INVESTIGATION OF THE APOPTOTIC EFFECTS OF CYCLOART-24-ENE-3β,26-DIOL FROM *Aglaia exima* ON SELECTED BREAST CANCER CELL LINES

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ABSTRACT

The main purpose of this study is to investigate the cytotoxic potential and anticancer mechanism of cycloart-24-ene-3β,26-diol isolated from the leaves of *Aglaia exima* of the Meliaceae family through a bioassay-guided fractionation. *In vitro* assays of this compound were conducted on two cancer cell lines—hormone-dependent breast adenocarcinoma cells (MCF-7) and hormone-independent breast adenocarcinoma cells (MDA-MB-231) in comparison with the normal human mammary epithelial cell line (hTERT-HME1). Cell viability was assessed using the MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium, inner salt) assay. Flow cytometry analysis was used to determine the mode of cell death and cell cycle arrest caused by the compound. Caspase 3/7 assay was performed to investigate caspase activation, while aromatase inhibitory activity was examined using the CYP19-MFC assay. The results showed that cycloart-24-ene-3β,26-diol is cytotoxic to MCF-7 and MDA-MB-231 in a dose- and time-dependent manner. Conversely, cycloart-24-ene-3β,26-diol did not significantly affect the viability of normal mammary cells within a similar concentration range. Flow cytometric analysis of annexin V/propidium iodide (PI) dual staining showed that cell death was through apoptosis. The apoptotic effects was further confirmed by caspase 3/7 activation. Cell cycle analysis showed that cycloart-24-ene-3β,26-diol caused G_1-S phase arrest in MCF-7. Besides, we found that cycloart-24-ene-3β,26-diol inhibited CYP19 (aromatase), suggesting a potential aromatase inhibitor. In conclusion, cycloart-24-ene-3β,26-diol, a natural compound from the leaves of *Aglaia exima* may have the potential to be further developed into a chemopreventive agent for breast cancer.

ABSTRAK

Tujuan utama kajian ini adalah untuk mengenal-pasti keupayaan sitotoksik dan mekanisme anti-kanser oleh cycloart-24-ene-3β,26-diol yang diekstrak daripada daun *Aglaia exima* dari keluarga tumbuhan Meliaceae melalui bioassai berpandu pengasingan. Esei-esei *in vitro* bagi sebatian ini telah dilakukan terhadap dua jenis sel-sel kanser iaitu sel-sel payudara bergantung hormon (MCF-7), sel-sel payudara tidak bergantung hormon (MDA MB-231) dengan perbandingan bersama sel-sel normal manusia payudara epithelia (hTERT-HME1). Keupayaan sel untuk meneruskan kelansungan hidup dinilai dengan menggunakan esei MTS. Analisis aliran sitometrik digunakan untuk menentukan jenis kematian sel dan penangkapan kitaran sel yang disebabkan oleh sebatian itu. Esei caspase 3/7 telah dijalankan untuk menyiasat pengaktifan caspase manakala, aktiviti anti-aromatase diperiksa dengan menggunakan esei CYP19-MFC. Keputusan menunjukkan bahawa cycloart-24-ene-3β,26-diol adalah sitotoksik terhadap MCF-7 dan MDA-MB-231 dalam keadaan yang bergantung kepada dos and tempoh rawatan. Sebaliknya, cycloart-24-ene-3β,26-diol tidak memberi kesan yang penting terhadap kelangsungan hidup sel-sel normal payudara di dalam julat kepekatan yang sama. Analisis aliran sitometrik dengan menggunakan pewarna gabungan annexin V dan propidium iodide (PI) telah menunjukkan kematian sel disebabkan oleh apoptosis. Kesan-kesan apoptosis juga disahkan dengan pengaktifan caspase 3/7. Analisis kitaran sel menunjukkan bahawa cycloart-24-ene-3β 26-diol menyebabkan G_1-S penangkapan fasa dalam MCF-7. Selain itu, kami mendapati bahawa cycloart-24-ene-3β 26-diol menghalang CYP19 (aromatase), mencadangkan potensi perencat aromatase. Kesimpulannya, cycloart-24-ene-3β,26-diol, suatu sebatian semula jadi dari daun pokok *Aglaia exima* yang mungkin mempunyai potensi untuk dikembangkan seterusnya sebagai suatu agent kimia mencegah kanser payudara.

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LIST OF SYMBOLS AND ABBREVIATIONS

^{13}C NMR	13-Carbon Nuclear magnetic resonance
α	Alpha
β	Beta
δC	Carbon chemical shift
^{o} C	Degree celsius
m/z	Mass per charge
±SD	Mean standard deviation
µ	Micro
µg/ml	Micrograms per mililitre
µl	Microlitre
µM	Micromolar
$[M]^{+}$	Molecular ion
1D-NMR	One dimension nuclear magnetic resonance
%	Percent
±	Plus-minus
+ve	Positive control
^{1}H NMR	Proton nuclear magnetic resonance
2D-NMR	Two dimension nuclear magnetic resonance
(v/v)	Volume per volume
(w/v)	Weight per volume
A	Absorbance
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease-activating factor-1

ATCC	American Tissue Culture Collection
ATP	Adenosine triphosphate
Bax	Bcl-2 associate X protein
Bcl-2	B-cell lymphocyte 2
BD	Becton Dickenson
BH	Bcl-2 homology domain
Bim	Bcl-2 interacting mediator
BPE	Bovine pituitary extract
CA	California
CARD	Caspase recruitment domains
Caspase	Cystein aspartate protease
$CDCl_3$	Deuterated chloroform
CDK	Cyclin dependent kinase
CKI	Cyclin kinase inhibitor
Cm	Centimeter
cm^2	Cemtimeter square
CO_2	Carbon dioxide
d	Doublet
dATP	Deoxy adenosine triphosphate
DCM	Dichloromethane
DEPT	Distortioness enhancement by polarization transfer
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR3	Death receptor 3
EDTA	Ethlene diamine tetra acetic acid

et al.	and other
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fas	FS9 associated surface antigen
FITC	Fluorescence isothiocyanate
G0	Quiescent state
G1	Gap 1
G2	Gap 2
GI	Growth inhibition
g	gram
h	Hour
HCl	Hydrochloric acid
hEGF	Human recombinant epidermal growth factor
HEPES	N-2-hydroxylethyl-piperazine-N-2-ethane sulfonoc
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantun correlation
Hz	Hertz
IAP	Inhibitor of apoptotic protein
IC50	50% Inhibitory concentration
Inc.	Incorporation
IR	Infrared
L	Litre
M	Multiplet
m	Meter
M	Mol
MD	Maryland

MEGM	Mammary epithelia growth media
Mg	Milligram
Min	Minimum
MFC	7-methoxy-4-trif- luoromethylcoumarin
mins	Minutes
mL	Mililiter
mM	Milimolar
MS	Mass spectroscopy
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt
MW	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NCI	National Cancer Insitute
NCR	National Cancer Registry
Ng	Nanogram
nM	Nanomolar
NMR	Nuclear magnetic resonance
TNFR1	Tumour necrosis factor receptor 1
PARP	Poly (ADP-ribose) polymerase
PRAD1	Parathyroid hormone 1 gene
RLU	Relative luminescence unit
USA	Unites States of America

CHAPTER 1

INTRODUCTION

Breast cancer is a heterogeneous disease characterized by abnormal cell proliferation and deregulations of the apoptosis process. This unregulated growth is usually triggered by an accumulation of genetic material alterations within the cell that regulate normal cell division and function (Schedin et al., 1996; Krajewski et al., 1999; Hanahan and Weinberg, 2011). In Malaysia, a total of 18,219 of new cancer cases were diagnosed in 2007 and breast cancer is one of the ten leading cancers among the Malaysian population in 2007 (Ariffin & Saleha, 2011).

Apoptosis is a regulated death process occurring in all normal cells. Upon receiving apoptosis signals, a series of proteins will activate other proteins in this process. It will create a cascading pathway that will involve several proteins including the pro-apoptotic and anti-apoptotic proteins. Then, these proteins will activate caspases, which are proteases and finally lead to apoptotic morphological changes (Kumar, 2006; Lamkanfi et al., 2006). However, this process is evaded by a majority of cancer cells through deregulation of the apoptosis regulatory proteins. Furthermore, some cancer cells could drive the production of growth factors to allow unlimited proliferation (Sporn & Roberts, 1985). Studies also showed that there is an imbalance in the production of anti-apoptotic and pro-apoptotic proteins in cancer cells (Juin et al., 2004; Vogler et al., 2008). Therefore, proper regulation of apoptosis is important for normal proliferation in cells and preventing it from turning into cancerous cells.

Malaysian rainforests consists of 15,000 flowering plant species, which is important as a source of natural medicine with approximately 1920 plants species are reported to have medicinal values (Othman et al., 2011). Natural products and their

derivatives represent part of the current anti-cancer drugs in clinical use. In fact, anti-cancer drugs derived from natural products and their products represent approximately 34.4% of all anti-cancer drugs available in the market (Newman and Cragg, 2012). For example, taxol isolated from the Pacific yew tree, *Taxus brevifolia* in 1971 and later approved by the Food and Drug Administration (FDA) for the treatment of cancer (Wani et al., 1971). The unique mode of action for this compound was found to be the stabilization of microtubule assembly. In 1992, Bristol-Myers Squibb received approval to market taxol for the treatment of refractory ovarian cancer, and subsequently, it was approved for the treatment of metastatic breast and lung cancers, and Kaposi's sarcoma (Oberlies & Kroll, 2004). Recently, romidepsin is another example of a natural product approved by FDA in 2009 for cutaneous T-cell lymphoma and in 2011 for peripheral T-cell lymphoma (Butler et al., 2014).

The tropical plant *Aglaia exima* from the Meliaceae family is a tree that can grow up to 15 m in height with rounded crown (Mabberly & Pannel, 1989). In tropical countries, some of *Aglaia* species has been used as a form of traditional medicine against several diseases including cancer (Cui et al., 1997; Qiu et al., 2001). Plants from this family have been known to be a rich source of secondary metabolites including various terpenoids, alkaloids and sterols with medicinal properties such as antiviral, anti-inflammatory, anti-cancer, anti-malarial and antifungal agents (Joycharat et al., 2010; Zhang et al., 2010; Phongmaykin et al., 2011; Awang et al., 2012; Yodsaoue et al., 2012).

Triterpenoids are universally distributed throughout the plant kingdom. A number of plant-derived triterpenoids has been reported to exhibit anti-neoplastic activity against various cancer cells and preclinical animal models (Setzer and Setzer, 2003; Petronelli et al., 2009). Triterpenoids have been shown to inhibit tumour cell proliferation in a number of ways including, suppression of inflammation, reduction of

oxidative stress, regulation of cell cycle, inhibition of cell proliferation, and induction of apoptosis. Also, triterpenoids have proven to be a promising antineoplastic agent in treating and preventing breast cancer (Bishayee et al., 2011).

In this study, a bioassay-guided fractionation using human breast cancer cell lines (MCF-7 and MDA-MB-231) led to the isolation of the most cytotoxic compound, cycloart-24-ene-3β,26-diol (Figure 1.1). This natural triterpenoid isolated from the leaves of *Aglaia exima* was further assessed on its selective cytotoxicity against the selected human breast cancer cell lines in comparison to normal human breast cell line (hTERT-HME). Then, the apoptotic and cytostatic potential of cycloart-24-ene-3β,26-diol were determined. Lastly, chemical structure similarity of this compound with the drug, exemestane, an inhibitor of aromatase (CYP19) led to the investigation of the inhibition activity against the enzyme.

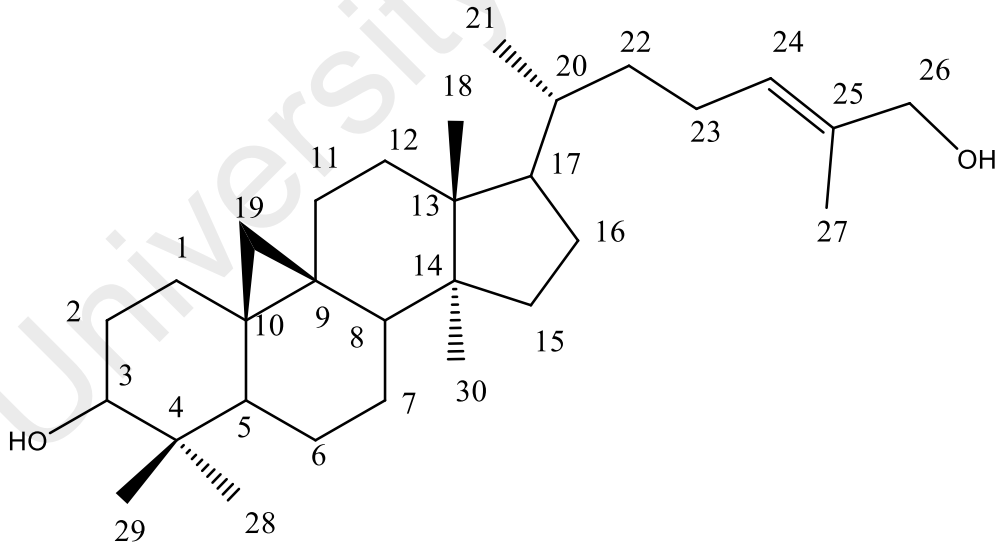


Figure 1.1: Chemical structure of cycloart-24-ene-3β,26-diol isolated from the leaves extact of *Aglaia exima* (Meliaceae).

AIM AND OBJECTIVES

The aim of this study was to isolate the most active compound from leaves extract of *Aglaia exima* (Meliaceae) and investigate the compound's mechanism of action in selected human breast cancer cell lines.

The objectives of the research were as follows:

i. To isolate bioactive compound from leaves extract of *Aglaia exima* using bioassay-guided fractionation techniques.

ii. To characterize the isolated bioactive compound using spectroscopic methods.

iii. To assess selective cytotoxicity of the most active compound against breast cancer cell lines in comparison to normal breast cell line.

iv. To determine the mechanism of action elicited by the most active compound on selected breast cancer cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of cancer

Approximately 12.7 million cancer cases and 7.6 million cancer deaths were estimated to have occurred in 2008 worldwide (Jemal et al., 2011), making it the leading cause of death in both developed and developing countries. The risk of cancer burden still accounts for more than half of the world's cancer burden in developing nations (Thun et al., 2010). Therefore, developing countries contribute a high percentage of cancer occurrences worldwide. In Malaysia alone, 18,219 cancer cases were diagnosed in 2007 as registered by the National Cancer Registry (NCR), with breast cancer the most common cancer incidence among Malaysians, followed by colon and lung cancer. In 2007, the National Cancer Registry reported 3,492 breast cancer, 2,236 colourectal cancer, and 1,865 lung cancer cases (Ariffin & Saleha, 2011).

Cancer is perceived differently under different circumstances; while the public sees it as a modern day plague, a cancer patient considers it a terrifying, alien entity invading his or her body, treatable only with medicines during the early stages. On the other hand, scientists view cancer as a collection of well-established cells with defective genome that allows it to proliferate endlessly. This has led to the extensive compendium of cancer genotypying done over the recent decades (Green & Evan, 2002).

Hanahan and Weinberg (2000) proposed that most of the cancer genome abnormalities in cancer cells are due to six alterations, namely "self-sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth) signals and evasion of programmed cell death (apoptosis), unlimited replication potential, sustained angiogenesis, tissue invasion, as well as metastasis". About a decade later, the authors

added two new concepts, which include the "reprogramming of energy metabolism and evading immune destruction" into their list of cancer hallmarks (Hanahan & Weinberg, 2011) (Figure 2.1). It is also accepted that cancer arises from two conditional events that permit uncontrolled cell expansion, which are abnormal cell proliferation and reduced apoptosis. In order for cancer cells to undergo cell proliferation unlimitedly, these two processes must occur together. Therefore, cancer cells proliferate endlessly when they receive interlocking signals that promote cell proliferation and suppression of apoptosis (Evan & Vousden, 2001).

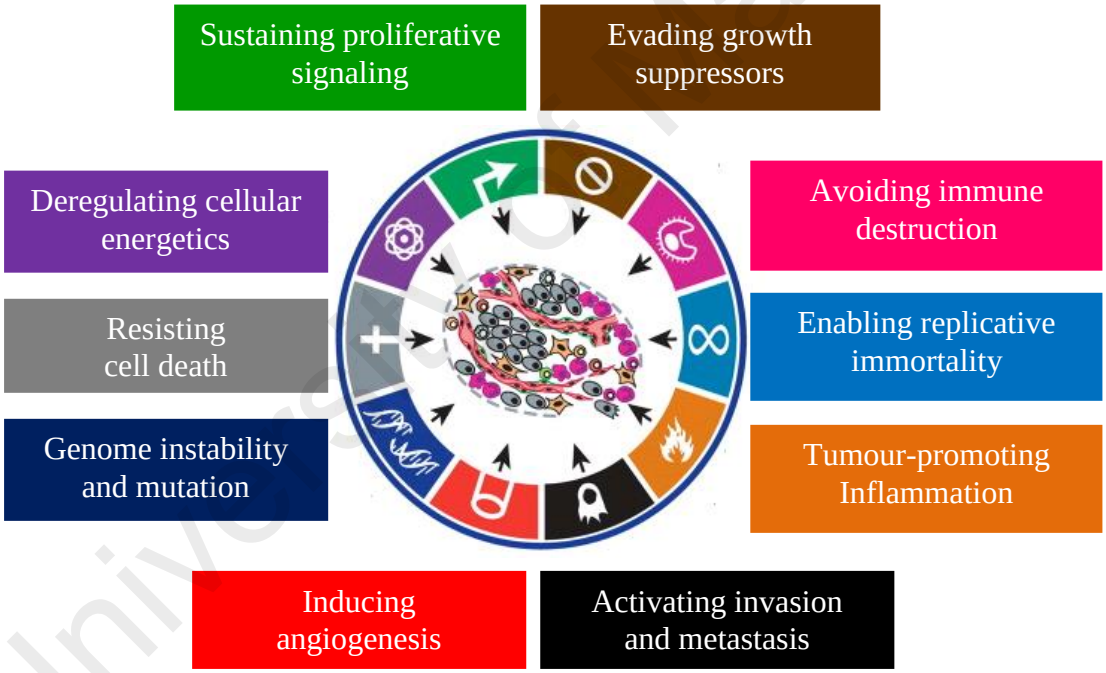


Figure 2.1: The hallmarks of cancer (Adapted from Hanahan & Weinberg, 2011).

Generally, cancer development is a multistep process involving initiation, promotion and progression. Initiation starts when normal cells are exposed to carcinogenic substances and their DNA becomes damaged to a state of unrepairable. Exposure to carcinogenic substances, which is subsequently distributed to organs to

undergo metabolic activation leads to the production of reactive species. These reactive species bind to the DNA and cause coding errors at the time of replication, leading to mutation. The somatic mutation is then reproduced during mitosis forming clones of mutated cells. The next stage of carcinogenesis process, promotion, is when these damaged cells keep expanding to form an actively proliferating multi-cellular premalignant tumour. The last stage known as progression is an irreversible process that produces a new clone of tumour cells with increased proliferative capacity, invasiveness, and metastasis (Pitot & Dragan, 1991).

Current treatments for cancer include surgery, radiation, immunotherapy, hormonal therapy and chemotherapy (Brunelle & Letai, 2009; Florescu et al., 2011). However, Huang and colleagues (2011) showed that radiation treatments for cancer patient have stimulate a recurrence of cancer cells. On the other hand, chemotherapy designed to kill cancer cells in the body cause negative side effects that include temporary hair loss and digestive disturbance (Vo & Nelson, 2012). To avoid these adverse effects, new drug discovery for chemotherapy is still of great interest, particularly in finding new compounds that may offer better treatment for cancer patient. Another important field of research is identifying compounds that can prevent cancer, known as chemoprevention. This includes the administration of pharmaceutical or dietary constituents known to have anti-cancer effects. The development of chemopreventive agents may help in the inhibition of one or more stages of carcinogenesis (Walaszek et al., 2004).

2.1.1 Breast cancer

Breast cancer is the most common and leading cause of cancer death among women worldwide, with an estimation of 1,383,500 new cases and 458,400 deaths annually (Jemal et al., 2011). Currently, the NCR reported that breast cancer is the most

commonly diagnosed type of cancer among females in Peninsular Malaysia, with a prevalence of 3,242 cases that constitute 32.1% of all female cancer cases reported in year 2007. Breast cancer is also common regardless of ethnic groups and the age group most vulnerable to develop breast cancer is at the age of 50-59 (Ariffin & Saleha, 2011).

Breast cancer initially derives from the epithelial lining of ducts or lobules are known as ductal and lobular carcinomas, respectively. There are also sarcomas, but its occurrence is less than 1% of all breast cancer types. The most common risk factors are associated with early menarche, nulliparity, late menopause, late pregnancies, obesity, and increase in the total number of menstrual cycles in a woman's life. Approximately 5% of breast cancer cases are due to the BRCA1 and BRCA2 gene mutations. But, many of these risk factors are nevertheless correlated with hormone linked pathways, where estrogens are found to play a crucial role in breast carcinogenesis of all stages (Bernstein & Ross, 1993; Key et al., 2001; Travis & Key, 2003). Estrogens are believed to stimulate cell proliferation and reduce the time available for DNA repair in breast cancer (Santen & Harvey, 1999). However, estrogens are also involved in various normal physiological processes, including the growth and perpetuation of the female reproductive organs, reproductive cycle, and various neuroendocrine functions (Brueggeimeier et al., 2005).

The correlation between breast cancer and estrogen has been acknowledged for more than 100 years since Sir George Beatson demonstrated that bilateral oophorectomy in premenopausal women with breast cancer induced tumour diminution (Beatson, 1986). The primary source of estrogens in premenopausal women is the ovaries. Even though estrogen biosynthesis by the ovaries ceases after menopause, estrogens continue to be produced by peripheral tissues such as muscles and adipose stromal in postmenopausal women (Figure 2.2) (Bulun et al., 2005). However, the tissue estrogen concentration is higher compared to the circulating estrogen levels because of

the body weight and circulating estrogens become positively correlated after menopause. Therefore, adipose tissues are the major sources of estrogens in postmenopausal women. Interestingly, breast tumour is also known to be another important source of estrogens in breast cancer patients. About 70% of breast cancers produce estrogens *in vitro* and this may be sufficient to maintain local levels of estrogen within the tumour (Szymczak et al., 1998; Miller, 2003; Choueiri et al., 2004; Lonning, 2004).

Estradiol has been known to be the most potent endogenous estrogen. It is biosynthesized from androgen substrates via cytochrome P450 enzyme complex called aromatase (Brueggemeier et al., 2005). The biological relevance of *in situ* estrogen-production by aromatase has been demonstrated by xenograft experiments that compared tumours with and without the aromatase. Human breast cancer cells that have been transfected permanently to express the aromatase enzyme are compared with cells transfected with irrelevant DNA. In these experiments, transfected tumours expressing aromatase have higher amount of estrogens and grow faster than those transfected with irrelevant DNA. Furthermore, these experiments showed that local production of estradiol in the tumour is a greater source of estrogen than those uptakes from plasma. In addition, further studies using a transgenic mouse model, whereby aromatase is over-expressed in mammary tissues, indicated that the *in situ*-produced estrogen plays a more important role than circulating estradiol in breast tumour promotion. Overall, these studies supported the importance of *in situ* estrogen-production in breast tumours and suggested that aromatase inhibitors may be sufficient to block intratumoural aromatase activity in lowering the estrogen production (Yue et al., 1994; Santen & Harvey, 1999).

