ANTIPROLIFERATIVE ACTIVITIES OF CHALEPIN AND RUTAMARIN ISOLATED FROM *Ruta angustifolia* ON SELECTED CANCER CELL LINES

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FACULTY OF SCIENCE
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ANTIPROLIFERATIVE ACTIVITIES OF CHALEPIN AND RUTAMARIN ISOLATED FROM *Ruta angustifolia* ON SELECTED CANCER CELL LINES

ABSTRACT

Chalepin and rutamarin isolated from the chloroform fraction of *Ruta angustifolia* were screened against selected cancer lines namely the human hormone-dependent breast cancer cell (MCF7), human non-hormone-dependent breast cancer cell (MDA-MB-231), human colon cancer cell (HT29), human colon carcinoma cell (HCT116) and a normal lung fibroblast cell line (MRC-5). Phytochemical investigation on the active chloroform extract led to the isolation of chalepin and rutamarin using HPLC. These compounds were then, identified by GC-MS and NMR analysis. This was followed by cytotoxicity screening using SRB assay. Based on the IC_{50} at the lower time point, chalepin was further investigated for its apoptotic induction on MCF7 cell through morphological analysis using both phase contrast and fluorescence microscopy; and established biochemical assays. Western blot analysis was also conducted on MCF7 treated with chalepin. For HT29 cells, rutamarin treatment followed by downstream study on protein profiling by LC-MS approach as well as western blot analysis was performed as there were no previously reported study. The active chloroform extract showed relatively higher cytotoxic activity against MCF7, MDA-MB-231, and HT29, but no activity against MRC5 (IC_{50} > 100μg/mL). Chalepin displayed remarkable cytotoxicity against all tested cancer cell lines but no activity against MRC-5. Rutamarin on the other hand, showed remarkable cytotoxic activity only on MCF7 and HT29, whereas no activity against MDA-MB-231 and MRC5 was observed. In this study, morphological examinations identified typical apoptotic features such as membrane blebbing, chromatin condensation, DNA fragmentation, and apoptotic body formation. Phosphatidylserine externalisations, DNA fragmentation, and caspase-3 activity significantly increased whereas mitochondrial membrane potential significantly decreased in chalepin treated MCF7 cells

as compared to untreated cells. Western blots results showed that the expression of pro-apoptotic proteins such as caspases, Bid and P53 were upregulated whereas cell cycle regulatory proteins such as CDK2, CDK4, cyclin A, and cyclin D were downregulated. Similarly, EGFR and its downstream cascades; (PI3K-AKT; JAK-STAT3 and Erk pathways) were also downregulated. The apoptotic effect of chalepin against MCF7 was a dose and time-dependent manner. On the other hand, Western blot results on HT29 treated with rutamarin shows that the expression of pro-apoptotic proteins such as caspases, Bid, P21, P27, and P53 was upregulated whereas cell cycle regulatory proteins such as CDK2, CDK4, cyclin A, and cyclin D were downregulated. Similarly, EGFR and its downstream cascades (PI3K-AKT; JAK-STAT3 and Erk pathways) were also downregulated. Results from proteomic profiling indicates that 2056 proteins were identified from both untreated and 6 hours rutamarin treated HT29. Following filtrations, at various levels, only 756 proteins were used for the analysis. Consequently, two sample t-test show that only one protein; mitochondrial carrier homolog 2 (MTCH2) (Q9Y6C9) was identified to be upregulated in 6 hours, whereas profile plot analysis indicated 20 proteins are having a similar pattern including the differentially expressed protein. These initial results, therefore suggest that chalepin and rutamarin may serve as potential anticancer agents that warrant further in-depth investigations.

Keywords: Chalepin, rutamarin, MCF7, HT29, apoptosis

AKTIVITI ANTIPROLIFERATIF BAGI CHALEPIN DAN RUTAMARIN DIASIYLAN DAN *Ruta angustifolia* DALAM GARISAN SEL KANSER TERPIUH

ABSTRAK

Chalepin dan rutamarin yang diasingkan dari pecahan chloroform *Ruta angustifolia* telah diuji aktivitinya terhadap garisan sel kanser terpilih garisan sel kanser payudara yang bergantung kepada hormon manusia (MCF7), garisan sel kanser payudara yang tidak bergantung kepada hormon manusia (MDA-MB-231), garisan sel sel kanser kolon manusia (HT29), garisan sel karsinoma kolon manusia (HCT116) dan garison sel sel fibroblast paru-paru biasa (MRC-5). Ekstrak kloroform aktif menunjukkan aktiviti sitotoksik yang lebih tinggi terhadap MCF7, MDA-MB-231, dan HT29, tetapi tiada aktiviti terhadap MRC5 (IC50 > 100 μg / mL). Penyelidikan fitokimia ke atas ekstrak kloroform aktif telah berjaya mengasingkan chalepin dan rutamarin menggunakan HPLC. Kompaun kemudiannya telah dikenalpasti menggunakan analisis GC-MS dan NMR. Ini telah diikuti dengan penyaringan sitotoksiksiti menggunakan ujian SRB. Berdasarkan IC50 pada titik masa lebih rendah, chalepin telah dikaji dengan lebih lanjut berkenaan induksi apoptotiknya dalam garisan sel MCF7 melalui analisis morfologi menggunakan kedua-dua mikroskopi fasa knotras dan berpendarfluor; serta ujian biokimia. Analisis Western blot juga dijalankan pada MCF7 yang dirawat dengan chalepin. Bagi sel HT29, rawatan degan rutamarin telah diikuti oleh kajian pemprofililan protein menggunakan pendekatan LC-MS serta analisa Western blot memangdangkan tiada kajian yang dilaporkan sebelum ini. Ekstrak kloroform aktif menunjukkan aktiviti sitotoksik yang lebih tinggi terhadap MCF7, MDA-MB-231, dan HT29, tetapi tiada aktiviti terhadap MRC5 (IC50 > 100 μg / mL). Chalepin menunjukkan sitotoksisiti yang luar biasa terhadap semua sel kanser yang diuji tetapi tiada aktiviti menentang MRC-5. Sebaliknya, Rutamarin menunjukkan aktiviti sitotoksik yang luar biasa hanya pada MCF7 dan HT29,

sedangkan tidak ada aktiviti menentang MDA-MB-231 dan MRC5. Dalam kajian ini, pemeriksaan morfologi bagi mengenal pasti ciri-ciri apoptotik yang biasa seperti pembengkakkan membran, kondensasi kromatin, fragmentasi DNA, dan pembentukan badan apoptotik. Eksternalisasi fosfatidilserin, fragmentasi DNA, dan aktiviti kaspase-3 telah meningkat dengan ketara manakala potensi membran mitokondria berkurangan dengan ketara dalam sel MCF7 yang dirawat dengan chalepin berbanding dengan sel yang tidak dirawat. Keputusan Western blot menunjukkan bahawa ekspresi protein pro-apoptosis seperti kaspase, Bid, P21, P27, dan P53 telah menigkat manakala protein pengawalan kitar sel seperti CDK2, CDK4, siklin A, dan siklin D telah turun ekspresinya. Begitu juga, EGFR dan cascades hilirannya; (PI3K-AKT; JAK-STAT3 dan laluan Erk) juga dikurangkan. Kesan apoptosis chalepin terhadap MCF7 adalah cara dos dan bergantung kepada masa. Sebaliknya, hasil blot Barat pada HT29 yang dirawat dengan rutamarin menunjukkan bahawa ungkapan protein pro-apoptosis seperti caspase, Bidaan, P21, P27, dan P53 adalah regulasi sementara protein regulasi kitaran sel seperti CDK2, CDK4, cyclin A, dan Cyclin D dikurangkan. EGFR dan lata hilirannya (PI3K-AKT; JAK-STAT3 dan laluan Erk) juga turun ekxpresinya. Hasil daripada profil proteomik menunjukkan bahawa 2056 protein telah dikenal pasti dari kedua-dua sampel HT29 yang tidak dirawat dan yang telah dirawat selama 6 jam dengan rutamarin. Selepas penapisan, pada pelbagai peringkat, hanya 756 protein telah digunakan untuk analisis. Natijahnya, t-test dual sampel menunjukkan bahawa hanya satu protein; mitokondria homolog 2 (MTCH2) (Q9Y6C9) telah dikenal pasti dikawal naik dalam masa 6 jam, manakala analisis plot profil menunjukkan bahawa 20 protein mempunyai corak yang sama termasuk protein yang dinyatakan secara berbeza. Keputusan awal ini mencadangkan bahawa chalepin dan rutamarin mungkin berfungsi sebagai agen antikanker potensial yang menjamin penyelidikan lebih mendalam.

Kata Kunci: Chalepin, rutamarin, MCF7, HT29, apoptosis

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cm	:	Centimetre
CO_2	:	Carbon dioxide
^{13}C	:	Carbon-13
^{o}C	:	Degree Celsius
^{1}H	:	Proton
µg	:	Microgram
µm	:	Micromile
µg/ml	:	Microgram Per millilitre
g	:	Gram
>	:	greater than
≥	:	greater than or equal to
<	:	Less than
≤	:	Less than or equal to
%	:	Percentage
ACN	:	Acetonitrile
AIF	:	Apoptosis-inducing factor
ALS	:	Autosampler
AMC	:	7-amino-4-methylcoumarin
ANOVA	:	Analysis of variance
Apaf1	:	Apoptosis proteases activating factor-1
APS	:	Ammonium persulfate
ATCC	:	American Type Culture Collection
BD	:	Becton Dickinson
BID	:	Bcl-2 interacting protein
Bcl-2	:	B-cell lymphoma 2

bp	:	Base pair
BrdU	:	Bromodeoxyuridine
BrdUTP	:	5-bromo-2'-deoxyuridine 5'-triphosphate
BSA	:	Bovine serum albumin
CDK	:	Cyclic dependent kinase
DAD	:	Diode Array Detector
DDA	:	Data Detection Acquisition
DMEM	:	Dulbecco's Modified Eagles Medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic acid
DOX	:	Doxorubicin
DR	:	Death receptors
DTT	:	Dithiothreitol
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptor
EMEM	:	Eagle's Minimum Essential Media
FA	:	Formic Acid
FBS	:	Fetal Bovine Serum
FC-AS	:	Fraction collector
FCCP	:	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FDR	:	False Discovery Rate
FITC	:	Fluorescein isothiocyanate
FNI	:	Function not identified
GCMS	:	Gas Chromatography-Mass Spectrometry
HCD	:	High Collision Dissociation
HPLC	:	High-Performance Liquid Chromatography

HRP	:	Horseradish peroxidase
IAA	:	2-iodoacetamide
IAPs	:	Inhibitor of Apoptosis Proteins
IC_{50}	:	Inhibitory concentration at 50%
INH	:	Inhibitor
JAK	:	Janus kinase
JC-1	:	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarcocyanine
JNK	:	c-Jun N-terminal kinase
KDa	:	Kilo Dalton
Kg	:	Kilogram
L	:	Liter
LC	:	Liquid Chromatography
LFQ	:	Label-Free Quantification
m	:	Meter
MAPK	:	Mitogen-activated protein kinase
mL	:	Milliliter
mm	:	Millimetre
MMP	:	Mitochondrial Membrane Potential
MOM	:	Mitochondrial Outer Membrane
MS	:	Mass spectrometry
mTOR	:	Mechanistic target of rapamycin
m/z	:	Mass to charge ratio
NCI	:	National Cancer Institute
NF-κB	:	Nuclear factor-kappa B
nm	:	Nanometer

NMR	:	Nuclear Magnetic Resonance
OH	:	Hydroxyl
PAGE	:	Polyacrylamide Gel Electrophoresis
PARP	:	Poly adenosine diphosphate (ADP)-ribose polymerase
PBS	:	Phosphate buffer saline
PI	:	Propidium iodide
PS	:	Phosphatidylserine
PSM	:	Peptide Spectrum Matching
Ra	:	*Ruta angustifolia*
rpm	:	Revolution Per Minute
SD	:	Standard Deviation
SDS	:	Sodium Dodecyl
SRB	:	Sulphorhodamine
SMAC	:	Second Mitochondrial-Derived Activator of Caspases
STAT	:	Signal transducers and activators of transcription
TBST	:	Tris-buffered Saline with Tween-20
TCA	:	Trichloroacetic Acid
TCC	:	Thermostatted Column Compartment
TFA	:	Trifluroacetic Acid
TLC	:	Thin Layer Chromatography
TMS	:	Tetramethylsilane
TNF	:	Tumour Necrosis Factor
TRAIL	:	TNF-Related Apoptosis-Inducing Ligand
TUNEL	:	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UNSCEAR	:	United Nations Scientific Committee on Effects of Atomic Radiation

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Cancer has been defined as growth disturbance characterized by excessive proliferation of cells without apparent relation to the physiological demands of the organ involved. All cancers usually originated from one aberrant cell which continued to multiply and produce a tumor mass (Vasudevan, Sreekumari, & Kannan, 2017). Cancers are originated from multifactorial which includes genetics, hormonal, metabolic, physical, chemical and environmental factors. In addition, many chemicals such as mutagens and aflatoxins are known to be powerful carcinogens. These chemicals increase mutation thereby enhancing the rate of cancer incidence. Many of the human cancers are spontaneous in nature (Vasudevan et al., 2017).

However, transformation and progression were believed to be linked with the ordered enzymic and metabolic imbalance discovery resulted from molecular correlation concept and the key enzyme concept that coupled to the use of biologically meaningful tumor models and control systems (Weber, 2001). It has been observed and reported that alterations in enzymic pattern was due to reprogramming of gene expression which characterized neoplasia. In contrast, no similar pattern of imbalance was observed in any of the control normal, regenerating, or differentiating tissues. Consequently, gene reprogramming confers to cancer cells (Weber, 2001).

Furthermore, the pattern of biochemical imbalance discovery was linked to neoplastic transformation and progression which provided accurate measurable expected changes in enzymic and metabolic pattern of cancer cells. In this regard three key proposal were made which includes:

"1) The biochemical strategy of the genome in neoplasia can be identified by elucidating the pattern of gene expression as revealed in the activity, concentration, and isoenzyme program of the key enzymes.

2) Activities of key enzymes and metabolic pathways and concentration of strategic metabolites are stringently linked with neoplastic transformation and progression; those not stringently linked do not yield a pattern.

3) The linking of enzyme and metabolic imbalance with transformation and progression can be identified in a spectrum of tumors of graded malignancy, proliferative rate, and degree of differentiation" (Weber, 2001).

Another research also pointed out that, successive alteration of genetic and epigenetics induced cell hydration which served as basic mechanism of multistep carcinogenesis. Thus, the degree of cell hydration increased the degree of malignancy (McIntyre, 2007). The increased cell hydration has been implicated as a common factor for promoting both growth and metastasis of tumor, and that metastasis potential increases with the degree of cell hydration. Thus, acquired metastasis potential is the major cause of cancer mortality. It was also been postulated earlier that the increased ability of tumor cells to compete for nutrients with their counterparts was due to enhanced metabolic activity through increased cell hydration (McIntyre, 2007).

Therefore, understanding alterations in the enzymology and biochemistry of cancer cells could identify possible potential targets for anticancer chemotherapy.

Natural products, including from medicinal plant are reported as rich sources anticancer agents for drug discovery and development (Bishayee & Sethi, 2016). Moreover, new compounds isolated from medicinal plants are expected to be identified and developed into efficient and less toxic anticancer drugs (Bishayee & Sethi, 2016). Several natural compounds have been shown to exhibit bioactivity and are useful in prevention as well as in cancer therapy by targeting various signalling molecules and pathways (Bishayee & Sethi, 2016).

Several plant species are reported to contain bioactive compounds having various biological, pharmaceutical as well as therapeutic effects. Among these plant species is the

Ruta species. The *Ruta* species belongs to a common genus used in Italian traditional medicine, economic botany and folklife (Pollio, De Natale., Appetiti, Aliotta, & Touwaide, 2008). *Ruta* species contains various classes of phytochemicals which include alkaloids, coumarins, flavonoids, glycoside, lignans, saponins, and triterpenes. Abortive, depressant, antidotal, antifungal, anti-inflammatory, antioxidant and phytotoxic are among the biological activities exhibited by many of these compounds (Amar, Abdelwahab, & Noureddine, 2012).

The Rutaceae family contains several plant species of medicinal interest; which has ecological, economic and therapeutic values (Januário et al., 2009). Rutaceae family belongs to the order Sapindales and has over 1600 species with 150 genera. The species are widely distributed all over the tropical as well as temperate areas of the world. However, these plants are mostly abundant in countries like South Africa, Australia and tropical America (Albarici, Vieira, Fernandes, Silva, & Pirani, 2010).

Several Ruta species including *Ruta angustifolia* that belongs to this family has been used for cancer treatment by the Chinese community of Malaysia. They have been reported to contain various essential oils (Ferhat, Kabouche, & Kabouche, 2014; Joulain, Laurent, Fourniol, & Yaacob, 1991). However, a literature survey revealed little to no detailed biological or pharmacological investigation on *Ruta angustifolia* as well as it's isolated compounds; consequently, there is limited published information on the bioactivity of *Ruta angustifolia*, particularly on cytotoxicity. Generally, many plants were claim with various degree of biological activities including anticancer due to presence of several bioactive constituents. Though, many studies are ongoing to validate the efficacy and safety of many plants and their active components. However, most of the studies are still at clinical trials which needs further investigations to authenticate their used as anticancer agents for the effective treatment of various cancer types.

Proteins involve in maintaining homeostasis playing a significant role in almost all cellular physiological processes through biochemical catalytic reactions in intermediary metabolism to the processing and integration of internal and external signals (Schmidt, Forne, & Imhof, 2014). However, dysregulation of protein expressions is usually associated with several pathological conditions including cancer, metabolic imbalances, and neurodegenerative diseases (Schmidt et al., 2014). Therefore, it is essential for the understanding of molecular processes that mediate cellular physiology through identification, quantification, and characterisation of all proteins in the cells (Schmidt et al., 2014).

Over the last few decades, proteomics analysis of biological samples such as cells and tissues has become a relevant field to identify changes in protein abundance between two different conditions (Kammers, Cole, Tiengwe, & Ruczinski, 2015). Mass-spectrophotometry based experiments such as using LC/MS and Orbitrap becomes a fundamental task in detecting significant changes in proteins abundance when comparing treated to untreated cells, wildtypes to mutants, or samples from diseased or non-diseased subjects (Kammers et al., 2015).

Cancer been an abnormal and uncontrolled proliferation of cells that has ability to invade surrounding environments such as cells, tissues and even organs is a devastating disease, thus averting the disease could be of vital importance to human as well as public health benefit. It is, therefore, necessary to search for novel and safe anticancer agents due to cytotoxic and other side effect on healthy cells following treatment with existing drugs (Al-Mekhlafi et al., 2017; Patel, Raval, Karanth, & Patel, 2010). Herbal products are becoming more popular and fast progressing as potential sources of anticancer agents (Al-Mekhlafi et al., 2017; Patel et al., 2010).

1.2 Aims and objectives of the study

The study aimed to evaluate the antiproliferative activity of chalepin and rutamarin and their possible mechanism for cell death against MCF7 and HT29 cancer cell lines.

The research has the following specific objectives:

1. To isolate chalepin and rutamarin from *Ruta angustifolia*.
2. To investigate the cytotoxic activities of chalepin and rutamarin on selected cancer cell lines.
3. To investigate whether chalepin can induce apoptosis in MCF7.
4. To identify proteins in the cancer cell lines (HT29 and MCF7) that change in abundance following exposure to rutamarin and chalepin respectively.
5. To identify the molecular target involved in the mechanism of cell death induced by rutamarin in HT29.

1.3 Research approach

As illustrated, Figure 1.1 summarises extraction, fractionation and cytotoxicity screening of the plant extracts. It also highlighted the approached procedure for isolation, identification as well as cytotoxicity screening of the isolated compounds. This section outlined the phase I of the research methodology.

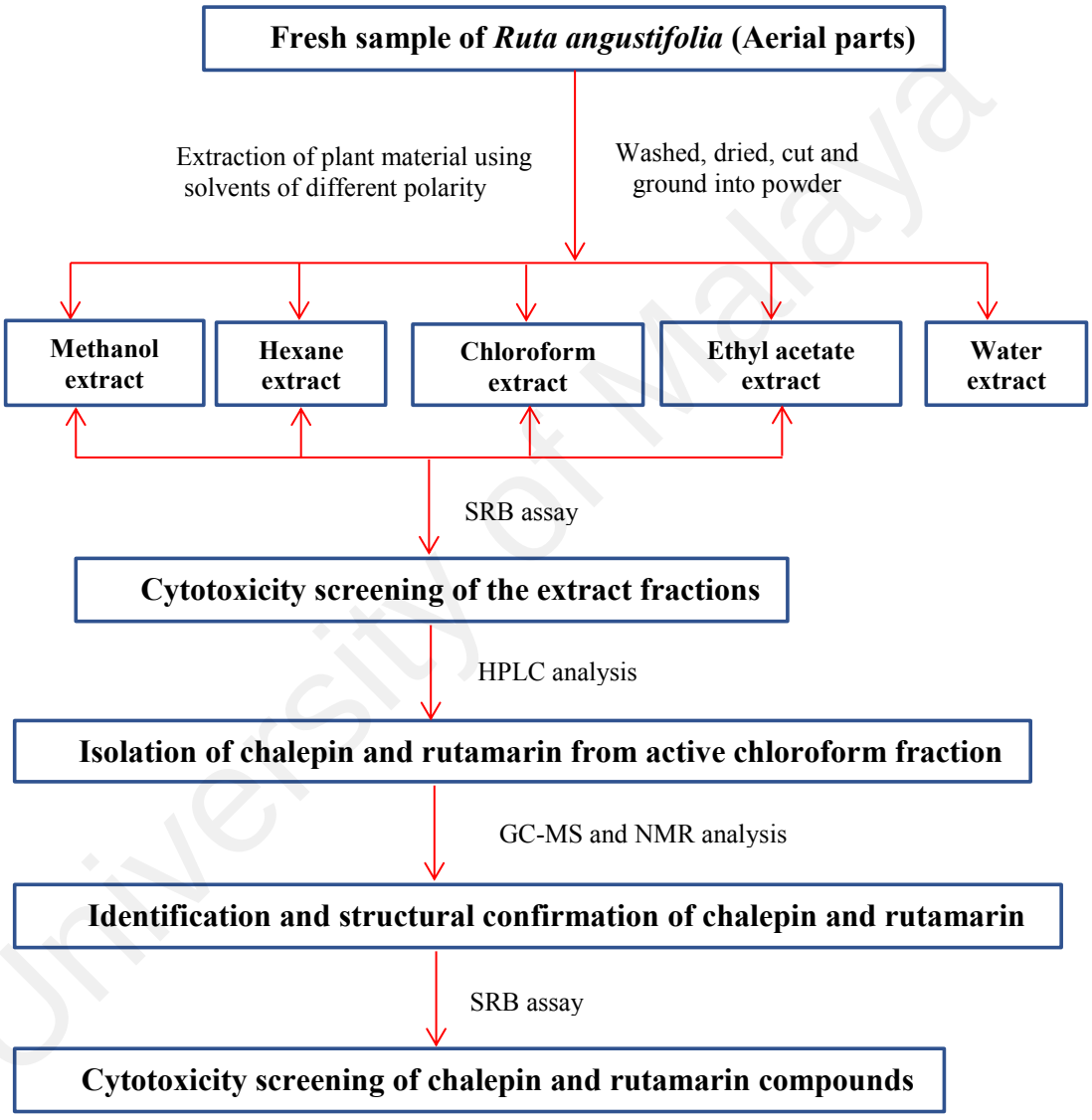


Figure 1.1: Overview of the phase I research methodology

The illustrations in Figure 1.2, summarises the apoptosis induction in MCF7 chalepin-treated using various detection assays. It also highlighted the procedure for proteins analysis using both western blot and LC/MS-MS. These sections outlined phase II and phase III of the research methodology.

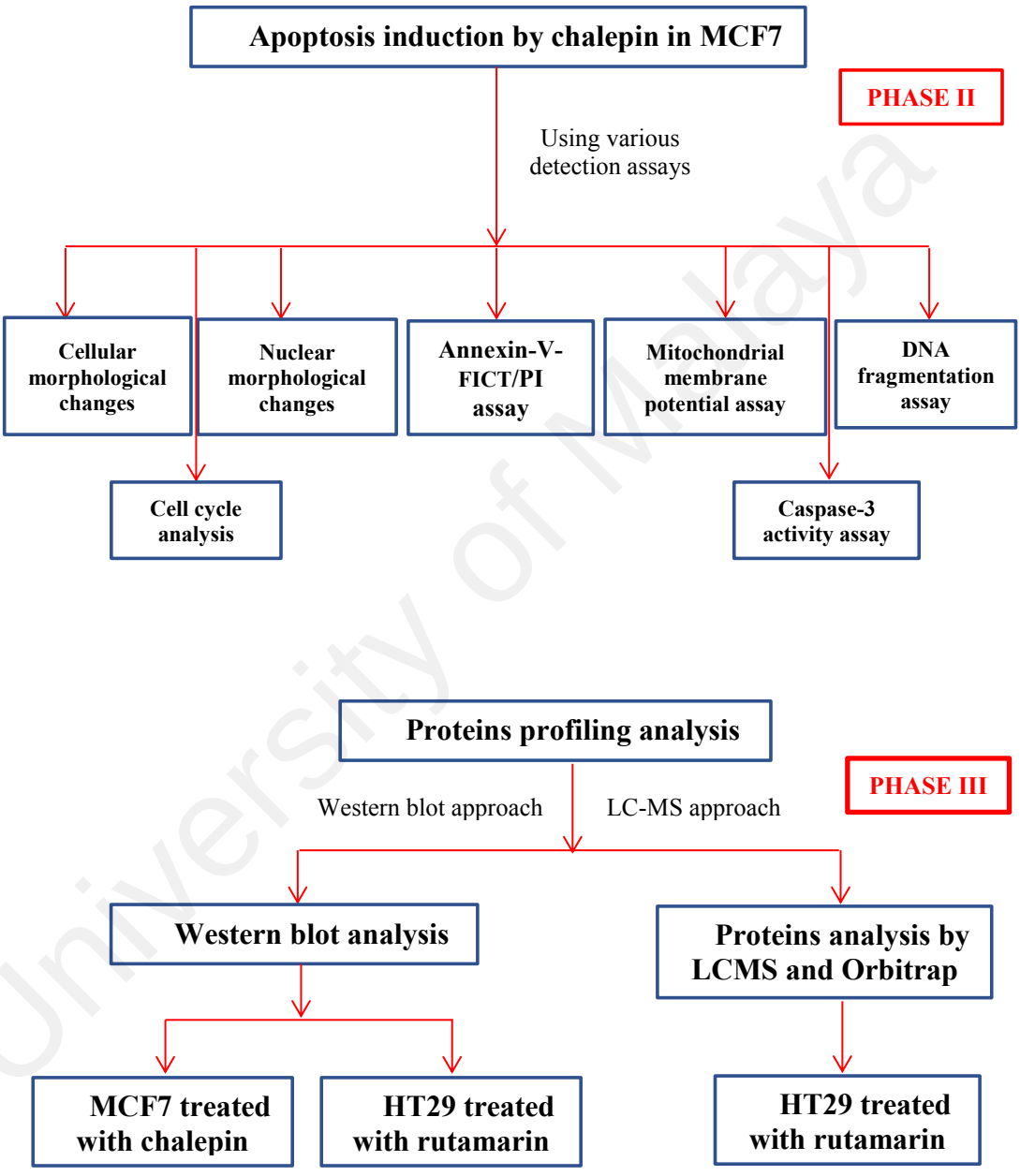


Figure 1.2: Overview of phase II and phase III research methodology

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer is an abnormal and uncontrolled proliferation of cells which can invade healthy surrounding tissues and organs within the body. Cancer cells may invade and destroy healthy cells (Madhuri & Pandey, 2009). In cancer cells, the normal regulatory growth control mechanism is typically partially or entirely lost. Thus, consequently disrupt the normal cellular cell division process as controlled by genetic material (DNA) (Becker, Kleinsmith, Hardin, & Raasch, 2003; Reddy, Odhav, & Bhoola, 2003). Subsequently, abnormal cell growth will occur in the body resulting in cancer stem cells due to damage on the DNA material. The DNA damage may be due to environmental factors which includes and not limited to contact with asbestos and smoking. Other factors are those from inherited mutated and damaged DNA material. If the damaged DNA is not repairable in healthy cells, it may ultimately lead to uncontrolled proliferation. Healthy cells can be rapidly overwhelmed by the growth of abnormal cells that spread all over the body (Latiff, 2010).

Cancer cells are less specialised and this allow them to grow out of control and continue to divide, thus becoming invasive. In contrast, normal cells usually mature to produce a distinct cell types performing a specific function (NCI., 2015). Additionally, cancer cells ignore signals that usually instruct stoppage of cellular division of the dividing cells and or starting a process for removal of the unwanted cells from the body known as apoptosis or programmed cell death. Consequently, cancer cells may influence the microenvironment, inducing surrounding normal cells to form blood vessels which provide tumours with oxygen and nutrients needed for their growth and survival (NCI., 2015).

There are about more than 100 different types of cancer that can develop in virtually any organ, tissue or body fluid (NCI., 2015). The main features of cancer cells are

uncontrolled cell proliferation, differentiation and death invading organs and tissues. Cancer may be described based on the cell type that formed them, such as epithelial or squamous cells and can further be divided into carcinomas, leukaemias, lymphomas, sarcomas and central nervous system cancers (NCI., 2015).

Some cancers can be trigged by chemicals (benzene or nickel) and radiation (UV-radiation or X-rays) which damage DNA, others results from spontaneous DNA mutations and replication errors. Some cellular fundamental processes including differentiation, proliferation as well as apoptosis are found to be regulated and influenced by mutations in some proto-oncogenes like Ras and P53 like-tumor suppressor genes (Scholzová, Malík, Ševčík, & Kleibl, 2007). Thus, mutations in DNA is the leading cause of cancer which can be inherited and expose the individual to the risk of cancer.

Cancer is one of major source of morbidity and mortality all over the world (Sylla & Wild, 2012). In 2008, about 12.7 million new cases of cancer and 7.6 million cancer-related deaths has been reported (Ferlay et al., 2010b). Similarly, in 2012 cancer death was estimated to be 8.2 million, whereas new case of cancer was estimated as 14.1 million occurred worldwide (Figure 2.1). Moreover, developed countries were reported to have less cancer burden as compared to other Nations (Torre et al., 2015). Among the cancer types; breast (1.67m), colorectal (1.36m) and lung (1.2m) are the most commonly diagnosed cancer (Ferlay et al., 2015). However, cancer death is commonly results from the cancers like lung (1.6m), liver (745,000), and stomach (723,000) (Ferlay et al., 2015).

Current statistics estimated 18.1 million new cancer cases (17.0 million excluding nonmelanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding nonmelanoma skin cancer). However, in both males and females lung cancer is the most commonly diagnosed caner accounting for 16.1% of the total cases and the leading cause of cancer death accounting for 18.4% of the total cases (Bray et al., 2018). Similarly, female breast cancer account for 11.6%, prostate cancer (7.1%), and colorectal (6.1%) for

incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality rate (Bray et al., 2018). Moreover, in males lung cancer remain the most frequent cancer and the leading cause of cancer death, followed by prostate and colorectal cancer for incidence, and liver and stomach cancer for mortality. Conversely, in females breast cancer remain the most frequently diagnosed and the leading cause of cancer death, followed by colorectal and lung cancer for incidence and vice versa for mortality. In addition, cervical cancer was the fourth for both incidence and mortality (Bray et al., 2018). Developing countries were found to contain about 56% of these new cases; conversely, by 2030, 70% of all new case has been projected to be from developing countries (Boyle & Levin, 2008). The increased cancer incidence is mostly due to population growth and a rise in life expectancy (Lyerly et al., 2011). The projection of the global cancer burden will be doubled in the next two decades, which can cause significant increased investment in the health system, thereby posing a real medical problem (Vineis & Wild, 2014).

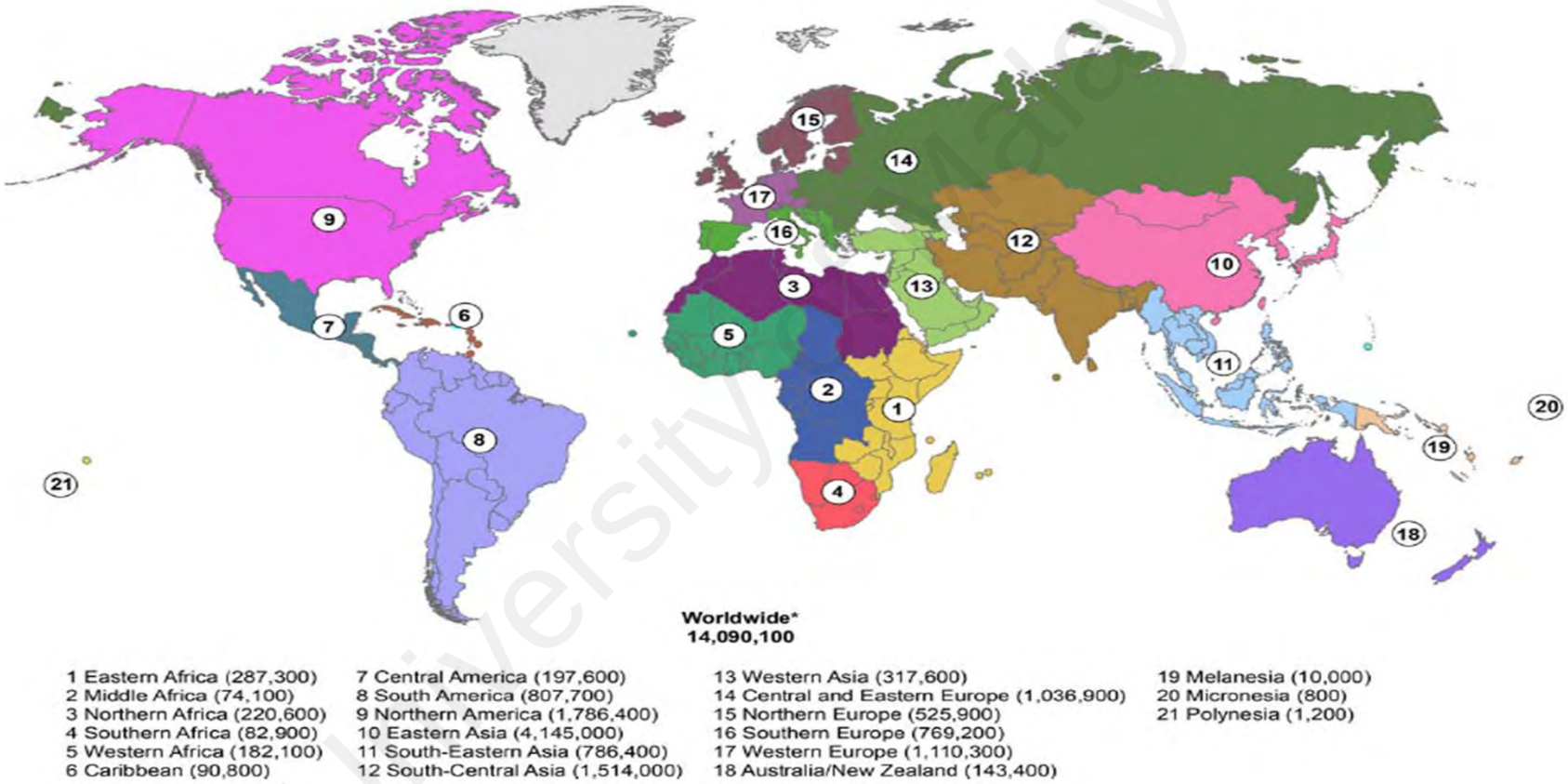


Figure 2.1: Rate of cancer new case estimated over 21 areas in the world. Torre et al., (2015), and reproduced with permission from John Wiley and Sons

In Malaysia, cancer incidence also has been reported with an estimated 30,000 cases annually (Lim, 2002). The cause of the increase is similar to that observed in the developed world (Lim & Lim, 1993; Lim, 2002). A report from regional cancer registry data identified ten main cancers in males: bladder, colon, larynx, liver, lung, nasopharynx, non-Hodgkins's lymphoma, oesophagus, rectum, stomach, and uninary (Lim, 2002). Equally, in females breast, cervix, colon, ovary, lung, nasopharynx, non-Hodgkin'slymphoma, oesophagus, rectum, and thyroid were among the leading cancers (Lim, 2002).

Breast and lung which account for the main reasons of death due to cancer in women and men respectively, as found in less developed countries and worlwide at large. However, in advanced developed countries, the most frquently diagnosed in men is prostate cancer whereas the principal cause of death in women resulting from cancer is due to lung cancer (Torre et al., 2015).

2.2 Carcinogenesis

Carcinogenesis is a multistep process of cancer development. This indicate a sign of genetic alterations by which healthy cells transformed progressively to highly malignant tumours. Carcinogenesis is divided into initiation, promotion and progression stages (Figure 2.2) (Devi, 2004; Ito et al., 1995). Though various cancer types exist, the multistep processes are similar in all cancer types. These stages have been studied extensively during the past decades in different animal models (Tanaka, 1997; Tanaka, 2009).

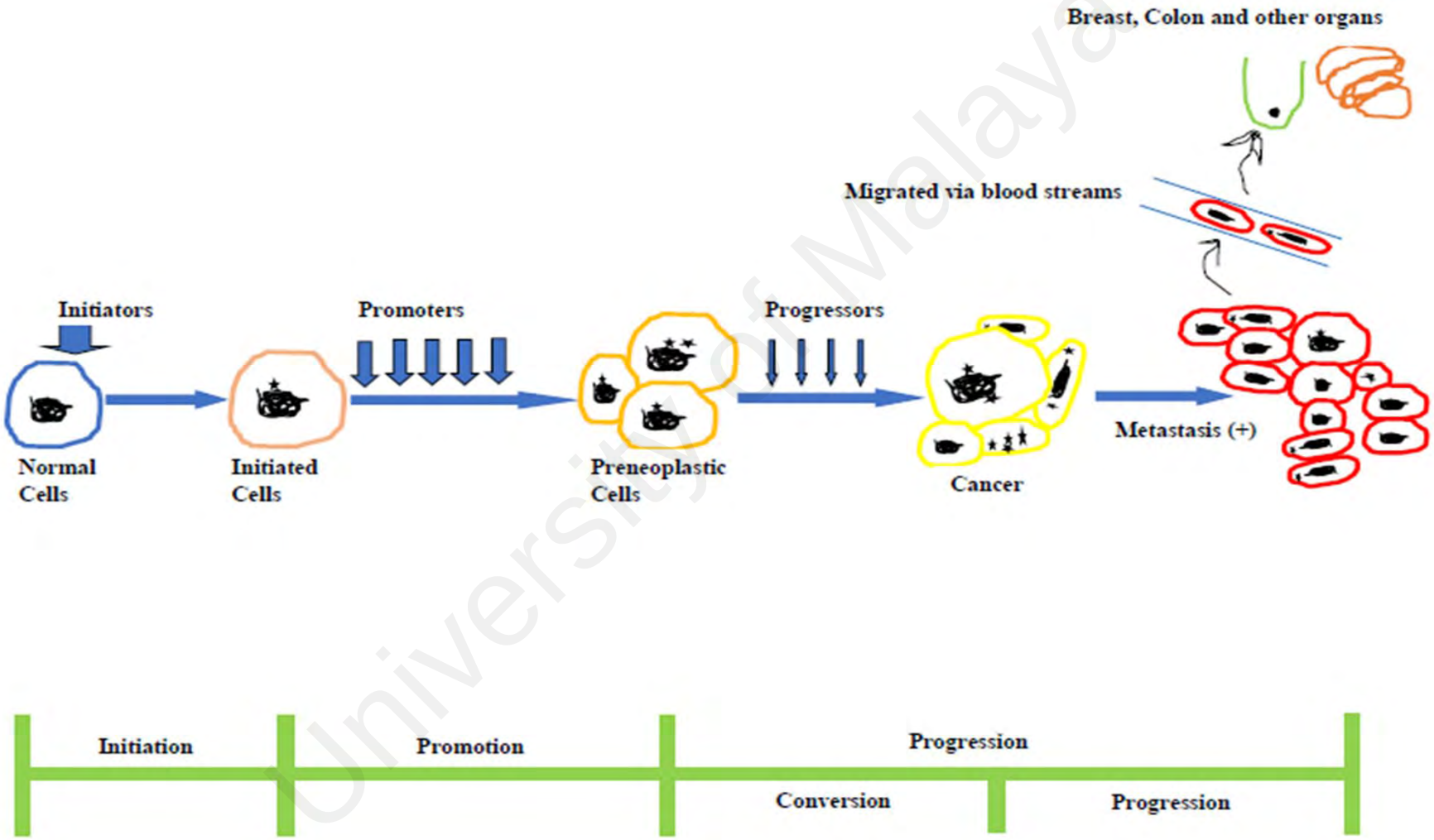


Figure 2.2: Multistep cancer development. Adopted from Tanaka (1997)

Initiation in carcinogenesis is the first stage of carcinogenesis which is an irreversible change that occurs in target cells. It involves some cellular changes resulting from spontaneous or induced contact to a carcinogen, that subsequently causes mutation of the genetic material (DNA) (Devi, 2004; Latiff, 2010). This initiate neoplastic development with concomitant effect on the cell and its offspring making it susceptible to consequent neoplastic alteration (Devi, 2004).

Promotion is the second and longest stage in carcinogenesis. The cells transformed (initiated) in this stage remains harmless until further proliferation is stimulated which then causes cellular imbalance (Devi, 2004). Subsequently, variations in the initiated cell result in neoplastic alteration that may involve several steps which require continued and extended contact to promoting stimuli (Devi, 2004; Upton, Albert, Burns, & Shore, 1986). The promotion stage is further divided into two; cell proliferation as well as expression of the altered phenotypes (Ito et al., 1995). During this stage, repeated application of irritating and promoting agents are involved that affects expression of genetic material by various mechanisms such as cell surface receptors interactions, cytoplasm and nuclear receptors interactions and through alterations of other cellular functions or components (Ito et al., 1995; Laconi, Doratiotto, & Vineis, 2008; Latiff, 2010).

The last stage of carcinogenesis is progression. This stage has an array of consequences for the tumour cell. Successive progression changes in the neoplasm lead to increasing sub-population of the malignant. Though molecular mechanisms of tumor progression are not completely understood, mutations and anomalies in and mutations are likely to be implicated in the process (Devi, 2004). This stage involved conversion as the first phase and sometimes known as a neoplastic conversion by which cells are transformed from pre-neoplastic to a more committed state of malignant development (Devi, 2004). Furthermore, gene mutations may be involved, with increased

accumulation in the neoplastic cell clone (Devi, 2004; UNSCEAR, 2000). Conversion process always shows nuclear morphological changes which initiates due to the invasion of the cells into the surrounding environment from outside the normal tissue boundaries (Kulesz-Martin, 1997).

During the progression stage, genetic material is unstable, fragile and thus becomes more prone to additional genetic alterations (mutations). This leads to various consequences such as rearrangements, translocation, amplification or deletion of chromosomes or genes. It may also cause proto-oncogenes activation as well as inactivation of p53 tumour suppressor gene (Devi, 2004; Ito et al., 1995; Ruddon, 2007). Recurring exposures may accelerate the process to carcinogenic stimuli. Hence, cancer cells acquire malignancy and survive in the human body due to the genetic alterations. Subsequently, tumour size increases at a faster rate due to initiated cell proliferation. Thus, the continued growth of tumour size may eventually results to increased heterogeneous cell population due to induced cells undergoing further mutation (Devi, 2004; Ruddon, 2007).

2.2.1 Breast cancer

Breast cancer is a worldwide health problem (Baeshen, Elkady, Abuzinadah, & Mutwakil, 2012). It is the most commonly diagnosed cancer that causes death in women all over the world (Baeshen et al., 2012; Chen et al., 2011; Dandawate, Subramaniam, Jensen, & Anant, 2016; Sinha, Sarkar, Biswas, & Bishayee, 2016). Recent statistics indicated an annual worldwide occurrence of about 1.3 million people with 465, 000 deaths (Baeshen et al., 2012; Elangovan, Hsieh, & Wu, 2008). In the United States, breast cancer is the most common cancer and second-top cancer-related cause of death (Dandawate et al., 2016; DeSantis, Ma, Bryan, & Jemal, 2014). Conversely, it is the most common cancer in Malaysia across all ethnic groups which accounts for 11% of all medically certified deaths (Hadi, Hassali, Shafie, & Awaisu, 2010; Yip, Taib, &

Mohamed, 2006). A significant number of breast cancer cases has also been reported in Nigeria (Adesunkanmi, Lawal, Adelusola, & Durosimi, 2006; Anyanwu, Egwuonwu, & Ihekwoaba, 2011). Similarly, breast cancer remains the foremost cause of death in females all over less developed countries due to risk factors associated with overweight/obesity and physical inactivity (Torre et al., 2015). Breast cancer risk increases as a result of associated prolonged exposure to estrogen. However, mammary carcinogenesis has been inhibited through both oxidative stress-induced estrogen and estrogen receptor (ER) signalling inhibition by various natural products as well as bioactive compounds (Bishayee & Sethi, 2016). There are some established factors associated with risk of breast cancer which includes age, family history, genetic defects, lifestyle, mammogarphic density of breast, menstrual and menuposal history, as well as exposure to radiation. Notably, breast cancer risk can also be increased by estrogen and progesterone hormones (Bak, Das Gupta, Wahler, & Suh, 2016). The global incidence, mortality rate, and prevalence of breast cancer have been well documented (Ferlay, Héry, Autier, & Sankaranarayanan, 2010a; Yip et al., 2006).

However, chemotherapy is still the primary tratment for breast cancer. Although initially treated cells respond to treatment, breast cancer eventually become resistant and can survive the treatment. This leads to the treatment becoming ineffective in many patients causing them to suffer the disease due its adverse effect (Baeshen et al., 2012; Chen et al., 2011).

2.2.2 Colorectal cancer (CRC)

One of the most common globally reported cancer is colorectal cancer. CRC begins either in the colon or rectum and is commonly found on epithelial lining in the large intestine or the rectum (Hong, Hong, Lee, Yaacob, & Malek, 2016). It is reported worldwide as the third most common malignancy and fourth most common death-caused from cancer. In 2012, 1.4 million cases of newly diagnosed CRC were estimated with

693,000 of mortality rate (Torre et al., 2015). CRC occurs in both men and women with an estimated 746, 000 cases annually and is the third most common in men whereas is the second most common with 614,000 cases in women worldwide (Ferlay et al., 2015). The global incidence rate of CRC varied due to vast geographical differences. In developed countries like Australia, Europe, New Zealand, and the United States, the incidence rate was higher in comparison to the developing countries (Ng & Wong, 2013). However, the incidence rate was found to be decreasing in the United States due to increasing awareness and screening rates. However, several historically low-risk countries, especially in Asia including Thailand, China, Japan, Korea and Singapore, were reported to have a rapidly increasing incidence of CRC (Sano et al., 2016; Torre et al., 2015).

Colorectal cancer in Malaysia has been identified and reported by the National Cancer Registry in 2007 as the second most common cancer with its incidence varied among the ethnic groups. Thus, higher incidence was found among Chinese, then Malays whereas low incidence was observed among the Indians (Abu Hassan et al., 2016). Increasing incidence of CRC has been reported globally following fast habitual adoption of western lifestyle which includes highly nutritional caloric intake coupled with low physical activity. Peoples living in the newly industrialised countries were also increasingly affected by CRC (Weidner et al., 2015). However, the risk and reoccurrences of CRC for patient cancer stage can be reduce using chemotherapy, radiotherapy as well the combined treatments to treat various tumour stages. Though, effective CRC treatment is usually hindered by some factors. These include diagnosis of new cases at late-stage of CRC, litle or no compliance to screening recommendations, severity of chemotherapy and toxicity of radiotherapy, resistance to therapy as well as reoccurrences of cancer (Weidner et al., 2015).

Studies also pointed out that targeted therapy using antibody inhibitors of the EGFR (cetuximab and panitumumab) and the VEGF (bevacizumab, regorafenib and ramucirumab) is another treatment option for CRC (Van Schaeybroeck et al., 2014). Several signalling pathways have been reported as targeted therapy for CRC. These include tyrosine kinase receptors [epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF1R), and MET]. Other affected signalling pathways are Akt, PI3K, P53 and the transforming growth factor (TGF)-β1 receptor (Palma, Zwenger, Croce, Abba, & Lacunza, 2016). Additional studies also highlighted some novel agents from natural products which can target numerous anti-apoptotic and pro-apoptotic proteins, executioner caspases, and cell cycle proteins (Chen & Chen, 2018; Hong et al., 2016; Phang, Karsani, & Malek, 2017; Rouhollahi et al., 2015a). The identification of many targeted biomarkers and novel agents with anticancer activity form various plant products will no doubt contribute significantly to the treatments and reduced risk of CRC incidence and reoccurrences.

2.3 Current trends in cancer diagnosis, prevention and treatments

Different kinds of anticancer treatments such as chemotherapy, radiation therapy and surgery are available but are usually associated with some side effects including bone marrow depression, internal bleeding, nausea, secondary infections and vomiting (Ekowati, Prasasti, & Rastuti, 2011; Reddy, Chandrasekhar, & Sadiq, 2015).

A number of approved available anticancer drugs are used for various cancer treatments as documented by USA National Cancer Institute Drug Repository (Liu, Delavan, Roberts, & Tong, 2017). However, there are still challenges related to anticancer drugs research and development with a high likelihood of failure of the drugs. Furthermore, only a small percentage of compounds ever developed get into drugs (Liu et al., 2017). Cancer patients with prolonged chemotherapeutic treatment frequently developed drug resistance. Therefore, among the essential features for development of

effective cancer therapeutic strategies is to overcome the resistance phenomenon in order to make therapy available to these patients (Singh, Krishnakumar, Kanwar, Cheung, & Kanwar, 2015).

Immune-checkpoint blockage (ICB) is a cancer immunotherapy currently being considered as a promising strategy for treatment of cancer (Chen, Ye, Lu, Sun, & Liu, 2017). ICB-based cancer immunotherapy provides promising opportunities for effective cancer treatment as compared to the traditional therapeutics strategies. It is aimed at inhibiting tumour-mediated suppression of anticancer immune responses thereby improving T-cell activities (Chen & Mellman, 2017; Nishino, Ramaiya, Hatabu, & Hodi, 2017; Topalian, Taube, Anders, & Pardoll, 2016). Developing inhibitors that target these checkpoints by blocking interactions between T cells and tumour cells; and restoring anticancer immune response could be considered a promising immunotherapy strategy for cancer patients (Chen & Mellman, 2017; Chen et al., 2017; Nishino et al., 2017).

2.3.1 Mechanism of action of doxorubicin drug

Doxorubicin is an anthracycline compound also known as Adriamycin (Yang, Teves, Kemp, & Henikoff, 2014). It is widely used anthracycline used as an anti-tumor agent with great efficacy in killing cancer cells for both solid and liquid tumors (Xu et al., 2018; Yang et al., 2014). Doxorubicin is approved by Food and Administration and regarded as one of the most potent chemotherapeutic drugs. Thus, its ability in combating rapidly dividing cells and slowing the progression of disease has been widely acknowledged for many decades (Tacar, Sriamornsak, & Dass, 2013). Despite its common use as anticancer drug in clinics, the molecular mechanisms by which doxorubicin cause cell death or cardiotoxicity is still not clear (Yang et al., 2014). However, several mechanisms by which doxorubicin causes cell death has been proposed and reported. These includes topoisomerase II poisoning, DNA adduct formation, oxidative stress and ceramide overproduction. Most importantly, it intercalates between DNA molecules thereby

inhibiting the activity of topoisomerase II (AbuHammad & Zihlif, 2013; Kubiliūtė, Šulskytė, Daniūnaitė, Daugelavičius, & Jarmalaitė, 2016; Yang et al., 2014).

Although doxorubicin is effective for cancer treatment, however, it uses is limited due to its toxicity on the noncancerous cells in the human body. These results because the drug is a nonselective class I anthracycline containing aglyconic and sugar moieties in its structure (Tacar et al., 2013). Many researches attributed the limited uses of doxorubicin due to resistance developed by various cancer types. Abuhammad and Zihlif (2013), demonstrated a multi-factorial process for the development of doxorubicin resistance in MCF7 cells, where various genes such as CYP1A1, CYP1A2, TOP2A and other crucial genes for cell cycle, apoptosis and DNA repairs were involved. Similarly, some molecular features such as altered expression of the EMT genes, cell adhesion and motility, chemoresistance-related genes, downregulation of TET1 as well as changes in the methylation status of p16 gene were responsible for doxorubicin-resistance development in colorectal cancer CX-1 cell line (Kubiliūtė et al., 2016). Recently, it has been evidently demonstrated that microRNAs (miRNAs) are involved in chemoresistance and tumorigenesis where doxorubicin resistance and tumorigenesis is mediated by microRNA-501-5p-suppressed BLID as a novel mechanism (Xu et al., 2018).

