ANTIPROLIFERATIVE, ANTIMETASTATIC PROPERTIES OF PHYLLANTHUS SPECIES ON BREAST AND LUNG CANCER CELL LINES AND THEIR TARGETTED SIGNAL TRANSDUCTION PATHWAYS

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ABSTRACT

Cancer is the second leading cause of death worldwide and among them, lung and breast cancers have mortality rates of 1.4 million and 400,000 deaths each year respectively. This is due to the current chemotherapies that are still far from ideal and hence, alternative treatments founded in a 'back-to-nature' approach might yield improved treatment avenues. The genus *Phyllanthus* is a rainy season weed that has been exploited for various medicinal usages due to the presence of abundant polyphenol compounds in the plant that can be broadly grouped into ellagitannins, gallotannins, flavonoids, and phenolic acids. Therefore, the aim of the current study was to evaluate the antimetastatic potential of four *Phyllanthus* species (*P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*) on lung (A549) and breast (MCF-7) carcinoma cell lines.

Phyllanthus exhibited antiproliferative activity by causing selective toxicity on A549 and MCF-7 cells with IC_{50} values ranging from 50-470μg/ml, without the involvement of cell cycle arrest. At these IC_{50} concentrations, *Phyllanthus* successfully halted the endothelial-mesenchymal transition process by increasing cell aggregation and cell-cell adhesion. *Phyllanthus* also reduced the invasion of A549 and MCF-7 cells through the extracellular matrix layer by 40% and 10%, respectively. It was also observed that *Phyllanthus* inhibited almost 60% cells adhesion to the matrix layer while suppressing up to 80% migration of these cells through an 8μm pore size polycarbonate membrane. This ability of *Phyllanthus* to inhibit A549 and MCF-7 cells' invasion, migration, and adhesion were even enhanced at increasing treatment concentrations and were associated with their capacity to repress the expressions of matrix metalloproteinases 2, 7, and 9 in cancer cells that function to hydrolyze the basement membrane during cell metastasis. In addition, *Phyllanthus* induced apoptosis in A549

and MCF-7 cells in conjunction to its antimetastatic action with the activation of caspases-3 and -7 as well as DNA fragmentation. Among the four *Phyllanthus* species, *P. urinaria* inhibited cancer cells growth and metastasis most effectively, closely followed by *P. watsonii*.

Following this, we screened the ten major cellular signaling pathways and recognized that *Phyllanthus* exerted its antimetastatic and apoptosis-inducing activities by inhibiting ERK1/2 and hypoxia pathways, attributed to the repression of signaling transduction components such as G proteins, G protein receptors, and serine/threonine-protein kinases. ERK1/2 pathway inhibition by *Phyllanthus* subsequently led to downregulated expression of cytoskeletal proteins for cell invasion and mobility, protein synthesis and transcriptional proteins for cell growth, as well as antiapoptotic protein for cell survival. Meanwhile, inhibition of the hypoxia pathway repressed expression of angiogenic proteins that are essential for cell angiogenesis and migration as well as various glucose uptake and glycolytic enzymes for cell growth and metabolism. *Phyllanthus* also suppressed drug detoxification enzymes and tumor defense mechanism proteins including gluthathione transferase, gluthathione synthetase, metallothionein, and annexins.

In summary, *Phyllanthus* could be a valuable candidate as a therapeutic agent for metastatic cancers since it does not only stop spreading of cancer cells, but it also inhibits cell growth and induces apoptosis.

ABSTRAK

Kanser adalah pembunuh kedua di seluruh dunia dan di antaranya, kanser peparu (1.4 juta) dan payudara (400,000) merangkumi jumlah kematian yang tertinggi pada setiap tahun. Hal ini kerana kemoterapi pada masa kini masih tidak memuaskan disebabkan oleh ‘multidrug resistance’ fenomena, pelbagai kesan sampingan yang dialami oleh pesakit-pesakit kanser, serta kemampuan tumor malignan untuk merebak. Oleh kerana itu, rawatan alternatif yang bertemakan 'back-to-nature' mungkin boleh menjadi penawar kanser yang lebih efektif. Genus Phyllanthus adalah merupakan sejenis rumpai yang tumbuh semasa musim hujan dan tumbuhan ini telah digunakan untuk merawat pelbagai jenis penyakit disebabkan ia mengandungi polifenol-polifenol yang boleh dikelompokkan kepada ellagitannins, gallotannins, flavonoids dan phenolic acids. Oleh itu, objektif kajian ini adalah untuk menilai potensi empat *Phyllanthus* species (*P. niruri*, *P. urinaria*, *P. watsonii*, dan *P. amarus*) untuk mengelak perebakan kanser sel peparu (A549) dan payudara (MCF-7).

Phyllanthus telah menunjukkan bahawa ia menghindari pertumbuhan sel-sel A549 dan MCF-7 secara spesifik dengan nilai IC_{50} berkisar antara 50-470µg/ml, tanpa pembantutan kitaran sel. Pada nilai-nilai IC_{50} tersebut, *Phyllanthus* berjaya untuk menghentikan proses perubahan endothelial-mesenchymal melalui perangsangan agregasi serta adhesi sel-sel. Selain itu, ia juga berupaya untuk mengencatkan 40% dan 10% invasi sel-sel A549 dan MCF-7 masing-masing melalui lapisan matriks ekstraselluler. *Phyllanthus* juga terbukti berkebolehan untuk mengencatkan 60% adhesi sel-sel ke lapisan matriks tersebut sementara membantutkan 80% migrasi sel-sel A549 dan MCF-7 melalui membran polikarbonat yang mempunyai liang-liang bersaiz 8µm. Kebolehan *Phyllanthus* untuk mengelak invasi, migrasi, dan adhesi juga meningkat

apabila dos yang diuji dinaikkan dan hal ini mungkin disebabkan oleh kebolehan *Phyllanthus* untuk mencegah ekspresi matriks metalloproteinase 2, 7 serta 9 yang bertangungjawab untuk memusnahkan membran semasa metastasis. Selain itu, *Phyllanthus* juga mampu untuk menginduksikan apoptosis dengan pengaktifan caspases-3 dan -7 berserta fragmentasi DNA. Di antara empat spesies *Phyllanthus* yang diuji dalam kajian ini, *P. urinaria* menunjukkan aktiviti yang paling efektif untuk menghalang pertumbuhan dan metastasis sel kanser, diikuti oleh *P. watsonii*.

Berikutannya, transduksi isyarat-isyarat di dalam sel yang digunakan oleh *Phyllanthus* untuk mengelakkan perebakan sel-sel kanser dikaji dan pemerhatian menunjukkan bahawa *Phyllanthus* menyebabkan aktiviti 'antimetastatic' melalui perencatan transduksi isyarat ERK1/2 dan hipoksia. Kejayaan *Phyllanthus* untuk menghalang transduksi isyarat tersebut kemungkinan besar disebabkan oleh kemampuannya untuk menghindari ekspresi pelbagai komponen transduksi isyarat seperti G proteins, G protein receptors serta serine/threonine-protein kinases. Penghambatan transduksi isyarat ERK1/2 menyebabkan pencegahan ekspresi protein-protein cytoskeletal untuk invasi dan mobiliti, transkripsi serta sintesis protein untuk pertumbuhan sel-sel, dan antiapoptosis protein untuk meneruskan kehidupan sel-sel. Sementara itu, penghambatan transduksi isyarat hipoksia menyebabkan pencegahan ekspresi protein angiogenik untuk pertumbuhan kapilari-kapilari darah dan migrasi sel-sel serta pelbagai enzim glikolitik untuk pertumbuhan dan metabolism sel-sel. Tambahan pula, *Phyllanthus* berjaya menghambatkan enzim mendetoksifikasi ubat serta protein untuk mekanisme pertahanan tumor seperti gluthathione transferase, gluthathione synthetase, metallothionein dan annexins.

Semua penemuan di atas membuktikan bahawa *Phyllanthus* adalah calon yang baik untuk mengubati penyakit kanser metastasis kerana ia bukan sahaja mengelakkan

perebakan sel-sel kanser, tetapi ia juga menghentikan pertumbuhan sel-sel serta menginduksikan apoptosis.

DEDICATION

I would like to dedicate this Thesis with much love and gratitude to

My own determination and persistence

My late grandfather, YOW MUN YEW

My grandmother, CHAM AH MOY

My father, LEE AH FAH

My mother, YOW LAI YIN

MY sisters

My family members

And all my friends

Without your patient, continuous support and encouragements, this project would not have possibly been a success.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Description
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
SCLC	Small cell lung cancer
NSCLC	Non small cell lung cancer
BRCA	Breast cancer type 1 susceptibility protein
P53	Tumor protein 53
ER	Estrogen receptor
Bcl-2	B-cell lymphoma 2
PI3K/AKT	Phosphoinositide 3-kinase/protein kinase B
pTEN	Phosphatase and tensin homolog
mm	Millimeter
ECM	Extracellular matrix
Cdk	Cyclin-dependent kinases
pRB	Retinoblastoma protein
MMP	Matrix metalloproteinase
ATP	Adenosine triphosphate
TNFR	Tumor necrosis factor receptor
AIF	Apoptosis inducing factor
TRAIL	TNF-related apoptosis-inducing ligand
EMT	Endothelial-mesenchymal transition
E-cadherin	Epithelial cadherin
N-cadherin	Neuronal cadherin
SDF-1	Stromal cell-derived factor-1
VEGF	Vascular endothelial growth factor
AP-1	Activator protein 1
NF-κB	Nuclear factor-kappa B
MAPK	Mitogen-activated protein kinase
TIMP	Tissue inhibitor of metalloproteinase
Ets-1	E26 transformation-specific sequence-1
PEA3	Poly-oma enhancer activator
SNP	Single-nucleotide polymorphism
u-PA	Urinary plasminogen activator
Hdm2	Human double minute 2
ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinases
MAPKAPK	MAP Kinase Activated Protein Kinase
SAPK	Stress-activated protein kinase
ATF	Activating transcription factor

HIF	Hypoxia inducible factor
PHD	Prolyl hydroxylase
HRE	Hypoxia responsive element
mRNA	Messenger ribonucleic acid
IκB	NF-kappa B inhibitor
mg	Mmilligram
ml	Milliliter
kg	Kilogram
HCV	Hepatitis C virus
NS1	Non-structural protein 1
µm	Micrometer
PBS	Phosphate buffered saline
DMSO	Dimethyl sulfoxide
HPLC	High performance liquid chromatography
MS-MS	Tandem mass spectrometry
µl	Microliter
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
rpm	Revolutions per minute
MTS/PMS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]/phenazine methosulfate
PI	Propidium iodide
RNase	Ribonuclease
IC_{50}	Half maximal inhibitory concentration
BSA	Bovine serum albumin
SDS	Sodium dodecyl sulfate
V	Volt
cm^2	Centimeter squared
HCl	Hydrochloric acid
M	Molar
LDH	Lactate dehydrogenase
nm	Nanometer
iNOS	Inducible nitric oxide synthase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
VEGF	Vascular endothelial growth factor
HUVEC	Human umbilical vein endothelial cells
°C	Degree celcius
kDa	Kilodalton
cm	Centimeter
IPG	Immobilized pH gradient
mA	Miliampere
TBST	Tris-buffered saline with tween 20

DAB	3,3′-Diaminobenzidine
Elk1	E twenty-six (ETS)-like transcription factor 1
RSK	Ribosomal s6 kinase
NL	Nonlinear
DTT	Dithiothreitol
IAA	Iodoacetamide
ACN	Acetonitrile
UV	Ultraviolet
µg	Microgram
TGFβ	Transforming growth factor beta
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
ADP	Adenosine diphosphate
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
2DE	Two dimensional gel electrophoresis
hnRNP	Heterogeneous nuclear ribonucleoprotein
tRNA	Transfer ribonucleic acid
CaM	Cell Adhesion Molecules
Hsp	Heat shock protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
NO	Nitric oxide
DHAP	Dihydroxyacetone phosphate
H_2O_2	Hydrogen peroxide
SCID	Severe combined immunodeficiency
Thr	Threonine
Tyr	Tyrosine
Glu	Glutamate

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 CANCER

Cancer is a complex group of heterogeneous diseases due to accumulation of gene mutations that inhibit the activity of regulatory genes that usually restrain cell proliferation while at the same time enhancing the activity of proteins that stimulate it (Williams & Stoeber 2007). This disease is in fact an exceedingly old disease although it is often mistaken as a disease of the developed world. This is because cancer has been discovered even in the Egyptian mommies and hence it is sensible to consider that cancer has constantly been a part of the pluricellular life (Sasco 2008).

The absolute number of cancer cases has increased tremendously with an accelerating trend in most of the countries worldwide (Sasco 2008). It is currently the second leading cause of death after cardiovascular diseases with more than 11 million deaths every year. By 2020, the number of new cancer cases could increase up to 16 million each year. Globally, mortality from cancer is predicted to be continuously increasing with an estimated 9 million and 11.4 million people dying from cancer in 2015 and 2030 respectively (Sardari et al. 2009). This estimation of cancer incidences and mortality is in accordance with the world population evolution whereby there is a projected gradual decline in fertility with an increase in life expectancy. This implies that there will be a continually decreasing population of children with a concurrent increase in elderly population. The predicted 80 million increase of people each year will produce 7.5 billion human population by year 2020, and is expected to increase to 8.9 billion by 2050 of which the elderly group constitutes 16% (Parkin et al. 2001). Hence, age is becoming a powerful determinant of cancer risk as cancer is one of the common causes of disability and death in the elderly population, since more than 50%

of malignant neoplasms occurs in people aged above 70. This could be associated to several hypotheses such as the development of malignant neoplasm (carcinogenesis) is said to occur over several years and therefore, is more likely to manifest in older individuals; a higher prevalence of cancer in older people reflecting their prolonged exposure to environmental carcinogens and the progressive changes in the internal milieu of the individuals as they age, hence providing a favorable condition for the initiation of new neoplasms or development of the existing latent malignant cells (Anisimov 2009).

Different individuals irrespective of their age and gender are exposed to varying risk of numerous cancer diseases. Some of the cancer diseases such as acute lymphoblastic leukemia and acute myeloid leukemia are more likely to manifest in children (Dorak et al. 2007) while lung, colorectal, and bladder cancers are more common in men (Parkin et al. 2001). This information is made available through collection of statistics on cancer incidences and deaths that are commonly used to monitor the trends as well as for the epidemiologic studies for the etiology, prevention, and control of cancer diseases (German et al. 2011). Different types of cancers worldwide present varying profiles in terms of their incidences and mortality as shown in Figure 1.1. Meanwhile, the projected number of cases and deaths worldwide at different future times are shown in Table 1.1.

1.1.1 LUNG CANCER

Among cancer diseases, lung cancer is by far the most common cause of cancer-related mortality in the world. Despite the advances in diagnostic imaging and therapeutic improvements over the decade, lung cancer has a poor prognosis with a 5-year patient survival rate. This disease is more frequent in men, comprising 75% of the world total (Parkin et al. 2001, Beadsmoore & Screaton 2003). Lung cancer occurrence

patterns are greatly affected by past exposure to tobacco smoking, depending on several factors including number of cigarettes smoked per day, degree of inhalation, and the age at initiation. A family history of lung cancer may also increase the risk in individuals due to genetic polymorphisms in carcinogen-metabolizing enzymes such as glutathione-S-transferase-1 (GSTM1) deletion homozygote which enhances susceptibility to tobacco smoke. Other factors increasing risk of lung cancer are occupational exposures to asbestos, metals, radon, and ionizing radiations (Parkin et al. 2001).

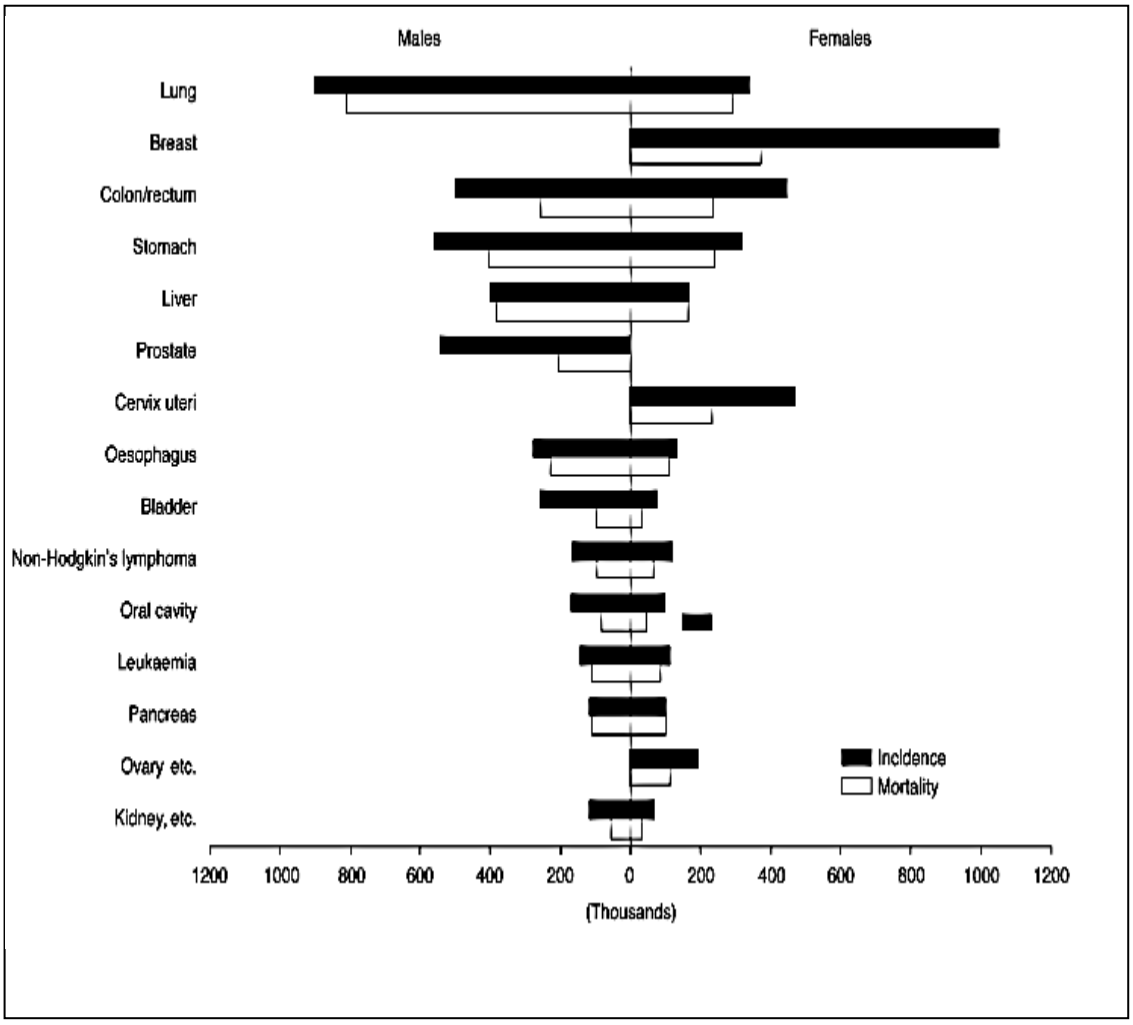


Figure 1.1: Number of new cases and deaths worldwide for the 15 most common cancers, 2000, cited from (Parkin et al. 2001)

Table 1.1: Projected demographic effects on cancer burden: Incidence

		Number (thousands)			
		2000	**2010**	**2020**	**2050**
Stomach cancer	Incidence	880	1110	1440	2440
	Mortality	650	810	1060	1900
Colorectal cancer	Incidence	940	1140	1390	2080
	Mortality	490	590	740	1160
Lung cancer	Incidence	1230	1540	2030	3160
	Mortality	1110	1360	1740	2860
Female breast cancer	Incidence	1050	1250	1480	1970
	Mortality	370	450	540	770
Cervical cancer	Incidence	470	600	740	1130
	Mortality	230	300	390	620
Prostate cancer	Incidence	540	660	820	1250
	Mortality	200	250	320	580

Adapted from (Parkin et al. 2001)

The most important distinction among lung cancers is that between a non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), due to their differences in biological and clinical characteristics. SCLC is a clinically aggressive tumor that accounts for 20% of all lung cancers, with patients presenting with extensive disease and hence is rarely amenable to surgery with curative objective. Meanwhile, NSCLC can be further subdivided into three histologic subtypes, namely adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Chang et al. 2004, Mollah et al. 2009, Saeed & Anderson 2011). The major clinicopathologic features for each type of lung cancer are listed in Table 1.2.

Prognosis for lung cancer patients is dependent on the anatomical staging and histological classification of the tumor as well as clinical factors. SCLC is a rapidly growing tumor that commonly spreads to the extra thoracic region at the time of

Table 1.2: Histological subtypes of NSCLC and SCLC and their clinical features

Tumor	Location	Features	Comments
Adenocarcinoma (45%)	Peripheral (75% cases)	Lymph node metastases are common; Differentiation from secondary tumors may be difficult	Bronchoalveolar carcinoma is a subtype: grows along alveolar septae, often synchronous, multifocal lesions
Squamous cell (30%)	Located centrally near hilum or major bronchi (70% cases)	Prominent endobronchial component; Propensity for local invasion	Most likely to produce malignant cells on sputum cytology; Usually associated with a history of heavy smoking
Undifferentiated large cell (5-10%)	Peripherally located	Poorly differentiated tumors often cavitate	Poor prognosis: early spread to distant sites (e.g. brain and mediastinum are common)
Small cell (oat cell) (20%)	Central (80% cases)	Chest X-ray – classically large central tumor with bulky mediastinal lymphadenopathy; Can express neuroendocrine neurotransmitters, hormones or paracrine regulators; Adrenocorticotropic hormone (ACTH) and antidiuretic hormone (ADH) commonly overproduced; Most frequently associated with preneoplastic syndromes	Clinically aggressive tumors with high rates of cell proliferation and tendency to early dissemination; Usually associated with a history of heavy smoking; Majority of patients present with extensive disease: median survival 8-12 months

Cited from (Saeed & Anderson 2011)

diagnosis. This type of tumor has dismal prognosis and even the minority SCLC patients who have prolonged survival are at a high risk to develop a second malignancy. Meanwhile, squamous carcinoma has the most optimistic prognosis compared to other NSCLCs, mostly attributed to its localization to the chest. This is followed by adenocarcinoma, which can be grouped into bronchoalveolar and adenosquamous carcinomas. Generally, large cell carcinoma has a poor prognosis (Beadsmoore & Screaton 2003, Zhang et al. 2009).

1.1.2 BREAST CANCER

According to the cancer statistics, breast cancer is the second most common cancer in the world with 999,000 new cases and 375,000 deaths each year (Parkin et al. 2001). It is the leading cause of morbidity and mortality in women, not only in developed countries but increasingly also in developing countries. Several studies have shown that the number of cases in North America and North Europe is considerably higher than in Asia, the Far East, African, and South America (Jo et al. 2005, Kanaan et al. 2009). The high incidence of breast cancer could be due to various important factors, including differences in the endogenous reproductive and hormonal factors, particularly high levels of free estradiol. Hence, the risk to develop breast cancer increases with early menarche, late age at first birth, low parity, as well as late menopause. Besides that, the risk also increases proportionally with the aging process, but it slows down when a woman reaches approximately 50 years that mostly represents the onset of menopause characterized by a change in hormonal environment involving a decrease in estrogen levels. Lifestyle and environmental factors such as diet also play a role in increasing the risk, as a large weight gain after the age of 18 years has been shown to be a strong independent risk factor. Alcohol consumption has also been shown to amplify the risk (Parkin et al. 2001). On top of that, genetic predisposition may confer

susceptibility of an individual to breast cancer, especially among the relatives of young breast cancer cases. Several genes presenting susceptibility to breast cancer include BRCA1 on chromosome 17q, BRCA2 on chromosome 13q, germline mutations in TP53 gene as well as polymorphism of genes involved in estrogen metabolism such as cytochrome P450c17α, 17β hydroxysteroid dehydrogenase 1 gene, and the estrogen receptor gene (Parkin et al. 2001, Kanaan et al. 2009).

One of the complexities of breast cancer is due to the presence of distinct classes of tumors that have distinct proliferative responses towards hormonal signals as well as other environmental cues. Among the breast cancer patients, approximately 70% of them express estrogen receptor (ER) and hence, are responsive to the estrogen-dependent progression of the disease (Al-Dhaheri et al. 2006, Jump et al. 2008). ER is classified in the nuclear receptor superfamily of ligand-dependent transcription factors and is activated via binding of estrogen that leads to the initiation of breast cancer cells' proliferation (Al-Dhaheri et al. 2006).

Breast cancer is curable if it is detected at an early stage. Since estrogen plays a major role in breast cancer development and growth in the estrogen responsive breast cancers, they can be controlled with adjuvant therapies that act either directly such as non-steroidal antiestrogen tamoxifen, or indirectly such as aromatase inhibitors. Effects of tamoxifen depend greatly on the specific promoter, cell, and ER subtypes (Al-Dhaheri et al. 2006). Recent improvement in therapy and diagnosis greatly increases the survival chances of women with estrogen-dependent breast cancer. Unfortunately, breast cancer cells can eventually survive and develop a resistance to the drug despite the fact that the tumors initially did respond to the chemotherapy (Yang et al. 2006). Contrarily, the treatment options available for estrogen-independent tumors are not as satisfactory, therefore, having a poorer prognosis. The only available treatment options

for estrogen-independent breast cancer mainly involve surgery, general chemotherapy, and radiation therapy (Kanaan et al. 2009).

1.1.3 TNM STAGING

In cancer medicine, the main concern for patients and physicians involved prognostication of recovery or survival from the disease. In order to do so, the endpoints were often correlated with the anatomical extent of the tumor. Hence, the surgeons from the early days proposed the staging concept that is done by retrospective analysis of a large number of patients' scale with a certain tumor and its demographic, clinical, anatomical, radiological, and pathological characteristics (van Meerbeeck 2001). The TNM classification was first proposed by Denoix in 1946 as an anatomical basis to unify staging. The T component of the classification expresses the extent of the primary tumor in terms of size and local invasion. Meanwhile, the N component denotes the involvement of regional lymph nodes and the M component informs the presence or absence of metastases. Various combinations of T, N, and M define different clinical or surgical-pathological stages (IA-IV) characterized by different survival characteristics (van Meerbeeck 2001, Beadsmoore & Screaton 2003, Saeed & Anderson 2011). An ideal cancer staging is critically important to provide an accurate and reproducible description of the extent of the disease that could be readily communicated to other people. Besides that, it allows the selection of an optimal therapeutic approach, whether it is surgical or non-surgical, curative or non-curative. The pathological stage also gives the most precise information for estimation of the prognosis as well as calculation of the result of a treatment (van Meerbeeck 2001, Gross et al. 2011).

For lung cancer, there is a precise classification developed for NSCLC while a relatively broader classification was used for SCLC. The latest version of the International system to stage lung cancer was adopted by the American Joint Committee

for Cancerstaging (AJCC) and Union Internationale Contre le Cancer (UICC) in 1997 (van Meerbeeck 2001). The various procedures used to diagnose and stage lung cancer can be divided into invasive (transbronchial needle biopsy, transthoracic needle biopsy, mediastinoscopy, and open lung biopsy) or non-invasive (sputum cytology, chest computed tomography, positron emission tomography, and magnetic resonance imaging) (Saeed & Anderson 2011). The most critical lung cancer stage is stage 4 or T4, whereby any size of the lung tumor that has invaded into the mediastinum, heart, great vessels, tracheas, esophagus, vertebral body, and/or carina, the tumor presents with malignant pleural and/or pericardial effusion, as well as the presence of satellite tumor nodules within an ipsilateral primary tumor (van Meerbeeck 2001, Saeed & Anderson 2011).

Similarly, the TNM classification was created to allow recognition of breast cancer stages by indicating the extent of local, regional, and general extension of the disease at the time of primary treatment. However, the previous classification failed to relate the size of the primary tumor, degree of axillary involvement, and the indicators for endocrine responsiveness (Veronesi et al. 2006). Therefore, the new TNM staging systems of breast cancer introduced at the beginning of last century (Guth et al. 2007) has considered skin invasion as a morphologic parameter. This is because patients with the classical picture of breast cancer are often characterized as having visible tumors of considerable size that often cause entire breast deformation, as well as distant metastases. In the current TNM classification, all breast carcinomas with skin involvement are grouped together under T4 category, which relates to tumor of any size with direct extension to chest wall or skin (Guth et al. 2007).

1.2 MULTISTEP CARCINOGENESIS

Cancer cells grow out of our normal body cells through carcinogenesis, the process in which cancer is generated because of somatic mutations accumulation in a single cell over a period that causes a gradual phenotypic change from a normal to a preneoplastic cell, which moves on to become neoplastic (Russo 2007). Carcinogenesis is often considered as an active phenomenon induced in a living organism through various chemical or physical agents that can be categorized into four rather distinct groups: chemical, radiation, biological, and genetic. Both biological and genetic carcinogenesis are mostly due to the informational macromolecules (DNA or RNA) carried by the carcinogens. Radiation carcinogenesis is attributed to the direct or indirect action of high-energy photons or particles, while chemical carcinogenesis usually causes a permanent chemical change in the DNA structure. Table 1.3 shows some representative examples of carcinogens for individual categories with their respective range of molecular masses (Pitot & Dragan 1991).

Nevertheless, passive or spontaneous carcinogenesis may also occur in organisms without any active introduction of carcinogenic agents, although there is not a distinction between the two types of carcinogenesis (Pitot & Dragan 1991). This is because DNA damage is a common phenomenon that occurs daily in a living cell due to the replication machinery that does not have true 100% efficiency. Substantially, our body has certain complex mechanisms to detect and repair this DNA damage, or trigger its own suicide program if the DNA damage is too severe. However, occasionally the cell’s repair mechanism fails to correct the DNA damage and does not trigger the cell's suicide apparatus, hence giving rise to a constant low level of spontaneous mutation (Minamoto et al. 1999).

Extensive experimental observations in carcinogenesis have showed that this process can be divided into three distinct steps which require different lengths of time to accomplish, namely initiation (days), promotion (several years), and progression (1-5 years) (Klaunig et al. 2011).

Table 1.3: General classification of carcinogenic agents

Class	**Examples**	**Relative molecular mass (Da)**
I. Chemical	Polycyclic hydrocarbons, aromatic amines and halides, diet, hormones, metals, and polymer surfaces	$5 \times 10^1 – 5x\ 10^4$
II. Radiation	Ionizing (X and γ ray, particle radiation) and ultraviolet radiation	$<<< 1\text{-}1^+$
III. Biological	Viruses (papova, herpes, retro, and hepadna viruses)	$3 \times 10^6 – 170 \times 10^6$ (viral genomes)
IV. Genetic	Transgenic by enhancer-promoter-oncogene constructs; selective breeding	$\sim 10^6 - 10^8$

Cited from (Pitot & Dragan 1991)

Initiation is the first step of the process involving an irreversible change in the initiated cells as a result of mutation in a critical gene (Avila et al. 2004). Some of the most harmful cancer-causing mutations are mutations in the genes that regulate DNA repair and apoptosis, which enhance cancer survival rate (Minamoto et al. 1999). These genetic damages can be divided into two categories: (1) a dominant mutation involving mainly proto-oncogenes, which normally results in a gain of function and (2) a recessive mutation involving tumor suppressor genes, growth suppressor genes, recessive oncogenes, or antioncogenes that normally cause a loss of function. Tumor suppressor genes are genes whose normal function is to inhibit the cell cycle. Cancer will develop once both copies of this gene have lost their function resulting in the release of the cell cycle from inhibition (Avila et al. 2004, Sullivan & Lu 2007, Anisimov 2009). The

efficiency of initiation step is often associated with the cellular replicative DNA synthesis and cell division process (Pitot & Dragan 1991). At this stage, the initiated population does not or produces minimal observable changes in their appearance and they will not develop into a tumor without additional stimulus (Vincent & Gatenby 2008). Nevertheless, they are already at an advantage as compared to normal cells, permitting them to evolve towards fitter and more tumorigenic phenotypes. These mutant cells will increase slowly in number and line the basement membrane for an indefinite length of time (Vincent & Gatenby 2008).

This is followed by the promotion step that is constantly modulated by various environmental factors, depending on the frequency of the promoting agent administration, age, composition and amount of diet. The major distinct characteristic of promotion that distinguishes it from other stages of carcinogenesis is its operational reversibility. The prior initiation event usually will not be tumorigenic without the pressure by secondary environmental agitations such as wounding, inflammation, hypoxia, and acidosis, which produce a harsh condition that increases the selection pressure for the growth of the passively initiated cells (Pitot & Dragan 1991, Hennings et al. 1993). One example would be the limitation of nutrients for the population of initiated cells during promotion stage, hence resulting in their growth reaching a plateau state. Therefore, they begin to evolve by adapting to the tissue growth constraints that allows them to switch to anaerobic metabolism, hence promoting a phenotypic change that becomes increasingly insensitive to the proliferation constraints as well as with heritable changes in oncogenes and tumor suppressor genes (Vincent & Gatenby 2008, Klaunig et al. 2011).

Although the mutated cells continue to grow after initiation and promotion stage, intact basement membrane causes them to remain malnourished as the nutrients and oxygen must diffuse over a long distance to the deeply buried blood vessels resulting in

severe hypoxia of the cells (Vincent & Gatenby 2008). At the same time, the lactic acid accumulated from the aerobic and anaerobic glycolysis is not eliminated, causing an acidic extracellular environment (Helmlinger et al. 2002). This therefore enhances the fitness of the mutated cells that have been adapted to their own acid-induced toxicity that normally kills a large number of normal cells. Due to the rapid death of these normal cells, the mutated cells are accessible to a larger amount of nutrients. As the tumor increases in size, it has the ability to breech the basement membrane that confers to its invasiveness (Vincent & Gatenby 2008). This progression stage is irreversible mostly due to the unstable alterations of the cell's genome and its evolution, leading to the final stage of carcinogenesis with six essential alterations in cell physiology. They collectively describe the malignant growth, including evasion of cell death, infinite replicative potential, sustained angiogenesis, self-sufficiency in growth signals, insensitivity to antigrowth signals, as well as tissue invasion and metastasis (Pitot & Dragan 1991, Russo 2007).

A complete carcinogenic process will give rise to two types of neoplasms or tumors depending on their characteristics. Tumors that only proliferate and enlarge at their site of origin are benign tumors. This type of tumor lacks the ability to spread to other parts of the body and is usually not harmful to their host. Besides that, cells in the benign tumor are well-differentiated resembling the normal cells, encapsulated with a membrane, and grow at a slower rate. They rarely cause death of their surrounding tissue. They only cause a medical problem when its sheer bulk interferes with the normal bodily functions or when they secrete abundant biological substances such as hormones. Unlike benign tumors, malignant tumors that are better known as cancer are dangerous when they metastasize, start growing and dividing elsewhere in the body after they had overcome the strict growth factors and adhesive requirements. These cells are less differentiated with continuously changing properties, not membrane-bound, as

well as growing rapidly resulting in the destruction of the neighboring tissue (Darnell et al. 1986, Lee et al. 2011a).

1.3 HALLMARKS OF CANCER

Observations from human cancers and animal models suggest that the development of tumor occurs through a process similar to Darwinian's theory of evolution, where each succession of genetic changes contributes a particular type of growth advantage that leads to the progressive conversion of normal human cells into cancer cells (Hanahan & Weinberg 2000). Figure 1.2 depicts the six essential changes in cell physiology which collectively dictate tumor growth; infinite replicative potential, evasion of cell death, sustained angiogenesis, tissue invasion and metastasis, self-sufficiency in growth signals, and insensitivity to antigrowth signals (Hanahan & Weinberg 2000, Pietras & Ostman 2010).

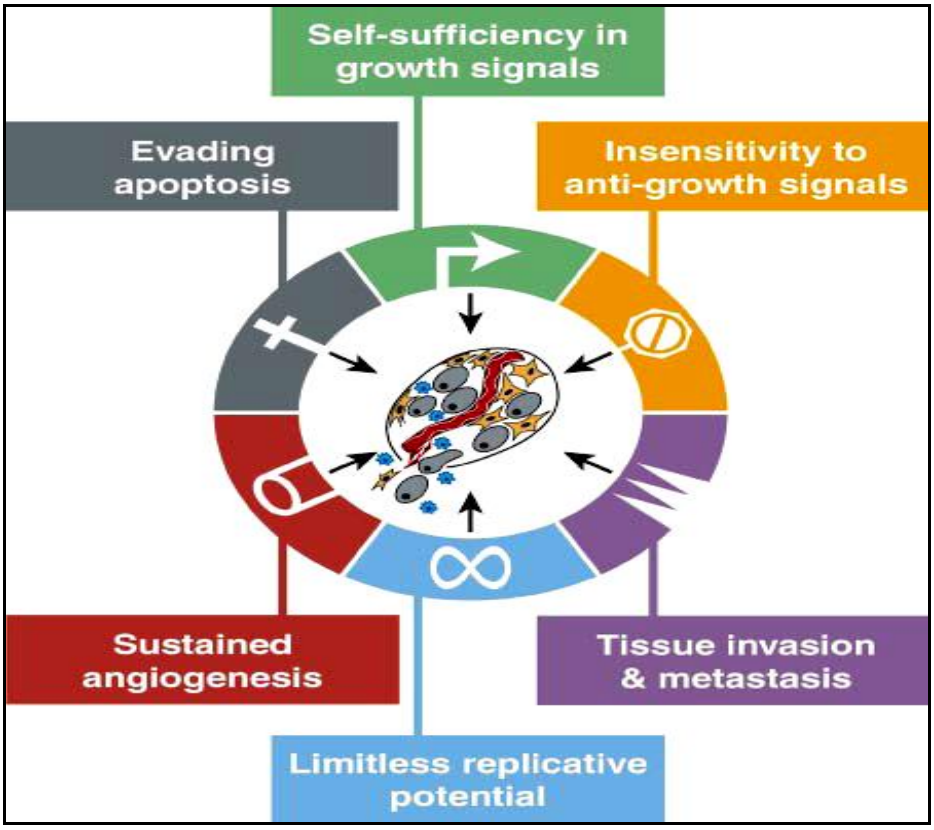


Figure 1.2: Acquired capabilities of cancer, cited from (Hanahan & Weinberg 2000)

1.3.1 INFINITE REPLICATIVE POTENTIAL

Normal cells are confined to a finite replicative potential, as their growth will be halted once they have undergone a certain number of doublings, a process known as senescence. The counting device that determines the number of cell divisions is the telomere (ends of chromosome) which is made up of several thousand repeats of short 6bp sequence element (Hanahan & Weinberg 2000). In the absence of telomerase that functions to replicate the 3' ends of chromosomal DNA during each S phase, the telomere is shortened after each cell division and once the telomere length has ended, the cell no longer has the ability replicate and will eventually die (Hanahan & Weinberg 2000, Anisimov 2009).

However, senescence does not seem to occur in cancer cells, hence suggesting that the evolving premalignant cell populations have breached the mortality barrier during the course of multistep tumor progression. One of the crucial processes in order for a cancer cell to become immortal is to constantly reactivate the telomerase activity that adds hexanucleotide repeats onto the ends of telomeric DNA to maintain the telomere length above a critical threshold (Hanahan & Weinberg 2000, Rha et al. 2000). Alternatively, cancer cells might activate a mechanism known as ALT which maintains telomeres length via recombination-based-interchromosomal exchanges of sequence information (Hanahan & Weinberg 2000). This in turn allows the unlimited multiplication of the descendant cells through a continuous series of events known as cell cycle.

Cell cycle is defined as a cell division process by which the two replicas of genome are partitioned into the daughter cell. This division process can be divided into five phases (Raven et al. 2004) as shown in Figure 1.3. G_1, S, and G_2 are grouped together as interphase, forming the portion of the cell cycle between cell divisions. G_1 is

the primary growth phase that comprises the major portion of the cell's life span. S is the phase in which the cell synthesizes a replica of the genome. G_2 is the second growth phase, in which preparations are made for genomic separation (Weinberg 2007). Meanwhile, M (for Mitosis) is the essential phase of the cell cycle for separation of the two daughter genomes (Raven et al. 2004). Each subsequent phase is activated only if the previous stage has been properly progressed and completed where the cells that have temporarily or reversibly stopped dividing will usually enter a quiescent state known as G_0 phase (Pecorino 2005).

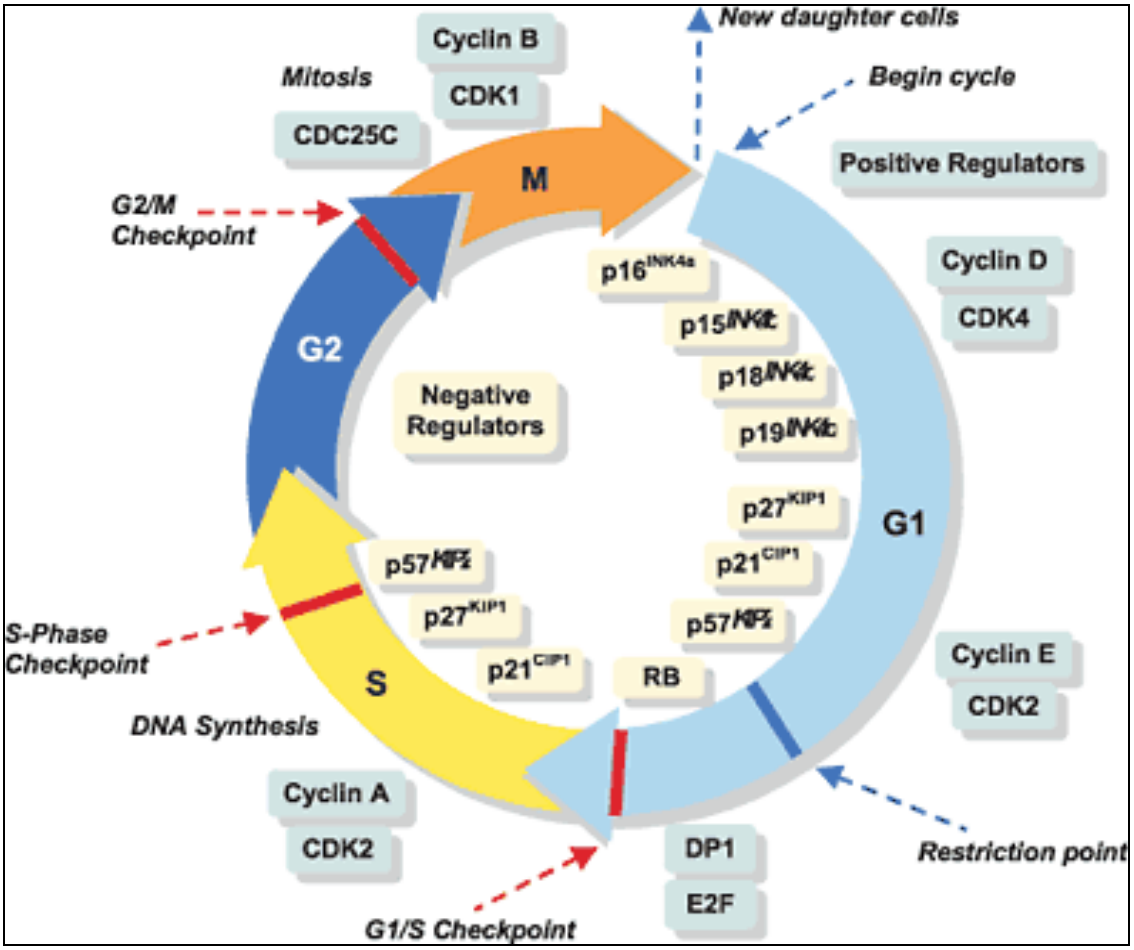


Figure 1.3: Cell cycle process, cited from (Kong et al. 2003)

Normally, the cell cycle is tightly regulated to prevent uncontrolled cell proliferation that may lead to various diseases. For instance, progress into mitosis will be halted during DNA replication, and sister-chromatid separation is delayed until all

kinetochores are attached to the spindle (Vogelstein & Kinzler 2002, Schönthal 2004). There are also three main checkpoints to assess the internal state of cell and integrate external signals, which are the G_1/S, G_2/M, and Spindle Checkpoint. The G_1/S checkpoint is the primary point at which the cell decides whether to divide, mainly governed by the Cyclin D/Cdk-4/6. The activated complex will increase phosphorylation of Rb protein that in turn releases bound transcription factors such as E2F-1 that subsequently activates genes transcription whose products are necessary for cells entry into S phase (Moon et al. 2000, Chou & Huang 2002). Meanwhile, G_2/M checkpoint, which is controlled by the Cyclin A/Cdk-2, assesses the success of DNA replication and can stall the cycle if DNA has not been accurately replicated. The last spindle checkpoint ensures that all of the chromosomes are attached to the spindle in preparation for anaphase. This process depends on the presence of Cyclin B/A/Cdk-2 (Raven et al. 2004, Schönthal 2004).

At the same time, inhibitory proteins, Cdk inhibitors (CKIs) also help to regulate Cdks activity throughout the cell cycle process. CKIs identified in mammalian cells can be broadly grouped into two categories. The first group consists of $p21^{WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$ that work by binding and inhibiting all the G_1 cyclin/Cdk complexes. Among this group, $p21^{CIP1/WAF1}$ is the key regulator of cell cycle arrest as it stops G_1/S transition in cell cycle by blocking complex formation between cyclins A to D1, D2, and E with Cdk2. On the other hand, the second group of CKIs that includes $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$ specifically inhibits Cyclin D/Cdk4 or Cyclin D/Cdk6 (Moon et al. 2000, Watson et al. 2008).

1.3.2 EVASION OF CELL DEATH

Besides bypassing the proliferation constraints imposed in a normal cell, cancer cell also has the ability to evade death. Accumulation of evidence from past researches

showed that the programmed cell death machinery is present in almost all the cell types of the body in a latent form. This program will unfold in a precisely choreographed order once it is stimulated by a variety of signals. The cell death mechanism is essentially divided into two groups of components: sensors and effectors. Sensors function to monitor both the extracellular and intracellular environment for any normality or abnormality conditions that may influence the survival of a cell. The information received will then be interpreted via regulation of the effectors of the cell death machinery (Hanahan & Weinberg 2000).

The resistance of cell death acquired by cancer cells is mostly attributed to the survival signals supplied by the stromal component because of several genetic alterations in cellular death pathway components (Sledge & Miller 2003, Pietras & Ostman 2010). For instance, when bcl-2 was co-expressed with a myc oncogene, the bcl-2 gene was found to be able to promote formation of B cell lymphomas via inducing lymphocyte survival by stimulating myc-stimulated proliferation. Besides that, p53 tumor suppressor protein (DNA damage sensor) which is a crucial member of the apoptotic signaling pathway was frequently inactivated, thus leading to a rapidly growing tumor with minimal number of dead cells (Hanahan & Weinberg 2000, Sledge & Miller 2003). Additionally, the survival signaling circuit such as PI3 kinase-AKT/PKB could be activated by a number of extracellular signals such as IGF-1/2 or IL3, intracellular signals originating from Ras, as well as loss of pTEN tumor suppressor that usually stops AKT survival signal. Stimulation of such survival pathways in turn mitigates cell death program in a substantial number of human tumors (Hanahan & Weinberg 2000).

The two fundamental modes of cell death in nucleated eukaryotic cells are apoptosis, which is also known as programmed cell death, and necrosis. Apoptosis is a Greek word that means dropping or falling of leaves from a tree. This term was coined

in 1972 by John Kerr, Andrew Wyllie and Sir Alistair Currie to explain the morphologic events during apoptosis, involving a controlled cell removal phenomenon which seemed to play a complementary but opposite role to mitosis in the regulation of animal cell population (Kerr et al. 1972). Meanwhile, necrosis is derived from the word “oncos” that means swelling, in accordance with cell swelling which is a characteristic feature occurring during necrosis (Proskuryakov et al. 2003). Apoptosis is a genetically regulated process of cell death that is ATP-dependent and it requires time to take place after the initial insult. In contrary, necrosis is an ATP-independent process and it occurs rapidly after the initial insult. Hence, the intracellular ATP concentration is an important factor in the selection of the pathway of cell death. Both of these modes have distinct morphological and biochemical features (Kiechle & Zhang 2002, Woo et al. 2008).

1.3.2.1 APOPTOSIS

Apoptosis, a highly organized physiological process, plays an important role to eliminate superfluous, aged, and damaged cells without inducing local inflammatory response that would likely damage adjacent cells. During apoptosis, the undesired cells will undergo several biochemical changes to prepare themselves to be phagocytized by macrophages. Ingestion of the cellular components by the macrophages therefore avoids the leakage of noxious proteolytic enzymes or reactive oxygen metabolites which otherwise would harm surrounding cells (Vinatier et al. 1996, Yang et al. 2006, Manu & Kuttan 2008). Apoptosis is also important during embryonic development and adult tissue homeostasis to remove unwanted and potentially harmful cells such as self-reactive lymphocytes that may cause autoimmune diseases during development of reproductive organs and for remodeling of vascular pattern (Vinatier et al. 1996). In addition, apoptosis also plays a crucial role as a protective mechanism against certain pathological conditions such as cancer (Del Bello et al. 2004, Choi et al. 2006). Induction of apoptosis is a highly desirable mode as a therapeutic strategy in cancer

control. Currently, most of the chemopreventive agents exert their anticancer activities by inducing apoptosis in order to suppress tumor promotion and progression (Chen et al. 2004, Kim et al. 2005).

Several remarkable stereotyped morphologies are the outstanding features of apoptosis. They include plasma membrane blebbing, loss of cell volume, loss of positional organization of organelles in the cytoplasm, breakdown of nuclear DNA with subsequent nucleosomal fragmentation, followed by cleavage of nucleus and cytoplasm by calpains into multiple membrane-enclosed bodies known as apoptotic bodies containing chromatin fragments (Taraphdar et al. 2001, Yang et al. 2006, Manu & Kuttan 2008, Lee et al. 2011a). A highly complex biochemical process involving various events that trigger the activation of the cellular machinery of apoptosis often accompanies these morphological characteristics (Taraphdar et al. 2001, Yang et al. 2006). Nevertheless, activation of catabolic caspases seem to be the one that is directly accountable for the various molecular and structural modifications during apoptosis.

Upon activation of effector caspases, the phosphatidylserine (PS) which normally resides at the inner leaflet of the plasma membrane is translocated to the outer leaflet. Since the apoptotic cell debris will eventually be phagocytized by macrophages or surrounding cells, this translocation is necessary to facilitate their recognition by the macrophages (Kiechle & Zhang 2002). The first structural or regulatory cellular protein targeted by effector caspases includes poly ADP-ribose polymerase (PARP), a DNA repair enzyme which is cleaved from its full length of 116kDa into an inactive 85kDa form. At the same time, the effector caspases activate the inactive caspase-activate deoxyribonuclease by removing its inhibitor. Without the presence of PARP, the nuclear DNA will be cleaved at inter-nucleosomal sites resulting in the production of DNA fragments with 180-200 bp. These DNA fragments show a typical “DNA ladder” configuration when they were analyzed using agarose gel electrophoresis (Vinatier et al.

1996, Kiechle & Zhang 2002). Meanwhile, fragmentation of cells to multiple membrane-bound apoptotic bodies is due to the cleavage of cytoskeletal elements and membrane proteins by calpains, a calcium-binding and thiol-containing enzyme (Taraphdar et al. 2001).

The activation of apoptosis can be divided into three phases, which are initiation, execution, and degradation. Apoptosis can be initiated by a variety of environmental and physiological stimuli, such as growth factor deprivation, ionizing radiation, chemotherapeutic drugs, ultraviolet radiation, as well as activation of cell death receptor. Activation of this programmed cell death will then trigger the signalling for apoptosis which may occurs via several independent pathways, depending whether the initial assault is triggered from outside of the cell [death receptor or extrinsic pathway] or within the cell [mitochondrial or intrinsic pathway] (Manu & Kuttan 2008). Nevertheless, apoptosis has several common "cytoplasmic regulator" irrespective of the biochemical pathways being initiated (Kiechle & Zhang 2002, Lee et al. 2011a). Figure 1.4 illustrates the regulation of the integrated apoptosis via both the intrinsic and extrinsic cell death pathways.

Activation of death receptor or extrinsic pathway involves the binding of a ligand to a death receptor resided on the cell's surface whereby apoptotic signals will be transmitted upon their ligation. This death receptors are grouped under the tumor necrosis factor receptor (TNFR) gene superfamily, which includes Fas(CD95), tumor necrosis factor receptor type 1 (TNF-R1), and TRAIL-receptor (Chen et al. 2004, Hung et al. 2010). Activation of these receptors by their respective ligands will stimulate the oligomerization of their intracellular death domain resulting in the recruitment of signal transducing molecules, namely TNFR 1-associated death domain protein (TRADD) and Fas-associated death domain (FADD). Consequently, they will activate the procaspases-8 and -10 to form a death-inducing signalling complex (DISC), which in turn leads to

the activation of the caspases. The activated caspases-8 and -10 will then cleave and activate other downstream procaspases, triggering the caspase cascade that eventually lead to the apoptotic cell death (Kiechle & Zhang 2002, Hung et al. 2010).

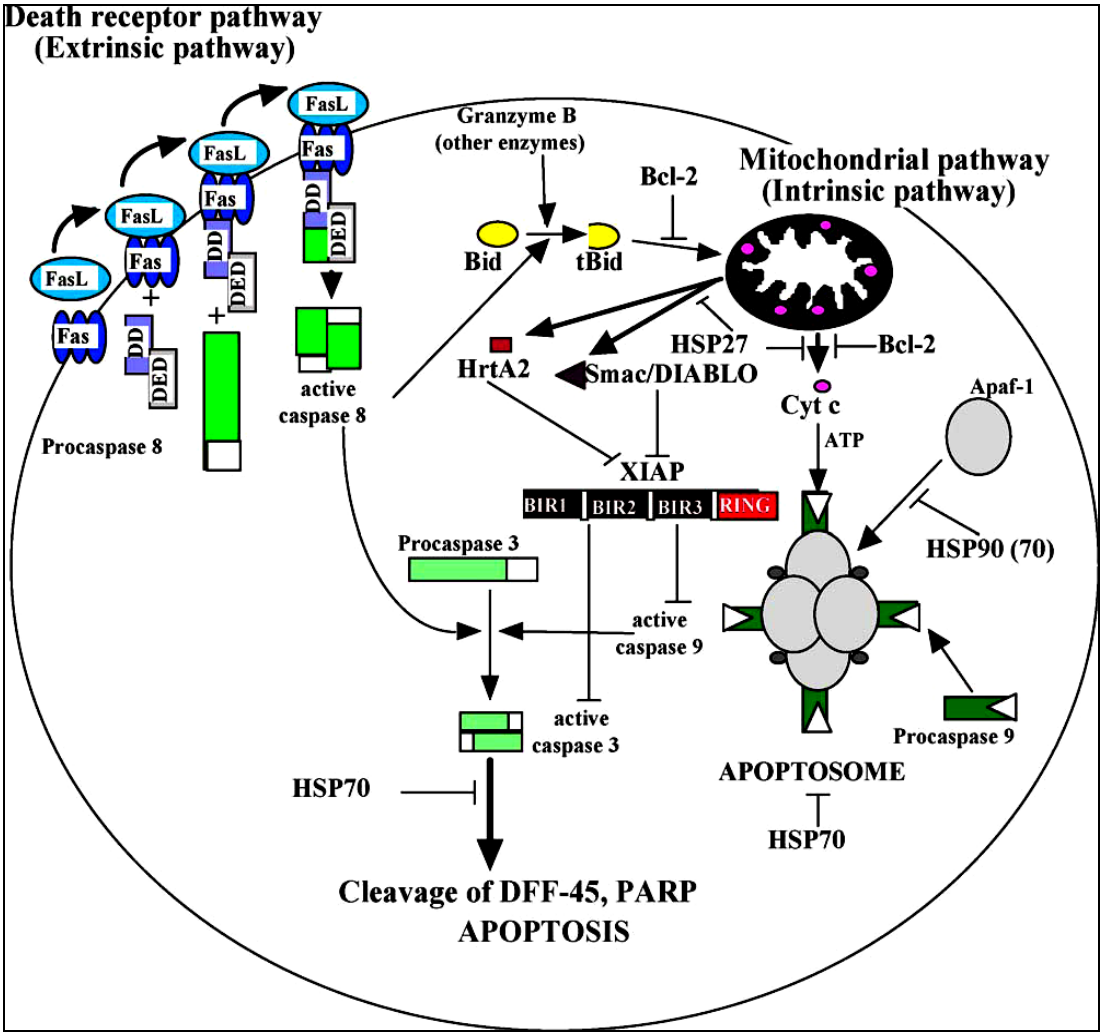


Figure 1.4: Regulation of apoptosis via intrinsic and extrinsic cell death pathways, cited from (Oliver & Vallette 2005)

Nonetheless, activated caspase 8 may also induce apoptosis by cleaving Bid (Kiechle & Zhang 2002), the only molecule which forms a link between death receptor pathway and mitochondrial pathway. Cleavage of the cytoplasmic Bid (p21) forms a truncated form, tBid (p15) which then associates with another Bax-related protein such as Bak to induce the release of cytochrome c. It in turn activates the initiator procaspase-9 into caspase-9 that subsequently proteolytically initiates the caspases

cascade which includes downstream effector caspases such as caspases-3 and -7, leading to the death of cell (Kuo et al. 2006).

Comparing with the extrinsic pathway, amplification of the death signals induced by a variety of stimuli via the mitochondrial or intrinsic pathway occurs most frequently in majority of the cell lines to induce apoptosis. With regard to this pathway, mitochondria act as the central control point for apoptosis by releasing apoptogenic or death-promoting proteins which are normally confined to the intermembrane space of mitochondria, including cytochrome c, Smac/DIABLO, apoptosis-inducing factor (AIF), and endonuclease G (Kiechle & Zhang 2002, Wu et al. 2009). These mitochondrial proteins are normally released as a response to an increase in the inner mitochondrial transmembrane potential due to opening of the permeability transition pore (PTP) during permeability transition (PT) process. As a consequence of the megachannel pores opening, there is an osmotic swelling of the matrix owing to its hypertonic condition. Since the inner membrane is made up of numerous cristae contributing to a large surface area, its expansion upon matrix swelling breaks the outer mitochondrial membrane. This in turn triggers the release and irreversible dilution of the intermembrane content into the cytoplasm (Desagher & Martinou 2000, Huang et al. 2009).

Apoptosis is said to be time-consuming due to the delay between the change in the inner mitochondrial membrane potential and the release of cytochrome c which usually takes approximately 5 minutes upon the initiation of the process (Kiechle & Zhang 2002). Once cytochrome c is released into the cytoplasm, it interacts with apoptotic protease-activating factor-1 (Apaf-1). This complex in turn undergoes self-oligomerization in the presence of dATP or ATP through the CED-4-like domains. Subsequently, this complex further binds with two procaspase-9 molecules, forming a complex known as apoptosome to begin caspase-9 auto-activation via “proximity-

induced action". This activated caspase-9 will then activate other downstream effector caspases, including caspases-3 and -7, which cleave a variety of cellular proteins and cause cell death (Del Bello et al. 2004, Lv et al. 2008). As for the remaining apoptogenic proteins, Smac/DIABLO acts mainly as a caspase activator by interacting with the inhibitors of apoptosis proteins (IAPs) to interfere with their ability to inhibit the caspase enzymes (Kiechle & Zhang 2002). Meanwhile, AIF and endonuclease G works in concert to directly cause chromatin condensation and DNA fragmentation to induce apoptotic morphological changes (Wu et al. 2009).

1.3.2.2 NECROSIS

Theoretically, it is desirable for most chemotherapeutic agents to induce apoptosis as the main mode of cell death in the cancer cells. In practical terms however, it may not always occur this way for some of the drugs. Some of the chemotherapeutic drugs such as cladribine, cisplatin, and doxorubicin have the ability to activate both the apoptotic and necrotic cell death pathways (Tang et al. 2010, Lee et al. 2011a). Moreover, both apoptosis and necrosis share some common pathways and hence, have the possibility to occur concurrently (Proskuryakov et al. 2003, Woo et al. 2008).

Necrosis has a different set of defined morphological characteristics as compared to apoptosis. This includes an early phase mitochondrial and organelle swelling, cytoplasmic swelling, mottled chromatin condensation, loss of membrane asymmetry, loss of plasma membrane integrity, followed by leakage of intracellular cytoplasmic constituents and rapid cell lysis which eventually result in an induction of inflammation (Suin et al. 2008). Unlike apoptosis that could be induced by a variety of stimuli, necrosis is usually induced by severe external damage resulting in accidental cell death via unregulated processes of membranes, cytoplasmic structures, and nucleus destruction. Therefore, necrosis is often thought as a form of passive cell death

(Proskuryakov et al. 2003). Nevertheless, some of the TNF receptor family such as TNF, FAS, and TRAIL are able to initiate both the apoptotic and necrotic cell deaths. An example would be the induction of the necrosis-like cell death via Fas-FasL system in the presence of caspase inhibitors that evokes the mitochondria swelling. Binding of TRAIL ligand to death receptors (DR4 and DR5) can also induces caspase-independent necrosis (Proskuryakov et al. 2003, Ohno et al. 2008). Another protein which also specifically triggers necrotic cell death is BNIP3, a member of the Bcl-2 family (Proskuryakov et al. 2003).

Necrotic membrane damage can be divided into four events: (1) activation of phospholipases (such as phospholipase A2) which degrade the phospholipids into free fatty acids (such as arachidonic acid) and lysophospholipids, (2) surfactant effect of these products and other amphipathic lipid species, (3) disruption of the membrane-associated cytoskeletal scaffold, as well as (4) direct attack by reactive oxygen species (ROS), free radicals, and lipid peroxides (Raffray & Cohen 1997). Meanwhile, DNA degradation during necrosis usually occurs randomly whereby caspase-independent DNase I and II are probably involved, resulting in a 'smear' formation on agarose gels (Proskuryakov et al. 2003, Lee *et al.*, 2011). Other possible explanations could be due to the pH-associated chromatin changes that aid non-enzymatic disintegration of high order structures. This is because intracellular acidification or acidosis has been known as a common change in cells undergoing necrosis due to oxidative phosphorylation inhibition, lactic acid and inorganic phosphate build-up, as well as end-stage release of acidic groups by degradative enzymes (Raffray & Cohen, 1997).

1.3.3 TISSUE METASTASIS AND SUSTAINED ANGIOGENESIS

Tissue invasion and metastasis eventually occur as tumor cells progress into a malignant state, as the primary tumor masses develop the capability to invade adjacent

tissues as well as basement membrane and hence travel to distant locations via lymphatic or blood vessels to form new colonies. In order to do so, they need to acquire a migratory phenotype as well as extensively remodelling the surrounding extracellular matrix and blood vessels (Hanahan & Weinberg 2000, Sledge & Miller 2003, Pietras & Ostman 2010).

The major alteration in cell-to-environment interactions in cancer involves E-cadherin whose function is lost mainly as a result of mutational inactivation of E-cadherin or β-catenin genes, transcriptional repression, or proteolysis of extracellular cadherin domain (Hanahan & Weinberg 2000, Pietras & Ostman 2010). Another parameter for invasive and metastatic cells is their ability to secrete extracellular proteases such as the matrix metalloproteinases (MMPs) which degrade the basement membrane and surrounding stroma. Expression of these protease genes is normally upregulated while the expression of the protease inhibitor genes is downregulated. On top of that, inactive zymogen forms of these proteases will be stimulated and converted into active enzymes. In addition to MMPs, some tumors may also produce other proteases such as membrane-associated urokinase-type plasminogen activator (uPA) which binds to the urokinase receptor (uPAR) expressed on the cancer cells (Hanahan & Weinberg 2000, Sledge & Miller 2003).

The proteases expression is also crucial during vascular remodelling. Normal cell function and survival depends very much on the adequate supply of oxygen and nutrients by the vasculature. This necessity obligates almost all cells in a tissue to grow within 100μm of a capillary blood vessel (Hanahan & Weinberg 2000, Sledge & Miller 2003). Hence, the developing neoplasias must acquire the ability to stimulate angiogenesis in order for them to grow into a larger tumor size (Sledge & Miller 2003). Tumors that are well-vascularized have the capability to expand locally and metastasize but avascular tumors grow only within a diameter of 2-3 mm (Song et al. 2008). A

common strategy to alter the angiogenic switch involves a modification of gene transcription to increase the expression of vascular endothelial growth factor (VEGF), which is the most potent angiogenic peptide (Hanahan & Weinberg 2000, Sledge & Miller 2003). Other examples of angiogenic peptide is basic fibroblast growth factor (bFGF1/2) and inducible nitric oxide synthase (iNOS). Alternatively, the expression of endogenous inhibitors such as thrombospondin-1 or β-interferon can be downregulated to induce angiogenesis. Suppression of plasmin (a pro-angiogenic component of the blood clotting system) that has the ability to cleave itself to form angiostatin, an angiogenesis inhibitor, also increases angiogenesis. The bioavailability of these angiogenic inducers and inhibitors can be regulated by a variety of proteases stored in the extracellular matrix (ECM) (Hanahan & Weinberg 2000).

Malignant tumors are often fatal due to the ability of the cancer cells to invade other tissues and spread to other organs. For the majority of the cancer patients, metastasis to the regional lymph node or distant organs has occurred by the time of diagnosis of the primary tumor, hence it is responsible for 90% of cancer-associated mortality (Folgueras et al. 2004, Yang et al. 2008, Chaffer & Weinberg 2011). Metastasis of a cancer cell from its primary tumor must accomplish the following steps in the correct sequence for a successful dissemination (Figure 1.5): reduced adhesion ability via epithelial-mesenchymal transition (EMT), damaging intercellular interaction and detachment from their matrix, secretion of proteolytic enzymes for matrix degradation, invasion of surrounding tissues and blood vessels, translocation via circulatory or lymphatic stream to microvessels of distant tissues, extravasation, survival, and adaptation to the microenvironment of distant tissues, and finally recruitment of blood vessels for nourishment to establish a secondary tumor (Nelson et al. 2003, Yang et al. 2008, Bourboulia & Stetler-Stevenson 2010, Chaffer & Weinberg 2011). Throughout this entire interrelated sequential events, a metastasizing cell may

face the possibility of being eliminated from the process if there is failure in any of the steps (Xie & Huang 2003, van Zijl et al. 2011).

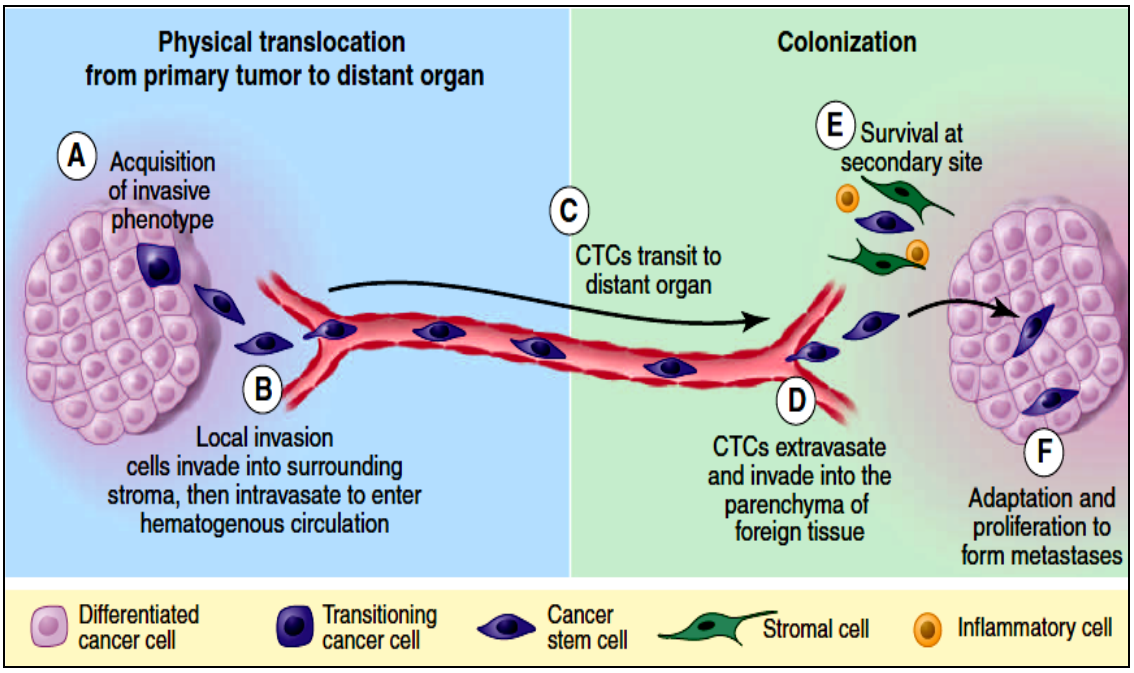


Figure 1.5: The metastatic cascade, cited from (Chaffer & Weinberg 2011)

1.3.3.1 EPITHELIAL-TO-MESENCHYMAL TRANSITION

The population of cells within a malignant tumor is hierarchically structured, with self-renewing cancer stem cells, progenitor cancer cells, and fully differentiated end-stage cancer cells. Cancer stem cells have superior tumor-initiating potential as compared to other cancer cells within a tumor. Features such as motility, invasiveness, and self-renewal that are essential in a malignant tumor could be owing to the inherent capabilities of the cancer stem cells subpopulation within a larger population of neoplastic cells (Chaffer & Weinberg 2011). However, epithelial-to-mesenchymal transition (EMT) has been shown to possess the potential to induce those non-cancer stem cells into cancer stem cell-like state by conferring polarized, epithelial cells with traits that enable them to detach from primary tumors and metastasize. An example would be a heightened resistance to apoptosis, a critical criterion of carcinoma cells to survive the harsh environments throughout their journey from their primary tumor to

dissemination locations for initiation of new cancer cells colonies (Chaffer & Weinberg 2011). Besides that, EMT is characterized by epithelial loss with a concurrent gain of mesenchymal gene expression program. During this process, there would be a reduction or loss of epithelial E-cadherin, whose function is to bind its extracellular domain to an E-cadherin molecule of the neighbouring epithelial cells to stabilize cell-to-cell contact (Spaderna et al. 2006, Jang et al. 2011). Meanwhile, successive up-regulation of mesenchymal markers such as vimentin and neuronal-cadherin (N-cadherin) cause the rearrangement of cytoskeleton as well as formation of lamellopodia and filopodia. This cadherin switch (E-cadherin to N-cadherin) leads to a more efficient movement of EMT-transformed cells after detaching from their epithelial cell clusters that allows them to move like a single mesencymal-like cells (Palmer et al. 2011, van Zijl et al. 2011).

1.3.3.2 INVASION

Although not all tumor cells have the ability to detach from its primary tumor and disseminate into blood or lymphatic circulation, this is a pre-requisite for metastasis. Therefore, EMT provides these cells with an upper hand for invasion with cytopathological changes which no longer constraint them to contact inhibition so that they are capable to cross barriers that are non-permissive for normal cells (Hsiao et al. 2007, Palmer et al. 2011). Tumor cells that are located closely to a blood vessel have the brightest chances to disseminate since they can easily access the on-site growing vessels with immature vascular structure and high interstitial pressure (Xie & Huang 2003). Cancer cells that grow far apart from the blood vessels will have to go through a complex maneuver of invasion (Hsiao et al. 2007).

Invasion of cancer cells may occur either by moving collectively as epithelial sheets or detached clusters, or as single cells in the form of mesenchymal or amoeboid

cell types (Painter et al. 2010, van Zijl et al. 2011). Invasion of tumor cells usually incorporates cell attachment to the basement membrane with succeeding proteolytic degradation of the barrier to allow migration through the extracellular matrix (Hsiao et al. 2007). This is achieved via production of a specific type of protease, namely matrix metalloproteinase (MMP) by cancer and stroma cells into the peritumoral environment to degrade the extracellular matrix. On top of that, they also synthesize different cytokines that form a positive feedback loop to enhance the MMP expression. The concentration of these MMP proteins is often associated with more advanced tumor stage and depth that involve lymph node and distant metastases (Lukaszewicz-Zajac et al. 2011).

1.3.3.3 MOTILITY

Although epithelial cells are generally stationary, tightly interconnected sheets of cells, they can also be mobilized depending on their developmental differentiation, environmental stimuli and surrounding tissue architecture. This is because cell motility is another fundamental process occurring while an embryo develops, repairing of wound, and during inflammatory responses (Ho et al. 2010, Palmer et al. 2011). The migration of adherent cells is described as the movement of cells from one location to another and it involves five processes, namely polarization, protrusion, adhesion, cell body translocation and finally rear retraction. In order to do so, integration of numerous molecular mechanisms is crucial so that a tumour cell can direct the configuration of new adhesions while disengaging existing adhesions and concomitantly applying force to move the cell body (Palmer et al. 2011).

The role of motility in the evolution of a metastatic phenotype is supported by evidence of the primary tumor re-seeding itself in the context of circulating tumor cells (Palmer et al. 2011). Many cancer patients with advanced primary carcinomas contain

circulating cancer cells in their blood that are believed to be in transit to their future sites of metastasis (Chaffer & Weinberg 2011). Although blood circulatory system is considered as the main mode for metastasis, the lymphatic system can also claim a role during metastatic spread, as lymph capillaries are only thin-walled single layers of endothelial cells that lack inter-endothelial tight junctions and basement membrane. Therefore, it provides an easier means of access for the metastasizing cancer cells (van Zijl et al. 2011). Different routes of dissemination may pose different fates for the migrating cell. In contrary to pulmonary dissemination, portal vein dissemination would provide the cells a greater chance of survival since the circulation is much slower and its microvessels lack extracellular matrix, offering a less shear-force stress condition and easier extravasation (Xie & Huang 2003).

Nonetheless, various factors in both the disseminating tumor cells and host may cause the death of the circulating cells. First, the tumor cells might die quickly while circulating because of non-specific factors such as hemodynamic turbulent forces. Besides that, the presence of T-lymphocyte cells, natural killer cells, endothelial cells and macrophages activity may destroy the blood-borne tumor emboli. In addition, intravascular death of disseminating tumor cells could also be due to oxygen-derived free radicals such as superoxide anion (Xie & Huang 2003). Therefore, highly metastatic cells must acquire the ability to survive the challenges of the blood stream in terms of physical constraints and host immune system. Enhanced homotypic tumor cell aggregation such as platelet-cancer cell association and formation of tumor embolus could be the survival factor for the disseminating tumor cells (Xie & Huang 2003, Laubli & Borsig 2010, Chaffer & Weinberg 2011, van Zijl et al. 2011).