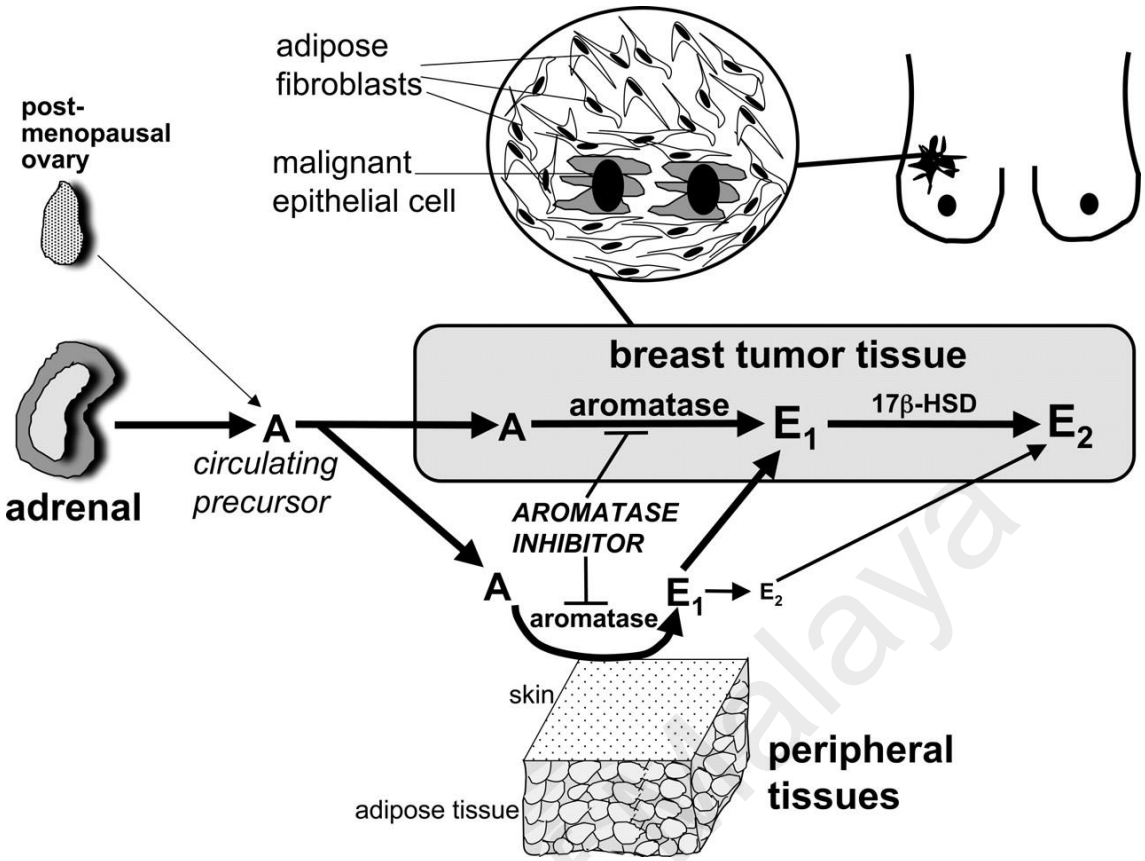


Figure 2.2: Sources of estrogen in postmenopausal woman. Androstenedione (A) is an estrogen precursor that originate primarily from the adrenal in the postmenopausal woman. The conversion of circulating A to Estrone (E1) in undifferentiated breast adipose fibroblasts happens around malignant epithelial cells and subsequent conversion of E1 to Estradiol (E2) in malignant epithelial cells provides high concentrations of E2 for tumour growth (Adapted from Bulun et al., 2005).

2.1.1.1 Role of aromatase in breast cancer

Aromatase is an enzyme that belongs to the cytochrome P450 family. In addition, it is a product of the CYP19 gene positioned on chromosome 15 (Thompson & Siiteri, 1974; Chen et al., 1988). Aromatase plays the role of a catalyst in a rate-limiting final step of estrogen production that converts androgen to estrogens. Androstenedione and testosterone are two major androgens that serve as substrates for aromatase (Simpson et al., 1996; Sasano & Harada, 1998). This enzyme consists of a complex bound in the endoplasmic reticulum of the cell that contains cytochrome P450

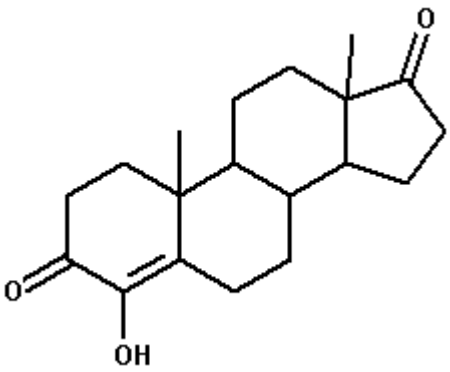
hemoprotein and flavoprotein NADPH cytochrome P450 reductase. The cytochrome P450 converts C19 steroids (androgens) into C18 steroids (estrogens), while the NADPH-cytochrome P450 reductase transfers a proton to cytochrome P450 (Kellis & Vickery, 1987; Simpson et al., 1994). Humans have the highest levels of aromatase expression per tissue compared to other species (Bulun et al., 2005; Chumsri et al., 2011). However, this enzyme is expressed in a tissue-specific manner, especially in the granulosa cells of ovarian follicles in premenopausal women and the placenta of pregnant women (Santen & Harvey, 1999; Brueggeimeier et al., 2005). Conversely, in postmenopausal women and men, this enzyme is present in the non-granular cells such as connective tissue, adipose tissue, bone marrow, liver, muscle, skin, as well as benign and malignant breast tissue (Miller, 1991; Smith & Dowsett, 2003; Lonning, 2004). In *in vitro* environment, the aromatase activity was found not only in the epithelial cells, but also in the stromal cells of breast cancer (Reed et al., 1993; Miller et al., 1997; Quinn et al., 1999). Furthermore, James et al. (1987) reported that aromatase activity is higher in breast tumours than in the surrounding adipose tissue. In addition, real-time PCR analysis on the adipose tissue showed higher aromatase mRNA than nonmalignant adipose tissue (Bulun et al., 1993). Altogether, these studies supported the magnitude of aromatase activity in breast cancer and suggested that inhibiting the aromatase is a strategy for therapeutic intervention in estrogen-dependent breast cancers.

Research on aromatase inhibitors began in the 1970s and have resulted in the development of various aromatase inhibitors used in the clinic (Santen et al., 1987; Brodie et al., 1993; Simpson et al., 1994; Simpson., 2001). The foremost aromatase inhibitors discovered were aminoglutethimide and testololactone, but the latter was not as potent as the former. Therefore, aminoglutethimide is widely used as an aromatase inhibitor in the treatment of advanced breast cancer in postmenopausal women. Aminoglutethimide, known as the first generation inhibitor, is also the first aromatase

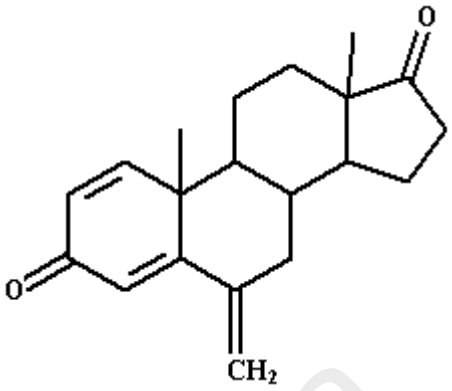
inhibitor to be studied in patients (Santen & Harvey, 1999; Buzdar et al., 1999; Brueggemeier et al., 2005). Initially, it was used as an anti-epileptic drug to suppress adrenal steroid production, which was developed as a "medical adrenalectomy". However, in the late 1970s, it was discovered that aminoglutethimide was effective in treating breast cancer via the inhibition of aromatase enzyme. It was later used in the clinics to treat patients with advanced breast cancer. Aminoglutethimide not only inhibited the aromatase, but it induced multiple metabolic effects on other enzymes such as thyroxine synthase, 11-beta hydroxylase, and aldosterone synthase (Santen & Harvey, 1999). Therefore, this non-selective aromatase activity led to the inhibition of the biosynthesis of cortisol, aldosterone, and thyroid hormone. This necessitated the co-administration of glucocorticoid such as hydrocortisone to efficiently treat breast cancer patient. However, it causes a number of significant side effects, including drowsiness, skin rash and ataxia. In addition, standard daily dose of 1000 mg aminoglutethimide will also inhibit other cytochrome P450-mediated steroid hydroxylations, particularly those involving the cholesterol side-chain cleavage enzymes (Santen & Harvey, 1999; Smith & Dowsett, 2003; Santen et al., 2009; Chumsri et al., 2011).

Recently, several potent and specific aromatase inhibitors have been identified and utilized in breast cancer treatment. In the chronological order of their clinical development, these agents can be classified into first-generation (e.g. aminoglutethimide), second-generation (e.g. formestane and fadrazole), and third-generation (e.g. anastrozole, letrozole, and exemestane) inhibitors (Figure 2.3). In addition, these inhibitors are classified as type 1 or type 2 according to their mechanism of action.

Type 1 Inhibitors - Steroidal inactivators

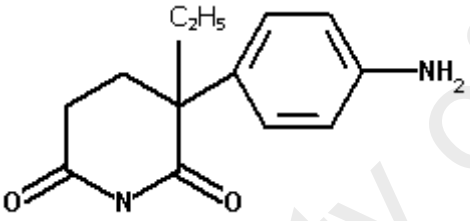


Formestane

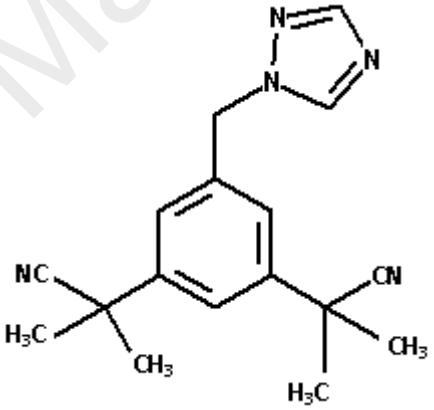


Exemestane

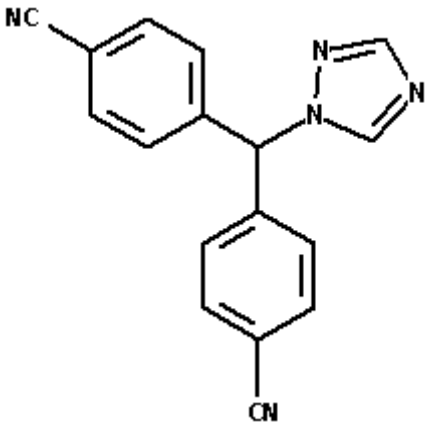
Type 2 inhibitors- Nonsteroidal inhibitors



Aminogluthimide



Anastrozole



Letrozole

Figure 2.3: Structures of the main aromatase inhibitors (Adapted from Smith & Dowsett, 2003).

Type 1 inhibitors have a more steroidal structure similar to androstenedione and bound to the catalytic site in the aromatase (Figure 2.3). But unlike the androstenedione, they bind irreversibly because of their conversion to reactive intermediates by the aromatase (Figure 2.4) (Carpenter & Miller, 2005). Therefore, they are also known as enzyme inactivators. Type 2 inhibitors are non-steroidal and bind reversibly to the heme group of the enzyme (Mokbel et al., 2002; Carpenter & Miller, 2005). The third-generation aromatase inhibitors are capable of inhibiting the levels of aromatase to a greater extent than the first- and second-generation inhibitors. This is most readily shown *in vitro*, where aromatase activity is effectively inhibited in both cultures of breast cancers and mammary adipose tissue fibroblasts, with the newer inhibitors being at least 100-fold more potent than aminoglutethimide. While micromolar concentrations are required with aminoglutethimide, only nanomolar concentrations are required with letrozole, anastrozole and exemestane. While these *in vitro* studies show that aromatase inhibitors work, evidence of effectiveness came from *in vivo* use of the aromatase inhibitors, whereby the newer drugs produced almost complete inhibition of the whole-body aromatase activity. Among the third generation, letrozole is the most potent and selective aromatase inhibitor. It has also shown a negligible effect on mineralocorticoid or glucocorticoid synthesis, which is a contrast to the earlier first- and second-generation aromatase inhibitors. Of the three agents, exemestane is the most recent FDA-approved and may be distinct because its steroidal structure potentially protects bone and lipid metabolism from estrogen ablation. Currently, the third-generation aromatase inhibitors is the first-line therapy for postmenopausal women with metastatic estrogen-dependent breast cancer (Miller, 1999 & 2003; Choueiri et al., 2004; Lonning, 2004; Brueggemeier et al., 2005).

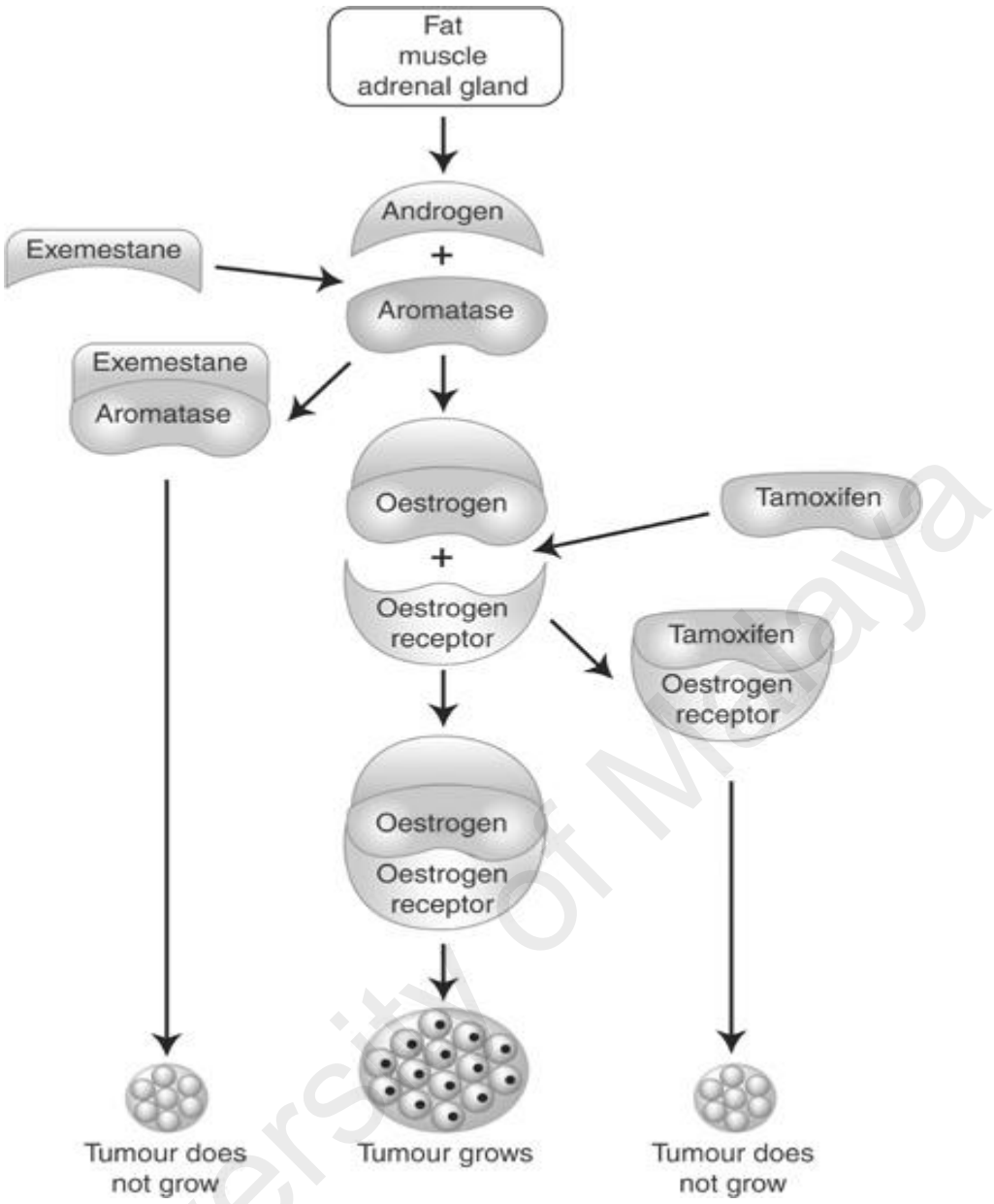


Figure 2.4: Mechanism of actions of exemestane and tamoxifen (Adapted from Carpenter & Miller, 2005).

Drug discovery research frequently utilizes *in vitro* cell cultures and in anticancer drug discovery for breast cancer, two of the most common cancer cell lines investigated are MCF-7 and MDA-MB-231. The MCF-7 cell line is derived from the primary human breast cancer cells and an estrogen receptor positive human breast adenocarcinoma cells. On the other hand, MDA-MB-231 cell line is derived from

metastatic human breast cancer cells and usually formed at the late stage of breast cancer (Brooks et al., 1973; Cailleau et al., 1978).

In 1992, Kitawaki and his colleagues demonstrated that by converting androgen to estrogen by the aromatase in the MCF-7 breast cancer cells, it stimulated DNA synthesis, which was abolished when aromatase inhibitor was administered. In another study, nude mice inoculated with the aromatase transfected MCF-7 cells in a Matrigel was utilized to demonstrate that local production of estrogen by intratumoural aromatase stimulated tumour growth. In addition, the tumours of the mouse model were dependent on estrogens for their growth from an endogenous nonovarian source (Kitawaki et al., 1992).

2.2 Mechanisms of cell death

2.2.1 Apoptosis

The term "Apoptosis" is derived from the ancient Greek word used to describe the "falling off" of the petals from a flower or leaves from a tree (Duque-Parra et al., 2005). The first use of the term 'apoptosis' in medicine is accredited to Hippocrates of Cos, who used this word to describe a physiological form of cell or tissue death (Diamantis et al., 2008). Interestingly, the term "Apoptosis" was coined by Kerr et al., 1972 in the early 1970s to describe a distinct mode of cellular death with ultra-structural features that were consistent with an active and inherently-controlled phenomenon. Apoptosis is an essential physiologic process required for embryogenesis, organ metamorphosis, removal of damaged cells, and tissue homeostasis. Deregulations of apoptosis often contribute to the pathogenesis of a wide variety of diseases such as cancer, viral infections, neurodegenerative disease, and autoimmune disorder (Saikumar et al., 1999). However, in some conditions insufficient apoptosis occurred, whereas others feature excessive apoptosis. Cancer is an example where the normal mechanisms

of apoptosis and/or cell cycle regulation become dysfunctional, with either an over-proliferation of cells or less cell death (Elmore, 2007).

Generally, apoptosis can be characterized by the morphological and biochemical alterations. The morphological changes include cell shrinkage, chromatin condensation, and membrane blebbing (Duprez et al., 2009). Most of the morphological changes observed by Kerr et al., 1972 were caused by a set of cysteine proteases activated specifically in apoptotic cells known as the caspases. Biochemical features associated with apoptosis include caspases activation, DNA, and protein breakdown, as well as the presentation of phosphatidylserine on the outer surface of the cell membrane (Zimmermann et al., 2001). As a result, cells are broken down into apoptotic bodies, which are subsequently recognized and engulfed by surrounding cells and phagocytes without the induction of an inflammatory response (Zimmermann et al., 2001; Duprez et al., 2009).

Apoptosis can be triggered by various extracellular and intracellular stimuli that will result in the coordinated activation of caspases (Figure 2.5). As of today, more than 14 caspases have been characterized in the mammalian systems. They can be categorized into two major subgroups based on their role in apoptosis (Devarajan et al., 2002; Fan et al., 2005). The caspase precursors, known as procaspase, are activated by proteolytic processing at the internal caspase recognition sites. These suggest that caspases can process themselves or other precursor caspases to generate active enzymes *in vivo*. The upstream or initiator caspases are activated by apoptotic signals, resulting in the activation of the downstream or executioner caspases. Two main caspase cascades have been delineated in mammalian cells. The first pathway links caspase-8 to death receptors expressed at the cell surface, including Fas, TNFR1, and DR3. In the second pathway, caspase-9 is activated by a variety of death stimuli from both outside and inside the cell (Kaufmann & Fusseneggar, 2005). Following their activation, the

executioner caspases, which include caspase-3, -6, and -7, can subsequently cleave distinct cellular proteins such as PARP [poly(ADP-ribose) polymerase], lamin, fodrin, and also Bcl-2, leading to apoptotic morphological changes (Devarajan et al., 2002; Fan et al., 2005).

Among the caspases identified, caspase-3, -6 and -7 are the central convergence of apoptosis signaling activated by numerous death signals (Jänicke et al., 1998; Duprez et al., 2009) (Figure 2.5). Recent evidence suggested that caspase-3 plays an important role for several key events during apoptosis such as nuclear and DNA fragmentation, and membrane blebbing in a cell type-specific and stimulus-specific manner. Furthermore, caspase-3 was reported to play the role of an amplifier of the apoptotic signals, i.e. by the cleavage of Bcl-2 (Blanc et al., 2000). Studies in caspase-3 knockout mice showed that this protease is also essential for brain development (Jänicke et al., 1998).

The substrates of caspase-3 include procaspase-3, procaspase-6, procaspase-7, and so on. Because all substrates of caspase-3 have DEVD amino acids sequence in common, artificially synthesized tetrapeptides, Ac-DEVE-AMC and Ac-DEVE-CHO, are usually used as specific substrate and inhibitor of caspase-3 respectively. Caspase-6 and caspase-7 are highly homologous to caspase-3. The former can be activated by caspase-3 by a positive feedback pathway. The other substrates for caspase-3 includes PARP, lamin and procaspase-3. On the other hand, procaspase-7's substrates include PARP, procaspase-6, and steroid response element-binding protein (Fan et al., 2005).

Apoptosis is widely studied among the different mode of cell death such as necrosis. At the molecular level, the discovery of caspase activation as key elements of apoptosis has provided significant insight into the understanding of the molecular basis of cancer. In normal cells, limited activation of the caspase cascade may be essential in

cellular differentiation and/or replication, thus making the preservation of caspase control essential. However, in cancer cells, it is often observed that caspase activation pathways are impaired or absent, thereby preventing cells from dying when they should. Since the loss of caspase activation appears to be central to the prevention of most cell death events in cancer, finding strategies to overcome caspase inhibition may be of value for the development of effective cancer treatments (Fan et al., 2005).

