CYTOTOXIC EFFECT OF HELICHRYSETIN ON CANCER CELL LINES AND ITS MECHANISMS

HO YEN FONG

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Ho Yen Fong

Matric No: SHC 120123

Name of Degree: Doctor of Philosophy (Ph.D.)

Title of Thesis ("this Work"):

"Cytotoxic Effect of Helichrysetin on Cancer Cell Lines and Its Mechanisms"

Field of Study: Biochemistry

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

(Ho Yen Fong)

Subscribed and solemnly declared before,

Witness's Signature Date:

Name: Assoc. Prof. Dr Saiful Anuar Karsani

Designation: Supervisor

CYTOTOXIC EFFECT OF HELICHRYSETIN ON CANCER CELL LINES AND ITS MECHANISMS

ABSTRACT

Helichrysetin is a naturally-occurring compound from the group of chalcones that is found in the Chinese ginger, seeds of some *Alpinia sp.* and flowers of *Helichrysum sp.*. Previous studies have shown that helichrysetin exhibits several biological activities such as antiplatelet aggregation, antioxidant, and anti-cancer activity. This compound has been found to be effective in the growth inhibition of human breast, liver and cervical cancer cell lines but no study on the molecular mechanisms has been performed. Hence, this research aims to study the growth inhibitory effect of helichrysetin on four selected cancer cell lines and it shows the highest activity on A549 and Ca Ski cell lines. The effect of helichrysetin on A549 cell line has been selected for further molecular investigation. In this study helichrysetin was found to inhibit A549 cells through the induction of apoptosis by triggering mitochondrial-mediated apoptotic pathway. Apoptotic cellular and nuclear morphological features were observed in the A549 cells after exposure to helichrysetin. Treatment with helichrysetin also resulted in the changes in the structure of cell plasma membrane, disruption in the mitochondrial membrane potential, cell cycle arrest and damage of DNA mainly internucleosomal DNA fragmentation in A549 cells. Proteomic study was performed to further understand the signaling cascades in the cells. The proteomic study has revealed the ability of helichrysetin to initiate cell death in A549 cells potentially by stimulating oxidative stress proven by the stimulation of oxidative stress markers HMOX1 and NRF2. Presence of oxidative stress in cells will result in DNA damage. In response to DNA damage, DNA damage response and cell cycle arrest are commonly triggered to allow for DNA repair. Results from this study showed that helichrysetin causes the suppression of proteins related to DNA damage repair such as p-ATM, BRCA1, and

FANCD2 and Rb1 hence contributing to the impairment of DNA damage response in A549 cells. The inability of the cells to perform DNA repair will trigger the induction of apoptosis in the cells. Findings from this study have therefore proven the potential of helichrysetin as anti-cancer agent for the treatment of human lung cancer. The discovery of its mechanism of action on human lung cancer cells provides a better understanding and important information for the development of helichrysetin for future targeted-cancer therapy.

KESAN SITOTOKSIK HELICHRYSETIN PADA SEL-SEL KANSER DAN MEKANISMENYA

ABSTRAK

Helichrysetin adalah kalkon semulajadi yang boleh didapati daripada halia cina, biji benih *Alpinia sp.*, dan sesetengah bunga *Helichrysum sp.*. Hasil kajian saintifik menunjukkan helichrysetin mempunyai aktiviti biologi seperti aktiviti antiplatelet, antioksidan dan antikanser. Kompaun ini boleh merencat pertumbuhan sel-sel kanser payudara, hati, dan serviks. Sehingga kini, tiada kajian mekanisme molekular yang pernah dijalankan berkaitan aktiviti antikanser helichrysetin. Objektif penyelidikan ini adalah untuk mengkaji aktiviti sitotoksik helichrysetin terhadap empat jenis sel-sel yang terpilih. Keputusan kajian menunjukkan helichrysetin adalah efektif dalam perencatan pertumbuhan sel-sel kanser serviks, Ca Ski dan sel-sel kanser paru-paru, A549. A549 dipilih untuk kajian yang lebih lanjut bagi memahami mekanisme molekular sitotoksik helichrysetin. Kajian menunjukkan helichrysetin boleh merencat pertumbuhan sel-sel A549 melalui induksi apoptosis dengan mengaktifkan laluan intrinsik apoptosis yang melibatkan mitokondria di dalam sel. Helichrysetin juga boleh mengakibatkan perubahan dari segi ciri-ciri morfologi sel dan nukleus. Selain itu, helichrysetin juga menyebabkan perubahan biokimia dalam sel-sel A549 seperti perubahan struktur membran plasma, perubahan potensial membran mitokondria, pemberhentian kitaran sel, dan kerosakan DNA. Kajian proteomik telah dilakukan untuk memahami perubahan mekanisme molekular di dalam sel. Keputusan kajian proteomik menunjukkan helichrysetin berpotensi untuk merangsang stres oksidatif di dalam sel dan ini telah dibuktikan melalui kehadiran protein-protein yang berkaitan dengan stres oksidatif seperti protein HMOX1 dan NRF2. Stres oksidatif boleh menyebabkan kerosakan DNA di dalam sel dan ini boleh merangsang mekanisme kerosakan DNA dan pemberhentian kitaran sel bagi membolehkan sel membaiki DNA yang rosak. Keputusan menunjukkan

helichrysetin telah mengurangkan tahap ekpresi protein yang berkaitan dengan mekanisme kerosakan DNA seperti protein p-ATM, BRCA1, FANCD2 dan Rb1. Ini boleh menyebabkan kemerosotan respon kepada kerosakan DNA di dalam sel. Ketidakupayaan sel-sel untuk membaiki DNA yang rosak akan mencetuskan induksi apoptosis dalam sel. Kajian ini telah membuktikan potensi helichrysetin sebagai agen anti-kanser untuk rawatan kanser paru-paru. Penemuan mekanisme sitotosiknya terhadap sel-sel kanser paru-paru manusia memberikan maklumat yang penting dalam penggunaan helichrysetin sebagai agen terapi kanser untukrawatan kanser pada masa yang akan datang.

ACKNOWLEDGEMENTS

First and foremost, praises and thanks to God Almighty for His blessings throughout the study that enable me to complete this research work successfully.

I would like to express my deepest gratitude to both my supervisors, Professor Datin Dr Sri Nurestri Abd Malek and Associate Professor Dr Saiful Anuar Karsani for giving me the opportunity to undertake this research and providing me invaluable guidance throughout the whole research. I am extremely grateful for all the facilities and resources that were offered to me for completion of this research.

I am extremely grateful to my family for their love, patience, care and sacrifices for preparing and educating me for my future. Their understanding and unconditional love have given me the strength to go through all the difficulties.

I am extending my thanks to my research colleagues from HIR Functional Molecules group, Yong Wai Kuan, Foo Yiing Yee, Dr Syarifah Nur Syed Abdul Rahman, Jaime Stella Richarson, Zarith Shafinaz, Phang Chung Weng, Shafinah Suhaimi, Dr Hong Sok Lai, and Dr Lee Guan Serm for being with me, and giving me help and guidance throughout the whole journey.

I express my special thanks to Professor Shyur Lie-Fen from Agriculture Biotechnology Research Center (ABRC), Academia Sinica, Taiwan for providing me the opportunity to gain invaluable research experience and knowledge which enable me to move further in my research. I would like to convey my heartfelt gratitude to all lab members of A728, ABRC for the help, encouragement and genuine support during my time in A728 lab.

Finally, my thanks go out to Institute of Biological Sciences, Faculty of Science and University of Malaya for providing me the facilities and financial support.

HO YEN FONG

January 13, 2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

53BP1	p53 binding protein1
AIF	apoptosis inducing factor
APAF	apoptotic protease activating factor
ARE	antioxidant response element
ARF	ADP ribosylation factor
ATCC	American type culture collection
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3 related
BAAT	BRCA1-associated ATM activator
BID	Bcl2-interacting protein
BRCA1	breast cancer type 1 susceptibility protein
CAT	catalase
CDC7	Cell division cycle 7
CDK	cyclin dependent kinase
CHK2	checkpoint kinase 2
CHOP	CCAAT-enhancer-binding protein homologous protein
DAPI	4′, 6-diamino-2-phenylindole
DDR	DNA damage response
DISC	death inducing signaling complex
DMSO	dimethyl sulfoxide
DSB	double strand break
EGFR	epidermal growth factor receptor
EMEM	Eagle's minimum essential media
ER	endoplasmic reticulum

ESI	electrospray ionisation
FA	Fanconi's anemia
FBS	fetal bovine serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate
GADD	Growth Arrest and DNA Damage
GO	gene ontology
HCD	high collision dissociation
HDMEC	human dermal microvascular endothelial cell
HMOX1	heme oxygenase 1
Hsp90	heat shock protein 90
IAP	inhibitor of apoptosis
IPA	ingenuity pathway analysis
IRE	iron responsive element binding protein
iTRAQ	isobaric tags for relative and absolute quantitation
JAK	Janus kinase
JC1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide
KCl	potassium chloride
KDR	kinase insert domain receptor
Keap1	Kelch like-ECH associated protein 1
KOH	potassium hydroxide
LC	liquid chromatography
LDH	lactate dehydrogenase
MDM2	mouse double minute 2 homolog
MDR	multi-drug resistance

MOMP	mitochondrial outer membrane permeabilization
MS	mass spectrometry
mTOR	mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa-light chain enhancer of activated B cell
Nrf2	nuclear factor erythroid 2-related factor
NuMA	nuclear mitotic apparatus protein
ORAC	oxygen radical absorbance capacity
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PI	propidium iodide
PK	protein kinase
PS	phosphatidylserine
PSM	peptide spectrum matching
PTP	permeability transition pore
RB1	retinoblastoma 1
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell park memorial institute
SD	standard deviation
SFTPB	surfactant protein B
Smac	second mitochondria-derived activator of caspase
SSB	single strand break
STAT	signal transducers and activators of transcription

TNF	tumor necrosis factor
TP53	tumor protein p53
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis inducing ligand receptor
TUNEL	terminal deoxy transferase transferase-mediated dUTP nick end labelling
UV	ultraviolet
VEGF	vascular endothelial growth receptor
WFDC2	WAP Four-Disulfide Core Domain 2
WHO	World Health Organisation

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of study

1.1.1 Cancer incidence and death

Cancer is one of the leading causes of death worldwide with 8.2 million deaths in 2012 according to World Health Organization (WHO). A project by The International Agency for Research on Cancer, GLOBOCAN 2012 showed that there were 14.1 million new cancer cases while 8.2 million were accounted for cancer deaths with lung cancer being the most highly diagnosed cancer followed by breast and colorectal cancer worldwide (Ferlay et al., 2015). Lung cancer accounts for 1.8 million new cancer cases and 1.6 million cancer deaths globally. The second most common cancer, breast cancer followed by colorectal cancer has 1.7 million new cancer cases and 0.52 million cancer deaths and 1.4 million new cases and 0.69 million deaths respectively. In Malaysia, there are 37,400 people who are newly diagnosed with cancer for both male and female in 2012. The number of cancer mortality is 21,700 in 2012 and the 5 most frequently diagnosed cancers for both male and female in Malaysia are breast cancer, followed by colorectal cancer, lung cancer, cervical cancer and nasopharynx cancer.

Table 1.1: Number of new cancer cases and cancer deaths worldwide in 2012 for the top 3 cancer types(Ferlay et al., 2015).

Cancer type	New cancer cases (thousand)	Cancer deaths (thousand)
Lung cancer	1,800	1,600
Breast cancer	1,700	520
Colorectal cancer	1,400	690
Cervical cancer (female)	528	266

Table 1.2: Malaysia cancer statistics. Number of new cancer cases, deaths and ranking of cancers in Malaysia for both male and female (Ferlay et al., 2015).

MALAYSIA	**Male**	**Female**	**Male & Female**
Population (thousands)	14862	14459	29321
Number of new cancer cases (thousands)	18.1	19.3	37.4
Number of cancer deaths (thousands)	11.3	10.4	21.7
5 most common cancers			
1)	Lung	Breast	Breast
2)	Colorectal	Cervix uteri	Colorectal
3)	Nasopharynx	Colorectal	Lung
4)	Prostate	Lung	Cervix uteri
5)	Stomach	Ovary	Nasopharynx

1.1.2 Natural products and its role in cancer

Natural product has been an important medicinal source for different types of diseases and illnesses since ancient times. During the ancient times, people have been using natural products as medicines for illnesses such as fever, asthma, constipation, obesity, cough, infections and others in the form of traditional medicines, ointments, potions, and remedies.

According to The World Medicine Situation 2011 report, by World Health Organization (WHO), 70-95% of populations from developing countries including Asia, Latin America, Middle East and Africa are relying on traditional medicines for primary care. The development in natural product chemistry has brought to light the importance of bioactive metabolites especially secondary metabolites to treat different diseases. This has led to the isolation of secondary metabolites from natural resources that become prominent in pharmaceuticals. In 1971, natural products have been used for the first time to treatcancer (Hartwell, 1971). Paclitaxel (Weaver, 2014), camptothecin derivatives (Venditto et al., 2010), vincristine and vinblastine (Mann, 2002) are

clinically approved drugs that have long been used and well-known for the treatment of different cancers. Naturally occurring compounds and their derivatives have been proven to be able to cause inhibition to cancer cells and have cytotoxic effects to cancer cells. The groups of different compounds include the phenolic compounds, flavonoids, terpenoids, alkaloids, polysaccharides, lectins and many more.

Researchers have shown that natural products and the components can result in modification to the cancer cells and also initiate cell death in cancer cells. Their actions include the modification of gene expression, inducing cell cycle arrest, DNA damage, inhibition of cell proliferation, and other characteristics that can cause the induction of apoptosis in the treated cancer cells. Hence, natural products are becoming important in drug discovery and phytochemicals have become an important component in the search for anti-cancer agents. Natural products that are being studied extensively include curcumin (Shehzad et al., 2013), resveratrol (Singh et al., 2015), and isothiocyanates (Wu et al., 2009) and these bioactive compounds have the potential to become the new drug candidates for cancer treatment.

1.1.3 Discovery of chalcone as potent anti-cancer agent

Chalcone is biological compound reported to be the precursor of flavonoids and isoflavonoids. The structure of chalcone varies in different conjugated forms with two aromatic rings joined by a three-carbon unsaturated carbonyl bond. A large number of chalcone derivatives can be yielded and these derivatives showed promising biological activities like anti-neoplastic, anti-inflammatory, anti-oxidant, anti-hypertensive andother biological activities. Hence, these studies have proven that chalcones have potential as anti-cancer agents. This group of biological compounds has the ability to inhibit the degradation of tumor suppressor protein, pathways related to tumor invasion and multi-drug resistance for chemotherapy, to block the process of angiogenesis, and to suppress cell proliferation by acting on the cell proliferative signaling pathways.

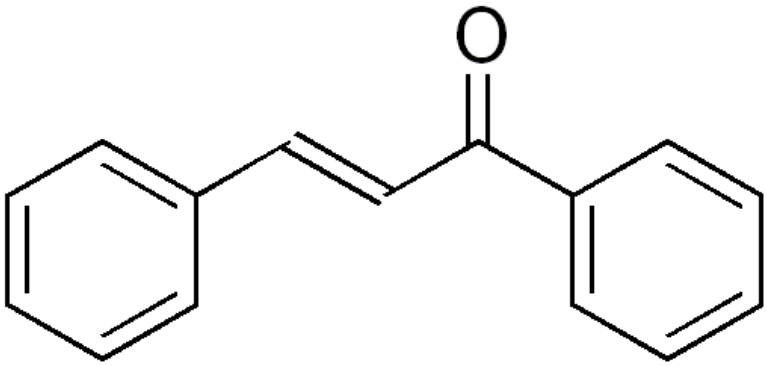


Figure 1.1: Basic chemical structure of chalcone

Helichrysetin is categorized as chalcone three hydroxyl groups, and one methoxy group. Helichrysetin is a naturally occurring chalcone that can be found in rhizomes of *Boesenbergia pandurata*, some plants from seeds of *Alpinia* sp., and flowers of *Helichrysum* sp. Several studies have proven that this compound exhibits cytotoxic activity towards different cancer cell lines. However, no further scientific research has been done to investigate the molecular mechanisms related to the inhibitory activity of helichrysetin on cancer cells.

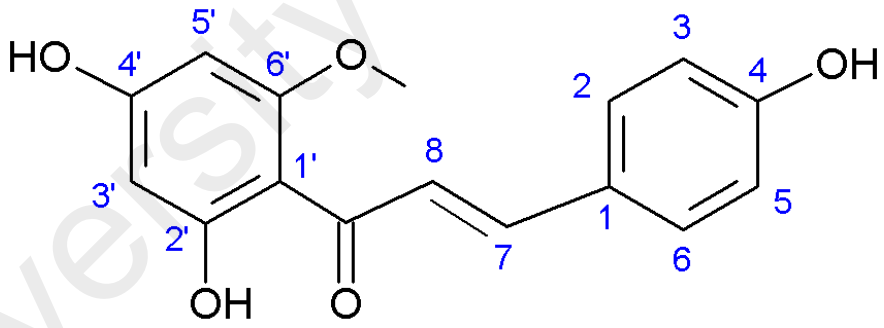


Figure 1.2: Chemical structure of helichrysetin

The purpose of this study is to evaluate the growth inhibitory activity of helichrysetin on selected cancer cell lines and normal fibroblast cell line, A549, Ca Ski, HT-29, MCF-7 and MRC-5 cell lines and to detect apoptotic features and changes to the cell cycle progression upon treatment with helichrysetin. In addition, proteomic study is performed to investigate the molecular mechanisms involved in the action of helichrysetin in causing cancer cell death.

1.2 Aim and objectives

As stated, helichrysetin is a naturally occurring compound with potential to be developed as an anti-cancer agent due to its effective growth inhibitory and cytotoxic activity on cancer cell lines. This study serves as part of the effort to investigate the potential of helichrysetin as a novel anti- cancer drug with minimal undesirable side-effects, high therapeutic efficiency, and active against some of the untreatable cancer.

In order to reveal its potential as anti-cancer agent, the following investigations have been carried out:

1) Evaluation of growth inhibitory activity of helichrysetin on four selected human cancer cell lines and human normal cell line and observation of the effect of helichrysetin on the cellular and nuclear morphology
2) Detection of the occurrence of apoptosis in cancer cell line upon exposure to helichrysetin by detecting its apoptotic features and cell cycle progression.
3) Investigation of the molecular mechanisms involved in the action of helichrysetin on cancer cell lines using bottom up proteomic approach to observe the protein changes in the cells.

1.3 Hypothesis

The following hypotheses have been considered prior to the beginning of the study:

1) Helichrysetin can inhibit the growth of the selected human cancer cell line and helichrysetin is not active towards human normal cell line.
2) This compound is able to induce apoptotic cellular and nuclear morphological changes in human cancer cells.
3) Upon exposure to helichrysetin, biochemical apoptotic features will be induced in the cancer cells and helichrysetin can induce cell cycle arrest.

4) Helichrysetin triggers apoptotic signaling response through the intrinsic or extrinsic apoptotic pathways in effect of cellular changes in the cells such as DNA damage and cellular stress.

1.4 Thesis structure

The thesis was written in 6 chapters. Chapter One presents the general introduction of this study including the background, objectives and hypothesis of the study. Chapter Two includes literature review on cancer, natural products, chalcone, helichrysetin, apoptosis and cell death molecular mechanisms. Chapter Three describes the Methodology employed through the whole study. Chapter Four shows all the results and findings obtained from this study while Chapter Five contains the discussion and conclusion of this study. Chapter 6 concludes the finding and describes the fulfillment of the three main objectives of this study.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer can be defined as the result of uncontrolled proliferation of cells in the body. The development of cancer occurs through the abnormal tissue growth that no longer displays proper cell organization, namely, dysplasia which can later develop into neoplasia. As neoplasm emerges, the rate of cell division and cell differentiation becomes imbalance. This result in the great number of new cells being produced and these cells can further develop into invasive cancer that can migrate and metastasize to neighbouring tissues and organs hence disrupting the normal function of the tissue or organ. According to Hanahan and Weinberg, there are six biological capabilities that are acquired by cells in the process of tumor development. The capabilities include sustaining proliferative signaling, evading growth suppressors, cell death resistance, activation of replicative immortality, angiogenesis, and activating metastasis and invasion (Hanahan et al., 2011).

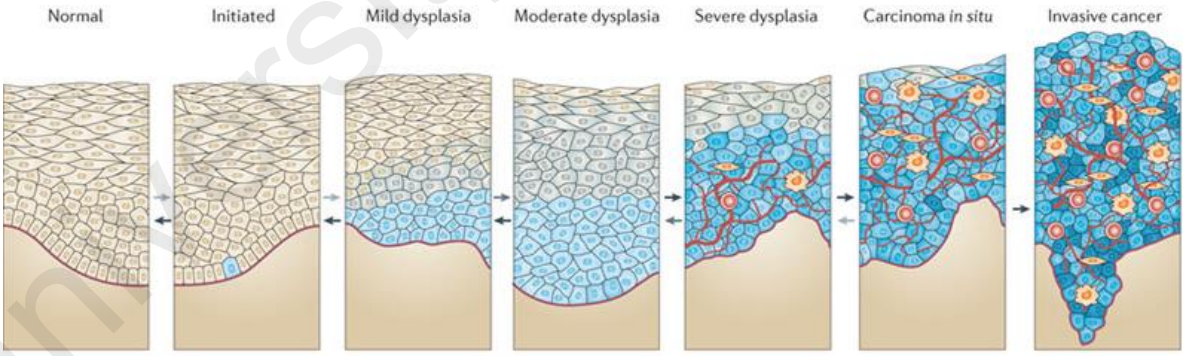


Figure 2.1: The development of normal tissue to invasive cancer. Cancer progression occurs at different points and gene mutations contribute to the progression from normal tissue to dysplasia, carcinoma in situ and to invasive cancer. (Source: Umar et al.,2012)

Carcinogenesis or cancer development can be divided into different stages, tumor initiation, tumor promotion, and tumor progression. Genetic mutation has been widely known as the early event in carcinogenesis; however, it has been found that epigenetics also contributes to the development of cancer. Chemical carcinogen can trigger genetic error by changing the structure of DNA which will be inherited through DNA synthesis and replication and this is done by the formation of adduct between the carcinogen with DNA (Yuspa et al., 1988). Initiated cells will undergo clonal expansion in the tumor promotion stage.

Tumor promoters are able to cause biological effects without affecting the metabolic process and they are commonly non-mutagen or carcinogen. Tumor promoters are able to cause tumor formation by increasing the number of tumors in a tissue in a short latency period (Verma et al., 1980). In the tumor progression stage, malignant phenotype starts to be expressed and malignant cells will acquire aggressive characteristics with the tendency for genomic instability and uncontrolled growth (Lengauer et al., 1998).

Cancer can be distinguished as either benign or malignant tumors. The growth of benign tumor is restricted to its local area while malignant tumor invades the neighbouring tissue and spread throughout the body via the circulatory system. Cancers are commonly categorized in to three main groups namely carcinoma, sarcoma and lymphoma. Carcinoma, sarcoma and lymphoma are of epithelial, connective tissues and blood or lymphatic origins respectively.

2.2 Natural products and cancer

Natural products play a big role in the effort of discovery and development of drugs for different diseases. People from different cultures have used a variety of natural products as traditional medicine and some proven to have potent anticancer effects. Prominent

naturally-derived anticancer drugs include campthothecin, vincristine, vinblastine, and paclitaxel (Bhanot et al., 2011).

The vinca alkaloids, vincristine and vinblastine are the first agents that participitated in the clinical use for the treatment of cancer including lung cancer, leukemia, breast cancer, and Karposi's sarcoma and these alkaloids are derived from the Madagascar periwinkle (Chen et al., 2012; Devita et al., 1970).

While paclitaxel that comes from the bark of Pacific yew tree is another proven success of the usage of natural products as anti-cancer agent. Paclitaxel is effective towards breast cancer, non-small and small cell lung cancer, and ovarian cancer. It targets on the tubulin of cells and stabilizes the structure of microtubule that leads to the blockage in the progression of mitosis (Demidenko et al., 2008). Prolonged mitosis will result in the occurrence of apoptosis and reversion of the cell cycle.

Camptothecin is extracted from the plant *Camptotheca acuminate* which exhibits anti-cancer activity towards different types of cancer cells and it acts by inhibiting DNA topoisomerase I causing death to cancer cells (Gaur et al., 2014). However, due to its poor solubility and stability, camptothecin derivatives have been developed such as irinotecan and topotecan. These two compounds have been clinically approved for use on metastatic colorectal cancer, ovarian cancer, cervical cancer and small-cell lung cancer respectively (Venditto et al., 2010). Studies are also being conducted to use camptothecin derivatives in late-stage cancer therapy and also in combination with other drugs (Harasym et al., 2007).

2.3 Chalcone

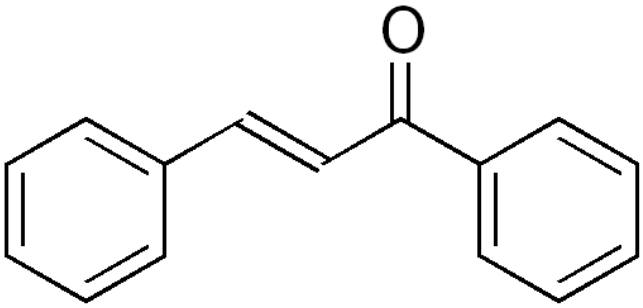


Figure 2.2: Chemical structure of chalcone

Chalcone, trans-1, 3-diaryl-2-propen-1-ones, consists of two aromatic rings with a diverse array of substituents. It belongs to the flavonoid family and is the precursors of open chain flavonoids and isoflavonoids that can be found abundantly in plants (Rahman, 2011). The two aromatic rings in chalcones are connected by an electrophilic three carbon α, β-unsaturated carbonyl system (Awasthi et al., 2009). It has developed a vast interest for medicinal chemist because it has displayed a wide spectrum of pharmacological effect such as anticancer, antioxidant (Vogel et al., 2008), antimicrobial, antifungal (Bag et al., 2009), anti-inflammatory (Hamdi et al., 2011), antibacterial activities (Yadav et al., 2011).

Chalcones have been found to inhibit multi-drug resistance (MDR) channels part of being chemosensitizers by improving the absorption of cancer chemotherapeutic drugs that are poorly absorbed in the intestine (Vasiliou et al., 2009). Chalcones also act as inhibitors for the degradation of tumor suppressor proteins (Issaenko et al., 2012), p53 which prevents cancer by regulating cell cycle, mediates apoptosis, DNA repair, genome stability and inhibition of angiogenesis. It's been reported that chalcone derivaties inhibited the JAK/STAT signaling pathway which when activated can caused abnormal growth of malignant cancer and neoplastic transformation (Pinz et al., 2014). Zhu et al. had demonstrated the ability of 2,4-dihydroxy-6-methoxy-3,5-

dimethylchalcone to inhibit the phosphorylation of KDR tyrosine kinase, a vascular endothelial growth receptor (VEGF) resulting in the inhibition of the growth of human vascular endothelial HDMEC cells consequently blocking the process of angiogenesis (Zhu et al., 2005). Cell proliferation can be suppressed by the action of chalcones due to its inhibition on Cathepsin-K (Ramalho et al., 2013) that contributes to tumor invasion, tubulin assembly in cell cycle, mTOR signaling pathway (Sun et al., 2010), NF-KB inhibition (Orlikova et al., 2012), and also its cytotoxic activity (Nakhjavani et al., 2014)

2.3.1 Helichrysetin

Helichrysetin,1-(2,4-dihydroxy-6-methoxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one was isolated as orange-yellow crystal with the melting point of 248 ºC and molecular weight of 286 g/mol. It was isolated from the flowers of *Helichrysum odoratissimum* (Van Puyvelde et al., 1989), *Helichrysum foetidum* (Zanetsie Kakam et al., 2011), rhizomes of *Boesenbergia pandurata* (Tewtrakul et al., 2009), seeds of *Alpinia blepharocalyx.*

Synthesis of helichrysetin was also done by Puyvelde *et al.* Helichrysetin was synthesized by the condensation of 1,3-dihydroxy-5-methoxy-benzene with MeCN to produce ketimine hydrochloride. It was the hydrolysed to yield 2,4-dihydroxy-6-methoxyacetophenone. Finally, it was condensed with aldehyde in 60% aqueous KOH containing some ethanol to give helichrysetin (Van Puyvelde et al., 1989).

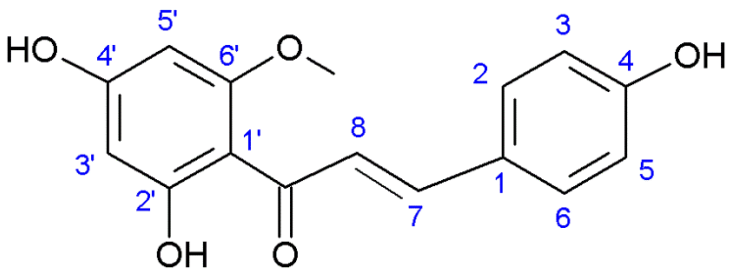


Figure 2.3: Chemical structure of helichrysetin

Helichrysetin isolated from *Alpinia blepharocalyx* have been shown to have strong platelet aggregation inhibition (Doug et al., 1998). Helichrysetin had shown a high cytotoxic effect on HeLa cell line in the MTT cell proliferation assay with the IC_{50} value of 5.2 ± 0.8 µM (Vogel et al., 2008).

This natural compound isolated from the seeds of *Alpinia katsumadai* showed inhibition against human liver cancer cell line HEPG2 and human breast cancer cell lines MCF-7 and MDA-MB-435 with IC_{50} values of 14.64 µg/ml, 24.22 µg/ml and 1.83 µg/ml respectively (Hua et al., 2008).

It was reported that substitution of hydroxyl group, electron donating group at the ortho and para position of benzene can enhance tumour reducing activity (Anto et al., 1995). Chalcone that was found to have high antitumour activity also possess the antioxidant activity. Hence, the antioxidant activity of helichrysetin has also been investigated with the remarkable activity of 4.4 ± 0.6 Trolox equivalents in ORAC-fluorescein assay.

2.4 Development of cancer and cell death

Clinicians and researchers have been striving to look for novel targets for cancer to improving existing cancer therapies. The development of cancer or tumorigenesis has been highly related to the changes in genetic information of cells and biological processes play an essential role in sustaining tumorigenesis. Review by Hanahan and Weinberg discussed the biological processes involved in tumorigenesis and the resistance of cell death is proposed as an important mechanism that sustain tumorigenesis and malignant transformation (Hanahan et al., 2011).

Hence, the deregulation of apoptosis has come into the light as one of the hallmarks of cancer. This deregulation is facilitated by the occurrence of mutations in oncogenes and tumor suppressors. The tumor suppressor gene TP53 is highly mutated in human cancer which has been linked to apoptosis (Amundson et al., 1998). Even though there is a

huge number of biological processes and therapeutics agents known and available, most of these agents are still depending on the induction of apoptosis to kill cancer cells. Therefore, it is important to discover potential cancer drugs that causes cancer cell death through apoptosis.

Other components that are frequently deactivated in human cancer are MDM2, ARF, RB1 which are responsible for regulation of DNA damage checkpoint pathways (Møller et al., 1999). Ras gene which is involved in kinase signaling pathways for cell growth and differentiation is also an oncogene that is commonly mutated in cancer such as colon, lung, pancreas, breast, liver and ovarian cancer (Schubbert et al., 2007). Hence, this protein encoded by Ras gene and those important for the formation of cancer cells become important drug target over the years.

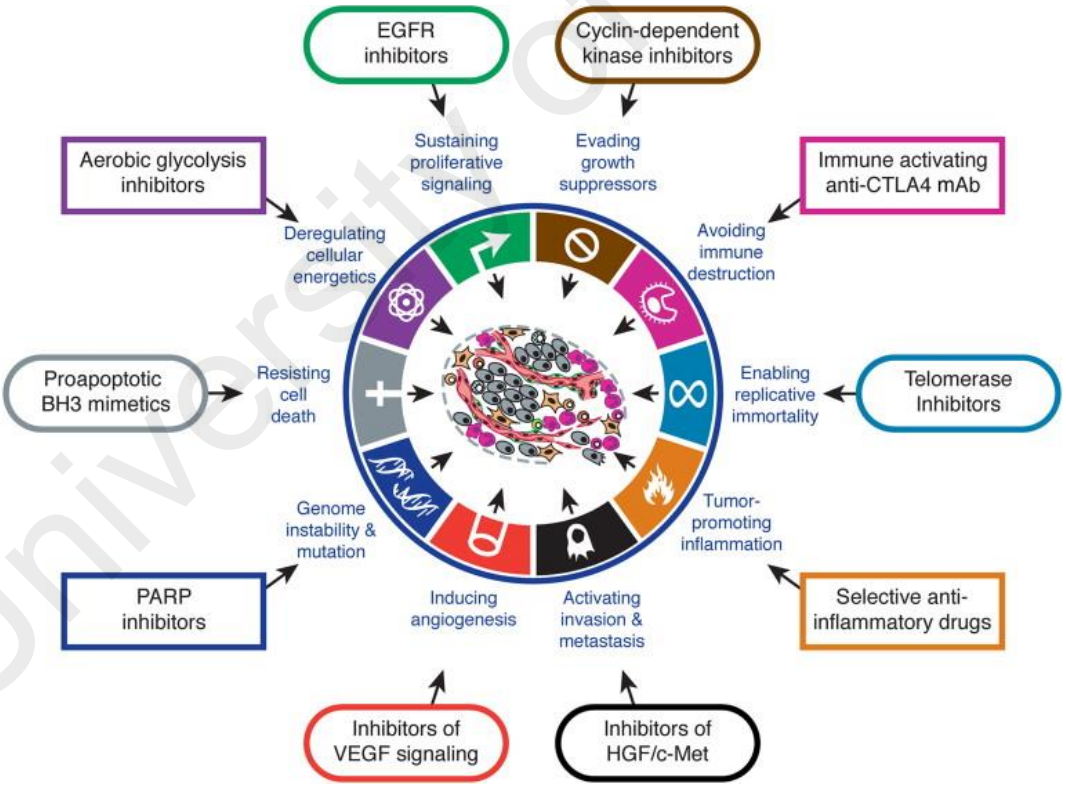


Figure 2.4: The hallmark of cancer displaying the characteristics of cancer cells for development of cancer and drugs that specifically interfere with each of the ability of cancer for tumor growth and progression that have been clinically-approved or in clinical trials. (Source: Hanahan and Weinberg,2011)

2.4.1 Cell viability assays

The commonly used method to screen molecules for its effect on cell proliferation is by using cell-based assays. The amount of live cells that remain at the end of the experiment will be measured. Cell-based assays can help to measure specific events in the cells upon exposure to treatments, such as cell organelle activities, cellular components trafficking, and different types of signal transductions events (Quent et al., 2010). Cell-based assays can be performed using different methods such as multi-well formats, flow cytometry, and high content imaging (Kepp et al., 2011).

