CYTOTOXIC EVALUATION OF *Leptospermum javanicum* AND *Baeckea frutescens* ALONG WITH THE ANTI-CANCER ACTIVITIES OF THE ACTIVE ISOLATE, BETULINIC ACID

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CYTOTOXIC EVALUATION OF *Leptospermum javanicum* AND *Baeckea frutescens* ALONG WITH THE ANTI-CANCER ACTIVITIES OF THE ACTIVE ISOLATE, BETULINIC ACID

ABSTRACT

The usage of natural products as precursors for pharmacologically significant outputs has longed yielded positive outcomes. Along these lines, this study chose to look into *Leptospermum javanicum* and *Baeckea frutescens*, both found in mountainous region in Peninsular Malaysia and have limited biological investigations. The aim of the present study was to assess the cytotoxicity of both plants against lung cancer cells, followed by evaluating the anti-cancer and anti-metastatic properties of the active extracts and their compound. In addition to this, the protein expression of the active compound was evaluated to obtain a better understanding into the mechanism of action. The crude methanol extracts of both plants showed no signs of acute oral toxicity when tested on Sprague-Dawley rats. The cytotoxic potentials of the extraction yields (methanol, hexane, ethyl acetate and water extracts as wells as a semi pure fraction, LF1 from *L. javanicum*) were evaluated against two human non-small cell lung carcinoma cell lines (A549 and NCI-H1299) using the MTT assay, with LF1 being the most promising. LF1 treatment resulted in the manifestation of a sub-G_1 region in the cell cycle analysis while also causing presences of apoptotic morphologies in cells stained with acridine orange and ethidium bromide (AO/EB). Treatment with LF1 resulted in an apoptotic population in cells that were evaluated using the Annexin V/ propidium iodide assay. Blockage of cell cycle progression was also observed in LF1-treated cells. Following this, LF1 underwent isolation and purification to yield a white powder which was identified as Betulinic acid (BA) *via* NMR, LCMS and IR spectroscopy. The isolate, BA, which produced an encouraging cytotoxic effect against A549 and NCI-H1299 cells through the MTT assay, was further assessed with AO/EB staining, TUNEL assay, sub-G_1 population

quantification as well as activated caspase-3 detection. The data pointed towards the induction of apoptosis as a result of increasing concentrations of BA, regardless of the p53 status in both cell lines. Treatment with BA also prevented an effective attachment of the invasive A549 cells onto a new culture surface, in addition to diminishing the migratory and invasive potential of treated cells across an uncoated porous membrane and a Matrigel-coated membrane, respectively. Further investigation through the ELISA detection method and gelatin zymography showed an adverse effect to production of matrix metalloproteinase-2 (MMP-2). Protein expression of BA-treated cells showed decreased levels of p-Akt, ERK1/2 and cyclin D1 followed by a slight increase in p21 and p27 expression, all of which is believed to have contributed to the G_0/G_1 arrest. In addition to this, alteration in NF-κB, IκB-α, STAT3 and JNK phosphorylation due to treatment with BA could have been a contributing factor to its apoptotic and anti-metastatic properties. Taken together, the results showed that the *L. javanicum* isolate, BA, possess anti-cancer effects through induction of apoptosis, restriction of cell cycle progression and anti-metastatic effects on human lung cancer cells. The ability of BA to not only eradicate but control the spread of lung cancer cells makes it a promising lead compound in the treatment of lung cancer.

Keywords: *Leptospermum javanicum*, *Baeckea frutescens*, Betulinic acid, apoptosis, cell cycle arrest, metastasis.

PENILAIAN SITOTOKSIK *Leptospermum javanicum* DAN *Baeckea frutescens* SERTA AKTIVITI ANTI-KANSER KOMPONEN AKTIF, ASID BETULINIK

ABSTRAK

Penggunaan produk semula jadi sebagai pelopor dalam bidang farmakologi telah lama membuahkan hasil yang positif. Berlandaskan ini, kajian ini telah memilih untuk mengkaji *Leptospermum javanicum* dan *Baeckea frutescens*, dua tumbuhan yang dijumpai di kawasan pergunungan Semenanjung Malaysia dan mempunyai siasatan biologi yang terhad. Tujuan kajian ini adalah untuk menilai aktiviti sitotoksik kedua-dua tumbuhan pada sel-sel kanser paru-paru dan kemudiannya menilai sifat-sifat anti-kanser dan anti-metastatik ekstrak-ekstrak aktif dan konstituennya. Tambahan lagi, kesan ke atas ekspresi protein yang disebabkan konstituen aktif telah dinilai untuk mendapatkan pemahaman yang lebih baik terhadap mekanisme tindakannya. Ekstrak-ekstrak mentah metanol kedua-dua tumbuhan tidak menunjukkan sebarang tanda toksiksiti akut apabila diuji pada tikus Sprague-Dawley. Potensi sitotoksik hasil pengekstrakan (ekstrak metanol, heksana, etil asetat dan air serta fraksi separa tulen, LF1 dari *L. javanicum*) telah dinilai terhadap dua sel kanser paru-paru (A549 dan NCI-H1299) menggunakan esei MTT, dimana LF1 adalah yang paling efektif. Sel yang dirawat dengan LF1 menghasilkan populasi sub-G_1 dalam analisis kitaran sel disamping menyebabkan kehadiran morfologi apoptotik dalam sel-sel berlumuran dengan akridin jingga dan etidium bromida (AO/EB). Rawatan dengan LF1 menunjukkan populasi apoptotik dalam sel ketika dinilai dengan menggunakan esei Anesin V/ propidium iodide. Disamping itu, kehadiran kaspase-3 yang diaktifkan dikesan akibat rawatan LF1. Sekatan perkembangan kitaran sel juga diperhatikan dalam sel-sel yang dirawat oleh LF1. Berikutan ini, LF1 telah melalui proses pengasingan dan penulenan untuk mengdapatkan serbuk putih yang telah dikenal pasti sebagai Asid Betulinik (BA) melalui spektroskopi NMR, LCMS dan IR. BA menghasilkan kesan sitotoksik yang menggalakkan terhadap sel-sel A549 dan

NCI-H1299 melalui esei MTT, dan kemudiannya dinilai dengan AO/EB, esei TUNEL, kuantifikasi populasi sub-G_1 serta pengesanan kaspase-3 yang diaktifkan. Data menunjukkan bahawa induksi apoptosis dengan peningkatan kepekatan BA, tanpa mengira status p53 di kedua-dua titisan sel. Rawatan dengan BA juga menghalang lampiran berkesan sel-sel A549 invasif ke permukaan kultur baru, di samping mengurangkan potensi migrasi dan invasif sel-sel yang dirawat BA di seluruh membran berliang tidak bersalut dan membran Matrigel bersalut. Siasatan lanjut melalui kaedah pengesanan ELISA dan gelatin zimografi menunjukkan kesan yang negatif kepada penghasilan matriks metaloproteinase-2 (MMP-2). Siasatan ke atas ekspresi protein sel-sel yang dirawat BA menunjukkan penurunan tahap p-Akt, ERK1/2 dan siklin D1 diikuti oleh sedikit peningkatan dalam ekspresi p21 dan p27, semuanya dipercayai telah menyumbang kepada sekatan kitaran sel di fasa G_0/G_1. Tambahan lagi, perubahan pemfosforilan NF-κB, IκB-α, STAT3 dan JNK akibat rawatan BA berkemungkinan menjadi faktor yang menyumbang kepada sifat-sifat apoptotik dan anti-metastatik BA. Secara keseluruhan, keputusan menunjukkan bahawa konstituen *L. javanicum*, BA, mempunyai kesan-kesan anti-kanser melalui induksi apoptosis, sekatan perkembangan kitaran sel dan kesan anti-metastatik pada sel-sel kanser paru-paru manusia. Keupayaan BA bukan sahaja untuk membasmi tetapi mengawal penyebaran sel-sel kanser paru-paru menjadikannya kompaun yang berpotensi dalam rawatan kanser paru-paru.

Kata Kunci: *Leptospermum javanicum*, *Baeckea frutescens*, Betulinic acid, apoptosis, sekatan kitaran sel, metastatik.

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MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
AO	:	Acridine orange
APAF	:	Apoptotic protease activating factor
BCE	:	Before the Common Era
BCA	:	Bicinchonic acid assay
BSA	:	Bovine serum albumin
JNK	:	c-Jun N-terminal kinases
CDK	:	Cyclin-dependent kinase
CDKI	:	Cyclin-dependent kinase inhibitor
DISC	:	Death-inducing signalling complex
^{o}C	:	Degree Celsius
DNA	:	Deoxyribonucleic acid
DDT	:	Dichlorodiphenyltrichloroethane
DMSO	:	Dimethyl sulfoxide
ELISA	:	enzyme-linked immunosorbent assay
EMT	:	Epithelial-mesenchymal transition
EB	:	Ethidium bromide
ECM	:	Extracellular matrix
ERK	:	Extracellular signal–regulated kinase
FBS	:	Foetal bovine serum
FITC	:	Fluorescein isothiocyanate
FDA	:	Food and Drug Administration
GRP	:	Gastrin releasing peptide
g	:	Gram

IC_{50}	:	half maximal inhibitory concentration
HIV	:	Human immunodeficiency virus
HCl	:	Hydrochloric acid
IR	:	Infra-red
kDa	:	Kilodalton
Kg.	:	Kilograms
LCMS	:	Liquid chromatography–mass spectrometry
MMP	:	Matrix metalloproteinases
LD_{50}	:	Median lethal dose
MHz	:	Megahertz
µg	:	Microgram
µl	:	Microlitre
ml	:	Millilitre
MiMP	:	Mitochondrial membrane permeabilization
MAPK	:	Mitogen-activated protein kinase
NSCLC	:	Non-small cell lung cancer
NMR	:	Nuclear magnetic resonance
OECD	:	Organisation for Economic Co-operation and Development
%	:	Percentage
PBS	:	Phosphate-buffered saline
PS	:	Phosphatidylserine
±	:	Plus-minus
PCB	:	Polychlorinated biphenyl
PI	:	Propidium iodide
PAB	:	Pseudolaric acid B
RIPA	:	Radio immunoprecipitation assay

STAT	:	Signal transducer and activator of transcription
SCLC	:	Small cell lung cancer
SD	:	Standard deviation
SAPK	:	Stress activated protein kinase
TUNEL	:	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TLC	:	Thin Layer Chromatography
×	:	Times
TIMP	:	Tissue inhibitors of metalloproteinases
TRAIL	:	TNF-related apoptosis-inducing ligand
TGF	:	Transforming growth factor
TBS	:	Tris-buffered saline
TBST	:	Tris-buffered Saline with Tween 20
TNF	:	Tumour necrosis factor
Vs.	:	Versus
V	:	Volts
WHO	:	World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Cancer has overwhelmed humans since the beginning of recorded history. It is a persistent ailment where more than a million people are diagnosed with this disease each year and has a significant social impact and considerable economic burden on our healthcare system. Cancer was the fourth largest cause of reported mortality among Malaysians. The National Cancer Registry Report 2007-2011, Malaysia (Abdul Manan et al., 2016; Omar & Ibrahim, 2011) reported that a total of 103,507 new cancer cases were diagnosed and registered at the National Cancer Registry from the year of 2007 to 2011.

One manifestation of this ailment that is severely disregarded is lung cancer that makes up for 19.8% of all medically certified deaths attributed to cancer in Malaysia (Sachithanandan & Badmanaban, 2012). Lung cancer arise from mutations present in the respiratory epithelium and can be grouped into two broad categories, small cell lung cancer (SCLC) (15% of diagnosed cases) and non–small cell lung cancer (NSCLC) (85% of diagnosed cases). NSCLC patients have a low survival outcome worldwide; less than 20% of patients survived beyond five years upon being diagnosed with the disease (Bartucci et al., 2011; Collins et al., 2007; Jemal et al., 2010).

The usage of standard chemotherapy may help to a certain extent but more often the survival benefits are at the expense of severe toxicity and unwanted side effects. Based on this reasoning, an ethical imperative arises to seek less toxic alternative to current chemotherapy drugs that is able to effectively combat and manage this disease and at the same time exclude severe toxicity and unwanted complications that are synonymous with current treatment.

Historically, scientists have been turning to natural products to seek cures against cancer. Stereo chemically complex molecules present in natural products appears to have a much higher specificity towards certain biological target when compared to synthetic drugs (Khazir et al., 2013). The continuous discovery of biological active secondary metabolites from natural product sources show that it is still a viable tool in drug discovery. More than half of the presently approved chemotherapy drug are directly or indirectly sourced from natural products (Khazir et al., 2013).

In order to look for possible alternative modalities against lung cancer, the plant family of Myrtaceae was looked into. The myrtle family of shrubs and trees, in the order Myrtales, contains about 150 genera and 3,300 species that are widely distributed in the tropics. *L. javanicum* and *B. frutescens* were selected for this study.

L. javanicum (known previously as *Leptospermum flavescens*) is a medium sized shrub in the Myrtaceae family with many twiggy angular branches and a reddish-brown bark. The flowers are solitary, numerous and white with a yellow tinge. *L. javanicum* has been used as a natural remedy by locals, either eaten raw or taken as a concoction brewed from fresh plants. The plant has been brewed as a tea and used as a local remedy for various ailments (Burkill, 1966; Jantan et al., 1995). Although *L. javanicum* is reported to be used in traditional medicine preparations, there is limited data on the biological and chemical investigations of the species.

B. frutescens is found in Peninsular Malaysia, Sumatra as well as the coastal areas of southern China and Australia. In Peninsular Malaysia, *B. frutescens* is found both on the mountain tops and sandy coasts. The entire plant, except the roots, has been traditionally used by the Chinese, Malaysians and Indonesians to treat a list of common ailments, ranging from fevers to sunstrokes. In addition, the essential oil of this plant has been used in the treatment of rheumatism (Herbal Medicine Research Centre, 2002). At

the beginning of this research, there has only been limited published literature on *B. frustescens*. Investigation by Fujimoto et al. (1996) reported a strong cytotoxic activity against L1210 leukaemia cells while Makino and Fujimoto (1999) managed to isolate three new flavanones from *B. frustescens*. A later report by Hwang et al. (2004) indicated the anti-cariogenic, anti-malarial and anti-babesial activity of *B. frustescens*.

This study aimed to determine the possibility of these two plants possessing compound which may be developed into a potent anti-cancer agent, specifically lung cancer. A comprehensive treatment encompassing restriction of growth, inhibition of metastasis and ultimately cancer cell death was sought out through this study.

In order to achieve the above, this study was initiated through the solvent extraction process of *L. javanicum* and *B. frutescens.* The resulting crude methanol extracts were subjected to the acute oral toxicity test to determine the toxic level of the plants. In addition to this, the extracts were subjected to cytotoxic evaluation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The active fraction from this test was subsequently used in apoptotic evaluation studies (such as Acridine orange/ Ethidium bromide [AO/EB] assay, the Annexin V/PI assay, Caspase-3 detection assay) as well as cell cycle arrest analysis. The active component present in the above active fraction was isolated and purified using column chromatography and thin layer chromatography (TLC). This was followed by an identification process that incorporated three different techniques, Nuclear magnetic resonance (NMR), Infrared (IR) and Liquid chromatography–mass spectrometry (LCMS). The effect of the isolated compound on treated lung cancer cell was evaluated using the AO/EB assay, the caspase-3 detection assay and the TUNEL assay. The capabilities of the compound to induce cell cycle arrest was also evaluated. To further study the capabilities of the isolated compound in suppressing aspects of metastasis, the adhesion, migration and invasion assays were

carried out. This was followed up by evaluating the levels of MMP-2 and MMP-9 production through the gelatin zymogram and the Enzyme-linked immunosorbent assay (ELISA). Lastly, in an attempt to uncover possible targets of the isolated compound, the cell lysate of treated cells was subjected to western blot analysis as well as ELISA detection. This was done with the hope of obtaining a better understanding of the mechanisms affected due to treatment with the isolated compound. As summary of the procedures carried out is outlined in Figure 1.1.

Objectives of study

The main objectives of the present study are as follows:

i. to evaluate the cytotoxic activities of *L. javanicum* and *B. frutescens* extracts on selected human lung cancer cell lines;

ii. to isolate and identify the chemical constituents from the cytotoxic extracts;

iii. to assess the cytotoxic activities of the identified chemical constituents on selected human lung cancer cell lines;

iv. to evaluate the anti-invasive properties of the cytotoxic chemical constituents on metastatic cancer cells; and

v. to determine the cell death mechanisms induced by the cytotoxic chemical constituents.

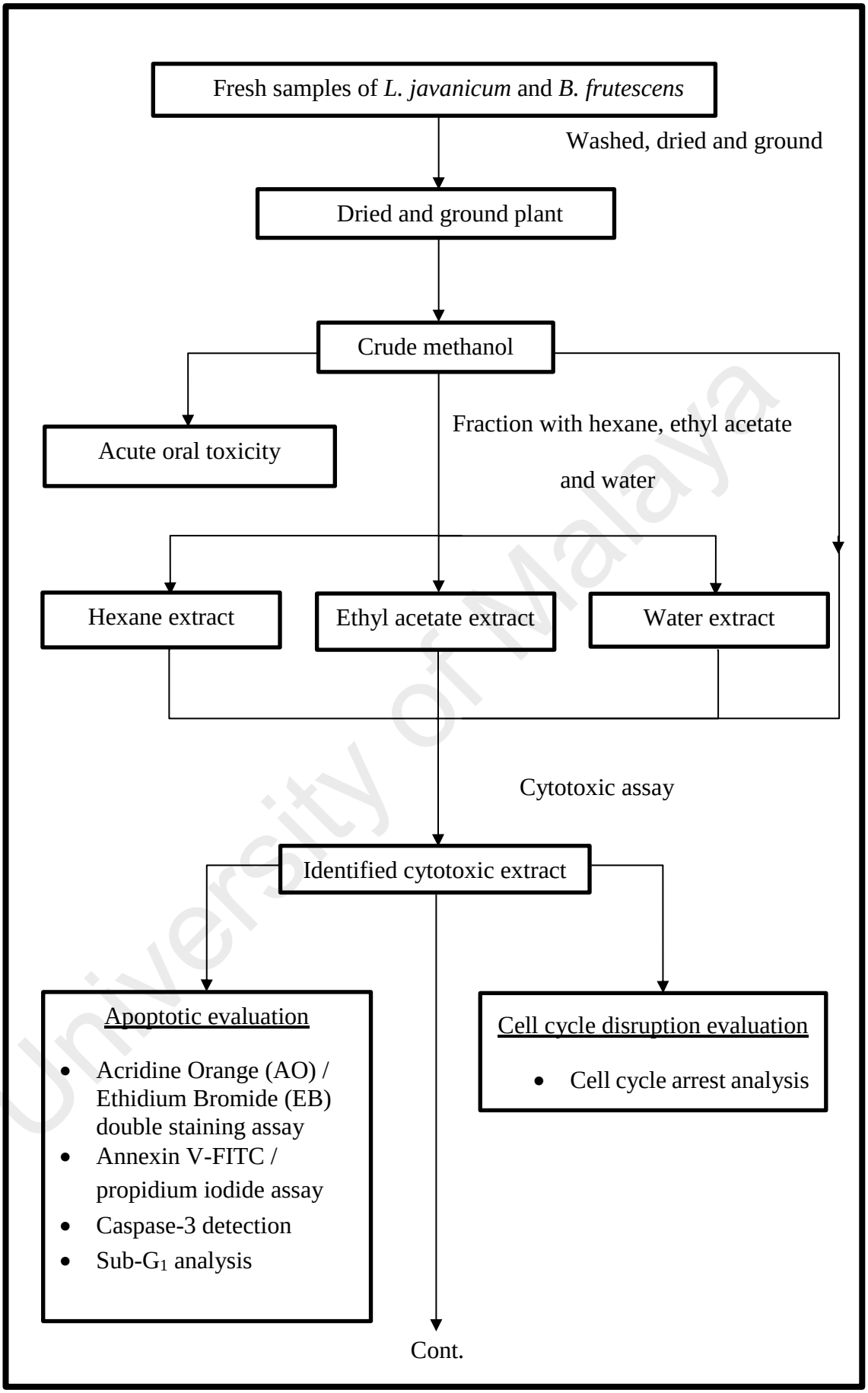


Figure 1.1: Outline of the experimental design for the study.

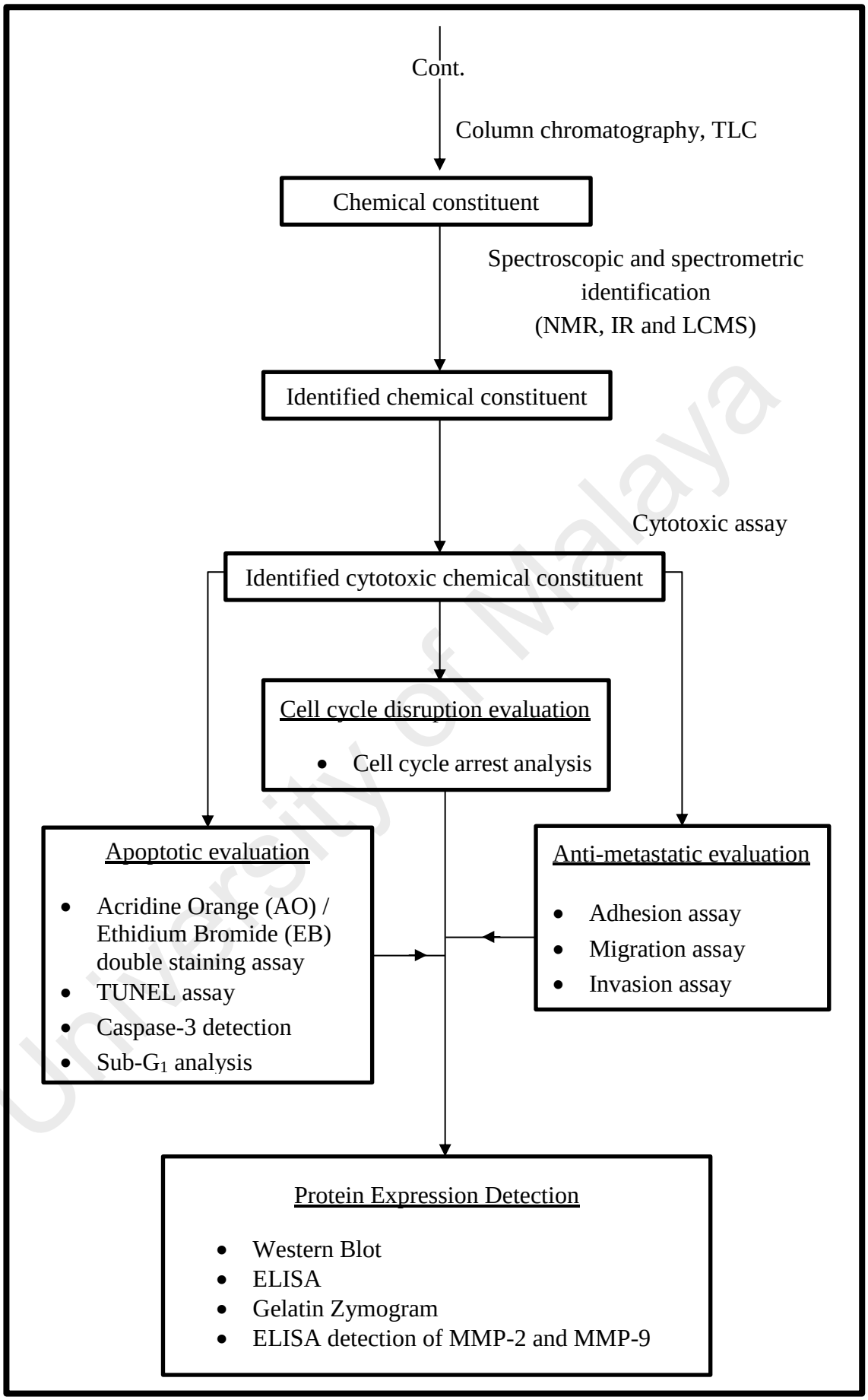


Figure 1.1: continued

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer can be defined as a set of diseases characterized by unregulated cell growth leading to invasion of surrounding tissues and spread (metastasis) to other anatomical sites in the body (King & Robins, 2006). The development of cancer occurs when normal cells in a particular part of the body begin to grow out of control; continuing to grow, divide and re-divide instead of dying, leading to the formation of new abnormal cells (King & Robins, 2006). Up to a certain point, certain types of cancer undergo transformation which leads to its movement through the blood circulation system or the lymphatic vessels before finally "settling down" in a new site. In other words, the tumour is considered to have developed into a cancer once it metastasizes to other parts of the body (Hanahan & Weinberg, 2011). Although being a disease associated with mankind for such a long time, this disease continues to plague humans to this day; the absolute number of cases worldwide has greatly increased and this trend is accelerating in many countries around the globe (Sasco & Nikulin, 2008).

One of the first descriptions of cancer can be found in ancient Egyptian papyri dating between 3000 B.C and 1500 B.C (Sasco, 2008). Hippocrates, often regarded as the father of medicine, described cancer in detail and used the Greek terms "carcinos" and "carcinoma" to refer to chronic ulcers or growths that seemed to be malignant tumours and "squirr(h)e" to refer to a type of cancer (epithelium) with a hard consistency due to the predominance of a sclerosis with a tissue retraction (Hajdu, 2011).

In the late 1970s, the discovery of oncogenes as well as tumour suppressor genes greatly increased the efficiency of cancer treatment as specific target were available to either suppress, repair, control or kill the cell afflicted with this condition. Briefly, oncogenes are activated genes that function to promote cancer cell proliferation while tumour suppressor genes contribute to cancer development through loss of its function; essentially acting as an "emergency brake" to avoid inappropriate cell division (Boyle & Levin, 2008).

2.1.1 Cancer epidemiology

To put the seriousness of cancer into perspective, one in seven reported deaths worldwide was due to a failed battle with cancer. Among high income nations, the disease is responsible for the second most cause of death (preceded only by cardiovascular disease), while being the third highest cause of death in developing countries. In fact, the United Nations has even gone as far as estimating that cancer related deaths have suppressed all coronary heart disease or all stroke (World Health Organization, 2015). According to a report by International Agency for Research on Cancer (IARC), there were a total of 14.2 million new cancer cases reported in 2012, not including non-melanoma skin cancer (Ferlay et al., 2015). In the same year, there were about 8.2 million cancer related deaths reported (around 22,000 per day), with a higher incidence in developing countries compared to developed nations (5.3 million against 2.9 million cases). By only taking into account the growth and aging trends of the current global population, 21.7 million new cases and 13 million cancer related deaths are expected by the year 2030 (Torre et al., 2015). This is an optimistic expectation at most, as it does not take into factor the rising adoption of unhealthy lifestyles such as smoking, physical inactivity, a poor diet as well as fewer number of pregnancies. In fact, the types of cancer that are directly linked with these living behaviours, such as colorectal, breast and lung cancers are already shooting in numbers among economically transitioning countries (Torre et al., 2015).

Looking a little deeper, incidence rate of all cancer combined was higher in developed countries (such as Europe, Northern America, Australia/New Zealand, and Japan) as compared to developing countries (such as Africa, Latin America and the Caribbean, Melanesia, Micronesia, and Polynesia) (Alwan, 2011). This could be attributed to prevalence of the above mentioned unhealthy lifestyles in high income nations. At the same time, the high number could also be attributed to an increased efficiency in detecting the disease due to better diagnostic method as well as a better awareness in developed nation. The mortality rate in both developed and developing countries are similar. The variations among cancer incidence and cancer related-mortality is dependent the type of cancers involved as well as the availability and accessibility of services catering to early detection and their subsequent treatment modalities.

Three of the most diagnosed type of cancers in economically developed countries were prostate, lung, and colorectal among males, and breast, colorectal, and lung among females. On the other hand, in economically developing countries, the three most commonly diagnosed cancer types were lung, liver, and stomach in males, and breast, cervix uteri, and lung in females. In both economically developed and developing countries, the three most common cancer sites were also the three leading causes of cancer death. Lung and breast cancer have the highest worldwide incidence and mortality rates among men and women, respectively. Worryingly, with respect to lung cancer, the number of deaths is just slightly below the number of newly reported cases (1,241,600 incidence vs 1,098,700 deaths for men and 583,100 incidence vs 491,200 deaths for women) (Ferlay et al., 2015).

Looking a little closer at home, cancer contributed to 13.56% of all deaths that occurred in Malaysian government hospitals in 2015. 103,507 new cancer cases were diagnosed in Malaysia during the period of 2007 to 2011 whereby 46,794 (45.2%) were reported in males while 56,713 (54.8%) in females (Abdul Manan et al., 2016). The five most common cancers among males were cancers of the colorectal (16.3%), followed by lung (15.8%), nasopharynx (8.1%), lymphoma (6.8%) and prostate (6.7%). Among females, the five most common were cancers of the breast (32.1%), colorectal (10.7%), cervix uteri (7.7%), ovary (6.1%) and lung (5.6%) (Abdul Manan et al., 2016).

2.1.2 Lung cancer

One form of cancer that is severely overlooked is lung cancer which accounts for 19.8% of all medically certified deaths due to cancer in Malaysia (Sachithanandan & Badmanaban, 2012). On a global perspective, new cancer diagnoses largely consist of lung cancer cases, whereby 1.8 million or 13% of total new cancer cases were contributed from lung cancer. Lung cancer attributes to the highest cause of cancer related deaths in men (1.1 million), while being the second most cause of death in women (491,200) with an estimated total of 1.6 million worldwide in the year 2012. However, lung cancer has surpassed breast cancer as the leading cause of cancer deaths among women in developing countries (Ferlay et al., 2015).

Mutations in the respiratory epithelium gives rise to lung cancer which can be grouped into two broad categories, small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC). About 15% of all lung cancer cases fall under SCLC, which is an extremely malignant form of tumour originating from cells that exhibits neuroendocrine characteristics. The remainder 85% cases fall into the NSCLC category which can be broken down into three more groups based on their pathologic subtypes, large cell carcinoma, adenocarcinoma, and squamous cell carcinoma (Bi et al., 2014). Worldwide,

less than 20% of those afflicted with NSCLC survived beyond five years after being diagnosed with the disease, contributing to the fact that this disease has a low survival rate (Bartucci et al., 2011; Collins et al., 2007; Jemal et al., 2010).

Increasing trends in the number of new lung cancer cases, especially Asia, are expected in the years to come (Dela Cruz et al., 2011). In fact, the figure of lung cancer cases has climbed by 51% since the year 1985, mostly in women, whereby a rise of 76% in new patients have been recorded (Dela Cruz et al., 2011). Both of these occurrences are grounded on a global rise in the usage of tobacco, which is identified as the chief risk factor for lung cancer and is responsible for a large share of all pulmonary carcinomas. Non-smokers are also affected by this ever prominent illness, mainly due to one or a combination of causes comprising genetic factors (Gorlova et al., 2007), asbestos, air pollution (Kabir et al., 2007), second-hand smoking and also Radon exposure (Catelinois et al., 2006) which is responsible as the second highest cause of lung cancer.

2.1.3 Lung cancer cell lines

In order to assess the effects of the test subjects on lung cancer, two established lung cancer cell lines were utilized, A549 and NCI-H1299. The main differentiating characteristic of these cells would be the presence or absence of an active p53 gene in the cells. This would serve as an ideal method allowing for initial assessment to the dependence of the test material towards the tumour suppressor gene, p53.

The A549 non-small lung cancer cell line was first established in 1972 by Giard et al. (1973) through explanation and continuous sub culturing of a human alveolar-cell carcinoma which originated from 58-year-old Caucasian male. These alveolar cells are squamous shaped and play a role in the movement of substances such as water and electrolytes across the alveoli of lungs (Sundararajan et al., 2014).

Studies by Lieber et al. (1976) validated that the cell line had indeed originated from a single cell and was of human origin. An epithelioid in morphology is present in A549 cultures. In addition to that, they also have the presence of multiple small cytoplasmic granules that can be observed through bright field and phase microscopy (Lieber et al., 1976). A549 possess a wild-type p53 expression, meaning the native expression of the protein is not affected through mutation.

NCI-H1299 is a carcinoma that has been derived from the metastatic lymph node of a 43-year-old male Caucasian that had received prior radiation therapy. These cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein (Leroy et al., 2014). Giaccone et al. (1992) has reported that these cells are able to synthesize the neuromedin B peptide, but not the gastrin releasing peptide (GRP). The NCI-H1299 cells also possess a mutation at the NRAS gene (Neuro RAS gene) (Ohashi et al., 2013). The NRAS gene provides instructions for making a protein called N-Ras that is involved primarily in regulating cell division.

NCI-H1299 has been used in a variety of experimentations in the study of p53 and its effect into the absence and presence of the gene, particularly in tandem with A549 which as mentioned earlier, has the wild type p53 present in it. Studies such as that done by Yao et al. (2016) have used this varying characteristic between NCI-H1299 and A549 for that very purpose, in this case to show that p53 activation is essential to the induction of pseudolaric acid B (PAB) induced senescence in human lung cancer cells.

2.1.4 Carcinogenesis

Cancer is a complex disease that is variable in its presentation, development and outcome from one patient to another. Part of this arises from the fact that cancer is a multi-step process, allowing numerous opportunities for variability to take place. Profound metabolic and behavioural changes which leads to excessive proliferation and the ability

to evade the scrutiny of the immune system which ultimately ends up with invasive tissue formation through metastasis (Merlo et al., 2006). The accumulation of modification through genetic mutation that plays a role in the way cells proliferate and interact with neighbouring cell, will result in the formation of deregulated cells that contravene rules governing normal cell growth and behaviour. Asymptomatic condition in the initial phase of cancer development will eventually give rise to a myriad of symptoms as a result impairment of physiological functionality of the tissue wherein the tumour is located. The final and usually most lethal step of cancer growth is spread of cancer to other sites within the organism.

2.1.5 Cancer development

The development of cancer as a result to exposure of carcinogen or cancer-causing substances, can be typically divided into a few different steps. The first step is called 'initiation' during which the carcinogen cause mutation in the DNA sequences of normal cells. The initial stages of the cancer formation cause irreversible genetic damage. The DNA contains 23,000 genes of which several thousands of these genes (3,000 - 5,000) are possibly targets of deregulation in cancer cells (Boyle & Levin, 2008). Cancer may be initiated by changes caused through mutation in the DNA sequence; as small as a single base changes that leads to formation of defective protein due to a substitution of a single amino acid. Studies have implicated epigenetic changes as an early event in carcinogenesis as well (Jones & Baylin, 2002). In short, initiation involves one or more stable cellular changes arising spontaneously or induced by exposure to a carcinogen.

The tumour promotion step comprises of the selective clonal expansion of initiated cells (Kufe et al., 2003). Upon stimulation to undergo further proliferation, the transformed cells which was harmless up to this point, increases its population, causing an upset in the cellular balance as well as increasing the possibility of genetic alterations as well as malignant conversion (Kufe et al., 2003). The subsequent changes of an initiated cell leading to neoplastic transformation may involve more than one step and requires repeated and prolonged exposures to promoting stimuli (Devi, 2004).

Tumour promoters, which are often non-mutagenic or carcinogenic by themselves, have ability to reduce the latency period for tumour formation after exposure of cells to a tumour initiator. In other words, they function to increase the number of tumour formation in the exposed tissue. Besides that, tumour promoters are also able to aide cancer initiation in cells exposed to low doses of initiators that are deemed too low to be considered a carcinogen. Some of the better-known tumour-promoting agents are dioxins, phenols, dichlorodiphenyltrichloroethane (DDT), cigarette-smoke condensate, polychlorinated biphenyls (PCBs), estrogens and other hormones, bile acids as well as ultraviolet light (Kufe et al., 2003).

The next and final phase would be the progression of pre-neoplastic cells into malignant cancer cells. Further mutations in the expanding pre-neoplastic clones are required for the expression of malignant phenotypes. These genetic and epigenetic modifications confer the cells a growth advantage as well the capacity to successfully spread and colonize distant sites during the metastatic process (Boyle & Levin, 2008). The conversion to malignant cells is accelerated by a higher rate of cell division as well as an increased quantity of dividing cells in the benign tumour or pre-neoplastic lesion. As the tumour progression advance, cells have a diminished capacity to adhere to the matrix as well as among each other. This leads to detachment from the tumour mass

followed by invasion to the neighbouring tissue. In most cases, the cells are also able to secrete proteases that aids in the invasion beyond the immediate primary tumour location. Through the lymph and bloodstream, these metastatic cells are able to travel to distant organs and develop into secondary tumours at the new sites. These forms distant metastases, resulting in widely spread cancers. It is the metastatic process and tumour spreading that are mainly responsible for the lethal effects of many common human tumours. Accumulation of gene mutations is believed to be the driving force for tumour metastasis (Devi, 2004).

