CLEARANCE OF APOPTOTIC CELLS BY MACROPHAGE IN MCF-7 CELL LINE TREATED WITH METHANOL EXTRACT OF *CENTELLA ASIATICA* (MECA)

NOR 'ATIQAH MUHAMAD

UNIVERSITI SAINS MALAYSIA

2018

CLEARANCE OF APOPTOTIC CELLS BY MACROPHAGE IN MCF-7 CELL LINE TREATED WITH METHANOL EXTRACT OF *CENTELLA ASIATICA* (MECA)

by

NOR 'ATIQAH MUHAMAD

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science (Biomedicine) Mixed Mode

JANUARY 2018

ACKNOWLEDGEMENT

All praises to Allah, I could not have finished this thesis without His blessings. First of all, I would like to express my gratitude to my main supervisor, Associate Professor Dr. Rapeah Binti Suppian for her endless motivation and support throughout the study of my Master's degree. Her constant positive mind-set drove me to be very determined to finish the research project with good results.

I also would like to thank Associate Professor Dr. Hasmah Binti Abdullah for her guidance and her personal generosity that have contributed a big encouragement for me to complete the research project. I would like to mention, Associate Professor Dr. See Too Wei Cun and Associate Professor Dr. Few Ling Ling as they offered to help me regarding any issues during my Master's degree.

Not forgotten are my helpful seniors, Anis Fadhilah Binti Zulkipli and Nor Munirah Binti Zakaria. Their guidance has made me to be adapted in using laboratory techniques and procedures that were new for me.

I would love to thank Universiti Sains Malaysia for providing research equipment and facilities for me to perform my research project. I also would like to thank all the laboratory staffs for the information in all the aspects of the laboratories.

I also would love to express my gratitude to my family for their continuous support especially to my parents for providing financial supports during my time as a Master's degree student. Last but not least, I express sincere gratitude to my fiancé, Mohammad Shahrizal Bin Mohammad Umar for his loving inspiration, motivation, and guidance for me to finish the research project for my Master's degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACS	American Cancer Society
AECA	Aqueous extract of *Centella asiatica*
AI	Aromatase inhibitor
Apaf-1	Apoptotic protease activating factor-1
Apo2L/TRAIL	Apoptosis ligand 2/tumour necrosis factor-related inducing ligand
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AV/PI	Annexin V/propidium iodide
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 homologous antagonist killer
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
BID	BH3 interacting domain death agonist
BRCA	Breast cancer gene
BSC	Biosafety cabinet
CDC	Centre for disease control and prevention
CFS	Chronic fatigue syndrome
CHD	Coronary heart disease
CO_2	Carbon dioxide
CT-scan	Computerized tomography (CT)-scan
CX_2CL1	Chemokine lignand-1
dH_2O	Distilled water
DCIS	Ductal carcinoma *in situ*

DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
ER	Estrogen-receptor
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
HCl	Hydrochloric acid
ICAM-3	Intercellular adhesion molecule-3
IGF-1	Insulin-like growth factor-1
IL-2	Interleukin-2
JNK	c-Jun N-terminal kinase
J774A.1	Mouse BALB/C macrophage
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen orthophosphate
LDL	Low-density lipoprotein
lysoPC	Lysophosphatidylcholine
MBA	Methylene blue assay
MCF-7	Human breast adenocarcinoma cell line established by Michigan Cancer Foundation-7

MCL-1	Myeloid leukemia sequence-1
MDA-MB-231	Human Caucasian breast adenocarcinoma
MECA	Methanol extract of *Centella asiatica*
MOI	Multiplicity of infection
NaCl	Sodium chloride
Na_2HPO_4	Di-sodium hydrogen phosphate anhydrous
NCI	National Cancer Institute
NIH3T3	Embryo fibroblast cell line
NOXA	Phorbol-12-myristate-12-acetate-induced protein 1
OD	Optical density
PBS	Phosphate buffer saline
PS	Phosphatidylserine
PUMA	p53-upregulated modulator of apoptosis
p53	Tumor protein
p38 MAPK	p38 mitogen-activated protein kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.E.M	Standard error mean
SERM	Selective estrogen receptor modulator
SMAC/DIABLO	Mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein
TGF-ß	Transforming growth factor-ß
TNF-α	Tumor necrosis factor-alpha
UK	United Kingdom
USA	United State of America

UTI	Urinary tract infections
UTP	Uridine triphosphate
VLDL	Very-low density lipoprotein
WHO	World Health Organization

LIST OF SYMBOLS

%	Percentage
<	Less than
>	More than
≤	Less and or equal to
≥	More than or equal to
±	About
~	Approximately
ºC	Degree celcius
et al.	et alii – 'And others'
cells/mL	Cells per millilitre
cm	Centimetre
cm^2	Centimetre square
L	Litre
M	Molar
µg	Microgram
µL	Microlitre
mL	Millilitre
mm	Millimetre
mM	Millimolar
µg/mL	Microgram per mililitre
mg/mL	Milligram per mililitre
xg	Relative centrifugal force