1.3.3.4 ATTACHMENT AND LOCALIZATION

Circulating tumor cells that have survived transport must arrest as a function of hemodynamic properties vasculature and identify endothelium of various tissues (Stafford et al. 2008). Although mechanical forces are important for tumor cells' delivery to secondary sites, not all of these circulating cells present in the vessels of various organs would give rise to metastatic disease. Stephen Paget has hypothesized in 1889 a "seed-to-soil" theory explaining a disseminating cancer cell as a seed that grows only if it had found an appropriate organ or soil at a secondary site (Kauffman et al. 2003, Palmer et al. 2011). A successful engraftment and growth at a clinically relevant metastatic site is dependent on the receptive microenvironment that is normally optimized to create a "landing dock" before the arrival of the cancer cells. This process is known as the pre-metastatic niche formation that explains the tendency of solid tumors to home preferentially at distinct organs (Peinado et al. 2011). For example, lung and brain are the preferred metastatic sites for melanoma while bone is the preferred metastatic site for prostate carcinomas. This pre-metastatic niche usually attracts the disseminating tumor cells by releasing factors such as SDF-1, S1000A8, and S100A9 (Chaffer & Weinberg 2011, Peinado et al. 2011). Otherwise, tumor cells can release factors such as VEGF-A, PIGF, and PSAP that educate specific distant sites to prepare before their arrival (Chaffer & Weinberg 2011).

Cells that have completed all metastatic steps will eventually extravasate through the endothelial walls into the organs' parenchyma and proliferate to colonize ectopic tissue. Tumor cells have developed various ways for extravasation: (1) inducing endothelial cell's death or retraction, (2) generating reactive oxygen species resulting in an irreversible damage to the endothelial cells, (3) inducing angiogenesis or vascular remodeling, and (4) stimulating proliferation of arrested tumor cells intravascularly to

grow into large metastases without undergoing extravasation that eventually erupts through the basement membrane (Stafford et al. 2008, Shibue & Weinberg 2011).

Once the tumor cell has extravasated, tumor-host interactions within the target organs' parenchyma became the major contributing factor for a successful colonization of the metastatic cell (Shibue & Weinberg 2011). This includes the ability of tumor cell to trigger mitogenic stimulation from growth factors and cytokines (Chaffer & Weinberg 2011), vascularization and immune surveillance (Shibue & Weinberg 2011). The surviving metastatic cells can exist in one of the three alternative forms: (1) as solitary viable cancer cells in a quiescent, non-proliferative dormant state, (2) actively growing macrometastatic lesions allowing net increases in cell number, and (3) remaining as a small lesion due to a balance between proliferation and apoptosis (Chaffer & Weinberg 2011, Shibue & Weinberg 2011).

1.3.3.5 PROTEOLYTIC ENZYME – MATRIX METALLOPROTEINASES

Basement membrane degradation has been found to be a crucial process during metastasis for migration and invasion of cancer cells. This process is normally associated with overexpression and activation of proteolytic enzymes that provide an access for cancer cells into the vascular or lymphatic system, constituting a getaway route for distribution. More than 500 genes that encode for proteases or protease-like proteins were discovered in human genome (Folgueras et al. 2004). However, the main proteolytic enzymes involved in tumor invasion include the members of matrix metalloproteinase (MMP) family that have the potential to cleave almost all components in the extracellular matrix and basement membrane (Folgueras et al. 2004, Ho et al. 2010, Kim et al. 2010).

MMPs are grouped in a family of zinc-dependent endopeptidases that degrades a variety of proteins such as fibrillar and non-fibrillar collagens, fibronectin, laminin, as

well as glycoproteins in the extracellular matrix and basement membrane by cleaving their internal peptide bonds (Bourboulia & Stetler-Stevenson 2010, Lukaszewicz-Zajac et al. 2011). The human MMPs family comprises of 26 endopeptidases that can be subdivided depending on their cellular localization, either secreted or membrane bound, as well as based on their sequence homology and substrate specificity (Figure 1.6).

Similar to other proteolytic enzymes, MMPs are synthesized as inactive enzymes or zymogens which require additional activation steps to form a functional MMP form. Most of the MMPs are activated outside the cell, requiring participation of other proteases such as serine proteinases, furin, plasmin, and others (Verma & Hansch 2007, Lukaszewicz-Zajac et al. 2011). An example would be urokinase-type plasminogen activator (u-PA), a serine proteinase that converts plasminogen into plasmin that in turn cleaves and activates MMPs (Hsiao et al. 2007).

Among the various MMPs, MMP2 and MMP9 are greatly associated with tumor invasion and metastasis, and can be attributed to their capability to degrade the type-IV collagen, the main component in basement membrane. Both these MMPs work via different mechanisms, as MMP2 promotes cleavage of extracellular matrix proteins while MMP9 alters vascular endothelium permeability (Hsiao et al. 2007, Yang et al. 2008, Lukaszewicz-Zajac et al. 2011). Other roles of MMPs include regulation of tumor growth by favoring release of cell proliferation factors such as insulin-like growth factors, as well as targeting and activating growth factors whose precursors are attached to cell surface or sequestered in the peritumor extracellular matrix (Folgueras et al. 2004).

Under normal conditions, MMPs activities are maintained at low levels and are tightly monitored at several steps, namely transcriptional (through AP-1 or NF-κB via mitogen activated protein kinase (MAPK) or PI3K/Akt pathways), post-transcriptional,

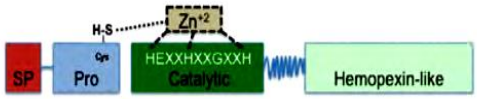
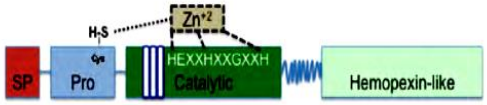
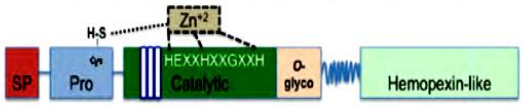
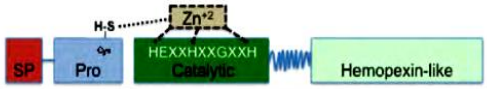
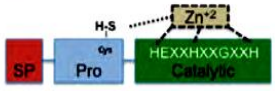
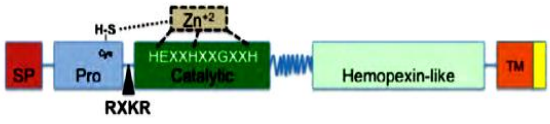
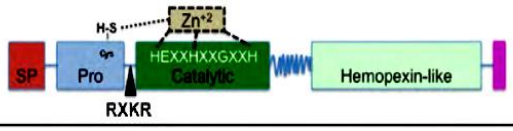
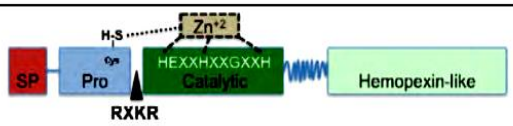
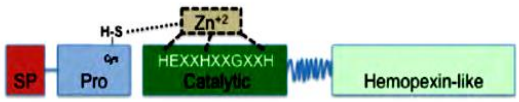
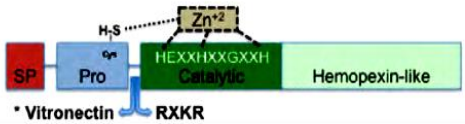
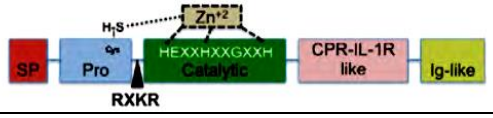
MMP Member	Common name/ Synonym	Cellular Localization	Domain structure
MMP1	Collagenase-1/ Interstitial collagenase	Secreted	
MMP8	Collagenase-2/ Neutrophil collagenase		
MMP13	Collagenase-3		
MMP2	Gelatinase A/ 72kDa gelatinase		
MMP9	Gelatinase B/ 92kDa gelatinase		
MMP12	Macrophage metalloelastase		
MMP7	Matrilysin/ Uterine		
MMP26	Matrilysin-2/ Endometase		
MMP14	MT1-MMP	TM type I	
MMP15	MT2-MMP		
MMP16	MT3-MMP		
MMP24	MT5-MMP		
MMP17	MT4-MMP		
MMP25	MT6-MMP/ Leukolysin		
MMP3	Stromelysin-1/ progelatinase	Secreted	
MMP10	Stromelysin-2		
MMP11	Stromelysin-3		
MMP28	Epilysin		
MMP18	Collagenase 4		
MMP19	RASI-1		
MMP20	Enamelysin		
MMP27			
MMP21	MMP-23A		
MMP22	MMP23B	TM type II	

Figure 1.6: Matrix metalloproteinases (MMPs) domain structures and functions, cited from (Bourboulia & Stetler-Stevenson 2010)

post-translational, and through the involvement of their endogenous inhibitors (TIMPs). Tissue inhibitors of metalloproteinases (TIMPs) can be found in most tissues and body fluids. Each TIMP molecule consists of one structural and one functional domain, known as the N- and C-terminals. The N-terminal region acts as the main inhibitor of all MMPs by interacting with the enzyme's catalytic domain. Meanwhile, the C-terminal region contains at least two binding sites for enzyme as well as for interaction with pro-forms of MMP2 and MMP9 to stabilize the complex (Lukaszewicz-Zajac et al. 2011). Four known human forms of TIMPs have the capability to inhibit almost all MMPs since they do not seem to differentiate much between various MMPs (Folgueras et al. 2004, Snoek-van Beurden & Von den Hoff 2005). TIMPs inhibit MMPs activity mainly by forming a MMP/TIMP complex in a 1:1 stoichiometric pattern (Snoek-van Beurden & Von den Hoff 2005, Lukaszewicz-Zajac et al. 2011). Regulation of the MMPs promoters and inhibitors affect the balance of matrix degradation or synthesis that subsequently determines the invasive potential of cancer cells (Curran & Murray 2000, Snoek-van Beurden & Von den Hoff 2005, Lee et al. 2010, Lukaszewicz-Zajac et al. 2011).

1.3.4 SELF-SUFFICIENCY IN GROWTH SIGNALS AND INSENSITIVITY TO ANTIGROWTH SIGNALS

Cell proliferation is strictly regulated during growth, differentiation and subsequent life of the normal cells as they only move from a quiescent state into an active proliferative state when the mitogenic growth signals (GS) are present (Hanahan & Weinberg 2000, Sledge & Miller 2003). Nevertheless, cancer cells have escaped the normal growth control through mutations that lead to an overexpression of differentially regulated growth factors, hormones, and cytokines such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), stromal derived factor (SDF)-1α and

interleukin-6 (IL6) (Sledge & Miller 2003, Pietras & Ostman 2010). Besides that, many of the oncogenes in cancer cells act by mimicking normal growth signaling. They produce various growth factors that they themselves are responsive towards, hence removing their dependence for growth stimulation from their normal tissue microenvironment (Hanahan & Weinberg 2000). Overexpression of receptors is another key factor that enables the cancer cells to be hyperresponsive towards ambient levels of growth factors that otherwise would not stimulate cell proliferation. Besides that, cancer cells also have the capability to selectively express their favourable extracellular matrix receptors (integrins) which transmit progrowth signals (Hanahan & Weinberg 2000, Sledge & Miller 2003).

On the other hand, many and perhaps most of the anti-proliferative signals are channeled through retinoblastoma protein (pRb) and its two relatives, p107 and p130. pRb normally blocks proliferation by altering the function of E2F transcription factors that govern genes expression critical for G_1 progression into S phase. Disruption of this pRb pathway in cancer cells will lift its control on E2Fs and thus allowing cell proliferation, rendering the cells become insensitive to anti-growth factors (Hanahan & Weinberg 2000).

Therefore, activation of the signaling machineries by different growth signals or stimuli could either cause an induction or inhibition effect in different cell types and even within the same cell. This is because the protein components for the cell death program were already expressed in the cells and are maintained in association with their respective inhibitors (Vinatier et al. 1996, Kiechle & Zhang 2002). Similarly, each of the metastatic processes are dependent on various factors, broadly grouped into two categories known as metastasis promoters and metastasis suppressors (Shevde & Welch 2003). The balance and complex interplay of both the metastasis promoters and suppressors in each step will determine whether a tumor cell can establish a successful

distant metastasis (Iiizumi et al. 2008). Hence, analysis of a large network of upstream complex signaling pathways that control cell death and metastasis remains to be proven as a routine tool for clinicopathological assessment (Williams & Stoeber 2007).

1.3.4.1 TUMOR SUPPRESSOR, P53

The p53 is a well-known tumor suppressor protein that is also known as the "guardian of the genome". It protects cells against numerous physiological stresses via relay of signals through the p53 signaling network into the nucleus (Dey et al. 2010). The p53 gene is mapped on the short arm of human chromosome 17, encoding for a 53kDa protein that consists of 393 amino acids (Shu et al. 2007, Machado-Silva et al. 2010). The p53 signaling pathway is made up of p53 gene with its downstream genes which responds to various intrinsic and extrinsic stimuli to control cell survival, DNA damage repair, chromosome segregation, metabolic adaptation, cell senescence, aging and cell death (Shu et al. 2007, Hock & Vousden 2010, Solozobova & Blattner 2010). P53 protein functions are controlled by its post-translational modifications that affect its stability and transcriptional activity. Hock and Vousden depicts the outcomes of p53 protein after several modifications such as neddylation, sumoylation, and ubiquitination (Hock & Vousden 2010). Other modification processes include acetylation, methylation, and phosphorylation (Brooks & Gu 2011).

Since the presence of p53 has a negative effect in cell proliferation, their levels are usually kept low in a normal healthy cell by rapid degradation through ubiquitin-proteasome pathway. One of the key regulators would be human double minute 2 (Hdm2), the predominant and crucial E3 ubiquitin ligase which mediates p53 ubiquitination via a RING domain by ubiquitinating p53 at six key lysine residues (K370, K372, K373, K381, K382, and K386) present at the C terminal of the protein. Working in a negative feedback loop, p53 drives the transcription of Hdm2 that

stimulates its degradation to maintain its low level at times of normal homeostasis (Hock & Vousden 2010, Solozobova & Blattner 2010, Brooks & Gu 2011).

In response to DNA damage, ribosomal stress, hypoxia, or oncogene activation, p53 level will be stabilized to allow its accumulation through various mechanisms (Ryan 2011). Firstly, Hdm2 has the ability to target itself for degradation via autoubiquitination after inactivation of its own E3 ligase activity (Hock & Vousden 2010). Besides that, phosphorylation of p53, enzymatic process of p53 acetylation at ubiquitination sites, as well as binding of tumor suppressor p14ARF to Hdm2 also prevents Hdm2-p53 interactions (Brooks & Gu 2011).

Once p53 proteins are activated, they bind to their p53 responsive element to initiate one of the three transcriptional programs, including cell cycle arrest, DNA repair, or cell death. One of the primary target genes is the cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ which interacts and inhibits with cyclin-dependent kinases, resulting in phosphorylation of retinoblastoma protein (pRb) that leads to cell cycle arrest (Shu et al. 2007, Ryan 2011). The p53 has also been implicated in DNA repair by initiating repair mechanisms such as nucleotide excision repair (NER), base excision repair (BER), non-homologous end-joining (NHEJ) and homologous recombination (HR) (Shu et al. 2007). However, if the damage is beyond repair, the cell will be subjected to programmed cell death whereby p53 may activates both the intrinsic and extrinsic cell death pathways (Ryan 2011).

Nevertheless, the loss of p53 function is implicated in carcinogenesis and hence rendering tumor cells resistant to chemotherapy and radiotherapy. Contrary to other tumor suppressors that contain large deletion or frameshift mutations, p53 is normally altered via missense mutations in the conserved DNA binding core domain of the protein (Machado-Silva et al. 2010, Schilling et al. 2010). This results in the inability of

the p53 proteins to produce a proper response in the presence of any physiological stresses, leading to uncontrolled cancer cell growth and survival.

1.3.4.2 MAP KINASES

Protein phosphorylation is a crucial mechanism utilized by a large number of proteins that are mainly enzymes, receptors, transporters, docking and scaffolding proteins to control their activity that regulate a variety of biological processes from cell growth and differentiation to apoptosis and disease. The human genome may encode as many as 2000 protein kinases to phosphorylate amino acid residues serine, threonine and tyrosine present in proteins of various tissues, cells, and organelles (Farooq & Zhou 2004). The MAPK family consists of highly conserved proline-directed, protein-serine/threonine kinases that transduce extracellular signals or physical stresses such as osmotic shock, ischemic injury, radiation, growth factors, cytokines, and neurotransmitters via different receptor types for intracellular cell regulation. The wide range of cellular processes modulated by MAPK cascades include cell proliferation, differentiation, survival, division, movement, immune responses, development, and cell death (Farooq & Zhou 2004, Zohrabian et al. 2009, Porta et al. 2011). Transmission of the extracellular signals into their intracellular targets is mainly mediated through a network of interacting proteins, in a cascade module known as "the central three-tiered core" since it consists of three sequentially activating enzyme cascades of phosphorylation-dephosphorylation reaction. It begins with an active MAPK kinase kinase (MAPKKK such as Raf and mos) that activates MAPK kinase (MAPKK such as Mek and MKK) and subsequently activates a particular MAPK (such as ERK, JNK, p38, or p42). In this case, the terminal activated MAPK functions as an effector of a unique pathway that regulates the gene expression and protein activity to carry out a precise physiological response (Mutalik & Venkatesh 2006, Porta et al. 2011). Depending on the terminal MAPK activated, MAP kinases are divided into three major

subfamilies as shown in Figure 1.7, namely extracellular signal-regulated kinases (ERK-1/2), c-Jun N-terminal kinases (JNK-1/2/3) and p38 proteins (p38-α/β/γ/δ). Upon activation, various membrane-associated and cytoplasmic proteins are phosphorylated and translocated to the nucleus to activate specific transcription factors resulting in immediate gene transcription of important cellular proteins and cytokines (Farooq & Zhou 2004, Porta et al. 2011).

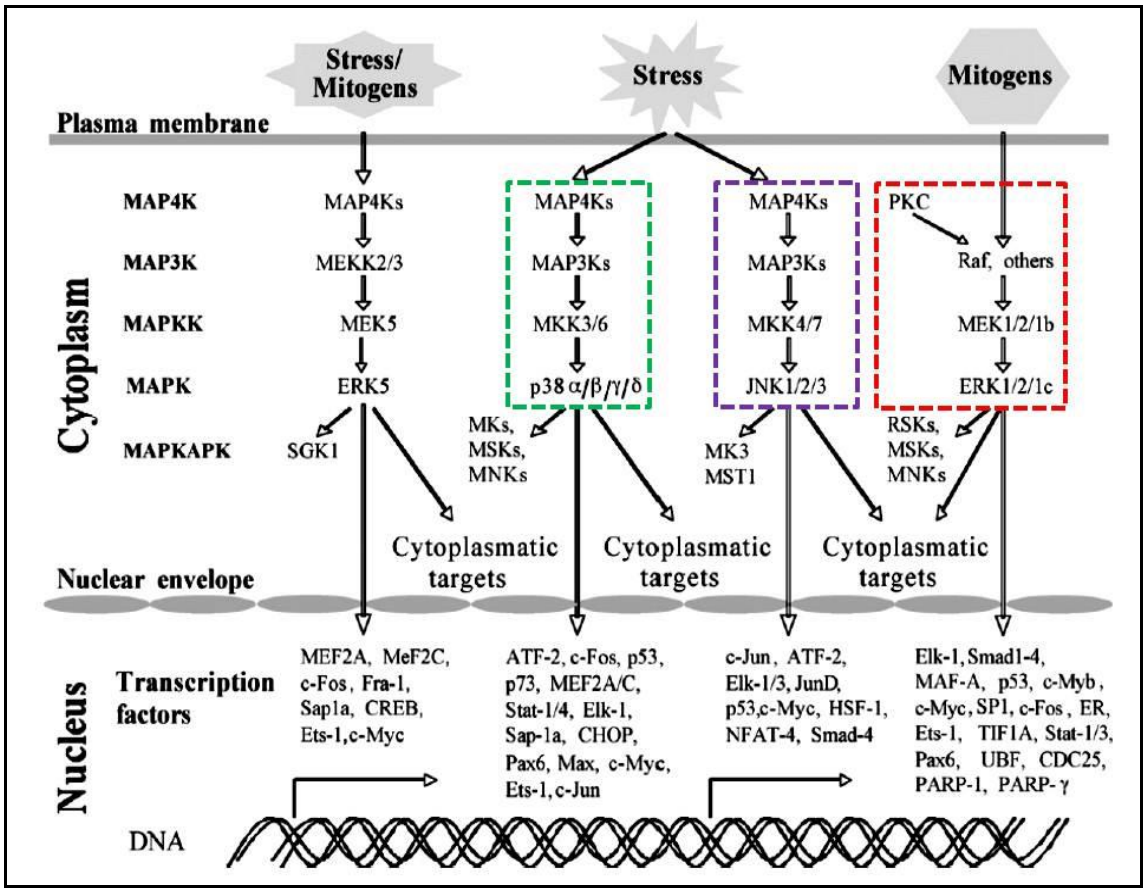


Figure 1.7: Schematic representation of MAP kinase cascade and their nuclear targets, cited from (Plotnikov et al. 2011)

The first MAPK pathway elucidated was extracellular signal-regulated kinases (ERK-1/2) cascade since ERK-1/2 proteins are ubiquitously expressed. ERK-1/2 phosphorylation cascade is normally initiated due to activation of GTPase Ras at the plasma membrane by insulin or other growth factors, leading to activation of the MAPKKK tier of the cascade (mainly Raf-1 and B-raf). The signal is then transmitted

to MEK1 and MEK2 (MAPKKs) via phosphorylation of two serine residues in their activation loop. Subsequently, ERK1 and ERK2 receive the signal and are activated via phosphorylation of the regulatory Thr and Tyr residues in the Thr-Glu-Tyr domain (Plotnikov et al. 2011). The final destination of the signal would be the MAPKAPK components (such as RSKs, MNKs, and MSKs) or other substrates localized either in cytoplasm, nucleus, or cellular organelles such as cytoskeletal (Porta et al. 2011). Mutations of signalling components upstream or within the cascade of this pathway have been observed in many cancer types (Plotnikov et al. 2011). Constitutive activation of this pathway leads to increased expression of transcriptional products such as cyclin D1 that resulted in cell cycle progression. In addition, genes that inhibit proliferation are suppressed, while genes involved in angiogenesis, migration, invasion, and metastasis are upregulated (Zohrabian et al. 2009).

c-Jun N-terminal kinases (JNK) was initially also named as stress-activated protein kinase (SAPKs) cascade since it is a mediator of intra- or extra-cellular stresses including UV irradiation, growth factor deprivation, and DNA damaging agents. It was found out later on that JNK pathway can also be activated in response to other stress-independent stimuli and growth factors such as cytokines (Plotnikov et al. 2011, Porta et al. 2011). Those stress and other stimuli transduce their signal via GTPases such as Cdc42 and Rac1, which subsequently activate “the central three-tiered core” (Plotnikov et al. 2011). JNK pathway is identified as the regulator for transcription factor c-Jun since activated JNKs phosphorylate and activate the N-terminus of c-Jun protein, hence stabilizing and increasing the activity of c-Jun as a transcription factor (Krens et al. 2006). Most of the JNK targets are nuclear hormone receptors and nuclear transcriptional regulators, including ATF2, NF-AT4, and p53, which in turn mediate cellular processes such as apoptosis, innate immune response after activation of various Toll-like receptor family proteins, neuronal activity, and insulin signalling.

Dysregulation of JNK has also been implicated in several cancer types (Plotnikov et al. 2011, Porta et al. 2011).

The p38 cascade is another SAPK pathway that can be stimulated by environmental stress and inflammatory cytokines. Upon activation of the receptors, the signals are transmitted to four isoforms of p38, each having specific functions that are determined by the specificity of the upstream activators as well as the identities and functions of the downstream substrates (Plotnikov et al. 2011, Porta et al. 2011). Alternatively, p38 can also be autophosphorylated in a MAPKK-independent manner, induced either by; (1) stimulated interaction with adaptor proteins Tab1, (2) phosphorylation of Tyr323 by ZAP-70, or (3) interaction with lipidic phosphatidyl inositol analogues (Plotnikov et al. 2011). Dysregulation of this cascade is usually noticed in pathological conditions such as inflammation, autoimmune diseases, neurodegenerative diseases, diabetes, as well as cancer due to cancer-associated inflammation or dysregulation of cell cycle (Krens et al. 2006, Plotnikov et al. 2011).

1.3.4.3 HYPOXIA INDUCIBLE FACTOR (HIF)

Hypoxia, defined as a state of reduced oxygen level below normal values, usually exists within solid tumors due to the disordered perfusion and unregulated growth (Joung et al. 2008, Kaluz et al. 2008, Manolescu et al. 2009). Although mild-hypoxia is considered pro-survival, prolonged hypoxia will lead to cell death. If the tumor cells survive the hypoxic insult, they may develop drug therapy resistance, DNA over-amplification, enhanced malignancy and metastatic potential (Ho et al. 2006, Sermeus et al. 2008).

The hypoxic condition normally activates hypoxia inducible factor (HIF) pathway which regulates post-translational modifications of the hypoxia inducible factor 1 (HIF-1) through two mechanisms, whereby the first controls the abundance of

HIF-1α and the second controls its transcriptional activity (Kaluz et al. 2008). HIF-1 is a heterodimeric transcription factor made up of two subunits, HIF-1α (120kDa) that is continuously produced and degraded in cells while HIF-1β (91-94kDa) presents in the cell irrespective of oxygen tension changes and therefore is important for hypoxia-induced transcriptional changes mediated by HIF-1 heterodimer (Lee et al. 2006, Kaluz et al. 2008, Klaunig et al. 2011). Oxygen dependent degradation of HIF-1 is regulated by prolyl hydroxylases (PHDs) in the presence of iron and 2-oxoglutarate and oxygen, whereby HIF-1 is hydroxylated either via capturing by von Hippel-Lindau (pVHL) through prolyl hydroxylation or preventing transactivator recruitment through asparaginyl hydroxylation (Maxwell 2005, Sermeus et al. 2008).

Elevated HIF-1 expression has been implicated in various human tumors with poor outcome, such as head and neck cancer, colorectal, nasopharyngeal carcinoma, breast, osteosarcoma, pancreatic and others. The signaling pathway is activated in response to reactive oxygen species (ROS) and other cellular oxidants level (Klaunig et al. 2011). This leads to stabilization of HIF-1α, which is an essential step so that it can assemble with HIF-1β to form a functional HIF-1 complex on the hypoxia responsive element (HRE) in the regulatory regions of its target genes to recruit different proteins that participates in the adaptation to hypoxic environment (Ruas & Poellinger 2005, Lee et al. 2006). Some of the adaptations induced by HIF-1 include: (1) stimulating protein synthesis to promote metabolic changes into anaerobic glycolysis in hypoxic tissues (Sermeus et al. 2008, Manolescu et al. 2009), (2) inducing neovascularization via transcriptional activation of a number of growth factors such as VEGF to ensure an adequate supply of oxygen (Manolescu et al. 2009, Ndubuizu et al. 2010), (3) increasing expression of transferrin receptor and transferring genes to replenish iron deficiency (Lee et al. 2006), and (4) increasing expression of drug export pumps such as MDR1 (Sermeus et al. 2008).

1.3.4.4 B CELL LYMPHOMA-2 GENE ENCODED PROTEIN (BCL-2)

Bcl-2 (B cell lymphoma-2 gene encoded protein) was first discovered in human B-cell lymphomas as a gene translocated from its normal position on chromosome 18 to chromosome 14 (Vinatier et al. 1996). It has been established to be a proto-oncogene since the translocation causes it to become an oncogene that helps to prolong cell survival by inhibiting apoptosis (Manu & Kuttan 2008). The various Bcl-2 proteins constitute a protein family consisting at least 22 members that have similar α-helical sequences, known as Bcl-2 homology (BH) domains (Martin & Vuori 2004). Bcl-2 family proteins play a pivotal role as the main regulators of apoptosis as well as for induction of caspase activation. Its family members can be grouped into two categories, whether they are proapoptotic or antiapoptotic molecules. They may contain up to four conserved Bcl-2 homology domains, from BH1 to BH4. Most of the antiapoptotic members of Bcl-2 family that includes Bcl-2, Bcl-X_L, Mcl-1, and Bcl-w possess sequence conservation in all four domains. On the other hand, the proapoptotic members of Bcl-2 family can be subdivided into two groups. The first group shares sequence homology from BH1 to BH3 domains but not in BH4, and it consists of Bax, Bak, and Bok. Whereas, the second group shares only sequence homology in BH3 domain and they are Bid, Bim, Bad, Bik, Bmf, Hrk, Noxa, Puma, BNIP3 and Spike (Komarasamy 2011).

The crucial function of Bcl-2 family members is their involvement in the direct control of mitochondrial membrane permeability that regulates the release of apoptosis-inducing factors from the intermembrane space into the cytoplasm (Jin et al. 2006). Bcl-2 is the key regulator among the family members that control cell homeostasis. It usually forms a partner with Bax (its death promoting partner) protein to constitute a "pre-set rheostat" within a cell, where the ratio of Bcl-2 to Bax often decides whether a cell receiving a death stimulus to accept or ignore it (Vinatier et al. 1996). If the

antiapoptotic Bcl-2 protein is in excess in the cell, it heterodimerizes with Bax and hence averts the conformational changes of Bax. Under these circumstances, the cell is able to survive (Hao et al. 2007). Another member of the Bcl-2 family that has a similar role is Bcl-X that is divided into two types, Bcl-X_L and Bcl-X_S after differential splicing. Both of these proteins form another competing pair, whereby excess amount of Bcl-X_S will result in cell death (Vinatier et al. 1996).

1.3.4.5 CASPASES CASCADE

The shift of the balance between Bcl-2 family proteins will in turn induce activation of cysteine-containing, aspartate-specific proteases known as caspases that form the central component of the apoptotic mechanism (Choi et al. 2006, Graf et al. 2007). Caspases are usually synthesized as pro-enzymes or zymogens that consist of an N-terminal pro-domain, linked with a small linker sequence to a 20kDa (p20) subunit followed by a 10kDa (p10) subunit (Graf et al. 2007, Zhu et al. 2007). Catalytically active caspase is normally released upon cleavage of the prodomain. To date, fourteen human caspases have been identified, with twelve caspases (caspases 1-10, 12, and 14) of human origin while caspase 11 is of bovine origin and caspase 13 is of murine origin. These human caspases can be subdivided into three groups, based on their prodomain structure, substrate specificity and functions (Oliver & Vallette 2005, Graf et al. 2007) as shown in Table 1.4.

The first group of the caspase family is involved in the maturation of cytokines, but their involvement in the apoptosis is not straightforward. Meanwhile, the other caspases who are participating in the induction and execution of cell death are grouped into initiator and effector caspases. The initiator caspases are made up of long prodomains with recognizable homotypic protein-protein interaction motifs, such as caspase recruitment domain (CARD) or a death effector domain (DED). These caspases

transduce various signals into proteolytic activity. In contrast to the initiator caspases, the effector caspases do not contain a large prodomain and lack intrinsic enzymatic activity. These procaspases are activated after proteolytic cleavage by initiator caspases into large and small subunits (Oliver & Vallette 2005, Graf et al. 2007). Figure 1.8 shows a schematic representation of the events leading to caspases activation.

Table 1.4: The caspase family and their functions

Group	**Function**	**Caspase**	**Optimal tetrapeptide sequence**
Group I	Inflammation	Caspase 1	WEHD
		Caspase 4	(W/L)EHD
		Caspase 5	(W/L)EHD
		Caspase 12	Not known
		Caspase 14	WEHD
Group II	Effector	Caspase 3	DEVD
		Caspase 7	DEVD
		Caspase 6	VEHD
Group III	Initiator	Caspase 2	DEHD
		Caspase 8	LETD
		Caspase 9	LEHD
		Caspase 10	LEXD
		Caspase 12	Not known

Adapted from (Oliver & Vallette 2005, Graf et al. 2007)

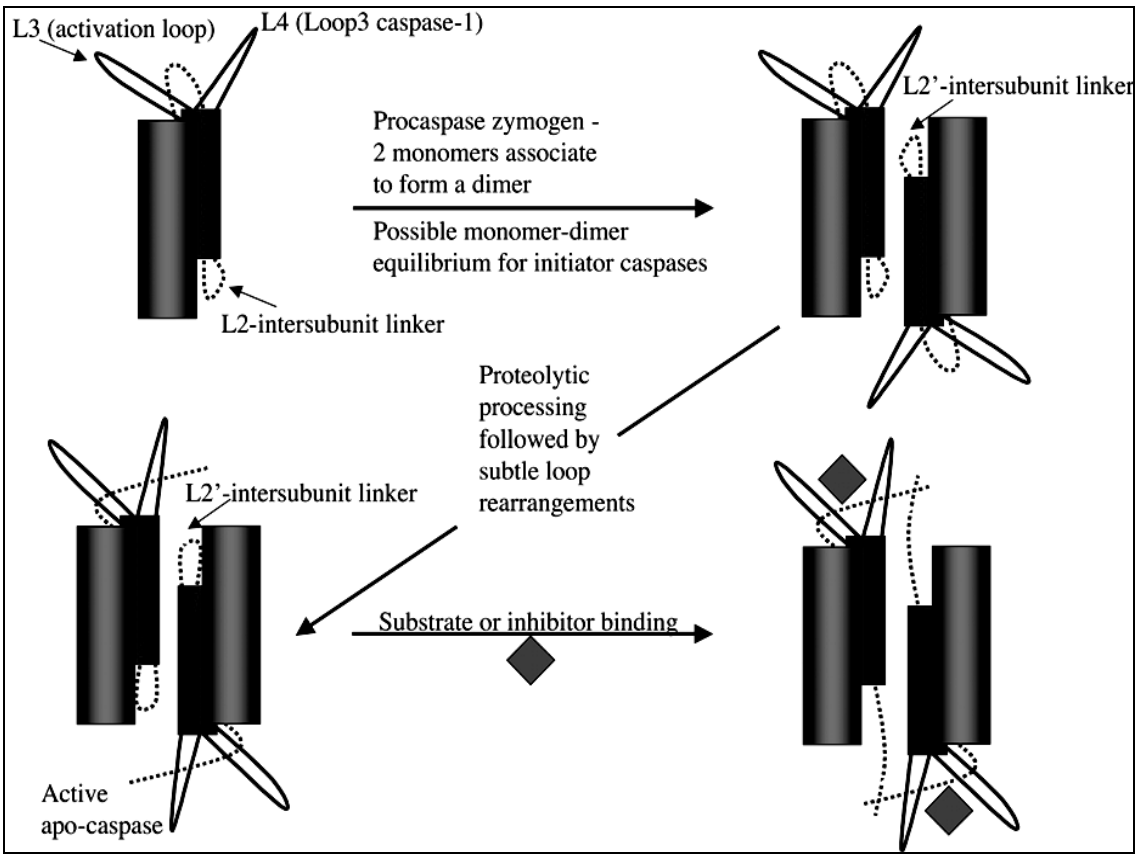


Figure 1.8: Schematic diagram of caspase activation, cited from (Donepudi & Grutter 2002)

1.3.4.6 C-MYC

The c-myc protein is a transcription factor made up of 439 amino acids, which regulates 15% of genes in genomes from flies to humans. Regulation of the transcription is done through several mechanisms, such as recruitment of histone acetylases, chromatin modulating proteins, basal transcriptional factors, and DNA methyltransferase (Dang et al. 2006, Wang et al. 2012). It usually heterodimerizes with its partner protein Max, forming a c-myc/Max complex which binds to a consensus enhancer box (E-box) DNA element to act as a transcription factor that regulates various aspects of cell behavior such as cell proliferation, cell cycling, apoptosis, adhesion, and tumorigenesis (Sanders & Gruppuso 2005, Cho et al. 2010).

Since c-myc has a role in cellular proliferation and growth, its expression is normally higher in proliferating cells and is downregulated during differentiation. This change in expression level is regulated at multiple stages, such as transcription initiation, elongation, mRNA stability, and protein stability (Sanders & Gruppuso 2005). However, genetic alterations of myc genes were discovered in abundant human malignancies and have been considered as one of the most frequently activated oncogenes involved in the initiation of several cancer types (Luscher 2001, Cho et al. 2010). Some of the consequences of myc proteins overexpression include: (1) promoting cell cycle progression, (2) increasing protein synthesis, (3) allowing neoplastic transformation of susceptible cell lines, (4) increasing glucose uptake and glycolysis, (5) up-regulating transferrin receptor (TFRC1), (6) enhancing nucleotide synthesis and metabolism, and (7) regulating stability or translational efficiency of target mRNAs (Luscher 2001, Dang et al. 2006).

1.3.4.7 ACTIVATED PROTEIN-1 (AP-1)

Activated protein-1 (AP-1) is a redox-sensitive basic leucine zipper (bZIP) transcription factor, hence regulating genes expression involved in cell differentiation, proliferation, migration, transformation, oxidative response, inflammation, apoptosis and immune response (Jang & Surh 2005, Vesely et al. 2009). It is a sequence-specific transcription factor consisting of either homodimers or heterodimers proteins of jun (c-jun, junB, junD), fos (c-fos, fosB, fra-1, fra-2), Jun dimerization partners (JDP1, JDP2), Maf (musculoaponeurotic fibrosarcoma), and the closely related activating factors subfamilies (ATF2, LRF1/ATF3, B-AT) (Jang & Surh 2005, Klaunig et al. 2011).

Activation of AP-1 is regulated by cis-elements in the promoters of AP-1-encoding genes and mediated mainly via the MAP kinase cascades (Vaiopoulos et al. 2010). One of the obvious effects of AP-1 activation involves an elevated cell

proliferation since both c-fos and c-jun are positive regulators of cell proliferation. This is due to the binding of AP-1 at its binding site in cyclin D1 promoter, hence activating cyclin-dependent kinase that promotes progression of cell division cycle. Alternatively, expression of cyclin-dependent kinase inhibitor, $p21^{WAF}$ that inhibits cell cycle is suppressed (Klaunig et al. 2011). In addition, overexpression of AP-1 also results in an increase of matrix metalloproteinase (MMP) expression, therefore enhancing the cellular invasion since they share a consensus AP-1 binding site at position-66 to -72 in their promoters (Ozanne et al. 2000). JunB, although it is another member of the jun family proteins, has a distinct function from c-jun due to its negative regulation of c-jun-induced cell proliferation activity by transcribing the cell cycle inhibitor, p16INK4A. Similarly, junD is also shown to inhibit intestinal epithelial cell proliferation via activation of p21 promoter and reducing tumor angiogenesis by protecting cells from oxidative stress. Therefore, AP-1 activation is greatly depending on the relative abundance and composition of AP-1 dimers, as well as cell types, stimuli, and cellular environment (Klaunig et al. 2011).

1.3.4.8 E2F

E2F proteins are implicated in cell cycle control, DNA licensing and synthesis, mitosis, DNA repair, and apoptosis due to the presence of E2F responsive site in the promoters of the genes. E2F expression is regulated via multiple mechanisms, mainly through the pocket protein interaction involved in the E2F cell cycle activity. The pocket protein family consists predominantly of retinoblastoma protein (pRb), as well as p107 and p130, all having the capability to regulate cell cycle progression via arresting cells in G1 phase when their expression are upregulated. This is possible as hypophosphorylated pocket proteins (p130) will bind themselves to E2F proteins (E2F4 and E2F5) rendering them inactive (Stevaux & Dyson 2002, Stevens & La Thangue 2004). An increased oncogenic capacity measured by unscheduled proliferation could

be due to; (1) higher E2F protein levels in tumor cell, as well as (2) higher extent of phosphorylated pRb proteins with E2F colocalization which suggest overexpression of E2F that corresponds to the free and active form (Tsantoulis & Gorgoulis 2005).

1.3.4.9 NFκB

NFκB is an ubiquitously expressed nuclear transcription factor that is generally known as an antiapoptotic factor (Manu & Kuttan 2008, Klaunig et al. 2011). NFκB is a dimeric transcription factor made up of homodimerization or heterodimerization of different proteins in the Rel family, including p50 (NFκB1), p52 (NFκB2), c-Rel, v-Rel, Rel-A (p65), and Rel-B (Klaunig et al. 2011). In majority of the cells, it consists of a heterodimer of p65 and p50 or p52 subunits that are the most frequent components of an active NFκB molecule. This is because the p65-containing complexes have the ability to bind to the consensus DNA sequences at p65/c-Rel with high affinity, resulting in transcriptional activation (Kiechle & Zhang 2002, Manu & Kuttan 2008). At normal conditions, these dimers are sequestered in the cytoplasm in an inactive form via binding to its inhibitory counterpart IκB proteins such as IκBa, IκBb, and IκBe (Kundu & Surh 2004, Klaunig et al. 2011).

NFκB can be activated by intracellular mediators in response to various external stimuli, including cytokines, oxidative stress, oncogenes, and DNA damage. For example, cytokines binding to their receptors will lead to interaction with TNF receptor-associated factors, hence activating the NFκB-inducing kinase through activation of Tat-associated kinase-1. Subsequently, activated NFκB-inducing kinase phosphorylates and activates IκB kinase which in turn phosphorylates two critical serine residues (Ser32 and Ser36) in the inhibitory NFκB binding protein IκB, leading to their degradation. Thus, the active NFκB will be released and translocated into the nucleus for binding on the κB-regulatory elements, modulating the expression of a number of

genes that sustain cell survival (Kiechle & Zhang 2002, Romano et al. 2004, Klaunig et al. 2011). NFκB activation has been implicated in various malignant cancers such as breast, colorectal, pancreatic adenocarcinoma, and T-cell leukemia due to its critical roles in: (1) growth factor mediated cell proliferation, (2) tumor cell invasion, (3) angiogenesis, (4) tumor cell metastasis, and most importantly (5) promoting cell survival by inducing antiapoptotic gene expression (Kiechle & Zhang 2002, Manu & Kuttan 2008, Klaunig et al. 2011).

1.4 CANCER TREATMENTS

Cancers that are left untreated ultimately results in serious illness and most often, death. Therefore, adopting appropriate conventional treatments such as surgery, radiation therapy and chemotherapy drugs can help to control most of these cancers (Jemal et al. 2008). Most of the time, these conventional therapies are used in compliment to each other as none of those therapies can be used as a standalone therapy for cancer due to their limitations (Avila et al. 2004).

Surgery has been the basis of treatment for patients with solid cancers. It was considered as having the central role when it concerns cancer management since it is often the most effective therapy to achieve cure. Surgery has a role in different aspects of cancer treatment (King & Primrose 2003). More than half of the people diagnosed with cancer will have some type of surgery or operation at some point as it can be used to remove tumours confined to a small space. Biopsy is a type of surgery, and is preferable for diagnosis of malignancy because more information can be gained from a core biopsy as compared to fine-needle aspiration cytology, hence allowing a more reliable diagnosis of invasive disease (Reed 2009). Besides that, surgery may also benefit cancer patients with gastrointestinal and pancreatic tumours that cause

obstruction of the gastrointestinal tract and the biliary tree, which can only be overcomed by performing a bypass surgery. Additionally, it can also be used to reduce the size of large tumours such as ovarian cancer so that subsequent treatment by radiation therapy or chemotherapy will be even more effective (King & Primrose 2003).

Meanwhile, radiation therapy uses radiation or high-energy rays to kill or shrink tumour cells by damaging their genetic material, making these cells impossible to continuously grow and divide. Radiation therapy is the principal treatment for various skin cancers, cancers of the mouth, nasal cavity, pharynx and larynx, brain tumours, and many gynecological cancers, as well as lung and prostate cancer (Ota et al. 2007). However, the potential role of radiation therapy depends greatly on the relative risk of the disease to the patient. If a patient has large metastases that cause a greater risk to the patient's life, systemic chemotherapy is the best option. On the contrary, if a patient has relatively small mass of asymptomatic metastases, radiation therapy should be a better option (Saltz 2004).

On the other hand, there are several roles for cytotoxic chemotherapy in cancer treatment. Curative chemotherapy only works for a small number of chemosensitive tumours that may show response to an individual cytotoxic agent while adjuvant chemotherapy involves administration of chemotherapy after primary local treatment to improve the cure rate when chemotherapy alone does not ensure a curative solution. Palliative chemotherapy helps to relieve symptoms of bulky and metastatic tumour that has low cure possibility (Parnell & Woll 2003, Bhosle & Hall 2009). Chemotherapy can be grouped into several classes of drugs based on their chemical structure and modes of action: (a) topoisomerase I and II inhibitors that inhibit the action of DNA topoisomerases which break and rejoin DNA molecules to control the topological state of DNA, (b) platinum compounds that form complexes with DNA, hence inhibiting DNA polymerase which results in the blockage of replication and transcription

processes, (c) alkylating agents that act on DNA directly, resulting in cross-linking or breaking of DNA strands as well as abnormal base pairing, (d) antibiotics that bind to the DNA of cancer cells and prevent RNA (ribonucleic acid) synthesis which is an important step for the protein synthesis that is necessary for cell survival, (e) anti-metabolites that replace natural substances by acting as building blocks in DNA molecules, resulting in the alteration of the function of enzymes which is essential for cell metabolism and protein synthesis, (f) mitotic inhibitors that mainly affect tubulin, hence disrupting formation of the spindle for chromosomes migration during mitosis (Wijen et al., 2000; Calatayud et al., 2002; Parnell & Woll, 2003; Elisa et al., 2004; Kanaan et al., 2009).

1.4.1 PLANT-DERIVED CHEMOTHERAPEUTICS

Cancer chemotherapy began in the 1940s as a consequence of toxicological studies of nitrogen mustard-based war gas. It is approximated that more than 60% of the anticancer drugs applied in cancer chemotherapy have originated from natural source, whether they are original natural products, products derived semi-synthetically from natural products, or even synthetic products based on natural products models. For example, vincristine, irinotecan, and etoposide were derived from herbal sources while doxorubicin, bleomycin, and dactinomycin were extracted from marine sources (Sardari et al. 2009). The abundance of plants that have anticancer properties have been in existence for thousands of years in countries such as China, India, and Central America where they still play an essential role in primary health care of under-developed countries (Avila et al. 2004, Yineger et al. 2008).

In the past two decades, the National Cancer Institute (NCI) had established a system for scientific approach to develop chemopreventive agents. It began from epidemiological and basic laboratory data, stepwise clinical trials, and finally led to the

Food and Drug Administration (FDA) to apply the chemoprevention to human subjects (Russo 2007). Among the nature-derived anticancer drugs discovered, only 11% of the compounds that entered clinical trial successfully made their entry into the market place. Others were mostly withdrawn from subsequent studies mainly for issues related to their efficacy, toxicity, drug metabolism and pharmacokinetics (Sardari et al. 2009). Nevertheless, even those compounds that were marketed were not considered flawless. First, chemotherapy drugs are most effective against rapidly dividing cells such as cancer cells. However, certain body cells such as bone marrow cells, immune cells and hair follicle cells that rapidly divide were also targeted in a similar manner (Lupulescu 1999). Hence, this treatment may cause a range of side effects to the patient with varying degrees of severity (Parnell & Woll 2003, Bhosle & Hall 2009).

Second, inhibition of cancer cell proliferation as well as induction of apoptosis have been thought as the markers to evaluate the effectiveness of the anticancer drugs or cancer chemopreventive agents to eliminate genetically damaged or pre-neoplastic cells before manifestation of malignancy (Lv et al. 2008, Lee et al. 2011a). Hence, most of the currently available natural product-derived chemotherapeutic drugs kill cancer cells primarily by inducing apoptosis. Since malignancy of tumors is often also attributed to their invasive and metastatic ability, a chemotherapeutic agent that only possesses the ability to induce apoptosis may not be entirely effective (Lee et al. 2011a). Therefore, the use of chemotherapy in the metastatic disease most often is just to palliate symptoms and prolong survival (Bhosle & Hall 2009).

Moreover, tumor cells tend to acquire resistance towards apoptosis because of conventional chemotherapy (Soto-Cerrato et al. 2005). Chemoresistance can be divided into two types, primary and secondary resistance. Primary resistance occurs when the chemotherapy fails to show any effects on the tumor cells from the beginning of treatment as a result of biological resistance, physiological resistance, or

pharmacological resistance. Primary resistance could also be due to insufficient dosing or inappropriate administration route. Meanwhile, secondary resistance reflects the ability of tumor cells to handle the toxic metabolites or substances after exposure to the chemotherapy (Bhosle & Hall 2009). Therefore, this poses a great problem in cancer treatment and the search for a more effective natural product-based chemotherapy drug is an ongoing process.

1.4.2 *PHYLLANTHUS*

A successful chemotherapeutic agent should be able to interfere with more than one phase of the multistep carcinogenesis process (Lv et al. 2008). Various phytochemicals have been shown to be present in a diet rich in fruit and vegetables, therefore having the potential to act as chemopreventive agents. These components can be grouped into two categories, depending whether they are blocking the cancer or suppressing the cancer. Cancer-blocking agents basically stop the carcinogens from hitting their cellular targets to avoid the initiation process through several mechanism, either increase carcinogen detoxification, modifying carcinogen uptake and metabolism, scavenging reactive oxygen species, or enhancing DNA repair. On the other hand, cancer-suppressing agents stop cancer promotion and progression after pre-neoplastic cells formation via interference with cell cycle regulation, signal transduction, transcriptional, regulation and apoptosis (Russo 2007). Besides that, it also relies on their extent to which they would selectively induce tumor cell death while allowing the survival of normal tissue (Lv et al. 2008).

The genus *Phyllanthus* is a large genus of trees, shrubs, and rare herbs of the family *Euphorbiaceae,* and is one of the most widely distributed plants throughout the Amazon rainforests as well as other tropical and subtropical countries (Bagalkotkar et al. 2006, Eldeen et al. 2011, Lee et al. 2011a). The genus *Phyllanthus* consists of about

500 temperate and tropical species whereby most of them are used as medicine in various countries. They are actually a rainy season weed that can be found in both cultivated fields and wastelands, with height varying from 30 to 60 centimeters. All parts of the plant, including their roots, leaves, fruits, milky juice, and even the whole plant can be exploited for medicinal usages (Shakil et al. 2008).

The wide range of pharmacological activities exhibited by *Phyllanthus* can be attributed to the presence of varying phytochemicals within the plant. For instance, *P. emblica* is reported to contain ellagitannins, flavanoids, apigenin glucoside, norsesquiterpenoid glycosides, methyl ester, phenolics, gallic acid, chebulinic acid, quercetin, corilagin and isostrictiniin (Mehmood et al. 2011). Meanwhile, the major active components in *P. amarus* are lignans, flavanoids, terpenes, alkaloids, steroids, ellagitannins, hydrolysable and condensed tannins (Nayak et al. 2011). On the other hand, some of the active compounds present in *P. niruri* are flavanone glycosides, lignans, niranthin, nirtetralin, phyltetralin, acyclic triterpene, glycoflavones and flavones sulfonic acid (Shakil et al. 2008).

Numerous research studies on *Phyllanthus spp.* began in the late 1980's when *P. niruri* showed clinical efficacy against Hepatitis B virus (Lee et al. 2011a). Due to their wide therapeutic usage in folk medicine, wide distribution, as well as presence of diverse secondary metabolite entities, various pharmacological properties of different *Phyllanthus* species became focals point for studies (Eldeen et al. 2011). In addition, *Phyllanthus* has shown to be either non-toxic or to exert minimal toxicity when tested *in vitro* against a number of normal cell lines in various studies, including MRC5-fibroblast cells, 184B5-breast epithelial, NL20-epithelial cells, CCD-1127Sk-skin cells, as well as RWPE-1-prostate epithelial (Ngamkitidechakul et al. 2010, Tang et al. 2010, Lee et al. 2011a).

1.4.2.1 ANTIBACTERIAL ACTIVITY

Aqueous and methanolic extracts of *P. amarus* aerial parts were active against numerous bacteria tested, including *E. coli*, *P. aeruginosa*, *S. typhii*, *S. aureus*, and *C. albicans*. Moreover, methanolic *P. amarus* demonstrated broad-spectrum antimicrobial activity with a minimum inhibitory concentration of 1.56mg/ml (Alli et al. 2011). Besides that, both extend spectrum β-lactamase producing *E. coli* isolated from the stool samples of HIV sero-positive patients with or without diarrhea showed susceptibility to varying doses of ethanolic *P. amarus* extracts (Akinjogunla et al. 2010). Essential oils and fractions from leaves and seeds of *P. amarus* also demonstrated activity against 11 microorganisms that include yeast, gram-positive as well as gram-negative bacterias (Oluwafemi & Debiri 2010).

1.4.2.2 ANTIOXIDANT ACTIVITY

Streptozotocin-induced diabetic male Wistar albino mice were fed with 200mg/kg of aqueous extract of *P. amarus* to evaluate its antioxidant activity and there was a significant decrease in the renal LPO as well as protein oxidation while an increase in the antioxidant enzymes such as GR, GPX, GST, and GSH (Karuna et al. 2011). Moreover, there were high phenolic contents in *Phyllanthus* extracts that have a strong correlation with free radical-scavenging activities and lipid peroxidation inhibiting capacity (Guha et al. 2010). Boiling water extracts were also shown to exhibit stronger antioxidant potential due to greater solubility of compounds, breakdown of tannins and cellular constituents (Lim & Murtijaya 2007). Nevertheless, Kumaran and Karunakaran found that *P. debilis* possessed greatest antioxidant activity among the five species tested while *P. amarus* showed the weakest activity (Kumaran & Joel Karunakaran 2007).

1.4.2.3 ANTIVIRAL ACTIVITY

Water-alcohol extracts of *P. amarus* leaves had the ability to block HIV-1 attachment *in vitro* as well as inhibiting HIV-1 integrase, reverse transcriptase, and protease enzymes to varying degrees due to the presence of gallotannin containing fraction, isolated ellagitannins geraniin and corilagen (Notka et al. 2003). Besides that, inhibitory actions of methanolic extract of root and leaves of *P. amarus* were tested against NS3 and NS5B enzymes of hepatitis-C virus (HCV). Their results indicated that the root extract showed significant inhibition on HCV-NS3 while leaves extract showed better inhibition on HCV-NS5B protease enzyme. Nevertheless, both of them were capable of inhibiting replication of HCV monocistronic replicon RNA and HCV H77S viral RNA in HCV cell culture system (Ravikumar et al. 2011). On top of that, antiviral activity of aqueous *P. amarus* extract was observed against white spot syndrome virus in shrimp at 150mg/kg of animal body weight (Balasubramanian et al. 2007). Moreover, 25 compounds isolated from *P. multiflorus*, *P. amarus*, *P. tenelus*, and *P. virgatus* suppressed both hepatitis B surface and effective antigens' expression at a non-toxic concentration of 50µm (Huang et al. 2003). However, *P. amarus* was not effective against duck hepatitis virus as shown in two different group of studies (Munshi et al. 1993a, Munshi et al. 1993b).

1.4.2.4 ANTIAMNESIC ACTIVITY

Aqueous extract of stems and leaves of *P. amarus* was tested in male Swiss albino mice on their cognitive functions and brain cholinesterase activity. Results obtained showed that *P. amarus* successfully reversed the amnesia induced by a combination of scopolamine and diazepam as well as reducing the brain cholinesterase activity. In addition, the memory scores of both young and older mice were also improved (Joshi & Parle 2010).

1.4.2.5 DIURETIC ACTIVITY

Administration of 400mg/kg of *P. sellowianus* into rats was shown to significantly increase the urinary volume as compared to the placebo group after 8 hours (Hnatyszyn et al. 1999) while *P. corcovadensis* increased urinary volume 8 hours after its administration (Ribeiro Rde et al. 1988). Meanwhile, in a human clinical study, 40-60 years old subjects were required to ingest *P. amarus* for 10 days and their urinary volume was found to be significantly increased (Srividya & Periwal 1995, Wright et al. 2007).

1.4.2.6 ANTIINFLAMMATORY ACTIVITY

The 75% methanolic extract of whole plant *P. amaru*s significantly inhibited carrageenan, bradykinin, serotonin and prostaglandin E1-induced paw edema at a dose of 250mg/kg body weight (Mahat & Patil 2007). Paw edema suppression was also observed after treatment with the hexane and methanolic extracts of *Phyllanthus* (Raphael & Kuttan 2003, Kassuya et al. 2005). In a separate study, aqueous, ethanolic and hexane extracts of *P. amarus* also inhibited LPS-induced production of nitric oxide and secretion of TNF-α in RAW264.7, KC and human blood (Kiemer et al. 2003).

1.4.2.7 ANTICANCER ACTIVITY

Cytotoxicity of methanolic and aqueous extracts of four *Phyllanthus* (*P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*) species were shown to selectively cause toxicity to various cancer cell lines (MCF-7, A549, PC-3, and MeWo) with little effect on normal cell lines (NL20, 184B5, RWPE-1, and CCD-1125Sk). These *Phyllanthus* extracts were capable of inducing apoptosis in all those cell lines with the presence of DNA fragmentation and increased caspase activity. In addition, a dose-dependent antimetastatic effect was observed on A549 and MCF-7 (Tang et al. 2010, Lee et al. 2011a). In a separate study, *P. urinaria* induced apoptosis in human osteosarcoma 143B

cells by triggering Fas ligand binding with its receptor through intracellular adaptor protein (FADD) to activate caspase 8 (Wu et al. 2012). Hari Kumar and Kuttan also demonstrated that elevated cytochrome P450 activity in vitro and in vivo was reduced by alcoholic extract of *P. amarus* (Hari Kumar & Kuttan 2006). In another in vivo experiment, oral administration of 75% methanolic extract of *P. amarus* aerial parts enhanced the life span of leukemia harboring animals with decreased anemia incidence by reducing the infiltration of leukemic cells into the sinusoidal space (Harikumar et al. 2009). Other animal studies via oral administration of *Phyllanthus* extracts also exhibited their ability to reduce myelosuppression while improving white blood cell count (Kumar & Kuttan 2005), protect against carcinogenesis (Joy & Kuttan 1998, Kumar & Kuttan 2004), as well as prolonging the life span of DLA and EAC bearing mice and reduce the volume of the solid tumors (Rajeshkumar et al. 2002).

1.4.2.8 OTHER THERAPEUTIC AND PHARMACOLOGICAL ACTIVITIES

Some of the other therapeutic functions of *Phyllanthus* include hypoglycemic, treatment of digestive disorders, jaundice and coughs. From pharmacological point of view, *Phyllanthus* could inhibit micronuclei formation, clastogenecity, sister chromatid exchanges, cytoprotective and immunomodulating activities. In addition, *Phyllanthus* can be used to treat gastroenteritis, urethritis, wound healing, intestinal infections, diabetes, antihypertensive, antidiarrheal and antilithic (Lin et al. 2008, Agyare et al. 2011, Brusotti et al. 2011, Mehmood et al. 2011, Thaweboon & Thaweboon 2011, Woottisin et al. 2011).

1.5 JUSTIFICATION AND OBJECTIVES OF STUDY

Cancer has been the second leading cause of death of the human population after cardiovascular disease with more than 11 million deaths every year. Lung cancer is by

far the most common cause of cancer-related mortality in the world. Despite the advances in diagnostic imaging and therapeutic improvements over the decade, lung cancer has a poor prognosis with the 5-year patient survival rate. Meanwhile, breast cancer is the second most common cancer in the world and is the leading cause of morbidity and mortality in women. Currently available chemotherapy drugs expose cancer patients to a wide range of side effects with varying degrees of severity. Inhibition of cancer cell proliferation as well as induction of apoptosis has been thought as the ideal phenomenon to evaluate the effectiveness of the anticancer drugs or cancer chemopreventive agents. However, tumor cells tend to acquire resistance towards apoptosis because of conventional chemotherapy. Moreover, malignancy of tumors is often attributed to their invasive and metastatic ability, therefore a chemotherapeutic agent that only possesses the ability to induce apoptosis renders less useful.

Thus, the anticancer properties of *Phyllanthus* were being evaluated in this study since it was previously shown to possess antiproliferative activity on cancer cells with minimal effect on normal cells. Although certain *Phyllanthus* species have been demonstrated to exhibit antiproliferative and apoptotic-induction activity, the detailed mechanisms on how *Phyllanthus* exerts its anticancer activities are not fully elucidated yet. To begin with, the toxicity of four *Phyllanthus* species (*P. niruri*, *P. urinaria*, *P. watsonii*, and *P.amarus*) were being tested on two cancer cell lines (lung carcinoma – A549 and breast carcinoma – MCF-7) and two normal cell lines (lung epithelial – NL20 and breast epithelial – 184B5) to confirm the selective antiproliferative activity against cancer cells. Subsequently, the mode of cell deaths induced by *Phyllanthus* in cancer cells was being determined. In addition, the antimetastatic activity of Phyllanthus on the cancer cell lines was being assessed. To further study on how the anticancer activities were being exerted, signaling pathway studies were being conducted to determine the upstream signaling events activated.

The objectives of my study include:

1. To determine the antiproliferative effect of four *Phyllanthus* spp. (*P. niruri*, *P. urinaria*, *P. watsonii*, *P. amarus*) *in vitro* on two cancer cell lines (A549 – lung carcinoma; MCF-7 – breast carcinoma) and two normal cell lines (NL20 – lung epithelium; 184B5 – breast epithelium).

2. To study the mechanism of cell death in cancer cells upon treatment with *Phyllanthus spp*.

3. To determine the antimetastatic properties of *Phyllanthus spp*.

4. To determine the signal transduction pathways involved in anticancer activities induced by *Phyllanthus spp*.

CHAPTER 2: METHODOLOGY

2.1 TEST COMPOUNDS

2.1.1 *PHYLLANTHUS* PLANT EXTRACTS

The crude extracts (aqueous and methanolic) and their two fractions of each *Phyllanthus spp.,* namely *P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*, were obtained from the Malaysian Agriculture and Research Development Institute (MARDI), Malaysia. The aqueous extracts and fractions were prepared by dissolving 10mg in 1ml of sterile phosphate buffered saline (PBS) (final concentration 10mg/ml), whereas, the methanolic extracts were prepared by dissolving 40mg in 1ml of DMSO (final concentration 40mg/ml). The tubes containing the extracts were wrapped with aluminium foil and stored at -20^{o}C until use. A single batch of extracts was used for all the experiments.

The polyphenol contents of *Phyllanthus* were identified by performing High Performance Liquid Chromatography (HPLC) coupled with Electron Spray Ionization (ESI) and Mass Spectrometry (LC-MS-MS) analysis. Supernatant of the aqueous extract sample was dried using a vacuum concentrator (Concentrator 5301 Eppendorf, Germany). For LC-MS-MS analysis, the lyophilized sample was redissolved into 20mg/ml with 30% methanolic. Meanwhile, the total supernatant of the methanolic extract samples were evaporated using a rotary evaporator (Rotavapor RII, BUCHI, Switzerland) and redissolved in 20% methanol. The resulting products were then subjected to a solid phase extraction (SPE) column (LiChrolut RP-18 1000mg/6ml, Merck Millipore, Germany) with mobile phase of 60% and 70% methanol. All eluates

collected were concentrated to 0.5ml, and then diluted 8 times with 40% methanol before the LC-MS-MS analysis was performed.

The HPLC system used consisted of a HPLC binary pump, diode array detector (DAD) and an auto-sampler injector compartment (1200 series, Agilent Technologies, Germany). For separation, C-18, 150mm x 4.6mm i.d, 5µm particle size Thermo Hypersil GOLD column (Thermo Fisher Scientific, USA) was chosen as the reverse phase while the mobile phase was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with the gradient setting of solvent B: 5% (5min), 5-90% (60 min), 5% (4min) at a flow rate of 1ml/min. Detection wavelengths were both set at 280nm and 360nm with constant injection volume at 20µl. A 3200 QTrap LC/MS/MS system (Applied Bioscience–MDS Sciex) was used for the mass spectrometry analysis, with the iron source and voltage maintained at 500 ˚C and -4.5 kV for negative ionization, respectively. The nitrogen generator was set at 60 psi curtain gas flow, 60 psi exhaust gas flow, and 90 psi source gas flow. The scanning modes chosen were Enhance Mass Spectrometer (EMS) and Enhance Ion Product (EIP) for full scan mass spectra that ranged from mass to charge ratio (*m/z*) of 100-1200.

2.1.2 STANDARD DRUGS

The standard drugs used in this study were Cisplatin and Doxorubicin (Merck Millipore, Germany). These standard drugs were prepared by dissolving 1mg in 1ml of sterile PBS to achieve a stock concentration of 1mg/ml. They were wrapped with aluminium foil and stored at -20ºC until use.

2.2 CELL CULTURE

2.2.1 CELL LINES

The cancer cell lines used in this study included human lung carcinoma (A549) and human breast carcinoma (MCF-7), whereas the normal cell lines used were human bronchus epithelium (NL20) and human breast epithelium (184B5). All cells were purchased from American Type Culture Collection (ATCC, USA). A549 and MCF-7 were grown in RPMI-1640 (Roswell Park Memorial Institute) and DMEM (Dulbecco's modified Eagles Medium), respectively, while NL20 and 184B5 were grown in F-12 K (ATCC, USA) and Mammary Epithelial Growth Medium (Lonza, Switzerland) respectively. Upon receipt of the frozen vials of cells, they were thawed in a water bath at 37ºC. Subsequently, the cells were transferred into different T-25 flasks (Nunc, Denmark) containing 5ml of their respective growth medium and left overnight in a 5% CO_2 incubator at 37ºC to allow their attachment to the flask surface. To ensure growth and viability of the cells, the growth mediums were supplemented with 10% fetal bovine serum (FBS) (JR Scientific, US). On the following day, the cells were checked for their viability. Viable cells were adhered onto the surface while dead cells remained suspended in the medium. The used medium containing dead cells was replaced with fresh growth medium and the culture flask was returned into the incubator for cell growth.

The A549 tumor-cell line, a human alveolar cell carcinoma, was initiated by D. J. Giard et. al. through explant culture of lung carcinomatous tissue from a 58 year old Caucasian male. It has been continuously passaged *in vitro* for more than 3 years and exhibits a human karyotype and appears to have been derived from a single parent cell. Examination of A549 cells by electron microscopy at both early and late passage levels

showed that they contain multilamellar cytoplasmic inclusion bodies commonly present in type II alveolar epithelial lung cells (Lieber et al. 1976, Honma et al. 1996).

A stable cell line, MCF-7, was derived from the free-floating cells of a primary culture, 734B, that was grown from a pleural effusion of a 69 year old Caucasian woman with metastatic mammary carcinoma. It has been maintained for over 90 weekly passages and maintained several features of differentiated mammary epithelium, including the ability to display discrete multilayered post-confluent growth or foci in the presence of 17-β-estradiol. The observed foci are solid cell aggregates with a morula-like structure on a confluent monolayer background during post-confluent growth (Soule et al. 1973, Bradley et al. 2008).

2.2.2 CELL MAINTENANCE

Used medium was removed from the culture flask containing cells of about 70 – 80% confluence. Two milliliters of phosphate buffered saline (PBS) (Oxoid, England) were added to rinse the cells and remove the traces of serum that may inhibit the action of the trypsin enzyme. Subsequently, 1ml of trypsin-EDTA (HyClone, Thermo Fisher Scientific, USA) was added into the flask and incubated in the 5% CO_2 incubator at 37ºC for about 5 minutes. After the cells had completely detached from the flask surface, 2ml of fresh growth medium was then added into the flask to stop the action of trypsin enzyme and to flush the cell clumps to separate the cells. Half a milliliter of the cell suspension was then dispensed into a new culture flask containing 5ml of growth medium and kept at 37ºC in a 5% CO_2 incubator. Cells were checked daily and repeatedly maintained until they were required for use in experiments. Cells with eighty percent confluence were at their optimal condition and hence were used for all the experiments.

2.2.3 CELL COUNTING

The trypsinized cell suspension was gently resuspended to disperse the cells into a single-cell suspension that is desirable to ensure an accurate cell count. A hemocytometer was cleaned with 70% ethanol before a cover slip was affixed onto it and 10µl of the cell suspension was mixed thoroughly with 10µl of 0.4% trypan blue solution. Subsequently, approximately 10µl of the mixture was loaded at the edge of the cover slip. The total number of cells at four large corner squares were counted under the inverted microscope (Olympus) using the 10× objective lens. Viable cells appeared unstained, clear, and surrounded by a refractile ring. In contrary, dead cells were stained blue and non-refractive. The number of viable cells can be determined using the following formula:

Total Viable Cells =

Dilution factor × Average viable cell number in one set of corner square × 10^4 ×

Cell suspension volume

2.2.4 CELL CRYOPRESERVATION

Cells at low passage numbers resembled most closely to their original phenotype. Therefore, stocks of these cells were kept by cryopreserving them in cell freezing media before storing them in a liquid nitrogen tank. The freezing media was made up of 50% FBS, 40% growth medium, and 10% dimethyl sulfoxide (DMSO) (Sigma, US). Eighty percent confluence cells were trypsinized and pelleted by centrifuging for 5 minutes at 1500rpm. The cell pellet was gently resuspended with the freezing media at 1×10^7 viable cells/ml and aliquoted into cryotubes (Nunc, Denmark). The cells were first kept in a Mr. Frosty (Nunc, Denmark) at -80ºC for 24 hours before transferring them into a liquid nitrogen tank for long-term storage.

2.3 ANTIPROLIFERATIVE EFFECTS OF *PHYLLANTHUS*

The antiproliferative effects of *Phyllanthus* were examined by performing both cytotoxicity and cell cycle analysis. This is because *Phyllanthus* could exert its inhibitory action on cells' proliferation either by causing a cytotoxic or a cytostatic effect. Cytotoxicity is defined as the cumulative effect of a compound on a particular cell number over a specific period, usually due to apoptosis or necrosis (Xia et al. 2008). Meanwhile, cytostatic agents inhibit cell growth and multiplication by inhibiting signal transduction and they lack obvious cytotoxic properties (Petit et al. 1997). In addition, the effects of *Phyllanthus* on cellular morphologies were also studied.

2.3.1 MORPHOLOGICAL ANALYSIS

Cells were cultured overnight in 6-well plates using medium supplemented with 10% FBS for overnight. After 24 hours, cultured cells were treated with various aqueous and methanolic *Phyllanthus* extracts. Their effects on the cells were observed under a light microscope (Olympus) and images were taken at a magnification of 200×.

2.3.2 CYTOTOXICITY ANALYSIS

For each *Phyllanthus* extract, fraction and standard drug, a serial dilution was performed in order to determine the cytotoxic effectiveness of each test compounds at varying concentrations. The desired serial concentrations for each extract and fraction were prepared up to 1000µg/ml and subsequently diluted using a two-fold serial dilution. Therefore, from the sub stock solution which contained 1000µl at a concentration of 1000µg/ml, 500µl was dispensed into a new 1.5ml tube containing 500µl of fresh medium and resuspended to prepare a diluted extract of 500µg/ml. The re-suspension steps were repeated for the subsequent tubes containing 500µl medium each to prepare the final desired concentrations. Sub stock solution for standard drugs

were prepared at 20µg/ml for Cisplatin and 2µg/ml for Doxorubicin and further diluted using the same serial dilution method.

Eighty percent confluence cells were trypsinized and pelleted by centrifugation at 1500rpm for 5 minutes. The cell pellet was then resuspended with fresh medium, counted using a hemocytometer, and adjusted to 1×10^5 cells/ml. One hundred microliters (1×10^4 cells) of this cell suspension were then dispensed into each well of a 96-well microtiter plate and incubated overnight to allow cell attachment. After that, they were treated with *Phyllanthus* fractions as well as both the aqueous and methanolic *Phyllanthus* extracts at a 6-point serial dilution up to a final concentration of 1000µg/ml. Vehicle-control wells with cells only and compound-control wells with extracts only were included. Each of these treatment conditions and controls was performed in triplicates. The plates were incubated at 37ºC, 5% CO_2 and 100% humidity for 24, 48, and 72 hours.

At the end of each incubation period, the cell viability was determined using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA) in accordance to the manufacturer's protocols. This assay contains phenazine methosulfate (PMS) that acts as an eletron coupling reagent as well as a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) which was bioreduced by dehydrogenase enzymes present in viable cells into formazan product that is soluble in tissue culture medium. The amount of formazan product is measured using GloMax Multi Detection System (Promega, USA) at a wavelength of 490nm with a reference wavelength of 750nm and is directly proportional to the number of viable cells. The background absorbance was corrected by subtracting the average absorbance for compound-control wells from the average absorbance for other treatment conditions and vehicle-control wells. A graph of corrected absorbance (Y axis) versus concentration of test compounds (X axis) was

plotted and the respective Half Maximal Inhibitory Concentration [IC_{50}] (Budzinski et al. 2000, DeBonis et al. 2004) values at 24, 48, and 72 hours incubation for individual plant extracts, fractions, and standard drugs were determined and used in succeeding assays.

2.3.3 CELL CYCLE ANALYSIS – PROPIDIUM IODIDE STAINING

Cell cycle analysis allows separation of cells at different stages of the cell cycle based on their DNA content. Propidium iodide (PI) dye is frequently used for DNA content analysis due to its high capability of binding to DNA with low specificity through intercalation process between the major grooves of double stranded DNA (Riccardi & Nicoletti 2006). Cells at different phases of its cell cycle contain a distinct amount of DNA content as shown in Figure 2.1. Cells were seeded at 10^5 cells/well, treated with extracts at their IC_{50} values, and incubated for various time points from 0 to 72 hours. At the end of each incubation period, cells treated with or without *Phyllanthus* extracts were harvested by trypsinization and fixed with ice-cold 70% ethanol for at least 1 hour at -20ºC. Cells were then pelleted, washed once with PBS, resuspended in the PI solution [10µg/ml PI (Sigma-Aldrich, USA) and 1mg/ml RNase A (Sigma-Aldrich, USA) in PBS], and incubated in a 37ºC water bath for 30 minutes. Data acquisition was performed using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, USA) and the CellQuest software. The results were subsequently analyzed using WinMDI 2.9 software. The distribution of cell percentages in each cell cycle phase was determined by setting gates based on their amount of DNA content.

