EVALUATION OF ANTICANCER EFFECTS OF *Dioscorea esculenta* TUBER EXTRACTS ON BREAST CANCER CELL LINE AND TOXICITY TESTING

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by

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CERTIFICATE

This is to certify that the dissertation entitles "Evaluation of Anticancer Effects of *Dioscorea esculenta* Tuber Extract on Breast Cancer Cell Lines and Toxicity Testing" is fide record of research work done by Ms. Nor Amira Binti Ismail during the period February 2018 to December 2018 under my supervision.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other masters at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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-	negative
+	positive
%	percentage
<	less than
>	more than
±	approximately
°C	degree Celsius
µm	micrometer
µg	microgram
µg/ml	microgram per milliliter
µl	microliter
ATCC	American Type Culture Collection
Bcl-2	anti-apoptotic protein Bcl-2
BSLA	brine shrimp lethality assay
Ca^{2+}	calcium ion
CD95	cluster of differentiation 95 (tumor necrosis factor receptor)
cm	centimeter
CO_2	carbon dioxide
DCIS	ductal carcinoma *in situ*
DE	*Dioscorea esculenta* (Lour.) Burkill
DEAE	*Dioscorea esculenta* aqueous extract
DEME	*Dioscorea esculenta* methanol extract
DMEM	Dulbecco's Modified Eagles Medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
FBS	fetal bovine serum
$FeCl_3$	ferum (III) chloride
FITC	fluorescein isothiocyanate
g	gram
g/rcf	relative centrifugal force
G_0	gap 0 phase
GAE	gallic acid equivalent
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
HDI	human development index
HER2	human epidermal growth receptor 2
HNO_3	nitric acid
IAPs	inhibitor of apoptosis protein
IARC	International Agency for Research on Cancer
IC_{50}	inhibition concentration at 50% population
ICP-MS	inductively coupled plasma mass spectrometry
IDC	infiltrating ductal carcinoma
IU/ml	international unit per milliliter
KOH	potassium hydroxide
LCIS	lobular carcinoma *in situ*

M	molar
MCF-7	human breast adenocarcinoma cells
MDA-MB-231	human breast adenocarcinoma cells
mg	milligram
mg/ml	milligram per milliliter
ml	milliliter
mM	milli molar
mm	millimeter
MRI	magnetic resonance imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	sodium carbonate
NH_4OH	ammonium hydroxide
NIH 3T3	normal fibroblast cell
nm	nanometer
p53	tumor suppressor protein p53
$Pb(C_2H_3O_2)_2$	lead (II) acetate
PBS	phosphate buffered saline
Pen-strep	penicillin streptomycin
PI	propidium iodide
ppm	parts per million
PR	progesterone receptor
QE	quercetin equivalent
rpm	rotation per minute
SE	standard error
siRNA	small interfering RNA

SMACs	second mitochondria-derived activator of caspases
T47D	human ductal carcinoma cells
TAE	tannic acid equivalent
TNF1	type 1 tumor necrosis factor
USA	United State of America
v/v	volume/volume percent
WHO	World Health Organization

ABSTRAK

Tumbuhan ubatan telah memainkan peranan penting dalam perkembangan budaya manusia dan juga dikenali sebagai sumber yang kaya untuk ubat tradisional. Pada masa kini, tumbuhan ini digunakan untuk mengasingkan sebatian bioaktif di samping menyediakan garis panduan untuk penyelidikan kanser. Kanser adalah antara punca utama penyakit dan kematian di seluruh dunia. Penemuan agen antikanser yang berkesan dan kesan sampingan yang sedikit amat diperlukan. Usaha telah dilakukan melalui kajian ini untuk menilai kesan antikanker ekstrak ubi *Dioscorea esculenta* (DE) terhadap sel kanser payudara, ujian toksik dan pemeriksaan fitokimia. Ekstrak metanol (DEME) dan air (DEAE) DE telah disediakan oleh siri pengekstrakan menggunakan teknik makerasi dengan eter petrolium dan dietil eter. Sebatian-sebatian fitokimia yang terdapat di dalam DE ekstrak telah dinilai dan dikira. Analisis logam berat telah dilakukan melalui ICP-MS analisis dan ujian toksik menggunakan 'brine shrimp lethality assay'. Aktiviti antiproliferasi dinilai melalui ujian MTT terhadap sel fibroblast normal (NIH-3T3) dan dua sel kanser payudara (MDA-MB-231 dan MCF-7) manakala kesan apoptosis ekstrak yang menunjukkan nilai IC_{50} terendah dengan perencatan berkesan terhadap kanser payudara dianalisis oleh Annexin V / FITC PI apoptosis asai. Hasil keputusan menunjukkan bahawa DE ekstrak mengandungi banyak kandungan saponin dan ICP-MS analisa menunjukkan DE mempunyai kandungan toksik logam berat yang rendah. DE ekstrak mempunyai nilai LD_{50} > 1000 ppm yang dianggap tidak toksik terhadap nauplii udang. Sel MDA-MB-231 menunjukkan perencatan yang paling berkesan dengan nilai IC_{50} yang paling terendah apabila dirawat dengan DEME selama 72 jam. Tambahan pula, DE ekstrak menunjukkan tidak mempunyai kesan sitotoksisiti terhadap sel normal. Analisis flowsitometri telah mengesahkan bahawa MDA-MB-231 sel yang dirawat

dengan DEME telah mengakibatkan apoptosis dengan ketara apabila dibandingkan dengan kontrol. Oleh itu, DEME menunjukkan aktiviti antiproliferasi dalam MDA-MB-231 sel dengan induksi apoptosis.