ABSTRAK

Kanser payudara adalah salah satu daripada penyumbang terbesar dalam kalangan penyakit tidak berjangkit di seluruh dunia. Sehingga kini, banyak bukti menunjukkan bahawa rintangan terhadap apoptosis oleh sel-sel kanser dan eferositosis yang tidak cekap oleh sel-sel fagositik menyebabkan perkembangan penyakit lain dalam kalangan pesakit kanser, seperti penyakit autoimun. Kajian terdahulu menunjukkan bahawa ekstrak *Centella asiatica* (*C. asiatica*) menyebabkan apoptosis kepada sel kanser tetapi kajian mengenai eferositosis terhadap sel-sel kanser amatlah terhad. *C. asiatica* mempunyai potensi dalam menjadi rawatan alternatif untuk mendorong apoptosis dan eferositosis oleh sel-sel fagositik dalam pesakit kanser. Aktiviti anti-proliferatif ekstrak metanol *C. asiatica* (MECA) terhadap sel kanser payudara (MCF-7) diperolehi dengan menjalankan ujian metilina biru (MBA). Dalam kaedah ini, DMSO berfungsi sebagai kawalan negatif manakala tamoxifen berfungsi sebagai kawalan positif. Selepas itu, mod kematian sel dalam sel-sel yang dirawat MECA ditentukan oleh pewarnaan Hoechst 33342 dan selanjutnya disiasat oleh analisis aliran sitometri. Penemuan MBA menunjukkan bahawa MECA adalah sitotoksik kepada MCF-7, namun sebaliknya, ia tidak toksik kepada sel normal (NIH3T3). Di samping itu, MCF-7 yang dirawat dengan MECA mengalami peningkatan apoptosis yang ditunjukkan oleh pendarfluor yang lebih cerah, perubahan morfologi nuklear dan pemecahan DNA yang diperhatikan melalui pewarnaan Hoechst. Untuk mengkaji aktiviti eferositosis oleh makrofaj (J774A.1), sel MCF-7 telah dikultur bersama sel J774A.1 dengan nisbah kepanjangan jangkitan (MOI) 1: 2. Selepas rawatan dengan MECA, data aliran sitometri menunjukkan peningkatan ketara sel-sel apoptosis yang lewat disebabkan oleh apoptosis J774A.1 selepas memakan sel-sel MCF-7. Kesimpulannya, MECA dapat meningkatkan aktiviti

apoptosis dan eferositosis dalam sel MCF-7, dengan itu ia dicadangkan sebagai rawatan alternatif yang bersesuaian untuk kanser payudara.

ABSTRACT

Breast cancer is one of the major contributions in non-communicable diseases worldwide. To date, much evidence has shown that resistance to apoptosis by cancer cells and inefficient efferocytosis by phagocytic cells in cancer patients foster the development of other diseases in cancer patients, such as autoimmune diseases. Previous studies have shown that *Centella asiatica* (*C. asiatica*) extract induced apoptosis in cancer cell line but there was a lack of study regarding efferocytosis against cancer cells. *C. asiatica* has the potential to be an alternative treatment to induce apoptosis and efferocytosis by phagocytic cells in cancer patients. The anti-proliferative activity of methanol extract of *C. asiatica* (MECA) against breast cancer cell line (MCF-7) was obtained by performing methylene blue assay (MBA). In this method, DMSO served as a negative control while tamoxifen served as a positive control. After that, the mode of cell death in MECA-treated cells was determined by Hoechst 33342 staining and was further investigated by flow cytometry analysis. The findings on the MBA suggested that MECA was cytotoxic on MCF-7 and not toxic on normal cells (NIH3T3). Furthermore, MCF-7 treated with MECA undergone increased apoptosis indicated by brighter fluorescence, alteration of nuclear morphology and DNA fragmentation observed in Hoechst staining. To study efferocytosis activity by macrophages (J774A.1), MCF-7 cells were cultivated in J774A.1 cells with multiplicity of infection (MOI) of 1:2. After treatment with MECA, flow cytometry data showed a significant increase of late apoptotic cells due to apoptosis of J774A.1 after engulfing MCF-7 cells. In conclusion, MECA was able to improve the activities of apoptosis and efferocytosis in MCF-7 cells, thus suggesting a promising alternative treatment for breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Study background

Cancer has dominated and remained as a major contributor to mortality in society worldwide and one of the major contributors is breast cancer (Ab Manan *et al*., 2016). Today, the increase in the number of deaths caused by breast cancer is higher than coronary heart disease (CHD) and stroke (Ferlay *et al*., 2015). This is because, breast cancer is very complicated and it is a heterogeneous disease that is closely related to pathology, biochemistry and etiology, making breast cancer the leading cause of death worldwide (Hoshyar *et al*., 2015). The increase in breast cancer statistics is caused by several biological capabilities including sustaining proliferative signaling, evading growth suppressors, resisting cell death (apoptosis), enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2011).

Nowadays, there are several treatments for breast cancer, which is it usually requires a multimodal approach such as medical, surgical and radiological. However, all these approaches are impossible to cure breast cancer (Hoshyar *et al*., 2015). Besides that, recent treatments in breast cancer including chemotherapy, hormonal treatment and targeted therapy are sophisticated, expensive, ineffective as well as they are not widely available in certain countries (Hoshyar *et al*., 2015).

Among the treatments mentioned before, chemotherapy is the most preferred option because it is most convenient and involves the selection of several medications to

control the critical stage of malignant tissue and metastatic diseases (Fernando and Jones, 2015). Despite the success of chemotherapy treatments in dealing with cancer problems, conventional drugs that commonly used in treating breast cancer such as tamoxifen, usually have serious side effects especially in developing kidney failure (Mahassni and Al-Reemi, 2013). Moreover, chemotherapy medications also can exert toxic effects on normal cell division, for example bone marrow and mucus membrane (Fernando and Jones, 2015). In addition, chemotherapy medications will also cause adverse effects to some of the organ systems and may also cause resistance to medications that currently remain the main obstacle to achieve benefits and success in clinical treatment (Mahassni and Al-Reemi, 2013).

