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***In vitro pro-apoptotic and anti-migratory effects of
Marantodes pumilum (Blume) Kuntze and Ficus deltoidea L.
extracts on prostate cancer cell lines.***

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Declaration Statement

I Mohd Mukrish Mohd Hanafi confirm that the work presented in this thesis is my own. This work has been conducted at the School of Pharmacy, University College London between June 2013 and October 2016 under the supervision of Dr. Jose Prieto-Garcia and Prof. Simon Gibbons. I certify that research described is original and have written all the text and that all source materials, which have already appeared in publication, have been clearly acknowledged by suitable citations.

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To Ayah, Mak, & Ina

Abstract

This thesis evaluates the *in vitro* pro-apoptotic and anti-migratory effects of *Marantodes pumilum* Blume Kuntze and *Ficus deltoidea* L. plants on prostate cancer cells, characterising both their mechanism of actions on some of the main Hallmarks of Cancer, and their chemistry with a view to contribute to future chemopreventive strategies. Plant materials of *M. pumilum* (MP) *F. deltoidea* var. *angustifolia* (FD1) and *F. deltoidea* var. *deltoidea* (FD2) were obtained from dedicated farms in Southern Malaysia. The crude methanolic extract was partitioned into *n*-hexane (MPh, FD1h, FD2h) chloroform (MPc, FD1c, FD2c) and aqueous extracts (MPa, FD1a, FD2a). Active fractions (GI50<30 μ g/mL) based on prostate cancer cell line, PC3, Sulforhodamine B staining were further fractionated. Active compound/s were identified using spectroscopic methods. *In vitro* mechanistic studies on PC3 cells were conducted to investigate the mode of death of PC3 cells and effects of the active extracts on PC3 cells migration and invasion. MPc, FD1c and FD2c extracts induced cell death via apoptosis as evidenced by nuclear DNA fragmentation, accompanied by a significant increase in MMP depolarization ($P<0.05$), activation of caspases 3 and 7 (MPc $P<0.01$; FD1c and FD2c $P<0.05$) in both PC3 and LNCaP cell lines. All active plant extracts up-regulated Bax and Smac/DIABLO and down-regulated Bcl-2 ($P<0.05$). Only MPc inhibited the expression of ALOX-5 mRNA gene expression ($P<0.001$). None resulted cytotoxic against normal human fibroblast cells (HDFa) at the tested concentrations. All active plant extracts inhibited both migration and invasion of PC3 cells (MPc; $P<0.01$, FD1c and FD2c; $P<0.05$), achieved by down-regulation of both VEGF and CXCL-12 gene expressions ($P<0.001$). A monounsaturated 5-alkyl resorcinol was isolated as the active compound present in the MPc extract. LC-MS dereplication identified isovitexin in FD1c; and oleanolic acid, moretenol, betulin, lupenone and lupeol in FD2c. In conclusion, evidence gathered in this study suggests a role for interaction of MPc, FD1c and FD2c in three of the Hallmarks of Cancer in PC3 cells: (1) apoptosis by activating of the intrinsic pathway, (2) inhibition of both migration and invasion by modulating the CXCL12-CXCR4 axis, and (3) inhibiting angiogenesis by modulating VEGF-A expression. The compounds identified and dereplicated in this study will be further characterized and used for the standardization of the active extracts in the future.

Impact Statement

In 2011, a “Herbal Development Office” has been formed under the Ministry of Agriculture (MoA) in Malaysia. This office is assigned with the task of outlining the strategic direction, policies and regulation of research and development (R&D) clusters focusing on the discovery, crop production and agronomy, standardization and product development, toxicology/pre-clinical and clinical studies, and processing technology. Preference has been given to a subset of 11 traditional plant species with high economic potential. This list includes the two plants selected for this project namely *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var *pumila*) and *Ficus deltoidea* L. This project is designed to promote and protect the growth of a local herbal industry, as well as producing high value herbal supplements and remedies. We have successfully characterised the mechanism of actions that lead to prostate cancer cells death and inhibition of both prostate cancer cells migration and invasion, which are induced by the active extracts and fractions from these 2 plants species. This is the first time such characterisation has been done; therefore the output of this project would serve as a new knowledge to academia. Since more work needs to be done before the output of this project can be used for human application, the immediate impact of it, is to use the data collected in this project for research grant applications because funding is essential in drug development. In the long run, based on the potential of the standardized extracts of these plants, they could be further developed into herbal remedies or supplements and then produced commercially. This will benefit everyone in the supply chain starting from the farmers to entrepreneurs and the end-product users.

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List of Abbreviations

5-LOX	5-lipoxygenase
ACE	Angiotensin-converting Enzyme
ADP	Adenosine di-phosphate
AICR	American Institute for Cancer Research
AIF	Apoptosis-inducing Factor
AIF	Apoptosis-inducing Factor
AR	Androgen Receptor
ASAP	Atypical small acinar proliferation
ATCC	American Type Culture Collection
BIR	Baculoviral IAP Repeat
BPH	Benign Prostate Hyperplasia
CAD	Caspase-activated DNase
CAM	Complimentary and Alternative Medicine
CDK	Cyclin-dependent Protein Kinase
cDNA	Complementary Deoxyribonucleic acid
COSY	Correlation Spectroscopy
COX	Cyclooxygenase
CRPC	Castration-resistant Prostate Cancer

DAPI	4'-6-Diamidino-2-phenylindole
DEPT	Distortionless enhancement by polarization transfer
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSHEA	Dietary Supplement, Health and Education Act
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELSD	Evaporative Light Scattering Detector
EMEM	Eagle's Minimum Essential Medium
ER	Estrogen Receptor
ESI-MS	Electrospray Ionisation Mass Spectrometry
EU	European Union
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FIM	Foundation for Innovation in Medicine

FSQD	Food Safety and Quality Division
FT-IR	Fourier Transformed Infrared Spectroscopy
gDNA	Genomic Deoxyribonucleic acid
GI50	Growth Inhibitory concentration 50
HDFa	Human Dermal Fibroblast adult
HDL	High-density Lipoprotein
HGPIN	High Grade Intraepithelial Neoplasia
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Bond Correlation
HPLC-DAD	High Performance Liquid Chromatography-Diode Array
	Detector
HRES-MS	High Resolution-Mass Spectrometry
IAP	Inhibitor of Apoptosis Proteins
IARC	International Agency for Research on Cancer
IBD	Institute of Bioproduct Development
IGF-1	Insulin-like Growth Factor 1
IGF-2	Insulin-like Growth Factor 2
IGFBP	Insulin-like Growth Factor Binding Protein
IND	Investigational New Drug

lncRNA	Long non-coding RNA
LDL	Low-density Lipoprotein
LHRH	Luteinizing Hormone-releasing Hormone
<i>m/z</i>	mass-to-charge-ratio
miRNA	micro RNA
MMP	Matrix Metalloproteases
MMP	Mitochondrial Membrane Potential
MNTC	Maximum non-toxic Concentration
MoA	Ministry of Agriculture
MOH	Ministry of Health
MPT	Mitochondrial Permeability Transition
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
MTT	(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NCI	National Cancer Institute
NCE	New chemical entity
NDA	New drug application
NEAA	Non-essential Amino Acids