2.3.2 Anticancer agents from natural products

Broad range of old and new strategies are now covered by complementary and alternative medicine which offers options to prevent and treat diseases such as cancer (Harvey, Edrada-Ebel, & Quinn, 2015; Wang, He, Wang, & Yuan, 2012).

Noteworthy, recent research are geared towards finding small molecules that can serve as potential anticancer agents which are useful, less costly and less toxic from natural plant products. Natural products are found to be essential source for therapeutic agents which are rich source for the discovery and development of cancer preventing and anticancer drugs (Bishayee & Sethi, 2016; Hait, 2009). It has been reported that 80% of

the drugs approved by United State Food and Drug Administration are derived from natural compounds (Bishayee & Sethi, 2016; Cragg, Grothaus, & Newman, 2009; Newman & Cragg, 2016).

Medicinal plants contain various bioactive compounds that poses therapeutic properties. Anti-inflammatory, antimalarial, antiviral, analgesic, and antitumor are among the collection of therapeutic effects of medicinal plants (Raina, Soni, Jauhari, Sharma, & Bharadvaja, 2014). Several plants and plant products have been reported to be used in disease prevention, management and treatment since ancient time by the various communities due to their medicinal properties (Al-Mekhlafi et al., 2017).

2.3.3 Plants used for cancer treatments

Plants remain historically significant as essential sources of medicinal agents (Balandrin, Kinghorn, & Farnsworth, 1993). It continues to be one of the most reliable and useful sources of raw material for new plant-derived compounds for the development of new clinical candidate for world-class medicine (Itokawa, Morris-Natschke, Akiyama, & Lee, 2008).

Nowadays, thousands of plants and plant products have been reported to possess anticancer activity. Similarly, many other plants and their products have yet to be explored and are currently under investigation. It has been reported that diverse classes of natural compounds play a role in inducing non-canonical cell death pathway that may have therapeutic value to patients suffering from cancer disease (Bishayee & Sethi, 2016; Diederich & Cerella, 2016). Some of the plants reported with anticancer activity as presented in Table 2.1.

Table 2.1: Plants with cytotoxicity activity against various cancer cell lines

Plant Species	Part Used	Cancer cell lines	Reference
Salvia miltiorrhiza	Roots	MCF7, ZR-71-1, SK-BR-3	(Wang et al., 2004)
Marila pluricostata	Leaves	MCF7, H-480, SF-268	(Lopez-Perez et al., 2005)
Withania somnifera	Roots	AGS, MCF7, SF-268, HCT116, NCI-H460	(Subbaraju et al., 2006)
Boenninghausenia sessilicarpa	Whole plant	A549, Bel-7402, HepG2, HCT8, KB	(Yang, Tian, & Fang, 2007)
Cymbopogo flexuosus	Essential oils	HL-60	(Kumar et al., 2008)
Pereskia bleo (Kunth) DC.	Leaves	KB, MCF7, CasKi, HCT116, A549	(Malek, Shin, Wahab, & Yaacob, 2009)
Dellinea indica L.	Fruits	U937, HL60, K562	(Kumar, Mallick, Vedasiromoni, & Pal, 2010)
Schinus molle L. & *Schinus terebinthifolius*	Raddi berries essential oils	MCF7	(Bendaoud, Romdhane, Souchard, Cazaux, & Bouajila, 2010)
Mammea Americana	Stem bark	T47D, PC-3	(Du, Mandi, Jekabsons, Nagle, & Zhou, 2011)
Punica granatum L.	Fruits	MCF7	(Dikmen, Ozturk, & Ozturk, 2011)
Phyllanthus watsonii	Leaves	SKOV-3, CaSki, HT29	(Ramasamy, Wahab, Zainal Abidin, Manickam, & Zakaria, 2012)
Plucea indica (L.) Less.	Leaves & root	GBM8401, HeLa	(Cho et al., 2012)
Psolarea corylifolia	Seeds	HepG2, Hep3B	(Song, Yang, & Yuan, 2013)
Mikania hirsutissima	Leaves	U87	(Lizarte Neto et al., 2013)
Amorphophallus campanulatus (Roxb.) Bl.	Tubers	HCT-15	(Ansil, Wills, Varun, & Latha, 2014)
Stellera Chamaejasme L.	Roots	SMMC-7721, HepG2, A549, MG63, U2OS, KHOS, HCT116, HeLa, Bel-7402	(Li, Zhang, Pang, ZhengChen, & Gan, 2014; Wang et al., 2014b)
Nigella sativa	Seeds	ACHN, L929, MCF7, HepG2, HeLa, Hep-2	(Elkady, 2012; Reddy et al., 2015; Tabasi et al., 2015)

Table 2.1: Continued.

Curcuma purpurascens	Rhizomes	HepG2, HT29, MDA-MB-231, PC-3, WRL-68	(Rouhollahi et al., 2015b)
Tabernaemontana corymbosa	Leaves	MCF7, MDA-MB-468, T47D, U87MG, HT29, HCT116, A549	(Abubakar, Lim, Kam, & Loh, 2016; Chung et al., 2017)
Corydalis saxicola	Ariel parts	MGC-803, HepG2, T24, NCI-H460, Spca-2, HL-7702	(Zhang et al., 2016)
Dioscorea membranacea Pierre	Rhizomes	HepG2, KKU-M156	(Thongdeeying, Itharat, Umehara, & Ruangnoo, 2016)
Curcuma mangga	Rhizomes	HT29, CCD-18Co, MRC5	(Foo, Periasamy, Kiew, Kumar, & Malek, 2017; Hong et al., 2016)
Ruta angustifolia	Leaves	A549, HT29	(Richardson, Aminudin, & Malek, 2017; Suhaimi, Hong, & Malek, 2017)
Azadirachta indica L.	Leaves & Seeds	HeLa	(Shilpa et al., 2017)
Artemesia herba alba	Shoots	MCF7, HeLa	(Bourgou et al., 2017)
Myristica fatua hautt	Leaves	MCF7	(Fajriah et al., 2017)
Stegnosperma halimifolium (Benth 1844)	Leaves & Stems	L-929, HeLa, A549, LS-180, PC-3, RAW 264.7, M12.C3. F6	(Meneses-Sagrero et al., 2017)
Artemisia nilagirica	Leaves & stem	DLD-1, HT29, A549, MCF7	(Sahu et al., 2018)
Fruska gora	Leaves & root	MCF7, T47D, MDA-MB-231, MDA-MB-361	(Vlaisavljevic et al., 2018)
Linum usitatissimum	Root	HT29, A549, MDA-MB-231	(Safarpoor et al., 2018)
Uncaria tomentosa	Bark	HaCaT, A431, SCC011, Scc013, SCC022	(Ciani et al., 2018)
Tinospora cordifolia	Stem	A549, PC-3, SF-269, MDA-MB-435, HCT116, MCF7	(Sharma et al., 2018)
Ficus burtt-davyi	Fruit	MCF7, Caco-2	(Ogunlaja, Moodley, Singh, Baijnath, & Jonnalagadda, 2018)
Eleutherococcus senticosus	Stems	A549, HT29	(Kim et al., 2018)

2.3.4 Rutaceae

Rutaceae are flowering plants which come from rue family and belongs to the order Sapindales. It is has about 160 genera with approximately 2,070 species (Tamokou, Mbaveng, & Kuete, 2017). Rutaceae are found as woody shrubs and trees with some as herbaceous perennials. They are widely distributed worldwide and primarily found in the tropical as well as warm temperate regions. Most substantial members of this family are found in mostly semi-arid woodlands of Africa and Australia. This family contains some economically essential fruits and trees as well as several ornamental species (Tamokou et al., 2017). The Rutaceae family contains a high variety of secondary metabolites including many group sources responsible for structural diversity. Some of these secondary plant metabolites that exhibit significant biological activity with potential medicinal values include flavonoids, coumarins, alkaloids, limonoids and terpenes (Nebo et al., 2014; Tan & Luo, 2011). *Ruta angustifolia* is among the principal plants belonging to this family with claimed biological activity.

2.3.5 *Ruta angustifolia (Ra)*

Ruta angustifolia belongs to the family Rutaceae. It is commonly known as Rue in English, "Garuda" in Malay and "luru" in Vietnam. The plant is a strongly scented evergreen shrub and about 0.2 – 0.6 meters tall (Figure 2.3). It can be found in the Mediterranean region, Macaronesia and Southeast Asia (Ulubelen & Ozturk, 2006). The leaves are green to strong glaucous blue-green, and appear feathery and are either bipinnate or tripinnate. It's has yellow flower with 4 – 5 petals. The fruits have 4 – 5 lobed capsules, with numerous seeds.

Figure 2.3: *Ruta angustifolia* plant

Various classes of phytochemicals such as alkaloids and coumarins have been reported to be isolated from many *Ruta species* including *Ruta angustifolia* by several researchers (Del Castillo, Luis, & Secundino, 1984; Orlita et al., 2008; Richardson et al., 2016; Suhaimi et al., 2017; Ulubelen & Ozturk, 2006; Wu et al., 2003).

The plant and its isolated compounds is reported to posses numerous biological activity. Rutamarin isolated from *Ruta angustifolia* has earlier been reported to induced both GLUT4 translocation and expression in mice (Zhang et al., 2012b). It also exhibits antiviral activity by inhibiting topoisomerase II activity (Xu et al., 2014). Recently, Suhaimi et al. (2017); reported that rutamarin induced apoptotic cell death in HT29. Similarly, chalepin has been reported to inhibit glycerol-3-phosphate dehydrogenase activity (Pavão et al., 2002); it also inhibits hepatitis C virus replication (Wahyuni et al., 2014). Furthermore, chalepin isolated from the plant has recently been reported to induce

apoptosis and cell cycle arrest in lung cancer cell (A549) (Richardson et al., 2017; Richardson et al., 2016).

2.4 Cell cycle

The cell cycle is a mechanism that mediates cellular replication through various stages. It is a vital process during differentiation, proliferation and development of eukaryotic cells in both plants and animals. Appropriate and timely activation and inactivation of cell cycle controlling proteins is a crucial factor for regulating cell cycle progression. Thus, any alteration(s) in cell cycle events may result in cellular dysfunction or even death. Importantly, dysregulation in this mechanism has been implicated in cancers (Wang et al., 2009). In dividing cells, the DNA molecules are usually duplicated and distributed such that the two new resulting daughter cells will have an equal and complete set of genetic information. In achieving this task, a cell passes through a series of different cell cycle stages known as phases which are G_1, S, G_2 and M phases (Kinjyo, Weninger, & Hodgkin, 2015; Kleinsmith, 2006). Collectively, these four phases were tightly linked and regulated forming a complete cell cycle mechanism as illustrated (Figure 2.4).

Biochemically, in dividing cells, cell cycle is associated with main features which includes, DNA synthesis occur only in S phase, the rate of RNA synthesis was roughly constant during the cycle, except during mitosis where RNA synthesis ceases, and the rate of protein synthesis is higher during S phase and lower during mitosis (Baserga, 1968). Many biochemical events were found to occur during the cell cycle; the continues flow of the dividing cells from G1 to S phase appears to require synthesis of RNA and proteins, as well as special enzymes such thymidine kinase (Baserga, 1968). It has been reported that DNA made early in S phase is necessary for both survival of the cell and another round of DNA replication. It is also clear that DNA synthesis completely depends on proteins synthesis. Thus, inhibition of protein synthesis may eventually stop DNA synthesis with ultimate effect on the cell cycle progression. In addition, decreased

alkaline phosphatase activity was found during S phase as another biochemical event during S phase of cultured cells (Baserga, 1968). Furthermore, several biochemical events such as inhibition of transfer RNA which is necessary for proteins synthesis may result in both G2 and mitotic inhibition during cell cycle, since protein synthesis is required for preceding mitosis has been clearly indicated (Baserga, 1968).

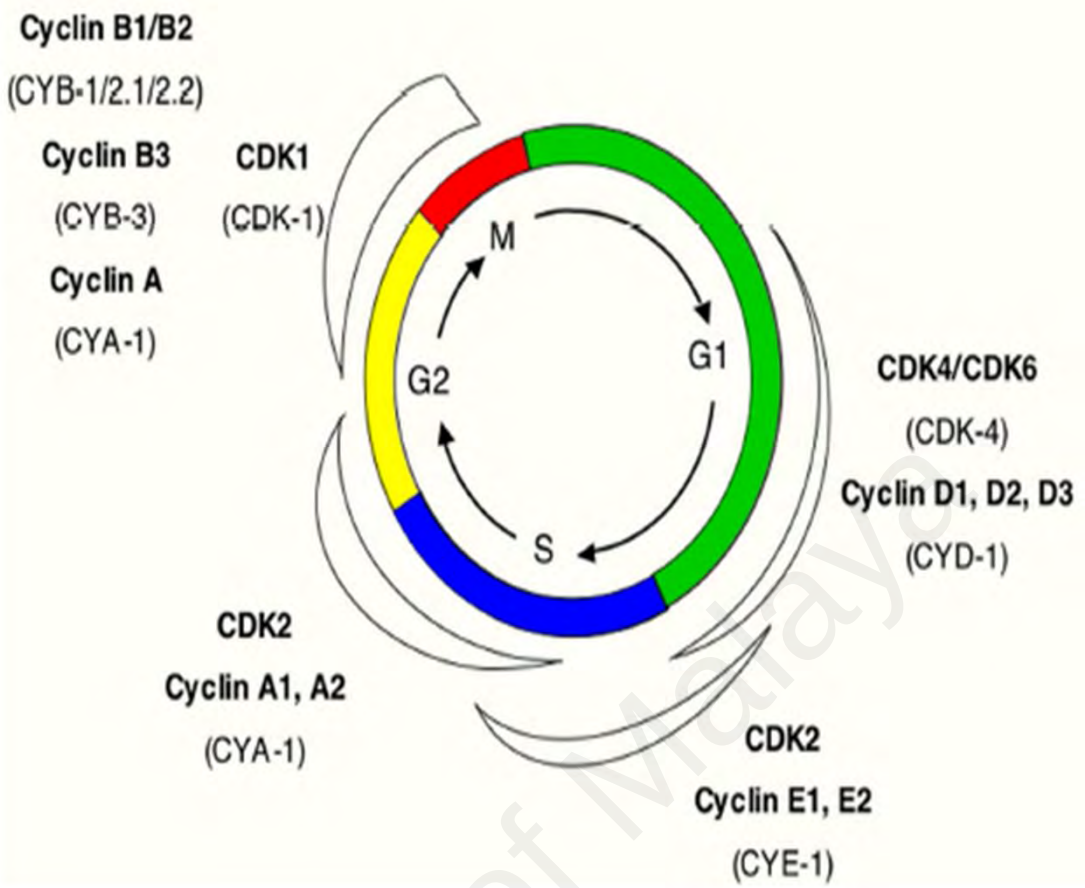


Figure 2.4: Cell cycle regulatory mechanisms. van den Heuvel, (2005) and reproduced with permission from wormbook.org.

The first phase in the cell cycle after a cell divides is the gap 1 phase (G_1-phase) which varies in length. It is the most critical period where a cell decides to proliferate or to enter a quiescent phase (G_0). In the G_1-phase the cell is committed to entering the cell cycle and prepares for DNA duplication process in the S-phase (DiPaola, 2002); it connects the end of M-phase to the beginning of S-phase at the commencement next cycle (van den Heuvel, 2005). As a phase for growth, requirements for cellular metabolism is higher in G_1-phase than the other phases. Similarly, G_1 remains the primary determinant for the whole cell cycle process since the duration of all other phases is constant. However, it was found that slow dividing cells may be arrested at G_1-phase for a more extended period or enter the G_0-phase (Kleinsmith, 2006). This situation results depending on the environmental and developmental signals causing cells in G_1-phase to withdraw from the cell cycle temporarily or permanently and then enter the G_0 (van den Heuvel, 2005).

The second phase in cell cycle mechanism is the synthesis, S-phase and is typically a DNA synthesizing phase. The replication of the chromosomal DNA also occurs in this phase and is roughly no longer than 6 – 8 hours. However, the length of time may considerably extend due to DNA damage or any hindered replication of the DNA (Kleinsmith, 2006).

The third phase is also another gap 2 phase (G_2-phase) which separates the S and M phases (van den Heuvel, 2005). In this phase, preparations for cell division is finally concluded as well as completion of the DNA replication process through the appropriate packaging of chromosomes and sister chromatids. It represents more than a passive 'gap' with continues cell growth and concomitant proteins synthesis required for mitosis (Kleinsmith, 2006).

The last phase is the mitosis phase (M-phase) which last only for a short period and is about 5% of the total duration of the cell cycle period. M-phase concludes the whole process of the cell cycle where events such as nuclear division as well as cytoplasmic

division occurred. This process leads to formation of two daughter cells by cytokinesis. Subsequently, the newly formed daughter cells enter the G_1-phase, and another cell cycle begins (Kleinsmith, 2006).

Collectively, G_1, S and G_2 referred to as interphase which is a period for cell growth, in addition to providing enough time for making copies of DNA molecules by the cells. Interphase dominates about 95% of a typical cell cycle period while mitosis (M-phase) as the primary cell division process account for only about 5% (Kleinsmith, 2006).

2.4.1 Cell cycle regulatory mechanisms

The cell cycle consists of various regulatory systems known as checkpoints. In this system, the checkpoint is a critical control point where signals to stop, and go-ahead are regulated in the cell cycle by both internal and external controls. Generally, three fundamental checkpoints exist in cell cycle mechanisms which are G_1, G_2 and M checkpoints. In most cells, the most critical checkpoint is the G_1-checkpoint, in this case, once a cell receives a signal to go-ahead in G_1-checkpoint the cycle will complete, and the cell divides. However, if there is no signal to go-ahead at this point, the cell will automatically exit the cycle which then switched to a state of non-dividing cells called G_0-phase.

Healthy cells usually decide to replicate DNA and replicate itself under the influence of extracellular signals. Once DNA replication starts, it must finish. Thus, external signals for instance growth factors (GF) does not and are not required to regulate the progression of S-phase. Following successful DNA synthesis in the S-phase, cells proceed to G_2-phase to prepare for mitosis. Importantly, control of G_2-phase does not needs growth factors, since internal signalling events such as DNA damage are checked at this time (Blagosklonny & Pardee, 2002).

Similarly, the progression of the cell cycle through mitosis is GF-independent phase instead it is controlled by signalling pathways which monitor the integrity of microtubule

functions that ensure the fidelity of chromosomes segregation is complete. Moreover, only restriction point requires GF for initiation and maintening transition via G_1-phase to S-phase, atbthis point the cell is committed to complete cell cycle with no requirement for growth factors any longer (Blagosklonny & Pardee, 2002).

Checkpoints mechanisms usually act by negative intracellular signals that arrest cell cycle, instead of positive removal of the signals which usually stimulates cell cycle progression (Alberts et al., 2002). Many proliferative and anti-proliferative signals were reported to strictly regulate the quality of cell division at specific cell cycle checkpoint. The checkpoints played a central role to ensure nuclear DNA replication is precise by details assessment of DNA damage (Thornton & Rincon, 2009). It has been demonstrated that progression of cell cycle to cell division depends on motivated activation and inactivation of cyclin-dependent kinases (CDKs) which are small serine/threonine proteins, as well as interaction with their activating cyclin subunits (DiPaola, 2002; Malumbres et al., 2009; van den Heuvel, 2005). The subsequent cell cycle transition from one phase to another was found to be triggered by cyclin-dependent kinases (CDKs) (van den Heuvel, 2005). The suppression or expression of some regulators can halt cell division via cell cycle arrest as well as induction of apoptosis resulting from persistence emergence of unrepair DNA damage (Malumbres et al., 2009).

There are four classes of cyclins found at a different stage of the cell cycle which function by binding to CDKs. These include G_1/S-cyclins that commited cell to DNA replication by binding to Cdks at the end of G_1, S-cyclins which is required to initiate DNA replication during S-phase via binding to Cdks and M-cyclins requires for promoting mitosis events. These three classes of cyclins are usually required in all eukaryotic cells. However, in most cells the fourth class G_1-cyclins usually assist in promoting passage during start or restriction point at the late G_1-phase (Alberts et al., 2002). Interestingly, researches emerge in drug discovery and development of agents

targeting various checkpoints responsible for controlling progression of cell cycle through different phases. It is also well evident that quality and rate of cell division is regulated by cell cycle checkpoints and many agents that can either increase or decrease the degree of cell cycle checkpoints are now under development (DiPaola, 2002). The control of cell cycle at various checkpoints in many cancer types has been studied and could be an essential therapeutic target in different stages of various cancers. This can be achived through regulated activities of cyclin-cdk complexes which are influenced by several mechanisms such as phospholyration of cdk subunit, the binding of special inhibitory proteins (CKIs) proteolysis of cylins, and aleterations in the transcription genes encoding Cdk regulators (Alberts et al., 2002). Other crucial component of the cell cylcle control system also include the SCF and APC complexes that induces proteolysis of specific cell cycl regulators by ubiquitalating them and triggered several critical events in the cycle. These can be achieved using agents that interfere with and downregulates the activity of Cdks, cyclins as well as Cdk/cyclins complexes at different stages of many cancer types (Alberts et al., 2002).

2.5 Apoptosis

Apoptosis is a programmed cell death which is profoundly coordinated via structured pathways in the cells. It is an important mechanism that regulates cell growth and tissue homeostasis by eliminating older, damaged and unwanted cells from the biological system (Bhatia, Mandal, Nevo, & Bishayee, 2015). In the apoptosis process, a cell destined to die activates enzymes which degrade the cell's nuclear membrane, nuclear DNA and cytoplasmic proteins. However, plasma membrane is not damaged, instead the apoptotic cell membrane becomes distorted thus making cell and its fragments susceptible to phagocytic attack (Kumar, Abbas, & Aster, 2013). Subsequently, dead cells are speedily removed before leakage of their contents. Thus inflammatory reactions are not usually elicited in the host by this pathway. In contrast, common inflammatory reactions

were observed in the host as characterised by necrosis resulting from loss in membrane integrity, enzymatic digestion of the cells and outflow of cellular contents (Kumar et al., 2013). Apoptosis and necrosis may coincide in some pathological conditions as illustrated in cellular apoptotic and necrotic pathways (Figure 2.5).

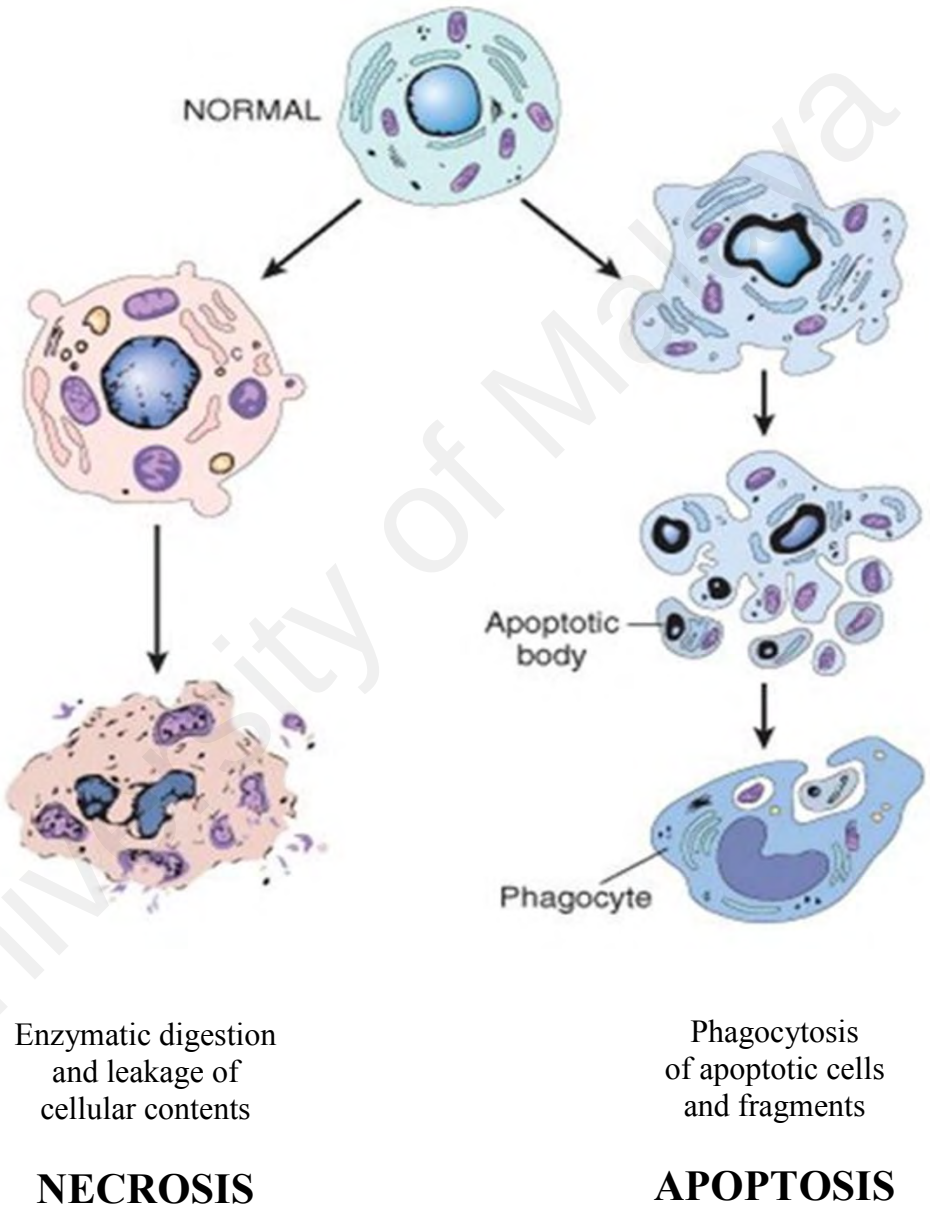


Figure 2.5: Formation of necrosis (left) and apoptosis (right) from a normal cell. Kuma et al. (2013) and reproduced with permission from S. Karger, Basel, Switzerland

Homeostasis in eukaryotic cells is strongly correlated with the equilibrium between survival and death signals originating from the external domains. Thus, cellular self-destruction processes are critical for immune regulation, organ development, tissue remodelling and many other disease conditions (Bhatia et al., 2015). However, dividing tumour cells do not initiate apoptosis even though their DNA is damaged which has been one of the silent characteristics of tumours during carcinogenesis (Thompson, 1995). Hence, effective measures for cancer control could be achievable by developing approaches to reinstate apoptosis machinery selective in tumour cells (Denmeade & Isaacs, 1996). Most anticancer drugs primarily exert their actions in neoplastic cells through apoptosis induction. Similarly, the emergence of transformed cells during carcinogenesis process results from apoptosis induction as a promising anticancer or cancer preventive properties by many natural agents (Bhatia et al., 2015; Bishayee & Darvesh, 2012; Mukhtar, Mustafa Adhami, Khan, & Mukhtar, 2012).

Apoptosis mechanisms have been studied extensively in the last two decades and caspases such as caspase-3, -8 and -9 are found to play an established crucial role in apoptosis (Sawai & Domae, 2011). The activation of caspases as the critical enzymes in apoptosis cleaves several targets leading to ultimate activation of nucleases which degrades DNA and other enzymes necessary for the destruction of nuclear and cytoskeletal proteins. This activation depends on the regulated equilibrium between pro-apoptotic and anti-apoptotic proteins in their molecular pathways (Kumar et al., 2013). Two primary mechanisms of apoptotic pathways leading to caspases activation was identified and described.

2.5.1 Intrinsic apoptotic pathway

In mitochondrial-mediated (intrinsic) mechanism, the mitochondria contain some proteins such as cytochrome c and some endogenous cytosolic antagonizing apoptotic inhibitors that are released into the cytosol to induced apoptosis (Fadeel & Orrenius,

2005; Kumar et al., 2013). The cytochrome c released usually binds Apaf-1, an adaptor molecule which then oligomerized thus forming complex with procaspase-9 known as "apoptosome" making active. The active caspase-9 then activate the execution caspase-3 which initiate the hallmarks degradation of apoptosis induction (Brentnall, Rodriguez-Menocal, De Guevara, Cepero, & Boise, 2013; Fadeel & Orrenius, 2005). However, this mechanism depends solely on the mitochondrial permeability arising from intracellular signals that initiate events in the cells causing the release of cytochrome c, AIF, Smac/DIABLO, endonuclease G, HtrA2/Omi, and CAD from intermembrane space into the cytosol (Mukhtar et al., 2012). Smac/DIABLO as well as HtrA2/Omi acts in promoting apoptosis through inhibition of the activity of inhibitors of apoptosis proteins (IAP) (Schimmer, 2004; van Loo et al., 2002). Conversely, Bcl-2 family proteins were found to regulate the mitochondrial membrane permeability thus controls and regulates mitochondrial-mediated apoptosis. These family proteins can be pro-apoptotic including Bax, Bak, Bid and Bad or anti-apoptotic such Bcl-2, Bcl-x, Bcl-xS and Bcl-xl. The balance between these proteins ultimately determines the fate of a cell. Moreover, most apoptosis situations are due to the mitochondrial apoptotic pathway (Kumar et al., 2013; Mukhtar et al., 2012) (Figure 2.6).

2.5.2 Extrinsic apoptotic pathway

In death receptor-mediated (extrinsic) mechanism, molecules known as death receptors are usually expressed on the cell surface by many cells which trigger apoptosis. These molecules mainly belong to the members of a tumour necrotic (TNF) superfamily that contains a conserved "death domain" in their cytoplasmic region (Kumar et al., 2013; Mukhtar et al., 2012). This pathway is initiated when a death ligand binds its death receptors at the cell surface forming a death domain. The intracellular death domain will then transmit signals from cell surface through the recruitment of adaptor proteins to intracellularly signalling pathways (Mukhtar et al., 2012; Wong, 2011). Several death

receptors were identified. However, the notable ones are tumour necrosis factor 1 (TNF1) and Fas (CD95) receptors and their respective ligands known as TNF and Fas ligand (FasL) (Hengartner, 2001; Kumar et al., 2013). The binding of TNF and FasL to their respective TNF1 and Fas receptors subsequently recruited the; TNF receptor-associated death domain (TRADD) adaptor as well as Fas-associated death domain (FADD) adaptor thus associating with procaspase-8 and death inducing-signalling complex (DISC) (Mukhtar et al., 2012; Wong, 2011). Consequently, caspase-8 will be activated which initiate apoptosis by activated cleavage of other downstream pro-apoptotic proteins such as Bcl-2 family (Bid), thus nourishing the mitochondrial-mediated apoptotic mechanism (Kumar et al., 2013); or activating the executioner caspases such as caspase-3 (Wong, 2011). In contrast, FLIP protein was found to binds and antagonized the activities of both caspase-8 and FADD which subsequently inhibited the death receptor-mediated apoptotic pathway (Kumar et al., 2013; Mukhtar et al., 2012) (Figure 2.6).

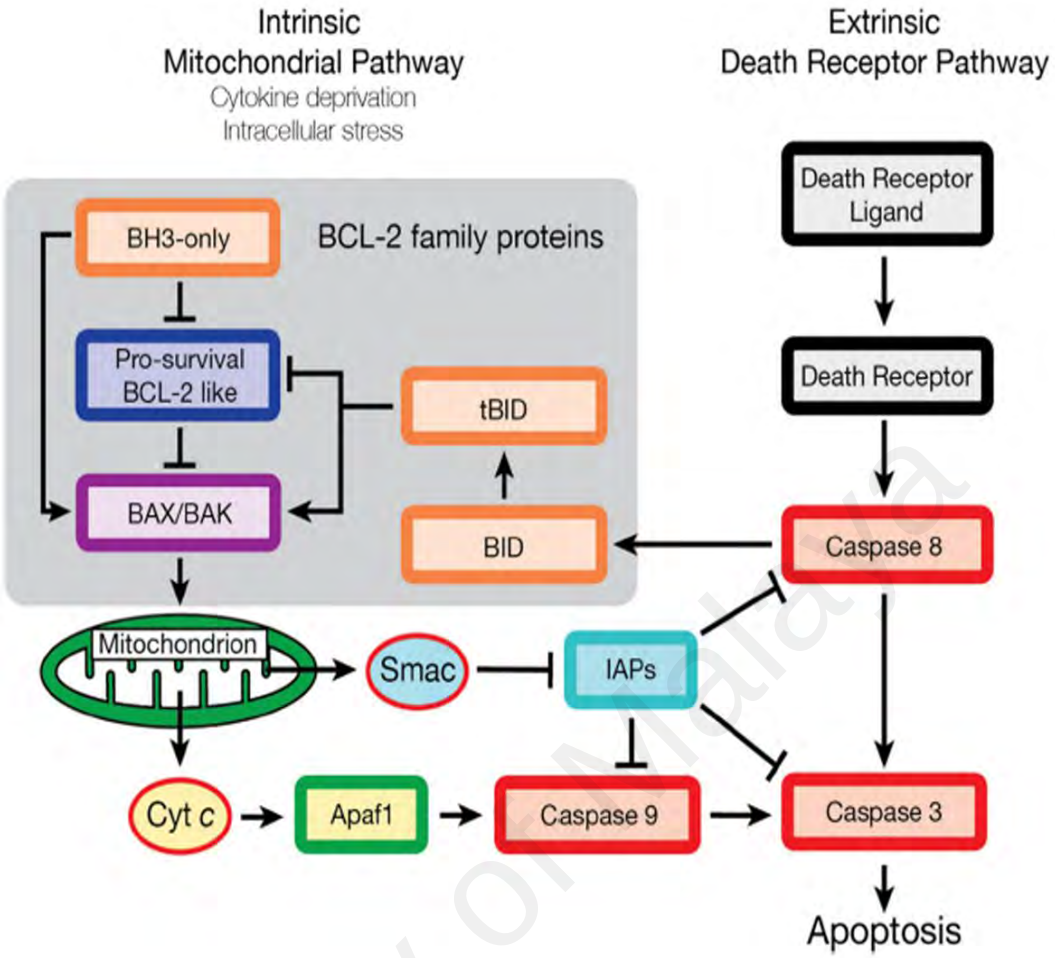


Figure 2.6: Mitochondrial and death receptor-mediated apoptotic signalling cascades. Birkinshaw and Czabotar, (2017), and reproduced with permission from Academic Press

2.6 Epidermal growth factor receptor (EGFR) signalling pathway as cancer therapeutic target

The epidermal growth factor receptor (EGFR) is a family of Erb/HER type 1 tyrosine kinase receptor and is activated upon binding by its endogenous epidermal growth factor (EGF) (Chen et al., 2016). Many cellular activities including adhesion, migration, cell proliferation and apoptosis are regulated by the transmembrane receptor known as tyrosine kinase receptors (RTKs). RTKs are reported to be overexpressed or overactivated in diverse cancer types including breast, lung, ovarian and prostate (Singh & Bast, 2015). It comprises of many protein receptors such as EGF, VEGF, IGF1, and insulin receptors (Singh & Bast, 2015). Conversely, there are 4 classes of receptor tyrosine kinases (RTKs): EGFR (ERBB1), HER2 (ERBB2), HER3 (ERBB3), and HER4 (ERBB4) with each receptor having 3 different domains: intracellular tyrosine kinase (TK), transmembrane and extracellular ligand-binding domains (Bar & Onn, 2012).

The emergence of EGFR as well as its family members as useful biomarkers and therapeutic; leads to current developments target EGFR signalling pathway which could be used for treating epithelial cancers (Seshacharyulu et al., 2012). In cancers such as non-small lung cancer cell, neck, and head, prostate, and breast; cell membrane EGFR is found to be overexpressed and over-activated (Singh & Bast, 2015). Moreover, activation of EGFR upon binding by its ligand EGF and its subsequent phosphorylation will consequently initiate signal transductions of kinase cascades. These cascades of kinases reactions activate and phosphorylate several other proteins including Erk, Akt, PI3K, and STAT3 and so on. These proteins are found in various downstream signalling pathways that are involved in cancer metastasis and were investigated in the earlier decades (Duan, Qu, & Shou, 2014). The pathways included MAPK/Erk, PI3K/Akt, and Jak/Stat pathways which played essential role in various in cancer cells during several cellular processes

such as growth, survival, metabolism, and motility (Duan et al., 2014; Singh & Bast, 2015).

Several signalling proteins, protein complexes, suppressor proteins and transcription factors associated with the EGFR downstream signalling pathways can either be activated or inhibited upon binding of EGF and other related external stimuli that initiate autophosphorylation on the EGFR. This results in activation of many other downstream Signalling pathways that brings about various cellular responses at DNA level within the nucleus including cell proliferation, survival, angiogenesis, tumorigenesis, cell cycle and apoptosis (Figure 2.7).

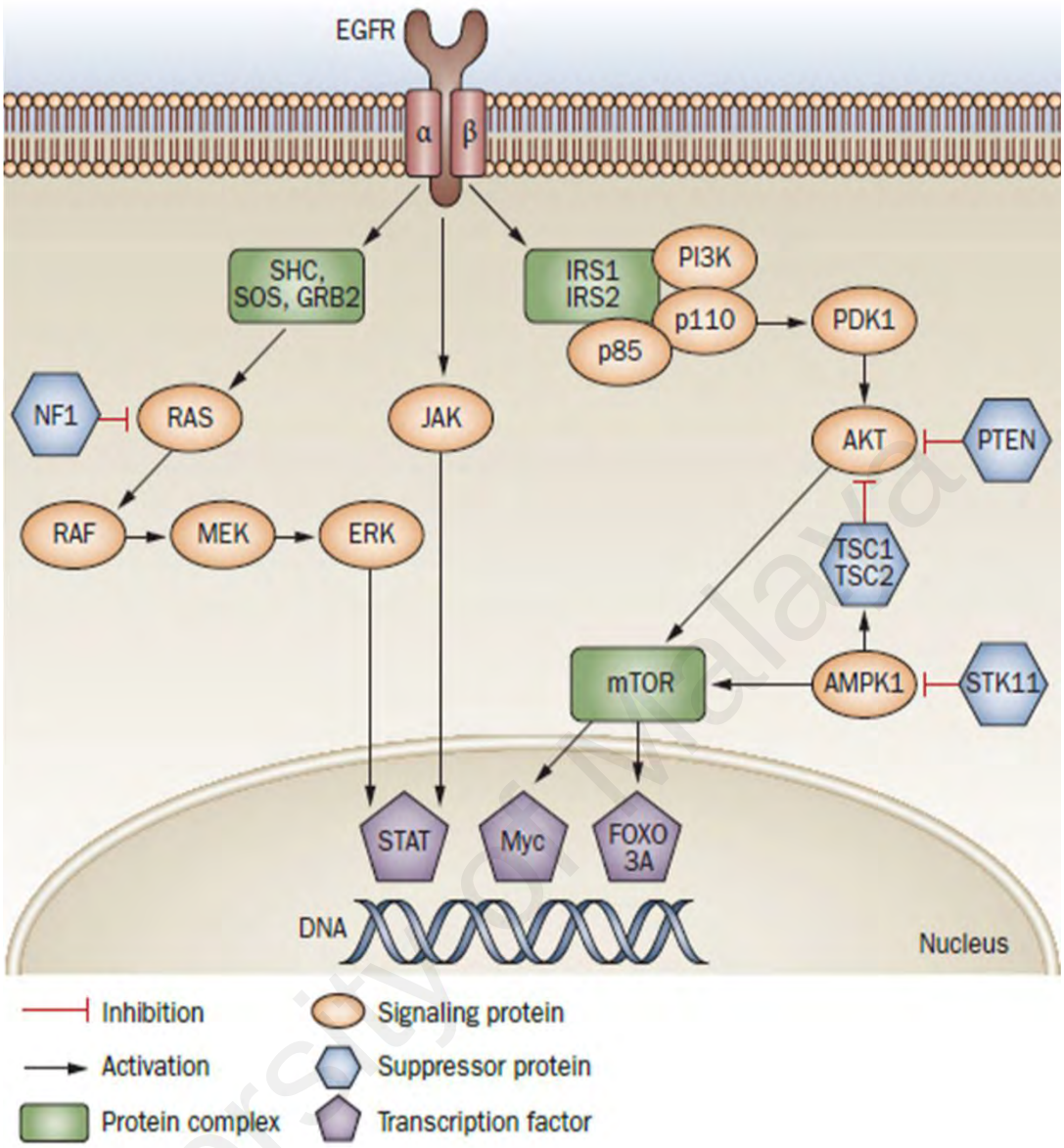


Figure 2.7: EGFR signalling and related pathways. Harris & Mcmacormick, (2010) and reproduced with permission from Nature Publishing Group

However, the development of various EGFR tyrosine kinase inhibitors becomes an essential area in the field of cancer research which encompasses diagnosis, prevention, management, treatments and prognosis of different cancer types. For instance, the use of gefitinib and erlotinib provide significant benefits to non-small lung cancer (NSCLC) patient. Though these agents show effectiveness, only subset of patient responds to therapy and those that respond developed resistance to treatment in NSCLC patients, thus limiting their clinical benefits (Bar & Onn, 2012). Interestingly, many types of research are ongoing to evaluate putative molecular mechanisms of EGFR TKIs resistance by various cancer types and to search more agents for clinical development that may address variable resistance to EGFR TKIs. Many of the novel agents developed were already undergoing clinical trial (Beck, Ismail, & Tolomeo, 2014).

In the past decade, focus on targeting EGFR emerged as an essential strategy for many cancer treatments. In line with this strategy erlotinib and gefitinib were earlier approved as the two EGFR tyrosine kinase inhibitors (TKIs) used for second-line treatments of the advanced stage in NSCLC patients. Moreover, the European Union recommended erlotinib as first-line treatment for patients with EGFR mutation-positive (Bar & Onn, 2012). Other investigation in preclinical models, shows that buparlisib in combination with mTOR inhibitors (rapamycin) synergistically inhibited NSCLC cancer growth. Similarly, combination of buparlisib and everolimus inhibited growing of lung cancer cells *in vitro* aa well as murine lung cancer xenograft models (Beck et al., 2014; Ren et al., 2012). Furthermore, phase II studies (BASALT-1 and BASALT-2) is ongoing using buparlisib, docetaxel, pemetrexed, carboplatin and paclitaxel for first-line treatment in patients having pre-treated metastatic NSCLC and activated PI3K. Several other agents such as Akt inhibitors, pictilisib, mTOR inhibitors, sirolimus, dual PI3L/mTOR inhibitors and many more were currently undergoing clinical trial at various stages of cancer and phases of the trial (Beck et al., 2014).

Despite the development and emergence of various reversible EGFR TKIs, many patients were found to develop an acquired resistance following treatments (Bar & Onn, 2012; Moerkens, Zhang, Wester, van de Water, & Meerman, 2014). It was evident that almost all patients respond to agents' treatments initially but eventually experience progression of the disease after a period, possibly due to mutation of the EGFR a mutation-positive tumour and some other unclear mechanisms (Bar & Onn, 2012; Moerkens et al., 2014).

Various signalling pathways were also implicated as a downstream target by many agents for useful EGFR targeted therapy. Bian et al. (2013) demonstrated that tumour cell death mediated by PI3K/Akt and AMPK-PRAS40-mTOR signalling pathways as integrated by P2X7. The mitogen-activated extracellular signal-regulated kinase (MEK) pathway has also been highlighted to play a role in cancer biology (Bian et al., 2013). The pathway triggers growth factors or activates key oncogenic proteins for instance Ras and Raf. It was found to play an essential role in carcinogenesis as well as maintenance of numerous cancers including colorectal, pancreatic, lung, melanoma and breast cancers (Bian et al., 2013).

Interestingly, novel therapy targeting this MEK using MEK inhibitors (MEKi) is already on clinical trials where three models (Ras-Erk driven tumours, BRaf-mutant and KRas-mutant) were proposed to be the targets for MEKi with consideration as a potential target in cancer treatment (Neuzillet et al., 2014). Similarly, the Jak/Stat pathway has been reported to be a critical downstream pathway in EGFR signalling. Moreover, EGFR-STAT3 signally pathway has been activated in bisphenol treated MCF7 breast cancer cells. Thus, inhibiting STAT3 could eventually deactivate EGFR-STAT3 signally pathway which is an essential therapeutic mechanism in carcinogenesis (Zhang et al., 2012a).

2.7 Proteomic approach in cancer research and therapy

Studies on the human genome usually measure the sequence and copy number changes in genes expression which can provide significant information about biomarkers, therapeutic targets and mechanism of cancer development. However, more profound understanding about cancer can only be provided by investigation of protein products since protein expressions indicate strong regulation of protein levels and function such as translation and post-translational modifications.

Proteomic approaches widely encompass elucidation of protein function which might involve the study of protein-protein interactions or identification of the complexes protein components. However, proteins identification does not provide adequate information to evaluate the protein pathway but remains the first necessary step in such strategies (Steen & Pandey, 2002). Proteome project provides an overview of genes associated with the various human disease. However, the understanding of some genes functions is usually incomplete due to lack of experimental evidence at proteins level (Legrain et al., 2011). Noteworthy, understanding the gene function through studies on the proteome derived from the translation of all protein-coding genes by a cell, tissue or organism could provide a significant understanding of a protein at the cellular level.

Protein is implicated in almost all cellular biological functions, as such they reflect the actual status of the cells. They function as a catalyst in the intermediary metabolism during biochemical reactions and are involved in integration as well as processing signalling stimuli from both internal and external cellular environments (Schmidt et al., 2014).

By understanding the biological mechanism and pharmacological effect of drugs, proteomics becomes valuable means of enhancing drug development process, thus making it more efficient clinical designs (Savino, Paduano, Preianò, & Terracciano, 2012). Furthermore, detection of physiological conditions, system changes in response to

external stimuli, mutations and adaptations are all now detected by emergences of proteomics. Many studies conducted on cancer patients using biospecimens also support the understanding and monitoring of pathogenesis and detection of novel targets for cancer treatment (Hudler, Kocevar, & Komel, 2014; Jin et al., 2018; Kammers et al., 2015; Sallam, 2015). Interestingly, several potential tumour-associated protein biomarkers were discovered by proteomic approach especially from serum and urine (Hudler et al., 2014). Therefore, one essential goal of proteomic technique application is to adopt this for regular diagnostic and prognostic usage in the clinical laboratories and assessment of different cancer therapeutic regimens (Sallam, 2015).

The current proteomics techniques are useful for investigation in changes of global protein profile as well as protein alteration as potential drug targets. Similarly, the identification of post-translation modifications and sequence variants are also possible by using this technique (Zhang & Ge, 2011). Many analytical tools for proteomic analysis already developed which includes 1-dimensional and 2-dimensional gel electrophoresis, as well as mass spectrometry-based application such as LC-MS/MS and MALDI-TOF that allows qualitative and quantitative analysis of the whole proteome in diseased related samples (Altaf Hussain & Huygens, 2012). Thus, expression of many proteins can be measured simultaneous by the current development of high-throughput technology.

Generally, bottom-up and top-down proteomic approach is currently applied in most proteomic analysis. In the top-down approach, intact proteins were analysed whereas, the bottom-up approach allows the identification of proteins based on its peptides resulting from proteolytic digestion of the intact proteins. Most of the samples analysed by top-down approach use the 2-dimensional gel electrophoresis as a traditional method for this approach followed by mass spectrometric analysis of the identified proteins, while samples analysis using bottom-up approach can be analysed directly by mass spectrometry after proteolytic digestion.

However, due to some limitations of the top-down approach such as low detection limits, difficult fragmentation of large analytes, resolution problem of proteins at certain pH and protein solubility; nowadays, bottom-up has become the most useful approach for protein identification. Currently, the use of multi-dimensional LC-MS/MS coupled to orbitrap accurate mass analyser becomes more useful for proteolysis of complex proteins and its subsequent identification. These technique separate peptides using chromatography technique and then sequencing it by MS/MS fragmentation. Accordingly, MS/MS fragmentation of the selected ions from the spectral data will be used for sequencing and identification of the proteins since data-dependent acquisition could sequence peptides (Kalli, Smith, Sweredoski, & Hess, 2013; Schmidt et al., 2014). Therefore, the current development of this high-throughput technique significantly allows the simultaneous identification of many proteins in various physiological states and becomes a useful application in many diseases such as cancer.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Ruta angustifolia whole plant was purchased at a Nursery in Sungai Buloh, Selangor, Malaysia; on 15/09/2015. The plant sample was identified at the Institute of Biological Sciences, Faculty of Science, University of Malaya by a Botanist, Dr. Sugumaran Manickam where a voucher specimen (Reference no. KLU48128) was deposited at Herbarium of Rimba Ilmu, University Malaya, Kuala Lumpur, Malaysia.

3.1.2 Chemicals and reagents

Analytical and high performance liquid chromatography (HPLC) grade solvents and thin layer chromatography plates, glycerol, acetic acid glacial, formic acid, formaldehyde, β-mercaptoethanol, 2-iodoacetamide (IAA), ammonium thiosulfate, ammonium persulfate, sodium dodecyl sulfate, sodium carbonate, sodium citrate, Triton-X-100 and RNase A solution were obtained from Merck KGaA Darmstadt (Germany). Dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), glycine, urea, silver nitrate, Coomassie brilliant blue, bromophenol blue, dithiothreitol (DTT), thiourea, Tris(hydroxymethyl) aminomethane and phosphate buffer saline (PBS) were obtained from Nacalai Tesques, Inc (Japan). Sulphorhodamine B (SRB), Hoechst 33342 dye, propidium iodide (PI) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (USA). Fetal bovine serum (FBS) and sodium pyruvate were purchased from Sigma-Aldrich (USA). Cell culture media accutase, penicillin/streptomycin, amphotericin B, L-glutamine and non-essential amino acids were purchased from Nacalai Tesques, Inc (Japan). Trypsin was obtained from Promega. Annexin-V and caspase-3 assay kits were obtained from BD Bioscience. JC-1 mitochondrial membrane potential assay kit was obtained from Abcam PLC, Cambridge Science, UK, APO-BrdU TUNEL assay kit was obtained from life technologies, USA. Bradford reagent, bovine standard and PBS-T were obtained from

Biorad Laboratories, Inc, USA. RIPA lysis buffer and protease inhibitors were from Thermo Scientific. Other chemicals such as antibodies against β-actin, Bax, Bcl-2, and secondary antibodies were obtained from Pierce, USA. The anti-PARP-1 antibody was purchased from Genetex, USA. Several other primary antibodies for western blot were purchased from cell signalling technologies, Inc. USA. Similarly, the chemiluminescent detection reagents kit was obtained from advansta Inc. Bering Drive, San Jose, CA, USA.

3.1.3 Cell lines

Human hormone-dependent breast cancer (MCF7), Human non-hormone-dependent breast cancer (MDA-MB-231), human colon adenocarcinoma cancer (HT29), human colon carcinoma cancer (HCT116) and normal lung fibroblast (MRC-5) cell were obtained from American Type Culture Collection (ATCC, USA). MCF7 and MDA-MB-231 were cultured in Dulbecco's modified Eagles medium (DMEM) augmented with 10% v/v FBS, 2% v/v penicillin/streptomycin, 1% v/v amphotericin B, HT29 and HCT116 were cultured in RPMI 1640 augmented using 10% v/v FBS, 2% v/v penicillin/streptomycin, 1% v/v amphotericin B, while MRC-5 were cultured in EMEM augmented with 10% v/v FBS, 2% v/v penicillin/streptomycin, 1% v/v amphotericin B.

3.2 Methods

3.2.1 Preparations of plant material

Ruta angustifolia (Ra) Pers. aerial parts were cut, washed, dried and milled into powder (176.34 g). The powdered sample was extracted using 90% aqueous methanol for 72 hours at room temperature, to obtain a methanol extract (63.29 g; 35.89%). The methanol extract (63.29 g) was extracted further with hexane to obtained hexane-soluble fraction (1.63 g; 2.58%) and hexane insoluble residue. The hexane-insoluble residue was then partitioned between chloroform-water (1:1) yielding a chloroform-soluble extract (14.58 g; 23.04). The water fraction was then partitioned using ethyl acetate to obtained ethyl acetate-soluble extract (1.58 g; 2.50%) and water extract (45.49 g; 71.88%) (Figure

3.1). The methanol extract and other fractions of the extract (hexane, chloroform and ethyl acetate) were constituted in DMSO as the stock solutions of 40 mg/ml; considerably, final DMSO concentration was ≤ 0.5% v/v in each test well.

