CYTOTOXIC EFFECTS OF PINNATANE A EXTRACTED FROM *Walsura pinnata* (MELIACEAE) ON LIVER CANCER CELLS

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ABSTRACT

The main objective of the study is to identify the cytotoxic activity of pinnatane A, a glutinane type triterpene extracted from bark of *Walsura pinnata* (Meliaceae). MTT assay was used to analyse the selectivity of pinnatane A in inducing cell death in cancer and normal cells. Various assays were carried out to analyse the anti-cancer properties of pinnatane A, such as Live/Dead assay for microscopic visualization of cell death; cell cycle analysis using propidium iodide (PI) to identify the cell cycle arrest phase; annexin V- fluorescence isothiocyanate (FITC)/PI flow cytometry assay to measure percentage of cell populations at different stages of apoptosis and necrosis; and DNA fragmentation assay to verify the late stage of apoptosis. Preliminary MTT assay demonstrated dose-dependent effects of pinnatane A against twelve cancer cell lines and one normal cell line. Pinnatane A was found to induce prominent time-dependent cytotoxicity in both liver cancer cell lines, Hep3B and HepG2 with minimal effects on normal cell line, MRC-5. Live/Dead assay visualized the disruption of cell integrity leading to cell death. Cell cycle analysis indicated cell arrest at G_0/G_1 phase, and annexin V-FITC/PI dual staining demonstrated that pinnatane A triggered apoptosis in Hep3B cells and necrosis in HepG2 cells. DNA fragmentation assay visualized DNA laddering in Hep3B cells while DNA smearing was observed in HepG2 cells to confirm the induction of apoptosis and necrosis in the respective cell lines. In conclusion, pinnatane A, a natural product from the Malaysian flora exhibited potential pharmaceutical use for cancer treatment.

Keywords: anti-cancer, apoptosis, cell cycle arrest, necrosis, triterpene

KESAN-KESAN SITOTOKSIK PINNATANE A DIEKSTRAK DARIPADA *Walsura pinnata* (MELIACEAE) PADA SEL KANSER HATI

ABSTRAK

Objektif utama kajian ini adalah untuk mengenalpasti aktiviti sitotoksik pinnatane A, triterpena jenis glutinan yang diekstrak daripada kulit batang *Walsura pinnata* (Meliaceae). Esei MTT telah digunakan untuk menganalisa selektiviti pinnatane A dalam menginduksi kematian sel-sel kanser dan normal. Pelbagai esei telah dilakukan bagi menganalisa ciri-ciri anti-kanser pinnatane A, seperti esei "Live/Dead" untuk visualisasi mikroskopik sel yang mati, analisa kitaran sel menggunakan propidium iodida (PI) bagi mengenal pasti pemberhentian fasa kitaran sel, esei aliran sitometri annexin V-FITC/PI digunakan untuk mengira peratusan populasi sel dalam pelbagai peringkat apoptosis dan nekrosis, dan esei fragmentasi DNA digunakan untuk mengenalpasti peringkat akhir apoptosis. Kajian awal esei MTT menunjukkan kesan melalui kebergantungan dos pinnatane A terhadap dua belas jenis sel kanser dan satu jenis sel normal. Pinnatane A didapati menginduksi sitotoksik ketara yang bergantungan dengan masa bagi kedua-dua sel kanser hati Hep3B dan HepG2 dengan kesan minimal terhadap sel normal, MRC-5. Esei "Live/Dead" menunjukkan gangguan integriti membran yang membawa kepada kematian sel. Analisa kitaran sel menunjukkan sel mengalami pemberhentian kitaran sel pada fasa G_0/G_1, manakala penggunaan dwi perwarnaan annexin V-FITC/PI menunjukkan pinnatane A mengakibatkan sel Hep3B menjalani apoptosis manakala sel HepG2 menjalani nekrosis. Esei fragmentasi DNA menunjukkan pembelahan DNA dalam sel Hep3B manakala kaburan DNA dalam sel HepG2 membuktikan berlakunya apoptosis dan nekrosis dalam sel-sel tersebut. Kesimpulannya, pinnatane A, produk semula jadi flora Malaysia mempunyai potensi dalam farmaseutikal untuk terapi kanser.

Kata kunci: anti- kanser, apoptosis, pemberhentian kitaran sel, nekrosis, triterpina

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LIST OF SYMBOLS AND ABBREVIATIONS

A	:	Absorbance
α	:	Alpha
bp	:	Base pair
β	:	Beta
Ca^{2+}	:	Calcium ion
δ_C	:	Carbon chemical shift
CO_2	:	Carbon dioxide
^{13}C	:	Carbon-thirteen
cm	:	Centimetre
cm^2	:	Centimetre square
δ	:	Chemical shift
^oC	:	Degree Celsius
$CDCl_3$	:	Deuterated chloroform
D	:	Dilution factor
dd	:	Doublet of doublet
g	:	Gram
g/L	:	Gram per litre
g mol^{-1}	:	Gram per mole
Hz	:	Hertz
h	:	Hour
H_2O_2	:	Hydrogen peroxide
kb	:	Kilobase
kg	:	Kilogram
<	:	Less than
m/z	:	Mass per charge
MHz	:	Megahertz

m	:	Metre
µg/mL	:	Microgram/millilitre
µL	:	Microlitre
µm	:	Micrometre
µM	:	Micromolar
mA	:	Milliampere
mg/L	:	Milligram per litre
mL	:	Millilitre
mm	:	Millimetre
mM	:	Millimolar
min	:	Minutes
>	:	More than
m	:	Multiplet
nm	:	Nanometre
ppm	:	Part per million
%	:	Percentages
±	:	Plus-minus
P	:	Positive control
pH	:	Potential of hydrogen
^{1}H	:	Protium
δ_H	:	Proton chemical shift
P	:	*P*-value of significant statistical data
®	:	Registered trademark
rpm	:	Revolutions per minute
s	:	Singlet
×	:	Times
ND	:	Total number of dead cells
NV	:	Total number of viable cells

V	:	Total volume (mL)
V	:	Volt
v/v	:	Volume per volume
w/v	:	Weight per volume
1D-NMR	:	One dimensional nuclear magnetic resonance
2D-NMR	:	Two dimensional nuclear magnetic resonance
AIF	:	Apoptosis inducing factor
Akt	:	Protein kinase B
AM	:	Acetoxymethyl
ANOVA	:	Analysis of variance
ATCC	:	American Tissue Culture Collection
Bak	:	Bcl-2 antagonist or killer
Bax	:	Bcl-2 associated X protein
Bcl-2	:	B cell lymphoma 2
BD	:	Becton Dickenson
bFGF	:	Basic fibroblast growth factor
BH	:	Bcl-2 homology domain
Bim	:	Bcl-2 Interacting mediator
CAD	:	Caspase dependent DNAse
Caspase	:	Cysteine-aspartic proteases
CDK	:	Cyclin dependent kinase
COSY	:	Homonuclear correlation spectroscopy
DAMP	:	Damage-associate molecular pattern
DCM	:	Dichloromethane
DEPT	:	Distortioness enhancement by polarization transfer
DISC	:	Death-inducing signalling complex
DMEM	:	Dulbecco modified Eagle medium
DMSO	:	Dimethyl sulfoxide

DNA	:	Deoxyribonucleic acid
DNAse	:	Deoxyribonuclease
$DPPH^2$	:	2,2-diphenyl-1-picrylhydrazyl
EDTA	:	Ethylenediaminetetraacetic acid
EMEM	:	Minimum Essential Medium Eagle
EMT	:	Epithelial to mesenchymal transition
ERK	:	Extracellular regulated kinase
et al.	:	Et alia; and others
EtBr	:	Ethidium bromide
etc.	:	Et cetera; and so on
EthD-1	:	Ethidium homodimer
EtOAc	:	Ethyl acetate
FAS	:	Fatty acid synthase
FITC	:	Fluorescence isothiocyanate
G_0 phase	:	Quiescent state phase
G_1 phase	:	Gap 1 phase
G_2 phase	:	Gap 2 phase
HBV	:	Hepatitis B virus
HBx	:	Hepatitis B viral protein
HCC	:	Hepatocellular carcinoma
HCV	:	Hepatitis C virus
Hh	:	Hedgehog
HMBC	:	Heteronuclear multiple bond correlation
HMQC	:	Heteronuclear multiple-quantum correlation
HUVEC	:	Human umbilical vein endothelial cells
IC_{50}	:	Inhibition concentration for 50%
IFNγ	:	Interferon gamma
JAK	:	Janus protein tyrosine kinase

M	:	Marker
MEK	:	Mitogen activated protein kinase-extracellular regulated kinase
MEM-α	:	Minimum Essential Medium Alpha
MMP	:	Matrix metalloproteinases
mRNA	:	Messenger RNA
MTT	:	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MW	:	Molecular weight
NA	:	Not applicable
NAFLD	:	Non-alcoholic fatty liver disease
NASH	:	Non-alcoholic steatohepatitis
NC	:	Not calculated
NF-κB	:	Nuclear factor-kappa B
NMR	:	Nuclear magnetic resonance spectroscopy
PBS	:	Phosphate buffered solution
PCD	:	Programmed cell death
PI	:	Propidium iodide
PS	:	Phosphatidylserine
R point	:	Restriction point
RAF	:	Rat sarcoma–activated factor
RAS	:	Rat sarcoma
Rb	:	Retinoblastoma
RNA	:	Ribonucleic acid
RNAse A	:	Ribonuclease A
RPMI-1640	:	Roswell Park Memorial Institute 1640 medium
S phase	:	Synthetic phase
SD	:	Standard deviation
SHH	:	Sonic Hedgehog

SI	:	Selectivity index
SMAC	:	Second mitochondria-derived activator of caspases
SMI	:	Small molecule inhibitor
SOCS	:	Suppressors of cytokine signalling
STAT	:	Signal transducers and activator of transcription
TAE	:	Tris base, acetic acid and EDTA
Tbid	:	Truncated BID
TLC	:	Thin layer chromatography
TM	:	Trademark
TNF	:	Tumour necrosis factor
TRAD	:	TNFR-1 associated death domain protein
TUNEL	:	Terminal deoxynucleotidyl dUTp nick end labelling
USA	:	United State of America
UV	:	Ultra violet
VEGF	:	Vascular endothelial growth factor
XIAP	:	X-linked inhibitor of apoptosis proteins

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Cancer is an illness that are universally identified for the aggressive unrestrained proliferation of abnormal cells and the ability of these cells to invade and metastasise to local or distant tissue region to develop malignancies (Weinberg, 2007). This chromosomal disease is triggered by carcinogens or occurs spontaneously to result in altered level of proteins and chromosomes with oncogenic karyotypes (Duesberg et al., 2005; Nicholson & Duesberg, 2009).

According to the global cancer report by World Health Organization in 2014, approximately 14 million new cases with over 8 million cases death reported in 2012, placing cancer as one of the major contributor in morbidity and mortality worldwide (Stewart & Wild, 2016). In America, the death rates among cancer patients of liver and uterine cancers continuously increase yearly in rapid pace (Siegel et al., 2017). In Malaysia, liver cancer rank eighth in both sexes, sixth among males and about 65% cases detected at stage 4 for both sexes with the majority incidence rate among Chinese (Azizah et al., 2016).

Programmed cell death (PCD) is a cellular mechanism that encourages cell suicide mechanism. Apoptosis is one important mechanism of PCD in the human body that involves the balance of tissue homeostasis, pathogenesis and development (Fuchs & Steller, 2015). Apoptosis involves biochemical modification such as protein cleavage and linking, DNA damages and phagocytic recognition (Elmore, 2007). Apoptosis minimises the release of damage-associated molecular patterns (DAMPs) in addition to facilitate recognition of phagocytes to allow engulfment of apoptotic cells (Linkermann et al., 2014; Tait et al., 2014). The morphological attributes of cells undergoing apoptosis includes rounding up in shape, pseudopods retracted, diminished in cell volume,

chromatin condensation, nuclear fragmentation and subtle changes in ultra-structural modifications of cytoplasmic organelles (Kroemer et al., 2009).

Cells that undergo apoptosis are considered as efficient way for cell to die without causing traumatic effects such as inflammation and immunogenic death. However, deregulated expression of pro-apoptotic genes and proteins will result in signalling imbalance in cancer cells to allow the escape of death and encourage growth and proliferation (Hanahan & Weinberg, 2011). Furthermore, many cancer cells manage to establish a distinct mechanism of autocrine loop to synthesise growth factor that promote constitutive pathways activation for growth and proliferation in tumours (Witsch et al., 2010). Thus, potential anti-cancer drugs are needed to induce a proper apoptosis regulation to counter the escape death of cancer cells in order to treat patient efficiently with minimal negative side effects.

Necrosis is another type of cell death that eventually leads to cell membrane destruction and spillage of intracellular proteins to evoke a powerful inflammatory response. This is an attribute not preferable in cancer treatment due to adverse side effects (Amaravadi & Thompson, 2007; Proskuryakov & Gabai, 2010).

In cancer treatment industries, natural products as anti-cancer drug approximately dominate 62% of drugs approved worldwide since 1983. The inability of producing sufficient natural compounds as such drugs resulted in its modifications into semi-synthetic compounds and to reduce undesired problems such as over-cytotoxicity (Newman et al., 2000).

Triterpenes are one big class of natural compound from the plant kingdom with around 20,000 different types of triterpenoid identified. Wide-range variety of triterpenes

structure synthesised in plants from simple and ubiquitous linear isoprenoid substrate 2,3-oxidosqualene via oxidosqualene cycles (Thimmappa et al., 2014).

Pentacyclic triterpenes have been found to have enormous potential as anti-cancer agents. This include triterpenes types with a wide spectrum of multi-target agents such as lupeol, betulin, betulinic acid, oleanic and ursolic acid which are advantageous to tackle the tumour environment and induced the immune system (Laszczyk, 2009). Triterpenes behaved as pro-apoptotic, cell cycle arrest, anti-inflammatory, anti-angiogenesis by affecting different molecular targets in cellular regulatory pathways (Jagan & Chinthalapally, 2012). Importantly, triterpenes as anti-cancer agents have selective cytotoxicity towards cancer cells with relatively moderate cytotoxicity towards normal cells, for example, 200.00 μM oleanic acids only induced 26.2% cytotoxicity towards HaCat keratinocytes cell after treatment for 72 h (George et al., 2012).

The Meliaceae family are commonly abundant with triterpenes and have shown beneficial anti-cancer activities (Awang et al., 2012). *Walsura pinnata* from the Meliaceae family is a mahogany tree with a wide distribution area across South East Asia. *W. pinnata* bark obtained from Pahang, Malaysia have been reported to produce two major compound with basic structure of triterpenes, that is, betulonic acids and pinnatane A. Betulonic acid is a lupane type triterpenoid reported to have an anti-cancer properties towards leukaemia stem cells by inducing the intrinsic apoptosis pathway (Leong et al., 2017).

Pinnatane A (Figure 1.1) is a rare glutinane type triterpenoid recently discovered in 2009 and its biological activity is largely unknown. In a previous preliminary study, the hexane crude extract of *W. pinnata* containing pinnatane A showed cytotoxic activity against four human cancer cells, that is, HepG2 (liver), MCF-7 (breast), HSC-2 (oral) and

Ca Ski (cervical) (Yusoff, 2012). Thus, to explore the potential of pinnatane A as an anti-cancer agent, this study focused on the cytotoxic effects and mode of cell death induced.

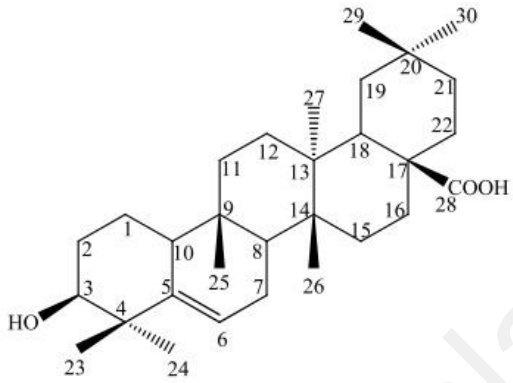


Figure 1.1: Chemical structure of 3*β*-hydroxy-5-glutinen-28-oic acid (pinnatane A) isolated from *Walsura pinnata*.

1.1 Hypothesis and Problem Statements of Study

Pinnatane A is hypothesized to be a potential anti-cancer agent due to its skeletal structure of triterpenes and the preliminary cytotoxic analysis (Yusoff, 2012). Thus, the research questions are:

i. Can treatment with pinnatane A induce high cytotoxic activity with various cancer cell lines?
ii. What are the suitable doses and durations of treatment for pinnatane A on the cancer cell lines?
iii. Does pinnatane A cause cell cycle arrest to the cancer cell lines?
iv. What is the type of cell death induced by pinnatane A in the cancer cell lines?

1.2 Objectives of Study

The objectives of this study are:

i. To investigate the concentration and time dependent cytotoxic effects of pinnatane A on prominent cancer cell lines.

ii. To identify the selectivity of pinnatane A inducing cytotoxic activity in cancer cell lines with minimal effects in normal cell line.

iii. To analyse the induction of cell cycle arrest on prominent cancer cell lines across different time of treatment with pinnatane A.

iv. To observe different mechanism of cell death, apoptosis and/or necrosis on prominent cancer cell lines across different duration of treatment with pinnatane A.

CHAPTER 2: LITERATURE REVIEWS

2.1 Cancer Overviews

Cancer is a disease that is initiated from uncontrolled growth and proliferation of abnormal cells and eventually capable to invade and metastasise to other tissues. Gene expression balance in cells are important to generate the optimum number of cells that our body need by controlling the amount and frequency of cell division. Uncontrolled proliferation of cells due to faulty gene expression leads to development of a lump of abnormal cells known as benign tumour. Faulty cells that invade and metastasise to different sites are called malignant tumour or cancer. The survival of tumour cells depends on regulation of oncogenes expression for survival and at the same time loss-of-function of the tumour suppressor genes to avoid death.

Oncogenes in cancer cells encourage continuous growth and proliferation. In normal cells, proto-oncogene is responsible in controlling the rate of proliferation to escape cell death but faulty proto-oncogene stimulates up regulation of oncogene expression that causes imbalance in growth. Tumour suppressor genes are able to detect mutation in DNA and control cell division from over proliferation by halting cell cycle and inducing cell death. In cancer cells, mutation in tumour suppressor genes encourage the cells to proliferate and escape PCD even though the cell had multiple genes mutation which leads to variation in defective gene expression that may result in further development of different diseases.

2.1.1 Incidence of Liver Cancer

Cancer is the second main cause of death worldwide and the foremost cause of fatal among women aged 40 to 79 years and amongst men aged 45 to 79 years (Fitzmaurice et al., 2017; Siegel et al., 2017). Liver cancer is prominent in less developed countries

especially in low socio-demographic index countries and is placed fourth in incidence and first for mortality (Ferlay et al., 2015; Fitzmaurice et al., 2017).

Globally liver cancer is placed fifth in men (7.5%) and ninth in women (3.4%) out of overall cancer cases in which 1 in 45 men and 1 in 113 women before age 79 years suffer from the disease (Ferlay et al., 2015; Fitzmaurice et al., 2017). In America, rates of fatality from liver cancer are three times higher in men compared to women (Siegel et al., 2017). Incidence rates for liver cancer continues to increase rapidly which arose about 3% in women and 4% in men annually (Siegel et al., 2017). Despite the success in lowering the overall incidence of cancer, death rates among liver cancer patients increased rapidly from year 2010 to 2014 for about 4% annually and this was found to be strongly associated with obesity (Siegel et al., 2017).

In Malaysia, liver cancer is placed at eighth place at 4% of all cancer cases from 2007 to 2011 and more common among men where the lifetime risks of liver cancer patient in Malaysia was 1 out of 144 for males compared to 1 out of 418 for females (Azizah et al., 2016). According to Malaysian Health Ministry report, most of the cases were detected at late stage with more than 45% for both sexes (Azizah et al., 2016).

2.1.1.1 Hepatocellular Carcinoma

Among primary liver cancers that occurred worldwide, hepatocellular carcinoma (HCC) accounts for 70% to 85% out of total cases (Jemal et al., 2010). Both incidence and mortality rates are higher in male subjects (Schutte et al., 2009). HCC is a complex disease associated with many risk factors that further developed to chronic diseases such as inflammation and cirrhosis (Schutte et al., 2009; Venook et al., 2010). Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), cirrhosis, alcoholic and non-alcoholic fatty liver disease are some identified key risk factors for HCC (El-Serag, 2011; Farazi & DePinho, 2006; Venook et al., 2010). Data from epidemiologic studies suggested that

global deviations in HCC incidence involves geographic differences, races and ethnic groups (El-Serag, 2011; Schutte et al., 2009; Venook et al., 2010). In short, HCC has a higher risk on elderly male, family history associated with HCC, alcohol or tobacco consumption and co-infected with virus such as HBV and HCV (El-Serag, 2011).

