KOENIMBIN INDUCES APOPTOSIS IN MCF7 BREAST CANCER CELLS WITH ANTIPROLIFERATION EFFECT ON MCF7-DERIVED CANCER STEM CELLS ($CD44^{+}/CD24^{-/LOW}$) *IN VITRO*

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Abstract

Apoptosis is a fundamental cellular process in the pathogenesis of cancer, and other human diseases including neurodegeneration, diabetes and coronary disease. The origin of cancer is initiated through deregulated cellular proliferation and the inhibition of apoptotic signaling processes. Breast cancer is one the most common form of malignancy in women across the world. Breast cancer stem cells (BCSCs) involve a subpopulation of tumoral cells, expressing the stem cell-associated surface markers that have a high capacity to form tumor *in vivo*. Inhibition of breast cancer stem cells (CSCs) revealed effective and therapeutic strategies for cancer prevention. Here, we evaluated the efficacy of Koenimbin**(K)**, isolated from *Murraya koenigii*(L) Spreng, to inhibit proliferation of MCF7 breast cancer cells and target MCF7 breast cancer stem cells (CSCs) through apoptosis *in vitro*. Cell viability of Koenimbin-induced MCF7 was evaluated using MTT assay. Nuclear condensation, cell permeability, mitochondrial membrane potential (MMP) and cytochrome c release were observed using high content screening (HCS). Cell cycle arrest was examined using flow cytometry, while human apoptosis proteome profiler assays investigated the mechanism of apoptosis. Protein expression levels of Bax, Bcl2, and HSP70 were confirmed using Western blotting. The Caspase-7, -8 and -9 levels were measured, and the NF-κB activity was assessed using high content screening (HCS) assay. Aldefluor and mammosphere formation assays were respectively used to evaluate the inhibition effect of Koenimbin on number and size of mammospheres and enzymatic ALDH activity inMCF7 breast CSCs *in vitro*. It was found that Koenimbin-induced apoptosis in MCF7 cells was is mediated by cell death-transducing signals regulating MMP through down-regulating Bcl2 and up-regulating Bax, due to cytochrome *c* release from the mitochondria to the cytosol. Koenimbin significantly ($p<0.05$) induced sub-G0

phase arrest in breast cancer cells. Cytochrome *c* release triggered caspase-9 activation, which then activated caspase-7, leading to apoptotic changes. This form of apoptosis is closely associated with the intrinsic pathway and inhibition of NF-κB translocation from the cytoplasm to the nucleus. Koenimbin significantly ($p<0.05$) decreased the aldehyde dehydrogenase–positive cell population in MCF7 CSCs and significantly ($p<0.01$) decreased the size and number of MCF7 CSCs in primary, secondary and tertiary mammospheres *in vitro*. Koenimbin has potential for future chemoprevention studies, which may lead to the discovery of more cancer management strategies by reducing cancer resistance and recurrence and improving patient survival for clinical evaluation.

KOENIMBIN MENGARUH APOPTOSIS DALAM SEL KANSER PAYUDARA MCF7 DENGAN KESAN ANTIPROLIFERASI KEATAS SEL TERBITAN KANSER STEM MCF7 (CD44 + / CD24- /RENDAH) *IN VITRO*

Abstrak

Apoptosis adalah satu proses selular asas dalam patogenesis kanser serta penyakit manusia lain, termasuk degenerasi saraf, kencing manis dan penyakit jantung. Asal-usul kanser dimulai dengan proliferasi selular yang tidak teratur dan perencatan proses pemberian isyarat apoptosis. Kanser payudara adalah salah satu kanser yang paling lazim di antara wanita di seluruh dunia. Sel induk kanser payudara (BCSCs) melibatkan satu subpopulasi sel tumor yang memaparkan penanda permukaan sel induk, yang mempunyai kapasiti tinggi untuk membentuk tumor *in vivo*. Perencatan sel induk kanser payudara (CSCs) mendedahkan strategi-strategi efektif dan terapeutik untuk pencegahan kanser. Di sini, kita menilai keberkesanan Koenimbin (K) yang diperolehi daripada *Murraya koenigii*(L) Spreng, untuk merencat sel kanser payudara MCF7 dan menyasar sel induk kanser payudara MCF7 (CSCs) melalui apoptosis *in vitro*.

Kedayahidupan sel yang dirangsang oleh Koenimbin telah dinilai menggunakan ujian MTT. Kondensasi nuklear, keberasapan sel, potensi membrane mitokondria (MMP) dan pelepasan sitokrom *c* telah diperhatikan menggunakan saringan kandungan tinggi (HCS). Pemberhentian kitar sel telah dikaji menggunakan sitometri aliran, manakala ujian pemprofilan proteome apoptosis manusia menyiasat mekanisme apoptosis. Aras ekspresi protin Bax, Bcl2 dan HSP70 telah disahkan menggunakan 'Western blotting'. Aras Caspase-7, -8 dan -9 telah diukur dan aktiviti NF-κB telah dinilai menggunakan ujian HCS. Ujian pembentukan aldefluor dan mammosfera telah digunakan untuk menilai efek perencatan Koenimbin ke atas bilangan dan saiz mammosfera dan aktiviti ALDH enzimatik dalan CSCs payudara MCF7 *in vitro*.

Telah didapati bahawa apoptosis dalam sel MCF7 yang dirangsang oleh Koeninbim telah dikawal oleh isyarat transduksi kematian sel yang mengawal MMP melalui pengurangan

aras Bcl2 dan peninggian aras Bax, disebabkan pelepasan sitokrom c daripada mitokondria ke dalam sitosol. Koenimbin merangsang perencatan fasa sub-G0 secara signifikan (p<0.05) dalam sel kanser payudara. Pelepasan sitokrom c mencetuskan pengaktifan caspase-9, yang mengaktifkan caspase-7, yang menyebabkan perubahan apoptosis. Bentuk apoptosis ini berkaitan dengan laluan intrinsic dan perencatan translokasi NF-κB daripada sitoplasma ke nukleus. Koenimbin mengurangkan populasi sel aldehid nyahhidrogenasi-positif dalam CSCs MCF7 secara signifikan (p<0.05) serta mengurangkan saiz dan bilangan CSCs MCF7 dalam mammosfera primer, sekunder dan tertier *in vitro* secara signifikan (p<0.01).

Koenimbin mempunyai potensi untuk kajian pencegahan kemo di masa hadapan, yang boleh menjurus kepada menemuan lebih banyak strategi pengurusan kanser dengan mengurangkan rintangan dan pengulangan kanser serta meningkatkan kemandirian pesakit untuk penilaian klinikal.

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List of symbols and abbreviations

µg	:	Microgram
µl	:	Microlitre
µM	:	Micrometer
ACS	:	American Cancer Society
ALDH	:	Aldehyde dehydrogenase
AML	:	Acute myeloid leukemia
AP	:	Alkaline Phosphatase
ASR	:	Age Standardized Ratio
ATCC	:	American Type Culture Collection
ATP	:	Adenosine triphosphate
BCIP	:	5-Bromo-4-Chloro-3-Indolyl Phosphate p-Toluidine
BSA	:	Bovine Serum Albumin
CSC	:	Cancer stem cell
CDK	:	Cyclin-Dependent Kinase
cm	:	Centimeter
CMP	:	Cell membrane permeability
CO2	:	Carbon Dioxide
Da	:	Dalton
DAPI	:	4',6-Diamidino-2-Phenylindole
DMEM	:	Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic Acid
EDTA	:	Ethylenediamine Tetra-Acetic acid
EGF	:	Epidermal Growth Factor

EtOH	:	Ethanol
FBS	:	Fetal Bovine Serum
FCM	:	Flow Cytometry
FCS	:	Fetal Calf Serum
GSH	:	Glutathione
HCC	:	Hepatocellular carcinoma
HCS	:	High-content screening
HSP70	:	Heat Shock Protein 70
Kb	:	Kilobase
LCIS	:	Lobular Carcinoma In Situ
M	:	Molar
mA	:	Milliampere
mM	:	Millimolar
MMP	:	Mitochondrial membrane permeability
MPT	:	Mitochondrial permeability transition
M-phase	:	Mitosis phase
mRNA	:	Messenger RNA
NBT	:	Nitro-Blue Tetrazolium Chloride
NF-kB	:	Nuclear factor kappa beta
nDNA	:	Nuclear DNA
ng	:	Nanogram
PAGE	:	Polyacrylamide Gel Electrophoresis
PBS	:	Phosphate Buffered Saline
PI	:	Propidium Iodide
PN	:	Passage Number
PR	:	Progesterone Receptor

PVDF	:	Polyvinylidene Fluoride
Rb	:	Retinoblastoma
rpm	:	Revolutions Per Minute
RS	:	Replicative senescence
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEER	:	Surveillance, Epidemiology, and End Results
SEM	:	Standard Error of the Mean
SF	:	Scatter Factor
TBE	:	Tris-Borate-EDTA
TE	:	Tris EDTA Buffer
TEMED	:	N,N,N',N'-Tetramethyl-Ethylenediamine
TNF-α	:	tumor necrosis factor alpha
V	:	Volt
WS	:	Werner's syndrome

Chapter 1

Introduction

The medicinal plants are widely used for treatment and healing of various human diseases due to the different phytochemical compounds (Nostro, Germano, D'angelo, Marino, & Cannatelli, 2000). The Phytochemicals from different part of medicinal plants including flowers and blooms, fruits, leaves, stems, seeds and roots are used with individuals for inhibition of various diseases. Phytochemicals are classified into primary and secondary bioactive constituents, in which chlorophyll, proteins and sugars are included in primary compounds, while alkaloids, terpenoid and phenolic compounds are included in secondary compounds (Krishnaiah, Sarbatly, & Bono, 2007). Alkaloids and terpenoids indicate different important pharmacological properties such as anticancer, anti-inflammatory, anti-viral, inhibition of cholesterol synthesis, anti-malarial and anti-bacterial and anesthetic properties (Herouart, Sangwan, Fliniaux, & Sangwan-Norreel, 1988; Kappers et al., 2005; Mahato & Sen, 1997).

Murraya koenigii(L) *Spreng* (Sanskrit name: Surabhinimba) is a plant belonging to the Rutaceae family, and is most commonly found in the South Asian region where it is known locally as curry tree (Nakamura et al., 2013). The applications of *Murraya koenigii* are broad and varied. Its leaves, for instance, are used in the manufacturing of food condiments (Ma et al., 2013). Whereas in alternative medicine, various parts of *Murraya koenigii* are essential ingredients in the production of herbal tonics to be used to treat dyspepsia, dysentery, chronic fever, mental disorders, nausea,dropsy and diarrhea (Nakamura et al., 2013), as well as diabetes (Arulselvan & Subramanian, 2007; Arulselvan & Subramanian, 2007; Vinuthan, Kumar, Narayanaswamy, & Veena, 2007). Moreover, certain biologically active carbozole alkaloids are extracted from

M. koenigii to be used as ingredients of insect repellants (Ito et al., 2006; Nakamura et al., 2013; Rao, Ramalakshmi, Borse, & Raghavan, 2007).

The use of cell lines calls upon a paradigm shift in modern genetic, biological, drug resistance and chemotherapeutic research. Cell lines are cost-effective, and they allow many characteristics of derived cancer tissues to be retained for laboratorical studies (Burdall, Hanby, M. Lansdown, & V. Speirs, 2003; Kamalidehghan et al., 2012). Recent studies (both *in vitro* and *in vivo*) suggest that phytochemicals (biologically active compounds found in plants) possess anti-oxidant properties as well as anti-proliferative, and pro-apoptotic effects on cancer cells found in certain organs such as the brain, the breats, the colon, the human pancreas, the prostate and the skin (Arbab et al., 2013; Christensen & LeBlanc, 1996; Ibrahim MY, 2014; Ichikawa, Nakamura, Kashiwada, & Aggarwal, 2007; Isa et al., 2013; Karmakar, Banik, & Ray, 2007; Lev-Ari et al., 2007; Muhammad Nadzri et al., 2013; Paydar et al., 2014; Salim et al., 2013). The greatest virtue of these phytochemicals lies in their accessibility. As such, not only are they highly abundant in plant-based food and can therefore be easily added to an individual's diet, they are also well-tolerated by the human physiology. With such benefits, phytochemical could potentially be an effective long-term solution in the inhibition of primary tumor growth or in the prevention of tumor recurrence (Aggarwal, Sethi, Baladandayuthapani, Krishnan, & Shishodia, 2007).

Various types of cancer originate from cancer stem cells (CSCs), as recently published studies have shown (Korkaya et al., 2009; S. Liu, Dontu, & Wicha, 2005). Like normal stem cells, this small population of CSCs continually differentiate and renew themselves (possibly aided by the same signaling pathways which drive the self-renewal of normal stem cells); and this capacity for self-renewal possessed by the CSCs is what gives rise to

and sustains a tumor (Korkaya et al., 2009; Liu et al., 2006; S. Liu et al., 2005; Reya, Morrison, Clarke, & Weissman, 2001). The works of (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Allan, Vantyghem, Tuck, & Chambers, 2007; D. Fang et al., 2005; Kawasaki, Hurt, Mistree, & Farrar, 2008; Lapidot et al., 1994; C. Li et al., 2007; O'Brien, Pollett, Gallinger, & Dick, 2006; Patrawala et al., 2006; Singh et al., 2004) pioneered the idea of using phytochemicals as a deterrent against CSCs. Further studies have identified the Wnt/β-catenin, Hedgehog and Notch signaling pathways (among others) as salient regulators of CSC self-renewal (Dontu et al., 2004; Liu et al., 2006). The risks of tumor resistance/relapse is inadequately addressed by modern cancer treatments (chemo and radiation therapy) since these treatments eliminate only the cells of tumor tissues and not the CSCs (Hambardzumyan, Squartro, & Holland, 2006; Korkaya et al., 2009; Shafee et al., 2008).

Also, bioactive compounds which target the self-renewing pathways of CSCs, such as curcumin (Jaiswal, Marlow, Gupta, & Narayan, 2002; Wang, Zhang, Banerjee, Li, & Sarkar, 2006), quercetin, and epigallocatechin-gallate (Pahlke et al., 2006), may reduce the risks of tumor resistance/relapse (Liu et al., 2005).

This study therefore presents an evaluation of the efficacy of Koenimbin (**K**) (extracted from *Murraya koenigii*(L) Spreng) in the following roles: a) the inhibition of MCF7 breast cancer cells, and b) the targeting of MCF7 CSCs through apoptosis. The study will be conducted *in vitro*.

1.1 Problem Statement and significance of the Study

The development of chemotherapeutic medicines from natural sources is essential to prevent the development of cancer. Chemotherapy is a complicated procedure for treatment of different cancers, carrying a high risk because of drug toxicities and usually the more effective drugs revealed more toxicity (Comis & Carter, 1974). There are many factors for chemotherapeutic agents in order to determine its success or failure. Problems associated with side effects of chemotherapeutic agents still exist and individuals under treatment with different type of cancers have to tolerate the severe side effects and sacrifice their quality of life. The efficacies of chemotherapeutic agents are associated with many factors, comprising the type of drugs, the precondition of the patient and the specific required dosages. According to previous studies, many chemotherapeutic agents are highly hydrophobic, and therefore are insoluble in water and some other pharmaceutical solvents (Ansell et al., 2008; Ragnhammar, Hafström, Nygren, & Glimelius, 2000). Additionally, adjuvant therapies are also used for the clinical administration to maximize the effectiveness of chemotherapeutic agents and may cause and develop serious life-threatening side effects (Jones & Buzdar, 2004). Past studies have shown CSCs to be the root of various types of cancers (Korkaya et al., 2009; Liu et al., 2005). Moreover, the presence of CSCs is also the main cause of tumor resistance/relapse, since they aren't adequately dealt with by conventional cancer therapies such as chemotherapy and radiation therapy (Hambardzumyan et al., 2006; Korkaya et al., 2009; Shafee et al., 2008).

The CSC self-renewal and differentiation process (the root cause of tumor development) is regulated and sustained by the Wnt/β-catenin, Hedgehog and Notch signaling pathways

(Dontu et al., 2004; Korkaya et al., 2009; S. Liu et al., 2006; Liu et al., 2005; Reya et al., 2001).

There is a limited literature review and evidence to describe the anticancer effect of natural compounds from plants on human breast cancer cells, breast cancer stem cells and normal breast cell line and the efficacy of phytochemicals to inhibit the proliferation of cancer stem cells has been neglected for further researches (Dontu et al., 2004; S. Liu et al., 2006). In this study, the anticancer effect of Koenimbin, isolated from *Murraya koenigii*(L) Spreng, was examined on human breast cancer cell and derived human breast cancer stem cell and normal breast cell line.

1.2 Objectives

The primary objective of this paper is to study the anticancer properties of the Koenimbin extracted from *Murraya koenigii* (L) Spreng on human breast cancer cell lines.

The specific objectives of this *in vitro* study were as follows:

1- To determine the intrinsic/extrinsic apoptotic signaling pathways using Koenimbin on human MCF7 breast cancer cells.

2- To isolate the human breast cancer stem cells from MCF-7 cells

3- To evaluate the effectiveness of Koenimbin in curbing the proliferation of human breast cancer CSCs from MCF7 cells.

Chapter 2

Literature Review

2.1 *Murraya koenigii* Description and Traditional Uses

Murraya koenigii(L) Spreng (Sanskrit name: Surabhinimba) is a plant species belonging to the Rutaceae family, which includes over 150 genera and 1600 species (Saini & Reddy, 2015). It is widely distributed in South Asia (Nakamura et al., 2013) where it is commonly known as curry tree. *M. koenigii* leaves (curry leaves) found their use in the food industry, especially in the production of food condiments and flavouring agents (Ma et al., 2013). Furthermore, the stomachic properties of *M. koenigii* render them superb ingredients in the production of tonic medicine, many of which are used to treat dyspepsia, dysentery, chronic fever, mental disorders, nausea, dropsy and diarrhea (Nakamura et al., 2013), and for the management of diabetes (Arulselvan & Subramanian, 2007; Arulselvan & Subramanian, 2007; Vinuthan et al., 2007).

The bark and root of the plant is externally used for treatment of eruptions and bites of poisonous animals. Leaves and roots are also traditionally used for treatment of anthelmintic, analgesic, curing piles, inflammation, itching, leucoderma and blood disorders. Various systematic scientific review and experiments have been conducted concerning the efficacy of different parts of plant for the treatment of different diseases (Kirtikar & Basu, 1918; Nadkarni, 1994). Several chemical constituents and carbazole alkaloids with significant biological activities have been isolated from different part of *M. koenigii* (Ito et al., 2006; Nakamura et al., 2013; Parthasarathy, Zachariah, & Chempakam, 2008; Rao et al., 2007).

2.2 Origins of Cancer

Cellular multiplication is a process in which strict regulations are critical to ensure its proper completion. In particular, cellular multiplication occurs when the rate of cellular response to different survival molecular signaling pathways is higher than the rate of cellular death. The established condition is thus characterized by an equipoise between cell proliferation and cell apoptosis. However, there exist conditions in which the regulators of cell proliferation is inhibited while the cells continue to divide with varying time intervals. Under such conditions, in which the progenies of these cells are allowed to replicate in the absence of proper regulatory mechanisms, there is a distinctly high risk for the occurrence of neoplasia, which are abnormalities found in the formation of new tissues. It should be noted, however, that not all neoplasia will lead to health problems (Lodish et al., 2004).

Some mutation result in cancer but there are two important differences between cancer and genetic disorders. The first difference is that somatic cell mutations are primarily result in carcinogenesis, but in genetic disorders, the germ line mutations are as a detrimental factor at birth that may cause certain types of cancer in individuals. The second is that single mutation is unable to cause cancer, while accumulation of at least three and at most twenty mutations, in regulatory genes that are involved in cell replication mainly resulting in different types of cancer (Lengauer, Kinzler, & Vogelstein, 1998; Vogelstein & Kinzler, 2004). The effects of these accumulated mutations are observable only after an extensive period of time, which is why cancer is mainly diagnosed during an invididual's senescence (Lodish et al., 2004; Lodish et al., 2000).

Carcinogenesis is multiple steps of cellular process converting a normal cell to a cancer cell, results in different pre-neoplastic and neoplastic phenotypes and genotypes. The

multiple steps of carcinogenesis are including hyperplasia, dysplasia, abnormal morphology, anaplasia, drug- and multi-drug resistance, immortality, altered histocompatibility including even transplant ability to some heterologous species and viruses, abnormal metabolism, autonomous growth, invasiveness, and metastasis (Abelev & Eraiser, 2008; Duesberg & Li, 2003; Shabad, 1967).

Weinberg has described three signaling pathways for the occurrence of precancers including emergence and replication of precursor cells of some tumours, tumor-causing genetic alterations, and the various pathways which trigger tumoural vascularization (such as the synthesis of extracellular matrices, GFs and etc.) (Weinberg, 2006).

2.3 Breast Cancer

Breast cancer is the most common cancers among women throughout the world. The number of newly-diagnosed individuals and deaths rate were approximately 1.5 million and 460,000 worldwide, respectively (Zimonjic, Brooks, Popescu, Weinberg, & Hahn, 2001). Additionally, breast cancer is the most common cancer among three main ethnic groups including Malay, Chinese and Indian with the age range of 50-59 years among individuals in (Hisham & Yip, 2004). Moreover, Malaysian women demonstrated larger tumour size and the late stages 3 and 4, in comparison with other ethnic groups (Hisham & Yip, 2003). Although advancements in breast cancer prognosis, diagnosis and treatment have effectively been improved, the survival and death rates is still significantly high in women with breast cancer.

2.3.1 Incidence by Age

The main factors of breast cancer risk in women are including family history, age at first menstruation and genetics (Risendal et al., 2008). Data from previous studies reveal an

infrequency of breast cancer among women of age thiry. Notwithstanding, the risks of breast cancer are higher in younger women than in older women (Doll & Peto, 1981; Thomas & Kargas, 1996).

The peak age of the three Malaysian ethnic groups including Malays, Chinese, and Indians is ranged from 50 to 59 years (Hisham & Yip, 2003, 2004). The age distributions of women from each of Malaysia's three major ethnic groups (Malay, Chinese and Indian) have means of 48.1, 54.1 and 52.3 years respectively, all of which has over 50% of breast cancer patients below the age of 50 (Yip, Taib, & Mohamed, 2006).

2.3.2 Incidence by Ethnicity

Women with risk factors may develop breast cancer, and chances of developing breast cancer are higher with advancing age in which 77% of breast cancers occur in women above 50 years of age. According to the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute (USA), women belonging to certain ethnic groups, namely the Whites, the Hawaiians and the African-Americans are more susceptible to the development of invasive breast cancer. However, Vietnamese, American Indian and Korean women revealed the lowest risk of breast cancer. The African-American women within the age range of 30-69 years demonstrated the highest death rates, in which white women with age of 70 years, indicated higher mortality rates of breast cancer in comparison with African-American women (Doll & Peto, 1981; Thomas & Kargas, 1996; Woolcott et al., 2010). In 2004, Malaysia's National Cancer Registry (NCR) reported the following statistics: a) 1 in 16 Chinese women has breast cancer, b) 1 in 16 Indian women has breast cancer, and c) 1 in 28 Malay women has breast cancer (NCR, 2004).

2.3.3 Incidence by Geographical Variation

The multitude of factors associated with the occurences of breast cancer includes dietary regime, the number of pregnancies, and cultural factors. Studies which collected data across a wide variety of geographical locations (Doll & Peto, 1981; Thomas & Kargas, 1996) identify environmental, lifestyle and hereditary factors as the main causes of cancer. In those studies, researchers have examined all relevant data from analytical and clinical studies and have thus presented the number of cancers that are linked to nutrition, alcohol consuptiom, smoking and other factors. Their findings, however, did not include plausible explanations to account for the prevalence of certain types of cancer in certain geographical regions (Doll & Peto, 1981; Thomas & Kargas, 1996).

In Malaysia, the occurrence of Stage 4 breast cancer is linked to geographical differences as well (Zarihah, Mohd Yusoff, & Devaraj, 2003). For instance, approximately 50-60% of breast cancer patients in Hospital Kuala Lumpur (HKL) are in the late stages. It is worth mentioning that the majority of patients in HKL were Malays. Interestingly, most breast cancer patients in the University of Malaya Medical Center (UMMC) were Chinese. Evidently, there are notable variations in the stages of the breast cancer cases reported across different hospitals and regions in the country (Hisham & Yip, 2003).

2.3.4 Cell Cycle

All living organisms demonstrated the cell proliferation and division for over billion years. Rudolf Virchow (1858) explained, for the first time, the cell theory. The cell cycle is a cellular process involved in cell proliferation and division as well as DNA duplication. There are two particular stages in the cell cycle including interphase and mitosis. The length of the cell cycle phases in human cells is considerably over a period of 24 hours

including the G_1 phase (11 hrs), S phase (8 hrs), G_2 (4 hrs), and M phase (1 hr) (Gutkind, 2000).

The cell cycle involves the accurate contribution of cellular macromolecule synthesis, anabolic and catabolic processes. The cell cycle is including DNA replication and the chromosomes condensation, segregation and de-condensation. The spindle pole duplication, separation and migration is necessary for next events. Checkpoint controls monitor the cell cycle transition through specific cellular events. Internal and external signaling pathways contribute to the cell cycle with the aid of cyclin-dependent kinases (CDKs) (Carnero, 2000).

CDK activity is primarily regulated by the cyclin box whose function is to bind and activate the CDK (Kobayashi et al., 1992; Lees & Harlow, 1993). However, the binding and activation of CDKs can be inhibited through various mutations. Table 1 shows the interactions of each CDKs with a specific subset of cyclins (Lew, Duli, & Reed, 1991; Pines & Hunter, 1989, 1991).

Table 1: Cyclin-dependent kinases and associated Proteins (Carnero, 2000)**.**

Kinase	Regulatory Subunits	Putative Substrates	Proposed Function
CDC2	Cyclin A, B	pRb,NF, Histone H1	G2/M
CDK2	Cyclin A, E, D	pRb, p27	G1/S, S
CDK3	Cyclin E	E2F1/DP1	G1/S
CDK4	Cyclin D1, D2 ,D3	pRb	G1/S
CDK5	p35, cyclin D1, D3	NF, Tau	Neuronal differentiation
CDK6	Cyclin D1, D2, D3	pRb	G1/S
CDK7	Cyclin H	CDC2, CDK2/4/6	CAK
CDK8	Cyclin C	RNA pol II	Transcriptional regulation
CDK9	Cyclin T	pRb, MBP	G1/S

A complete cell cycle can be broken down into 4 phases:

G1-phase: The first and longest phase so-called gap phase 1, commencing after completion of mitosis and cytokinesis. The cell replicates either its DNA or enter into an inactive G0- phase,

S-phase: The chromosome replication is restricted to the S-phase of interphase with length of 8 hrs. The S-phase marks the commencement of DNA synthesis, in which each chromosome replicates to produce a pair of sister chromatids and centrioles.