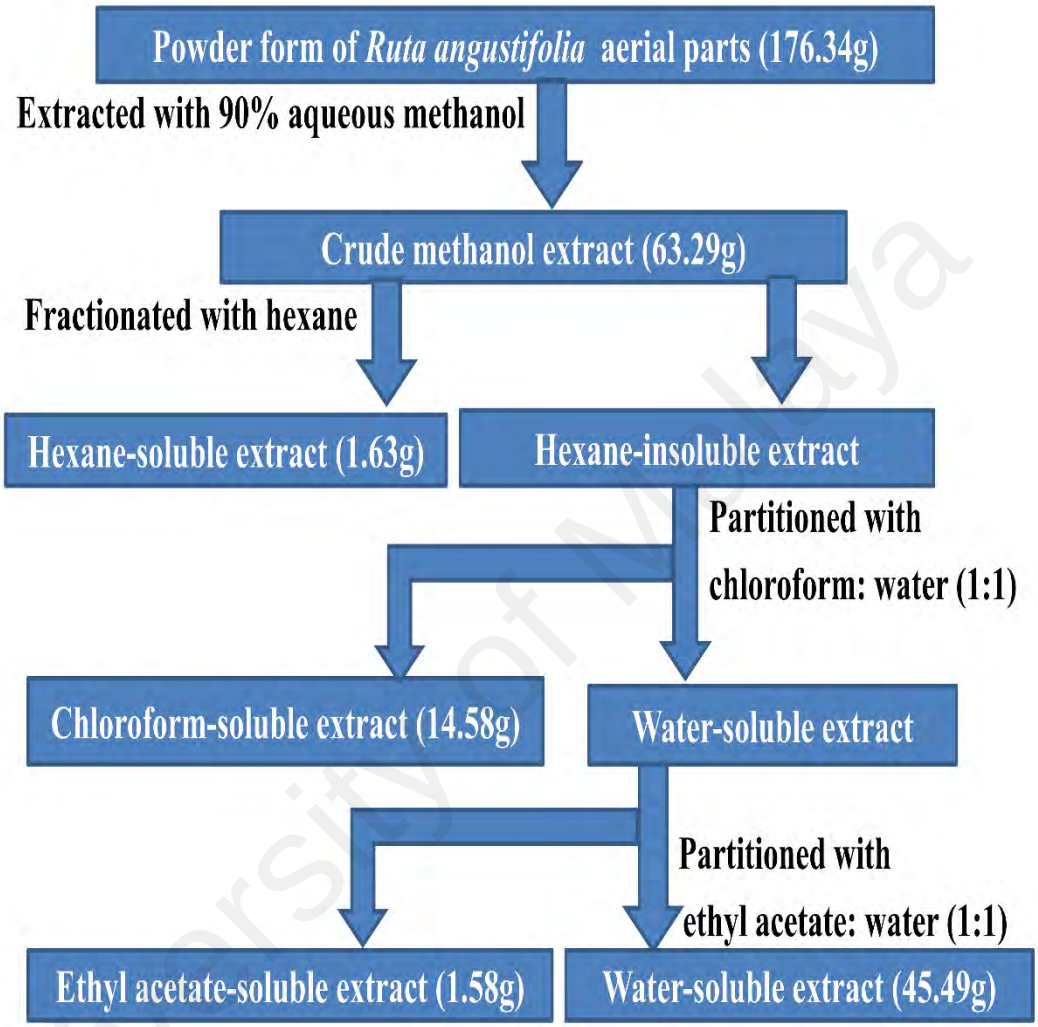


Figure 3.1: Extraction of plant material using solvents of different polarity

3.2.2 Chlorophyll free chloroform fraction preparation

A total of 10 mg of chloroform fraction was dissolved in 50 ml of chloroform followed by the addition of one teaspoon of activated charcoal and mixed thoroughly. The mixture was immediately filtered via a filter paper to obtain the chlorophyll free fraction.

3.2.3 Isolation of Pure Compounds (Chalepin and Rutamarin)

The analysis was performed by analytical HPLC using an Agilent 1260 infinity HPLC system equipped with a quaternary pump and 1260 autosampler (ALS). The system has a 1260 thermostat and thermostatted column compartment (TCC), with 1260 diode array detector (DAD VL+), and 1260 fraction collector (FC-AS). Similarly, the system has Agilent OpenLAB CDS Chemstation for LC software.

Analysis was done by means of a dual eluent system of chromatographic grade acetonitrile (ACN) and ultrapure water using gradient conditions as follows: 0 – 20 minutes isocratic 30% ACN; 20 – 25 minutes linear gradient from 30 – 60% ACN; 25 – 35 minutes linear gradient from 60 – 100% ACN; 35 – 40 minutes isocratic 100% ACN and 40 – 45 minutes isocratic 30% ACN at a flow rate 1.0 ml/min. ZORBAX Eclipse XDB-C18 (4.6 × 250 mm, 5 µm) column set at a temperature of 30^{o}C was used. The *Ruta angustifolia* chloroform fraction was filtered through activated charcoal to obtain less chlorophyll-chloroform fraction after drying filtrate using a rotary evaporator. Methanol was used to prepare 5 mg/ml concentration of the extract. The extract was then filtered using a membrane filter (0.45 µm, Sartorius). The column was then injected with 5.0 µl of the sample through which peaks were detected and monitored at 200 nm absorbance of the UV lamp. Consequently, a sample extract at 50 mg/ml concentration was prepared in methanol and filtered using a membrane filter (0.45 µm, Sartorius). Following peaks selection to be isolated, Agilent Semi-Prep XDB-C18 column (9.4 × 250 mm, 5 µm), was then injected with 50 µl of the sample at a flow rate of 5 ml/min and the fractions were collected in fraction collector.

Although several peaks were observed, only peaks unique to chalepin and rutamarin were continually collected in the fraction collector. The collected peak portions were pooled to two fractions; based on the similarity of TLC against the pre-isolated and identified compounds of chalepin and rutamarin. The mobile phase was vaporised at 40^{o}C via rotary evaporator. The weights of the dried sample fractions were then recorded. The two fractions were later analysed using analytical HPLC to determine their purity. Mass spectral and NMR data of the isolated compounds were obtained and confirmed using Gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectrometry (NMR) respectively.

The GC-MS analysis was done using Agilent Technologies 6980 N gas chromatography. The system was equipped with a 5979 Mass Selective Detector (70eV direct inlet), and capillary column HP-5 ms (5% phenyl methyl siloxane) was used; with an initial temperature set at 100^{o}C and dimension of (30.0 m × 25 mm × 25 μm). The temperature was increased to 300^{o}C at a rate of 5^{o}C per minute and then maintained for 10 minutes using a helium carrier gas at a flow rate of 1 ml/min. ChemStation auto-integration was done to obtain the total ion chromatogram, and the components were identified by comparison with the accompanying spectral database (Wiley, Mass Spectral Library, USA). The Bruker Advance III (Germany) 600 MHz NMR spectrometer was used to obtained ^{1}H and ^{12}C NMR data. Deuterated chloroform (Merck) with Tetramethylsilane (TMS) as internal standards were used to dissolve the pure compounds isolated. The NMR chromatograms data obtained were analysed using the ACD/NMR Processor Academic Edition software.

3.2.4 *In vitro* cytotoxicity assay

The cytotoxicity assay was done by adopting a sulphorhodamine (SRB) assay as described by Houghton et al. (2007) with modifications. Cells at a density of 40,000/ml for HT29, 30,000/ml for HCT116, 50,000/ml for MCF7, MDA-MB-231 and MRC-5

respectively were seeded onto a sterile 96-well flat bottom plates. The plates were incubated for 24 hours allowing adherence of the cells. The media was substituted with new media containing extract fractions (methanol, hexane, chloroform and ethyl acetate) and isolated compounds of different concentrations (1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/ml). The plates treated with extract were incubated for 72 hours, while plates treated with isolated compounds were incubated for 24, 48 and 72 hrs in 37ºC, 5% CO_2 incubator. The cells that served as negative control were not treated with extract or compound. Following each of the incubation periods, the cells were fixed by addition of 50 µl of 40% ice-cold TCA to the cells and then incubated at 4ºC for 1 hour. After that, the media containing TCA was removed, and the cells washed via deionised water. Then, 50 µl of 0.4% SRB in 1% acetic acid was then added to each well, incubated for 30 minutes at room temperature (28ºC) to stain the cells. SRB solution was later discarded, and the cells were washed with 1% v/v acetic acid before adding 10 mM Tris base to solubilise the stain. Finally, the plates were incubated for 5 minutes at 500 rpm using a microtiter plate shaker. Absorbance was then recorded at 492 nm and 620 nm as a background using microplate reader (Biotek Synergy H1 Hybrid). The percentage inhibition, as well as cell viability of each sample, was calculated using the following expressions.

$$\textbf{\% inhibition} = \frac{\textbf{Absorbance}_{\textbf{Control}} - \textbf{Absorbance}_{\textbf{Sample}}}{\textbf{Absorbance}_{\textbf{Control}}} \times \textbf{100}$$

$$\textbf{\% Cell Viability} = \frac{\textbf{Absorbance}_{\textbf{Sample}}}{\textbf{Absorbance}_{\textbf{Control}}} \textbf{ x 100}$$

The concentration of the test agent which causes 50% inhibition or cell death (IC_{50} value) was determined from dose-dependent response curves of each cell line (Appendix A). The experiment was performed in triplicates.

3.2.5 Cellular morphological changes by phase contrast microscopy

MCF7 cells with a density of 3×10^5 cells/ml were plated into sterile culture plate and then incubated overnight. Treatments were done with chalepin at concentrations of 10, 20 and 30 µg/ml and further incubated for 24 and 48 hours at 37ºC, 5% CO_2 incubator. The untreated cells served as a control. Changes in cellular morphology of the cells were observed under phase contrast microscopy at ×40 magnification (Zeiss Axio Vert. A1, Singapore).

3.2.6 Nuclear morphological changes by Hoechst 33342/PI staining fluorescence microscopy

MCF7 cells at a density of 3×10^5 cells/ml were plated into sterile culture plate and incubated overnight. Treatments were done with chalepin using concentrations of 10, 20 and 30 µg/ml and further incubated for 24 and 48 hours at 37ºC, 5% CO_2 incubator. The untreated cells served as a control. After incubation periods, both the floating and attached cells were harvested and washed using cold PBS. The cells were resuspended in media followed by addition of Hoechst 33342 solution (100 µg/ml) and then incubated for 7 minutes at 37ºC and 5% CO_2. The cells were then stained with propidium iodide (PI) (100 µg/ml); after the incubation period. Cells were further incubated at room temperature (28ºC) in the dark for 15 mins. Stained cells were then placed on a slide and covered with a coverslip. Nuclear morphology was examined under a Leica fluorescence microscope at ×40 magnification (DM6000B, Germany).

3.2.7 Detection of apoptosis by Annexin V binding using flow cytometry

MCF7 cells with a density of 5×10^5 cells/ml were plated into a culture plate and incubated overnight. Treatments were done with chalepin at concentrations of 10, 20 and 30 µg/ml and further incubated at 37ºC and 5% CO_2 for 48 hours. Untreated cells served as a negative control, while positive control cells were treated with doxorubicin. After the incubation periods, cells were harvested and washed with cold PBS. The cells were then

resuspended in Annexin V binding buffer, double stained with Annexin V/FITC and PI solutions; and incubated at room temperature (28^{o}C) in the dark for 15 minutes. BD FACS CANTO II flow cytometer was used for apoptosis detection. Quadrant statistics was used to analysed cell population distribution in different quadrants. Viable cells were represented in the lower left quadrant, early apoptotic cells in the lower right quadrant, late apoptotic/secondary necrotic cells in the upper right quadrant represents, and dead cells were represented in the upper left quadrant.

3.2.8 Evaluation of alteration in mitochondrial membrane potential (MMP) (Δψm)

Alteration in MMP was assayed with JC-1 mitochondrial membrane potential kit (Abcam) by adopting protocol as instructed in the manufacturer's manual. MCF7 cells with a density of 15000 cells/well were seeded in a sterile 96- well black culture plate and incubated overnight. The sample cells were later treated with chalepin of different concentrations (10, 20 and 30 µg/ml) for 24 and 48 at 37^{o}C and 5% CO_2; while untreated cells served as control. Four hours before completion of the treatment period, 1 mM of FCCP was added to wells containing only the cells assigned as positive control. Thirty minutes before the completion of the treatment period, JC-1 was overlaid on top of the treated and untreated cells and incubated for 15 – 30 minutes. Following incubation periods, the cell samples were washed using ×1 dilution buffer twice, and the fluorescent of the plate was read at excitation/emission of 495/590 for aggregates (Red fluorescent) and 495/530 for monomer (green fluorescent) using Fluorescent microplate reader (BioTek, Synergy H1 Hybrid). The fluorescent counts, as well as the ratio of aggregate/monomer, were obtained using the following expressions.

$$\textbf{\% Fluorescent count} = \frac{\textbf{Sample}_{(A590)}}{\textbf{Control}_{(A590)}} \times 100$$

$$\textbf{Ratio} = \frac{\textbf{Aggregate}}{\textbf{Monomer}} \times 100$$

3.2.9 Assessment of caspase-3 activity

MCF7 cells with a density of 5×10^5 cells/ml were plated into culture plate and then incubated overnight. Treatments were done with chalepin at concentrations of 10, 20 and 30 µg/ml and further incubated at 37°C, using 5% and CO_2 incubator for 24 and 48 hours. Untreated cells served as a negative control, while doxorubicin was used to treat cells serving as positive control. Following incubation periods, the cells were harvested and washed using cold PBS. The samples were later resuspended using cell lysis buffer and incubated on ice for 30 minutes. The assay was conducted by adopting the procedure described in the manufacturer's instructions manual. The amount of AMC fluorescent was read at an excitation wavelength of 380 nm with an emission wavelength of 420-460 nm using a microplate reader (BioTek, Synergy H1 Hybrid).

3.2.10 Cell cycle analysis

MCF7 cells with a density of 1×10^6 cells/ml were seeded into sterile culture plate and then incubated overnight. Treatment was done with chalepin using concentrations of 10, 20 and 30 µg/ml and further incubated for 24 and 48 hours at 37°C, 5% CO_2 incubator. The untreated cells served as a control. After the incubation periods, cells were harvested and washed using cold PBS. 70% ethanol was then used to fix the cells overnight at -20°C. The fixed cells were centrifuged, washed using ice-cold PBS and resuspended in staining solution containing 100 µg/ml RNase, 50 µg/ml PI, 0.1% Triton-X-100 and 0.1% sodium citrate. The cells were then incubated at room temperature (28°C) for 30 minutes and then analysed by BD FACS CANTO II flow cytometer. The data from flow cytometer was analysed using ModFit software (LT V3.3.11, Win).

3.2.11 Detection of DNA fragmentation by Tunnel assay using flow cytometry

MCF7 cells with a density of 1×10^6 cells/ml were plated into a culture flask and incubated overnight. Treatments were done with chalepin at concentrations of 10, 20 and 30 µg/ml and further incubated at 37°C, 5% and CO_2 for 48 and 72 hours. Untreated cells

served as a negative control, while doxorubicin was used to treat cells serving as positive control. Following incubation periods, the cells samples were then harvested, washed using cold PBS, fixed in 1% formaldehyde for 1 hour on ice and later fixed in 70% ethanol overnight to permeabilize the cells. Following fixation, DNA labelling and staining was done by adopting the manufacturer's instructions. Percentage DNA fragmentation was analysed using BD FACS CANTO II flow cytometer.

3.2.12 Protein extraction and quantification

Cells at a density of 2×10^6 cells/ml were seeded in a culture flask overnight, after which treatment was done using 20 µg/ml of chalepin on MCF7 for 6, 12, 18, 24 and 48 hours. Similarly, HT29 was treated using 10 µg/ml of rutamarin for 6, 12, 18 and 24 hours. All samples were incubated at 37°C and 5% CO_2. Afer each treatment period, cells were harvested and washed twice using cold PBS; afterwards RIPA buffer was used to lysed the cell samples (Thermo Scientific, USA). The lysate was then centrifuged using a 4°C centrifuge at 14,000 rpm for 30 minutes; after which supernatant was collected. Bradford method using Bradford reagent (Bio-Rad Laboratories, USA) was then adopted to estimate protein concentrations present in each sample. The proteins were kept at -80°C until required for analysis such as western blot or proteomics analysis.

3.2.13 Western blot analysis

Based on the estimated concentration in each sample, protein was denatured on a heating block at 100°C for 5 minutes and equal amounts of protein (50 µg) were loaded on a 12% gel for 1-D gel electrophoresis. A precision plus protein standard (Bio-Rad) was also loaded on the same gel and run together with the samples to determine the approximate size of the protein running in the electrophoretic gel. The sample was later electroblotted on a nitrocellulose membrane (Bio-Rad Laboratories, USA). Blocking one (Nacalai Tesque, Inc., USA) was then used to block the membrane and probed by specific primary antibodies (1:1000 dilutions in blocking buffer) overnight at 4°C. After overnight

incubation using specific primary antibodies, the membrane was washed three times using Tris-buffered saline containing 0.1% Tween-20 (TBST, Bio-Rad) to remove unbound antibody and then incubated with the HRP-conjugated secondary antibody (1: 10,000 dilutions in blocking buffer) at room temperature (28ºC) for 1 hour. The proteins band was captured with enhanced chemiluminescence reagent (WesternBright Sirius, Advansta) and images were caputred on Gel documentation system, FusionCapt Advance (FX-7 imaging system). Subsequently, different primary antibodies were used to reprobed the membranes after the membranes have been stripped using stripping buffer as necessary. Densitometry quantification of the band was performed using ImageJ software, and the result was presented as fold variation comparative to the control sample when normalised using β-actin as an internal standard.

3.2.14 Overview of the proteomics analysis in HT29 treated with rutumarin

Quantification of proteins abundance in the cell by detection of associated peptides using mass spectrophotometry is an essential strategy to analysed cellular proteins of various conditions which can be evaluated from diseased or healthy cells, mutant or wild-type and treated or untreated cells.

The overall protocol for the proteomics analysis on HT29 treated with rutamarin to identify proteins using LCMS and Orbitrap fusion as outlined in figure 3.2.

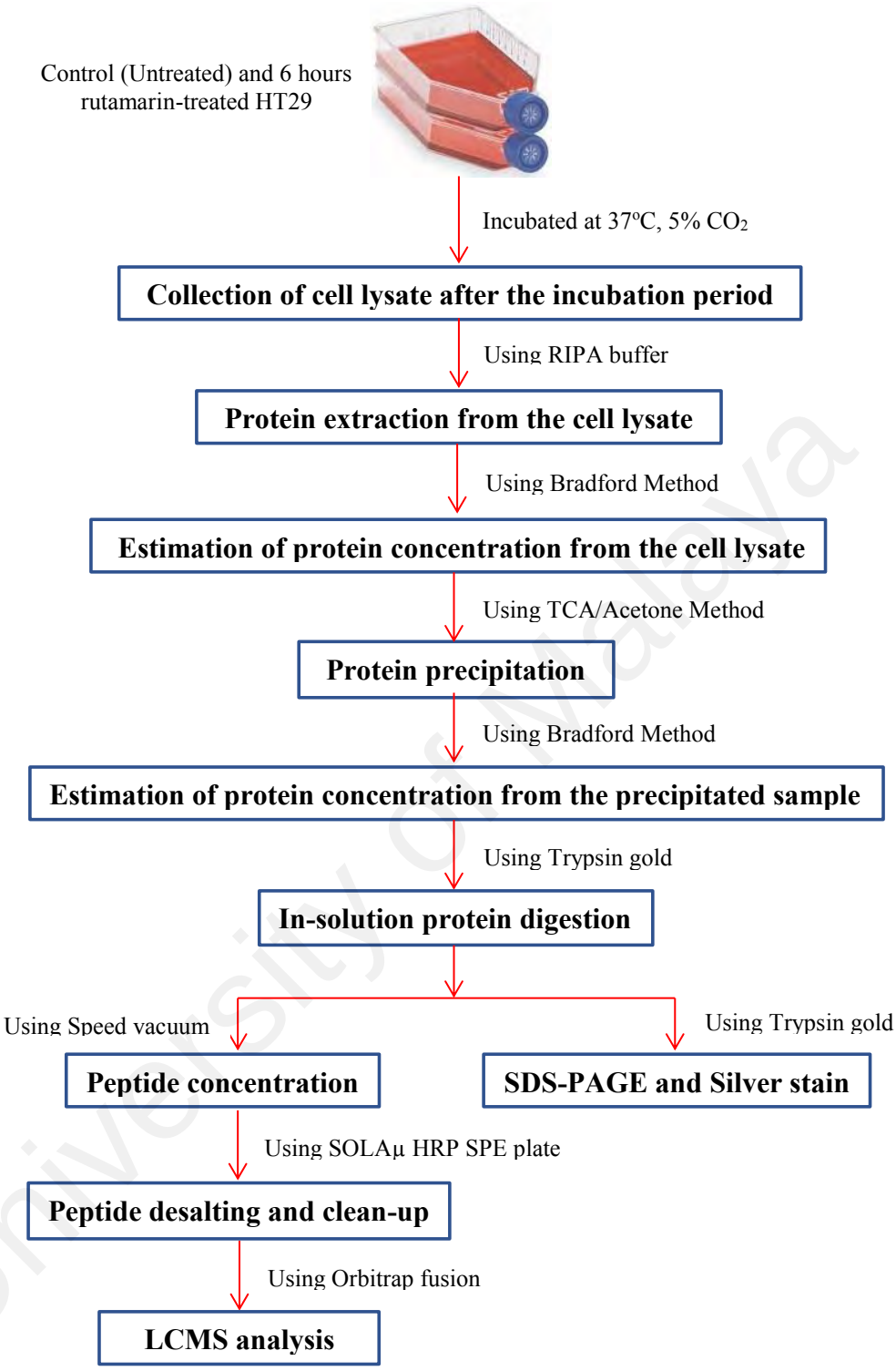


Figure 3.2: Overall protocol for proteomics analysis in HT29 treated with rutamarin

3.2.15 Protein precipitation

The method described by Duan et al. (2009) with some modifications was adopted. Briefly, 150 µg of the protein sample was precipitated by stepwise addition of 50 µl each for 3 times aliquots of frozen TCA/acetone with continued vortexing. The mixture was incubated overnight at -20ºC and then centrifuged for 15 minutes at 14000 rpm and 4ºC. The supernatant was removed while the pellet was washed twice with ice-cold acetone and centrifuged for 15 minutes at 14000 rpm and 4ºC. The supernatant was removed, whereas pellet was air dried for 3 minutes. The dried pellet was re-solubilised in 8 M urea with gentle shaking and concentrations of protein in the precipitated samples was later determined using Bradford assay. The samples were kept in -80ºC until required for further analysis.

3.2.16 Protein in-solution digestion

Protein was digested using trypsin, mass spectrophotometry grade according to the manufacturer's guide. In-solation digestion was done as described by Duan et al. (2009) with some modifications. Briefly, 100 µg of the precipitated protein sample was reduced with 100 mM dithiothreitol (DTT) to a final concentration of 10 mM, the was lightly mixture vortexed, spun and incubated for 1 hour at 37ºC. It was then alkylated with 500 mM iodoacetamide (IAA) to a final concentration of 50 mM IAA, vortexed lightly, spun and incubated at room temperature (28ºC) in the dark for 30 minutes. More 100 mM DTT was added to react with the excess IAA and 50 mM Tris-HCl (pH 8.0) was also added to reduce the concentration of urea to 0.8 M. The mixture was then vortexed lightly, spun and the pH was adjusted to 7.8 – 8.5. Trypsin was added at a ratio of 1:50 (w/w) for enzyme:substrate, the mixture was vortexed lightly, spun and incubated overnight at 37ºC. The digestion stopped following addition of 0.1% formic acid (FA) to the mixture, and this was subjected to speed vacuum and later clean-up using SOLAµ HRP SPE plate according to the manufacturer's guide.

3.2.17 Sodium Dodecyl Sulphate Polyacrylamide electrophoresis (SDS-PAGE) and Silver staining

Following digestion of the protein sample, 1 µg of the digested protein was analysed by SDS-PAGE electrophoresis, followed by silver staining to assess protein digestion.

3.2.18 Standard Nano-LC/MS-MS Analysis

The digested protein samples were analysed using Easy-nLC1000 connected to Orbitrap Fusion (Thermo Scientific, USA) for acquisition of the raw data in a data-dependent acquisition mode. The peptide samples were injected at a volume 3 µl for each sample and separated on an easy-spray column (PepMap® RSLC, C18, 2µm, 100; 75µm 500mm) (Thermo Scientific, USA). The solvents used were 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B) to achieved separation of the peptides at a gradient of 5% to 95% solvent B for 125 minutes at a flow rate 300 nL/min. Full scan of MS spectra were acquired in the orbitrap between 400 – 1600 *m/z* with resolution of 60,000 at *m/z* 400. The top 10 ions were isolated for HCD MS/MS fragmentation was done at a resolution of 7500, detected in the orbitrap having an isolation window of 1.2 *m/z* and a dynamic exclusion of 60s. The automatic gain control (AGC) target allowed accumulation up to 5×10^5 ions and activation time of 3s for full scan. The normalized collision energy of 35% and fragmented mass tolerance of 0.2 Da was used for fragmentation.

3.2.19 Protein identification and Xcalibur analysis

The raw data obtained from Orbitrap fusion was subjected to Xcalibur software to obtained the total ion chromatogram (TIC) (Thermo Scientific, USA). Subsequently, protein identification and quantification were done using Proteome Discoverer software (PD 2.1, Thermo Scientific, USA) against a UniProt homo sapiens human protein database (v. July, 2016) to identify the peptides as well as the protein groups present in each sample. The protein search was performed from SEQUEST databases with trypsin

two missed cleavage allowed. For target-decoy PSM validator, FDR strict target and the relaxed target was set at 0.01 and 0.05 respectively.

3.2.20 Proteomics data analysis using Perseus Software

Following proteins identification using PD 2.1.; the proteins were filtered to select proteins with ≥ 2 unique peptides. The resulting proteins groups obtained from PD 2.1 were analysed adopting a label-free quantification (LFQ) workflow as highlighted in the Perseus website; to obtained various visual data presentation and statistical results using Perseus software 1.6.0.7 (Max Planck Institute of Biochemistry, Germany).

3.2.21 Statistical analysis

Data were presented as a mean ± standard deviation of three (3) replicates. Statistically significant comparison between the means was determined using one-way analysis of variance (ANOVA) and Turkey *post-hoc* test at 5% confidence limit ($P<0.05$) using SPSS version 23.

CHAPTER 4: RESULTS

4.1 Extraction and fractionation of plant material

From the extraction results, water extract recorded the highest percentage yield, while ethyl acetate has the lowest percentage yield (Table 4.1).

Table 4.1: Percentage yield of *Ruta angustifolia* extract fractions

Extracts	Weight (g)	Percentage Yield (%)
Methanol	63.29	35.89
Hexane	1.63	2.58
Chloroform	14.58	23.04
Ethyl acetate	1.58	2.50
Water	45.49	71.88

4.2 Isolation and identification of the compounds of interest

Chalepin and rutamarin were the two compounds of interest for the study. The fraction **1** (retention time = **28.890)** corresponded to chalepin compound whereas fraction **2** (retention time = **32.342**) corresponded to rutamarin compound (Figure 4.1). The isolated compounds were spotted on TLC plate and both compounds showed single spot when compared with their corresponding pre-isolated and identified compounds (Figure 4.2). In addition, the relative yields of the isolated compounds were 35.30 mg/100 rounds for chalepin and 25.32 mg/100 rounds for rutamarin. GC-MS analysis identified structures of chalepin (retention time = **32.754**) and rutamarin (retention time = **34.407**) (Figure 4.3) with molecular weights of 314 and 356 respectively (Appendix A & B). Similarly, NMR analysis (Appendix C & D) further confirmed the structures of the isolated compounds with the following chemical shifts; chalepin: ^{1}H NMR (600MHz, CHLOROFORM-d) δ = 7.48 (3H, s, M12), 7.26 (3H, s, M11), 7.20 (3H, s, M10), 6.71 (3H, s, M09), 6.13 - 6.21 (3H, m, M08), 5.02 - 5.13 (6H, m, M07), 4.72 (3H, t, J = 8.8 Hz, M06), 3.19 (3H, d, J = 9.4 Hz, M05), 3.21 (3H, d, J = 8.4 Hz, M13), 2.04 (1H, s, M04), 1.47 (21H, s, M03), 1.36 (11H, s, M02), 1.11 - 1.31 (20H, m, M01). ^{13}C NMR (151MHz, CHLOROFORM-d) δ = 162.2, 160.2, 154.7, 145.6, 138.1, 130.9, 124.6, 123.2, 113.2, 112.1, 97.2, 90.9, 77.2,

77.0, 76.8, 71.7, 40.3, 29.6, 26.1, 24.2. Rutamarin: ^{1}H NMR (600MHz, CHLOROFORM-d) δ = 7.48 (3H, s, M15), 7.26 (4H, s, M14), 7.19 (3H, s, M13), 6.71 (3H, s, M12), 6.13 - 6.22 (3H, m, M11), 5.02 - 5.15 (10H, m, M10), 3.20 - 3.29 (3H, m, M09), 3.17 (3H, dd, J = 7.5 Hz, J = 1.1 Hz, M08), 1.99 (10H, s, M07), 1.65 (8H, br. s., M06), 1.56 (11H, s, M05), 1.51 (10H, s, M04), 1.47 (21H, s, M03), 1.25 (5H, br. s., M02), -0.01 (1H, br. s., M01). ^{13}C NMR (151MHz, CHLOROFORM-d) δ = 170.3, 162.4, 160.2, 154.7, 145.6, 138.0, 130.9, 123.9, 123.1, 113.1, 112.1, 97.2, 88.3, 82.2, 77.2, 77.0, 76.8, 40.3, 29.7, 26.1, 22.3, 22.0, 21.0, 0.0.

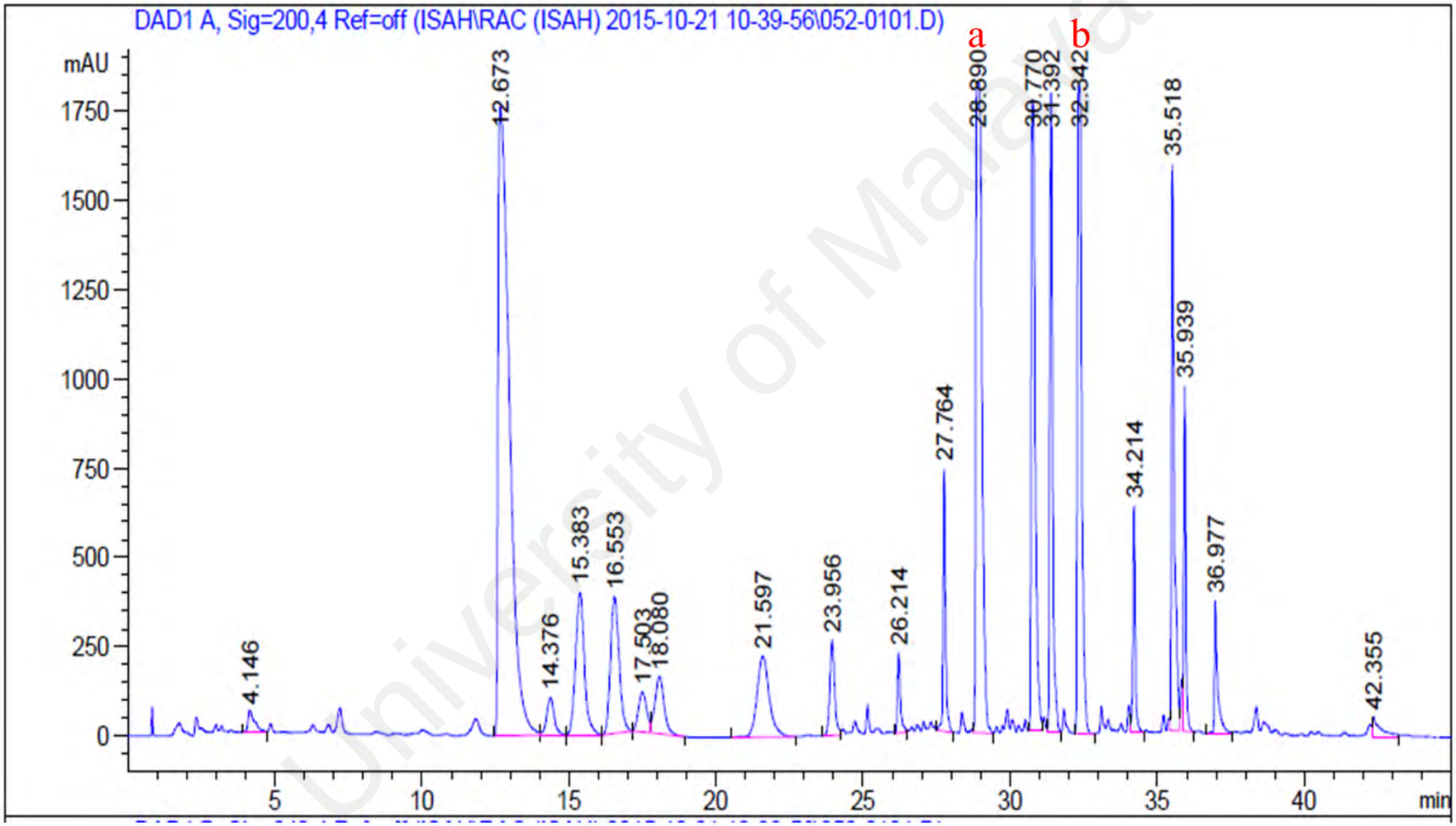


Figure 4.1: HPLC profile of charcoaled chloroform extract from *Ruta angustifolia* (Pers.) showing **(a)** Chalepin and **(b)** Rutamarin

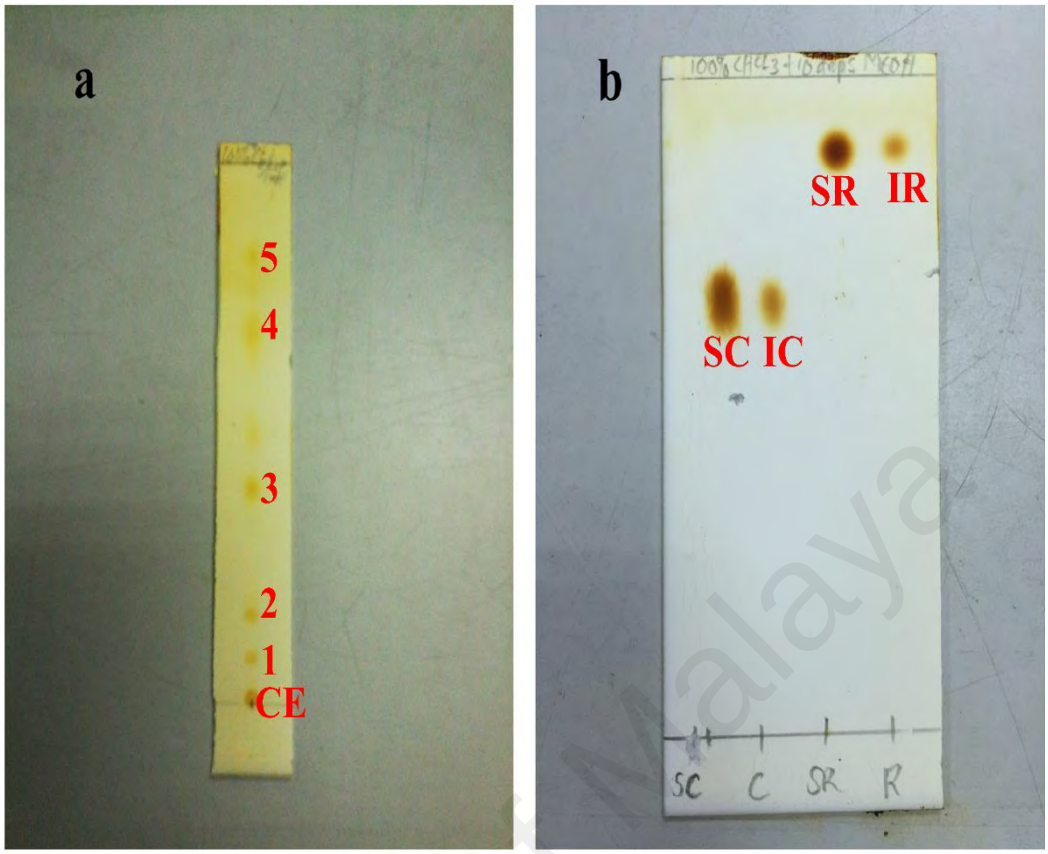


Figure 4.2: Thin layer chromatography (TLC) of **(a)** chloroform extract after charcoaled **(b)** chalepin and rutamarin spotted against their respective standards

Legend:
CE – Spot of chloroform extract, and
1, 2, 3, 4 and 5 are various unidentified compounds present in the chloroform extract on figure (a)
SC – Standard chalepin
IC – Isolated chalepin
SR – Standard rutamarin, and
IR – Isolated rutamarin on figure (b)

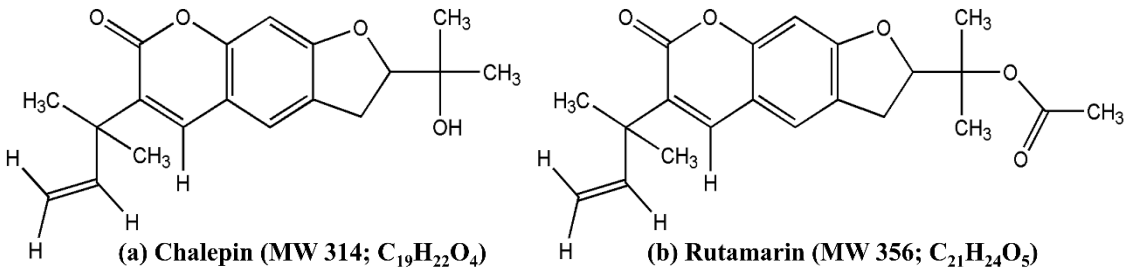


Figure 4.3: Structure of the isolated compounds

4.3 Cytotoxicity activity of methanol, hexane, chloroform and ethyl acetate extracts

The chloroform fraction showed lower IC_{50} values across all the tested cancer cell lines (Table 4.2). The percentage of cell growth inhibition increases in a dose-dependent manner across all the fractions against the tested cancer cell lines. Consequently, chloroform extract has the highest percentage cell growth inhibition whereas ethyl acetate has lowest percentage cell growth inhibition across the tested cancer cell lines (Figure 4.4). Interestingly, the growth of the normal cell was not inhibited by all the extract fractions (IC50 > 100 µg/ml).

Table 4.2: *In vitro* cytotoxicity activity of *Ruta angustifolia Pers*. extracts against selected cancer cell after 72 Hours treatment

	IC_{50} (µg/ml)				
Extracts	HT29	HCT116	MCF7	MDA-MB-231	MRC-5
Methanol	11.6 ± 0.3	61.0 ± 2.4	33.7 ± 2.4	>100	>100
Hexane	25.3 ± 1.2	32.6 ± 1.3	20.3 ± 1.6	>100	>100
Ethyl acetate	>100	>100	>100	>100	>100
Chloroform	7.5 ± 0.3	17.2 ± 1.0	12.0 ± 0.8	39.9 ± 1.7	>100
Doxorubicin	0.18 ± 0.01	0.28 ± 0.02	0.10 ± 0.00	0.07 ± 0.01	0.35 ± 0.01

Values are presented as the mean ± standard deviation of 3 replicate from 3 experiments

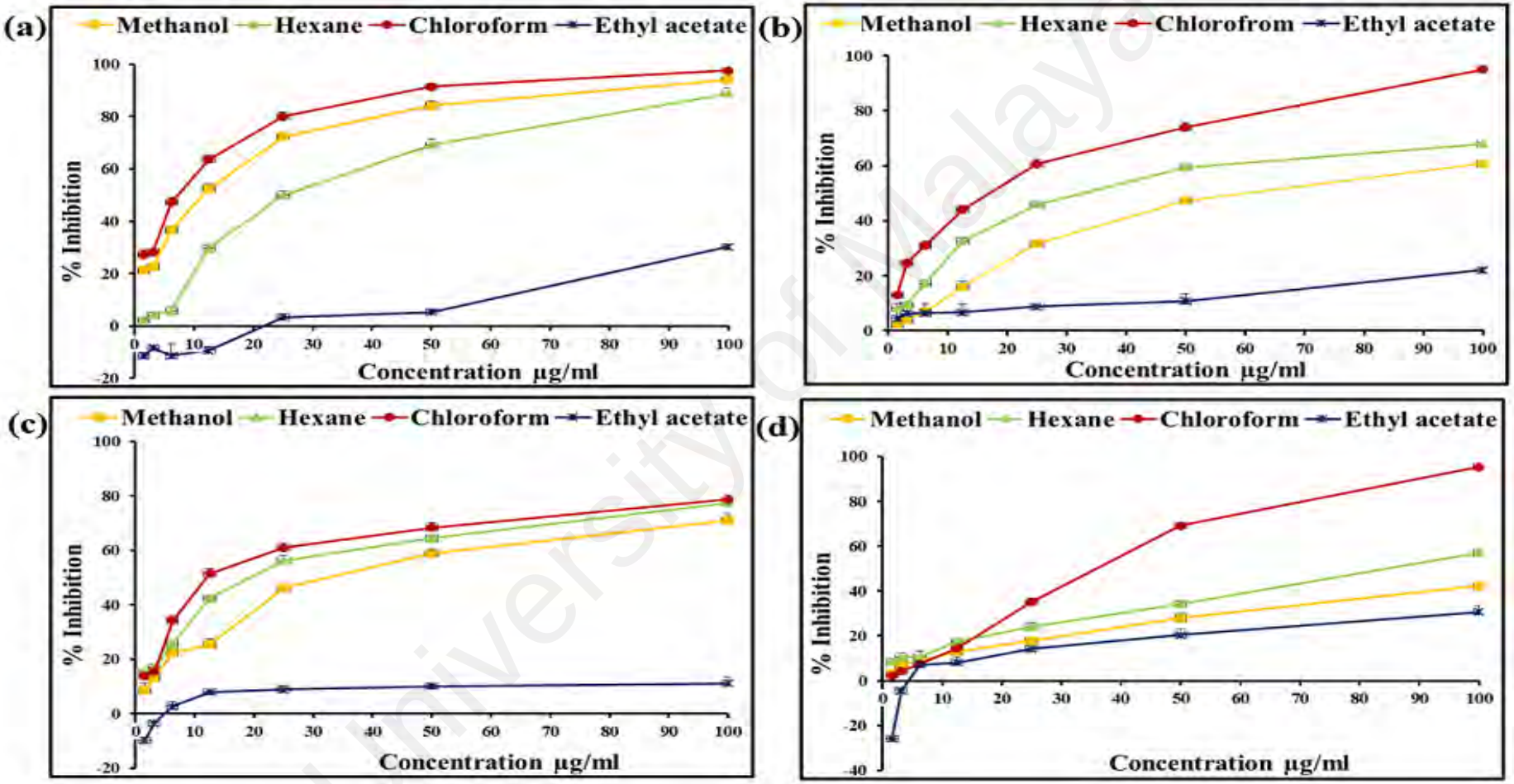


Figure 4.4: Percentage inhibition of the fractions in **(a)** HT29; **(b)** HCT116; **(c)** MCF7 and **(d)** MDA-MB231 after treatments for 72 hours. Data are presented as mean ± SD (n=3)

4.4 Cytotoxicity activity of chalepin and rutamarin

The result indicates lowest IC50 at 72 hours treatment of the compounds against the tested cancer cell lines (Table 4.3). There was also an increased percentage cell growth inhibition observed following both chalepin and rutamarin treatment across the tested cancer cell lines (Figure 4.5). Interestingly, the growth of the normal cell was not inhibited by both compounds (IC50 > 100 μg/ml).

Table 4.3: *In vitro* cytotoxic activity of Chalepin and Rutamarin isolated from *Ruta angustifolia Pers*. against selected cancer cell after 24, 48 and 72 hours incubation time

Cell lines	Time of incubation (hours)	IC_{50} (µg/ml)		
		Chalepin	Rutamarin	Doxorubicin (72 hours)
HT29	24	65.3 ± 4.0	>100	0.18 ± 0.01
	48	17.5 ± 0.1	5.1 ± 0.5	
	72	5.6 ± 0.3	2.6 ± 0.3	
HCT116	24	43.6 ± 1.1	>100	0.28 ± 0.02
	48	18.6 ± 0.9	9.9 ± 0.5	
	72	7.0 ± 0.9	3.1 ± 0.6	
MCF7	24	36.0 ± 3.3	>100	0.10 ± 0.00
	48	21.8 ± 1.9	90.2 ± 7.6	
	72	8.5 ± 1.4	4.3 ± 0.3	
MDA-MB-231	24	95.8 ± 1.3	>100	0.07 ± 0.01
	48	48.9 ± 1.0	>100	
	72	19.8 ± 0.8	>100	
MRC-5	72	>100	>100	0.35 ± 0.01

Values are presented as the mean ± standard deviation (n = 3)

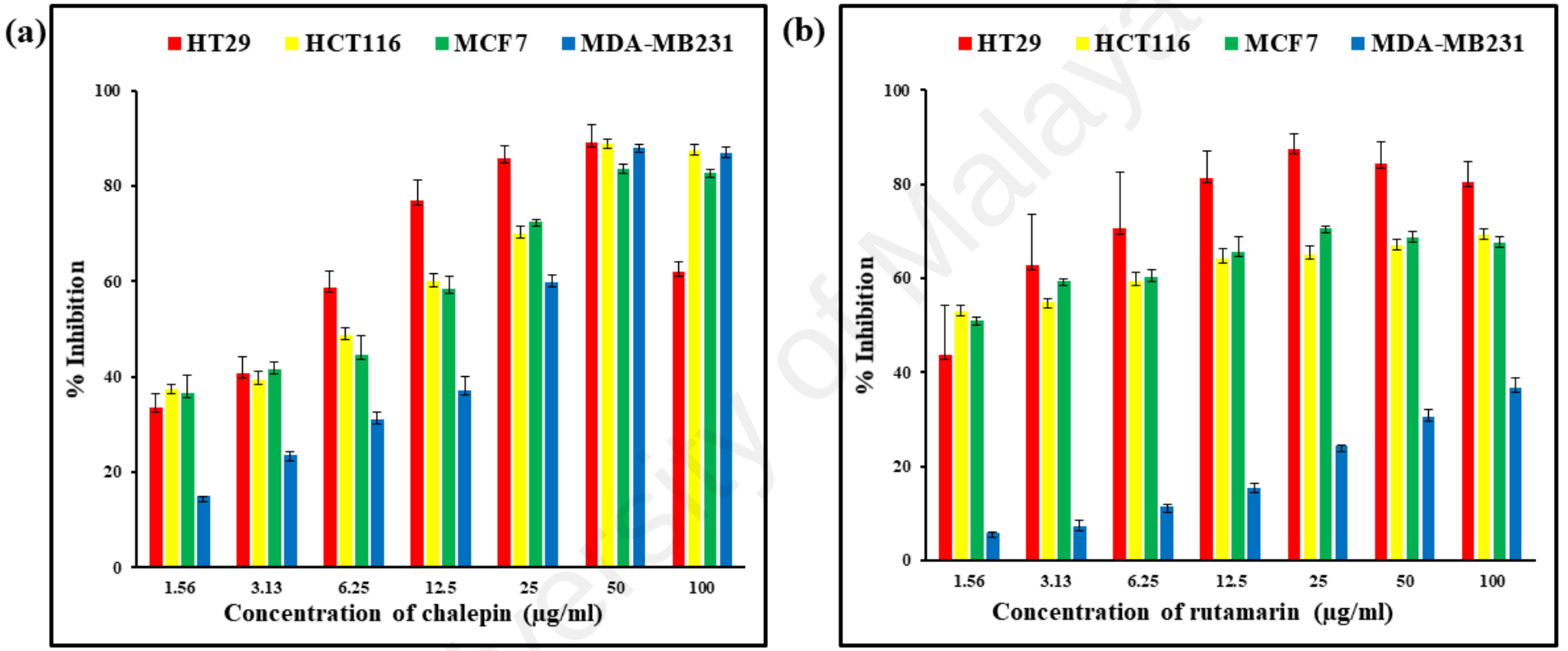


Figure 4.5: Percentage inhibition of **(a)** Chalepin and **(b)** Rutamarin against HT29, HCT116, MCF7 and MDA-MB231 after treatment for 72 hours Data are presented as mean ± SD (n=3)

4.5 Cell viability test

The results of the viability test for MCF7 treated with chalepin and HT29 treated with rutamarin were obtained from three (3) different experiments (Figure 4.6). The result indicated that cell viability decreased with increasing concentration and time point for both MCF7 treated and HT29 treated cells. However, the growth of the untreated cells from both cell lines were not affected by the compounds. Thus the cells continue to grow and multiply with increasing concentration and time point.

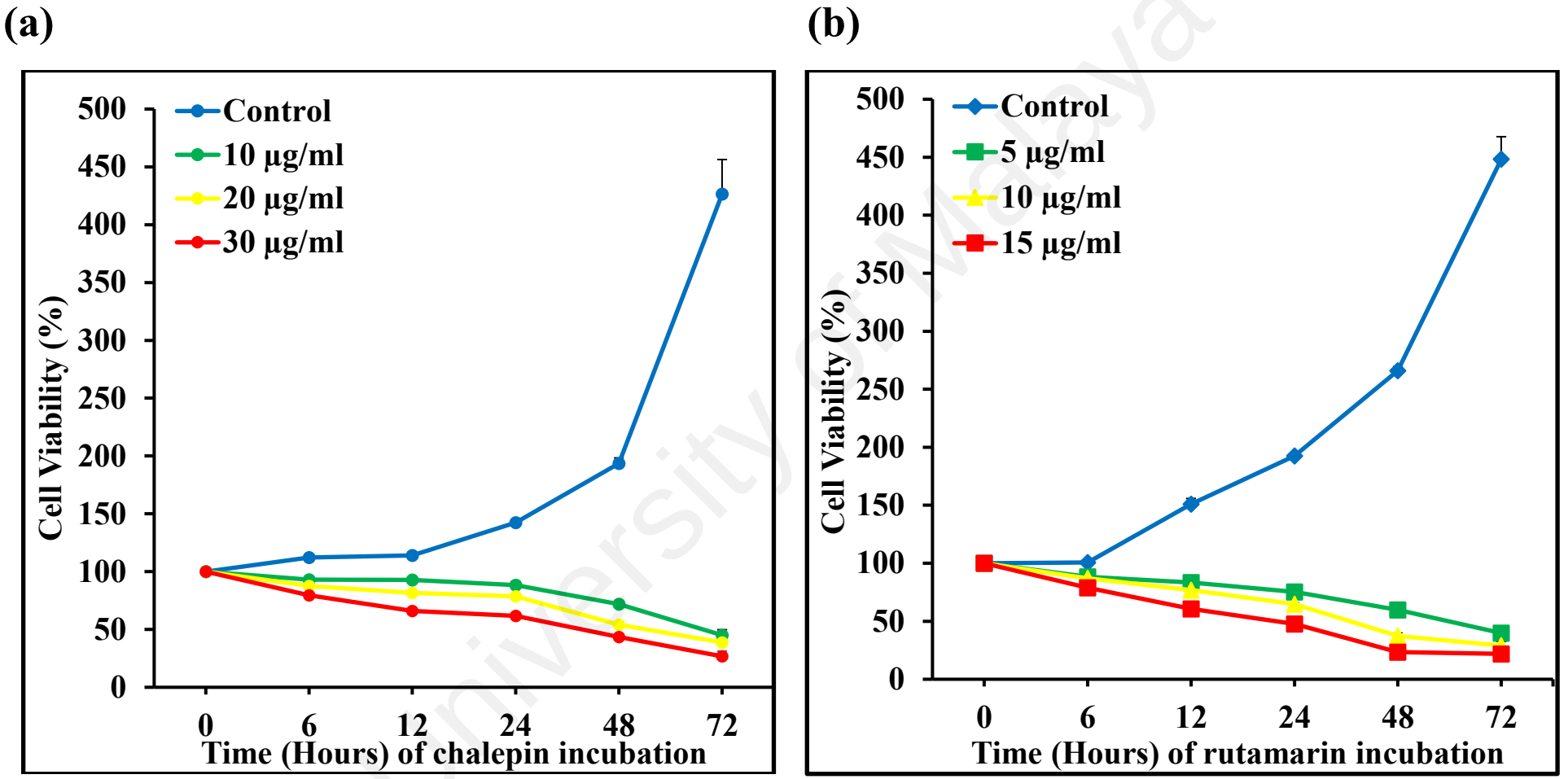


Figure 4.6: Percentage cell viability of MCF7 treated with chalepin **(a)** and HT29 treated with rutamarin **(b)**

4.6 Cellular morphological changes induced by chalepin in MCF7 observed under phase contrast microscope

Phase contrast microscopy examination indicated that chalepin treatment induced remarkable cellular morphological changes in MCF7 cells when compared with untreated cells. The result shows apparent early apoptosis features including membrane blebbing, cell shrinkage, rounding of cells, cell detachment from the surface of the plates and apoptotic bodies formation in a dose- and time-dependent pattern. In contrast, the untreated cells maintained their original cellular integrity with intact cellular membrane and adherent to culture plates (Figure 4.7).