Cell viability can be measured using different indicators such as fluorescence, absorbance, and luminescence detection methods and this is often measured by quantifying parameters such as membrane integrity, esterase activity, ATP levels, or simply by counting the cell nucleus (Gilbert et al., 2011). The most commonly used cell viability assays forin vitro toxicology studies are MTT assay, neutral red assay, LDH leakage, and protein assays upon exposure to potential toxic substances (Fotakis et al., 2006).

2.4.2 Morphological characteristics of cell death

Cell death in animal cells is discriminated into three main forms, apoptosis, necrosis and autophagic cell death (Kroemer et al., 2005). Morphological features of apoptosis are pyknosis, chromatin condensation, karyorhexis, plasma membrane blebbing and cell rounding (Kerr et al., 1972). The initiation of apoptosis is induced by different types of stimuli and its occurrence requires a series of signal transduction and downstream process (Ziegler et al., 2004).

In autophagic cell death, there is an increase autophagosomes which will later fuse with lysosomes for degradation process (Levine et al., 2004). Autophagy is normally seen as a pro-survival mechanism but it is also involved in promoting cell death.

Necrosis is a mechanism of cell death that is often stress-induced. In the past, necrosis has been seen as a process without proper mechanisms, however in recent years, researchers have shown that the process of necrosis will involve a series of mechanisms too. Necrosis is characterized by swollen organelles in the cells, rupture of plasma membrane, increase in cell volume, and exposure of intracellular components into the outer environment (Denecker et al., 2001).

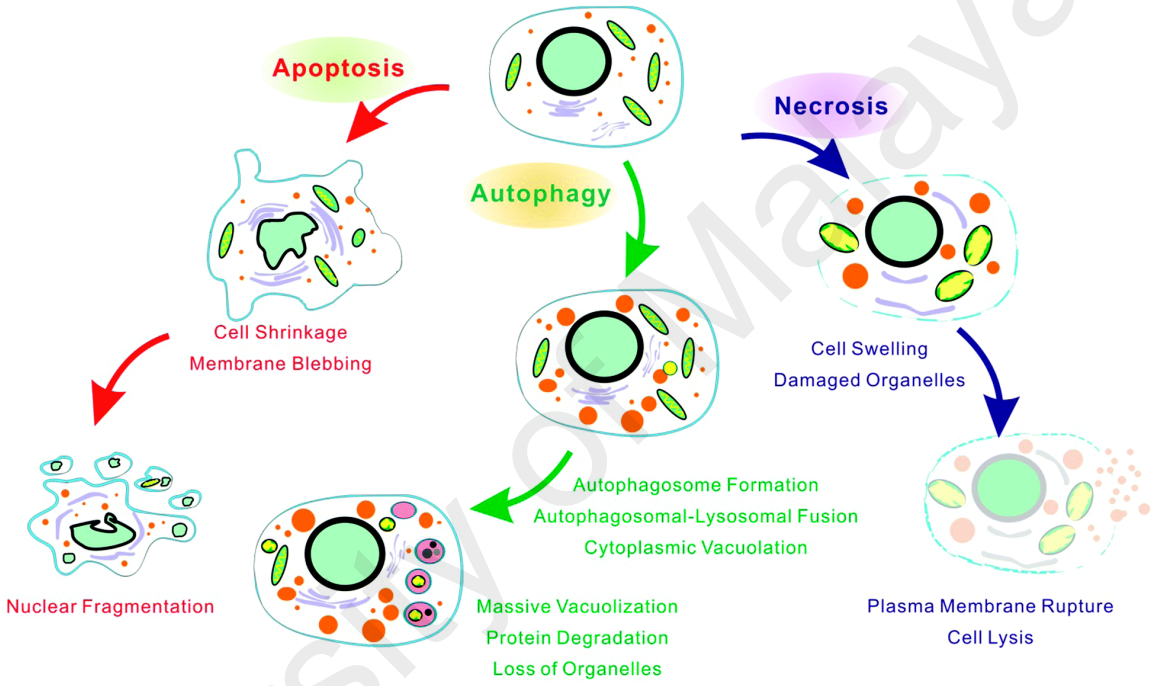


Figure 2.5: The morphological features of three main cell death processes, apoptosis, autophagy and necrosis. (Tan et al., 2014)

2.4.3 Apoptosis

Apoptosis is described as programmed cell death responsible for the tissue homeostasis in multicellular organisms by maintaining the balance between cell death and cell proliferation. Apoptosis can occur under normal physiological or pathological settings. The failure to regulate the mechanisms of cell suicide will result in the development of diseases (Fadeel et al., 2005).

In some pathological conditions when there is too much apoptosis, it will initiate the development of degenerative diseases. In the case of cancer, there is too little apoptosis

which results in the uncontrolled growth of malignant cells. Since the deregulation of apoptosis is one of the main reasons for the development of diseases including cancer when cells are resistant to programmed cell death, hence, a better understanding of this condition is important to help develop possible therapeutic strategies for the treatment of cancer.

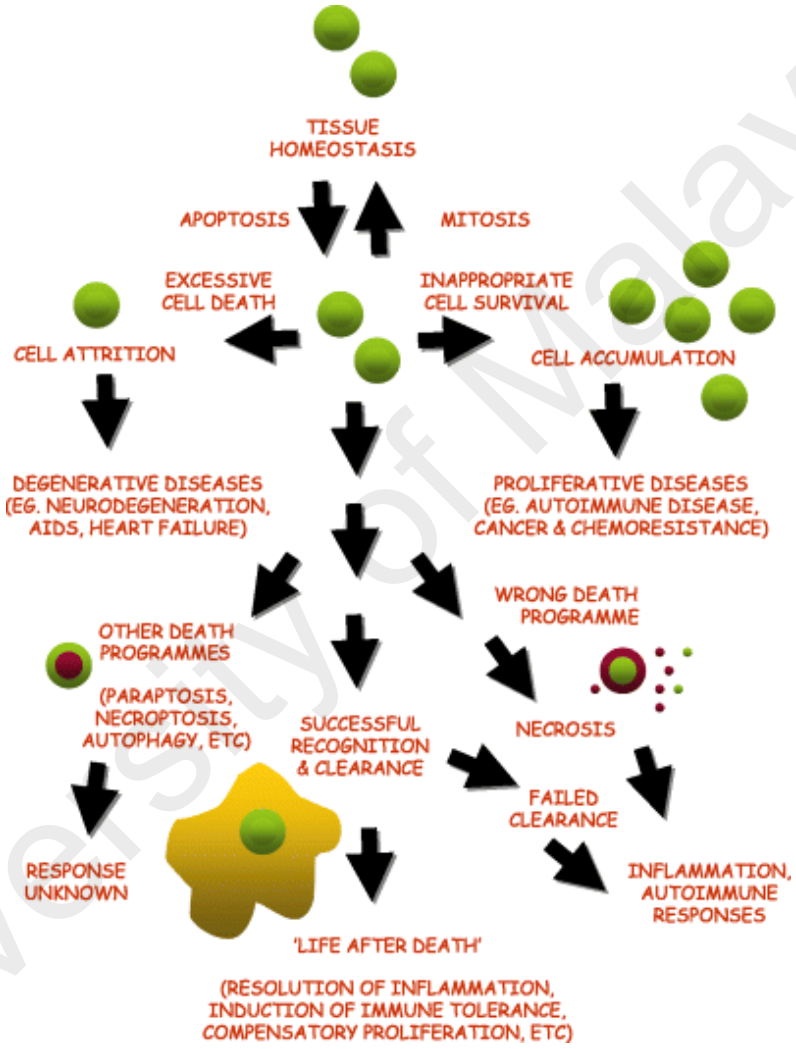


Figure 2.6: Role of apoptosis in human disease. Apoptosis is responsible for tissue homeostasis and its disruption will contribute to the pathogenesis of some human diseases. (Fadeel and Orrenius, 2005)

2.4.4 Features of apoptosis

Morphological characteristics of apoptosis in the nucleus are fragmentation of nucleus, and chromatin condensation together with the changes in the cellular morphology by the rounding of the cells, and reduction in the cell volume (Kroemer et al., 2005). Upon

chromatin condensation that occurs at the periphery of nuclear membrane, chromatin will continue to condense until it breaks up in the cell while the membrane remains intact. This phenomenon is termed karyorhexis (Majno et al., 1995).

In the later stage of apoptosis, extensive membrane blebbing leads to separation of cell content through the formation of apoptotic bodies. This stage involved the disruption in the plasma membrane integrity and also structural modification of organelles in the cytoplasm. Then, the apoptotic bodies from the dead cells will be engulfed through phagocytosis (Krysko et al., 2006). In the case where apoptotic cells are not engulfed by phagocytosis, they turn to secondary necrotic cells (Silva, 2010).

The occurrence of apoptosis also involved a few types of biochemical changes. As discussed earlier, in late apoptosis, disruption of plasma membrane integrity occurs and in order for this to happen in the earlier stage apoptosis, phosphatidylserine is flipped to the outer layer of plasma membrane. Following this, dead cells will be recognized by macrophages for degradation by phagocytosis (Kumar et al., 2010). Upon membrane blebbing and chromatin condensation, chromosomal DNA will be cleaved sequentially to oligosomal DNA fragments with the size of 50 to 300 kilobases pair and then to multiples of 180 to 200 base pairs (Vaux et al., 2003).

The breaking down of apoptotic protein is also a specific feature of apoptosis. A group of enzymes under the family of cysteine proteases named caspases will be activated. Caspases are enzymes that cleave aspartic acid residues by cysteine in its active site in many important cellular proteins involved in the process of apoptosis which include the proteins that support the structure of nucleus and cell cytoskeleton (Fan et al., 2005).

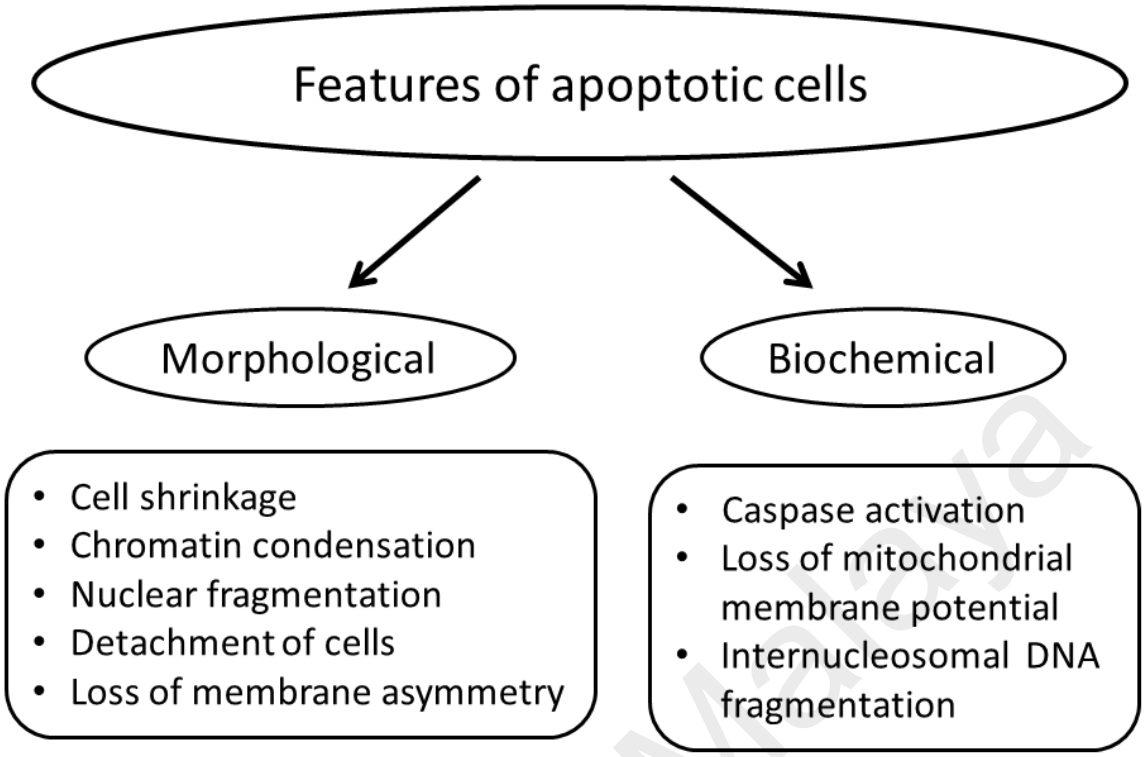


Figure 2.7: Features of apoptosis. Apoptosis causes changes to morphological and biochemical characteristics to cells.

Techniques performed to study the features of apoptosis should allow quantification, observe the qualitative differences in different experimental conditions, distinguish different stages of apoptosis, and revelation of molecular changes in different conditions (Archana et al., 2013). Changes in the morphology of the cells can be identified by light microscopy specifically during the early apoptosis when the cells start to shrink and pyknosis occur (Ziegler et al., 2004). A better view of the subcellular changes can be defined by applying electron microscopy which shows distinct changes like chromatin condensation and nuclear material aggregating under the nuclear membrane (Louagie et al., 1998), extensive membrane blebbing and formation of apoptotic bodies (Lawen, 2003).

One of the features of apoptosis is the fragmentation of DNA in the cells and this can be observed using the gel electrophoresis. Gel electrophoresis is a reliable technique to

visualize the high molecular weight DNA fragments approximately about 300kb (Higuchi, 2004) (Singh, 2000).

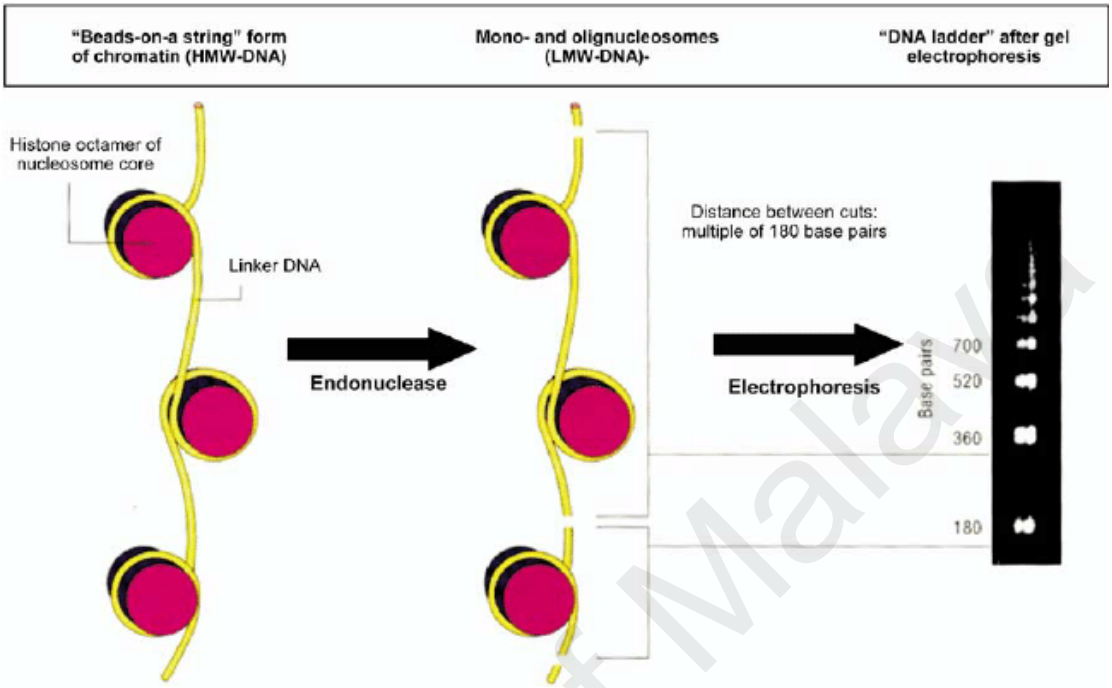


Figure 2.8: Structure of high molecular weight DNA fragment and formation of low molecular weight DNA fragment. DNA fragments base pairs detected using gel electrophoresis to visualize DNA ladder (Scarabelli et al., 2006).

For accurate quantification of apoptotic cells, components in the cells can be stained with fluorochromes to detect apoptotic features using flow cytometry. DNA fragmentation in the cells can be quantified using flow cytometry detection by terminal deoxy transferase transferase-mediated dUTP nick end labelling (TUNEL), propidium iodide (PI), 4′, 6-diamino-2-phenylindole (DAPI). To detect the loss of membrane integrity, cells can be stained with annexin-V fluorochrome to quantify number of cells that have undergone phosphatidylserine externalization that occurs in the calcium dependent manner (Vermes et al., 1995).

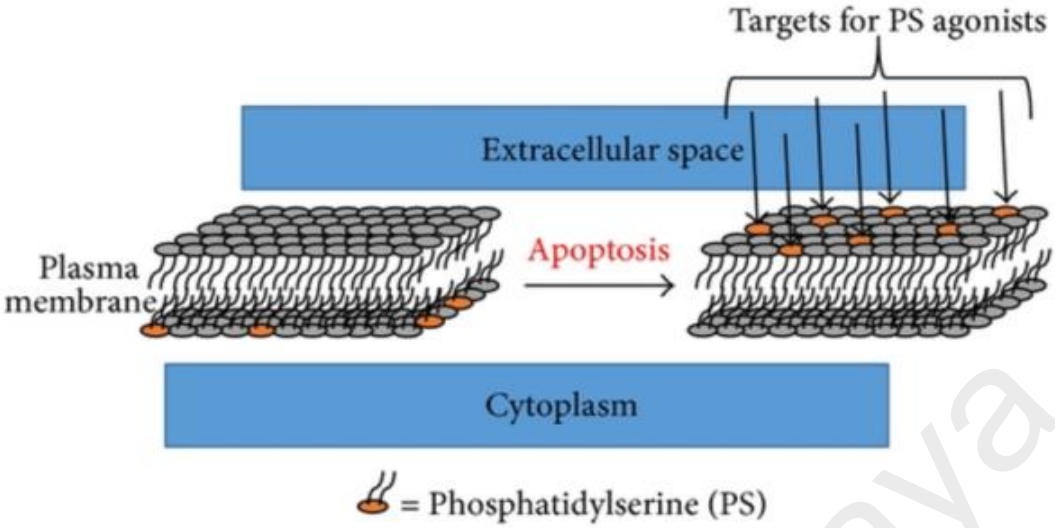


Figure 2.9: Externalization of phosphatidylserine (PS) to the extracellular space of the cells during the occurrence of apoptosis (Sogbein et al., 2014).

2.4.5 Mechanisms of apoptosis

It is important to understand the mechanisms underlying the process the apoptosis in order to know the pathological conditions of deregulated apoptosis in the cells. This will provide insights in the development of drugs which will target particular pathways or genes related to apoptosis for cancer therapy.

Apoptosis consists of upstream regulators and downstream effector components. The initiation pathways that are frequently described are the intrinsic and extrinsic apoptotic pathways that are triggered in mitochondrial and by death receptors respectively (Fulda, 2010). Apoptosis also can be triggered by intrinsic endoplasmic reticulum pathway, a pathway that is not commonly known (Breckenridge et al., 2003).

Intrinsic mitochondrial pathway is triggered by the internal stimuli for example oxidative stress, irreparable genetic damage, hypoxia and high concentrations of Ca^{2+} in the cytosol (Karp et al., 2008). Upon stimulation of this pathway, the permeability of mitochondria will be increased and pro-apoptotic molecules will be released from the mitochondria to the cytoplasm (Danial et al., 2004). Bcl-2 family proteins are important

proteins that regulate the intrinsic apoptotic pathway and the proteins are separated into pro-apoptotic and anti-apoptotic Bcl-2 family proteins (Siddiqui et al., 2015). The balance of these two groups of protein is important to determine the initiation of apoptosis in the cells. Anti-apoptotic proteins consist of four Bcl-2 homology (BH) domains that serve as protection to the cell from apoptotic stimuli. Anti-apoptotic proteins include Bcl-2, Bcl-xL, Mcl-1, Bcl-w and others, while the BH-3 only proteins include Bid, Bim, Bad, Puma, Noxa and others that are restricted to BH-3 domain only. Pro-apoptotic proteins are activated upon stimulation by cellular stress such as DNA damage, ER stress and growth factor depletion, and they contain all four BH domains and these proteins are Bax, Bak and Bok/Mtd proteins (Dewson et al., 2010).

The initiation of intrinsic pathway will induce other apoptotic factors such as apoptosis inducing factor (AIF), direct IAP Binding protein with Low pI (DIABLO), and second mitochondria-derived activator of caspase (Smac) (Kroemer et al., 2007). These apoptotic factors are responsible to bind to the inhibitor of apoptotic proteins which block the interference of IAPs to caspases (LaCasse et al., 2008).

The extrinsic apoptotic pathway is triggered by the binding of the death ligands to the death receptor. The most characterized death receptors are CD95 (Fas) receptor, TNF receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2 (Walczak et al., 2000). Death receptors consist of death domains that transmit death signals from the surface of cell membrane into the cell. Death domains will be recruited to the death receptor and this will cause the formation of death-inducing signaling complex (DISC) which will activate caspase 8 and result in the activation of downstream apoptotic pathways (Ganten et al., 2004).

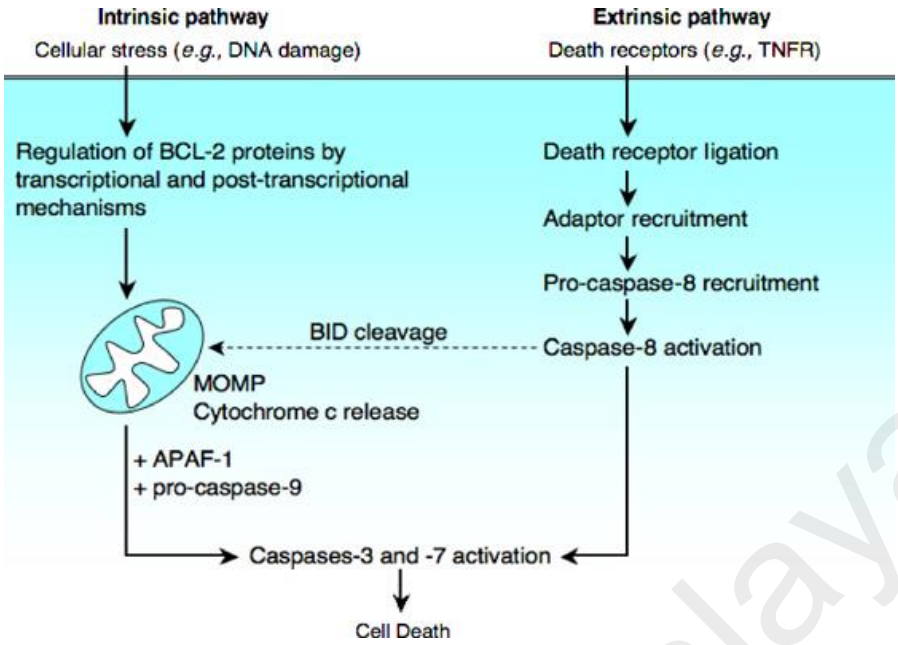


Figure 2.10: Intrinsic and extrinsic apoptosis cascades. Intrinsic pathway triggered by cellular stress while extrinsic pathway triggered by death receptor that cause apoptotic cell death. (Chipuk & Green, 2006)

In the intrinsic endoplasmic reticulum pathway, ER stress is coupled with the activation of caspase-12 and caspase 12 is localized at the cytosolic side of ER to prepare for the respond to ER stress and a signaling molecule (Nakagawa et al., 2000). When ER is exhausted by cellular stresses such as hypoxia, oxidative stress or glucose depletion, there will be reduction of protein synthesis and unfolding of proteins which will cause the dissociation of TNF receptor associated factor 2 (TRAF2) from procaspase 12 subsequently activating the caspase 12 (O'Brien et al., 2008).

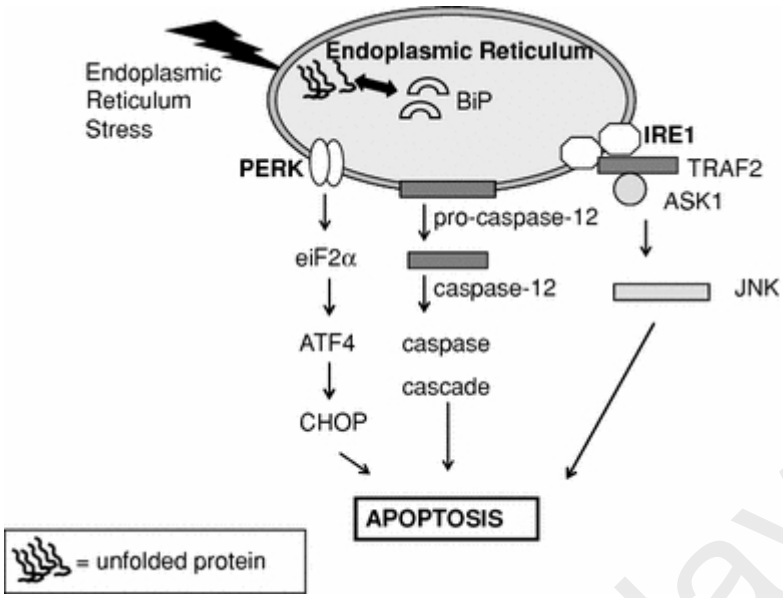


Figure 2.11: Endoplasmic reticulum (ER) stress induced apoptosis. ER stress-induced apoptosis is triggered by three main pathways, the proapoptotic pathway of CHOP/GADD153 transcription factor, IRE1 mediated-activation of apoptosis and activation of ER localized cysteine proteases. (van der Kallen et al., 2009)

In relation to this, p53, a well-known tumor suppressor protein plays a role as the central control governing the fate of the cells to undergo proliferation or apoptotis. Despite p53 being a tumor suppressor, p53 is often mutated in the cancer cells which contribute to the oncogenic property of cancer (Muller et al., 2013). Upon stimulation of p53 by intracellular cell stress and abnormality sensors such as excessive genome destruction, or growth-promoting signals, nucleotide pools and oxygenation being at lower than optimal level, p53 will stop the cell cycle progression and trigger apoptosis (Hanahan et al., 2011). More than half of the human cancers are linked to the mutation in the p53 protein and this protein is involved in many cellular processes such as apoptosis, cell cycle, cell differentiation, cellular senescence and others (Oren et al., 1999). One of the studies has found that when p53 mutant was silenced, it reduced the growth of cancer cells and this is found to be due to the occurrence of apoptosis in the cells (Vikhanskaya et al., 2007). The importance of understanding the molecular mechanisms has led researchers to use Western blotting to monitor the changes in the

molecular mechanisms of apoptosis in certain experimental conditions (Chandra et al., 2009).

2.5 Mitochondria as a target for cancer therapy

Mitochondria play an important role in cellular processes. Its actions involve the regulation of ATP production via oxidative phosphorylation in the inner mitochondrial membrane (Leist et al., 1997). The disruption in mitochondrial function can result in the development of diseases. Mitochondria participate in the mechanisms cell death because they play important role in cellular apoptotic responses.

Studies have shown the efficacy of compounds that inhibit mitochondrial function such as tigecycline that significantly inhibits mitochondrial respiration, disrupts mitochondrial membrane potential and increases reactive oxygen species in non-small cell lung cancer (Jia et al., 2016). Gamitrinib, a small molecule Hsp90 inhibitor has been proven to induce acute mitochondrial dysfunction in advanced prostate cancer cells with loss of membrane potential, cytochrome c release, and activation of caspase activity (Kang et al., 2010).

2.5.1 Mitochondria and apoptosis

Mitochondrial outer membrane permeabilization is an indicator of an irreversible event where cells commit suicide. During the onset of apoptosis, there is normally a dissipation of the mitochondrial inner transmembrane potential (Green et al., 2004). Commonly, upon mitochondrial outer membrane permeabilization, cytochrome c is released into the cytosol of the cells. It's been found that in apoptotic cells, after the release of cytochrome c and the activation of caspases will feed back to the permeabilized mitochondria to destroy the mitochondrial transmembrane potential and this will subsequently result in the generation of ROS by the action of caspases in electron transport chain (Ricci et al., 2003).

Since the collapse of mitochondrial membrane is highly related to its membrane potential, apoptosis in the cells can be quantified using flow cytometry method using a cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide). This dye can be used to detect the polarization and depolarization of the mitochondrial membrane as an indication of apoptosis (Perelman et al., 2012).

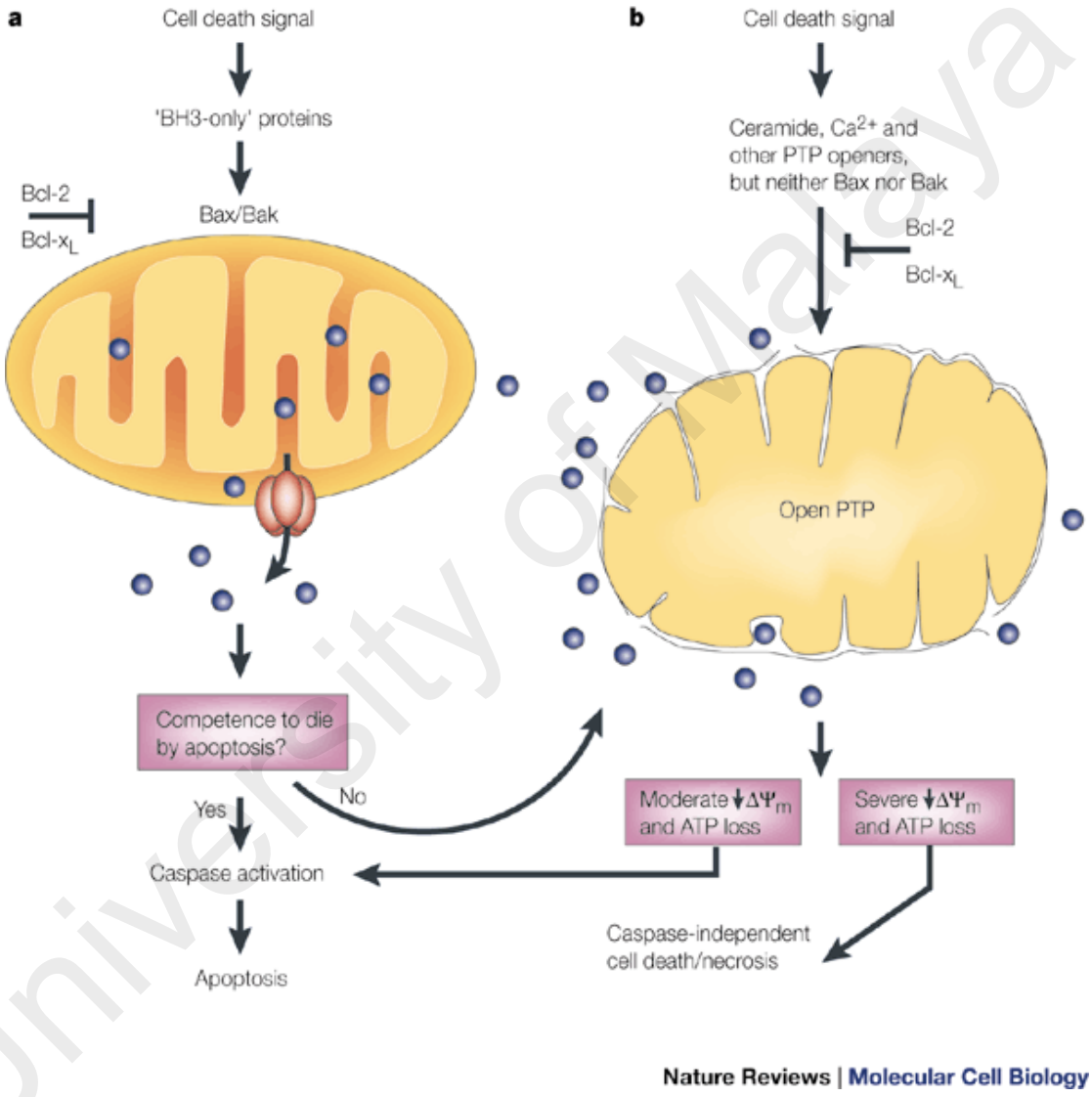


Figure 2.12: Models displaying the permeabilization of mitochondrial outer membrane. a) BH3-only proteins activate Bax proteins that leads to the formation of pores in the mitochondrial outer membrane. b) Death signals activate the opening of the permeability transition pore (PTP) leading to the disruption of mitochondrial membrane potential. (Martinou & Green, 2001)

Cytochrome c binds with adaptor molecule APAF-1 in the cytosol leading to an extensive conformational change that causes it to form a heptameric structure which we

called it apoptosome. Subsequently, apoptosome formed will recruit pro-caspase 9 and activate it which will then cleave and activate two executioner caspases, caspase 3 and caspase 7 (Taylor et al., 2008). Hundreds different substrates will be cleaved by caspase 3 and 7 which will effectively kill the cells within a few minutes.

2.5.2 Role of Bcl-2 proteins in mitochondrial-related apoptosis

Mitochondrial membrane potential change is largely regulated by the members of Bcl-2 protein family. These proteins can be separated into three divisions, pro-apoptotic effector proteins, pro-apoptotic BH3-only proteins, and anti-apoptotic proteins. The proteins that belong to these divisions are as followed:

Table 2.1: Bcl-2 family protein names categorized into its sub-divisions (Hardwick & Soane, 2013).

Bcl-2 protein family	Protein names
Pro-apoptotic effector proteins	Bax, Bak
Pro-apoptotic BH3-only proteins	Bid, Bim, Puma, Noxa, Hrk, Bik, Bmf, Bad
Anti-apoptotic Bcl-2 proteins	Bcl-2, Bcl-Xl, Mcl-1, A1, Bcl-B, Bcl-W

As an effect of apoptotic stress or cell damage, Bcl-2 family proteins will be activated. These proteins cause the activation of the pro-apoptotic proteins, Bax and Bak in mitochondria. Bax and Bak are gathered to form oligomers which contribute to the formation of pores in the outer membrane of mitochondria, subsequently the release of cytochrome c and activation of caspases for apoptosis (Certo et al., 2006).

The mechanism of mitochondrial outer membrane permeabilization ultimately represents an irreversible action. BH3-only proteins consist of activators or sensitizers that neutralize the effect of anti-apoptotic Bcl-2 proteins, hence, most of the drugs can

act as BH3-only proteins finally activating apoptosis in the targeted cells(Lopez et al., 2015).