Due to this matter, many researchers seek to find the best treatment for anti-cancer agents. In this quest, natural products have attracted many researchers such as pharmacologists and biochemists in producing potentially therapeutic agents in treating cancer (Baker *et al*., 2007). The use of natural products is becoming popular today as most of them have been optimized to become drug-like molecules and remain the best source of drugs (Newman and Cragg, 2012). Historically, extraordinary story of the discovery and use of natural products has proven that it affects the advancement in biology and the discovery of drug therapy (Shen, 2015). Therefore, natural products are among the consistent products and sources of success in medicine as it has become an important source in contributing to the basic needs of everyday life. The use of natural products is not limited to food sources but it is more than that because it plays an important role in traditional medicine in treating several diseases (Thomas *et al*., 2001).

Over the past few decades, the use of natural products as an alternative medicine has been detected (Thomas *et al*., 2001) and today, most research focuses on herbs and

aromatherapy products, where some of them have proven therapeutic to cancer (Mans *et al*., 2000; Yin *et al*., 2013). In this current study, natural herbs known as *Centella asiatica* (*C.* asiatica) has been selected because it can be used extensively, especially in traditional treatments worldwide and it has been claimed to possess anti-cancer properties (Jamil *et al*., 2007). Furthermore, there are some scientific evidences that *C. asiatica* has been used since prehistoric of Ayurveda and aborigines as therapeutic agents including wound healing, rheumatoid arthritis, aging, fertility and as well as an anti-cancer remedy (Kim *et al*., 2011a). In Malaysia, *C. asiatica* is also widely practiced and eaten freshly as "ulam" among Malaysians especially in women (Reihani and Azhar, 2012).

Previous study conducted by Michael (2016) has shown that *C. asiatica* can stimulate innate immune system by producing high anti-inflammatory cytokines and toxic mediators such as nitric oxide in macrophage. However, there was no study conducted to investigate the clearance of apoptotic materials in cancer cells. As apoptosis is one of the hallmarks in developing cancer, impaired of apoptosis can lead to a main obstacle to effective treatment (Adams and Cory, 2007). Inefficient clearance of apoptotic materials can lead to abnormal immune responses including accumulation of autoantigens in tissues that cause several diseases such as chronic inflammatory diseases, autoimmune diseases and developmental abnormalities (Yoon *et al*., 2015). Therefore, this present study is carried out to investigate the effectiveness of *C. asiatica* in triggering clearance of apoptotic cells by macrophage.

1.2 Objectives of the study

1.2.1 General objectives

The objective of this study is to determine the ability of methanol extract of *C. asiatica* (MECA) on apoptosis activity of MCF-7 and efferocytosis in J774A.1 macrophage.

1.2.2 Specific objectives

a) To determine the cytotoxicity activity of the MECA-treated against MCF-7 and NIH3T3 cell lines by using methylene blue assay (MBA) technique.

b) To determine the apoptotic activities of the MECA-stimulated cells by using Hoechst staining.

c) To determine the clearance of apoptotic materials stimulated with MECA against MCF-7 cell line by J774A.1 macrophage through flow cytometry.

1.3 Hypothesis

MECA stimulates the ability of apoptosis activities in treated cell lines and also stimulates J774A.1 macrophage to clear the apoptotic materials of the MCF-7 treated cancer cells. It is also induced macrophage apoptosis in J774A.1.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is an uncontrolled proliferation of abnormal cells beyond their normal boundaries, which it can metastasize to the adjacent parts of the body and other organs (WHO, 2017a). There are several factors that caused cancer; internally and externally. The internal factors include inherited mutations, hormones, immune system conditions and mutations that occur from metabolism (Rieger, 2004). However, both internal and external factors can act and interact with each other to promote carcinogenesis. For example, external factors such as physical, chemical and biological carcinogens can alter person's genetic factor (Rieger, 2004).

There are two types of tumor classifications; benign and malignant tumor (Table 2.1). A benign tumor (primary cancer) is not a cancerous tumor, which it does not invade its surrounding tissue or spread around the body. Primary cancer is a tumor mass that presents at the site of initial conversion of a normal cell to a tumor cell (Oppenheimer, 2006). Meanwhile, a malignant tumor is a tumor that may invade its surrounding tissue or spread around the body. In some cases, benign tumor may cause the development of metastasis (secondary cancer) if it not treated (Kangawa *et al*., 2015). Cancer cells that spread to areas that is indirectly adjacent to the primary cancer is called as metastasis through invasion of blood or lymph vessels or by penetrating the cancer cells into the body cavity or spaces of surrounding organs (Kitamura *et al*., 2015).

Table 2.1: Differences between benign and malignant tumors (Oppenheimer, 2006).

	Benign	**Malignant**
Cells	Relatively normal and mature.	Little resembles to normal, poorly differentiated, atypical in size and shape, non-uniform and immature.
Growth	Slow and restricted. Non-invasive of surrounding tissue, expansive and pushing aside normal tissue.	Rapid and unrestricted. Invasive of surrounding tissue.
Recurrence	Rare	Frequent
Spread	Localized and capsulated.	Metastasize via blood and lymph stream.
Threat host	Prognosis favorable. The effects are depending on the size and location. It may cause pressure on vital organs and obstruct a passageway, which is usually corrected by surgical excision of neoplasms.	Threatens life due to its local vicious proliferation and formation of secondary neoplasms in other structures. Prognosis more favorable with early diagnosis and treatment when cells show less departure from the normal and there is no metastasis.

According to Oppenheimer (2006), there are several stages of metastasis such as detachment of cells from the primary tumor, migration of tumor cells, penetration (invasion) of these cells into lymph vessels or blood vessels and dissemination of the cells or cell clusters to distant areas. Other than that, lodging of tumor cells in blood vessels of distant organs, invasion of tumor cells through the vessel walls and into the tissue of secondary sites and growth of secondary tumor at the secondary sites are also can be considered as the stage of metastasis.