G2-phase: Entering the G2-phase, the cells prepare themselves for mitosis. This phase proceeds, once DNA synthesis and chromosome replication is successfully completed. The length of this phase takes approximately 1 hrs. The distinction of multiplied individual chromosomes is difficult due to the form of chromatin fibers. In G2-phase, the cells are prepared for mitosis, initiating by prophase. Additionally, a checkpoint control distinguishes which cells are able to enter M-phase for division and assures that a cell with damaged DNA is inhibited from entering mitosis. The importance of this phase is to study the checkpoint in order to prevent spreading of damaged cells and investigation of the cellular and molecular basis of cancer.

M phase: The M-phase begins as the mitosis and cytokinesis processes are imbricated. It includes five crucial subphases: prophase, prometaphase, metaphase, anaphase, and telophase. In the anaphase, cytokinesis commences, which would only terminate upon the completion of mitosis (Lodish et al., 2004; Murray, Andrew, & Tim, 1993).

2.3.5 Breast Cancer Cell lines

Breast cancer cell lines are considerably a noteworthy tool to demonstrate the cellular and molecular processes involved in drug target in cancer chemotherapy (Correa, Bertollo, & Goes, 2009). The isolation and establishment of human breast cell lines play remarkable key roles to describe the important biological signaling pathways improvement and better prediction of the efficacy of novel chemotherapeutic natural and synthetic compounds. The human breast cancer cell line is commonly used in breast cancer *in vitro* model is mainly derived from Caucasians and African-Americans. The use of human breast cancer cell lines arehighly advantageous as they can be easily accessed and replaced from frozen stocks; however, it is worthwhile to note that there are risks of genotypic and phenotypic divergences within these cell lines, especially with procedures involving consecutive cultures (Burdall et al., 2003; Osborne, Hobbs, & Trent, 1987). Majority of *in vitro* studies on well-featured human breast cancer cell lines comprising MCF-7, MDA-MB-231, T-47D and ZR-75-30 were derived from tumoral metastasis site especially pleural effusions but not derived from primary tumoral site for over 40 years (Engel & Young, 1978; Keydar et al., 1979; Soule, Vazquez, Long, Albert, & Brennan, 1973). Additionally, human breast cancer cells derived from metastatic site have a prominent chance of establishment in comparison to established cell lines derived from primary breast tumoural site (Cailleau, Olivé, & Cruciger, 1978).

2.3.5.1 Immortalisation of human cancer cell lines

Leonard Hayflick and his colleague in 1961 identified that human embryonic cells have a determined capacity for division in culture (Hayflick & Moorhead, 1961). The cell culture phases were classified into three important stages including Phases I, II and III. Phase I, also known as the Lag phase, is characterized by the proliferation of the primary cells over the surface of the cell culture flask. During Phase I, the cells gradually grow as they begin acquiring all essential nutrients from the media. Phase II, also known as the Log or Exponential phase, marks the commencement of rapid cell division within the culture. In this phase, cells divide exponentially. In Phase III, cell proliferation diminishes as cell division plateaus. By increasing number of cells, the competitions among cells are initiated for the supplements and nutrients and then their exponential growth is restrained and is finally stabilized (Hayflick, 1975, 1998). Hayflick and his colleague identified a phenomenon known as Hayflick's limitation, Phase III phenomenon, or replicative senescence (RS) that cell divisions stopped after cell subculture (passage) number of fifty.

Hayflick and his colleague carried out their study on connective tissue-derived fibroblast cells. Additionally, RS were identified in different types of cells including keratinocytes, embryonic tissues, endothelial cells, lymphocytes, adrenocortical cells, vascular smooth muscle cells and chondrocytes as well as a number of animals including mice, chickens and Galapagos tortoises (Hayflick, 1998). Previous studies indicated the passaging number of 110 and 15 timesin Galapagos tortoise and murine cells, respectively (Goldstein, 1974; Röhme, 1981; Stanley, Pye, & MacGregor, 1975). Furthermore, the population doubling time for cells derived from patients with Werner's syndrome (WS) is less than normal cells (Salk, Bryant, Au, Hoehn, & Martin, 1981). However, cell lines including embryonic germ and HeLa cells never reach RS, but overcome RS, nominating "immortal" (Brunmark, Collins, Thaw, & Brunk, 1986; Chen & Yu, 1994; Pera,

Reubinoff, & Trounson, 2000). Moreover, there are evidence suggesting that certain type of rat cells appear unaffected by RS (Mathon, Malcolm, Harrisingh, Cheng, & Lloyd, 2001; Tang, Tokumoto, Apperly, Lloyd, & Raff, 2001).

2.3.6 Advantages and Disadvantages of Cell Lines

Established human breast cancer cells are widely employed for *in vitro* model to investigate many prospects of the cellular and molecular mechanisms of disorders and chemotherapeutic effect of natural and synthetic drugs in cancer research. The benefits of using cell lines include the ease of access and study, high abundance in cell sources, high degree of homogeneity, and also the ease of replacement from frozen stocks. Drawbacks of the use of cell lines include risks of genotypic and phenotypic divergences, especially in procedures involving consecutive cultures (Osborne, K. Hobbs, & J. Trent, 1987). Currently, different breast cancer cell lines including MCF-7 and MDA-MB-231 are the most commonly used and available cell lines in laboratory research, based on their origins, pathologies and properties (Burdall, Hanby, Lansdown, & Speirs, 2003; Lacroix & Leclercq, 2004).

2.4 Apoptosis

The apoptosis was first used by Kerr, Wyllie, and Currie in 1972 to explain a morphologically specific form of programmed cell death (Kerr, 2002; Paweletz, 2001). The mechanisms of apoptosis process in mammalian cells were developed from the investigation on the nematode *Caenorhabditis elegans* (Horvitz, 1999).

Apoptosis is a homeostatic mechanism to maintain number of cells during development and aging process (Norbury & Hickson, 2001). A wide variety of stimulants and pathological and physiological conditions may trigger apoptosis, but not all cells

necessarily die in response to the same stimulus. For instances, cancer chemotherapeutic agents cause DNA damage, resulting in apoptotic death via a *p53*-dependent pathway. Corticosteroid hormone may lead to apoptotic death or even proliferation in some cells. Expression of Fas or TNF receptors in some cells, results in apoptosis through ligand binding and protein cross-linking (Hirsch et al., 1997).

2.4.1 Distinguishing Apoptosis from Necrosis

There are differences between two important apoptosis and necrosis processes that may occur independently, sequentially, or simultaneously (Hirsch et al., 1997; Zeiss, 2003). Different factors such as heat, radiation, hypoxia and anticancer drugs result in apoptosis at low doses. However, those mentioned factors at higher doses can lead to necrosis. Therefore, apoptosis event is often energy-dependent mode involving in the activation of caspases and a complex cascade of events linking, to programmed cell death. Necrosis, which is considered as a toxic and degradative process as well as energy-independent mode of cell death (Levin et al., 1999; Majno & Joris, 1995). Necrosis is a process that results in karyolysis and cell swelling while apoptosis results in cell death with cell shrinkage, pyknosis, and karyorrhexis. Additionally, necrosis is an uncontrolled and passive process that usually affects large fields of cells while apoptosis is controlled and energy-dependent and can affect individual or clusters of cells (Denecker, Vercammen, Declercq, & Vandenabeele, 2001; Levin et al., 1999; Majno & Joris, 1995).

2.4.2 Mechanisms of Apoptosis

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy dependent cascade of molecular events. To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked

and that molecules in one pathway can influence the other (Igney & Krammer, 2002; Martinvalet, Zhu, & Lieberman, 2005).

2.4.2.1 Intrinsic Pathway

The intrinsic signaling pathways (Figure 1a) are involved in mitochondrial initiated events producing intracellular signals that may act in either a positive or negative fashion. All of these stimuli cause changes in the inner mitochondrial membrane that leads to the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of first group of proteins including cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (Du, Fang, Li, Li, & Wang, 2000; Garrido et al., 2006; van Loo et al., 2002) as well as second group of proteins including pro-apoptotic proteins, AIF, endonuclease G and CAD from the intermembrane space of mitochondria into the cytosol (Saelens et al., 2004). The first group of proteins activate the caspase-dependent mitochondrial pathway, in which cytochrome *c* binds and activates Apaf-1 and procaspase-9 (Hill, Adrain, Duriez, Creagh, & Martin, 2004) that results in caspase-9 activation (Schimmer, 2004; van Loo et al., 2002).

The second group of proteins are released from the mitochondria during late event of apoptosis and causes DNA fragmentation (Joza et al., 2001). The control and regulation of these apoptotic mitochondrial events is via members of the Bcl-2 family of proteins (Cory & Adams, 2002). The Bcl-2 family of proteins controls mitochondrial membrane permeability (MMP) and includes either pro-apoptotic or anti-apoptotic. The anti-apoptotic proteins are including Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and pro-apoptotic proteins are including Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. One of the important mechanisms of the Bcl-2 family is the regulation of cytochrome *c* release from the mitochondria through alteration of mitochondrial membrane permeability

(MMP). The activation of caspase-9 is triggered by the activation of anti-apoptotic Bcl-2 family members (Oda et al., 2000).

2.4.2.2 Extrinsic Pathway

The extrinsic signaling pathways (Figure 1b) that initiate apoptosis involve in the death receptors pathway of apoptosis is mediated by the caspase-8 cleavage of Bid in which, once caspase-8 is activated, the execution phase of apoptosis is triggered. The sequence of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models (Degli Esposti, 2002). This death domain including FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways (Ashkenazi & Dixit, 1998; Chicheportiche et al., 1997; Rubio-Moscardo et al., 2005; Suliman, Lam, Datta, & Srivastava, 2001).

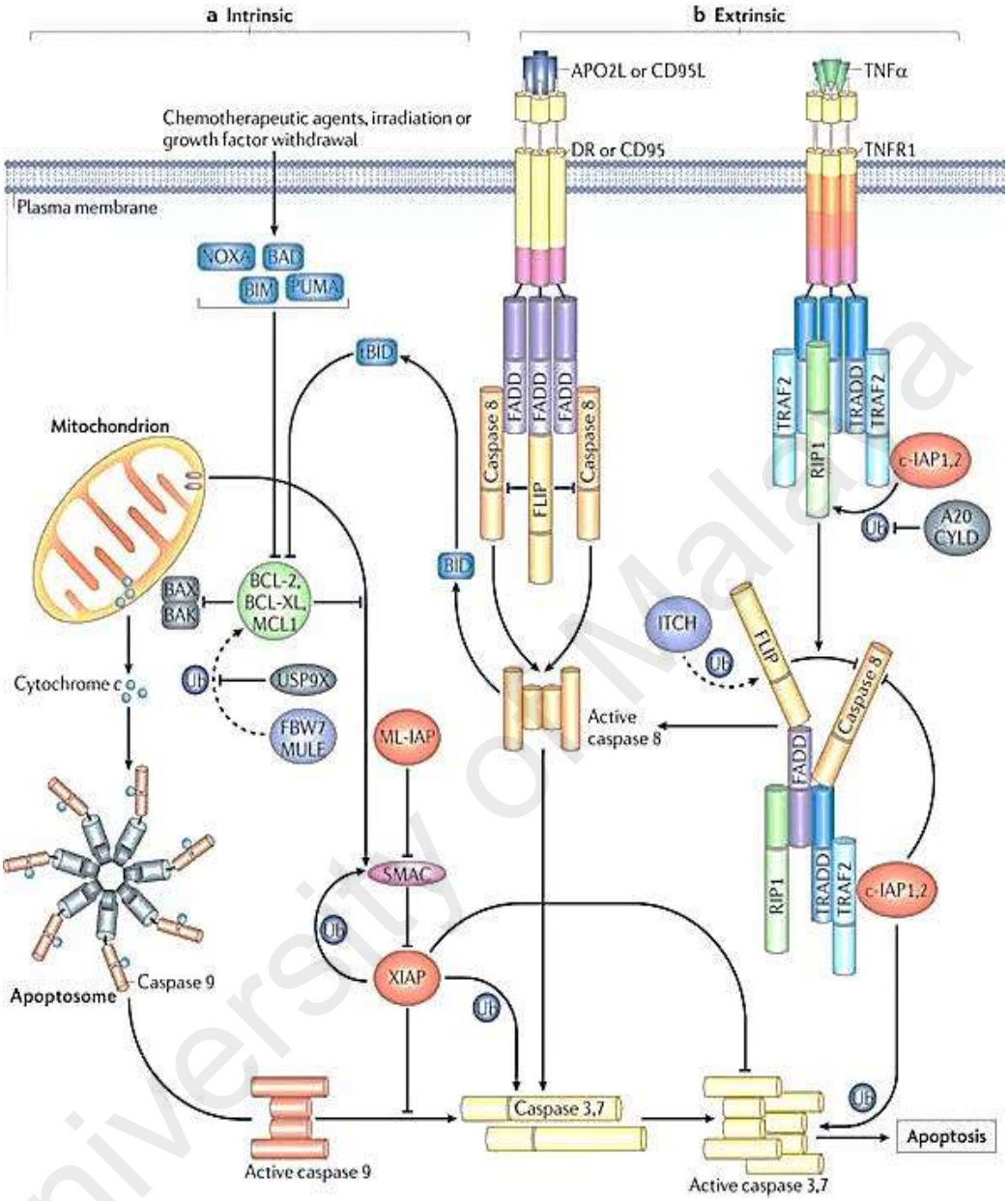

Figure 1: Intrinsic (a) and extrinsic (b) apoptosis pathways. The figure was taken from Vucic *et al* study (Vucic, Dixit, & Wertz, 2011).

2.4.3 Anti-apoptotic NF-KB Signaling Pathway

Nuclear factor kappa beta (NF-kB) is a nearly ubiquitous pathway regulating the expression of many genes involved in a variety of processes including inflammation, apoptosis, tumorigenesis, and auto-immune diseases. The pathway is activated by a variety of stimuli including cellular stress, cytokines, free radicals, UV radiation, oxidized LDL, and bacterial/viral infection. Activated NF-kB is translocated into the nucleus and binds to specific sequences of DNA in which, this DNA/NF-kB complex then recruits RNA polymerase and other co-activators, which transcribe downstream DNA into mRNA, for protein synthesis (Ghosh, Duyne, Ghosh, & Sigler, 1995; O'Neill & Kaltschmidt, 1997).

2.4.4 Morphology of Cell Lines in apoptosis

Cellular morphology is the easiest and the most direct methodology available to determine the states of cells within a culture. In cellular morphology, the cell cultures are magnified usig various optical tools, after which they are observed directly. Cell morphology is governed by a multitude of factors: cell health, cell differentiation, cell density, as well as the concentrations of media and sera. The recommended practice is that researchers remain highly alert during any periodical morphology checks. This is due to the fact that cell morphology only gives the best results when frequent and brief observations are made (Hay, 1992).

At the morphological level, apoptosis is characterized by membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation, and formation of dense bodies. The early and late apoptotic cells or necrotic cells following treatment with a specific compound can be observed using light and electron microscopies or flow cytometry-based methodologies (Susin et al., 2000).

2.5 Chemotherapy

The era of chemotherapeutic drugs was in the 1940s, initiating with experiments on the cytotoxic impacts of nitrogen mustards and folic acid antagonist drugs to induce the tumor regression. Multiple therapies are used for cancer treatment, usually a combination of chemotherapeutic drugs with surgery, and/or radio therapy to give a more effective result (Chabner & Roberts Jr, 2005). Cancer chemotherapy implicates the use of anticancer drugs to treat malignant diseases. Chemotherapy with combination of cytotoxic drugs is the first-line treatment (Faderl et al., 2010), contributing to tumor regression with different mechanisms (Chabner & Roberts Jr, 2005; Hanahan & Weinberg, 2011). There are several cytotoxic compounds for a specific cancer type, but most anticancer drugs lack tumor specificity, resulting in side effects and damage to normal cells (Hanahan & Weinberg, 2011).

2.5.1 Classical Anticancer Agents and their Mechanisms of Action

The classical anticancer agents are categorized into alkylating agents, topoisomerase inhibitors, antimetabolite and tubulin acting agents. The alkylating agents are able to generate reactive molecules, forming covalent bonds with deoxyribonucleic acid (DNA) bases, lead to DNA cross-linking and strand breaks. For instances, cyclophosphamide, melphalan, platinum-based alkylating agents, cisplatin, carboplatin, and oxaliplatin result in DNA strand breaks (Cheung-Ong, Giaever, & Nislow, 2013; Siddik, 2002). Methotrexate and 5-fluorouracil are as antimetabolite acting agents, blocking nucleic acid synthesis (Warrick, Gritsenko, & Aquino, 2015). Doxorubicin and irinotecan are as topoisomerase inhibitor agents involved in DNA replication (H. Wang et al., 2015; Warrick et al., 2015). Tubulin-acting agents interfere with dynamics of mitotic spindle, lead to mitotic inhibition (Dall'Acqua, 2014; Tran et al., 2014). For instances, the Vinca alkaloids including vincristine, vinblastine, and derivative vinorelbine as well as taxanes

including paclitaxel and docetaxel are two classes of compounds, acting on tubulin (Dumontet & Jordan, 2010; M. A. Jordan & Wilson, 2004). Thus, these anticancer agents are cell cycle dependent and involved in cell proliferation, but affect the normal cell proliferation, resulting in limitation of drug dosage.

2.5.2 New Targets for Chemotherapy

Today, the understanding of molecular mechanism of chemotherapeutic agents in cancer cells has increased rapidly, resulting in development of drugs with more specific cellular targets including signaling molecules, cell cycle proteins, apoptotic modulators, growth factors and angiogenic-associated molecules (Chabner & Roberts Jr, 2005; Hanahan & Weinberg, 2011; Van Meter & Kim, 2010). Cellular and molecular regulations are complex network, comprising several interconnected signaling molecules. The antibody bevacizumab (Avastin) is used to treat metastatic cancers and prevent angiogenesis through inhibition of the vascular endothelial growth factor receptor (VEGFR) (Van Meter & Kim, 2010). The monoclonal antibody rituximab (Mabthera) targets CD20 lymphoma cells (Martelli, Ferreri, & Agostinelli, 2013). The imatinib (Gleevec) and gefitinib (IRESSA) are tyrosine kinase inhibitors, acting on Bcr/Abl and epithelial growth factor receptor (EGFR) respectively (Arora & Scholar, 2005; London, 2009). The bortezomib (Velcade) is a proteasome inhibitor that approved as anticancer drugs (Goy et al., 2005; Kathawala et al., 2015).

2.5.3 Cancer Drug Discovery and Development

Anticancer drug discoveries are focused on determining the better strategies for cancer treatment with minimum side effect and drug resistance. During the past decade, high-throughput screening (HTC) is a common method to manifest drugs with anticancer activities in which thousands of compounds can be examined against a specific cell target

in a short period of time. The HTC process is highly automatized and compound libraries are commercially available to screen the compounds against selected drug targets for cytotoxicity and antiproliferative activities *in vitro* (Harvey & Cree, 2010; Rickardson et al., 2006; Rickardson, Wickström, Larsson, & Lövborg, 2007). Drug that are selected using HTC are further investigated by determination of concentration-effect analysis, mode of action, computerized *in silico* method and chemical global positioning system including natural products (ChemGPS-NP) for identification of new drugs (Larsson, Gottfries, Muresan, & Backlund, 2007). The ChemGPS-NP model is useful for prediction of molecular properties and activities and mechanism of action of anticancer drugs (Rosén, Rickardson, et al., 2009). Microarray technology is a very useful method for identification of drug action at a genetic level. The Connectivity Map (CMap) is a database including genome-wide transcriptional expression data from different treated human cell lines. The connectivity map is used to determine the connection of compound mechanism with intracellular targets (Fayad et al., 2009; Hieronymus et al., 2006; Larsson et al., 2007) Immortalized human cell lines play remarkable roles in cancer drug discovery, providing the opportunity for repeatable experiments while it is not possible using derived primary cells from cancer patients (Dhar et al., 1996; Shoemaker, 2006)

2.5.4 Natural Products as Anticancer Agents

Natural products play an important role as anticancer agents in which some of effective drugs used clinically today against cancers are derived from plants (Cragg, Grothaus, & Newman, 2009) For instances, the Vinca alkaloids from plant Vinca rosea, and taxanes from the bark of the Western yew are commonly used for cancer treatment (Cragg et al., 2009; Gordaliza, 2007). The irinotecan (Campto) as a topoisomerase inhibitor is a semisynthetic compound that derived from quinolone alkaloid camptothecin from the tree Camptoteca acuminate (Cragg et al., 2009; Gordaliza, 2007). Therefore, the molecular

mechanism of natural products makes them very useful in cancer drug development (Cragg et al., 2009) as natural products have pleiotropic effects, targeting different cellular and molecular signaling pathways (Efferth et al., 2008; Morphy, Kay, & Rankovic, 2004). The United States National Cancer Institute (NCI) was established in 1989 and then developed as an anticancer drug screening for evaluation of cytotoxic activities of natural, semisynthetic and synthetic compounds using a panel of 60 human cancer cell lines (Shoemaker, 2006). Many plant extracts were previously screened for anticancer activities, and several active compounds were isolated with anticancer activities followed by in-depth mechanistic studies (Peters, Cejka, Harris, Kleinschmidt, & Baumeister, 1993; Shoemaker, 2006). Additionally, it has been reported that natural compounds occupied a larger part of the medicinal and pharmaceutical chemistry than synthetic compounds (Harvey, Clark, Mackay, & Johnston, 2010; Rosén, Gottfries, Muresan, Backlund, & Oprea, 2009). The main components of natural medicinal plants including alkaloids, flavonoids and saponins have indicated anti-cancer activities, triggering apoptosis in different types of cancers.

2.5.4.1 Alkaloids

Plants with chemotherapeutic properties against cancer were found to contain substantial amounts of alkaloids (Hartwell, 1976). The typical chemical structure of these alkaloids is that of a nitrogen atom bound to a heterocyclic ring of relatively low toxicity (Lu, Bao, Chen, Huang, & Wang, 2012). Evidence has shown certain alkaloids to possess noteworthy anti-cancer, anti-inflammatory, anti-bacterial as well as anti-diabetic properties (Han, Lin, & Huang, 2011; Ji, 2011; H.-H. Yu et al., 2005). There are several semi-synthetic anti-cancer alkaloids including vinblastine, vinorelbine, vindesine, and vincristine that were approved and well-developed in the United States and Europe (Moudi, Go, Yien, & Nazre, 2013). For instance, matrine, a quinolizidine alkaloid found

in Sophora flavescens Aiton (Y. Liu et al., 2014), aids in the activation of caspase-3, -8, and -9 in human osteosarcoma MG-63 and MNNG/HOS, U-2OS and Saos-2 (Liang et al., 2012). This is mainly due to the stimulating effects matrine possesses on the up-regulation of Fas/FasL and Bax, as well as the down-regulation of Bcl-2 (Liang et al., 2012). The overall diminution of the Bcl-2/Bax protein and mRNA levels will boot the rate of cell cycle arrest in cancer cells (Yu et al., 2009). The increased expression of Bax and decreased expression of Bcl-2 were observed in matrine-induced human medulloblastoma D341 cells via caspase-3 and -9 mediated apoptotic signaling pathway (Zhou, Ji, Mao, & Bai, 2014). The oxymatrine-induced human lung cancer A549 cells demonstrated a remarkable increase of Bax expression and decrease of Bcl2 expression, resulting in apoptotic signaling pathways (Wang, Han, & Zhu, 2015). Proteomic analysis indicated that oxymatrine has capability to induce apoptosis in HeLa cells through inhibition of mitochondrial inosine monophosphate dehydrogenase type II (IMPDH2) proteins (M. Li et al., 2014). A study demonstrated that tetrandrine, a natural alkaloid, has immunosuppressive, anti-inflammatory and anti-cancer activities (Wu, Chen, Chen, Lin, & Tseng, 2010). It has been discovered as well that H1, a derivative of tetradrine, inhibits the proliferation of human cancer cells such as human monoblastic leukemic U937, esophageal ECal109 and ECal109-C3. More specifically, tetrandrine promotes the upregulation of apaf-1, Bax, Bad, and Bak, increases the downregulation of Bcl-2, prompts the release of cytochrome *c*, as well as activates caspases -3 and -9 in the mitochrondrail apoptic signaling pathway (Qin et al., 2013). The sheer prominence of tetrandrine's role in the activation of these intrinsic apoptosis signaling pathways no doubt renders it an excellent candidate as as therapeutic agent. Low concentration of vinblastine was shown to inhibit mitosis in HeLa and BSC cells (Panda, Jordan, Chu, & Wilson, 1996). On the other hand, Vinblastine, a common drug used in chemotherapy, is

highly effective in the treatment of relapsed anaplastic large-cell lymphoma with a five-year survivability rate of 65% (Brugieres et al., 2009).