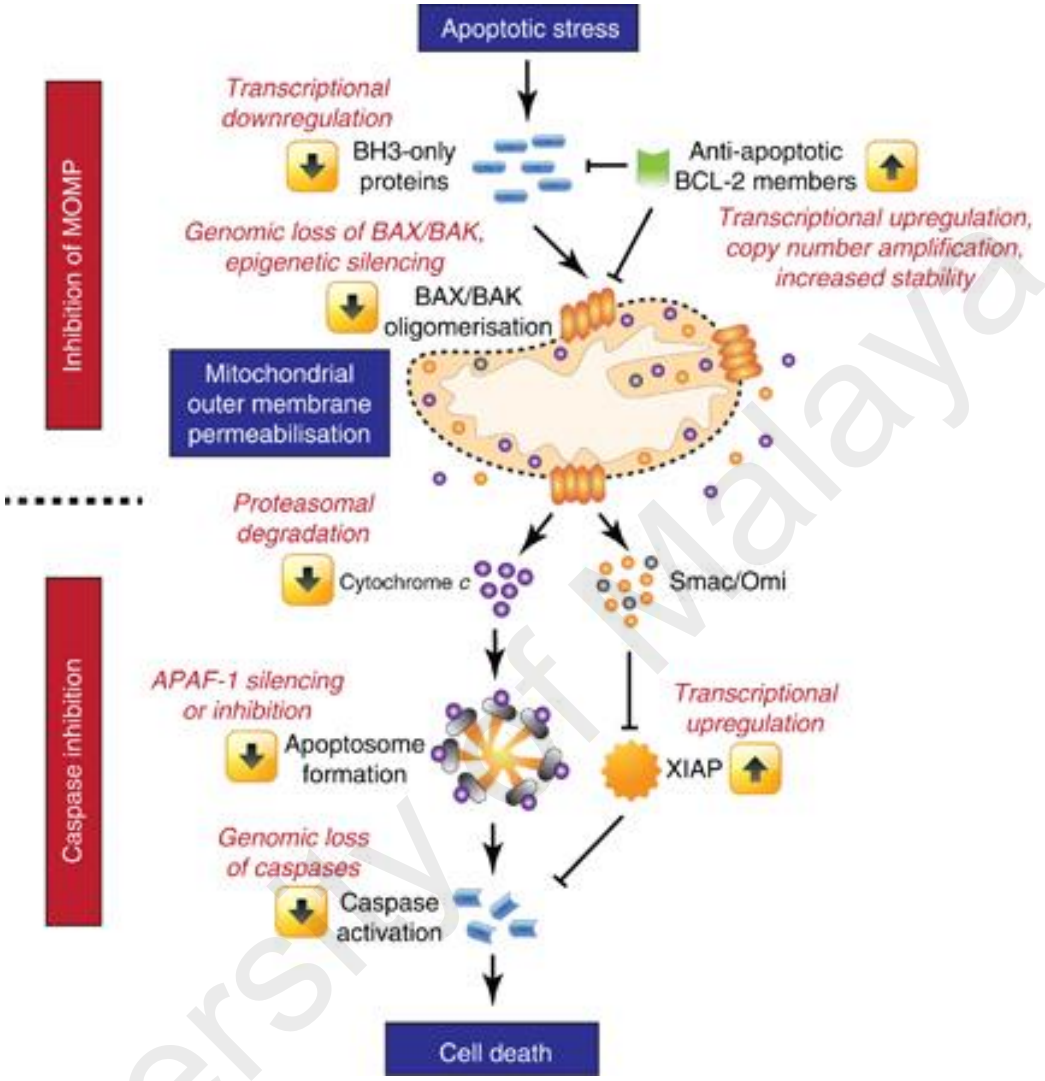


Figure 2.13: Execution of mitochondrial-mediated apoptosis by the involvement of BH3-only proteins to activate apoptosis through the occurrence of mitochondrial outer membrane permeabilization (Lopez & Tait, 2015).

2.6 Cell cycle regulation

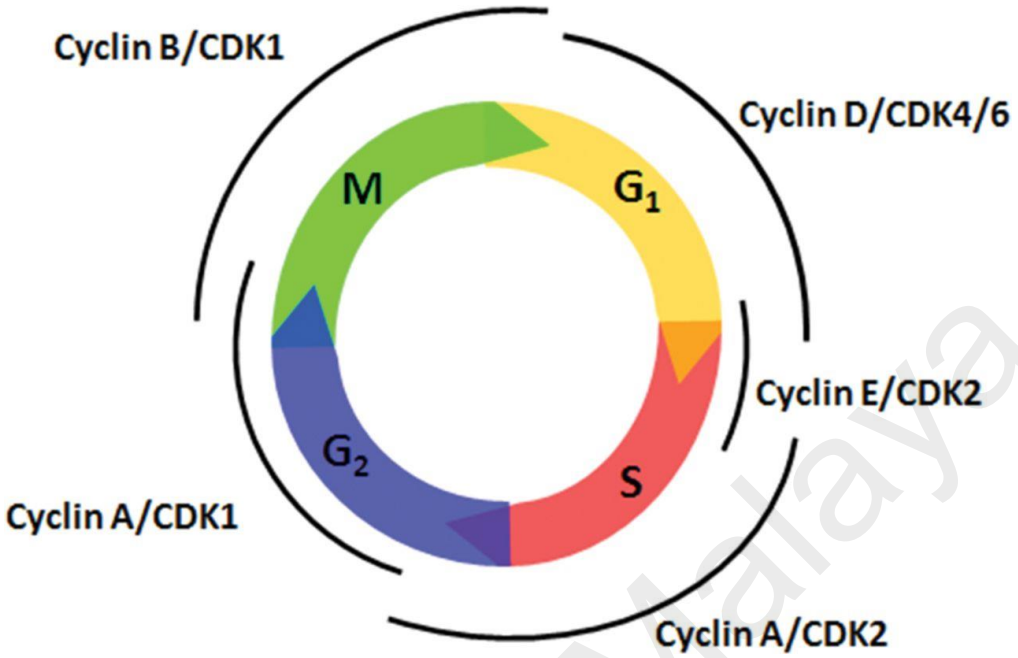


Figure 2.14: The regulation of mammalian cell cycle by cyclin-CDK complexes. Cell cycle is made up of the S phase (DNA synthesis), M phase (mitotic phase) that are separated by G1 and G2 phase of the cell cycle. (Suryadinata et al.,2010)

Regulation of cell cycle is important in maintaining the development of multicellular organisms. The deregulation of cell cycle can cause the rise of different diseases and this includes the development of cancer.

Cell cycle mainly consists of four phases G_1, S, G_2 and M phase and its progression is highly driven by cyclin-dependent kinases (CDK) in combination with cyclins. Cell cycle progression through each phase of the cell cycle is monitored by checkpoints which will ensure the accuracy of the cell cycle events (Hartwell et al., 1989).

When problems occurred during cell cycle, cell cycle checkpoints will be activated to induce cell cycle arrest and at this point, the fate of the cells will be determined by undergoing DNA repair or the cells will undergo cell death (Viallard et al., 2001). When the cell cycle checkpoints are disrupted, it can result in the development of cancer. Uncontrolled proliferation of cells is the main player in the development of cancer;

hence, it has been proposed and tested by researchers to use anti-cancer agents to target on cell cycle checkpoints.

The activation of the mitogenic signaling cascade will allow cells entry into the regulated steps in cell cycle. In the S phase of cell cycle, genome duplication occurs at which there is synthesis of DNA in the cells. In G1 phase, cells will commit into entering the new cell cycle (Senderowicz, 2004). This stage is controlled by the G1-S transition at which cyclin-dependent kinases (CDKs) complexes will be activated to allow entry into S phase. The regulation at this point is controlled by the G1/S cell cycle checkpoint. G2 checkpoint will help prevent cells with damaged DNA to enter the M phase for cell division (Paulovich et al., 1997). At this point, damaged cells will stop proliferating and some will undergo DNA repair.

2.6.1 Cyclin-dependent kinases and cyclins in cell cycle regulation

Cyclin-dependent kinases (CDK) are heterodimeric serine/threonine protein kinases that are made up of two subunits known as CDK which has catalytic function and cyclin which has regulatory function (Malumbres et al., 2007). CDKs play very important role in the mechanisms of cell cycle progression. To perform their function in cell proliferation, CDKs need to form complexes with cyclins. A total of twenty CDKs have been found in mammals that play different roles in cell cycle events or transcriptional regulation while some CDKs functions remain unknown (Malumbres et al., 2009). CDKs, cyclins and CDK inhibitors are important to maintain cellular homeostasis and the dysregulation of these substrates will cause the development of cancer (Maddika et al., 2007). CDKs are highly altered in tumors; therefore, many studies have been going into searching for therapeutics strategy against cancer by targeting CDKs. Cells proliferate uncontrollably when CDKs become overactive and cause the tumor suppressor genes to become dysfunctional.

CDK4 and CDK6 act as the interphase cyclin dependent kinases that regulate cell cycle progression through G_1 phase. These two CDKs are activated by the D-type cyclins to form complexes and cyclin Ds are highly produced upon activation by mitogenic signalling (Zhang et al., 2002). Then, phosphorylation will occur which will partially inactivate retinoblastoma (Rb) proteins that are responsible to suppress transcription of genes related to DNA replication (Sheppard et al., 2013).

Following the phosphorylation of Rb protein, E-type cyclins bind and activate CDK2 then activating E2F that allow the entry into S phase of cell cycle (Ortega et al., 2002) then CDK2 combines with cyclin A to allow the transition from S phase to G_2 phase. Towards the end of S phase, CDK1 form complexes with cyclin B to initiate mitosis (M) in the cells at the same time phosphorylating a large number of regulatory and structural proteins required for the process of mitosis (Castedo et al., 2002).

2.6.2 Cell cycle and cancer therapy

Given the importance of CDKs and cyclins in the regulation of cell cycle, cancer has also been deduced as a disease of cell cycle whencancer cells proliferate uncontrollably without proper regulation. In addition, alteration of cell cycle machinery is highly linked to the development of cancer and most molecules related to cell proliferation have been associated with malignant transformation.

There are studies that look into the inhibition of cell cycle proteins that work together with CDKs in different phases of cell cycle such as targeting the CDC7 kinase which is involved in the regulation of S-phase progression and this has become a major target of drug development by major pharmaceuticals company such as Pfizer, Roche, Novartis, Nerviano Medical Sciences, and Bristol Myers Squibb. The inhibitors displayed antitumor activity in pre-clinical studies hence these inhibitors such as NMS-1116354 by Nerviano and BMS-863233 by Bristol Myers Squibb have entered the phase I-II

clinical trials (Swords et al., 2010). There are also a number of drugs frequently used to target CDK activities in chemotherapy. These drugs commonly affect the cell cycle machinery and transcriptional CDKs to cause cell cycle arrest eventually cell death (Shapiro, 2006).

Cell cycle checkpoints are the supervisors and signalling pathways that are responsible for the coordination of DNA repair with cell cycle transitions. Upon the occurrence of DNA damage, checkpoint proteins will be recruited to the DNA which will activate checkpoint response (Bartek et al., 2007). Defects in DNA damage checkpoints will result in over activation of CDK, progression of cell cycle with the damaged DNA, which will finally developed into cancer. Cells with genome instability and presence of mutants will cause the cells to acquire malignant characteristics. CHK2 is one of the tumor suppressors that are commonly altered for example in colon and breast cancer and this caused the high expression of E2F1 transcription factor (Stawinska et al., 2008).

Studies have shown that a natural compound β-lapachone and its derivatives can elevate the formation of reactive oxygen species (ROS) and DNA damage in cancer cells that causes cancer cell death (Rios-Luci et al., 2012) and also stabilization of E2F1 (Li et al., 2003). Hence, targeting CDKs is a therapeutic approach with good potential for the treatment of cancer.

2.7 Oxidative stress and cancer cell death

DNA and cells are persistently exposed to attacks of oxidative stress and free radicals. Oxidative stress can come from different sources, exogenous and endogenous. Exogenous oxidative stress comes from the environment such as radiations and oxidizing chemicals while endogenous stress also known as intracellular oxidative stress produced by cellular signalling or in metabolic processes (Sedelnikova et al., 2010). Endogenous oxidative stress can result in a high level of DNA lesions in the cells and

this damage is primarily caused by the induction of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radical and peroxyl radical while reactive nitrogen species (RNS) consists of nitric oxide, peroxynitrite and nitrogen dioxide.

In normal cells, there exists an equilibrium between antioxidant and pro-oxidant regulated by ROS-metabolizing enzymes. However, when there is an imbalance or disturbance in this equilibrium, oxidative stress will happen that brings impact to some biological and pathological processes (Trachootham et al., 2008). Disturbance in oxidative balance is usually overcome by cell's own mechanism but prolonged imbalance of oxidative condition will result in cell death (Genestra, 2007).

Intracellular ROS are commonly produced by mitochondria as a byproduct of electron transport system. Even though ROS production is acceptable in the cellular metabolism aspect, when it reacts with the other components such as proteins, DNA, carbohydrate, lipids and other cellular components, it can damage the cells. Cells usually adapt to oxidative stress with the help of two proteins Kelch like-ECH associated protein 1 (Keap1) and transcription factor NFEL2L2 (Nrf2). Nrf2 levels are maintained in the low basal levels in the absence of oxidative stress (Zhang et al., 2003). When oxidative stress occurs, Keap1 is not able to target Nrf2 which will increase the level of Nrf2 proteins causing the transcription of antioxidant response gene such as NAD(P)H dehydrogenase, quinone 1 (NQO1), catalase (CAT) and heme oxygenase 1 (HMOX1) (Malhotra et al., 2010). ROS can induce several types of DNA damage which include, single strand DNA breaks (SSB), double strand DNA breaks (DSB), oxidized purines and pyrimidines, and apurinic/apyrimidinic DNA sites (Kryston et al., 2011).

DNA damage caused by free radical can have deleterious effect on the biology of the cells which will lead to formation of cancer. However, high intracellular level ROS in

the cells can also cause the cell cycle arrest as a result of DNA damage and this will subsequently trigger apoptosis. Oxidative stress can cause the release of cytochrome c from the mitochondria which then lead to the occurrence of apoptosis (Ueda et al., 2002).

Free radicals can trigger a series of biological events and one of the events is the ROS-mediated JNK activation of cell death that includes apoptosis and necrosis (Shen et al., 2006). This phenomenon provides opportunity for the exploitation of cellular mechanisms and to be one of the cancer therapeutics method and some studies have shown that there are natural products that can induce the production of ROS in cancer and subsequently caused DNA lesions and death to the cancer cells (Ahsan et al., 1998).

Example of a naturally occurring compound, melatonin which is found in plants, fungi, bacteria and animals is a prominent antioxidant but studies have found that melatonin have pro-oxidant ability. It has been shown that melatonin can stimulate the production of ROS which later melatonin promoted Fas-induced apoptosis (Wölfler et al., 2001). This compound has also been found to cause cytotoxicity in human leukemia cells with significant ROS generation (Büyükavcı et al., 2006).

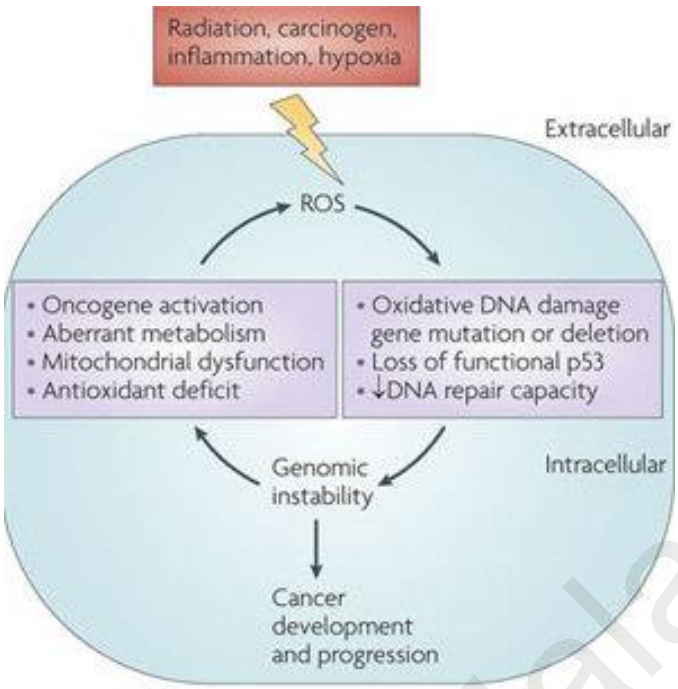


Figure 2.15: The cycle of reactive oxygen species induced by external stimuli and its fate in the intracellular environment. (Trachootham et al.,2009)

2.8 DNA damage response

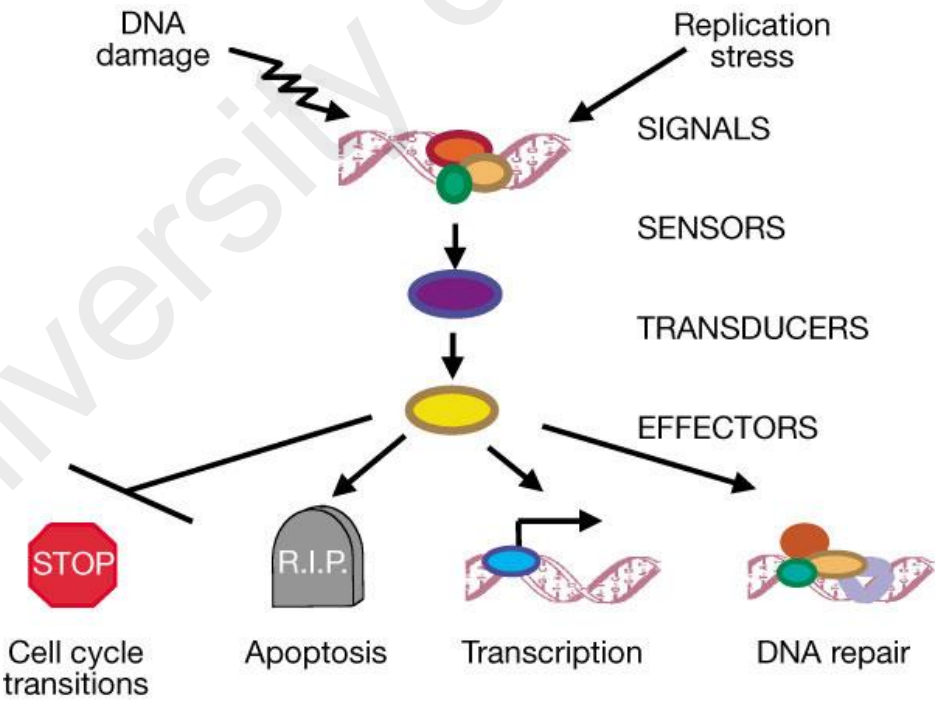


Figure 2.16: The overview of the DNA damage response signal. DNA damage triggers the signals followed by sensors, transducers and finally to the effectors that caused the occurrence of cell cycle disruption, apoptosis, transcription process and DNA repair mechanism. (Zhou and Elledge,2000)

Human body can have tens of thousands of DNA lesions each day (Lindahl et al., 2000). Damage to the DNA can result in the blockage of gene replication and transcription when DNA are not being repaired. Unrepaired DNA can lead to mutations and serious abnormality to the cells survival.

Hence, in order to fight the threats caused by DNA damage, cells can react in different ways when DNA damage occurred in the cells. Commonly, the mechanisms of DNA repair and cell cycle checkpoints will be activated as to arrest the cells in certain phases of cell cycle and perform DNA repair on the damaged DNA. However, cells that possess damaged DNA and cannot be repaired in this stage will be eliminated from the population by programmed cell death (Roos et al., 2006).

Different molecular mechanisms of apoptosis can be stimulated based on the chemical basis of DNA lesion and the way it is process and detected by the cells (Roos et al., 2013). DNA damage triggers the most upstream DNA damage response kinases, ATM (ataxia-telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related) and DNA-dependent protein kinases (DNA-PKcs) in mammalian cells where ATM and DNA-PKcs are activated by double-stranded DNA breaks (DSBs) while ATR responds to broad range of DNA damage which include DSBs and other types of DNA lesions. (Marechal et al., 2013).

53BP1 and BRCA1 are prominent mediators of ATM-controlled responses that are involved in cell cycle S-phase checkpoint, G_2/M phase, apoptosis, cell proliferation and DNA damage repair (Deng, 2006; Ward et al., 2003). Proteins such as Chk2 will be phosphorylated by ATM kinase which will further phosphorylate protein phosphatase CDC25A that leads to cell cycle arrest in response to DNA damage in the cells (Furnari et al., 1997).

One of the characteristics that contribute to the development of cancer is the impairment of DNA damage response which highlighted the roles of DDR genes as tumor suppressor genes (Hanahan et al., 2011). The expression of DDR elements in cancer cells have resulted in the failure of DNA-damaging chemo- and radiotherapy due to the reason that cancer cells are highly dependent on these DDR elements for survival.

Hence, the inhibition of DDR provides a platform for the exploitation of cancer cells that are highly dependent to the action of DDR elements for therapeutic purpose and also helps to solve the problem related to the resistance of tumour cells to DNA-damaging chemotherapy (Curtin, 2013). This exploitation provides the potential to use DDR inhibitors in combination with DNA-damaging therapeutics agents in radio- and chemo- therapy to improve the efficacy of DNA-damaging therapeutics agents in its antitumor activity (O'Connor, 2015). However, there are various challenges involved in the approach to combine DDR inhibitors and DNA-damaging therapeutics agents for cancer therapy.

2.9 Proteomics in cancer research

Genomic studies have provided us new information about biomarkers, therapeutics targets and mechanisms of cancer development by measuring the sequence, copy number changes in gene expression. However, investigation of proteins products can provide us with deeper understanding about cancer because protein expression shows significant translational and post-translational regulation of protein levels and functions.

Proteome is the set of proteins derived from the translation of all protein coding genes by a cell, tissue or organism. It provides us with the understanding of the gene functions. Proteomics can be categorized into three areas, large scale protein micro characterization for the identification of proteins and its post-translational modifications, differential expression of proteins for different application in diseases, and protein-

protein interactions study. These understandings will provide a better understanding on the proteins biochemistry and mechanisms which help in different aspects of a certain disease.

Proteomics has emerged as an important tool in detecting physiological condition, change in a system in response to external stimuli, mutations and adaptations. Studies on the biospecimens from cancer patients can help us to understand and monitor tumor pathogenesis and detection of novel targets for cancer treatment. One of the essential goals of proteomic technique application is to adapt this technique for regular diagnostic and prognostic usage in the clinical laboratories and for the assessment of different cancer therapeutic regimens (Sallam, 2015).

This technique can be used to investigate changes in global protein profiles and protein alteration as potential drug targets. Proteomics also allow the identification of post-translational modifications, and sequence variants (Mann et al., 2003; Zhang et al., 2011). Different proteomic analytical tools have been developed such as 1-dimensional and 2-dimensional gel electrophoresis, and mass-spectrometry based application which allows quantitative and qualitative analysis of diseases related whole proteome (Altaf Hussain et al., 2012). Development of protein microarray, a high-throughput technology has been really helpful to measure the expression of thousands of proteins simultaneously.

In general, two different types of proteomic analysis methods have applied which include, top-down proteomic approach and bottom-up proteomic approach. The top-down proteomic involved the analysis of intact proteins while bottom-up approach will help to identify proteins based on its peptides, a result of proteolytic digestion. An established method in top-down approach is the two-dimensional gel electrophoresis which uses the protein staining intensity to observe the changes in protein profiles. Then,

protein of interests will be excised from the gel for proteolytic digestion for analysis by mass spectrometry. Data from the peptide mass and fragmentation patterns will be used for protein database search for protein identification. However, a top-down strategy has its own limitations such as the low detection limit, difficulty in fragmenting large analytes, problems in resolving proteins of certain pH, and problems with protein solubility.

Hence, the bottom-up proteomic approach is used for protein identification. This involved the usage of multi-dimensional LC/MS/MS by performing proteolysis of complex proteins then separation of peptides using chromatography technique prior to MS/MS sequencing. Current improvement in this method allows the sequencing of peptides by data-dependent data acquisition. Spectra from the MS/MS fragmentation of selected ions will be used in the sequencing and identification of proteins.

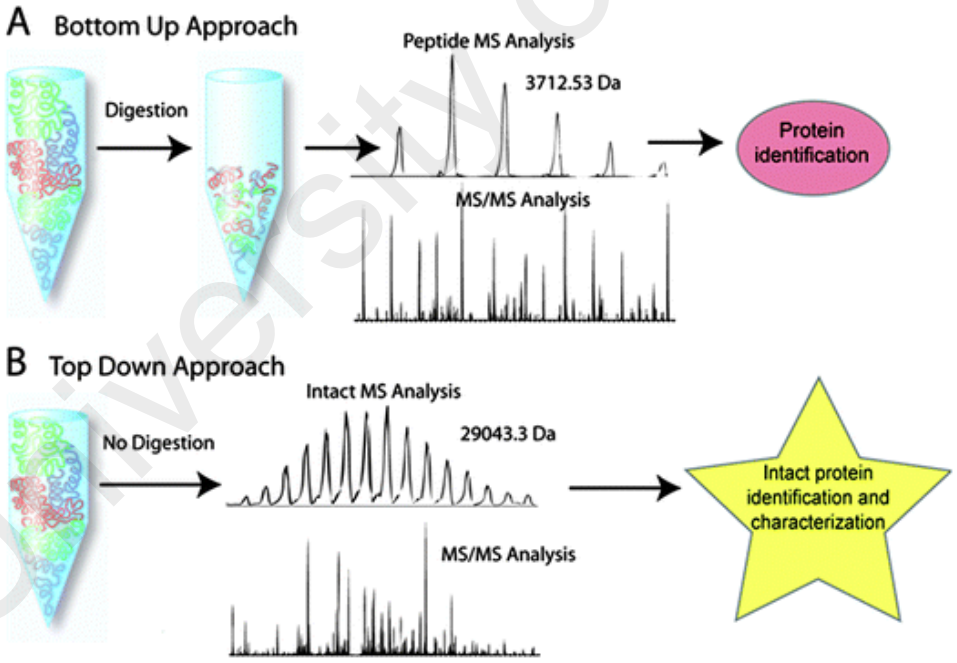


Figure 2.17: A) Bottom up approach and B) Top down approach of protein identification in LC/MS/MS proteomic approach. (Kellie et al.,2016)

Proteomic studies offer new opportunities in cancer biomarker discovery, monitoring cancer progression, investigating the efficacy of cancer treatment, discovering new therapeutic targets and understanding the mechanisms underlying the disease. Plasma samples from mouse models and human have been used to study the lung cancer plasma protein signatures and study has shown that the mouse models are relevant to human lung cancer of the lung cancer plasma protein signatures (Taguchi et al., 2011). EGFR signature was identified in the plasma with other biomarkers, SFTPB and WFDC2. These biomarkers are significantly different in lung cancer models in comparison with controls.

Another study on ovarian cancer also showed that proteomics has the potential to be used as a tool for routine screening of ovarian cancer by creating a template for proteomic-based cancer biomarkers discovery, for early detection of epithelial ovarian cancer and observing the treatment effect of epithelial ovarian cancer (Elschenbroich et al., 2011). A recent study has also applied proteomic technique to detect autoantibodies using microarray for the detection of ovarian biomarkers (Anderson et al., 2015).

In this study, proteomics technique is being applied to study the relative protein abundance that reflects the protein expression in cancer cells as an effect of the action of helichrysetin. The information provided by this technique will help in the discovery of new therapeutic target of this potential anti-cancer agent.

CHAPTER 3: MATERIALS AND METHODS

3.1 Pure compound

Helichrysetin was obtained from BioBioPha Co. Ltd (China) that is isolated from the plant of *Alpinia galanga*.

3.2 Cell culture

Human lung adenocarcinoma cell line (A549), human cervical carcinoma cell line (Ca Ski), human breast adenocarcinoma cell line (MCF-7), human fetal lung fibroblast cell line (MRC-5), and human colorectal adenocarcinoma cell line (HT 29) were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 (Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (FBS), 2% penicillin/streptomycin, and 1% amphotericin- B for A549, Ca Ski and MCF-7 cells while McCoy's 5A and Eagle's Minimum Essential Media (EMEM) were used for HT-29 and MRC-5 cells respectively with supplement stated above. Cells were maintained in 5% carbon dioxide ($CO_{2)}$ atmosphere at temperature of 37°C, humidified incubator in tissue culture flasks.

3.3 MTT assay

Cells were seeded in 96-well sterile culture plate at a concentration of 3 X 10^4 cells/ml. The cells seeded were incubated overnight for adherence of cells to the surface of the culture plate. After the overnight incubation, media was removed from the culture plate wells and fresh media (with 0.5% DMSO as a vehicle) containing helichrysetin with concentration of 1.56 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml were added to the cells for treatment. Treatments were done for 24 hours, 48 hours, and 72 hours. Doxorubicin was used as positive control. MTT assay was carried out as described by Mosmann with modifications. Upon treatment, 20 µl of MTT solution was added into 96 wells with 4 hours incubation in condition of 5% CO_2 and at

temperature of 37°C. The media was later discarded and 150 µl DMSO was added to dissolve the formazan crystals. Absorbance was measured as below using Synergy H1 Hybrid microplate reader:

Abs_{570nm}- Abs_{630nm}= $Abs_{treatment/control}$

The percentage of inhibition by helichrysetin was calculated as:

$$\frac{Abs_{control}-Abs_{treatment}}{Abs_{control}} \times 100 = \_\_\_\_\_ \%$$

IC_{50} values were obtained by determining the concentration at which 50% of the cell population is inhibited by helichrysetin.

3.4 Phase contrast microscopy

Cells were seeded at the concentration of 5 X 10^4 cells/ ml into a sterile tissue culture plate and incubated at 5% CO_2 and 37°C, overnight for cell adhesion. After overnight adhesion, cells were treated with helichrysetin at three time points, 24 hours, 48 hours and 72 hours. Then, the cells were observed under the phase microscope (Zeiss Axio Vert. A1) for changes in morphological features such as changes in cell volume, detachment, and rounding.

3.5 Nuclear morphological assessment by DAPI staining

Cells were plated in tissue culture plate at a concentration of 5 X 10^4 cells/ ml into a sterile tissue culture plate and incubated at 5% CO_2 and 37°C, overnight for cell adhesion. Then, cells were treated with helichrysetin at concentration of 15 µg/ml for 24 hours and harvested for staining by 4',6-diamidino-2-phenylindole (DAPI) staining. Cells were washed with phosphate buffered saline (PBS) and fixed in 4% formaldehyde. After fixing, cells were resuspended in DAPI solution (0.2 µg/ml), 0.1% Triton X 100 and incubated for 5 min in the dark. The cells were then spotted on the slide and air

dried. Nuclear morphological changes such as chromatin condensation and nuclear fragmentation were examined under the fluorescence microscope (Leica) at 40X magnification.

3.6 Annexin V FITC assay for apoptosis detection

Annexin V FITC assay was performed using the FITC Annexin V Apoptosis Detection Kit by BD Biosciences, USA. 4 X 10^5 cells/well were plated in culture plates and left overnight for adhesion. Then, the treatment was done to examine the effect of helichrysetin on the cells in a time and dose- dependent manner. After treatment, the cells were harvested and washed with PBS. Cell pellet was resuspended in 1X Annexin V binding buffer, then, stained with Annexin V and propidium iodide (PI) for 15 min at room temperature in the dark. The analysis was performed using Accuri C6 flow cytometer. Cell population was observed by its quadrant statistics given the lower left quadrant as the viable cells population, lower right quadrant as the early apoptotic cells, upper right quadrant as the secondary necrotic or late apoptotic cells and upper left the primary necrosis population.

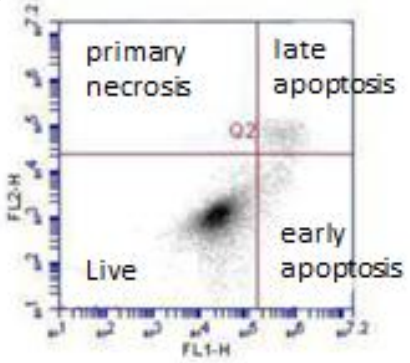


Figure 3.1: Cell population quadrant from flow cytometry analysis

3.7 Assay for Mitochondrial Membrane Potential

Assay for mitochondrial membrane potential was performed using the BD Mitoscreen Mitochondrial Membrane Potential Kit. Cells were plated in sterile culture plate at a concentration of 8 X 10^4 and incubated for 24 hours for cell adherence. Dose and time-dependent effect of helichrysetin on cells were evaluated. The cells were harvested and washed with PBS and then stained with JC-1 BD Mitoscreen Kit for 15 min at 37°C. Unbound dye was removed by washing with 1X assay buffer, then, the cell pellet was resuspended in 1X assay buffer for analysis with flow cytometer (Accuri C6). The density plot of this assay was divided into two regions, apoptotic and non-apoptotic cells.

3.8 TUNEL assay

Internucleosomal DNA fragmentation was detected using the APO-BrDU TUNEL assay kit (Invitrogen). Cells were plated overnight and treated at different time intervals and different concentrations of helichrysetin. At the end of treatement, cells were harvested and washed with PBS. Then, the cells were fixed with 1% (w/v) paraformaldehyde on ice for 15 min. Cells were then spinned and washed with PBS twice. Ice cold ethanol was added to the cells for fixation for at least 30 min in -20°C. After fixation, ethanol was removed and the cells were washed with wash buffer. Cells were incubated in DNA-labeling solution for 60 min at 37°C, rinsed and then labelled with antibody solution for 30 min at room temperature. Before analysis, propidium iodide/RNase A staining buffer was added to the sample and analysed using flow cytometry (Accuri C6).

3.9 Cell cycle analysis

8 X 10^4 cells/mL were plated in the sterile cell culture plate and allowed to adhere overnight. The cells were treated with helichrysetin for 24, 48 and 72 hours. After treatment, the cells were harvested and washed with cold PBS. Then, the cells were fixed with 70% ethanol overnight at -20°C. Cells were then pelleted and washed with cold PBS then stained with propidium iodide staining solution containing 0.1% Triton-X- 100, 0.1% sodium citrate, 100 µg/mL RNase and 50 µg/mL propidium iodide for 30 minutes in the dark. Then, the cells were separated using the cell strainer and analysed using Accuri C6 flow cytometer. Results were analysed using ModFit software.

3.10 Western blotting

50 µg of total cell lysates were separated by 5% stacking gel and 12% separating gel in acrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane and blocked for 2 hour with bovine serum albumin at room temperature. The membrane was incubated with primary antibody (1:1000) at 4°C overnight. Then, the membrane was incubated with peroxidase-conjugated secondary antibody (1:2000) and visualized using chemiluminescence.

3.11 In solution digestion

50 µg of cell protein extract was reduced with 10 mM dithiotreitol in 8M urea and 50mM Tris buffer at 37°C for 1 hour and then alkylated with 50 mM iodoacetamide 8M urea and 50mM Tris buffer for 30 minutes in the dark at room temperature finally stopped with dithiotreitol. A little benzonase was added into the solution and incubated for 2 hours at room temperature. Then, Lys-C was added (1:200) to the solution and incubated for 4 hours at room temperature and finally trypsin (1:50) was added and incubated overnight at 37°C.

3.12 iTRAQ labeling of digested peptides

Salts and detergents were removed from the resulting peptides of digested proteins using Sep-Pak C18 cartridges and dried in vacuum. Peptides were labeled according to the manufacturer's instructions with 114, 115, 116 and 117 isobaric tag 4-plex iTRAQ reagents for control, 6 hours, 24 hours and 48 hours treated sample respectively. Four samples were pooled together and dried with vacuum concentrator.

3.13 Strong cation exchange chromatography of peptide samples

Pooled labeled peptides were resuspended in 200 µL 10mM KH_2PO_4, pH 2.65, 25% acetonitrile (Buffer A) and loaded onto Polysulfoethyl A, 4.6 × 200mm column (Poly LC) containing 5 µm polysufoethyl aspartamide beads with 300Å pore size and at a flow rate of 1ml/min. Peptides were detected with UV at 214nm. Gradient was developed in 20 minutes to 15% Buffer B (1M KCl in Buffer A, pH 2.65), 10 minutes to 30% Buffer B, 5 minutes to 50% Buffer B, 1 minute to 100% Buffer B. Fractions were collected every 30 seconds and desalted using Sep-Pak C18 cartridges then dried with vacuum concentrator.

