ELUCIDATION OF ANTIPROLIFERATIVE MECHANISM OF *Dendrophthoe pentandra* METHANOLIC EXTRACT ON BREAST CANCER CELLS (MCF-7)

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2016

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by

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Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

JULY 2016

ACKNOWLEDGEMENTS

First and foremost, I would like to express my highest and deepest gratitude to All-Mighty; Allah SWT who gives me enough strength and time to accomplish this thesis.

My heartiest appreciation to my supervisors, Dr Mohd Dasuki Hj Sul'ain and Dr Yusmazura Zakaria for encouragement, guidance and critics. Without their support, this thesis would not have been the same as presented here. Their comments have also guided me in the preparation of this thesis.

My deepest thankful goes to my beloved mother, Nafisah Abdullah who gave me continuous support and motivational advices during my ups and downs throughout finishing this research. She is my strongest motivation and inspiration that keep me focused to finish my study.

My sincere appreciation also extends to my dear friends, Hidayah, Raizulnasuha, Saeida, Farah and Salmi who have provided assistance at various occasions. Their views and opinions are very useful indeed. Not forgetting staffs of Culture Laboratory, Biomedicine Laboratory, Puan Roslina and Encik Aminorddin from Science Laboratory Management Unit and Encik Jamaruddin from Department of Immunology, PPSP whom assist me during finishing my laboratory works.

Last but not least, my sincere thanks goes to Majlis Amanah Rakyat (MARA), MyBrain15-MyMaster from Ministry of Higher Education (MOHE), USM Graduate Assistance Scheme and MOHE Fundamental Research Grant Scheme (FRGS 203/PPSK/6171150) for providing financial assistance for my study.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

-	negative
%	percentage
+	positive
<	less than
>	more than
±	approximately
µg	microgram
µg/ml	microgram per millilitre
µl	microliter
µM	micromolar
A549	human lung carcinoma cells
ATCC	American Type Culture Collection
Bax	pro-apoptotic protein Bax
Bcl-2	anti-apoptotic protein Bcl-2
CaCo-2	human colorectal adenocarcinoma cells
cm^3	centimetre square
CO_2	carbon dioxide
DCIS	ductal carcinoma in situ
dH_20	distilled water
DMEM	Dulbecco Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DP	*Dendrophthoe pentandra*

DPME	*Dendrophthoe pentandra* methanol extract
EDTA	ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
$FeCl_3$	ferric chloride
FITC	fluorescein isothiocyanate
g	gram
G1	gap 1 phase
H_2O	water
HCl	hydrochloride acid
HCT-116	human colorectal adenocarcinoma cells
HeLa	human adenocarcinoma cervical cell
HepG2	human liver carcinoma hepatocellular cell
HER2	human epidermal growth factor receptor 2
HL-60	human leukaemia cells
HT-29	human colorectal adenocarcinoma cells
IC_{50}	inhibition concentration at 50% population
KOH	potassium hydroxide
L-929	normal subcutaneous fibroblast cells
M	mitosis phase
MCF-7	human breast adenocarcinoma cells
MDA-MB-231	human breast adenocarcinoma cells
MDCK	normal Madin Darby canine kidney cells
mg	miligram
mg/ml	miligram per millilitre

$MgCO_3$	magnesium carbonate
ml	millilitre
mm	millimetre
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NCSM	National Cancer Society Malaysia
NH_4OH	ammonium hydroxide
nm	nanometre
^oC	degree Celsius
p53	tumor suppressor protein p53
PBS	phosphate buffered saline
PC-3	human prostate adenocarcinoma cells
PE	phycoerythrin
PI	propidium iodide
PS	phosphatidylserine
ROS	reactive oxygen species
rpm	rotation per minute
S	synthesis phase
SD	standard deviation
SH-SY5Y	human neuroblastoma cells
SK-MEL-28	human melanoma cells
SW620	human colorectal adenocarcinoma cells

TAMO	tamoxifen
U-2 OS	human bone osteosarcoma cells
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organization

HURAIAN MEKANISME ANTIPROLIFERASI EKSTRAK METANOL *Dendrophthoe pentandra* TERHADAP SEL KANSER PAYUDARA (MCF-7)