2.5.4.2 Flavonoids

Flavonoids, found abundantly in fruits and vegetables, are secondary plant metabolites with a tpical chemical configuration of 16-carbon skeleton arranged around a heterocyclic oxygen ring (Yao et al., 2004). Evidence from past studies has revealed the various benefits of flavonoids. For instance, flavonoids seem to exhibit inhibitive properties pertaining to the following processes: cell proliferation, cell cycle arrest, angiogenesis, reverse multi-drug resistance, and finally in the induction of apoptotic signaling pathways (Chahar, Sharma, Dobhal, & Joshi, 2011). Accordingly, trifolirhizin, a pterocarpan flavonoid that is found in Sophora flavescent Aiton, is exemplary apropos of this discussion. Indeed, it has been demonstrated in a study (which uses experimentally lipopolysaccharide (LPS) – stimulated mouse J774A.1 macrophage) that trigolirhizin diminishes the expression of various pro-inflammatory cytokines such as TNF-α, cyclooxygenase-2 (COX-2), and IL-6 (Zhou, Lutterodt, Cheng, & Yu, 2009). Furthermore, the proliferation of certain human cancer cells lines *in vitro*, such as the human ovarian A2780, oral carcinoma SCC2095, and also the lung H23, are notably suppressed in the presence of trifolorhizin (Yin et al., 2015). A synergistic effect of trifolirhizin with combination of maackiain revealed the apoptotic induction through DNA degradation into oligonucleosome-size fragments in human leukemia HL-60cells (Aratanechemuge, Hibasami, Katsuzaki, Imai, & Komiya, 2004). Curcumin, a substance found in Curcuma longa, has been shown to play a role in several important molecular signaling pathways (H. Fang, Chen, Guo, Pan, & Yu, 2011; Ravindran, Prasad, & Aggarwal, 2009) such as apoptotic induction, autophagy pathways, the inhibition of NF-κB, caspase activities, DNA fragmentation and also the inhibition of COX-2 and 5 LOX

that selectively targets cancer cells *in vitro* (Ravindran et al., 2009). Moreover, curcumin promotes the apoptosis of breast cancer cells, as can be shown in a proteomic analysis in which an augmented upregulation of curcumin-induced MCF7 cells, ERP29, and 3-PGDH can be observed accompanied by a notable downregulation of TDP-43, SF2/ASF, and eIF3i (H. Fang et al., 2011). One limitation of curcumin, however, is its lack of solubility, which, in practice, would compel its administration in high-concentration dosages for it incur any significant chemotherapeutic benefits (B. C. Jordan, Mock, Thilagavathi, & Selvam, 2016). Notwithstanding the sheer volume of studies and experimentations conducted in the past, most claims on the chemotherapeutic effects of curcumin remain inconclusive (Baker, 2017; Nelson et al., 2017). Another common flavonoid is Quercetin, which is abundantly found in foods such as onions. Quercetin possesses anti-oxidant, anti-cancer and anti-inflammatory properties (Sak, 2014). It affects the cell cycle at the G1/S or G2/M phases, and it does so via the stimulation of p21 CDK inhibitors and the suppression of pRb phosphorylation, which in turn inhibits a main transcription factor of DNA synthesis, E2F1 (Sak, 2014). A study indicated that quercetin induces apoptosis through cell cycle arrest at G0/G1 phase and downregulation of survivin in MCF7 breast cancer cells (Deng, Song, Zhou, Yuan, & Zheng, 2013). In another study, proteomic analysis indicated that quercetin induces cell cycle arrest and downregulation of IQGAP1 and β-tubulin in HepG2 cells (Zhou et al., 2009).

2.5.4.3 Saponins

Saponins are a wide family of amphiphilic glucosides of steroids and triterpenes identified in plants and some marine organisms. Saponins are secondary metabolites with potential biological activities against different cancers through cell cycle arrest and triggering apoptosis (Man, Gao, Zhang, Huang, & Liu, 2010). An example of this is the Chikusetsusaponin IVa butyl ester (CS-IVa-Be) which is found in Acanthopanas

gracilistylus, and has demonstrated the capacity of inducing cell cycle arrests (in the G0/G1 phase) in various cancer cell lines (such as MT-2, Raji, HL-60, TMK-1, and HSC-2 cell lines) (B. E. Shan, Zeki, Sugiura, Yoshida, & Yamashita, 2000). In the MDA-MB-231 breast cancer cells, for instance, the presence of Chikusetsusaponin IVa butyl ester (CS-IVa-Be) promotes apoptosis via the following actions: a) inhibiting the activities of the IL-6 family induced STAT3, b) stimulating tumor necrosis factor-related apoptosis-inducing Ligands (TRAIL). The former is achieved via the IL-6/JAK/STAT3 signaling pathway whereas the latter through the upregulation of death receptor 5 (DR5) (Yang et al., 2016). Another example would be the polyphyllin D obtained from the plant Paris polyphylla. Polyphyllin D exhibits cytotoxic properties as it causes mitochondrial dysfunctions and the loss of membrane integrity in human breat MCF7 and MDA-MB-231 cells (Lee et al., 2005). The chemical compound diosgenin has demonstratedthe capacity to reduce both the mRNA and protein expressions of 3-hydroxy-3-methylglutaryl CoA reductase to trigger an apoptotic signaling pathway, making it a highly effective substance against the HCT-116 human colon cancer cells (Raju & Bird, 2007). Other functions of diosgenin include the inhibition of the FAS expressions (and the mammalian target of rapamycin, mTOR) in HER2 overexpressing human AU565 breast cancer cells; diosgenin also induces cell-cycle arrest at the sub-G1 phase (Chiang, Way, Tsai, & Lin, 2007). Macranthoside B (MB) promotes mitochondrial mediated apoptosis, which in turn brings about an increase in Bax to Bcl-2 ratio, altogether rendering it highly effective against different cancer cells (Wang et al., 2009). Apart from that, MB is also linked with a decrease in mTOR, and an increase in reactive oxygen species (ROS), in human ovarian cancer A2780 cells (Y. Shan et al., 2016). MB can be extracted from Lonicera macranthoids (Wang et al., 2009).

2.6 Normal Stem Cells and Cancer Stem Cells (CSCs)

It should be noted that there are differences between normal stem cells and cancer stem cells. Stem cells have three remarkable features comprising self-renewal, differentiation and homeostatic control. However, cancer stem cells (CSCs) revealed lack of multipotency (Dalerba, Cho, & Clarke, 2007; Hill & Perris, 2007) and lineage of cell development. Thus, CSCs have been designated as "cancer initiating cells" or "cancer stem-like cells" or "tumor initiating cells", while the origin of CSCs is mainly unknown (Dalerba, Cho, et al., 2007; Hill & Perris, 2007). In contrast to brain cancer stem cells, there are no evidences for cell development in other CSCs (Hemmati et al., 2003; Singh et al., 2003; Yuan et al., 2004). CSCs originated from normal stem cells or progenitor cells after multiple mutations. This hypothesis is supported by similar surface markers of normal stem cells and CSCs in brain cancer stem cells and leukemia stem cells (Cozzio et al., 2003; Fialkow, 1989; Krivtsov et al., 2006). Interestingly, other studies indicated that some types of CSCs and stem cells showed tightly regulated self-renewal Notch (Fan et al., 2006; Günther et al., 2008), Wnt (Chiba et al., 2006; Jamieson et al., 2004) and sonic hedgehog (Shh) (Bar et al., 2007; Peacock et al., 2007) pathways, suggesting possible origin of CSCs from the stem cell (Behbod & Rosen, 2005; Bjerkvig, Tysnes, Aboody, Najbauer, & Terzis, 2005; Reya et al., 2001). CSCs may also be originated from mature cells (Bjerkvig et al., 2005) where this hypothesis has been supported by a study on both breast and brain cancers (Ince et al., 2007). Glioma stem cells from glioma cancer do not express surface CD133marker, supporting this hypothesis that CSCs were derived from specific types of tumours may show discordance to express the same surface marker (Beier et al., 2007; Günther et al., 2008).

2.6.1 Surface Markers of Cancer Stem Cell (CSCs)

CSCs are a small subgroup of cancer cells with a self-renewal ability to produce the heterogeneous lineages of cancer cells (Clarke et al., 2006). The definition and concept of CSCs is not new (Cohnheim, 1867; Sell, 2004). Previous studies indicated that a few murine lymphoma cells and mouse plasma cell tumour were capable of proliferation *in vivo* (Bergsagel & Valeriote, 1968; Bruce & Van Der Gaag, 1963). In another study, leukemia and multiple myeloma cells had capability to form colony on soft agar (Hamburger & Salmon, 1977; Park, Bergsagel, & McCulloch, 1971; Salmon & Hamburger, 1983). These studies demonstrated that all cancer cells are not the same that might be due to existence of CSCs.

CSCs were first used as the best choice to isolate based on expression of specific surface markers of CSCs. The important advantage of this method is to isolate a pure population of CSCs with right surface markers while, there are several limitations with the current surface markers. For instance, *in situ* immunohistochemistry analysis of surface markers of CSCs, including CD44 or CD24 for breast, colon or pancreatic carcinomas, CD133 for glioblastomas or colon and prostate, CD20 for melanoma, unable to reveal defined localization patterns specific for CSCs from tumoral cells (Hill & Perris, 2007). There is a discrepancy in tumorigenicity of tumoral cells expressing these markers and those that do not, due to the different growth pattern and host immune reaction to these cells (Hill & Perris, 2007). Another limitation to isolate pure CSCs is using proteolytic enzymes destroying some surface markers expressed in tumoral cells and CSCs (Clarke et al., 2006; Masters, Foley, Bisson, & Ahmed, 2003). The CD44+CD24-surface makers were reported for breast cancer (Al-Hajj et al., 2003). A study demonstrated that there is a tumorigenic difference in cells expressing CD44+CD24- surface markers and non-expressing cells (Abraham et al., 2005; Al-Hajj et al., 2003; Liu et al., 2007).

A study indicated that a few CSCs with surface CD34+CD38- marker from acute myelogenous leukemia (AML) could cause leukemic proliferation in mice while, CSCs with surface CD34+CD38+ marker failed to cause leukemic proliferation (Bonnet & Dick, 1997; Lapidot et al., 1994). In contrast to leukemic CSCs, a significant progress was made in the first solid tumor cancer cells, where CSCs expressing CD44+CD24- surface markers from breast cancer cells showed a very high capacity to form tumours in NOD/SCID mice (Al-Hajj et al., 2003). Interestingly, more than ten thousands of the CSCs with surface CD44-CD24+ marker cells could not form tumour. However, as few as one hundred surface CD44+CD24- marker were able to induce tumours due to involvementwith different signaling pathways, such as Wnt, Hedgehog, and Notch as well as activation of chemokine receptor CXCR4 resulting in metastasis and tumor formation (Al-Hajj et al., 2003; Campbell & Polyak, 2007). Since then, CSCs have been largely examined and characterized in many different human cancer cells, comprising brain tumours (Galli et al., 2004; Hemmati et al., 2003; Singh et al., 2003; Singh et al., 2004; Yuan et al., 2004), multiple myeloma (Matsui et al., 2004), colon cancer (Dalerba, Dylla, et al., 2007; O'Brien, Pollett, Gallinger, & Dick, 2007; Ricci-Vitiani et al., 2007), prostate cancer (Collins, Berry, Hyde, Stower, & Maitland, 2005), head and neck cancer (Prince et al., 2007), melanoma (D. Fang et al., 2005), hepatocellular carcinoma (HCC) (Ma et al., 2007), pancreatic cancer (C. Li et al., 2007) and lung cancer (Eramo et al., 2008). The CSCs have attracted considerable recent interest, and many review papers on this topic have been published (Al-Hajj, Becker, Wicha, Weissman, & Clarke, 2004; Behbod & Rosen, 2005; Blagosklonny, 2007; Campbell & Polyak, 2007; Clarke et al., 2006; Gilbertson, 2006; Reya et al., 2001).

2.6.2 Aldehyde dehydrogenase (ALDH) as a marker for cancer stem cells

Recent evidence suggests that enhanced aldehyde dehydrogenase (ALDH) activity is a hallmark of cancer stem cells (CSC) measurable by the aldefluor assay (Jackson et al., 2011). Aldehyde dehydrogenase (ALDH) has been identified as highly expressed as a marker for cancer stem cells (CSCs) isolated from bone marrow (Keller, 2009), brain (Rasper et al., 2010), prostate (van den Hoogen et al., 2010), lung (Serrano et al., 2011) and breast cancer (Tanei et al., 2009). It has been known that ALDH activity is important for multiple biological activities including drug resistance, cell differentiation, and oxidative stress response. ALDH expression is especially considered as clinically relevant prognostic marker for CSCs. Additionally, based on previous studies, the subset of CSCs is relatively insusceptible to chemo and radiotherapy. For this reason, the subset of CSCs can be a therapeutic target for poor-prognostic, treatment-resistant and recurrence of cancer (Mieog et al., 2012; Rasheed et al., 2010; Salnikov et al., 2010).

Chapter 3

Materials and Methods

3.1 Materials

In this study, the Koenimbin, isolated from the leaves of *M. koenigii,* with a purity of 98.5% was a kind gift from Prof. Mohamed Aspollah Sukari from the Faculty of Science, Universiti Putra Malaysia (UPM).

3.2 Cell culture

The cell lines used in this research (MCF-7 and MCF-10A) originate from the American type culture collection (ATCC).

All cell lines were first cultured in flasks, and subsequently kept in a humidified environment with 5% CO2 concentration. The temperature of the medium was maintained at 37°C. For the human breast adenocarcinoma MCF7 cells, an RPMI-I640 medium (Sigma-aldrich, USA) was used with further supplementation of: 10% heat-inactivated fetal bovine serum (FBS; Sigma-aldrich, USA) and approximately 1% penicillin and streptomycin (Pen-Strep; Sigma-aldrich, USA). As for the non-tumorigenic normal ephithelial MCF-10A cell lines (the control), an MEGM medium (Sigma-aldrich, USA) was used together with growth factors obtained from Lonza/Clonetics Corporation (MEGM Kit, Catalog No. CC-3150, Lonza, USA). To fulfill all experimental objectives of this study, the cell lines with a confluency rate of 70 to 80% in the exponential growth phase were examined.

3.3 MTT cell viability assay

MTT, a yellow tetrazole is converted into the insoluble purple formazan, through mitochondrial reductase (Figure 2).

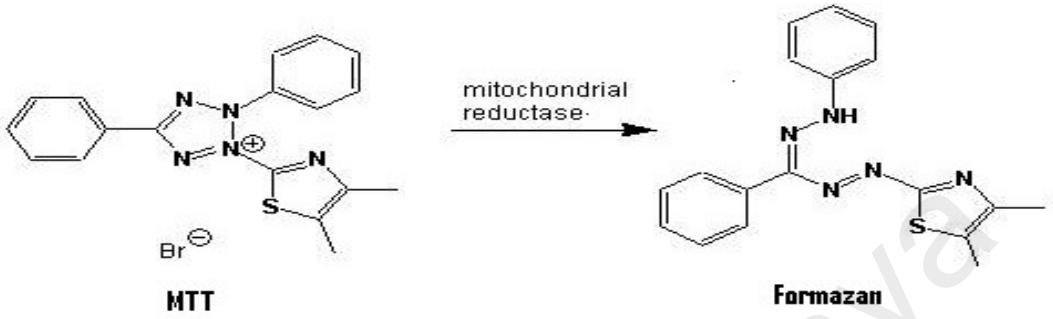

Figure 2: Conversion of MTT into insoluble Formazan in live cells. Image created by Jenpen 21 September 2006, Source: http://en.wikipedia.org/wiki/File:Mttscheme.png

The MTT assay is examined to identify the viability of Koenimbin-induced cells in this study. The experimental procedure commenced with the culturing of 1.0×10^4 cells in a 96-well plate. The cell cultures were then kept overnight in a CO2 incubator under the following physical conditions: 37°C constant temperature, and 5% CO2. The cell cultures were treated with Koenimbin (at various concentrations) the day following the overnight incubation before being subjected to further incubation at 37°C in 5% CO2 for durations of 24, 48 and 72 hours. Fresh MTT solution with a concentration of 2mg/mL (Sigma-aldrich, USA) was added two hours following the second incubation. To ensure dissolution of formazan crystals, DMSO solution (Sigma-aldrich, USA) was introduced into the cell cultures. The plates were then loaded into a microplate plate reader (Tecan Infinite M200 Pro) to be read at 570 nm absorbance wavelength. The percentage of cell viability were measured for each sample. Comparisons were made between the cell vianbility of cultures that had undergone Koenimbin-treatment for 24, 48 and 72 hours (Gummadi et al., 2013). The IC_{50} value is a concentration of the natural or synthetic compounds to decrease the absorbance of treated cells to 50% of the DMSO-induced control cells. The experiment was performed in triplicates (n=3).

3.4 Isolation of candidate breast cancer stem cells

The derivation of the MCF7 breast cancer stem cells with $CD44^{+}/CD24^{-/low}$ surface markers from the human breast cancer MCF7 cells was achieved via a technique known as magnetic cell sorting method (Oliveras-Ferraros et al., 2012). 20 µl of the CD44 antibody plus 20 µl of the CD24 antibody [(CD44 Mouse Anti-Human mAb (clone MEM-85), Fluorescein (FITC) Conjugate, CD24 Mouse Anti-Human mAb (clone SN3), PE Conjugate, Mouse IgG2b, (FITC), Mouse IgG1, (R-PE)] were used to stain the MCF CSCs. All chemicals used were procured from BD Biosciences (Mississauga, CA). The $CD44^{+}/CD24^{-/LOW}$ cell selection procedures are including two steps. First step is involved in removal of $CD24^{+}$ cells using negative selection in which a single-cell suspension of 1×10^{7} MCF7 CSCs containing $CD44^{+}/CD24^{-/LOW}$ target cells. The cells were suspended with 1X MagCellect Plus Buffer and 25 µL of human CD24 antibody were then added and gently mixed well, avoiding bubble formation and incubated for 15 min at 4°C. The cell suspension was centrifuged at 300×g for 8 min and supernatant was completely removed and 50 µL of Streptavidin were added to the cell suspension and mixed well and incubated for 15 min at 4°C. After incubation, the cell suspension was centrifuged at 300 ×g for 8 min and supernatant were removed and cell pellet was resuspended using 1X MagCellect Plus Buffer. The reaction tube was placed in MagCellect magnet that has been positionized horizontally and incubated 6 min at RT. Magnetically tagged $CD24^{+}$ cells migrated toward the magnet, leaving the untagged $CD24^{-/LOW}$ cells in supernatant and untagged $CD24^{-/LOW}$ cells were carefully aspirated using a sterile Pasteur pipette and placed in new tube. The collected $CD24^{-/LOW}$ cells were used in second step for positive selection of $CD44^{+}/CD24^{-/LOW}$ cells. The obtained $CD24^{-/LOW}$ cells from step 1 were centrifuged at 300 ×g for 8 min and the pellet resuspended in 0.5 mL of 1X MagCellect Plus Buffer, then 10 µL of human CD44 biotinylated antibody were gently mixed with suspension and incubated for 15 min at 4°C. After incubation, 3 mL of 1X MagCellect

Plus Buffer and centrifuged at 300×g for 8 min. The supernatant was completely removed and then resuspended gently with 0.5 mL of 1X MagCellect Plus Buffer and 50 µL of Streptavidin were added to cell suspension and incubated for 15 min at 4°C. After incubation, the cell suspension was centrifuged at 300×g for 8 min and supernatant were completely removed and resuspended with 2 mL of 1X MagCellect Plus Buffer. Then, the reaction tube was horizontally placed in the MagCellect magnet for 6 min at RT. Magnetically tagged $CD44^+$ cells migrated toward the magnet leaving the untagged $CD44^-$ cells in supernatant and removed carefully the untagged $CD44^-$ cells using a sterile Pasteur pipette. Then, the tube containing the magnetically selected $CD44^+/CD24^{-/LOW}$ cells from the magnet and resuspended the cells by adding 500 µL of 1X MagCellect Plus Buffer. Then, 5 µL of the FITC-conjugated human CD24 antibody and 7 µL of the PE-conjugated human CD44 were added to100 µL of selected $CD44^+/CD24^{-/LOW}$ cells and incubated in the dark place at room temperature for 45 min. The $CD44^+/CD24^{-/low}$ cell population were washed twice with PBS and were then resuspended in 400 µL of 1X MagCellect Plus Buffer for final flow cytometric analysis.

3.5 Non-adherent mammosphere formation assay

The 6-well ultra-low attachment plates (TPP, Fisher Scientific, USA) were utilized in the culturing of the MCF CSCs (Ponti et al., 2005). The culture concentration was maintained at 1000 cells per mL of culture medium (Ponti et al., 2005). The culture medium, which comprised of serum free DMEM/F12 media (Lonza, USA), together with a mixture of: B27 (Invitrogen, USA), 1% antibiotic-antimyocotic, 1 µg/mL hydrocortisone (Gibco, USA), 20 ng/mL b-FGF (Gibco, USA), 4 µg/mL gentamicin, 5 µg/mL insulin (Gibco, USA), and 20 ng/mL EGF (Gibco, USA), provides the cells with the capacity to thrive, thus promoting the formation of mammospheres. The culture media were replaced once every two or three days. The replacements will include the 1mL of supplements that were

added to each well. Incubation of the CSCs was conducted to simulate mammosphere-forming conditions. For the inbucation, Koenimbin was introduced into the cultures at concentrations 0, 1, 2, and 4 μg/mL. The subsequent step involves deriving secondary and tertiary cultures from the existing MCF7 CSCs cultures. This was accomplished through the subculturing of the MCF7 CSCs Koenimbin-induced primary mammospheres, in which the secondary and tertiary cultures were obtained from each respective group in the absence of Koenimbin. The number and size of mammospheres were recorded after 5 to 7 days. The data were compared with those of control. Photos were taken with the aid of MetaMorph 7.6.0.0 with a Nikon Eclipse TE2000-S microscope.

3.6 Aldefluor enzymatic assay (ALDH)

ALDH of MCF7 CSCs from MCF7 was evaluated to determine the high level of ALDH enzyme in MCF7 CSCs in comparison to normal cells. MCF7 CSCs demonstrate the high aldehyde dehydrogenase (ALDH) enzyme activity for enrichment of mammary stem cells (S. Liu et al., 2006). An ALDH substrate, together with bodipy-aminoacetaldehyde (1 umol/L per 10^6 cells) were used in the examination of the aldefluor enzymatic assay. (StemCell Technologies, CA). Techniques of flow cytometry were employed to determine the physical and chemical characteristics of the cell cultures. More specifically, single-cell suspensions of MCF7 CSCs were first cultured and then incubated for 45 minutes (at 37°C in 5% CO2) before they were analyzed using a flow cytometer.

3.7 Cell cycle analysis

The MCF7 cell cultures were incubated overnight (37°C with 5% CO_2), after which they were induced with 0, 2.5, 5 and 10μg/mL of Koenimbin for an incubation period of 12 hours. The staining of cells was conducted with a BD CycletestPlus DNA reagent kit (BD,

USA) in accordance with the manufacturer's protocol. A Guava easyCycle 8HT benchtop flow cytometer was employed in the analysis of cell cycle results (Merck, USA).

3.8 Multiple Cytotoxicity Assay

Examinations on the Cellomics Multiparameter Cytotoxicity 3 Kit (Cellomics, USA) allows the simultaneous determination of the 6-independent parameters: cytochrome *c* release, cell loss, morphological changes, nuclear size, MMP changes, and also changes in cell membrane permeability (Mohan et al., 2012). Overnight incubation (at at 37°C with 5% CO_2) was performed on the MCF7 cell cultures after which they were induced with Koenimbin for 24 hours. After treatment, 100 µl of the 2X solution of staurosporine (2 µM) was added to the cells and incubated for 4 hrs at 37°C. Approximately, 65 µl of 16% formaldehyde was added to each well followed by a 15-minute incubation period. Dyes (MMP dye and cell permeability dye) were applied to the MCF7 cells following the aspiration of formaldehyde. The wells were once again inbubated at 37°C for 30 minutes. Then, 100 µl of 1X Wash Buffer and 100 µl of 1X Permeabilization Buffer was added to each well and incubated for 15 min at room temperature (RT). After aspiration of solutions, 100 µl of 1X Blocking Buffer supplemented with 2% FBS was added to each well and incubated for 30 min at RT and then 50 µl of primary antibody solution was added and incubated for 1 hr at RT. The following were done after the aspiration of the blocking buffer and the primary cytochrome c antibody was ascertained: a) adding 50 µl of secondary DyLight 649 conjugated goat anti-mouse IgG antibody/staining solution, b) a 45-minute incubation at room temperature with minimal light exposure, c) double washing with 200 µl/well of 1X wash buffer. For the nuclear staining process, the Hoechst 33342 staining solution (2 µg/mL; Sigma-Aldrich, USA) was used. A total of 1000 cells (stained) were inspected, and this was carried out with the ArrayScan high-content screening (HCS) system (Cellomics, USA) which provides data regarding the intensity

and distribution of fluorescence in the cells. This system determines stained cells and indicates the intensity and distribution of fluorescence in individual cells. Photos were taken for each fluorescence channel by particular filters. All data obtained were stored in a Microsoft SQL database before they were analyzed using the ArrayScan II Data Acquisition and Data Viewer version 3.0 (Cellomics, USA). The data collected comprised of the following: a) photographic images, b) data regarding the intensity and texture of the fluorescence within individual cells, c) the average fluorescence of the entire cell population in the wells.

3.9 Bioluminescent Assays for Caspase-7, -8 and -9 enzymatic Activities

Caspase-7, caspase-8, and caspase-9 enzymatic activities were performed in triplicates and in a dose-dependent manner on a specific white 96-well microplate. This was accomplished via the Caspase-Glo®-7, -8 and -9 (Promega, Madison, WI) assay kits. A totality of 1×10^4 cells were cultured for each well and subsequently incubated at various concentrations of Koenimbin for 24 hours. All activities of the caspases were then closely examined with the Tecan Infinite200Pro microplate reader (Tecan, Männedorf Switzerland) (Mohan et al., 2012; Ng et al., 2013). 100 µl of caspase-Glo reagent was applied and incubated at room temperature for 30 minutes. Due to the presence of active caspases from the Koenimbin-induced apoptotic cells, the aminoluciferin-labeled synthetic tetrapeptide were cleaved. The substrates for the luciferase enzymes were released as a result.

3.10 NF-κB Translocation

In a 96-well plate, approximately 1.0×10^4 cells were cultured, and whose contents were then subjected to overnight incubation at 37ºC with 5% CO_2. For 3 hours, the cells were induced with different concentratious of Koenimbin. The TNFα were diluted to 75 ng/ml

in RPMI culture medium. Then, 50 µl of TNFα solution were added to each well and incubated for 30 min at 37°C in 5% CO2 incubator. Following the aspiration of the culture medium, the following was performed: a) the addition of 100 µl of pre-warmed Fixative Solution into each well, b) incubation at room temperature for 15 minutes. After aspiration of fixative solution, the plate was washed twice with 100 µl/well of 1X Wash Buffer. After Wash Buffer aspiration, 100 µl of 1X Permeabilization Buffer was added to each well and incubated for 15 min at RT for 15 minutes at RT. The permeabilization buffer was then removed and 100 µl of 1X Blocking Buffer was added to each well and incubated for 15 min. The Blocking Buffer was aspirated and 50 µl of primary antibody solution was added to each and incubated for 45 min. The primary antibody solution was doubly washed with 100 µl 1X Blocking Buffer before 50 µl of Staining Solution was introduced into each well. The wells were then incubated in a dark room for 45 minutes. The Staining Solution was then washed twice with 100 µl 1X Wash Buffer per well. An Array Scan HCS Reader was employed in the evaluation of plates. The cytoplasmic and nuclear NF-κB intensity ratio was obtained with the aid of the Cytoplasm to Nucleus Translocation BioApplication software. The intensity ratios obtained were averaged over 200 cells per well to be compared with the TNF-α-stimulated, treated, and untreated cells (Ismail Adam Arbab, 2012). The experiment was conducted according the procedures of the Cellomics nucleus factor-κB (NF-κB) activation kit (Thermo Scientific, USA).