NMR	Nuclear Magnetic Resonance
NOD-SCID	Non-obese Diabetic Severe Combined Immunodeficiency
NOESY	Nuclear Overhauser Effect Spectroscopy
OD	Optical Density
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PDGF-B	Platelet-derived Growth Factor B
PI	Propidium Iodide
PIN	Prostatic Intraepithelial Neoplasia
PPM	Parts per million
PS	Phosphatidylserine
PSA	Prostate Specific Antigen
PTP	Permeability Transition Pore
R&D	Research and Development
RLU	Relative Light Unit
RMA	Robust Multiarray Average
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute

rpm	rotation per minute
RT-qPCR	Quantitative Reverse Trancription Polymerase Chain Reaction
rRNA	ribosomal Ribonucleic Acid
SDF-1	Stromal-derived Factor 1
SFDA	State Food and Drug Administration
SRB	Sulforhodamine B
TCA	Trichloroacetic Acid
TdT	Terminal deoxynucleotidyl Transferase
TGF	Transforming Growth Factor
TGF β	Tumour Growth Factor β
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TNF	Tumour Necrosis Factor
TNM	Tumour, Node and Metastasis
t_R	Retention time
TRAIL	TNF-related Apoptosis Inducing Ligand
tRNA	transfer Ribonucleic acid

TUNEL	Terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling
UHPLC	Ultra High Performance Liquid Chromatography
UPM	Universiti Putra Malaysia
UTM	Universiti Teknologi Malaysia
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organization

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Chapter 1

Introduction

1 Introduction

1.1 Research Background

Cancer is a broad group of various diseases characterized by unregulated cell growth (Ochwang'i, Kimwele et al. 2014). In cancerous cells, growth and division are uncontrollable that could result in the formation of tumours which can become malignant by metastasizing other parts of the body. Current treatment regimes for cancer include chemotherapy, radiotherapy and surgery (Vickers 2004). However, toxic effects on other non-target tissues often limit the effectiveness of these treatments such as chemotherapy. It is usual that cancer patients resort to the use of complementary and alternative therapies to palliate these toxic effects or to extend their chances of survival. In the last decade, the population has become more 'health conscious' and an expanding range of complementary and alternative medicines (CAMs) is now globally available leading to a dramatic increase in the use of such approaches in recent years.

Since ancient times, almost all cultures and communities have considered plants as a valuable source of bioactive compounds for treating many diseases, including cancer (Mohan, Bustamam et al. 2011). History shows that the use of herbal drug preparations for medicinal purposes evolved for thousands of years and reached its peak hundreds of years ago. The 19th century brought about the application of modern chemistry to therapy; facilitating the isolation and characterization of plant compounds which in turn have been the building blocks of modern drug discovery and development.

In the last 30 years of the 20th century, the scientific community embarked into a giant quest for anticancer drugs with a focus on plants. This sparked a wider global research applying modern methods in a scale never seen before. As a result, it is believed that up to 70,000 species of plants have been screened for their medicinal use, most of them were selected based on their ethnopharmacological uses (Veeresham 2012). According to Newman and Cragg (2012), about 50% of the approved drugs during the last 30 years (1981-2010) are derived either directly or indirectly from natural products. In the field of cancer treatment, over the time frame from around 1940s to date, 85 from the 175 drugs used are being directly or indirectly derived from natural products. This clearly shows the importance of natural products in drug discovery and they will continue to do so in many years ahead.

Prostate cancer is one of the most common types of cancer worldwide, especially in Western societies. In the last 5 to 10 years, the incidence of prostate cancer is significantly increasing in Asian countries including Malaysia. Initially, this phenomenon was linked to the influx of Western food restaurants and franchises in most of the Asian continent that have led to the change of the normal dietary pattern of Asians. However, numerous studies made clear that even though diet could be one of the risk factors for prostate cancer, it only has a weak association with the disease. Direct risk factors such as age, ethnicity, family history and genetic conditions are reported to play major role in the onset of prostate cancer.

Traditional herbal medicines are still very important for Malaysians both from a nutritional and a medicinal point of view. Malay traditional medicine is largely driven to gender-related groups of preparations: women's health (*Jamu* and/or *Meroyan* remedies) and men's health (tonics or *makjun*) which work by "cleansing the blood" and improving virility (Zakaria and Mohd 2010). In the

same way that daily administration of phytoestrogens has been put forward as a potential chemopreventive approach for prostate cancer, (Morrissey and G Watson 2003) plants traditionally used for women's health may be a source of such compounds for prostate cancer therapy.

The use of plant-derived products in prevention and/or treatment of cancer may take two different pathways, in the one hand the identification and characterization of active extracts for use as food supplements (nutraceuticals) with the purpose of 'contributing to a healthy bodily function' which is in fact a hidden chemopreventive claim and in the other hand the isolation and identification of active molecules as lead compounds to develop drugs for future chemotherapy. Each option has extremely different regulatory frameworks, which lead to different research and development processes as explained in more details in section 2.3. The sponsor of this project retains that the first option is more realistic in the context of current market trends, nevertheless they did not want to compromise on the quality; therefore they decided to gather preclinical data even if this is not strictly necessary for this class of final product.

With this in mind, the main aims of this research are to investigate both the mechanisms of cytotoxic effect and the chemistry of the active plant extracts from two Malaysian plant species, predominantly used by the women namely *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var *pumila*) and *Ficus deltoidea* L. This study will inform the development of herbal preparations for prostate health standardized in the identified/dereplicated phytochemicals by Universiti Teknologi of Malaysia.

1.2 Problem Statement

Prostate cancer is the fourth most common type of cancer worldwide, and the second most common cancer in men. Prostate cancer is the fifth leading cause of death from cancer in men. The metastatic type of prostate cancer, which is often lethal, has no effective therapeutic management to date. Therefore, the finding of any new lead compounds from other sources including plants is important to improve the current management available for this condition.

1.3 Hypothesis

The active extracts of *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var *pumila*) and *Ficus deltoidea* L. plants induce prostate cancer cell death and inhibit both prostate cancer cell migration and invasion.

1.4 Objectives of study

This project aims to investigate the effect of *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var *pumila*) and *Ficus deltoidea* L. plant extracts on prostate cancer cells, characterize the phytochemistry and anti-cancer mechanism of actions based on the main Hallmarks of Cancer with a view to develop chemopreventive therapies. This aim will be achieved by following an array of different strategies including:

- Characterizing the prostate cancer cells mode of death by using *In vitro* mechanistic studies
- Characterizing the molecular pathways involved in the inhibition of both prostate cancer cells migration and invasion induced by the cytotoxic fractions or principles of the plants
- Bioguided isolation and identification of bioactive compounds from the plants with a view to future standardisation of the extracts

Chapter 2

Literature Review

2 Literature Review

2.1 Prostate Cancer

Prostate cancer is also known as the carcinoma of the prostate. It occurs when cancerous cells develop in the prostate which is a gland in the male reproductive system.