Liver cirrhosis results from different mechanisms of liver injury that lead to necroinflammation and fibrosis (Nishikawa & Osaki, 2015). Liver cirrhosis reduced the efficiency of anticancer therapy of liver and non-hepatic malignancies (Pinter et al., 2016). This is as a result of exhaustion of the regenerative capability of the liver due to an increase in fibrous tissue and destruction of liver cells that induces the advance of cancerous nodules (Sanyal et al., 2010).

In viral related cirrhosis, HBV/HCV co-infection increases the HCC risk up to 6-fold (Fattovich et al., 2004). Also the heavy consumption of alcohol increases the risk factor for HCC both independently and in combination with HCV infection and to a lesser extent with HBV infection (El-Serag, 2011). Alcohol induces chromosomal loses, oxidative stress, decreased retinoic acid level in the liver, altered DNA methylation and genetic susceptibility to promote hepato-carcinogenesis by increasing the synthesis of pro-inflammatory cytokines through monocyte activation and the level of circulating endotoxin and activating Küpffer cells with adverse effects against normal cells (Farazi & DePinho, 2006; Morgan et al., 2004).

HCC incidence particularly in developed countries is highly associate with obesity and diabetic because both diseases are implicated in the development of non-alcoholic steatohepatitis (NASH), the severest form of non-alcoholic fatty liver disease (NAFLD) that is believed to prime HCC development via progression of cirrhosis and fibrosis (Farazi & DePinho, 2006). Several factors are associated with the development of HCC via NASH and NAFLD, such as, insulin resistance, hyperinsulinemia, steatosis, oxidative

stress, imbalance adipokine/cytokine interplay, increased in TNF signalling pathways and alteration in cellular lipid metabolism (Baffy et al., 2012; Petta & Craxi, 2010).

Potentially curative treatments have become available depending on different stages of HCC that involve advance surgical instrumentation and the development of molecular target drugs. Treatment of HCC at early stage depends on the "Milan criteria" where a tumour with diameter less than 2 cm, is recommended to undergo surgical resection or radiofrequency ablation while nodules with diameter around 5 cm are recommended to undergo liver transplantation (Lin et al., 2012). Surgical resection and liver transplantation are generally considered as first-line choices since they result in prolonged survival and even excellent outcomes in patients with early disease (Ye et al., 2010). Intermediate stage HCC treatment regime is considered for patients that have Child-Pugh A and B liver function without cancer-related symptoms, such as, macrovascular invasion or extrahepatic spread. The recommended treatment involves trans-arterial chemoembolization (Lencioni et al., 2010; Lin et al., 2012). Final stage of HCC related with cancer symptoms, the recommended therapeutic option is through oral sorafenib treatment. However, the prognosis for patients with late stage HCC is still poor with high drug symptom burden and cost with minimal benefit (Lin et al., 2012; Sanoff et al., 2016).

2.1.1.2 Liver Cancer Cell Lines HepG2 and Hep3B

The liver cancer cell lines HepG2 and Hep3B are often used in the same experimental *in vitro* study. Even though both cells are categorized as HCC but pharmacological studies between the two cell lines resulted with significantly different outcomes. Differential gene expressions become the major contributor responsible for pharmacology differences between HepG2 and Hep3B cell lines. One such gene is the HBV genes. HepG2 cell line is HBV negative and non-tumorigenic while Hep3B cell line is HBV positive and

tumorigenic (Knowles et al., 1980), where 11 out of the 19 genes associated with HBV are differentially expressed especially HBx (Qiu et al., 2015).

There are three cellular signalling pathways during hepatocarcinogenesis known to respond differently between HepG2 and Hep3B cell lines which are, rat sarcoma/ rat sarcoma–activated factor/ mitogen activated protein kinase-extracellular regulated kinase/ extracellular regulated kinase (RAS/RAF/MEK/ERK) pathway; the Janus protein tyrosine kinase/signal transducers and activator of transcription (JAK/STAT) pathway and Hedgehog (Hh) pathway (Arzumanyan et al., 2012; Ng & Lee, 2011).

In RAS/RAF/MEK/ERK pathway, the content of RAS is much higher in Hep3B compared to HepG2 cell line, which influences the activities of inhibitor related with the pathways (Bose et al., 2011). A study involved in the inhibition of ERK phosphorylation using ERK specific inhibitors, PD098059 and U0126 have shown that the effects on HepG2 cell line were more profound compared to Hep3B cell line (Wiesenauer et al., 2004). However, the same study also revealed Hep3B cell line had higher degree of inhibition when the expression of the ERK protein was down regulated using antisense knockdown compared to HepG2 cell line.

The JAK/STAT pathway is another therapeutic drug target for the HepG2 and Hep3B cell lines. The inability of cells to inhibit constitutive activation of STAT stimulates the growth of tumours. Dysfunction of cellular STAT members, such as suppressors of cytokine signalling (SOCS), results in sensitivity of treatment and the outcome on the tumour cells, for example HepG2 cells are wild type SOCS-1 while Hep3B are null type SOCS-1. Treatment using AG490, a JAK2 specific inhibitor responsible for phosphorylation of STAT3, have different effects against HepG2 and Hep3B cell lines. There is an induction of G_0/G_1 phase arrest in HepG2 cells while marked apoptosis is seen in the Hep3B cells (Kusaba et al., 2007). The inhibitions of Src family members are also

capable in disrupting the constitutive activation of STAT3 in various malignancies. Treatment using specific Src inhibitor, KX2-391 and Dasatinib revealed that HepG2 cells were less sensitive towards the inhibitors compared to Hep3B cells (Lau et al., 2009).

A similar circumstance is observed with the Hh pathway, where aberrant activation correlated to growth, survival and adult stem cell maintenance can be interrupted during binding of Hedgehog proteins at the receptor, PTCH1. Unoccupied PTCH1 bind to SMO, a proto-oncogene and represses activation of transcription factors such as GL1 (Theunissen & de Sauvage, 2009). Therefore, manipulation of elements in the Hh pathway may cause different reaction between HepG2 and Hep3B cells due to the fact that level of Sonic Hedgehog (SHH) in HepG2 is lower than in Hep3B cells. Inhibitor for Hedgehog pathway, such as SHH neutralizing antibodies, KAAD-cyclopamines and SMO antagonist blocks growth of Hep3B cells and triggers apoptosis, which is in contrast with HepG2 cells that are resistant towards the treatment (Huang et al., 2006; Sicklick et al., 2006).

Gene expression of *p53* and its regulatory pathways were extensively and intensively studied to compare between wild type HepG2 and null type Hep3B cells. An anthraquinone from the aloe plant, aloe-emodin is seen to induce apoptosis via p53-dependent in HepG2 cells but via p21-dependent in Hep3B cells (Kuo et al., 2002). Fatty acid synthase (FAS) inhibitor, C75 triggers p53 overexpression and G_2 phase arrest in HepG2 cells but in contrast, C75 induces G_1 phase arrest in Hep3B cells and remained unchanged even after by overexpression of *p53* gene via p53 plasmid transfection (Gao et al., 2006). Thus, from both studies, p53 is not the critical reason behind the different pharmacological effects of therapeutic treatment against HepG2 and Hep3B cell lines.

Importantly the dissimilarities of the two cell lines may stem from the different ethnic origin of the cell source. First time reported in 1979, the cell lines were obtained from

biopsies on liver tumour of 15 years old male Caucasian Argentines in 1975 and 8 years old Black American in 1976. The cells were deposited under the names HepG2 and Hep3B cell lines, respectively (Aden et al., 1979). The morphology of the cells also contributed to the sensitivity towards drugs, where HepG2 cells remains as hepatocyte - like features while Hep3B cells with fibroblast structures and prevailing with expression of mesenchymal proteins, suggesting the process of epithelial to mesenchymal transition (EMT) (Slany et al., 2010).

A protein profile of over 3000 protein groups related to drug metabolizing enzymes were quantified in the liver cancer cell lines. There were significant differences in metabolism and toxicity which suggested the need to be cautious in designing hepatic drugs (Shi et al., 2018).

2.2 Cell Death

Tissue homeostasis is vital in living organisms to check function of organ, preserved building of tissue and maintained composition of cells in our body by controlling cell division and death. In healthy humans, cell turnover activities depend on organ compartment and their functions. The hematopoietic system, intestinal epithelium and skin have high rates of cell turn over due to continuous cells death and replacement whereas organs such as brain and cardiac muscle exhibit much lower rates due to inadequate regenerative potential in response to injury (Nalapareddy & Rudolph, 2010). Various endocrine and paracrine factors play key roles in physiological cell turnover either directly or altering the expression of genes that control proliferation or death (Medh & Thompson, 2000).

Apoptosis is one of the normal way for cells to die in order to maintain tissue homeostasis with hallmarks such as cytoplasmic shrinkage, nuclear condensation and retention of membrane and organelle integrity (Elmore, 2007; Kerr et al., 1972). Cancer

cells growth requires survival by escaping apoptosis that is influenced by unregulated oncogene expression and limited supply of growth factors, oxygen and nutrients (Evan & Littlewood, 1998; Harrington et al., 1994). Necrosis is another type of cell death which leads to karyolysis, pyknosis, karyorrhexis and cellular swelling (Kumar et al., 2015).

Identification of cell death type are based on several characteristics based on morphological appearance of lethal process, enzymological criteria, functional aspects and immunological attributes (Galluzzi et al., 2007). The type of cell death induced may vary depending on type of cancer and the treatment (Okada & Mak, 2004). The apoptotic and necrotic have cell unique morphological event, attributes and mechanisms as shown Table 2.1.

Table 2.1: Differential comparison of apoptosis and necrosis cell death. Table adapted from Tchounwou, 2008.

Attributes	Apoptosis	Necrosis
Histologic changes	Scattered individual cells throughout the affected tissue	Cells affected throughout whole fields
	Rounded bodies, often within a halo	Cell border loss with irregular fragmentation
	Chromatin condensation into “caps” or “crescent”, rounded nuclear bodies; preservation of nuclear envelope.	Irregular chromatin clumping, pyknosis, karyorhesis, and/or karyolysis; rupture of nuclear envelope
Gross changes	Insignificant or deteriorate without scarring	Utterly apparent with disorder of normal tissue structure and detail, scarring if long term
Morphological changes	Begins with shrinking of cytoplasm and condensation of nucleus	Begins with swelling of cytoplasm and mitochondria and
	Formation of membrane-bound apoptotic bodies; mitochondria become permeable	Disintegration/swelling of organelles
	Membrane blebbing, but remain in integrity; aggregation of chromatin at nuclear membrane	Loss of membrane integrity
	Ends product as smaller fragmented bodies	Ends with total cell lysis
Sequelae	Stromal collapse but retaining of intracellular enzymes within the apoptotic bodies prevent scarring	Discharge of intracellular enzymes into extracellular environment activate inflammation that causing scarring.
	Consumption by tissue macrophages or other cells	Ingress of neutrophils and further by macrophages

Table 2.1, continued.

Attributes	Apoptosis	Necrosis
Biochemical features	Strict regulated steps involving activation and enzymatic pathways such as release of cytochrome C, AIF from mitochondria into cytoplasm and caspase cascade activation	Ion homeostasis regulation imbalance
	Energy (ATP) dependent; active process	Zero energy; passive process
	Organized mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis) occur as prelytic event	Random digestion of DNA (smear of DNA after agarose gel electrophoresis) occur as postlytic event
	Phosphatidylserine translocation	Absent in phosphatidylserine translocation

2.2.1 Apoptosis

Apoptosis is a mechanism with highly regulated complex progression that involves several orders to execute a proper cell death (Figure 2.1). Apoptosis regulate cell turnover replacing old cells with newly divide cell through proliferation and it happen in the human body everyday (Medh & Thompson, 2000). Apoptosis is also important in defence by eliminating infected cells and to assist immune system to disseminate intracellular pathogens and induce immunosuppression against pathogens (Hatice, 2015). Damaged DNA or presence of DNA damaging genotoxic agent may activate apoptotic membrane death receptor or endogenous mitochondrial damage pathways (Kaina, 2003).

Some of the hallmarks of apoptosis are membrane blebbing, cell shrinkage, chromatin condensation, fragmentation into apoptotic bodies and perforated mitochondrial (Tchounwou & Centeno, 2008). Apoptotic cells experience major endonuclease DNA fragmentation leaving a laddering pattern in electrophoresis (Gavrieli et al., 1992;

Tchounwou & Centeno, 2008). Another important trait of apoptosis is the exposure of phosphatidylserine (PS) leaflet on the surface of apoptosis cells during early stage of apoptosis (Fadok et al., 1992). The apoptotic cell membrane remains intact and does not release their cellular constituents into surrounding interstitial tissue and are rapidly phagocytosed by macrophages or neighbouring normal cells to evade chemostatic signals that might ultimately induced inflammation (Elmore, 2007).

Apoptosis takes place in both human and animal tissues and integrating the concept of apoptosis between them helps to understand the mechanism of apoptosis. The studies on hermaphrodite nematode *Caenorhabditis elegans* model aids investigators to understand on the molecular machinery of apoptosis (Diamantis et al., 2008) that revealed three central genes named as *ced-3*, *ced-4* and *ced-9*. The *Ced-3* gene has substantial homology to cysteine-aspartic proteases (caspase), caspases-3 and caspases-8 (Fernandes-Alnemri et al., 1994; Miura et al., 1993; Yuan et al., 1993). *Ced-4* gene has a sequence homology to the cytosolic protein Apaf-1 in mammals, where the protein participates in caspase-9 activation in general apoptosis pathway and involves in apoptosome formation (Cecconi et al., 1998; Zou et al., 1997). *Ced-9* gene required for cell protection from PCD and has an analogous sequence and structural amino acid protein to the mammalian proto-oncogene *bcl-2* gene (Hengartner & Horvitz, 1994). The proteins that encoded by *ced-3*, *ced-4* and *ced-9* show significant similarity with proteins involves in vertebrates PCD, suggesting that the molecular cell death pathway has been conserved between nematodes and vertebrates.

In mammalian cells, there are two major apoptotic signalling pathways, known as intrinsic and extrinsic pathways. Intrinsic signal pathway involves activation of Bcl-2 associated X protein (BAX) or Bcl-2 antagonist or killer (BAK) expression due to intracellular stimuli such as DNA damage or endoplasmic reticulum stress to cause

permeabilisation of mitochondrial outer membrane and released of cytochrome c to activate downstream caspases pathway and trigger apoptosis (Elmore, 2007; Tait & Green, 2010).

The extrinsic pathway involves the binding of extracellular death receptor ligand to the members of TNF receptor gene superfamily (Locksley et al., 2001). Ligation of these receptors leads to the establishment of the death-inducing signalling complex (DISC), made up of death receptors, adaptor proteins and pro-caspase-8. Pro-caspase 8 then dimerizes, resulting in its cleavage and activation (Jiang et al., 2011). The cleaved caspase 8 activates a cascade reaction of downstream caspases, eventually activating the effector caspase-3 and caspase-7 (Locksley et al., 2001). In Fas apoptosis signal pathway, BID cleaved by caspase-8 resulted in a truncated BID (tBID), which then translocate into mitochondria and causing a crosstalk between extrinsic and intrinsic pathways (Li et al., 1998; Tait & Green, 2010).

X-linked inhibitor of apoptosis proteins (XIAPs) is an important protein for the cells to avoid cell death and retain survival by controlling caspases activity and caspase-activating platform formation by binding directly on caspases but could be counter by second mitochondria-derived activator of caspases (SMAC), that is released from the mitochondria (Berthelet & Dubrez, 2013; Tait & Green, 2010).

Bcl-2 family act as a crucial protein family related to critical life-death decision by regulating the anti-apoptotic and pro-apoptotic protein members (Tsujimoto, 1998). The Bcl-2 family members are categorized based on the regions of sequence and the structural homology. Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains designates BH1, BH2, BH3 and BH4, which correspond to α-helical segments. The anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w have sequence homology in four domains, whereas the pro-apoptotic proteins Bax and Bad have sequence homology in

three domains, contributing to their role in regulating apoptosis (Gross et al., 1999). Pro-apoptosis sub-members such as Bid and Bim share homologous domain only within BH3 domains and act as effectors of canonical mitochondrial apoptosis (Lomonosova & Chinnadurai, 2008; Tsujimoto, 1998).

Apoptosis is mainly achieved by activated form of caspases, which are originated from the inactive zymogens (Donepudi & Grutter, 2002). All caspases have a high degree of specificity in cleaving proteins following aspartic acid residues and a recognition sequence at least four amino acids N-terminal to the cleave site (Grütter, 2000). There are two major groups of caspases involve with apoptosis process which are initiator and effector caspases. Initiator caspases which include caspase-2, -8, -9, -10 and -12, have long pro-domains with noticeable homotypic protein-protein interaction motifs, such as the death effector domain or the caspase recruitment domain that contribute to the transduction of innumerable signals into proteolytic activity (Oliver & Vallette, 2005). The initiator caspases activate effector caspases including caspase-3, -6 and -7 and activated effector caspases are accountable for the proteolytic cleavage of a wide range of cellular targets eventually leading to cell death (Shi, 2002).

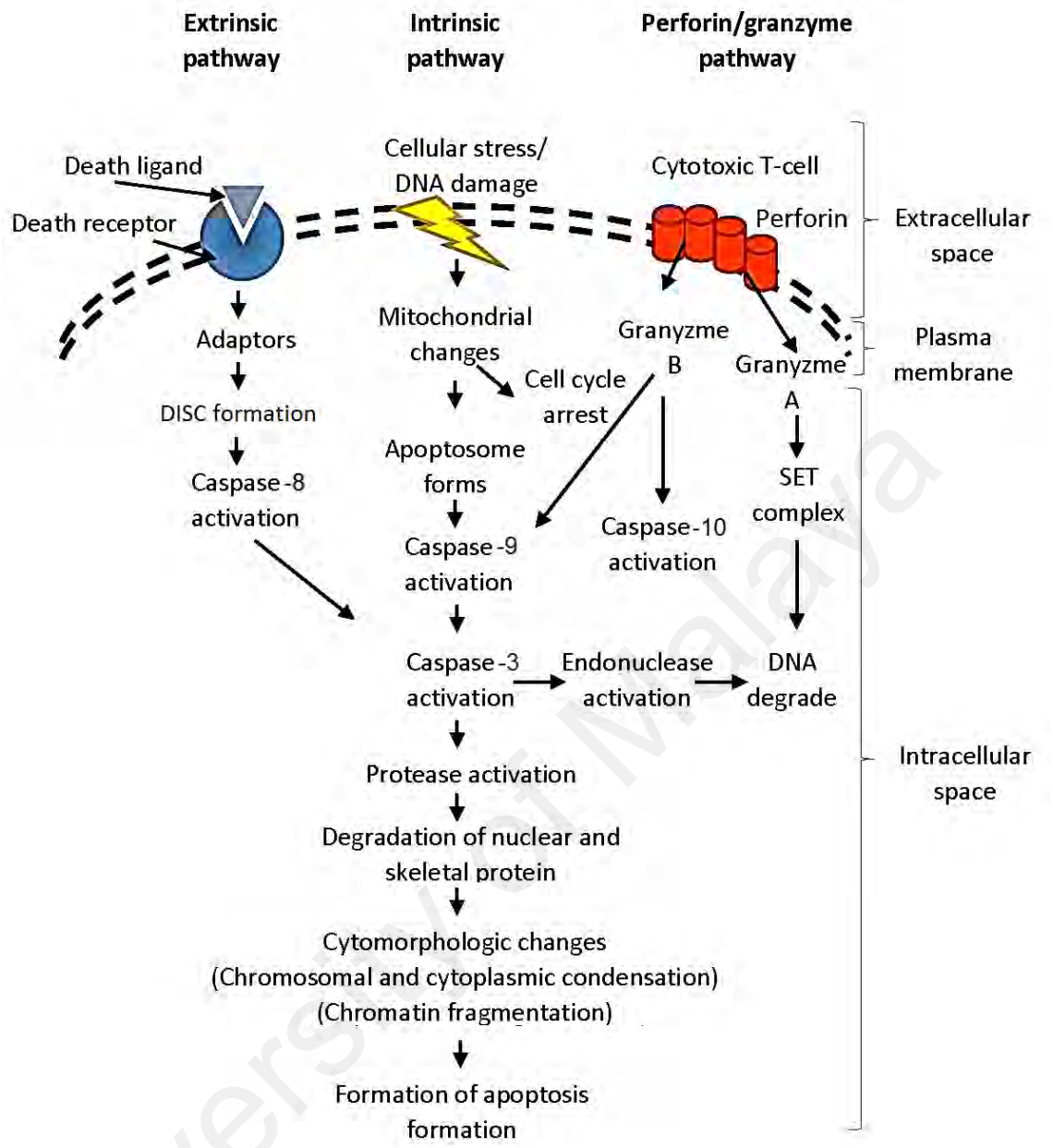


Figure 2.1: Schematic illustration of apoptotic events of extrinsic, intrinsic and perforin/granzyme pathway. The extrinsic and intrinsic apoptotic pathways and perforin/granzyme pathways execute energy-dependent cascade of molecular events through activation by specific signals. Each pathway involves different initiator caspases (caspase-8, -9 or -10), which eventually activate the executioner caspase (caspase-3). Additionally, granzyme A is also capable of caspase-independent apoptosis. The execution of apoptosis results in apoptotic cytomorphological features such as cell shrinkage, chromatin condensation, cytoplasmic blebbing and formation of apoptotic bodies. Figure adapted from Morad, 2010.

