PHYLLANTHUS INHIBITS PROLIFERATION, METASTASIS, ANGIOGENESIS AND INDUCES APOPTOSIS OF HUMAN MELANOMA (MEWO) AND PROSTATE ADENOCARCINOMA (PC-3) CELLS THROUGH MODULATION OF MULTIPLE CELL SIGNALLING PATHWAYS

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ABSTRACT

Modern cancer treatment therapies such as surgery, chemotherapy and immunotherapy are deemed relatively unsuccessful due to their ineffectiveness, safety issues and costliness. As not all cancer patients respond positively to current anticancer agents, mortality rates of cancer are on a continuous rise including melanoma and prostate cancer.

Natural product-based traditional medicine, often overshadowed by modern medicine, has returned to be a holistic approach for health care in many societies. This "back to basic" approach is due to its long history of usage in disease treatment and the pharmacological/nutritional value of these products which are believed to be able to halt/delay cancer progression.

In this study, the anticancer properties of four *Phyllanthus* (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) were studied against on human melanoma, MeWo and prostate adenocarcinoma, PC-3 cells. The main aims of this study were to identify the anti-proliferation, anti-metastasis, anti-angiogenesis properties, apoptosis induction and mechanisms of inhibition of *Phyllanthus* plant extracts on these cancer cells.

Phyllanthus extracts have significantly inhibited the growth of MeWo and PC-3 cells at IC_{50} values at the range of 155.0-260.0 μg/ml and 54.2-153.3 μg/ml for aqueous and methanolic extracts, respectively. This growth inhibition was due to cell cycle arrest at G1-phase in PC-3 and S-phase in MeWo cells, concurrent with the accumulation of apoptotic cells at Sub-G1. Induction of apoptosis was further implicated with the activation of caspase-3/7, presence of TUNEL positive cells and DNA fragmentations as well as increased pro-apoptotic Bax proteins activity in treated cancer cells. Low level of LDH was detected in treated MeWo and PC-3 cells as the result of damage to

the cytoplasmic membrane, indicative of late apoptosis or necrosis. In contrast, *Phyllanthus* exerted low cytotoxicity in human normal cell lines (CCD-1127Sk, RWPE-1 and HUVECs).

The anti-metastatic and anti-angiogenic effects of *Phyllanthus* extracts were observed when *Phyllanthus* extracts inhibited several essential steps during metastasis and angiogenesis; (i) adhesion, (ii) migration, (iii) invasion, (iv) transendothelial migration and (v) microcapillary-like tube formations. These observations were most likely due to reduction in activities of matrix metalloproteinase-2, -7 and -9 in treated cells as was noted.

These observed anticancer properties of*Phyllanthus* extracts are believed to be due to the plants inhibitory effects on multiple signalling pathways; MAPKs, Wnt, Myc/Max, Hypoxia and NFκB, via alteration on their intracellular signalling activities including pan-Ras, c-Raf, Akt, Elk1, RSK, c-Jun, JNK1/2, β-catenin, GSK3β, c-myc, HIF-1α, VEGF, NFκB p50 and p52. In addition, various other proteins involved in proliferation, metastasis and apoptosis were found to be differentially expressed in treated MeWo and PC-3 cells.

Taken together, the results showed that *Phyllanthus* extracts possess anticancer effects through inhibition in proliferation, metastasis and angiogenesis as well as induction of apoptosis on human melanoma, MeWo and prostate adenocarcinoma, PC-3 cells. Thus, *Phyllanthus* is a promising candidate for the development of future anticancer agents and could possibly be introduced as a part of diet to prevent cancer development.

ABSTRAK

Terapi rawatan kanser moden seperti pembedahan, kemoterapi, dan imunoterapi telah dianggap tidak berhasil kerana ketidakberkesanan, isu-isu keselamatan dan kos yang tinggi. Oleh sebab tidak semua pesakit kanser bertindak balas secara positif kepada ejen antikanser, kadar kematian kanser semakin meningkat termasuk “melanoma” dan kanser prostat.

Perubatan tradisional yang berasaskan penggunaan produk semulajadi yang sering dibayangi oleh perubatan moden, telah kembali menjadi salah satu pendekatan dalam penjagaan kesihatan masyarakat. Pendekatan "kembali kepada asas" ini adalah disebabkan oleh sejarah panjang penggunaannya dalam rawatan penyakit dan nilai farmakologi/nutrisi produk ini yang dipercayai dapat menghentikan/melambatkan perkembangan kanser.

Dalam kajian ini, aktiviti antikanser dari empat *Phyllanthus* (*P.amarus, P.niruri, P.urinaria* dan *P.watsonii*) telah dikaji terhadap sel melanoma, MeWo dan kanser prostat, PC-3. Matlamat utama kajian ini adalah untuk mengenalpasti sifat-sifat “anti-proliferation”, “anti-metastasis”, “anti-angiogenesis”, induksi apoptosis dan mekanisme ekstrak tumbuhan *Phyllanthus* terhadap sel-sel kanser ini.

Ekstrak *Phyllanthus* telah menghalang pertumbuhan sel MeWo dan PC-3 pada nilai IC_{50} pada julat 155.0-260.0 μg/ml bagi ekstrak “aqueous” dan 54.2-153.3 μg/ml bagi ekstrak “methanolic”. Penghalangan pertumbuhan sel-sel ini adalah disebabkan oleh penyekatan di kitaran sel pada fasa G1 dalam sel PC-3 dan fasa S dalam sel MeWo, serentak dengan pengumpulan sel-sel apoptotik di Sub-G1. Induksi apoptosis sekaligus dikaitkan dengan pengaktifan “caspase-3/7”, kehadiran sel positif TUNEL dan “DNA fragmentations” serta dengan peningkatan aktiviti protein Bax pro-apoptotik

di dalam sel-sel kanser. Tahap rendah LDH telah dikesan dalam sel MeWo dan PC-3 yang diakibatkan oleh kerosakan pada “cytoplasmic membrane”, yakni, menunjukkan tanda apoptosis lewat atau nekrosis. Selain itu, *Phyllanthus* menunjukan tahap rendah “cytotoxicity” terhadap sel-sel manusia biasa (CCD-1127Sk, RWPE-1 dan HUVECs).

Aktiviti “anti-metastatic” dan “anti-angiogenic” *Phyllanthus* telah diperhatikan apabila ekstrak *Phyllanthus* menghalang beberapa langkah penting semasa metastasis dan angiogenesis; (i) “adhesion”, (ii) “migration”, (iii) “invasion”, (iv) transendothelial migration” dan (v) “microcapillary-like tube formation”. Ini adalah disebabkan oleh pengurangan aktiviti “matrix metalloproteinase”-2, -7 dan -9 dalam sel-sel yang dirawati *Phyllanthus*.

Aktiviti antikanser dalam ekstrak *Phyllanthus* dipercayai berpunca daripada kesan penghalangan tumbuhan tersebut dalam pelbagai “signalling pathways”; MAPKs, Wnt, Myc/Max, Hypoxia dan NFκB, melalui perubahan aktiviti “intracellular signalling” seperti pan-Ras, c-Raf, Akt, Elk1, RSK, c-Jun, JNK1/2, β-catenin, GSK3β, c-myc, HIF-1α, VEGF, NFκB p50 dan p52. Disamping itu, pelbagai protein lain yang terlibat dalam “proliferation”, “metastasis” dan “apoptosis” juga didapati berbeza dalam sel-sel MeWo dan PC-3 setelah dirawati oleh *Phyllanthus*.

Kesimpulannya, hasil penyelidikan menunjukkan bahawa ekstrak *Phyllanthus* mempunyai aktiviti antikanser melalui penghalangan dalam proliferasi, metastasis dan angiogenesis serta induksi apoptosis pada sel melanoma, MeWo dan prostat kanser, PC-3. Dengan itu, *Phyllanthus* berkemungkinan dapat dijadikan agen antikanser pada masa depan dan diperkenalkan sebagai sebahagian daripada diet untuk menghalang perkembangan kanser.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Description
µg	Microgram
m/z	Mass- to charge- ratio
°C	Degree Celcius
vs	Versus
ATP	Adenosine triphosphate
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EDTA	Ethylene diamine Tetra-acetic Acid
FDA	Food and Drug Administration
FBS	Fetal bovine serum
HPLC	High-performance liquid chromatography
Hsp	Heat Shock Proteins
LDH	Lactate dehydrogenase
NCI	National Cancer Institute
MMP	Matrix Metalloproteinase
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-. (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
MS/MS	Tandem mass spectrometer
PAGE	Polyacrylamide grl electrophoresis
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate
WHO	World Health Organization
NCI	National Cancer Institute
MMP	Matrix Metalloproteinase

CHAPTER 1: INTRODUCTION

1.1 CANCER

1.1.1 Overview

The first description of cancer is recorded in an ancient Egyptian papyrus dated between 3000 and 1500 B.C., about eight cases of breast tumours that were treated by cauterization. However, the origin of the word "Cancer" was first described by the ancient Greek physician, Hippocrates (460-370 B.C.), known as the "Father of Medicine". Hippocrates was the first person who clearly recognized and differentiated between benign and malignant tumours after noticing the presence of blood vessels only in malignant tumours. Carcinos and carcinoma were the terms used by Hippocrates to describe non-ulcer forming and ulcer-forming tumours, respectively. In Greek, both words refer to a crab, most likely applied to the disease where the finger-like spreading projections from a cancer mimic claws of a crab. Later, the Roman physician, Celsus (28-50 B.C.), translated these terms into the Latin word, "Cancer" which is also known as crabs and until today is still widely used in medicine (Cohen, 1997; MedicineWorld.Org, 2012).

Cells are the building blocks of all living things in this world and all cells are derived from pre-existing cells. Every cell carries out respiration, reproduction (meiosis) and growth (mitosis), which are controlled by regulating gene expression at the DNA level. In a normal cell, disruptions or mutations in their DNA will initiate a repair system on the involved DNA or leading to cell death (Anagnostopoulos *et al.*, 2008). However, in cancer cells, neither the DNA is repaired nor cell death occurs; instead, the mutated cell will continuously generate new mutated cells, which all have the same damaged DNA as the first cell does. These mutated cells may have acquired

various abnormalities, including aneuploidy, chromosomal rearrangements, amplifications, deletions, gene rearrangements, and loss- or gain-of-function mutations (Mills, 2003). Most cancer cases are genetically inherited because the mutated DNA can be passed along the generations. Most mutation in DNA can occur during cell replication and/or caused by carcinogens such as cigarette smoking, diet or oncoviruses such as Epstein–Barr virus (Kim *et al.*, 1994; Vincent & Gatenby, 2008; Cancer Research UK, 2012).

Cancer is a name given to a group of diseases that arise from a single (mutated) cell when it starts to grow abnormally in an uncontrollable manner to form a group of undifferentiated cells, known as a tumour. Tumours are classified into two categories, benign or malignant. Not all benign tumours are cancerous but all malignant tumours are (Hanahan and Weinberg, 2000). The main difference is that benign tumour lacks metastatic ability, grows locally and is less harmful. However, some benign tumours can transform into malignant tumours that possess metastatic ability to invade and spread to other parts of the body via the blood or lymphatic circulation and form secondary tumours and eventually cause death (Vincent & Gatenby, 2008; Hanahan & Weinberg, 2011).

1.1.2 Development of Cancer (Carcinogenesis)

Cancer develops through a multistep process known as carcinogenesis, where by normal cells are transformed into cancerous cells. The process involves cellular and genetic changes and eventually reprograms a cell, thus forming a malignant mass. Carcinogenesis involves three significant steps; initiation, promotion and progression (Figure 1.1) (Van der Kamp & Jaspers, 1984; Pitot, 2006). An initiation stage is a permanent and irreversible event, which involves one or more cellular changes arising upon exposure to carcinogens, which leads to alteration in DNA and may result in a

mutated cell to divide rapidly (hyperplasia). These transformed (initiated) cells can remain harmless, unless exposed to a stimulator, which enhances the tumour to grow into a larger mass. This is a reversible process, known as the promotion stage. The progression stage is an irreversible conversion of a benign tumour to become a malignant tumour. The progression stage includes increased growth rate, invasiveness and metastatic capability of the malignant cells (Pitot, 2006). This carcinogenesis process usually takes 10 years or more to develop and usually depends on the internal (biological) and external (environmental) factors of the patient (Pitot, 2006; Hanahan & Weinberg, 2011).

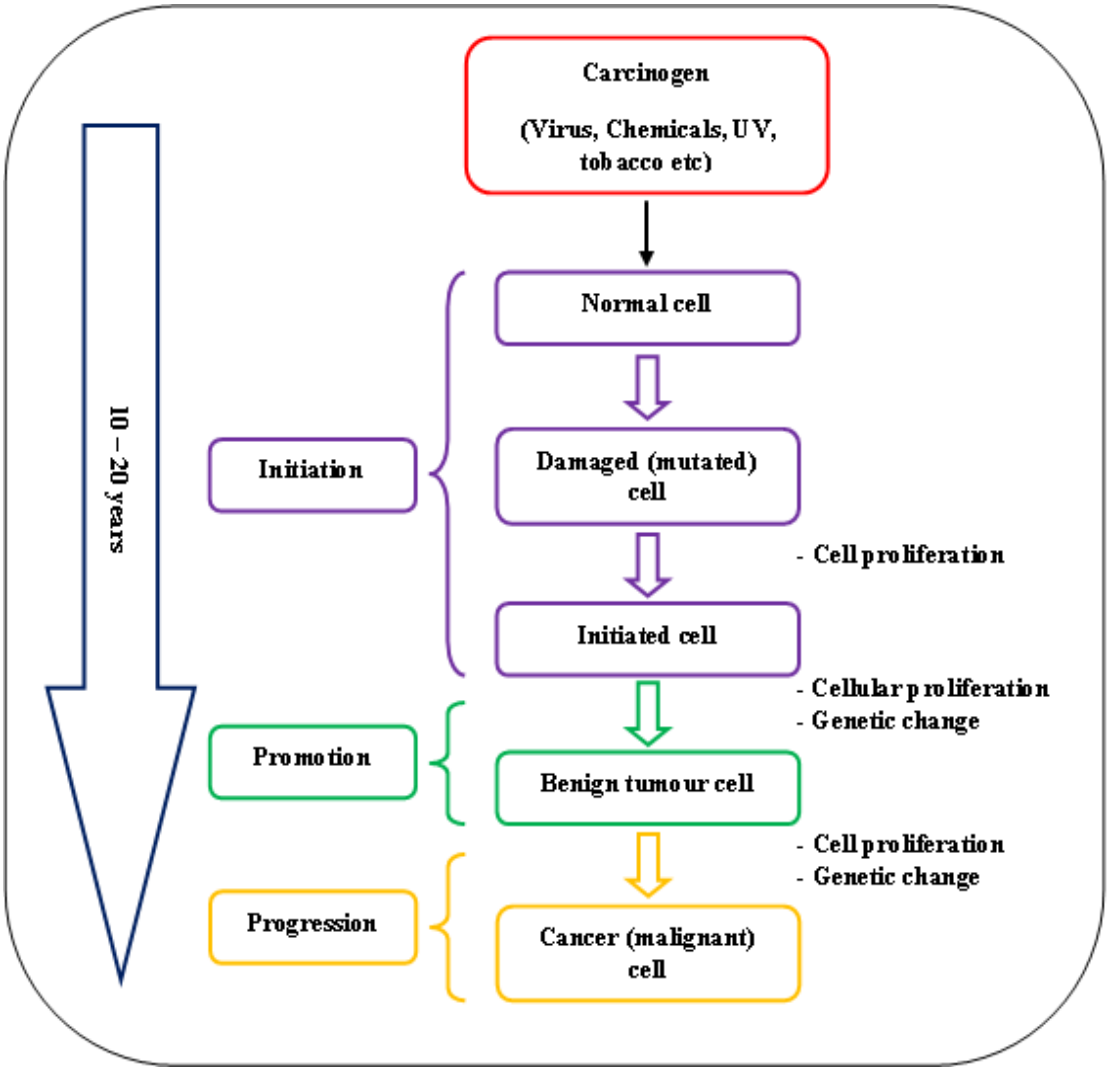

Figure 1.1: Schematic representative of carcinogenesis development (Tang & Sekaran, 2011).

1.1.3 Prostate Cancer

1.1.3.1 Anatomy and Physiology of Prostate Gland

The prostate gland is a compound tubuloalveolar exocrine gland of the male reproductive system, which is responsible for the production and storage of seminal fluids. These fluids are enriched with zinc, citric acid, choline, and various proteins and hormones that provide a protective medium for sperms as they make their way through the vagina for fertilization. Without this protective medium, most sperm would die soon after ejaculation. Before ejaculation, sperms will enter the vas deferens that is connected to the urethra, a tube that extends from the bladder to the tip of penis and builds the opening for semen and urine. During ejaculation, the muscular contractions in the prostate gland help to expel the semen out of the body through the urethra. It also produces a protein known as prostate-specific antigen (PSA) that turns the semen into liquid. The normal adult human prostate is about the size of a walnut and is located at the neck of the urinary bladder and surrounds part of the urethra (National Cancer Institute, 2009). Within the prostate, there is a prostatic urethra, an extension of the urethra coming from the bladder, and merges with the two ejaculatory ducts.

The prostate gland is classified in two different ways, by lobe or by zone (National Cancer Institute, 2009). The zone classification is often used in medicine so that physicians are able to classify the complications based on the different zones in the prostate and hence able to deliver proper treatment. In zone classification, the prostate gland is divided into four zones. The outermost zone is called peripheral zone (PZ) and it covers about 70% of the normal prostate gland in an adult. Most of prostate cancers (70-80%) originate from this zone. The central zone (CZ) covers 25% of the normal gland and surrounds the ejaculatory ducts. About 2.5% of prostate cancers originate from this zone and tend to be more aggressive and likely to invade into the seminal

vesicle. The third zone known as transition zone (TZ) covers 5% of normal prostate volume and this region is associated with prostate enlargement problems. The last zone known as anterior fibro-muscular zone or stroma, is devoid of any glandular parts but composed mainly of muscles and fibrous tissues.

In anatomy, prostate gland is divided into four lobes; anterior lobe, posterior lobe, lateral lobes and median lobe (National Cancer Institute, 2009). Anterior lobe refers to the anterior portion of the gland lying in front of the urethra. This lobe lacks glandular tissue and completely composed of fibromuscular tissue. Median lobe is a cone-shaped portion of the gland situated between the two ejaculatory ducts and the urethra. The lateral lobes form the main mass of the gland and are continuous posteriorly. There are two lobes (right and left lobes) separated by the prostatic urethra. The last part is the posterior lobe, which is used by anatomists to describe the posteromedial part of the lateral lobes that can be palpated through the rectum during a digital rectal exam (DRE).

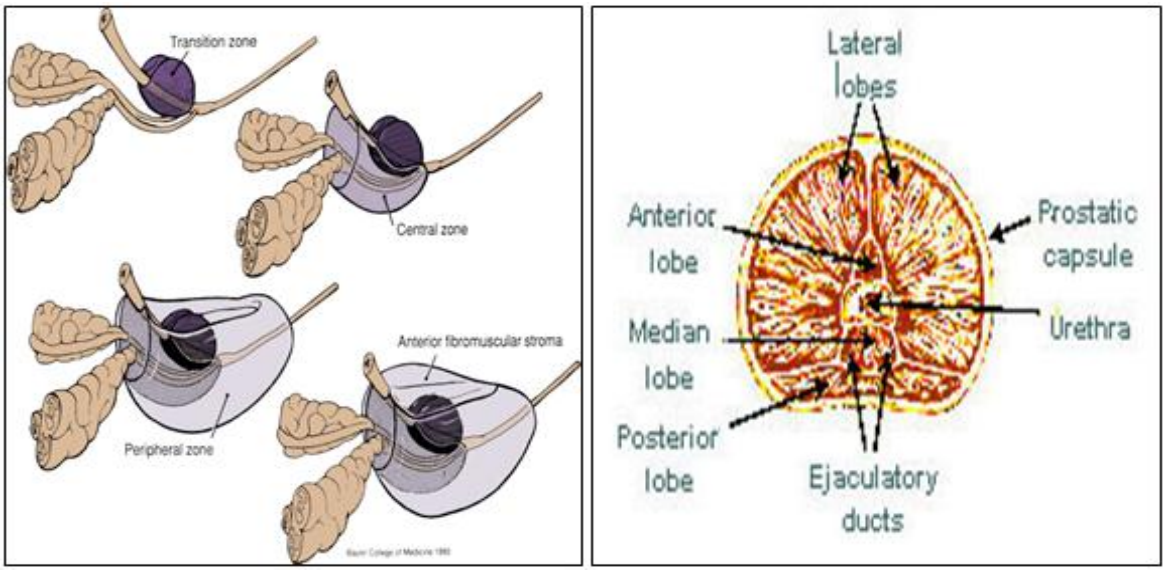

Figure 1.2: Zones (left) and lobes (right) classifications of prostate gland (National Cancer Institute, 2009).

1.1.3.2 Prostate Cancer

In 2008, according to GLOBOCAN report, there were about 12.7 million newly diagnosed cancer cases with 7.6 million cancer deaths (Ferlay *et al.*, 2010). Prostate cancer is the second most frequently diagnosed cancer after lung cancer. The incidence and mortality rates of prostate cancer are increasing in Asia as well as in the United States over the past few decades (Ferlay *et al.*, 2010), while in Malaysia, prostate cancer is the 6th most common cancer among men (Hew, 2012).

Prostate cancer, also knowns as adenocarcinoma, develops when the semen-secreting prostate gland cells are transformed into cancer cells. The schematic representation in Figure 1.3 shows the evolution of prostate cancer which starts with alterations at the genetic level, followed by a series of molecular changes, eventually resulting in an immortal cancer cell. The accumulation of genetic and molecular changes over the years allows visible histopathology of cancer cells. The malignancy of prostate tumour is a slow-growing process, and hence likely to be detected among the elderly. Prostate tumours can only be detectable when they reach relatively larger in size (0.3 cm^3) by screening of prostate-specific antigen (PSA), or via digital rectal exam (DRE) and/or transrectal echography of the prostate. It is important to mention that when diagnosis has become possible by screening, approximately 60% of the prostate cancers have metastasized outside of the prostate and are no longer organ confined (Labrie *et al.*, 2000; National Cancer Institute, 2009).

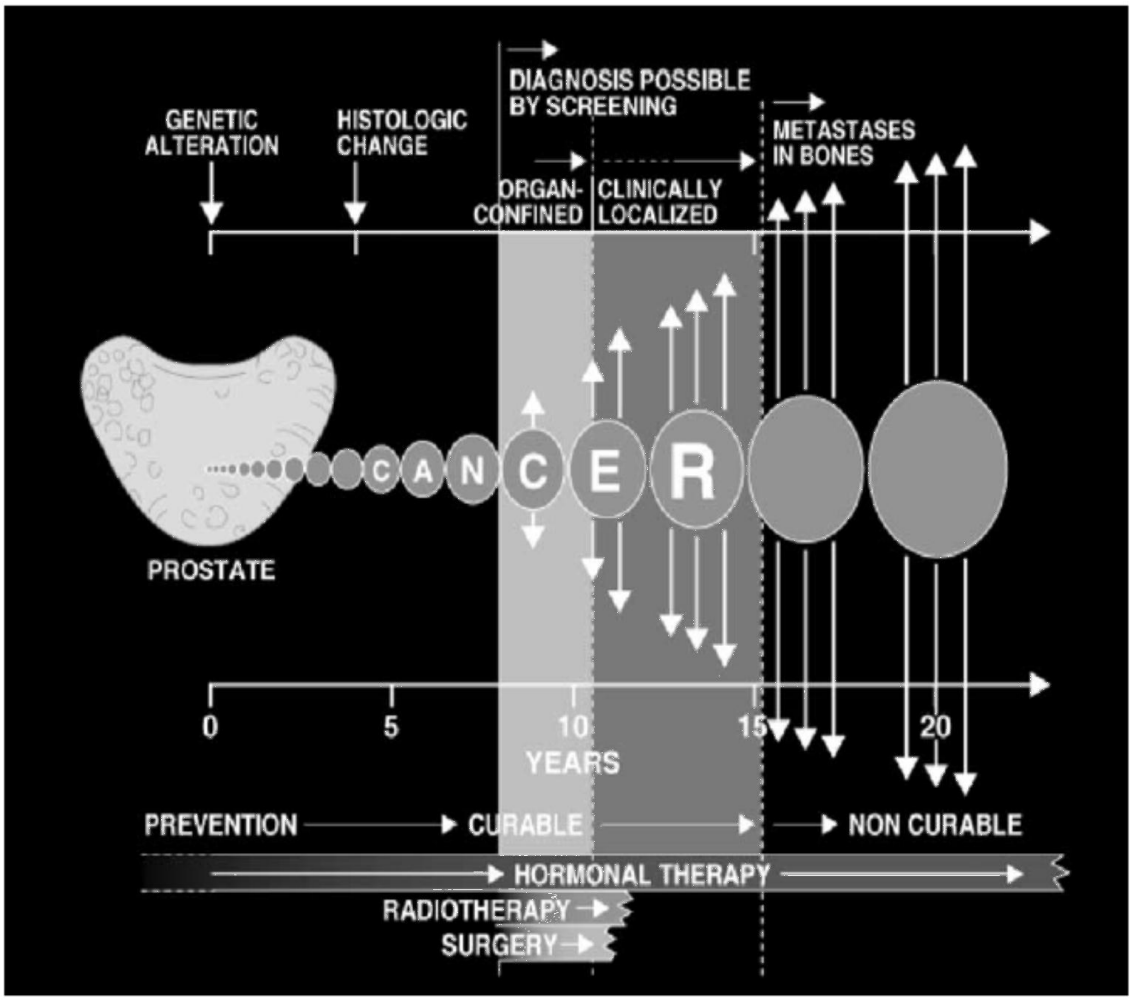

Figure 1.3: Schematic representation of prostate cancer progression. Depending upon the genes involved, the degree of prostate cancer growth is variable between individuals and the scale shown is an estimated average. Adapted from (Labrie *et al.*, 2000).

1.1.3.3 Symptoms and Diagnosis

The high mortality rate in prostate cancer patients is due to late detection as prostate cancer is usually asymptomatic or the symptoms appear only during the advanced stage of disease. Patients will experience difficulties during urination such as nocturia, hematuria, dysuria, pain, and may also have limited sexual functions and performance. The presence of pain in one or more bones such as in flares in the pelvis, the lower back, the hips, or the upper legs; may indicate the occurrence of metastases. In rare cases, patient may experience significant unexplained weight loss and fatigue. About 50% of prostate cancer patients are usually diagnosed with bone metastasis (National Cancer Institute, 2009).

There are several methods to diagnose prostate cancer in male patients, such as, detection of prostate tumour marker, prostate specific antigen, transrectal ultrasonography, digital rectal examination and biopsy (National Cancer Institute, 2009). Prostate-specific antigen (PSA) is a specific protein produced by prostate gland. PSA occurs in the serum of healthy men is in small quantities, and therefore abnormally high levels of serum PSA indicate the presence of prostate cancer. Federal Drug Administration (FDA) has approved the screening of the PSA in conjunction with a digital rectal exam (DRE) in men for early detection. A digital rectal examamination (DRE) is to check for abnormal growth or enlargement of the prostate gland in men as presence of prostate tumour can be felt as a hard lump. Transrectal ultrasonography (TRUS) uses high energy ultrasound to provide qualitative as well as quantitative measurements of prostate tissue stiffness. Biopsy is the removal small pieces of prostate tissue through transrectal (rectum) or transperinal (between scrotum and rectum) for microscopic examination by urologists and oncologists.

1.1.3.4 Stages and Treatments

It is important to determine the stage of prostate tumour in order to choose and deliver the best modalities of treatment to patientw. There is a differential response to treatment in the different stages of prostate cancer. Several tests will be conducted in order to determine the stage of prostate tumour in patients, that includes CT (computerised tomography) scan, MRI (magnetic resonance imaging), PSA (prostate-specific antigen) test and tumour biopsy (National Cancer Institute, 2009).

There are four stages in prostate tumour (Figure 1.4) (National Cancer Institute, 2009). Stage I is where cancer cells are found in prostate gland with low PSA levels (<10 ng/ml). In stage II, PSA levels are between 10-20 ng/ml and it is further subdivided into two groups; IIA and IIB. In stage IIA, cancer cells may be found in one-half or less than one lobe, but in stage IIB, cancer cells may be seen in both lobes of the prostate gland. In stage III, cancer could spread beyond the outer layer of prostate gland and may spread to the seminal vesicles with PSA values ranging from 2-10 ng/ml. In stage IV, the PSA can be any level because cancer has spread to nearby or other organs such as rectum, bladder and bone.

Currently, there are four standard treatments for prostate cancer; watchful waiting, surgery, radiation therapy and hormone therapy. In watchful waiting, a patient will be closely monitored by doctors and no treatment will be given until symptoms start to appear. While in surgery, the prostate gland from patients will be removed before the cancer cells spreads to organs in the vicinity. In radiation therapy, high energy X-rays will be used to kill cancer cells. Antiandrogens, estrogens and luteinizing hormone-releasing hormone agonists, are the few examples used in hormone therapy to stop the growth of cancer cells. Other treatments are includes chemotherapy and proton-beam radiation therapy (National Cancer Institute, 2009).

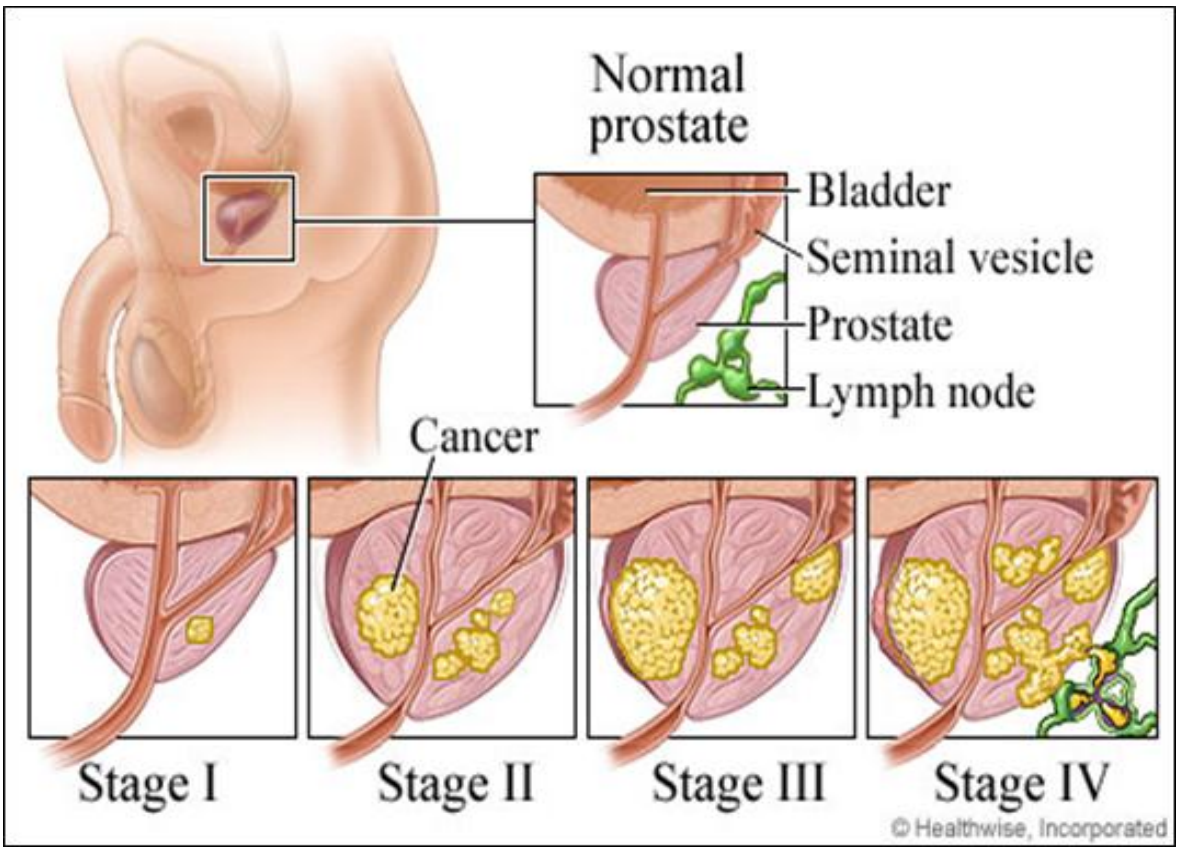

Figure 1.4: Stages of prostate cancer (National Cancer Institute, 2009).

1.1.4 Malignant Melanoma

1.1.4.1 Anatomy and Organization of Human Skin

The skin is the soft outermost covering of the human body. In mammals, the skin is the largest organ of the integumentary system, composed of multiple layers of ectodermal tissue. The functions of skin includes protection of the underlying muscles, bones and internal organs, sensation, heat regulation, control of evaporation, excretion, absorption and water resistance (Miller & Mihm, 2006).

1.1.4.2 Skin Cancer (Nonmelanoma and Melanoma)

Skin cancer is the most commonly diagnosed type of cancer. There are three main types of skin cancers; basal cell carcinoma, squamous cell carcinoma, and malignant melanoma (Table 1.5) (National Cancer Institute, 2012). The less common skin cancers accounting for less than 1% of nonmelanoma skin cancers includes dermatofibrosarcoma protuberans, merkel cell carcinoma, kaposi's sarcoma, keratoacanthoma, sebaceous carcinomas, microcystic adnexal carcinoma, Pagets's disease of the breast, atypical fibroxanthoma, leimyosarcoma, and angiosarcoma (Skin Cancer Info Line, 2012). Each cancer is named based on the type of skin cell from which it arose.

Nonmelanoma skin cancer (basal cell carcinoma and squamous cell carcinoma) is the most common type of skin cancer, whereas melanoma is least common, but is the most aggressive and serious type of skin cancer. Basal cell carcinoma presents as a raised, smooth, pearly bump on the sun-exposed skin of the head, neck, back, chest, or shoulders. It is considered as the least deadly form when compared to others skin cancers. Squamous cell carcinoma is the second most common skin cancer and appears

as red, scaling, thickened patch on sun-exposed skin and ulceration and bleeding may occur.

Melanoma is a tumour arising from the melanocyte, which are melanin-producing cells located in the basal layer of the epidermis (Figure 1.6). The main function of this cell is to produce melanin through a process called melanogenesis. Melanin provides protection to the hypodermis from ultraviolet rays (DNA photodamage). Fair-skinned populations are more susceptible to melanoma development as compared to dark-skinned populations due to the low activity and number of melanocytes in their skin. Malignant melanoma is an aggressive and life-threatening skin cancer because it has a high tendency to metastasize to other parts of the human body. In most of the cases, melanomas are typically pigmented (having a brown or dark colour), but in rare cases, this pigment can be flesh-coloured and this is known as amelanotic melanoma (Miller & Mihm, 2006).

Most of the melanoma cases start from an uncontrolled proliferation of melanocytes into benign nevus, or mole, in the human epidermis skin layer (Figure 1.5D). These pre-existing benign nevi will continue to progress into dysplastic nervus. However, there are exceptions where some melanomas will begin from dysplastic nevus, which are not really invasive malignancies, but rather just moles that have some aberrant growth. A radial growth phase melanoma is where the melanoma starts to grow through the epidermis into the upper region of dermis, where blood vessel and lymphatics are located. The melanoma continues to progress in a vertical growth phase and spreads deeper into the dermis and starts invading the blood vessels and/or lymphatic systems. Eventually, the metastasized melanoma forms a secondary tumour at a distant site in the body (lung, liver or brain) (Miller & Mihm, 2006).

The American Cancer Society has estimated 76,690 individuals will be diagnosed with melanoma with 9,480 expected deaths in 2013 (Siegel *et al.*, 2013). Australia and New Zealand have the highest incidence rate of skin cancer in the world (Cancer Council Australia, 2012). In 2010, there were 12,818 new cases of malignant melanoma diagnosed in the United Kingdom alone (Cancer Reserach UK, 2012).

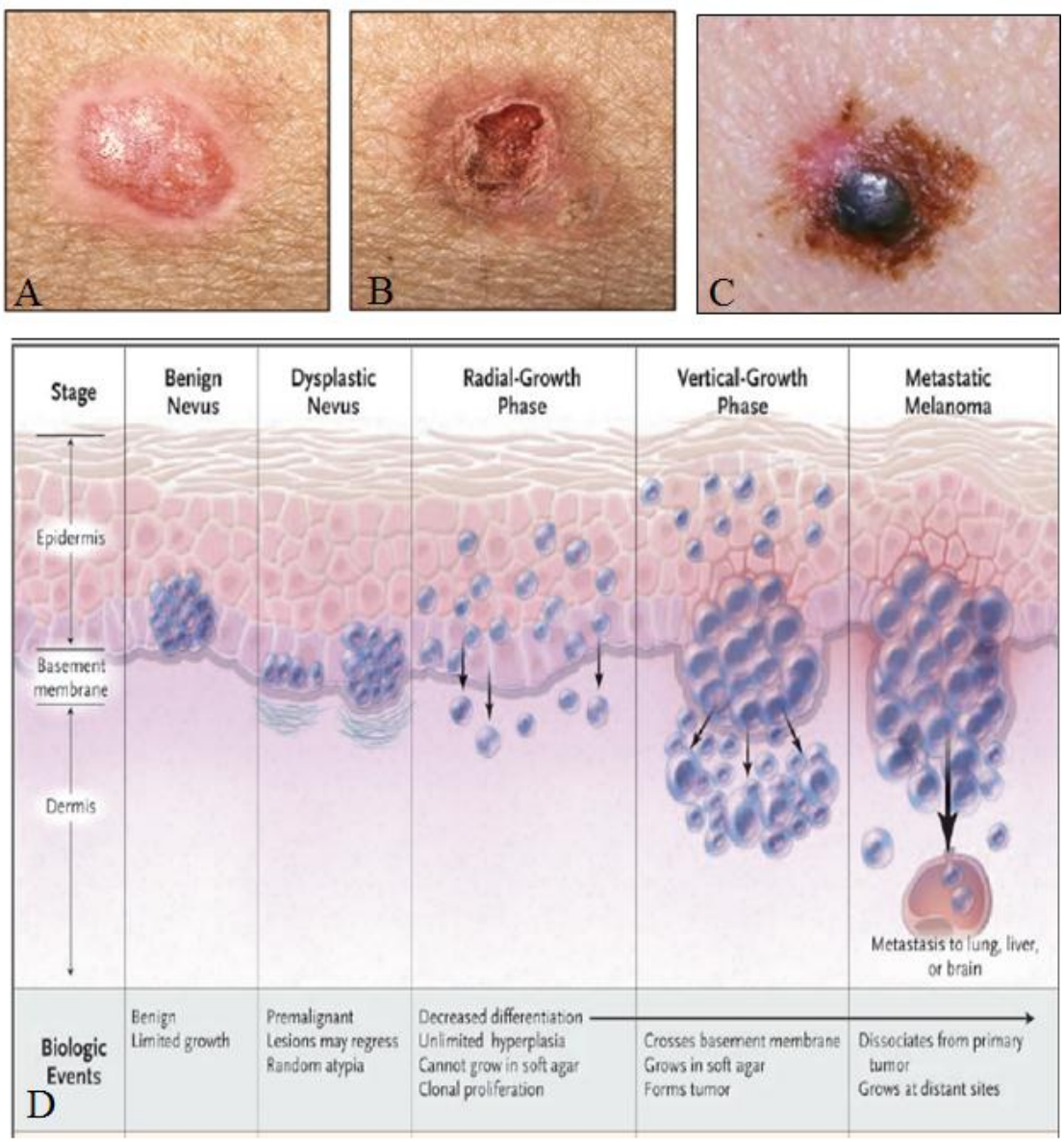

Figure 1.5: Three most common types of skin cancer; (A) Basal cell carcinoma (B) Squamous cell carcinoma (Romito & Burr, 2011), and (C) Malignant melanoma (Skin Cancer Specialists, 2012). (D) Progression of malignant melanoma, adapted from (Miller & Mihm, 2006).

1.1.4.3 Types of Melanoma

In general, melanomas are categorized based on their clinical characteristics and histologic at features. Melanoma can be divided into two main categories, *in situ* and invasive. *In situ* melanoma is the least dangerous type of melanoma. *In situ* melanoma rarely spread and is curable if removed early with a 5 mm margin of normal skin. However, invasive melanomas are not confined only to the epidermis, and are able to grow into the dermis layer of the skin. The deeper the growth of melanoma into the dermis, the greater the risk that it may spread to distant sites as there are blood vessels and lymphatic systems, which aids melanoma to metastasize. There are four basic types of invasive melanoma, which differ in occurrence and distribution in the human body; superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALM) (de Braud *et al.*, 2003) (Figure 1.6).

Superficial spreading melanoma (SSM) is the most common form of cutaneous melanoma accounting for about 70% of all diagnosed melanoma cases. It may occur at any age and body site. It usually appears flat, and is irregular in shape and colour, with various shades of black and brown. SSM is characterized by a noticeable intraepidermal proliferation (Lemon & Burns, 1998; de Braud *et al.*, 2003). It exhibits a slow-growing radial growth pattern that may progress into a nodular appearance. The regressions of patches are frequently seen in an amelanotic area. It then becomes an asymmetrically raised patch with sculpted edges and is irregularly pigmented with colours varying from pale blue and pink to mottled brown-black variegation, sometimes completely black (de Braud *et al.*, 2003).

Nodular melanoma (NM) is the most aggressive type of melanoma and accounts for 15 to 30% of all diagnosed melanoma patients (de Braud *et al.*, 2003). NMs are

more common in men and often seen in non-weight-bearing areas such as trunk, head and neck regions. It appears as a nodule with sharply demarcated borders on the skin, often shiny with a slightly infiltrated base. The colour of NM is generally darkly pigmented. However, some can be light brown or even colourless (non-pigmented) and more uniform than SSM (Sagebiel, 1993). These lesions are characterized by a relatively rapid vertical growth phase, which is capable of growing more rapidly in thickness (penetrate into the skin) rather than growing radially (growth in diameter to form a peduncle). It usually has a well-defined border as compared to superficial SSM (Sagebiel, 1993; Lemon & Burns, 1998). Ulceration and bleeding may occur in NM patients.

Lentigo maligna melanoma (LMM) accounts for approximately 5% of all melanoma cases (Weinstock & Sober, 2006). It is often diagnosed in elderly patients who are more than 50 years of age and appears in sun-exposed areas such as the face, forearms, head and neck. LLM arises from a pre-existing lentigo with irregular edges, rather than a mole. The colour of the lesion varies between dark to brown-black variegation (Weinstock & Sober, 2006). The growth pattern of LLM is more radial than other types of melanomas, remain *in situ* for up to approximately 20 years, thus resulting in decreased incidence of metastasis (Lemon & Burns, 1998). However, this horizontal non-invasive growth phase may divert to vertical growth. The vertical growth phase usually involves spindle-like cells invading into the reticular dermis surrounded by fibrotic stroma (dermoplastic) or may form fascicles displaying neural features infiltrating the perineural structures of the skin (de Braud *et al.*, 2003).

Acral lentiginous melanoma accounts for less than 5% of all diagnosed melanomas. As its name dictated, it mainly occurs on non-hairy skin of the acra (palm, sole, nailbed), and generally appears as a plantar lesion on the weight-bearing surface of the foot (Piliang, 2009). The appearance of this lesion may be vary due to the skin

thickness at these sites (de Braud *et al.*, 2003). This is the most common type of melanoma seen in dark-skinned people (African Americans, Hispanics, and Asians), and rarely occurs in white people (de Braud *et al.*, 2003; Piliang, 2009). This aggressive type of melanoma has the ability to metastasize much like nodular melanomas (Lemon & Burns, 1998).

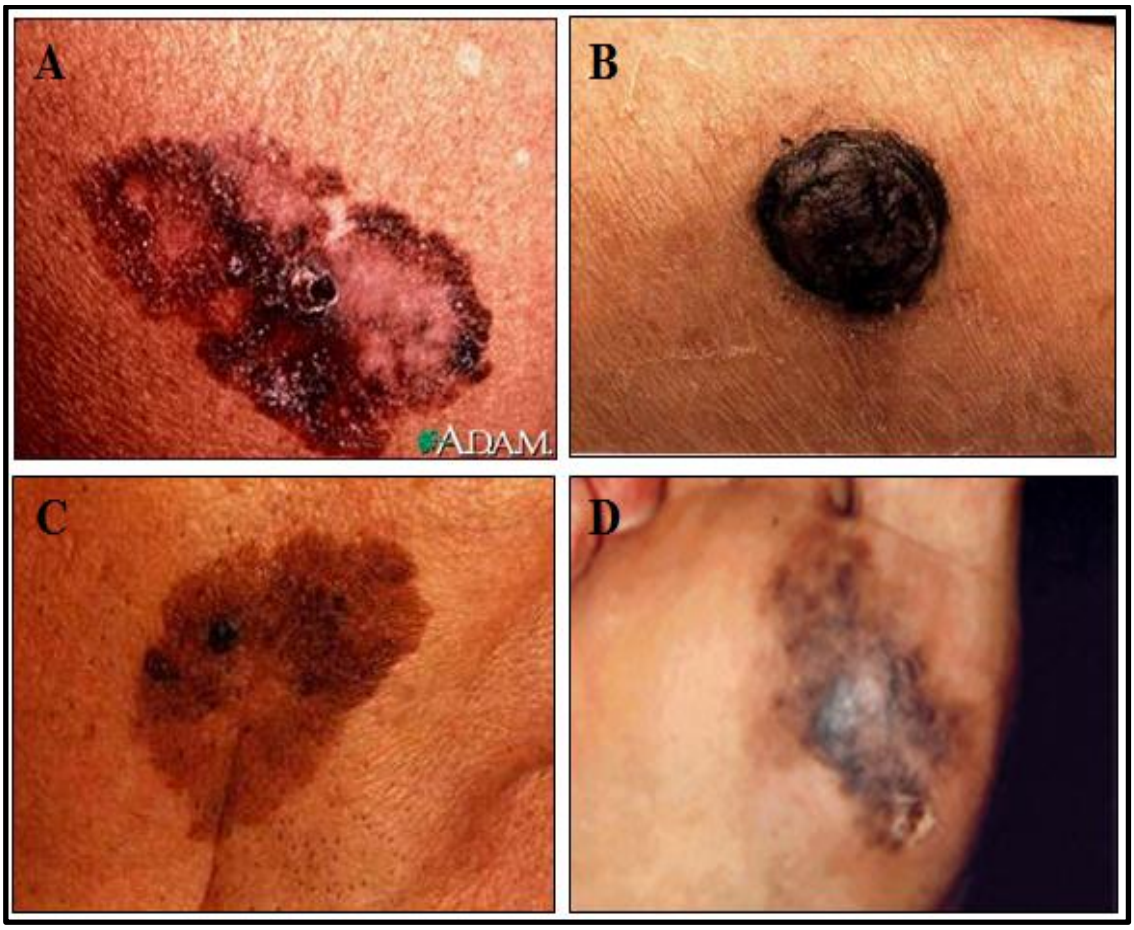

Figure 1.6: Types of invasive melanoma: (A) superficial spreading melanoma (Lehrer, 2011) , (B) nodular melanoma (Gupta, 2012), (C) lentigo maligna melanoma (Oakley, 2012), and (D) acral lentiginous melanoma (Swetter *et al.*, 2004).

1.1.4.4 Diagnosis

Malignant melanoma is diagnosed according to its pigmentation. “A-B-C-D-E” acronym is a guideline used in the identification of pigmented lesions evaluated with biopsy (Figure 1.7) (Abbasi *et al.*, 2004). Asymmetry (A) is where two halves of median line through a mole is not a match of each other. Border (B) is where the borders of an early melanoma tend to be irregular or uneven. The edges may be scalloped or notched. Having a variety of colour (C) is another warning signal for melanoma. The mole or lesion is not one uniform colour but rather a combination ranging from light brown to dark brown, black, red, blue or white. Diameter (D) of melanoma usually greater than 1/4 inch or 6 mm. Evolution (E) refers to any noticeable change occurring in a mole or lesion over time regardless of size, shape, colour, elevation, or any new symptoms such as bleeding, itching or crusting (Bono *et al.*, 1999).

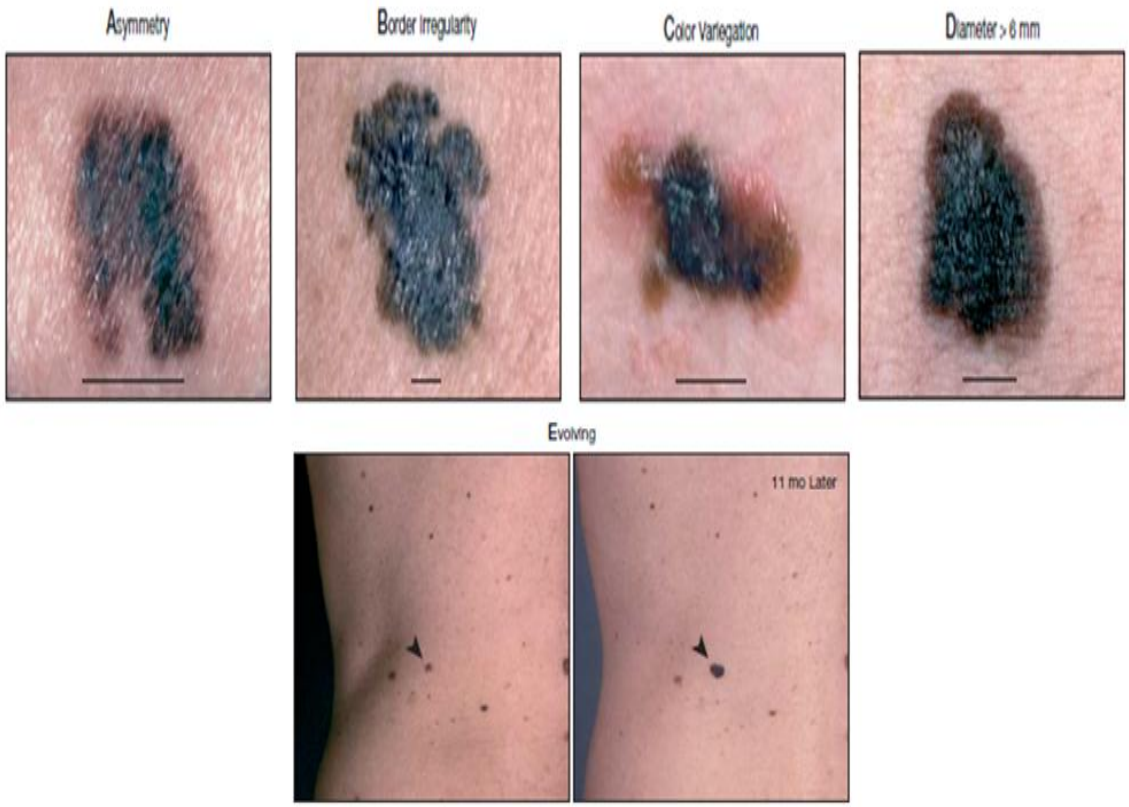

Figure 1.7: Diagnosis of melanoma according "A-B-C-D-E" acronym (Abbasi *et al.*, 2004).

1.1.4.4.1 Stages and Treatments

It is important to determine the depth of melanoma growth into the skin and its degree of metastasis. This information is important to determine the appropriate treatment to be delivered, risk of reoccurrence, and the ability to metastasize into the nearby lymph nodes. Most melanomas occurring at an early stage have high cure rates. However, the effectiveness of treatment is diminished in advanced melanoma and has a 50% survival rate, ranging from 2 to 8 months with only 5% generally surviving for 5 years or more (Jemal *et al.*, 2011).

The American Joint Committee on Cancer (AJCC) TNM classification is the most often classification for melanoma staging. TNM classification is based on the size of primary tumour (T), number of regional lymph nodes (N) and presence or absence of

distant metastases (M) (Balch *et al.*, 2009). The T stage is based on vertical thickness of the lesions in millimeters (Breslow's thickness) and the anatomic level of invasion into the dermal layers of skin and subcutaneous fat (Clark's classification). The N stage is determined by the distance of metastasized melanoma to nearby lymph nodes. The M stage is simply based on the presence or absence of metastasis to distant sites.

Melanoma is further grouped into different stages according to the revised TNM staging system. The combinations of T, N, and M groups will give an overall stage, using Roman numerals I to IV (Table 1.1). This process is known as stage grouping (Kim *et al.*, 2002; Balch *et al.*, 2009). In general, the patients with lower stage cancers have high chances of cure. Therefore, correct staging is important because best modalities of treatment to be delivered to patient are generally based on this parameter.

Table 1:1: Treatment of melanoma according stages (Melanoma Institute Australia, 2012; National Cancer Institute, 2012; Sosman, 2012).

Stages of Melanoma	**Description**	**Treatments**
Stage 0 (in situ)	• Appears as abnormal discoloured or dark coloured mole. • Only in the epidermis and has not spread into dermis layer	• Surgery, or • Cream on sensitive areas on the face
Stage I	• Stage IA: thickness is less or about 1 mm, with no ulceration and no mitoses • Stage IB: thickness is less or about 1 mm, with ulceration or mitoses	• Surgery (depends on the thickness of the melanoma)

Stage II	• Stage IIA:
	  • thickness is 1 - 2 mm with ulceration or thickness is 2 - 4 mm with no ulceration
	  • No spread into nearby lymph nodes or distant metastasis
	
	• Stage IIB:
	  • thickness is 2 - 4 mm with ulceration or greater than thickness 4 mm with no ulceration
	  • No spread into nearby lymph nodes or distant metastasis
	• Stage IIC:
	  • thickness is greater than thickness 4 mm with ulceration
	  • No spread into nearby lymph nodes or distant metastasis
		• Surgery, or
		• Adjuvant therapy with interferon after surgery

Stage III	• Stage IIIA:
	  • thickness ranges from less that 1 mm to more than 4 mm with no ulceration
	  • Micrometastasis into 1 – 3 nearby lymph nodes
	  • No distant metastasis
	
	• Stage IIIB:
	  • thickness is ranging from less
		• Surgery
		• Lymph node dissection,
		• Adjuvant therapy (radiation therapy) with interferon after surgery
		• Injections with Bacille Calmette-Guerin (BCG) vaccine or interleukin-2 directly into the melanoma or

	that 1 mm to more than 4 mm with or without ulceration • Macrometastasis into 1 – 2 nearby lymph nodes • No distant metastasis • Stage IIIC: • thickness is ranging from less that 1 mm to more than 4 mm with or without ulceration • Macrometastasis into 1 – 2 nearby lymph nodes • Metastasis in 4 or more lymph nodes and present of matted lymph nodes • No distant metastasis	• Topical immunotherapy imiquimod • Chemotherapy, immunotherapy with cytokines, or both combined (biochemotherapy).
Stage IV	• Melanoma has metastasized into other organs such as the liver, lungs and brain. • High levels of LDH	• Surgery • Immunotherapy drugs (Ipilimumab, interferon or interleukin-2), and • Chemotherapy drugs (Dacarbazine, temozolomide) • Radiation therapy

There are four standard treatments for melanoma; surgery, chemotherapy, immunotherapy and radiation therapy (Table 1.1). Surgery is the usual recommended treatment of choice for malignant melanomas (Lemon & Burns, 1998). If melanomas are detected at an early stage, surgical excision itself is sufficient with lower chances of reoccurrence (Garbe *et al.*, 2008). Patients with intermediate thickness of melanomas (0.76-4.00 mm) who received wide excision and elective lymph node dissection (ELND) usually have a significantly higher survival rate than those who received wide excision alone (Krag *et al.*, 1995; Balch *et al.*, 1996; Agnese *et al.*, 2003). ELND is the removal of all lymph nodes from the area surrounding the primary melanoma (Balch *et al.*, 1996).

Chemotherapeutic agents have been widely used in the treatment of different types of cancer including malignant melanoma. Chemotherapeutic agents that have shown anticancer activities against malignant melanoma include dacarbazine, cisplatin, carboplatin, temozolomide, vinca alkaloids, platinum compounds, nitrosoureas and taxanes (Cocconi *et al.*, 1992; Grossman & Altieri, 2001; Mouawad *et al.*, 2010). The first anticancer agent for malignant melanoma treatment approved by FDA was dacarbazine with the highest response rate compared to other drugs (Cocconi *et al.*, 1992). Polytherapy or combination therapy has been evaluated to improve the efficiency of chemotherapeutic agents (Sosman, 2012). The most widely used combinations including (i) cisplatin, vinblastine and dacarbazine; (ii) cisplatin, dacarbazine, carmustine and tamoxifen; (iii) bleomycin, vincristine, carmustine and dacarbazine; and (iv) carmustine, hydroxyurea and dacarbazine (Cocconi *et al.*, 1992; Jilaveanu *et al.*, 2009; Mouawad *et al.*, 2010).

Immunotherapy is a form of treatment that directly or indirectly, uses the host immune responses to fight cancer cells in patients (National Cancer Institute, 2012). Interferon alpha (IFN-α) has shown moderate activity against stage IV melanoma with 10% to 22% overall response rates (Chowdhury *et al.*, 1999; Jilaveanu *et al.*, 2009). In addition, IFN-α also possesses immunomodulatory effects to enhance the functions of natural killer cells, macrophages and T-lymphocyte functions as well as inhibit angiogenesis (Chowdhury *et al.*, 1999; Mouawad *et al.*, 2010). Another immunotherapeutic agent is interleukin-2 (IL-2), and has been approved by the FDA for therapy of patients with advanced stage melanoma. It acts through a variety of mechanisms including activation of cytotoxic T-cells and natural killer cells as well as production of other cytokines (Enk *et al.*, 1998; Chowdhury *et al.*, 1999; Atkins *et al.*, 2000). However, the application of these immunotherapeutic agents is limited due to their high toxicity. Therefore, these biological agents have been used in smaller doses in

combination with chemotherapy (biochemotherapy) to increase efficacy and to reduce toxicity (Yee *et al.*, 2000; Jilaveanu *et al.*, 2009).

1.2 Hallmarks of Cancer

The hallmarks of cancer is comprised of at least eight biological capabilities acquired during the multistep development of human tumours (Hanahan & Weinberg, 2011). Each of these hallmarks is derived upon changes in the normal cell's physiology and interacts with each other to promote malignant growth. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. This includes sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and metastasis, reprogramming of energy metabolism and evading immune destruction.

1.2.1 Sustaining Proliferative Signalling

Normal cells are carefully monitored for the production and release of growth-promoting signals to ensure a balance or homeostasis between cell numbers and cell death. Generally, growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF) and tumour necrosis factor alpha (TNF-α), binds to specific receptors on the surface of their target cells, typically containing intracellular tyrosine kinases. This engagement transmits signals via branched intracellular signalling pathways to regulate progression of cell cycle as well as cell growth (Aaronson, 1991). The conversion of a normal cell to a transformed cell usually starts from mutations in DNA, which causes the cells to no longer depend on growth signals, thus promoting uncontrolled growth and proliferation. (Hanahan & Weinberg, 2011).

There are several alternatives by which cancer cells acquire the ability to sustain proliferative signalling: (1) autocrine proliferative stimulation where cancer cells

produce their own growth factors to which they respond in autocrine manner via expression of cognate receptors (Lippman *et al.*, 1986; Iihara *et al.*, 2006), (2) paracrine proliferative stimulation where cancer cells may send signals to stimulate normal cells in its vicinity to produce various growth factors required for cancer cells (Bhowmick *et al.*, 2004; Cheng *et al.*, 2008), (3) increases the number of cell surface receptor at the cancer cell to hyperrespond to growth factors, or (4) structural alteration in the surface receptors of cancer cells to mediate ligand-independent response (Hanahan & Weinberg, 2011).

There are various cellular signalling pathways in a cell that are interconnected to form complex networks (Hanahan & Weinberg, 2011). A cell will integrate all information from each signal to regulate diverse functions, such as protein synthesis and cell growth, motility, differentiation, and cell death. The ability of intracellular signalling networks to integrate and distribute regulatory information requires a signalling molecule to respond to multiple inputs and output signals. However, the same signalling molecules will control different processes within different signalling complexes or at different intracellular locations. In addition, in different cell types, the activation of a signalling molecule may also have distinct consequences (Martin, 2003; Bazigou & Rallis, 2007).

During tumour progression, cancer cells acquire a number of alterations at both the cellular and genetic levels. All cancer cells derive an advantage by either dysregulating, upregulating, or deregulating signalling pathways that confer these cells the capacities to grow independently of growth and anti-growth signals, metastasis, initiate an angiogenetic response, and evade from apoptosis (Bazigou & Rallis, 2007).

1.2.1.1 Mitogen Activated Protein Kinase (MAPK) Signalling

Mitogen-activated protein kinase (MAPK) pathway contains conserved kinases that transmit extracellular signals to the host cell machinery that controls important cellular processes such as growth, proliferation, differentiation, migration and apoptosis. Three-tier kinases have been well characterized and widely studied in mammalian cells; a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK (Johnson and Lapadat, 2002). In addition, at least six members of the MAPK family have been discovered; extracellular signal-regulated kinase: ERK1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase/stress activated protein kinases (JNK1/2/3; also called SAPKs) with many splice variants (Fang & Richardson, 2005; Wagner & Nebreda, 2009; Rodríguez-Berriguete *et al.*, 2011) and the p38 kinases (p38 α/ß/γ/δ) (Olson & Hallahan, 2004; Wagner & Nebreda, 2009) In general, MAPK/ERK pathway is activated by binding of a growth factor to cell surface receptors, whereas the MAPK/JNK and p38 pathways are activated by stress, inflammatory cytokines and growth factors. Aberrant regulation of MAPK signalling contributes to development and progression of cancer including cancer cell proliferation, survival, migration and invasion (Fecher *et al.*, 2008).