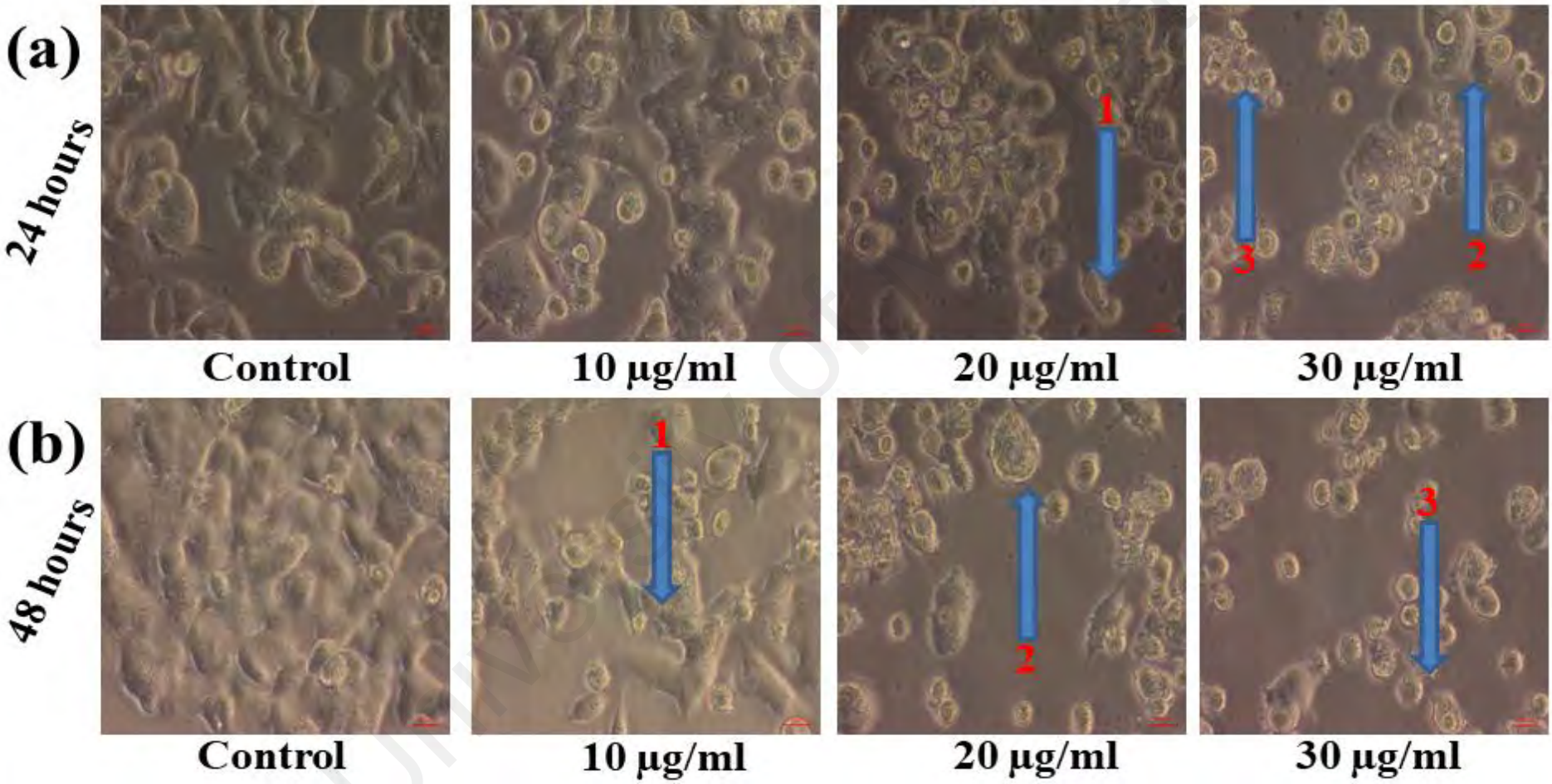


Figure 4.7: Cellular morphological changes observed under a phase contrast microscope **(×40 Magnification)** following Chalepin treatment against MCF7 at different concentration for **(a)** 24 hours and **(b)** 48 hours. Cell shrinkage **(1)**, membrane blebbing **(2)** and apoptotic bodies formation **(3)** were observed in MCF7

4.7 Nuclear morphological changes induced by chalepin in MCF7 observed under a fluorescence microscope

Fluorescent microscopy observations indicate that chalepin treatment induces significant nuclear morphological changes in MCF7 cells when compared with untreated cells as indicated by typical apoptotic features. Low blue colour intensity was observed in the untreated cells whereas a bright blue colour and pink colour with condense and fragmented DNA was observed in the treated cells. The result showed loss of viable cells, cell shrinkage, nuclear fragmentation and chromatin condensation as observed in MCF7-chalepin treated in a dose- and time-dependent pattern. In contrast, the untreated cells maintained their original nuclear integrity with intact nuclear membrane and adherent to culture plates (Figure 4.8).

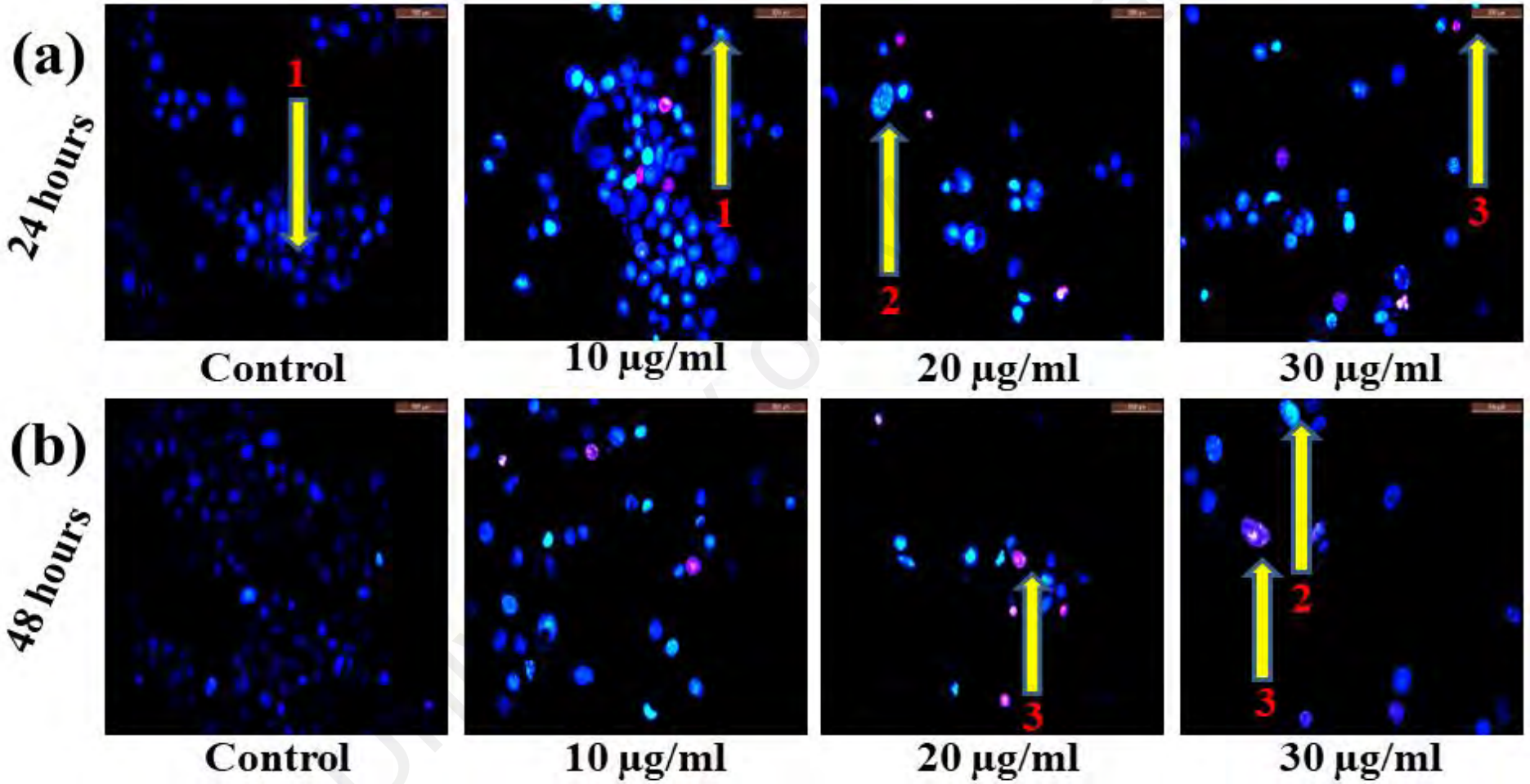


Figure 4.8: Nuclear morphological changes observed under phase fluorescence microscope **(40× Magnification)** following chalepin treatment against MCF7 at different concentration for **(a)** 24 hours and **(b)** 48 hours. Viable cells **(1)**, cells with condensed chromatin **(2)** and dead cells with fragmented DNA **(3)** are observed in MCF7

4.8 Detection of apoptosis through externalisation of phosphatidylserine induced by chalepin in MCF7

The results showed that chalepin treatment increases the number of cells that are positive to Annexin-V/FITC only, thereby increasing the density of cell population in the lower right quadrant (early apoptotic cells). It also increases the number of cells that are positive to both Annexin-V/FITC and PI, thus increasing the density of cell population in the upper right quadrant (late apoptotic/secondary necrotic cells); as the concentration of chalepin increases. In the present study, chalepin significantly ($P < 0.05$) increased the number of apoptotic cells in MCF7-treated cells when compared with the untreated cells in a dose-dependent manner as indicated by increased total Annexin-V/FITC positive cells. This observation was similar to that of the doxorubicin-treated MCF7 cells (Figure 4.9). In contrast, the untreated cells only showed low or negative staining intensity to both Annexin-V/FITC and PI with most cell population found in lower left quadrant (viable cells).

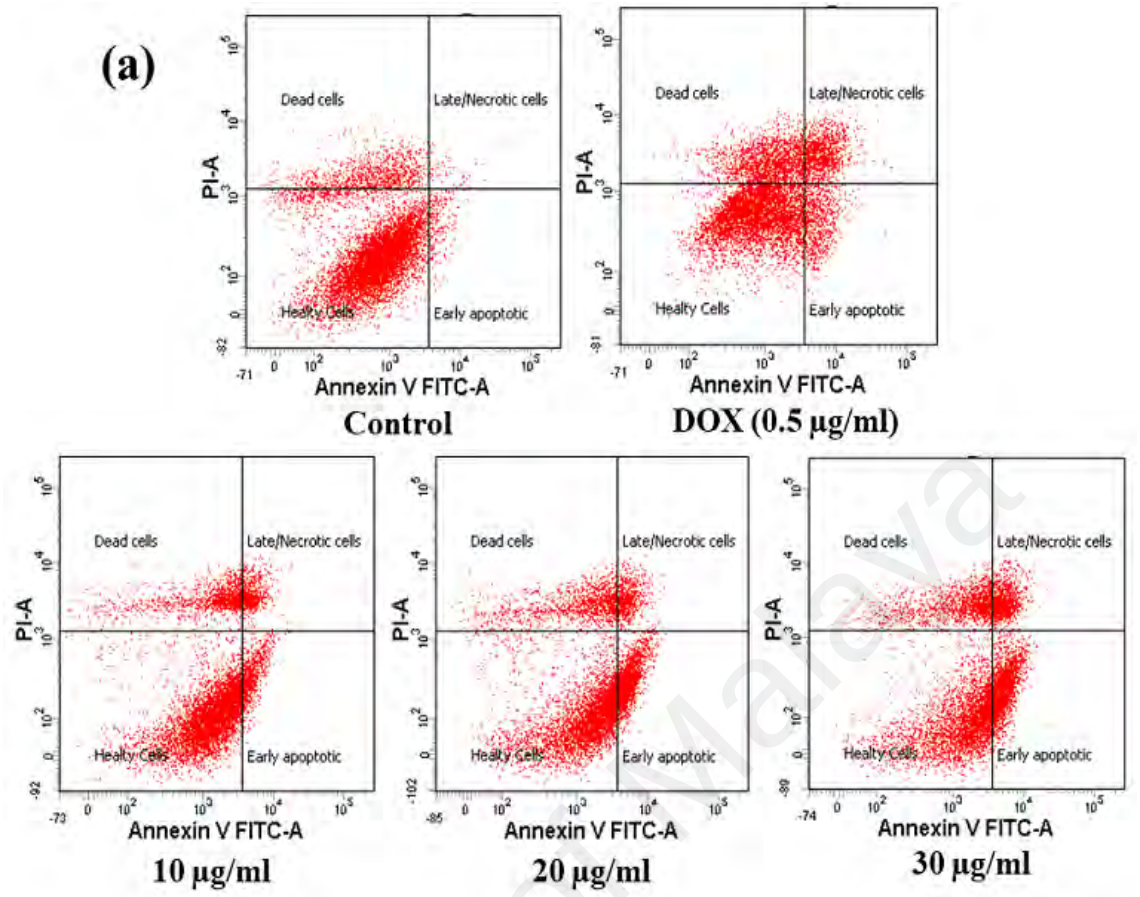
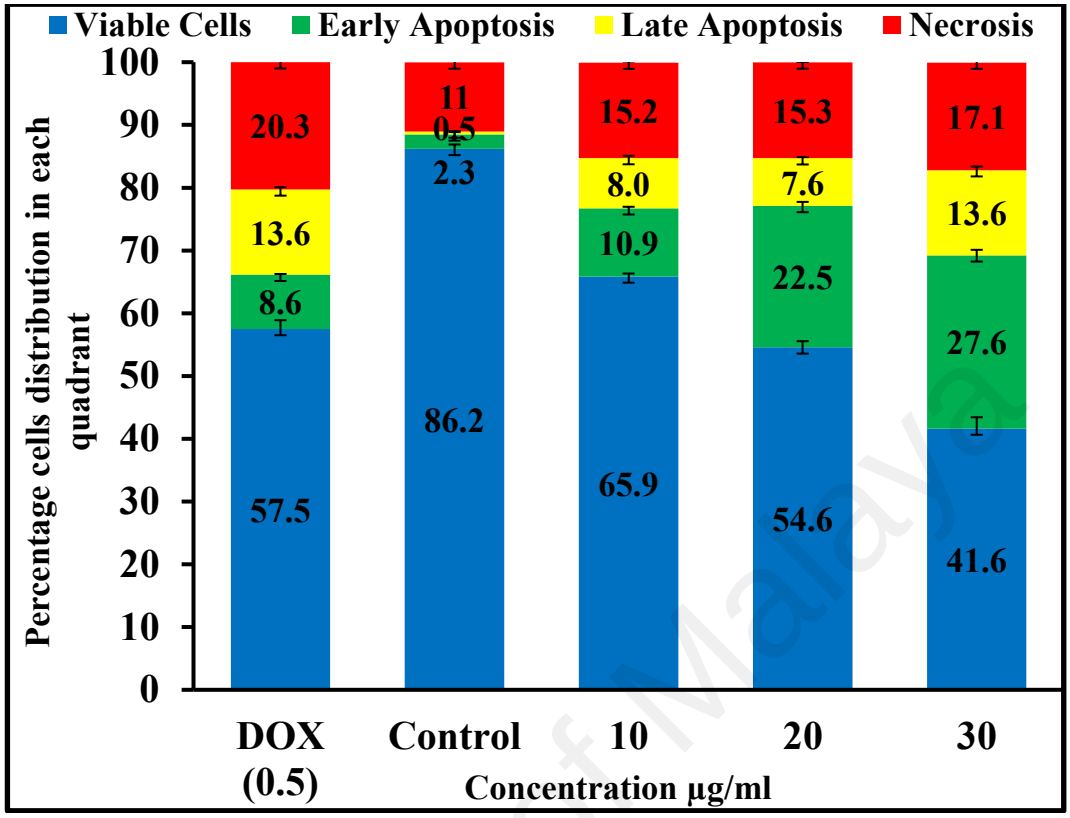


Figure 4.9: Annexin V/FITC analysis in chalepin treated MCF7**. (a)** Annexin V density plot, **(b)** Percentage cell distribution for each quadrant, and **(c)** Percentage total Annexin V positive cells; in MCF7 following treatment with Chalepin at different concentrations for 48 hours. Data are presented as mean ± SD for (n=3). Data carrying asterisk are significantly different from control ($P < 0.05$)

(b)



(c)

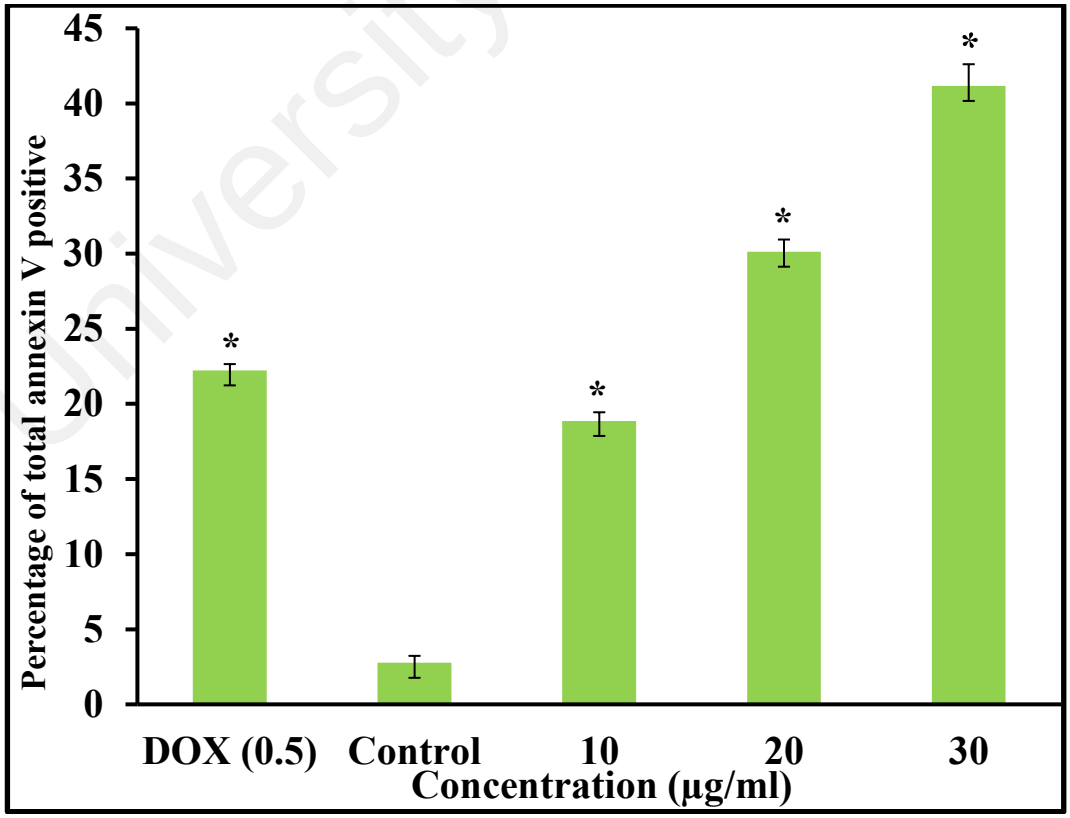


Figure 4.9, Continued.

4.9 Assesment of mitochondrial membrane potential (MMP) (Δψm) alterations induced by chalepin in MCF7

The result indicates decreased fluorescence emission intensity in chalepin-treated MCF7 in comparison to untreated cells. It also increased green fluorescence of JC-1 monomer, thus decreasing the fluorescence intensity ratio of aggregate to monomer for the treated cells in comparison to untreated cells. The result of the present study, therefore, revealed significantly decreased ($P < 0.05$) in mitochondrial membrane potential following chalepin treatment in MCF7 cells when compared with untreated cells (Figure 4.10).

(a)

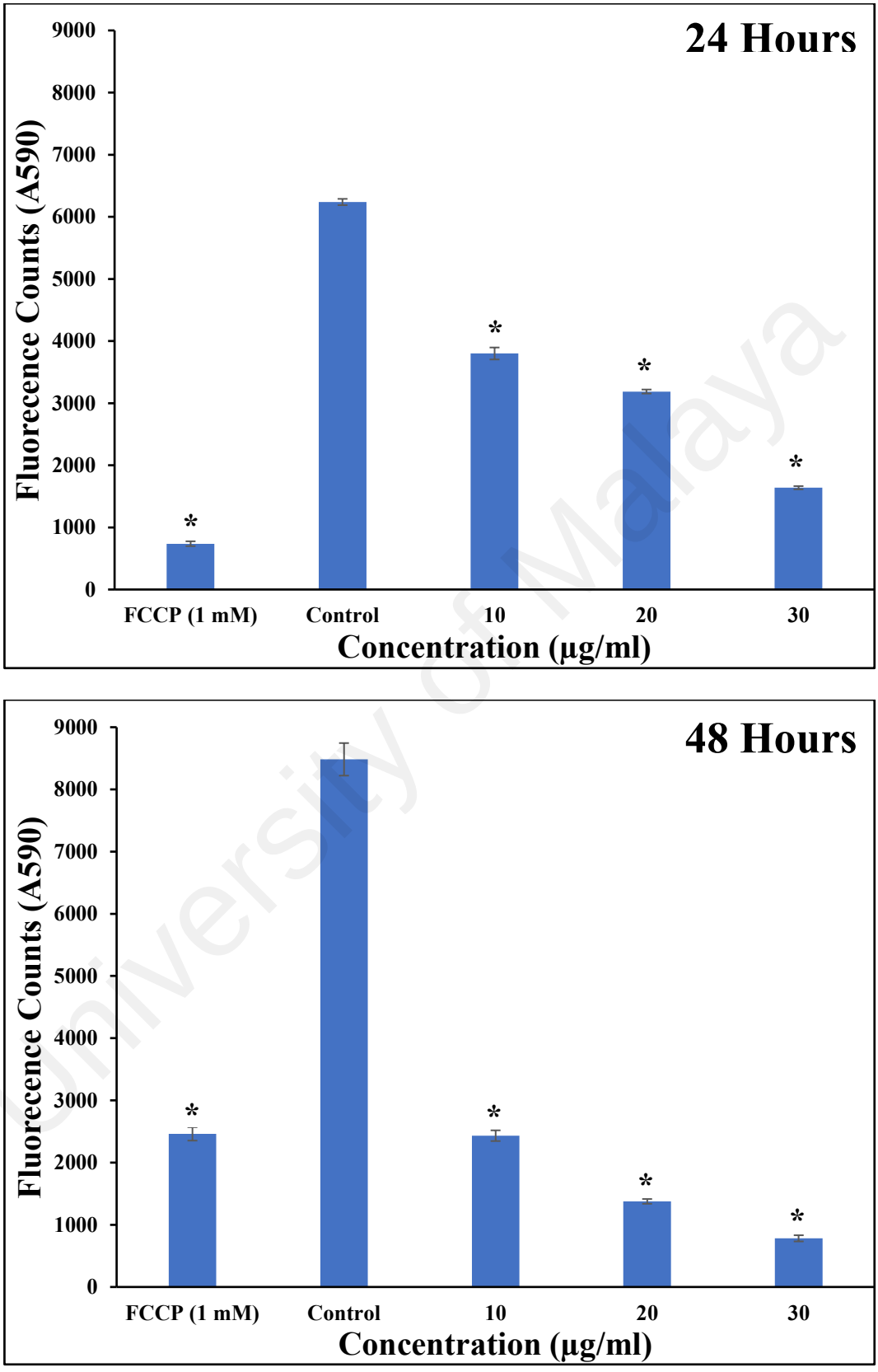
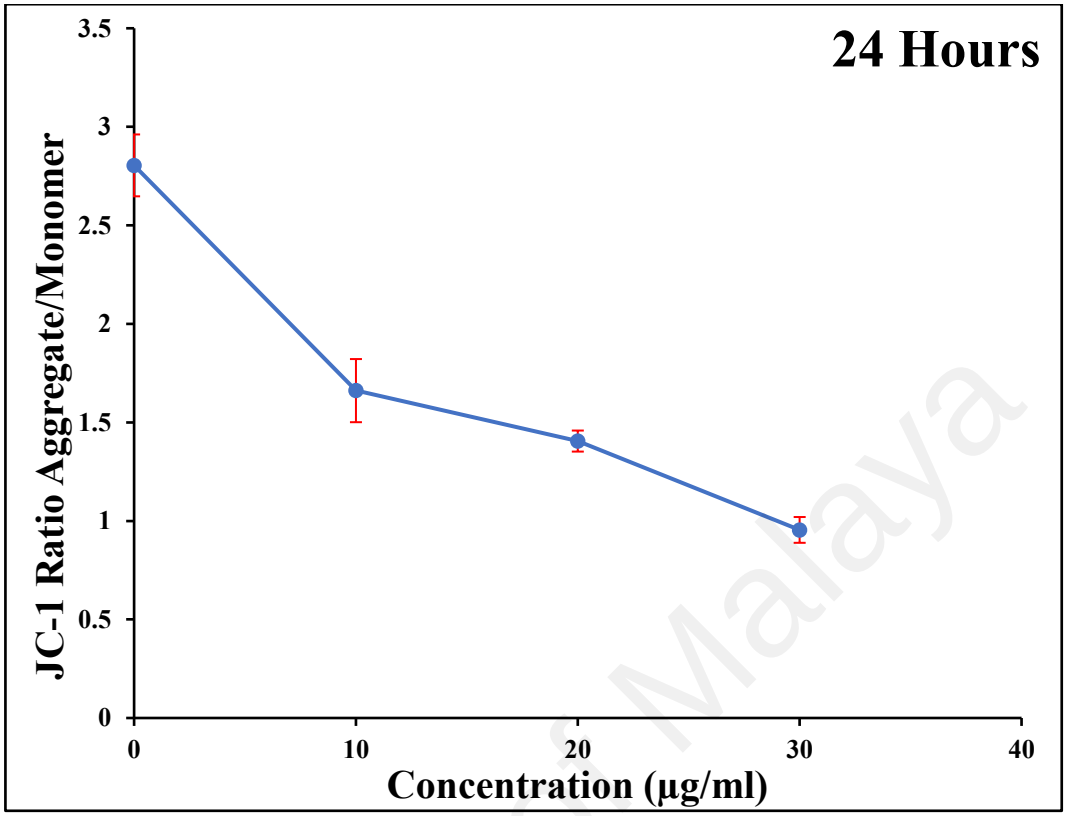


Figure 4.10: Mitochondrial membrane potential in chalepin treated MCF7**. (a)** Fluorescence count, and **(b)** JC-1 ration; in MCF7 following treatment with Chalepin at various concentrations for 24 and 48 hours. Data were presented as mean ± SD (n=3). Data carrying asterisk are significantly different from control ($P < 0.05$)

(b)



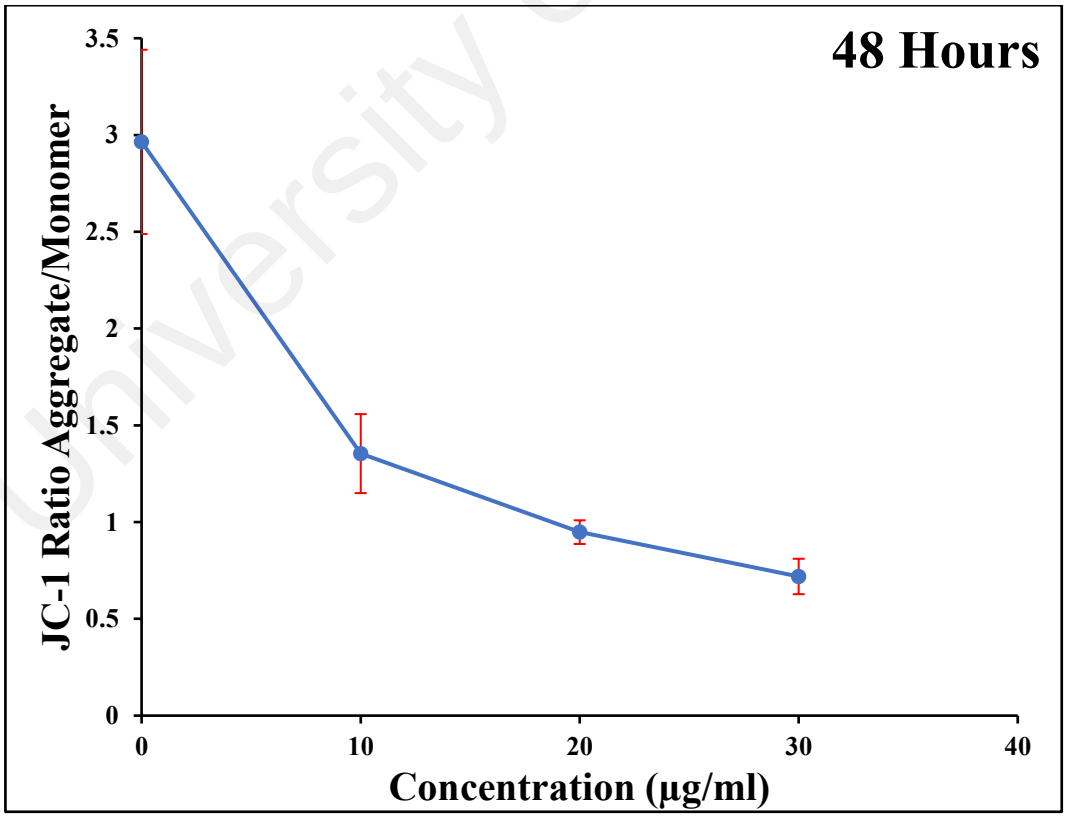


Figure 4.10, Continued.

4.10 Increased caspase-3 activity induced by chalepin in MCF7

The result indicates increased fluorescence count due to continues cleavage of the fluorogenic substrate resulted from increased caspase-3 activity in MCF7 cells undergoing apoptosis after chalepin treatments when compared with the untreated cells. Conversely, chalepin significantly ($P < 0.05$) increased caspase-3 activity (Figure 4.11) following chalepin treatment in MCF7 cells when compared with untreated cells in a dose and time-dependent manner. The pattern of increase in caspase-3 activity after treatment with chalepin was similar to that produced by the reference drug, doxorubicin in the present study.

(a)

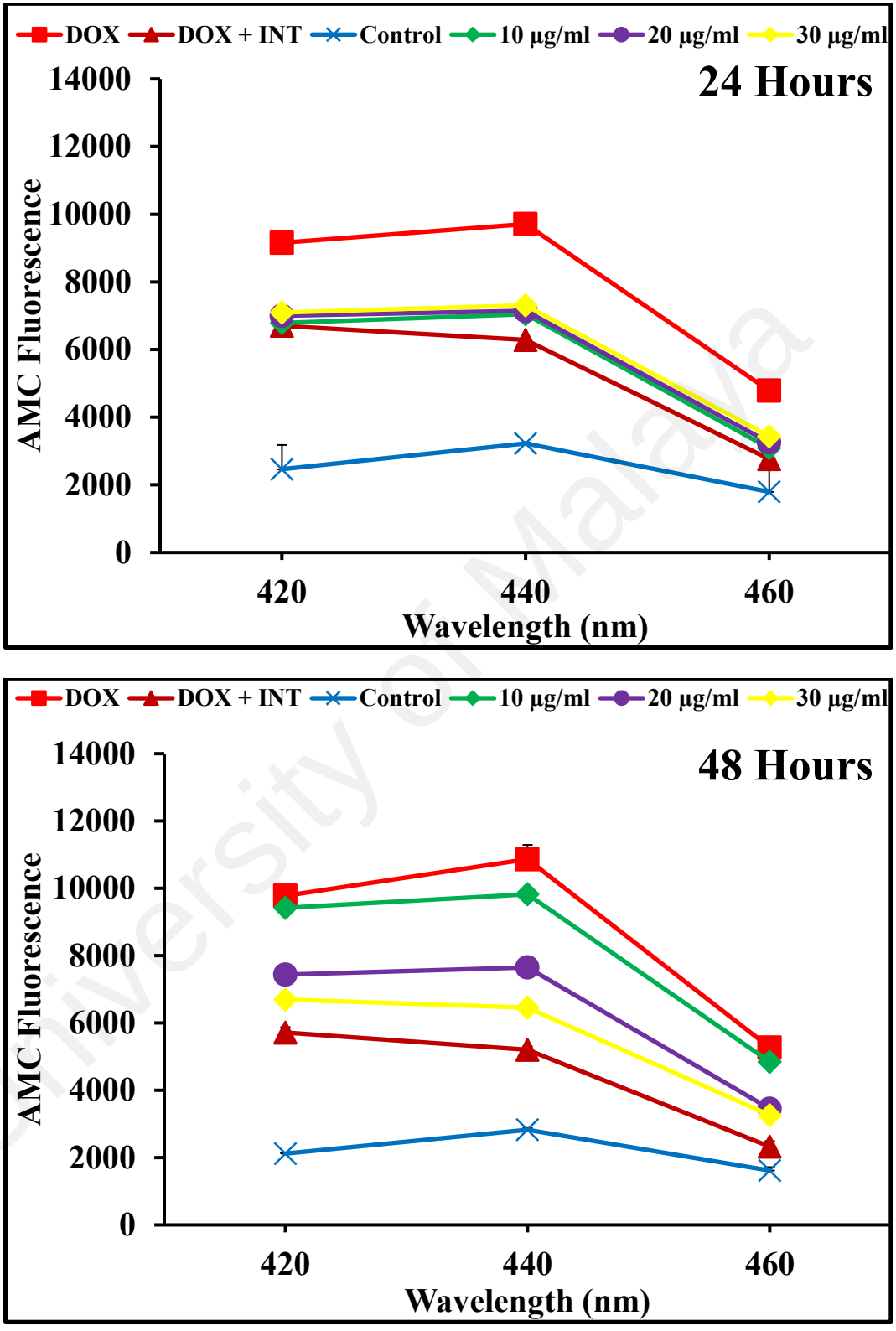
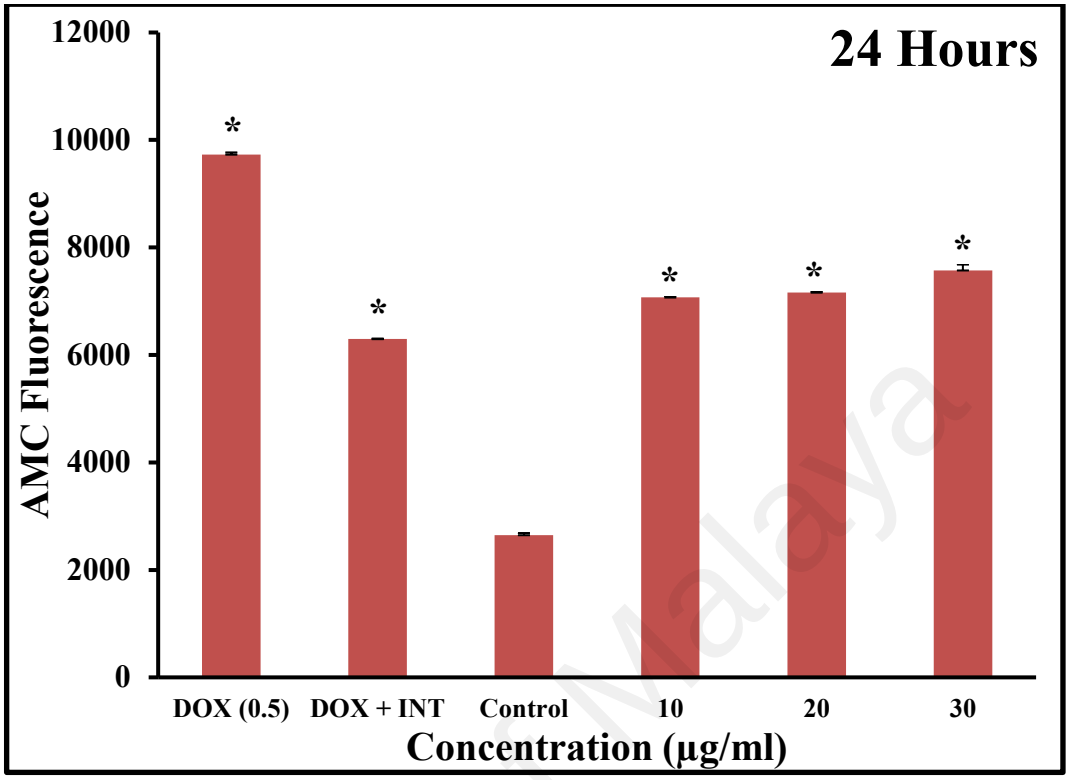


Figure 4.11: Cacspase-3 activity analysis in chalepin treated MCF7. **(a)** Caspase-3 substrate-fluorescence count, and **(b)** Increased caspase-3 activity, in MCF7 following treatment with Chalepin at different concentrations for 24 and 48 hours. Data were measured at excitation of 380 and emission of 420, 440 and 460 nm and presented as mean ± SD (n=3). Data carrying asterisk are significantly different from control ($P<0.05$)

(b)



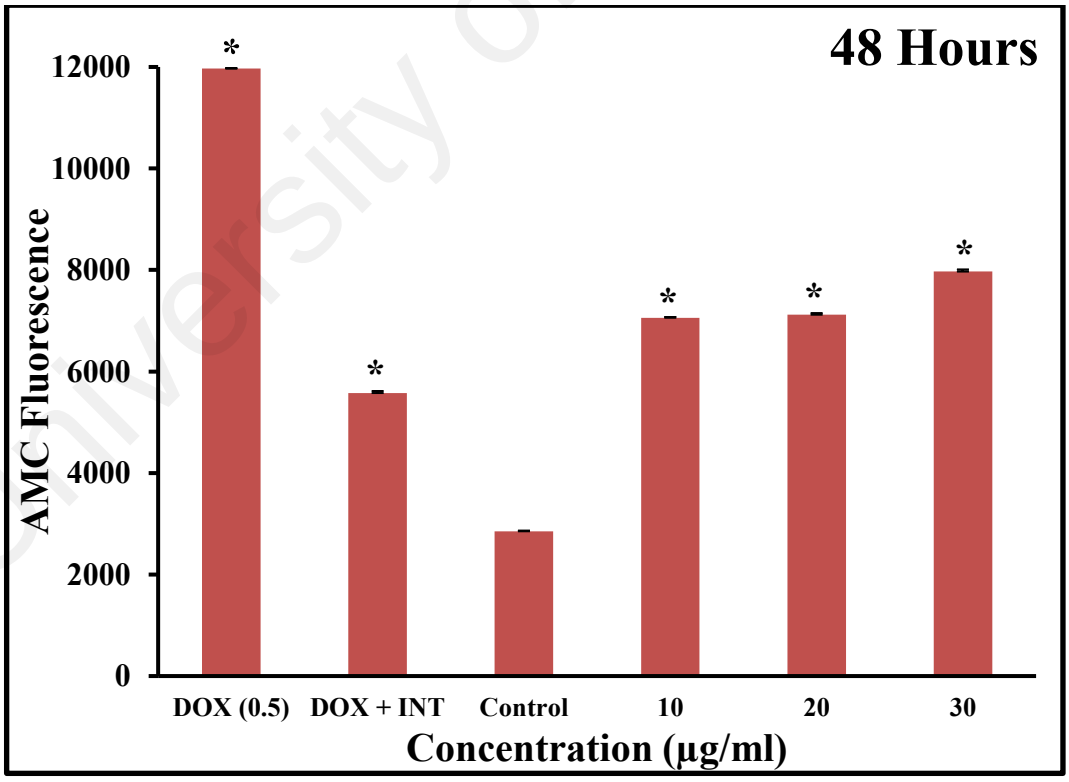


Figure 4.11, Continued.

4.11 Chalepin induced cell cycle arrest in MCF7

As illustrated in (Figure 4.12), chalepin significantly ($P > 0.05$) decreased the percentage of S-phase cells in MCF7-treated cells with consequent accumulation of cells in the G1 phase when compared with untreated cells for 24 and 48 hours. However, there was also an apparent significant increase in the percentage of cells in G2 phase in MCF7-chalepin treated cells as compared to untreated cells at 48 hours. These observations were in a dose and time-dependent manner.

(a)

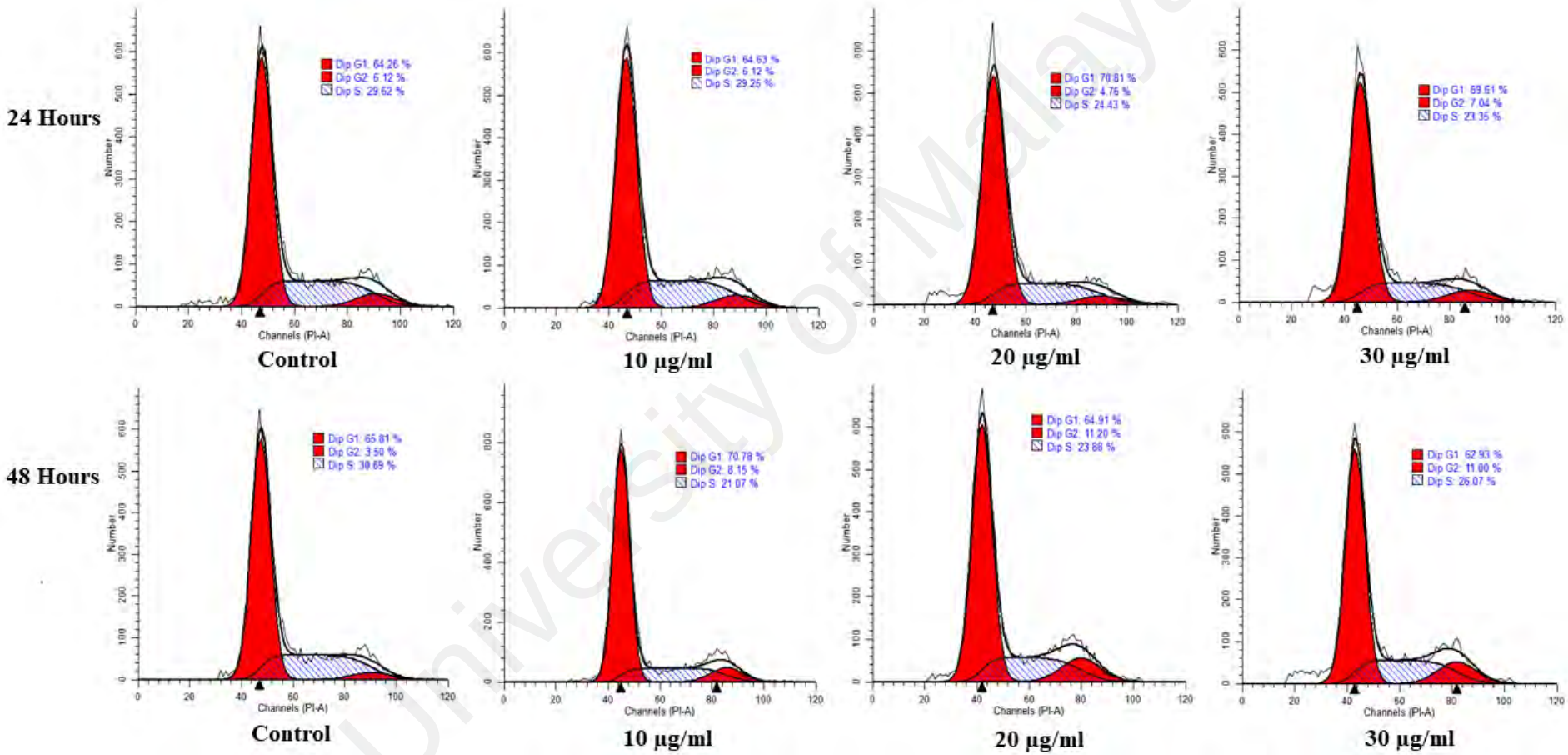
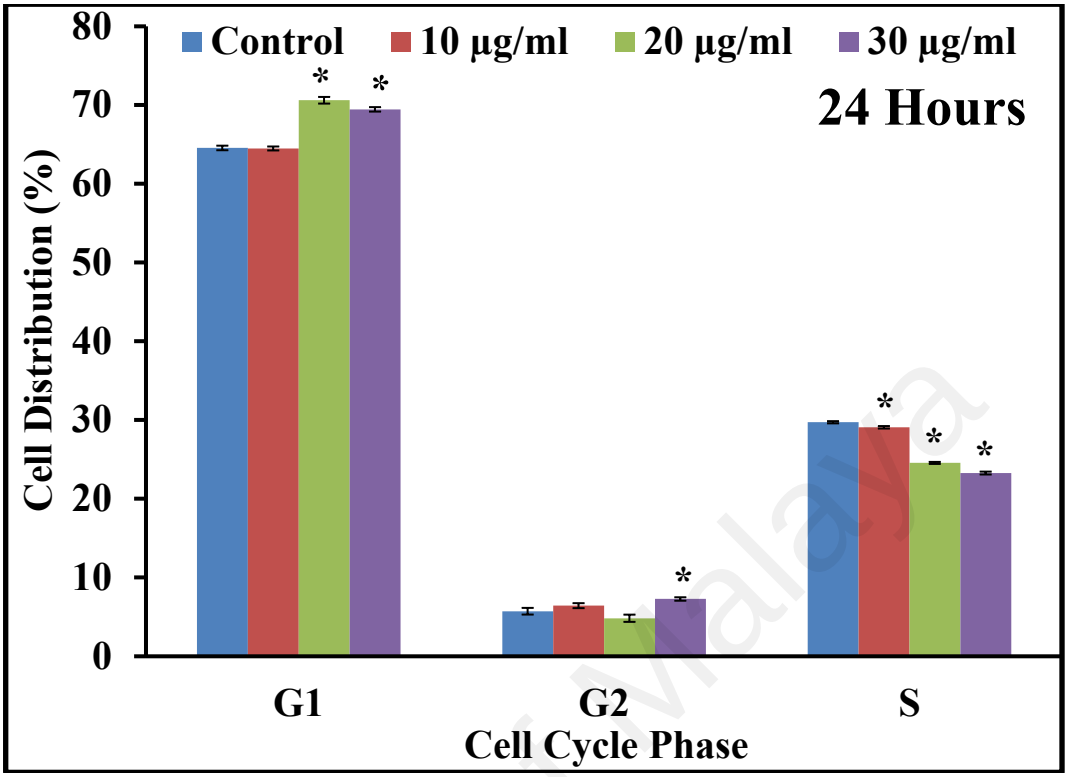


Figure 4.12: Cell cycle analysis in chalepin treated MCF7 **(a)** Cell cycle histogram, and **(b)** Cell distribution of each phase; in MCF7 following treatment with Chalepin at different concentrations for 24 and 48 hours. Data are presented as mean ± SD (n=3). Data carrying asterisk are significantly different from control ($P<0.05$)

(b)



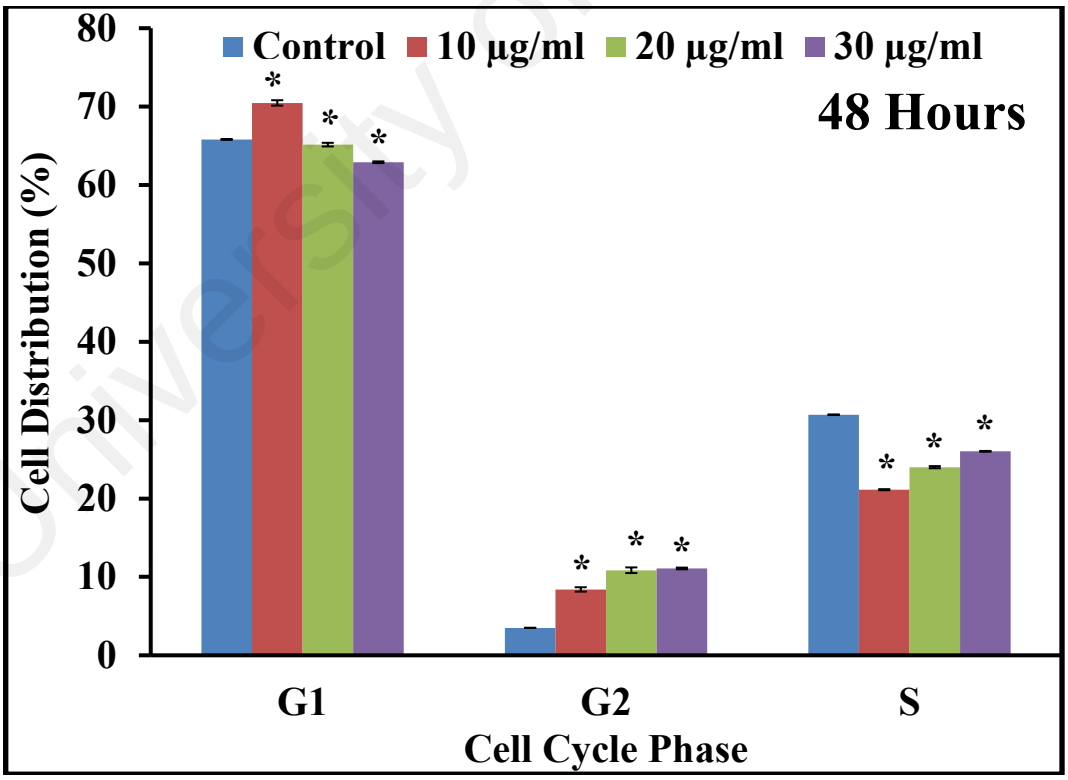


Figure 4.12, Continued.

4.12 Chalepin induced DNA fragmentation in MCF7

In this study, chalepin caused movement of some cell population to the upper section of the density plot as the DNA fragments were labelled. Thus, the upper section of the density plot constitutes cells having fragmented DNA in the treated cells. However, the untreated cell population with non-fragmented and unlabeled DNA remained in the lower region of the density plots. The result therefore as illustrated in (Figure 4.13) indicated that chalepin significantly ($P < 0.05$) increased the DNA fragmentation in MCF7-treated cells when compared with the untreated cells in a dose and time-dependent manner.