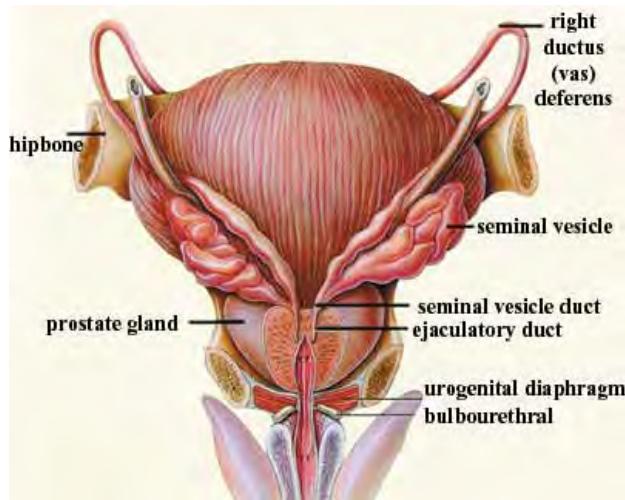


Figure 2.1 Posterior view of the Prostate gland. Adapted from Ballacchino (2015)

Prostate cancer is classified as adenocarcinoma or glandular cancer. It begins when some normal cells of the semen-secreting prostate glands mutate into cancerous cells. Prostate cancers are usually slow growing tumour cells, however in rare cases some prostate cancer cells can grow relatively fast. Adenocarcinoma cells are commonly found in the peripheral zone of the prostate gland where they initially form small clumps of cancer cells that remain confined to otherwise normal prostate glands. This condition is known as

carcinoma in situ or prostatic intraepithelial neoplasia (PIN). As mentioned earlier, most prostate carcinomas are slow growing tumours, therefore it will take time for these cancer cells to begin multiplying and spreading to the surrounding prostate tissue (the stroma) in order to form a full blown tumour. Eventually, the tumour has the ability to grow large enough to invade nearby organs such as the seminal vesicles or the rectum. Apart from that, given suitable conditions, the tumour cells could also develop the ability to travel in the bloodstream and lymphatic system. Therefore, because of these abilities, prostate cancer is considered as a malignant tumour, which is able to metastasize to other parts of the human body. The most common parts where prostate cancer metastasizes include the bones, lymph nodes, and local organs such as bladder and lower ureters (Roodman 2012). The tumour cells can spread to the bones using a specialized route known as the prostatic venous plexus, which connect directly to the vertebral veins (Casimiro, Guise et al. 2009).

2.1.1 Incidence

Prostate cancer is the fourth most common type of cancer worldwide, and the second most common cancer in men. In 2012, an estimated 1.1 million men worldwide were diagnosed with prostate cancer, which account for almost 15% of the cancers diagnosed in men. 70% of these cases occurred in more developed nations and this could be due to the practice of prostate specific antigen (PSA) testing and subsequent biopsy that has become widespread in this part of the world. 307,000 estimated deaths were reported in 2012 which make prostate cancer as the fifth leading cause of death from cancer in men (6.6% of the total men deaths) (Ferlay, Soerjomataram et al. 2013). In the United Kingdom, prostate cancer contributed to the 46,690 (13%) new cases and 11,287 deaths annually. 54% of prostate cancer cases in the UK are diagnosed in males aged 70 and over every year (Cancer Research UK 2016). According to the statistics provided by the National Cancer Registry (2011) of

Malaysia, prostate cancer ranked ninth overall and is the fourth most frequent cancer (7.3% of all cancers) diagnosed in men. Although the incidence of prostate cancer is more prevalent in the western countries, the number of prostate cancer cases has also grown rapidly in Asian countries such as Japan, and Hong Kong (Figure 2.2) (Wynder, Fujita et al. 1991, Liu 1993). These statistical data show that prostate cancer has no geographical boundaries and may be found in different parts of the world.

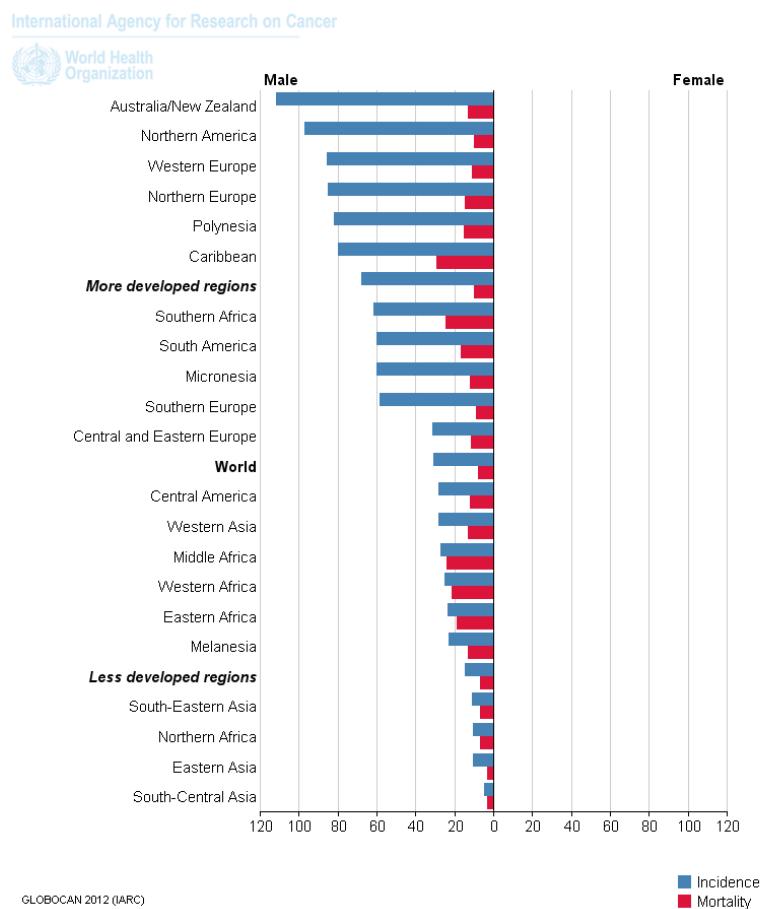


Figure 2.2 Worldwide incidence and mortality rate of prostate cancer. Adapted from Ferlay, Soerjomataram et al. (2013)

2.1.2 Risk Factors

Many studies have been conducted to determine the risk factors that are related to the onset of prostate cancer. However, a complete understanding of the cause of prostate cancer remains elusive. No modifiable and preventable factors have been conclusively linked with prostate cancer risk due to the complexity in interpreting the epidemiological evidence around prostate cancer (Giovannucci, Liu et al. 2007, Cogliano, Baan et al. 2011). The International Agency for Research on Cancer (IARC) (2014) together with the American Institute for Cancer Research (AICR) (2014) have underlined several risk factors for prostate cancer.