The MAPK/ERK pathway is well-studied as its dysregulation can be found in approximately one-third of all human cancers (Figure 1.8). The biological consequences of active ERK pathway including the enhancement of cancer cells' proliferation, differentiation, angiogenesis, motility and invasiveness. Upon activation by the binding of growth factors such as TNF-α, the extracellular signal is transmitted to small GTP binding proteins (Ras), which in turn activates the kinase cascades; starting from MAPKKK (Raf) to MAPKK (MEK1/2), and lastly to MAPK (ERK). Cancer cells usually will create an autocrine feedback loop in promotion of Ras-mediated transformation and Raf-mediated gene expression changes. Therefore, an aberrant

activation of Ras and Raf proteins can be found in different types of human cancers (breast, prostate, lung, colon cancers, etc) (Hilger *et al.*, 2002; Gollob *et al.*, 2006; Roberts & Der, 2007).

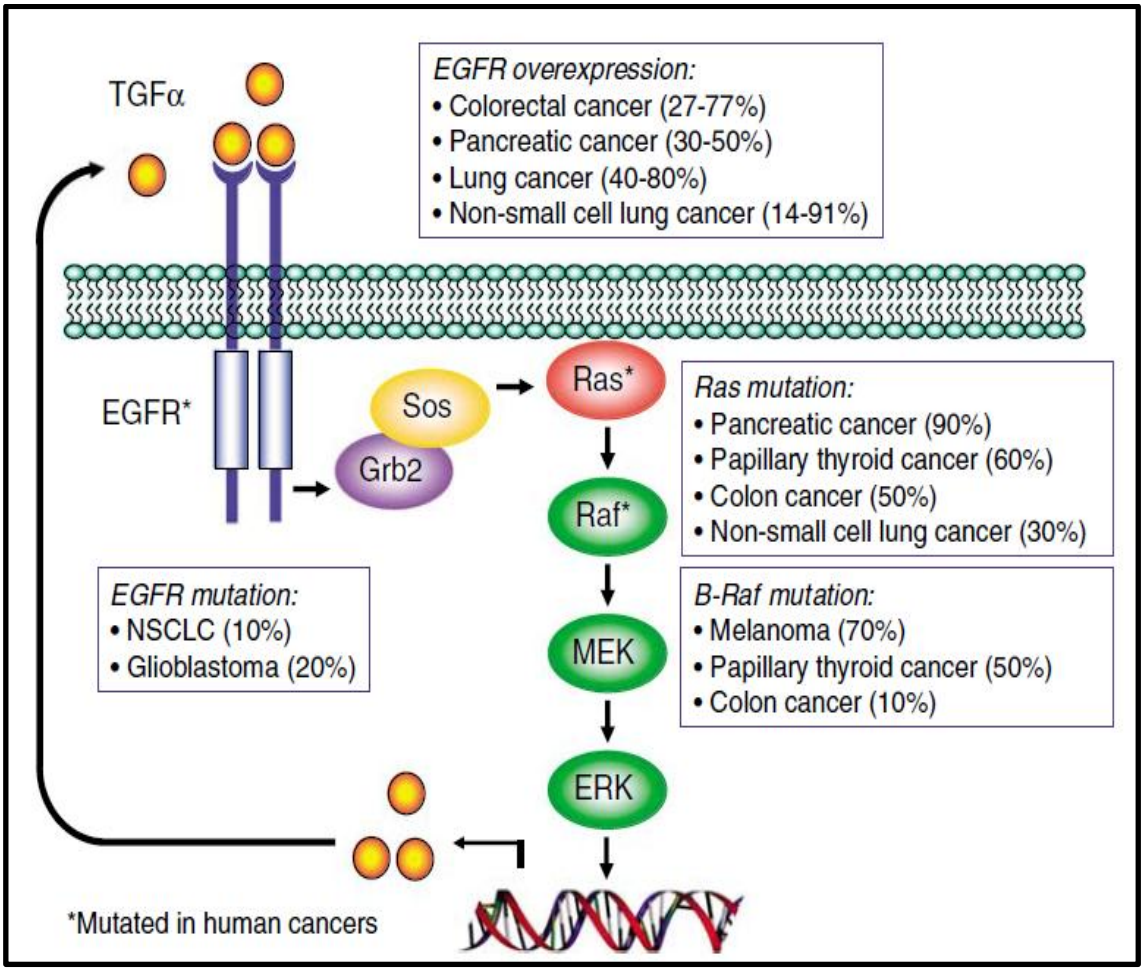

Figure 1.8: Oncogene activation of the MAPK/ERK cascade (Roberts & Der, 2007).

The JNK family of MAP kinases is mainly activated in response to diverse stimuli including inflammatory cytokines, ultraviolet (UV) and gamma radiation, growth factor deprivation, DNA-damaging (cytotoxic) agents, and certain G-protein coupled receptors (Figure 1.9) (Weston & Davis, 2002; Hui *et al.*, 2007). There are three JNK isoforms – JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously expressed in all types of cells, whereas JNK3 expression is only localized in brain, heart and testis. JNK activation requires dual phosphorylation on tyrosine and threonine residues by MEK4 and MEK7. Both MEK4 and MEK7 are activated by several MAPKKKs, including MEKK1–4, MLL2 and 3, YTpl-2, DLK, TAO1 and 2, TAK1 and ASK1 and 2. Once activated, JNK will be translocating from the cytoplasm into the nucleus where it regulates transcription factors including AP-1, Elk-1, c-myc, p53, as well as anti-apoptotic proteins such as Bcl-2 and 14-3-3 (Heasley & Han, 2006; Bode & Dong, 2007; Turjanski *et al.*, 2007). The JNK also plays a part in the regulation of p53 transcriptional activity and stability (Buschmann *et al.*, 2000; She *et al.*, 2002b; Wu, 2004).

Several studies showed an aberrant activation of JNK activity in various types of cancers including prostate, breast, pancreas and lung (Lin, 2002; Xu *et al.*, 2006; Wagner & Nebreda, 2009). The main aim of JNK signalling is the formation a complex known as activating protein-1 (AP-1) via combination of c-Jun and c-Fos proteins. This transcription factor controls cellular physiology including cell proliferation, apoptosis, differentiation and developmental processes. (Eferl & Wagner, 2003; Kennedy *et al.*, 2003). Several studies have shown that the deficiency of c-Jun protein in fibroblasts could inhibite transformation of normal cells to cancer cells (Dérijard *et al.*, 1994; Johnson *et al.*, 1996; She *et al.*, 2002a), highlighting the importance of JNK signalling pathway in the regulation of proliferation in tumourigenesis (Schreiber *et al.*, 1999; Eferl & Wagner, 2003). In addition, JNK has also been shown to promote cancer

metastasis by activating matrix metalloproteinases, MMP-2 and -9 (Hong *et al.*, 2005; Liang *et al.*, 2007). Therefore, aberrant activation of JNK/c-Jun/AP-1 is implicated in tumour development (Eferl & Wagner, 2003; Kennedy *et al.*, 2003).

In mammals, p38 isoforms are strongly activated by physical and chemical stress signals, oxidative stress, hypoxia, inflammatory cytokines, heat and osmotic shock (Figure 1.9). The four isoforms of p38, namely p38 α, β and γ and δ are characterized by the presence of the conserved Thr-Gly-Tyr (TGY) phosphorylation motifs in their activation loop with some differences in their functions (Kumar *et al.*, 2003). The expression of p38 α and p38 β are ubiquitous in all cells, whereas, p38γ and p38δ are confined in certain tissues such as muscle, testis, lung and kidney. These conserved motif is a target of phosphorylation by MEK3 and MEK6. However, in some cases, p38 can also be activated by MEK4, an activator kinase of JNK. Once activated, cytoplasmic p38 proteins will translocate into the nucleus where they regulate several genes including NFκB, Elk-1, p53 and Max, that are involved in apoptosis, cell cycle progression, growth and differentiation (Dhillon *et al.*, 2007).

Several studies have established that p38 plays an important role in the progression of leukemia, lymphoma, breast, prostate, gastric and lung cancers (Park *et al.*, 2003; Uzgare *et al.*, 2003; Olson & Hallahan, 2004; Hui *et al.*, 2007). The expression of upstream kinases (PAK1, MEK6, and MEK4) and p38 are found in prostate cancer cells (Park *et al.*, 2003; Uzgare *et al.*, 2003; Xu *et al.*, 2006). Besides, p38 plays a vital role in a hypoxic environment that increases survival, clonogenecity, migration and invasiveness of prostate cancer cells by increasing expression of matrix metalloproteinases (Uzgare *et al.*, 2003; Khandrika *et al.*, 2009; Kwon *et al.*, 2009).

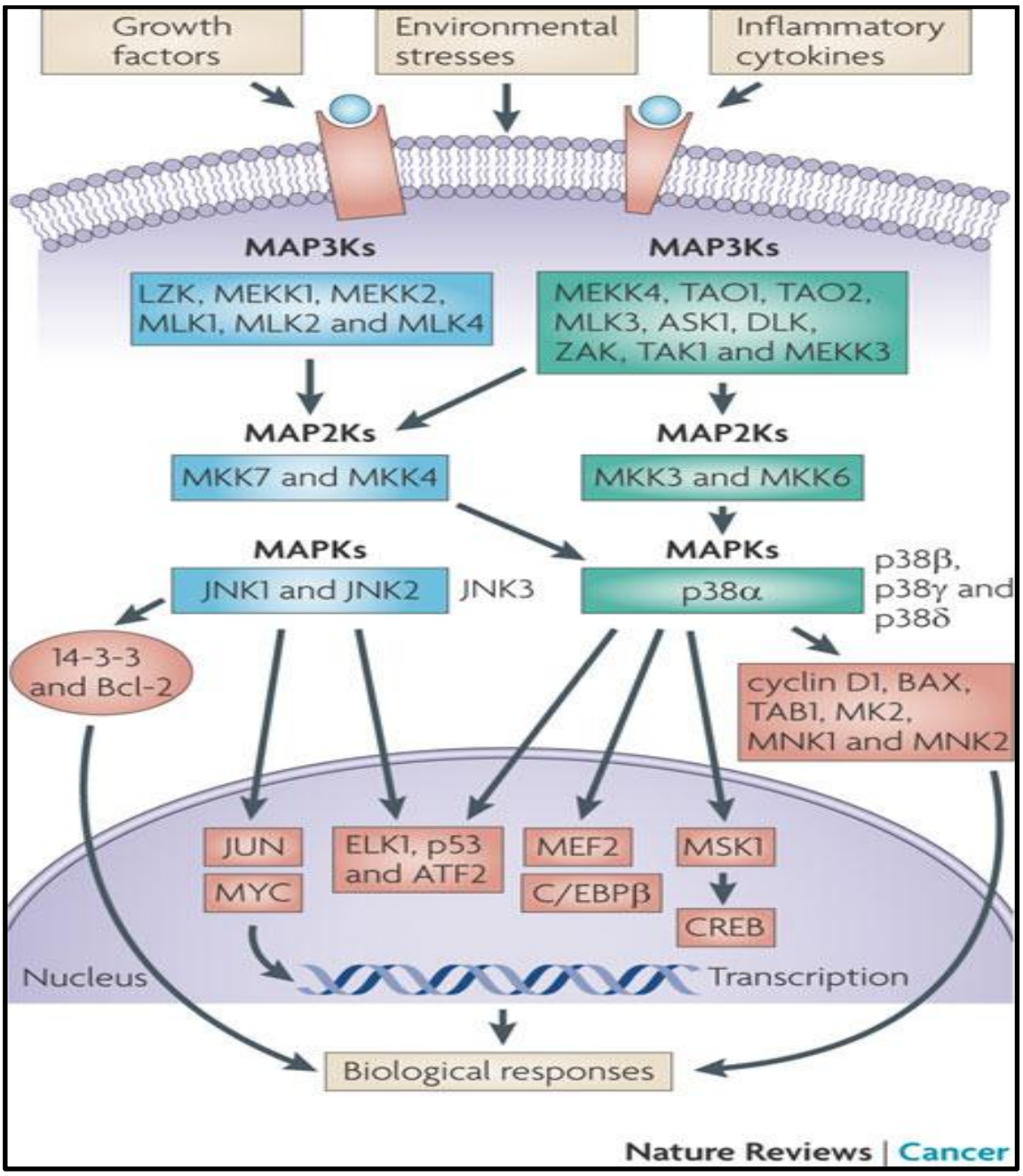

Figure 1.9: Overview of activation JNK and p38 MAPK pathways (Wagner & Nebreda, 2009).

1.2.1.2 Wnt Signalling

Wnt signalling pathway regulates cellular processes including proliferation, differentiation, motility, as well as survival and/or apoptosis. It plays an important role during embryogenesis and maintain homeostasis in mature tissues (Polakis, 2000). There are at least three distinct Wnt pathways: (1) the canonical (β-catenin) pathway, which activates target genes in the nucleus; (2) non-canonical (planar cell polarity, PCP) pathway, which involves JNK and cytoskeletal rearrangements; and (3) Wnt/Ca^{2+} pathway (Kolligs *et al.*, 2002; Polakis, 2007) (Figure 1.10).

In the canonical Wnt pathway, the binding of Wnt ligand to receptor complex comprised of Frizzled/low-density lipoprotein receptor-related protein (FZ/LLP) and cytoplasmic disheveled (Dsh) to stabilize cytoplasmic β-catenin through the inhibition of the β-catenin degradation complex (Figure 1.10). This binding will leads to activation of Dsh protein and in turn, inhibits glycogen synthase kinase 3β (GSK3β) from phosphorylating its substrates such as axin, APC and targeting β-catenin for degradation (Itoh *et al.*, 1998; Kishida *et al.*, 1999; Barker & Clevers, 2000; Giles *et al.*, 2003). In normal cells, unphosphorylated β-catenin will translocate into nucleus where it binds to transcription factors such as T-cell transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors and activates Wnt-regulated genes such as c-myc and cyclin D (Morin, 1999; Shtutman *et al.*, 1999; Barker & Clevers, 2000; van de Wetering *et al.*, 2002). The activity of the Wnt signalling pathway is tightly regulated by the amount of β-catenin. Normally, β-catenin is present in the cytoplasm and is maintained at a low level through ubiquitin-proteasome-mediated degradation, which is regulated by a multiprotein "destruction" complex containing axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK3β) (Kolligs *et al.*, 2002; Polakis, 2007).

In the non-canonical (Planar cell polarity, PCP) pathway, this pathway will leads to the activation of the small GTPases RHOA (RAS homologue gene-family member A) and RAC1, allowing the activation of JNK and ROCK (RHO-associated, coiled-coil-containing protein kinase 1). This will cause changes in cell adhesion and motility, and allows remodelling of the cytoskeleton. The Wnt/Ca^{2+} pathway, meanwhile, is mediated by G proteins and phospholipases (PLC) and leads to a brief increase in free calcium in cytoplasm and subsequently activates the PKC (protein kinase C), CAMKII (calcium calmodulin mediated kinase II) and phosphatase calcineurin. The activation of cell division control protein 42 (Cdc42) PKC allows cell adhesion, migration, and tissue separation (Polakis, 2000; Moon *et al.*, 2004; Katoh, 2005).

Mutations in APC and Axin have been found in some tumours lead to the dysregulation of this pathway in human cancers (Polakis, 2000; Moon *et al.*, 2004; Polakis, 2007). In addition, alteration of Wnt5a, a tumour suppressor gene, could also lead to tumour formation, amplification or overexpression of positive regulators of components of this pathway are observed in several type of cancers (Nagahata *et al.*, 2003; Okino *et al.*, 2003). Besides that, Frizzled-related protein 1 (FRP1/ FRZB), a secreted Wnt inhibitor is frequently absent in human cancers (Ugolini *et al.*, 1999).

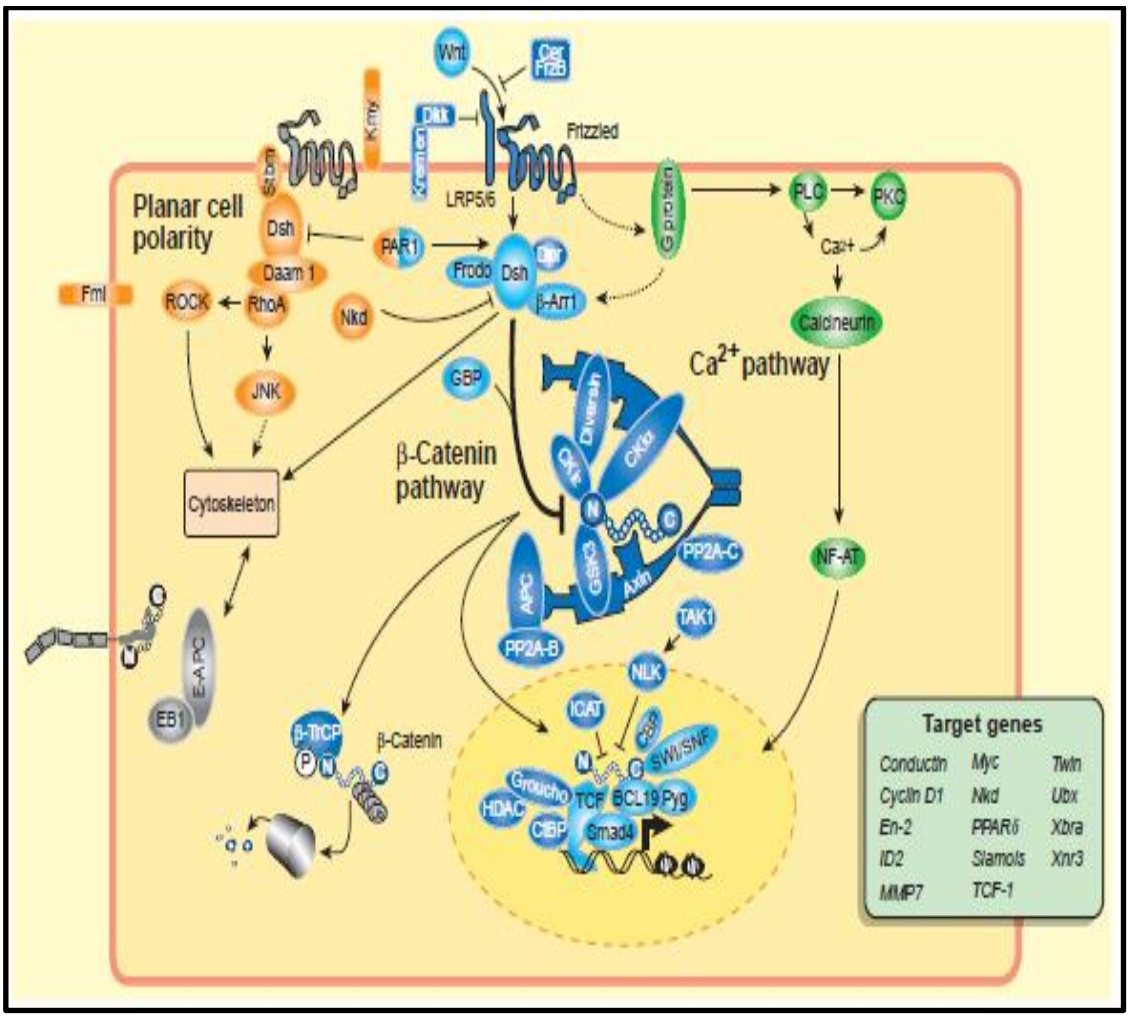

Figure 1.10: The Wnt signalling pathway. Three distinct pathways: (1) the canonical (β-catenin, blue) pathway, (2) non-canonical (planar cell polarity, PCP, orange) pathway, and (3) Wnt/Ca^{2+} pathway (green) (Huelsken & Behrens, 2002).

1.2.1.3 Myc/Max Signalling

MYC gene was first identified as a viral oncogene (v-myc) in the acutely transforming MC29 virus, which induces myelocytomatosis and tumours in chickens (Sheiness *et al.*, 1978; Dalla-Favera *et al.*, 1982; Watson *et al.*, 1983). The linkage between MYC gene and tumour formation is related by several homologs of myc in tumours such as c-myc in Burkitt's lymphoma and N-myc in neuroblastoma (DePinho *et al.*, 1991; Evan & Littlewood, 1993).

The MYC gene is located on chromosome 8 in the human genome and encodes for c-myc proteins (Battey *et al.*, 1983). Normally, expression of c-myc is only transient and at low levels in response to mitogenic signalling. The dimerization of c-myc with Max protein is necessary to induce expression of a number of genes through it binding on consensus sequences; Enhancer Box sequences (E-boxes). It is a key regulator for cell growth, proliferation, metabolism, differentiation, and apoptosis (Hermeking, 2003b; Xu *et al.*, 2010) (Figure 1.11).

High levels of c-myc were found in different types of cancer; breast, ovaries, lung, prostate, and skin, as well as leukemias and lymphomas (Hermeking, 2003b; Xu *et al.*, 2010). In cancer cells, the constitutively active c-myc promoter integrates diverse mitogenic signalling cascades and promotes cell proliferation by regulating the expression of cell survival genes. Cell cycle inhibitors such as p21, p15, and p27 will be suppressed by c-myc, thus allowing unrestricted cell cycle progression and uncontrolled proliferation of cancer cells (Hermeking, 2003b).

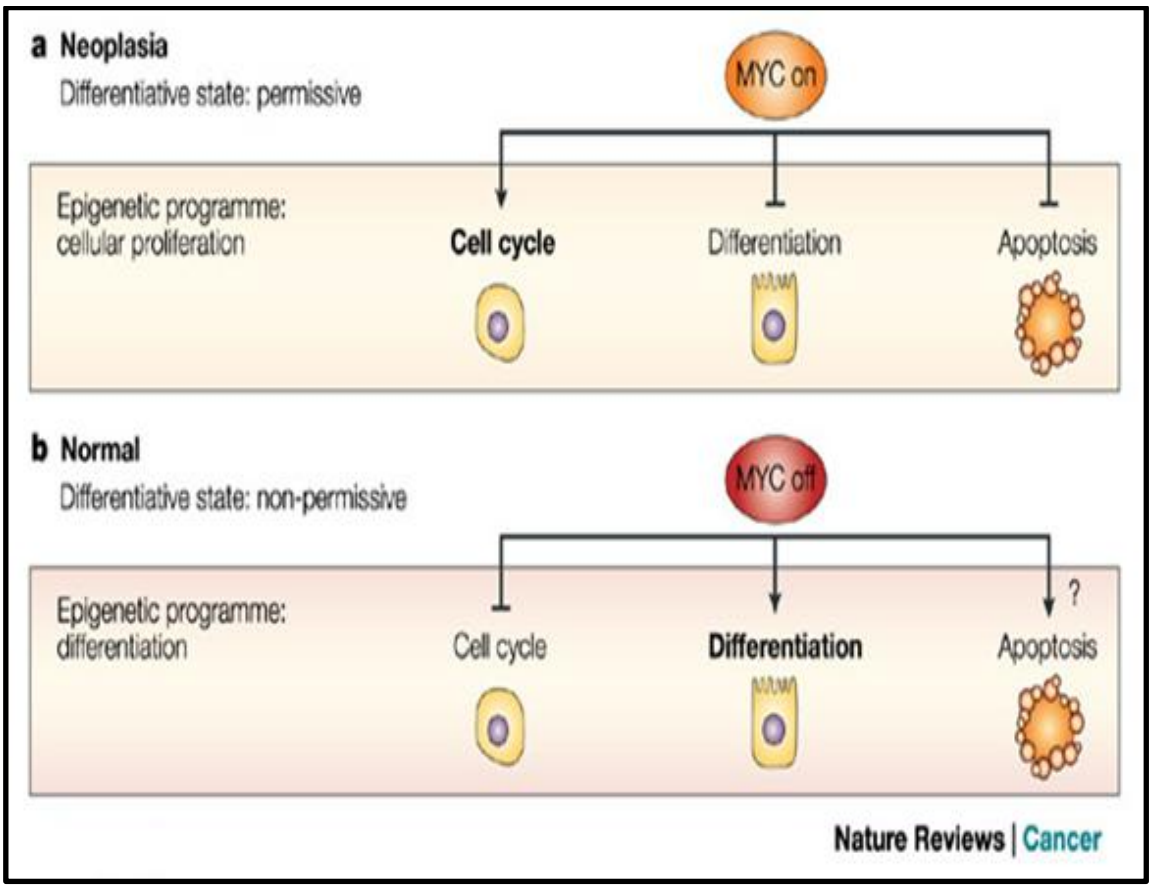

Figure 1.11: Impact on the epigenetic program of cells with Myc/Max. Adapted from (Felsher, 2003).

1.2.1.4 Nuclear Factor Kappa Beta (NFκB)

The nuclear factor kappa B (NFκB) is a family of transcription factors involved in the regulation of immune responses and inflammation. Incorrect regulation of NFκB has been linked to a number of disease including septic shock, viral infection inflammatory and autoimmune diseases as well as cancer (Migliazza *et al.*, 1994; Mukhopadhyay *et al.*, 1995; Caamaño *et al.*, 1996; Shukla *et al.*, 2004; Dolcet *et al.*, 2005; Prusty *et al.*, 2005; Bindhu *et al.*, 2006).

NFκB is not a single gene but a family of closely related transcription factors, p50 (NFκB1), p52 (NFκB2), RelA (p65), c-Rel and RelB (Karin & Lin, 2002; Moynagh, 2005). These five transcription factors shared a domain in their sequence, known as Rel Homology Domain (RHD). This domain allows (1) dimerization to occur (homodimers and/or heterodimers), (2) interacttion with their specific inhibitors, IκBs and (3) mediation of DNA binding. Only RelA (p65), c-Rel and RelB contains C-terminal transcriptional activation domains (TADs) to regulate their target genes. Both p50 and p52 can participate in their target gene regulations by forming heterodimers with RelA, c-Rel, or RelB. In addition, p50 and p52 homodimers form a complex with nuclear protein Bcl-3 to act as a transcriptional activator (Moynagh, 2005).

In normal cells, cytoplasmic NFκB dimer interacts with inhibitors of NFκB (IκBs) and therefore remains in an inactive state in the cytoplasm. There are four members of IκB; IκBα, IκBβ, IκBε and Bcl-3. These IκB members contain two conserved serines that become targets of phosphorylation by the IκB kinases (IKK). The IKK complex contains highly homologous kinase subunits, catalytic subunits (IKKα and IKKβ) and the regulatory subunit (IKKγ or NEMO). Upon phosphorylation, IkB members dissociate from NFκB and undergo proteasome-dependent degradation (Karin & Lin, 2002; Moynagh, 2005).

There are two signalling pathways in NFκB; the canonical pathway (or classical pathway) and the non-canonical pathway (or alternative pathway) (Figure 1.12) (Karin & Lin, 2002; Moynagh, 2005; Gilmore, 2006). In the canonical signalling pathway, ligand-receptor interactions (such as TRAF) will recruit and activate the IkB kinase (IKK) complex. Activation of IKK complex will mediate phosphorylation-induced proteasomal degradation of the IkB inhibitor. This subsequently allows the active NFκB transcription factor subunits to translocate into the nucleus and regulate their target genes. The canonical pathway activates NFκB dimers comprised of RelA, c-Rel, RelB and p50 (Gilmore, 2006; Hayden & Ghosh, 2008).

The non-canonical pathway involves the activation of p100/RelB complexes. The IKK complex in this pathway is only comprised of two catalytic IKKα subunits, but not NEMO. Upon receptor binding, NFκB-inducing kinase (NIK) will be activated and phosphorylated to activate the IKK complex. In turn, this complex phosphorylates p100 or p105 resulting in p52/RelB or p50/RelA active heterodimer, respectively, (Moynagh, 2005; Hayden & Ghosh, 2008), then is tranlocated into the nucleus to regulate target genes.

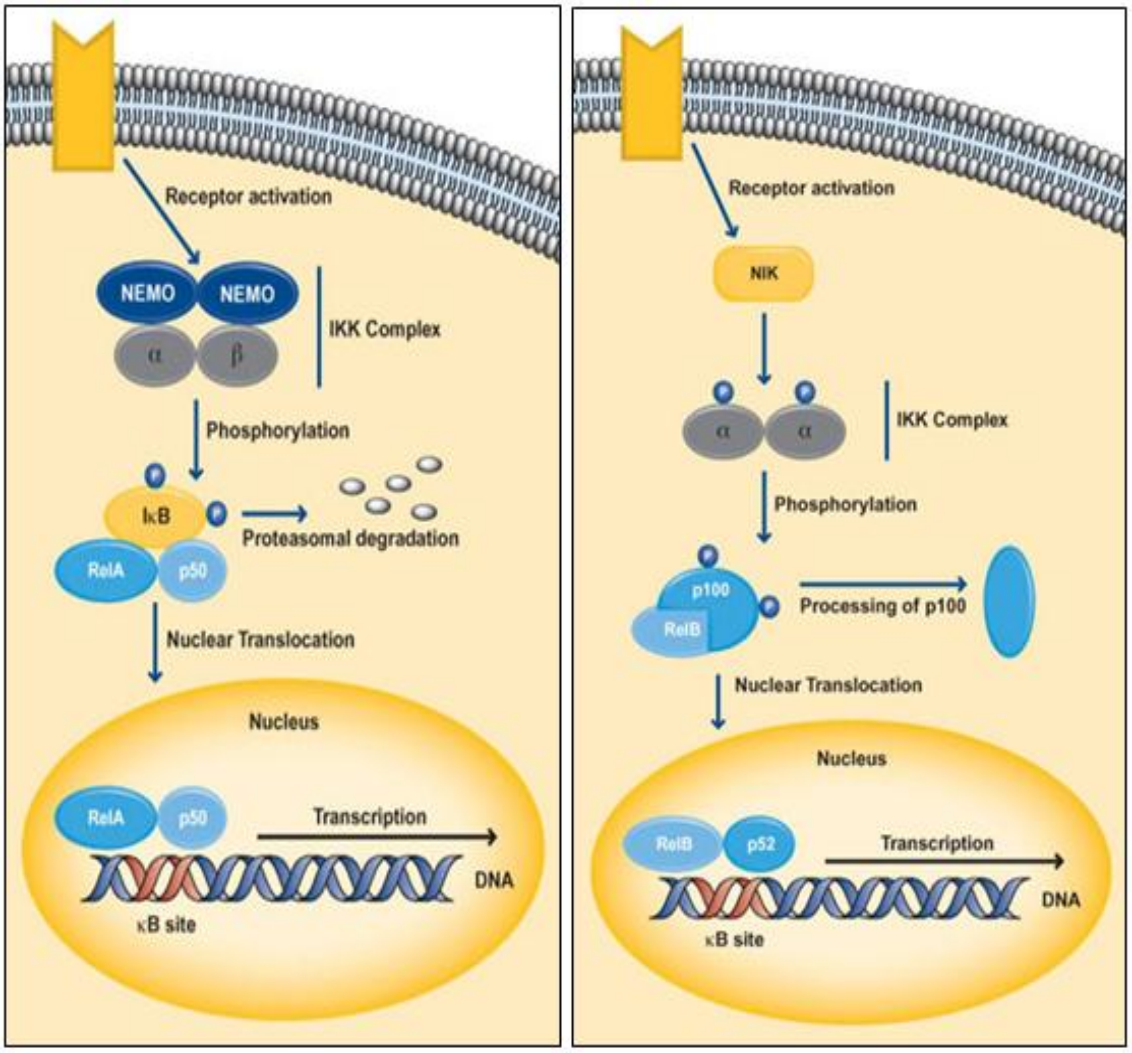

Figure 1.12: Overview of the cannonical (Left) and non-cannonical (Right) of NFκB pathways (Hooper, 2012).

1.2.2 Evading Growth Suppressors

Cancer cells negatively regulate cell proliferation by inhibition of tumour suppressor genes. There are a number of tumour suppressors in a cell and their main function is to protect cells from transforming to cancer cells. One of the well-studied tumour suppressor proteins is p53 that governs both cell proliferation and activate apoptotic programs (Hanahan & Weinberg, 2011).

A TP53 gene is a tumour suppressor gene located on chromosome 17 and encodes p53 protein. The p53 protein is a transcription factor that interacts with a large number of proteins and plays an important role in regulating cell cycle, growth, proliferation, and apoptosis (Vogelstein *et al.*, 2000). Upon DNA damage, p53 protein will bind to DNA, to induce production of p21 protein which is a potent cyclin-dependent kinase inhibitor (CKI). The p21 protein inhibits cell cycle progression by inhibiting the activity of cyclin-CDK2 or -CDK1 complexes until damaged DNA is repaired. If the extent of DNA damage exceeds the capacity of the repair mechanism, the cell will be destroyed via apoptosis. However, deregulation of p53 proteins in cancers causes p53 to no longer bind to DNA in an effective way, and as a consequence the p21 protein is not available to act as the 'stop signal' for cell division. Therefore, cells divide uncontrollably, and form tumours. Loss of p53 in many cancers leads to genome instability, impaired cell cycle regulation, and inhibition of apoptosis (Nikolova *et al.*, 2000; Joerger & Fersht, 2007; Song *et al.*, 2007).

This tumour suppressor protein is also activated in response to oncogene activation, hypoxia and DNA damage, leading to growth arrest and/or apoptosis by stimulating the expression of various p53 target genes including p21, Bax, Puma, Noxa, Apaf-1, Fas and DR5 (Vousden & Lu, 2002) or by repressing the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL (Xu *et al.*, 2001; Hoffman *et al.*, 2002).

Besides involvement at the nuclear level, p53 can also translocate into mitochondria where it interacts with Bcl-XL, resulting in permeability transition and release of cytochrome *c* (Findley *et al.*, 1997; Mihara *et al.*, 2003).

Mouse double minute 2 (Mdm2) (HDM2 in humans, henceforth as Mdm2) oncoprotein is a negative regulator of p53 (Figure 1.13). Mdm2 is a transcription target of p53, thus producing an auto regulatory feedback loop (Picksley & Lane, 1993; Vogelstein *et al.*, 2000; Lahav *et al.*, 2004). Mdm2 will promote the ubiquitination and rapid degradation of p53 to keep p53 at a low level. Mdm2 activity is positively regulated by serine/threonine kinase (Akt) which is activated in response to survival signals from growth factor receptors (Gottlieb *et al.*, 2002). Akt will promote Mdm2-mediated suppression of p53 by suppressing the action of the adenosine diphosphate-ribosylation factor (ARF), a tumour suppressor gene. ARF expression is dependent on the activity of adenovirus E2 gene promoter region binding factor 1 (E2F-1) and by the action of oncogenes such as c-myc, Ras and β-catenin (Henriksson *et al.*, 2001). Increased ARF activity leads to activation of p53 and induction of apoptosis. Aberrant accumulations of proto-oncogenes such as β-catenin, Ras, Myc and E1a have been documented in melanoma, breast, colorectal and prostate cancers (Sherr & Weber, 2000).

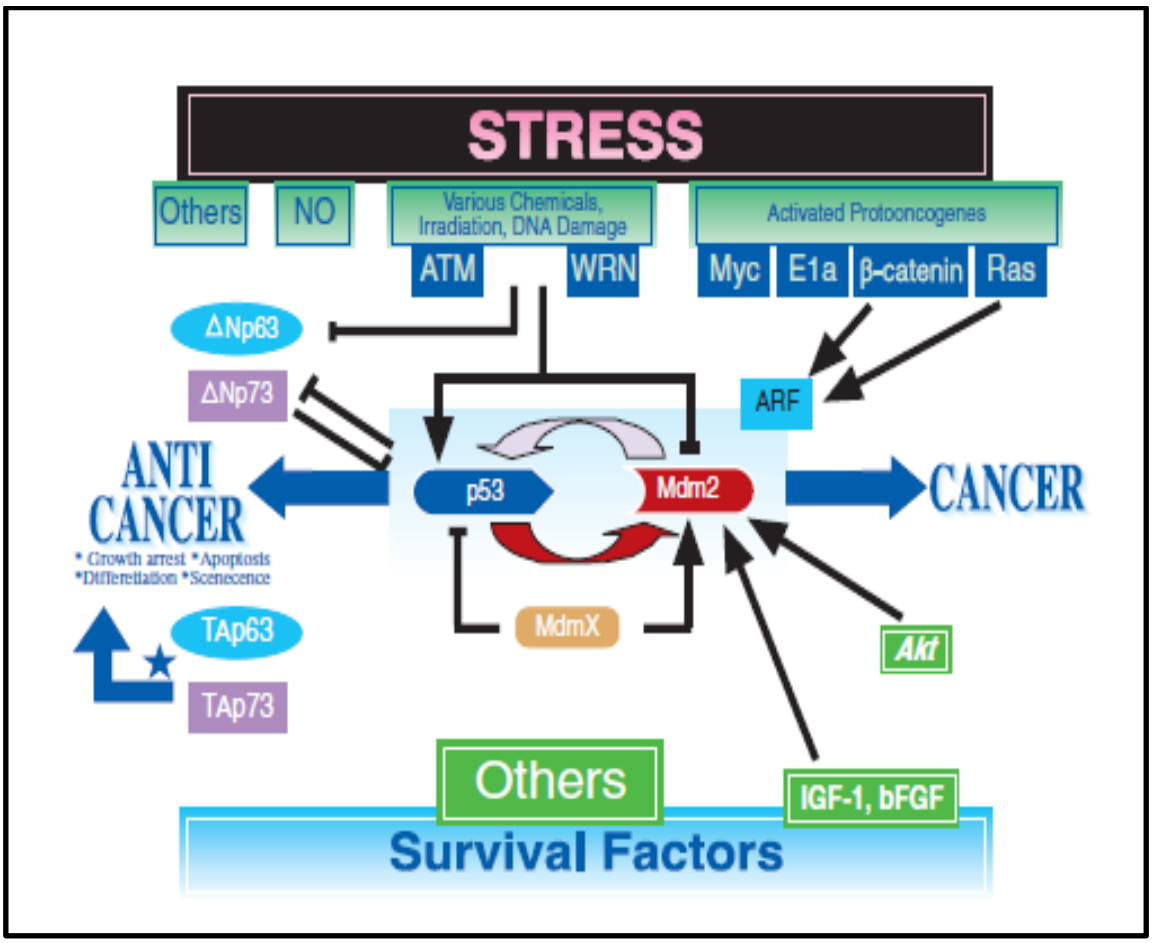

Figure 1.13: The p53-Mdm2 interactions. Arrows indicate positive inputs; horizontal bars indicate inhibitory inputs (Oren *et al.*, 2002).

1.2.3 Resisting Cell Death

Inhibition of apoptotic cell death in cancer cells allows the cells to expand their number during carcinogenesis. Apoptosis is known as one of the most important types of cell death in response to cytotoxic treatment. The administration of anticancer drugs from natural products has shown to be capable of inducing apoptotic death of cancer cells (HemaIswarya & Doble, 2006). This offers new opportunities in identifying new targets for therapeutic intervention and pharmacological manipulation in cancer research.

1.2.3.1 Bcl-2 family

The Bcl-2 (B-cell lymphoma 2) family consists of regulatory proteins that tightly regulates apoptosis including pro-apoptotic (Bax and Bak), and anti-apoptotic proteins (Bcl-xL, Bcl-w, Mcl-1, A1) (Reed, 1998; Korsmeyer, 1999). When anti-apoptotic proteins, Bax and Bak are activated, the integrity of the outer mitochondrial membrane is changed, causing the release of cytochrome *c*. The released cytochrome *c* activates a cascade of caspases that act via their proteolytic activities to induce the multiple cellular changes associated with the apoptotic program (Chao & Korsmeyer, 1998; Gross *et al.*, 1999; Korsmeyer, 1999).

1.2.3.2 Caspases

Caspases (cysteinyl, aspartate-specific protease) are a family of cysteine proteases that plays an important role in cell death. Caspases are present as inactive pro-forms or zymogens within a cell. There are two types of caspases: initiator caspases (caspase-2,-8,-9,-10) and effector caspases (caspase-3,-6,-7). Caspases are activated either by the mitochondrion-mediated (intrinsic) pathway or death receptor-mediated (extrinsic) pathway (Cohen, 1997; Stennicke & Salvesen, 2000). The activated initiator caspases will activate the effector caspases in a cascade by cleaving their inactive pro-

forms. In turn, the effector caspases will cleave other important cellular protein within the cell that lead to the morphological and biochemical features associated to apoptosis (Cohen, 1997; Earnshaw *et al.*, 1999). One of the downstream substrates of caspases is caspase-activated deoxyribonuclease (CAD), which induce DNA fragmentation in apoptotic cells. In normal conditions, CAD will bind to its inhibitor (ICAD) to form a complex in the nucleus. During apoptosis, activated caspase-3 will dissociate CAD from the complex and DNA fragmentation to take place. In addition, caspases target structural proteins such as lamin A, actin, Gas2 and fodrin, to produce apoptosis morphological characteristics including membrane blebbing, cell shrinkage, chromatin condensation and apoptotic body formation (Fan *et al.*, 2005; Herrera-Esparza *et al.*, 2011).

1.2.3.3 Apoptosis

Apoptosis or programmed cell death plays an important role in physiological growth and tissue homeostasis. In anticancer research, apoptotic cell death is a crucial strategy to kill tumour cells via activation of apoptosis signal transduction pathways: intrinsic and/or extrinsic pathways (Bold *et al.*, 1997; Fulda & Debatin, 2006).

The intrinsic pathway occurs in response to cellular signals resulting from DNA damage, a defective cell cycle, hypoxia, loss of cell survival factors, or other types of severe cell stress as well as chemotherapeutic drugs (Figure 1.14) (Kaufmann & Earnshaw, 2000; Johnstone *et al.*, 2002). Upon cellular stress, the equilibrium of activity between pro- and anti-apoptotic members of the Bcl-2 superfamily of proteins in a cell will be disrupted. In turn, these pro-apoptotic proteins will be activated and induce the opening of mitochondrion permeability transition pores (MPTPs). Cytochrome *c* will be released into the cytosol and it will bind to the adapter apoptotic protease activating factor-1 (Apaf-1). The recruitment of cytochrome *c*, Apaf-1, pro-

caspase 9, dATP (deoxyadenosine triphosphate) or ATP will form a multi-protein complex, known as apoptosome (Tsujimoto, 2001; Adams & Cory, 2002; Marsden *et al.*, 2002). Apoptosome aids the cleavage of procaspase-9 to form active caspase-9, and later activates caspase-3 and -7, ultimately resulting in cell death (Figure 1.14A).

The extrinsic pathway begins outside the cell through the activation of specific pro-apoptotic receptors on the cell surface, known as the death receptor such as Fas or TNFR-1 (Tumour Necrosis Factor receptor-1) (Figure 1.14). These death receptors are activated by specific molecules known as pro-apoptotic ligands. These ligands include Apo2L/TRAIL and CD95L/FasL which will bind to their cognate receptors DR4/DR5 and CD95/Fas, respectively (Fulda & Debatin, 2006). This binding induces receptor clustering and recruitment of the adaptor protein Fas-associated death domain (FADD) and procaspase-8, forming a death-inducing signalling complex (DISC). Formation of the DISC will cause an autocatalytic processing in procaspase-8 to form active caspase-8. Alternatively, caspase-8 will activate effector caspase-3, -6, and -7, thereby converging on the intrinsic pathway (Fulda & Debatin, 2006; Liu *et al.*, 2006a). In addition, caspase-8 triggers mitochondrion-mediated pathway by cleaving Bid (a pro-apoptotic Bcl-2 family member) into its active form, truncated Bid (tBid), which translocates into mitochondria. The combination of tBid with other proapoptotic Bcl-2 family members, Bax and Bak, will induce the release of cytochrome *c* and apoptosis-inducing factor (AIF) into cytosol. AIF will induce DNA fragmentation and chromatin condensation (Figure 1.14B) (Fan *et al.*, 2005; Fulda & Debatin, 2006).

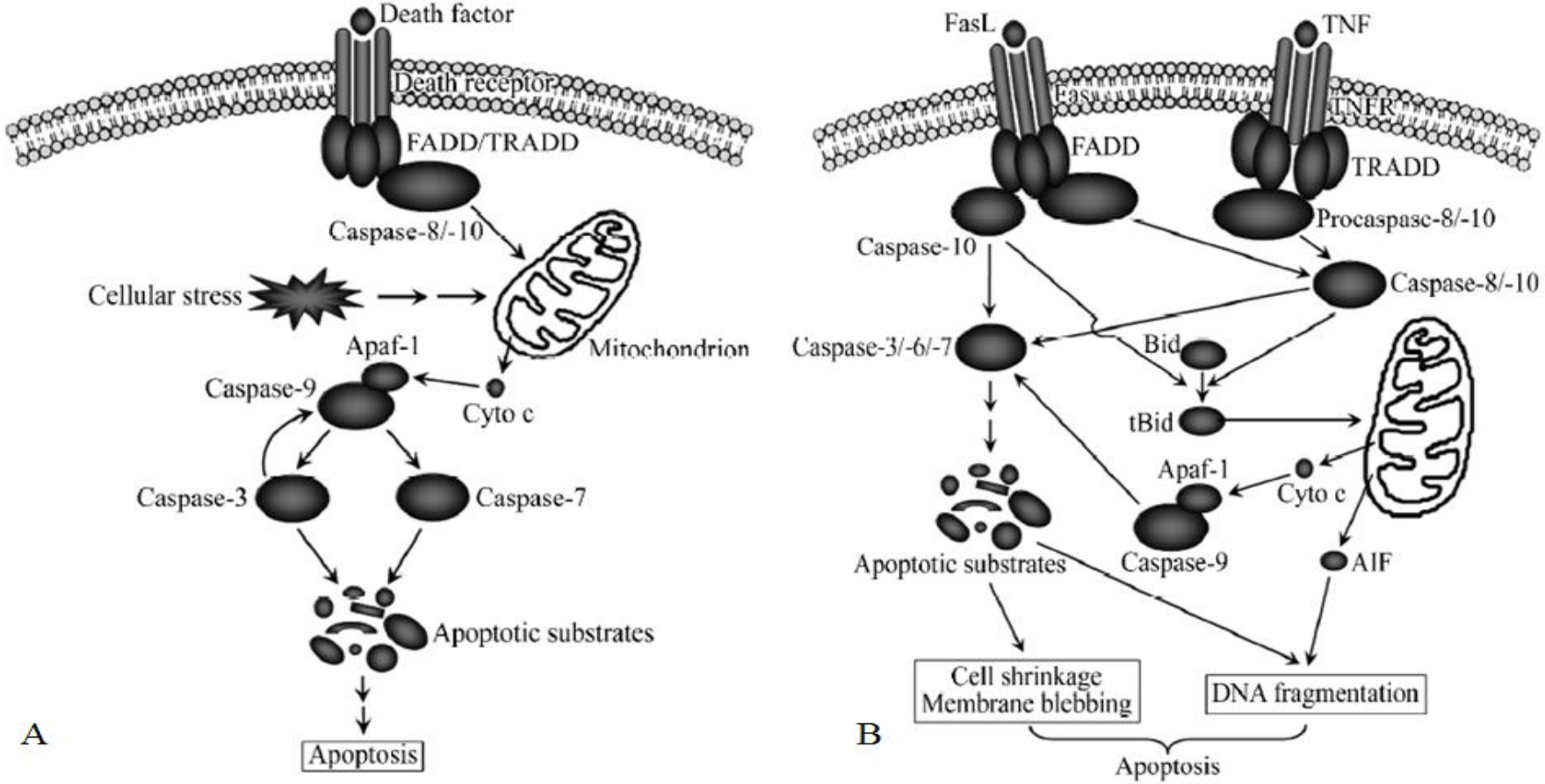

Figure 1.14: Schematic diagram illustrating (A) intrinsic and (B) extrinsic pathways of apoptosis (Fan *et al.*, 2005).

1.2.3.4 Necrosis

Necrosis is another type of cell death in multicellular organisms and is derived from the Greek “nekros” for corpse. It is a passive form of cell deaths. Necrosis also plays a role in some physiological processes such as inner ear development (Zong & Thompson, 2006), tissue renewal of small and large intestines (Barkla & Gibson, 1999; Murdoch *et al.*, 1999), follicular maturation during oogenesis and loss of interdigital cells in the mouse embryo (Chautan *et al.*, 1999; Murdoch *et al.*, 1999).

It can be initiated by external factors (viruses, bacteria, protozoa, toxins) and internal factors such as complements, activated natural killers and peritoneal macrophages (Bortner *et al.*, 1997; Dong *et al.*, 1997; Blom *et al.*, 1999; Arantes *et al.*, 2000; Shimizu *et al.*, 2000; Warny *et al.*, 2000). In addition, inadequate levels of cytokines, nitric oxide (NO) and reactive oxygen species (ROS) secretion in some pathological conditions also causes necrotic cells death (Proskuryakov *et al.*, 2003).

Although apoptosis and necrosis have been defined based on their distinguishable morphological criteria, however, they may involve several common signalling and execution mechanisms (Leist *et al.*, 1999b). The caspase-independent cell death is likely to resemble necrosis as it lacks the typical features of apoptosis (Table 1.2) (Holler *et al.*, 2000; Leist & Jaattela, 2001; Fan *et al.*, 2005). Adenosine triphosphate (ATP) levels also play critical decisive regulatory roles between apoptosis and necrosis. If the amount of ATP is below a certain critical level, it can either switch apoptotic cell death to necrosis or initiate necrosis by itself (Eguchi *et al.*, 1997; Leist *et al.*, 1999a; Proskuryakov *et al.*, 2003). In contrast to apoptosis that requires potassium (K^+) and calsium (Ca^{2+}) ions efflux

to induce cell shrinkage, necrosis requires sodium ion (Na^+) influx to induce cell swelling (Table 1.2) (Bortner *et al.*, 1997).

Table 1:2: Differential features of apoptosis and necrosis (Bold *et al.*, 1997; Bortner *et al.*, 1997; Cohen, 1997)

Apoptosis	**Necrosis**
Biochemical characteristic	
Tightly regulated by physiological homeostasis	Initiate by nonphysiological trama
Activation of caspase cascades	No caspases are require
Active process (ATP-dependent)	Passive process (no energy require)
Apoptotic DNA fragmentation (demonstrates a "ladder" pattern at ~180bp intervals on agarose gels)	Random DNA fragmentation (demonstrates a smear pattern on agarose gels)
Morphological characteristic	
Formation of membrane blebbing, but no loss of integrity	Loss of membrane integrity
Formation of apoptotic bodies	Swelling of organelles and eventually cell lysis
Lysosome still intact	Lysosomal leakage
Physiological features	
Death of individual cells	Death of groups of cell
No inflammatory response	Inflammatory response evoked
Phagocytosis by nearby cells and macrophages	Phagocytosis by macrophages

1.2.4 Enabling Replicative Immortality

Cell cycle is a series of events controlled by complex signalling pathways leading to cell DNA replication and division to form new cells. This process possesses checkpoints to ensure fidelity or errors (mutated DNA) are corrected, otherwise, the cells will be initiated to undergo cell death. In cancer, due to genetic mutations, this regulatory process can malfunction and cause uncontrolled cell proliferation (Hartwell & Kastan, 1994; Evan & Vousden, 2001). Cell cycle can be divided into four distinct phases: G1-, S-, G2- and M-phases (mitosis). M phase is composed of two tightly coupled processes: mitosis and cytokinesis. Mitosis is where the cell's chromosomes are divided into the two 'daughter' cells, while cytokinesis is where the cell's cytoplasm divides in half to forms distinct cells (Hartwell & Kastan, 1994; Shapiro & Harper, 1999).

There are two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs) that determine a cell's progress through the cell cycle (Figure 1.15). During the G1 phase, cyclin D will form a complex with CDKA; cyclin D/CDKA. This complex will phosphorylate their negative regulator, the retinoblastoma protein (pRB), to release E2F transcription factors. The free E2F will regulate genes needed for the G1 to S transition. To enter the S phase, cyclin D will be replaced by cyclin E to from cyclin E/CDKA complex. During S phase, cyclin A will be produced and its binding with CDKA is important for DNA synthesis. At the late S phase, cyclin B will be synthesized and is likely to form a complex with CDKA. This complex will promote the cell to enter G2 phase (Figure 1.15) (Hartwell & Kastan, 1994; Shapiro & Harper, 1999; Evan & Vousden, 2001). CDKIs such as p21 and p27 also play essential roles in controlling cell cycle progression by negatively regulating CDK activities (Toyoshima & Hunter, 1994; Coqueret, 2003).

The relationship between cell cycle and cancer are obviously related, since the cell cycle machinery controls cell proliferation while cancer is a disease of inappropriate cell proliferation. Progression through the cell cycle is tightly regulated by activity of cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CDKIs), growth factors (GFs), their receptors (GFRs), and inhibitory (GFR-I) factors. Thus, disrupting these controlling factors might cause cell cycle arrest (Waldman *et al.*, 1997). Many of the anticancer agents from natural products and chemotherapy agents exert their effects via interrupting cell cycle progression (Shapiro & Harper, 1999).

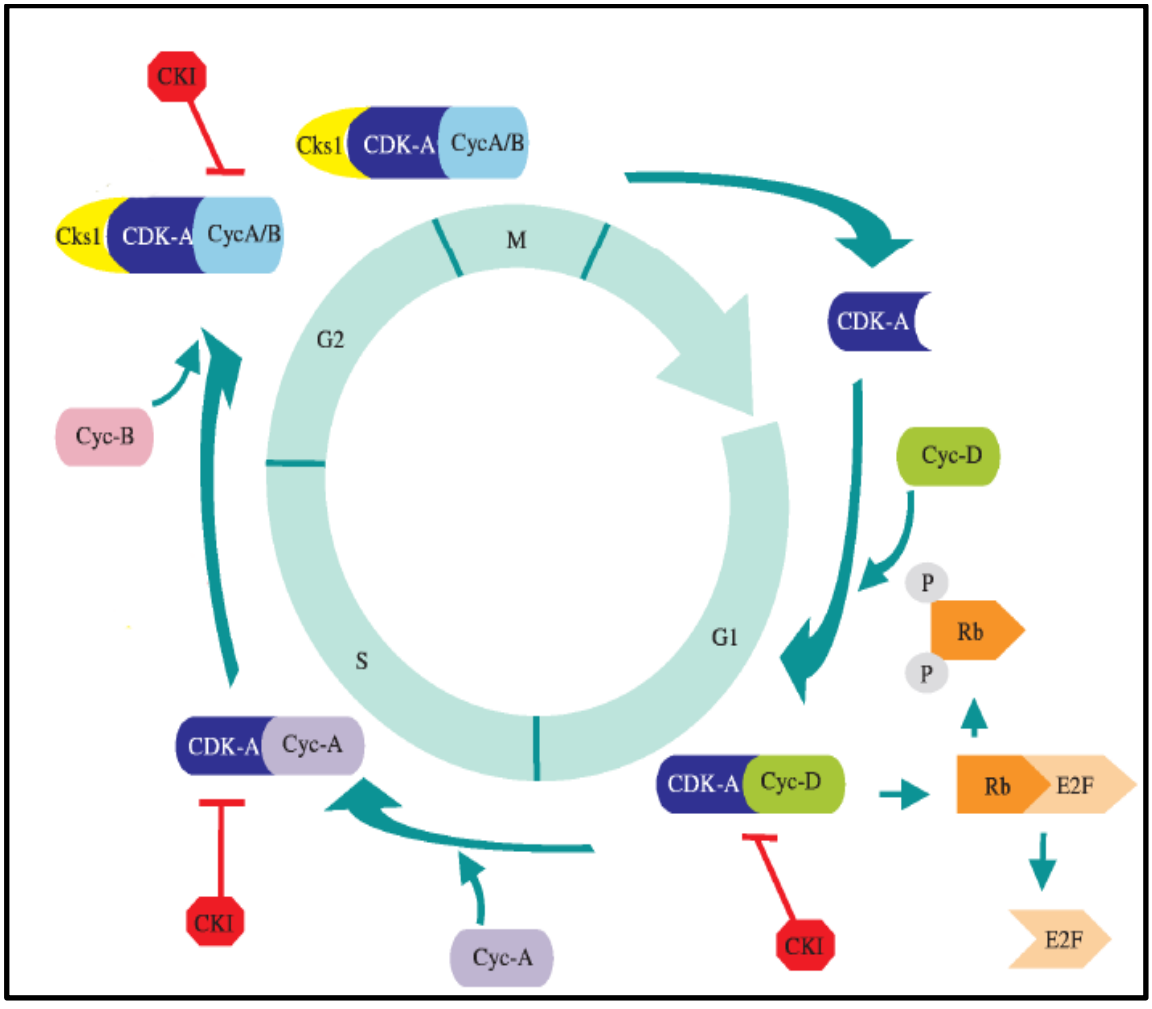

Figure 1.15: Schematic representation of cell cycle regulation (Andrietta *et al.*, 2001).

1.2.5 Invasion and Metastasis

About 90% of cancer-associated mortality is caused by cancer metastasis and yet it still remains poorly understood in cancer pathogenesis (Hanahan & Weinberg, 2000; Gupta & Massagué, 2006). It is one of the most challenging complications in cancer treatment. Metastasis is a complex and multistep process whereby cancer cells spread throughout the body, forming secondary tumours at a distance from its origin (primary tumour). Only small fractions from primary tumour are capable of metastasis. Metastatic cascades start from; (a) detachment of cancer cells from the primary tumour mass, (b) invasion to local tissue stroma, (c) penetration into nearby local lymphatic and/or blood vessels, (d) survive within the circulation, (e) arrested in capillaries or venules at other organs, (f) penetrate through corresponding parenchyma, (g) adapt to the new local microenvironment, and (h) divide to form the new secondary tumour (Figure 1.16) (Gupta & Massagué, 2006).

During metastasis, metastatic cancer cells must acquire five distinct functions including interaction with the local microenvironment, migration, invasion, resistance to apoptosis, and induction of angiogenesis (Figure 1.16) (Roy *et al.*, 2009). The secretion of matrix metalloproteinases is a critical component for metastatic tumour cell to migrate to different tissues. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are capable of degrading and remodeling extracellular matrix (ECM) process (Gialeli *et al.*, 2011). MMPs have been divided into four distinct groups based on their substrate specificity; collagenases, gelatinases, stromelysins and matrilysins. The activity of MMPs is tightly regulated by its inhibitor, tissue inhibitors of metalloproteinases (TIMPs). The balance between MMPs and TIMPs is largely responsible for the remodeling of tissues associated with various physiological processes such as embryonic development, angiogenesis, and tissue repair.

Dysregulation of this balance is implicated in various diseases including cancer invasion and metastasis (Figure 1.16). Thus, MMP have been implicated in progression and metastasis of different tumours (Bacac & Stamenkovic, 2008; Roy *et al.*, 2009; Gialeli *et al.*, 2011).

Several members of MMP family have been implicated in the degradation of ECM and associated with tumour growth and angiogenesis such as MMP-2 and MMP-9. Tumour cells always overexpress of MMP enzymes in order to degrade the basement membrane, invade a nearby lymphatic and/or blood vessel (intravasation) and extravasate at a distant site and invading the surrounding tissue in order to form secondary tumours. Besides ECM components, MMPs also can degrade other molecules including growth factor precursor (e.g. VEGF, FGF) to induce angiogenesis. Activation of growth factors and cleavage of adhesion molecules are believed to attribute to MMP-induced EMT (Bacac & Stamenkovic, 2008; Roy *et al.*, 2009). Epithelial–mesenchymal transition or transformation (EMT) is a hallmark of cancer progression to metastasis. It is the process that allows an epithelial cell to lose its interaction with the basement membrane and possesses a mesenchymal cell phenotype, which includes enhanced migratory ability, invasiveness, and resistance to apoptosis (Yilmaz & Christofori, 2009; Singh & Settleman, 2010). In addition, degradation of E-cadherin by MMPs could disrupt cell-cell interactions and integrins between tumour cells and ECM which enhances the migration and invasion of tumour cells (Noë *et al.*, 2001; Bacac & Stamenkovic, 2008; Bourboulia & Stetler-Stevenson, 2010).