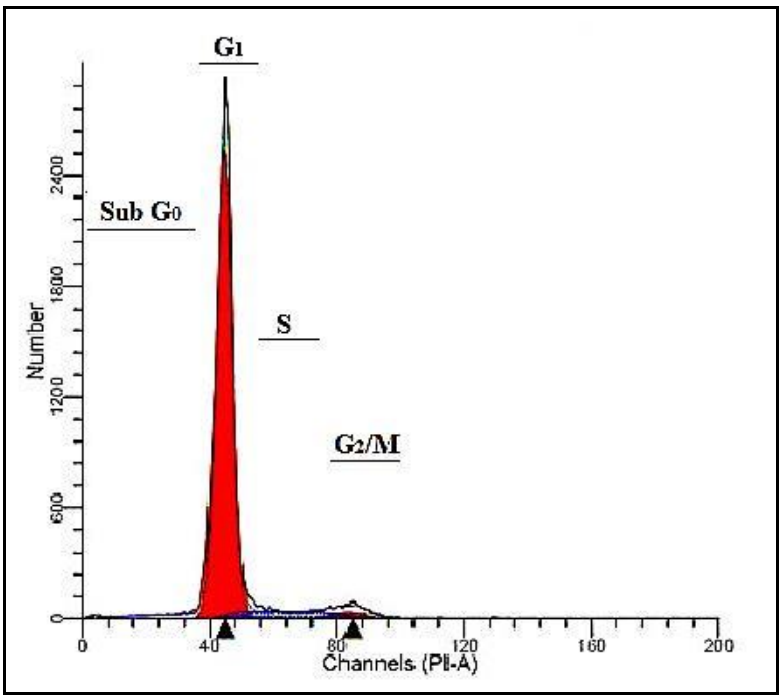


Figure 2.1: Histogram for cell population distribution based on cell DNA content

2.4 ANTIMETASTASIS EFFECTS OF *PHYLLANTHUS*

Metastasis involves a series of complex processes governed by complicated mechanisms, beginning with the detachment of tumor cells, invasion, motility, adhesion and reestablishment of growth at a distant site (Lee et al. 2010). This process is often associated with the destruction of the extracellular matrix (ECM) and the basement membrane components by proteolytic enzymes such as matrix metalloproteinases (MMPs) (Yang et al. 2008). Hence, various assays were performed to find out the ability of *Phyllanthus* to halt metastasis, including cell invasion assay, cell migration assays, cell adhesion assays, as well as zymography assay to observe the MMPs expression in response to *Phyllanthus* treatment.

2.4.1 CELL INVASION ASSAY

Cell invasion was determined using 24-well transwell chamber with 8µm pore polycarbonate filter coated with basement membrane extracts according to the

manufacturer's instructions provided by Cultrex, Trevigen (USA). This assay was carried out to screen the compounds that may influence the cell's invasive potential through extracellular matrices or basement membrane, which is fundamental to tumor metastasis. The basement membrane extract was purified from Engelbroth-Holm-Swarm (EHS) tumor and consists of major components in the basement membrane such as laminin I, collagen IV, entactin and heparin sulfate proteoglycan. A transwell chamber was utilized. It has a simplified Boyden chamber design with upper and lower compartments. Cells were seeded into the upper compartment at a concentration of 2×10^6 cells/ml in a volume of 100µl/well. The lower compartment contained 500µl of medium supplemented with extracts and 10% FBS as chemoattractants while serum-free medium was used as control. After 48 hours incubation at 37ºC, cells that had passed through the filters were detached using Cell Detachment Solution (Thermo Fisher Scientific, USA) mixed with calcein-acetoxymethylester supplied in the kit. Calcein-AM is originally a non-fluorescent but cell permeant compound, which is converted by intracellular esterases into calcein that is an anionic fluorescent (Uggeri et al. 2004). The free calcein emits a bright fluorescence and was used to quantitate the number of cells that have invaded through the artificial basement membrane and 8µm pore. Fluorescence intensity was measured with excitation wavelength at 485 nm and emission wavelength at 520 nm. Invasion inhibition rate was calculated using the following formula:

Cell Invasion (%) =

Mean fluorescence of (experimental wells – medium control wells) / Mean fluorescence of (cell control wells – medium control wells) × 100

2.4.2 CELL MIGRATION ASSAY

MultiScreen-MIC 96-well plates with 8µm polycarbonate membranes (Merck Millipore, Germany) were used in this migration experiment to study the chemotaxis of cancer epithelial cells. Cells were seeded into the upper compartment of the transwell chamber at a concentration of 1×10^5 cells/ml in a volume of 100µl/well. Medium for the experimental and control groups were added into the lower compartment of the transwell chamber at 500µl/well. At the end of 24 hours incubation, cells that did not penetrate the polycarbonate membrane to the bottom of chamber were scraped off using a cotton sticker. The chamber plate was then placed onto a new 96-well feeder tray containing 150µl of prewarmed Cell Detachment Solution in wells and incubated at 37ºC for 30 minutes. During the incubation, the plate was gently tilted several times to facilitate dislodgement of cells. One hundred microliters of 2× CyQuant NF (Invitrogen, USA) dye binding solution were then added into each well and further incubated for 1 hour to allow dye-DNA binding to produce a stable fluorescent endpoint. Fluorescence intensity was measured with excitation wavelength at 485 nm and emission wavelength at 530 nm. Migration inhibition rate was calculated using the following formula:

Cell Migration (%) =

Mean fluorescence of (experimental wells – medium control wells) / Mean fluorescence of (cell control wells – medium control wells) × 100

2.4.3 SCRATCH MOTILITY ASSAY

This assay was also performed in tissue culture research to estimate the migration and proliferation rates of different cells in response to variable experimental culture conditions. Cells were seeded in a 24-well microtiter plate at 1×10^5 cells/well and were allowed to grow overnight to reach confluence. The monolayer was then

scratched with a pipette tip, washed with PBS twice to remove floating cells and treated with extracts at their respective IC_{50} values. At the end of each incubation (0 – 48 hours), the cells that migrated into the scratched area were photographed and counted at 5 randomly selected fields. The migrated cells were expressed as mean value per field.

2.4.4 CELL ATTACHMENT ASSAY

Cell adhesion is a critical factor for the maintenance of tissue structure, promotion of cell migration, as well as for transduction of microenvironment information across the plasma membrane. Cells that were unable to attach themselves are prone to death fate. Briefly, cells were seeded at 1×10^5 cells/well in a 24-well microtiter plate, treated with extracts at their respective IC_{50} concentrations and incubated for 72 hours. After that, cells treated with *Phyllanthus* extracts were detached using 0.5% trypsin-EDTA and plated back onto a new culture plate. After each incubation period of 4 to 24 hours, the cell attachment status and morphology were observed and photographed.

2.4.5 CELL-MATRIX ADHESION ASSAY

Cells usually interact with the extracellular matrix (ECM) that is composed of a complex variety of proteins and carbohydrates found in spaces between cells. It forms a layer beneath epithelial and endothelial cells and surrounds all connective tissue cells. Therefore, this assay tests the adhesion ability of cells to ECM that has an essential role in regulating cellular activities. ECM gel (Sigma-Aldrich, USA) was thawed overnight at 4°C before being diluted 2× with cold medium. One hundred microliters of diluted ECM gel were then added into each well of a 96-well microtiter plate and incubated for 2 hours at 37°C. The coated wells were subsequently washed once with PBS and blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich, USA) dissolved in

serum free medium. Three wells not coated with ECM gel were also blocked with 2% BSA to check for non-specific cell adhesion. Meanwhile, cells were harvested, washed with PBS and resuspended at 1×10^5 cells/ml in freshly prepared serum free medium containing various *Phyllanthus* extracts. One hundred microliters of the respective cell suspension was then added into the ECM gel-coated wells. After 2 hours, the supernatant in each well was discarded and the wells were washed with 200µl of PBS to remove unattached cells. The number of adhered cells was determined using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA) according to the manufacturer's instructions. Absorbance was measured using GloMax Multi Detection System (Promega, USA) at a wavelength of 490nm with a reference wavelength of 750nm. Adhesion inhibition rate was calculated using the following formula:

Cell – Matrix Adhesion (%) =

Mean absorbance of (experimental wells – medium control wells) / Mean absorbance of (cell control wells – medium control wells) × 100

2.4.6 ZYMOGRAPHY ASSAY

Zymography is a technique employed to study extracellular matrix-degrading proteases such as MMPs, from various biological samples including cell cultures. It is a simple and sensitive method to identify MMPs based on their substrate specificity and molecular weight. Briefly, cells were seeded at 1×10^5 cells/well in a 24-well microtiter plate, treated with extracts at their respective IC_{50} concentrations and incubated for 72 hours. At the end of the incubation period, the supernatants were collected and centrifuged to remove the debris. After that, they were stored at -20°C to be used as conditioned media. The conditioned media was mixed with one part of 2× Tris-Glycine

SDS Sample Buffer and was incubated for 10 minutes at room temperature. The prepared samples were subsequently loaded onto a 12.5% SDS-polyacrylamide gels that had been copolymerized with 0.1% gelatin or 0.2% casein. The gel was then run with 1× Laemmli running buffer at approximately 125V for about 60 minutes. When the proteins were completely resolved, the gel was washed twice with renaturing buffer on a shaker at room temperature, 1 hour for each washing. Next, the gel was incubated with developing buffer overnight at 37°C before it was stained with 0.1% Coomasie blue for 1 hour. Finally, the gel was destained with destaining solution and the presence of matrix metalloproteinase enzyme was indicated as an opaque, unstained band against a dark blue background.

2.5 MECHANISM OF CELL DEATH INDUCED BY *PHYLLANTHUS*

There are two distinct types of cell death that could be induced by *Phyllanthus* in cancer cells, either apoptosis or necrosis. In order to study the apoptotic mode of cell death induced by *Phyllanthus*, three different assays were conducted including caspases assay, TUNEL assay and DNA fragmentation assay. Meanwhile, lactate dehydrogenase assay was carried out to observe whether *Phyllanthus* induces necrosis in the cancer cells.

2.5.1 APOPTOSIS

2.5.1.1 CASPASE-GLO 3/7 ASSAY

Caspases activity was determined using Caspase-Glo 3/7 Assay (Promega, USA) according to the manufacturer's instructions. This assay contains a luminogenic caspase-3/7 substrate for cleavage by the caspases to generate a stable luminescence signal that is proportional to the amount of caspase activity present. Cells were seeded, treated with extracts at their respective IC_{50} values and incubated at 37°C, 5% CO_2 and

100% humidity for 72 hours. Lyophilized Caspase-Glo 3/7 substrate was resuspended in its buffer and 100µl of this reagent were added into each well. The contents of the wells were mixed gently and incubated at room temperature for 1 hour. Luminescence of each sample was measured using Glomax-Multi Detection System (Promega, USA). The caspases-3/7 activity can be determined by using the following formula:

Caspase Activity (%) =

Mean luminescence of (experimental wells – medium control wells) / Mean luminescence of (cell control wells – medium control wells) × 100

2.5.1.2 TUNEL ASSAY

Terminal Deoxynucleotidyl-Transferase mediated dUTP Nick End Labelling (TUNEL) assay was performed using ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon® International, Merck Millipore, Germany). In this assay, the DNA strand breaks generated upon DNA fragmentation were detected *in situ* by enzymatically labeling the free 3'-OH termini with modified nucleotides. One hundred thousand cells were harvested, fixed in 1% paraformaldehyde in PBS, pH 7.4 and dried on a silanized microscope slide. The specimen was then post-fixed in pre-cooled ethanol/acetic acid (2:1) and quenched in 3% hydrogen peroxidase in PBS. Excess liquid was tapped off before 75µl/5cm^2 of equilibration buffer was immediately applied on the specimen. Next, 55µl/5cm^2 of working strength terminal deoxynucleotidyl transferase (TdT) enzyme were added and incubated at 37ºC for 1 hour. After incubation, the specimen was placed in a coplin jar containing working strength stop/wash buffer followed by an addition of 65µl/5cm^2 of antidigoxigenin peroxidase conjugate. Specimen was washed in four changes of PBS, stained with 75µl/5cm^2 of peroxidase substrate, counterstained in 0.5% (w/v) methyl green followed by several washes with distilled water, n-butanol, and xylene. Finally, the specimen was mounted

under a glass coverslip in Permount fluid and observed under a light microscope (Olympus BX51) at a magnification power of 200×. Images were captured using Olympus U-CMAD3 at three fields per slide.

2.5.1.3 DNA FRAGMENTATION ASSAY

The ultimate DNA fragments exist in multimers of about 180bp nucleosomal units that often appear as DNA ladder on a standard agarose electrophoresis gels. In order to demonstrate this, DNA had to be first extracted from the apoptotic cells. Five hundred microliters of 5×10^5 treated cells were lysed in 55µl DNA lysis buffer [1M Tris-HCI (pH 8.0), 0.5M EDTA, and 100% Triton X-100] and incubated at 4ºC for 30 minutes. DNA was extracted from the supernatant with an equal volume of phenol/choloroform/isoamyl alcohol (25:24:1). Samples were spun and the upper aqueous layer transferred to a new tube, to which an equal volume of ice-cold 100% ethanol and 1/10 volume of 3M sodium acetate (pH 5.2) were added and incubated overnight at -20ºC. After spinning the sample, supernatant was decanted, the pellet air dried, and then dissolved in deionized water-RNase solution [10mg/ml RNase I] and incubated at 37ºC for 30 minutes. Equal amounts of DNA (10µg/well) were electrophoresed in 1.2% agarose gel impregnated with ethidium bromide at 5V for the first 5 minutes and increased to 100V for 1 hour. DNA fragments were then visualized and imaged using a UV transilluminator.

2.5.2 NECROSIS

Lactate Dehydrogenase (LDH) is a cytosolic enzyme released into cell culture supernatant due to compromised membrane integrity, which is associated with necrotic cell death. The extent of its activity in converting tetrazolium salt into red formazan product is proportional to the number of necrotic cells (Lee et al. 2011a). Detection of LDH leakage was achieved using CytoTox-ONE® Homogeneous Membrane Integrity

Assay (Promega, USA) in accordance to the manufacturer's instructions. This assay measures the amount of LDH released from non-viable cells without damaging the viable cells by detecting the fluorescent resorufin products generated from the enzymatic conversion of resazurin substrate. Cells were seeded, treated, and incubated for 72 hours. No-cell control, untreated cells control, and maximum LDH release control wells were included in each plate. At the end of incubation, lysis solution was added to positive wells and further incubated for 30 minutes to generate maximum LDH release. Subsequently, an equal volume of CytoTox-ONE® reagent (50µl) was added into each well and incubated at room temperature for 10 minutes with a subsequent addition of stop solution (50µl). Fluorescence was recorded with an excitation wavelength of 560nm and an emission wavelength of 590nm within 1 hour to avoid increased background fluorescence. The percentage of LDH released can be determined by using the following formula:

LDH Released (%) =

Mean fluorescence of (experimental wells – medium control wells) / Mean fluorescence of (maximum LDH released wells – medium control wells) × 100

2.6 EFFECTS OF *PHYLLANTHUS* ON CELL'S ANGIOGENIC ABILITY

Expressions of inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) are two indexes for tumor angiogenesis. Their high expressions increase the microvascular density that is involved in the advancement of tumor metastasis (Song et al. 2002). Therefore, the effects of *Phyllanthus* on the expressions of these angiogenic components were studied by performing a cell-based ELISA assay. In addition, a western blot assay was performed for VEGF as a confirmatory test.

2.6.1 PRODUCTION OF VASOACTIVE AGENT, INOS

Inducible nitric oxide synthase (iNOS) is often upregulated in tumor cells and is an important regulator for vascularization and angiogenesis. In order to measure the total iNOS in whole cells, a cell-based ELISA, Human Total iNOS Immunoassay (R&D Systems, USA) was used. One hundred microliters (approximately 10000 cells) of cell suspension were seeded into each well of a black 96-well microplate with clear bottom and incubated overnight at 37°C. After that, the cells were treated with varying *Phyllanthus* extracts at their respective IC_{50} concentrations. At the end of treatment, the medium was discarded and replaced with 100µl of 4% formaldehyde diluted in PBS followed by incubation for 20 minutes at room temperature. Next, the cells were washed with wash buffer three times, 5 minutes for each wash with gentle shaking. Subsequently, 100µl of quenching buffer were added and incubated at room temperature for 20 minutes. After 20 minutes, the cells were washed again three times, followed by the addition of blocking buffer for 1 hour at room temperature. After that, washing steps were performed three times before primary antibody mixture (total iNOS and total GADPH antibody) was added into each well and incubated overnight at 4°C. Then, the primary antibody mixture was removed, washed, and replaced with secondary antibody mixture for 2 hours at room temperature. Next, the secondary antibody mixture was discarded and the cells were washed twice each with wash buffer and PBS. Finally, substrate F1 was added into each well and incubated for 60 minutes at room temperature, followed by addition of substrate F2 for 40 minutes. Both substrates F1 and F2 were provided in the kit and contain the fluorogenic substrate for horseradish-peroxidase (HRP) and alkaline phosphatase (AP) respectively. The plate was then first measured using GloMax Multi Detection System (Promega, USA) with excitation at 450nm and emission at 600nm, followed by a second measurement with excitation at 360nm and emission at 450nm. The readings at 600nm represent the amount of total

iNOS in the cells while readings at 450nm represent the amount of total GADPH in the cells. Normalized results were obtained by dividing the total iNOS fluorescence at 600nm by the total GADPH fluorescence at 450nm in each well.

2.6.2 PRODUCTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF is an essential angiogenic growth factor to induce vessel sprouting from existing capillary bed for metastasis. Production of vascular endothelial growth factor (VEGF) in cells was measured by performing two assays. First, the quantification for VEGF production in whole cell was carried out using a cell-based ELISA assay that is similar to the assay described in Section 2.6.1. Subsequently, expression of VEGF by cells was also determined by carrying out western blot assay as described in Section 2.8.4. Anti-VEGF mouse monoclonal antibody used in both assays was purchased from Merck Millipore, Germany.

2.7 EFFECTS OF *PHYLLANTHUS* ON CELL'S ENDOTHELIAL TO MESENCYHMAL TRANSITION (EMT)

Epithelial to mesenchymal transition (EMT) is the fundamental mechanism to diversify the cells found in complex tissues and is also a well-recognized mechanism to initiate metastasis in epithelial cancers (Kalluri & Neilson 2003). During this process, epithelial cells lose their polarity and cell-cell contacts while acquiring migratory behavior to travel away from their community (Xu et al. 2009). Thus, cell aggregation and cell-cell adhesion assays were performed to determine the capability of *Phyllanthus* to suppress this EMT process.

2.7.1 HANGING DROP AGGREGATION ASSAY

Transformed cells produce bigger cell aggregates than its corresponding normal cells when suspended in liquid media, a characteristic that is associated with tumorigenicity (Rhim 1983). Therefore, a cell aggregation assay was perfomed to evaluate the tumorigenic potential of the malignant cells. Cells used for this assay were passaged at least two to three times. Cells were first trypsinized and washed twice with PBS. Subsequently, the cells were counted and adjusted to 2.5×10^5 cells/ml in medium containing 10% FBS as control and varying *Phyllanthus* extracts with 10% FBS for experiments. About 20µl (5000 cells) of each cell suspension was added onto the inner surface of the lid of a 24-well microtiter plate. The lid was then carefully placed back onto the plate to ensure that the droplets of cell suspension were hanging from the lid with cells suspended within them. Eight milliliters of serum-free culture medium was added into each well to eliminate evaporation from the droplets. Cells were incubated for up to 60 hours at 37°C and were photographed every 12 hours interval using a Nikon ECLIPSE TE2000-E UV/Phase contrast microscope at 400× magnification.

2.7.2 CELL-CELL ADHESION ASSAY

Adhesion of cells to one another is a primary feature of the architecture of many tissues. Successful metastasis usually requires the disruption of cell-cell adhesion to release the neoplastic cells from the primary tumor. In this assay, human umbilical vein endothelial cells (HUVEC) which were passaged for at least two to three times were used. Firstly, HUVEC were cultured in a 96-well microtiter plate so that it will reach monolayer confluency after 24 hours. The cells were then kept in the incubator until it was used the next day. On the day of use, A549 or MCF-7 cells were trypsinized, centrifuged and resuspended in serum free Opti-MEM I reduced serum medium (Invitrogen, USA). Cells were carefully prepared to ensure single-cell suspension.

These cells were then counted and adjusted to 3×10^5 cells/ml in freshly prepared Opti-MEM medium containing 2% FBS and various *Phyllanthus* extracts. The cells and medium-containing extracts mixture were incubated for 2 hours before they were centrifuged to collect the extracts-treated cell pellet. This cell pellet was subsequently incubated with BCECF AM (Invitrogen, USA) solution for 30 minutes to stain the cells. Cell pellet was collected and resuspended in Opti-MEM medium containing 2% FBS. After these cells were prepared, the medium for HUVEC cells cultured on the day before was discarded and 100µl of cell suspension were added onto the monolayer of HUVEC. The plate was then further incubated for 30 minutes at 37°C before fluorescence reading was determined at excitation wavelength 488nm and emission wavelength 535nm. The percentage of cell-cell adhesion was calculated using the following formula:

Cell – cell Adhesion (%) =

Mean fluorescence of (experimental wells – medium control wells) / Mean fluorescence of (cell control wells – medium control wells) × 100

2.8 EFFECTS OF *PHYLLANTHUS* ON CELLULAR SIGNALLING TRANSDUCTION PATHWAYS

Cancer is a group of diseases strongly correlated with defects in signal transduction proteins whereby various key signaling pathways were implicated in human tumorigenesis. Therefore, Cignal Finder Cancer 10-pathway Reporter Array kit (SABiosciences, QIAGEN, USA) was used to screen the signaling pathways affected by *Phyllanthus* to halt the uncontrolled proliferation and metastasis of malignant tumor cells. Findings from this array were further confirmed by performing western blot analysis. Subsequently, two-dimensional gel electrophoresis assay was carried out to

study the differences in protein expressions as a result of perturbations in cellular signaling pathways by *Phyllanthus*. Prior to western blot and two-dimensional gel electrophoresis assays, cytoplasmic protein lysate was prepared and quantified to ensure consistency.

2.8.1 SIGNALING PATHWAY ANALYSIS USING CANCER 10-PATHWAY REPORTER ARRAY

Transient transfection was performed using TransIT-LT1 (Mirus Bio, USA). Plasmid DNAs for respective signalling pathways provided in the Cignal Finder Cancer 10-pathway Reporter Array kit (SABiosciences, QIAGEN, USA) and TransIT-LT1 was diluted using Opti-MEM I reduced serum medium (Invitrogen, USA) and incubated at room temperature for 5 minutes. Plasmid DNAs and the TransIT-LT1 prepared earlier were mixed and further incubated for 20 minutes at room temperature to allow TransIT-LT1/DNA complex formation. During the incubation period, cells were trypsinized and adjusted to 1.1×10^5 cells/ml. Subsequently, 90µl (approximately 10000 cells) of the cell suspension were mixed with 10µl of the complex and added into the designated wells of a 96-well cell culture white microplate (Nunc, Thermo Fisher Scientific, USA). The culture plate was rocked for 5 minutes on a rocker to ensure even distribution of the complexes before it was incubated overnight in a 5% CO_2 incubator at 37ºC.

After the transfection of cells with various plasmid DNAs for the respective signaling pathways, the cells were incubated with different *Phyllanthus* extracts at their IC_{50} concentrations for another 24 hours. On the next day, results were developed using Dual-Glo® Luciferase Assay System (Promega, USA) which contains genes for both firefly and Renilla luciferases. Dual-Glo Luciferase reagent was added into each well and incubated at room temperature for 10 minutes. This reagent induces cell lysis and acts as a substrate for firefly luciferase to produce a stable firefly luminescent signal.

The firefly luminescence generated was taken using GloMax Multi Detection System (Promega, USA). This was followed by the addition of Dual-Glo Stop & Glo reagent to all wells. Addition of this reagent quenches luminescence from the firefly reaction by at least 10000 fold while providing substrates for Renilla luciferase. Similarly, renilla luminescence reading was obtained after the plate was incubated for another 10 minutes. The firefly constructs monitor changes in the activity of a key transcription factor that is a downstream target of a particular signalling pathway. Meanwhile, renilla construct acts as an internal control for transfection efficiencies normalization as well as to monitor cell viability. Luminescence for each wells were determined by calculating the ratio of its firefly to renilla luminescence.

Luminescence Ratio =

Firefly luminescence for individual wells / Renilla luminescence for individual wells

Gene Expression (%) =

(Experimental ratio – Medium control ratio) / (Cell control ratio – Medium control ratio) × 100

2.8.2 PREPARATION OF CYTOPLASMIC PROTEIN LYSATE

Protein lysates were prepared for western blotting and proteomic analysis for both *Phyllanthus*-treated and untreated samples. Cells treated with *Phyllanthus* extracts were detached from the culture plates using 0.5% trypsin-EDTA and washed twice with phosphate buffered saline (PBS), centrifuging at 1500rpm for 5 minutes. For extraction of cytoplasmic proteins, 200µl of cell lysis buffer were added to the cell pellet and incubated on ice for 30 minutes before centrifuging at 14000rpm for 15 minutes at 4°C. Supernatant is then collected into a new 1.5ml tube followed by addition of 4× sample volume of iced-cold acetone. After that, the sample was incubated for at least 4 hours at

-20°C and centrifuged at 14000rpm for 15 minutes at 4°C. After centrifugation, the supernatant was discarded while the cytoplasmic protein pellet was diluted in 150µl of rehydration buffer. The cytoplasmic protein lysate can either be kept at -80°C for storage or proceed with sample quantification for subsequent experiments.

Protein lysate concentration was determined with 2-D Quant kit (GE Healthcare, USA) following manufacturer's instructions to ensure equal protein amount used in each experiments. Prior to starting with the quantification steps, working color reagent was prepared by mixing 100 parts of color reagent A with one part of color reagent B. A standard curve was prepared according to Table 2.1 using the 2mg/ml BSA standard solution supplied in the kit. Subsequently, 3µl of each protein sample were pipetted into different microcentrifuge tubes. The quantification began with addition of 500µl precipitant into each tube (standards and samples), followed by vortexing and 2 – 3 minutes incubation at room temperature. Next, 500µl of co-precipitant were added, briefly mixed and centrifuged at 14000×g for 5 minutes. After that, supernatant was completely removed so that there was no visible liquid remained in the tubes. One hundred microliters of copper solution and 400µl of deionized water were then added into each tube and thoroughly vortexed to dissolve the precipitated protein. Finally, 1ml of working color reagent prepared earlier was pipetted into each tube and incubated for 20 minutes. Absorbance at 480nm was read for each samples and standards using GloMax Multi Detection System (Promega, USA). Protein concentrations of samples were estimated by comparison to the standard curve drawn.

Table 2.1: Preparation of standard curve for protein quantification

Tube Number	**1**	**2**	**3**	**4**	**5**
Volume (µl)	0	5	10	15	20
Protein Quantity (µg)	0	10	20	30	40

2.8.3 WESTERN BLOT ASSAY

Western blotting is a technique that allows the production of a replica of proteins separated through sodium dodecyl sulfate–polyacrylamide gel onto a nitrocellulose membrane and subsequently probed with various antibodies to detect the presence or relative abundance of specific proteins (MacPhee 2010).

The Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, USA) was used for western blot assay. Two sequential gels (resolving and stacking gels) were involved in western blot described in Laemmlli method (Laemmli 1970). Glass plates with and without spacers were cleaned with 70% ethanol to remove the debris and contaminants before they were assembled using a setting rig. Resolving gel mixture was then added between the glass plates, leveled with deionized water, and left on the bench for about 30 minutes to polymerize. Once set, the water was poured off followed by addition of stacking gel mixture onto the resolving gel. After inserting the comb, the stacking gel was left to set for another 30 minutes. When the stacking gel had polymerized, the comb was removed while the wells were washed with Laemmli buffer to wash off excess acrylamide at the sides of the wells. Subsequently, the gel was fixed into the running rig, placed in the running tank and filled with 1× Laemmli buffer until the plates were completely covered.

One hundred and fifty micrograms protein of each sample were mixed with 4× sample buffer before they were loaded into a 12.5% of SDS-polyacrylamide gel. Five microliters of 10 – 170kDa PageRuler™ Prestained Protein Ladder (Thermo Fisher

Scientific, USA) were added to monitor the proteins separation. The proteins were separated at 100V for approximately 1 hour. Alternatively, protein samples were separated by two-dimensional (2D) gel electrophoresis method using 7cm IPG gel strips with pH 3–11 NL range (GE Healthcare, USA) as described in Section 2.8.4.

After electrophoresis, the gel was disassembled and the stacking gel was truncated from the front of the resolving gel. The "sandwich stack" was then assembled using a black and white cassette, sponge, blotting paper, and nitrocellulose membrane (GE Healthcare, USA) pre-soaked in 1× transfer buffer according to Figure 2.2. Once assembled, the "sandwich" was placed into the transfer tank and completely filled with iced-cold transfer buffer. The proteins were transferred at 250mA for 1 hour. Once the protein transfer was completed, the membrane was checked by Ponceaus S stain before being blocked at room temperature for 2 hours with Tris buffered saline buffer containing 0.1% Tween 20 (TBST) and 5% dry milk. Subsequently, the membranes were incubated with various primary antibodies diluted in blocking buffer at 4°C overnight. After that, the membrane was washed three times with TBST before further incubation with peroxidase-conjugated secondary antibodies at a dilution of 1:2000 in blocking solution. The immune-reacted proteins were detected via chromogenic method by addition of DAB substrate that was converted by Horseradish peroxidase enzyme into insoluble, coloured products that precipitated onto the membrane to form a protein band. Color development was terminated when the noise ratio was satisfied by placing the membrane in deionized water. Anti-pan-Ras (~ 21kDa), anti-c-Raf (~ 74kDa), anti-c-Myc (~ 60 – 67kDa), anti-Bcl-2 (~ 24 – 26kDa), anti-Hif-1α (~ 120kDa), anti-c-Jun/AP-1 (~ 39kDa), anti-p53 (~ 53kDa), anti-Elk1 (~ 47 – 60kDa), anti-JNK1/2 (~ 49kDa JNK1 and ~ 55kDa JNK2), anti-VEGF (~ 42kDa), goat anti-mouse IgG peroxidase conjugate, and goat anti-rabbit IgG peroxidase conjugate antibodies were purchased from Merck Millipore, Germany while anti-RSK (~ 100kDa) antibody was

purchased from Thermo Fischer Scientific, USA. These antibodies were chosen based on their essential roles in the pathways modulated by *Phyllanthus* as detected using Cignal Finder Cancer 10-pathway Reporter Array. Meanwhile, p53 antibody was included to find if p53 pathway was involved in the response to *Phyllanthus* treatment.

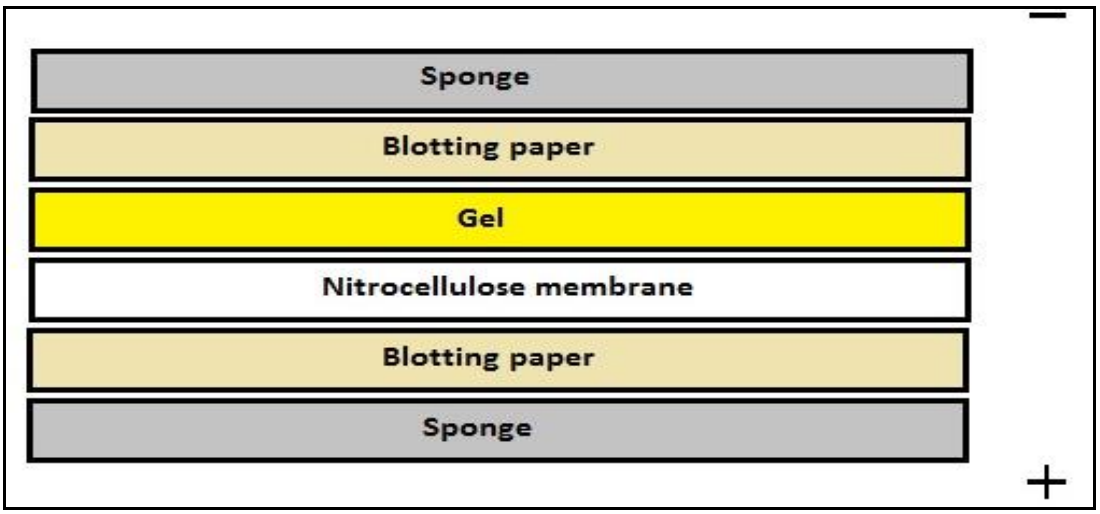


Figure 2.2: Assembly of western blot "Sandwich Stack"

2.8.4 TWO-DIMENSIONAL (2D) GEL ELECTROPHORESIS ASSAY

High resolution 2D electrophoresis gel is necessary for quantitative analysis of differentially expressed proteins and this could be achieved by separation of proteins extracted from cell sample based on their charge and mass via isoelectric focusing (IEF) and polyacrylamide gel electrophoresis (PAGE) respectively (Liao & Huang 2011).

Protein concentrations for each sample were quantitated, followed by overnight rehydration of the same protein amounts for both treated and untreated samples at room temperature. Five hundred microgram of proteins for both A549 and MCF-7 cells were rehydrated on 13cm IPG gel strips with pH 3–11 NL range (GE Healthcare, USA). Rehydrated strips were then transferred into IPG chambers and covered with mineral oil. Electrodes were placed over the hydrated electrode pads positioned at the ends of IPG strips. Proteins were then focused on an Ettan IPGphor Isoelectric Focusing unit

(GE Healthcare, USA), set at 20°C with a current of 50 mA per strip. The parameter setting began with 500V for 1 hour, then inclined to 8000V over 3.5 hours, and finally maintained at 8000V for another 30 minutes. Meanwhile for 7cm IPG gel strips with 3–11 NL range, it began with 300V for 30 minutes, then ramped to 5000V over 1 hour 50 minutes, and finally maintained at 5000V for an additional 25 minutes. The completely focused strips were transferred into equilibration tubes to be stored at -80°C.

Before proceeding with SDS-PAGE, the strips were first subjected to a two-step equilibration procedure for 15 minutes each. First, they were equilibrated with an SDS-PAGE equilibration base buffer composed of 6M urea, 75mM Tris-HCl (pH 8.8), 30% glycerol, 2% SDS, and 2% (w/v) dithiothreitol (DTT). This was followed by equilibration with a similar buffer composition, replacing DTT with 2.5% (w/v) iodoacetamide (IAA). The strips were then placed onto a 12.5% SDS-PAGE gel and sealed with 0.5% agarose sealing solution. Second dimensional separation was carried out using Ettan Dalt*twelve* Separation Unit (GE Healthcare, USA) with an initial low voltage at 100V for 45 minutes, followed by a constant 400V until the separation was completed. The gels were then stained with hot 0.1% Coomasie blue dye for 30 minutes and subsequently fixed using gel fixative solution for another 30 minutes. Finally, the gel was destained with destaining solution and kept in ultrapure water until they were imaged. The gels with stained protein spots were scanned using an Ettan DIGE Imager (GE Healthcare, USA). Three independent gels were run for each treatment ($n = 3$). Gel images were analyzed using PDQuest 2-D Analysis Software (Bio-Rad Laboratories, USA) which performed background removal, normalization, and automatic matching of the detected protein spots. Protein spots with more than 2-fold differential expression that showed significant difference ($p < 0.05$) were selected and excised for mass spectrometry analysis.

2.8.5 PROTEIN IDENTIFICATION BY MATRIX-ASSISTED LASER DESORPTION IONISATION-TIME OF FLIGHT MASS SPECTROMETRY

Protein spots excised from polyacrylamide gels were first destained using 50% acetonitrile (ACN) in 50mM ammonium bicarbonate. When the gel plugs became clear, they were incubated with 10mM DTT in 100mM ammonium bicarbonate for 30 minutes at 60°C followed by incubation with 55mM iodoacetamide in 100mM ammonium bicarbonate for 20 minutes in the dark. The gel plugs were then washed with 50% ACN in 100mM ammonium bicarbonate 3 times, each time for 20 minutes. After that, they were incubated with 100% ACN for 15 min on a shaker and dried using SpeedVac. Next, the dried gel plugs were incubated with 6ng/µl trypsin in 50mM ammonium bicarbonate overnight at 37°C. On the next day, the gel plugs were vortexed briefly and spun down before 50% ACN were added to them and shaked for 15 minutes. The supernatant of the gel spot was then transferred into a fresh tube. Subsequently, the gel plug was incubated with 100% ACN and shakened for another 15 minutes. Similarly, the supernatant was also transferred into the previous tube before the digested samples were completely dried using SpeedVac. The samples can be kept at -20°C until further use. Otherwise, extracted peptides were concentrated or desalted using ZipTip C18 microcolumns (Merck Millipore, Germany).

Prior to MALDI-TOF/TOF analysis, 3µl of each extracted peptide sample solution was mixed with 3µl of alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, USA) matrix solution dissolved in 50% aqueous ACN containing 0.1% trifluoroacetic acid (Sigma-Aldrich, USA). A volume of 0.7µl of each sample was applied onto a MALDI plate and was allowed to air dry at room temperature. Analysis was performed with ABSCIEX 4800 MALDI-TOF/TOF (AB SCIEX, USA) operated in the reflector

for MALDI-TOF/TOF with fully automated mode using the 4800 Series Explorer software at an accelerating voltage of 20kV. Calibration was performed using Mass Standards Kit for Calibration of AB SCIEX TOF/TOF^{TM} Instruments (AB SCIEX, USA). Data collected from the MALDI-TOF/TOF were submitted to the SwissProt database using the MASCOT search algorithm (version 2.1.0, Matrix Science, London, UK). Typical search parameters for both search engines were defined as follows: trypsin digestion allowing up to two tryptic-mass cleavages, variable modifications of oxidation and carbamidomethyl, maximal mass tolerance of 0.1Da, precursor tolerance of 100ppm, and taxonomy *Homo sapiens*. Protein scores greater than 55 were considered significant ($p < 0.05$). The protein with the highest number of peptides was considered as those corresponding to the spot if multiple proteins were identified in a single spot. The proteins identified were then compared with Uniprot KB/Swiss-Prot database and grouped according to the Eukaryotic Orthologous Group of Classifications (COGs).

2.9 DATA ANALYSIS

Results were expressed as the mean ± Standard Error Mean (SEM) of data obtained from three independent experiments. All data were analyzed using one way ANOVA, followed by Dunnett's test for pairwise comparison. $P < 0.05$ was considered statistically significant for all tests.

CHAPTER 3: RESULTS

3.1 POLYPHENOLS IDENTIFICATION IN PHYLLANTHUS *SPP.*

Tables 3.1 and 3.2 show the polyphenol compounds present in both aqueous and methanolic-soluble extracts obtained from four species of *Phyllanthus* (*P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*) after analysis by HPLC coupled with photodiode array (PDA) and MS-MS detection. Twelve main compounds were identified based on their retention times, UV spectra, parent mass spectra and secondary fragmentation patterns. These compounds include gallic acid, galloylglucopyronside, digalloylglucopyronside, trigalloylglucopyronside, tetragalloylglucopyronside, corilagen, geraniin, rutin, quercetin glucoside, quercetin diglucoside, quercetin rhamnoside, and caffeolquinic acid. Generally, aqueous extracts of *Phyllanthus* were found to contain a higher number of polyphenol compounds with the presence of ten compounds out of the twelve compounds detected, as compared to methanolic extracts that contain only four compounds. Meanwhile among the four *Phyllanthus* species, the polyphenol contents in both aqueous (9/10) and methanolic (3/4) *P. urinaria* extracts were found to be the highest. This was followed by *P. watsonii* (aqueous - 9/10 and methanolic – 1/4), *P. niruri* (aqueous - 8/10 and methanolic – 2/4), and *P. amarus* (aqueous - 8/10 and methanolic – 1/4).

Table 3.1: Polyphenol Compounds Detected in Aqueous Extracts of *Phyllanthus* species

Compounds	Retention time	[M-H] m/z	MS-MS fragmentation	*Phyllanthus* species
Gallic acid	3.8	169	125,169	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Galloylglucopyronside	2.8	331	125, 169, 211, 271	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Corilagen	18.0	633	301, 125, 169	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Geraniin	22.0	951	301, 125, 169, 463	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Rutin	26.0	609	301, 179, 151	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Quercetin glucoside	27.0	463	301, 179, 151	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Caffeolquinic acid	23.0	353	191	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Digalloylglucopyronside	15.0	483	125, 169, 211, 271, 313	*P. amarus, P. niruri, P. watsonii*
Quercetin rhamnoside	30.0	447	301, 151	*P. urinaria, P. watsonii*
Trigalloylglucopyronside	23.0	635	125, 169, 211, 271, 313, 465	*P. urinaria*

Table 3.2: Polyphenol Compounds Detected in Methanolic Extracts of *Phyllanthus* species

Compounds	Retention time	[M-H] m/z	MS-MS fragmentation	*Phyllanthus* species
Geraniin	12.0	951	301, 125, 169, 463	*P. amarus, P. niruri, P. urinaria, P. watsonii*
Quercetin diglucoside	9.0	625	463, 301	*P. niruri*
Trigalloylglucopyronside	13.0	635	125, 169, 211, 271, 313, 465	*P. urinaria*
Tetragalloylglucopyronside	15.0	787	169, 211, 313, 465	*P. urinaria*

3.2 ANTIPROLIFERATIVE EFFECTS OF TEST COMPOUNDS

Cellular morphological alterations are usually a result of some biological modifications in the cells' internal environment. Treatment of the cancer cells with *Phyllanthus* extracts produced some changes in the cellular morphologies and these alterations were most probably associated to the antiproliferative effects that the *Phyllanthus* exerted on the A549 and MCF-7 cells, attributed to either a cytotoxic or a cytostatic (cell cycle arrest) effect.

3.2.1 MORPHOLOGICAL ANALYSIS OF *PHYLLANTHUS*-TREATED CELLS

Upon treatment with *Phyllanthus* extracts for 72 hours, both A549 and MCF-7 cells displayed significant morphological changes. Observations showed that some of the cells were already detached from the monolayer and some were rounded up (Figure 3.1A). This suggests that those cells have lost their viability since they no longer have the adherence capability. Furthermore, some of the cells showed some granulation and vacuolation (Figure 3.1B), a sign that the cells were undergoing stress in response to *Phyllanthus* treatment. They also possessed condensed chromatin (Figure 3.1C) and displayed membrane blebbing with the presence of apoptotic bodies (Figure 3.1D), some of the characteristic morphological features in cells that were undergoing the programmed cell death machinery.

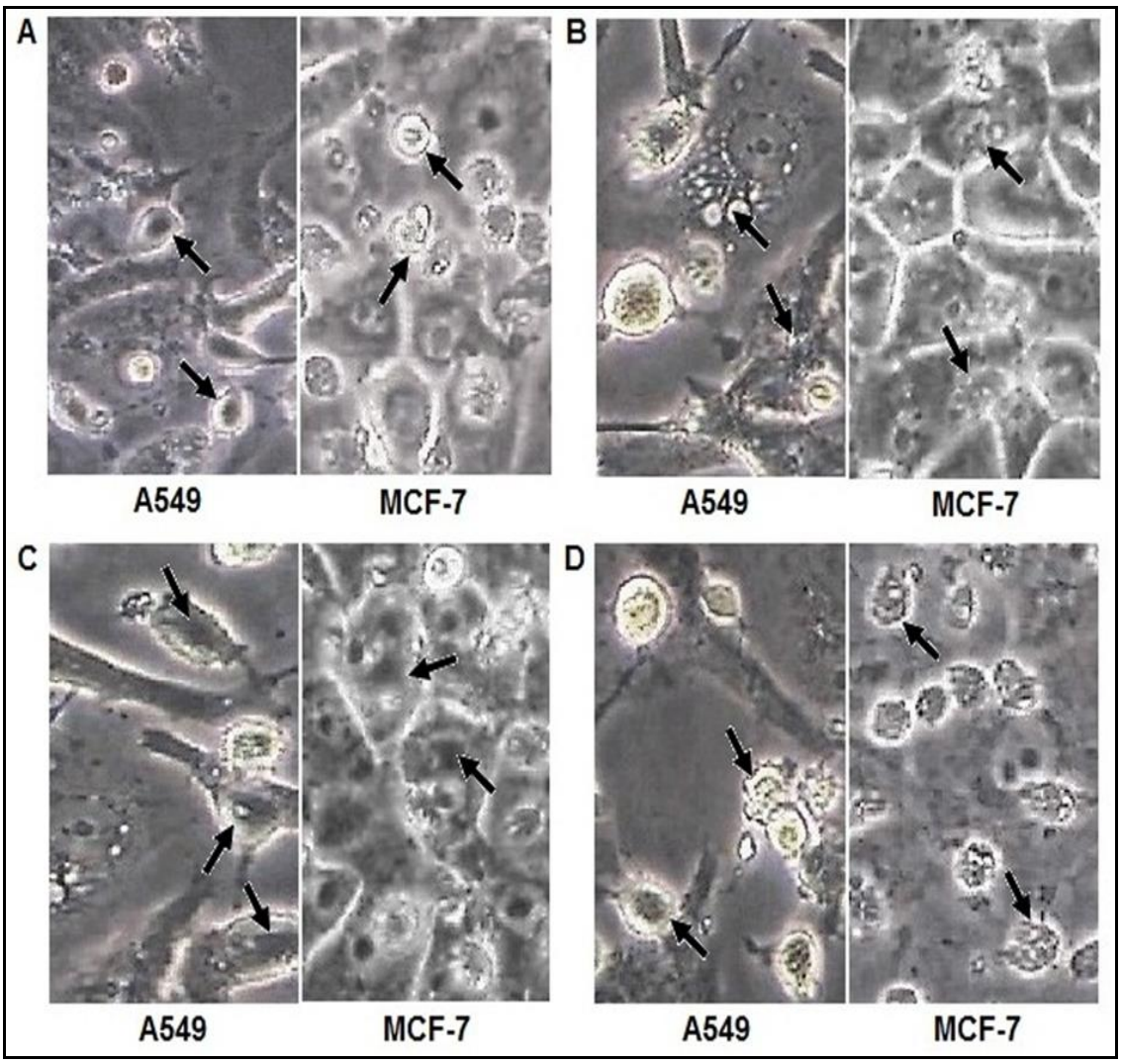


Figure 3.1: Morphological changes of treated A549 and MCF-7 cells.

Red arrows are pointing to each of these morphologies; (A) detached and rounded cells; (B) granulated and vacuolated cells; (C) cells with condensed chromatin; (D) membrane blebbing or apoptotic bodies. (Magnification power = 200×)

3.2.2 CYTOTOXIC EFFECTS OF *PHYLLANTHUS* CRUDE EXTRACTS, FRACTIONS, AND STANDARD DRUGS

The MTS assay was used to investigate the potential cytotoxic effects of *Phyllanthus* extracts and fractions on different cell lines, where the cells were treated at increasing concentrations up to 1000µg/ml for 24, 48, and 72 hours. Two standard drugs, namely Cisplatin and Doxorubicin were used as positive controls in this study, where the cells were treated at increasing concentrations up to 10µg/ml. The effects of aqueous and methanolic *Phyllanthus* extracts as well as standard drugs against A549 and MCF-7 cells were shown in Figures 3.2 to 3.6 while their effects against NL20 and 184B5 were depicted in Figures 3.7 to 3.11. Their cytotoxicity was recorded as IC_{50} (µg/ml) values, which resembles the concentration of extracts, fractions or drugs that kills or inhibits the growth of 50% of the population.

Data obtained showed that *Phyllanthus* extracts have the potential to inhibit growth of A549 and MCF-7 in a time- and dose-dependent manner with minimal effect on NL20 and 184B5. This is because the percentage of cell viability dropped to approximately 25% for A549 (Figures 3.2A, 3.3A, 3.4A, and 3.5A) and 10% for MCF-7 (Figures 3.2B, 3.3B, 3.4B, and 3.5B) after treatment with the aqueous extracts of four *Phyllanthus* species at 500µg/ml for 72 hours. At this similar treatment conditions, the cell viability of NL20 and 184B5 remained above 80% in response to aqueous *P. niruri* (Figures 3.6A and 3.6B) and *P. amarus* (Figures 3.9A and 3.9B) extracts, while their cell viability remained above 60% in response to aqueous *P. urinari*a (Figures 3.7A and 3.7B) and *P. watsonii* (Figures 3.8A and 3.8B) extracts. Likewise, cell viability of NL20 (Figures 3.6C, 3.7C, 3.8C, and 3.9C) and 184B5 (Figures 3.6D, 3.7D, 3.8D, and 3.9D) remained above 50% when they were treated with 250µg/ml of the methanolic extracts of four *Phyllanthus* species for 72 hours, which had caused 100% lethality on both

A549 and MCF-7 cancer cells. This showed that *Phyllanthus* extracts selectively inhibited cancer cells growth without causing major effects on the normal cells.

On the other hand, both Doxorubicin and Cisplatin showed strong cytotoxicity on A549 and MCF-7 cells with IC_{50} values < 10μg/ml. Complete death of cancer cells were observed at 2μg/ml for Doxorubicin (Figures 3.10A and 3.10B) while 20μg/ml for Cisplatin (Figures 3.10C and 3.10D). They were also very toxic to the NL20 and 184B5 normal cell lines (Figure 3.11) with IC_{50} values comparable to the IC_{50} values for cancer cells. One hundred percent NL20 cells were killed at 1.25μg/ml of Doxorubicin and 5μg/ml of Cisplatin, while 100% 184B5 cells lethality was observed at 5μg/ml for Doxorubicin and 10μg/ml for Cisplatin.

Apart from that, the four *Phyllanthus* species displayed different growth inhibitory patterns on both A549 and MCF-7 cells. For A549 cells, they responded towards the presence of the extracts as early as 24 hours post-treatment since IC_{50} values were obtained for three of the aqueous *Phyllanthus* extracts (*P. urinaria* - 820μg/ml; *P. watsonii* - 780μg/ml; *P. amarus* - 840μg/ml) except *P. niruri* (Figures 3.2A, 3.3A, 3.4A, and 3.5A). Contrarily, no IC_{50} values were available for MCF-7 cells after treatment with the aqueous extracts at 1000μg/ml for 24 hours. Nevertheless, upon prolonged incubation for 48 and 72 hours, the cell viability of MCF-7 cells drastically decreased to approximately 20% and 10% respectively (Figures 3.2B, 3.3B, 3.4B, and 3.5B). However, the drop in A549 cell viability became slower at 48 and 72 hours post-treatment. This showed that A549 cells responded more quickly towards *Phyllanthus* but slowed down after the initial exposure, while MCF-7 cells requires a longer exposure time to elicit an abrupt response.

The data also demonstrated that methanolic extracts of *Phyllanthus* exhibited greater cytotoxicity compared to the aqueous extracts. This is because 100% lethality of

both A549 and MCF-7 were achieved after the cells were treated with 250µg/ml of the methanolic extracts. Nonetheless, treatment with aqueous extracts at 1000µg/ml (highest treatment concentration applied in this study) resulted only about 90% growth inhibition.

Among the four *Phyllanthus* species, *P. watsonii* showed the strongest cytotoxicity with lowest IC_{50} values obtained for both aqueous and methanolic extracts on A549 and MCF-7 respectively, followed by *P. urinaria*, *P. amarus*, and *P. niruri* (Table 3.3). In contrast, fractions of *Phyllanthus* were not as effective as the *Phyllanthus* extracts. Fraction 1 showed little growth inhibitory activity against both cancer cell lines due to the high IC_{50} values. Since IC_{50} is defined as the concentration of the test compound that causes 50% growth inhibition of the cell population, a higher IC_{50} value signifies the need of a greater amount of the test compound to exert a similar 50% growth inhibition and is therefore rendered less effective. Although fraction 2 was relatively more toxic to cancer cells compared to fraction 1, but it is not as effective as *Phyllanthus* extracts as a whole. In addition, fraction 2 also displayed toxicity to the normal cells that renders them less useful.

In order to evaluate the pharmacological effects of *Phyllanthus* extracts on A549 and MCF-7 cells, it was necessary to choose a non-toxic but effective dose. From the toxicity testing, we obtained three sets of IC_{50} values at three different incubation time points (24, 48, and 72 hours). However, IC_{50} values at 72 hours incubation as shown in Table 3.3 were chosen for subsequent experiments (highlighted in yellow) since the cells' viability remained above 80% when the cells were treated at these concentrations for 24 and 48 hours. The maximum incubation time for most of the pharmacological assays was only up to 48 hours.

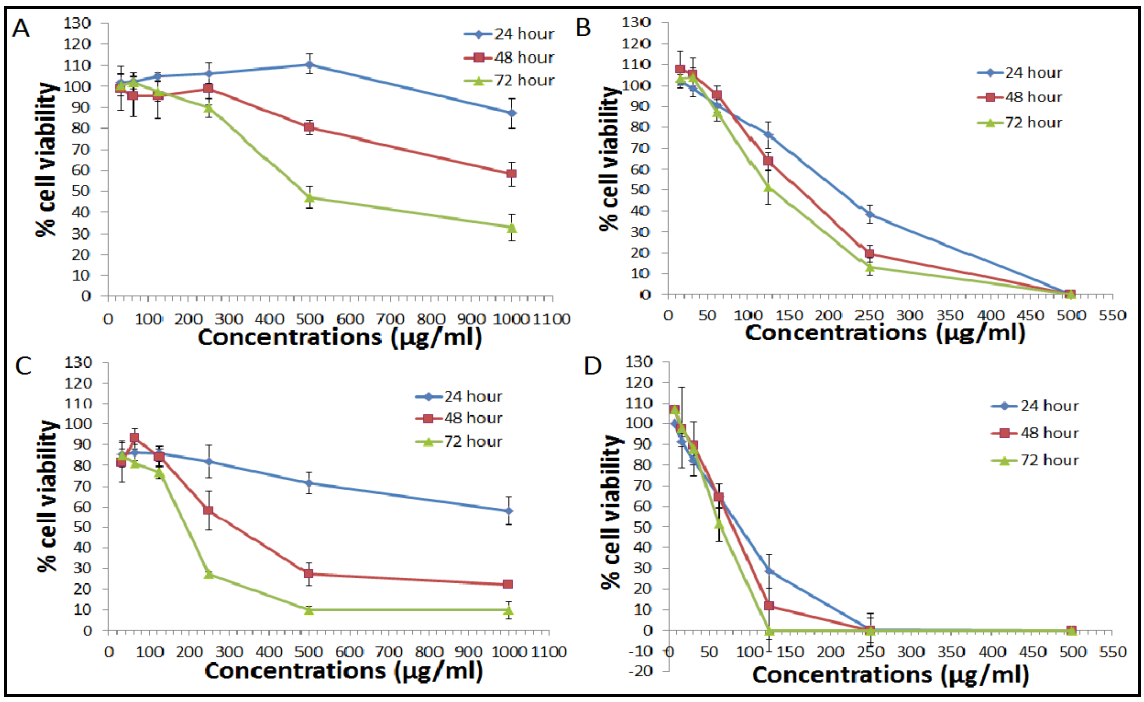


Figure 3.2: Growth inhibition effect of *P. niruri* on A549 and MCF-7.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on A549; (C) aqueous extracts and (D) methanolic extracts on MCF-7. Error bar indicates the standard error mean of three independent experiments.

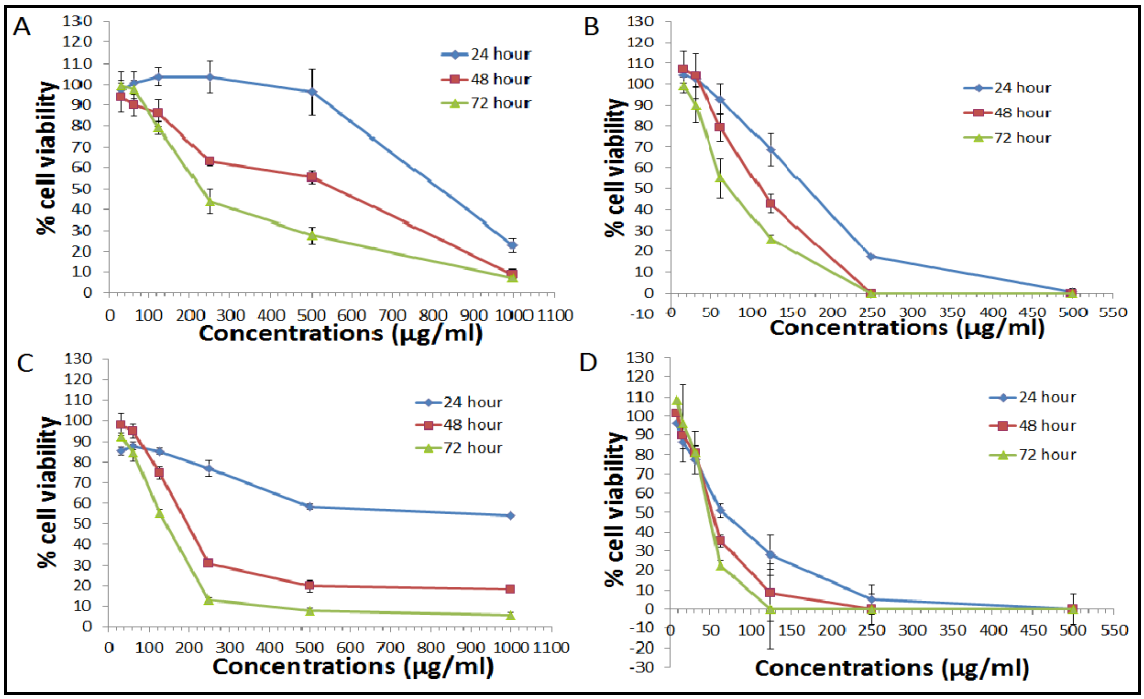


Figure 3.3: Growth inhibition effect of *P. urinaria* on A549 and MCF-7.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on A549; (C) aqueous extracts and (D) methanolic extracts on MCF-7. Error bar indicates the standard error mean of three independent experiments.

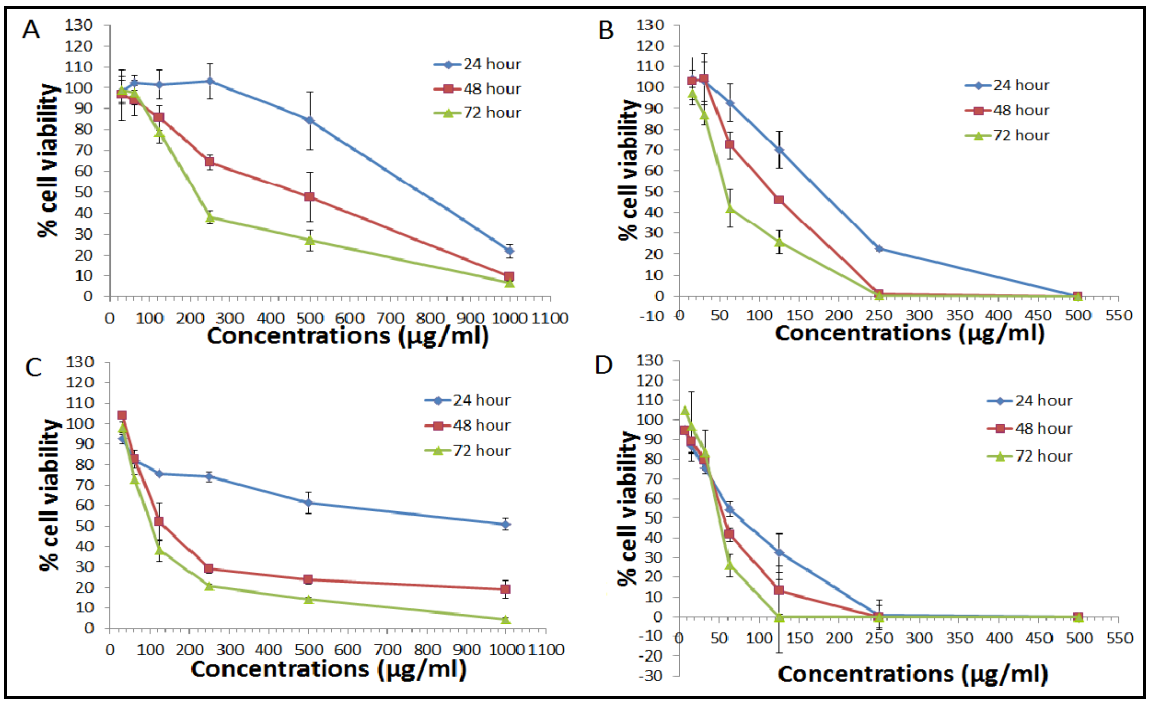


Figure 3.4: Growth inhibition effect of *P. watsonii* on A549 and MCF-7.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on A549; (C) aqueous extracts and (D) methanolic extracts on MCF-7. Error bar indicates the standard error mean of three independent experiments.

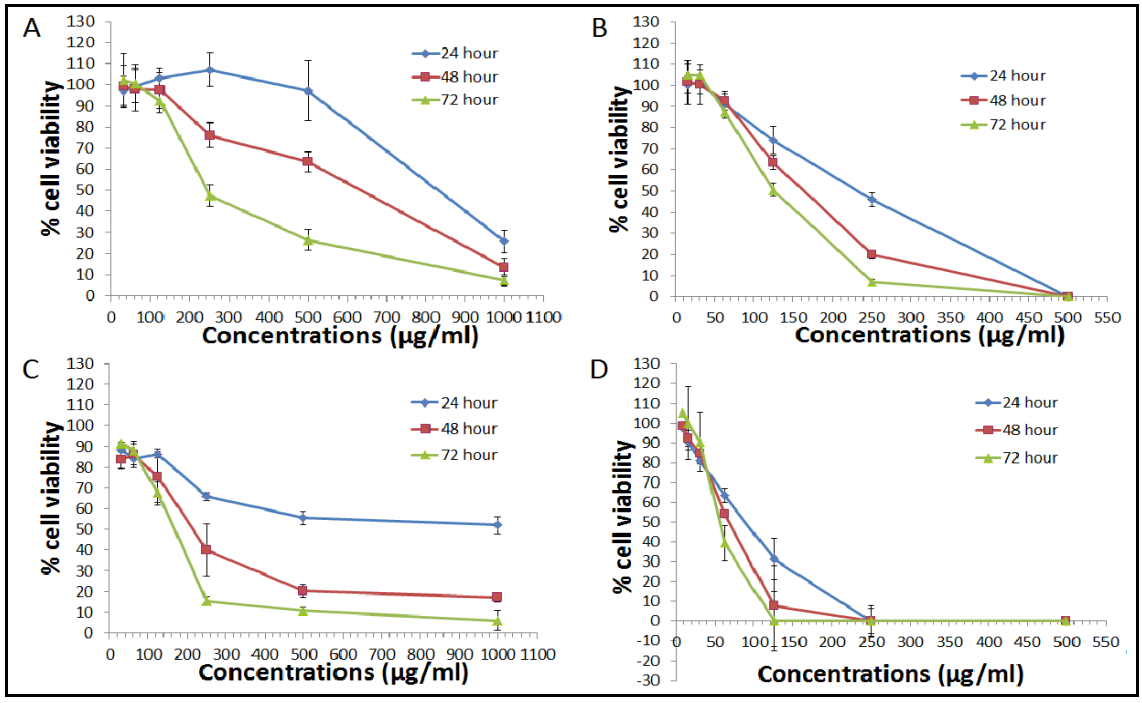


Figure 3.5: Growth inhibition effect of *P. amarus* on A549 and MCF-7.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on A549; (C) aqueous extracts and (D) methanolic extracts on MCF-7. Error bar indicates the standard error mean of three independent experiments.

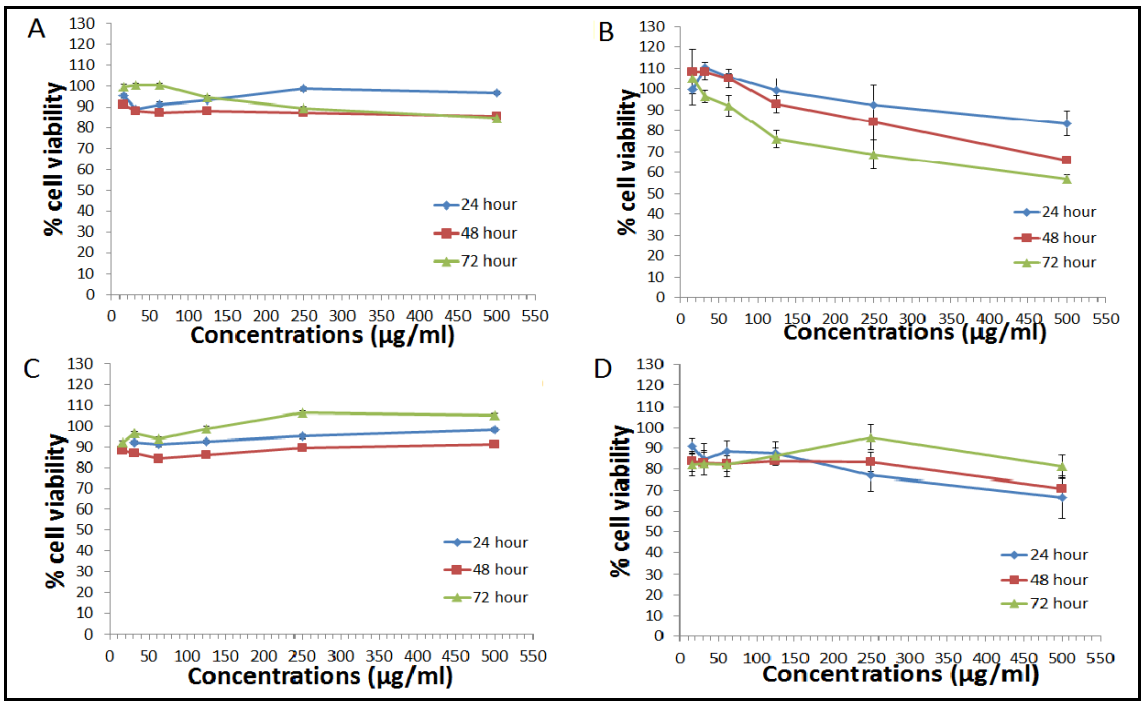


Figure 3.6: Growth inhibition effect of *P. niruri* on NL20 and 184B5.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on NL20; (C) aqueous extracts and (D) methanolic extracts on 184B5. Error bar indicates the standard error mean of three independent experiments.

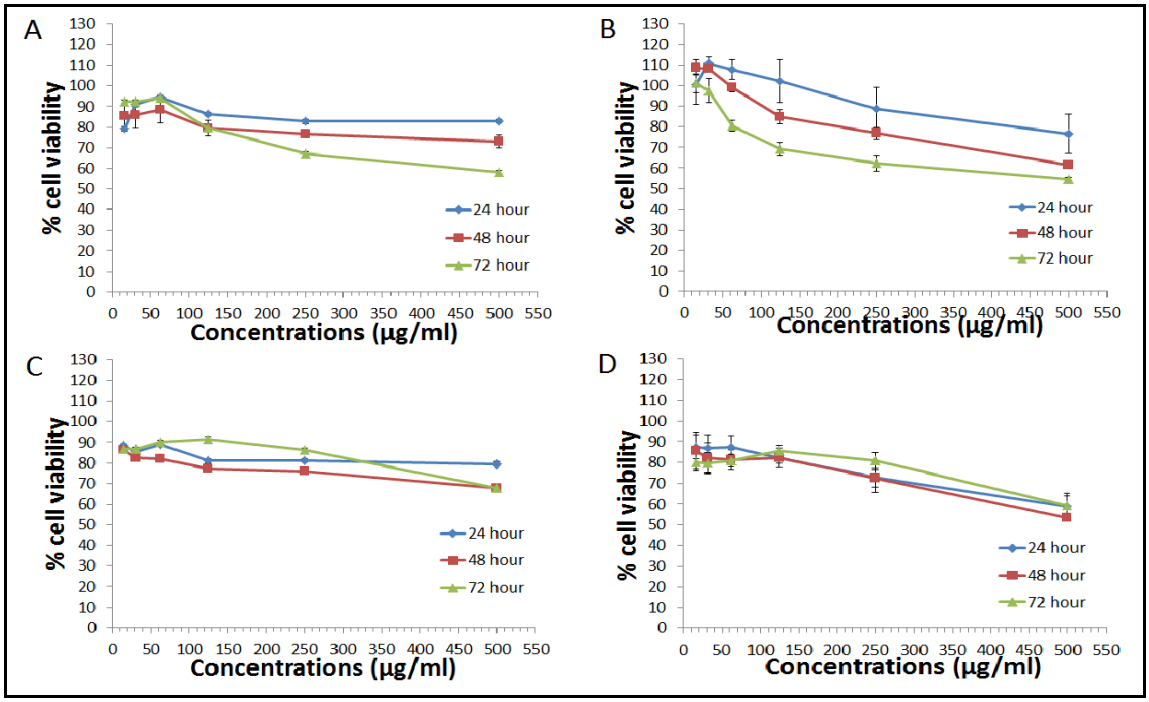


Figure 3.7: Growth inhibition effect of *P. urinaria* on NL20 and 184B5.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on NL20; (C) aqueous extracts and (D) methanolic extracts on 184B5. Error bar indicates the standard error mean of three independent experiments.

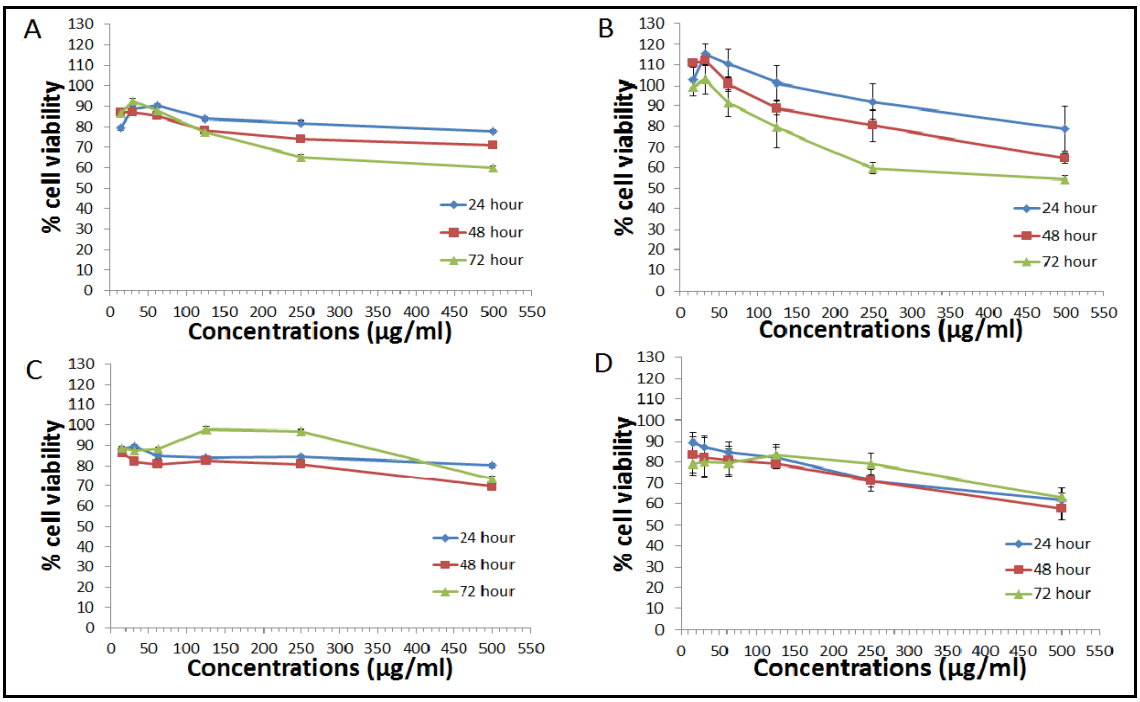


Figure 3.8: Growth inhibition effect of *P. watsonii* on NL20 and 184B5.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on NL20; (C) aqueous extracts and (D) methanolic extracts on 184B5. Error bar indicates the standard error mean of three independent experiments.

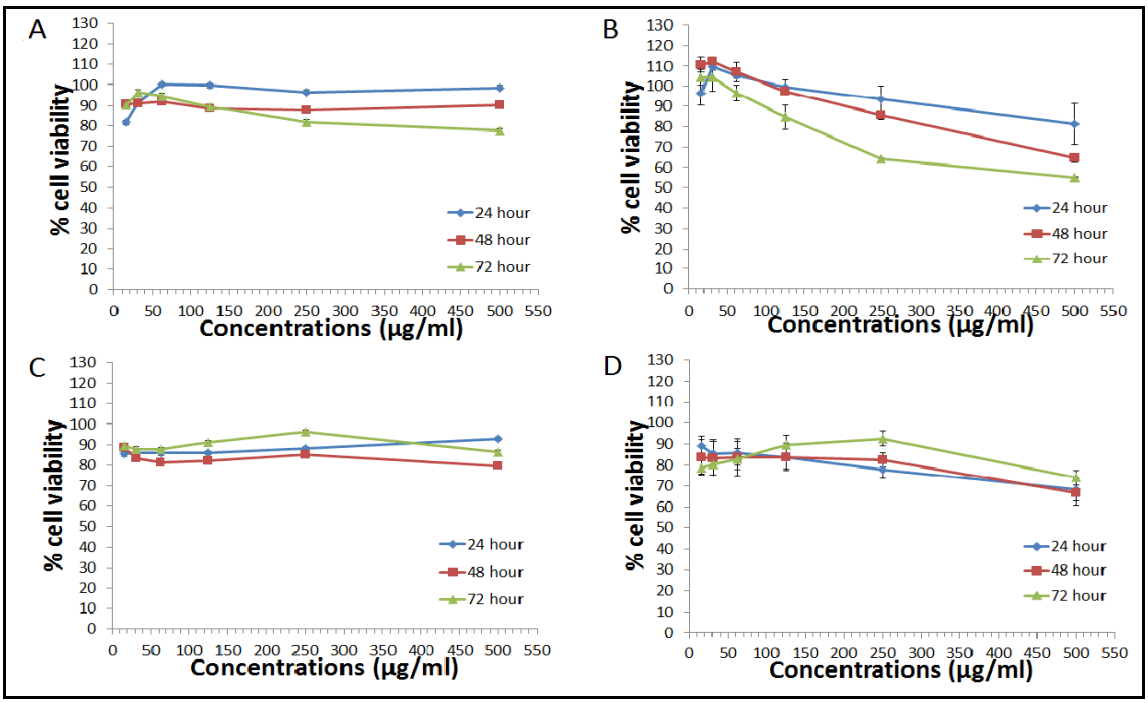


Figure 3.9: Growth inhibition effect of *P. amarus* on NL20 and 184B5.

Figure above shows the effects of (A) aqueous extracts and (B) methanolic extracts on NL20; (C) aqueous extracts and (D) methanolic extracts on 184B5. Error bar indicates the standard error mean of three independent experiments.