2.1.6 Sustainability of cancer cells

In order to ensure the survival of cancer cells, a few fundamental cellular rules have to be violated (Hanahan & Weinberg, 2000). Firstly, the cells should proceed to divide only when they receive appropriate signals. To circumvent this requirement, cancer cells will permanently activate cell division by switching on the circuits that become activated normally when the cell is stimulated by a hormone or a growth factor (Boyle & Levin, 2008). The second rule states that in an event of stressful condition or irreparable DNA damage, the cells are required to undergo a self-destruct sequence to prevent the DNA replication and further accumulation errors in the DNA sequence. Tumour suppressor genes such as Rb1 and p53, which function as safety brakes to prevent aberrant or excessive cell division, are typically removed or deactivated in cancer cells, thus not only preventing the suicide sequence from being initiated but also ensuring the continual division of abnormal cells. This would eventually lead to the formation of tumour mass. The third and last rule states that normal cells are only allowed to undergo cell division a limited, fixed number of times. The number of DNA replication and cell division in normal cells are limited to a finite number of times due to telomeres, the structure present at the end of each chromosome. The telomere is made of small repeats of DNA sequence which become eroded each time the cell divides (Boyle & Levin, 2008).

The cells enter a state of senescence upon depletion of all the repeats, crippling the ability of cells to divide. The activation of the enzyme telomerase in cancer cells permits the addition of new repeats the chromosome ends. This in turn allows the cancer cell to continuously replicate way past its programmed limited number. This process is equivalent to the acquisition of a form of "permanent cell youth" (Shay & Wright, 2011). Through the evasion of these three safety nets programmed in normal cells, cancerous cells are able to thrive and lead to the subsequent advancement of this disease.

Metastatic cells that have evolved well enough to migrate from organ to organ are rather good at adapting to changing condition due to the acquired characteristic throughout the carcinogenesis process (Boyle & Levin, 2008). Natural selection has allowed only the fittest and most aggressive cells to thrive, taking the place of other less disorganized cells. It is due to robust nature of these cells that attempts to kill them through cytotoxic drugs as well radiation treatment has a limited effectiveness. This is the reason why most cancer conditions are best detected and treated at early stages, at times when cancer cells possess a limited adaptive capacity that would have allowed it to resist the effects of the treatment.

2.1.7 Limitation of current treatments

Advancement in healthcare has not only allowed better patient care but also given us many avenues in combating cancer. Nevertheless, certain drawback occurs with currently available treatment. Among them are the limitations of conventional chemotherapeutic agents. Existing chemotherapeutic drugs are toxic to both cancer as well as normal cells. The administration of these drugs, although effective in most cases, do have a profound effect on the normal cells of the patient, which in turn leads to unwanted side effects, and in some cases even death (Chakraborty & Rahman, 2012).

Another limitation lies in the fact that metastasis poses a huge problem in cancer treatment. The metastatic nature of cancer cells further curtails the effectiveness of chemotherapeutic drugs. The asymptomatic cancer at initial stages prevents early detection, thus preventing the ability to control the disease while it is still localized (Chakraborty & Rahman, 2012). When cancer metastasizes, the treatment is not only directed towards the primary cancer but also needs to eliminate the secondary ones as well.

It would be imperative to identify a compound that would be able to be developed into an anti-cancer drug that not only would target a single pathway, but be able to combat the disease on multiple fronts with an array of capabilities, including ability to cause cell death (apoptosis), restrict further proliferation of tumour cells (cell cycle arrest) as well as preventing the spread of cancer to distant sites within the patient (anti-metastatic).

2.2 p53 tumour suppressor

2.2.1 Introduction and general characteristics

The tumour suppressor, p53, was first discovered during the peak of tumour virus research, whereby it was presented as a 53 kDa host protein that was bound to the simian virus T Antigen (Lane & Crawford, 1979; Linzer & Levine, 1979). It is one of the more widely present defects in tumours cells, whereby it is present in more than 50 % of cancer cells (Lane & Levine, 2010). As of 2017, there has been more than 80,000 articles published on this regulatory gene, highlighting the significance of this protein in the understanding of cancer treatment (Nguyen et al., 2017). Inactivation of TP53 is often times associated with a poor prognosis in cancer patients (Olivier et al., 2010).

The wild type p53, which is encoded by TP53 gene, consist of two N-terminals trans activation domain that interacts with basal transcription factors and regulatory proteins, a central DNA binding domain, a conserved proline-rich domain, a C-terminus that encodes the nuclear localization signal as well as an oligomerization domain that is required for transcription activities (Kastenhuber & Lowe, 2017).

2.2.2 Functionality

The p53 is triggered by stressful stimuli which includes oxidative stress, nutrient deprivation, hypoxia, DNA damage, telomere attrition, oncogenic expression and ribosomal dysfunction (Meek, 2015; Zhang & Lu, 2009; Zhou et al., 2012). Upon activation, the proteins express protective capabilities through induction of cell cycle arrest at the G1 or G2 phase, apoptosis through interaction with mitochondrial proteins such as BAX and PUMA, cellular senescence, DNA repairs as well as inhibition of angiogenesis and metastasis (Bieging & Attardi, 2012; Levine & Oren, 2009; Sengupta & Harris, 2005).

The ability of p53 to induce cell cycle arrest is purposed with giving the cells the opportunity to repair the damaged DNA, thus preventing the propagation of oncogenic mutation which would eventually lead to the formation of cancer (Mello & Attardi, 2017). At the same time, the apoptotic response of p53 was suggested as a means to remove damaged or neoplastic cells that are beyond the point of recovery.

Aside from it commonly known functions, p53 also plays a role in a number of cellular responses. These includes maintaining genetic and epigenetic stability (Sengupta & Harris, 2005; Tovy et al., 2017), repressing oncogenic signalling (Sachdeva et al., 2009) and inducing non-apoptotic cell death such as ferroptosis an iron dependent non-apoptotic cell death (Jiang et al., 2015). In addition to this, p53 also restrains stemness and promotes differentiation (McConnell et al., 2016; Olivos & Mayo, 2016) while

inhibiting motility and invasiveness by attenuating epithelial mesenchymal transformation (EMT) (Chang et al., 2011; Kim et al., 2011). The versatility of the p53 protein in causing multiple effects that would inconvenience cancer cells is the reason why p53 is more commonly silenced in cancer cells as compared to p53 effector targets.

2.2.3 Regulation of p53

The p53 has a rather short half-life, whereby it has a rather low level of expression in unstimulated cells (Kubbutat et al., 1997). This is mainly due to regulator proteins such as MDM2 that negatively regulates their stability and activity of the protein (Haupt et al., 1997). Through interactions with MDM2, p53 is translocated from the nucleus to the cytoplasm, thus limiting its capabilities to interact with the transcription machinery of the cells (Haupt et al., 1997). In addition to that, MDM2, which is the primary negative regulator of p53, is also able to target p53 for proteosomal degradation through ubiquitination of specific lysine residue on the p53s C-terminus (Kubbutat et al., 1997).

Activation of p53 occurs when the relevant stressors causes a disruption in interaction between p53 and MDM2, often times using different mechanisms (Kruse & Gu, 2009). For examples, DNA damages triggers activation of kinases such as Chk1/Chk2 which leads to a phosphorylation of the N-terminus of p53, lowering the effective binding of MDM2 to that site (Shieh et al., 2000). On the other hands, activation of mitogenic signals such as c-Myc and k-RAS causes a build-up of p14ARF, which is a tumour suppressor that is capable of binding with MDM2, thus freeing up the p53 from MDM2 repression (Weber et al., 1999; Zhang & Xiong, 1999).

2.2.4 p53 in cancer cells

Over 50% of tumours has a mutation at TP53, resulting in defects in the protein or post translational modification that ends up abolishing its activity (Toledo & Wahl, 2006). The majority of TP53 related mutation in cancer cells occur at the central DNA binding domain, whereby 80% of such mutation are missense mutations (Soussi & Lozano, 2005). As a result of this, the transcribed p53 is either unable to effectively bind or fail to properly fold into the correct 3D structure, thus making the resultant p53 inactive (Joerger & Fersht, 2008).

Another mode of p53 inactivation in cancer cells involves the disruption of its regulators. In these cancer cells, the wild type p53 is retained but is unable to carry out its functionality due to an upregulation of MDM2 which, as mentioned earlier, actively represses the p53 (Nicholson et al., 2001). Epigenetic inactivation of p14ARF is another example of disruption of p53 regulators that is commonly present in cancer cells (Saporita et al., 2007).

2.2.5 p53 as a viable target in cancer therapy

Restoration of p53 functionality, be it in mutant or wild type p53 cells, will allow a reversal in tumour growth. Molecules that are able restore p53 activity, either through releasing the protein from active repression by MDM2, which is a known oncotarget, or restoration of wild type functionality in mutant p53 cells, will allow us to harness the p53 ability to cause cell cycle arrest or apoptosis, eventually leading to the cancer cells death (Chene, 2003). Some of the known examples of agents that have proven to restore mutant p53 to its native states includes molecules from the thiosemicarbazone family, PhiKan083, PRIMA-1 as well as MIRA-1 (Hientz et al., 2017). On the other hand, small molecules such as Nutlins and Benzodiazepinediones as well as natural products such as

α-Mangostin and gambogic acid have shown potential as agents that are able to effectively disrupt the interactions of p53 and MDM2 (Hientz et al., 2017).

2.3 Apoptosis

2.3.1 What is apoptosis?

Apoptosis is a process that has been widely recognized as a crucial form of programmed cell death that is reliant on a gene-directed action, eventually leading to the elimination of the affected cell. The term apoptosis was first coined by Kerr, Wyllie, and Currie in 1972 to describe a particular set of morphological changes commonly associated with a distinct form of cell death, some of which have been described earlier on (Kerr, 2002; Kerr et al., 1972).

Apoptosis plays a role in embryonic and adult development as well as aging of an organism *via* tissue homeostasis, whereby the process ensures an ideal cell population is maintained in the tissue (Henson & Hume, 2006). In addition to this, apoptosis plays a role in the body's defence mechanism, either as an immune reaction or in instances of cell damaged caused by noxious agents (Norbury & Hickson, 2001).

Although generally perceived as the prime mode of active programmed cell death responsible in the removal of cells, recent reviews have suggested several alternative forms of cell death, mostly related to autophagy (Assuncao & Linden, 2004; Hetz et al., 2005; Proskuryakov et al., 2003).

The main advantage of apoptosis is the absence of an inflammatory reaction. This is achieved by design of the process, which among others does not involved the release of cellular content into the surrounding interstitial tissue. The prevention of secondary necrosis through the quick and efficient removal of apoptotic cells through phagocytosis by neighbouring cells, a process that hardly produces pro-inflammatory or anti-

inflammatory cytokines, leads to an almost null response (Kurosaka et al., 2003; Savill & Fadok, 2000).

2.3.2 Symptoms of apoptosis

Upon initiation of apoptosis, the cell undergoes a number of morphological changes at the early stages, including cell shrinkage and pyknosis, both of which can be visually assessed using a light microscope (Hacker, 2000; Kerr et al., 1972). In the former, cells overall size is visibly reduced, which causes the organelles to become more tightly packed as well as an increase cytoplasmic density (Fink & Cookson, 2005). Pyknosis on the other hand is a result of chromatin condensation, which is one of the most commonly used characteristic in the identification of apoptotic cells (Elmore, 2007). In this process, the nucleus condenses, followed by chromatin marginalization and fragmentation to form large and mostly consistent chromatin clumps, which later on end up being packaged into apoptotic bodies (Hou et al., 2016). Histological examination of apoptotic tissue using haematoxylin and eosin stain will reveal rounded and ovular shaped cells (as a result of cell shrinkage) with a dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments, which corresponds to the above mentioned condensed chromatins (Elmore, 2007).

Apoptotic bodies that were mentioned earlier are formed through extensive plasma membrane blebbing followed by the distribution of cell fragment into these buds that will eventually form the apoptotic bodies (Elmore, 2007). The buds are then subsequently removed through phagocytosis by a number agent, including macrophages and parenchymal cells.

One of the markers responsible for tagging the apoptotic bodies for destruction via phagocytosis is the phosphatidylserine (PS) protein which is present in the plasma membrane, typically located in the inner leaflet. Upon activation of effector caspases, these proteins are translocated to the outer portion of the membrane, thus facilitating the recognition of the apoptotic cell debris that are then engulfed either by surrounding cells or macrophages (Kiechle & Zhang, 2002). This is a common physiological marker used in the detection of apoptotic cells.

2.3.3 Activation mechanism

The apoptotic reaction is initiated through two distinct pathways, the extrinsic and intrinsic pathway, each with its own distinct set of stimulators. Both these pathways eventually result in activation of the protease activity of the effector caspases, that is essential for the morphological and biochemical hallmarks of apoptosis (Taylor et al., 2008).

As the name suggest, the extrinsic pathway is triggered through external stimulation of cellular death receptors located at the plasma membrane belonging to the tumour necrosis factor (TNF) receptor superfamily (Schulze-Osthoff et al., 1998). The activation of these receptors by death ligands, primarily the TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) or the CD95 Ligand, leading to the formation of death-inducing signalling complexes (DISC) (Fischer & Schulze-Osthoff, 2005). The recruitment of the DISC subsequently leads to the activation of procaspase-8 and procaspase-10, which in turns initiates proapoptotic cascade of the caspase proteins (Baig et al., 2016).

Despite initial promise of TNF and CD95L as potential therapeutic targets, a severe systemic toxicity of TNF and CD95L agonist in mice and human lead to its failure as an efficient cancer therapy (Daniel et al., 2001). In spite of the shortcomings of these two targets, another death receptor ligand, TRAIL appears to restore some hope with regards to utilizing the extrinsic pathway as a possible therapeutic target. Cancer stricken mice that have been injected with TRAIL resulted in the induction of tumour cell apoptosis, suppression of tumour growth as well as an improvement in survival sustainability (Roth et al., 1999; Walczak et al., 1999).

A majority of cancer treatment involved in the induction of apoptosis in tumour cells are focused on the intrinsic pathway (Fischer & Schulze-Osthoff, 2005). This pathway which is dominantly controlled by the Bcl-2 protein family, is stimulated by a number of stress-inducing stimuli that are sensed intracellularly; cytokine deprivation, endoplasmic reticulum (ER) stress and DNA damage to name a few (Czabotar et al., 2014). These apoptotic stresses converge to cause mitochondrial dysfunction that eventually leads mitochondrial membrane permeabilization (MiMP), which in turns causes the release of cytochrome C from the mitochondrial intermembrane (Tait & Green, 2010). Upon release, the cytochrome C proceed to form a multiprotein caspase activating complex called an apoptosome that is responsible for activating the procaspase-9 (Taylor et al., 2008). The activation of effector caspase-3/7 by the active caspase-9 completes the induction process of the intrinsic apoptotic pathway (Baig et al., 2016).

2.3.4 Current treatments

The Bcl-2 family which plays a crucial role in regulating the apoptotic process, consist of three sub families; the anti-apoptotic Bcl-2 proteins, the pro-apoptotic BH3-only proteins and the pro-apoptotic effectors proteins (BAX, BAK and BOK) (Ichim & Tait, 2016). BH3-only proteins convey the stress signals towards the activation of BAX and BAK, which in turns causes MiMP. On the other hand, the Bcl-2 proteins function by binding with the BH3-only proteins or the activated effector proteins (BAX and BAK), thus preventing the MiMP, ultimately blocking the apoptosis process from occurring (Ichim & Tait, 2016). This particular balance between the Bcl-2 and BH3-only proteins serves as the mechanistic basis for therapeutics solution currently being explored, namely the BH-3 mimetics. The fact that small-molecules BH3-mimicking agents, such as Obatoclax, Gossypol, ABT-263 and ABT-199, are undergoing clinical studies at various phases is a testament to the considerable potential of these mode of treatment (Baig et al., 2016).

Despite the increasing success of apoptotic-inducing treatments over the years, there have been instance of whereby apoptosis is responsible for effects that may be tumour-promoting, as suggested by Ichim and Tait (2016). They suggested an improved understand of the apoptotic process and its effects, especially in primary patient samples, holds the key into fully harnessing its potential as a therapeutic target against cancer.

2.4 Cell cycle

The cell cycle is an ordered sequence of events that spans from the time a cell is first formed from a dividing parent cell until its own division into two new cells. The cell cycle consists of two broad stages; the interphase, wherein cell roughly duplicates its cytoplasmic components as well as precisely duplicates it chromosomal DNA; and the mitotic phase (or M phase), which is the actual cell division (Campbell et al., 2006).

Looking a little deeper, the interphase can be further divided into the G_1 (the first gap between M and S phase), the S (or synthesis phase) and G_2 phase (the second gap that connects the S phase to the initiation of the M phase) (Figure 2.1).

The various cell cycle events are highly coordinated to occur in a defined order and with an exact timing, requiring precise control mechanisms as well as appropriate check and balances (King & Robins, 2006). Key aspect of the cell cycle includes ensuring sufficient factors and components for cell growth, survival of the cells through regulation of cell survival signals to aid in preventing apoptosis (unless damage to cell is beyond repair, which results in a role reversal form custodian to executioner) and cell proliferation through receiving and processing the appropriate proliferation-promotion signal which leads to cell division process (Krauss, 2014).

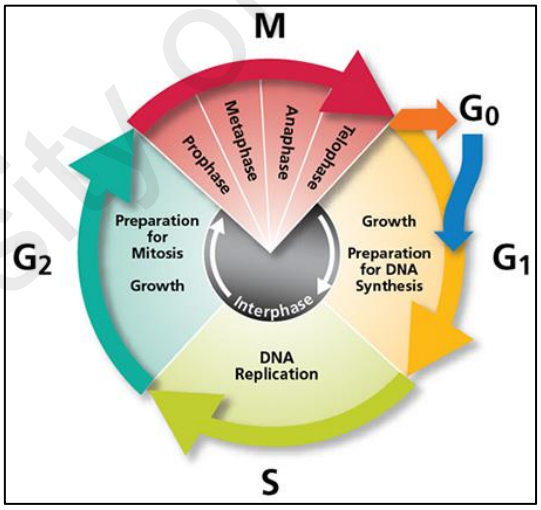


Figure 2.1: Cell cycle phases

(Taken from http://www.bdbiosciences.com.)

2.4.1 The different phase and its checkpoints

In order for the cell to commit to a mitotic proliferation, restriction points have to be overcome. The first barrier lies in between the progression from the G_1 to the S phase. This restriction point functions as a check and balance to ensure that cells are ready to undergo cell division, be it from the perspective of necessary cellular components and growth factors needed for a complete division or the absence of DNA damage originating from external factors. Failure to bypass this restriction point results in cell cycle arrest at the G_0/G_1 phase. The blockade can only be overcome by a synergistic effect of several mitogenic signals, such as hormones and growth factors (Sherr & Roberts, 1999).

Cyclins function as regulatory protein that form heterodimers with Cyclin-dependent kinases (CDKs), the master regulators of the individual cell cycle phases (Gopinathan et al., 2011; Lim & Kaldis, 2013). As each phase of the cell cycle is closely associated with a distinct cyclin-CDK pair, the progression of the cell cycle is dependent on the synthesis of cyclins, the formation of the complexes and also on the post-translational modification of the CDK to transform the enzymes into an active form (Lim & Kaldis, 2013).

The cyclin/CDK complexes are in turn regulated by inhibitory proteins which are identified as cyclin-dependent kinase inhibitors (CDKIs). These proteins, which negatively regulates the cell cycle progression, carry out their function by forming complexes with both CDKs as well as cyclins and are produced only on demand and are rapidly destroyed afterwards (Morgan, 2007). CDKI are primarily classified in to three main groups, the WAF1/CIP1 family (p21), the KIP family (p27, p57) and the INK4 family (p16, p15, p18), each with its own distinct structural and functional properties (Quereda et al., 2016).

The high presence of CDKI (such as p27) during the dormant G_0 phase with low levels of G_1 cyclins (such as Cyclin D1), is reversed as a result of mitogenic signals exposure which trigger the down regulation of CDKI and up regulation of cyclins, thus allow the cycle to progress onwards to the S phase (Chim et al., 2006). Similarly, the progression into the M phase into the S phase is controlled by the cyclin B/CDK1 complex (Castedo et al., 2002). The proteins regulated by this complex includes lamins in the nuclear membrane as well as condensation proteins and histone 1 which subsequently initiates chromosome condensation (Van Horn et al., 2010).

Throughout the cell cycle process, the genome is susceptible to damage either through exposure to stress factors, such as radiation or genotoxic chemicals, or even due to errors during the DNA replication process (Lodish et al., 2000). Such damages may cause gene mutations which either end up with the immediate death of the cell or in the worst-case scenario, becoming cancerous. As such, the cell cycle is slowed down, through upregulation of CDKI as well as degradation of cyclin, to enable DNA repair or elimination of an irreversible damage. Subsequent activation of the p53 transcription factor play a role in either deciding if cell goes into cell cycle arrest through the synthesis of $p21^{KIP}$ or proceed to apoptosis by activation of pro-apoptotic factors such as BAX, Noxa, PUMA, APAF-1 and Fas (Marks et al., 2009).

2.4.2 Cell cycle and cancer

Cancer has often been characterized as a disease of the cell cycle. This is based on the fact that cancer cells tend to thrive in an uncontrolled manner as opposed to normal cells which proliferate in response to growth stimuli and specific mitogenic signals in a regulated fashion (Diaz-Moralli et al., 2013). Modification to the cell cycle components typically associated with tumour cells arises from chromosomal alteration, which is achieved through amplification and translocation of oncogene as well as deletion of

tumour suppresser genes. Another cause of modification is through epigenetic inactivation through methylation of tumour suppressor promoters (Diaz-Moralli et al., 2013).

Overexpression of CDK and cyclins as well as a loss of CDKI (such as p21 and p27) and pRB functionality are often present in neoplasia (Malumbres & Barbacid, 2001; Shapiro, 2006). Unchecked CDK causes a constantly active mitogenic signals and flawed responses towards anti-mitogenic cytokines. This in turn leads to unregulated proliferation as well as genomic or chromosomal instability (Malumbres & Barbacid, 2009; Massague, 2004). Defects in the G_1 to S phase transition are quite prevalent in human cancer cells, with data suggesting that 80 -90% of tumours have a irregulated CDK 2,4 and 6, all of which play a role in this transition step (Malumbres, 2011; Malumbres & Barbacid, 2001; Tetsu & McCormick, 2003).

2.4.3 Current developments and treatments

CDK dependent pathways as well as metabolic adaptations have been proposed and tested as anti-cancer targets with different degree of success. Inhibition of oncogenes as well as tumour suppressor genes such as checkpoint kinase have shown encouraging outcomes with regards to impeding cancer cell proliferations (Malumbres, 2011).

The high incidence of CDK modification in cancer cells has directed research towards utilizing this component as a possible focus on cancer treatments. Along those lines, there has been pre-clinical data which suggests that inhibiting cyclin D-dependent kinases has a promising prospect to be used as therapeutic target (Ely et al., 2005; Landis et al., 2006; Ortega et al., 2002). Mice expressing a mutant cyclin D1, that was able to bind to its corresponding kinases (CDK4/6) without activating their catalytic domain, failed to develop mammary tumour despite being triggered by the ErbB-2 oncogene (Yu

et al., 2006). Moreover, CDK4 downregulation in ErbB-2 stimulated mammary tumour cells prevent tumour formation in mice (Yu et al., 2006).

Despite the above, there have been other studies that point out absence of the cyclin D1 did not necessarily cause any adverse effects, even after induction with Myc and Wnt1 oncogenes (Yu et al., 2001). This would suggest that other kinases may be able to compensate the absence of cyclin D1 and its corresponding kinase (Barriere et al., 2007; Berthet et al., 2003). This incidence also manages to highlight the importance of determining the type of cancer characteristics in order to choose the best therapy with the expectation of the best possible outcome.

A variety of drugs have been utilized in chemotherapeutic treatment with a particular focus on targeting CDK activities. These treatments target the cell cycle mechanism as well as the transcription of CDK proteins in order to cause an cell cycle arrest and cell death of the tumour cells (Diaz-Moralli et al., 2013). CDK inhibitors that have been developed hitherto can be typically divided into the broad range inhibitors (consisting of lavopiridol, olomoucine, R-roscovitine and kenpaullone to name a few) and the selective inhibitor CDKs (Fascaplysin, Ryuvidine, Purvalanol A and NU2058 among others) (Diaz-Moralli et al., 2013).

CDK inhibitors that were developed at the earlier stages, although provided promising pre-clinical data (Alessi et al., 1998; McClue & Stuart, 2008; Meijer et al., 1997), but failed to produce encouraging outcomes in the clinical trials due to low level activities as well as an incidence of toxicity (Bible et al., 2012; Lapenna & Giordano, 2009). The following generation of CDK inhibitors that were more potent, focused more on interacting with the catalytic active site of the kinases, either by competing with ATP or blocking its binding (Johnson et al., 2012; Johnson et al., 2010; Shafiq et al., 2012). An important note for evaluation of new CDK inhibitors would be that certain tumour

types might display different sensitivity to a different CDK inhibitor, depending on its pathogenic spectrum of mutation (Malumbres & Barbacid, 2009).

Some of the earlier chemotherapeutic drugs worked through the disruption of metabolic process of the cancer cells. These drugs include antifolates (aminopterin and methotrexate) as well as 5-fluorouracil (Farber et al., 1948; Heidelberger et al., 1957). Metabolite have been used as activators of CDKIs. For example, p21 responds to sodium butyrate to induce apoptosis in human breast cancer MCF-7 cells (Chopin et al., 2004). At the same time, human colon carcinoma HCT116 and in human lung adenocarcinoma A549 cell lines that were treated with antimetabolites of folate or folate synthesis blockers resulted in a metabolite depletion. Under those stressful conditions, p53 activated p21, which mediated a cell cycle arrest. Under the same condition, with the exception of the absence of p21, PUMA activation by p53 led to an apoptotic cells death (Hoeferlin et al., 2011). In short, p21 active cells responded to the metabolite blockers by inducing p21-dependent cell cycle arrest, while p21 absent cells underwent apoptosis through the activation of PUMA.

2.5 Metastasis

2.5.1 General features and steps involved

Cancer metastasis remains a poorly understood aspect of cancer pathogenesis in spite of making up 90% of cancer-related deaths (Gupta & Massague, 2006; Hanahan & Weinberg, 2000). It is due to this limitation, it remains as a huge stumbling block when concerned with cancer treatment. Through a complex multistep process, metastasis causes the spread of cancer cells which ends up forming secondary tumours at a distant site from its point of origin, which refers to the primary tumour site. Only transformed cells that have acquired the necessary capabilities are able to undergo metastasis (King & Robins, 2006).

Upon acquiring metastasis abilities, the malignant cells proceed to detach itself from neighbouring cells before undergoing localized invasion across the basement membrane. Upon breaching the basement membrane, which is also referred to as the extracellular matrix (ECM), the 'rouge' cells then proceed to intravasate into blood micro-vessels or the lymphatic system. Through the body's circulation system, the cancer cells are transported to distant anatomical sites, where they are lodged in the blood vessels through physical trapping or in some cases with the aid of surface receptors such as integrins. The next step would be for the cancer cells to extravasate through penetration into the tissue surrounding the blood vessel and subsequently the formation of dormant micrometastases. Upon acquiring further colonizing capabilities, these cancer cells are then able to stimulate the growth of new micro blood vessels through angiogenesis, thus allowing them to flourish and develop into macroscopic metastasis (Bracken et al., 2009; Ma & Weinberg, 2008).

2.5.2 Role of matrix metalloproteinase (MMP) in metastasis

In order to be prepared to undergo metastasis, cancer cells a required to obtained five key functionalities before qualifying to be called metastatic cancer cells: the ability to interact with local microenvironment, the potential to undergo migration, ability to undertake invasion, resist apoptosis and lastly the aptitude to induce angiogenesis (Roy et al., 2009). Among these process, the matrix metalloproteinases (MMP) remain a key factor for cancer cells to migrate across different tissues. These endopeptidases are zinc-dependent enzymes that possess the capabilities of degrading as well as remodelling the extracellular matrix or commonly known as the ECM (Gialeli et al., 2011).

Based on their corresponding substrate specificity and structure, the MMPs can be further classified into a few groups: stromelysins, collagenases, gelatinases, matrilysins, membrane-type MMPs, and other non-classified MMPs (Jabłońska-Trypuć et al., 2016). Although traditionally associated with incidence of metastasis, there have been evidence presented that highlight the multiple functionally of MMPs in all stages of cancer (Chambers & Matrisian, 1997; Mannello et al., 2005).

Physiological process such as angiogenesis, tissue repairs as well as embryonic development, all of which have an association with tissue remodelling, are tightly regulated by maintaining a balancing act between the MMPs and their competing tissue inhibitors of metalloproteinases (TIMPs) (Jabłońska-Trypuć et al., 2016). The alterations in balance of these two components has been associated with a number of disease that include cancer cell invasion among others. It is based on this that development and spread of metastatic cancer cells are often linked with MMPs (Bacac & Stamenkovic, 2008; Gialeli et al., 2011; Roy et al., 2009).

A number of MMPs have been linked with the damage of the basement membrane that eventually leads to cancer invasion, two of which are MMP-2 and MMP-9, both member of the gelatinase subset of MMPs. In addition to this, these two enzymes have also been implicated in angiogenesis and tumour growth, both processes essential to survival and expansion of malignant cancer cells (Jabłońska-Trypuć et al., 2016). Overexpression of these enzymes by tumour cells leads to the degradation of the basement membrane. This is preceded by invasion of tumour cells to lymphatic or blood vessels that are located near to primary tumour site, before invading a new site that eventually leads to the formation of a secondary tumour. Besides the ability to degrade E-cadherin, MMPs are able to interact with integrins and other cell adhesion molecules, thus further

enabling the migration and invasion of metastatic cancer cells (Bacac & Stamenkovic, 2008; Bourboulia & Stetler-Stevenson, 2010; Noe et al., 2001).

2.6 Natural products

Natural products are in great demand in developing countries for primary healthcare not only due to their low cost, but a lot to the general faith that natural products are considered relatively safe as a result of their natural sources. The factor of cultural acceptability also plays a role in the popularity of natural products in these parts of the worlds (Nema et al., 2013).

Humans have long been dependent on naturally occurring substances as source of medicinal provision for a wide spectrum of diseases. Egyptians medicine dates back to 2900 BCE; Ebers Papyrus (1500 BCE) provides documentation of over 700 types of drugs, mostly of plant origin. Many of the 1000 plant derived substance from Mesopotamia (2600 BCE) are still used today for treatment of ailments ranging from coughs and colds to parasitic infection and inflammation (Cragg et al., 2009).

Traditional Chinese Medicine has also been extensively documented, dating far back to 1100 BCE; Wu Shi Er Bing Fang, Shennong Herbal and the Tang Herbal to name a few. Likewise, documentations of the Indian Ayurveda system from 1000 BCE (in ancient Sanskrit text such as Charaka, Sushruta and Samhitas) are still being used by traditional practitioners today. Scientific research into the validity of these natural products has actually shown promising anti-tumour effects, adding to the relevance to the field of ethnopharmacology (Saad et al., 2017).

2.6.1 Development of medical approach to natural products

With the development and progress of chemistry in the 19th century, plants started to be examined much closer in an attempt to better understand their medically useful nature. Sertürner purified morphine from opium, serving as an analgesic in the 1804. This led to others seeking the active potential present in traditional medicinal plants, leading to a surge in notable discovery; quinine (Borchardt, 1996), cocaine (Gay et al., 1975), and many other plants. The involvement of the US National Cancer Institute (NCI) in the study of plant sources as a potential anti-cancer drug during the 1960s ignited further interest in the field. Drugs such as Taxol and Camptothecin analogues were the most prominent developments from the program; the former rapidly becoming a favoured cancer therapy upon its release.

Almost three quarters of the anti-tumour compounds used in medicine are directly or indirectly sourced from natural products. About 60% of anti-cancer drugs approved since the 1940s can be traced back to a natural product; 57% of drugs that underwent clinical trial for cancer in the year 2000 were either from natural products or their derivatives (Cragg & Newman, 2000).

2.6.2 Advantages of natural products as drug source

The ability of natural products to synthesize a variety of structurally diverse bioactive compounds adds to the significant advantage held by naturally sourced drugs. Evolution has provided a vast source of stereo chemically complex molecules that seem to have a high degree of specificity towards particular biological targets, an act that synthetic drugs have tried to emulate but met with less success (Nema et al., 2013).

The perception that secondary metabolites were by-products or wastage of plants had rapidly shifted with the better understanding of the plants physiology. Metabolic energy and the genetic cost of making a small molecule requires that the molecule provides some benefit to the organism; be it as a defensive tool against predator, communication medium among its population or as mean to interfere with competing organisms. The ability of secondary metabolites to reach and interact with receptor present on and in the human cells serves as a major advantage to the usage of natural products in drug discovery. The number of pure natural products which have been found to interact with specific mammalian protein receptors testifies to the inherent bioactivity and biocompatibility of natural products (Beutler, 2001).

Examples of drugs that have been sourced from natural products included actinomycin D, paclitaxel and vincristine, which is one of the most commonly used chemotherapeutic agent in cancer treatment. Drugs such as topotecan hydrochloride, dexamethasone, etoposide and tamoxifen are mimics of natural product compounds (Ovadje et al., 2015).

2.7 The Myrtaceae family

Myrtaceae, which has 17 tribes, consist of approximately 140 genera and 5,500 species (Biffin et al., 2010; Thornhill & Crisp, 2012). The fleshy-fruited Myrtoideae and the dry capsular-fruited Leptospermoideae were initially recognised as the two sub-families of Myrtaceae (Thornhill & Crisp, 2012). Through molecular phylogenetic that were much more recent, there appeared to be two distantly related tribes containing fleshy-fruited species, but also consisted of dry-fruited species: Myrteae and Syzygieae (Biffin et al., 2010; Thornhill & Crisp, 2012; Wilson et al., 2005).

Plants from this family are mostly distributed in the southern hemisphere, concentrated in Australia, South-East Asia as well as in South America (Thornhill & Crisp, 2012). Species from the Xanthostemoneae, Myrteae and Syzygieae are commonly found in the tropical rainforests of Australia and South-east Asia (Brophy et al., 2006), whereas species of Eucalypteae, Melaleuceae and Leptospermeae are common in forest woodlands from the dry tropics through to the temperate zones (Brophy & Southwell, 2002).