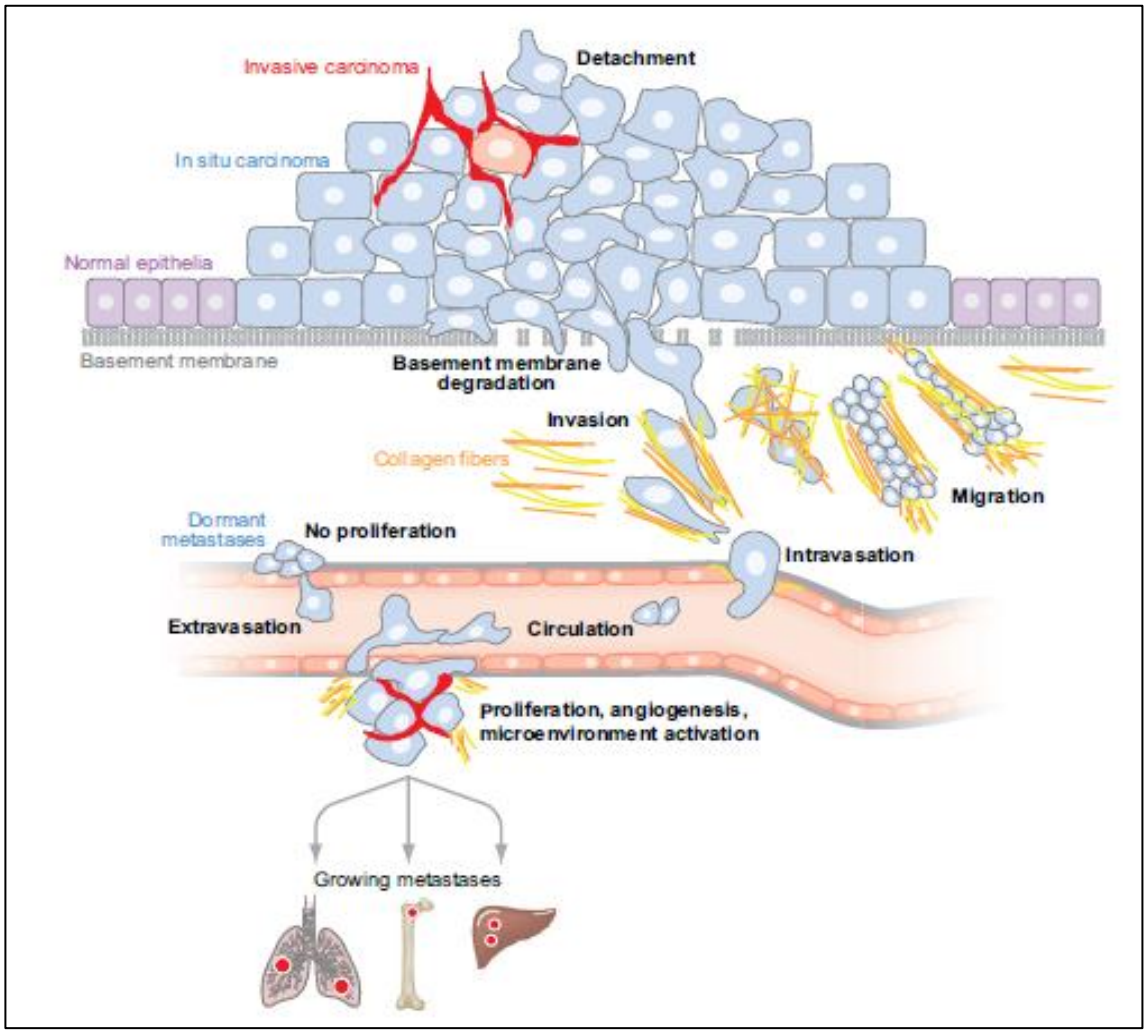

Figure 1.16: Schematic representative of cascade steps in cancer metastasis (Bacac & Stamenkovic, 2008).

1.2.6 Angiogenesis

Angiogenesis, or neovascularisation, is a term referring to the physiological process of new blood vessels formation as an extension from the pre-existing blood vessel. Angiogenesis plays a fundamental role in tumour growth and metastasis and has been labelled as one of the hallmarks of cancer. Therefore, tumour growth and metastasis depends on angiogenesis and remains a main cause of high cancer morbidity and mortality rates. Besides providing oxygen and nutrients for tumour growth, angiogenesis also provides a path to allow the tumour cells to metastasize (Weidner *et al.*, 1991; Zetter, 1998; Kerbel, 2000).

A tumour will produce and release angiogenic growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and angiopoietin. These angiogenic growth factors will bind to their respective receptors on the surface of endothelial cells (EC) in nearby pre-existing blood vessels. This binding will activate ECs to proliferate and start producing several proteases including MMP enzymes. ECs will migrate out through the degraded basement membrane (BM) of vessel walls by MMP enzymes, towards the tumour mass. Adhesion molecules such as integrins (αvß3 and αvß5) will guide the sprouting new blood vessel (remodeling of EC) to sprout forward to the tumour. Besides degradation of ECM, MMPs also degrade other components in the tissue in front of the sprouting vessel tip in order to provide space for new vessel to grow forward. As the new blood vessel extends, the surrounding tissue is remolded around the vessel and the sprouting EC will roll up to form a blood vessel tube. Combination of individual blood vessel tubes will form blood vessel loops that can circulate blood. Finally, newly formed blood vessel tubes will be stabilized by specialized muscle cells (smooth muscle cells and pericytes) that provide structural support (Figure 1.17) (Li *et al.*, 2012).

In the absence of angiogenesis, tumours cannot grow exceeding the size of 2 mm diameter and this small size is limited for simple diffusion of nutrients and oxygen and the removal of waste from the tumour. Therefore, this limitation of growth leads to tumour dormancy for many years in human body (Folkman, 1974; Weidner *et al.*, 1991). Tumour-induced blood vessels are weaker than normal vessels because tumour-associated endothelial cells are abnormal in shape and grow on top of each other, and thus lead the tumour vessels to be highly disorganized, thin walled, leaky, an irregular diameter with less supporting pericytes and smooth muscle cells. Therefore, tumour angiogenesis is now an active area of promoting research for cancer therapy.

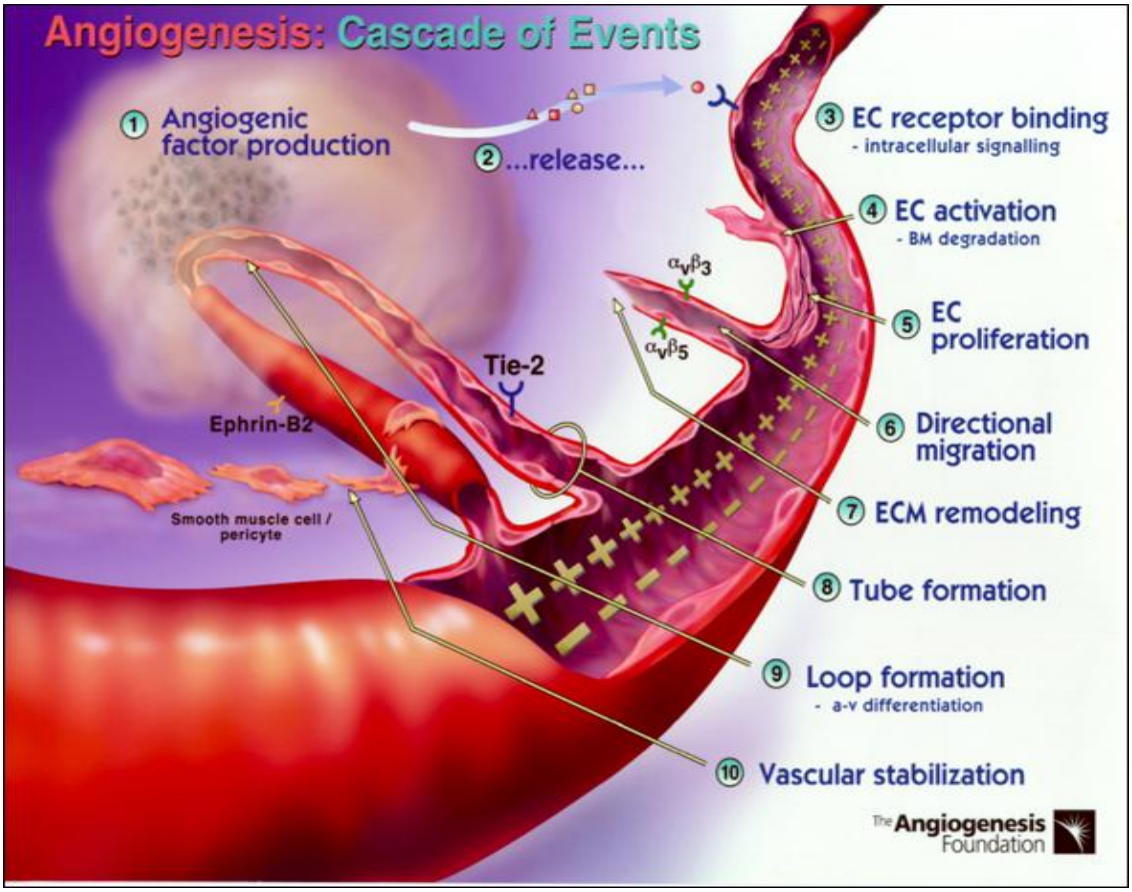

Figure 1.17: Schematic representation of the tumour angiogenesis cascade (Li *et al.*, 2012).

1.2.7 Reprogramming of Energy Metabolism

A fast-growing cancer cell requires an adjustment in its energy metabolism to continuously supply the building blocks required for continuously growth and division. Under aerobic conditions, glucose is the main energy source that will convert to pyruvate via glycolysis and the subsequently released free energy is used to generate ATP and NADH in mitochondria. However, under anaerobic conditions, normal cells will undergo apoptosis due to stress on the mitochondria. In contrast, cancer cells can reprogram their glucose metabolism and energy production, by limiting their energy metabolism in mitochondria. This reprogramming of energy metabolism in cancer cells is known as the "Warburg effect" after Otto Warburg (Warburg, 1956; Kim & Dang, 2006; Vander Heiden *et al.*, 2009).

This reprogramming of energy metabolism has allowed cancer cells to reduce their ATP production 18-folds than in normal cells. To compensate the efficiency of ATP production, cancer cells have up-regulated their glucose transporters, notably GLUT1 to increase glucose intake into the cytoplasm (Airley *et al.*, 2001; Jones & Thompson, 2009; Veronica *et al.*, 2010). The markedly increase of glucose uptake by human tumours is observed with a radiolabeled analog of glucose (18F-fluorodeoxyglucose, FDG), used as a reporter in positron emission tomography (PET) (Gillies *et al.*, 2008).

The rapid growth of cancer cells usually outpaces the new blood vessel generation, and hence results in insufficient blood supply/oxygen to the tumour tissues, and thus causes the cancer cells fall into a hypoxic, or oxygen deprivation state. There are two major regulators in hypoxic stress; hypoxia-inducible factors (HIF-1α and HIF-1β). The importance of these transcription factors in tumour cell survival is reflected in the finding where the levels of HIF-1α in glioma tumour cells is proportional to the

grade of the tumour (Zagzag *et al.*, 2000). During tumour progression, HIF-1 induces several glycolytic enzymes and glucose transporters such as aldolase A and pyruvate kinase M to produce energy in hypoxic environments (Schäfer *et al.*, 1997; Discher *et al.*, 1998). In addition, HIF-1 decreases mitochondrial oxygen consumption by regulating pyruvate dehydrogenase kinase I and inhibiting the citric acid cycle (Papandreou *et al.*, 2006).

During activation of the hypoxia pathway, the mRNA of HIF-1α is transcribed by binding of specificity protein 1(Sp1), P300, and HIF-1β on HIF-1α gene in the nucleus. In normal cells, HIF-1α is either hydroxylated and ubiquinated, or mostly degraded by proteasomes (under normal oxygen conditions) (Discher *et al.*, 1998). However, during hypoxic condition, HIF-1α will re-enter the nucleus and form a transcription complex with the HIF-1β subunit (Schäfer *et al.*, 1997). This complex will regulate its target genes such as vascular endothelial growth factor (VEGF) and cathepsin D to induce new blood vessels production, or angiogenesis to supply more oxygen and nutrients for tumour growth (Figure 1.18).

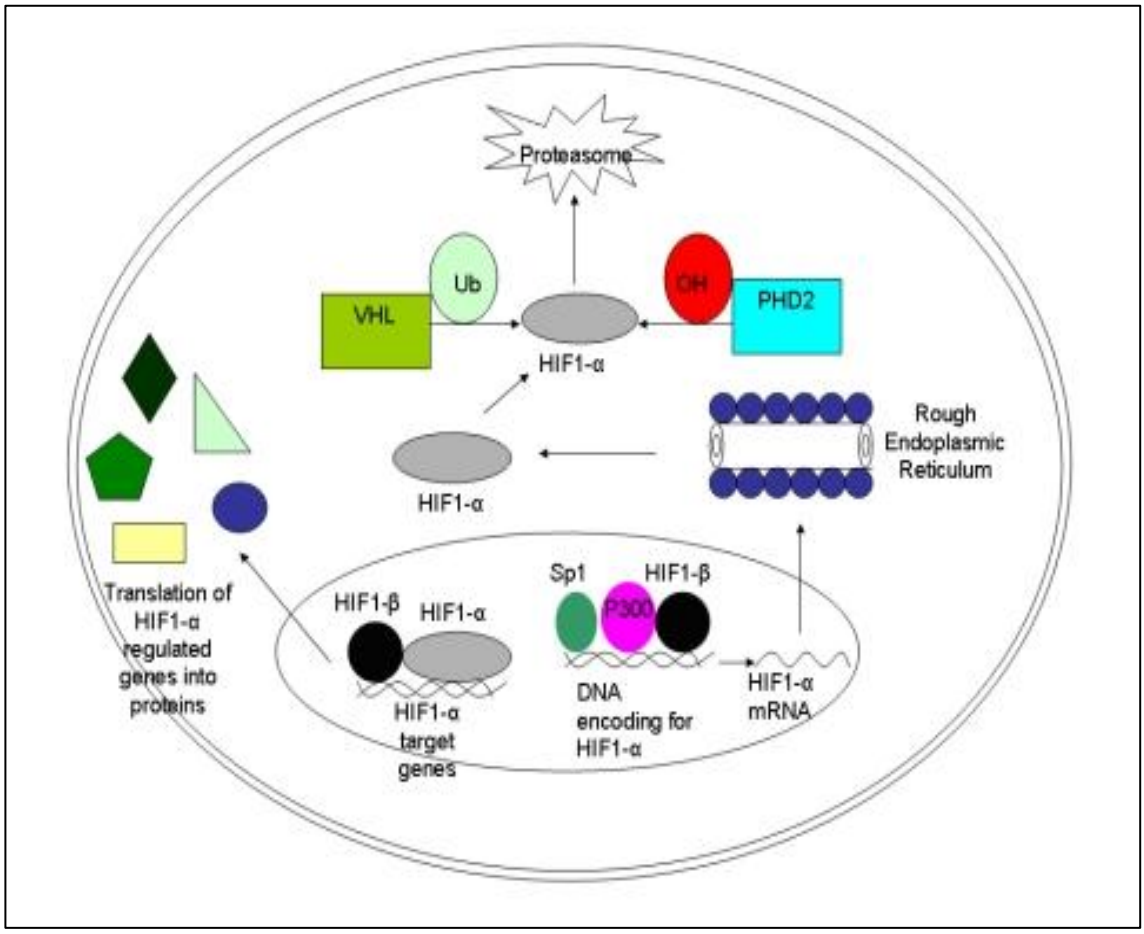

Figure 1.18: Schematic representation of the hypoxia-inducible factor (HIF)-1 pathway (Ziello *et al.*, 2007).

1.2.8 Evading Immune Destruction

The immune system is the defense system in the human body that monitors tissue homeostasis by providing protection against infectious pathogens and eliminates damaged cells including cancer cells. However, the interaction of immune system and tumour progression is still poorly understood. A tumour is believed to possess the capability of avoiding detection by the immune system or have been able to limit the extent of immunological killing, thereby evading eradication (Vajdic & van Leeuwen, 2009; Hanahan & Weinberg, 2011).

Studies found that immunodeficient mice that lack different components of the immune system are likely to develop tumours than immunocompetent mice (Hanahan & Weinberg, 2011). Several studies have showed that tumours arose more frequently and/or grew more rapidly in the immunudeficient mice, particularly lacks of CD8+ cytotoxic T lymphocytes (CTLs), CD4+ Th1 helper T cells, or natural killer (NK) cells, relative to immunocompetent mice (Kim *et al.*, 2007; Teng *et al.*, 2008). These studies indicate that both the innate and adaptive cellular immune system plays a significant role in immune surveillance and thus eradication of the tumour (Kim *et al.*, 2007; Nelson, 2008; Teng *et al.*, 2008).

A better prognosis is seen in colon and ovarian tumour patients which is heavily infiltrated with CTLs and NK cells compared to those that lack these cells (Nelson, 2008). Cancer cells may evade from destruction by inhibiting some components in the human immune system and/or modulated their surface receptors to escape from immune surveillance. For example, cancer cells can inhibit CTLs and NK cells through the secretion of TGF-β or other immunosuppressive factors (Shields *et al.*, 2010; Yang *et al.*, 2010) or by activation of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) which inhibits the actions of cytotoxic lymphocytes.

1.3 Natural Products

1.3.1 History and background of the use of natural products as therapeutics agents

Since the dawn of human history, humans have relied on plants and herbs as vital source for survival. Besides being a mainstay of nutrition, plants and herbs are also plays an important role in the treatment of illness. The earliest records on the usage of natural products in the treatment is including cuneiform in Mesopotamia, Chinese herbs guide documents and Ayurvedic hymns (Cragg & Newman, 2005; Ji *et al.*, 2009). One of the best examples is the discovery and development of Artemisinin (antimalaria drug) from Artemisia annua L, which was used in China for almost 2000 years for treating various infectious and chronic conditions (van Agtmael *et al.*, 1999).

By definition, a natural product refers to a compound that is present in or produced naturally and not man-made or artificial. Natural product can originate from microbes or plants sources (Table 1.3). Generally, a natural product is the common term for herbs, dietary supplements, traditional Chinese medicine, or alternative medicine (Cragg & Newman, 2005).

Natural product-based traditional medicine, often overshadowed by modern medicine, has returned to be an alternative approach for health care in many societies. The World Health Organization (WHO) estimated about 80% of world's populations still relies on traditional medicines as primary health care. Although the number of natural products based drugs introduced to market is low, but the interest of these drugs has not dropped in worldwide pharmaceutical companies (WHO, 2008). This is because the degree of chemical diversity and novelties of molecular structures found in natural products are broader than any sources. In addition, natural products are sometimes

preferred in medicine due to the fact that they can balance the combination of therapeutic effects with minimum side effects. The importance of natural products as therapeutic agents has stated by N.R. Farnsworth, an eminent scientist in ethnobotanical field; “each plant is a unique chemical factory capable of synthesizing an unlimited number of highly complex and unusual chemical substances whose structures could escape the imagination of synthetic chemists could” (Farnsworth *et al.*, 1985).

The plant-derived natural products have widely used in medicine including as antipyretics, neurotransmission modulators, antihypertensive agents and anti-inflammatory agents. Narcotic morphine is the first commercial pure natural product which was marketed by Merck in 1826 (Newman *et al.*, 2000). In 1899, Bayer introduced the first semisynthetic pure drug based on a natural product, aspirin (Spainhour, 2005).

1.3.2 Plants as source of anticancer agents

Chemotherapy is deemed relatively unsuccessful due to its ineffectiveness, safety issues and costliness. As not all cancer patients respond positively to current anticancer agents, mortality rates of cancer are on a continuous rise. Therefore, scientists have begun to focus on natural-products as alternatives to produce new therapeutic agents for cancer treatments. Herbs and plants-derived medicines have a long history of use in various treatments and now, they still remain as important sources for the development of anticancer drugs. More than 3000 plants species have been reported to be involved in the development of anticancer drugs (Shoeb, 2006). The exploration of anticancer agents from plant sources started in the 1950s. Since then, extensive research has been conducted and has led to the discovery and development of several anticancer agents derived from plants such as taxol, vinblastine and vincristine. From 1940-2006, more that 40% of drugs in the market are anticancer agents and 65%

of these anticancer drugs mimic natural compounds (Cragg & Newman, 2005). Some example of anticancer agents derived from natural products include zapotin (*Casimiroa edulis*, apoptotic inducer); apigenin (*Mezoneuron cacullatum*, antimutagenesis); and resveratrol (*Cassia quinquangulata*, cyclooxygenase inhibitor) (Table 1.3) (Holt & Chandra, 2002; Shoeb, 2006).

Besides as a direct medical application as drug, natural products could also serve as templates for the design, synthesis, and semisynthesis of new compounds. Paclitaxel and vincristine are the two examples of semisynthetic compounds used in the treatment of human cancers. Natural product provides those treating cancer with new avenue of treatment of the most aggressive forms of the disease (Farnsworth *et al.*, 1985; Cragg & Newman, 2005; Shoeb, 2006).

Table 1:3: Natural products-plant derived anticancer drugs (Pezzuto, 1997; da Rocha *et al.*, 2001; Amin *et al.*, 2009).

Compound	Trade name	Action	Cancer	Plant Source
1. Docetaxel	Taxotere	Anti-mitotic	Breast, ovarian, and prostate lung cancers	*Taxus brevifolia*
2. (a) Etoposide (b) Teniposide	Eposin, VP-16, Vumon, VM-26	Inhibits topoisomerase II	Lung, prostate, testicular cancers, lymphoma, leukemias, Ewing's sarcoma and brain tumour. Restinoblastoma, ALL.	*Podophyllum peltatum*
3. (a) Irinotecan (b) Topotecan	Camptosar, Campto Hycamtin	Inhibits topoisomerase I	Colon, ovarian, cervical and rectal cancers, small lung cancer cell (SLSC)	*Camptotheca acuminate*
4. Paclitaxel	Taxol, Onxal	Anti-mitotic	Ovarian, breast, melanoma, lung, bladder, prostate, head and neck cancers,	*Taxus brevifolia*
5. (a) Vinblastin (b) Vincristine	Velban, velbe Oncovin	Anti-mitotic	Lung, breast and testicular cancer, leukemia lymphoma	*Catharanthus roseus*
6. Combresastin	Combretastatin A4	Inhibits topoisomerase I	Liver, prostate, breast and lung cancers	*Combretum caffrum*
7. Homoharring-tonine	Myelostat, Ceflatonin	Anti-angiogenesis, apoptosis inducer	Leukemia	*Cephalotaxus harringtonia*

1.4 *Phyllanthus*

1.4.1 History and conventional usage of *Phyllanthus*

Phyllanthus is the largest genus in the family of the *Phyllanthaceae* (*Euphorbiaceae* s.l.). It is widely distributed throughout the tropical and subtropical regions of world including Malaysia, India, Brazil, Cuba and the Amazon (Lee *et al.*, 1996; Etta, 2008). To date, there are more than 700 species of *Phyllanthus* have been reported (Unander *et al.*, 1990) that includes *P. amarus, P. elegans, P. emblica L., P caroliniensis P. flexuosus, P. reticulatus, P.discoideus, P. muellerianus, P. multiflorus Willd., P. tenellus Roxb, P. virgatus Forst. f., P. urinaria L., P. niruri, P. reticulatus, P. conami Sw, P. lathyroides, P. casticum and P. madagascariensi*. In different countries, *Phyllanthus* is known by different names such as "Dukung Anak" in Malaysia, "Hsieh-hsia Chu" in China, "Quebra Pedra" in Brazil and in India, it known as "Pitirishi" and/or "Budhatri" (Taylor, 2003).

This plant has a long history in the medical herbalism system. It is mainly used to treat kidney and urinary bladder disturbances. In Ayurvedic medicine, *Phyllanthus* has a long tradition of use to treat jaundice, gonorrhea, frequent menstruation, dysentery and diabetes as well as skin ulcers, sores, swelling, and itchiness (Calixto *et al.*, 1998). In Traditional Chinese Medicinal (TCM), *Phyllanthus* has been used for generations to eliminate gallstones and kidney stones, as well as an immune system stimulator (Taylor, 2003).

Due to different climates and geographically regions, *Phyllanthus* grows in different forms such as annual and perennial herbs, shrubs, and pachycaulous succulents in order to survive. It can grow up to 12-24 inches in height and blooms with many greenish white flowers. One of the famous *Phyllanthus* species is *P.niruri.* In Amazon,

it is named as “stone breaker” due to its effectiveness in the elimination of gallstones and kidney stones and other treatments including diabetes and intestinal infections (Taylor, 2003). Table 1.4 shows the various usage of *Phyllanthus niruri* in different societies.

Figure 1.19: *P.urinaria* (Fito Pharma 2011), *P.amarus* (Find me cure, 2011) and *P.niruri* (GardenSeed, 2011).

Table 1:4: Worldwide ethnobotanical uses of *P. niruri* (Taylor, 2003).

Region	**Uses**
Amazonia	Anodyne, apertif, blennorrhagia, carminative, colic, diabetes, digestive, diuretic, dropsy, dysentery, dyspepsia, emmenagogue, fever, flu, gallstones, gonorrhea, itch, jaundice, kidney aliments, kidney stones, laxative, malaria, proctitis, stomachache, stomachic, tenesmus, tonic, tumour, vaginitis, vermifuge
Bahamas/ Caribbean	Antihepatotoxic, antispasmodic, appetite stimulant, antiviral, aperitif, bactericidal, cold, constipation, diuretic, fever, flu, hypoglycemic, laxative, stomachache, typhoid
Brazil	Abortifacient, ache (joint), albuminuria, analgesic, antibacterial, anticancerous, antidiabetic, anti-inflammatory, antilithic, antispasmodic, antiviral, aperient, arthritis, biliary conditions, bladder problems, bladder stones, calculi, catarrh (liver and kidney), chologogue, cystitis, deobstruent, diabetes, diaphoretic, digestion stimulant, diuretic, fever, gallbladder, gallstones, gastritis, gastrointestinal problems, gout, hepatitis, hepatoprotective, hydropsy, hypertension, hypoglycemic, jaundice, kidney colic, kidney pain, kidney stones, liver, m alaria, muscle relaxant, obesity, prostatitis, purgative, renal colic, renal problems, stomachic, sudorific, tonic, uric acid excess, urinary problems, uterine relaxant
Haiti	Carminative, colic, digestive, diuretic, fever, indigestion, malaria, spasmolytic, stomachache, stomachic, tenesmus
India	Anemia, asthma, astringent, bronchitis, conjunctivitis, cough, deobstruent, dropsy, diabetes, diarrhea, diuretic, dysentery, fevers, eye disorders, galactagogue, genitourinary disorders, gonorrhea, hepatitis, jaundice, leucorrhea, menorrhagia, oligogalactia, ringworm, scabies, stomachic, thirst, tuberculosis, tumour (abdomen), urogenital tract infections, warts
Malaysia	Caterpillar sting, dermatosis, diarrhea, diuretic, emmenagogue, itch, miscarriage, piscicide, purgative, renosis, syphilis, vertigo
Peru	Calculus, diuretic, emmenagogue, gallstones, hepatitis, kidney pain, kidney problems, kidney stones, renal problems, urinary infections, vermifuge
United States	Analgesic, bronchitis, chologogue, deobstruent, diabetes, fever, gallbladder problems, gallstones, gout, hepatitis, hypertension, kidney problems, kidney stones, liver disease, uric acid excess, urinary tract infections
Elsewhere	Analgesic, antipyretic, appetite stimulant, blennorrhagia, bruises, chologogue, cough, cuts, diabetes, diarrhea, diuretic, dropsy, dysentery, dyspepsia, emmenagogue, eye diseases, fever, gallstones, gonorrhea, itch, jaundice, kidney disease, kidney stones, laxative, malaria, menorrhagia, menstrual problems, poultice, purgative, rectitis, stomachache, tonic, tuberculosis, urinary tract infections, vaginitis, venereal diseases

1.4.2 Scientifically proven pharmacological activities of *Phyllanthus* and its constituents

Phyllanthus is believed to have originated from India and by the late 1980s, these plants have gained attention from scientists worldwide. One of its species, *P. niruri* has showed clinical efficacy against viral Hepatitis B (Calixto *et al.*, 1998; Padma & Setty, 1999). Therefore, substantial studies of this genus regarding its chemistry, pharmacological activity and clinical effectiveness have been extensively carried out. The extract of these plants had been reported to have a variety of pharmacological effects and is listed in Table 1.5.

Table 1:5: Various pharmacological activities of *Phyllanthus* species.

Pharmacological activities	**Description**
Antiviral	• Inhibits cellular proliferation, DNA polymerase, hepatitis B surface antigen (HBsAg) gene expression (Venkateswaran *et al.*, 1987; Blumberg *et al.*, 1990; Yeh *et al.*, 1993; Ott *et al.*, 1997; Huang *et al.*, 2003; Lee *et al.*, 2003a) • Growth inhibitions of HIV-1 and HIV-2 (Venkateswaran *et al.*, 1987; Naik & Juvekar, 2003)
Antibacterial	• Growth inhibitions of *Escherichia coli, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium smegmatis, Bacillus stearothermophilus, Bacillus subtilis, Micrococcus leuteus, Salmonella typhi, Enterobacter aerogenes, Proteus mirabilis,* and *Proteus vulgaris* (Mensah *et al.*, 1990; Mazumder *et al.*, 2006; Komuraiah *et al.*, 2011)
Hepatoprotective and gastroprotective	• Reduces liver infiltration, focal necrosis and intracellular level of reactive oxygen species (Lee *et al.*, 2006a; Naaz *et al.*, 2007) • Protects liver tissues against oxidative damage and improves liver repair mechanism (Bhattacharjee & Sil, 2006) • Gastroprotective (Shokunbi & Odetola, 2008)

Hypoglycaemia or anti-diabetic	• Hypoglycemic effect (Raphael *et al.*, 2002; Adeneye *et al.*, 2006; Kumar *et al.*, 2008)
Anticancer and antitumour	• Anticancer properties against liver, renal, breast, colon, lung, cervical, ovarian, colorectal, melanoma and leukemia cancer cells (Pettit *et al.*, 1984; Jeena *et al.*, 1999; Rajeshkumar & Kuttan, 2000; Huang *et al.*, 2004b; Rajkapoor *et al.*, 2007; Ratnayake *et al.*, 2008; Ngamkitidechakul *et al.*, 2010) • Antitumour properties against sarcoma, Dalton's Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma (EAC), hepatocarcinoma, and skin tumour (Rajeshkumar & Kuttan, 2000; Rajeshkumar *et al.*, 2002; Ngamkitidechakul *et al.*, 2010)
Others	• Diuretic and hypotensive effects (Srividya & Periwal, 1995) • Antioxidant properties (Khopde *et al.*, 2001; Kumaran & Joel Karunakaran, 2007) • Lipid lowering activity (Khanna *et al.*, 2002) • Anti-inflammatory effect (Ihantola-Vormisto *et al.*, 1997; Kiemer *et al.*, 2003) • Antinociceptive effect (Santos *et al.*, 1995; Santos *et al.*, 2000) • Analgesic effect (Santos *et al.*, 1994) • Antimalaria (Totte *et al.*, 2001) • Antimutagenic and antigenotoxic properties (Sripanidkulchai *et al.*, 2002) • Anti-babesial and anti-plasmodialactivity (Totte *et al.*, 2001; Subeki *et al.*, 2005)

1.5 Justification and Objectives of the Study

Melanoma is the most fatal form of skin cancer, with higher morbidity and mortality rates in fair-skinned populations, whereas, prostate cancer remains one of the major life-threatening cancers in men. Typically, tumour metastasis is the main cause for high morbidity and mortality rates in cancer patients. Currently, the best treatments for melanoma and prostate cancers include patient management as well as standard treatments to control metastasis such as chemotherapeutic drugs, surgery and/ or radiation therapy. Conversely, these treatments are often accompanied by undesirable side effects such as vomiting, nausea and alopecia. Distressingly, not all cancer patients respond positively to current anticancer agents and the mortality rates have increased inspite of treatment. Hence, intense effort is required to acquire anticancer agents that have minimal side effects and could also target cancer metastasis.

Natural product-based traditional medicine, is often overshadowed by modern medicine, has returned to be a holistic approach for health care in many societies. This "back to basic" approach is due to its long history of usage in disease treatment and the pharmacological/nutritional value of these products are believed to be able to halt/delay cancer progression. The well-known examples of plant-derived anticancer drugs include taxol (antimicrotubule agent) and vincristine (mitotic inhibitor) (Shoeb, 2006). Herb- and plant-derived medicines that are widely used in traditional cultures have gained popularity in modern society as natural alternatives to produce new potential therapeutic compounds (Shoeb, 2006).

Phyllanthus plant is widely distributed in subtropical and tropical regions. A variety of pharmacological effects have been reported including antiviral, antibacterial, anti-hepatotoxic as well as having anti-diabetic properties. These effects are mainly attributed to the presence of various of bioactive compounds in *Phyllanthus* plant such

as gallic acid, ellagic acid, corilagen and rutin. Most of these bioactive compounds have been well-documented for their pharmacological effects. However, as no individual class of components could be fully responsible for the activity/effect produced by a whole extract, therefore it is more meaningful to assess the activity of *Phyllanthus* extract as a whole mixture of bioactive compounds rather than as their individual compounds.

Hence, in this study the anticancer properties of four species of *Phyllanthus* (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) against two high metastatic potential cancer cell lines; a human melanoma (MeWo) and prostate adenocarcinoma (PC-3), were evaluated. The main aims of this study were to identify the anti-proliferation, anti-metastasis, anti-angiogenesis properties, apoptosis induction and mechanisms of inhibition of *Phyllanthus* plant extracts on these cancer cells.

Hence the objectives of this study were:

1. To investigate the anti-proliferative effects of four plants species of *Phyllanthus* (*P.amarus, P. niruri, P.urinaria* and *P. watsoni*) on two human cancer cell lines; namely MeWo and PC-3 cells and their respective normal cell lines (CCD-1127Sk and RWPE-1).
2. To study the mechanism of cell death induced by *Phyllanthus* extracts.
3. To investigate the anti-metastasis properties of *Phyllanthus* extracts
4. To investigate the anti-angiogenesis properties of *Phyllanthus* extracts
5. To investigate the underlying anti-carcinogenic mechanisms (cell signalling pathway and protein-protein interaction) of *Phyllanthus* extract.

CHAPTER 2: METHODOLOGY

2.1 Cell Culture and Maintenance

2.1.1 Normal and Cancer Cell Lines

In this study, human skin melanoma cells, MeWo (ATCC: HTB-65) and prostate adenocarcinoma cells, PC-3 (ATCC: CRL-1435), normal human skin cells, CCD-1127Sk (ATCC: CRL-2565) and normal human prostate cells, RWPE-1 (ATCC: CRL-11609) as well as human umbilical vein endothelial cells, HUVECs (ATCC: CRL-2873) were used. All these cell lines were purchased from American Type Culture Collection (ATCC) and cultured with different media, EMEM (Eagle's minimum essential medium) for MeWo cells, RPMI-1640 (Roswell Park Memorial Institute) for PC-3 cells, keratinocyte growth medium-chemically defined, KGM™-CD medium (CC-4455, Lonza, USA) for RWPE-1 cells, DMEM (Dulbecco's modified Eagle Medium) for CCD-1127Sk cells and endothelial growth media, EGM-2 Bullet Kit (CC-3162, Lonza, USA) for HUVECs. Growth media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Flowlab, Australia). All cells were grown in 5 ml in 25 cm^2 flasks or 20 ml in 75 cm^2 flasks (Nunc, Denmark). Cells were maintained at 37°C under humidified air with 5% CO_2. Cells were harvested using 0.25% trypsin-EDTA (Hyclone) when they reach 70–80% confluency in culture flasks. Cells undergoing exponential growth were used throughout the experiments.

2.1.2 Culture Medium

KGM™-CD (Chemically Defined) and EGM-2 BulletKit media were purchased from Lonza and were supplemented with SingleQuots™ Kit, consisting of essential growth factors, cytokines, and supplements. Culture media (EMEM, DMEM and

RPMI-1640) were purchased from Flowlab, Australia and stored at 4°C until further reconstitution for use. The powder was reconstituted by dissolving the medium together with 3.7 g of sodium bicarbonate and 4.7 g of HEPES (Sigma-Aldrich, Ireland Ltd) in one liter of sterile double-distilled water or milliQ water. After complete dissolution of the powder, the medium was then filter-sterilized with 0.2 μm membrane filter (Nalgene, USA) using air pressure from a machine diaphragm pump. The medium was stored at 4°C till further use. Fetal Bovine Serum (FBS, Flowlab, Australia) was heat inactivated at 56°C for 30 minutes, aliquoted into 50 ml tubes (Nunc, Germany) and stored at -20°C till required for reconstitution as complete growth medium. Heat-inactivated FBS was added freshly into medium at 10% concentration just before use.

2.1.3 Culture Technique

All cell culture procedures were conducted under sterile conditions in a vertical flow hood (Microflow, Birmingham, UK) to avoid contaminations. Cell passaging is the process of splitting cultured cells to yield large or low number of cells from pre-existing cells, depending on the purpose. Briefly, old growth media was discarded, rinsed with sterile PBS and trypsinized with trypsin-EDTA (0.25%) for 5 minutes. The detached cells were resuspended in serum-containing growth medium and the suspension was gently aspirated few times to avoid cell clumps. At ratio 1:1, cell suspension was mixed with 0.2% trypan blue solution (Sigma-Aldrich, Ireland Ltd) and loaded into the haemocytometer chamber. The cells were counted under an inverted microscope (Olympus CK-40).

The calculation of cell number in 1ml of solution is as following:

Cell number/1 ml = A x B x C

Where,

A = average number of cells counted in four large corner squares,

B = dilution factor is 2 (1:1 dilution of cell suspension with trypan blue), and

C = volume of 0.1 mm^3 square is equivalent to 10^{-4} ml but substitute with 10^4 in calculation as for conversion factor to convert 10^{-4} ml to 1 ml.

Cryoperservation of the cells was performed to store the cells in liquid nitrogen for future use. The freezing medium consists of culture medium, pure fetal bovine serum (FBS) and the freeze substitute, dimethyl sulphoxide (DMSO, Sigma-Aldrich, Ireland Ltd) at 6:3:1 ratio. Briefly, cell suspension was spun down at 1,500 rpm (Eppendorf Centrifuge 5810R, Germany) for 10 minutes at 4°C. Cell pellet was resuspended with freezing medium and transferred to a cryogenic vial (Nunc, Denmark) at $2x10^6$ cell/vial. The vial was tightly capped, properly labeled with cell line, passage number, and date of freeze down, and placed in a freezing container which was then stored at -80°C overnight before transferring it into the liquid nitrogen tank for long term storage.

Cryopreserved cells from the liquid nitrogen were thawed when required for experiments. Briefly, cryogenic vial was thawed by rapid agitation of in a 37°C water bath (Memmert). Contents in cryovial were transferred to a 25 cm^2 culture flask containing warm growth media and incubated overnight. On the following day, the flask was rinsed with PBS to remove any floating dead cells and residual DMSO and replaced with new growth medium.

2.2 Preparation of Serial Dilutions of Samples and Standard Anticancer Drugs

Aqueous and methanolic extracts of four different species of *Phyllanthus* (*P.amarus, P.niruri, P.urinaria, and P.watsonii*) were prepared and provided by Dr. Indu Bala, Biotechnology Centre, Malaysian Agricultural Research and Development Institute (MARDI). The freshly harvested whole plants (*P.amarus, P.niruri, P.urinaria, and P.watsonii*) except the root part were collected, washed, and dried at room temperature. The dried materials were cut into smaller pieces and freeze dried with liquid nitrogen into powder form. For the aqueous extract, the dried sample was mixed with extraction buffer (ultra pure water, diethyldithiocarbamic acid and formic acid), while absolute methanol was used for the methanolic extract. The mixtures were then centrifuged at 8,900 rpm at 4°C for 5 minutes. The supernatant was collected after three rounds of extraction and filtered with 15 WHATMAN No.4. The powder form of each extract was prepared after vacuum dried and kept at -20°C. For the experiment, the master stock of aqueous extract (10 mg/ml) and methanolic extract (40 mg/ml) were prepared by dissolving into sterile miliQ water and DMSO, respectively. Standard anticancer drugs, Doxorubicin (Dox) and 5-Fluorouracil (5'FU) were used as positive control in this study. Master stock of plant extracts and standard drugs were aliquoted into 1 ml tubes and kept at -20°C.

2.3 High performance liquid chromatography coupled with electronspray ionization (ESI) and mass spectrometry (LCMS- MS) analysis

For aqueous extracted samples, 2 ml of supernatant was dried in a vacuum concentrator (Concentrator 5301 eppendorf, Germany) and re-dissolved into 20 mg/ml with 30% methanol before being subjected for LC-MS-MS analysis. For those samples extracted with methanol, total supernatant was evaporated using rotary evaporator

(Rotavapor RII, BUCHI, Switzerland) and re-dissolved again with 20% methanol. Samples were then separated with solid phase extraction (SPE) column (LiChrolut RP-18, 1000 mg/6 ml, Merck, Germany) with mobile phase of 60% methanol and 70% methanol. All elutes were concentrated to 0.5 ml, then diluted 8 times with 40% methanol before being subjected for LC-MS-MS analysis.

Samples were separated using HPLC system comprising of a HPLC binary pump, an autosampler injector compartment and diode array detector (DAD) (1200 series, Agilent Technologies, Germany). Separations were carried out using a reverse phase C- 18, 150 mm X 4.6 mm i.d, 5 µm particle size Thermo Hypersil GOLD column (Thermo Scientific, UK). Separation was developed using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with a gradient setting of solvent B: 5% (5 min), 5–90% (60 min), 5% (4 min) at flow rate of 1 ml/min. The injection volume was set at 20µl and the detections were both at 280 nm and 360 nm. For mass spectrometry analysis, 3200 QTrap LC/MS/MS system (Appiled Bioscience – MDS Sciex) was used with the iron source and voltage was maintained at 500°C and -4.5 kV for negative ionization, respectively. Nitrogen generator was set to be operated at 60 psi curtain gas flow, 90 psi source gas flow and 60 psi exhaust gas flow. Two types of scanning modes were chosen: enhance mass spectrometer (EMS) and enhance ion product (EPI) for a full scan mass spectra ranging from m/z 100–1200.

2.4 Assessment of Cancer Cell's Proliferation

The anti-proliferative activity of *Phyllanthus* was measured using a colourimetric MTS assay, which is composed of solutions of a novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt, MTS and an electron coupling reagent (phenazine

methosulphate; PMS) (Promega, Madison, WI). This assay is based on the cleavage of the yellow dye MTS to purple formazan crystals by dehydrogenase activity in mitochondria of viable cells (Husoy et al., 1993). Cancer (MeWo and PC-3) and normal (CCD-1127Sk and RWPE-1, HUVECs) cells in cultured flasks were washed thoroughly with sterile PBS (1X), collected by addition of trypsin-EDTA (1X) solution and re-suspended in the culture medium. The cancer and normal cell lines were then counted and seeded at $1x10^5$ cells per well in a 96-wells flat-bottom plate. For endothelial cells (HUVECs), cells were seeded at $5x10^3$ cells per well into a fibronectin pre-coated 96-well plate. The seeded cells were incubated overnight at 37°C for cell attachment.

The master stock solution of *Phyllanthus* crude extracts were serially diluted and added to the cells to reach a final concentration at a range of 31.3–500.0 μg/ml and further incubated for 72 hours. The percentage of DMSO in working concentration of methanolic extracts was not exceeding 1.3% to avoid toxicity to cell. Anticancer drugs, Doxorubicin (Calbiochem, USA) and 5'Fluorouracil (Duchefa, The Netherlands) were used as positive controls for PC-3 and MeWo cells, respectively. In addition, the fractions of crude aqueous extract of *Phyllanthus* species, fraction 1 and 2 were also tested for their anti-proliferative effect on normal and cancer cells for comparison with the crude extracts. After 72 hours, the cytotoxicity screening was performed using CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, USA), according to the manufacturer's instructions. Briefly, an equal volume of MTS/PMS solution was added in each well and the plate was stored in dark for an hour before determining their absorbance at 490 nm with the reference wavelength at 600 nm using GloMax Multi Detection System (Promega, USA). The absorbance is directly proportional to the number of viable cells in the culture. At least three replications for each sample were used to determine the anti-proliferative activity. The percentage of

cell viability of *Phyllanthus* extracts on both human cancer and normal cell lines were determined using following formula:

$$\text{Percentage of cell viability} = \frac{[\text{Mean OD of the tested cells} - \text{Mean OD of the medium}]}{[\text{Mean OD of the untreated cells} - \text{Mean OD of the medium}]} \times 100\%$$

Half-maximal inhibitory concentration (IC_{50}) value is a concentration of tested sample that kills 50% of cell population as compared to the untreated control. The IC_{50} was determined as cut-off values of each *Phyllanthus* plant extracts to have anti-cancer properties according to US National Institute of Health (NIH) plant screening program and will be used in subsequent experiments.

2.5 Assessment of Cell Cycle Distribution

Assessing the damaged DNA of cancer cells resulting from exposure to anticancer agents is important in cancer biology and toxicology studies. The susceptibility and variation of DNA to damage can differ between cell types. Thus, assessment of anti-proliferative activity of *Phyllanthus* was determined by measuring the amount of cellular DNA damage in a cancer cell population (MeWo and PC-3) through flow cytometric application.

Flow cytometric analysis allows the measurement of changes in cellular DNA content during the various phases of the cell cycle. Optimization of flow cytometric DNA was performed to increase the sensitivity in detecting levels of DNA damage (strand breaks) and allowed the measurement of the extent of DNA damage by providing adequate resolution, linearity and sensitivity to distinguish single cells from aggregates in flow cytometry. In addition, optimization of flow cytometry is to ensure stability of the system performance and reproducibility of the results. Propidium iodide

(PI) dye was used as it intercalates with DNA and allows evaluation of cell viability and DNA content at excitation/emission wavelength the 480/490 nm.

Melanoma (MeWo) and prostate cancer cells (PC-3) were seeded at $2.5x10^5$ cells per well in a 6-wells plate and allowed to incubate overnight. Cells treated with respective IC_{50} value of *Phyllanthus* extracts were incubated at different time intervals; 24, 48, 60 and 72 hours. The floating and trypsin-detached cells were collected and fixed in 70% ethanol. The ethanol-fixed cells were spun down and washed twice with ice-cold PBS. The cell pellet was stained with propidium iodide (10 μg/ml) (Sigma-Aldrich, Ireland Ltd) in PBS (Flowlab) containing RNase A (1 mg/ml) and was incubated in a 37°C water bath (Memmert) for 30 minutes in the dark before being analyzed on a FASCalibur flow cytometer (Becton Dickinson, USA). Cell cycle distribution is presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence. The percentage of cells in sub-G1, G0/G1, S and G2/M phases of cell cycle was quantitated using WinMDI software. The percentage of hypodiploid cells (Sub-G1) over total cells was calculated and expressed as percentage of apoptotic cells.

2.6 Apoptosis Assays

Inhibition of apoptosis in cancer is a major causative factor leading to malignant transformation of the cancer cells, metastasis and resistance to anticancer agents. The induction of apoptosis in cancer cells is important to halt cancer progression. Therefore, induction of apoptosis by *Phyllanthus* plant extracts in cancer cells (MeWo and PC-3) was studied using caspases detection, TUNEL and DNA fragmentation.

2.6.1 Caspase-3/7 Detection

Caspases (caspase-3 and -7) are member of executioners in apoptosis induction. Their activity was measured by caspase-Glo-3/7 assay (Promega, Madison, WI). This is a luminescent assay that allows measurement of caspase-3 and -7 activities by providing proluminescent caspase–3/-7 substrate, Z-DEVD. The cleavage of this Z-DEVD by these caspases will produce aminoluciferin that generate luminescent signals.

Cancer (MeWo and PC-3) cells were seeded at a density of $1x10^4$ of cells/well in 96-wells white-walled flat bottom plates and incubated overnight for cell attachment. Cells were then treated with the respective IC_{50} value of *Phyllanthus* extracts for 72 hours. Control well consists of cells with culture medium without extracts while blank wells contained culture medium only. The Z-DEVD substrate reagent was added at 1:1 ratio to the cells. After an hour of incubation at room temperature, caspase-3/7 activity of treated cells was determined by measuring luminescence signal using GloMax Multi Detection System (Promega, USA). The percentage of caspase-3/7 of *Phyllanthus* extract-treated cell was determined, where the background luminescence associated with the culture media and assay reagent (blank reaction) was subtracted from the experimental values of treated cells. The percentage activity of caspase-3/7 level was presented as mean of three independent experiments conducted in triplicates.

Percentage of caspase-3/7 activity

$$= \frac{\text{[Mean RLU of the tested cells – Mean RLU of the medium]}}{\text{[Mean RLU of the untreated cells – Mean RLU of the medium]}} \text{ X } 100\%$$

Where,

RLU = Relative Luminescence Units

2.6.2 TUNEL Assay and Apoptotic Index

DNA fragments produced by *Phyllanthus* treatment was determined using the ApopTag@Plus Peroxidase *In Situ* Apoptosis Detection Kit (Cemicon International, USA) based on the terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) assay according to the manufacture's instruction. Cancer cells (MeWo and PC-3) were treated with respective IC_{50} values of *Phyllanthus* extracts for 72 hours. The cells were fixed with 1% paraformaldehyde in PBS (pH 7.4) and dried on a silanized glass slide. Pre-cooled ethanol:acetic acid (2:1 v/v) was used for post-fixation of cells on the slide for 5 minutes at -20°C. Endogenous peroxidase was quenched with 3% H_2O_2 at room temperature for 5 minutes. The apoptotic DNA fragments were labelled with digoxigenin antibody with TdT (terminal deoxynucleotidyl transferase) enzyme and then conjugated with anti-digoxigenin antibody. The labelled peroxidase-apoptotic DNA fragments were then treated with peroxidase substrate, diaminobenzidine (DAB) to produce permanent and localized brown-coloured stain. Methyl green (Sigma-Aldrich, Ireland Ltd) was used to counterstain the cells to further differentiate the apoptotic cells from viable normal cells. The slide was observed under light microscope (Olympus BX41) and photographed with Olympus U-CMAD3 camera. The number of apoptotic cells were calculated from a total of at least 1000 apoptotic cells at 100x magnification and presented as an apoptotic index. Apoptotic cells were identified in TUNEL assay by brown coloured cells from blue coloured viable cells.

2.6.3 DNA Fragmentation

During apoptosis, chromatin DNA were cleaved by the activated endogenous endonucleases into internucleosomal fragments with 180 base pairs (bp) and multiples thereof (360, 540, etc.) which can be detected using agarose gel electrophoresis assay.

Cancer cells (MeWo and PC-3) were treated with respective IC_{50} values of *Phyllanthus* extract for 72 hours. After incubation, both floating and trypsin-detached cells were collected and then centrifuged at 1,500 rpm for 5 minutes at 4°C. The cell pellet was resuspended with sterile PBS and then lysed with ice-cold lysis buffer (1 M Tris-HCl, pH 8.0, 0.5 M EDTA, 100% Triton X-100 and distilled water) for 35 minutes at 4°C. The cell lysates were centrifuged at 7,800 rpm for 30 minutes at 4°C and supernatant was collected. The DNA sample was extracted from supernatant with 25:24:1 (v/v/v) equal volume of neutral phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, Ireland Ltd). The DNA sample was precipitated by adding two volumes of isopropanol (Fisher Scientific, Canada) and 0.1 volume of 3 M sodium acetate (pH 5.2) into the supernatant and left undisturbed at -20°C overnight. The precipitate containing the DNA fragment was centrifuged at 7,800 rpm for 30 minutes at room temperature. The RNA in the sample was digested with 30 µl of deionized water-RNase solution (10 mg/ml RNase, Sigma, Ireland Ltd) for 30 minutes at 37°C. The DNA was loaded and electrophoresed on 1.2% agarose gel (Vivantis, UK) containing 0.5 µg/ml of ethidium bromide (Invitrogen, Canada). DNA fragments were visualized under UV light using UV transilluminator (Vilbert loumat, France) at 312 nm and photographed using Olympus C-5060 camera.

2.7 Necrosis Assay

The leakage of LDH enzyme from damaged membrane cells may indicate for late stages of apoptosis or necrosis (Edinger & Thompson, 2004). The measurement of LDH levels in *Phyllanthus* treated cells was determined by CytoTox-One Homogenous Membrane Integrity Assay kit (Promega, USA). Cancer cells (MeWo and PC-3) were seeded at $1x10^4$ cells per well in black-coloured 96-wells flat bottom plate and incubated for overnight. The cells were treated with respective IC_{50} value of

Phyllanthus extract for 72 hours. The negative control wells were cell with culture medium only whereas blank wells contained culture medium. The CytoTox-One reagent containing diaphorase was added into cells and incubated at room temperature for 10 minutes. The generated fluorescence signal indicated LDH activity was measured at an excitation wavelength of 560 nm and an emission wavelength of 640 nm by using GloMax Multi Detection System (Promega, USA). The LDH levels were presented as the mean of three independent experiments conducted in triplicate manner.

Percentage of LDH level

$$= \frac{[\text{Mean RFU of the tested cells} - \text{Mean RFU of the medium}]}{[\text{Mean RFU of the untreated cells} - \text{Mean RFU of the medium}]} \times 100\%$$

Where,

RFU = Relative Fluorescence Units

2.8 Anti-Metastasis Assays

Cell migration is defined as the movement of cells from one area to another in response to chemoattractant and it is one of the important phenotype changes in cancer cells during metastasis (Zetter, 1998). Cell invasion is similar to cell migration except in cell invasion; the cells have to invade through an extracellular matrix (ECM) barrier by enzymatically degrading this barrier in order to become established in a new site (Weidner *et al.,* 1991). Besides cancer cells, endothelial cells migration is essential during tumour-induced angiogenesis. During angiogenesis, endothelial cells migrate from existing vessels, invade surrounding tissue into new areas, proliferate and assemble into new capillaries that supply oxygen and nutrients to tumour mass (Delgado *et al.*, 2011). The transwell migration/invasion assay is a commonly used assay to study the migratory/invasion response of cell to chemical signals. This assay is

also known as the Boyden or modified Boyden chamber assay. Migration and invasion assays were performed using transwell filter culture plate, which has an upper chamber containing polycarbonate filters of 8.0 μm pore size (Corning, USA). The wound healing assay was performed to confirm findings from transwell migration assay. The anti-metastatic effect of *Phyllanthus* in cancer cells (MeWo and PC-3) and HUVECs was studied in terms of migration, invasion and adhesion.

2.8.1 Transwell Migration assay

Both cancer (MeWo and PC-3, $2.5x10^5$ cells/well) and HUVECs ($2x10^5$ cells/well) were seeded in 6-wells plate and incubated overnight. After incubation, the cells were treated with various concentrations of *Phyllanthus* extracts (31.3-500.0 μg/ml) for 72 hours. After 72 hours, pre-treated cells were suspended with serum-free culture media and added to the upper chamber and the lower chamber was filled with culture media supplemented with chemoattractant (10% FBS for cancer cells and 10 ng/ml of VEGF (Sigma-Aldrich, Ireland Ltd) for HUVECs). After 12 hours incubation at 37°C, the cells at the upper side of the upper chamber filter were removed using a cotton-tipped swab. The cells at the lower chamber were fixed with methanol and stained with 0.5% crystal violet. The migrated cells were examined under the light microscope (Olympus BX41) and were photographed with Olympus U-CMAD3 camera. Number of cells at the lower side of the upper chamber was calculated in five random fields under 100X magnification.

$$\text{Percentage of Migrated Cells} = \frac{\text{[Total number of treated (migrated) cells]}}{\text{[Total number of untreated (migrated) cells]}} \text{ X } 100\%$$

2.8.2 Transwell Invasion assay

For invasion assay, the same protocol was performed as migration assay with the exception that the filter surface of upper chamber was coated with (1) 10.0 mg/ml of extracellular matrix (ECM) (Sigma-Aldrich, Ireland Ltd) for cancer cells (MeWo and PC-3) and (2) 50.0 µg/ml of fibronectin (Roche, Germany) for endothelial cells (HUVECs). Both cancer (MeWo and PC-3, $2.5x10^5$ cells/well) and HUVECs ($2x10^5$ cells/well) were seeded in 6-wells plate and incubated overnight. After incubation, the cells were treated with various concentrations of *Phyllanthus* extracts (31.3-500.0 µg/ml) for 72 hours. After 72 hours, pre-treated cells were collected, suspended with serum-free culture media and added to the upper chamber while the lower chamber was filled with culture media supplemented with chemoattractant (10% FBS for cancer cells and 10 ng/ml of VEGF for HUVECs). After 12 hours incubation at 37°C, the cells at the upper side of the upper chamber filter were removed by using a cotton-tipped swab. The cells at the lower side of the upper chamber were fixed with methanol and stained with 0.5% crystal violet. The invaded cells were examined under the light microscope and were photographed with Olympus U-CMAD3 camera. Number of cells at the lower side of the upper chamber was calculated in five random fields under 100X magnification.

$$\text{Percentage of Invaded Cells} = \frac{\text{[Total number of treated (invaded) cells]}}{\text{[Total number of untreated (invaded) cells]}} \text{ X } 100\%$$

2.8.3 Wound healing assay

The wound migration assay is a simple and inexpensive method which allows dual studies; cancer cell migration and cell-cell interactions. This assay was performed

to confirm previous findings in transwell migration assay and allows investigation of cancer cell-cell interaction after *Phyllanthus* treatment. Cancer cells (MeWo and PC-3) were seeded at $1.0x10^5$ cells per well in a 24-wells plate (Corning, USA) and cultured until sub-confluence (80-90%). The cells were washed with PBS before substitution of serum free media. After 24 hours, the plates were scraped firmly with the tip of a disposable pipette to generate an acellular 1-mm-wide lane per well, known as denuded zone or wound on the cell monolayer. After washed with sterile PBS, cells were further incubated with various concentrations of *Phyllanthus* extract for 24 hours for PC-3 cells and 48 hours for MeWo cells at 37°C. At the end of incubation time, the cells were photographed (Olympus μ1040) and the numbers of migrated cells into the denuded zone were calculated using ImageJ software. At least five different random fields were quantified for cell migration.

Percentage of Migrated Cells Acrossed into the Denuded Zone

$$= \frac{\text{[Total number of treated migrated cells]}}{\text{[Total number of untreated migrated cells]}} \text{ X } 100\%$$

2.8.4 Cancer Cell (Cell-Matrix) Adhesion assay

The interaction of cancer cells with extracellular matrix (ECM) proteins such as fibronectin, laminin and collagen is important to enables cancer cells to adhere, invade and metastasize (Hay, 1991). Disruption of these interactions could halt cancer progression. Cell-matrix adhesion assay was performed to study the interaction of PC-3 cells to type-IV collagen and MeWo cells to fibronectin after treatment with *Phyllanthus* extracts. Ninety-six-well plate was pre-coated with adhesion molecules; (1) 50 μg/ml of type-IV collagen (Sigma-Aldrich, Ireland Ltd) for PC-3 cells and (2) 50

μg/ml of fibronectin for MeWo cells, for 2 hour at 37°C. The pre-coated paltes were incubated with 1% bovine serum albumin (BSA) (Sigma-Aldrich, Ireland Ltd) to block nonspecific binding, for an hour prior to the experiment. The *Phyllanthus*-treated and untreated cancer cells were harvested, suspended into new media and seeded into the pre-coated plates and incubated for an hour at 37°C. The non-adherent cells were then removed with two washes of sterile PBS. The number of adhered cells was then quantified with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, USA). Background values of the seeded cells with fibronectin (for MeWo cells) and type-IV collagen (for PC-3 cells) was subtracted from with the wells coated with adhesion molecule alone (blank).

$$\text{Percentage of Adherent Cells} = \frac{[\text{Mean OD of the test cells} - \text{Mean OD of the blank}]}{[\text{Mean OD of the untreated cells} - \text{Mean OD of the blank}]} \times 100\%$$

2.8.5 Tumour Transendothelial Migration Assay

Adhesion and migration of tumour cells on and across the vascular endothelium, known as transendothelial migration is a critical step of the metastatic cascade (Lee *et al.*, 2003b). This cascade step was studied via the transendothelial migration assay. Fibronectin (50 μg/ml) was pre-coated onto the upper chamber of transwell plate for 2 hours at 37°C. HUVECs ($5x10^4$ cells/well) were then added onto the fibronectin-coated chamber and cultured for 72 hours to allow formation of cell monolayer. Pre-treated cancer (MeWo and PC-3) cells were suspended in serum-free media and were added onto HUVECs monolayer and allowed to incubate for 16 hours before analysis of transmigration. The lower chamber was filled with growth culture media with 10% FBS

used as chemoattractant. The cells on the upper side of the upper chamber filter were removed by cotton-tipped swab. The cells at lower side of the upper chamber were fixed and stained with 0.5% crystal violet as previously described and were examined under the light microscope (Olympus BX41) and photographed with Olympus U-CMAD3 camera. Number of transendothelial migrated cancer cells was calculated from five random fields under 100X magnification.

$$\text{Percentage of Transendothelial Migrated Cells} = \frac{\text{[Total number of treated transendothelial migrated cells]}}{\text{[Total number of untreated transendothelial migrated cells]}} \times 100\%$$

2.8.6 Gelatin and casein Zymographies

Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix (ECM) during the tumour angiogenesis and metastasis (Zetter, 1998). MMPs can be analysed with substrate zymography techniques depending on the degradation of their preferential substrate (Cockett *et al.*, 1998). The activities of MMP-2 and MMP-9 were assessed by gelatin zymography and MMP-7 by casein zymography.

Cancer cells (MeWo and PC-3) cells and HUVECs were pre-treated with various concentrations of *Phyllanthus* extracts (31.3-500.0 μg/ml) for 72 hours. After 72 hours, pre-treated cells were washed twice with sterile PBS and incubated with serum-free culture media for 48 hours. Supernatant was collected and centrifuged at 1,500 rpm for 20 minutes at 4°C to remove cellular debris. The supernatant was collected and stored at -80°C until further analysis. Mixture of supernatant and sample buffer (0.5 M Tris-HCI (pH 6.8), 50% glycerol, 10% SDS and 0.1% bromophenol blue) in ratio 1:1 were loaded and electrophoresis was conducted under non-reducing conditions on 10% SDS

polyacrylamide separating gel containing 0.1% gelatin (for gelatin zymography) or 0.2% of casein (casein zymography). For casein zymography, the gel was electrophoresed twice; first without any samples to remove the excess casein and the second was loaded with samples. After electrophoresis, the gels were washed with 2.5% Triton-X100 for 1 hour to remove SDS. Subsequently, the gels were incubated in renaturing buffer (50 mM Tric-HCI, pH 6.8; 200 mM $NaCI_2$; 5 mM $CaCI_2$) for 16 hours at 37°C. The gels were stained with 0.5% Coomasie Blue for 1 hour at room temperature and then destained with destaining solution (Methanol: acetic acid: water; 2:1:7) until proper contrast was achieved. The detected MMPs bands were measured using ImageJ software and the percentage of MMP activitiy was determined using following formula.

$$\text{Percentage of MMP activity} = \frac{\text{[Mean of treated band intensity]}}{\text{[Mean of untreated band intensity]}} \times 100\%$$

2.9 *In vitro* anti-angiogenesis assay – ECM gel-induced capillary tube formation

The extracellular matrix (ECM) gel-induced capillary tube formation assay is one of the most widely used *in vitro* assays to mimic the reorganization phase of angiogenesis. This assay measures the ability of endothelial cells to form capillary-like tube structures, with the support of extracellular matrix. The resulting tubes will eventually form hollow lumens and become guidance pathways that facilitate tumour cell metastasis (Akhtar *et al.*, 2002). Thus, ECM gel-induced capillary tube formation assay was used to assess the anti-angiogenic effects of *Phyllanthus* extracts. The extracellular matrix (ECM) gel is derived from Engelbreth Holm-Swarm mouse sarcoma and contains various proteins such as laminin, collagen, heparan sulfate

proteoglycan, entactin and other minor components (Sigma-Aldrich, Ireland Ltd). Extracellular-matrix was coated onto sterile 24-well culture plates prior the experiment. Two hundred microliter of ECM (10 mg/ml) was added into each well of a 24-well plate and incubated for 2 hours prior to the experiment.