2.2.2 Necrosis

Necrosis is a passive form of unavoidable cell death due to exposure of extreme environment condition, adverse stimuli or involvement of deleterious mutation in genetic materials (Syntichaki & Tavernarakis, 2002). Necrotic cells undergo swelling due to influx of water, disruption of cell membrane integrity, cisternae dilation and deformation of specialized surface feature that allow the release of intercellular components to cause inflammation and recruitment of phagocytes to the affected site (Tchounwou & Centeno, 2008).

2.3 Deoxyribonucleic Acid

Deoxyribonucleic acid (DNA) is the genetic materials that serve as genetic information repository in cells and is composed of macromolecules with basic structure of nucleotides consisting of deoxyribose phosphate linked to either adenine-thymine or cytosine-guanine (Hardin et al., 2012). In central dogma envisioned by Watson in 1965, DNA genetic information conveyed to ribonucleic acid (RNA) through transcription and then serve as protein templates via translation process (Thieffry & Sarkar, 1998). Combination of DNA damage and faulty DNA repair are responsible for development of uncontrolled cell proliferation due to failure to halt or stall cell cycle before the defective DNA is passed on to daughter cells (Griffiths et al., 2006).

2.3.1 DNA in Cell Death

In order to restrict the survival of faulty cells, the entire genome will be degraded to make sure a proper cell death is executed. However, the DNA degradation mechanism in apoptosis and necrosis differs in their metabolic pathway and outcomes. Humoral and cellular system act as vital components in cleansing and keeping in check the homeostasis of extracellular DNA that are being released by dead cells to avoid induction of disease (Pisetsky & Fairhurst, 2007).

In apoptosis, DNA degradation involves two different methods of cutting the DNA strand, leading to the formation of high molecular weight fragment (> 50,000 bp) and nucleosome sized (180 to 200 bp). In contrast to apoptosis, DNA degradation in necrotic cells occurs as post cell death, where proteases and endonucleases destroy the histones in chromatin and expose the entire length of DNA to the nucleases leaving a random length of DNA fragments.(Duvall & Wyllie, 1986). Under gel electrophoresis, apoptotic cells leave a laddering effect while necrotic cells induced a smearing pattern.

2.4 Cell Cycle

Cell cycle involves a complex metabolic machinery in order to sustain growth and proliferation of cells, organismal development, regulation of DNA damage repair, tissue hyperplasia and diseases (Schafer, 1998). Regulatory proteins associated with cell cycle are responsible to ensure proper replication and segregation of chromosomes to form new daughter cells (Vermeulen et al., 2003). Cell cycle is driven by a number of positive and negative regulatory phosphorylation and dephosphorylation events, involving protein kinases, protein phosphatases, cyclin, cyclin-dependent kinases and cyclin-dependent inhibitors, that ultimately influence the activity of transcription factors (Dictor et al., 1999). The cell cycle consists of an initial growth phase (G_1), DNA replication (S), a gap phase (G_2) and mitosis (M). The cell may further differentiate or enter the resting state (G_0) (Figure 2.2).

During G_1 phase, stream of cues from metabolic, stress and environmental conditions influence cell decision and fate whether to proceed, rest or exit the cell cycle (Massague, 2004). Cells experience S phase when DNA undergoes entire genome replication without any errors in a timely fashion (Takeda & Dutta, 2005). Cells in G_1 phase are diploid (2N) and during S phase, the DNA content doubled, forming aneuploidy from 2N to 4N (Cooper, 2007). In order to make sure the DNA content is properly replicated, cells further

undergo G_2 phase before entering mitosis state where genomic stability are controlled and repaired. Any defects in DNA such as damaged or not properly replicated, the cells will be inhibited from proliferation and entering mitosis phase (Stark & Taylor, 2006). M phase involves five phases namely prophase, prometaphase, metaphase, anaphase and telophase that occurs in a strict sequential order, while cytokinesis begins in anaphase and continues through telophase (Alberts et al., 2007). Differentiated cells with specific purposes will remain quiescence where the cells enter G_0 phase, an extension of G_1 phase (Behl & Ziegler, 2014).

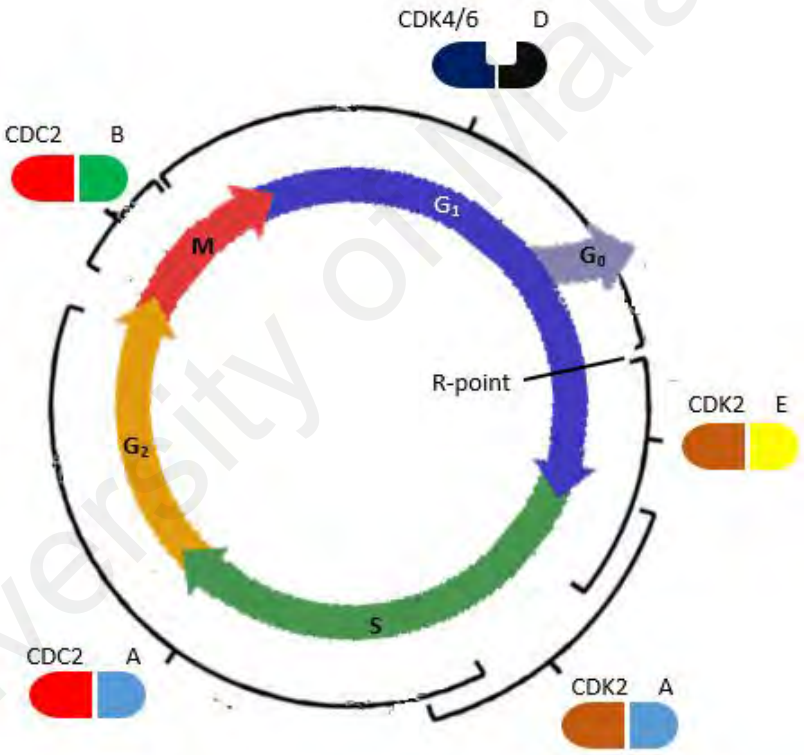


Figure 2.2: Cell cycle phases and the respective pair of cyclin-CDK complex that responsible in allow or prevent further division of cells. Cell cycle checkpoints act as quality control to ensure each step is properly executed before advancing to the next phase. Mammalian cell cycle is divided into four phases, which are G_1, S (DNA replication phase), G_2, and M (mitosis). G_0 phase denotes the withdrawal from active cell cycle. Figure adapted from Weinberg, 2007.

2.4.1 Cell Cycle Checkpoint and Restriction Point

Cells pass into a resting state or G_0 phase at each successive cell cycle under appropriate conditions such as high density of cells population, lack of growth factors, nutrients deficiency and serum starvation (Baserga, 1994; Behl & Ziegler, 2014; Cooper, 1998). Cells transition from G_0 to G_1 phase mainly depends primarily on extracellular growth factors by stimulating activation of an upstream positive feedback loop to promote growth and proliferation (Pfeuty et al., 2008). Cells with insufficient cues from growth factors tend to revert back to G_0 phase state.

The important regulatory (R) point is called as restriction point where the checkpoint is more sensitive in withdrawal of growth factors before the R point will prevents the onset of S phase and protecting the cell by lowering metabolic activity (Blagosklonny & Pardee, 2002; Pardee, 1974). When cell passes through R-point, the cell commits to the cell cycle events and proceeds to DNA synthesis without any cues from mitogen (Calzone, 2013). Defects in regulating R-point encourage avoidance of quiescent state or cell deaths as seen in cancer cells (Campisi et al., 1982).

2.4.2 Cell Cycle in Cancer Development

Most important hallmark of cancer associated with faulty cell cycle machinery is unscheduled cell proliferation in either stem or progenitor cells, modulated by over activation of CDKs (Malumbres & Barbacid, 2009). Amplification or mutation of genes encoding cyclins or CDK or deletion, mutation or hypermethylation of genes encoding endogenous inhibitors of CDKs, ultimately results in deregulated CDK activity and loss of cell cycle control (Johnson & Shapiro, 2010). Cancer arises from a complex and unique genes mutation that drive oncogenes to develop small-molecule inhibitors (SMIs), targeting cell-surface receptors and signalling molecules (Williams & Stoeber, 2012). The

level of expression of pro-survival or inhibitor of proliferation is important to determine the onset of cell division especially before R point (Figure 2.3).

Each type of cancer cells have different response and sensitivity towards chemotherapeutic agents due to different capacity in DNA repairs, cell cycle checkpoints and apoptosis control (Sakaue-Sawano et al., 2011).

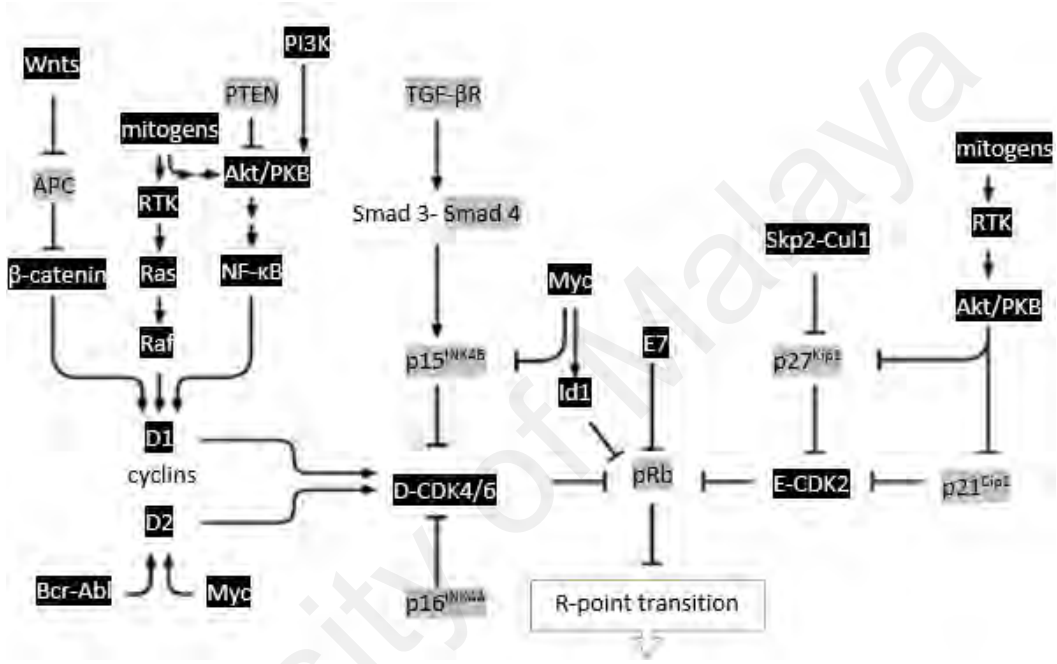


Figure 2.3: Perturbation of the R-point transition in human tumours. The decision to advance through the R-point transition can be perturbed in a variety of ways in human tumours. Tumour cells prone to shows hyperactivity of the activation of the agents that favour (black) transition or inactivation of the agents that responsible to block (grey) this advance. Figure adapted from Weinberg, 2007.

2.5 Meliaceae Family

The Meliaceae family is universally known as Mahogany trees and mostly with scented wood. About 650 species recorded from 50 different genera are classified under the Meliaceae family from a vast variety of habitat including humid, subtropical and intermittently warm temperate region of both hemispheres (Hua et al., 2008). A majority of the species are hard trees or shrubs with a few being herbaceous or mangroves plants (Busia, 2016; Powell, 1998). The distribution region is mainly in the tropical regions of

Africa, Asia, Australia and South America (Nelson, 2010). In Malaysia, there are more than 50 species from 16 different genera and mostly found in the rainforest. They are locally known by the natives as sentol family (Corner, 1988).

Different parts of the tree in the Meliaceae family create a variety of functions and economic values. The seed extracts forms an oil that are used in the production of soap (Hua et al., 2008). High demand in the production of furniture and agriculture implements encourage the exploitation of the high quality timber from Meliaceae trees (Kannan et al., 2016). Interestingly, the Meliaceae trees have numerous functions in biological activities such as anti-bacteria, anti-cancer, anti-feedant, anti-inflammatory, anti-oxidant, anti-plasmodia, anti-protozoa and treatment for certain disease such as asthma, diarrhoea, dyslipidemia, hyperglycaemia, skin diseases and chronic fever (Yadav et al., 2015).

2.5.1 The Genus *Walsura*

The genus *Walsura* (Meliaceae) is categorized under the Trichilieae tribe with up to 40 species and are predominantly sub-canopy trees (Holmes, 1956). The species lack exudate of any kind in slash bark and woods with height of up to 40 m (Ridley, 1952). The bark morphology is sparse but seems to have an important taxonomic value in the furniture industries. Figure 2.4 shows the botanical classification of genus *Walsura*.

Kingdom: Plantae (plants)
Subkingdom: Tracheobionta (vascular)
Superdivision: Spermatophyta (seed)
Division: Magnoliophyta (flowering)
Class: Magnoliopsida (dicotyledon)
Order: Sapindales (stamens inserted on a disk)
Family: Meliaceae (mahogany tree)
Genus: *Walsura*

Figure 2.4: Botanical classification of genus *Walsura*.

2.5.1.1 *Walsura pinnata*

Walsura is one of the diverse genus under Meliaceae with over 40 species across South East Asia such as China, India, Vietnam, Indonesia, Malaysia and New Guinea (Peng & Mabberley, 2008). *Walsura pinnata* is locally known as "lantupak mata kucing", The evergreen tree species distribution centred on subtropical climate and lowland forest region and hills up to 600 m above sea level with height up to 40 m tall (Soepadmo et al., 2002). The name *W. pinnata* refers to Latin word "*pinnatus"* meaning feather, due to its feather-like leaves. *W. pinnata* has the largest variation range within species of leaf, leaflet size and to some extent, leaflet number (Figure 2.5).

Figure 2.5: Morphological diagram of *Walsura pinnata*. A: tree; B: leaves and flowers; C: bark; D: fruit. Figures adapted from Mahdzir et al. 2017.

2.6 Natural Products as Anti-cancer Agents

Natural products have been in the spotlight for the pharmaceutical industry as a source for therapeutic agents in anti-cancer treatment. Many natural and synthetically modified natural products have been successfully developed for clinical use to treat human diseases in almost all therapeutics areas. Anti-cancer drugs derived from natural and semi-synthetic products represent approximately 17.1 % and 38.22 % respectively of all anti-cancer drugs discovered from 1981 to 2014 (Newman & Cragg, 2016). Some examples of plant derived compounds that are currently important in treatment of cancers are arglabin, paclitaxel, masoprocol, solamargine, vinblastine and vincristine (Tan et al., 2006).

Initiative in identifying natural products initially through identification of plant by a botanist or ethnobotanist, ethnopharmacologist or plant ecologist. Next, plant extracts are being screened biologically by a phytochemist to identify the potential therapeutic activity and isolation of the active compound. Finally molecular biology studies also known as pharmacognosy are required to reveal the mode of action and relevant molecular targets (Fridlender et al., 2015).

2.6.1 Bioactivities of Chemical Constituents from *Walsura* Species

The *Walsura* species are wildly dispersed and diverge in species with limited phytochemical information reported. To date, only eight species under genus *Walsura* were reported for its phytochemicals properties, namely, *W. chrysogyne*, *W. cochinchinensis*, *W. piscidia*, *W. robusta*, *W. trichostemon*, *W. trifoliate W. tubulata* and *W. yunnanensis. Walsura* species were known for its biological properties from various type of chemical constituents, such as, apotirucallane, tirucallane, cycloartane and damarane and were shown to exhibit anti-bacterial, anti-cancer, anti-feeding and anti-oxidant properties.

Research studies have been carried out to evaluate anti-cancer activity of limonoids purified from bark extract of *Walsura chrysogyne*. Walsogynes B – D and G were reported to have moderate cytotoxic activity against human cancer cell lines, HepG2 liver, A549 lung, HL-60 myeloid leukaemia and MCF-7 breast cancer with a range of 7.70 – 42.40 μM (Nugroho et al., 2013).

A total of 41 compounds were successfully purified from *W. yunnanensis* but approximately half of them were tested for its biological activities as cytotoxic agent against cancer cell lines. Two new compounds, yunnanolide A and 11β-hydroxyisowalsuranolide extracted from the leaves and twigs of *W. yunnanensis* were found to induce significant potent cytotoxic activities against A549, HL-60, MCF-7, SMMC-7721 endocervical and SW480 colon cancer cell lines from 2.20 to 4.20 μM (Ji et al., 2014). Another compound, 11β-hydroxycedrelone also extracted from the same parts exhibited moderate inhibitory activity against the HL-60 cell line with IC_{50} value of 8.90 μM (Jiang, 2015).

In the analysis of *W. robusta*, two new limonoids compounds, walsuroid D and E extracted from leaves and twigs were discovered to have potent cytotoxic activity against several human cancer line. The IC_{50} values ranges from 2.70 to 4.50 μM against A549, HL-60, MCF-7, SMMC-7721 and SW480 cancer cell lines (Ji et al., 2016).

Phytochemical investigations of *W. trifoliata* founded through the practise as traditional remedy were reported to have cytotoxicity activity. Two novel apotirucallane type triterpenoids, piscidinone A and B extracted from leaves showed moderate cytotoxic activity against various cancer and normal cell lines namely A549 lung, HT-29 colon, PC-3 prostate, B-16 mouse skin cancer, IEC-6 rat normal small intestine, L6 rat skeletal muscle and with IC_{50} values ranging from 14.33 to 50.63 μg/mL (Rao et al., 2012).

2.6.2 Anti-cancer Properties of Triterpenoids

Tritepenes are terpenoids with 30 carbon biosynthesized mainly from oxidosquelene through cyclisation reaction involving oxidosqualene cyclase catalysation that result in 100 different unique triterpenes skeleton found in nature, predominantly from plants (Kushiro & Ebizuka, 2010). Triterpenes are compounds known for its versatility in biological activity including anti-cancer, anti-inflammatory and anti-oxidant (Mokhtari et al., 2015; Saleem, 2009). Its potential as anti-cancer agents are highlighted by its ability to target multiple cancer types with minimal effects on normal cell lines (Zuco et al., 2002). The anti-cancer attributes of triterpenoids involve anti-inflammatory mechanism, inhibition of proliferation, induction of apoptosis, and prevention of invasion, metastasis and angiogenesis (Zhang et al., 2014b).

The ability of triterpenoids to interrupt the transcription factor, nuclear factor-kappa B (NF-κB) that mediates inflammation induced by the innate immune system makes it an important anti-inflammatory agent. Avivins, a saponin triterpenoid compound inhibits the NF-κB signalling and up regulated the NF-E2-related factor 2 pathway to help in stress adaptation by redox regulation (Haridas et al., 2001; Haridas et al., 2004). Through interruption of NF-κB signalling, other pro-inflammatory gene expression would likewise be affected. Ursolic acid was found to down regulate the production of inducible nitric oxide synthase and inducible cyclooxygenase-2 via inhibition of NF-κB in mouse macrophages cell line, RAW246.7 (Suh et al., 1998).