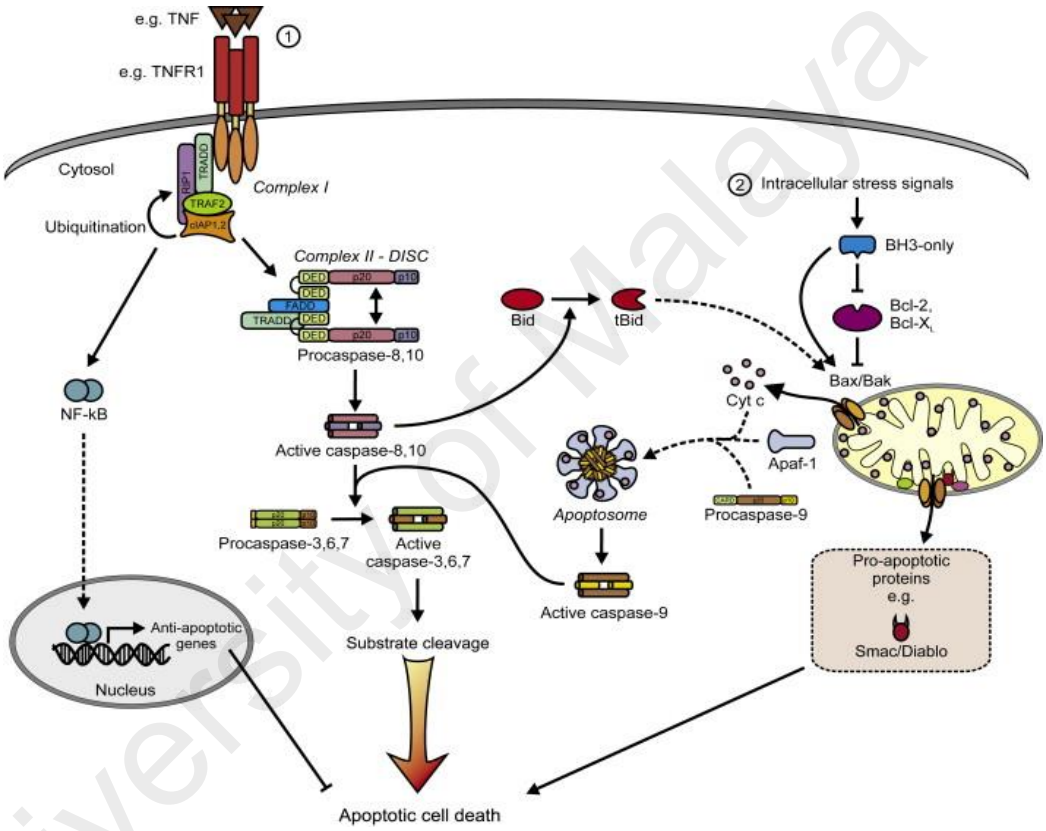


Figure 2.5: Representation of extrinsic and intrinsic apoptotic signaling (Adapted from Duprez et al., 2009).

2.2.1.1 MTS Assay

A viability assay is an assay to determine the ability of cells to survive or recover their viability, usually after a particular experimental procedure (e.g: cytotoxic treatment, irradiation, immunological attack). Thus, the viability assay can assess the number or percentage of living cells by measuring certain biochemical or morphological parameters connected with cell homeostasis and living processes, such as dye uptake or exclusion, the total cellular protein or metabolism of added compound (Riddell, et al 1986; Alley et al., 1988).

To assess for preliminary antitumour activity in terms of cell viability, the MTS *in vitro* cytotoxicity assay is considered as one of the most economic, reliable and convenient method. This is based on their ease of use, accuracy and rapid indication of toxicity, as well as their sensitivity and specificity (Malich et al., 2008). In the MTS assay, a bioconversion takes places utilising 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS and an electron coupling reagent phenazine ethosulfate, PES. MTS is bio-reduced by cells into a formazan product that is soluble in cell culture medium (Baltrop et al., 1991). The absorbance of the formazan at 490nm can be measured directly from 96-well assay plates without additional processing (Cory et al., 1991). The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of the formazan product is directly proportional to the number of live cells in the culture since MTS reagent can only be reduced to formazan by metabolically active cells.

2.2.1.2 Annexin FITC/PI Assay

Annexin V was first reported by Inaba et al., 1984, who isolated the protein from human placenta and called it placental protein 4 (PP4), and by Reutelingsperger et al., 1985, who isolated it from the umbilical cord by virtue of its anticoagulant activity and called it vascular-anticoagulant-α.. Andree et al., 1990 found that a protein, vascular anticoagulant α, bound to phospholipid bilayers in a calcium dependent manner. The protein was later renamed as Annexin V. Phosphatdylserine (PS) is a negatively charged phospholipid, that is normally present in membrane leaflets facing the cytosol and cells undergoing apoptosis break up the phospholid asymmetry of their plasma membrane to expose PS (Fadok et al., 1992).

Since PS translocation occurs early in apoptosis when cell membrane integrity is still intact, flow cytometric analysis using fluorescene in isothiocyanate-(FITC) labeled annexin V is useful as a quantitative measure of apoptosis (Vermes et al., 1995; Koopman et al., 1994). Annexin V/PI assay is a commonly used approach for studying apoptotic cells. Propidium iodide (PI) is widely used in conjuction with Annexin V to determine if cells are viable, apoptotic, or necrotic through differences in plasma membrane integrity and permeability. The ability of PI to enter a cell is dependent upon the permeability of the membrane; PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, cells that are considered viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive (Vermes et al., 1995). The Annexin V assay is also useful for measuring the kinetics of apoptotic death in relation to the cell cycle (Martin et al., 1995).

2.2.2 Necrosis

Necrosis is another form of cell death that occurs when the cells experience physicochemical stress. Necrotic cell death is usually described as accidental and uncontrolled, lacking the underlying signaling events presence in other types of natural cell death (Festjens et al., 2006; Golstein & Kroemer, 2007). Necrotic cell is morphologically characterized by vacuolation of the cytoplasm, breakdown of the plasma membrane, swelling of organelles, and induction of inflammation around the dying cell (Edinger & Thonpson, 2004; Kroemer et al., 2009). Several experimental models suggested a sequence of intracellular events specific to necrotic cell death, including signs of mitochondrial dysfunction that enhanced reaction oxygen species, ATP depletion, failure of Ca^{2+} homeostasis, perinuclear clustering of organelles, activation of a few proteases, lysosomal rupture, and early membrane rupture (Golstein & Kroemer, 2007).

Necrotic cell death is an alternative death mode for cells lacking in apoptosis signaling. It was suggested that the activation of DNA damage response pathways induce necrotic cell death in cancer cells. PARP activation triggers rapid depletion of NAD+ and ATP levels, leading to the inhibition of glycolysis (Figure 2.6). As a result, cancer cells that are dependent on glycolysis for ATP production quickly become depleted of ATP leading to necrotic death (Edinger & Thompson, 2004). However, PARP activation by chemotherapy can induce an immediate metabolic crisis by depleting NAD+ and ATP. On the contrary, cancer cells treated with therapeutic stress can increase intracellular calcium concentration and ROS, which then activates phospholipase A2 and subsequently leads to irreversible necrotic cell death (Amaravadi & Thompson, 2007).

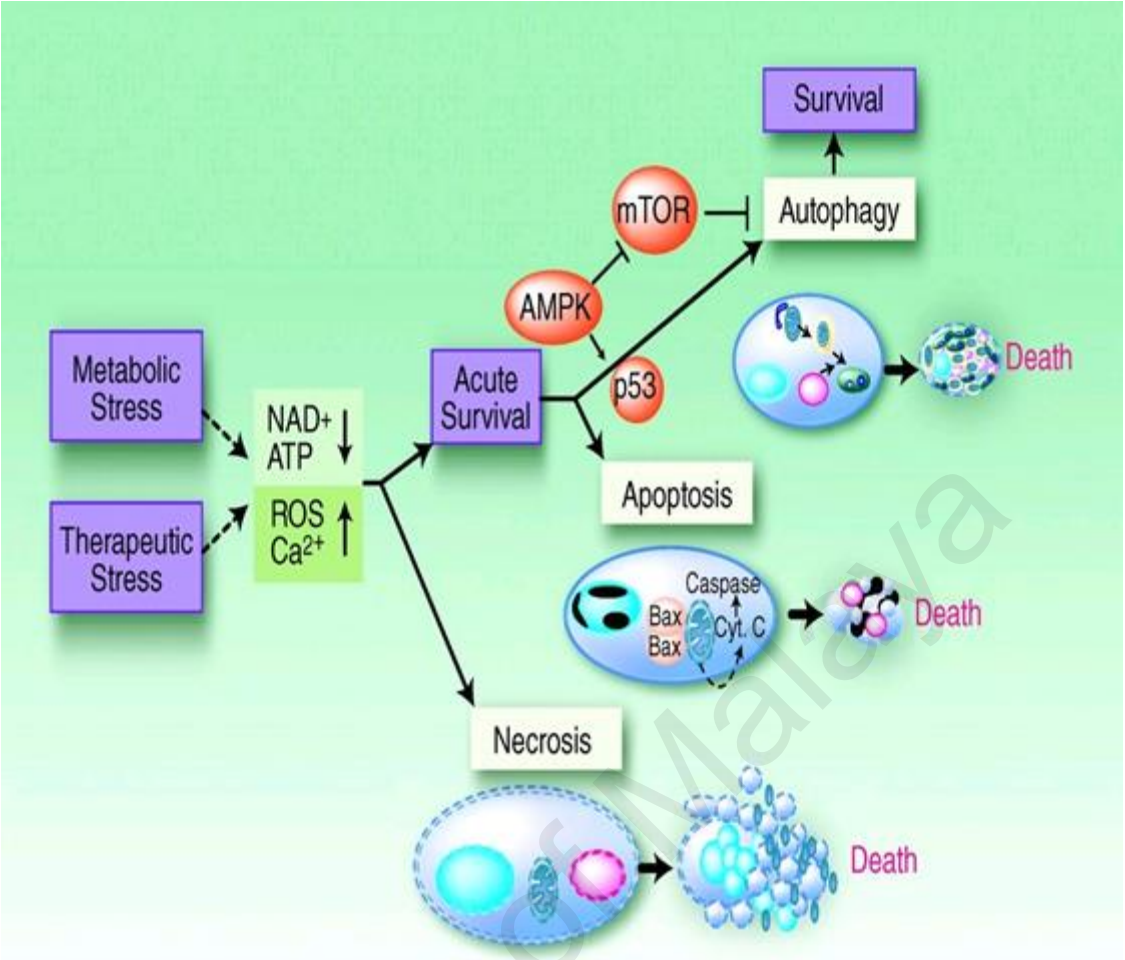


Figure 2.6: The relationship between necrosis, apoptosis and autophagy cell deaths induced by therapeutic and metabolic stress (Adapted from Amaravadi & Thompson, 2007).

2.3 Cell cycle

2.3.1 Overview of cell cycle

Cell growth and proliferation depend on a highly regulated process known as cell cycle. It is a complex process and involves numerous regulatory proteins that direct the cell through a specific set of events leading to mitosis (Schafer, 1998). Basically, the main function of the cell cycle is to divide into two daughter cells.

Morphologically, the cell cycle can be subdivided into the interphase and the mitotic (M) phases (Figure 2.7). Mitotic phases include prophase, metaphase, anaphase, and telophase, while G_1, S, and G_2 phases are the phases of interphase (Schafer, 1998; Mitchison & Salmon, 2001). The G_1 and G_2 phases of the cycle are the "gaps" in the

cell cycle placed between two obvious landmarks: DNA synthesis and mitosis. During the G_1 phase, a cell makes the decision to proceed, pause, or exit the cell cycle based on the received mitogenic and growth inhibitory signals. The S phase is defined as the stage in which DNA synthesis occurs. Therefore, S phase cells have aneuploid DNA content between 2n and 4n. The G_2 phase is the second gap in between the cell cycle, whereby the cell prepares for the process of division or the M phase. In the M phase, in order for the cells to form two daughter cells, the replicated chromosomes are segregated into separate nuclei and undergo the cytokinesis process. In addition to G_1, S, G_2, and M, the term G_0 is used to describe cells that have exited the cell cycle and become quiescent (Schafer, 1998; Mitchison & Salmon, 2001; DiPaola, 2002).

Cyclin dependent kinases (CDKs) were identified to have key roles in the cell cycle progression (Figure 2.7). The active forms of CDKs are a complex of at least two proteins, a kinase, and a cyclin. These complexes undergo changes in the kinase and cyclin components that are believed to drive the cell from one stage of the cell cycle to another. In mammalian cells, a succession of kinase subunits (CDK1, 2, 4, and 6) is expressed along with a succession of cyclins (cyclins A, B, D and E), as the cells progress from G_1 to mitosis. CDK4 and CDK6 complexed with one of several D-type cyclins in the G_1 phase, in response to growth factors. CDK2 that complexed with cyclin E, cyclin A, or both is essential for the G_1-S transition and DNA replication respectively. CDK1 that complexed with cyclin A and cyclin B is essential for mitosis (Park & Lee, 2003).

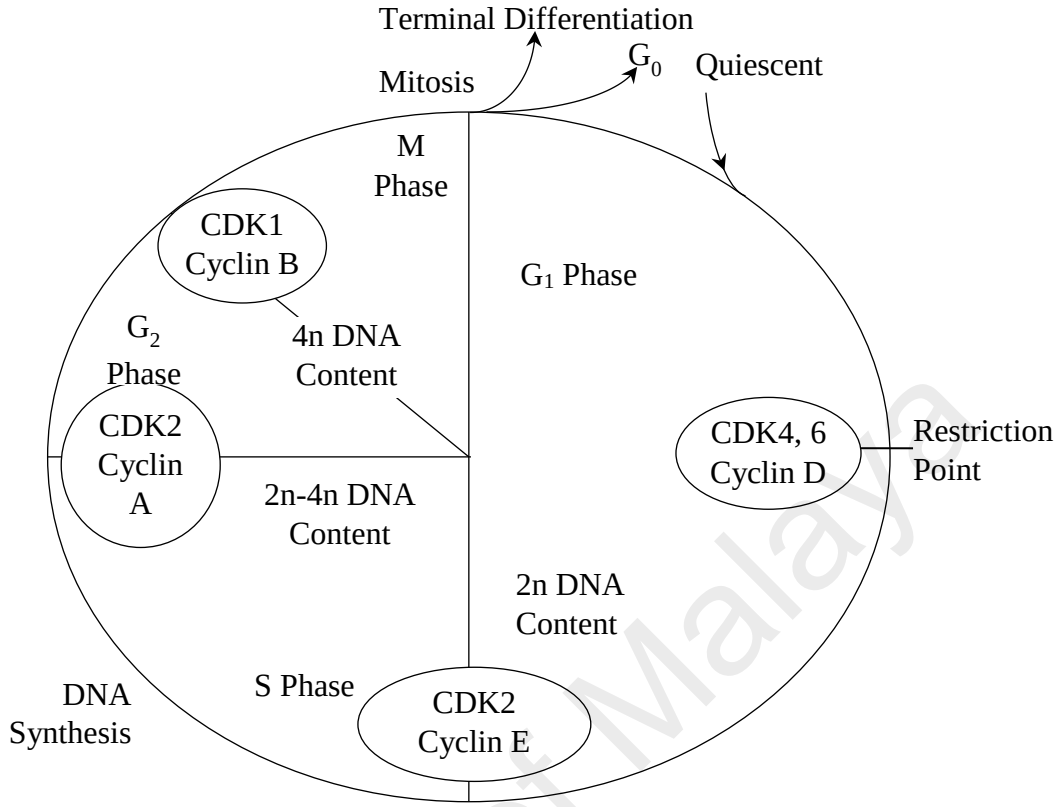


Figure 2.7: Normal cell cycle progression regulated by various CDK-cyclin complexes (Adapted from Park & Lee, 2003).

2.3.2 Cell cycle checkpoints and restriction point

Eukaryotic cells undergo cell division using a mechanism known as cell cycle checkpoints. The mechanism ensures the integrity of cell cycle events by preventing complications as it progresses into each events. As an example, the completion of cellular DNA replication is a checkpoint that must be passed before the chromosome separation process in mitosis can begin. Although the term checkpoint and the above example suggest a discrete point or time in the cell cycle, checkpoint is often more indistinct (Pardee, 1974; Hartwell & Weinerty, 1989).

Most cells stop the cell cycle and enter a resting state called G_0 in response to suboptimal growth conditions such as high cell density, serum deprivation, limitation of some nutrients, and the presence of certain drugs. It is also shown that extracellular signals, such as growth factors, act primarily on cells in the G_0 and G_1 phases. They stimulate cells from the G_0 phase to enter the cell cycle and stimulate cells in the G_1 phase to replicate DNA. However, once the DNA replication starts, growth factors have no control over the S phase progression (Blagosklonny & Pardee, 2002; Foster et al., 2010).

Early in the G_1 phase, the removal of growth factors will result in cells returning to the G_0 phase. However, when the cells reach a certain point in the G_1 phase, they become committed to complete the cell cycle. This important regulatory point in the G_1 phase, at which cells no longer require growth factors, has been termed the restriction point (Campisi & Pardee, 1984; Hartwell & Weinert, 1989; Assoian & Zhu, 1997). Therefore, this checkpoint is responsible for determining whether cells are irreversibly committed to initiating DNA synthesis and undergo cell division or to enter a resting state. Arthur Pardee was the first person who described the restriction point and suggested that the position to be anywhere between middle and late G_1 phase depending on the cell type (Pardee, 1974). The restriction point control is mediated by cyclin D and cyclin E-dependent kinases (Park & Lee, 2003). More importantly, Pardee also found that the restriction point is defective in cancer cells, providing a possible target in cancer treatment.

Cell cycle arrest, also referred to as delay, is produced by a variety of factors that may be intrinsic (e.g.: cell size) or extrinsic (e.g.: nutrition factors) and may affect any checkpoint (Hilakivi-Clarke et al., 2004; Jorgenson & Tyers, 2004). Other factors, such as DNA-damaging agents, can also trigger checkpoints that produce arrest in the G_1 and G_2 phases of the cell cycle. Cells can also be arrested during the S phase, resulting in a

prolonged S phase and slowed DNA synthesis. Arrest in the G_1 allows repair before DNA replication, whereas arrest in G_2 allows repair before chromosome separation in mitosis (Mikhailov et al., 2002).

2.3.3 Cell cycle and cancer

The proliferation of normal cells occurs in response to developmental or other mitogenic signals, whereas the proliferation of cancer cells proceeds essentially unchecked. For that reason, the uncontrolled cell cycle is considered as one of the fundamental aspects of cancer. There is a direct link between the deregulation of cell cycle control and cancer (Castedo et al., 2002). In recent years, it has become apparent that tumourigenesis is frequently associated with mutations or abnormalities in the expression of various cyclins, CDKs, and CKIs in several types of human cancers. In 1991, a presumptive oncogene that is composed of the parathyroid hormone gene (PRAD1) fused to the gene that encodes cyclin D1 was identified in a human parathyroid adenoma. This discovery provided the first clue that cyclins might be directly involved in some human cancers. Subsequent evidence was discovered linking cyclins to other types of human cancer cells. These included the B-cell lymphoma breast, gastric, colon and esophageal carcinomas, as well as several other types of cancers. The increased expression of cyclin D1 is indeed one of the most frequent abnormalities in human breast cancer. The cyclin E gene, which acts in the late G_1, is also overexpressed and dysregulated in a variety of human cancers (Park & Lee, 2003).

Although dysregulated cell cycle has frequently been observed in cancer, the accumulation of abnormal cancer cells was found even in the small population of cycling cells. The reason for this phenomenon is an apoptosis suppression that has recently been recognized and accepted as another central event in the development and

progression of cancer (Malumbres & Barbacid, 2009). The comparison of the normal mammalian and cancer cell cycle is depicted in Figure 2.8.

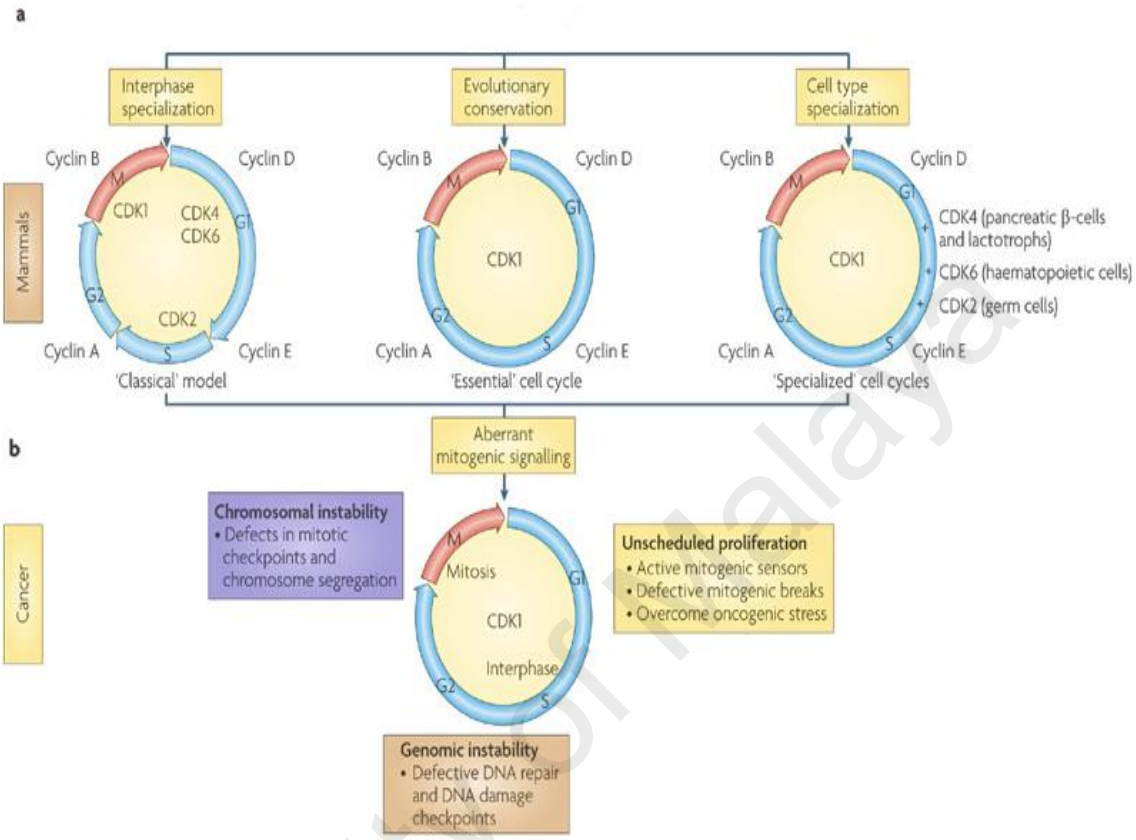


Figure 2.8: Comparison of the (a) normal mammalian and (b) cancer cell cycle (Adapted from Malumbres & Barbacid, 2009).

2.4. Natural products as anticancer agents

Natural products is a potential source of new drug discovery. In fact, many natural products and synthetically modified derivatives have been successfully developed for clinical use to treat various human diseases. From the 1940s to 2010, a total of 128 anticancer agents were approved, with 34.4% classified as either natural products or directly derived from natural product-lead compounds by semi-synthesis (Newman & Cragg, 2012). In fact, plant-derived compounds have played an important role in treatment of cancers and some of the most promising and effective drugs were discovered from plants, such as Taxol®, Camptothecin, Combrestatin,

Epipodophyllotoxin, Vinblastine and Vincristine. Apart from these, many plant-derived compounds are now in clinical trials as anticancer agents (Saklani & Kutty, 2008). Recently, Romidepsin, a natural product was approved by FDA in 2009 for cutaneous T-cell lymphoma, and in 2011, for peripheral T-cell lymphoma (Butler et al., 2014). Therefore, natural products has a proven track record serving as a major source of chemical diversity in offering potential anticancer drug discovery over the past century.

2.4.1 Bioassay-guided fractionation

Bioassay-guided fractionation is a method to isolate bioaactive compounds, capable of curing or improving a human or animal ailment and which can either be ultimately developed as established drugs directly or which can provide interesting structural leads (Atta-ur-Rahman & Thomsen, 2001). Firstly, crude extract separated into a few fractions using simple fractionation method such as chromatography. The second and most characteristic part is the biological part, which has to select or indicate compounds, which interact with the biological entity. The final part is a detection step, which can be combined with various kinds of structural analysis, such as mass spectroscopy, NMR, IR (Weller, 2002).