HUVECs were seeded ($2x10^5$ cells/well) at 6-wells plate for overnight. After incubation, HUVECs were treated with different concentrations of *Phyllanthus* extracts (31.3-500.0 μg/ml) and further incubated for 72 hours. *Phyllanthus*-treated and untreated HUVECs were harvested and seeded into the ECM-coated 24-well culture plate at concentration of $1x10^4$ cells/well and incubated for 16 hours. The formation of microcapillary-like tube structures by HUVECs on ECM gel were observed and photographed (Olympus μ1040) after 16 hours under a phase contrast microscope. The complete formations of microcapillary-like tubes were counted using ImageJ software and percentage of microcappillary-like tubes formed according to following formula.

$$\text{Percentage of Microcapillary-like Tubes Formed} = \frac{\text{[Total number of micro-capillary tubes formed in treated cells]}}{\text{[Total number of micro-capillary tubes formed in untreated cells]}} \times 100\%$$

2.10 Molecular Mechanisms of Anti-carcinogenic Properties of *Phyllanthus* extracts

2.10.1 Dual luciferase pathway reporter transient transfection

Many vital biological processes including uncontrolled proliferation, metastasis, angiogenesis and inhibitions of apoptosis, are tightly regulated by complex signalling networks and signal transduction in cancer cells to ensure its malignancy. Ten different

cancer-related pathways were studied using the Cignal Finder 10-Pathway Reporter Arrays (SA Biosciences, QIAGEN, USA).

Reverse transfection protocol was implemented for this assay. Prior to transfection, 2 µl of Lipofectamine® 2000 transfection reagent (Invitrogen, USA) was diluted into 25 µl of Opti-MEM® Reduced Serum Media and incubated for 5 minutes. Meanwhile, 100 ng of plasmid construct of transcription factor-responsive reporter of each pathway and control were mixed with 25 µl of Opti-MEM® Reduced Serum Media (Invitrogen, USA). After 5 minutes, the diluted plasmid construct of each pathway and control were mixed with diluted Lipofectamine® 2000 in ratio 1:1 and further incubated for 20 minutes at room temperature. Meanwhile, cancer cells (MeWo and PC-3) were washed with PBS, trypsinized with trypsin-EDTA (0.25%) and resuspended to $4x10^5$ cells/ml in Opti-MEM® containing 5% of fetal bovine serum and 1% NEAA. After the 20 minutes incubation time, 50 µl of transfection cocktail was added to $4x10^4$ cells/well and further incubated for 16 hours at 37°C. After transfection, the old media was discarded and the transfected cells were treated with *Phyllanthus* extracts at their respective IC_{50} values in complete growth medium (MEM supplemented with 10% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin) and further incubated for another 24 hours. Each transfection condition was carried out in triplicate.

Each of the pathways/reporters consist an inducible transcription factor responsive firefly luciferase reporter and constitutively expressing *Renilla* construct. *Renilla* construct is to act as an internal control for normalizing transfection efficiencies and monitor cell viability. After 24 hours of *Phyllanthus* treatment, the changes in expression of each pathway were determined by measuring the generated firefly and *Renilla* luminescent signals using the Dual-Glo Luciferase Assay system (Promega, Madison, WI) on Glomax machine (Promega, USA). The fold changes of each

reporter's expression between *Phyllanthus*-treated and untreated cells were calculated from the relative luciferase units, generated from the firefly/*Renilla* luciferase activity ratio.

2.10.2 Western Blot Analysis

After identifying the cancer-related pathways altered by *Phyllanthus* in cancer cells (MeWo and PC-3), the underlying molecular mechanism was investigated. The intracellular signalling molecules in MAPK (pan-Ras, c-Raf, Elk, RSK, c-Jun, JNK1/2, Akt and p38), Wnt (GSK3β, β-catenin and DSH), Myc/Max and Hypoxia (c-myc, HIF-α, and VEGF) and NFκB (p50 and p52) pathways as well as apoptotic (Bcl-2 and Bax) proteins were chosen for western blot analysis to determine their expression upon *Phyllanthus* treatment in cancer cells. All these antibodies were purchased from Merck (USA).

Cancer cells were cultured in 25 cm^2 culture flask until 80-90% confluency. The old medium was discarded and treated with respective IC_{50} value of *Phyllanthus* extracts and incubated for 72 hours. After 72 hours, the floating and trypsin-detached treated cells were collected and spun down at 1,500 rpm for 5 minutes. The cell pellet was lyzed with lysis buffer on ice for 30 minutes. Supernatant was collected after centrifuged at 12,000 rpm for 10 minutes at 4C° to remove cellular debris, and kept at -80°C until further analysis.

Total amount of proteins was determined as described in section 2.11.1, prior to the experiment. Twenty micrograms of protein lysates were mixed with sample buffer in ratio 1:1 and resolved on a 12% SDS-PAGE gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes. Nonspecific binding of the membrane was blocked with Tris-buffered saline (TBS) containing 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for 2 hours. Membranes were washed with TBST for

three times, each with 10 minutes and incubated with appropriate dilution of specific primary antibodies overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated with appropriate secondary antibodies (horseradish-conjugated goat anti-mouse or anti-goat IgG) for 1 hour. Membranes were washed three times with TBST for 10 minutes; and the band was visualized after incubation with chromogenic substrate containing 4-chloro-1-napthol and hydrogen peroxide. Reaction was stopped by washing with distilled water. The membrane was then scanned and ImageJ software was used to measure the band intensity.

$$\text{Percentage of protein expression} = \frac{[\text{Mean of treated band intensity}]}{[\text{Mean of untreated band intensity}]} \times 100\%$$

2.11 2-Dimensional Gel Electrophoresis for Protein Identification

2.11.1 Sampel Preparation and Protein Quantification

Total proteins were extracted from untreated and treated groups by incubation with lysis buffer on ice for 30 minutes. The protein pellet was re-solubilized in rehydration solution (8 M urea, 2% CHAPS, 40 mM DTT, 0.5% IPG buffer pH 3-11NL, bromophenol blue) and kept at -80°C until further analysis. Total amount of proteins was determined using 2-D Quant kit (GE Healthcare Bio-Sciences, USA). This assay is based on the specific binding of copper ions to protein. The colour intensity is directly proportional to the protein concentration. The working colour reagent was prepared by mixing colour reagent A and B in ratios 1:100.

Table 2:1: Standard curve for protein quantification

Tube number	1	2	3	4	5	6
Volume of 2 mg/ml BSA standard solution	0 µl	5 µl	10 µl	15 µl	20 µl	25 µl
Protein quantity	0 µg	10 µg	20 µg	30 µg	40 µg	50 µg

Six tubes were prepared accoding to Table 2.1 to construct a standard curve for protein quantification. Meanwhile, 10 µl of tested sample was aliquoted into a tube and labelled as sample tube. Five hundred microliters of precipitant was added into each standard and sample tube, and incubated for 2-3 minutes at room temperature. Five hundred microliter of co-precipitant was added into each tube. Tubes were then centrifuged at 10,000xg for 5 minutes and supernatant was completely removed. Five hundred microliters of diluted copper solution was added into each tube to dissolve the protein pellet. Working colour reagent was prepared by diluting concentrated copper solution into de-ionized water in ratios 1: 4. One milliliter of working colour reagent was added to each tube and mixed. The tubes were then allowed to stand at room temperature for 15 minutes. One hundred microliters of samples was transferred into a well of 96-wells flat bottom plate. At least three replications of each sample were prepared and absorbance was read at 480 nm. A standard curve for absorbance of the standard against protein amount was generated and used to determine the protein amount in sample.

2.11.2 First and Second Dimensional Separation

A total of 500 mg of total proteins were rehydrated into 13 cm immobilized pH gradient (IPG) strips (pH 3-11 nonlinear) (GE Healthcare, USA). The first dimension was run on the IPGphor III machine (GE Healthcare, USA) at 20°C with the following

settings: step 1 at 500V for 1 hour; step 2 at 500-1000V for 1 hour; step 3 at 1000-8000V for 2.5 hours, and step 4 at 8000V for 0.5 hour.

Upon completion of first dimensional separations, the strip was equilibrated as following; first reduction with 64.8 mM of dithiothreitol-SDS equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) for 15 minutes, followed by alkylation with 135.2 mM of iodoacetamide-SDS equilibration buffer for another 15 minutes. The second dimension electrophoresis was performed by electrophoresing the samples in 12.5% SDS acrylamide gels by using the SE600 Ruby system (GE Healthcare) at 25°C in an electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% [wt/vol] SDS) with the following settings: step 1 at 100V/gel for 45 minutes; step 2 at 300V/gel until the run is completed. After electrophoresis, the gels were fixed with destaining solution for 30 minutes, followed by staining with hot Coomasie blue for 30 minutes. Lastly, the gels were scanned using Ettan DIGE Imager (GE Healthcare). Gel images were analyzed using PDQuest 2-D Analysis Software (Bio-Rad, USA) and only protein spots which showed significant differences (more than 1.0 fold) were selected for mass spectrometry analysis.

2.11.3 Protein Digestion, Desalting and MALDI-TOF/TOF Analysis

The significant protein spots were manually excised from polyacrylamide gels and were kept in sterile 1.5 ml eppendorf tubes. Excised spots (gel plugs) were washed with destaining solution (50% Acetonitrile (ACN) in 50 mM NH_4HCO_3) until the gel plugs are clear. The destaining solution was discarded and the gel plugs were then incubated with reducing solution (100 mM ammonium bicarbonate (NH_4HCO_3) containing 10 mM dithiothreitol (DTT)) for 30 minutes at 60°C. Then, the gel plugs were alkylated with 100 mM NH_4HCO_3 containing 55 mM iodoacetamine for 20 minutes in the dark and followed with three times washing with 50% acetone in 100

mM NH_4HCO_3 for 20 minutes each. The gel plugs were rehydrated with 100% ACN and dried in speed vacuum concentrator (Savant SpeedVac®, Thermo Scientific, USA). In-gel digestion using trypsin gold (Promega, Mass Spectrometry Grade) in 50 mM NH_4HCO_3 was added into gel plug and incubated overnight in 37°C. Proteins were extracted from gel plugs and purified by Ziptip (Ziptip C18, Millipore, Bedford, MA, USA). The eluted proteins were mixed with MATRIX solution and spotted on MALDI plate using dry droplet method and analysed using Ab Sciex Tof/Tof™ instruments. The generated peptides were blasted with MASCOT Search Algorithm (Version 2.1.0) to identify the possible proteins.

2.12 Statistical analysis

From all the experiments, results were expressed as mean ± standard error (SEM) of data obtained from three independent experiments using SPSS software (version 16). The Student *t*-test, followed by Dunnett's test were used where values of $*p<0.05$ and $**p<0.01$ were considered significant.

CHAPTER 3: RESULTS

3.1 Identification of Bioactive Compounds

The bioactive compounds in both aqueous and methanolic extracts of four species of *Phyllanthus* plant were identified by subjecting the extracts to HPLC (High Performance Liquid Chromatography) analysis coupled with photodiode array (PDA) and MS/MS detection. Twelve main bioactive compounds were identified based on their retention time, UV spectra, parent mass spectra and secondary fragmentation patterns. These bioactive compounds are comprised of ten water-soluble compounds and four methanol-soluble compounds (Table 3.1).

In aqueous crude extracts of *Phyllanthus*, all the species consist of at least eight bioactive compounds and seven of them are identical; gallic acid, galloylglucopyronoside, corilagen, geraniin, rutin, quercetin glucoside and caffeolquinic acid. Among these identified bioactive compounds, geraniin is the only compound which can be found in both aqueous and methanolic extracts (Table 3.1). *P.urinaria* contains the highest number of bioactive compounds, followed by *P.watsonii, P.amarus* and *P.niruri*.

Table 3:1: Bioactive compounds in aqueous and methanolic extracts of *Phyllanthus* species (*P.A: P.amarus, P.N: P.niruri; P.U: P.urinaria* and *P.W: P.watsonii*) (Tang *et al.*, 2010).

	Compound	**Retention time**	**[M-H] m/z**	**MS-MS Fragment-ation**	***Phyllanthus species***
Aqueous extract	Gallic acid	3.8	169	125,169	*P.A, P.N, P.U, P.W*
	Galloyl-glucopyronoside	2.8	331	125,169,211, 271	*P.A, P.N, P.U, P.W*
	Corilagen	18	633	301, 125, 169	*P.A, P.N, P.U, P.W*
	Geraniin	22	951	301, 125, 169, 463	*P.A, P.N, P.U, P.W*
	Rutin	26	609	301, 179,151	*P.A, P.N, P.U, P.W*
	Quercetin glucoside	27	463	301, 179,151	*P.A, P.N, P.U, P.W*
	Caffeolquinic acid	23	353	191	*P.A, P.N, P.U, P.W*
	Digalloyl-glucopyronoside	15.0	483	125,169,211, 271, 313	*P.A, P.N, P.W*
	Quercetin rhamnoside	30	447	301, 151	*P.U, P.W*
	Trigalloyl-glucopyronoside	23	635	125,169,211, 271, 313,465	*P.U*
Methanolic extract	Geraniin	12	951	301, 125, 169, 463	*P.A, P.N, P.U, P.W*
	Trigalloyl-glucopyronoside	13	635	125,169,211, 271, 313,465	*P.U*
	Tetragalloyl-glucopyronoside	15	787	169,211, 313, 465	*P.U*
	Quercetin diglucoside	9	625	463, 301	*P.N*

3.2 Anti-proliferative effect of *Phyllanthus* extracts, fractions and standard anticancer drugs on growth of cells

The MTS assay was used to investigate the potential anti-proliferative (cytotoxic) effect of *Phyllanthus'* crude extracts and their fractions on different cancer (MeWo and PC-3) and normal cells (RWPE-1, CCD-1127Sk and HUVECs), where the cells were treated at different concentrations ranging 31.3 to 500.0 µg/ml for 72 hours. In this study, two standard anticancer drugs namely, 5'Flurouracil (5'FU) and Doxorubicin (Dox) were used as positive controls.

3.2.1 Human melanoma (MeWo) cells

For aqueous extracts, the IC_{50} values were in the range of 160.0-260.0 µg/ml and 56.2-153.3 µg/ml for methanolic extracts of *Phyllanthus*. As presented in Table 3.2, methanolic extracts showed stronger anti-proliferative effect on MeWo cells, as their IC_{50} values were lower than aqueous extracts. *P.urinaria* showed the strongest anti-proliferative effect on MeWo cells with the lowest IC_{50} value at 160.0 µg/ml ($p<0.05$) for aqueous and 56.2 µg/ml ($p<0.05$) for methanolic extracts. As compared to standard anticancer drugs, both 5'Fluorouracil and doxorubicin showed much stronger anti-proliferative effect on both MeWo and normal skin cells (CCD-1127Sk) with an IC_{50} values of 2.5 µg/ml ($p<0.05$) and 1.0 µg/ml ($p<0.05$), respectively.

Fractions (fraction 1 and 2) of crude aqueous extract of *Phyllanthus* were also evaluated for its cytotoxicity. The IC_{50} values of fraction 1 of *P.niruri*, *P.urinaria* and *P.watsonii* were in the range of 433.0–460.0 µg/ml on MeWo cells, and *P.amarus* did not show any anti-proliferative effect. On the other hand, fraction 2 of all *Phyllanthus* species exhibited lower IC_{50} values (220.0-240.0 µg/ml) as compared to fraction 1. Both

fractions exhibited toxicity towards the normal skin cell line (CCD-1127Sk) but not affected by both aqueous and methanolic extracts of *Phyllanthus* as shown in Table 3.2.

Table 3:2: The IC_{50} values of *Phyllanthus* extracts on human skin cancer (melanoma, MeWo) and normal (CCD-1127Sk) cell lines. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

		IC_{50} ± SEM (µg/ml)	
Phyllanthus species	Extracts	Skin cells	
		Cancer (MeWo)	Normal (CCD-1127Sk)
P.amarus	Aqueous	193.3 ± 1.3	> 500
	Methanolic	133.3 ± 2.9	> 500
P.niruri	Aqueous	260.0 ± 2.4	> 500
	Methanolic	153.3 ± 2.6	> 500
P.urinaria	Aqueous	193.3 ± 1.1	> 500
	Methanolic	56.2 ± 3.2	> 500
P.watsonii	Aqueous	160.0 ± 3.2	> 500
	Methanolic	100.7 ± 2.0	> 500
Positive control	5'Fluorouracil	2.3 ± 0.5	0.8 ± 0.5
	Doxorubicin	2.5 ± 0.5	1.0 ± 0.2
Fraction 1 (Aqueous)	*P.amarus*	> 500	> 500
	P.niruri	433.3 ± 16.9	376.2 ± 21.2
	P.urinaria	460.0 ± 16.4	392.2 ± 26.2
	P.watsonii	437.3 ± 23.4	394.2 ± 31.3
Fraction 2 (Aqueous)	*P.amarus*	248.2 ± 21.4	114.2 ± 9.4
	P.niruri	243.9 ± 21.3	99.2 ± 14.2
	P.urinaria	220.2 ± 10.2	108.4 ± 14.4
	P.watsonii	225.2 ± 22.0	119.3 ± 21.4

3.2.2 Human Adenocarcinoma (PC-3) cells

Aqueous extracts of *Phyllanthus,* exhibited IC_{50} values in the range of 155.0-178.3 µg/ml ($p<0.05$), while for the methanolic extracts, the IC_{50} values were at ranged 54.2-117.7 µg/ml ($p<0.05$) (Table 3.3). In comparison, methanolic extracts showed stronger anti-proliferative effect on PC-3 cells as their IC_{50} value were lower than that of aqueous extracts. Overall, *P.urinaria* showed the strongest anti-proliferative effect on PC-3 cells as compared to other *Phyllanthus* species. Both 5'Fluorouracil and doxorubicin showed stronger anti-proliferative effect than *Phyllanthus* on PC-3 and normal prostate cells (RWPE-1) with IC_{50} values of 2.5 µg/ml ($p<0.05$) and 1.0 µg/ml ($p<0.05$), respectively.

Semi-purification of crude aqueous extract of *Phyllanthus* yielded two fractions (fraction 1 and 2). Both fractions were evaluated for their cytotoxicity on PC-3 and RWPE-1 cell lines. The IC_{50} values of fraction 1 for *P.niruri*, *P.urinaria* and *P.watsonii* were in the range of 456.0–497.7 µg/ml, but undetected in *P.amarus*-treated cells. On the other hand, the IC_{50} values of fraction 2 for all *Phyllanthus* species were lower compared to fraction 1 which at ranged 185.4–228.4 µg/ml. The normal prostate cell line (RWPE-1) was affected by fraction 1 and 2 but not affected by both aqueous and methanolic extracts of *Phyllanthus* as shown in Table 3.3.

Table 3:3: Cytotoxicity (IC_{50} ± SEM) values of *Phyllanthus* extracts on human prostate adenocarcinoma (PC-3) and normal (RWPE-1) cell lines. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

		IC_{50} ± SEM (µg/ml)	
Phyllanthus species	Extracts	Prostate cells	
		Cancer (PC-3)	Normal (RWPE-1)
P.amarus	Aqueous	178.3 ± 2.8	> 500
	Methanolic	84.3 ± 1.1	> 500
P.niruri	Aqueous	155.0 ±1.2	> 500
	Methanolic	117.7 ± 2.1	> 500
P.urinaria	Aqueous	155.7 ± 2.1	> 500
	Methanolic	54.2 ± 2.1	> 500
P.watsonii	Aqueous	156.7 ± 2.4	> 500
	Methanolic	100.5 ± 1.2	> 500
Positive control	5'Fluorouracil	1.0 ± 0.3	1.0 ± 0.5
	Doxorubicin	2.5 ± 0.5	1.0 ± 0.1
Fraction 1 (Aqueous)	*P.amarus*	> 500	> 500
	P.niruri	483.3 ± 21.1	302.5 ± 21.2
	P.urinaria	456.0 ±31.2	315.6 ± 13.2
	P.watsonii	497.7 ±2 2.1	323.4 ± 17.6
Fraction 2 (Aqueous)	*P.amarus*	228.4 ± 9.4	108.4 ± 21.4
	P.niruri	214.1 ± 12.5	116.2 ± 26.2
	P.urinaria	185.4 ± 21.4	132.6 ± 25.7
	P.watsonii	191.5 ± 13.0	115.2 ± 22.0

3.2.3 Human Umbilical Vein Endothelial Cells (HUVECs)

Endothelial cells play an important role in the development of new blood vessels. The excessive proliferation and transformation of endothelial cells would lead to pathological angiogenesis, which is described as one of the hallmarks of cancer. The anti-proliferative effect of *Phyllanthus* extracts on HUVECs was exerted in the range of 125.0-500.0 µg/ml. However, *Phyllanthus* showed low cytotoxicity against HUVECs was observed at the maximal dose of all extracts (both aqueous and methanolic) 500.0 µg/ml (Figure 3.1).

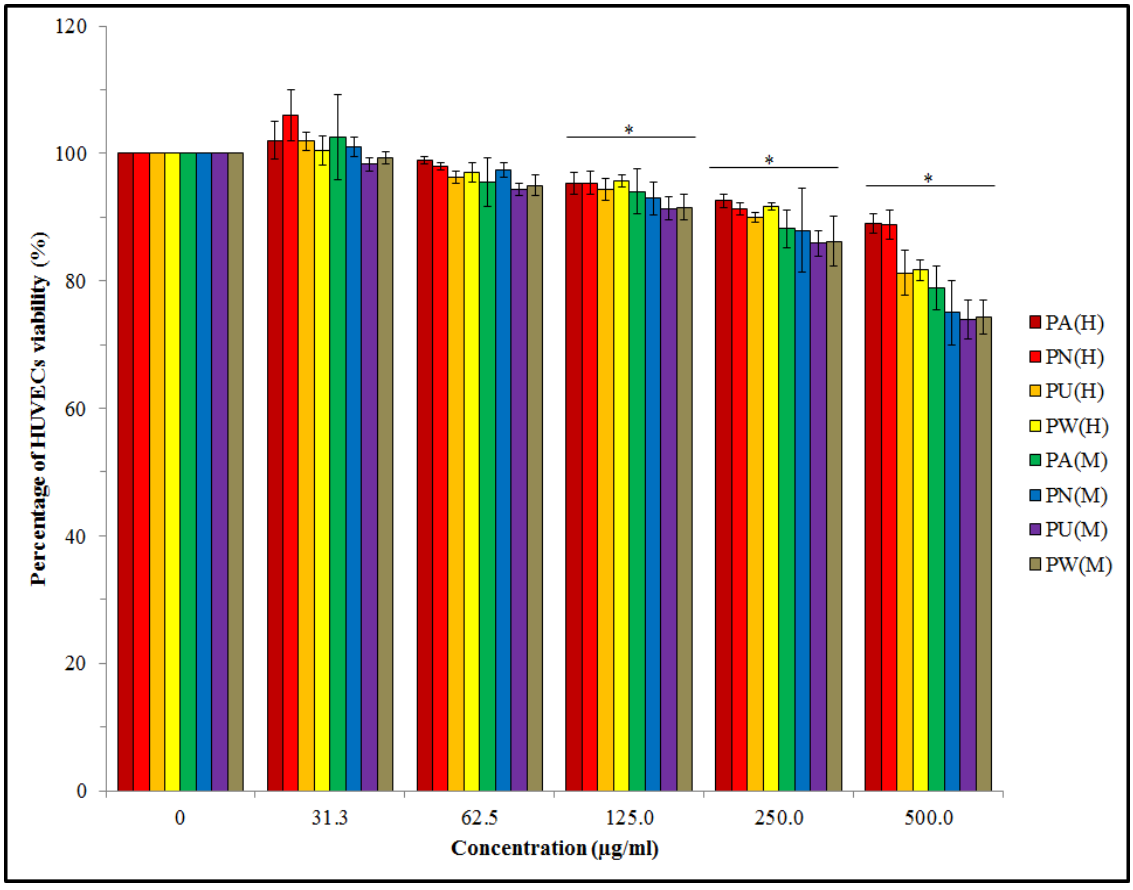

Figure 3.1: Anti-proliferative effect of *Phyllanthus* extracts on the growth of HUVECs.

Figure shows HUVECs were treated with various concentrations of *Phyllanthus*. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated and *Phyllanthus*-treated cells, *p<0.05.

3.3 Induction of Cancer Cell Cycle Arrest

Cell cycle is a biological process that involves a sequence of molecular events to ensure correct transmission of the genetic material to subsequent generations. Defects in cell cycle can lead to genetic modification (mutation) and hence develop uncontrolled cell proliferation. An uncontrolled proliferation and the ability to evade apoptosis by cancer cells are the hallmarks of cancer (Hanahan & Weinberg, 2011). Targeting the cell cycle could be an approach for anticancer agents to halt the uncontrolled proliferation of cancer cells and initiate them to undergo apoptosis.

The growth arrest on cell cycle and apoptotic induction by *Phyllanthus* extracts on cancer (MeWo and PC-3) cells were assessed using propidium iodide DNA incorporation with flow cytometry. Both MeWo and PC-3 cells were treated with respective IC_{50} value of *Phyllanthus* extracts for 24, 48, 60 and 72 hours. Changes in the distribution in cell cycle were observable by 24 hours after being treated with *Phyllanthus* extracts for both cell lines.

3.3.1 S-phase arrest in human melanoma (MeWo) cells

Phyllanthus extracts exhibited anti-proliferative effect on MeWo cells by growth arrest at the S-phase at 24 hours and remained evident after 72 hours of treatment. This was accompanied by accumulation of cells in Sub-G1 (apoptotic cells) phase for both aqueous and methanolic extracts (Figure 3.2). The percentage of apoptotic cells had increased in a time-dependent manner from 1.8% at 24 hours to 6.1% at 72 hours as compared to the untreated cells. Meanwhile, the percentage of cells at the S-phase of treated MeWo cells was elevated to 15.0% above the controls at 72 hours of treatment. Furthermore, the percentage of cells at Go/G1 and G2/M phases decreased with time upon treatment with *Phyllanthus* extracts due to the fact that treated cells have been

arrested at S-phase and subsequently accumulated at Sub-G1 (apoptosis) phase. However, the potency of *Phyllanthus* extracts to induce S-phase arrest was not as strong as the standard anticancer drug (5'Fluorouracil), with a 22.1% difference at 72 hours post treatment.

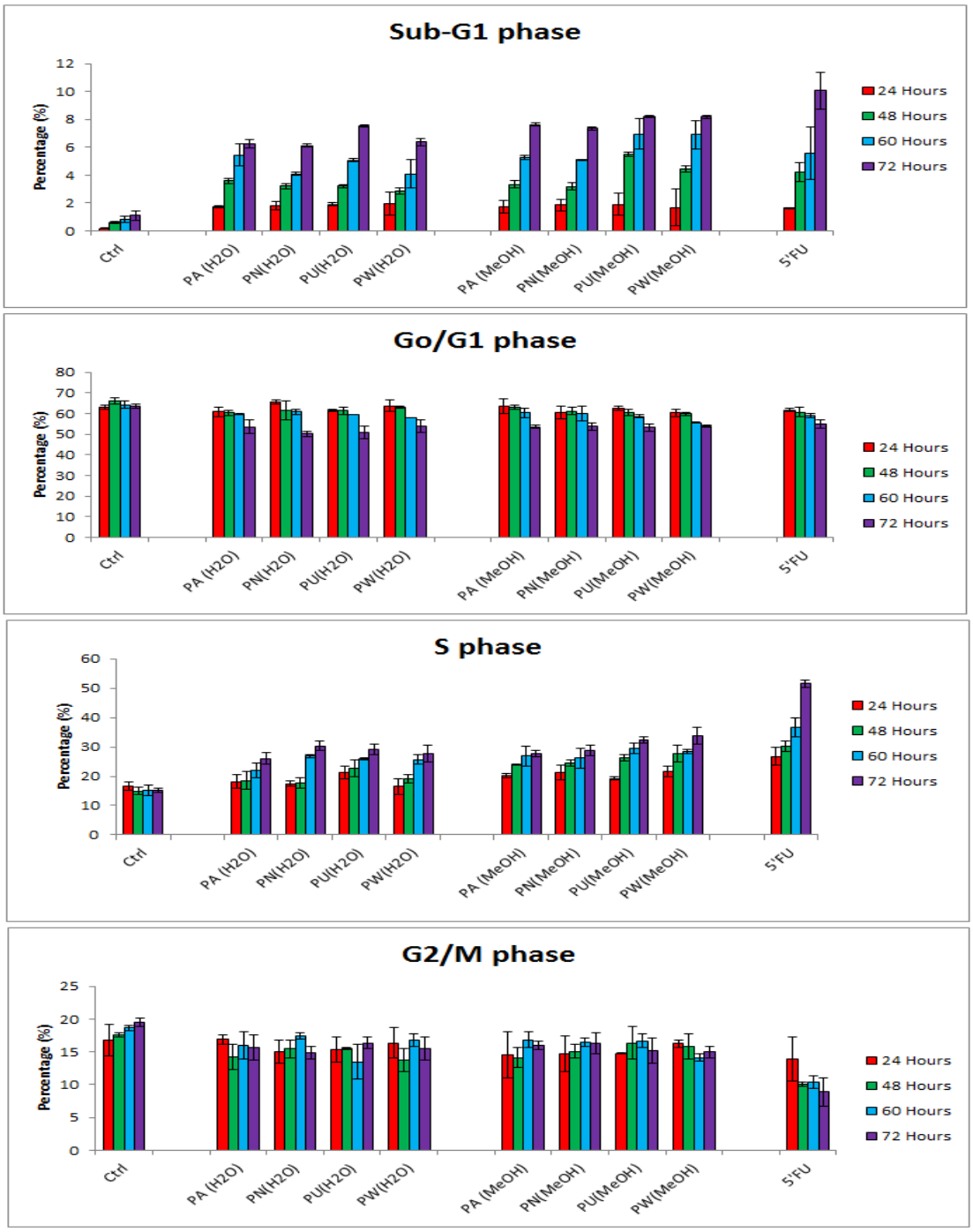

Figure 3.2: The kinetic of cell cycle distribution of *Phyllanthus* extracts-treated MeWo cells.

Figure shows the percentage of *Phyllanthus* extracts-treated cells at Sub-G1, Go/G1, S, G2/M phases of MeWo cells at different time intervals (24, 48, 60 and 72 hours) of treatment. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

3.3.2 G1-phase arrest in human prostate adenocarcinoma (PC-3) cells

Phyllanthus extracts exhibited anti-proliferative effect by disrupting the cell cycle distribution of treated PC-3 cells and arresting them at the G1-phase with an accumulation of apoptotic cells at the Sub-G1 (apoptosis) phase (Figure 3.3). The accumulation of apoptotic cell was 3.4% at 24 hours increased up to 7.4% at 72 hours ($p<0.05$), as compared to the untreated cells. The percentage of treated PC-3 cells at Go/ G1-phase was 13.7% at 24 hours and this has increased to 18.8% at 72 hours as compared to the untreated cells ($p<0.05$). However, the percentage of treated-PC-3 cells at the S and G2/M phases decreased with time of treatment due to the fact that treated PC-3 cells were arrested at Go/G1 phase and subsequently accumulated at Sub-G1 phases. The standard drug, doxorubicin showed a G2/M phase arrest on PC-3 cells at 24 hours and remained evident after 72 hours of treatment.

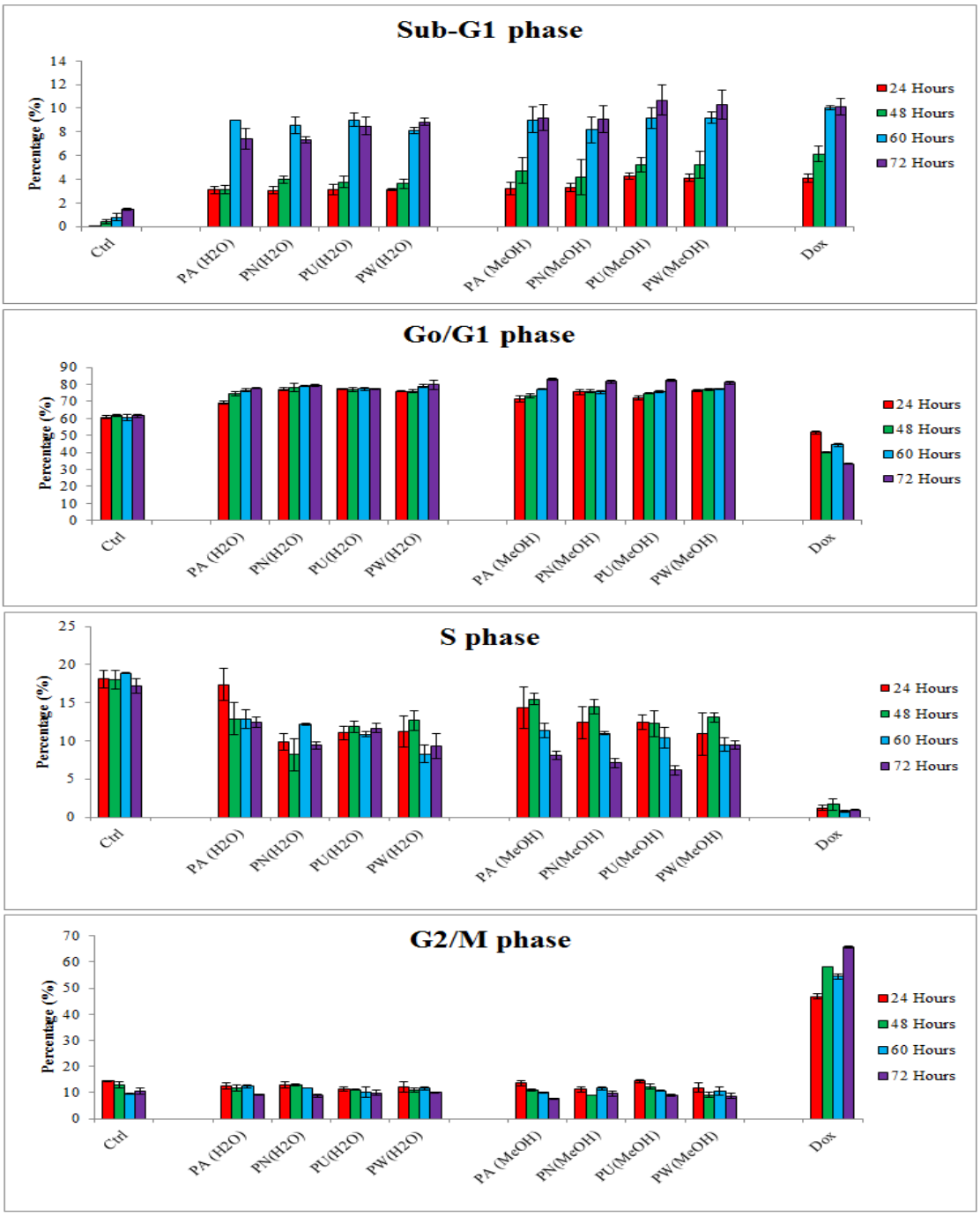

Figure 3.3: The kinetic of cell cycle distribution of *Phyllanthus* extracts-treated PC-3 cells.

Figure shows the percentage of *Phyllanthus* extracts-treated cells at Sub-G1, Go/G1, S, G2/M phases of PC-3 cells at different time intervals (24, 48, 60 and 72 hours) of treatment. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

3.4 Mechanism (s) of Cancer Cell Death

3.4.1 *Phyllanthus* induced Caspases-3/7 Activation

Activation of caspases (aspartate specific cysteine protease) is one of the biochemical changes that occur during apoptosis. Caspase-3 and -7 were chosen to be studied because they can be activated by both the death receptor and mitochondrial pathways (Cohen, 1997). Furthermore, they are the major executor class of caspases and both are essential for the induction of DNA fragmentation as well as apoptosis (Nagata, 2000). *Phyllanthus* extrcats induced caspases-3/7 activation in both PC-3 and MeWoc ells. The levels of caspases-3/7 induced by *Phyllanthus* treatment were markedly increased (3–4 folds) as compared to the control group (Figure 3.4) for both extracts of *Phyllanthus*. The level of caspases-3/7 induced by standard drugs (5'Fluorouracil and doxorubicin) in MeWo and PC-3 cells were 6-folds and 0.5-folds higher than control group (untreated cells) and *Phyllanthus*-treated cells, respectively. These indicates that apoptosis induced by *Phyllanthus* extracts was mediated via activation of caspases.

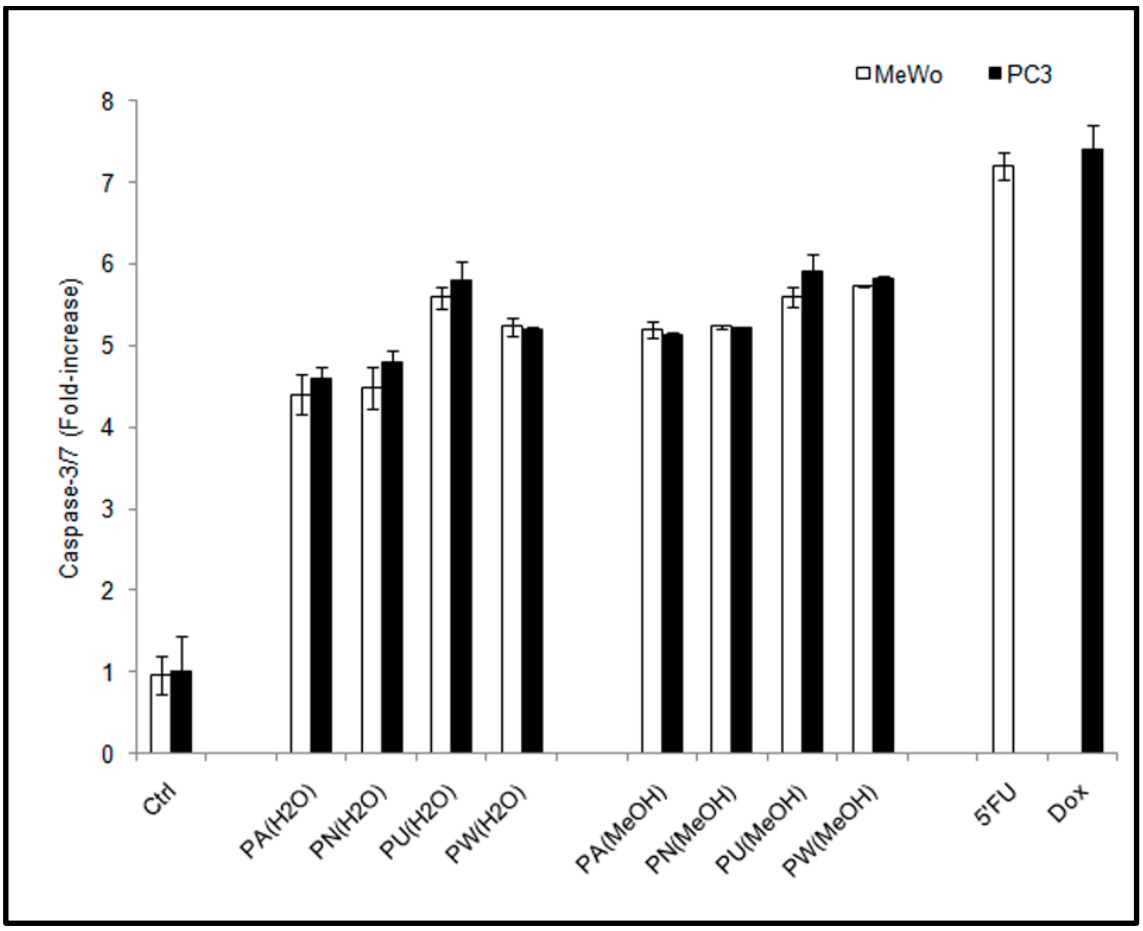

Figure 3.4: The level of caspases-3/7 in cells treated with *Phyllanthus* extracts and standard drugs).

Figure shows the data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

3.4.2 *Phyllanthus* induced DNA Fragmentation in Cancer Cells

Caspases activation is followed by DNA fragmentation (Nagata, 2000). Therefore, early and late detections of DNA fragmentation in treated cancer cells were performed with TUNEL assay and agarose gel electrophoresis, respectively.

3.4.2.1 TUNEL labeling of DNA Fragmentation

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay is a technique to allow detection of apoptotic cells by labelling the free end of apoptotic DNA with a marker which can be visualized under light microscope. Apoptotic cells were observed in *Phyllanthus* extracts-treated MeWo (Figure 3.5A, red arrow) and PC-3 (Figure 3.5B, red arrow) cells as brown-colour cells and its appearance was similar to apoptotic cells were present in positive control, apoptotic-inductive anticancer drugs (5'Fluorouracil and Doxorubicin). Viable cells were stained in blue colour.

The populations of cell death can be calculated and expressed in as an apoptotic index. The percentage of apoptotic cells (AI) of treated-MeWo and PC-3 cells were markedly increased up to 50% compared to the untreated cells (control group) at 72 hours of treatment with *Phyllanthus* extracts ($p<0.05$) (Figure 3.6). Only 8% difference in AI values between *Phyllanthus* and standard drugs ($p<0.05$) were noted indicating that the potential of causing apoptotic cell death by *Phyllanthus* was close to standard anticancer drugs.

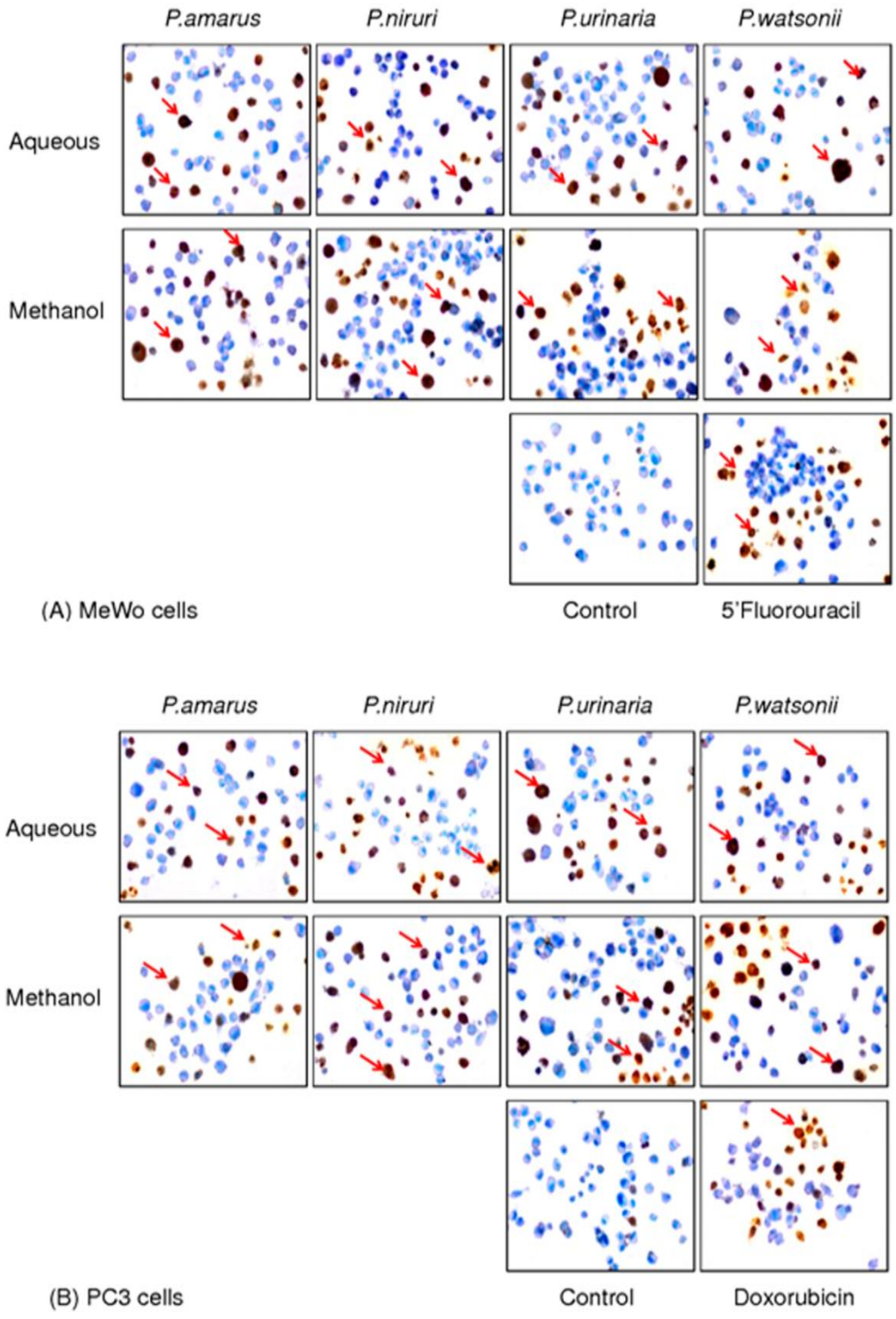

Figure 3.5: TUNEL analysis of MeWo and PC-3 cancer cells after treatment with *Phyllanthus* extracts with magnification 100X.

Figure shows TUNEL-positive (apoptotic) cells were observable as brown staining cells (red arrow) in *Phyllanthus* extract-treated (A) MeWo and (B) PC-3 cells and normal viable cells stain as blue colour (Tang *et al.*, 2010).

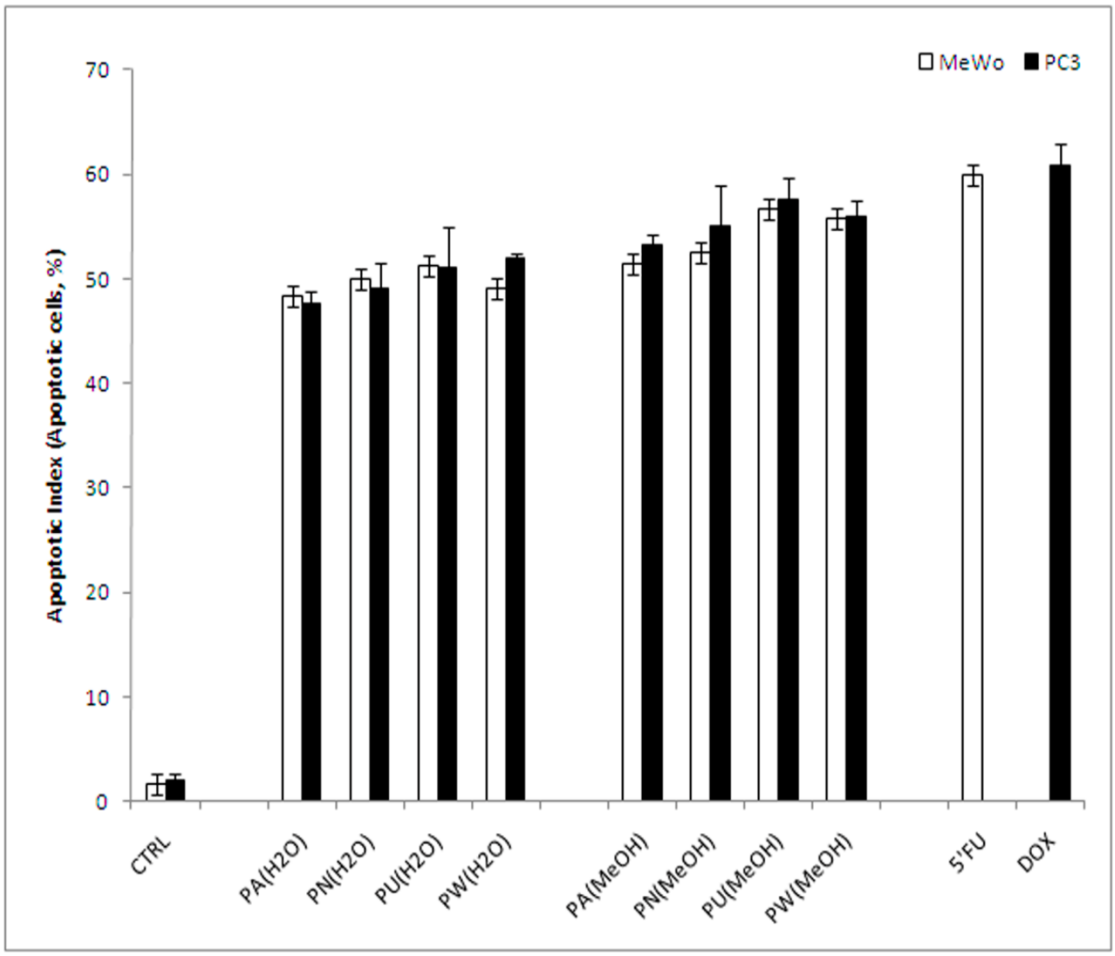

Figure 3.6: Percentage of apoptotic index (%) of untreated and treated (*Phyllanthus* extracts and anticancer drugs) of MeWo and PC-3 cancer cells from TUNEL analysis.

Figure shows the data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

3.4.2.2 DNA ladder formation

The late detection of DNA fragmentation in cells treated with *Phyllanthus* extracts was studied using agarose gel electrophoresis. Analysis of DNA fragments in apoptotic cells by agarose gel electrophoresis will produce a characteristic DNA ladder which is described as biochemical hallmarks of apoptosis (Cohen, 1997; Nagata, 2000).

DNA content from *Phyllanthus* treated cells was extracted and electrophoresed on agarose gel. The typical ladder DNA fragments were observed in *Phyllanthus* extracts-treated MeWo (Figure 3.7A) and PC-3 (Figure 3.7B) cells on agarose gel under a UV transiluminator. This similar pattern was seen with standard anticancer drugs, which possess apoptotic induction, indicating the ability of *Phyllanthus* extracts to induce apoptosis on MeWo and PC-3 cells.

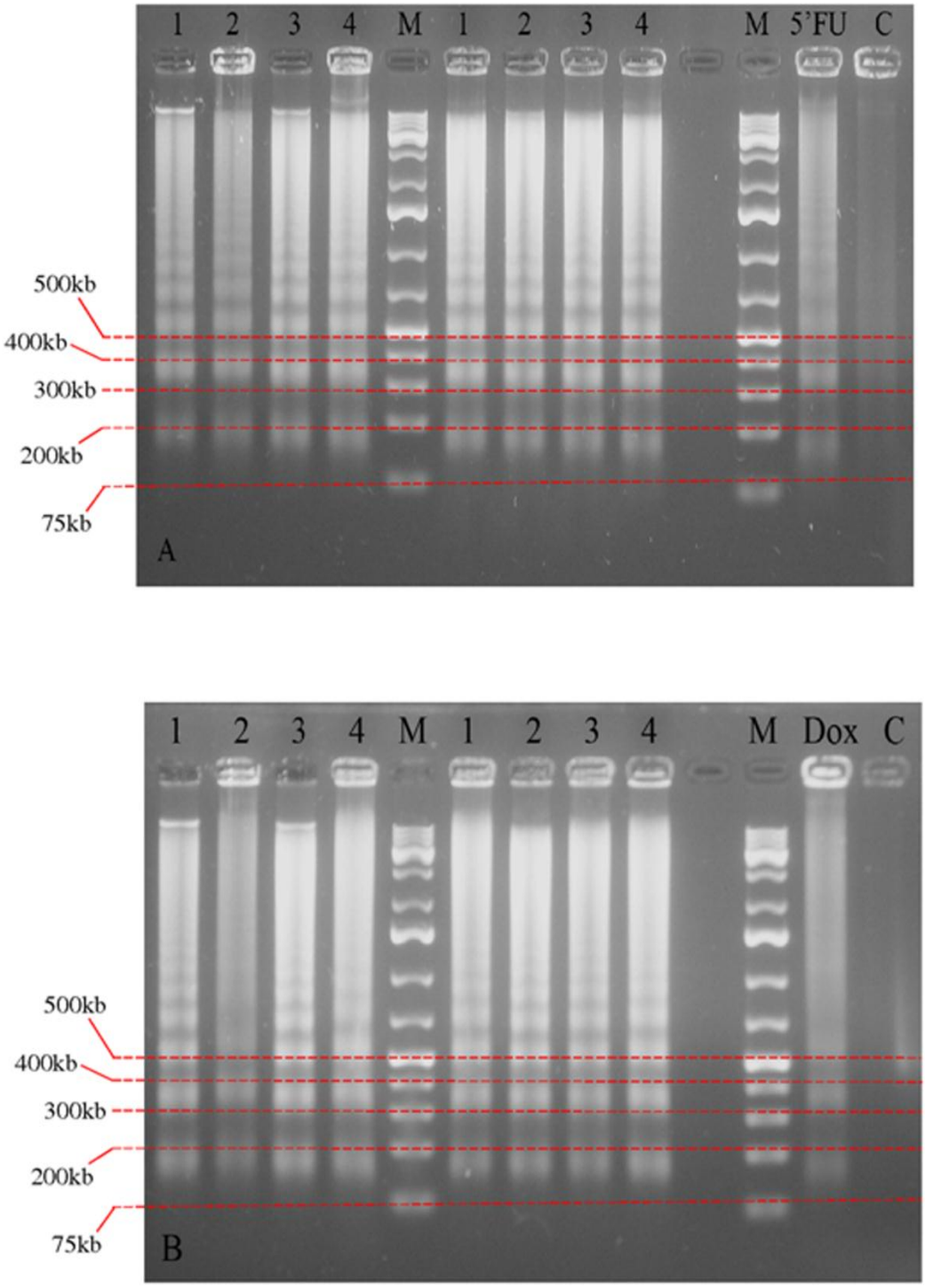

Figure 3.7: Apoptotic DNA fragmentation was observable in *Phyllanthus* extracts-treated (A) MeWo and (B) PC-3 cells.

Figure shows Lane 1 -4: aqueous extracts and Lane 6 – 9: methanol extracts for *P.amarus, P.niruri, P.urinaria and P.watsonii*, orderly. Lane 5 and 10: 1kb DNA marker, Lane 11: standard drugs, where (A) 5'Fluorouracil for MeWo and (B) Doxorubicin for PC-3 cells. Lane 12: untreated cells (Tang *et al.*, 2010).

3.4.3 *Phyllanthus* induced changes in membrane integrity by LDH release

One of the biochemical changes during necrosis is leakage of lactate dehydrogenase (LDH) enzyme. The determination of necrotic-induction of *Phyllanthus* was performed by measuring LDH levels in treated cancer cells by using CytoTox-One Homogeneous Membrane Integrity Assay. This is a fluoremetric assay to allow measurement of the release of LDH from cells with damaged membrane which is associated with necrotic cell death (Loo & Rillema, 1998; Denecker *et al.*, 2001). LDH released into the culture medium was measured based on the conversion of resazurin into resorufin through enzymatic activity.

3.4.3.1 LDH Levels in MeWo cells

In untreated cells, the LDH level was measured at 1.2% ($p<0.05$). However, LDH levels were noticed to increase in MeWo cells after treatment with *Phyllanthus* extracts (Figure 3.8). For aqueous extracts, the LDH levels in MeWo cells were induced by *P.niruri*, *P.urinaria* and *P.watsonii* in the range from 8.5 to 9.0% ($p<0.05$) and 5.1% by *P.amarus* ($p<0.05$). This indicates that the aqueous extracts of *P.amarus* possessed the lowest necrotic effect among aqueous extracts in MeWo cells.

For methanolic extracts, the LDH leakage induced by *P.amarus*, *P.urinaria* and *P.watsonii* ranged from 7.3 to 7.8%, and 8.3% by *P.niruri* ($p<0.05$). The methanolic extract of *P.niruri* possessed strongest necrotic effect among the methanolic extracts on MeWo cells. However, the percentage of LDH levels induced by 5'FU was much stronger at 30.0% ($p<0.05$).

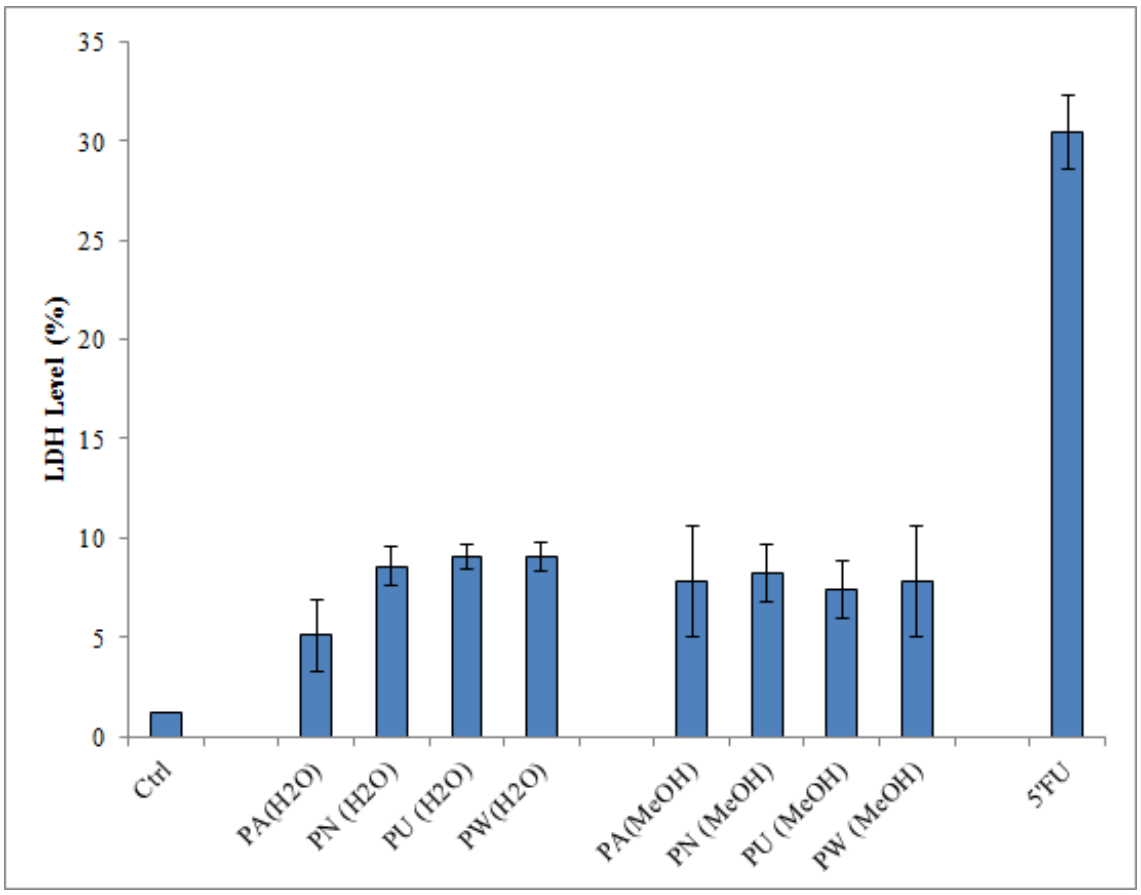

Figure 3.8: The percentage of LDH levels in treated and untreated MeWo cells.

Figure shows the LDH levels in the treated group was higher than the untreated (control) group. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

3.4.3.2 LDH Levels in PC-3 cells

The LDH levels produced by untreated PC-3 cells were 2.1%. ($p<0.05$) (Figure 3.9) This LDH level was increased in PC-3 cells upon treatment with *Phyllanthus* extracts. In both aqueous and methanolic extracts of *Phyllanthus*, the percentage of LDH levels in PC-3 cells induced by all four *Phyllanthus* species ranged from 10.0-12.0% ($p<0.05$). For the standard drug, doxorubicin, the LDH levels induced was 26.0% ($p<0.05$). Hence, the necrotic effect of doxorubicin seems to be stronger than *Phyllanthus* extracts with a 10.0% difference ($p<0.05$).