Previous report suggested that the anti-inflammatory ability of triterpenoid are associated with other biological activity such as cell death. Oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid suppressed the production of inflammatory cytokines that are able to induce apoptosis at high dose against various cancer cell line (Suh et al., 1999).

Triterpenoids are able to mediate anti-proliferation activity by inducing cell cycle arrest through regulatory elements such as cyclins, CDKs and growth factors. Boswellic acid was reported to induce G_1 phase arrest in colon cancer cells via p21-dependent pathways and inhibit proliferation by decreasing cyclin D1 and E, CDK 2 and 4 and involved in phosphorylation of Rb (Liu et al., 2006). Ganedorial F induced G_1 phase arrest cell in HepG2 cells through up regulation of CDK inhibitor P16 through MAPK/EKR signalling pathway (Chang et al., 2006). This proves that triterpenoids able to inhibit tumour proliferation in different phases through multiple regulatory pathways.

Triterpenoids also capable to induce cell arrest and death at the same time. Oleanic acid induces apoptosis and G_2/M phase arrest through down regulation of cyclin B/Cdc2 activity in HepG2 cell line (Wang et al., 2013). However, the effects of each triterpenoid differ depending on the type of cell line. Betulinic acid triggers G_0/G_1 phase arrest and apoptosis in IGROV-1 ovarian cancer cells but fails to regulate cell arrest in Me665/2/21 melanoma cells (Zuco et al., 2002).

Another important characteristic to consider in developing therapeutic drugs is the ability to induce apoptosis. Triterpenoids are able to induce up regulation of pro-apoptotic Bax and down regulation of anti-apoptotic Bcl-2 protein expressions. Oleanic acid induced apoptosis in NB4 acute promyelocytic leukemia cell line by increasing Bax and decreasing Bcl-2 expression level which leads to the activation of caspase-3 and -9 that are involved with the apoptosis machinery (Li et al., 2013). Triterpenoids induces apoptosis through multiple pathways depending on type of cancer cells. Induction of apoptosis in HepG2 liver cancer cells by ursolic acid is via inhibition of PI3K/Akt/survivin pathway (Tang et al., 2009) while cucurbitacin B inhibites JAKT/STAT signalling pathway both in *in vitro* and *in vivo* models in Mia PaCa-2, PL45, and PANC-1 pancreatic cancer cell lines (Thoennissen et al., 2009). Apoptosis also occurs

via elevation of intracellular Ca^{2+} levels and reactive oxygen species that leads to dysregulation of p53 proteins (Lee et al., 2002; Wang et al., 2013). Study also showed cucurbitacin B ability to induce apoptosis through p53-independent pathway through up regulation of $p21^{CIP1/WAF1}$ in PANC-1 pancreatic cancer cells (Zhang et al., 2010). The multi-target properties of triterpenoids allow broader prospect for cancer treatment improvement especially in term of effectiveness and efficiency.

Triterpenoids capable to control the spread of cancer by inhibiting cell invasion and metastasis. Cucurbitacin 1 induces programmed cell death via anoikis by down regulation of STAT3 gene expression to reduce the invasiveness of HK1 and CNE-2 nasopharyngeal carcinoma cell lines (Lui et al., 2009).

Metastatic elements that provide anchorage and interaction with cells at the new site could be manipulated to disturb the establishment of cancer cells at the new site. Matrix metalloproteinases (MMP) plays an important role in controlling the remodelling of extracellular matrix that are largely responsible in cancer cell invasion and triterpenoids have been proven to influence the expression of MMP in cancer cells. Ursolic acid are able to halt the invasion and migration of HO-8910PM ovarian carcinoma cells by decreasing the expression of the MMP-2 and MMP-9 proteins and mRNAs (Yu et al., 2010). Ursolic acid and oleanic acid are proven to have anti-invasion and anti-metastatic ability as seen by inhibition of adhesion, migration and cathepsin B secretion in highly metastatic PGCL3 lung cancer cells (Huang et al., 2003). Ginsenoside Rh2 successfully down regulated the expression of intercellular junction adhesion molecules in transplanted tumour mice. The adhesion molecules function in cell-cell contact formation, cell migration and mitotic spindle formation (Ebnet, 2017; Wang et al., 2008). The action by ginsenoside Rh2 inhibited cell growth, angiogenesis and lymphangiogenesis of tumour

cells. Thus, prevention of cancer cells from invading and metastasize help to prevent cancer progression that will lead to end stage of cancer.

Angiogenesis is a crucial stage for a cancer cells to survive at a new site by development of blood vessels growth from the existing vasculature for source of nutrients and metabolites supply. Triterpenoids able to inhibit the activation of vascular endothelial growth factor (VEGF) by halting the angiogenesis stage of cancer cells. Ursolic acid was found to suppress the human umbilical vein endothelial cells (HUVECs) growth through inhibition of angiogenic factors including VEGF-A and basic fibroblast growth factor (bFGF) involving multiple pathways such as, SHH, STAT3, protein kinase B (Akt) and p70S6k pathways (Lin et al., 2013). Hence, suggesting that triterpenoids can induces anti-angiogenesis in numerous pathways.

CHAPTER 3: MATERIALS AND METHODS

3.1 Extraction of Pinnatane A

3.1.1 Plant Material

The plant materials of *Walsura pinnata* were collected within the range of 243 km from Kuala Lipis, Pahang to Gua Musang, Kelantan in Peninsular of Malaysia. The sample was identified by botanist, Mr. Tarelli O. and deposited at the Herbarium of the Chemistry Department, Faculty of Science, University of Malaya, Malaysia with the voucher specimen KL 4571.

3.1.2 Reagents and Chemicals

Dichloromethane (DCM), ethyl acetate (EtOAc), n-hexane, deuterated chloroform ($CDCl_3$) (deuteration degree minimum 99.80%), silica gel (Merck 60, 230 - 400 mesh) and thin layer chromatography (TLC) (Merck 60 GF254) were purchased from Merck, Germany. Each solvent was of analytical grade and distilled prior to use for extraction, isolation and characterization of pinnatane A.

3.1.3 Extraction and Purification of Pinnatane A from *Walsura pinnata*

Dried ground bark of *Walsura pinnata* (2.30 kg) was defatted using simple maceration method with n-hexane for 72 h. The solvent was periodically stirred throughout the duration to increase the extraction yield. After 72 h, the solvent was filtered using a filter paper and was concentrated using rotary vacuum evaporator at 40 ^{o}C to obtain dark brown gummy crude extract (25.00 g). The hexane crude extract (10.00 g) was then subjected to open column chromatography with solvent system of n-hexane:ethyl acetate and ethyl acetate:methanol by gradually increasing the polarity of mobile phase to yield 24 major fractions. The 24th fraction was eluted with n-hexane:ethyl acetate producing 2.00 g of product that was further purified by column chromatography using n-hexane:acetone

(94:4) to yield white powder crystal of pinnatane A (5.00 mg). Structural elucidation was established through several spectroscopic methods: 1D (^{1}H, ^{13}C and DEPT) NMR, 2D (COSY, HMQC, HMBC) NMR, etc.

3.1.4 Preparation of Stock and Working Solution

The pinnatane A (10.00 mg) obtained was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 50.00 mM and stored at -20 ^{o}C to prevent decomposition or crystallization. The stock solution was further diluted in DMSO to prepare 10.00 mM working solution and kept at 4 ^{o}C for use in cell treatment.

3.2 Cell Lines

3.2.1 Reagents

Dulbecco Modified Eagle Medium (DMEM) supplemented with 4.50 g/L glucose and 300.00 mg/L L-glutamine was purchased from Hyclone Laboratories, USA. Roswell Park Memorial Institute 1640 medium (RPMI 1640) was purchased from Thermo Fisher Scientific, USA. Minimum Essential Medium Alpha (MEM-α), ribonuclease A (RNase A) and propidium iodide (PI) were purchased from Nacalai Tesque, Japan. Minimum Essential Medium Eagle (EMEM), foetal bovine serum (FBS) and sodium pyruvate were purchased from Sigma-Aldrich, USA. Ethanol was purchased from Merck, Germany. DMSO was purchased from Fisher Scientific, USA. Cisplatin and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent were purchased from EMD Chemicals, USA. ApoTarget™ LIVE/DEAD® Viability/Cytotoxicity kit for mammalian cells and Quick Apoptotic DNA Ladder Detection Kit was purchased from Invitrogen, USA. FITC Annexin V Apoptosis Detection Kit I BD Pharmingen™ was purchased from Becton, Dickinson & Co, USA. RedSafe Nucleic Acid Staining solution was purchased from iNtRON Biotechnology, South Korea.

3.2.2 Cell Culture

A total of twelve cancer and one normal human cell lines were used in this study, which are summarized in Table 3.1. For routine maintenance: HeLa S3, HepG2 and SiHa cell lines were cultured in DMEM; Hep3B and MRC-5 cell lines were cultured in EMEM supplemented with 1% (v/v) sodium pyruvate; SK-LU-1 cell line was cultured in MEM-α; while A549, DU 145, EJ-28, MCF-7, MDA-MB-231, PC-3 and RT-112 were cultured in RPMI-1640. All culture media were supplemented with 10% (v/v) FBS to encourage the growth of cells. Cells were allowed to grow as monolayer at 37 ^{o}C in incubator with 5% CO_2 and 95% humidified atmosphere air.

3.2.3 Cell Sub-culture

All cell lines were split regularly every three days or when the cell culture are around 80% surface confluency. Spent media was discarded and cells were washed using 4.0 mL of 1× PBS to remove the residual serum that could inactivate trypsinization of cells. The spent 1× PBS was discarded and cells were exposed with 1.0 mL of 0.25% (v/v) Trypsin-EDTA solution in incubator at 37 ^{o}C for 8 mins to detach cells from the surface of T-25cm^2 culture flask. In order to avoid further trypsinization of cells, 3.0 mL of media supplemented with 10% (v/v) was added and the flask was tapped gently to detach the cells completely. Cell culture suspension was then transferred into 15.0 mL centrifuge tube and centrifuged at 500× g for 8 mins. The supernatant was discarded and the cell pellet was re-suspended in fresh complete media and four flasks were seeded for further usage.

Table 3.1: Summary of cell lines sources and culture media used for cultivation.

Cell line name	Histotype of human cell	Source	Culture media[a]
MRC-5	Normal lung fibroblast	ATCC, USA	EMEM[b]
MCF-7	Breast adenocarcinoma	ATCC, USA	RPMI-1640
MDA-MB-231	Breast adenocarcinoma	AseaCyte, Malaysia	RPMI-1640
EJ-28	Bladder carcinoma	ATCC, USA	RPMI-1640
RT-112	Bladder carcinoma	ATCC, USA	RPMI-1640
HeLa S3	Cervical adenocarcinoma	ATCC, USA	DMEM
SiHa	Cervical carcinoma	ATCC, USA	DMEM
Hep3B	Hepatocellular carcinoma	ATCC, USA	EMEM[b]
HepG2	Hepatocellular carcinoma	ATCC, USA	DMEM
A549	Lung adenocarcinoma	ATCC, USA	RPMI-1640
SK-LU-1	Lung adenocarcinoma	AseaCyte, Malaysia	MEM-α
DU 145	Prostate carcinoma	ATCC, USA	RPMI-1640
PC-3	Prostate adenocarcinoma	ATCC, USA	RPMI-1640

[a]Culture media supplemented with 10% (v/v) FBS.

[b]Culture media supplemented with 1% (v/v) sodium pyruvate.

3.2.4 Cell Counting

Cell count from cell suspension was via dye exclusion viability assay using haemocytometer by calculating number of cells present in a specific area of population (Strober, 2001). Monolayer cells were trypsinized, centrifuged and re-suspended in fresh complete media prior to cell counting. Approximately 20.0 µL of cell suspension was mixed with 20.0 µL of 0.04% (v/v) trypan blue (Merck, Germany) dye solution. The cells suspension was mixed for 3 mins, and 10.0 µL of the solution was transferred to a haemocytometer and spread evenly in the counting chamber and the cells were counted using the inverted fluorescence microscope (Nikon, Japan) under 10× magnification. The mean number of cells was calculated by counting the unstained live cells in each of the four-square grid corners of haemocytometer. Haemocytometer contains four square grid with each square grid represent 10^{-4} mL volume and the concentration of cells suspension was calculated using Equation 3.1 with dilution factor of two. In order to calculate the viability of cells, both viable (unstained) and dead (stained) cells were counted independently and calculated using Equation 3.2. The desired concentration of cell suspension was calculated every time before cell plating. The haemocytometer and glass cover slip used in cell counting were immediately rinsed and cleaned with 70% ethanol (Merck, Germany) between samples and sterilized prior to being kept to avoid contamination.

$$\text{Concentration of cells} = \frac{N}{V} \times D \quad (3.1)$$

$$\text{Cells viability (\%)} = \frac{NV}{NV + ND} \times 100\% \quad (3.2)$$

Where; N = Mean number of cells

V = Total volume (mL)

D = Dilution factor

NV = Total number of viable cells

ND = Total number of dead cells

3.3 MTT Viability Assay

Cytotoxicity activity of pinnatane A against selected cell lines was conducted according to the metabolic reaction with MTT reagent solution in viable cells (Mosmann, 1983). MTT reagent (5.00 mg/mL) solution was prepared by diluting 50.0 mg of MTT with 10.0 mL of 1× PBS solution and vortexed until completely dissolved. MTT was stored in 15.0 mL centrifuge tube and covered with aluminium foil at 4 ^{o}C for long storage and room temperature (25 ^{o}C) for working solution. Cytotoxic effect of pinnatane A on cell lines were determined by measuring the uptake and metabolism of MTT reagent by viable cells. Briefly, cancer and normal cell lines were washed with 1× PBS and trypsinized using 0.25% (v/v) trypsin (SAFC Bioscience, USA)/EDTA (Gibco, USA) solution. Detached cells were collected and centrifuged at 1,500 rpm for 5 mins and resuspended in a fresh media. Determination of viable cells count was done using trypan blue solution according to Equation 3.1 to obtain desired cell concentration of 1.00×10^5 cells/mL. A total of 1.0×10^4 cells was seeded with 100.00 µL/well in 96 flat bottom well plates and incubated in incubator for 24 h at 37 ^{o}C to allow cells to attach at bottom of the well. Pinnatane A treatments were at various concentrations (0, 10, 20, 40, 60, 80, 100 µM) and incubated for 24 h. Wells treated with DMSO were prepared as solvent control to ensure the cytotoxic effects were not influenced by the solvent used. Standard curve

was plotted for each independent replicate during MTT assay to estimate the cell numbers based on the optical density of known number of cells. Standard curves for quantification was constructed by measuring optical density readings from cell concentrations seeded in descending order (10,000 cells, 5,000 cells, 2,500 cells, 1,250 cells and 0 cells/well) through serial dilution. After treatment with pinnatane A or DMSO, 20.0 µL of 5.00 mg/mL MTT solution (Calbiochem, USA) was added into each well and allowed to mix gently before incubated in dark for 1 h at 37 ^{o}C to allow formation of the purple formazan precipitate. The spent media were carefully aspirated, and 200.0 µL of DMSO (Merck, Germany) was added to each well to dissolve the purple formazan precipitate. The plate was left on shaker in dark condition for 15 mins to allow an evenly dilution and colour stabilization of the formazan solution. The absorbance value of each wells were measured at 570 nm wavelength with a 650 nm reference wavelength using Tecan Sunrise microtiter plate reader (Tecan, Switzerland) and data tabulation quantification was expressed using Magellan version 6.3 (Tecan, Switzerland) software. All steps were repeated for Hep3B, HepG2 and MRC-5 cell lines with treatment times at 12, 48 and 72 h to observe the time-dependent effects of pinnatane A. The 50% inhibitory concentration (IC_{50}) values were determined from dose response curve of cell viability at half population. The selectivity index (SI) values of pinnatane A were calculated using Equation 3.3 by comparing IC_{50} values of normal cells and cancer cells. All treatments were carried out with three independent replicates, and each independent replicate is the average of three technical replicates.

$$SI = \frac{IC_{50} \text{ of normal cells}}{IC_{50} \text{ of cancer cells}} \tag{3.3}$$

3.4 Live/Dead assay

Observation on cell viability and morphology after treatment with pinnatane A was done using LIVE/DEAD® Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen, USA) according to manufacturer's protocol (Slepian et al., 1996). Cells with concentration of 1.00×10^5 cells/mL were cultured as monolayer cells on sterile coverslip glass in 6 wells culture plate overnight. After the cells were completely adherence on glass coverslip, cells were treated with pinnatane A with IC_{50} value at 12 h to avoid false result where cells detach from coverslip due to long time exposure of treatment. Untreated and DMSO-treated cells were controls to ensure the cytotoxic activity was induced by pinnatane A treatment only. After 12 h of treatment period, spent media were aspirated and cells were washed with 1× PBS twice to remove the esterases from serum that would restrict the reaction hydrolization of the AM fluorescent dye in cells. Dual fluorescent dye staining was used using calcein-AM (Invitrogen, USA) which emits green fluorescent upon cleavage by intracellular esterases in viable cells and EthD-1 (Invitrogen, USA) which emits red fluorescent upon entering dying or dead cells to bind to nucleic acids. Prior use, 150.0 µL of each dye from 2.00 µM calcein-AM and 4.00 µM EthD-1 were mixed and spread evenly on the surface of glass coverslip adhered with treated cells and incubated in dark room at room temperature for 45 mins to allow complete interaction of fluorescent dyes with cells. Following incubation, excess dyes were removed and cells were washed with 1× PBS. Carefully, coverslip was mounted on microscopes slides and sealed with nailed polish to avoid evaporation and allowed to dry at room temperature for 10 mins. Visualization of samples were done using a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Japan) with excitation and emission wavelengths of both fluorescents set at 494/517 nm for calcein-AM and 528/617 nm for EthD-1 respectively. Visualization was done at 100× magnification with separate pass filter for viewing of both stains and

percentages of viable cells were calculated as in Equation 3.2 from four random field of view.

3.5 Cell Cycle Assay

3.5.1 Fixation of Cells

Liver cancer cells were grown as monolayer in a 6 wells plate. Cells were left untreated and treated with pinnatane A at IC_{50} value of 48 h and incubated at 12, 24 and 48 h at temperature 37 ^{o}C in 5% CO_2 and 95% humidified air. Following treatment, both treated and untreated cells were collected through trypsinization, centrifuged and diluted with ice-cold 1×PBS to a final concentration of approximately 1.00×10^6 cells/mL and aliquots of 1.0 mL of cell suspension in a 15.0 mL centrifuge tube. Fixation of cells were done by adding 3.0 mL of ice cold 70% (v/v) ethanol (Merck, Germany) in a drop wise manner while vortexed in order to prevent formation of cells clump. Fixed cells were stored at 4 ^{o}C before stained with PI solution (Larsen et al., 1986).

3.5.2 Cell Cycle Analysis

Fixed cells were centrifuged at 2,000 rpm for 5 mins and the supernatant was discarded. The cell pellet was washed using 5.0 mL ice cold 1× PBS twice to eliminate any traces of ethanol and filtered using cell strainers (Fisher scientific, USA) to allow isolation of single cells from flocculent cells that may result in false result during flow cytometry. Filtered cells were then centrifuged and the supernatant were discarded. The cell pellets were re-suspended in 1.0 mL of ice-cold PI solution (50.00 µg/mL) with 50.0 µL of RNase A solution (10.00 mg/mL) and incubated in the dark at 37 ^{o}C for 40 mins (Larsen et al., 1986). After incubation, samples were stored in the dark at 4 ^{o}C until analysed by flow cytometry. Cell cycle analyses were carried out using MACSQuant® Analyzer 10 flow cytometry with MACSQuantify™ version 2.10 software (Miltenyi

Biotec, Germany). All treatments were carried out with three independent replicates, and each independent replicate is the average of three technical replicates.

3.5.3 Data Analysis using Macsquantify™ Software

Total population of 1.0×10^4 cells were counted for each sample and analysed using FSC-parameter and SSC-parameter based on gated populations. Cells detected were expressed in a histogram as total percentages of cells from four different cell cycle phases (sub-G_0/G_1, G_0/G_1, S and G_2/M phase) depending on DNA content of cells expressed by PI signals. PI signals were detected by blue laser (488 nm wavelength) and channel B3 with filter 655 – 730 nm.