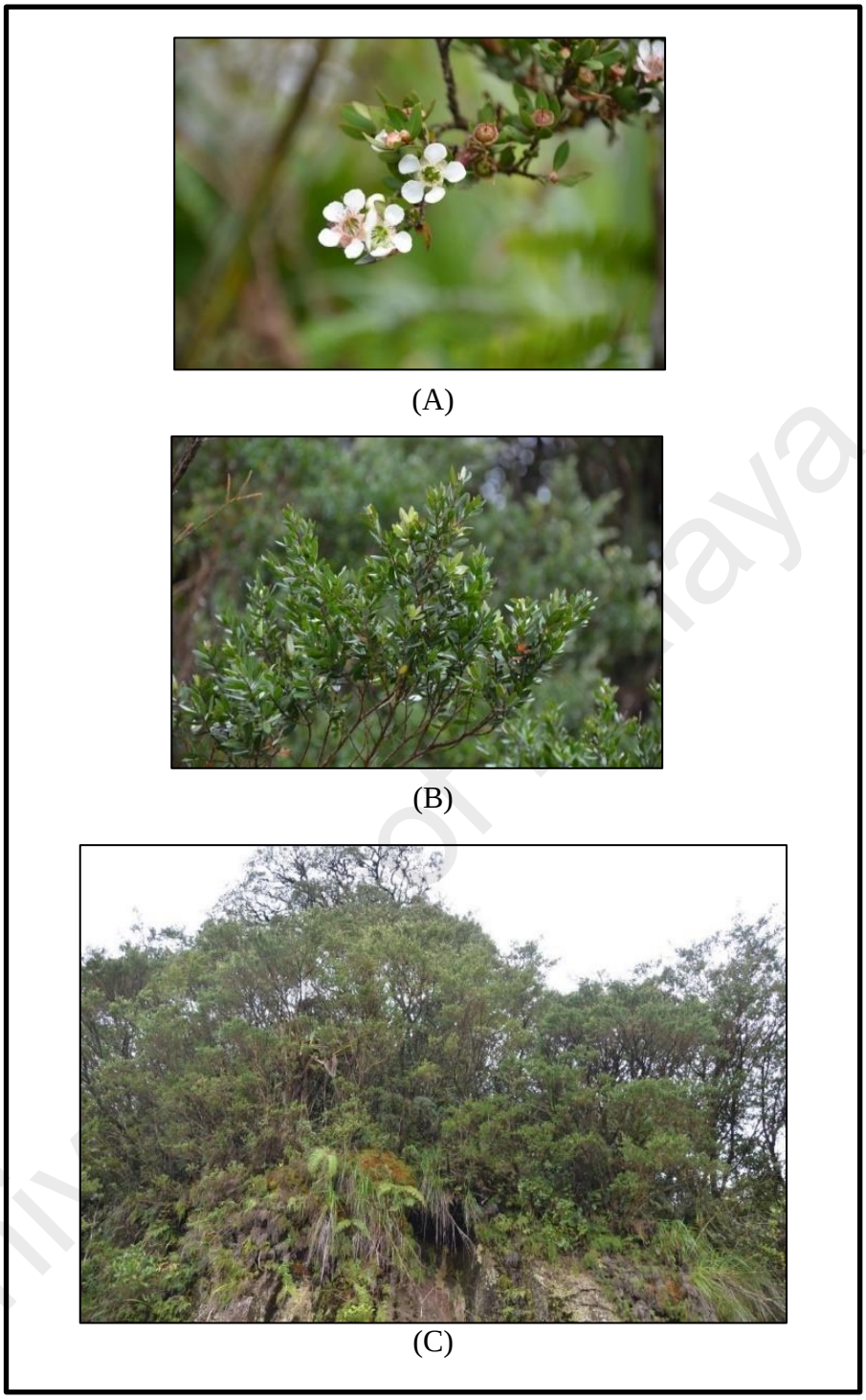
The family is often characterized by having stringy bark, mostly having an inferior ovary, opposite leaves tilled with aromatic essential oils in translucent 'gland dots', and often with a secondary marginal vein (Govaerts et al., 2008). Plants from this family are used for many industrial purposes, for example tea tree oil is extracted from *Melaleuca alternifolia* (Butcher et al., 1994; Carson et al., 2006), eucalyptus oil is extracted from the 1,8-cineole rich leaves of several *Eucalyptus* species (Boland et al., 1991), spices from *Pimenta* as well as *Syzygium* and guava (the fruit of *Psidium guajava*) is a common food crop and important in traditional south east Asian medicines (Chen et al., 2007). Eucalyptus is commonly found naturalized and invasive throughout the tropics. *Psidium* species are also invasive in certain tropical localities.

2.7.1 The *Leptospermum javanicum*

Leptospermum javanicum (syn. *Leptospermum flavescens*), a medium sized shrub in the Myrtaceae family that has been brewed as a tea and used as a local remedy for various ailments (Burkill, 1966; Jantan et al., 1995). The plant has also been reported to be used for medicinal purposes in Indonesia (Brophy et al., 2000). It has a reddish to brown bark with numerous twiggy angular branches. The flowers are solitary, frequent and has a white colouration with a yellow tinge (Figure 2.2).

The plant that was described as *L. flavescens* in Navanesan et al. (2015), has undergone a change in nomenclature to *L. javanicum*. In the past, *L. flavescens* encompassed species distributed from Myanmar to the Australian region. With increased taxonomic studies focusing on these plants, obvious morphological differences were observed with materials collected in its distributional range. As *L. flavescens* could no longer be classified as a true species, the name has been reduced to a synonym and the plants have been renamed to reflect their localized distribution. Examples such as *L. petersonii, L. liversidgei* and *L. polygalifolium* which are found in Australia were all once called *L. flavescens*. In line with that, the locally (Peninsular Malaysia) found variation of *L. flavescens* had been renamed as *L. javanicum*. This change of this name has been highlighted by Thompson (1989) and Ashton (2011).

To date, there have been limited number of studies on this plant with respect to its biological activities, including Jantan et al. (1995) which highlights the essential oil composition from this plant. The plant contains an oil the composition of which may be dependent on altitude or collecting season or both (Jantan et al., 1995) One sample from Malaysia contained α- and β-pinene as the major components whereas another two samples were rich in terpinen-4-ol. All samples contained small amounts of 4-hydroxy-4-methylpentan-2-one (Jantan et al., 1995). *L. javanicum* is from the same genus which are indigenous to Australia and New Zealand, such as *Leptospermum polygalifolium* and *Leptospermum scoparium* that have been studied for its healing properties (Cooper et al., 2001; Visavadia et al., 2008). On prominent product of *Leptospermum* genus is the Manuka honey from *L. scoparium,* which is widely believed to possess anti-bacterial properties (George & Cutting, 2007).



(A)

(B)

(C)

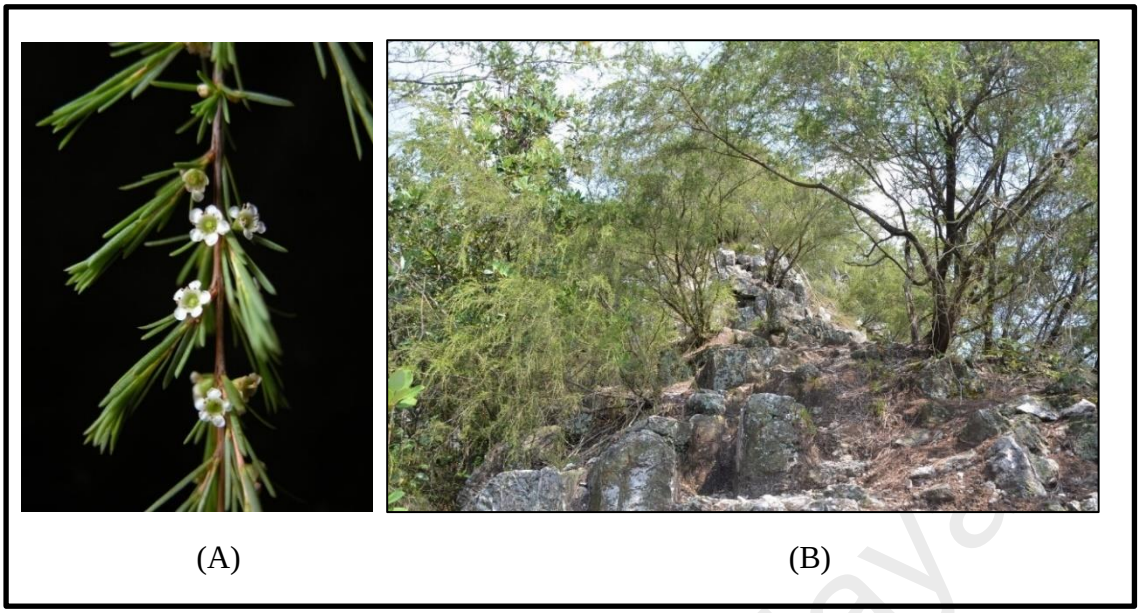
Figure 2.2: The appearance of *L. javanicum*

White solitary flowers (A) as well as the intact branches with the small leaves (B). The plant in its natural habitat. The images were taken from the collection site in the Genting Highlands area.

2.7.2 The *Baeckea frutescens*

Baeckea frutescens is found in Peninsular Malaysia, Sumatra as well as the coastal areas of southern China and Australia. In Peninsular Malaysia, *B. frutescens* is found both on the mountain tops and sandy coasts. The leaves release an aromatic fragrance when crushed. The flowers are solitary and white in colour with a pink centre. The entire plant, except the roots, is used as an antibacterial, anti-dysentery, antipyretic and a diuretic (Herbal Medicine Research Centre, 2002). It is claimed to be effective in treating influenza, coryza, epistaxis, fever, headache, measles, colic, abdominal pain, dyspepsia, jaundice, haemorrhagic dysentery and irregular menstrual cycles. (Herbal Medicine Research Centre, 2002). The Chinese use the leaves as remedy for sunstroke and fever. In Malaysia and Indonesia, they are one of the ingredients used in traditional medicines given to women during confinement. The flowers of this plant are used to treat dyspepsia and menstrual problems. They are also used in massaging postpartum women for the treatment of body aches and numbness of the limbs. The essential oil extracted from this plant is used in the treatment of rheumatism.

To our knowledge, there is only limited published literature on *B. frustescens*. An investigation by Fujimoto et al. (1996) reported that the ethanolic leaf extract of *B. frutescens* exhibited strong cytotoxic activity against L1210 leukaemia cells. The phytochemical study conducted by Makino and Fujimoto (1999) reported the isolation of three new flavanones from *B. frustescens* leaf extracts, which showed promising cytotoxicity against L1210 leukaemia cells. A later report by (Hwang et al., 2004) indicated the anti-cariogenic, anti-malarial and anti-babesial activity of *B. frustescens*.



(A) (B)

Figure 2.3: Appearance of *B. frutescens.*

Flowers with the branch (A) and the plant in its natural habitat (B).

2.8 Betulinic acid (BA)

2.8.1 General characteristics

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) is a naturally occurring pentacyclic triterpenoid. It has a double bond at position 20(29) as well as 3beta-hydroxy and 28-carboxy substituents. Betulinic acid (BA) is a white crystalline solid that exhibits limited solubility in organic alcohols such as MeOH and EtOH, $CHCl_3$, and ether. BA has low solubility in H_2O, petroleum ether, DMSO, and benzene. However, BA is highly soluble in pyridine and acetic acid (Cichewicz & Kouzi, 2004).

This triterpenoid with interesting pharmacologic activities has been extracted from a wide range of plant species. This ranges from carnivorous plants such as *Sarracenia flava* (Sarraceniaceae) (Kingston & Munjal, 1978) to trees and shrubs such as *Diospyros spp.* (Ebenaceae), *Inga punctata* (Fabaceae) (Trumbull et al., 1976), *Ziziphus spp*. (Rhamnaceae), *Vauquelinia corymbosa* (Rosaceae) (Eiznhamer & Xu, 2004) and *Syzygium spp*. (Myrtaceae) (Cichewicz & Kouzi, 2004; Yogeeswari & Sriram, 2005). The

reduced congener of BA, betulin (3b-lup-20(29)-en-3,28-diol), was one of the first natural products identified and isolated from plants in 1788. Quantitatively, betulin is extracted in higher amounts than BA (Cichewicz & Kouzi, 2004; Alakurtti et al., 2006). One of the most widely reported sources of BA is the birch tree (*Betula spp*., Betulaceae) where both BA and betulin can be obtained in substantial quantities (O'Connell et al., 1988).

2.8.2 BA as anti-cancer agent

BA has proven to be a promising anti-cancer agent against multiple tumour (Alakurtti et al., 2006; Fulda, 2008; Pisha et al., 1995; Yogeeswari & Sriram, 2005) primarily through the induction of intrinsic apoptosis. This done by directly reacting with the mitochondria causing a decrease in the mitochondrial membrane permeability complex which leads to the enhancement of cytochrome C release which is aided by the formation of reactive oxygen species (ROS) (Fulda et al., 1998; Fulda, 2008). This is followed by the cleavage of caspase-3 and -8 (Fulda et al., 1999) which precedes poly(ADP-ribose) polymerase cleavage (Wick et al., 1999). Zanon et al. (2004) reported that BA 2 promoted enzymatic activity of caspases 2, 3, 8 and 9 independently of APAF-1 (apoptosis protease activator protein-1) status in melanoma cells. The independence of BA from the p53 tumour suppressor gene status and death inducing ligand/receptor CD95/CD95L pairs further confirms the induction of apoptosis through the intrinsic pathway (Fulda et al., 1997; Wick et al., 1999; Selzer et al., 2000; Zuco et al., 2002).

With regards to complementary role with currently available chemotherapy drugs, BA has managed to induce apoptosis in SHEP neuroblastoma cells when combined with doxorubicin, VP16, Taxol and actinomycin D. BA also managed to sensitized medulloblastoma (Daoy), glioblastoma (A172) and melanoma (Mel-Juso) cells for anticancer drug doxorubicin-induced apoptosis, without affecting normal human fibroblasts (Fulda & Debatin, 2005). However, combination with cisplatin did not exhibit

any signs of a synergistic effect on head and neck cancer cell lines (SCC25 and SCC9) (Eder-Czembirek et al., 2005).

BA was shown to cause an increase in phosphorylation that lead to an activation of p38 and JNK/SAPK MAPKs, while at the same time not effecting the levels of phosphorylation of ERKs (Tan et al., 2003). Contrary to this, Qiu et al., 2005 reported that BA manages to induce ERK activation in melanoma cells. In that same study, melanoma cells appeared to be less sensitive to BA due to the activation through phosphorylation of EGFR and Akt, expression of survivin as well as the weak activation of KNK/SAPK and p38. NF-κB, a key mediator of the cellular stress response which typically engages survival pathways (Zhang & Chen, 2004;Karin et al., 2004), is suppressed as a result of inhibition of IκBα kinase phosphorylation and degradation as well as the subsequent suppression of p65 phosphorylation and nuclear translocation (Alakurtti et al., 2006). Takada and Aggarwal (2003) also mentioned a similar reaction towards to BA, whereby the triterpenoid managed restraint of NF-κB activation leading to a suppression of its regulated gene products such as cyclooxygenase-2 and mealloprotease-9. However, contrary to the above to statements, Kasperczyk et al. (2005) managed to show that BA activation of NF-κB promotes BA-induced apoptosis in a cell type-specific manner. This experiment also provided evidence of p65 subunit nuclear translocation as well increased IKK activity and phosphorylation of IκBα, revealing that revealed that NF-κB activated by BA is transcriptionally active.

Downregulation of specificity protein (Sp) transcription factor and EGFR, which are commonly upregulated in bladder tumour cells, was a repercussion of treatment with BA (Chadalapaka et al., 2010). A semi-synthetic derivative of BA, B10, decreased the phosphorylation of Akt and also induced autophagic cell death in glioblastoma cells (Gonzalez et al., 2012). Treatment of multiple myeloma cell lines with BA resulted in a

downregulation of STAT-3 regulated gene products, including Bcl-xL, Bcl-2, cyclin D1 and survivin (Pandey et al., 2010). BA has been shown to regulate the cell cycle through G_0/G_1 arrest, thereby inhibiting cell proliferation, but at the same time having no such effect in certain glioma and ovarian cancer cell lines (Wick et al., 1999; Zuco et al., 2002).

Treatment with BA increased the survival of ovarian cancer xenografted animals with no systematic toxicities or weight loss (Zuco et al., 2002). In a separate study, BA treatment in cervical carcinoma (U14) tumour bearing mice caused a suppression of tumour growth, induction of apoptosis as well inhibition of expression of Bcl-2 and Ki-67 protein (Wang et al., 2012). A C-3 modified BA has also managed to enhance in vivo anti-tumour efficacy and pharmacokinetics properties when compared with treatment with BA (Rajendran et al., 2008). Liby et al. (2007) reported the bioavailability of synthetic BA derivative were distinctly higher in liver, lung, brain and plasma when compared to the parent compound, BA.

In relation to an earlier mentioned point, the independence of BA action from p53 serves as an advantage as nearly one-half of tumours in humans have a deactivated p53 pathway, decreased activation or possess a p53 mutation (Green & Kroemer, 2009); making treatment motilities depended on p53 activation less optimal in its action. The direct targeting of mitochondrial-related apoptotic induction circumvents the requirement of p53, thus making this treatment method less susceptible to drug resistance.

Despite the above statement appears to be promising, the role of p53 in BA treatment does appear to be contradictory. Although the general consensus agrees with findings, such as that of Fulda (2015) and Raisova et al. (2001), that suggest a p53-independent mechanism of apoptotic induction, there appears to be a differing view as suggested by (Rieber & Strasberg-Rieber, 1998). They proposed that BA exerts an

inhibitory effect on human metastatic melanoma partly by escalating p53 levels. As such, further attention should be directed to determine the variation in these findings.

2.8.3 Other properties of BA

Besides the above, BA extracted from *Vitex negundo* plant leaves and Brazilian medicinal plant *Zizyphus jaazerio* stem bark has shown antibacterial (Cichewicz & Kouzi, 2004; Kim et al., 2002; Steele et al., 1999), antimalarial (Krogh et al., 1999; Recio et al., 1995), anthelminthic (Tezuka et al., 2000) activity, as well as anti-inflammatory actions. A BA derivative, PA-457 (bevirimat dimeglumine), was identified as a new class of antiretroviral compound and its antiviral activities were due to the disruption of viral maturation (Li et al., 2003; Salzwedel et al., 2007). The compound is at the most advanced stage of development (a multicentre open-label, randomized, parallel-group phase 2 trial in HIV-1-infected patients receiving administered bevirimat dimeglumine as monotherapy at the beginning for 14 days and as part of an optimized background treatment regimen for up to 72 weeks) (Ali-Seyed et al., 2016).

CHAPTER 3: MATERIALS AND METHODS

3.1 Source of plant material

Plant samples of *Leptospermum javanicum* was obtained from Genting Highlands, Pahang, Malaysia; while *Baeckea frutescens* was collected from Klang Gates area in Ampang, Kuala Lumpur, Malaysia. The collected plant materials were identified by Dr Sugumaran Manicakam of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia and assigned voucher specimen numbers: KLU 47798 (deposited as *L. flavescens*, but here on referred to as *L. javanicum*) and KLU 47909 (*B. frutescens*). The voucher specimens were deposited at the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

3.2 Preparation of plant samples

Collected plant materials were washed and cleaned to remove any excess dirt or insects that may have accumulated during the sample collection. The cleaned materials were then dried by placing them in a dry oven at 40 ºC until a consistent weight was obtained. Following the removal of water content through drying process, the plant materials were ground into fine powder, which was then weighed and recorded.

3.3 Preparation of crude methanol extract and its fractions

The resulting powder from Section 3.2 was then soaked in analytical grade methanol (~100%) with a ratio of 1:3, referencing to powder:methanol. The mixture was left for three days at room temperature under dark conditions before filtration. This process was repeated another two times to ensure maximal yield of the crude methanol extract. The solvent present in the resulting filtrate was removed *via* evaporation under reduced pressure by utilizing a rotary evaporator (Rotavapor R-200, Buchi).

The crude methanol extract was left in the oven at 40 ºC overnight before recording the weight. Part of the extract was reserved for the acute oral toxicity and cytotoxic evaluation. At the same time, the remainder of the extract was subjected to fractionation using hexane to produce a hexane-soluble extract and hexane insoluble residue. The insoluble residue was further partitioned by utilizing ethyl acetate and water in a 1:1 ratio (100 ml: 100 ml) to yield ethyl acetate extract as well as water extract; the former evaporated using a rotary evaporator while the water present in the latter was removed through the freeze-drying process.

The resulting extracts were collected, weighed and kept in a refrigerator at 4 ºC prior to further experimentations. This method was used for both *L. javanicum* and *B. frutescens*. Prior to testing, dimethyl sulfoxide (DMSO) was used to dissolve the obtained extracts, with the exception of the water extract which was instead dissolved in sterilized distil water to for the stock solution.

3.4 Acute oral toxicity

Twelve healthy male Sprague-Dawley rats (aged 8–12 weeks, weighing 200–250 g) which were obtained from Laboratory Animal Centre, Faculty of Medicine, University of Malaya, Malaysia were used in the current acute oral toxicity study. The weight variation in the rats did not exceed ± 20% standard deviation (SD) of the mean weight. The experiment protocol was approved by the Institutional Animal Care and Use Committee, University of Malaya (UMIACUC) with ethical number ISB/29/06/2012/SKS (R) before the acute oral toxicity assay was carried out.

Procedures outlined by the Organization for Economic Cooperation and Development (OECD) guideline 423 (OECD) was used as a reference for this experimentation. The crude methanol extract of *L. javanicum* and *B. frutescens* was diluted to three different dosage (300, 2000 and 5000 mg of crude methanol extract per kg of body weight) in 0.3% carboxymethyl cellulose which was used as vehicle. The male rats were randomly assigned to four different groups, three treatment groups and one control group. A ventilated room was used to place the rat-containing stainless-steel cages with 12 hours light/dark cycle at room temperature (approximately 25 ^{o}C) with constant humidity. The rats were given a week before experimentation to acclimatize to the room condition and free access to water and food was given. Prior to dosing the rats with the treatment, the rats were fasted for 12 hours by withhold food and only allowing access to water.

Following the 12 hours of fasting period, the rats were weighed and the dosage was calculated using the body weight as a reference and the volume of extract given to rats was 1 ml/100 g of the body weight. A syringe attached to a stainless-steel ball-tipped gavage needle was used to orally administer the dosage to rats. Food was provided to the rats four hours after the treatment. The rats were weighed and observed for signs of toxicity effects (mortality, changes in behaviour and physical appearances) within the first four hours after treatment and daily for a further period of 14 days. The experiment was repeated twice to ascertain the reproducibility of results.

3.5 Human cell lines

Two human lung adenocarcinomas (A549 and NCI-H1299) and a normal human lung fibroblast (MRC-5) cells were purchased from American Type Culture Collection (ATCC, USA). The cancer cells were chosen based on their variation in the p53 status; A549 being positive for p53, while NCI-H1299 possessing a partial deletion of the gene leading to a lack of p53 expression. The A549 and NCI-H1299 cells were cultured in RPMI 1640 medium while MRC-5 cells used Eagle's Minimum Essential Medium (EMEM). All media included 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (100×) and 0.5% amphotericin B; an additional 1% sodium pyruvate and 20% instead of 10% FBS was supplemented for the EMEM which was used in the maintenance of MRC-5 cells. The cells were cultured at 37 ºC in CO_2 incubator. The working culture of cells were maintained at a low passage-number for the experimentation process in order to avoid the incidence of genetic drift.

3.6 MTT cytotoxicity assay

Cytotoxicity was determined through the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay that was performed according to Mosmann (1983), with modifications. Prior to the experimentation, the optimal cell number to be used was determine through an optimization step. Cells were plated at various concentrations (ranging 1×10^6 to 1×10^3) before leaving them to incubate for 72 hours. Through comparison of the obtained absorbance from each dilution group, the optimal number of cells was chosen to be used in the MTT assay. As for the actual experimentation utilizing the extracts and compound, the cells were plated in 96-well microplate (7,000 cells per well) and left to grow overnight at 37 ºC in a humidified 5% CO_2 incubator. The cells were then exposed to the treatments that were diluted with 0.5% DMSO at varying concentrations (ranging from 100 to 5.62 µg/ml for the extracts as well as the semi-pure

compound, and 40 to 2.5 µg/ml for the pure compounds) for a period of 72 hours, following which 20 µl of the MTT reagent (5 mg/ml) was added to each well. The plate was incubated for another four hours prior to solubilizing the formazan crystals with dimethyl sulfoxide (DMSO). The optical density of each well was then measured using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 570 and 650 nm as reference wavelength. The IC_{50} values were determined by plotting percentage of viability against the concentration of treatment on a logarithmic scale. Cisplatin was used as a positive control.

3.7 Acridine Orange/Ethidium Bromide (AO/EB) staining

Qualitative assessment to the occurrence of apoptosis was evaluated using the Acridine Orange/Ethidium Bromide (AO/EB) double staining method. Ribble et al., 2005 was used as a reference for this experimentation with minor modification to better suit this study. Cells were plated onto a 6-well plate with a density of 100,000 cells per well. The cells were left overnight to adhere to the plate surface and treated with the relevant treatment for 24 hours before being detached with an EDTA-based detachment solution, Versene (Thermo Fisher Scientific Inc., Waltham, MA, USA). The detached cells were collected and centrifuged at 300 × g for five minutes to concentrate them and were subsequently diluted in 25 µl of phosphate buffered saline (PBS) (Sigma, MO, USA).

A dye mixture containing Acridine Orange(AO) (Sigma, MO, USA) and Ethidium Bromide (EB) (Sigma, MO, USA) mixed in a 1 to 1 ratio was prepared. A volume of 2 µl dye mix was added into the diluted cell suspension and resuspended in order to get a good distribution of dye. The suspension (15 µl) was then placed on a glass slide before overlaying a coverslip. The slide was observed under a fluorescence microscope (Olympus IX73) using a blue excitation mirror unit at 400 × magnification. Signs consistent with apoptosis including chromatin condensation, membrane blebbing and cell

shrinkage were observed and images were captured using the Image D (Olympus, TYO, JPN). In the event a Lab-Tek 2 chamber slide (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used instead of a 6-well plate, the cells were plated at a density of 15,000 cells in each chamber. Cell detachment was not required in this case and a dye mixture using the same ratio as above was used (25 parts of PBS: 2 parts AO/EB dye mixture).

3.8 Annexin V/PI staining

The apoptotic effect of the compound of interest on treated cancer cells were assessed by means of the Annexin V-FTIC/ Propidium Iodide (PI) staining method. The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) was used for this purpose and the experimentation was done according to the protocol outlined in the manufacturer's guidelines.

The cancer cells were first plated at a density of 300,000 cells per 60 mm dish and left overnight. The medium was then replaced with fresh medium containing the treatment at predetermined concentrations. The concentration used was determined by using the IC_{50} values as a reference point. The cells were incubated with the cells at three different time points, 24, 48 and 72 hours, before being harvested. To ensure minimal damage on the surface proteins externalized due to harsh detachment conditions which may lead to a false positive result, a gentle non-enzymatic cell dissociation reagent, Versene (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used instead of trypsin or accutase. As part of the detachment process, the dishes were washed twice with Calcium-free PBS before adding 1 to 2 ml of Versene. The dishes were left for 15 minutes in the CO_2 incubator and checked from time to time for signs of detachment. The dishes were lightly tapped to aid the detachment process. Once detached, the cells were collected and transferred into a 15 ml centrifuge tube, which was then subjected to centrifugation at 300 × g for five minutes. The supernatant was discarded and the pellets were resuspended in

PBS. This was repeated two times as part of the washing step. The concentration of cells was then adjusted to 100,000 cells per ml. A volume of 5 µl Annexin V-FITC conjugate and 5 µl of PI were added and the cells were further incubated for 15 minutes in dark condition. Lastly, the stained cells were analysed using a BD FACS Canto II flow cytometer (Beckton-Dickinson, USA). A minimum of 10,000 events were recorded and the data was interpreted based on the dot plot graph obtained.

3.9 Cell cycle analysis

Disruption in the cell cycle distribution due to exposure with the compound of interest was assessed using the PI staining method which utilized a flow cytometry analysis to collect and interpret the resulting data. The BD Cycletest™ Plus DNA Reagent Kit (Beckton-Dickinson, USA) was used for this purpose and the experimentation was carried following the recommended procedure outlined by the manufacturer.

Briefly, cells were plated onto a 60-mm dish at concentration of 300,000 cells per dish and left overnight, allowing proper adherence. The cells were treated with the samples at predetermined concentrations thorough supplementation of the growth medium. The concentration used was determined by using the IC_{50} values as a reference point. Following a 24-hour incubation, Accutase® Cell Detachment Solution cell detachment solution (Sigma, MO, USA) was used to detach the cells, which were then washed twice using PBS. The cells were subsequently resuspended in the provided buffer solution and adjusted to a concentration of 1,000,000 cells per ml. The solutions provided were then used to dissolve the membrane and release as well as stabilize the nuclear chromatin present in the treated cells. PI was used to subsequently stain the clean, isolated nuclei which were subsequently detected with a BD FACS Canto II flow cytometer (Beckton-Dickinson, USA).

The resulting signals were acquired at a low flow rate to make certain the optimal resolution of the data was obtained. At least 10,000 events were collected. The data was then analysed utilizing the ModFit software (Verity Software House, USA). The collected data was presented as histogram which was analysed to detect the presence of abnormal DNA stem line (DNA aneuploidy). In addition to this, the resulting histogram was used to determine the presence of a sub-G_1 population in order to supplement the other apoptotic assays.

3.10 Caspase-3 assessment

Activated caspase-3, which is a chemical bio-maker used for positive identification of activated apoptosis in cells, was detected using flow cytometry methods in current study. The FITC Active Caspase-3 Apoptosis Kit (BD Biosciences, San Jose, CA, USA) was utilized to enumerate the incidence of activated caspase-3 in treated cells.

Briefly, cells were plated at a density of 300,000 cells per 60 mm dish and left overnight to allow attachment of the adherent cells onto the culture surface. The cells were then exposed to predetermined concentration of the treatment and left for another 24 hours. The concentration used was determined by using the IC_{50} values as a reference point. Following this, treated cells were detached using Accutase® Cell Detachment Solution (Sigma-Aldrich, MO, USA) and collected in a centrifuge tube. Cells were washed twice with PBS, centrifuged at 300 × g for five minutes each time. Fixation and permeabilization of cells were done with the Cytofix/Cytoperm solution provided with the above-mentioned kit. The cells were suspended in this solution for 20 minutes on ice which was then removed through centrifugation at 300 × g for five minutes. The cells were washed with the provided BD perm/wash buffer followed by incubation with the FITC Rabbit anti-active caspase-3 antibody for 30 minutes at room temperature. The

stained cells were once again washed with the BD perm/wash buffer and placed on ice prior to analysis using the flow cytometer.

The events were acquired at a high flow rate and subsequently expressed as a histogram of relative cell number against FITC signal strength. The data was then summarized and presented in a bar chart representing the percentage of positive FITC signal relative to the population of cells acquired for that particular treatment group. The experiment was repeated three times to ensure reproducibility.

3.11 TUNEL assay

The TUNEL assay was performed to identify the presence of DNA strand breaks as a result of exposure to the treatment used. The in-Situ Cell Death Detection Kit (Fluorescein) (Roche, Basel, CHE) was used for this purpose. The protocols used were based on the methods recommended by the kit manufactures with modification from Darzynkiewicz et al. (2008).

3.11.1 Treatment and preparation of cell samples

Cells were plated at a density of 1,000,000 cells in 100-mm dish and left overnight to allow them to attach to the culture surface. Following that, the cells were treated with pre-determined concentrations of the treatment in 10% FBS complete medium and left to incubate for 24, 48 or 72 hours. The concentration used was determined by using the IC_{50} values as a reference point. The treated cells were detached with Accutase® Cell Detachment Solution and centrifuged at 400 ×g for five minutes, before being washed twice in PBS. The cell suspension was then adjusted to a concentration of 1×10^7 cells/ml. Subsequently, cells were incubated with the prepared fixation solution consist of 4% paraformaldehyde in PBS at a pH of 7.4. The suspension was left to incubate at room temperature (15-25ºC) for 60 minutes. The process was done on an orbital shaker to avoid formation of clumps which may interfere during the acquisition using the flow cytometer.

Fixed cells were centrifuged and washed twice with PBS before being permeabilized to allow for the stains to enter the cells. The permeabilization solution, which was prepared by adding 0.1% Triton-X 100 in 0.1% sodium citrate, was incubated with the cells for two minutes on ice and once again washed twice in PBS.

3.11.2 Cell staining and analysis

Breaks in DNA strands present in the above prepared cells were labelled through incubation with the labelling and enzyme solutions provided in the earlier mentioned kit. The incubation process was done for 60 minutes at 37ºC in a dark, humidified atmosphere, as instructed by the kit manufacturer. Following this, stained cells were washed twice in PBS to remove excess dyes to avoid appearance of a false positive population. Lastly, the stained cells were scrutinized using a BD FACS Canto II flow cytometer (Beckton-Dickinson, USA). At least of 10,000 events were recorded and the data was presented as a histogram of relative cells number against the signal strength of fluorescein.

3.12 Isolation, purification and identification of active compounds

3.12.1 Column chromatography

Column chromatography was performed using Silica gel 60 (0.015-0.040 mm) (Merck KGaA, Darmstadt, Germany) with particle size 0.015-0.040 mm. A plug of cotton wools was placed at the bottom of column to prevent the silica from flowing out the column. Caution was taken not to pack the cotton too tightly as it may prevent the eluent from dripping at an acceptable rate.

The column was then filled with one third of the solvent before adding the slurry of silica that was prepared by mixing the dry silica with the same solvent used above. While adding the slurry, the bottom tap was opened to allow the excess solvent to drain out. The column was gently tapped to encourage bubbles to rise and the silica to settle. A layer of sea sand was added to the top surface of the stationary phase which aids in even

loading of sample. After the sample was introduced to the column, solvent with increasing polarity gradient was used to elute the chemical compounds from the column. Fractions collected were monitored by Thin Layer Chromatography (TLC) and appropriate fractions were combined and where necessary subjected to further separation.

3.12.2 Thin Layer Chromatography (TLC)

TLC was used to monitor the separation of fractions during column chromatography by using pre-coated silica gel 60 F_{254} plates (0.2 mm thick, Merck KGaA, Darmstadt, Germany). The TLC plates were spotted with a piece of fine glass capillary tube and then developed in saturated chromatography tanks with various solvent systems at room temperature. The spots were visualized by examination of the TLC plates under UV light (254 and/or 343 nm), followed by exposure iodine vapour.

3.12.3 Identification of chemical constituent

The isolated constituent was identified using a Bruker AVANCE 270 MHz spectrometer operating at 270 MHz for ^{1}H NMR (Nuclear magnetic resonance spectroscopy) and 67.8 MHz for ^{13}C NMR. Internal standards used in ^{1}H NMR spectra was TMS (δ: 0.00) for $CDCl_3$; in ^{13}C NMR was $CDCl_3$ (δ: 77.0). The mass of the constituent was analysed using AB Sciex 3200QTrap LCMS/MS with Perkin Elmer FX 15 uHPLC system (Perkin Elmer, USA) utilizing chemical ionisation. An Agilent Zorbax C18 – 150 mm × 4.6 mm × 5 uM column was used. To identify the structural features of the isolate, it was mixed with potassium bromide (1:10), pressed into approximately 1 mm pallet disc and subjected to Infrared (IR) spectroscopy analysis. Spectrum was recorded at the absorbance mode between 4000–400 cm^{-1} on Perkin Elmer Spectrum RX-1 FT-IR spectrophotometer (Waltham, Massachusetts, USA).

3.13 Migration assay

The transwell membranes were used to assess the anti-migration capabilities of the isolated compound. The protocol was based on Tan et al. (2014) and Siripong et al. (2012) with modifications. The experimentation procedures cover the preparation of the consumables (plates and inserts), treatment and plating of cells, the subsequent staining procedure of the migrated cells which was finally followed by their quantification.

3.13.1 Plate preparation and treatment of cells

The 8.0 µm sized pore inserts suited for 24 well plates (SPL Life Sciences Co., Ltd., Gyeonggi, KOR) were used for this experimentation. The inserts were removed from the packaging and immersed in serum-free medium for 10 minutes to allow the membrane present in the inserts to assimilate to the culture conditions.

A549 cells were serum starved overnight and subsequently detached using Versene (Thermo Fisher Scientific Inc., Waltham, MA, USA), a serum-free cell dissociation solution. The cells were pelleted and adjusted to a concentration of 70,000 cells per well before being mixed with serum-free growth medium containing the isolated compound at 7.5, 5.0 and 2.5 µg/ml. The cells that were left untreated served as a negative control for this experimentation and was used as a baseline to determine the effectiveness of the treatment in preventing migration of the treated cells. The inserts that were soaked earlier were transferred into a new 24 well plate and used immediately. The cell suspension (100 µl) was transferred into the culture inserts before swirling the plate gently to allow for uniform distribution of the cells. Following this, the lower chamber (receiver wells) were filled with 600 µl of 10% FBS supplemented growth medium, which serves as a chemo-attractant to encourage cell migration across the porous membrane. The plates were then incubated for 48 hours at 37 ºC in a humidified CO_2 incubator.

3.13.2 Staining of migrated cells with crystal violet

A fixing and staining solution containing 0.5% crystal violet dissolved in 30% methanol was prepared. Following the incubation period, the inserts were removed and placed in new wells and stained with the prepared solution for 30 minutes at room temperature. This was followed by a washing step which involved the immersion of the inserts in distilled water to remove the excess stains. The inserts were thoroughly rinsed with distilled water until the water appeared clear, indicative of the complete removal of the crystal violet stain. A cotton swab was then used to remove cells which had not migrated from the inner chamber of the inserts. Caution was taken to do this gently to avoid puncturing the membrane. The membranes were left to dry in an inverted position under dark conditions prior to performing the analysis.