One of the risk factors include age and ethnicity which are strongly related to prostate cancer. The highest incidence of prostate cancer cases occur in older men. In the UK between 2009 and 2011, an average of 36% of cases were diagnosed in men aged 70 years and over while only 1% were diagnosed in the under-50s (Northern Ireland Cancer Registry , Welsh Cancer Intelligence and Surveillance Unit) and this number has increased steadily over time as 54% of prostate cancer cases were diagnosed in the UK between the period of 2012 to 2014 in men aged 70 and over (Cancer Research UK 2016). Ethnicity also play an important role in prostate cancer incidence as age-standardised rates for White males with prostate cancer range from 96.0 to 99.9 per 100,000. Rates for Asian males are significantly lower, ranging from 28.7 to 60.6 per 100,000 whereas the rates for Black males are significantly higher, ranging from 120.8 to 247.9 per 100,000 (National Cancer Intelligence and Cancer Research UK). In Malaysia, the overall age standardized incidence is 12 per 100,000 populations, with the highest incidence of prostate cancer reported among Chinese (15.8 per 100,000) followed by Indians (14.8 per 100,000) and the lowest in Malays (7.7 per 100,000).

Apart from that, family history and genetic conditions also play important roles in prostate cancer, for example men whose father has/had the disease has 2.1-2.4 times higher risked in developing prostate cancer (Johns and Houlston 2003, Watkins Bruner, Moore et al. 2003, Kicinski, Vangronsveld et al. 2011) and Men whose brother has/had prostate cancer in the family has 2.9-3.3 times higher risk of developing the disease (Johns and Houlston 2003, Watkins Bruner, Moore et al. 2003, Kicinski, Vangronsveld et al. 2011). However, prostate cancer is not associated with a breast cancer in a sister (Hemminki and Chen 2005, Chen, Page et al. 2008). Prostate cancer risk is not associated with prostate cancer in adoptive parents or adoptive family (Zöller, Li et al.); this has further supported the family history and genetic link hypothesis. Men with BRCA2 mutation has up to 5 times higher risk in developing prostate cancer when compared to the general population and this risk could increase to 7 times higher in men aged under 65 (The Breast Cancer Linkage Consortium, 1999). BRCA1 mutation in men could also increase prostate cancer risk but the evidence remains unclear (Thompson, Easton et al. 2002, Fachal, Gómez-Caamaño et al. 2011). Lynch syndrome which is an autosomal dominant genetic condition could also increase the risk of prostate cancer by 2.1-4.9 times higher when compared to the general population as shown by a meta-analysis and a cohort study (Haraldsdottir, Hampel et al. 2014, Ryan, Jenkins et al. 2014) Apart from that, endogenous hormone such as insulin-like growth factor-1 (IGF-1) has been reported to increase prostate cancer risk by 38-39% higher in men with highest level of IGF-1 (Renéhan, Zwahlen et al. , Roddam, Allen et al. 2008). However, studies have shown that prostate cancer risk is not associated with both insulin-like growth factor-2 (IGF-2) and insulin-like growth factor binding protein (IGFBP) (Roddam, Allen et al. 2008, Rowlands, Gunnell et al. 2009), but the risk may vary between IGFBPs. These are the main risk factors associated with prostate cancer, other risk factors with weaker association include diet, occupational exposures, exposure to ionizing radiation and obesity.

2.1.3 Stages

Cancer staging is very important as it will help to determine the extent to which a cancer has developed by spreading. There are a few different staging system used for prostate cancer. Two of the most commonly used systems are the 'TNM' staging system and the 'number staging' system. TNM is an abbreviation for tumour (T), node (N), and metastasis (M). TNM system shows how far the cancer has spread in and around the prostate, it also shows how far the cancer has spread to the nearby lymph nodes and evaluate whether the cancer has already spread to other parts of the body such as the bones. The 'number staging' for prostate cancer is divided into 4 stages (I,II,III and IV) (Figure 2.3). Primary prostate cancer is under stages I and II. In these two stages the cancer cells are still localised in the prostate. Stage II prostate cancer means that the cancer cells are at an advanced stage compared to stage I but are still localised in the prostate. Patients with stage I and stage II prostate cancer has low or intermediate risk of having a metastasis. Stage III and stage IV prostate cancer cells are characterized by the spreading of the cells to other bodily parts. In stage III prostate cancer, the cells have spread beyond the outer layer of prostate and may have spread to the seminal vesicles whereas in stage IV, the cancer cells have spread beyond the seminal vesicles to nearby tissues or organs such as the rectum, bladder or pelvic wall and also to distant parts of the body. Therefore stage IV represents the worst stage of prostate cancer and patients at this stage only have 28% of 5-year relative survival rate according to the American Cancer Society (2016).

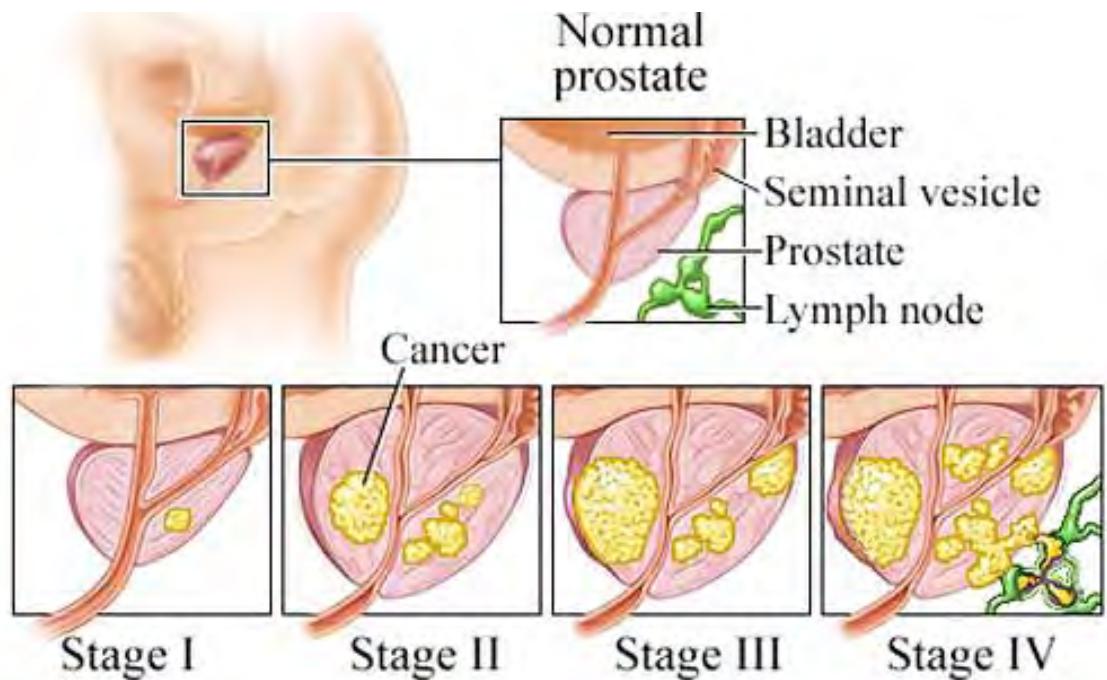


Figure 2.3 Stages of prostate cancer. Adapted from Healthfavo (2014)

2.1.4 Pathophysiology of Prostate Cancer

Many abnormalities at cellular, molecular and genetics levels have been associated with the occurrence of prostate cancer. These include chromosomal abnormalities, the presence of oncogenes and overexpression of certain proteins.