3.14 Shotgun proteomic identifications

NanoLC−nanoESI-MS/MS analysis was performed on a nanoAcquity system (Waters, Milford, MA) in data-dependent mode, connected to the LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Electron, Bremen, Germany) equipped with PicoView nanospray interface (New Objective, Woburn, MA). Peptide samples were loaded onto a 75 µm ID, 25 cm length C18 BEH column (Waters, Milford, MA) with a pore of 130 Å. Peptides were separated with gradient from 5% to 40% solvent B (acetonitrile with 0.1% formic acid) for 120 min at a flow rate of 300 nL/min. 0.1% formic acid in water was used as solvent A. Full scan of MS spectra were acquired in the orbitrap (*m/z* 350–

1600) with 60K resolution at *m/z* 400. The top 10 ions were isolated for HCD MS/MS fragmentation and detected in the orbitrap with dynamic exclusion of 60s. MS/MS analysis was done with resolution of 7500, isolation window of 2 *m/z* and a target value of 50000 ions**. N**ormalized collision energy of 40% and activation time of 0.1 ms were used for fragmentation. Unrecognized and single charged ions were excluded from the analysis.

3.15 Protein identification and quantitation

Protein identification and quantitation were carried out using Thermo Proteome Discoverer (PD 1.4.1.14.) software against Swiss-Prot human protein database (Human_uniprot2015001, 27 October 2015, 148,986 entries). The search was performed with two trypsin missed cleavage allowed, fixed modications of carbamidomethylation of cysteine residues, iTRAQ 4plex at lysine residues and N-terminal proteolytic peptides, variable modifications of oxidation at methionine residues. For target decoy PSM validator, strict target FDR was set at 0.01 and relaxed target FDR at 0.05.

3.16 Statistical analysis

Proteins present in both biological replicates were identified with calculations of 114:115, 114:116, and 114:117 protein ratios. Average protein ratios of each protein were normalized by conversion to $\log_2$ average protein ratio and then converted to z-score using the formula as given below. Z-score determines the standard deviation of protein ratio from the mean and the proteins were then selected for further analysis with the cut-off, z-score >1.960σ or <-1.960σ.

$$\text{Z-score} = \frac{\log_2 \text{ protein ratio} - \text{average of } (\log_2 \text{ all protein ratio})}{\text{Standard deviation of } (\log_2 \text{ all protein ratio})}$$

3.17 Functional annotation

Differentially expressed proteins derived from the calculation of Z-score of proteins from each time point were categorized based on their gene names. These genes were annotated based on its biological processes, molecular functions and cellular components by gene ontology human genome wide annotation for human, org.Hs.eg.db package (Carlson et al., 2013) using an R package, clusterProfiler (v. 3.0.5) (Yu et al., 2012) (http://www.bioconductor.org) with p-value 0.01 using the Benjamini Hochberg method to control the false discovery rate. To perform semantic comparison of gene ontology terms in order to eliminate GO terms redundancy, GoSemSim package (v.2.0.3) (Yu et al., 2010) was used in R.

3.18 Ingenuity Pathway Analysis (IPA)

Data from the statistical analysis of significantly altered proteins were selected for elucidation of pathway involved in the action of helichrysetin using Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). Datasets were analyzed using the gene identifiers and its $\log_2$ protein ratios to represent the protein expression value. Top 10 canonical pathways were identified from the library of IPA. The top 10 canonical pathways represent the datasets that are most significantly matched to pathways involved in the database. The significance of the matched dataset to the canonical pathways from the library of IPA was determined by the ratio of the number of genes from the dataset that mapped to the canonical pathways in IPA database divided by the total number of genes involved in the particular canonical pathways. The *p*-value was calculated using the Fischer's exact test which determines the probability of an association that occur by chance. To further understand the changes of the expression in this dataset, upstream regulators were also predicted using IPA. Results from the upstream regulator prediction and determination of the canonical pathways were

combined to elucidate the biological activities that were involved in the action of helichrysetin. To better display the molecular mechanisms involved in the action of helichrysetin on cancer cells, the biological pathways were built using the IPA Pathway Builder.

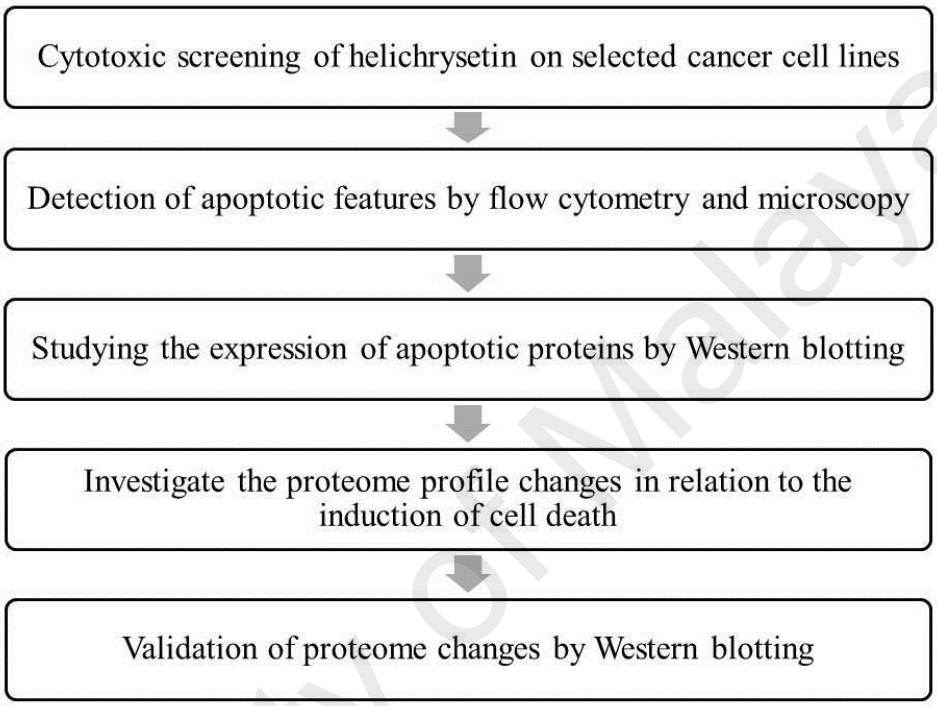


Figure 3.2: Summary of workflow involving the study of helichrysetin on cancer cell lines.

CHAPTER 4: RESULTS

4.1 Growth inhibition activity of helichrysetin on selected cancer cell lines

Four selected cancer cell lines were treated with helichrysetin at different concentrations, 1.56 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml for 72 hours to evaluate the growth inhibition of helichrysetin using the MTT assay. Results showed, as the concentration of helichrysetin increased, the percentage of growth inhibition also increased significantly in a dose-dependent manner (Figure 4.1). This growth impairment is in comparison with its negative control. No significant cell inhibitory activity was observed in the vehicle/DMSO- treated cells. The results of MTT assay revealed that helichrysetin inhibits the cells with the IC_{50} values of 50.72 ± 1.26 µM, 31.02 ± 0.45 µM, 97.35 ± 1.71 µM, and 102.94 ± 2.20 µM on A549, Ca Ski, MCF-7 and HT-29 cell line respectively. Results showed that helichrysetin is most effective towards A549 and Ca Ski cell lines with its IC_{50} values of 50 µM and below. A549 cell line was selected for further investigation.

The inhibitory activity of helichrysetin was also tested on MRC-5 cell line which is human normal lung fibroblast and the result showed IC_{50} value of 123.66 ± 0.05 µM that is below the active concentration. Next, we determine the growth inhibitory activity by helichrysetin on A549 cell line at time points, 6, 12, 24 and 48 hours. The percentage of inhibition on A549 cell line increased in a time-dependent manner from 12.28%, 25.95%, 38.57% and 51.69% when treated at the concentration of 50 µM (15 µg/ml) (Table 4.2).

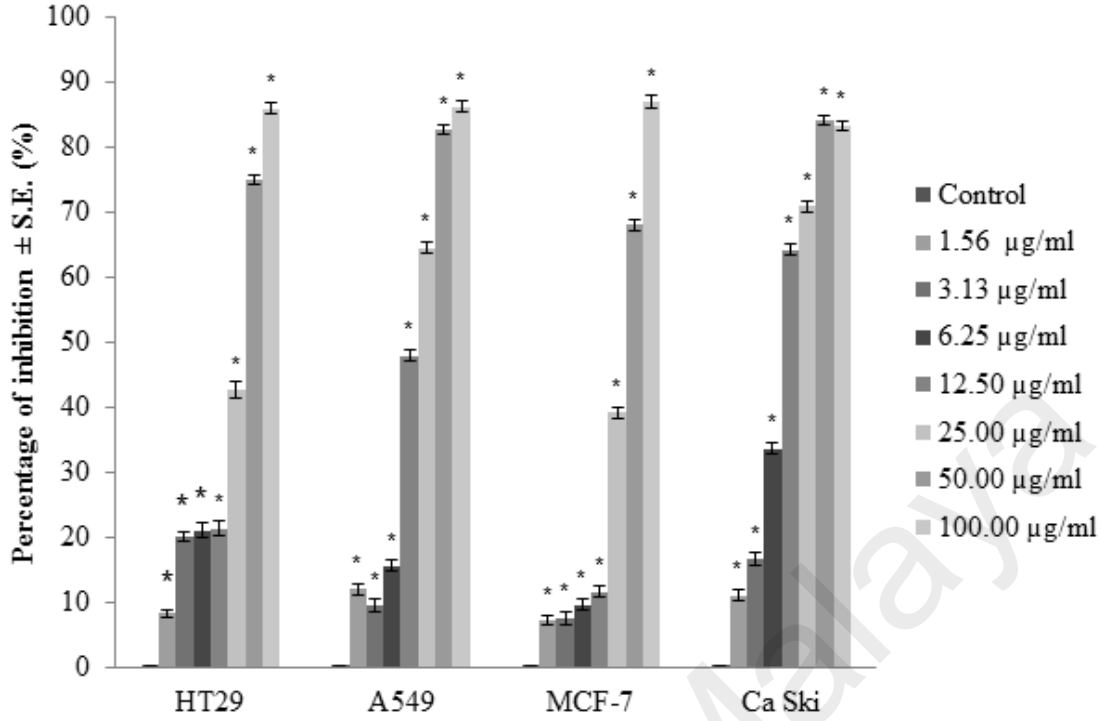


Figure 4.1: MTT assay to assess growth inhibitory activity of helichrysetin on four selected cancer cell lines. Cells were treated with helichrysetin from 1.56 µg/ml to 100.00 µg/ml and the percentage of inhibition was calculated in comparison with untreated control. Data represents the means ± SD. The results shown represent three independent experiments. *p value < 0.05 vs untreated control.

Table 4.1: IC_{50} values for helichrysetin by MTT assay in selected cancer cell lines

Cell Line	Cell Type	Half maximal inhibitor concentration IC_{50} (µM)
A549	Human lung adenocarcinoma	50.72 ± 1.26 (14.52 ± 0.36 µg/ml)
Ca Ski	Human cervical carcinoma	31.02 ± 0.45 (8.88 ± 0.13 µg/ml)
MCF-7	Human breast adenocarcinoma	97.35 ± 1.71 (29.47 ± 0.63 µg/ml)
HT-29	Human colon adenocarcinoma	102.94 ± 2.20 (27.87 ± 0.49 µg/ml)
MRC-5	Human lung fibroblast	123.66 ± 0.05 (35.40 ± 1.47 µg/ml)

Table 4.2: Time-dependent inhibition of helichrysetin in A549 cell line by MTT assay.

Treatment duration (hour)	Percentage of inhibition
6	12.38
12	25.95
24	38.57
48	51.69

4.2 Helichrysetin-treated A549 cells morphological study

Cellular morphology and cell nuclear morphology changes were assessed using phase contrast microscopy and fluorescence microscopy with DAPI staining.

4.2.1 Cell morphological assessment by phase contrast microscopy

The effect of helichrysetin on the A549 cells morphology was evaluated using phase contrast microscopy. A549 cells were treated at concentration 5 µg/ml, 15 µg/ml and 20 µg/ml helichrysetin and at time point 24 hours, 48 hours and 72 hours. Phase contrast microscopy showed that cells treated with helichrysetin detached from the substratum of the culture surface that caused the increase in the number of floating cells (Figure 4.2). Total cell from the population that detached from culture surface increased as the concentration of the helichrysetin increased from untreated samples, 5 µg/ml, 15 µg/ml and 20 µg/ml of helichrysetin. Detachment of cells also occurred in a time-dependent manner all at time 24 hours, 48 hours and 72 hours in comparison with untreated cell samples.

Results of the time-dependent study (Figure 4.3) showed that cells start to shrink after 6 hours of treatment with 15 µg/ml helichrysetin. After 12-24 hours of treatment, A549 cells become rounded and some of the cells started to lose its attachment to the flask displayed by the bright cells. Finally after 48 hours of treatment, cells detached from the flask appearing as rounded and bright cells that loss its anchoring to the bottom of the flask.

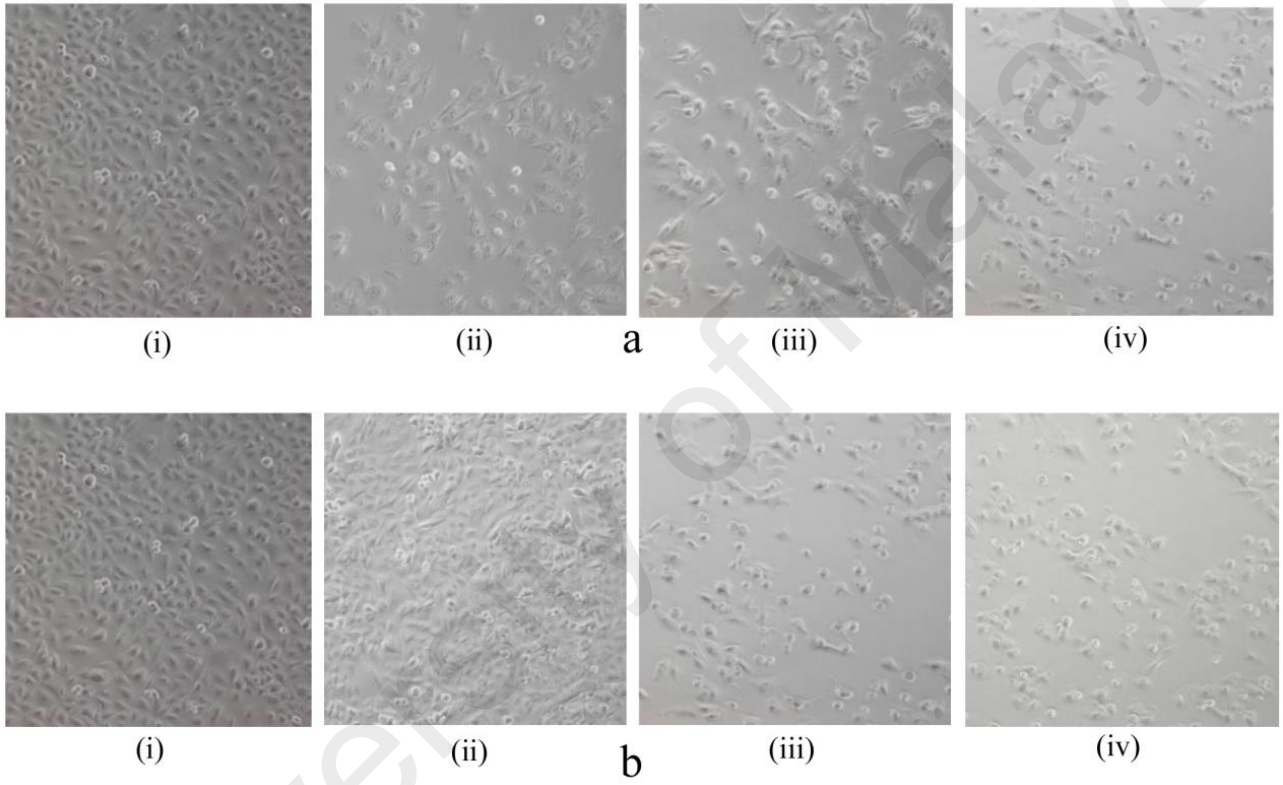


Figure 4.2: Cell morphological assessment of A549 cells treated with helichrysetin at different time points (a) and concentrations (b). a) Time-dependent study i) Untreated sample ii) 24 hours treatment iii) 48 hours treatment iv) 72 hours treatment b) Dose-dependent study i) Untreated A549 cells ii) A549 cells treated with 5 µg/ml helichrysetin iii) A549 cells treated with 15 µg/ml helichrysetin iv) A549 cells treated with 20 µg/ml helichrysetin.

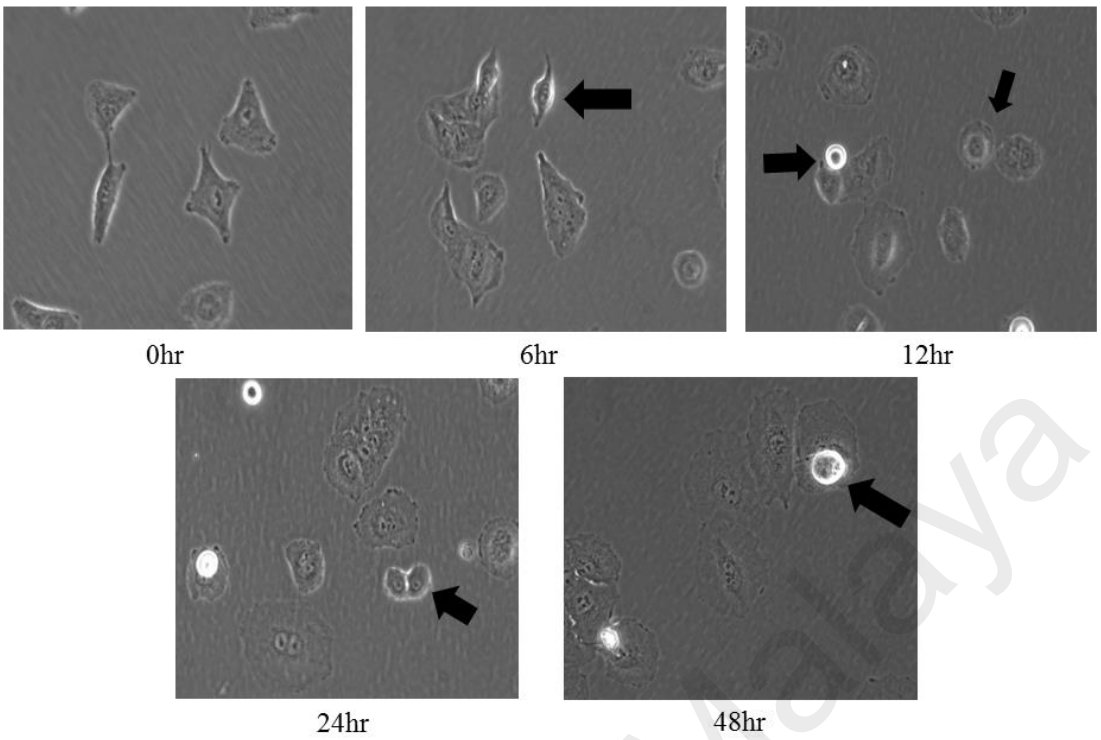


Figure 4.3: Cell morphological assessment at 40× magnification, a time-dependent study. Black arrows indicate cells that showed morphological changes due to treatment of helichrysetin at 15µg/ml at different time points.

4.2.2 Fluorescence microscopy evaluation of nuclear morphological changes by DAPI staining

A549 cells were treated with 15 µg/ml helichrysetin for 24 hours to observe its effect on the cell nuclear morphology. Figure 4.4a showed the untreated A549 cells stained with DAPI fluorescent stain while Figure 4.4b displayed the nuclear morphology of helichrysetin-treated A549 cells. As shown in the fluorescence microscopy image in Figure 4.4, there were no visible bright blue cells in the untreated samples (Figure 4.4a) and the bright blue fluorescence can be seen in A549 cells treated with 15 µg/ml helichrysetin at time point of 24 hours (Figure 4.4b).

A549 cells cultured in the absence of helichrysetin showed intact nuclei due to the low permeability of the DAPI stain through the cell membranes of healthy cells hence contributed to the low fluorescence in the untreated A549 cells. Bright blue cells in Figure 4.4b are present due to the highly concentrated nature of the cell DNA in

helichrysetin-treated cells which prove that the cells exhibited nuclear fragmentation and chromatin condensation. This observation clearly displays the occurrence of apoptotic cell death in helichrysetin-treated cells.

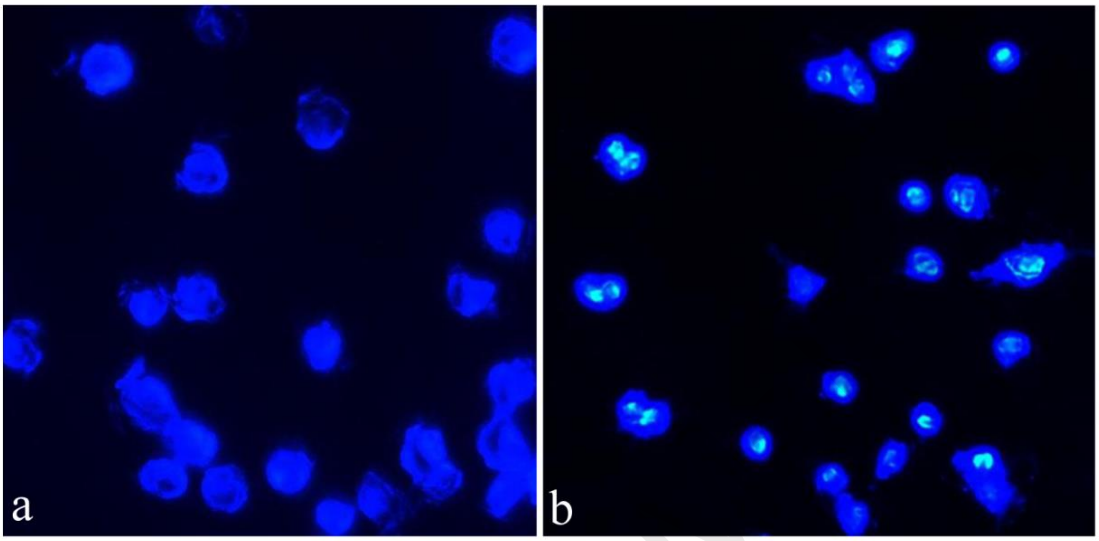


Figure 4.4: Nuclear morphological assessment of A549 cells treated with 50 µM of helichrysetin for 24 hours by DAPI staining. Cells with nuclear morphological changes are indicated by bright blue cells in the treated sample (b). a) Untreated A549 cells b) A549 cells treated with 50 µM helichrysetin.

4.3 Flow cytometry study of apoptosis and cell cycle analysis

4.3.1 Detection of early and late apoptosis by evaluation of cell membrane integrity

Figure 4.5 and 4.6 showed the density plot from flow cytometry analysis of apoptotic induction effect of helichrysetin on A549 cells in a dose- and time- dependent study. Percentage of the live, early apoptotic, late apoptotic and necrotic cells are represented in four different quadrants in the density plot, lower left, lower right, upper left and upper right quadrant respectively. The percentage of cell population in each quadrant is summarized into bar chart in Figure 4.7 and 4.8.

As shown in Figure 4.7, percentage of live cells in the dose-dependent study decreased as the concentration of helichrysetin increased from 5 µg/ml to 20 µg/ml in comparison with untreated control. While the percentage of cell in the early apoptotic, late apoptotic,

and necrotic quadrants increased in a dose-dependent manner. The percentage of early apoptotic cells increased from 2.65 ± 0.31% (control) to 2.78 ± 0.21%, 14.98 ± 0.79%, and 28.55 ± 1.19% as the concentration increased from 5 μg/ml to 20 μg/ml. Annexin V/PI double stained cells increased from 3.74 ± 0.17% (control) to 4.39 ± 0.60%, 8.40 ± 1.02%, and 18.29 ± 2.58% for dose-dependent study. This proves that helichrysetin has the ability to induce apoptosis in A549 cells and its effect is more pronounced at the concentration of 15 μg/ml and 20 μg/ml causing more cells to undergo early and late apoptosis.

Upon treatment of A549 cells at 15 μg/ml, the percentage of viable cells decreased in a time-dependent manner in comparison with untreated control. Helichrysetin caused great effect on treated A549 cells by inducing early apoptosis at IC_{50} in a time-dependent manner. However, its effect is not pronounced in inducing late apoptosis in A549 cells given the increase in the percentage of cells in late apoptotic stage from 24 hours to 48 hours. After treatment with helichrysetin for 24 hours, 48 hours and 72 hours, results showed increase of early apoptotic cells from 2.03 ± 0.18% (control) to 11.15 ± 3.53%, 15.73 ± 1.18%, and 26.92 ± 1.38% while percentages of late apoptotic cells are 3.99 ± 0.30% to 6.33 ± 0.65%, 11.70 ± 0.90% and 11.87 ± 1.05% respectively.

The percentage of cells in both early and late apoptotic stages were combined and summarized in Figure 4.9 and 4.10 for dose- and time- dependent studies. The results are summarized as the percentage Annexin-V positive cells indicating all cells that undergo apoptosis upon treatment with helichrysetin. As shown in Figure 4.9, the percentage of Annexin-V positive cells increased in a dose-dependent manner and it is significantly different compared to untreated control at the concentration of 15 μg/ml and 20 μg/ml. This shows that the effect of helichrysetin to induce apoptosis in A549 cells is significant at 15 μg/ml and 20 μg/ml upon treatment for 72 hours. At IC_{50}, the

effect of helichrysetin on A549 cells is significant at time 24 hours, 48 hours and 72 hours.

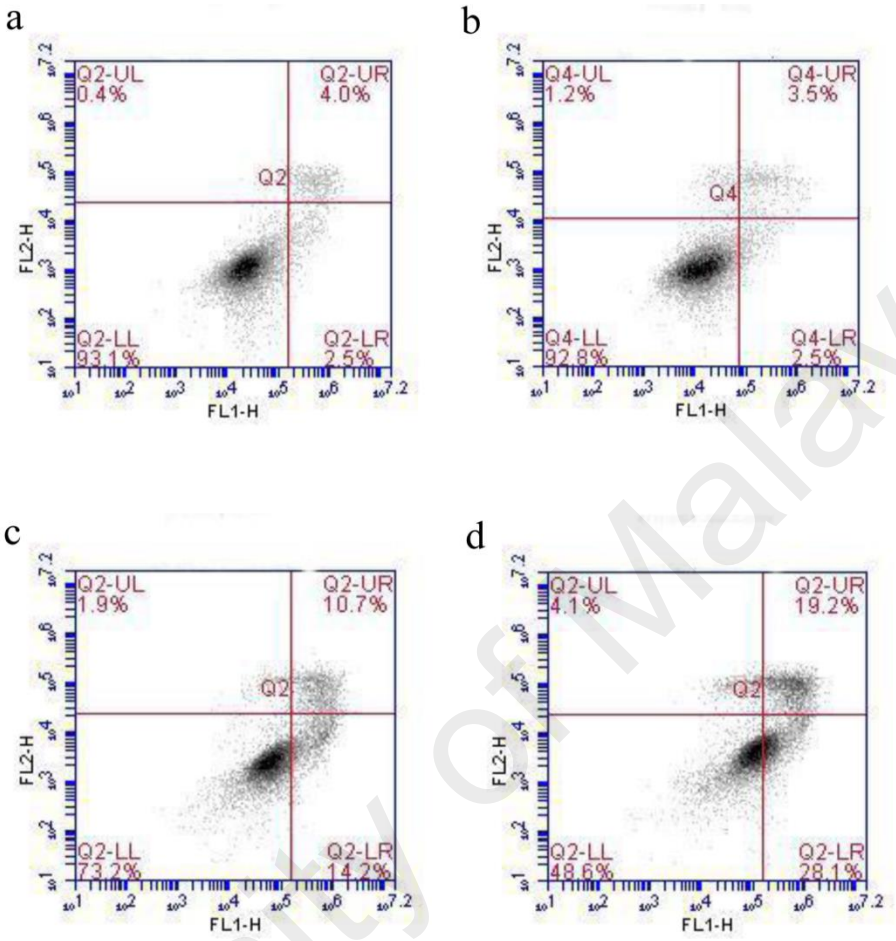


Figure 4.5: Apoptotic induction effect of helichrysetin on A549 cells in a dose-dependent study. Density plots showed the cell population in different quadrants, LL- Live cells, LR- early apoptotic cells, UR- late apoptotic cells, UL- secondary necrotic cells. a) Untreated A549 cells b) A549 cells treated with 5 µg/ml helichrysetin c) A549 cells treated with 15 µg/ml helichrysetin d) A549 cells treated with 20 µg/ml helichrysetin

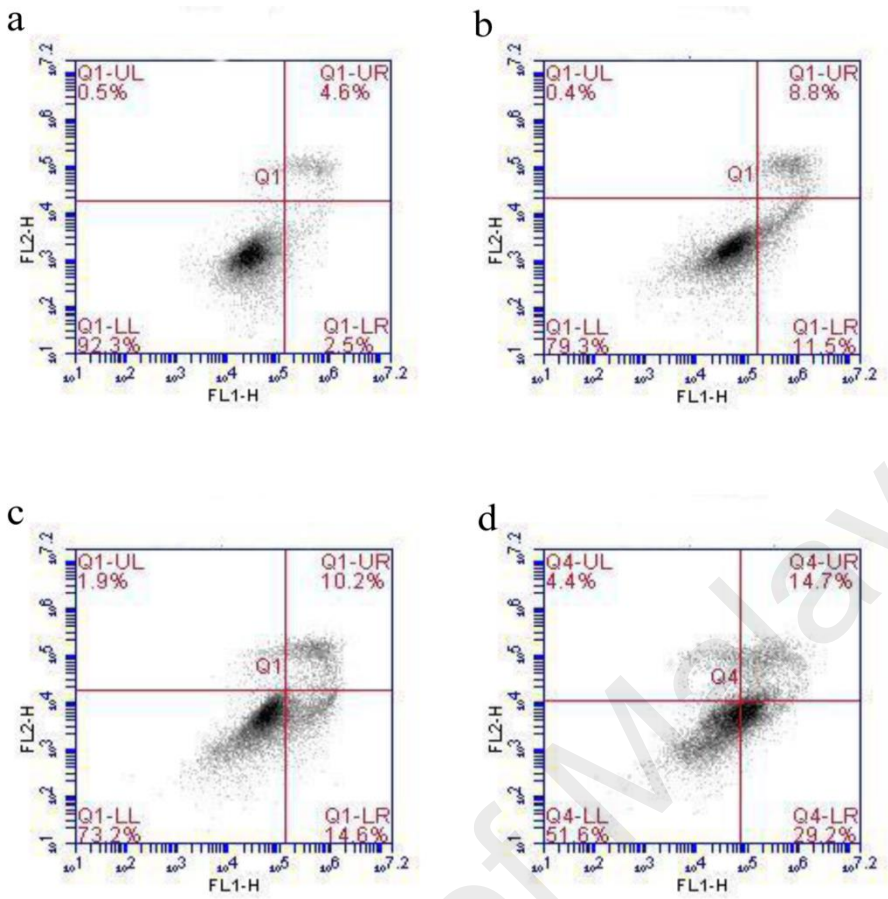


Figure 4.6: Apoptotic induction effect of helichrysetin on A549 cells in a time-dependent study. Density plots showed the cell population in different quadrants, LL- Live cells, LR- early apoptotic cells, UR- late apoptotic cells, UL- secondary necrotic cells. a) Untreated A549 cells b) A549 cells treated with 15 µg/ml helichrysetin for 24 hours c) A549 cells treated with 15 µg/ml helichrysetin for 48 hours d) A549 cells treated with 15 µg/ml helichrysetin for 72 hours

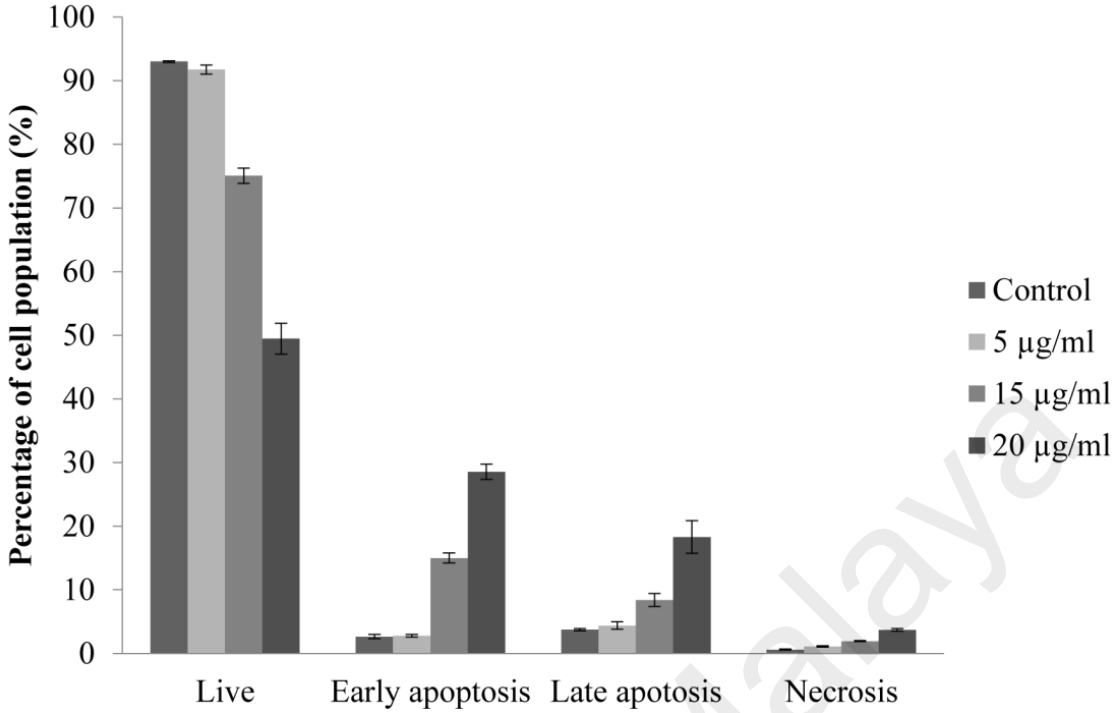


Figure 4.7: Percentage of cell population that consists of live cells, early apoptotic cells, late apoptotic cells and necrotic cells in dose-dependent study detected by Annexin V-FITC/PI assay. Cells were treated with 5 µg/ml, 15 µg/ml and 20 µg/ml of helichrysetin for 72 hours. Data represents the means ± SD. The results shown represent three independent experiments.