(a)

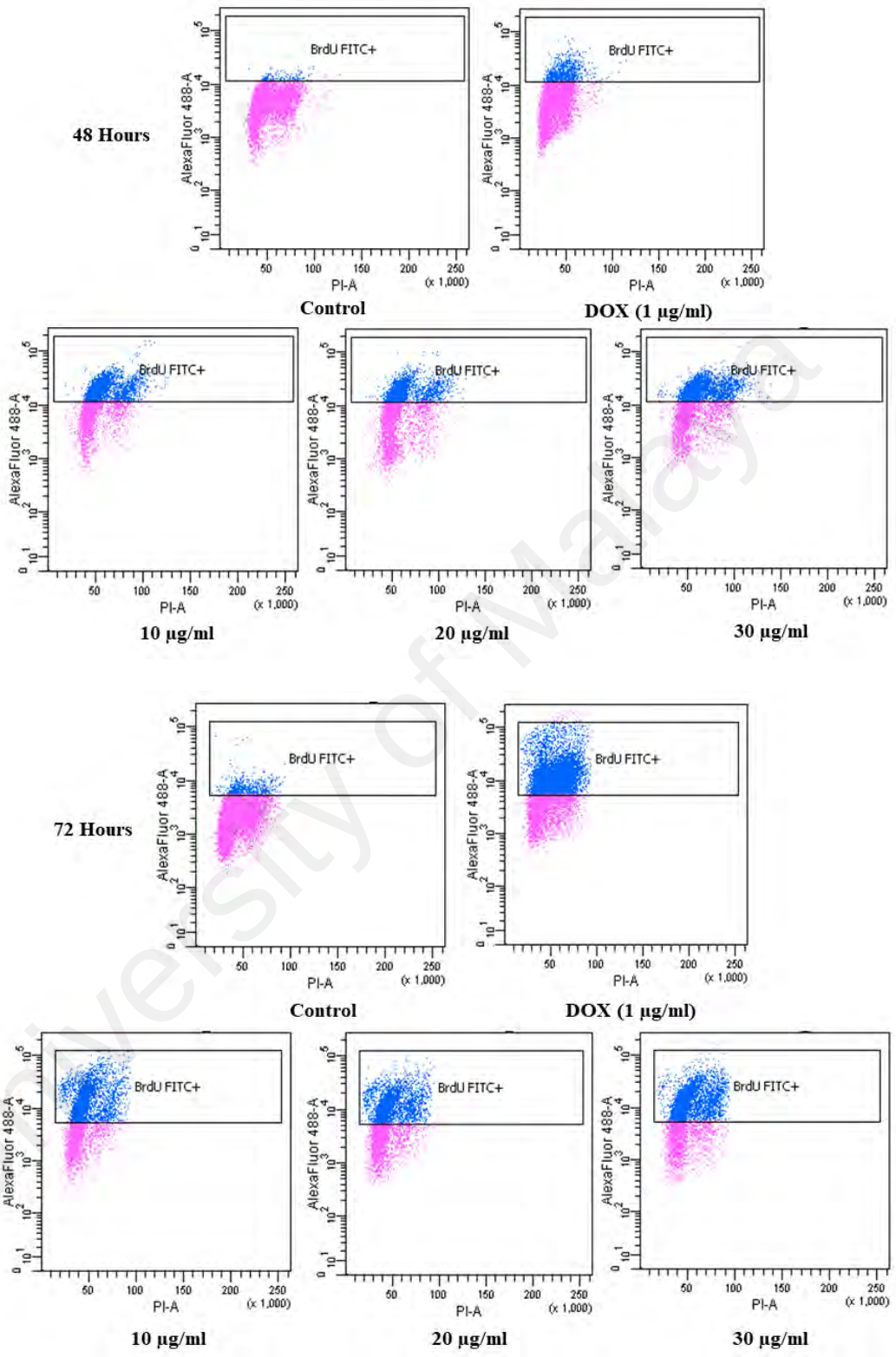
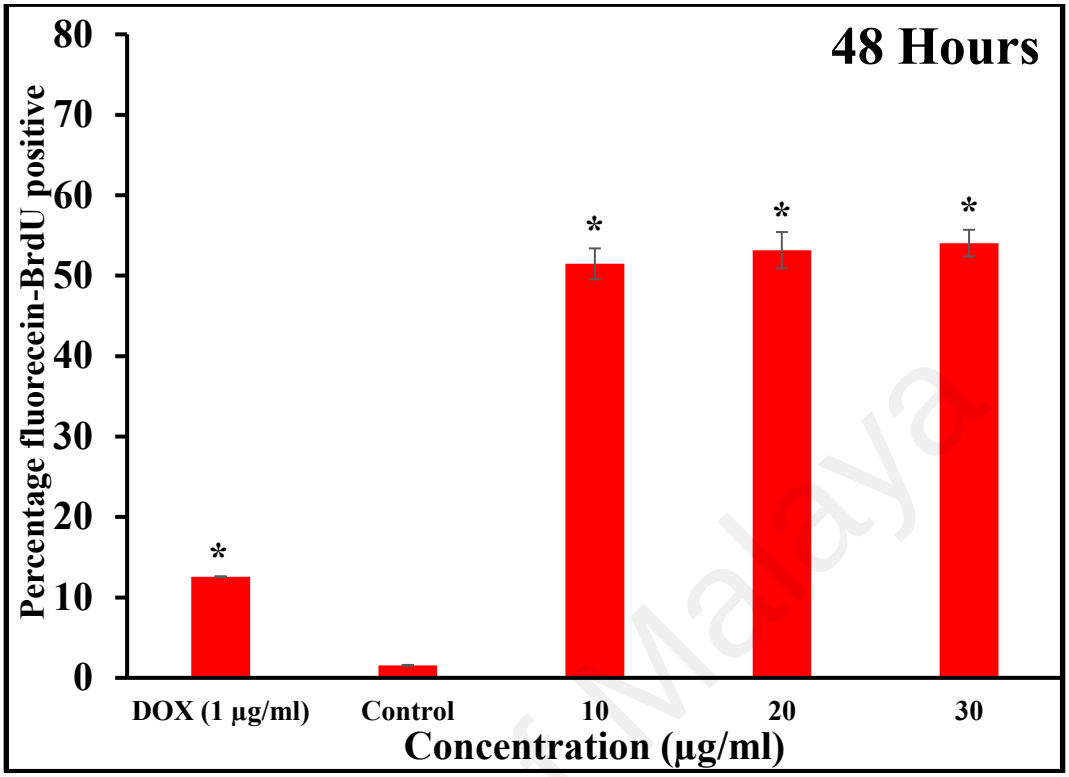


Figure 4.13: DNA fragmentation in chalepin treated MCF7. **(a)** TUNEL assay dot plots, and **(b)** Percentage DNA fragmentation; in MCF7 following treatment with Chalepin at different concentrations for 48 and 72 hours. Data were presented as mean ± SD (n=3). Data carrying asterisk are significantly different from control (P<0.05)

(b)



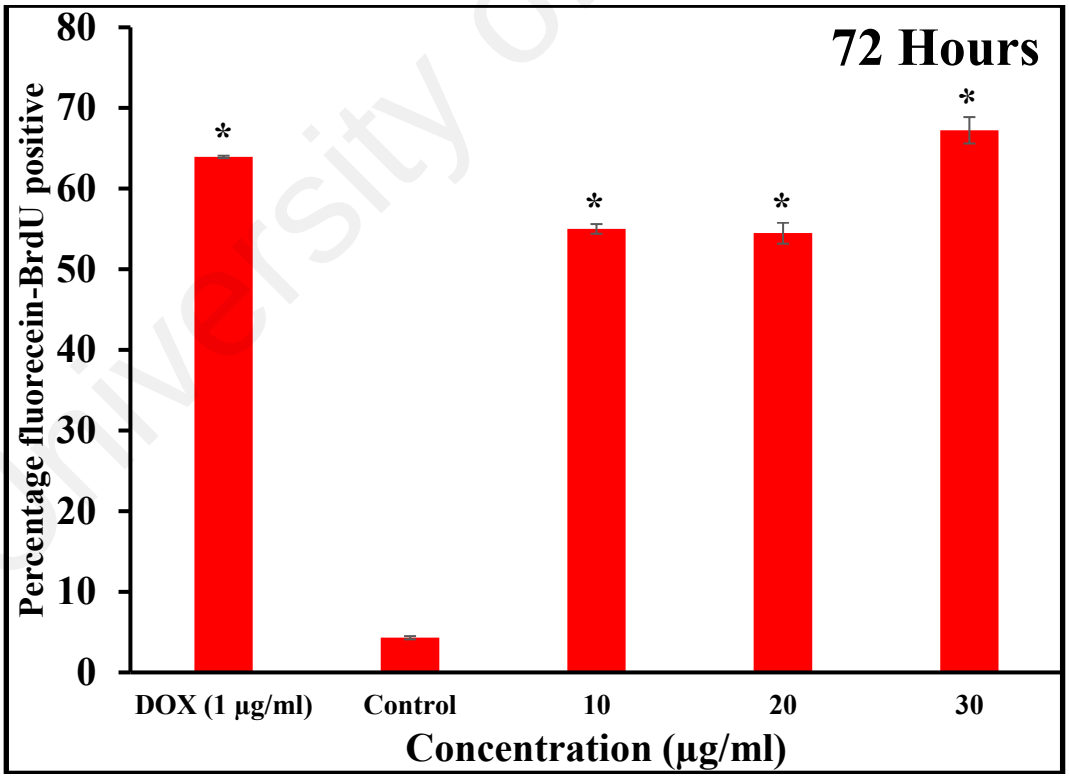


Figure 4.13, Continued.

4.13 Western blot results on MCF7

4.13.1 Effect of chalepin on some apoptotic proteins in MCF7

In the present study, chalepin treatment on MCF7 resulted in down-regulation of anti-apoptotic protein Bcl2. In contrast, pro-apoptotic protein Bid was upregulated, interestingly Bax expression was decreased in the cytosol as it was translocated to mitochondrial membrane to stimulate cytochrome C released which can leads to apoptotic cell death (Figure 4.14a). Similarly, cleaved caspase-8 and caspase-9 were upregulated, whereas PARP1 was downregulated with upregulation of cleaved PARP1 (Figure 4.14b). On the other hand, the expression of phosphorylated P38 was also upregulated (Figure 4.14c). The dynamic regulations observed were all in a time-dependent manner.

4.13.2 Effect of chalepin on cell cycle regulatory proteins in MCF7

In this study, chalepin treatment on MCF7 resulted in increased expressions of P21, P27 and P53 proteins (Figure 4.15a). However, expressions of CDK2, CDK4, cyclin A and Cyclin D were all down-regulated (Figure 4.15b). The observed expressions of these proteins were all in a time-dependent manner.

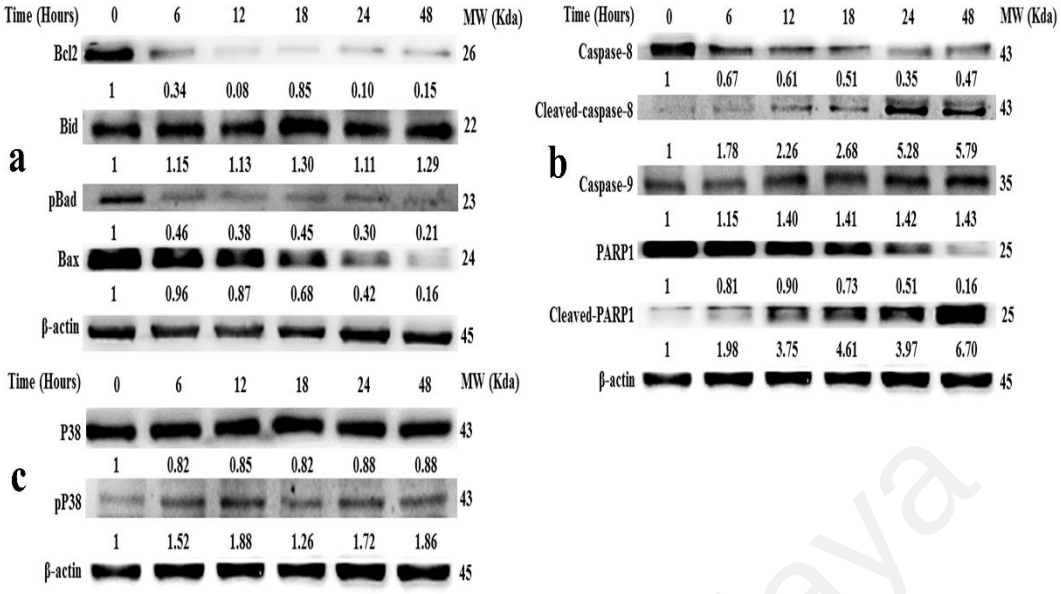


Figure 4.14: Effect of chalepin treatment on apoptotic proteins in MCF7 at 6, 12, 18, 24, and 48-hours incubation periods **(a)** Bcl2 family proteins expressions **(b)** Caspases expression **(c)** P38 expression

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **G**.

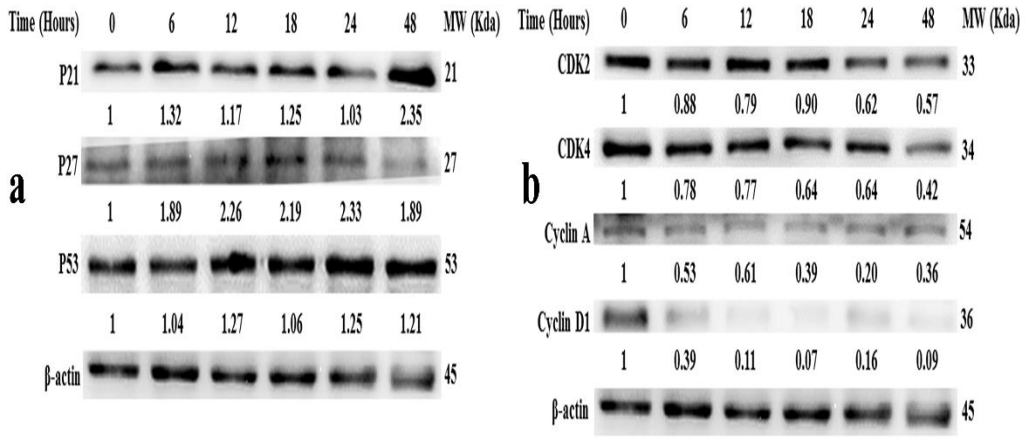


Figure 4.15: Effect of chalepin treatment on cell cycle regulatory proteins in MCF7 at 6, 12, 18, 24 and 48 hours incubation periods **(a)** Cyclin kinase inhibitors expressions **(b)** Cyclins and cyclin-dependent kinases expression

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **G**.

4.13.3 Effect of chalepin on EGFR and its downstream cascades proteins in MCF7

The results of this present study revealed that chalepin treatment on MCF7 induced an alternating effect on EGFR and its downstream cascades. The expressions of the non-phosphorylated forms of the proteins were either down-regulated or remain unchanged. Similarly, the phosphorylated forms of the proteins were down-regulated across all the pathways (Figure 4.16). In contrast, the expression of PTEN a protein that inhibits the PI3K pathway was upregulated (Figure 4.16d). These observations were all time-dependent.

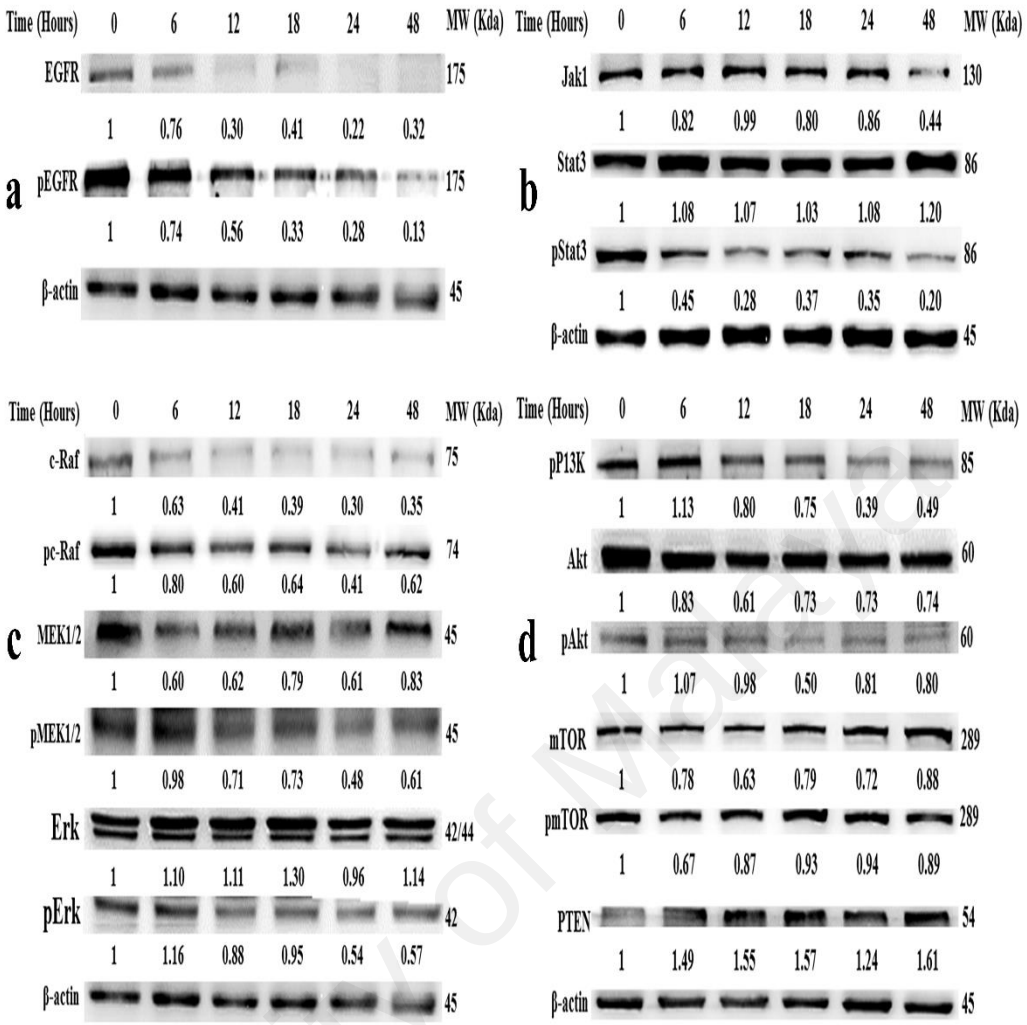


Figure 4.16: Effect of chalepin treatment on EGFR and its downstream cascades proteins in MCF7 for 6, 12, 18, 24 and 48 hours incubation periods **(a)** EGFR expressions **(b)** Jak/Stat pathway **(c)** Erk pathway **(c)** Akt pathway

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **G**.

4.14 Western blot results on HT29

4.14.1 Effect of rutamarin on some apoptotic proteins in HT29

In this study, rutamarin treatment on HT29 resulted in downregulation of anti-apoptotic protein Bcl2. In contrast pro-apoptotic protein Bid, pBad and Bax were upregulated (Figure 4.17a). Similarly, inactive caspase-3, caspase-8 and caspase-9 were cleaved into active cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 respectively with consequence cleavage of PARP1 (Figure 4.17b). On the other hand, the expression of DR4, DR5 and Survivin were downregulated whereas expression of pSAPK/JNK was upregulated (Figure 4.17c). The alternating regulations observed were all in a time-dependent manner.

4.14.2 Effect of rutamarin on cell cycle regulatory proteins in HT29

The result of this study revealed that, rutamarin treatment on HT29 resulted in increased expressions of P21, P27 and P53 proteins (Figure 4.18a). However, expressions of CDK2, CDK4, cyclin A and Cyclin D were all down-regulated (Figure 4.18b). The observed expressions of these proteins were all in a time-dependent manner.

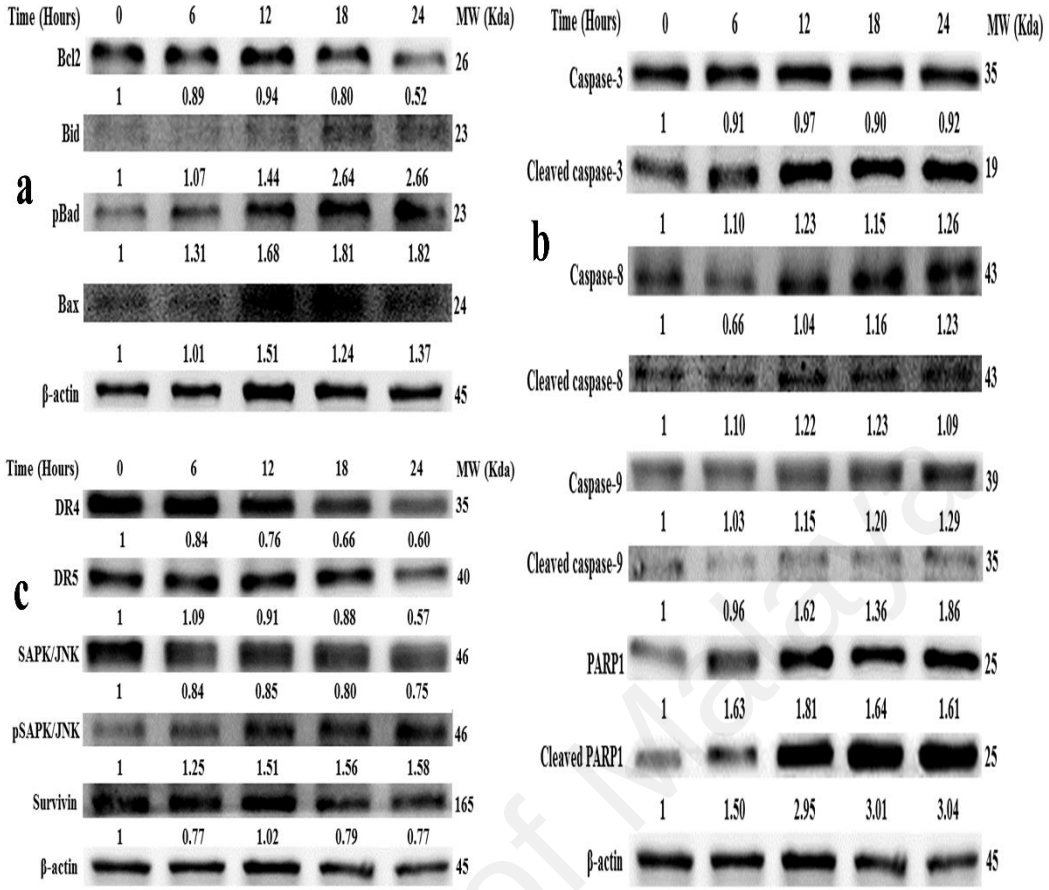


Figure 4.17: Effect of rutamarin treatment on apoptotic proteins in HT29 at 6, 12, 18 and 24 hours of incubation periods **(a)** Bcl2 family proteins expressions **(b)** Caspases expression **(c)** Death receptor expression

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **H**.

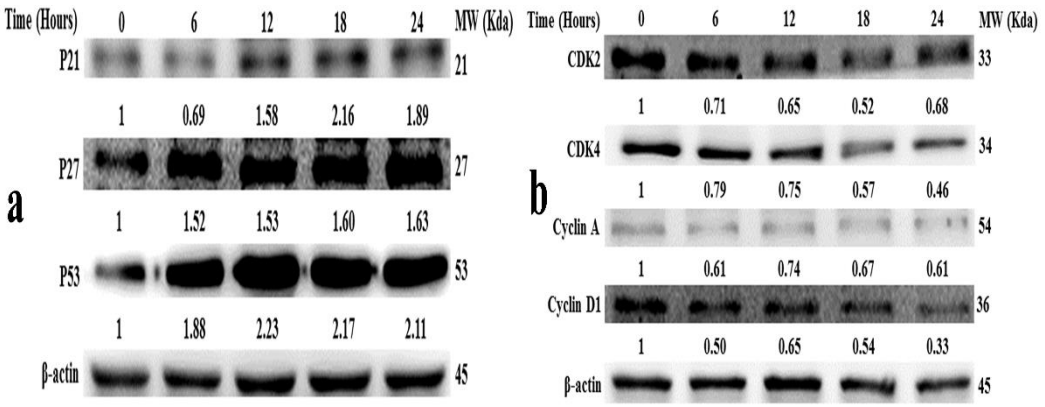


Figure 4.18: Effect of rutamarin treatment on cell cycle regulatory proteins in HT29 at 6, 12, 18 and 24 hours incubation periods **(a)** Cyclin kinase inhibitors expressions **(b)** Cyclins and cyclin-dependent kinases expression

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **H**.

4.14.3 Effect of rutamarin on the NF-κB pathway, COX-2 and P38 proteins in HT29

In the present study, rutamarin treatment on HT29 resulted in decreased expressions of NF-κB, IKKα and IKKβ, whereas expression of IκBα protein were upregulated (Figure 4.19a). However, expressions of COX-2 protein was down-regulated in HT29-treated cells (Figure 4.19b). The result of this study also revealed the upregulation of the phosphorylated P38 (Figure 4.19c). The observed expressions of these proteins were all in a time-dependent manner.

4.14.4 Effect of rutamarin on EGFR and its downstream cascades proteins in HT29

The result of this present study revealed that rutamarin treatment on HT29 induced an alternating effect on EGFR and its downstream cascades. In this study, the expressions of the non-phosphorylated forms of the proteins were either down-regulated or remain unchanged. Similarly, the phosphorylated forms of the proteins were down-regulated across all the pathways (Figure 4.20). In contrast, the expression of PTEN a protein that inhibits the PI3K pathway was upregulated (Figure 4.20d). These observations were all in a time-dependent manner.

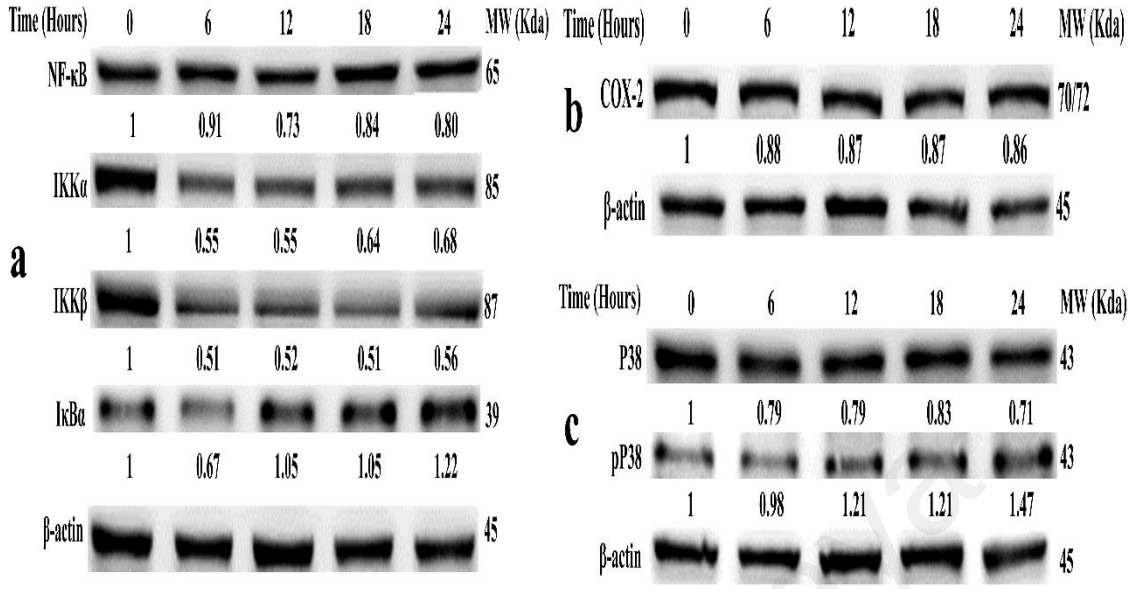


Figure 4.19: Effect of rutamarin treatment on the NF-κB pathway in HT29 at 6, 12, 18 and 24 hours incubation periods **(a)** NF-κB family proteins expressions **(b)** COX2 expression **(c)** P38 expression

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **H**.

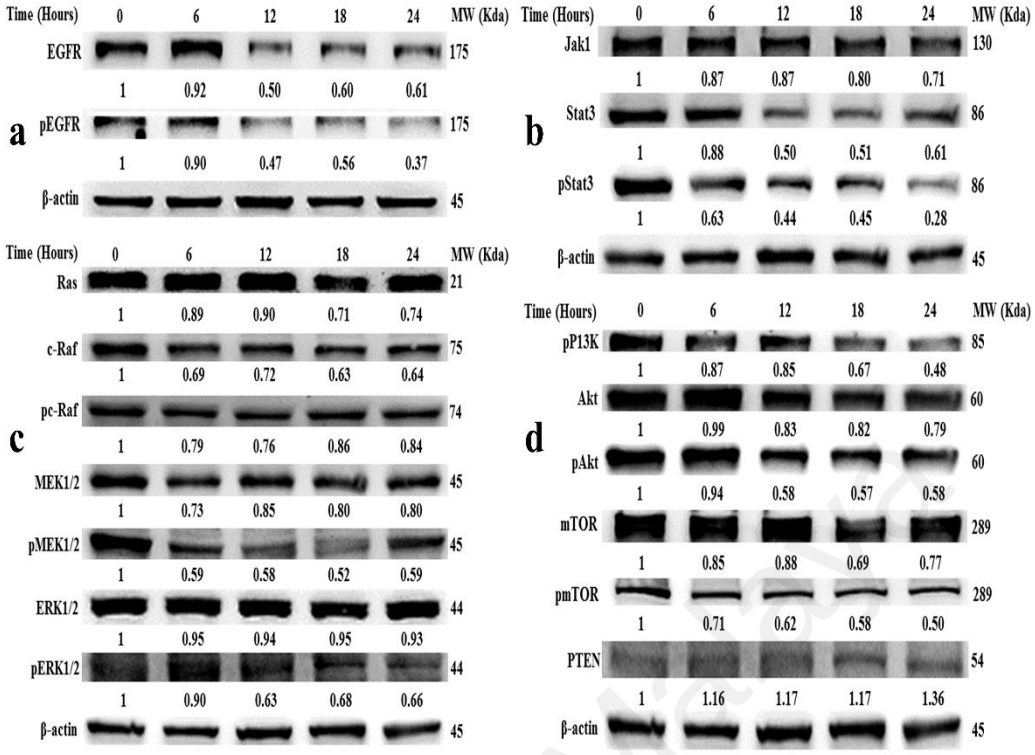


Figure 4.20: Effect of rutamarin treatment on EGFR and downstream cascades proteins in HT29 at 6, 12, 18 and 24 hours incubation periods **(a)** EGFR expressions **(b)** Jak/Stat pathway **(c)** Erk pathway **(c)** Akt pathway

Note: Each protein was normalized based on β-actin to obtain values for the fold change. However, representative of the original western blot images has been presented in appendix **H**.

4.15 Proteomic analysis in HT29 using LC-MS/MS approach

LC-MS/MS detected and identified enormous number of various protein groups from the samples based on the charge to mass ratio m/z of the fragmented peptides.

4.15.1 Total ion chromatogram (TIC)

The TIC was on the average of 3.89 × E9 and 3.24 × E9 for untreated 0 hour and 6 hours rutamarin-treated HT29 respectively (Figure 4.21).

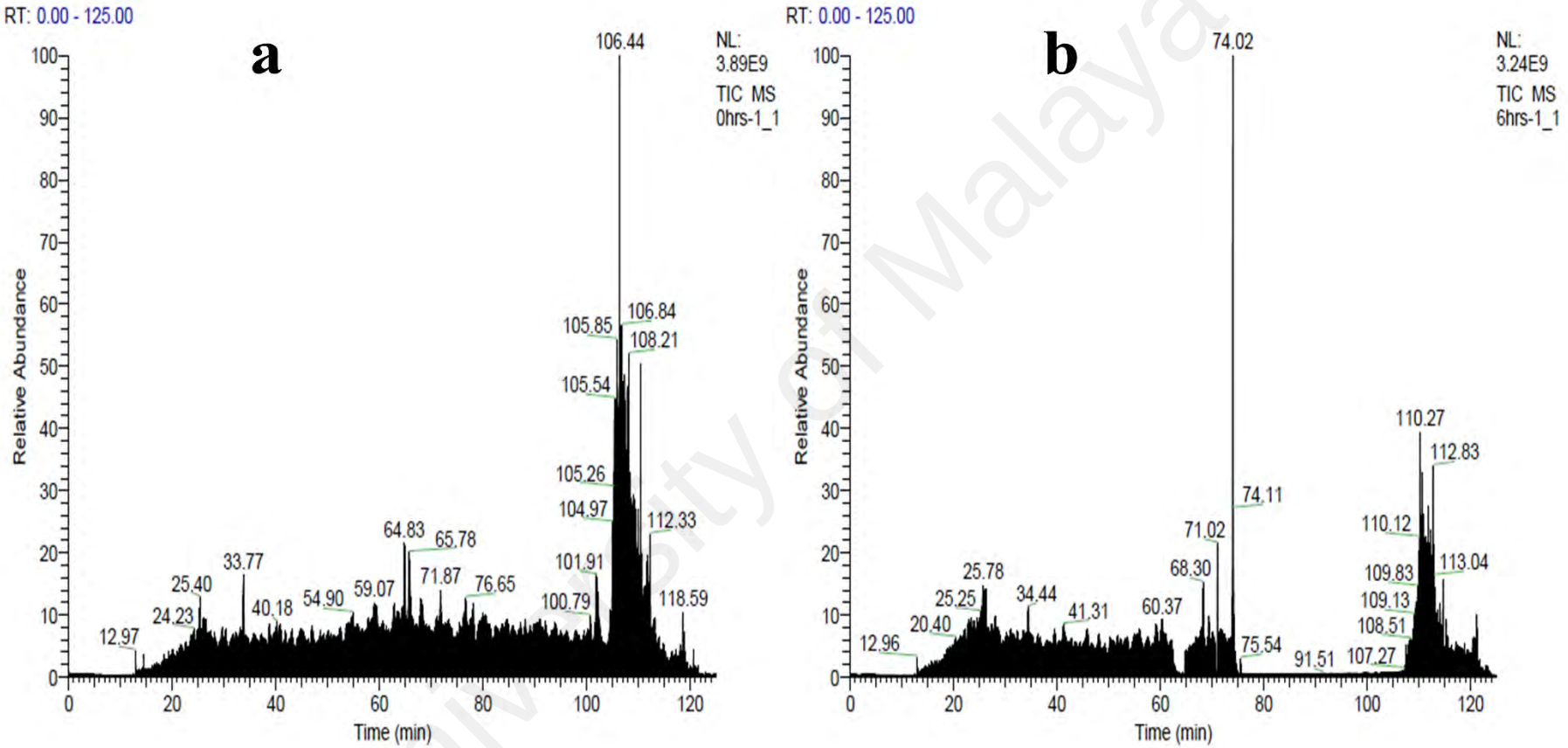
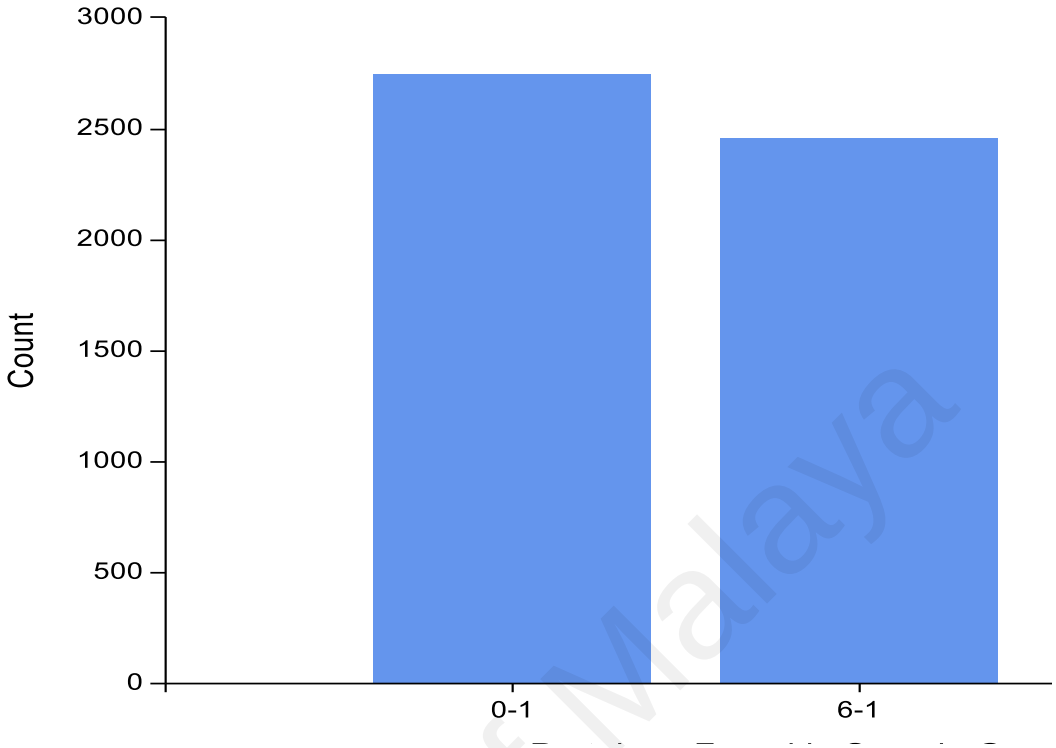


Figure 4.21: Total ion chromatogram of a representative HT29 samples **(a)** Untreated 0-hour HT29 and **(b)** 6-hours rutamarin-treated HT29 sample

4.15.2 Protein identification, visualisation and functional characterisation

In ttis study, the total number of proteins identified using Proteome Discoverer (PD 2.1) in both untreated (0 hours) and 6 hours rutamarin-treated HT29 samples were 3060, 3087 and 2984 from 1, 2 and 3 biological replicates respectively. The result indicated that similar protein amounts were identified from the 3 biological replicates. However, bar chart analysis indicated a reduction in protein groups that were identified at a higher rate in 6 hours rutamarin-treated HT29 sample as compared to untreated (0 hours) sample (Figure 4.22a). The primary biological processes that involved these proteins were metabolic process, regulation of biological process, response to a stimulus, transport; cell organisation and biogenesis (Figure 4.22b). Most of the proteins were localise to membrane, cytoplasm, nucleus, cytosol and mitochondria (Figure 4.22c). On the other hand, pie chart analysis indicated that a major percentage of the proteins were involved in metabolism and regulation of biological processes (Figure 4.23a); it also showed that a lesser percentage of identified proteins were found in mitochondria (Figure 4.23b). However, the molecular functional analysis revealed that a major percentage of the proteins identified involved in protein binding and catalytic activity (Figure 4.23c).

(a)



(b)

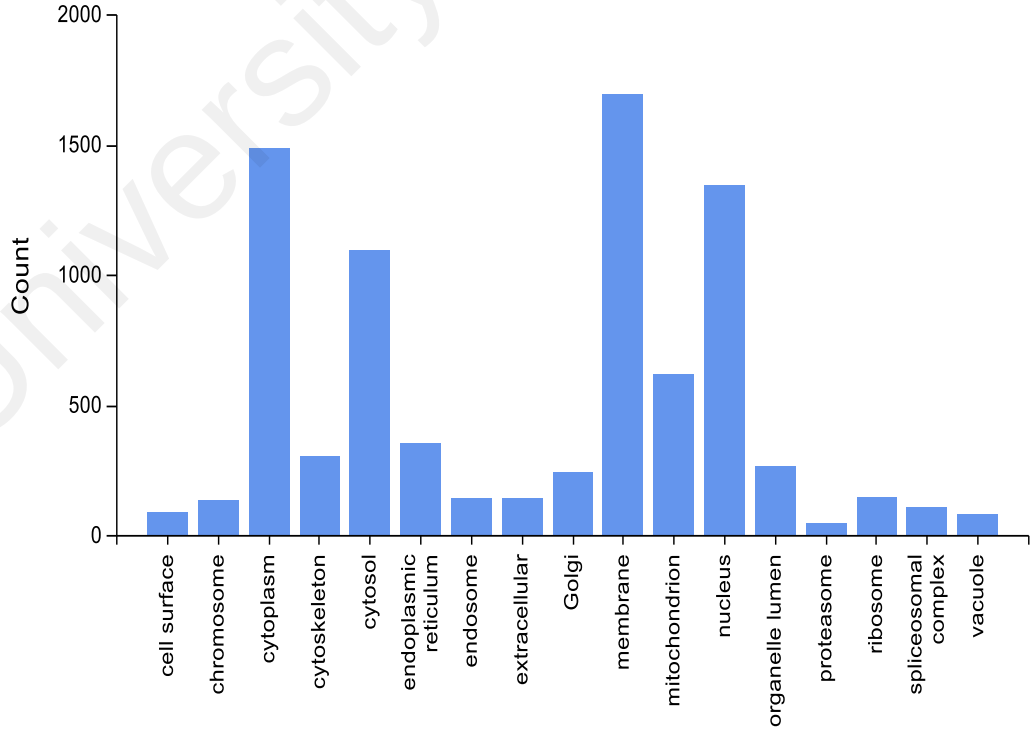


Figure 4.22: Bar chart presentation of proteins identified using PD 2.1 software: **(a)** Representative amount of protein groups identified in 0-hour untreated and 6-hours rutamarin-treated HT29, **(b)** Protein groups identified based on cellular compartment, **(c)** Protein groups identified based on biological process

(c)

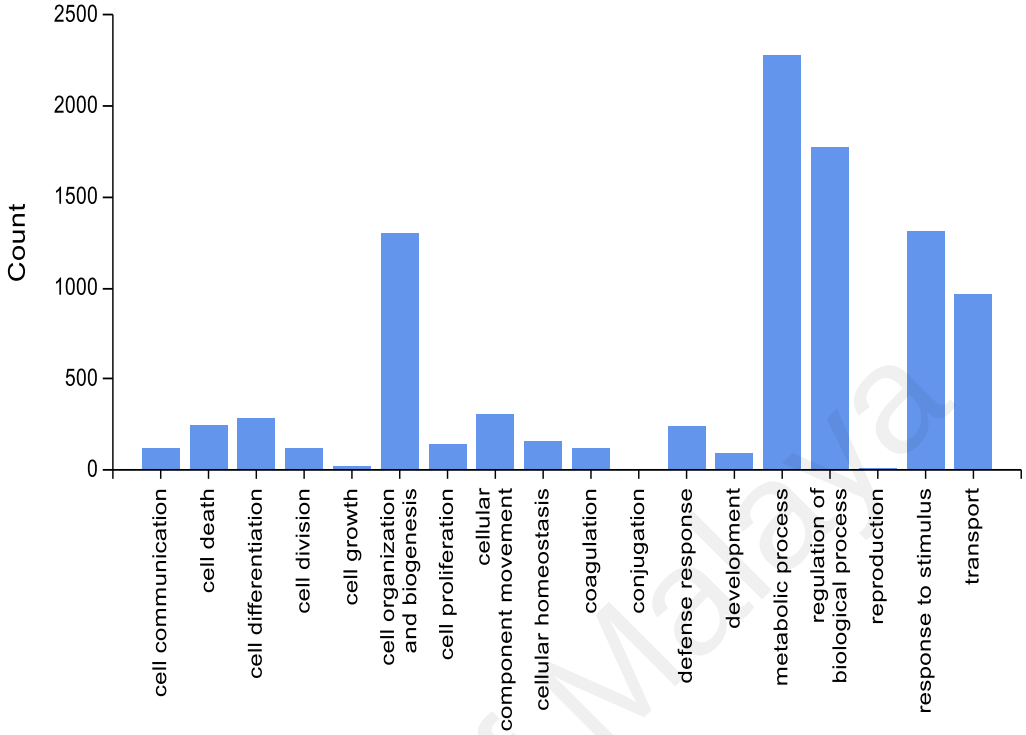
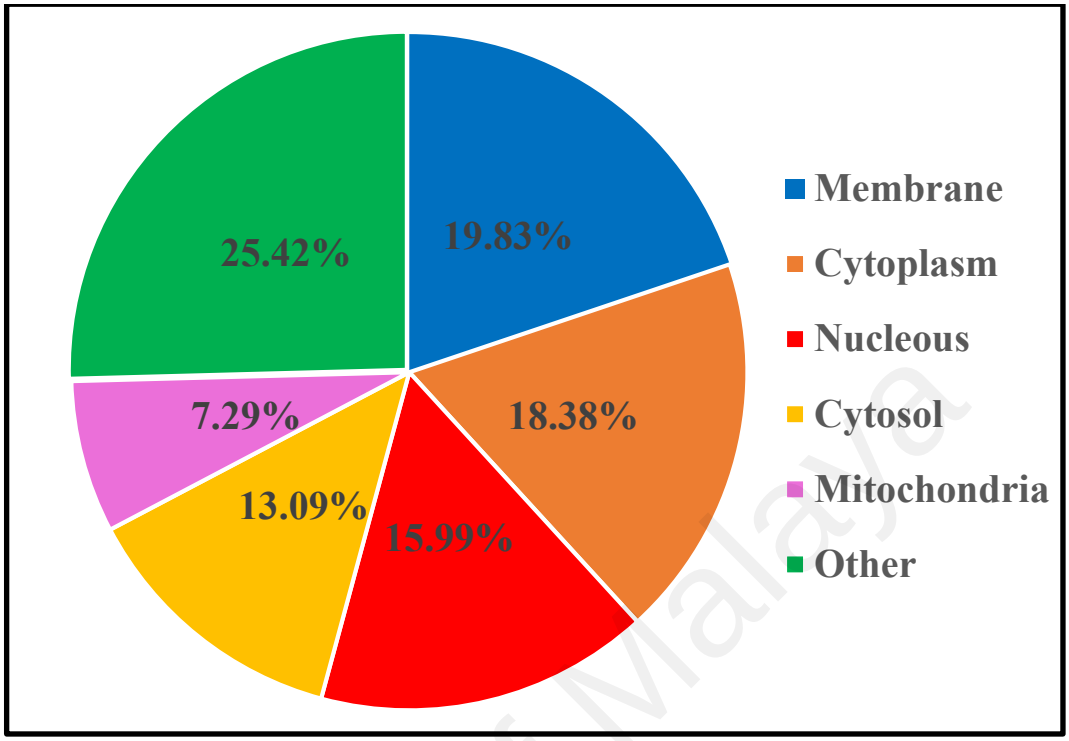


Figure 2.22, Continued.

(a)



(b)

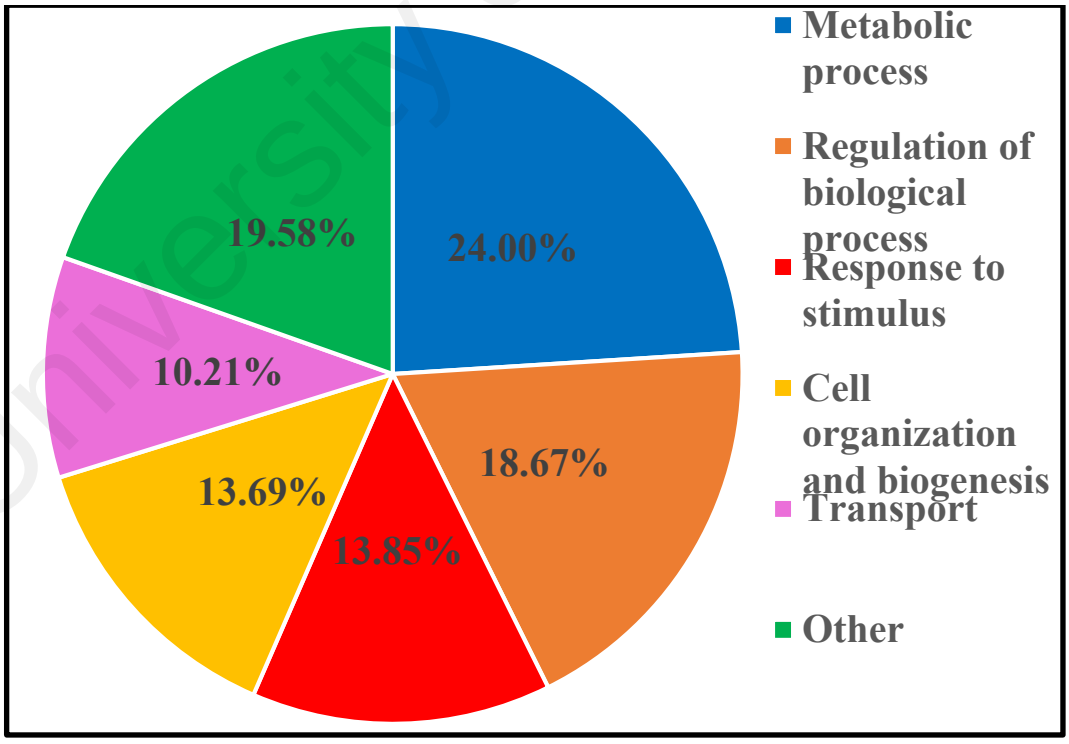


Figure 4.23: Pie chart presentation of proteins identified using PD 2.1 software: **(a)** Percentage of protein groups identified based on cellular compartment, **(b)** Percentage of protein groups identified based on biological process, and **(c)** Percentage of protein groups identified based on molecular function

(c)

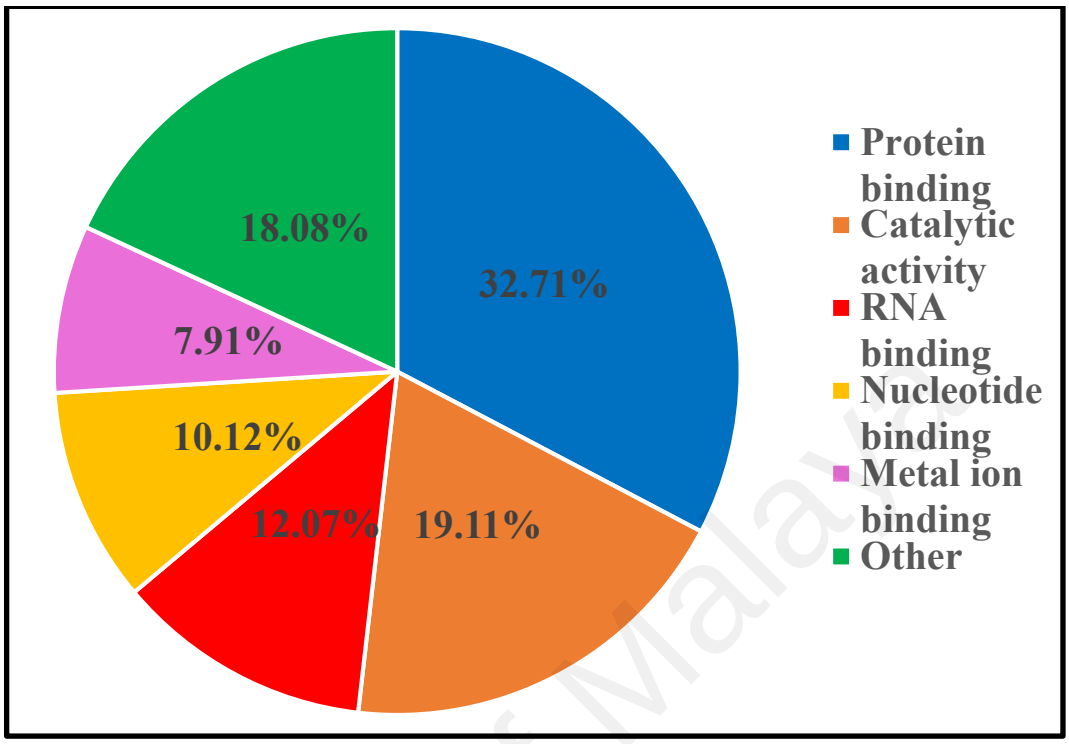


Figure 4.23, Continued.

4.15.3 Analysis of identified proteins using Perseus software

From the histogram, the result indicated that the intensities of the samples were normally distributed. Importantly, the imputed values did not form a separate normal distribution, started around same point in the replicates and were narrower than the distributions of the measured values (Figure 4.24). Heat map analysis also indicated that some proteins had higher intensities whereas others had lower intensities (Figure 4.25).

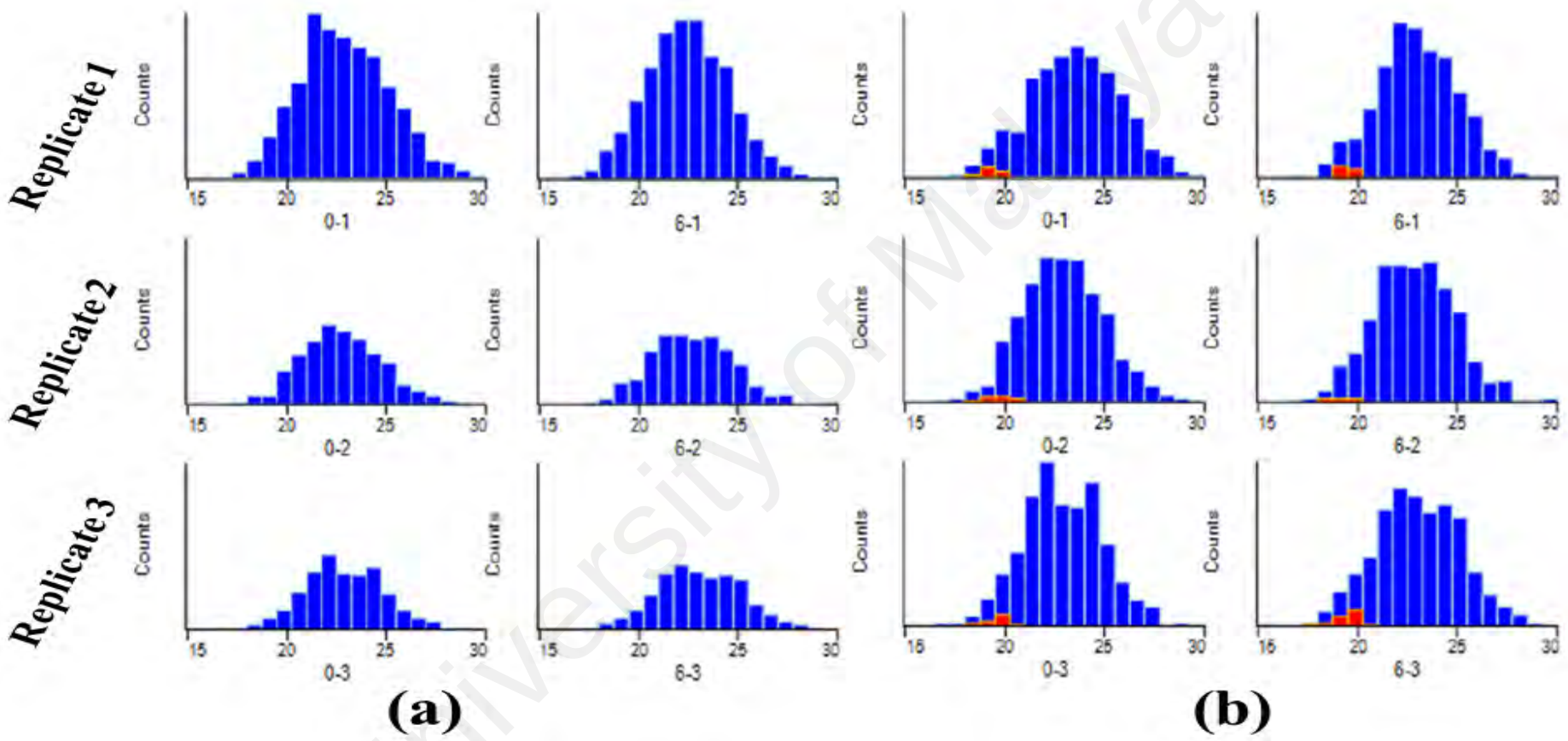


Figure 4.24: Distribution of the replicates sample intensities **(a)** Before imputation **(b)** After imputation for untreated and 6-hours rutamarin-treated HT29 of the 3 replicates

Legend: 0-1 --- Untreated sample replicate 1, 0-2 --- Untreated sample replicate 2, 0-3 --- Untreated sample replicate 3, 6-1 --- Treated sample replicate 1, 6-2 --- Treated sample replicate 2, 6-3 --- Treated sample replicate 3

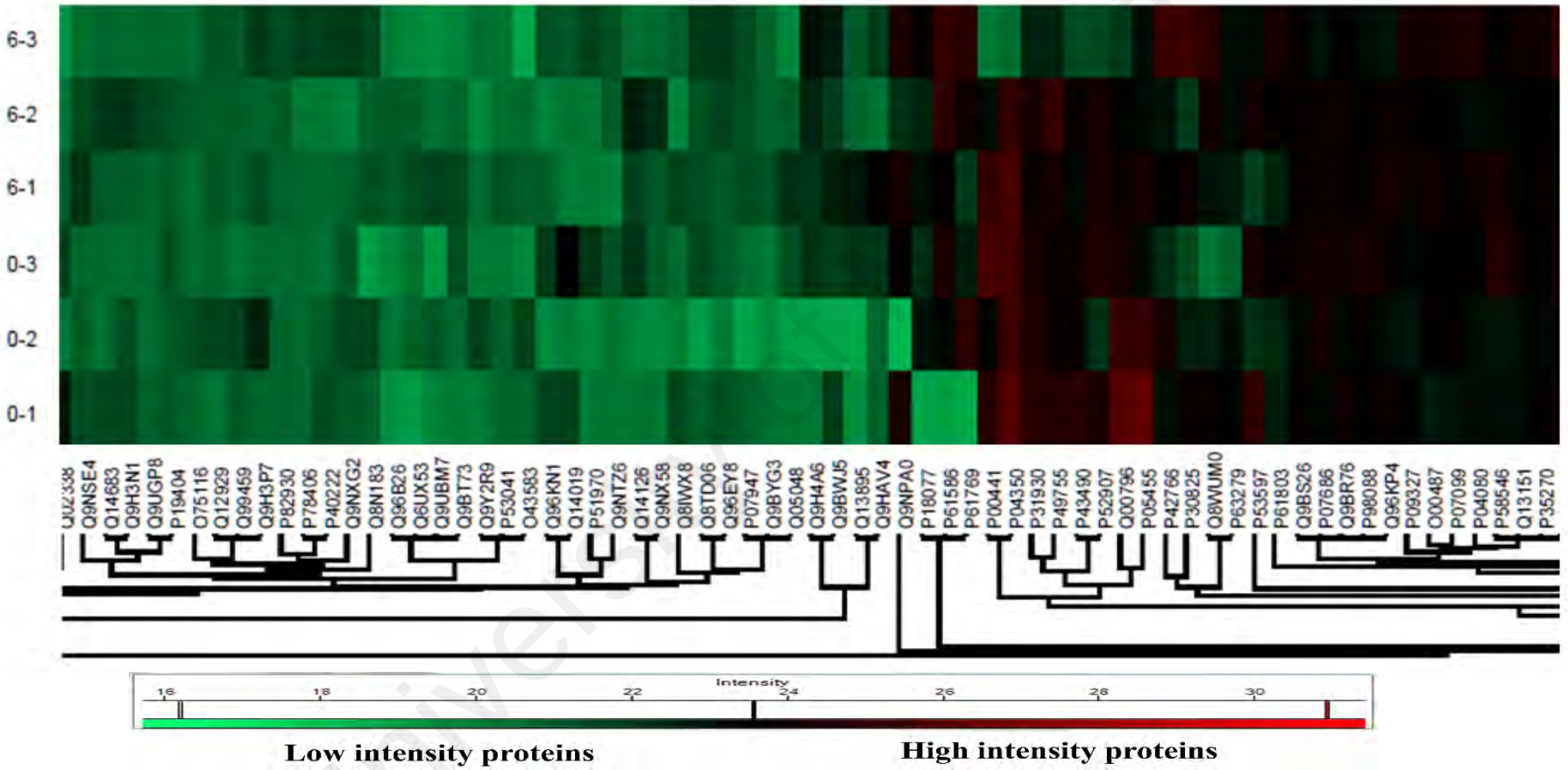


Figure 4.25: Heat map of the identified proteins indicating a representatives of low and hihg intensity proteins for untreated and 6-hours rutamarin-treated HT29 of the 3 replicate

Legend: 0-1 --- Untreated sample replicate 1, 0-2 --- Untreated sample replicate 2, 0-3 --- Untreated sample replicate 3, 6-1 --- Treated sample replicate 1, 6-2 --- Treated sample replicate 2, 6-3 --- Treated sample replicate 3

4.15.3.1 Correlation and statistical analysis of identified differentially expressed proteins

The samples were analysed by Pearson correlation using Perseus software 1.6.0.7. The result from multiscatter plot showed a strong positive correlation between all the samples with a Pearson correlation coefficient > 0.5 across all the samples (Figure 4.26). Two sample t-test indicated that only one protein; mitochondrial carrier homolog 2 (MTCH2) (Q9Y6C9) was significantly ($P < 0.05$) differentially expressed in 6 hours (Figure 4.27). However, a profile plot analysis indicated that 20 proteins were having a similar pattern including the differentially expressed protein (Figure 4.28 and Table 4.4).