2.1.4.1 Chromosomal abnormalities and oncogenes

Chromosomal abnormalities associated with prostate cancer includes the deletion of 8p, 10q, 13q, and 16q as well as the gains of 7p, 7q, 8q and Xq chromosomes. These allelic abnormalities has been reported by Nupponen and Visakorpi (1999), Hughes, Murphy et al. (2005) and Shi, Sun et al. (2013). Apart from that other allelic loss has been reported by Rubin and Rubin (1998), Shen

and Abate-Shen (2010) and Fraser, Sabelnykova et al. (2017) which include the loss of 6q,7q,17p and 18q.

The MYC is a well-known oncogene which plays an important role in the regulation of cellular proliferation, differentiation and apoptosis. This oncogene is located at 8q24 and also at other amplified region of 8q (Visakorpi, Kallioniemi et al. 1995, Cher, Bova et al. 1996, Nupponen, Kakkola et al. 1998, Taylor, Schultz et al. 2010). The overexpression and amplification of this oncogene have been detected in prostate cancer cells especially in the metastatic stage (Bubendorf, Kononen et al. 1999, Taylor, Schultz et al. 2010, Rickman, Beltran et al. 2017). Besides MYC, the RAS family oncogenes are the most common oncogenes in human cancer. However, in prostate tumours, mutations in the ras genes (HRAS, KRAS and NRAS) are relatively uncommon (Min, Zaslavsky et al. 2010, Zeng, Hu et al. 2014), except in the rare ductal form of the disease. Morote, de Torres et al. (1999), has reported that overexpression of ERBB2 gene is a frequent event in prostate cancer. The ERBB2 gene, commonly referred to as Her-2/neu belongs to a family of genes that provide instructions for producing growth factors receptors. These growth factors are important in stimulating cell growth and division. ERBB2 gene amplification will result in the overproduction of ErbB2 protein and this can cause cells to grow and divide continuously leading to uncontrolled cell division, which is one of the hallmarks of cancerous tumour progression (Pignon, Koopmansch et al. 2009, Yan, Parker et al. 2014). Another oncogene that plays a very important role in the progression of prostate cancer is Bcl-2. Several authors have reported the overexpression of Bcl-2 especially in recurrent tumours (McDonnell, Troncoso et al. 1992, Colombel, Symmans et al. 1993, Krajewska, Krajewski et al. 1996, Pienta and Bradley 2006, Delbridge, Grabow et al. 2016), however, this event did not seem to happen due to the amplification of the genes (Nupponen and Visakorpi 1999, Pienta and Bradley 2006). Bcl-2 inhibits apoptosis of the prostate cancer cells

subjected to androgen deprivation therefore allowing the cancerous cells to survive even without the presence of required hormone.

2.1.4.2 Androgen receptor

Androgen receptor (AR) is a type of nuclear receptor that is encoded by a single copy gene located on the X-chromosome (Xq11.2-q12), which consists of 919 amino acids in length, but this can vary depending on the poly-glutamine, poly-glycine, and poly-proline repeats of variable lengths (Velcheti, Karnik et al. 2008). AR signalling is very important as it plays critical role not only for prostate function and differentiation but also for the growth and progression of prostate cancer (Velcheti, Karnik et al. 2008, Yuan, Cai et al. 2014). AR activity is regulated by two major ligands, testosterone and dihydrotestosterone (DHT). DHT, which has 10 times higher binding affinity to AR is the primary androgen bound to AR. The binding of DHT to AR promotes the recruitment of proteins kinases, which lead to the phosphorylation of several serine residues. This process is very important as it serves many functions such as protection from proteolytic degradation, stabilization, and transcriptional activation (Edwards and Bartlett 2005). The transactivation of AR is vital as it regulates specific gene targets that are involved in cells growth and survival (Chmelar, Buchanan et al. 2007). The rate of cells proliferation and the rate of cells apoptosis are balanced in normal prostate epithelium, however, the balance is lost in prostate cancer that leads to the formation of tumour cells (Denmeade, Lin et al. 1996). Since prostate cancer growth is highly dependent on androgen, androgen-ablation therapy has always been the most effective treatment for prostate cancer at an early stage. However, this therapy only manages to delay tumour progression by 18-24 months followed by the development of a lethal drug-resistant stage known as castration-resistant prostate cancer (CRPC). Visakorpi, Kallioniemi et al. (1995), has reported that in CRPC patients, the frequent amplification of chromosome Xq in recurrent tumours has led to the overexpression of AR after

androgen deprivation therapy. This type of chromosomal amplification is rarely seen in the primary tumours. The overexpression of AR overcomes the decreased levels of circulating androgens in hormone-independent prostate cancer thus allowing the cancer cells to continue growing even in very low level of androgen left in serum after castration (Visakorpi, Kallioniemi et al. 1995, Tan, Li et al. 2015). The overexpression of AR also produce a receptor that is more sensitive to a low androgen levels or that can be activated by other types of steroids such as adrenal androgens, estrogens, progestins, as well as anti-androgens that used in the management of the disease (Scher and Sawyers 2005).

2.1.4.3 Metastasis

Metastasis is defined as the spread of tumour cells from their primary site to distant organs and their ability to grow and survive at the new sites remains the most fearsome aspect of cancer. Metastasis has been the subject of study for more than 100 years. However, only until recently that researchers have gained important insight into the mechanism by which metastatic cells arise from primary tumour and the reasons why certain tumours tend to spread or metastasize to specific organs (Fidler 2003). Back in 1889, an English surgeon proposed the ‘seeds and soil’ hypothesis that says metastasis depends on cross-talk between selected cancer cells (the ‘seeds’) and the specific microenvironments (the ‘soils’) surrounding them (Paget 1889). Although this hypothesis still holds forth today, it is now understood that the ability of tumour cells to metastasize depend on several elements including its interactions with the homeostatic factors that promote tumour-cell growth, angiogenesis, survival, invasion and metastasis (Fidler 2003). Figure 2.4 shows the general process of cancer metastasis, which consists of a long series of sequential and inter-related steps. Each of these steps can be rate-limiting, therefore a failure or

insufficiency at any steps could lead to the collapse of the whole process (Poste 1980, Fidler 2002).