ABSTRAK

Kanser adalah antara punca kematian di seluruh dunia. Matlamat utama rawatan kanser ialah untuk membuang sel malignan sepenuhnya tanpa merosakkan sel normal. Penemuan penawar yang selamat dan berkesan untuk kanser dengan kadar yang segera adalah sangat diperlukan dan ianya kekal menjadi cabaran besar pada masa kini. Dalam sejarah perubatan, tumbuhan herba telah terbukti menjadi salah satu sumber berharga dalam merawat kanser seperti *Dendrophthoe pentandra* (DP). Dikenali sebagai dedalu, tumbuhan ini merupakan tumbuhan separa parasit dengan latar belakang perubatan tradisional untuk merawat kanser. Namun begitu, mekanisme yang menjadi asas kepada aktiviti antikanser adalah tidak jelas. Oleh itu, DP telah dipilih untuk memahami aktiviti antiproliferasi dan mekanisme kematian sel dalam rawatan kanser tersebut. Pengekstrakan DP menggunakan pelarut metanol (DPME) telah dijalankan. DPME dinilai bagi mengetahui sebatian fitokimia yang hadir. Tannin hadir sebagai sebatian fitokimia yang paling banyak terdapat dalam DPME. Aktiviti antiproliferasi oleh DPME terhadap HeLa, MCF-7, MDA-MB-231, HepG2 dan U-2 OS telah dinilai oleh MTT Asai dan graf tindak balas dos kemudiannya diplot untuk menentukan nilai IC_{50}. Sel MCF-7 menunjukkan perencatan pertumbuhan yang paling berkesan dengan nilai IC_{50} terendah apabila dirawat dengan DPME. Aktiviti sitotoksisiti terhadap sel buah pinggang yang normal, MDCK dan sel fibroblast yang normal, L-929 juga dinilai untuk menentukan kesan sitoselektif oleh DPME. Pewarnaan nukleus oleh Hoechst 33258 menunjukkan

kondensasi kromatin, fragmentasi nuklear dan pembentukan badan apoptotik apabila dirawat dengan DPME mengikut tempoh masa tertentu. Analisis flowsitometri mengesahkan bahawa rawatan DPME menghalang proliferasi sel MCF-7 apabila perkembangan kitaran sel terhenti pada fasa G1/S. Mekanisme MCF-7 kematian sel yang disebabkan oleh DPME melalui tapak jalan apoptosis telah dinilai oleh pewarnaan berganda Annexin V-FITC dan propidium iodida. Kajian ini juga mendapati bahawa mekanisme apoptosis tersebut telah dicetuskan oleh DPME melibatkan pengawalaturan tumor p53 protein penindas yang membawa kepada pembebasan cytochrome C. Mekanisme tapak jalan apoptosis juga berkaitan dengan perencatan protein antiapoptotik, Bcl-2 dan pengaktifan protein proapoptotik, Bax. Kesimpulannya, DPME menunjukkan aktiviti antiproliferasi sel MCF-7 secara aruhan kematian apoptosis dan ini membuktikan pendekatan yang positif untuk rawatan kanser payudara.

ELUCIDATION OF ANTIPROLIFERATIVE MECHANISM OF *Dendrophthoe pentandra* METHANOLIC EXTRACT ON BREAST CANCER CELLS (MCF-7)

ABSTRACT

Cancer is one of the major causes of death, worldwide. The ultimate goal of cancer treatment is to remove the malignant cells completely without harming healthy cells. There is an urgency to find a safe and highly effective treatment for cancer disease and it remains a big challenge nowadays. Throughout medical history, herbal plants have been shown to be one of valuable sources in combating cancer such as *Dendrophthoe pentandra* (DP). DP or Mistletoe is a semi-parasitic plant with traditional claims for anticancer property. However, the mechanism underlying anticancer activity is unclear and need to be explored. Therefore, DP was selected in order to evaluate its antiproliferative activity and mode of cell death in cancer treatment. The extraction of DP leaves were carried out using methanol (DPME) by maceration technique. Phytochemicals compound present in DPME were screened and quantified. Tannin is the most abundance phytochemical present in DPME. The antiproliferative activities of DPME towards HeLa, HepG2, MCF-7, U-2 OS and MDA-MB-231 cell lines have been examined by MTT Assay and IC_{50} values were obtained. MCF-7 cells showed the most effective growth inhibition with lowest IC_{50} value upon treatment with DPME. The cytotoxicity activities towards normal kidney MDCK and normal fibroblast L-929 cells were evaluated to determine the cytoselectivity property of DPME. The nuclear staining by Hoechst 33258 displayed the chromatin condensation, fragmented nuclei and formation of apoptotic bodies

upon treatment with DPME according to certain period of time. Flowcytometric analysis using Annexin V/PI double staining has confirmed that DPME-treated MCF-7 arrested cell cycle distribution at G1/S phase and induced apoptosis. The mechanism of action was further confirmed by determination of protein involved in apoptosis pathway; Bcl-2, Bax, p53 and cytochrome C. The results found out that the increased of p53 was followed by an increment of pro-apoptotic, Bax and decreased of anti-apoptotic, Bcl-2. Activation of Bax and inactivation of Bcl-2 triggered release of cytochrome C which leads to apoptosis event. In conclusion, DPME demonstrated antiproliferative activity in MCF-7 cells by induction of apoptosis. Therefore, DP has a promising approach for breast cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of study

In general, recent trends showed that Malaysia as a developing countries is going through rapid transformation of social life and economic changes. This situation has led to a typical lifestyles improvement that usually occur to other industrialized countries. In consequences of the situation, there are rising cases of cancers associated with reproductive and dietary (International Agency for Research on Cancer, 2013). The report also stated that aging and growth of the global population would affect some significant increase up to 19.3 million new cancer cases per year by 2025.