3.13.3 Quantification of migrated cells

The air-dried inserts were placed onto a clean glass slide and observed using an inverted microscope. This served as a qualitative assessment for this assay. To obtain a consistent quantitative evaluation to the number of migrated cells, the inserts were placed in a new, clean well and 30% of acetic acid was added to bleach the stains from the cells attached to the lower membrane surface. The cells were exposed to the de-staining solution for 30 minutes to ensure complete bleaching of the crystal violet. Following this, the inserts were removed and the remaining solution in the wells were thoroughly resuspended prior to transferring 200 µl of the suspension to a well in a 96-well plate. The vigorous resuspension was done to ensure an even distribution of the bleached dye, thus ensuring a consistent reading later on. The absorbance was read at 590 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the relative percentage (%) of migrated cells were obtained through comparison with the untreated controls.

3.14 Invasion assay

The methods utilized in the invasion assay were similar to the migration assay, except the inclusion of a Matrigel coating on the transwell membrane inserts. The protocol was based on Tan et al. (2014) and Siripong et al. (2012) with modifications. The experimentation procedures cover the preparation of the consumables (plates and inserts), treatment and plating of cells, the subsequent staining procedure of the migrated cells which was finally followed by their quantification.

3.14.1 Plate preparation

Similarly, to the migration assay, the invasion assay employed the usage of a Boyden Chamber with the additional coating of Matrigel across the membrane which serves as a barrier to migrating cells. Cells that have reached confluency of 70 to 80% were plated in a 100-mm dish at a density of 1,000,000 cells per dish. The cells were subsequently incubated with serum free medium for 24 hours before being detached using an enzyme-free detachment solution, Versene. The cells were counted using Trypan blue method and the cell number was adjusted to 700,000 cells/ml using serum free medium. Following this, the cells were mixed with serum-free growth medium containing the isolated compound at 7.5, 5.0 and 2.5 µg/ml and left to stand for approximately 30 minutes or so.

During this time, the Matrigel pre-coated cell culture inserts were removed from the packaging and placed in a sterile 24-well plate. Pre-warmed serum free medium was placed both in the upper and lower chamber of the inserts in order activated the Matrigel which was frozen at -20 ºC. The inserts that have been warmed up were then placed in new wells and the treated cell suspension mentioned earlier on was placed in the upper chamber. Care is taken to avoid introduction of serum into this cells suspension as it may decrease the invasive potential of the cells. The lower chamber was then filled with 10%

serum supplemented growth medium which served as a chemoattractant to the potential invading cells. The plates were left to incubate in a CO_2 incubator under normal cell culture conditions for 48 hours to allow the invasion process to take place.

3.14.2 Staining

Following the incubation period, the inserts were removed from the plate and placed in new wells. A fixation/dye solution containing 0.5% crystal violet dissolved in 30% methanol was prepared and added into the upper and lower chambers of the cell culture inserts and left to stand for 30 minutes. The fixative and dye were removed by dipping the inserts into beakers containing deionized water. The water was replaced after each washing step and was repeated until the dye was completely removed. A cotton swab was used remove the layer of uninvaded cells from the inner chamber of the inserts. Care was taken to ensure that the process was done as gently as possible to avoid puncturing the membrane. The washed and swabbed inserts were then left to air dry at room temperature overnight.

3.14.3 Quantification of invaded cells

The dried inserts were then placed on a glass slide and observed under an inverted light microscope. The inserts were further analysed by placing them in a 30% acetic acid solution to bleach the dyes from the cells. After an incubation period of 30 minutes, the resulting solution was repeatedly pipetted to ensure a uniform mixture. 200 µl of the solution were then transferred into a 96-well plate and was read at an absorbance of 590 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the relative percentage (%) of invaded cells were obtained through comparison with the untreated controls.

3.15 Cell attachment assay

The ability of the isolated compound to inhibit adhesion of cells was assessed using the protocol outlined by Xia et al. (2005) with modifications. Briefly, cells were treated with varying concentrations (2.5, 5.0 and 7.5 µg/ml) of the isolated compound prior to detachment and re-plating onto a new culture plate. The plates were incubated for 6 and 24 hours, after which the cell attachment status and morphology was examined and compared to the untreated controls. The images were captured using an inverted confocal light microscope (Leica Microsystems GmbH, Wetzlar, DEU).

3.16 Gelatin zymography

3.16.1 Treatment and collection of test samples

Cells were plated with 1,000,000 cells on a 100-mm dish and left overnight for attachment. Cells were then starved for 24 hours in 0.5% FBS supplemented medium before being treated with the isolated compound at predetermined concentrations (ranging from 1.25 to 7.50 µg/ml). A volume of 100 picograms of Transforming growth factor beta 1 (TGF-β1) was supplemented into the medium to promote gelatinase production. Cells were left for 48 hours before the condition medium was collected and clarified through centrifugation at 400 × g for five minutes before being stored at -80ºC.

3.16.2 Protein quantification

A portion of the conditioned medium was used to quantify the total protein content in the solution for standardization and dilution purposes. The samples were quantified using the bicinchoninic acid assay (BCA assay). The procedure was done in accordance with the manufacturers protocol (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a standard curve was used to determine the estimated amount of protein present in the samples. Each sample was adjusted in order to contain equal amounts of protein.

3.16.3 Zymogram

The gelatin zymogram gel was prepared and run according to the protocols outlined by Toth et al. (2012) with modifications. Firstly, the short plate and spacer plates were cleaned with 70% ethanol before being assembled onto the casting stand. The 10% resolving gel solution (Appendix G) was prepared and loaded into the glass plates, followed by adding a layer of deionized water to ensure and even polymerization of the gel. Once the resolving gel was solidified, the deionized water was decanted and the stacking gel solution (Appendix G) was layered on the resolving gel and the comb was inserted.

The polymerized gel was placed in the electrode assembly and the protein samples that were mixed with 4× sample buffer (Appendix G) were loaded into the wells. A protein ladder was also loaded into one of the wells to aid in the identification of the resolved bands. Running buffer (Appendix G) was poured into the chamber to the appropriate mark and was run at 125V for approximately two hours or until the marker dyes had reached the bottom of the gel. Once completed, the gels were removed from the assembly and placed in 100 ml of renaturing buffer (Appendix G) for 30 minutes to activate the gelatinase enzymes. A quick wash of the gel with 300 ml of deionized water was followed by incubation in developing buffer (Appendix G) for 30 minutes. The buffer was replaced with 100 ml of fresh developing buffer and left for 16 hours at 37 ºC in order for the gelatinase enzymes to digest the gelatin present in the gel.

The developed gels were then stained with comassie blue (Appendix G) for an hour with gentle agitation followed by removal of excess stains with a de-staining solution (Appendix G). The de-staining solution containing the gel were microwaved at high power for 30 minutes followed by gentle agitation for 10 minutes to speed up the de-staining process. This step was repeated until clear bands were present against a backdrop

of Coomassie blue stained gelatin gel. The de-stained gels were stored in a solution of 5% acetic acid at 4 ºC until imaging was done. A gel scanner was used to obtain an image which was used to analyse BA's effect on the enzyme activity of MMP-2 and MMP-9.

3.17 Enzyme-linked immunosorbent assay (ELISA) for MMP detection

The presence of MMP-2 and MMP-9 were detected using the enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's protocols (Abcam, Cambridge, UK). The MMP-2 Human ELISA Kit and MMP-9 Human ELISA Kit were used for the detection of MMP-2 and MMP-9, respectively. The cells were plated at a density of 1,00,000 cells per 100-mm culture dish and incubated for 24 hours at 37°C, ahead serum starvation for 24 hours and then adding treatments at various concentrations (ranging from 1.25 to 7.5 µg/ml). The cells were also exposed to 100 picograms/ml of TGF-β1 to induce the production of the gelatinase. The conditioned medium was collected and clarified through centrifugation at 400×g for 10 minutes following which 100 µl of the prepared conditioned medium was loaded into the pre-coated wells overnight. The medium was discarded and the wells were washed with the provided 1× wash buffer for a total of four times. 100 µl of Biotinylated MMP-2 Detection Antibody was added into each well and left to incubate for an hour with gentle agitation. The wells were once again washed four times with the wash buffer, following which 100 µl of 1× HRP-Streptavidin solution was added and left for 45 minutes. Another washing step was followed by addition of 100 µl of TMB One-Step Substrate Reagent and was then incubated for 30 minutes. Finally, 50 µl of Stop Solution was added to each well and the absorbance was read at 450 nm using a microplate spectrophotometer reader. The absorbance value obtained was compared with a standard curve to determine the concentration of MMP proteins present in the sample.

3.18 Western blot analysis

3.18.1 Treatment of cells

To begin with, 1,000,000 cells were plated on to 100-mm dish and left overnight to attach, followed by serum starvation with serum-free medium for a further 24 hours. The synchronized cells were then subjected to treatment with the isolated compound at 5.0, 7.5 and 10µg/ml that were diluted in complete growth medium for an additional 24 hours under normal growth conditions.

3.18.2 Harvesting and lysing cells

Following exposure to the treatment, the medium was removed and the cells were washed twice with cold PBS to remove any debris or dead cells that may have still present in the dish. The residual PBS was pipetted out. Radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with protease and phosphatase inhibitor was then used to lyse the cells. In order to ensure a concentrated protein yield, 100 µl of the extraction buffer was placed onto the dish and a cell scrapper was used to remove the layer of cells towards the buffer solution present in the dish. The suspension was carefully aliquot into a micro centrifuge tube and left on ice for 20 minutes. The protein suspension was then sonicated using an ultrasonic probe to shear the chromosomal DNA which could hamper the separation process later on. The cell lysates were placed on ice during the sonication process, which was done in short burst to minimized heat damage of the protein in the lysate. Finally, the suspension was centrifuged at 14,000 × g for 20 minutes at 4ºC. The supernatant was collected and transferred to a new, clean micro centrifuge tube before being stored at -80ºC.

3.18.3 Protein quantification

The resulting protein lysate was quantified using the Pierce™ BCA Protein Assay Kit. 25 µl of the lysate were placed in a 96-well plate followed by the addition of 200 µl of the BCA reagent provided in the kit. The plates were left to incubate for 30 minutes at 37ºC, after which it was allowed to cool down to room temperature. The absorbance was read at 562 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the resulting data was interpolated from a previously prepared standard curve using the protein samples provided in the kit.

3.18.4 Protein separation through SDS-Page

The SDS-Page was performed using the Mini PROTEAN tetra cell (Bio Rad, CA, USA). The casting apparatus was setup by placing a 70% ethanol cleaned short and spacer glass plates into the casting frame. The separating gel solution (Appendix H) was poured into the casting apparatus and was layered with distilled water before being left to set for 30 minutes. Caution was taken to avoid introduction of air bubbles which could disrupt the separation process later on. Following the separating gel solidification and removal of the distilled water, the stacking gel solution (Appendix H) was poured to the brim of the apparatus. A 10-well comb was immediately placed and left for 15 minutes or so to solidify. The gel cassette was removed and placed in to the electrode assembly which was then filled with running buffer (Appendix H). 40 µg of the protein samples were mixed with 4× loading buffer (Appendix H) and heated at 95ºC for five minutes after which they were kept on ice prior to loading. Once the samples were ready, the comb was removed and the samples were loaded into the wells. A pre-stained protein ladder as well as a biotin ladder was included to serve as molecular weight markers that would aid in the band identification later on. The assembly was hooked up the power unit and ran at 180 V until the dye front reaches the bottom which took approximately one hour.

3.18.5 Blotting

The resolved gel was removed from the cassette using a prying tool and placed in the transfer buffer (Appendix H) for 15 minutes to equilibrate the gel to the transfer buffer. At the same time, the thick filter paper, nitrocellulose membrane as well as the fibre pads was soaked in the transfer buffer as well. Following this, the transfer sandwich was prepared by utilizing the gel holder cassette.

The prepared sandwich was then placed in a buffer tank containing ice-cold transfer buffer. A cooling unit was placed into the tank to maintain a low temperature, ensuring minimal heat damage to the proteins being transferred. In order to improve the transfer of separated proteins from the gel to the membrane, 0.037% of SDS was added into the buffer system. The transfer process was begun by hooking up the tank to the power unit and ran at 100 V for two hours. Subsequently, the membrane was removed from the sandwich and placed in 25 ml TBS (Appendix H) and incubated with gentle rocking for five minutes to ensure traces of the transfer buffer were completely removed. The next step was to decant the TBS and replace it with 25 ml of blocking buffer (Appendix H) which contained 5% of non-fat Dry Milk (Cell Signalling Technologies, MA, USA) to prevent nonspecific binding of the antibodies. Following an incubation period of one hour at room temperature with gentle rocking, the membrane was washed with 25 ml of TBST (Appendix H) for five minutes.

3.18.6 Detection with antibodies

The TBST was decanted and replaced with the primary antibody diluted in 10 ml of 5% BSA in TBST according to the manufacturer's recommendation. The membrane was left overnight (16 hours) at 4 ºC before being washed with 15 ml of TBST for five minutes. This was repeated a further two time to ensure complete removal of unbounded antibodies which may cause a background. The membrane was then immersed in

horseradish peroxidase secondary antibody solution in blocking buffer at room temperature for 1 hour with gentle rocking which was followed by a 5-minute washing step with TBST that was done three times. The washed membrane was flooded with SuperSignal West Pico chemiluminescent substrate and viewed with ChemiDoc Chemi-luminescent Gel documentation system (Bio-Rad, CA, USA) with exposure times ranging from 15 seconds to 5 minutes (depending on the intensity of the bands). The resulting bands were analysed using the Image Lab imaging software (Bio-Rad, CA, USA).

3.19 ELISA detection of proteins from cell lysate

The samples were prepared mentioned in Section 3.18.1 and 3.18.2. Briefly, the RIPA buffer was used to lyse the treated cells and the resulting lysate was collected, quantified using the BCA protein quantification method (Section 3.18.3) and stored at -80 ºC until the experimentation was carried out. The concentration of the lysates was diluted in order to have equal amounts of proteins for each sample (treated and untreated). A volume of 100 µl of each sample was loaded into the pre-coated wells and left to incubate at 4 ºC overnight. The medium was discarded and the wells were washed with the 1 × washing buffer for a total of four times. 100 µl of Detection Antibody was then added into each corresponding well and left to incubate for an hour at 37 ºC with gentle agitation. The wells were once again washed four times with the wash buffer, following which 100 µl of HRP-linked secondary antibody solution was added to the its respective well and left for 30 minutes at 37 ºC. Another washing step was followed by addition of 100 µl of TMB Substrate to each well and incubated for an additional 30 minutes at 25 ºC. Finally, 100 µl of Stop Solution was added to each well and the absorbance was read at 450 nm using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The absorbance values were used to calculate the percentage of protein expression change through comparison with their respective untreated controls.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Yields of extraction from *L. javanicum* and *B. frutescens*

Extraction can be defined as a process that utilizes selective solvents and standard extraction methods to separate medicinally significant component of plans or animal tissue from their inert or inactive portions. The maceration technique was used as it is simpler, more convenient and less costly in terms of instrumentation (Vongsak et al., 2013).

The plant samples that were obtained were subjected to drying prior to any further extraction processes. This particular step was performed in this study with the intention of inhibiting degradation of the compounds, which would subsequently cause variations in the composition between different batches of the plants. This would ensure the quality and consistency of the bioactive compounds extracts that would be further used in this study.

In order to ensure that the medicinal ingredient of the dried *L. javanicum* and *B. frutescens* were exposed to the extraction solvent, the plant samples were subjected to grinding, which would cause the rupture its organ, tissue and cell structures. In addition to this reasoning, the reduction in sample size would maximise the exposed surface area, which in turn enhances the mass transfer of active principle from both *L. javanicum* and *B. frutescens* to the solvent. During the grinding process, caution was taken to prevent over grinding the previously dried plant samples, as a coarse powder eased the subsequent clarification process.

In this study, methanol was utilized as a primary solvent due to its known ability to extract most polar compounds as well as a number of non-polar compounds as well. This is in addition to its relatively low boiling point, which eases its removal and concentration of the crude extracts using a rotary evaporator.

As shown in Table 4.1, methanol extraction for *L. javanicum* and *B. frutescens* produced a yield of 16.3% and 46.02%, respectively. *B. frutescens* appeared to have a higher methanol extraction output compared to *L. javanicum*, whereby almost half of the dried material was recovered. *L. javanicum* yielded a crude methanol extract that appeared to be a dry pasty substance with a brown colour, while *B. frutescens* crude methanol extract produced a dry dark brown extract that had a brown colour with a greenish tinge (Figure 4.1).

Following the methods outlined in Section 3.3, hexane was used in order to separate out the non-polar constituents from the crude methanol extract. Both plants seemed to have a lower yield for this category of compounds, as shown in Table 4.2, indicative of low amount non-polar substance in the crude methanol extract. Both plants produced semi-liquid substance that had an oily texture with a greenish brown colour as an end product of the hexane extraction (Figure 4.1). Two-phases separations of the hexane insoluble fraction of *L. javanicum* with ethyl acetate and water managed to produce two distinct fractions, the former producing a rather solid and dry fraction with a deep green pigmentation while the water fraction appeared as a semi-solid, caramel like texture that was dark brown. *B. frutescens* on the other hand produced a similar solid green ethyl acetate fraction while a fine brown powder was obtained as the water fraction. The respective yields of the fractions of both the plants in question are listed in Table 4.2, while the images of the resulting extracts are displayed in Figure 4.1.

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The resulting extracts were subjected to a pre-screening using a number of cancer cell lines (A549, HCT-15, MDA-MB-231 and MCF7). Based on that pre-screening, lung cancer was chosen to further the study as it showed the most promising outcome (refer to Appendix A).

Table 4.1: Yield of extraction for *L. javanicum* and *B. frutescens*

Plants	**Samples / Extract**	**Weight (g) (%)**
L. javanicum	Dried and ground plant material	1000.00
	Crude methanol extract	163.00 (16.3%)
B. frutescens	Dried and ground plant material	500.00
	Crude methanol extract	230.10 (46.02%)

Table 4.2: Yield of extracts fractionated from crude methanol extract

Plant	**Extract**	**Yield of extract (g)**	**Percentage (%)**
L. javanicum (extracted from 153.00 g of crude methanol extract)	Hexane	6.30	4.12
	Ethyl acetate	30.00	19.61
	Water	29.40	19.22
B. frutescens (extracted from 220.10 g of crude methanol extract)	Hexane	17.50	7.95
	Ethyl acetate	16.80	7.63
	Water	12.90	5.86

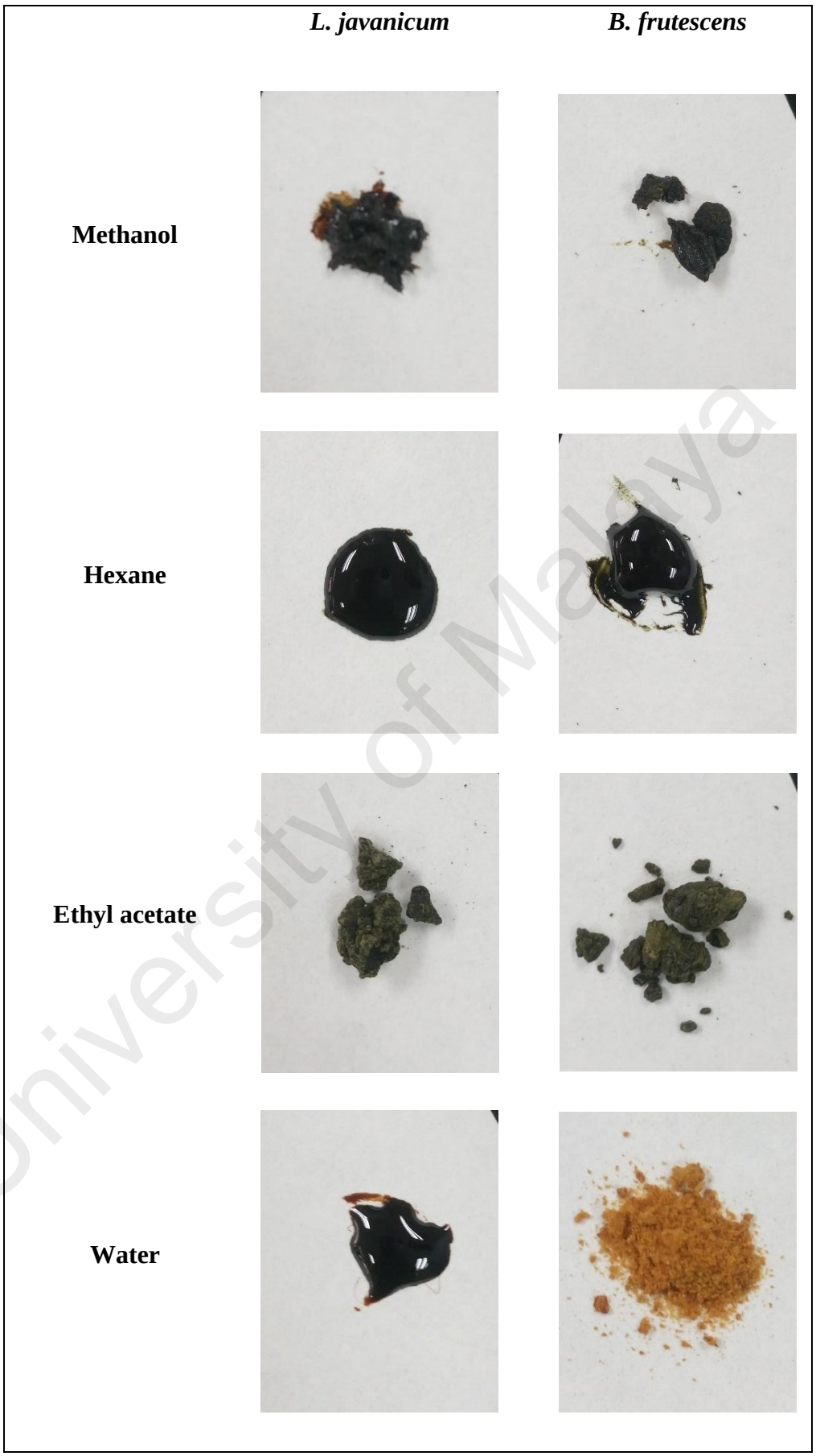

Figure 4.1: Crude methanol extracts and its fractions obtained from *L. javanicum* and *B. frutescens*

4.2 Absence of acute oral toxicity in crude methanol extracts

Toxicity is a state of adverse effects due to interactions between toxicants from poisonous substance with the living cells (Jothy et al., 2011). Evaluating the toxic potential of particular substance is crucial as exposure to toxicant may lead to adverse or hazardous effects on human beings. Thus, it is highly important to ensure the substance is safe before exposing it to humans through consumption.

In the current study, an acute oral toxicity testing was conducted on Sprague-Dawley rats in order to determine the toxicity of *L. javanicum* and *B. frutescens* crude methanol extracts. As mentioned in Section 3.4, the rats were exposed to 300, 2000 and 5000 mg/kg of the crude methanol extracts. No toxic symptoms were observed on the test subjects throughout the whole experimentation which lasted for 14 days. Both extract-treated group and vehicle-treated group did not exhibit any abnormalities in behaviour, breathing, disruption in food and water consumption, skin effects and hair loss. All the rats showed no sign of distress or displayed any symptoms associated with toxicity or death. The increase in body weight of the rats gradually with time indicates normal growth (Figure 4.2). This is a clear indication that the extract tested has no significant amount of toxicity in reference with the growth of the rats. Thus, it can be concluded that the methanol extract of *L. javanicum* and *B. frutescens* were non-toxic towards the rats.

This study also indicates the Lethal Dose (LD_{50}) value for the extracts were more than 5000 mg/kg. In accordance with OECD guideline for testing of chemicals, the methanol extracts of *L. javanicum* and *B. frutescens* fall in category 5, where the LD_{50} exceeds 5000 mg/kg (OECD). This is the lowest class of toxicity outlined by OECD. Any substance with a LD_{50} which exceeds 5000 mg/kg, which was administered orally to the animal, is considered as safe and non-toxic (Kennedy et al., 1986).

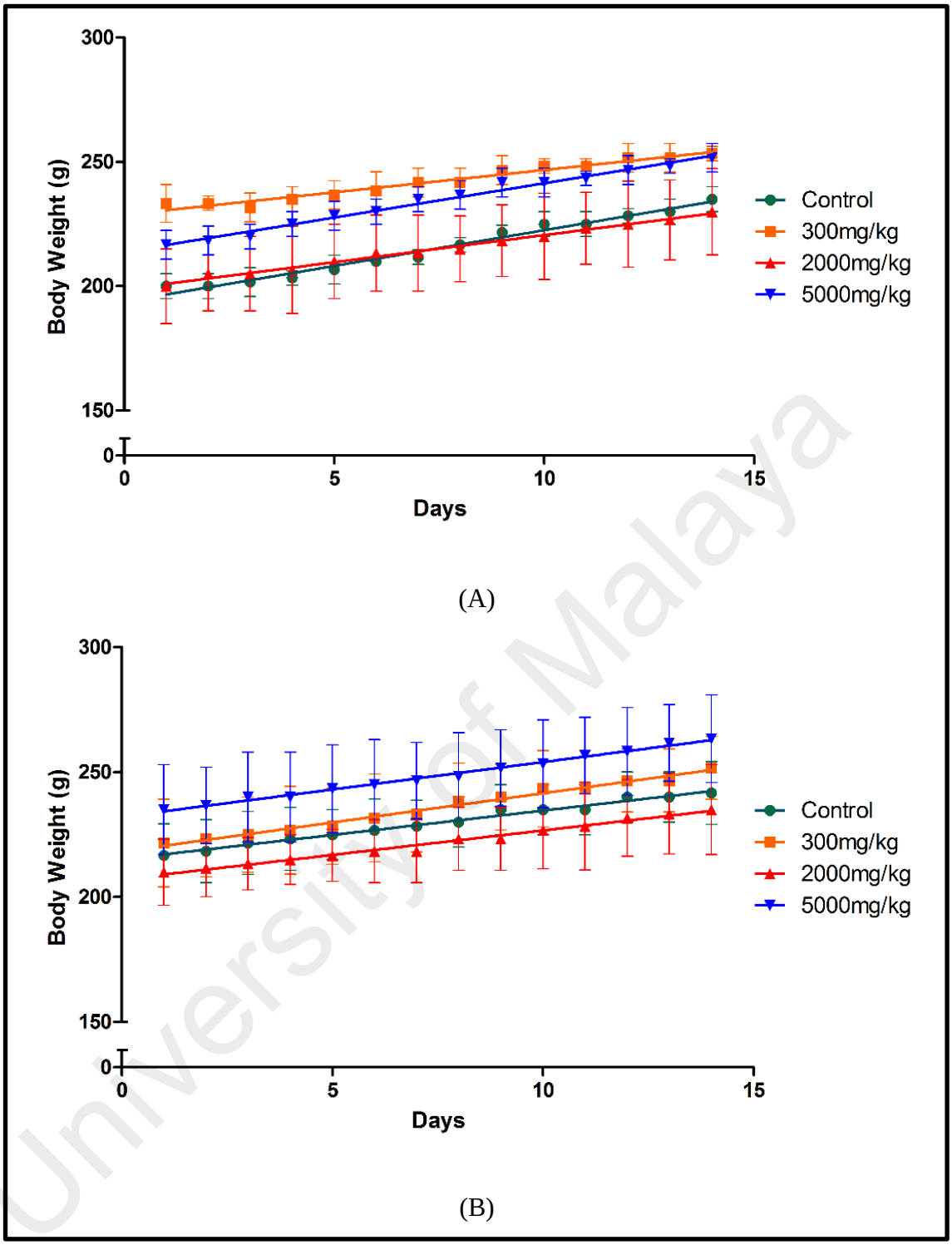

Figure 4.2: The effect of *L. javanicum* (A) and *B. frutescens* (B) crude methanol extracts on rat body weight.

The graph indicates the average body weight (n = 3) of the test subjects (rats) throughout the experiment, grouped by the amount of concentration (0, 300, 2000 and 5000 mg/kg) administered. A gradual increase in weight is observed in all treatment groups indicative of healthy test subjects.

To the best of our knowledge, this is the first of any report or study into the toxicity of *L. javanicum*. At the same time, only one other study was found to have studied the toxicity of *B. frutescens* (Gray et al., 2003), whereby the chromone constituents of the plant was found to be toxic against brine shrimp *Artemia salina*, contradicting our findings. As it may be so, the study on the simple zoological organisms involves a particular isolated compound as opposed to our experiment which involves the total crude extract of *B. frutescens*. One of the main reasons given for isolating active compounds in the classical pharmaceutical development process is the elimination of potentially toxic but inactive compounds (Rasoanaivo et al., 2011). As such it would be premature to eliminate *B. frutescens* based on the toxicity of one type of compounds; with the possibility, the plant could possess other components that might be less toxic that fits our intended objective.

4.3 Cytotoxicity evaluation of extracts and fractions

Once the crude methanol extracts were found to be non-toxic by means of acute oral toxicity on a fully functional organism, the plant materials were subjected to a more specific toxicity study on the cellular level *via* the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. The MTT assay was performed to evaluate if the extracts tested have anti-proliferative effect on the cancer cells. Quantification of viable cells is crucial in research involving cell culture. Assays involving tetrazolium salts such as MTT cell proliferation assay are one of the methods broadly used in cell proliferation and cytotoxicity assays, enzyme assays, histochemical procedure and bacterial screening (Ferrari et al., 1990; Mosmann, 1983). This mode of quantitative measurement is convenient for assessing a population of cell's reaction to external factors which may trigger an alternation in cell growth (Van de Loosdrecht et al., 1994).

Intact metabolically active cells will cleave the yellow tetrazolium salt (MTT) into purple formazan and solubilized through the addition of a detergent (Mosmann, 1983). These active cells accomplish this using the 'succinate-tetrazolium reductase' system which belongs to the respiratory chain of the mitochondria. This activity is only possible in metabolically active cells.

The intensity of the coloration caused by the formazan is linearly related to the number of active cells present. Using a spectrophotometer, the intensity of the colouration was quantified, thus establishing a linear relationship between the absorbance and the number of active cells present in the sample. Based on this connection, the effects of the external factor on the proliferation of the cells concerned were accurately quantified.

Following the procedures from Section 3.6, the extracts were examined against three human lung cells, two of which were cancerous (A549 and NCI-H1299) and one was a normal cell (MRC-5). One of the distinguishing characteristic of the cancerous cells used was that A549 possessed a functional wild type p53 expression while a deficient p53 was present in NCI-H1299. The variation in the p53 status served as additional screening criteria, whereby the dependence of utilizing this tumour suppressor by the treatment could be assessed thorough the reaction of the cells. A normal human lung fibroblast cell (MRC-5) was used to establish if there was any negative influence on non-carcinoma cells.

In the US National Cancer Institute (NCI) Plant Screening Program, crude extracts are considered to have *in vitro* cytotoxicity should the IC_{50} value be less than 20 μg/ml, upon incubation of 48 to 72 hours, (Mahavorasirikul et al., 2010).Referring to Table 4.3 which presents the MTT assay data, none of the extracts nor their fractions managed to exert a significant cytotoxic effect which met the criteria set forward by the NCI Plant Screening Program which was used as benchmark in the selection of an active extract.

Table 4.3: Cytotoxic activity (IC_{50} values) of extracts

Plant / Control	Extract	Cytotoxicity (IC_{50}) in µg/ml		
		A549	**NCI-H1299**	**MRC-5**
L. javanicum	Methanol	96.38 ± 4.03	> 100	> 100
	Hexane	40.91 ± 0.97	90.44 ± 1.55	69.11 ± 5.15
	Ethyl acetate	55.77 ± 2.59	77.10 ± 2.81	62.20 ± 1.45
	Water	> 100	> 100	> 100
B. frutescens	Methanol	> 100	93.20 ± 22.60	41.34 ± 0.60
	Hexane	56.24 ± 0.03	26.70 ± 0.23	6.08 ± 0.17
	Ethyl acetate	> 100	> 100	31.53 ± 0.13
	Water	> 100	> 100	> 100
Cisplatin *	-	8.70 ± 1.30	21.41 ± 3.99	1.27 ± 0.06

*Positive reference standard. Values are expressed as mean ± standard deviation (n = 3).

4.4 Fractionation and isolation of chemical constituents from L. *javanicum*

During the extraction process, a fraction (LF1) was obtained from the methanol extract of *L. javanicum*. The substance obtained was a white powder with a slight greenish tinge. The powder was fine in texture and did not dissolve in methanol. This could indicate the substance is not stable in methanol, thus explaining its precipitation from the crude methanol extract.

The fraction LF1 was then subjected to the cytotoxicity assay using the MTT reagent on the three human cells lines which were used during the extract screening process. LF1 was dissolved in DMSO for the experimentation. The results from MTT assay are presented in Table 4.4.

Table 4.4 : Cytotoxic activity (IC_{50} values) of LF1

Plant	Fraction	Cytotoxicity (IC_{50}) in µg/ml		
		A549	**NCI-H1299**	**MRC-5**
L. javanicum	LF1	7.12 ± 0.07	9.62 ± 0.50	6.66 ± 0.17
Cisplatin*	-	8.70 ± 1.30	21.41 ± 3.99	1.27 ± 0.06

*Positive reference standard. Values are expressed as mean ± standard deviation (n = 3).

LF1 managed to exert an encouraging cytotoxicity against both the cancer cell lines used. Although LF1 may appear to be harmful towards the normal cell MRC-5, Cisplatin, which is a drug approved by US Food and Drug Administration (FDA) for treatment of lung cancer, showed a much more potent effect. In fact, when comparing LF1 with Cisplatin, the former appeared to be five times less toxic against MRC-5. Moreover, treatment with LF1 produced a much lower IC_{50} value towards the A549 and NCI-H1299 cells when compared to the values produced by Cisplatin. This indicates that LF1 is much more efficient than the FDA approved drug (Cisplatin) when it comes to eliminating the cancer cells. Among the extracts and fractions obtained from *L. javanicum* and *B. frutescens*, LF1 showed to be the most promising as an anti-tumour agent. Based on these results, the ability of LF1 to stimulate cell death was further evaluated.

4.5 Assessing apoptotic-inducing capabilities of LF1

4.5.1 Qualitative evaluation of apoptosis induction

Following the MTT assay, effectiveness of the active LF1 in cell death was further scrutinised employing the AO/EB double staining technique. This staining method (as outlined in Section 3.7) was used to determine if the cytotoxic effect upon treatment with the fraction has any association with apoptosis. This mixture of dyes is regularly utilized

in the recognition and distinction of live, apoptotic as well as necrotic cells. Acridine orange (AO) stains both viable and nonviable cells and emits a green fluorescence when intercalated with DNA while ethidium bromide (EB) is taken up only by non-viable cells, emitting a red fluorescence (Ariffin et al., 2009).

Morphological observations commonly associated with apoptosis such as chromatin condensation, fragmented nuclei as well as membrane blebbing were used to categorize the cells as apoptotic or otherwise. Four different types of cellular states were determined by the fluorescence emission and also the morphological observations, especially those concerning the chromatin condensation. Viable cells have uniform bright green nuclei with organized structure while early apoptotic cells have green nuclei, but chromatin condensation is visible as bright green patches. Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin and necrotic cells have a uniformly orange to red nuclei with organized structure (Baskić et al., 2006).

In all LF1-treated A549 and NCI-H1299 cells (5, 15 and 25 µg/ml), chromatin condensation was present in cell undergoing both early and late apoptosis (Figure 4.3). Moreover, at the lowest concentration of LF1, 5 µg/ml, both types of cancer cells exhibited membrane blebbing which is another classic symptom of early apoptosis. Treated cells appear to have shrunk in size, mainly those which have been stained orange when compared to the viable cells. This can be interpreted that the dying cells have taken the apoptosis pathway as necrotic cells tend to swell.