ABSTRACT

Medicinal plants have played an essential role in the development of human cultures and also known as rich resources for traditional medicines. Nowadays medicinal plants are used for isolation of bioactive compounds which also provide a promising line for cancer research. Cancer is among the top causes of morbidity and mortality worldwide. Discovery of new efficient anticancer agents with reduced side effects are really needed. Effort has been made through this study to evaluate anticancer effects of *Dioscorea esculenta* (DE) tuber extract against breast cancer cell lines, toxicity testing and phytochemical screening. Methanol (DEME) and aqueous (DEAE) extracts of DE were prepared by serial extraction of maceration technique with petroleum ether and diethyl ether. Phytochemical compounds present in DE extracts were screened and quantified. The toxicity heavy metal analysis was done through ICP-MS analysis while toxicity testing by using brine shrimp lethality assay. Antiproliferative activity was evaluated by MTT assay against normal fibroblast (NIH-3T3) and two breast cancer (MDA-MB-231 and MCF-7) cell lines while apoptotic effect of extract that showed the lowest IC_{50} value with effective inhibition of breast cancer was analysed by using Annexin V/FITC PI apoptosis assay. The results revealed that DE extracts contained abundance of saponin and ICP-MS data showed DE have low concentration of trace toxic heavy metal. The DE extracts have LD_{50} values > 1000 ppm which considered non-toxic against shrimp nauplii. MDA-MB-231 cells demonstrated the most effective inhibition with the lowest IC_{50} value upon treatment with DEME for 72 hours. In addition, DE extracts showed no cytotoxicity effect towards the normal cells. Flowcytometric analysis has confirmed that MDA-MB-231 cells treated with DEME was significantly induced apoptosis incomparable with

controls. Thus, DEME demonstrated antiproliferative activity in MDA-MB-231 cells by induction of apoptosis.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer is the most prominent disease and it causes the most mortality in Malaysia and worldwide. Economical changes and transformation in social life contribute to the high incidence of cancer cases in most developing countries. Cancer may affect all people worldwide either men or women but it is noticed that different type of cancers may give a different outcome of incidence rate among the people. According to the World Health Organization (WHO), breast cancer is a common cancer among women worldwide and in 2018, it was estimated 2.1 million of new breast cancer cases have been diagnosed (International Agency for Research on Cancer (IARC), 2018) which accounting for almost 1 to 4 breast cancer cases among women (Bray *et al.*, 2018). This uncontrolled proliferation of cells which produce cell accumulation within tissues and transform into malignant cells have threatened the thousands live of women around the globe.

In Malaysia, there are 7, 593 new cases of breast cancer been reported in 2018 which represents about 17.3% of the total incidence of all type of cancers (IARC, 2018). For mortality cases, breast cancer contributed 2, 894 cases (11%) of the total death caused by cancer in 2018. However, the actual number of breast cancer incidence in Malaysia was difficult to determine because of lack data on cancer registry (Hisham & Yip, 2004). Breast cancer incidence showed the highest rate among Chinese as compared to Malay and Indian. There are multiple factors associated with the occurrence of breast cancer including decrease in childbearing and breastfeeding, increase in exogenous hormone receptor as well as dietary and lifestyle changes (Hisham & Yip, 2004). Breast cancer can be classified based on distinct histological and molecular features. Early detection of

breast cancer such as mammography is very important in order to prevent the development of malignant cells from metastasise.

Apoptosis is a programmed cell death that is an important mechanism in both physiological and pathological conditions. Apoptosis can be triggered by DNA damage, signaling imbalance or lack of oxygen supply which the cellular functions cannot be maintained and cause cell death. One of the carcinogenesis hallmarks is an evasion of apoptosis in which the malignant cells can reduce the apoptosis process and also produce apoptosis resistance. Previous studies have demonstrated that more than 50% of neoplasm occurred because of the abnormalities in apoptosis (Hotchkiss *et al.*, 2009) and recently, many of studies have been conducted to investigate the relationship between apoptosis with the development and prevention of cancer (Wong, 2011). Targeting abnormalities along the apoptosis pathway are pivotal role in the treatment of carcinogenesis and many recent therapeutic strategies have been focusing on this area. These therapeutic strategies were applied to restore the normality of apoptotic signaling pathways that give potential effect in cancer treatment.

As for breast cancer, there are many methods been practiced as treatment strategies including surgery, radiotherapy, immunotherapy, chemotherapy and monoclonal antibodies therapy. Besides high treatment cost and difficulty to access, breast cancer therapies always accompanied by the severe side effects like limited bioavailability, toxicity, non-specificity, fast clearance and restriction in metastasis (Mukherjee & Patra, 2016). Many efforts have been made to minimise the harmful side effects of drugs which were used during the process of breast cancer therapy like preventing side effects on the neighboring cells and tissues. Until today, even there are many efforts have been done, breast cancer still remains as an aggressive killer among women around the world. Therefore, the development of new, effective and affordable

anticancer drugs become high in demand and most of the researchers are focusing on medicinal plants and claimed that these plants have least of side effects (Solowey *et al.*, 2014).

Medicinal plants serve as an important part to human which help to pursue a better life. Medicinal plants have received high attention over the past 30 years for their potential as an anticancer candidate. There are approximately 60% of drugs that been used as anticancer currently derived from medicinal plants (Gordaliza, 2007) since plants are the most significant source which is easy obtain. Medicinal plants contain many natural compounds that have significant value in inhibiting the progression of breast cancer. These natural compounds can be isolated from different parts of plants like flower, flower stigmas, pericarp, sprouts, fruits, seeds, roots, rhizomes, stem, leaf, tuber, bark (Preeti, 2013). Several natural compounds including alkaloids, flavonoids, lignans, saponins, terpenes, taxanes, vitamins, minerals, glycosides, gums, oils, biomolecules and others play a major role in cancer prevention and development.

In this research, *Dioscorea esculenta* (Lour.) Burkill (DE) or locally known as "ubi itik" has been chosen as a plant of interest. There are claims from old folks with the scientific studies showed that this yam species has the potential as an anticancer agent (Dina Fatmawati & Sumarno, 2012). This plant contains a high saponin (Senanayake *et al*., 2011) which is a natural compound that has been claimed as a potential anticancer (Beit-Yannai *et al*., 2011; Subramanyam & Immanuel, 2016). Traditionally, this plant has also been used as the treatment for several diseases such as beriberi, rheumatism, swelling, ulcer, inflammation, fatigue and others (Olayemi & Ajaiyeoba, 2007). DE comes in abundant source and it is already been utilised in certain other action as a medicinal plant. Since targeting apoptosis is one of strategies in cancer prevention and treatment, the potential of DE as anticancer candidate towards breast cancer,

phytochemical profile and toxicity properties have been reported throughout this study. The findings of this study can provide support to the traditional knowledge of local people about using DE in cancer treatment. The mechanism of action of DE towards human breast carcinogenesis can be used as a guide for future medicinal practice especially in targeting apoptosis for breast cancer treatment.