The choice of the bio-screening approach to be adopted generally depends on the targeted disease as well as on the available information about the targeted organism to be studied. For example neem (*Azadirachta indica*) has ethnopharmacological history of uses as traditional medicine against various human diseases (Varma, 1976). Therefore, based on ethnopharmacological data, nimbolide was isolated from neem and possesses antibacterial activity against *S. aureus, S. coagulase* (Rojanapo et al., 1985) and antimalarial activity against *Plasmodium falciparum* (Rochanakij et al., 1985). Bio-rational selection is based on the knowledge of plants and animals and their behavior in certain circumference. For example, certain

plants may exhibit resistance against insect attack. Therefore, they can be screened for insectidal compounds using specific bioassay. According to chemotaxonomic studies, plant species from similar taxa are usually assumed to have similar chemical properties. Zofou et al., 2013 described antimalarial and antiprotozoal compounds isolated from members of Meliaceae includings *Azadirachta indica*, *Cedrela odorata*, *Dysoxylum fraserianum* and *Trichilla glabra*. Finally, basic knowledge on certain plant species found in literature also can be used as source of knowledge in the discovery of plant derived drugs. Prior research conducted by Awang et al., 2012 regarding the cytotoxic activity of *Aglaia exima* has inspired Leong et al., 2016 to isolate active compound responsible for the cytotoxic activity.

2.4.2 Botanical aspect of Meliaceae

Meliaceae, or the mahogany family, consists of more than 550 species in 51 genera of woody plants located in the tropical region of Asia, Africa, Australia and South American (Nelson, 2011). Within the family, two major subfamilies were designated: Cedreloideae and Meliodeae (Kubitzki, 2010) (Figure 2.9). They have been documented based on their wood anatomy and seed morphology (Harms, 1940) and more recently, molecular data (Muellner et al., 2008). Meliaceae is found as common trees of the canopy and understory of the lowland primary forest. In Peninsula Malaysia, there are 91 species in 17 genera of Meliaceae compared to the whole of Africa, which contains 84 species (Keng, 1978; Kubitzki, 2010). Plants in the Meliaceae family have attracted great interest in the field of chemistry and pharmacology research, as they have been found to contain many bioactive compounds with antineoplastic, antifungal, antifeedant and insecticidal properties, as well as many members of this family are used in traditional medicine (Wiart, 2006). Generally, Meliaceae can be classified into different subfamilies by its specific features of leaves that are mostly pinnate,

imparipinnate or paripinnate, trifoliolate and unifoliolate. Leaflets usually crenate or serate, while the fruits of the Melioideae are commonly characterized as loculicidal capsules that are fleshy, membranous, leathery or even woody. They also do not contain columella. Meanwhile, the Cedreloideae fruits are septifragal capsules, usually woody with a ridged or angled columella. Seeds are normally arillate, winged or wrapped in a woody outer covering. The flowers are mostly free petals, but more specifically, the pseudo-tubular androecium with filaments are variously united into a tube that sometimes resembles a corolla. Stamens usually consist from eight to ten in a filament tube on the base of a disc with or without lobes, whereas the ovary is locular, with each locule having one or many ovules (Styles & White, 1991; Mabberley et al., 1995; Kubitzki, 2011; Nelson, 2011).

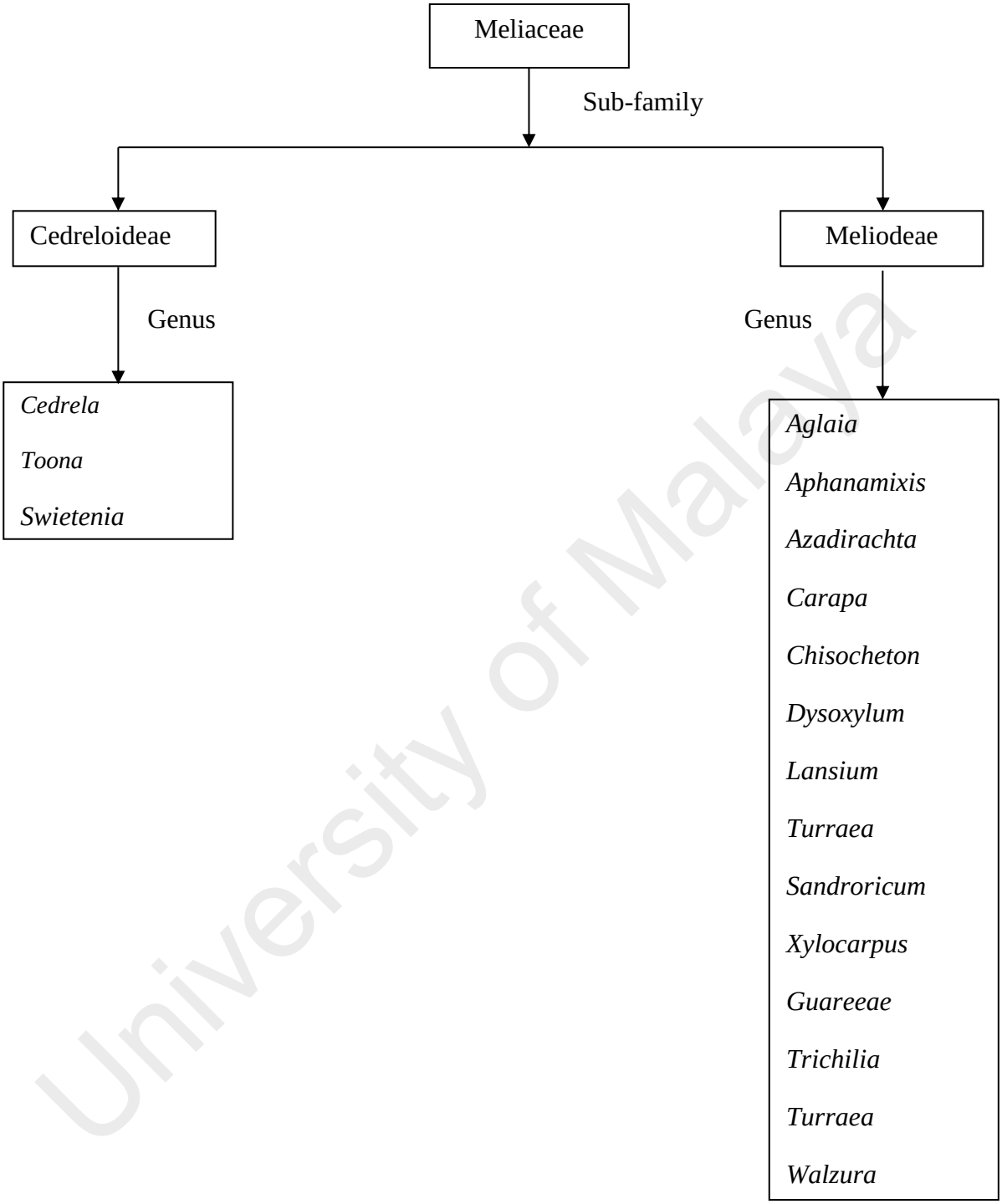


Figure 2.9: Classification of the Meliaceae family (Kubitzki, 2011).

2.4.3 *Aglaia exima*

Aglaia exima was first collected by Teysmann, J.E. and verified by Pannell, C.M. in September 1984 in Sumatera, Indonesia. It is a tree that grows up to 15m with a rounded crown. The leaves measured up to 135cm in length and 50cm in width, while the flowers measured up to 2mm in diameter with the Calyx densely covered with brown stellate hairs. The seeds usually have a thin aril, whereby the aril is in white, pale yellow, or pink in colour. *Aglaia exima* trees are found in primary forests, secondary forests, and the lowlands, sometimes on limestone at a sea level of up to 2000m in altitude (Mabberley et al., 1995).

2.4.4 Chemical constituents of the *Aglaia* species

The *Aglaia* species is used in various traditional ways: for example, the *Aglaia lawii* found in Vietnam is used by the locals as an antibacterial and antitumour treatment (Qiu et al., 2001). In the Philippines, the bark of the *Aglaia elliptica* is boiled and used to treat tumours, whereas the leaves are applied to treat wounds (Cui et al., 1997). In Indonesia, the *Aglaia exima* bark is used to reduce fever, moisturize the lungs, and treat contused wound, coughs and skin diseases (Harneti et al., 2014).

There are various reports on the bioactivities of compounds isolated from the *Aglaia* species (Table 2.1). The *Aglaia exima* was reported to show potent cytotoxic activity among eight cancer cell lines screened. The compounds isolated from this plant are mainly triterpenoids and steroids. The isolated new cycloartane triterpenoids, cycloart-24-ene-26-ol-3-one showed cytotoxic activity against HT-29 colon cancer cell line (IC_{50} 11.5 μM) (Awang et al., 2012). Later, the isolation of the bark of the *A. eximia* reported new stigmastane steroid, 3,4-epoxy-(22R,25)-tetrahydrofuran-stigmast-5-en, together with the cytotoxic activity of the isolated compounds against the P-388

murine leukemia cells (Harneti et al., 2014). However, the mechanism of action investigating the apoptosis effects of compounds from the *Aglaia exima* has not been reported so far. Therefore, this is the first investigation on the apoptotic effects of the most potent compound, cycloart-24-ene-3β,26-diol using a bioassay-guided fractionation against breast cancer cell lines.

Table 2.1: Occurrence of some selected chemical compounds with bioactivities in various species of *Aglaia*

***Aglaia* species**	**Compound class**	**Compound**	**Bioactivities**	**References**
Aglaia abbreviata	Dammarane	Aglaiabbreviatin F	Antitumour	Zhang et al., 2010
Aglaia crassinervia	Glabretal	Aglaiaglabretol B	Antitumour	Su et al., 2006
Aglaia ellipfolia	Cyclopentabenzofuran	Aglafolin Rocaglamide	Antitumour Antiplatelet	Wu et al., 1997
Aglaia elliptica	Cyclopentabenzofuran	4'-demethoxy-3',4'-methylene-dioxy-methylrocaglate 1-O-formyl-4'-demethoxy-3',4'-methylenedioxy-methylrocaglate	Antitumour	Cui et al., 1997
Aglaia erythrosperma	Cyclopentabenzofuran	4-demethoxy-3,4-methlenedioxy-methylrocaglate	Antimalarial antitumour	Phongmaykin et al., 2011
Aglaia exima	Cycloartane Dammarane	24(E)-cycloart-24-ene-26-ol-3-one Dammar-20,25-diene-3β,24-diol	Antitumour	Awang et al., 2012 Harneti et al., 2014
Aglaia foveolata	Cyclopenta[b]benzofuran	Silvestrol	Antitumour	Kim et al., 2007; Hwang et al., 2004
Aglaia perviridis	Cyclopentabenzofuran	Rocaglaol	Antitumour	Pan et al., 2013
Aglaia ponapensis	Cyclopenta[bc]benzopyran	Ponapensin	Antitumour	Salim et al., 2007
Aglaia odorata	Dammarane	Aglaiaxiflorin D Odorine 4',5,7-trimethoxydihydroflavonol	Anti-inflammatory	Yodsaoue et al., 2012

CHAPTER 3

MATERIALS AND METHODS

3.1 Bioassay-guided fractionations and characterization of the leaves of *Aglaia exima*

3.1.1 Plant material

Leaves from *Aglaia exima* were collected from H.S. Kg. Kepayang, Pahang, Malaysia on November 1997. The plant material was identified and authenticated by a botanist. The voucher specimen (KL4762) has been deposited in the Herbarium of Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

3.1.2 Gravity column chromatography of DCM leaves extract from *Aglaia exima*

The crude leaves extract was subjected to a bioassay-guided fractionation using 0.040-0.063mm silica gel (Merck, USA) column chromatography. The column was plugged at the bottom with a cotton plug to prevent loss of the absorbent material. The ratio of extract to silica gel (Merck, USA) used was 1:30. Then, silica gel (Merck, USA) was made into a slurry mixture with hexane and carefully poured into a column. After which, the column was allowed to settle and 500.0 mL of hexane were eluted through the column. The column was left overnight to ensure proper packing before proceeding to the isolation process. Approximately 17.0 g of DCM crude leaves extract was dissolved in DCM and loaded onto the packed column. After absorption of extract onto the column, first eluting solvent n-hexane was slowly added to the top of the column. The column was eluted by a stepwise gradient solvent system where the polarity of the solvent system was gradually increased by mixing a higher percentage of ethyl acetate. The collected fractions were analyzed using silica gel 60 F254 TLC plates (Merck, USA) and fractions of similar pattern were pooled to give 21 fractions (F1-F21). A MTS colourimetric assay resulted in the localization of the active fraction of the DCM extract, fraction 19, which was further subjected to separation over a silica gel column,

eluted under an increasing gradient polar solvent system of *n*-hexane-EtOAc to give the most cytotoxic compound, compound 1.

3.1.3 Characterization of compound 1 isolated from the leaves of *Aglaia exima*

^{1}H NMR spectra were taken by dissolving compound 1 in deuterated chloroform in an NMR sample tube and analyzed with the JEOL JNM-LA 400 FT-NMR system. The ^{13}C, DEPT, HSQC and HMBC spectra were obtained from JEOL ECA 400 system. Chemical shifts are given in ppm on δ scale. Mass spectra were carried out using the Shimadzu LCMS-IT-TOF Liquid Chromatography Mass Spectrometer. The analysis of all the spectra enable the identification of the compound as cycloart-24-ene-3β,26-diol (Figure 1.1).

3.1.4 Preparation of stock and working solutions

The crude extract, fractions, tamoxifen, vinblastine and cycloart-24-ene-3β,26-diol were first dissolved in DMSO to make stock solutions. To make 2 mM of crude extract stock solution, 2.0 mg of crude extract was dissolved in 100.0µl of DMSO and diluted with cell culture media. Similar preparation methods of stock solutions were repeated with all the fractions. Drug standards, tamoxifen and vinblastine stock solutions were prepared by dissolving 7.4 mg and 8.1 mg in 100.0 µL of DMSO to obtain a final concentration of 20 mM and 10 mM, respectively while 0.9 mg of cycloart-24-ene-3β,26-diol (MW of 442.38 g/mol) was dissolved in 100.0 µL of DMSO to get 2 mM stock solution of cycloart-24-ene-3β,26-diol. All the solutions were then gently vortexed to ensure all test compounds have completely dissolved in the DMSO. Before it is introduced into cell cultures, the stock solutions were diluted with cell culture media to a final DMSO concentration of 0.05% (v/v) or less, at which the DMSO solvent concentration did not induced cytotoxic effect to the cultured cells.

3.2 Cell lines

3.2.1 Cell lines and culture conditions

The human breast epithelial cancer cell lines, MCF-7 and MDA-MB-231, and normal mammary epithelial cell line, hTERT-HME1 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). For regular continuance of cell growth, MCF-7 and MDA-MB-231 were cultured in complete DMEM culture media while hTERT-HME1 was cultured in complete MEGM culture media.

3.2.2 Preparation of complete cell culture media

3.2.2.1 Dulbecco's modified Eagle's medium (DMEM)

DMEM powder and sodium bicarbonate were purchased from United States Biological, Swampscott, MA, USA and British Drug Houses Laboratory Supplies, Radnor, Pennsylvania, respectively. Heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin and l-glutamine were obtained from Life Technologies, USA. According to the manufacturer protocol, the complete cell culture media for MCF-7 and MDA-MB-231 cell lines was prepared by dissolving 12.9 g of DMEM powder, 3.7 g of sodium bicarbonate, 100.0 mL fetal bovine serum 100.0 U/ml of penicillin and 100.0 µg/ml of streptomycin, 2.0 mM l-glutamine in 1.0 L of autoclaved water. After that, the pH of the medium was adjusted to pH 7.2 - 7.4 by adding 2N hydrochloric acid. The medium was filter sterilized through a 0.2 µm polyethersulfone membranes filtration unit (Thermo Fisher Scientific, USA) into a sterile Schott bottle and kept at 4ºC until further use.

3.2.2.2 Mammary epithelial growth medium (MEGM)

Mammary Epithelium Basal Medium (MEBM) and Single Quot additives consisting of bovine pituitary extract (BPE), hydrocortisone, human recombinant epidermal growth factor (hEGF) and insulin were purchased from Lonza Walkersville, USA. According to the manufacturer protocol, the complete MEGM medium is prepared by aseptically combining 500.0 mL filtered-sterilized MEBM media, 52.0 μg/mL BPE, 0.5 μg/mL hydrocortisone, 10.0 ng/mL hEGF and 5.0 μg/mL insulin. The prepared medium was stored in 4 ºC for further usage.

3.2.3 Thawing cell lines

Cryopreserved cells were removed from the liquid nitrogen storage tank and thawed immediately in a water bath at 37ºC for approximately 1 minute. Every 1.0 mL of thawed cell suspension was then diluted 10 times with 10.0 mL of growth media and centrifuged at 1500 rpm for 5 minutes. The supernatant containing cryoprotective agent, DMSO (Sigma-Aldrich, USA) was discarded and the pellet was resuspended in fresh growth media. Resuspended cells were split into two T-25cm^2 flasks and incubated at 37ºC in a humidified atmosphere at 5% CO_2 (McAteer & Douglas, 1979; Freshney, 1984).

3.2.4 Cell culture

As for healthy cell growth maintenance, the growth medium was changed when the colour had turned orangy-yellow or every 3 to 4 days. The old media was discarded and replenished with 15.0 mL of fresh growth media. The cells were subcultured when it has attained 80 - 90% confluent. For subculturing, the spent media was discarded and the cells were gently rinsed with 5.0 mL of PBS (Life Technologies, USA) twice to remove all traces of FBS. Then, 1.0 mL of 0.25% trypsin-EDTA (Life Technologies, USA) was added into the flask and incubated around 4 minutes or until the cells

rounded up and detached from flask surface through a light microscope observation. Following that, 10.0 mL of fresh media was added into the flask to inactivate the trypsin activity. Then, the cell suspension was collected in 15.0 mL centrifuge tube and centrifuged for 5 minutes at 1500 rpm. After that, the supernatant was discarded and the cell pellet was re-suspended in fresh growth media and, split into culture flasks as desired for further usage (McAteer & Douglas, 1979; Freshney, 1984).

3.2.5 Cryopreservation of cell lines

Cryogenic preservation of cell cultures is widely used in the storage of cell lines to ensure sufficient supplies of cell culture stock. Using a sterile pipette, the old culture media was discarded. For T-75cm^2 flask, the cells were rinsed with 5.0 mL of PBS (Life Technologies, USA) twice to remove all traces of FBS. Then 1.0 mL of trypsin-EDTA (Life Technologies, USA) was added into the flask and incubated at 37^{o}C. Once most of the cells have rounded up, 10.0 mL of fresh media was added to the cell suspension and the cells were collected in a 15.0 mL centrifuge tube. Then, the cell suspension was centrifuged at 1500 rpm for 5 minutes to obtain a cell pellet. The supernatant was discarded and the resulting pellet was suspended in 1 mL of cryopreservation medium containing 90% New Calf Serum and 10% DMSO to give a final cell concentration of 2.0×10^6 cells/mL. The cell suspension was transferred into cryovials, put in a freezing container (Nalgene ® Mr. Frosty) containing isopropanol and frozen at -80^{o}C. After 24 hours, the cells were transferred into a liquid nitrogen freezer for permanent storage (Shannon & Macy, 1973; Schroy & Todd, 1976).

3.2.6 Cell counting

A trypan blue dye exclusion assay was employed to determine the number of viable cells present in a cell suspension. The basis for this assay relies on the principle that live cells possess intact cell membranes, which exclude trypan blue staining while dead cells

do not. A total of 50.0 µL cell suspension and 50.0 µL of 0.25% (v/v) trypan blue dye solution was diluted with 400 µL of cell culture media. After that, 7.5 µL of the solution was spread evenly by capillary action in a hemacytometer counting chamber. Using an inverted light microscope at 20 X magnification, the number of unstained viable cells in each of the five square grids were counted and the average number of cells was obtained. The concentration of cells was determined as shown below (Equation 3.1) with a dilution factor of 10. To determine the percentage of viable cells, viable (unstained) cells and dead (stained) were counted separately, and calculated as shown below (Equation 3.2). For cell seeding, the desired concentration of cell suspension (number of cells/ml) was calculated as required. After usage, the haemacytometer slide and glass coverslip was immediately rinsed and cleaned with 70% (v/v) ethanol (Strober, 2001).

$$C = A \times D \times 10{,}000 \qquad \text{(Equation 3.1)}$$

$$\text{Viability (\%)} = (AV/AV + AD) \times 100\% \qquad \text{(Equation 3.2)}$$

Where: C = cell concentration

A = Average number of cells counted

D = Dilution factor

AV = Total number of viable cells

AD = Total number of dead cells

3.3 Cytotoxicity assays

3.3.1 MTS assay

The cytotoxic effects of crude, fractions and compound on all cell lines were determined by MTS assay. Briefly, both cancer and normal cells were washed with 1 × PBS (Life Technologies, USA) free of magnesium and calcium. PBS solution was aspirated, and cells were detached with 0.25% (v/v) trypsin (Gibco, USA) solution. Cell pellets obtained by centrifugation at 1500 rpm for 5 minutes were resuspended in media to make a single cell suspension. Determination of viable cell count was done using the trypan blue dye exclusion method according to section 3.2.6, to give the desired cell density. A total of 1.0 x 10^4 cells were plated in triplicates at 100.0 µL/well in a 96-flat bottom well plate and incubated for 24 hours at 37^{o}C to allow adherence of the cells to the bottom of the wells. The crude extract and fractions F1-F21 were tested at a concentration range of 1.0 – 200 µg/ml for 48 h incubation, while cycloart-24-ene-3β,26-diol was tested at a concentration of 1.0 – 200 µM for different incubation periods (24, 48 and 72 h). Wells containing cells and media only were used as negative controls, whereas solvent controls containing 0.05% DMSO were conducted to ensure that cytotoxicity was not due to the solvent used to dissolve the samples. After the incubation period, culture media was carefully refreshed with 100 µL of fresh growth media and 20 µL of 317 µg/mL MTS reagent (Celltiter 96® AQueous One Solution, Promega, Madison, WI, USA) was added into each well. The reagent was mixed by gentle tapping of the plate and incubated for 1 – 2 h in the dark at 37 ^{o}C until a reddish colour formazan product was clearly visible in control wells. The absorbance of each well was measured at 490 nm with 690 nm as the reference wavelength. All measurements were conducted using the Tecan Infinite 200 microplate reader (Tecan, Switzerland) and quantified using the Magellan Version 7.0 (Tecan, Switzerland)

software. Cell viability (%) was determined using the following equation (Barltrop et al., 1991; Cory et al., 1991).