2.1.1 Breast cancer

Breast cancer occurs when abnormal cells in the breast proliferates out of control in one or both breasts and invade nearby tissues thus form a mass called as malignant tumor. The malignant tumor or also known as cancerous tumor can metastasize to the lymph nodes and other parts of the body (ACS, 2016). Majorly, breast cancer is caused by the mutation in genes, which are present in nucleus of all cells in body known as *BRCA1* and *BRCA2* (Majeed *et al*., 2014).

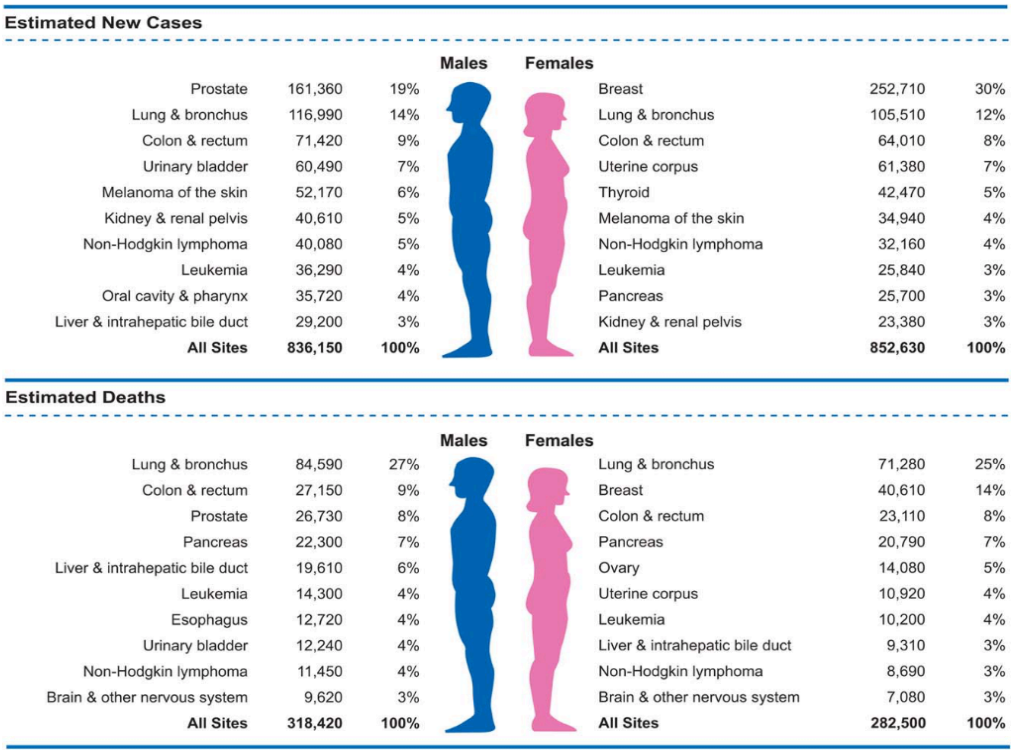
2.1.2 Prevalence of breast cancer

Breast cancer is one of the most important undeniable non-communicable disease worldwide. In 2008, about 1.38 million new breast cancer cases were diagnosed with almost half of all breast cancer cases. Previous study by Lancet (2009) and Shulman *et al*. (2010) stated that, an estimated 1.7 million women will be diagnosed with breast cancer in 2020.

The percentage of breast cancer deaths is higher in low- and middle-income countries due to constraint in resource and infrastructure thus it challenges the aim to improve breast cancer fallouts by early detection, diagnosis and treatment (Anderson *et al*.,

2008). Moreover, poor health awareness and education, lack of screening programs because lacking of governmental support and insufficient funds, social barriers to early diagnosis and treatment due to low priority for women health issues in predominantly patriarchal developing societies, fear of loss of employment and the social taboo of cancers and misconceptions about cancer treatment and outcomes, lack of standardized treatment protocols with diversity of clinical practice, health care standards and infrastructure, and finally poor follow up data and the lack of mortality data (Tfayli *et al*., 2010).

In high income countries such as United States, breast cancer is the most commonly diagnosed cancer among American women compared to other types of cancers such as lung and skin cancers (CDC, 2016). Approximately 252710 of estimated new cases for women will be diagnosed with invasive breast cancer and 40610 will be die due to breast cancer in 2017 (Figure 2.1) (Siegel *et al*., 2017). The prevalence of estimated new cases and estimated deaths for breast cancer in 2017 is increasing compared to 2016, where the estimated cases were 246660 and the estimated deaths were 40450 (Siegel *et al*., 2016). Breast cancer is increasing particularly in developing countries, where the majority of cases are diagnosed in late stages (WHO, 2017b). Ferlay *et al*. (2010) also stated that approximately 60% of breast cancer related deaths are occurring in low- and middle-income countries.