World Health Organization (WHO) stated that breast cancer is a common cancer among women worldwide (National Breast Cancer Foundation, 2015). Every year, the lives of thousands females has been threaten due to breast cancer. Pathological assessment of the breast cancer should be evaluated routinely since it is essential for accurate decision making upon treatment. Usually, breast cancer patients encountered certain side effects of treatment such as infertility and early menopause. Fear of reoccurrence and physical appearance also one of the physiological issue faced by breast cancer patients. Since 2008, Malaysia has experienced in increment of breast cancer prevalence by more than 20 % and death was up by 14 % (Fernandez, 2014). Public awareness about breast cancer are still

poor and below par. Lack of cancer awareness and incapability to seek basic health care are among major obstacle to optimum cancer care (Yusof, 2013a). This scenario also had been attributed to a robust faith toward traditional claim on effectiveness of herbal medicine, the negative perception of the disease as well as poverty and poor education (Hisham and Yip, 2004).

1.2 Rationale of study

One of the most common cancer type in Malaysia is breast cancer. Surgery remain the most common option for breast cancer treatment with the help of radiotherapy as well as chemotherapy. Surgery such as mastectomy is a crucial process due to certain disfiguring operation. The cost, access and fear of further surgery remain as remarkable hindrance in these type of breast cancer treatment. Chemotherapy has been proved to alter survival in women with breast cancer. However, there are certain cases where the patient's immune system has been compromised with this treatment (Yip et al, 2014). Effectiveness and side effects of chemotherapy, surgery and radiation are limited by toxicity effects to healthy tissues in body and costly operation.

Most common alternative medicine for treatment of cancer are dietary supplements, Malay traditional medicine and followed by praying (Shaharudin et al, 2011; Yip et al, 2014). Usage of traditional medicine such as herbal medicine have been utilized extensively by many Malaysian to improve survivorship regardless of scanty evidence of their effectiveness (Soon et al, 2009; Benzie and Wachtel-Galor, 2011; Sooi and Keng, 2013). There is an overwhelming interest in search for herbal

plant-derived anticancer agents since the old folks believed that they are less toxic. Therefore by consuming herbal medicine, it probably can reduce toxicity effect of commercial medicine on human (Abdullah et al, 2013; Shaharudin et al, 2011; Boik, 1996). This proved that there is an urgency to find a safe and highly effective cure for cancer disease.

With this current situation, it is important to establish as well as initiate clinical trials especially for the common herbal medicine that has been utilized locally. In this research, *Dendrophthoe pentandra* (DP) has been chosen based on traditional claim from old folks and previous research that identified *D.pentandra* methanol extracts (DPME) has highest total phenolic content compared to water extract and petroleum ether extract of DP (Dasuki et al, 2012). DP come in abundant source, easily available and it is already been utilized in certain other action as medicinal plant. DP is considered as unwanted plant to economically important horticultural plant (Artanti et al, 2012). Since targeting apoptosis which is one of a good alternative strategy for cancer prevention and treatment, the potential of DP as an anticancer agent towards breast cancer, its mechanism and basic phytochemical profile have been reported throughout this study. Although there are several research reported on the potential of DP as an anticancer agent but the studies regarding the phytochemical profile, antiproliferative effect and its mechanism of cell death has not yet been fully elucidated.

The findings of this research could have provide guidance and support the traditional knowledge of local people in using DP for cancer treatment especially breast cancer. It is also could deliver new knowledge about certain new species of

herbal plant that having medicinal properties of anticancer. With increasing concern of cancer chemoprevention from herbal medicinal plant, the utilization of unwanted part of medicinal plant in Malaysia such as DP might have beneficial effect towards cancer treatment. The mechanism of action of DP extract towards human breast carcinogenesis can be used as a guide for future medicinal practice in the treatment of breast cancer as apoptosis has been plays a vital task in developmental processes, maintenance of homeostasis and removal of damaged cells.

1.3 Objectives of study

1.3.1 General objective

The general objective for this research is to evaluate the antiproliferative effect of DPME and identify its mechanism of action as a potential anticancer agent.