3.6 Annexin V-FITC/ PI Flow Cytometry Assay

3.6.1 Annexin V-FITC/PI Staining

Liver cancer cells were treated with pinnatane A at IC_{50} value at 48 h and incubated for 12, 24 and 48 h. After treatment, spent media containing detached cells were aspirated and kept in 15.0 mL centrifuge tube. Adherent cells were washed with 1× PBS, trypsinized and neutralized using media supplemented with 10% (v/v) FBS and centrifuged at 1,500 rpm for 5 mins. Supernatants were discarded and cell pellet were washed twice with 1.0 mL ice cold 1× PBS and centrifuged at 1,500 rpm for 5 mins to remove traces of media and serum. The cell pellet was then re-suspended with 1× Binding Buffer (BD Biosciences, USA) at concentration of 1.00×10^6 cells/mL. For each sample, 1.0×10^5 cells in 100.0 µL of PBS was transferred into a 2.5 mL centrifuge tube and stained with 5.0 µL of PI (BD Biosciences, USA) and 5.0 µL of annexin V-FITC solution (BD Biosciences, USA) (Lakshmanan & Batra, 2013). The centrifuge tubes were then kept in the dark at room temperature for 15 mins. After incubation, 400.0 µL of 1× binding buffer (BD Biosciences, USA) was added to each tube and the samples were kept in ice with dark environment before analysed using flow cytometry within 1 h to avoid false

result. All treatments were carried out with three independent replicates, and each independent replicate is the average of three technical replicates.

3.6.2 Data Analysis using Macsquantify™ Software

For each sample, 1.0×10^4 cells were analysed through flow cytometry. The cells were gated using the pre-set FSC- and SSC gating parameters for each cell line to exclude non-cell particles from the analysis. The gated cells were then presented on a four quadrants scatter plot, with annexin V-FITC signal intensity on the x-axis and PI on the y-axis. Annexin V-FITC and PI signals were detected using blue laser (488 nm wavelength) and detection of annexin V-FITC solely using B1 channel (525/50 nm wavelength filter) while PI solely using B3 channel (655 – 730 nm wavelength filter). Single dye signal controls of each sample were used to quantitatively analyse cells that undergo early apoptosis (annexin V-FITC-stained cells), late apoptosis (annexin V-FITC- and PI stained cells) and necrosis (PI-stained cells).

3.7 DNA Fragmentation Assay

3.7.1 DNA Extraction

Confirmation of apoptotic cells mediated by pinnatane A was assessed using ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen Corp., USA) by evaluating DNA fragmentation in dead cells (Park & Patek, 1998). Hep3B and HepG2 cells were grown as monolayer in 6-well plate until 80% of confluency before being treated with pinnatane A at IC_{50} value at 48 h. Hep3B treated with cisplatin was used as positive control. DMSO-treated Hep3B and HepgG2 cells were used as solvent control. After treatment, both adherent and detached cells were collected and washed with 1× PBS and trypsinization. Cell pellets were obtained and lysed with 35.0 µL of TE Lysis Buffer (Invitrogen Corp., USA). After pipetting the crude lysate, 5.0 µL of enzyme A solution (Invitrogen Corp., USA) was added to each sample and gently vortexed before incubated

at 37 ^{o}C for 10 mins. Following the incubation, 5.0 µL of enzyme B solution (Invitrogen Corp., USA) was added to each sample and further incubated at 50 ^{o}C water bath for 10 mins. The cell lysate was then added with 5.0 µL ammonium acetate solution (Invitrogen Corp., USA) and 100.0 µL of -20 ^{o}C absolute ethanol (Merck, USA) to each sample. All samples were vortexed and kept at -20 ^{o}C for 15 mins to allow formation of DNA precipitation. The samples were centrifuged at 12,000 rpm for 10 mins and the supernatant were discarded to collect the DNA precipitate. DNA pellets were washed using 0.5 mL of 70% cold ethanol (Merck, USA) and re-centrifuged at 12,000 rpm for 10 mins. Supernatants were discarded and DNA pellets were allowed to dry at room temperature for 10 mins. DNA pellets were re-suspended in 30.0 µL in DNA Suspension Buffer (Invitrogen Corp., USA) and pipetted carefully until the DNA was fully dissolved. DNA suspension were kept in -20 ^{o}C freezer until prior used for gel electrophoresis. All treatments were carried out with three independent replicates.

3.7.2 Quantification of DNA

Assessment and quantification of total purified DNA extracted from samples were conducted using Nanodrop Spectrophotometer Model 2000 (Thermo Fisher Scientific, USA). A total of 5.0 µL DNA sample was diluted to make a final volume of 500.0 µL with 495.0 µL distilled water with the ratio of 1:99, forming DNA samples with dilution factor of 100. Distilled water was used as the blank control. All samples were measured by pipetting 1.0 µL of each sample on Nanodrop Spectrophotometer. DNA quantification was carried out by measuring the absorbance values at 260 nm with conversion factor of 50. DNA purity was assessed by examining the absorbance ratio of A_{260}/A_{280} for protein contamination while A_{260}/A_{230} for solvent contamination (Desjardins & Conklin, 2010). All samples were normalized with DNA Suspension Buffer (Invitrogen Corp., USA) to be uniformed in sample concentration before being evaluated with gel electrophoresis.

3.7.3 Agarose Gel Electrophoresis

DNA visualization of pinnatane A-treated cells for both Hep3B and HepG2 were done using agarose gel electrophoresis (Lee et al., 2012). Gel preparation was done by adding 0.45 g of agarose powder with low electro-endoosmosis (Fisher Scientific, USA) into 30.0 mL of 1× Tris base, acetic acid and EDTA (TAE) buffer, prepared from 10× TAE buffer (Fisher Scientific, USA) to make 1.5% (w/v) of agarose gel. The mixture was heated in microwave (Panasonic, Malaysia) for 3 mins. The mixture was let to cool until lukewarm and added with 1.5 µL RedSafe Nucleic Acid Staining solution (iNtRON Biotechnology, South Korea) and stirred well. The mixture were poured into 5.5 × 12.0 cm^2 gel casting tray (Baygen, China) and attached with gel comb with 8-wells and 1.5 cm spacing between wells (BayGen, China). The gel mixture was allowed to solidify at room temperature for 30 mins. The gel comb was removed and the solidified gel with casting tray was immersed in 1× TAE buffer solution inside electrophoresis chamber (Baygen, China) with the wells positioned near to the negative charge electrode (cathode). A total of 10.0 µL of DNA sample was mixed with 2.0 µL of 6× orange DNA loading dye (Fermentas, USA). DNA molecular weight ladder marker used was 5.0 µL of O'Gene Ruler^{TM} 1 kb DNA Ladder (Fermentas, USA). The electrophoresis was set at 120 V and 80 mA and run for 1 h to allow the DNA to separate and form bands. The gel was viewed and photographed using UV transillumination at 307 nm.

3.8 Statistical Analysis

Results were expressed as mean values ± standard deviation (SD). All data collected from experiments were performed in three replicates and analysed using the one-way analysis of variance (ANOVA) at significance level of $P < 0.05$ and indicated by *.

CHAPTER 4: RESULTS

4.1 Characterization of Pinnatane A

Pinnatane A is chemically recognized as 3*β*-hydroxy-5-glutinen-28-oic acid. It was extracted and purified and further analysed and characterized by Mr. Azrul Mohd Mahdzir from IFM Nat-Pro laboratory, Department of Chemistry, University of Malaya through several analyses via ultraviolet visible (UV), infrared (IR), nuclear magnetic resonance spectroscopy (NMR), correlation and heteronuclear multi-bond correlation spectroscopy.

4.1.1 Ultraviolet-visible and Infrared Spectroscopy

Pinnatane A was obtained as white crystals with a melting point of 306 ^{o}C. It was assigned with a molecular formula of $C_{30}H_{48}O_3$ as deduced from its negative HRESIMS ($[M-H]^-$, m/z 456.3603; calcd. for $C_{30}H_{47}O_3$; 455.3525), consistent with seven degrees of unsaturation. The IR spectrum exhibits a broad band for hydroxyl (3396 cm^{-1}) and olefinic (2933 cm^{-1}) structures. Both strong and medium intensity bands for carbonyl (1695 cm^{-1}) and C-O stretch (1180 cm^{-1}) were observed.

4.1.2 Nuclear Magnetic Resonance

The ^{1}H NMR spectrum (Table 4.1 and Figure 4.1) showed seven singlets of methyl groups resonated in close proximity at δ 0.82 (Me-25), 0.93 (Me-26), 0.94 (Me-30), 0.98 (Me-29), 1.04 (Me-23), 1.04 (Me-27) and 1.14 (Me-24). An olefinic methine with oxymethine signals were observed at δ 5.64 (*d*, *J* = 5.8 Hz, H-6) and at δ 3.47 (*dd, J* = 3.2, 2.3 Hz) respectively. The olefinic methine was observed as doublet signal because the coupling constant is too small compared to the width of the doublet peak. Therefore, it was observed as doublet signal rather than doublet of doublet (*dd*) signal.

Table 4.1: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of pinnatane A in $CDCl_3$.

Position	δ_H (ppm), *J* (Hz)	δ_C (ppm)
1	1.45, *m* 1.50, *m*	18.3
2	1.67, *m* 1.85, *m*	27.8
3	3.47, *dd* (3.2, 2.3)	76.3
4	-	40.8
5	-	141.6
6	5.64, *d* (5.8)	121.7
7	1.77, *m* 1.95, *m*	23.5
8	1.52, *m*	47.7
9	-	35.1
10	2.00, *m*	49.4
11	1.36, *m* 1.53	34.5
12	1.39, *m* 1.47, *m*	30.9
13	-	38.7
14	-	37.2
15	1.21, *m* 1.25, *m*	32.5
16	1.50, *m*	35.8
17	-	44.7
18	2.43, *dd* (13.2, 4.5)	37.8
19	1.17, *m* 1.31, *m*	34.9
20	-	28.5
21	1.47, *m*	32.8
22	1.67, *m* 2.29, *dd* (14.9, 9.7)	29.4
23	1.04, *s*	28.9
24	1.14, *s*	25.4
25	0.82, *s*	15.6
26	0.93, *s*	20.3
27	1.04, *s*	18.2
28	-	182.6
29	0.98, *s*	34.3
30	0.94, *s*	29.8

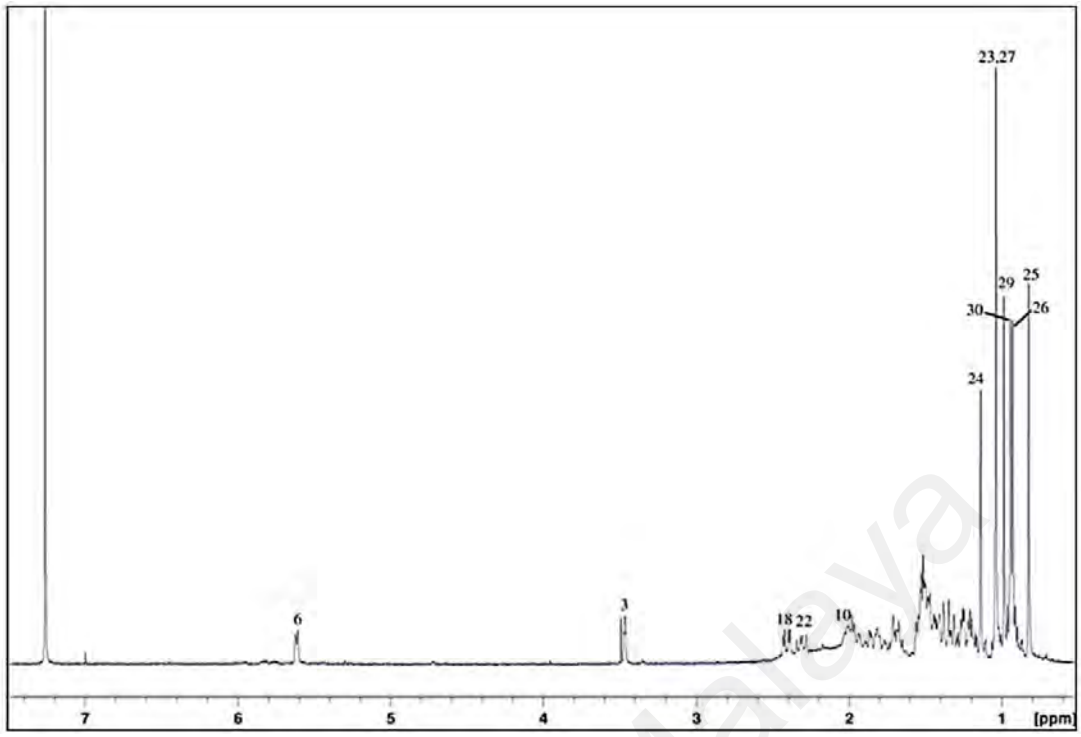


Figure 4.1: ^{1}H-NMR spectrum of pinnatane A.

The ^{13}C NMR (Figure 4.2), DEPT (Figure 4.3) and HSQC experiment of pinnatane A revealed the presence of thirty carbon signals, among which seven methyls resonated at δ 15.6 (C-25), 18.2 (C-27), 20.3 (C-26), 25.4 (C-24), 28.9 (C-23), 28.9 (C-30) and 34.3 (C-29). A hydroxyl, olefinic moiety (methine 36 and quaternary carbon) and carbonyl carbon were identified resonated at δ 76.3 (C-3), δ 121.7 (C-6), δ 141.6 (C-5) and δ 182.6 (C-28).

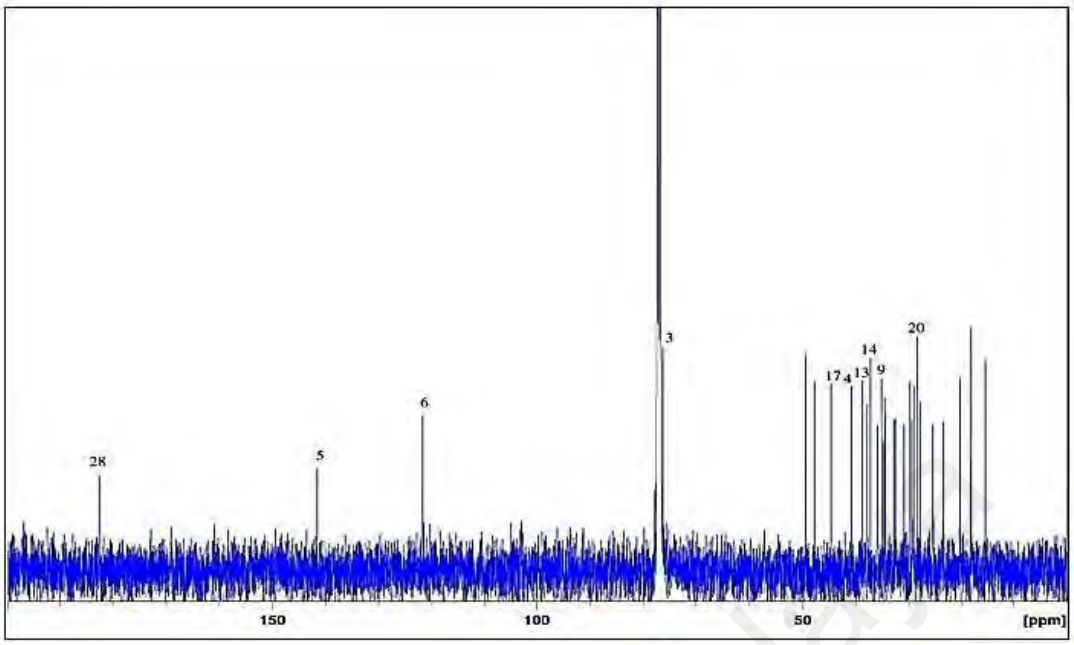


Figure 4.2: ^{13}C (150 MHz) NMR spectrum of pinnatane A.

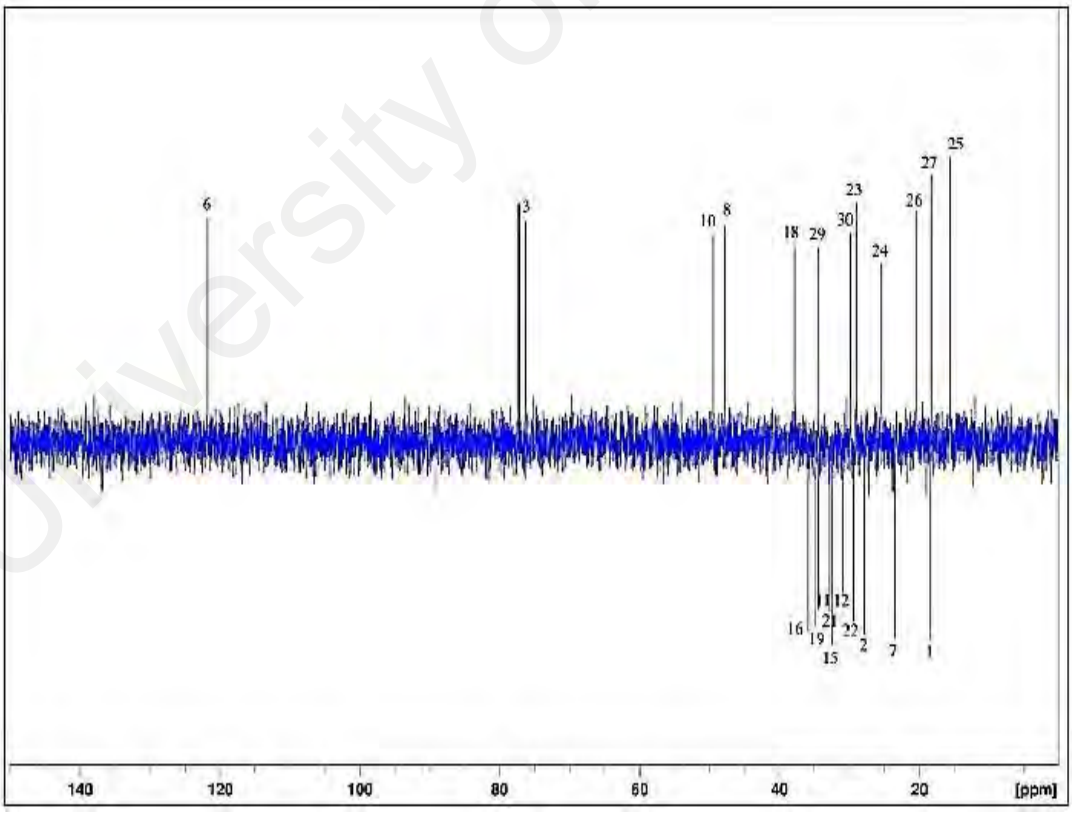


Figure 4.3: DEPT-135 spectrum of pinnatane A.

4.1.3 Correlation Spectroscopy

The correlation in COSY spectrum (Figure 4.4 and Figure 4.5) between H-10 (δ 2.00, *m*) and H-1 (δ 1.45, *m* and δ 1.50, *m*) confirmed that C-10 was not methylated thus suggesting that pinnatane A possess a glutinane triterpenoid skeleton (Atta ur et al., 2002). Other correlations observed were H-3/H-2, H-6/H-7 and H-18/H-19.

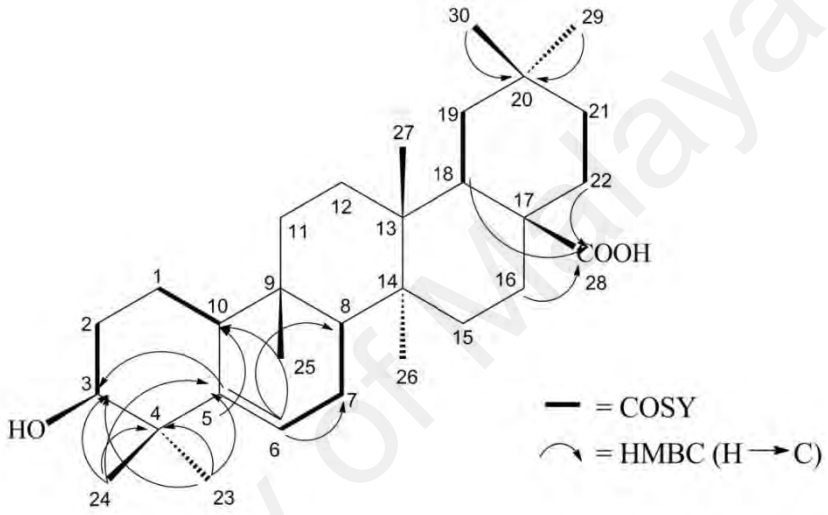


Figure 4.4: Selected COSY and HMBC correlations of pinnatane A.