1.2 Problem statement

Cancer is the second leading cause of death after heart disease which also contributes to global attention in the healthcare system. Breast cancer shows the most common cancer that causes high mortality among people worldwide (Bray *et al*., 2018) and requires effective treatment strategies. Plants are reservoirs for novel chemical entities and provide a promising line for research on cancer. Nowadays, there are many natural plants that thought to be a rich unexplored source of potent anticancer agents including yam species. In response to these problems, this study was conducted to evaluate anticancer effect of *D. esculenta* tuber extracts on breast cancer cell line and the toxicity testing.

1.3 Objectives

1.3.1 General objective

The general objective of this study is to evaluate anticancer effect of *D. esculenta* tuber extracts on breast cancer cell line and the toxicity testing.

1.3.2 Specific objectives

The specific objectives of this study are:

1) To evaluate the phytochemical compounds in DE extracts by using qualitative and quantitative analysis.
2) To investigate the heavy metal content in DE sample using inductively coupled plasma mass spectrometry (ICP-MS) and toxicity of DE extracts using brine shrimp lethality assay (BSLA).
3) To evaluate antiproliferative activity of DETE on breast cancer cell lines (MDA-MB-231 and MCF-7) and normal fibroblast cell line (NIH 3T3).
4) To investigate the apoptotic effect of DE extract that showed lowest IC_{50} value on breast cancer cell line with the most effective inhibition.

1.4 Hypothesis

The hypothesis of this study is both methanol and aqueous extract of *D. esculenta* rich with phytochemical compounds that can be used as anticancer agent. This plant contains low amount of trace toxic heavy metal and DE extracts not toxic against shrimp nauplii. Besides, DE extracts also exhibit anticancer effect against breast cancer cell line.

1.5 Significance of study

Investigating anticancer properties of DE could have provide guidance and support of the traditional usage by local people in using DE for cancer treatment. It also provides a wide range of cancer treatment with minimum of side effects. Moving towards the natural medicine development, this study could profound scope for discovery of new molecules with pharmacological significance towards the management of cancer.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

For most of the century, the leading incidence and mortality cases in the world is heart disease and followed by cancer. Cancer is the most common disease that developed among the people worldwide and thus, recently, many of studies related with cancer have been conducted by researchers. This disease become global burden to the world since its incidence and mortality continues to increase rapidly year by year.

2.1.1 Statistics of cancer

2.1.1.1 World

Based on Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) database by International Agency for Research on Cancer (IARC), 2018, there are around 18 million cancer cases around the world in 2018 and from these, 9.5 million cases were in men and 8.5 million among women. It has been estimated that the number of cancer incidence in the world will increase to more than 11 million by 2040. Global cancer incidence shows that lung (11.6%) and breast (11.6%) cancer are the most common cancers developed among the people worldwide for both sexes in 2018 (Figure 2.1). Colorectal cancer is the third most common cancer with the statistic 1.8 million new cases in 2018. In men, lung cancer is the top cancer occurred which contributes 14.5% of the total number of new cases diagnosed in 2018 followed by prostate (13.5%) and colorectal (10.9%) cancer. It has been reported that breast cancer is the most common cancer among women that contributed 24.2% of the total number of new cases diagnosed in 2018. The other cancers that occurred in women are colorectal (9.5%), lung (8.4%), cervix uteri

(6.6%) and thyroid cancer (5.1%). (International Agency for Research on Cancer (IARC), 2018)

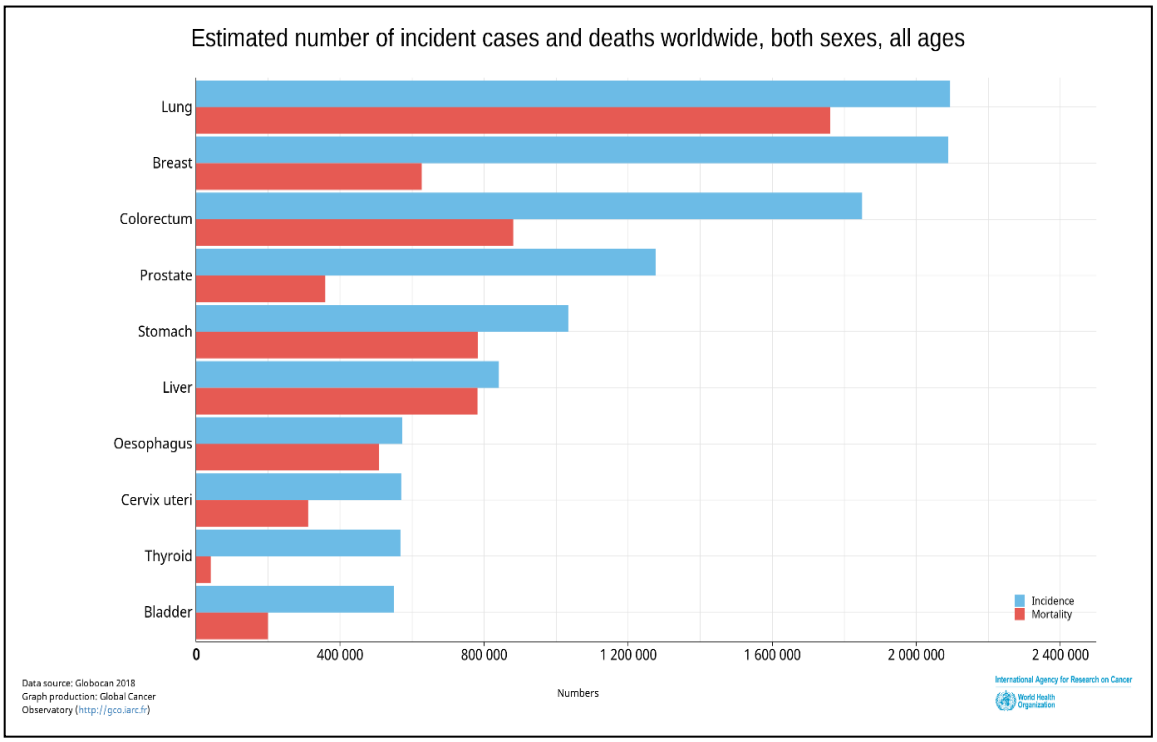

Figure 2.1: Estimated number of new cases and death cause by all type of cancers in 2018 worldwide for both sexes and all ages. Lung cancer is the most common cancer and also the cause of death among the cancer patient worldwide (Source: IARC, 2018).