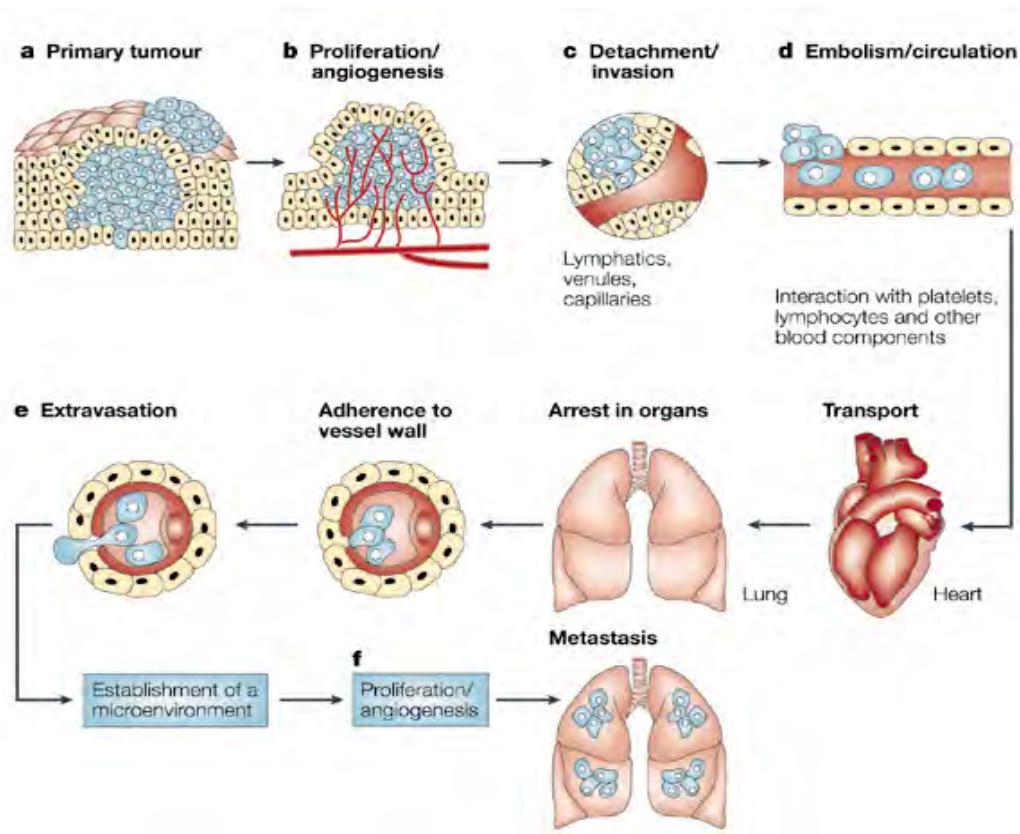


Figure 2.4 The process of cancer metastasis. Adapted from “The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited” by I. J. Fidler, 2003. a. Growth of primary tumours require nutrient that is initially supplied by simple diffusion. b. The growth of tumour mass exceeding 1-2 mm in diameter requires extensive vascularization. c. Lymphatic channels, which are characterized by their thin-walled venules, act as the most common route for tumour-cell entry into the circulation. d. Tumour cells that survived the circulation become trapped in the capillary bed of distant organs by adhering either to exposed capillary endothelial cells or subendothelial basement membrane. e. Extravasation occurs. f. Proliferation of the tumour cells at distant organ accompanied by micrometastasis. *Nature Reviews Cancer* 3, 453-458. Copyright 2003 by Nature Publishing Group. Adapted with permission.

Prostate cancer is one of the most malignant types of cancer in men. It has the ability to spread to other distant sites including bones, lymph nodes, lungs, liver and brain. However, prostate cancer frequently metastasizes to the bone marrow.(Roudier, Morrissey et al. 2008) This is evident as almost 90% of advanced prostate cancer patient suffer from pathologic fractures, spinal cord compression, and pain due in part of deregulated cycles of osteoblastic and osteolytic resorption/formation driven by the growing tumour mass (Rubens 1998, Fizazi, Carducci et al. 2011). Although the mechanisms that account for the tendency of prostate cancer cells to metastasize to bone have not yet been elucidated but it may include direct vascular pathway, highly permeable sinusoids, chemotactic factors produced by bone marrow stromal cells and the synthesis of growth factors important to support cells survival, and proliferation of 'seeded' cancer cells (Diel 1994, Geldof 1996). Taichman, Cooper et al. (2002) hypothesize that metastatic prostate carcinomas may use the hematopoietic model to localize to the bone barrow. In this model, chemokines, which are a group of molecules known to play significant roles as activators and chemoattractants, including CXC chemokine such as CXCL12 and its receptor CXCR4 appear to be critical molecular determinants for the events in this model (Aiuti, Tavian et al. 1999, Kim and Broxmeyer 1999). In their study, Taichman, Cooper et al. (2002) showed that the CXCL12/CXCR4 chemokine axis was activated in prostate cancer metastasis to the bone. They also confirmed that CXCR4 expression is related to increasing tumour grade, as well as showing that CXCL12 signalling through CXCR4 triggers the adhesion of prostate cancer cells to bone marrow endothelial cells (Sun, Wang et al. 2003). Similar studies conducted by other researchers also suggest that CXCL12/CXCR4 axis play parallel roles in other tumours that also metastasize to the marrow. Müller, Homey et al. (2001) reported that CXCL12/CXCR4 axis play central roles in regulating metastasis by showing that normal breast tissues express little CXCR4 receptors compared to breast neoplasms, which express high levels of CXCR4. Furthermore, this study also shows that the use of antibody that could

block CXCR4 receptor was able to prevent the spread of tumours cells to lungs and lymph nodes.

Apart from that, the metastatic progression of prostate cancer is also closely associated with two genes namely E-cadherin (CDH1) and KAI1 genes. The expressions of both genes are significantly reduced in metastatic prostate cancer cells (Umbas, Schalken et al. 1992, Morton, Ewing et al. 1993). However, this was not caused by allelic loss but rather by post-transcriptional events regulated by p53. Therefore loss of p53 function in the late stages of tumour progression could cause down-regulation of these two genes with subsequent metastasis (Mashimo, Watabe et al. 1998).

2.1.4.4 Arachidonic acid metabolism

Besides the abnormalities in chromosomes, as well as the presence of oncogenes and the overexpression of specific receptors, malignant cancer cells commonly overexpress key enzymes of the arachidonic acid metabolism (mainly COX-2 and 5-LOX). The COX-2 is overexpressed in practically every premalignant and malignant condition involving the colon, liver, pancreas, breast, lung, bladder, skin, stomach, head, neck and oesophagus (Eberhart, Coffey et al. 1994, Hida, Yatabe et al. 1998, Hwang, Byrne et al. 1998, Koga, Sakisaka et al. 1999, Mohammed, Knapp et al. 1999, Tucker, Dannenberg et al. 1999, Aggarwal and Shishodia 2006, Wolfesberger, Walter et al. 2006). Interestingly, human prostate cancer cells are known to generate 5-lipoxygenase (5-LOX) instead. 5-LOX is a type of enzyme in human encoded by the ALOX-5 gene. Ghosh and Myers (1997) reported that chemical constituents such as arachidonic acid, an omega-6, polyunsaturated fatty acid was found to stimulate prostate cancer cell growth via the 5-LOX pathway. This has been recently corroborated by Yang, Cartwright et al. (2012), who also point towards

12-LOX. The expression of 5-LOX is normally restricted to specified immune cells such as neutrophils, eosinophils basophils and macrophages whereas the vast majority of non-immune body cells do not express 5-LOX unless at the onset of certain diseases such as asthma, arthritis, psoriasis and cancer (Fürstenberger, Krieg et al. 2006, Werz and Steinhilber 2006, Rådmark, Werz et al. 2007, Ghosh 2008). 5-LOX plays a very important role in chemotaxis in these cells (Werz and Steinhilber 2006). Ghosh and Myers (1997), reported that the inhibition of 5-LOX would block the production of 5-LOX metabolites and triggers apoptosis in prostate cancer cells. The expression of 5-LOX in normal prostate glands is almost undetectable, however 5-LOX is heavily expressed in prostate tumour tissues. Therefore, this finding is very important for the development of future therapeutic approaches for prostate cancer as 5-LOX plays a critical role in the survival of prostate cancer cells. This leads to the concept that 5-LOX may play a major role in the development and progression of prostate cancer and could be used as a promising target in prostate cancer therapy. Other abnormalities including the amplification and overexpression of certain genes mentioned earlier could also be used as potential target in future prostate cancer therapy.