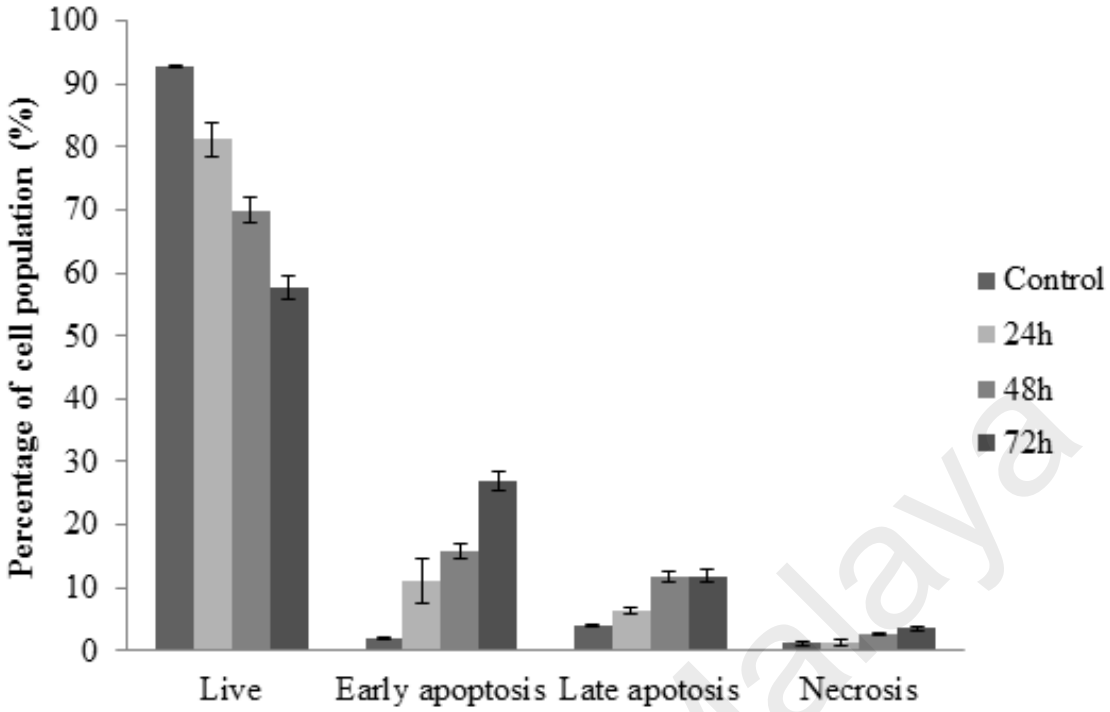


Figure 4.8: Percentage of cell population that consists of live cells, early apoptotic cells, late apoptotic cells and necrotic cells in time-dependent study detected by Annexin V-FITC/PI assay. Cells were treated with 15 µg/ml of helichrysetin at 24 hours, 48 hours and 72 hours. Data represents the means ± SD. The results shown represent three independent experiments.

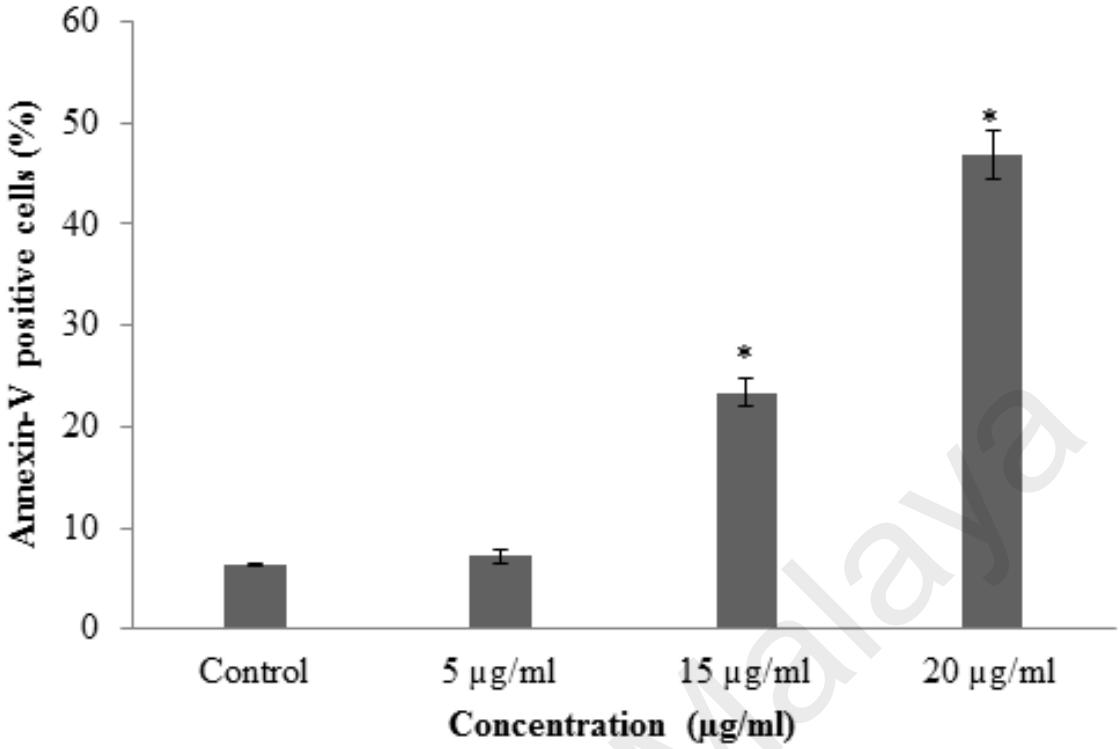


Figure 4.9: Percentage of apoptotic cells represented by the Annexin V positive-stained cells that consists of early and late apoptotic cells in dose-dependent study detected by Annexin V-FITC/PI assay. Cells were treated with 5 µg/ml, 15 µg/ml and 20 µg/ml of helichrysetin for 72 hours. Data represents the means ± SD. *p value < 0.05 vs untreated control. The results shown represent three independent experiments.

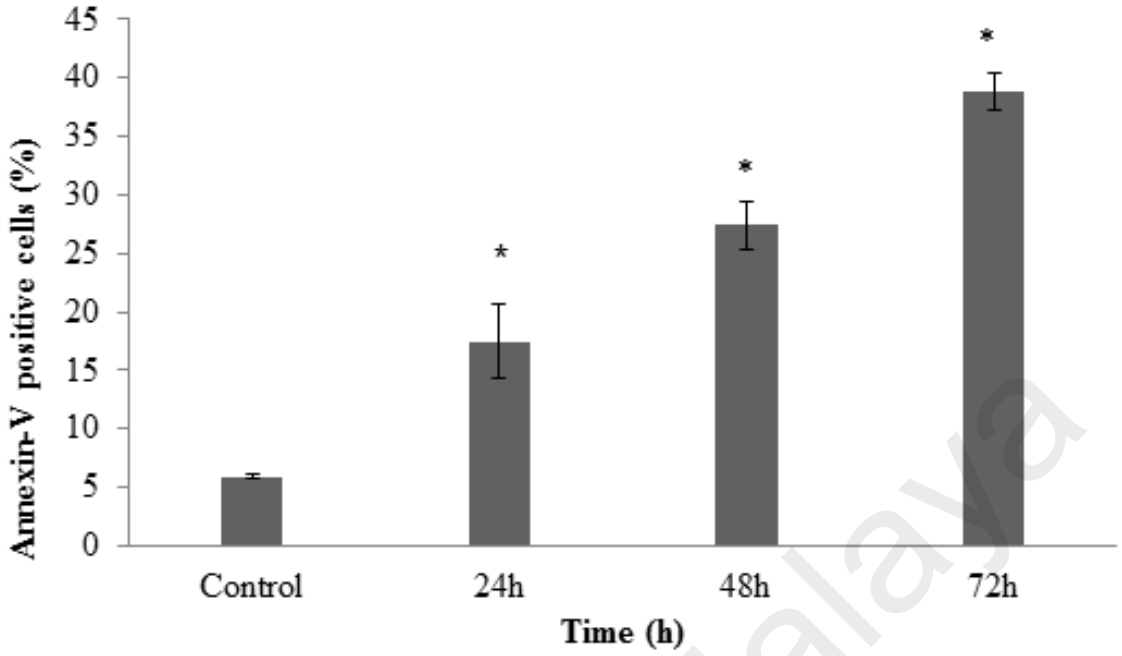


Figure 4.10: Percentage of apoptotic cells represented by the Annexin V positive-stained cells that consists of early and late apoptotic cells in time-dependent study detected by Annexin V-FITC/PI assay. Cells were treated with 15 μg/ml of helichrysetin at 24 hours, 48 hours and 72 hours. Data represents the means ± SD. *p value < 0.05 vs untreated control. The results shown represent three independent experiments.

4.3.2 Analysis of mitochondrial membrane potential for detection of apoptosis in A549 cells

Evidence for the effect of helichrysetin on the mitochondrial membrane potential of A549 cells was obtained using JC-1 mitochondrial membrane potential assay analyzed by flow cytometer. Density plot in Figure 4.11 and Figure 4.12 displayed the distribution of viable (top region) and apoptotic cells (bottom region) in the mitochondrial membrane potential analysis. Apoptotic cells are indicated by cells that have collapsed mitochondrial membrane potential which is related to the mitochondria-mediated apoptosis.

In the dose-dependent experiment, the percentage of cells in the apoptotic cells (bottom region) increased when the dosage of helichrysetin increased from 5 μg/ml to 20 μg/ml. The increase in the percentage of cells in the apoptosis region indicates the dose-dependent decrease in mitochondrial membrane potential. Collapse of mitochondrial membrane potential is a result of increase fluorescence intensity of monomeric JC-1 in the cytosolic part of A549 cells contributing to the increase in the green fluorescence in flow cytometry analysis. The percentage of apoptotic cells increased drastically at concentration of 15 μg/ml and 20 μg/ml.

Treatment with helichrysetin at IC_{50} at different time points showed that exposure of A549 cells to helichrysetin will cause the reduction in the mitochondrial membrane potential in the time-dependent manner. The percentage of viable cells decreased upon 24 hours and 48 hours treatment. Approximately one-half of the cell populations undergo apoptosis after exposure with helichrysetin for 48 hours. This suggests that helichrysetin is capable of inducing mitochondria-mediated apoptosis in A549 cells indicates by the changes of mitochondrial membrane potential.

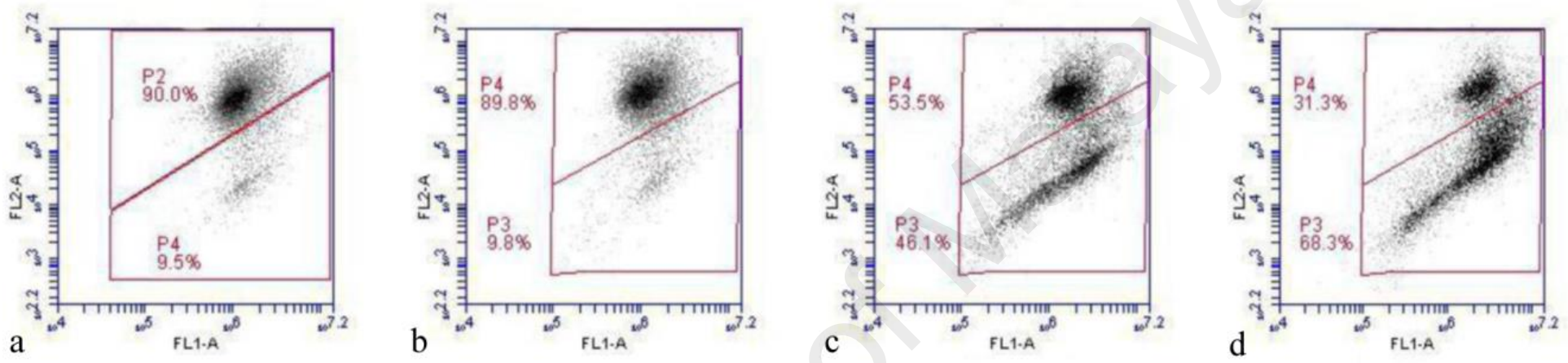


Figure 4.11: Treatment of A549 cells with varying concentrations of helichrysetin causes the collapse of mitochondrial membrane potential. Cell distribution in mitochondrial membrane potential assay analysed with flow cytometer in a dose-dependent study. Cells were exposed to helichrysetin at 5 µg/ml, 15 µg/ml and 20 µg/ml for 72 hours a) Untreated A549 cells b) A549 cells treated with 5 µg/ml helichrysetin c) A549 cells treated with 15 µg/ml helichrysetin d) A549 cells treated with 20 µg/ml helichrysetin

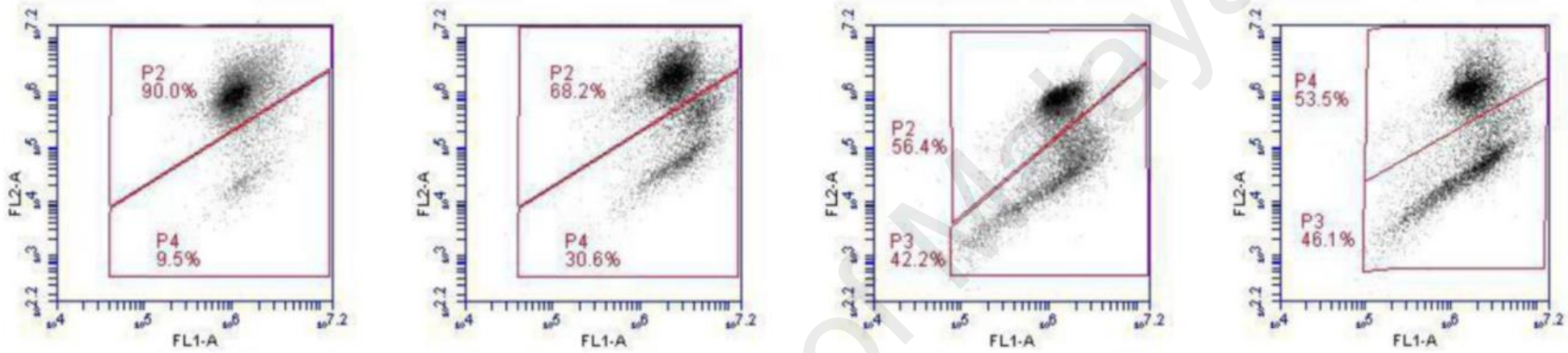


Figure 4.12: Exposure of A549 cells at different duration with helichrysetin at IC_{50} concentration causes the collapse of mitochondrial membrane potential. Cell distribution in mitochondrial membrane potential assay analyzed with flow cytometer in a time-dependent study. Cells were exposed to 15 µg/ml helichrysetin for 24 hours, 48 hours and 72 hours. a) Untreated A549 cells b) A549 cells treated with 15 µg/ml helichrysetin for 24 hours c) A549 cells treated with 15 µg/ml helichrysetin for 48 hours d) A549 cells treated with 15 µg/ml helichrysetin for 72 hours

4.3.3 Detection of apoptotic DNA fragmentation by TUNEL assay

Apoptotic DNA fragmentation was detected using TUNEL assay by flow cytometer. Figure 4.13 and Figure 4.14 displayed the results of apoptotic DNA fragmentation of A549 cells upon treatment with helichrysetin at different concentrations and time points. Plots in Figure 4.15 showed the distribution of live cells and apoptotic cells with fragmented DNA for the dose-dependent study and it is cleared that the increase in the concentration of helichrysetin from 5 μg/ml to 20 μg/ml caused increase DNA fragmentation in A549 cells. As shown in Figure 4.16, the treatment of helichrysetin at IC_{50} at time 24 hours, 48 hours and 72 hours resulted in the increase of apoptotic cells with DNA fragmentation.

There is a significant increase in the portion of TUNEL positive cells which are the apoptotic cells with fragmented DNA from 0.61% to 1.28%, 42.63% and 82.34% as the concentration of helichrysetin increased (Figure 4.13). When treated at IC_{50} for 24 hours, 48 hours and 72 hours, the percentage of TUNEL positive cells showed a significant elevation from 0.61% to 2.76%, 15.16%, and 41.29% (Figure 4.14). This proves that helichrysetin can cause the occurrence of DNA strand break in A549 cells and its effect is significant with increase dosage and exposure time.

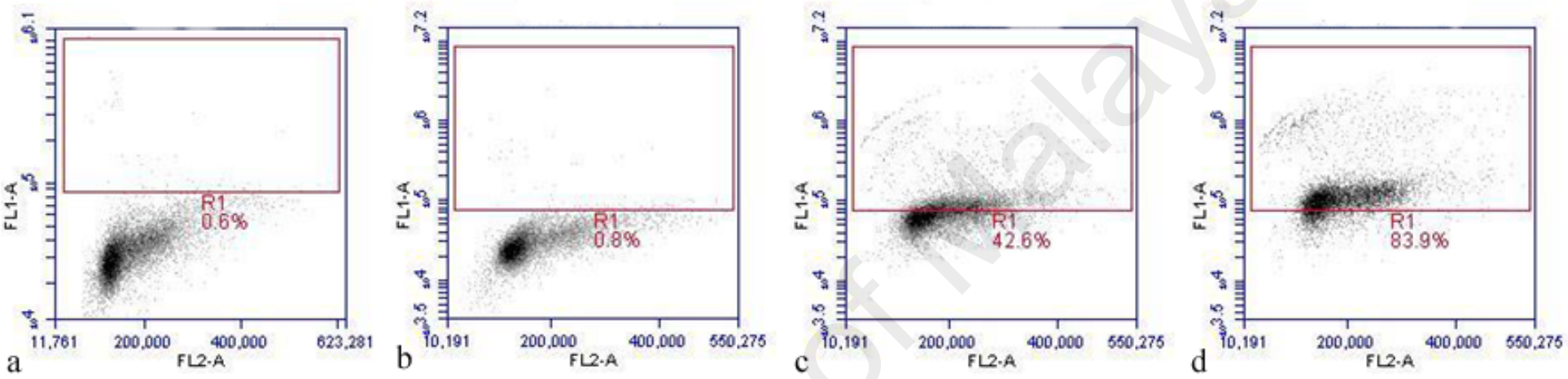


Figure 4.13: Detection of fragmented DNA from apoptotic cells by flow cytometric TUNEL assay. Density plot with cell distribution of live cells and apoptotic cells in a dose-dependent study. A549 cells were exposed to 5 µg/ml, 15 µg/ml and 20 µg/ml helichrysetin for 72 hours. a) Untreated A549 cells b) A549 cells treated with 5 µg/ml helichrysetin c) A549 cells treated with 15 µg/ml helichrysetin d) A549 cells treated with 20 µg/ml helichrysetin. (Each figure is representative of 3 experiments)

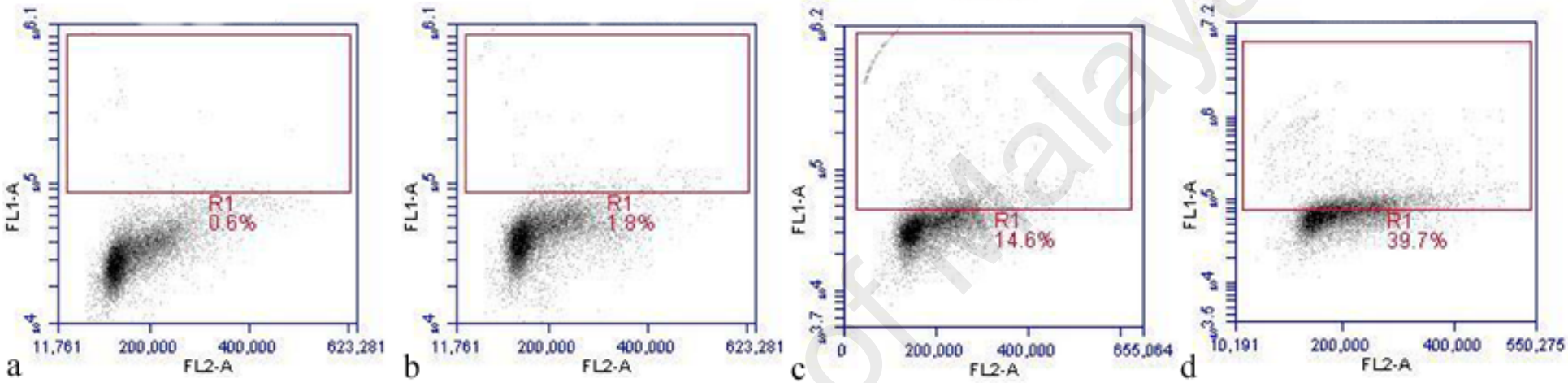


Figure 4.14: Detection of fragmented DNA from apoptotic cells by flow cytometric TUNEL assay. A549 cells were exposed to helichrysetin at IC_{50} at different duration. a) Untreated A549 cells b) A549 cells treated with 15 µg/ml helichrysetin for 24 hours c) A549 cells treated with 15 µg/ml helichrysetin for 48 hours d) A549 cells treated with 15 µg/ml helichrysetin for 72 hours. (Each figure is representative of 3 experiments)

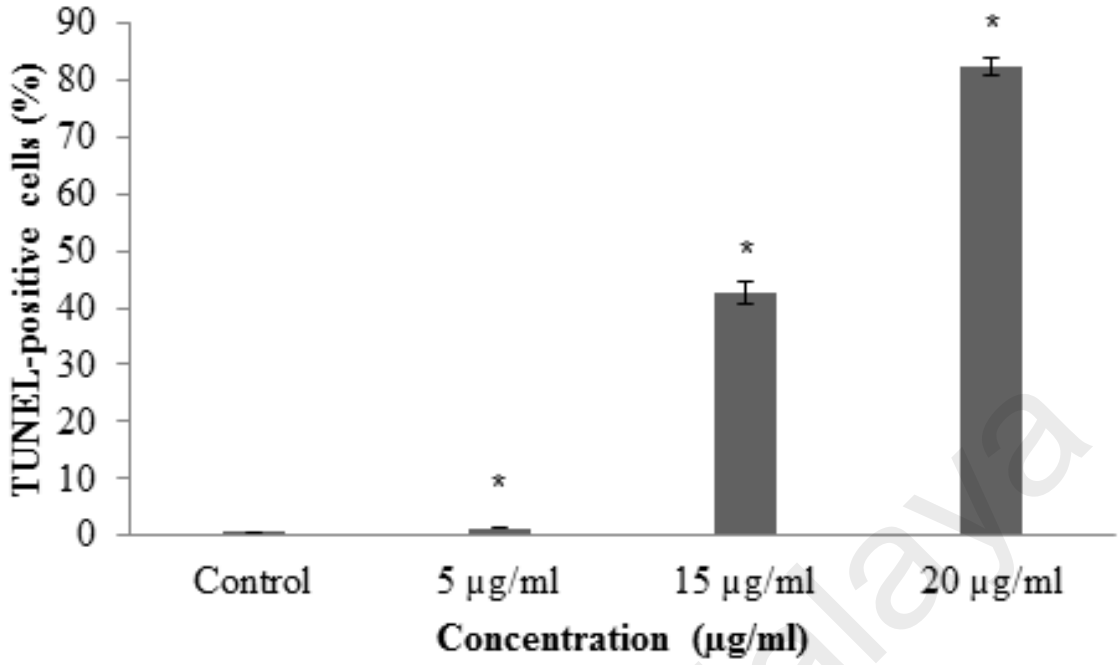


Figure 4.15: Percentage of apoptotic cells with fragmented DNA represented by percentage of TUNEL-positive cells. Cells were treated with 5 µg/ml, 15 µg/ml and 20 µg/ml of helichrysetin for 72 hours. Data represents the means ± SD. *p value < 0.05 vs untreated control. The results shown represent the findings from three independent experiments.

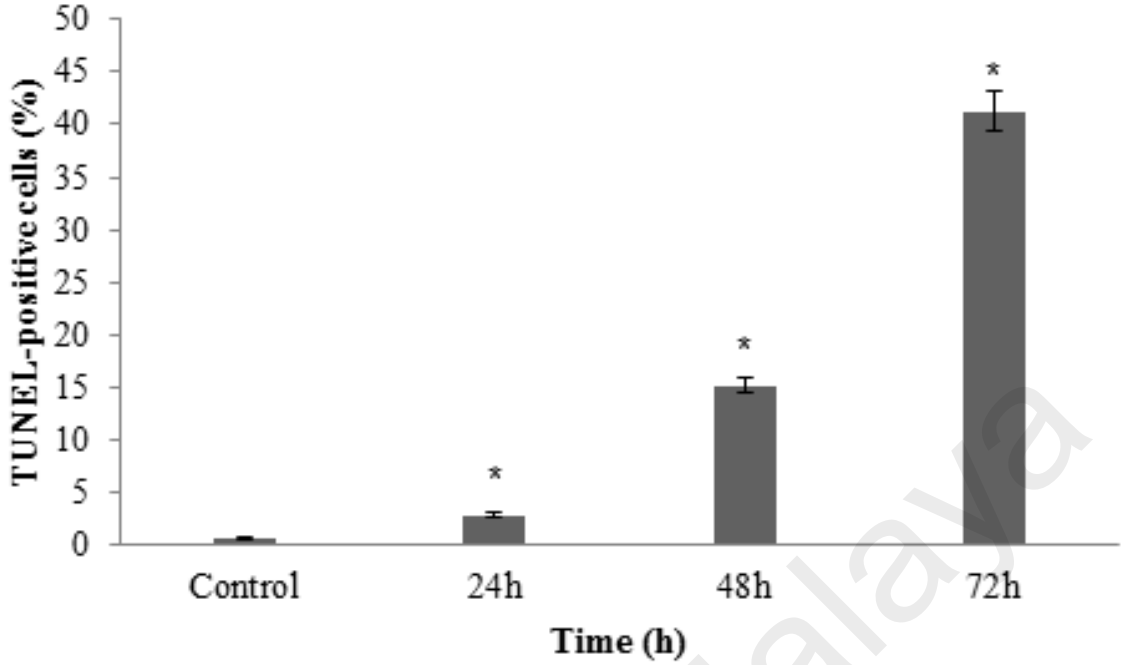


Figure 4.16: Percentage of apoptotic cells with fragmented DNA represented by percentage of TUNEL-positive cells. Cells were exposed to helichrysetin at IC_{50} for 24 hours, 48 hours and 72 hours. Data represents the means ± SD. *p value < 0.05 vs untreated control. The results shown represent the findings from three independent experiments.

4.3.4 Cell cycle analysis of helichrysetin-treated A549 cells

In cell cycle analysis, A549 cells were exposed to different dosage of helichrysetin, 5 µg/ml, 15 µg/ml and 20 µg/ml for 72 hours. Results from cell cycle analysis showed that helichrysetin caused the accumulation of cells in the S phase of the cell cycle (Figure 4.17). This is shown by the significant increase in the percentage of A549 cells in the S phase of cell cycle.

The elevation in the percentage of cells in S phase occurred simultaneously with a significant decrease of cell percentage in G0/G1 phase of the cell cycle (Figure 4.18). The percentage of cells in S phase increased from 16.69 ± 2.99% (untreated control) to 26.47 ± 1.56%, 38.29 ± 0.89%, and 46.91 ± 2.62% in a dose-dependent fashion. The percentage of cells in G0/G1 phase reduced from 73.38 ± 3.57% (untreated control) to 69.43 ± 0.49%, 49.03 ± 1.43%, and 43.33 ± 2.05% in a dose-dependent manner.

Results from cell cycle analysis revealed that helichrysetin has an effect on the cell cycle of A549 cells by causing the accumulation of cells in the S phase and the reduction of cells in the G0/G1 phase of the cell cycle.

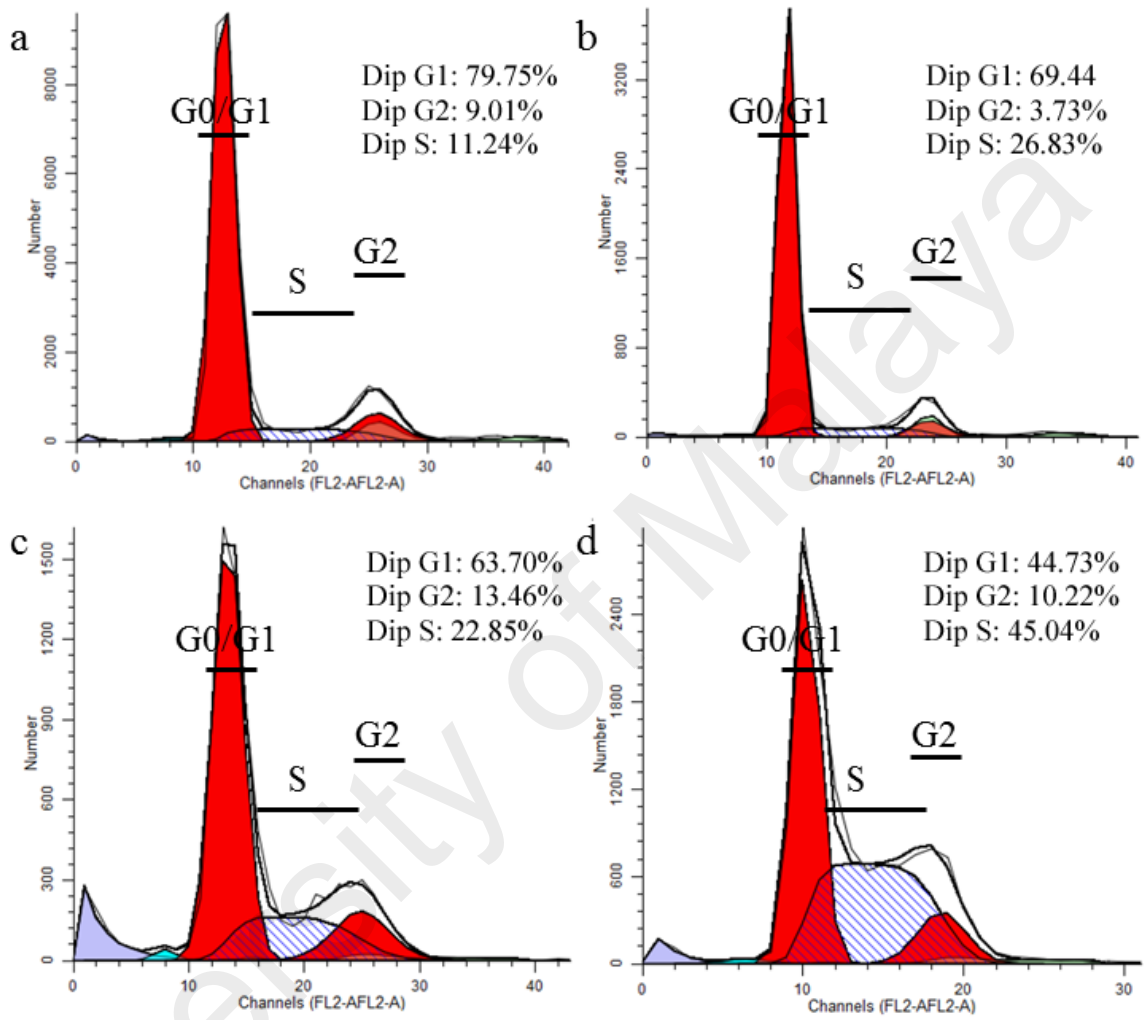


Figure 4.17: Cell cycle analysis of A549 cells treated with helichrysetin. Histogram showed the distribution of cells at different phases of cell cycle was determined using flow cytometer upon treatment with helichrysetin at different concentrations. a) Untreated A549 cells b) A549 cells treated with 5 µg/ml helichrysetin c) A549 cells treated with 15 µg/ml helichrysetin d) A549 cells treated with 20 µg/ml helichrysetin

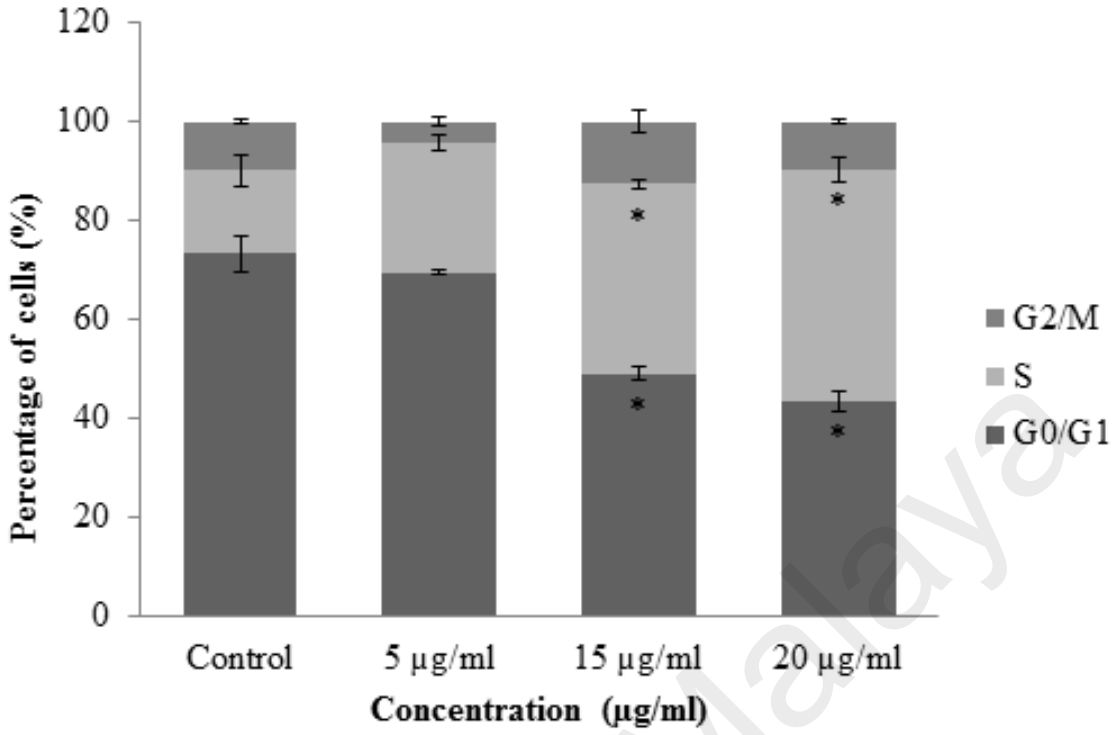


Figure 4.18: Bar chart displayed the A549 cells distribution at different phases of cell cycle in cell cycle analysis. Cells were exposed to helichrysetin at different concentrations, 5 µg/ml, 15 µg/ml and 20 µg/ml for 72 hours. Data represents the means ± SD. *p value < 0.05 vs untreated control. The results represent the findings from three independent experiments.

4.3.5. Apoptotic proteins detection in helichrysetin-treated and untreated A549 cells with western blotting

A549 cells were treated with helichrysetin at concentration of 15 µg/ml for 6 hours, 24 hours and 48 hours to detect specific apoptotic proteins changes. Caspases are protease enzymes that play important roles in the mechanisms of programmed cell death or apoptosis. Expression of caspase 3, caspase 8 and caspase 9 was investigated upon treatment with helichrysetin in comparison with untreated cells.