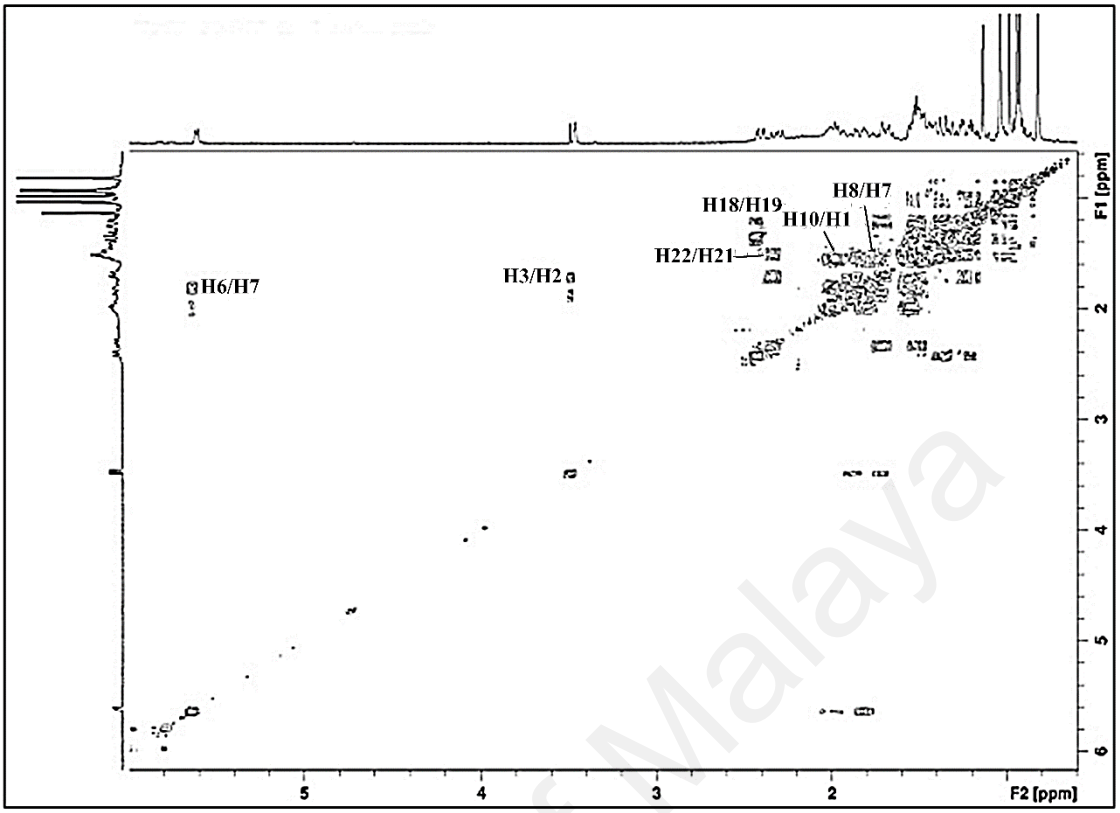


Figure 4.5: COSY spectrum of pinnatane A.

4.1.4 Heteronuclear Multi-bond Correlation Spectroscopy

Through analysis of HMBC experiments (Figure 4.4 and Figure 4.6), the location of hydroxyl group at H-3 (δ 3.47, *dd*, *J* = 3.2, 2.3 Hz) was supported from correlation with C-23 (δ 28.9), C-24 (δ 25.4) and C-5 (δ 141.6). Both sets of methyls Me-23 (δ 1.04, s)/Me-24 (δ 1.14, *s*) and Me-29 (δ 0.98, *s*)/Me-30 (δ 0.94, *s*) was observed as geminal on cross correlation between them together with C-4 (δ 40.8) and C-20 (δ 28.5) respectively. A double bond at C-5(6) was confirmed on the basis correlation of C-5 with H-10 (δ 2.00, *m*), H-3, Me-23 and Me-24; and C-6 (δ 121.7) with H-7 (δ 1.77, *m* and δ 1.95, *m*), H-8 (δ 1.52, *m*) and H-10. The existence of carboxyl moiety at C-28 (δ 182.6) position was verified on basis correlation with H-16 (δ 1.50, *m*), H-18 (δ 2.43, *dd*, J = 13.2, 4.5 Hz) and H-22 (δ 1.67, *m* and δ 2.29, *dd*, J = 14.9, 9.7 Hz.

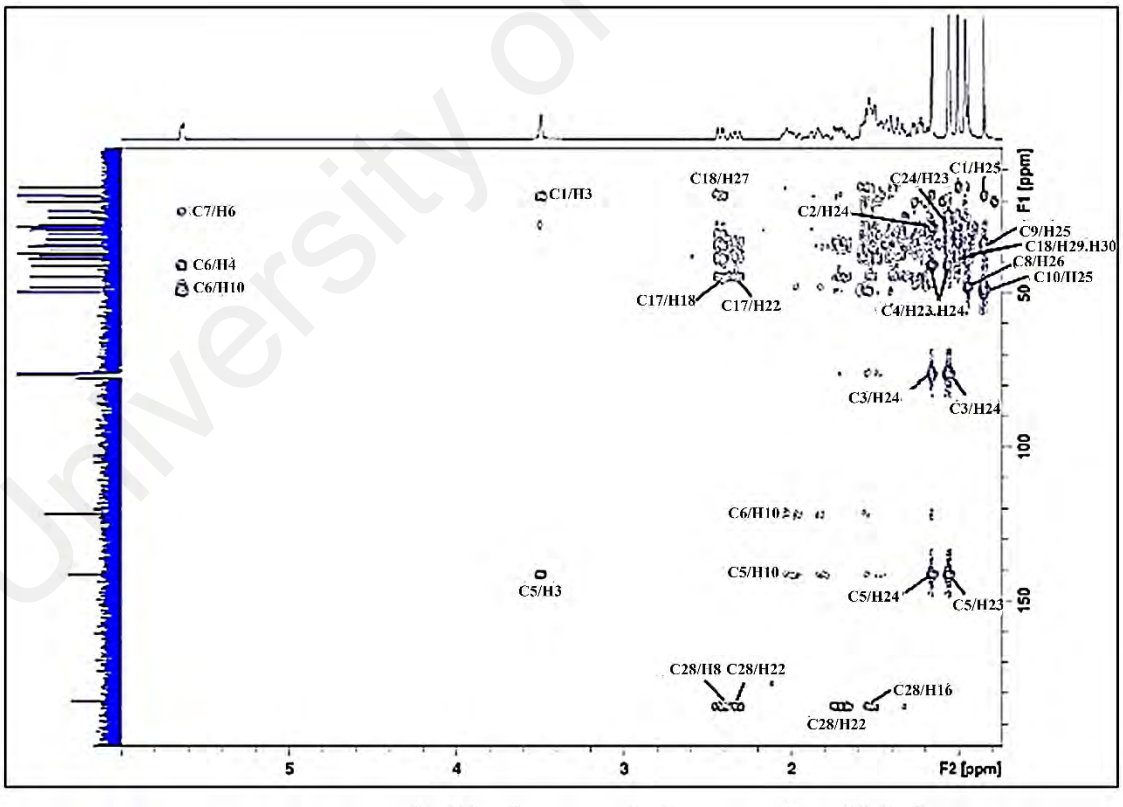


Figure 4.6: HMBC spectrum of pinnatane A.

4.2 Cytotoxicity Assay

4.2.1 Pinnatane A Triggers Cytotoxicity on Various Cancer Cell Lines

Preliminary study of pinnatane A cytotoxic activity was done using the MTT assay by measuring metabolic reaction rate in pinnatane A treated cells at various dose for 24 h. Pinnatane A was treated on six pairs of cancer cell lines which are bladder (EJ-28 and RT-112), breast (MCF-7 and MDA-MB-231), cervical (HeLa S3 and SiHa), hepatocellular (HepG2 and Hep3B), lung (A549 and SK-LU-1) and prostate (PC-3 and DU 145). The various cancer cell lines were used to investigate the potential of pinnatane A in inducing cell death in different cancer types. Many cancer cell lines from different cell types were used initially including lung cancer cell lines, A549 and SK-LU-1 to screen and identify effective cytotoxic activity of pinnatane A. A normal control cell line was used to calculate the SI value to determine suitable treatment time for downstream assay. One normal lung fibroblast cell line, MRC-5 was used to measure the selectivity of pinnatane A in inducing cytotoxicity in cancer cells compared to normal cells by calculating the SI value. The MRC-5 cell line was chosen as a control as it was used in the previous studies investigating cytotoxicity of pinnatane A and commonly used in cytotoxic assay for natural products (Elfita et al., 2009; Mahdzir et al., 2017).

The results indicated that pinnatane A induced cytotoxic activity in dose dependent manner in all the cell lines tested (Figure 4.7). However, each cell lines had different degree of sensitivity towards pinnatane A causing wide range of IC_{50} values (Table 4.2). Among all, two cell lines did not achieve 50% total viable cell population even at 100 μM pinnatane A treatment, namely, SiHa and SK-LU-1 cell lines. In comparison to IC_{50} value of MRC-5 cell line (48.75 ± 1.01 μM), only three cell lines had lower IC_{50} values, that is, Hep3B (19.04 ± 0.51 μM), EJ-28 (33.93 ± 3.79 μM) and RT-112 (48.03 ± 4.61 μM). However, only Hep3B cell line had SI value more than 2, that is, 2.56. SI value less

than 2 are considered as generally toxic towards normal cells (Koch et al., 2005). The greater the SI value, the higher is the selectivity in inducing cytotoxicity in cancer cells without harming normal cells. Since Hep3B was the only cell line with SI value more than 2, hence it was selected with another liver cancer cell line, HepG2 for further test to compare cytotoxic activity between these two cell lines.

MTT assay was carried out at various time of treatment (12, 48 and 72 h) to investigate the time-dependent effects of pinnatane A on Hep3B, HepG2 and MRC-5 cell lines. Results showed that pinnatane A triggered time-dependent cytotoxicity, the percentages of viable cell decreased in both Hep3B and HepG2 cell lines as the time of treatment increased (Figure 4.8). After 48 h of treatment, Hep3B and HepG2 cell lines had an IC_{50} of 13.47 ± 1.58 μM and 17.06 ± 2.14 μM respectively, lesser than the recommended biological activity assay endpoint for pure compound which are 25.00 μM (Table 4.3) (Cos et al., 2006). The MTT assay result of MRC-5 indicated that pinnatane A did not cause significant changes in percentages of viable cells for treatment time between 24 h to 72 h and the IC_{50} values remained between 40.00 to 60.00 μM. The SI values of both liver cancer cell lines treated for 42 h exceeded 3, with 3.96 for Hep3B and 3.12 for HepG2. These values were considered as good selectivity in causing cancer cell death in the same time with less adverse effects on normal cells for a pure compound.

From the MTT assay results, it was concluded that liver cancer cells were the most suitable for further testing to analyse cytotoxicity activity of pinnatane A. Pinnatane A was proven to induced dose- and time-dependent cytotoxicity against Hep3B and HepG2 cell lines with optimal treatment at 48 h. Further testing was required to investigate the cell death mechanism induced by pinnatane A. All further experiments were carried out based on IC_{50} values as obtained from MTT assay results and summarized in Table 4.3.

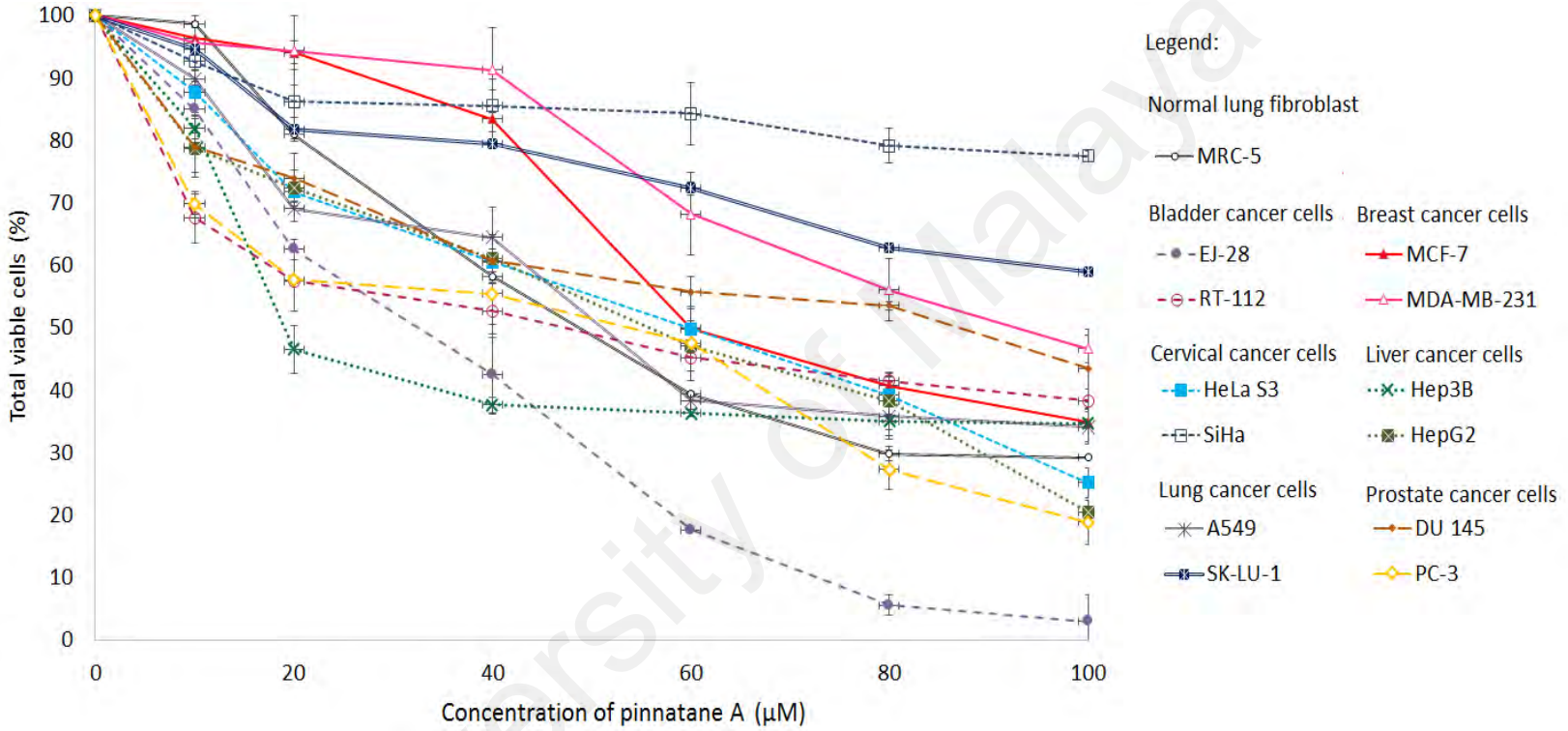


Figure 4.7: MTT assay for dose-dependent cytotoxicity activity of pinnatane A. Comparison of total viable cells (%) between various types of cancer and one normal (MRC-5) cell lines were investigated after treated with pinnatane A at various doses of treatment (0 to 100 µM) for 24 h of treatment time. All results were expressed as total percentages of viable cells. Each value point indicated mean ± SD from triplicates of data.

Table 4.2: The effects of pinnatane A treatment for 24 h on various cell lines.

Human cell lines	IC_{50} (μM)	SI[a]
Normal lung fibroblast (MRC-5)	48.75 ± 1.01	N.A.[c]
Breast adenocarcinoma (MCF-7)	60.93 ± 2.29	0.80
Breast adenocarcinoma (MDA-MB-231)	92.85 ± 3.06	0.52
Bladder carcinoma (EJ-28)	33.93 ± 3.79	1.44
Bladder carcinoma (RT-112)	48.03 ± 4.61	1.02
Cervical adenocarcinoma (HeLa S3)	59.73 ± 0.93	0.82
Cervical carcinoma (SiHa)	> 100[b]	N.C.[d]
Hepatocellular carcinoma (Hep3B)	19.04 ± 0.51	2.56
Hepatocellular carcinoma (HepG2)	55.79 ± 2.31	0.87
Lung adenocarcinoma (A549)	50.93 ± 3.05	0.95
Lung adenocarcinoma (SK-LU-1)	> 100[b]	N.C.[d]
Prostate carcinoma (DU 145)	87.23 ± 2.13	0.56
Prostate adenocarcinoma (PC-3)	55.30 ± 3.90	0.88

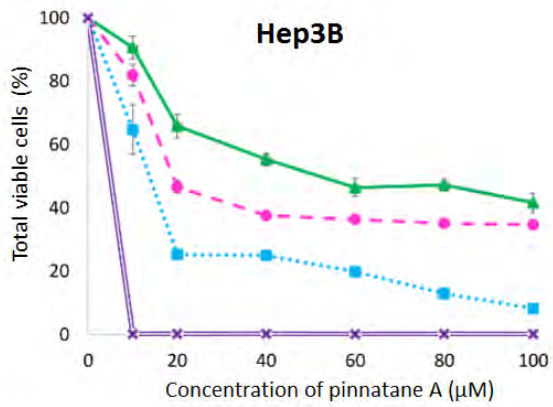
Note: [a]SI = IC_{50} value of normal cells/IC_{50} value of cancer cells.

[b] denotes an overall cell viability level of > 50% after treatment with pinnatane A at 100 μM for 24 h.

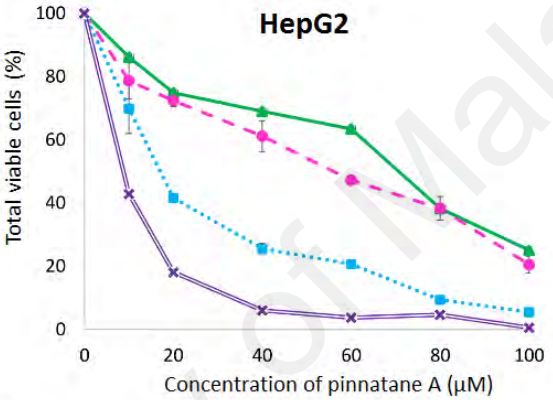
[c]N.A.: Not applicable

[d]N.C.: Not calculated because IC_{50} value was not determined.

(A)



(B)



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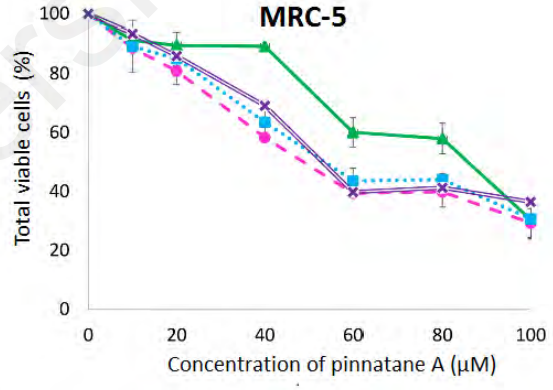

Figure 4.8: MTT assay for time-dependent cytotoxic activity of pinnatane A. Comparison of total viable cell (%) between two liver cancer cell lines, (A) Hep3B and (B) HepG2 and one normal cell line, (C) MRC-5 after treatment with pinnatane A for various doses (0 to 100 µM) for different post-treatment time (12, 24, 48 and 72 h). All results were expressed as total percentages of viable cells. Each value point indicated mean ± SD from triplicates of data.

Table 4.3: The time-dependent effects of pinnatane A on liver cancer cell lines.

Time	MRC-5	Hep3B		HepG2	
	IC_{50} (μM)	IC_{50} (μM)	SI [a]	IC_{50} (μM)	SI [a]
12 h	86.04 ± 4.50	52.72 ± 5.49	1.63	70.66 ± 0.48	1.21
24 h	48.62 ± 1.15	19.04 ± 0.51	2.56	55.79 ± 2.31	0.87
48 h	53.29 ± 4.64	13.47 ± 1.58	3.96	17.06 ± 2.14	3.12
72 h	53.05 ± 0.83	5.01 ± 0.00	10.59	8.79 ± 0.63	6.04

Note: [a] SI = IC_{50} value of normal cells/IC_{50} value of cancer cells.

4.2.2 Pinnatane A Caused Membrane Disruption in Liver Cancer Cells

Limitation and lack in sensitivity of MTT assay caused the need for alternative assays that would validate the cytotoxic effects of pinnatane A (Van Tonder et al., 2015). To validate the induction of cell death by pinnatane A, Live/Dead assay was used to visualize the appearances of cells upon treatment.