1.3.2 Specific objectives

The specific objectives of this research study are:

1. To evaluate the phytochemical compounds of DPME using qualitative and quantitative analysis.
2. To investigate antiproliferative activity of DPME-treated on selected cancerous cell lines.
3. To determine the cell cycle arrest in the cell line that showed most effective inhibition by DPME

4. To confirm the mechanism of cell death for the cell line that showed most effective inhibition by DPME-induced apoptosis pathway, with observation of p53, Bax, Bcl-2 and cytochrome c

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer has been associated as major factor of death in the world. In 2013, International Agency Research on Cancer (IARC), the specialized cancer agency of the World Health Organization in its online database, GLOBOCAN, reported that more than 14 million new cancer cases and almost 10 million cancer-related deaths. Compared to 2008, there were approximately 12.7 million new cases and 7.6 million deaths related to cancer cases. The report also disclosed that lung cancer was the most commonly diagnosed cancers worldwide, followed by breast and colorectal. More than half of all cancer cases which is 56.8 % and cancer deaths which is 64.9 % in 2012 alone, occurred in non-industrialized countries in the world. These percentage has been predicted to be increase by the year 2025 (International Agency for Research on Cancer, 2013).

In Malaysia, there are about 37, 000 new cancer incidents and 22, 000 cancer deaths were reported throughout the whole nation, every year (Fernandez, 2014). Based on this statistical data, National Cancer Society Malaysia (NCSM) foresaw that there will be more Malaysians diagnosed with cancer in upcoming years. Developing countries such as Malaysia will bear the burden when there is an estimation more than 20 million new cancer incidents arises yearly by 2030.

2.2 Breast cancer

Breast cancer is among major causes of deaths worldwide that has provoked 40, 000 lives in 2013 (Majumdar et al, 2014). Breast cancer has been reported as most frequent diagnosed cancer among women in 140 out of 184 countries. This scenario can be regarded as 1 : 4 of all cancers diagnosed in women (International Agency Research on Cancer, 2013). In Asia countries including Malaysia, the prevalence of breast cancer was reported increased. (Park et al, 2011; Abdullah et al, 2013).

In Malaysia, breast cancer is one of three common cancer cases in Malaysian women (Dahlui et al, 2011). The International Agency for Research on Cancer estimated that age-standardised rate of breast cancer in Malaysia as 38.7 per 100, 000 with 5, 410 new cases (Yip et al, 2014). By comparing Malaysian women and women in Western countries, the breast cancer case present at earlier age in Malaysian women. This happened could be due to Malaysia has a younger demographic with median age of 26 years old, compared to 39.8 years old in United Kingdom. As reported by Abdullah et al (2013), patients with breast cancer were mostly Malay (53.8 %) followed by Chinese (27.1 %) and Indian (9.6 %). From all of above alarming situation, there is an urgent need to prevent increment of breast cancer case by developing potent and economical approaches for the early detection and treatment of breast cancer among women in Malaysia.

2.2.1 Characteristics and classification of breast cancer cells

Generally, each type of cancer cells can be distinguished into 3 main categories, by the cells which it originates (Kleinsmith, 2006). Firstly, carcinomas that appears from epithelial cells which form covering layers of external and internal body. Secondly, sarcomas which originates in supportive tissue such as bone and muscles. The last one is correlated with lymphoma and leukaemia. The lymphoma can be defined as lymphocytes cancer which mainly grow as solid masses of tissue. The leukaemia can be simplified as cancers in blood cells that grow rapidly in bloodstream. Breast cancers are mostly carcinomas that initiates in the epithelial cells of breast organ. Other than that, it could originates in glandular tissue of breast and it is known as adenocarcinoma.

Despite origin sites of cancer, each type of cancer behaves differently. Cassidy et al (2010) reported that characteristics of cancer cells are including proliferation in the lack of growth factors, failure to respond to normal brakes on proliferation; for example growth inhibitors, cell cycle control check points, resistance to apoptosis and senescence, ability to invade surrounding tissues and metastasize and ability to recruit blood vessels. Normal cell growth showed well-mannered balance between promoting and restraining signals so that the proliferation happened only when required. This balance is tilted when increased cell numbers are required. Differentiation of the cells during this process take places in proper regulation and proliferation discontinues when no longer needed (Cassidy et al, 2010). Cancer cell growth showed distortion of above process where cell keep on proliferate continuously and causing loss of differentiation could be seen clearly.

Cancer itself can be defined as a pathological disturbance of growth, characterized by excessive and unnecessary proliferation of living cells (Elmore, 2007).

Breast cancer cells can be classified as either benign or malignant (Table 2.1). Benign breast cancer are generally slow-growing expansive masses that grow and compress in a confined local area rather than invade surrounding tissue of healthy breast cells. They are often encapsulated, grow by expansion and does not invade or spread to nearby tissue (Kleinsmith, 2006). However, there are some benign cancer that could have malignant potential and these should be removed before malignancy develops thus thrusts on vital structures such as nearby blood vessels or nerves. In other cases, breast cells may grow, invade surrounding and divide with complete disregard for the needs of the body which is called malignant cells. These type of cancer are potent to grow into large masses known as lumps and spread to other areas of the body becoming metastases. Metastases means the development of secondary malignant growth at a distance from a primary breast cancer site. This condition allow the malignant breast cells continues to grow rapidly without any limitation. Generally, these types of cells are not encapsulated, tend to infiltrate, invade and destroy surrounding tissue. Malignant breast cancer are able to colonize other organ which often involving vital organs.