Results in Figure 4.19 showed that the expression of pro-caspase 3 (6hr: 1.00; 24hr: 1.07; 48hr: 1.33, Treated: 6hr: 1.01; 24hr: 0.89; 48hr: 0.73) was diminished upon treatment with helichrysetin in a time-dependent manner from 6 hours to 48 hours and the cleaved caspase 3 increased from 6 hours to 48 hours (6hr: 1.00; 24hr: 0.73; 48hr: 0.53, Treated: 6hr: 1.08; 24hr: 2.29; 48hr: 3.75). Pro-caspase 8 (6hr: 1.00; 24hr: 0.78;

48hr: 0.83, Treated: 6hr: 0.70; 24hr: 0.97; 48hr: 0.92) and cleaved caspase 8 (6hr: 1.00; 24hr: 0.82; 48hr: 0.64, Treated: 6hr: 0.69; 24hr: 1.15; 48hr: 1.84) also showed the similar trend where both proteins showed increase expression upon treatment for 6 hours, 24 hours and 48 hours. For caspase 9, the pro-caspase 9 showed decreased in expression after 6hours. 24 hours and 48 hours treatment (6hr: 1.00; 24hr: 0.96; 48hr: 0.82, Treated: 6hr: 0.77; 24hr: 0.46; 48hr: 0.41) while cleaved caspase 9 showed increased expression from 6 hours to 48 hours treatment (6hr: 1.00; 24hr: 2.24; 48hr: 2.59, Treated: 6hr: 3.11; 24hr: 3.53; 48hr: 3.58).

Poly (ADP-ribose) polymerase (PARP) is one of the commonly used markers for the detection of apoptosis. As shown in Figure 4.19, the full length PARP protein expression decreased after treatment with helichrysetin and this effect occurs in a time-dependent fashion (6hr: 1.00; 24hr: 0.84; 48hr: 0.80, Treated: 6hr: 0.96; 24hr: 0.78; 48hr: 0.27). As the expression of full length PARP protein decreased, the expression of cleaved PARP increased upon helichrysetin treatment in a time-dependent manner (6hr: 1.00; 24hr: 0.95; 48hr: 1.00, Treated: 6hr: 0.95; 24hr: 1.20; 48hr: 1.21).

Upon treatment with helichysetin, there is also a release of cytochrome c into the the cytosol with the increase in the protein expression at 6 hours, 24 hours and 48 hours of treatment in comparison with vehicles at each time point. (6hr: 1.00; 24hr: 1.00; 48hr: 1.36, Treated: 6hr: 1.58; 24hr: 1.35; 48hr: 1.67).

Apoptosis is regulated by Bcl-2 family proteins and the proteins that are being investigated are Bcl-2, and Bax. Results in Figure 4.19 displayed the protein band intensity of Bcl-2 protein (6hr: 1.00; 24hr: 1.11; 48hr: 1.31, Treated: 6hr: 1.20; 24hr: 0.81; 48hr: 0.54) decreasing after 24 hours and 48 hours treatment. While for Bax which is a pro-apoptotic protein, the expression of Bax protein increased in a time-dependent

manner from 6hours treatment to 24 hours (6hr: 1.00; 24hr: 1.81; 48hr: 2.47, Treated: 6hr: 2.46; 24hr: 2.63; 48hr: 2.47).

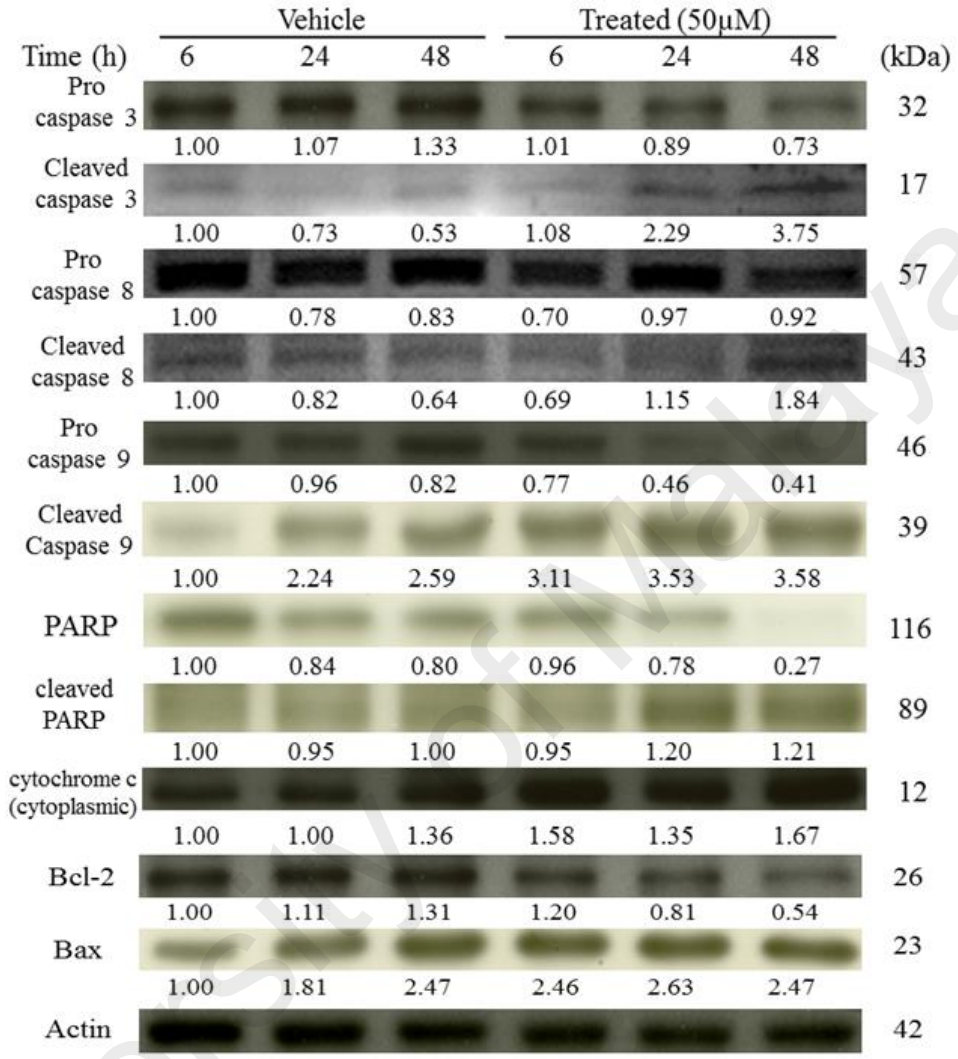


Figure 4.19: Western blotting analysis of apoptotic markers. Effects of helichrysetin on A549 cells were evaluated by western blotting after treatment with 50 μM helichrysetin at 6, 24 and 48 hours and without treatment (vehicle) at 6, 24 and 48 hours.

4.4 Application of proteomic to investigate mechanism of action of helichrysetin

4.4.1 Proteomic analysis of whole cell proteome from helichrysetin-treated A549 cell

To investigate the molecular mechanisms involved in the induction of cell death in A549 cancer cells by helichrysetin, quantitative proteomic analysis was performed using the iTRAQ labeling. Duplicate protein samples from 4 different treatments, untreated (control), 6 hours helichrysetin-treated sample, 24 hours helichrysetin-treated sample, and 48 hours helichrysetin-treated sample were labeled with iTRAQ labels 114, 115, 116, 117, pooled and analyzed.

A total of 3708 and 3561 proteins were identified and quantified in both first and second biological replicates respectively. These proteins are selected by at least 2 unique peptides with 1% FDR. 2645 proteins were identified and identical in both replicates which correspond to 1% FDR. 95% confidence level which corresponds to z-score of $>1.960\sigma$ or $< -1.960\sigma$ were used to determine differentially regulated proteins. z-score was calculated as followed:

$$\text{z-score (protein)} = \frac{\text{Log}_2 \text{ protein ratio} - \text{Mean of } (\log_2 \text{ of each protein ratio})}{\text{Standard deviation of } (\log_2 \text{ of each protein ratio})}$$

With this criterion, differentially expressed proteins were selected from the 2645 identified and identical proteins in both replicates from each sample.

Following this criterion, 125 proteins, 129 proteins and 141 proteins were identified as differentially expressed after treatment with helichrysetin for 6 hours, 24 hours, and 48 hours respectively. From the 6 hours helichrysetin-treated sample (115:114), 60 proteins were up-regulated and 65 proteins were down-regulated, the 24 hours helichrysetin-treated sample (116:114), 45 proteins were up-regulated and 84 proteins were down-

regulated and the 48 hours helichrysetin-treated sample (117:114), 70 proteins were up-regulated and 71 proteins were down-regulated.

Table 4.3: Number of proteins that are differentially up- and down- regulated in three samples treated with helichysetin.

No. of proteins	6hr (115:114)	24hr (116:114)	48hr (117:114)
Upregulated	60	45	70
Downregulated	65	84	71
Total	125	129	141

The differentially expressed proteins were analyzed using Ingenuity Pathway Analysis (IPA) software to determine canonical pathways and upstream regulators that are significantly linked to these proteins. Figure 4.20 showed the analysis result of top 10 canonical pathways from comparison analysis for 6 hours, 24 hours and 48 hours helichrysetin-treated results that are most significant to the data set and the proteins involved in the pathways.

Top 10 canonical pathways relevant to the given set of data are assembly of RNA polymerase III complex, cell cycle: G2/M DNA damage checkpoint regulation, NRF2-mediated oxidative stress response, granzyme B signaling, asparaginase biosynthesis I, ATM signaling, GADD45 signaling, hereditary breast cancer signaling, aryl hydrocarbon receptor signaling and γ-linolenate biosynthesis II.

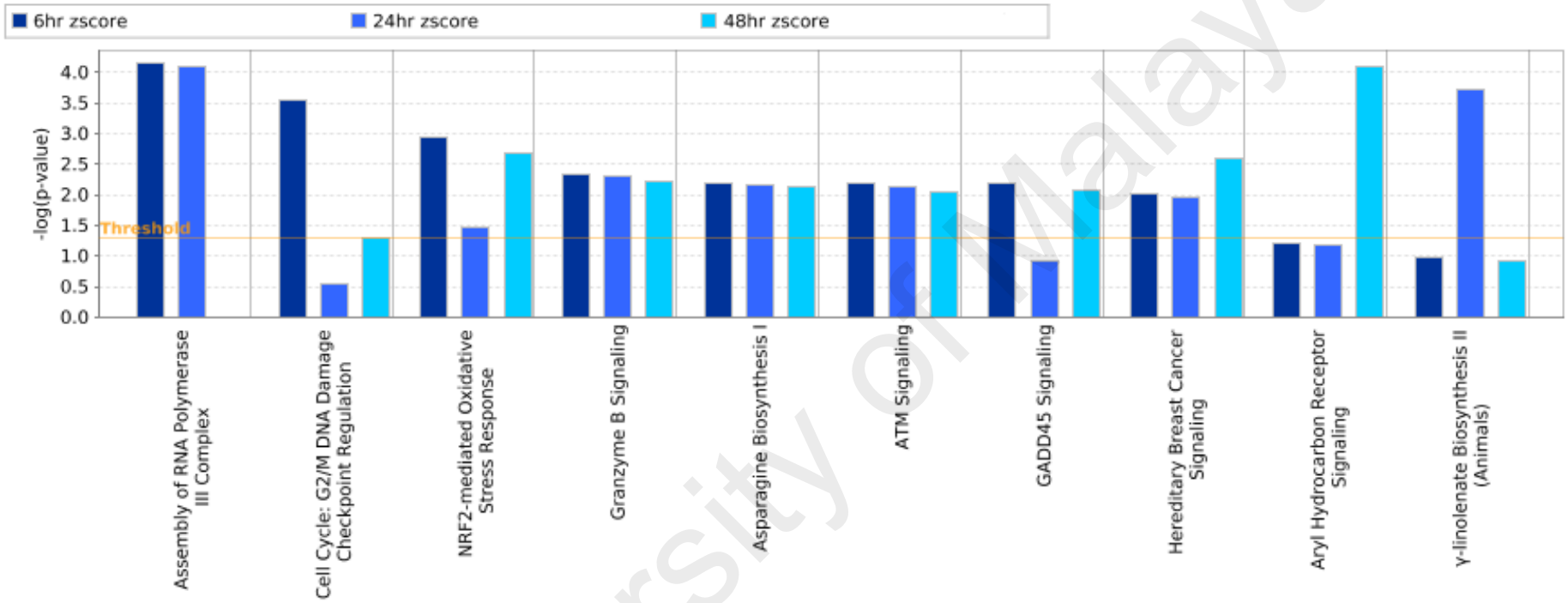


Figure 4.20: Ingenuity Pathway Analysis for significantly regulated proteins. Top 10 canonical pathways from comparison IPA analysis for 6, 24, and 48 hours with significance of p value < 0.05 (-log (p-value)>1.3)

This result revealed the important components involved in cell cycle: G_2/M DNA damage checkpoint regulation, NRF2-mediated oxidative stress response, ATM signaling, GADD45 signaling and hereditary breast cancer signaling, and will be further investigated in this study (Table 4.4). Gene ontology analysis was performed based on the biological processes for all the differentially expressed proteins using R package, clusterProfiler and GoSemSim. Result is displayed in Figure 4.21.

Table 4.4: List of top 10 canonical pathways and proteins that are significantly altered in association with the pathways.

Top 10 canonical pathways	Proteins involved (Gene name)		
	6h	24h	48h
Assembly of RNA Polymerase III Complex	GTF3C1 GTF3C3 GTF3C5	GTF3C1 GTF3C3 GTF3C5	-
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	YWHAG YWHAE SFN CCNB1	CDKN1A	CDKN1A CCNB1
NRF2-mediated Oxidative Stress Response	HMOX1 FTL EPHX1 ERP29 ABCC1 GSTK1	HMOX1 FTL DNAJB1 EPHX1	HMOX1 FTL ERP29 EPHX1 GSTK1
Granzyme B Signaling	LMNB1 LMNB2	LMNB1 LMNB2	LMNB1 LMNB2
Asparagine Biosynthesis I	ASNS	ASNS	ASNS
ATM Signaling	BRAT1 FANCD2 CCNB1	BRAT1 FANCD2 CDKN1A	FANCD2 CCNB1 CDKN1A
GADD45 Signaling	CDK4 CCNB1	CDKN1A	CDKN1A CCNB1
Hereditary Breast Cancer Signaling	FANCD2 CDK4 SFN CCNB1	FANCD2 CDKN1A CDK6	FANCD2 RB1 CDKN1A CDK6 CCNB1

Table 4.4 Continued

Aryl Hydrocarbon Receptor Signaling	CDK4 GSTP1 PTGES3 CTSD RB1 ALDH3B1 GSTK1	POLA1 CDKN1A CDK6	CTSD RB1 ALDH3B1 GSTK1 POLA1 CDKN1A CDK6
γ-Linolenate Biosynthesis II (Animals)	FADS2	ACSL3 FADS2 FADS1	ACSL3

Protein alterations were further evaluated with the analysis of the potential upstream regulator. The activation and inhibition of upstream regulators are determined by its activation and inhibition z-score, >2.0 or <-2.0. Based on the data set obtained, p53 protein, nuclear factor (erythroid-derived 2)-like 2, and cyclin D1 proteins and these upstream regulators are important to predict the regulatory cascades involved with the related biological activities (Table 4.5). The activation scores showed that p53 protein and nuclear factor (erythroid-derived 2)-like 2 were activated while cyclin D1 protein was inhibited.

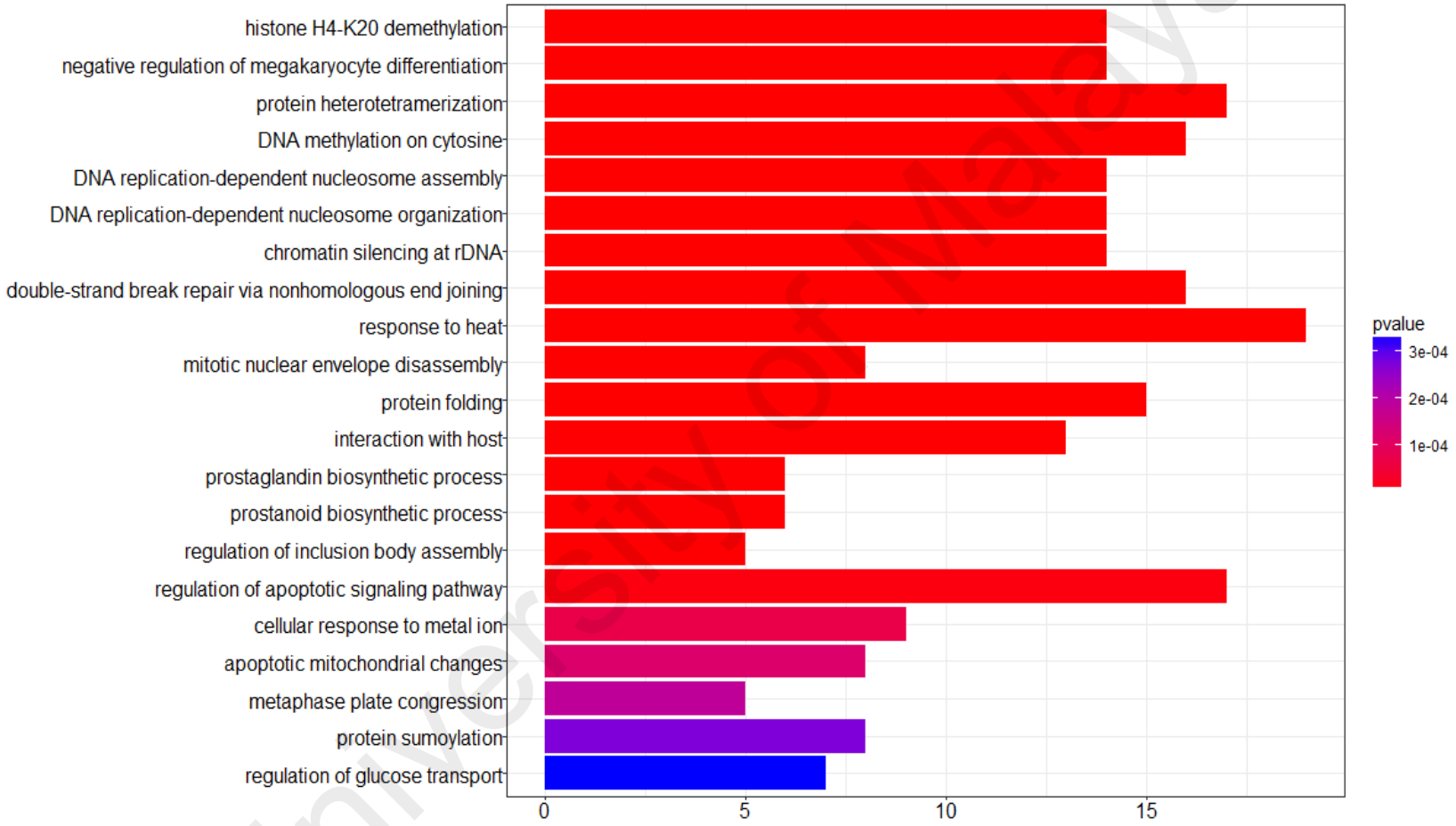


Figure 4.21: Differentially expressed proteins in A549 cells treated with helichrysetin for 6 hours, 24 hours, and 48 hours that were categorized based on its biological processes using Gene Ontology (GO) database searchers using R package, clusterProfiler (v. 3.0.5) and removing GO terms redundancy using GOSemSim (v.1.30.3).

Table 4.5: Upstream regulator analysis of differentially regulated proteins in A549 cells that are predicted to be activated and inhibited as determined by IPA.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
p53	transcription regulator	Activated	2.404	3.13E-11
NRF2	transcription regulator	Activated	2.776	1.39E-03
Cyclin D1	transcription regulator	Inhibited	-2.449	8.08E-05

4.4.2 Validation of proteins related to DNA damage response, oxidative stress and cell cycle regulation pathways by Western blotting.

A549 cells were treated with 15 μg/ml helichrysetin for 6 hours, 24 hours and 48 hours to investigate the expression of DNA damage response, oxidative stress and cell cycle related proteins. p21 protein, which is also known as cyclin-dependent kinase inhibitor 1 (CDKN1A) was evaluated for its protein expression and there is an increase in the expression of p21 after treatment at 24 hours and 48 hours (6hr: 15.00; 24hr: 0.39; 48hr: 0.52, Treated: 6hr: 0.91; 24hr: 0.50; 48hr: 1.13).

The expression of heme-oxygenase 1 (HMOX1) was increased upon treatment with helichrysetin (6h: 1.00; 24hr: 1.30; 48hr: 1.19, Treated: 6hr: 1.23; 24hr: 1.80; 48hr: 1.57). Nrf2 was investigated for its expression to validate result from the upstream regulator (6h: 1.00; 24hr: 0.95; 48hr: 1.11, Treated: 6hr: 0.92; 24hr: 1.23; 48hr: 0.62).

Rb1 (6h: 1.00; 24hr: 1.02; 48hr: 0.66, Treated: 6hr: 0.97; 24hr: 0.50; 48hr: 0.56) and FANCD2 (6h: 1.00; 24hr: 0.64; 48hr: 0.67, Treated: 6hr: 0.15; 24hr: 0.49; 48hr: 0.15) proteins are the two proteins that are involved in the cell cycle progression and DNA repair and they are down-regulated after treatment with helichrysetin from 6 hours to 48 hours. Studying the expression of ATM and BRCA1 proteins are important to understand its association with the expression of BRCA1-associated ATM activator in

ATM signaling pathway (Table 4.4). Results showed that BRCA1 (6h: 1.00; 24hr: 0.73; 48hr: 0.46, Treated: 6hr: 1.10; 24hr: 0.50; 48hr: 0.47) was up-regulated at 6 hours treatment and subsequently reduced after 24 hours treatment while p-ATM (6h: 1.00; 24hr: 0.87; 48hr: 0.68, Treated: 6hr: 0.80; 24hr: 0.64; 48hr: 0.72) was reduced after helichrysetin treatment. The expression of p53 tumor suppressor showed an increase after treatment with helichrysetin (6hr: 1.00; 24hr: 1.25; 48hr: 0.65, Treated: 6hr: 0.62; 24hr: 0.57; 48hr: 1.51). Rad51 protein was up-regulated after 6 hours and 24 hours treatment and at 48 hours the expression was down-regulated (6hr: 1.00; 24hr: 1.01; 48hr: 1.34, Treated: 6hr: 1.14; 24hr: 1.53; 48hr: 1.22).

Cyclin D1, cyclin E, cyclin A and cyclin-dependent kinase 2 (cdk2) proteins are essential proteins in cell cycle regulation. Results showed that after treatment with helichrysetin, cyclin D1 protein showed increased expression after 6 hours treatment and subsequently decreased after 24 and 48 hours (6hr: 1.00; 24hr: 0.84; 48hr: 1.21, Treated: 6hr: 1.55; 24hr: 1.18; 48hr: 1.25). For cyclin E (6hr: 1.00; 24hr: 0.98; 48hr: 1.14, Treated: 6hr: 0.71; 24hr: 0.60; 48hr: 0.72) and cyclin A (6h: 1.00; 24hr: 0.80; 48hr: 0.72, Treated: 6hr: 0.75; 24hr: 0.72; 48hr: 0.55), the expression were down regulated after treatment with helichrysetin for 6 hours, 24 hours and 48 hours while cdk2 protein expression was down-regulated with helichrysetin treatment. (6hr: 1.00; 24hr: 0.64; 48hr: 0.86, Treated: 6hr: 0.63; 24hr: 0.42; 48hr: 0.56).

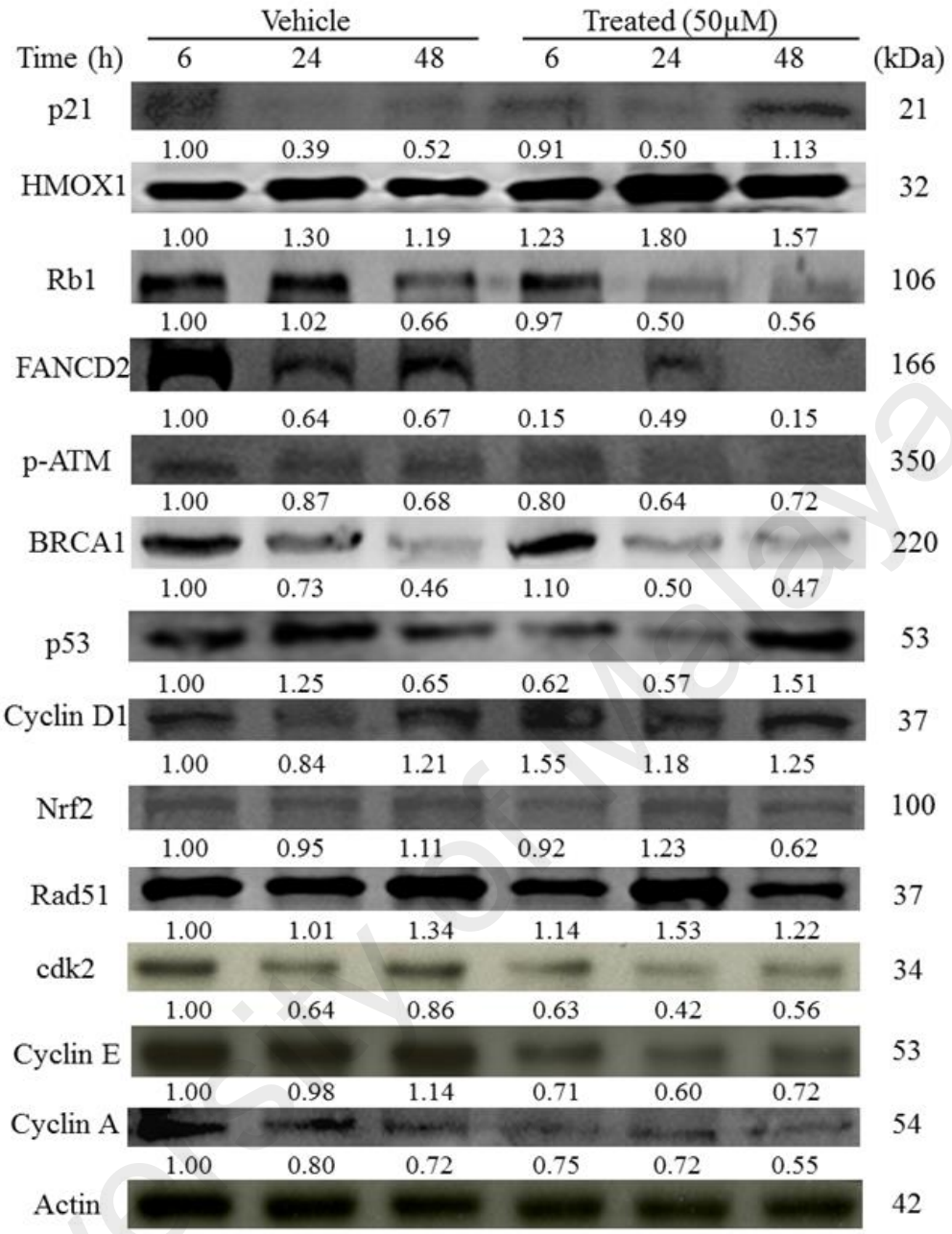


Figure 4.22: Western blotting analysis. Effects of helichrysetin on A549 cells were evaluated by western blotting after treatment with 50 μM helichrysetin at 6, 24 and 48 hours and without treatment (vehicle) at 6, 24 and 48 hours.

CHAPTER 5: DISCUSSION

5.1 Helichrysetin inhibits growth of A549 cells and causes changes to the cell and nuclear morphology

Based on the previous studies, helichrysetin showed effective anti-proliferative activity on human breast cancer cell line (6.5μM), human liver cancer cell line (51.1 μM) (Hua et al., 2008), human cervical cancer cell line (5.2 μM) (Vogel et al., 2008) and human fibrosarcoma cell line (40.1μM) (Ali et al., 2001) with active IC_{50} readings of approximately 50 μM and below.

In this study, we reported the results of growth inhibitory activity of helichrysetin on five selected cell lines that include four human cancer cell lines and one human normal cell line. Helichrysetin has been proven to be active against A549 and Ca Ski cell lines with its highest inhibition on Ca Ski cells with IC_{50} of 14.52 ± 0.36 μg/ml and 8.88 ± 0.13 μg/ml respectively. A compound that acts directly on cancer cells is considered effective when the IC_{50} range between 1-50 μM (Boik, 2001). Hence, helichrysetin has effective cytotoxicity on A549 and Ca Ski cell lines.

Since this is the first finding of the action of helichrysetin on human lung cancer cells, the study further proceeds to focus on A549 cells. Time-course action of helichrysetin on A549 cell line is performed to understand its cytotoxicity at different time points. The time-dependent inhibition of helichrysetin on A549 cells showed that helichrysetin can inhibit the proliferation of cells up to 50% at 48 hours of treatment. The percentage of inhibition also increases in a time-dependent manner. This suggested that when cells are exposed to helichrysetin, the cell growth of the cells will be inhibited and this reflects the reduction in the cellular metabolic activity because MTT assay performs measurement of the cell population's total mitochondrial activity related to its number of viable cells where the yellow tetrazolium salt, MTT will be cleaved to form soluble

blue formazan by the mitochondrial enzymes reflecting the activity of mitochondria in the cells (Sylvester, 2011).

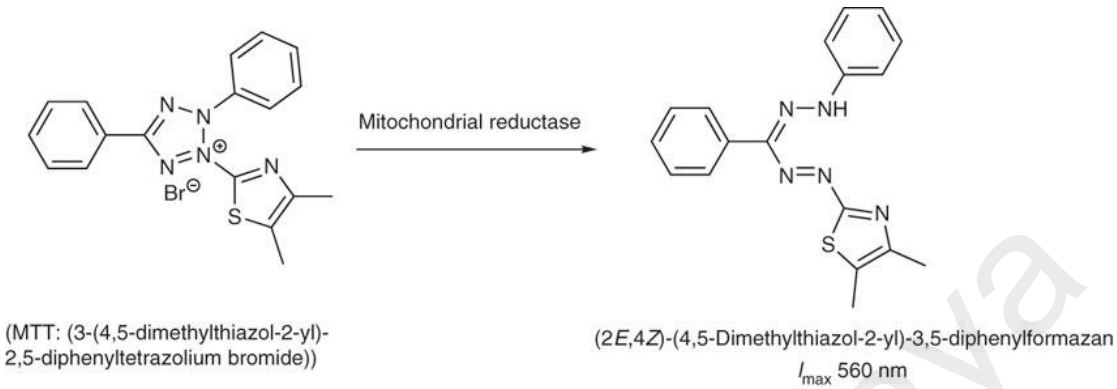


Figure 5.1: Cleavage of MTT to blue formazan product by mitochondrial reductase (Ebada et al., 2008)

Result from the growth inhibitory assay revealed that helichrysetin can partially inhibit the mitochondrial activity in A549 cells (van Meerloo et al., 2011). IC_{50} reading for MRC-5, human lung fibroblast showed the highest value that is approximately 3 times higher than the active concentration, 50 μM showing that helichrysetin is not cytotoxic towards normal cells in comparison with cancer cells which prove it beneficial for the treatment of human lung cancer.

The detection of unique morphological characteristics of cells upon stimulation by drugs or compounds is important in determining the mode of cell death occurred in the cells (Ziegler et al., 2004). Apoptotic treatments applied to cells can induce morphological alterations specific to the occurrence of apoptosis. Cells altered due to the occurrence of apoptosis exhibit shrinkage, rounding and blebbing of plasma membrane (Rello et al., 2005). Upon treatment of A549 cells with helichrysetin at increasing dosage, results showed that helichrysetin can cause the rounding and loss in volume/shrinkage of A549 cells under the microscope. A549 cells were found to be floating in the culture medium which implied that upon treatment with helichrysetin, the cells started to round up and losses its attachment from the surface of the culture

substratum. These are the morphological characteristics of cells that are specific to the occurrence of apoptosis (Gomez-Angelats et al., 2002; Marushige et al., 1998).

Normally, uponstimulation of apoptosis, cells started to lose its contact with the neighboring cells in the early stage of apoptosis. Shrinkage of cells will lead to the formation of apoptotic bodies that contain cellular organelles, then engulfed by surrounding cells through phagocytosis (Hengartner, 2001). It has been shown in some studies that the apoptotic morphological changes are the results of proteases that cleave cell cytoskeleton proteins such as actin when cleaved will induce cellular shrinkage (Mashima et al., 1999). However, this action is insufficient to cause apoptotic cellular fragmentation. When apoptosis occurs, caspases cleave other proteins that are involved in the attachment, organization, maintenance of the cytoskeletons (Kothakota et al., 1997). The induction of apoptotic cell morphology degeneration has also been observed in other studies related to natural compound (Koparal et al., 2003) such as studies on curcumin (Taraphdar et al., 2001), genistein (Qi et al., 2010), resveratrol (Clement et al., 1998) and others.

Nuclear morphological changes are very distinct and predominant in the process of apoptosis and the morphological hallmarks in nucleus are nuclear fragmentation and chromatin condensation (Ziegler et al., 2004). Observation from this study showed the effect of helichrysetin on nuclear morphology by fluorescence staining, DAPI stain and the result revealed that helichrysetin can induce chromatin condensation, subsequently the breaking of the condensed chromatin into nuclear fragments in A549 cells. These changes in the nuclear chromatin and the occurrence of nuclear fragmentation are the commonly recognized markers of apoptosis (Dini et al., 1996; Liu et al., 1998). DAPI stain is used commonly to stain by binding to the minor groove of double-stranded DNA, hence, suitable for the visualization of chromatin condensation and nuclear fragmentation (Chazotte, 2011).

Chromatin condensation usually occurs along the nuclear membrane forming crescent-like structure and finally breaks up in the cells. Fragmented nuclear components eventually are packaged into apoptotic bodies at the final stage of apoptosis (Toné et al., 2007). The phenomenon of the nuclear fragmentation in an intact cell is described as karyorhexis (Majno et al., 1995). Curcumin which is one of the major components in *Curcuma longa* rhizome a naturally derived phenolic component has also showed similar nuclear morphological changes as observed in this study (Lee et al., 2009).

These changes in the nuclear morphology can be initiated by the cleavage activity of caspase 3 (Eidet et al., 2014). Caspase 3 can act by cleaving various proteins that are important to maintain the structural integrity of cell nuclei such as lamin and nuclear mitotic apparatus protein (NuMA). For chromatin condensation and the formation of apoptotic bodies to occur during the fragmentation of cell nuclei, the disintegration of nuclear matrix is required. NuMA and lamins served as the important markers for the structure of nuclear matrix (Merdes et al., 1996; Nickerson, 2001). Therefore, when apoptosis occurs and caspase 3 is initiated, it cleaves lamins and NuMA that supports the structure of cell nuclei and this will result in the occurrence of chromatin condensation and nuclear fragmentation following the initiation of apoptosis (Kivinen et al., 2005).

The preliminary screening for the growth inhibitory effect of helichrysetin showed that helichrysetin can inhibit the growth of A549 cells in a time- and dose- dependent manner. Growth inhibition is the result of induction of cell death in A549 cells and this is characterized by the cellular and nuclear morphological changes in the cells observed by fluorescence and phase contrast microscopy. The features observed in the microscopy study showed apoptotic features, cell shrinkage, detachment, chromatin condensation and nuclear fragmentation in helichrysetin-treated A549 cells. Hence, helichrysetin has the ability to cause the occurrence of apoptotic features in the cells.