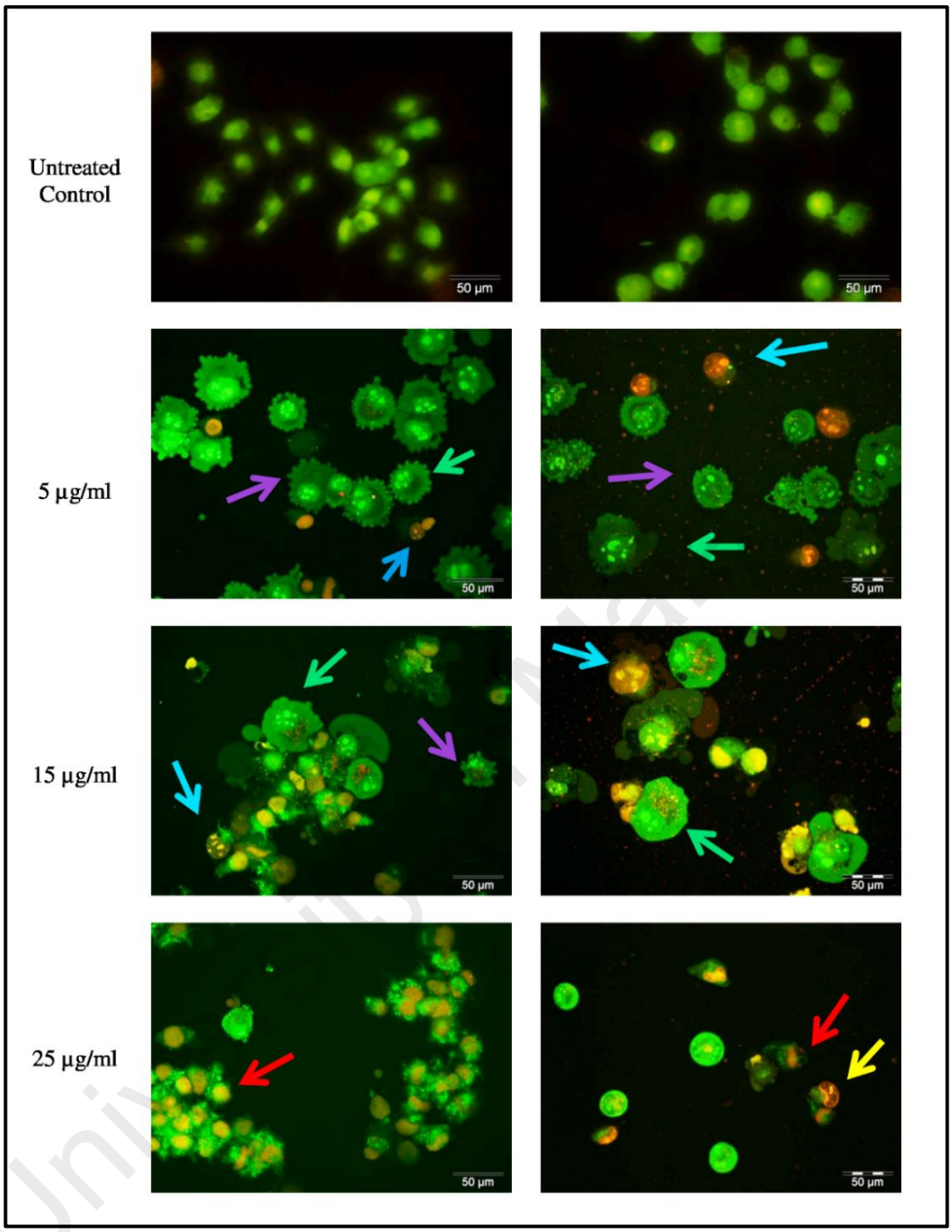

Figure 4.3: Morphological observation of LF1-treated A549 and NCI-H1299 using AO/EB staining at 400 ×magnification**.**

A549 and NCI-H1299 were treated without (negative control) and with LF1 at different concentrations (5, 15 and 25 µg/ml). Green arrows indicate early apoptotic cells (chromatin condensation stained green); blue arrows are late apoptotic cells (chromatin condensation stained orange); purple arrows show membrane blebbing; yellow arrows are for cells which appear shrunken and red arrows indicate loss of membrane shape.

During treatment with 25 µg/ml, which was the highest concentration used, cells seemed to be more consistently stained orange suggesting that at higher concentrations of LF1, the cells undergoing necrosis as opposed to apoptosis. In addition to this, the cells also appear to have lost their membrane integrity, highlighted by the lack of defined shape of the treated cells. According to Sancho-Martinez et al. (2011), the concentration of various cytotoxic molecules and stimuli, including a number of anticancer drugs, was the determinant in the manner of cell death, being either apoptosis or necrosis. The higher concentration of the fraction which triggered necrosis in the treated cells could have been initiated by general toxicity of foreign compound towards the cells. In short, the higher the concentration of LF1 utilized, the less controlled pathway taken in the initiation of death in the cancer cells.

4.5.2 LF1 treatment causes Sub-G_1 manifestation

Exposure to substances that may elicit an apoptotic reaction causes the emergence of a subpopulation in a DNA histogram upon staining with propidium iodide (PI) (Vermes et al., 2000). This DNA population which sits ahead of the diploid (2N) region that corresponds to DNA content in the G_0/G_1 phase, is classified as the sub-G_1 region and is scored as the presence of apoptosis in the sample (Kajstura et al., 2007). Due to the initiation of apoptosis, endogenous endonuclease are activated which subsequently causes the progressive generation of hypo-diploid DNA particles which are commonly referred to as the Sub-G_1 population (Arends et al., 1990).

The DNA content is measured upon permeabilization or fixation of the treated cells. This process does not fully retain highly degraded DNA within the cell which usually exits the apoptotic cell during the rinsing and staining process. As a result, stained apoptotic cells contain fractional DNA content which corresponds to the incidence of the hypo-diploid DNA content in the DNA content frequency histogram, indicating a deficit

in the normal DNA content, which was mentioned earlier. Necrotic cells do not show immediate reduction of DNA stainability as compared to apoptotic cells and this factor can be used to distinguish between the two types of cells (Vermes et al., 2000). The results are conveyed as percentages relative to the total acquired gated population during the acquisition process which utilized the flow cytometer. The sub-G_1 population analysis is commonly performed in tandem with the cell cycle analysis, as highlighted in Section 3.9. A549 cells treated with LF1 manifested a sub-G_1 population that corresponds to the occurrence of apoptosis. At low concentrations (5 µg/ml), the treatment yielded a very low and insignificant sub-G_1 region when compared with the untreated control. As the concentration of LF1 was increased from 5 µg/ml to 10 µg/ml, so did the prominence of the sub-G_1 population obtained during the acquisition process (from 1.2 ± 0.53% to 10.7 ± 0.97%). This may be taken as an early cue that LF1 does indeed cause apoptosis and the effect of the treatment escalates with the increasing concentration used. Treatment with 7.5 as well as 10 µg/ml of LF1 was statistically significant when compared to the untreated control.

A similar observation was seen with the NCI-H1299 treated cells. Treatment with 5 µg/ml of LF1 yielded an insignificant difference when compared with negative control (Figure 4.4). This may indicate that such a low concentration was insufficient to elicit a significant activation of the apoptotic pathway in the treated cells. Similar to the A549 treated cells, an increase in concentration from 5 µg/ml to 10 µg/ml seemed to have positive effect with regards to the induction of cell death through apoptosis (from 3.0 ± 0.70% to 9.0 ± 1.59%). A statistically significant sub-G_1 population was seen when treating NCI-H1299 cells at 7.5 µg/ml and 10 µg/ml (Figure 4.4).

Looking a little deeper into the results obtained, LF1 does not seem to have any preference towards the p53 activation route in inducing apoptosis in treated cells. The p53 functions by co-ordinately blocking cell proliferation, stimulating DNA repair and stimulating apoptotic cell death in cells that are damaged (King & Robins, 2006). Both cell lines seem to have a similar effect on the treatment used suggesting that the apoptosis pathway triggered was not p53 specific. This observation is consistent with the results obtained during the MTT assay (Table 4.4) whereby both cells produced a similar cytotoxic response towards LF1 regardless if the cell possessed an active p53 gene.

According to Darzynkiewicz et al. (2009), a common misconception is that the frequency of apoptotic cells in the histogram is a reflection of 'how many' cell underwent apoptosis. Although this method has been used as the sole indicator of apoptosis in some studies, such action is not a recommended practise as there as several pitfalls. Physical disintegrated cells which may appear in the sample and the fractional DNA from these cells may present themselves in the sub-G_1 population, leading to a false positive result.

Fragmented DNA from apoptotic cells in the S and G_2/M phase may end up in the G_1 or early S phase in the DNA content histogram, contrary to the general assumption that all fractional DNA presents itself in the sub-G_1 region. Fractional DNA in these cases may be masked as an arrest at the G_1 or S phase due to its larger size fragments. Moreover, this method also fails to detect apoptosis in cell that do not undergo internucleosomal DNA fragmentation (Kajstura et al., 2007).

The sub-G_1 population detection is a convenient assay in assessing the potential apoptotic capabilities of LF1 but has to be coupled with tests that are specific towards the apoptotic markers. The test would best serve as preliminary screening into the incidence of apoptosis in LF1 treated cells. This would then provide validation to further assess the treatment capabilities via test that monitor distinct signs of controlled cell death

activation. This leads us to the following point of this investigation; the assessment of apoptosis utilizing the Annexin V /Propidium Iodide staining technique.

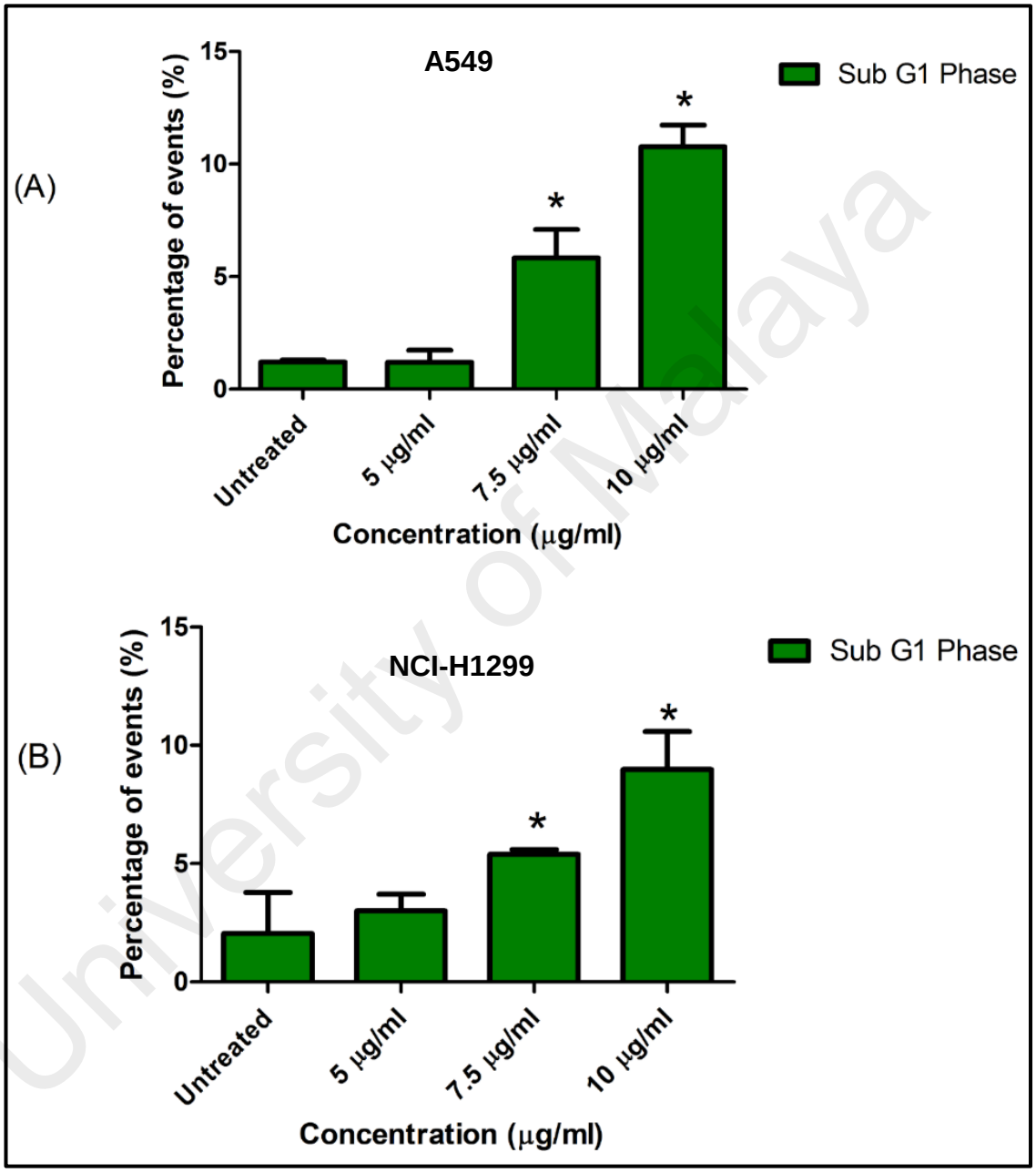

Figure 4.4: Sub-G_1 population of LF1-treated cells.

A549 (A) and NCI-H1299 (B) cells were treated with 5, 7.5 and 10 µg/ml of LF1. The consequential sub-G_1 population showed an increase with the increasing concentrations of LF1 used.

4.5.3 LF1 causes phosphatidylserine (PS) externalization

Under typical physiological circumstances, cells preserve an asymmetrical distribution of phospholipids amongst the internal and external leaflet of the plasma membrane (Higgins, 1994). An example of this asymmetry distribution is the almost exclusive presence of phosphatidylserine (PS) in the cytosolic side of the membrane. According to Vermes et al. (2000), this asymmetry may be maintained through a set of enzymes which translocate any PS present in the outer layer back into the inner layer of the membrane. Under specific conditions, such as the induction of apoptosis, this asymmetric architecture is lost and leads to the continual occurrence of the PS in the outer leaflet of the plasma membrane (Fadok et al., 1992). This action is said by Schutte et al., (1998), to serve a physiological role in the identification and consequent elimination of the dying cell through phagocytosis. This unique feature can be used as a discriminatory marker to discriminate between viable as well as apoptotic cells.

Employing the FITC-conjugated Annexin V, which has a high affinity towards the PS region, with the concurrent usage of propidium iodide (PI) as an exclusion dye to probe the membrane integrity, the early apoptotic (Annexin V^{+}/ PI^{-}) cells can be differentiated from secondary necrotic cells and necrotic (Annexin V^{+}/ PI^{+}) as well as damaged cells (Annexin V^{-}/ PI^{+}) (Baskić et al., 2006; Schutte et al., 1998). This experiment permits a quantitative analysis into the occurrence of apoptosis in cells treated with LF1. Being an operator independent technique, this method has the benefit of being more precise as there would be an absence of user bias. In addition to this, the larger sample size obtained using this method (typically 10,000 events), gives it a higher degree of statistical significance compared to more conventional methods such as microscopy.

The A549 cells were treated with 5.0, 7.5 and 10.0 µg/ml of LF1 while 10.0, 15.0 and 20.0 µg/ml was used on NCI-H1299 cells, as outlined in Section 3.8. Flow cytometric examination of Annexin V-FITC and PI stained A549 and NCI-H1299 cells showed a significant rise in the early apoptotic and secondary necrotic cell population with the increasing concentration of LF1 used (Figure 4.5). The quantity of viable cells experienced a steep reduction in population for both cells when compared with the untreated control (from 93.4 ± 1.9 to 40.7 ± 5.3 % for A549; and 91.4 ± 2.3 to 44.6 ± 3.3% for NCI-H1299) (Figure 4.5).

The translocation of PS to the outer leaflet of the lipid bilayer, which occurs very early in the apoptotic process, serves as an apoptotic cell-associated molecular patterns (ACAMPs) (Franc et al., 1999). These expressions on the surface of apoptotic cells are recognized by phagocytes to carry out phagocytosis (Erwig & Henson, 2007). In the absence of clearance of apoptotic cells through these phagocytic scavengers, apoptotic cells proceed to cellular disintegration phase called secondary necrosis characterized by similar features of necrotic cell death (Krysko et al., 2006). This autolysis was called secondary necrosis by Wyllie et al. (1980), intending to distinguish this mode of cell elimination from "cellular necrosis occurring *ab initio*", which should be called "primary necrosis". Among the features, cytoplasmic membrane permeabilization plays a part in this assay as staining with PI would lead to positive signal (Silva, 2010). As such, both necrotic and secondary necrotic cells would stain positive with PI, leading to an inability to distinguish between the two.

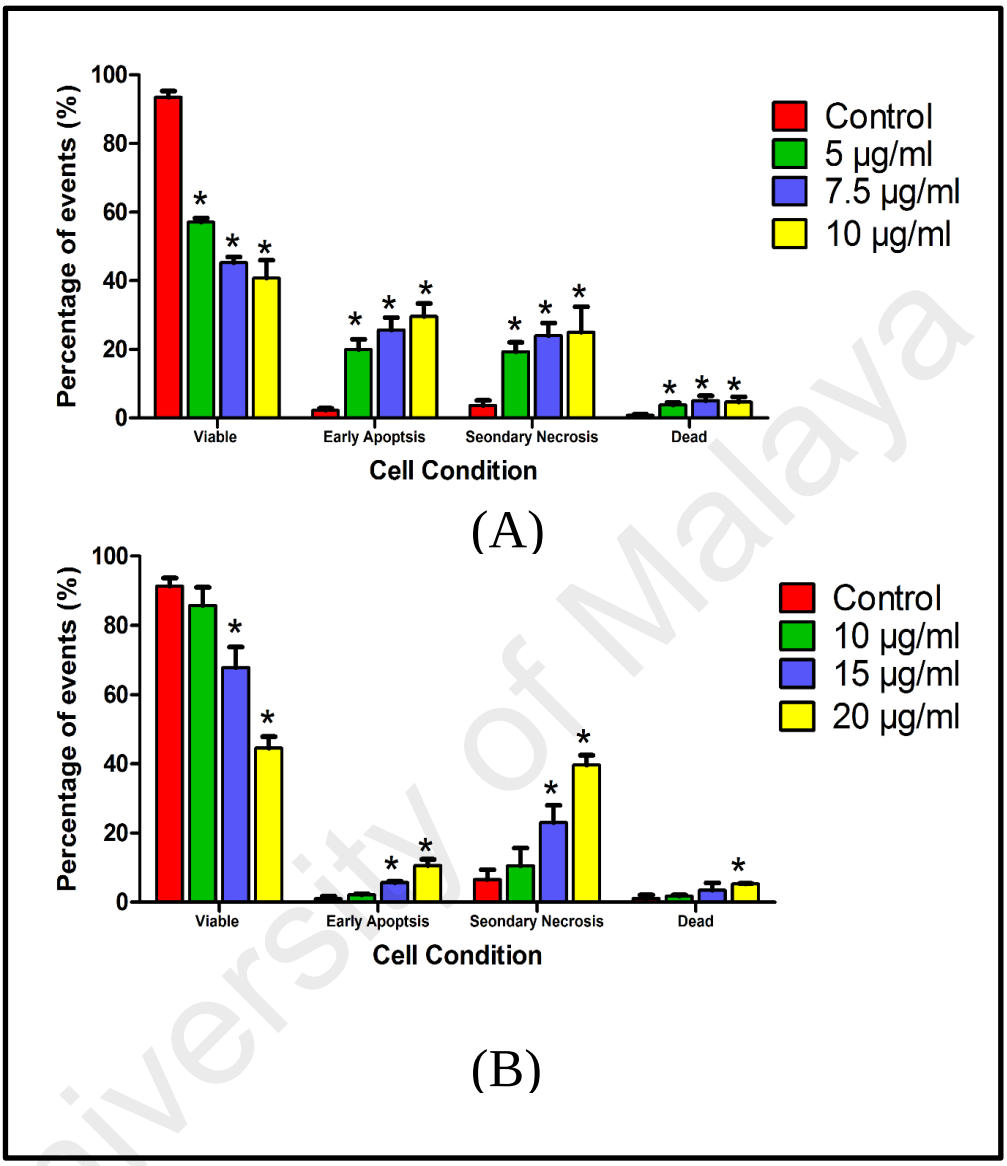

Figure 4.5: Effects of exposure to LF1 for 24 hours on the externalization of PS in A549 and NCI-H1299 (Dose – dependent).

Summary of the results for 24 hours treatment with LF1 was presented in a bar chart (A) for A549 and bar chart (B) for NCI-H1299. A significant increase in the early apoptotic and secondary necrotic cell population is observed with the increasing concentration of LF1 in both cell lines.

In order to overcome this shortcoming, a time point assay was done using the same Annexin V/PI method. In the event of increasing early apoptotic cells followed by an increase in double positive cell with the progression of time, the cells are assumed to be undergoing apoptosis. On the other hand, an increase on the population in the 2nd quadrant that bypass the 4th quadrant with increasing exposure time would lead to a conclusion that the cells are actually undergoing necrosis as opposed to secondary necrosis, which as mentioned earlier is one of the endpoints of the apoptotic pathway.

The results for cells exposed with 10 µg/ml of LF1 were selected to do a comparison on the effects caused by LF1 across different time points (Figure 4.6). The population of early apoptosis in stained A549 cells increased from 29.6 ± 3.8% at 24 hours to 39.9 ± 3.7% when exposed to the treatment for 72 hours. At the same time, H1299 increased from 5.7 ± 0.4% to 19.6 ± 1.5% and finally to 37.4 ± 4.2% upon exposure to 24, 48 and 72 hours, respectively. Another observation that is worth pointing out is the longer exposure period managed to exhibit a much more clearer distinction between the early apoptotic population and the secondary necrotic population of treated A549 cells. A generally increasing and decreasing trend is also observed for late apoptotic cells and viable cells, respectively. In this study, both cells lines showed a rise in early apoptotic cells over time, followed by an increase in double positively stained cells (Figure 4.6). This indicates that the cells have indeed endured apoptosis as an effect of being exposed to LF1. In addition to that, a decrease in the number of viable cells suggest a time dependent effect of LF1 in the induction of apoptosis in treated cells.

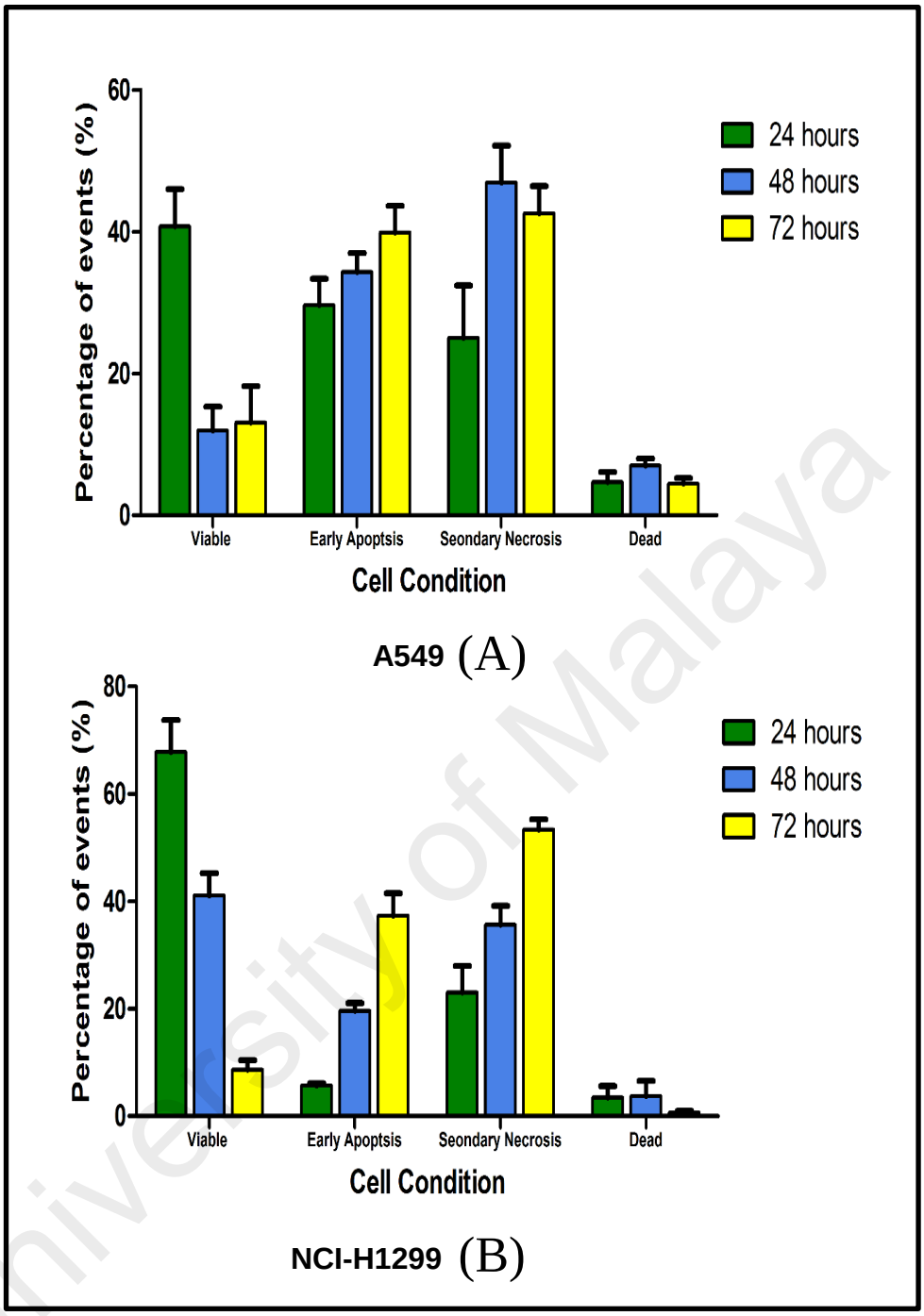

Figure 4.6: Effects of exposure to LF1 on the externalization of PS in A549 (A) and NCI-H1299 (B) at different time points (Time – dependent).

Cells were treated with 10 µg/ml (A549) and 15 µg/ml (NCI-H1299) of LF1 for 24 hours, 48 hours and 72 hours. For both cell lines, an increase in early apoptotic cells over time that was then followed by an increase in double positively stained cells indicate an apoptotic pathway was taken by LF1-treated cells.

The increasing presence of early and late apoptotic cells with the progression of time and increasing concentrations of treatment suggest that LF1 managed to induce a controlled demolition at the cellular level *via* the apoptotic pathway, as opposed to the un-programmed necrotic route. The results from this assay also confirms the outcome of the AO/EB procedure earlier on (Figure 4.3), whereby the visual presence of classic apoptotic characteristic in LF1-treated cells pointed towards the apoptotic pathway. Moreover, the lack of novelty in the reaction towards either one of the cells used (wild p53-A549 and p53-deficient NCI-H1299) indicate no preference tied to the presence of the p53 gene. Based on this observation, it can be stated with a certain degree of confidence that LF1 induces apoptosis through a p53 independent pathway.

4.5.4 Increase in activated Caspase-3 due to LF1 treatment

The cysteine-dependent aspartate driven proteases (caspases), which consist of several proteases, are major executors of apoptosis which are interconnected in a series to form a proteolytic cascade (Porter & Janicke, 1999). Caspases are typically divided into initiator caspases (caspase-2, -8, -9, and -10) which are activated upon interaction with adaptor protein, and the effector caspase (caspase-3, -6, and -7) that act directly on specific cellular substrates involved in the disablement of key proteins of the cell upon activation by the effector caspases (Parrish et al., 2013).

Among these enzymes, caspase-3 is a death protease that is frequently activated, triggering the specific cleavage of many key cellular proteins involved in apoptosis (Porter & Janicke, 1999). Activation of caspase-3 may occur through the intrinsic pathway which involves the release of cytochrome c and the initiator caspase-9 or via the extrinsic pathway that includes activation of death receptors as well as caspase-8 (Cullen & Martin, 2009; Parrish et al., 2013). As such, caspase-3 is a perfect biomarker to affirm the role of LF1 in inducing apoptosis among treated cells as it is detectable in apoptotic cells regardless of the pathway used.

The activated caspase-3 binds covalently with the FITC-conjugated antibody and was expressed as percentage relative to the total acquired gated-population using the flow cytometer. The procedures were done as outlined in Section 3.10. As shown in Figure 4.7, an increasing presence of activated caspase-3 is observed with the increasing treatment of LF1 in both A549 and NCI-H1299, although a stronger effect is seen in the former. A shift in the histogram towards the right is seen as higher concentrations of LF1 are used against both cell lines. Treatment with 5 µg/ml of LF1 did not seem to elicit significant caspase activation as compared to the untreated control on both cell lines. On the other hand, a significant increase ($p < 0.05$) from 0.13 ± 0.15% to 53.90 ± 2.36% of active caspase-3 signal was observed in A549 treated with 20 µg/ml of LF1 whilst NCI-H1299 cells experienced a smaller jump from 0.40 ± 0.26% to 20.47 ± 0.80% under the same conditions.

The results obtain reflect an increasing presence of the activated caspase-3 enzyme, suggesting a rise in the intensity of apoptotic activity in LF1-treated cells. Moreover, the concentration-dependent characteristic exhibited by LF1 in the activated caspase-3 quantification is consistent with the data from the other apoptotic assessment within this study.

Increment of LF1 concentration showed a similar trend of increased apoptotic activity that was observed in the Annexin V/PI assay, Sub-G_1 quantification and the morphological observation with AO/EB stains. Caspase-3 plays a central role in the proteolytic cleavage of several proteins and is responsible for the apoptosis-associated chromatin condensation (observed in the AO/EB staining), DNA fragmentation (present in the Sub-G_1 analysis), and nuclear collapse of the affected cells (Jeruc et al., 2006). The elevated presence of active caspase-3 serves as a biochemical validation of the ability of LF1 in inducing apoptosis in treated cells.

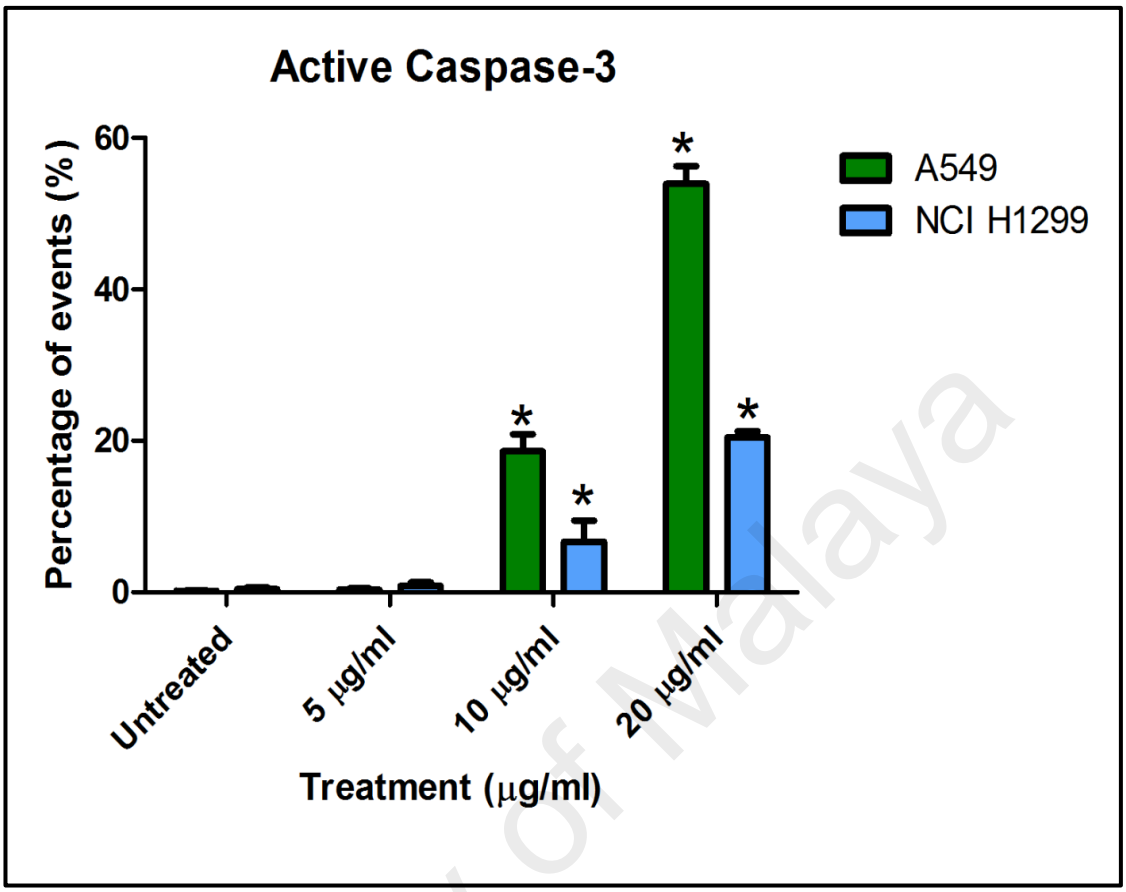

Figure 4.7: Presence of active caspase-3 in LF1-treated A549 and NCI-H1299. A549 and NCI-H1299 cells were treated with 5, 10 and 20 µg/ml of LF1 for 24 hours. An increase in the presence of cleaved caspase-3 was detected in both cell lines in a concentration-dependent manner, indicating an apoptotic pathway was taken by LF1-treated cells.

4.6 Disruption in cell cycle distribution upon exposure to LF1

The growth development process in multicellular organisms is a complex process and need to be done in an appropriate and orderly fashion to avoid accumulated errors which may translate into unwanted situation, i.e. formation of cancer cells. For this purpose, the cell employs a regulatory network of genes and proteins which serves as a check and balance in the development process to avoid formation of mutant cells. Such a

mechanism has been incorporated into the basic cell cycle machinery to control where and when is it ideal for the cells to divide in a proper manner (Parker et al., 1995). When this system fails, overridden or bypassed due to external factors, the cells require 'outside' help to re-arm the system in order to kill of the defective (cancer) cells.

Chemotherapeutic drugs have been shown to be capable of disrupting the cell cycle system in cancer cells. These drugs disrupt the developmental process in the cancer cell by halting the cell cycle at a particular stage which results in the suppression of tumour proliferation before activating the apoptotic pathway leading to the demise of the cancer cells (Hseu et al., 2014). Directing drugs to specifically target regulatory genes has and is being developed as a treatment mode in the fight against cancer (Schwartz & Shah, 2005).

As it has been shown in our study thus far that LF1 is able to induce apoptosis in cancer cells, the cell cycle analysis was performed, as outlined in Section 3.9, in the hopes of determining if cell death caused by LF1 through apoptosis involved arresting the cell cycle at a specific stage (Song et al., 2006). By doing so, the specific targets (genes or proteins) of LF1 can be narrowed and this in turns allows a better understanding into the nature of the isolated fraction.

Referring to the summary of the result from this assay (Figure 4.8), treatment of A549 with LF1 caused the percentage of cells in the G_0/G_1 phase to increase from 59.12 ± 3.4% to 87.54 ± 1.21% with the increasing concentrations used (from 0 to 10 µg/ml). A reverse was observed in the S and G_2/M phase population, whereby the number of events recorded depleted (29.66 ± 2.97% to 8.99 ± 1.20% for the S phase and 11.23 ± 0.48% to 3.45 ± 0.25% for the G_2/M phase) with the escalating concentration of LF1 used (Figure 4.8). These observations were done with reference to the untreated controls and the differences with the control were statistically significant in most cases.