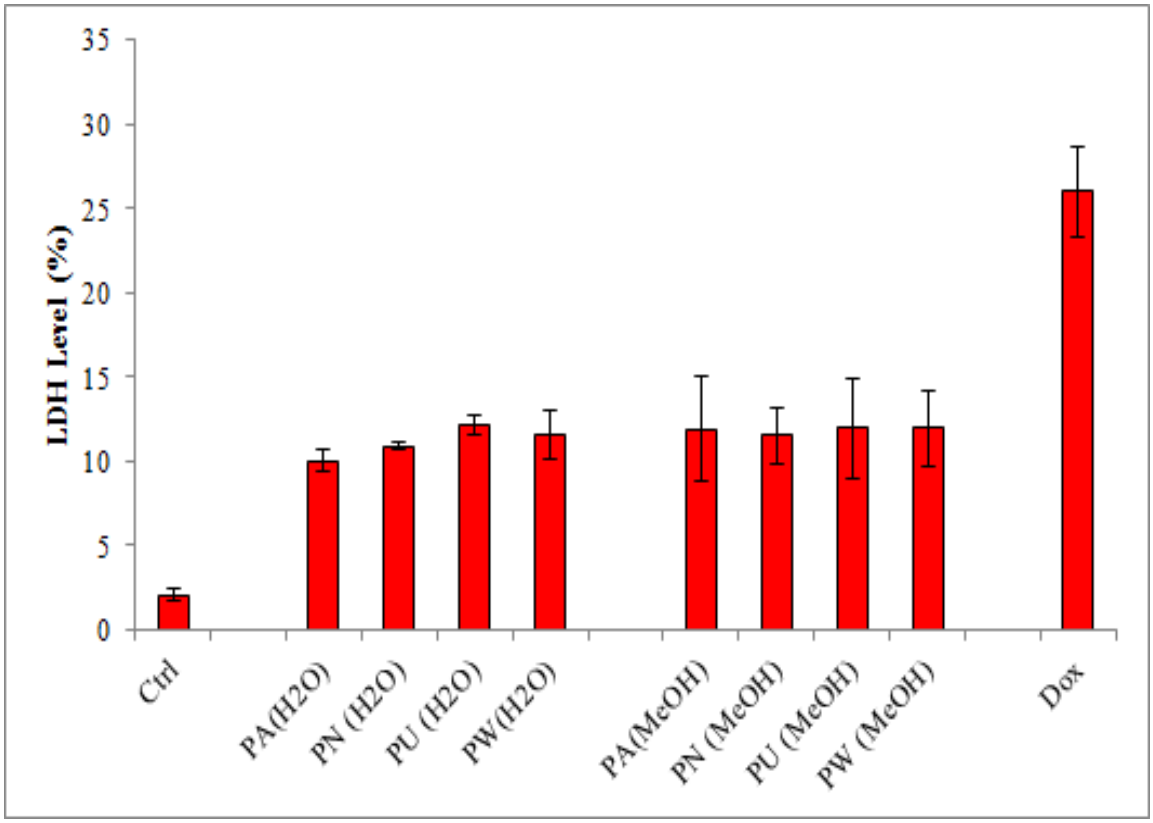

Figure 3.9: The percentage of LDH levels in the treated and untreated PC-3 cancer cells.

Figure shows the LDH levels in the treated group were higher than that of the untreated (control) group. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. All showed significant difference between untreated cells and *Phyllanthus*-treated cells, $p<0.05$ (Tang *et al.*, 2010).

3.5 *Phyllanthus* exerts Anti-Metastatic Effect

Tumour metastasis is a multistep process which includes adhesion of tumour cells to the extracellular matrix (ECM) of basement membrane, degradation of ECM by secreted proteolytic enzymes, migration and invasion of cancer cells through the systemic and/or lymphatic system (Zetter, 1998). Therefore, the anti-metastatic effect of *Phyllanthus* was studied by assessing several assays; migration, invasion, cell-matrix adhesion, transendothelial migration and zymographies.

3.5.1 Inhibition of Cancer and Endothelial Cells' Migration

The transwell assay was performed to quantify the migratory potential of cancer (MeWo and PC-3) and endothelial (HUVECs) cells after treatment with *Phyllanthus* at different concentrations. 31.3-500.0 μg/ml.

3.5.1.1 Inhibition of MeWo Cells' Migration

The migration activity of MeWo cells was decreased after treatment with *Phyllanthus* extracts ($p<0.05$) (Figure 3.10). At 31.3 μg/ml of aqueous extracts of *Phyllanthus*, the migrated MeWo cells ranged from 81.3-88.9% and reduced to 12.0-25.6% at 500.0 μg/ml ($p<0.05$). For methanolic extracts, the migrated MeWo cells ranged from 83.3-92.9% at 31.3 μg/ml and reduced to a range of 4.4-12.6% at 500.0 μg/ml ($p<0.05$). Notably the anti-migration effect of methanolic extracts of *Phyllanthus* was greater than aqueous extracts. *P.urinaria* showed the strongest anti-migration activity in both aqueous and methanolic extracts, followed by *P.watsonii*, *P.niruri* and *P.amarus.*

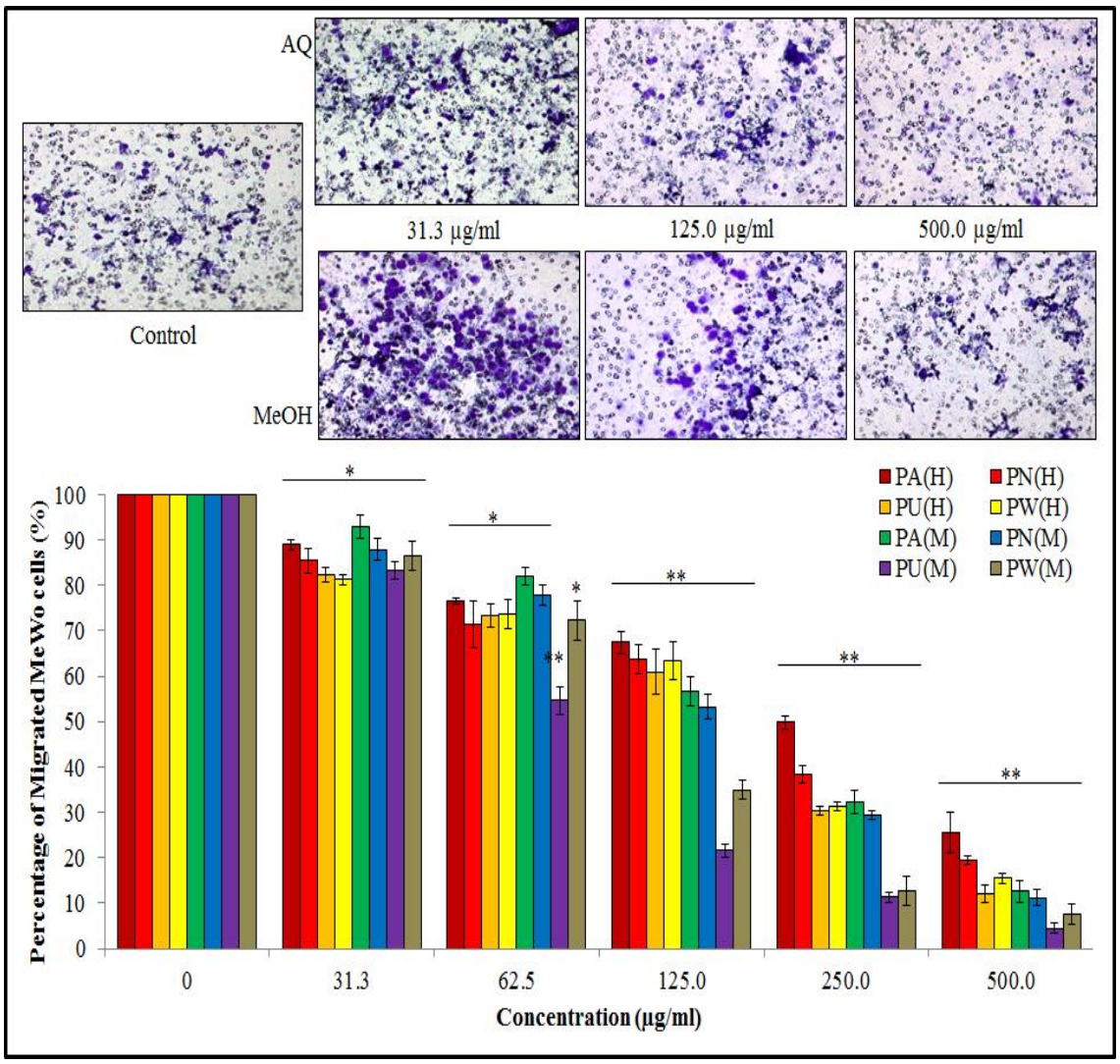

Figure 3.10: Effect of *Phyllanthus* extracts on the migration ability of MeWo cells.

Figure shows **(Upper)** MeWo cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Migration ability of treated MeWo cells was quantified and represented the mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.1.2 Inhibition of PC-3 Cells' Migration

The migration activity of PC-3 cells was decreased after treatment with *Phyllanthus* extracts (Figure 3.11). At 31.3 µg/ml of aqueous extracts of *Phyllanthus*, the migrated PC-3 cells ranged from 84.0-91.8% and reduced to 20.0-28.6% at 500.0 µg/ml ($p<0.05$). For methanolic extracts, the migrated PC-3 cells ranged from 43.1-66.0% at 31.3 µg/ml and reduced to 1.6-6.5% at 500.0 µg/ml ($p<0.05$). *P.urinaria* showed the strongest anti-migration activity in both aqueous and methanolic extracts, followed by *P.watsonii*, *P.niruri* and *P.amarus.*

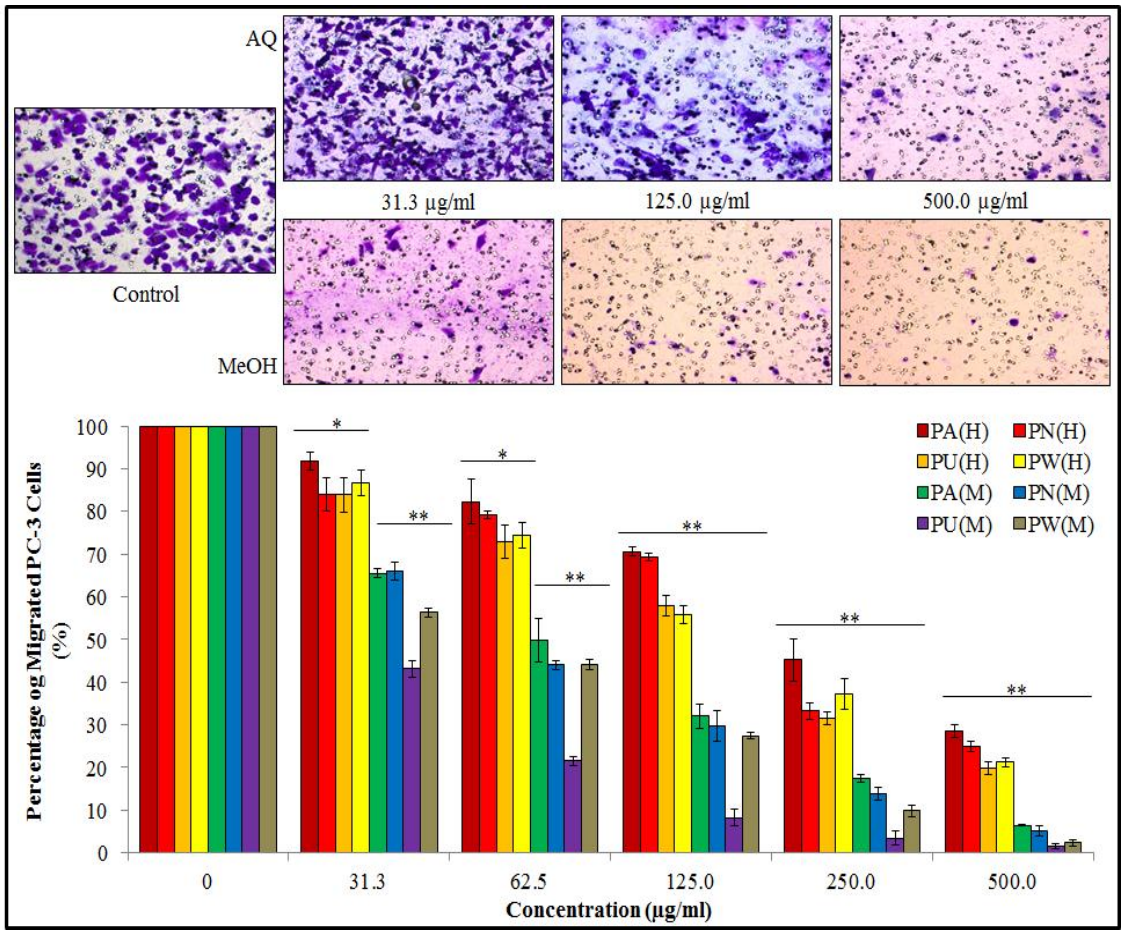

Figure 3.11: Effect of *Phyllanthus* extracts on the migration ability of PC-3 cells.

Figure shows **(Upper)** PC-3 cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Migration ability of treated PC-3 cells was quantified and represented the mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.5.1.3 Inhibition of HUVECs' Migration

The migration activity of *Phyllanthus* against HUVECs was found to be dose-dependent (Figure 3.12). At 31.3 µg/ml of *Phyllanthus* aqueous extracts, the migrated HUVECs were ranged from 93.3-97.2% and reduced to a range of 64.9-73.6% at 500.0 µg/ml ($p<0.05$). For methanolic extracts of *Phyllanthus,* the migrated HUVECs ranged from 93.2-97.7% at 31.3 µg/ml and reduced to 69.8-70.0% at 500.0 µg/ml ($p<0.05$). As noted, *P.amarus* showed the strongest anti-migration activity in both aqueous and methanolic extracts, followed by *P.urinaria*, *P.watsonii*, and *P.niruri*.

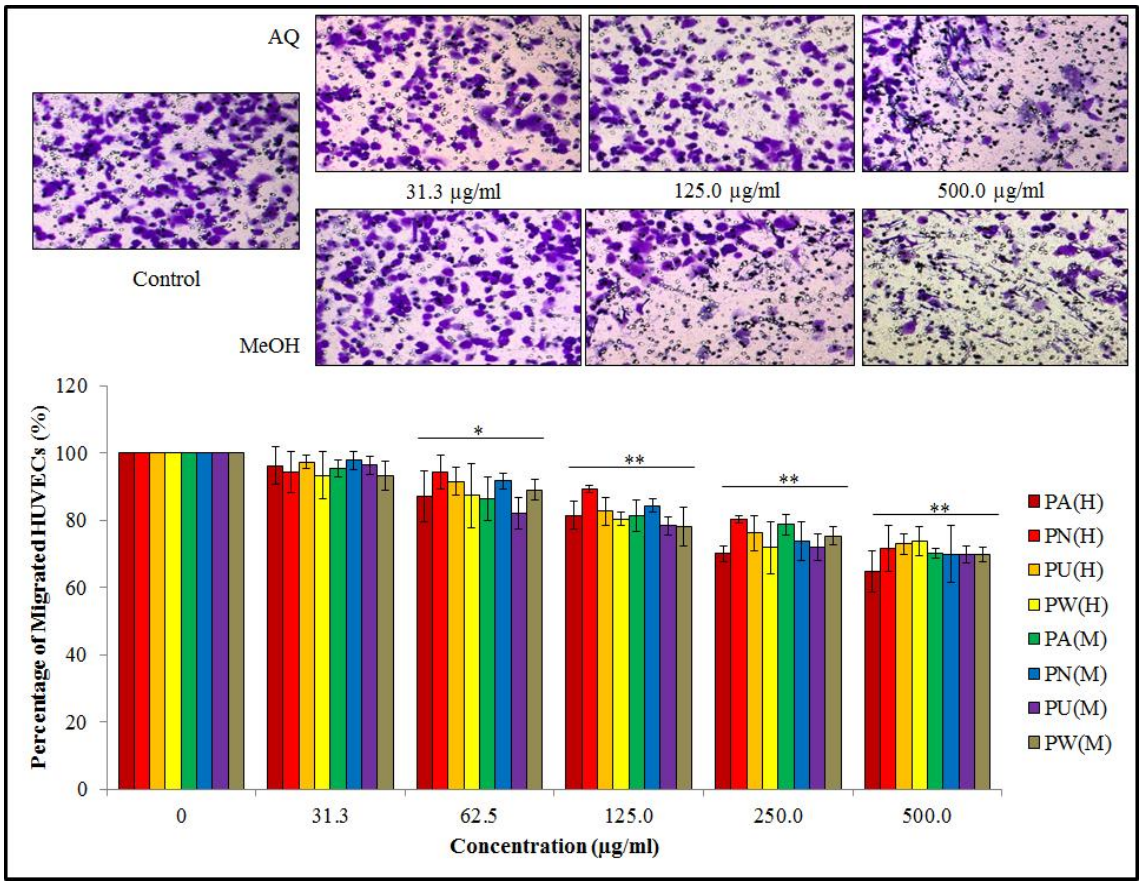

Figure 3.12: Effect of *Phyllanthus* extracts on the migration ability of HUVECs.

Figure shows **(Upper)** HUVECs cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Migration ability of treated HUVECs was quantified and represented the mean ± SEM of three independent experiments each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.5.2 Inhibition of Cancer Cell's Motility

The wound migration assay is a simple and inexpensive method which allows dual studies; cancer cell migration and cell-cell interactions. This assay was performed to confirm previous findings in transwell migration assay and allows investigation of cancer cell-cell interaction after *Phyllanthus* treatment. This assay was performed only on cancer (MeWo and PC-3) cells, as it requires to create a "wound" or "denuded zone" in a cell monolayer and not suitable for HUVECs which needs adhesion molecules on the flask for growth.

3.5.2.1 Inhibition of MeWo Cells' Motility

The anti-migration effect of *Phyllanthus* extracts on MeWo cells by the wound migration assay is shown in Figure 3.13. The reduction in migration activity of MeWo cells was clearly observed after treatment with different concentrations of *Phyllanthus* extracts. The migrated MeWo cells ranged from 88.9-93.3% by aqueous extracts treatment at 31.3 µg/ml and further reduced to a range of 28.0-31.0% at 500.0 µg/ml ($p<0.05$). For methanolic extracts, the migrated MeWo cells ranged from 84.0-91.5% at 31.3 µg/ml and further reduced to a range of 11.0-21.9% at 500.0 µg/ml ($p<0.05$). *P.urinaria* showed the strongest anti-migration activity in both aqueous and methanolic extracts, followed by *P.watsonii*, *P.niruri* and *P.amarus*.

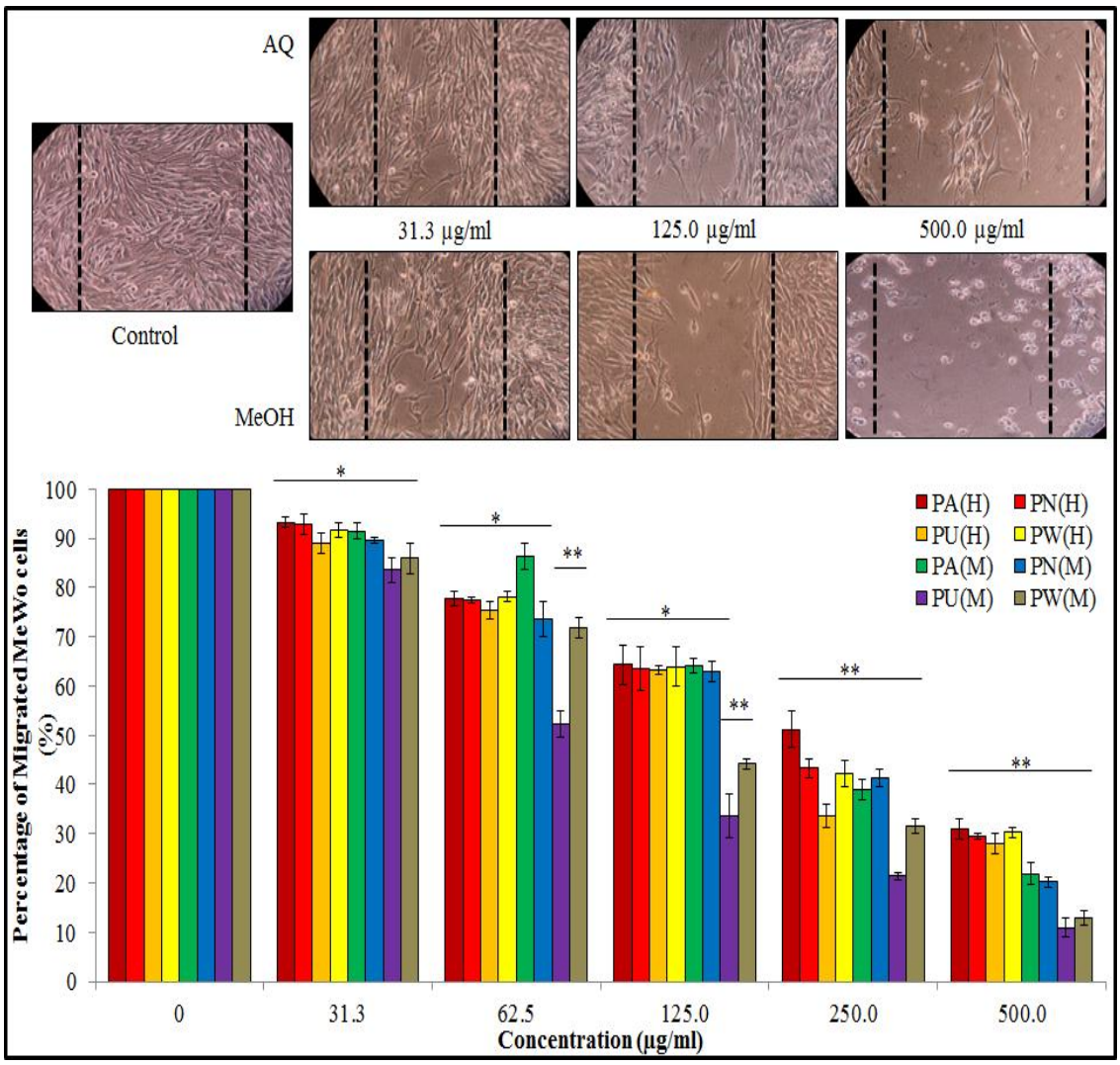

Figure 3.13: Effect of *Phyllanthus* extracts on the motility of MeWo cells.

Figure shows **(Upper)** MeWo cell monolayer was scraped, and the cells were treated with different concentrations of *Phyllanthus* extracts for 48 hours. **(Lower)** Quantitative assessments of migrated cells are expressed as mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

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3.5.2.2 Inhibition of PC-3 Cells' Motility

For the PC-3, the anti-migration effect of *Phyllanthus* extracts was observed in wound migration assay (Figure 3.14). The migrated PC-3 cells were around 90.6-93.6% at 31.3 µg/ml for the the aqueous extracts treatment, and reduced to a range of 20.7-35.0% at 500.0 µg/ml. For the methanolic extracts, the percentage of migrated PC-3 cells was 74.6-85.9% at 31.3 µg/ml and further reduced to a range of 20.3-23.9% at 500.0 µg/ml. As noted again, *P.urinaria* showed the strongest anti-migration activity in both aqueous and methanolic extracts, followed by *P.watsonii, P.niruri* and *P.amarus*.

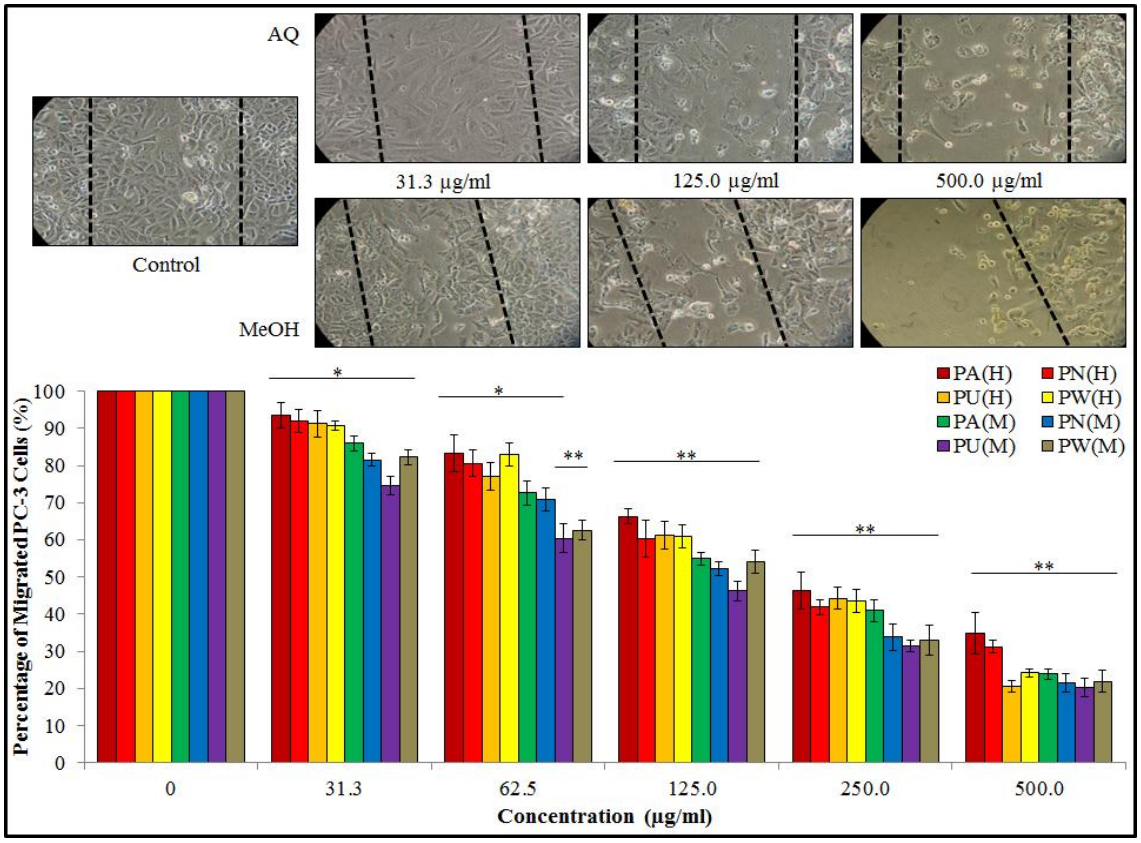

Figure 3.14: Effect of *Phyllanthus* extracts on the motility of PC-3 cells.

Figure shows **(Upper)** PC-3 cell monolayer was scraped to create denuded zone and treated with different concentrations of *Phyllanthus* extracts for 24 hours. **(Lower)** Quantitative assessments of migrated cells are expressed as mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.3 Inhibition of Cancer and Endothelial Cells' Invasion

The invasion ability of cancer cells allows cancer cells to invade beyond the constraints of the normal tissue from which they originate and to enter the circulation that leads them to reach distant organs/tissues and eventually form secondary tumours, called metastases (Mareel & Leroy, 2003). Invasion also plays an important role during tumour angiogenesis as endothelial cells from pre-existing blood vessels need to invade surrounding tissues to form new blood vessels towards solid tumour (Carlevaro *et al.*, 1997).

Thus, the anti-invasion activity of *Phyllanthus* extracts on MeWo and PC-3 cells as well as HUVECs was studied with transwell invasion assay after treatment with *Phyllanthus* at different concentrations (31.3-500.0 μg/ml).

3.5.3.1 Inhibition of MeWo Cell' Invasion

The invasion activity of MeWo cells was noted to decrease after treatment with *Phyllanthus* extracts (Figure 3.15). The invasion ability of MeWo cells were significantly inhibited by both aqueous and methanolic extracts of *Phyllanthus* from 31.3-500.0 μg/ml ($p<0.05$). At 31.3 μg/ml of aqueous extracts, the invaded MeWo cells were 83.9-91.9% and reduced to a range of 13.6-31.8% at 500.0 μg/ml ($p<0.05$). For methanolic extracts, the invaded MeWo cells ranged from 84.6-92.9% at 31.3 μg/ml and reduced to a range of 8.4-15.3% at 500.0 μg/ml ($p<0.05$). *P.urinaria* showed the strongest anti-invasion activity in both aqueous and methanolic extracts, followed by *P.watsonii, P.niruri* and *P.amarus*.

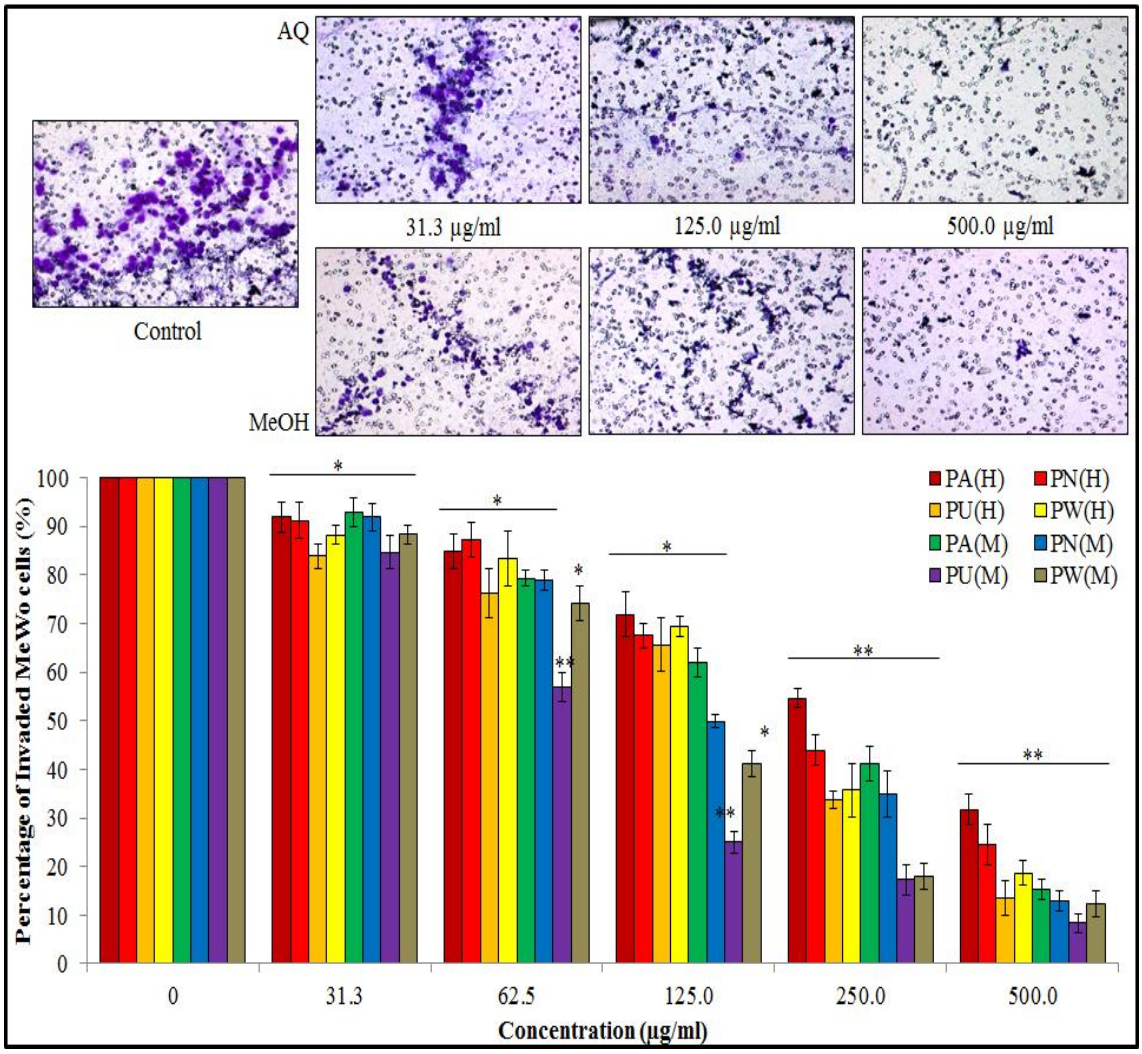

Figure 3.15: Effect of *Phyllanthus* extracts on the invasion ability of MeWo cells.

Figure shows **(Upper)** MeWo cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Invasion ability of treated MeWo cells was quantified and represented the mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.5.3.2 Inhibition of PC-3 Cell' Invasion

The invasion activity of PC-3 cells was decreased after treatment with *Phyllanthus* extracts (Figure 3.16). At 31.3 µg/ml of aqueous extracts, the invaded PC-3 cells ranged from 83.2-93.6% and reduced to 20.9-31.8% at 500.0 µg/ml ($p<0.05$), while for methanolic extracts, the invaded PC-3 cells ranged from 60.6-91.2% at 31.3 µg/ml and reduced to 11.1-21.1% at 500.0 µg/ml ($p<0.05$). As noted before, *P.urinaria* showed the strongest anti-invasion activity in both aqueous and methanolic extracts, followed by *P.watsonii*, *P.niruri* and *P.amarus*.

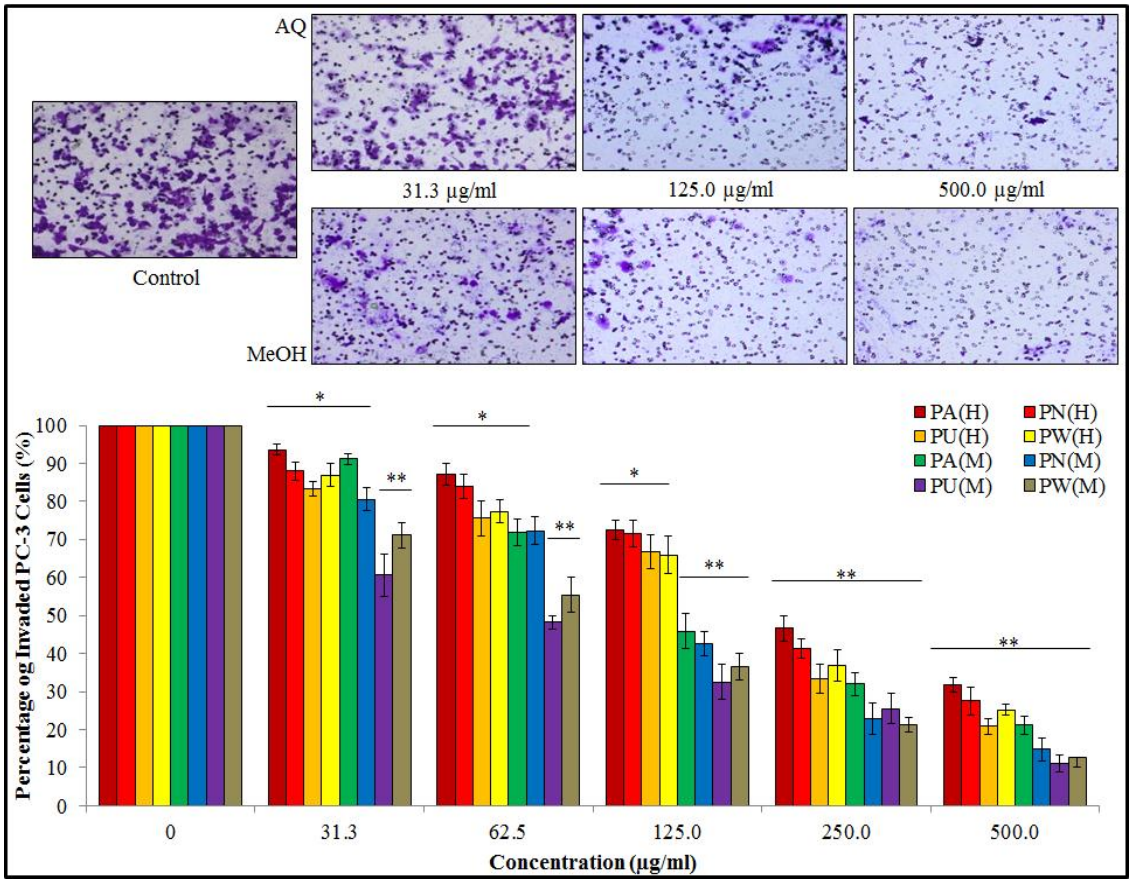

Figure 3.16: Effect of *Phyllanthus* extracts on the invasion ability of PC-3 cells.

Figure shows **(Upper)** PC-3 cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Invasion ability of treated PC-3 cells was quantified and represented the mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.5.3.3 Inhibition of HUVECs' Invasion

For HUVECs, the invasion activity reduced upon treatment with *Phyllanthus* extracts (Figure 3.17). At 31.3 µg/ml of aqueous extracts of *Phyllanthus*, the invaded HUVECs ranged from 93.6-98.9% and reduced to 51.2-52.6% at 500.0 µg/ml ($p<0.05$), while for methanolic extracts, the percentage of invaded HUVECs were 90.3-93.9% at 31.3 µg/ml and reduced to a range of 48.3-55.9% at 500.0 µg/ml ($p<0.05$). The results showed that both aqueous and methanolic extracts of *Phyllanthus* showed similar extent of anti-invasion effects on HUVECs for all *Phyllanthus* species.

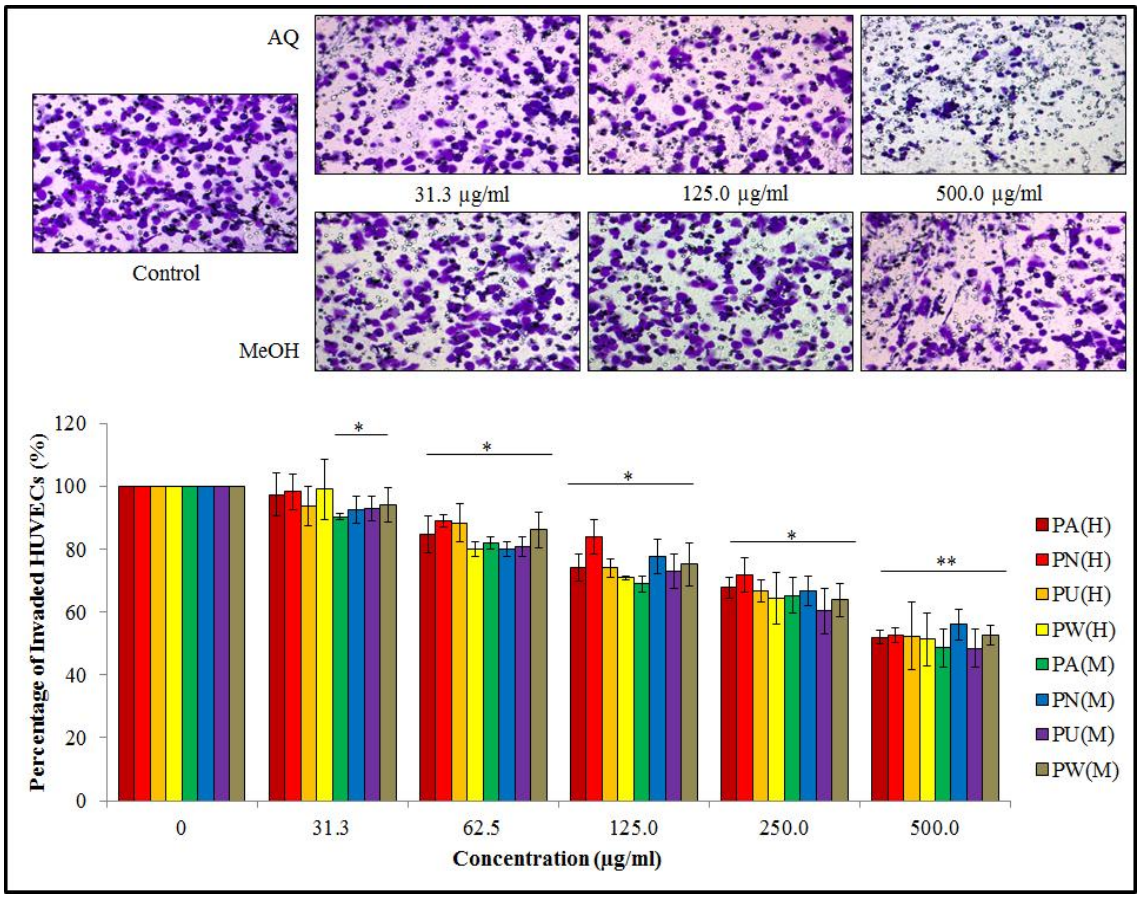

Figure 3.17: Effect of *Phyllanthus* extracts on the invasion ability of HUVECs.

Figure shows **(Upper)** HUVEC cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Invasion ability of treated HUVECs was quantified and represented the mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.5.4 Cell-Extracellular Matrix Interactions in Cancer Cells

The interaction of cancer cell with ECM proteins such as fibronectin, laminin and collagen is important to enable cancer cells to adhere, invade and metastasize. Disruption of these interactions could halt cancer progression. Cell-matrix adhesion assay was performed to study the interaction of PC-3 cells to type-IV collagen and MeWo cells to fibronectin after treatment with *Phyllanthus* extracts

3.5.4.1 Inhibition on MeWo Cells-Extracellular Matrix Interactions

Figure 3.18 shows the adhesion activity of MeWo cells to fibronectin was significantly reduced after treatment with *Phyllanthus* extracts. At 31.3 μg/ml of aqueous extracts, the adherent MeWo cells ranged from 64.7-71.6% and reduced to 3.6-16.2% at 500.0 μg/ml ($p<0.05$), while for methanolic extracts, the adherent MeWo cells were 61.1-68.8% at 31.3 μg/ml and further reduced to 2.0-4.6% at 500.0 μg/ml ($p<0.05$). *P.urinaria* again showed the strongest inhibitory effect on adhesion activity of MeWo cells in both aqueous and methanolic extracts, followed by *P.watsonii, P.amarus* and *P. niruri*.

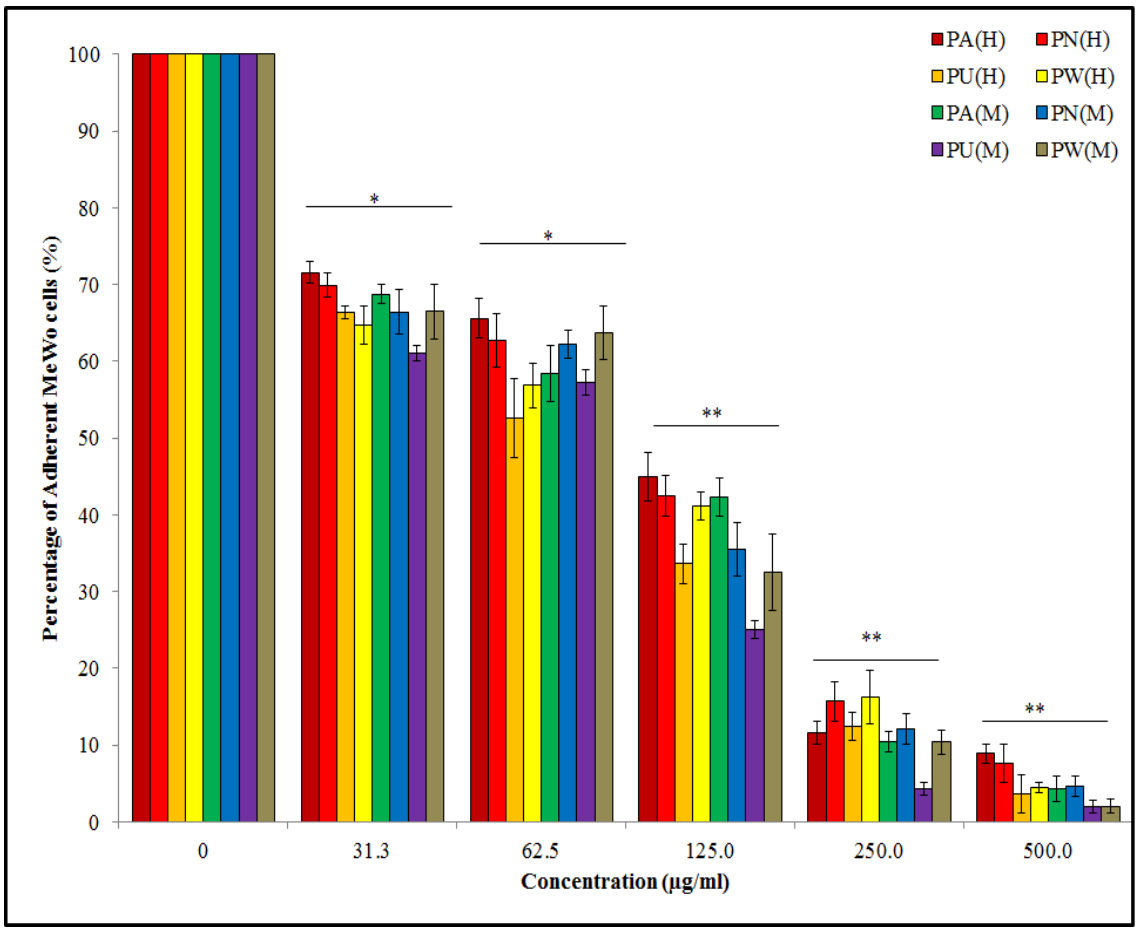

Figure 3.18: Effects of *Phyllanthus* on MeWo cells adhesion activity.

Figure shows the adhesion ability of treated MeWo cells towards fibronectin were exhibited a dose-dependent manner of *Phyllanthus* concentrations. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.5.4.2 Inhibition on PC-3 Cells-Extracellular Matrix Interactions

The adhesion ability of PC-3 cells towards type-IV collagen was reduced after treatment with *Phyllanthus* extracts (Figure 3.19). In the aqueous extracts treatment, the adherent PC-3 cells were 82.6-90.6% at 31.3 µg/ml, and reduced to a range of 10.6-11.7% at 500.0 µg/ml, while for methanolic extracts, the adherent PC-3 cells were 70.7-81.2% at 31.3.0 µg/ml and reduced to 3.8-4.4% at 500.0 µg/ml. Again *P.urinaria* showed the strongest inhibitory effects in adhesion activity of PC-3 cells, followed by *P.watsonii, P.amarus* and *P.niruri* in both aqueous and methanolic extracts.

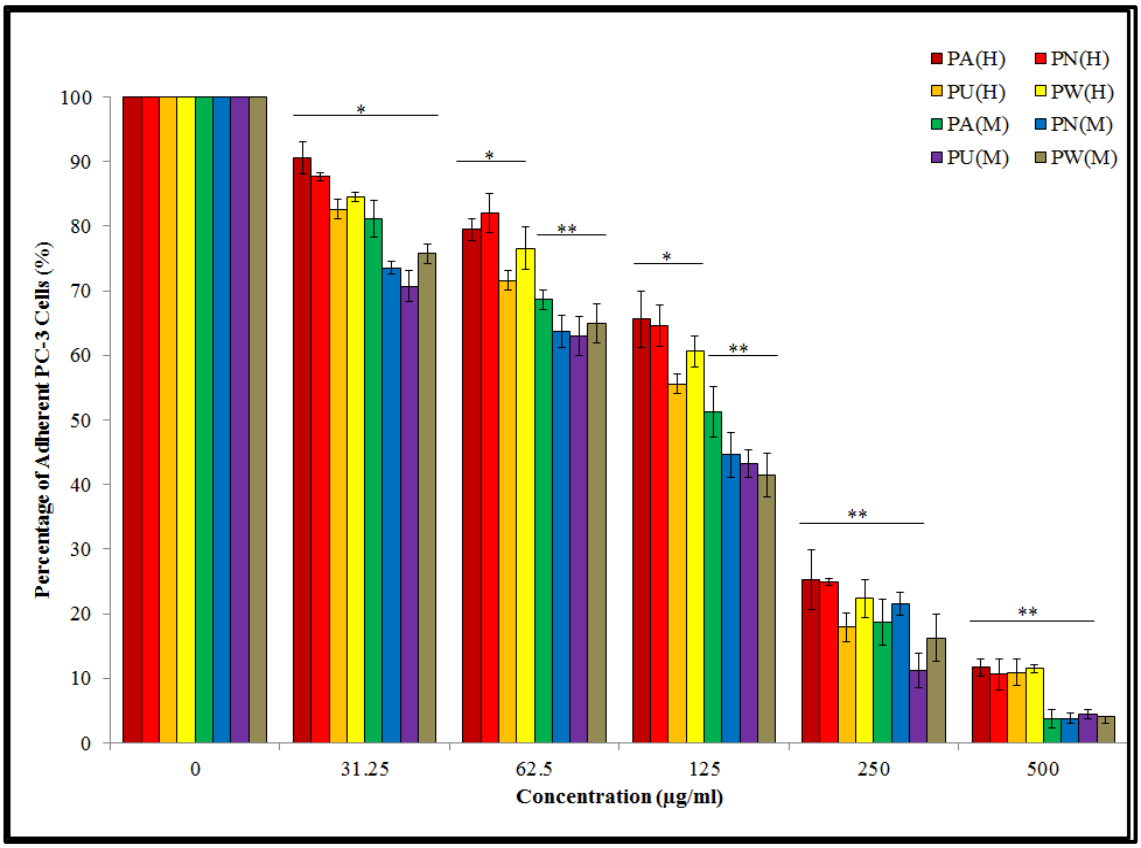

Figure 3.19: Effects of *Phyllanthus* on PC-3 cells' adhesion activity.

Figure shows the adhesion ability of treated PC-3 cells towards type-IV collagen were exhibited a dose-dependent manner to *Phyllanthus* concentrations. Data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.5 Inhibition of Cancer Cells' Transendothelial Migration

During metastasis, cancer cells will transvasate into blood vessels, circulate in the blood stream and transmigrate out from the vessels into a new site of the body to form secondary tumours. In order to study cancer cells-endothelium interaction and transmigration, tumour transendothelial migration assay was performed.

3.5.5.1 Inhibition of MeWo Cells' Transendothelial Migration

As shown in Figure 3.20, *Phyllanthus* extracts significantly inhibited transendothelial migration of MeWo in a dose-response manner at all concentration ($p<0.05$). At 31.3 μg/ml of aqueous extracts, the transendothelial migrated MeWo cells ranged from 73.8-81.3% and reduced to 4.2-21.6% at 500.0 μg/ml. For methanolic extracts, the transendothelial migrated MeWo cells were around 72.9-82.6% at 31.3 μg/ml and reduced to 2.0-4.8% at 500.0 μg/ml. Again, *P.urinaria* showed the strongest inhibitory effects on transendothelial migration ability of MeWo cells, followed by *P.watsonii, P.niruri* and *P.amarus* in both aqueous and methanolic extracts.

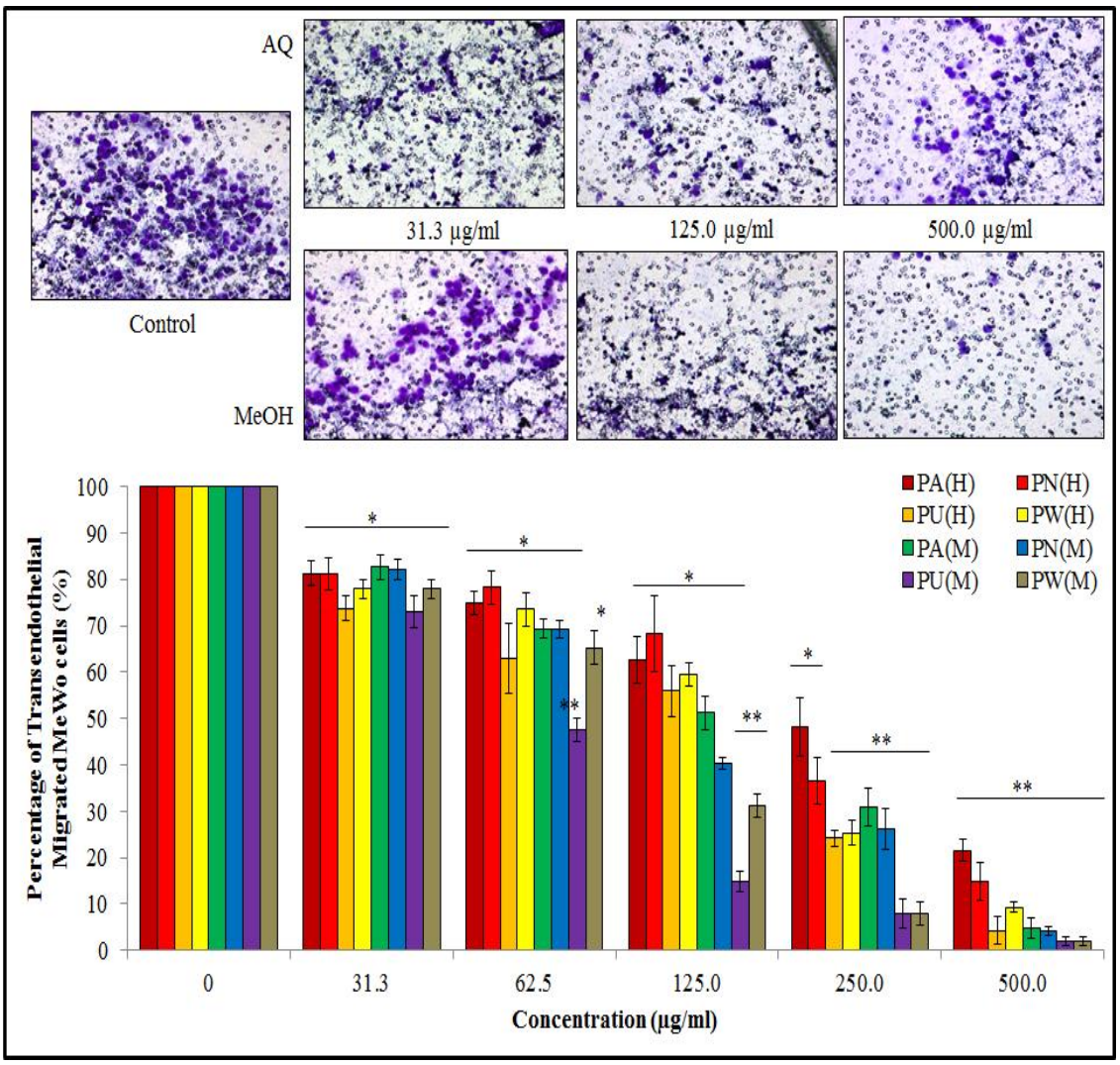

Figure 3.20: Effects of *Phyllanthus* on transendothelial migration of MeWo cells.

Figure shows **(Upper)** MeWo cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Transendothelial migration ability of treated MeWo cells was quantified and represented the mean ± SEM of three independent experiments each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.5.2 Inhibition of PC-3 Cells' Transendothelial Migration

Phyllanthus extracts also exhibits the inhibiton effect on transendothelial migration of PC-3 cells in a dose-response manner (Figure 3.21). At 31.3 µg/ml of aqueous extracts of *Phyllathus*, the transendothelial migrated PC-3 cells ranged from 72.2-83.6% and reduced to 10.3-21.3% at 500.0 µg/ml, while for methanolic extracts, the transendothelial migrated PC-3 cells were 51.9-80.3% at 31.3 µg/ml and reduced to 3.3-13.3% at 500.0 µg/ml. *P.urinaria* again showed the strongest inhibitory effects on transendothelial migration ability of PC-3 cells, followed by *P.watsonii*, *P.niruri* and *P.amarus* in both aqueous and methanolic extracts.

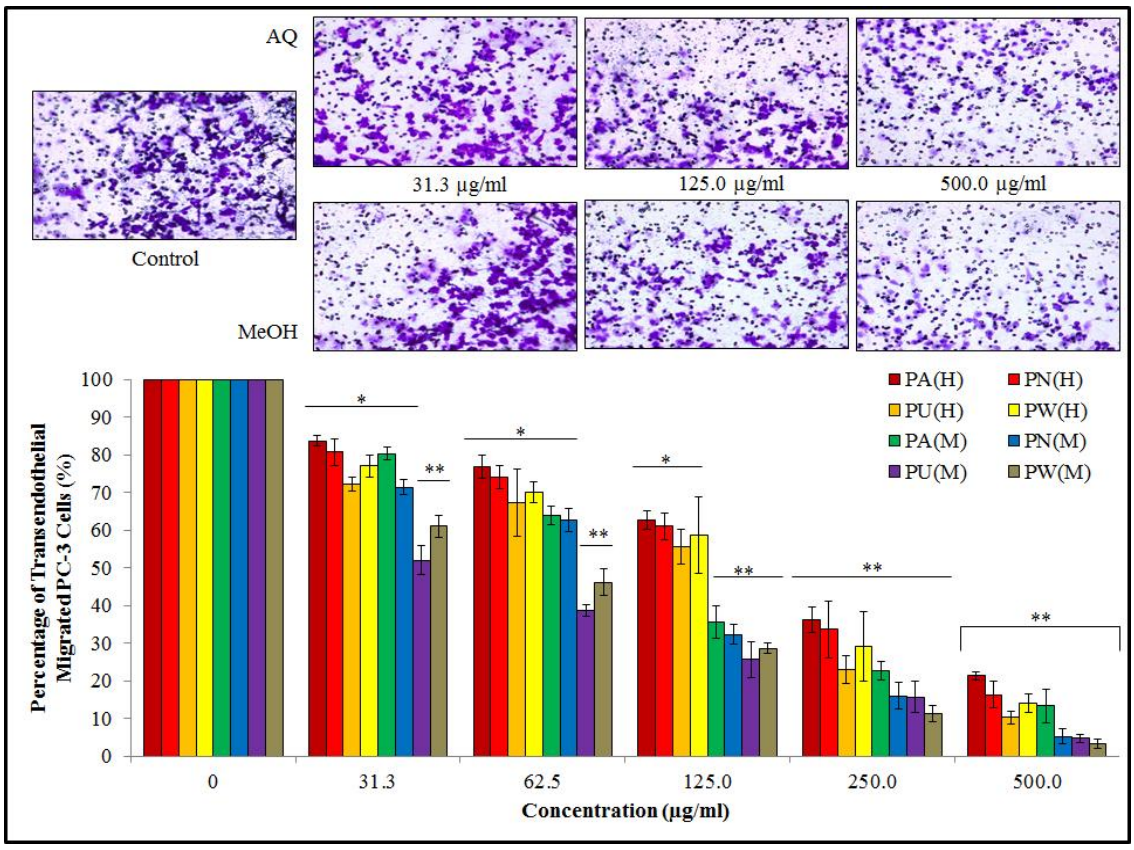

Figure 3.21: Effects of *Phyllanthus* on transendothelial migration of PC-3 cells.

Figure shows **(Upper)** PC-3 cells were treated with different concentrations of *Phyllanthus* extracts for 72 hours. **(Lower)** Transendothelial migration ability of treated PC-3 cells was quantified and represented the mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.6 Inhibition on Matrix Metalloproteinase (MMP) Enzymes

Matrix metalloproteinases (MMPs) are zinc-dependent matrix proteases which are involved in ECM destruction, cell growth, apoptosis, angiogenesis, invasion and migration. Inhibition of these enzymes is believed to prevent endothelial and cancer cells' invasiveness, subsequently, decreasing the incidence of tumour metastasis and halts the formation of secondary tumours. Gelatinase (MMP-2 and -9) and matrilysins (MMP-7 and -26) metalloproteases were detected in MeWo, PC-3 and HUVECs by zymographies and their activities were noted to be altered upon *Phyllanthus* treatment.

3.5.6.1 Inhibition of MMP-2 and MMP-7 in MeWo cells

As shown in Figure 3.22, MMP-2 was detected at 64kDa in MeWo cells. At 31.3 μg/ml of aqueous extracts of *Phyllathus*, the MMP-2 levels ranged from 89.2-95.3% ($p<0.05$) and reduced to 21.4-33.3% at 500.0 μg/ml ($p<0.01$). For methanolic extracts, the MMP-2 levels were around 81.4-94.2% at 31.3 μg/ml ($p<0.05$) and reduced to a range of 13.3-21.4% at 500.0 μg/ml ($p<0.01$).

Besides that, MMP-7 was also detected at 64kDa in MeWo cells (Figure 3.22). At 31.3 μg/ml of aqueous extracts of *Phyllathus*, the MMP-7 levels ranged from 89.4-93.3% ($p<0.05$) and reduced to 26.3-34.2% at 500.0 μg/ml ($p<0.01$). For methanolic extracts, the MMP-2 levels ranged from 80.4-91.4% at 31.3 μg/ml ($p<0.05$) and reduced to 29.4-33.4% at 500.0 μg/ml ($p<0.01$). *P.urinaria* showed the strongest inhibitory effects on MMP-2 and -7 activities of MeWo cells, followed by *P.watsonii*, *P.niruri* and *P.amarus* in both aqueous and methanolic extracts.