Estimated New Cases

Males				Females		
Prostate	161,360	19%		Breast	252,710	30%
Lung & bronchus	116,990	14%		Lung & bronchus	105,510	12%
Colon & rectum	71,420	9%		Colon & rectum	64,010	8%
Urinary bladder	60,490	7%		Uterine corpus	61,380	7%
Melanoma of the skin	52,170	6%		Thyroid	42,470	5%
Kidney & renal pelvis	40,610	5%		Melanoma of the skin	34,940	4%
Non-Hodgkin lymphoma	40,080	5%		Non-Hodgkin lymphoma	32,160	4%
Leukemia	36,290	4%		Leukemia	25,840	3%
Oral cavity & pharynx	35,720	4%		Pancreas	25,700	3%
Liver & intrahepatic bile duct	29,200	3%		Kidney & renal pelvis	23,380	3%
All Sites	**836,150**	**100%**		**All Sites**	**852,630**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	84,590	27%		Lung & bronchus	71,280	25%
Colon & rectum	27,150	9%		Breast	40,610	14%
Prostate	26,730	8%		Colon & rectum	23,110	8%
Pancreas	22,300	7%		Pancreas	20,790	7%
Liver & intrahepatic bile duct	19,610	6%		Ovary	14,080	5%
Leukemia	14,300	4%		Uterine corpus	10,920	4%
Esophagus	12,720	4%		Leukemia	10,200	4%
Urinary bladder	12,240	4%		Liver & intrahepatic bile duct	9,310	3%
Non-Hodgkin lymphoma	11,450	4%		Non-Hodgkin lymphoma	8,690	3%
Brain & other nervous system	9,620	3%		Brain & other nervous system	7,080	3%
All Sites	**318,420**	**100%**		**All Sites**	**282,500**	**100%**

Figure 2.1: Ten leading cancer types for the estimated new cancer cases and deaths by sex, United States 2017 by Siegel *et al*. (2017).

In Malaysia, breast cancer is one of the most important undeniable non-communicable diseases that caused 13.56% of all death in 2015 (Ab Manan *et al*., 2016). The percentage of breast cancer is highest which is 17.7% (Figure 2.2) compared to other most common cancers in Malaysia such as colorectal and lung cancers (Ab Manan *et al*., 2016). It is also dominating the percentage of common cancers in females by 32.1% (Table 2.2) (Ab Manan *et al*., 2016; The Star Online, 2016). The incidence increases gradually from the age of 30 and peaks at 50 to 59 years old. The racial distribution is 41% Chinese, 33% Malay and 26% Indian and almost half of the cases are already Stage II when diagnosed (The Star Online, 2016). This event happens due to the increase of growth and aging of the population as well as an increasing prevalence of established risk factors such as smoking, overweight, physical inactivity and changing reproductive patterns associated with urbanization and economic development (Ab Manan *et al*., 2016).

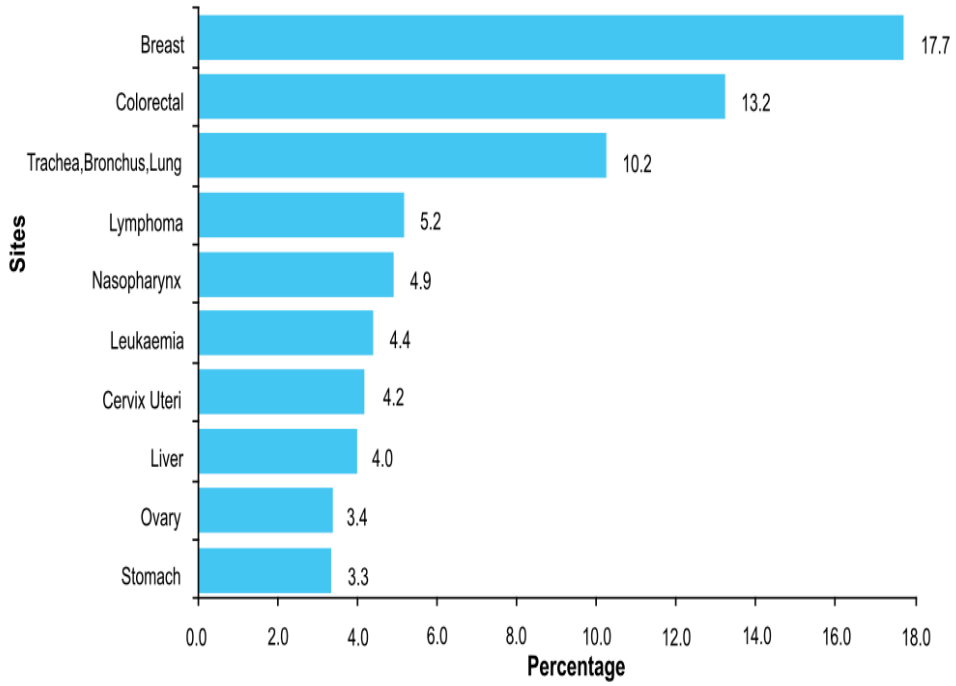


Figure 2.2: Percentage of ten most common cancers in Malaysia by Ab Manan *et al*. (2016).

Table 2.2: Ten most common cancer in females by Ab Manan *et al*. (2016).

Sites	Number	Percentage (%)
Breast	18206	32.1
Colorectal	6047	10.7
Cervix uteri	4352	7.7
Ovary	3472	6.1
Trachea, bronchus, lung	3193	5.6
Lymphoma	2203	3.9
Corpus uteri	2181	3.8
Leukemia	2024	3.6
Thyroid	1723	3.0
Stomach	1447	2.6
Others	11865	20.9
TOTAL	**56713**	**100.0**

2.1.3 Signs and symptoms of breast cancer

Signs and symptoms of the breast cancer may include formation of a new lump or mass. According to American Cancer Society (2016), usually, a painless and hard mass, which has irregular edges is more likely to be cancerous. Other possible symptoms of breast cancer such as a change in the breast shape, which is swelling of all or part of a breast, dimpling of the skin, having pain in the breast or nipple, nipple is turning inward (retraction), redness, thickening of the nipple or breast skin as well as having nipple discharge, which means the fluid comes out is other than breast milk (Healthline, 2017).

2.1.4 Risk factors of breast cancer

The risk factors for developing breast cancer including being a woman, increased in age, having a family history of breast cancer, radiation exposures and unhealthy lifestyles such as drinking alcohol and being overweight (obese) (Shah *et al*., 2014).