3.11 Human apoptosis proteome profiler array

With the Proteome Profiler Array (RayBio® Human Apoptosis Antibody Array Kit, Raybiotech, USA), it is possible to determine the Koenimbin-induced apoptotic signaling pathways. The cells were induced with Koenimbin at a concentration of 10 µg/mL for 24 hrs. The supernatants were removed from the T-25 flask containing the cell cultures, after which the cells were doubly washed using cold 1X PBS. After that, lysis buffer containing

protease inhibitor cocktail was added to flask and incubated for 30 min at 4°C. The protein extract was transferred to microfuge tube and centrifuged for 10 min at 14000 ×g. The protein concentration was determined using Bradford Assay (Bio-Rad, USA). Approximately, 300 µg of total protein diluted with 1mL of Blocking Buffer was added to each membrane and incubated at 4°C overnight on a shaker. After incubation, the membrane was washed 3 times and 2 times with 1X Wash Buffer I and II, respectively. The cocktail of biotin-conjugated antibody (1 mL) was added to each membrane and incubated at 4°C overnight. After washing the cocktail of biotin-conjugated antibody, 1.5 mL of 1X HRP-conjugated Streptavidin was added to each membrane and incubated at RT for 2 hrs. After washing the 1X HRP-conjugated Streptavidin, 250 µl of Detection Buffer-C and 250 µl of Detection Buffer-D were added to each membrane and incubated for 2 min. A BiospectrumAC ChemiHR 40 (UVP, Upland, CA) and the ImageJ analysis software were utilized for the signal-detection and membrane image files analysis respectively.

3.12 Protein expression of apoptotic markers

In T-25 flasks, the MCF7 cells were cultured before they were induced with Koenimbin at varying concentrations. With a cell lysis buffer (50 mM Tris-HCL pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM PMSF), the protein extracts were lysed. 40 µg of the protein extracts were loaded onto a 10% SDS-PAGE. The extracts were then transferred into the polyvinylidenedifluoride (PVDF) membrane (Bio-Rad, CA) which was then partially blocked using milk colloids (5% non-fat milk) in TBS-Tween buffer 7 (0.12 M Tris-base, 1.5 M NaCl, 0.1% Tween20) for 40 minutes at room temperature. Following that was a series of consecutive incubations subjected to the PVDF membrane. The first incubation was performed on the specific primary antibody for a duration of 12 hours at 4°C. Immediately after that was the incubation of the membrane with alkaline phosphatase

(AP)-conjugated secondary antibody. The second incubation, which lasted for 40 minutes, was carried out at room temperature. Upon the completion of the incubations, the membrane was washed with TBST buffer. The primary antibodies for identification of apoptotic protein markers (Isa et al., 2013; Muhammad Nadzri et al., 2013), including β-actin (1:5000), Bcl2 (1:1000), Bax (1:1000), and HSP70 (1:1000) originated from Santa Cruz Biotechnology, Inc, CA, USA. The membranes were further incubated at room temperature for an hour with alkaline phosphatase (i-DNA, USA) conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:5000 ratio). Following the incubation, the membranes were doubly washed with TBST (10 minutes each time for three times). The washing was carried out on an orbital shaker. The detection of the blots was carried out using the BCIP/NBT (Santa Cruz, USA) solution for 5 to 30 minutes. The targeted protein bands appeared as dark-blue precipitates.

3.13 Statistical Analysis

Experimental data were expressed statistically as the mean values ± standard deviation (SD) over three independent trials (n=3). Moreover, various assumptions such as those of normality and homogeneity of variance were adequately checked. Among the statistical tools used in all analyses were the SPSS-16.0 package and the GraphPad prism 3.0. The definition of statistical significance is that of $p<0.05$.

Chapter 4

Results

4.1 MTT cell viability assay

MTT assays were used in the assessment of Koenimbin's anticancer effects. The assays, which involved various human breast cell lines inclusing MCF7 cells and non-invasive normal MCF-10A cells, were all performed on a dose-dependant basis (Table 2; Figures 3 and 4). The IC_{50} value of human breast cancer MCF7 cells 24, 48 and 72 hours following Koenimbin treatment were determined as 9.42 ± 1.05 µg/mL, 7.26 ± 0.38 µg/mL and 4.89 ± 0.47 µg/mL, respectively (Figure 3).

Table 2: IC_{50} values of Koenimbin on MCF7 and MCF-10A cells. Results were represented during 24, 48 and 72 hours as means± SD for three independent experiments (n=3).

Cell line	**IC_{50} ± SD (µg/mL)**		
	24 h	**48 h**	**72 h**
MCF7	9.42 ± 1.05	7.26 ± 0.38	4.89 ± 0.47
MCF-10A	31 ± 0.78	27 ± 1.02	21 ± 0.35
MDA-MB-231	13.54 ± 0.83	10.32 ± 0.24	6.25 ± 0.14

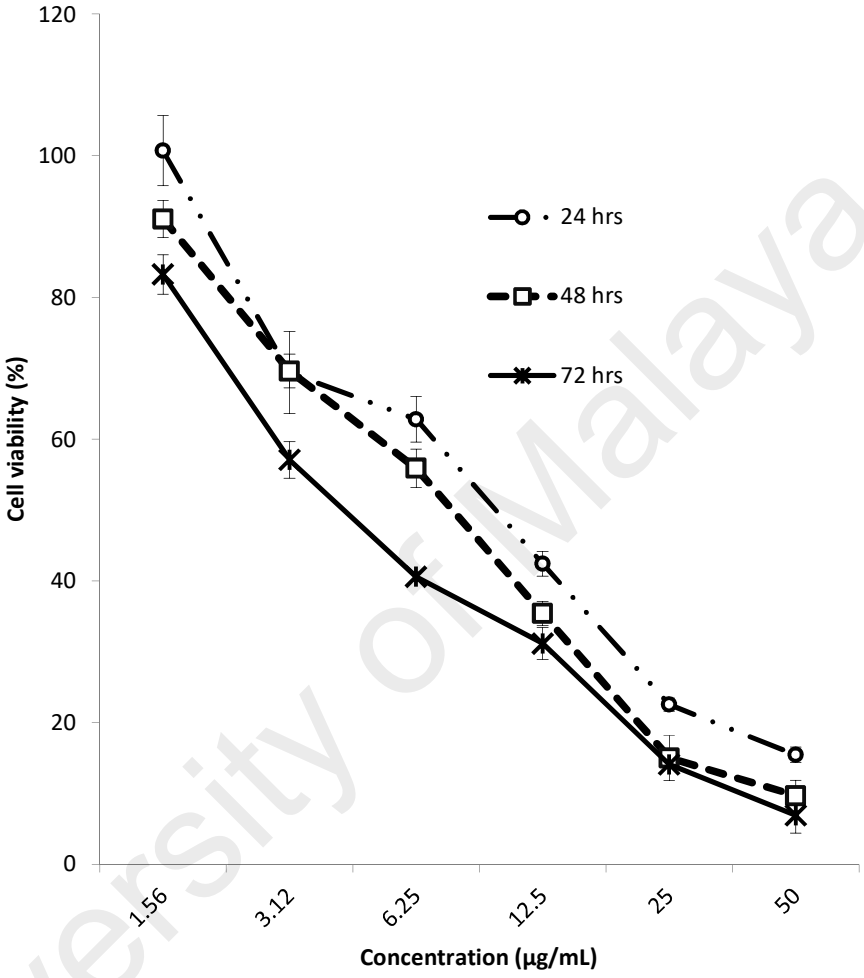

Figure 3: MTT assay of Koenimbin-induced MCF7. The growth curves of the MCF7 cells after Koenimbin treatment of varying durations: 24, 48 and 72 hours.

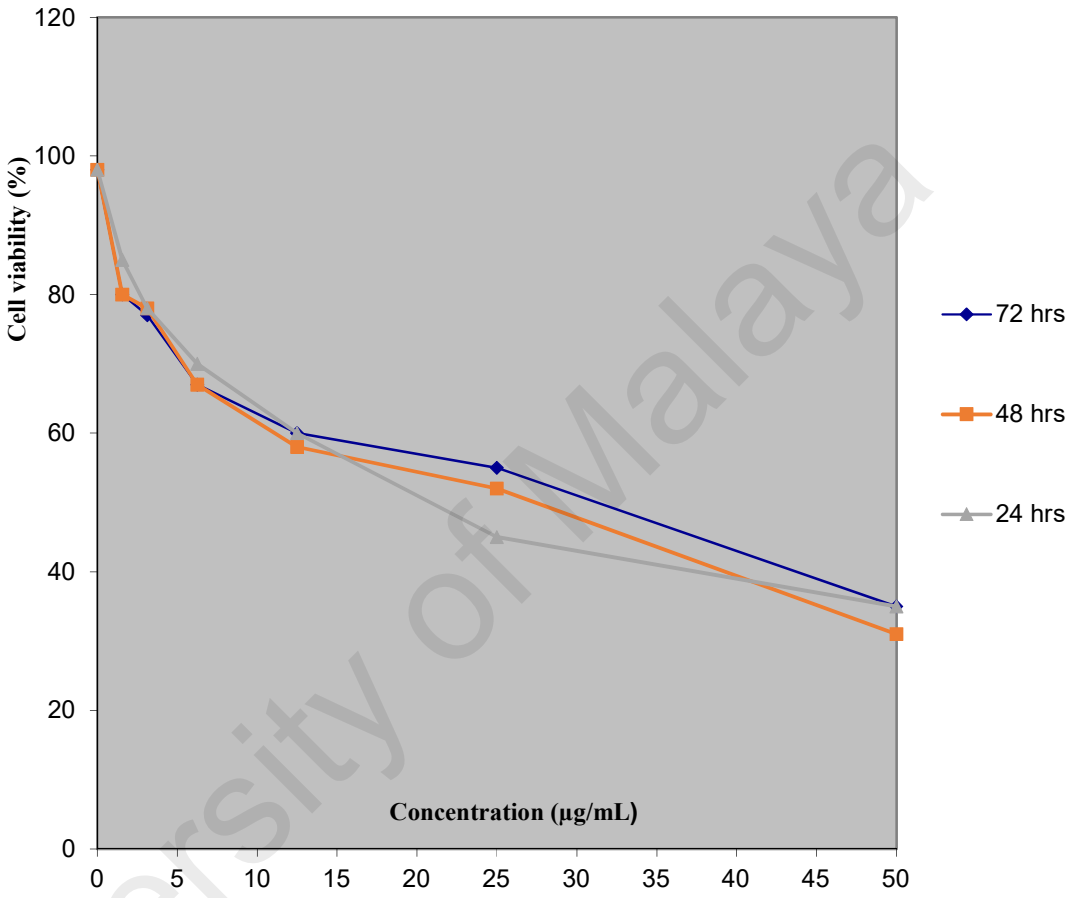

Figure 4: MTT assay of Koenimbin-induced MCF-10A. The growth curves of MCF-10A cells after subjected to Koenimbin treatment of varying durations: 24, 48 and 72 hours.

4.2 Breast cancer stem cells with $CD44^{+}/CD24^{-/low}$ surface markers

In this study, derived breast cancer stem cells were first isolated from human breast cancer MCF7 cells by sorting based on cell $CD44^{+}/CD24^{-/low}$ surface marker expressions (Figure 5) and subsequently they were cultured in specific media for mammosphere type. Derived breast cancer stem cells from MCF7 were determined by the CD44 cell surface marker expression and a weak expression of CD24 surface marker.

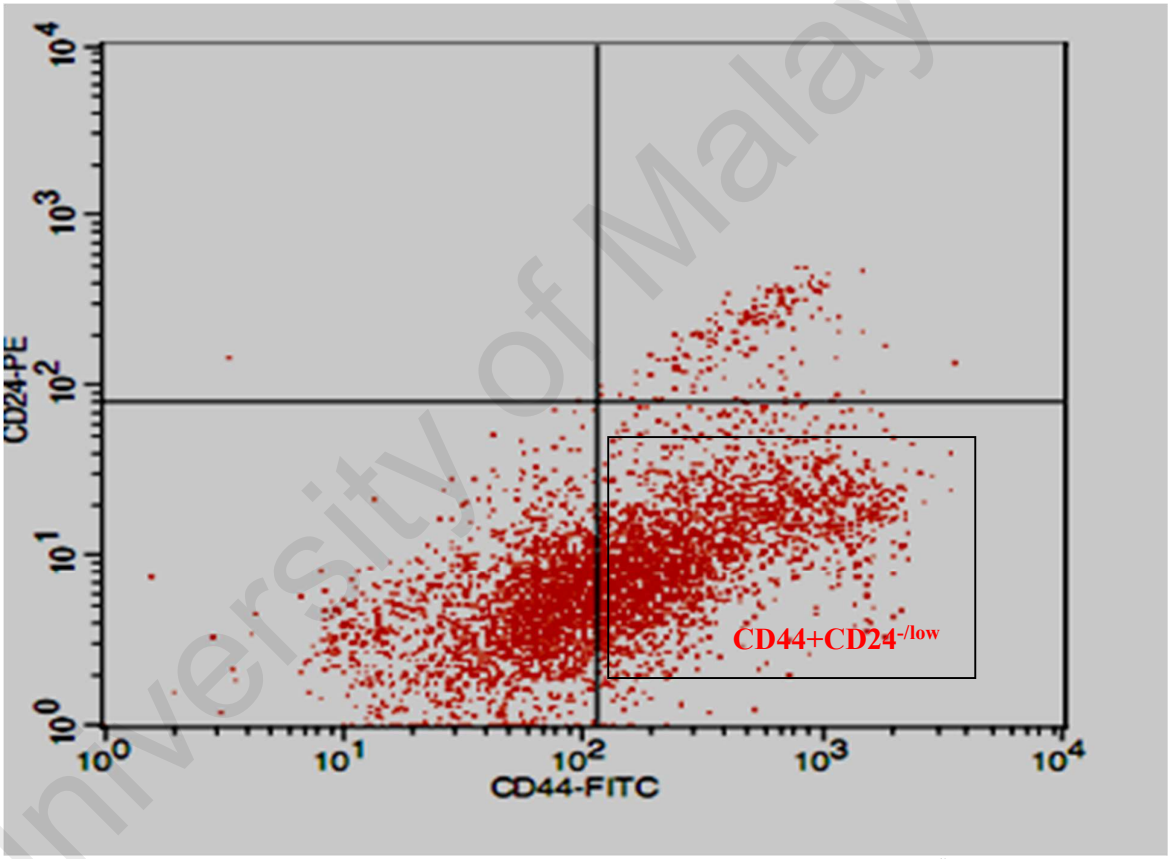

Figure 5: Expression of $CD44^{+}$ surface marker and dim expression of $CD24^{-/low}$ surface marker The presence of human breast cancer stem cells (derived from MCF7 cells) were detected through a quadrant analysis ($CD44^{+}/CD24^{-/low}$). Each experiment was performed thrice (n=3).

4.3 Inhibitory effect of Koenimbin on mammosphere formation

Specific serum-free media were used in the culturing of the mammosphere cells. Photographic images of the mammospheres were acquired and processed with the aid of

a Nikon Eclipse TE2000-S microscope and the MetaMorph 7.6.0.0. image analysis software. The number and size of mammospheres were recorded (Figures 6 and 7). Mammospheres, which are non-adherent spherical clusters of cells, have been demonstrated to act as cell-enrichment sites for the mammary stem/progenitor cells (Dontu et al., 2004). The derived breast cancer stem cells from MCF7 with $CD44^{+}/CD24^{-/low}$ surface marker expressions were then segregated from the rest of the cultures. Then, MCF7 CSCs were treated using Koenimbin with various concentrations of 1, 2 and 4 µg/mL less than IC50 of breast cancer MCF7 cells during 72 hrs in order to determine the minimal impact of Koenimbin on MCF7 CSCs. This is due primarily to long-term proliferation of MCF7 CSCs in serum-free media for approximately 5-7 days in comparison to MCF7 cells and limited numbers of passaging of CSCs, whence it follows that different concentrations were used in the Koenimbin treatment of the MCF7 CSC spheres to enable a comprehensive evaluation of Koenimbin's effect in the suppression of *in vitro* mammosphere formation. Our results suggest that Koenimbin does indeed suppresses the non-adherent spherical clusters *in vitro*. In fact, our data shows that the presence of Koenimbin prevents these cells from forming secondary spheres and prevents them from differentiating along multiple lineages. Our findings are detailed in Figures 6 and 7, which clearly shows a significant decrease in the number of spheres ($p < 0.01$) with increasing Koenimbin concentrations.

4.4 Inhibitory effect of Koenimbin on ALDH positive population

The Aldefluor assay was employed in the evaluation of human breast cancer MCF7 populations with: a) high ALDH activities, b) enriched breast stem/progenitor cells, and c) elevated levels of self-renewal in the derived MCF CSCs. Indeed, our data indicates a marked decline in the ALDH-positive population of MCF7 CSCs following the Koenimbin treatment. More specifically, for Koenimbin concentrations of 1, 2 and 4

µg/mL used in the treatment, the percentage reductions are 25, 50 and 80% respectively (see Figure 8). It is worth mentioning, however, that the same effects were not observed when Koenimbin treatment was provided to the MCF-10A cell populations, which further hints at the specificity of Koenimbin's cancer suppressing effects. Our data all points to the fact that Koenimbin's cancer suppressing properties are effectual only for MCF7 CSCs derived from MCF7 cells.

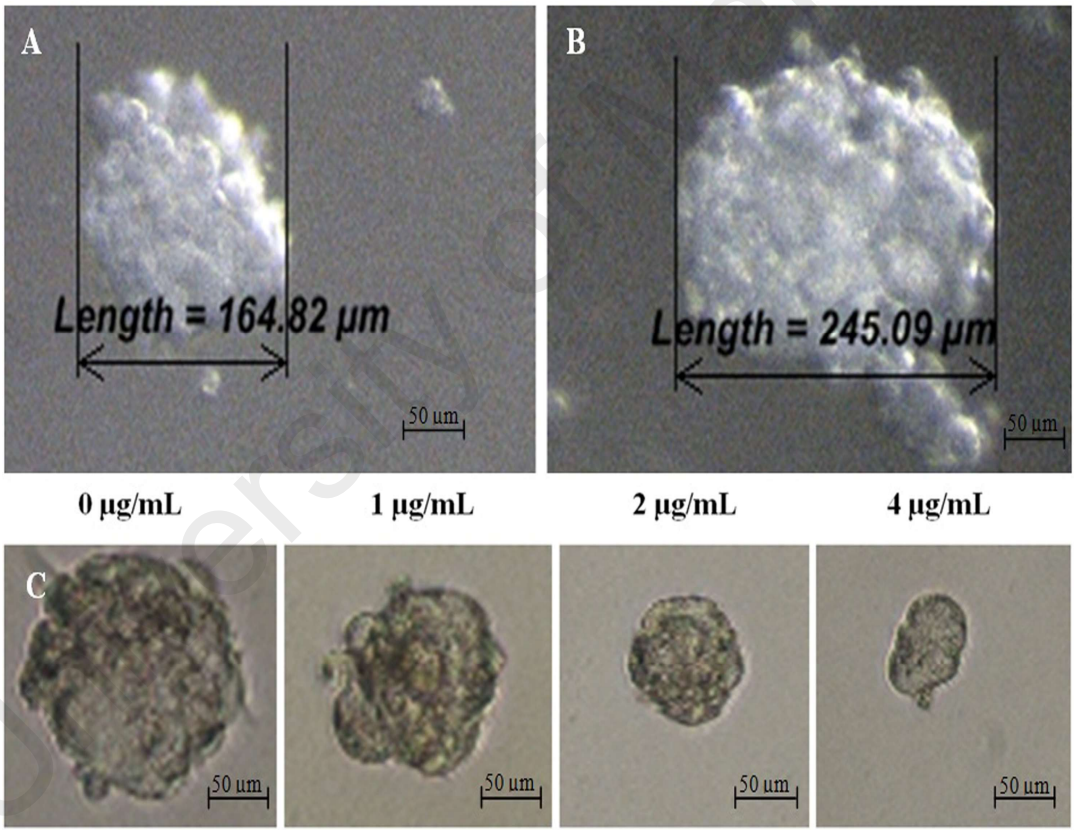

Figure 6: Mammosphere development of the MCF7 CSCs derived from MCF7 cells. Mammosphere size at day 5 (A), and day 7 (B). Primary mammospheres of the Koenimbin-induced MCF7 CSCs (C). From (C), the reduction in the size of the primary mammospheres is evident.

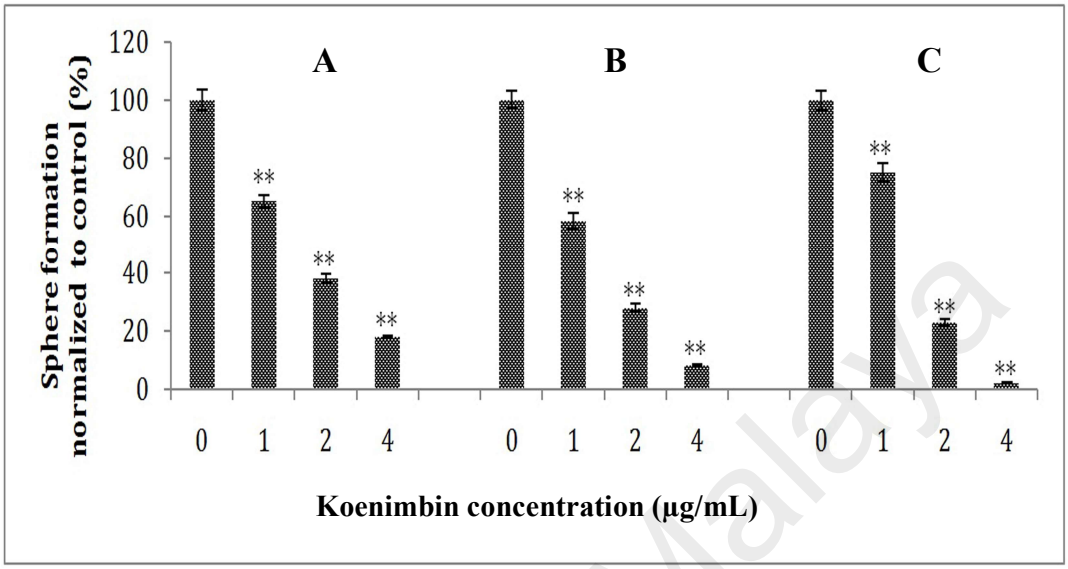

Figure 7: Comparisons between the sizes of mammospheres relative to control. Evidently, the spheres generated from the second and third passages (derived from Koenimbin-induced primary mammospheres) were smaller in size (volumetric size, $V=(4/3)\pi R^3$) than those of control. The conclusion is thus demonstrative, that Koenimbin-induced MCF7 CSCs suppresses mammospherical formation and inhibits self-renewing process of: primary mammosphere forming cells (A), the secondary mammosphere forming cells derived from first passage of MCF7 CSCs (B), and tertiary mammosphere forming cells that derived from second passage of MCF7 CSCs (C). Statistically, all data are represented as the mean values ± SD with n = 3. ** $p<0.01$ *vs* control.

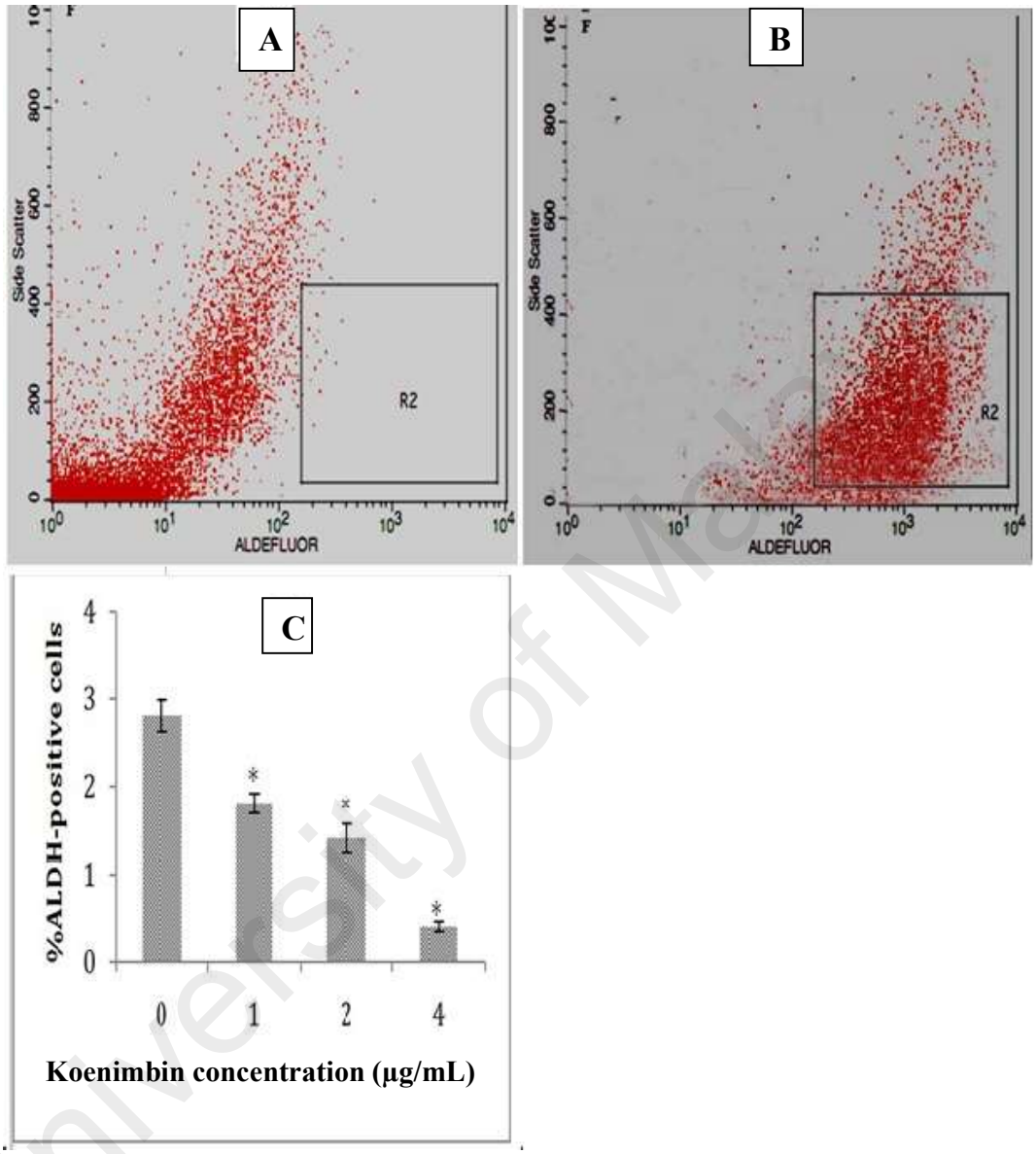

Figure 8: Aldefluor assay of MCF7 cells prior to isolation of MCF7 CSCs (A). Aldefluor assay of derived MCF7 CSCs from MCF7 (B). Separated cell from cell cultures were incubated for 50 min at 37°C in an Aldefluor assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde (1 µmol/L per 1×10^6 cells). A cell population (R2) represented a high aldehyde dehydrogenase (ALDH) enzyme activity in order to enrich mammary stem/progenitor cells. Inhibition impact (B) of Koenimbin on ALDH-positive cell populations. Derived MCF7 CSCs from MCF7 were induced with Koenimbin at concentration of 1, 2 and 4 µg/mL for 4 days were examined for Aldefluor assay using flow cytometry analysis. Koenimbin reduced the percentage of ALDH-positive cells. Data are means ± SD (n = 3). * *p<0.05 vs* control; ** *p<0.01 vs* control.