2.1.1.2 Malaysia

In Malaysia, there are about 43,837 new cancer incidents (Figure 2.2) and 26,395 cancer death in 2018. Among the cancer incidences, breast cancer is the most common cancer among the people in Malaysia that contributed 17.3% of total number of cancer new cases. The second leading cancer in Malaysia for both sexes is colorectal cancer (14%) and followed by lung cancer (10.7%). Based on GLOBOCAN database by International Agency for Research on Cancer (IARC), 2018, it is estimated that new cases of cancer in Malaysia will increase to more than 40 thousand by 2040. Men contributed 20,000 while women contributed 23,000 of the total number of cancer incidence. The report from IARC, 2018 also disclosed that lung cancer was the most commonly diagnosed cancer among men followed by colorectal (16.2%) and prostate cancer (8.8%). Besides, breast cancer was common cancer occurred among women in Malaysia and it contributed 32.7% of the total number of cancer incidence in 2018. The other type of cancers that are commonly occurred among women in Malaysia are colorectum (12%), cervix uteri (7.2%) and ovary (5.5%). These percentage has been predicted to be arise by the year 2040. (International Agency for Research on Cancer (IARC), 2018)

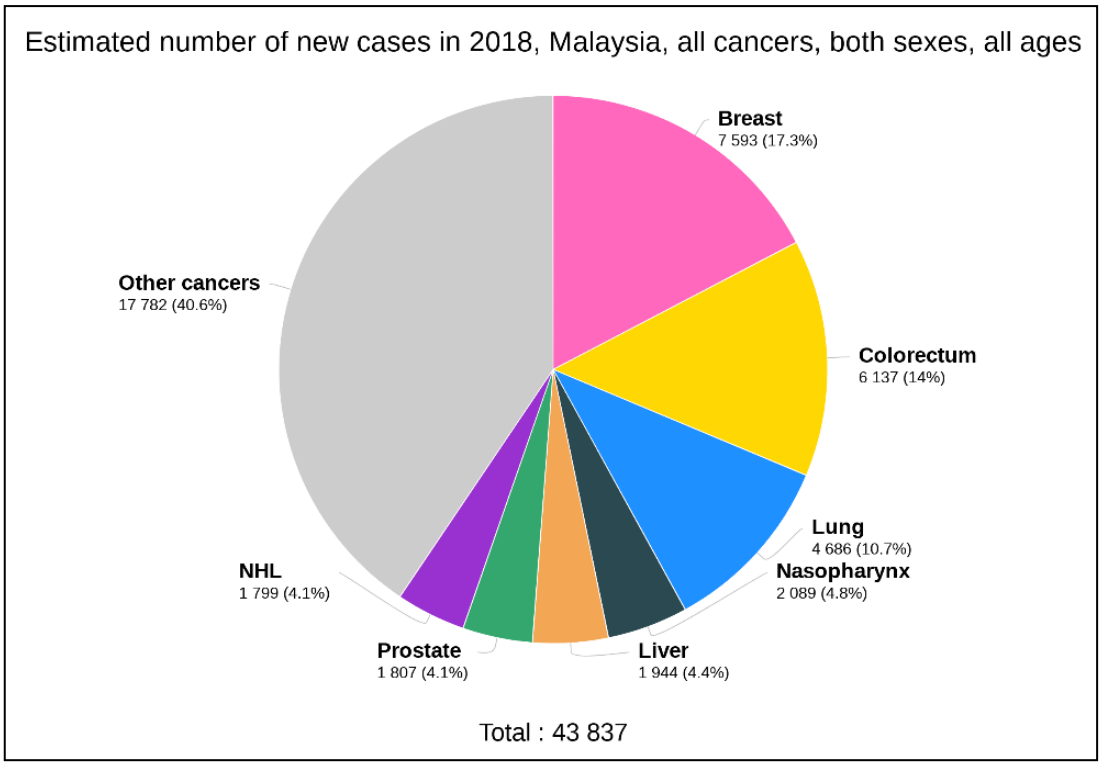

Figure 2.2: Estimated number of cancer incidence in 2018 among men and women of Malaysia population, all ages. Breast cancer is the top leading cancer among the other type of cancers (Source: IARC, 2018).

2.1.2 Definition of cancer

World Health Organization (WHO) defines cancer as the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs (WHO, 2018). Cancer was first named by the Greek physician Hippocrates, Father of Medicine that applied the Greek words "carcinoma" and "Karakinos" to describe a tumor (Amrish *et al.*, 2018; Sudhakar, 2009). Cancer is the second leading disease that cause death after cardiovascular disease (Bray *et al.*, 2018). The most common types of cancer among men are lung, prostate, colorectal, stomach and liver cancer while in women including breast, colorectal, lung, cervix and thyroid cancer (WHO, 2018). According to the National Cancer Institute, when cancer start to grows, the cells grow abnormally and can divide without stopping which may form growths known as tumour (National Cancer Institute, 2015). Most of the cancerous tumours are malignant by which they can spread to nearby tissues and some of them can travel to distant part of body through blood vessels and lymph system to form new tumours far from the original tumour (National Cancer Institute, 2015).

There are also tumours that called benign tumour which this type of tumor does not spread or invade to the nearby tissues (National Cancer Institute, 2015). The majority causes of cancer are due to environmental factors (Amrish *et al.*, 2018). Environmental factors include lifestyle and behavioural exposures. Exposure to chemicals or substances in tobacco smoke, radiation and ultraviolet rays can lead to the development of cancer. However, cancer also is a genetic disease which can develop due to heredity factor (National Cancer Institute, 2015). Cancer can be caused by the changes in genes that play important roles in controlling the cell function and growth. These genetic changes can be inherited from family or arise from the errors that occur during cell division DNA

damage. Mutation in type of genes like proto-oncogenes, tumour suppressor genes and DNA repair genes may cause cancer to develop (Amrish *et al.*, 2018).