Breast cancer in men is a rare disease and it accounts ~1% of all breast cancer cases (Rashid *et al*., 2017). However, men also can have breast cancer but it is usually most common in women compared to men due to less of estrogen and progesterone levels in men (Allen *et al*., 2013). Both of these hormones can promote growth of cancer cell in the breast. Allen *et* al. (2013) also stated that, breast cancer is 100 times common in women compared to men whereas American Cancer Society (2016) stated that breast cancer accounts for over 32% of all invasive cancers in women and only 1% in men.

Increased of growth and aging of the population is also contributing in getting breast cancer. As a person increases in age, the risk of getting breast cancer is also high. Breast cancer is rarely found in people under 20 years old, but the incidence rates of the breast cancer is increasing and become significant after 50 years old (Allen *et al*., 2013). In

addition, invasive breast cancer is mostly found in women age 60 and older (Siegel *et al*., 2017).

Women who have a close relative or family history of the breast cancer have higher risk of developing breast cancer (Colditz *et al*., 2012). Moreover, it is important to notice that women who have a first-degree relative; mother, sister or daughter have a double-risk in getting breast cancer. Meanwhile, by having two first-degree relatives will increase women's risk to have breast cancer about three-fold (ACS, 2016).

Radiation exposure from various sources such as CT-scan and x-ray especially having radiation to the chest increases the risk of getting breast cancer (Henderson *et al*., 2010). Other than that, women who are having another type of disease such as Hodgkin's disease are at higher risk to get breast cancer (Guibout *et al*., 2005). Besides that, radiation effects on development of breast cancer in females also been proved in Japan post nuclear attack on Hiroshima and Nagasaki (Preston *et al*., 2007).

Alcoholic person has been claimed to have higher risk associated with breast cancer and the risk of getting breast cancer is directly proportional to the amount of alcohol consumption (Yip *et al*., 2006). Being physical inactive is also one of the risk factor contributing in breast cancer, where it can lead to overweight or obese (Wu *et al*., 2013). Postmenopausal women are more common to be obese because during this time the ovaries stop making estrogen and in most of a woman, estrogen comes from fat tissue, thus, the estrogen level increased and the chance of having breast cancer is also increased (Lahmann *et al*., 2004; Ritte *et al*., 2012).

2.2 Cancer chemotherapy

The word ‘chemotherapy’ is defined to any system in which chemical substances are used to treat a disease (Schnitzer and Hawking, 1964). The modern era of cancer chemotherapy began in the 1940s with the initial trial of chemical warfare known as nitrogen mustard during World War I (Gilman, 1963). Over past decades, 50 licensed drugs in the clinical practice was used for the management of malignant disease have been introduced (Fernando and Jones, 2015). The study of adjuvant chemotherapy is facilitated by the ability of combination chemotherapy to cure acute childhood leukemia and advanced Hodgkin’s disease in the 1960s and early 1970s, thus it prevailed pessimism about the ability of chemotherapy drugs to cure advanced cancers (DeVita and Chu, 2008).

Chemotherapy drugs can induce apoptosis (programmed cell death), either by directly inhibiting DNA or by targeting key proteins required for cell division (Johnstone *et al*., 2002). However, chemotherapy drugs can give toxic effects to normal cells (Fernando and Jones, 2015). In general, there are two types of chemotherapy drugs, where, they are classified by their cell cycle effects or by biochemical properties. Chemotherapy drugs that are in the same biochemical class have a similar mechanism of action (Table 2.3). Meanwhile, by classifying them based on their cell cycle specificity is crucial because it gives consequences how drugs are schedule and combined to produce maximal effect (Fernando and Jones, 2015).

Table 2.3: Biochemical classification of chemotherapy drugs by Fernando and Jones (2015).

Drug class	Mechanism of action	Examples
Alkylating agents	Impair cell function by forming covalent bonds on important molecules in proteins, DNA and RNA. Classified by their chemical structure and mechanism of covalent bonding.	Platinums (cisplatin, carboplatin, oxaliplatin) nitrogen mustards (chlorambucil, melphalan) oxazophosphorines (cyclophosphamide, ifosfamide).
Anti-metabolites	Structural analogues of naturally occuring metabolities involved in DNA and RNA synthesis. They either substitute for a metabolite that is normally incorporated in to DNA or RNA or compete for the catalytic site of a key enzyme.	Pyrimidine analogues (gemcitabine, 5-fluorouracil, capecitabine) anti-folates (methotrexate, raltitrexed).
Anti-tumour antibiotics	Intercalate DNA at specific sequences, creating free radicals which cause strand breakage. Anthracyclines are products of the fungus Streptomyces, also have mechanism of action of topoisomerase I and II, required for the uncoiling of DNA required for DNA synthesis.	Anthracyclines (doxorubicin, epirubicin), bleomycin, mitoxantrone.
Topoisomerase inhibitors	Topoisomerases are enzymes that control the 3D structure of DNA. Topoisomerase I and Topoisomerase II are enzymes responsible for the uncoiling of DNA during replication.	Topoisomerase I inhibitors (irinotecan, topotecan),

		topoisomerase II inhibitors (etoposide).
Tubulin binding drugs	Vinca alkaloids bind to tubulin, and prevent the formation of the microtubule, which is important during mitosis, but also for cell shape, intracellular transport and axonal function. Taxoids prevents the disassembly of the microtubules, thereby inhibit normal function.	Vinca alkaloids (vincristine, vinorelbine), Taxanes (paclitaxel, docetaxel).