4.5 Cell cycle analysis

The cell cycle results suggest that at a concentration of 10 µg/mL, Koenimbin imposes significant ($p < 0.05$) alterations to various phases of the cell cycle (see Table 3). Such alterations include: a) a remarkable increase of the sub-G0 phase, b) noticeable DNA fragmentations, and c) a decrease in the Koenimbin-treated cells at 10µg/mL. All these alterations were observed in the G0/G1, S and G2/M phases. However, no significant difference was determined in Koenimbin-treated MCF7 cells at concentration of 2.5 and 5 µg/mL (Figures 9 and 10). Therefore, the cell cycle analysis demonstrated the significant cell cycle arrest in G0/G1 phases of Koenimbin-induced MCF7 cells at concentrations of 10 µg/mL.

Table 3: Data obtained from the analyses performed on Koenimbin-induced MCF7 cells at various cell cycle phases. The first column shows the various concentrations of Koenimbin used for the treatment (0, 2.5, 5 and 10 µg/mL). A 24-hour incubation period was initiated immediately after the Koenimbin treatment. Each value in the table refers to the percentages of cell cycle arrest following the Koenimbin treatment. The percentages were recorded at each cell cycle phases and are summarized in Table 3 below. A '*' denotes a significant difference ($p<0.05$). All data tabulated are represented as the mean values means±SD with n=3.

Concentration/Phase	Sub-G0	G0-G1	S	G2/M
Control	5.92±1.62	60.65±2.94	9.00±0.30	24.43±1.15
2.5	5.75±1.06	61.60±2.02	11.45±0.46	21.20±1.47
5	6.38±1.65	60.40±1.58	10.57±0.49	22.65±1.38
10	76.12±2.49*	17.17±2.08*	2.29±0.24*	4.42±0.32*

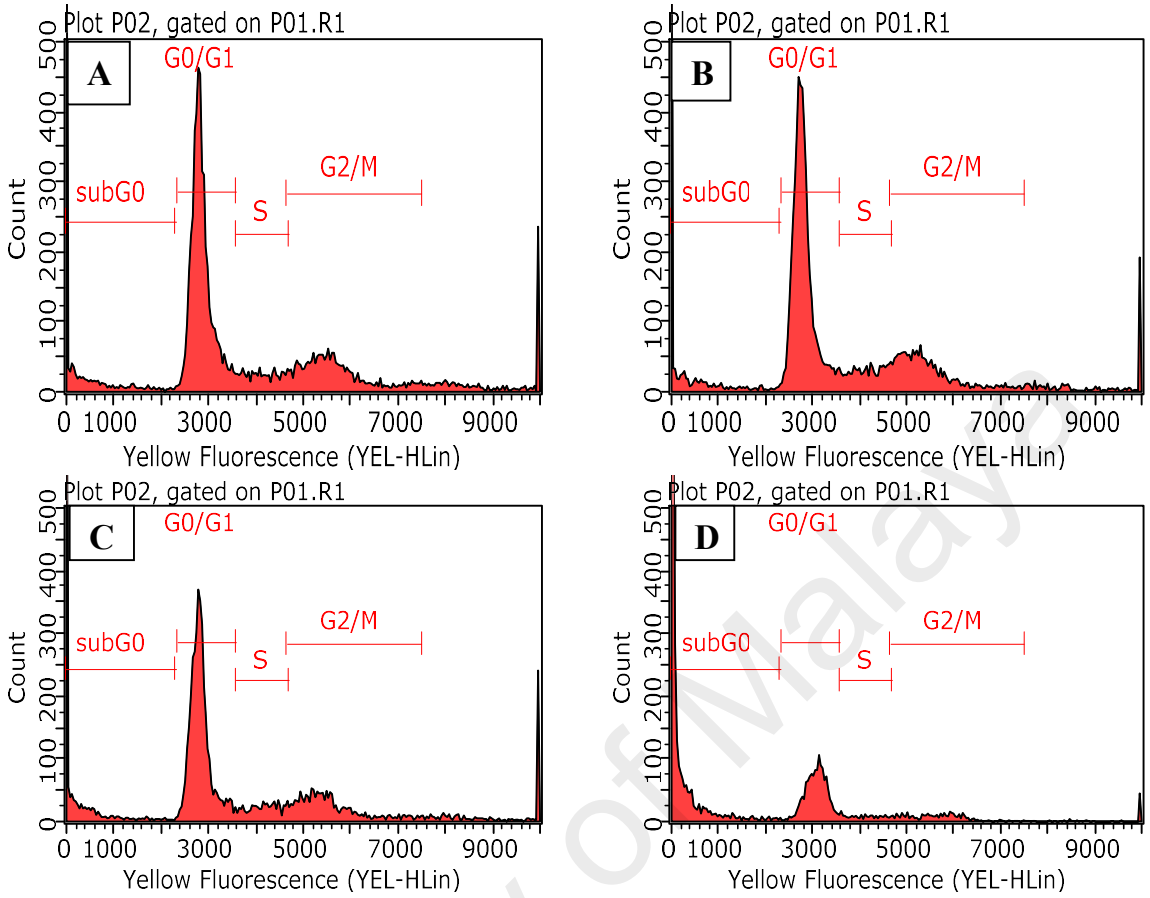

Figure 9: Cell cycle histograms of Koenimbin-induced MCF7 at various concentrations. Cell cycle histograms of Koenimbin-induced MCF7 over the course of 12 hours: control (A), 2.5 µg/mL Koenimbin (B), 5 µg/mL Koenimbin (C), and 10 µg/mL Koenimbin (D).

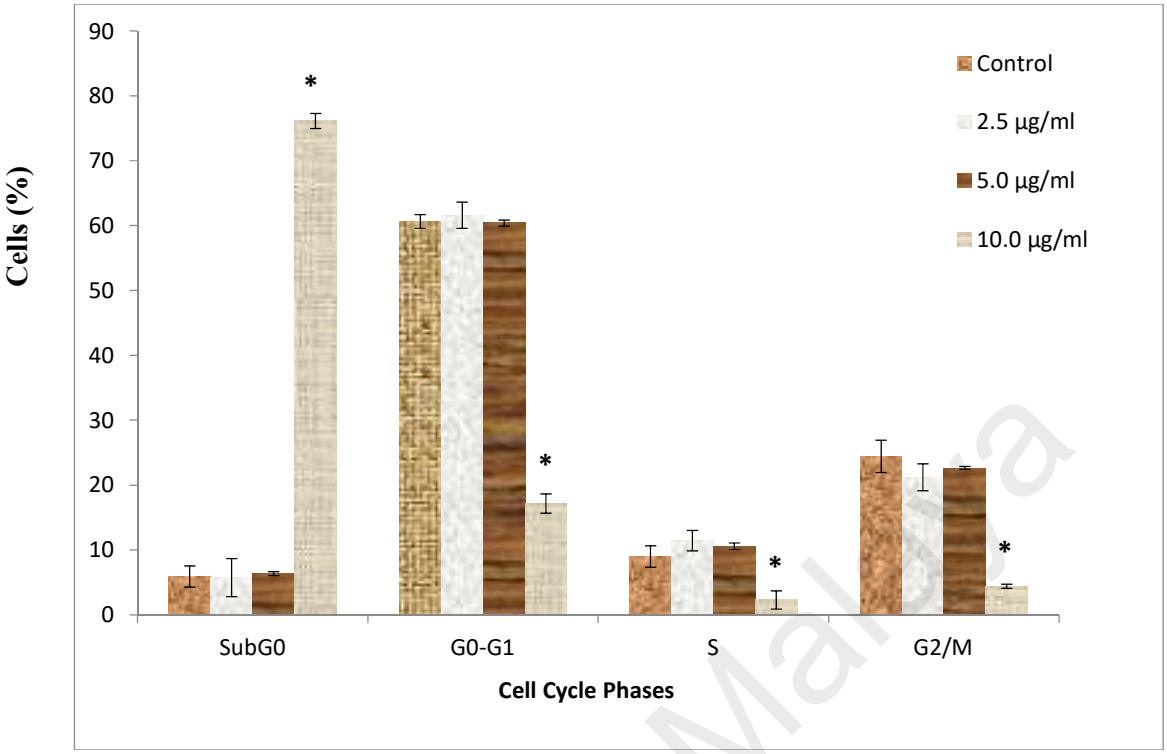

Figure 10: Cell cycle analysis and progression of the control and Koenimbin-induced MCF7 cells. Data are represented as mean values ± SD with n = 3. A * denotes a $p<0.05$ *vs* control.

4.6 The effects of Koenimbin on mitochondrial membrane potential (MMP), membrane permeability, and Cytochrome *c* release

The mitochondria plays a vital role in modulating the survival as well as the apoptosis of cells due to it being the main source of ATP and cellular ROS for all cells. Fluorescent probes were used to collect data on the mitochondrial membrane potential (MMP). The MMP data enables the proper identification of the role of mitochondria in both the Koenimbin-treated and -untreated MCF7 cells. Over the course of 24 hours, untreated MCF7 cells were stained with MMP dye (concentration of 9 µg/mL), after which they were compared to the Koenimbin-treated MCF7 cells (see Figure 11). A drop in the MMP fluorescence intensity implies the demolishment of MMP in the Koenimbin-treated MCF7 cells. A significant spike in cell membrane permeability was also observed in the Koenimbin-treated MCF7 cells after 24 hours. The Koenimbin-treated MCF7 cells also exhibited an increased level of cytochrome *c* into the cytosol compared to control (Figure 11).

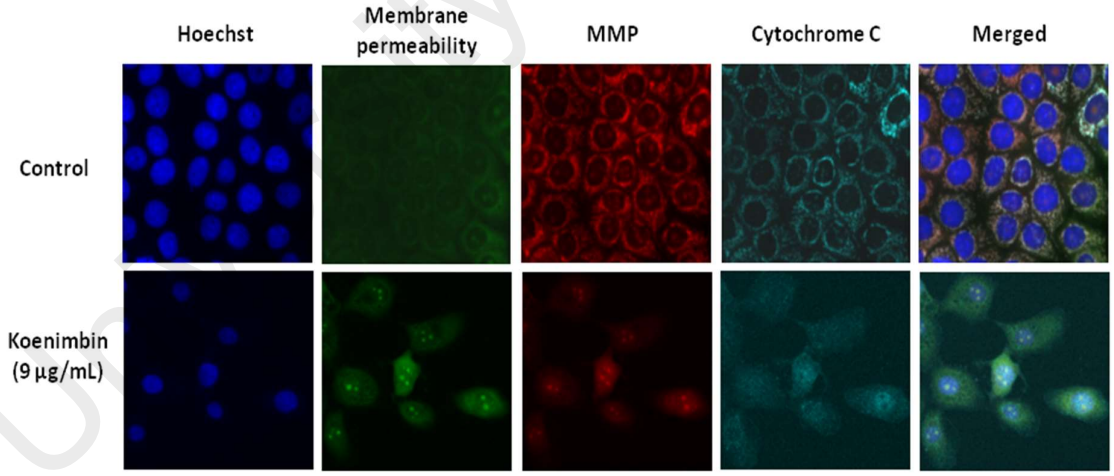

Figure 11: Representative images of Koenimbin-induced MCF7 cells with concentration of 9 µg/mL and medium alone, and were stained with Hoechst for the nuclei, cell permeability dye, MMP dye and cytochrome *c* dye. The obtained images from each row are photographed from the same field of the same treatment sample (Magnification 20×).

4.7 Bioluminescent Assays for Caspase-7,-8 and -9 Activities

All data hitherto indicates an activation of apoptotic signaling pathway in MCF7 cells following the Koenimbin treatment. One plausible hypothesis is that the Koenimbin treatment, which induces a redundant production of ROS from the mitochondria and the collapse of MMP, may cause downstream caspase molecules to be activated, hence resulting in an apoptotic signaling pathway. To put this hypothesis to test, the bioluminescent intensities associated with caspase-7, -8, -9 activities in the Koenimbin-induced MCF7 cells were monitored and measured over the course of 24 hours. Once again, the test was done for various concentrations of the Koenimbin used in the prior treatment. Indeed, a discernible enhancement was observed in the activities of caspase-7, and caspase -9 among the Koenimbin-treated MCF7 cells, whereas the activity of caspase-8 appeared unaffected by the prior Koenimbin treatment (see Figure 12). This suggests that the Koenimbin-triggered apoptotic signaling pathways in MCF7 cells is mediated via the intrinsic mitochondrial-caspase -9 signaling pathway and not the extrinsic caspase-8 signaling pathway.

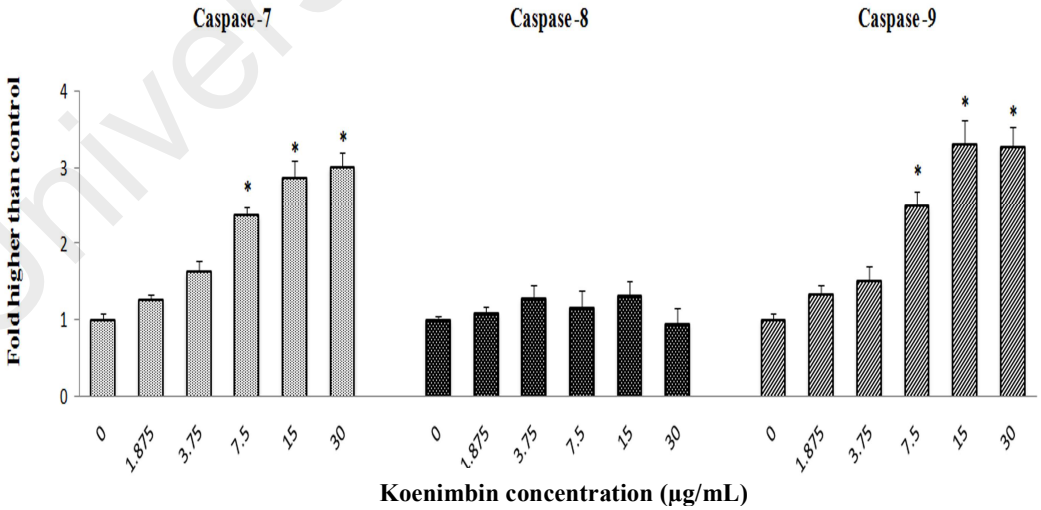

Figure 12: The relative bioluminescence data of caspase-7, caspase-8 and caspase-9 in Koenimbin-induced MCF7 cells at varying concentrations of Koenimbin. All data is represented as the mean values ± SD over three independent experiments (n=3). The statistical significance is expressed as *p< 0.05.

4.8 NF-κB Translocation

A vital transcription factor for the cytokine gene expression is the nuclear factor kappa B, NF-κB. Activation of NF-κB is involved in inflammatory cytokines response including tumour necrosis factor-α (TNF-α), mediating nuclear movement for activity of DNA-binding and alleviate target gene expression. The presence of Koenimbin impeded the translocation of NF-κB into the nucleus (see Figure 13A). A marked reduction in nuclear NF-κB translocation in the Koenimbin-induced TNF-α-stimulated MCF7 cells clearly implies Koenimbin's inhibitory effect on the nuclear translocation process of NF-κB (Figures 13A and B). Furthermore, Curcumin as a NF-κB inhibitor was served as a positive control for treatment of MCF7 cells in this experiment.

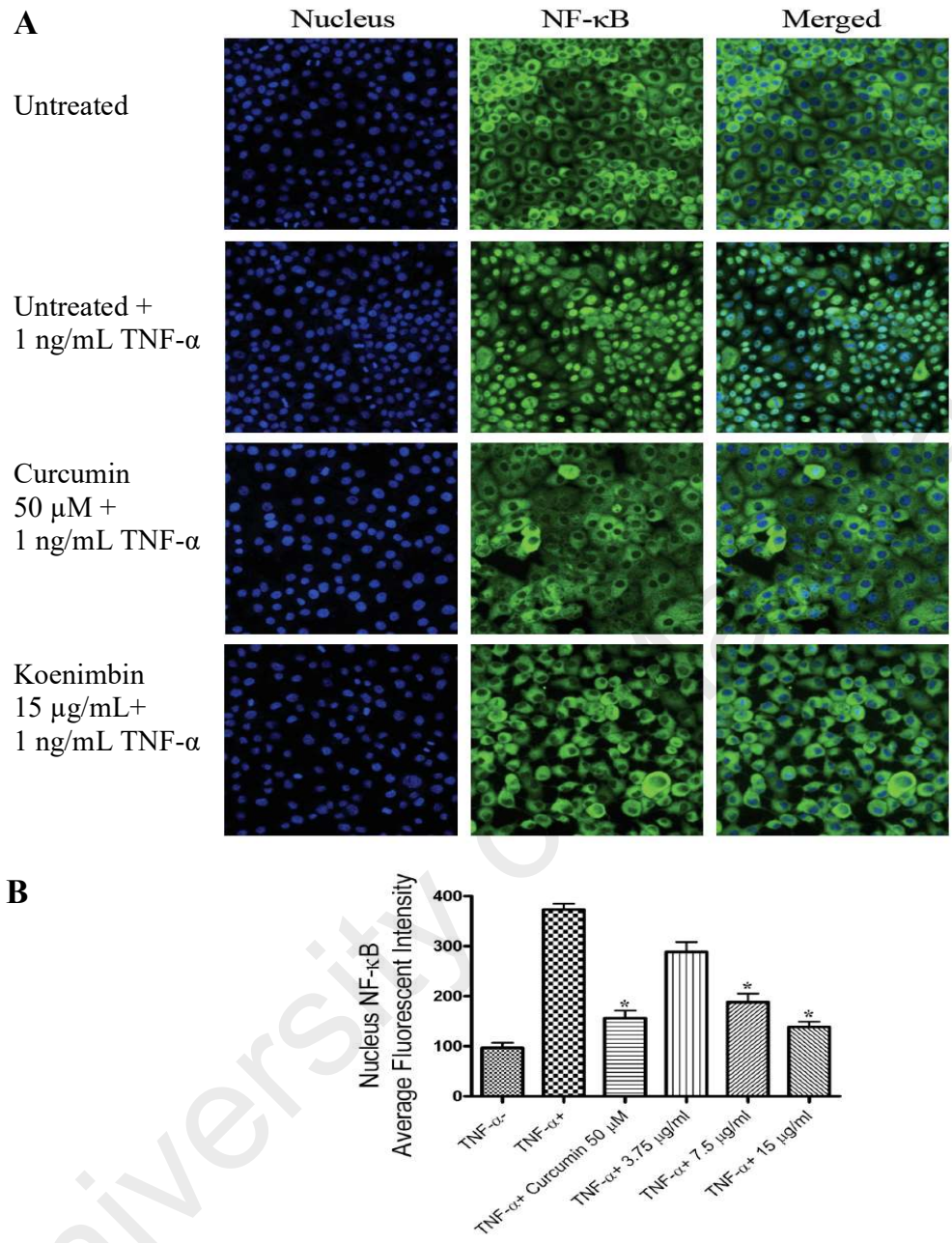

Figure 13: (A) Images of intracellular targets of stained Koenimbin-treated MCF7 cells for 3 hrs and then stimulated for 30 min with 1 ng/mL of TNF-α in order to activation of NF-κB (scale bar, 50 µm). Curcumin as a NF-κB inhibitor was used as a positive control for treatment of MCF7 cells in this experiment. (B) Bar chart portrating a significant decline in the average of fluorescent intensity of nuclei NF-κB. All data are represented as the mean values ± SD over three independent experiments (n=3).*p<0.05 *vs* control.

4.9 Effect of Koenimbin on apoptotic protein markers using protein array

The Koenimbin-induced MCF7 cells were subjected to lysis after 24 hours, after which commenced the process of protein extraction. The apoptotic protein markers were then evaluated via the human apoptotic protein arrays. The resulting data illustrates all alterations of the apoptotic markers within both the Koenimbin-treated and untreated MCF7 cells (see Figures 14A-C, 15). Indeed, our data has shown that most of the prominent markers associated with apoptosis signaling pathways (Bax, Bcl2, caspase-7, caspase-8, and cytochrome *c*) were induced in response to the Koenimbin treatment. Moreoever, there were also observable signs of down-regulation of HSP70, a prominent chaperone protein associated with apoptosis.

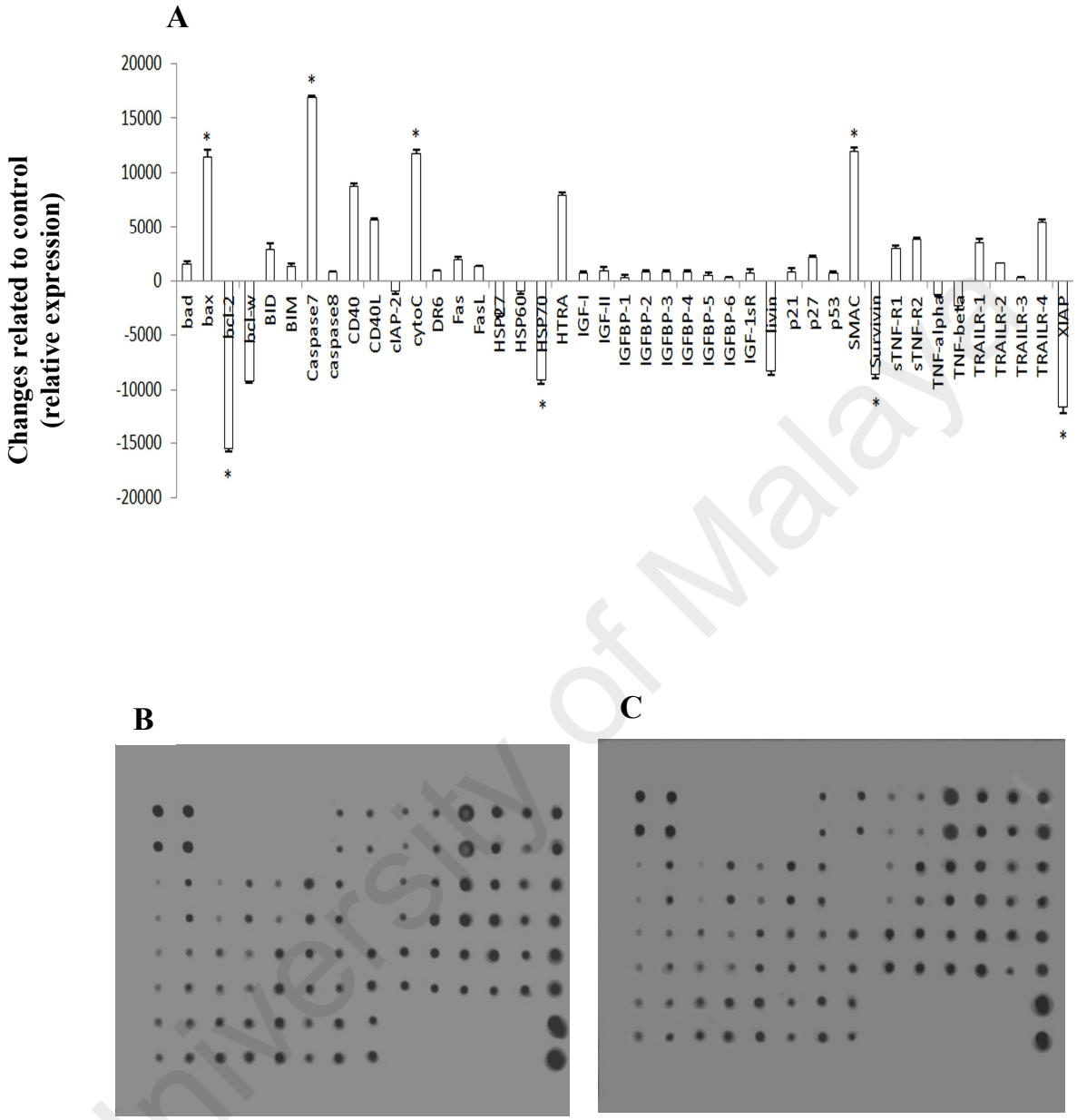

Figure 14: Data delineating the differences in the human apoptotic protein markers between the Koenimbin-treated MCF7 cells and the untreated MCF7 cells (control) (see graph in Figure 14A). Photographic images to represent the apoptotic protein array for the untreated control cells (B), and the Koenimbin-treated MCF7 cells (C). The Koenimbin-treatment provided to the MCF7 cells has the following parameters: the use of 10 µg/mL of Koenimbin for a treatment period of 24 hours. 300 µg of each treated and untreated samples were used for the human apoptotic protein array. All results are represented as the means ± SD over three independent experiments (n=3). "∗" shows a significant difference compared to control (P<0.05).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase7	caspase8
2	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase7	caspase8
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R2
7	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	sTNF-R3
8	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	sTNF-R4

Figure 15: The exact list of protein name in each dot of the protein array.