Live/Dead assay differentiated the state of cells being viable or dead using two fluorescent dyes, calcein-AM and EthD-1. Calcein-AM, a non-fluorescent dye that are able to passively cross the cell membrane and be converted by intracellular esterases to calcein, produces bright green fluorescence (excitation/emission at 495/515 nm) that are retained in the intact membranes of viable cells (Bratosin et al., 2005). EthD-1 is a positively charged compound that can pass through damaged cell membranes and are intercalated between double stranded nucleic acids and produces 40-fold enhancement of bright red fluorescence upon complexation (excitation/emission at 495/635 nm) (Moore et al., 1990).

Based on the observations, cytotoxic activity was significantly induced by pinnatane A after 12 h of treatment in both Hep3B and HepG2 cell lines (Figure 4.9). Respectively, viable cells of Hep3B were reduced from 98.77 ± 0.37% to 43.94 ± 0.68% with *P*-value

of 5.93×10^{-8} while HepG2 decreased from 98.68 ± 0.49% to 35.76 ± 1.83% with *P*-value of 1.24×10^{-6}. Control cells treated with DMSO remained viable at more than 90.00% for both Hep3B (97.11 ± 0.92%) and HepG2 (91.70 ± 3.18%) that indicated the quantity of solvent used does not influence the significant cytotoxic activity. Visualization of cells also showed that cell membrane integrity was disrupted in dying cells that lost the green fluorescence due to lack of ability in retaining the calcein dye.

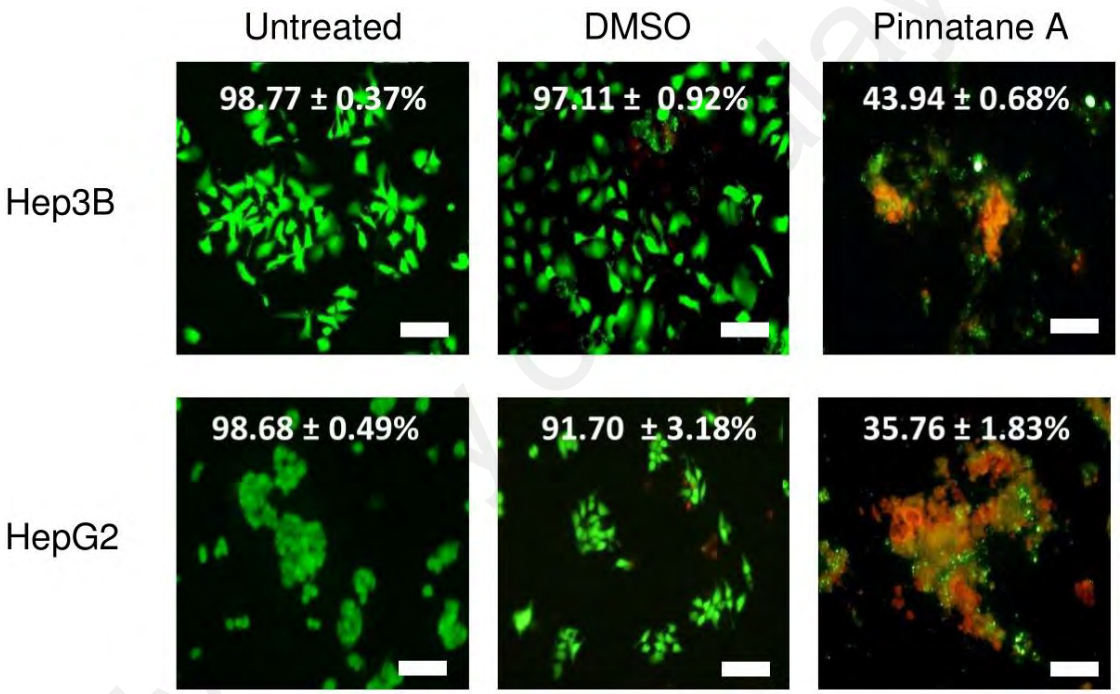

Figure 4.9: Live/Dead assay after treatment with pinnatane A and DMSO for 12 h. Green fluorescence denotes viable cells stained with calcein-AM, while reddish orange fluorescence represents dead cells stained with ethidium homodimer. All results were expressed as a total percentage of viable cells from random four fields with mean ± SD of three independent determinations. Scale bar = 100 μm.

4.3 Cell Cycle Arrest

Pinnatane A was proven to induce cytotoxicity in Hep3B and HepG2 cell lines by inducing disruption of cell membrane that allowed dyes to enter and interact with intercellular nucleic acids. Treated cells were fixed and cell cycle progression were analysed using flow cytometry based on univariate analysis of cellular nucleic acids that was stained by PI dye stoichiometrically. Cell cycle analysis by flow cytometry expressed the phases of cell by detecting diploid cells (G_0/G_1 phase), tetraploid cells (G_2/M phase) while S phase located between diploid and tetraploid states.

Cell cycle profiles in Hep3B cell line showed a significant increase of cell population in sub-G_0/G_1 phase (region I) from 2.64 ± 0.33% to 6.81 ± 1.14% after 24 h treatment and increased tremendously to 57.19 ± 1.50% after 48 h (Figure 4.10 A – B). The significant increase was consistent with the decreased in G_0/G_1 phase (Region II) from 57.59 ± 0.19% to 28.39 ± 0.27% after 48 h of treatment. Hep3B cell population also decreased at stage S and G_2/M phases. This indicated that pinnatane A induced G_0/G_1 phase and cells eventually became hypodiploid cells as seen in apoptotic cells.

On other hand, HepG2 cell line cell population significantly increased in G_0/G_1 phase from 46.58 ± 1.29% to 59.93 ± 0.95% after 48 h (Figure 4.10 A – C). The cell population in S and G_2/M phase eventually decreased over time that indicated pinnatane A halted the cell cycle at G_0/G_1 phase. However, null significant changes in sub-G_0/G_1 phase population suggested that the cytotoxicty activity induced in Hep3B and HepG2 cells probably were not the same and further analysis was needed to identify the cell death mechanism induced.

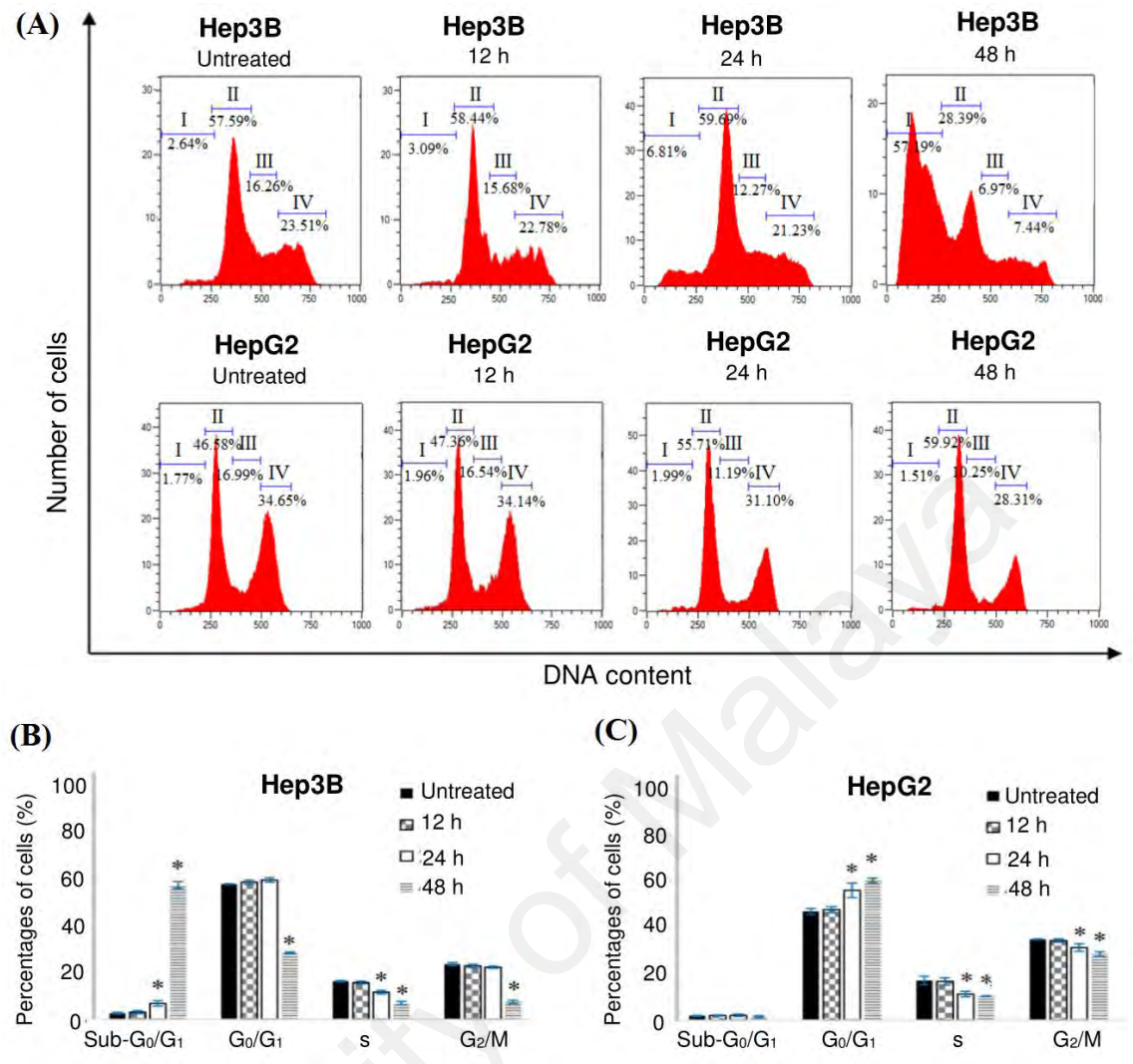

Figure 4.10: Cell cycle analysis of pinnatane A-treated Hep3B and HepG2 cell lines for 12, 24 and 48 h using flow cytometry after staining with PI fluorescent dye. (A) Histogram of cell population distribution categorized into four phases which are I: Sub-G_0/G_1; II: G_0/G_1; III: S; and IV: G_2/M. The cell arrest phase of (B) Hep3B and (C) HepG2 cell population distribution were expressed in the histogram as total percentages of cells from four different quadrants with mean ± SD of three independent determinations and significant differences are indicated by * for $P < 0.05$.

4.4 Determination of Cell Death Mechanism

4.4.1 Pinnatane A Induced Dual Cell Death Mechanisms

Cell cycle analysis revealed that pinnatane A induced different cytotoxicity activity in Hep3B and HepG2 cell lines. The cell death mode was determined using the dual stains of annexin V-FITC/ PI fluorescent dyes by measuring the cell population distribution across different stages of apoptosis or necrosis with the flow cytometer. The cells stained with annexin V-FITC denote PS externalization while with PI indicates cell membrane integrity disruption in dying or dead cells.

Pinnatane A induced apoptosis in Hep3B cell line (Figure 4.11 A – B) with early apoptosis cell population from 3.34 ± 0.79% increased significantly to 34.93 ± 4.46% and late apoptosis from 3.58 ± 0.40% to 18.96 ± 1.92% after treatment for 48 h. The viable cell population decreased in number across the time duration of treatment which suggested the cytotoxic activity of pinnatane A remains active and continues triggering apoptosis in cells.

In contrast, pinnatane A triggered necrosis in HepG2 cell line (Figure 4.11 A, C). Cell population increased significantly in early necrosis for 24 h of treatment from 2.37 ± 1.27% to 14.67 ± 1.40% but the number of cell population decreased to 6.73 ± 0.18% after 48 h of treatment. However, cells in late necrosis consistently increased across time of treatment and significantly from 4.81 ± 2.99% to 23.89 ± 1.02% after 48 h of treatment. The percentages of viable cell population between 24 and 48 h treatment did not show significant difference which implied that the increased of cell population in late necrosis was due to the shift of cell population from early necrosis and not from the viable cell population. Furthermore, non-significant changes occurred across different post-time treatment in early apoptosis quadrant confirmed the induction of necrosis in the HepG2 cell line.

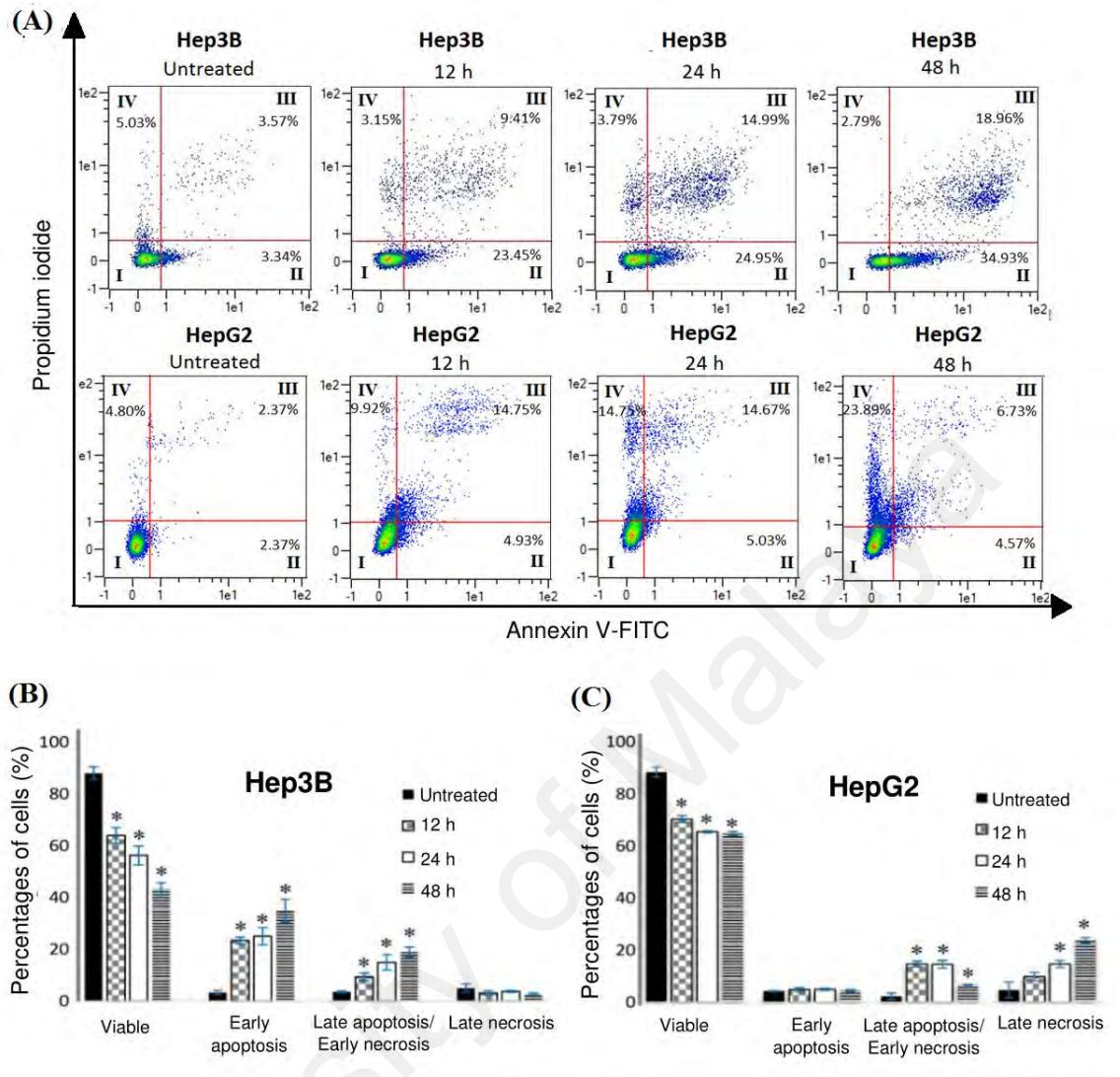

Figure 4.11: Pinnatane A induced apoptosis in Hep3B and necrosis in HepG2 cells. (A) Scatter plot of pinnatane A treated Hep3B and HepG2 cell lines after 12, 24 and 48 h post treatment using annexin V-FITC/PI flow cytometry assay. Quadrants were denoted as follows, I: viable cells; II: early apoptosis; III: late apoptosis or early necrosis; and IV: late necrosis. The death stages of (B) Hep3B and (C) HepG2 cell population distribution were expressed in the histogram as total percentages of cells from four different quadrants with mean ± SD of three independent determinations and significant differences are indicated by * for $P < 0.05$.

4.4.2 Pinnatane A Stimulated DNA Fragmentation in Dead Cells

In this study, pinnatane A triggered death in both Hep3B and HepG2 cell lines which were interpreted as late apoptosis or early necrosis respectively using annexin V/PI flow cytometry analysis. Further study was needed to confirm the induction of late stage apoptosis in Hep3B cells, rather than false positive with the existence of necrotic cells. As chromatin condensation and DNA fragmentation is a unique major hallmark in late stages of apoptosis, it is normally used as a confirmatory test.

Pinnatane A triggered different cell degradation pattern under gel electrophoresis (Figure 4.12). In Hep3B cell line, DNA laddering that resulted from apoptotic mechanism stimulated by activated endonucleases to cleave sites in between nucleosomes leaving DNA fragments of approximately between 180 to 200 bp intervals, was induced in 24 h and became clearer after 48 h of treatment. There was no induction of DNA fragments in the first 12 h of treatment.

In contrast, no DNA laddering was seen in HepG2 cell line, instead a smear was triggered. Similarly, the DNA smear was only visualized after 48 h of treatment with pinnatane A without any changes after 12 and 24 h post-treatment. DNA smear is usually seen in necrotic cells due to random degradation of DNA by simultaneous activity of proteases without apparent regard to chromatin structures which confirmed the earlier flow cytometry results on the induction of necrosis by pinnatane A in HepG2 cell line.

Positive control of Hep3B cells were treated with a Food and Drug Administration approved anti-cancer drug, cisplatin, to compare the laddering pattern induced by apoptosis mechanism with pinnatane A. From the results, the dose and time used to treat Hep3B and HepG2 cell lines to induce enzymatic reaction and caused cell population to undergo late stages of death, was adapted for further downstream study.

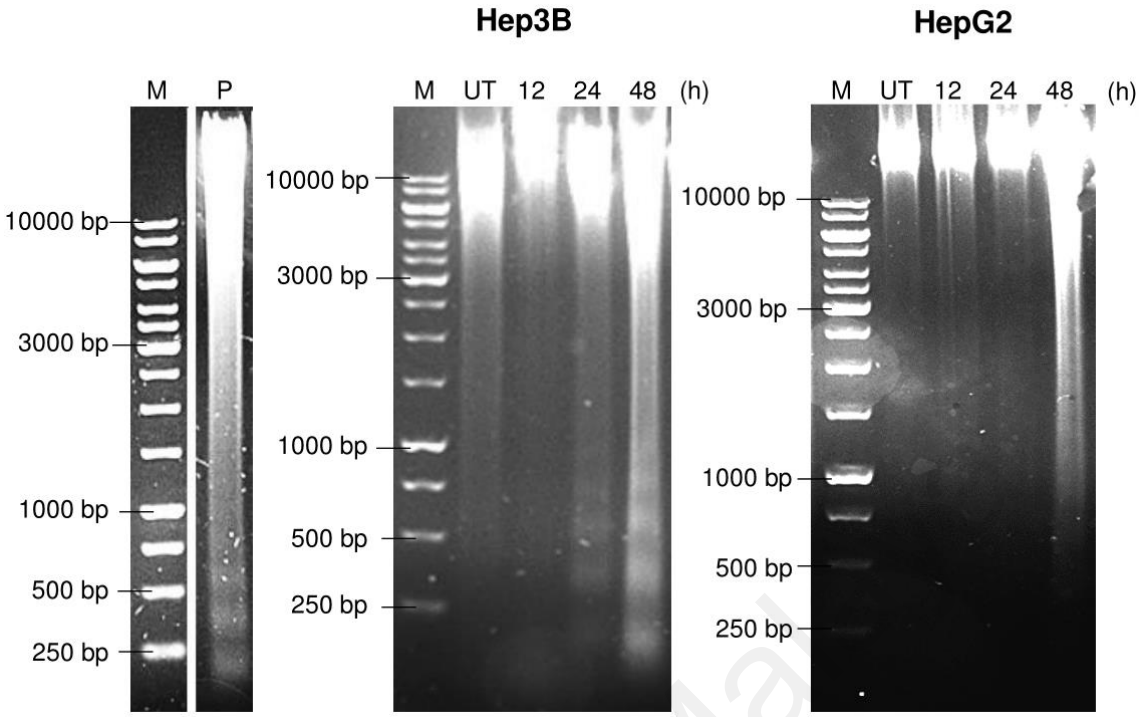

Figure 4.12: DNA fragmentation assay by gel electrophoresis on liver cancer cells DNA induced by pinnatane A across 12, 24 and 48 h time of treatment. Confirmation of apoptosis in Hep3B cells by visualization of 180 to 200 bp DNA laddering while necrosis in HepG2 cells by observable DNA smear pattern. M: marker; P: Positive control using Hep3B cells treated with cisplatin.

CHAPTER 5: DISCUSSION

The aim of present study was to investigate the potential of rarely found glutinane type triterpene, pinnatane A, isolated from *Walsura pinnata* bark in inducing cytotoxic activity in cancer cells. The cytotoxic activity analyses of pinnatane A treatment involved identifying cancer cell lines that have significant IC_{50} values, the effects on normal cell line, optimal treatment time and dose, visualization of cell membrane integrity, analysis on cell cycle arrest, identifying cell death mode and observation on DNA integrity.

Natural products have been used as traditional remedies or commercial drugs in treatment of various types of diseases. The uniqueness of natural products producing innumerable structural diversity provides opportunities to develop multiple drugs in order to tackle various health issues in human. Secondary metabolites synthesized to serve as protection and survival of plants were discovered to give benefit to humans including providing various biological effects on several high risk diseases such as cancer (Seca & Pinto, 2018), Alzheimer (Orhan, 2012), heart diseases (Abdalla, 2003), tuberculosis (Kumar et al., 2010) and diabetes (Bahmani et al., 2014).

The ability of natural products to be developed as chemotherapeutic agents are due to their molecular scaffold that helps in identifying genetic materials (Hamilton & Arya, 2012) and proteins (Drahl et al., 2005), mediating protein-protein interaction within cells (Jin et al., 2018) and targeting molecular pathways (Hong, 2011).

Small molecule natural products tend to have suitable molecular size to pass through cell membrane and largely biosynthesized through protein-based machinery that further add on to benefits for interaction with cellular proteins such as enzymes and receptors (Wilson & Danishefsky, 2006). Based on the "Rule of 5" by Lipinski's research team, compounds with molecular weight more than 500 g/mol are considered as unsuitable

drugs due to poor permeability in the gut and central nervous system (Pollastri, 2010). Large compounds also give challenges in excretion system due to minimal clearance via renal and biliary excretion system (Taft, 2009). Thus, to search the compounds with suitable molecular weights and active biological activities are important in drug development to design a safe and effective therapeutic agent.

In this study, pinnatane A, a rare glutinane type triterpenoid extracted from *Walsura pinnata* was investigated for its cytotoxic activity in cancer cells. Since the molecule weight of pinnatane A is 455.35 g mol^{-1}, thus the compound has a suitable size as a drug. Triterpenes are seen to be potent against a vast variety of cancer cells (Chudzik et al., 2015) but there are limited study on cytotoxic activity of glutinane type triterpene and the mechanism of its action still remains ambiguous (Mahdzir et al., 2017).

An *in vitro* approach was used in the present preliminary study due to advantages such as low cost and direct effects assessment (Polli, 2008). However, *in vitro* approach lack in representation of real conditions in host (*in vivo*). Further testing in animal models will help in study of bioactivity of compounds in natural setting but limited bioavailability of compound and potential adverse effects on host pushes the need for compound usage optimization. Thus, the present study focused on identifying the optimal use of pinnatane A on potent cancer cell lines that may help in future biological model studies.

Normal cell line, MRC-5 was used in the present study to observe the selectivity of cytotoxic effects of pinnatane A in causing cell death in cancer cells with minimal effects on normal cell line. MRC-5 cell line originated from human diploid lung fibroblast that was used in cytotoxic study of triterpene due to its ability in maintaining normal diploid karyotype *in vitro* for a long time (Zhang et al., 2014a). Previous cytotoxic study using pinnatane A against MCF-7 breast cancer and SK-OV-3 ovarian cancer had moderate

cytotoxic activity. However both cell lines were recorded with poor SI value against MRC-5 cell line (Mahdzir et al., 2017).

Hence, in order to fully exploit and understand the cytotoxicity activity of pinnatane A, this study was designed with several initial considerations, firstly, identifying the most potent cancer cell line; secondly, assessing the effects of pinnatane A in dose and time dependent manner: thirdly, identifying IC_{50} values which is important for down-stream assays and fourthly, determined the selectivity of pinnatane A to determine the optimal treatment for further analysis.

Preliminary study against several types of cancer cells using MTT assay treated for 24 h with various dose was to identify the most potent cell line. Six pairs of cancer cell lines from human bladder, breast, cervical, liver, lung and prostate showed that all cell lines were dose dependent with different degree of sensitivity. The Hep3B liver cancer cell line was shown to have the lowest IC_{50} value and the only cell line with SI value more than two. Thus, Hep3B cell line was denoted as prominent cell line for further downstream assays. HepG2 cell line also was selected for further analysis to assess the discrete differences in cytotoxic effects of pinnatane A.

Pinnatane A had significant time-dependent effects against Hep3B and HepG2 cell lines where the viability of cells kept decreasing over time. The IC_{50} values of both cell lines were below 25.00 μM after 48 h of treatment. Notably, the IC_{50} value of Hep3B after 72 h treatment was 2.28 μM that is considered as potent for cytotoxicity induced by pure compound (Boik, 2001; Kuete & Efferth, 2010).

The different time-dependency of pinnatane A cytotoxicity between cancer and normal cells had influenced in the determination of SI values. The SI values of Hep3B and HepG2

cell lines exceeded 3 and was considered as good selectivity indices after 48 h of treatment.

The discovery of dose- and time-dependent cytotoxicity effects of a compound helps in drug design. Treatment with low doses with extension of time helps improving treatment progression without causing adverse side effects on non-cancerous cells. (Awang et al., 2010).

Triterpernoids extracted from several plant species were reported to induce cytotoxicity against Hep3B and HepG2 cell lines with different degree of effectiveness. Triterpenoids extracted from *Camptotheca acuminate* react differently against HepG2 and Hep3B cell lines, where the most active triterpenoid induced cytotoxic activity in HepG2 cell lines with IC_{50} value of 29.60 µM as compared to 47.50 µM for Hep3B cell line (He et al., 2018). Ursolic acid, another triterpenoid commonly found in apple peel and edible plants, halted proliferation in Hep3B cells by inhibiting P-STAT3 induced by interleukin-6 while in HepG2 cells was via inhibiting constitutive STAT3 phosphorylation (Yan et al., 2010). Thus, downstream analysis of each treated cell line is required to identify the mode of action with pinnatane A.

From the MTT assay results, pinnatane A potential benefits were specifically against hepatocellular carcinoma cell lines. The results clearly underlined the rate of cell killing was varied between cell lines, seen by the different IC_{50} values with the same doses of treatment. The disparity of pinnatane A cytotoxic activity against different type of cell lines allowed the ability to comprehend drug efficacy and toxicity (Lledó, 1993). Tumour interaction between cells, extracellular matrix and soluble factors creates microenvironment are known to influence the sensitivity on drug (Morin, 2003). Also, drug resistance may be due to different abilities of cells in DNA repair, to inactivate or inhibit drug, alter drug target and drug efflux (Housman et al., 2014). High dose of drug

treatment are required in aggressive type cancer cells with accumulated alterations in tumour suppressor genes and oncogenes (Yokota, 2000). Therefore, there are many reasons to the difference of sensitivity in the cancer cell lines against pinnatane A.

In the MTT assay, pinnatane A was proven to induce significant cell death in Hep3B and HepG2 cell lines. The dead cells are represented by the bright red fluorescent dye by EthD-1 that pass through loosely membrane integrity and bind to the nucleic acids even with damaged DNA that might have occurred after treatment (Gokduman et al., 2018).

Previous studies reported that the interaction between triterpenoids and cell membrane may influence the molecular mechanism influencing biological activities through modification at the molecular level of cell membrane structure. Early study on the investigations of the membrane activity induced by saponins discovered the capability of triterpenoids to integrate into cellular membrane transiently and induce pore like structures which change the membrane permeability associated with modifications of intracellular and extracellular homeostasis (Melzig et al., 2001). Plant pentacyclic acids from orujo olive oil such as oleanic acid, masclinic acid and ursolic acid alter the structural order in membranes without destabilizing the lipid bilayer, strengthening the assumption of biophysical properties of cell membranes as the key roles in their wide-ranging molecular mechanism of biological effects (Prades et al., 2011). The activation of programmed cell death induced by avicins, saponins from *Acacia victorariae,* is via regulation of innate stress response in human cells through formation of channels in membranes and altering the potential across mitochondrial membrane potential and the pH of intermembrane, favouring the induction of apoptosis (Li et al., 2005).

The Nicoletti method was adapted for cell cycle analysis of pinnatane A treated Hep3B and HepG2 using PI fluorescent dye that bind to DNA content and detected by flow cytometry. This method takes advantage of DNase activation and DNA fragmentation

that happen in late stage of apoptosis to detect weak signals from hypodiploid DNA, that is represented by sub-G_0/G_1 phase (Ghavami et al., 2005). It also enables the discerning apoptosis from necrosis, as the DNA content of necrotic cells, which resembles normal cells, is markedly different from apoptotic cells (Berghe et al., 2004; Ghavami et al., 2005).

Previous reports also suggested the ability of triterpenoids in inducing liver cancer cell cycle arrest. However, each type of triterpene has different cytotoxicity nature against the cancer cell lines. Escin, saponins mixture extracted from *Aesculus wilsonii* Rehd., induced G_1 phase that led to execution of apoptosis in HepG2 cell line (Zhou et al., 2009). Ginsenoside RK1, a saponin type triterpenoid also induced G_1 phase arrest in HepG2 cell line but with the induction of autophagy and apoptosis (Ko et al., 2009). Ganoderiol F, a tetracyclic triterpene induced senescence in liver cancer cell line, HepG2 and Huh7 by inducing G_1 phase arrest without significantly affecting Hep3B and MRC-5 cell lines (Chang et al., 2006). Thus, triterpenes are also capable of selective senescence without causing cytotoxic effects against the cell line.

Identification of cell mode death is important in designing therapeutic drug to determine the fate of cells and probable effects towards patients. Apoptosis is an important type of cell death to maintain the cellular homeostasis operating important cellular process involved in wide functions from defence system to organ sculpting during embryogenesis (Robertson et al., 2009). Cancer cells de-regulate the apoptosis machinery, by activating oncogene and deactivating tumour suppressor gene, allowing emissions from cell death that leads to uncontrolled cell division, resistance towards drugs and recurrence of tumour (Mohammad et al., 2015). Necrosis is a type of cell death that induce inflammation due to release intracellular materials that causes harm to neighbouring cells (Elkon, 2013). Cancer therapies are preferred to promote apoptosis

rather than necrosis to halt the progression of tumour with limited concurrent adverse effects on normal cells (Gerl & Vaux, 2005). Two assays were carried out to determine the cell death mode induced by pinnatane A in Hep3B and HepG2 cells that were annexin V-FITC/PI flow cytometry assay and DNA fragmentation assay.

Annexin V-FITC/PI flow cytomery assay applying the knowledge based on the PS leaflet translocation from inner leaflet to outer leaflet of cell plasma membrane during early apoptosis. The PS leaflet can be detected in both apoptotic and necrotic cells that may lead to the false positive results due to the common pathological condition but with appropriate markers and time of treatment, the cell death mode could be determined. By using the combination of annexin V-FITC/PI staining dyes, the cells can be recognized as viable (unstained), early apoptosis (annexin V-FITC stained only), late apoptosis or early apoptosis (annexin V-FITC and PI stained) and necrosis (PI stained only) across various time of treatment to observe the shift of cells towards different stages of death.

The recent results suggested that pinnatane A induced apoptosis in Hep3B cell line by significantly and constantly increasing cell population in early and late apoptosis for the 48 h of treatment. In HepG2 cell line, pinnatane A induced necrosis cell death by inducing significant population increase in late stage of necrosis after 48 h of treatment. Cell population increased significantly in early necrosis after 24 h of treatment but decreased after 48 h which suggest the shift to late necrosis. Non-significant differences between viable cells population for 24 and 48 h of treatment indicated that the cell death triggered was not from the viable cells population. Apoptosis can be distinguished from necrosis by analysing the flow of events that exist in dying cells. Apoptotic cells are characterized by the lag phase between annexin V-FITC and PI positivity, due to exposure of PS towards extracellular of cells before the disruption in cell membrane. In contrast, necrotic cells undergo both events simultaneously (Berghe et al., 2004).

In HepG2, triterpenoids such as asiatic acid (Lee et al., 2002), echinocystic acid and escin (Tong et al., 2004) have been reported to induce apoptosis. Meanwhile in Hep3B, the cytotoxicity of triterpene has been demonstrated, although the information on the mechanism of cell death is limited. On the other hand, some compounds such as oleanolic and ursolic acids have been reported to induce caspase-dependent apoptosis in both Hep3B and HepG2 cell lines (Yan et al., 2010).

DNA fragmentation was used in present study to confirm the induction of apoptosis and necrosis. At the moment, the conventional method of DNA fragmentation assay through gel electrophoresis remained as the golden technique in differentiating between apoptosis and necrosis. Despite this method there is more advanced technique in identifying induction of apoptosis through molecular level as a marker such as Terminal deoxynucleotidyl dUTp nick end labelling (TUNEL) and Comet assay, however distinguishing apoptosis from necrosis is hardly confirmed due to limitation of the techniques. Thus, false positive results are most likely to happen that can lead to the incorrect future analysis (Daryl & Kim, 1995; Grasl-Kraupp et al., 1995). As such, annexin V/PI flow cytometry and DNA fragmentation assays were carried out to differentiate apoptosis from necrosis and avoid false positive result.

The results from DNA assay were consistent with cell cycle analysis and annexin V-FITC/PI flow cytometry assay which validated the induction of apoptosis and necrosis in Hep3B and HepG2 cell lines, respectively. Saikosaponin, a triterpenoid saponine, in human CEM lymphocytes also reported to induced apoptosis by inducing DNA fragmentation associated with *c-myc*, *p53* and *bcl-2* mRNA (Hsu et al., 2000). Thus, the report recognizes the triterpene ability in triggering apoptotic internucleosomal DNA fragmentation.

The different outcomes of the treatment with pinnatane-A in Hep3B and HepG2 may be attributed to different mechanism of action within these cell lines. Similar findings have been observed in a study on cytotoxic activity of triterpenoid asiatic acid against human glioblastoma and colon cancer cells. Asiatic acid was found to pre-dominantly induce necrosis in glioblastoma cells although it was also being shown to induce apoptosis in colon cancer cells (Cho et al., 2006).

Hep3B and HepG2 cell lines are categorized as hepatocellular carcinoma but both cells have different background and genetic profiles. Both cell lines originated from different stages in liver cell lineage and the morphological differences observed in Hep3B cells have a fibroblast structure with more mesenchymal protein properties while HepG2 cells retain as hepatocyte related features (Qiu et al., 2015).

Comparison study between Hep3B and HepG2 cell lines has become cell lines of choice in drug development research related to hepatocellular carcinoma treatment. The most notable difference in term of gene expression between Hep3B and HepG2 cell lines is the p53 status. Hep3B cell line is a null type p53 which are partial mutation while HepG2 cells has a wild type p53 gene (Bressac et al., 1990). The dissimilar status of gene expression may change the mode of cell death. A study on the properties of etoposide towards p53 inhibition in human kidney proximal tubule cells showed that p53 silencing changes the mode of death from necrosis to apoptosis due to mitochondrial superoxide accumulation and mitochondrial damage which generates the initiation of caspase-3 expression and thus explains the role of p53-mediated anti-apoptosis in promoting necrosis (Kwon et al., 2015).

Disruption in molecular pathways would lead to different destiny on how the cell will die. Other differences may be due to alteration of other gene or origin of the cell itself. The status of hepatitis B virus DNA integrated in Hep3B cells also creates a distinct

difference in gene expression due to intervention of HBx protein with host gene especially related to proliferation and inflammation (Kew, 2011; Tang et al., 2006). HBx protein induced typical chronic inflammation in which generates a tumour-supporting microenvironment that encourages the growth of cancer cells.

Thus, it is suggested that pinnatane A has the potential to be developed as a chemotherapeutic agent due to its properties of anti-proliferation and pro-apoptotic ability in Hep3B cells.

The use of necrosis mechanism in chemotherapeutic treatment may rely on methods such as conducting a genetic detection or combine with other therapeutic strategies that may important to combat cancer with deficiency in the apoptosis machinery (Su et al., 2016).

The combination of drugs mediating different mechanism of cell death may result in a more effective way in treatment of cancers. A study of treatment using honokiol, a small molecule that is competent in inducing apoptosis and combined with chemotherapy drugs that induce necrosis was seen to induce a synergy in combating multidrug resistance breast cells (Tian et al., 2013).

This knowledge is crucial for the development of drugs especially in targeted therapies, hence giving a wide pharmaceutical spectrum and opportunities in therapeutic treatments while minimizing the adverse side effects in order to have better results and to improve quality of life.

CHAPTER 6: CONCLUSION

The main objective of the present study was to determine the potential of pinnatane A, a rare naturally occurring triterpenoid extracted from *Walsura pinnata* bark as chemo-preventive agent against liver cancer cells. To the best of my knowledge, this is the first *in vitro* study that investigated cell cycle arrest and cell death mode identification triggered against human liver cancer cell lines. The highlight of this study was the selective cytotoxic activity of pinnatane A in inducing significant cell death with minimal adverse effects on normal cell line and the dual mechanism of cell death against liver cancer cells.

These results demonstrated the cytotoxic, cell cycle arrest and apoptotic or necrotic abilities of pinnatane A in liver cancer cells. The discovery of pinnatane A inducing potent cytotoxic activity against cancer cells without adverse effects on normal MRC-5 cells was noteworthy for development of chemotherapeutic drug. The findings also expand the probability in developing more diverse treatment by combining with other therapeutic regime and drugs.

Modifications of chemical structure also advocate more possibility to increase efficient and effective outcomes. Further elucidations on the molecular pathways of cell death execution, *in vivo* studies and clinical trials involving patients are needed to discover the true potential of pinnatane A as a potential therapeutic agent.

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Cytotoxic Effects of Pinnatane A Extracted from *Walsura pinnata* (Meliaceae) on Human Liver Cancer Cells

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Abstract: Background: Pinnatane A from the bark of *Walsura pinnata* was investigated for its anti-cancer properties by analyzing the cytotoxic activities and cell cycle arrest mechanism induced in two different liver cancer cell lines. Methods: A 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to analyze the pinnatane A selectivity in inducing cell death in cancer and normal cells. Various biological assays were carried out to analyze the anti-cancer properties of pinnatane A, such as a live/dead assay for cell death microscopic visualization, cell cycle analysis using propidium iodide (PI) to identify the cell cycle arrest phase, annexin V-fluorescein isothiocyanate (annexin V-FITC)/PI flow cytometry assay to measure percentage of cell populations at different stages of apoptosis and necrosis, and DNA fragmentation assay to verify the late stage of apoptosis. Results: The MTT assay identified pinnatane A prominent dose- and time-dependent cytotoxicity effects in Hep3B and HepG2 cells, with minimal effect on normal cells. The live/dead assay showed significant cell death, while cell cycle analysis showed arrest at the G_0/G_1 phase in both cell lines. Annexin V-FITC/PI flow cytometry and DNA fragmentation assays identified apoptotic cell death in Hep3B and necrotic cell death in HepG2 cell lines. Conclusions: Pinnatane A has the potential for further development as a chemotherapeutic agent prominently against human liver cells.

Keywords: anti-cancer; apoptosis; cell cycle arrest; necrosis; triterpene

1. Introduction

Treatment of hepatocellular carcinoma with chemotherapeutic drugs tested in randomized controlled trials has shown only moderate survival benefit for intermediate grade tumour and poor first-line treatment for advanced diseases [1–3]. Currently, sorafenib, a multi-target kinase inhibitor, is the recommended Food and Drug Administration (FDA)-approved drug to improve survival by controlling tumour progression in patients with advanced hepatocellular carcinoma [4–6]. However, adverse side effects on patient's health and a high-dose drug burden with increased treatment costs necessitate the development of more effective treatment options [7,8]. Therefore, it is desirable to