In clinical practice, most of the combination chemotherapy consists of a few agents from various classes, thus there are several principles to achieve an effective combination chemotherapy including different phases of the cell cycle (Figure 2.3), which are targeted to achieve a maximal cell kill and there is less likelihood of resistance emerging (Fernando and Jones, 2015). Other than that, the combination of drugs that used should have action against the tumour and drugs which exert maximal effects are most preferred (Fernando and Jones, 2015). In order to achieve the most effective combination chemotherapy, the mechanism of action also should be distinct to grant addictive or synergistic effects (Fernando and Jones, 2015). In addition, the toxicities must have least overlapping to cut down the exposure of life threatening toxicity to a single organ system (Fernando and Jones, 2015).

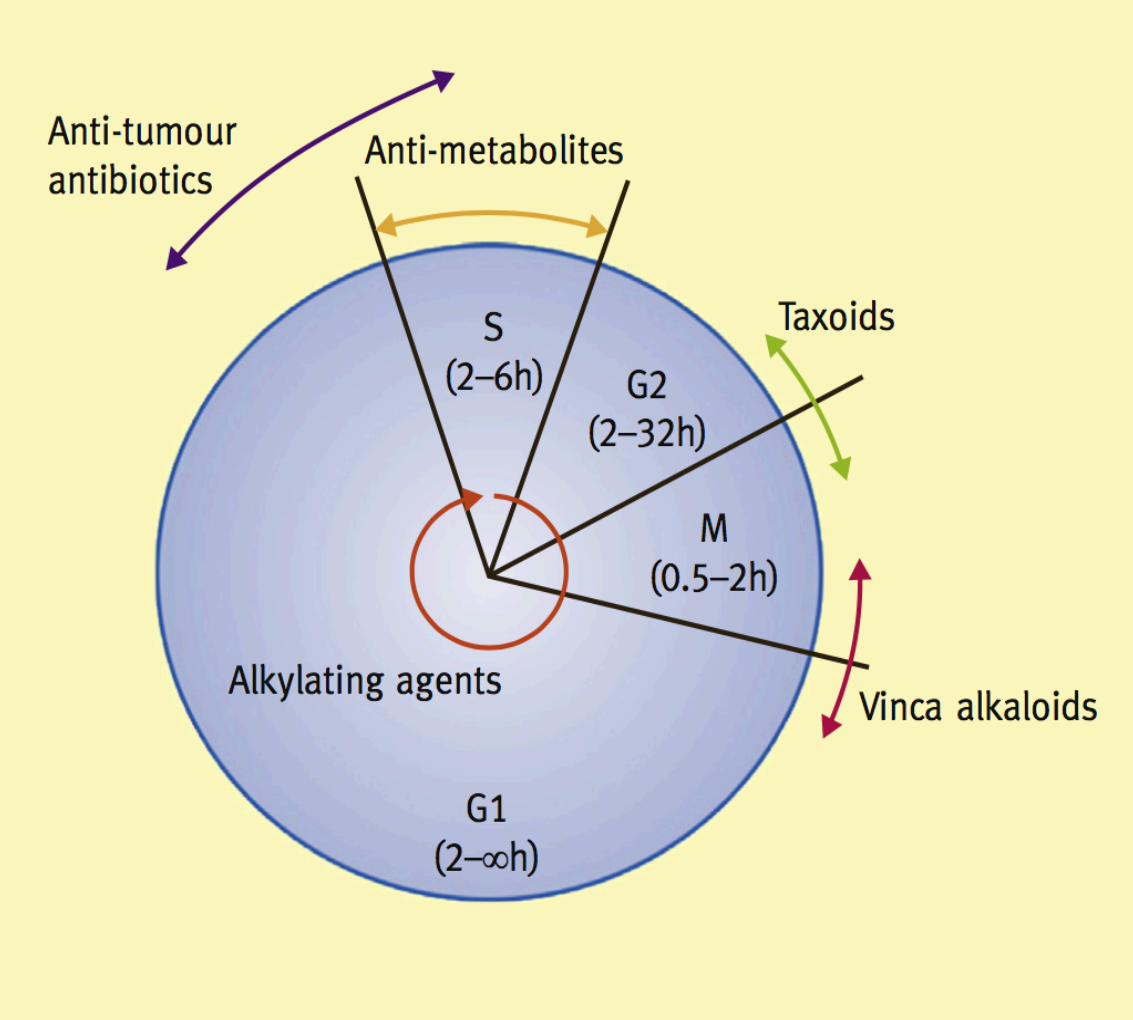


Figure 2.3: Actions of chemotherapy agents on the cell cycle by Fernando and Jones (2015).

Despite of their successful in becoming one of the way of cancer treatment, the side effects of chemotherapy have been noticed and it is based on the specific agent, dose, route and schedule of administration and several patient factors, which may be known and unknown (Fernando and Jones, 2015). The most common adverse events that occur due to chemotherapy include neutropenia, stomatitis, mucositis, diarrhea, and emesis (Hauner *et al*., 2017). However, these adverse events can be considerable if the patients are carefully educating and monitor during treatment. The competence of chemotherapy can be enhanced by doing good prophylaxis, standardized management of toxicities and limit the doses where threshold is known for the late side effects of chemotherapy (Fernando and Jones, 2015; Hauner *et al*., 2017).

2.2.1 Tamoxifen

Tamoxifen (Figure 2.4) is a non-steroidal anti-estrogen, which is the first targeted therapy using the tumor estrogen receptor (ER) as the target (Jordan, 2014). Usually, it is used to decrease the risk of early-stage, hormone-receptor-positive breast cancer after coming back from surgery (Breast Cancer, 2017). Moreover, tamoxifen is also used in treating the advanced-stage of breast cancer such as metastatic breast cancer and ductal carcinoma *in situ* (DCIS) (Breast Cancer, 2017).