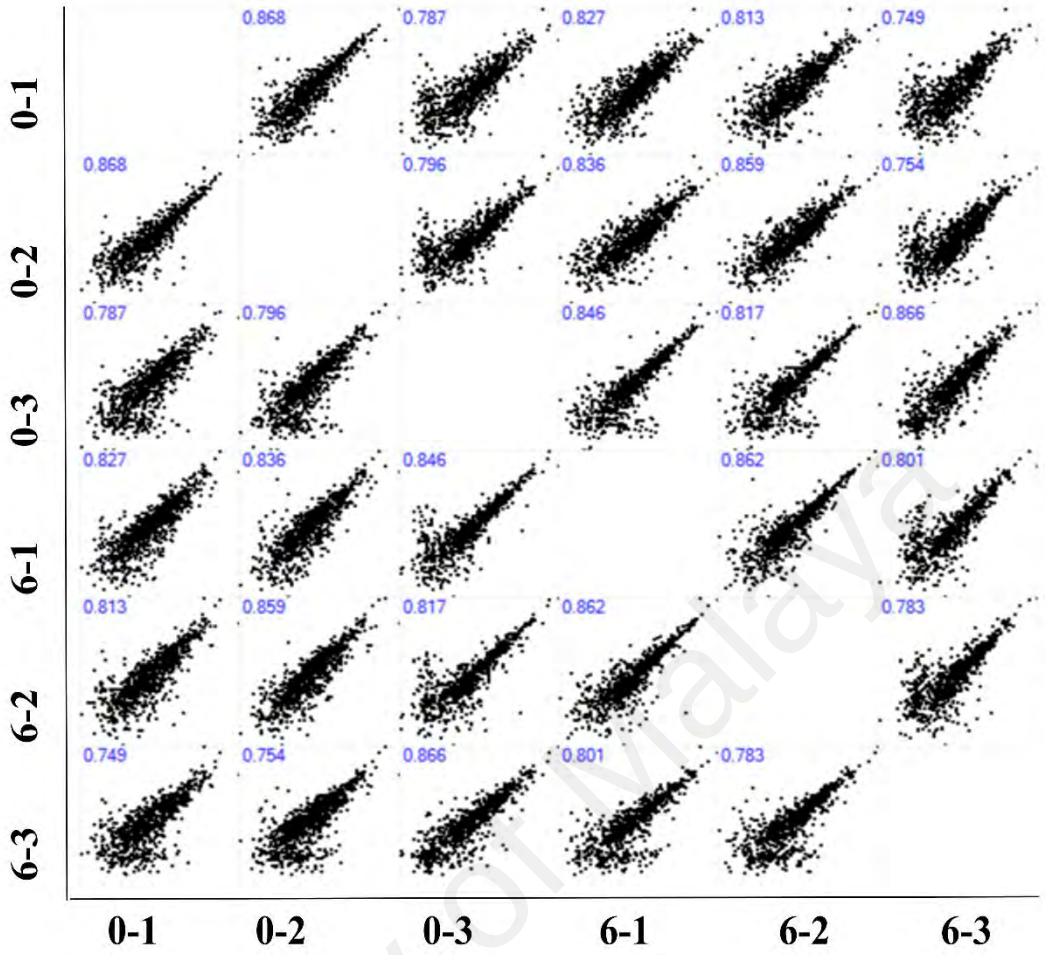


Figure 4.26: Pearson correlation analysis plotted against all the sample replicates for untreated and 6 hours rutamarin-treated HT29

The value on the chart indicated Pearson coefficient between different sample

Legend:

0-1 --- Untreated sample replicate 1
0-2 --- Untreated sample replicate 2
0-3 --- Untreated sample replicate 3
6-1 --- Treated sample replicate 1
6-2 --- Treated sample replicate 2
6-3 --- Treated sample replicate 3

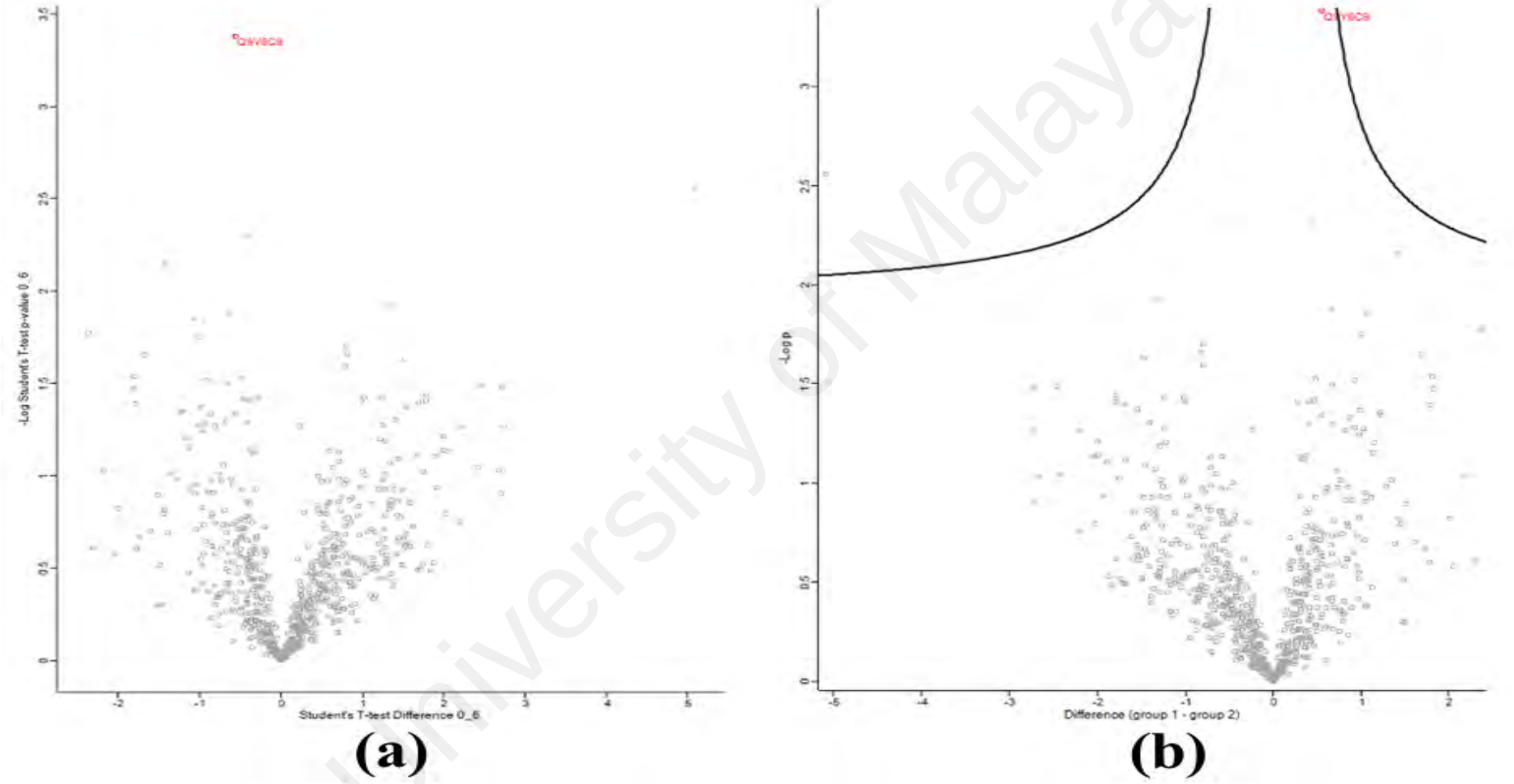


Figure 4.27: Statistical differentially expressed protein **(a)** Scatter plot of student t-test difference **(b)** Volcano plot of difference; between untreated and 6 hours HT29-rutamarin treated showing differentially expressed protein at ($P < 0.05$)

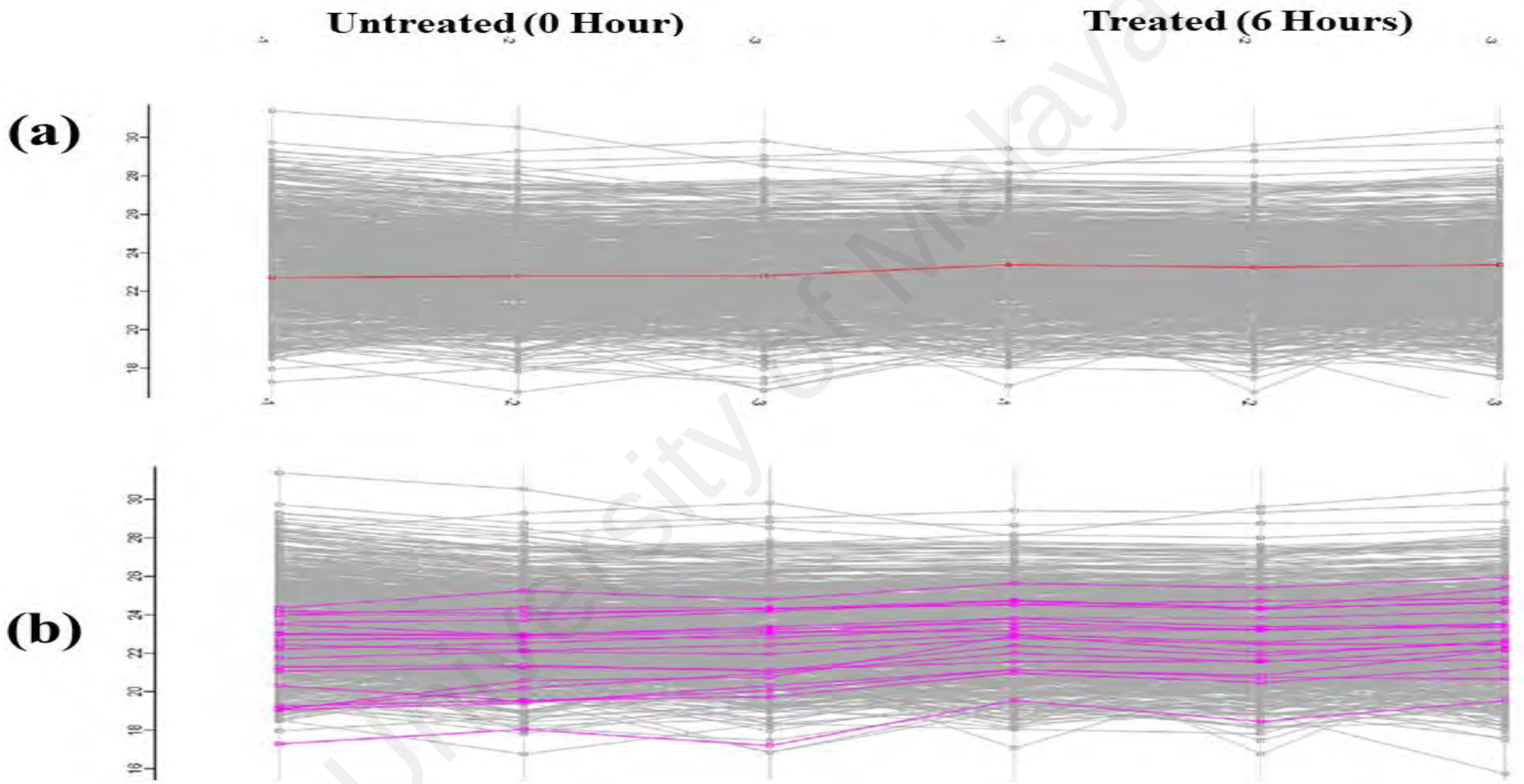


Figure 4.28: Profile plot of the differentially expressed proteins**. (a)** The expression pattern of Mitochondrial carrier homolog 2 (MTCH2) (Q9Y6C9); differentially expressed protein **(b)** The expression patterns of the 20 proteins inclusive of differentially expressed protein for the untreated and 6 hours HT29-rutamarin treated

Table 4.4: Proteins identified with similar expression pattern based on the profile plot using Perseus Software (1.6.0.7)

Accession	Gene ID	Protein Name	Biological Process	Cellular Component	Molecular Function	MW [kDa]	Unique Peptides	P-value
P09327	VIL1	Villin-1	Cell death; Cell differentiation; Cell organisation and biogenesis; Cellular component Movement; Regulation of biological process; Response to Stimulus; Transport	Cytoplasm; Cytoskeleton; Membrane	Enzyme regulator activity; Metal ion binding; Protein binding	92.64	26	1.50
Q9Y6N5	SQRDL	Sulfide: quinone oxidoreductase, mitochondrial	Metabolic process	Membrane; Mitochondrion	Catalytic activity	49.93	13	1.34
O00515	LAD1	Ladinin-1	**FNI**	Extracellular	Structural molecule activity	57.10	11	1.42
Q53GQ0	HSD17B12	Very-long-chain 3-oxoacyl-CoA reductase	Cell organization and biogenesis; Metabolic process; Regulation of biological process	Endoplasmic reticulum; Membrane	Catalytic activity; Protein binding	34.30	10	1.12
*Q9Y6C9	MTCH2	Mitochondrial carrier homolog 2	Cell death; Cell organization and biogenesis; Regulation of biological process; Transport	Membrane; Mitochondrion; nucleus	**FNI**	33.31	9	3.38
Q13162	PRDX4	Peroxiredoxin-4	Cell organization and biogenesis; Cellular homeostasis; Metabolic process; Regulation of biological process	Cytoplasm; Cytosol; Endoplasmic reticulum; Extracellular; Mitochondrion; Nucleus	Antioxidant activity; Catalytic activity; Protein binding	30.52	9	1.75

Table 4.4, Continued.

Accession	Gene ID	Protein Name	Biological Process	Cellular Component	Molecular Function	MW [kDa]	Unique Peptides	P-value
P29373	CRABP2	Cellular retinoic acid-binding protein 2	Metabolic process; Regulation of biological process; Response to stimulus; Transport	Cytoplasm; Cytosol; Endoplasmic reticulum; Nucleus	Protein binding; Transporter activity	15.68	8	1.29
P35914	HMGCL	Hydroxymethylglutaryl-CoA lyase, mitochondrial	cell organisation and Biogenesis; Metabolic process; Response to stimulus	Membrane; Mitochondrion; Organelle lumen	Catalytic activity; Metal ion binding; Protein binding	34.34	6	1.47
Q03252	LMNB2	Lamin-B2	**FNI**	Membrane; Nucleus	Catalytic activity; Motor activity; Structural molecule activity	69.91	6	1.85
P62244	RPS15A	40S ribosomal protein S15a	Cell organisation and biogenesis; Metabolic process; Regulation of biological process; Response to stimulus; Transport	Cytoplasm; Cytosol; Membrane; Ribosome	Protein binding; RNA binding; Structural molecule activity	14.83	5	1.37
Q8WUY1	THEM6	Protein THEM6		extracellular	**FNI**	23.85	5	1.88
P62979	RPS27A	Ubiquitin-40S ribosomal protein S27a	Cell communication; Cell death; Cell organisation and biogenesis; Cellular component movement; Defense response; Metabolic process; Regulation of biological process; Response to stimulus; Transport	Cytoplasm; Cytosol; Membrane; Nucleus; Ribosome	Metal ion binding; Protein binding; RNA binding; Structural molecule activity	17.95	5	1.41

Table 4.4, Continued.

Accession	Gene ID	Protein Name	Biological Process	Cellular Component	Molecular Function	MW [kDa]	Unique Peptides	P-value
P60903	S100A10	Protein S100-A10	Cell organisation and biogenesis; Regulation of biological process	Membrane	Metal ion binding; Protein binding	11.20	5	1.33
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	Cell organisation and biogenesis; Metabolic process; Regulation of biological process; Transport	Cytoplasm; Cytoskeleton; Cytosol; Membrane; Nucleus; Vacuole	Protein binding	31.54	4	1.54
Q9BYG3	MKI67IP; NIFK	MKI67 FHA domain-interacting nucleolar phosphoprotein	Cell organisation and biogenesis; Metabolic process; Regulation of biological process	Chromosome; Cytoplasm; Nucleus	Nucleotide binding; Protein binding; RNA binding	34.20	4	1.52
O75940	SMNDC1	Survival of motor neuron-related-splicing factor 30	Cell death; Metabolic process	Cytoplasm; Nucleus; Spliceosomal complex	protein binding; RNA binding	26.69	3	2.15
P49207	RPL34	60S ribosomal protein L34	cell organisation and biogenesis; Metabolic process; Transport	Cytoplasm; Cytosol; Mitochondrion; ribosome	RNA binding; Structural molecule activity	13.28	3	1.42
P37059	HSD17B2	Estradiol 17-beta-dehydrogenase 2	Metabolic process; Response to stimulus	Membrane	Catalytic activity	42.76	3	2.31

Table 4.4, Continued.

Accession	Gene ID	Protein Name	Biological Process	Cellular Component	Molecular Function	MW [kDa]	Unique Peptides	P-value
Q5T653	MRPL2	39S ribosomal protein L2, mitochondrial	Cell organization and biogenesis; Metabolic process	Membrane; Mitochondrion; Ribosome	catalytic activity; RNA binding; Structural molecule activity	33.28	2	1.78
Q9Y2U8	LEMD3	Inner nuclear membrane protein Man1	Cell differentiation; Cell organization and biogenesis; Cellular component movement; Regulation of biological process	Membrane; Nucleus	DNA binding; Nucleotide binding; Protein binding	99.94	2	1.65

Legend: *Differentially expressed protein, Biological process signifies the biological activities involved by each protein, Cellular compartment identifies the localization of each protein, Molecular function signifies the function performed by each protein at molecular level, Unique peptides identified number of peptides specific only to each protein, p-value is a software specific value indicating fold change of each protein

FNI: Function not identified by PD

CHAPTER 5: DISCUSSIONS

Industrial development new pharmaceutical drugs, has dramatically improved through natural product research to provide marvellous number of lead compounds suitable for new drugs. The identification of the "lead" compound through screening a variety of natural products was among the earliest stages during process of drug discovery and development. The desired new drug charactering lead compounds can be utilized as model in chemical modification (Borris, 1996).

Though there are many drugs available used in prevention and treatment of several ailments including cancer, many of the drugs are toxic and costly. Similarly, some of the drugs become ineffective as a result of resistance developed by cancer cells possibly due to mutations (Galustian & Dalgleish, 2010; Gurunathan, Raman, Malek, John, & Vikineswary, 2013). It is famous that most chemotherapeutic drugs are synthesised to recognise and target a specific protein or gene associated with cancer proliferation or survival pathway (Galustian & Dalgleish, 2010). The specificity of the chemotherapeutic drugs could also be a reason why the drugs are ineffective since multiple signalling pathways are usually channelled toward oncogenic events through alterations of normal cellular homeostasis during tumour initiation and progression (Rai, Mishra, Suman, & Shukla, 2016).

However, various agents from natural products or semi-synthetic compounds of natural origin such as docetaxel, etoposide, irinotecan, teniposide, topotecan, paclitaxel, vinblastine and vincristine have been reported to be used for cancer chemotherapy (Mann, 2002). It has been reported that for the last three decades, about 80% of all approved drugs for cancer therapy by the United States Food and Administration are from natural products or based from there as well as simulated natural product in different forms. Therefore, studies on the natural products and chemical agents can be an essential source of anticancer and chemotherapeutic agents (Bishayee & Sethi, 2016). Thus, many

molecules are in clinical trials, and some already shown promising therapeutic efficacy either administered alone or in combination with standard drugs. Hence, it facilitates crucial chemopreventive and chemotherapeutic drug development from natural sources (Bishayee & Sethi, 2016). It is a common perception that consumption of natural products is safe and always consumers assumed that there is no associated risk using the natural product as they are from "natural source". Evidence also indicates that natural products may have potential chemopreventive activities with low toxicity (Sanders, Moran, Shi, Paul, & Greenlee, 2016). Thus more interest is geared towards the use of natural products as chemopreventive agents (Sanders et al., 2016). In the present, chalepin and rutamarin compounds were isolated from *Ruta angustifolia* and evaluated for their cytotoxic, apoptotic and signalling transduction activity in cancer cells.

5.1 Extraction, fractionation and isolation of plant material/compounds

In the present study, the cold extraction method was adopted where the extraction process was done at room temperature (28^{o}C) which enables effective extraction of the unstable or heat-labile lead compound. However, using hot extraction method may have destroyed these compounds. Interestingly this method allows successive extraction of various fractions from *Ruta angustifolia* plant material using solvents of different polarity as well as effective isolation of chalepin and rutamarin from the chloroform fraction. The result of this study indicated that the cold extraction method employed was suitable to disrupt the plant cell wall effectively thereby enabling effective release of considerable number and amount various chemical constituent of the plant sample into the different solvents used in this study. Furthermore, various phytochemicals were found to be present in the different fractions including the chloroform fraction with little to no destruction on the major constituents present in the plant sample. However, the major constituents include alkaloids and coumarins. Similarly, the chalepin and rutamarin been both

furanocoumarins in nature were successfully and efficiently isolated from the chloroform fraction used for downstream analysis.

Cold extraction method has been extensively employed to extract different phytochemicals from plant material using different solvent. The method is frequently applied for extraction of grounded plant material based on desired solvents of increasing polarity. Consequently, sugars, amino acids and glycosides can be extracted effectively using methanol; waxes, fats and volatile oils using hexane, whereas alkaloids, aglycones and glycoside can be extracted using ethyl acetate (Houghton & Raman, 1998). Also, more excellent and efficient extraction of plant chemical compounds is usually achieved using organic solvents because of their ability to degrade plant cell wall thereby extracting a more considerable amount of endocellular material unlike using water (Lim & Murtijaya, 2007).

5.2 Identification and characterisation of chalepin and rutamarin

Though there are several other compounds previously isolated and identified, in this study, only chalepin and rutamarin were isolated and identified following repeated collection from HPLC analysis. It has been reported that comparison of their MS can elucidate structures of isolated compounds (Song et al., 2013; Zhang et al., 2018). Therefore, the compounds chalepin and rutamarin were identified and confirmed through their mass spectral and NMR data which were consistent with the published literature (Del Castillo et al., 1984; Massanet, Pando, Rodriguez-luis, & Salva, 1987; Orlita et al., 2008; Wu et al., 2003). Other compounds isolated, identified and reported recently from the same plant included arborinine, bergapten, chalepensin,graveoline, kokusaginine, methoxsalen, moskachan B, moskachan D psoralene, neophytadiene scopoletin, γ-fagarine and Pseudane IX (Richardson et al., 2016; Suhaimi et al., 2017; Wahyuni et al., 2014).

5.3 Cytotoxicity activity of extract fractions and isolated compounds

In this study, the cytotoxicity and apoptosis-inducing ability of chalepin and rutamarin in selected human cancer cells were explored. The result, therefore, indicates a dose and time-dependent cytotoxic activity of both chalepin and rutamarin against the tested cancer cell lines. Moreover, developing a new therapeutic product requires an evaluation of the *in vitro* cytotoxicity of the compound as an essential step. Consequently, our study suggests that various fractions exerted growth inhibitory activity against the tested cancer cells. Among the fractions, chloroform showed more promising cytotoxicity and hence selected for isolation of the compounds of interest.

However, doxorubicin which is an established drug used for cancer treatment affect both cancer and the healthy cells, indicating that chalepin could be explored as a better complementary therapy for cancer treatments as it causes no observable toxicity against the healthy cells. It has been reported earlier that most therapeutic drugs used nowadays for cancer treatment remarkably cause damage to healthy cells (Sak, 2014). The present study is consistent with the report of Richardson et al. (2016); who reported remarkable cytotoxicity of chalepin against A549. Similarly, Gurunathan et al. (2013) reported that silver nanoparticles (AgNP) synthesized from *Ganoderma neo-japonicum* Imazeki-induced cytotoxicity in MDA-MB-231 breast cancer cells. Recently, Suhaimi et al., remarkable cytotoxicity of rutamarin a similar compound to chalepin also isolated *Ruta angustifolia* has been observed and reported following treatment on HT29 colon carcinoma cancer cells (Suhaimi et al., 2017).

Interestingly many compounds targeting proteins associated with apoptosis are now developed for cancer treatment. It is essential that any candidate anticancer drug can induce apoptosis in tumour cells and even better if a molecule can be specific in differentiating between cancer and healthy cells (Frankfurt & Krishan, 2003; Kishore & Sathees, 2011).

The present study, therefore, suggests that the cytotoxicity and anti-proliferative activity of the fractions, as well as the isolated compounds, could lead cell death via apoptotic induction and other signalling transduction pathways. It has been well-known that apoptosis is an essential mechanism by which damage or old cells died, and cellular debris are eliminated from the biological system without affecting other tissues. However, cancer cells resist cell death since they avoided apoptosis and continued to proliferate. Therefore, apoptosis manipulation using chemotherapy could be an essential strategy to treat cancer and any compound with cytotoxic ability to overcome apoptosis resistance by cancer cells could be used as potential agent for treatment of cancer.

5.4 Effect of chalepin on cellular morphological changes in MCF7

Cells undergoing apoptosis typically show distinct morphological changes such as membrane blebbing, nuclear fragmentation, condensation of chromatin with associated chromatin margination to the nuclear membrane, as well as formation of apoptotic bodies (Krysko, Berghe, D'Herde, & Vandenabeele, 2008). Our study, therefore, suggested that chalepin treatment promotes apoptosis in MCF7 as observed using phase contrast. Following 24 and 48 hours treatments at different concentrations of chalepin incubation; typical apoptotic features such as reduced number of cells, cell shrinkage, vacuolisation, chromatin condensation, cytoplasmic, nuclear fragmentation; with ultimate formation of apoptotic bodies were well observed in MCF7 cells (Figure 4.7). However, untreated cells were tightly-packed and retained their typical morphology with no noticeable apoptotic features indicating cellular morphology was not affected in the untreated cells.

The cellular morphological changes observed in the chalepin-treated MCF7 was more evident as the concentration increases following 24 and 48 hours treatment. These indicates that the apoptotic induction by chalepin was in a dose- and time-dependent manner. The results obtained in the present study agreed with the report of Richardson et al. (2016), who reported decreased number of cells, vacuolisation, nuclear condensation,

echinoid spikes and apoptotic bodies formation in lung cancer cells (A549) upon treated at different concentration of chalepin. Similarly, cellular morphological changes in HT29 treated with *Phyllanthus watsonii* has been reported (Ramasamy et al., 2012). Suhaimi et al. (2017), has also recently observed and reported cellular morphological alterations following treatment of HT29 with various concentrations of rutamarin at different incubation time. Many other researchers reported typical observable apoptotic characteristics including cell number reduction, vacuolisation, cell shrinkage, detachment of cells from the surface of the culture well and rounding of cells in various cancer cells treated with different extracts and isolated or synthetic compounds (Dikmen et al., 2011; Ho, Karsani, Yong, & Malek, 2013; Jaudan, Sharma, Malek, & Dixit, 2018; Kritsanawong, Innajak, Imoto, & Watanapokasin, 2016).

5.5 Effect of chalepin on nuclear morphological changes in MCF7

Alterations in nuclear morphology were obvious as observed under fluorescence microscopy in Hoechst 33342 and PI doubled stained cells. The chalepin treatment was well correlated with nuclear morphological changes, especially condensation and fragmentation of nuclei, and loss of plasma membrane integrity observed in chalepin-treated MCF7 cells. Similar results were observed and reported in another research using same compound but another cell line (Richardson et al., 2016). Another similar compound (rutamarin) also isolated from *Ruta angustifolia* was reported to induced nuclear morphological changes in HT29 (Suhaimi et al., 2017).

Similarly, chalepin caused nuclear morphological changes resulting in concomitant cell number reductions, the existence of cells with condensed chromatin and dead cells with fragmented DNA. It has been well-known that cells undergoing apoptosis typically shows nuclear fragmentation, condensation of chromatin with associated chromatin magination towards nuclear membrane, as well as formation of apoptotic bodies. It is, therefore, suggested that chalepin treatment promoted apoptosis in MCF7 through these

nuclear morphological alterations which were well observed after 24 and 48 hours incubation (Figure 4.8). These observations were similar to the report of Richardson et al. (2016), who reported mitochondrial mediated apoptosis in lung carcinoma cells induced by chalepin isolated from *Ruta angustifolia*; as well as the report by Shiau et al. (2017), who reported ROS-mediated exosomal activity and protein functions inhibited by phytoagent deoxyelephantopin and its derivative in breast cancer cells.

The nuclear morphological alterations observed in the chalepin-treated MCF7 was more evident as the concentration increases following 24 and 48 hours treatment. These indicated that the apoptotic induction by chalepin in MCF7 was found to be a dose- and time-dependent fashion.

5.6 Chalepin causes increased phosphatidylserine externalisation in MCF7

Typically, cells undergoing early apoptosis usually have their phosphatidylserine exposed to the outer leaflet of the plasma membrane as one of the earliest markers of apoptotic cell death (Sawai & Domae, 2011). .

During apoptosis, phosphatidylserine located in the inner leaflet is translocated to outer leaflet of the plasma membrane thus exposing the apoptotic cells to macrophages attack to initiate early recognition and quick phagocytosis by the neighbouring cells with limited effect to the surrounding tissues (Richardson et al., 2016). Our present study, therefore, suggests that externalisation of phosphatidylserine increases at both early and late apoptosis with increased concentration of chalepin in MCF7-treated while it remained intact in the untreated cells with the uncompromised plasma membrane (Figure 4.8). Furthermore, phosphatidylserine exposed on the outer leaflet resulted in mitochondrial membrane potential alterations that causes protein release from the intermembrane space, caspases activations, and translocation of caspase-activated DNase leading to cleavage of the vital substrate such as DNA and PARP1 as some of the biochemical features that characterized apoptosis (Krysko et al., 2008). The result

suggests dose-dependent early apoptosis increased due to chalepin treatment. It also increased the total Annexin-V positive cells, thus validating the apoptotic inducing ability of chalepin. These observations were consistent with the published reports on mitochondrial mediated apoptosis in lung carcinoma cells induced by chalepin isolated from *Ruta angustifolia* (Richardson et al., 2016), and apoptotic cell death induced by rutamarin isolated from *Ruta angustifolia* on HT29 colon adenocarcinoma cell line (Suhaimi et al., 2017).

The observed phosphatidylserine translocation in the present study also suggests that extensive damage occurred on the cellular membrane with subsequent resulting nuclear staining which further confirmed apoptosis induction due to chalepin treatment in MCF7 (Kishore & Sathees, 2011). The result also summitted that chalepin-cytotoxic activity was mainly due it apoptotic cell death-inducing ability in MCF7. This agreed with the report on the potential cancer chemopreventive and anticancer activity of various chemical constituents isolated from *Ficus hispida* fruits against selected cancer cell lines published by Zhang et al. (2018).

5.7 Chalepin decreased mitochondrial membrane potential (Δψm) in MCF7

Mitochondrial membrane potential (MMP) alteration is a vital biochemical event in the intrinsic pathway which indicates apoptosis. Hence, apoptotic factors are released into the cytosol due to increased mitochondrial membrane permeability (Phang, Karsani, Sethi, & Malek, 2016; Tait & Green, 2010). Mitochondrial-mediated apoptotic mechanism typically causes cellular death through cell self intitiated signal. Conversely, permeability of mitochondrial outer membrane (MOM) has been a critical event which occurs during mitochondrial pathway that leads to apoptosis. This sophisticated event has been shown to be governed by interactions between Bcl-2 family proteins. Therefore, the interplay between both pro-apoptotic and anti-apoptotic Bcl-2 proteins family is believed to facilitate intrinsic apoptotic pathway through controls of both mitochondrial outer

membrane (MOM) integrity as well as neuronal ca^{2+} homeostasis and energetics (Birkinshaw & Czabotar, 2017; D'Orsi, Mateyka, & Prehn, 2017; Zeestraten et al., 2013).

In apoptosis, a decrease in JC-1 signals may indicate either mitochondrial depolarisation or cell death which results in reduced aggregates fluorescent counts in the treated cells. Results from this study revealed significant reduction in fluorescent counts of the aggregates following chalepin treatment on MCF7 cells in comparison to the untreated cells with a resulting loss of MMP (Figure 4.9). It is, therefore, suggested that chalepin treatment on MCF7 may lead to disruption and total loss of mitochondrial membrane integrity. Such activity can result in increased permeability of mitochondrial membrane thereby causing mitochondrial release of pro-apoptotic proteins which includes cytochrome C, Smac/DIABLO and apoptosis-inducing factor (AIF) which subsequently induced binding of apoptotic protease activating factor-1 (Apaf1) to activated caspase-9. The activated caspase-9 consequently results in downstream activation of executioner caspase-3 that triggered cell death through apoptosis in MCF7 chalepin-treated through the intrinsic apoptotic pathway (Kritsanawong et al., 2016; Phang et al., 2016).

The loss of MOM integrity caused by chalepin on MCF7 observed in the present study results as a consequence of mitochondrial membrane potential depolarization which was found to be a time and dose-dependent pattern. The present results substantiate the reports on loss of mitochondrial membrane potential following treatment with various chemicals, plants and plants isolated compounds in different cancer cell types as reported by many researchers (Ansil et al., 2014; Phang et al., 2017; Sharma et al., 2018; Shilpa et al., 2017; Turan et al., 2017).

5.8 Chalepin increased caspase-3 activity in MCF7

Apoptosis mechanism is typically associated with activation of an aspartame-specific class of proteases known as caspases which are usually expressed in an inactive zymogens

form and becomes active upon cleaved by other caspases via activation of caspase cascades (Janicke, 2009). Caspases are classified into two (2): initiators including caspase-2, -8, -9 and -10 and executioners including caspase-3, -6 and -7 caspases that involved in cell death. Among these, caspase-3 is the most crucial in apoptosis induction as it is involved in both mitochondrial and death receptor mediated apoptotic mechanism; and cleavage of the majority of substrates related to apoptosis (Janicke, 2009; Maiese, Chong, Shang, & Wang, 2012; Ouyang et al., 2012). The data from this study shows that caspase-3 activity increased in chalepin-treated MCF7 due to continued cleavage of AMC fluorogenic substrate thereby increasing fluorescent count in chalepin-treated MCF7 cells (Figure 4.11). Our result, therefore suggests that chalepin may induce activation of both caspase-8 and caspase-9 leading to consequent activation of caspase-3 that results to continues cleavage of PARP to cleaved-PARP. Subsequently, cleaved-PARP initiated irreversible apoptosis in MCF7 treated as compared to untreated (Figure 4.17b). Though, it has earlier been reported that MCF7 did not express caspase-3 because of CASP-3 gene deletion (Janicke, 2009; Janicke, Sprengart, Wati, & Porter, 1998). However, other executioners such as caspase-6 or -7 may be involved in the increase of caspase-3-like activity of chalepin in MCF7. The increased expression also confirmed the activation of cleaved-caspase-8, caspase-9, and cleaved-PARP through western blot analysis (Figure 4.17b). A significant dose-dependent increase in the activities of caspase-3, -8 and -9 has also been reported following salidroside treatment in MCF7 breast cancer cells (Ramasamy, 2012; Zhao, Shi, Fan, & Du, 2015). Thus, their reports agree with the result of this study. Caspases including caspase-3, 8 and 9 were found to play vital role for initiation and execution of apoptosis. Thus activation of initiator and executioner caspases results in mitochondrial and death receptor-mediated apoptotic mechanisms (Bedner, Smolewski, Amstad, & Darzynkiewicz, 2000; Maiese et al., 2012; Ouyang et al., 2012). The increased caspase-3 activity observed in the present study also agreed with the earlier

reports (Phang et al., 2017; Richardson et al., 2016; Suhaimi et al., 2017). The result may also suggest that chalepin induces mitochondrial membrane potential alterations causing the release of cytochrome c with subsequent binding to apoptotic proteases activating factor 1 (Apaf1) which further activates executioner caspase-3 and subsequently triggered induction of intrinsic apoptotic pathway. Accordingly, chalepin-induced apoptosis mediated through caspase-3 activation might be associated with both extrinsic and intrinsic apoptotic pathways activation due to caspase-8 and caspase-9 expressions respectively. Similar trends of increase in caspase-3 activity have also been reported (Gurunathan et al., 2013; Lizarte Neto et al., 2013; Sagar et al., 2014).

5.9 Chalepin induces cell cycle arrest in MCF7

Many currently used therapeutic drugs for breast cancer exert their action by various mechanisms which includes specific hormone receptors inhibition, growth factor receptors inhibition and induced cell cycle arrest (Alabsi, Lim, Paterson, Ali-Saeed, & Muharram, 2016; Ang, Nguyen, Sim, Putti, & Lim, 2009; Hu, Zhang, Qiu, Yu, & Lin, 2010). Cytotoxic effect of anticancer drugs is identified to induced cell cycle arrest at specific checkpoints, thus inducing apoptosis as a common mechanism . Most anticancer agents were reported to arrest cell cycle at various stages (G0/G1, S and G2/M phases), with consequence cell death induced through apoptosis (Alabsi et al., 2016). The result of this study, therefore, suggests that chalepin arrest cell cycle in MCF7 cell at S-phase after 24 and 48 hours treatments, with increased accumulated cells in G1 phase (Figure 4.12). It also proposed a relationship between MCF7 cell cycle arrest at S-phase and G2 as well as apoptosis induction which agreed with the earlier reports of Alabi et al, (2016) ; Phang et al, (2016) .

In cell cycle deregulation as a hallmark of cancer, processes coming from internal and external signals are usually coordinated by interactions of various proteins which then decides the fate of a cell to proliferate or differentiate (Suhaimi et al., 2017; Weinberg,

2013). This cellular process is regulated at the cell cycle checkpoints, by which one or more of these checkpoints may be lost in cancer. In this regards, cells are usually arrested at these checkpoints temporarily to repair damaged cells, or if the damaged cell is irreparable, then apoptosis will be induced on the cell (Pietenpol & Stewart, 2002; Suhaimi et al., 2017; Weinberg, 2013). Thus, the result of this study signifies arrest of MCF7 cells at S and G2 phases following chalepin treatment at increasing concentration and time point. The arrest at G2 was more prominent at 48 hours treatment than in 24 hours of treatment.

These results indicate a dose and time dose-dependent pattern with increased accumulated cells at G2-phase that was more evident at 48 hours treatment and a decreased S-phase accumulated cells at 24 and 48 hours in comparison to the untreated cells. Furthermore, results from the present study validate the notion that chalepin causes cell cycle arrest in MCF7-treated cells that might have lead to apoptotic cell death. Thus, this result agreed with the reports on the anticancer activities of salidroside and chalepin on lung cancer; as well as inhibition of human breast cancer by salidroside as earlier published (Richardson et al., 2017; Wang, Li, Lu, Zhang, & Li, 2014a; Zhao et al., 2015).

5.10 Chalepin increased DNA fragmentation in MCF7

Generally, cells undergoing apoptosis are characterized by fragmented DNA as one of the apoptotic features considered to be biochemical hallmark of apoptosis (Zhang & Xu, 2002). It has been observed that early morphological signs of apoptosis are relatively correlated to the formation of DNA ladder and is a widely used marker for detection of apoptosis processes (Kerr, Wyllie, & Currie, 1972; Ramasamy, 2012).

Result from this study has shown that the DNA-laddering pattern in chalepin-treated MCF7 corresponds to the amount of DNA fragmented (Figure 4.13). This result suggests that chalepin could induce stress in MCF7 resulting in DNA damage and fragmentation which is among the typical characteristics of cells undergoing apoptosis. The amount of

fragmented DNA observed commensurate with the amount of TUNEL positive cells which also corresponds to the rate of apoptosis induction in chalepin-treated MCF7 cells. However, there were little or no apoptotic cells with fragmented DNA observed in the untreated cells; in contrast, a similar pattern of DNA fragmentation was observed in doxorubicin (DOX)-treated cells. Apoptosis in cells associated with one of its biochemical hallmarks known as DNA degradation. In this case, endonucleases cleave DNA into double-stranded oligonucleosomal DNA fragments with sizes of about 180 – 200bp (Kyryachenko, Kyrylkova, Leid, & Kioussi, 2012; Kyrylkova, Kyryachenko, Leid, & Kioussi, 2012; Suhaimi et al., 2017).

Therefore, data from this study supported that, the apoptotic inducing ability of chalepin in MCF7 and commensurate with both increased cellular and nuclear morphological alterations, cell cycle arrest, Annexin-V positive cells, loss of mitochondrial membrane potential, caspase-3 activity with consequence release of numerous apoptotic factors. Moreover, several signalling pathways such as proliferation, cell survival, differentiation, angiogenesis and cell death were also affected in MCF7 chalepin-treated which collectively indicates that, cytotoxic effect of chalepin treatment observed was associated with apoptosis induction in MCF7. Thus, the result of this study agreed with the published reports on apoptotic cell death induced by rutamarin isolated from *Ruta angustifolia* on HT29 colon adenocarcinoma cell line (Suhaimi et al., 2017); and inhibition of melanoma cells proliferation by polysaccharide extracted from *Umbilicaria esculenta* through ROS-activated mitochondrial apoptotic pathway (Sun et al., 2018).

5.11 Molecular mechanisms involved in cell death signalling cascades following chalepin and rutamarin treatments against MCF7 and HT29 respectively

Numerous cellular signalling cascades are involved in cell death via apoptosis induction. Apoptosis in cells was strongly regulated by numerous pro- and anti-apoptotic

family proteins. Hence, the balance between dynamic expression changes of these proteins may often trigger apoptosis induction thut determined the fate of a cell to survive or die through induction of apoptosis. In intrinsic apoptotic pathway, the typical released of cytochrome C from the mitochondria is a process that is strongly controlled by Bcl-2 proteins by which anti-apoptotic Bcl-2 protein inhibits cytochrome C release whereas pro-apoptotic Bax protein stimulates cytochrome C release from the mitochondria (Burlacu, 2003; Elkady, 2012). The present study indicates that expressions of the pro-apoptotic family proteins such as Bax, pBad and Bid were increased while Bcl-2 expression was decreased thereby favouring cell death through apoptotic induction in both treated cancer cells (Figure 4.14a & Figure 4.17a).

An earlier study on neocarzinostatin treatment against MCF7 also caused condensation and fragmentation of cellular nuclei with consequent release of cytochrome C into the cytosol from mitochondrial. This result in apoptosis due to decreased level of Bcl-2 with corresponding increased level Bax (Banerjee et al., 2017; Liang, Yan, & Schor, 2001). The result of the study also indicates that pBad, Bid, caspases-3, -8 and -9; and PARP were cleaved suggesting that chalepin and rutamarin triggered apoptosis by stimulating interplay between intrinsic and extrinsic mechanisms.

Previous studies identified two major apoptosis-inducing pathways; intrinsic which induces the cytochrome C released from mitochondrial and extrinsic which involves activation death receptors on the cell surface (Sun et al., 2018). The released cytochrome C usually binds Apaf1 which then activates initiator caspase-9 in the intrinsic mechanism, whereas in the death receptor pathway, activated caspase-8 activates one of the critical death receptors known as Fas/FasL. Finally, the combined activation effect of caspase-8 and caspases-9 activates caspase-3 which disintegrating the cell by various strategies such as inactivating inhibitors of proteins and destroying cellular structures, thus promoting apoptosis (Brentnall et al., 2013; Sun et al., 2018).

The data from this study indicates that chalepin and rutamarin induced stress on the treated cancer cells which might serve as an initiating factor that causes increased expressions and further activate caspases leading to eventual apoptotic cell death. These observations were a time-dependent manner (Figure 4.14b & Figure 4.17b). The present observations also agrees with the observed morphological changes, alteration of mitochondrial membrane potential, DNA fragmentation in this study on MCF7 chalepin-treated; and the earlier report on HT29 rutamarin-treated (Suhaimi et al., 2017). The decreased Bcl-2 family proteins with the corresponding increased pro-apoptotic family proteins as well caspases activation leading to cleavage of various critical cellular substrates such as PARP signifies cell death through apoptosis induction in the treated cells which agree with the published report (Song, Jang, & Kang, 2018).

In this study activation of caspases by both chalepin and rutamarin in the treated cells significantly facilitated cell deaths by apoptosis due to subsequent cleavage of various vital cellular substrates including PARP. These results from activated deadly initiator caspases might be responsible for the activation of the execution caspases with later cleavage of substrates by proteases, thus destroying the treated cells. These caspases activation could indicate both cell surface as well as mitochondrial apoptosis signalling mechanisms due to the treatments. However, the caspases activity in the untreated cells remains significantly unaffected when compared to the treated cells.

The mitochondrial-mediated apoptotic mechanism as triggered by damaged DNA and other cellular stresses are also regulated by an inhibitor of apoptosis proteins (IAPs) which can affect caspases directly (Vaux & Silke, 2005; Yang, Wilson, & Ashkenazi, 2010). IAPs are reported to be overexpressed in various tumours regulating cancer development at different stages. The notable IAPs (c-IAP1 and c-IAP2) typically binds to both caspase-3 and caspase-8 thereby inhibiting their activities. Thus IAPs serves as

antiapoptotic proteins by inhibiting downstream activation of caspase cascades (Richardson et al., 2017; Schimmer, 2004).

Another vital member of apoptosis inhibitor protein family is survivin. The expression of this protein has been found to be downregulated in this study (Figure 4.17c), and the result may imply rutamarin ability for the growth inhibition of HT29 cells which enhance cell death via apoptosis. Survivin has been reported earlier to inhibit caspases activity and other pro-apoptotic signalling molecules including Fas and Bax with consequent apoptosis suppression that stimulates carcinogenesis due to various mechanisms such as prolonging the lifespan of the cells (Kawasaki et al., 1998; Tamm et al., 1998). It is also believed that apoptosis inhibition often associated with tumour aggressiveness and expression of survivin gene which can provide prognosis insight in colorectal carcinomas (Sarela, Macadam, Farmery, Markham, & Guillou, 2000).

Death receptors like Fas as well as the tumour necrosis factors (TNF)-related apoptosis-inducing ligand (TRAIL) receptors; DR4 (TRAIL-R1), and DR5 (TRAIL-R2) usually trigger death signals upon binding to their natural ligands (Wilson et al., 2007). Interestingly, TRAIL-induced apoptosis hallmarks by both extrinsic and intrinsic death pathway which can be regulated at cell surface and mitochondrial level (Mellier, Huang, Shenoy, & Pervaiz, 2010). Specifically, death receptors such as Fas (Tillman, Petak, & Houghton, 1999) and DR5 (Herbeuval et al., 2003) are found to be expressed in colon cancer. Although DR5 is implicated in GANT61-induced cytotoxicity via upregulated mRNA of DR5, the mechanism of transcriptional regulation of DR5 is still unknown (Mazumdar et al., 2011).

In this study, DR4 and DR5 expressions were found to be down-regulated in rutamarin-treated HT29 (Figure 4.17c). This result suggests that death receptors (DR4 and DR5) signalling might not be implicated in the apoptotic-induced cell death in HT29 cell by rutamarin. In contrast, DR4 and DR5 levels have been shown to increase in γ-

humulene-treatment which contributes to apoptosis-induction in HT29 (Lan et al., 2011). Furthermore, a pro-apoptotic agonist of DR4 and DR5 has been implicated in apoptosis induction signalling with consistent selectivity on malignant over healthy cells (Ashkenazi, 2008; Wilson, Dixit, & Ashkenazi, 2009; Yang et al., 2010).

Various cellular, behavioural response to external stimuli is reported to be interceded by mitogen-activated protein kinases (MAPKs) (Koul, Pal, & Koul, 2013). The MAPKs family, SAPK/JNK and P38 are found to be activated in response to stress which then conferred their involvement in apoptosis. Although, apoptosis regulations by MAPKs remains controversial due to its complexity than initially assumed (Wada & Penninger, 2004). Thus the activation of MAPKs such as SAPK/JNK and P38 signalling transduction in this study could be one of the targeted mechanism of chalepin and rutamarin induced cell death in MCF7 and HT29 respectively. Apoptosis is also extremely coordinated by the involvement of some signalling molecules including Bcl-2 proteins family, caspases, and stress kinases such as stress activated-protein kinase/c-Jun N-terminal kinase (SAPK/JNK) which is a member of mitogen-activated protein kinases, (MAPK) family (Verma & Datta, 2012).

SAPK/JNK is critically believed to mediate apoptotic phenomena, though its involvement is quite controversial due to its role as mediator for both pro-apoptotic as well as anti-apoptotic signals which is yet to be completely understood (Sun et al., 2017; Verma & Datta, 2012). In this study, the activation of SAPK/JNK (Figure 4.17c), by rutamarin might suggest its possible participation in the HT29-treated cell death mediated apoptosis signalling pathways. The SAPK/JNK activation may also phosphorylate and regulates other transcription factors which includes NF-κB and p53 towards favouring induction of apoptosis in cancer. Xie *et al.,* (2015), also reported that MAPKs activation involved in dimethyl fumarate (DMF)-induced necroptosis in colon cancer cells. The result agreed with several other earlier reports on the involvement of SAPK/JNK

mediated cell death through apoptosis induction (Chen, 2012; Kong, Kim, Kim, & Seo, 2016; Reyes-Zurita et al., 2011).

Like SAPK/JNK, the activation of p38 signalling in MCF7-treated and HT29-treated (Figure 4.14c & Figure 4.19c) may suggest apoptotic cell death due to stress induced by chalepin and rutamarin in the treated cells. The activation of p38 commensurate with the cleavage of caspases and PARP as observed in this study. It also enhances phosphorylation of Bcl-2 family of anti-apoptotic proteins thereby inhibiting their activity, whereas mitochondrial translocation of the pro-apoptotic proteins such as Bax was promoted. These combined effects resulted in cell death via apoptosis in the treated cancer cells observed in the present study. The activation which was a time-dependent manner, and this agreed with the published report (Liu et al., 2014; Zhou et al., 2014).