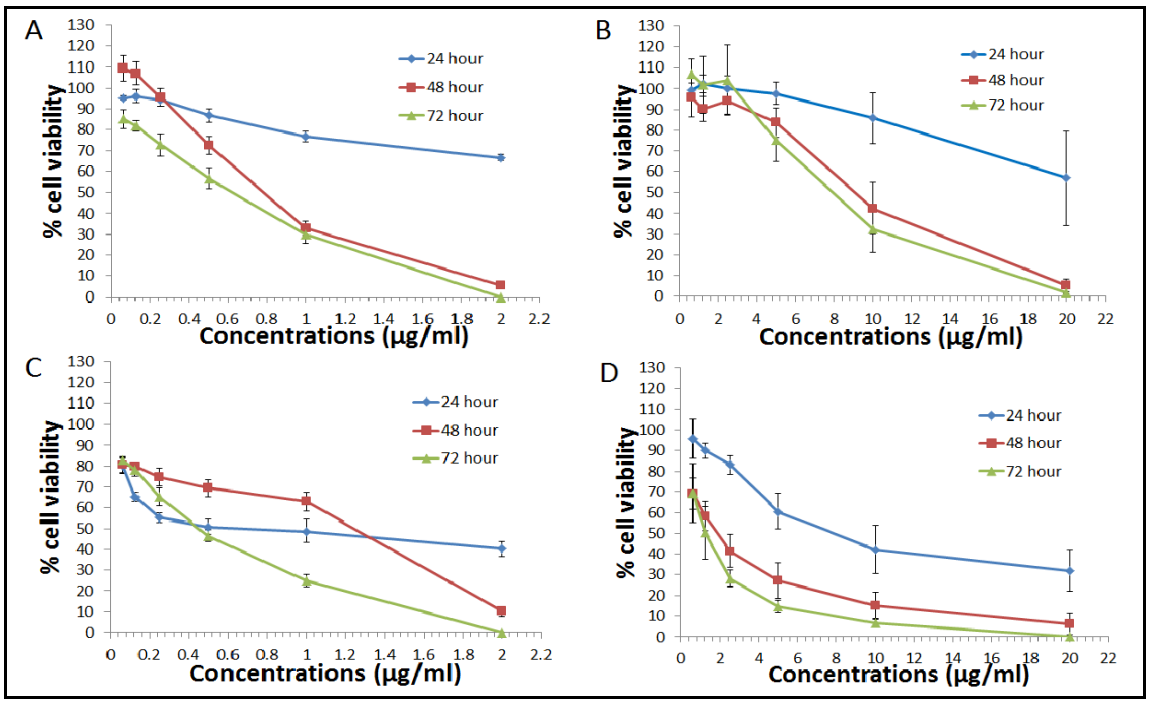


Figure 3.10: Growth inhibition effect of standard drugs on A549 and MCF-7.

Figure above shows the effects of (A) doxorubicin and (B) cisplatin on A549; (C) doxorubicin and (D) cisplatin on MCF-7. Error bar indicates the standard error mean of three independent experiments.

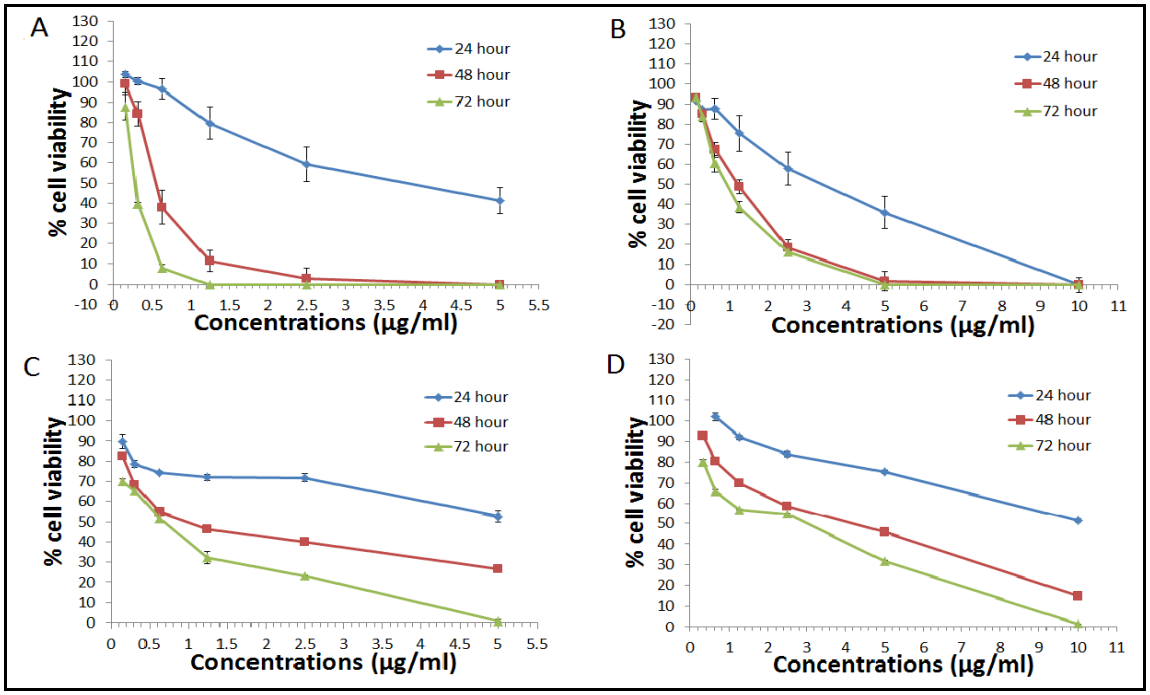


Figure 3.11: Growth inhibition effect of standard drugs on NL20 and 184B5.

Figure above shows the effects of (A) doxorubicin and (B) cisplatin on NL20; (C) doxorubicin and (D) cisplatin on 184B5. Error bar indicates the standard error mean of three independent experiments.

Table 3.3: Cytotoxic effect (IC_{50}) of *Phyllanthus* extracts, fractions, and standard drugs.

Table below showed the IC_{50} (µg/ml) values of various test compounds against two cancer (A549, MCF-7) and two normal cell lines (NL20, 184B5) after 72 hours incubation. Data is expressed as a mean of three independent experiments ± Standard Error Mean (SEM).

			IC_{50} (µg/ml) ± SEM			
			Cancer Cell Lines		Normal Cell Lines	
		Solvents	A549	MCF-7	NL20	184B5
Plant Extracts	*P. niruri* (*P.n*)	Aqueous	466.7 ± 41.63	179.7 ± 0.58	> 500.0	> 500.0
		Methanolic	128.3 ± 17.56	62.3 ± 9.07	> 500.0	> 500.0
	P. urinaria (P.u)	Aqueous	215.0 ± 21.79	139.3 ± 1.16	> 500.0	> 500.0
		Methanolic	69.0 ± 11.53	48.7 ± 10.02	> 500.0	> 500.0
	P. watsonii (P.w)	Aqueous	198.3 ± 10.41	104.0 ± 10.39	> 500.0	> 500.0
		Methanolic	61.3 ± 16.17	49.0 ± 8.19	> 500.0	> 500.0
	P. amarus (P.a)	Aqueous	240.0 ± 26.46	156.7 ± 5.77	> 500.0	> 500.0
		Methanolic	126.7 ± 7.64	56.3 ± 6.66	> 500.0	> 500.0
Standard Drugs	Cisplatin		7.6 ± 1.10	1.4 ± 0.54	0.9 ± 0.05	3.0 ± 0.03
	Doxorubicin		0.6 ± 0.08	0.4 ± 0.05	0.3 ± 0.02	0.6 ± 0.03
Fraction 1	*P. niruri (P.n)*	Aqueous	380.0 ± 18.03	438.3 ± 11.55	266.7 ± 41.93	283.3 ± 25.17
	P. urinaria (P.u)	Aqueous	> 500.0	> 500.0	> 500.0	231.7 ± 18.93
	P. watsonii (P.w)	Aqueous	395.0 ± 8.66	376.7 ± 2.89	241.7 ± 20.21	230.0 ± 26.46
	P. amarus (P.a)	Aqueous	> 500.0	> 500.0	> 500.0	> 500.0
Fraction 2	*P. niruri (P.n)*	Aqueous	228.3 ± 5.77	81.7 ± 16.07	108.3 ± 5.77	230.0 ± 50.74
	P. urinaria (P.u)	Aqueous	225.0 ± 13.23	61.7 ± 12.58	95.0 ± 5.00	230.0 ± 13.23
	P. watsonii (P.w)	Aqueous	225.0 ± 43.30	46.7 ± 10.41	105.0 ± 5.00	201.7 ± 20.21
	P. amarus (P.a)	Aqueous	264.3 ± 45.24	70.0 ± 17.32	106.7 ± 7.64	213.3 ± 54.85

3.2.3 *PHYLLANTHUS* DID NOT MODULATE CELL CYCLE

Cell cycle study was carried out by staining the DNA contents of cells with fluorescent propidium iodide dye. In repeated experiments, our data did not demonstrate any cell cycle phase arrest in both the A549 and MCF-7 cells treated with various *Phyllanthus* extracts since the percentage of gated cells for each cell cycle phases (G_0/G_1, S, and G_2/M) did not change significantly ($p > 0.05$) between the untreated and extracts-treated cells (Figures 3.12 and 3.13). However, at increasing incubation time points (24, 48, and 72 hours), the percentage of gated cells for each of the cell cycle phases decreased gradually for both A549 and MCF-7 cell lines with a concurrent increase in the percentage of dead cells (increase in Sub G_1 phase). A Sub G_1 phase increase was considered as one of the indicators for the presence of apoptotic cells.

On the other hand, there is an accumulation of cells at G_2/M phase for both A549 and MCF-7 treated with Cisplatin and Doxorubicin drugs for 72 hours (Figures 3.12C and 3.13C). Nonetheless, a cell cycle arrest was more profound in MCF-7 cells since the G_2/M phase increase compared to untreated control was already significant after 24 hours incubation (Cisplatin – 17%; Doxorubicin – 34%), and continuously increased after 48 hours (Cisplatin and Doxorubicin – 37%) and 72 hours (Cisplatin – 45%; Doxorubicin – 43%). Contrarily, the increase in G_2/M phase of treated A549 cells was only significant after 48 hours (Cisplatin – 10%; Doxorubicin – 32%) and 72 hours (Cisplatin – 27%; Doxorubicin – 41%) incubation. Similarly, an increase in the number of cells arrested at Sub G_1 phase was noted.

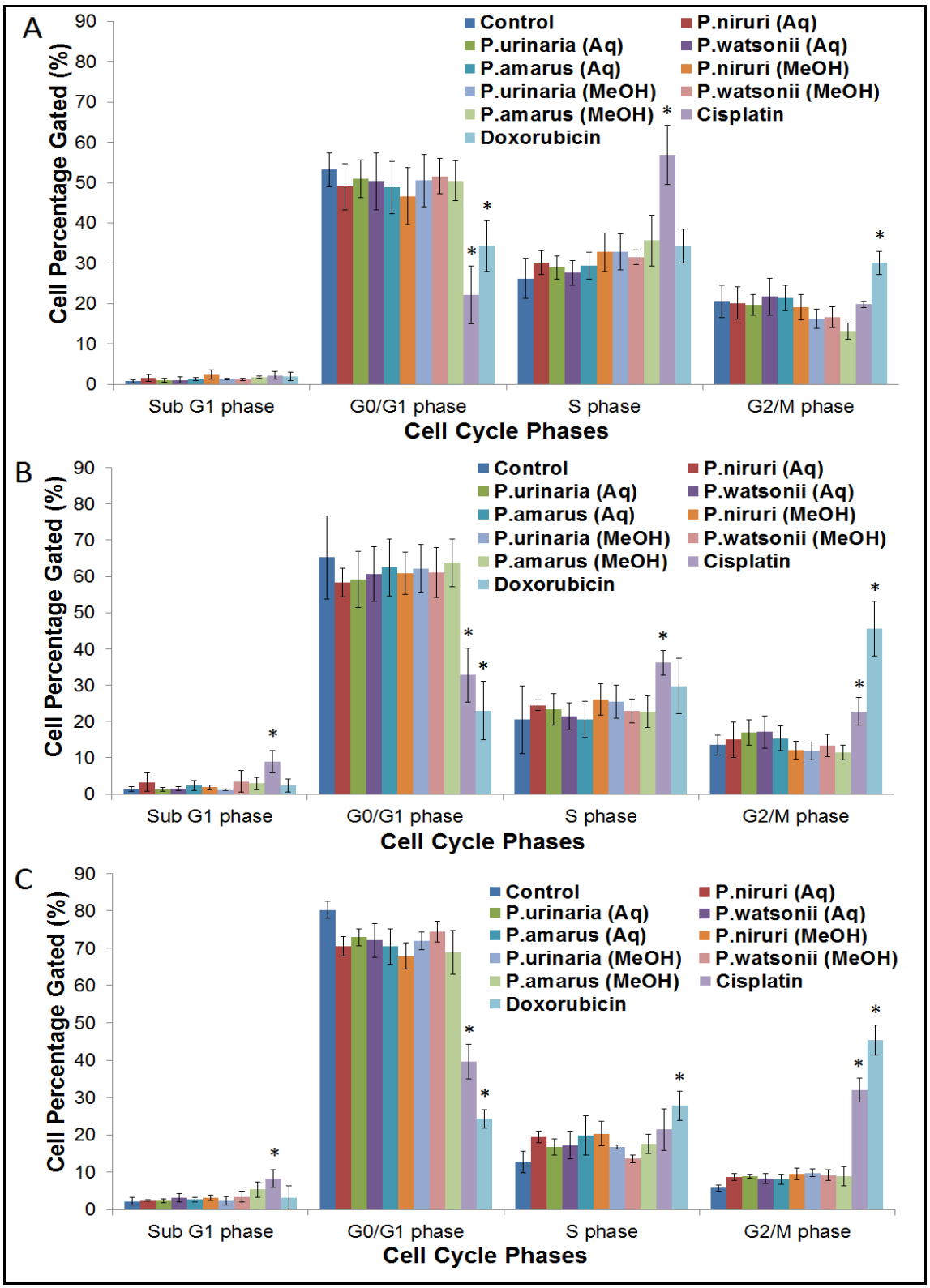


Figure 3.12: Cell cycle phase distribution of A549 in response to *Phyllanthus* extracts.

Figure above shows the percentage distribution of A549 cells in different phases of the cell cycle after treatment with both aqueous and methanolic *Phyllanthus* extracts and standard drugs at their IC_{50} (µg/ml) concentrations for (A) 24 hours, (B) 48 hours, and (C) 72 hours. Error bar indicates the standard error mean of three independent experiments. Aq – Aqueous; MeOH – Methanolic; Control – untreated cells. *$P < 0.05$ vs untreated control.

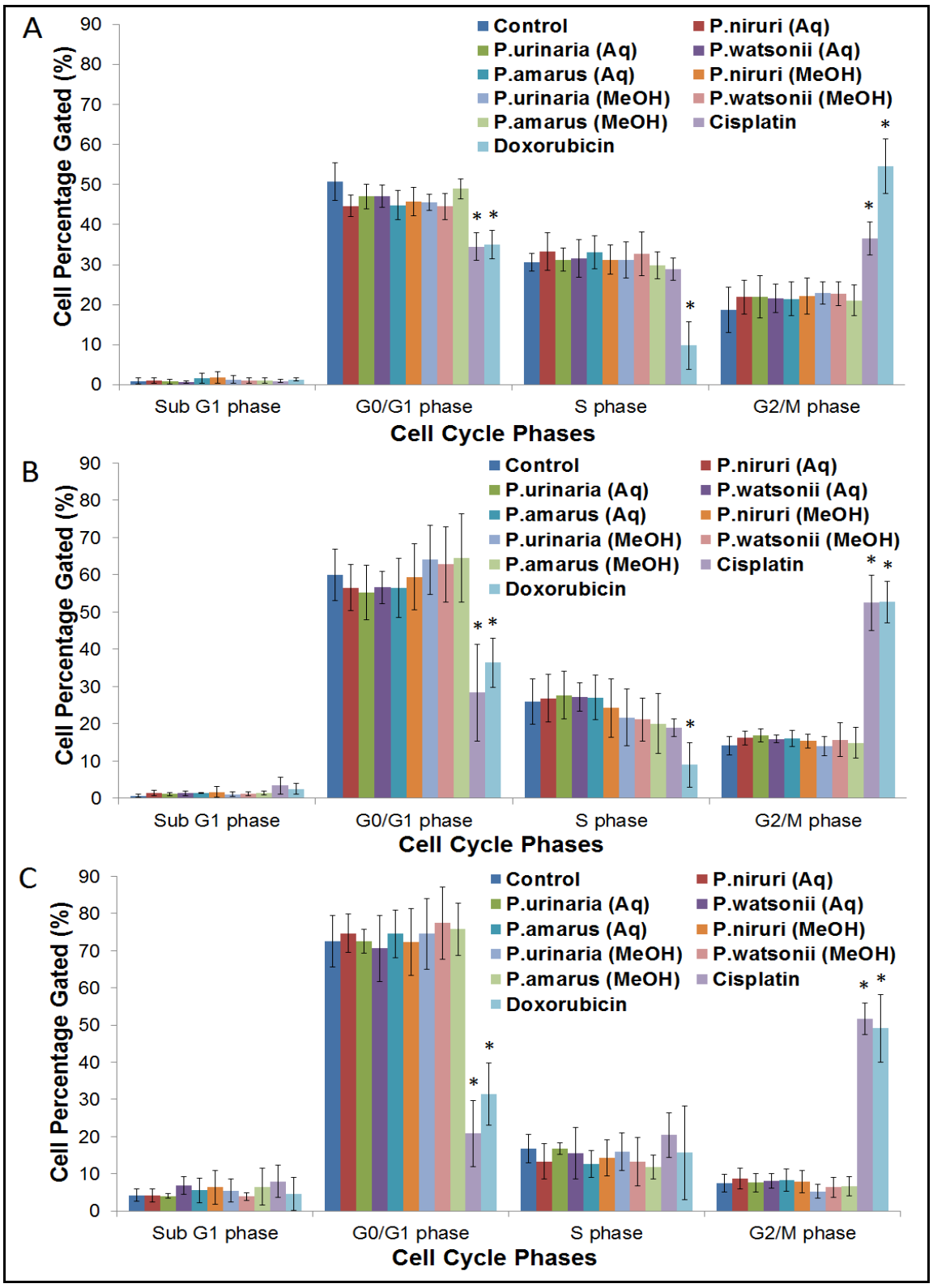


Figure 3.13: Cell cycle phase distribution of MCF-7 in response to *Phyllanthus* extracts.

Figure above shows the percentage distribution of MCF-7 cells in different phases of the cell cycle after treatment with both aqueous and methanolic *Phyllanthus* extracts and standard drugs at their IC_{50} (µg/ml) concentrations for (A) 24 hours, (B) 48 hours, and (C) 72 hours. Error bar indicates the standard error mean of three independent experiments. Aq – Aqueous; MeOH – Methanolic; Control – untreated cells. *$P < 0.05$ vs untreated control.

3.3 ANTIMETASTASIS EFFECTS OF *PHYLLANTHUS*

Metastasis is often the pivotal factor that determines the survival of a cancer patient. It is a multistep process beginning with cell invasion, migration, adhesion, as well as tumor establishment at a secondary site, and these steps are often associated with their ability to express matrix metalloproteinase enzymes. Treatment with *Phyllanthus* had demonstrated its ability to halt these various stages in metastasis, hence blocking the spreading of cancer cells from its primary site to other parts of the body.

3.3.1 EFFECTS OF *PHYLLANTHUS* EXTRACTS ON CELL INVASION

The antiinvasive effect of *Phyllanthus* was studied by using a transwell chamber coated with basement membrane extract that occludes the pores of the membrane. This is to avoid non-invasive cells from migrating through the membrane while allowing the invasive cells to detach themselves from surrounding cells and invade through the matrix in response to a chemoattractant. Therefore, the number of cells that were able to pass through the matrix and 8μm pore into the lower well emulates their invasive potential.

As shown in Figure 3.14, the *Phyllanthus* extracts prevented the invasion of both A549 and MCF-7 cells in a dose-dependent manner ($P < 0.05$). When the cells were treated with extracts at their respective IC_{50} concentrations, they inhibited invasion of A549 cells (20% - 40%) more strongly as compared to MCF-7 cells (less than 10%). At higher concentrations however, both cancer cells' invasion was inhibited to a greater extent (40% - 60% for A549 cells and 30% - 50% for MCF-7 cells). Among the four *Phyllanthus* species, *P. watsonii* and *P. amarus* showed better inhibitory activity on A549 cells invasion while aqueous *P. niruri* and methanolic *P. urinaria* is more effective in inhibiting MCF-7 cells invasion.

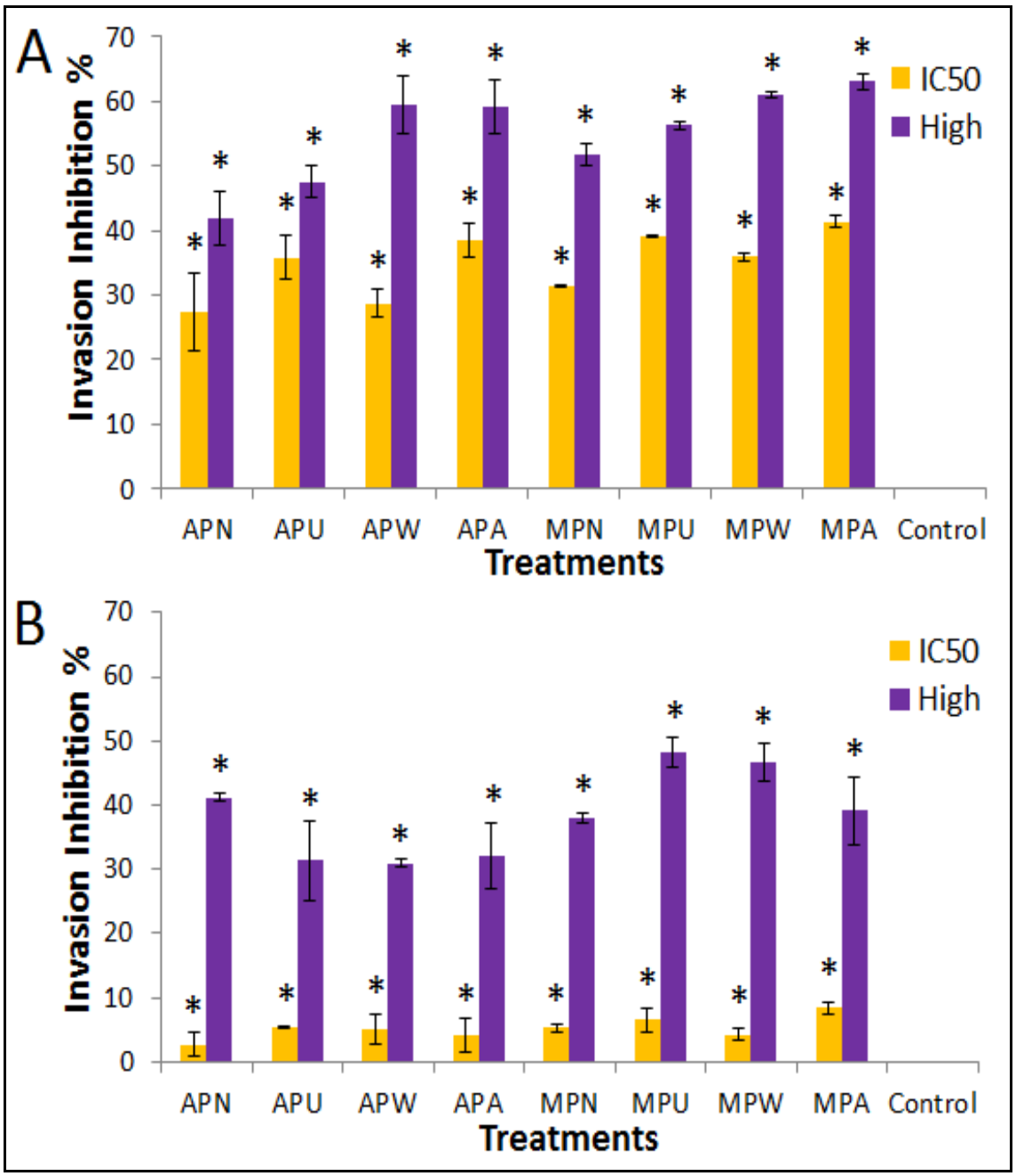


Figure 3.14: Invasion inhibition percentage induced by aqueous and methanolic extracts of *Phyllanthus* on (A) A549 and (B) MCF-7 cells treated at varying concentrations.

High – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; Control – untreated cells. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

3.3.2 EFFECTS OF *PHYLLANTHUS* EXTRACTS ON CELL MOTILITY

The effect of *Phyllanthus* on A549 and MCF-7 cell motility was determined by cell migration and scratch motility assays. From the cell migration assay, *Phyllanthus* exhibited a reduction in the migration of A549 and MCF-7 cells in a dose-dependent manner (Figure 3.15). The migration of cells was significantly decreased ($P < 0.05$) when treated with increasing concentrations of aqueous extracts (50 - 500μg/ml) and methanolic extracts (20 - 200μg/ml). Even at the lowest concentration tested, migration inhibition exerted by *Phyllanthus* was greater than 20% for A549 cells and greater than 40% for MCF-7 cells. Meanwhile at the highest concentration tested, inhibition of A549 and MCF-7 cells migration was greater than 70% and 80% respectively.

When the A549 cells were treated at the lowest and highest concentrations, there was not a great difference between the activities of the four *Phyllanthus* species and the two types of extracts. However, A549 cells treated at the respective IC_{50} concentrations of methanolic extracts showed a lesser capability to migrate than the aqueous extracts. This phenomenon was not observed in MCF-7 cells treated with the respective IC_{50} concentrations of the aqueous and methanolic *Phyllanthus* extracts.

Figures 3.16, 3.17, 3.18, 3.19, 3.20, and 3.21 shows the wound closure activity of A549 and MCF-7 cells in the presence of the four *Phyllanthus* species. From the figures, both the untreated A549 and MCF-7 cells exhibited a complete wound closure activity after 24 and 48 hours incubation respectively. A shorter duration needed by A549 cells to close the wound revealed that they have a higher capability to migrate than MCF-7 cells. Nonetheless, the *Phyllanthus*-treated cells exhibited decreased ability to close the wound at the end of their respective incubation time by forming asymmetric lamellipodial protrusions into the denuded zone as marked by the red arrows. Generally, methanolic *Phyllanthus* extracts had a greater inhibitory activity on the A549 cells

migration since the gap of cells treated with methanolic extracts was more clearly visible compared to the aqueous extracts after 24 hours. Meanwhile, both aqueous and methanolic extracts were equally effective to inhibit MCF-7 cells migration since only a limited closure of wound was noticed after 48 hours incubation.

The numbers of migrated cells were calculated from five randomly selected fields per sample and were represented as migration inhibition rate for A549 and MCF-7 cells in Figures 3.22A and 3.22B respectively. As the incubation time increased, the migration inhibition rate were also significantly increased ($P < 0.05$). A more rapid migration inhibitory action of *Phyllanthus* was observed at 6 hours post-treatment in A549 cells and 12 hours post-treatment in MCF-7 cells. Approximately 85% of A549 cells migration and 95% of MCF-7 cells migration were inhibited at the end of their 24 and 48 hours incubation respectively. Among the four *Phyllanthus* species, *P. urinaria* and *P. watsonii* displayed enhanced activity compared to the other two species.

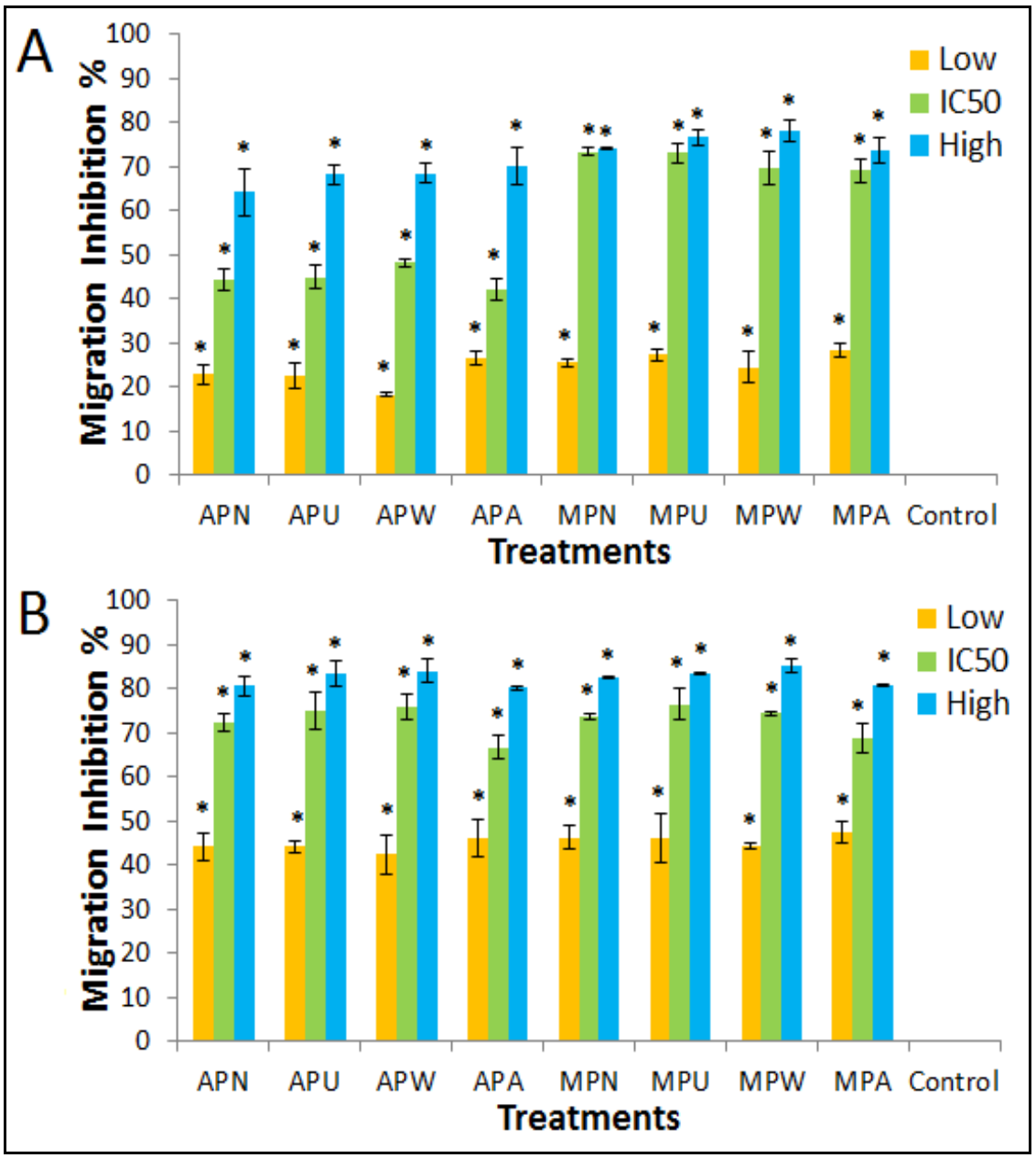


Figure 3.15: Migration inhibition percentage of aqueous and methanolic extracts of *Phyllanthus* on (A) A549 and (B) MCF-7 cells treated at varying concentrations.

High – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; Low – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; Control – untreated cells. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

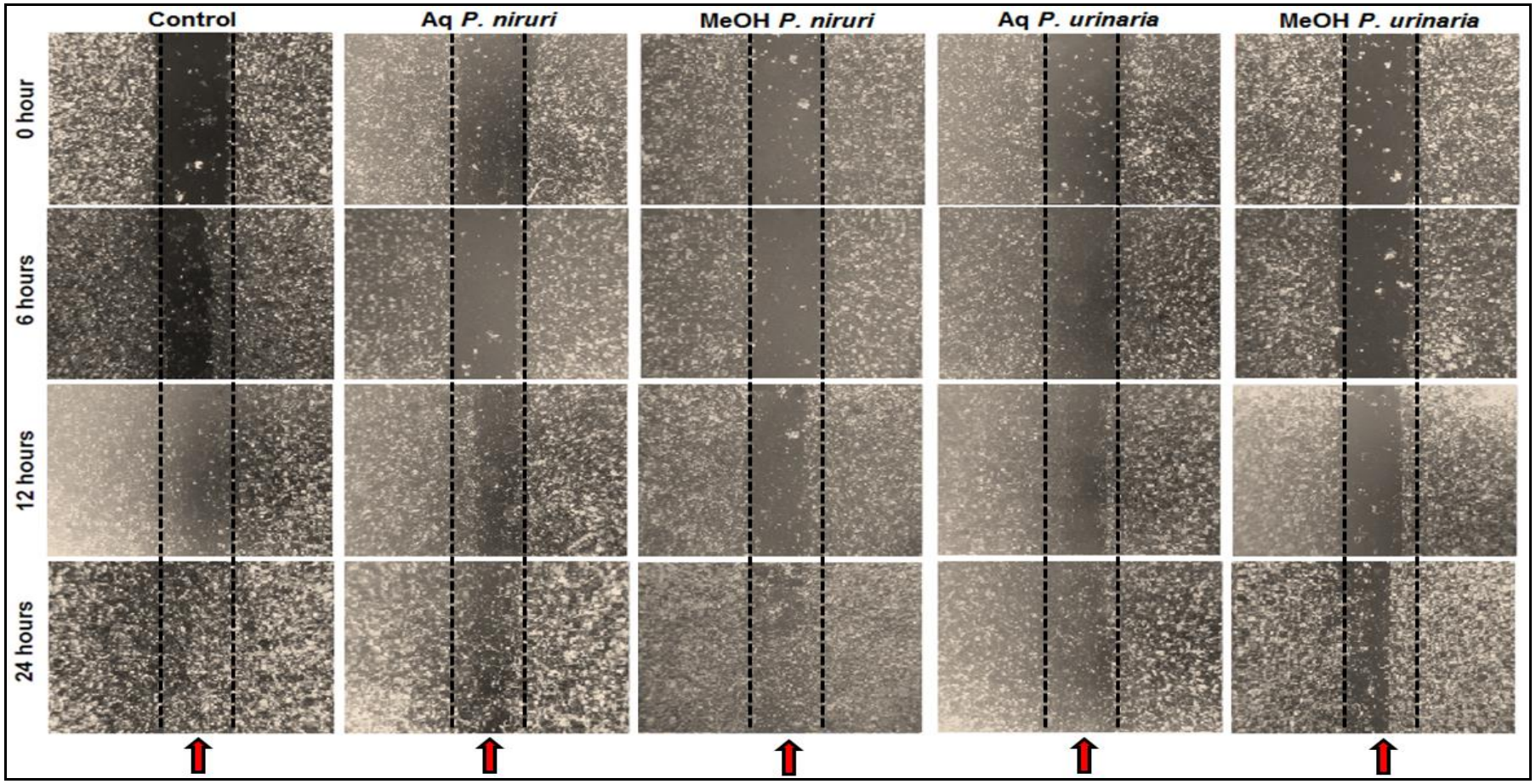


Figure 3.16: Wound closure activity of A549 in response to *P. niruri* and *P. urinaria*.

Figure above shows the migration activity of A549 cells across the wound after treated with aqueous (Aq) and methanolic (MeOH) *P. niruri* and *P. urinaria* extracts for 24 hours. Typical result from three independent experiments is shown. (Magnification power: 40×)

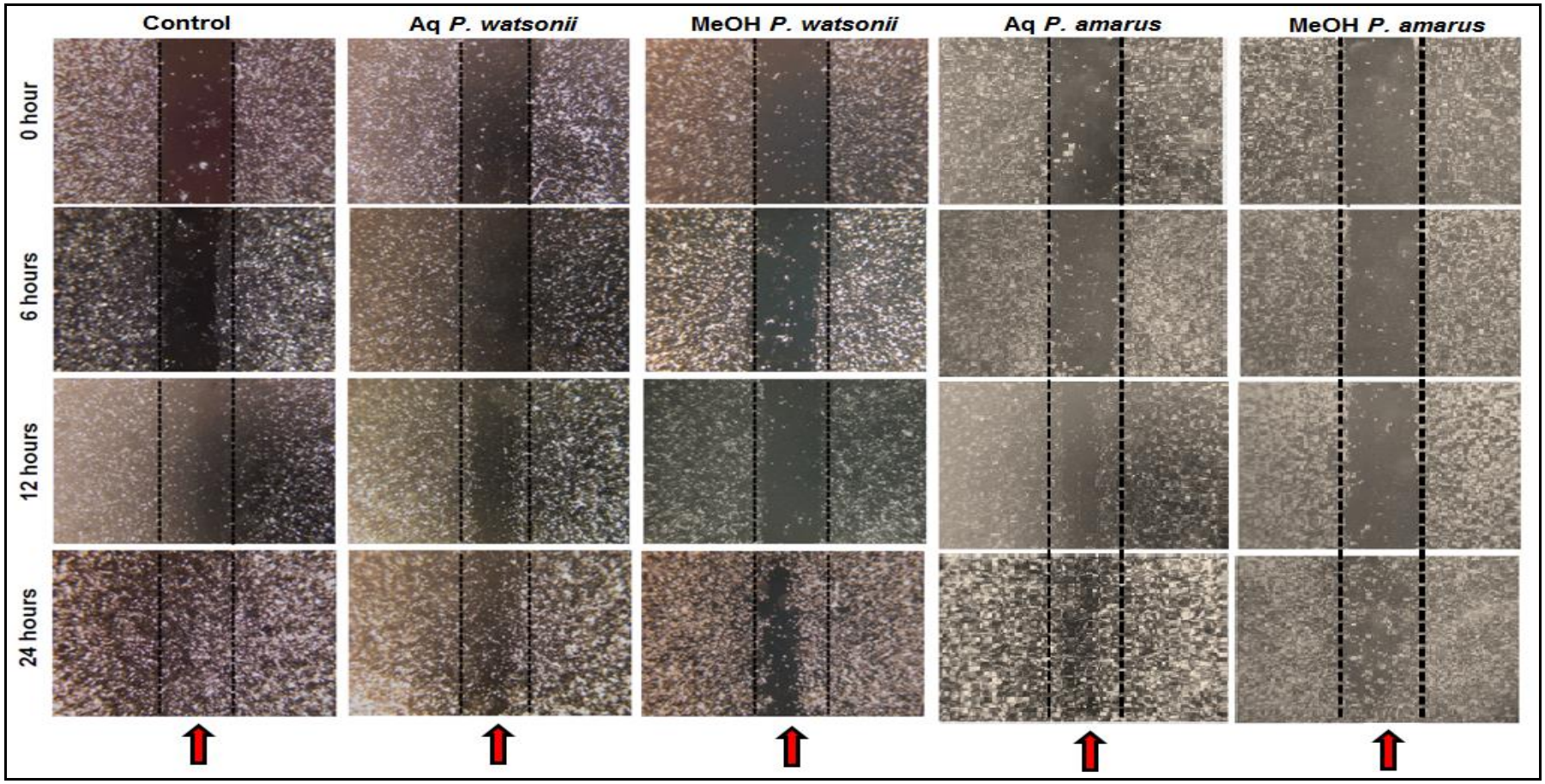


Figure 3.17: Wound closure activity of A549 in response to *P. watsonii* and *P. amarus.*

Figure above shows the migration activity of A549 cells across the wound after treated with aqueous (Aq) and methanolic (MeOH) *P. watsonii* and *P. amarus* extracts for 24 hours. Typical result from three independent experiments is shown. (Magnification power: 40×)

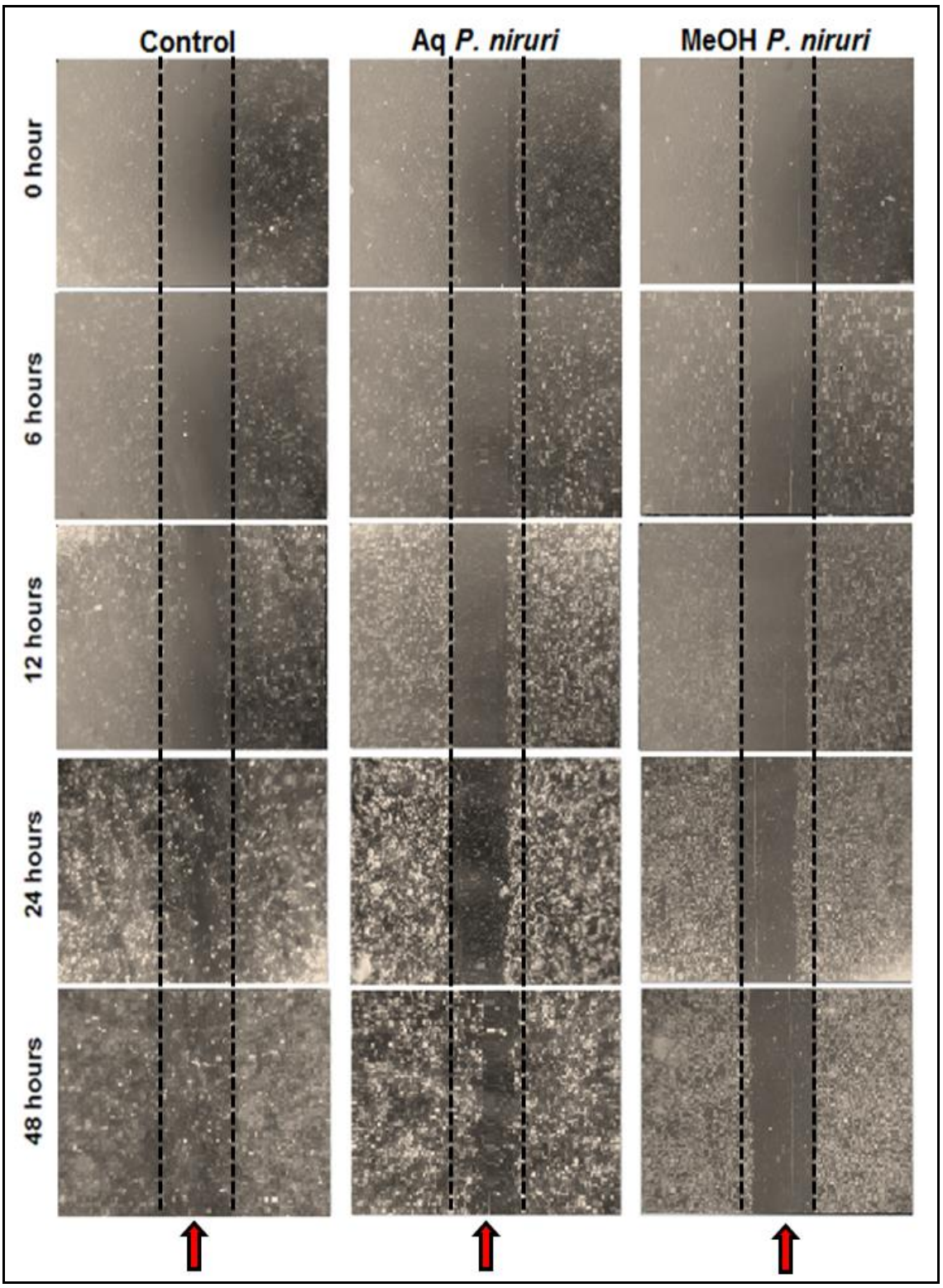


Figure 3.18: Wound closure activity of MCF-7 in response to *P. niruri*.

Figure above shows the migration activity of MCF-7 cells across the wound after treated with aqueous (Aq) and methanolic (MeOH) *P. niruri* extracts for 48 hours. Typical result from three independent experiments is shown. (Magnification power: 40×)

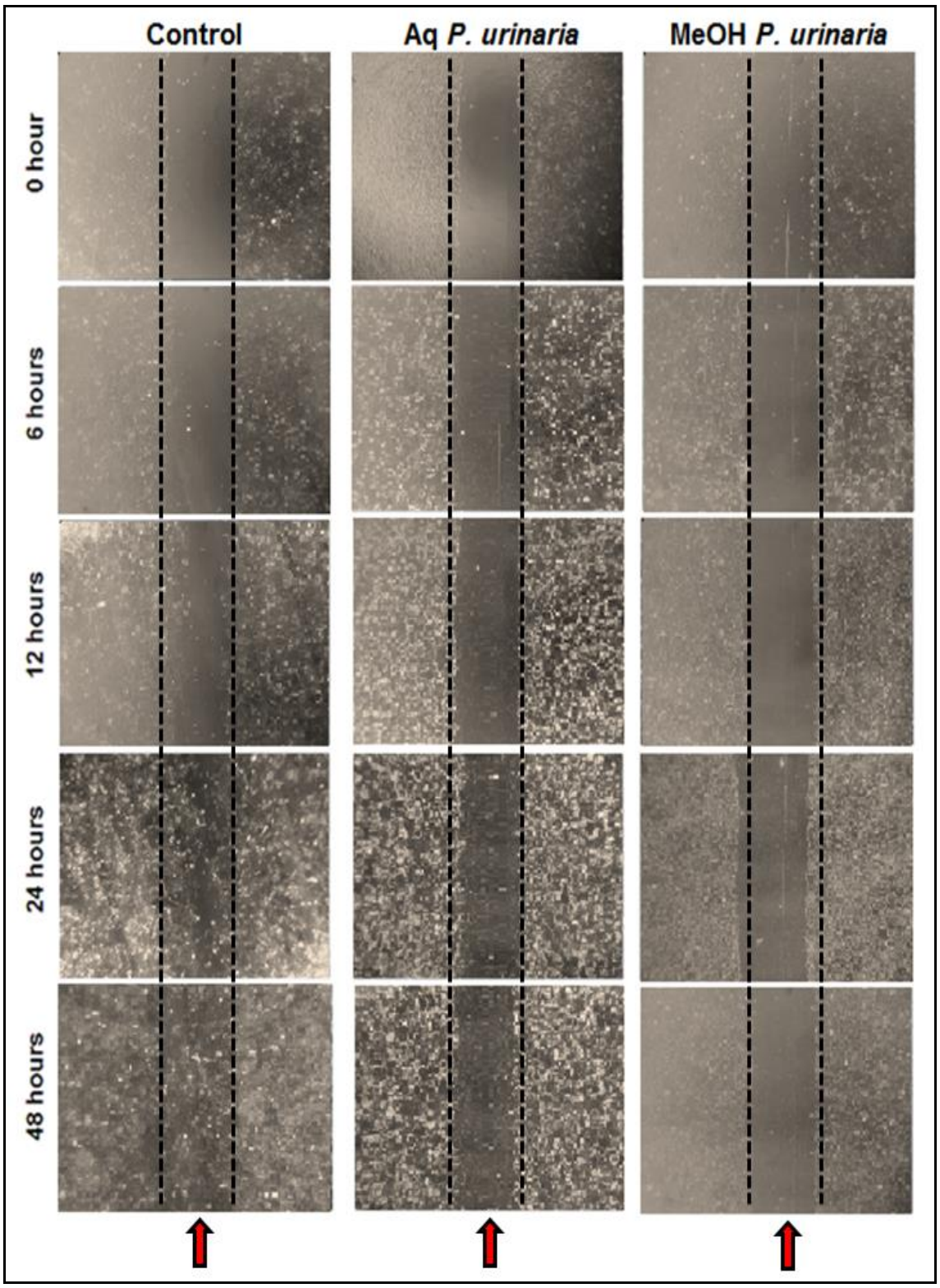


Figure 3.19: Wound closure activity of MCF-7 in response to *P. urinaria*.

Figure above shows the migration activity of MCF-7 cells across the wound after treated with aqueous (Aq) and methanolic (MeOH) *P. urinaria* extracts for 48 hours. Typical result from three independent experiments is shown. (Magnification power: 200×)

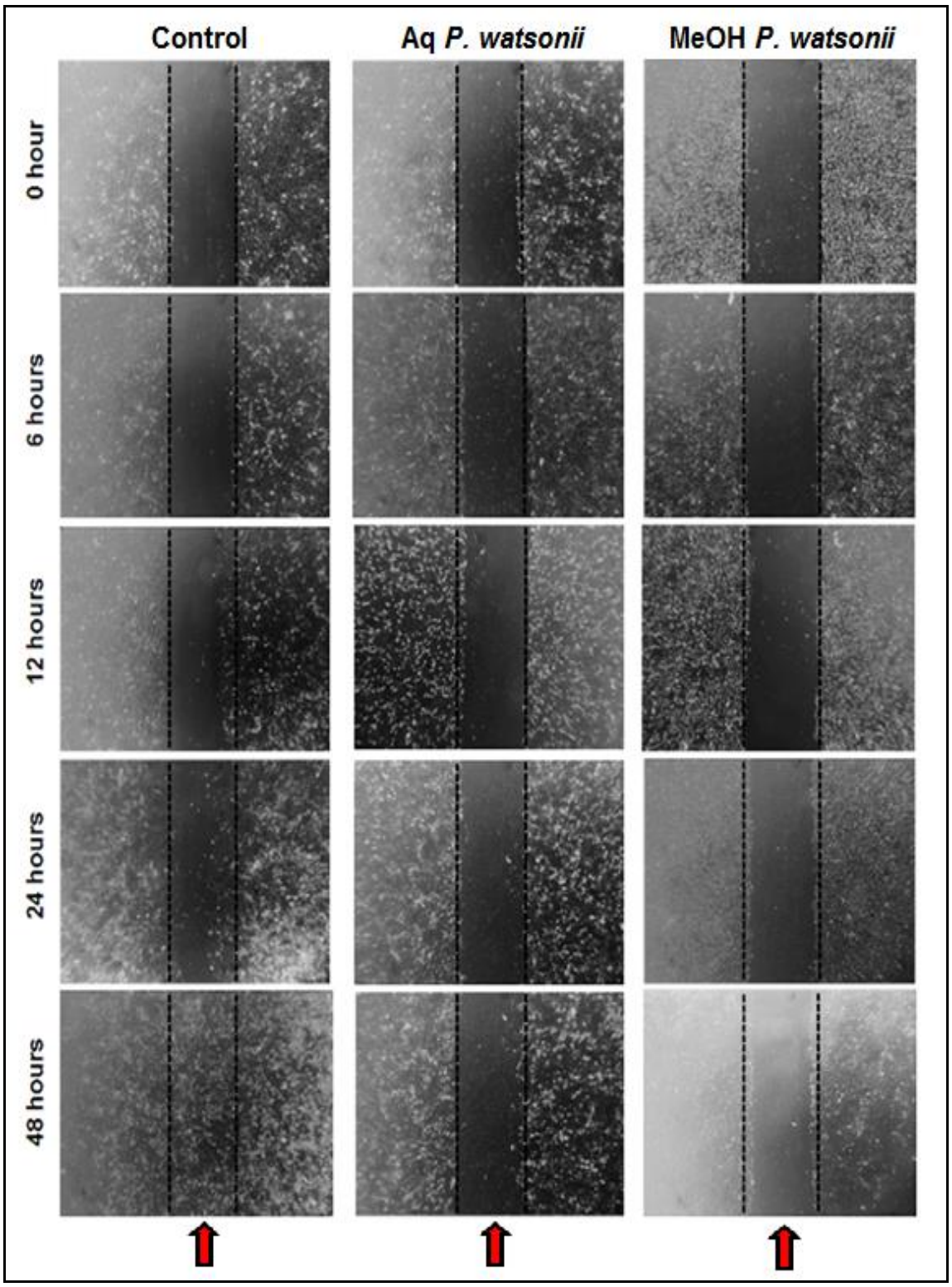


Figure 3.20: Wound closure activity of MCF-7 in response to *P. watsonii*.

Figure above shows the migration activity of MCF-7 cells across the wound after treated with aqueous (Aq) and methanolic (MeOH) *P. watsonii* extracts for 48 hours. Typical result from three independent experiments is shown. (Magnification power: 200×)

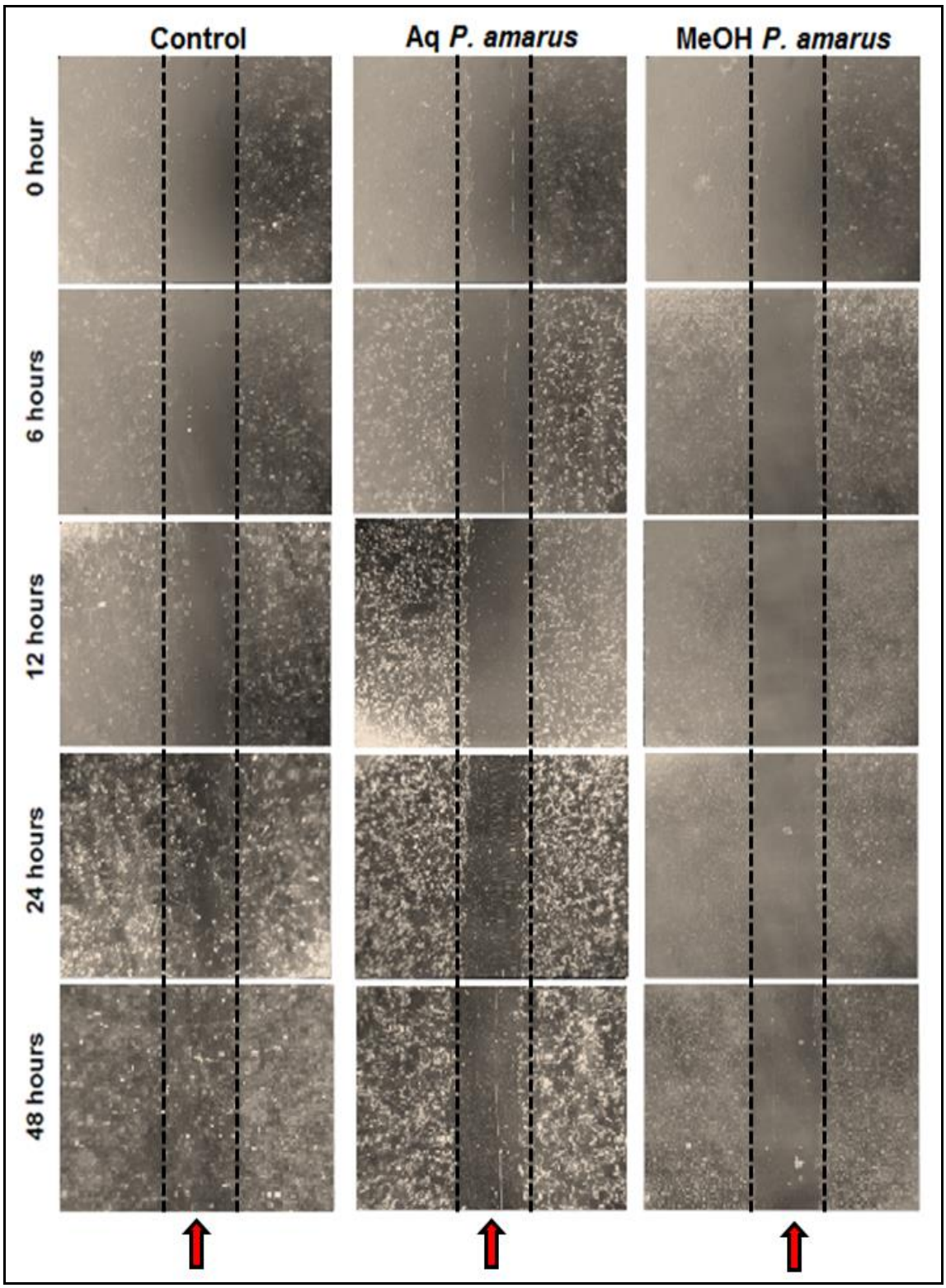


Figure 3.21: Wound closure activity of MCF-7 in response to *P. amarus*.

Figure above shows the migration activity of MCF-7 cells across the wound after treated with aqueous (Aq) and methanolic (MeOH) *P. amarus* extracts for 48 hours. Typical result from three independent experiments is shown. (Magnification power: 200×)

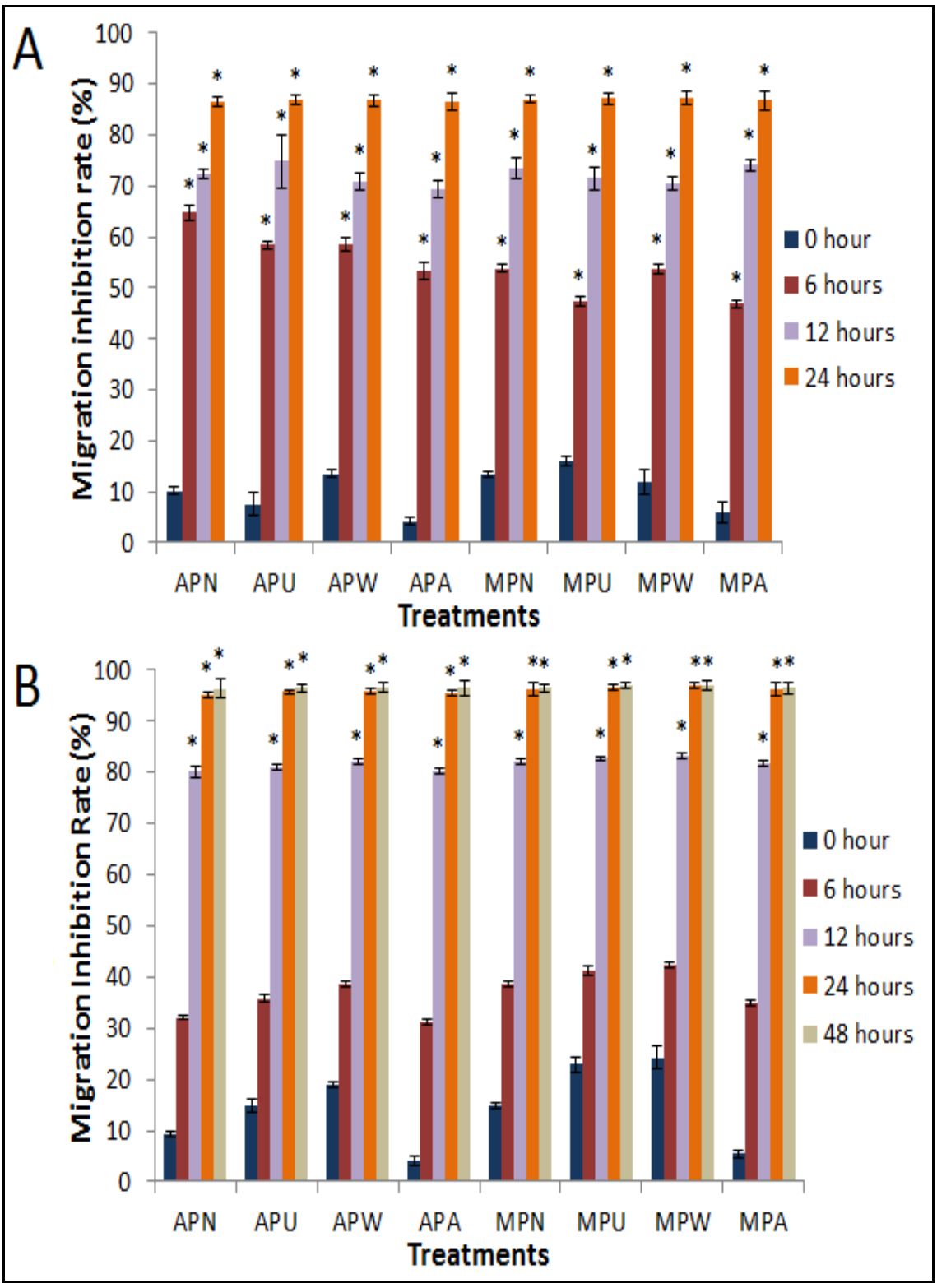


Figure 3.22: Quantitative assessment of migration inhibition rate of aqueous and methanolic extracts of *Phyllanthus* on (A) A549 and (B) MCF-7 cells.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

3.3.3 EFFECT OF *PHYLLANTHUS* EXTRACTS ON CELL ATTACHMENT

The effect of *Phyllanthus* on cell adhesion was examined by detaching the treated cells from the cultured flasks and plating them onto a new culture plate with the same number of viable treated cells in each group. The rounded cells represent the unattached cells, but all cells ultimately will attach themselves to the plate. Therefore, the higher number of rounded (unattached) cells at a given time point as compared to the untreated control signifies a delay or defect in their attachment.

Figures 3.23 and 3.24 showed the different attachment ability between the *Phyllanthus*-treated A549 cells and the untreated control cells. Meanwhile, the attachment ability of MCF-7 cells treated with various *Phyllanthus* extracts was illustrated in Figures 3.25 and 3.26. As can be seen from those figures, most of the untreated cells (both A549 and MCF-7) have begun to adhere to the plate after 6 hours of incubation. They even began to multiply and formed a monolayer after incubation for 24 hours, as marked by the red box in the figures. In contrast, most of the treated A549 and MCF-7 cells remained in their suspension form after incubation for 6 hours and began to adhere slightly only after incubation for 12 hours. Twenty-four hours later, some of the treated cells were still unattached as indicated by the red arrows, hence suggesting that the attachment capability of the *Phyllanthus*-treated cells was retarded.

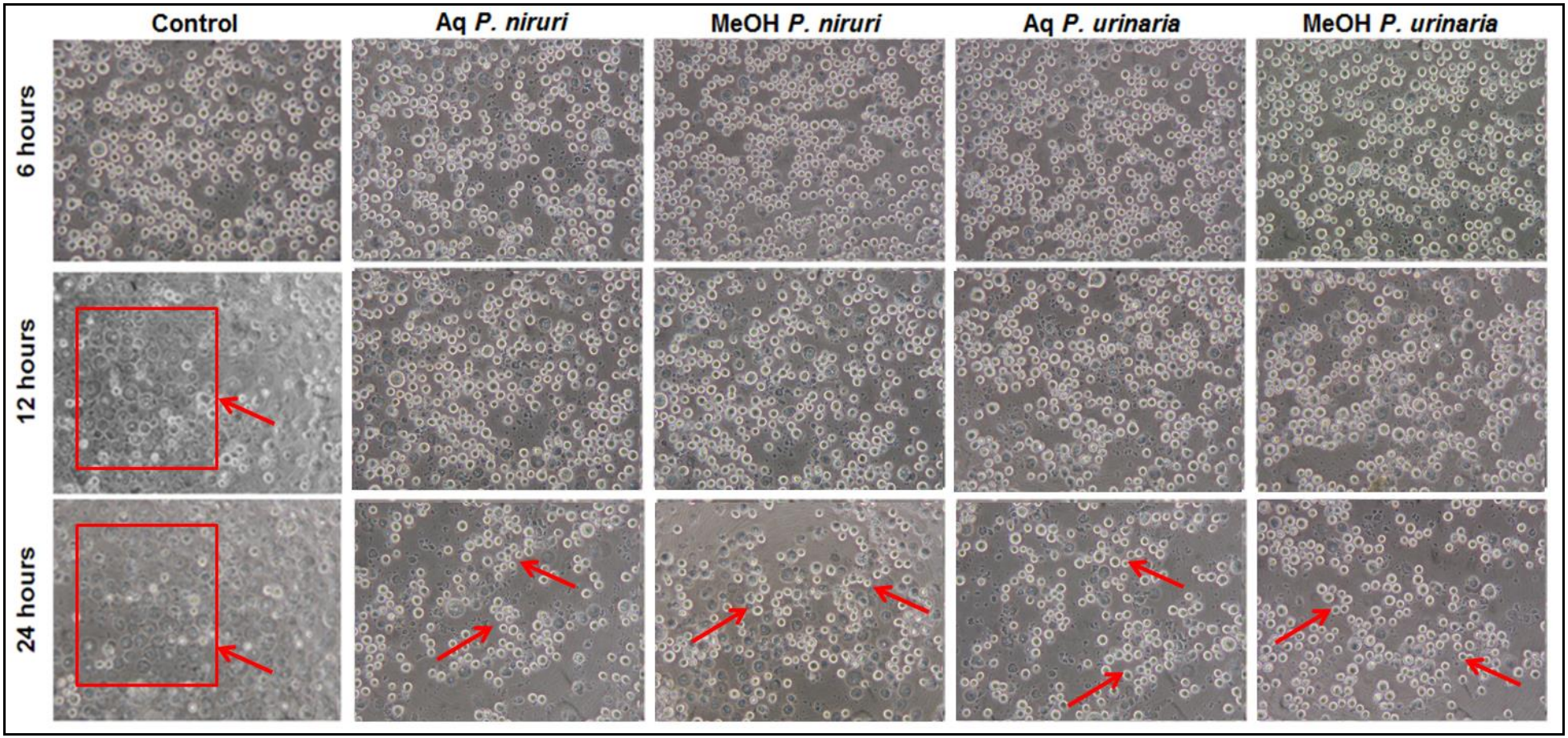


Figure 3.23: Cell attachment status of A549 in response to *P. niruri* and *P. urinaria*.

Figure above shows the attachment of A549 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. niruri* and *P. urinaria* extracts after 24 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 100×)

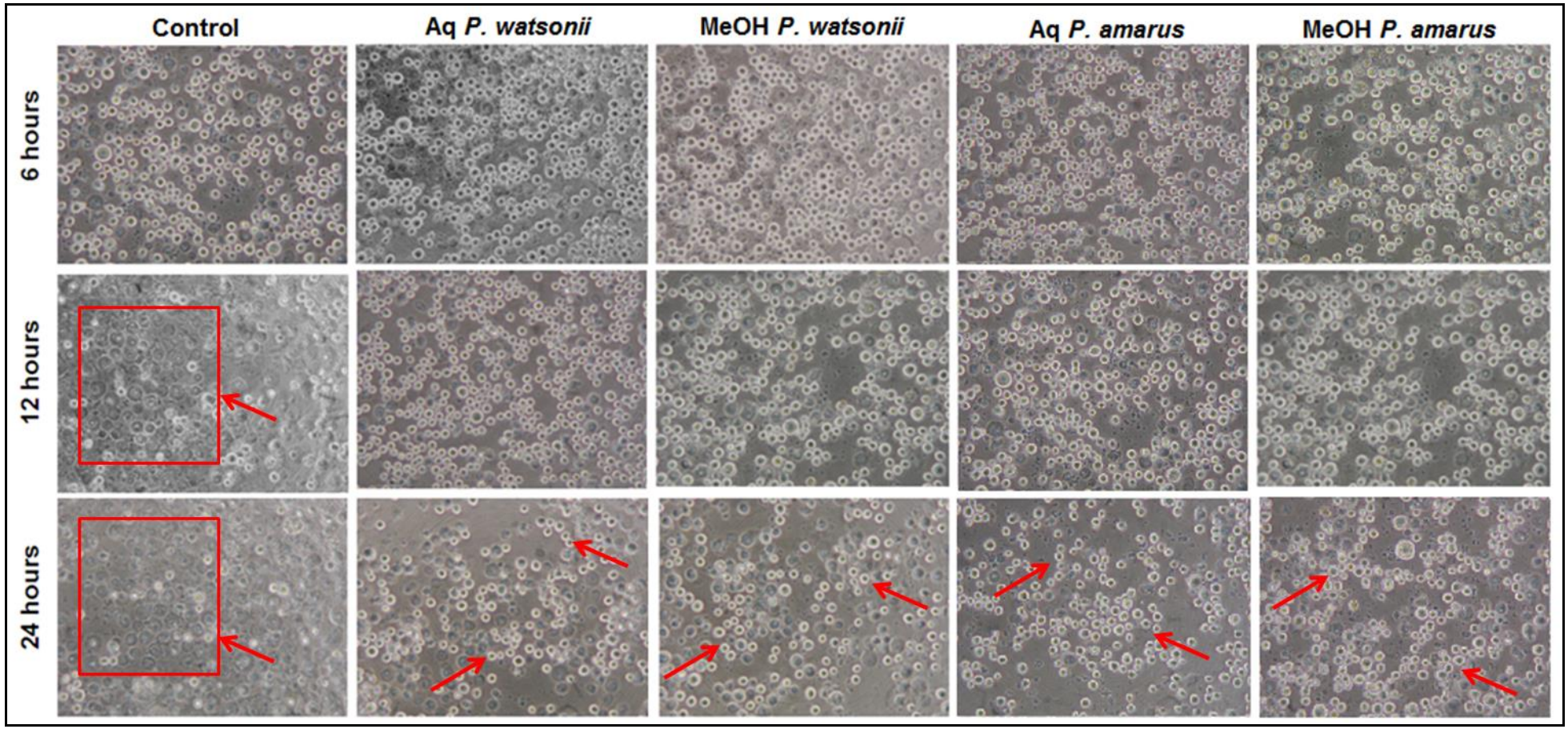


Figure 3.24: Cell attachment status of A549 in response to *P. watsonii* and *P. amarus*.

Figure above shows the attachment of A549 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. watsonii* and *P. amarus* extracts after 24 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 100×)

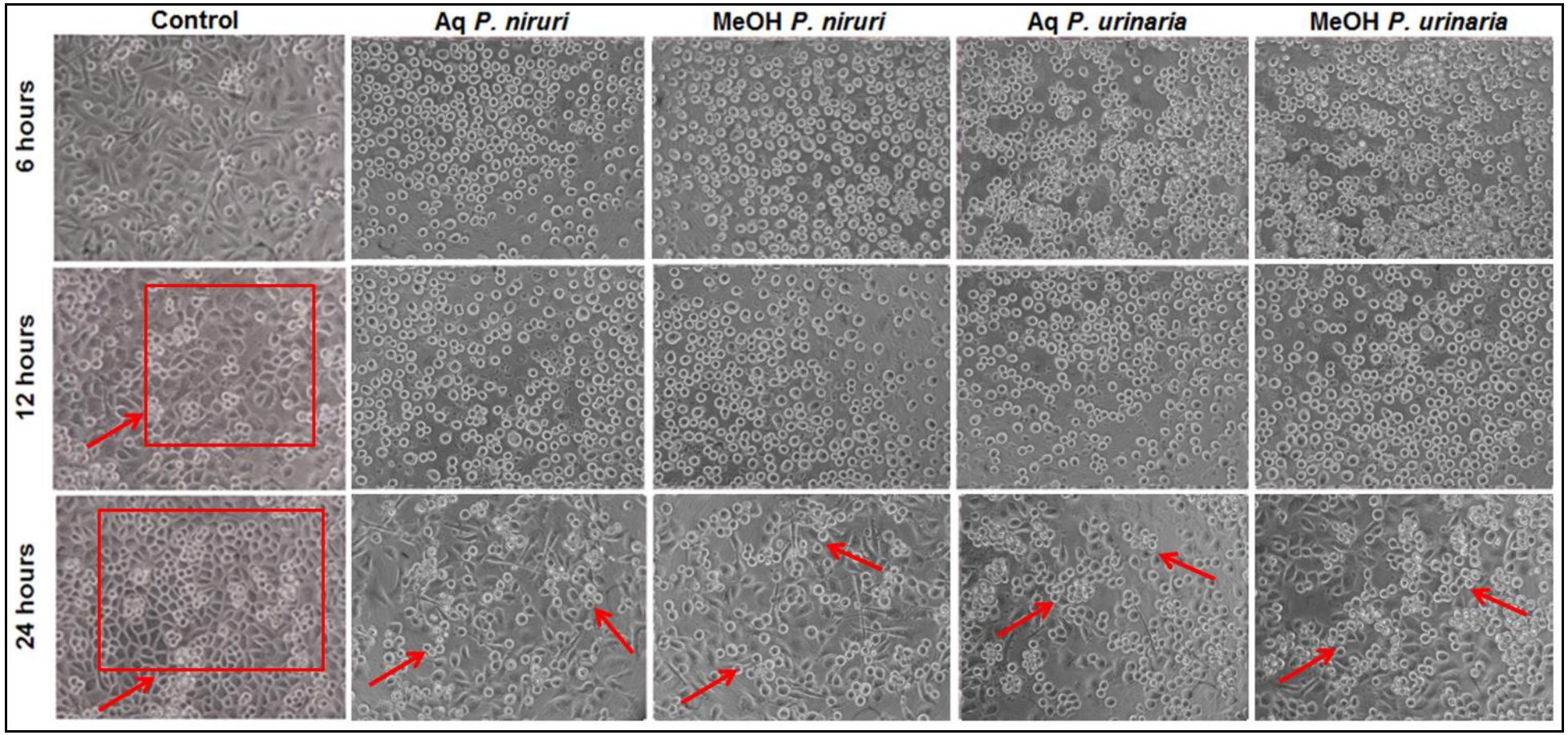


Figure 3.25: Cell attachment status of MCF-7 in response to *P. niruri* and *P. urinaria*.

Figure above shows the attachment of MCF-7 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. niruri* and *P. urinaria* extracts after 24 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 100×)

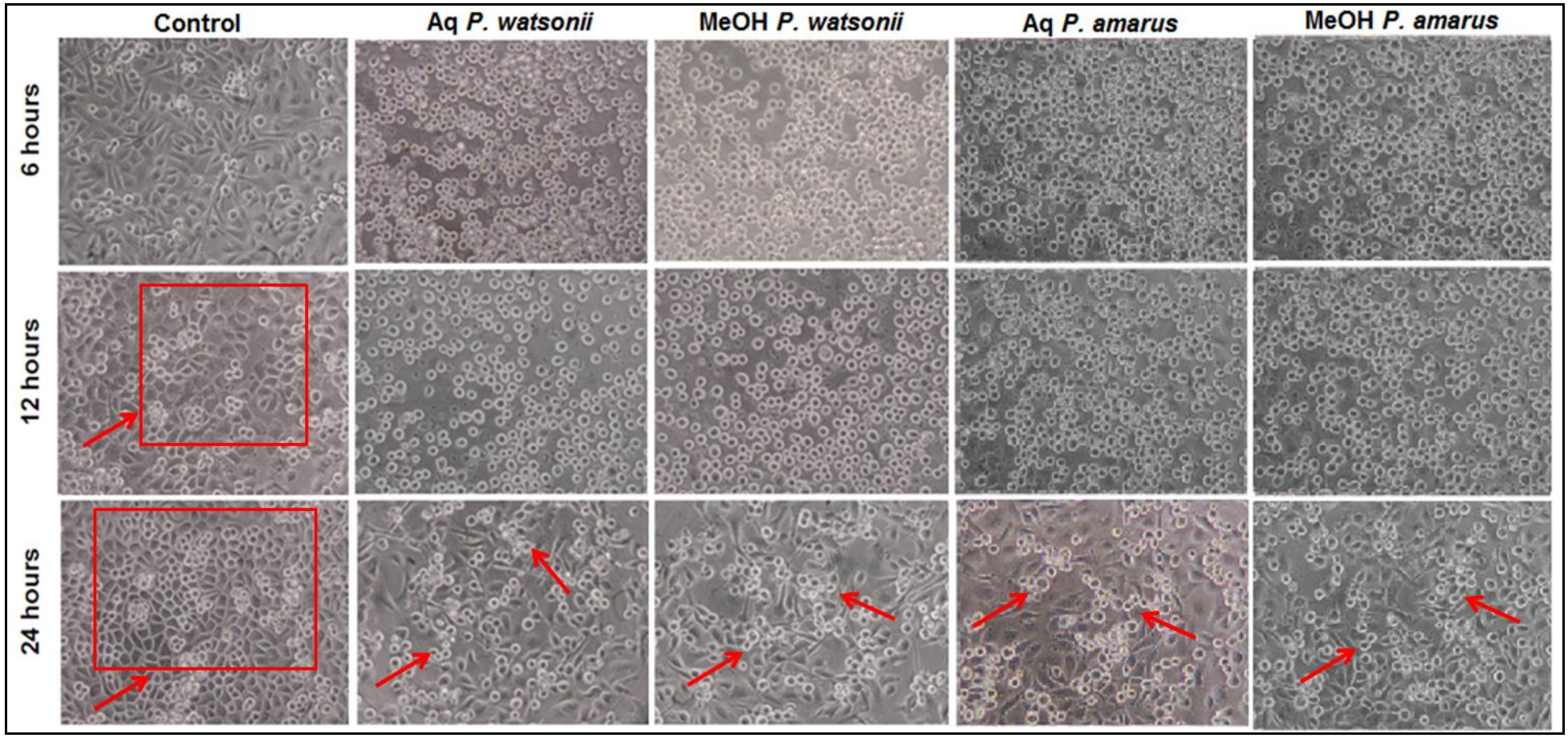


Figure 3.26: Cell attachment status of MCF-7 in response to *P. watsonii* and *P. amarus*.

Figure above shows the attachment of MCF-7 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. watsonii* and *P. amarus* extracts after 24 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 100×)

3.3.4 EFFECTS OF *PHYLLANTHUS* ON CELL-MATRIX ADHESION

In order to examine further the effects of *Phyllanthus* on cell's adhesion ability, cell-matrix adhesion assay is necessary since attachment of cancer cells to basement membrane or extracellular matrices is the first barrier for successful metastasis. As shown in Figure 3.27, the adhesion ability of A549 and MCF-7 cells to matrix decreased when they were treated at increasing concentrations of *Phyllanthus* extracts.

At low concentrations of the aqueous and methanolic *Phyllanthus* extracts, there was only about 20 – 30% adhesion inhibition of A549 cells. However, when the extracts concentrations were increased, the adhesion inhibition increased up to 40 – 50% (IC_{50} treatments) and 55 – 62% (treatment at 500µg/ml for aqueous and 200µg/ml for methanolic). Among the four *Phyllanthus* species, *P. watsonii* showed the best inhibition on A549 cells' adhesion to the matrix. Meanwhile, Cisplatin and Doxorubicin caused 46% and 54% suppression on A549 cell-matrix adhesion.

For MCF-7 cells, there was 13 – 30% cell-matrix adhesion inhibition when they were treated at low extracts concentrations. This inhibition percentage increased when MCF-7 cells were treated at IC_{50} (40 – 52%) and higher (66 – 92%) concentrations. Generally, methanolic *Phyllanthus* extracts caused greater inhibition of MCF-7 cells' adhesion to matrix as compared to aqueous extracts. Cisplatin and Doxorubicin also caused 33% and 42% cell-matrix adhesion inhibition on MCF-7 respectively.

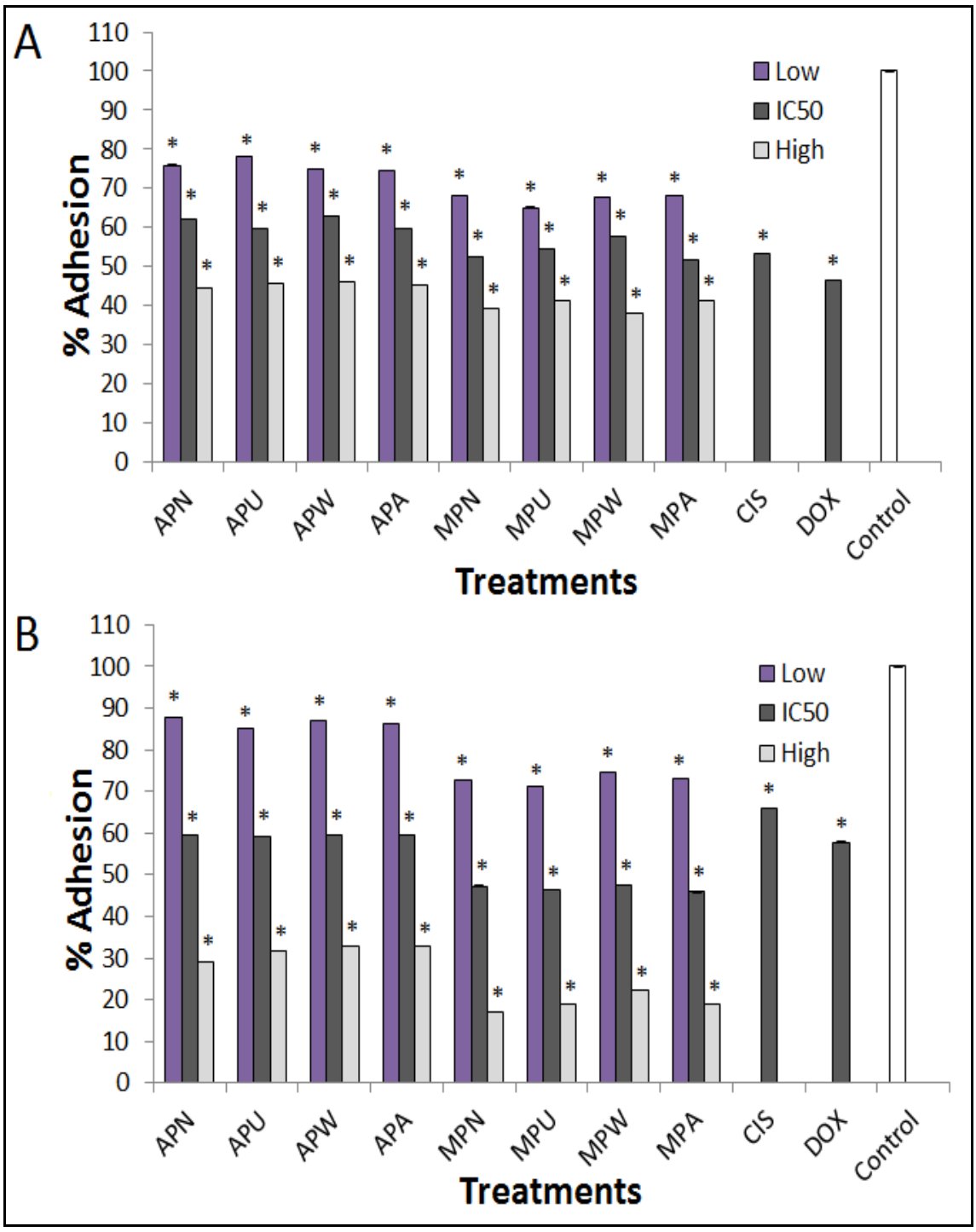


Figure 3.27: Cell-matrix adhesion percentage of (A) A549 and (B) MCF-7 cells treated with *Phyllanthus* extracts at varying concentrations.

Low – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; High – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; CIS – Cisplatin; DOX – Doxorubicin; Control – untreated cells. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

3.3.5 EFFECTS OF *PHYLLANTHUS* ON MATRIX METALLOPROTEINASES (MMPs) EXPRESSION

MMPs play an important role during tumor metastasis and angiogenesis since its expression level is often correlated with the tumor invasiveness. Among the variety of MMPs, MMP2, MMP7, and MMP9 were more commonly associated with cancer metastasis as they have the ability to degrade collagen type IV that is the major component of basement membrane.

In A549 cells, expression of all three MMPs (MMP2, MMP7, and MMP9) was detected as shown in Figure 3.28. Among these MMPs, MMP7 probably plays a greater role in A549 metastasis since its expression was higher with brighter and clearer bands as compared to MMP2 and MMP9. Nonetheless, their expressions decreased in a dose-dependent manner. Subsequent quantitative assessment of these bands intensity further confirmed the gradual reduction in MMPs expression when the cells were treated at increasing extracts concentrations (Figure 3.29). Generally, methanolic *Phyllanthus* extracts demonstrated greater inhibition on the MMPs' expression than aqueous extracts, with *P. urinaria* showing the greatest inhibitory activity compared to the other *Phyllanthus* species. Contrarily, only MMP-2 and MMP-9 were expressed in MCF-7 cells, with MMP-2 expression higher than MMP-9, therefore suggesting a more prominent role of MMP-2 in MCF-7 cells' metastasis. Nevertheless, comparison of the untreated control and *Phyllanthus*-treated bands' intensity in Figure 3.30 demonstrated that the expression for both MMP2 and MMP9 in MCF-7 cells was reduced in a dose-dependent manner. Similarly, quantitative assessment of the bands' intensity as shown in Figure 3.31 revealed that methanolic *Phyllanthus* extracts exerted greater inhibition on the MMP2 and MMP9 expression. Among the four *Phyllanthus* species, *P. urinaria* showed the greatest inhibition, followed by *P. watsonii*, *P. niruri*, and *P. amarus*.

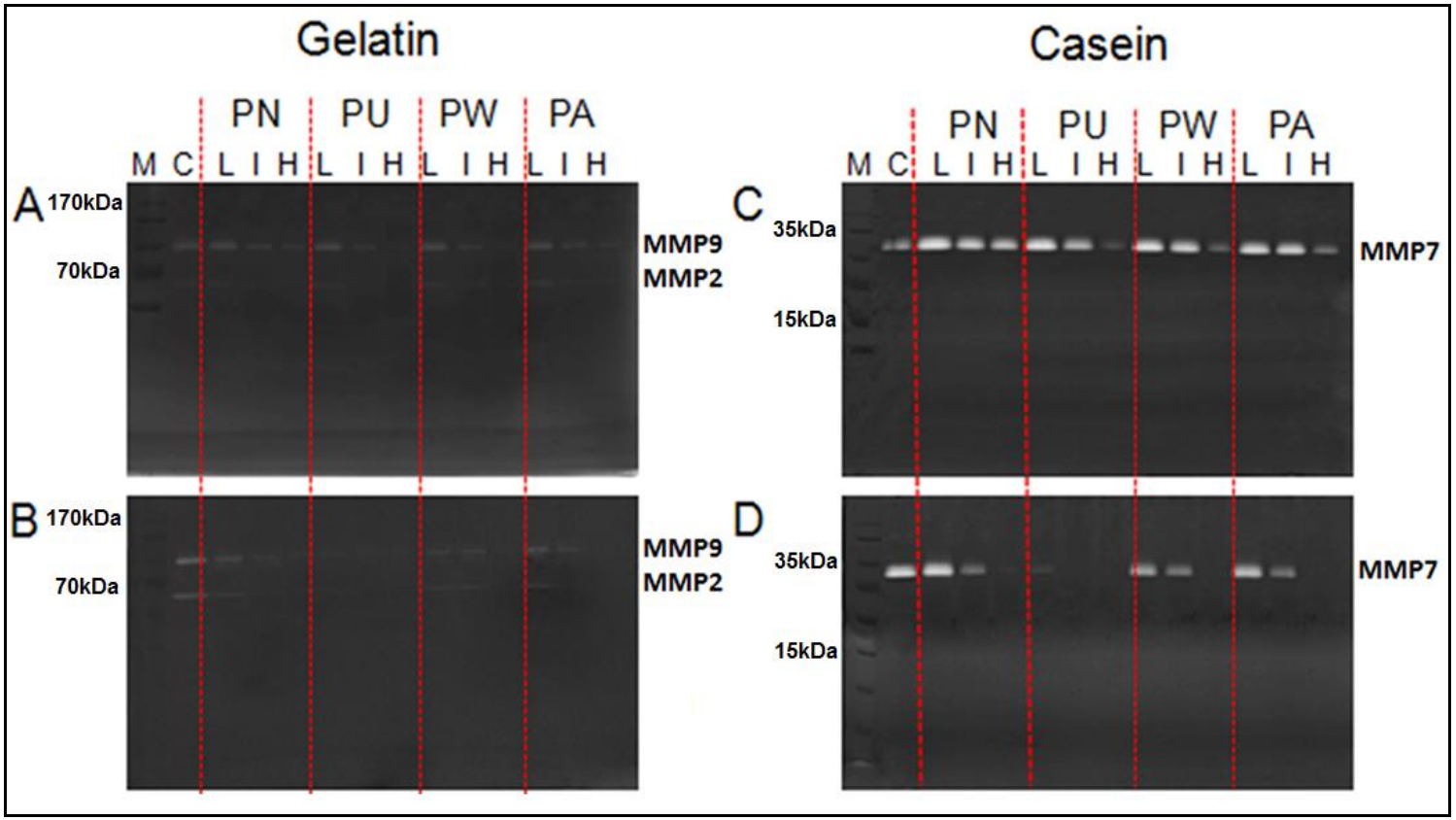


Figure 3.28: Matrix metalloproteinases 2, 7, and 9 (MMP2, MMP7, and MMP9) expression levels in A549 cells in response to *Phyllanthus* extracts.

Figure above shows the MMP2, MMP7, and MMP9 expressions in A549 cells treated with (A and C) aqueous *Phyllanthus* extracts and (B and D) methanolic *Phyllanthus* extracts. M – protein marker; C – untreated control; L – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; I – treatment at their respective IC_{50} concentrations; H – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; PN – *P. niruri*; PU – *P. urinaria*; PW – *P. watsonii*; PA – *P. amarus*.

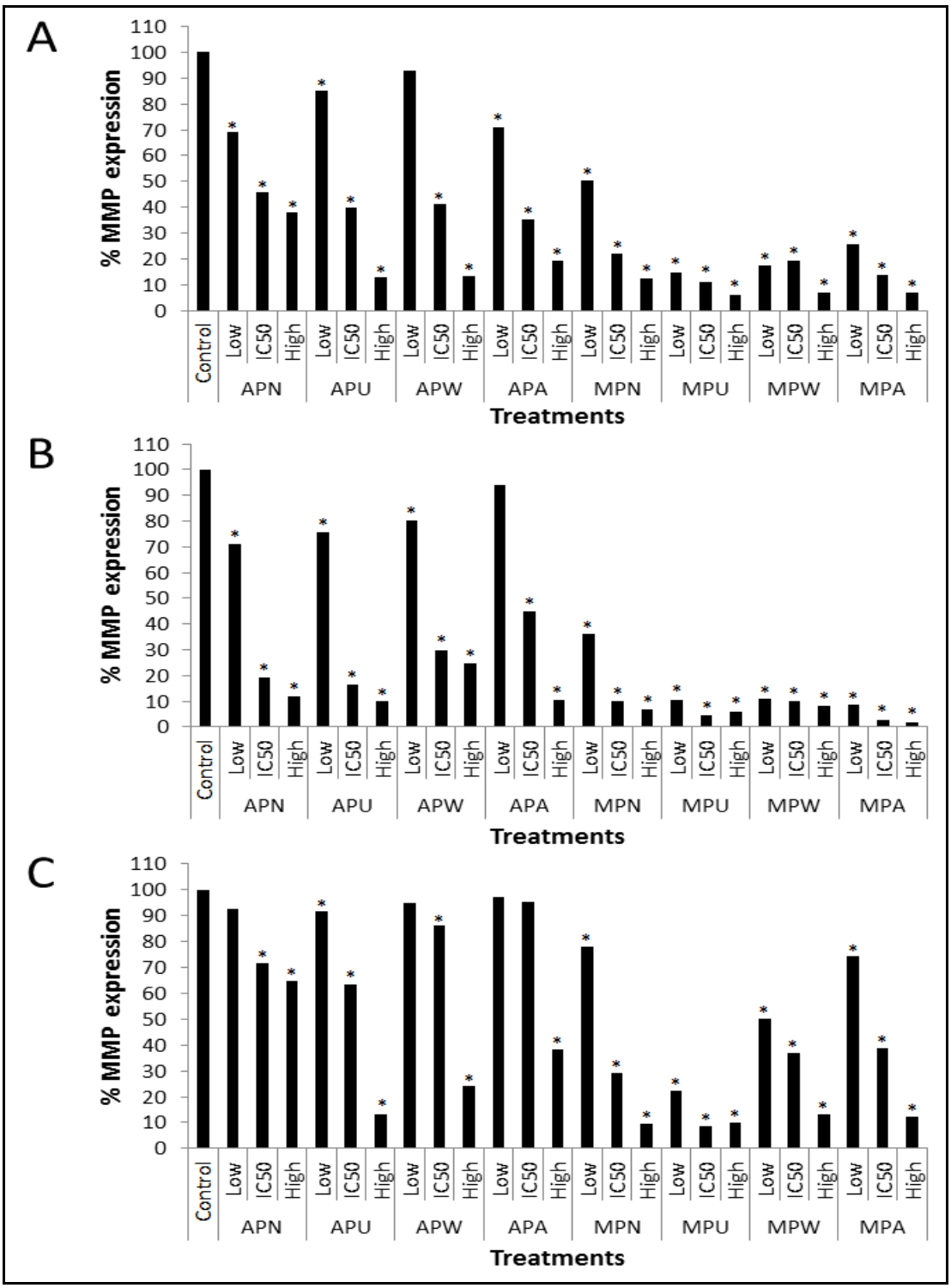


Figure 3.29: Quantitative assessment of (A) MMP2, (B) MMP7, and (C) MMP9 expressions in A549 cells in response to aqueous and methanolic *Phyllanthus* extracts.

Low – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; High – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*. Error bar indicates the standard error mean of three independent experiments. **P* < 0.05 vs untreated control.

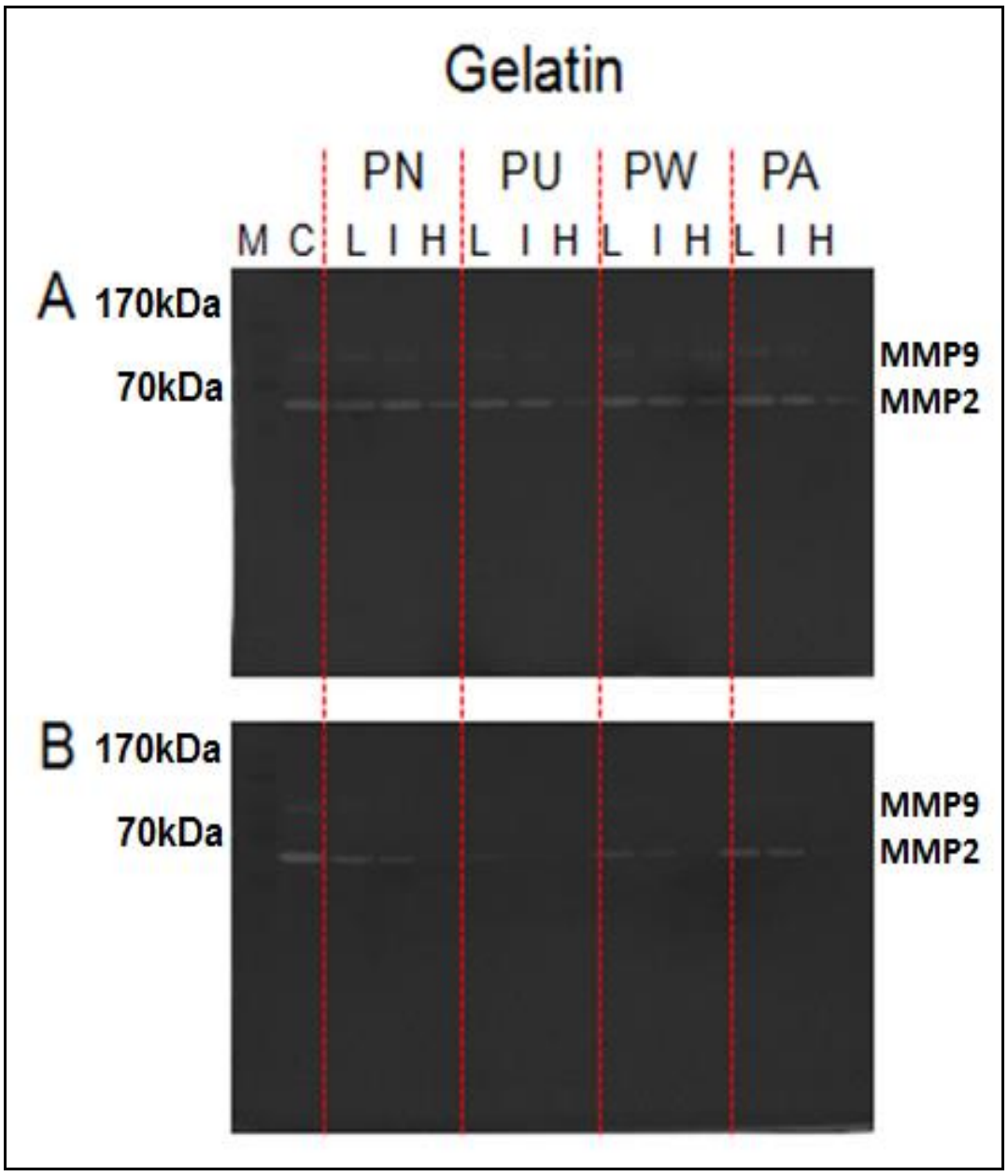


Figure 3.30: Matrix metalloproteinases 2 and 9 (MMP2 and MMP9) expression levels in MCF-7 in response to *Phyllanthus* extracts.

Figure above shows the MMP2 and MMP9 expressions in MCF-7 cells treated with (A) aqueous *Phyllanthus* extracts and (B) methanolic *Phyllanthus* extracts. M – protein marker; C – untreated control; L – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; I – treatment at their respective IC_{50} concentrations; H – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; PN – *P. niruri*; PU – *P. urinaria*; PW – *P. watsonii*; PA – *P. amarus*.

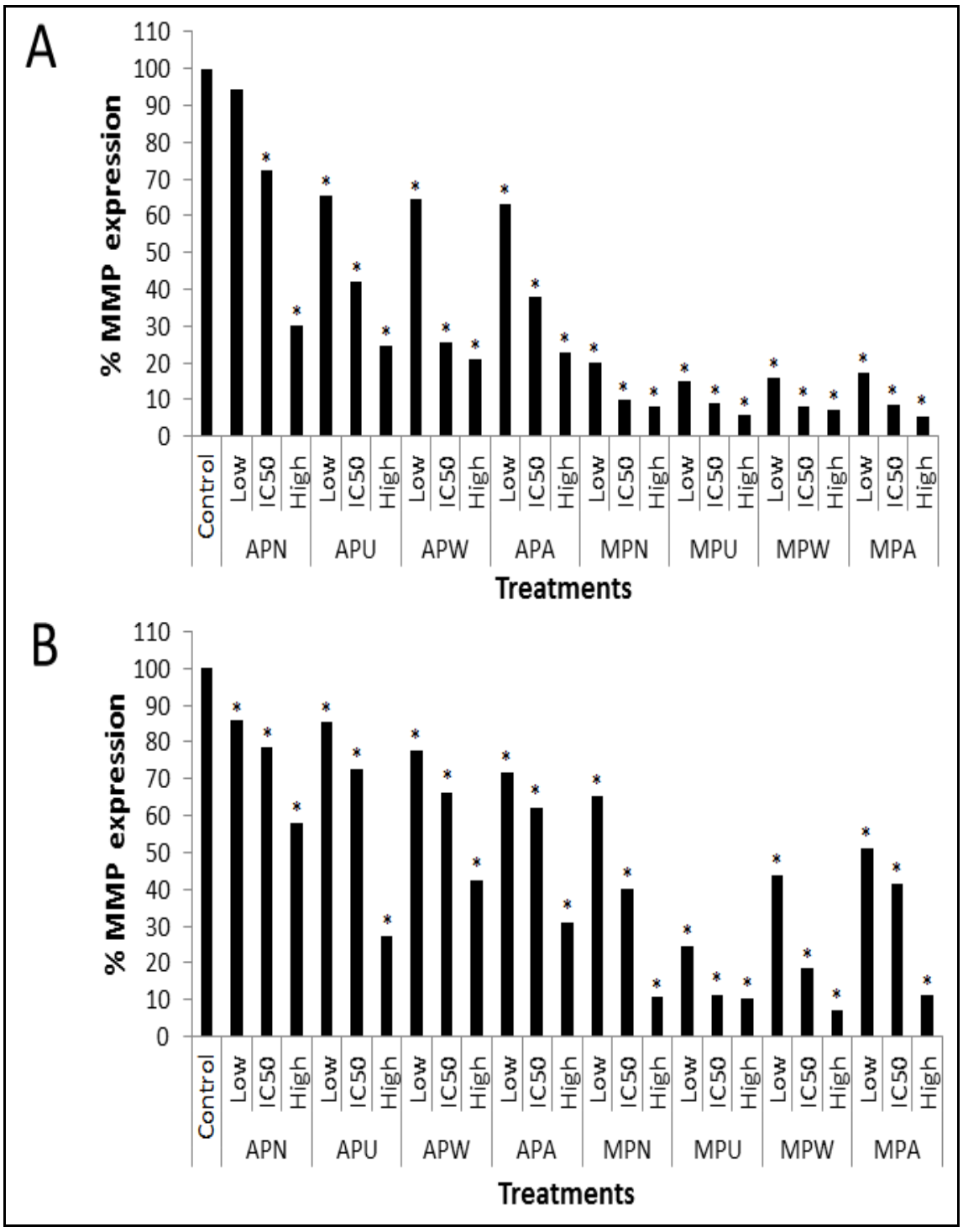


Figure 3.31: Quantitative assessment of (A) MMP2 and (B) MMP9 expressions in MCF-7 cells in response to aqueous and methanolic *Phyllanthus* extracts.

Low – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; High – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*. Error bar indicates the standard error mean of three independent experiments. **P* < 0.05 vs untreated control.

3.4 MECHANISM OF CELL DEATH INDUCED BY *PHYLLANTHUS*

Treatment with chemotherapeutic agents would normally induce one of the two distinct modes of cell death in cancer cells, via either apoptosis or necrosis. Apoptosis is a preferable mode of cell death in cells since it does not provoke an inflammatory response unlike necrosis. Nevertheless, some of the agents have the capability to stimulate both cell death machineries, which include *Phyllanthus* that mainly induces apoptosis in cancer cells with little involvement of necrotic cell death.

3.4.1 EFFECTS OF *PHYLLANTHUS* EXTRACTS ON CASPASE-3 AND -7 ACTIVITIES

Caspase-3 and -7 play crucial roles as early apoptosis biochemical markers in mammalian cells. Caspase-Glo® 3/7 Assay uses a luminogenic substrate containing the DEVD sequence that is selective for caspase-3 and -7. The caspases activity in both untreated and *Phyllanthus*-treated cancer cells were measured after 72 hours and are shown in Figure 3.32. Our findings showed that caspase-3 and -7 activities were detected in untreated A549 and MCF-7 control cells, which corresponded to the portion of apoptotic cells present in the naturally growing population due to natural aging. Therefore, the caspases fold change in treated cells was calculated by assuming the caspases activity present in untreated cells as the basal level and was set to zero. Results obtained revealed that activities of caspase-3 and -7 increased to a range from 3-fold to 5-fold ($P < 0.05$) over basal levels in treated cells, hence signifying an activation of these caspases in both A549 and MCF-7 cells treated with *Phyllanthus* extracts. Besides that, A549 and MCF-7 cells treated with standard drugs also showed a greater fold increase in their caspase-3 and -7 activity (Cisplatin – 6 fold increase; Doxorubicin – 7 fold increase).

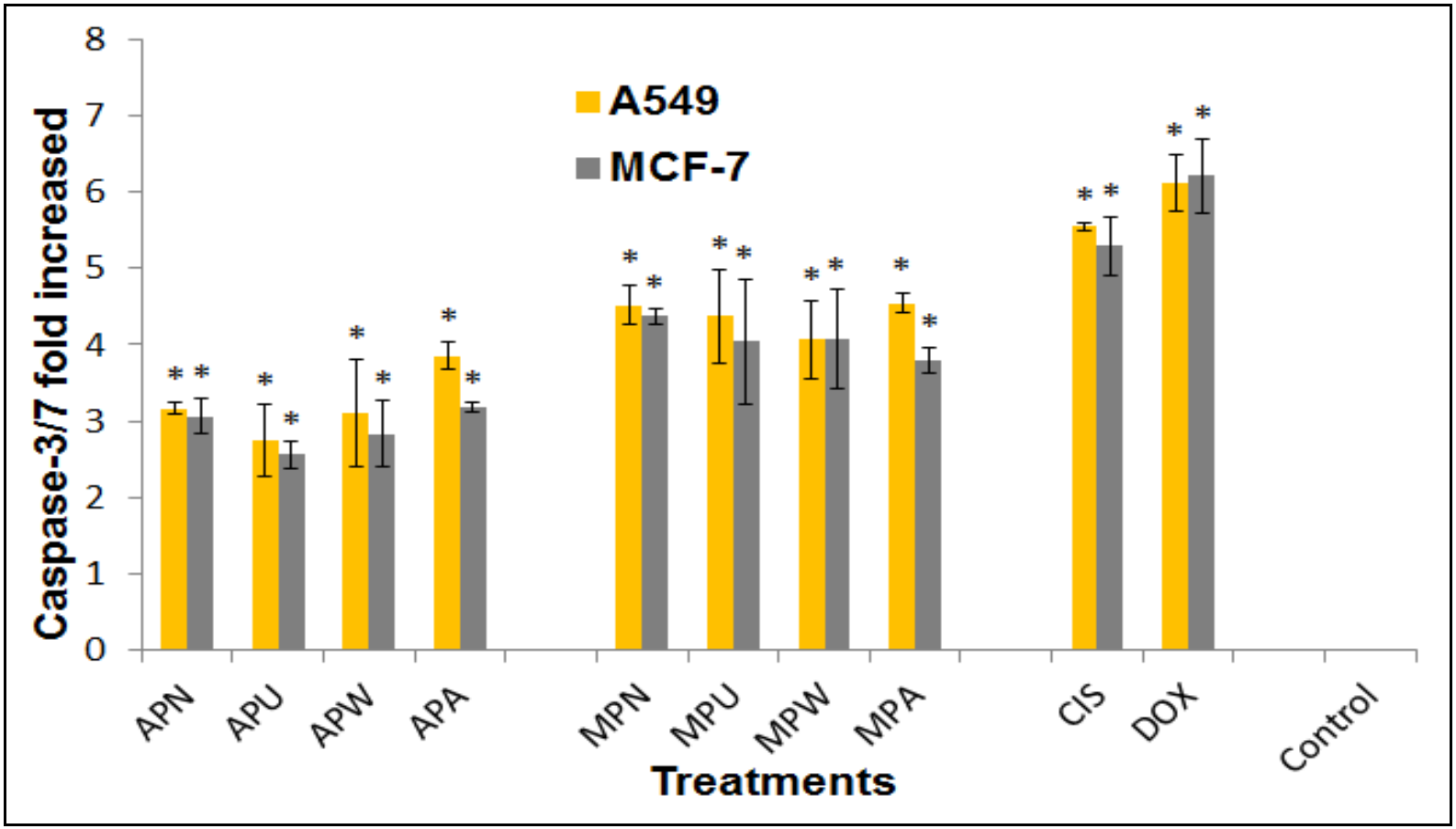


Figure 3.32: Caspase-3 and -7 levels released from A549 and MCF-7 cells treated with aqueous and methanolic *Phyllanthus* extracts.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; CIS – Cisplatin; DOX – Doxorubicin; Control – untreated cells. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

3.4.2 EFFECTS OF *PHYLLANTHUS* EXTRACTS ON CELLULAR MEMBRANE INTEGRITY

Lactate Dehydrogenase (LDH) is a cytosolic enzyme released into cell culture supernatant due to compromised membrane integrity, which is associated with necrotic cell death. The extent of its activity in converting tetrazolium salt into red formazan product is proportional to the number of necrotic cells. Figure 3.33 shows the percentage of LDH released from A549 and MCF-7 cells after 72 hours of treatment with various aqueous and methanolic extracts of *Phyllanthus*. As the positive control for this assay, maximal LDH release was induced in the cells by addition of a lysis compound provided by the supplier that lysed the cell membrane. From the data, the LDH amount released by *Phyllanthus*-treated cells remained at low levels (less than 20%) that is comparable to the level of LDH released from the untreated cells (< 10%). This therefore suggests that *Phyllanthus* induces minimal cytotoxicity by disrupting membrane integrity that led to necrosis. On the other hand, Cisplatin-treated A549 and MCF-7 cells released 30 – 35% of LDH from its compromised membrane while 20 – 25% LDH was detected from Doxorubicin-treated cells.

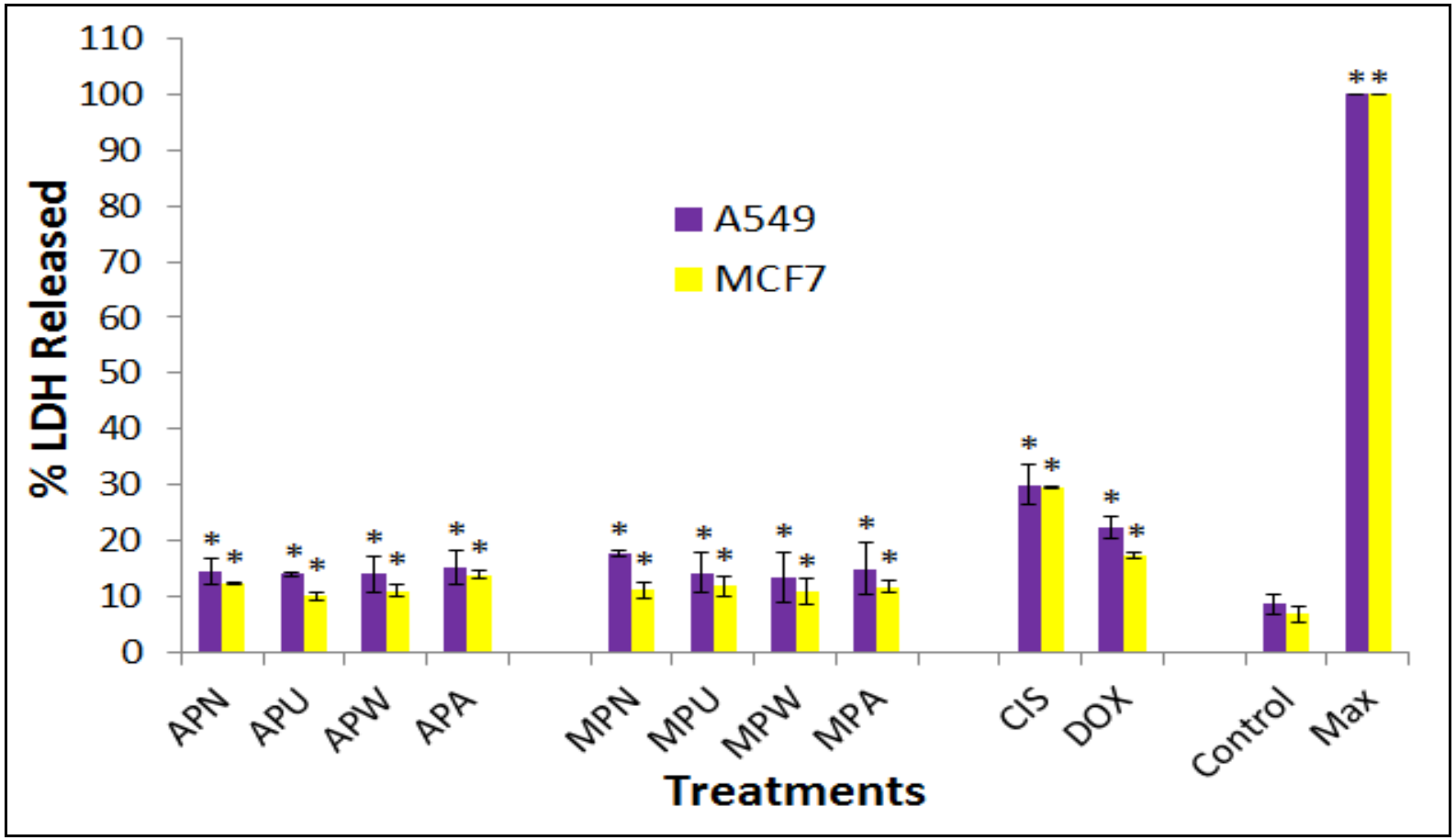


Figure 3.33: Lactate dehydrogenase (LDH) released from A549 and MCF-7 cells treated with aqueous and methanolic *Phyllanthus* extracts.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; CIS – Cisplatin; DOX – Doxorubicin; Control – untreated cells; Max – maximal LDH released. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

3.4.3 EFFECTS OF *PHYLLANTHUS* EXTRACTS ON NUCLEAR FRAGMENTATION

DNA fragmentation in condensed chromatin and formation of apoptotic bodies are some of the events of late apoptosis. In order to demonstrate the ability of *Phyllanthus* extracts to induce apoptosis, DNA fragmentation assay was used to verify the presence of apoptotic cells in the treated A549 and MCF-7 cells. Isolation of DNA from the treated cells and their subsequent separation on an agarose gel presented a typical ladder-like pattern of multiples of approximately 180 - 200 bps DNA fragments, one of the hallmarks of apoptosis (Figures 3.34A and 3.34B).

Additionally, cell apoptosis was determined *in situ* based on the enzymatic labelling of free 3'-OH terminus of non-random DNA single-stranded and double-stranded breaks with modified nucleotides, resulting in the brown staining of the apoptotic cells. Figures 3.35, 3.36, 3.37, and 3.38 show the TUNEL-positive A549 and MCF-7 cells after treatment with various aqueous and methanolic extracts of four *Phyllanthus* species. After 72 hours of extracts treatment, the percentage of apoptotic cells in both A549 and MCF-7 increased tremendously as compared to the untreated control cells. Since the cells were treated with the IC_{50} concentrations of each extracts, the mean percentage of apoptotic cells observed from 3 views per slide varied from 30% up to 55%.

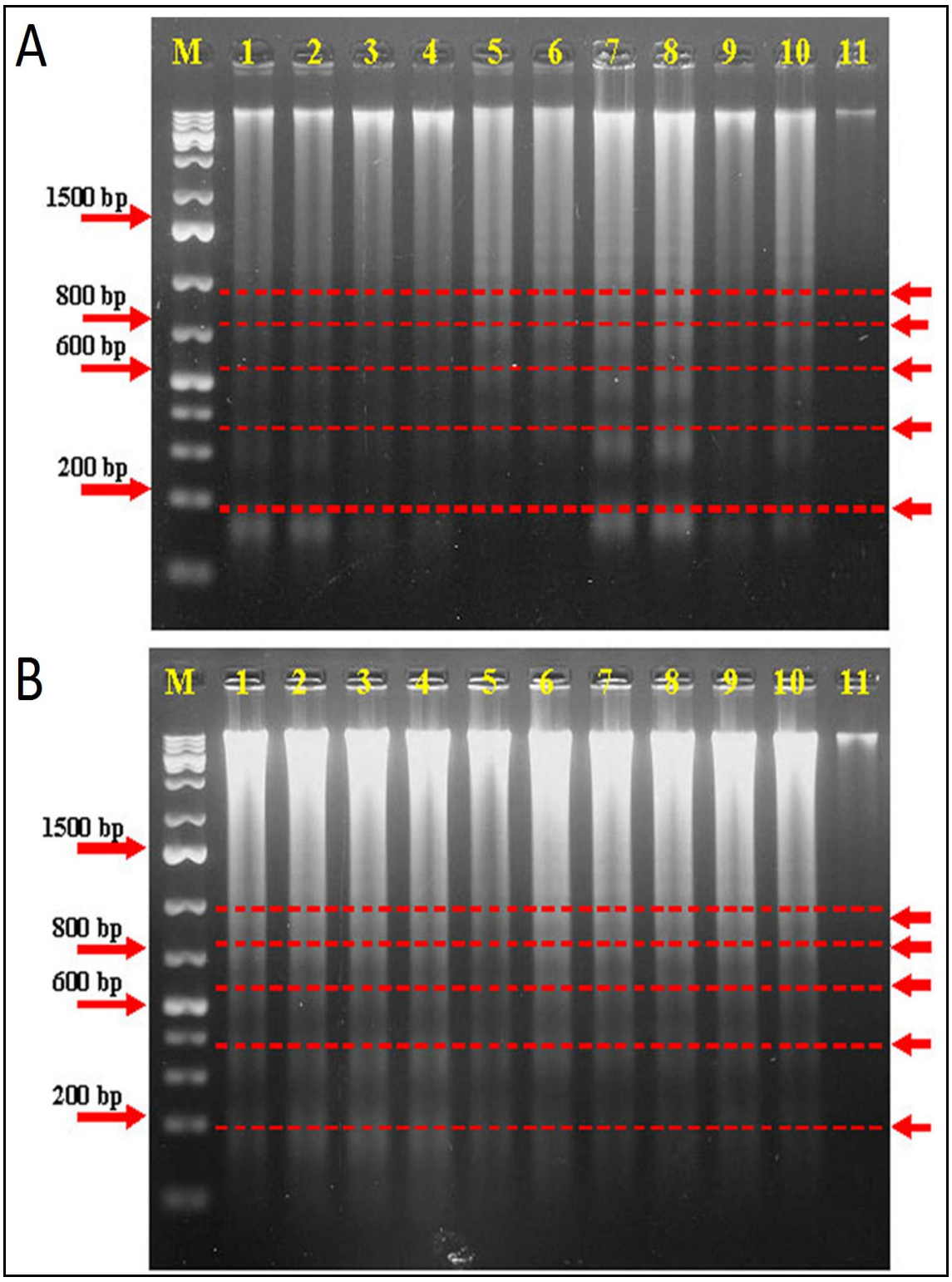


Figure 3.34: Agarose gel electrophoretic analysis of DNA fragmentation in (A) A549 and (B) MCF-7 cells in response to *Phyllanthus* extracts and standard drugs.

Red arrows at the right are pointing to the bands of DNA fragments. M: Molecular-weight marker; Lane 1 - 4: aqueous extracts of *P. niruri, P. urinaria, P. watsonii,* and *P. amarus*; Lane 5 – 8: methanolic extracts of *P. niruri, P. urinaria, P. watsonii,* and *P. amarus*; Lane 9: Cisplatin; Lane 10: Doxorubicin; Lane 11: Untreated control. Typical result from three independent experiments is shown.

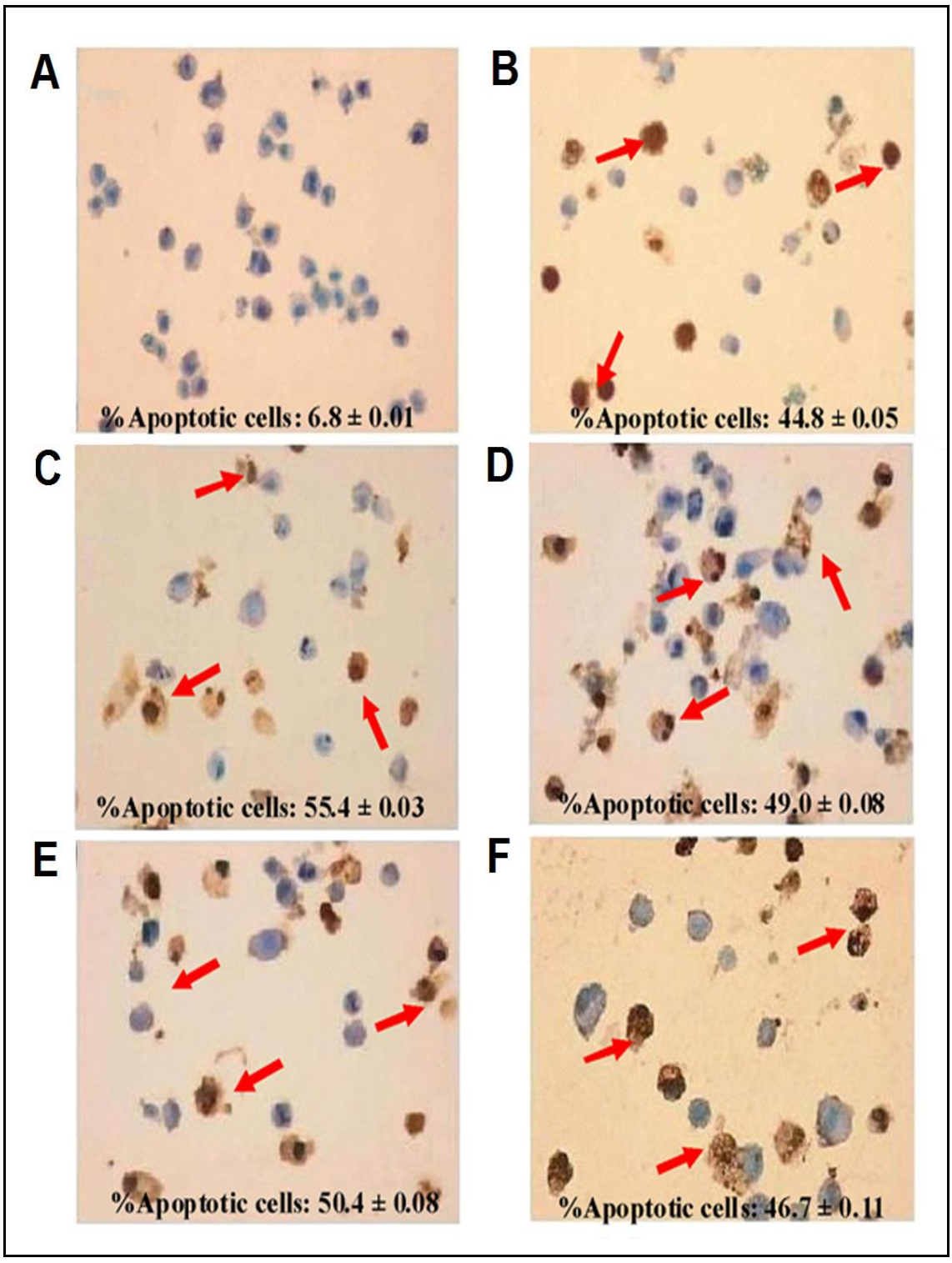


Figure 3.35: TUNEL-positive A549 in response to aqueous *Phyllanthus* extracts.

Red arrows showing (A) untreated A549 cells and TUNEL-positive cells treated with (B) aqueous *P. niruri*; (C) aqueous *P. urinaria*; (D) aqueous *P. watsonii*; (E) aqueous *P. amarus*; (F) Cisplatin. Typical results from three independent experiments are shown. (Magnification power: 200×)

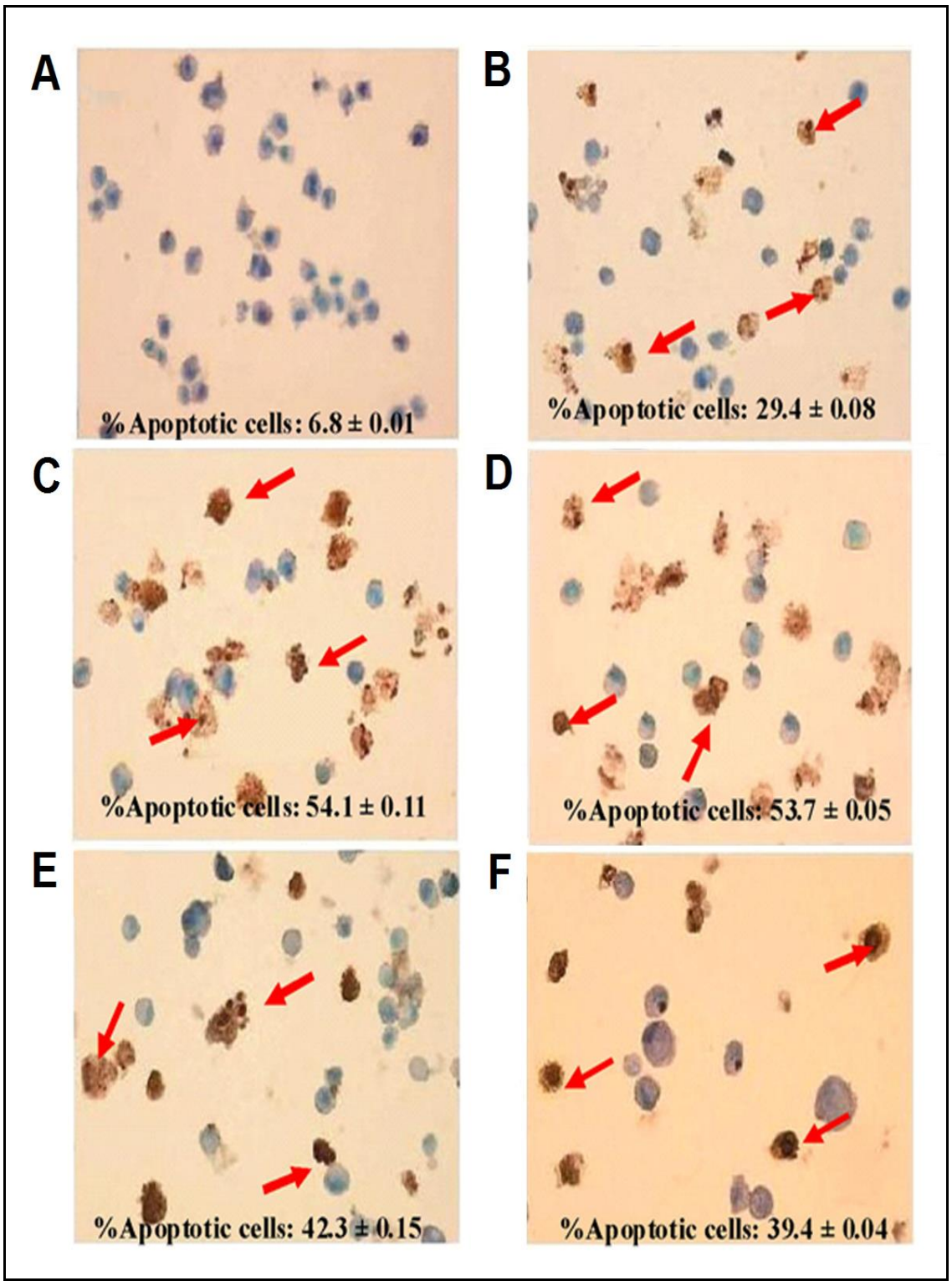


Figure 3.36: TUNEL-positive A549 in response to methanolic *Phyllanthus* extracts.

Red arrows showing (A) untreated A549 cells and TUNEL-positive cells treated with (B) methanolic *P. niruri*; (C) methanolic *P. urinaria*; (D) methanolic *P. watsonii*; (E) methanolic *P. amarus*; (F) Doxorubicin. Typical results from three independent experiments are shown. (Magnification power: 200×)

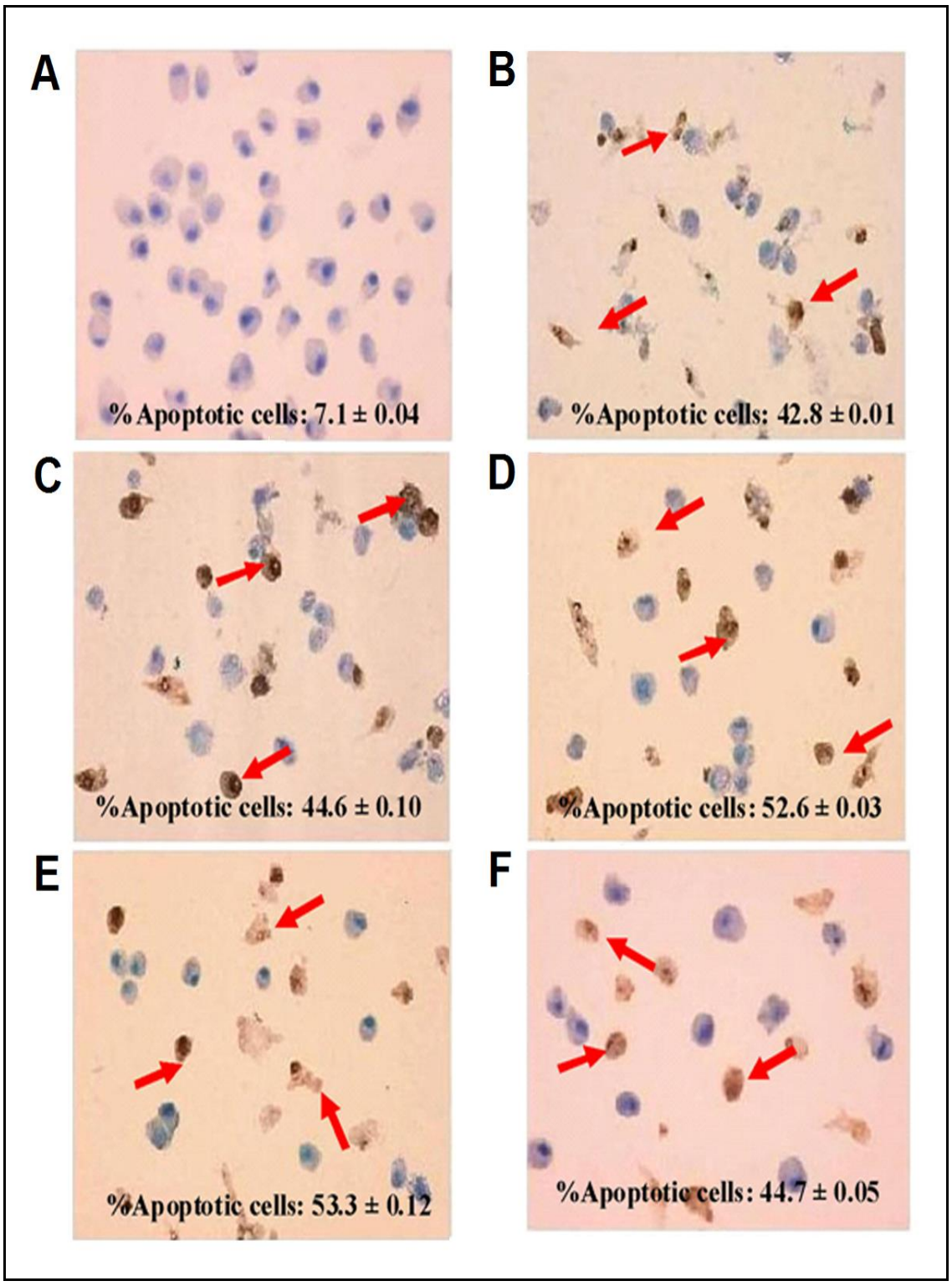


Figure 3.37: TUNEL-positive MCF-7 in response to aqueous *Phyllanthus* extracts.

Red arrows showing (A) untreated MCF-7 cells and TUNEL-positive cells treated with (B) aqueous *P. niruri*; (C) aqueous *P. urinaria*; (D) aqueous *P. watsonii*; (E) aqueous *P. amarus*; (F) Cisplatin. Typical results from three independent experiments are shown. (Magnification power: 200×)

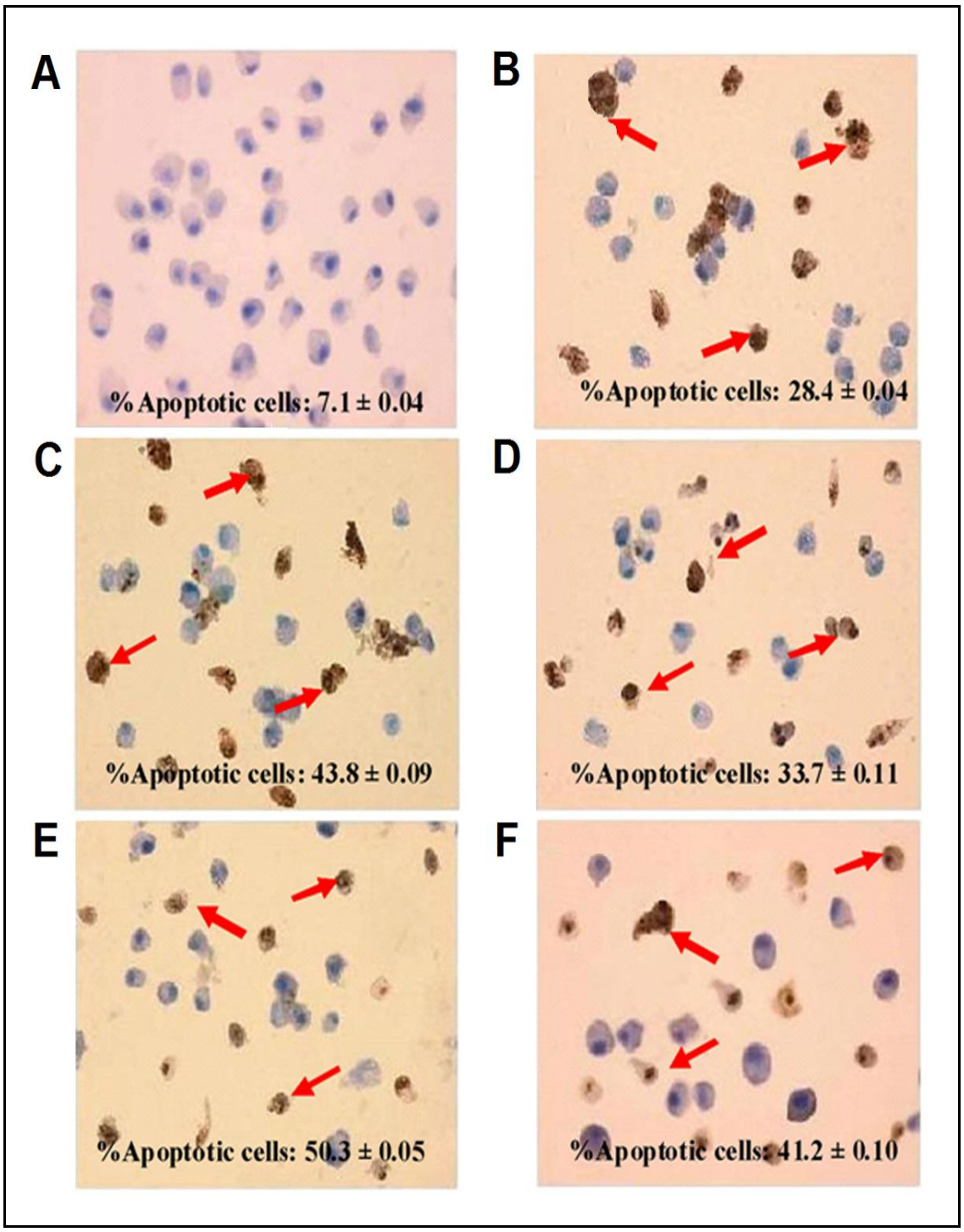


Figure 3.38: TUNEL-positive MCF-7 in response to methanolic *Phyllanthus* extracts.

Red arrows showing (A) untreated MCF-7 cells and TUNEL-positive cells treated with (B) methanolic *P. niruri*; (C) methanolic *P. urinaria*; (D) methanolic *P. watsonii*; (E) methanolic *P. amarus*; (F) Doxorubicin. Typical results from three independent experiments are shown. (Magnification power: 200×)

3.5 EFFECTS OF *PHYLLANTHUS* ON CELL'S ANGIOGENIC ABILITY

Angiogenesis or blood vessel formation is the key process for tumor's survival and metastasis in hypoxic environment. During hypoxia, cytoplasmic HIF-1α subunit stabilization will lead to the activation of various genes to promote their survival, including vascular endothelial growth factor (VEGF) and inducible nitric oxide synthases (iNOS). This was verified with the high expression of both VEGF and iNOS detected in the untreated-control A549 and MCF-7 cells (Figures 3.39, 3.40, 3.41, and 3.42) using cell-based ELISA and western blotting assays.

After A549 treatment with various *Phyllanthus*, both the aqueous and methanolic extracts for all four species were observed to demonstrate inhibition on iNOS (Figure 3.39A), whereby most of their expressions dropped markedly to approximately 20 - 30% except for methanolic *P. urinaria* that retained 40% iNOS expression. Generally, *P. urinaria* (78% reduction) scored highest iNOS inhibition among aqueous extracts while *P. watsonii* (82% reduction) exhibited strongest activity among methanolic extracts. This suppression ability was comparable to Cisplatin and Doxorubicin effects, with 15% and 10% iNOS expression remained after their treatment respectively. Meanwhile, methanolic *Phyllanthus* extracts showed better suppression on VEGF expression with 60 – 80% reduction compared to aqueous extracts that caused 20 – 50% reduction (Figure 3.39B). Among the four methanolic extracts, *P. amarus* displayed better VEGF repression with only 20% VEGF expression. In addition to this ELISA assay, expression of VEGF was also confirmed by western blot assay as shown in Figure 3.40. The bands' intensity for *Phyllanthus*-treated A549 cells was thinner as compared to the untreated cells, with methanolic extracts exhibiting a better suppression on VEGF. Similarly, VEGF expression dropped 30 – 75% after A549 cells were treated with various aqueous and methanolic *Phyllanthus* extracts.

Meanwhile, iNOS and VEGF expression falls significantly to a range from 15 – 40% and 20 – 65% respectively after the MCF-7 cells were treated with various *Phyllanthus* extracts (Figure 3.41). Among the four species, aqueous *P. urinaria* (16% expression) and methanolic *P. urinaria* (20% expression) showed better inhibition on iNOS expression. This iNOS suppression ability was comparable to the Cisplatin and Doxorubicin that reduced iNOS expression in MCF-7 cells to 26% and 20% respectively. On the other hand, methanolic *P. amarus* (19% expression) was the most effective among the different *Phyllanthus* extracts and species in suppressing VEGF expression. Likewise, methanolic *Phyllanthus* extracts showed better inhibition on VEGF expression compared to the aqueous extracts. This observation was further confirmed using western blotting as shown in Figure 3.42 whereby the VEGF bands' intensity were decreased for *Phyllanthus*-treated MCF-7 cells. Quantitative assessment also revealed the drop in VEGF expression to 5 – 55% after MCF-7 cells were treated with various aqueous and methanolic *Phyllanthus* extracts.

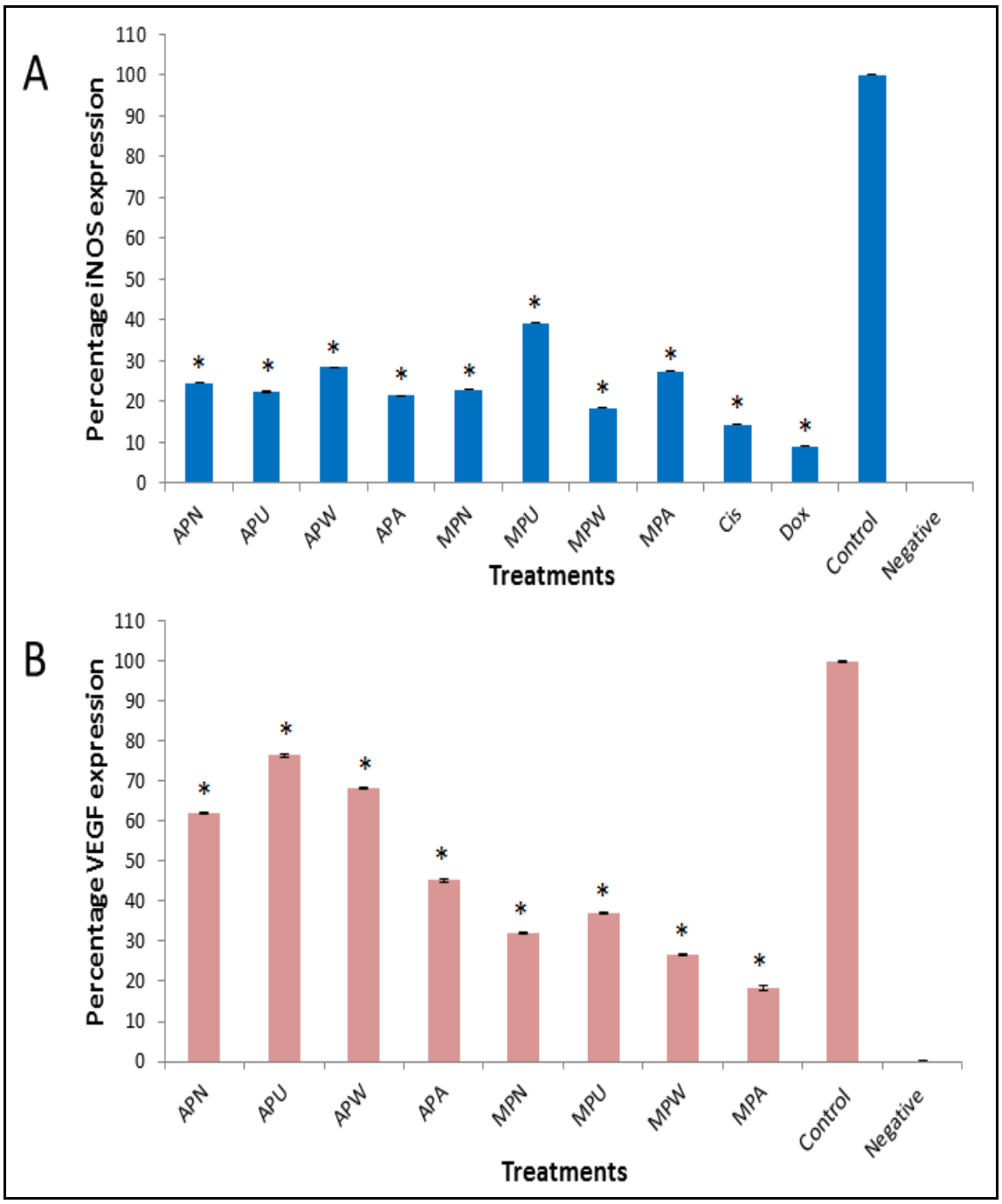


Figure 3.39: Expression levels of (A) iNOS and (B) VEGF in untreated and *Phyllanthus*-treated A549 cells.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; Cis – Cisplatin; Dox – Doxorubicin. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

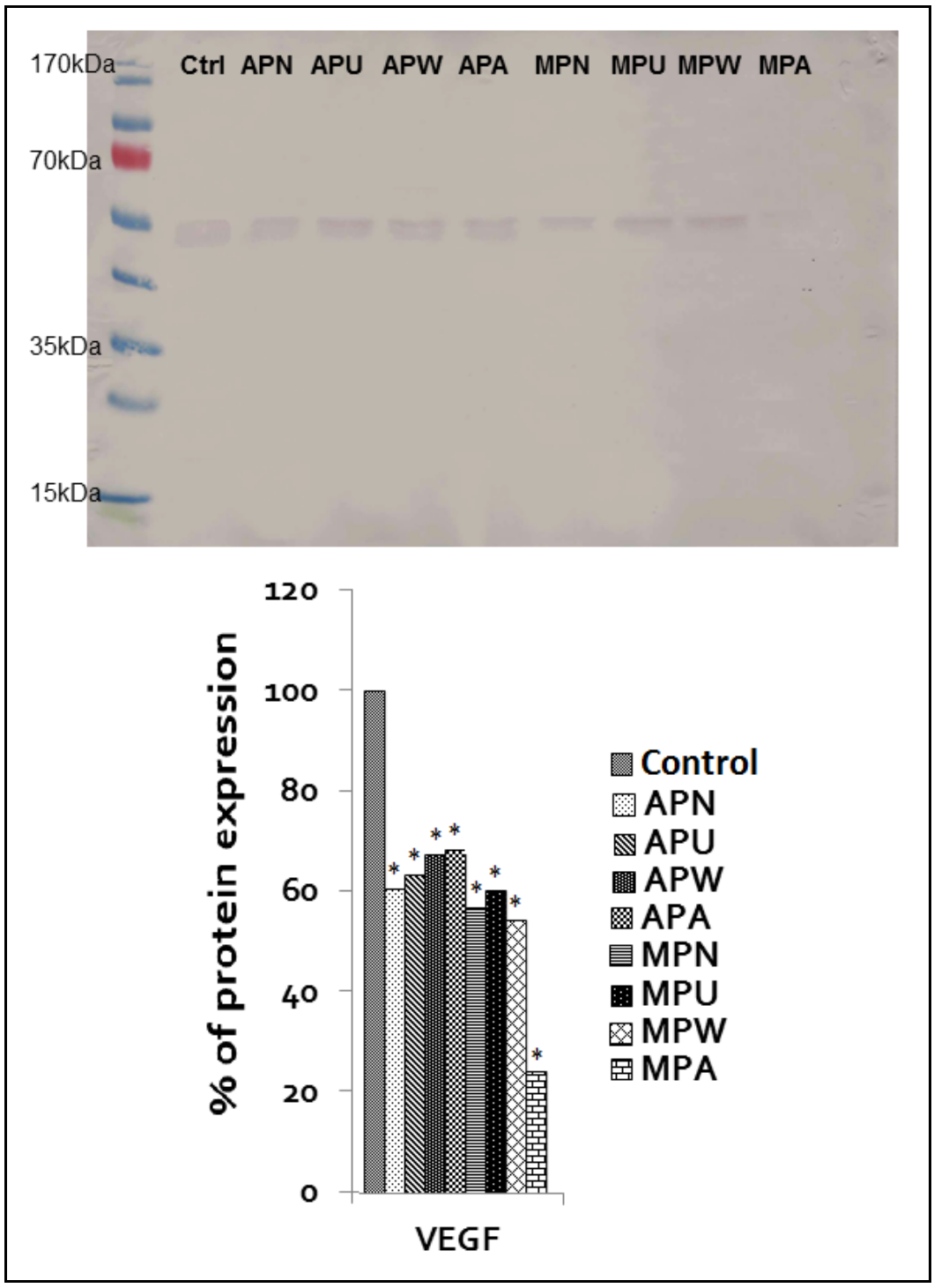


Figure 3.40: Western blotting of VEGF expression in A549 in response to *Phyllanthus* extracts.

Figure above shows western blot and expression level of VEGF protein in untreated control and *Phyllanthus*-treated A549 cells analyzed using Image J software. APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*. **P* < 0.05 vs untreated control.

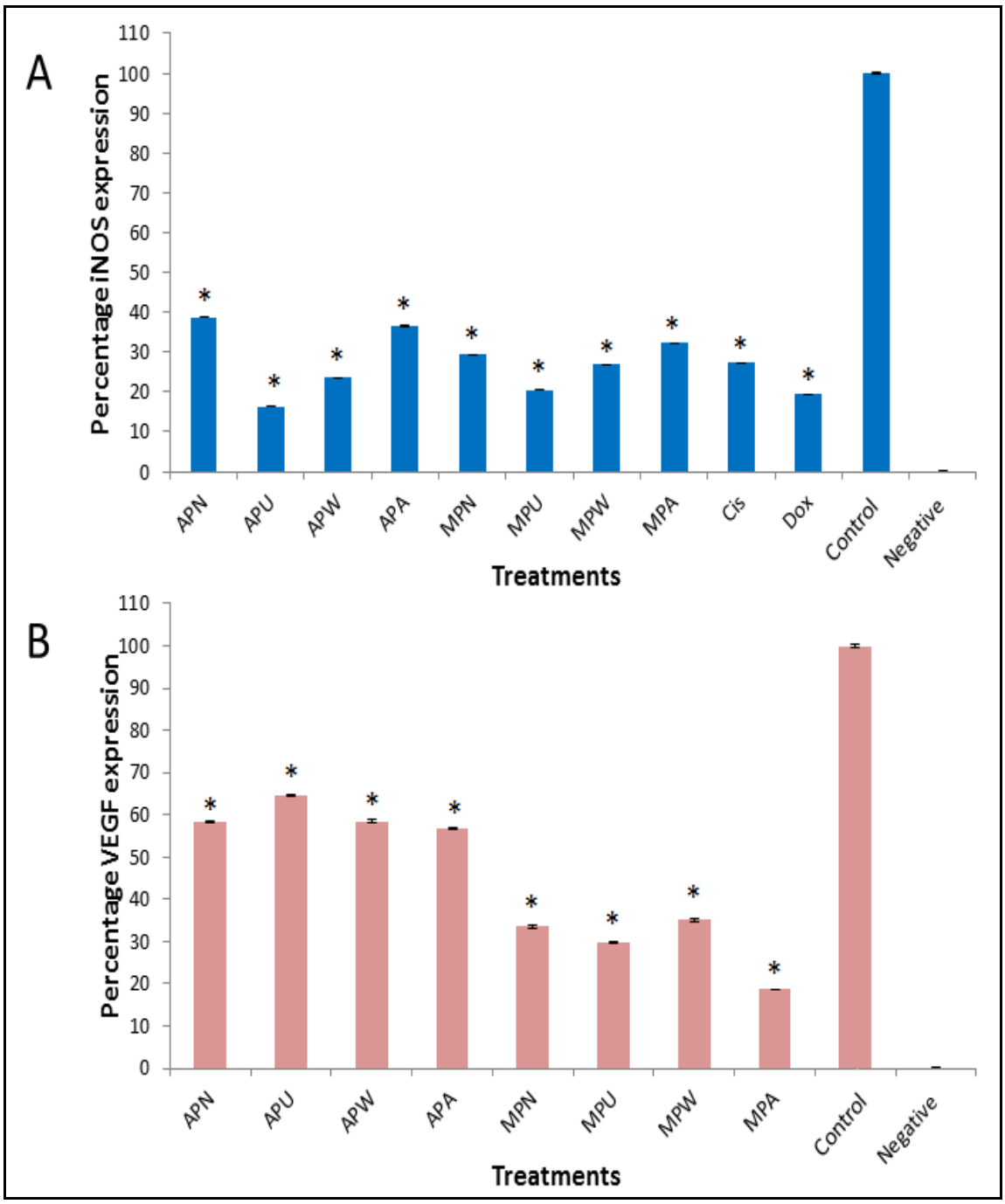


Figure 3.41: Expression levels of (a) iNOS and (b) VEGF in untreated and *Phyllanthus*-treated MCF-7 cells.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; Cis – Cisplatin; Dox – Doxorubicin. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

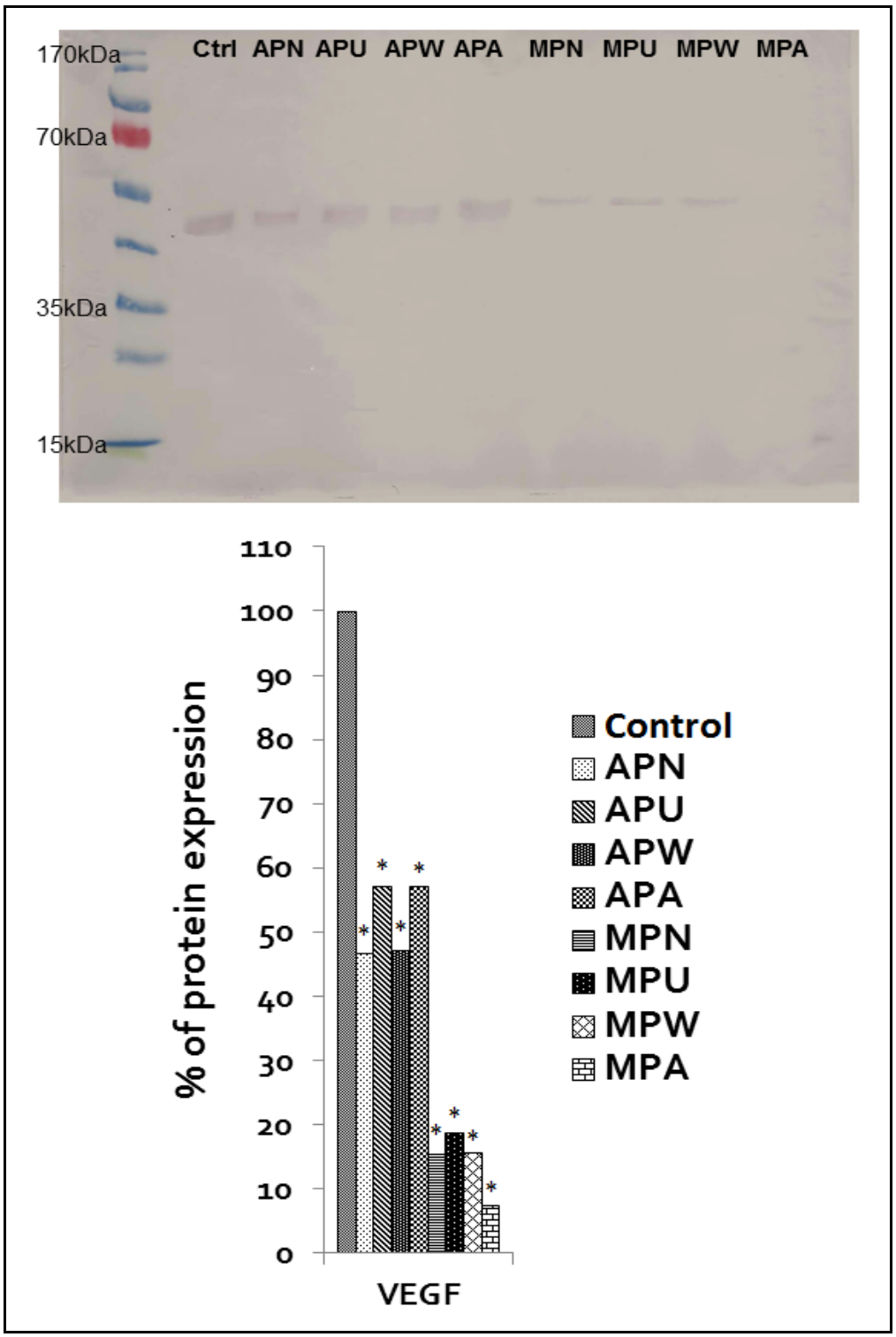


Figure 3.42: Western blotting of VEGF expression in MCF-7 in response to *Phyllanthus* extracts.

Figure above shows western blot and expression level of VEGF protein in untreated control and *Phyllanthus*-treated MCF-7 cells analyzed using Image J software. APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*. **P* < 0.05 vs untreated control.

3.6 EFFECTS OF *PHYLLANTHUS* ON CELL'S ENDOTHELIAL TO MESENCHYMAL TRANSITION (EMT)

Enhancement of cell motility for metastasis requires the conversion of an epithelial cell to a mesenchymal-like cell via epithelial-mesenchymal transition (EMT) process to release the epithelial tumor cells from its neighbouring tissue. Treatment of the cancer cells with *Phyllanthus* had showed some reversal of this EMT process to reduce their motility mainly via enhanced adhesion between cells.

3.6.1 EFFECTS OF *PHYLLANTHUS* ON CELL AGGREGATION

Metastatic cancer cells are usually characterized by loss of cell polarity as well as epithelial tight junctions to break their adhesions with the surrounding cells. This phenomenon was apparent in the untreated A549 (Figures 3.43, 3.44, 3.45, and 3.46) and MCF-7 (Figures 3.47, 3.48, 3.49, and 3.50) cells that remained in their individual cell suspension form up to 36 hours. However, they began to die after 48 hours incubation as shown by the clumped cells due to the absence of a flat surface for them to adhere onto and grow. In order to determine whether *Phyllanthus* could reverse this EMT process, cells were treated with the various aqueous and methanolic extracts of the four *Phyllanthus* species. A successful EMT reversion is signified by increased cell adhesions to each other to form cell aggregates. Indeed, the extracts-treated cells began to show appearance of cell aggregation after 24 hours of treatment as indicated by the red arrows. The cell aggregates became even more obvious after 36 hours incubation. Nevertheless, the cells started to die and formed clumps at 48 and 60 hours post-treatment, similar to the untreated control cells due to their inability to attach and grow.

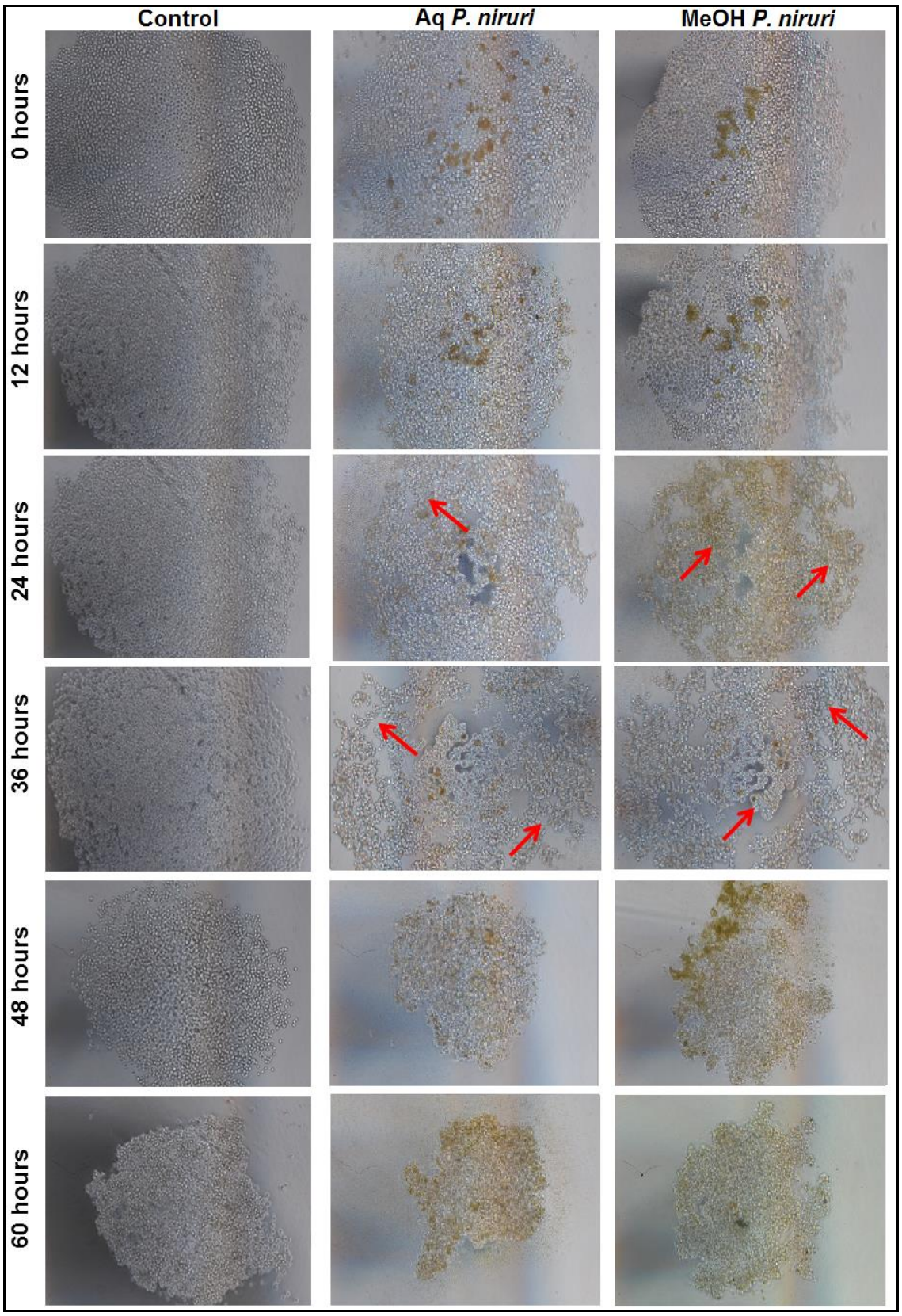


Figure 3.43: Cell aggregation status of A549 in response to *P. niruri*.

Figure above shows aggregation of A549 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. niruri* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

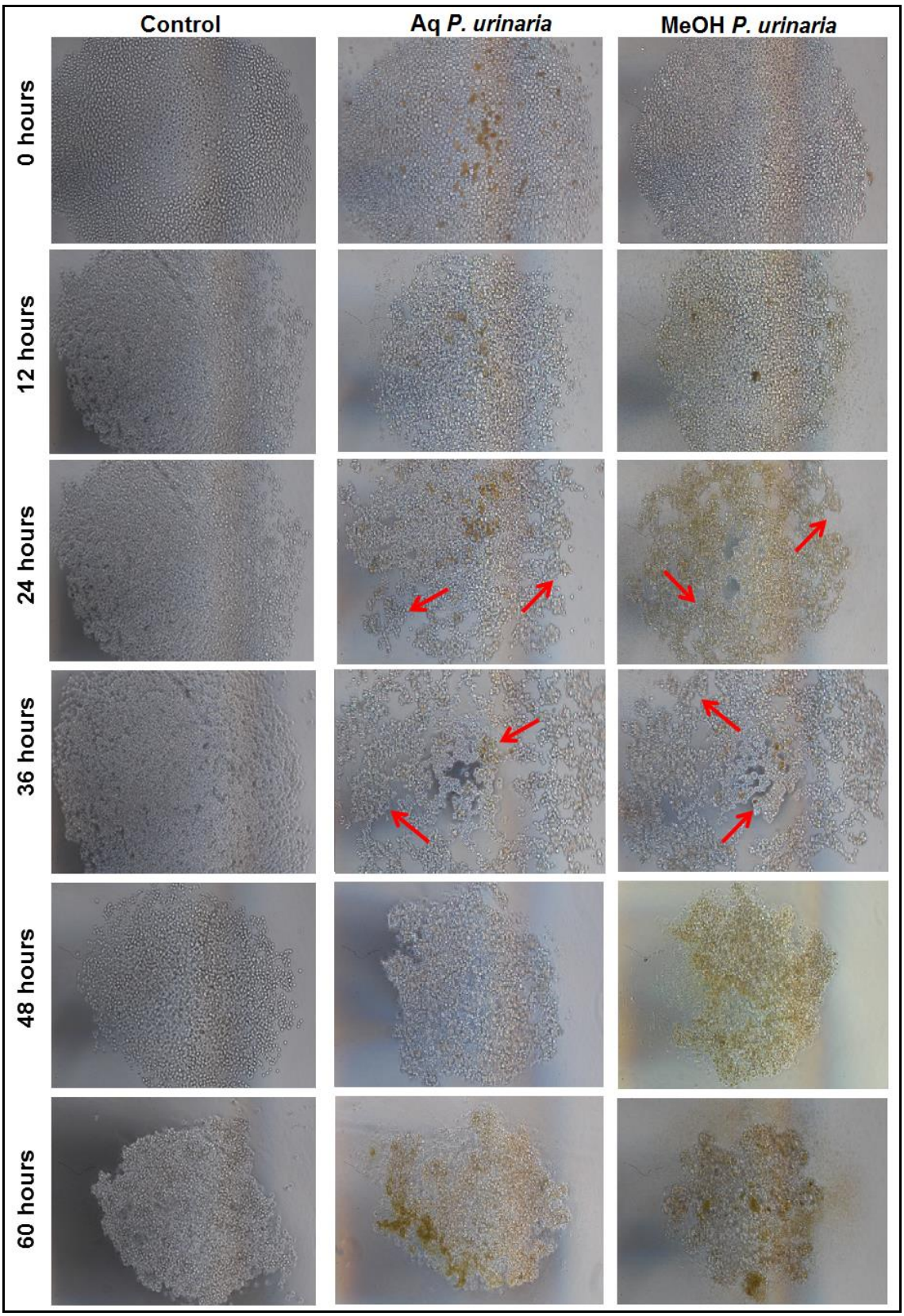


Figure 3.44: Cell aggregation status of A549 in response to *P. urinaria*.

Figure above shows aggregation of A549 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. urinaria* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

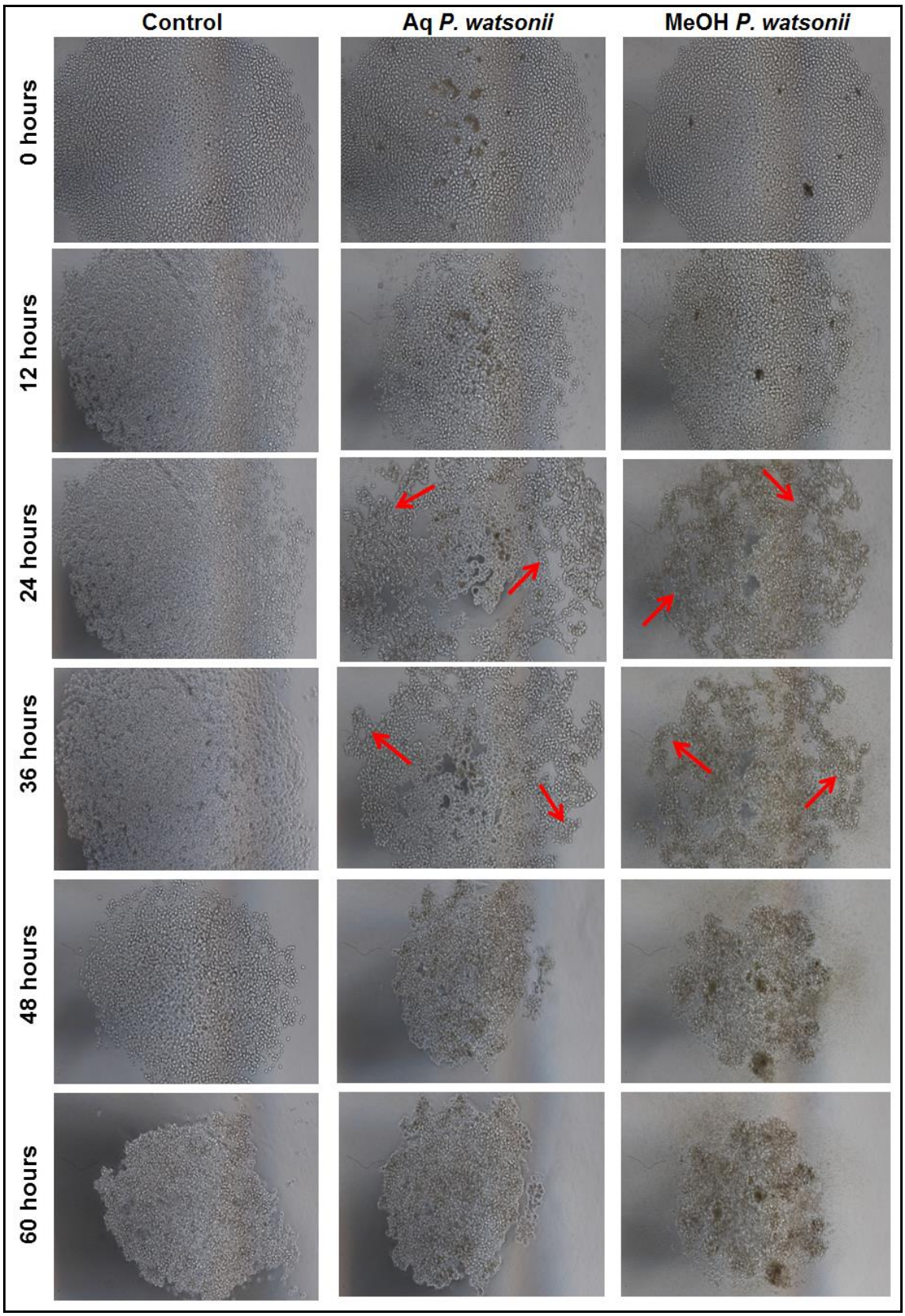


Figure 3.45: Cell aggregation status of A549 in response to *P. watsonii*.

Figure above shows aggregation of A549 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. watsonii* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

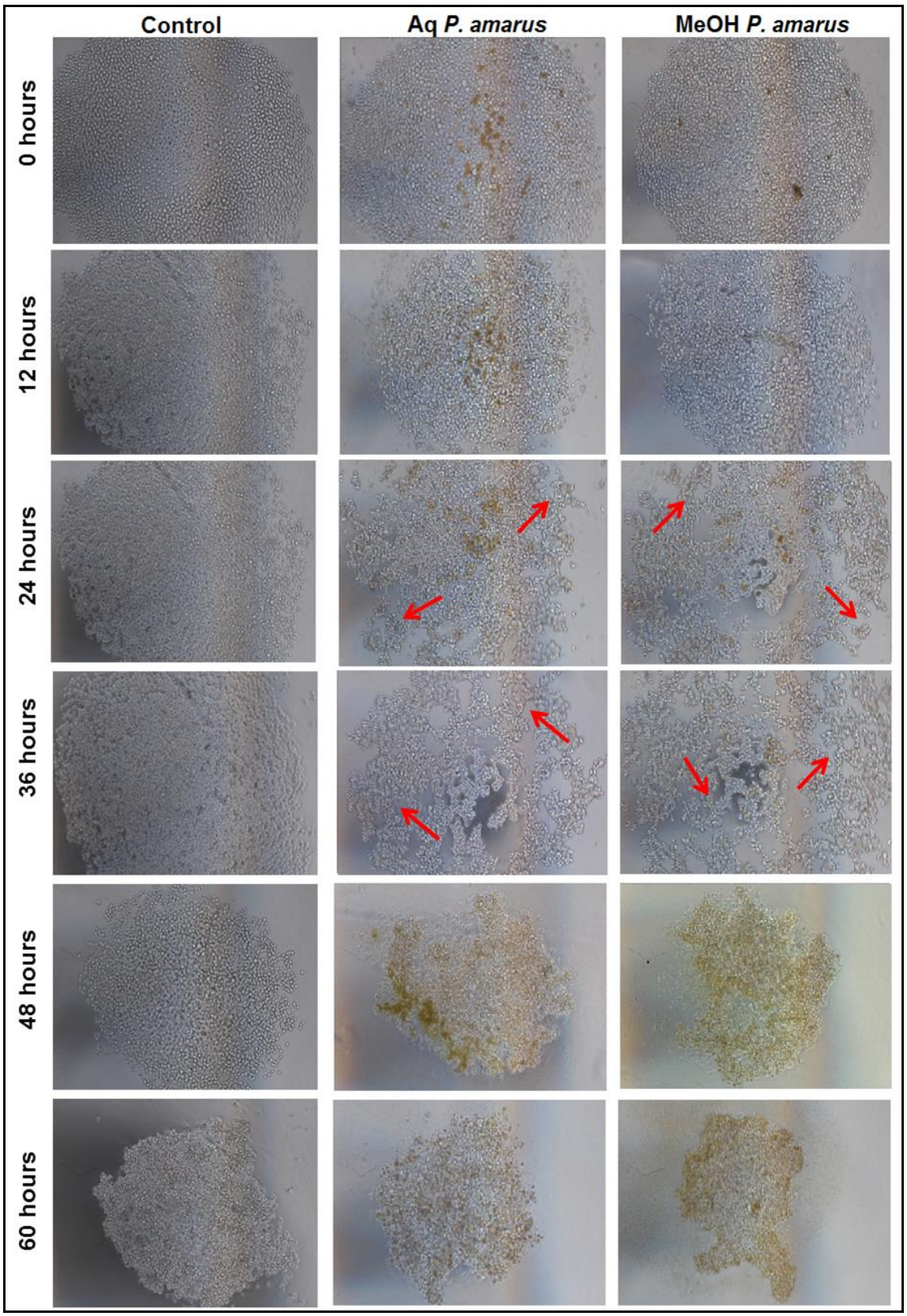


Figure 3.46: Cell aggregation status of A549 in response to *P. amarus*.

Figure above shows aggregation of A549 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. amarus* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

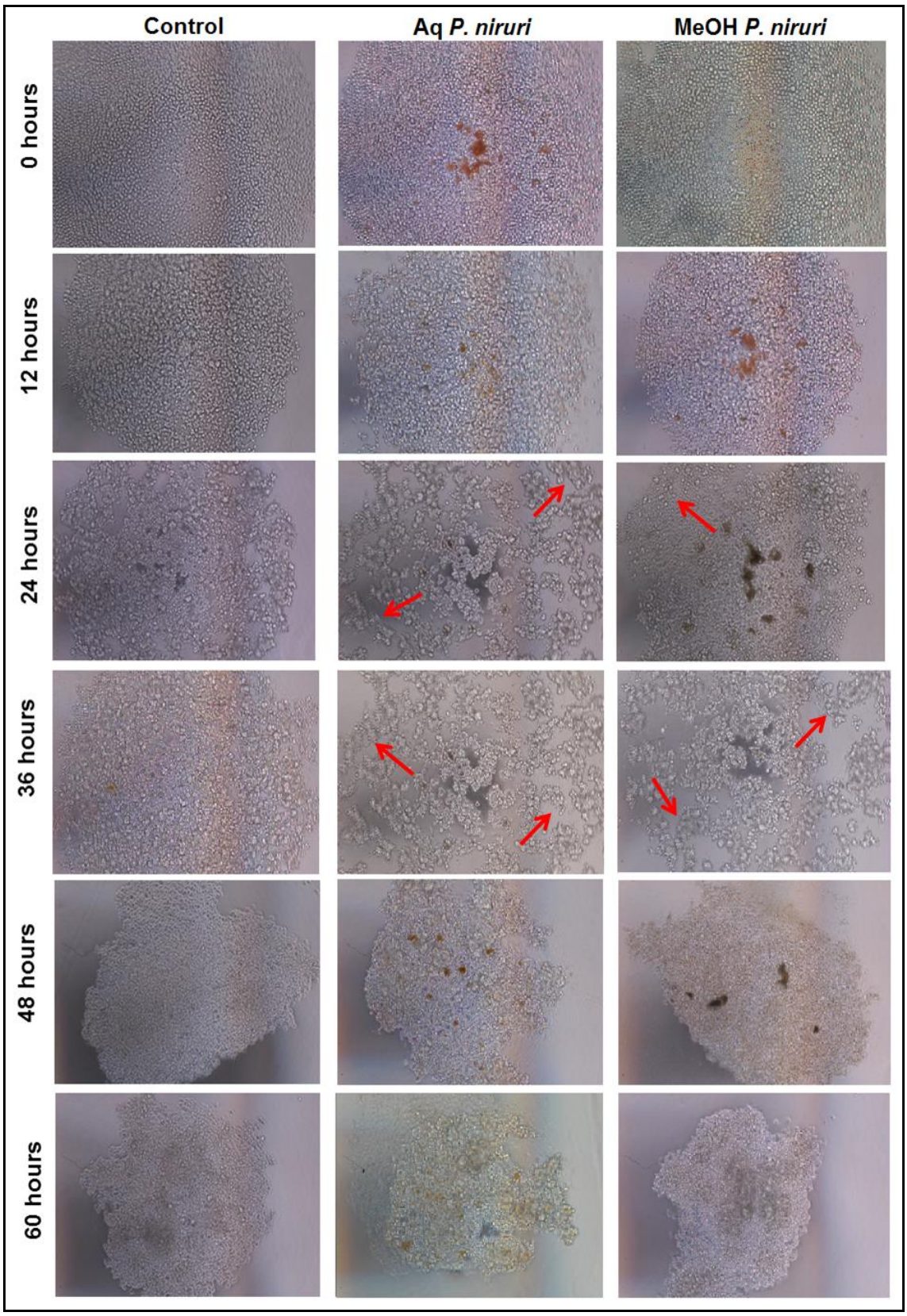


Figure 3.47: Cell aggregation status of MCF-7 in response to *P. niruri*.

Figure above shows aggregation of MCF-7 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. niruri* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

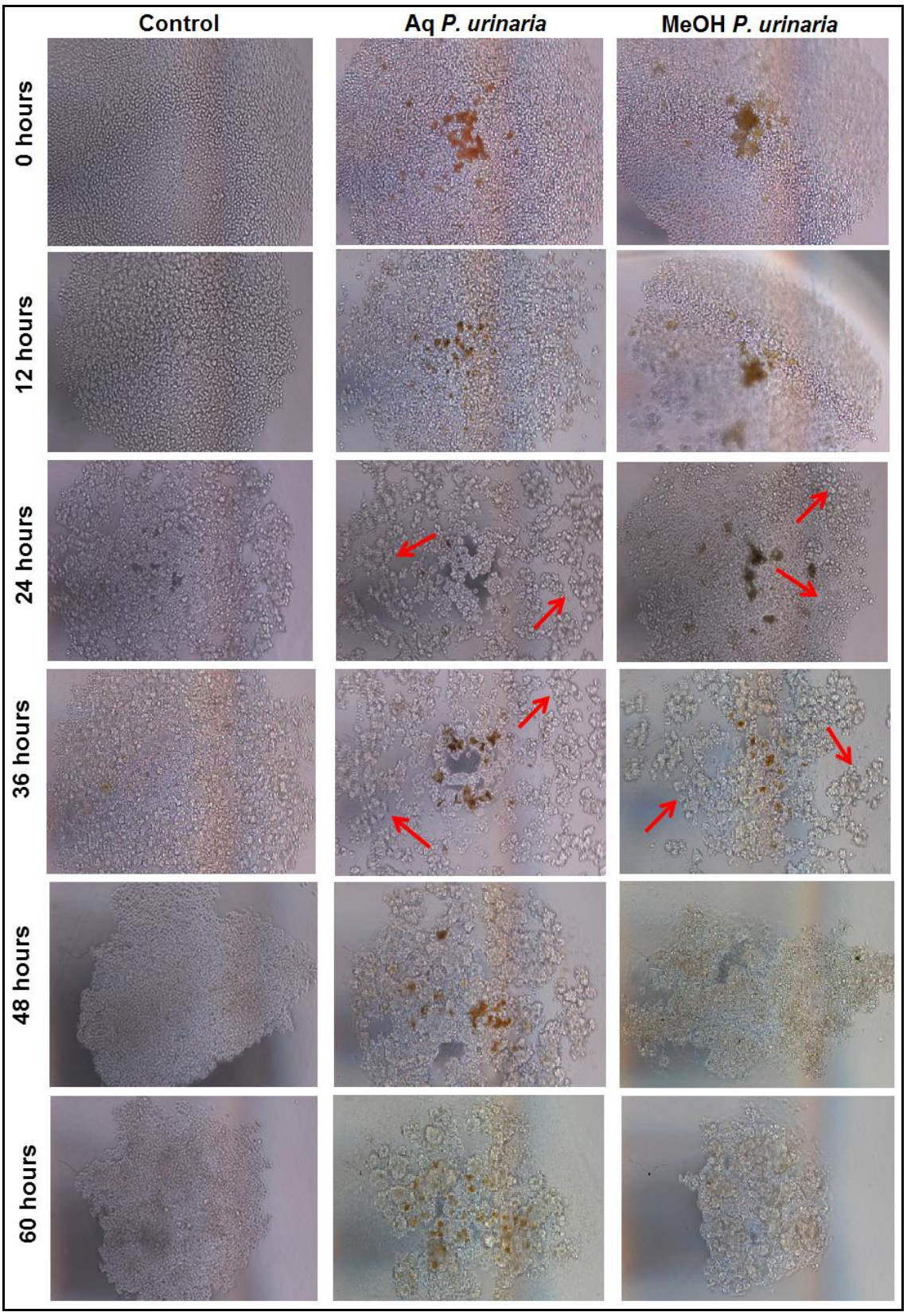


Figure 3.48: Cell aggregation status of MCF-7 in response to *P. urinaria*.

Figure above shows aggregation of MCF-7 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. urinaria* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

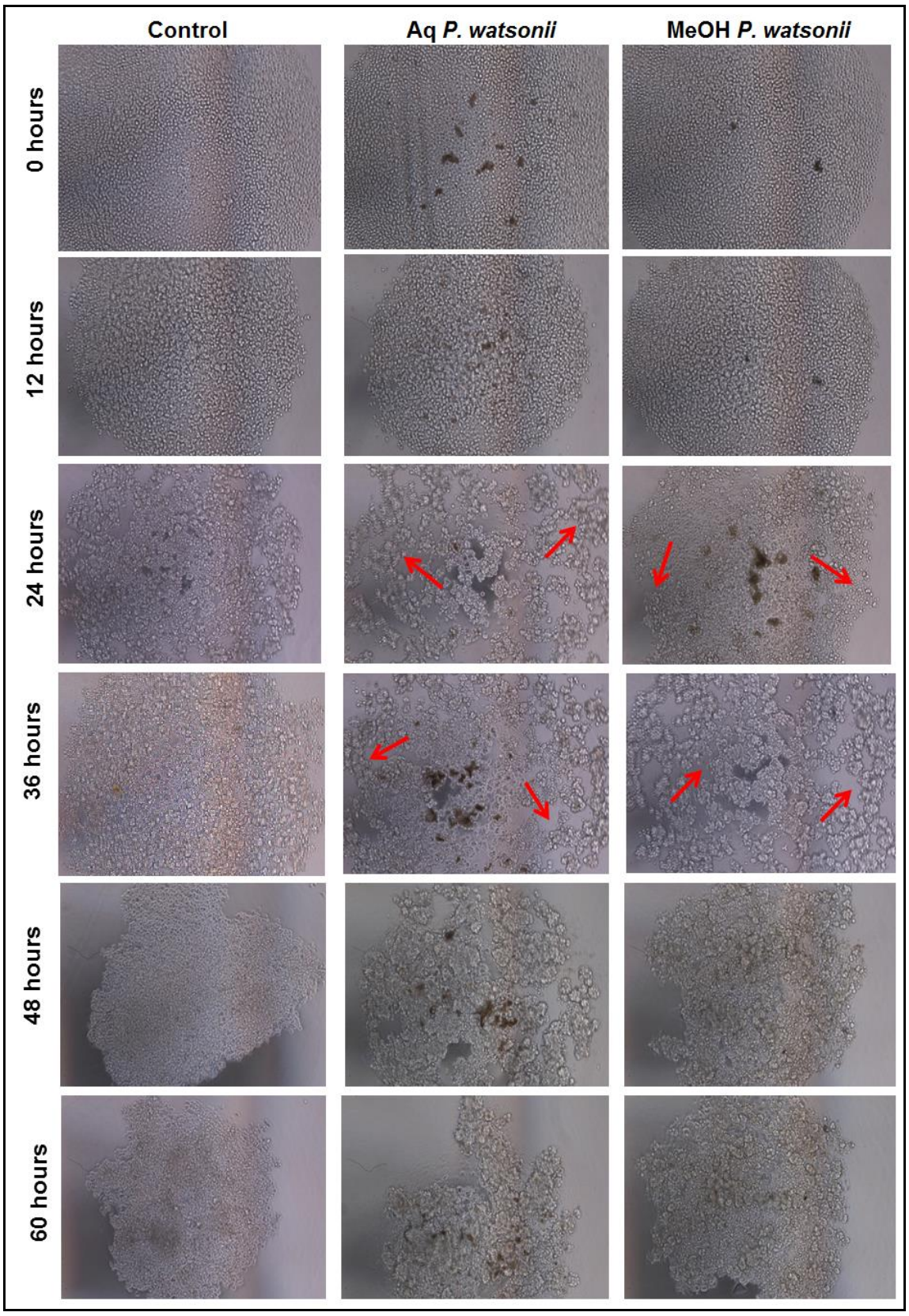


Figure 3.49: Cell aggregation status of MCF-7 in response to *P. watsonii*.

Figure above shows aggregation of MCF-7 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. watsonii* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

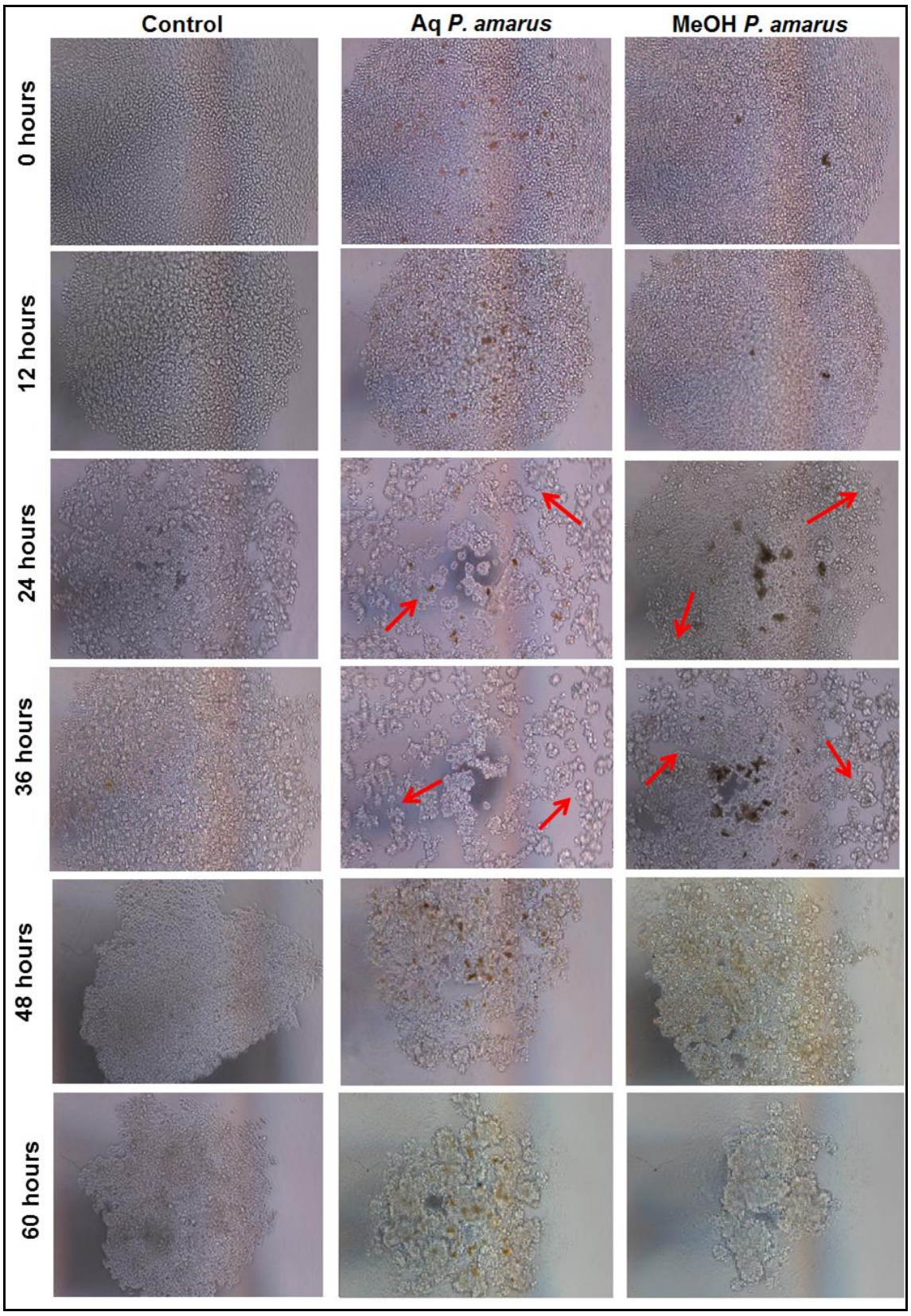


Figure 3.50: Cell aggregation status of MCF-7 in response to *P. amarus*.

Figure above shows aggregation of MCF-7 cells (indicated by red arrows) treated with aqueous (Aq) and methanolic (MeOH) *P. amarus* extracts after 60 hours incubation. Typical result from three independent experiments is shown. (Magnification power: 40×)

3.6.2 EFFECTS OF *PHYLLANTHUS* ON CELL-CELL ADHESION

Cell-cell adhesion is mediated by the homotypic interactions of the cadherin molecules located on their cell surface. Expression of cadherins is mainly tissue type specific, as E-cadherins are usually confined to the epithelial cells. Hence, this assay was performed to determine whether *Phyllanthus* could increase the E-cadherin expression on the A549 and MCF-7 cells that is correlated to the suppression of cell metastasis. Figure 3.51 shows the cell-cell adhesion percentage between the untreated cells and the cells treated with various *Phyllanthus* extracts.

For A549, the percentage of cell-cell adhesion of the untreated cells to HUVEC cells was 20%. When the cells were treated at low concentrations, only the aqueous extracts showed approximately 10% increased cell-cell adhesion activity. However, when the extracts concentrations were increased, cell-cell adhesion activity was notably enhanced (increased approximately 40 – 50% adhesion activity) hence showing a dose-dependent effect (Figure 3.51A). Among the extracts, aqueous *Phyllanthus* has greater cell-cell adhesion promoting capability compared to methanolic extracts, in particular *P. niruri*, followed by *P. amarus*, *P. watsonii*, and *P. urinaria*. However, the cell-cell adhesion was not increased greatly when the A549 cells were treated with standard drugs due to their low adhesion percentage (Cisplatin – 32% and Doxorubicin – 25%).

For MCF-7 cells, the percentage of cell-cell adhesion of the untreated MCF-7 cells to HUVEC cells was approximately 35% (Figure 3.51B). Treatment of the cells with low concentrations of extracts did not promote the cell-cell adhesion activity except for aqueous *P. niruri* and *P. amarus*. Nevertheless, the cell-cell adhesion activity increased about 40% when the cells were treated with higher concentrations of the extracts. Likewise, aqueous *Phyllanthus* extracts especially *P. niruri* had higher effectiveness while treatment with standard drugs barely increased cell-cell adhesion.

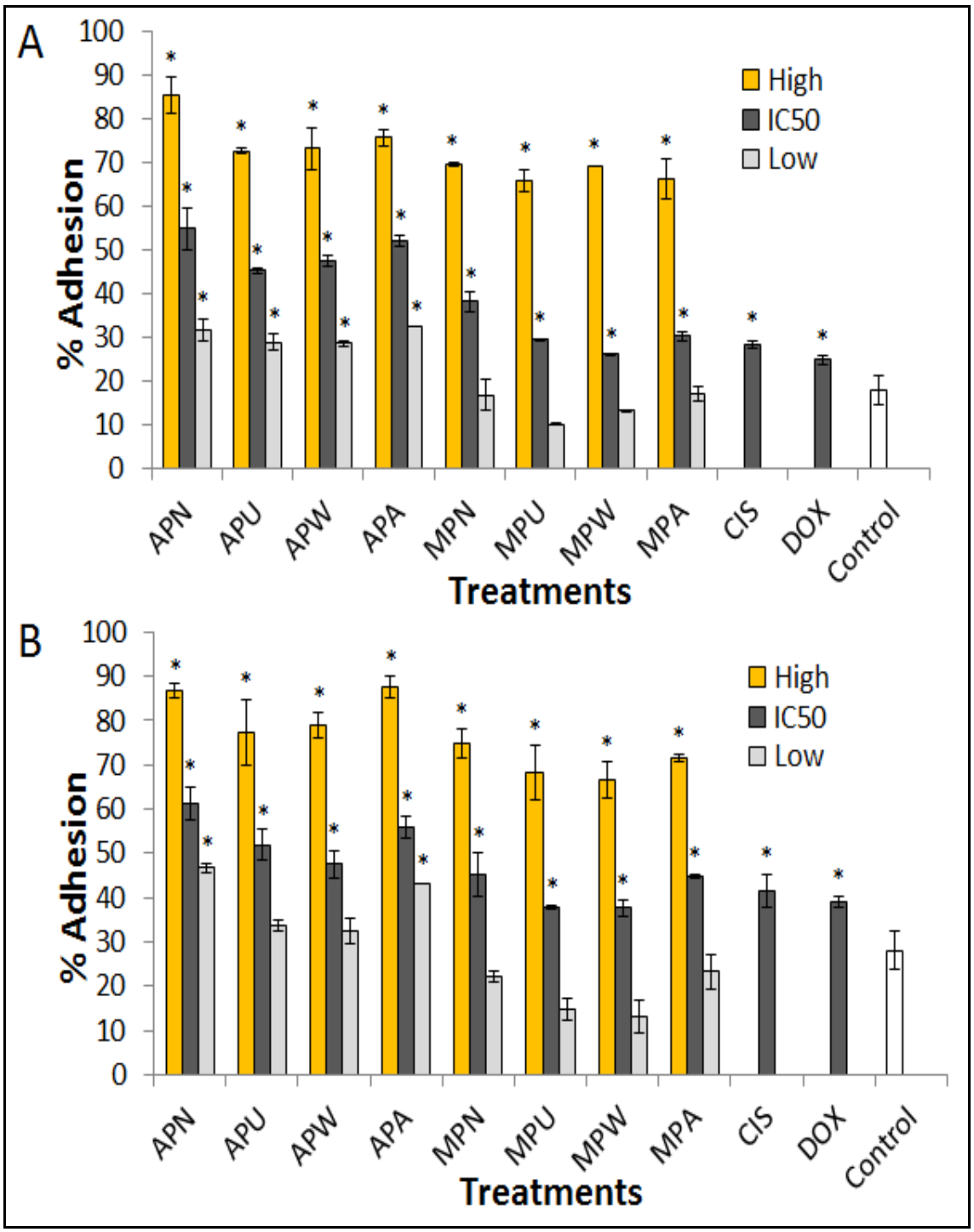


Figure 3.51: Cell-cell adhesion percentage of (A) A549 and (B) MCF-7 cells treated with *Phyllanthus* extracts at varying concentrations.

Low – treatment at 50µg/ml and 20µg/ml for aqueous and methanolic extracts respectively; High – treatment at 500µg/ml and 200µg/ml for aqueous and methanolic extracts respectively; APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; CIS – Cisplatin; DOX – Doxorubicin; Control – untreated cells. Error bar indicates the standard error mean of three independent experiments. **P* < 0.05 vs untreated control.

3.7 SIGNALING PATHWAYS ANALYSIS

Antiproliferative, antimetastatic, and induction of apoptosis is usually the result of a series of events known as signal transduction. Treatment with *Phyllanthus* will usually lead to receptor binding which in turn stimulates or inhibits a particular signaling cascade that is made up of various components to exert a biological response. Various signaling pathways could be activated or suppressed at the same time to cause the different inhibitory activities in cancer cells.

3.7.1 DETERMINATION OF SIGNALING PATHWAYS AFFECTED BY *PHYLLANTHUS*

In order to find out the pathways that were affected by *Phyllanthus* to exert its anticancer activities, a cignal finder cancer 10-pathway reporter array was used as it permits the concurrent screening of 10 major cellular pathways. The pathways included in this array include Wnt, Notch, p53/DNA damage, TGFβ, Cell cycle/pRb-E2F, NFκB, Myc/Max, Hypoxia, MAPK/ERK, and MAPK/JNK, with GFP construct plasmid DNA as the positive control for this array. As shown in Figures 3.52 (aqueous and methanolic extracts-treated A549) and 3.53 (aqueous and methanolic extracts-treated MCF-7), the expression of GFP construct was consistent in both the extracts-treated and untreated control cells, hence the results obtained were deemed valid.

Among the 10 pathways studied, NFκB, Myc/Max, Hypoxia, and MAPK (ERK and JNK) pathways were highly expressed in untreated A549 as compared to the other pathways (Figure 3.52), therefore suggesting their major role to ensure A549 cells growth and survival. Upon treatment with various aqueous and methanolic extracts, most of these pathways' expression decreased significantly ($p < 0.05$) except for NFκB which indicate *Phyllanthus* probably did not modulate this pathway to cause cell growth

or metastasis inhibition. However, aqueous *Phyllanthus* extracts showed better inhibitory activity on the expression of both ERK (20 - 43% reduction) and JNK (44 - 50% reduction) pathways, while the methanolic extracts showed enhanced inhibition on the expression of Hypoxia (42 - 76% reduction) and Myc/Max (92 - 97% reduction) pathways. Among the four plant species, *P. watsonii* exhibited greatest suppression on Hypoxia (aqueous – 60% and methanolic – 86%), ERK (aqueous – 47% and methanolic – 27%), and JNK (aqueous - 50% and methanolic – 26%) pathways, followed by *P. urinaria*, *P. amarus*, and *P. niruri*. On the other hand, other pathways including Wnt, Notch, p53/DNA damage, TGFβ as well as cell cycle/pRb-E2F could be playing insignificant roles in A549 since they were lowly expressed in both the treated and untreated cells. From the flow cytomery-based cell cycle analysis (Section 3.2.1.3), we did not observe cell cycle phase arrest in the A549 cells treated with *Phyllanthus* extracts since the percentage of gated cells for each cell cycle phases (G_0/G_1, S, and G_2/M) did not change significantly ($p > 0.05$) between the untreated and extracts-treated cells. Thus, this further explained the low expression level of cell cycle/pRb-E2F pathway obtained in this assay for both the untreated and treated A549 cells.

Likewise, Myc/Max, Hypoxia, and JNK pathways were highly expressed in untreated MCF-7 as compared to the other pathways (Figure 3.53). Thus, these pathways are most likely constitutively activated by MCF-7 cells to guarantee their continuous growth and survival. However, treatment with various aqueous and methanolic *Phyllanthus* extracts reduced the expression of these pathways significantly ($p < 0.05$), leading to an inhibition of cell growth and metastasis. Comparing the effect of both extracts, methanolic *Phyllanthus* generally demonstrated approximately 10 – 20% increased inhibitory activity on those pathways than the aqueous extracts. There were no significant differences ($p > 0.05$) between the four *Phyllanthus* species although *P. urinaria* might appear to be a little more effective in inhibiting those

pathways. NFκB is probably another pathway utilized by MCF-7 to ensure their growth due to its high expression in the untreated cells. Yet, data obtained suggests that both aqueous and methanolic *Phyllanthus* did not modulate this pathway to exert antiproliferative action except for methanolic *P. niruri* and *P. watsonii* that showed significant differences ($p < 0.05$) than the untreated cells. Besides that, cell cycle/pRb-E2F pathway was also observed to be expressed in untreated MCF-7 and showed reduction after extracts treatment. However, their percentages of expression were noticed to be lower than the GFP construct expression and for that reason, its contribution in suppression of cell growth and survival was inconclusive. Nevertheless, results obtained from flow cytometry-based cell cycle analysis (Section 3.2.3) confirmed that *Phyllanthus* did not exhibit cell cycle phase arrest in the MCF-7 cells. Other pathways including Wnt, Notch, p53/DNA damage, as well as TGFβ were also not involved in MCF-7 to ensure their growth and survival due to low expressions in both the untreated and extracts-treated cells.

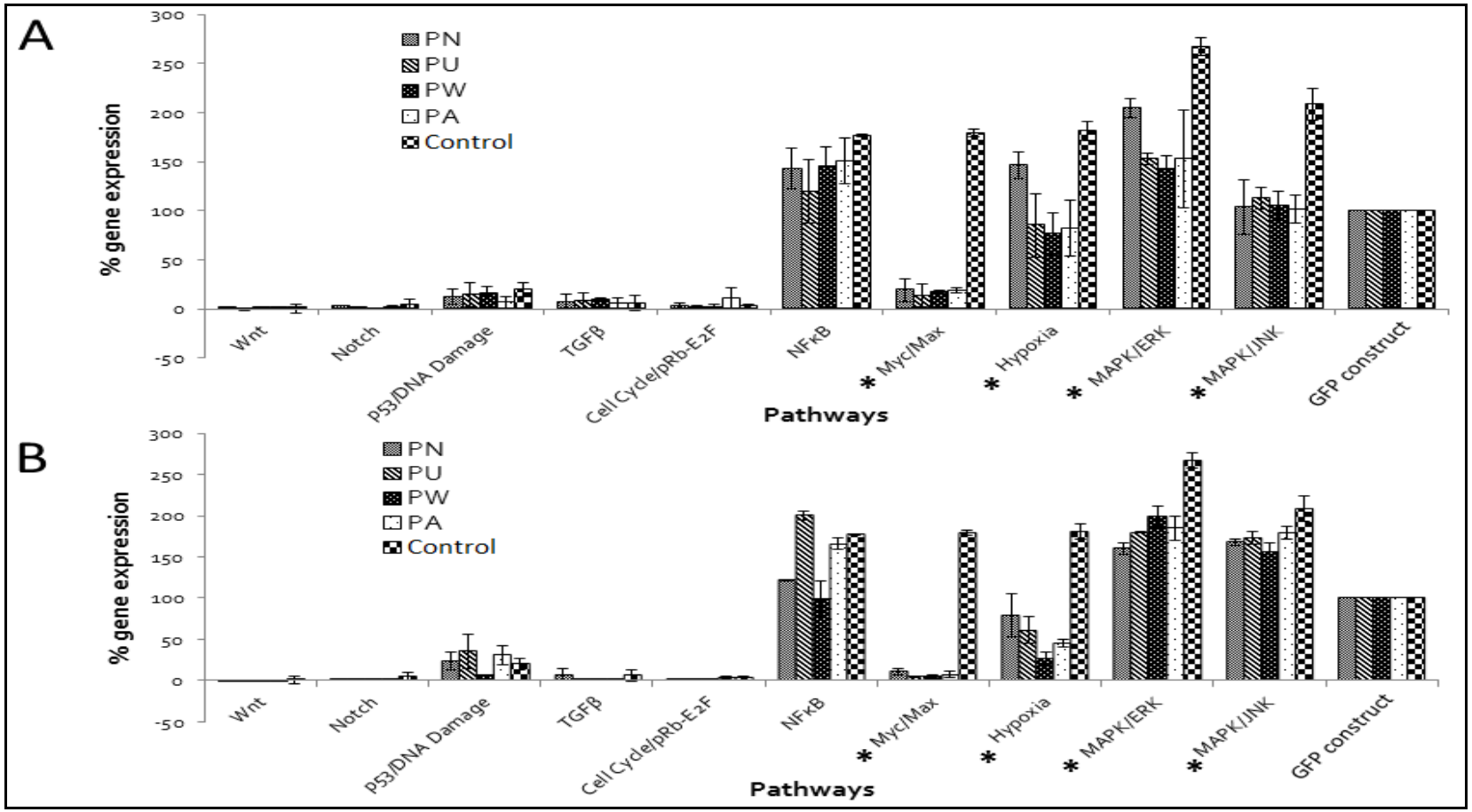


Figure 3.52: Expression level of ten cellular signalling pathways in A549 as determined by cignal finder cancer 10-pathway reporter array.

Figure above shows the expression level of pathways in A549 cells treated with (a) aqueous *Phyllanthus* extracts and (b) methanolic *Phyllanthus* extracts. PN – *P. niruri*; PU – *P. urinaria*; PW – *P. watsonii*; PA – *P. amarus*. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

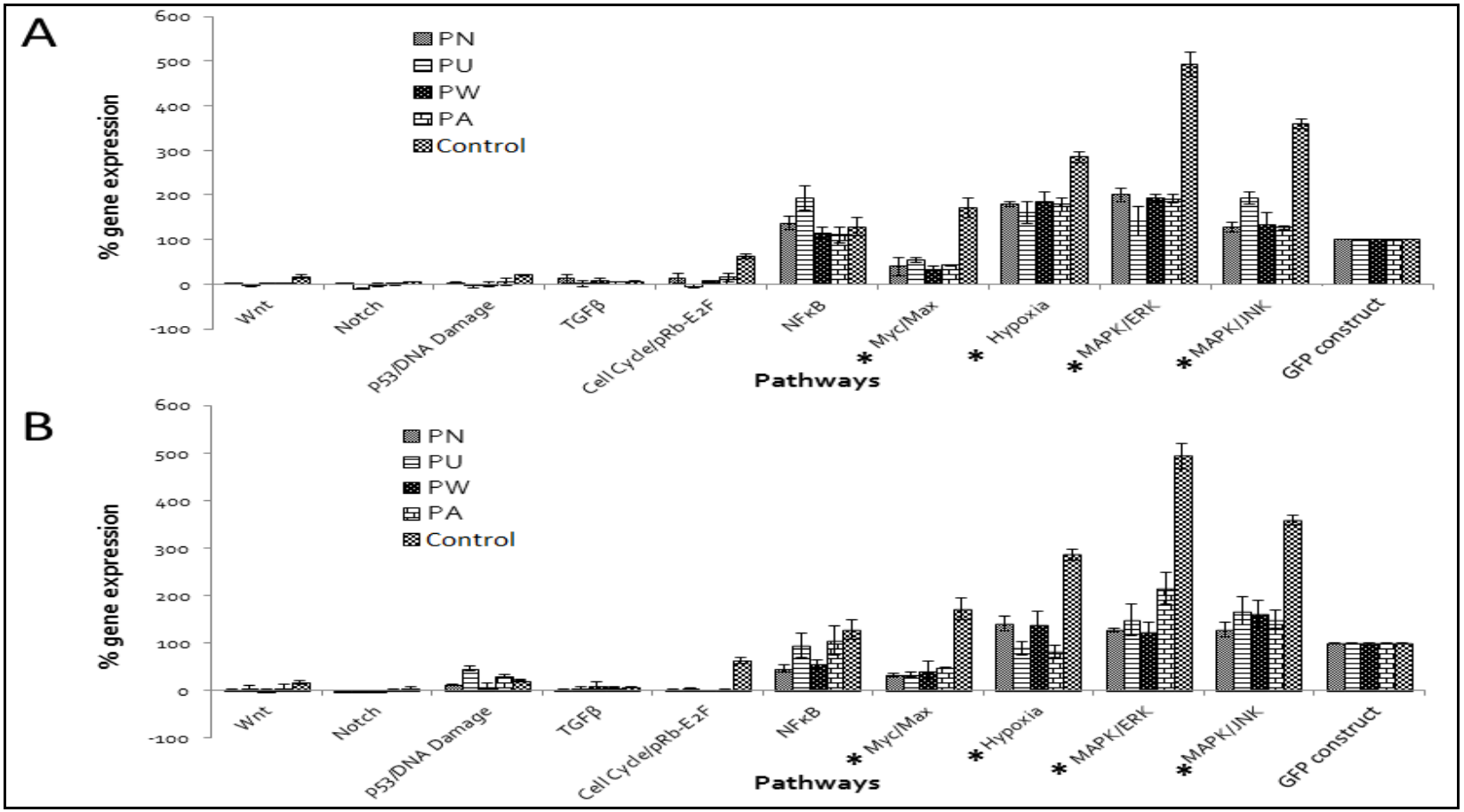


Figure 3.53: Expression level of ten cellular signalling pathways in MCF-7 as determined by cignal finder cancer 10-pathway reporter array.

Figure above shows the expression level of ten pathways in MCF-7 cells treated with (a) aqueous *Phyllanthus* extracts and (b) methanolic *Phyllanthus* extracts. PN – *P. niruri*; PU – *P. urinaria*; PW – *P. watsonii*; PA – *P. amarus*. Error bar indicates the standard error mean of three independent experiments. *$P < 0.05$ vs untreated control.

3.7.2 WESTERN BLOTTING OF SIGNALING PATHWAYS AFFECTED BY *PHYLLANTHUS*

Phyllanthus was shown to suppress MAP kinases and Hypoxia pathways to inhibit A549 and MCF-7 cell's growth, survival, and metastasis. However, it was unclear as to which specific protein within the pathway was targeted by *Phyllanthus* to suppress its activation since the pathways cascade involves many different kinases and proteins (Figure 3.54). So, western blots were performed with available antibodies (Anti-pan-Ras, anti-c-Raf, anti-c-Myc, anti-Bcl-2, anti-Hif-1α, anti-c-Jun/AP-1, anti-p53, anti-Elk1, anti-JNK1/2, anti-VEGF, and anti-RSK) to determine the specific targets of *Phyllanthus*, whether it affects during the early or late stages of the cascade. Figure 3.55 shows the blots for untreated A549 control as well as the A549 treated with various aqueous and methanolic extracts while Figure 3.56 depicted the expression level of each protein. Similarly, Figure 3.57 represents the blots for untreated MCF-7 control as well as the cells treated with various aqueous and methanolic extracts while Figure 3.58 illustrated the expression level of each protein. In both untreated A549 and MCF-7 cells, the proteins detected were Pan-Ras, c-Raf, c-Jun/AP-1, Elk-1, c-Myc, and HIF-1α. The presence of these proteins reflects the specific involvement of MAPK/ERK and Hypoxia pathways in regulating the cells growth and survival. In addition, Bcl-2 protein was also detected in both untreated A549 and MCF-7 which explains its role as an antiapoptotic agent in cancer cells to ensure their survival (Teijido & Dejean 2010).

As expected, Pan-Ras, c-Raf, c-Jun/AP-1, Elk-1, c-Myc, HIF-1α, Bcl-2, and FUSE-binding proteins' expressions were decreased when A549 was treated with various *Phyllanthus* extracts (Figures 3.55 and 3.56). Among the four species, *P. urinaria* shows better inhibition on those proteins, followed by *P. amarus*, *P. watsonii*, and *P. niruri* for both the aqueous and methanolic extracts. However, expression of Pan-

Ras and Elk-1 proteins were only mildly affected as compared to the other proteins. Besides that, FUSE-binding proteins were also detected although antibody specific to this protein was not included in this experiment. This could be attributed to its role for proper regulation of the c-Myc protooncogene (Weber et al. 2008), hence having certain percentage of similarity with c-Myc protein and was therefore detected when c-Myc antibody was used. P53 expression was also hardly seen in both the treated and untreated A549 cells via western blot technique and this confirmed the finding obtained from the previous cancer 10-pathway array that demonstrated to be low p53 expression in A549.

Likewise, the expressions of Pan-Ras, c-Raf, c-Jun/AP-1, Elk-1, c-Myc, HIF-1α, and Bcl-2 proteins were decreased when MCF-7 was treated with different aqueous and methanolic *Phyllanthus* extracts (Figures 3.57 and 3.58). Generally, methanolic extracts exhibited better suppression on these proteins compared to the aqueous extracts, in particular methanolic *P. watsonii* and *P. amarus*. Similar phenomenon was also observed among aqueous extracts whereby both *P. watsonii* and *P. amarus* showed enhanced activity than the other two species. Decreased expression of HIF-1α might also contribute to the suppression of cell metastasis and survival that is further supported by the evidence of decreased Bcl-2 expression that predisposes MCF-7 to apoptosis. Nonetheless, p53 expression remained undetected in both treated and untreated MCF-7 cells.

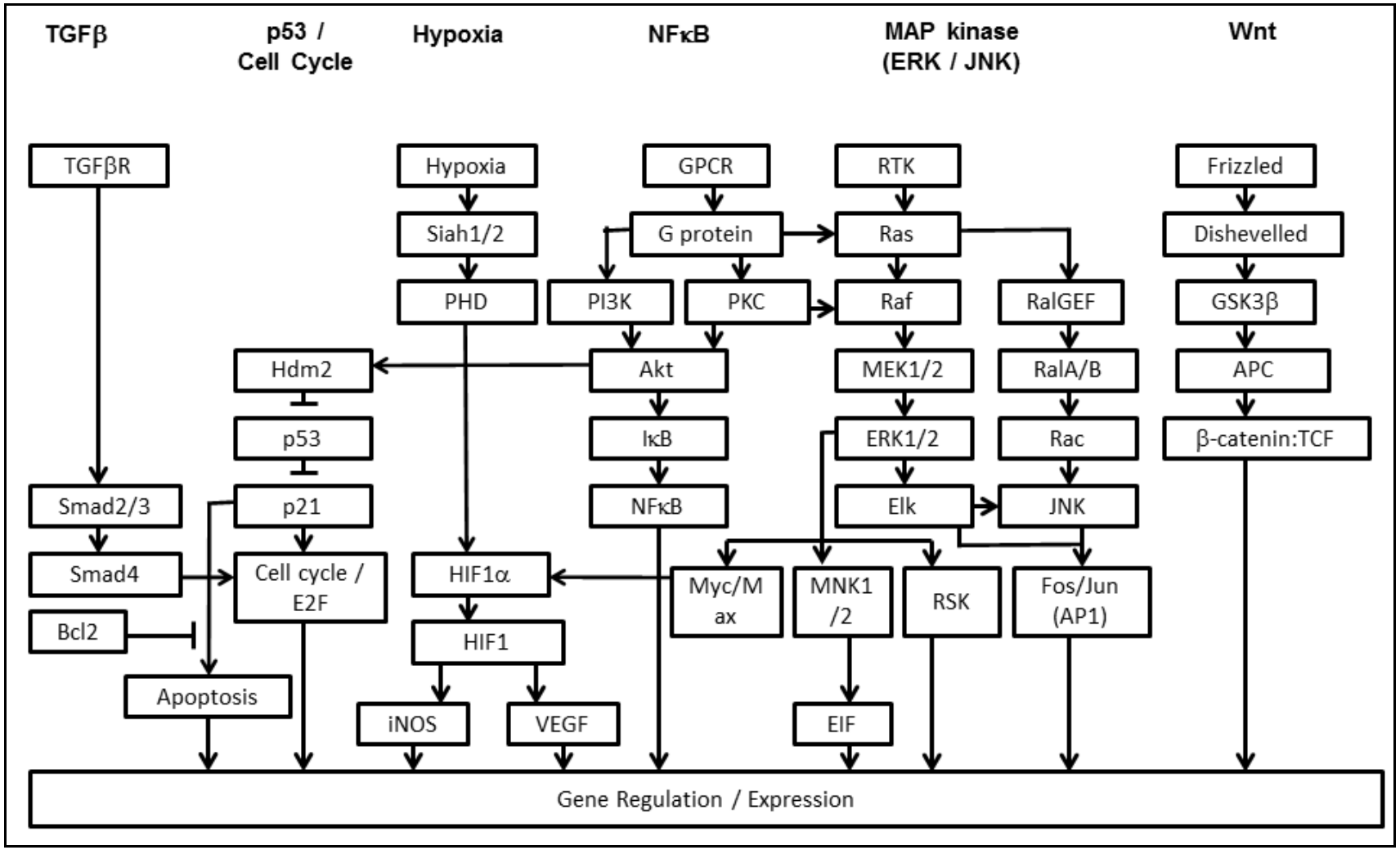


Figure 3.54: Pathways cascade activation.

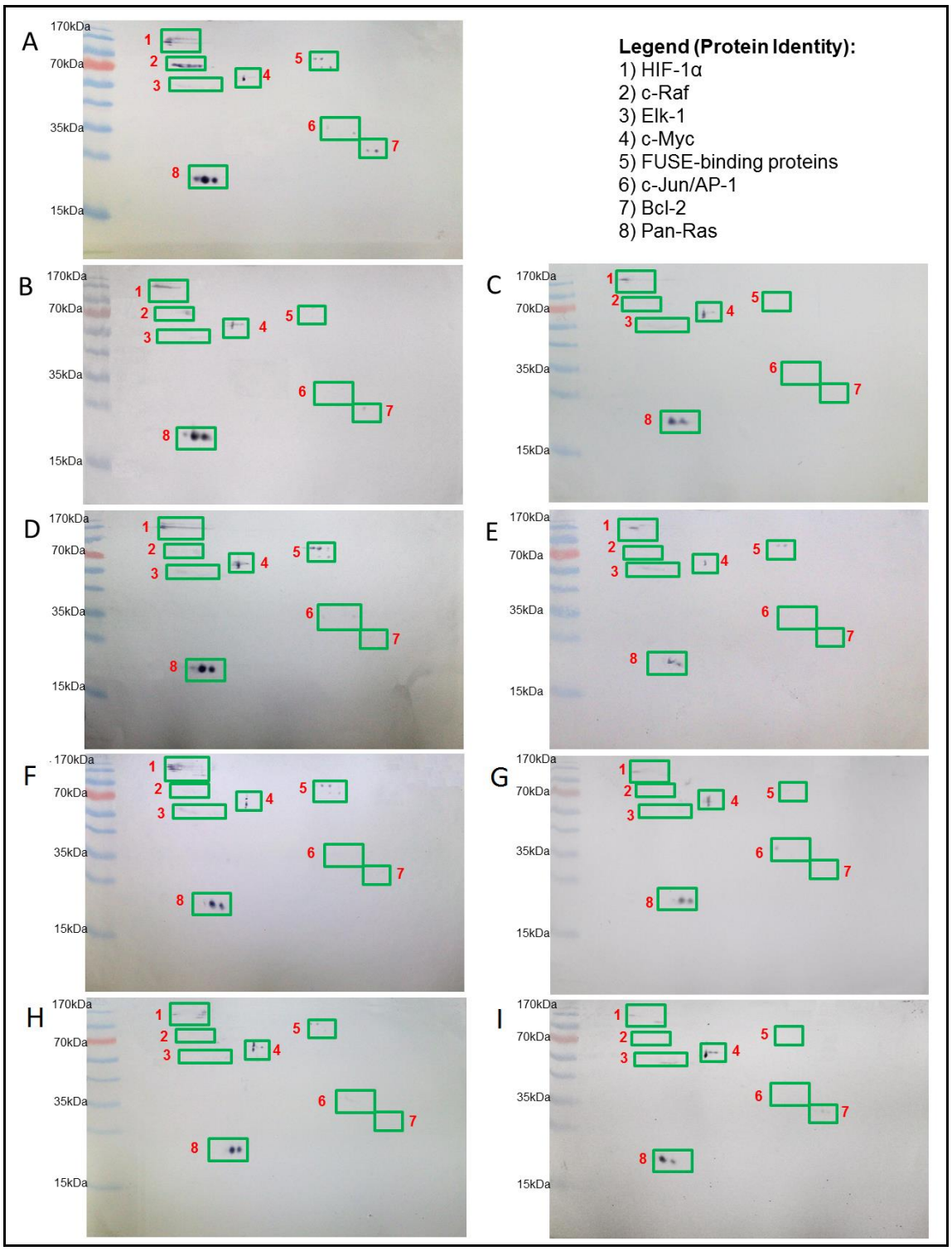


Figure 3.55: Western blotting of pathways activated in A549 in response to *Phyllanthus* extracts.

Expression levels of Pan-Ras, c-Raf, c-Jun/AP-1, Elk-1, c-Myc, HIF-1α, Bcl-2, and FUSE-binding proteins in (A) untreated A549 cells and cells treated with (B) aqueous *P. niruri*; (C) aqueous *P. urinaria*; (D) aqueous *P. watsonii*; (E) aqueous *P. amarus*; (F) methanolic *P. niruri*; (G) methanolic *P. urinaria*; (H) methanolic *P. watsonii*; (I) methanolic *P. amarus*. Green boxes indicate the protein spots corresponding to the proteins that are numbered. Typical results from three independent experiments are shown.

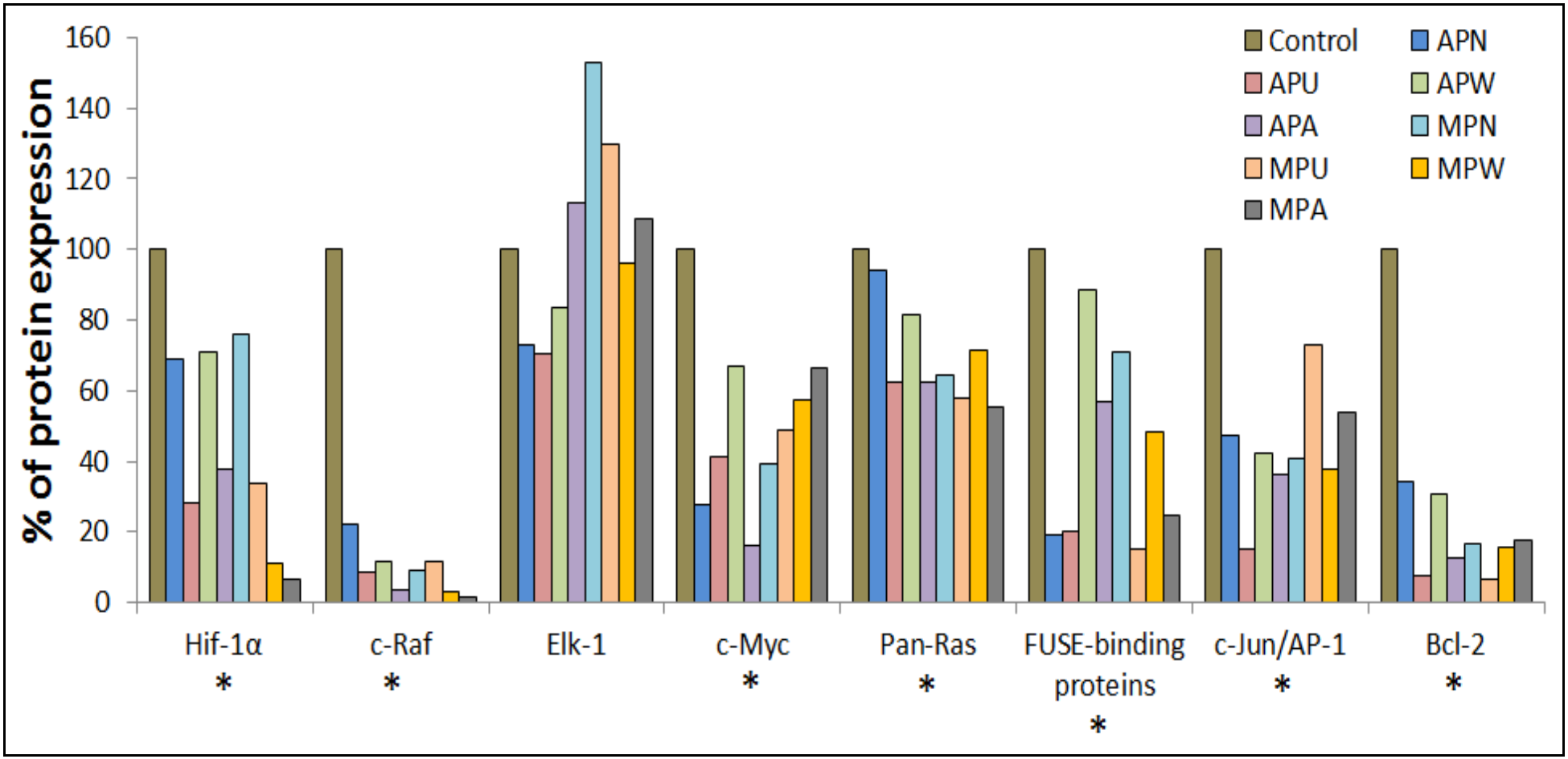


Figure 3.56: Percentage of individual protein expressions in untreated and *Phyllanthus*-treated A549 analyzed using Image J software.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; Control – untreated cells. *$P < 0.05$ vs untreated control.

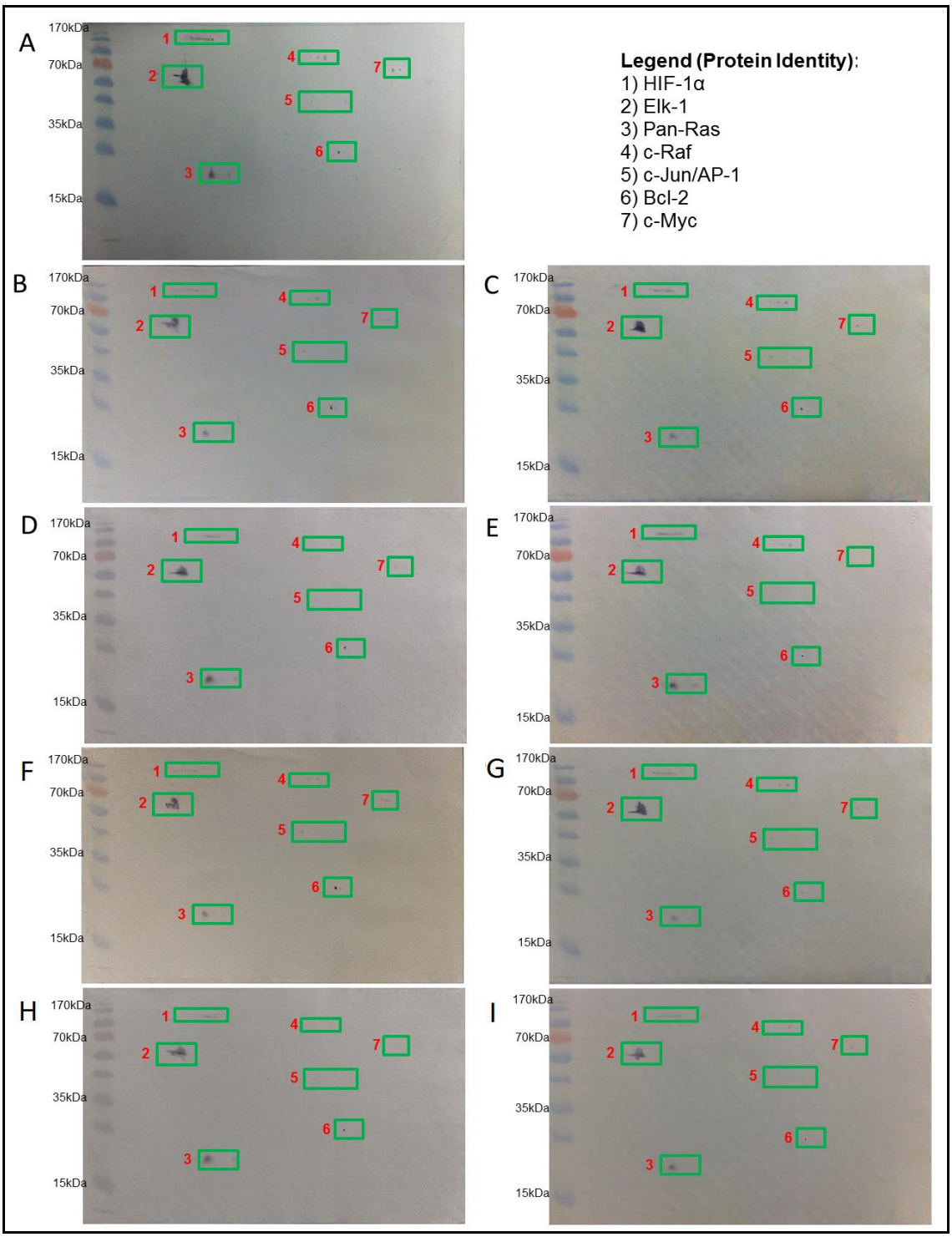


Figure 3.57: Western blotting of pathways activated in MCF-7 in response to *Phyllanthus* extracts.

Expression level of Pan-Ras, c-Raf, c-Jun/AP-1, Elk-1, c-Myc, HIF-1α, and Bcl-2 proteins in (A) untreated MCF-7 cells and cells treated with (B) aqueous *P. niruri*; (C) aqueous *P. urinaria*; (D) aqueous *P. watsonii*; (E) aqueous *P. amarus*; (E) aqueous *P. amarus*; (F) methanolic *P. niruri*; (G) methanolic *P. urinaria*; (H) methanolic *P. watsonii*; (I) methanolic *P. amarus*. Green boxes indicate the protein spots corresponding to the proteins that are numbered. Typical results from three independent experiments are shown.

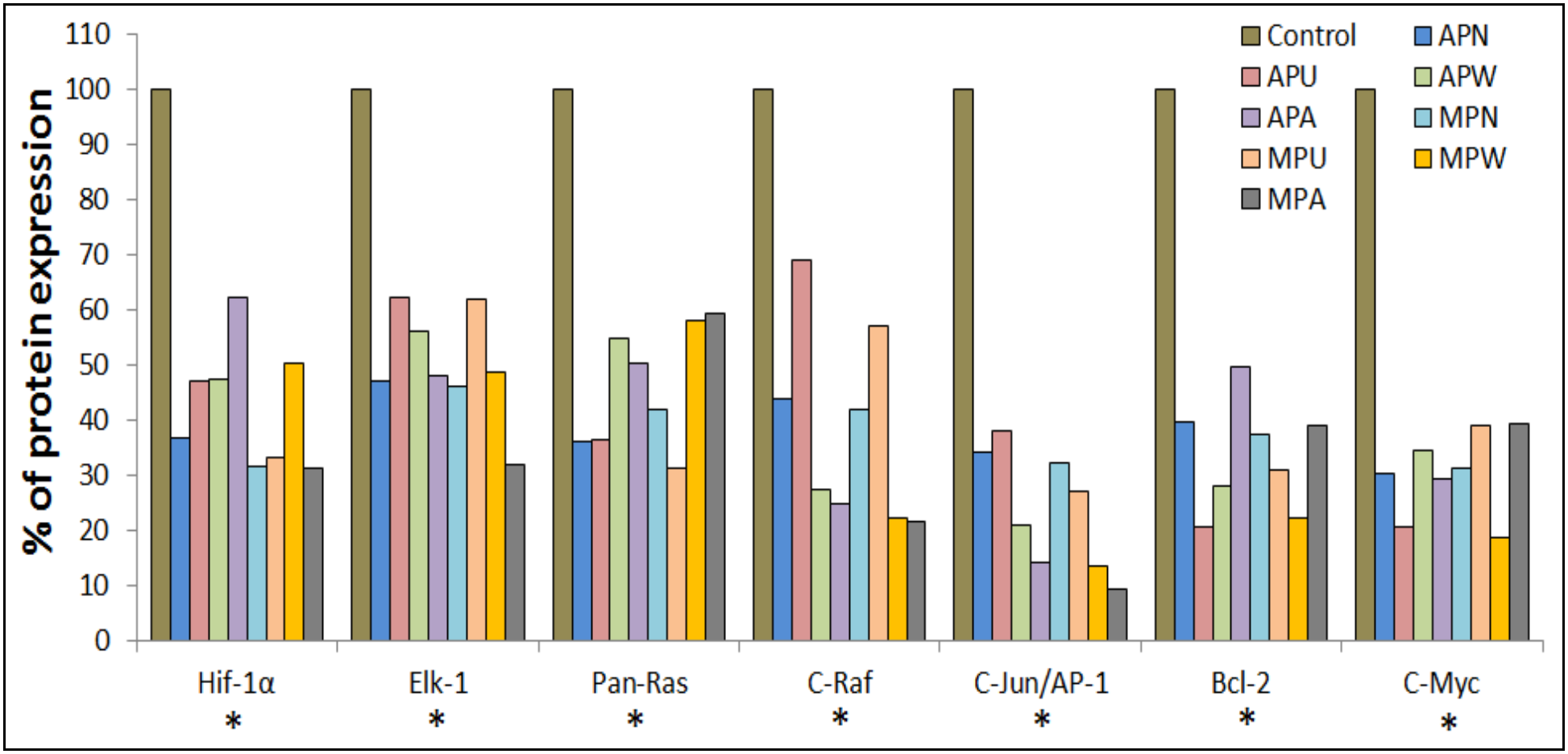


Figure 3.58: Percentage of individual protein expressions in untreated and *Phyllanthus*-treated MCF-7 analyzed using Image J software.

APN - aqueous *P. niruri*; APU - aqueous *P. urinaria*; APW - aqueous *P. watsonii*; APA - aqueous *P. amarus*; MPN - methanolic *P. niruri*; MPU - methanolic *P. urinaria*; MPW - methanolic *P. watsonii*; MPA - methanolic *P. amarus*; Control – untreated cells. *$P < 0.05$ vs untreated control.

3.7.3 DIFFERENTIALLY EXPRESSED PROTEINS IN *PHYLLANTHUS*-TREATED CELLS

MAPK/ERK and Hypoxia cellular signaling pathways were shown to be targeted by *Phyllanthus* to exert an inhibitory effect on cancer cells growth and metastasis. Perturbation of these pathways will eventually lead to the expression of a differential array of proteins in the treated cancer cells as demonstrated by the two-dimensional gel electrophoresis analysis. Figures 3.59 and 3.60 presented 2D-PAGE gels for untreated control, aqueous *Phyllanthus*-treated, and methanolic *Phyllanthus*-treated A549 samples. PD-Quest software analysis picked out 68 and 79 protein spots differentially expressed in aqueous and methanolic extracts-treated groups respectively. Subsequent mass spectrometry analysis and database examination using MASCOT identified 52 protein spots significantly downregulated by aqueous *Phyllanthus* extracts. Figure 3.61A depicts the categorization of these proteins according to Clusters of Orthologous Groups (COGs) classification while Table 3.4 lists the important proteins that play an essential role in post-translational modification, protein turnover, and chaperones (17%), cytoskeleton (11%), energy production and conversion (11%), transcription (4%), as well as drug detoxification and survival (6%). Meanwhile, 64 proteins significantly suppressed by methanolic *Phyllanthus* extracts were grouped as shown in Figure 3.61B and Table 3.5 where most of these proteins fell into the category of signal transduction mechanisms (16%), transcription (11%), drug detoxification and survival (9%), energy production and conversion (3%), as well as cytoskeleton (2%).

Likewise, Figures 3.62 and 3.63 showed representative 2D-PAGE gels for untreated control, aqueous *Phyllanthus*-treated, and methanolic *Phyllanthus*-treated MCF-7 samples. Proteomic analysis identified 62 and 61 protein spots differentially expressed in MCF-7 cells treated with aqueous and methanolic extracts respectively.

Examination of these proteins recognized 42 protein spots that were significantly downregulated by aqueous *Phyllanthus* extracts. These protein spots were consequently classified according to Clusters of Orthologous Groups (COGs) classification (Figure 3.64A) where majority of them fell into the category of energy production and conversion (31%), cytoskeleton (24%), post-translational modification, protein turnover, and chaperones (12%), RNA processing and modification (10%), as well as drug detoxification and survival (2%) as tabulated in Table 3.6. For methanolic *Phyllanthus* extracts, 59 protein spots significantly suppressed in MCF-7 cells were categorized (Figure 3.64B) with most of these proteins falling into the category of cytoskeleton (32%), energy production and conversion (17%), post-translational modification, protein turnover, and chaperones (13%), RNA processing and modification (13%), as well as drug detoxification and survival (2%) as shown in Table 3.7.

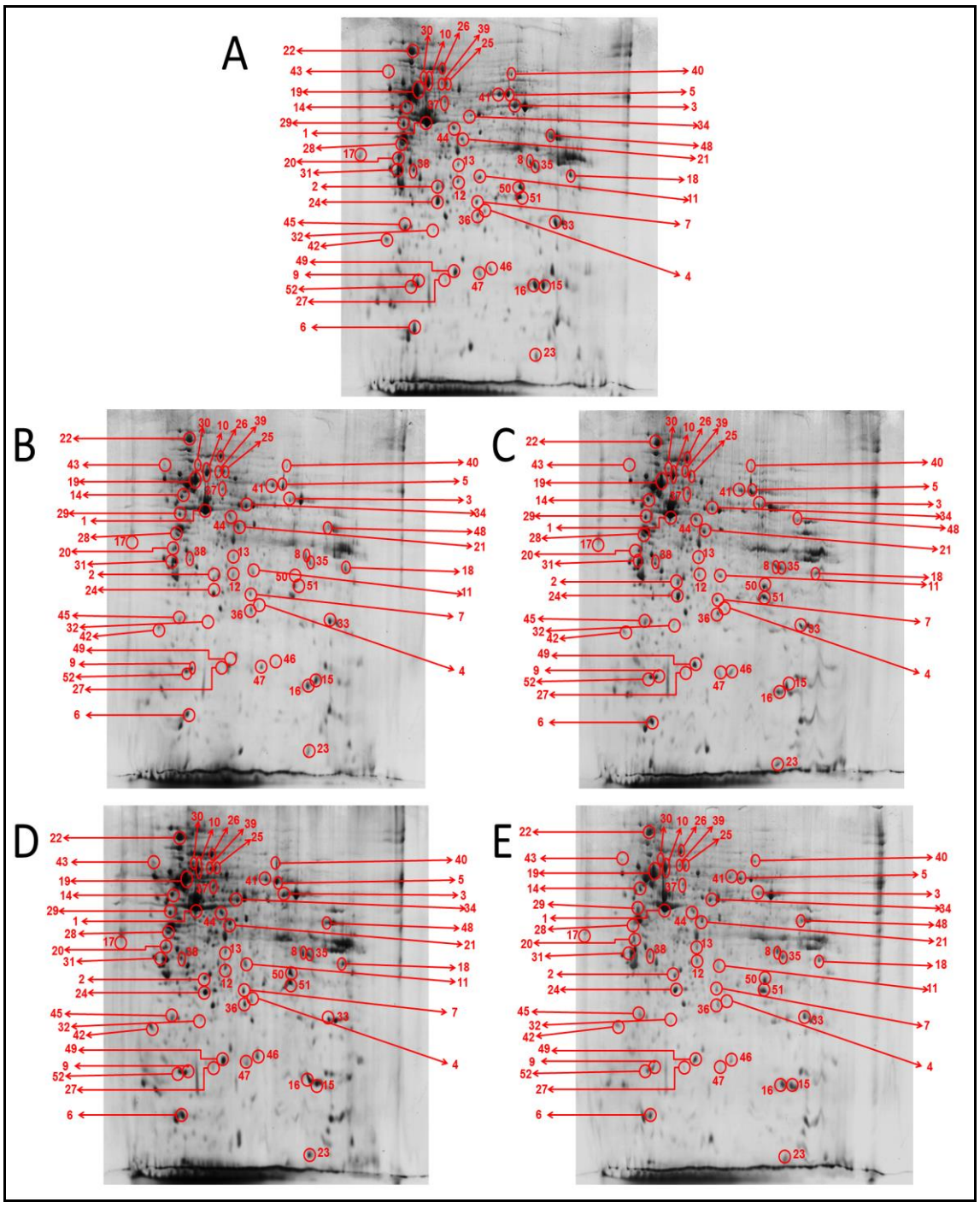


Figure 3.59: 2D-PAGE gels for A549 treated with aqueous *Phyllanthus* extracts.

Figure above is showing the representative gels for (A) untreated A549 cells and cells treated with (B) aqueous *P. niruri*; (C) aqueous *P. urinaria*; (D) aqueous *P. watsonii*; (E) aqueous *P. amarus*. Typical result from three independent experiments is shown.

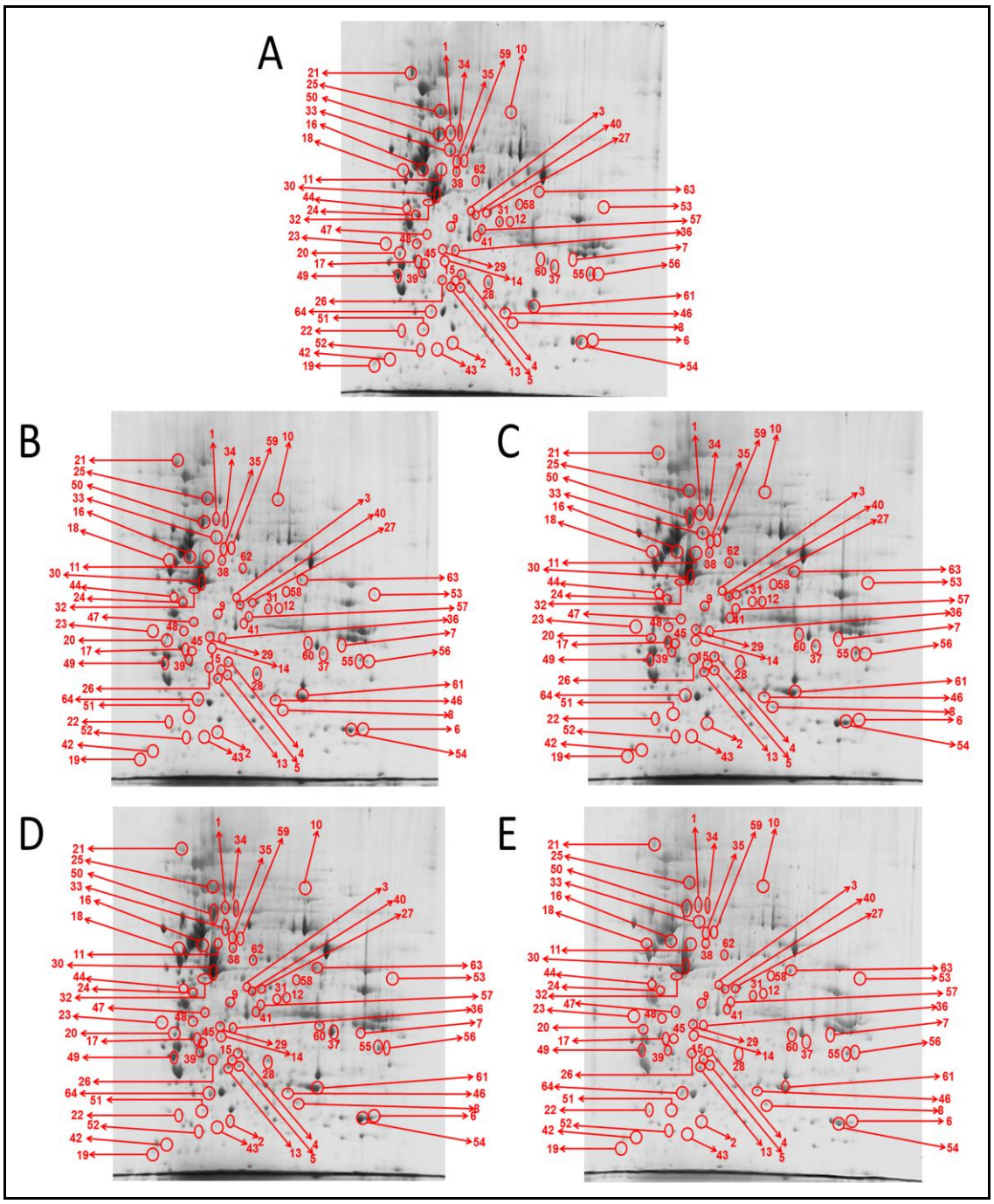


Figure 3.60: 2D-PAGE gels for A549 treated with methanolic *Phyllanthus* extracts.

Figure above is showing the representative gels for (A) untreated A549 cells and cells treated with (B) methanolic *P. niruri*; (C) methanolic *P. urinaria*; (D) methanolic *P. watsonii*; (E) methanolic *P. amarus*. Typical result from three independent experiments is shown.

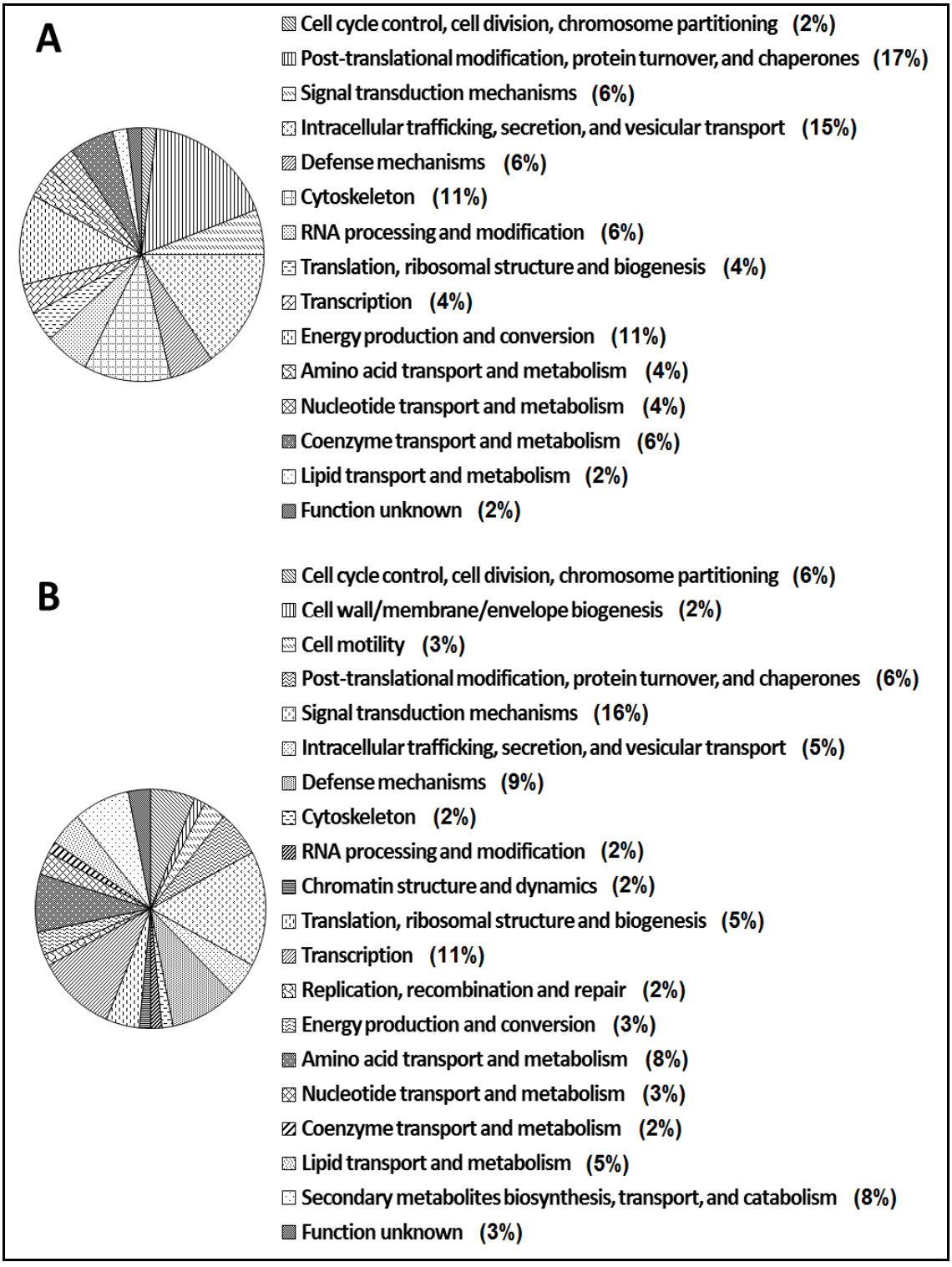


Figure 3.61: Clusters of Orthologous Groups (COGs) classification of identified proteins in A549 cells.

Grouping of differentially expressed proteins in A549 cells treated with (A) aqueous *Phyllanthus* extracts and (B) methanolic *Phyllanthus* extracts according to COGs classification.

Table 3.4: Classification of identified proteins of A549 downregulated (-) in the presence of aqueous *Phyllanthus* extracts

Spot ID	Proteins	Fold change			
		APN	APU	APW	APA
Post-translational modification, protein turnover, and chaperones					
4	Proteasome subunit beta type 3	-1.00	-0.20	N/A	-1.00
7	Heat-shock protein beta-1	-0.12	-0.22	-0.32	-0.10
10	60 kDa heat shock protein, mitochondrial precursor	-0.46	-0.82	-0.90	-0.75
15	Peptidyl-prolyl cis-trans isomerase A	-0.13	-0.36	-1.00	-0.42
16	Peptidyl-prolyl cis-trans isomerase A	-1.00	-1.00	-0.32	N/A
26	Stress-70 protein, mitochondrial precursor	-0.19	-0.22	N/A	N/A
39	T-complex protein 1 subunit epsilon	-0.89	N/A	-0.20	-0.97
40	Stress-induced-phosphoprotein 1	-0.54	-0.62	-0.44	-0.21
Cytoskeleton					
1	Actin, cytoplasmic 1	-0.81	-0.88	-0.95	-0.84
14	Vimentin	-0.91	-0.82	-0.24	-0.79
19	Tubulin alpha-8 chain	-0.80	-0.37	N/A	-0.80
27	Actin-related protein 2/3 complex subunit 5	-1.00	-1.00	-1.00	-1.00
46	Cofilin-1	-0.76	N/A	-0.85	-0.38
Energy production and conversion					
3	Alpha-Enolase	-0.62	-0.57	-0.48	-0.58
8	Glyceraldehyde-3-phosphate dehydrogenase	-0.30	-0.93	-0.99	-0.91
17	Glyceraldehyde-3-phosphate dehydrogenase	-1.00	-0.50	-1.00	-0.71
47	ADP-ribosylation factor 1	-1.00	-0.21	-0.15	-1.00
48	Fructose-bisphosphate aldolase A	-1.00	-1.00	-1.00	-1.00
50	Phosphoglycerate mutase 1	-1.00	-1.00	-1.00	-1.00
51	Triosephosphate isomerase	-1.00	-1.00	-1.00	-1.00
Transcription					
9	Eukaryotic translation initiation factor 5A-1	-0.32	-0.39	-0.27	-0.80
25	40S ribosomal protein S19	-0.30	-0.30	-0.15	-0.38
30	40S ribosomal protein S24	-1.00	-1.00	-1.00	-1.00
52	Eukaryotic translation initiation factor 5A-1	-1.00	-1.00	-1.00	-1.00
Drug detoxification					
11	Glutathione transferase omega-1	N/A	-0.45	N/A	-0.22
13	Annexin A4	-0.29	-0.29	-0.21	-0.65
Survival					
33	Peroxiredoxin-1	-0.08	-0.56	-1.00	-0.33

APN – aqueous *P. niruri*; APU – aqueous *P. urinaria*; APW – aqueous *P. watsonii*, APA – aqueous *P. amarus*; N/A – Not Affected.

Table 3.5: Classification of identified proteins of A549 downregulated (-) in the presence of methanolic *Phyllanthus* extracts

Spot ID	Proteins	Fold change			
		MPN	MPU	MPW	MPA
Signal transduction mechanisms					
13	Probable G-protein coupled receptor 179 precursor	-0.21	-0.15	-1.00	N/A
23	Serine/threonine-protein kinase 6	-1.00	-0.96	-0.19	-1.00
27	Putative Ras-related protein Rab-42	-1.00	N/A	-0.97	-0.80
35	GTPase HRas precursor	-0.60	-0.66	-0.59	-0.61
63	Guanine nucleotide-binding protein gamma-5-like subunit	-1.00	-1.00	-1.00	-1.00
Transcription					
10	DNA-directed RNA polymerase II 16 kDa polypeptide	-0.46	-0.59	-0.43	-0.93
11	39S ribosomal protein L40, mitochondrial precursor	-1.00	-1.00	-1.00	-1.00
12	Zinc finger protein 174	-1.00	-1.00	-1.00	-1.00
20	Proliferating cell nuclear antigen	-1.00	-1.00	-1.00	-1.00
22	Eukaryotic translation initiation factor 3 subunit 12	-1.00	-1.00	-1.00	-1.00
31	DNA-directed RNA polymerase II 16 kDa polypeptide	-0.42	-0.48	-0.48	-0.45
33	Transcription elongation factor B polypeptide 1	-0.58	-0.59	-0.56	-0.79
52	DNA-directed RNA polymerase II 16 kDa polypeptide	-1.00	-1.00	-1.00	-1.00
Drug detoxification					
7	Glutathione synthetase	-0.38	-0.79	-1.00	-0.51
34	Metallothionein-1M	-0.55	-0.52	-0.48	-0.48
47	Metallothionein-2	-0.71	-0.17	-0.14	-0.53
49	Metallothionein-1H	-0.52	-0.48	N/A	-0.29
50	Metallothionein-2	-0.34	-0.59	-0.49	-1.00
59	Glutathione synthetase	-0.50	-0.45	-0.38	-0.61
62	Metallothionein-1L	-0.86	-0.79	-0.96	-0.44
Energy production and conversion					
39	ADP-ribosylation factor-like protein 6	-0.58	-0.52	-1.00	-0.29
40	Alpha-enolase	-0.27	-0.51	N/A	-0.56
Survival					
4	Peroxiredoxin-1	-0.78	-1.00	-1.00	-1.00
16	Bcl-2-like protein 11	-0.25	-1.00	-1.00	-0.70
Cytoskeleton					
60	Actin-related protein 10	-0.94	-0.70	-0.13	-0.73

MPN – methanolic *P. niruri*; MPU – methanolic *P. urinaria*; MPW – methanolic *P. watsonii*, MPA – methanolic *P. amarus*; N/A – Not Affected.

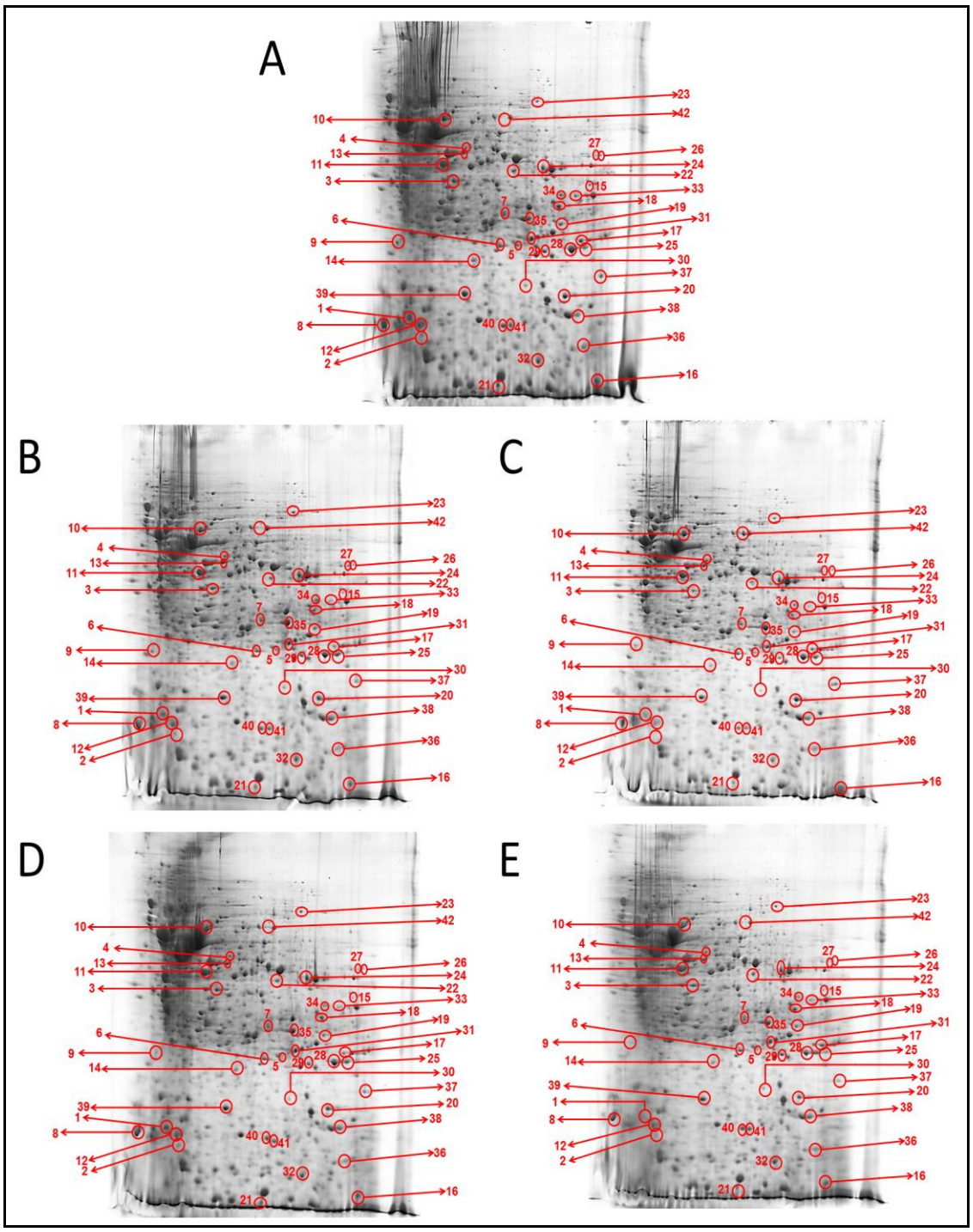


Figure 3.62: 2D-PAGE gels for MCF-7 treated with aqueous *Phyllanthus* extracts.

Figure above is showing the representative gels for (A) untreated MCF-7 cells and cells treated with (B) aqueous *P. niruri*; (C) aqueous *P. urinaria*; (D) aqueous *P. watsonii*; (E) aqueous *P. amarus*. Typical result from three independent experiments is shown.

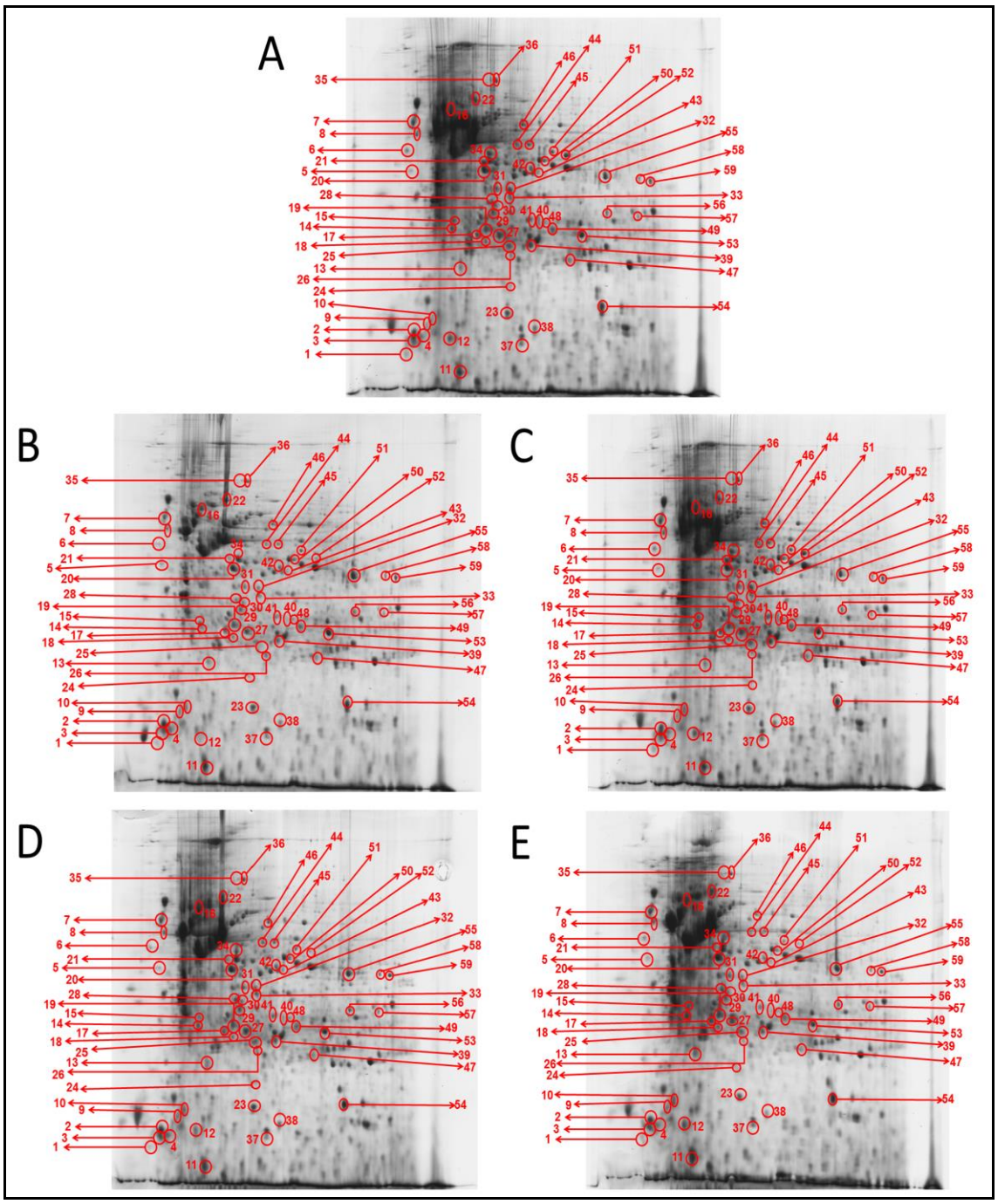


Figure 3.63: 2D-PAGE gels for MCF-7 treated with methanolic *Phyllanthus* extracts.

Figure above is showing the representative gels for (A) untreated MCF-7 cells and cells treated with (B) methanolic *P. niruri*; (C) methanolic *P. urinaria*; (D) methanolic *P. watsonii*; (E) methanolic *P. amarus*. Typical result from three independent experiments is shown.

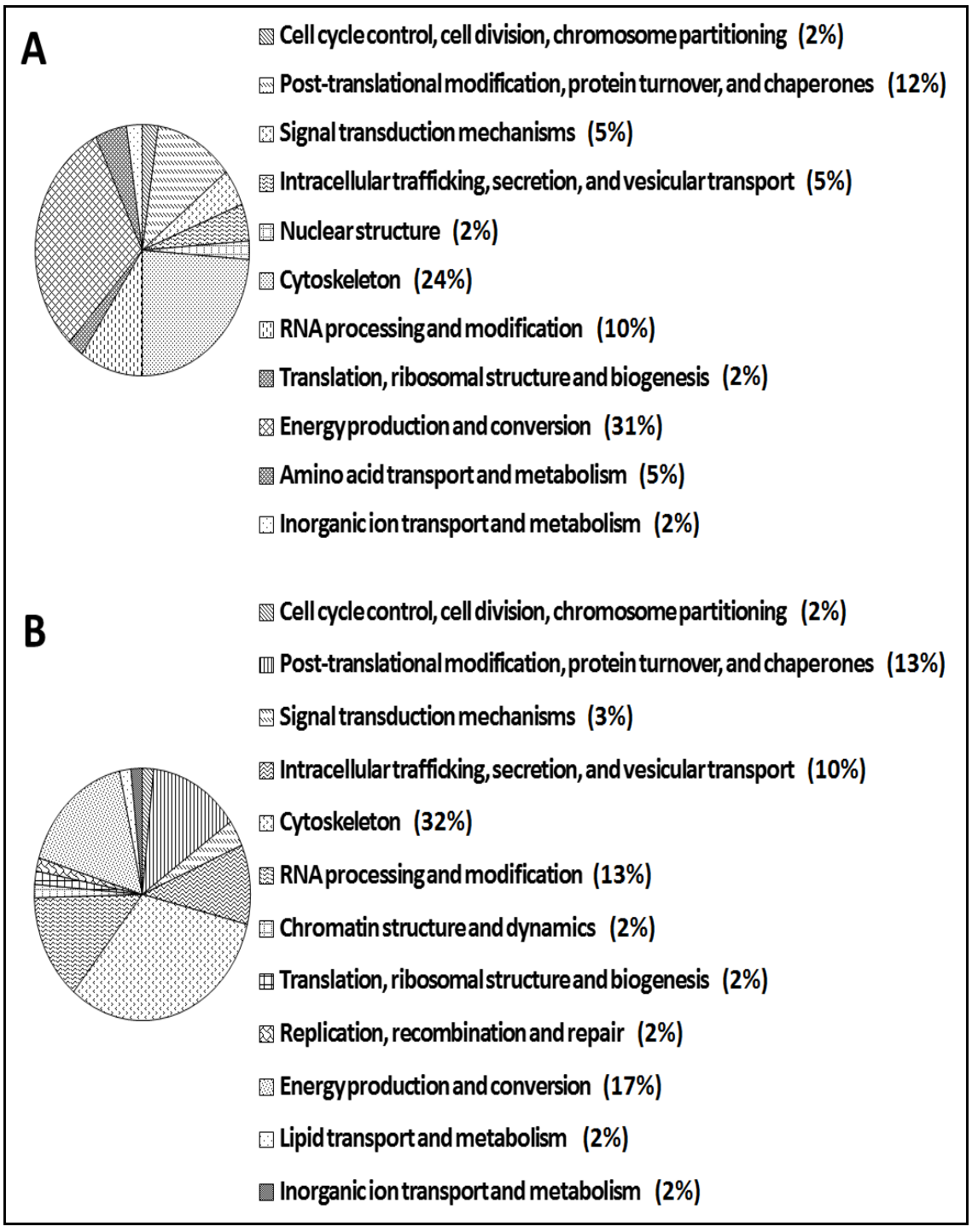


Figure 3.64: Clusters of Orthologous Groups (COGs) classification of identified proteins in MCF-7 cells.

Grouping of differentially expressed proteins in MCF-7 cells treated with (A) aqueous *Phyllanthus* extracts and (B) methanolic *Phyllanthus* extracts according to COGs classification.

Table 3.6: Classification of identified proteins of MCF-7 downregulated (-) in the presence of aqueous *Phyllanthus* extracts

Spot ID	Proteins	Fold change			
		APN	APU	APW	APA
Energy production and conversion					
1	Pyruvate kinase isozymes M1/M2	-0.96	-0.42	-0.70	-0.67
3	Alpha-Enolase	-0.41	-0.64	-0.47	-0.34
6	ATP synthase subunit alpha, mitochondrial precursor	-0.32	-0.47	-0.10	-0.53
22	Pyruvate kinase isozymes M1/M2	-0.34	-0.21	-0.32	-0.25
24	Glyceraldehyde-3-phosphate dehydrogenase	-0.72	-0.75	-0.20	-0.57
27	Glyceraldehyde-3-phosphate dehydrogenase	-0.83	-0.38	-0.96	-0.13
34	Glyceraldehyde-3-phosphate dehydrogenase	-0.50	-0.60	-0.39	-0.64
35	Triosephosphate isomerase	-0.98	-0.83	-0.74	-0.76
40	Glyceraldehyde-3-phosphate dehydrogenase	-1.00	-1.00	-1.00	-1.00
41	L-lactate dehydrogenase A chain	-1.00	-1.00	-1.00	-1.00
42	Beta-Enolase	-1.00	-1.00	-1.00	-1.00
Cytoskeleton					
4	Tubulin beta-5 chain	-0.11	-0.84	-1.00	-0.34
10	Actin, cytoplasmic 2	-1.00	-0.55	-0.88	-1.00
11	Tubulin beta-5 chain	-0.32	-0.49	-0.33	-0.21
12	Actin, alpha cardiac muscle 1	-0.62	-0.12	N/A	-0.28
20	Cofilin-1	-0.59	N/A	-0.15	-0.21
21	Annexin A2	-0.14	-1.00	-1.00	N/A
36	Cofilin-1	-1.00	-1.00	-1.00	-1.00
39	Stathmin	-1.00	-1.00	-1.00	-1.00
Post-translational modification, protein turnover, and chaperones					
14	Heat shock protein HSP 90-beta	-0.39	-0.55	-0.30	-0.37
16	10 kDa heat shock protein, mitochondrial	-0.57	-1.00	-0.51	-1.00
18	Heat shock cognate 71 kDa protein	-0.49	-0.57	-0.30	N/A
RNA processing and modification					
15	Heterogeneous nuclear ribonucleoprotein A1	-0.40	-0.45	-0.39	N/A
19	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.45	-0.24	-0.19	-0.10
26	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.28	N/A	-0.30	N/A
30	Heterogeneous nuclear ribonucleoprotein A1	N/A	-1.00	-0.15	N/A
Drug detoxification					
2	Annexin A2	-1.00	-1.00	-1.00	-1.00
Survival					
17	Peroxiredoxin-1	-0.50	-0.25	-0.37	-0.26
29	Peroxiredoxin-1	-1.00	-1.00	-0.25	-0.85
33	Galectin-3	-0.66	-0.58	-0.46	-0.53

APN – aqueous *P. niruri*; APU – aqueous *P. urinaria*; APW – aqueous *P. watsonii*, APA – aqueous *P. amarus*; N/A – Not Affected.

Table 3.7: Classification of identified proteins of MCF-7 downregulated (-) in the presence of methanolic *Phyllanthus* extracts

Spot ID	Proteins	Fold change			
		MPN	MPU	MPW	MPA
Cytoskeleton					
9	Tubulin alpha-ubiquitous chain	-0.43	N/A	-0.30	-0.78
10	Tubulin alpha-ubiquitous chain	-0.15	N/A	-0.58	-0.24
12	Actin, alpha cardiac muscle 1 precursor	-0.38	N/A	-0.13	-0.45
16	Tubulin beta-5 chain	-0.96	-0.85	-0.95	-0.62
19	Actin, alpha cardiac muscle 1 precursor	-0.41	-0.70	-0.25	-0.10
20	Tubulin beta-5 chain	-0.41	N/A	-0.15	-0.84
21	Tubulin beta-5 chain	-0.67	-1.00	-0.44	-0.36
22	Actin, alpha cardiac muscle 1 precursor	-0.87	-0.34	-0.74	-0.81
23	Stathmin	-0.56	N/A	-0.38	-0.20
34	Tubulin alpha-ubiquitous chain	-1.00	-0.65	-0.65	-1.00
44	Tubulin alpha-ubiquitous chain	-0.19	-0.11	-0.13	-0.26
49	Filamin-A	-0.53	-0.61	-0.54	-0.86
Energy production and conversion					
18	Phosphoglycerate kinase 1	-0.25	N/A	-0.28	-0.41
31	Alpha-Enolase	-0.70	-0.09	-0.58	-0.68
42	Aldose reductase	-1.00	-0.82	-0.77	-0.96
43	Pyruvate kinase isozymes M1/M2	-0.50	-0.09	-0.24	N/A
46	Pyruvate kinase isozymes M1/M2	-0.22	-0.49	-0.51	N/A
47	Phosphoglycerate mutase 1	-0.29	-0.09	-0.21	-0.24
52	Phosphoglycerate kinase 1	-0.85	-0.50	-0.86	-0.87
53	Triosephosphate isomerase	-0.26	N/A	-0.12	-0.34
54	Glyceraldehyde-3-phosphate dehydrogenase	-0.48	-0.69	-0.65	-0.78
55	Glyceraldehyde-3-phosphate dehydrogenase	-0.87	-0.51	-0.22	-0.47
56	Glyceraldehyde-3-phosphate dehydrogenase	-0.55	N/A	-0.67	-0.82
Post-translational modification, protein turnover, and chaperones					
27	Heat-shock protein beta-1	-0.12	N/A	-0.29	-0.21
35	Stress-70 protein, mitochondrial precursor	-0.53	N/A	-0.54	-0.47
36	Stress-70 protein, mitochondrial precursor	-0.51	-0.55	-0.20	-0.47
57	60 kDa heat shock protein, mitochondrial precursor	-0.09	N/A	-0.14	-0.21
RNA processing and modification					
24	Splicing factor, arginine/serine-rich 3	-0.38	N/A	-0.46	-0.50
26	Heterogeneous nuclear ribonucleoprotein H	N/A	N/A	-0.10	-0.43
30	Heterogeneous nuclear ribonucleoprotein H	-0.35	N/A	-0.26	-0.17
48	Heterogeneous nuclear ribonucleoprotein H3	-1.00	N/A	-0.39	-1.00
50	Elongation factor 2	-0.36	N/A	-0.31	-0.21
58	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.81	-0.83	-0.81	-0.57
59	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.59	N/A	-0.24	-0.60

‘Table 3.7, continued’

Survival					
11	Thioredoxin	-0.43	-0.26	-0.39	-0.41
38	Peroxiredoxin-5, mitochondrial precursor	-0.18	N/A	-0.46	-0.66
Drug detoxification					
13	Annexin A2	-0.70	-0.44	-0.33	-0.40
17	Annexin A2	-0.45	-0.97	-0.51	-0.48
25	Annexin A2	-0.34	-0.32	-0.39	-0.21
28	Annexin A8	-0.21	N/A	-0.29	-0.14
29	Annexin A2	-0.64	-0.09	-1.09	-0.47

MPN – methanolic *P. niruri*; MPU – methanolic *P. urinaria*; MPW – methanolic *P. watsonii*, MPA – methanolic *P. amarus*; N/A – Not Affected.

CHAPTER 4: DISCUSSION

Statistics on cancer burden in terms of incidence, prevalence, and mortality are generally used to observe trends as well as for epidemiologic studies on the cause, prevention, and control measures of cancer. Results from these studies will guide the design, implementation, and assessment of comprehensive control programs for cancer disease (German et al. 2011). In 2008 alone, the number of new cancer cases and deaths worldwide were reported to be 12.7 million and 7.6 million respectively (Duffy et al. 2012). This global cancer burden was projected to keep increasing and the most common cancers measured in relation to number of new cases were lung, breast, colorectal, stomach, and liver (Parkin et al. 2001). Lung cancer constitutes the greatest incidence and mortality rates with 1.6 million new cases and 1.4 million deaths each year, with less than 15% lung cancer patients reaching five years survival (Ye et al. 2012). Breast malignancy is the most common malignancy in women with more than one million cancer incidences and 400,000 deaths each year (Yang et al. 2012).

Cancer disease mainly poses a great deal of problems due to its capability to proliferate uncontrollably leading to the production of a bulky tumor mass that disrupts the normal organ functions (Raven et al. 2004). Following a cancer diagnosis, chemotherapy is the most frequently implemented treatment in conjunction with local therapies such as radiotherapy and surgery (Yang et al. 2012). Examples of the commonly used chemotherapeutic drugs for treatment of breast and lung cancers are Doxorubicin and Cisplatin, respectively. Doxorubicin (Adriamycin) is classified as a topoisomerase II poison as it inhibits the activity of this enzyme resulting in the genomic DNA breakage. Other mechanisms include intercalation between the DNA bases resulting in the blockage of DNA synthesis and transcription. It may also act

through sequestering irons followed by the generation of free radical. All these mechanisms lead to disruption of DNA that ultimately causes toxicity to cell (Swift et al. 2006). Meanwhile, Cisplatin causes formation of DNA adducts, which is the formation of complexes with DNA, resulting in the blockage of replication, transcription and repair mechanisms. The halting of the replication process is a result of the inhibitory action of the DNA adduct on the DNA polymerase, leading to cell toxicity (Zamble et al. 1998). Nevertheless in our study, Cisplatin and Doxorubicin not only showed strong cytotoxicity against both cancer cells (A549 and MCF-7), but also towards normal cells (NL20 and 184B5) with IC_{50} values generally lesser than 10μg/ml. Therefore, they pose a wide variety of side effects such as nausea, vomiting, myelosuppression, neurological, mucositis, infertility, as well as lethargy, which were graded as mild, moderate, severe, life threatening, and death (Bhosle & Hall 2009). Moreover, cancer cells that normally respond to the chemotherapeutic agents initially will slowly develop drug resistance upon prolonged exposure leading to a more aggressive phenotype that is often capable of migration and distant metastasis (Ryu et al. 2011), hence resulting in high death rates every year which turns out to be the leading cause of cancer-related deaths (Ju et al. 2011, Kabir et al. 2011). Therefore, this therapy is still far from ideal since most of the cancer patients only benefit from their short-term efficacy (Karroum et al. 2010, Ju et al. 2011).

Thus, the search for novel chemotherapeutic agents with higher selectiveness and effectiveness that surpass drug resistance and metastasis is crucial to improve the survival of patients with advanced or recurrent lung and breast cancers that have poor therapeutic efficacy and prognostic survival (Natarajan et al. 2011). In this connection, natural-product based drugs are gaining their popularity as preventive medicines or for health management and hence have spurred an intensive search for naturally occurring plant-derived anticancer compounds (Wong et al. 2012). Over the years, the plants of

the genus *Phyllanthus* from the family *Euphorbiaceae*, have gained reputation in folk and traditional medicine including ayurveda, siddha and traditional Chinese medicine for their myriad of healing properties. Numerous research studies on *Phyllanthus spp*. began in the late 1980's with the clinical efficacy of *Phyllanthus niruri* against viral Hepatitis B being observed (Paranjpe 2001). In the current study, we aimed to investigate the antimetastatic activity of *Phyllanthus* on cancer cells. Prior to that, an effective dose which is non-toxic to the cells had to be determined. Hence, we initially evaluated the toxicity of both aqueous and methanolic crude extracts of four different species of *Phyllanthus* plants, namely *P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*, on two human cancer cell lines (A549 and MCF-7) and two normal human cell lines (184B5 and NL20). Our data showed that the crude *Phyllanthus* extracts were able to exhibit selective cytotoxicity against MCF-7 and A549 human cancer cells. Recently, pure natural products and roughly fractionated extracts are gaining their popularity in drug discovery as they would speed up the whole process from screening to a validated lead. Besides that, the cost will be greatly reduced when pure natural compound libraries are used as basic raw materials (Bindseil et al. 2001). Thus, the effects of two *Phyllanthus* fractions were also tested on both cancer and normal cell lines. Nevertheless, the first fraction was nontoxic or showed very little toxicity to cancer cells while the second fraction showed appreciable toxicity to both cancer cells and normal cells. Sun and Liu reported that not 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 is more meaningful as well as prudent to assess the activity of *Phyllanthus* as a complete mixture of interacting bioactive compounds rather than evaluating them as a breakup of their individual components.

The cytotoxic activities exhibited by natural products are mainly attributed to the presence of different bioactive compounds within the plant extracts (Issa et al. 2006,

Russo 2007, Gopalakrishnan & Tony Kong 2008). High Performance Liquid Chromatography (HPLC) analysis revealed the presence of various polyphenol compounds in both aqueous and methanolic *Phyllanthus* extracts. These polyphenol compounds were broadly classified into four categories; ellagitannins, gallotannins, flavonoids, and phenolic acids (Tang et al. 2010, Lee et al. 2011a). Different species of *Phyllanthus* plants have a variation in the quantity of each bioactive component, thus giving rise to the different extents of cytotoxicity to cancer cells. Among the four *Phyllanthus* species studied, *P. urinaria* and *P. watsonii* exhibited almost similar level of cytotoxicity to both A549 and MCF-7 cells *in vitro*, probably in part to the higher amount of polyphenol compounds present in their extracts.

The half-maximal inhibitory (IC_{50}) concentrations determined for each *Phyllanthus* crude extracts were then applied as the treatment conditions for the subsequent antimetastatic experiments. Malignant tumor progression depends largely on its capability to invade, metastasize, as well as to induce angiogenesis. Cancer metastasis is a complex process that normally begins with degradation of extracellular matrix of basement membrane, cell invasion, cell migration, and eventually tumor growth at distant metastatic sites. The crucial factor that affects the invasion and metastasis of tumor is the integrity of the basement membrane that holds the tumor cells together (Wang et al. 2009). During invasion process, the extracellular matrix (ECM) and the basement membrane components are often destroyed by a synergistic action of a number of proteolytic enzymes including the matrix metalloproteinases (MMPs), cysteine proteases, and serine proteases (Woodward et al. 2007, Yang et al. 2008a). From our data, *Phyllanthus*-treated cells exhibited greater difficulties to invade the extracellular matrix as compared to the untreated cells, hence suggesting the ability of *Phyllanthus* to inhibit the production of proteolytic enzymes, and thus limiting the invasive and metastatic capabilities of tumor cells.

Cell motility and adhesion are the next critical processes in metastasis upon the successful invasion of tumor cells into the blood or lymph capillaries. Since the lungs are the first organ that the detached tumor cells come upon most frequently, they become the main location for tumor metastasis (Kim et al. 2010). Data obtained from cell migration assays displayed that *Phyllanthus* has the ability to reduce migration of cancer cells. It can be argued that the reduction in cellular migration could be due to the cytotoxic effect exerted by *Phyllanthus* at high concentrations. However, a significant decrease of cellular mobility was also observed at IC_{50} and lower concentrations in which there were minimal cell death, indicating its ability to suppress and limit cell motility. Once the tumor cell is arrested at a particular organ, it must be able to adhere strongly before it can colonize and establish a secondary tumor at the new site. In this study, we showed that the *Phyllanthus*-treated cells had a diminished capacity to attach at a new location compared to the untreated cells. This ability of *Phyllanthus* to inhibit cell motility and adhesion can also be correlated with its ability to inhibit the invasiveness of cells since inhibition of proteolytic enzyme activities had been shown to be capable of reducing cells' migration (Ha et al. 2004, Tanimura et al. 2005).

In addition, we were interested to further investigate how *Phyllanthus* had caused the cells to lose their normal ability to metastasize, where our main hypothesis was that the cancer cells were dying due to *Phyllanthus*. A disseminating tumor cell faces the possibility of losing its viability during anytime throughout the metastasis process (Xie & Huang 2003). They could have died simply due to mechanical destruction during the invasion or migration process (necrosis), or by *Phyllanthus* triggered cell death (apoptosis or necrosis). Apoptosis typically involves a series of events (Yang et al. 2004), beginning with the release of cytochrome c from mitochondria, activation of a cascade of caspases, degradation of poly ADP-ribose polymerase (PARP), and finally the fragmentation of chromosomal DNA (Yang et al.

2006, Pojarova et al. 2007). As opposed to apoptosis, necrosis is usually associated with external damage leading to accidental cell death, resulting in mitochondrial and cytoplasmic swelling, followed by compromised membrane integrity that will eventually burst, releasing its cytoplasmic contents (Qiu et al. 1998, Woo et al. 2008). Based on our data, apoptosis occurred in the cells treated with *Phyllanthus* as the levels of these execution caspases were increased many fold over the basal level of untreated cells. This could be due to the presence of tannins (such as gallic acid and geraniin) in the *Phyllanthus* extracts which had been shown to be able to induce apoptosis in several human cancer cells (Lee et al. 2008, Harikumar et al. 2009). Activation of caspase-3 will subsequently trigger the proteolytic cleavage of poly ADP-ribose polymerase resulting in DNA fragmentation that usually occurs during late apoptosis (Yang et al. 2006, Wu et al. 2009). These DNA fragments appear as DNA ladder on an agarose gel instead of a randomized DNA breakdown which is observed as a smear in necrosis. However, internucleosomal DNA fragmentation is not universal, as it may not always occur during apoptosis (Vinatier et al. 1996). Nevertheless, further *in situ* staining of the DNA breaks confirmed the induction of apoptosis by *Phyllanthus* with the presence of TUNEL-positive cells.

Although the data obtained suggest apoptosis as the mode of cell death, the complex phytochemical mixture of *Phyllanthus* species allows a possibility of necrosis as the other mechanism of action. Some of the cytotoxic agents have the ability to activate both apoptotic and necrotic cell death pathways (Woo et al. 2008). In addition, cells might also have died via necrosis as they invade or migrate through the membrane pores. Therefore, in order to differentiate between the dominant modes of cell death, release of lactate dehydrogenase (LDH) that is an indicator of necrosis was assessed in *Phyllanthus*-treated cells. Our data revealed that LDH levels released in *Phyllanthus*-

treated cells remained low. Therefore, the possibility of necrosis as the main mode of cell death can be excluded.

Even though the exact bioactive compounds in *Phyllanthus* exerting the antimetastatic and antiproliferative effects are not known yet, it is definite that *Phyllanthus* contains abundant flavonoids, phenolic acids and ellagitannins. Many of these bioactive compounds have been shown to exert antimetastatic and apoptosis-inducing effects through activation of various mechanisms. For instance, gallic acid prevents the metastasis of AGS and U87 cells via inhibition of NF-κB activity, suppression of metalloproteinases activities, as well as downregulation of Ras/PI3K/AKT and Ras/MAPK signaling pathways (Ho et al. 2010, Lu et al. 2010). In addition, flavones and plant polyphenols have been shown to exert antimetastatic and antiinvasion activities by inhibiting matrix-degrading proteases (Tanimura et al. 2005, Yang et al. 2008b). Another phenolic compound, 5-caffeoylquinic acid isolated from *Euonymus alatus* has also been shown to be a strong MMP-9 inhibitor (Jin et al. 2005). Hence, the presence of flavonoids, phenolic acids, or ellagitannins in *Phyllanthus* plays a crucial role in its antimetastatic actions.

Therefore, the subsequent phase in this study is to determine the underlying mechanisms that confer the antimetastatic and apoptosis-inducing abilities of *Phyllanthus*. Using 2DE-based proteomic approach, numerous differentially expressed proteins were identified in both A549 and MCF-7 cells in response to *Phyllanthus* treatments. In order to have a clearer understanding on how these affected proteins inhibit metastasis and proliferation of A549 and MCF-7 cells, a diagram was illustrated based on the findings obtained as shown in Figure 4.1. Both aqueous and methanolic *Phyllanthus* extracts were shown to modulate expression of different sets of proteins. Among the four *Phyllanthus* species, *P. urinaria* generally demonstrated the greatest inhibitory activity, closely followed by *P. watsonii*, *P. amarus*, and *P. niruri*. This could

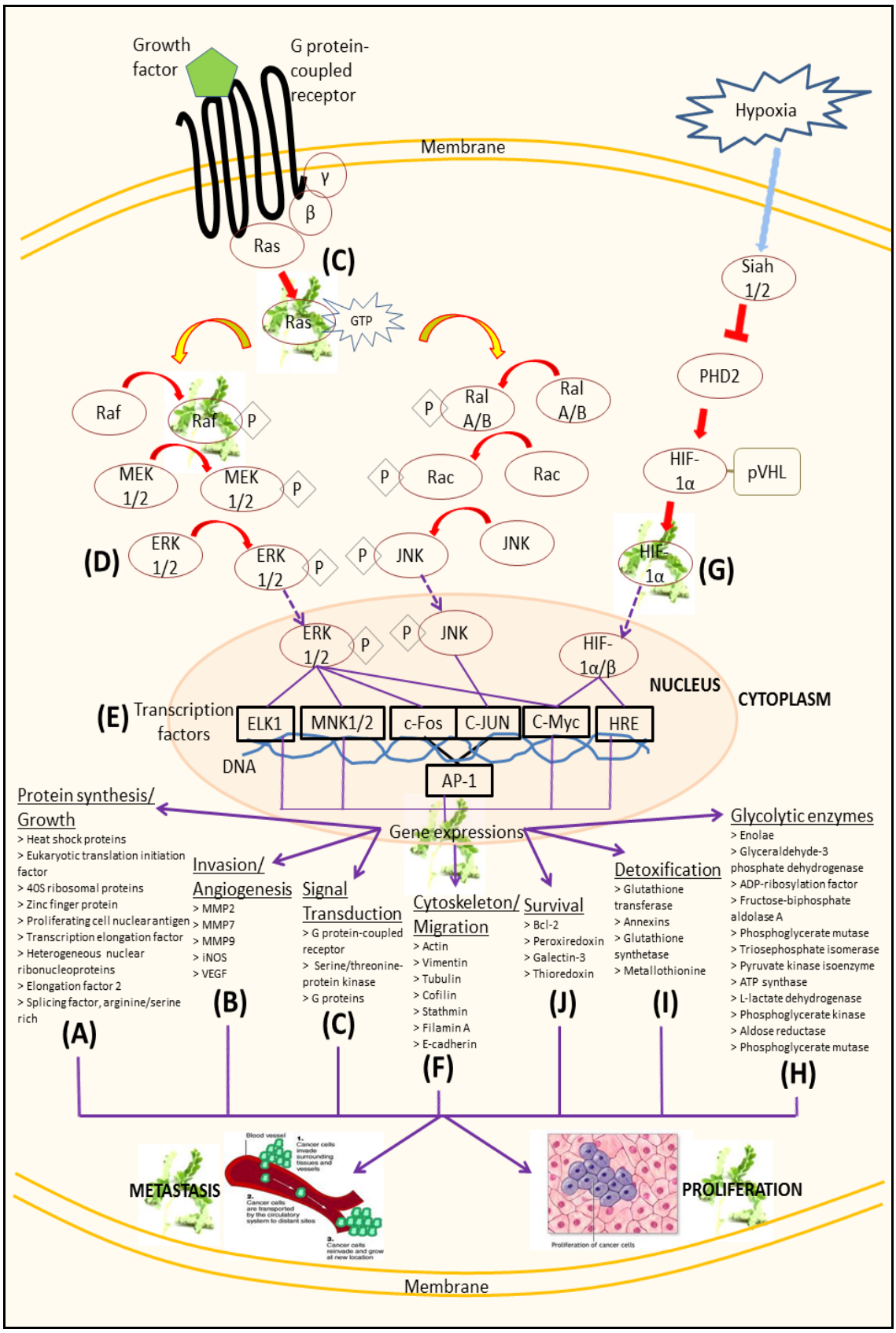


Figure 4.1: Antiproliferative and antimetastatic mechanisms of *Phyllanthus* in A549 and MCF-7 cancer cells

Phyllanthus plant at the background signifies an inhibition of the signaling molecules or cellular processes.

be delineated by the higher number of polyphenol compounds present in both the aqueous (9 out of 10 polyphenols) and methanolic (3 out of 4 polyphenols) *P. urinaria* extracts (Tang et al. 2010, Lee et al. 2011a), hence having a higher capability to cause antimetastatic activities on A549 and MCF-7 cells. Some of those proteins were represented by more than one spot, which represents different splicing forms of the same protein as a result of post-translational modification (Afjehi-Sadat et al. 2005).

Thus, protein turnover, chaperones, and post-translational modification are indeed crucial to produce many variants of the common amino acid that possess distinctive structures and functions essential for tumor growth (Nalivaeva & Turner 2001, Walsh & Jefferis 2006). In order to ensure uninterrupted cell growth, continuous protein synthesis is necessary. An accurate protein translational activity begins with RNA processing and modification, an important step immediately after transcription before translation into functional proteins or polypeptides (Alberts 2002). Hence, proteins that facilitate this particular step would be crucial as listed in Figure 4.1(A), such as heterogeneous nuclear ribonucleoproteins (hnRNPs), which are the RNA-binding proteins required during mRNA maturation. Besides that, hnRNPs also interact with telomeres to promote reactivation of telomerase that is crucial during carcinogenesis (Hooven & Baird 2008). Another protein is arginine/serine-rich family of proteins which consist of essential pre-mRNA splicing factors that are critical for pre-mRNA splicing as well as in the consequent stages of post-transcriptional gene expression (Fu 1995, Sanford et al. 2005). The next step during protein synthesis is the translation of mRNA into proteins with the presence of a eukaryotic ribosome and its associating proteins. Therefore, 40S ribosomal protein is normally upregulated in the tumor cell as it is an essential component of the higher eukaryotic ribosome necessary for proper protein translational function (Ruvinsky & Meyuhas 2006). Eukaryotic translation initiation factor 3 subunit 12 is another important component as it binds to

poly(A)-binding protein to initiate translation process (Martineau et al. 2008). Meanwhile, elongation factor 2 functions to translocate peptidyl tRNA from the A site to the P site in the ribosome upon phosphorylation by CaM kinase III (Collodoro et al. 2012). Downregulation of these proteins by *Phyllanthus* will lead to production of aberrant proteins that resulted in cell growth attenuation.

Protein folding is the subsequent critical process upon successful protein translation to ensure formation of functional proteins as well as to ensure that they could pass through membranes and be integrated into their respective cellular organelles (Khalil et al. 2011). This step usually requires the presence of variety molecular chaperone proteins. One of these proteins is Hsp60 whose primary role is to guide the folding of mitochondrial proteins while facilitating proteolytic degradation of denatured or misfolded proteins in an ATP-dependent manner (Khalil et al. 2011). Activity of Hsp60 is normally regulated by its co-chaperone Hsp10 to control ATPase activity and substrate binding (Myung et al. 2004). Besides that, Hsp90 which is overexpressed in many tumor cell lines (Myung et al. 2004) is crucial to prevent misfolding and aggregation of pre-existing proteins under stress conditions (Martin & Hartl 1997). The stress-70 family of proteins such as Hsp70 also has a principal role in prevention of the premature misfolding of nascent polypeptides by cooperating with Hsp40 and Hip to help the activation of glucocorticoid receptors (Hartl & Martin 1995). Cells overexpressing Hsp70 are also protected against apoptosis via interaction with AIF to avoid chromatin condensation of induced nuclei (Ravagnan et al. 2001). Other chaperones include peptidyl-prolyl cis-trans isomerase A and T-complex protein 1 subunit epsilon that also assist the proper folding of proteins (Yoo et al. 2001, Justice et al. 2005). Besides facilitating protein folding, chaperones also guide the proper assembly of other proteins for them to carry out their activities. An important example is stress-induced phosphoprotein 1 that facilitates association of molecular chaperones

Hsp70 and Hsp90 which were implicated in MMP2 activity that leads to increased invasiveness (Walsh et al. 2009). Suppression of these proteins by *Phyllanthus* is therefore one of the key processes for cancer cell growth and metastasis inhibition to reduce functional protein synthesis and suppress MMP expression.

In addition to MMP2, suppression of other MMPs in response to *Phyllanthus* treatment was also observed including MMP7 and MMP9 (Figure 4.1(B)). The MMPs are a family of highly homologous, zinc- and calcium-dependent endopeptidases (Tanimura et al. 2005, Song et al. 2008). A genomic study done by Puente et al. discovered 24 distinct genes which encode for various MMPs (Puente et al. 2003), where MMP-2 and MMP-9 were more deeply associated with cancer invasion and metastasis. This is because their elevated expression has increased the metastatic potential of tumor cells and they had been known to be able to degrade type IV collagen-rich basement membrane of vessel wall (Jin et al. 2005, Tanimura et al. 2005) to access the vasculature to develop distant metastases (Chen et al. 2005). MMP2 is constitutively expressed and secreted in most tumor cells as a latent proenzyme form since they have the characteristics of housekeeping genes (Liu et al. 2002). Upon cleavage of its N-terminal propeptide by membrane-associated MMPs (MT1-MMP), MMP2 is activated leading to the proteolytic destruction of the basement membrane (Sen et al. 2009). Unlike MMP2, MMP9 is synthesized only when it was required and upon stimulation by various stimuli such as epidermal growth factors through their transmembrane receptors (Kondapaka et al. 1997). Its secretion was found to be upregulated particularly in multidrug resistant cancer that led to aggravation of its tumor invasiveness (Liu et al. 2002, Kim et al. 2009b, Karroum et al. 2010). MMP7 (28 kDa) is another member of the MMP family with broad substrate specificity against ECM components such as elastin, type IV collagen, fibronectin, vitronectin, aggrecan, and

proteoglycans (Liu et al. 2007). Their reduced expression hence explained the decreased aggressiveness of A549 and MCF-7 cells' invasion upon treatment with *Phyllanthus*.

A constant activation of various growth-promoting signaling pathways is also required to ensure continuous cell growth and survival (Figure 4.1(C)). This activation often involves numerous proteins that require some form of modifications such as phosphorylation for their biological role. Thus, post-translational modification process that alters the proteins properties is important (Li & Shang 2007, Crow & Xue-Bian 2010). The first step for pathways activation often requires the presence of reception molecules on the cell surface for ligand binding. So, guanine nucleotide-binding protein, putative Ras-related protein Rab-42, GTPase HRas precursor protein, and ras-related protein Rab-11B which can be broadly grouped as G-proteins, as well as probable G-protein coupled receptor 179 precursor expressions are usually elevated in the tumor cells to accommodate the large amount of extracellular signals to be transduced into the cells (Tuteja 2009). Both G-protein coupled receptor and G-proteins form the G-protein mediated signaling cascade whereby binding of ligands to receptors will lead to activation of G-proteins by promoting GDP/GTP exchange, which in turn regulates many effector molecules such as protein kinases (Tuteja 2009). Therefore, the subsequent upregulated protein in the tumor cell is serine/threonine-protein kinase 6 which is a family of kinases including MAP kinase (Cross et al. 2000), which functions to turn on the downstream kinases via serine/threonine phosphorylation (Nishida & Gotoh 1993). Suppression of this cell signaling proteins by *Phyllanthus* will therefore decelerates or stops the constitutive activation of the growth-promoting pathways, ERK1/2 and hypoxia.

ERK1/2 pathway is one of the MAP kinase subgroups that was frequently found to be inhibited in A549 and MCF-7 to repress cells' continuous growth and metastasis as presented in Figure 4.1(D). In a study by Shih et al., α-tomatine found in tomatoes

was shown to be capable of inactivating extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway to inhibit metastasis occurrence in A549 cell line (Shih et al. 2009). Similarly, A549 metastasis was inhibited by Silibinin isolated from *Silybum marianum* which suppresses ERK1/2 pathway that led to a reduced expression of MMP2 and u-PA concomitantly with a significant inhibition on cell invasion (Chen et al. 2005). In a separate study by Kim et al., Silibinin also caused inactivation of Raf/MEK/ERK pathway to prevent MMP9 and VEGF expression in MCF-7 cells (Kim et al. 2009b). Indeed, our study also showed that *Phyllanthus* inhibited A549 and MCF-7 metastasis via targeting specifically ERK1/2 pathway. ERK1/2 module is often thought as a linear pathway since ERK is the effector of an evolutionarily conserved signaling component that is triggered exclusively by the Raf serine/threonine kinases (Hindley & Kolch 2002, Roberts & Der 2007). Thus, *Phyllanthus* might possibly downregulate Raf protein at the early stage of the pathway as demonstrated in A549 cells. Nevertheless, GTP-binding protein Ras is another upstream activator of the ERK1/2 pathway that has been shown to be a potent stimulus for MMPs (Liu et al. 2002). Montague et al. related the blockage of Ras/Rho/MAP kinase pathway to the inhibition of MMP expression since inhibition of Ras *in vitro* has been shown to stop MMPs production (Montague et al. 2004). So, *Phyllanthus* could also probably target inhibition of Ras protein as shown in MCF-7 cells. Both Ras small GTPase and Raf kinase are the most commonly mutated oncogenes in a number of human cancers, hence explaining the importance of ERK1/2 pathway in human oncogenesis (Roberts & Der 2007). Repression of these proteins by *Phyllanthus* therefore resulted in the subsequent suppression of other proteins' expression down the pathway.

One of the downstream transcription factors of ERK1/2 pathways suppressed include AP-1 (Figure 4.1(E)), which is a major transcription factor that has been associated with cancer metastasis via regulation of a number of genes involved in

apoptosis, cell proliferation, and MMPs production (Lee et al. 2011b). AP-1 is a dimeric protein consisting of either homodimers of c-Jun proteins or heterodimers of c-Jun and c-Fos proto-oncogenes (Scodelaro Bilbao et al. 2010). As shown via western blot analysis (Figures 3.55 and 3.57), suppression of AP-1 in both A549 and MCF-7 cells by *Phyllanthus* mainly involves inhibition of c-Jun proteins induction. Another downstream target gene of ERK1/2 includes the *c-myc* oncogene that is very commonly overexpressed in a variety of human cancers including lung and breast carcinomas. Downregulation of this gene could affect apoptosis as well as repressing cell cycle progression (Bocca et al. 2007). As a result, various biological activities controlled by ERK1/2 pathway to increase cell growth and malignancy are repressed, including regulation of transcriptional, cell cycle, apoptosis, and metastasis (Bachmann & Moroy 2005).

Transcription is an important biological activity in a cell as it is the first step for the transmission of genetic information from DNA into RNA to be translated into proteins (Solomon et al. 2008). With the suppression of the essential transcriptional proteins for eukaryotic chromosomal DNA replication such as DNA-directed RNA polymerase II 16kDa polypeptide, transcription elongation factor B polypeptide 1, and proliferating cell nuclear antigen, transcription initiation and elongation process becomes inefficient (Naryzhny 2008). Besides that, zinc finger protein 174 is a DNA binding protein by acting as a cofactor for transcription factor. Its downregulation will lead to increased transcription of proteins such as E-cadherin that in turn represses cell invasion (Comijn et al. 2001, Scicchitano et al. 2004). E-cadherin is a type of adherens junctions expressed on the epithelial cells to enable them to form a sheet or layers of cells that are tightly attached laterally (Yang & Weinberg 2008). However, they are often functionally inactivated or silenced by various mechanisms such as somatic mutations, transcriptional repressions or downregulation of gene expression during

tumor progression (Huber et al. 2005) in order for the carcinoma cells to release themselves from neighboring cells for invasion into adjacent cell layers (Yang & Weinberg 2008). Concurrent with the reduced E-cadherin expression, there is usually an increased N-cadherin expression and this phenomenon is known as epithelial to mesenchymal transition (Gravdal et al. 2007). Loss of E-cadherin in tumors was demonstrated to be one of the contributing factors for metastatic dissemination (Onder et al. 2008) while N-cadherin transcription in a prostate carcinoma cell line was found to be activated during metastasis (Alexander et al. 2006). Nevertheless, our results showed that *Phyllanthus* had the ability to reverse this epithelial to mesenchymal transition process with an increased cell-cell adhesion capability, probably due to enhanced expression of functional E-cadherin proteins on the cells' surface.

On the other hand, cell cycle disorder plays a critical role in cancer progression. So, modulation of cell cycle by phytochemicals from natural product sources is gaining worldwide attention to control carcinogenesis (Abdolmohammadi et al. 2008). However, our findings showed that cell cycle pathway was not modulated with the flow cytometric data showing insignificant shifts in each cell cycle phases for the cells treated with *Phyllanthus* extracts. Hence, cell cycle arrest was ruled out as one of the mechanism of actions of the extracts. Since *Phyllanthus* did not inflict cell cycle arrest on both A549 and MCF-7, the only approach to inhibit cells' continuous growth is by causing toxicity. As shown previously, *Phyllanthus* does induce apoptosis in the cells with more than three-fold increase of caspases-3 and -7, the presence of DNA-fragmentation and TUNEL-positive cells (Lee et al. 2011a). In order to determine whether *Phyllanthus* induces extrinsic or intrinsic apoptotic pathway, Bcl-2 expression was examined using western blot analysis since it is one of the main regulators of the mitochondrial outer membrane permeabilization which initiates intrinsic apoptotic cell death (Chipuk & Green 2008, Brunelle & Letai 2009). Agreeing to our hypothesis, the

data suggests that *Phyllanthus* probably activates the intrinsic pathway of apoptosis by inhibiting antiapoptotic Bcl-2 protein to release cytochrome c for caspases activation. In addition to this, proteomic analysis also observed downregulation of Bcl-2-like protein 11 which is similar to bcl-2 protein that has a role as an antiapoptotic protein (Brunelle & Letai 2009).

Furthermore, *Phyllanthus* extracts also suppressed cytoskeletal proteins such as actin, vimentin, tubulin alpha chain, actin-related protein, stathmin, and cofilin-1/2 (Figure 4.1(F)). Besides being the components of the cytoskeleton, both actin and tubulin-binding proteins are also mediators of motility during cell migration and vascular remodelling (Murphy et al. 2008). Structure, conformational dynamics, and mechanical properties of actin filaments are mainly controlled by cofilin (Pfaendtner et al. 2010). On the other hand, tubulin dynamics was regulated by stathmin and transfection of this gene into lung cancer cell has been shown to increase their sensitivity to vinca alkaloids (Nishio et al. 2001). Meanwhile, vimentin constitutes the intermediate filaments of the cytoskeleton that stabilizes cytoskeletal interactions as well as affecting cell motility and movement. Elevated expression of vimentin in several invasive cell lines suggests the possibility of it being a representative marker for epithelial to mesenchymal transition (Walsh et al. 2009). Therefore, the significant inhibitory effects of *Phyllanthus* on the A549 cell's cytoskeleton most probably involve the alteration of the microfilament organization and function, therefore suppressing motility, angiogenesis and metastasis (Fang et al. 2011).

Hypoxia is largely perceived as another major obstacle to cancer therapy as increasing evidence in the cancer therapy-related literature suggests the involvement of proangiogenic factors in the tumor progression (Lin et al. 2011). Angiogenesis is essential for tumor growth and survival due to the imbalance of nutrient and oxygen supplies to solid tumors larger than $1mm^3$, resulting in tumor hypoxia (Dachs & Tozer

2000). In reaction to oxygen deprivation, transcription factor hypoxia-inducible factor-1 (HIF-1), which is the chief mediator of angiogenesis and energy metabolism is normally upregulated (Li et al. 2006). HIF-1α is one of the major regulatory components of HIF (Figure 4.1(G)) and is observed in many solid tumors to ensure cell survival and to promote angiogenesis (Lin et al. 2011). This is in agreement with our findings that displayed high HIF-1α expression in the untreated A549 and MCF-7 cells. Therefore, HIF-1α could possibly be the target for the development of novel anticancer agents. Lin et al. demonstrated suppression of lung tumor angiogenesis and metastasis by andrographolide isolated from *Andrographis paniculata* which downregulates HIF-1α (Lin et al. 2011, Ye et al. 2012). In addition, inhibition of HIF-1α pathway by *HIF-1α–siRNA* displayed a direct correlation with A549 cellular proliferation and angiogenesis, a prerequisite for metastasis (Hanze et al. 2003). Meanwhile, Lycium barbarum polysaccharides was found to be inhibiting angiogenesis in MCF-7 cells by reducing HIF-1α and VEGF expression levels (Huang et al. 2011). Downregulation of VEGF in MCF-7 by epigallocate-chin-3-gallate was also reported to suppress MMP2 activation, an essential gelatinase during blood vasculature formation or remodeling (Sen et al. 2009). In agreement to these studies, our results obtained also showed the reduction of HIF-1α expression in both A549 and MCF-7 cells treated with *Phyllanthus* extracts. This in turn led to the suppression of various target genes controlled by HIF-1α via hypoxia-responsive-element (HRE) (Hanze et al. 2003), including vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS).

VEGF is often overexpressed in malignant and nonmalignant cells as a response to numerous extracellular stimuli such as cytokine, oxygenous oxidants, growth factors, inflammation, and most importantly hypoxia (Kim et al. 2009b). It is a crucial angiogenic growth factor, which induces endothelial cell proliferation from the pre-existing capillary bed for wound healing, tumor growth, and metastasis. Its expression is

therefore increased prior to an invasive and metastatic phenotype (Dachs & Tozer 2000). Also iNOS, one of the three distinct isoforms of NOS which is widely expressed and often upregulated in multiple tumor tissues (Fitzpatrick et al. 2008) is expressed in tumor cells associated with vascularization and hence, is probably another important regulator of angiogenesis (Dachs & Tozer 2000, Fitzpatrick et al. 2008). It generates a generally higher amount of nitric oxide (NO) which contains tumor growth induction ability when present at low concentration (Loibl et al. 2006). Nitric oxide (NO) produced have been shown to affect vascular permeability, induces extracellular matrix degradation, triggers VEGF production, as well as stimulating endothelial cell proliferation and migration (Cullis et al. 2006). Hence, inhibition of VEGF and iNOS by *Phyllanthus* can greatly reduce A549 and MCF-7 angiogenesis, resulting in tumor cells malnutrition and hypoxia thereby preventing tumor growth, survival, and metastasis.

In addition to its role as an angiogenic inducer, HIF-1α also upregulates glucose uptake and glycolytic enzymes as shown in Figure 4.1(H) to increase energy production in response to hypoxia, since one of the amazing characteristics of tumor is its ability to alter its metabolism including increased dependence on glucose utilization via glycolysis in order to produce ATP (Li et al. 2006). Adenosine 5'-triphosphate (ATP), a major source of energy for cells and its involvement in a variety of cellular activities that are ATP-dependent is often increased in tumor cells (Shigenaga et al. 1994). Downregulation of these enzyme activities by *Phyllanthus* causes cellular energy deficit that results in cancer cell death. This includes phosphoglycerate kinase 1 (PGK1) which converts 1,3-diphosphoglycerate to 3-phosphoglycerate. In addition to its role as a glycolytic enzyme, PGK1 gene is known to contain hypoxia responsive element (HRE) region which could transfer the hypoxic inducibility to other promoters both in vitro and in vivo (Bando et al. 2003). Knockdown of HIF-1α can therefore prevent the expression

of PGK1 during hypoxia (Li et al. 2006). Another downregulated protein was pyruvate kinase isozymes M1/M2 which is also a glycolytic enzyme induced by HIF-1 (Bando et al. 2003). This enzyme metabolizes phosphoenolpyruvate to sustain the high rate of glycolysis in cancer cells by donating the phosphate group to phosphoglycerate mutase (Chiaradonna et al. 2012) which subsequently catalyzed the conversion of 3-phosphoglycerate to 2-phosphoglycerate (Pathania et al. 2009). Meanwhile, enolase catalyzes conversion of 2-phosphoglyceric acid (PGA) to phosphoenolpyruvate (PEP) in the anabolic pathway during gluconeogenesis to enhance aerobic glycolysis in cancer cells (Liu & Shih 2007). Elevated ATP synthase subunit alpha was also reported since it synthesizes most of the ATP in eukaryotes by transforming most of the energy from a gradient of ions across the membrane (Müller & Grüber 2003). Other glycolytic enzymes suppressed include lactate dehydrogenase A which converts pyruvate into lactate (Pelicano et al. 2006), glyceraldehyde-3-phosphate dehydrogenase that catalyzed glyceraldehydes-3-phosphate conversion into 1,3-biphosphoglycerate, as well as triosephosphate isomerase that facilitates reversible conversion of DHAP and glyceraldehyde-3-phosphate (Pathania et al. 2009). Suppression of these glucose uptake and glycolytic enzymes in cancer cells after *Phyllanthus* treatment could possibly deprive the cells of energy that lead them to their death fate.

Besides exploiting cellular signaling pathways for their growth and metastasis, tumor cells possess various efficient drug detoxification (Figure 4.1I) and defense mechanisms (Figure 4.1J) to remove compounds that may be fatal to them. This include upregulation of glutathione transferase omega-1 that catalyzes binding of glutathione to various anticancer compounds such as cisplatin, thereby decreasing production of platinum-DNA adducts and rendering them useless while glutathione synthetase catalyzes production of glutathione substrate for the detoxifying activity (Polekhina et al. 1999, Stewart 2010). Similarly, metallothionein also plays a role in chemotherapy

binding and detoxification since its elevated expression was noticed in several cisplatin-resistant lung cancer cell lines (Stewart 2010). In addition, Annexins are normally associated with chemoresistance in part by enhancing endocytosis, exocytosis, as well as ion channel activity for drug efflux (Zhang & Liu 2007, Kim et al. 2009a). Meanwhile, one of the protective mechanisms involves increased expression of galectin-3, a β-galactoside-binding protein that was associated with angiogenesis, cell proliferation, tumor progression, apoptosis, and metastasis. It is translocated into nucleus upon stimulation by apoptotic stimulus to inhibit cytochrome c release (Hooven & Baird 2008). Another defense mechanism include upregulation of peroxiredoxins which are antioxidant enzymes that eliminate reactive oxygen species such as H_2O_2 using thioredoxin as immediate electron donor (Zhang & Liu 2007). Hence, thioredoxins in mammalian cells were also increased to ensure a reducing intracellular redox state by acting as reducing agents using thiol groups (Lillig & Holmgren 2007). Inhibition of these detoxification and protective enzymes expression in A549 and MCF-7 after treatment with *Phyllanthus* as demonstrated from the proteomic analysis therefore advocates their reduced drug-resistance capability resulting in their susceptibility to death-inducing compounds.

Apart from that, it is interesting to note that both A549 and MCF-7 are wild type p53-expressing cell lines (Sandhya & Mishra 2006, Sun et al. 2008). P53 is a versatile protein that triggers apoptosis via downregulating survival factors while upregulating proapoptotic factors (Fridman & Lowe 2003). Its activity is normally triggered in response to various cellular stresses such as chemo- or radiation-induced DNA damages, oncogene assaults, and oxidative stress to prevent cell survival (Chen et al. 2010). For instance, epigallocatechin-3-gallate was shown to exclusively activate p53-dependent pathway in A549 cells to induce apoptosis (Yamauchi et al. 2009). Furthermore, Gefitinib and zinc sulfate respectively demonstrated a phosphorylation of

p53 at Ser15 to stimulate tumor cell apoptosis (Chang et al. 2008, Nakagawa & Matsuoka 2008). Moreover, benzo(α)pyrene and selenocystine was shown to induced MCF-7 cell apoptosis via p53 phosphorylation at Ser392 in two separate studies (Tampio et al. 2008, Chen & Wong 2009). So, p53 involvement to inhibit cells' survival was expected after treatment with *Phyllanthus* extract and yet, their expression was not significantly affected. This could be owing to the suppression or Hdm2-regulated degradation of p53 proteins via other cellular pathways which renders the p53 pathway nonfunctional (Wade et al. 2010). For example, dephosphorylation of p53 often leads to increased binding to its negative regulator, Hdm-2 resulting in enhanced ubiquitination and proteolysis of p53 (Vousden & Lane 2007). Otherwise, it could be attributed to the aberrant posttranslational modification of p53 that produces defective p53 proteins (Vousden & Lane 2007). Nevertheless, further research is necessary to confirm this hypothesis.

4.1 CONCLUSION

As presented in Figure 4.1, the anticancer activities demonstrated by *Phyllanthus* most probably is due to its capacity to suppress activation of ERK1/2 and hypoxia pathways in both A549 and MCF-7 cells. *Phyllanthus* suppresses ERK1/2 signaling mainly by preventing the stimulation of Ras and Raf kinase protein during the early stages of the pathway, hence leading to the reduced expression of other protein kinases downstream of the pathways including MEK1/2 and ERK1/2. Similarly, *Phyllanthus* also blocked the activation of HIF-1α subunit during hypoxia events, which in turn inhibited the association of HIF-1α and HIF-1β in the nucleus to form an active HIF-1 protein. Decreased translocation of the activated ERK1/2 protein as well as a reduced amount of active HIF-1 protein in the nucleus will therefore block the activation of numerous transcription factors such as AP-1, c-Myc, and various promoters containing

hypoxia responsive element (HRE). These transcription factors govern a wide range of cellular biological activities and hence their diminished stimulation in response to *Phyllanthus* results in reduced expression of abundant genes.

The first category of gene expression downregulated by *Phyllanthus* include the signal transduction components such as G proteins, G-protein coupled receptors and serine/threonine-protein kinase. Reduced expression of these receptors and their associated components on the cell surface produces a positive feedback loop phenomena, as increasingly lesser amounts of growth stimuli can be transmited into the cell. Inhibition of ERK1/2 pathway by *Phyllanthus* also leads to downregulation of various invasion and mobility proteins that are essential during cell metastasis while suppression of protein synthesis and transcriptional proteins expression cause the inability of the cells to proliferate continuously due to the lack of crucial cell replicative proteins. Meanwhile, inhibition of hypoxia pathway causes repression of the angiogenic proteins, VEGF and iNOS, resulting in the failure of the cancer cells to build new blood vessels that eventually reduces their chance to metastasize. Besides that, *Phyllanthus* also impair expression of various glucose uptake and glycolytic enzymes so that the cancer cells will become energy-deficient and die. Suppression of drug detoxification enzyme in cancer cells such as gluthathione transferase, gluthathione synthetase, metallothionein, and annexins also increases sensitivity of A549 and MCF-7 to *Phyllanthus* treatment. In addition, *Phyllanthus* also represses several proteins involved in the tumor's survival mechanism, including antiapoptotic protein (Bcl-2), peroxiredoxins, thioredoxins, and galectin 3.

Since *Phyllanthus* decreased the expression of numerous proteins in the cancer cells, they will not be able to grow uncontrollably and spread to the other organs. Devoid of the ability to proliferate nonstop, the cancer cells will not be able to thrive and will ultimately activate the cell death programme. Unlike the currently available

chemotherapeutic drugs, *Phyllanthus* have shown selectiveness where they target only the cancer cells without affecting the growth of normal cells, by mainly inducing apoptosis in the cancer cells that is associated with the activation of caspases-3 and -7 as well as DNA fragmentation. Meanwhile, the ability of *Phyllanthus* to stop metastasis is correlated with their inhibitory effects on the critical steps in metastasis, including cell invasion, migration, and adhesion. Furthermore, *Phyllanthus* had demonstrated the ability to reverse the endothelial-mesenchymal transition process, the foremost decisive factor for the cell metastasizing events. Generally, the antiproliferative and antimetastatic potential of *Phyllanthus* is most possibly attributed to the presence of abundant polyphenol compounds. Among the four *Phyllanthus* species tested in this study, *P. urinaria* was found to be the most effective to inhibit A549 and MCF-7 growth and metastasis, closely followed by *P. watsonii*.

Database search revealed only two reports that have reported the antimetastatic property of *Phyllanthus* thus far, and this study is one of those two. The study reported by Tseng et al. focused only on the effects of *Phyllanthus urinaria* on two different lung cancer cells (A549 and Lewis lung carcinoma), where their metastasis was inhibited by the suppression of MMP2 and MMP9 (Tseng et al. 2012). In contrary, the current study reported the effects of four *Phyllanthus* species on two different cancer cells, both lung (A549) and breast (MCF-7) cancers, thereby showing a broader spectrum of antimetastatic activity exerted by *Phyllanthus*. In addition to MMP2 and MMP9, MMP7 was also found to be repressed in A549 cells to cause metastasis inhibition. Moreover, the underlying mechanisms in A549 and MCF-7 cells targeted by *Phyllanthus* to suppress the MMP expressions and eventually stop the cell metastasis and proliferation were also discovered. The molecular mechanisms of *Phyllanthus* reported here might aid those who are trying to develop this or other species as a potential antiproliferative or antimetastatic agent.

4.2 FUTURE WORK

Phyllanthus could be a valuable candidate in the treatment of metastatic cancers. However, the main concern before application of *Phyllanthus* as an antimetastatic or antiproliferative agent is its *in vivo* effect. Hence, further testing of the extracts activity *in vivo* is necessary to investigate the antitumor effects of *Phyllanthus* on a tumor-bearing mouse. The results obtained would provide information on the safe use of this plant for the development of potential anticancer compounds.

The suitable mouse strain to be used in this antitumor study would be severe combined immunodeficient (SCID) mice. Numerous solid human tumors and hematological neoplasms for both cell lines and fresh biopsy tissues have been engrafted into SCID mice after the first report indicating the successful engraftment of SCID mice with human tumors was revealed. This human-SCID mouse chimeric model has been exploited for evaluation of a wide variety of anticancer compounds or therapies (Bankert et al. 2001, Belizário 2009). Preliminary work has identified low toxicity of *Phyllanthus* in SCID mice as a dosage greater than 50g/kg is needed to cause 100% acute death in the mice tested. Meanwhile, the maximal non-toxic dose was determined to be 10g/kg. Development of a tumor-bearing mice model is still in progress before efficacy testing can be carried out.

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APPENDICES

APPENDIX 1: PREPARATION OF CULTURE MEDIA, REAGENTS, AND CHEMICALS

1. Culture Media Preparation

<table>
<tr><td>DMEM (HyClone)
(13.4gram)</td><td>RPMI (HyClone)
(10.4gram)</td><td rowspan="4">F-12 K
(ATCC)</td><td rowspan="4">Mammary Epithelial Growth Medium (Lonza)</td></tr>
<tr><td>Sodium bicarbonate
(Sigma Aldrich)
(3.7gram/liter)</td><td>Sodium bicarbonate
(Sigma Aldrich)
(2.0gram/liter)</td></tr>
<tr><td colspan="2">Dissolve in 1 liter of deionized water</td></tr>
<tr><td colspan="2">Filter sterilization through 0.2µm pore filter unit</td></tr>
<tr><td colspan="4">Growth media: Add 10% fetal bovine serum (JR Scientific)
Maintenance media: Add 2% fetal bovine serum (JR Scientific)</td></tr>
</table>

2. Common Use Reagents/Chemicals

0.1% Coomasie Blue Dye (400ml)	
Chemicals	**Volume**
Coomasie blue R-350 (GE Healthcare)	1 tablet
Deionized water	80.0ml
Methanol	120.0ml
20% Acetic acid in deionized water	200.0ml

Destaining Buffer (500ml)	
Chemicals	**Volume**
95% Ethanol	250.0ml
100% Acetic acid	50.0ml
Deionized water	200.0ml

10% (w/v) Sodium dodecyl sulfate (SDS)	
Chemicals	**Volume**
SDS powder	10.0g
Deionized water	100.0ml

30% Acrylamide/Bisacrylamide	
Chemicals	**Volume**
Acrylamide	30.0g
N,N’-methylenebisacrylamide	0.8g
Deionized water	100.0ml

Cell Lysis Buffer	
Chemicals	**Volume**
Urea	12.6g
Thiourea	4.5g
2% CHAPS	0.6g
Triton X-100	1% (v/v)
Dithiothreitol (DTT)	185.0mg
Deionized water	30.0ml

12.5% SDS Polyacrylamide Resolving Gel		
Chemicals	**Volume**	**Volume**
30% Acrylamide/Bisacrylamide	4.2ml	187.5ml
1.5M Tris-HCl, pH8.8	2.5ml	112.5ml
10% (w/v) SDS	100.0µl	4.5µl
10% (w/v) APS	100.0µl	4.5µl
100% TEMED	5.0µl	620.0µl
Deionized water	3.5ml	140.5ml

4% SDS Polyacrylamide Stacking Gel	
Chemicals	**Volume**
30% Acrylamide/Bisacrylamide	670µl
0.5M Tris-HCl, pH6.8	1.3ml
10% (w/v) SDS	50.0µl
10% (w/v) APS	50.0µl
100% TEMED	5.0µl
Deionized water	3.1ml

10× Laemmli/SDS Electrophoresis Buffer	
Chemicals	**Volume**
Tris base	30.0g
Glycine	144.0g
SDS	10.0g
Deionized water	Top up to 1000.0ml
To prepare 1× Laemlli buffer, mix one part 10× Laemlli buffer with nine parts deionized water	

Agarose Sealing Solution	
Chemicals	**Volume**
1× Laemmli buffer	10.0ml
Agarose powder	50.0mg
1% Bromophenol blue	20.0µl

3. Reagents/Chemicals for DNA Fragmentation Assay

Triton X-100 Lysis Buffer	
Chemicals	**Volume**
0.5M EDTA	40.0ml
1M Tris-Cl buffer, pH8.0	5.0ml
Triton X-100	5.0ml
Deionized water	50.0ml

4. Reagents/Chemicals for Zymography Assay

2× Tris-Glycine SDS Sample Buffer	
Chemicals	**Volume**
0.5M Tris-HCl, pH6.8	2.5ml
87% Glycerol	2.0ml
10% (w/v) SDS	4.0ml
0.1% Bromophenol blue	0.5ml
Deionized water	10.0ml

12.5% SDS Polyacrylamide Resolving Gel with Gelatin or Casein	
Chemicals	**Volume**
30% Acrylamide/Bisacrylamide	4.2ml
1.5M Tris-HCl, pH8.8	2.5ml
10% (w/v) SDS	100.0µl
10% (w/v) APS	100.0µl
100% TEMED	5.0µl
10% Gelatin or 20% Casein in deionized water	1.0ml
Deionized water	2.5ml

Renaturing Buffer (1000ml)	
Chemicals	**Volume**
100% Triton X-100	25.0ml
Deionized water	975.0ml

Developing Buffer	
Chemicals	**Amount**
Tris base	12.1g
Tris-HCl	63.0g
Sodium chloride	117.0g
Calcium chloride	7.4g
Brij35	0.2% (v/v)
Deionized water	top up to 1000.0ml

5. Reagents/Chemicals for Western Blot Assay

10× Tris-Buffered Saline (TBS) Buffer	
Chemicals	**Volume**
Tris-HCl	24.3g
Sodium chloride	80.1g
6N Hydrochloric acid (HCl)	adjust to pH7.6
Deionized water	top up to 1000.0ml

5% Blocking Buffer	
Chemicals	**Volume**
1× TBS buffer	100.0ml
Skimmed milk powder	5.0g

TBS-Tween 20 Washing Buffer	
Chemicals	**Volume**
10× TBS buffer	100.0ml
Tween 20	1.0ml
Deionized water	900.0ml

10× Transfer Buffer	
Chemicals	**Volume**
Tris-HCl	30.3g
Glycine	144.1g
Deionized water	top up to 1000.0ml
To prepare 1× transfer buffer, mix one part 10× transfer buffer with two parts methanol and seven parts deionized water	

10× Ponceau S Dye	
Chemicals	**Volume**
Ponceau S	2.0g
100% Acetic acid	10.0ml
Deionized water	90.0ml
Dilute one part 10× Ponceau S with nine parts deionized water before use	

DAB Substrate	
Chemicals	**Volume**
Substrate A	
4-chloro-1-napthol	0.15g
Methanol	40.0ml
1× TBS buffer	80.0ml
Substrate B	
30% Hydrogen Peroxide	250.0µl
1× TBS buffer	120.0ml
To prepare working DAB substrate solution, mix one part of substrate A with one part of substrate B	

6. Reagents/Chemicals for 2-Dimensional Gel Electrophoresis Assay

Rehydration Buffer	
Chemicals	**Volume**
Urea	4.2g
Thiourea	1.5g
CHAPS	0.4g
Triton X-100	1% (v/v)
1% Bromophenol blue	20.0µl
Deionized water	10.0ml
Add 5.0µl IPG buffer and 2.8mg DTT to each milliliter of rehydration buffer before use	

Gel Fixative Solution	
Chemicals	**Volume**
95% Ethanol	400.0ml
100% Acetic acid	100.0ml
Deionized water	500.0ml

SDS Equilibration Buffer	
Chemicals	**Volume**
1.5M Tris-HCl, pH8.8	10.0ml
Urea	72.1g
87% Glycerol	69.0ml
SDS	4.0g
1% Bromophenol blue	400.0µl
Deionized water	top up to 200.0ml
Add 50.0mg dithiothreitol (DTT) or 125.0mg iodoacetamide (IAA) to 5.0ml of equilibration buffer before use	

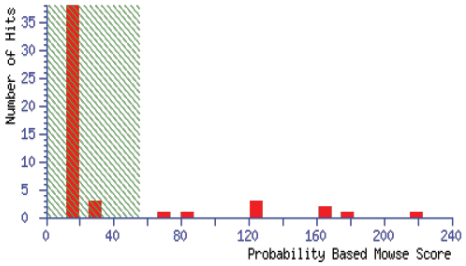
APPENDIX 2: EXAMPLE OF MASCOT SEARCH RESULT

Mascot Search Results

```
User           :
Email          :
Search title   : SampleSetID: 1301, AnalysisID: 2955, MaldiWellID: 117934, SpectrumID: 219291, Path=\280911\msms\lsh
Database       : Sprot 080308 (261513 sequences; 95638062 residues)
Taxonomy       : Homo sapiens (human) (16040 sequences)
Timestamp      : 29 Sep 2011 at 08:26:43 GMT
Warning        : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score      : 219 for TBB5_HUMAN, Tubulin beta chain (Tubulin beta-5 chain) - Homo sapiens (Human)
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 55 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein Summary Report

Format As: Protein Summary (deprecated) — Help

Significance threshold p< 0.05 — Max. number of hits 10

Standard scoring / MudPIT scoring — Ions score cut-off 0 — Show sub-sets

Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	TBB5_HUMAN	49639	219	Tubulin beta chain (Tubulin beta-5 chain) - Homo sapiens (Human)
2.	TBB2A_HUMAN	49875	177	Tubulin beta-2A chain - Homo sapiens (Human)
3.	TBB2B_HUMAN	49921	168	Tubulin beta-2B chain - Homo sapiens (Human)
4.	TBB2C_HUMAN	49799	162	Tubulin beta-2C chain (Tubulin beta-2 chain) - Homo sapiens (Human)
5.	TBB3_HUMAN	50400	127	Tubulin beta-3 chain (Tubulin beta-III) (Tubulin beta-4) - Homo sapiens (Human)
6.	TBB4_HUMAN	49554	127	Tubulin beta-4 chain (Tubulin 5 beta) - Homo sapiens (Human)
7.	TBB6_HUMAN	49825	120	Tubulin beta-6 chain - Homo sapiens (Human)
8.	TBB1_HUMAN	50295	79	Tubulin beta-1 chain - Homo sapiens (Human)
9.	TBB4Q_HUMAN	48403	65	Tubulin beta-4q chain - Homo sapiens (Human)
10.	PCDAB_HUMAN	103235	28	Protocadherin alpha 11 precursor (PCDH-alpha11) - Homo sapiens (Human)

Results List

1. TBB5_HUMAN **Mass:** 49639 **Score:** 219 **Expect:** 2e-018 **Queries matched:** 19
Tubulin beta chain (Tubulin beta-5 chain) - Homo sapiens (Human)

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
1039.64	1038.63	1038.59	0.05	310	318	0	---	R.YLTVAAVFR.G
1077.58	1076.58	1076.53	0.05	155	162	1	18	K.IREEYPDR.I
1077.58	1076.58	1076.53	0.05	155	162	1	---	K.IREEYPDR.I
1130.65	1129.64	1129.59	0.05	242	251	0	34	R.FPGQLNADLR.K
1130.65	1129.64	1129.59	0.05	242	251	0	---	R.FPGQLNADLR.K

APPENDIX 3: IDENTIFIED PROTEINS OF A549 AND MCF-7 DOWNREGULATED (-) IN THE PRESENCE OF *PHYLLANTHUS* EXTRACTS

Table I: Identified proteins of A549 downregulated (-) in the presence of aqueous *Phyllanthus* extracts

APN – aqueous *P. niruri*; APU – aqueous *P. urinaria*; APW – aqueous *P. watsonii*, APA – aqueous *P. amarus*; N/A – Not Affected.

Spot ID	Possible Proteins	Fold change				UNIPROT KB/SWISS-PROT Acc. Number	Database
		APN	APU	APW	APA		
1	Actin, cytoplasmic 1	-0.81	-0.88	-0.95	-0.84	P60709	MASCOT;UNIPROT KB/SWISS-PROT;COGS
2	Nicotinamide N-methyltransferase	-0.50	N/A	-0.28	-0.10	P40261	MASCOT;UNIPROT KB/SWISS-PROT;COGS
3	Alpha-Enolase	-0.62	-0.57	-0.48	-0.58	P06733	MASCOT;UNIPROT KB/SWISS-PROT;COGS
4	Proteasome subunit beta type 3	-1.00	-0.20	N/A	-1.00	P49720	MASCOT;UNIPROT KB/SWISS-PROT;COGS
5	Retinal dehydrogenase 1	-0.23	N/A	-0.13	N/A	P00352	MASCOT;UNIPROT KB/SWISS-PROT;COGS
6	Galectin-1	-1.00	-0.13	-0.57	-0.38	P09382	MASCOT;UNIPROT KB/SWISS-PROT;COGS
7	Heat-shock protein beta-1	-0.12	-0.22	-0.32	-0.10	P04792	MASCOT;UNIPROT KB/SWISS-PROT;COGS
8	Glyceraldehyde-3-phosphate dehydrogenase	-0.30	-0.93	-0.99	-0.91	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
9	Eukaryotic translation initiation factor 5A-1	-0.32	-0.39	-0.27	-0.80	P63241	MASCOT;UNIPROT KB/SWISS-PROT;COGS
10	60 kDa heat shock protein, mitochondrial	-0.46	-0.82	-0.90	-0.75	P10809	MASCOT;UNIPROT KB/SWISS-PROT;COGS
11	Glutathione transferase omega-1	N/A	-0.45	N/A	-0.22	P78417	MASCOT;UNIPROT KB/SWISS-PROT;COGS
12	Proteasome activator complex subunit 1	-0.19	-0.18	N/A	-1.00	Q06323	MASCOT;UNIPROT KB/SWISS-PROT;COGS
13	Annexin A4	-0.29	-0.29	-0.21	-0.65	P09525	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table I, continued’

14	Vimentin	-0.91	-0.82	-0.24	-0.79	P08670	MASCOT;UNIPROT KB/SWISS-PROT;COGS
15	Peptidyl-prolyl cis-trans isomerase A	-0.13	-0.36	-1.00	-0.42	P62937	MASCOT;UNIPROT KB/SWISS-PROT;COGS
16	Peptidyl-prolyl cis-trans isomerase A	-1.00	-1.00	-0.32	N/A	P62937	MASCOT;UNIPROT KB/SWISS-PROT;COGS
17	Glyceraldehyde-3-phosphate dehydrogenase	-1.00	-0.50	-1.00	-0.71	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
18	Voltage-dependent anion-selective channel protein 1	-0.37	-0.19	-0.20	N/A	P21796	MASCOT;UNIPROT KB/SWISS-PROT;COGS
19	Tubulin alpha-8 chain	-0.80	-0.37	N/A	-0.80	Q9NY65	MASCOT;UNIPROT KB/SWISS-PROT;COGS
20	Protein FAM24B precursor	-0.51	-0.54	-0.66	-0.88	Q8N5W8	MASCOT;UNIPROT KB/SWISS-PROT;COGS
21	Interferon alpha-6 precursor	-0.41	N/A	-0.38	-0.75	P05013	MASCOT;UNIPROT KB/SWISS-PROT;COGS
22	ATP-dependent DNA helicase Q5	-1.00	-1.00	-0.93	-0.79	O94762	MASCOT;UNIPROT KB/SWISS-PROT;COGS
23	Sorting nexin-3	-0.25	-1.00	-1.00	-1.00	O60493	MASCOT;UNIPROT KB/SWISS-PROT;COGS
24	Gap junction beta-5 protein	-0.36	-0.16	-0.32	-0.41	O95377	MASCOT;UNIPROT KB/SWISS-PROT;COGS
25	40S ribosomal protein S19	-0.30	-0.30	-0.15	-0.38	P39019	MASCOT;UNIPROT KB/SWISS-PROT;COGS
26	Stress-70 protein, mitochondrial precursor	-0.19	-0.22	N/A	N/A	P38646	MASCOT;UNIPROT KB/SWISS-PROT;COGS
27	Actin-related protein 2/3 complex subunit 5	-1.00	-1.00	-1.00	-1.00	O15511	MASCOT;UNIPROT KB/SWISS-PROT;COGS
28	Sorting nexin-3	-0.36	-0.35	-0.37	-1.00	O60493	MASCOT;UNIPROT KB/SWISS-PROT;COGS
29	Complement receptor type 1 precursor	-0.19	N/A	-0.41	-1.00	P17927	MASCOT;UNIPROT KB/SWISS-PROT;COGS
30	40S ribosomal protein S24	-1.00	-1.00	-1.00	-1.00	P62847	MASCOT;UNIPROT KB/SWISS-PROT;COGS
31	Corticotropin-lipotropin precursor	-0.73	-0.88	-1.00	-1.00	P01189	MASCOT;UNIPROT KB/SWISS-PROT;COGS
32	Inosine triphosphate pyrophosphatase	-1.00	-0.67	-1.00	-1.00	Q9BY32	MASCOT;UNIPROT KB/SWISS-PROT;COGS
33	Peroxiredoxin-1	-0.08	-0.56	-1.00	-0.33	Q06830	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table I, continued’

34	Protein memo	-0.95	-0.88	-0.56	-0.47	Q9Y316	MASCOT;UNIPROT KB/SWISS-PROT;COGS
35	Voltage-dependent anion-selective channel protein 2	-1.00	-0.31	-0.46	-0.21	P45880	MASCOT;UNIPROT KB/SWISS-PROT;COGS
36	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	-0.28	-0.19	-0.27	N/A	P30048	MASCOT;UNIPROT KB/SWISS-PROT;COGS
37	RuvB-like 2	-0.24	N/A	-0.43	-0.11	Q9Y230	MASCOT;UNIPROT KB/SWISS-PROT;COGS
38	EF-hand domain-containing protein 2	-0.36	-0.17	-0.37	-0.29	Q5JST6	MASCOT;UNIPROT KB/SWISS-PROT;COGS
39	T-complex protein 1 subunit epsilon	-0.89	N/A	-0.20	-0.97	P48643	MASCOT;UNIPROT KB/SWISS-PROT;COGS
40	Stress-induced-phosphoprotein 1	-0.54	-0.62	-0.44	-0.21	P31948	MASCOT;UNIPROT KB/SWISS-PROT;COGS
41	Retinal dehydrogenase 1	-0.33	-0.79	-0.58	-0.61	P00352	MASCOT;UNIPROT KB/SWISS-PROT;COGS
42	Prostaglandin E synthase 3	-0.26	-0.36	-0.97	-0.16	Q15185	MASCOT;UNIPROT KB/SWISS-PROT;COGS
43	Trypsin-1 precursor	-0.37	-1.00	-0.28	-0.35	P07477	MASCOT;UNIPROT KB/SWISS-PROT;COGS
44	Serpin B9	-0.51	-0.14	-0.21	-0.51	P50453	MASCOT;UNIPROT KB/SWISS-PROT;COGS
45	N-acylneuraminate cytidylyltransferase	-0.64	-0.57	-0.50	-0.28	Q8NFW8	MASCOT;UNIPROT KB/SWISS-PROT;COGS
46	Cofilin-1	-0.76	N/A	-0.85	-0.38	P23528	MASCOT;UNIPROT KB/SWISS-PROT;COGS
47	ADP-ribosylation factor 1	-1.00	-0.21	-0.15	-1.00	P84077	MASCOT;UNIPROT KB/SWISS-PROT;COGS
48	Fructose-bisphosphate aldolase A	-1.00	-1.00	-1.00	-1.00	P04075	MASCOT;UNIPROT KB/SWISS-PROT;COGS
49	Sorting nexin-3	-1.00	-1.00	-1.00	-1.00	O60493	MASCOT;UNIPROT KB/SWISS-PROT;COGS
50	Phosphoglycerate mutase 1	-1.00	-1.00	-1.00	-1.00	P18669	MASCOT;UNIPROT KB/SWISS-PROT;COGS
51	Triosephosphate isomerase	-1.00	-1.00	-1.00	-1.00	P60174	MASCOT;UNIPROT KB/SWISS-PROT;COGS
52	Eukaryotic translation initiation factor 5A-1	-1.00	-1.00	-1.00	-1.00	P63241	MASCOT;UNIPROT KB/SWISS-PROT;COGS

Table II: Identified proteins of A549 downregulated (-) in the presence of methanolic *Phyllanthus* extracts

MPN – methanolic *P. niruri*; MPU – methanolic *P. urinaria*; MPW – methanolic *P. watsonii*, MPA – methanolic *P. amarus*; N/A – Not Affected.

Spot ID	Possible Proteins	Fold change				UNIPROT KB/SWISS-PROT Acc. Number	Database
		MPN	MPU	MPW	MPA		
1	Protein S100-A8	-0.47	-0.10	-0.48	-0.55	P05109	MASCOT;UNIPROT KB/SWISS-PROT;COGS
2	Transmembrane protein 35	-0.82	-0.78	-1.00	-0.50	Q53FP2	MASCOT;UNIPROT KB/SWISS-PROT;COGS
3	Adenylate kinase isoenzyme 6	-0.32	-0.62	-0.81	-0.73	Q9Y3D8	MASCOT;UNIPROT KB/SWISS-PROT;COGS
4	Peroxiredoxin-1	-0.78	-1.00	-1.00	-1.00	Q06830	MASCOT;UNIPROT KB/SWISS-PROT;COGS
5	Prolactin-releasing peptide precursor	-0.59	-0.29	-0.23	-0.39	P81277	MASCOT;UNIPROT KB/SWISS-PROT;COGS
6	Putative protein SSX6	-0.46	-0.96	-1.00	-0.75	Q7RTT6	MASCOT;UNIPROT KB/SWISS-PROT;COGS
7	Glutathione synthetase	-0.38	-0.79	-1.00	-0.51	P48637	MASCOT;UNIPROT KB/SWISS-PROT;COGS
8	Nucleoside diphosphate-linked moiety X motif 16	-0.61	-0.63	-0.98	-0.94	Q3MHX9	MASCOT;UNIPROT KB/SWISS-PROT;COGS
9	E3 ubiquitin-protein ligase ZNRF1	N/A	N/A	-0.47	-0.36	Q8ND25	MASCOT;UNIPROT KB/SWISS-PROT;COGS
10	DNA-directed RNA polymerase II 16 kDa polypeptide	-0.46	-0.59	-0.43	-0.93	Q9VEA5	MASCOT;UNIPROT KB/SWISS-PROT;COGS
11	39S ribosomal protein L40, mitochondrial precursor	-1.00	-1.00	-1.00	-1.00	Q9NQ50	MASCOT;UNIPROT KB/SWISS-PROT;COGS
12	Zinc finger protein 174	-1.00	-1.00	-1.00	-1.00	Q15697	MASCOT;UNIPROT KB/SWISS-PROT;COGS
13	Probable G-protein coupled receptor 179 precursor	-0.21	-0.15	-1.00	N/A	Q6PRD1	MASCOT;UNIPROT KB/SWISS-PROT;COGS
14	Histatin-1 precursor	-0.24	-0.66	-0.78	-0.27	P15515	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table II, continued’

15	U6 snRNA-associated Sm-like protein LSm5	-0.33	-0.57	-0.19	-0.54	Q9Y4Y9	MASCOT;UNIPROT KB/SWISS-PROT;COGS
16	Bcl-2-like protein 11	-0.25	-1.00	-1.00	-0.70	O43521	MASCOT;UNIPROT KB/SWISS-PROT;COGS
17	Contactin-2 precursor	-0.29	-0.60	-0.42	-0.39	Q02246	MASCOT;UNIPROT KB/SWISS-PROT;COGS
18	Bis(5'-adenosyl)-triphosphatase	-1.00	-1.00	N/A	-1.00	P49789	MASCOT;UNIPROT KB/SWISS-PROT;COGS
19	Trypsin-1 precursor	-1.00	-0.98	-1.00	-1.00	P07477	MASCOT;UNIPROT KB/SWISS-PROT;COGS
20	Proliferating cell nuclear antigen	-1.00	-1.00	-1.00	-1.00	P12004	MASCOT;UNIPROT KB/SWISS-PROT;COGS
21	Endoplasmin precursor	N/A	-1.00	-1.00	-0.37	P14625	MASCOT;UNIPROT KB/SWISS-PROT;COGS
22	Eukaryotic translation initiation factor 3 subunit 12	-1.00	-1.00	-1.00	-1.00	Q9UBQ5	MASCOT;UNIPROT KB/SWISS-PROT;COGS
23	Serine/threonine-protein kinase 6	-1.00	-0.96	-0.19	-1.00	O14965	MASCOT;UNIPROT KB/SWISS-PROT;COGS
24	Phenylalanyl-tRNA synthetase beta chain	-1.00	-1.00	-1.00	-1.00	Q9NSD9	MASCOT;UNIPROT KB/SWISS-PROT;COGS
25	Neutrophil defensin 1 precursor	-0.78	-0.49	-0.20	-0.88	P59665	MASCOT;UNIPROT KB/SWISS-PROT;COGS
26	Proto-oncogene protein Wnt-3 precursor	-0.58	N/A	-0.34	-0.55	P56703	MASCOT;UNIPROT KB/SWISS-PROT;COGS
27	Putative Ras-related protein Rab-42	-1.00	N/A	-0.97	-0.80	Q8N4Z0	MASCOT;UNIPROT KB/SWISS-PROT;COGS
28	UPF0404 protein C11orf59	-0.19	-0.36	N/A	-0.36	Q6IAA8	MASCOT;UNIPROT KB/SWISS-PROT;COGS
29	Neutrophil defensin 1 precursor	-0.31	-0.25	-1.00	-0.87	P59665	MASCOT;UNIPROT KB/SWISS-PROT;COGS
30	Agouti-signaling protein precursor	-0.33	-0.67	-0.67	N/A	P42127	MASCOT;UNIPROT KB/SWISS-PROT;COGS
31	DNA-directed RNA polymerase II 16 kDa polypeptide	-0.42	-0.48	-0.48	-0.45	Q9VEA5	MASCOT;UNIPROT KB/SWISS-PROT;COGS
32	Beta-defensin 107A precursor	-0.21	N/A	-0.62	-0.61	Q8IZN7	MASCOT;UNIPROT KB/SWISS-PROT;COGS
33	Transcription elongation factor B polypeptide 1	-0.58	-0.59	-0.56	-0.79	Q15369	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table II, continued’

34	Metallothionein-1M	-0.55	-0.52	-0.48	-0.48	Q8N339	MASCOT;UNIPROT KB/SWISS-PROT;COGS
35	GTPase HRas precursor	-0.60	-0.66	-0.59	-0.61	P01112	MASCOT;UNIPROT KB/SWISS-PROT;COGS
36	Neuromedin-B precursor	-0.24	-0.32	-0.23	-0.34	P08949	MASCOT;UNIPROT KB/SWISS-PROT;COGS
37	Protein-tyrosine sulfotransferase 2	-0.36	N/A	N/A	-0.33	O60704	MASCOT;UNIPROT KB/SWISS-PROT;COGS
38	Apolipoprotein A-II precursor	-0.23	-0.23	-0.13	-0.37	P02652	MASCOT;UNIPROT KB/SWISS-PROT;COGS
39	ADP-ribosylation factor-like protein 6	-0.58	-0.52	-1.00	-0.29	Q9H0F7	MASCOT;UNIPROT KB/SWISS-PROT;COGS
40	Alpha-enolase	-0.27	-0.51	N/A	-0.56	P06733	MASCOT;UNIPROT KB/SWISS-PROT;COGS
41	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	-1.00	-1.00	-0.44	-0.71	P62140	MASCOT;UNIPROT KB/SWISS-PROT;COGS
42	N-acylneuraminate cytidylyltransferase	-1.00	-1.00	-1.00	-1.00	Q8NFW8	MASCOT;UNIPROT KB/SWISS-PROT;COGS
43	Inosine triphosphate pyrophosphatase	-1.00	-1.00	-1.00	-1.00	Q9BY32	MASCOT;UNIPROT KB/SWISS-PROT;COGS
44	Alkyldihydroxyacetonephosphate synthase	-1.00	-0.55	-1.00	-1.00	O00116	MASCOT;UNIPROT KB/SWISS-PROT;COGS
45	Microsomal signal peptidase 18 kDa subunit	-1.00	-0.57	-1.00	-1.00	P67812	MASCOT;UNIPROT KB/SWISS-PROT;COGS
46	Tumor suppressor candidate 2	-0.89	-0.75	-0.67	-0.21	O75896	MASCOT;UNIPROT KB/SWISS-PROT;COGS
47	Metallothionein-2	-0.71	-0.17	-0.14	-0.53	P02795	MASCOT;UNIPROT KB/SWISS-PROT;COGS
48	Protein FAM3B precursor	-0.57	-0.48	-0.39	-0.48	P58499	MASCOT;UNIPROT KB/SWISS-PROT;COGS
49	Metallothionein-1H	-0.52	-0.48	N/A	-0.29	P80294	MASCOT;UNIPROT KB/SWISS-PROT;COGS
50	Metallothionein-2	-0.34	-0.59	-0.49	-1.00	P02795	MASCOT;UNIPROT KB/SWISS-PROT;COGS
51	Protein BEX5	-0.48	-0.33	-0.30	-0.48	Q5H9J7	MASCOT;UNIPROT KB/SWISS-PROT;COGS
52	DNA-directed RNA polymerase II 16 kDa polypeptide	-1.00	-1.00	-1.00	-1.00	Q9VEA5	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table II, continued’

53	Cytochrome c oxidase polypeptide VIa-liver	-0.93	-0.24	-1.00	-0.14	P12074	MASCOT;UNIPROT KB/SWISS-PROT;COGS
54	Putative protein 15E1.2	N/A	-0.94	-1.00	-0.30	-	MASCOT;COGS
55	Voltage-dependent anion-selective channel protein 1	-0.15	-0.28	-0.35	-0.27	P21796	MASCOT;UNIPROT KB/SWISS-PROT;COGS
56	Voltage-dependent anion-selective channel protein 1	-0.47	-0.13	-1.32	N/A	P21796	MASCOT;UNIPROT KB/SWISS-PROT;COGS
57	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	-0.64	-0.76	-0.43	-0.32	P62136	MASCOT;UNIPROT KB/SWISS-PROT;COGS
58	Acetyl-CoA acetyltransferase, cytosolic	-0.95	-1.00	-0.17	-1.00	Q9BWD1	MASCOT;UNIPROT KB/SWISS-PROT;COGS
59	Glutathione synthetase	-0.50	-0.45	-0.38	-0.61	P48637	MASCOT;UNIPROT KB/SWISS-PROT;COGS
60	Actin-related protein 10	-0.94	-0.70	-0.13	-0.73	Q9NZ32	MASCOT;UNIPROT KB/SWISS-PROT;COGS
61	Enteropeptidase precursor	-0.63	-0.19	-1.00	-0.70	P98073	MASCOT;UNIPROT KB/SWISS-PROT;COGS
62	Metallothionein-1L	-0.86	-0.79	-0.96	-0.44	Q93083	MASCOT;UNIPROT KB/SWISS-PROT;COGS
63	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-5-like subunit	-1.00	-1.00	-1.00	-1.00	P63218	MASCOT;UNIPROT KB/SWISS-PROT;COGS
64	Small inducible cytokine B14 precursor	-1.00	-1.00	-1.00	-1.00	O95715	MASCOT;UNIPROT KB/SWISS-PROT;COGS

Table III: Identified proteins of MCF-7 downregulated (-) in the presence of aqueous *Phyllanthus* extracts

APN – aqueous *P. niruri*; APU – aqueous *P. urinaria*; APW – aqueous *P. watsonii*, APA – aqueous *P. amarus*; N/A – Not Affected.

Spot ID	Possible Proteins	Fold change				UNIPROT KB/SWISS-PROT Acc. Number	Database
		APN	APU	APW	APA		
1	Pyruvate kinase isozymes M1/M2	-0.96	-0.42	-0.70	-0.67	P14618	MASCOT;UNIPROT KB/SWISS-PROT;COGS
2	Annexin A2	-1.00	-1.00	-1.00	-1.00	P07355	MASCOT;UNIPROT KB/SWISS-PROT;COGS
3	Alpha-Enolase	-0.41	-0.64	-0.47	-0.34	P06733	MASCOT;UNIPROT KB/SWISS-PROT;COGS
4	Tubulin beta-5 chain	-0.11	-0.84	-1.00	-0.34	P07437	MASCOT;UNIPROT KB/SWISS-PROT;COGS
5	Tryptophanyl-tRNA synthetase, cytoplasmic	-0.18	-0.54	-0.72	-0.70	P23381	MASCOT;UNIPROT KB/SWISS-PROT;COGS
6	ATP synthase subunit alpha, mitochondrial precursor	-0.32	-0.47	-0.10	-0.53	P25705	MASCOT;UNIPROT KB/SWISS-PROT;COGS
7	Proteasome subunit alpha type 6	-0.44	-0.62	-0.35	-0.81	P60900	MASCOT;UNIPROT KB/SWISS-PROT;COGS
8	Calmodulin	-0.37	N/A	-0.47	-1.00	P62158	MASCOT;UNIPROT KB/SWISS-PROT;COGS
9	Nucleolin	-1.00	-0.31	-0.25	-1.00	P19338	MASCOT;UNIPROT KB/SWISS-PROT;COGS
10	Actin, cytoplasmic 2	-1.00	-0.55	-0.88	-1.00	P63261	MASCOT;UNIPROT KB/SWISS-PROT;COGS
11	Tubulin beta-5 chain	-0.32	-0.49	-0.33	-0.21	P07437	MASCOT;UNIPROT KB/SWISS-PROT;COGS
12	Actin, alpha cardiac muscle 1	-0.62	-0.12	N/A	-0.28	P68032	MASCOT;UNIPROT KB/SWISS-PROT;COGS
13	Tubulin alpha-6 chain	-0.83	-0.63	N/A	-0.12	Q9BQE3	MASCOT;UNIPROT KB/SWISS-PROT;COGS
14	Heat shock protein HSP 90-beta	-0.39	-0.55	-0.30	-0.37	P08238	MASCOT;UNIPROT KB/SWISS-PROT;COGS
15	Heterogeneous nuclear ribonucleoprotein A1	-0.40	-0.45	-0.39	N/A	P09651	MASCOT;UNIPROT KB/SWISS-PROT;COGS
16	10 kDa heat shock protein, mitochondrial	-0.57	-1.00	-0.51	-1.00	P61604	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table III, continued’

17	Peroxiredoxin-1	-0.50	-0.25	-0.37	-0.26	Q06830	MASCOT;UNIPROT KB/SWISS-PROT;COGS
18	Heat shock cognate 71 kDa protein	-0.49	-0.57	-0.30	N/A	P11142	MASCOT;UNIPROT KB/SWISS-PROT;COGS
19	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.45	-0.24	-0.19	-0.10	P22626	MASCOT;UNIPROT KB/SWISS-PROT;COGS
20	Cofilin-1	-0.59	N/A	-0.15	-0.21	P23528	MASCOT;UNIPROT KB/SWISS-PROT;COGS
21	Annexin A2	-0.14	-1.00	-1.00	N/A	P07355	MASCOT;UNIPROT KB/SWISS-PROT;COGS
22	Pyruvate kinase isozymes M1/M2	-0.34	-0.21	-0.32	-0.25	P14618	MASCOT;UNIPROT KB/SWISS-PROT;COGS
23	Lamin-A/C	-0.37	N/A	-0.47	N/A	Q6UYC3	MASCOT;UNIPROT KB/SWISS-PROT;COGS
24	Glyceraldehyde-3-phosphate dehydrogenase	-0.72	-0.75	-0.20	-0.57	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
25	Transgelin-2	-0.81	-0.33	-0.86	-0.57	P37802	MASCOT;UNIPROT KB/SWISS-PROT;COGS
26	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.28	N/A	-0.30	N/A	P22626	MASCOT;UNIPROT KB/SWISS-PROT;COGS
27	Glyceraldehyde-3-phosphate dehydrogenase	-0.83	-0.38	-0.96	-0.13	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
28	Transgelin-2	-0.59	-1.00	-0.83	-1.00	P37802	MASCOT;UNIPROT KB/SWISS-PROT;COGS
29	Peroxiredoxin-1	-1.00	-1.00	-0.25	-0.85	Q06830	MASCOT;UNIPROT KB/SWISS-PROT;COGS
30	Heterogeneous nuclear ribonucleoprotein A1	N/A	-1.00	-0.15	N/A	P09651	MASCOT;UNIPROT KB/SWISS-PROT;COGS
31	Glyceraldehyde-3-phosphate dehydrogenase	-0.73	-0.22	N/A	-0.83	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
32	Glyceraldehyde-3-phosphate dehydrogenase	N/A	-0.42	-0.12	N/A	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
33	Galectin-3	-0.66	-0.58	-0.46	-0.53	P17931	MASCOT;UNIPROT KB/SWISS-PROT;COGS
34	Glyceraldehyde-3-phosphate dehydrogenase	-0.50	-0.60	-0.39	-0.64	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
35	Triosephosphate isomerase	-0.98	-0.83	-0.74	-0.76	P60174	MASCOT;UNIPROT KB/SWISS-PROT;COGS
36	Cofilin-1	-1.00	-1.00	-1.00	-1.00	P23528	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table III, continued’

37	Peptidyl-prolyl cis-trans isomerase B precursor	-1.00	-1.00	-1.00	-1.00	P23284	MASCOT;UNIPROT KB/SWISS-PROT;COGS
38	Peptidyl-prolyl cis-trans isomerase A	-1.00	-1.00	-1.00	-1.00	P62937	MASCOT;UNIPROT KB/SWISS-PROT;COGS
39	Stathmin	-1.00	-1.00	-1.00	-1.00	P16949	MASCOT;UNIPROT KB/SWISS-PROT;COGS
40	Glyceraldehyde-3-phosphate dehydrogenase	-1.00	-1.00	-1.00	-1.00	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
41	L-lactate dehydrogenase A chain	-1.00	-1.00	-1.00	-1.00	P00338	MASCOT;UNIPROT KB/SWISS-PROT;COGS
42	Beta-Enolase	-1.00	-1.00	-1.00	-1.00	P13929	MASCOT;UNIPROT KB/SWISS-PROT;COGS

Table IV: Identified proteins of MCF-7 downregulated (-) in the presence of methanolic *Phyllanthus* extracts

MPN – methanolic *P. niruri*; MPU – methanolic *P. urinaria*; MPW – methanolic *P. watsonii*, MPA – methanolic *P. amarus*; N/A – Not Affected.

Spot ID	Possible Proteins	Fold change				UNIPROT KB/SWISS-PROT Acc. Number	Database
		MPN	MPU	MPW	MPA		
1	Myosin regulatory light chain 2, nonsarcomeric	-0.90	-0.71	-0.98	-0.91	P19105	MASCOT;UNIPROT KB/SWISS-PROT;COGS
2	Myosin light polypeptide 6	-0.13	-0.37	-0.22	-0.17	P60660	MASCOT;UNIPROT KB/SWISS-PROT;COGS
3	Myosin light polypeptide 6	-1.00	-1.00	-0.55	-0.57	P60660	MASCOT;UNIPROT KB/SWISS-PROT;COGS
4	Myosin light polypeptide 6	-0.76	-0.67	-0.31	-0.23	P60660	MASCOT;UNIPROT KB/SWISS-PROT;COGS
5	Nascent polypeptide-associated complex subunit alpha	-1.00	-1.00	-0.29	-0.15	Q13765	MASCOT;UNIPROT KB/SWISS-PROT;COGS
6	Protein SET	-1.00	-1.00	-0.51	-1.00	Q01105	MASCOT;UNIPROT KB/SWISS-PROT;COGS
7	Calreticulin precursor	-0.42	N/A	-0.35	-0.45	P27797	MASCOT;UNIPROT KB/SWISS-PROT;COGS
8	Calumenin precursor	-0.73	-0.22	-0.46	-0.30	O43852	MASCOT;UNIPROT KB/SWISS-PROT;COGS
9	Tubulin alpha-ubiquitous chain	-0.43	N/A	-0.30	-0.78	P68363	MASCOT;UNIPROT KB/SWISS-PROT;COGS
10	Tubulin alpha-ubiquitous chain	-0.15	N/A	-0.58	-0.24	P68363	MASCOT;UNIPROT KB/SWISS-PROT;COGS
11	Thioredoxin	-0.43	-0.26	-0.39	-0.41	P10599	MASCOT;UNIPROT KB/SWISS-PROT;COGS
12	Actin, alpha cardiac muscle 1 precursor	-0.38	N/A	-0.13	-0.45	P68032	MASCOT;UNIPROT KB/SWISS-PROT;COGS
13	Annexin A2	-0.70	-0.44	-0.33	-0.40	P07355	MASCOT;UNIPROT KB/SWISS-PROT;COGS
14	Calpain small subunit 1	-0.28	-1.00	-0.53	-0.37	P04632	MASCOT;UNIPROT KB/SWISS-PROT;COGS
15	Protein disulfide-isomerase precursor	-0.65	N/A	-0.66	-1.00	P07237	MASCOT;UNIPROT KB/SWISS-PROT;COGS
16	Tubulin beta-5 chain	-0.96	-0.85	-0.95	-0.62	P07437	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table IV, continued’

17	Annexin A2	-0.45	-0.97	-0.51	-0.48	P07355	MASCOT;UNIPROT KB/SWISS-PROT;COGS
18	Phosphoglycerate kinase 1	-0.25	N/A	-0.28	-0.41	P00558	MASCOT;UNIPROT KB/SWISS-PROT;COGS
19	Actin, alpha cardiac muscle 1 precursor	-0.41	-0.70	-0.25	-0.10	P68032	MASCOT;UNIPROT KB/SWISS-PROT;COGS
20	Tubulin beta-5 chain	-0.41	N/A	-0.15	-0.84	P07437	MASCOT;UNIPROT KB/SWISS-PROT;COGS
21	Tubulin beta-5 chain	-0.67	-1.00	-0.44	-0.36	P07437	MASCOT;UNIPROT KB/SWISS-PROT;COGS
22	Actin, alpha cardiac muscle 1 precursor	-0.87	-0.34	-0.74	-0.81	P68032	MASCOT;UNIPROT KB/SWISS-PROT;COGS
23	Stathmin	-0.56	N/A	-0.38	-0.20	P16949	MASCOT;UNIPROT KB/SWISS-PROT;COGS
24	Splicing factor, arginine/serine-rich 3	-0.38	N/A	-0.46	-0.50	B2R6F3	MASCOT;UNIPROT KB/SWISS-PROT;COGS
25	Annexin A2	-0.34	-0.32	-0.39	-0.21	P07355	MASCOT;UNIPROT KB/SWISS-PROT;COGS
26	Heterogeneous nuclear ribonucleoprotein H	N/A	N/A	-0.10	-0.43	P31943	MASCOT;UNIPROT KB/SWISS-PROT;COGS
27	Heat-shock protein beta-1	-0.12	N/A	-0.29	-0.21	P04792	MASCOT;UNIPROT KB/SWISS-PROT;COGS
28	Annexin A8	-0.21	N/A	-0.29	-0.14	P13928	MASCOT;UNIPROT KB/SWISS-PROT;COGS
29	Annexin A2	-0.64	-0.09	-1.09	-0.47	P07355	MASCOT;UNIPROT KB/SWISS-PROT;COGS
30	Heterogeneous nuclear ribonucleoprotein H	-0.35	N/A	-0.26	-0.17	P31943	MASCOT;UNIPROT KB/SWISS-PROT;COGS
31	Alpha-Enolase	-0.70	-0.09	-0.58	-0.68	P06733	MASCOT;UNIPROT KB/SWISS-PROT;COGS
32	Splicing factor, proline- and glutamine-rich	-0.48	N/A	-0.45	-0.39	P23246	MASCOT;UNIPROT KB/SWISS-PROT;COGS
33	Splicing factor, proline- and glutamine-rich	-0.21	-0.52	-0.44	-0.76	P23246	MASCOT;UNIPROT KB/SWISS-PROT;COGS
34	Tubulin alpha-ubiquitous chain	-1.00	-0.65	-0.65	-1.00	P68363	MASCOT;UNIPROT KB/SWISS-PROT;COGS
35	Stress-70 protein, mitochondrial precursor	-0.53	N/A	-0.54	-0.47	P38646	MASCOT;UNIPROT KB/SWISS-PROT;COGS
36	Stress-70 protein, mitochondrial precursor	-0.51	-0.55	-0.20	-0.47	P38646	MASCOT;UNIPROT KB/SWISS-PROT;COGS
37	Ubiquitin-conjugating enzyme E2 N	-0.36	-0.09	-0.37	N/A	P61088	MASCOT;UNIPROT KB/SWISS-PROT;COGS
38	Peroxiredoxin-5, mitochondrial precursor	-0.18	N/A	-0.46	-0.66	P30044	MASCOT;UNIPROT KB/SWISS-PROT;COGS

‘Table IV, continued’

39	Ras-related protein Rab-11B	-0.21	-0.17	N/A	N/A	Q15907	MASCOT;UNIPROT KB/SWISS-PROT;COGS
40	Myosin-9	-1.00	-0.36	-0.11	-1.00	P35579	MASCOT;UNIPROT KB/SWISS-PROT;COGS
41	Protein disulfide-isomerase A3 precursor	-0.21	-0.23	-0.18	-1.00	P30101	MASCOT;UNIPROT KB/SWISS-PROT;COGS
42	Aldose reductase	-1.00	-0.82	-0.77	-0.96	P15121	MASCOT;UNIPROT KB/SWISS-PROT;COGS
43	Pyruvate kinase isozymes M1/M2	-0.50	-0.09	-0.24	N/A	P14618	MASCOT;UNIPROT KB/SWISS-PROT;COGS
44	Tubulin alpha-ubiquitous chain	-0.19	-0.11	-0.13	-0.26	P68363	MASCOT;UNIPROT KB/SWISS-PROT;COGS
45	Protein disulfide-isomerase A6 precursor	N/A	N/A	-0.13	-0.48	Q15084	MASCOT;UNIPROT KB/SWISS-PROT;COGS
46	Pyruvate kinase isozymes M1/M2	-0.22	-0.49	-0.51	N/A	P14618	MASCOT;UNIPROT KB/SWISS-PROT;COGS
47	Phosphoglycerate mutase 1	-0.29	-0.09	-0.21	-0.24	P18669	MASCOT;UNIPROT KB/SWISS-PROT;COGS
48	Heterogeneous nuclear ribonucleoprotein H3	-1.00	N/A	-0.39	-1.00	P31942	MASCOT;UNIPROT KB/SWISS-PROT;COGS
49	Filamin-A	-0.53	-0.61	-0.54	-0.86	P21333	MASCOT;UNIPROT KB/SWISS-PROT;COGS
50	Elongation factor 2	-0.36	N/A	-0.31	-0.21	P13639	MASCOT;UNIPROT KB/SWISS-PROT;COGS
51	Macrophage capping protein	-0.23	-0.22	-0.34	-0.42	P40121	MASCOT;UNIPROT KB/SWISS-PROT;COGS
52	Phosphoglycerate kinase 1	-0.85	-0.50	-0.86	-0.87	P00558	MASCOT;UNIPROT KB/SWISS-PROT;COGS
53	Triosephosphate isomerase	-0.26	N/A	-0.12	-0.34	P60174	MASCOT;UNIPROT KB/SWISS-PROT;COGS
54	Glyceraldehyde-3-phosphate dehydrogenase	-0.48	-0.69	-0.65	-0.78	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
55	Glyceraldehyde-3-phosphate dehydrogenase	-0.87	-0.51	-0.22	-0.47	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
56	Glyceraldehyde-3-phosphate dehydrogenase	-0.55	N/A	-0.67	-0.82	P04406	MASCOT;UNIPROT KB/SWISS-PROT;COGS
57	60 kDa heat shock protein, mitochondrial	-0.09	N/A	-0.14	-0.21	P10809	MASCOT;UNIPROT KB/SWISS-PROT;COGS
58	Heterogeneous nuclear ribonucleoproteins A2/B1	-0.81	-0.83	-0.81	-0.57	P22626	MASCOT;UNIPROT KB/SWISS-PROT;COGS
59	Heterogeneous nuclear ribonucleoproteins A2	-0.59	N/A	-0.24	-0.60	P22626	MASCOT;UNIPROT KB/SWISS-PROT;COGS

APPENDIX 4: ADDITIONAL OUTPUTS OF THE PHD PROJECT

1. PUBLICATIONS

i. Lee SH, Jaganath IB, Wang SM, Sekaran SD (2011) Antimetastatic Effects of *Phyllanthus* on Human Lung (A549) and Breast (MCF-7) Cancer Cell Lines. PLoS ONE 6(6): e20994. doi:10.1371/journal.pone.0020994

ii. Lee SH, Tang YQ, Rathkrishnan A, Wang SM, Ong KC, Manikam R, Payne BJ, Jaganath IB, Sekaran SD (2013) Effects of Cocktail of Four Local Malaysian Medicinal Plants (*Phyllanthus spp.*) against Dengue Virus 2. BMC Complementary and Alternative Medicine 13:192. doi:10.1186/1472-6882-13-192.

iii. Inhibition of Raf-MEK-ERK and Hypoxia pathways by *Phyllanthus* Prevents Metastasis in Human Lung (A549) Cancer Cell Line (Submitted to BMC Complementary and Alternative Medicine)

iv. *Phyllanthus* Suppresses ERK1/2 and Hypoxia Pathways to Inhibit Metastasis in Human Breast (MCF-7) Cancer Cell Line (Submitted to Current Cancer Drug Targets)

2. PATENT APPLICATIONS

i. Patent application for anticancer properties of *Phyllanthus* (Under processing), Sekaran SD, Lee SH, Tang YQ

3. ORAL PRESENTATIONS

i. Induction of apoptosis in A549 and MCF-7 cell lines treated with *Phyllantus spp.* extracts, 17th International Student Congress of Medical Sciences, 8th – 11th June 2010, University Medical Center Groningen, The Netherlands

4. POSTER PRESENTATIONS

i. Potential anti-proliferative effect of *Phyllantus spp.* on human cancer cell lines, UK-Malaysia Symposium on Cancer Drug Discovery & Development, 24th – 25th February 2010, Grand Dorsett Subang Hotel, Subang, Malaysia

ii. Potential anti-proliferative effect and apoptosis induction on human lung and breast carcinoma cell lines by *Phyllanthus spp*., Innovation and Creativity Expo, University Malaya, 1st – 3rd April 2010

iii. Effects of Malaysia medicinal plant (*Phyllanthus*) against dengue virus type 2, 9th Asia-pacific congress of Medical Virology, 6th – 8th June 2012, Adelaide, Australia

iv. Inhibitory potential of Malaysian medicinal plant (*Phyllanthus*) against dengue virus type 2 (DENV2), National Postgraduate Seminar, 11th July 2012, IPS Building, University of Malaya, Malaysia

5. AWARDS

i. National Science Fellowship (NSF) from July 2009 to June 2012

ii. University Malaya research grant (PPP) for the project entitled "Anti-cancer effect of *Phyllanthus spp.* on human cancer cell line's cellular signalling", Project number: PS180/2010A, PV053/2011B

iii. ISCOMS Travel Grant to attend the 17th International Student Congress of Medical Sciences, 8th – 11th June 2010, University Medical Center Groningen, The Netherlands

iv. Best Oral Presentation for "Genetics and Molecular Mechanisms" Session, 17th International Student Congress of Medical Sciences, 8th – 11th June 2010, University Medical Center Groningen, The Netherlands

6.WORKSHOPS AND TRAINING

i. Flow Cytometry Training – BD FacsCanto II, 30th June – 3rd July 2009, Department of Medical Microbiology, Faculty of Medicine, University of Malaya

ii. BD FACS Calibur Flow Cytometry Basic Training, 21st – 23rd October 2009, Blood Bank, University of Malaya Medical Center (UMMC) and Department of Medical Microbiology, Faculty of Medicine, University of Malaya

iii. Basic Principle and Application of Real Time PCR - CFX96 Real Time PCR System, 10th February 2010, Department of Medical Microbiology, Faculty of Medicine, University of Malaya

iv. 60th Meeting of Nobel Laureates, 27th June – 2nd July 2010, Lindau, Germany

v. Basic Course in Care and Use of Laboratory Animals Research, 17th July 2010, Laboratory Animal Center, Faculty of Medicine, University of Malaya

vi. Proteomics Seminar Series: Current Trends of 2D Proteomics & Beyond, 29th September 2010, The Royale Bintang Damansara Hotel, Malaysia

vii. In-vitro to In-vivo- The Path to Discovery, 2nd December 2010, DKSH, Petaling Jaya, Malaysia

viii. Confocal Laser Scanning Microscope Workshop Electron Microscopy Unit, 5th and 6th September 2012, IPS Building, University of Malaya, 50603 Kuala Lumpur, Malaysia

ix. Animal Experimental Unit (AEU) Induction Course, 18th October 2012, AEU Facility Unit, University of Malaya, 50603 Kuala Lumpur, Malaysia

x. Basic and Clinical Immunoloy Course, 23rd – 27th July 2013, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

7. ATTACHMENTS

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PLoS one

Antimetastatic Effects of *Phyllanthus* on Human Lung (A549) and Breast (MCF-7) Cancer Cell Lines

Sau Har Lee[1], Indu Bala Jaganath[2], Seok Mui Wang[1], Shamala Devi Sekaran[1]*

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Abstract

Background: Current chemotherapeutic drugs kill cancer cells mainly by inducing apoptosis. However, they become ineffective once cancer cell has the ability to metastasize, hence the poor prognosis and high mortality rate. Therefore, the purpose of this study was to evaluate the antimetastatic potential of *Phyllanthus* (*P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*) on lung and breast carcinoma cells.

Methodology/Principal Findings: Cytotoxicity of *Phyllanthus* plant extracts were first screened using the MTS reduction assay. They were shown to inhibit MCF-7 (breast carcinoma) and A549 (lung carcinoma) cells growth with IC_{50} values ranging from 50–180 μg/ml and 65–470 μg/ml for methanolic and aqueous extracts respectively. In comparison, they have lower toxicity on normal cells with the cell viability percentage remaining above 50% when treated up to 1000 μg/ml for both extracts. After determining the non-toxic effective dose, several antimetastasis assays were carried out and *Phyllanthus* extracts were shown to effectively reduce invasion, migration, and adhesion of both MCF-7 and A549 cells in a dose-dependent manner, at concentrations ranging from 20–200 μg/ml for methanolic extracts and 50–500 μg/ml for aqueous extracts. This was followed by an evaluation of the possible modes of cell death that occurred along with the antimetastatic activity. *Phyllanthus* was shown to be capable of inducing apoptosis in conjunction with its antimetastastic action, with more than three fold increase of caspases-3 and -7, the presence of DNA-fragmentation and TUNEL-positive cells. The ability of *Phyllanthus* to exert antimetastatic activities is mostly associated to the presence of polyphenol compounds in its extracts.

Conclusions/Significance: The presence of polyphenol compounds in the *Phyllanthus* plant is critically important in the inhibition of the invasion, migration, and adhesion of cancer cells, along with the involvement of apoptosis induction. Hence, *Phyllanthus* could be a valuable candidate in the treatment of metastatic cancers.

Citation: Lee SH, Jaganath IB, Wang SM, Sekaran SD (2011) Antimetastatic Effects of *Phyllanthus* on Human Lung (A549) and Breast (MCF-7) Cancer Cell Lines. PLoS ONE 6(6): e20994. doi:10.1371/journal.pone.0020994

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Introduction

Tumors can be divided into two types; benign and malignant. Benign tumor is mainly localized and lacks the ability to spread to other parts of the body. Hence, they are rendered to be less harmful. On the other hand, malignant tumor which is more commonly known as cancer, had overcome the strict growth factors and adhesive requirements for their motility or metastatic ability [1]. Metastasis involves a series of complex processes governed by complicated mechanisms, beginning with the detachment of tumor cells, invasion, motility, adhesion to endothelial cells, and reestablishment of growth at a distant site [2]. Cells which are detached from the extracellular matrix often undergo apoptosis. Any resistance of these cells towards apoptosis will allow a successful metastatic dissemination. Cancer cells usually contain several mutations in the genes that regulate apoptotic process, therefore allowing them to evade programmed cell death. This superior resistance to apoptosis provides an advantage for the metastatic cells [3–6].

The metastasizing ability of malignant tumors is accountable for the poor prognosis and high mortality rate in cancer patients. Hence, metastasis is still a major clinical challenge for medical practitioners worldwide in cancer treatment [7]. Currently, there is still no absolute cure for cancer and its many devastating presentations [8]. Most cancers can be controlled by adopting appropriate conventional treatments such as surgery, radiation therapy and chemotherapy. However, these treatments have the potential to cause a range of side effects; hence the importance of conventional therapies may decline [9]. Alternative treatments founded in a 'back-to-nature' approach might yield improved treatment avenues with fewer or no undesirable side effects. In the search for these new treatments, natural products are carving a path as prospective anticancer agents.

The genus *Phyllanthus* is one of the most widely distributed plants throughout the Amazon rainforests as well as other tropical and subtropical regions. Numerous research studies on *Phyllanthus spp.* began in the late 1980's with the clinical efficacy of *Phyllanthus niruri*

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RESEARCH ARTICLE Open Access

Effects of cocktail of four local Malaysian medicinal plants (*Phyllanthus spp.*) against dengue virus 2

Sau Har Lee[1], Yin Quan Tang[1], Anusyah Rathkrishnan[1], Seok Mui Wang[2], Kien Chai Ong[3], Rishya Manikam[4], Bobby Joe Payne[5], Indu Bala Jaganath[6] and Shamala Devi Sekaran[1*]

Abstract

Background: The absence of commercialized vaccines and antiviral agents against dengue has made the disease a major health concern around the world. With the current dengue virus transmission rate and incidences, the development of antiviral drugs is of vital need. The aim of this project was to evaluate the possibility of developing a local medicinal plant, *Phyllanthus* as an anti-dengue agent.

Methods: Cocktail (aqueous and methanolic) extracts were prepared from four species of *Phyllanthus* (*P.amarus, P.niruri, P.urinaria,* and *P.watsonii)* and their polyphenolic compounds were identified via HPLC and LC-MS/MS analysis. MTS assay was then carried out to determine the maximal non-toxic dose (MNTD) of the extracts, followed by screening of the *in vitro* antiviral activity of aqueous cocktail extracts against DENV2 by means of time-of -addition (pre-, simultaneous and post-) using RT-qPCR. The differentially expressed proteins in the treated and infected cells were analysed with two dimensional gel electrophoresis experiments.

Results: Several active compounds including gallic acid, geraniin, syringin, and corilagen have been identified. The MNTD of both aqueous and methanolic extracts on Vero cells were 250.0 μg/ml and 15.63 μg/ml respectively. *Phyllanthus* showed strongest inhibitory activity against DENV2 with more than 90% of virus reduction in simultaneous treatment. Two-dimensional analysis revealed significantly altered levels of thirteen proteins, which were successfully identified by tandem MS (MS/MS). These altered proteins were involved in several biological processes, including viral entry, viral transcription and translation regulations, cytoskeletal assembly, and cellular metabolisms.

Conclusions: *Phyllanthus* could be potentially developed as an anti-DENV agent.

Keywords: *Phyllanthus*, Dengue, Antiviral

Background

Dengue virus (DENV), of the family *Flaviviridae*, is the causative agent for the high morbidity rated disease-dengue. Being a tropical/sub-tropical disease, dengue is currently endemic in more than 100 countries around the world. It is estimated that 36 million dengue fever cases and another 2.1 million cases of severe dengue occur yearly [1]. These numbers are predicted to increase over the years, mainly due to global warming and the increased frequencies of migration, local and international travelling [2] as this arthropod-borne virus is mainly transmitted by mosquitoes, *Aedes aegypti* and *Aedes albopictus.*

DENV is a single-stranded positive sense RNA virus with a genome of approximately 11 kb [3] which encodes for 3 structural proteins (capsid (C), envelope (E) and membrane (M)) and 7 non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Despite extensive research on dengue, many ambiguities lie in the functions on the various DENV proteins. Nevertheless, the NS3 protein has been shown to be multi-functional with protease activity at its N-terminal

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