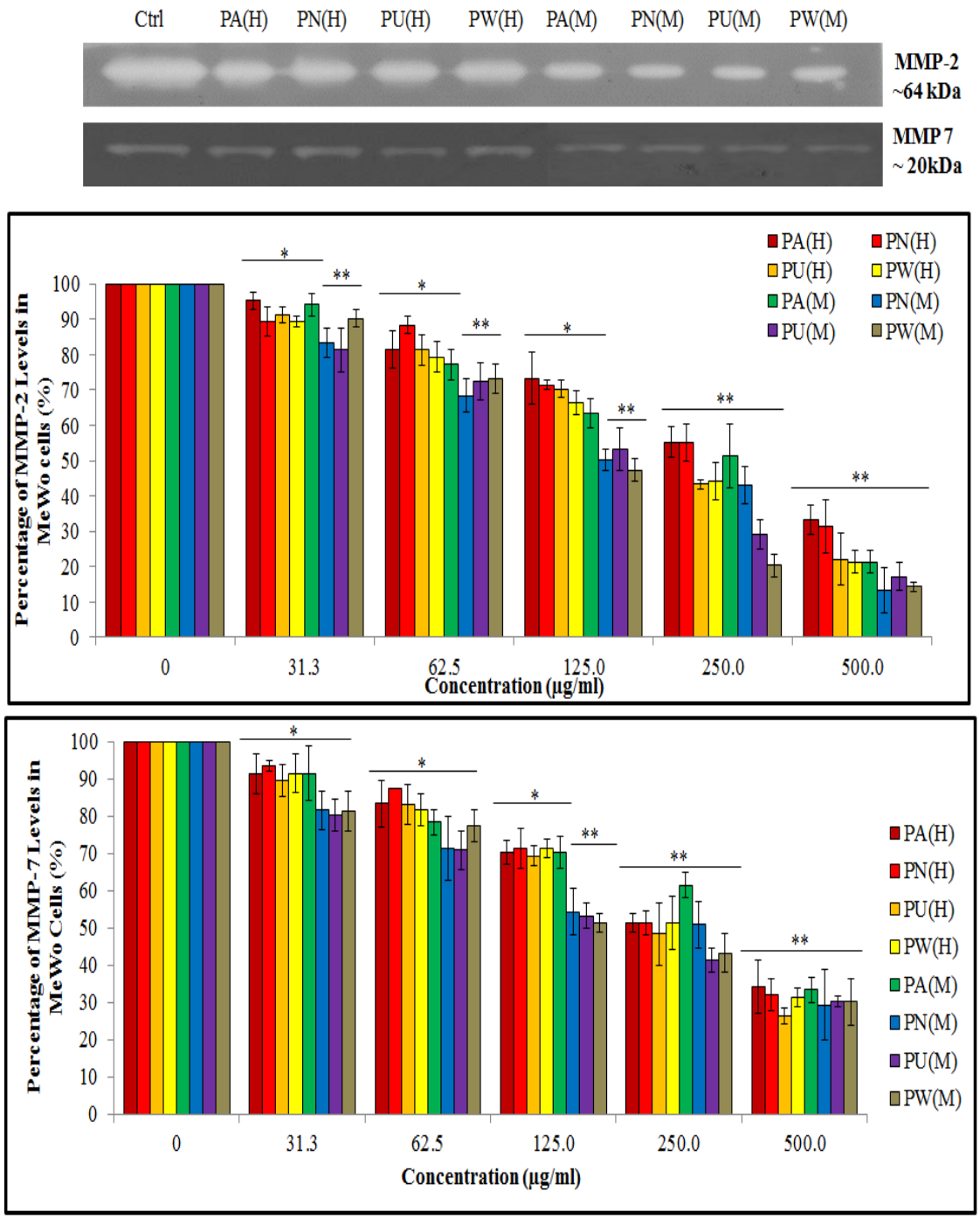

Figure 3.22: Zymography analysis of MeWo cells. MMP-2 and -7 were detected at 64KDa and 20kDa, respectively.

Figure shows the MMPs activities were measured and expressed as mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.6.2 Inhibition of MMP- 7, -9 and -26 in PC-3 cells

Four different MMPs were detected in PC-3 cells; MMP-7 at 20kDa, pro-MMP 9 at 92kDa, active MMP-9 at 82kDa and MMP-26 at 18kDa (Figure 3.23). As shown in Figure 3.23, MMP-7 levels ranged from 80.3-91.4% ($p<0.05$) at 31.3 μg/ml and reduced to 20.3-31.3% at 500.0 μg/ml ($p<0.01$) of aqueous-treated cells. For methanolic extracts, the MMP-7 levels were around 79.3-81.3% at 31.3 μg/ml ($p<0.05$) and reduced to a range of 26.8-37.2% at 500.0 μg/ml ($p<0.01$).

The MMP-26 activity was reduced by *Phyllanthus* extracts in treated PC-3 cells and its levels ranged from 71.3-81.4% ($p<0.05$) at 31.3 μg/ml and were reduced to 43.2-60.2% at 500.0 μg/ml ($p<0.01$) in aqueous-treated PC-3 cell. For methanolic extracts, the MMP-26 levels were 65.3-71.4% at 31.3 μg/ml ($p<0.05$) and reduced to 34.3-49.2% at 500.0 μg/ml ($p<0.01$). As MMP-26 acts as an activator of pro-MMP-9 to activate MMP-9, reduction of MMP-26 activity is followed by increased levels of pro-MMP 9. The pro-MMP-9 levels ranged from 21.4-25.7% ($p<0.05$) at 31.3 μg/ml and increased to 86.3-91.7% at 500.0 μg/ml ($p<0.01$) of aqueous-treated PC-3 cells. For methanolic extracts, the pro-MMP-9 levels ranged from 10.4-21.4% at 31.3 μg/ml ($p<0.05$) and increased to 83.2-91.5% at 500.0 μg/ml ($p<0.01$). As consequence from this, active MMP-9 activity in PC-3 cells was reduced after treatment with *Phyllanthus*. At 31.3 μg/ml of aqueous extracts of *Phyllathus*, the active MMP-9 levels were 80.2-88.2% ($p<0.05$) and reduced to 17.3-23.8% at 500.0 μg/ml ($p<0.01$). For methanolic extracts, the MMP-9 levels ranged from 79.2-87.4% at 31.3 μg/ml ($p<0.05$) and reduced to a range of 9.4-14.2% at 500.0 μg/ml ($p<0.01$).

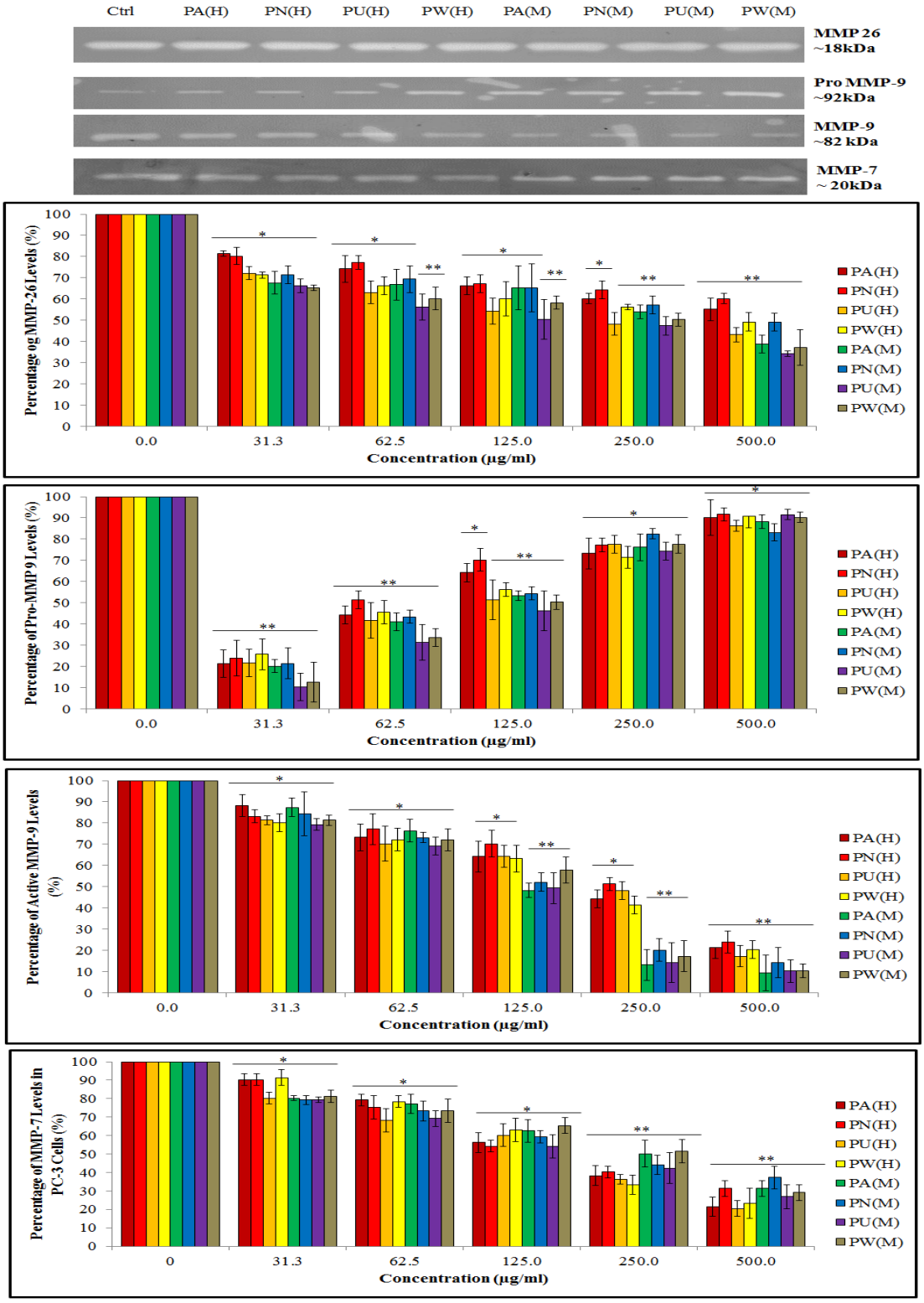

Figure 3.23: Zymography analysis of PC-3 cells**.**

Figure shows four different MMPs; MMP-7, pro-MMP-9, active MMP-9 and MMP-2, were detected, measured and expressed as mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.5.6.3 Inhibition of MMP- 2 in HUVECs

As shown in Figure 3.24, MMP-2 is detected at 64kDa in HUVECs. At 31.3 µg/ml of aqueous extracts of *Phyllathus*, the MMP-2 levels ranged from 88.2-93.3% ($p<0.05$) and this was reduced to 43.2-51.4% at 500.0 µg/ml ($p<0.01$). For methanolic extracts, the MMP-2 levels were around 79.3-89.3% at 31.3 µg/ml ($p<0.05$) and reduced to 40.3-45.3% at 500.0 µg/ml ($p<0.01$). *P.urinaria* showed the strongest inhibitory effects on MMP-2 activity on PC-3 cells, followed by *P.watsonii*, *P.niruri* and *P.amarus* in both aqueous and methanolic extracts.

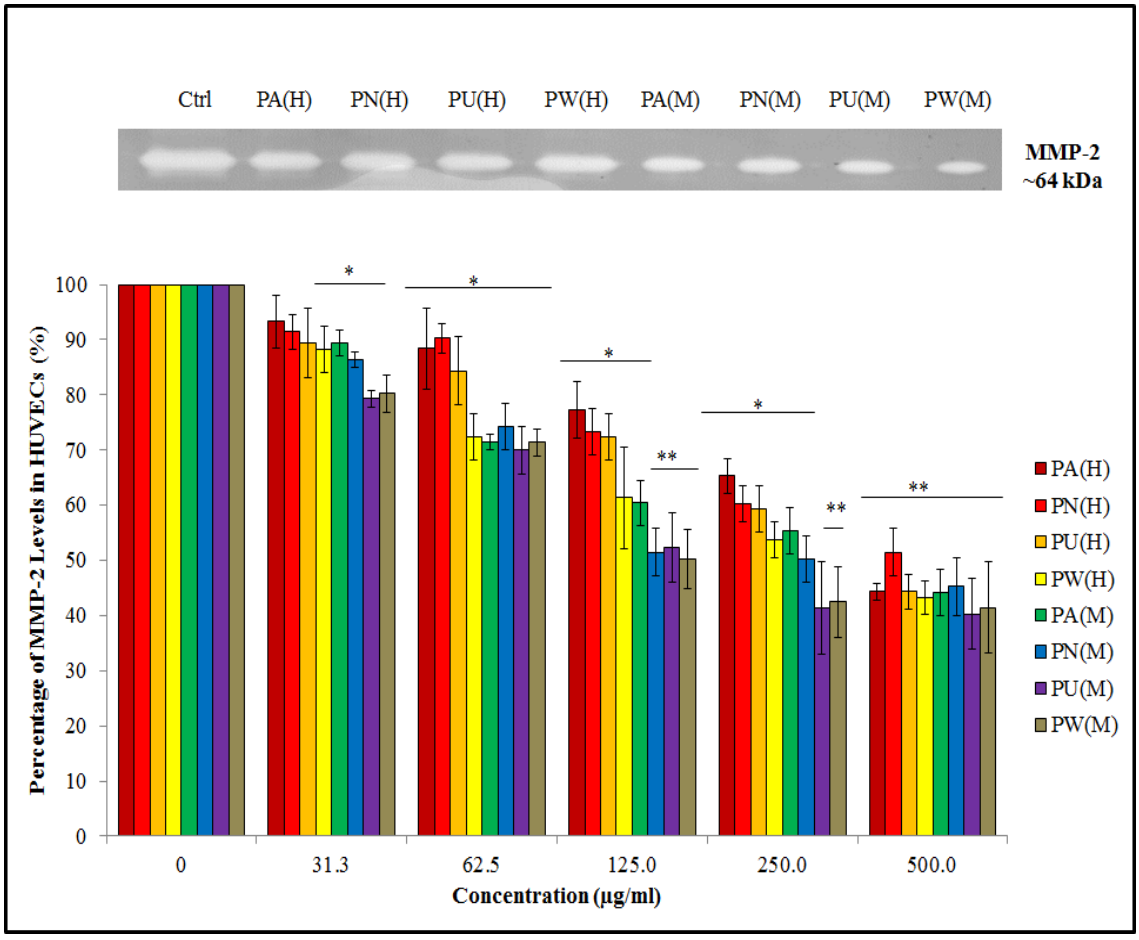

Figure 3.24: Zymography analysis of HUVECs. MMP-2 was detected at 64kDa.

Figure shows the levels of MMP-2 activity was measured and expressed as mean ± SEM of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.6 *Phyllanthus* exerts Anti-Angiogenic Effect

The extracellular matrix (ECM) gel-induced capillary tube formation assay is one of the most widely used *in vitro* assays to mimic the reorganization phase of angiogenesis. This assay measures the ability of endothelial cells to form capillary-like tube structures with the the support of extracellular matrix. The resulting tubes will eventually form hollow lumen and become guidance pathways that facilitate tumour cell metastasis. Thus, ECM gel-induced capillary tube formation assay was used to assess the anti-angiogenic effects of *Phyllanthus* extracts

Without any treatment, endothelial cells become elongated and form microcapillary-like tube structures, organized by larger number of HUVECs on ECM gel (Figure 3.25). The inhibition in gel-induced microcapillary-like tube structures by *Phyllanthus* extracts was noticed from 31.3-500.0 μg/ml. At 31.3 μg/ml of aqueous extracts of *Phyllanthus*, the percentage of tube formation ranged from 90.8-93.3% ($p<0.05$) and was further reduced to 31.3-37.4% at 500.0 μg/ml ($p<0.05$). For methanolic extracts, the percentage of tube formation was around 90.1-92.3% at 31.3 μg/ml and reduced to 24.6-30.6% at 500.0 μg/ml ($p<0.05$). *P.urinaria* again showed the strongest inhibitory effect on microcapillary-like tube structures of HUVECs, followed by *P.watsonii, P.amarus* and *P.niruri* in both aqueous and methanolic extracts.

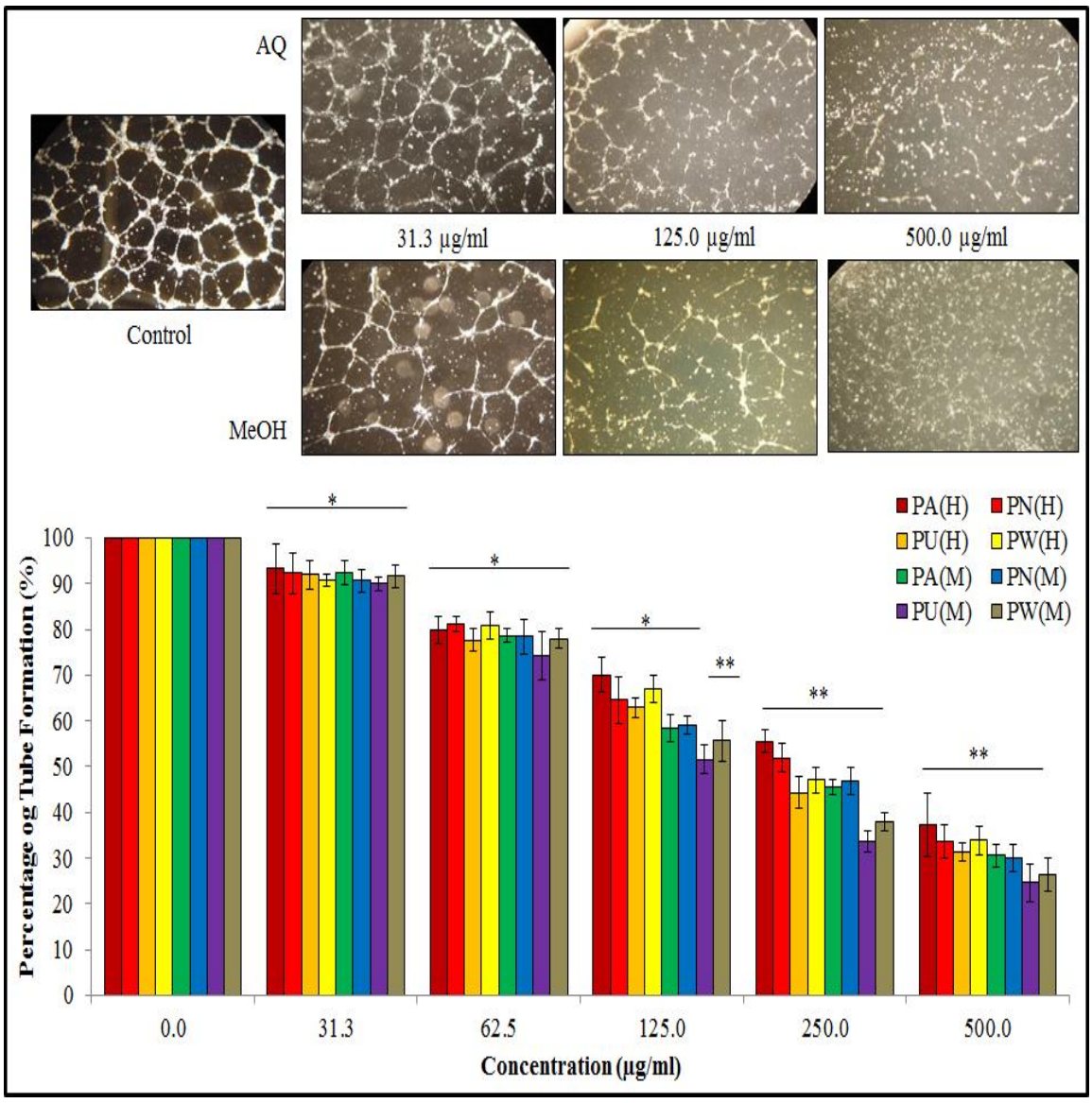

Figure 3.25: Effect of *Phyllanthus* extracts on microcapillary-like tube formation of HUVECs.

Figure shows **(Upper)** Microcapillary-like tube formation of HUVECs at different concentrations of *Phyllanthus* extracts. **(Lower)** The completed microcappilary-like tube formation was calculated and expressed as mean ± SEM of three experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.7 Disruption of Cancer Cell Signalling Pathways by *Phyllanthus*

From the results, *Phyllanthus* extracts have exhibited anti-proliferation, anti-metastasis, anti-angiogenesis and induction of apoptosis on both melanoma (MeWo) and prostate (PC-3) cancer cell lines. Further investigations to study the underlying molecular mechanisms of these anticancer effects of *Phyllanthus* on MeWo and PC-3 cells was carried out. The cancer ten-pathway reporter array was performed to profile the changes in the activities of ten different signalling pathways relevant to cancer that is influenced by *Phyllanthus.* The ten-related cancer pathways that were screened included Wnt, Notch, p53/DNA damage, TGFβ, cell cycl3/pRb-E2F, NFκB, Myc/Max, hypoxia, MAPK/ERK and MAPK/JNK.

3.7.1 Alterations on Multiple Signalling Pathways in MeWo cells

Figure 3.26 showed the differential expression of each cancer-related pathway in treated and untreated MeWo cells. It was noted that in the untreated MeWo cells, six (p53/DNA damage, NFκB, Myc/Max, Hypoxia, MAPK/ERK and MAPK/JNK) investigated pathways were expressed to regulate the cell growth and survival. It is observed that out of these six pathways, four pathways (NFκB, Myc/Max, Hypoxia and MAPK/ERK) showed significantly down-regulation in treated MeWo cells ($p<0.05$). Contrarily, the expression of p53/DNA damage and MAPK/JNK pathways showed significant up-regulation in the treated cells ($p<0.05$). Other pathways investigated were found to be not significantly affected by *Phyllanthus* extracts ($p>0.05$).

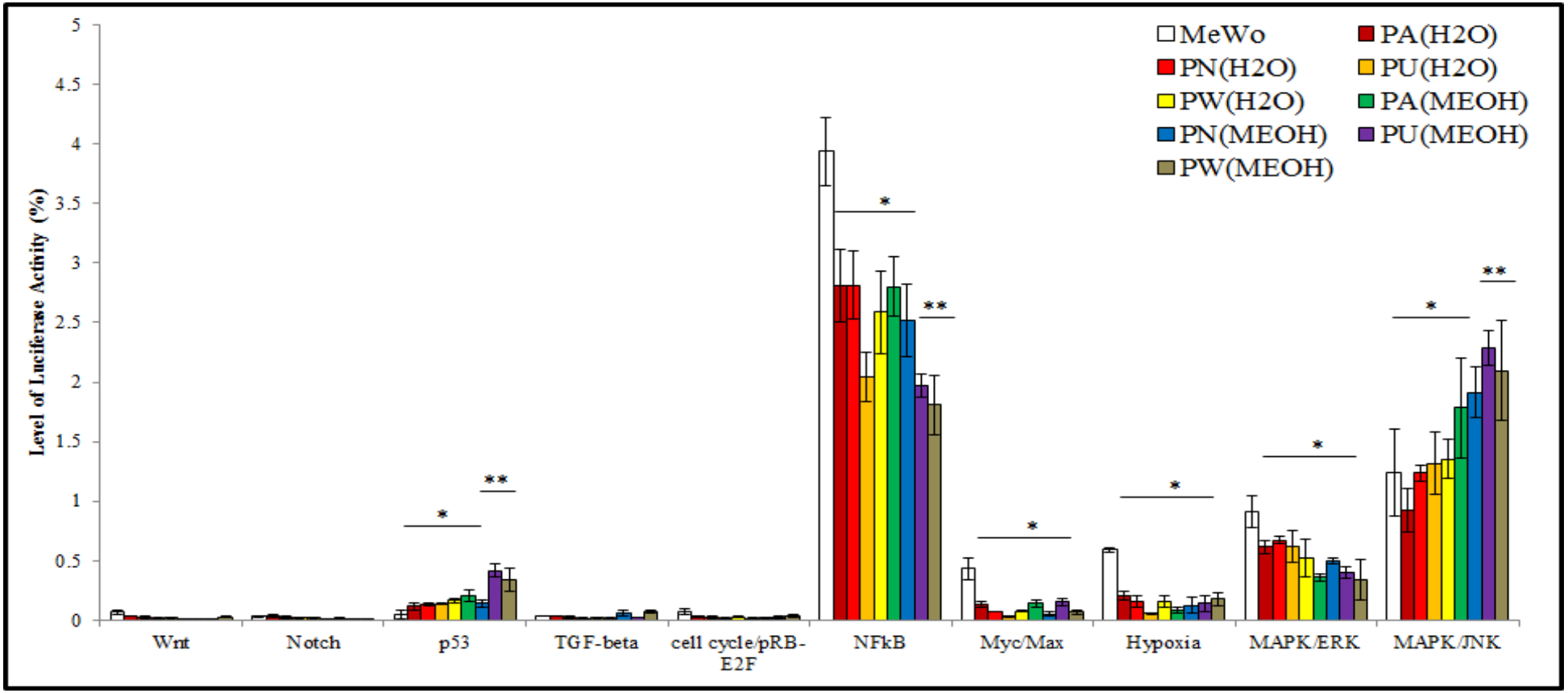

Figure 3.26: Alterations in ten cancer-related pathways upon *Phyllanthus* treatment in MeWo cells.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.7.2 Alterations on Multiple Signalling Pathways in PC-3 cells

Figure 3.27 depicts the differential expression of Wnt, Notch, p53, TGF-β, cell cycle/pRB-E2F, NFκB, Myc/Max, Hypoxia, MAPK/ERK and MAPK/JNK pathways affected by *Phyllanthus* species on PC-3 cells. It was noted that in the untreated PC-3 cells, all ten investigated pathways were expressed to regulate the cells growth and survival. Upon treatment with the different *Phyllanthus* species, the cells showed a significant down regulation of six pathways; Wnt, NFκB, Myc/Max, Hypoxia, MAPK/ERK and MAPK/JNK, suggesting that the plant extracts exerted its properties by targeting these pathways ($p<0.05$). The other pathways were not significantly affected by *Phyllanthus* extracts ($p>0.05$).

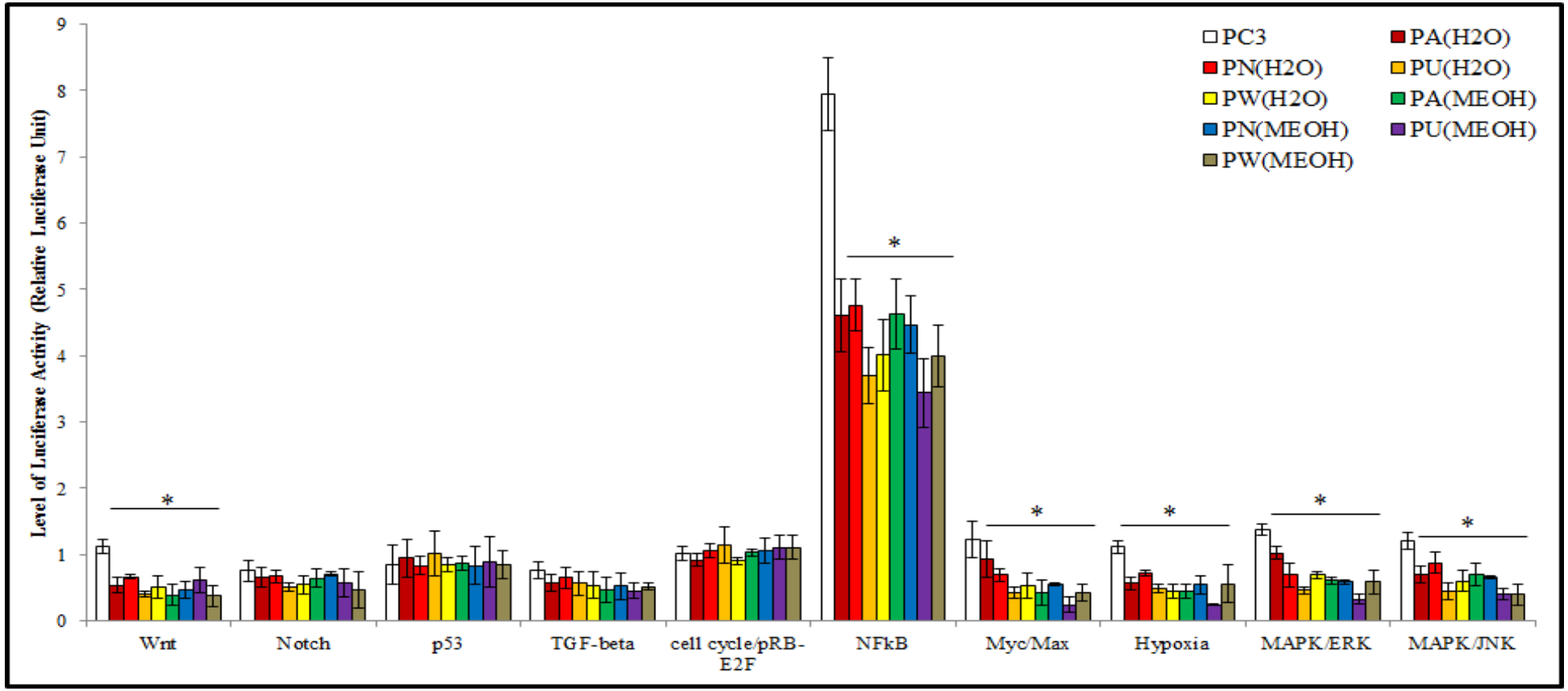

Figure 3.27: Alterations in ten cancer-related pathways upon *Phyllanthus* treatment in PC-3 cells.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$.

3.7.3 *Phyllanthus* disrupted anti-apoptotic/pro-apoptotic balance in cancer cells

One of the hallmarks of cancer is the inhibition of apoptosis. This can be achieved by suppressing the expression of pro-apoptotic protein, Bax and stimulating the expression of anti-apoptotic protein, Bcl-2. As shown in Figure 3.28, Bax and Bcl-2 proteins were detected at 23kDa and 26kDA, respectively.

In Figure 3.28A, it is observed the levels of the pro-apoptotic Bax proteins in treated MeWo was significantly increased with a concurrent decreased in anti-apoptotic Bcl-2 protein in treated MeWo cells as compared to untreated MeWo cells ($p<0.05$). Besides that, another apoptosis related protein; p53, was also detected at 53kDa. The p53 levels were significantly up-regulated in MeWo cells after treatment with *Phyllanthus* extracts ($p<0.05$). These observations indicate that *Phyllanthus* was able to induce apoptosis through the activation of p53 pathway and disrupted the anti-apoptotic/pro-apoptotic balance in MeWo cells.

A similar phenomenon was observed in treated PC-3 cells (Figure 3.28B). The graph shows a significant increase of Bax protein in the *Phyllanthus*-treated cells ($p<0.05$) with a concurrent decrease in Bcl-2 ($p<0.05$). P53 protein was not found in both untreated and treated PC-3 cells, suggesting *Phyllanthus* induced apoptosis via p53-independent pathway.

Among the species of *Phyllanthus*, both aqueous and methanolic extracts of *P.watsonnii* showed the most significant changes on Bax and Bcl expression ($p<0.01$), followed by *P.urinaria*, *P.niruri* and *P.amarus*.

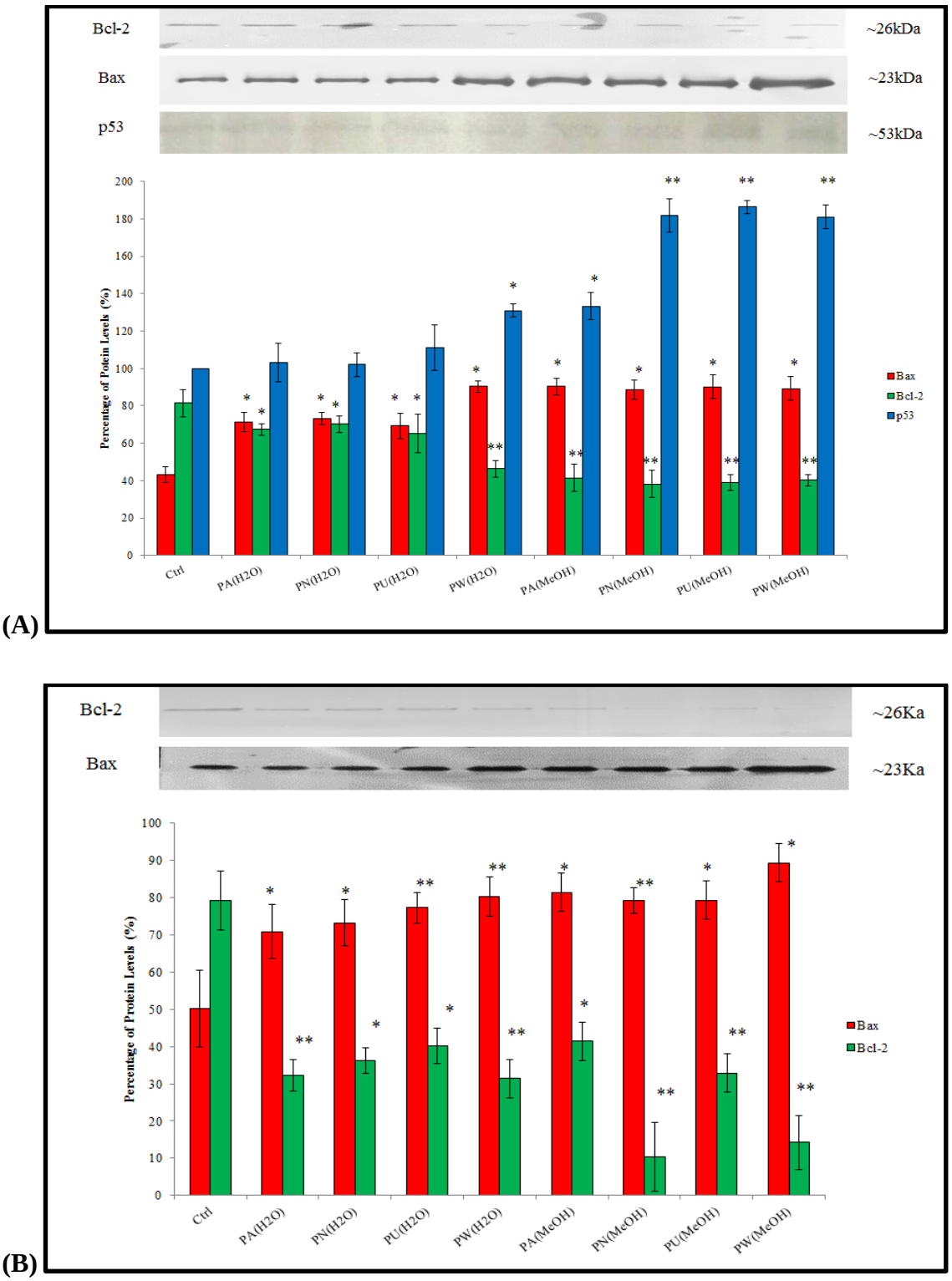

Figure 3.28: Effects of *Phyllanthus* extracts on pro-apoptotic/anti-apoptotic balance in (A) MeWo and (B) PC-3 cells.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *$p<0.05$ and **$p<0.01$.

3.7.4 Alterations of MAPK Pathways by *Phyllanthus* extracts

The high expressions of MAPK signalling in melanoma and prostate cancers impinges on most signalling pathways and plays a critical role in the progression of cancer such as tumour metastasis and angiogenesis. Three different major MAPK were studied; ERK, JNK and p38 MAPK pathways.

3.7.4.1 Down-Regulation of MAPK/ERK, p38 MAPK and PI3K/Akt Pathways in MeWo cells

Three up-stream activator molecules in MAPK and PI3K/Akt pathways; pan-Ras, c-Raf and Akt were highly expressed in the untreated MeWo cells. The constitutive activations of these molecules can activate their downstream targets including MAPK/ERK (RSK, Elk1, c-Jun/AP-1), MAPK/JNK (JNK1/2) and p38 (p38 MAPK). As shown in Figure 3.29, the expressions of all these intracellular signalling molecules were detected in untreated MeWo cells indicating their involvement in regulating MeWo cells' growth. However, the expression of all these intracellular signalling molecules had notably been down-regulated expression ($p<0.05$) except for JNK1/2 protein which showed a slight increase in expression in treated MeWo cells.

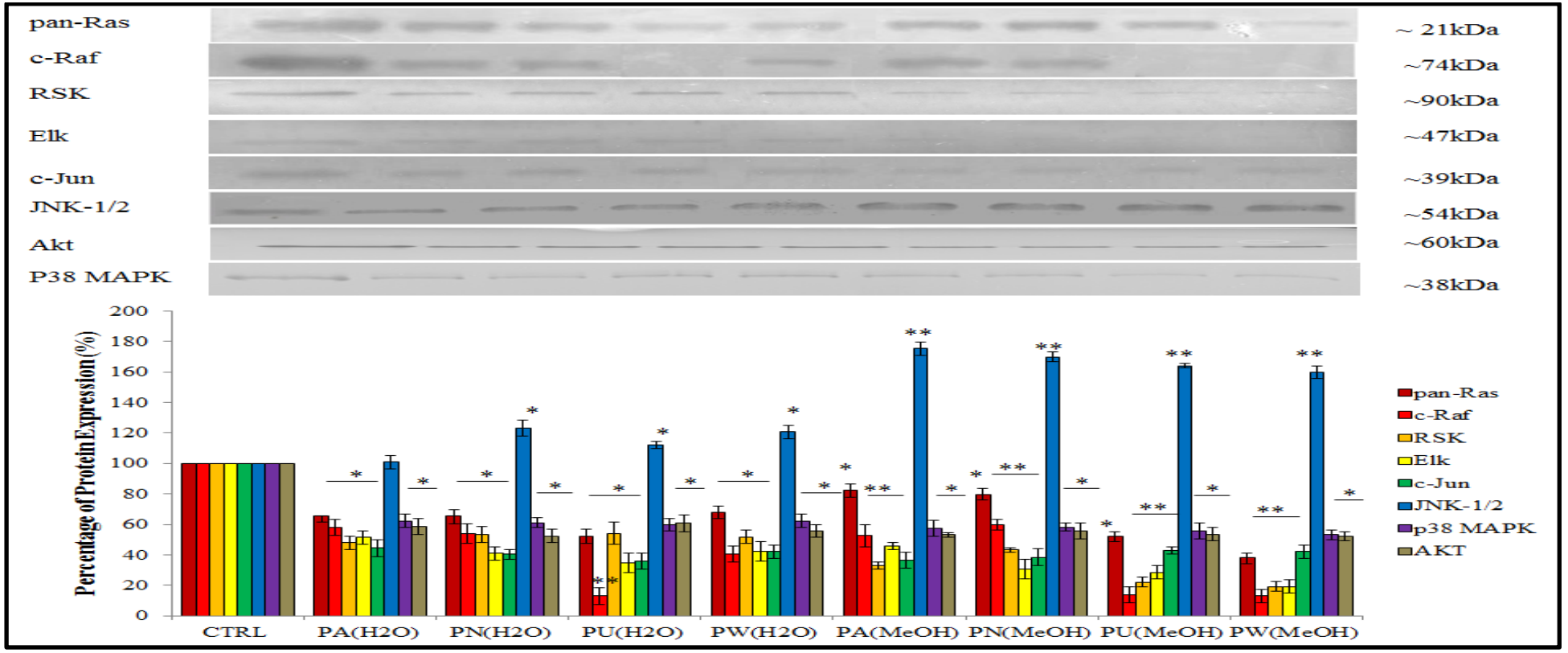

Figure 3.29: Alterations in activities of intracellular signalling molecules of MAPK and PI3K/Akt pathways upon *Phyllanthus* treatment in MeWo cells.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.7.4.2 Down-Regulation of MAPK and PI3K/Akt Pathways in PC-3 cells

In untreated PC-3 cells, two upstream activators, pan-Ras and c-Raf were highly expressed to ensure constitutive activation of MAPK pathways (Figure 3.30). Another upstream activator of Akt was also found to be highly expressed in PC-3 cells. The constitutive activations of pan-Ras, c-Raf and Akt can activate their downstream targets in three different MAPK pathways; MAPK/ERK (RSK, Elk1, c-Jun/AP-1), MAPK/JNK (JNK1/2) and p38 (p38 MAPK). As shown in Figure 3.30, the detected expressions of all these intracellular signalling molecules in untreated PC-3 cells indicate their involvement in regulating PC-3 cells' growth. Nevertheless, expression all these signalling molecules proteins had notably been down-regulated in PC-3 cells treated with the different species of *Phyllanthus* ($p<0.05$).

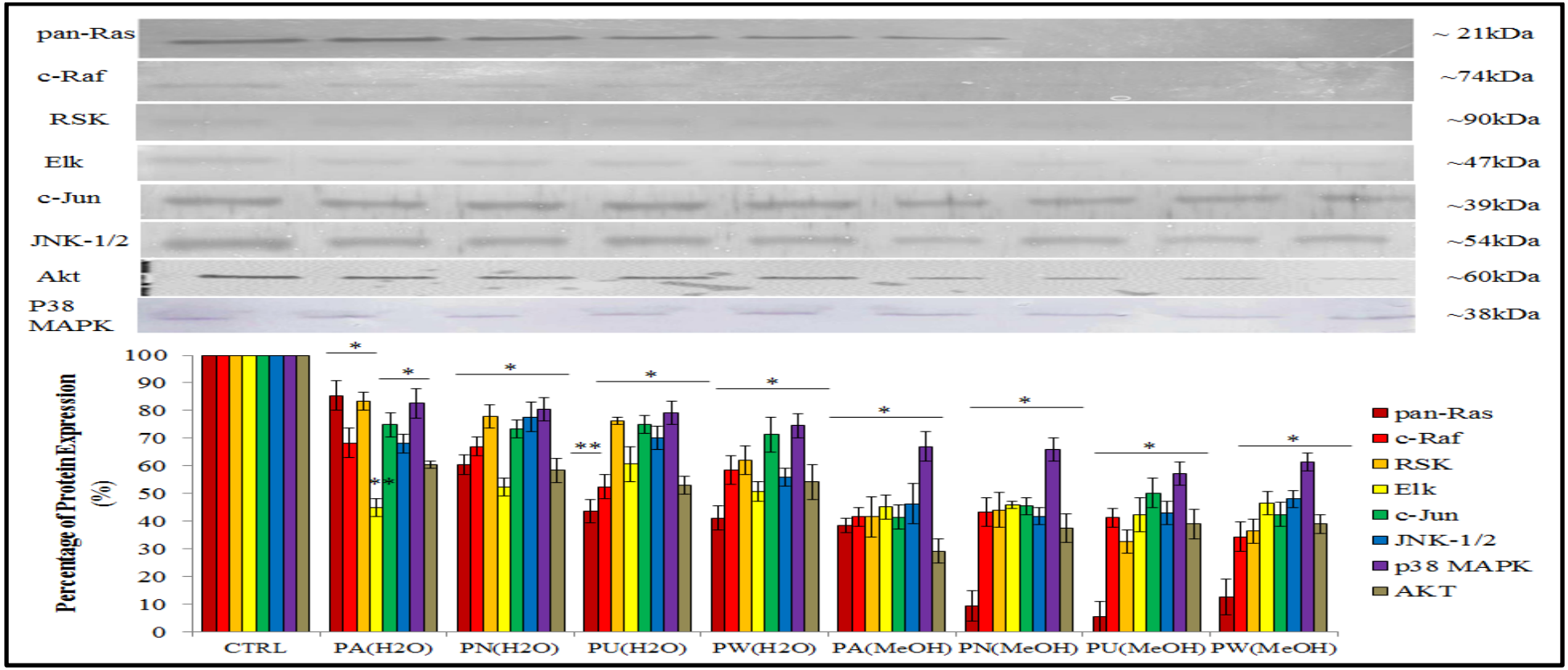

Figure 3.30: Alterations in activities of intracellular signalling molecules of MAPK and PI3K/Akt pathways upon *Phyllanthus* treatment in PC-3 cells.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.7.5 Alterations of Myc/Max and Hypoxia Pathways by *Phyllanthus* extracts

The high expression of Myc/Max and hypoxia pathways in melanoma and prostate cancers was observed in untreated MeWo and PC-3 cells (Figure 3.26 and 3.27), suggesting their crosstalk activities to regulate tumour metastasis and angiogenesis. In these pathways, three intracellular signalling molecules were detected by western blot; c-myc, HIF-1α, VEGF and GSK3β. However, their expressions were altered after treatment with *Phyllanthus*.

3.7.5.1 Down-Regulation of Myc/Max and Hypoxia Pathways in MeWo cells

As shown in Figure 3.31, the expression of the c-myc was detected at 65kDa and its expression was noticed significantly decreased by aqueous- ($p<0.05$) and methanolic-*Phyllanthus* ($p<0.01$) treated MeWo cells as compared to untreated cells. The downstream target of c-myc; HIF-1α and VEGF were detected at 120kDa and 50kD, respectively. Their expression was significantly down-regulated in treated MeWo cells as compared to untreated cells ($p<0.05$). A negative regulator of c-myc, glycogen synthase kinase 3-beta (Gsk3β) was detected at 51kDa and its expression was significantly up-regulated in treated MeWo cells as compared to untreated MeWo cells ($p<0.01$).

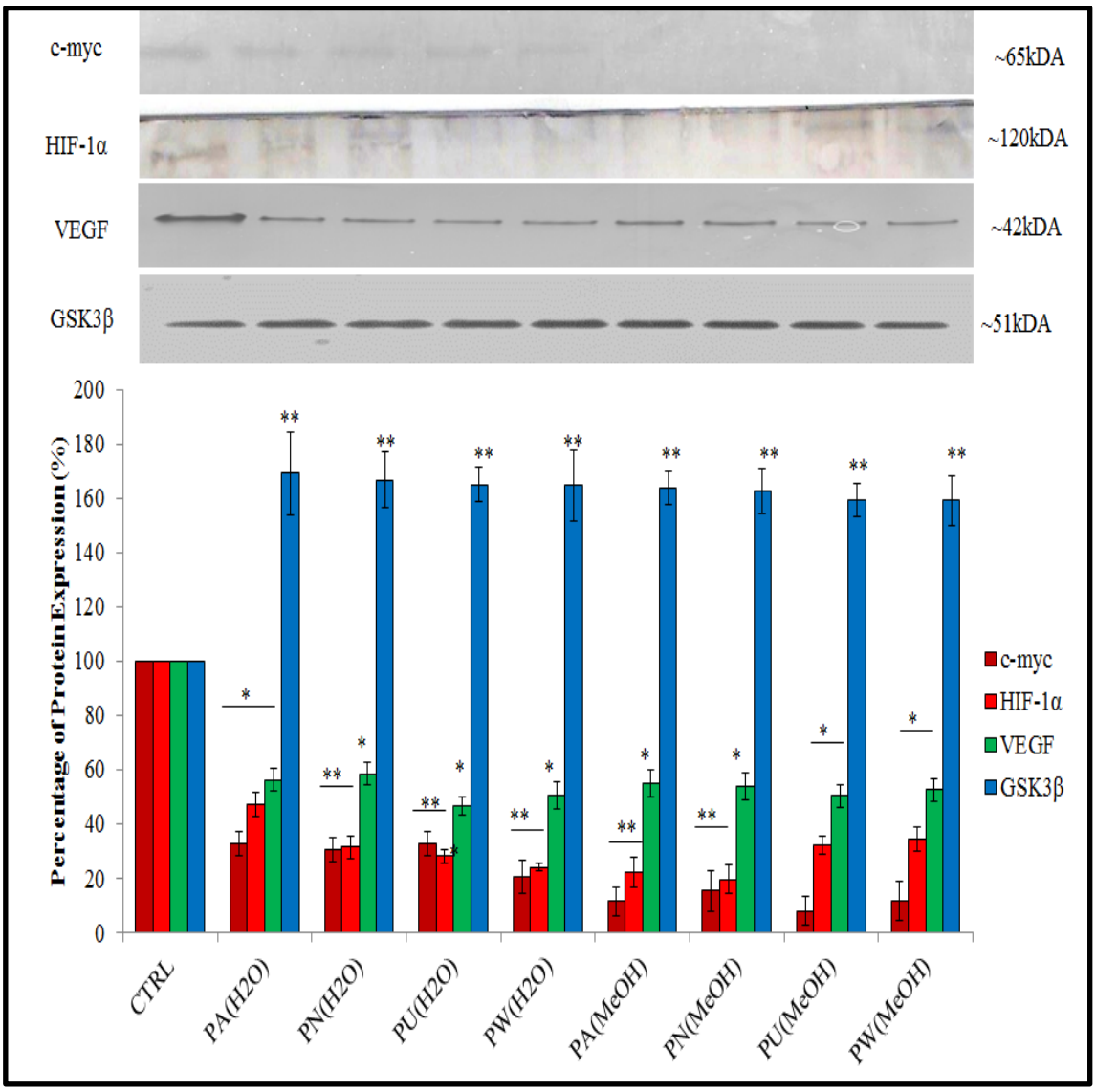

Figure 3.31: Alterations in activities of intracellular signalling molecules of Myc/Max and Hypoxia pathways upon *Phyllanthus* treatment in MeWo cells.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.7.5.2 Down-Regulation of Wnt, Myc/Max and Hypoxia Pathways in PC-3 cells

The expression of Wnt signalling pathway was detected at increased levels in PC-3 cells (Figure 3.27) with high percentage down-regulation when treated with *Phyllanthus*. Investigation of the downstream molecules in this pathway, revealed expression of three intracellular signalling molecules; Dishevelled (DSH) at 95kDA, Gsk3β at 47kDA and β–catenin at 65kDA. After treatment with aqueous ($p<0.05$) and methanolic ($p<0.01$) extracts of *Phyllanthus* species as shown in Figure 3.32A, the expression of DSH and β–catenin were noted significantly down-regulated. Contrarily, the expression of Gsk3β was significantly up-regulated in treated PC-3 cells as compared to untreated PC-3 cells ($p<0.01$).

In the Myc/Max and hypoxia pathways, three different intracellular signalling molecules were detected by western blot. As shown in Figure 3.32B, the expression of the c-myc was detected at 67kDA and its expression was noted to be down-regulated in aqueous- ($p<0.05$) and methanolic-treated ($p<0.01$) PC-3 cells. The downstream targets of c-myc; HIF-1α and VEGF were detected at 120kDa and 50kDa, respectively. The expression of HIF-1α and VEGF in PC-3 was significantly down-regulated by *Phyllanthus* extracts ($p<0.05$).

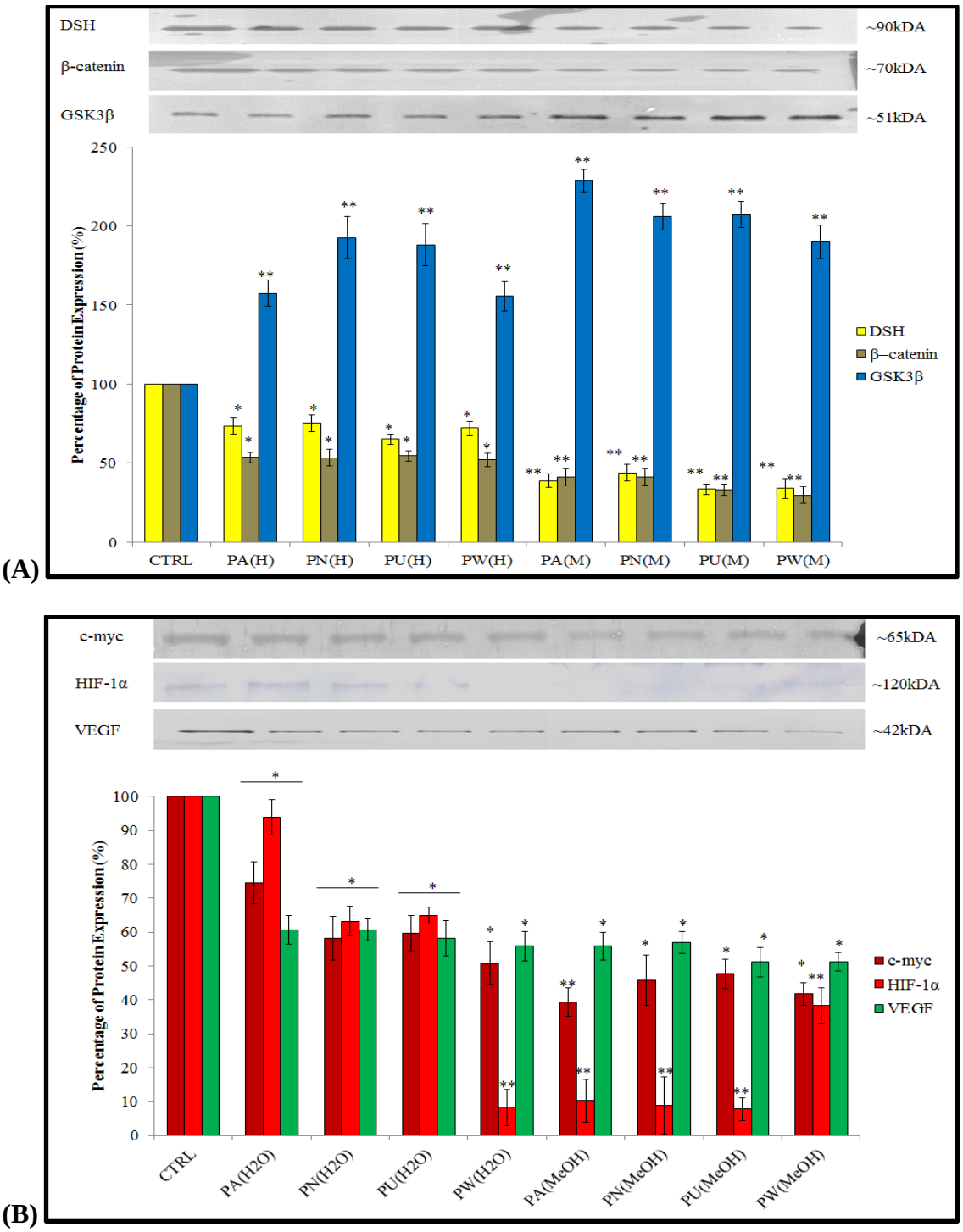

Figure 3.32: Alteration in intracellular signalling molecules of (A) Wnt and (B) Myc/Max and Hypoxia pathways in PC-3 cells after treatment with *Phyllanthus* extracts.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.7.6 Alterations of NFκB Pathway by *Phyllanthus* extracts in MeWo and PC-3 cells

Two members of NFκB signalling pathway proteins were detected in MeWo and PC-3 cells; NFκB p50 and NFκB p52. As shown in Figure 3.33, both proteins were significantly down-regulated in MeWo and PC-3 cells after treatment *Phyllanthus* extracts as compared to untreated cells. The level of both NFκB p50 and p52 expressions were down-regulated to a range of 56.4-72.3% ($p<0.05$) by aqueous and 44.5-57.9% by methanolic extracts in treated cells ($p<0.01$) in both cells.

Among the *Phyllanthus* species, *P.urinaria* showed strongest inhibitory effects on these intracellular signalling molecules in NFκB pathways, followed by *P.watsonii*, *P.amarus* and *P.niruri* for both aqueous and methanolic extracts.

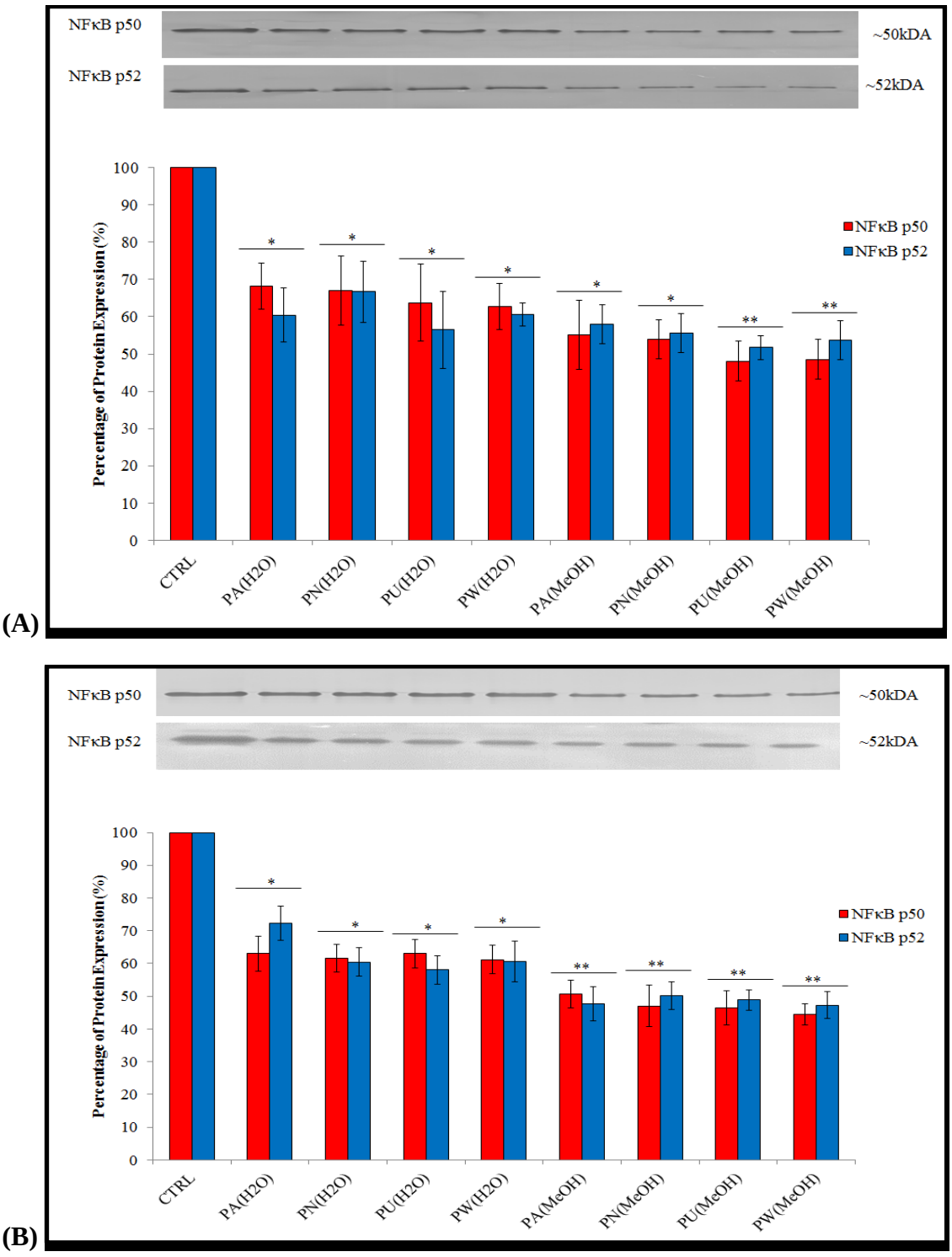

Figure 3.33: Alterations of activities intracellular signalling molecules in NFκB pathway in (A) MeWo and (B) PC-3 cells after treatment with *Phyllanthus* extracts.

Figure shows data represent the mean (± SEM) of three independent experiments, each performed in triplicate. The asterisk indicates a significant difference between untreated (control) and *Phyllanthus*-treated cells, *p<0.05 and **p <0.01.

3.8 Proteomic profiling of the differentially expressed proteins in *Phyllanthus* treated MeWo and PC-3 cells

The interaction of protein in a cell is complex and provides a comprehensive picture of cellular function and biological processes. In cancer, this interaction usually is tightly regulated to ensure malignancy of cancer. The dysregulation of some proteins and/or interactions may halt the growth of cancer. Therefore, studies of cancer-related proteins in cancer cells after treatment with *Phyllanthus* plant extracts could explain its anticancer properties via regulation in the protein and/or interaction networks of cancer.

A 2D electrophoresis approach was performed to compare the proteomic profiles of an untreated cell with that treated cancer cells to identify differentially expressed proteins that may be involved in the anticancer activity of *Phyllanthus* plant extracts. The proteomic profiles of untreated and treated MeWo (Figure 3.34) and PC-3 (Figure 3.35) cells were obtained from ImageMaster 2D scanner (GE Healthcare Life Sciences). A two-dimensional electrophoresis gel analytical software, PDQuest version 7.1 (Bio-Rad, USA) was used to to evaluate and identify the differentially expressed proteins between untreated and treated groups.

Differentially expressed proteins were statistically defined based on two criteria: 1) degree of intensity >1.0 fold (Protein scores of greater than 70 are considered significant, $p<0.05$) and 2) reoccurrence of the same proteins in the three repeated experiments.

3.8.1.1 Proteomic profiling of human melanoma, MeWo cells

According to these criteria, 52 proteins in treated MeWo cells were identified by MS/MS and grouped in four biological processes; Group 1 (cell adhesion, migration, invasion and metastasis), Group II (proliferation, cell cycle and apoptosis), Group III (glycogenesis and glycolysis) and Group IV (protein synthesis and energy

metabolisms), based on their functions described in UniProtKB/Swiss-Prot protein database (Table 3.4).

In Group I, 8 proteins were found to be differentially expressed in treated MeWo cells. Of these, type I cytoskeletal 13 keratin was found to be up-regulated ($p<0.05$). Its expression was significantly up-regulated about 1.4-1.8 folds higher than untreated cells. Proteins that were down-regulated in treated cells were identified as Ephrin-B1, alpha-centractin, bystin, superoxide dismutase, tubulin beta chain, F-actin-capping protein subunit alpha-2 and partitioning defective 3 homolog.

In Group II, 21 proteins were significantly down-regulated in MeWo cells after treatment with *Phyllanthus* extracts. Among these down-regulated proteins, HCLS1-associated protein X-1 and casein kinase II subunit alpha proteins showed the greatest reduction at 1.5-2.3 folds in their expression as compared to untreated cells ($p<0.05$).

In Group III, 5 enzymes were down-regulated in treated MeWo cells. These identified enzymes were glucosamine--fructose-6-phosphate aminotransferase, protein phosphatase 1 regulatory subunit 3D, alpha-enolase, pyruvate kinase isozymes M1/M2 and phosphoglycerate kinase 1.

In Group IV, 15 proteins were found to be down-regulated at the range of 1.3-1.9 in *Phyllanthus* treated MeWo cells as compared to untreated cells. Eight of these proteins; E3 ubiquitin-protein ligase ARIH2 and RNF115, aspartate aminotransferase, 26S protease regulatory subunit 8, putative elongation factor 1-alpha-like 3, mitochondrial inner membrane organizing system protein 1 (MINOS), zinc finger protein, Sec1 and trimethyllysine dioxygenase have been known to be involved involved in regulation of mitochondrial integrity and stability of cellular proteisn.