4.10 Protein expression of apoptotic markers using western blot

There are many remarkable apoptotic proteins including Bcl2, Bax and HSP70 that are related with apoptotic signaling pathway were remarkably up or down- regulated in treated and untreated MCF7 cells using membrane-based protein antibody arrays. Analyses performed on the mitochondrial function in the regulation of apoptotic protein markers yield the following findings for the Koenimbin-treated MCF7 cells: 1) an up-regulation in the expression of the pro-apoptotic protein Bax, 2) a down-regulation in the expression of the anti-apoptotic protein Bcl2, 3) a down-regulation (in a dose-dependent manner) in the protein expression of HSP70 (Figure16).

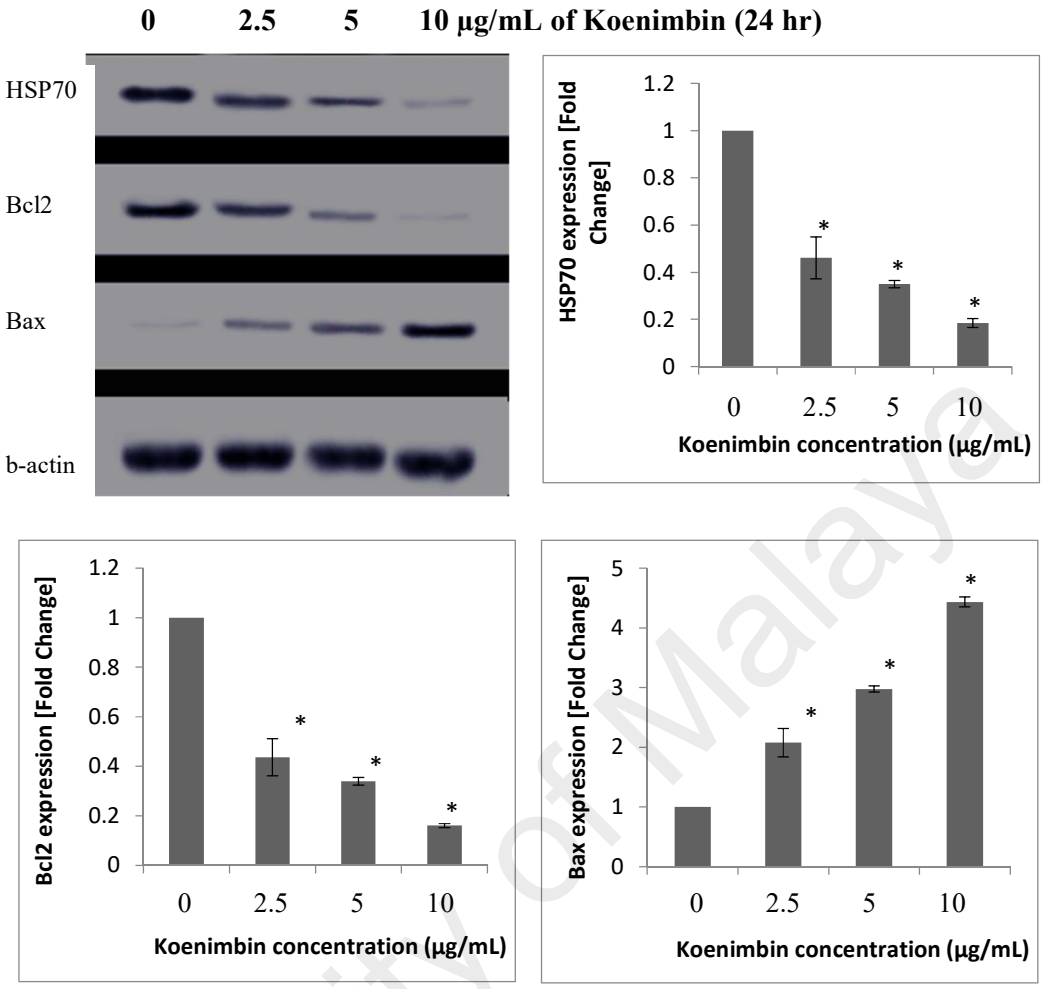

Figure 16: Western blot analysis of Koenimbin-induced MCF7 cells in selected remarkable apoptotic protein markers.Western blot analyses indicating a significant increase in the Bax apoptotic marker, and a significant decrease in the Bcl2 apoptotic marker for the Koenimbin-treated MCF7 cells. All observed effects are dose-dependent. There is a significant alteration of the Koenimbin-induced MCF7 cells at concentrations 4 and 8 µg/mL of the Koenimbin used in treatment, as indicated by the evident down-regulation of the HSP70 protein expressions. The blot densities are expressed as folds of control. Data are means ± SD (n=3). *p<0.05 *vs* control.

Chapter 5

Discussion

Apoptosis plays a role in several cellular and molecular alterations in all human cells, such as changes in mitochondrial potentials, nuclear fragmentations, or even the regulations of different caspases (Hunter, LaCasse, & Korneluk, 2007). This research serves as a pioneer in modern cancer research in that it is the first ever attempt to investigate (*in vitro*) the anti-cancer effects of Koenimbin (a natural compound found in *Murraya koenigii*(L) Spreng) against MCF7 cells and the stem cells/progenitors derived from them. What we have discovered through all relevant data is that Koenimbin does indeed inhibit the proliferation of MCF7 cells (and the stem cells/progenitors derived from them) whereas the normal non-invasive breast MCF-10A cells appear more resistant to any Koenimbin-induced anti-proliferative activity. It is worth mentioning, however, that several chemotherapy drugs have plant origins, such as vincristine, vinblastine, and taxol (Cragg & Newman, 2005), all of which has been shown to affect normal cells (Preedy, 2004). Thus, the implied advantage of using Koenimbin as a chemotherapy drug is clear, in that normal, healthy cells are more resistant to it.

In agreement with previous study, Koenimbin-induced MCF7 indicated a remarkable increase in cell membrane permeability, cytochrome *c* release and chromatin condensation and a noteworthy reduction in mitochondrial membrane potential (MMP) due to activating apoptotic signaling pathways through enhancement of intracellular calcium and plasma membrane alterations (Bailey et al., 2009). Despite of activation of the cellular apoptotic program, mitochondria have been depicted to play an important key role in the apoptotic process (Wang & Youle, 2009). Therefore, the apoptosis in MCF-7 cells is examined by the alterations in mitochondrial membrane permeability (MMP), as

it is assumed that its disruption is the +onset of mitochondrial membrane transition pore (MPTP) formation (Zamzami & Kroemer, 2003). The role of Koenimbin in inducing apoptosis can be validated via a High Content Screening (HCS) analysis, in which a loss of MMP in the mitochondria due to Koenimbin can be observed. This loss of MMP (due to the Koenimbin) is what ultimately leads to apoptosis. The cytochrome *c* as key apoptotic protein is relocalized as a result of decrease in MMP and MPTP processes (Ajenjo et al., 2004).

Various complicated extrinsic and intrinsic signalings trigger the apoptotic signaling pathway involved in apoptotic regulation or cell survival. There are two remarkable apoptotic signaling pathways including caspase-dependent and caspase-independent apoptotic pathway (Thayyullathil, Chathoth, Hago, Patel, & Galadari, 2008). Furthermore, mitochondria play the central role to connect the variety of apoptotic processes and signaling pathways (Wang & Youle, 2009). Caspase-dependent apoptosis is comprising the caspase-3/7, capase-8, caspase-9 and caspase-12 cascades and important involved receptors including the TNF-alpha, FasL, TLR and death receptors in apoptotic signaling pathways (Pradelli, Bénéteau, & Ricci, 2010). TNF-alpha triggered caspase-8-dependent pathway through death complexes and activation of caspase-8, and therefore the activating Bcl-2 protein, resulting in alterations in the mitochondrial membrane and the release of cytochrome *c*. Translocation of mitochondrial cytochrome *c* into cytosol is the proapoptotic signaling pathway, resulting in activation of the caspase cascade and finally enter to apoptotic phase (Nakagawa & Yuan, 2000). Furthermore, UV light and X-ray radiation cause remarkably mitochondrial depolarization, MMP, cytochorme *c* translocation, and therefore activate caspase-9 and caspase-3, resulting in apoptosis (Godar, 1999). Additionally, other pathogenic infections including herpesvirus and Mycobacterium tuberculosis (MT) trigger the apoptotic signaling pathway via

caspase-8 and -12 dependent pathways, respectively (Lim et al., 2011; Obregón-Henao et al., 2012). However, in caspase-independent signaling pathway that plays key role in apoptosis, several molecular ligands lead to mitochondria membrane potential (MMP) alteration, resulting in high level production of ROS (Martinvalet et al., 2005). Another proapoptotic factor in caspase-independent signaling pathway is AIF, in which movement of AIF from mitochondria into the nuclear leads to apoptosis via the cleavage of nuclear DNA (Kang et al., 2004). Furthermore, ROS plays a central role to associate both caspase-dependent and –independent apoptotic signaling pathways *in vivo* (Misirlic Dencic et al., 2012). Despite of the AIF and ROS, there are other important ligands including lysosomal membrane permeabilization (LMP) (Johansson et al., 2010), viral protein (Teodoro & Branton, 1997), p53 suppression tumoral factors associated with apoptosis via caspase independent signaling pathway (El-Deiry et al., 1993; Symonds et al., 1994).

The activations of different caspase cascades play a remarkable regulatory role in apoptotic signaling pathway (Vaux & Korsmeyer, 1999). Following the intrinsic signaling pathway, the activation of various effector caspases such as caspases -3, -6 and -7 is triggered by the release of mitochrondrial cytochrome *c* into the cytosol (Krajewski et al., 1999; Z. Li et al., 2010; Susin et al., 1999). An evidence indicated that the Bcl2 protein family members are important key modulators of cytochrome *c* release involved in apoptotic signaling pathway (Green, 2006; Kluck, Bossy-Wetzel, Green, & Newmeyer, 1997). Indeed, it has been established that the release of Bax into the mitochondria not only promotes the release of cytochrome *c* into cytosols as well as the loss of MMP, but it would aso trigger various changes in mitochondria permeability (Xiang, Chao, & Korsmeyer, 1996). The results of this study have shown that the presence of Koenimbin causes the activation of caspases -7, and -9, as well as the release of mitochondrial cytochrome *c* into cytosols, thus cementing the strong connection between Koenimbin

and the intrinsic apoptotic pathway. On a different note, the downstream effector caspases (-3 and -7) are activated by means of the extrinsic apoptotic signaling pathway wherein cellular death receptors (Fas and TNFα) activates caspase-8 as well as the associated signal transduction (Earnshaw, Martins, & Kaufmann, 1999; Mohan et al., 2012). Previous studies have confirmed the profound association between the activation of caspase-8 with the extrinsic apoptosis signaling (Li et al., 2010). Similarly, the cleavage of the Bid to tBid suggests an interrelation between the mitochondrial signaling pathways and the activation of caspase-8 (Gu et al., 2005). Moreover, our data has shown that the presence of tumor necrosis factor alpha (TNF-α) triggers the release of cytoplasmic NF-κB into nuclei, which is in accord with the results of previously published research (Ibrahim MY, 2014; Mohan et al., 2012). Our data further indicates the potency of Koenimbin on TNF-α-related apoptosis-inducing ligands on human breast cancer cells via the following mechanisms: 1) the NF-*κ*B-induced anti-apoptotic signaling pathway, 2) the movement of mitochondrial cytochrome *c* into the cytosol, 3) the activation of caspase-7 and caspase-9, 4) the up-regulation of Bax protein expressions, and 5) the down-regulation of Bcl-2 protein expressions.

It has been established hitherto that CSCs play a potent role in tumor resistance, tumor relapses, as well as cancer recurrences (Sakariassen, Immervoll, & Chekenya, 2007; Tang, Chua, & Ang, 2007). Moreover, the cancer stem cell (CSC) theory posits that a small proportion of CSCs would give rise to different types of cancers. This postulate has various consequences, one of which would ultimately lead to a novel approach to cancer therapy and prevention through the targeting of CSCs (Kakarala & Wicha, 2008; Reya et al., 2001). Indeed, having treatments capable of targeting both the cancer cells and CSCs would prove highly advantageous to modern cancer treatment since conventional chemotherapies fall short in this regard (Lippman, 2000; Reya et al., 2001; Williams et

al., 1987). This isn't a new conception either, as several attempts have already been made in the past to prove that certain natural dietary compounds such as curcumin (Jaiswal et al., 2002; Z. Wang et al., 2006) and sulforaphane (Y. Li et al., 2010) exhibit chemotherapeutic properties on CSCs. The sole focus of this research, however, is on the anticancer activities of Koenimbin. In particular, this study examined, *in vitro*, the effects of Koenimbin on breast CSCs in the hopes of identifying the full chemotherapeutic effects of Koenimbin, as well as confirming the postulates of CSC theory.

Conventional methods of isolating and characterizing breast CSCs *in vitro* includes mammosphere cell culture, stem cell surface markers and aldehyde dehydrogenase assays. The mammary stem/progenitor cells (Dontu et al., 2003) using mammosphere cell culture are isolated and proliferated in serum-free media, selecting the stem/progenitor cells (Charafe-Jauffret et al., 2008). The results of MTT and mammosphere formation assays, the IC50 values of MCF7 cells during 24, 48 and 72 hrs were less in comparison to the IC50 of non-invasive normal breast MCF-10A cells, and minimal impact of Koenimbin on MCF7 CSCs using mammosphere assay was observed less than IC50 of human breast cancer MCF7 cells during 72 hrs, suggesting Koenimbin barely impacted the non-invasive normal breast MCF-10A cells in comparison with MCF7 cells and derived MCF7 CSCs. Furthermore, in this research, we identified the minimal impact of Koenimbin on derived MCF7 CSCs rather than determination of IC50 value. This is due primarily to long-period proliferation of MCF7 CSCs in serum-free media for approximately 5-7 days in comparison to MCF7 cells and limited numbers of passaging of CSCs. Consistent with previous study (Grimshaw et al., 2008), the mammospheres are composed primarily of cancer stem cells, our observations indicated that Koenimbin remarkably inhibited the mammosphere formation of breast CSCs, indicating that Koenimbin may inhibit derived breast cancer stem cells from MCF7. An alternative

technique to identify mammary stem/progenitor cells from differentiated cancer cells is to use surface makers of cancer stem cells, e.g., $CD44^{+}/CD24^{-/low}$ and ALDH positive (Al-Hajj et al., 2003; Charafe-Jauffret et al., 2008; Ginestier et al., 2007). As few as 500 ALDH-positive cells suffice to effectively form a breast tumor within 40 days, whereas 50,000 ALDH-negative cells showed no signs of tumor formation (Ginestier et al., 2007). ALDH-positive cells and the $CD44^{+}/CD24^{-/low}$ surface marker possess the highest tumorigenic capacity, as they are capable of forming tumors from as few as 20 cells (Ginestier et al., 2007). On the other hand, 1,500 of ALDH-positive cells without the $CD44^{+}/CD24^{-/low}$ surface markers will lead to tumor formation, whereas ALDH-negative cells with 50,000 $CD44^{+}/CD24^{-/low}$ surface marker were unable to form tumors (Ginestier et al., 2007). Previous study on identification of selective inhibitors of breast cancer stem cell demonstrated that salinomycine has capability to decrease the population of CSCs by IC50 approximately 10-fold comparably lower than IC50 of salinomycine as determined using MTT assay. Salinomycine decreased the proportion of CSCs >100-fold compared to paclitaxcel that is used as a chemotherapeutic drug for breast cancer treatment. Interestingly, salinomycine treatment, but not paclitaxcel, indicated the loss of expression of breast cancer stem cell and the relative size of the $CD44^{+}/CD24^{low}$ fraction was therefore 360-fold lower following treatment with salinomycine compared to paclitaxel (Gupta et al., 2009). In agreement with previous study, sulforaphane, a dietary compound of broccoli/broccoli sproutus inhibited breast CSCs with IC50 approximately 10-fold lower compared to MCF7 and SUM159 breast cancer cell lines indicating anti-proliferative effects in MTT assay (Y. Li et al., 2010). Sulforaphane inhibited the ALDH-positive cells and mammosphere formations at 10-fold comparably lower concentration than IC50 of sulforaphane on breast cell lines using MTT assay. In consistence with previous studies (Gupta et al., 2009; Y. Li et al., 2010), An examination of the aldefluor assays allows the determination of Koenimbin's effect on breast cancer stem cells. Our

results indicate that Koenimbin does indeed selectively suppress the ALDH-positive breast cancer stem cells *in vitro*. Importantly, Koenimbin effectively suppress breast cancer stem cells in lower IC50 concentration in comparison with breast MCF7 cell line in both non-adherent mammosphere formation and ALDH-positive breast cancer stem cells using MTT assay, demonstrating the potency of Koenimbin for targeting the breast cancer stem cells. In this research, Koenimbin effectively manifested the anticancer impact on MCF7 cells and MCF7 CSCs in comparison with non-invasive normal MCF-10A cells. Another anticancer impact of Koenimbin against cancer cells might be due to cause of reactive radical formation, inhibiting the proliferation of cancer cells or may increase the amount of the protective molecule called glutathione (GSH) in the normal cells (Neesse & Hessmann, 2015), protecting the normal cells from any damages by anticancer natural and synthetic compounds.

Chapter 6

Conclusion

Koenimbin exhibits the capacity to trigger apoptosis signaling pathway in human breast cancer cells *in vitro*. Apoptosis in Koenimbin-induced MCF7 breast cancer cells takes place via a few mechanisms: a) through cell death-transducing signals, b) through the modulation of MMP caused by the down-regulation of Bcl2 and the up-regulation Bax, resulting in triggering translocation of mitochondrial cytochrome *c* into the cytosol. Upon the release of cytochrome *c,* caspases -9 and -7 were activated, which will lead to the cleaving of specific substrates, finally leading to apopsotis via intrinsic apoptosis signaling pathway. In addition, Koenimbin has capability to target derived MCF7 CSCs as determined by specific experiments including the mammosphere formation assay and Aldefluor assay. To conclude this reseach, we hereby assert our experimental validation of the chemotherapeutic value of Koenimbin as applied to breast cancer treatment and prevention. Strategies will be proposed with regards to future preclinical and clinical treatments.

Chapter 7

Future recommendations

Different aspects of this study need to be further investigated in order to confirm the observed activity. The MTT assay used in this study indicated anticancer activities of Koenimbin on different human cancer cell lines such as breast cancer triple negative cell lines and prostate cancer cell line. These results demonstrated the anticancer potential of Koenimbin on cancer cell lines. To confirm the anticancer activities of the Koenimbin, more in-depth *in vivo* studies are required to examine the interactions between Koenimbin and cancer cells (and cancer stem cells). Also, we highly recommend comparing results between *in vivo* and *in vitro* studies in order to better understand the expression of some important protein markers involved in pro-apoptotic and anti-apoptotic mechanisms such as BAX, BCL2, HSP70, P53 and ErbB2.

Chapter 7

REFERENCES

Abelev, G., & Eraiser, T. (2008). On the path to understanding the nature of cancer. *Biochemistry (Moscow), 73*(5), 487-497.

Abraham, B. K., Fritz, P., McClellan, M., Hauptvogel, P., Athelogou, M., & Brauch, H. (2005). Prevalence of CD44+/CD24−/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. *Clinical Cancer Research, 11*(3), 1154-1159.

Aggarwal, B. B., Sethi, G., Baladandayuthapani, V., Krishnan, S., & Shishodia, S. (2007). Targeting cell signaling pathways for drug discovery: an old lock needs a new key. *Journal of cellular biochemistry, 102*(3), 580-592.

Ajenjo, N., Cañón, E., Sánchez-Pérez, I., Matallanas, D., León, J., Perona, R., & Crespo, P. (2004). Subcellular localization determines the protective effects of activated ERK2 against distinct apoptogenic stimuli in myeloid leukemia cells. *Journal of Biological Chemistry, 279*(31), 32813-32823.

Al-Hajj, M., Becker, M. W., Wicha, M., Weissman, I., & Clarke, M. F. (2004). Therapeutic implications of cancer stem cells. *Current opinion in genetics & development, 14*(1), 43-47.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences, 100*(7), 3983-3988.

Allan, A. L., Vantyghem, S. A., Tuck, A. B., & Chambers, A. F. (2007). Tumor dormancy and cancer stem cells: implications for the biology and treatment of breast cancer metastasis. *Breast disease, 26*(1), 87-98.

Ansell, S. M., Johnstone, S. A., Tardi, P. G., Lo, L., Xie, S., Shu, Y., . . . Bermudes, D. (2008). Modulating the therapeutic activity of nanoparticle delivered paclitaxel by manipulating the hydrophobicity of prodrug conjugates. *Journal of medicinal chemistry, 51*(11), 3288-3296.

Aratanechemuge, Y., Hibasami, H., Katsuzaki, H., Imai, K., & Komiya, T. (2004). Induction of apoptosis by maackiain and trifolirhizin (maackiain glycoside) isolated from sanzukon (Sophora Subprostrate Chen et T. Chen) in human promyelotic leukemia HL-60 cells. *Oncology reports, 12*(6), 1183-1188.

Arbab, I. A., Abdul, A. B., Sukari, M. A., Abdullah, R., Syam, S., Kamalidehghan, B., . . . Mohd Ali, H. (2013). Dentatin isolated from< i> Clausena excavata</i> induces

apoptosis in MCF-7 cells through the intrinsic pathway with involvement of NF-< i> κ</i> B signalling and G0/G1 cell cycle arrest: A bioassay-guided approach. *Journal of ethnopharmacology, 145*(1), 343-354.

Arora, A., & Scholar, E. M. (2005). Role of tyrosine kinase inhibitors in cancer therapy. *Journal of Pharmacology and Experimental Therapeutics, 315*(3), 971-979.

Arulselvan, P., & Subramanian, S. (2007). Effect of Murraya koenigii leaf extract on carbohydrate metabolism studied in streptozotocin induced diabetic rats. *Int J Biol Chem, 1*(1), 21-28.

Arulselvan, P., & Subramanian, S. P. (2007). Beneficial effects of< i> Murraya koenigii</i> leaves on antioxidant defense system and ultra structural changes of pancreatic β-cells in experimental diabetes in rats. *Chemico-biological interactions, 165*(2), 155-164.

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science, 281*(5381), 1305-1308.

Bailey, R. W., Nguyen, T., Robertson, L., Gibbons, E., Nelson, J., Christensen, R. E., . . . Bell, J. D. (2009). Sequence of physical changes to the cell membrane during glucocorticoid-induced apoptosis in S49 lymphoma cells. *Biophysical journal, 96*(7), 2709-2718.

Baker, M. (2017). Deceptive curcumin offers cautionary tale for chemists. *Nature, 541*, 144-145.

Bar, E. E., Chaudhry, A., Lin, A., Fan, X., Schreck, K., Matsui, W., . . . Olivi, A. (2007). Cyclopamine-Mediated Hedgehog Pathway Inhibition Depletes Stem-Like Cancer Cells in Glioblastoma. *Stem cells, 25*(10), 2524-2533.

Behbod, F., & Rosen, J. M. (2005). Will cancer stem cells provide new therapeutic targets? *Carcinogenesis, 26*(4), 703-711.

Beier, D., Hau, P., Proescholdt, M., Lohmeier, A., Wischhusen, J., Oefner, P. J., . . . Beier, C. P. (2007). CD133+ and CD133− glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer research, 67*(9), 4010-4015.

Bergsagel, D., & Valeriote, F. (1968). Growth characteristics of a mouse plasma cell tumor. *Cancer research, 28*(11), 2187-2196.

Bjerkvig, R., Tysnes, B. B., Aboody, K. S., Najbauer, J., & Terzis, A. (2005). The origin of the cancer stem cell: current controversies and new insights. *Nature Reviews Cancer, 5*(11), 899-904.

Blagosklonny, M. V. (2007). Cancer stem cell and cancer stemloids: from biology to therapy. *Cancer biology & therapy, 6*(11), 1684-1690.

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine, 3*(7), 730-737.

Bruce, W., & Van Der Gaag, H. (1963). A quantitative assay for the number of murine lymphoma cells capable of proliferation in vivo.

Brugieres, L., Pacquement, H., Le Deley, M.-C., Leverger, G., Lutz, P., Paillard, C., . . . Lamant, L. (2009). Single-drug vinblastine as salvage treatment for refractory or relapsed anaplastic large-cell lymphoma: a report from the French Society of Pediatric Oncology. *Journal of Clinical Oncology, 27*(30), 5056-5061.

Brunmark, A., Collins, V., Thaw, H., & Brunk, U. (1986). Lipofuscin accumulation in cultured non-dividing cells as a function of time and oxygen tension. *Scanning electron microscopy*(Pt 1), 189.

Burdall, S., Hanby, A., Lansdown, M., & Speirs, V. (2003). Breast cancer cell lines: friend or foe? *Breast Cancer Research, 5*(2), 89-89.

Burdall, S. E., Hanby, A. M., Lansdown, M., & Speirs, V. (2003). Breast cancer cell lines: friend or foe? *Breast Cancer Research, 5*(2), 89-89.

Cailleau, R., Olivé, M., & Cruciger, Q. V. (1978). Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In vitro, 14*(11), 911-915.

Campbell, L. L., & Polyak, K. (2007). Breast tumor heterogeneity: cancer stem cells or clonal evolution? *Cell Cycle, 6*(19), 2332-2338.

Carnero, A. (2000). Cell cycle and Cancer. *Clinical and Translational Oncology, 2*(1), 12-22.

Chabner, B. A., & Roberts Jr, T. G. (2005). Chemotherapy and the war on cancer. *Nature reviews. Cancer, 5*(1), 65.

Chahar, M. K., Sharma, N., Dobhal, M. P., & Joshi, Y. C. (2011). Flavonoids: a versatile source of anticancer drugs. *Pharmacognosy reviews, 5*(9), 1.

Charafe-Jauffret, E., Monville, F., Ginestier, C., Dontu, G., Birnbaum, D., & Wicha, M. S. (2008). Cancer stem cells in breast: current opinion and future challenges. *Pathobiology, 75*(2), 75-84.

Chen, J., & Yu, B. (1994). Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radical Biology and Medicine, 17*(5), 411-418.

Cheung-Ong, K., Giaever, G., & Nislow, C. (2013). DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. *Chemistry & biology, 20*(5), 648-659.