2.2 Breast cancer

Breast cancer is the most common cancer that occurred in women and it is the second cancer that newly diagnosed and the cause of death among the other cancers type (Bray *et al.*, 2018). There are increasing in number of women that were diagnosed with breast cancer in most of the developed countries worldwide. Although breast cancer still remains arises in developed countries, breast cancer still continues to emerge as a major health problem for women in the world.

2.2.1 Breast cancer statistic

2.2.1.1 World and Asian Region

Worldwide, there are about 2.1 million of breast cancer new cases been diagnosed in 2018 (IARC, 2018) and accounting for almost 1 to 4 breast cancer cases among women (Bray *et al.*, 2018). Breast cancer is the common type of cancer been diagnosed in the majority of developed countries such as United States, China and Australia (154 of 185) in the world (Bray *et al.*, 2018). Besides, it is also the top cause of cancer death in over 100 countries worldwide (Bray *et al.*, 2018). Breast cancer incidence rates in Australia/New Zealand, Northern Europe, Western Europe, Netherlands, Southern Europe and Northern America show high incidences as compared with the other part of the world (Figure 2.3) (IARC, 2018). Age-standardised incidence shows that Australia/ New Zealand have the highest rate which is 94.2 per 100,000 (IARC, 2018). According to World Health Organization, age-standardisation rate is a weighted average of the age-

specific rate per 100,000 persons which the weights are the proportions of persons in the corresponding age groups of the WHO standard population.

In higher human development index (HDI) countries, there are elevated breast cancer incidences in 2018 which may be contributed by few factors including menstruation (early age at menarche, later age at menopause), reproduction (nulliparity, late age at first birth, and fewer children), exogenous hormone intake (oral contraceptive use and hormone replacement therapy), nutrition (alcohol intake), and anthropometry (greater weight, weight gain during adulthood, and body fat distribution) (Bray *et al.*, 2018). Human development index is an index published by the United Nations Development Program which is lists of countries in order of human achievement. This index is a summary that measure of average achievement of human development which including a long and healthy life, being knowledgeable and have a decent standard of living. The purpose of this index is to simulate global, regional and national policy discussions on issues that are relevant to human development.

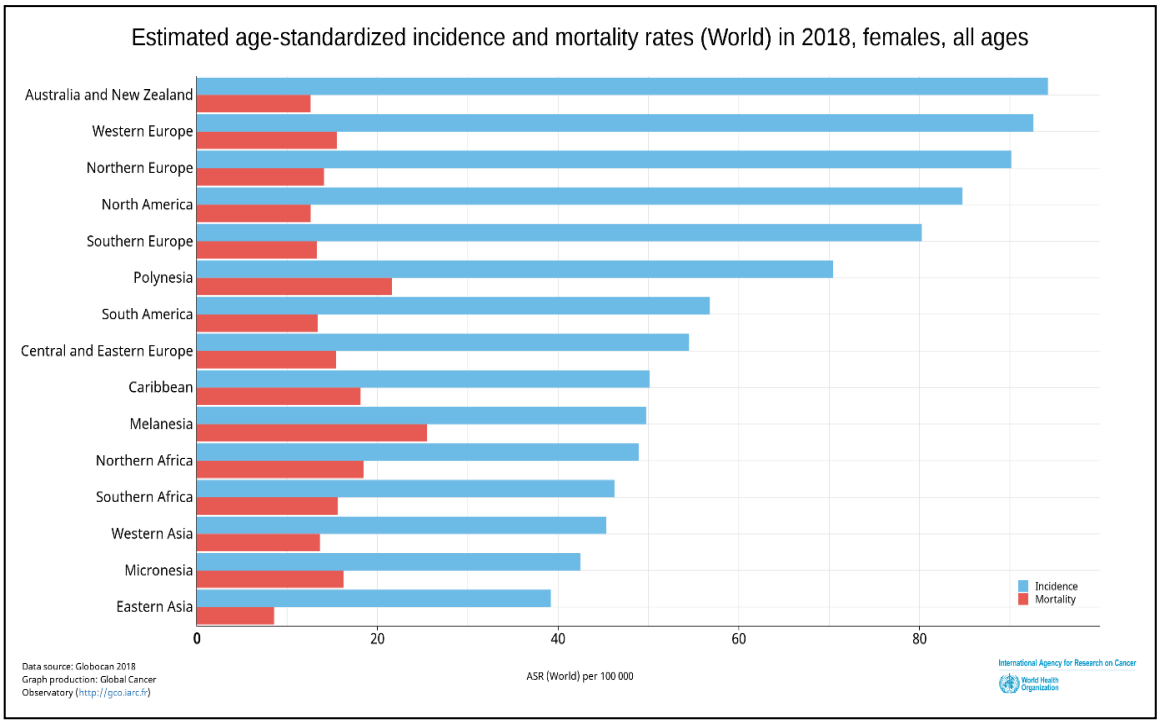

Figure 2.3: Region-specific incidence and mortality age-standardised rates for female breast cancer worldwide in 2018 (Source: IARC, 2018).

Recently, breast cancer incidence is rapidly increasing in Asian populations. In India and China, the incidence rate of breast cancer increased over the last 10 years while in Japan and Korea, there are doubled or tripled rates of newly cases diagnosed in recently as compared with the past few decades (Coughlin & Ekwueme, 2009). Based on GLOBOCAN database by the International Agency for Research on Cancer (IARC), 2018, the age-standardised incidences of breast cancer 2018 which recorded the highest rate in Asia region is Korea (310.6 per 100 000) followed by Brunei (232.8 per 100,000), Singapore (223.2 per 100,000), Japan (220.5 per 100,000) and China (182.6 per 100,000) (IARC, 2018). However, the incidence of breast cancer in Asian women are still lower compared with the other Western counterpart across all age groups (Figure 2.4) (IARC, 2018). In the future, there will be a dramatic increase of breast cancer incidence rates in Asian populations due to majority of breast cancer patients worldwide are from the Asian ethnicity (Coughlin & Ekwueme, 2009).