AB = Average absorbance read at the reference wavelength

AC = Average absorbance of control wells

AS = Average absorbance of treated wells

$$\text{Viability (\%)} = \frac{AS - AB}{AC - AB} \times 100\%$$

3.4 Flow cytometry-based assays

3.4.1 Apoptosis assay

Detection and differentiation of the various apoptosis stages were conducted using the Annexin V-FITC Apoptosis Detection Kit (Becton Dickson, San Jose, CA, US). Stock solutions of cycloart-24-ene-3β,26-diol was diluted in culture media as outline in Section 3.1.4 to yield final concentrations of 8.78 µM ($IC_{12.5}$), 17.55 µM (IC_{25}) and 35.10 µM (IC_{50}) for MCF-7 cell line and 10.95 µM ($IC_{12.5}$), 21.90 µM (IC_{25}) and 43.80 µM (IC_{50}) for MDA-MB-231 cell line, whereas tamoxifen at 24.30 µM (IC_{50}) and 44.50 µM (IC_{50}) for the respective cell lines. Cancer cell lines were maintained in the growth media and used when they are at the log phase of growth through harvesting by trypsinization and centrifugation at 1500 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 2 mL of fresh culture media. The cell plating for this assay was done by first counting the number of cells harvested as described in Section 3.2.6, and the concentration of cells was adjusted to 1×10^6 cells/mL using culture media in each T_{25} flask. The cells were then left overnight in the cell incubator for proper attachment of the cells. Then, the used media was discarded and cells were treated with the prepared cycloart-24-ene-3β,26-diol and tamoxifen concentrations for 24 hours. Untreated tumour cells was used as the control. After the

treatment time, used media containing detached cells were collected in 15 ml tubes. The remaining adherent cells were trypsinized, neutralized by growth media and centrifuged at 1500 rpm for 5 minutes. Then, supernatants were discarded and the resulting cell pellets were washed twice with 1 mL of ice-cold PBS and re-suspended in 1 mL of 1x binding buffer following the kit's protocol. The binding buffer contains 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1.4 M of NaCl and 25 mM of $CaCl_2$. Then, 100 µL of cell suspension (~ 1×10^5 cells) was transferred to a 2 mL tube (Beckton Dickinson, USA) and mixed with 5 µL Annexin V-FITC and 5 µL PI solution (10 mg/mL). The cells were gently mixed and incubated for 15 minutes in the dark at room temperature. A total of 400 µL of the 1 x binding buffer was added to each tube and the samples were analyzed on a BD FACSCanto 11 TM flow cytometer (Becton Dickinson, USA) within an hour. A work list was created from the Annexin V-FITC assay and the samples were acquired automatically using the BD FACS Diva Software with acquisition criteria of 10, 000 events for each tube. The data was analyzed by the same software.

3.4.2 Cell cycle assay

Cancer cells, in exponential growth phase, were seeded into a T-25cm^2 flask at 1.0×10^6 cells/mL and cultured in growth media. After 24 hours, the used media was discarded and the cells treated with IC_{50} concentration of cisplatin and cycloart-24-ene-3β,26-diol. These flasks were then incubated in the 5 % CO_2 incubator for 12, 24 and 48 hours. Untreated cells were used as negative control and incubated in the 5 % CO_2 incubator for 12, 24 and 48 hours. At each time point stated above, the used media containing cells were completely transferred into a 15.0 ml tube while the adherent cells were freshly harvested as mention in Section 3.2.4. Then, cell suspensions were centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and cell pellets

were washed twice with 1.0 mL of the buffer solution provided in the BD Cycle test Plus DNA reagent kit and then re-suspended in 1.0 mL of buffer solution. This buffer solution contains sodium citrate, sucrose, and DMSO for the collection and freezing of cell suspensions. Approximately 300 µL of this suspension was transferred into a microcentrifuge tube and 700.0 µL ice cold absolute ethanol (Merck, Germany) was added dropwise while gently vortexing the tube. These tubes were kept in the -80ºC freezer. Before analysis, cell suspensions were centrifuged at 400 g for 5 minutes at room temperature. Cell pellets were washed twice with ice-cold buffer solution and stained according to the BD Cycle test Plus DNA reagent kit. Stained cells were analyzed within an hour on a BD FACSCanto 11 TM flow cytometer (Becton Dickinson, USA) with a laser emission at 488 nm and the PI fluorescence detected at the FL2 channel in linear mode. A dot plot with Forward Scatter (FSC) on the x axis and Side Scatter (SSC) on the y-axis was constructed and then a gate was drawn to include all DNA plots. Next, a histogram was set up with FL2 channel on x-axis and cell population on the y-axis to analyze gated populations of living cells. Data was collected using BD FACS Diva software to acquired automatically 30,000 events for each tube. BD DNA QC particles were also initially acquired to set up the BD FACS Canto 11 TM flow cytometer for DNA analysis according to instructions in the BD FACSuite Software Research Assays Guide. The report was automatically generated and analyzed using the Mod Fit Software analysis program (Mod Fit 3.3, Verity Software House).

3.5 Caspase assay

Confirmation of apoptosis mediated cell death induced by the compound was performed using Caspase-Glo 3/7 kit (Promega, Madison, WI, USA). This assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, where luminescence signal is proportional to the amount of caspase activity present. Approximately 1.0 x 10^5 cells were plated in triplicates at 100.0 µL/well in a 96 well plate and incubated for 24 hours at 37ºC. These cells were treated with 50.0 µL of cycloart-24-ene-3β,26-diol at IC_{50} concentrations for 0, 1 ,3, 6,12, 18, 24, 36 h with inclusion of untreated cells as control. At each time point, the plate was allowed to equilibrate to room temperature where 50.0 µL buffer containing the substrate was added into each well and incubated for 30 minutes in the dark. The luminescence signal was measured by a microplate luminometer (Infinite 200, Tecan, Switzerland) and quantified using the Magellan Version 7.0 (Tecan, Switzerland) software. The amount of caspase activity was calculated using the equation as shown below.

LB: Average of luminescent signal for blank wells

LS: Average of luminescent signal for treated wells

LN: Average of luminescent signal for untreated wells

Amount of caspase activity (Relative Luminescent Unit) = AS - AB / AN-AB

3.6. Aromatase assay

3.6.1 Preparations of test compound and standard drugs

Each of test compounds and drugs were dissolved in acetronitrile (Acros Organics, USA) to prepare 2 mM of stock solutions. The solutions were vortexed to ensure the compounds and drugs dissolved properly.

3.6.2 Preparations NADPH-cofactor mix and cofactor-acetonitrile solutions

According to the manufacturer protocol (BD Gentest™ CYP19 kit), NADPH-cofactor mix was prepared by dissolving 187.5 µL of cofactors, 150 µL of G6PDH, and 100 µL of control protein to 14.56 mL of 37°C deionized water. To make cofactor/acetonitrile mix, 400 µL of acetonitrile was added to 9.6 mL NADPH - cofactor mix.

3.6.3 Preparations of enzyme-substrate solution

Following the manufacturer protocol (BD Gentest™ CYP19 kit), 2 ml of buffer was pre-warmed in the water bath. Then, 7.83 mL of 37°C deionized water, 150 µL of HTS-760, and 20 µL of 25 mM MFC was added to 2 mL of the pre-warmed buffer and mixed well by vortexing.

3.6.4 CYP19 inhibitor assay

The aromatase inhibition assay was performed according to the BD Gentest™ CYP19 kit's instruction. The kit uses CYP19 enzyme and MFC as fluorometric substrate wherein ketoconazole, letrozole and exemestane were used as reference drugs. This fluorescence - based assay measures the rate at which recombinant human aromatase (baculovirus/insect cell-expressed) converts the substrate MFC into a fluorescent product (λex = 409 nm, λem = 530 nm) in a NADPH regenerating system. To start the assay, 144 µL of NADPH-cofactor was mixed together with 6 µL of test compound and reference drugs. Then, 100 µL of cofactor/acetonitrile mix was added to each well. The solutions in each well was mixed by pipetting up and down for 3 to 5 times. In each row, 50 µL was serial diluted with buffer and then, the plate was covered with aluminum foil and incubated at 37°C for 10 minutes. After incubating the plate for 10 minutes, 100 µL of the CY19/P450 reductase/substrate solution (67.5 µg protein/mL enzyme; 50 µM MFC; 20 mM phosphate buffer, pH 7.4) was added to each well. The

plate was covered and incubated for 30 minutes at 37°C. After the incubation time, 75 µL of 0.5 M tris buffer was added to stop the reaction and the fluorescence of the formed de-methylated MFC was measured with a plate reader (Infinite 200, Tecan, Switzerland) and quantified using the Magellan Version 7.0 software.

3.7 Statistical Analysis

All the assays were performed in triplicates of independent experiments. All the data were presented as mean ± standard deviation (SD). One way ANOVA and Student's t-test was used to determine whether the results had statistical significance, where the threshold was set at $p < 0.05$ value**.**

CHAPTER 4

RESULTS

4.1 Bioassay-guided fractionations of the leaves of *Aglaia exima*

In the present study, the cytotoxic activity of crude leaves extract of *Aglaia exima* was screened in an *in vitro* assay system for growth inhibition against two cancer cell lines, namely hormone-dependent breast cancer cell (MCF-7) and hormone-independent breast cancer cell (MDA-MB-231). In addition, the cytotoxicity of the crude extract against normal human mammary epithelial cell (hTERT-HME1) was also examined. Dose response curves where constructed and IC_{50} values (µg/mL) of the crude DCM extract on two breast cancer cells and normal mammary cell were determined and summarized in Table 4.1. Stock solution of the *Aglaia exima* (2mg/ml) extract was dissolved in 10% DMSO and further subjected to 10-folds serial dilution. The highest final concentration of the DMSO used was 0.5% (v/v), and this concentration was found to be non-toxic to the cells.

The crude extract of the *Aglaia exima* showed a concentration-dependent increase in growth inhibition up to 200 µg/mL treatment regime in a 48 h incubation period (Figure 4.1). The DCM crude extract exhibited IC_{50} values of 44.6±1.3 µg/mL and 89.9±4.0 µg/mL when tested on MCF-7 and MDA-MB-231 cells respectively. The viability of normal hTERT-HME1 cells was much higher after treatment with the crude extract of *Aglaia exima* (IC_{50} = 143.3±1.2).

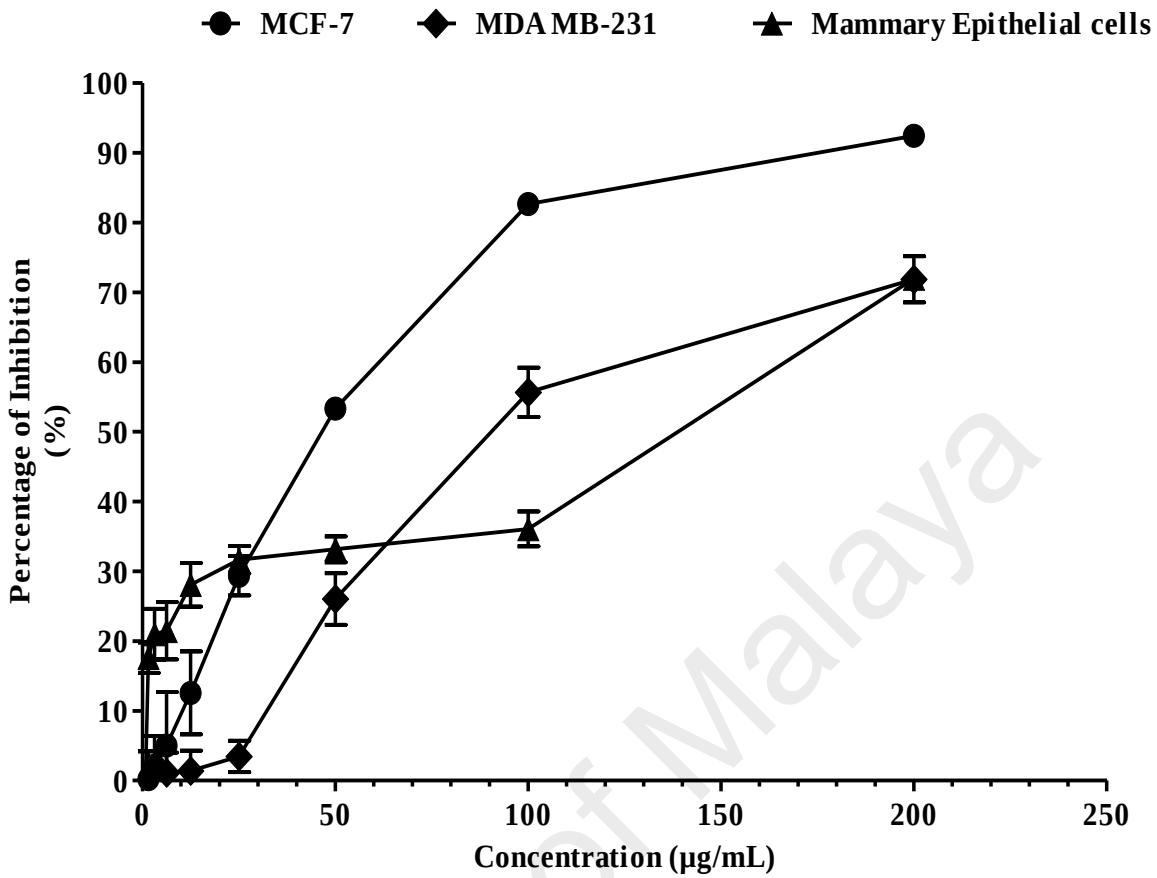


Figure 4.1: The *in vitro* growth inhibitions of various human cell lines by crude DCM extract of *Aglaia exima* (leaves) determined by MTS cytotoxicity assay. Each point is the mean± standard deviation (S.D) of three replicates.

The cytotoxic effect of the *Aglaia exima* extract was more pronounced towards malignant cells, with less toxicity to nonmalignant cells. The crude extract was further subjected to column chromatography to fractionate the bioactive compound. Gravity column chromatography of the crude extract yielded 168 fractions. Based on TLC profiles, the fractions were pooled into 21 fractions.

Fractions F1-F21 were evaluated for cytotoxicity by MTS assay on MCF-7, MDA-MB-231 and hTERT-HME1 cells, and the results are summarized in Table 4.1. Amongst fractions screened, F15 and F19 showed the greatest cytotoxicity towards malignant cells with an IC_{50} value of 47.7±1.0 µg/mL and 43.6±2.2 µg/mL in MCF-7 cells respectively. Similarly, IC_{50} value of 86.4 µg/mL and 87.7 µg/mL were recorded

in MDA-MB-231 cells. However, F19 was more selective towards cancerous cells than noncancerous cell. Interestingly, F19 demonstrated similar cytotoxic activity as the crude leaves extract. As a consequence, other fractions were excluded from the following assays and F19 was chosen for further purification and characterization of the active compound.

Table 4.1: Summary of IC_{50} values of leaves extract and fractions on MCF-7, MDA-MB-231 and hTERT-HME1 cells obtained from MTS cell viability assays after 48 h exposure.

Test samples	IC_{50} values (µg/mL) in different cell lines [a]		
	MCF-7	MDA-MB-231	hTERT-HME1
Leaves extract	44.6±1.3	89.9±4.0	143.3±1.2
F1	>200	>200	>200
F2	>200	>200	>200
F3	>200	>200	>200
F4	>200	>200	>200
F5	>200	>200	>200
F6	>100	>200	82.2±2.4
F7	71.8±8.9	>200	46.2±1.4
F8	>100	>200	55.1±0.2
F9	>200	>200	50.1±7.3
F10	108.5±3.7	>200	45.5±12.5
F11	102.7±5.2	>200	17.8±3.8
F12	90.1±4.3	71.0±5.1	21.4±2.1
F13	108.9±2.1	>200	22.8±0.6
F14	109.0±9.5	>200	28.2±1.4
F15	47.7±1.0	87.7±0.7	16.9±0.7
F16	59.9±7.7	>200	26.9±2.3
F17	>200	>200	30.6±2.3
F18	>200	>200	29.9±6.6
F19	43.6±2.2	86.4±5.6	120.7±6.9
F20	64.4±3.0	70.7±6.6	11.3±3.1
F21	>200	>200	11.7±0.3
Vinblastine	18.0±0.9	52.4±2.7	57.9±1.4
Tamoxifen	17.0±1.4	30.6±1.8	34.5±1.5

[a] All data are presented as mean ±SD of three independent experiments. MCF-7 is a hormone-dependent breast cancer; MDA-MB-231 is a hormone-independent breast cancer; hTERT-HME1 is a normal mammary epithelial cell line.

4.2 Identification of active compound from *Aglaia exima*

As aforementioned, F19 was purified by repeated column chromatography and compound 1 was isolated as a colourless crystal with molecular formula of $C_{30}H_{50}O_2$. A molecular ion peak [M+Na] 465.37075 was shown in EI-MS. Its IR spectrum exhibited bands for hydroxyl group (3327 cm^{-1}) and a cyclopropane methylene (3041 cm^{-1}).

The ^1H NMR (Figure 4.2 and Table 4.2) spectrum showed the characteristics signal of cyclopropane methylene, a pair of doublet at upfield region of 0.55 ppm (^1H, d, J= 4.2Hz) and 0.33 ppm (^1H, d, J=4.2 Hz). The ^1H NMR spectrum showed a triplet at 5.40 ppm (^1H, t, J_1=7.1Hz, J_2=13.2Hz) of H-24 indicating the presence of olefinic proton. In addition, the ^1H NMR showed a multiplet and singlet signals at 4.00 ppm (H-26) and 3.28 ppm (H-3) respectively, which revealed the presence of hydroxyl group in compound 1.

The ^{13}C NMR (Figure 4.3, 4.4 and Table 4.2) showed compound 1 was composed of six methyls, twelve methylenes, six methines and six quaternary carbons. Peak at 127.2 ppm (C-24) and 134.4 ppm (C-25) represented the double bond in the compound 1. The presence of hydroxyl groups was revealed by the peak at the position of 78.9 ppm (C-3) and 69.1 ppm (C-26). The carbons of the six methyls gave signals at 18.2, 18.0, 13.6, 25.4, 19.4 and 19.3 ppm for C-18, C-21, C-27, C-28, C-29 and C-30 respectively. The HMBC correlation (Figure 4.5 and Table 4.2) confirmed the methyl proton (H-27) position in relation to C-24 and C-25, which is a double bond. Besides, the proton (H-26) attached to a hydroxyl group correlated with C-24 and C-25. Morever, the correlation of H-24, the olefinic proton to C-22, C-23, C-26 and C-27; H-26 correlated to C-27 confirmed that the assignation of side chain. The HMBC correlation showed the correlation of H-2 with C-3 which is a hydroxyl carbon. In addition, the methylene proton H-19 was found having correlation with C-1, C-9, C-10 and C-11 which confirmed its position. Spectral analysis confirmed that compound 1

was identical to cycloart-24-ene-3β,26-diol (Figure 1.1) as reported in literatures (Anjaneyulu et al., 1985; Zhang et al., 2012; Awang et al., 2012).

Table 4.2: ^{1}H NMR, ^{13}C NMR, HMBC data of cycloart-24-ene-3β,26-diol in $CDCl_3$

Position	δ_H (ppm)	δ_C (ppm)	HMBC (H-C)
1	1.26 (1H, *m*) 1.51 (1H, *m*)	31.9	3
2	1.44 (1H, *m*) 1.74 (1H, *m*)	30.3	3
3	3.28 (1H, *m*)	78.9	28
4	-	40.5	-
5	1.29 (1H, *m*)	47.1	-
6	1.53 (1H, *m*) 0.78 (1H, *m*)	21.1	-
7	1.24 (1H, *m*) 1.90 (1H, *m*)	28.1	-
8	1.41 (1H, *m*)	48.0	-
9	-	20.0	-
10	-	26.1	-
11	1.06 (1H, *m*) 1.29 (1H, *m*)	26.0	-
12	1.04 (1H, *m*) 1.36 (1H, *m*)	35.5	-
13	-	45.3	-
14	-	48.8	-
15	1.55 (1H, *m*) 1.34 (1H, *m*)	32.9	-
16	1.58 (1H, *m*) 1.96 (1H, *m*)	26.4	-
17	1.54 (1H, *m*)	52.2	-
18	0.97 (3H, *s*)	18.2	13,15,17
19	0.33 (1H, d, J=4.2 Hz) 0.55(1H, d, J=4.2Hz)	29.9	1,9,10,11
20	1.04 (1H, *m*)	36.0	-
21	0.89 (3H, d, J= 6.4 Hz)	18.0	17,22
22	1.02(1H, *m*) 1.23 (1H, *m*)	35.9	-
23	2.05(1H, *m*) 1.92 (1H, *m*)	24.5	-
24	5.40 (1H, t, J1=7.1 Hz, J2= 13.2 Hz)	127.4	22,23,26,27
25	-	134.4	-
26	4.00 (2H, s)	69.1	24,25,27
27	1.59 (3H, m)	13.6	24,25,26
28	0.97 (3H, s)	25.4	3
29	0.81 (3H, s)	19.4	3,5
30	0.89 (3H, s)	19.3	8,14,13