P38-MAPK signalling pathways have been earlier reported to involved in various biological processes with different and complicated outcomes of cellular response. These include promotion of cell death, enhancing survival, cell growth, differentiation, migration and invasion (Koul et al., 2013; Wada & Penninger, 2004). Similar to SAPK/JNK, an NF-κB dependent transcription can be regulated by p38 inside the nucleus. It is also required for phosphorylation and activation of Stat1 transcription factor (Zhang & Liu, 2002). Koul *et al*., (2013), reported that a p38 tumour inhibitory effect is associated with both activations of p53 protein and p53-induced apoptosis in addition to its negatively regulating cell cycle progression.

Various cellular insult such as environmental stress, oxidative stress and toxic chemical insults may favourably activate MAP kinases (SAPK/JNK and p38) which can lead to cellular-induced signals. It is also believed that apoptosis regulation may involve phosphorylating both pro- and anti-apoptotic Bcl-2 proteins family by MAP kinases (Cai, Chang, Becker, Bonni, & Xia, 2006). In the present study, chalepin and rutamarin were found to activate MAP kinases resulting from their chemical nature that subsequently

induce apoptosis in the treated cells by regulating both pro- and anti-apoptotic Bcl-2 proteins family and other related apoptosis proteins.

5.12 Cell cycle regulatory mechanism following chalepin and rutamarin treatments against MCF7 and HT29 respectively

One of the focus of the present study was to explore the potential of natural plant agents "chalepin and rutamarin" in cancer therapy and the prognostic role of cell cycle control mechanisms. The progression of cell cycle is a crucial biological mechanism with effective control regulation in normal cells but having becomes dysregulated in almost all transformed and cancer cells (Birjandian, Motamed, & Yassa, 2018; Singh & Agarwal, 2006). Various coordinated and highly conserved proteins regulate it in different species (Goodger, Gannon, Hunt, & Morgan, 1997). The cell cycle regulatory mechanisms determine cellular fate to die or proliferate. Among the hallmarks in cancer cells is the alteration or loss of cell cycle regulatory mechanism which leads to unrestricted cell proliferation (Li et al., 2006; Wang, Wuu, Savas, Patwardhan, & Khan, 1998). The proteins involve include p21, p27, p53, cyclin A, cyclin D as well as cyclin-dependent kinases (CDK2 and CDK4). Therefore, dysregulations of these proteins may eventually lead to abnormal cell cycle progression which can be arrested at different checkpoints thereby halting cellular differentiation and proliferation.

In the present study, chalepin and rutamarin treatments significantly upregulated the expressions of P21, P27 and the tumor suppression protein P53 (Figure 4.15a & Figure 4.18a), whereas the expressions of cyclin A, cyclin D, and the cyclin-dependent kinases (CDK2 and CDK4), were found to be downregulated (Figure 4.15b & Figure 4.18b) in MCF7 and HT29 treated cells respectively. P21, as a direct transcriptional factor of p53, is highly induced by wild-type p53 due to DNA damage (Li et al., 2006). The P21 activation in the present study may mediate growth suppression effect of p53, thus arresting cell cycle at G1/S which stimulate apoptosis induction in the chalepin and

rutamarin treated MCF7 and HT29 respectively. Similarly, downregulation of P21 may be involved in other cellular mechanisms such cellular senescence, terminal differentiation and p53-independent apoptosis (Li et al., 2006; Xiong et al., 1993; Zeng & El-Deiry, 1996). P21 activation may also be responsible for downregulating the cyclins and cyclin-dependent kinases observed in this study (Figure 4.15b & Figure 4.18b) due to its increased stoichiometry in P21-cyclin D1-CDK4 complexes (Li et al., 2006). Moreover, P21 protein was earlier reported with wide range of binding activity against cyclin/CDK complexes with CDK2 complexes having a higher susceptibility to P21 binding inhibition activity (Harper et al., 1995).

The S-phase arrest couple with accumulated cell in the G1-phase in this study might also be related to increased expression of P27 in both chalepin, and rutamarin treated cancer cells (Figure 4.15a & Figure 4.18a). The study indicates that increased expression of P27 might regulate cell growth particularly progression of cells from G1 to S via its ability to induce apoptosis (Wang, Gorospe, Huang, & Holbrook, 1997). Consequently, the result might suggest that chalepin and rutamarin treatments cause S-phase to arrest in the treated cancer cells, hence resulted to cell death through apoptosis due to the treatments.

Wang et al. (1997), reported that $P27^{kip1}$ as a family member of Cip/Kip family is involved in stimulating G1 arrest due to many growths inhibitory signalling responses. The growth inhibitory activity of chalepin and rutamarin treatments might suggest that a tumour suppressive function of $P27^{kip1}$ could be associated with other properties of the protein. This result also suggested increased P27 expression as one of the cyclin-dependent kinase (CDK) inhibitors might have formed complexes through stoichiometric interactions with CDKs like that of P21 thereby decreasing CDKs expressions thus regulating cell cycle progression in the treated cancer cells as observed in this study.

In general, the re-entry of cells into the cell cycle is essentially regulated by CDKs and P21. In addition, P27 plays a crucial role for regulating G1 and S phases cell cycle regulatory checkpoints (Lee, Rhee, Kim, & Pyo, 2018). Cell cycle analysis on MCF7 chalepin-treated in this study and on HT29 rutamarin-treated (as reported by Suhaimi et al., (2017), indicates that cell cycle arrest at S-phase and G1-phase may be mediated via increased expression of cyclin-kinase inhibitors (CKI) (P21 and P27). It is clear from this study that P21 and P27 expressions were upregulated thus regulating cyclins and cyclin-dependent kinases by inhibiting their expressions in the chalepin and rutamarin treated cancer cells. In fact the involvement of P21, P27, P53, cyclins and cyclin-dependent kinases in the regulation of cell cycle mechanism and its relation to apoptosis induction has been well documented where P21 and P27 were reported to form complexes with cyclins and CDKs thus inactivates and inhibits their activity leading to concomitant cell cycle arrested at various checkpoints (Cerqueira et al., 2014; Karimian, Ahmadi, & Yousefi, 2016; Kawauchi et al., 2002).

Inactivation or mutation of the tumour suppressor protein gene p53 is one of the most common alterations identified in breast cancer cell (Lahiry et al., 2010). However, this study suggests chalepin and rutamarin may induce mitochondrial cell death cascades leading apoptosis induction via increased expression of the tumour suppressor protein p53 in both chalepin-treated MCF7 and rutamarin-treated HT29 cancer cells (Figure 4.15a & Figure 4.18a). P53 as a main transcription regulator of P21 has been reported to be upregulated due to many stresses such as oxidative stress and DNA damage which subsequently increases P21 expression (Karimian et al., 2016). It is, therefore, possible that chalepin and rutamarin causes some stress in the treated cancer cells.

Therefore, this study indicates that P53 activity was increased due to chalepin and rutamarin treatments in the treated cancer cells and this might correlate with the increased expressions of both P21 and P27 proteins. The cell cycle arrest is a consequence of P53

activation which then resulted in the expression of $P21^{WAF1/CIP1}$ and P27 via P53-dependent mechanism (Cho et al., 2012; Seoane, Le, & Massagué, 2002). However, the expression of both P21 and P27 potentially inhibited cyclin-dependent kinases (CDKs) by the formation of complexes, hence the decreased expression of both cyclins as well as cyclin-dependent kinases in this study (Figure 4.15b & Figure 4.18b). The suppression of cell proliferation through induction of P53, P21 and P27 in the treated cancer cells in this study agreed with the earlier reports (Birjandian et al., 2018; Cho et al., 2012).

Various cyclins and CDKs were reported earlier to be isolated and synthesized from different species at a different phase of cell cycle progression checkpoints. These proteins were implicated in cell cycle regulation at specific checkpoints (Goodger et al., 1997). The cyclins were found to be positive regulators of cell cycle progression, serving as a positive regulatory subunit of a notable class of proteins, cyclin-dependent kinases (CDKs). The CDKs were identified as the principal regulators of major cell cycle transition in various eukaryotic systems (Resnitzky & Reed, 1995).

The result of this study indicates that cyclin A, cyclin D, CDK2 and CDK4 expressions were all down-regulated due to chalepin and rutamarin treatments on MCF7 and HT29 respectively (Figure 4.15b & Figure 4.18b). The results, therefore, might suggest that the increased P53-dependent expression of P21 and P27 in the treated cancer cells may have interacted and inhibited the activation of cyclins A, cyclin D and the cyclin-dependent kinases (CDK2 & CDK4) analysed. The inhibition resulted from the stoichiometric formation of complexes between either P21 or P27 with the cyclins and CDKs thus affecting the expressions and activation of cyclins and CDKs in the treated cancer cells. The down-regulation, however, was a time-dependent manner.

Birjandian et al. (2018), pointed out that uncontrolled cell division resulted from activated cyclins that binds to CDK leading to cell cycle arrest towards S-phase. It is also thought that CDK activity mainly causes progression of cancer and their function is

strongly regulated by P21 and P27 as cyclin kinase inhibitors (CKI). Based on the present findings, chalepin and rutamarin treatments on the cancer cells inhibited the cyclins-CDK activity since P21 is a universal inhibitor of CDKs and P27 is usually upregulated due to signals from antiproliferative responses such as treatment of chalepin and rutamarin on cancer cells.

The synthesis of cyclin A has been found to occur during S-phase, and the onset of S-phase is associated with activation of cyclin A promoter. In addition, DNA synthesis is also well correlated with the appearance of cyclin A in the cell. Cyclin A is required for S-phase as well as the entry of G2 into mitosis. Similarly, cyclin D is found to be one of the G1 cyclin (Goodger et al., 1997). Therefore, the inactivation of the cyclin A and D observed in the present study might suggest antiproliferative activity of both chalepin and rutamarin treatments on the treated cancer cells which occur through blockage of the cell cycle arrested at different checkpoints.

Since Cip/Kip family of cyclin kinase inhibitors (CKIs) are essential regulators of the cell cycle that inhibit cyclin-CDK complexes activity. The result of the present study revealed overexpression of P21 and P27 due to chalepin and rutamarin treatments both led to G1-phase cell cycle arrest with a consistent role in negative regulation of the G1-phase in the cell cycle (Tang et al., 2018). Furthermore, active cyclin-CDK complexes are required for effective cell cycle progression, thus the overexpression of CKIs may have blocked S-phase entry by decreasing the activity of cyclin D-CDK4, and cyclin A-CDK2 complexe levels in the chalepin and rutamarin treated cancer cells. It has been clearly stated that cell cycle regulatory proteins are fully implicated in the control of cellular progression or arrest via the cell cycle. Hence, the proliferation of cells towards completion of the cell cycle can be achieved by binding of cyclins to activates specific CDKs in each cell cycle phase. Contrary, cell cycle arrest that halted cellular proliferation could be achieved through inhibition of cyclin-CDK complexes by cyclin kinase

inhibitors (Shankland, 1999). Therefore, data from this study might suggest that overexpression of the CKIs such as P21, P27 and the p53 tumour suppressor protein coupled with the inactivation of cyclins. CDKs and cyclin-CDK complexes regulatory mechanisms could lead to cell cycle arrested at different checkpoints, thus halting cellular proliferation with consequent apoptosis induction in the chalepin and rutamarin treated cancer cells which could be an essential prognostic mechanism in cancer therapy.

5.13 Molecular mechanisms involved in cell survival signalling cascades following chalepin and rutamarin treatments against MCF7 and HT29 respectively

Numerous cellular signalling cascades are implicated in regulating cell survival through various coordinated mechanisms. These mechanisms comprise of epidermal growth factor receptor (EGFR) signalling pathway and many downstream cascades which eventually regulate several transcription factors responsible for various cellular signalling responses such as cell proliferation, survival, oncogenesis, angiogenesis, tumorigenesis, inhibition of apoptosis, gene expression and cell cycle progression (Chen et al., 2016; Moerkens et al., 2014). The activation of proteins kinase activity in EGFR due to dimerisation of the receptor results from binding of epidermal growth factor to EGFR pathway has been involved in breast cancer aa a major therapeuthic target for treatment of breast cancer. Several studies reported overexpression of EGFR in many cancer types (Harris & McCormick, 2010; Moerkens et al., 2014), and in most cancers, metastasis resulted in most of the cancer-associated death with many signalling pathways implicated in this process (Duan et al., 2014). In evaluate these mechanisms, the expression of various signalling proteins related to 3 of the signalling pathways were analysed following chalepin and rutamarin treatment in MCF7 and HT29 cancer cells respectively.

Data from the present study implies that the downregulation of EGFR was due to chalepin and rutamarin treatment in the treated cancer cells (Figure 4.16a & 4.20a) which

inhibited phosphorylation of EGFR, thus serving as tyrosine kinase inhibitors thereby stopping the signal transduction by EGFR tyrosine kinase signalling following binding of its epidermal growth factor (EGF) on the receptors. Many studies have been shown to implicate EGFR signalling pathways as an important therapeutic target in many cancer types through the development and recruitment of EGFR tyrosine kinase inhibitors (Cho, 2013; Rolfo et al., 2014; Vecchione, Jacobs, Normanno, Ciardiello, & Tejpar, 2011).

The data also suggests that chalepin and rutamarin inhibited EGFR downstream signalling pathways such as Jak/Stat, Akt/PI3K/mTOR and Erk pathways in MCF7-treated as well as HT29-treated. The compounds acted by inhibiting the phosphorylation of most signalling proteins involved in all the 3 downstream pathways including Jak/Stat pathway (Figure 4.16b & 4.20b), Erk pathway (Figure 4.16c & 4.20c) and PI3K/Akt/mTOR pathway (Figure 4.16d & 4.20d) in both treated cancer cell types. The inhibition of various signalling proteins involved in the signalling pathways ultimately inhibited the activity of many proteins complexes and transcription factors thereby stopping several cellular signalling responses including proliferation, survival, tumorigenesis, invasion, metastasis, angiogenesis, Cell cycle progression and apoptosis inhibition. However, the inhibitory effects exerted by both chalepin and rutamarin on the expression of the signalling proteins in MCF7 and HT29 treated might boost apoptotic cell death in both treated cancer cells. A similar pattern of inhibition of various signalling proteins associated with the EGFR downstream signalling cascades has been observed and reported (Chen et al., 2016; Singh & Bast, 2015; Wu, Wang, Miao, & Gao, 2015).

This study shows that inhibition of PI3K/AKT/mTOR pathway in MCF7 and HT29 treated cells can consequently halt the acquired resistance to EGFR-TK inhibitors as present in adenocarcinoma patients carrying EGFR activating mutations. Chalepin and rutamarin alone or in combination with EGFR TKIs could potentially play a role as irreversible EGFR inhibitors, thus disabling and averting the reversible EGFR TKIs

resistance. It has been reported that site located near the nuclear localisation signal which induced cytoplasmic retention of cell cycle inhibitors can be phosphorylated by AKT thus directly antagonising the functions of the cell cycle inhibitors such P21WAF1 and P27Kip1 (Testa & Bellacosa, 2001). Therefore, chalepin and rutamarin pro-apoptotic and antiproliferative activities in the treated cells might result from induced activation of P53 and P21 by downregulating phosphorylated AKT causing cell cycle arrest, thus eventually leads to apoptotic cell death in the treated cancer cells (Cho et al., 2012).

Similarly, Saiprasad et al. (2014), found that hesperidin treatment modulates the Bax/Bcl-2 ratio, enhancing the released of cytochrome C and activates caspase-3 and -9. Moreover, the P53-P21 axis was enhanced leading to decreased cell cycle regulator. Furthermore, significant PTEN up-regulation with consequence of inhibition of p-PI3K, p-AKT and mTOR were observed due to hesperidin treatment in colon cancer (Saiprasad et al., 2014). An earlier report also pointed out that targeting PI3K, AKT and mTOR using various inhibitor agents in combination with other treatments may perhaps lead to total inhibition of PI3K/Akt/mTOR signalling pathway and can be an evolving treatment strategy for squamous lung cell carcinoma (Beck et al., 2014; Fumarola, Bonelli, Petronini, & Alfieri, 2014). The inactivation of PI3K/Akt/mTOR signalling pathway observed might correlate with the activation of P53 and P21 causing dysregulation of cell cycle regulators.

The study further found that chalepin and rutamarin induced the expression of PTEN (Figure 4.16d & 4.20d) which is an inhibitor of AKT thereby downregulating its expression that might also lead to inactivation of AKT signalling pathway in the treated cancer cells. AKT is a promising targeted candidate. Therefore inhibitors of kinases such as AKT can provide potentially new treatments in cancers. Also, its inhibition obstructs cell proliferation through apoptotic induction. Importantly, activation of AKT has been linked to chemotherapy resistance in lung cancer (Dobashi et al., 2012). Hence, AKT

might be sensitized to chalepin and rutamarin inhibition in the treated cancer cells which then downregulated its activity as observed in this study. Furthermore, signals initiated by PI3K was found to be inhibited by PTEN. However, various mechanisms such as mutation can lead to loss of PTEN activity (De Roock, De Vriendt, Normanno, Ciardiello, & Tejpar, 2011).

Interestingly, Ras, Raf, Mek and Erk (Figure 4.16c & 4.20c) phosphorylation and activation were inhibited following chalepin and rutamarin treatments proposing that these compounds might cause inhibition of cell survival by targeting Erk signalling pathway in the treated cancer cells. Another study reported that KRas, BRaf, PI3K and PTEN mutations were implicated as a targeted therapy in metastatic colorectal cancer (De Roock et al., 2011; Dobashi et al., 2012). Therefore, targeting these signalling proteins with small molecules such as chalepin and rutamarin alone or in combination with other EGFR TKIs could be a promising treatment for various cancer types including colon and breast cancer. Specifically, chalepin and rutamarin inhibition of cRaf observed might binds and induced reduction in Bcl-2 and BAD phosphorylation, thus leading to apoptosis by mitochondrial localisation (Smalley et al., 2009). Neuzillet et al., (2014), higlighted that some regulatory molecules for cell cycle including P16, P15 and P21 can be altered by the Ras-Erk pathway. Thus, the activation of P21 in this study may correlate with inhibition of the Erk signalling pathway observed.

The result of the current study further suggested that chalepin and rutamarin treatment leads to Jak/Stat signalling pathway inactivation (Figure 4.16b & 4.20b) in the treated cancer cells. The inactivation of the signalling proteins will eventually lead to deactivation and block the induction of the transcription factors that cause nuclear gene expression and various possible cellular responses which includes proliferation, survival, proliferation, angiogenesis, invasion, metastasis, and apoptosis inhibition (Rosen et al., 2010). The inactivation of the Jak/Stat signalling pathway is connected to inhibition of

phosphorylation and dimerisation of Stat3 in the treated cancer cells. This stops the nuclear translocation of Stat3 proteins and DNA binding thereby halting the activation of several gene transcriptions implicated in progression of cell cycle (Kusaba et al., 2005; Rosen et al., 2010). Jak is a family of kinases which are activated by many ligands such as interferons and growth factors. The activated Jak kinases family can then activate a class of tyrosine phosphorylated transcription factors known as STATs (Kusaba et al., 2005). One of the STATs family; Stat3 has been reported to be activated by IL-6 or INFα (Darnell, Kerr, & Stark, 1994).

Data in this study confirmed that chalepin and rutamarin inhibited phosphorylation of Stat3 (Figure 4.16b & 4.20b) which is a crucial gene in cell proliferation. Thus, based on this result cell proliferation inhibition was induced by chalepin and rutamarin in both MCF7 and HT29 treated cells through inactivation of the Jak1 kinase which causes inactivation of Stat3 with concomitant decreased expression of phosphorylated Stat3. In contrast, bisphenol A was found to up-regulate Stat3 expression, and its activation stimulate cell growth and proliferation in MCF7 (Zhang et al., 2012a). The data from this study might also suggest that inactivation NF-κB can be attributed to downregulation of the EGFR signalling cascades in the HT29 treated with rutamarin which inhibited the tyrosine kinase activity of EGFR. The overall data suggested that chalepin and rutamarin inhibited cell proliferation and survival in the treated cancer cells through interaction with various signalling proteins thus causing deactivation of the EGFR downstream signalling pathways. This scenario was found to be correlated and interrelated with various activation of pro-apoptotic and deactivation of anti-apoptotic proteins as well as regulation of cell cycle regulatory proteins. These compounds impede cell survival and enhanced apoptotic cell death in the treated cells, and therefore, can be used as anticancer agents.

5.14 Effect of rutamarin treatment on the NF-κB pathway and COX-2 protein in HT29

Nuclear factor κB (NF-κB) is a transcription factor consisting of a various family of transcription factors. It regulates a broad number of biological responses including immune responses, inflammation and oncogenesis. It plays a role in cancer development by the promotion of tumour cells proliferation, suppression of apoptosis, attracts angiogenesis and facilitates metastasis via transition of epithelial-mesenchymal cells (Dolcet, Llobet, Pallares, & Matias-Guiu, 2005; Xia, Shen, & Verma, 2014). Therefore, suppression of NF-κB in tumour cells usually revert a tumour and the NF-κB pathway has earlier been reported to be an important and interesting targeted therapy in cancer therapeutics (Dolcet et al., 2005; Xia et al., 2014). NF-κB usually achieved its biological regulatory activities through interactions with other signalling molecules and pathway such as the transcription factors (STAT3 and P53); and the kinases (GSK3-β, P38 and PI3K) (Hoesel & Schmid, 2013).

The activation of NF-κB that is usually induced by carcinogens and inflammatory agents was found to be suppressed by rutamarin treatment in HT29 cancer cells as observed in this study. Rutamarin was found to act by interacting with IKKα and IKKβ which then inhibited their kinase activity to phosphorylate NF-κB thus inactivating the entire pathway. It also acted through its ability to inhibit phosphorylation of IκBα which is an inhibitor of NF-κB thus causing the pathway to be inactive. However, phosphorylation and proteasome-dependent degradation of IκBα can lead to activated NF-κB pathway. Eventually, the free NF-κB dimers translocate into the nucleous from the cytoplasm and then binds DNA to regulates transcription genes. The findings from this study suggested that rutamarin downregulated the expressions NF-κB, IKKα, IKKβ whereas IκBα was upregulated making it available to interact with NF-κB thereby causing it to be transcriptionally inactive (Figure 4.19a). These eventually halted the entire NF-

κB pathway to be inactive, thus expressing potentiality of rutamarin as antiproliferative agent in HT29 cancer cells. The present result agrees with the report of Richardson et al., (2017), who reported suppression of nuclear factor (NF-κB) following chalepin treatment in non-small lung cancer carcinoma.

It has been reported that some signalling pathways that are triggered by cytokines, growth factors or tyrosine kinases can activate the NF-κB pathway (Xia et al., 2014). Some of which are implicated for the activation of NF-κB pathway include members of tumour necrosis factor receptor, epidermal growth factor receptor, insulin growth factor receptor as well as like Ras/MAPK and PI3k/Akt signalling pathways (Dolcet et al., 2005; Xia et al., 2014). Interestingly, data from this study found out that inactivation of the NF-κB pathway might be responsible for the downregulation and inhibition of the EGFR downstream signalling cascades. The expression of the NF-κB regulated proliferative gene products such as cyclin D1, and COX-2 were also found to be downregulated which implies that rutamarin may help in causing cell death role played by NF-κB pathway (Batool, Aziz, Tan, & Mahmood, 2017; Richardson et al., 2017).

Cyclooxygenases (COXs) and other metabolic enzymes including phospholipase A_2s (PLA_2s) and lipogeneses (LOXs) as well as their metabolic products like prostaglandin and leukotrienes are reported to be considered as a novel targeted therapy for cancer treatment (Yarla et al., 2016). Cyclooxygenases (COX) are enzymes catalysing arachidonic acid conversion to prostaglandins and thromboxane at the rate-limiting step and play important role in inflammation and cancer (Sobolewski, Cerella, Dicato, Ghibelli, & Diederich, 2010). Two isoenzymes of COX were identified; a constitutive form, COX-1 and an inducible form, COX-2. It has been noted that COX-1 and COX-2 inhibition by a well-known inhibitor such as non-steroidal anti-inflammatory drugs (NSAIDs) can decrease the risk of colon cancer (Sobolewski et al., 2010; Vainio, 2001).

COX-2 was found to be induced by proinflammatory and mitogenic stimuli, cytokines, growth factors and tumour enhancers like phorbol esters. COX-2 has been described to control cell proliferation and apoptosis primarily in solid tumours and its expression was reported to increase in colorectal cancers, bladder, cervical, lung, pancreas well as a subset of breast cancers (Morgan & Vainio, 1998; Sobolewski et al., 2010; Vainio, 2001). However, experimental studies in animal models have shown that COX-2 inhibitors can reduce tumour formation in many organs and tissues including bladder, breast, intestine, lung, skin and tongue (Vainio, 2001).

According to data from this study, it is noteworthy that rutamarin might act by inhibition of COX-2 (Figure 4.19b), thus suppressing prostaglandin E2 (PGE2) production which subsequently inhibited cell proliferation, enhance cell death and stops tumour invasion in HT29-treated cancer cells. The reported associated increased of PGE2 resulted from COX-2 overexpression and activation which controls cell death, cell proliferation and tumour invasion in many cancers including breast, colon and lung. The PGE2 can act through various membrane receptors known as EP receptors (EP1, EP2, EP3 and EP4) which triggers diverse signalling pathways (Regulski et al., 2016; Sobolewski et al., 2010). The downregulation of COX-2 by rutamarin in this study (Figure 4.19b), may correlate with the inhibition of NF-κB since NF-κB was found to induced cycloxygenase-2 (COX-2) expression. The COX-2 was repoted to be important in various tumours such as colon carcinoma. It is an immune response and inflammatory-related gene which is associated with cell survival and metastasis (Dolcet et al., 2005; Salehifar & Hosseinimehr, 2016).

In apoptosis, many transcription genes that suppress cell death by both intrinsic as well as extrinsic apoptotic mechanism may be activated by NF-κB (Xia et al., 2014). Moreover, the expression of proteins that interfere with the death receptor apoptotic pathway may also be up-regulated by NF-κB. FLICE-like inhibitory protein (FLIP) is one

of the target protein which has similar homology with caspase-8, although lacking protease activity still competitively binds to Death-Inducing Signaling Complex (DISC) against caspase-8 (Dolcet et al., 2005; Xia et al., 2014). Thus, increased expression of FLIP stops affinity of caspase-8 to the DISC which has been found in several tumours and believed to be responsible for resistance exhibited by some tumours to death receptor apoptosis (Dolcet et al., 2005).

Many other proteins targeted and induced by NF-κB include TNF-α signalling cascades (TRAF2 and TRAF6), Inhibitors of Apoptosis (IAP) (c-IAP1, c-IAP2 and XIAP) and some of the anti-apoptotic Bcl-2 proteins family (Batool et al., 2017; Dolcet et al., 2005; Xia et al., 2014). The induced expressions of these targeted proteins by NF-κB could eventually enhance cancer survival and inhibit apoptosis. However, it is noteworthy data obtained from this study indicates that rutamarin induced remarkable inhibition on NF-κB and its related subunits and other proteins associated with NF-κB activation thereby halted the entire NF-κB pathway. Thus, confirming the anti-proliferative activity and apoptotic enhancing activity of rutamarin in HT29 treated cancer cells.

5.15 Effect of rutamarin treatment on protein profiling in HT29

The result indicates repeatability in the total ion chromatogram (Figure 4.21a & Figure 4.21b) of the sample replicates across both untreated, and the 6-hours treated cancer cells with a remarkable relative abundance attributed to the variance in proteoform signal intensity across the level of samples analysed. Based on the data, it was found that rutamarin treatment significantly decreased the total amount of proteins detected in the 6-hours treated-HT29 as compared to untreated (Figure 4.22a), suggesting that the treatment might have affected the expression of many proteins leading to their absent or low detection in the treated group. However, the identified proteins primary biological processes include metabolic process, regulation of biological process, response to a stimulus, transport, cell organisation and biogenesis (Figure 4.22b). Similarly, most of

the proteins were localised in membrane, cytoplasm, nucleus, cytosol and mitochondria (Figure 4.22c). Moreover, a significant percentage of the proteins were involved in metabolic and regulation of biological process (Figure 4.23a); the data observed also implies that lesser percentage of identified proteins were found in mitochondria (Figure 4.23b). However, the data suggest that proteins identified might play a role in molecular function since significant percentage were found to be involved in protein binding and catalytic activity (Figure 4.23c).

It is noted that proteins are involved in almost all aspect of cellular physiology catalyzing intermediary metabolism within the biochemical reactions or processing and integration of signals from both internal and external stimuli. However, pathological states such as cancer, neurodegenerative disease and metabolic imbalance usually occur due to misregulations of protein expression (Schmidt et al., 2014).

From the histogram, the normal distribution of the samples with the imputed values within the normal distribution and started around the same point in the replicates which are narrower than the distributions of the measured values (Figure 4.24), further supported the reproducibility and reliability of the data obtained from the LC-MS/MS analysis. Heatmap data also indicates that rutamarin treatment in HT29 might have altered some proteins expression such that some proteins have higher intensities whereas others have lower intensities (Figure 4.25). This could have correlated with high and low abundant proteins detected in the samples analysed. The proteins abundance can reflect the dynamic balance among the biological processes which are linked to cellular protein production and maintenance as required by various biological processes (Vogel & Marcotte, 2012).

The Pearson correlation coefficient on multiscatter plot indicates a strong positive correlation between all the samples (Figure 4.26), which can contribute to the reproducibility and reliability of the protein data obtained. The identification of MTCH2

proteins as differentially expressed protein and other proteins with similar expression pattern in the treated cancer cells may be due to rutamarin treatment which can be an essential chemotherapeutic target in colon cancer. Importantly, MTCH2 could be a potential biomarker for colon cancer treatment.

The identification of MTCH2 as the only statistically differentially expressed protein in the rutamarin-treated HT29 cancer cells may be due to the treatment concentration since many other proteins have similar expression pattern based on the profile plot. It is also possible that there are some interferences during sample preparation steps, since sample preparation is fundamental for achieving dependable results as label free proteomic analysis does not usually employ internal standards (Duan et al., 2009; Ono et al., 2006). In label free expression profiling, processing samples in a quantitative manner remains a fundamental challenge due to many reasons (Le Bihan et al., 2006). Effective extraction of proteins biomarkers as well as solubility of some proteins such as membrane proteins often required strong solvents containing substantial amount of detergents necessary to disrupt protein-lipid interaction, dissolve membrane, and achieved efficient protein solubility (Cañas, Piñeiro, Calvo, López-Ferrer, & Gallardo, 2007).

However, removing the detergent and other buffer component used during sample preparations remains a challenge and often causes proteins loss that can harshly compromise quantitative LC/MS analysis. Similarly, some endogenous nonprotein components may be extracted and co-eluted together with the digested protein peptides during LC/MS analysis. This usually, causes unwanted effects including ion suppression and/or interference as well as compromising chromatographic reproducibility. Thus, removing the interfering compounds prior to analysis is critical for achieving reproducible label free quantification (Duan et al., 2009).

Mitochondrial carrier homolog 2 (MTCH2) is a protein that is widely expressed and belongs to mitochondrial carries (MCs) family proteins due to its conserved mito-carrier

domain (Zhang et al., 2000). It is a novel protein found on the outer mitochondrial membrane serving as a receptor-like protein for the BH3-interacting death domain agonist (tBid). It has been shown that MTCH2 play a critical role as receptor-like proteins in Fas-induced apoptosis by recruiting tBid to the mitochondrial with consequence activation of Bax and Bak as well as activation of caspase-3 (Goldman et al., 2015; Robinson, Kunji, & Gross, 2012). Similarly, the conformational change of tBid at the outer mitochondrial membrane (OMM) which is required for Bax activation as well as permeabilization of OMM was found to be facilitated by MTCH2 (Shamas-Din et al., 2013; Shamas-Din et al., 2014).

The differential expression of MTCH2 in the rutamarin-treated HT29 cancer cells suggest that it might contribute to activation of some pro-apoptotic Bcl-2 family proteins (Bid and Bax) and caspases activation (caspase-3, 8 and 9) which causes subsequent cleavage PARP leading to apoptotic cell death observed in this study. Goldman et al. (2015), also reported an observed differential expression of MTCH2 in rat testes which was mainly associated with apoptosis of spermatocytes. However, tumorigenicity and increase invasiveness has been associated with down-regulation of MTCH2 in some cancer types (Arigoni et al., 2013; Huang et al., 2013). Therefore, rutamarin treatment in HT29 might suggest that overexpression of MTCH2 identified can induce apoptotic stimulated cell death in HT29 through tBid recruitment to mitochondria since MTCH2 downregulation impaired tBid recruitment to mitochondria in apoptotic stimulated Hela cells (Raemy et al., 2016).

In addition to apoptosis stimulation through tBid activation, MTCH2 induction might also cause cell growth arrest and loss of tumorigenicity in HT29- treated cancer cells. Thus, the available data may suggest MTCH2 be considered as a novel therapeutic target for cancer treatment (Zhang, Hu, Wang, & Cai, 2017). However, the mechanisms by which MTCH2 induction facilitates apoptosis and cell growth inhibition in rutamarin-

treated HT29 required further study as the current finding of the present study was not sufficient to validate its therapeutic involvement in cancer such as HT29.

CHAPTER 6: CONCLUSION

6.1 General conclusion

In summary, our study demonstrated the cytotoxicity of chalepin and rutamarin against MCF7 breast cancer and HT29 colon cancer cells in a dose- and time-dependent manner. These compounds showed remarkable anti-proliferative and apoptosis inducing ability in the treated cancer cells. Furthermore, the apoptotic cell death-inducing ability of chalepin against MCF7 cancer cell was achieved through loss of mitochondrial membrane potential, increased Annexin-V possible cells, caspase-3 activity, and DNA fragmentation. Chalepin was also found to induce cell cycle arrest at S-phase with an accumulation of cells in G1-phase in MCF7-treated cancer cells. The rate of apoptosis significantly affected the lifespan of both healthy and cancer cells. Therefore, apoptosis regulation and manipulation could be an essential strategy in cancer prevention, management and therapy.

Similarly, the present study also indicated that the anti-proliferative and apoptosis inducing ability was demonstrated by inhibition and activation of various signalling molecules and pathways in both chalepin and rutamarin treated cancer cells. Some molecules and signalling pathways were significantly modulated due to the treatments. These includes various pro-apoptotic proteins activations, downregulated anti-apoptotic proteins, inactivation of the cell cycle proteins (cyclins and CDKs). Others include downregulation of the EGFR signalling cascades and NF-κB pathway.

Furthermore, mitochondrial carrier homolog 2 (MTCH2) (Q9Y6C9) was identified to be differentially expressed in HT29 rutamarin-treated by label-free quantification using LCMS/MS spectrometry. In the same vein, another 19 more proteins were identified with a similar pattern of expression to MTCH2 based on the profile plot indicating that the expressions these proteins might also be affected by rutamarin treatment in HT29 cancer cells.

Therefore, “Chalepin” and “rutamarin” isolated from *Ruta angustifolia* might be considered as potential candidates for development as an anticancer agent in complementary with the current conventional anticancer drugs used for the treatment of various cancer types. However, due to mutations of various signalling molecules among individual cancer patients; and based on the genetic profiling of individual tumours, personalised cancer medicine is regarded as the treatment strategy for the future.

6.2 Further study

The current study explores and explains various mechanisms involved in the antiproliferation and apoptotic activity of chalepin and rutamarin *in vitro*. Therefore, further research is needed to investigate their activity *in vivo* in an animal model to further buttress and support the notion that these compounds could be used as a potential source of anticancer agents.

The differentially expressed protein identified (MTCH2) and other related proteins found to have similar expression according to profile plot in this study can be validated using techniques such as western blot and qPCR. Furthermore, the proteins can be evaluated as potential biomarkers for HT29 colon cancer cell diagnosis and management/treatment. Proteomics analysis using both label-free and labelling quantification could also be conducted on the cancer cells treated with these compounds to investigate proteomic profiling and networks implicated in cell death mechanisms, as this study does not perform in-depth research on this aspect.

REFERENCES

Abu Hassan, M. R., Ismail, I., Suan, M. A. M., Ahmad, F., Khazim, W. K. W., Othman, Z., . . . Mohammed, N. S. (2016). Incidence and mortality rates of colorectal cancer in Malaysia. *Epidemiology and Health, 38*, e2016007.

Abubakar, I. B., Lim, K.-H., Kam, T.-S., & Loh, H.-S. (2016). Synergistic cytotoxic effects of combined δ-tocotrienol and jerantinine B on human brain and colon cancers. *Journal of Ethnopharmacology, 184*, 107-118.

AbuHammad, S., & Zihlif, M. (2013). Gene expression alterations in doxorubicin resistant MCF7 breast cancer cell line. *Genomics, 101*(4), 213-220.

Adesunkanmi, A., Lawal, O., Adelusola, K., & Durosimi, M. (2006). The severity, outcome and challenges of breast cancer in Nigeria. *The Breast, 15*(3), 399-409.

Al-Mekhlafi, F. A., Abutaha, N., Mashaly, A. M. A., Nasr, F. A., Ibrahim, K. E., & Wadaan, M. A. (2017). Biological activity of *Xanthium strumarium* seed extracts on different cancer cell lines and *Aedes caspius*, *Culex pipiens* (Diptera: Culicidae). *Saudi Journal of Biological Sciences, 24*(4), 817-821.

Alabsi, A. M., Lim, K. L., Paterson, I. C., Ali-Saeed, R., & Muharram, B. A. (2016). Cell cycle arrest and apoptosis induction via modulation of mitochondrial integrity by Bcl-2 family members and caspase dependence in *Dracaena cinnabari*-treated H400 human oral squamous cell carcinoma. *BioMed Research International, 2016*, 1-13.

Albarici, T. R., Vieira, P. C., Fernandes, J. B., Silva, M. F. G., & Pirani, J. R. (2010). Cumarinase alcaloides de Rauia resinosa (Rutaceae). *Química Nova, 33*, 2130–2134.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Components of the Cell-Cycle Control System. In *Molecular Biology of the Cell* (4th ed., pp. 351-352). New York: Garland Science.

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

Amar, Z., Abdelwahab, B. B., & Noureddine, G. (2012). Environmental impact on chemical composition and yield of essential oils of Algerian *Ruta montana* (Clus.) L and their antioxidant and antibacterial activities. *Advances in Environmental Biology, 6,* 2684 – 2688.

Ang, E. Z. F., Nguyen, H. T., Sim, H. L., Putti, T. C., & Lim, L. H. K. (2009). Annexin-1 regulates growth arrest induced by high levels of estrogen in MCF-7 breast cancer cells. *Molecular Cancer Research, 7*(2), 266-274.

Ansil, P. N., Wills, P. J., Varun, R., & Latha, M. S. (2014). Cytotoxic and apoptotic activities of *Amorphophallus campanulatus* (Roxb.) Bl. tuber extracts against human colon carcinoma cell line HCT-15. *Saudi Journal of Biological Sciences, 21*(6), 524-531.

Anyanwu, S. N., Egwuonwu, O. A., & Ihekwoaba, E. C. (2011). Acceptance and adherence to treatment among breast cancer patients in Eastern Nigeria. *The Breast, 20*, S51-S53.

Arigoni, M., Barutello, G., Riccardo, F., Ercole, E., Cantarella, D., Orso, F., . . . Merighi, I. (2013). miR-135b coordinates progression of ErbB2-driven mammary carcinomas through suppression of MID1 and MTCH2. *The American Journal of Pathology, 182*(6), 2058-2070.

Ashkenazi, A. (2008). Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nature Reviews Drug Discovery, 7*(12), 1001.

Baeshen, N. A., Elkady, A. I., Abuzinadah, O. A., & Mutwakil, M. H. (2012). Potential anticancer activity of the medicinal herb, *Rhazya stricta*, against human breast cancer. *African Journal of Biotechnology, 11*(37), 8960.

Bak, M. J., Das Gupta, S., Wahler, J., & Suh, N. (2016). Role of dietary bioactive natural products in estrogen receptor-positive breast cancer. *Seminars in Cancer Biology, 40-41*, 170-191.

Balandrin, M. F., Kinghorn, A. D., & Farnsworth, N. R. (1993). *Plant-derived natural products in drug discovery and development: An overview*. In: ACS Publications.

Banerjee, P. P., Bandyopadhyay, A., Harsha, S. N., Policegoudra, R. S., Bhattacharya, S., Karak, N., & Chattopadhyay, A. (2017). Mentha arvensis (Linn.)-mediated green silver nanoparticles trigger caspase 9-dependent cell death in MCF7 and MDA-MB-231 cells. *Breast Cancer-Targets and Therapy, 9*, 265-278.

Bar, J., & Onn, A. (2012). Overcoming molecular mechanisms of resistance to first-generation epidermal growth factor receptor tyrosine kinase inhibitors. *Clinical Lung Cancer, 13*(4), 267-279.

Baserga, R. (1968). Biochemistry of the cell cycle: A review. *Cell Tissue Kinetics, 1*(2), 167-191.

Batool, R., Aziz, E., Tan, B. K.-H., & Mahmood, T. (2017). *Rumex dentatus* inhibits cell proliferation, arrests cell cycle, and induces Apoptosis in MDA-MB-231 cells through suppression of the NF-κB pathway. *Frontiers in Pharmacology, 8*, 731.

Beck, J. T., Ismail, A., & Tolomeo, C. (2014). Targeting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway: An emerging treatment strategy for squamous cell lung carcinoma. *Cancer Treatment Reviews, 40*(8), 980-989.

Becker, W. M., Kleinsmith, L. J., Hardin, J., & Raasch, J. (2003). *The world of the cell. (Vol. 6).* San Francisco: Benjamin Cummings.

Bedner, E., Smolewski, P., Amstad, P., & Darzynkiewicz, Z. (2000). Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): Correlation with DNA fragmentation. *Experimental Cell Research, 259*(1), 308-313.

Bendaoud, H., Romdhane, M., Souchard, J. P., Cazaux, S., & Bouajila, J. (2010). Chemical composition and anticancer and antioxidant activities of *Schinus molle* L. and *Schinus terebinthifolius* Raddi Berries essential oils. *Journal of Food Science, 75*(6), C466-C472.

Bhatia, D., Mandal, A., Nevo, E., & Bishayee, A. (2015). Apoptosis-inducing effects of extracts from desert plants in HepG2 human hepatocarcinoma cells. *Asian Pacific Journal of Tropical Biomedicine, 5*(2), 87-92.

Bian, S., Sun, X., Bai, A., Zhang, C., Li, L., Enjyoji, K., . . . Wu, Y. (2013). P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. *PLoS ONE, 8*(4), e60184.

Birkinshaw, R. W., & Czabotar, P. E. (2017). The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. *Seminars in Cell and Developmental Biology, 72*, 152-162.

Bishayee, A., & Darvesh, A. S. (2012). Pomegranate-derived constituents as inducers of cell death: implications in cancer prevention and therapy. In *Natural compounds as Inducers of Cell Death* (pp. 33-47). Springer.

Bishayee, A., & Sethi, G. (2016). Bioactive natural products in cancer prevention and therapy: Progress and promise. *Seminars in Cancer Biology, 40*, 1-3.

Blagosklonny, M. V., & Pardee, A. B. (2002). The restriction point of the cell cycle. *Cell Cycle, 1*(2), 102-109.

Borris, R. P. (1996). Natural products research: Perspectives from a major pharmaceutical company. *Journal of Ethnopharmacology, 51*(1-3), 29-38.

Bourgou, S., Rebey, I. B., Mkadmini, K., Isoda, H., Ksouri, R., & Ksouri, W. M. (2017). LC-ESI-TOF-MS and GC-MS profiling of *Artemisia herba-alba* and evaluation of its bioactive properties. *Food Research International, 99*, 702-712.

Boyle, P., & Levin, B. (2008). *World cancer report 2008*. IARC Press, International Agency for Research on Cancer.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *A Cancer Journal for Clinicians, 68*(6), 394-424.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E., & Boise, L. H. (2013). Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biology, 14*(1), 32.

Burlacu, A. (2003). Regulation of apoptosis by Bcl-2 family proteins. *Journal of Cellular and Molecular Medicine, 7*(3), 249-257.

Cai, B., Chang, S. H., Becker, E. B., Bonni, A., & Xia, Z. (2006). p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. *Journal of Biological Chemistry, 281*(35), 25215-25222.

Cañas, B., Piñeiro, C., Calvo, E., López-Ferrer, D., & Gallardo, J. M. (2007). Trends in sample preparation for classical and second generation proteomics. *Journal of Chromatography A, 1153*(1-2), 235-258.

Cerqueira, A., Martín, A., Symonds, C. E., Odajima, J., Dubus, P., Barbacid, M., & Santamaría, D. (2014). Genetic characterization of the role of the Cip/Kip family of proteins as cyclin-dependent kinase inhibitors and assembly factors. *Molecular and Cellular Biology, 34*(8), 1452-1459.

Chen, D. S., & Mellman, I. (2017). Elements of cancer immunity and the cancer-immune set point. *Nature, 541*(7637), 321-330.

Chen, F. (2012). JNK-induced apoptosis, compensatory growth, and cancer stem cells. *Cancer Research, 72*(2), 379-386.

Chen, K., Ye, H., Lu, X. J., Sun, B. C., & Liu, Q. (2017). Towards *in Sslico* prediction of the immune-checkpoint blockade response. *Trends in Pharmacological Sciences, 38*(12), 1041-1051.

Chen, W.-Y., Hsieh, Y.-A., Tsai, C.-I., Kang, Y.-F., Chang, F.-R., Wu, Y.-C., & Wu, C.-C. (2011). Protoapigenone, a natural derivative of apigenin, induces mitogen-activated protein kinase-dependent apoptosis in human breast cancer cells associated with induction of oxidative stress and inhibition of glutathione S-transferase π. *Investigational New Drugs, 29*(6), 1347-1359.

Chen, Y. C., & Chen, B. H. (2018). Preparation of curcuminoid microemulsions from *Curcuma longa* L. to enhance inhibition effects on growth of colon cancer cells HT-29. *RSC Advances, 8*(5), 2323-2337.

Chen, Y. J., Lin, K. N., Jhang, L. M., Huang, C. H., Lee, Y. C., & Chang, L. S. (2016). gallic acid abolishes the EGFR/Src/Akt/Erk-mediated expression of matrix metalloproteinase-9 in MCF-7 breast cancer cells. *Chemico-Biological Interactions, 252*, 131-140.

Cho, J. J., Cho, C. L., Kao, C. L., Chen, C. M., Tseng, C. N., Lee, Y. Z., . . . Hong, Y. R. (2012). Crude aqueous extracts of *Pluchea indica* (L.) Less. inhibit proliferation and migration of cancer cells through induction of p53-dependent cell death. *BMC Complementary and Alternative Medicine, 12*, 11.

Cho, W. C. S. (2013). Targeting signaling pathways in lung cancer therapy. *Expert Opinion on Therapeutic Targets, 17*(2), 107-111.

Chung, F. F. L., Tan, P., Raja, V. J., Tan, B. S., Lim, K. H., Kam, T. S., . . . Leong, C. O. (2017). Jerantinine A induces tumor-specific cell death through modulation of splicing factor 3b subunit 1 (SF3B1). *Scientific Reports, 7*, 13.

Ciani, F., Tafuri, S., Troiano, A., Cimmino, A., Fioretto, B. S., Guarino, A. M., . . . Calabro, V. (2018). Anti-proliferative and pro-apoptotic effects of Uncaria tomentosa aqueous extract in squamous carcinoma cells. *Journal of Ethnopharmacology, 211*, 285-294.

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

D'Orsi, B., Mateyka, J., & Prehn, J. H. M. (2017). Control of mitochondrial physiology and cell death by the Bcl-2 family proteins Bax and Bok. *Neurochemistry International, 109*, 162-170.

Dandawate, P. R., Subramaniam, D., Jensen, R. A., & Anant, S. (2016). Targeting cancer stem cells and signaling pathways by phytochemicals: Novel approach for breast cancer therapy. *Seminars in Cancer Biology, 40-41*, 192-208.

Darnell, J. E., Kerr, I. M., & Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science, 264*(5164), 1415-1421.

De Roock, W., De Vriendt, V., Normanno, N., Ciardiello, F., & Tejpar, S. (2011). KRAS, BRAF, PIK3CA, and PTEN mutations: Implications for targeted therapies in metastatic colorectal cancer. *The Lancet Oncology, 12*(6), 594-603.

Del Castillo, J. B., Luis, F. R., & Secundino, M. (1984). *Angustifolin*, a coumarin from *Ruta angustifolia*. *Phytochemistry, 23*(9), 2095-2096.

Denmeade, S. R., & Isaacs, J. T. (1996). Programmed cell death (apoptosis) and cancer chemotherapy. *Cancer Control, 3*(4), 303-309.

DeSantis, C., Ma, J., Bryan, L., & Jemal, A. (2014). Breast cancer statistics, 2013. *A Cancer Journal for Clinicians, 64*(1), 52-62.

Devi, P. U. (2004). Basics of carcinogenesis. *Health Administration, 17*(1), 16-24.

Diederich, M., & Cerella, C. (2016). Non-canonical programmed cell death mechanisms triggered by natural compounds. *Seminars in Cancer Biology, 40-41*, 4-34.

Dikmen, M., Ozturk, N., & Ozturk, Y. (2011). The Antioxidant Potency of *Punica granatum* L. fruit peel reduces cell proliferation and induces apoptosis on breast cancer. *Journal of Medicinal Food, 14*(12), 1638-1646.

DiPaola, R. S. (2002). To arrest or not to G2-M Cell-cycle arrest. *Clinical Cancer Research, 8*(11), 3311-3314.

Dobashi, Y., Kimura, M., Matsubara, H., Endo, S., Inazawa, J., & Ooi, A. (2012). Molecular alterations in AKT and its protein activation in human lung carcinomas. *Human Pathology, 43*(12), 2229-2240.

Dolcet, X., Llobet, D., Pallares, J., & Matias-Guiu, X. (2005). NF-kB in development and progression of human cancer. *Virchows Archives, 446*(5), 475-482.

Du, L., Mandi, F., Jekabsons, M. B., Nagle, D. G., & Zhou, Y. D. (2011). Natural and semisynthetic mammea-type isoprenylated dihydroxycoumarins uncouple cellular respiration. *Journal of Natural Products, 74*(2), 240-248.

Duan, H. Y., Qu, L. K., & Shou, C. C. (2014). Activation of EGFR-PI3K-AKT signaling is required for *Mycoplasma hyorhinis*-promoted gastric cancer cell migration. *Cancer Cell International, 14*, 9.

Duan, X., Young, R., Straubinger, R. M., Page, B., Cao, J., Wang, H., . . . Qu, J. (2009). A straightforward and highly efficient precipitation/on-pellet digestion procedure coupled with a long gradient nano-LC separation and Orbitrap mass spectrometry for label-free expression profiling of the swine heart mitochondrial proteome. *Journal of Proteome Research, 8*(6), 2838-2850.

Ekowati, H., Prasasti, E., & Rastuti, U. (2011). The Active Fraction from *Nigella sativa* and its Activity against T47D Cell Line. *Indonesian Journal of Chemistry, 11*(3), 217-222.

Elangovan, S., Hsieh, T.-C., & Wu, J. M. (2008). Growth Inhibition of Human MDA-MB-231 Breast Cancer Cells by Δ-Tocotrienol is Associated With Loss of Cyclin D1/CDK4 Expression and Accompanying Changes in the State of Phosphorylation of the Retinoblastoma Tumor Suppressor Gene Product. *Anticancer Research, 28*(5A), 2641-2647.

Elkady, A. I. (2012). Crude extract of *Nigella Sativa* inhibits proliferation and induces apoptosis in human cervical carcinoma Hela cells. *African Journal of Biotechnology, 11*(64), 12710-12720.

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.

Fajriah, S., Darmawan, A., Megawati, Hudiyono, S., Kosela, S., & Hanafi, M. (2017). New cytotoxic compounds from *Myristica fatua houtt* leaves against MCF-7 Cell Lines. *Phytochemistry Letters, 20*, 36-39.

Ferhat, M., Kabouche, A., & Kabouche, Z. (2014). Comparative compositions of essential oils of three *Ruta species* growing in different soils. *Journal of Material and Environmental Sciences 5*(3), 735-738.

Ferlay, J., Héry, C., Autier, P., & Sankaranarayanan, R. (2010a). Global burden of breast cancer. In *Breast Cancer Epidemiology* (pp. 1-19). New York, NY: Springer.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2010b). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer, 127*(12), 2893-2917.

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), 359-386.

Foo, Y. Y., Periasamy, V., Kiew, L. V., Kumar, G. G., & Malek, S. N. A. (2017). *Curcuma mangga*-mediated synthesis of gold nanoparticles: Characterization, stability, cytotoxicity, and blood compatibility. *Nanomaterials, 7*(6), 14.

Frankfurt, O. S., & Krishan, A. (2003). Apoptosis-based drug screening and detection of selective toxicity to cancer cells. *Anti-Cancer Drugs, 14*(7), 555-561.

Fumarola, C., Bonelli, M. A., Petronini, P. G., & Alfieri, R. R. (2014). Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. *Biochemical Pharmacology, 90*(3), 197-207.

Galustian, C., & Dalgleish, A. G. (2010). The power of the web in cancer drug discovery and clinical trial design: Research without a laboratory?. *Cancer Informatics, 9*, 31.