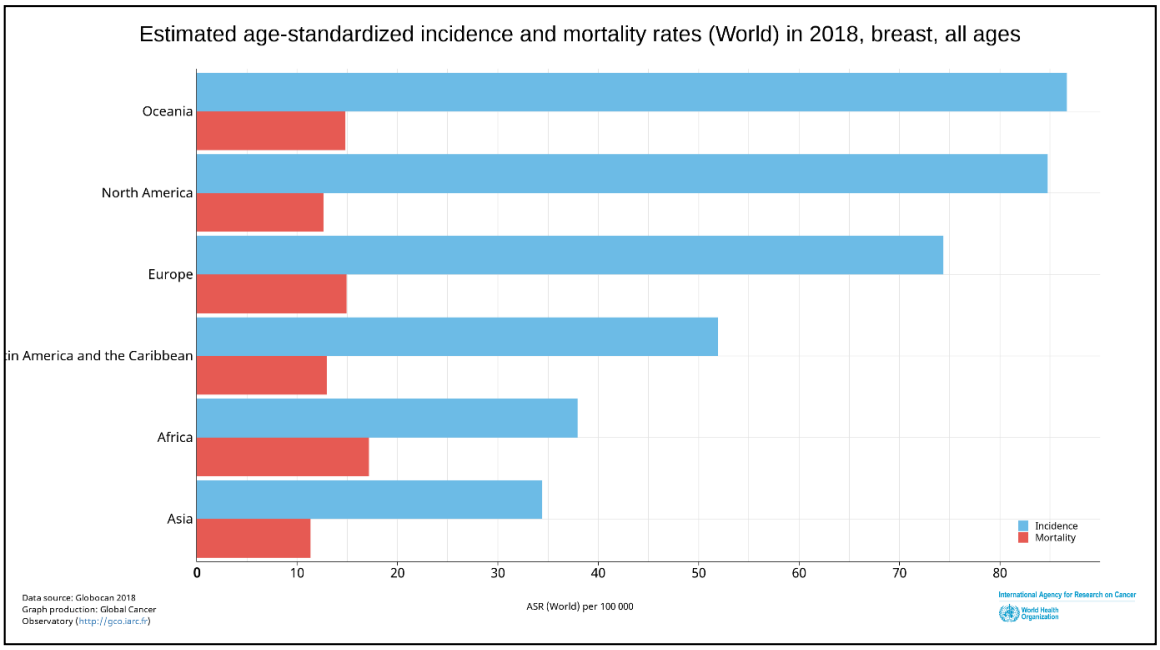

Figure 2.4: Estimated age-standardised incidence and mortality based on region-specific worldwide for female breast cancer in 2018 (Source: IARC, 2018).

2.2.1.2 Malaysia

Breast cancer is the most common cancer that occurred in women worldwide including Malaysia. According to IARC, 2018, there are 7,593 new cases of breast cancer reported in 2018 which contributed 17.3% of the total incidence of all type of cancers (Figure 2.5) (IARC, 2018). The mortality number of breast cancer is 2,894 cases (11%) of the total death 26,395 cases in 2018 (IARC, 2018). The age-standardised rate incidence of breast cancer in Malaysia has been estimated to be 47.5 per 100,000 population (IARC, 2018). However, there is some difficulty in determining the actual number of breast cancer incidence in Malaysia because of the lack of cancer registry (Hisham & Yip, 2004). Based on National Cancer Registry Malaysia, Chinese have the highest incidence of breast cancer as compared with Malay and Indian ethnicity. The different rates of breast cancer incidence among Chinese, Malay and Indian can be explained by several factors including having fewer children, having their first child later, breast-feeding for shorter periods and also higher socioeconomic status and diet (Har Yip *et al.*, 2005). Breast cancer commonly occurred among younger Malaysian women, aged between 40 to 49 years as compared to the Western women which the peak prevalence is in the aged of 50 to 59 years (Hisham & Yip, 2004). Some of Malaysian women having a strong belief in traditional medicine to treat the breast cancer compared seeking for available treatment from medical team (Hisham & Yip, 2004).

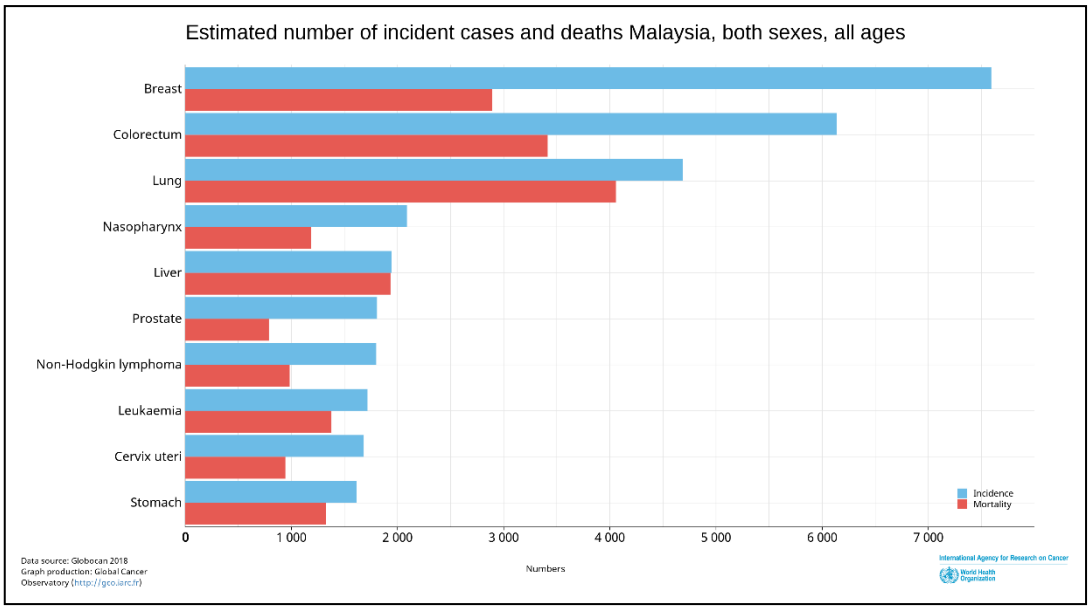

Figure 2.5: Estimated number of all type of cancers incidence and mortality among Malaysian populations in 2018. Breast cancer is the top leading cancer that been diagnosed and the cause of death (Source: IARC, 2018).

2.2.2 Breast cancer

According to the American Cancer Society, (2017), breast cancer is a disease which tumour (cancer) cells form in the breast tissues and the cells start to grow out of control (Figure 2.6). The tumor is malignant cells that invade to the surrounding tissues or spread (metastasise) to the other parts of body. Breast cancer is the most common cancer among women worldwide and it becomes a global burden since it is cause high mortality for past few decades until now. Most of the breast cancers begin in the part of breast tissue that are made up of gland for milk production (lobules) and ducts which connect the lobules to the nipple (ductal) (American Cancer Society, 2017). Historically, the physicians like Hippocrates, Galen provide description about abnormal growth in breast which refer to breast cancer (Lukong, 2017). Hippocrates believed that accumulation of black bile formed in liver during hemostasis that was not eliminated by spleen can cause breast cancer (Lukong, 2017) since most of nipple discharge or secretions are the symptoms of breast cancer (Chen *et al.*, 2012).

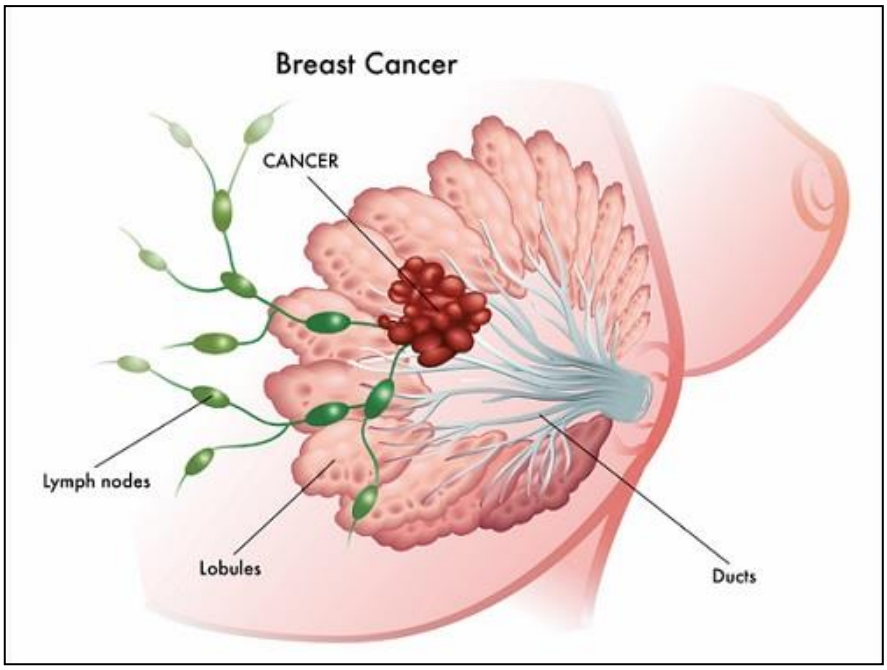

Figure 2.6: Female breast structure. Abnormal cells start to grow out of control and accumulation of cells in the breast tissues form a tumour and start to spread to the other neighbouring tissues (Source: American Cancer Society, 2017).

Breast cancer usually can be detected during screening examination either the symptoms are not developed yet or there is a lump on breast. Based on the World Health Organization, early detection is critical which can be categorised into two type, early diagnosis and screening. Both diagnosis methods are used to detect the presence of breast cancer at the early stage. For example, mammography is uses to detect the masses and identify the abnormalities in the breast through low-energy X-rays (WHO, 2018). When a patient is diagnosed with breast cancer, the breast tissues is taken by using fine needle aspiration or biopsy for further microscopic analysis. When the tumor is small, the breast cancer patient usually does not show any symptoms until the tumor becomes big or metastasised.

2.2.3 Classification of breast cancer

Breast cancer is a heterogeneous disease with histological and biological differences. Different types of breast cancer exhibit variable responses toward cancer treatment which as the outcome, it will influence breast cancer prognosis (Viale, 2012; Eliyatkin, 2015; Dai *et al.*, 2016). In the past few decades, breast cancer was divided into two subtypes based on the status of estrogen receptor (ER) (Dai *et al.*, 2016). The aim of breast cancer classification is to aid in the treatment strategies and prognosis (Malhotra *et al.*, 2010). There are 20 major types and 18 minor subtypes of breast cancer that have been categorised and included in the WHO classification (Eliyatkin, 2015). Generally, there are two types of breast cancer classifications which are histopathological and molecular classification. The implementation of these classifications make the target therapies and especially individualised treatment programs become more possible (Eliyatkin, 2015).

2.2.3.1 Histological classification

Histological classification of breast cancer is based on the morphological features of the tumour and where the tumour originated either from inner epithelial lining of ducts or lobules that supply ducts with milk (Viale, 2012; Makki, 2015). Morphological study of breast cancer showed that breast cancer can be limited to the epithelial component (*in situ* carcinoma) or invaded to other parts of tissues and become invasive carcinoma. Therefore, breast cancer is broadly divided into two types, *in situ* carcinoma and invasive carcinoma (Figure 2.7). Breast cancer then is sub-classified as either ductal or lobular and also its cytological features (Malhotra *et al.*, 2010). Ductal carcinoma *in situ* (DCIS) are epithelial cells have neoplastic proliferation limited to the ducts in which it been categorized by potential malignant capacity, cellular and nuclear atypia and non-obligate tendencies to develop invasive breast cancer (Makki, 2015).

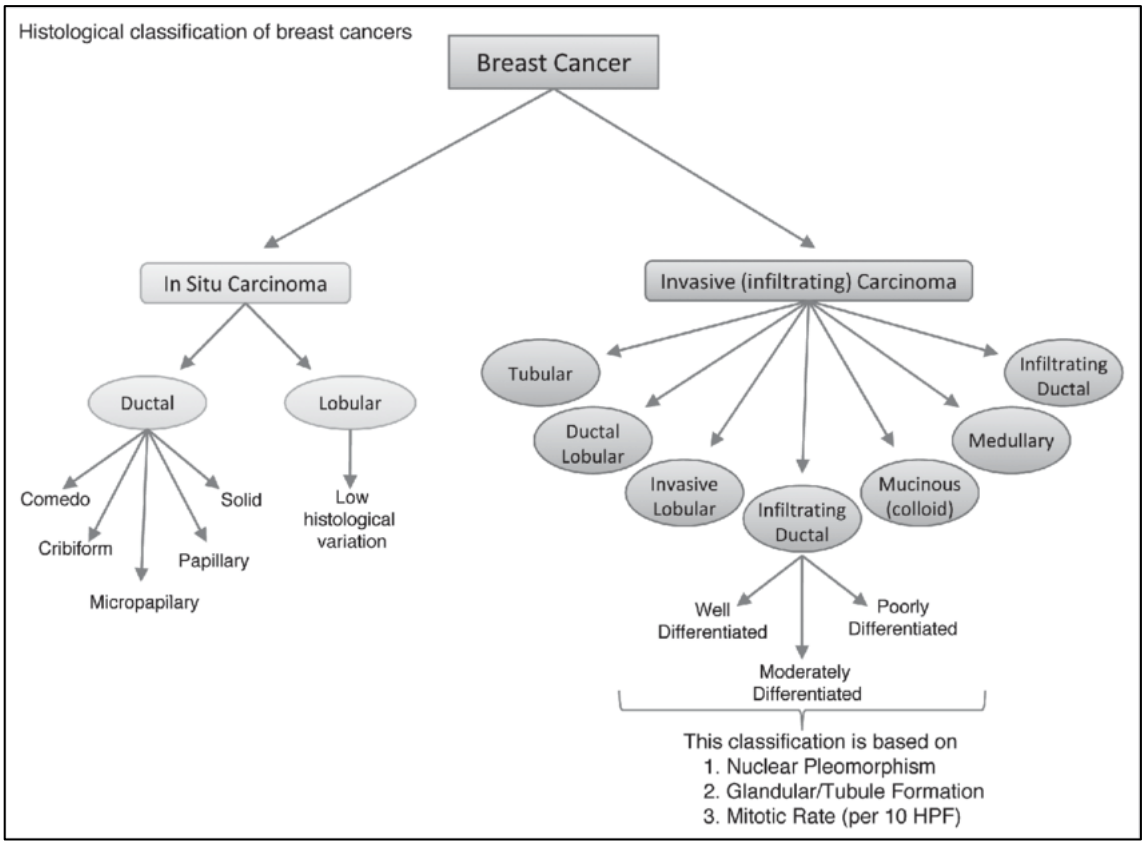

Figure 2.7: Histological classification of breast cancer. Breast cancer are classified based on the architectural features and growth patterns (Source: Malhotra *et al.*, 2010).

DCIS is considered as a precursor lesion for continuous formation of invasive breast cancer. Traditionally, DCIS has been divided into few sub-classes which based on the architectural hallmarks of tumour and they are comedo, cribiform, micropapillary, papillary and solid (Malhotra *et al.*, 2010; Makki, 2015). The grade of DCIS has been widely used to determine the severity of DCIS and there are three grades, low-grade DCIS (small, micropapillae, cribiform or solid pattern with uniform size of nucleus and regular chromatin), intermediate-grade DCIS (cytological similar with low-grade DCIS, duct contain intraluminal necrosis and coarse chromatin) and high-grade DCIS (lesion is larger than 2 mm, consists of one layer of highly atypical cells, forming micropapillae, cribiform or solid pattern with irregular contour and prominent of nucleoli) (Lakhani *et al.*, 2012; Makki, 2015). The risk of invasive carcinoma development is proportional to the grade of DCIS (Makki, 2015). According to IARC, DCIS is extremely rare to cause death and most of death due to undetected invasive component or recurrence of invasive lesion after treatment (Lakhani *et al.*, 2012).

Lobular carcinoma *in situ* (LCIS) is small intralobular proliferation and the cells are loosely cohesive (Makki, 2015). According to IARC, LCIS can be a risk factor and a non-obligatory precursor for the development of invasive carcinoma based on the long term follow-up for women with LCIS (Lakhani *et al.*, 2012). LCIS leaves the underlying architecture intact which known as lobule and the cells are small to medium size with normochromatic nuclei filling the distended lobules (Makki, 2015). The prevalence of patients with LCIS is 25% to 35% (about 1% per year) of patients that been observed for more than 20 years (Lakhani *et al.*, 2012). The other subtype of breast cancer is invasive carcinoma which are tumor heterogeneous groups differentiated into histological subtypes. There are seven major subtypes of invasive carcinoma and they are infiltrating ductal, invasive lobular, ductal/lobular mucinous (colloid), tubular, medullary and

papillary carcinomas (Malhotra *et al.*, 2010). From all these subtypes, infiltrating ductal carcinoma (IDC) is the most common subtype among women worldwide which contributed 70% to 80% of total breast cancer cases (Malhotra *et al.*, 2010; Eliyatkin, 2015; Makki, 2015). IDC is breast cancers having malignant ductal proliferation together with stroma invasion in the presence or absence of DCIS (Makki, 2015). IDC can be sub-classified into few groups which are well-differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3) where these classifications are based on level of nuclear pleomorphism, formation of glandular/tubule and mitotic index (Malhotra *et al.*, 2010; Makki, 2015).

2.2.3.2 Molecular classification

At the beginning of the new century, many efforts have been concentrated to unveil the molecular basis of breast cancer which can provide better prediction of tumor behaviour to improve therapeutic strategies. Perou *et al.*, (2000), reported that breast cancers can be classified into few distinct subgroups which based on similarities in the gene expression profiles by using microarray technology. This new approach has been accepted by medical and scientific community and this approach provides new insights into biology of breast cancer and give better effect in treatments. There are four defined subgroups of breast cancer which are luminal A, luminal B, HER2 overexpression and basal like (Perou *et al.*, 2000; Malhotra *et al.*, 2010; Makki, 2015). Luminal A is a breast cancer with estrogen receptor (ER) and progesterone receptor (PR) positive and human epidermal growth receptor 2 (HER2) negative (Perou *et al.*, 2000; Eliyatkin, 2015; Makki, 2015). The histological features that associated with luminal A breast cancer are tubular carcinoma, cribiform carcinoma, low grade invasive ductal carcinoma and classic lobular carcinoma (Eliyatkin, 2015). Luminal A accounts for 50% of the total invasive