Chiang, C.-T., Way, T.-D., Tsai, S.-J., & Lin, J.-K. (2007). Diosgenin, a naturally occurring steroid, suppresses fatty acid synthase expression in HER2-overexpressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation. *FEBS letters, 581*(30), 5735-5742.

Chiba, T., Kita, K., Zheng, Y. W., Yokosuka, O., Saisho, H., Iwama, A., . . . Taniguchi, H. (2006). Side population purified from hepatocellular carcinoma cells harbors cancer stem cell–like properties. *Hepatology, 44*(1), 240-251.

Chicheportiche, Y., Bourdon, P. R., Xu, H., Hsu, Y.-M., Scott, H., Hession, C., . . . Browning, J. L. (1997). TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *Journal of Biological Chemistry, 272*(51), 32401-32410.

Christensen, J. G., & LeBlanc, G. A. (1996). Reversal of multidrug resistance in vivo by dietary administration of the phytochemical indole-3-carbinol. *Cancer research, 56*(3), 574-581.

Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H., Jones, D. L., . . . Wahl, G. M. (2006). Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer research, 66*(19), 9339-9344.

Cohnheim, J. (1867). Ueber entzündung und eiterung. *Virchows Archiv, 40*(1), 1-79.

Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., & Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer research, 65*(23), 10946-10951.

Comis, R. L., & Carter, S. K. (1974). A review of chemotherapy in gastric cancer. *Cancer, 34*(5), 1576-1586.

Correa, C., Bertollo, C., & Goes, A. (2009). Establishment and characterization of macl-1 and mgso-3 cell lines derived from human primary breast cancer. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics, 17*(10), 473-482.

Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer, 2*(9), 647-656.

Cozzio, A., Passegué, E., Ayton, P. M., Karsunky, H., Cleary, M. L., & Weissman, I. L. (2003). Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes & development, 17*(24), 3029-3035.

Cragg, G. M., Grothaus, P. G., & Newman, D. J. (2009). Impact of natural products on developing new anti-cancer agents. *Chemical reviews, 109*(7), 3012-3043.

Cragg, G. M., & Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of ethnopharmacology, 100*(1), 72-79.

Dalerba, P., Cho, R. W., & Clarke, M. F. (2007). Cancer stem cells: models and concepts. *Annu. Rev. Med., 58*, 267-284.

Dalerba, P., Dylla, S. J., Park, I.-K., Liu, R., Wang, X., Cho, R. W., . . . Simeone, D. M. (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences, 104*(24), 10158-10163.

Dall'Acqua, S. (2014). Natural products as antimitotic agents. *Current topics in medicinal chemistry, 14*(20), 2272-2285.

Degli Esposti, M. (2002). The roles of Bid. *Apoptosis, 7*(5), 433-440.

Denecker, G., Vercammen, D., Declercq, W., & Vandenabeele, P. (2001). Apoptotic and necrotic cell death induced by death domain receptors. *Cellular and Molecular Life Sciences CMLS, 58*(3), 356-370.

Deng, X.-H., Song, H.-Y., Zhou, Y.-F., Yuan, G.-Y., & Zheng, F.-J. (2013). Effects of quercetin on the proliferation of breast cancer cells and expression of survivin in vitro. *Experimental and therapeutic medicine, 6*(5), 1155-1158.

Dhar, S., Nygren, P., Csoka, K., Botling, J., Nilsson, K., & Larsson, R. (1996). Anti-cancer drug characterisation using a human cell line panel representing defined types of drug resistance. *British journal of cancer, 74*(6), 888.

Doll, R., & Peto, R. (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst 66*, 1191-1308.

Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., & Wicha, M. S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & development, 17*(10), 1253-1270.

Dontu, G., Jackson, K. W., McNicholas, E., Kawamura, M. J., Abdallah, W. M., & Wicha, M. S. (2004). Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res, 6*(6), R605-R615.

Du, C., Fang, M., Li, Y., Li, L., & Wang, X. (2000). Smac, a mitochondrial protein that promotes cytochrome c–dependent caspase activation by eliminating IAP inhibition. *cell, 102*(1), 33-42.

Duesberg, P., & Li, R. (2003). Multistep carcinogenesis: a chain reaction of aneuploidizations. *Cell cycle (Georgetown, Tex.), 2*(3), 202.

Dumontet, C., & Jordan, M. A. (2010). Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nature reviews Drug discovery, 9*(10), 790-803.

Earnshaw, W. C., Martins, L. M., & Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annual review of biochemistry, 68*(1), 383-424.

Efferth, T., Konkimalla, V. B., Wang, Y.-F., Sauerbrey, A., Meinhardt, S., Zintl, F., . . . Volm, M. (2008). Prediction of broad spectrum resistance of tumors towards anticancer drugs. *Clinical Cancer Research, 14*(8), 2405-2412.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., . . . Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell, 75*(4), 817-825.

Engel, L. W., & Young, N. A. (1978). Human breast carcinoma cells in continuous culture: a review. *Cancer research, 38*(11 Part 2), 4327-4339.

Eramo, A., Lotti, F., Sette, G., Pilozzi, E., Biffoni, M., Di Virgilio, A., . . . De Maria, R. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death & Differentiation, 15*(3), 504-514.

Faderl, S., O'brien, S., Pui, C. H., Stock, W., Wetzler, M., Hoelzer, D., & Kantarjian, H. M. (2010). Adult acute lymphoblastic leukemia. *Cancer, 116*(5), 1165-1176.

Fan, X., Matsui, W., Khaki, L., Stearns, D., Chun, J., Li, Y.-M., & Eberhart, C. G. (2006). Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. *Cancer research, 66*(15), 7445-7452.

Fang, D., Nguyen, T. K., Leishear, K., Finko, R., Kulp, A. N., Hotz, S., . . . Herlyn, M. (2005). A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer research, 65*(20), 9328-9337.

Fang, H., Chen, S., Guo, D., Pan, S., & Yu, Z. (2011). Proteomic identification of differentially expressed proteins in curcumin-treated MCF-7 cells. *Phytomedicine, 18*(8), 697-703.

Fayad, W., Fryknäs, M., Brnjic, S., Olofsson, M. H., Larsson, R., & Linder, S. (2009). Identification of a novel topoisomerase inhibitor effective in cells overexpressing drug efflux transporters. *PLoS One, 4*(10), e7238.

Fialkow, P. (1989). Stem cell origin of human myeloid blood cell neoplasms. *Verhandlungen der Deutschen Gesellschaft fur Pathologie, 74*, 43-47.

Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., De Vitis, S., . . . Vescovi, A. (2004). Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research, 64*(19), 7011-7021.

Garrido, C., Galluzzi, L., Brunet, M., Puig, P., Didelot, C., & Kroemer, G. (2006). Mechanisms of cytochrome c release from mitochondria. *Cell Death & Differentiation, 13*(9), 1423-1433.

Ghosh, G., Duyne, G. V., Ghosh, S., & Sigler, P. B. (1995). Structure of NF-kB p50 homodimer bound to a kB site. *Nature, 373*(6512), 303-310.

Gilbertson, R. J. (2006). Brain tumors provide new clues to the source of cancer stem cells: does oncology recapitulate ontogeny? *Cell cycle, 5*(2), 135-137.

Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., . . . Liu, S. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell stem cell, 1*(5), 555-567.

Godar, D. E. (1999). UVA1 radiation triggers two different final apoptotic pathways. *Journal of investigative dermatology, 112*(1), 3-12.

Goldstein, S. (1974). Aging in vitro* 1:: Growth of cultured cells from the Galapagos tortoise. *Experimental cell research, 83*(2), 297-302.

Gordaliza, M. (2007). Natural products as leads to anticancer drugs. *Clinical and Translational Oncology, 9*(12), 767-776.

Goy, A., Younes, A., McLaughlin, P., Pro, B., Romaguera, J. E., Hagemeister, F., . . . Wang, M. (2005). Phase II study of proteasome inhibitor bortezomib in relapsed or refractory B-cell non-Hodgkin's lymphoma. *Journal of Clinical Oncology, 23*(4), 667-675.

Green, D. R. (2006). At the gates of death. *Cancer cell, 9*(5), 328-330.

Grimshaw, M. J., Cooper, L., Papazisis, K., Coleman, J. A., Bohnenkamp, H. R., Chiapero-Stanke, L., . . . Burchell, J. M. (2008). Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Res, 10*(3), R52.

Gu, Q., De Wang, J., Xia, H. H., Lin, M. C., He, H., Zou, B., . . . Lam, S. K. (2005). Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer. *Carcinogenesis, 26*(3), 541-546.

Gummadi, V. R., Rajagopalan, S., Yeng, L. C., Paydar, M., Renukappa, G. A., Ainan, B. R., . . . Raghuramachandran, S. (2013). Discovery of 7-azaindole based anaplastic lymphoma kinase (ALK) inhibitors: Wild type and mutant (L1196M) active compounds with unique binding mode. *Bioorganic & medicinal chemistry letters*.

Günther, H., Schmidt, N., Phillips, H., Kemming, D., Kharbanda, S., Soriano, R., . . . Lamszus, K. (2008). Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. *Oncogene, 27*(20), 2897-2909.

Gupta, P. B., Onder, T. T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R. A., & Lander, E. S. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. *cell, 138*(4), 645-659.

Gutkind, J. (2000). *Signaling Networks and Cell Cycle Control*. Totowa: Humana Press.

Hambardzumyan, D., Squartro, M., & Holland, E. C. (2006). Radiation resistance and stem-like cells in brain tumors. *Cancer cell, 10*(6), 454-456.

Hamburger, A., & Salmon, S. E. (1977). Primary bioassay of human myeloma stem cells. *Journal of Clinical Investigation, 60*(4), 846.

Han, J., Lin, H., & Huang, W. (2011). Modulating gut microbiota as an anti-diabetic mechanism of berberine. *Medical science monitor: international medical journal of experimental and clinical research, 17*(7), RA164.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *cell, 144*(5), 646-674.

Hartwell, J. (1976). Types of anticancer agents isolated from plants. *Cancer treatment reports, 60*(8), 1031-1067.

Harvey, A. L., Clark, R. L., Mackay, S. P., & Johnston, B. F. (2010). Current strategies for drug discovery through natural products. *Expert opinion on drug discovery, 5*(6), 559-568.

Harvey, A. L., & Cree, I. A. (2010). High-throughput screening of natural products for cancer therapy. *Planta medica, 76*(11), 1080-1086.

Hay, R. (1992). *ATCC quality control methods for cell lines*: Amer Type Culture Collection.

Hayflick, L. (1975). Cell biology of aging. *Bioscience, 25*(10), 629-637.

Hayflick, L. (1998). How and why we age. *Experimental Gerontology, 33*(7-8), 639-653.

Hayflick, L., & Moorhead, P. (1961). The serial cultivation of human diploid cell strains* 1. *Experimental cell research, 25*(3), 585-621.

Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., & Kornblum, H. I. (2003). Cancerous stem cells can arise from pediatric brain tumors. *Proceedings of the National Academy of Sciences, 100*(25), 15178-15183.

Herouart, D., Sangwan, R., Fliniaux, M., & Sangwan-Norreel, B. (1988). Variations in the Leaf Alkaloid Content of Androgenic Diploid Plants of Datura innoxia. *Planta medica, 54*(1), 14-17.

Hieronymus, H., Lamb, J., Ross, K. N., Peng, X. P., Clement, C., Rodina, A., . . . Raj, S. M. (2006). Gene expression signature-based chemical genomic prediction identifies a novel class of HSP90 pathway modulators. *Cancer cell, 10*(4), 321-330.

Hill, M. M., Adrain, C., Duriez, P. J., Creagh, E. M., & Martin, S. J. (2004). Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *The EMBO journal, 23*(10), 2134-2145.

Hill, R. P., & Perris, R. (2007). "Destemming" cancer stem cells. *Journal of the National Cancer Institute, 99*(19), 1435-1440.

Hirsch, T., Marchetti, P., Susin, S. A., Dallaporta, B., Zamzami, N., Marzo, I., . . . Kroemer, G. (1997). The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene, 15*(13), 1573-1581.

Hisham, A., & Yip, C. (2003). Spectrum of breast cancer in Malaysian women: overview. *World journal of surgery, 27*(8), 921-923.

Hisham, A., & Yip, C. (2004). Overview of breast cancer in Malaysian women: a problem with late diagnosis. *Asian Journal of Surgery, 27*(2), 130-133.

Hisham, A. N., & Yip, C.-H. (2004). Overview of breast cancer in Malaysian women: a problem with late diagnosis. *Asian Journal of Surgery, 27*(2), 130-133.

Hisham, A. N., & Yip, C. H. (2003). Spectrum of breast cancer in Malaysian women: overview. *World journal of surgery, 27*(8), 921-923.

Horvitz, H. R. (1999). Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer research, 59*(7 Supplement), 1701s-1706s.

Hunter, A. M., LaCasse, E. C., & Korneluk, R. G. (2007). The inhibitors of apoptosis (IAPs) as cancer targets. *Apoptosis, 12*(9), 1543-1568.

Ibrahim MY, H. N., Mohan S, Abdulla MA, Kamalidehghan B, Ghaderian M, Dehghan F, Ali LZ, Arbab IA, Yahayu M, Lian GE, Ahmadipour F, Ali HM. (2014). α-Mangostin from Cratoxylum arborescens demonstrates apoptogenesis in MCF-7 with regulation of NF-κB and Hsp70 protein modulation in vitro, and tumor reduction in vivo. *Drug Design, Development and Therapy, 8*, 1629-1647.

Ichikawa, H., Nakamura, Y., Kashiwada, Y., & Aggarwal, B. B. (2007). Anticancer drugs designed by mother nature: ancient drugs but modern targets. *Current pharmaceutical design, 13*(33), 3400-3416.

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. *Nature Reviews Cancer, 2*(4), 277-288.

Ince, T. A., Richardson, A. L., Bell, G. W., Saitoh, M., Godar, S., Karnoub, A. E., . . . Weinberg, R. A. (2007). Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. *Cancer cell, 12*(2), 160-170.

Isa, N. M., Abdul, A. B., Abdelwahab, S. I., Abdullah, R., Sukari, M. A., Kamalidehghan, B., . . . Mohan, S. (2013). Boesenbergin A, a chalcone from< i> Boesenbergia rotunda</i> induces apoptosis via mitochondrial dysregulation and cytochrome c release in A549 cells< i> in vitro</i>: Involvement of HSP70 and Bcl2/Bax signalling pathways. *Journal of Functional Foods, 5*(1), 87-97.

Ismail Adam Arbab, C. Y. L., Ahmad Bustamam Abdul, Foo Kit Cheah, Won Fen Wong, Mohd Aspollah Sukari, Rasedee Abdullah, Syam Mohan, Suvitha Syam, Aditya Arya, Manal Mohamed Elhassan Taha, Bushra Muharram, Mohd Rais Mustafa, Siddig Ibrahim Abdelwahab. (2012). Dentatin induces apoptosis in prostate cancer cells via Bcl-2, Bcl-xL, Survivin downregulation, caspase-9, -3/7 activation and NF-kB inhibition. *Evidence-Based Complementary and Alternative Medicine*, 856029.

Ito, C., Itoigawa, M., Nakao, K., Murata, T., Tsuboi, M., Kaneda, N., & Furukawa, H. (2006). Induction of apoptosis by carbazole alkaloids isolated from< i> Murraya koenigii</i>. *Phytomedicine, 13*(5), 359-365.

Jackson, B., Brocker, C., Thompson, D. C., Black, W., Vasiliou, K., Nebert, D. W., & Vasiliou, V. (2011). Update on the aldehyde dehydrogenase gene (ALDH) superfamily. *Human genomics, 5*(4), 283.

Jaiswal, A. S., Marlow, B. P., Gupta, N., & Narayan, S. (2002). Beta-catenin-mediated transactivation and cell-cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. *Oncogene, 21*(55), 8414-8427.

Jamieson, C. H., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., . . . Keating, A. (2004). Granulocyte–macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *New England Journal of Medicine, 351*(7), 657-667.

Ji, Y. (2011). Active ingredients of traditional Chinese medicine: Pharmacology and application. *People's Medical Publishing Hourse Cp., LTD*.

Johansson, A.-C., Appelqvist, H., Nilsson, C., Kågedal, K., Roberg, K., & Öllinger, K. (2010). Regulation of apoptosis-associated lysosomal membrane permeabilization. *Apoptosis, 15*(5), 527-540.

Jones, K. L., & Buzdar, A. U. (2004). A review of adjuvant hormonal therapy in breast cancer. *Endocrine-Related Cancer, 11*(3), 391-406.

Jordan, B. C., Mock, C. D., Thilagavathi, R., & Selvam, C. (2016). Molecular mechanisms of curcumin and its semisynthetic analogues in prostate cancer prevention and treatment. *Life sciences, 152*, 135-144.

Jordan, M. A., & Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nature reviews. Cancer, 4*(4), 253.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., . . . Ravagnan, L. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature, 410*(6828), 549-554.

Kakarala, M., & Wicha, M. S. (2008). Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. *Journal of Clinical Oncology, 26*(17), 2813-2820.

Kamalidehghan, B., Houshmand, M., Kamalidehghan, F., Jafarzadeh, N., Azari, S., Akmal, S. N., & Rosli, R. (2012). Establishment and characterization of two human breast carcinoma cell lines by spontaneous immortalization: Discordance between Estrogen, Progesterone and HER2/neu receptors of breast carcinoma tissues with derived cell lines. *Cancer Cell Int, 12*, 43.

Kang, Y.-H., Yi, M.-J., Kim, M.-J., Park, M.-T., Bae, S., Kang, C.-M., . . . Rhee, C. H. (2004). Caspase-Independent Cell Death by Arsenic Trioxide in Human Cervical Cancer Cells Reactive Oxygen Species-Mediated Poly (ADP-ribose) Polymerase-1 Activation Signals Apoptosis-Inducing Factor Release from Mitochondria. *Cancer research, 64*(24), 8960-8967.

Kappers, I. F., Aharoni, A., Van Herpen, T. W., Luckerhoff, L. L., Dicke, M., & Bouwmeester, H. J. (2005). Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. *Science, 309*(5743), 2070-2072.

Karmakar, S., Banik, N. L., & Ray, S. K. (2007). Molecular mechanism of inositol hexaphosphate-mediated apoptosis in human malignant glioblastoma T98G cells. *Neurochemical research, 32*(12), 2094-2102.

Kathawala, R. J., Gupta, P., Ashby, C. R., Chen, Z.-S., Shefet-Carasso, L., & Benhar, I. (2015). Over the past decade, the proteasome inhibitor bortezomib (Velcade) has not only gained a cornerstone position in the treatment of hematological malignancies, particularly multiple myeloma and mantle cell lymphoma, but also in experimental therapeutics of acute leukemia. However, the therapeutic efficacy of bortezomib is hampered by the emergence of acquired resistance, for which multifactorial mechanisms. *Drug Resistance Updates, 21*(22), 20-29.

Kawasaki, B. T., Hurt, E. M., Mistree, T., & Farrar, W. L. (2008). Targeting cancer stem cells with phytochemicals. *Molecular interventions, 8*(4), 174.

Keller, L. H. (2009). Bone Marrow–Derived Aldehyde Dehydrogenase–Bright Stem and Progenitor Cells for Ischemic Repair. *Congestive Heart Failure, 15*(4), 202-206.

Kerr, J. F. (2002). History of the events leading to the formulation of the apoptosis concept. *Toxicology, 181*, 471-474.

Keydar, I., Chen, L., Karby, S., Weiss, F., Delarea, J., Radu, M., . . . Brenner, H. (1979). Establishment and characterization of a cell line of human breast carcinoma origin. *European Journal of Cancer (1965), 15*(5), 659-670.

Kirtikar, K. R., & Basu, B. D. (1918). Indian medicinal plants. *Indian Medicinal Plants.*

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., & Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science, 275*(5303), 1132-1136.

Kobayashi, H., Stewart, E., Poon, R., Adamczewski, J., Gannon, J., & Hunt, T. (1992). Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits. *Molecular Biology of the Cell, 3*(11), 1279.

Korkaya, H., Paulson, A., Charafe-Jauffret, E., Ginestier, C., Brown, M., Dutcher, J., . . . Wicha, M. S. (2009). Regulation of mammary stem/progenitor cells by PTEN/Akt/β-catenin signaling. *PLoS biology, 7*(6), e1000121.

Krajewski, S., Krajewska, M., Ellerby, L. M., Welsh, K., Xie, Z., Deveraux, Q. L., . . . Fiskum, G. (1999). Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. *Proceedings of the National Academy of Sciences, 96*(10), 5752-5757.

Krishnaiah, D., Sarbatly, R., & Bono, A. (2007). Phytochemical antioxidants for health and medicine–A move towards nature. *Biotechnol Mol Biol Rev, 1*(4), 97-104.

Krivtsov, A. V., Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., Faber, J., . . . Gilliland, D. G. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL–AF9. *Nature, 442*(7104), 818-822.

Lacroix, M., & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast cancer research and treatment, 83*(3), 249-289.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., . . . Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.

Larsson, J., Gottfries, J., Muresan, S., & Backlund, A. (2007). ChemGPS-NP: tuned for navigation in biologically relevant chemical space. *Journal of natural products, 70*(5), 789-794.

Lee, M.-S., Chan, J. Y.-W., Kong, S.-K., Yu, B., Eng-Choon, V. O., Nai-Ching, H. W., . . . Fung, K.-P. (2005). Effects of polyphyllin D, a steroidal saponin in Paris polyphylla, in growth inhibition of human breast cancer cells and in xenograft. *Cancer biology & therapy, 4*(11), 1248-1254.

Lees, E., & Harlow, E. (1993). Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. *Molecular and cellular biology, 13*(2), 1194.

Lengauer, C., Kinzler, K. W., & Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature, 396*(6712), 643-649.

Lev-Ari, S., Vexler, A., Starr, A., Ashkenazy-Voghera, M., Greif, J., Aderka, D., & Ben-Yosef, R. (2007). Curcumin augments gemcitabine cytotoxic effect on pancreatic adenocarcinoma cell lines. *Cancer investigation, 25*(6), 411-418.

Levin, S., Bucci, T. J., Cohen, S. M., Fix, A. S., Hardisty, J. F., Legrand, E. K., . . . Trump, B. F. (1999). The nomenclature of cell death: recommendations of an ad hoc Committee of the Society of Toxicologic Pathologists. *Toxicologic pathology, 27*(4), 484-490.

Lew, D., Duli, V., & Reed, S. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell, 66*(6), 1197-1206.

Li, C., Heidt, D. G., Dalerba, P., Burant, C. F., Zhang, L., Adsay, V., . . . Simeone, D. M. (2007). Identification of pancreatic cancer stem cells. *Cancer research, 67*(3), 1030-1037.

Li, M., Su, B.-S., Chang, L.-H., Gao, Q., Chen, K.-L., An, P., . . . Li, Z.-F. (2014). Oxymatrine induces apoptosis in human cervical cancer cells through guanine nucleotide depletion. *Anti-cancer drugs, 25*(2), 161-173.

Li, Y., Zhang, T., Korkaya, H., Liu, S., Lee, H.-F., Newman, B., . . . Wicha, M. S. (2010). Sulforaphane, a dietary component of broccoli/broccoli sprouts, inhibits breast cancer stem cells. *Clinical Cancer Research, 16*(9), 2580-2590.

Li, Z., Jo, J., Jia, J.-M., Lo, S.-C., Whitcomb, D. J., Jiao, S., . . . Sheng, M. (2010). Caspase-3 activation via mitochondria is required for long-term depression and AMPA receptor internalization. *Cell, 141*(5), 859-871.

Liang, C. Z., Zhang, J. K., Shi, Z., Liu, B., Shen, C. Q., & Tao, H. M. (2012). Matrine induces caspase-dependent apoptosis in human osteosarcoma cells in vitro and in vivo through the upregulation of Bax and Fas/FasL and downregulation of Bcl-2. *Cancer chemotherapy and pharmacology, 69*(2), 317-331.

Lim, Y.-J., Choi, J.-A., Choi, H.-H., Cho, S.-N., Kim, H.-J., Jo, E.-K., . . . Song, C.-H. (2011). Endoplasmic reticulum stress pathway-mediated apoptosis in macrophages contributes to the survival of Mycobacterium tuberculosis. *PLoS ONE, 6*(12), e28531-e28531.

Lippman, M. E. (2000). High-dose chemotherapy plus autologous bone marrow transplantation for metastatic breast cancer. *The New England journal of medicine, 342*(15), 1119.

Liu, R., Wang, X., Chen, G. Y., Dalerba, P., Gurney, A., Hoey, T., . . . Clarke, M. F. (2007). The prognostic role of a gene signature from tumorigenic breast-cancer cells. *New England Journal of Medicine, 356*(3), 217-226.

Liu, S., Dontu, G., Mantle, I. D., Patel, S., Ahn, N.-s., Jackson, K. W., . . . Wicha, M. S. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer research, 66*(12), 6063-6071.

Liu, S., Dontu, G., & Wicha, M. S. (2005). Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast Cancer Research, 7*(3), 86.

Liu, Y., Xu, Y., Ji, W., Li, X., Sun, B., Gao, Q., & Su, C. (2014). Anti-tumor activities of matrine and oxymatrine: literature review. *Tumor Biology, 35*(6), 5111-5119.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., . . . Darnell, J. (2004). *Molecular Cell Biology*. New York: Freeman W.H.

Lodish, H., Berk, A., Zipursky, S., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). *Molecular Cell Biology*. New York: Freeman, W.H.

London, C. A. (2009). Tyrosine kinase inhibitors in veterinary medicine. *Topics in companion animal medicine, 24*(3), 106-112.

Lu, J.-J., Bao, J.-L., Chen, X.-P., Huang, M., & Wang, Y.-T. (2012). Alkaloids isolated from natural herbs as the anticancer agents. *Evidence-Based Complementary and Alternative Medicine, 2012*.

Ma, Q., Tian, J., Yang, J., Wang, A., Ji, T., Wang, Y., & Su, Y. (2013). Bioactive Carbazole Alkaloids from< i> Murraya koenigii</i>(L.) Spreng. *Fitoterapia*.

Ma, S., Chan, K. W., Hu, L., Lee, T. K. W., Wo, J. Y. H., Ng, I. O. L., . . . Guan, X. Y. (2007). Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology, 132*(7), 2542-2556.

Mahato, S. B., & Sen, S. (1997). Advances in triterpenoid research, 1990–1994. *Phytochemistry, 44*(7), 1185-1236.

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The American journal of pathology, 146*(1), 3.