Table 2.1 Some properties of benign and malignant cancers (summarized from Kerr et al, 1972; Elmore, 2007; MacDonald et al, 2004; Kamal, 2014).

	Benign	**Malignant**
Growth Pattern	Local growth only	Spreads by invasion and metastasis
Life Threatening	Rare	Often
Proliferation	Slow	Rapid
State of differentiation	Well differentiated	Variable

2.2.2 Treatment of breast cancer

In general, treatment of cancer is a multidisciplinary effort. It depends on the locality of cancer, cancer categories and stages of the disease which include whether it is metastasis or not. Based on this factor, most cancer can be treated and cured. The histology examination of cancer tissue are usually performed whenever definitive diagnosis are carried out. Once the definitive diagnosis are done, the cancer is usually treated with incorporation of operation, chemotherapy and radiotherapy. Besides that, hormonal therapy, immune therapy, monoclonal antibody therapy, supportive therapy and symptomatic therapy can be considered (Acton, 2013). Currently, the treatments becomes particular for that type of cancer pathologically, and in this study, particularly towards breast cancer cells. The ultimate aim of any cancer treatment is to remove the cancer tissue completely without affected to the rest of the body. To remove the cancer with the goal to reduce the risk of reoccurrence of diseases can be such a major problem to achieve.

The most common method for breast cancer treatment is surgery. Majority patients with breast cancer diseases choose surgery as their first treatment. However, the limitation of this option is the tendency of cancers to invade nearby tissue and spread to distant location by microscopic metastatic. Patient with invasive breast cancer are offered to be treated with chemotherapy or hormonal therapy to shrink the cancer prior to surgery (National Breast Cancer Foundation, 2015).

Other than that, hormone therapy also should be considered if belongs to hormone receptor positive (Acton, 2013). Otherwise, if the cancer cell is hormone

receptor negative, then this therapy have no benefit for patient in treatment of cancer disease. Hormone therapies function by blocking the effect of hormones on breast cancer cells. There are patients that have been given hormone therapy before the surgery was carried out due to reduce size of the cancer or if surgery is not an option for the patients. Anastrozole, letrozole, exemestane and goserelin are the hormone therapy drugs commonly used to treat primary breast cancer (National Cancer Institute, 2015).

Another common option in combating breast cancer disease is radiotherapy. This treatment utilizes high energy x-rays to demolish cancer cells that left at the breast area after surgery. Radiotherapy has been given to lessen the risk of the cancer returning in the breast. Magnetic radiation imaging (MRI) is an example of new diagnostic tool for breast cancer treatment in Malaysia. However, MRI is not suitable for routine screening even though it is also practical to differentiate between malignant and benign breast tumor (Hisham and Yip, 2004). This types of radiation can cause damage to normal tissue and affects healthy cells. Damage to non-cancerous cells can be reduced by providing small doses frequently which eventually can increase the time for cancer treatment to accomplish.

Apart of that, the targeted therapy has been used by blockage of cancer growth through inhibiting specific molecules that involved in growth of cancer cells (Yusof, 2013b). For example, molecule known as HER2 protein that present excessively in breast cells lead to disorderly growth thus promoting the formation of malignant cells. The drug known as trastuzumab acts against HER2 protein. It is

considered as an ideal therapy for HER2 positive breast cancer. The main disappointment is the treatment with trastuzumab involved high cost (Yusof, 2013b).

Chemotherapy is a treatment using anticancer drug that behaving as cytotoxic drugs. It can be provided before surgery which referred as neo-adjuvant chemotherapy or after surgery which known as adjuvant chemotherapy. The neo-adjuvant can helps in reducing the size of a cancer. There are many different types of chemotherapy. The chemotherapy drugs can be utilized in several different combinations or as individually. It also can be given intravenously or orally. However, the effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. The usage of chemotherapy drugs are controversial, mainly related to drugs resistance where the immune system of patients has been used with chemotherapy routine treatment (Yip et al, 2014). The most common side effects are nausea, sore mouth, hair loss or thinning and tiredness.

The most ubiquitous chemotherapy drugs for breast cancer treatment is tamoxifen, which have been widely used as antiestrogen. Since 1973, tamoxifen has been used since 1973 as an adjuvant therapy (Osborne, 1998). It has been accepted by Food and Drug Administration (FDA) in 1977 to treat both early and later stages of breast cancer. Almost 70 % of all breast cancers cases are categorized as estrogen receptor alpha positive (Dixon, 2014). However, patients that undergo treatment with tamoxifen is developing a risk towards endometrial cancer (Fisher et al, 1994) due to genotoxic DNA damage (Kim et al, 2004). Growth of some cancer may initially be weakened by treatment of tamoxifen but become immune to continued treatment (Clemons et al, 2002). Early Breast Cancer Triallists' Collaborative Group (2005)

reported that one third of breast cancer patients which have been treated with tamoxifen for 5 years will have reoccurrence within 15 years. Although there are many cancer respond to chemotherapy drugs, breast cancer cells eventually build resistance towards chemotherapy. The hitch of treatment with chemotherapy have pave the way for alternative drugs that grant maximum effect and are less harmful for cancer treatment (Yaacob et al, 2013).

2.3 Mechanism of cell death

Inhibition of cell proliferation that have been induced by certain agents which cause cell death can be simplified as antiproliferative activity. Mode of cell death can be implemented through apoptotic, necrotic death, aging and so on. There is certain overlap properties between these apoptosis and necrosis even both of mechanism are different (Elmore, 2007). Therefore, it is really important to utilize more than one distinguishable assay to confirm the mechanism of cell death that occurs in the pathway. It has become a general routine involving early detection of apoptotic events and another detection could target for later execution event, which this later assay can be double check the presence of apoptosis.

2.3.1 Necrosis

Necrosis is a toxic process where the cells are put in passive situation, uncontrolled and energy-independent mode of death. In necrotic cell death, external factors such as hypoxia, excessively high concentration of chemical toxins and injury could damage the cell irreversibly. During necrosis, there are changes of cell

morphology. The cell swelling, formation of cytoplasmic vacuoles, formation of blebbed cytoplasm and condense swollen mitochondria are among typical morphological changes that occur in necrosis (Kerr et al, 1972; Elmore 2007; Zakaria et al, 2009). There is an influx of water and ions after which the cells and its organelles swell and rupture. Necrotic cell lysis induces acute inflammatory responses owing to the release of lysosomal enzymes into extracellular environment. Lysosomal proteases released into the cytosol cause widespread degradation. There is a rise in cytosolic calcium, increased reactive oxygen species (ROS), intracellular acidification and ATP depletion (Kumar and Clarks, 2012).

2.3.2 Apoptosis

Apoptosis is tightly controlled and energy-dependent process (Fridman and Lowe, 2003). It is a normal genetically programmed process that occurs during embryonic development, maintenance of tissue homeostasis for cell population, under pathological conditions and aging process. It is originally called shrinkage necrosis, it was renamed apoptosis as ‘dropping off as leaves from tree’ (Kerr et al, 1972; Elmore, 2007). Morphological changes that occur during apoptosis can be identified by light and electron microscope. During early apoptosis, the breakdown of nucleus occur. This include the chromatin condensation and DNA fragmentation that are visible under light microscope. When this happened, the organelles are more tightly packed and plasma membrane blebbing occurs followed by cell fragmented into apoptotic bodies. Apoptosis has specific characteristic features of membrane-bound vesicles called apoptotic bodies and absence of inflammatory response (Elmore, 2007) due to phagocytosed by surrounding cells (Kurosaka, 2003). There

are no inflammatory reaction during process of apoptosis since there are no release of cellular contents to nearby tissue. The apoptosis event eliminates cancerous cell without damaging normal cells and surrounding tissues (Rahman et al, 2013). Table 2.2 summarized the comparison between apoptosis and necrosis.

Table 2.2 Comparison of apoptosis and necrosis (summarized from Elmore, 2007; Rang et al, 2007; Kumar and Clarks, 2012)

Apoptosis	**Necrosis**
Programmed cell death	Uncontrolled cell death
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convulation	Cell swelling
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present
Non – toxic process	Toxic process
Non – degradative process	Degradative process
Active process	Passive process
Energy dependent	Energy independent

Apoptotic cells display a few number of biochemical modifications such as protein cleavage and DNA breakdown (Hengartner, 2000). The mechanisms of apoptosis pathway involves an energy-dependent cascade of molecular events (Elmore, 2007). Many chemotherapy and radiotherapy regimens work by triggering apoptotic pathways in the cancer cells (Kumar and Clarks, 2012). These common regimens cause DNA damage in cells which lead to apoptosis event through a p53-dependent pathway (Elmore, 2007). There are two main signalling pathway involve in apoptosis which are extrinsic and intrinsic pathways. Rang et al (2007) reported that extrinsic pathways is known as death-receptor pathways signals from outside of the cell whereas intrinsic apoptotic pathways or popularly known as mitochondrial pathways signed for internal signals such as DNA damage. The extrinsic pathway that commence apoptosis involve transmembrane receptor-mediated interactions (Elmore, 2007). These involve death receptors that are members of the tumour necrosis factor (TNF) receptor gene. Members of TNF bind to extrinsic ligands and transduce some intracellular signals that cause destruction of the cell (Elmore, 2007). The extrinsic pathways can be amplified by induction of the intrinsic pathway.