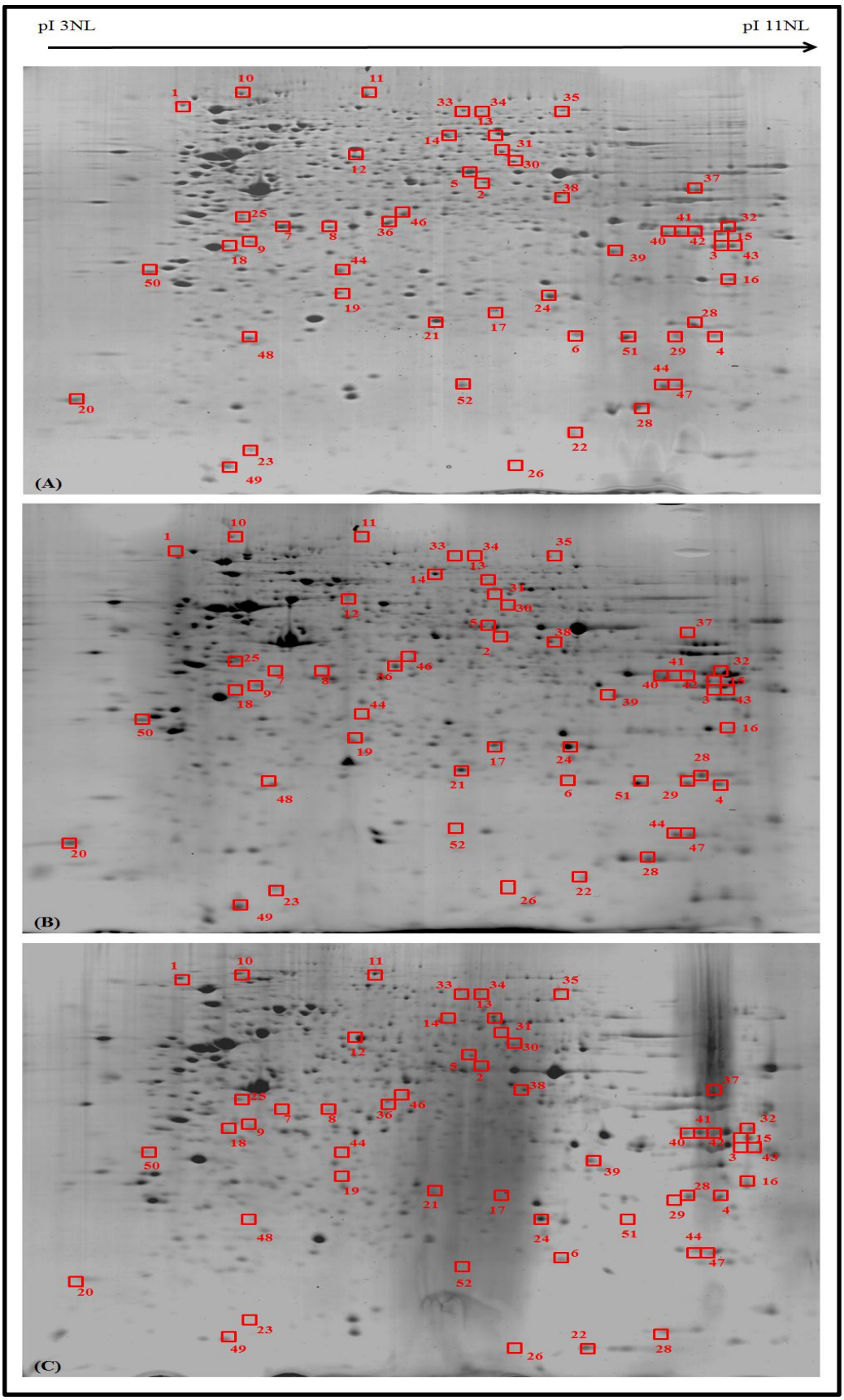

Figure 3.34: The proteomic profiles of (A) untreated, aqueous- (B) and methanolic- (C) *Phyllanthus* treated MeWo cells.

Table 3:4: The differentially expressed proteins in treated MeWo cells.

	Differentially expressed proteins in *Phyllanthus*-treated MeWo cells			
	Down-regulated		Up-regulated	
Group I (Cell adhesion, migration, invasion and metastasis)	1. 2. 3. 5. 6. 7. 8. 9.	Ephrin –B1 Alpha-centractin Bystin Vimentin Superoxide dismutase [Mn], mitochondrial Tubulin beta chain F-actin-capping protein subunit alpha-2 Partitioning defective 3 homolog	4.	Keratin, type I cytoskeletal 13
Group II (Proliferation, cell cycle and apoptosis)	Down-regulated			
	10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20.	Casein kinase II subunit alpha Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical] Mitochondrial import inner membrane translocase subunit Tim8 Structure-specific endonuclease subunit SLX1 E3 ubiquitin-protein ligase TRAF7 Humanin-like protein 9 Galectin-3 Heterogeneous nuclear ribonucleoprotein H Annexin A5 Transcription elongation factor A protein-like 3 Nuclear body protein SP140-like protein	21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32.	14-3-3 protein theta Peptidyl-prolyl cis-trans isomerase A Zinc finger protein 169 GRB2-related adapter protein HCLS1-associated protein X-1 Methyl-CpG-binding domain protein 4 Glutathione transferase omega – 1 Peroxiredoxin-1 Serpin B9 E3 ubiquitin-protein ligase ZNRF1 E3 ubiquitin-protein ligase ARIH2 E3 ubiquitin-protein ligase RNF115

	Down-regulated			
Group III (Glycogenesis and glycolysis)	33. 34. 35.	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 2 Protein phosphatase 1 regulatory subunit 3D Alpha-enolase	36. 37.	Pyruvate kinase isozymes M1/M2 Phosphoglycerate kinase 1
	Down-regulated			
Group IV (Protein synthesis and energy metabolisms)	38. 39. 40. 41. 42. 43. 44.	Aspartate aminotransferase, cytoplasmic Small proline-rich protein 3 Patatin-like phospholipase domain-containing protein 4 Ethanolamine kinase 1 26S protease regulatory subunit 8 Putative elongation factor 1-alpha-like 3 Mitochondrial inner membrane organizing system protein 1	45. 46. 47. 48. 49. 50. 51. 52.	Cathepsin D AP-4 complex subunit sigma-1 Adenylate kinase Metallothionein-1H Zinc finger protein 534 Mitochondrial inner membrane protease ATP23 homolog Sec1 family domain-containing protein 1 Trimethyllysine dioxygenase, mitochondrial

3.8.1.2 Proteomic profiling of human prostate adenocarcinoma, PC-3 cells

There were 72 differentially expressed proteins identified in treated PC-3 cells (Table 3.5). In Group I, 10 proteins were found to be differentially expressed in *Phyllanthus* treated PC-3 cells. Of these, three keratin proteins (type II cytoskeletal 8, type I cytoskeletal 9, and keratin-associated protein 3-1) were observed to be up-regulated. Notably, these up-regulated proteins were derived from same family, keratin and their expressions were about 1-2 folds higher than untreated cells. The 7 other proteins found to be down-regulated were Ephrin-B1, actin, EH domain-binding protein 1, heat shock protein 1, vimentin, tubulin alpha-8 chain and MEMO1.

In Group II, 28 proteins were significantly down-regulated in treated PC-3 cells. Among these altered proteins, five namely gluthathione S-transferease P, protein Wnt-5a, proto-oncogene Wnt-3, putative Ras-related protein Rab-42 and GTPAse HRas precursor showed the greatest reduction in their expression with a range of 1.7-2.2 folds higher than untreated cells ($p<0.05$).

In Group III, 7 down-regulated proteins were identified in the treated PC-3 cells and five of them were enzymes; phosphoglycerate kinase-1, alpha-enolase, glyceraldehyde-3-phosphate dehydrogenase (G3PD), fructose-biphosphate aldolase and triosephosphate isomerase.

In Group IV, 27 proteins were differentially expressed with only one being significantly up-regulated in PC-3 after treatment with *Phyllanthus* extracts; voltage-dependent anion-selective channel protein 1 in the range of 1.5-1.8 folds higher. Among the down-regulated proteins detected, four have been associated with calcium regulation were detected; 39S ribosomal protein L51, calumenin, calreticulin and 78 kDa glucose regulated protein.

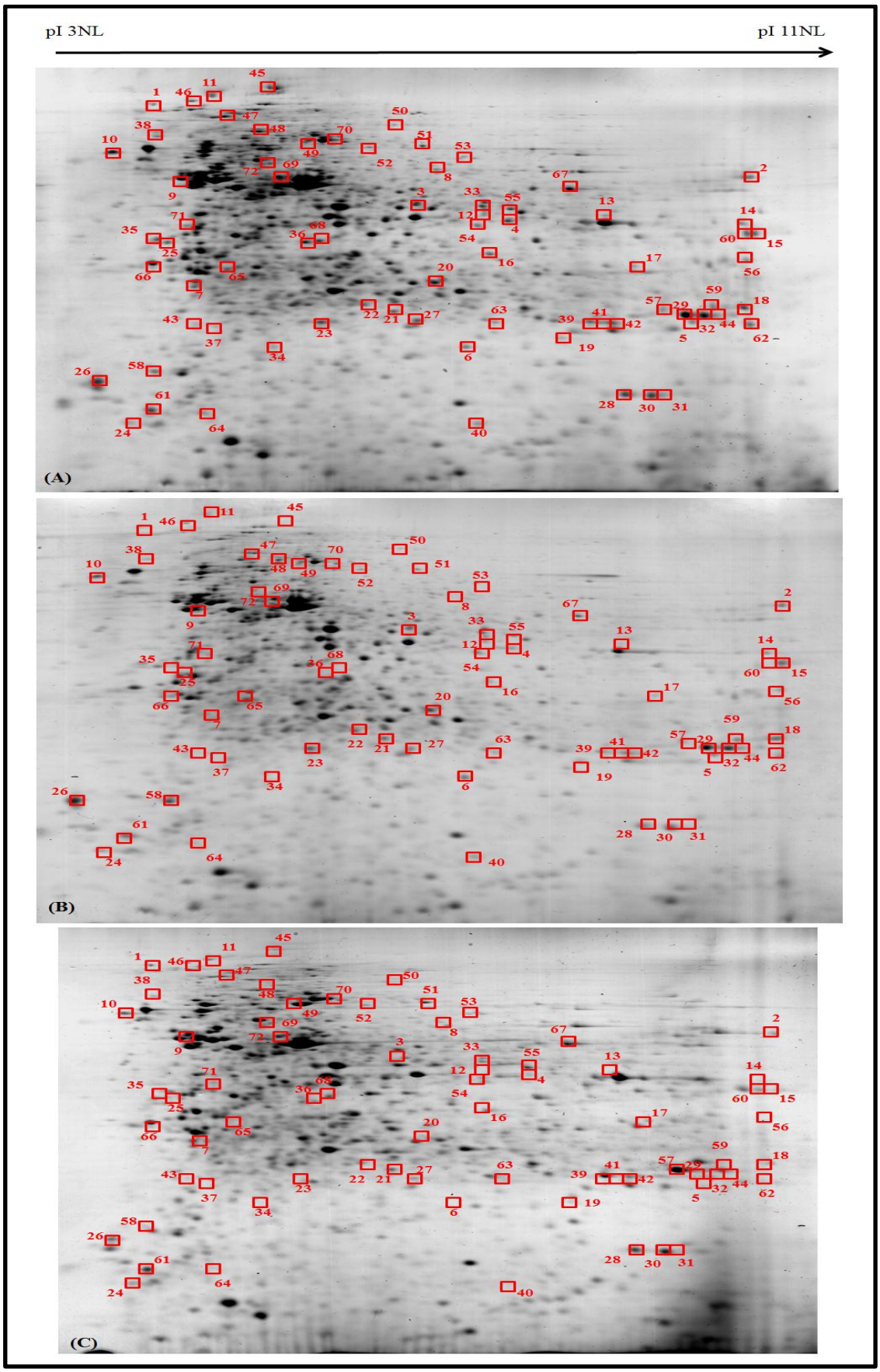

Figure 3.35: Proteomic profiles of (A) untreated, aqueous- (B) and methanolic- (C) *Phyllanthus* treated PC-3 cells.

Table 3.5: The differentially expressed proteins in treated PC-3 cells.

	Differential expressed proteins in *Phyllanthus*-treated PC-3 cells			
Group I (Cell adhesion, migration, invasion and metastasis)	Down-regulated		Up-regulated	
	1. 3. 4. 5. 8. 9. 10.	Ephrin –B1 Actin, cytoplasmic 2 EH domain-binding protein 1 Heat shock protein beta-1 Vimentin Tubulin alpha-8 chain Protein MEMO1	2. 6. 7.	Keratin, type II cytoskeletal 8 Keratin, type I cytoskeletal 9 Keratin-associated protein 3-1
Group II (Proliferation, cell cycle and apoptosis)	Down-regulated			
	11. 12. 13. 14. 15. 16. 17. 18. 19. 20. 21. 22. 23. 24.	DNA damage-binding protein 2 Transcription factor 23 Proliferation-associated protein 2G4 Growth factor receptor-bound protein 2 Gremlin-1 14-3-3 protein theta 14-3-3 protein gamma Annexin A1 Coiled-coil domain-containing protein 74B Heterogeneous nuclear ribonucleoprotein H Glutathione S-transferase P Protein Wnt-5a Transmembrane protein 222 Sperm protein associated with the nucleus on the X chromosome N3	25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38.	ATP-dependent DNA helicase Q1 Galectin-1 Heat shock protein beta-1 Glutathione transferase omega – 1 Peroxiredoxin-1 Thioredoxin-dependent peroxide reductase, mitochondrial RuvB-like 2 Serpin B9 E3 ubiquitin-protein ligase ZNRF1 Probable G-protein coupled receptor 179 Bcl-2-like protein 11 Proto-oncogene Wnt-3 precursor Putative Ras-related protein Rab-42 GTPase HRas precursor
Group III (Glycogenesis and glycolysis)	Down-regulated			
	39. 40. 41. 42.	Urocortin-3 Phosphoglycerate kinase 1 Alpha-enolase Glyceraldehyde-3-phosphate dehydrogenase	43. 44. 45	Fructose-bisphosphate aldolase a Triosephosphate isomerase Neuroglobin

Group IV (Protein synthesis and energy metabolisms)	Down-regulated			
	45.	39S ribosomal protein L51	59.	Protein disulfide-isomerase A3
	46.	Betaine--homocysteine S-methyltransferase 1	60.	Elongation factor Tu, mitochondrial
	47.	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	61.	Mitochondrial inner membrane organizing system protein 1
	48.	Galactose-3-O-sulfotransferase 3	62.	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial
	49.	Type 2 lactosamine alpha-2,3-sialyltransferase	63.	ADP-ribosylation factor-like protein 6
	50.	Calumenin	64.	Vacuolar protein sorting-associated protein 26A
	51.	Protein disulfide-isomerase A4	65.	BTB/POZ domain-containing protein KCTD5
	52.	Calreticulin	66.	Dynamin-1-like protein
	53.	Heat shock protein HSP 90-alpha	67.	Nicotinamide N-methyltransferase
	54.	ADP/ATP translocase 1	68.	Proteasome subunit beta type-3
	55.	6-phosphogluconate dehydrogenase, decarboxylating	69.	Speckle-type POZ protein-like
	56.	5'-AMP-activated protein kinase subunit beta-1	71.	Adenylate kinase isoenzyme 6
	57.	78kDa glucose-regulated protein	72.	Eukaryotic translation initiation factor 3 subunit 12
	58.	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12		
	Up-regulated			
	70.	Voltage-dependent anion-selective channel protein 1		

CHAPTER 4: DISCUSSION

The integration of human knowledge and intelligence in science and technology has improved the quality of human health with earlier diagnosis, different types of treatments and reductions in hospitalization. However, the global burden of cancer has not decreased but has seen a continuous rise (Ferlay *et al.*, 2010). Modern cancer treatment therapies such as surgery, chemotherapy and immunotherapy are deemed relatively unsuccessful due to their ineffectiveness, side effects, safety issues and costliness. In addition, humankind is confronted with increasing implementation of cancer-associated lifestyle choices such as smoking and physical inactivity, contributing to an increase in the number of deaths caused by cancer annually (Hanahan & Weinberg, 2011). As not all cancer patients respond positively to current anticancer agents, mortality rates of certain cancers continue to rise, including melanoma and prostate cancer.

Herbs and plants are the basic remedies in many traditional medicine systems and have been extensively used throughout the world for thousands of years and still continue to offer mankind with alternatives for disease treatment. The exploration of anticancer agents from plant sources began in the 1950s and is still active, with the successful discoveries of plant-derived anticancer drugs such as taxol, vinblastine and vincristine (Cragg & Newman, 2005; Shoeb, 2006). The “back to basics” approach is due to both its long history of usage in disease treatment and the pharmacological/nutritional value of these products which are believed to be able to halt/delay cancer progression (Deorukhkar *et al.*, 2007).

In this study, the anticancer properties of four *Phyllanthus* (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) were studied on human melanoma, MeWo and prostate

adenocarcinoma, PC-3 cells. The main aims of this study were to identify the various of ways by which *Phyllanthus* plant extracts exert their anti-cancer properties on these two cancer cell lines

4.1 Bioactive Compounds in *Phyllanthus*

Food is the main source of essential nutrients to support the nutritional needs of our body. Plant-based foods contain a wide range of non-nutrient bioactive compounds for vital biological functions including the defense system. These bioactive compounds are normally non-toxic in the human body and are important for health promotion. Several epidemiologic studies have shown that a plant-based diet provided protective effects on chronic diseases such as cancer (Duthie *et al.*, 2000; Talalay & Fahey, 2001). In cancer treatment, approximately 74% of anticancer agents are naturally derived products (Tan *et al.*, 2006).

Phyllanthus extracts were subjected to high performance liquid chromatography (HPLC) coupled with electronspray ionization (ESI) and mass spectrometry (LCMS-MS) analysis to identify the presence of bioactive compounds. The results revealed that *Phyllanthus* extracts consists of twelve bioactive compounds; gallic acid, galloylglucopyronside, digalloylglucopyronside, trigalloylglucopyronside, tetragalloylglucopyronoside, corilagen, geraniin, rutin, quercetin glucoside, quercetin diglucoside, quercetin rhamnoside, and caffeolquinic acid. (Table 3.1) Some of these bioactive compounds such as geraniin, corilagen and gallic acid have been proven to possess anticancer effect on different cancers (Alía *et al.*, 2006; Guruvayoorappan & Kuttan, 2007; Lee *et al.*, 2008a; Chen *et al.*, 2009).

Geraniin is the main tannin in *Euphorbiaceae* and its hydrolysation produces corilagin (Okuda *et al.*, 1980). Both of these compounds were identified in all four

Phyllanthus species. In addition, geraniin is the only bioactive compound that can be found in both aqueous and methanolic extracts of four *Phyllanthus* species. Geraniin and corilage have been shown to contribute to growth arrest and induction of apoptosis in several cancer cells (Pan *et al.*, 2000; Huang *et al.*, 2004b; Chu *et al.*, 2007; Lee *et al.*, 2008a; Wang & Jin, 2010; Xiong & Qing, 2010). Besides this, gallic acid has been shown to exhibit anti-proliferative effects on several cancer cell lines such as lung (Ohno *et al.*, 1999; Choi *et al.*, 2009), stomach, colon (Yoshioka *et al.*, 2000), prostate (Chen *et al.*, 2009) and cervical (Choi *et al.*, 2009).

As no individual class of components could be fully responsible for the activity/effect produced by a whole extract (Sun & Hai Liu, 2006), it was therefore more meaningful to assess the activity of *Phyllanthu*s extracst as a whole mixture of bioactive compounds rather than as their individual compounds. Semi-purification of crude extract of *Phyllanthus* yielded two fractions (fraction 1 and 2). Both fractions were evaluated for their cytotoxicity as comparison to crude extracts of *Phyllanthus* species.

4.2 Anti-Proliferative Effect of *Phyllanthus*

Anti-proliferative effect or cytotoxicity screening was conducted on human skin melanoma (MeWo) and prostate (PC-3) cancer cell lines. The half-maximal inhibitory concentration (IC_{50}) value is a parameter used to assess dose/concentration of anticancer drug, which has an impact on the proliferation of cancer cells. In the present study, crude (aqueous and methanolic) extracts of four plant species of *Phyllanthus* displayed different IC_{50} values on human skin melanoma (MeWo) (Table 3.2) and prostate (PC-3) (Table 3.3) cell lines. The variations in the IC_{50} values of *Phyllanthus* extracts against melanoma and prostate cancer cells might be due to the differing levels of bioactive

compounds present in each *Phyllanthus* species. Among the four *Phyllanthus* species, *P. urinaria* showed the strongest anti-proliferative effect with the lowest IC_{50} values compared to other species. This could be associated to the presence of higher content of bioactive compounds in both extracts.

As noted, the methanolic extracts of *Phyllanthus* seemed to have more pronounced anti-proliferative effects compared to the aqueous extracts as their effect was exhibited at a relatively low dose. Geraniin can be found in both aqueous and methanolic extracts. However, geraniin is present together with other bioactive compounds in aqueous extracts. This mixture might have reduced the killing effect of geraniin on cancer cells, thus resulting in the aqueous extracts exerting the same anti-proliferative effect at a higher dose as compared to methanolic exracts. Several studies reported that the organic-soluble compounds are likely to inhibit or are lethal to cancerous cells than water-soluble compounds based solely on its toxicity effect (Cai *et al.*, 2004; Saetung *et al.*, 2005).

Phyllanthus extracts have exhibited low cytotoxic effects on normal human skin (CCD- 1127Sk) and prostate (RWPE-1) cell lines as well as the human umbilical vein endothelial cells (HUVECs). These findings correlate with studies carried out by Huang et al. in which *Phyllanthus* plants likely displayed selective killing against cancer cells (Huang *et al.*, 2004a). This selective anti-proliferative or cytotoxic effect of *Phyllanthus* is important because currently available anticancer drugs can target normal cells and cancer cells, resulting in serious side effects. Thus, *Phyllanthus* may be applied in cancer treatments as it has much lower undesirable side effects.

The anti-proliferative effect of *Phyllanthus* species could be due to the presence of different bioactive compounds in aqueous extract including galic acid, rutin, and quercetin, as well as geraniin in both aqueous and methanolic extracts (Table 1). These

bioactive compounds have been reported to possess anti-proliferative effects associated with their natural antioxidant activity (Aruoma *et al.*, 1993; Lamson & Brignall, 2000; Yen *et al.,* 2002; Alía *et al.,* 2006; Lin *et al.,* 2008; Ito, 2011). The roles of these bioactive compounds against cancer have been well documented as they can reduce the chance of cancer development by preventing mutation caused by free radicals in normal cells (Rao & Agarwal, 2000; Hayes & McMahon, 2001). Therefore, the low anti-proliferative effect of *Phyllanthus* extracts on normal cells (CCD- 1127Sk, RWPE-1 and HUVECs) is believed due to presence of these naturally occurring bioactive compounds with antioxidative properties in *Phyllanthus* plant extracts.

Phyllanthus plant extracts exhibited selective cytotoxicity against MeWo and PC-3 human cancer cells. In comparison, the plant extracts did not show any significant cytotoxicity on normal human skin (CCD-1127Sk) and prostate (RWPE-1) cells. In contrast, semi-purification of all *Phyllanthus'* aqueous crude extract; fraction 1 and 2 have displayed cytotoxicity to both normal and cancer cell lines. As mentioned earlier, no any individual class of components in an extract could be entirely held accountable for the activity produced by the whole extract itself (Sun & Hai Liu, 2006). Therefore, it was more meaningful to assess the anticancer activity of *Phyllanthus* as a complete mixture of bioactive compounds in crude extracts rather than fractionation of *Phyllanthus* species.

4.3 Regulation of Cancer Cells Proliferation by *Phyllanthus*

Cell cycle is the main regulator in cell proliferation and growth. Deregulation of this process will drive cancer cells into uncontrolled proliferation, which has been described as one of the hallmark traits of cancer (Evan and Vousden, 2001). This deregulation of cell cycle in human cancer appears to be the ultimate targets for possible

therapeutic interventions. Several studies have showed that the anti-proliferative effects of cytotoxic agents are attributed to cell cycle arrest at different phases (G0/G1, S or G2/M) and lead to apoptosis induction (Hsieh & Wu, 1999; Evan & Vousden, 2001).

Phyllanthus extracts induced cell cycle arrest at S-phase in MeWo cell (Figure 3.2) indicating that DNA synthesis was interupted, halting the progression of cell cycle at S-phase and leading to apoptosis induction with evidence of apoptotic cell accumulation of Sub-G1 phase. The induction of apoptosis in MeWo cells was further implicated with caspases-3/7 activation, TUNEL-positive cells and DNA fragmentation (Figure 3.4-3.7). The S-phase arrest in *Phyllanthus* treated MeWo cells were due to an alteration in MAPK/JNK and PI3K/Akt (Figure 3.29). Shishodia et al. has shown the the suppression of PI3K/Akt and activation of JNK/c-Jun would lead to activation of cyclin-dependent kinase inhibitor (CKI), p21 to induce S-phase arrest (Shishodia *et al.*, 2007). In addition, the cell cycle arrest at S-phase was further imposed with the activation of p53 in treated MeWo cells (Figure 3.28A).The involvement of p53 to induce cell cycle arrest at S-phase was observed as p21 is a major target for transactivation by p53 (Radhakrishnan *et al.*, 2004). The activation of p21 will induce cell cycle arrest at S-phase through two mechanisms; (1) interaction of p21 with the proliferating cell nuclear antigen (PCNA) (Waga *et al.*, 1994; LaBaer *et al.*, 1997; Levine, 1997) and/or (2) p21 directly inhibiting the activity of cyclin E/CDK2 complexes (Harper *et al.*, 1995; Kuwajerwala *et al.*, 2002; Shishodia *et al.*, 2007). Beyond its involvement with cyclin/CDKs, p21 also involved in regulation of various DNA-binding proteins that contribute to growth arrest including NFκB, Myc, E2F, STAT3, and estrogen receptor (Perkins, 2002; Coqueret, 2003; Fritah *et al.*, 2005). Therefore, the down-regulations of c-myc (Figure 3.31) and NFκB (Figure 3.33A) proteins were observed in MeWo cells after treatment with *Phyllanthus* extracts (Figure 4.1C and 4.1D).

Phyllanthus extracts have exerted their growth arrest on treated-PC-3 cells by accumulating the cells at Go/G1-phase (Figure 3.3), indicating that *Phyllanthus* extracts may interfere with protein synthesis in PC-3 cells. The induction of apoptosis by *Phyllanthus* was observed with accumulation of apoptotic cells at Sub-G1 phase, and further implicated with caspases-3/7 activation, TUNEL-positive cells and DNA fragmentation (Figure 3.4-3.7). The G1-phase arrest in *Phyllanthus* treated PC-3 cells was believed due to the down-regulation of MAPKs, Wnt, NFκB and Myc/Max pathways (Figure 3.30, 3.32 and 3.33). The down-regulation of these pathways could lead to the activation of cyclin-dependent kinase inhibitor (CKI), p27, which in turn, the activated p27 proteins could inhibit the activation of cyclin E/CDK2 and/or cyclin D/CDK4 complexes that halted cell cycle progression at G1 phase (Collins *et al.*, 2005; Ollinger *et al.*, 2007). In addition, down-regulation of Wnt and Myc/Max pathways by *Phyllanthus* in PC-3 cells caused activation of GSK3β to degrade c-myc and β-catenin proteins (Figure 4.2C), which in turn, reduce production of cyclin D, which is an important initiator of cell cycle. Several studies have shown the involvement of Wnt and Myc/Max pathways in regulation of cyclin D to induce cell growth arrest (Massagué, 2004; Dolcet *et al.*, 2005; Tang *et al.*, 2009).

4.4 Induction of Apoptosis by *Phyllanthus*

Evasiveness of apoptosis is one of the hallmarks of cancer. This can be achieved by suppressing the expression of pro-apoptotic protein, Bax and stimulating the expression of anti-apoptotic protein, Bcl-2. However, the expression of Bcl-2 was greatly suppressed, accompanied by the up-regulation of Bax expression in *Phyllanthus*-treated MeWo and PC-3 cells (Figure 3.28). The reduction in Bcl-2 expression was due to the inhibitions in MAPK pathway by *Phyllanthus* extracts (Milella *et al.*, 2002; Xiao *et al.*, 2004) (Figure 3.29 and 3.30). The highly expressed Bax could induce cytochrome

c release from the mitochondria which can then induce proteolytic activation of procaspse-9. This in turn activates caspase-3 and -7 (Kirsch *et al.*, 1999; Fan *et al.*, 2005) as detected (Figure 3.4) and finally leads to apoptosis induction in MeWo and PC-3 cancer cells (Figure 4.1E and 4.2E).

Other pro-apoptotic factors, caspase-3 and -7 were also found to be up-regulated in treated MeWo and PC-3 cells (Figure 3.4). The actived caspase-3 and -7 can induce apoptosis via (1) cleaving and releasing of caspase-activated DNase (CAD) from its inhibitor in CAD/ICAD complex, which in turn, translocates into the nucleus to cause internucleosomal DNA fragmentation (Cohen, 1997; Fan *et al.*, 2005) and (2) inactivation of enzyme poly (ADP-ribose) polymerase (PARP) (Los *et al.*, 2002). Therefore, elevation of these caspases after *Phyllanthus* treatment produces the appearance of the morphological characteristics of apoptosis, allowing for DNA fragments to be seen on an agarose gel (Figure 3.7) and is further confirmed with the presence of TUNEL-positive cells (Figure 3.5). Hence, the anti-proliferative effect of *Phyllanthus* extracts on MeWo and PC-3 cancer cell lines was accompanied with apoptosis by triggering the activation of caspase-3 and -7. Several studies have reported the involvement of geraniin, rutin, quercetin and gallic acid that presence in *Phyllanthus* extracts to induce apoptosis via caspases activation (Serrano *et al.*, 1998; Ohno *et al.*, 1999; Shen *et al.*, 2003; Lee *et al.*, 2008a; Wang & Jin, 2010).

The PI3K/Akt pathway is an overactive intracellular signalling pathway that can be found in different cancers that involves in the regulation of apoptosis, cell cycle progression and cellular growth (Figure 4.1A and 4.2A) (Luo *et al.*, 2003; Osaki *et al.*, 2004; Vara *et al.*, 2004). However, supression of Akt protein by *Phyllanthus* was observed in both MeWo and PC-3 cells (Figure 3.29 and 3.30) and this suppression could induce apoptosis through activation of pro-apoptotic factors such as Bax, GSK3β, procaspase-9 and TRAIL/APO-2L (TNF-Related Apoptosis-Inducing Ligand) (Cross *et*

al., 1995; Datta *et al.*, 1997; Testa & Bellacosa, 2001). The activation of Bax and GSK3β were both detected in the treated cancer cells (Figure 3.28, 3.31 and 3.32). Induction of apoptosis by *Phyllanthus* will be further implemented when the transcription factor cyclic AMP response element-binding protein (CREB) and IκB kinase (IKK), a positive regulator of NFκB are dephosphorylated by Akt protein, thus possibly leading to a reduction in the expression of genes with anti-apoptotic activity (Kane *et al.*, 1999; Vara *et al.*, 2004).

Ras proteins are membrane bound GTPases responsible for transmitting extracellular signals into the nucleus to regulate gene-driven malignancy of cancer including proliferation, apoptosis, evasion, metastasis and angiogenesis (Figure 4.1B and 4.2B) (Roberts & Der, 2007). The observed down-regulation of pan-Ras proteins by *Phyllanthus* will then leads to suppression of its downstream target; c-Raf (Figure 3.29 and 3.30). The major downstream targets of c-Raf can be subdivided into three mitogen-activated protein kinase (MAPK) pathways; ERK1/2, JNK1/2 and p38 MAPK (Figure 4.1B and 4.2B). In cancer cells, the activate ERK1/2 molecules will activate RSK and Elk1 proteins that subsequently activate c-Jun and c-Fos proteins. Both c-Jun and c-Fos will then combine to form an activator protein (AP-1) which is a transcription factor that regulates survival genes (Figure 4.1B and 4.2B). Besides that, JNK1/2 and p38 MAPK pathways are also involved in enhancing AP-1 formation by also producing c-Jun and c-Fos (Roberts & Der, 2007). However, all these intracellular signalling molecules involved in the MAPK signalling were noted to be down-regulated in MeWo and PC-3 cells after treatment with *Phyllanthus* extracts (Figure 3.29 and 3.30). These observations could be due to presence of quercetin and gallic acid in *Phyllanthus* extracts that induce apoptosis via suppression of MAPK pathway as observed (Ishikawa & Kitamura, 2000; Nguyen *et al.*, 2004; Granado-Serrano *et al.*, 2006; Ho *et al.*, 2010).

Tumour necrosis factor-alpha (TNF-α) is a cytokine that and is capable of activating multiple downstream signalling pathways, including caspases, IκB kinase (IKK) and JNK (Baud & Karin, 2001; Lin, 2002). TNF-α does not usually induce apoptosis unless NFκB has been inactivated (Baud & Karin, 2001; Karin & Lin, 2002). During TNF-α induced apoptosis, caspases will be activated and IKK will be inactivated. The inactivation of IKK causes NFκB to bind to its inhibitor and is then unable to translocate into nucleus to regulate its target genes, which include inhibitors of apoptosis (IAPs) family (Baldwin, 2001; Karin & Lin, 2002). Therefore, *Phyllanthus* probably causes apoptosis in MeWo cells by regulating TNF-α induced apoptosis through activation of the JNK pathway (Figure 3.29) and inhibition of NFκB pathway (Figure 3.33A).

In different types of cancer, NFκB is constitutively active and is believed to play an anti-apoptotic role (Ahn *et al.*, 2007). The inhibition of NFκB was observed in MeWo and PC-3 cells after treatment with *Phyllanthus* extracts (Figure 3.33). The inhibitory effect of *Phyllanthus* extracts on NFkB pathway was conducted by measuring the activities of NFkB-1 (p50) and NFkB -2 (p52). Both p50 and p52 lack a transactivation domain, thus are required to form homodimers or heterodimers with other Rel subunits, in order to regulate its target genes (Figure 4.1D and 4.2D). The anti-apoptotic effects of active NFκB have been proposed as NFκB induces the expression of the Inhibitors of Apoptosis (IAPs) and some anti-apoptotic proteins. The IAPs can suppress the activation of effector caspases (caspases-3, -6, -7, and 9), whereas anti-apoptotic proteins (e.g. Bcl-2) can antagonize the function of the pro-apoptotic proteins (e.g. Bax). Therefore, the observed down-regulation of NFκB pathways in treated cancer cells (Figure 3.33) is believed to inhibit proliferation and by suppressing anti-apoptotic proteins (Bcl-xl and cIAP) as well as by inducing

programmed cell death in MeWo and PC-3 cells (Figure 4.1D and 4.2D) (Levine *et al.*, 2003; Aggarwal, 2004).

The highly conserved cellular processes of apoptosis cannot be regulated by a single protein; instead they are usually controlled by a group of tightly regulated proteins. Uncontrolled proliferation of cancer cells can cause many of these proteins to be differentially expressed to pace with the tumour growth. With the treatment of *Phyllanthus*, many of these proteins were found to be altered in MeWo (Table 3.4) and PC-3 cells (Table 3.5) such as keratin, Ephrin-B1, alpha-centractin, bystin, tubulin, actin, vimentin and protein MEMO1 (Figure 4.1G and 4.2G).

Two differentially expressed proteins showed greatest reduction in their expression in treated cells after treatment with *Phyllanthus*; HCLS1-associated protein X-1 (HAX1) and casein kinase II subunit alpha (CK2). HAX-1 is a 35kDa protein that interacts with HS1 and the α-subunit of G13 heterotrimeric G protein for cell migration (Suzuki *et al.*, 1997; Radhika *et al.*, 2004). HAX-1 is highly expressed in hypoxic tumour progression, metastatic pancreatic cancer and oral squamous cell carcinoma, liver, lung, and breast cancer (Velculescu *et al.*, 1995; Jiang *et al.*, 2003; Ramsay *et al.*, 2007). The down-regulation of HAX-1 is believed to inhibit cell migration and induce apoptosis as it shares partial sequence similarity with pro-apoptotic proteins in Bcl-2 family (Suzuki *et al.*, 1997; Klein *et al.*, 2006). CK2 is a highly conserved protein serine/threonine kinase and is highly expressed in different cancers (Landesman-Bollag *et al.*, 2001; Litchfield, 2003; Izeradjene *et al.*, 2005). Several studies showed that the involvement of CK2 has anti-apoptotic function through (1) inhibition on Max which is a transcriptional partner of the c-myc, from caspase-mediated degradation (Krippner-Heidenreich *et al.*, 2001), (2) deactivation of Bid, a pro-apoptotic protein (Desagher *et al.*, 2001), and (3) through protection from Fas- and drug-triggered apoptosis (Desagher *et al.*, 2001; Guo *et al.*, 2001). Therefore, down-regulation of CK2 in treated MeWo

cells could elicit apoptosis induction and could increase the susceptibility of MeWo cells to apoptotic inducer chemotherapeutic agents (Faust & Montenarh, 2000; Ravi & Bedi, 2002; Ruzzene *et al.*, 2002).

Another differentially expressed protein that showed reduction in its expression in treated cells after treatment with *Phyllanthus* was galectin. Galectin is a multifunctional protein, which can act as (1) an apoptosis inhibitor (Yang & Liu, 2003; Hoyer *et al.*, 2004), (2) mRNA splicing promoter (Dagher *et al.*, 1995; Vyakarnam *et al.*, 1997), and (3) as an adhesion molecule (Glinsky *et al.*, 2001) to promote cancer progression and metastasis (Takenaka *et al.*, 2002; Liu & Rabinovich, 2005). Therefore, the down-regulation of galectins in treated MeWo and PC-3 cells is believed to inhibit metastasis and trigger programmed cell death.

Besides this, *Phyllanthus* extracts also disrupted cancer-related proteins involved in cell cycle to halt the growth of MeWo and PC-3 cells. These altered proteins include DNA damage-binding protein 2, transcription factor 23, gremlin-1, proliferation-associated protein 2G4, growth factor receptor-bound protein 2, 14-3-3 proteins, annexin A1, glutathione, galectin-1, heat shock proteins, peroxiredoxin, RuvB-like 2 and Serpin B9, in treated cancer cells (Table 3.4 and 3.5). Among these proteins, the expressions of glutathione S-transferases showed the greatest reduction compared to other proteins. Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in redox homeostasis. These enzymes are highly expressed in cancer cells and are believed to limit the efficacy of chemotherapeutic agents (Tew, 1994; Townsend *et al.*, 2003). This occurs via detoxification whereby the agents are conjugated with reduced gluthathione, causing them to be more water-soluble and enhancing the elimination of the agents (Hayes & McMahon, 2001; Michael & Doherty, 2005). Therefore, the suppression of GSTs in treated PC-3 cells could possibly bypass

detoxification of *Phyllanthus* extracts, allowing them to be circulated to target cancer areas.

Another down-regulated protein by *Phyllanthus* was Damaged DNA Binding protein 2 (DDB2). It is a 48kDa subunit protein of the damage-specific DNA-binding heterodimeric complex (DDB) and is involved DNA repair mechanism, transcription and cell cycle regulation in normal cells (Datta *et al.*, 2001; Martinez *et al.*, 2001; Takimoto *et al.*, 2002; Stoyanova *et al.*, 2009). During cell cycle, high levels of DDB2 in G1-phase are observed (Stoyanova *et al.*, 2009). It has been proposed that DDB2 plays an important role in regulation of cell cycle and apoptosis induction rather than in the DNA repair mechanism (Kulaksız *et al.*, 2005). Therefore, the low level of DDB2 proteins in treated cells is believed to attribute to cell cycle arrest and the triggering apoptosis induction.

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is produced by the mitochondria and present in all tissues. It plays an important role in oxidation of lactate while reducing pyruvate in carbohydrate metabolism during anaerobic glycolysis (Nathan *et al.*, 2006). LDH has been widely used as a general indicator and monitors for acute or chronic tissue damage, heart attack, kidney and liver disease as well as cancer. (Goldberg *et al.*, 1989; Ribeiro *et al.*, 1999; Danpure, 2004). The potential of LDH as a tumour biomarker was identified in several cancers such as melanoma (Ugurel *et al.*, 2009), lung (Jørgensen *et al.*, 1989; Molina *et al.*, 2004), breast (Seth *et al.*, 2003; Duffy & Crown, 2008) and prostate cancers (Albers *et al.*, 2008; Scher *et al.*, 2009). During necrosis, LDH enzyme will be secreted due to damaged cell membrane of necrotic cells and causes an inflammatory reaction (Drent *et al.*, 1996). However, the changes in the cytoplasmic membrane integrity during late apoptosis also resulting secretion of LDH (Rauen *et al.*, 1999; Jiang *et al.*, 2007). Measurements of LDH enzyme as an indicator of necrosis, demonstrated that *Phyllanthus* extracts besides having apoptotic activity,

also possess minimal capacity for inducing necrotic cell death on both MeWo and PC-3 cells (Figure 3.8 and 3.9). Taken together, the results indicate that the *Phyllanthus* plant possesses a dual-capability for cell death.

4.5 Anti-Metastatic Effect of *Phyllanthus*

Typically, tumour metastasis is the main cause for high morbidity and mortality rates in cancer patients. Most melanoma and prostate cancer patients' exhibits clinical evidence of tumour metastases such as lung, breast and bone metastases (Jemal *et al.*, 2011). The mortality associated with metastasized tumours, accounting for 90% of all cancer deaths (Gupta & Massagué, 2006). Metastases has caused poor prognosis in these cancer patients and is a major clinical challenge in cancer treatment.

Cancer cell interaction with the basement membrane is an important step in the initiation of the metastatic cascade (Poste & Fidler, 1980; Gupta & Massagué, 2006). The basement membrane is a thin layer that underlies the epithelium of vessels, capillaries, cavities and organs. It is composed of glycoproteins such as collagen and laminin (Okegawa *et al.*, 2004). However, adhesion activity of MeWo and PC-3 cells after treatment with *Phyllanthus* extracts to fibronectin and type-IV collagen respectively, was disrupted (Figure 3.18 and 3.19). In addition, *Phyllanthus* extracts have significantly inhibited MeWo and PC-3 cells' migration and invasion ability in a dose-dependent manner (Figure 3.10-3.17). Migration of cancer cells across the endothelium is important for secondary tumour development and P*hyllanthus* extracts significantly inhibited the transendothelial migration of MeWo (Figure 3.20) and PC-3 cells (Figure 3.21). All these findings indicate that *Phyllanthus* exhibits its anti-metastatic activity through inhibition of several crucial steps of tumour-invasion which include attachment of tumour cell to the membrane, migration, invasion and

transendothelial migration of MeWo and PC-3 cells and could possibly lessen the chances of secondary tumour formation. Epithelial-mesenchymal transition (EMT) is a cellular process that allows immotile epithelial cells to become motile mesenchymal cells, promoting carcinoma invasion and metatstasis as well as resistance to apoptosis (Voulgari & Pintzas, 2009). Several signalling networks including PI3K/Akt- (Figure 4.1A and 4.2A), Ras/MAPK- (Figure 4.1B and 4.2B), Wnt- (Figure 4.1C and 4.2C) and NFκB-dependent (Figure 4.1D and 4.2D) pathways in EMT were found to be down-regulated in treated MeWo and PC-3 cells, and are thus believed to inhibit metastasis of cancer cells (Klymkowsky & Savagner, 2009). The inhibition on metastasis was further imposed with the up-regulation of keratin and concurrent down-regulation of vimentin in MeWo and PC-3 cells after treatment with *Phyllanthus*. The exact mechanism of up-regulation of keratin during EMT is unclear but its down-regulation in treated cells can restrict the motility of cells, thus halting metastasis (Paccione *et al.*, 2008).

In cell cytosol, Akt protein protects vimentin from caspase-induced proteolysis, and in *Phyllanthus*-treated cells, Akt was down-regulated and thus down-regulates production of vimentin. The overexpression of vimentin is always correlated with tumour growth and invasion, where vimetin is believed to regulate various intracellular signalling pathways and cell surivial (Zhu *et al.*, 2010). This is done by (1) stabilization of the ERK protein, allowing it to be translocated into the nucleus (Perlson *et al.*, 2006; Satelli & Li, 2011) and (2) preventing assembly of the Raf-14-3-3 complex, allowing Ras protein to be continuously expressed and thus regulate signalling pathways in cells (Tzivion & Avruch, 2002). Therefore, down-regulation of vimentin could decrease melanoma and prostate cancer growth, adhesion and invasion as well as apoptosis induction via suppression on ERK- and Ras-dependent pathways as observed.

A phosphoserine/phosphothreonine binding protein, 14-3-3 is involved in protein kinase signalling that regulates several vital biological processes in cancer

including cell cycle, DNA damage checkpoint, activation of MAPK, prevention of apoptosis, and coordination of integrin signalling and cytoskeletal dynamics (Hermeking, 2003a; Tzivion *et al.*, 2006). This can all be achieved by association of 14-3-3 with (1) oncogenic proteins (Ras, Bcr and Bcr-Abl), (2) cell survival factors (Bad, Bax, ASK-1), (3) tumour suppressors (p53, TSC2, p27) and (4) cell cycle regulators (Cdc25 A, B and C, Wee1, Chk1) as well as (5) proteins involved motility (p130 Cas, integrins β1, β2, β3 and Ron) (Hermeking, 2003a; Wilker & Yaffe, 2004; Tzivion *et al.*, 2006). However, all these vital biological processes in MeWo and PC-3 cells are believed to be interrupted due to the down-regulation of the 14-3-3 protein.

4.6 Anti-Angiogenesis Effect of *Phyllanthus*

Tumour-induced angiogenesis is the growth of new blood capillaries from pre-existing blood vessels and involves multiple steps including endothelial cell (EC) activation, disruption of vascular basement membranes, and migration and proliferation of ECs (Delgado *et al.*, 2011). Tumour-induced metastasis and angiogenesis are intrinsically connected as highly vascular tumours may have a higher metastatic potential than angiogenic tumours (Zetter, 1998). Besides providing oxygen and nutrients for tumour growth, angiogenesis also provides a path to allow the tumour cells to metastasize (Weidner *et al.*, 1991).

Tumour-induced angiogenesis has similar cytophysiological changes in endothelial cells as in metastatic cells. The activated endothelial cells will migrate out from parental vessels, invade basement membrane, proliferate and assemble into new capillaries to supply oxygen and nutrients to tumours (Zetter, 1998). Therefore, the anti-angiogenesis activity of *Phyllanthus* was studied by assessing cell viability, migration, invasion and microcapillary-like tube structure formation using HUVECs.

The migration and invasion capabilities of endothelial cells are the basis in angiogenesis. Their motility capability is initiated by pro-angiogenic factors including VEGF, secreted by the tumour (Carmeliet & Jain, 2000). In addition, this factor can increase proliferation and differentiation of endothelial cells into capillaries to form new blood vessels for tumour growth and metastasis. Results show that *Phyllanthus* extracts have effectively inhibited the migration and invasion ability of HUVECs (Figure 3.12 and 3.17). In addition, the low expression of VEGF and its regulator, HIF-1α in MeWo and PC-3 cells were detected after treatment with *Phyllanthus* extracts (Figure 3.31 and 3.32) and further explaines that *Phyllanthus* extracts have reduced the expression and/or secretion of VEGF of cancer cells via suppression on HIF-1α to inhibit angiogenesis (Figure 4.1C and 4.2C). Therefore, these findings suggested that *Phyllanthus* extracts can significantly reduce migration and invasion of endothelial cells, indicating the plants potential as an anti-angiogenic agents and was shown previously by Huang et al (Huang *et al.*, 2006). On the ECM gel, HUVECs assembled into microcapillary–like tube structures or lumen and this formation was inhibited when treated with *Phyllanthus* extracts (Figure 3.25).

Besides that, suppression on Wnt and Myc/Max pathways in MeWo (Figure 4.1C) and PC-3 (Figure 4.2C) cells by *Phyllanthus* extracts can be attributed to down-regulation of VEGF and HIF-1α. This can achieve by activate GSK3β protein to degrade β-catenin and c-myc proteins, thus down-regulating the expression of VEGF and HIF-1α (Figure 3.31 and 3.32). In addition, suppression on MAPK and PI3K/Akt pathways in MeWo (Figure 4.1A and 4.2B) and PC-3 (Figure 4.2A and 4.2B) cells by *Phyllanthus* extracts could also lead to inactivation of VEGF, thus inhibiting tumour-induced angiogenesis (Adya *et al.*, 2008).

Besides VEGF, the Ephrin-B1 protein also plays an important role in tumour-induced angiogenesis. Ephrin-B1 can act as a ligand to bind and activate Eph receptor

tyrosine kinases that induce migration and integrin-mediated αvβ3 and α5β1 attachment (Huynh-Do *et al.*, 2002; Sawai *et al.*, 2003; Surawska *et al.*, 2004; Kuijper *et al.*, 2007; Tanaka *et al.*, 2007). High expression of Ephrin-B1 is found in different types of advanced cancer such as studies gastric, colorectal, breast, ovarian, neuroblastoma, and lung cancers (Sawai *et al.*, 2003; Herath *et al.*, 2006; Cortina *et al.*, 2007; Tanaka *et al.*, 2007; Vaught *et al.*, 2008). Therefore, down-regulation of of Ephrin-B1 by *Phyllanthus* extracts could inhibit cancer progression via inhibition on modulation integrin-mediated cell attachment, migration and angiogenesis (Huynh-Do *et al.*, 2002).

Several plant-derived compounds identified to possess anti-angiogenesis activity includes sanguinarine isolated from *Sanguinaria canadensis*, vinca alkaloids from *Catharanthus roseus* and pterogynidine from *Alchornea glandulos* (Eun & Koh, 2004; Kruczynski *et al.*, 2006; Flávia *et al.*, 2009). The anti-angiogenic effect of *Phyllanthus* against endothelial cells could be due to the presence of bioactive compounds in the plant such as gallic acid, rutin and quercetin (Donnini *et al.*, 2006; Guruvayoorappan & Kuttan, 2007; Chen *et al.*, 2009). These compounds have been reported to reduce the sensitivity of endothelial cells towards VEGF via inhibitions on several signalling pathways including PI3k/Akt, NFκB and Ras/MAPK pathways (Figure 3.29, 3.30 and 3.33) (Shiojima & Walsh, 2002; Donnini *et al.*, 2006; Liu *et al.*, 2006b; Adya *et al.*, 2008; Kitamura *et al.*, 2008; Lu *et al.*, 2010). All these findings indicate that *Phyllanthus* can inhibit tumour growth through the suppression of blood vessel development and has a high potential to be developed as an anti-angiogenesis agent. Furthermore, *Phyllanthus* could also possibly prevent other angiogenesis-mediated disorders such as diabetic blindness, age-related macular degeneration, rheumatoid arthritis and stroke.

4.7 Activation of Matrix Metalloproteinases by *Phyllanthus*

In metastasis, matrix proteolytic enzymes such as matrix metalloproteinases (MMPs) are known to be crucial for degradation of extracellular matrix components and promoting both endothelial and tumour cellular invasion *in vitro* and *in vivo* (Huang *et al.*, 2006; Bourboulia & Stetler-Stevenson, 2010). Overexpression of MMP enzymes is always associated with poor prognosis (Sier *et al.*, 1996; Yoshizaki *et al.*, 2001; Pellikainen *et al.*, 2004; Liu *et al.*, 2005). MMP-2 and MMP-9 are always found highly expressed in various cancers malignancies, including cervical, melanoma, breast, colon and prostate cancers (Stearns & Stearns, 1996; Luca *et al.*, 1997; Kondapaka *et al.*, 1998; Nielsen *et al.*, 1998).

In this study, different MMP enzymes including MMP-2, -7, -9 and -26 were detected in HUVECs, MeWo and PC-3 cell lines (Figure 3.22-3.24). *Phyllanthus* extracts showed inhibitory effects on expression of MMP-2 in HUVECs and MeWo cells. Human matrilysin-2, also known as MMP-26 is an activator for pro-MMP-9 (Uría & López-Otín, 2000; Marchenko *et al.*, 2001). MMP-9 and MMP-26 are commonly found in the human prostate carcinoma tissue samples than in prostatitis, benign prostate hyperplasia (BSH), and normal prostate tissue (Lee *et al.*, 2006b). However, PC-3 cells showed reduction of both active MMP-26 and MMP-9 after treatment with *Phyllanthus* extracts; in conjuction with increasing levels of pro MMP-9 level (Figure 3.23). Another down-regulated MMP enzyme in treated MeWo and PC-3 cells was MMP-7 which has a broad proteolytic activity against a variety of extracellular matrix substrates, including collagens, proteoglycans, elastin, laminin, fibronectin, and casein (Wilson & Matrisian, 1996; Adachi *et al.*, 1999). The reduction of active MMP-2, -7 and -9 after treatments with *Phyllanthus* extracts partially explain the inhibition of

migration and invasion of endothelial cell as well as melanoma and prostate cancer cells.

In *Phyllanthus*, several bioactive compounds have been identified (Tang *et al.*, 2010). Some of these bioactive compounds have been suggested to inhibit activities of MMPs including gallic acid and 5-caffeoylquinic acid. Gallic acid has been shown to inhibit migration and invasion of several cancer cells through the reduction of MMP activities (Ho *et al.*, 2010; Lu *et al.*, 2010) and 5-caffeoylquinic acid has also been shown to be a strong inhibitor on MMP-9 (Jin *et al.*, 2005). In addition, these reduced activities of MMPs upon treatment with *Phyllanthus* could be due to alteration in signal transduction in PI3K/AKT, Ras-Raf-MAP kinase, Wnt, NFκB pathways in the regulation of MMP expression (Figure 4.1 and 4.2) (Montague *et al.*, 2004; Ho *et al.*, 2010). In addition, both Wnt5a and Wnt-3 proteins are involved in Wnt signalling pathway and regulation of MMPs, were found to be down-regulated by *Phyllanthus* extracts (Masckauchán *et al.*, 2006; Pukrop *et al.*, 2006).

There are several proteins involved in regulations of MMPs that were found to be altered in treated cancer cells such as superoxide dismutase and heat shock protein. The down-regulated mitochondrial superoxide dismutase protein could suppress MMP action and alters intracellular ROS and nitric oxide levels via inhibition on the MAPK/ERK pathway (Ranganathan *et al.*, 2001; Zhang *et al.*, 2002). In addition, down-regulation of heat shock protein beta-1 Hsp27 protein could affect MMP-9 activity, thus inhibits metastasis and angiogenesis (Hansen *et al.*, 2001; Aldrian *et al.*, 2002; Lee *et al.*, 2008b).

4.8 Alteration in Energy Metabolism of Cancer cells

The rapid uncontrolled proliferation of cancer cells usually outpaces new blood vessels generation, hence resulting in insufficient bloods supply/oxygen to tumour tissues. In this hypoxic condition, the cancer cells are forced to up-regulate the expression of genes and enzymes which are involved an anaerobic glycolytic pathway as the main route of energy production, and this phenomenon is known as Wasburg effect (Warburg, 1956; Hsu & Sabatini, 2008) (Vander Heiden *et al.*, 2009). The expression of hyoxia-inducible factor (HIF) is activated during low oxygen level (Guppy, 2002; Xu *et al.*, 2005). The active HIF will mediate activation genes involved in angiogenesis (e.g. VEGF), cell survival (e.g. IGF-1) and metastasis (e.g. LOX, PAI-1) and drives tumour progression (Poon *et al.*, 2009). This metabolic adaptation in response to these alterations is believed to be associated with resistance to therapeutic agents (Carmeliet *et al.*, 1998).

Phyllanthus extracts were noted to inhibit the glycolytic pathway and energy production in MeWo and PC-3 by down-regulating HIF-1α protein. The deactivated HIF-1α protein will reduce the production VEGF and thus inhibit tumour angiogenesis, glycolytic pathway and energy production and thereby decrease cancer progression (Figure 3.31 and 3.32). The synthesis of HIF-1α protein in the Wasburg effect is regulated by activation of the PI3K/Akt and Ras-MAPK (Poon *et al.*, 2009). Therefore, both PI3K/Akt and Ras-MAPK pathways were also noted to be suppressed by *Phyllanthus* plant extracts (Figure 3.29 and 3.30). In addition, several glycolytic enzymes were significantly down-regulated in MeWo and PC-3 cells after treatment with *Phyllanthus* extracts (Table 3.4 and 3.5, Group III) such as glucosamine-fructose-6-phosphate aminotransferase, fructose-bisphosphate aldolase protein phosphatase 1

regulatory subunit 3D, alpha-enolases, pyruvate kinase isozymes M1/M2, urocortin-3, alpha-enolase, GAPDH and phosphoglycerate kinases.

The mitochondrion is involved in protein synthesis and energy metabolism for cancer cell survival, transformation, invasion and metastasis (Wallace, 1999). Thirteen mitochondria-related components (E3 ubiquitin-protein ligase ARIH2 and RNF115, aspartate aminotransferase, 26S protease regulatory subunit 8, putative elongation factor 1-alpha-like 3, mitochondrial inner membrane organizing system protein 1 (MINOS), zinc finger protein, Sec1, trimethyllysine dioxygenase, 39S ribosomal protein L51, calumenin, calreticulin and 78 kDa glucose regulated protein) were found to be down-regulated in *Phyllanthus*-treated MeWo (Table 3.4, Group IV) and PC-3 (Table 3.4, Group IV) cells. This down-regulation is believed to be attributed to alteration in intracellular calcium, energy production (e.g. ATP) and stability of cellular proteins in treated (MeWo and PC-3 cells, thus triggering apoptotic cell death and halting cancer growth (Henderson *et al.*, 1982; Franklin *et al.*, 1997; Yabe *et al.*, 1997; Ferrell *et al.*, 2000; Sreedhar & Csermely, 2004; Zhang *et al.*, 2004; Thornburg *et al.*, 2008; Alkhaja *et al.*, 2012; Sun *et al.*, 2012) .

Among the affected mitochondria-related components in *Phyllanthus*-treated cells, only one protein was significantly up-regulated; voltage-dependent anion-selective channel protein 1. Voltage-dependent anion-selective channel protein 1 (VDAC) is a mitochondrial outer membrane protein that regulates ATP/ADP exchange and respiratory control (Hiller *et al.*, 2008). VDAC has been shown to be pro-apoptotic by regulation of Bak and Bax (Tsujimoto & Shimizu, 2002; Rostovtseva *et al.*, 2005) as well as activation of caspase-8 to induce extrinsic apoptosis pathway (Madesh & Hajnóczky, 2001; Roucou *et al.*, 2002). Therefore, the up-regulation of VDAC by *Phyllanthus* extracts was able to initiate apoptotic cell death in cancer cells (Figure 4.2F).

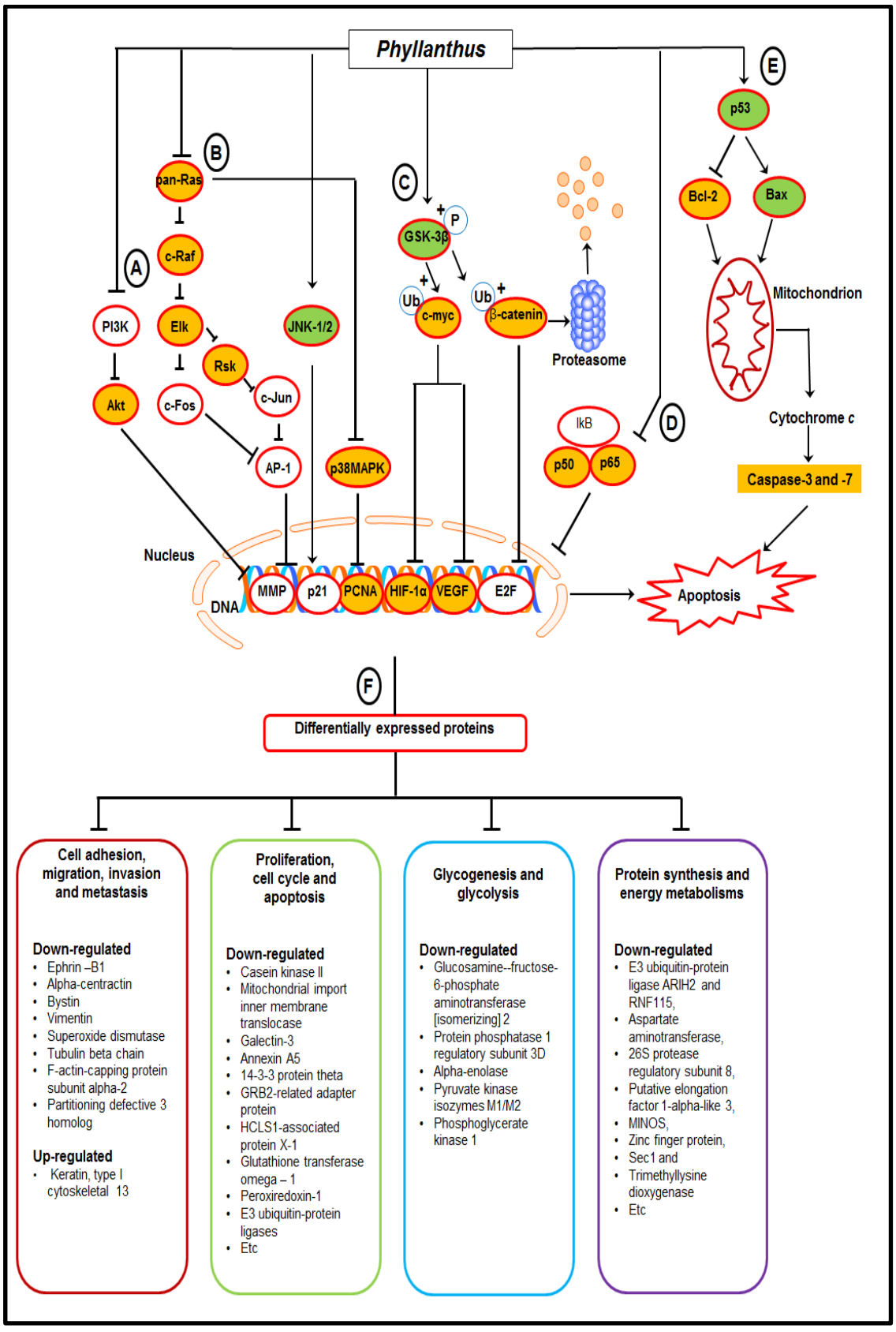

Figure 4.1: Schematic diagram illustrating *Phyllanthus* regulates multiple signalling [MAPKs (A), PI3K/Akt (B), Myc/Max and Hypoxia (C), NFκB (D) and p53 (E)] pathways and protein activities (G) in MeWo cells.