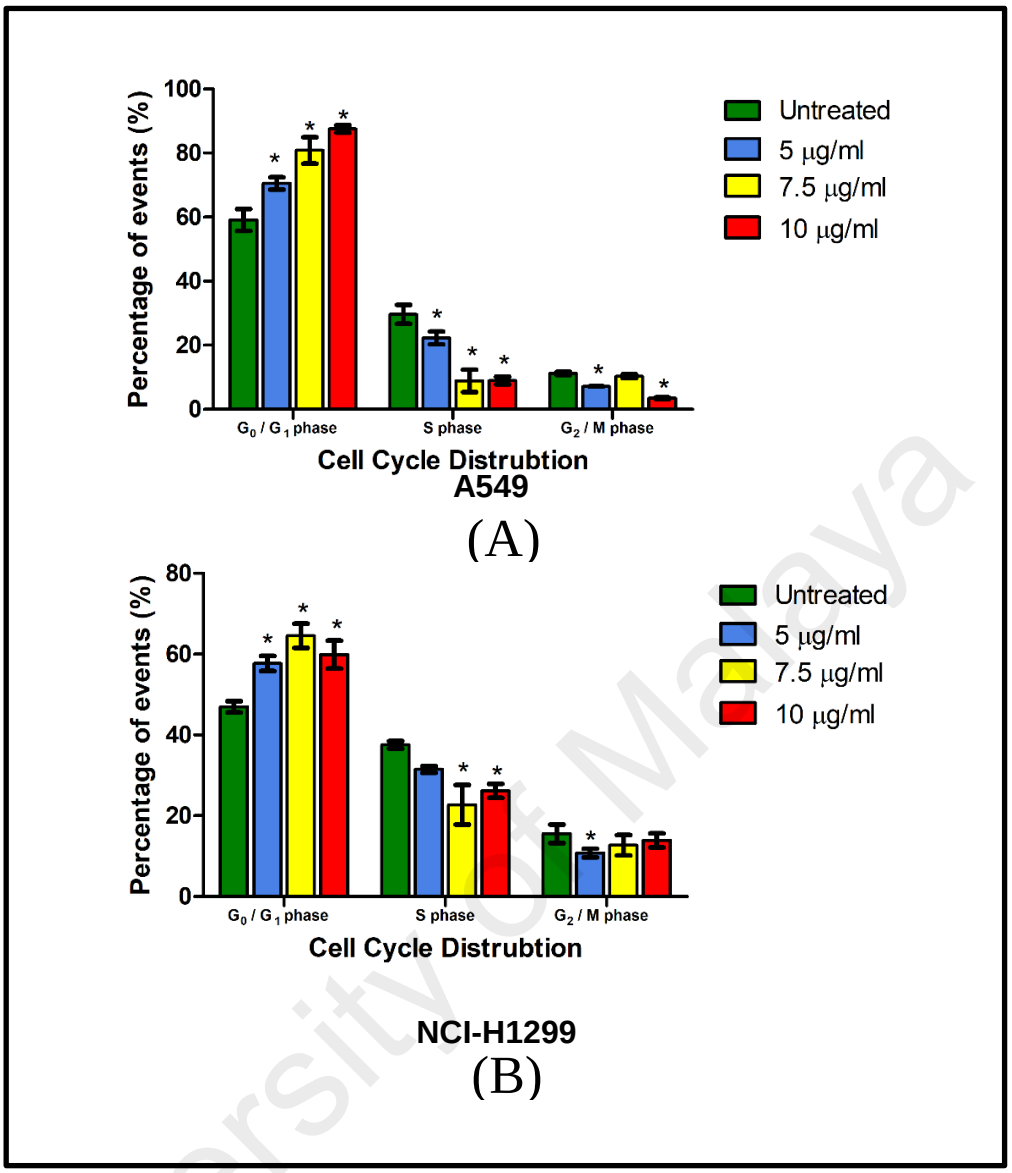

Figure 4.8: Effects of LF1 on cell cycle distribution in A549 (A) and NCI-H1299 (B) cells.

Cells were incubated in absence (control) and presence of LF1 at 5, 7.5 and 10 µg/ml for 24 hours. Summary of results indicate an increase in G_0/G_1 population with increasing concentrations of LF1 used.

A similar pattern was observed in LF1-treated NCI-H1299 cells (Figure 4.8). A statistically significant increase of the cell population in G_0/G_1 phase (46.95 ± 1.40% to 59.91 ± 3.47%) was seen with increasing concentration when compared with the negative control. A marked decrease in the S phase population (37.57 ± 0.95% to 26.17 ± 1.76%)

was followed by minor drop in the G_2/M phase population (15.55 ± 2.29% to 13.92 ± 1.72%).

The findings in this experiment suggest that LF1 managed to cause an arrest of the cell cycle at the G_0/G_1 phase in treated cells, thus preventing the transition from the G_1 phase to the S phase. The treatment managed to inhibit the proliferation and subsequent death of the cancer cell most likely through the suppression of the cell cycle process. The consistent pattern observed in the apoptotic detection as well as the cell cycle arrest strongly suggests a correlation between the two processes. Moreover, the general similarity between the activation of apoptosis and induction of cell cycle arrest, with respect to the lack of discrimination towards the p53 absent NCI-H1299, allows us to downplay the role of p53 as a prime target for LF1 to a certain extent.

4.7 Isolation and purification of LF1 isolates

Upon establishment of LF1 as a viable candidate to be used against lung cancer as both a tool to cause controlled cell death as well as to restrict the ability in cell division, the next step would involve the separation and purification of the possible compound(s) responsible for this action.

Column chromatography is the oldest form of chromatographic technique. The sample mixture (LF1 in this study) is placed in a mobile phase and allowed to elude through the stationary phase under gravity. The constituent of mixtures moves at different rates through the column as bands. Eventually they reach the end of the column and elute in solution in the mobile phase.

The column chromatography was preferred due to flexible nature with regards to the type and quantity of mixture to be separated. In addition to this, a wider choice of mobile phase is available, allowing greater freedom in tweaking the setup to maximise the separation yield and purity. The resulting eluent was concentrated using rotary evaporator and monitored for purity using Thin Layer Chromatography (TLC). Fractions with similar composition were combined. The process was repeated until a clear single spot was present the TLC strips.

As highlighted in Section 3.12, the separation process was initiated by flushing the column once with hexane to remove the presence of any lipids that may be present in LF1. This was followed by using a dichloromethane: methanol solvent system, with gradually increasing the polarity of the mobile phase. The collected eluents were spotted on a TLC plate and fractions with similar spotting pattern were pooled together. As a result, seven different isolates were obtained, duly named LF1-1 to LF1-7. The ranges of weight obtained are as high as 402 mg to as low as 1 mg. The isolation diagram is drawn out in Figure 4.9.

LF1-4 was chosen to undergo further isolation as it had the most distinctive spotting pattern with the highest yield (402 mg) as well as having a clear distinction of a possible pure compound as seen through the NMR analysis (not shown), although there were quite a number of impurities present. LF1-4 was then subjected through another column chromatogram, similar to the previously used, which the exception of a dichloromethane: diethyl ether solvent system (Figure 4.9). The eight isolates were obtained upon assessment with TLC and combination of similar patterned eluents. The isolates were named LF1-4-1 to LF1-4-8. The highest yield was obtained from LF1-4-3 with a total weight of 102 mg that a white powdered appearance.

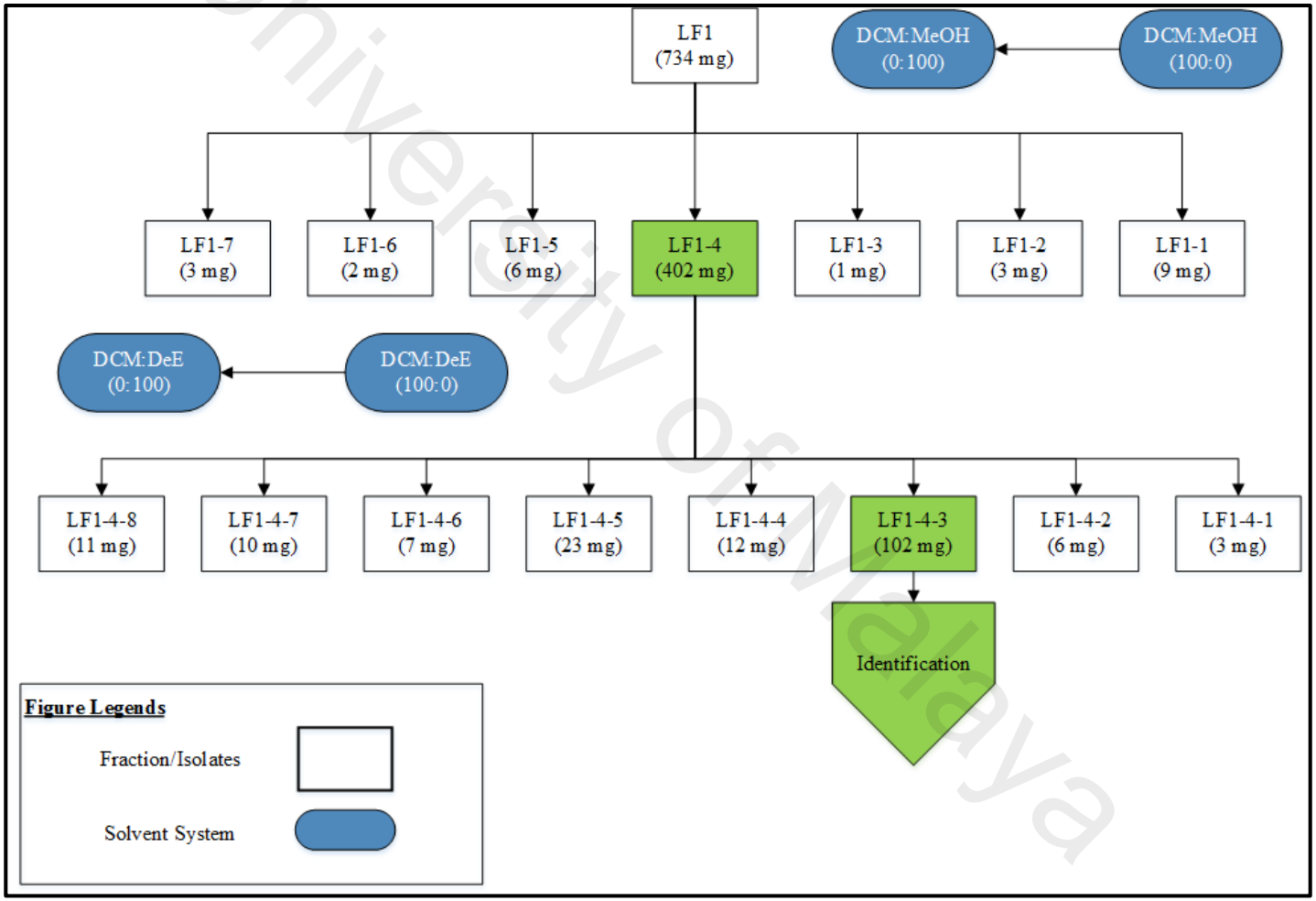

Figure 4.9: Isolation and purification diagram for LF1.

4.8 Identification of LF1-4-3 through spectroscopy methods

In order to identify the isolate LF1-4-3, three different methods mentioned in Section 3.12.3 were employed, NMR, LCMS and IR spectroscopy. The three methods complement each other by providing different data which will eventually accumulate in the identification of the isolate, the NMR providing the carbon and hydrogen structure; the MS giving the total mass from which the molecular formula can be derived and lastly the IR spectroscopy which functions to identify the functional groups in the isolate.

Through the LCMS which utilized the chemical ionisation mass spectroscopy (CIMS) method, the molecular ion peak $[M-H]^-$ at 455m/z corresponds to the molecular formula $C_{30}H_{48}O_3$. Generally characterised as a milder form of ionization, CIMS is commonly used in analytical mass spectrometry to obtain simplified mass spectra of compounds, often one species spectra, which can be used for quantitative analysis of mixtures (Munson, 2006). The technique is useful for the analysis of complex molecules of biomedical interest (Fales et al., 1970).

The IR spectra of LF1-4-3 revealed an intense absorption ribbon indicative of a hydroxyl group at 3456 cm^{-1}. The absorbance bands at 1685 cm^{-1} and 1643 cm^{-1} were due to the presence of carbonyl carboxylic acid and carbon-carbon double bond functional groups respectively.

The ^1H NMR spectrum showed the presence of two terminal methylene protons at δ 4.73 (H-29a) and δ 4.60 (H-29b). A doublet of doublets was observed at δ 3.19 (J = 4.3Hz, 9.4Hz) due to the presence of a hydroxymethine group at H-3. In addition to that, multiplets in the region of δ 1.00 to 3.00 for the methylene and methine protons along with the six methyl groups, δ 1.68, 0.97, 0.96, 0.93, 0.82 and 0.75, were also observed.

The ^{13}C NMR spectrum exhibited six methyl signals (δ 27.97, 19.35, 16.14, 16.00, 15.33, 14.67), 10 methylene peaks (δ 38.67, 37.01, 34.28, 32.12, 30.15, 29.67, 27.36, 25.45, 20.82, 18.26), five methine carbons (δ 55.30, 50.47, 49.22, 6.87, 38.36) and five quaternary carbons (δ 56.27, 42.40, 40.65, 38.84, 37.17). The peak at δ 180.32 corresponds to a carboxyl group while δ 78.37 represented an oxygenated methine. The chemical shifts of δ 180.32, 150.41 and 109.7 are characteristic peaks for a Betulinic type of skeleton, assigned to C-28, C-20 and C-29 respectively (Munson, 2006).

Based on the data from the three spectroscopic methods, as well as comparison with previously reported data (Baek et al., 2010; Haque et al., 2013; Kovac-Besovic et al., 2009; Peng et al., 1998; Robinson & Martel, 1970; Siddiqui et al., 1988), the identity of LF1-4-3 was deduced to be Betulinic acid (BA), a triterpenoid which was isolated for the first time from this plant. The pentacyclic triterpenoid of plant origin that is widely distributed across the plant kingdom (Tan et al., 2003). The white birch bark, a known source of BA, has been used by Native Americans as folk remedy (Fulda, 2008). BA has shown cytotoxicity towards melanoma, neuroblastoma and glioblastoma cells among others (Pisha, Chai, Lee, Chagwedera, Farnsworth, et al., 1995; Schmidt et al., 1997; Wick et al., 1999). In addition to this, anti-HIV and anti-inflammatory properties of BA have been exhibited in various experimental systems (Bache et al., 2011). The structure of BA is shown in Figure 4.10.

Betulinic acid IR (KBr) (ν, cm^{-1}): 3456, 2869, 1685, 1643, 1450, 1376, 1190, 1043, 882 cm^{-1}. CIMS m/z $[M-H]^-$, 455 = 456 -1. The ^1H NMR (270 MHz, $CDCl_3$): δ 4.73 (s, H-29b); 4.60 (s, H-29a); 3.19 (dd, H-3, J = 4.3Hz, 9.4Hz); 3.00 (m, H-19); 1.68 (s, H-30); 0.97 (s, H-27); 0.96 (s, H-26); 0.93 (s, H-23); 0.82 (s, H-25); 0.75 (s, H-24). ^{13}C NMR (67.8 MHz, $CDCl_3$): δ 180.32 (C-28); 150.41 (C-20); 109.70 (C-29); 78.97 (C-3); 56.27 (C-17); 55.30 (C-5); 50.47 (C-9); 49.22 (C-19); 46.87 (C-18); 42.40 (C-14); 40.65 (C-8); 38.84 (C-4); 38.67 (C-1); 38.36 (C-13); 37.17 (C-10); 37.01 (C-22); 34.28 (C-7); 32.12 (C-16); 30.51 (C-15); 29.67 (C-21); 27.97 (C-23); 27.36 (C-2); 25.45 (C-12); 20.82 (C-11); 19.35 (C-30); 18.26 (C-6); 16.14 (C-26); 16.00 (C-25); 15.33 (C-24); 14.67 (C-27).

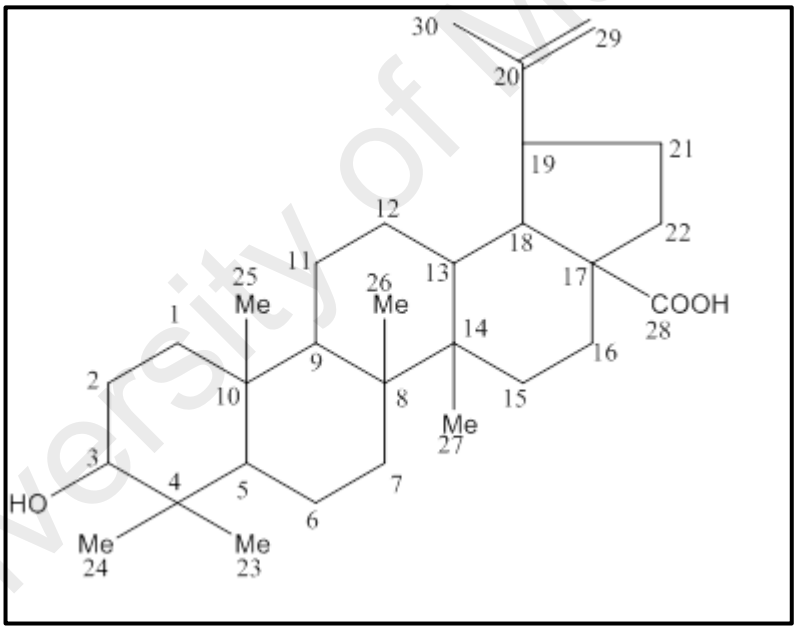

Figure 4.10: Structure of Betulinic acid (BA).

4.9 Cytotoxic evaluation of BA

Utilizing the same MTT methods as Section 4.3, the cytotoxicity of LF1 and it isolate, BA, was presented in Table 4.5 as IC_{50} values. Similar to the LF1 assessment (Table 4.4), three different human lung cell lines (A549, NCI-H1299 and MRC-5) were used to determine the concentration of the treatment in which the population was reduced by half relative to the untreated cells (IC_{50} value). BA managed to cause a significant reduction of viable cancer cells with IC_{50} values of 4.54 ± 0.09 µg/ml and 4.67 ± 0.12 µg/ml against the population of A549 and NCI-H1299 cells, respectively.

The MTT assay results showed a concentration-dependent trend in the cytotoxicity towards both cancer cell lines. Although BA may seem to be toxic towards the normal fibroblast cell, MRC-5, the IC_{50} value is still two times less toxic when compared with the FDA approved drug for the treatment of non-small lung cancer, Cisplatin. Moreover, the toxicity against cancer cells is much more potent in BA as compared to cisplatin, with respect to both A549 and NCI-H1299. When compared to its parent compound, LF1, BA performed marginally better, producing a lower IC_{50} value against both cancer cells. Like its predecessor, BA caused toxicity towards the cancer cells and less toxic to normal cell when compared to the positive control drug, suggesting its potential as an alternative to the current commercially available treatments.

When comparing the results of both cancer cell lines with each other, a similar trend is observed whereby IC_{50} values between 4 and 5 µg/ml was obtained. Based on this observation alone, the cytotoxic effect of BA seems to impartial to the presence of p53, a similar characteristic when to its parent compound. Comparing the above results with cytotoxic data from other studies (Kessler et al., 2007; Zuco et al., 2002), BA managed to induce a similar or much more potent effect on A549 and NCI-H1299

compared to other cancer cell lines (lung, colorectal, breast, prostate and cervical cancer cells).

Table 4.5 : Cytotoxic activity (IC_{50} values) of BA

Fraction	Cytotoxicity (IC_{50}) in µg/ml		
	A549	**NCI-H1299**	**MRC-5**
BA	4.54 ± 0.09	4.67 ± 0.12	3.27± 0.24
LF1	7.12 ± 0.07	9.62 ± 0.50	6.66 ± 0.17
Cisplatin *	8.70 ± 1.30	21.41 ± 3.99	1.27 ± 0.06

*Positive control, Cisplatin was used as a reference standard. Values are expressed as mean ± standard deviation (n = 3).

4.10 Evaluating the ability of BA in inducing apoptosis

4.10.1 Morphological detection of cell death caused by BA

BA-treated lung cancer cells showed morphological changes associated with apoptosis upon staining with AO/EB as outlined in Section 3.7. The criteria set out in Section 4.5.1 were used in the interpretation of the results obtained. Untreated cells for both A549 and NCI-H1299 appeared to be principally stained green, lacking dense bright spots (Figure 4.11). As the concentration used increased, treated A549 cells presented a rise in intense bright patches synonymous with chromatin condensation. However, at the concentrations of 2.5 µg/ml, NCI-H1299 cells seem to be similar in appearance to the negative control, suggesting a lack of apoptosis activation. As the concentration of BA was increased, so did the presence of yellow to orange stained cells, corresponding to an increase in apoptotic cells. A sizeable population of cells at the 7.5 µg/ml treatment group appear to have shrunk in sized compared to the untreated cells; signs of escalated presence of apoptosis.

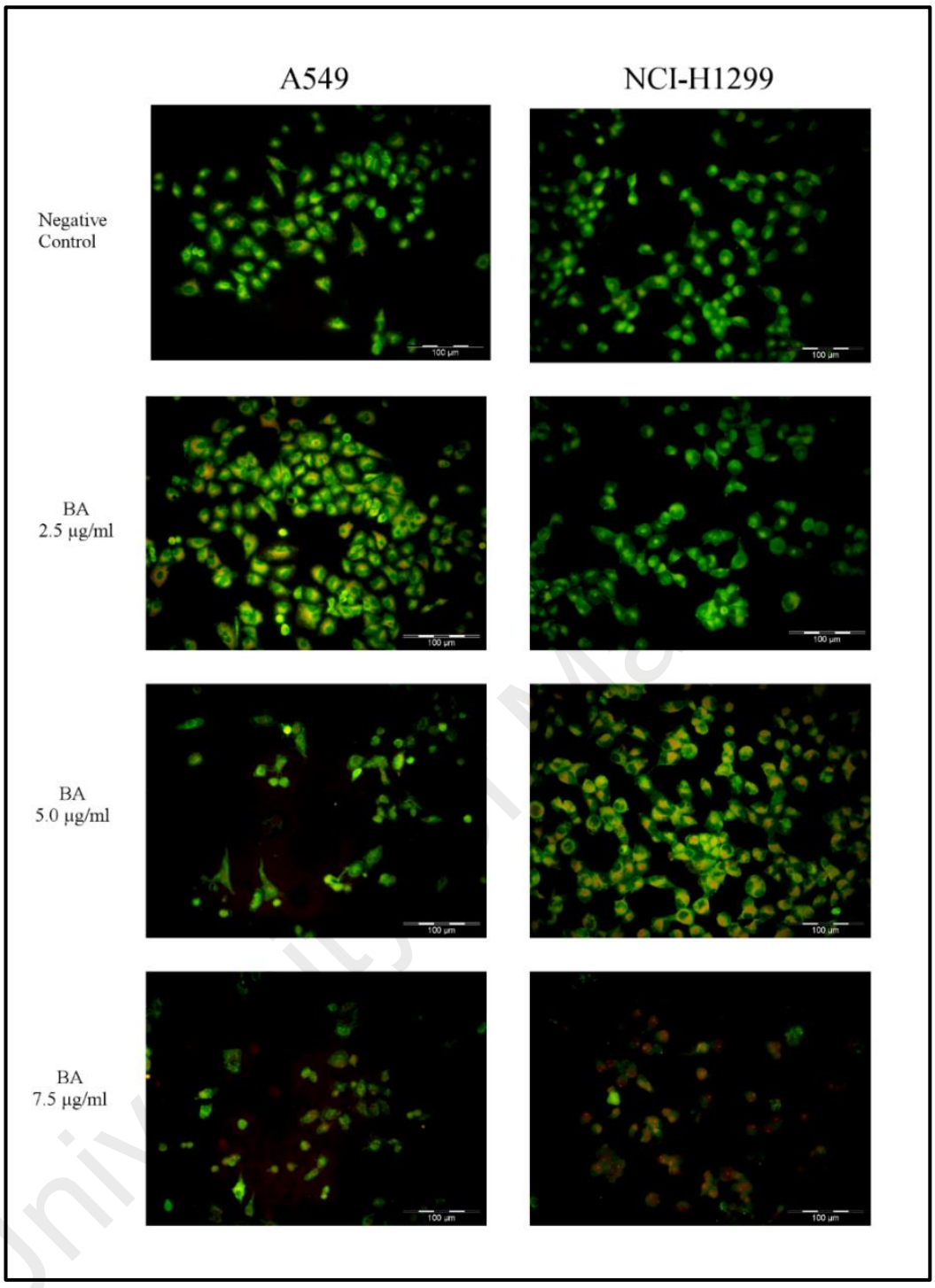

Figure 4.11: Morphological observation of BA-treated A549 and NCI-H1299 using AO/EB staining at × 200 magnifications.

A549 and NCI-H1299 were treated without (untreated control) and with BA at different concentrations. Lower concentrations of BA appeared to be indifferent compared to the untreated controls for NCI-H1299 treated cells; increased concentrations cause an increase in apoptotic cells.

The density of cells at 5.0 and 7.5 μg/ml seems to be lower compared to the control group, possibly due to the washing step which remove the detached cells that have either advanced to later stages of apoptosis or undergone cell death.

Through the AO/EB double staining method, the prominence of apoptotic cells increased with the concentration of BA, suggesting a concentration-dependent relationship between BA and the induction of apoptosis. Contrary to the findings in the cytotoxic assay (Table 4.5), both cell lines did not act similarly between identical treatment groups. For instance, cells treated with the lowest concentration of BA (2.5 μg/ml) managed to elicit an apoptotic reaction in A549 cells, evident through the presence of chromatin condensation, but no such feedback was obtained in NCI-H1299 treated cells. NCI-H1299 cells treated with the lowest concentration of BA appeared to be similar to untreated controls; producing uniformly stained green cells. This observation suggests that genotype differences between the two types of cells may be the contributing factor. Our attention is once again drawn towards the p53 gene, which is the prime variation between A549 and NCI-H1299 cells. The initial assumption would be that the presence of p53 does have an influence into the degree of apoptotic induction, especially in lower range of concentrations.

4.10.2 Sub-G_1 population analysis of BA-treated cells

The sub-G_1 population, which serves as an initial detection of apoptosis, was quantified in tandem with the cell cycle analysis (as outlined in Section 3.9). The results were expressed as percentage relative to the total acquired gated-population. An accumulation of sub-G_1 population was present in the DNA histogram of treated cells with the increasing concentrations of BA used (Figure 4.12). The percentage of cells in the sub-G_1 population significantly increased ($p < 0.05$) from 0.25 ± 0.25% in untreated cells to 24.02 ± 2.90% in A549 cells exposed with 10 μg/ml of BA, while similar

conditions caused a less drastic, but significant increase ($p < 0.05$) from 0.91 ± 0.67 to 8.05 ± 0.14% in NCI-H1299 cells.

In apoptotic cells, DNA is cleaved by a calcium-dependent endogenous endonuclease that fragments the chromatin into nucleosomal units with multiples of about 180-bp oligomers, which causes the characteristic DNA-ladder pattern commonly seen in electrophoresis (Hengartner, 2000). Fractional DNA that is fixed and stained with a nuclear stain such as PI will lead to a population of hypodiploid cells that manifests as a Sub-G_1 population. BA-treated cells appeared to have an increase in this subpopulation with increasing concentrations, suggesting a concentration-dependent relationship. Similar to observations present in morphological assessment of treated cells (Figure 4.11), NCI-H1299 cells appeared to be less responsive to BA, but nevertheless apoptotic induction was present.

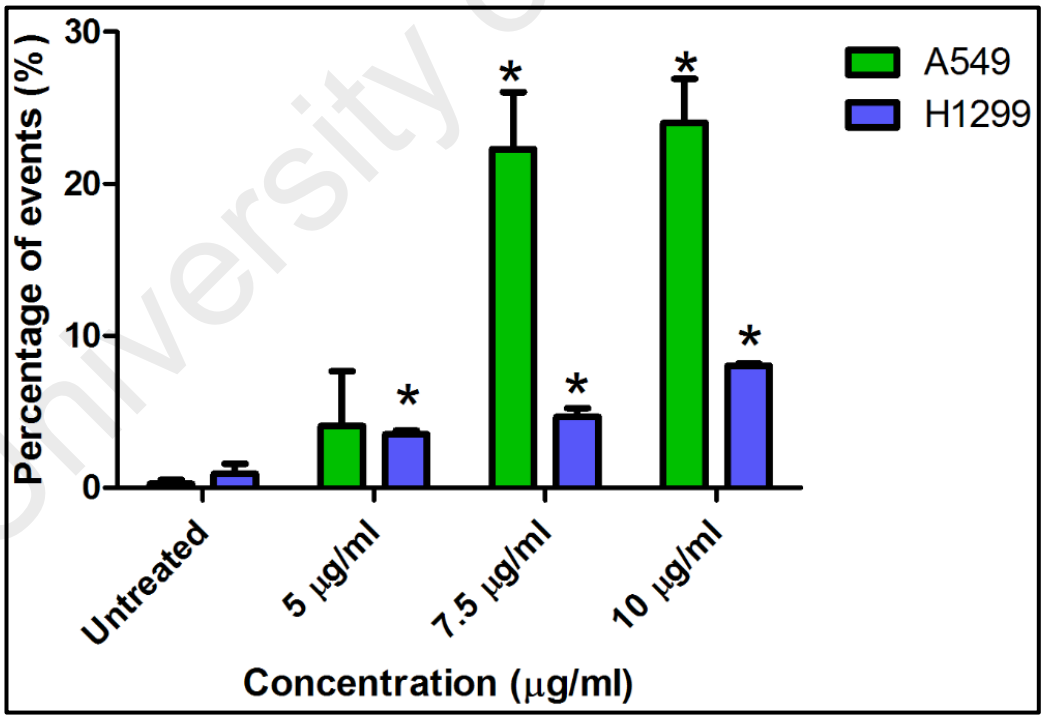

Figure 4.12: Sub-G_1 population of BA-treated cells.

A549 (A) and NCI-H1299 (B) cells were treated with 5, 7.5 and 10 µg/ml of BA. The resultant sub-G_1 population show an increase with the increase in concentration of BA used.

4.10.3 BA causes DNA strand breaks in A549 cells

Terminal deoxynucleotidyl transferase (TdT) identifies DNA strand breaks by labelling free 3'-OH termini with modified nucleotides, which is the basis of the TdT-mediated dUTP nick end labelling (TUNEL) reaction. As mentioned in Section 4.10.2, DNA fragmentation is present in apoptotic cells due to the activation of calcium-dependent endogenous endonuclease. The effect of BA in inducing apoptosis was done through the quantitative detection of DNA strand breaks at regular intervals *via* TUNEL assay, using the procedures outlined in Section 3.11. The results showed a general increase in the presence of Fluorescein-labelled DNA strand breaks, indicative of an increasing incidence of apoptosis. Compared with the untreated control, a slight increase to 4.46 ± 3.72% in cells undergoing apoptosis was detected in 5.0 µg/ml treatment group, which then increased to 31.03 ± 8.77% and 41.57 ± 4.48 % of apoptotic cells as a result of exposing A549 cells to 7.5 µg/ml and 10.0 µg/ml of BA, respectively (Figure 4.13). Exposure to 15.0µg/ml of BA resulted in 50.25 ± 0.49% of fragmented DNA from the population. In addition, A549 cells exposed to 10.0 µg/ml for 48 and 72 hours yielded a population of 79.73 ± 2.21% and 85.60 ± 4.56% respectively, which is a steep increase compared to the 24-hour exposure of the same BA concentration (Figure 4.13).

An increasing population of apoptotic cells with rising concentrations of BA show that the treatment acted in a concentration-dependent manner, consistent with the observation in the Sub-G_1 identification. In addition to this, BA was also assessed at 48 and 72 hours to evaluate the effects of prolong exposure on the cells. Although only managing to provide a mild response at 24 hours, increased treatment time of up to 72 hours caused a rapid escalation in the presence of detected fragmented DNA. This incidence of increased apoptotic outcome over prolonged exposure can be explained by the fact that cleavage of DNA in apoptotic cells is a late- stage event (Collins et al., 1997). Based on this, it can be inferred that the detrimental effect of high concentration of BA

can be reduced through a regiment of low concentration with prolonged treatment time. As such, an interplay between concentration and treatment time could be used to maximise the effectiveness of BA. This would especially be a useful point to consider should BA be applied as a treatment for cancer one way or another.

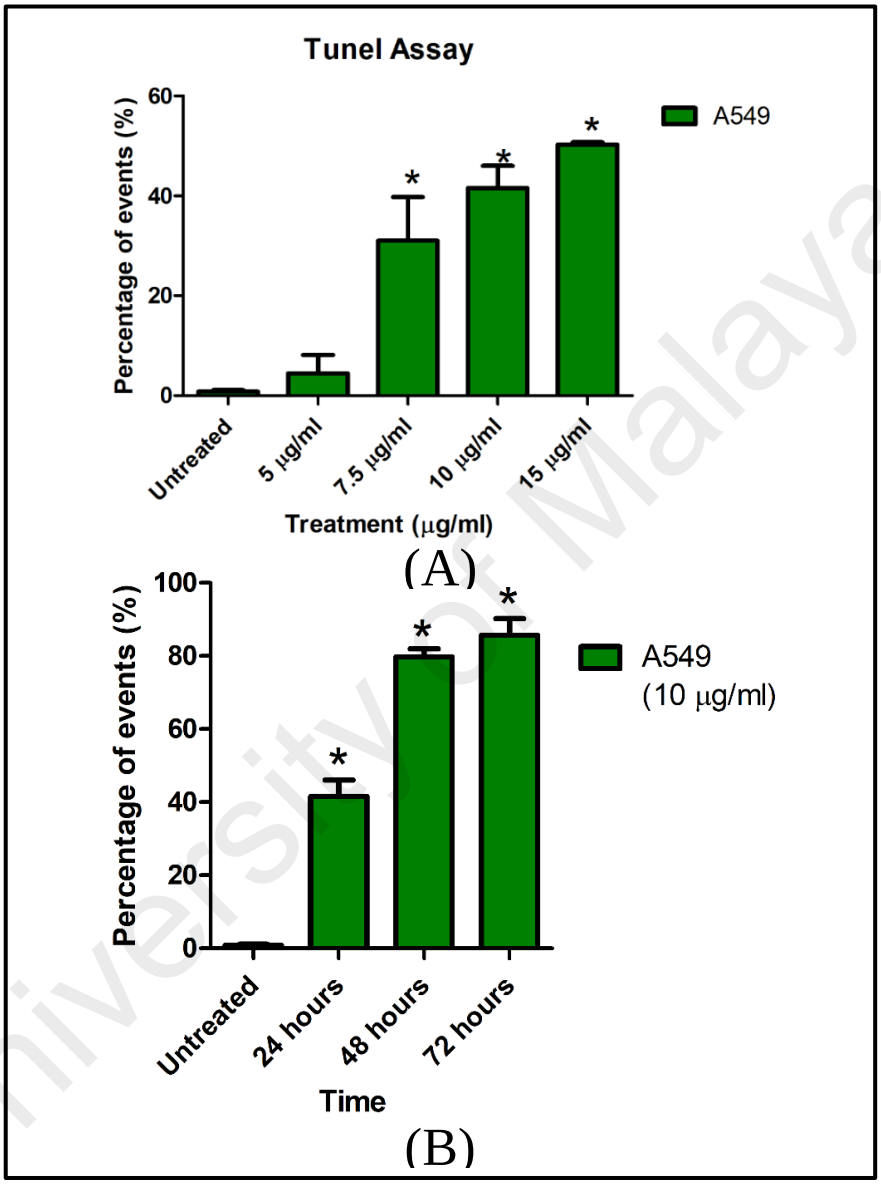

Figure 4.13: Concentration and time dependent formation of fragmented DNA in BA-treated cells.

A549 was treated with 5, 7.5,10 and 15 µg/ml of BA for 24 hours (A). An increase in presence of labelled DNA strand breaks was present in a concentration dependent manner. A549 treated with BA for 48 and 72 hours appeared to experience a rise in apoptotic incidence (B). (*) is indicative of a statistically significant result ($p < 0.05$).

4.10.4 BA increases presence of active caspase-3

In a bid to obtain better understanding of the concentration-response relationship of BA against treated cells, caspase-3 was quantified using flow cytometry methods, as was done for the parent compound, LF1 in Section 4.5.4. Referring to Figure 4.14, a gradual and significant increase ($p < 0.05$) of activated caspase-3 was observed with increasing concentrations of BA. The highest population of apoptotic signals was obtained in the 10 µg/ml treatment group, 42.20 ± 0.14% for A549 cells and 20.60 ± 0.57% for NCI-H1299 cells. Although an increasing pattern is observed in both cells lines, this occurrence however is less prominent in NCI-H1299 cells in which the number of apoptotic events was roughly half of that present in treated A549 cells.

As shown in Figure 4.14, BA-treated cells appeared to have a steady increase in activated caspase-3, suggesting a concentration-dependent reaction. This observation which was consistent with previous mention data (Sections 4.10.1, 4.10.2 and 4.10.3) is not surprising as caspases have been known to be involved in most of the reported observation in this study. Previous studies have indicated the general involvement of caspase-3 in the cleavage of lamins which cause nuclear shrinkage (Kivinen et al., 2005), loss of overall cell shape by cleavage of cytoskeletal protein such as fodrin and gelsolin (Nishio & Matsumori, 2009; Porter & Janicke, 1999), and activation of caspase-activated DNase (CAD) which is responsible for DNA fragmentation (Nagata, 2000).