Goldman, A., Rodriguez-Casuriaga, R., Gonzalez-Lopez, E., Capoano, C. A., Santinaque, F. F., & Geisinger, A. (2015). MTCH2 is differentially expressed in rat testis and mainly related to apoptosis of spermatocytes. *Cell and Tissue Research, 361*(3), 869-883.

Goodger, N., Gannon, J., Hunt, T., & Morgan, P. (1997). Cell cycle regulatory proteins: An overview with relevance to oral cancer. *Oral Oncology, 33*(2), 61-73.

Gurunathan, S., Raman, J., Malek, S. N. A., John, P. A., & Vikineswary, S. (2013). Green synthesis of silver nanoparticles using *Ganoderma neo-japonicum* imazeki: A potential cytotoxic agent against breast cancer cells. *International Journal of Nanomedicine, 8*, 4399.

Hadi, M. A., Hassali, M. A., Shafie, A. A., & Awaisu, A. (2010). Evaluation of breast cancer awareness among female University Students in Malaysia. *Pharmacy Practice, 8*(1), 29.

Hait, W. N. (2009). Targeted cancer therapeutics. *Cancer Research, 69*(4), 1263-1267.

Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L.-H., Zhang, P., . . . Swindell, E. (1995). Inhibition of cyclin-dependent kinases by p21. *Molecular Biology of the Cell, 6*(4), 387-400.

Harris, T. J., & McCormick, F. (2010). The molecular pathology of cancer. *Nature Reviews Clinical Oncology, 7*(5), 251.

Harvey, A. L., Edrada-Ebel, R., & Quinn, R. J. (2015). The Re-emergence of natural products for drug discovery in the genomics era. *Nature Reviews Drug Discovery, 14*(2), 111-129.

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

Herbeuval, J.-P., Lambert, C., Sabido, O., Cottier, M., Fournel, P., Dy, M., & Genin, C. (2003). Macrophages from cancer patients: Analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis. *Journal of the National Cancer Institute, 95*(8), 611-621.

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

Hoesel, B., & Schmid, J. A. (2013). The complexity of NF-κB signaling in inflammation and cancer. *Molecular Cancer, 12*(1), 86.

Hong, G. W., Hong, S. L., Lee, G. S., Yaacob, H., & Malek, S. N. A. (2016). Non-aqueous extracts of *Curcuma mangga* rhizomes induced cell death in human colorectal adenocarcinoma cell line (HT29) via induction of apoptosis and cell cycle arrest at G 0/G 1 phase. *Asian Pacific Journal of Tropical Medicine, 9*(1), 8-18.

Houghton, P., & Raman, A. (1998). *A laboratory manual for the fractionation of natural extracts*. London: Chapman & Hall.

Houghton, P., Rui, F., Isariya, T., Glyn, S., Peter, J. H., & Lee, C. C. (2007). The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods, 42* 377–387.

Hu, X. L., Zhang, X. Q., Qiu, S. F., Yu, D. H., & Lin, S. X. (2010). Salidroside induces cell-cycle arrest and apoptosis in human breast cancer cells. *Biochemical and Biophysical Research Communications, 398*(1), 62-67.

Huang, B., Belharazem, D., Li, L., Kneitz, S., Schnabel, P., Rieker, R., . . . Müller-Hermelink, H. K. (2013). Anti-apoptotic signature in thymic squamous cell carcinomas–functional relevance of anti-apoptotic BIRC3 expression in the thymic carcinoma cell line 1889c. *Frontiers in Oncology, 3*, 316.

Hudler, P., Kocevar, N., & Komel, R. (2014). Proteomic approaches in biomarker discovery: New perspectives in cancer diagnostics. *The scientific World Journal, 2014*. 1-18.

Ito, N., Hasegawa, R., Imaida, K., Hirose, M., Asamoto, M., & Shirai, T. (1995). Concept in multistage carcinogenesis. *Oncology/Hematology, 21*, 105-133.

Itokawa, H., Morris-Natschke, S. L., Akiyama, T., & Lee, K.-H. (2008). Plant-derived natural product research aimed at new drug discovery. *Journal of Natural Medicines, 62*(3), 263-280.

Janicke, R. U. (2009). MCF-7 breast carcinoma cells do not express caspase-3. *Breast Cancer Research and Treatment, 117*(1), 219-221.

Janicke, R. U., Sprengart, M. L., Wati, M. R., & Porter, A. G. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *Journal of Biological Chemistry, 273*(16), 9357-9360.

Januário, A. H., Vieira, P. V., Da Silva, M. F. G. F., Fernandes, J. B., Silva, J. J. B., & Conserva, L. M. (2009). Alcaloides β-indolopiridoquinazolínicos de *Esenbeckia grandiflora* mart. (Rutaceae). *Química Nova, 32*, 2034–2038.

Jaudan, A., Sharma, S., Malek, S. N. A., & Dixit, A. (2018). Induction of apoptosis by pinostrobin in human cervical cancer cells: Possible mechanism of action. *PLoS ONE, 13*(2), 23.

Jin, J., Son, M., Kim, H., Kim, H., Kong, S. H., Kim, H. K., . . . Han, D. (2018). Comparative proteomic analysis of human malignant ascitic fluids for the development of gastric cancer biomarkers. *Clinical Biochemistry, 56*, 55-61.

Joulain, D., Laurent, R., Fourniol, J., & Yaacob, K. B. (1991). Novel moskachan related compounds in the essential oil of *Ruta angustifolia* Pers. from Malaysia. *Journal of Essential Oil Research, 3*(5), 355-357.

Kalli, A., Smith, G. T., Sweredoski, M. J., & Hess, S. (2013). Evaluation and optimization of mass spectrometric settings during data-dependent acquisition mode: Focus on LTQ-Orbitrap mass analyzers. *Journal of Proteome Research, 12*(7), 3071-3086.

Kammers, K., Cole, R. N., Tiengwe, C., & Ruczinski, I. (2015). Detecting significant changes in protein abundance. *EuPA Open Proteomics, 7*, 11-19.

Karimian, A., Ahmadi, Y., & Yousefi, B. (2016). Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair, 42*, 63-71.

Kawasaki, H., Altieri, D. C., Lu, C.-D., Toyoda, M., Tenjo, T., & Tanigawa, N. (1998). Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Research, 58*(22), 5071-5074.

Kawauchi, S., Goto, Y., Ihara, K., Furuya, T., Oga, A., Tsuneyoshi, M., . . . Sasaki, K. (2002). Survival analysis with p27 expression and apoptosis appears to estimate the prognosis of patients with synovial sarcoma more accurately. *Cancer, 94*(10), 2712-2718.

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

Kim, C. G., Castro-Aceituno, V., Abbai, R., Lee, H. A., Simu, S. Y., Han, Y., . . . Yang, D. C. (2018). Caspase-3/MAPK pathways as main regulators of the apoptotic effect of the phyto-mediated synthesized silver nanoparticle from dried stem of *Eleutherococcus senticosus* in human cancer cells. *Biomedicine and Pharmacotherapy, 99*, 128-133.

Kinjyo, I., Weninger, W., & Hodgkin, P. D. (2015). Shedding light on cell cycle control by T and B lymphocytes. *Cell Cycle, 14*(15), 2381.

Kishore, K. C., & Sathees, C. R. (2011). A natural compound, methyl angolensate, induces mitochondrial pathway of apoptosis in Daudi cells. *Invest New Drugs, 29*, 583–592.

Kleinsmith, L. J. (2006). *Principles of cancer biology*. CA: Pearson Benjamin Cummings. pp. 22–29.

Kong, C. S., Kim, Y. A., Kim, H., & Seo, Y. (2016). Evaluation of a furochromone from the halophyte Corydalis heterocarpa for cytotoxic activity against human gastric cancer (AGS) cells. *Food and Function, 7*(12), 4823-4829.

Koul, H. K., Pal, M., & Koul, S. (2013). Role of p38 MAP kinase signal transduction in solid tumors. *Genes and Cancer, 4*(9-10), 342-359.

Kritsanawong, S., Innajak, S., Imoto, M., & Watanapokasin, R. (2016). Antiproliferative and apoptosis induction of α-mangostin in T47D breast cancer cells. *International Journal of Oncology, 48*(5), 2155-2165.

Krysko, D. V., Berghe, T. V., D'Herde, K., & Vandenabeele, P. (2008). Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods, 44*(3), 205-221.

Kubiliūtė, R., Šulskytė, I., Daniūnaitė, K., Daugelavičius, R., & Jarmalaitė, S. (2016). Molecular features of doxorubicin-resistance development in colorectal cancer CX-1 cell line. *Medicinal, 52*(5), 298-306.

Kulesz-Martin, M. F. (1997). Biological aspects of multistage carcinogenesis as studied in experimental animals and cell culture models. In: Sipes, I.G., McQueen, C.A., Gandolfi, A.J., Bowden, G.T. and Fischer, S.M. In *Comprehensive Toxicology.*: (Eds). Elsevier Science Ltd., UK. pp.7-30.

Kumar, A., Malik, F., Bhushan, S., Sethi, V. K., Shahi, A. K., Kaur, J., . . . Singh, J. (2008). An essential oil and its major constituent isointermedeol induce apoptosis by increased expression of mitochondrial cytochrome c and apical death receptors in human leukaemia HL-60 cells. *Chemico-Biological Interactions, 171*(3), 332-347.

Kumar, D., Mallick, S., Vedasiromoni, J. R., & Pal, B. C. (2010). Anti-leukemic activity of *Dillenia indica* L. fruit extract and quantification of betulinic acid by HPLC. *Phytomedicine, 17*(6), 431-435.

Kumar, V., Abbas, A., & Aster, J. (2013). Cell injury, cell death, and adaptations. *Robbins Basic Pathology 8th*, 1 - 30.

Kusaba, T., Nakayama, T., Yamazumi, K., Yakata, Y., Yoshizaki, A., Nagayasu, T., & Sekine, I. (2005). Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors. *Journal of Clinical Pathology, 58*(8), 833-838.

Kyryachenko, S., Kyrylkova, K., Leid, M., & Kioussi, C. (2012). Immunohistochemistry and detection of proliferating cells by BrdU. *Odontogenesis: Methods and Protocols*, 33-39.

Kyrylkova, K., Kyryachenko, S., Leid, M., & Kioussi, C. (2012). Detection of apoptosis by TUNEL assay. *Odontogenesis: Methods and Protocols*, 41-47.

Laconi, E., Doratiotto, S., & Vineis, P. (2008). The microenvironments of multistage carcinogenesis. *Seminars in Cancer Biology, 18*(5), 322-329.

Lahiry, L., Saha, B., Chakraborty, J., Adhikary, A., Mohanty, S., Hossain, D. M. S., . . . Das, T. (2010). Theaflavins target Fas/caspase-8 and Akt/pBad pathways to induce apoptosis in p53-mutated human breast cancer cells. *Carcinogenesis, 31*(2), 259-268.

Lan, Y.-H., Wu, Y.-C., Wu, K.-W., Chung, J.-G., Lu, C.-C., Chen, Y.-L., . . . Yang, J.-S. (2011). Death receptor 5-mediated TNFR family signaling pathways modulate γ-humulene-induced apoptosis in human colorectal cancer HT29 cells. *Oncology Reports, 25*(2), 419-424.

Latiff, A. A. M. (2010). *Cytotoxic and anti-human papillomavirus activities in selected vegetables.* (Unpublished M. Sc. thesis), University of Malaya, Kuala Lumpur, Malaysia.

Le Bihan, T., Goh, T., Stewart, I. I., Salter, A. M., Bukhman, Y. V., Dharsee, M., . . . Wiśniewski, J. R. (2006). Differential analysis of membrane proteins in mouse fore-and hindbrain using a label-free approach. *Journal of Proteome Research, 5*(10), 2701-2710.

Lee, H. W., Rhee, D. K., Kim, B. O., & Pyo, S. (2018). Inhibitory effect of sinigrin on adipocyte differentiation in 3T3-L1 cells: Involvement of AMPK and MAPK pathways. *Biomedicine and Pharmacotherapy, 102*, 670-680.

Legrain, P., Aebersold, R., Archakov, A., Bairoch, A., Bala, K., Beretta, L., . . . Costello, C. E. (2011). The human proteome project: current state and future direction. *Molecular & Cellular Proteomics, 10*(7), M111. 009993.

Li, J., Zhang, J.-J., Pang, X.-X., ZhengChen, X.-L., & Gan, L.-S. (2014). Biflavanones with anti-proliferative activity against eight human solid tumor cell lines from *Stellera chamaejasme*. *Fitoterapia, 93*, 163-167.

Li, W., Sanki, A., Karim, R. Z., Thompson, J. F., Soon Lee, C., Zhuang, L., . . . Scolyer, R. A. (2006). The role of cell cycle regulatory proteins in the pathogenesis of melanoma. *Pathology, 38*(4), 287-301.

Liang, Y., Yan, C., & Schor, N. F. (2001). Apoptosis in the absence of caspase 3. *Oncogene, 20*(45), 6570.

Lim, A. K. H., & Lim, G. C. C. (1993). *The burden of advanced cancer in Malaysia.* Paper presented at the Proceedings of the National Hospice Conference, Penang, 13-18.

Lim, G. C. C. (2002). Overview of cancer in Malaysia. *Japanese Journal of Clinical Oncology, 32*(suppl 1), S37-S42.

Lim, Y., & Murtijaya, J. (2007). Antioxidant properties of *Phyllanthus amarus* extracts as affected by different drying methods. *LWT-Food Science and Technology, 40*(9), 1664-1669.

Liu, J., Wu, N., Ma, L.-N., Zhong, J.-T., Liu, G., Zheng, L.-H., & Lin, X.-K. (2014). p38 MAPK signaling mediates mitochondrial apoptosis in cancer cells induced by oleanolic acid. *Asian Pacific Journal of Cancer Prevention, 15*(11), 4519-4525.

Liu, Z., Delavan, B., Roberts, R., & Tong, W. (2017). Lessons learned from two decades of anticancer drugs. *Trends in Pharmacological Sciences, 38*(10), 852-872.

Lizarte Neto, F., Tirapelli, D., Ambrosio, S., Tirapelli, C., Oliveira, F., Novais, P., . . . Tirapelli, L. (2013). Kaurene diterpene induces apoptosis in U87 human malignant glioblastoma cells by suppression of anti-apoptotic signals and activation of cysteine proteases. *Brazilian Journal of Medical and Biological Research, 46*(1), 71-80.

Lopez-Perez, J. L., Olmedo, D. A., del Olmo, E., Vasquez, Y., Solis, P. N., Gupta, M. P., & Feliciano, A. S. (2005). Cytotoxic 4-phenylcoumarins from the leaves of *Marila pluricostata*. *Journal of Natural Products, 68*(3), 369-373.

Lyerly, H. K., Abernethy, A. P., Stockler, M. R., Koczwara, B., Aziz, Z., Nair, R., & Seymour, L. (2011). Need for global partnership in cancer care: Perceptions of cancer care researchers attending the 2010 Australia and Asia Pacific Clinical Oncology Research Development Workshop. *Journal of Oncology Practice, 7*(5), 324-329.

Madhuri, S., & Pandey, G. (2009). Some anticancer medicinal plants of foreign origin. *Current Science, 96*(6), 779-783.

Maiese, K., Chong, Z. Z., Shang, Y. C., & Wang, S. (2012). Targeting disease through novel pathways of apoptosis and autophagy. *Expert Opinion on Therapeutic Targets, 16*(12), 1203-1214.

Malek, S. N. A., Shin, S. K., Wahab, N. A., & Yaacob, H. (2009). Cytotoxic components of *Pereskia bleo* (Kunth) DC. (Cactaceae) leaves. *Molecules, 14*(5), 1713-1724.

Malumbres, M., Harlow, E., Hunt, T., Hunter, T., Lahti, J. M., Manning, G., . . . Wolgemuth, D. J. (2009). Cyclin-dependent kinases: A family portrait. *Nature Cell Biology, 11*(11), 1275 - 1276.

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

Massanet, G., Pando, E., Rodriguez-luis, F., & Salva, J. (1987). Synthesis of 3-(1′, 1′-Dimethylallyl) coumarins: Gravelliferone (I), Chalepin (IIa), and Rutamarin (IIb). *ChemInform, 18*(51).

Mazumdar, T., DeVecchio, J., Shi, T., Jones, J., Agyeman, A., & Houghton, J. A. (2011). Hedgehog signaling drives Cellular Survival in Human Colon Carcinoma Cells. *Cancer Research, 71*(3), 1092-1102.

McIntyre, G. I. (2007). Increased cell hydration promotes both tumor growth and metastasis: A biochemical mechanism consistent with genetic signatures. *Medical Hypotheses, 69*(5), 1127-1130.

Mellier, G., Huang, S., Shenoy, K., & Pervaiz, S. (2010). TRAILing death in cancer. *Molecular Aspects of Medicine, 31*(1), 93-112.

Meneses-Sagrero, S. E., Navarro-Navarro, M., Ruiz-Bustos, E., Del-Toro-Sanchez, C. L., Jimenez-Estrada, M., & Robles-Zepeda, R. E. (2017). Antiproliferative activity of spinasterol isolated of *Stegnosperma halimifolium* (Benth, 1844). *Saudi Pharmaceutical Journal, 25*(8), 1137-1143.

Moerkens, M., Zhang, Y., Wester, L., van de Water, B., & Meerman, J. H. (2014). Epidermal growth factor receptor signalling in human breast cancer cells operates parallel to estrogen receptor α signalling and results in tamoxifen insensitive proliferation. *BMC Cancer, 14*(1), 283.

Morgan, G., & Vainio, H. (1998). Barrett's oesophagus, oesophageal cancer and colon cancer: an explanation of the association and cancer chemopreventive potential of non-steroidal anti-inflammatory drugs. *European Journal of Cancer Prevention, 7*(3), 195-199.

Mukhtar, E., Mustafa Adhami, V., Khan, N., & Mukhtar, H. (2012). Apoptosis and autophagy induction as mechanism of cancer prevention by naturally occurring dietary agents. *Current Drug Targets, 13*(14), 1831-1841.

NCI. (2015). *About cancer*. Retrieved on 23 March, 2018 from https://www.cancer.gov/about-cancer/understanding/what-is-cancer.

Nebo, L., Varela, R. M., Molinillo, J. M., Sampaio, O. M., Severino, V. G., Cazal, C. M., . . . Macias, F. A. (2014). Phytotoxicity of alkaloids, coumarins and flavonoids isolated from 11 species belonging to the Rutaceae and Meliaceae families. *Phytochemistry Letters, 8*, 226-232.

Neuzillet, C., Tijeras-Raballand, A., de Mestier, L., Cros, J., Faivre, S., & Raymond, E. (2014). MEK in cancer and cancer therapy. *Pharmacology and Therapeutics, 141*(2), 160-171.

Newman, D. J., & Cragg, G. M. (2016). Natural products as sources of new drugs from 1981 to 2014. *Journal of Natural Product, 79*(3), 629-661.

Ng, S. C., & Wong, S. H. (2013). Colorectal cancer screening in Asia. *British Medical Bulletin, 105*(1), 29-42.

Nishino, M., Ramaiya, N. H., Hatabu, H., & Hodi, F. S. (2017). Monitoring immune-checkpoint blockade: Response evaluation and biomarker development. *Nature Reviews Clinical Oncology, 14*(11), 655-668.

Ogunlaja, O. O., Moodley, R., Singh, M., Baijnath, H., & Jonnalagadda, S. B. (2018). Cytotoxic activity of the bioactive principles from *Ficus burtt-davyi*. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes, 53*(4), 261-275.

Ono, M., Shitashige, M., Honda, K., Isobe, T., Kuwabara, H., Matsuzuki, H., . . . Yamada, T. (2006). Label-free quantitative proteomics using large peptide data sets generated by nanoflow liquid chromatography and mass spectrometry. *Molecular and Cellular Proteomics, 5*(7), 1338-1347.

Orlita, A., Sidwa-Gorycka, M., Kumirska, J., Maliński, E., Siedlecka, E. M., Gajdus, J., . . . Stepnowski, P. (2008). Identification of *Ruta graveolens* L. metabolites accumulated in the presence of abiotic elicitors. *Biotechnology Progress, 24*(1), 128-133.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: A review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation, 45*(6), 487-498.

Palma, S., Zwenger, A. O., Croce, M. V., Abba, M. C., & Lacunza, E. (2016). From molecular biology to clinical trials: toward personalized colorectal cancer therapy. *Clinical Colorectal Cancer, 15*(2), 104-115.

Patel, P. R., Raval, B. P., Karanth, H. A., & Patel, V. R. (2010). Potent antitumor activity of *Rubia cordifolia*. *International Journal of Phytomedicine, 2*(2010), 44-46.

Pavão, F., Castilho, M., Pupo, M., Dias, R., Correa, A., Fernandes, J., . . . Oliva, G. (2002). Structure of Trypanosoma cruzi glycosomal glyceraldehyde-3-phosphate dehydrogenase complexed with chalepin, a natural product inhibitor, at 1.95 Å resolution. *FEBS Letters, 520*(1), 13-17.

Phang, C.-W., Karsani, S. A., & Malek, S. N. A. (2017). Induction of apoptosis and cell cycle arrest by flavokawain C on HT-29 human colon adenocarcinoma via enhancement of reactive oxygen species generation, upregulation of p21, p27, and Gadd153, and inactivation of inhibitor of apoptosis proteins. *Pharmacognosy Magazine, 13*(Suppl 2), S321.

Phang, C.-W., Karsani, S. A., Sethi, G., & Malek, S. N. A. (2016). Flavokawain C inhibits cell cycle and promotes apoptosis, associated with endoplasmic reticulum stress and regulation of MAPKs and Akt signaling pathways in HCT 116 human colon carcinoma cells. *PLoS ONE, 11*(2), e0148775.

Pietenpol, J. A., & Stewart, Z. A. (2002). Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis. *Toxicology, 181*, 475-481.

Pollio, A., De Natale., A., Appetiti, E., Aliotta, G., & Touwaide, A. (2008). Continuity and change in the Mediterranean medical tradition: *Ruta spp*. (rutaceae) in Hippocratic medicine and present practices. *Journal of Ethnopharmacology, 116*, 469 – 482.

Raemy, E., Montessuit, S., Pierredon, S., Van Kampen, A., Vaz, F., & Martinou, J. (2016). Cardiolipin or MTCH2 can serve as tBID receptors during apoptosis. *Cell Death and Differentiation, 23*(7), 1165.

Rai, G., Mishra, S., Suman, S., & Shukla, Y. (2016). Resveratrol improves the anticancer effects of doxorubicin *in vitro* and *in vivo* models: A mechanistic insight. *Phytomedicine, 23*(3), 233-242.

Raina, H., Soni, G., Jauhari, N., Sharma, N., & Bharadvaja, N. (2014). Phytochemical importance of medicinal plants as potential sources of anticancer agents. *Turkish Journal of Botany, 38*(6), 1027-1035.

Ramasamy, S. (2012). *Cytotoxic and apoptotic activities in selected Phyllanthaceae species of Malaysia.* (Unpublished Ph.D. thesis), University of Malaya, Kuala Lumpur, Malaysia.

Ramasamy, S., Wahab, N. A., Zainal Abidin, N., Manickam, S., & Zakaria, Z. (2012). Growth inhibition of human gynecologic and colon cancer cells by *Phyllanthus watsonii* through apoptosis induction. *PLoS ONE, 7*(4), 15.

Reddy, L., Odhav, B., & Bhoola, K. (2003). Natural products for cancer prevention: A global perspective. *Pharmacology and Therapeutics, 99*(1), 1-13.

Reddy, Y. P., Chandrasekhar, K., & Sadiq, M. J. (2015). A study of *Nigella sativa* induced growth inhibition of MCF and HepG2 cell lines: An anti-neoplastic study along with its mechanism of action. *Pharmacognosy Research, 7*(2), 193.

Regulski, M., Regulska, K., Prukała, W., Piotrowska, H., Stanisz, B., & Murias, M. (2016). COX-2 inhibitors: a novel strategy in the management of breast cancer. *Drug Discovery Today, 21*(4), 598-615.

Ren, H., Chen, M., Yue, P., Tao, H., Owonikoko, T. K., Ramalingam, S. S., . . . Sun, S.-Y. (2012). The combination of RAD001 and NVP-BKM120 synergistically inhibits the growth of lung cancer *in vitro* and *in vivo*. *Cancer Letters, 325*(2), 139-146.

Resnitzky, D., & Reed, S. I. (1995). Different roles for cyclins D1 and E in regulation of the G1-to-S transition. *Molecular and Cellular Biology, 15*(7), 3463-3469.

Reyes-Zurita, F. J., Pachon-Pena, G., Lizarraga, D., Rufino-Palomares, E. E., Cascante, M., & Lupianez, J. A. (2011). The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer, 11*, 13.

Richardson, J. S. M., Aminudin, N., & Malek, S. N. A. (2017). Chalepin: A compound from Ruta angustifolia L. pers exhibits cell cycle arrest at S phase, suppresses nuclear factor-kappa B (NF-κB) pathway, signal transducer and activation of transcription 3 (STAT3) phosphorylation and extrinsic apoptotic pathway in non-small cell lung cancer carcinoma (A549). *Pharmacognosy Magazine, 13*(Suppl 3), S489.

Richardson, J. S. M., Sethi, G., Lee, G. S., & Malek, S. N. A. (2016). Chalepin: Isolated from *Ruta angustifolia* L. Pers induces mitochondrial mediated apoptosis in lung carcinoma cells. *BMC Complementary and Alternative Medicine, 16*(1), 389.

Robinson, A. J., Kunji, E. R., & Gross, A. (2012). Mitochondrial carrier homolog 2 (MTCH2): The recruitment and evolution of a mitochondrial carrier protein to a critical player in apoptosis. *Experimental Cell Research, 318*(11), 1316-1323.

Rolfo, C., Giovannetti, E., Hong, D. S., Bivona, T., Raez, L. E., Bronte, G., . . . Germonpre, P. (2014). Novel therapeutic strategies for patients with NSCLC that do not respond to treatment with EGFR inhibitors. *Cancer Treatment Reviews, 40*(8), 990-1004.

Rosen, D. B., Putta, S., Covey, T., Huang, Y.-W., Nolan, G. P., Cesano, A., . . . Fantl, W. J. (2010). Distinct patterns of DNA damage response and apoptosis correlate with Jak/Stat and PI3kinase response profiles in human acute myelogenous leukemia. *PLoS ONE, 5*(8), e12405.

Rouhollahi, E., Moghadamtousi, S. Z., Paydar, M., Fadaeinasab, M., Zahedifard, M., Hajrezaie, M., . . . Awang, K. (2015a). Inhibitory effect of *Curcuma purpurascens* BI. rhizome on HT-29 colon cancer cells through mitochondrial-dependent apoptosis pathway. *BMC Complementary and Alternative Medicine, 15*(1), 15.

Rouhollahi, E., Moghadamtousi, S. Z., Paydar, M., Fadaeinasab, M., Zahedifard, M., Hajrezaie, M., . . . Mohamed, Z. (2015b). Inhibitory effect of *Curcuma purpurascens* BI. rhizome on HT-29 colon cancer cells through mitochondrial-dependent apoptosis pathway. *BMC Complementary and Alternative Medicine, 15*, 12.

Ruddon, R. W. (2007). Cause of cancer. In *Cancer biology* (4th ed., pp. 17-61). New York: Oxford University Press, Inc.

Safarpoor, M., Ghaedi, M., Yousefinejad, M., Javadian, H., Asfaram, A., Ghasemi, Z., . . . Rahimi, D. (2018). Podophyllotoxin extraction from *Linum usitatissimum* plant and its anticancer activity against HT-29, A-549 and MDA-MB-231 cell lines with and without the presence of gold nanoparticles. *Applied Organometallic Chemistry, 32*(2), 13.

Sagar, S., Esau, L., Moosa, B., Khashab, N. M., Bajic, V. B., & Kaur, M. (2014). Cytotoxicity and Apoptosis Induced by a Plumbagin Derivative in Estrogen Positive MCF-7 Breast Cancer Cells. *Anti-Cancer Agents in Medicinal Chemistry, 14*(1), 170-180.

Sahu, N., Meena, S., Shukla, V., Chaturvedi, P., Kumar, B., Datta, D., & Arya, K. R. (2018). Extraction, fractionation and re-fractionation of *Artemisia nilagirica* for anticancer activity and HPLC-ESI-QTOF-MS/MS determination. *Journal of Ethnopharmacology, 213*, 72-80.

Saiprasad, G., Chitra, P., Manikandan, R., & Sudhandiran, G. (2014). Hesperidin induces apoptosis and triggers autophagic markers through inhibition of Aurora-A mediated phosphoinositide-3-kinase/Akt/mammalian target of rapamycin and glycogen synthase kinase-3 beta signalling cascades in experimental colon carcinogenesis. *European Journal of Cancer, 50*(14), 2489-2507.

Sak, K. (2014). Cytotoxicity of dietary flavonoids on different human cancer types. *Pharmacognosy Reviews, 8*(16), 122.

Salehifar, E., & Hosseinimehr, S. J. (2016). The use of cyclooxygenase-2 inhibitors for improvement of efficacy of radiotherapy in cancers. *Drug Discovery Today, 21*(4), 654-662.

Sallam, R. M. (2015). Proteomics in cancer biomarkers discovery: Challenges and applications. *Disease Markers, 2015*, 1-12.

Sanders, K., Moran, Z., Shi, Z. X., Paul, R., & Greenlee, H. (2016). Natural products for cancer prevention: Clinical update 2016. *Seminars in Oncology Nursing, 32*(3), 215-240.

Sano, Y., Byeon, J. S., Li, X. B., Wong, M. C., Chiu, H. M., Rerknimitr, R., . . . Iwatate, M. (2016). Colorectal cancer screening of the general population in East Asia. *Digestive Endoscopy, 28*(3), 243-249.

Sarela, A., Macadam, R., Farmery, S., Markham, A., & Guillou, P. (2000). Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. *Gut, 46*(5), 645-650.

Savino, R., Paduano, S., Preianò, M., & Terracciano, R. (2012). The proteomics big challenge for biomarkers and new drug-targets discovery. *International Journal of Molecular Sciences, 13*(11), 13926-13948.

Sawai, H., & Domae, N. (2011). Discrimination between primary necrosis and apoptosis by necrostatin-1 in Annexin V-positive/propidium iodide-negative cells. *Biochemical and Biophysical Research Communications, 411*(3), 569-573.

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

Schmidt, A., Forne, I., & Imhof, A. (2014). Bioinformatic analysis of proteomics data. *BMC Systems Biology, 8*(2), S3.

Scholzová, E., Malík, R., Ševčík, J., & Kleibl, Z. (2007). RNA regulation and cancer development. *Cancer Letters, 246*(1), 12-23.

Seoane, J., Le, H.-V., & Massagué, J. (2002). Myc suppression of the p21 Cip1 Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature, 419*(6908), 729.

Seshacharyulu, P., Ponnusamy, M. P., Haridas, D., Jain, M., Ganti, A. K., & Batra, S. K. (2012). Targeting the EGFR signaling pathway in cancer therapy. *Expert Opinion on Therapeutic Targets, 16*(1), 15-31.

Shamas-Din, A., Bindner, S., Zhu, W., Zaltsman, Y., Campbell, C., Gross, A., . . . Fradin, C. (2013). tBid undergoes multiple conformational changes at the membrane required for Bax activation. *Journal of Biological Chemistry*, M113. 482109.

Shamas-Din, A., Satsoura, D., Khan, O., Zhu, W., Leber, B., Fradin, C., & Andrews, D. (2014). Multiple partners can kiss-and-run: Bax transfers between multiple membranes and permeabilizes those primed by tBid. *Cell Death and Disease, 5*(6), e1277.

Shankland, S. J. (1999). Cell cycle regulatory proteins in glomerular disease. *Kidney International, 56*(4), 1208-1215.

Sharma, N., Kumar, A., Sharma, P. R., Qayum, A., Singh, S. K., Dutt, P., . . . Vishwakarma, R. (2018). A new clerodane furano diterpene glycoside from *Tinospora cordifolia* triggers autophagy and apoptosis in HCT-116 colon cancer cells. *Journal of Ethnopharmacology, 211*, 295-310.

Shiau, J.-Y., Chang, Y.-Q., Nakagawa-Goto, K., Lee, K.-H., & Shyur, L.-F. (2017). Phytoagent deoxyelephantopin and its derivative inhibit triple negative breast cancer cell activity through ROS-mediated exosomal activity and protein functions. *Frontiers in Pharmacology, 8*, 398.

Shilpa, G., Renjitha, J., Saranga, R., Sajin, F. K., Nair, M. S., Joy, B., . . . Priya, S. (2017). Epoxyazadiradione purified from the *Azadirachta indica* seed induced mitochondrial apoptosis and inhibition of NFB nuclear translocation in human cervical cancer cells. *Phytotherapy Research, 31*(12), 1892-1902.

Singh, N., Krishnakumar, S., Kanwar, R. K., Cheung, C. H. A., & Kanwar, J. R. (2015). Clinical aspects for survivin: A crucial molecule for targeting drug-resistant cancers. *Drug Discovery Today, 20*(5), 578-587.

Singh, P., & Bast, F. (2015). Screening and biological evaluation of myricetin as a multiple target inhibitor insulin, epidermal growth factor, and androgen receptor; *in silico* and *in vitro*. *Investigational New Drugs, 33*(3), 575-593.

Singh, R. P., & Agarwal, R. (2006). Natural flavonoids targeting deregulated cell cycle progression in cancer cells. *Current Drug Targets, 7*(3), 345-354.

Sinha, D., Sarkar, N., Biswas, J., & Bishayee, A. (2016). Resveratrol for breast cancer prevention and therapy: Preclinical evidence and molecular mechanisms. *Seminars in Cancer Biology, 40-41*, 209-232.

Smalley, K. S., Xiao, M., Villanueva, J., Nguyen, T. K., Flaherty, K. T., Letrero, R., . . . Nathanson, K. L. (2009). CRAF inhibition induces apoptosis in melanoma cells with non-V600E BRAF mutations. *Oncogene, 28*(1), 85.

Sobolewski, C., Cerella, C., Dicato, M., Ghibelli, L., & Diederich, M. (2010). The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies. *International Journal of Cell Biology, 2010*, 1-21.

Song, H. S., Jang, S., & Kang, S. C. (2018). Bavachalcone from *Cullen corylifolium* induces apoptosis and autophagy in HepG2 cells. *Phytomedicine, 40*, 37-47.

Song, P., Yang, X. Z., & Yuan, J. Q. (2013). Cytotoxic constituents from *Psoralea corylifolia*. *Journal of Asian Natural Products Research, 15*(6), 624-630.

Steen, H., & Pandey, A. (2002). Proteomics goes quantitative: Measuring protein abundance. *Trends in Biotechnology, 20*(9), 361-364.

Subbaraju, G. V., Vanisree, M., Rao, C. V., Sivaramakrishna, C., Sridhar, P., Jayaprakasam, B., & Nair, M. G. (2006). Ashwagandhanolide, a bioactive dimeric thiowithanolide isolated from the roots of *Withania somnifera*. *Journal of Natural Products, 69*(12), 1790-1792.

Suhaimi, S. A., Hong, S. L., & Malek, S. N. A. (2017). Rutamarin, an active constituent from *Ruta angustifolia* Pers., induced apoptotic cell death in the HT29 colon adenocarcinoma cell line. *Pharmacognosy Magazine, 13*(Suppl 2), S179.

Sun, W., Wu, X. X., Gao, H. W., Yu, J., Zhao, W. W., Lu, J. J., . . . Chen, X. P. (2017). Cytosolic calcium mediates RIP1/RIP3 complex-dependent necroptosis through JNK activation and mitochondrial ROS production in human colon cancer cells. *Free Radical Biology and Medicine, 108*, 433-444.

Sun, Y. H., Li, J., Zhang, Y., Tu, Y. T., Huang, C. Z., Tao, J., . . . Yang, L. (2018). The polysaccharide extracted from *Umbilicaria esculenta* inhibits proliferation of melanoma cells through ROS-activated mitochondrial apoptosis pathway. *Biological and Pharmaceutical Bulletin, 41*(1), 57-64.

Sylla, B. S., & Wild, C. P. (2012). A million africans a year dying from cancer by 2030: What can cancer research and control offer to the continent?. *International Journal of Cancer, 130*(2), 245-250.

Tabasi, N., Mahmoudi, M., Rastin, M., Sadeghnia, H. R., Mashhadi, M. H., Rabe, S. Z. T., & Rad, A. K. (2015). Cytotoxic and apoptogenic properties of *Nigella sativa* and thymoquinone, its constituent, in human renal cell carcinoma are comparable with cisplatin. *Food and Agricultural Immunology, 26*(1), 138-156.

Tacar, O., Sriamornsak, P., & Dass, C. R. (2013). Doxorubicin: An update on anticancer molecular action, toxicity and novel drug delivery systems. *Journal of Pharmacy and Pharmacology, 65*(2), 157-170.

Tait, S. W., & Green, D. R. (2010). Mitochondria and cell death: Outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology, 11*(9), 621-632.

Tamm, I., Wang, Y., Sausville, E., Scudiero, D. A., Vigna, N., Oltersdorf, T., & Reed, J. C. (1998). IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Research, 58*(23), 5315-5320.

Tamokou, J., Mbaveng, A., & Kuete, V. (2017). Antimicrobial activities of African medicinal spices and vegetables. *Medicinal spices and vegetables from Africa: Therapeutic Potential against Metabolic, Inflammatory, Infectious and Systemic Diseases*, 207.

Tan, Q.-G., & Luo, X.-D. (2011). *Meliaceous limonoids*: Chemistry and biological activities. *Chemical Reviews, 111*(11), 7437-7522.

Tanaka, T. (1997). Chemoṕrevention of human cancer: Biology and therapy. *Critical Reviews in Oncology/Hematology, 25*(3), 139-174.

Tanaka, T. (2009). Colorectal carcinogenesis: Review of human and experimental animal studies. *Journal of Carcinogenesis, 8*, 5.

Tang, X. F., Zhou, X. L., Zhang, Q., Chen, P., Lu, C., & Pan, M. H. (2018). Bombyx mori cyclin-dependent kinase inhibitor is involved in regulation of the silkworm cell cycle. *Insect Molecular Biology, 27*(3), 404-413.

Testa, J. R., & Bellacosa, A. (2001). AKT plays a central role in tumorigenesis. *Proceedings of the National Academy of Sciences, 98*(20), 10983-10985.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science, 267*(5203), 1456-1462.

Thongdeeying, P., Itharat, A., Umehara, K., & Ruangnoo, S. (2016). A novel steroid and cytotoxic constituents from *Dioscorea membranacea Pierre* against hepatocellular carcinoma and cholangiocarcinoma cells. *Journal of Ethnopharmacology, 194*, 91-97.

Thornton, T. M., & Rincon, M. (2009). Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *International Journal of Biological Sciences, 5*(1), 44 - 52.

Tillman, D. M., Petak, I., & Houghton, J. A. (1999). A Fas-dependent component in 5-fluorouracil/leucovorin-induced cytotoxicity in colon carcinoma cells. *Clinical Cancer Research, 5*(2), 425-430.

Topalian, S. L., Taube, J. M., Anders, R. A., & Pardoll, D. M. (2016). Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nature Reviews Cancer, 16*(5), 275-287.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., & Jemal, A. (2015). Global cancer statistics, 2012. *A Cancer Journal for Clinicians, 65*(2), 87-108.

Turan, I., Demir, S., Kilinc, K., Burnaz, N. A., Yaman, S. O., Akbulut, K., . . . Deger, O. (2017). Antiproliferative and apoptotic effect of *Morus nigra* extract on human prostate cancer cells. *Saudi Pharmaceutical Journal, 25*(2), 241-248.

Ulubelen, A., & Ozturk, M. (2006). Alkaloids and coumarins from *Ruta species*. *Natural Product Communications, 1*(10), 851-857.

UNSCEAR. (2000). *Sources and effects of ionizing radiation* (9211422388). Retrieved from United Nations Scientific Committee on the Effects of Atomic Radiation, 2000 Report to the General Assembly, United Nations. New York. United Nations Publications.

Upton, A. C., Albert, R. E., Burns, F. J., & Shore, R. E. (1986). *Radiation Carcinogenesis*. New York: Elsevier. pp. 1-10.

Vainio, H. (2001). Is COX-2 inhibition a panacea for cancer prevention? *International Journal of Cancer, 94*(5), 613-614.

van den Heuvel, S. (2005). Cell-cycle regulation. In (September 21, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.28.1, http://www.wormbook.org.

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

Van Schaeybroeck, S., Kalimutho, M., Dunne, P. D., Carson, R., Allen, W., Jithesh, P. V., . . . Blayney, J. (2014). ADAM17-dependent c-MET-STAT3 signaling mediates resistance to MEK inhibitors in KRAS mutant colorectal cancer. *Cell Reports, 7*(6), 1940-1955.

Vasudevan, D., Sreekumari, S., & Kannan, V. (2017). Biochemistry of Cancer. In *Textbook of Biochemistry for Medical Students* (8th ed., pp. 661-671). New Delhi, India: Jaypee Brothers Medical Publishers.

Vaux, D. L., & Silke, J. (2005). IAPs, RINGs and ubiquitylation. *Nature Reviews Molecular Cell Biology, 6*(4), 287.

Vecchione, L., Jacobs, B., Normanno, N., Ciardiello, F., & Tejpar, S. (2011). EGFR-targeted therapy. *Experimental Cell Research, 317*(19), 2765-2771.

Verma, G., & Datta, M. (2012). The critical role of JNK in the ER - mitochondrial crosstalk during apoptotic cell death. *Journal of Cellular Physiology, 227*(5), 1791-1795.

Vineis, P., & Wild, C. P. (2014). Global cancer patterns: causes and prevention. *The Lancet, 383*(9916), 549-557.

Vlaisavljevic, S., Sibul, F., Sinka, I., Zupko, I., Ocsovszki, I., & Jovanovic-Santa, S. (2018). Chemical composition, antioxidant and anticancer activity of licorice from *Fruska gora* locality. *Industrial Crops and Products, 112*, 217-224.

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics, 13*(4), 227.

Wada, T., & Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene, 23*(16), 2838.

Wahyuni, T. S., Widyawaruyanti, A., Lusida, M. I., Fuad, A., Soetjipto, Fuchino, H., . . . Hotta, H. (2014). Inhibition of hepatitis C virus replication by chalepin and pseudane IX isolated from *Ruta angustifolia* leaves. *Fitoterapia, 99*, 276-283.

Wang, C.-Z., He, H., Wang, X., & Yuan, C.-S. (2012). Trends in scientific publications of Chinese medicine. *The American Journal of Chinese Medicine, 40*(06), 1099-1108.

Wang, J., Li, J. Z., Lu, A. X., Zhang, K. F., & Li, B. J. (2014a). Anticancer effect of salidroside on A549 lung cancer cells through inhibition of oxidative stress and phospho-p38 expression. *Oncology Letters, 7*(4), 1159-1164.

Wang, S., Wuu, J., Savas, L., Patwardhan, N., & Khan, A. (1998). The role of cell cycle regulatory proteins, cyclin D1, cyclin E, and p27 in thyroid carcinogenesis. *Human Pathology, 29*(11), 1304-1309.

Wang, W., Bu, B., Xie, M., Zhang, M., Yu, Z., & Tao, D. (2009). Neural cell cycle dysregulation and central nervous system diseases. *Progress in Neurobiology, 89*(1), 1-17.

Wang, X., Gorospe, M., Huang, Y., & Holbrook, N. J. (1997). p27 Kip1 overexpression causes apoptotic death of mammalian cells. *Oncogene, 15*(24), 2991.

Wang, X. H., Bastow, K. F., Sun, C. M., Lin, Y. L., Yu, H. J., Don, M. J., . . . Lee, K. H. (2004). Antitumor agents. 239. isolation, structure elucidation, total synthesis, and anti-breast cancer activity of neo-tanshinlactone from *Salvia miltiorrhiza*. *Journal of Medicinal Chemistry, 47*(23), 5816-5819.

Wang, Z.-x., Cheng, M.-c., Zhang, X.-z., Hong, Z.-l., Gao, M.-z., Kan, X.-x., . . . Xiao, H.-b. (2014b). Cytotoxic biflavones from *Stellera chamaejasme*. *Fitoterapia, 99*, 334-340.

Weber, G. (2001). Ordered biochemical program of gene expression in cancer cells. *Biochemistry-Moscow, 66*(10), 1164-1173.

Weidner, C., Rousseau, M., Plauth, A., Wowro, S., Fischer, C., Abdel-Aziz, H., & Sauer, S. (2015). *Melissa officinalis* extract induces apoptosis and inhibits proliferation in colon cancer cells through formation of reactive oxygen species. *Phytomedicine, 22*(2), 262-270.

Weinberg, R. A. (2013). *p53 and apoptosis: Master guardian and executioner* (2nd ed.). UK: Garland Science.

Wilson, N. S., Dixit, V., & Ashkenazi, A. (2009). Death receptor signal transducers: Nodes of coordination in immune signaling networks. *Nature Immunology, 10*(4), 348.

Wilson, T. R., McLaughlin, K. M., McEwan, M., Sakai, H., Rogers, K. M. A., Redmond, K. M., . . . Longley, D. B. (2007). c-FLIP: A key regulator of colorectal cancer cell death. *Cancer Research, 67*(12), 5754-5762.

Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental and Clinical Cancer Research, 30*(1), 87.

Wu, T. S., Shi, L. S., Wang, J. J., Iou, S. C., Chang, H. C., Chen, Y. P., . . . Tenge, C. M. (2003). Cytotoxic and antiplatelet aggregation principles of *Ruta graveolens*. *Journal of the Chinese Chemical Society, 50*(1), 171-178.

Wu, Y. T., Wang, B. J., Miao, S. W., & Gao, J. J. (2015). Picropodophyllin inhibits the growth of Ewing's sarcoma cells through the insulin-like growth factor-1 receptor/Akt signaling pathway. *Molecular Medicine Reports, 12*(5), 7045-7050.

Xia, Y., Shen, S., & Verma, I. M. (2014). NF-κB, an active player in human cancers. *Cancer Immunology Research, 2*(9), 823-830.

Xie, X., Zhao, Y., Ma, C. Y., Xu, X. M., Zhang, Y. Q., Wang, C. G., . . . Dong, D. L. (2015). Dimethyl fumarate induces necroptosis in colon cancer cells through GSH depletion/ROS increase/MAPKs activation pathway. *British Journal of Pharmacology, 172*(15), 3929-3943.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature, 366*(6456), 701.

Xu, B., Wang, L., González-Molleda, L., Wang, Y., Xu, J., & Yuan, Y. (2014). Antiviral activity of (+)-rutamarin against Kaposi's sarcoma-associated herpesvirus by inhibition of the catalytic activity of human topoisomerase II. *Antimicrobial Agents and Chemotherapy, 58*(1), 563-573.

Xu, Y.-c., Liu, X., Li, M., Li, Y., Li, C.-y., Lu, Y., . . . Mao, L.-m. (2018). A Novel Mechanism of Doxorubicin Resistance and Tumorigenesis Mediated by MicroRNA-501-5p-Suppressed BLID. *Molecular Therapy Nucleic Acids, 12*, 578-590.

Yang, A., Wilson, N. S., & Ashkenazi, A. (2010). Proapoptotic DR4 and DR5 signaling in cancer cells: toward clinical translation. *Current Opinion in Cell Biology, 22*(6), 837-844.

Yang, F., Teves, S. S., Kemp, C. J., & Henikoff, S. (2014). Doxorubicin, DNA torsion, and chromatin dynamics. *Biochimica et Biophysica Acta Reviews on Cancer, 1845*(1), 84-89.

Yang, Q. Y., Tian, X. Y., & Fang, W. S. (2007). Bioactive coumarins from *Boenninghausenia sessilicarpa*. *Journal of Asian Natural Products Research, 9*(1), 59-65.

Yarla, N. S., Bishayee, A., Sethi, G., Reddanna, P., Kalle, A. M., Dhananjaya, B. L., . . . Duddukuri, G. R. (2016). Targeting arachidonic acid pathway by natural products for cancer prevention and therapy. *Seminars in Cancer Biology, 40-41*, 48-81.

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

Zeestraten, E. C., Benard, A., Reimers, M. S., Schouten, P. C., Liefers, G. J., Van de Velde, C. J., & Kuppen, P. J. (2013). The prognostic value of the apoptosis pathway in colorectal cancer: A review of the literature on biomarkers identified by immunohistochemistry. *Biomarkers in Cancer, 5*, S11475.

Zeng, Y.-X., & El-Deiry, W. S. (1996). Regulation of p21WAF1/CIP1 expression by p53-independent pathways. *Oncogene, 12*(7), 1557-1564.

Zhang, B., Huang, R. Z., Hua, J., Liang, H., Pan, Y. M., Dai, L. M., . . . Wang, H. S. (2016). Antitumor lignanamides from the aerial parts of *Corydalis saxicola*. *Phytomedicine, 23*(13), 1599-1609.

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

Zhang, J., & Xu, M. (2002). Apoptotic DNA fragmentation and tissue homeostasis. *Trends Cell Biology, 12*(2), 84-89.

Zhang, J., Zhu, W. F., Xu, J., Kitdarnrongtham, W., Manosroi, A., Manosroi, J., . . . Feng, F. (2018). Potential cancer chemopreventive and anticancer constituents from the fruits of *Ficus hispida* L.f. (Moraceae). *Journal of Ethnopharmacology, 214*, 37-46.

Zhang, Q.-H., Ye, M., Wu, X.-Y., Ren, S.-X., Zhao, M., Zhao, C.-J., . . . Lu, G. (2000). Cloning and functional analysis of cDNAs with open reading frames for 300 previously undefined genes expressed in CD34+ hematopoietic stem/progenitor cells. *Genome Research, 10*(10), 1546-1560.

Zhang, T., Hu, Y., Wang, T., & Cai, P. (2017). Dihydroartemisinin inhibits the viability of cervical cancer cells by upregulating caveolin 1 and mitochondrial carrier homolog 2: Involvement of p53 activation and NAD (P) H: quinone oxidoreductase 1 downregulation. *International Journal of Molecular Medicine, 40*(1), 21-30.

Zhang, W., Fang, Y., Shi, X., Zhang, M., Wang, X., & Tan, Y. (2012a). Effect of bisphenol A on the EGFR-STAT3 pathway in MCF-7 breast cancer cells. *Molecular Medicine Reports, 5*(1), 41-47.

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

Zhang, Y., Zhang, H., Yao, X.-g., Shen, H., Chen, J., Li, C., . . . Hu, L. (2012b). (+)-Rutamarin as a dual inducer of both GLUT4 translocation and expression efficiently ameliorates glucose homeostasis in insulin-resistant mice. *PLoS ONE, 7*(2), e31811.

Zhao, G., Shi, A. P., Fan, Z. M., & Du, Y. (2015). Salidroside inhibits the growth of human breast cancer *in vitro* and *in vivo*. *Oncology Reports, 33*(5), 2553-2560.

Zhou, Y. Q., Zhao, W., Xie, G. B., Huang, M. F., Hu, M. J., Jiang, X., . . . Zhang, X. K. (2014). Induction of Nur77-dependent apoptotic pathway by a coumarin derivative through activation of JNK and p38 MAPK. *Carcinogenesis, 35*(12), 2660-2669.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. **Musa, I. F.,** Malek, S. N. A., & Karsani, S. A. (2019). Induction of apoptosis by chalepin through phosphatidylserine externalisations and DNA fragmentation in breast cancer cells (MCF7). *Life Sciences, 220,* 186-193.

PAPERS PRESENTED

1. **Musa, I. F.,** Karsani, S. A., & Malek, S. N. A. (2017). *Phosphatidylserine externalizations result in increased caspase-3 activity and DNA fragmentation in chalepin induced apoptotic MCF7 breast cancer cell line.* Paper presented at Cancer Research Conference (CRC), Universiti Tunku Abdul Rahman, Sungai Long Campus, Malaysia. 29th – 30th November 2017.

2. **Musa, I. F.,** Karsani, S. A., & Malek, S. N. A. (2017). *Chalepin induced cell cycle arrest and apoptosis in MCF7 cancer cell line*. Paper presented at 2nd International Conference on Molecular Biology and Biotechnology (ICMBB), PAUM Clubhouse, University of Malaya, Kuala Lumpur, Malaysia. 1st – 2nd November 2017.

3. **Musa, I. F.,** Karsani, S. A., & Malek, S. N. A. (2017). *Chalepin and rutamarin isolated from Ruta angustifolia inhibit cell growth in selected cancer cell lines (MCF7, MDA-MB-231, HT29 and HCT116)*. Paper presented at International Research Conference (IRC2017), Holiday Villa Hotel, Kota Bharu Kelantan Malaysia. 27th – 28th August 2017.

4. **Musa, I. F.,** Malek, S. N. A., & Karsani, S. A. (2016). *Apoptotic induction of rutamarin and chalepin isolated from Ruta angustifolia against selected cancer cell lines (MCF7, MDA-MB-231, and HT29)*. Paper presented at 21st Biological Science Graduate Congress (BSGC), University of Malaya, Kuala Lumpur, Malaysia. 15th – 17th December 2016.