Man, S., Gao, W., Zhang, Y., Huang, L., & Liu, C. (2010). Chemical study and medical application of saponins as anti-cancer agents. *Fitoterapia, 81*(7), 703-714.

Martelli, M., Ferreri, A., & Agostinelli, C. (2013). New drugs for aggressive B-cell and T-cell lymphomas. *Crit Rev Oncol Hematol, 87*(2), 146-171.

Martinvalet, D., Zhu, P., & Lieberman, J. (2005). Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity, 22*(3), 355-370.

Masters, J., Foley, C., Bisson, I., & Ahmed, A. (2003). Cancer stem cells. *BJU international, 92*(7), 661-662.

Mathon, N., Malcolm, D., Harrisingh, M., Cheng, L., & Lloyd, A. (2001). Lack of replicative senescence in normal rodent glia. *Science, 291*, 872-875.

Matsui, W., Huff, C. A., Wang, Q., Malehorn, M. T., Barber, J., Tanhehco, Y., . . . Jones, R. J. (2004). Characterization of clonogenic multiple myeloma cells. *Blood, 103*(6), 2332-2336.

Mieog, J. S. D., de Kruijf, E. M., Bastiaannet, E., Kuppen, P. J., Sajet, A., de Craen, A. J., . . . Liefers, G.-J. (2012). Age determines the prognostic role of the cancer stem cell marker aldehyde dehydrogenase-1 in breast cancer. *BMC cancer, 12*(1), 42.

Misirlic Dencic, S., Poljarevic, J., Vilimanovich, U., Bogdanovic, A., Isakovic, A. J., Kravic Stevovic, T., . . . Grguric-Sipka, S. (2012). Cyclohexyl analogues of ethylenediamine dipropanoic acid induce caspase-independent mitochondrial apoptosis in human leukemic cells. *Chemical research in toxicology, 25*(4), 931-939.

Mohan, S., Abdelwahab, S. I., Kamalidehghan, B., Syam, S., May, K. S., Harmal, N. S. M., . . . Rahmani, M. (2012). Involvement of NF-κB and Bcl2/Bax signaling pathways in the apoptosis of MCF7 cells induced by a xanthone compound Pyranocycloartobiloxanthone A. *Phytomedicine, 19*(11), 1007-1015.

Morphy, R., Kay, C., & Rankovic, Z. (2004). From magic bullets to designed multiple ligands. *Drug discovery today, 9*(15), 641-651.

Moudi, M., Go, R., Yien, C. Y. S., & Nazre, M. (2013). Vinca alkaloids. *International journal of preventive medicine, 4*(11), 1231.

Muhammad Nadzri, N., Abdul, A. B., Sukari, M. A., Abdelwahab, S. I., Eid, E. E., Mohan, S., . . . Syam, S. (2013). Inclusion complex of zerumbone with hydroxypropyl-β-cyclodextrin induces apoptosis in liver hepatocellular HepG2 cells via caspase 8/bid cleavage switch and modulating Bcl2/Bax ratio. *Evidence-Based Complementary and Alternative Medicine, 2013*.

Murray, Andrew, & Tim, H. (1993). *The Cell Cycle: An Introduction*. Oxford: Oxford University Press.

Nadkarni, K. M. (1994). *[Indian materia medica]; Dr. KM Nadkarni's Indian materia medica: with Ayurvedic, Unani-Tibbi, Siddha, allopathic, homeopathic, naturopathic & home remedies, appendices & indexes. 1*: Popular Prakashan.

Nakagawa, T., & Yuan, J. (2000). Cross-talk between two cysteine protease families Activation of caspase-12 by calpain in apoptosis. *The Journal of cell biology, 150*(4), 887-894.

Nakamura, S., Nakashima, S., Oda, Y., Yokota, N., Fujimoto, K., Matsumoto, T., . . . Nishida, S. (2013). Alkaloids from Sri Lankan curry-leaf (< i> Murraya koenigii</i>) display melanogenesis inhibitory activity: Structures of karapinchamines A and B. *Bioorganic & medicinal chemistry, 21*(5), 1043-1049.

NCR. (2004). Second report of the National Cancer Registry, Cancer incidence in Malaysia 2003.

Neesse, A., & Hessmann, E. (2015). Electron transfer-based compounds: a novel weapon in the cancer battlespace? *EBioMedicine*.

Nelson, K. M., Dahlin, J. L., Bisson, J., Graham, J., Pauli, G. F., & Walters, M. A. (2017). The Essential Medicinal Chemistry of Curcumin: Miniperspective. *Journal of medicinal chemistry, 60*(5), 1620-1637.

Ng, K.-B., Bustamam, A., Sukari, M. A., Abdelwahab, S. I., Mohan, S., Buckle, M. J. C., . . . Hadi, A. H. A. (2013). Induction of selective cytotoxicity and apoptosis in human T4-lymphoblastoid cell line (CEMss) by boesenbergin a isolated from boesenbergia rotunda rhizomes involves mitochondrial pathway, activation of caspase 3 and G2/M phase cell cycle arrest. *BMC complementary and alternative medicine, 13*(1), 41.

Norbury, C. J., & Hickson, I. D. (2001). Cellular responses to DNA damage. *Annual review of pharmacology and toxicology, 41*(1), 367-401.

Nostro, A., Germano, M., D'angelo, V., Marino, A., & Cannatelli, M. (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Letters in applied microbiology, 30*(5), 379-384.

O'Neill, L. A., & Kaltschmidt, C. (1997). NF-kB: a crucial transcription factor for glial and neuronal cell function. *Trends in neurosciences, 20*(6), 252-258.

O'Brien, C. A., Pollett, A., Gallinger, S., & Dick, J. E. (2006). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature, 445*(7123), 106-110.

O'Brien, C. A., Pollett, A., Gallinger, S., & Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature, 445*(7123), 106-110.

Obregón-Henao, A., Duque-Correa, M. A., Rojas, M., García, L. F., Brennan, P. J., Ortiz, B. L., & Belisle, J. T. (2012). Stable extracellular RNA fragments of Mycobacterium tuberculosis induce early apoptosis in human monocytes via a caspase-8 dependent mechanism. *PLoS ONE, 7*(1), e29970.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., . . . Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science, 288*(5468), 1053-1058.

Oliveras-Ferraros, C., Corominas-Faja, B., Cufí, S., Vazquez-Martin, A., Martin-Castillo, B., Iglesias, J. M., . . . Menendez, J. A. (2012). Epithelial-to-mesenchymal transition (EMT) confers primary resistance to trastuzumab (Herceptin). *Cell Cycle, 11*(21), 4020-4032.

Osborne, C., Hobbs, K., & Trent, J. (1987). Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast cancer research and treatment, 9*(2), 111-121.

Osborne, C. K., Hobbs, K., & Trent, J. M. (1987). Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast cancer research and treatment, 9*(2), 111-121.

Pahlke, G., Ngiewih, Y., Kern, M., Jakobs, S., Marko, D., & Eisenbrand, G. (2006). Impact of quercetin and EGCG on key elements of the Wnt pathway in human colon carcinoma cells. *Journal of agricultural and food chemistry, 54*(19), 7075-7082.

Panda, D., Jordan, M. A., Chu, K. C., & Wilson, L. (1996). Differential effects of vinblastine on polymerization and dynamics at opposite microtubule ends. *Journal of Biological Chemistry, 271*(47), 29807-29812.

Park, C., Bergsagel, D., & McCulloch, E. (1971). Mouse myeloma tumor stem cells: a primary cell culture assay. *Journal of the National Cancer Institute, 46*(2), 411-422.

Parthasarathy, V., Zachariah, T. J., & Chempakam, B. (2008). 23 Curry Leaf. *Chemistry of Spices*, 413.

Patrawala, L., Calhoun, T., Schneider-Broussard, R., Li, H., Bhatia, B., Tang, S., . . . Claypool, K. (2006). Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene, 25*(12), 1696-1708.

Paweletz, N. (2001). Walther Flemming: pioneer of mitosis research. *Nature Reviews Molecular Cell Biology, 2*(1), 72-75.

Paydar, M., Kamalidehghan, B., Wong, Y. L., Wong, W. F., Looi, C. Y., & Mustafa, M. R. (2014). Evaluation of cytotoxic and chemotherapeutic properties of boldine in breast cancer using in vitro and in vivo models. *Drug design, development and therapy, 8*, 719.

Peacock, C. D., Wang, Q., Gesell, G. S., Corcoran-Schwartz, I. M., Jones, E., Kim, J., . . . Beachy, P. A. (2007). Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. *Proceedings of the National Academy of Sciences, 104*(10), 4048-4053.

Pera, M., Reubinoff, B., & Trounson, A. (2000). Human embryonic stem cells. *Journal of cell science, 113*, 5.

Peters, J.-M., Cejka, Z., Harris, J. R., Kleinschmidt, J. A., & Baumeister, W. (1993). Structural features of the 26 S proteasome complex. *Journal of molecular biology, 234*(4), 932-937.

Pines, J., & Hunter, T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell, 58*(5), 833-846.

Pines, J., & Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *The Journal of cell biology, 115*(1), 1.

Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., . . . Daidone, M. G. (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer research, 65*(13), 5506-5511.

Pradelli, L. A., Bénéteau, M., & Ricci, J.-E. (2010). Mitochondrial control of caspase-dependent and-independent cell death. *Cellular and molecular life sciences, 67*(10), 1589-1597.

Preedy, V. R. (2014). *Cancer: Oxidative Stress and Dietary Antioxidants*: Academic Press.

Prince, M., Sivanandan, R., Kaczorowski, A., Wolf, G., Kaplan, M., Dalerba, P., . . . Ailles, L. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proceedings of the National Academy of Sciences, 104*(3), 973-978.

Qin, R., Shen, H., Cao, Y., Fang, Y., Li, H., Chen, Q., & Xu, W. (2013). Tetrandrine induces mitochondria-mediated apoptosis in human gastric cancer BGC-823 cells. *PLoS One, 8*(10), e76486.

Ragnhammar, P., Hafström, L., Nygren, P., & Glimelius, B. (2000). A systematic overview of chemotherapy effects in colorectal cancer. *Acta oncologica (Stockholm, Sweden), 40*(2-3), 282-308.

Raju, J., & Bird, R. P. (2007). Diosgenin, a naturally occurring furostanol saponin suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression and induces apoptosis in HCT-116 human colon carcinoma cells. *Cancer letters, 255*(2), 194-204.

Rao, L., Ramalakshmi, K., Borse, B., & Raghavan, B. (2007). Antioxidant and radical-scavenging carbazole alkaloids from the oleoresin of curry leaf (< i> Murraya koenigii</i> Spreng.). *Food Chemistry, 100*(2), 742-747.

Rasheed, Z. A., Yang, J., Wang, Q., Kowalski, J., Freed, I., Murter, C., . . . He, X. (2010). Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. *Journal of the National Cancer Institute, 102*(5), 340-351.

Rasper, M., Schäfer, A., Piontek, G., Teufel, J., Brockhoff, G., Ringel, F., . . . Schlegel, J. (2010). Aldehyde dehydrogenase 1 positive glioblastoma cells show brain tumor stem cell capacity. *Neuro-oncology*, noq070.

Ravindran, J., Prasad, S., & Aggarwal, B. B. (2009). Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *The AAPS journal, 11*(3), 495-510.

Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *nature, 414*(6859), 105-111.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., & De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature, 445*(7123), 111-115.

Rickardson, L., Fryknäs, M., Haglund, C., Lövborg, H., Nygren, P., Gustafsson, M. G., . . . Larsson, R. (2006). Screening of an annotated compound library for drug activity in a resistant myeloma cell line. *Cancer chemotherapy and pharmacology, 58*(6), 749.

Rickardson, L., Wickström, M., Larsson, R., & Lövborg, H. (2007). Image-based screening for the identification of novel proteasome inhibitors. *Journal of biomolecular screening, 12*(2), 203-210.

Risendal, B., Hines, L., Sweeney, C., Slattery, M., Giuliano, A., Baumgartner, K., . . . Byers, T. (2008). Family history and age at onset of breast cancer in Hispanic and non-Hispanic white women. *Cancer Causes and Control, 19*(10), 1349-1355.

Röhme, D. (1981). Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. *Proceedings of the National Academy of Sciences of the United States of America, 78*(8), 5009.

Rosén, J., Gottfries, J., Muresan, S., Backlund, A., & Oprea, T. I. (2009). Novel chemical space exploration via natural products. *Journal of medicinal chemistry, 52*(7), 1953-1962.

Rosén, J., Rickardson, L., Backlund, A., Gullbo, J., Bohlin, L., Larsson, R., & Gottfries, J. (2009). ChemGPS-NP Mapping of Chemical Compounds for Prediction of Anticancer Mode of Action. *Molecular Informatics, 28*(4), 436-446.

Rubio-Moscardo, F., Blesa, D., Mestre, C., Siebert, R., Balasas, T., Benito, A., . . . Schilhabel, M. (2005). Characterization of 8p21. 3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood, 106*(9), 3214-3222.

Saelens, X., Festjens, N., Walle, L. V., Van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene, 23*(16), 2861-2874.

Saini, S. C., & Reddy, G. B. S. (2015). A Review on Curry Leaves (Murraya koenigii): Versatile Multi-Potential Medicinal Plant. *Advanced Journal of Phytomedicine and Clinical Therapeutics, 3*(4), 363-368.

Sak, K. (2014). Site-specific anticancer effects of dietary flavonoid quercetin. *Nutrition and cancer, 66*(2), 177-193.

Sakariassen, P. Ø., Immervoll, H., & Chekenya, M. (2007). Cancer stem cells as mediators of treatment resistance in brain tumors: status and controversies. *Neoplasia (New York, NY), 9*(11), 882.

Salim, L. Z. A., Mohan, S., Othman, R., Abdelwahab, S. I., Kamalidehghan, B., Sheikh, B. Y., & Ibrahim, M. Y. (2013). Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. *Molecules, 18*(9), 11219-11240.

Salk, D., Bryant, E., Au, K., Hoehn, H., & Martin, G. (1981). Systematic growth studies, cocultivation, and cell hybridization studies of Werner syndrome cultured skin fibroblasts. *Human Genetics, 58*(3), 310-316.

Salmon, S. E., & Hamburger, A. W. (1983). Primary bioassay of human tumor stem cells: Google Patents.

Salnikov, A. V., Gladkich, J., Moldenhauer, G., Volm, M., Mattern, J., & Herr, I. (2010). CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. *International Journal of Cancer, 126*(4), 950-958.

Schimmer, A. D. (2004). Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer research, 64*(20), 7183-7190.

Sell, S. (2004). Stem cell origin of cancer and differentiation therapy. *Critical reviews in oncology/hematology, 51*(1), 1-28.

Serrano, D., Bleau, A.-M., Fernandez-Garcia, I., Fernandez-Marcelo, T., Iniesta, P., Ortiz-de-Solorzano, C., & Calvo, A. (2011). Inhibition of telomerase activity preferentially targets aldehyde dehydrogenase-positive cancer stem-like cells in lung cancer. *Mol cancer, 10*(96), 10.1186.

Shabad, L. M. (1967). *Precancer in Experimental and Morphological Aspects (in Russian)*. Moscow: Meditsina.

Shafee, N., Smith, C. R., Wei, S., Kim, Y., Mills, G. B., Hortobagyi, G. N., . . . Lee, E. Y. P. (2008). Cancer Stem Cells Contribute to Cisplatin Resistance in Brca1/p53–Mediated Mouse Mammary Tumors. *Cancer research, 68*(9), 3243-3250.

Shan, B. E., Zeki, K., Sugiura, T., Yoshida, Y., & Yamashita, U. (2000). Chinese medicinal herb, Acanthopanax gracilistylus, extract induces cell cycle arrest of human tumor cells in vitro. *Cancer Science, 91*(4), 383-389.

Shan, Y., Guan, F., Zhao, X., Wang, M., Chen, Y., Wang, Q., & Feng, X. (2016). Macranthoside B Induces Apoptosis and Autophagy Via Reactive Oxygen Species Accumulation in Human Ovarian Cancer A2780 Cells. *Nutrition and cancer, 68*(2), 280-289.

Shoemaker, R. H. (2006). The NCI60 human tumour cell line anticancer drug screen. *Nature reviews. Cancer, 6*(10), 813.

Siddik, Z. H. (2002). Mechanisms of Action of Cancer Chemotherapeutic Agents: DNA-Interactive Alkylating Agents and Antitumour Platinum-Based Drugs. *The cancer handbook*.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., & Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer research, 63*(18), 5821-5828.

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., . . . Dirks, P. B. (2004). Identification of human brain tumour initiating cells. *nature, 432*(7015), 396-401.

Soule, H., Vazquez, J., Long, A., Albert, S., & Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute, 51*(5), 1409-1416.

Stanley, J., Pye, D., & MacGregor, A. (1975). Comparison of doubling numbers attained by cultured animal cells with life span of species.

Suliman, A., Lam, A., Datta, R., & Srivastava, R. K. (2001). Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and-independent pathways. *Oncogene, 20*(17), 2122-2133.

Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., . . . Prévost, M.-C. (2000). Two distinct pathways leading to nuclear apoptosis. *The Journal of experimental medicine, 192*(4), 571-580.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., . . . Kroemer, G. (1999). Mitochondrial release of caspase-2 and-9 during the apoptotic process. *The Journal of experimental medicine, 189*(2), 381-394.

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., & Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell, 78*(4), 703-711.

Tanei, T., Morimoto, K., Shimazu, K., Kim, S. J., Tanji, Y., Taguchi, T., . . . Noguchi, S. (2009). Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. *Clinical Cancer Research, 15*(12), 4234-4241.

Tang, C., Chua, C. L., & Ang, B. (2007). Insights into the cancer stem cell model of glioma tumorigenesis. *ANNALS-ACADEMY OF MEDICINE SINGAPORE, 36*(5), 352.

Tang, D., Tokumoto, Y., Apperly, J., Lloyd, A., & Raff, M. (2001). Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. *Science, 291*(5505), 868.

Teodoro, J. G., & Branton, P. E. (1997). Regulation of apoptosis by viral gene products. *Journal of virology, 71*(3), 1739.

Thayyullathil, F., Chathoth, S., Hago, A., Patel, M., & Galadari, S. (2008). Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and-independent apoptosis in L929 cells. *Free Radical Biology and Medicine, 45*(10), 1403-1412.

Thomas, D., & Kargas, M. (Eds.). (1996). *Migrant studies. In: Schottenfeld D, Fraumeni J, editors. Cancer epidemiology and prevention*. New York: Oxford University Press. pp. 236-266.

Tran, T. T. N., Gerding-Reimers, C., Schölermann, B., Stanitzki, B., Henkel, T., Waldmann, H., & Ziegler, S. (2014). Podoverine A—a novel microtubule destabilizing natural product from the Podophyllum species. *Bioorganic & medicinal chemistry, 22*(18), 5110-5116.

van den Hoogen, C., van der Horst, G., Cheung, H., Buijs, J. T., Lippitt, J. M., Guzmán-Ramírez, N., . . . Cecchini, M. G. (2010). High aldehyde dehydrogenase activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. *Cancer research, 70*(12), 5163-5173.

van Loo, G., Van Gurp, M., Depuydt, B., Srinivasula, S., Rodriguez, I., Alnemri, E., . . . Vandenabeele, P. (2002). The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell death and differentiation, 9*(1), 20-26.

Van Meter, M. E., & Kim, E. S. (2010). Bevacizumab: current updates in treatment. *Current opinion in oncology, 22*(6), 586-591.

Vaux, D. L., & Korsmeyer, S. J. (1999). Cell death in development. *Cell, 96*(2), 245-254.

Vinuthan, M., Kumar, V. G., Narayanaswamy, N., & Veena, T. (2007). Lipid lowering effect of aqueous leaves extract of Murraya koenigii (curry leaf) on alloxan-induced male diabetic rats. *Pharmacognosy Magazine, 3*(10), 112.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature medicine, 10*(8), 789-799.

Vucic, D., Dixit, V. M., & Wertz, I. E. (2011). Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. *Nature reviews Molecular cell biology, 12*(7), 439-452.

Wang, B., Han, Q., & Zhu, Y. (2015). Oxymatrine inhibited cell proliferation by inducing apoptosis in human lung cancer A549 cells. *Bio-medical materials and engineering, 26*(s1), S165-S172.

Wang, C., & Youle, R. J. (2009). The role of mitochondria in apoptosis*. *Annual review of genetics, 43*, 95-118.

Wang, H., Agarwal, P., Zhao, S., Xu, R. X., Yu, J., Lu, X., & He, X. (2015). Hyaluronic acid-decorated dual responsive nanoparticles of Pluronic F127, PLGA, and chitosan for targeted co-delivery of doxorubicin and irinotecan to eliminate cancer stem-like cells. *Biomaterials, 72*, 74-89.

Wang, J., Zhao, X.-Z., Qi, Q., Tao, L., Zhao, Q., Mu, R., . . . Guo, Q.-L. (2009). Macranthoside B, a hederagenin saponin extracted from Lonicera macranthoides and its anti-tumor activities in vitro and in vivo. *Food and chemical toxicology, 47*(7), 1716-1721.

Wang, Z., Zhang, Y., Banerjee, S., Li, Y., & Sarkar, F. H. (2006). Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. *Cancer, 106*(11), 2503-2513.

Warrick, A. B., Gritsenko, K., & Aquino, M. (2015). Chemotherapeutic Agents *Essentials of Pharmacology for Anesthesia, Pain Medicine, and Critical Care* (pp. 503-523): Springer.

Weinberg, R. (2006). *The Biology of Cancer, Chap. 8, Rb and Control of Cell Cycle Clock*. New York: Garland Science.

Williams, S. D., Birch, R., Einhorn, L. H., Irwin, L., Greco, F. A., & Loehrer, P. J. (1987). Treatment of disseminated germ-cell tumors with cisplatin, bleomycin, and either vinblastine or etoposide. *New England Journal of Medicine, 316*(23), 1435-1440.

Woolcott, C., Shvetsov, Y., Stanczyk, F., Wilkens, L., White, K., Caberto, C., . . . Goodman, M. (2010). Plasma sex hormone concentrations and breast cancer risk in an ethnically diverse population of postmenopausal women: the Multiethnic Cohort Study. *Endocrine-related cancer, 17*(1), 125.

Wu, J.-M., Chen, Y., Chen, J.-C., Lin, T.-Y., & Tseng, S.-H. (2010). Tetrandrine induces apoptosis and growth suppression of colon cancer cells in mice. *Cancer letters, 287*(2), 187-195.

Xiang, J., Chao, D. T., & Korsmeyer, S. J. (1996). BAX-induced cell death may not require interleukin 1β-converting enzyme-like proteases. *Proceedings of the National Academy of Sciences, 93*(25), 14559-14563.

Yang, J., Qian, S., Cai, X., Lu, W., Hu, C., Sun, X., . . . Cao, P. (2016). Chikusetsusaponin IVa Butyl Ester (CS-IVa-Be), a Novel IL6R Antagonist, Inhibits IL6/STAT3 Signaling Pathway and Induces Cancer Cell Apoptosis. *Molecular cancer therapeutics, 15*(6), 1190-1200.

Yao, L. H., Jiang, Y., Shi, J., Tomas-Barberan, F., Datta, N., Singanusong, R., & Chen, S. (2004). Flavonoids in food and their health benefits. *Plant foods for human nutrition, 59*(3), 113-122.

Yin, T., Yang, G., Ma, Y., Xu, B., Hu, M., You, M., & Gao, S. (2015). Developing an activity and absorption-based quality control platform for Chinese traditional medicine: Application to Zeng-Sheng-Ping (Antitumor B). *Journal of ethnopharmacology, 172*, 195-201.

Yip, C., Taib, N., & Mohamed, I. (2006). Epidemiology of breast cancer in Malaysia. *Asian Pacific Journal of Cancer Prevention, 7*(3), 369.

Yu, H.-H., Kim, K.-J., Cha, J.-D., Kim, H.-K., Lee, Y.-E., Choi, N.-Y., & You, Y.-O. (2005). Antimicrobial activity of berberine alone and in combination with ampicillin or oxacillin against methicillin-resistant Staphylococcus aureus. *Journal of medicinal food, 8*(4), 454-461.

Yu, P., Liu, Q., Liu, K., Yagasaki, K., Wu, E., & Zhang, G. (2009). Matrine suppresses breast cancer cell proliferation and invasion via VEGF-Akt-NF-κB signaling. *Cytotechnology, 59*(3), 219-229.

Yuan, X., Curtin, J., Xiong, Y., Liu, G., Waschsmann-Hogiu, S., Farkas, D. L., . . . John, S. Y. (2004). Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene, 23*(58), 9392-9400.

Zamzami, N., & Kroemer, G. (2003). Apoptosis: mitochondrial membrane permeabilization–the (w) hole story? *Current biology, 13*(2), R71-R73.

Zarihah, M., Mohd Yusoff, H., & Devaraj, T. (2003). Penang Cancer Registry Report 1994-1998. *Penang: Penang Cancer Registry*.

Zeiss, C. (2003). The apoptosis-necrosis continuum: insights from genetically altered mice. *Veterinary Pathology Online, 40*(5), 481-495.

Zhou, H., Lutterodt, H., Cheng, Z., & Yu, L. (2009). Anti-inflammatory and antiproliferative activities of trifolirhizin, a flavonoid from Sophora flavescens roots. *Journal of agricultural and food chemistry, 57*(11), 4580-4585.

Zhou, J., Liang, S., Fang, L., Chen, L., Tang, M., Xu, Y., . . . Wei, Y. (2009). Quantitative proteomic analysis of HepG2 cells treated with quercetin suggests IQGAP1 involved in quercetin-induced regulation of cell proliferation and migration. *OMICS A Journal of Integrative Biology, 13*(2), 93-103.

Zhou, K., Ji, H., Mao, T., & Bai, Z. (2014). Effects of matrine on the proliferation and apoptosis of human medulloblastoma cell line D341. *International journal of clinical and experimental medicine, 7*(4), 911.

Zimonjic, D., Brooks, M. W., Popescu, N., Weinberg, R. A., & Hahn, W. C. (2001). Derivation of human tumor cells in vitro without widespread genomic instability. *Cancer research, 61*(24), 8838-8844.

List of Publication

1. Ahmadipour F, Noordin MI, Mohan S, et al. Koenimbin, a natural dietary compound of Murraya koenigii (L) Spreng: inhibition of MCF7 breast cancer cells and targeting of derived MCF7 breast cancer stem cells (CD44(+)/CD24(-/low)): an in vitro study. *Drug Des Devel Ther.* 2015;9:1193-1208.