One key observation present in all apoptotic evaluation was the reduced effect of BA towards NCI-H1299 treated cells when compared to A549 cells. Early experiment *via* the MTT assay (Section 4.9) showed a lack of disparities between the two cells lines, but this was not the case in more specific assays focusing on the detection of apoptosis induction. This may be explained through longer exposure period of the MTT assay (72 hours) which may have masked the underlying mechanism involved in causing cell death. Although induction of apoptosis through treatment with BA occurs in both p53 present

A549 and p53 absent NCI-H1299, the apoptotic reaction appeared to intensify in A549, almost double in most cases. This would suggest BA has multiple targets and does not solely depend on the p53 to trigger apoptosis but may be more efficient at inducing apoptosis with the presence of the p53 gene. In other words, the presence of p53 has compounding effect with regards to induction of apoptosis; itself not being the main cause of cell death but does aid in intensifying the apoptotic effect. This would most likely be caused by the repression of active inhibitors of p53, such as MDM2, as a result of treatment with BA. Current studies have reported that BA induces apoptosis in a p53-independent manner (Fulda, 2008) with others suggesting otherwise (Rieber & Strasberg-Rieber, 1998). In addition to this, the milder effect towards the p53-mutant NCI-H1299 would suggest that BA does not have the capabilities of restoring wild-type functionality of mutant cells.

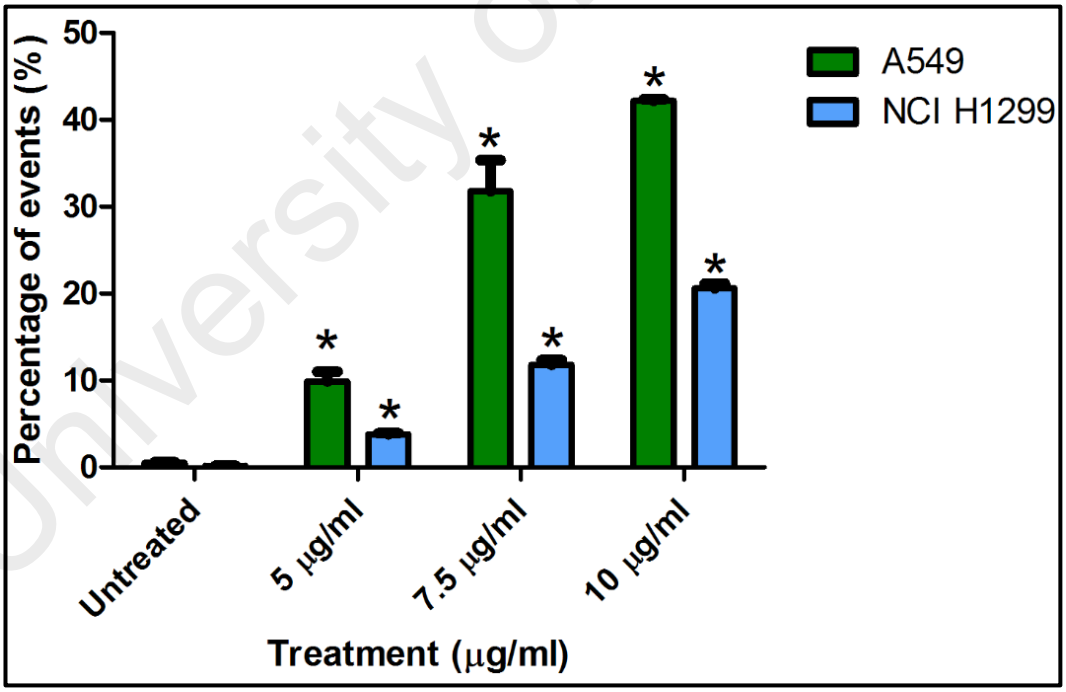

Figure 4.14: Presence of active caspase-3 in BA-treated A549 and NCI-H1299.

A549 and NCI-H1299 cells were treated with 5, 7.5 and 10 µg/ml of BA for 24 hours. An increase in the presence of cleaved caspase-3 was detected in both cell lines in a concentration-dependent manner, indicating an apoptotic pathway was taken by BA-treated cells.

4.11 BA has a disruptive effect the cell cycle in lung cancer cells

The cell cycle is a cellular apparatus that regulates the cell proliferation process. Due to defective communication between the signal processing network and the cell division mechanisms, an imbalance in tissue homoeostasis in favour of hyperproliferation occurs, which may eventually give rise to tumorigenesis. A link between apoptosis and cell cycle can be established based on the argument that both the process tend to share a set of regulatory proteins which causes apoptosis as well as are involved in cell cycle regulation (Pucci et al. 2000).

The disruption in cell cycle was next examined through flow cytometry analysis as outlined in Section 3.9, with the hope of determining the mechanism responsible for the induction of apoptosis in treated lung cancer cells. As shown in Figure 4. 15, an increase in the number of A549 cells in the G_0/G_1 phase (61.67 ± 1.64% to 74.29 ± 1.44) with increasing concentration of BA (from 0 to 10 µg/ml) was observed, while a similar trend was seen in NCI-H1299 (50.13 ± 1.75% to 67.64 ± 1.80). At the same time, a decrease in the S population was observed with escalating concentrations of BA. These observations were done with reference to the untreated controls and the differences were statistically significant ($p < 0.05$) in most cases.

The findings of this study suggest that BA causes cell cycle arrest at the G_0/G_1 phase in treated cells, preventing the cell from proceeding to the S phase which eventually leads to cell death. The concentration dependent pattern observed was consistent with the apoptosis and cytotoxic studies, suggesting a possible relationship between the three. A similar observation was seen by Sawada et al. (2004) in BA-treated B16F10 melanoma cells, whereby an accumulation in the G_0/G_1 phase was present in a concentration dependent manner.

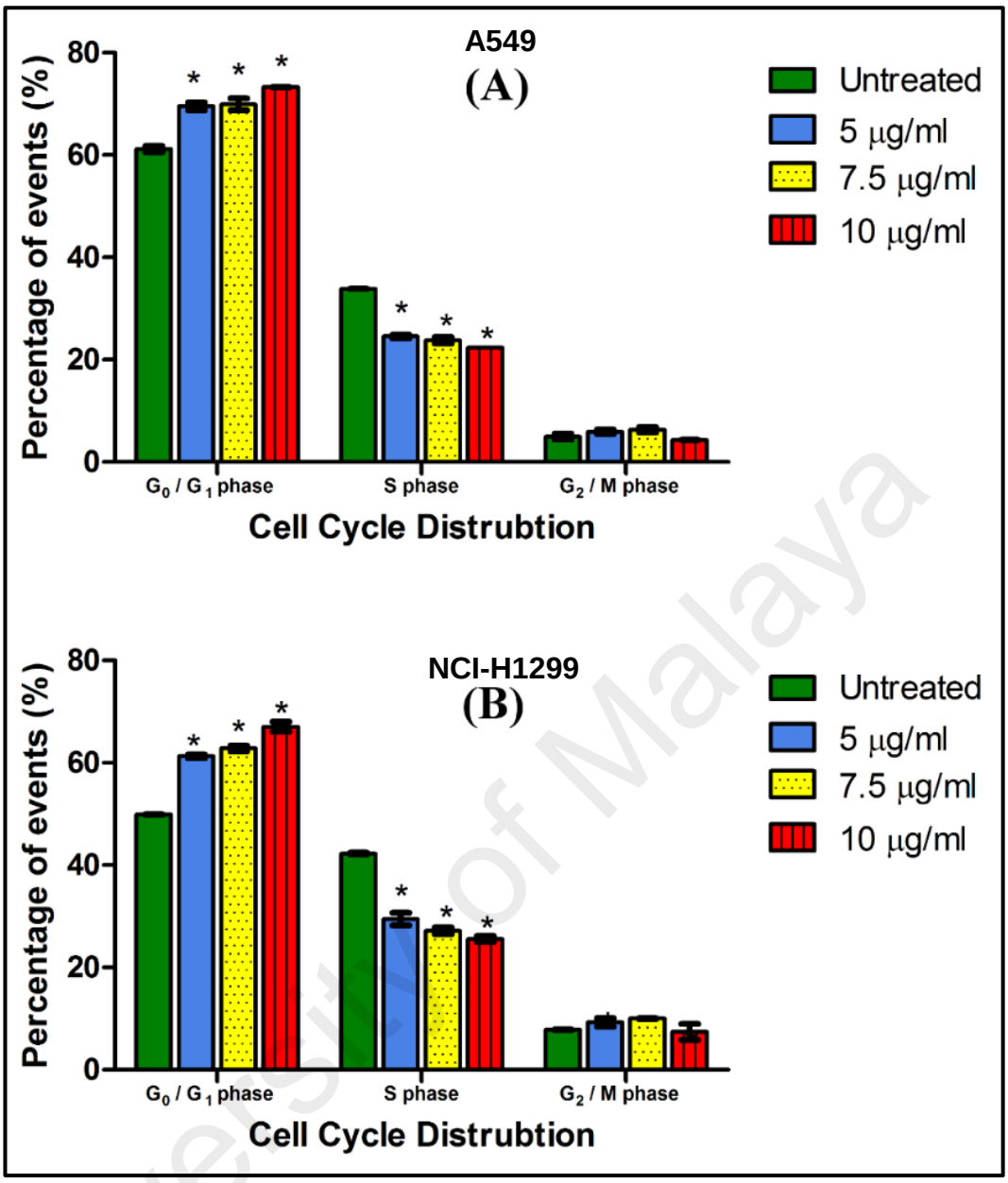

Figure 4. 15: Effects of BA on cell cycle distribution in A549 and NCI-H1299 cells. A549 (A) and NCI-H1299 (B) were incubated in absence (control) and presence of LF1 at 5, 7.5 and 10 μg/ml for 24 hours. Summary of results indicate an increase in G_0/G_1 population with increasing concentrations of BA used.

Another similarity with the apoptotic analysis is the milder effect of BA on NCI-H1299 when compared with A549 cells. Although the variation is not as apparent as in the apoptotic reaction, yet the response of NCI-H1299 towards BA does draw attention to the possibility of p53 involvement in enhancing both cell cycle disruption and apoptosis in BA-treated cells. The p53 is known to cause both cell cycle arrest as well as apoptosis. Following along the line of argument used in Section 4.10.4, BA may be targeting p53 regulators, such as MDM2, in addition to other cell cycle arrest inducing targets, explaining why both cells were affected, regardless of the p53 status. On the other hand, BA has been shown to react differently in causing cell cycle arrest towards different cell lines from the same cancer type, which could explain the above situation (Fulda, 2008).

4.12 Evaluating BAs ability to disrupt key process of metastasis

4.12.1 Hampered adhesion of BA-treated cells

Cancer metastasis consist of a complex cascade of biological events which include cellular adhesion, invasion across vessel walls and cell migration within the extracellular matrix to remote sites and establish new foci of growth (Bogenrieder & Herlyn, 2003). Upon arresting at a particular organ, invading cancer cells should be able to firmly adhere to the new site before colonization can take place. Disruption in such capabilities of invasive cells could retard the metastatic process and prevent the spread of cancer.

The effect of BA in disrupting the ability of treated A549 cells to adhere was examined through the adhesion assay which involves detaching treated cells and placing them onto a new culture surface, following the procedures outlined in Section 3.15. Cells which did not attach appeared to be rounded while attached cells retain their morphological shape. The untreated group, which was used as a reference point, appears to have mostly attached after a six hours' incubation period, with the exception of a few cells which remained rounded (Figure 4.16). Following a 24-hour incubation period, all

untreated cells completely attached with clear defined shapes. In contrast to the negative control, BA-treated cells appeared to have a less effective adhesion to the surface after six hours, with most of them still appearing to be in a suspension state. Although there is an increase in the number of attached cells after a 24-hour incubation period, these cells lack a clearly defined shape when compared to negative control. This is especially evident in the higher treatment groups, 5.0 and 7.5 μg/ml. As a result of treatment with BA, the adhesive efficiency of A549 cells appears to be impeded.

In this study, BA was shown to disrupt the ability of treated cells in attaching to a new location compared to untreated cells. The lack of ability to adhere to the coated culture surface suggests that BA might have prevented the production of key components such as ICAM-1 and integrin. It is interesting to note that exposure to BA to human umbilical vein endothelial cells (HUVEC) cells had a significant reduction in the expression of ICAM-1 (Yoon et al., 2010), providing further clues into its possible role in the results obtained.

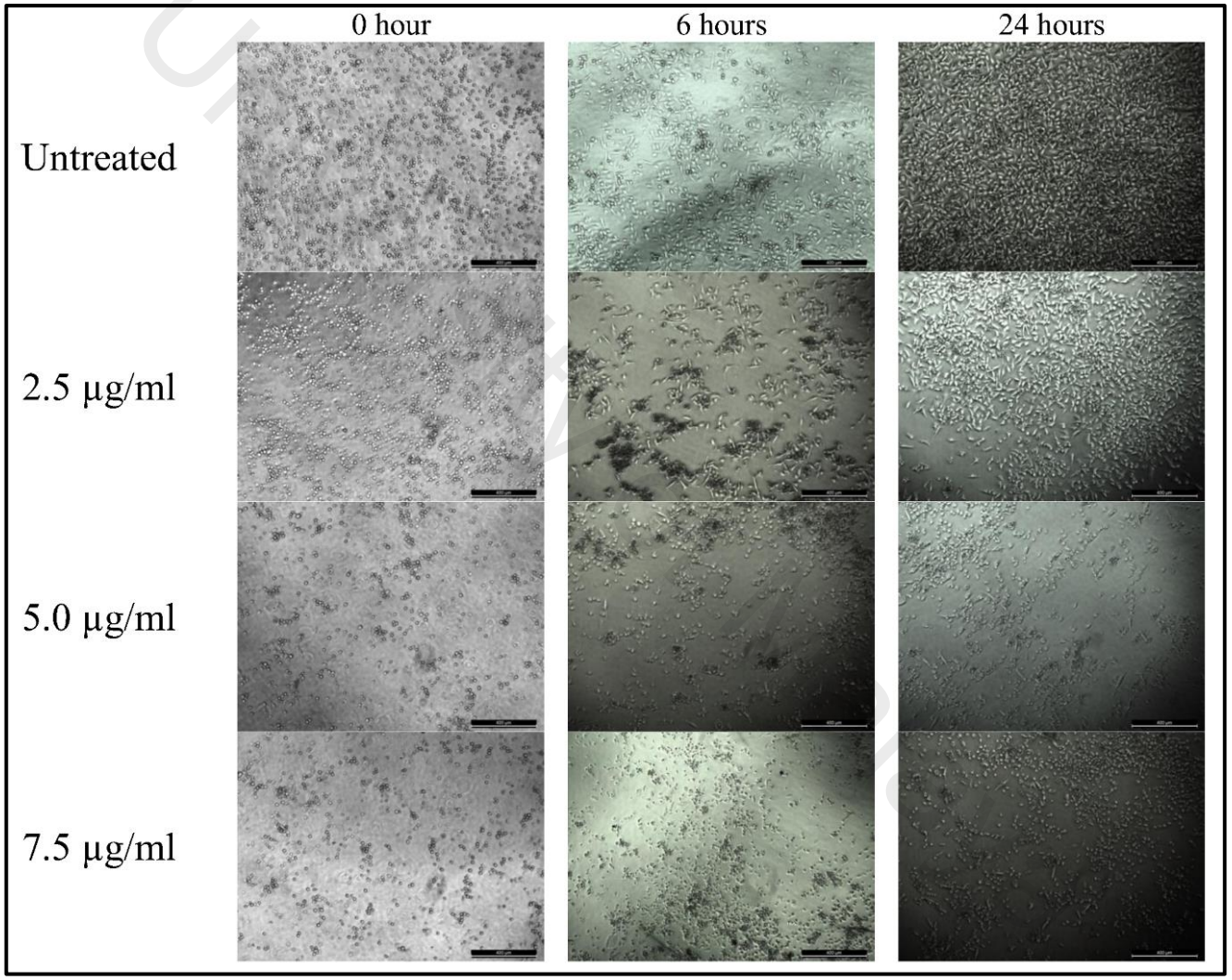

Figure 4.16: Disruption in adhesion of BA-treated A549 cells.

4.12.2 BA disrupts the movement of A549 across a porous membrane

Cell migration is the directed movement of cells from one area to another on a substrate such as basal membranes, ECM fibres or plastic plates in response to a chemical signal or chemoattractant. It is a crucial process which is central to a variety of functions, such as wound repair, cell differentiation, embryonic development and the metastasis of tumours. The ability to migrate is a prerequisite to invade; a cell cannot invade without migration but can move without invasion. Therefore, it is of our interest to study the response of metastatic cancer cells towards BA with aim of assessing its anti-migration potentials.

One of the common methods used in the study cell migration is the trans-well membrane migration assay, which is similar to the Boyden-Chamber assay (Section 3.13). The principle of this assay is based on two medium containing chambers separated by a porous membrane through which cells transmigrate as a result of enticement from the chemoattractant. The polycarbonate membrane serves as a barrier to discriminate migratory cells from non-migratory cells. Migratory cells are able to extend protrusions towards chemoattractant (via actin cytoskeleton reorganization) and ultimately pass through the pores of the polycarbonate membrane. It is a simple and quick assay which does not need specialized equipment or materials other than the cell culture inserts.

The results obtained indicate a general decrease in migrated A549 cells in response towards increasing concentrations of BA, indicated by the drop in the number of cells stained purple *via* crystal violet (Figure 4.17). The untreated cells appeared to be able to migrate across the porous membrane with no issue, as seen through the high density of migrated cells, with a reduction in the number of migrated cells with increasing concentrations of BA used to treat the cells.

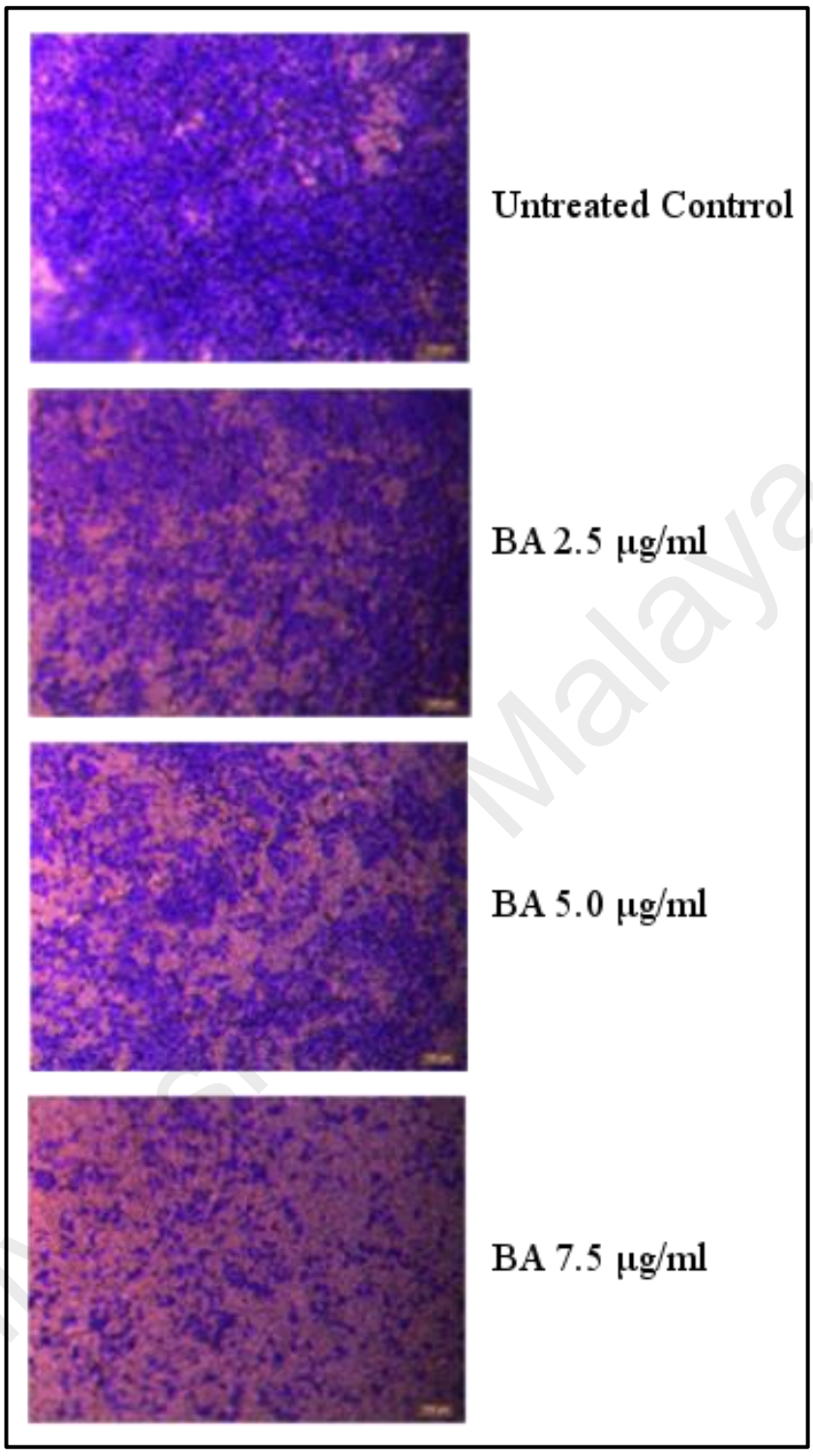

Figure 4.17: Migrated A549 cells after treatment with BA.

A declining number of cells managed to migrate post treatment with BA for 48 hours. This is shown through a decrease in purple colour. Images were taken after removing the layer of unmigrated cells from the upper chamber and stained with crystal violet.

To provide a little more clarity of the data through quantification, the dyes were bleached in 30% acetic acid and the subsequent solution's absorbance was quantified, with the summary of the data presented in a graphical format (Figure 4.18). As the concentration of BA used was increased from 2.5 µg/ml to 7.5 µg/ml, a decrease in migrated cells was observed (from 72.83 ± 1.07 to 41.51 ± 3.13%), suggesting an inverse relationship between the two factors. The trend in the two sets of data from the qualitative (Figure 4.17) and quantitative (Figure 4.18) evaluation methods are in agreement with each other, thus validating the microscopical observation.

Even at the lowest concentration used (2.5 µg/ml), which is below the IC_{25} value (3.75 µg/ml), a significant inhibition in the motility of treated cells were observed, imply that the reduction in cellular movement was not caused by loss in cell viability. The lowest concentration used in the apoptotic evaluation through caspase-3 activation detection (Section 4.10.4) was 5 µg/ml, whereby less than 10% of cells recorded a positive signal for apoptosis induction. At the same treatment concertation used in the migration assay, almost 40% of cells were prevented from migrating across the membrane. As such, the anti-migration effects of BA observed was not dependent on the apoptotic induction, especially when concerning with lower concentrations.

These results are consistent with previous studies reported by Rzeski et al. (2006) which highlighted that low concentrations of BA significantly inhibited migration of glioma (C6), lung carcinoma (A549) and medulloblastoma (TE671) cells through the scratch wound assay. The inability of BA-treated A549 cells to efficiently migrate across a porous membrane could be attributed to the suppression of chemical factors which have been implicated in the enhancement of tumour motility, including matrix metalloproteinase-2 (MMP-2) (Xu et al., 2005) among others.

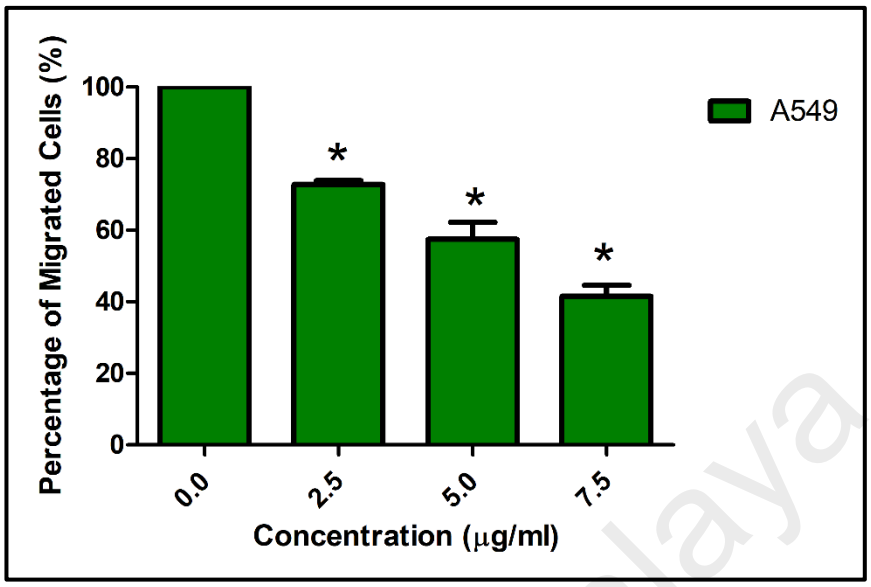

Figure 4.18: Effects of BA on Trans-well migration in treated A549 cells. A549 was treated in absence (control) and presence of BA at 2.5, 5.0 and 7.5 µg/ml for 48 hours. A decrease in migrated cells is observed with increasing concentrations of BA used.

4.12.3 BA upsets invasive potential of A549 treated cells

Cell invasion plays a crucial role in tumour metastasis which represents the fundamental difference between a benign tumour and a malignant cancer. Metastasis involves multiple processes such as infiltrative growth through the extracellular matrix (ECM), cell migration through blood or lymph vessels and rise of distant colonies, all of which require cell invasion and cell migration. Along those lines of reasoning, BA was tested to determine if it possessed an anti-invasive property in addition to its cytotoxic potential which has been shown through the pervious assays.

The principal technical setup of the Trans-well invasion assay is similar to the Trans-well migration assay, with the exception of the porous filter being overlaid by a thin layer of Matrigel, before seeding the cells into the top chamber. The Matrigel occludes the membrane pores, blocking non-invasive cells from migrating through. By

contrast, invasive cells can degrade the matrix and move through the Matrigel layer and adhere to the bottom of the filter. The assay was carried out according to the steps put forward in Section 3.14.

Similar to the migration assay, the untreated controls were used as a baseline to determine the effectiveness of BA in deterring the invasive nature of cancer cells. Based on the data from Figure 4.19, there appears to be a decreasing trend with respect to the percentage of cells that managed to move across the Matrigel coated membranes. Almost identical in its trend to the migration assay, the lowest concentration used, 2.5 µg/ml of BA, managed to yield an inhibition of 18.02 ± 2.07% of cells from invading through Boyden chamber, relative to the untreated control. The decreasing number of invaded cells was consistent with the movement pattern in the migration assay, allowing us to assume that the restrictive behaviour of BA is through a combination of inhibiting the motility as well as the ability to degrade the Matrigel. Using the same line of argument as those used in Section 4.12.2, lower concentrations of BA managed to cause an inhibitory reaction, which is not caused by cell death. Treatment with 2.5µg/ml, which is below the IC_{25} value, managed to reduce the invasive potential of treated cells. As such, the anti-invasive properties of BA appear to be impartial of the toxicity effects to certain degree. Consistent with these findings, human pancreatic adenocarcinoma cell lines FG and PANC-1 that have been exposed to BA have shown to significantly inhibit invasive and migratory activities.

As mentioned in Section 4.12.2, certain elements play a crucial role in the interplay of migration and invasion. One such protein is the MMP family, namely MMP-2 and MMP-9, which play a crucial role in cell motility as well as the degradation of Type IV Collagenase, which is a key component in the ECM (Xu et al., 2005). As such, these two enzymes that are commonly affected downstream as a reaction to anti-metastatic

compound, would serve as good evaluation point to determine the mode of action used by BA in reducing the invasive potential of treated cells.

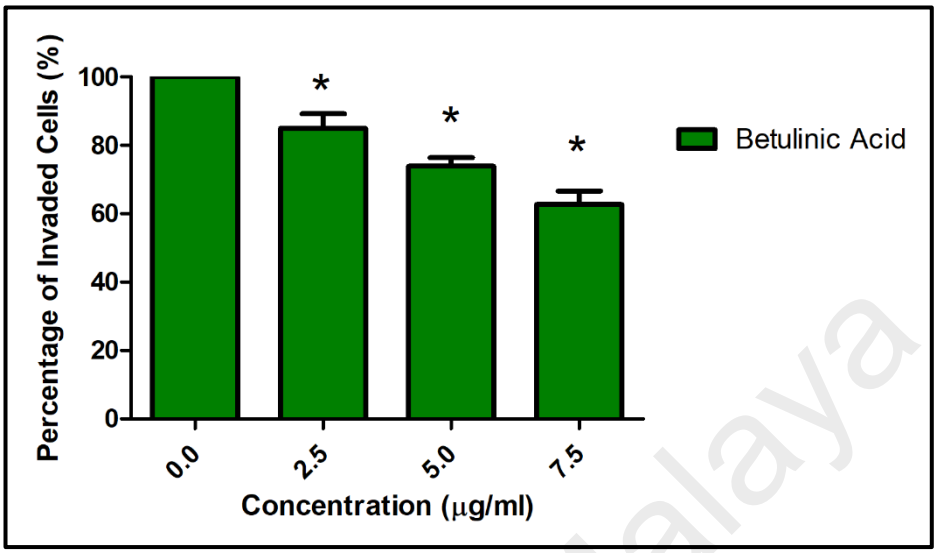

Figure 4.19: Effects of BA on Trans-well invasion in treated A549 cells.

A549 was treated in absence (control) and presence of BA at 2.5, 5.0 and 7.5 µg/ml for 48 hours. A decrease in migrated cells is observed with increasing concentrations of BA used.

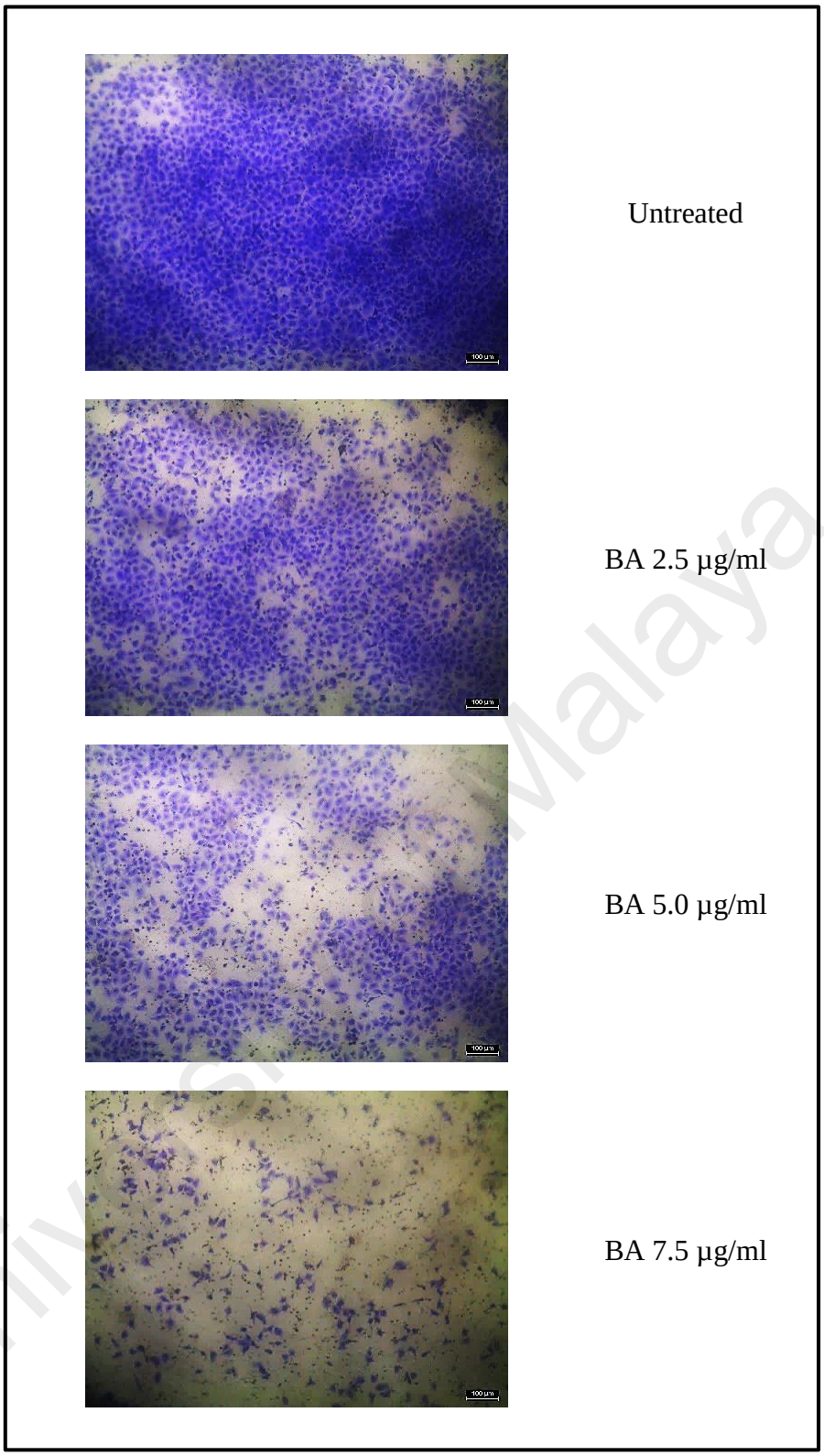

Figure 4.20: Invaded A549 cells after treatment with BA.

A decreasing number of cells managed to invade post treatment with BA for 48 hours. Images were taken after removing the layer of unmigrated cells from the upper chamber and stained with crystal violet. The images were taken at 100× magnification.

4.12.4 BA reduces the presence of MMP- 2

Following the results relating to the metastatic process as mentioned above (Sections 4.12.1, 4.12.2 and 4.12.3), MMPs were further assessed to get a better understanding into the reactions in response treatment with BA. MMPs are set of proteins which play an important role in the metastatic process. These enzymes are responsible for the extracellular matrix (ECM) breakdown, which serves as a barrier preventing the invasion of cancer cells in spreading to other site of the body (Mott & Werb, 2004). Particular interest has been given to MMP-2 and MMP-9, two types of IV collagenases which are responsible for the degradation collagen type IV, a major component of ECM and basement membrane (Gomes et al., 2012; Roomi et al., 2009). TGF-β1, which is a cytokine commonly associated with the gelatinase in focus (Krstic & Santibanez, 2014), was used in this experiment to induce the production of these MMPs in order to get a better visualization into the inhibitory effects of BA against the enzymes.

A gelatin zymogram was performed following the steps outlined in Section 3.16, to assess the enzymatic activity of the gelatinase (MMP-2 and MMP-9) present in samples. The increased expression of MMP-2 as a result of TGF-β1 activation was reduced in response to increasing concentrations of BA. This was evident through the lowered intensity of the bands corresponding to MMP-2, 58 kDa for the active MMP-2 and 45 kDa for the catalytic domain of MMP-2 (Figure 4.21). On the other hand, bands associated with MMP-9 did not exhibit any noticeable changes with regards to enzyme activity, be it in the activated or latent state. This was represented by the faint bands with almost identical intensities at 68 kDa (Active MMP-9) and 92 kDa (Pro MMP-9) (Figure 4.21). To further validate these findings, an ELISA was carried out using the methods in Section 3.17 to determine the amount of MMP-2 and MMP-9 expressed in the sample. The Quantification of MMP-2 via the ELISA method showed a gradual decrease (from 100.00% to 48.89 ± 3.51%) with increasing level of BA concentrations (0 to 7.5 µg/ml)

(Figure 4.21). At the same time, an obvious pattern was absent with regards to the effect of BA towards the presence of MMP-9 (Figure 4.21). The levels of enzyme present in treated cells appear to have increased when compared with the untreated control, which was used as a baseline for this experimentation.