2.1.4.5 Angiogenesis

Tumour normally consists of cancer cells and stromal cells. These stromal cells face a very hostile metabolic environment characterized by acidosis and hypoxia. Tumour development requires the supply of oxygen and nutrition, this is usually provided by the nearby blood vessels (Holmgren, O'Reilly et al. 1995). With this, the tumour growth is only limited to 1-2 mm in diameter (avascular phase) (Carmeliet 2003). Therefore in order for the tumour to exceed the size limit, the tumour needs an increased blood supply. This is mainly provided by the formation of new blood vessels from pre-existing capillaries and venules. This process is called angiogenesis (Carmeliet 2003).

The classical angiogenesis process is shown in Figure 2.5. Prostate tumours like other type of tumour cells overexpress vascular endothelial growth factor (VEGF) (Li, Younes et al. 2004). VEGF is a homodimeric glycoprotein that belongs to the first group of pro-angiogenic factors (Ferrara, Gerber et al. 2003). Other important pro-angiogenic factors include fibroblast growth factor (FGF) (Friesel and Maciag 1995), platelet-derived growth factor B (PDGF-B) (Battegay, Rupp et al. 1994), angiopoietins (Battegay, Rupp et al. 1994, Friesel and Maciag 1995), growth-related oncogenes (Wang, Hendricks et al. 2006), tumour growth factor β (TGF β) (Sankar, Mahooti-Brooks et al. 1996), and matrix metalloproteases (MMPs) (Egeblad and Werb 2002).

The overexpression of VEGF in prostate tumours will promote the development of tumour neovascularization and this overexpression also correlate with increasing grade, vascularity and tumorigenicity. Besides that, the receptor for VEGF, VEGFRs and $\alpha_5\beta$ integrin were expressed by prostate cancer cells in-vitro and prostate tumours in-vivo and their expression was elevated at sites of bones metastasis compared to original prostate tumour. He, Kozaki et al. (2002) reported that the angiogenic effect of VEGF in prostate tumour could be blocked by the inactivation of its receptor, VEGFR. Without VEGF or VEGFR, the prostate tumour cells would not be able to form sprouting capillaries. Therefore, this could be a potential target to stop the development and progression of prostate cancer.

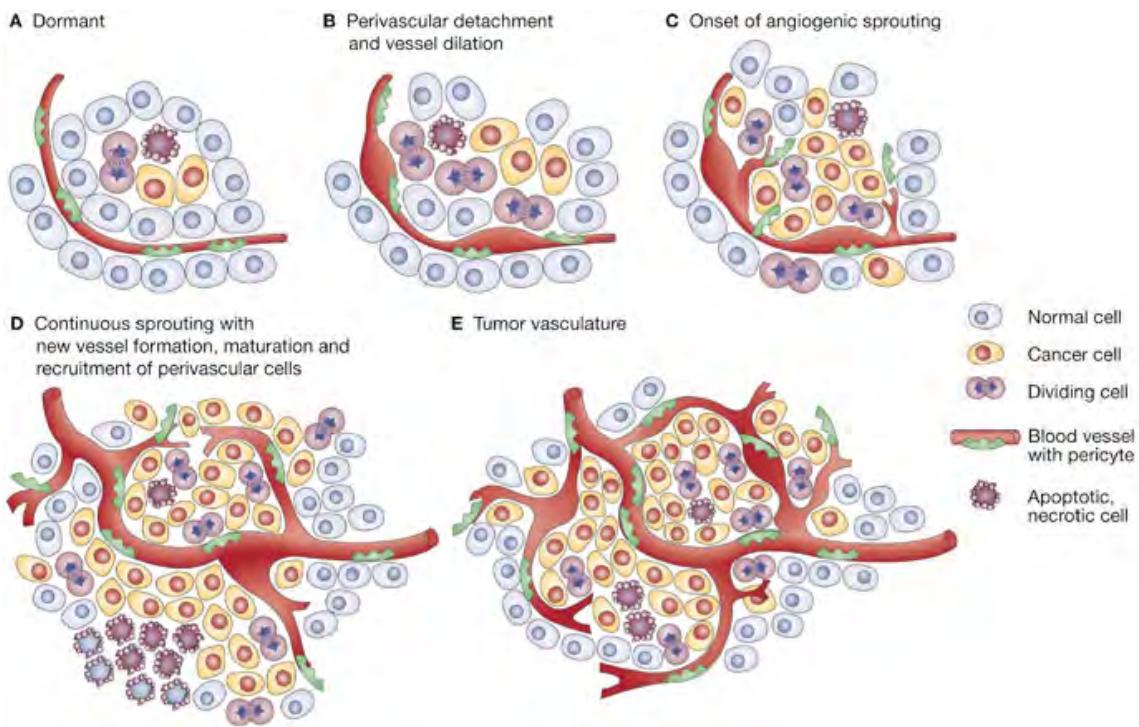


Figure 2.5 The classical angiogenic process. Adapted from “Circulating endothelial cells as biomarkers of prostate cancer” by H. D. Georgiou, B. Namdarian, N. M. Corcoran, A. J. Costello, and C. M. Hovens, 2008, *Nature Clinical Practice Urology* (2008) 5, 445-454. Copyright 2008 by Nature Publishing Group. Adapted with permission.

2.1.5 Treatment

2.1.5.1 Chemoprevention of Prostate cancer

The idea of chemoprevention was initially introduced in the mid 1970s (Sporn and Newton 1979, Brawley 2002). It is defined as the administration of agents or substances such as drugs or vitamins to try and reduce the risk or delay the development or recurrence of cancer. Carcinogenesis in cancer development is a complex process that normally requires a long period of time; therefore chemoprevention could play a major role in inhibition or slowing of this process before cancer growth becomes clinically significant (Kelloff, Lieberman et al. 1999). Chemoprevention is a type of preventive medicine and can be divided into primary, secondary and tertiary prevention. Primary prevention deals with the incidence of disease in an otherwise healthy individual. Secondary prevention aims to stop the progression of a disease to a clinically significant stage by focusing on the treatment given to an individual already with a premalignant condition. Tertiary prevention focuses on the prevention of recurrence or progression of disease in an individual previously treated with malignancy (Sandhu, Nepple et al. 2013). In the case of prostate cancer, chemoprevention is essentially aimed at primary and secondary prevention (Sandhu, Nepple et al. 2013).