The intrinsic apoptotic pathway can be distinguished by membrane permeability that causes mitochondrial swelling, rupture of outer membrane and release of pro-apoptotic factors (Kwon and Kalimuthu, 2013). The intrinsic pathway involves various non-receptor mediated intracellular signals and triggered cellular activities in mitochondria thus leads to apoptosis event. Cellular stresses such as growth factor withdrawal, p53-dependent cell cycle arrest and DNA damage induce expression of pro-apoptotic proteins, Bax and cytochrome C (Elmore, 2007). p53 has been named ‘the guardian of the genome’ due to its crucial role in protecting the

integrity of genetic information in response to various genotoxic injuries (Moll et al, 2005; Wong et al, 2013). Activation of apoptotic event by p53 is tightly regulated. Disruption of apoptosis activation can promote tumor progression, allowing the continual of cell proliferation and chemoresistance. The p53 activate DNA repair proteins when DNA has sustained damage, arrest growth of cancer cells at a checkpoint for DNA damage repair thus cause cells undergo apoptosis if the damage remained (Pietenpol and Stewart, 2002). As a transcription factor, p53 stimulates apoptosis though the transcription and regulation of its target genes such as Bcl-2 family members (Fridman and Lowe, 2003). This is due to cancer cells can build resistance towards apoptosis by the expression of anti-apoptotic proteins or by down-regulation of pro-apoptotic proteins.

2.4 Cell cycle

A cell actively proliferates and passes in and out of cell cycle. During cell proliferation, DNA replication and cell division are two important activities that occurred (Li et al, 2007). The DNA replication has to be ended before cells divide. Theoretically, cells division or known as mitosis resulting formation of two cells. Both cells received a complete copy of genome with all vital genetic information (Hung et al, 1996). Cell cycle has been divided into four sequential phases which is G1, S, G2 and M phase. The G1 defined as first gap phase that allow cell to prepare for another round of DNA replication. It is an evaluative point where "cell decide" whether should enter the cell progression or not. The S phase or known as the period of DNA synthesis occurred. In this phase, enzymes such as DNA polymerases, primases, helicases and topoisomerases are assemble to duplicate the genome (Hung

et al, 1996). The G2 phase is the second gap phase that allow cell to prepare for cell division. The M or mitosis is a phase where DNA segregation occur and the cell divide into new cells that genetically similar. Pietenpol & Stewart (2002) stated that the gaps between the end of cell division and DNA synthesis starts known as G1/S checkpoint whereas between DNA synthesis and mitosis begins known as G2/M checkpoint. These checkpoints signalling are important to ensure a proper sequence and timing of events.

2.5 Herbal medicine

2.5.1 Background

Herbal medicines can be defined as traditionally and naturally occurring, plant-derived substances with minimum or none involvement of any industry that intended to cure illness through therapeutic practices (Tilburt and Kaptchuk, 2008). Herbal plant have been used by human being since ages in traditional and complementary medicines due to their therapeutic potential. Herbal medicines nowadays are really popular to the public (Kennedy and Seely, 2010) and has been commonly used for various treatment of diseases and enhancement in quality of life (Benzie and Wachtel-Galor, 2011). The World Health Organization (2016) reported that approximately 80 % of the world population depend on herbal plants for their first-line medicines. Including Malaysia, the usage of herbal medicine as alternative medicine also has been favoured.

Herbal medicines are derived from various parts of herbal plants. It can be processed in different ways and formed into teas, ointments, rubs, tablets, essential oils, syrup and tablets that contain powdered form of herbs or its dried extract (Benzie and Wachtel-Galor, 2011). Old folks believed that the various form of herbal preparation can be utilised for treatment of chronic disease and diverse ailments. The biological activities of herbal plant may varied when different technique of extraction has been utilised during the herbal preparation. This may be due to herbal preparation contain natural compounds with different technique of extraction that may release certain remarkable biological activities. Among the common reason for using traditional herbal medicine are affordability and adverse effects of synthetic medicines.