5.2 Helichrysetin can induce apoptosis features in A549 cells

To further investigate the ability of helichrysetin to induce apoptosis in A549 cells, further study using the flow cytometry to observe biochemical hallmarks of apoptosis and cell cycle, and western blotting to understand the mechanisms of apoptosis have been performed. Results from this study showed that helichrysetin displayed time- and dose- dependent effect on A549 cells in most of the detection of biochemical hallmarks of apoptosis.

Firstly, the induction of apoptosis was detected by the changes in the plasma membrane integrity in A549 cells upon treatment with helichrysetin at increasing concentrations and time points. In the Annexin V-FITC/PI detection, Annexin V binds to phosphatidylserine that is translocated to the outer leaflet of cell membrane during the early stage of apoptosis (Martin et al., 1995). The percentage of Annexin V-positive cells that increases in both the time- and dose- dependent study showed that helichrysetin is capable of inducing apoptosis signify by the disruption of the cell membrane integrity hence the loss of membrane asymmetry (Fadok et al., 1992). The changes in the composition of the cell membrane through this process will serve as a signal for phagocytes to recognize dying cells for the clearance of apoptotic cells (Erwig et al., 2007).

Treatment with helichrysetin also caused the release of cytochrome c into the cytosol from mitochondria. Hence, the effect of helichrysetin on mitochondrial membrane potential was determined using JC-1, a mitochondrial-specific cationic dye which will measure the loss of mitochondrial membrane potential (Perry et al., 2011). In untreated cells, most A549 cells exhibit red fluorescence due to the formation of J-aggregates indicating the presence of more cells with intact mitochondrial membrane potential while the disruption of mitochondrial membrane potential occurred in treated cells

showed by the accumulation of JC-1 dye in the cytoplasm in the monomeric form with higher green fluorescence (Smiley et al., 1991).

Exposure of A549 cells with helichrysetin caused the loss of mitochondrial membrane potential in a dose- and time- dependent manner. The release of cytochrome c started as an early event after the exposure of the cells to helichrysetin at 6 hours treatment. Because of the cytochrome c release into the cytoplasm, it may involve the opening of permeability transition pore of the mitochondrial inner membrane which caused the collapse in the mitochondrial membrane potential (Vander Heiden et al., 1997). Opening of the pore also result in swelling of the mitochondria. Disruption and swelling of the mitochondrial have been described as events that occur during apoptosis when tumor cells are exposed to cancer chemopreventive agents (Nakamura et al., 2002).

TUNEL assay was performed to detect apoptotic cells with fragmented DNA. DNA will be cleaved by the nucleases during the process of apoptosis resulting in the exposure of 3´OH that serves as a site for Br-dUTP nick labeling (Darzynkiewicz et al., 2008).

As A549 cells were treated with helichrysetin, there is an increase in the percentage of cells in the apoptotic cells region indicating the increase of DNA fragmentation in the cells. Higher concentrations of helichrysetin and longer exposure resulted in the higher number of free DNA ends that are labeled with TUNEL assay (Jakob et al., 2011). This indicates that helichrysetin can cause the production of more DNA double strand breaks in the cells. DNA double strand breaks are one of the most severe forms of DNA damage and the failure of the DNA repair system will trigger apoptotic responses through the activation of mitochondrial intrinsic pathway (Kaina, 2003).

Cell cycle analysis showed that the increase in helichrysetin treatment caused the elevation in the percentage of cells in S phase of cell cycle. The accumulation of cells in S phase increased significantly at concentration of 15 μg/ml to 20 μg/ml and this may

contribute to the initiation of cell death in A549 cells. The effect of helichrysetin on cell cycle progression with S phase arrest is distinct only at high dosage, 15 μg/ml to 20 μg/ml.

Cell cycle arrest that occurs in the S phase can be due to the accumulation of DNA strand breaks in the cells (Wyllie et al., 1996). This is to prevent the damaged DNA to proceed further into the next phase of the cell cycle that will finally produce cells that have defective DNA. DNA damage response can be triggered at this stage to initiate DNA repair.

The progression of S phase in cell cycle is supported by the cell cycle related proteins, cyclin A, cyclin E, CDK2, and CDC25A (Collins et al., 1997) and it has been shown in some studies that the inhibition of these proteins will lead to S phase arrest (Hung et al., 2013). When cell cycle failed to progress through S phase, this will trigger the occurrence of apoptosis in A549 cells.

Flow cytometry studies have proven that helichrysetin can trigger apoptosis in A549 cells. Understanding the molecular mechanisms of action of helichrysetin that trigger cell death in A549 cells is important to develop potential therapeutic strategy. Hence, the protein expression of apoptotic related proteins was investigated to better understand the proteins that are involved in the action of helichrysetin.

Caspases are a group of cysteine proteases that are important in the process of apoptosis. Based on the observations from this study, helichrysetin served as the stimuli for the activation of caspase 3, 8 and 9. Exposure of A549 cells to helichrysetin resulted in the increase of cleaved caspase 3 proteins and decrease of pro-caspase 3. Caspase 3 is activated by the cleavage of pro-caspase 3 into cleaved caspase 3 by initiator caspase or other proteases under certain circumstances (Boatright et al., 2003). Activation of caspase 3 catalyzes the cleavage of proteins involved in cell death such as

retinoblastoma (Rb) protein, poly (ADP-ribose) polymerase (PARP), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to induce apoptosis in the cells (Janicke et al., 1998).

Caspase 8 and caspase 9 are both initiator caspases that play important roles in the initiation of extrinsic and intrinsic apoptotic pathways respectively. Results showed that both pro-caspase 8 and pro-caspase 9 are activated upon exposure to helichrysetin displayed by the increase in the cleaved-form protein expression of both caspases. The intrinsic pathway of apoptosis is activated upon the cleavage of caspase 9 following the treatment by helichrysetin. This matches the result where cytochrome c is shown to be released into the cytosol from the mitochondria which lead to the activation of caspase 9. This activation is reported to occur in a complex called the apoptosome (Zou et al., 1999). Apoptosome will then proceed to activate pro-caspase 3 by its proteolytic activity and finally cause the execution of apoptosis (Würstle et al., 2012).

Activation of caspase 8 is induced by the signaling by death-inducing signaling complex (DISC) where death receptors are involved (Medema et al., 1997). Caspase 8 subsequently induces the activation of downstream effector proteins caspase 3 and caspase 7 for the occurrence of apoptosis in the cells (Hirata et al., 1998). It's been reported that caspase 8 can also induce mitochondrial activated apoptosis by cleaving Bcl2-interacting protein (BID) which cause the translocation of BID to the mitochondria hence inducing the release of cytochrome c into the cytosol (Luo et al., 1998).

As a result of caspase 3 activation, PARP will be deactivated by the cleavage activity catalyzed by caspase 3 (Tewari et al., 1995). It can be observed in the results that there is an increase expression of cleaved PARP upon treatment with helichrysetin while full length PARP showed down regulation. This proved that helichrysetin triggered the downstream apoptotic pathways deactivating PARP for the execution of apoptosis in

A549 cells. Caspase 3 cleaved PARP to produce N-terminal DNA-binding domain and C-terminal catalytic fragment followed by the DNA repair blockage of N-terminal DNA-binding domain to promote the occurrence of apoptosis (D'Amours et al., 2001).

Study has demonstrated that helichrysetin can cause changes to Bcl-2 family proteins in A549 cells. After treatment with helichrysetin at different exposure time, the expression of Bax protein showed increment at 6, 24, and 48 hours treatment while Bcl-2 protein was reduced through these time points. This indicates helichrysetin can elevate the pro-apoptotic proteins in A549 cells, sensitizing the cells to apoptosis given the fact that Bcl-2 family proteins are primary regulator of apoptosis (Siddiqui et al., 2015). Bcl-2 protein is found to inhibit cell death by preserving the integrity of mitochondrial membrane as the hydrophobic carboxyl-terminal domain of this protein is linked to the outer membrane of mitochondria. Bcl-2 prevents the oligomerization of Bax/Bak proteins that will cause the release of apoptogenic substances from mitochondria (Tzifi et al., 2012). Hence, the inhibition of Bcl-2 can initiate the activation of Bax and finally apoptosis process.

When apoptosis is initiated, Bax will move from the cytosol to the membrane of mitochondrial to form mitochondrial outer membrane permeabilization (MOMP) complex, hence, triggering the critical apoptotic caspase cascade (Green et al., 2004). When Bax intergrates with mitochondria, it will oligomerizes with Bak to cause the release of cytochrome c (Petros et al., 2004). During the activation of Bax, some studies have proven that Bax will undergo conformational rearrangements to be associated with the mitochondrial membrane (Westphal et al., 2011).

Hence, the exposure of A549 cells to helichrysetin induces apoptosis in the cells detected by flow cytometry that showed biochemical hallmark of apoptosis such as loss of membrane asymmetry, DNA fragmentation, and disruption of mitochondrial

membrane potential. Helichrysetin induces the up-regulation of upstream and downstream pro-apoptotic proteins related to the mitochondrial-mediated apoptosis while the anti-apoptotic proteins were diminished upon treatment with helichrysetin.

5.3 Proteomic study to investigate the mechanism of action of helichrysetin on A549 cells

The mechanism underlying helichrysetin growth inhibitory activity against A549 cells has not been studied and this study provided the proteomics profiling results to find the differentially expressed proteins in A549 cells. To compare the proteome changes from untreated A549 cells versus 6 hours, 24 hours and 48 hours treated cells, proteins with 95% confidence level with z score >2.0 or <-2.0 were chosen as differentially regulated proteins.

Ingenuity Pathway Analysis (IPA) software was used to investigate the significantly altered pathways and activated upstream regulator. The top 10 significantly altered pathways are shown in the Figure 4.20. Of these canonical pathways, proteins that are highly regulated in the pathways, cell cycle: G2/M DNA damage checkpoint regulation, NRF2-mediated oxidative stress response, ATM signaling, and hereditary breast cancer signaling are the focus in this study for further elaboration on the mechanisms of action of helichrysetin in A549 cells. These pathways might contribute to the mechanisms of cell death in helichrysetin-treated A549 cells.

The proteomic study showed that this compound impaired the growth of A549 cancer cells by altering its DNA damage response, cell cycle regulation and inducing oxidative stress in the cells. p21 protein which is highly expressed in the cells upon treatment with helichrysetin plays an important role in inhibiting the activity of cyclin-CDKs complexes. p21 protein plays an important role in the growth arrest upon DNA damage and its overexpression can promote G_1 and G_2 or S-phase cell cycle arrest through the binding of p21 protein to CDKs in response to stimuli (Abbas et al., 2009).

By inhibiting CDKs and cyclins, p21 reduces the expression of both types of proteins. Besides, p21 also contains a carboxyl terminal binding site that matches PCNA that plays essential role in DNA replication and DNA repair including nucleotide excision repair, base excision repair and mismatch repair. The interaction of p21 with PCNA will block DNA synthesis facilitated by DNA polymerase δ (Waga et al., 1994). p21 is largely regulated by p53 the transcriptional level since one of its two p53 binding sites is responsive to p53 upon DNA damage (El-Deiry et al., 1995). With the help of p53, tumor suppressor protein, it will lead to the induction of both apoptosis and cell cycle arrest (Giono et al., 2006).

In conjunction with results from the activated upstream regulator from IPA analysis, p53 is one of the regulators activated with Z-score 2.404 and its expression is further proven with western blotting (Figure 4.22). p53 protein can be activated and stabilized in processes such as DNA damage, hypoxia, DNA repair, apoptosis and others in the presence of DNA-damaging agents, chemotherapeutic drugs, and irradiation (Giaccia et al., 1998). This happens when there is a phosphorylation of its transactivation domain, and acetylation and phosphorylation of its basic allosteric control region. The two important kinases involved in the activation of p53 are ATM and ATR (ATM related) kinases.

Result from western blot showed that p53 is highly expressed in the early exposure of helichrysetin late at 48 hours which is similar to the expression of p21 protein. It has been reported that upon DNA damage, p53 the upstream regulator induces p21 expression which then localized to nucleus for cell cycle arrest (El-Deiry et al., 1994). Besides, p53 also regulates the expression of apoptotic proteins such as Bcl-2 and TNF receptor families. The first apoptotic protein that is associated with p53 activation is Bax pro-apoptotic protein that contains BH3-domain and p53 also represses Bcl-2 which contributes to the blocking of survival signals (Miyashita et al., 1994).

ATM signaling is one of the top canonical pathways affected by the treatment of helichrysetin. This signaling pathway is involved in the cellular DNA damage response pathway and ATM, ataxia-telangiectasia mutated protein is a protein kinase that mediates responses to DNA double strand breaks in the cells (Kitagawa et al., 2005). BRCA1-associated ATM activator (BAAT) is responsible for the activation of ATM kinase that stimulate the DNA damage repair and regulate cell cycle checkpoints in cells (Minami et al., 2014).

Observation from the quantitative proteomic study showed that BAAT was down-regulated upon treatment with helichrysetin in comparison to its untreated control. The reduction in the expression of BAAT is also coupled with the western blotting results that show the reduction in the protein expression of BRCA1 protein and phospho-ATM at the given time points. ATM signals with MRN complex from DNA double strand breaks (DSBs) and activates the DNA damage checkpoint in the cells. ATM autophosphorylation has become an important readout for the activation of ATM. Therefore, the low expression of p-ATM in A549 cells treated with helichrysetin indicate that helichrysetin lowers the activation of ATM that is important for the stimulation of DNA damage repair subsequently causing the failure of DNA damage repair in A549 cells.

Fanconi's anemia (FA) is a genetic disorder characterized by its chromosomal instability, susceptibility to cancer, and hypersensitivity to DNA cross-linking agents. FA proteins form complexes that are important for the activation of FANCD2 that acts together with BRCA1 and BRCA2 proteins in DNA damage response (Garcia-Higuera et al., 2001). When DNA damage occurs, it activates the FA complex-dependent monoubiquitination of the FANCD2. FANCD2 protein plays a pivotal role in coordinating S phase in cell cycle and DNA double strand breaks repair (Ho et al., 2006).

The monoubiquitination of FANCD2 results in the relocation of FANCD2 from a nuclear compartment to S-phase-specific foci that contains Rad51, BRCA1 and other proteins related to DNA repair to facilitate repair of DNA in S phase of cell cycle (Taniguchi et al., 2002). FANCD2 and Rad51 complexes are involved in DNA repair by homologous recombination. However, FANCD2 protein is down-regulated upon treatment of helichrysetin in A549 cells. Hence, FANCD2 monoubiquitination will be disrupted and the DNA repair is diminished upon treatment with helichrysetin resulting in the presence of irreparable DNA in the cells. Damaged DNA that is failed to be repaired will cause cell cycle arrest and finally triggering the process of apoptosis in A549 cells.

In relation to the hereditary breast cancer signaling, retinoblastoma 1 (Rb1) is also one of the proteins affected by the treatment of helichrysetin. In this quantitative proteomic study, it is shown that expression of Rb1 protein is down-regulated upon treatment with helichrysetin in a time-dependent manner. Rb1 protein plays an important role in the DNA double-strand break (DSB) repair which will cause genetic instability (Manning et al., 2012). Non-homologous end joining is a mechanism where DNA double strand breaks are repaired during G_1 and S phase of cell cycle (Huang et al., 2016). Since Rb1 protein is important in the DNA repair mechanism, the loss of Rb1 protein upon treatment with helichrysetin impairs the DNA repair in the cells. Inability of the cells to repair DSBs causes severe DNA damage and eventually cell death (Cook et al., 2015).

When cells are exposed to DNA-damaging agents, RAD51 will increase in the nucleus. RAD51 is an important component of homologous recombination and it is recruited to the DNA break sites and bind to the single-stranded DNA break and then DNA will be repaired by DNA strand exchange with homologous chromosome for recovery of missing genes (Gildemeister et al., 2009).

Upon treatment with helichrysetin, the expression of RAD51 protein increases at 6 hours and 24 hours treatment and decreases after 48 hours exposure to helichrysetin. In relation to ATM kinase and BRCA1 protein, ATM will phosphorylate BRCA1 and c-ABL tyrosine kinase and subsequently phosphorylates RAD51 to form RAD51 repair protein complex following DNA damage (Chen et al., 1999). BRCA1 regulates RAD51 function upon DNA damage which is required for RAD51-mediated homologous recombination (Cousineau et al., 2005). Therefore, RAD51 might have been trigger in the early stage of the treatment for DNA repair but due to the lack of BRCA1 and ATM to facilitate the DNA repair, the mechanism might have failed too.

As observed in the results from the top canonical pathway from IPA, NRF2-mediated oxidative stress response was activated in A549 cells treated with helichrysetin. Heme-oxygenase 1 (HMOX1) is one of the antioxidant protective enzymes that are being up-regulated by helichrysetin in A549 cells which is due to the production of reactive oxygen species (ROS) in the mitochondria and endoplasmic reticulum of cells. The increase of HMOX1 could be a result of oxidative stress through the activation of Keap1 ubiquitination-degradation. During this process, there is a movement of Nrf2 transcription factor into the nucleus that stimulates HMOX1 (Gozzelino et al., 2010). The transactivation of HMOX1 is regulated by the binding of NRF2 transcription factor to antioxidant response element (ARE) (Reichard et al., 2007). The result from the IPA analysis on the upstream regulator shows that helichrysetin trigger the activation of NRF2 transcription factor and this is further validated using western blotting in Figure 4.22. Results showed that NRF2 and HMOX1 proteins were up regulated and this is highly related to the presence of ROS in A549 cells which can result in the induction of apoptosis (Huang et al., 2003).

Thus, the results from the proteomic study and western blotting showed that helichrysetin might be able to induce the production of reactive oxygen species in A549

cells. The production of reactive oxygen species is indicated by the stimulation of the antioxidant responses, NRF2 the master regulator and HMOX1 the antioxidant enzyme. The presence reactive oxygen species in the cells will cause the occurrence of DNA damage in A549 cells.

However, due to inhibitory activity of helichrysetin, DNA damage repair mechanism was repressed which can be observed by the down-regulation of ATM kinase, BRCA1, BAAT, Rb1 protein, and FANCD2. Tumor suppressor protein, p53 and p21, the inhibitor of cyclin-dependent kinase were elevated upon treatment with helichrysetin in A549 cells which imply the deregulation of cell cycle and the occurrence of S phase cell cycle arrest. Excessive damage to the DNA caused the occurrence of cell cycle arrest and eventually stimulated the mechanism of apoptosis in A549 cells.

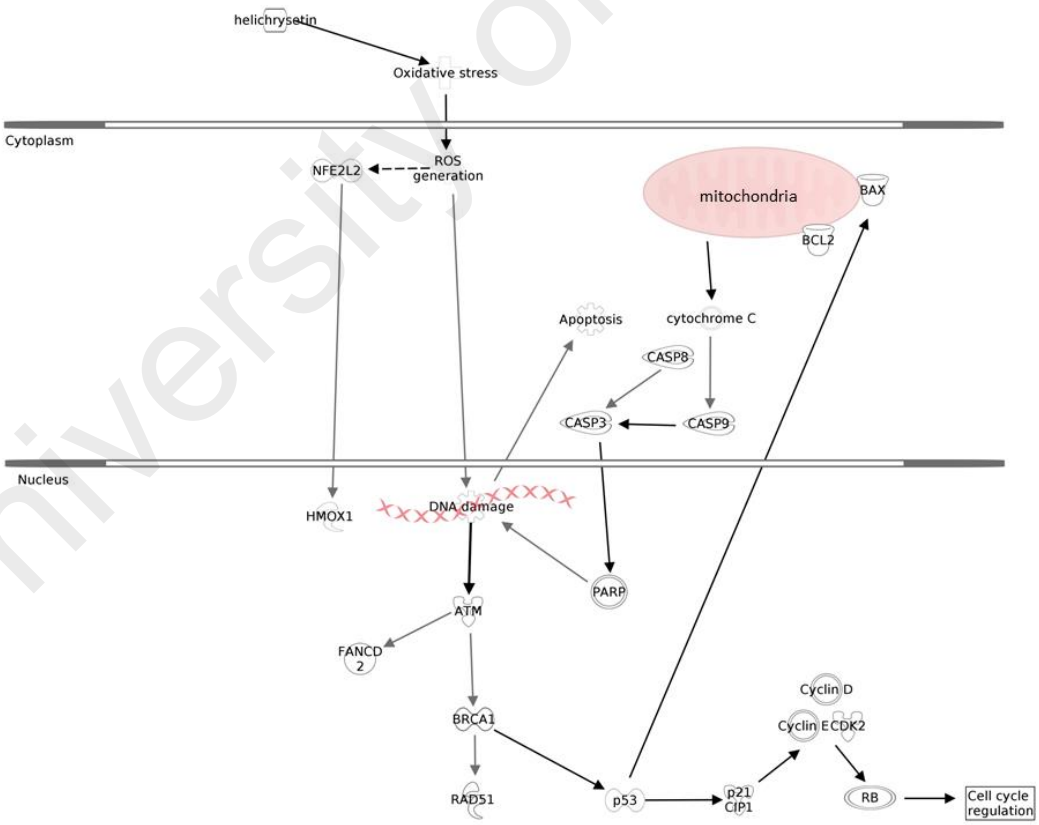


Figure 5.2: Illustration of the molecular mechanisms of action by helichrysetin on A549 cells. Helichrysetin caused oxidative stress in the cells that induce DNA damage, and this compound inhibits DNA damage response that leads to cell cycle arrest and subsequently stimulation of apoptosis in A549 cells. Pathway illustration is drawn using IPA software.

CHAPTER 6: CONCLUSION

In this thesis, helichrysetin a naturally occurring chalcone has been investigated for its growth inhibitory activity on four selected cancer cell lines and it is proven to be effective on human cervical carcinoma cell line, Ca Ski and human lung adenocarcinoma cell line, A549 with IC_{50} of 31.02±0.45 μM and 50.72±1.26 μM respectively. Since this is the first discovery on the activity of helichrysetin on human lung cancer, further investigations have been done on A549 cancer cells.

Helichrysetin is able to induce apoptotic morphological changes in A549 cells. This is indicated by the decrease in cell volume, cell rounding and the loss of cells support to the culture substratum. Helichrysetin also induces the changes in nuclear morphology shown by the occurrence of chromatin condensation and nuclear fragmentation in the cells. Flow cytometry studies have revealed that this compound can induce apoptosis in A549 cells defined by the changes in biochemical characteristics in the cells such as the externalization of phosphatidylserine, internucleosomal DNA fragmentation, collapse of the mitochondrial membrane potential and cell cycle arrest at the S phase. Helichrysetin also triggered the mitochondrial-mediated apoptotic pathway through the activation of caspase 3,8,9 and the cleavage of PARP. Cytochrome c which is a marker of apoptosis also found to be up-regulated in the helichrysetin-treated A549 cells. Changes in the expression of Bcl-2 family proteins, the major regulator of apoptosis can also be observed. Pro-apoptotic protein, Bax has been activated while the anti-apoptotic protein has been deactivated upon treatment with helichrysetin.

Proteomic studies further revealed the mechanisms involved in the induction of apoptosis in A549 cells in effect of helichrysetin treatment. It is shown that helichrysetin induces the antioxidant protective proteins, Nrf2 and HMOX1 that are commonly activated upon the production of ROS in the cells. This observation

suggested the production of ROS in the cells that might have contributed to the DNA damage in the cells which eventually leads to cell cycle arrest.

Cell cycle arrest functions as a process to allow for the repairing of damaged DNA in the cells. When DNA damage occurs in the cells, DNA damage response is responsible to trigger the signaling cascades to repair damaged DNA. This study showed that DNA damage response has been suppressed in A549 cells upon exposure to helichrysetin and this prevents the cells from undergoing DNA damage repair. The failure in the DNA damage repair will lead the cells into undergoing cell death and in this case, apoptotic cell death. Cells that possess the damaged DNA were arrested in the S phase of cell cycle to facilitate DNA repair. When excessive DNA damage was present in A549 cells, apoptosis was triggered in the cells to facilitate cell suicide.

Therefore, the finding of this study on the activity and the mechanism of action of helichrysetin on human lung adenocarcinoma cell, A549, provides an insight on the effective anti-cancer activity of helichrysetin on human lung cancer cells through its ability to cause changes to the process of DNA damage response in human lung cancer. The finding of the mechanism of action from this study will provide an opportunity for the development of helichrysetin as cancer therapeutic by targeting the DNA damage response elements specifically in lung cancer cells. Hence, the future direction of this research should include the genetic modification of the targeted genes related to the DNA damage response for further confirmation on the mechanism of action of helichrysetin in A549 cells. Future studies should also include the investigation of other types of human cancer cell line to broaden the understanding on the effect of helichrysetin on human malignancies.

REFERENCES

Abbas, T., & Dutta, A. (2009). p21 in cancer: Intricate networks and multiple activities. *Nature Reviews: Cancer, 9*(6), 400-414.

Ahsan, H., & Hadi, S. M. (1998). Strand scission in DNA induced by curcumin in the presence of Cu(II). *Cancer Letters, 124*(1), 23-30.

Ali, M. S., Tezuka, Y., Awale, S., Banskota, A. H., & Kadota, S. (2001). Six new diarylheptanoids from the seeds of *Alpinia blepharocalyx*. *Journal of Natural Products, 64*, 289-293.

Altaf Hussain, M., & Huygens, F. (2012). Proteomic and bioinformatics tools to understand virulence mechanisms in *Staphylococcus aureus*. *Current Proteomics, 9*(1), 2-8.

Amundson, S. A., Myers, T. G., & Fornace, A. J., Jr. (1998). Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene, 17*(25), 3287-3299.

Anderson, K. S., Cramer, D. W., Sibani, S., Wallstrom, G., Wong, J., Park, J., . . . LaBaer, J. (2015). Autoantibody signature for the serologic detection of ovarian cancer. *Journal of Proteome Research, 14*(1), 578-586.

Anto, R. J., Sukumaran, K., Kuttan, G., Rao, M. N. A., Subbaraju, V., & Kuttan, R. (1995). Anticancer and antioxidant activity of synthetic chalcones and related compounds. *Cancer Letters, 97*(1), 33-37.

Archana, M., , B., Yogesh, T., & Kumaraswamy, K. (2013). Various methods available for detection of apoptotic cells- A review. *Indian Journal of Cancer, 50*(3), 274-283.

Awasthi, S. K., Mishra, N., Kumar, B., Sharma, M., Bhattacharya, A., Mishra, L. C., & Bhasin, V. K. (2009). Potent antimalarial activity of newly synthesized substituted chalcone analogs *in vitro*. *Medicinal Chemistry Research, 18*(6), 407-420.

Bag, S., Ramar, S., & Degani, M. S. (2009). Synthesis and biological evaluation of α, β-unsaturated ketone as potential antifungal agents. *Medicinal Chemistry Research, 18*(4), 309-316.

Bartek, J., & Lukas, J. (2007). DNA damage checkpoints: From initiation to recovery or adaptation. *Current Opinion in Cell Biology, 19*(2), 238-245.

Bhanot, A., Sharma, R., & Noolvi, M. N. (2011). Natural sources as potential anti-cancer agents: A review. *International Journal of Phytomedicine, 3*(1), 9.

Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., . . . Salvesen, G. S. (2003). A unified model for apical caspase activation. *Molecular Cell, 11*(2), 529-541.

Boik, J. (2001). Natural compounds in cancer therapy. *Journal of Natural Products, 64*, 1605-1606.

Breckenridge, D. G., Germain, M., Mathai, J. P., Nguyen, M., & Shore, G. C. (2003). Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene, 22*(53), 8608-8618.

Büyükavcı, M., Özdemir, Ö., Buck, S., Stout, M., Ravindranath, Y., & Savaşan, S. (2006). Melatonin cytotoxicity in human leukemia cells: Relation with its pro-oxidant effect. *Fundamental & Clinical Pharmacology, 20*(1), 73-79.

Carlson, M., Falcon, S., Pages, H., & Li, N. (2013). org.Hs.eg.db: Genome wide annotation for human: R package version.

Castedo, M., Perfettini, J. L., Roumier, T., & Kroemer, G. (2002). Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death and Differentiation, 9*(12), 1287-1293.

Certo, M., Moore, V. D. G., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S. A., & Letai, A. (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell, 9*(5), 351-365.

Chandra, D., & Tang, D. G. (2009). Detection of apoptosis in cell-free systems. *Methods in Molecular Biology, 559*, 65-75.

Chazotte, B. (2011). Labeling nuclear DNA using DAPI. *Cold Spring Harbor Protocols, 2011*(1), pdb prot5556.

Chen, G., Yuan, S.-S. F., Liu, W., Xu, Y., Trujillo, K., Song, B., . . . Lee, E. Y. H. P. (1999). Radiation-induced assembly of Rad51 and Rad52 recombination complex requires ATM and c-Abl. *Journal of Biological Chemistry, 274*(18), 12748-12752.

Chen, J., Li, S., & Shen, Q. (2012). Folic acid and cell-penetrating peptide conjugated PLGA–PEG bifunctional nanoparticles for vincristine sulfate delivery. *European Journal of Pharmaceutical Sciences, 47*(2), 430-443.

Clement, M. V., Hirpara, J. L., Chawdhury, S. H., & Pervaiz, S. (1998). Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood, 92*(3), 996-1002.

Collins, K., Jacks, T., & Pavletich, N. P. (1997). The cell cycle and cancer. *Proceedings of the National Academy of Sciences, 94*(7), 2776-2778.

Cook, R., Zoumpoulidou, G., Luczynski, Maciej T., Rieger, S., Moquet, J., Spanswick, Victoria J., . . . Mittnacht, S. (2015). Direct involvement of retinoblastoma family proteins in DNA repair by non-homologous end-joining. *Cell Reports, 10*(12), 2006-2018.

Cousineau, I., Abaji, C., & Belmaaza, A. (2005). BRCA1 regulates RAD51 function in response to DNA damage and suppresses spontaneous sister chromatid replication slippage: implications for sister chromatid cohesion, genome stability, and carcinogenesis. *Cancer Research, 65*(24), 11384-11391.

Curtin, N. J. (2013). Inhibiting the DNA damage response as a therapeutic manoeuvre in cancer. *British Journal of Pharmacology, 169*(8), 1745-1765.

D'Amours, D., Sallmann, F. R., Dixit, V. M., & Poirier, G. G. (2001). Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. *Journal of Cell Science, 114*(20), 3771-3778.

Danial, N. N., & Korsmeyer, S. J. (2004). Cell death: Critical control points. *Cell, 116*(2), 205-219.

Darzynkiewicz, Z., Galkowski, D., & Zhao, H. (2008). Analysis of apoptosis by cytometry using TUNEL assay. *Methods, 44*, 250-254.

Demidenko, Z. N., Kalurupalle, S., Hanko, C., Lim, C. u., Broude, E., & Blagosklonny, M. V. (2008). Mechanism of G1-like arrest by low concentrations of paclitaxel: next cell cycle p53-dependent arrest with sub G1 DNA content mediated by prolonged mitosis. *Oncogene, 27*(32), 4402-4410.

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

Deng, C.-X. (2006). BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Research, 34*(5), 1416-1426.

Devita, V. T., Jr., Serpick, A. A., & Carbone, P. P. (1970). Combination chemotherapy in the treatment of advanced Hodgkin's disease. *Annals of Internal Medicine, 73*(6), 881-895.

Dewson, G., & Kluck, R. M. (2010). Bcl-2 family-regulated apoptosis in health and disease. *Cell Health and Cytoskeleton, 2*, 9-22.

Dini, L., Coppola, S., Ruzittu, M. T., & Ghibelli, L. (1996). Multiple pathways for apoptotic nuclear fragmentation. *Experimental Cell Research, 223*(2), 340-347.

Doug, H., Chen, S. X., Xu, H. X., Kadota, S., & Namba, T. (1998). A new antiplatelet diarylheptanoid from *Alpinia blepharocalyx*. *Journal of Natural Products, 61*, 142-144.

Eidet, J. R., Pasovic, L., Maria, R., Jackson, C. J., & Utheim, T. P. (2014). Objective assessment of changes in nuclear morphology and cell distribution following induction of apoptosis. *Diagnostic Pathology, 9*, 92-92.

El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., . . . Vogelstein, B. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Research, 54*(5), 1169-1174.

El-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescu, V. E., Burrell, M., . . . Vogelstein, B. (1995). Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Research, 55*(13), 2910.

Elschenbroich, S., Ignatchenko, V., Clarke, B., Kalloger, S. E., Boutros, P. C., Gramolini, A. O., . . . Kislinger, T. (2011). In-depth proteomics of ovarian cancer ascites: combining shotgun proteomics and selected reaction monitoring mass spectrometry. *Journal of Proteome Research, 10*(5), 2286-2299.

Erwig, L. P., & Henson, P. M. (2007). Clearance of apoptotic cells by phagocytes. *Cell Death and Differentiation, 15*(2), 243-250.

Fadeel, B., & Orrenius, S. (2005). Apoptosis: A basic biological phenomenon with wide-ranging implications in human disease. *Journal of Internal Medicine, 258*(6), 479-517.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., & Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *The Journal of Immunology, 148*, 2207-2216.

Fan, T. J., Han, L. H., Cong, R. S., & Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica et Biophysica Sinica, 37*(11), 719-727.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., . . . Bray, F. (2015). Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer, 136*(5), E359-E386.

Fotakis, G., & Timbrell, J. A. (2006). In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters, 160*(2), 171-177.

Fulda, S. (2010). Evasion of apoptosis as a cellular stress response in cancer. *International Journal of Cell Biology, 2010,* 370835.

Furnari, B., Rhind, N., & Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. *Science, 277*(5331), 1495-1497.

Ganten, T. M., Haas, T. L., Sykora, J., Stahl, H., Sprick, M. R., Fas, S. C., . . . Walczak, H. (2004). Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. *Cell Death and Differentiation, 11* Suppl 1, S86-96.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M. S., Timmers, C., Hejna, J., . . . D'Andrea, A. D. (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Molecular Cell, 7*(2), 249-262.

Gaur, S., Wang, Y., Kretzner, L., Chen, L., Yen, T., Wu, X., . . . Yen, Y. (2014). Pharmacodynamic and pharmacogenomic study of the nanoparticle conjugate of camptothecin CRLX101 for the treatment of cancer. *Nanomedicine: Nanotechnology, Biology and Medicine, 10*(7), 1477-1486.

Genestra, M. (2007). Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular Signalling, 19*(9), 1807-1819.

Giaccia, A. J., & Kastan, M. B. (1998). The complexity of p53 modulation: Emerging patterns from divergent signals. *Genes & Development, 12*(19), 2973-2983.

Gilbert, D. F., Erdmann, G., Zhang, X., Fritzsche, A., Demir, K., Jaedicke, A., . . . Boutros, M. (2011). A novel multiplex cell viability assay for high-throughput RNAi screening. *PLOS ONE, 6*(12), e28338.

Gildemeister, O. S., Sage, J. M., & Knight, K. L. (2009). Cellular redistribution of Rad51 in response to DNA damage: Novel role for Rad51C. *Journal of Biological Chemistry, 284*(46), 31945-31952.

Giono, L. E., & Manfredi, J. J. (2006). The p53 tumor suppressor participates in multiple cell cycle checkpoints. *Journal of Cellular Physiology, 209*(1), 13-20.