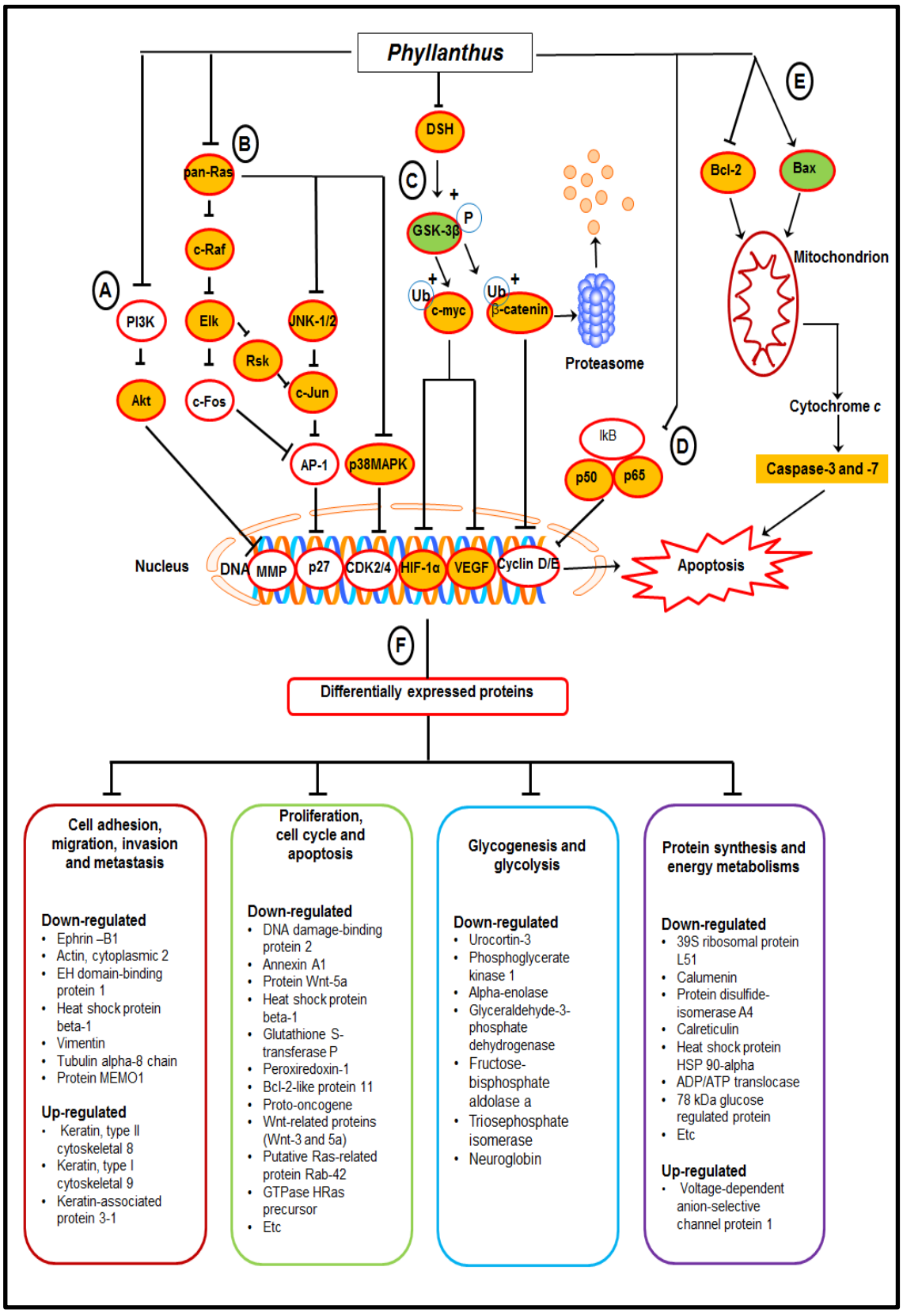

Figure 4.2: Schematic diagram illustrating *Phyllanthus* regulates multiple signalling [(MAPKs (A), PI3K/Akt (B), Wnt, Myc/Max and Hypoxia (C), NFκB (D), and apoptosis (E)] pathways and protein activities (G) in PC-3 cells.

CHAPTER 5: CONCLUSION

Modern therapies for cancer treatment such as surgery, chemotherapy and immunotherapy are deemed relatively unsuccessful due to their ineffectiveness, safety issues (side effects) and costliness. Although chemotherapy was advocated at one time, but recent studies have implied that these agents are no longer effective as they used to be, mainly these agents being cytotoxic to both normal and cancer cells. These treatments are often elicited undesirable side effects such as vomiting, nausea and alopecia. As not all cancer patients respond positively to current anticancer agents, the mortality rates are on a continuous rise including melanoma and prostate cancer.

Natural product-based traditional medicine, is often overshadowed by modern medicine, has returned to be a holistic approach for health care in many societies. This "back to basic" approach is due to its long history of usage in disease treatment and the pharmacological/nutritional value of these products which are believed to be able to halt/delay cancer progression. Therefore, the anticancer properties of *Phyllanthus* plant extracts were studied against human melanoma, MeWo and prostate adenocarcinoma, PC-3 cells.

In summary, the findings of the study suggested that *Phyllanthus* extracts possesses the ability to suppress the proliferation of MeWo and PC-3 directly without affecting the growth of normal cells; CCD-1127Sk, RWPE-1 and HUVECs. *P.urinaria* showed the strongest anti-proliferative effect on both cancer cell lines with an IC_{50} value ranged 54.2-56.2 μg/ml and 155.7-193.3 μg/ml for methanolic and aqueous extracts, respectively. The selective killing on cancer cells by *Phyllanthus* extracts was observed. However, the anti-proliferative effect of *Phyllanthus* extracts should be

further investigated on the other types of normal cells to further imposed its selective killing properties on cancer cells only.

The anti-proliferative effect of *Phyllanthus* extracts against MeWo and PC-3 cells is likely to be due to the induction of growth arrest and apoptosis. Treatment with *Phyllanthus* extracts induced significant different cell cycle arrest in treated MeWo (S-phase arrest) and PC-3 (G1-phase arrest). *Phyllanthus* extracts induces cell cycle arrest at S-phase in MeWo cell was due to an alteration in MAPK/JNK, PI3K/Akt and p53 pathways to interfere activities of the PCNA and/or cyclin E/CDK2 complexes. On the other hand, the G1-phase arrest in *Phyllanthus* treated PC-3 cells was due to suppression on MAPK, PI3K/Akt, Wnt and Myc/Max pathways to inhibit cyclin E/CDK2 and/or cyclin D/CDK4 complexes, halting cell cycle progression. However, a detailed investigation on the G1- and S-phases related proteins such as cyclin D, cyclin E, cyclin A, p21, and p27 as well as other cell cycle regulatory molecules are required to shed light on the actual molecular mechanisms of *Phyllanthus* extracts induced cell cycle arrest.

Phyllanthus extracts were capable to induce apoptosis in PC-3 and MeWo cells via activation of caspases-3/7 to induce DNA fragmentation. Caspase activation represents one of the earliest known markers for the onset of apoptosis while DNA fragmentation which occurs downstream of caspase cascade activation represents a late, dispensable step in the apoptotic process. Other pro-apoptotic factor, Bax was also found to be up-regulated in treated cancer cells. The activated Bax could induce cytochrome *c* release from mitochondria to initiate caspases-induce apoptosis. However, a detailed exploration of other pro-apoptotic and anti-apoptotic proteins as well as the regulatory molecules such as cytochrome *c* and PUMA needs to be carried out to explicate the exact molecular mechanism of *Phyllanthus*-induced apoptosis. Besides inducing apoptosis, *Phyllanthus* extracts were also showed to capable induce minimal

necrotic cell death. The induction of necrosis by *Phyllanthus* extracts was observed through the leakage of LDH enzymes from the treated cancer cells. All these findings were based on biochemical changes during apoptotic and necrotic mechanisms. Therefore, further investigation is necessary to investigate *Phyllanthus*-induce apoptosis or necrosis in the term of morphological changes. The typical apoptotic cells' morphology including chromatin condensation, cell shrinkage, membrane blebbing, and formation of apoptotic bodies can be investigated by staining methods includes Hoescht 33258, Acridine orange/ethidium bromide (AO/EtBr), and Annexin-V. Besides that, transmission electron microscopy (TEM) may provide a better and clear view of the morphological changes during apoptosis/necrosis.

Typically, tumour-induced metastasis and angiogenesis are the main cause for high morbidity and mortality rates in cancer patients. Both are intrinsically connected. Besides providing oxygen and nutrients for tumour growth, angiogenesis also provides a path to allow the tumour cells to metastasize. The anti-metastatic and anti-angiogenic effects of *Phyllanthus*extracts were observed when *Phyllanthus* extracts inhibited several essential steps during metastasis and angiogenesis; (i) adhesion, (ii) migration, (iii) invasion, (iv) transendothelial migration and (v) microcapillary-like tube formations. These observations were most likely due to reduction in activities of matrix metalloproteinase-2, -7, -9 and VEGF in *Phyllanthus*-treated cells. In addition, *Phyllanthus* extracts have impaired energy production and mitochondria integrity of MeWo and PC-3 cells via inhibition on hypoxia pathway and glycolytic enzymes.

These observed anticancer properties of*Phyllanthus* extracts are believed to be due to the plants inhibitory effects on multiple signalling pathways; MAPKs, Wnt, Myc/Max, Hypoxia and NFκB, via alteration on their intracellular signalling activities including c-Ras, pan-Raf, RSK, Akt, Elk1, RSK, c-Jun, JNK1/2, β-catenin, GSK3β, c-myc, HIF-1α, VEGF, NFκB p50 and p52.

The highly conserved cellular processes of invasion, metastasis and apoptosis cannot be regulated by a single protein; instead they are usually controlled by a group of tightly regulated proteins. Uncontrolled proliferation of cancer cells can cause many of these proteins to be differentially expressed to pace with the tumour growth. With the treatment of *Phyllanthus*, many of these vital proteins were found to be altered in MeWo and PC-3 cells and this may affects cellular functions and biological processes in MeWo and PC-3 cells, thus halts their progression.

In summary, this study revealed a comprehensive perspective of the possible mechanism behind the anticancer activity of *Phyllanthus* extracts by inspection of their regulation in multiple signalling pathways and protein-protein interaction in melanoma (MeWo) and prostate adenocarcinoma (PC-3) cells. This study not only shows that *Phyllanthus* could be developed as an alternative anticancer agent; the identified diffentially expressed proteins could become potential targets for development of new anticancer agents. Besides, *Phyllanthus* also possibly be part of plant-based diet to prevent cancer development. Further *in vivo* studies with *Phyllanthus* alone or in conjunction with existing chemotherapeutic drugs are needed to demonstrate the overall effect in a living subject (e.g. mice) in term of toxicity and efficacy as an anticancer/antitumour agent.

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APPENDICES

APPENDIX I: Reagent and media preparation

(1) Equipment used

- Vertical laminar flow cabinet (Microflow, Birmingham, UK)
- Water bath (SASTEC)
- Electronic balance (SASTEC)
- Analytical balance (SATEC)
- Ultracentrifuge (Eppendorf)
- GloMax®-Multi Detection Reader (Promega, USA)
- Inverted microscope (Olympus CK-40)
- Inverted microscope (Olympus BX41)
- Olympus µ-1040 camera
- UV transilluminator (Vilbert loumat, France)

(2) Preparation of EMEM, DMEM and RPMI-1640

- EMEM supplemented with L-glutamine stock (powder form), (Flowlab, Australia)
- DMEM supplemented with L-glutamine stock (powder form), (Flowlab, Australia)
- RPMI-1640 supplemented with L-glutamine stock (powder form), (Flowlab, Australia)
- Sterile Fetal Bovine Serum, FBS (Flowlab, Australia)
- Penicillin-Stretomycin, (Gibco-BRL, USA)
- HEPES buffer (Sigma-Aldrich, Ireland Ltd)
- Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Ireland Ltd)
- Filter unit with 0.2µm membrane filter, (Nalgene, USA)
- Autoclaved double-distilled water

Growth medium (GM) – 1000 ml

Stock media	13.39g
HEPES buffer	4.7g
Sodium bicarbonate	3.7g
Fetal Bovine Serum (inactivated)	50ml
Penicillin-streptomycin	0.2ml
Distilled water	Top up to 1000ml

- Mix all the chemicals into a 1000ml reagent bottle and then filter through a 0.2 µm of membrane filter.
- Store at 4°C.

(3) Preparation of freezing medium

- Prepared serum free EMEM/RPMI-1640
- Fetal Bovine Serum (Flowlab, Australia)
- DMSO (Sigma-Aldrich, Ireland Ltd)
- Autoclaved double distilled water
- Sterile 50ml falcon bottle (Corning Inc, USA)

Freezing medium – 10ml

Prepared serum free EMEM/RPMI-1640	6ml
Fetal Bovine Serum (inactivated)	3ml
DMSO	1ml

- Mix all the chemicals into a 10ml falcon bottle.
- Store at 4°C.

(4) Preparation of 1X PBS (100ml)

- 1 PBS tablet (Amresco, USA)
- Double distilled water (100ml)

- Dissolve the PBS tablet in the double distilled water
- Autoclave the 1X PBS buffer

(5) Reagents for MTS assay

CellTiter 96® Aqueous Non-Radioactive Cell Proliferation assay kit (Promega, Madison, WI)

Kit components
- 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H tetrazolium, inner salt (MTS) solution
- Phenazine methosulphate (PMS) solution
- Store at -20°C

(6) Reagents for DNA staining for cell cycle analysis

- Ice cold 70% ethanol
- PBS
- RNase (1 mg/ml)
- Propidium iodide (PI) (1 mg/ml)

(7) Reagents for DNA extraction

- PBS
- Lysis buffer
- Phenol/chloroform/isoamyl alcohol (24:25:1)
- 3M Sodium acetate (pH 5.2)
- Isopropanol
- Deionized water-RNase solution (10 mg/ml)

(8) Preparation of lysis buffer for DNA Fragmentation assay

0.5 M EDTA, pH7.0	40 ml
1 M Tris-Cl buffer, pH 8.0	5 ml
100 % Triton X-100	5 ml
Distilled water	50 ml

(9) Preparation of 0.5 M EDTA, pH7.0

- Disodium EDTA.2H2O (18.61 g)
- Double distilled water (100 ml)
- Fine adjust to the desired pH (7.0) with 1 M NaOH

(10) Preparation of 1 M NaOH

- NaOH (4 g)
- Double distilled water (100 ml)

(11) Preparation of 1M Tris-Cl, pH 8.0

- Tris base (12.11 g)
- Double distilled water (80 ml)
- Fine adjust to the desired pH (8.0) with concentrated HCI
- Distilled water (final volume 100 ml)

(12) Preparation of 3 M sodium acetate, pH 5.2

- Hydrated sodium acetate (20.4 g)
- Double distilled water (40 ml)
- Fine adjust to the desired pH (5.2)
- Distilled water (final volume 50 ml)

(13) Reagents for Capsase-3/7 assay

Caspase-Glo 3/7 Assay kit (Promega, Madison, WI)

Kit components
- Caspase-Glo 3/7 substrate
- Caspase-Glo 3/7 buffer

(14) Reagents for TUNEL Assay

ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (S7101), (Chemical International, USA).

Kit components
- Equilibration buffer
- Reaction buffer
- TdT enzyme
- Stop/Wash buffer
- Anti-digoxigenin-peroxidase
- DAB substrate
- DAB dilution buffer

Materials required but not supplied in the kit
- Deionized water (dH2O)
- Xylene
- Ethanol:absolute, 90 %, 70 % (diluted in dH2O)
- 100% n-butanol (1-butanol)
- Ethanol:acetic acid 2:1 (v:v)
- 1% paraformaldehyde in PBS (pH 7.4)
- PBS
- Hydrogen peroxide
- 0.5% methyl green
- Triton X-100
- Slide mounting medium
- Silanized glass slides
- Plastic coverslips
- Humidified chamber
- Microcentrifuge tubes
- 37 °C covered water bath
- Light microscope
- Filter through 0.2 μm filter unit
- Reagent bottles

(15) Preparation of 0.5% methyl green

- 0.1 M sodium acetate, pH 4.0
- 0.85% methyl green stock (0.425 g)
    - Filter through 0.2 μm filter unit

(16) Reagents for LDH release assay

CytoTox-One Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI)

Kit components
- Substrate mix
- Assay buffer

(17) Preparation of Adhesion Molecules

Fibronectin and Type-IV Collagen

- Mix the lypholized with sterile double distilled water
- Store at -20 °C

ECM (Extracellular Matrix)

- Store at -20°C
- Thaw overnight before use

(18) Preparation of Gels and Buffers for Western Blot Analysis

Preparation of Separting gel (12%) – 2 gels (10.0 ml)

30% Bis/Acrylamide Mix (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide)	4.0 ml
1.5M Tris buffer, pH 8.8	2.5 ml
10 % SDS (Sodium Dodecyl Sulfate)	100.0 µl
10% APS (Ammonium Persulfate)	50.0 µl
TEMED (N,N,N',N'-tetramethylethylenediamine)	5.0 µl
Double distilled water	3.4 ml

Preparation of Stacking gel – 2 gels (8.0 ml)

30% Bis/Acrylamide Mix (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide)	1.32 ml
1M Tris buffer, pH 6.8	2.52 ml
10 % SDS (Sodium Dodecyl Sulfate)	100.0 µl
10% APS (Ammonium Persulfate)	50.0 µl
TEMED (N,N,N',N'-tetramethylethylenediamine)	10.0 µl
Double distilled water	6.0 ml

Dilution of primary antibodies (All antibodies purchased from Merck, Millipore)

Anti-Pan-Ras (Ab-3) Mouse mAb	1:1000
PhosphoDetect™ Anti-c-Raf (pSer 6 2 ¹) Mouse mAb	1:1000
PhosphoDetect™ Anti-Elk1 (pSer383) Rabbit pAb	1:1000
Anti-p90RSK	1:500
Anti-c-Jun/AP-1 (Ab-3) Mouse mAb	1:2000
PhosphoDetect™ Anti-JNK1/2 (pThr¹83/Tyr¹85) Rabbit pAb	1:1000
Anti-Akt1 (88-100) Rabbit pAb	1:2500
PhosphoDetect™ Anti-p38 MAP Kinase (pThr¹80, pTyr¹82) Rabbit pAb	1:1000
PhosphoDetect™ Anti-GSK3β (pSer9) Mouse mAb	1:2000
Anti-β-Catenin Mouse mAb (9G10)	1:500
Anti-Dishevelled-3 Antibody (Rabbit)	1:1000
PhosphoDetect™ Anti-c-Myc (pThr58, Ser62) Rabbit pAb	1:1000
Anti-HIF-1α Antibody (Rabbit)	1:200
Anti-VEGF (Rabbit)	1:500
Anti-NFκB p50 Antibody (Rabbit)	1:1000
Anti-NFκB p52 Antibody (Mouse)	1:1000

Dilution of secondary antibodies (Purchased from Merck, Millipore)

Rabbit Anti-mouse IgG	1:10,000
Goat Anti-rabbit IgG	1:10,000

Lysis buffer
- 50 mM Tric, pH 8.0 SDS
- 150.0 mM NaCI
- 1.0% Triton X®-100
- Protease inhibitor cocktail (P9599, Sigma-Aldrich, Ireland Ltd)

Laemmli 2X sample Buffer
- 4% SDS
- 20% Glycerol
- 125.0 mM Tris, pH 6.8
- 0.02% Bromophenol blue
- 10%βME, β-mercaptoethanol (Add freshly, just before use)

10% SDS solution
-5.0 g SDS
- 50.0 ml of distilled water

10% APS (Ammonium Persulfate)
- 1.0 g APS
- 10.0 ml of double distilled water

Gel Electrophoresis Running Buffer
- 25.0 mM Tris base
- 190.0 mM Glycine
- 0.1% SDS

Transfer Buffer
-50.0 mM Tris base
- 380.0 mM Glycine
- 0.1% SDS
- 20% Methanol

TBS buffer
- 8.0 g NaCI
- 0.2 g KCI
- 3.0 g Tris base

    - Dissolve in 800 ml double distilled water
    - Adjust pH to 8.0 with 1 M HCI
    - Top up to final volume of 1 liter
    - Sterilize by autoclaving and store at room temperature

Washing buffer (TBST)
- TBS with 0.1% Tween® 20

Blocking buffer (5% Nonfat Dried Milk in TBST)
- 5.0 g nonfat dried milk powder
- 100.0 ml of TBST

(19) Preparation of Buffers in 2D Gel Dimentional

Sample preparation solution (with urea and thiourea)

	Final concentration	Amount
Urea	7.0 M	10.5 g
Thiourea	2.0 M	3.8 g
CHAPS	2.0% (w/v)	1.0 g
IPG buffer	2.0% (w/v)	500.0 µl
DTT	40.0 mM	154.0 mg
Double distilled water	-	to 25.0 ml

Thiourea rehydration stock solution

	Final concentration	Amount
Urea	7.0 M	10.5 g
Thiourea	2.0 M	3.8 g
CHAPS	2.0% (w/v)	0.5 g
IPG buffer	2.0% (w/v)	500.0 µl
1% bromophenol blue	0.002%	50.0 µl
Double distilled water	-	to 25.0 ml

- DTT is added prior to use; 7 mg DTT for 2.5 ml of rehydration stock solution

SDS equilibration buffer solution

	Final concentration	Amount
Urea	6.0 M	72.1 g
This-HCI, pH 8.8	75.0 mM	10.0 ml
Glycerol (87% w/w)	29.3% (v/v)	69.0 ml
SDS	2.0% (w/v)	4.0 g
1% bromophenol blue	0.002%	400.0 µl
Double distilled water	-	to 200.0 ml

- First equilibration: Add 100 mg of DTT in 10 ml of SDS equilibration buffer
- Second equilibration: Add 250 mg of Iodoacetamide 10 ml of SDS equilibration buffer

1X Laemmli SDS electrophoresis buffer

	Final concentration	Amount
Tris base	25 mM	30.3 g
Glycine	192 mM	144.0 g
SDS	0.1% (w/v)	10.0 g
Double distilled water	-	to 10.0 liter

30% T, 2.6% C monomer stock solution

	Final concentration	Amount
Acrylamide	30.0%	300.0 g
N,N'-methylenebisacrylamide	0.8%	8.0 g
Double distilled water	-	to 1 liter

- Filter solution through a 0.45 µm filter
- Store at 4°C in the dark

4X Resolvng gel buffer solution

	Final concentration	Amount
Tris base	1.5 M	181.7 g
Double distilled water	-	750.0 ml
HCI	-	Adjust to pH 8.8
Double distilled water	-	to 1 liter

Agarose sealing solution

	Final concentration	Amount
Laemmli SDS electrophoresis buffer		100.0 ml
Agarose	0.5%	0.5 g
1% Bromophenol blue	0.002% (w/v)	200.0 µl

Fixation solution

Ethanol	200.0 ml
Acetic acid, glacial	50.0 ml
Double distilled water	250.0 ml

5% Coomassie blue

Coomassie Blue G-250	0.5 g
Double distilled water	10.0 ml

APPENDIX II: The fold changes of differentially expressed proteins in treated MeWo cells.

No	UniProtKB/Swiss-Prot (Accession number)	Protein	*Phyllanthus*							
			PA(H)	PN(H)	PU(H)	PW(H)	PA(M)	PN(M)	PU(M)	PW(M)
I	**Cell Adhesion, Migration, Invasion and Metastasis and Angiogenesis**									
1	P98172	Ephrin-B1	-1.46	-1.47	-1.53	-1.48	-1.59	-1.58	-1.57	-1.65
2	P61163	Alpha-centractin	-1.42	-1.33	-1.57	-1.45	-1.58	-1.75	-1.58	-1.74
3	Q13895	Bystin	-1.43	-1.57	-1.34	-1.45	-1.46	-1.59	-1.48	-1.65
4	P13646	Keratin, type I cytoskeletal 13	1.42	1.47	1.46	1.57	1.74	1.58	1.57	1.64
5	P08670	Vimentin	-1.73	-1.92	-1.72	-2.01	-1.85	-1.73	-1.72	-1.92
6	P04179	Superoxide dismutase [Mn], mitochondrial	-1.29	-1.54	-1.57	-1.39	-1.58	-1.85	-1.65	-1.48
7	P07437	Tubulin beta chain	-1.42	-1.48	-1.67	-1.97	-1.57	-1.58	-1.59	-1.54
8	P47755	F-actin-capping protein subunit alpha-2	-1.34	-1.33	-1.63	-1.58	-1.68	-1.58	-1.53	-1.63
9	Q8TEW0	Partitioning defective 3 homolog	-1.32	-1.49	-1.32	-1.68	-1.50	-1.56	-1.68	-1.58
II	**Proliferation, Cell Cycle, and Apoptosis**									
10	P68400	Casein kinase II subunit alpha	-2.28	-1.97	-1.83	-2.08	-2.18	-2.08	-1.95	-2.07
11	P50583	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	-1.47	-1.53	-1.48	-1.37	-1.58	-1.74	-1.63	-1.75

12	O60220	Mitochondrial import inner membrane translocase subunit Tim8	-1.56	-1.35	-1.64	-1.62	-1.63	-1.65	-1.68	-1.58
13	Q9BQ83	Structure-specific endonuclease subunit SLX1	-1.37	-1.49	-1.57	-1.48	-1.93	-1.57	-1.74	-1.63
14	Q6Q0C0	E3 ubiquitin-protein ligase TRAF7	1.48	1.57	1.65	1.57	1.45	1.78	-1.69	-1.549
15	P0CJ76	Humanin-like protein 9	-1.56	-1.68	-1.58	-1.58	-1.62	1.67	1.73	1.78
16	P17931	Galectin-3	-1.58	-1.75	-1.63	-1.69	-1.68	-1.68	-1.64	-1.78
17	P31943	Heterogeneous nuclear ribonucleoprotein H	-1.49	-1.57	-1.34	-1.63	-1.56	-1.68	-1.58	-1.84
18	P08758	Annexin A5	-1.42	-1.57	-1.73	-1.57	-1.57	-1.47	-1.59	-1.47
19	Q969E4	Transcription elongation factor A protein-like 3	-1.32	-1.47	-1.57	-1.74	-1.62	-1.48	-1.68	-1.57
20	Q9H930	Nuclear body protein SP140-like protein	-1.32	-1.38	-1.55	-1.57	-1.58	-1.58	-1.57	-1.58
21	P27348	14-3-3 protein theta	-1.42	-1.47	-1.29	-1.58	-1.57	-1.67	-1.45	-1.34
22	P62937	Peptidyl-prolyl cis-trans isomerase A	-1.54	-1.23	-1.47	-1.54	-1.58	-1.62	-1.57	-1.46
23	Q14929	Zinc finger protein 169	-1.69	-1.57	-1.33	-1.84	-1.75	-1.55	-1.47	-1.56
24	O75791	GRB2-related adapter protein	-1.59	-1.43	-1.4	-1.83	-1.56	-1.48	-1.53	-1.28
25	O00165	HCLS1-associated protein X-1	-1.69	-1.72	-1.97	-1.66	-1.84	-1.86	-1.67	-1.78
26	O95243	Methyl-CpG-binding domain protein 4	-1.49	-1.63	-1.56	-1.57	-1.66	-1.54	-1.63	-1.64

27	P78417	Glutathione transferase omega - 1	-1.45	-1.53	-1.46	-1.57	-1.64	-1.53	-1.48	-1.58
28	Q06830	Peroxiredoxin-1	-1.45	-1.42	-1.46	-1.47	-1.64	-1.74	-1.53	-1.48
29	P50453	Serpin B9	-1.32	-1.31	-1.53	-1.56	-1.57	-1.45	-1.55	-1.63
30	Q8ND25	E3 ubiquitin-protein ligase ZNRF1	-1.48	-1.64	-1.67	-1.57	-1.56	-1.46	-1.53	-1.58
31	Q06210	E3 ubiquitin-protein ligase ARIH2	-1.48	-1.58	-1.64	-1.6	-1.57	-1.65	-1.45	-1.64
32	Q9Y4L5	E3 ubiquitin-protein ligase RNF115	-1.56	-1.43	-1.66	-1.73	-1.69	-1.58	-1.58	-1.56
III	**Glycogenesis and glycolysis**									
33	Q969E3	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 2	-1.57	-1.58	-1.58	-1.53	-1.65	-1.73	1.67	-1.48
34	P00558	Protein phosphatase 1 regulatory subunit 3D	-1.33	-1.58	-1.53	-1.64	-1.56	-1.37	-1.57	-1.74
35	P06733	Alpha-enolase	-1.42	-1.57	-1.74	-1.73	-1.58	-1.57	-1.65	-1.69
36	P04406	Pyruvate kinase isozymes M1/M2	-1.45	-1.67	-1.47	-1.63	-1.78	-1.36	-1.36	-1.75
37	P04075	Phosphoglycerate kinase 1	-1.57	-1.58	-1.61	-1.64	-1.48	-1.53	-1.52	-1.58
IV	**Protein Synthesis and Energy Metabolism**									
38	P17174	Aspartate aminotransferase, cytoplasmic	-1.26	-1.67	-1.68	-1.56	-1.57	-1.58	-1.53	-1.58
39	Q9UBC9	Small proline-rich protein 3	-1.63	-1.75	-1.74	-1.56	-1.67	-1.68	-1.73	-1.57

40	P41247	Patatin-like phospholipase domain-containing protein 4	-1.63	-1.32	-1.49	-1.66	-1.53	-1.53	-1.58	-1.64
41	Q9HBU6	Ethanolamine kinase 1	-1.39	-1.75	-1.57	-1.83	-1.48	-1.75	-1.68	-1.74
42	P62195	26S protease regulatory subunit 8	-1.53	-1.64	-1.57	-1.74	-1.75	-1.56	-1.64	-1.54
43	Q5VTE0	Putative elongation factor 1-alpha-like 3	-1.48	-1.64	-1.64	-1.57	-1.57	-1.64	-1.58	-1.85
44	Q5TGZ0	Mitochondrial inner membrane organizing system protein 1	-1.53	-1.63	-1.74	-1.56	-1.67	-1.68	-1.64	-1.75
45	P07339	Cathepsin D	-1.54	-1.54	-1.65	-1.46	-1.68	-1.68	-1.47	-1.58
46	Q9Y587	AP-4 complex subunit sigma-1	-1.33	-1.57	-1.56	-1.36	-1.68	-1.74	-1.58	-1.67
47	P00568	Adenylate kinase	-1.58	-1.39	-1.53	-1.57	-1.56	-1.63	-1.68	-1.58
48	P80294	Metallothionein-1H	-1.29	-1.74	-1.73	-1.45	-1.59	-1.58	-1.76	-1.68
49	Q76KX8	Zinc finger protein 534	-1.59	-1.46	-1.57	-1.64	-1.54	-1.63	-1.58	-1.58
50	Q9Y6H3	Mitochondrial inner membrane protease ATP23 homolog	-1.59	-1.56	-1.57	-1.67	-1.68	-1.67	-1.68	-1.63
51	Q8WVM8	Sec1 family domain-containing protein 1	-1.44	-1.56	-1.64	-1.54	-1.76	-1.65	-1.75	-1.64
52	Q9NVH6	Trimethyllysine dioxygenase, mitochondrial	-1.58	-1.48	-1.58	-1.54	-1.79	-1.68	-1.53	-1.66

(Up-regulation indicated with “+” symbol and down-regulation indicated with “-” symbol)

APPENDIX III: The fold changes of differentially expressed proteins in treated PC-3 cells.

No.	UniProtKB/Swiss-Prot (Accession number)	Protein	*Phyllanthus*							
			PA(H)	PN(H)	PU(H)	PW(H)	PA(M)	PN(M)	PU(M)	PW(M)
I	**Cell Adhesion, Migration, Invasion and Metastasis and Angiogenesis**									
1	P98172	Ephrin-B1	-1.53	-1.45	-1.21	-1.41	-2.14	-2.21	-2.01	-2.31
2	P05787	Keratin, type II cytoskeletal 8	1.45	1.32	1.38	1.21	1.86	1.74	1.92	1.62
3	P63261	Actin, cytoplasmic 2	-1.43	-1.53	-1.21	-1.53	-1.74	-1.79	-1.72	-1.53
4	Q8NDI1	EH domain-binding protein 1	-1.32	-1.32	-1.21	-1.13	-1.42	-1.32	-1.43	-1.43
5	P04792	Heat shock protein beta-1	-1.43	-1.32	-1.54	-1.54	-1.42	-1.43	-1.32	-1.25
6	P35527	Keratin, type I cytoskeletal 9	1.38	1.32	1.53	1.42	1.64	1.34	1.35	1.37
7	Q9BYR8	Keratin-associated protein 3-1	1.53	1.32	1.47	1.64	1.47	1.53	1.42	1.46
8	P08670	Vimentin	-1.43	-1.32	-1.32	-1.11	-1.25	-1.43	-1.32	-1.42
9	Q9NY65	Tubulin alpha-8 chain	-1.53	-1.43	-1.48	-1.35	-1.40	-1.43	-1.42	-1.73
10	Q9Y316	Protein MEMO1	-1.43	-1.42	-1.24	-1.32	-1.63	-1.32	-1.42	-1.43
II	**Proliferation, Cell Cycle, and Apoptosis**									
11	Q92466	DNA damage-binding protein 2	-1.42	-1.32	-1.43	-1.41	-1.42	-1.37	-1.43	-1.32
12	Q7RTU1	Transcription factor 23	-1.39	-1.33	-1.42	-1.43	-1.53	-1.63	-1.58	-1.42
13	Q9UQ80	Proliferation-associated protein 2G4	-1.37	-1.57	-1.42	-1.47	-1.63	-1.45	-1.53	-1.43
14	P62993	Growth factor receptor-bound protein 2	-1.42	-1.52	-1.43	-1.32	-1.42	-1.42	-1.43	-1.64

15	O60565	Gremlin-1	-1.43	-1.43	-1.33	-1.43	-1.56	-1.43	-1.54	-1.44
16	P27348	14-3-3 protein theta	-1.32	-1.43	-1.53	-1.42	-1.42	-1.21	-1.32	-1.42
17	P61981	14-3-3 protein gamma	-1.32	-1.32	-1.43	-1.35	-1.32	-1.42	-1.22	-1.32
18	P04083	Annexin A1	-1.52	-1.54	-1.42	-1.43	-1.64	-1.43	-1.43	-1.43
19	Q96LY2	Coiled-coil domain-containing protein 74B	-1.47	-1.34	-1.47	-1.46	-1.33	-1.33	-1.36	-1.57
20	P31943	Heterogeneous nuclear ribonucleoprotein H	-1.47	-1.33	-1.35	-1.21	-1.44	-1.56	-1.43	-1.53
21	P09211	Glutathione S-transferase P	-1.96	-1.84	-1.77	-2.05	-1.89	-1.87	-2.01	-1.97
22	P41221	Protein Wnt-5a	-1.75	-1.87	-1.87	-1.92	-1.74	-1.84	-1.85	-2.01
23	Q9H0R3	Transmembrane protein 222	-1.57	-1.52	-1.57	-1.42	-1.47	-1.62	-1.57	-1.46
24	Q5MJ09	Sperm protein associated with the nucleus on the X chromosome N3	-1.45	-1.57	-1.57	-1.57	-1.34	-1.45	-1.32	-1.46
25	P46063	ATP-dependent DNA helicase Q1	-1.57	-1.57	-1.36	-1.46	-1.56	-1.47	-1.46	-1.57
26	P09382	Galectin-1	-1.25	-1.46	-1.57	-1.47	-1.46	-1.33	-1.46	-1.46
27	P04792	Heat shock protein beta-1	-1.37	-1.47	-1.47	-1.46	-1.56	-1.46	-1.34	-1.47
28	P78417	Glutathione transferase omega - 1	-1.46	-1.46	-1.47	-1.47	-1.57	-1.54	-1.68	-1.57
29	Q06830	Peroxiredoxin-1	-1.57	-1.42	-1.29	-1.33	-1.48	-1.62	-1.54	-1.67
30	P30048	Thioredoxin-dependent peroxide reductase, mitochondrial	-1.45	-1.52	-1.12	-1.64	-1.58	-1.44	-1.57	-1.57
31	Q9Y230	RuvB-like 2	-1.46	-1.47	-1.38	-1.32	-1.75	-1.57	-1.64	-1.57
32	P50453	Serpin B9	-1.46	-1.50	-1.36	-1.38	-1.58	-1.67	-1.76	-1.63
33	Q8ND25	E3 ubiquitin-protein ligase ZNRF1	-1.56	-1.43	-1.47	-1.47	-1.58	-1.67	-1.82	-1.54

34	Q6PRD1	Probable G-protein coupled receptor 179	-1.45	-1.43	-1.47	-1.56	-1.36	-1.37	-1.54	-1.58
35	O43521	Bcl-2-like protein 11	-1.66	-1.52	-1.42	-1.56	-1.57	-1.73	-1.65	1.56
36	P56703	Proto-oncogene Wnt-3 precursor	-2.18	-1.92	-2.06	-2.34	-1.79	-1.97	1.83	-2.11
37	Q8N4Z0	Putative Ras-related protein Rab-42	-2.11	-2.03	-1.79	-1.98	-1.96	-2.1	-2.15	-1.94
38	P01112	GTPase HRas precursor	-2.12	-1.99	-1.83	-1.74	-1.85	-2.03	-2.05	-1.92
III	**Glycogenesis and glycolysis**									
39	Q969E3	Urocortin-3	-1.37	-1.42	-1.37	-1.46	-1.65	-1.73	1.67	-1.47
40	P00558	Phosphoglycerate kinase 1	-2.01	-2.12	-2.22	-1.93	-1.56	-1.37	-1.57	-1.36
41	P06733	Alpha-enolase	-1.42	-1.38	-1.37	-1.36	-1.58	-1.57	-1.65	-1.57
42	P04406	Glyceraldehyde-3-phosphate dehydrogenase	-1.92	-1.88	-1.92	-1.83	-1.78	-1.36	-1.36	-1.56
43	P04075	Fructose-bisphosphate aldolase a	-1.63	-1.42	-1.39	-1.36	-1.48	-1.53	-1.52	-1.57
43	P60174	Triosephosphate isomerase	-1.47	-1.46	-1.47	-1.76	-1.48	-1.53	-1.49	-1.49
44	Q9NPG2	Neuroglobin	-1.47	-1.47	-1.58	-1.47	-1.56	-1.63	-1.74	-1.74
IV	**Protein Synthesis and Energy Metabolism**									
45	Q4U2R6	39S ribosomal protein L51	-1.37	-1.47	-1.36	-1.53	-1.64	-1.74	-1.65	-1.67
46	Q93088	Betaine--homocysteine S-methyltransferase 1	-1.46	-1.47	-1.46	-1.48	-1.67	-1.48	-1.58	-1.57
47	P50583	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	-1.45	-1.57	-1.30	-1.42	-1.39	-1.47	-1.67	-1.87
48	Q96A11	Galactose-3-O-sulfotransferase 3	-1.69	-1.74	-1.67	-1.85	-1.93	-1.74	-1.82	-1.89
49	Q9Y274	Type 2 lactosamine alpha-2,3-sialyltransferase	-1.28	-1.52	-1.58	-1.44	-1.68	-1.64	-1.58	-1.57
50	O43852	Calumenin	-1.53	-1.47	-1.47	-1.42	-1.57	-1.65	-1.56	-1.53

51	P13667	Protein disulfide-isomerase A4	-1.47	-1.41	-1.44	-1.45	-1.58	-1.64	-1.45	-1.57
52	P27797	Calreticulin	-1.54	-1.32	-1.47	-1.48	-1.45	-1.53	-1.57	-1.64
53	P07900	Heat shock protein HSP 90-alpha	-1.64	-1.32	-1.34	-1.43	-1.55	-1.42	-1.32	-1.53
54	P12235	ADP/ATP translocase 1	-1.46	-1.38	-1.45	-1.35	-1.58	-1.68	-1.67	-1.47
55	P52209	6-phosphogluconate dehydrogenase, decarboxylating	-1.46	-1.49	-1.54	-1.45	-1.57	-1.52	-1.48	-1.56
56	Q9Y478	5'-AMP-activated protein kinase subunit beta-1	-1.38	-1.46	-1.47	-1.47	-1.67	-1.75	-1.47	-1.57
57	P11021	78kDa glucose-regulated protein	-1.46	-1.46	-1.47	-1.57	-1.57	-1.54	-1.57	-1.84
58	Q9UI09	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	-1.42	-1.36	-1.33	-1.33	-1.57	-1.73	-1.57	-1.46
59	P30101	Protein disulfide-isomerase A3	-1.20	-1.47	-1.37	-1.21	-1.57	-1.63	-1.56	-1.57
60	P49411	Elongation factor Tu, mitochondrial	-1.48	-1.45	-1.57	-1.33	-1.38	-1.54	-1.68	-1.47
61	Q5TGZ0	Mitochondrial inner membrane organizing system protein 1	-1.47	-1.46	-1.47	-1.49	-1.57	-1.47	-1.67	-1.65
62	P19404	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	-1.21	-1.32	-1.33	-1.43	-1.57	-1.57	-1.57	-1.74
63	Q9H0F7	ADP-ribosylation factor-like protein 6	-1.76	-1.50	-1.57	-1.54	-1.56	-1.54	-1.49	-1.70
64	O75436	Vacuolar protein sorting-associated protein 26A	-1.46	-1.54	-1.63	-1.76	-1.47	-1.38	-1.57	-1.57
65	Q9NXV2	BTB/POZ domain-containing protein KCTD5	-1.47	-1.47	-1.33	-1.50	-1.58	-1.65	-1.67	-1.66

66	O00429	Dynamin-1-like protein	-1.47	-1.63	-1.47	-1.57	-1.56	-1.57	-1.67	-1.73
67	P40261	Nicotinamide N-methyltransferase	-1.29	-1.22	-1.34	-1.28	-1.45	-1.48	-1.83	-1.42
68	P49720	Proteasome subunit beta type-3	-1.53	-1.22	-1.34	-1.47	-1.58	-1.53	-1.67	-1.57
69	Q6IQ16	Speckle-type POZ protein-like	-1.42	-1.67	-1.47	-1.47	-1.63	-1.64	-1.68	-1.47
70	P21796	Voltage-dependent anion-selective channel protein 1	1.57	1.63	1.52	1.79	1.67	1.84	1.77	1.76
71	Q9Y3D8	Adenylate kinase isoenzyme 6	-1.51	-1.33	-1.43	-1.49	-1.46	-1.64	-1.74	-1.76
72	Q14152	Eukaryotic translation initiation factor 3 subunit 12	-1.40	-1.37	-1.39	-1.49	-1.53	-1.67	-1.63	-1.53

(Up-regulations indicated with “+” symbol and down-regulation indicated with “-” symbol).

APPENDIX IV: Additional Outputs of the PHD Project.

Scientific Publications

Tang Y-Q, Jaganath IB, Sekaran SD (2013). *Phyllanthus spp. Exerts Anti-Angiogenic and Anti-Metastatic Effects through Inhibition on Matrix Metalloproteinase Enzymes.* Manuscript submitted to "PLoS ONE".

Tang Y-Q, Jaganath IB, Sekaran SD (2013). *Phyllanthus Suppresses MeWo Cell Proliferation and Induces Apoptosis through Modulation Multiple Signalling Pathways.* Submitted to "Cellular Signalling".

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Dengue Virus Type 2 (DENV2) .Presented at 9th Asia-Pacific Congress Medical Virology (APCMV), Adelaide, Australia (6th – 8th June 2012)

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Tang Yin Quan, Indu Bala Jaganath and Shamala Devi Sekaran. *Potential Anticancer Properties of Phyllanthus against Skin Melanoma and Prostate Cancer Cells.* Presented at Creativity and Innovation Expo University of Malaya 2010, Malaysia (April, 2010)

Yin-Quan Tang, Indu Bala Jaganath and Shamala Devi Sekaran. *Anticarcinogenic properties of extracts derived from medicinal plant, Phyllanthus on human skin and prostate cancer cells*. Presented at the UK-Malaysia Symposium on Drug Discovery and Development for Cancer, Kuala Lumpur, Malaysia (24-25 February 2010).

Wee-Chee Tan, Sau-Har Lee, **Yin-Quan Tang**, Indu-Bala Jaganath, Shamala-Devi Sekaran. *Phyllanthus spp. as potent antiviral agent against Dengue viruses.* Presented at 4th Asian Regional Dengue Research Network Meeting; DUKE-NUS Emerging Infectious Diseases Inauguration Symposium Meeting in Singapore (08 Dec 2009 to 11 Dec 2009).

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Phyllanthus spp. Induces Selective Growth Inhibition of PC-3 and MeWo Human Cancer Cells through Modulation of Cell Cycle and Induction of Apoptosis

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Abstract

Background: *Phyllanthus* is a traditional medicinal plant that has been used in the treatment of many diseases including hepatitis and diabetes. The main aim of the present work was to investigate the potential cytotoxic effects of aqueous and methanolic extracts of four *Phyllanthus* species (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) against skin melanoma and prostate cancer cells.

Methodology/Principal Findings: *Phyllanthus* plant appears to possess cytotoxic properties with half-maximal inhibitory concentration (IC_{50}) values of 150–300 µg/ml for aqueous extract and 50–150 µg/ml for methanolic extract that were determined using the MTS reduction assay. In comparison, the plant extracts did not show any significant cytotoxicity on normal human skin (CCD-1127Sk) and prostate (RWPE-1) cells. The extracts appeared to act by causing the formation of a clear "ladder" fragmentation of apoptotic DNA on agarose gel, displayed TUNEL-positive cells with an elevation of caspase-3 and -7 activities. The Lactate Dehydrogenase (LDH) level was lower than 15% in *Phyllanthus* treated-cancer cells. These indicate that *Phyllanthus* extracts have the ability to induce apoptosis with minimal necrotic effects. Furthermore, cell cycle analysis revealed that *Phyllanthus* induced a Go/G1-phase arrest on PC-3 cells and a S-phase arrest on MeWo cells and these were accompanied by accumulation of cells in the Sub-G1 (apoptosis) phase. The cytotoxic properties may be due to the presence of polyphenol compounds such as ellagitannins, gallotannins, flavonoids and phenolic acids found both in the water and methanol extract of the plants.

Conclusions/Significance: *Phyllanthus* plant exerts its growth inhibition effect in a selective manner towards cancer cells through the modulation of cell cycle and induction of apoptosis via caspases activation in melanoma and prostate cancer cells. Hence, *Phyllanthus* may be sourced for the development of a potent apoptosis-inducing anticancer agent.

Citation: Tang Y-Q, Jaganath IB, Sekaran SD (2010) *Phyllanthus* spp. Induces Selective Growth Inhibition of PC-3 and MeWo Human Cancer Cells through Modulation of Cell Cycle and Induction of Apoptosis. PLoS ONE 5(9): e12644. doi:10.1371/journal.pone.0012644

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Introduction

Cancer is a name given to group of diseases that arise from uncontrolled growth, spread of an abnormal cell and can result in death. It is extremely hard to treat due to several distinct classes of tumours that exhibit different responses to treatment and not all anticancer agents effectively give a positive response in every case [1]. Some have been reported to exhibit toxicity to normal cells, accompanied by undesirable effects such as vomiting, nausea and alopecia. Thus, ineffective anticancer agents have resulted in high death rates in cancer patients [2]. Melanoma is a type of skin cancer that arises from melanocytes, a pigment-producing tanning cell. Melanoma incidence and its mortality rate are high in fair-skinned populations in all parts of the world, including Australia, USA and UK [3–4]. Prostate cancer is the second leading cause of cancer deaths after lung cancer worldwide [3]. Currently, there are no effective treatments for both melanoma and prostate cancer, and as such intense research is required to obtain new anticancer agents for these cancers.

The high mortality in cancer patients has led many researchers to source for potential natural-product based therapeutic compounds [2]. Herbal plants and plant-derived medicines have been used as the source of potential anticancer agents in traditional cultures all over the world and are becoming increasingly popular in modern society [5]. The potential natural product-derived anticancer agents are known to possess various bioactive compounds such as roscovitine from red radish and flavopiridol from *Amoora rohituka*, a tropical tree that has shown tremendous effects in the treatment of cancers [6–8].

The plant of the genus *Phyllanthus* belongs to the family *Euphorbiaceae* and has been reported to have pharmacological effects such as antiviral activity against Hepatitis B and related hepatitis viruses [9–12], anti-bacterial activity [13,14], anti-hepatotoxic or liver-protecting activity [15–19] as well as anti-

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Evaluation of Phyllanthus, for Its Anti-Cancer Properties

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University of Malaya
Malaysia

1. Introduction

1.1 Cancer

Cancer is a name given to a group of diseases that arise from a single cell when it starts to grow abnormally in an uncontrollable manner to form a group of undifferentiated cells, known as tumour. Tumour can be classified into two categories; benign and malignant. Not all benign tumours are cancerous but all malignant tumours are (Hanahan & Weinberg, 2000). The main difference between these tumours is benign tumour lack the metastatic ability, grows locally and is less harmful. However, some benign tumours can transform into malignant tumours that possess the metastatic ability to invade and spread to other parts of the body via the blood or lymphatic circulation and form secondary tumour and eventually lead to death (Vincent & Gatenby, 2008).

1.2 Development of cancer (carcinogenesis)

Cancer develops through a multistep process known as carcinogenesis (Fig 1), which includes initiation, promotion and progression (Pitot, 2006). An initiation stage is a permanent and irreversible event, which involves one or more cellular changes arising upon exposure to carcinogens, which leads to alteration in DNA and may result in a mutated cell to divide rapidly (hyperplasia). These transformed (initiated) cells can remain harmless, unless exposed to a stimulator, which enhances the tumour to grow into a larger mass. This is a reversible process and is known as the promotion stage. The progression stage is an irreversible conversion of a benign tumour to become a malignant tumour. This carcinogenesis process usually takes 10 years or more to develop and usually depends on the internal (life style) and external (environmental) factors of the patient (Pitot, 2002).

1.3 Hallmarks of cancer

A transformed cell has to acquire six hallmarks in order to be developed into cancer (Fig 2) (Hanahan & Weinberg, 2000). Each of these hallmarks is derived upon changes in the normal cell's physiology and interacts with each other to promote malignant growth. The conversion from a normal cell to become a transformed cell usually starts from mutations in DNA which cause the cells no longer depend on growth signals, thus gaining uncontrolled growth and proliferation. The irresponsiveness or insensitivity to anti-growth signals in

ANTICARCINOGENIC PROPERTIES OF EXTRACTS DERIVED FROM MEDICINAL PLANT, *PHYLLANTHUS* ON HUMAN SKIN AND PROSTATE CANCER CELLS

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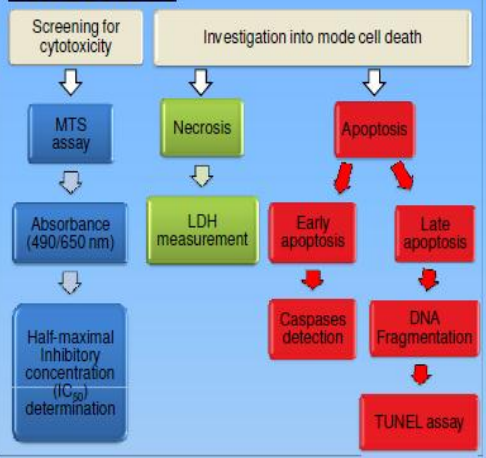
INTRODUCTION

Cancer is a group of diseases that are characterized by uncontrolled growth, spread of abnormal cells and can result in death. Current anticancer agents have resulted in a high death rate in patients and this caused the shift to source for potential natural-based therapeutic compounds. *Phyllanthus* is a medicinal plant belonging to *Euphorbiaceae* family and its anticancer effect had been studied against liver and leukemia cancer, however its effectiveness on cancer has not been fully elucidated. We are interested to investigate their potential anticarcinogenic (antiproliferative) effect on human skin (MeWo) and prostate (PC-3) cancer cells. Two normal human skin (CRL-2565) and prostate (RWPE-1) cells were used as comparison. Two extracts (aqueous and methanol) of four plant species of genus *Phyllanthus*, including *P. amarus (PA), P. niruri (PN), P. urinaria (PU)* and *P. watsonii (PW)*, are used as representatives.

METHODOLOGY



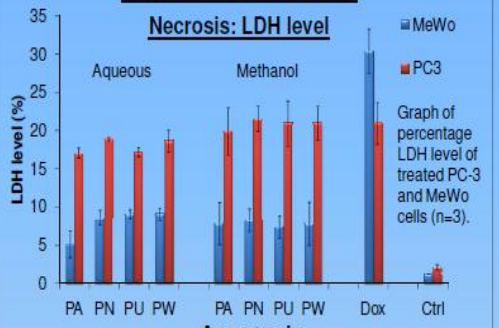
FINDINGS

CYTOTOXIC SCREENING

Phyllanthus		IC50 ±SD (µg/ml)		
Species	Ext	Cancer cells		Normal cells
		PC-3	MeWo	RWPE-1 CRL-2565
PA	H_2O	198.3 ± 1.3	178.3 ± 2.8	> 500
	MeOH	133.3 ± 2.9	84.3 ± 1.1	
PN	H_2O	260.0 ± 2.4	155.0 ± 1.2	
	MeOH	153.3 ± 2.6	117.7 ± 2.1	
PU	H_2O	193.3 ± 1.1	155.7 ± 2.1	
	MeOH	56.21 ± 3.2	54.2 ± 2.1	
PW	H_2O	160.0 ± 3.2	156.7 ± 2.4	
	MeOH	100.7 ± 2.0	100.5 ± 1.2	

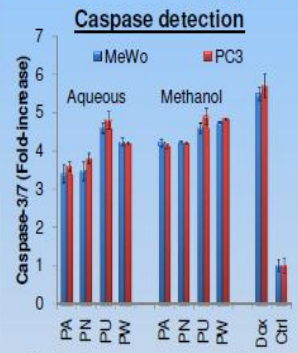
IC_{50} value of *Phyllanthus* on cancer and normal cells (n=3), where range 150–300 µg/ml for aqueous and 50–150 µg/ml for methonlic extract.

MODE OF CELL DEATH

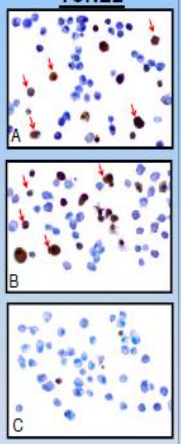


Graph of percentage LDH level of treated PC-3 and MeWo cells (n=3).

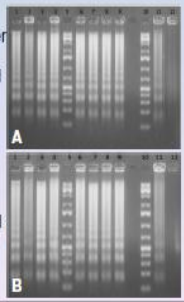
Apoptosis



The level of caspase-3/7 was higher than untreated cells.



DNA Fragmentation



Apoptotic DNA ladder was observable for treated (A) PC-3 and (B) MeWo cells, where lane 1-4 : aqueous extract and lane 6-9 : methanol extract for PA, PN, PU and PW. Lane 11:untreated cell and lane 12:Doxorubicin. Lane 5&10:1kb DNA marker.

Apoptotic cells were observable as brown colour (red arrow) after treated with aqueous (A) and methanol (B) extract of *P.U* on PC3 cells. Untreated cell (C) was stained with blue colour.

DISCUSSION AND CONCLUSION

Both *Phy.* extracts showed anticarcinogenic properties, where *PU* displayed stronger cytotoxicity among the spp. Importantly, the plant has not shown significant cytotoxicity effect on normal human lines, as comparison. *Phy.* plant also possessed the ability as apoptotic-inducer mediated via caspases activation, together with minimal necrotic effect. Although the underlying mechanism of selectivity of *Phy.* plant against skin and prostate cancer cells is still unclear, our findings indicate that *Phy.* plant exert its growth inhibition effect via apoptotic pathway through caspases activation and this information is useful to lead new anticancer compounds development.

ACKNOWLEDGEMENT

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THE ANTI-METASTASIS AND ANTI-ANGIOGENESIS PROPERTIES OF *PHYLLANTHUS* SPECIES

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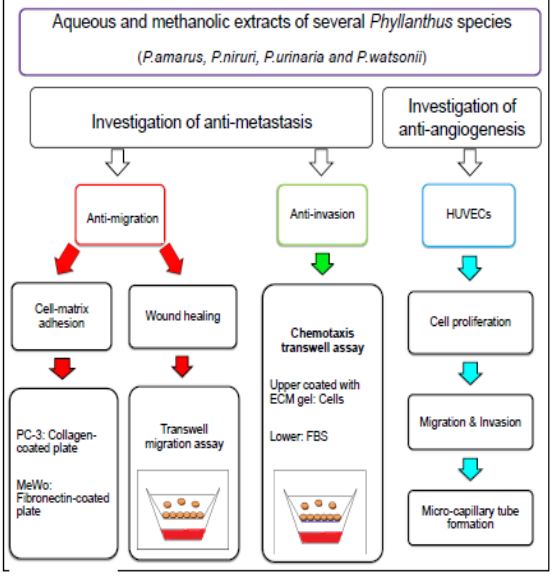
INTRODUCTION

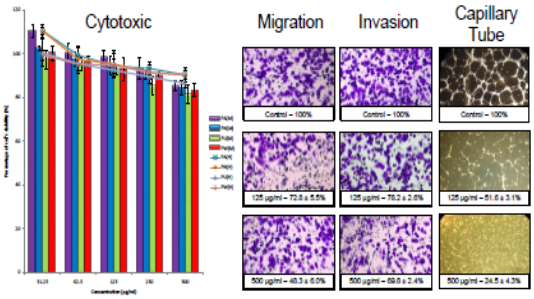
Cancer is one of the leading causes of death in the world. About 50% of advanced cancer patients are diagnosed with tumour metastasis. Tumour angiogenesis is a process where new blood vessels are generated to cancer cells and is critical for tumour growth and metastasis. Both tumour metastasis and angiogenesis are major cause of high mortality rate and they remain as a challenge in cancer treatment. *Phyllanthus* is a medicinal plant belonging to the *Euphorbiaceae* family and our previous findings indicated that the *Phyllanthus* extracts can induce growth arrest and apoptosis in prostate and skin cancer cells. In this study, the anti-metastatic and anti-angiogenesis properties of several *Phyllanthus* species against prostate (PC-3) and skin (MeWo) cancer cell were further investigated.

MATERIALS AND METHODS



RESULTS

ANTI-ANGIOGENIC EFFECTS



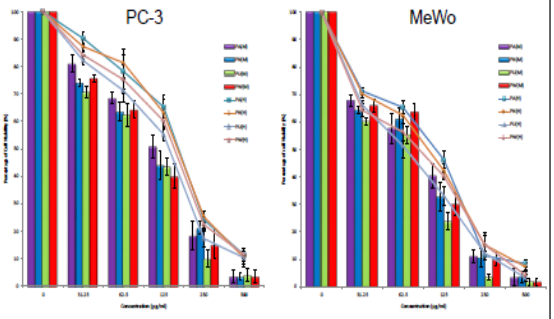
In the HUVECs, *Phyllanthus* extracts exhibited dose-dependent inhibition on both their migration and invasion ability without any perturbation cell proliferation. The anti-angiogenic effect of *Phyllanthus* was observable as it inhibit *in vitro* matrix-induced capillary tube formation of HUVECs.

RESULTS

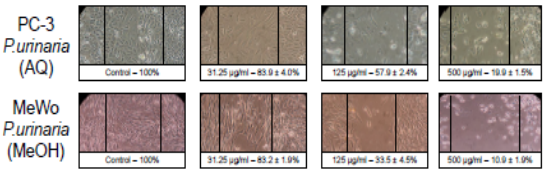
ANTI-METASTATIC EFFECTS

(I) Anti-migration

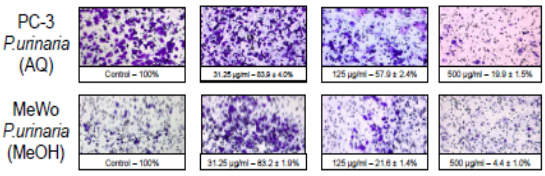
(a) Cell-matrix adhesion assay



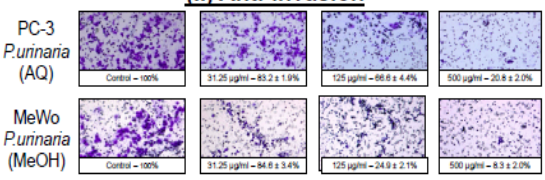
(b) Wound healing assay



(c) Transwell migration assay



(II) Anti-invasion



The result shows that *Phyllanthus* extracts reduced the adhesion ability of treated PC-3 and MeWo cells to collagen and fibronectin, respectively. *Phyllanthus* extracts also exhibited a dose-dependent inhibitory effects on PC-3 and MeWo cell's migration and invasion

CONCLUSION

Phyllanthus extracts exert anti-metastatic and anti-angiogenesis effects on skin melanoma and prostate cancer cells. Hence, *Phyllanthus* can be sourced as a potential anticancer drug against human malignant melanoma and prostate cancer upon further investigations.

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•Cragg, *et al. Anticancer agents from Natural products.* United States of America: Taylor & Francis Group. 2005: 12-43.
•Tang, *et al.*, (2010). *Phyllanthus* spp. Induces Selective Growth Inhibition of PC-3 and MeWo Human Cancer Cells through Modulation of Cell Cycle and Induction of Apoptosis. PLoS ONE 5(9): e12644. doi:10.1371/journal.pone.0012644

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