (BD Bioscience). Data obtained was analysed with FCS 7 software (De Novo).

### 5.2.5(d) Apoptosis Detection

Determination of the percentage of apoptotic cells of MDA MB 231 in the culture was done by apoptosis staining of the target cells using the AnnexinV-FITC/PI. The apoptosis assay was performed according to the manufacturer protocol, AnnexinV-FITC detection kit I (BD Bioscience). The collected cells (100µl) following the co-culture experiment, was washed twice with 1xPBS and then, 100µl of 1X binding buffer was added prior to mixing with 5µl of AnnexinV and 5µl of PI. This suspension was incubated for 15 minutes, at room temperature in dark. After the

incubation period, 500µl of 1X binding buffer was added into the tube. All samples readings were acquired with FACSCANTO II (BD Bioscience). Data obtained was analysed with FCS 7 software (De Novo). Percentage of NK cells cytotoxicity for each donor category was determined by the following formula: ([percentage of cell deaths by effector – percentage of cell deaths without effector] / [100 – percentage of cell deaths without effector]) x 100 (Nishimura *et al.*, 2017).

Table 5.1: Experimental design for NK cells co-culture with MDA-MB-231 cells

| No | Treatment Groups | Details |
|---|---|---|
| 1 | NK/MDA-MB-231/APME | NK cells were incubated with APME prior to treatment on MDA-MB-231 cells |
| 2 | NK/MDA-MB-231 | NK cells were incubated on MDA-MB-231 cells |
| 3 | NK/MDA-MB-231/DMSO | NK cells were incubated with DMSO prior to treatment on MDA-MB-231 cells |
| 4 | NK ONLY | NK cells were incubated on its own |
| 5 | APME/MDA-MB-231 | MDA-MB-231 cells were treated with APME |

### 5.2.5(e) ELISA (IL-2, IFN-γ, PRF-1, GzmB)

ELISA was performed to quantify the expression level of the cytokines, interleukin-2 (IL-2) and interferon gamma (IFN-γ); and cytotoxic granules protein, perforin (PRF-1) and granzymeB (GzmB). This assay was performed according to the manufacturer's protocol (Elabscience). In general, 100µl of sample/standard was added into a pre-coated ELISA plate and incubated for 90 minutes at 37°C. The samples or standards were diluted accordingly as needed. After the first incubation period, liquid from each well was removed and 100µl of Biotinylated Detection Ab was added into each well and incubated for 1h at 37°C. Then the liquid from each well was aspirated and washed with 350µl of wash buffer was added. The washing step was performed three times. 100µl of HRP conjugate was added after the washing steps and

the plate was incubated for 30 minutes at 37°C. Another five times of washing steps were performed after the incubation. Then, 90µl of substrate reagent was added into each well and was incubated for 15 minutes at 37°C in dark. 50µl of stop solution was added after the incubation time ended and the optical density (OD value) of each well was determined with a micro-plate reader at absorbance of 450nm.

### 5.2.6 Statistical Analysis

The data were expressed as mean ±SD of three repeated experiments. The level of statistical significance among the group was tested using repeated measure one-way ANOVA, followed by Tukey's multiple comparison test. Level of significance between two main groups were tested using paired or unpaired t-test. The difference was considered significant if $P<0.05$.The normality of each data set was determined by Shapiro-Wilk normality test. Analyses were all done using GraphPad Prism7 . (Graphpad Software, La Jolla, CA, USA)

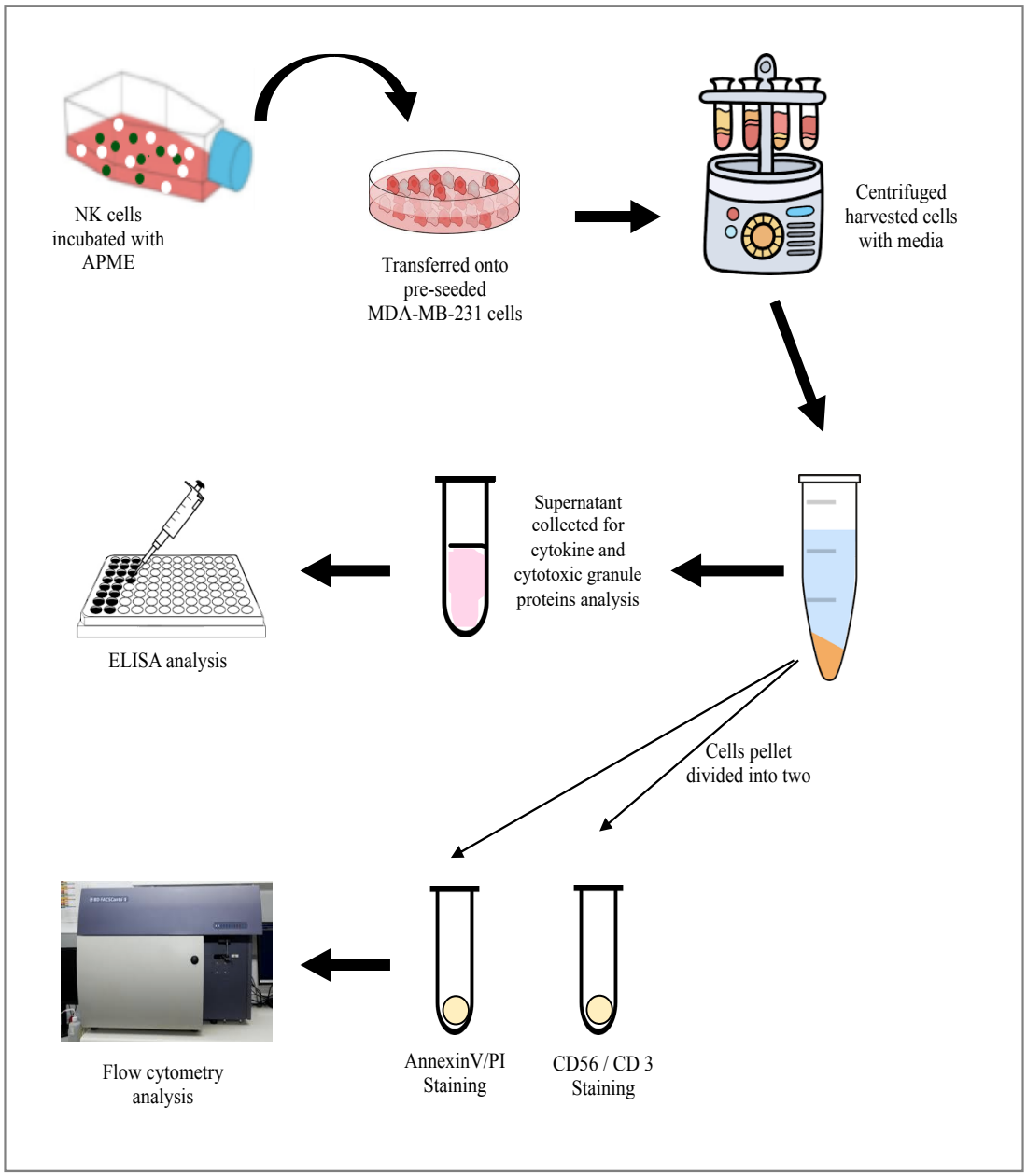



Figure 5.2: Graphical summary of the co-culture experiment of APME-treated NK cells with MDA-MB-231 cells.

## 5.3 RESULTS

### 5.3.1 NK Purification

NK cells were isolated from healthy and cancer donor using the Human NK cell isolation kit (Miltenyi Biotec). Isolated NK cells were stained with CD56 (NCAM (ERIC) - PE, Santa Cruz Biotechnology). Dot plot graph of the cell distribution events after acquiring by flow cytometry were as shown in Figure 5.3. About 85.04% of NK cells were successfully isolated using this NK isolation kit.

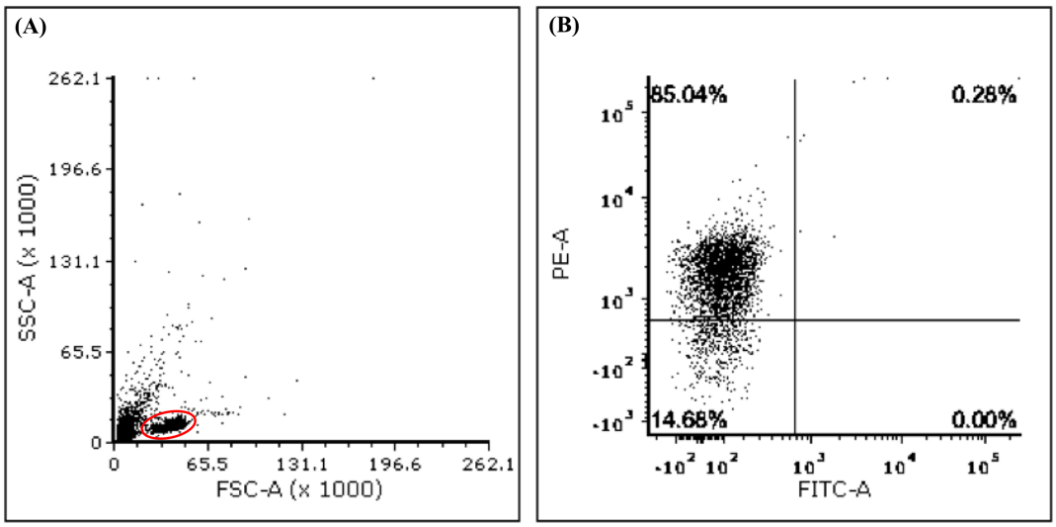



Figure 5.3: **Dot plot graphs representing the isolated NK cells using the Human NK cell isolation kit (Miltenyi Biotec).**

(A) The cell population gated as NK cells.
(B) The cells were tagged with CD56 and CD3.

### 5.3.2 Isolated NK Cell in Healthy and Cancer Donor

In this experiment, NK cells were isolated from three healthy donors and three cancer donors (breast cancer). Trypan blue exclusion assay was used to count the cells. These isolated NK cells were used in the subsequent analysis following the isolation. The average counts of NK cells isolated from healthy donor was significantly higher (7.72 x $10^5$ cells/ml) than NK isolated from cancer donor (3.8 x $10^5$ cells/ml).

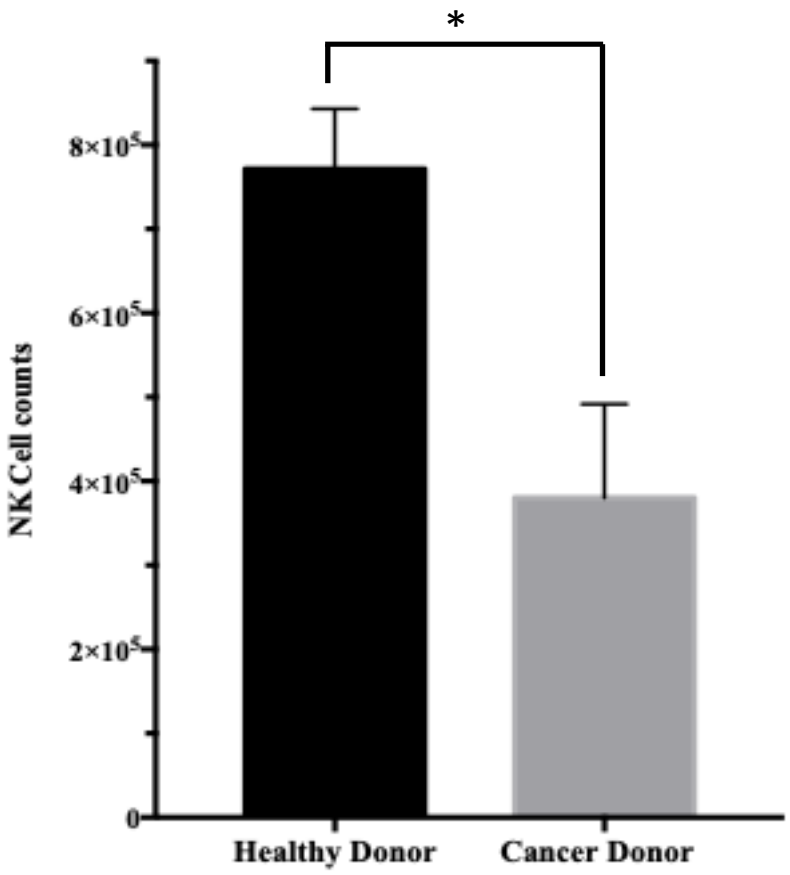
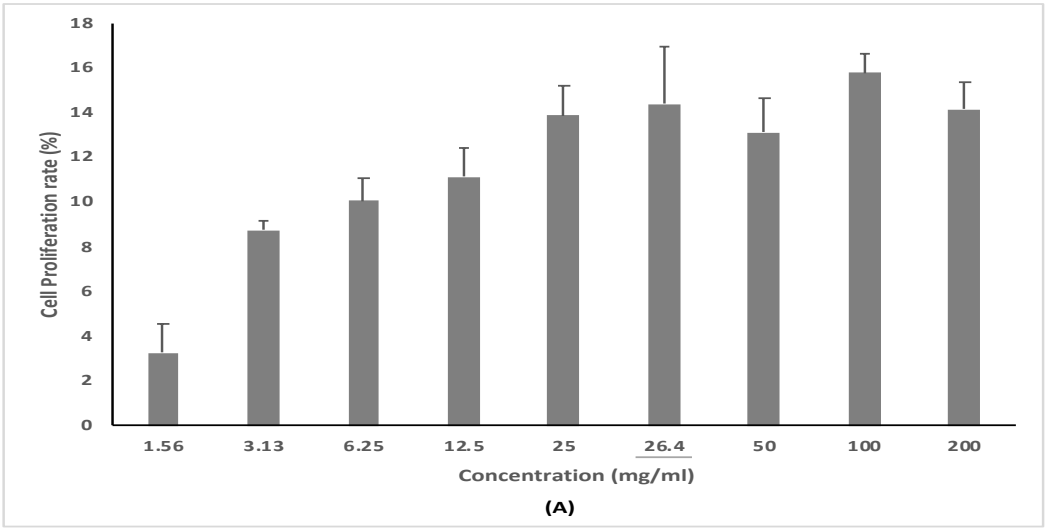


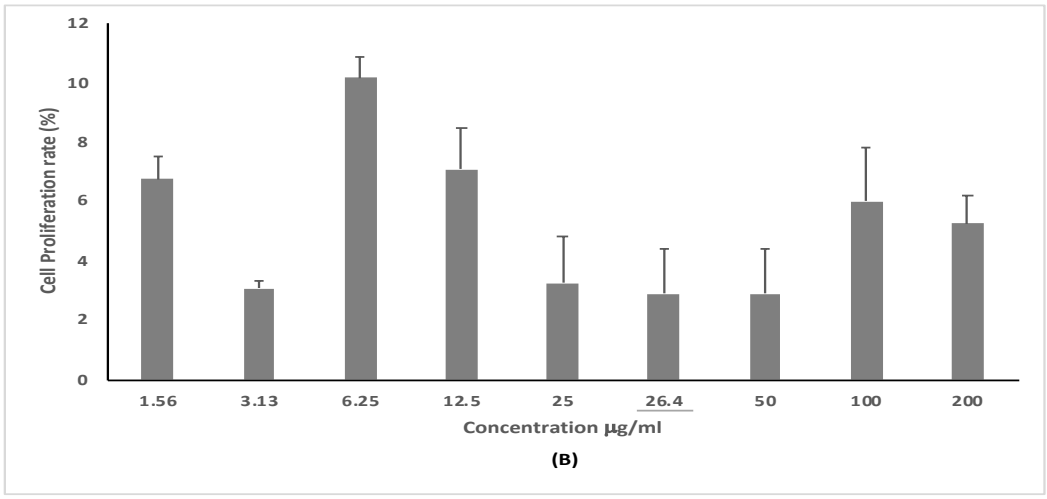

Figure 5.4: **NK cells counts isolated from healthy and cancer donor.**

The results were expressed as mean, ±SD of three independent donor for each group. P<0.05 is considered significance when comparing NK counts between healthy donor and cancer donor using unpaired t-test.

### 5.3.3 Ability of APME to induce NK cells proliferation

The ability of APME to induce NK cells proliferation, was analysed in this experiment. This was carried out in order to identify the optimum concentration of APME needed to be used in the subsequent analysis. APME with concentrations from 200µg/ml until 1.56µg/ml were added in the medium containing NK cells in order to observe the ability of the extract to induce NK proliferation. The APME at 26.4µg/ml was also added because it was the $IC_{50}$ value that was identified in previous chapter which was able to induce apoptosis in MDA-MB-231 cells. Isolated NK cells were seeded in a 96 wells plate and were incubated for 4h before adding the extract. Then, the cells were incubated for 24h, 48h and 72h. From Figure 5.5, it was found that the proliferation of NK cells was stable at 24h, and the proliferation pattern rate was fluctuating at 48h and 72h. This is because this NK cells were primary cells isolated from donors and no other stimulant was added to expand the cell line. The results also showed that at 24h, NK cells proliferation increased until 25µg/ml and 26.4µg/ml and started to decline at 50µg/ml and increase again at 100µg/ml. Since the cells proliferated well at APME $IC_{50}$ value, this concentration was chosen for the subsequent experiments since it was also used in the previous apoptosis experiments in Chapter 4.



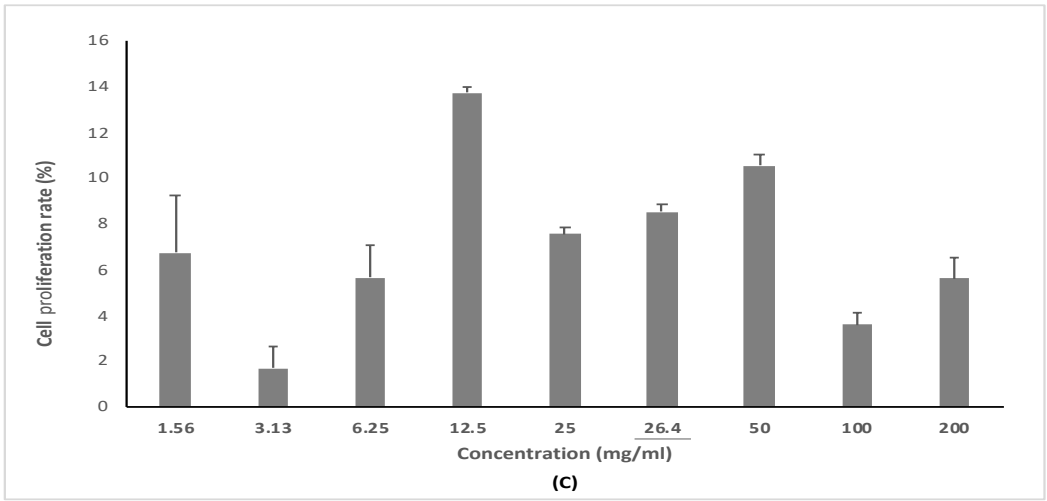




Figure 5.5: **NK cells proliferation treated with APME**

(A) 24h, (B) 48h, and (C) 72h. The results were expressed as mean, ±S.D of three independent experiments.

### 5.3.4 Effects of APME-induced NK cells on apoptosis of breast cancer cell MDA-MB-231; and measurement of cytokines and cytotoxic granules protein levels

NK cells from both healthy and cancer donor were treated in the same manner as listed in Table 5.1. NK Cells was incubated with APME (26.4µg/ml) for 4h prior to treatment on the MDA-MB-231 cells. Then this culture was further incubated for 20h. After the incubation period, the media were collected for cytokines and cytotoxic granules protein assay by ELISA and the remaining cells were harvested for CD56/CD3 staining and apoptosis assay with Annexin-V/PI staining. Figure 5.6 demonstrated the forward and side scatter of the MDA-MB-231 cells and NK cells population in the sample following co-culture experiment, acquired by flow cytometry. The size of the MDA-MB-231 cells were bigger compared to the size of the NK cells.

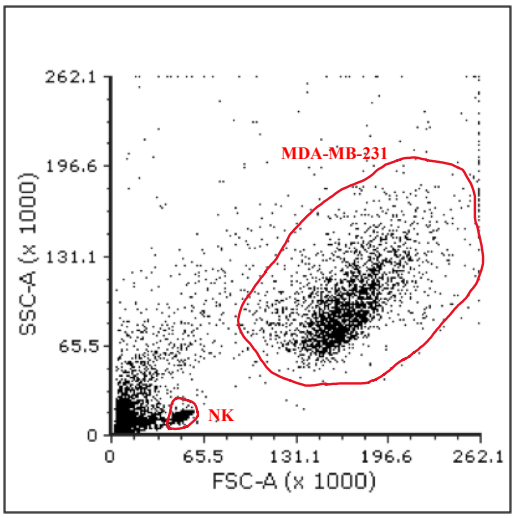



Figure 5.6: **The forward and side scatter of NK cell co-culture with MDA-MB-231 cells .**

Gating of the NK cell population and MDA-MB-231 cell population were marked in the circles.

- FSC (Forward Scatter)
- SSC (Side Scatter)

### 5.3.4(a) NK Cell Counts of Healthy and Cancer Donor after co-culture with MDA-MB-231 cells

Figure 5.7(A) showed the representative profiles of dot plot of NK cells counts in healthy donor and Figure 5.7(B) showed the results of the NK cells counts in different treatments in healthy donor. In healthy donor, NK cell count in Group 1 (NK/MDA-MB-231/APME) is 50.5±8.7%, Group 2 (NK/MDA-MB-231) is 37.6±5.5%, Group 3 (NK/MDA-MB-231/DMSO) is 40.0±9.7% and Group 4 (NK-Only) is 39.3±7.9%. NK cell count in Group 1 is significantly higher statistically when comparing to the rest of the groups. In cancer donor (Figure 5.8), the NK cell counts for all groups were slightly similar among each other except for Group 4 (NK-Only) which has a slight increase at 45.2±5.5%. NK cell counts for Group 1 (NK/MDA-MB-231/APME) is 41.9±5.0%, Group 2 (NK/MDA-MB-231) is 42.7±6.6% and Group 3 (NK/MDA-MB-231/DMSO) is 43.2±6.4%.

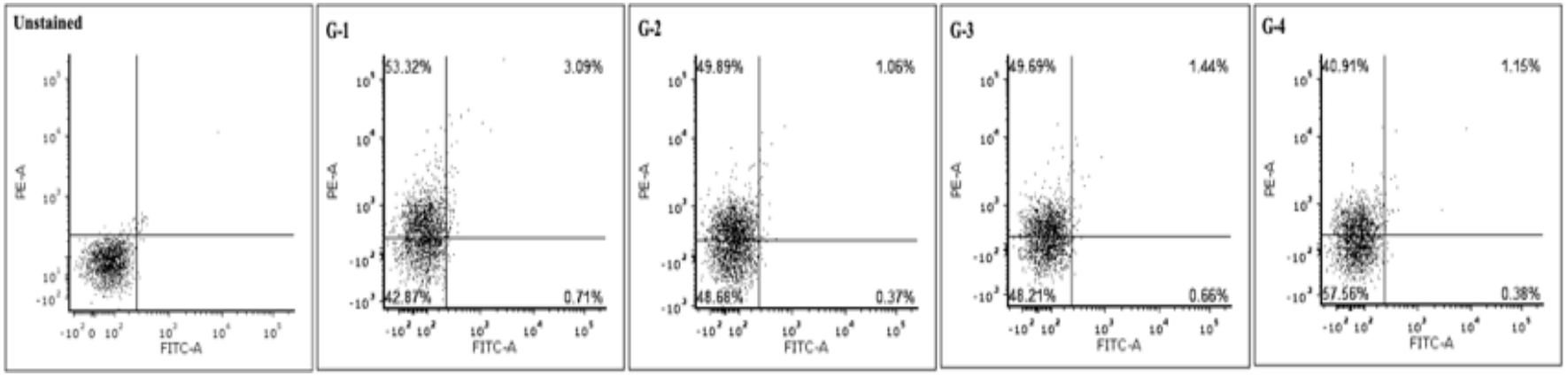
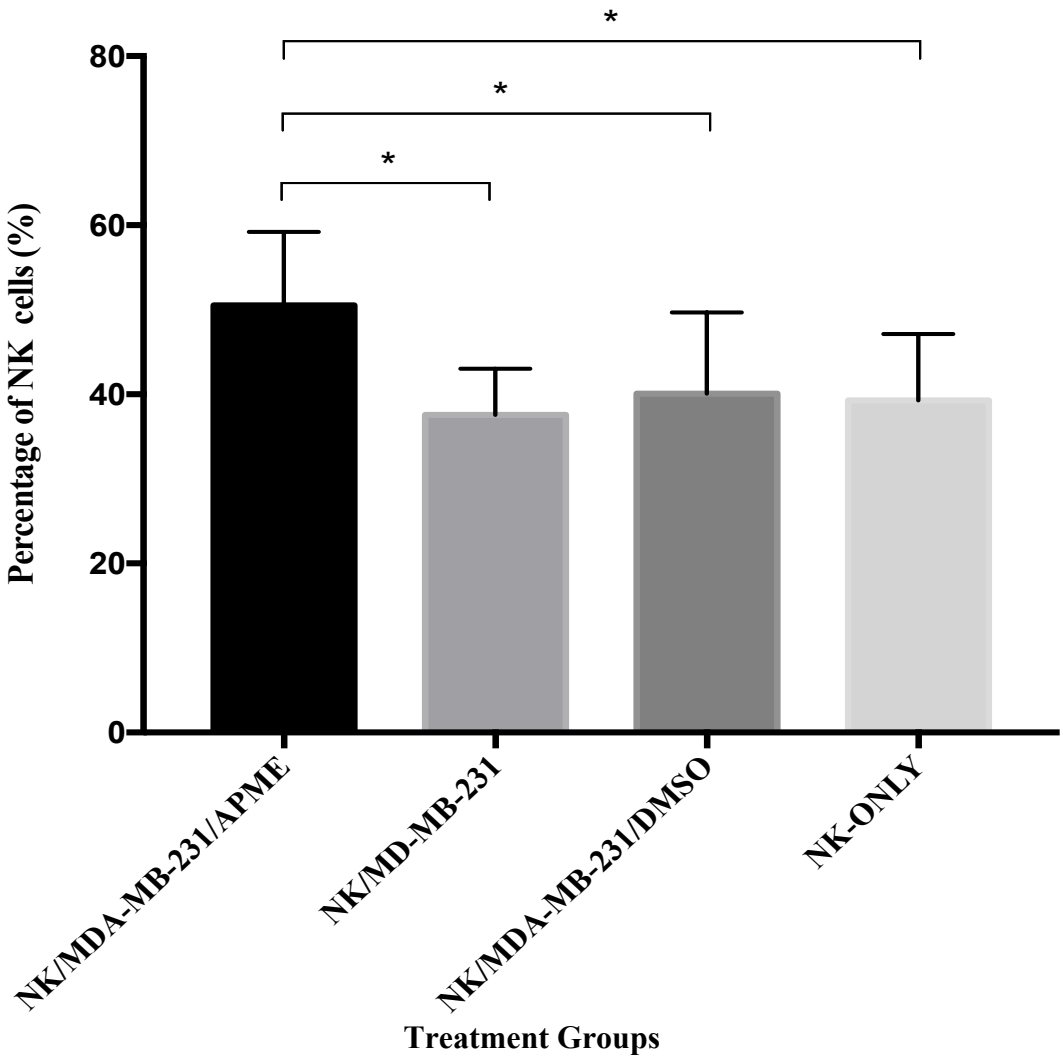



Figure 5.7(A): **Healthy donor NK cell counts in NK cell co-culture with MDA-MB-231 cells** .

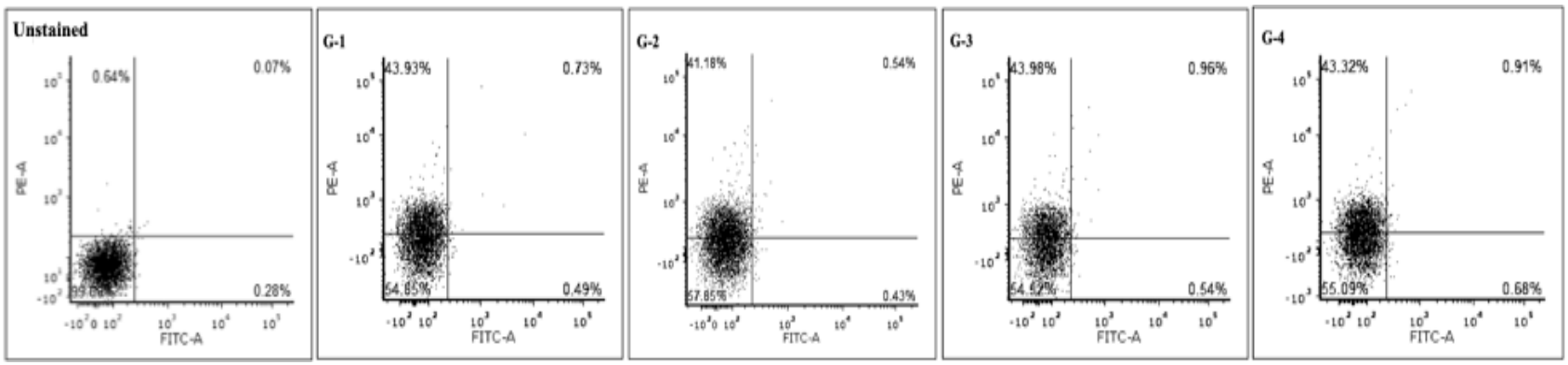
The representative dot plots where (G-1) NK/MDA-MB-231/APME, (G-2) NK/MDA-MB-231 (G-3) NK/MDA-MB-231/DMSO and (G-4) NK- ONLY. FITC-stained cells are represented at x-axis, while PE-stained cells at y-axis.




**(B)**

Figure 5.7(B): **Healthy donor NK cell counts in NK cell co-culture with MDA-MB-231 cells** .

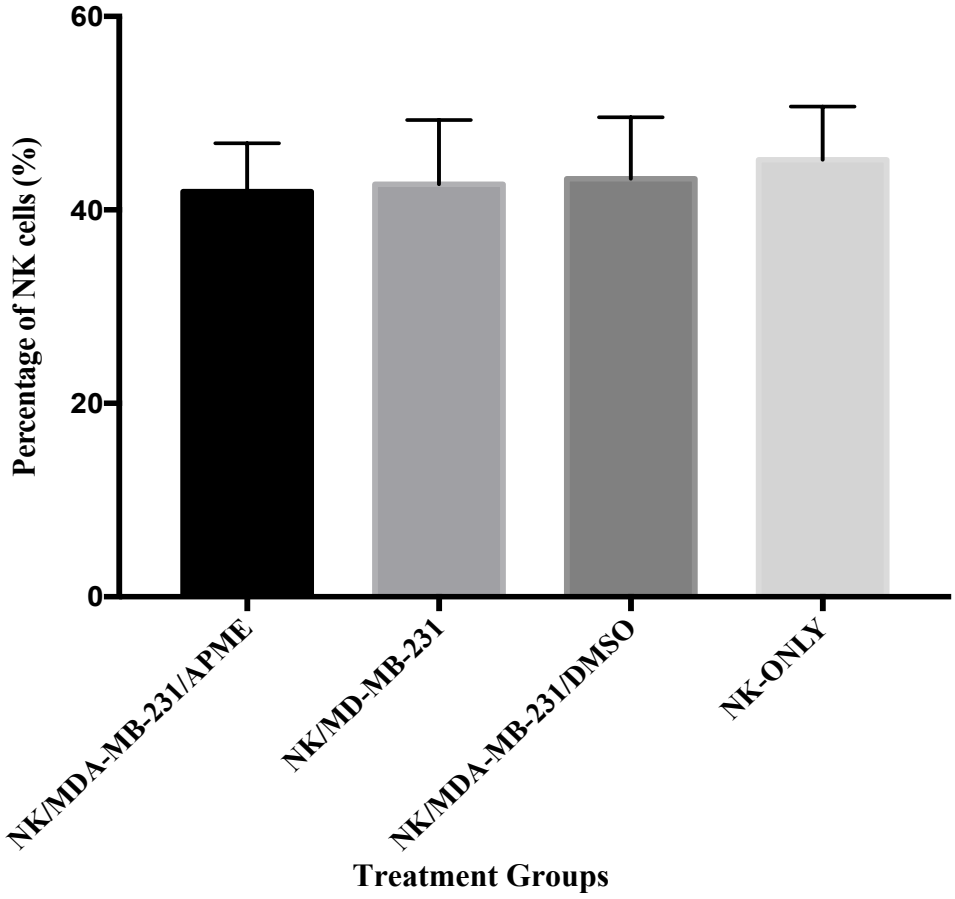
Graph representing the NK cell counts in Healthy Donor from the co-culture with MDA-MB-231 cells in different treatment groups. The results were expressed as mean, ±SD of three independent donor for each group and ($p<0.05$) is considered statistically significant.




**(A)**

Figure 5.8 (A): **Cancer donor NK cell counts in NK cell co-culture with MDA-MB-231 cells.**

The representative dot plots where (G-1) NK/MDA-MB-231/APME, (G-2) NK/MDA-MB-231 (G-3) NK/MDA-MB-231/DMSO and (G-4) NK- ONLY. FITC-stained cells are represented at x-axis, while PE-stained cells at y-axis.




**(B)**

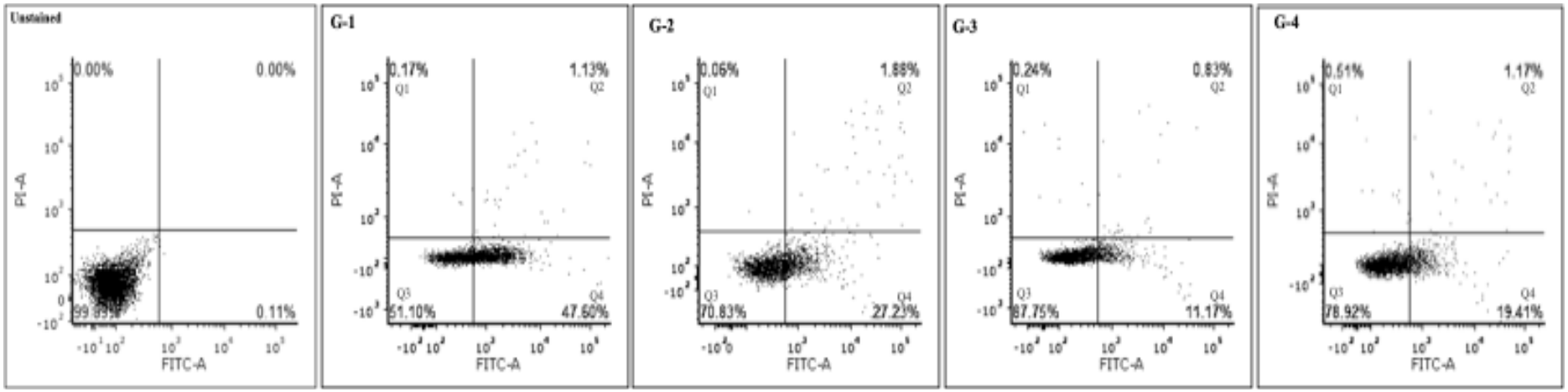
Figure 5.8 (B): **Cancer donor NK cell counts in NK cell co-culture with MDA-MB-231 cells.**

Graph representing the NK cell counts in Cancer Donor from the co-culture with MDA-MB-231 cells in different treatment groups. The results were expressed as mean, ±SD of three independent donor for each group and ($p<0.05$) is considered statistically significant.

### 5.3.4(b) Apoptosis Assay of MDA-MB-231 cells treated with APME-induced NK cells

The ability of APME-induced NK cells to promote apoptosis and cell death in MDA-MB-231 cells was assessed by staining with Annexin-V/PI. Figure 5.9(A) represents the dot plot of the treated MDA-MB-231 cells in healthy donor acquired by flow cytometry. Figure 5.9(B) shows the graph plotted from tabulated results of the apoptotic MDA-MB-231 cells following co-culture with NK cells from healthy donor. As presented in Figure 5.9(B), Group 1 (NK/MDA-MB-231/APME) showed 46.28±2.2% apoptotic cells, Group 2 (NK/MDA-MB-231) showed 30.50±1.8%, Group 3 (NK/MDA-MB-231/DMSO) showed 29.4±1.5% and Group 4 (APME/MDA-MB-231) showed 27.55±0.8%. These results showed that there were significantly higher apoptotic cells in Group 1 in the healthy donor category comparing to the rest of the groups in that category.

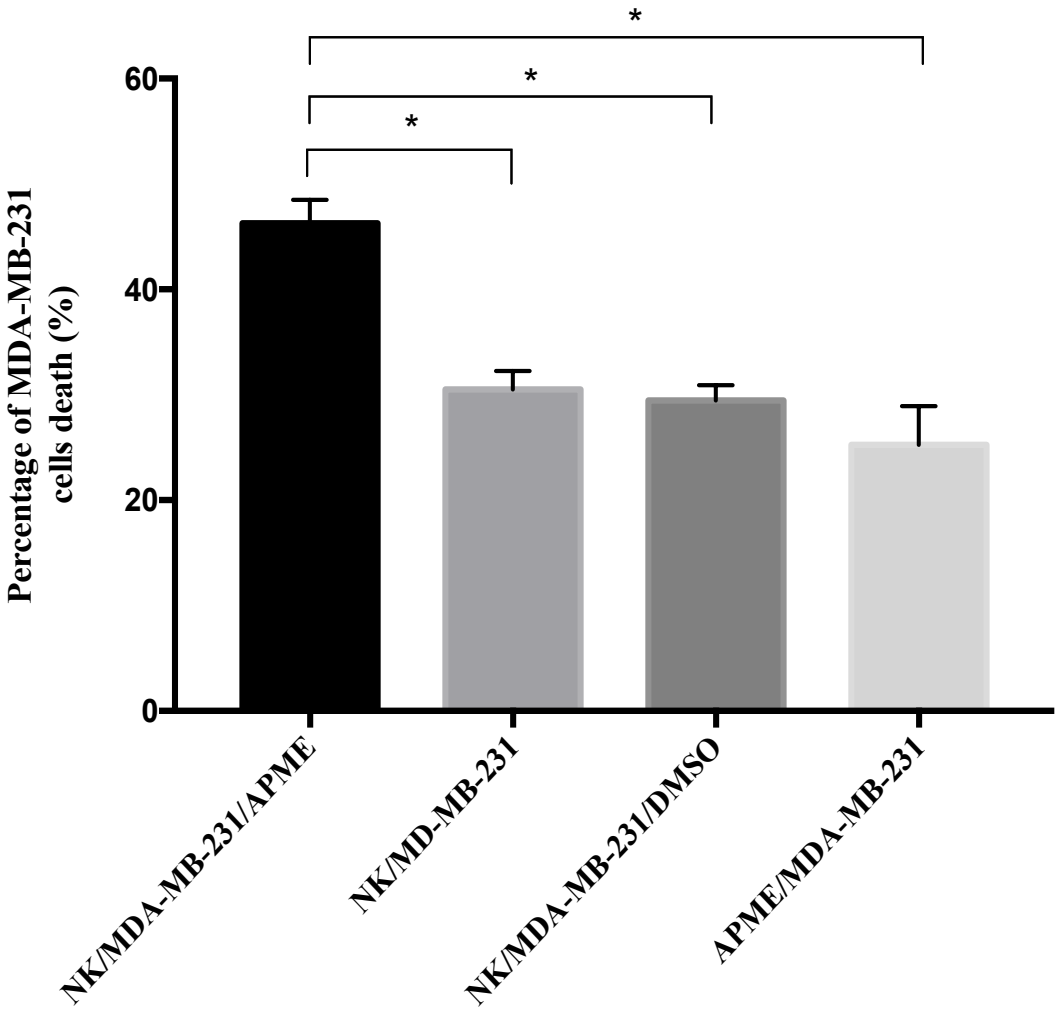
Figure 5.10(A) represents the dot plot of the treated MDA-MB-231 cells in cancer donor and Figure 5.10(B) shows the graph plotted from tabulated results of the apoptotic MDA-MB-231 cells following co-culture with NK cells from cancer donor. As presented in Figure 5.9(B), apoptotic cells in Group 1 (NK/MDA-MB-231/APME) was 26.32±6.0%, Group 2 (NK/MDA-MB-231) was 26.84±56.1%, Group 3 (NK/MDA-MB-231/DMSO) was 24.28±4.3% and Group 4 (APME/MDA-MB-231) was 20.68±2.0%. Group 1, 2 and 3, demonstrated higher apoptotic cell percentage comparing to Group 4. However, these differences were not statistically significant.




**(A)**

Figure 5.9(A): **MDA-MB-231 apoptotic cells from NK cell co-culture with MDA-MB-231 in Healthy Donor.**

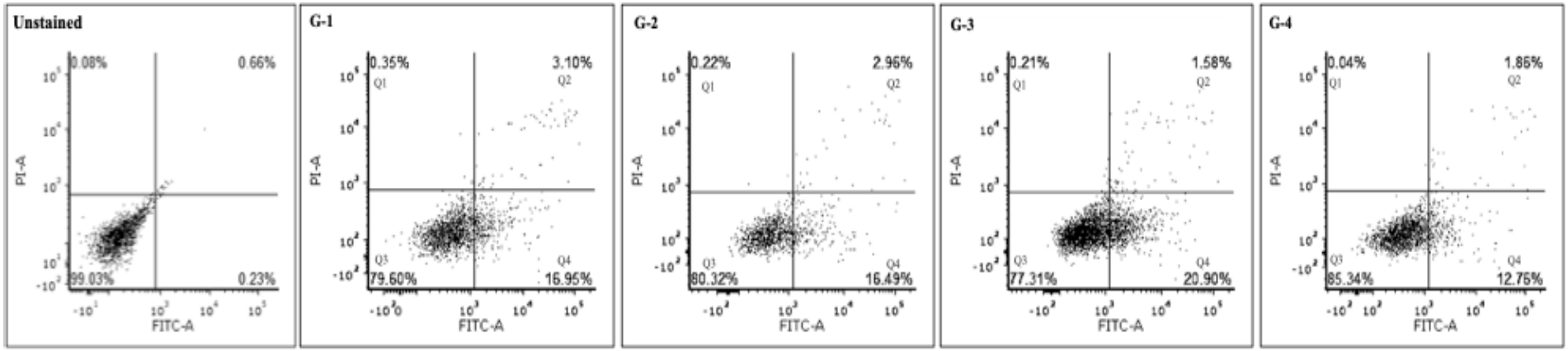
The representative dot plots where (G-1)NK/MDA-MB-231/APME, (G-2) NK/MDA-MB-231 (G-3) NK/MDA-MB-231/DMSO and (G-4) NK- ONLY.Quadrants represent the percentage of cell populations; Q1- Necrosis, Q2- Late apoptosis, Q4-Early apoptosis, Q3-Live cells.




**(B)**

Figure 5.9(B): **MDA-MB-231 apoptotic cells from NK cell co-culture with MDA-MB-231 in Healthy Donor.**

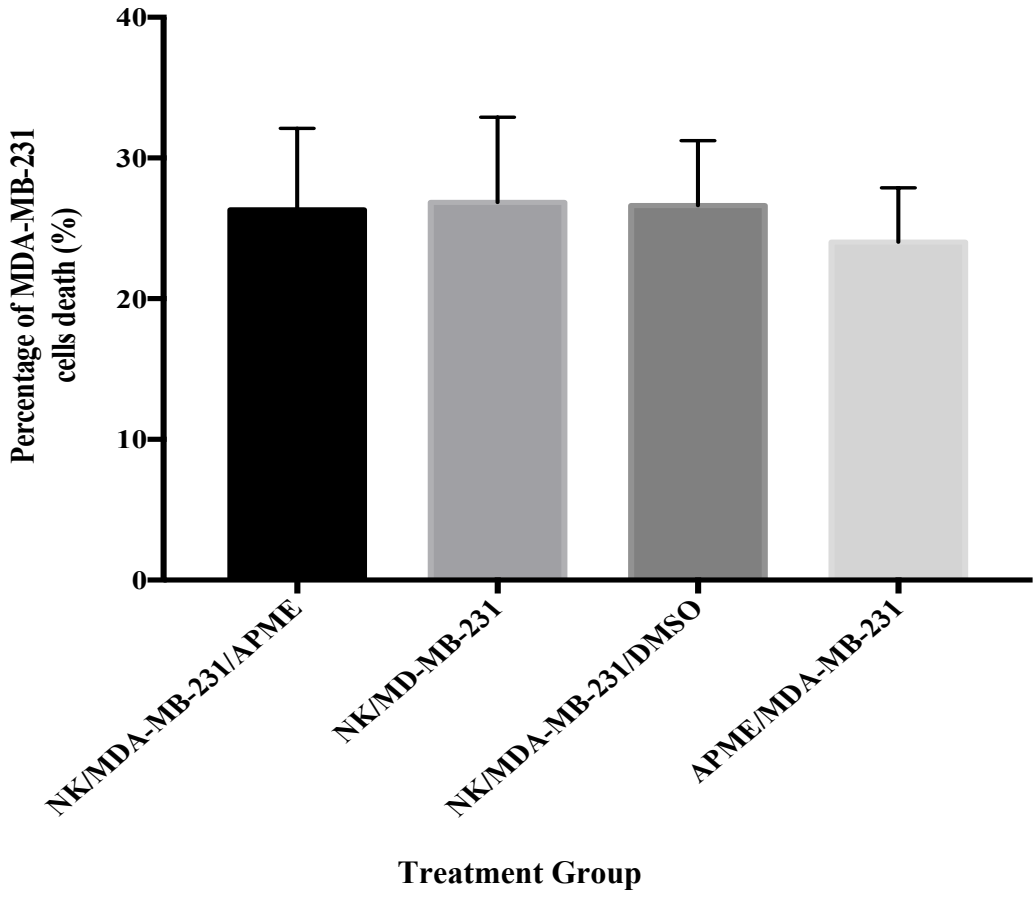
Graph representing the apoptotic cells of MDA-MB-231 co-culture with NK cells from healthy donor. The results were expressed as mean, ±SD of three independent donor for each group and ($p<0.05$) is considered statistically significant.




**(A)**

Figure 5.10(A) :**MDA-MB-231 apoptotic cells from NK cell co-culture with MDA-MB-231 in Cancer Donor.**

The representative dot plots where (G-1) NK/MDA-MB-231/APME, (G-2) NK/MDA-MB-231 (G-3) NK/MDA-MB-231/DMSO and (G-4) NK- ONLY.Quadrants represent the percentage of cell populations; Q1- Necrosis, Q2- Late apoptosis, Q3-Early apoptosis, Q4-Live cells.




**(B)**

Figure 5.10(B): **MDA-MB-231 apoptotic cells from NK cell co-culture with MDA-MB-231 in Cancer Donor.**

Graph representing the apoptotic cells of MDA-MB-231 co-culture with NK cells from cancer donor. The results were expressed as mean, ±S.D of three independent donor for each group and ($p<0.05$) is considered statistically significant.

Figure 5.11 showed the comparison of the target cell deaths between healthy and cancer donor. Percentage of the target cell death by APME-treated NK cells from the Healthy Donor were significantly higher than the cancer donor. This data inserted into the NK cell cytotoxicity formula gave the percentage of NK cell cytotoxic activity as shown in Figure 5.12. APME-treated NK cells from the healthy donor significantly showed higher percentage comparing to the other treatment groups and also between the APME-tretaed NK cells from the Cancer Donor.

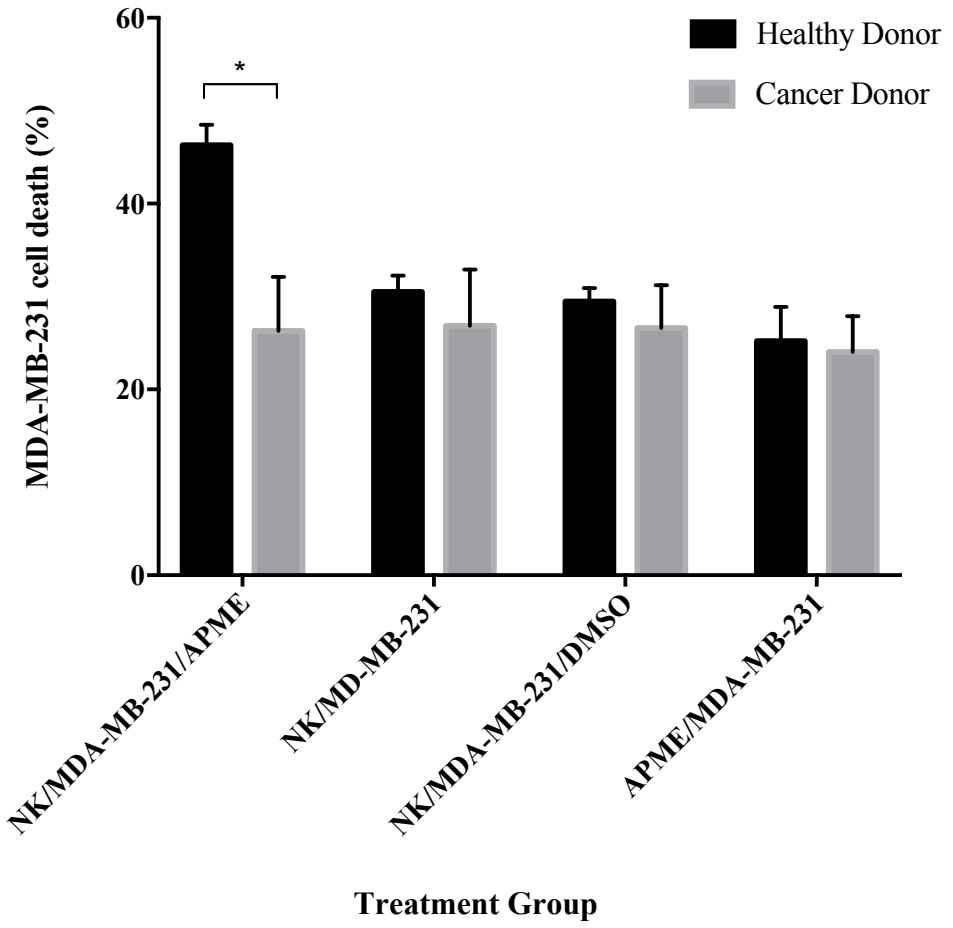



Figure 5.11: **MDA-MB-231 apoptotic cells from NK cell co-culture with MDA-MB-231 cells.**

Graph showing the comparison of MDA-MB-231 cell deaths between Healthy Donor and Cancer Donor. The results were expressed as mean, ±SD of three independent donor for each group and ($P$<0.05) is considered statistically significant, when comparing between healthy donor and cancer donor in each groups.

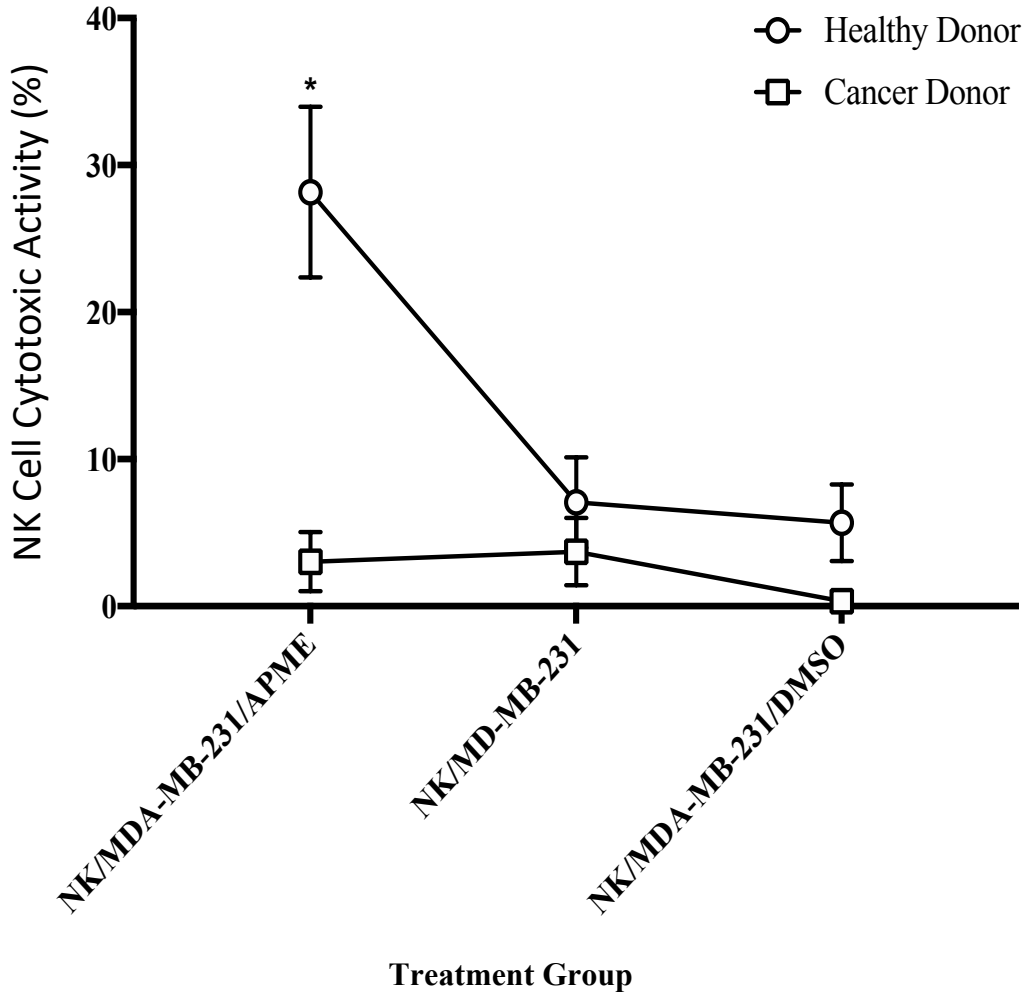



Figure 5.12: **Percentage of NK cells cytotoxic activity**

Graph showing the comparison of NK cells cytotoxic activity between Healthy Donor and Cancer Donor. The results were expressed as mean ±SD of three independent donor for each group and ($P$<0.05) is considered statistically significant when comparison was made between all groups.

### 5.3.4(c) IL-2, IFN-γ, PRF-1 and GzmB protein expression level following AMPE-induced NK cells co-culture with MDA-MB-231 cells

ELISA was performed to quantify the production of cytokines, interleukin-2 and interferon gamma (IFN-γ); and cytotoxic granule proteins (PRF-1 and GzmB) in the media collected following NK cells co-culture with MDA-MB-231 cells. Figure 5.13 presents the expression of interleukin-2 (IL-2), Figure 5.14 presents the expression of interferon gamma (IFN-γ), Figure 5.15 shows the expression of perforin (PRF-1) and Figure 5.16 presents the expression of granzyme B (GzmB).

In healthy donor, as presented in Figure 5.13(A), the IL-2 expression in Group 1 (NK/MDA-MB-231/APME) was 388.7±80.6pg/ml, Group 2 (NK/MDA-MB-231) was 353±56.5pg/ml, Group 3 (NK/MDA-MB-231/DMSO) is 356.9±57.33pg/ml, and Group 4 (NK-Only) is 8.13±0.8pg/ml. IL-2 expression in Group 1 was slightly higher but not significant than the expression in Group 2 and 3. In cancer donor, as presented in Figure 5.13(B), the IL-2 expression in Group 1 (NK/MDA-MB-231/APME) is 427.5±82pg/ml, Group 2 (NK/MDA-MB-231) is 436.9±118pg/ml, Group 3 (NK/MDA-MB-231/DMSO) is 439.1±71.7pg/ml, and Group 4 (NK-Only) is 9.09±0.8pg/ml. Figure 5.13(C) showed the comparison of IL-2 expressions between healthy donor and cancer donor in al treatment groups. The IL-2 expressions in cancer donor were higher but not significant in all treatment groups comparing to healthy donor.

Quantification of IFN-γ in healthy donor, as presented in Figure 5.14(A), in Group 1 (NK/MDA-MB-231/APME) was 812.5±82.3pg/ml, Group 2 (NK/MDA-MB-231) was 656.5.7±88.3pg/ml, Group 3 (NK/MDA-MB-231/DMSO) was 607.9±98.6pg/ml, and Group 4 (NK-Only) was 8.34±0.5pg/ml, however the value

obtained in Group 4 is below the lower cut-off point of the minimum concentration detected by the kit. Therefore, expression level in Group 4 is considered null or no expression. The expression level of IFN-γ was significantly higher in Group 1 of both healthy and cancer donor categories. In cancer donor, as presented in Figure 5.14(B), the expression of IFN-γ in Group 1 (NK/MDA-MB-231/APME) was $2.5x10^3 \pm 2.3x10^2$pg/ml, Group 2 (NK/MDA-MB-231) was $1.5x10^3 \pm 1.7x10^2$pg/ml, Group 3 (NK/MDA-MB-231/DMSO) was $1.4x10^3 \pm 4.5x10^2$pg/ml, and Group 4 (NK-Only) is 87.33±2.6pg/ml The expression IFN-γ in cancer donor for all treatment groups were significantly higher comparing to the expressions in healthy donor, as depicted in Figure 5.14(C).

Similarly to the cytokines expression analysis, the cytotoxic granule protein, perforin (PRF-1) and Granzyme B (GzmB) were all quantified using the ELISA. In healthy donor, as presented in Figure 5.15(A), the expression of PRF-1 in Group 1 (NK/MDA-MB-231/APME) is $5.7x10^3 \pm 3.3\ x10^2$pg/ml, Group 2 (NK/MDA-MB-231) is $4.6x10^3 \pm 8.5x10^2$pg/ml, Group 3 (NK/MDA-MB-231/DMSO) is $4.7x10^3 \pm 6.7x10^2$pg/ml, and Group 4 (NK-Only) is 248.8±12.6pg/ml, however, the value obtained in Group 4 was below the lower cut off point of the minimum concentration. Therefore, expression level in Group 4 is considered null. The expression level of PRF-1 was the highest in Group 1 and statistically significant comparing to the rest of the groups. In cancer donor, as presented in Figure 5.15(B), the expression of PRF-1 in Group 1 (NK/MDA-MB-231/APME) was $8.4x10^3 \pm 9.0x10^2$pg/ml, Group 2 (NK/MDA-MB-231) was $8.0x10^3 \pm 1.1x10^3$pg/ml, Group 3 (NK/MDA-MB-231/DMSO) was $7.7x10^3 \pm 7.5x10^2$ pg/ml, and Group 4 (NK-Only) is 299pg/ml, however, the value obtained in Group 4 was below the lower cut

off point of the minimum concentration of the standard curve. Therefore, expression level of PRF-1 in Group 4 is considered null. The expression level of PRF-1 was the highest in Group 1 from the cancer donor category however it was not statistically significant. The expression level of PRF-1 in cancer donor was overall significantly higher comparing to the corresponding expression level of the healthy donor as shown in Figure 5.15(C).

Another cytotoxic granule protein measured was the GranzymeB (GzmB). In healthy donor, as presented in Figure 5.16(A), the expression of GzmB in Group 1 (NK/MDA-MB-231/APME) was 14.82±1.7pg/ml, Group 2 (NK/MDA-MB-231) is 13.02±0.7pg/ml, Group 3 (NK/MDA-MB-231/DMSO) was 13.58±1.1pg/ml, and Group 4 (NK-Only) was 10.33±0.7pg/ml. In this category (healthy donor) Group 1 has the highest GzmB protein expression but it was not statistically significant. In cancer donor, as presented in Figure 5.16(B), the expression of GzmB in Group 1 (NK/MDA-MB-231/APME) was 12.57±2.4pg/ml, Group 2 (NK/MDA-MB-231) was 14.12±3.2pg/ml, Group 3 (NK/MDA-MB-231/DMSO) was 15.35±1.1pg/ml, and Group 4 (NK-Only) was 11.74±1.8pg/ml. In this category (cancer donor) Group 3 has the highest GzmB protein expression, followed by Group 2, Group 1 and Group 4. Nevertheless their GzmB protein concentration were all statistically insignificant. Similarly, the GzmB expression between healthy and cancer donor were all slightly similar and insignificant (Figure 5.16(C)).

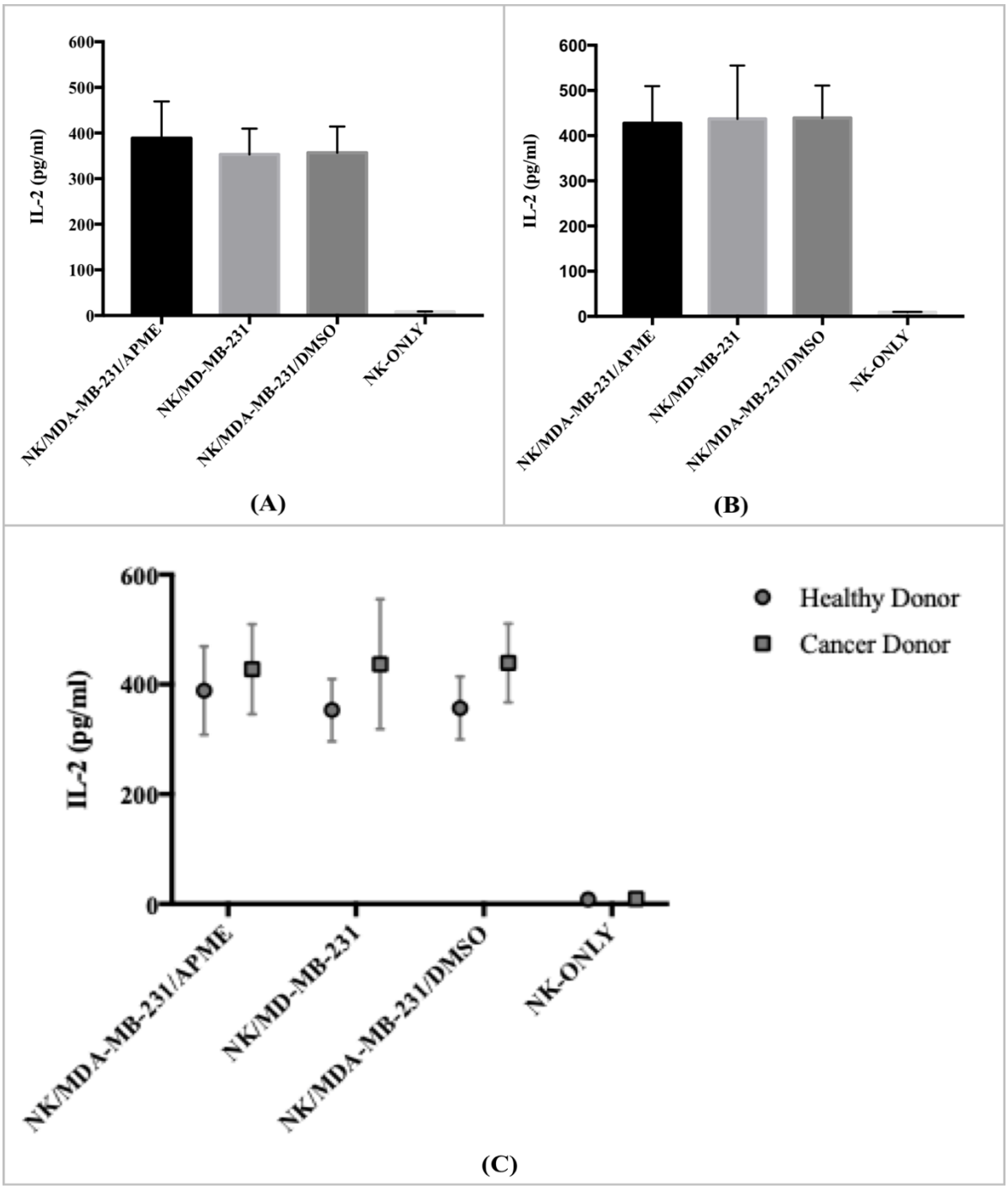



Figure 5.13: **The expression level of IL-2 in the co-culture experiment of NK cells with MDA-MB-231 cells.**

(A) The IL-2 expression level in Healthy Donor. (B) The IL-2 expression level in Cancer Donor. (C) General comparison of IL-2 expressions between Healthy and Cancer Donor. The results were expressed as mean, ±SD of three independent donor for each group.

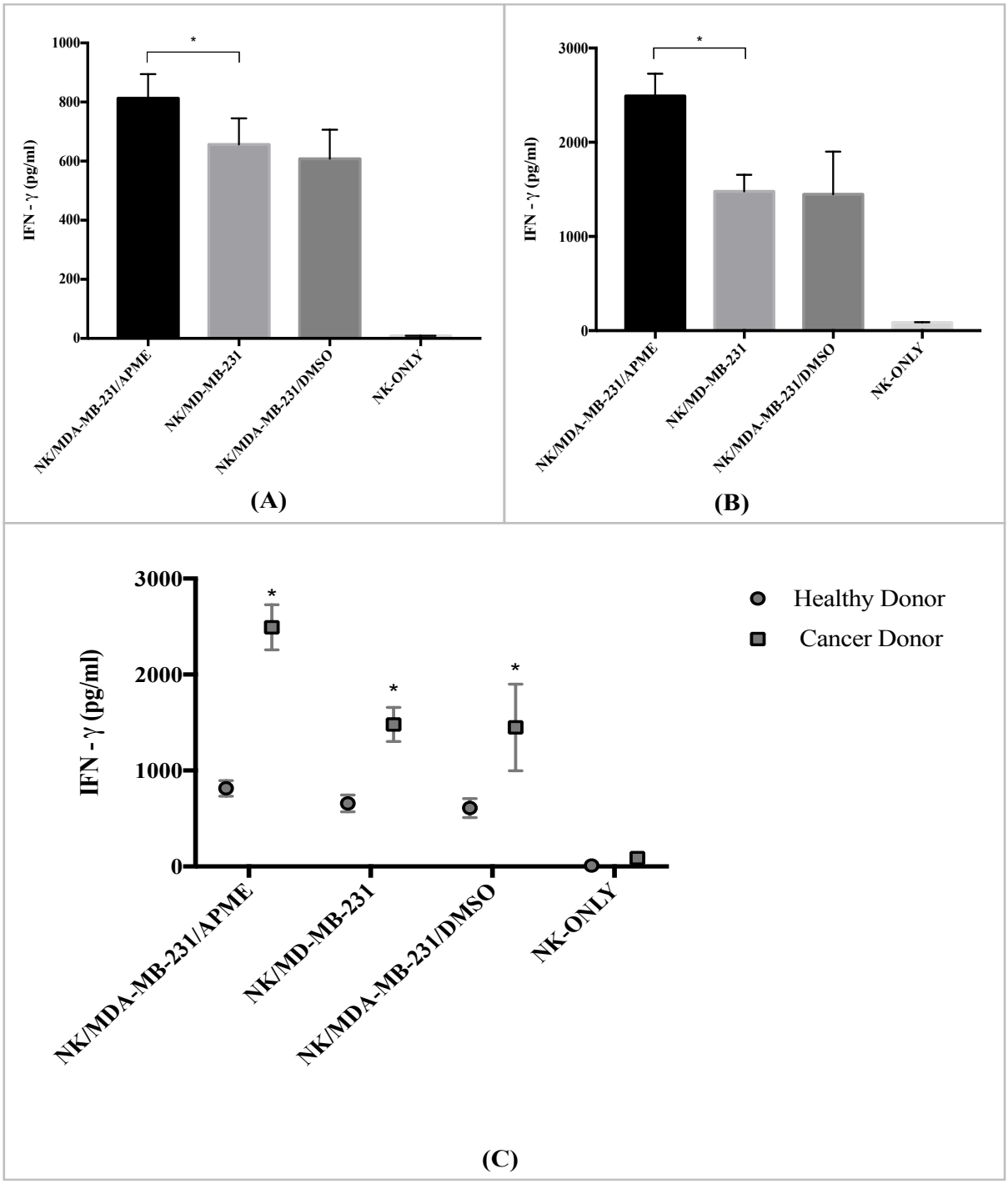



Figure 5.14: **The expression level of interferon gamma (IFN-γ) in the co-culture experiment of NK cells with MDA-MB-231 cells.**

(A)The IFN-γ expression level in Healthy Donor. (B) The IFN-γ expression level in Cancer Donor. (C) General comparison of IFN-γ expressions between Healthy and Cancer Donor. The results were expressed as mean, ±SD of three independent donor for each group and ($P$<0.05) is considered statistically significant, when comparing between groups in (A) and (B), and within the groups between healthy and cancer donor in (C).

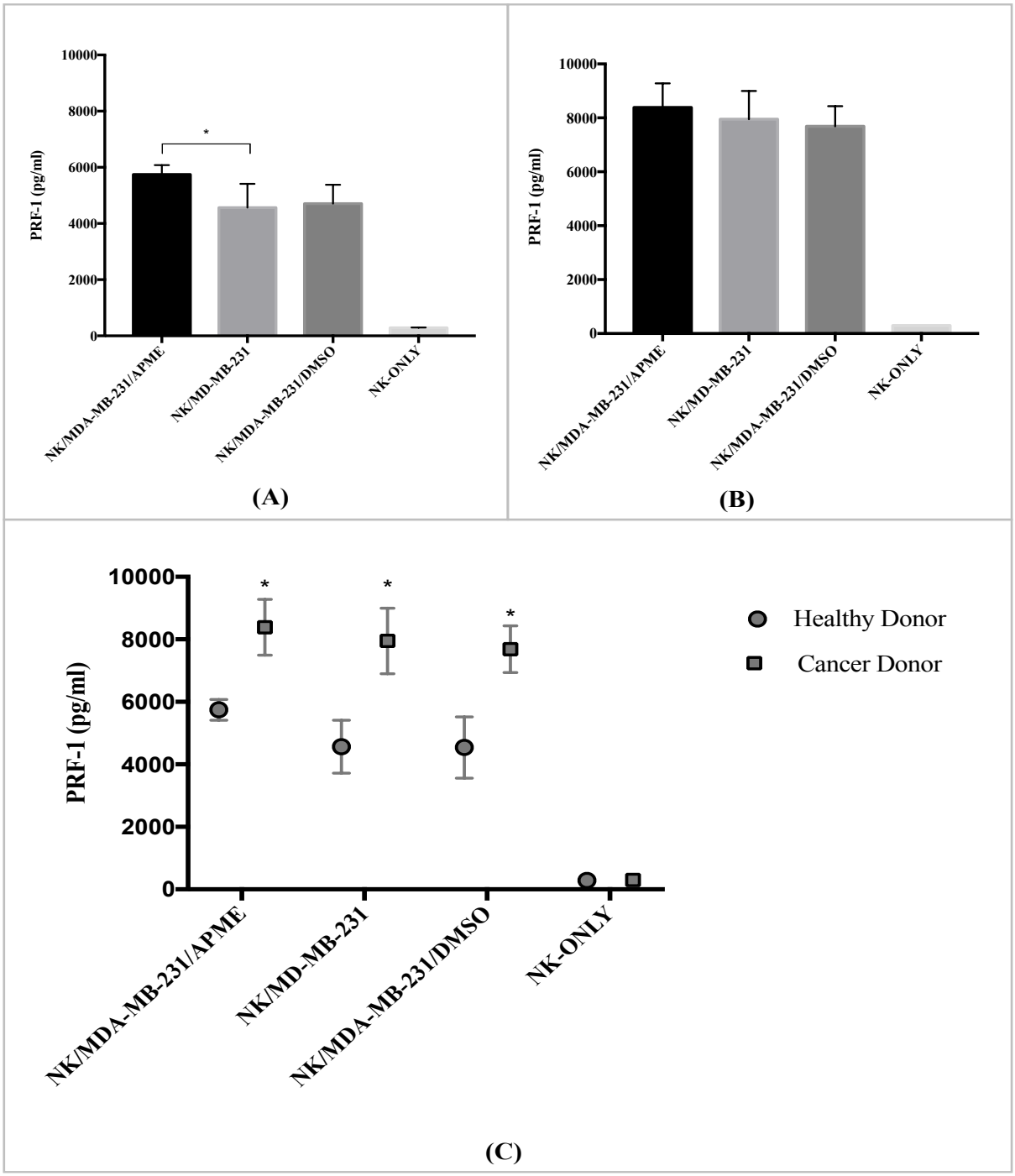



Figure 5.15: **The expression level of perforin (PRF-1) in the co-culture experiment of NK cells with MDA-MB-231 cells.**

(A) The PRF-1 expression level in Healthy Donor. (B) The PRF-1 expression level in Cancer Donor. (C) General comparison of PRF-1 expressions between Healthy and Cancer Donor. The results were expressed as mean, ±SD of three independent donor for each group and ($p<0.05$) is considered statistically significant, when comparing between groups in (A) and (B), and within the groups between healthy and cancer donor in (C).

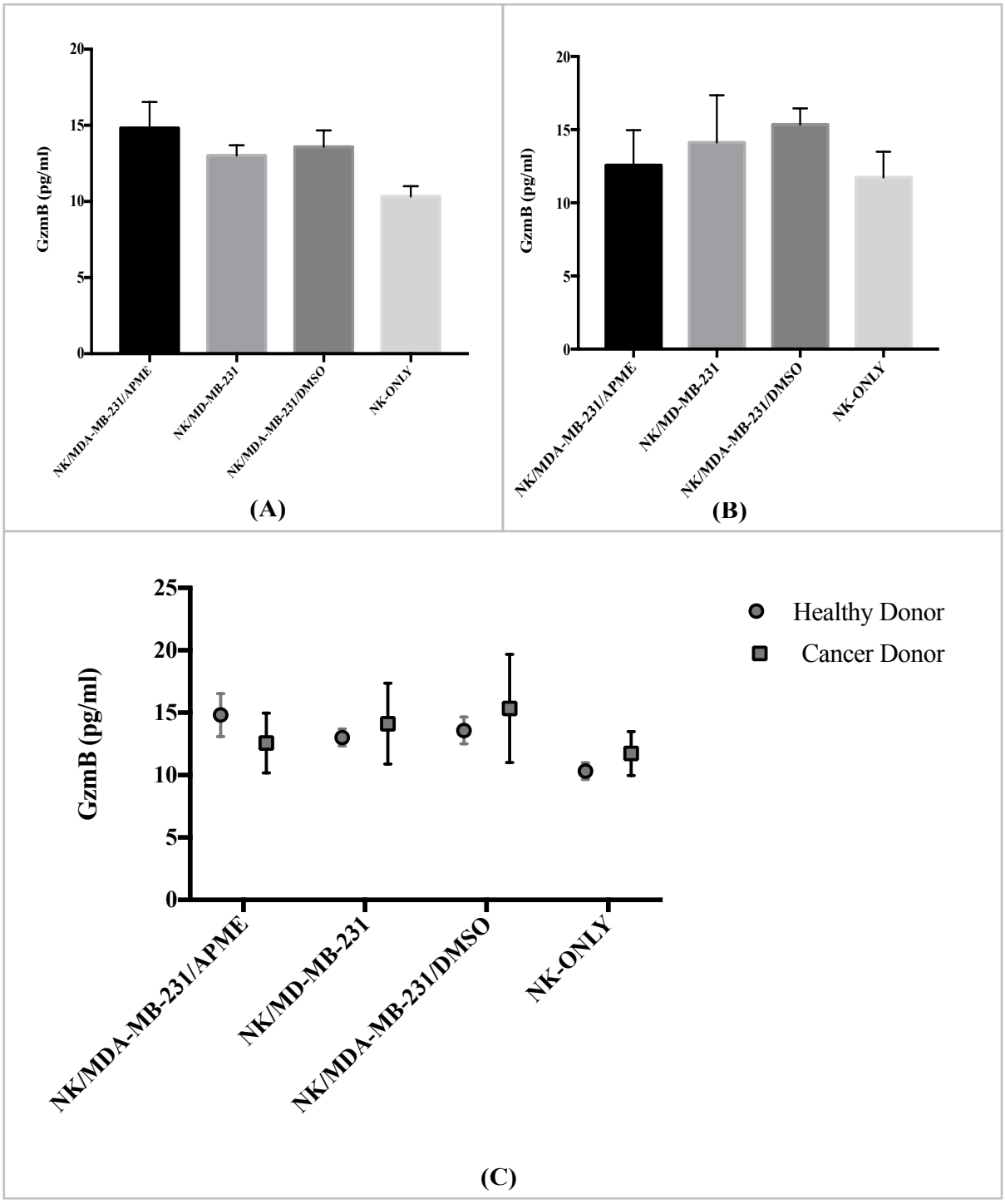



Figure 5.16: **The expression level of granzyme B (GzmB) in the co-culture experiment of NK cells with MDA-MB-231 cells.**

(A) The GzmB expression level in Healthy Donor. (B) The GzmB expression level in Cancer Donor. (C) General comparison of GzmB expressions between Healthy and Cancer Donor. The results were expressed as mean, ±S.D of three independent donor for each group and ($p<0.05$) is considered statistically significant.

## 5.4 DISCUSSION

Previous chapter have reported that *A. precatorius* methanol leaves extract (APME) was able to induce apoptosis in MDA-MB-231 cells. NK cell activation can be measured by cytototoxic analysis of the target cell death, quantification of soluble target cell death markers, quatification of cytokine relased upon NK cell activation and finally the evaluation of the degranulation indicators (Baran *et al.*, 2001; Blom and Albers, 2009; Rudnicka *et al.*, 2015; Wang *et al.*, 2010a). This chapter reported on the ability of APME to activate the NK cells activity upon co-culture with MDA-MB-231 cells. NK cells used in this study were freshly isolated from donors, three from healthy and three from cancer patients. This was done to observe if APME would have any effect on NK cells activation, thus only three healthy donors and three cancer donors were chosen. These donors were selected in accordance with the ethics approval guideline.

In the previous apoptosis study, APME was found to induce apoptosis in MDA-MB-231 cells, a breast cancer cell line, hence the donors chosen for this NK study were all females. Healthy donors must not have any known disease and must be healthy at the time of blood withdrawal. While cancer donors were females with diagnosed breast cancer but have not undergone any treatments. These three cancer donors were on the waiting list to undergo chemotherapy at the time of blood donation. They were at different stages of the disease where two of the donors were at stage 4 and one was at stage 3. NK cells were isolated from the peripheral blood using the negative sorting NK isolation kit from Miltenyi Biotec. This kit applied the concept of negative sorting that allows the tagging of non-NK cells and collect NK cells in the flow through. The collected NK cells were tested for purity by staining with CD56-PE conjugated and CD3-FITC conjugated, measured by flow cytometry. NK cells are

known to express $CD56^+$ and $CD3^-$. Lacking of CD3 expression differentiate NK from T lymphocyte (Grudzien and Rapak, 2018). By using this kit, about 85% of sample purity was achieved. A group recently published the acquisition of their isolated NK cells from PBMC was around 86% (Sugita *et al.*, 2018). Other study reported to obtain purification of NK cells up to 90% - 95% (Kanevskiy *et al.*, 2013), while another study managed to obtain around 60.6% (Klingemann and Martinson, 2004). By using the same kit, another study suggested the addition of MACS CD15 microbeads in order to reach purification until 98% by improving further depletion of granulocyte (Pesce *et al.*, 2017). In the study by Wang *et al.* (2017), only 34.4% of NK cells were able to be recovered and more granulocytes were present when using the kit. They also suggested addition extra microbeads in order to obtain higher sample purity.

As shown by Figure 5.2, the amount of NK cells isolated from the healthy donor were two folds higher than the amount of NK cells obtained from the cancer donor. An experiment on NK cells expansion from healthy and cancer donor showed that the NK cells counts in healthy donor was also higher than from cancer donor. The same trend was also reported where reduced numbers of NK cells from breast cancer patients were observed comparing to the healthy donor (Mamessier *et al.*, 2011; Shenouda *et al.*, 2017). Another study by Yunusova *et al.* (2018) also reported lower number of NK cells in the peripheral blood of ovarian cancer patients. Low NK cells counts in cancer patients could be the cause of tumour cells escape which eventually led to metastasis (Gulubova *et al.*, 2009). Other studies also revealed that cancer patients exhibiting advanced disease progression exhibited lower number of NK cells in their peripheral blood and normally have lower chance of survival (Ishigami *et al.*, 2000; Li *et al.*, 2016; Peng *et al.*, 2017; Tang *et al.*, 2020). NK cells from cancer patients exhibited inhibitory phenotype which is characterized by decreased activating

markers expression such as NKG2D and natural cytotoxicity receptors (NCRs). On the contrary, the inhibitory markers such as killer-cell immunoglobulin- like receptors (KIRs) and NKG2A were upregulated. These phenotype leads to the inability of NK cells from the cancer patients to exhibit cytotoxic activity comparing to the NK cells from healthy donors (Costello *et al.*, 2002; Pasero *et al.*, 2015).

Lymphocytes activation, survival, proliferation and differentiation are regulated by cytokines. Interleukin in particular IL-2, IL-15, IL-12, IL-18 and IL-21 are known to promote NK cells proliferation and improve their anti-tumour ability (Floros and Tarhini, 2015; Hu *et al.*, 2019; Srivastava *et al.*, 2013). However, in this study, freshly isolated unstimulated NK cells from healthy and cancer donor were used for the co-culture assay to measure the NK cells cytotoxicity with the target cells in the presence of plant extract or compound only (Ismail *et al.*, 2012). Therefore, prior to the co-culture assay with the target cell, the optimal concentration which APME could induce proliferation of NK cells was determined. The ability of APME to proliferate NK cells was evaluated by MTT assay (Lu *et al.*, 2016; Shpakova *et al.*, 2000; Zhang *et al.*, 1996). The experiment showed that the proliferation trends were stable at 24h and were unstable at 48h and 72h. At 24h, NK cell proliferation trend was seen to increase as the concentration increased. Thus $IC_{50}$ value of APME was chosen for the NK cells co-culture with MDA-MB-231 cells.

The ability of APME to induce NK cell cytotoxicity was assessed in the experiment of NK cells co-culture with MDA-MB-231 cells, in the presence of APME. NK cells were the effector and MDA-MB-231 cells were the target. The ability of APME to promote effector to induce cytotoxicity on the target cell was the main aim for this experiment. Three parameters were measured in this experiment, the first one was the effect on NK counts following the incubation; secondly the effect on cell death

measured by apoptosis AnnexinV/PI assay; and thirdly the concentration of interleukin-2 (IL-2), interferon gamma (IFN-γ), perforin (PRF-1) and granzyme B (GzmB).

Following the co-culture incubation, percentage of NK cells was determined by observing CD56/CD3 expression. In healthy donor, percentage of NK cells was significantly higher in the APME-treated NK cells comparing to the rest. However, no significant differences can be seen in the percentage of NK cells from cancer donor. At this point, the difference between APME-treated and untreated NK cells was only observed in the healthy donor. To evaluate the ability of APME to induce cytotoxicity, the cell death was measured by the apoptosis staining with AnnexinV/PI. The advantage of this staining is that the NK cytotoxic activity that caused target cell apoptosis or death could be determined.

In this study, APME-treated NK cells from healthy donor was able to induce a significant MDA-MB-231 cell death comparing to the non-treated NK cells. While in cancer donor, the percentage of cell deaths in all treatment groups were indifference. Especially the APME-treated NK cells from healthy donor exhibited significant cytotoxicity comparing to APME-treated NK cells from cancer donor. This is supported by the percentage of NK cytotoxicity which was calculated based on a formula as described previously (Mhatre *et al.*, 2014; Nishimura *et al.*, 2017). These values were obtained simply by deducting the value presented by non-effector treatment, which was the APME incubation with MDA-MB-231 cells only.

Overall, from these values, APME-treated NK cells from the healthy donor definitely has the highest percentage of NK cell cytotoxic activity comparing to the rest of the treatment groups, and the percentage of NK cells cytotoxic activity from

healthy donor were higher comparing to the NK cells from cancer donor. This demonstrated that APME was able to further promote NK cell cytotoxic activity in NK cells obtained from the healthy donor but not from the cancer donor. NK cells count from cancer patients were not only lesser comparing to the healthy donor as previously described (Mamessier *et al.*, 2011; Shenouda *et al.*, 2017), but these endogenous NK cells also have less cytotoxic activity might be due to the alteration of the NK cells receptors including decreased inhibitory receptors or/and increase activating receptors (Guillerey *et al.*, 2016). Because of that, in immunotherapy treatment involving NK cells, immune activation is facilitated by addition of cytokines and antibodies to sensitize the NK cells to assist its modulation towards the anti-tumour response (Hu *et al.*, 2019)

Medium from the co-culture experiment was collected to quantify the secretion of interleukin-2 (IL-2), interferon gamma (IFN-γ), perforin (PRF-1) and granzyme B (GzmB). Levels of IL-2 secretion in all treatment groups from both healthy and cancer donor showed insignificance differences. Presence of IL-2 indicated that the isolated NK cells were contaminted with other IL-2 secreting lymphocytes because the purity of NK cells obtained in this study was less than 90%. IL-2 is a 15K-kDa cytokine mainly produced by activated $CD4^+$/ $CD8^+$ T cells, however, there were reports on the scretion of IL-2 by dendritic cells, and NK cells (Gaffen, 2001; Granucci *et al.*, 2001). Nevertheless, the relevance of the IL-2 production by these cells remains ambiguos (Malek, 2008).

The results obtained showed that the level of IFN-γ production was significantly increased in APME-treated NK cells. These production of IFN-γ marked the activation of NK cells. IFN-γ, is the cytokine that mainly important in modulating

host immune response in particular controlling the spread of pathogen infection and defence against cancers. IFN-γ is also released by activated TH1 cells, $CD8^+$ T cells, and NK T cells, besides NK cells (Mah and Cooper, 2016; Schoenborn and Wilson, 2007). NK cells producing high level of IFN-γ exhibited low cytotoxicity while on the contrary, cytotoxic NK cells demonstrated low level of IFN-γ production (Björkström *et al.*, 2016; Caligiuri, 2008). This coincide with the results of this current study where even in extremely high level of IFN-γ expression found in cancer donor comparing to healthy donor, the cytotoxicity activity of NK cells from healthy donor were higher. It was also reported that $CD56^{dim}CD16^+$ NK cells were able to produce IFN-γ during their cytolytic activity. Activated NK cells producing intrinsic cytokines such as IFN-γ and TNF-α promotes target cell lysis and neutralization of these cytokines caused impaired function of the cell lysis (Wang *et al.*, 2012). From these results, APME was found to be able to stimulate NK cells from both healthy and cancer donor by producing higher IFN-γ levels comparing to non-tretated NK cells.

Perforin (PRF-1) and granzyme B (GzmB) are two granule proteins released by NK cells upon activation by stimuli from the target cells. The synergistic effect of PRF-1 and GzmB leads to the lysis of the target cell. PRF-1 is a potent pore-forming protein that allows the entrance of cytotoxic proteases such as GzmB into the target cell cytoplasm. This pore-forming process by PRF-1 is crucial in order to ensure the granzyme proteases able to enter the cytoplasm of the target cells. Defects in this pathway, particularly lacking of the perforin molecule will result in human disorder termed as perforinopathies, which include immunoproliferative disease Familial Haemophagocytic Lymphohistocytosis (FHL) type 2, haemotological malignancies and protracted viral infections (Spicer *et al.*, 2017). In this current study, the PRF-1 expression was significantly high in the APME-treated NK cells from healthy donor

and similar trend can be seen with its counterpart from the cancer donor, however this slight difference was insignificance. Nevertheless, the expression of PRF-1 in NK cells from the cancer donor were overall significantly increased comparing to the healthy donor. Even though the NK cells cytotoxic activity from cancer donor was lesser comparing to the NK cells from the healthy donor, their PRF-1 expression were extremely high. This was parallel to the production of the IFN-γ levels, where they were higher in NK cells from the cancer donor. This phenomena was probably caused by NK cells isolated from cancer donors have "memory" and were activated upon stimulation with the target cells. The review by Fehniger and Cooper (2016) mentioned that enhanced NK cells function was observed in antigen-specific stimulation and this is termed as "memory" or "memory-like" depending on how the NK cells were activated. NK cells memory would be beneficial use for anitumour immunotherapy, especially *in vitro* expansion capability (Capuano *et al.*, 2019).

GzmB induces cell deaths either through caspase-dependant or independent manner (Martinvalet, 2019). GzmB initially is expressed as an inactive pre-cursor protein that bears an N-terminal signal peptide. This terminal direct packaging of GzmB into secretory granules followed by removal of dipeptide Glysine and Glutamate by cysteine protease cathepsin C. Upon removal of those peptides, GzmB becomes activated and kept together in a lytic granules together with other granzymes and perforin. GzmB enters target cells after the activation of perforin, and instantly induce apoptosis either by caspase-dependant or independent manner (Afonina *et al.*, 2010). In this current study, it was found that GzmB were lowly expressed in both healthy and cancer donor. No conclusive remarks could be deduced from this finding because the detected GzmB expression levels in all groups were very low. Comparison between healthy and cancer donor also showed indifference expressions of GzmB.

AMPE-treated NK cells were unable to exhibit any significance differences in GzmB expression from this co-culture experiment. This experiment conducted *in vitro* might be the reason that very low amount of GzmB expressions were detected. Madakkannu and Ravichandran (2017) have successfully demonstrated that *Indigofera tinctoria* and Scoparia dulcis aqueous extracts were able to exhibit immunopreventive role in noise-stress rats by elevation of GzmB *in vivo*. Another study involving a plant known as sea buckthorn or *Hippophae rhamnoides* L. exhibited elevated level of GzmB and PRF-1 expression in chronic-stress rat model (Diandong *et al.*, 2016).

These results have demonstrated that APME was able to increase the proliferative capacity of NK cells, induce NK cell cytotoxic activity thus promoting cell death *via* apoptosis, and exhibited increased levels of IFN-γ and PRF-1 protein expressions by NK cells isolated from healthy donor. As for the cancer donor, although higher expressions of IFN-γ and PRF-1 were observed in comparison to the healthy donor, the NK cells cytotoxicity was impaired and APME was unable to further stimulate or better the activity of NK cells isolated from cancer donor. Therefore it is believed that in healthy individuals, APME could help promote their NK cells activity and this would benefit if *A. precatorius* could be introduce in their diet occasionally. Thus helping them to combat the emergence of cancer cells. However, this assumption needs to be scientifically proven.

## 5.5 CONCLUSION

In conclusion, it was found that APME was able to activate and promote better NK cells cytotoxic activity from healty donor but not in NK cells from cancer donor. It is suggested that more sample could be acquired especially from cancer donor in order to have better observation and conclusion. Probably details analysis of the donor such as their specific type of cells (example: breast – triple negative) will provide better understanding on this matter. Expansion of NK cells is also recommended to ensure better NK cells stability and viability. A better protocol to enhance NK purtity is also recommended such as using another specific microbeads tag to eliminate as much contaminant as possible. If screening a large number of donors, it is impossible to acquire large volume of blood. Therefore, another robust and simple method using MACSxpress® (Miltenyi) would solve this issue because isolation of NK cells is possible to achieve from as little as 2ml blood.

# CHAPTER 6

# GENERAL DISCUSSION, CONCLUSION AND FUTURE DIRECTION

## 6.1 GENERAL DISCUSSION

Since many decades or even centuries ago, human depends on medicinal plants for treatment of various health issues such as, high blood pressure, diabetes mellitus, infections, arthritis, and cancers. With advancement of the science and technology, the efficacy and mechanisms of the medicinal plants to treat these illnesses can be proven. Furthermore, scientific findings has also provided the identifications of bioactive compounds from these plants that have been tested *in vitro, in vivo* and even some are successfully tested in clinical settings and practices (Ortega and Campos, 2019). In this modern world, with high throughput research and technologies, many powerful drugs and treatment for cancer are established. However, many cancer patients are also relying on medicinal plants to help them battling with cancers and the side effect of chemotherapy. For that matter, it is crucial that more information on medicinal plants need to be studied in order to understand their effect and mechanisms of action.

The GC-MS phytochemical analysis of *A. precatorius* leaves extracted by different methods and different solvents in this current studies have exhibited various groups of phytocompounds including phenolic compound, terpenoid, sterols, vitamin, lipids, and carbohydrates. GC-MS is an interesting tool to use for the analysis of chemical compounds from medicinal plants, foods, cosmetics, drugs and pharmaceutical products (Kolb *et al.*, 2019). It is robust because the information obtained are based on identified compound in their libraries. Different solvents used in this study have different ability to extract different type of phytocompounds, and have also affected the extraction yield and the bioactive compound content. These

differences would affect the biological activity of the extract (Ngo *et al.*, 2017). Information of these phytochemicals profile is desirable because it can facilitate researcher to further explore any particular compound in relation to the bioactivity. Acknowledging these profiles in the beginning of any study on medicinal plants will avoid random sampling and selection of the plant organs and parts (Dirar *et al.*, 2019).

In this current study, the extracts obtained from different extraction processes were evaluated for their ability to inhibit cell proliferation. This was done by performing the MTT assay. Among the tested extracts, the *A. precatorius* methanol leaves extract (APME) obtained from Soxhlet extraction have exhibited the lowest $IC_{50}$ value on all cancer cells tested. This may be due to the presence of high phenolic and terpenoid compounds (Basli *et al.*, 2017; Huang *et al.*, 2012). Many other studies have demonstrated that the methanolic extract of the leaves of the medicinal plants tested showed high level of phenolic, flavonoid, alkaloid and terpenoid compounds (Kuppusamy *et al.*, 2015; Ruiz-Ruiz *et al.*, 2017; Truong *et al.*, 2019). Many dietary phenolics may block carcinogenesis through induction of apoptosis through different mechanisms. In vitro studies showed that curcumin, a phenolic compound, induced apoptosis through the modulation of the extrinsic pathway (Shankar *et al.*, 2007). Another phenolic compound, resveratrol, induced apoptosis via the intrinsic pathway in prostate cancer-derived cell lines by activation of caspase-9 and -3 (Benitez *et al.*, 2007). The anti-proliferative effect of this extract was the lowest on the MDA-MB-231 cells. However, this extract showed no toxicity on the normal cells used in this study.

Further confirmation of the anti-proliferative effect of APME was analysed by evaluating the cell cycle arrest and the ability to induce apoptosis. Various apoptosis protein markers have been evaluated in many studies to evaluate the ability of an

extract or compound to induce apoptosis. These proteins are involved in the cell cycle progression and apoptosis pathways and they include c-Myc, p53, Ras, pRb, protein kinase A (PKA), protein kinase C (PKC), CDK, Bcl-2 family proteins, cyclins and CK1. With stimulation, these proteins will able to promote or halt the proliferation of cells, induce cell cycle arrest or apoptosis (Vermeulen *et al.*, 2003). Most studies are looking into the ability of medicinal plants and their derived compound to induce cancer cell deaths but not the normal cell deaths. In this current study, APME has demonstrated to promote cell deaths through up regulation of p53 and Bax, which activated the apoptosis by increase Caspase-3 expression. APME were able to selectively induce cell deaths in cancer cells but not the normal cells.

Immunomodulation is a process of the immune system response to stimulation to prevent or suppress diseases. Immunomodulation mechanisms occur by macrophage activation, lymphoid stimulation, phagocytosis stimulation, enhancement of cellular immune function, increasing the production of antigen-specific immunoglobulin, increasing the non-specific immunity mediators and stimulation of the natural killer (NK) cells. Medicinal plants extracts that are able to facilitate the immunomodulation is desirable. Medicinal plants have not only demonstrated their ability to effect the target cells, but can also modulate, stimulate and activate the immune system. This ability is depending on the phytocompounds such as terpenoid, phenolics, polysaccharides, alkaloids, peptides, nucleotides and glycoproteins (Utami, 2019). As such, vitamin E has shown the ability to modulate the immune system especially the NK cells. Higher vitamin E content will increase the NK cells activity and lower vitamin E caused decrease NK cells activity (Mutalip, 2018).

In this study, the APME has shown the ability to increase NK cells activity from the healthy donor, while insignificance differences were observed with the NK

cells from the cancer donor. As outlined by (Hanahan and Weinberg, 2016) cancer cell exhibited eight hallmarks in order to survive, grow, proliferate, and metastasis. Therefore maintaining a healthy lifestyle might help our immune system to be able to perturb the formation of cancer. This includes eating foods that are rich in beneficial phytochemical. NK cells from healthy donor were able to be stimulated to induce cell deaths but not from the cancer donors. This shows that ingested phytochemical could halt the progression of cancer cells and tumour formation by stimulating the immune systems responses. Similarly as shown by Takeda and Okumura (2019), in a group of ten volunteers consuming 1000mg of ginseng per day for 14 days, showed no difference in NK cell size or CD56 expression but the cytotoxicity of the NK cells was significantly increased.

## 6.2 CONCLUSION

From the results obtained in this study it can be concluded that *A. precatorius* leaves extract possessed beneficial phytochemicals that are able to induce the anti-proliferative activity on cancer cells. It is found that the *A. precatorius* methanol extract from Soxhlet extraction showed the highest phenolic and terpenoid compounds while the ethyl acetate demonstrated the highest terpenoid compound. The methanol extract (APME) has shown the ability to moderately inhibit cancer cell proliferation in all cancer cells tested. APME was able to induce cell deaths in MDA-MB-231 cells *via* apoptosis. p53, Bax and Caspase-3 were activated and Bcl-2 protein was down-regulated. Finally, APME was also found to significantly promote NK cells (from healthy donor) cytotoxicity on MDA-MB-231 cells. NK cells activation was observed by the production of INF-γ and PRF-1. However, APME was unsuccessful to modulate NK cells activity obtained from the cancer donors *in vitro*.

These findings provide a better understanding of *A. precatorius* as an anticancer agent or better as chemopreventive agent. Different parts of *A. precatorius* have been widely studied for this bioactivity, especially the seeds. This current study adds the portfolio of the *A. precatorius* leaves. Since *A. preactorius* leaves has also exhibited higher sweetness profile than sucrose, it is recommended to utilise this plant as sugar substitute. Therefore, besides maintaining the "sweetness of life", *A. precatorius* could also maintain the general healthy state in human.

## 6.3 FUTURE RECCOMENDATION

Due to limited budget, this current study possesses many limitations therefore better approaches would give better findings of the study. Here are some future recommendations to improve the understanding of *A. precatorius* as an anticancer medicinal plant.

1. Study on the mechanism of cell death and NK activation of other extracts of *A. precatorius* exhibiting anti-proliferative effect.

2. Study on the ability of APME as a chemopreventive agent in animal models.

3. Study on other apoptosis proteins and markers such as cytochrome-c, caspase-9, and Fas ligand.

4. Increase the number NK cell donors especially from cancer patients in order to better understand the NK cell activity and provide a broader perspective.

5. Expand the isolated NK cells in order to have more stable NK cells.

6. Measure the effect of *A. precatorius* on NK cells directly in human by oral intake. Therefore, optimisation of the safe dosage per body weight need to be explored or the plant can also be consumed in the dosage as practised in traditional setting.

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# APPENDICES

## **Appendix A:** MCF10-A MEDIA RECIPE

**Expansion, subculturing and cryopreservation of MCF10a cells**

Complete growth medium reagents

1. DMEM F-12
2. 5% (v/v) horse serum
3. 10 µg/mL recombinant human insulin
4. 20 ng/mL recombinant human epidermal growth factor (hEGF)
5. 0.5 µg/mL hydrocortisone
6. 1% penicillin streptomycin

Subculturing reagents

1. Trypsin-EDTA (0.05% % (w/v) Trypsin/ 0.02% EDTA)
2. PBS: modified without calcium chloride and without magnesium chloride

Cryopreservation medium reagents

1. DMEM F-12
2. 5% (v/v) horse serum
3. 10 µg/mL recombinant human insulin
4. 20 ng/mL recombinant human epidermal growth factor (hEGF)
5. 0.5 µg/mL hydrocortisone
6. 10% (v/v) DMSO

Preparation of complete growth medium

- (DMEM F-12 + 5% (v/v) horse serum + 10 µg/mL recombinant human insulin + 20 ng/mL hEGF + 0.5 µg/mL hydrocortisone + 1% penicillin streptomycin)

The complete growth medium (500 mL) is prepared by aseptically combining:

- 25 mL horse serum, 1.25 mL recombinant human insulin (stock 4 mg/mL in 5 mL), 10 µl hEGF (stock 1 mg/mL), 5 ml hydrocortisone (stock 50 µg/mL) and 5 mL penicillin streptomycin to 463.74 mL of basal medium DMEM F-12.

**Appendix B:** LIST OF CHEMICALS / REAGENTS / KIT

| | |
|---|---|
| FITC-Annexin V Apoptosis Detection Kit 1 | BD Biosciences |
| BD Cyclotest Plus DNA | BD Biosciences |
| Lymhocyte Separation Media | Capricorn |
| Human IL-2 ELISA Kit | Elabscience |
| Human IFN-γ ELISA Kit | Elabscience |
| Human PRF-1 ELISA Kit | Elabscience |
| Human GzmB ELISA Kit | Elabscience |
| Trypsin EDTA | Gibco |
| Penicillin Strepromycin | Gibco |
| DMEM, powder, high glucose medium | Gibco |
| RPMI 1640 medium | Gibco |
| Horse Serum | Gibco |
| DMEM F12 | Gibco |
| Insulin human recombinant | Gibco |
| Hoechst 33258 | Invitrogen |
| Recombinant human epidermal growth factor | Invitrogen |
| MTT Formazan | Merk Milipore |
| n-hexane | Merk Milipore |
| Ethyl acetate | Merk Milipore |
| Methanol | Merk Milipore |
| MACS® BSA Stock Solution (NK Buffer*) | Miltenyi Biotec |
| autoMACS® Rinsing Solution (NK Buffer*) | Miltenyi Biotec |
| NK Cell Isolation Kit (human) | Miltenyi Biotec |
| Bax-PE (sc-7480) | SantaCruz |
| Bcl-2- Alexa Fluor 647 (sc-7382) | SantaCruz |
| p53- Alexa Fluor 488 (sc-126) | SantaCruz |
| Caspase-3 - Alexa Fluor 488 (sc-7272) | SantaCruz |
| NCAM (ERIC 1) - PE for CD56 | SantaCruz |
| CD3 - FITC | SantaCruz |
| PBS Tablets | Sigma |
| DMSO | Sigma |
| Hydrocortisone | Sigma |

*NK Buffer:

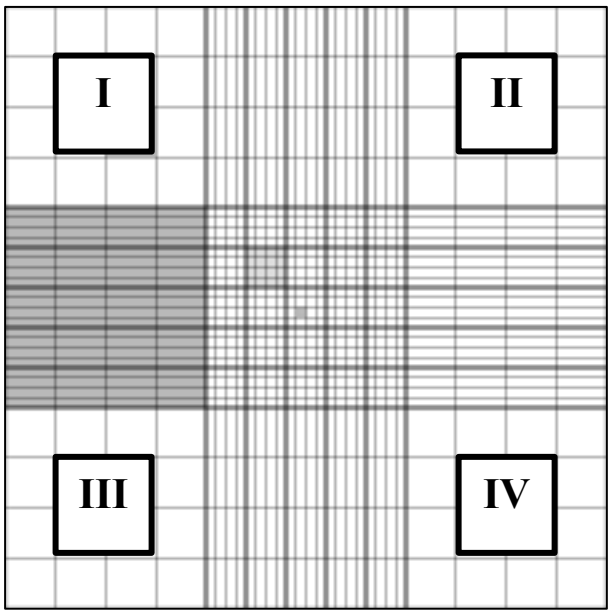
1:20 ratio of BSA: Rising Solution

**Appendix C :**CELLS USED IN THIS STUDY

| **Cell Name** | **Information** |
|---|---|
| HeLa<br>(Cancer) | Organism: Human<br>Tissue: Cervix<br>Cell type: Epithelial |
| MCF-7<br>(Cancer) | Organism: Human<br>Tissue: Marmary gland, breast; derived from metatastic site<br>Cell type: Epithelial |
| MDA-MB-231<br>(Cancer) | Organism: Human<br>Tissue: Marmary gland, breast; derived from metatastic site<br>Cell type: Epithelial |
| SW480<br>(Cancer) | Organism: Human<br>Tissue: Colon<br>Cell type: Epithelial |
| MCF10-A<br>(Normal) | Organism: Human<br>Tissue:Marmary gland, breast<br>Cell type: Epithelial |
| NIH (3T3)<br>(Normal) | Organism: Mouse<br>Tissue: embryo<br>Cell type: Fibroblast |

**Appendix D:** CELL COUNTING USING HEMOCYTOMETER

Cells were counted using the trypan blue exclusion assay. Following trypsinization, cells were centrifuged to obtain the cell pellet. The supernatant was discarded and the pellet was resuspended in 1ml complete medium. Then, 10µl of the cell culture was mixed with 10µl of 4% (w/v) trypan blue dye. 10µl of this mixture was decanted into the gap of the edge between the hemocytometer chamber and a cover slip. The cell suspension will diffuse into the calculating chamber as shown below.



Calculating chamber

Live cells were counted from each chamber **I**, **II**, **III**, and **IV**. Only live cells located in each chamber (16 small grid squares) were counted. Live cells can be differentiated from dead cells by the trypan blue colour. This dye will exclusively label only the dead cells.

Cell counting was performed under a light microscope at a 10X magnification. Total live cells were determined using the calculation formula as below:

$$\mathbf{C} = [(\mathbf{I} + \mathbf{II} + \mathbf{III} + \mathbf{IV}) \times ¼ ] \times \mathbf{df} \times \mathbf{10^4}$$

**C** = number of cells in 1ml (cells/ml)

**df**= dilution factor

**¼** = to obtain the mean of the cells in one area

$\mathbf{10^4}$ = volume of improved hemocytometer chamber

Therefore, to prepare $\boldsymbol{A} \mathbf{\times 10^4}$ cells, $\mathbf{N} = [(\delta \mathbf{\times 10^4} \times \mathbf{V})] / \mathbf{C}$

$\boldsymbol{A} \mathbf{\times 10^4}$ = number of cells needed for seeding

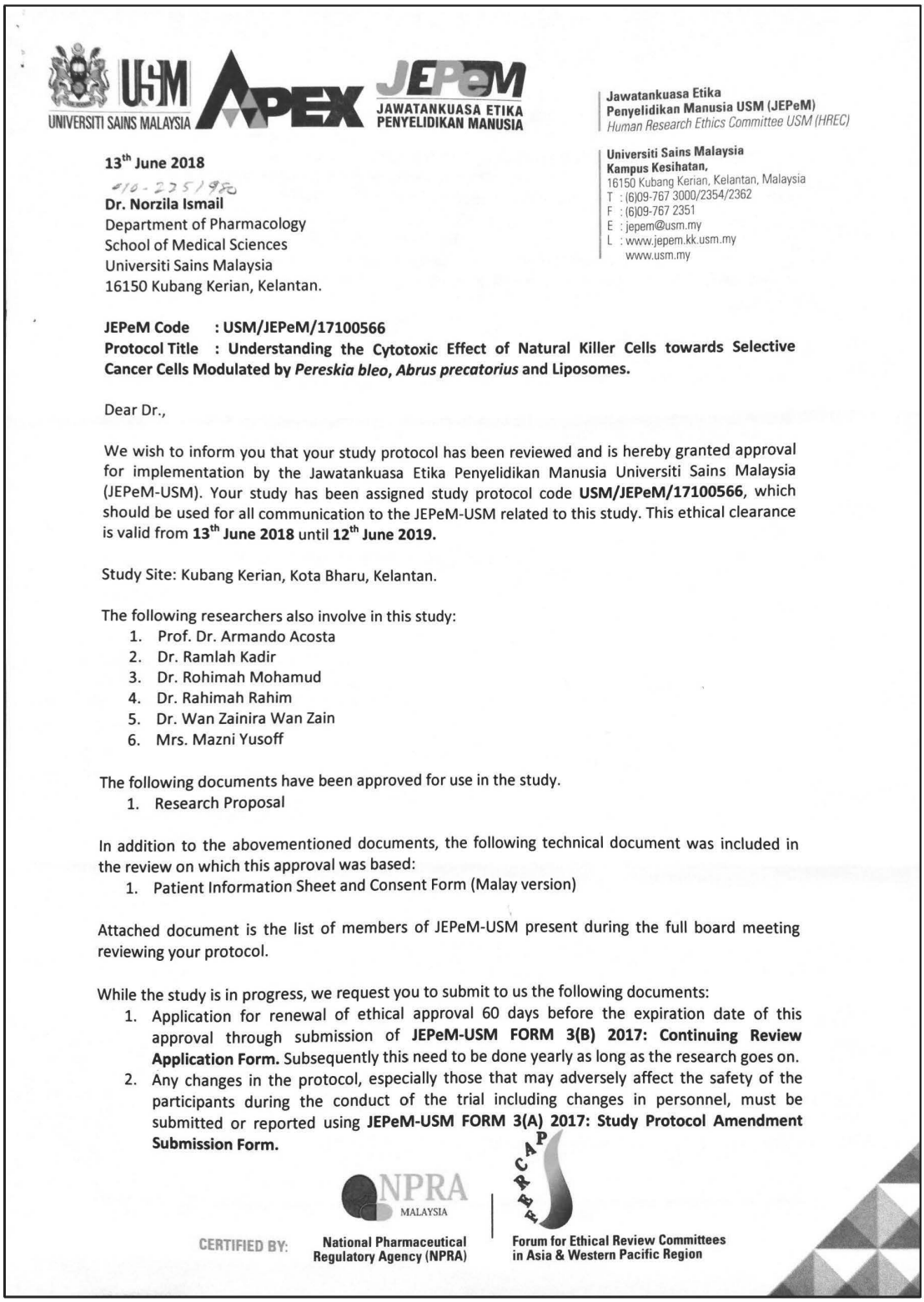
**N** = volume of cells needed from the cell suspension to obtain $\boldsymbol{A} \mathbf{\times 10^4}$

**V** = Volume of medium + cell suspension needed for seeding

**C** = number of cells in 1ml (cells/ml)

Lastly, **N** was topped up with **V** of complete medium prior to seeding.

**Appendix E**: ETHICS APPROVAL FOR BLOOD SAMPLE COLLECTION



UNIVERSITI SAINS MALAYSIA

JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA


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

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian, Kelantan, Malaysia
T : (6)09-767 3000/2354/2362
F : (6)09-767 2351
E : jepem@usm.my
L : www.jepem.kk.usm.my
www.usm.my


**13th June 2018**

910-225/98

**Dr. Norzila Ismail**
Department of Pharmacology
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

**JEPeM Code : USM/JEPeM/17100566**
**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 your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/17100566**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **13th June 2018** until **12th June 2019.**

Study Site: Kubang Kerian, Kota Bharu, Kelantan.

The following researchers also involve in this study:

1. Prof. Dr. Armando Acosta
2. Dr. Ramlah Kadir
3. Dr. Rohimah Mohamud
4. Dr. Rahimah Rahim
5. Dr. Wan Zainira Wan Zain
6. Mrs. Mazni Yusoff

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient Information Sheet and Consent Form (Malay version)

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2017: Continuing Review Application Form.** Subsequently this need to be done yearly as long as the research goes on.
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2017: Study Protocol Amendment Submission Form.**

CERTIFIED BY:

NPRA MALAYSIA
National Pharmaceutical Regulatory Agency (NPRA)

Forum for Ethical Review Committees in Asia & Western Pacific Region

**Appendix F:** EXTENSION PERIOD OF THE ETHICS APPROVAL

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

7th July 2019

**Dr. Norzila Ismail**
Department of Pharmacology
School of Medical Sciences
Universiti Sains Malaysia
16150, Kubang Kerian, Kelantan.

**Universiti Sains Malaysia**
**Kampus Kesihatan**
16150 Kubang Kerian, Kelantan, Malaysia
Tel. : +6 09-767 3000/2354/2362
Fax. : +6 09-767 2351
Emel : jepem@usm.my
Laman Web : www.jepem.kk.usm.my
www.usm.my

**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

JAWATANKUASA ETIKA
PENYELIDIKAN MANUSIA

**Appendix G:** TEMPLATE OF PARTICIPANT INFORMATION SHEET AND CONSENT FORM

**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)<br>(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**

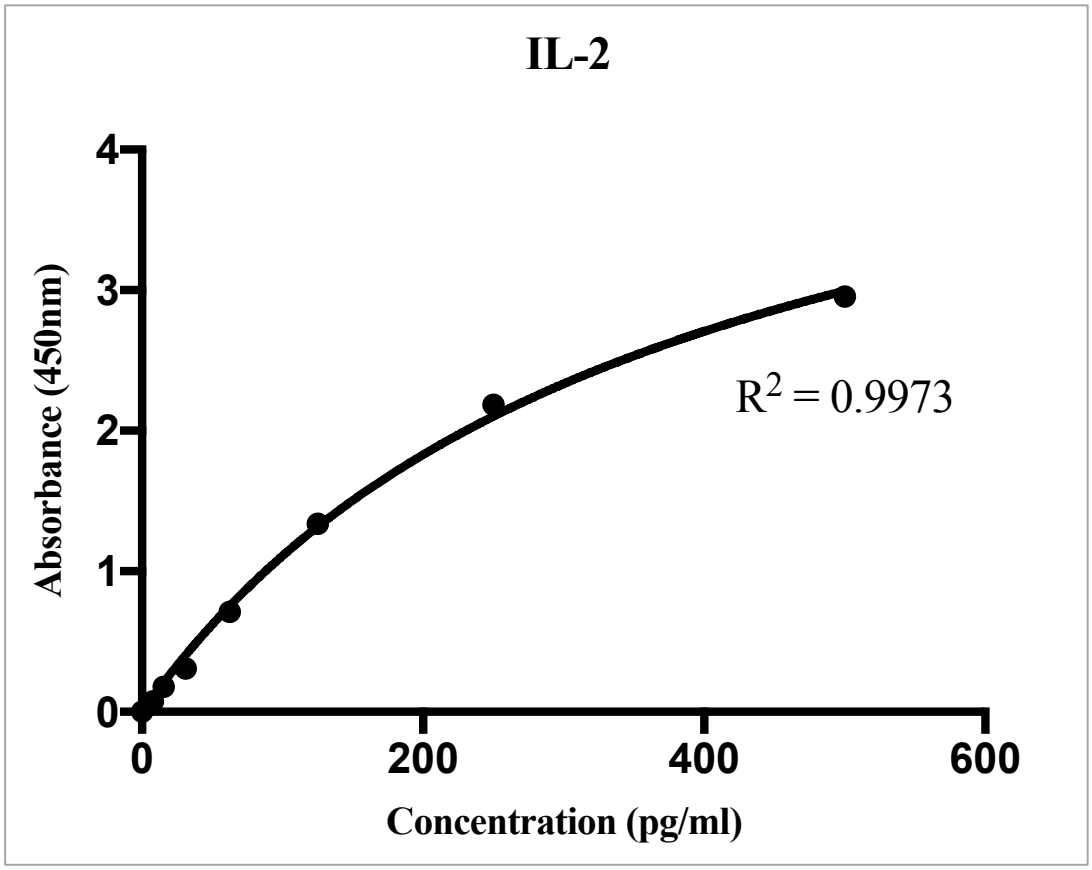
_____ _____ _____

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

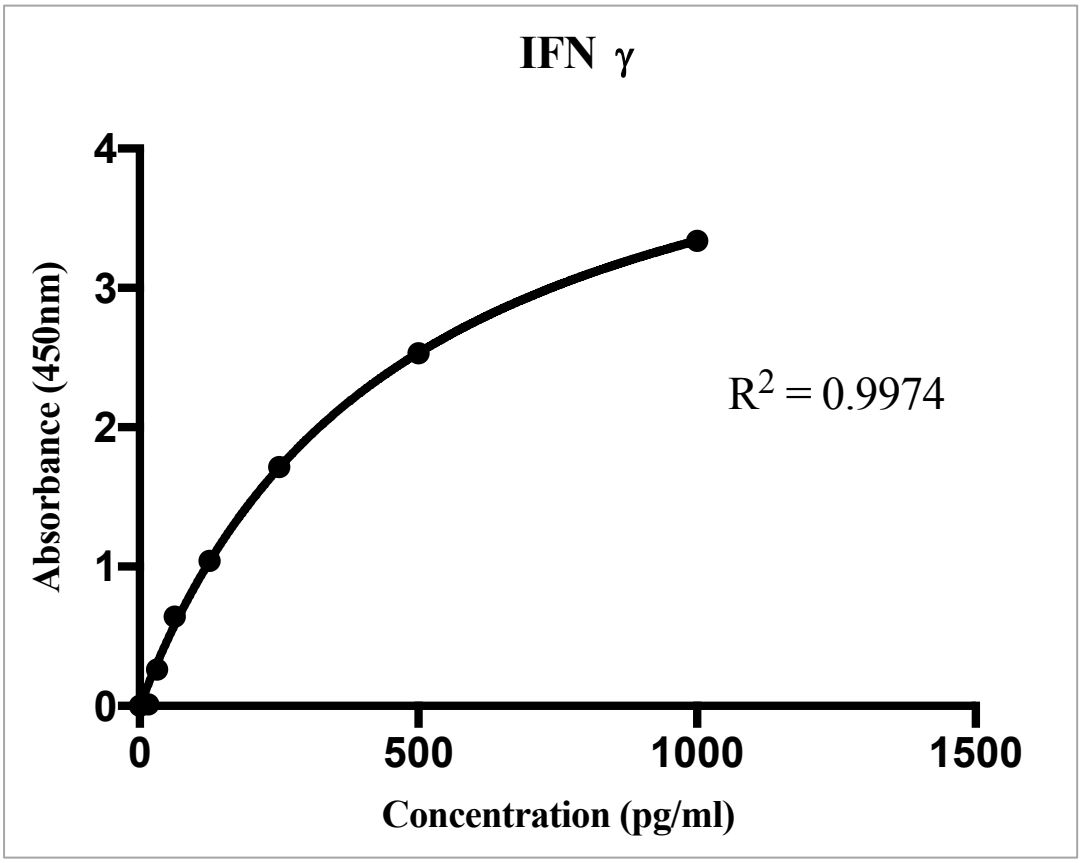
_____ _____

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

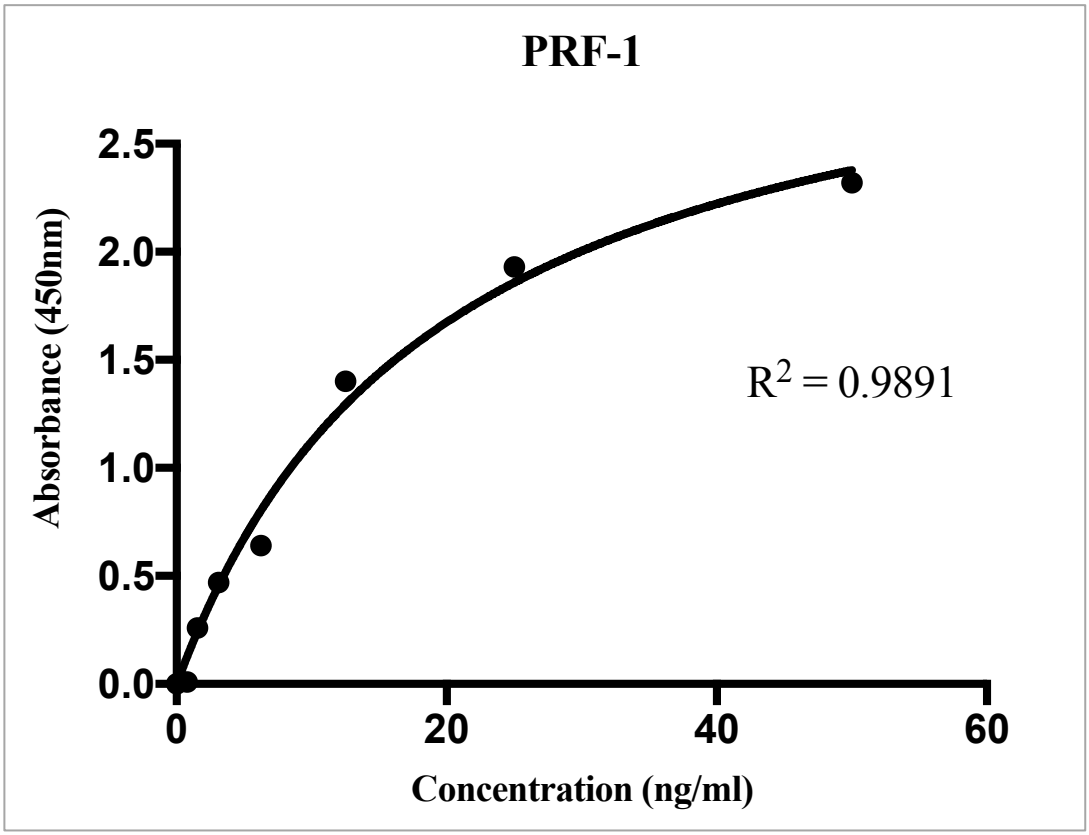
**Appendix H:** STANDARD CURVE OF INTERLEUKIN-2 FOR ELISA



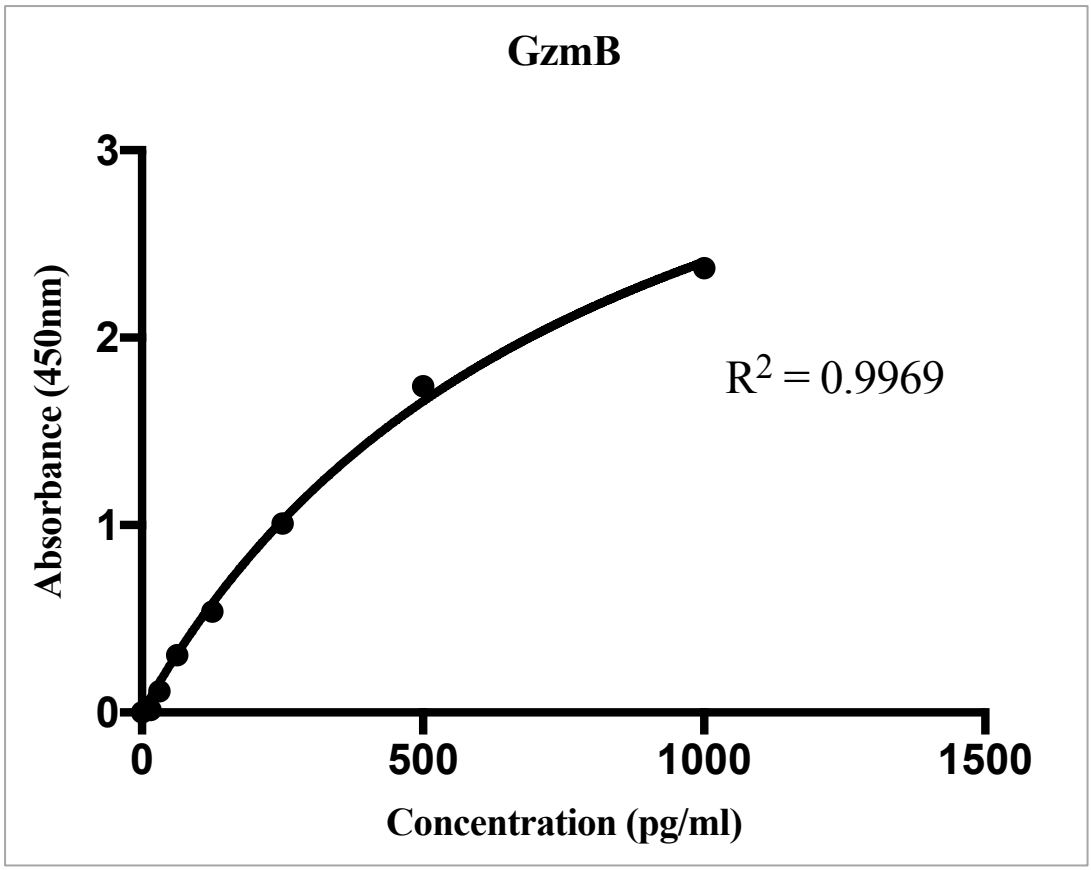
**Appendix I:** STANDARD CURVE OF INTERFERON GAMMA FOR ELISA



**Appendix J:** STANDARD CURVE OF PERFORIN FOR ELISA



**Appendix K:** STANDARD CURVE OF GRANZYME B FOR ELISA



**Appendix L:** PUBLICATION 1

Pertanika J. Trop. Agric. Sci. 41 (1): 241 - 250 (2018)

PERTANIKA

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

# GC-MS Analysis of Phytochemical Compounds in Aqueous Leaf Extract of *Abrus Precatorius*

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## ABSTRACT

*Abrus precatorius* is a flowering plant that belongs to the legume family, Fabaceae. In Malaysia, the leaves of *Abrus precatorius* are used traditionally to treat ailments such as fever, ulcer and mouth cancer. These traditional practices, however, have never been documented and usage of the plant is based on popular beliefs held by the local people. This work documented the phytocompounds that are present in the aqueous extract of *Abrus precatorius* leaves collected from a local area in Kota Bharu, Kelantan, Malaysia. The leaves were dried and then subjected to extraction using the decoction technique. The compounds were identified by gas chromatography with mass spectrometry analysis and characterised by comparison through the NIST02 and Wiley275 library search software. The GC-MS analysis showed that the classes of compounds identified in aqueous extracts of *Abrus precatorius* leaves were phenolic compounds, terpenoids and steroids.

*Keywords*: Abrus precatorius, aqueous extract, GC-MS

## INTRODUCTION

Medicinal plants are widely used as food and medicine in traditional practice. In Malaysia, such plants are consumed as an alternative treatment for illness or to maintain a healthy lifestyle. A huge reservoir of bioactive compounds exists in over 400,000 species of plants on Earth, but only a small percentage of these compounds have been examined in research. In many developed countries,

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**Appendix M:** PUBLICATION 2



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Original Article

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# Methanolic extract of *Abrus precatorius* promotes breast cancer MDA-MB-231 cell death by inducing cell cycle arrest at $G_0/G_1$ and upregulating Bax

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ABSTRACT

**Objective:** To determine the anti-proliferative activity of *Abrus precatorius* (*A. precatorius*) leaf extracts and their effect on cell death.

**Methods:** *A. precatorius* leaves were extracted successively with hexane, ethyl acetate and methanol by Soxhlet extraction. Aqueous extract was prepared by decoction at 50 ℃. Extracts of *A. precatorius* leaves were used to treat selected cancer and normal cell lines for 72 h. Furthermore, 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide assay was performed to determine cell viability. Analysis of cell cycle arrest, apoptosis assay and apoptosis protein expressions were determined by flow cytometry.

**Results:** Methanolic extract of *A. precatorius* leaves showed the lowest $IC_{50}$ on MDA-MB-231 cells at (26.40±5.40) μg/mL. Flow cytometry analysis revealed that cell arrest occurred at $G_0/G_1$ phase and the apoptosis assay showed the occurrence of early apoptosis at 48 h in MDA-MB-231 cells treated with methanolic extract of *A. precatorius* leaves. Methanolic extract of *A. precatorius* leaves induced apoptosis by upregulation of Bax, p53 and caspase-3 and downregulation of Bcl-2.

**Conclusions:** Methanolic extract of *A. precatorius* leaves promotes MDA-MB-231 cell death by inducing cell cycle arrest and apoptosis possibly *via* the mitochondrial-related pathway.

## 1. Introduction

Apoptosis is a characterized form of cell death and is mostly studied. Known commonly as programmed cell death, apoptosis is the packaging of dying cells into fragments that are easily consumed and eliminated by phagocytes without disturbing the normal function of surrounding tissues[1]. Equilibrium between cell death and cell proliferation is important to avoid disruption of the cellular balance. Excessive apoptosis or deficient apoptosis is the cause of many clinical diseases including cancer[2]. Apoptosis can be initiated through two separate pathways, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. In cancer management, apoptosis has become an important tool as a target by potent apoptosis-inducing agents, including both chemical and biological[3].

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## LIST OF PUBLICATIONS

1. **Wan-Ibrahim, W. S**., Ismail, N., Mohd-Salleh, S. F., Yajid, A. I., Wong, M. P.-K. & Hashim, M. N. M. (2019). Methanolic extract of Abrus precatorius promotes breast cancer MDA-MB-231 cell death by inducing cell cycle arrest at G0/G1 and upregulating Bax. *Asian Pacific Journal of Tropical Biomedicine,* **9(6)**, 249. [Web of Science][Q3]
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2. **Wan-Ibrahim, W. S.,** Ismail, T., Naim, T. N., Mohd-Salleh, S. F. & Ismail, N. (2018). GC-MS Analysis of Phytochemical Compounds in Aqueous Leaf Extract of Abrus Precatorius. *Pertanika Journal of Tropical Agricultural Science,* **41(1)**. [Scopus][Q3]

## LIST OF PRESENTATIONS

**April 2019** **The 3rd International Conference on Molecular Biology and Biotechnology (ICMBB),** UCSI University, Kuala Lumpur, Malaysia
*(Oral- Pecha Kucha Style)*
*Abrus precatorius* methanolic leaves extract induced apoptosis in MDA-MB-231 cells
**Wan Suriyani Wan Ibrahim,** Siti Farhanah Mohd Salleh, Norzila Ismail

**Oct 2018** **Monthly CME, PPSP, USM Health Campus, Malaysia**
*(Oral)*
Phytochemical profiling and Anti-proliferative activity of *Abrus precatorius* leaves extract
**Wan Suriyani Wan Ibrahim,** Norzila Ismail

**March 2018** **ICNP 2018, Penang, Malaysia**
*(Oral)*
Phytochemical Profiling & Anti-proliferative Activity of *Abrus precatorius* Leaves Extracts
**Wan Suriyani Wan Ibrahim**, Siti Farhanah Mohd Salleh, Tuan Nadrah Naim Tuan Ismail, Norzila Ismail