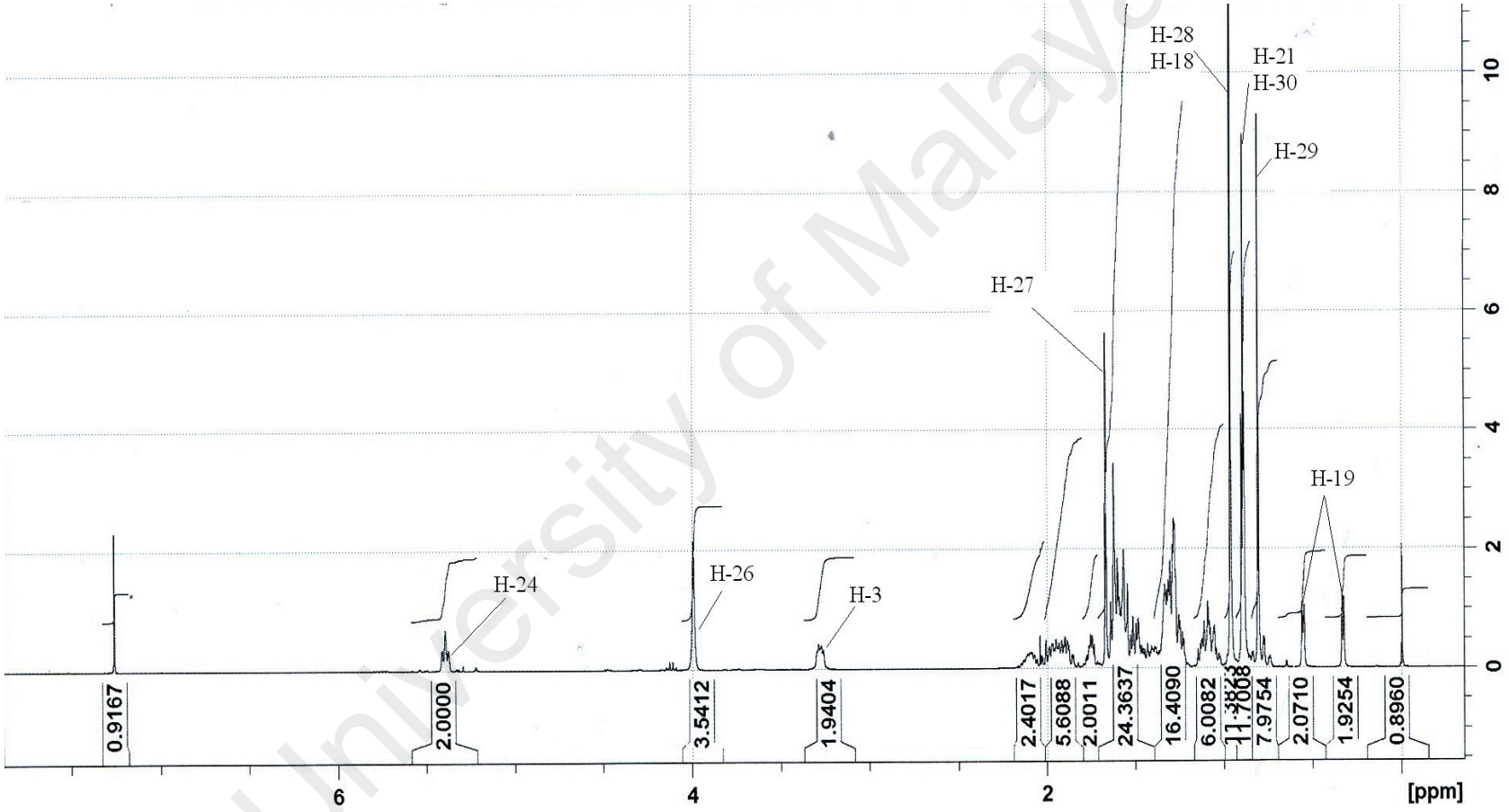


Figure 4.2: ^{1}H NMR spectrum of cycloart-24-ene-3β,26-diol in $CDCl_3$

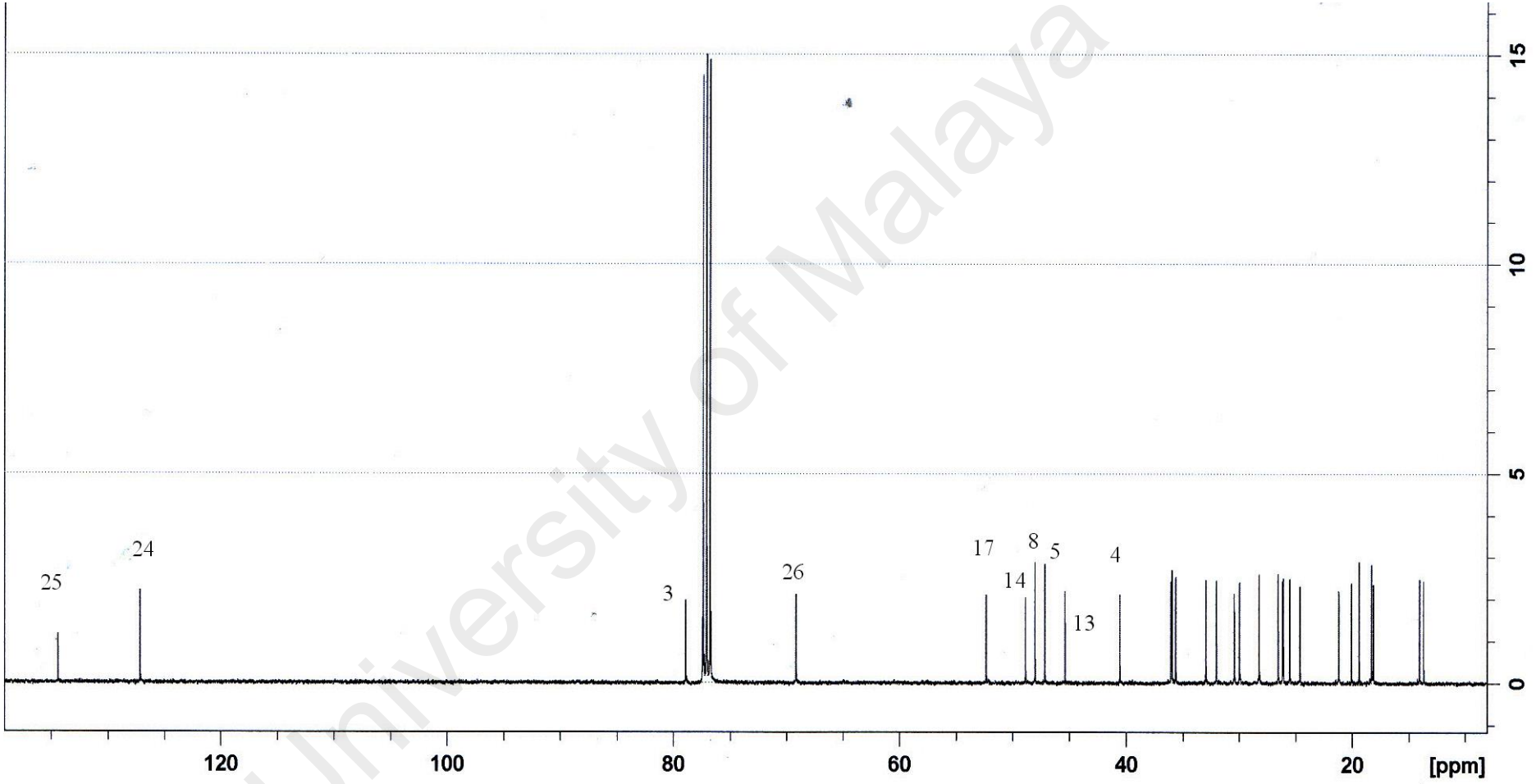


Figure 4.3: ^{13}C NMR spectrum of cycloart-24-ene-3β,26-diol in $CDCl_3$

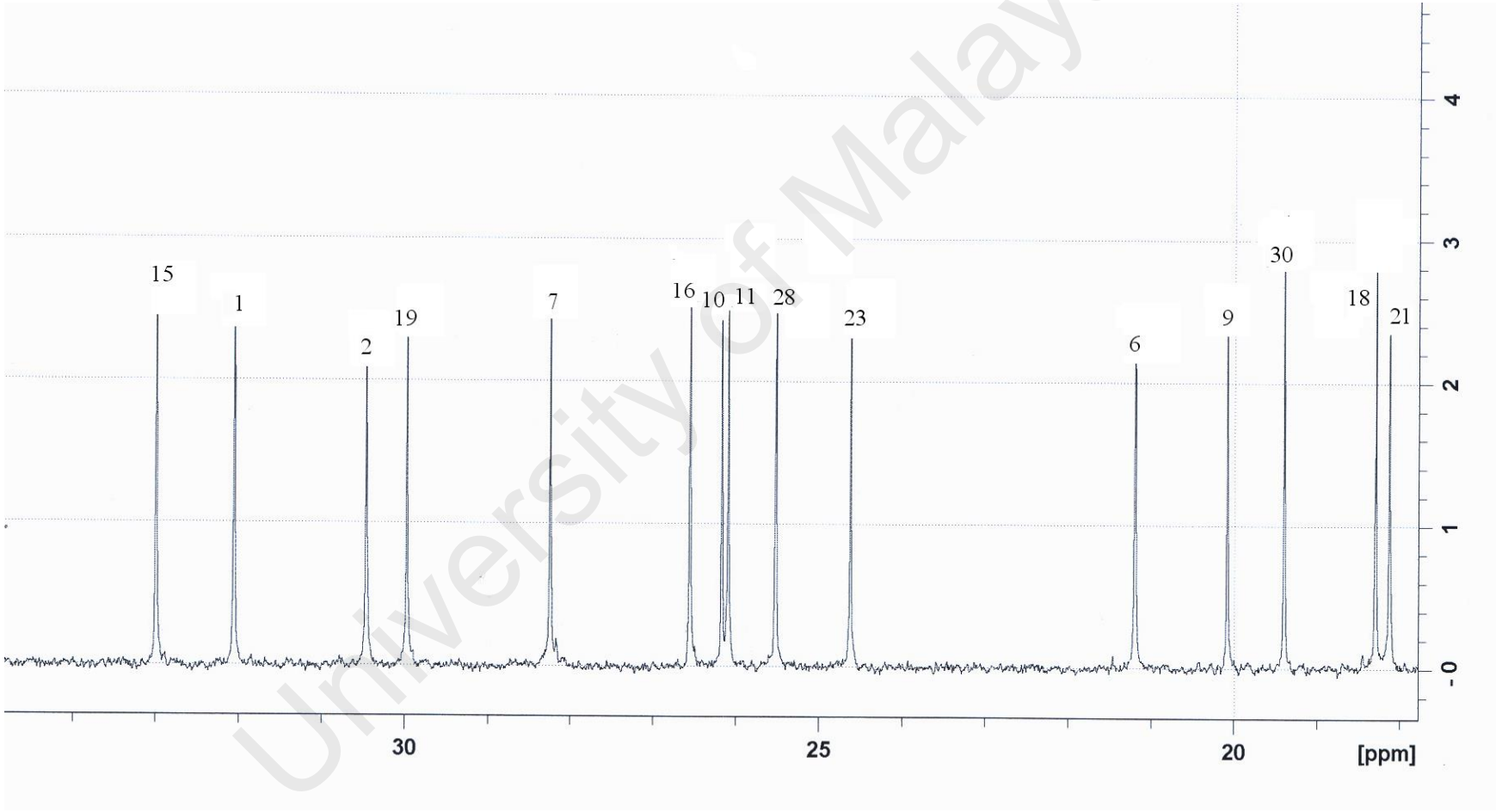


Figure 4.4: ^{13}C NMR spectrum of cycloart-24-ene-3β,26-diol in $CDCl_3$ magnified between 18 – 35ppm.

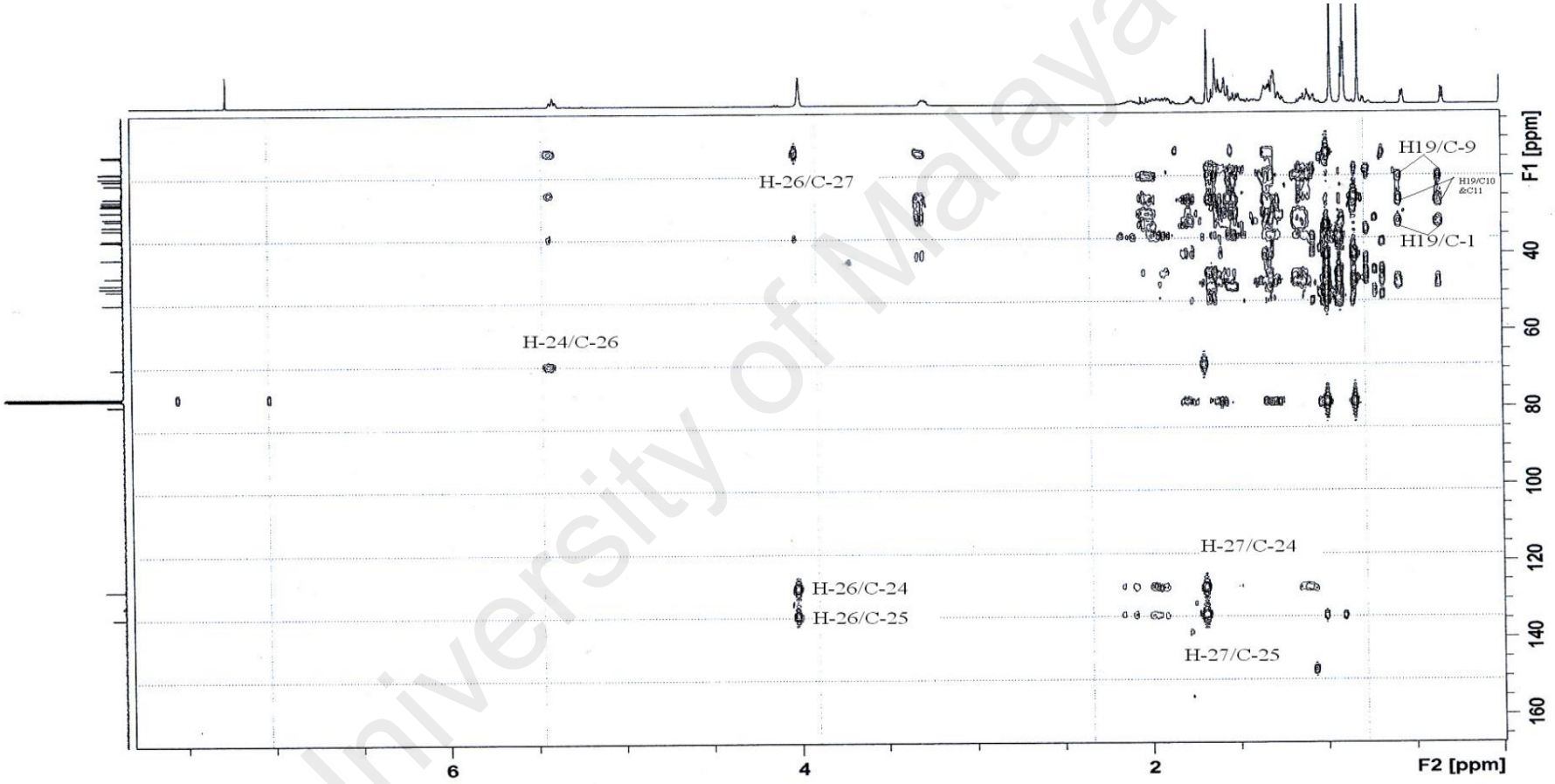


Figure 4.5: HMBC spectrum of cycloart-24-ene-3β,26-diol in $CDCl_3$

4.3 Cytotoxicity of cycloart-24-ene-3β,26-diol

Cycloart-24-ene-3β,26-diol was evaluated for cytotoxicity by MTS assay on two breast cancer cells (MCF-7 and MDA MB-231) and one breast normal cell (hTERT-HME1) at various incubation periods, which were 24 h, 48 h and 72 h. Cycloart-24-ene-3β,26-diol exhibited a time-dependent increase in inhibiting cell proliferation at concentration range of 1 - 200 µM (Figure 4.6).

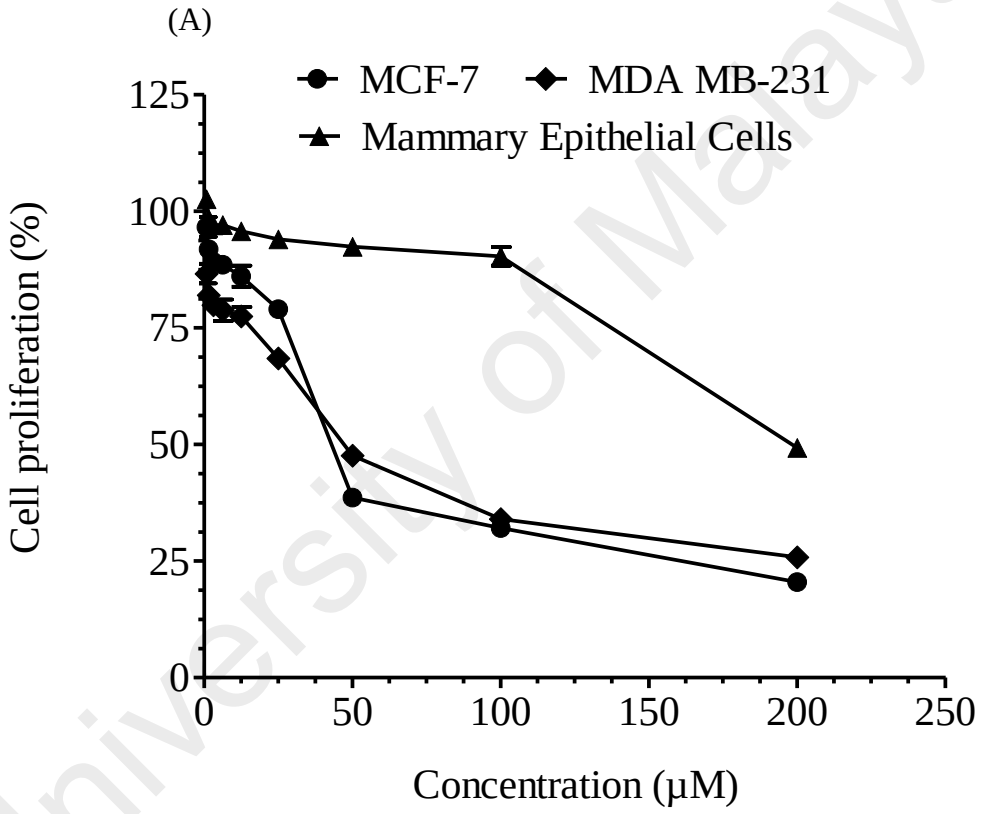


Figure 4.6: Cycloart-24-ene-3β,26-diol inhibited cell proliferation in human breast cancer cells at various incubation periods (A) 24 h & (B) 48 h and (C) 72 h. The data represent a mean of three independent experiments±SD.

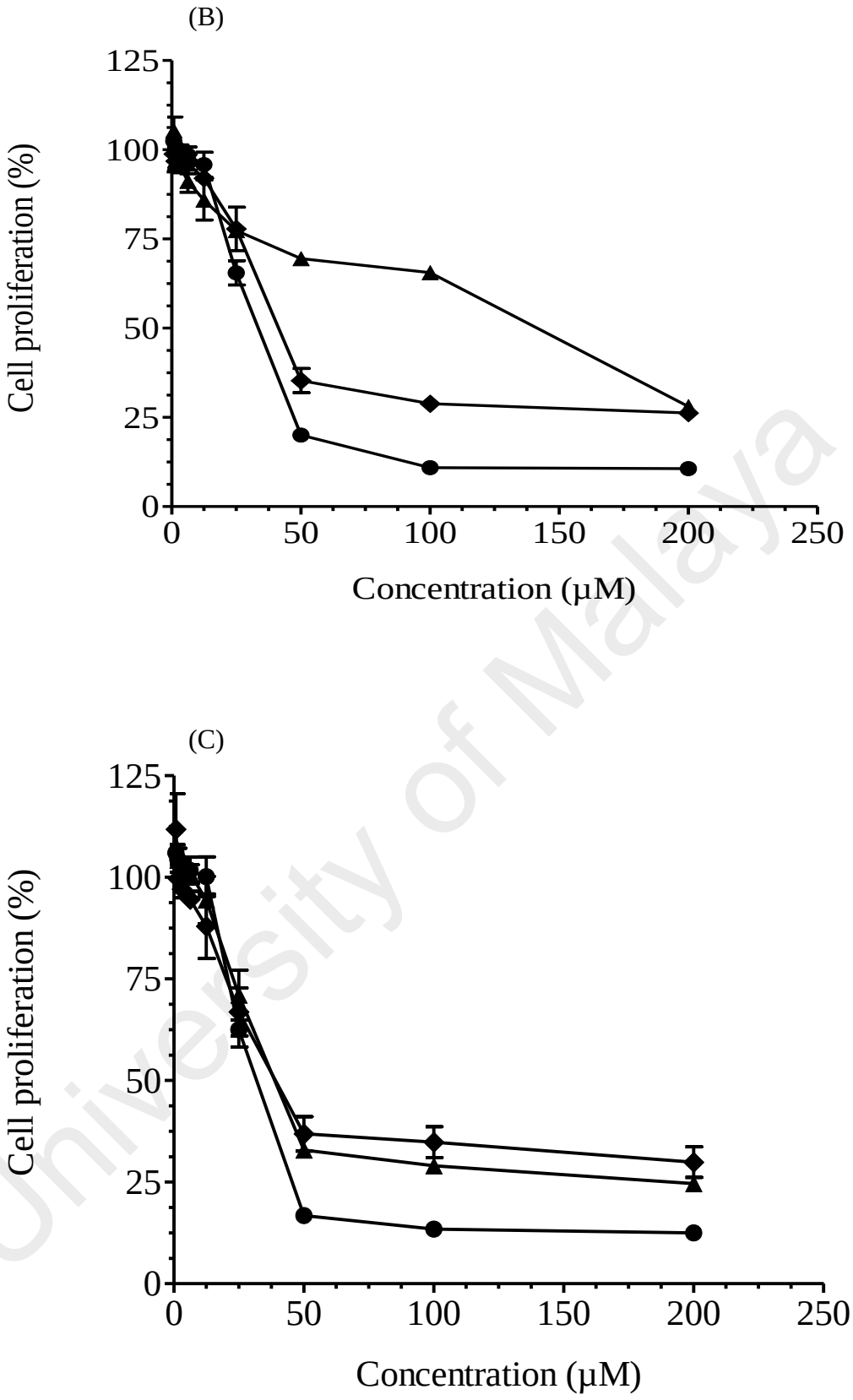


Figure 4.6 (continue): **C**ycloart-24-ene-3β,26-diol inhibited cell proliferation in human breast cancer cells at various incubation periods (A) 24 h & (B) 48 h and (C) 72 h. The data represent a mean of three independent experiments ±SD.

The IC_{50} values shown in Table 4.3 clearly indicated that the cytotoxic potency of cycloart-24-ene-3β,26-diol in an ascending order of 24 h > 48 h > 72 h for all the cell lines. At 24 h, cycloart-24-ene-3β,26-diol exhibited cytotoxicity with IC_{50} value of 35.1±1.9 µM and 43.8±0.3 µM, when tested against MCF-7 and MDA-MB-231 cells, respectively. However, at similar incubation period, cycloart-24-ene-3β,26-diol displayed an IC_{50} value more than 100 µM when tested on hTERT-HME1. In addition, MCF-7 and MDA-MB-231 cells treated with cycloart-24-ene-3β,26-diol for 48 h, the IC_{50} values obtained were 27.7±1.0 µM and 31.7±3.5 µM, respectively. Although growth inhibition was concentration dependent in all cell lines, it occurs earlier in MCF-7 than in MDA-MB-231 and hTERT-HME1cells. Furthermore, selectivity of the active compound was calculated by comparison of IC_{50} values of cycloart-24-ene-3β,26-diol against each malignant cells with that of the non-malignant cell (hTERT-HME1) and expressed as selectivity index (SI). The results are shown in the Table 4.4 and SI unit of more than three was considered as highly selective. Cycloart-24-ene-3β,26-diol appears to be most selective against MCF-7 at 24 h treatment.

Table 4.3: Summary of IC_{50} values (µM) of cycloart-24-ene-3β,26-diol on MCF-7, MDA-MB-231 and hTERT-HME1 cell lines obtained from MTS cell viability assays after 24 h, 48 h and 72 h treatments

Incubation time (h)	**IC_{50} values (µM)[a]**					
	MCF-7		MDA-MB-231		hTERT-HME1	
	Tamoxifen	Cycloart-24-ene-3β,26-diol	Tamoxifen	Cycloart-24-ene-3β,26-diol	Tamoxifen	Cycloart-24-ene-3β,26-diol
24	24.3±2.1*	35.1±1.9*	44.5±2.3	43.8±0.3*	46.1±0.8	117.1±0.7
48	17.0±1.21*	27.7±1.0*	30.6±1.0*	31.7±3.5*	34.5±1.5	46.3±6.5
72	14.9±1.32*	25.6±1.2	24.2±0.5*	24.2±3.7	27.1±0.9	25.1±0.3

[a] All data are presented as mean±SD of three independent experiements.
* shows IC_{50} values significantly different ($p<0.05$) between breast cancer cells (MCF-7 and MDA-MB-231) compared to normal breast epithelial cells hTERT-HME1.

Table 4.4: Cytotoxic selectivity index of cycloart-24-ene-3β,26-diol.

Incubation time (h)	**Selectivity index of cycloart-24-ene-3β,26-diol [a]**	
	hTERT-HME1/MCF-7	hTERT-HME1/ MDA-MB -231
24	3.3	2.7
48	1.7	1.5
72	1.0	1.0

[a]The selectivity index is the ratio of the IC_{50} values of compound on hTERT-HME1 cells to those in the cancer cell lines. Samples with SI greater than 3 were considered to have high selectivity.

4.4 Apoptosis determination

4.4.1 Cycloart-24-ene-3β,26-diol induced apoptosis mediated cell death

An early phenomenon of cell apoptosis is translocation of phosphatidylserine to the outer leaflet of the plasma membrane. In contrast, necrotic cell will lose the integrity of plasma membrane. To determine whether the cytotoxic compound decrease cell survival by the induction of apoptosis, cancerous cells were introduced to different concentrations, $IC_{12.5}$, IC_{25} and IC_{50} of cycloart-24-ene-3β,26-diol with untreated cells as the control. These cells were stained using Annexin V-FITC/PI method.

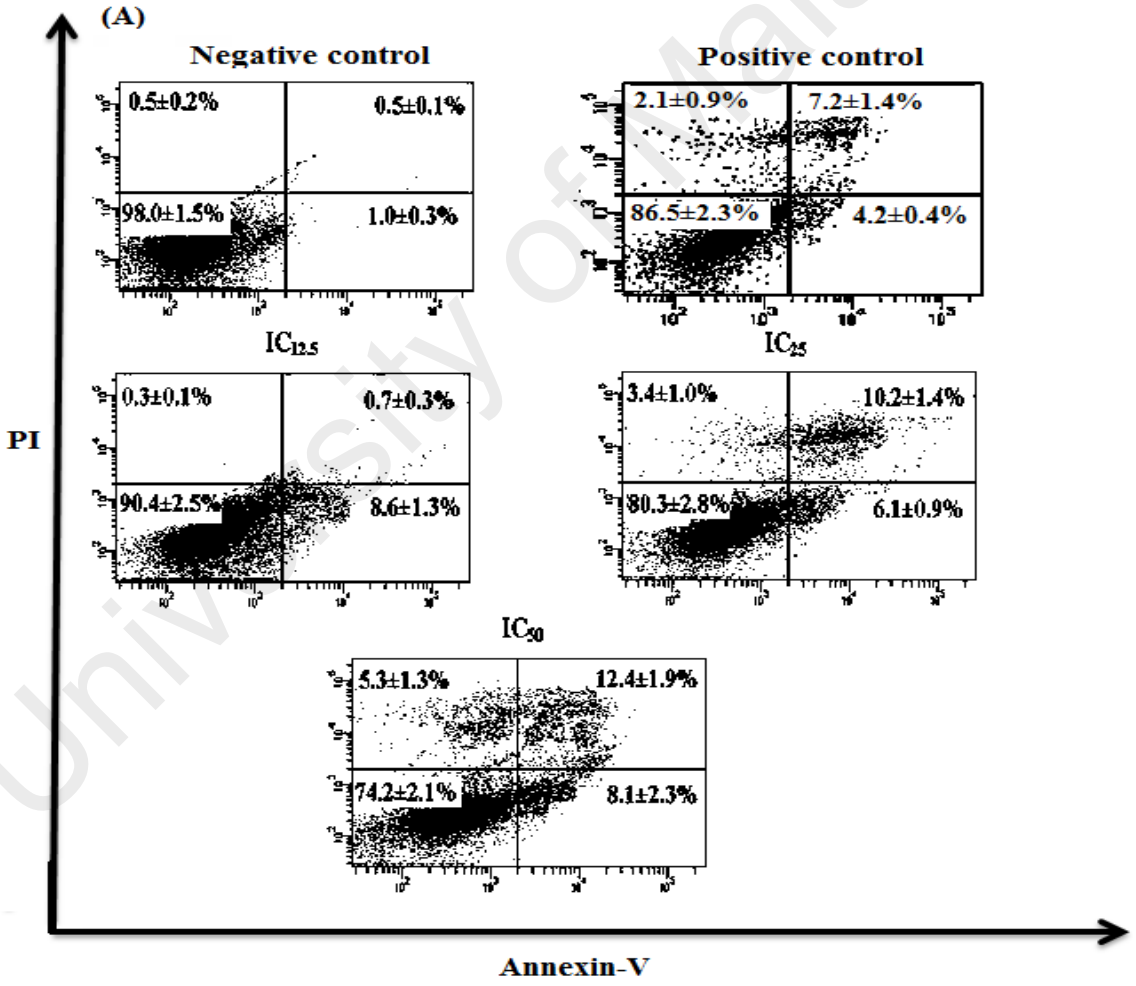


Figure 4.7: Cycloart-24-ene-3β,26-diol induced apoptosis in human breast cancer cells, (A) MCF-7 and (B) MDA-MB-231. MCF-7 and MDA-MB-231 cells were treated at $IC_{12.5}$, IC_{25} and IC_{50} concentrations of **c**ycloart-24-ene-3β,26-diol and positive control for 24 hours. The data are presented as mean±SD of three independent experiements.

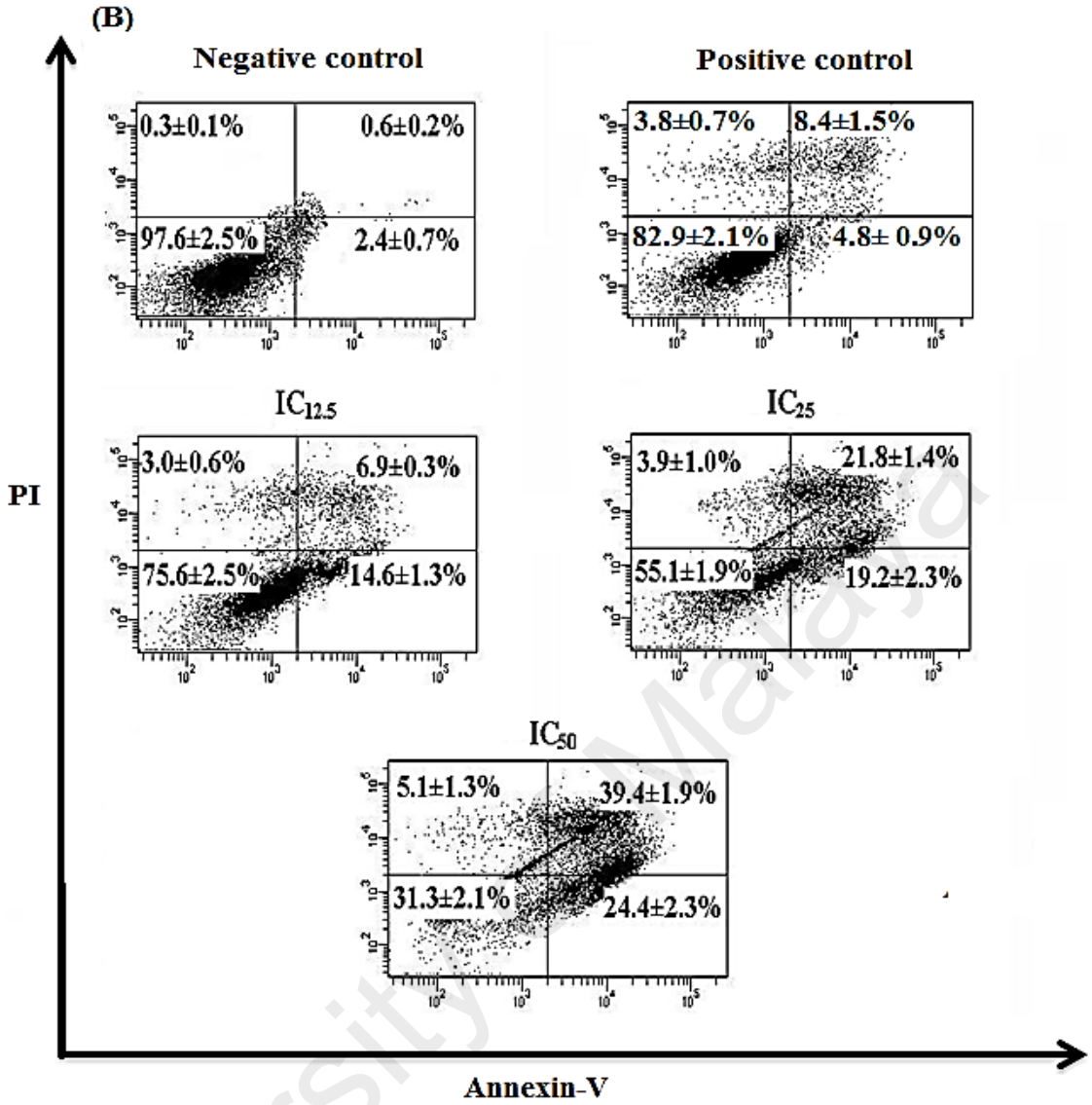


Figure 4.7 (continue): Cycloart-24-ene-3β,26-diol induced apoptosis in human breast cancer cells, (A) MCF-7 and (B) MDA-MB-231. MCF-7 and MDA-MB-231 cells were treated at $IC_{12.5}$, IC_{25} and IC_{50} concentrations of **c**ycloart-24-ene-3β,26-diol and positive control for 24 hours. The data are presented as mean±SD of three independent experiements.

In Figure 4.7A, more than 95% of untreated MCF-7 and MDA-MB-231 cells were negative for both Annexin-V-FITC and PI at the time point of analysis. The increase in Annexin-V-FITC positive and PI negative suggested that at $IC_{12.5}$, IC_{25} and IC_{50} concentrations of cycloart-24ene-3β,26-diol, an increase in the percent of apoptotic populations was dose dependent in both cancer cells. For MCF-7 cells treated with cycloart-24-ene-3β,26-diol, the population of viable cells decreased from 90.4% at

$IC_{12.5}$ concentration to 80.3% at IC_{25} concentration and it further decreased to 74.2% at IC_{50} concentration. Apoptotic cell populations increased from 9.3% at $IC_{12.5}$ concentration to 16.3% at IC_{25} concentration and further increased to 20.5% at IC_{50} concentration. For MDA-MB-231 cells treated with cycloart-24-ene-3β,26-diol (Figure 4.7B), the population of viable cells decreased from 75.6% at $IC_{12.5}$ concentration to 55.1% at IC_{25} concentration and further decreased to 31.1% at IC_{50} concentration. Apoptotic cells increased from 21.5% at $IC_{12.5}$ concentration to 41.0% at IC_{25} concentration and further increased to 63.8% at IC_{50} concentration. Secondary necrotic populations of the cancer cells treated with cycloart-24-ene-3β,26-diol was maintained at the low percentage in relation to apoptotic populations. These results suggested that cycloart-24-ene-3β,26-diol significantly ($p<0.05$) induced apoptotic cell death in both MCF-7 and MDA-MB-231 cells.

4.4.2 Cycloart-24-ene-3β,26-diol induced caspase-dependent apoptosis

Another important feature of cell apoptosis is caspase activation. Caspase plays a central role in mediating apoptotic responses. Caspase-3 and caspase-7 are major executioner caspases of the demolition phase of apoptosis. Therefore, we assessed the caspase activation through Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA). Caspase-Glo 3/7 assay is a luminescent cell-based assay measuring either caspase-3 or -7 or both. The luminescence is directly proportional to the amount of caspase activity present. The activation of caspase 3/7 in MCF-7 and MDA-MB-231 cells by cycloart-24-ene-3β,26-diol is shown in Figure 4.8.

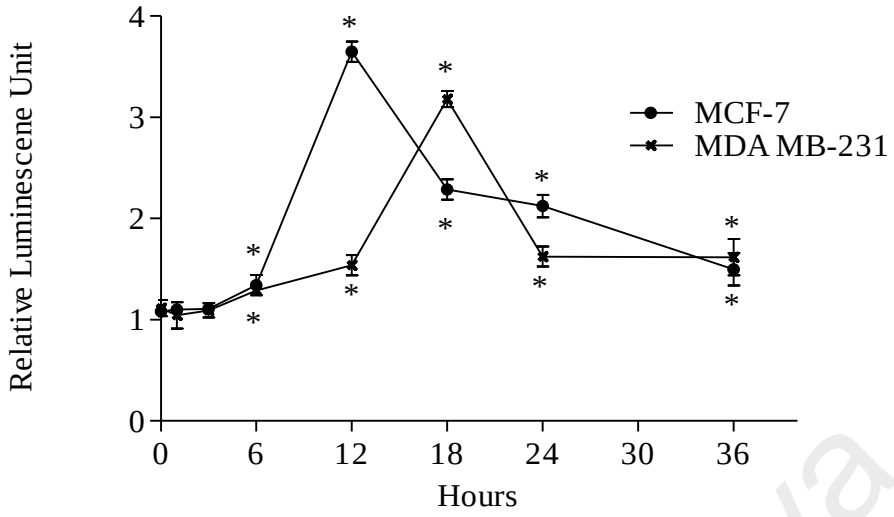


Figure 4.8: Caspase-3/7 activation in MCF-7 and MDA-MB-231 cells by cycloart-24-ene-3β,26-diol. * shows significantly different ($p<0.05$) compared to caspase-3/7 activity at 0 h. All data are presented as mean±SD of three independent experiements.

The caspase-3/7 activity for MCF-7 cells showed a baseline of 1.1 relative luminescence unit (RLU) at 0 h. The caspase 3/7 activity of MCF-7 cells after treatement by cycloart-24-ene-3β,26-diol peaked at the 12th hour with a 3.6 RLU. After that, the activity slowly subdued as the cells undergo apoptosis. The MDA-MB-231 cells also showed a baseline level of 1.1 RLU at 0 h. After treatment with cycloart-24-ene-3β,26-diol, MDA-MB-231 cells showed an increased in caspase-3/7 activity up to 3.2 RLU at the 18th hour. Similarly, the activity slowly reduced as apoptosis proceeded. Nevertheless, caspase activation occurs earlier in MCF-7 than MDA-MB-231 cells. The results suggest that cycloart-24-ene-3β,26-diol can induce apoptosis in both cell lines in a time-dependent manner, similar to the MTS results.

4.5 Cell cycle analysis

4.5.1 Cycloart-24-ene-3β,26-diol caused cell cycle arrest and death

In order to determine whether cycloart-24-ene-3β,26-diol affects cell growth, cell cycle was investigated, the cell distribution in the different phases of the cell cycle was studied by flow cytometry using the BD Cycle test Plus DNA reagent kit. Figure 4.9 shows the effect of the compound on the cell cycle of MCF-7 and MDA-MB-231 cells.

Initially, the stained DNA of untreated MCF-7 cells demonstrated that 55.0% of the cells were in the G_0/G_1 phase, 37.4% in the S phase and 7.6% in the G2/M phase (Figure 4.9A). After 12 h treatment with 35.1 µM (IC_{50} concentration) of cycloart-24-ene-3β,26-diol, stained DNA of MCF-7 cells demonstrated that 61.4% of the cells were in G_0/G_1 phase, 29.9% in the S phase and 8.7% in the G_2/M phase. The percentage of treated MCF- 7 cells in G_0/G_1 phase increased from 58.7 % (control) to 72.0 % whereas a significant decrease from 32.4% (control) to 18.0% in the S phase and minimal changes in G_2/M phase were observed after 24 h treatment. Similarly, stained DNA of MCF-7 cells after 48 h of treatment demonstrated that 78.9% of the cells were in the G_0/G_1 phase, 11.5% in the phase and 9.4 % in the G_2/M phase. These results provide evidence that cycloart-24-ene-3β,26-diol significantly ($p<0.05$) caused cell cycle arrest in G_0/G_1.

Surprisingly, in MDA MB-231 cells treated with cycloart-24-ene-3β,26-diol a dramatic accumulation of cells in sub G_0 phase and concomitant decrease of the percent of cells in S and G_2/M phase was observed (Figure 4.9B). This suggests that cycloart-24-ene-3β,26-diol induced MDA-MB-231 cell death. After 12 h, MDA MB-231 cells treated with the 43.8 µM (IC_{50} concentration) of cycloart-24-ene-3β,26-diol show increased in sub G_0 phase from 0 % (control) to 0.8 % whereas a minimal increase from

47.3% (control) to 48.7% in the G_0/G_1 phase and significant changes in S and G_2/M phase were observed. After 24 h, percent of treated cells in sub G_0 phase increased from 0.6% (control) to 16.5%. Similarly, cell populations in G_0/G_1 phase increased from 30.6% (control) to 52.2%. However, percent of cells in S and G_2/M phases decreased from 55.1% (control) to 37.6% and 14.3% (control) to 10.2% respectively. Nevertheless 48 h treatment showed all the cell population shifted to sub G_0 compared to control cells. These results suggested that cycloart-24-ene-3β,26-diol induced apoptotic cell death in MDA-MB-231 cells with the accumulation of cells in the sub G_0 phase overtime.

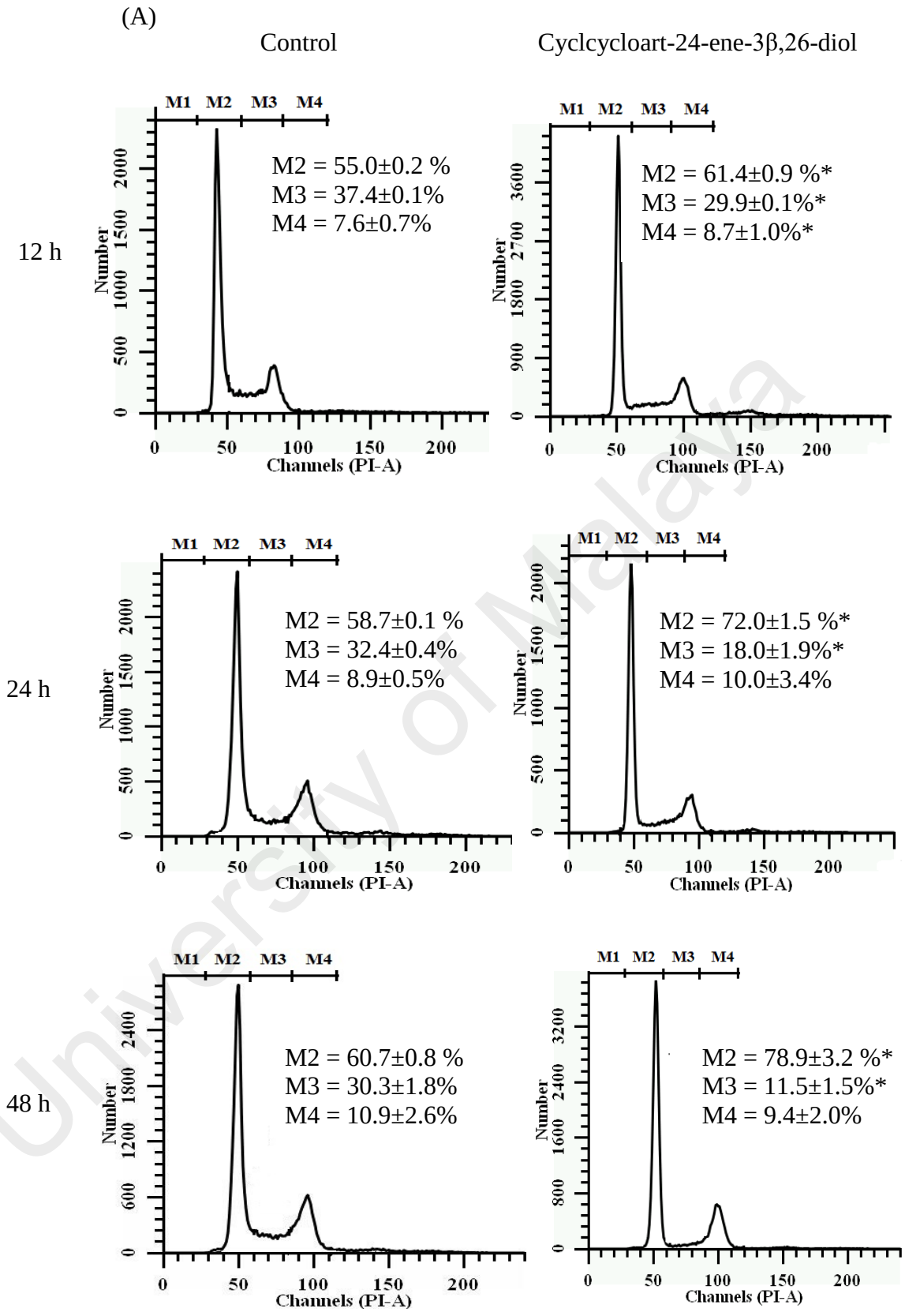


Figure 4.9: Histogram of cell cycle for (A) MCF-7 and (B) MDA-MB-231 between untreated control and treated with cycloart-24-ene-3β,26-diol. M1=Sub G_0, M2= G_0/G_1, M3=S, M4= G_2/M phases. * shows significantly different ($p<0.05$) compared to untreated control. All data are presented as mean±SD of three independent experiements.

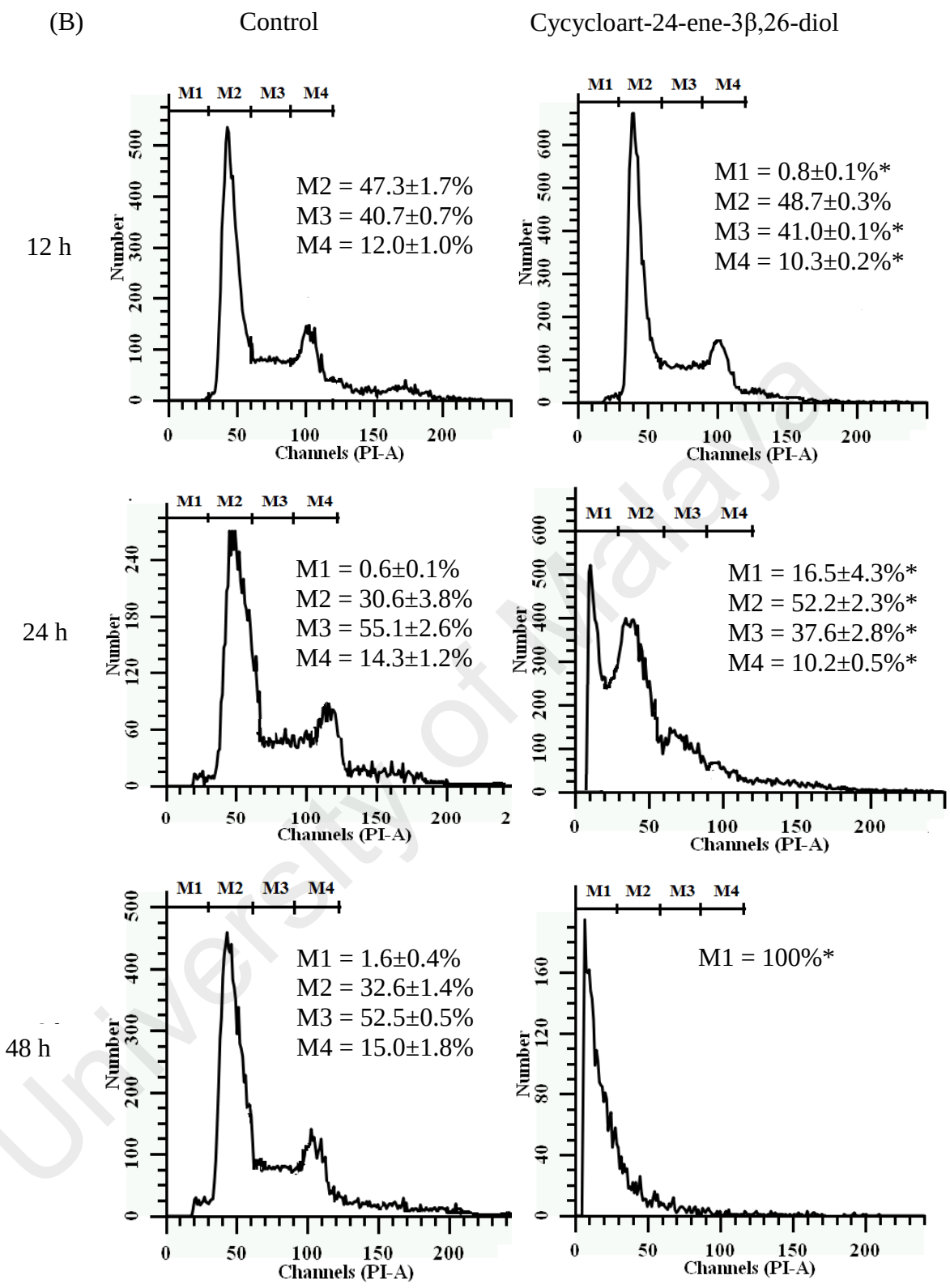


Figure 4.9 (continue): Histogram of cell cycle for (A) MCF-7 and (B) MDA-MB-231 between untreated control and treated with cycloart-24-ene-3β,26-diol. M1=Sub G_0, M2= G_0/G_1, M3=S, M4= G_2/M phases. * shows significantly different ($p<0.05$) compared to untreated control. All data are presented as mean±SD of three independent experiements.

4.6 Aromatase inhibition assay

4.6.1 Cycloart-24-ene-3β,26-diol inhibited aromatase

In addition to cytotoxic, cytostatic and apoptosis, cycloart-24-ene-3β,26-diol was tested for potential aromatase inhibition using the BD Gentest™ CYP19 kit. Aromatase inhibitors including ketoconazole, letrozole and exemestane were used as positive controls. The conversion of 7-methoxy-4-trifloromethyl coumarin (MFC) fluorescent substrate into 7-hydroxy-4-trifloromethyl coumarin (HFC) fluorescence product was measured in the presence of different concentrations of the compounds. The intensity of the fluorescence of HFC is directly proportional to the aromatase activity. The results are summarized in Table 4.5. By comparison, although cycloart-24-ene-3β,26-diol inhibits aromatase, but it was less effective than the other positive controls. To put in other words, inhibition activity of cycloart-24-ene-3β,26-diol was at micromolar levels while clinically used aromatase inhibitors for breast cancer treatment including letrozole and exemestane have nanomolar levels of inhibition. These results suggested that cycloart-24-ene-3β,26-diol is a less potent aromatase inhibitor than letrozole, exemestane and ketoconazole.

Table 4.5: The IC_{50} values of cycloart-24-ene-3β,26-diol, ketoconazole, letrozole and exemestane for aromatase inhibiton.

Test compounds	IC_{50} values (Mean±SD)
Ketoconazole	0.8±0.1 µM
Letrozole	7.0±1.8 nM
Exemestane	60.0±13.8 nM
Cycloart-24-ene-3β,26-diol	1.7±0.3µM

CHAPTER 5

DISCUSSION

The main objectives of this study were to isolate and characterize the most bioactive compound from the crude DCM leaves extract of *Aglaia exima* against two human cancer cells, namely estrogen-dependent breast cancer cell (MCF-7) and estrogen-independent breast cancer cell (MDA MB-231). Further selective cytotoxicity study was incorporated by comparing with normal mammary epithelial cell line, hTERT-HME1. Then, efforts were made to determine whether the bioactive compound (cycloart-24-ene-3β,26-diol) caused cytostatic, induced apoptosis and inhibited the aromatase activity.

Natural products has been known as a rich source of anti-cancer agents. Many chemotherapeutic agents have been isolated and characterized by screening natural products from plants, animals, marine organisms and microorganisms. Several plant-derived compounds that are being employed in cancer treatment include vincristine, irinotecan, etoposide and paclitaxel (Da Rocha et al., 2001). These bioactive compounds promote different physiological conditions by targeting various genes (Walsh & Fischbach, 2010), proteins (Bykov et al., 2002), enzymes (Jedinak et al., 2006) and biochemical pathways (Ancuceanu & Istudor, 2004) in mammalian cancer cells.

In the past years, several methods have been developed to evaluate the cytotoxicity of natural products (Cook & Mitchell, 1989). These include epidemiology, human clinical, animal and *in vitro* studies. In the present study, *in vitro* method was employed to assess the compound's biological activities, which include cytotoxicity, cytostatic potential, apoptosis and aromatase inhibition. Although this method is most convenient, inexpensive and useful in providing mechanistic information, there is a limitation where it does not represent the exact situation *in vivo*.

In this present study, a convenient *in vitro* MTS assay was conducted to screen the cytotoxicity potentials of crude extract of *Aglaia exima* against human breast cancer cell lines including MCF-7 and MDA-MB-231. MTS assay has been optimized for the use of microtiter plates (96-wells plate format) which allows many samples to be analyzed rapidly and simultaneously. MTS is reduced by living cells to yield a formazan product that can be assayed colourimetrically. With each cell line, the amount of formazan product was time-dependent and proportional to the number of viable cells. This assay can be used to quantify the number of viable cells after exposure to toxicants.

The crude extract of *Aglaia exima* was found to be more cytotoxic towards MCF-7 cells compared to MDA-MB-231 cells. This could be due to the molecular characteristic of the cell lines, in which the patterns of chemo sensitivity and gene expression profiles differ from one cell line to another (Szakacs and Gottesman, 2004). The inhibition of proliferation of the normal mammary epithelial cell line, hTERT-HME1 by the crude extract after 48 h was lower, indicating that the crude extract was less toxic to normal cells. Even though it is important for an anticancer agent to exhibit cytotoxicity, selective cytotoxicity towards cancer cells will be advantageous.

The current study showed the ability of *Aglaia exima* crude extract in inhibiting breast cancer cells proliferation. There are reports on the potential of extracts and compounds derived from *Aglaia* species as cytotoxic and anti-neoplastic agents in various cancer cell lines such as human oral epidermoid carcinoma (KB), human estrogen dependent breast cancer (MCF-7), human umbilical vein endothelial (HUVEC), human hormone-dependent prostate cancer (LNCaP) and P388 murine leukemia cells (Hwang et al., 2004; Rivero-Cruz et al., 2004; Zhang et al., 2010; Harneti et al., 2012). Different classes of organic compounds of medicinal interest have been isolated and characterized from various *Aglaia* species, including

benzo[*b*]oxepinesbisamides, cyclopenta[b]benzofurans (flavaglines), cyclopenta[b]benzopyrans, lignans, limonoids, pregnane steroids and triterpenoids (apotirucallane, cycloartane, dammarane, and tirucallane (Hwang et al., 2004; Rivero-Cruz et al., 2004; Su et al., 2006). Therefore, probably the presence of these compounds might be responsible for the cytotoxic actions reported in this study in relation to plants extracts of the species.

Bioassay-guided fractionation method was used to isolate the most effective constituent of *Aglaia exima* in inhibiting the growth of the human breast cancer cells. Bioassay-guided fractionation of medicinal plants is a process in which natural product extract is chromatographically fractioned and re-fractioned until a pure biologically active constituent(s) is isolated. As separations are performed on crude extracts, an *in vitro* assay can be used to find the potential antitumour agent (Atta-ur-Rahman, 2001).

In this study, the bioassay-guided fractionation of *Aglaia exima* extract led to the identification of a potent compound, cycloart-24-ene-3β,26-diol. Structurally, cycloart-24-ene-3β,26-diol is a tetracyclic triterpenoid, which was also isolated from various plant species including the stem bark of *Mangifera indica* (Anjaneyulu et al., 1985) and *Allophylus longipes* (Zhang et al., 2012). Several studies showed that many other cycloartanes have been reported as cytotoxic agents against several tumour cells (Öksüz et al., 1993; Banskota et al., 1998; Omobuwajo et al., 1996; Mohamad et al., 1997; Tanaka et al., 2000; Kim et al., 2001; Banskota et al., 2000). In fact, Smith-Kielland et al., 1996 reported that cycloartenediol and its isomer showed cell inactivating effects against a murine mammary tumour. So far, our research group have done the cytotoxic activity of *Aglaia exima* crude extract, which demonstrated cytotoxic potential against eight cancer cell lines; lung (A549), prostate (DU-145), skin (SK-MEL-5), pancreatic (BxPC-3), liver (Hep G2), colon (HT-29), breast MCF-7 and MDA-MB-231 (Awang et al., 2012). The study mainly investigated cell viability of cycloart-24-ene-3β,26-diol

treated cancer cell lines by MTS assay and no information was available on the antitumour mechanism. Therefore, this is the first report on the selective cytotoxicity and mechanism of action of cycloart-24-ene-3β,26-diol on human breast cancer cells.

Since cycloart-24-ene-3β,26-diol is a rare natural triterpenoid and the mechanism of action is not well understood, the *in vitro* method is the best approach to study its effects. In this study, the cells were incubated with different concentrations of the compound for different time intervals up until 72 h, which would provide information on how cancerous and non-cancerous cells respond to the compound. Based on the IC_{50} values, cycloart-24-ene-3β,26-diol inhibited the proliferation in MCF-7 better than in MDA-MB-231. Cycloart-24-ene-3β,26-diol showed 1.0 - 3.3-folds and 1.0 - 2.7-folds selectivity toward MCF-7 and MDA MD-231 respectively, in comparison to hTERT-HME1 cells. Therefore, the results indicated that cycloart-24-ene-3β,26-diol induced cell line selective cytotoxicity in a dose and time-dependent manner.

A wide variety of plant-derived compounds appear to possess significant cytotoxic as well as chemopreventive activity and many of these agents were reported to act via apoptosis, a programmed cell death. Many researchers consider the induction of apoptosis in cancer cells or malignant tissues as one of the key mechanisms for the targeted therapy of various cancers and has also been recognized as an efficient strategy in cancer chemotherapy (Vermeulen et al., 2005).

A number of methods exist for detecting apoptotic cells, but DNA integrity assays which provide quantitative data about apoptosis may lack specificity, are time consuming and usually require the destruction of cell intergrity (Vermes et al., 1995). In addition, the morphological changes and degradation of chromatin, which are the bases of such assays, occur rather late in apoptosis. Zhang and coworkers (1997) have shown

that annexin-V assay is easy, simple and non-invasive for early detection of apoptosis. The assay detects the evidence of apoptosis through staining of the phosphatidylserine during early apoptosis. Annexin-V binding assays have several advantages over other methods. For example, annexin-V binding requires only 5-10 minutes whereas DNA fragmentation based assays take 3-4 hours to complete. In addition, annexin-V binding is non-enzymatic and does not require fixation, so it allows one to score apoptotic cells with living, unfixed samples, which is not possible with DNA fragmentation assays.

Early stages of apoptosis mostly occur at the cell surface which include the expression of thrombospondin binding sites (Pytela et al., 1985), loss of sialic acid residues (Savill et al., 1993) and exposure of phosphatidylserine (PS) (Fadok et al., 1992). Plasma membranes are composed of neutral phosphatidylcholine and sphingomyeline which constitutively present in the outer leaflet and negatively charged phosphatidylserine are predominantly present in the membrane leaflets facing the cytosol. The translocation of phosphatidylserine to the outer leaflet of plasma membrane is an early phenomenon in the apoptosis process (Demchenko et al., 2013). Balasubramanian and Schroit (2003) have demonstrated that the exposure of PS is a universal process during early apoptosis which is independent of the species, cell type or apoptosis inducer.

Annexin-V was initially discovered as a vascular protein with strong anticoagulant properties (Reutelingsperger et al., 1985). Annexin-V is calcium dependent, phospholipid-binding protein that preferentially binds PS which is normally absent in the outer leaflet of plasma membrane and shows minimal binding to phospholipid species such as phosphatidylcholine and sphingomyeline (Vermes et al., 1995). Annexin-V can be conjugated with a flourochrome such as FITC which allows distinguishing between non-apoptotic and apoptotic cells and could be used to develop flow cytometric assay for apoptosis (Demchenko et al., 2013). Currently this assay is

frequently used to probe and quantify the early stage of apoptosis as well as to characterize the apoptotic cell population by flow cytometry (Boersma et al., 2005; van Engeland et al., 1998). Since PS exposure also occurs in necrotic cells, annexin-V binding assay is normally combined with a membrane-impermeable DNA stain, such as propidium iodide (PI) that will allow discrimination between early apoptotic cells with intact membranes and necrotic cells with leaky membranes (Vermes et al., 1995 and Demchenko et al., 2013).

In this study, Annexin-V-FITC/PI dual staining was used to evaluate apoptotic cells. Since annexin-V can bind to phosphatidylserine protein when cells undergo apoptosis, probes of annexin-V conjugated with FITC were used to measure apoptotic cells quantitatively by flow cytometry (Vermes et al., 1995; Zhang et al., 1997). By staining cells with combination of FITC-conjugated annexin-V and PI, it is possible to detect non-apoptotic live cells (FITC-negative/PI-negative), early apoptotic cells (FITC-positive, PI-negative), and late apoptotic or necrotic cells (FITC-positive, PI-positive) (Koopman et al., 1994; van England et al., 1998). Through flow cytometric methods employing annexin-V-FITC and PI dual staining, it was clearly shown that following treatment of breast cancer cell lines with the compound, the population of viable cancerous cells shifted toward apoptosis. Increasing annexin-V staining after incubation showed that the compound is capable of inducing apoptosis in human breast cancer cell lines. These results are in good agreement with previous studies reported on the cell death morphological changes induced by various plants species assessed using annexin-V staining method (Chiruvella et al., 2008; Luan et al., 2015).

Caspases activation plays an essential role in the execution of apoptotic cell death. Currently, 14 caspases have been recognized in mammals, but only few caspases have been identified to be involved in the regulation and execution of apoptosis (Budihardjo et al., 1999). Generally, caspases can be divided into two categories, the

initiator caspases, (caspase-2, caspase-8 and caspase-9) and the executioner caspases, (caspase-3, caspase-6 and caspase-7). Initiator caspases activates other downstream executioner caspases and the activated executioner caspases subsequently cleave distinct cellular proteins which lead to the characteristic apoptotic morphology (Fan et al., 2005).

Caspase-Glo-3/7 reagent is a universal indicator of apoptotic death. In this study, MCF-7 and MDA-MB-231 cells were treated respectively with 35.1 µM and 43.8 µM (IC_{50} concentrations) of cycloart-24-ene-3β,26-diol. The treated cells were exposed for various time points to scrutinize the caspase activity. CaspaseGlo 3/7 reagent is targeted by both caspase -3 and -7. Therefore, Caspase-Glo-3/7 reagent is cleavaged at the specific peptide sequence (DEVD) by caspase -3 and/or -7. These will result in an end product that is luminescence, measurable by a luminometer (Looi et al., 2013).

Recently, many plant extracts including *Aglaia* species extracts have been demonstrated to cause apoptosis in various cell types by inducing the activities of caspase-3 and -7 (Inayat-Hussain et al., 1999; Chakrabarty et al., 2002; Looi et al., 2013; Badmus et al., 2015; Hajiaghaalipour et al., 2015). In this study, caspases activity was shown to be increased with respect to the number of cells undergoing apoptosis. Cycloart-24-ene-3β,26-diol increased caspase-3/7 activities up to 12 h and 18 h incubation time and then decreased after that in MCF-7 and MDA-MB-231 cells respectively. The reduction of the caspase activities after the peaks is possibly due to the increasing number of cells approaching late stage of apoptosis and the accumulation of dead cell debris where cellular function ceased. Both MCF-7 and MDA-MB-231 treated cells expressed the highest level of caspase-3/7 activities at 12 h and 18 h, increasing by 3.6 and 3.2-folds, respectively, as compared to the levels in untreated cells. Caspase-3/7 activities in treated cells were statistically shown to increase significant compared to the untreated cells ($p < 0.05$).

The development of cancer involves disruption of the normal regulation of cell cycle progression and damage to cellular DNA. Normal cell cycle progressions depend on the cell's ability to translate extracellular signals, such as mitogenic stimuli and intact extracellular matrices. These ensure efficient DNA replication and cell division. Moreover, cells have several defensive mechanisms to deal with agents that attack the DNA. Nevertheless, the DNA-repair processes are not always perfect. Since various types of DNA damage can occur, a variety of different repair mechanisms exists. One of the ways cells respond to DNA damage is by halting cell-cycle progression or by undergoing apoptosis (Meeran & Katiyar, 2008). Therefore, the inhibition of cell cycle has become an appreciated target for management and treatment of tumour cells with cytotoxic agents (Shih and Stutman, 1996; Schwartz and Shah, 2005). To determine whether growth inhibition of the human breast cancer cells by cycloart-24-ene-3β,26-diol may be due to cell cycle arrest, the DNA content was analyzed by propidium iodide staining followed by flow cytometry. Flow cytometry (FCM) analysis of cellular DNA content has become an increasingly important research tool for the measurement and identification of abnormal cell populations. Examination of cellular DNA content can provide useful information in cell cycle analysis in cancer studies (Spyratos, 1993). This is based on the ability to stain the cellular DNA where the amount of stain is directly proportional to the amount of DNA within the cell. A variety of dyes are available to serve this function, all of which have high binding affinities for DNA. The location to which these dyes bind to the DNA molecule varies with the type of dye used. The most common DNA binding dye is Propidium Iodide (PI), which is an intercalating dye that binds to DNA and double stranded RNA. Therefore, PI is always used in conjunction with RNAse to remove RNA (Rabinovitch, 2010). The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the

dye. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content (Nunez, 2001).

The *in vitro* data indicated that treatment of estrogen-sensitive (MCF-7) cells with cycloart-24-ene-3β,26-diol resulted in significant G_1-phase cell cycle arrest. The results suggested that cycloart-24-ene-3β,26-diol was able to halt uncontrolled cell cycle progression of the cancer cells. However, this effect was not seen in an estrogen insensitive (MDA-MB-231) cell. The finding showed a significant increased sub-G_0 and decrease in S and G_2/M phases in MDA-MB-231 cells suggests that the compound inhibited cell proliferation through cell death rather than cell cycle arrest.

Estrogens play an important role in the development of breast malignances in both pre- and postmenopausal women. However, the number of patients with estrogen-dependent breast cancer is greater and the occurrence of breast cancer increases with age. Since aromatase mediates the rate-limiting step in the estrogen synthesis, aromatase inhibitors have been used in the treatment of estrogen-dependent breast cancers particularly in postmenopausal women (Brodie & Njar, 2000). Subsequently, new inhibitors are constantly being developed and used as first or second line therapy for breast cancer patients. Additionally, several natural compounds found in a wide diversity of plants includes strawberry, mangosteen, grape, soybeans, and celery (Balunas et al., 2008) were recognized as an inhibitor of aromatase. Therefore, natural compounds derived from plants might have malignancy prevention effects and their intake may justify the low cancer rates noticed in some people consuming mainly plant-based diets.

Herein, we evaluated the aromatase inhibitory activity of cycloart-24-ene-3β,26-diol, positive control; ketoconazole and third generation aromatase inhibitors; letrozole and exemestane. As expected, third generation aromatase inhibitors inhibited the

aromatase activity efficiently at low concentration in nM range. The IC_{50} values of letrozole, exemestane and ketoconazole are in good agreement with those previously reported on the aromatase inhibitory activity (Geisler & Lønning, 2006; Stresser et., 2000). Nevertheless, cycloart-24-ene-3β,26-diol do possess aromatase inhibitory activity, which is interesting as a compound capable of both apoptotic death induction and estrogen production suppression.

Cycloart-24-ene-3β,26-diol has a steroidal structure. Thus, we deduced from the structure activity relationship that the compound is closely related to known steroidal aromatase inhibitors such as exemestane. As seen in Figure 2.3, exemestane has carbonyl groups attached to C-3 and C-17 position, which are important as hydrogen bond acceptors and involved in the inhibition of aromatase. Carbonyl groups in C-3 and C-17 position of exemestane freely bind to the aromatase and inhibit the estrogen biosynthesis. Moreover, approximately seventy percentage of compounds having both C-3 and C-17 carbonyl groups is known to produce maximum inhibitory activity against the enzyme. This statement is further supported by the fact that elimination of C-3 carbonyl group significantly decreases the inhibitory activity (Petkov et al., 2009). Moreover, Gnoatto and colleagues, 2008 has reported that pentacyclictritepenoids, ursolic acid and oleanolic acid which has C-3 hydroxyl group configuration inhibited the aromatase. Hence, it is anticipated that a functional group which has the ability to form hydrogen bonding between the steroid nucleus and the aromatase cavity would increase the inhibitory activity (Petkov et al., 2009). Therefore, from the literature review and the results, it can be hypothesized that the presence of a steroid nucleus and a free hydroxyl group at C-3 in cycloart-24-ene-3β,26-diol seem to be the key functional groups for aromatase inhibition. However, it is lacking the carbonyl group at C-17, which explains the observation that aromatase inhibition did not reach a nanomolar level in the results.

CHAPTER 6

CONCLUSION

The aim of this research was to determine whether the most bioactive compound from crude extract of *Aglaia exima* can be used as a potential chemotherapeutic agent in the treatment and prevention of human breast cancer. Crude extract and fractions were found to exhibit potent cytotoxic activity against breast cancer cell lines with the greatest cytotoxicity observed in fraction 19. Further bioassay-guided fractionation of the fraction led to isolation of cycloart-24-ene-3β,26-diol as the active compound. Results demonstrated that cycloart-24-ene-3β,26-diol induced cytotoxic effects in MCF-7 and MDA-MB-231 cells *in vitro* via decreasing the proliferation of cells. In contrast, cycloart-24-ene-3β,26-diol showed some selective cytotoxic effects against these cancer cell lines in comparison to the normal cell line, hTERT-HME1.

Investigation on the mechanism of action of cycloart-24-ene-3β,26-diol showed that cell death was mediated by apoptosis with the involvement of caspases. *In vitro* results show the externalization of phosphatidylserine at the cell membrane and increased caspase 3/7 activities in the cytosol, which are the hallmarks of apoptosis. Since apoptosis is related to the balance of cell growth and proliferation that are regulated mainly by cell cycle events, investigation on the effect of cycloart-24-ene-3β,26-diol on cell cycle arrest was perfomed. The results showed that the compound arrested MCF-7 cells in G_0/G_1 phase while it caused apoptotic cell death in MDA-MB-231 cells by increasing sub-G_0 phase. Since breast cancer cell growth and proliferation is also dependent on female hormones such as estrogens, the study looked at whether cycloart-24-ene-3β,26-diol can inhibit the aromatase, which is a rate-limiting enzyme in the production of estrogens in the body. The compound showed inhibitory potential towards aromatase. In summary, cycloart-24-ene-3β,26-diol is a potential lead compound to be developed as a chemotherapeutic agent against breast cancer.

6.1 Future study

One of the limitations of this study is that it cannot explain why cycloart-24-ene-3β,26-diol showed selective toxicity towards malignant breast cancer cells (MDA-MB-231) compared to non-malignant breast cancer cells (MCF-7). In addition, the study of apoptosis and cell cycle arrest was performed only in breast cancer cell lines and it is uncertain whether the compound will cause similar response in normal breast cells. However, the simple apotosis detection assays used in this study showed the potential of cycloart-24-ene-3β,26-diol as an antitumour agent. In order to understand the pharmacological action of the compound, further investigation on the molecular mechanism of cycloart-24-ene-3β,26-diol by characterizing targeted apoptotic proteins can be done. Western blotting analysis of apoptosis allows for the detection, localization and quantification of proteins involved in apoptotic signalling. This method is widely used in *in vitro* cell culture studies. By using western blot, it can identify specific proteins from a complex mixture of proteins extracted from cells. The specifc markers of apoptosis detectible by western blot include phospholyrated B-cell lymphoma 2 (Bcl-2) family members, activated fragments of caspases, activation of death receptor such as FAS and tumour necrosis factor (TNF) and cleaved poly (ADP-ribose) polymerase-1 (PARP-1). In particular, detection of the smaller active fragments of caspase-8 or caspase-9 is a useful method for differentiating between extrinsic and intrinsic activation of apoptosis. PARP-1 is involved in DNA repair in response to environmental stress. During apoptotic signaling, PARP-1 is cleaved by caspase-3 inactivating its DNA repair ability (Looi et al., 2013). Finally, from the western blot study a detailed apotosis pathways can be mapped for cycloart-24-ene-3β,26-diol.

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