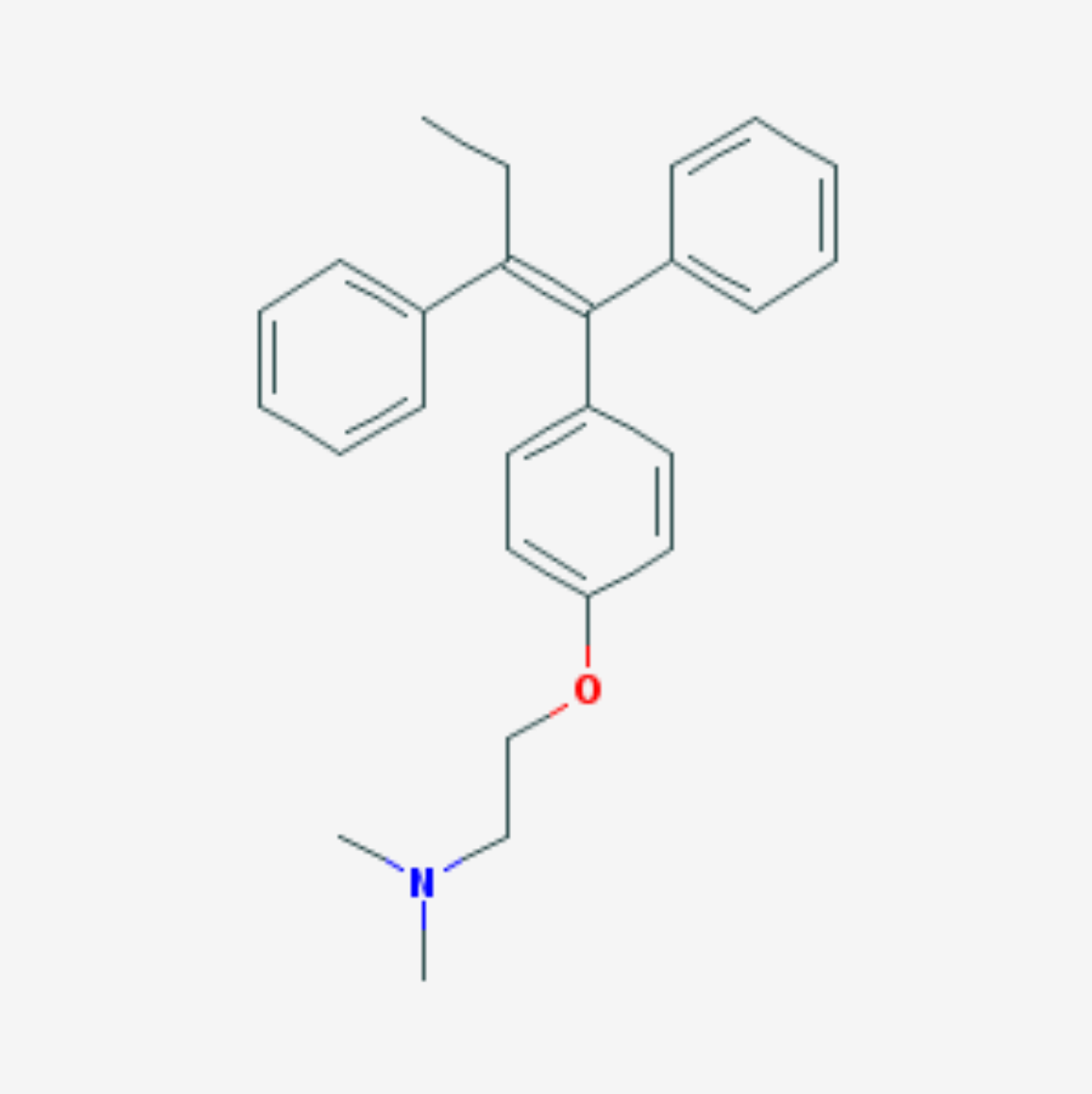


Figure 2.4: Chemical structure of tamoxifen by National Cancer Institute (2017).

There are two types of targeted approaches to anti-estrogenic therapy. Firstly, tamoxifen and its metabolites anticipate the tumor ER and it is also can avoid estrogen-stimulated growth (Maximov *et al*., 2014). Secondly, an aromatase inhibitor (AI) inhibits the production of estrogen in postmenopausal patients, therefore, the AI is considered as an adjuvant treatment of choice for postmenopausal breast cancer patients (Maximov *et al*., 2014). Generally, tamoxifen is given up to five years for longer therapy whereas a current recommendation for shorter therapy (standard therapy) is less than five years (Davies *et al.,* 2013). However, these recommendations do not increase the effectiveness or increase of patients' survival (Davies *et al.,* 2013).

Tamoxifen is one of the anti-neoplastic selective estrogen receptor modulators (SERMs), where it has specificity in tissue activities. SERM works by cutting off the response of estrogen in the breast tissue by adhering to the estrogen receptors in breast cells (Breast Cancer, 2017). The mechanism of tamoxifen begins when it completely inhibits the binding of estradiol to estrogen receptors, thus it blocks the receptor from binding to the estrogen-response element on DNA resulting in decreasing of DNA synthesis and cellular response to estrogen (NCI, 2017). The up-regulation of tamoxifen leads to the synthesis of transforming growth factor-ß (TGF-ß), a factor that constrains tumor cell growth. Moreover, tamoxifen is also can down-regulate a factor that stimulates breast cancer cell growth known as insulin-like growth factor-1 (IGF-1) (NCI, 2017).

Although tamoxifen has a potential role as a chemotherapeutic drug in treating patients with breast cancer, it has been limited by the lack of selectivity and specificity as well as numerous of adverse effects (Sultana *et al*., 2003). Gynecological side effects such as endometrial carcinoma, premalignant endometrial changes, vaginal dryness and sexual