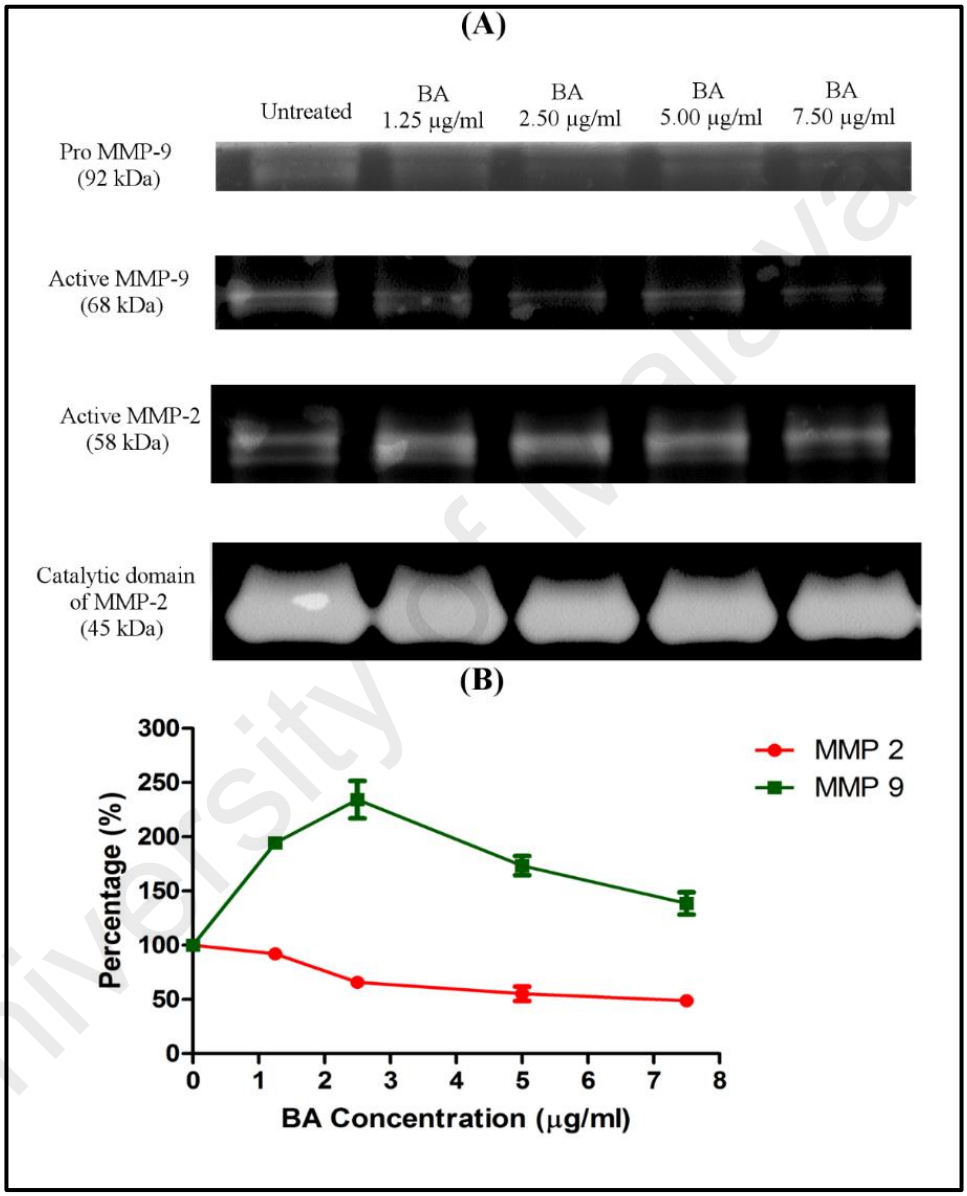

Figure 4.21: Reduction of MMP-2 production in TGF-β1 induced A549 caused by treatment with BA.

(A) Gelatin zymography revealed a decrease in MMP-2 concentration with a relatively constant presence of MMP-9 in treated A549 cells. At the same time, the (B) ELISA assay showed a reduction by half for MMP-2 and an increase in MMP-9 concentration compared to the untreated control in the same A549 cells.

Treatment with BA appeared to reduce the production of MMP-2 across the board, regardless if it is in the pro or active form of the enzyme. The pattern which was present in the gelatin zymogram was validated through the ELISA assay. A lack of pro-enzyme accumulation would also suggest that the inhibitory reaction could have been at the transcriptional level and not by preventing the activation of these enzymes. These pattern of MMP-2 expression has a similar trend to the results in the adhesion and migration assay mentioned above, suggesting a possible relationship between the three.

4.13 Protein expression and activation in response to BA treatment

Extracellular influences, that can be stimulatory (growth factors, hormones) or inhibitory (other cells, extracellular matrix, growth factors), may alter cellular functions via the receptor binding and signal transduction to the cell nucleus (King & Robins, 2006). The signal-transduction cascade is responsible in mediating the sensory and processing of these stimuli. These molecular circuits detect, amplify, and integrate diverse external signals to generate responses such as changes in enzyme activity, gene expression, or ion-channel activity (Berg et al., 2002).

In cancer cells, elements of the signal transduction are often overexpressed or mutated, leading to a state of constant activation or suppression, depending on mutated component (Lobbezoo et al., 2003). As a result, cancer cells attain a degree of autonomy from external regulatory signals that renders them less subjected to such signals than normal cells. This autonomy is reflected through the diminished necessity for growth stimulating molecules, allowing for constant growth and proliferation of the tumour cells; and a weakened response towards inhibitor signals capable of suppressing the growth of the cancer cells (King & Robins, 2006).

The improvement in the understanding with regards to the inner works of the signal transduction process has led to an evolution from the linear signalling pathway, whereby a single signal causes a limited number downstream products, to a much more comprehensive signalling network that takes into account the possibility of cross talk between multiple pathway (Milella et al., 2010). This reveals the possibility of drug design that targets specific proteins of the signal transduction cascade with the hope of overcoming the muted inhibitory response in cancer cells.

Based along these lines, this study proceeded to look into the effects of BA on common targets in the signal transduction process. As it has already been proven that BA does have the capabilities to induce apoptosis (Sections 4.10.1 to 4.10.4), cell cycle arrest (Section 4.11) as well restrict the metastatic process in lung cancer cells (Sections 4.12.1 to 4.12.4), the next step would be to obtain a better understanding into how the pure compound imparts its effect on the treated cells. This would provide the knowledge needed to better customize the treatment modalities in terms of concentrations or through combination with other drugs.

The protein expression of pathway component which may have been affected as a result of BA treatment was assessed *via* two commonly used methods, the western blotting process (Section 3.18) as well as the qualitative ELISA method (Section 3.19). The former utilizes an SDS-Page to separate the mixture of protein lysates according to molecular weight followed by a transfer and immobilization on a membrane prior to antibody-based detection; the latter on the other hand utilized a 96-well plate coated with the detection antibodies to perceive the presence of protein of interest. The untreated controls were used as a baseline to determine the extent of the detrimental effects of BA on the treated lung cancer cells.

The reduction in p-Akt band seen in Figure 4.22 was indicative of reduction in Akt phosphorylation in NCI-H1299 cells as a result of treatment with BA. At the same time, levels of Akt appeared to be similar across all treatment groups, implying that BA action is towards the activation and not on the transcription level of this protein.

The mitogen-activated protein (MAP) kinases are critical mediators of the signal transduction in mammalian cells (Kyriakis & Avruch, 2001). These kinases can be divided into three major groups: the extracellular signal regulated protein kinases (ERK), the p38 MAP kinases and the C-Jun NH_2- terminal kinase (JNK).

Expression of ERK 1/2 modules, which respond to mitogenic signals such as transmitted by growth factor receptors, was examined. From the results in Figure 4.22, the p44/42 MAPK, which corresponds to ERK1/2, appeared to decrease with increasing concentrations of BA in treated NCI-H1299 cells. This observation was seen in the base protein, which further suggests that BA's interference in the cancer cells expression of this protein could possibly lie in the transcription or translational phase, blocking the production of the protein thus reducing its influence on the cellular machinery of the cancer cells.

In addition to this, the levels of p38 MAP kinase in treated cancer cells was also observed. In contrast to the ERK group, p38 MAP kinases are activated by inflammatory cytokines and by the exposure of cells to environmental stress. As for the reaction towards treatment with BA, the levels of p38 MAP kinases appeared to follow a similar trend to the ERK1/2 expression intensities; a reduction was observed with increasing concentrations of BA used (Figure 4.22). Similar to ERK1/2, the decrease in presence of the p38 protein was with regards to the un-phosphorylated state of the protein, ruling out a post-translational modification by BA.

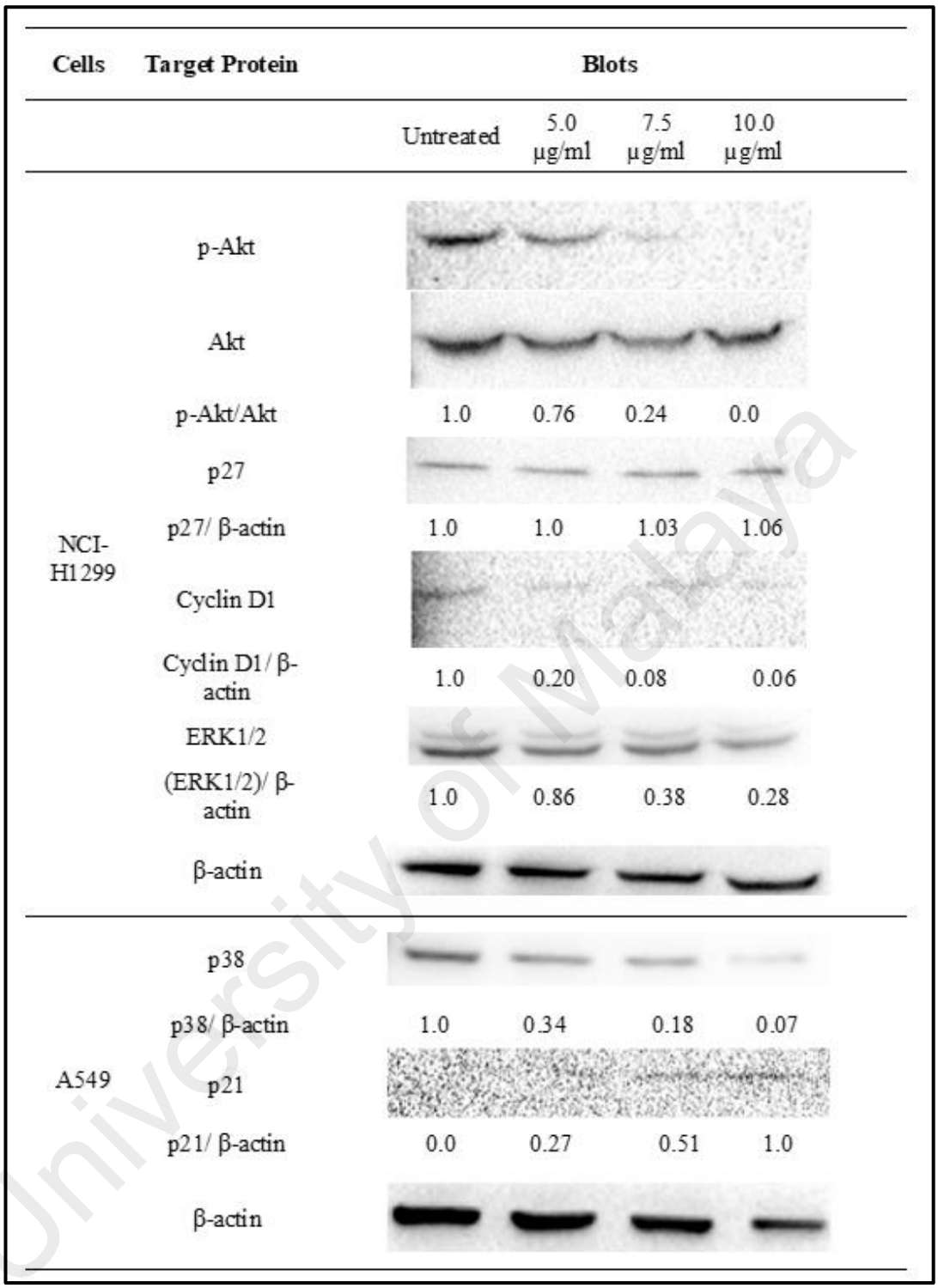

Figure 4.22: Effect of BA on expression of target proteins.

The cell lysate for BA-treated NCI-H1299 and A549 cells were analyzed through western blot. B-actin and Akt was used as a loading control. The intensities of the bands are shown relative to the untreated controls after normalization.

In order to get a better understanding into ability of BA to cause cell cycle arrest as shown in section 4.17, the expression of p27, p21 and cyclin D1 was determined (Figure 4.22). The first two corresponds to a class of important regulators of the cell cycle progression, the CIP/KIP (CDK or Kinase Inhibiting Proteins) family. Specifically, $p27^{KIP}$ plays a particularly important role in the control of tissue homeostasis by keeping it in a non-proliferative quiescence state, while $p21^{CIP}$ permanently regulates the cell cycle progression of proliferating cells. The inactivation of prevention of their *de novo* synthesis is crucial for the advancement of cell cycle progression. In the above experiment, both these proteins appeared to have an increased expression with increasing concentrations of BA, suggesting a reversal in the repression of these proteins. Although, it has to be noted the levels of $p21^{CIP}$ appeared to be rather low and was visible in the enhanced blot images. The band detection of this blot was performed through the automated band detection function in the ImageLab software.

Other than these two proteins, levels of cyclin D1 were investigated. Cyclins are heterogeneous proteins that are crucial in the progression of the cell cycle. They function by reacting with cyclin dependent kinases (CDK) to form a complex that would then allow the cell cycle to progress to the next phase. In relation to that, cyclin D1 plays a role in transition from the G_1 to the S phase through interactions with CDK4/6. BA-treated NCI-H1299 cells appeared to have a reduction in the amount of cyclin D1 present in the cell lysate when compared with the untreated controls.

Besides the above-mentioned proteins that were evaluated via the western blot method, a few more protein expression levels were determined using the ELISA method. The levels of proteins present in the lysate was compared to the untreated controls, which was used as a baseline to determine the basal level of that said protein (Figure 4.23). NF-kB p65 was used as a loading control to ensure all loaded protein lysates were of equal

amounts. Four different proteins were evaluated: Phospho-NF-kB p65, Phospho-IkB-α, Phospho-STAT3 and the Phospho-SAPK/JNK.

NF-kB is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biologicals processed such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. The multifunctional role of this protein makes it an ideal target for a number of treatments. In this experiment, p-NF-kB p65 saw an increase of 31.3% in BA-treated A549 cells when compared with the untreated controls; suggesting the BA manages to increase the rate of activation of NF-kB p65 (Figure 4.23).

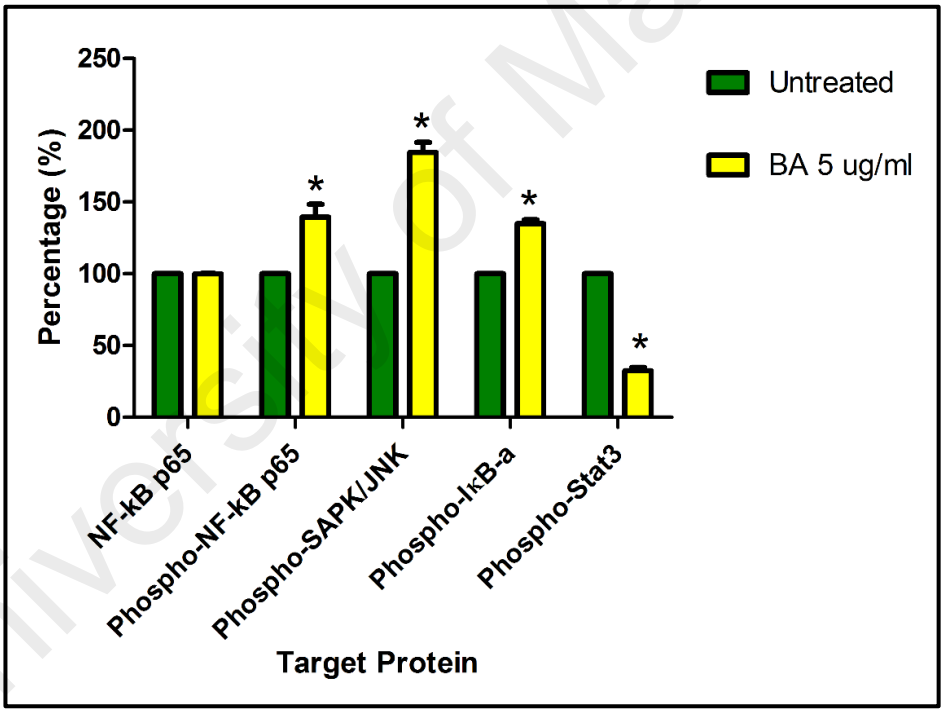

Figure 4.23: ELISA detection of target proteins.

The protein lysate from A549 cells that were treated with BA was collected and analysed using the ELISA method. NF-κB was used as a loading control. The (*) indicates a significantly different value when compared with the untreated controls ($p < 0.05$).

IkB-α, which is an inhibitory factor of NF-kB, constrains the transcription factors in the cytoplasm through the masking of their nuclear localization signals. Upon cellular stimulation, they become phosphorylated which promotes its ubiquitination and degradation, enabling NF-kB to translocate to the nucleus and activate transcription. Consistent with the result of p-NF-kB p65, p-IkB-α increased in A549 cells upon treatment with BA, almost by the same metric, 35.5% (Figure 4.23).

The JNK group of MAP kinases is activated by many of the same stimuli that causes activation of the p38 MAP kinases, including exposure of cells to inflammatory cytokines and environmental stress, as such both JNK and p38 MAP kinases are collectively named stress-activated protein kinase (SAPK). However, the activation mechanism in JNK does differ in a number of ways. Furthering on that, the expression of activated JNK in A549 cells was detected via ELISA detection of p-SAPK/JNK. The phosphorylated form of this kinase appeared to increase due to treatment with BA when compared with the untreated controls, an increment of 84.4% over the untreated control (Figure 4.23).

STAT3 is a key transcription factor that is primarily activated by the JAK-STAT pathway as wells as the MAP kinases pathway (Spitzner et al., 2014). The protein has been implicated to be involved in proliferation, differentiation, angiogenesis, metastasis, invasion as well as resistance to apoptosis (Siveen et al., 2014). In this study, BA managed to reduce the activation of STAT3 which can be seen through the reduction of 67.7% in the expression of p-STAT3 (Figure 4.23).

For a cell cycle to remain in the G_0/G_1 phase, the action of CDK inhibitors is required together with a shortage of G_1 cyclin such as cyclin C and D. The synergistic effect of several mitogenic signals that are able to supress the production of CDK inhibitors and at the same time stimulate the production of cyclin D, will allow the cells to proceed beyond the restriction point late in the G_1 phase. The disruption of this control mechanism is what that allows cancer cells to undergo unregulated proliferation through the cell cycle.

Among the CDK inhibitors, p27 is responsible primarily for keeping the cell in the G_0 phase which must be inactivated for mitogenic stimulation. The synthesis of p27 is inhibited and its degradation is promoted while the reverse holds true for cyclin D. Both occur via mitogenic signalling cascades, namely the MAP kinase and phosphatidylinositol 3-kinase-protein kinase B (PKB)/Akt pathways.

The phosphorylation of Akt, which relives the cell cycle from the restrains caused by CDK inhibitors, is significantly reduced as a result of treatment with BA. Under normal circumstances, Akt (or also known as PKB) phosphorylates and inactivates the inhibitor p27 and prevents its *de novo* synthesis by phosphorylating the corresponding transcription factor, in this case those of the Forkhead family that controls the p27 gene. To reinforce this action, Akt also activates the gene responsible for the production of subunit SKP2 of ubiquitin ligase SCF, which is responsible for triggering the degradation of p27. In addition to this, the MAP kinase ERK also plays a role in priming p27 for ubiquitylation and degradation.

The results obtained upon treatment with BA, as displayed in Figure 4.22 shows a decrease in p-Akt and ERK, which was followed by an increase in p27 expression. This is consistent with the above-mentioned reasoning as by downplaying the role of Akt in the cell cycle regulation, the inhibition and degradation of the CDK inhibitor p27 was prevented. In addition to this, the ERK enzyme, which was played a similar role as Akt in negatively effecting p27, was downregulated as a result of BA treatment.

The progression beyond the G_1 phase is also dependent on the activation of the gene transcribing cyclin D. The MAP kinase ERK not only is responsible for suppression of CDK inhibitors as mentioned earlier, but also plays a role in the synthesis of cyclin D. The sustained presence of ERK is required during the progression past the restriction point between the G_1 and S phase. GSK3β on the other hand, primes the cyclins for ubiquitylation and its subsequent degradation. Akt prevents this action by inactivating GSK3β, thus stabilizing the cyclins. Treatment of cells with BA that inhibits activation of Akt would reverse this reaction, allowing for GSK3β to initiate degradation of cyclin D. The repression of ERK expression as a result of exposing the cells to BA also prevented its ability to stimulate the synthesis of cyclin D, consequently halting the cell cycle progression at the G_1 phase. Consistent with this, the reduction of cyclin D1 was observed in Figure 4.22. In short, BA action in reducing cyclin D1 was twofold, through inhibition of its synthesis via ERK activation as well as stimulating its degradation through GSK3β that was not repress by Akt.

In addition to the above action of BA against the cell cycle, the effects of the p53 dependent p21 was determined. The p53 protein controls the expression of p21 by activating the genes responsible for the production of this CDK inhibitor. The p21, which is a member of the CIP/KIP family, binds to the CDK/cyclin complexes which prevents the kinase activation that subsequently leads to halt of the cell cycle at the G_1 phase. The

treatment with BA on the p53-positive A549 cells lead to a slight, but noticeable increase in p21 expression. Looking back, BA has a disruptive effect the cell cycle in lung cancer cells, whereby a slightly increased effect was seen in the p53-postive A549 as compared to the p53-negative NCI-H1299; it would seem that BA was partially better in A549 due to its effect on p21. The restriction of cell cycle does occur in NCI-H1299 through suppression of cyclin D1 and the upregulation of p27, but an increased effect would be explained by the increase of p21 which is only present in a p53-positive cell. This same line of argument could be used to suggest a possible explanation into the incidence of increased apoptotic effect of BA onto the p53-positive A549 cells. A prolong cell cycle arrest coupled with increase in other stress factors caused by BA, would then push the affected cells into cell suicide through apoptosis. In short, BA is not entirely dependent on the p53 gene, but does seem to react much more effectively in the presences of the tumour suppressor.

The summary of the proposed effects of BA on the cell cycle is shown in Figure 4.24.

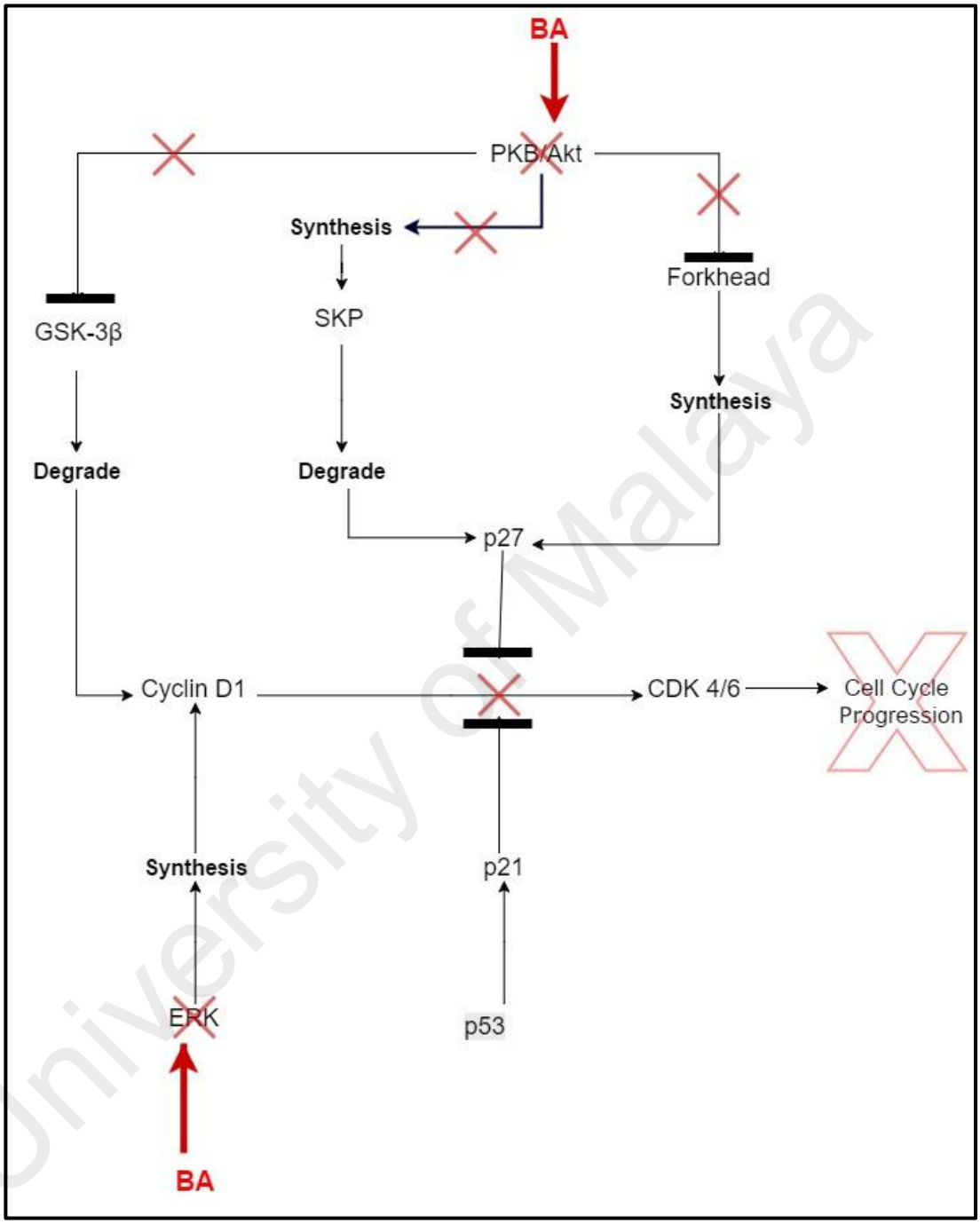

Figure 4.24: Proposed reaction diagram for cell cycle arrest caused by treatment with BA.

The transcription factor NF-κB is a key mediator of the cellular stress response that is involved in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. In the absence of input signals, they are mainly concentrated in the cytoplasm where they exist as complexes with their inhibitory subunit called IκBs, one of them being the IκB-α isoform. These inhibitors masked the nuclear localization sequence, thus preventing NF-κB from entering the nucleus and its subsequent DNA binding activities. In a conventional activation pathway, IκB-α is phosphorylated by IκB-kinases (IKKs) in response to activators. This leads to the release of NF-κB complex, that is able to be translocated into the nucleus, and subsequent degradation of the inhibitor IκB-α. Lastly, the translocated NF-κB is able to bind to specific DNA sequences present in the promoters of various genes.

Most studies that look into the functionality of NF-κB with regards to the apoptotic pathway contribute to the general perception that it is responsible in mediating the resistance towards programmed cell death by activating anti-apoptotic genes. However, there have been instance of the opposite occurring, whereby NF-κB was associated with pro-apoptotic genes as well (Hayden & Ghosh, 2004). In addition to this, p65, a subunit of NF-κB, has been reported to promotes apoptosis by actively repressing transcription of anti-apoptotic gene (Campbell et al., 2004).

Figure 4.23 indicates an increase in IκB-α and NF-κB phosphorylation in BA-treated A549 cells that are consistent with the above arguments where NF-κB being associated with the apoptotic pathway. An increase in p-IκB-α should follow a rise in p-NF-κB as the transcription factor has been released from its inhibitor, allowing its activation and subsequent DNA binding activities. The effect of BA on NF-κB and its inhibitor is in line with the finding of Kasperczyk et al. (2005), which reported that BA activated NF-κB in neuroblastoma (SHEP and SH-SY5Y), glioblastoma (LN229 and

U373) as well as melanoma (MeWo) cells, subsequently leading to apoptosis in a number of those cells.

The phosphorylation of STAT3 leads to its dimerization and translocation into the nucleus where it binds with DNA resulting in the regulation of several genes involved in cell proliferation, differentiation and apoptosis. The role of activated STAT3 has been closely associated with cell survival and proliferation in a number of human cancer cells. Some of the regulated products include Bcl-xL, Bcl-2, cyclin D1 and survivin. In fact, a study done by Pandey et al. (2010), showed a that BA managed to downregulate the mentioned proteins which are associated with STAT3. In this study, a decrease in STAT3 phosphorylation was observed as well as a reduction in the levels of cyclin D1, consistent with the above-mentioned findings of Pandey et al. (2010).

In addition to this, Lim and Cao (1999) managed to show that ERK and MAP kinase p38 did not play a role in STAT3 activation whilst, JNK1 managed to negatively regulate the tyrosine phosphorylation as well as DNA binding and transcriptional activities of STAT3. In the same manner, Figure 4.23 shows a decrease in STAT3 activation and was followed by an increase in JNK phosphorylation, while a decrease in ERK1/2 and MAP Kinase p38 was observed in Figures 4.22 and 4.23.

STAT3 has also been implicated in the metastatic and angiogenesis process (Demaria et al., 2012). Regulation of STAT3 has been shown to inhibit epithelial-mesenchymal transition (EMT) (Yadav et al., 2011). In addition to this, suppression of STAT3 regulation has also managed to inhibit tumour invasion in A549 cells (Jiang et al., 2016). As such, it would be possible to draw parallel between these finding and ours in which treatment with BA reduced the migration and invasion capabilities of A549, and at the same time BA managed to greatly reduce the activation of STAT3.

Reports have suggested that JNK triggered mechanism are capable of promoting apoptosis (Xia et al., 1995;Verheij et al., 1996) . Among the modes of action include the expression of FAS ligands as well as the modulation of the Bcl-2 protein family (Kolbus et al., 2000; Deng et al., 2001). The upregulation of JNK in BA-treated A549 cells could have caused apoptosis in this manner as the role of Bcl-2 in BA induced apoptosis has been previously established (Fulda, 2008).

Although p38 and JNK are said to be induced mostly by the same stimuli, the data in this study suggest otherwise. The p38 kinase was downregulated while the JNK activation was increased as a result of treatment with BA. The answer could lie in the fact that both these kinases do have some difference in the way they are activated (Davis, 2000). The JNK signalling module includes the MAP kinase kinases MKK4 and MKK7, whereby MKK7 is a specific activator of the JNK pathway. Of the two, only MKK4 is involved in both JNK and p38 (Davis, 2000).

The downregulation of p38 has been associated with the diminution of breast cancer metastasis through the usage of p38 inhibitors (Wu et al., 2014). It has also been suggested by Igea and Nebreda (2015) that p38 inhibitor may be used as a viable tool in order to sensitize drug resistant tumour cells to chemotherapeutic drugs and enhance the effectiveness of such treatment modalities in causing cell death. This idea rises from recent data that has strongly implicated p38α signalling in the resistance to chemotherapeutic treatments. As such, this provides a lead to develop BA as a possible adjuvant with chemotherapeutic drugs, especially when concerned with drug resistant tumour cells.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Summary of findings

Usage of natural products, either directly or indirectly, in the treatment of various aliments has thus far been a promising idea. The importance of seeking new and alternatives to current drugs is of more importance now due to the rise in the number of cases and increasing mortality related to cancer, especially lung cancer. Compounded with the increasing reports of drug resistance tumours, it is of paramount importance to seek new treatment modalities. In accordance with this statement, this study managed to evaluate the candidacy of *Leptospermum javanicum* and *Baeckea frutescens* to be developed as sources of an anti-tumour drug.

The cytotoxic evaluation of both plants managed to hint at a possibility of a promising candidate in the form of the semi-pure fraction LF1. The apoptotic capabilities of this mixture were also encouraging as it managed to present signs synonymous with apoptotic induction, both qualitatively and quantitatively. In addition to this, cell cycle arrest caused by LF1 compounded with the above observations, made it a suitable contender to be further evaluated, thus managing to fulfil the first objective of this study.

As for the second objective, the active LF1 was subjected to isolation, purification and identification through spectroscopic methods to reveal the identity of the major constituent as Betulinic acid (BA). This compound has been previously studied and been proven as an apoptotic agent against melanoma and neuroblastoma cells. This study chose to expand further on currently available research of BA by focusing on lung cancer and three key aspects in cancer progression and survival; apoptotic induction, cell cycle control as well as metastatic potential.

With respect to the cytotoxic potential, the pure compound indicated to be comparable with its parent compound, demonstrating itself as a better candidate in most cases. Its capabilities in inducing apoptosis were also verified through a series of assay focusing on the qualitative and quantitative aspect of apoptotic initiation. The altered protein phosphorylation of NF-κB, IκB-α, STAT3 and JNK, all of which have been implicated in previous studies to play a role in apoptotic induction, is believed to have contributed to apoptotic observation in lung cancer cells in response to BA. Although not dependent on its presence with regards to the activation of apoptosis, the p53 does appear to play a role in intensifying the incidence of the cell death mechanism activation.

In addition to this, BA showed capabilities in causing cell cycle arrest at the G_o/G_1 phase, possibly contributing to its apoptotic potential. The role of BA in this reaction was further confirmed through the reduced expression of p-Akt, ERK1/2 and cyclin D1 together with a slight increase in p27 and p21, all of which are known players in the cell cycle control mechanism, particularly in the passage from the G_1 to S phase.

Lastly, the anti-metastatic potential of BA against lung cancer cells was examined. The key feature of the metastasis process, adhesion, migration and invasion was evaluated in response to treatment to BA. The capabilities to move across an uncoated and Matrigel-coated membrane as well as the ability to adhere to a new culture surface were all diminished as a result of treatment with BA, even at lower concentrations. The reduction of MMP-2 expression (as seen through the gelatin zymogram and ELISA method), which plays a crucial role in the metastatic properties of invasive cancer cells, further strengthening the argument into the ability of BA to be developed as an anti-cancer drug.

5.2 Future works

In order to obtain much more comprehensive understanding into the ability of BA as an anticancer agent, it is suggested particular focus be given to the upstream targets of BA in order to map out the complete reaction pathway taken by BA in exerting its cancer inhibiting abilities. Combinational therapies have also been a promising area of research that would benefit from the inclusion of BA.

A major drawback of BA lies in its limited solubility which could prove to be a stumbling block, especially in the delivery system of the treatment. As such, it would be ideal to look into chemical modification of BA functional group that may at best improve the solubility of the terpenoid. There have been a few studies which have focused on this issue with some success, but nevertheless with room for improvement.

Moreover, it would be critical to evaluate BA on an *in vivo* model to obtain a grasp to reaction of BA in a systemic model as opposed to a tissue-based experimentation that was used in this study. The *in vivo* study could also be vital in understanding the probable acute and chronic side effects that may possibly occur in response to treatment with BA.

5.3 Conclusion

This study highlighted the ability of the *L. javanicum* isolate, BA in not only inducing apoptosis but also restricting the proliferation of lung cancer cells through cell cycle modulation as well as limits the metastatic capabilities of invasive lung cancer cells. The finding of this study provides validation to the potential of BA to be developed as an anti-cancer drug, thus expanding the treatment scope of BA to include lung cancer as a possible treatment modality.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications

Navanesan, S., Abdul Wahab, N., Manickam, S., & Sim, K.S (2015). *Leptospermum flavescens* constituent-LF1 causes cell death through the induction of cell cycle arrest and apoptosis in human lung carcinoma cells. *PLoS ONE*, 10(8), e0135995. (Impact Factor: 2.806, Q1)

Navanesan, S., Abdul Wahab, N., Manickam, S., & Sim, K.S (2015). Evaluation of selected biological capacities of *Baeckea frutescens*. *BMC Complementary and Alternative Medicine*, 15(1), 186. (Impact Factor: 2.288, Q1)

Navanesan, S., Abdul Wahab, N., Manickam, S., Cheow, Y.L., & Sim, K.S (2017). Intrinsic capabilities of *Leptospermum javanicum* in inducing apoptosis and suppressing the metastatic potential of human lung carcinoma cells, *Chemico-Biological Interactions*, 273, 37-47. (Impact Factor: 3.143, Q2)

List of presentations

Navanesan, S., Abdul Wahab, N., Manickam, S., Cheow, Y.L., & Sim, K.S. Investigating the potential usage of *Leptospermum flavescens Sm*. as a therapeutic agent against human lung carcinoma. 18th Biological Sciences graduate Congress (BSGC), 6th to 7th Jan 2014, University of Malaya, Malaysia.

Navanesan, S., Abdul Wahab, N., Manickam, S., Cheow, Y.L., & Sim, K.S. Exploring the potential of *Leptospermum flavescens* as a possible therapeutic agent against lung cancer. 2nd International Conference of Traditional and Complementary Medicine on Health (ICTCMH), 24-27th Oct 2015, GIS NTU Convention Center, Taipei, Taiwan.