2.5.2 Safety

In many countries, herbal medicines are not regulated as extensively as conventional medicine. In Malaysia, the usage of herbal medicines are mostly based on ethnic beliefs and ritual observation (Sooi and Keng, 2013). Many of herbal medicines claim to offer relief and prevention of adverse health conditions (Ibrahim, 2004). Comparing to the commercially available pharmaceutical medicine, majority of Malaysian believed that the content of herbal medicine does not harmful and free from side effects (Soon et al, 2009). More than 75 % of Malaysian females considered herbal medicines safe and effective due to herbs contain natural substances (Sooi and Keng, 2013). This elevation trend of prevalence regarding usage of herbal medicines in Malaysia may probably due to the assumption that herbs are safe to be utilized. There is concern for the lack of standardization of herbal

preparations to guarantee their safety, quality and efficacy (Ibrahim, 2004). This awareness is due to certain herbal may be contaminated and degraded during the herbal preparation (Benzie and Wachtel-Galor, 2011). Actual knowledge of herbs, information related to dosage and their properties should be established. To ensure quality, consistency and safety, the herbal preparation need to be replaced with more accurate and reproducible ways. Thus, there is an immediate need to improve the safety knowledge of herbal medicine with proper documentation.

2.6 Herbal medicine and breast cancer

From the early 1980's, the trend showed that there are roughly 60 % of anticancer agents are originated from herbal plant such as curcumin, a phenol compound derived from rhizome of Curcuma species that can inhibit carcinogenic effect in colorectal cancer and genistein, a phytosterol belongs to flavonoids that induced apoptosis in human promyelocytic leukemic cells (Safarzadeh et al, 2014; Rahman et 2013). There are more than 177 drugs that have been approved and registered for cancer treatment, for example vinca alkaloids including vinblastine and vincristine, taxanes including paclitaxel and docetaxel (Benzie and Wachtel-Galor, 2011; Safarzadeh et al, 2014). Those various natural substances that derived from plants have been acknowledged to be able induce apoptosis in several malignant cells. Recently, significant attention from plant-derived anticancer agents has gained to the apoptotic cell death events and the role of its mechanism in cancer treatment.

The use of herbal plant extracts as herbal medicines has become increasingly popular especially in breast cancer treatment. As example, *Vernonia amygdalina*, a

woody shrub that have been reported as anticancer agent that documented by Wong et al (2013). The plant demonstrated its potential as an anticancer agents for breast cancer treatment by G1/S phase cell cycle arrest in breast cancer cells, specifically only towards MCF-7 cells. Two types of alkaloids, pterogynine (PGN) and pterogynidine (PGD) from *Pterogyne nitens* extract could induce apoptosis in breast cancer cell line (Duarte et al, 2010). Hseu et al (2006) reported that antiproliferative action, growth suppression and promotion of apoptosis in breast cancer cells by treatment with *Antrodia camphorate*. The data obtained from Reddy et al (2013) revealed that the death of breast cancer cells incubated with cytotoxic extracts of *Alpinia scabra* may be due to apoptosis induction. Those reported plants also has been said contain various phytochemical such as alkaloid, flavonoid, tannin and others that could related to their pharmacological benefits (Kamal, 2014).

These medicinal values could no longer be ignored and their mode of action in the apoptosis pathway of those reported herbal plants should be well-described. Therefore, the need to find a safe and highly effective for breast cancer disease remains a big challenge in modern science. One of the most recognized herb to be developed as anticancer agent is mistletoes.

2.7 Mistletoes

Mistletoe is a semi - parasite of plant. It has been used since long time ago for various medicinal purposes (Becker, 1982). Mistletoe plants has been used as a ritual or medicinal plants from the past few thousand years. It was first introduced by Rudolf Steiner in the form of plant extract for the treatment of malignancies disease

(Mansky, 2002). It has been widely used form of complementary and alternative medicine for cancer treatment and research into its uses poses the challenges to implement this kind of approaches into clinical efficacy thus can be used in complex cancer treatment. From recent history, there are various confusing history regarding mistletoe extract for having ability to delay the cancer growth. Regardless the risks of toxicity, old folks have used mistletoe as a therapeutic for various sickness such as haemorrhage, syphilis, heart disease and gout (Hyun et al, 1998). Mistletoe preparations are harvested from several host-tree species for example pine, apple, oak, fur, maple, poplar and hawthorn (Mansky, 2002). In Europe, white berry of mistletoe have been used as traditional curative for cancer, hypertension and diabetes (Bussing, 2000). The anticancer property of mistletoe extract has been claimed due to presence of lectins (Franz, 1986), viscotoxins (Romagnoli et al, 2000) and alkaloids (Zhou et al, 2010).

According to Mabberley (2008), the name mistletoe is not only applicable to the genus *Viscum* but also to genus *Dendrophthoe*. Although several species in the Loranthaceae family occur in many rural and disturbed sites, often on exotic or cultivated hosts, *D. pentandra* (L.) Miq is the most common genera that can be found easily in urban parks and garden and on street trees as well as roadside tress of semi-rural areas (Start, 2011).

2.7.1 *Dendrophthoe pentandra*

Several different local names have been used in various countries such as “benalu” in Indonesia and “dedalu” in Malaysia regardless its common name is