Gomez-Angelats, M., & Cidlowski, J. A. (2002). Cell volume control and signal transduction in apoptosis. *Toxicologic Pathology, 30*(5), 541-551.

Gozzelino, R., Jeney, V., & Soares, M. P. (2010). Mechanisms of cell protection by heme oxygenase-1. *Annual Review of Pharmacology and Toxicology, 50*, 323-354.

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629.

Hamdi, N., Fischmeister, C., Puerta, M. C., & Valerga, P. (2011). A rapid access to new coumarinyl chalcone and substituted chromeno[4,3-c]pyrazol-4(1H)-ones and their antibacterial and DPPH radical scavenging activities. *Medicinal Chemistry Research, 20*(4), 522-530.

Hanahan, D., & Weinberg, R. A. (2011). Review hallmarks of cancer : The next generation. *Cell, 144*, 646-674.

Harasym, T. O., Tardi, P. G., Harasym, N. L., Harvie, P., Johnstone, S. A., & Mayer, L. D. (2007). Increased preclinical efficacy of irinotecan and floxuridine coencapsulated inside liposomes is associated with tumor delivery of synergistic drug ratios. *Oncology Research, 16*(8), 361-374.

Hartwell, J. L. (1971). Plants used against cancer. A survey. *Lloydia, 34*(4), 386-425.

Hartwell, L. H., & Weinert, T. A. (1989). Checkpoints: Controls that ensure the order of cell cycle events. *Science, 246*(4930), 629-634.

Hengartner, M. O. (2001). Apoptosis: Corralling the corpses. *Cell, 104*(3), 325-328.

Higuchi, Y. (2004). Glutathione depletion-induced chromosomal DNA fragmentation associated with apoptosis and necrosis. *Journal of Cellular and Molecular Medicine, 8*(4), 455-464.

Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., . . . Sasada, M. (1998). Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *The Journal of Experimental Medicine, 187*(4), 587-600.

Ho, G. P., Margossian, S., Taniguchi, T., & D'Andrea, A. D. (2006). Phosphorylation of FANCD2 on two novel sites is required for mitomycin C resistance. *Molecular and Cellular Biology, 26*(18), 7005-7015.

Hua, S.-Z., Wang, X.-B., Luo, J.-G., Wang, J.-S., & Kong, L.-Y. (2008). A pair of unique sesquiterpene–chalcone conjugates isolated from the seeds of *Alpinia katsumadai*. *Tetrahedron Letters, 49*, 5658-5661.

Huang, H.-L., Fang, L.-W., Lu, S.-P., Chou, C.-K., Luh, T.-Y., & Lai, M.-Z. (2003). DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation. *Oncogene, 22*(50), 8168-8177.

Huang, P. H., Cook, R., Zoumpoulidou, G., Luczynski, M. T., & Mittnacht, S. (2016). Retinoblastoma family proteins: New players in DNA repair by non-homologous end-joining. *Molecular & Cellular Oncology, 3*(2), e1053596.

Hung, F. M., Chen, Y. L., Huang, A. C., Hsiao, Y. P., Yang, J. S., Chung, M. T., . . . Chung, J. G. (2013). Triptolide induces S phase arrest via the inhibition of cyclin E and CDC25A and triggers apoptosis via caspase- and mitochondrial-dependent signaling pathways in A375.S2 human melanoma cells. *Oncology Reports, 29*(3), 1053-1060.

Issaenko, O. A., & Amerik, A. Y. (2012). Chalcone-based small-molecule inhibitors attenuate malignant phenotype via targeting deubiquitinating enzymes. *Cell Cycle, 11*(9), 1804-1817.

Jakob, B., Splinter, J., Conrad, S., Voss, K.-O., Zink, D., Durante, M., . . . Taucher-Scholz, G. (2011). DNA double-strand breaks in heterochromatin elicit fast

repair protein recruitment, histone H2AX phosphorylation and relocation to euchromatin. *Nucleic Acids Research*, 39(15), 6489-6499.

Janicke, R. U., Ng, P., Sprengart, M. L., & Porter, A. G. (1998). Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *Journal of Biological Chemistry, 273*(25), 15540-15545.

Jia, X., Gu, Z., Chen, W., & Jiao, J. (2016). Tigecycline targets nonsmall cell lung cancer through inhibition of mitochondrial function. *Fundamental & Clinical Pharmacology, 30*(4), 297-306.

Kaina, B. (2003). DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochemical Pharmacology, 66*(8), 1547-1554.

Kang, B. H., Siegelin, M. D., Plescia, J., Raskett, C. M., Garlick, D. S., Dohi, T., . . . Altieri, D. C. (2010). Preclinical characterization of mitochondria-targeted small molecule Hsp90 inhibitors, gamitrinibs, in advanced prostate cancer. *Clinical Cancer Research, 16*(19), 4779-4788.

Karp, G., Iwasa, J., & Marshall, W. (2008). *Karp's cell and molecular biology: concepts and experiments* (Vol. 5). John New Jersey: Wiley and Sons.

Kepp, O., Galluzzi, L., Lipinski, M., Yuan, J., & Kroemer, G. (2011). Cell death assays for drug discovery. *Nature Reviews: Drug Discovery, 10*(3), 221-237.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wideranging implications in tissue kinetics. *British Journal of Cancer, 26*(4), 239-257.

Kitagawa, R., & Kastan, M. B. (2005). The ATM-dependent DNA damage signaling pathway. *Cold Spring Harbor Symposia on Quantitative Biology, 70*, 99-109.

Kivinen, K., Kallajoki, M., & Taimen, P. (2005). Caspase-3 is required in the apoptotic disintegration of the nuclear matrix. *Experimental Cell Research, 311*(1), 62-73.

Koparal, A. T., & Zeytinoğlu, M. (2003). Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549. In K. Yagasaki, Y. Miura, M. Hatori & Y. Nomura (Eds.), *Animal cell technology: Basic & applied aspects: Proceedings of the fifteenth annual meeting of the japanese association for animal cell technology (JAACT), Fuchu, Japan, November 11–15, 2002* (pp. 207-211). Dordrecht: Springer Netherlands.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., . . . Williams, L. T. (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science, 278*(5336), 294-298.

Kroemer, G., El-Deiry, W. S., Golstein, P., Peter, M. E., Vaux, D., Vandenabeele, P., . . . Melino, G. (2005). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death and Differentiation, 12*(2), 1463-1467.

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiological Reviews, 87*(1), 99-163.

Krysko, D. V., D'Herde, K., & Vandenabeele, P. (2006). Clearance of apoptotic and necrotic cells and its immunological consequences. *Apoptosis, 11*(10), 1709-1726.

Kryston, T. B., Georgiev, A. B., Pissis, P., & Georgakilas, A. G. (2011). Role of oxidative stress and DNA damage in human carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 711*(1–2), 193-201.

Kumar, V., Abbas, A., Fausto, N., & Aster, J. (2010). *Robins and Cotran: Pathologic basis of disease* (Vol. 8, pp. 25-32). Philadelphia: Saunders Elsevier.

LaCasse, E. C., Mahoney, D. J., Cheung, H. H., Plenchette, S., Baird, S., & Korneluk, R. G. (2008). IAP-targeted therapies for cancer. *Oncogene, 27*(48), 6252-6275.

Lawen, A. (2003). Apoptosis—an introduction. *Bioessays, 25*(9), 888-896.

Lee, D. S., Lee, M. K., & Kim, J. H. (2009). Curcumin induces cell cycle arrest and apoptosis in human osteosarcoma (HOS) cells. *Anticancer Research, 29*(12), 5039-5044.

Leist, M., Single, B., Castoldi, A. F., Kühnle, S., & Nicotera, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *The Journal of Experimental Medicine, 185*(8), 1481-1486.

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

Levine, B., & Klionsky, D. J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental Cell, 6*(4), 463-477.

Li, Y., Sun, X., LaMont, J. T., Pardee, A. B., & Li, C. J. (2003). Selective killing of cancer cells by β-lapachone: Direct checkpoint activation as a strategy against cancer. *Proceedings of the National Academy of Sciences, 100*(5), 2674-2678.

Lindahl, T., & Barnes, D. E. (2000). Repair of endogenous DNA damage. *Cold Spring Harb Symposia on Quantitative Biology, 65*, 127-133.

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W. T., & Wang, X. (1998). The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. *Proceedings of the National Academy of Sciences, 95*(15), 8461-8466.

Lopez, J., & Tait, S. W. G. (2015). Mitochondrial apoptosis: Killing cancer using the enemy within. *British Journal of Cancer, 112*(6), 957-962.

Louagie, H., Cornelissen, M., Philippe, J., Vral, A., Thierens, H., & De Ridder, L. (1998). Flow cytometric scoring of apoptosis compared to electron microscopy in gamma irradiated lymphocytes. *Cell Biology International, 22*(4), 277-283.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., & Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell, 94*(4), 481-490.

Maddika, S., Ande, S. R., Panigrahi, S., Paranjothy, T., Weglarczyk, K., Zuse, A., . . . Los, M. (2007). Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. *Drug Resistance Updates, 10*(1–2), 13-29.

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *American Journal of Pathology, 146*(1), 3-15.

Malhotra, D., Portales-Casamar, E., Singh, A., Srivastava, S., Arenillas, D., Happel, C., . . . Biswal, S. (2010). Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Research, 38*(17), 5718-5734.

Malumbres, M., & Barbacid, M. (2007). Cell cycle kinases in cancer. *Current Opinion in Genetics & Development, 17*(1), 60-65.

Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: A changing paradigm. *Nature Reviews: Cancer, 9*(3), 153-166.

Mann, J. (2002). Natural products in cancer chemotherapy: Past, present and future. *Nature Reviews: Cancer, 2*(2), 143-148.

Mann, M., & Jensen, O. N. (2003). Proteomic analysis of post-translational modifications. *Nature Biotechnology, 21*(3), 255-261.

Manning, A. L., & Dyson, N. J. (2012). RB: mitotic implications of a tumor suppressor. *Nature Reviews. Cancer, 12*(3), 220-226.

Marechal, A., & Zou, L. (2013). DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harbor Perspective in Biology, 5*(9).

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., & Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *Journal of Experimental Medicine, 182*(5), 1545-1556.

Marushige, Y., & Marushige, K. (1998). Alterations in focal adhesion and cytoskeletal proteins during apoptosis. *Anticancer Research, 18*(1A), 301-307.

Mashima, T., Naito, M., & Tsuruo, T. (1999). Caspase-mediated cleavage of cytoskeletal actin plays a positive role in the process of morphological apoptosis. *Oncogene, 18*(15), 2423-2430.

Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., & Peter, M. E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *The EMBO Journal, 16*(10), 2794-2804.

Merdes, A., Ramyar, K., Vechio, J. D., & Cleveland, D. W. (1996). A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell, 87*(3), 447-458.

Minami, A., Nakanishi, A., Kitagishi, Y., Ogura, Y., & Matsuda, S. (2014). Roles of Estrogens in Prostate Cancer Development via the Modulation of DNA-Repair System. *Cancer Science & Research, 1*(2), 1-8.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., . . . Reed, J. C. (1994). Tumor suppressor p53 is a regulator of Bcl-2 and Bax gene expression *in vitro* and *in vivo*. *Oncogene, 9*(6), 1799-1805.

Møller, M. B., Gerdes, A.-M., Skjødt, K., Mortensen, L. S., & Pedersen, N. T. (1999). Disrupted p53 function as predictor of treatment failure and poor prognosis in B- and T-Cell Non-Hodgkin's lymphoma. *American Association for Cancer Research, 5*(5), 1085-1091.

Muller, P. A. J., & Vousden, K. H. (2013). p53 mutations in cancer. *Nature Cell Biology, 15*(1), 2-8.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., & Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature, 403*(6765), 98-103.

Nakamura, Y., Kawakami, M., Yoshihiro, A., Miyoshi, N., Ohigashi, H., Kawai, K., . . . Uchida, K. (2002). Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis. *Journal of Biological Chemistry, 277*(10), 8492-8499.

Nakhjavani, M., Zarghi, A., & H. Shirazi, F. (2014). Cytotoxicity of selected novel chalcone derivatives on human breast, lung and hepatic carcinoma cell lines. *Iranian Journal of Pharmaceutical Research, 13*(3), 953-958.

Nickerson, J. (2001). Experimental observations of a nuclear matrix. *Journal of Cell Science, 114*(3), 463-474.

O'Brien, M. A., & Kirby, R. (2008). Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease. *Journal of Veterinary Emergency and Critical Care, 18*(6), 572-585.

O'Connor, Mark J. (2015). Targeting the DNA damage response in cancer. *Molecular Cell, 60*(4), 547-560.

Oren, M., & Rotter, V. (1999). Introduction: p53--the first twenty years. *Cellular and Molecular Life Sciences, 55*(1), 9-11.

Orlikova, B., Schnekenburger, M., Zloh, M., Golais, F., Diederich, M., & Tasdemir, D. (2012). Natural chalcones as dual inhibitors of HDACs and NF-kappaB. *Oncology Reports, 28*(3), 797-805.

Ortega, S., Malumbres, M., & Barbacid, M. (2002). Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochimica et Biophysica Acta (BBA)- Reviews on Cancer, 1602*(1), 73-87.

Paulovich, A. G., Toczyski, D. P., & Hartwell, L. H. (1997). When checkpoints fail. *Cell, 88*(3), 315-321.

Perelman, A., Wachtel, C., Cohen, M., Haupt, S., Shapiro, H., & Tzur, A. (2012). JC-1: alternative excitation wavelengths facilitate mitochondrial membrane potential cytometry. *Cell Death & Disease, 3,* e430.

Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B., & Gelbard, H. A. (2011). Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *BioTechniques, 50*(2), 98-115.

Petros, A. M., Olejniczak, E. T., & Fesik, S. W. (2004). Structural biology of the Bcl-2 family of proteins. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1644*(2), 83-94.

Pinz, S., Unser, S., Brueggemann, S., Besl, E., Al-Rifai, N., Petkes, H., . . . Rascle, A. (2014). The synthetic alpha-bromo-2',3,4,4'-tetramethoxychalcone (alpha-Br-TMC) inhibits the JAK/STAT signaling pathway. *PLOS One, 9*(3), e90275.

Qi, F., Li, A., Inagaki, Y., Gao, J., Li, J., Kokudo, N., . . . Tang, W. (2010). Chinese herbal medicines as adjuvant treatment during chemo- or radio-therapy for cancer. *Bioscience Trends, 4*(6), 297-307.

Quent, V. M., Loessner, D., Friis, T., Reichert, J. C., & Hutmacher, D. W. (2010). Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research. *Journal of Cellular and Molecular Medicine, 14*(4), 1003-1013.

Rahman, M. A. (2011). Chalcone : A valuable insight into the recent advances and potential. *Chemical Sciences Journal, 2011,* 1-16.

Ramalho, S. D., Bernades, A., Demetrius, G., Noda-Perez, C., Vieira, P. C., Dos Santos, C. Y., . . . Mousinho, K. C. (2013). Synthetic chalcone derivatives as inhibitors of cathepsins K and B, and their cytotoxic evaluation. *Chemistry & Biodiversity, 10*(11), 1999-2006.

Reichard, J. F., Motz, G. T., & Puga, A. (2007). Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1. *Nucleic Acids Research, 35*(21), 7074-7086.

Rello, S., Stockert, J. C., Moreno, V., Gamez, A., Pacheco, M., Juarranz, A., . . . Villanueva, A. (2005). Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis, 10*(1), 201-208.

Ricci, J.-E., Gottlieb, R. A., & Green, D. R. (2003). Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *The Journal of Cell Biology, 160*(1), 65-75.

Rios-Luci, C., Bonifazi, E. L., Leon, L. G., Montero, J. C., Burton, G., Pandiella, A., . . . Padron, J. M. (2012). Beta-lapachone analogs with enhanced antiproliferative activity. *European Journal of Medicinal Chemistry, 53*, 264-274.

Roos, W. P., & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends in Molecular Medicine, 12*(9), 440-450.

Roos, W. P., & Kaina, B. (2013). DNA damage-induced cell death: From specific DNA lesions to the DNA damage response and apoptosis. *Cancer Letters, 332*(2), 237-248.

Sallam, R. M. (2015). Proteomics in cancer biomarkers discovery: challenges and applications. *Disease Markers, 2015*, 12.

Schubbert, S., Shannon, K., & Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. *Nature Reviews: Cancer, 7*(4), 295-308.

Sedelnikova, O. A., Redon, C. E., Dickey, J. S., Nakamura, A. J., Georgakilas, A. G., & Bonner, W. M. (2010). Role of oxidatively induced DNA lesions in human pathogenesis. *Mutation Research/Reviews in Mutation Research, 704*(1–3), 152-159.

Senderowicz, A. M. (2004). Targeting cell cycle and apoptosis for the treatment of human malignancies. *Current Opinion in Cell Biology, 16*(6), 670-678.

Shapiro, G. I. (2006). Cyclin-dependent kinase pathways as targets for cancer treatment. *Journal of Clinical Oncology, 24*(11), 1770-1783.

Shen, H.-M., & Liu, Z.-g. (2006). JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. *Free Radical Biology and Medicine, 40*(6), 928-939.

Shehzad, A., Lee, J., & Lee, Y. S. (2013). Curcumin in various cancers. *BioFactors, 39*(1), 56-68.

Sheppard, K. E., & McArthur, G. A. (2013). The cell-cycle regulator cdk4: an emerging therapeutic target in melanoma. *Clinical Cancer Research, 19*(19), 5320.

Siddiqui, W., Ahad, A., & Ahsan, H. (2015). The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. *Archives of Toxicology, 89*(3), 289-317.

Silva, M. T. (2010). Secondary necrosis: The natural outcome of the complete apoptotic program. *FEBS Letters, 584*(22), 4491-4499.

Singh, N. P. (2000). A simple method for accurate estimation of apoptotic cells. *Experimental Cell Research, 256*(1), 328-337.

Singh, C. K., Ndiaye, M. A., & Ahmad, N. (2015). Resveratrol and cancer: Challenges for clinical translation. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1852*(6), 1178-1185.

Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., . . . Chen, L. B. (1991). Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proceedings of the National Academy of Sciences, 88*(9), 3671-3675.

Stawinska, M., Cygankiewicz, A., Trzcinski, R., Mik, M., Dziki, A., & Krajewska, W. M. (2008). Alterations of Chk1 and Chk2 expression in colon cancer. *International Journal of Colorectal Disease, 23*(12), 1243-1249.

Sun, Y. W., Huang, W. J., Hsiao, C. J., Chen, Y. C., Lu, P. H., & Guh, J. H. (2010). Methoxychalcone induces cell-cycle arrest and apoptosis in human hormone-resistant prostate cancer cells through PI 3-kinase-independent inhibition of mTOR pathways. *Prostate, 70*(12), 1295-1306.

Swords, R., Mahalingam, D., O'Dwyer, M., Santocanale, C., Kelly, K., Carew, J., & Giles, F. (2010). Cdc7 kinase - a new target for drug development. *European Journal of Cancer, 46*(1), 33-40.

Sylvester, P. W. (2011). Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Methods in Molecular Biology, 716,* 157-168.

Taguchi, A., Politi, K., Pitteri, Sharon J., Lockwood, William W., Faça, Vitor M., Kelly-Spratt, K., . . . Hanash, Samir M. (2011). Lung cancer signatures in plasma based on proteome profiling of mouse tumor models. *Cancer Cell, 20*(3), 289-299.

Taniguchi, T., Garcia-Higuera, I., Andreassen, P. R., Gregory, R. C., Grompe, M., & D'Andrea, A. D. (2002). S-phase–specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood, 100*(7), 2414-2420.

Taraphdar, A. K., Roy, M., & Bhattacharya, R. K. (2001). Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Current Science, 80*(11), 1387-1396.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nature Reviews: Molecular Cell Biology, 9*(3), 231-241.

Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., . . . Dixit, V. M. (1995). Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell, 81*(5), 801-809.

Tewtrakul, S., Subhadhirasakul, S., Karalai, C., Ponglimanont, C., & Cheenpracha, S. (2009). Anti-inflammatory effects of compounds from *Kaempferia parviflora* and *Boesenbergia pandurata*. *Food Chemistry, 115*(2), 534-538.

Toné, S., Sugimoto, K., Tanda, K., Suda, T., Uehira, K., Kanouchi, H., . . . Earnshaw, W. C. (2007). Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. *Experimental Cell Research, 313*(16), 3635-3644.

Trachootham, D., Lu, W., Ogasawara, M. A., Valle, N. R.-D., & Huang, P. (2008). Redox regulation of cell survival. *Antioxidants & Redox Signaling, 10*(8), 1343-1374.

Tzifi, F., Economopoulou, C., Gourgiotis, D., Ardavanis, A., Papageorgiou, S., & Scorilas, A. (2012). The role of BCL2 family of apoptosis regulator proteins in acute and chronic leukemias. *Advances in Hematology, 2012*, 15.

Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M., & Yodoi, J. (2002). Redox control of cell death. *Antioxidants and Redox Signaling, 4*(3), 405-414.

van Meerloo, J., Kaspers, G. J. L., & Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. In A. I. Cree (Ed.), *Cancer cell culture: Methods and protocols* (pp. 237-245). Totowa, NJ: Humana Press.

Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., & Thompson, C. B. (1997). Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell, 91*(5), 627-637.

Van Puyvelde, L., De Kimpe, N., Costa, J., Munyjabo, V., Nyirankuliza, S., Hakizamungu, E., & Schamp, N. (1989). Isolation of flavonoids and a chalcone from *Helichrysum odoratissimum* and synthesis of helichrysetin. *Journal of Natural Products, 52*, 629-633.

Vasiliou, V., Vasiliou, K., & Nebert, D. W. (2009). Human ATP-binding cassette (ABC) transporter family. *Human Genomics, 3*(3), 281-290.

Vaux, D. L., & Silke, J. (2003). Mammalian mitochondrial IAP binding proteins. *Biochemical and Biophysical Research Communications, 304*(3), 499-504.

Venditto, V. J., & Simanek, E. E. (2010). Cancer therapies utilizing the camptothecins: a review of the in vivo literature. *Molecular Pharmaceutics, 7*(2), 307-349.

Verma, A. K., & Boutwell, R. K. (1980). Effects of dose and duration of treatment with the tumor-promoting agent, 12-O-tetradecanoylphorbol-13-acetate on mouse skin carcinogenesis. *Carcinogenesis, 1*(3), 271-276.

Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods, 184*, 39-51.

Viallard, J. F., Lacombe, F., Belloc, F., Pellegrin, J. L., & Reiffers, J. (2001). Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology. *Cancer Radiotherapy, 5*(2), 109-129.

Vikhanskaya, F., Lee, M. K., Mazzoletti, M., Broggini, M., & Sabapathy, K. (2007). Cancer-derived p53 mutants suppress p53-target gene expression--potential mechanism for gain of function of mutant p53. *Nucleic Acids Research, 35*(6), 2093-2104.

Vogel, S., Ohmayer, S., Brunner, G., & Heilmann, J. (2008). Natural and non-natural prenylated chalcones: synthesis, cytotoxicity and anti-oxidative activity. *Bioorganic & Medicinal Chemistry, 16*, 4286-4293.

Waga, S., Hannon, G. J., Beach, D., & Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature, 369*(6481), 574-578.

Walczak, H., & Krammer, P. H. (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Experimental Cell Research, 256*(1), 58-66.

Ward, I. M., Minn, K., van Deursen, J., & Chen, J. (2003). p53 binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. *Molecular and Cellular Biology, 23*(7), 2556-2563.

Weaver, B. A. (2014). How taxol/paclitaxel kills cancer cells. *Molecular Biology of the Cell, 25*(18), 2677-2681.

Westphal, D., Dewson, G., Czabotar, P. E., & Kluck, R. M. (2011). Molecular biology of Bax and Bak activation and action. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(4), 521-531.

Würstle, M. L., Laussmann, M. A., & Rehm, M. (2012). The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and activity on the apoptosome. *Experimental Cell Research, 318*(11), 1213-1220.

Wyllie, F. S., Haughton, M. F., Bond, J. A., Rowson, J. M., Jones, C. J., & Wynford-Thomas, D. (1996). S phase cell-cycle arrest following DNA damage is independent of the p53/p21(WAF1) signalling pathway. *Oncogene, 12*(5), 1077-1082.

Wölfler, A., Caluba, H.-C., Abuja, P. M., Dohr, G., Schauenstein, K., & Liebmann, P. M. (2001). Prooxidant activity of melatonin promotes Fas-induced cell death in human leukemic Jurkat cells. *FEBS Letters, 502*(3), 127-131.

Wu, X., Zhou, Q.-h., & Xu, K. (2009). Are isothiocyanates potential anti-cancer drugs? *Acta Pharmacologica Sinica, 30*(5), 501-512.

Yadav, H. L., Gupta, P., Pawar, R. S., Singour, P. K., & Patil, U. K. (2011). Synthesis and biological evaluation of anti-inflammatory activity of 1,3 diphenyl propenone derivatives. *Medicinal Chemistry Research, 20*(4), 461-465.

Yu, G., Li, F., Qin, Y., Bo, X., Wu, Y., & Wang, S. (2010). GOSemSim: an R package for measuring semantic similarity among GO terms and gene products. *Bioinformatics, 26*(7), 976-978.

Yu, G., Wang, L. G., Han, Y., & He, Q. Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS, 16*(5), 284-287.

Yuspa, S. H., & Poirier, M. C. (1988). Chemical carcinogenesis: from animal models to molecular models in one decade. *Advances in Cancer Research, 50*, 25-70.

Zanetsie Kakam, A. M., Franke, K., Ndom, J. C., Dongo, E., Mpondo, T. N., & Wessjohann, L. A. (2011). Secondary metabolites from *Helichrysum foetidum* and their chemotaxonomic significance. *Biochemical Systematics and Ecology, 39*(2), 166-167.

Zhang, D. D., & Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by

chemopreventive agents and oxidative stress. *Molecular and Cellular Biology, 23*(22), 8137-8151.

Zhang, H., & Ge, Y. (2011). Comprehensive analysis of protein modifications by top-down mass spectrometry. *Circulation: Cardiovascular Genetics, 4*(6), 711.

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research, 12*(1), 9-18.

Zhu, X.-F., Xie, B.-F., Zhou, J.-M., Feng, G.-K., Liu, Z.-C., Wei, X.-Y., . . . Zeng, Y.-X. (2005). Blockade of vascular endothelial growth factor receptor signal pathway and antitumor activity of ON-III (2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone), a component from chinese herbal medicine. *Molecular Pharmacology, 67*(5), 1444-1450.

Ziegler, U., & Groscurth, P. (2004). Morphological features of cell death. *Physiology, 19*(3), 124-128.

LIST OF PUBLICATION AND PAPERS PRESENTED

Publication

1. Ho, Y. F., Karsani, S.A., Yong, W. K., & Abd Malek, S. N. (2013). Induction of apoptosis and cell cycle blockade by helichrysetin in A549 human lung adenocarcinoma cells. *Evidence-Based Complementary and Alternative Medicine*, 2013(857257), 10 pages.

2. Ho, Y.F., Abd Malek, S.N., Hui, S.Y., Karsani, S. A. (2017). Helichrysetin triggers DNA-damage-induced JNK-mediated apoptosis in Ca Ski cells. *Pharmacognosy Magazine*, *13*(52), 607-612.

Presentations

1. Ho, Y. F., Abd Malek, S. N. (2012). Cytotoxic effect of helichrysetin on cancer cell lines and its mechanisms. Proceedings of the 17th Biological Sciences Graduate Congress. Abstract No. BC-OR-19, pp. 85.

2. Ho, Y. F., Abd Malek, S. N. (2013). Inhibitory effect of helichrysetin, a natural occurring chalcone, on selected cancer cell lines. Seminar Nasional Kimia.

3. Ho, Y.F., Karsani, S.A., Abd Malek, S.N. (2015). Induction of apoptosis by helichrysetin in human cervical epidermoid carcinoma, Ca Ski cells. Proceedings of the 2nd International Conference on Advances in Medical Science. Abstract No. RS 100090.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 857257, 10 pages
http://dx.doi.org/10.1155/2013/857257

Research Article

Induction of Apoptosis and Cell Cycle Blockade by Helichrysetin in A549 Human Lung Adenocarcinoma Cells

Yen Fong Ho, Saiful Anuar Karsani, Wai Kuan Yong, and Sri Nurestri Abd Malek

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Correspondence should be addressed to Sri Nurestri Abd Malek; srimalek@um.edu.my

Received 21 November 2012; Accepted 17 January 2013

Academic Editor: Alfredo Vannacci

Researchers are looking into the potential development of natural compounds for anticancer therapy. Previous studies have postulated the cytotoxic effect of helichrysetin towards different cancer cell lines. In this study, we investigated the cytotoxic effect of helichrysetin, a naturally occurring chalcone on four selected cancer cell lines, A549, MCF-7, Ca Ski, and HT-29, and further elucidated its biochemical and molecular mechanisms in human lung adenocarcinoma, A549. Helichrysetin showed the highest cytotoxic activity against Ca Ski followed by A549. Changes in the nuclear morphology of A549 cells such as chromatin condensation and nuclear fragmentation were observed in cells treated with helichrysetin. Further evidence of apoptosis includes the externalization of phosphatidylserine and the collapse of mitochondrial membrane potential which are both early signs of apoptosis. These signs of apoptosis are related to cell cycle blockade at the S checkpoint which suggests that the alteration of the cell cycle contributes to the induction of apoptosis in A549. These results suggest that helichrysetin has great potentials for development as an anticancer agent.

1. Introduction

Cancer is a disease caused by the uncontrolled growth of abnormal cells in the body. Lung cancer is one of the most commonly diagnosed cancers worldwide making up 12.7% of all cancer cases. It is also the most common cause of cancer death accounting for 18.2% of all cancer associated deaths [1]. Available literature suggested that natural compounds can be effective in cancer therapy [2, 3]. Helichrysetin, 2′,4,4′-trihydroxy-6′-methoxy chalcone (Figure 1(a)), is a naturally occurring chalcone that is found in the flower of *Helichrysum odoratissimum* [4] and the seeds of the *Alpinia* sp. such as *Alpinia blepharocalyx* [5], *Alpinia katsumadai* [6], and *Alpinia galanga* [7]. Chalcones substituted with OH groups exhibit maximum *in vitro* cytotoxicity against tumour cells and increase in antitumour activity [8].

Previous studies have reported that helichrysetin possessed antiproliferative and cytotoxic activity towards murine carcinoma and human fibrosarcoma [5], human cervical adenocarcinoma [9], human liver cancer and human breast cancer [6], and human colon sarcoma cell lines [10]. In addition, helichrysetin has also been shown to possess antiplatelet [11] and antioxidant activities [9]. These studies demonstrated the potential use of helichrysetin as an anticancer agent. However, the mechanism of cell death triggered by helichrysetin has not yet been elucidated.

One of the hallmarks of cancer is the resistance of cancer cells towards apoptosis which contributes to the ineffectiveness of anticancer therapies [12]. Apoptosis is characterized by several biochemical and morphological events, such as nuclear fragmentation, internucleosomal DNA fragmentation [13], cell shrinkage [14], chromatin condensation [15], formation of apoptotic bodies, loss of plasma membrane asymmetry [16], and disruption of mitochondrial membrane [17]. In an attempt to understand the mechanism(s) of action involved, we have investigated the effect(s) of helichrysetin on the viability of selected cancer cell lines. Furthermore, we elucidated, for the first time, the biochemical and molecular mechanisms of apoptosis in cancer cells caused by helichrysetin. Our results showed that helichrysetin inhibits the

Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Helichrysetin Induces DNA Damage that Triggers JNK-Mediated Apoptosis in Ca Ski Cells

Ho Yen Fong, Sri Nurestri Abd Malek, Hui Shin Yee, Saiful Anuar Karsani

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Submitted: 11-02-2017 Revised: 01-08-2017 Published: 13-11-2017

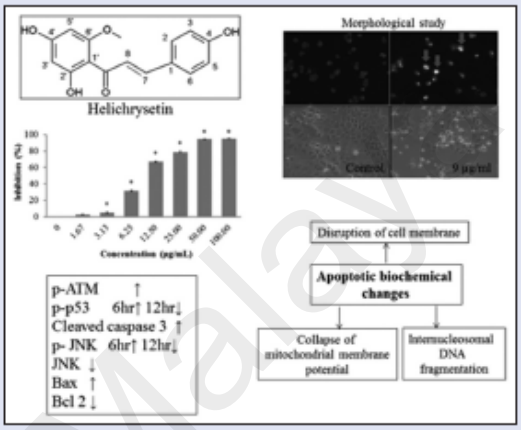
ABSTRACT

Background: Cervical cancer has become one of the most common cancers in women and currently available treatment options for cervical cancer are very limited. Naturally occurring chalcones and its derivatives have been studied extensively as a potential anticancer agent in different types of cancer and helichrysetin is naturally occurring chalcone that possess potent antiproliferative activity toward human cancer cells. **Materials and Methods:** Inhibitory activity of helichrysetin was evaluated at different concentrations. Ability of helichrysetin to induce apoptosis and its relation with c-Jun N-terminal kinase (JNK)-mediated mechanism of apoptosis was assessed using flow cytometry and Western blotting. **Results:** Helichrysetin inhibited Ca Ski cells at half maximal inhibitory concentration 30.62 ± 0.38 μM. This compound has the ability to induce DNA damage, mitochondrial membrane disruption, and loss of cell membrane integrity. We have shown that apoptosis was induced through the activation of JNK-mediated apoptosis by DNA damage in the cells then triggering p53-downstream apoptotic pathway with increased expression of pro-apoptotic proteins, Bax and caspase 3, and suppression of Bcl-2 anti-apoptotic protein. DNA damage in the cells also caused phosphorylation of protein ataxia-telangiectasia mutated, an activator of DNA damage response. **Conclusion:** We conclude that helichrysetin can inhibit Ca Ski cells through DNA damage-induced JNK-mediated apoptotic pathway highlighting the potential of this compound as anticancer agent for cervical cancer.

Key words: Apoptosis, Ca Ski, helichrysetin, c-Jun N-terminal kinase pathway

SUMMARY

- Helichrysetin induced DNA damage in Ca Ski cells
- DNA damage caused JNK-mediated phosphorylation of p53 resulting in p53-mediated apoptosis
- Helichrysetin is a potential DNA damage inducing agent through JNK activation to kill human cervical carcinoma cells.



Abbreviations used: ATM: Ataxia-telangiectasia mutated, DAPI: 4',6-diamidino-2-phenylindole, DMSO: Dimethyl sulfoxide, FITC: Fluorescein isothiocyanate, IC_{50}: Half maximal inhibitory concentration, JC1-5,5',6,6'-Tetrachloro: 1',3,3'-tetraethylbenzimidazolylcarbocyanine, iodide, JNK: c-Jun N-terminal kinase, MMP: Mitochondrial membrane potential, PBS: Phosphate-buffered saline, SRB: Sulforhodamine B, TUNEL: Terminal deoxynucleotidyl transferase dUTP nick labeling

Correspondence:

Dr. Saiful Anuar Karsani,
Faculty of Science, Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia.
E-mail: saiful72@um.edu.my

DOI: 10.4103/pm.pm_53_17

Access this article online
Website: www.phcog.com
Quick Response Code: