ANTICANCER STUDIES OF *Ximenia americana* AND *Catharanthus roseus* IN HUMAN BREAST CANCER CELL LINES MCF7 AND MDA-MB-231

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Degree Celsius
µ	Micro
<	Less than
≤	Less than or equal to
≥	More than or equal to

LIST OF ABBREVIATIONS

cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
DAVID	DAVID Database for Annotation, Visualization, and Integrated Discovery
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ddH_2O	Double-distilled water
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide
EtOAc	Ethyl acetate
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
GCO	Global Cancer Observatory
GO	Gene ontology
HCL	Hydrochloric acid
hEGF	Human epidermal growth factor
HRP	Horseradish peroxidase
LCMS	Liquid chromatography mass spectrometry
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration

KEGG	Kyoto Encyclopedia of Genes and Genomes
M	Molar
m/z	Mass-to-charge ratio
MCF7	Human breast cancer cell line (estrogen receptor-positive)
MCF 10A	Non-tumourigenic breast epithelial cell line
MDA-MB-231	Human breast cancer cell line (estrogen receptor-negative)
MeOH	Methanol
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymrthoxy-pheny)-2-(4-sulfopheny)-2H tetrazolium salt
MW	Molecular weight
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
pH	Potential of hydrogen
PI	Propidium iodide
ppm	Parts per million
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate

sec	Seconds
SEM	Standard error of mean
SPSS	Statistical Package for the Social Sciences
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TBE	Tris-borate- ethylenediaminetetraacetic acid
TLC	Thin layer chromatography
TNBC	Triple-negative breast cancer
VLC	Vacuum liquid chromatography
w/v	Weight over volume
WHO	World Health Organization

LIST OF APPENDICES

KAJIAN ANTIKANSER *Ximenia americana* DAN *Catharanthus roseus* KE ATAS TITISAN SEL PAYUDARA MANUSIA MCF7 DAN MDA-MB-231

ABSTRAK

Ximenia americana adalah tumbuhan ubatan yang terdapat di kebanyakan negara di benua Afrika. Tumbuhan ini digunakan secara tradisional untuk mengubati pelbagai penyakit, termasuk kanser. Kajian ini bertujuan untuk mengkaji kesan antiproliferatif ekstrak dan fraksi daun *X. americana* terhadap titisan sel kanser payudara, serta menentukan mekanisme tindakan dan mengenalpasti bahan bioaktif utama dalam fraksi paling aktif. Pemisahan berpandukan bioasai daun *X. americana* menggunakan kromotagrafi turus terbuka, dan asai MTT, menghasilkan SFr.6.2 sebagai subfraksi paling aktif dengan kesan antiproliferatif terhadap titisan sel MCF7 dan MDA-MB-231. SFr.6.2 menunjukkan kesan antiproliferatif bergantung dos dan masa terhadap kedua-dua titisan sel kanser dan lebih selektif kepada sel MCF7. Tambahan pula, kesan antiproliferatif SFr.6.2 ke atas sel-sel MCF7 lebih signifikan daripada drug standard, tamoxifen. Nilai IC_{50} SFr.6.2 dan tamoxifen pada sel-sel MCF7 ialah 3.02 ± 0.21 dan 5.97 ± 0.54 µg/ml, masing-masing. SFr.6.2 menunjukkan keberkesanan yang rendah terhadap titisan sel bukan tumor, MCF 10A dengan index selektif 3.14 berbanding 1.64 untuk tamoxifen. Analisis menggunakan aliran sitometri menunjukkan SFr.6.2 menghentikan kitaran sel bergantung dos pada fasa G2/M serta merangsang apoptosis sel-sel MCF7. Seterusnya kandungan kimia SFr.6.2 dianalisis menggunakan LCMS-Q-TOF. Kromatogram yang diperolehi menunjukkan kehadiran sebatian bioaktif bernilai yang mungkin bertanggungjawab terhadap aktiviti antikanser SFr.6.2. Pemisahan kromatografi ekstrak daun *X. americana* juga menghasilkan tiga sebatian iaitu lupeol (1), daucosterol (2) and avicularin (3). Kajian ini melaporkan

pemisahan dan pengenalpastian sebatian bioaktif daripada daun *X. americana* yang mempunyai kesan antiproliferatif yang poten dan selektif terhadap titisan sel MCF7. Kesan antiproliferatif sebatian tersebut melalui hentian fasa G2/M dan induksi apoptosis. Sepanjang pengetahuan kami, ini adalah kajian pertama yang melaporkan berkaitan pemencilan daucosterol dari genus Ximenia. Kajian ini juga mengkaji kesan anti-migrasi dan anti-invasif ekstrak metanol *Catharanthus roseus* terhadap titisan sel kanser payudara tripel negatif (TNBC) yang sangat agresif, MDA-MB-231 dan juga untuk menentukan mekanisme tindakan molekul. Dengan menggunakan asai-asai goresan dan Transwell, ekstrak *C. roseus* menghalang secara signifikan migrasi dan invasi sel-sel MDA-MB-231 pada kepekatan bukan sitotoksik. ELISA dan zimografi gelatin menunjukkan ekstrak mengurangkan rembesan dan aktiviti MMP2 dan MMP9 dalam cara bergantung dos. Kesan ekstrak *C. roseus* ke atas ekspresi 84 gen yang sering terlibat dalam motiliti sel dinilai menggunakan Human Cell Motility RT^2 Profiler PCR Array dan Real time-PCR (RT-PCR). Pendedahan sel MDA-MB-231 terhadap ekstrak *C. roseus* pada kepekatan 4 µg/ml selama 24 jam mengakibatkan pengurangan pengawalaturan menurun yang signifikan 52 gen. Gen-gen ini terlibat terutamanya dalam degradasi matriks ekstraselular, penyusunan semula sitoskeleton, pembentukan perekatan fokal dan pembentukan invadopodia. Analisis laluan Gen ontologi (GO) dan Ensiklopedia Genetik dan Genom Kyoto (KEGG) menunjukkan bahawa gen-gen yang dikawal atur secara menurun diperkayakan dalam proses biologi dan laluan yang berkait rapat dengan motiliti sel. Kesimpulannya, kajian ini mencadangkan bahawa ekstrak *C. roseus* merencat migrasi dan invasi sel-sel MDA-MB-231 melalui pengawalaturan menurun ekspresi pelbagai gen yang terlibat dalam beberapa aktiviti migrasi sel. Ekstrak ini berkemungkinan mengandungi sebatian-sebatian yang dapat menghalang secara sinergi ekspresi sasaran-sasaran terapeutik

kritikal yang boleh dikaji dengan lebih lanjut lagi bagi menghasilkan rawatan kanser payudara di masa hadapan. Kesimpulannya, kajian ini menghasilkan data mengenai potensi terapi daun *X. americana* dan *C. roseus* terhadap kanser payudara reseptor estrogen-positif dan tripel negatif.

ANTICANCER STUDIES OF *Ximenia americana* AND *Catharanthus roseus* IN HUMAN BREAST CANCER CELL LINES MCF7 AND MDA-MB-231

ABSTRACT

Ximenia americana is a medicinal plant found in most African countries. It has been used traditionally to treat numerous diseases, including cancer. This study aimed to investigate the antiproliferative effect of *X. americana* leaves extract and fractions against breast cancer cell lines, determine the mechanisms of action and identify the major bioactive principle (s) in the most active fraction. Bioassay-guided fractionation of *X. americana* leaves using repeated open column chromatography and MTT assay resulted in the separation of SFr.6.2 as the most active subfraction with antiproliferative effect against MCF7 and MDA-MB-231 cell lines. SFr.6.2 showed dose- and time-dependent antiproliferative effect against both cell lines with more selectivity towards MCF7 cells. Moreover, the antiproliferative effect of SFr.6.2 on MCF7 was more significant than the standard drug tamoxifen. The IC_{50} values of SFr.6.2 and tamoxifen on MCF7 cells were 3.02 ± 0.21 and 5.97 ± 0.54 µg/ml, respectively. SFr.6.2 exerted less potency on non-tumourigenic cell line MCF 10A with a selectivity index of 3.14 compared to 1.64 for tamoxifen. Using flow cytometry analysis, SFr.6.2 showed dose-dependent cell cycle arrest at G2/M phase and apoptosis induction on MCF7 cells. SFr.6.2 chemical constituents were further analysed by LCMS-Q-TOF. The obtained chromatogram demonstrates the presence of valuable bioactive compounds which could be responsible for the anticancer activity of SFr.6.2. In addition to that, the chromatographic separation of *X. americana* leaves extract yielded three compounds, lupeol (1), daucosterol (2) and avicularin (3). This study reported the separation and identification of bioactive compounds from *X. americana*

leaves with potent and selective antiproliferative effect against MCF7 cell line. The antiproliferative effect of the separated compounds was mediated through arresting G2/M phase and apoptosis induction. To the best of our knowledge, this is the first report of the isolation of daucosterol from Ximenia genus. The study also investigated the anti-migratory and anti-invasive effect of *Catharanthus roseus* methanolic extract on highly aggressive triple-negative breast cancer (TNBC) cell line, MDA-MB-231 and determined the molecular mechanisms of action. *C. roseus* extract significantly inhibited the migration and invasion of MDA-MB-231 cells at non-cytotoxic concentrations in scratch and Transwell assays. ELISA and gelatin zymography showed that the extract decreased the secretions and activities of MMP2 and MMP9 in a dose-dependent manner. The effect of *C. roseus* extract in the expression of 84 genes commonly involved in cell motility was assessed using Human Cell Motility RT^2 Profiler PCR Array and Real time-PCR (RT-PCR). Treatment of MDA-MB-231 cells by *C. roseus* extract at 4 µg/ml for 24 h resulted in significant downregulation of 52 genes. These genes are mainly involved in extracellular matrix degradation, cytoskeleton reorganization, focal adhesions and invadopodia formation. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the downregulated genes are enriched in biological processes and pathways closely related to cell motility. This study suggests that *C. roseus* extract inhibits migration and invasion of MDA-MB-231 cells via downregulation of the expression of various genes implicated in several motility cellular events. The extract may contain compound (s) that could synergistically inhibit the expression of critical therapeutic targets which could be explored further for future breast cancer treatment. In conclusion, this study provided data about the therapeutic potential of *X. americana*

and *C. roseus* against estrogen receptor-positive and triple-negative breast cancer, respectively.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cancer is a group of diseases that affects almost all parts of the body. The hallmarks characterizing cancer include aberrant cell proliferation, resistance to cell death signals and spread of malignant cells to other parts of the body which is the leading cause of cancer-related death. Cancer represents the second cause of death globally. According to the latest global estimates in 2018, cancer accounts for 18.1 million new cases and 9.6 million deaths (1 person in each 6) (Bray *et al*., 2018; WHO, 2018; IARC, GCO, 2019). Moreover, these figures are projected to increase to 29.5 million new cases and 16.4 million deaths, respectively, by 2040 (IARC, GCO, 2018). Around 70% of cancer deaths occur in people living in middle- and low-income countries (WHO, 2018). The mortalities are mainly due to the absence of high-quality registry data and evidence-based control programs of cancer in most of these countries (Bray *et al*., 2018).

Based on the affected organ, cancer can be classified into various types, including lung, breast, colorectal, stomach, liver and prostate cancer. The most common type of cancer and the leading cause of cancer-associated mortality vary between different countries. In each country, the cancer incidence and death depend on the level of economic development, behavioural factors and lifestyle (Bray *et al*., 2018). The most recent figures in 2018 indicate that among all types of cancers, lung cancer represents the most frequently diagnosed type and the leading cause of cancer deaths with 2.1 million (11.6%) new cases and 1.8 million (18.4%) deaths. The second

most common form of cancer is breast cancer, accounting for 2.1 million (11.6%) new cases and 626.679 (6.6 %) deaths (IARC, GCO, 2019; Bray *et al*., 2018). Breast cancer is most frequently diagnosed in women and is very rare to occur in men with less than 1% of the incidence and less than 0.5% of mortality (Harbeck *et al*., 2019). Breast cancer remains for several years the most diagnosed type of cancer and the leading cause of cancer-related mortality among women worldwide (Bray *et al*., 2018). Figure 1.1 shows the number of new cases and deaths of breast cancer patients among females in 2008, 2012 and 2018 worldwide (Ferlay *et al*., 2015; Ferlay *et al*., 2010; Bray *et al*., 2018).

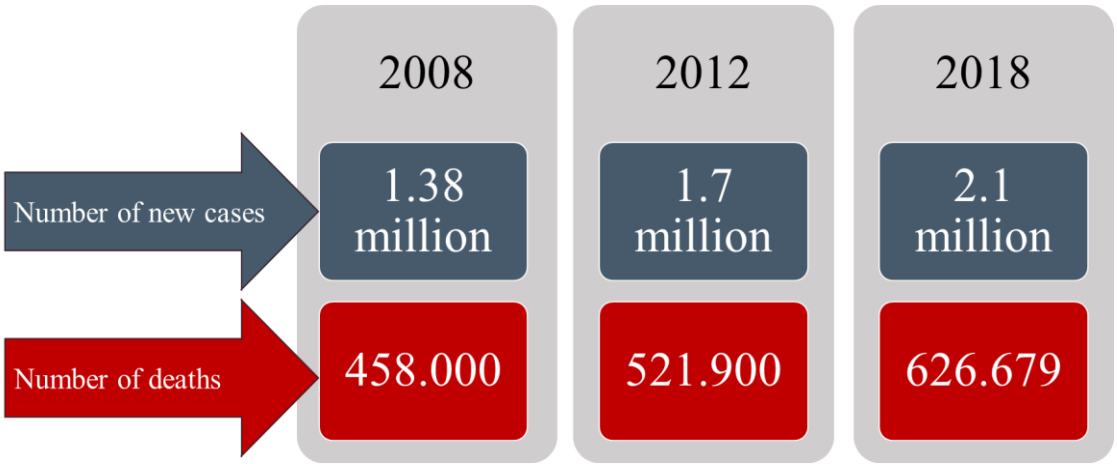

Figure 1.1 The number of new cases and deaths of breast cancer patients among women worldwide in 2008, 2012 and 2018.

In most Asian and African countries, the increase of breast cancer incidence and mortality is more rapid than the global trends and high-income countries (Azubuike *et al*., 2018). Most of the Asian countries, including Malaysia, reported an increase in breast cancer prevalence (Abdullah *et al*., 2013; Sajahan & Omar, 2018). In Malaysia, breast cancer is considered the most common type of cancer among females from all ethnic groups (Lim *et al*., 2008). In 2018 the number of new cases

and deaths among Malaysian females were 7593 (32.7 %) and 2894 (23.2 %), respectively (IARC, GCO, Malaysia, 2019). In Africa, in particular sub-Saharan African countries such as Sudan, the situation is even worse, and the incidence increase is alarming. The recent figures in 2018 of new cases and deaths were 5677 (36.6%) and 2935 (29.8%), respectively (IARC, GCO, Sudan, 2019). In Sudan, breast cancer patients are predominantly young, with about 70% being premenopausal (younger than 50 years). This is the reason why breast cancer is considered an emerging health problem in Sudan (Mariani-Costantini *et al*., 2017).

Based on the expression profile of certain markers (estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)), breast cancer can be categorised into luminal (A and B), HER-2 and basal-like subtypes (Perou *et al*., 2000). The basal-like subtype, also known as triple-negative breast cancer (TNBC), is considered the most aggressive form of breast cancer with early progression and very poor prognosis. More than 15% of breast cancer is attributed to TNBC. Treating TNBC is highly challenging due to the lack of therapeutic molecular targets (ER, PR and HER2 gene overexpression) (Yao *et al*., 2019). Patients who are diagnosed with TNBC have a high risk of developing metastasis (Yam *et al*., 2017; Yao *et al*., 2019) which is the leading cause of deaths amongst patients with breast cancer (Redig & McAllister, 2013; Hill *et al*., 2020).

The molecular heterogeneity of breast tumours is the main obstacle to effective treatment. Advances in molecular knowledge help to change the concepts of breast cancer treatment from conventional chemotherapy to targeted treatment which could help to reduce the drug-associated adverse effects. Moreover, the use of multimodal or combination therapies improves the overall outcome of patients with early breast

cancer and increases the probability of cure significantly to more than 70% (Harbeck *et al*., 2019). By contrast, patients diagnosed with metastatic breast cancer do not have sufficient options to cure their advanced disease. Despite improvements in systemic therapies over the last ten years, metastatic disease remains incurable and represents the prime cause of mortality among females with breast cancer (Hill *et al*., 2020). The current treatment strategies aim to limit metastasis-associated consequences, delay tumour growth and enhance the quality of life for patients (Harbeck *et al*., 2019).

The main approaches to treat breast cancer include surgery, radiotherapy and systemic therapies. Among different types of systemic therapies, chemotherapy represents the first-line breast cancer treatment. The main obstacles of current chemotherapeutic drugs are lack of selectivity, which causes several adverse effects, and development of various resistance mechanisms by tumour cells. The emergence of drug resistance in cancer treatment is responsible for more than 90% of cancer-related deaths (Yu *et al*., 2020; Si *et al*., 2019). The heterogeneous nature of breast cancer makes chemoresistance to be very rampant. Most of the chemotherapeutic drugs used to treat breast cancer patients may lead to treatment failure and death. Such drugs include paclitaxel, doxorubicin and 5-fluorouracil (Si *et al*., 2019). Therefore, it is imperative to search for new anticancer agents with more selectivity and specificity to improve the efficacy of breast cancer treatment. Several new approaches have been suggested to overcome chemoresistance in breast cancer. These approaches include gene therapy, immunotherapy, drug carriers, novel chemotherapeutic agents and combination treatment (Ji *et al*., 2019).

Medicinal plants represent a continuous rich source of numerous molecules with unique anticancer activities that could be effectively developed to treat cancer and reduce the adverse effects of current cancer therapies. Most of the successful discoveries of anticancer drugs were plant-derived agents that are currently in the market for the treatment of cancer patients (Amaral *et al*., 2019). Such examples include the alkaloids; vincristine and vinblastine from *Catharanthus roseus*, the alkaloid; paclitaxel from *Taxus brevifolia*, the lignan; podophyllotoxin from *Podophyllum peltatum* and the quinoline alkaloid; camptothecin from *Camptotheca acuminata* (Majumder *et al*., 2019; Xie & Zhou, 2017; Canel *et al*., 2000). In addition to the therapeutic properties of plant phytochemicals, plant derivatives represent essential substances in the field of cancer chemoprevention due to their ability to regulate various biomedical and molecular pathways associated with cell cycle; apoptosis, invasion, metastasis, angiogenesis and chronic inflammation (Zhao *et al*., 2018; Mitra & Dash, 2018). Exploring medicinal plants with ethnopharmacological knowledge to uncover the therapeutic or preventative potential of their phytochemicals is of great value. It may result in a new successful discovery to help cancer patients.

In the world, particularly in developing countries, various plant species are used to prevent and treat cancer development and progression (Greenwell & Rahman, 2015). *Ximenia americana* (*X. americana*) is a medicinal plant that has been consumed by locals in many African countries as a remedy to treat various ailments. The plant has ethnopharmacological importance in a variety of African countries like Sudan and Tanzania in treating multiple types of cancer (Adwan *et al*., 2014; Sawadogo *et al*., 2012; Monte *et al*., 2012). Case reports in a cancer clinic in Tanzania reported unexpected improvement of patients who had been diagnosed with advanced prostate cancer. The patients improved following taking a powder that has been used by

traditional healers to treat cancer. This plant material was investigated by a group of researchers to determine the source of the powder and the medicinal substances. The powder was identified to be derived from the plant *X. americana* and it was suggested to be a mixture of different parts of the plant (Voss *et al*., 2006a; Voss *et al*., 2006b; Adwan *et al*., 2014). The same authors identified the lectin riproximin as one of the active components of the plant material used in African traditional medicine (Voss *et al*., 2006b). Riproximin was found to be mainly present in the kernels of *X. americana*. Based on the above literature, *X. americana* is an important traditional cancer remedy, which has been claimed to treat patients with advanced cancer. The plant kernels of *X. americana* have been widely studied; however, very few data are available about the anticancer activities of the other parts of the plant. The systematic study of anticancer activity and bioactive chemical constituents of *X. americana* is still scarce. Information about cytotoxic activities of *X. americana* leaves and their bioactive chemical components and their mechanisms of action will help to increase the knowledge about the anticancer therapeutic properties of this important medicinal plant.

Catharanthus roseus (*C. roseus*), also known as Madagascar periwinkle, is a remarkable source of various medicinal substances. The discovery and development of Catharanthus alkaloids (vinca alkaloids) are crucial in the field of cancer therapy. Vinca alkaloids are used as chemotherapies to treat various malignancies, including breast cancer, ovarian cancer and non-small cell lung cancer (Lichota & Gwozdzinski, 2018). Vinca alkaloids act through binding, with high affinity, to tubulin causing microtubules disruption. Microtubule function and structure are crucial for cell morphology and motility. Thus, changes in microtubule dynamics can affect motility-related processes and signalling pathways. Studies have shown that the microtubule

targeting drugs such as taxanes, vinca alkaloids and colchicine are more than just antimitotic agents and they exert antiangiogenic and anti-metastatic properties (Mabeta & Pepper, 2009; Bates & Eastman, 2017). Microtubule destabilizing drugs affect angiogenesis by altering cellular contacts, suppressing sprout formation and cell motility (Mabeta & Pepper, 2009; Bijman *et al*., 2006). Moreover, microtubule destabilizing agents inhibited metastatic steps, such as cellular migration and invasion (Bijman *et al*., 2006; Su *et al*., 2016; Bates & Eastman, 2017). Although *C. roseus* has been studied extensively since the 1950s, its biological activities against the different cancer hallmarks are still not fully understood. Moreover, studies on the molecular mechanisms of anti-migratory and anti-invasive properties of *C. roseus* on cancer cell lines are lacking. Therefore, investigating the anti-migratory and anti-invasive properties of *C. roseus* crude extract and elucidation of molecular mechanisms of action on invasive types of cancer is intriguing.

1.2 Breast cancer

Breast cancer is a heterogeneous disease characterized by uncontrolled proliferation of cells that originated from mammary gland tissue. Based on the origin of cells that are involved, breast tumours are broadly classified into two categories; breast carcinomas and breast sarcomas. Breast carcinomas are the breast cancers that arise from the epithelial cells, and breast sarcomas are the types of breast cancers that arise from cells of connective tissues. Breast carcinomas represent the most frequent types of breast cancer, while breast sarcomas (phyllodes tumours and angiosarcomas) are very rare (Feng *et al*., 2018; Miyazaki *et al*., 2019). Breast carcinomas are of adenocarcinoma subtype, which is a subtype that initiates in glandular epithelial cells. The molecular mechanisms underlining the initiation of breast cancer is not yet fully elucidated. However, it is well known that the genetic alterations in breast cells drive

the carcinogenesis process. Knowledge about breast cancer development and progression is essential to fully understand the complexities of breast tumours, which will help to develop individualized therapies.

1.2.1 Breast cancer development and progression

The normal developed female breast is a mammary gland that is made up of numerous ducts and lobules surrounded by connective tissues (Figure 1.2). Normal breast cells can be transformed into malignant cells through the tumourigenesis process, in which cells keep proliferating out of signals control leading to tumour formation.

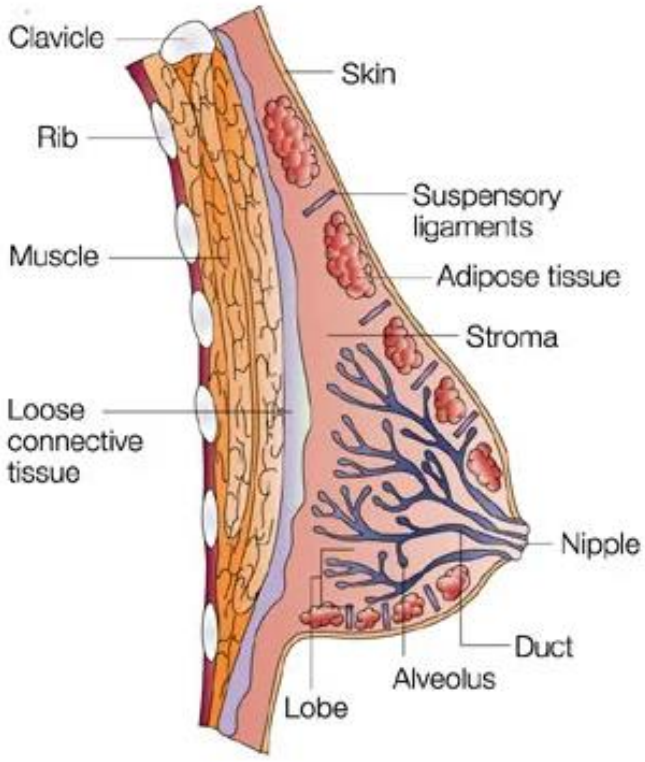

Figure 1.2 Structure of normal female breast. Adopted from Ali & Coombes, 2002.

Tumourigenesis is a complex process classified into three stages: initiation, progression and dissemination (Wang *et al*., 2017). Breast tumourigenesis can initiate in different parts of the breast, but most commonly arise in duct epithelial cells. The stages of breast cancer development and progression start with the formation and growth of the primary tumour. The primary tumour then invades the surrounding, intravasates into blood circulation, extravasates and establishes metastasis in distant sites (Mittal *et al*., 2018).

Breast tumour not only depends on its microenvironment components for its development and progression but also recruits various cells from distant sites into the stromal compartment to enhance and support the different steps of its dissemination to other sites (Hill *et al*., 2020). Moreover, tumour cells are capable of reprogramming the surrounding stromal cells, particularly innate and adaptive immune cells, to foster their survival and progression (Hinshaw & Shevde, 2019). In this contest, the transformed cells interact and cooperate with normal cells to acquire the various cancer hallmarks such as invasion and metastasis (Hill *et al*., 2020). As shown in Figure 1.3, the primary breast tumour comprises heterogeneous malignant cells surrounded by stromal cells and extracellular matrix (ECM) components. The stromal cells include normal and cancer-associated fibroblasts, immune cells, inflammatory cells and adipocytes. (Hanahan & Coussens, 2012; Mittal *et al*., 2018). The cellular paracrine interactions between these stromal components and malignant cells along with the specific genetic defect in primary transformed cells, determine the tumour characteristics and regulate its progression and spread. It has been documented that breast malignant cell migration and dissemination to other sites, often occur in the very early stage of tumour formation (Hosseini *et al*., 2016). Studies have shown that malignant tumours, including breast tumour, arrange for their dissemination and

engraft in distant secondary sites before their arrival by the formation of a suitable microenvironment, known as pre-metastatic niche (PMN), in these sites (Psaila & Lyden, 2009; Chin & Wang, 2016). PMN is the outcome of a sequence of events orchestrated by tumour secreted soluble factors (Müller *et al*., 2001) and tumour extracellular vesicles such as exosomes (Mittal *et al*., 2018). Tumour-produced exosomes enclose various molecules such as nucleic acids, MMPs, cytokines and growth factor receptors which play an essential role in breast cancer progression and dissemination (Hendrix & Hume, 2011; Mashouri *et al*., 2019).

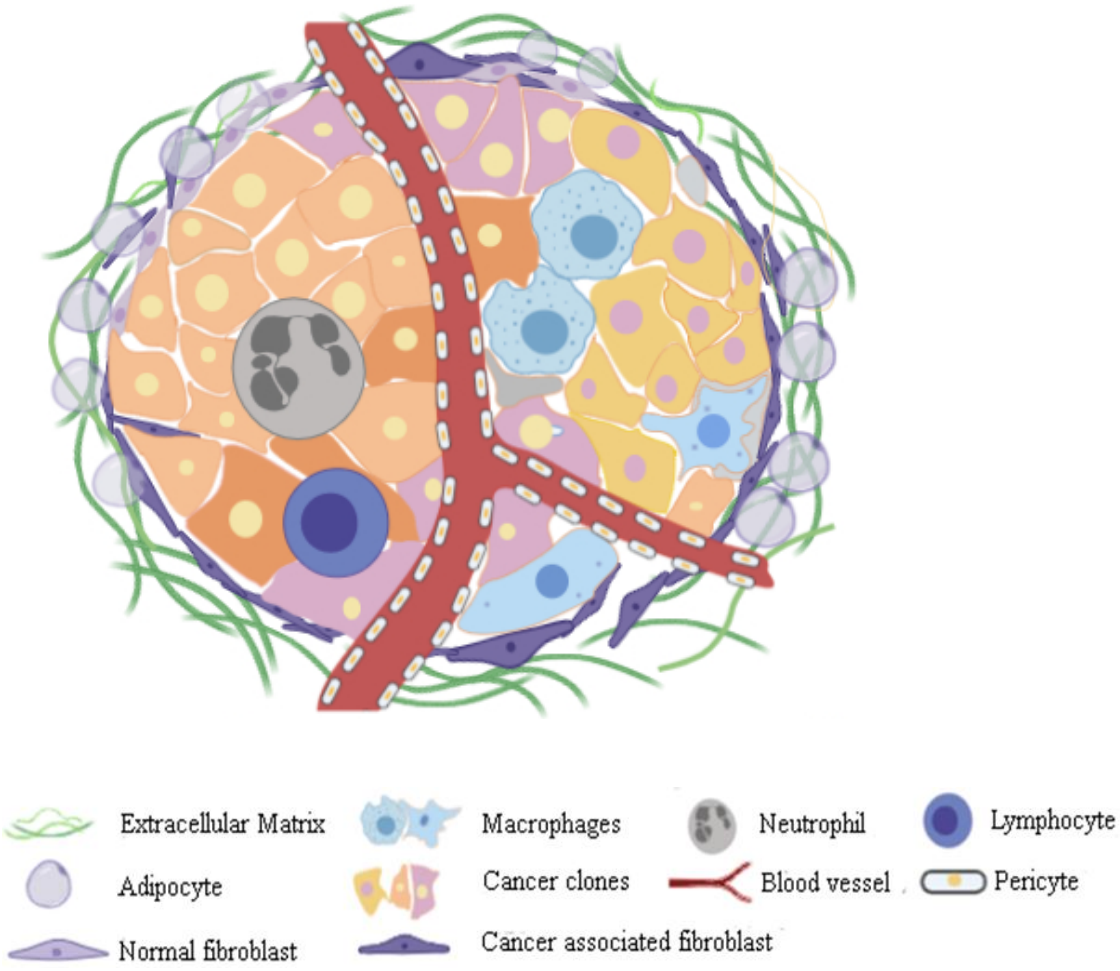

Figure 1.3 Breast tumour microenvironment components. Breast tumour consists of heterogeneous malignant cells surrounded by stromal cells. The diagram was drawn using BioRender software. The diagram was drawn based on Mittal et al., 2018.

During breast cancer progressions and following PMNs formation, malignant cells escape from the initial tumour and initiate metastasis steps by undergoing epithelial-to-mesenchymal transition (EMT) (Hanahan & Weinberg, 2011; Mittal *et al*., 2018). In this process, the cells lose their polarity and cell-cell adhesion, reduce E-cadherin expression and increase the expression of MMPs and gain migratory and invasive potential (Hanahan & Weinberg, 2011). As shown in Figure 1.4, the tumour cells move from the original tumour and intravasate into the blood vessels, as a single cell or a group of cells, and become circulating tumour cells (CTCs) (Alix-Panabières & Pantel, 2017). The hostile environment of the bloodstream makes it difficult for the CTCs to survive in the circulatory system. Only a few cells that successfully survive in the bloodstream and extravasate into other sites and become disseminated tumour cells (DTCs) (Vanharanta & Massagué, 2013). During this journey, the tumour cells interact with platelets, which provide protection to tumour cells and enhance their metastasis. The interaction between the platelets and CTCs activates TGF-β signalling pathway which promotes EMT as well as invasion and metastasis of malignant cells (Labelle *et al*., 2011; Drabsch & Ten, 2011; Xie *et al*., 2018). In addition to that, the activated platelets release different molecules to help cancer cells to maintain their viability and enhance the adhesion of cancer cells to the endothelium and facilitate extravasation (Bambace & Holmes, 2011; Tokyol *et al*., 2009). After successful extravasation, malignant cells must invade the tissue of the new site and adhere to the cells and matrix in the metastatic niche. Even after successful arrival to the metastatic niche, not all disseminated cells are capable of adapting to the new microenvironment; only a few cells, 0.01% of circulating cells, successfully colonize and initiate proliferation in secondary organs (Cheung & Ewald, 2016; Doglioni *et al*., 2019). The successfully metastasized cells locate to a unique microenvironment, the metastatic

niches, which support their growth and survival through interaction with microenvironment components. The preferable organs of metastasized breast cancer cells include bone, liver and brain (Mittal *et al*., 2018).

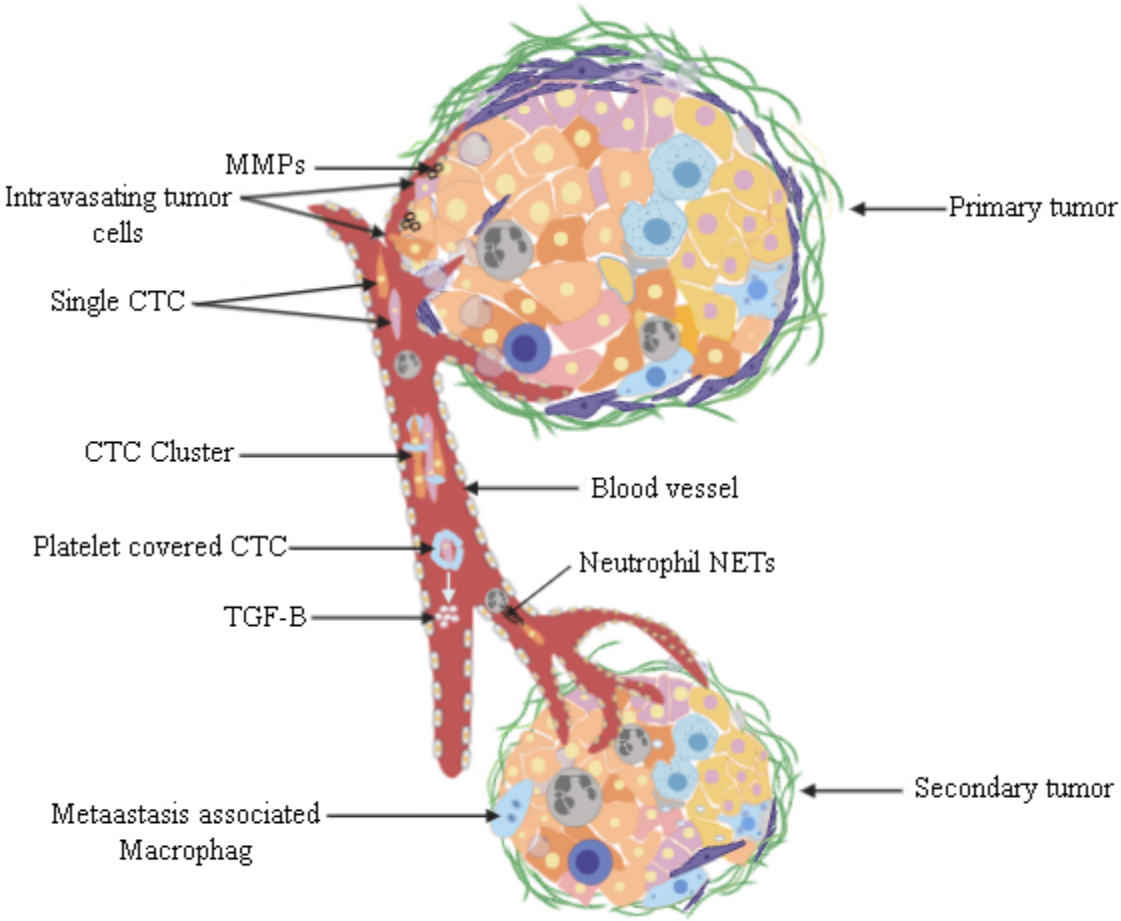

Figure 1.4 Metastatic steps and progression of breast cancer. Cancer cells intravasate into the blood vessel and become circulating tumour cells (CTCs). The CTCs interact with platelets and finally extravasate and colonize at distant secondary sites. The diagram was drawn using BioRender software. The diagram was drawn based on Mittal *et al*., 2018.

1.2.2 Breast cancer classification

Breast cancer is a highly heterogeneous disease with various morphological and molecular manifestations and several clinical presentations (Dai *et al*., 2016). The heterogeneity and complexity of breast tumours make them among the most clinically

challenging cancers in terms of diagnosis and management. Reliable classification of tumour facilitates personalised treatment which could contribute to decrease the global burden of the disease. Based on histological and pathological features, breast cancers can be classified as either preinvasive (or *in situ*) or invasive cancers. The most common histological subtypes of breast cancer (Harbeck *et al*., 2019) are shown in Figure 1.5.

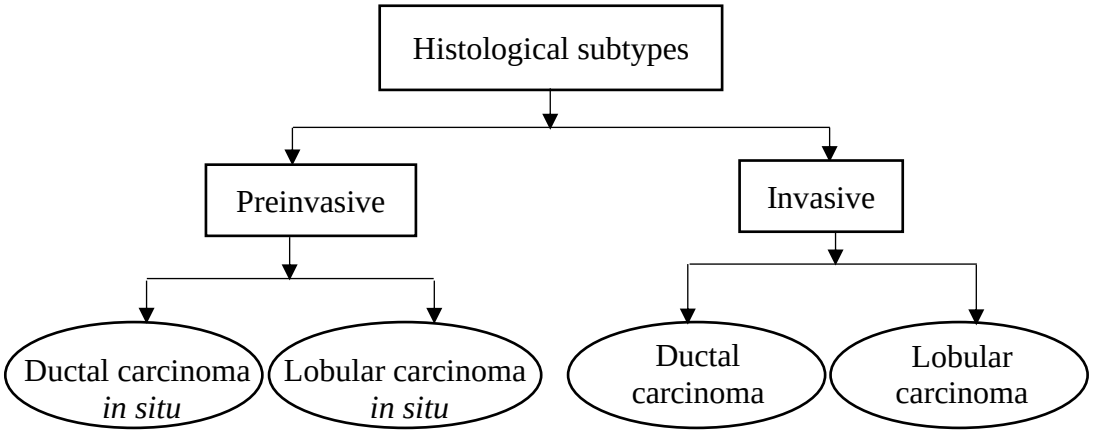

Figure 1.5 Breast cancer subtypes based on histological analysis.

Breast cancer is classified at the genomic level using intrinsic classification. This classification categorises breast cancer into four main subtypes, namely; basal-like, luminal A, luminal B and HER2-enriched (Perou *et al*., 2000). These subtypes can be distinguished based on the gene expression analysis using the Prediction Analysis of Microarray 50 (PAM50) classifier (Parker *et al*., 2009). However, the use of gene expression profiling is not practical due to the high expenses of the analysis. The more recent and current clinical practice in classifying breast cancer utilizes a surrogate classification, and it is based on immunohistochemistry analysis of hormone receptors (estrogen receptor (ER) and progesterone receptor (PR)), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67 (Harbeck *et al*.,

2019). The use of this approach enables classifying breast tumours into subtypes closely related to molecular categories. As shown in Table 1.1, the surrogate classification categorises breast tumours according to immunohistochemistry profiles into five subtypes (Harbeck., *et al*., 2019). The prognosis and percentage of incidence of each subtype are also shown in the Table.

Table 1.1 Breast cancer surrogate subtypes based on immunohistochemistry profile.

Surrogate subtype	Immunohistochemistry profile	Prognosis	Percentage (%)
Luminal A-Like	ER+, PR+, HER2-, low Ki67 index	Good	60-70%
Luminal B-Like HER2-	ER+, PR+, HER2- (ER, PR expression is lower than in luminal A-like), high Ki67 index	Intermediate	10-20%
Luminal B-Like HER2+	ER+, PR+, HER2+, high Ki67 index (ER, PR expression is lower than in luminal A-like)	Intermediate	13-15%
HER2-enriched (Non-luminal)	ER-, PR-, HER2+, high Ki67 index	Intermediate	13-15%
Triple-negative	ER-, PR-, HER2-, high Ki67 index	Poor	10-15%

(ER) estrogen receptor; (PR) progesterone receptor; (HER2) human epidermal growth factor receptor 2; (Ki67) proliferation marker.

1.2.3 Breast cancer risk factors

There are numerous well-documented factors associated with the probability of women to develop breast cancer during their lifetime. Some of these risk factors are beyond control and could not be avoided, such as gender, age, ethnicity, inherited gene mutations and family history. The most significant risk factors are being female and getting older. Females are 100 times more susceptible to initiate and develop breast cancer compared to men. The women's risk to be diagnosed with breast cancer increased with ageing as most breast cancer patients are above 55 years (Kamińska *et al*., 2015; Sun *et al*., 2017). Ethnicity is considered one of the important risk factors.

Generally, Caucasian women are slightly more likely to have breast cancer as compared to African-American women, while other races such as Native American, Hispanic and Asian women have lower rates of breast cancer cases (Yang *et al*., 2007; Kamińska *et al*., 2015; Feng *et al*., 2018).

On the other hand, inherited gene mutations and family history considered crucial risk factors. Inherited gene mutations contribute to about 5-10% of breast cancer cases. The most common parent inherited gene mutations associated with breast cancer are BRCA1 or BRCA2 gene mutations (Hulka, 1996; Mehrgou & Akouchekian, 2016). Women who have one of their close relatives diagnosed with breast cancer have a higher possibility to get the disease during their life (Sun *et al*., 2017; Ozsoy *et al*., 2017; Yang *et al*., 2007). It is of great importance for women aged over 55 or having a family history of breast cancer to have a regular mammography screening for prevention.

Another important group of factors that increases the susceptibility of developing breast cancer is the menstrual and reproductive factors. These factors include early menarche, having long menstrual cycles, using hormonal contraceptives, hormone replacement therapy, not having children and not breastfeeding (Howell *et al*., 2014; Feng *et al*., 2018).

Nowadays, modern personal lifestyle and behaviour such as alcohol consumption, obesity and lack of physical activities substantially increase the probability to be diagnosed with breast cancer (Sun *et al*., 2017; Feng *et al*., 2018). Lifestyle factors can be gradually modified to reduce the threat of breast cancer. It has been reported that regular physical activities reduce the probability of having breast cancer by 20-40% in females (Williams & Hord, 2005). The physical practices in both

premenopausal and postmenopausal women have been linked to decreases in the risk of breast cancer aetiology (Pizot *et al*., 2016; Hardefeldt *et al*., 2018). Thus, having a healthy lifestyle is a paramount factor to avoid breast cancer.

1.2.4 Breast cancer therapy

There are two main approaches to manage breast cancer which are locoregional therapies and systemic therapies. Locoregional therapies include surgery, which is the cornerstone for breast cancer treatment, and radiotherapy. Systemic therapies comprise hormonal (endocrine) therapy, targeted therapy, chemotherapy and immunotherapy. Figure 1.6 shows the main types of therapies for breast cancer patients.

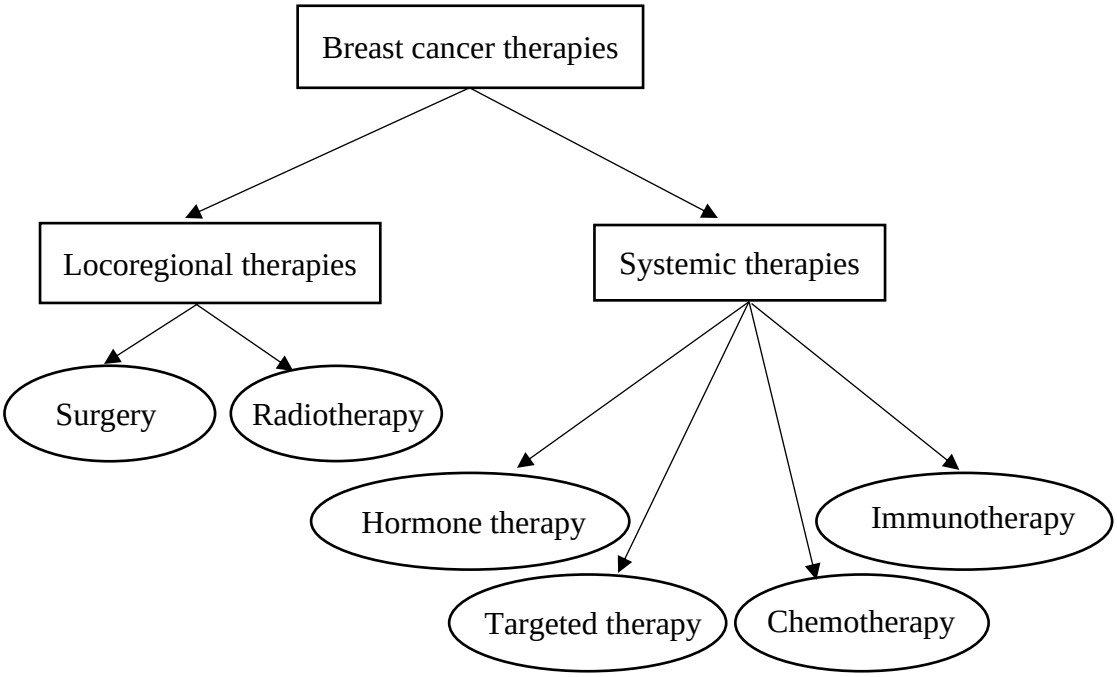

Figure 1.6 The main types of breast cancer therapies

Generally, the strategy of treating early breast cancer includes surgery (mastectomy or lumpectomy) followed by administration of radiotherapy or/and systemic therapeutic agents. Systemic therapies can be administrated before surgical operation (neoadjuvant) to minimize the tumour size or after the surgical procedure

(adjuvant) to eradicate the remaining malignant cells and prevent the relapse of the disease. The selection and effectiveness of systemic therapies mainly depend on the molecular profile of the patients. Hormone treatment or anti-estrogen drugs are the gold-standard therapy for patients with luminal breast cancers. They act through either blocking the estrogen receptors and therefore disturbing the estrogen function (tamoxifen) or inhibition of the estrogen production by body cells (aromatase inhibitors) (Szostakowska *et al*., 2019). Luminal tumours develop various mechanisms to resist these drugs which pose a new challenge in breast cancer battle (Szostakowska *et al*., 2019).

Targeted therapeutic drugs are small molecules or monoclonal antibodies that interact with specific molecular targets which are crucial for tumourigenesis processes and signalling pathways (Aggarwal, 2010; Joo *et al*., 2013). The current available targeted therapies include signal transduction inhibitors, apoptosis inducers, gene expression modulators and angiogenesis inhibitors (NCI, 2020a). Although targeted therapies are pioneering oncogenic drugs with low cytotoxicity to normal cells, however, development of resistance mechanisms by breast tumours limits their efficacy (Aggarwal, 2010; NCI, 2020a; Masoud & Pagès, 2017).

Immunotherapy or biological therapy is the use of therapeutic substances to enhance the body's immune system to fight cancer. Although the use of immunological agents to treat breast cancer patients is still early to be in clinical practice, some trials showed promising results. Recently, the use of immunotherapy such as pembrolizumab and atezolizumab significantly improves the overall outcome of breast cancer patients (Nanda *et al*., 2016; Schmid *et al*., 2017; Harbeck *et al*., 2019).

Chemotherapy is the use of cytotoxic agents such as anthracyclines, taxanes, and epipodophyllotoxins (Mackey *et al*., 2016, Nitz *et al*., 2019). Chemotherapy is used alone or along with other types of systemic therapies to improve the outcome of the patients. However, in specific breast cancer subtypes such as TNBCs, chemotherapy is the only option to minimize the breast cancer burden (Nitz *et al*., 2019). The cytotoxic drugs act through different mechanisms of action such as DNA alkylating, microtubule assembly disturbing, DNA synthesis interfering and topoisomerase suppression to kill the rapidly dividing cells (Dumontet & Jordan 2010; Magalhães *et al*., 2018). These molecules, despite their effectiveness, could fail to treat some patients due to the rapid development of resistance mechanisms. Moreover, chemotherapeutic drugs have a poor selectivity towards cancer cells and affect healthy cells as well leading to adverse effects (Baudino, 2015). Chemotherapy induces several adverse effects, including acute toxicities such as fatigue and nausea as well as chronic toxicities like cardiotoxicity, peripheral neuropathy, cognitive dysfunction and infertility (Harbeck *et al*., 2019). The chemotherapeutics-associated adverse effects could cause depression and impair the life quality of breast cancer survivors.

In summary, most of the currently available therapeutic drugs have significant drawbacks which mainly include drug resistance and adverse effect on healthy cells. These disadvantages limit the efficacy of current cancer treatment. Therefore, it is a necessity to search for new anticancer agents with better therapeutic properties to solve the problems associated with the management of breast cancer patients.

1.3 Cell cycle

The cell cycle is the process by which somatic cells divide and reproduce themselves. During the process, the cells grow, replicate their DNA and split into two

new daughter cells. In eukaryotic cells, the cell cycle comprises several phases which are Gap1(G1), DNA synthesis (S), Gap2 (G2) and Mitosis (M) (Nurse, 2002). The transitions of the dividing cell between the different phases are mediated by certain cyclins and cyclin-dependent kinases (Cdks). Each cell cycle phase is regulated by a specific cyclin/Cdk complex allowing the progression to the next stage (Figure 1.7). The sequence of the cell cycle phases is monitored by different checkpoints that respond to multiple internal/external stress to maintain the integrity of the genomic material of the new cells.

In the case of DNA damage due to exogenous or endogenous stimuli, the progression of the cell cycle can be paused by triggering the specific checkpoints. This pause, which is controlled by a variety of signals that suppress cyclin/Cdk effects, allows the affected cells to repair the DNA damage and get the required level of growth factors that enough to proceed to the next stage (Malumbres & Barbacid, 2009). In case of severe DNA damage, the dividing cell may trigger apoptotic signals and undergo apoptosis to prevent the transmission of the genetic defect to its new daughter cells. Thus, checkpoints act as monitors to ensure proper cell cycle program and the genomic stability of the generated cells (Bower *et al*., 2010; Hartwell & Kastan, 1994).

There are main four checkpoints in the cell cycle, one in each cell cycle phase (Figure 1.7). Upon DNA damage, the G1 phase checkpoint blocks the cell from entry into the S phase and prevents the damaged DNA from replication. S phase checkpoint suppresses DNA synthesis, while the G2 phase checkpoint prevents cells with damaged DNA from entering mitosis. The final checkpoint in the cell cycle is the spindle assembly checkpoint which allows cells to undergo mitosis only if the cell chromosomes are appropriately located to the spindle. Defect in these checkpoints and Cdks activities results in accumulation of DNA damage, mutations and genetic

instability which eventually lead to tumourigenesis (Kastan & Bartek, 2004; Sherr, 2000; Spoerri *et al*., 2015).

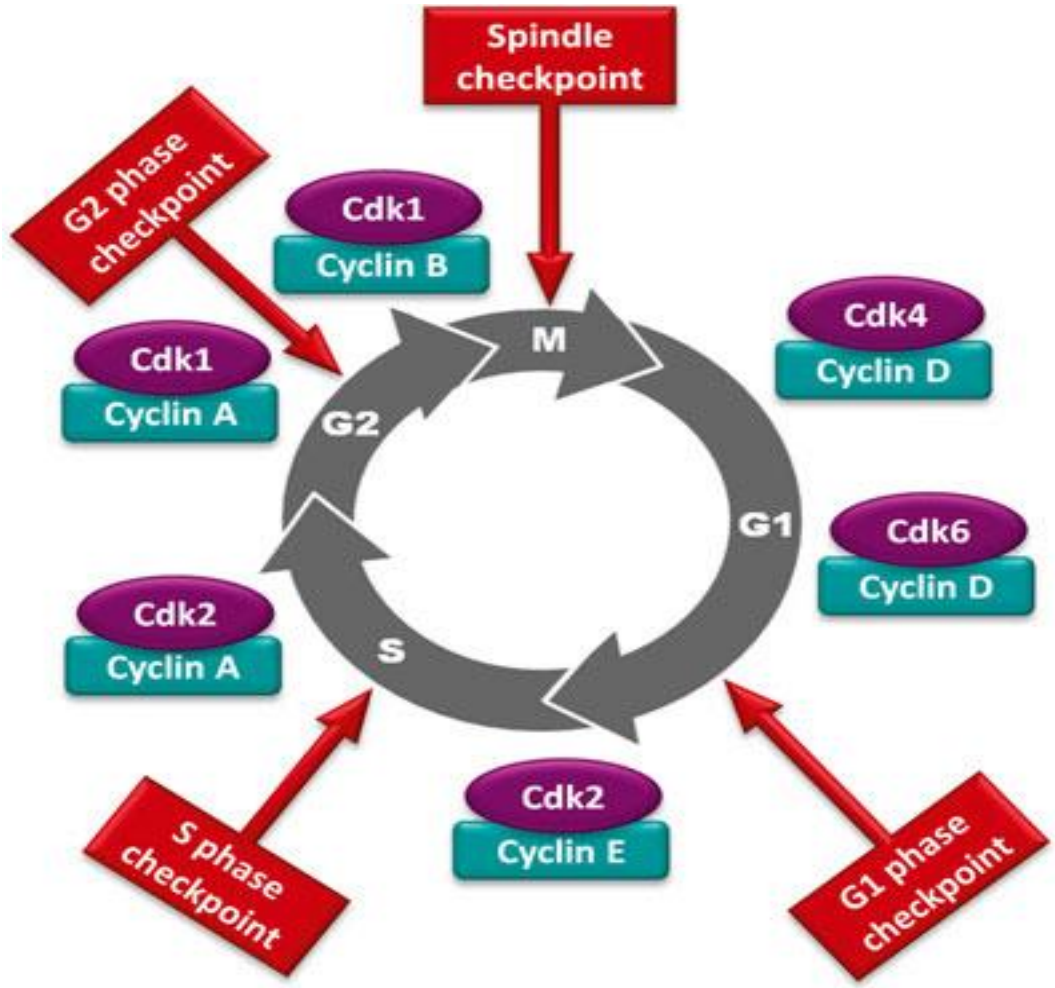

Figure 1.7 The cell cycle phases and their checkpoints. Adopted from Spoerri *et al*. (2015).

The cell cycle process and cell division are tightly controlled under normal conditions. However, in tumourigenesis cell cycle progression is dysregulated, leading to accelerated cell proliferation which is a hallmark of cancer (Hanahan & Weinberg, 2011). Increased Cdks activities due to cell cycle defect contribute significantly to cancer development and progression. The excellent knowledge about the molecular

mechanisms underlining cell cycle progression and control sheds light on specific therapeutic targets for cancer therapy. In comparison to normal cells, tumour cells are more dependent on G2 phase checkpoint to repair the damage in genomic DNA (Sun *et al*., 2018). G2 checkpoint represents an important therapeutic target for cancer therapy. Transmission of cells from G2 to M phase is mediated by B1/Cdk1(Cdc2) complex (Shangguan *et al*., 2014). Reduction in cyclin B1 and Cdk1 protein levels and suppression of B1/Cdk1 complex formation result in G2/M phase arrest (Sun *et al*., 2018).

Targeting the cell cycle mechanisms in breast cancer is a promising strategy and may lead to discover and develop a new anticancer drug. Some of the anticancer agents used for breast cancer therapy act by binding to the microtubule, arresting cells in the mitosis phase of the cell cycle and eventually lead to cell death. Examples of these agents include taxanes (paclitaxel and docetaxel), vinca alkaloids and eribulin (Dumontet & Jordan, 2010; Thu *et al*., 2018). Moreover, inhibitors of Cdks and other cell cycle regulators represent potential candidates in breast cancer therapeutics (Cai & Liu, 2017). Recently, anti-Cdk4/6 therapy using selective Cdk4/6 inhibitors (palbociclib, ribociclib and abemaciclib) has been successfully developed and approved by US FDA to treat patients with advanced ER-positive breast cancer (Wee *et al*., 2018; Lin *et al*., 2019).

1.4 Apoptosis

Apoptosis is an essential biological process that occurs in all tissues of multicellular organisms to eliminate unwanted or harmful cells in various developmental stages, homeostasis and some pathological conditions (Kerr *et al*., 1972; Elmore, 2007). Apoptosis is triggered by one of two different apoptotic routes,

the intrinsic and extrinsic signalling pathways. The intrinsic pathway, also known as the classical or mitochondrial-mediated pathway, is driven by the members of Bcl-2 family proteins. While the extrinsic pathway is mediated by cellular membrane death receptors and extracellular ligands, e.g. TNF-α and Fas (CD95L) ligands. As demonstrated in Figure 1.8, both apoptotic pathways eventually lead to the activation of the executioner caspases, caspase-3, -6 and -7. These effector caspases cleave numerous important substrates to execute the cell death process (McArthur & Kile, 2018; Baig *et al*., 2016).

Cells undergoing apoptosis can be distinguished by various morphological characteristics which include membrane blebbing, membrane protrusions and apoptotic body formation (Poon *et al*., 2014). These morphological features along with biochemical changes eventually lead to death and disassembly of an apoptotic cell into smaller cellular segments to facilitate its recognition and removal by phagocytes (Atkin-Smith & Poon, 2017; Tixeira *et al*., 2017). Understanding the molecular signalling pathways and regulatory molecules of the apoptosis process facilitates the development of apoptosis-based therapeutic molecules (Xu *et al*., 2019). In malignancy, cells escape apoptotic signals leading to the survival of malignant cells and proliferation in an uncontrolled manner (Moela & Motadi, 2015).

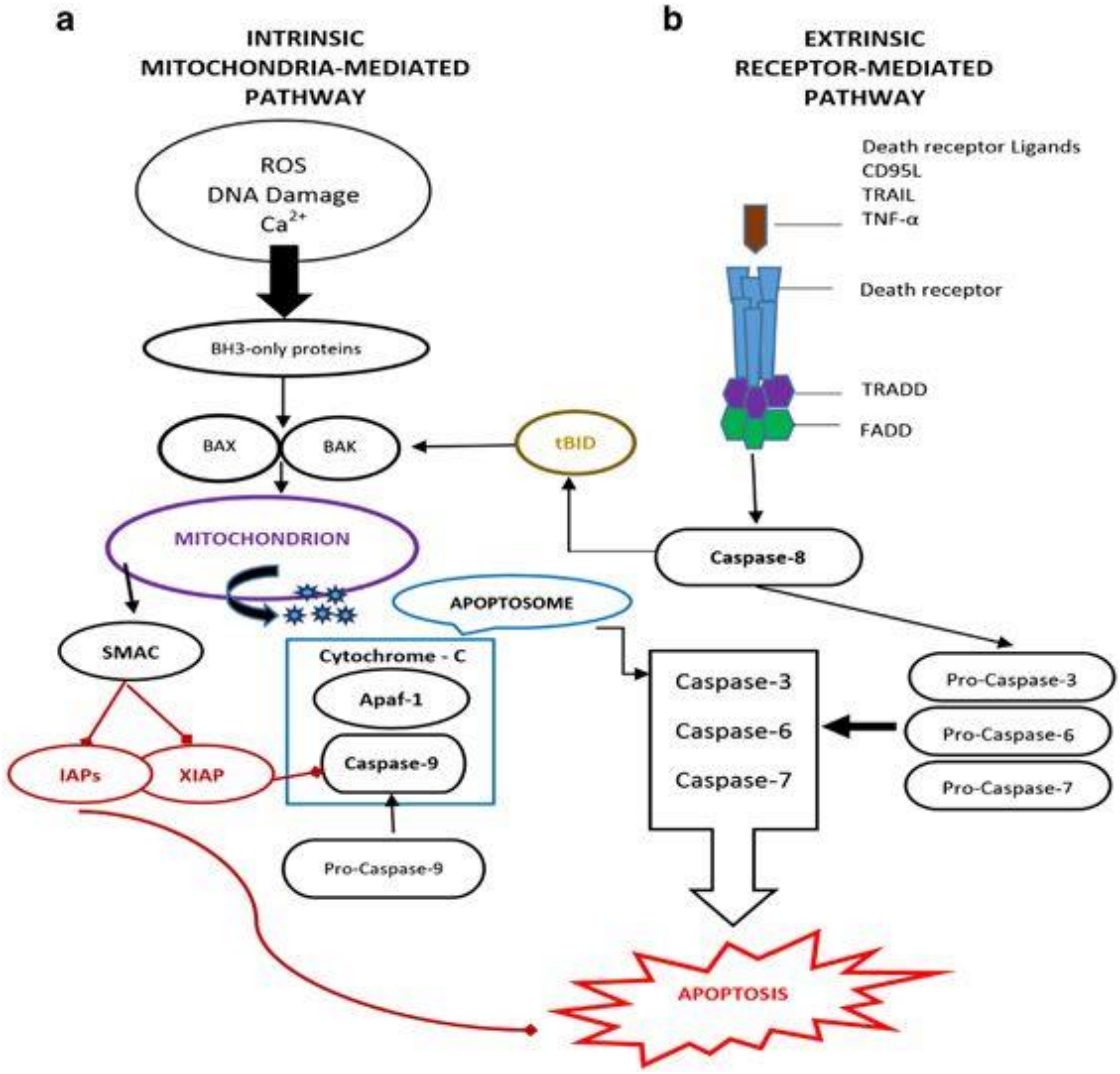

Figure 1.8 Apoptotic pathways; (a) the intrinsic pathway; (b) the extrinsic pathway. Adopted from Baig *et al*., 2016.

Malignant cells, including breast cancer cells, develop various strategies to circumvent or prevent triggering apoptotic signalling pathways, which is one of the cancer hallmarks (Hanahan & Weinberg, 2011). Such strategies include loss of P53 function, disruption of death receptors signalling pathway, suppression of caspase activities and change in the balance between pro-apoptotic and anti-apoptotic mediators (Hanahan & Weinberg, 2011; O'Brien & Kirby, 2008; Schneider & Tschopp, 2000; Wong, 2011). Several studies highlighted the significance of targeting

apoptosis-associated factors such as caspases and Bcl-2 family members in breast cancer treatment (Moela & Motadi, 2015). Targeting apoptosis signalling pathways using bioactive molecules could provide beneficial therapeutic means for cancer and other diseases (Poon *et al*., 2014)

It has been well-documented that natural compounds can promote apoptosis in breast cancer cell lines, thus represent potential therapeutic agents for breast cancer therapy. *In vitro* and *in vivo* studies reported the anticancer activity of curcumin, an active polyphenol isolated from *Curcuma longa*, in breast cancer cell lines which is primarily mediated through triggering different apoptotic pathways. Examples of curcumin targets molecules and signalling pathways include Bcl-2, Bax, P53, protein kinase B, phosphatidylinositol-3-kinase, Ras and Wnt-β catenin (Song *et al*., 2019; Lv *et al*., 2014). Furthermore, clinical studies have revealed that treatment of breast cancer patients with curcumin alone or in combination with other standard drugs showed promising improvement without exerting side effects (Song *et al*., 2019). Therefore, targeting apoptosis signalling pathways in breast tumours for therapeutic intervention is essential and might lead to the discovery and development of novel therapies.

1.5 Cell Migration and invasion

Cell migration is the movement of cells from their place to another location reacting to chemical signals. It is a fundamental procedure for different physiological processes, including morphogenesis during embryonic development, immune response and tissue maintenance (Yamaguchi & Condeelis, 2007). Cells migrate either as a group of cells (collective migration) or as a single cell (individual migration). Cellular migration consists of four main steps as illustrated in Figure 1.9. The cell migration begins with the formation of cell membrane protrusions (filopodia and

lamellipodia) at the leading edge which are temporally membrane structures regulated by actin polymerization (Bailly & Condeeli, 2002; Pollard & Borisy, 2003). Then the moving cells form focal adhesion and adhere to the ECM followed by translocation through actomyosin contraction. Finally, the dissociation of focal adhesions at the cell rear and retraction of the cell body result in cell locomotion towards the targeted direction (Ridley et al., 2003; Mattila & Lappalainen, 2008).

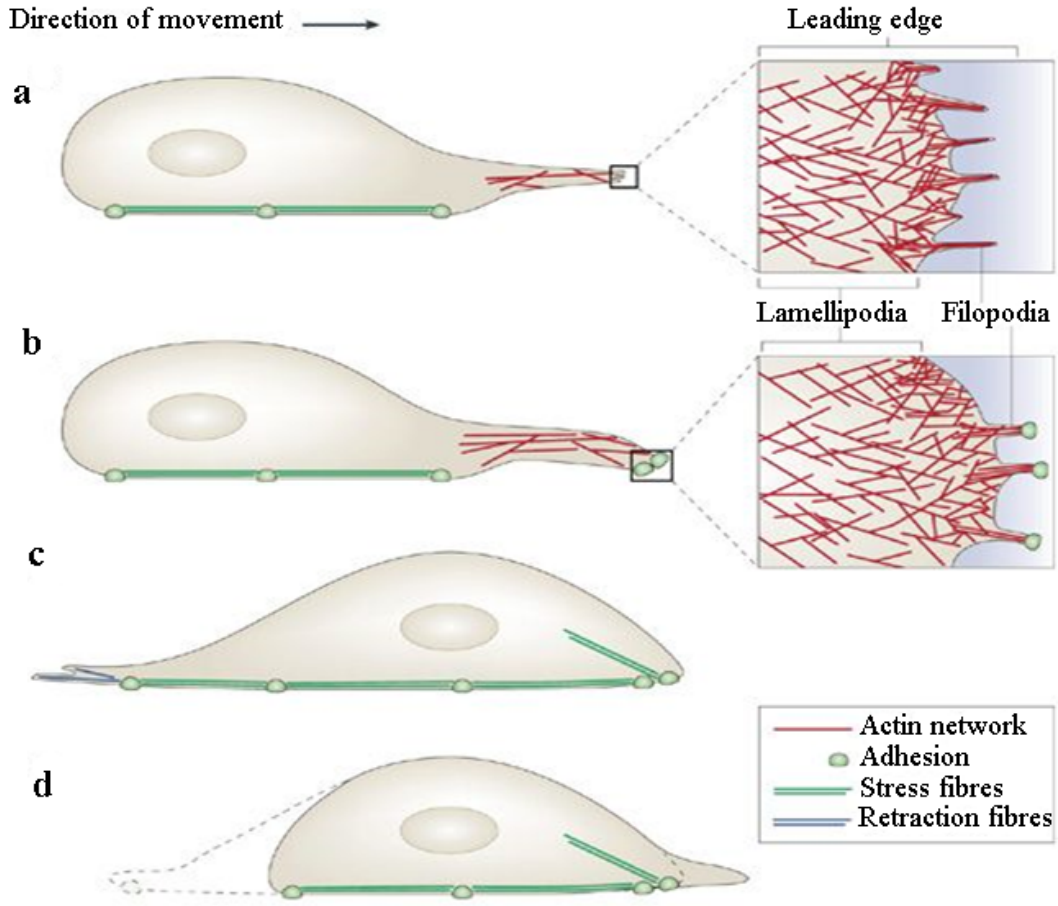

Figure 1.9 The steps of cell migration; a) formation of cell membrane protrusion (lamellipodia and filopodia); b) formation of new adhesions under the leading edge; c) translocation of the nucleus and the cell body forward through actomyosin-based contraction forces; d) disassembly of rear focal adhesions and retraction of the cell body. Adopted from Mattila & Lappalainen, (2008).

Uncontrolled cellular migration is associated with invasion and metastasis of malignant cells, including breast cancer cells (Lehtimäki *et al.*, 2016). Apparent