There are many reasons that support the use of chemoprevention as part of prostate therapy. As mentioned earlier in this section, carcinogenesis in cancer development occurs over time and this is also the case for prostate cancer. In fact, carcinogenesis in prostate cancer is thought to consist of a protracted multistep molecular processes affecting numerous pathways (Gonzalgo and Isaacs 2003). This molecular pathogenesis could lead to the

development of pre-cancerous characteristics such as atypical small acinar proliferation (ASAP) and high grade intraepithelial neoplasia (HGPIN) (Epstein and Herawi 2006). Both ASAP and HGPIN can be detected many years before the formation of the actual cancer mass in the prostate gland itself. These characteristics make prostate cancer as an ideal target for primary chemoprevention. Apart from that, prostate cancer also has a prolonged latent disease state. This simply means there is a long period between the development of the cancerous cells and their eventual manifestations by symptoms. Therefore increasing the incidence of prostate cancer in elderly men aged between 60-79 years old (Stamatiou, Alevizos et al. 2006). This specific characteristic of prostate cancer makes it suitable as a target for secondary chemoprevention.

In chemoprevention, it is vital to use agents or substances with low toxicity as it involves the treatment of healthy individual (Hong and Lippman 1994). In prostate cancer, chemoprevention can be used to prevent the transformation of normal cells or precursor lesions to cancerous cells (secondary prevention) as well as stopping the growth of the existing tumour cells (Walsh 2010). 5 Alpha-reductase inhibitors are one of the chemopreventives used in prostate cancer therapy. Inhibition of 5- α -reductase will decrease the amount of dihydrotestosterone (DHT) in prostate cancer tissues, thus lowering the androgenic stimulation to the prostate (Brawley 2002). Several studies showed that the inhibition of 5- α -reductase in prostate cancer tumours grafted into animal has significantly impeded tumour implantation and growth (Tsukamoto, Akaza et al. 1998, Roehrborn, Boyle et al. 1999). Finasteride is a type of 5- α -reductase inhibitor, which lowers intraprostatic DHT levels while causing testosterone level to increase slightly (Stoner 1994). It was the first 5- α -reductase inhibitor to enter human trial. Another 5- α -reductase inhibitor that is used for prostate cancer chemoprevention is dutasteride. Unlike finasteride, dutasteride is thought to have better efficacy in chemoprevention as

it can inhibit both isoforms of 5- α -reductase (Bramson, Hermann et al. 1997). Recent study conducted by Andriole, Bostwick et al. (2010) showed that dutasteride reduced the risk of prostate cancers and precursor lesions among men at increased risk of prostate cancer and for benign prostatic hyperplasia (BPH) while improving many outcomes related to BPH. Other agents such as vitamin C, vitamin B1, vitamin B2, vitamin B3, calcium, zinc, protein and selenium have been used in the hope of preventing the growth of prostate cancer, however no clear association exists between these common dietary factors and prostate cancer (Brawley 2002). In this study, characterization of the active extracts could lead to their use in prostate cancer chemoprevention in the future.

2.1.5.2 Management of Prostate cancer

Treatments and prostate cancer management depends on several factors such as the stage of the disease, age, general health, and a person's view about potential treatments and their possible side effects. Due to the aggressiveness and the annoying side effects (erectile and urinary dysfunction) of most treatment options, treatment discussion often focus on balancing the goals of therapy with the risk of lifestyle alterations. A combinations of treatment options is highly recommended for managing the disease (Lu-Yao, Albertsen et al. 2009, Mongiat-Artus, Peyromaure et al. 2009, Picard, Golshayan et al. 2012). The management of prostate cancer can be divided into three categories; 1) management of localised prostate cancer, 2) management of locally advanced prostate cancer, 3) management of metastatic prostate cancer.

Most patients diagnosed with localised prostate cancer will have an excellent prognosis. This is achieved by radical prostatectomy and/or radiation therapy. One-fifth of the patients opt out surgery and will undergo 'watchful waiting'. However, 20%-40% of the patients who went through primary therapy will experience biochemical relapse, with 30%-70% of those developing metastatic disease within 10 years after the introduction of local therapy (Pound and Pound 1999, D'Amico, Cote et al. 2002, Antonarakis, Trock et al. 2009, Uchio and Uchio 2010). Metastatic stage of prostate cancer is normally managed by the use of androgen-deprivation therapy or also known as hormone ablation therapy. A luteinizing hormone-releasing hormone (LHRH) agonist will improve the symptoms but tumours invariably become hormone independent (castration-resistant) and evolve to the progressive stage. Before 1990s, patients with castration-resistant prostate cancer (CRPC) were generally treated with palliative approaches, as no other life-prolonging options were available. As prostate cancer is often associated with elderly men with limited bone marrow reserve and concurrent medical conditions, the use of chemotherapy at this time was not recommended as it can further deteriorate the quality of life (Paller and Antonarakis 2011).

Tannock, Osoba et al. (1996) has reported that mitoxantrone (with prednisone) has the ability to improve quality of life and reduce bone pain besides lowering serum PSA level in men with CRPC. This important discovery has led to the use of intercalating agent mitoxantrone as the initial standard of care for such patients. The cytotoxic Docetaxel has replaced the use of mitoxantrone as the first line standard of care in 2004 after the Southwest Oncology Group reported that docetaxel (with estramustine) significantly extended progression-free and overall survival for CRPC patients when compared to mitoxantrone (with prednisone) (Petrylak, Petrylak et al. 2004). However, up until 2010, physicians have no other life-prolonging second-line options after the failure of docetaxel. On June 17 the same year, the US Food

and Drug Administration (FDA) approved the use of cabazitaxel for CRPC patients previously treated with a docetaxel-containing regime (US Food and Drug Administration) thus changing from stabilisation of microtubule to inhibition of the assembly as the underlying mechanism of therapeutic action. Until recently, other therapies including Enzalutamide (Xtandi®), Abiraterone (Zytiga®) and Radium-233 (Xofigo®) have been used for the management of CRPC patients. Both Enzalutamide and Abiraterone are a new type of hormone therapy for men with prostate cancer that is no longer responding to hormone therapy or chemotherapy. Enzalutamide works by blocking the hormone testosterone from reaching the prostate cancer cells whereas Abiraterone works by stopping the production of testosterone in the body. Without the availability of this hormone, prostate cancer cells will cease to grow wherever they are in the body.

2.2 Possible therapeutic targets for Prostate Cancer based on the major hallmarks of cancer

Figure 2.6 shows the 6 main hallmarks of cancer originally proposed by Hanahan and Weinberg (2000) which include sustaining proliferative signaling, evading growth suppressors, resisting cell deaths, activating invasion and metastasis, inducing angiogenesis and enabling replicative immortality. The same authors later extended this list after considering the new progresses made in the past decade by adding two new hallmarks comprising the reprogramming of energy metabolism and the evasion of immune destruction (Hanahan and Weinberg 2011). These are the biological capabilities acquired by the tumor cells during their multistep development that enable them to become tumorigenic and ultimately malignant. The understanding of these hallmarks is very important in order to find suitable therapeutic targets that can be used more effectively in cancer treatment. Blandin, Renner et al. (2015) has reported that β 1 integrins which is a type of matricellular receptors (extracellular matrix

component), plays important role in the modulation of these cancer hallmarks. Therefore, it can be used as one of the suitable therapeutic targets for cancer therapy. This study aims to find bioactive compound/s from plants that could modulate some of the cancer hallmarks namely resisting cell death or evading apoptosis, activating invasion and metastasis and inducing angiogenesis. These are the three main hallmarks of cancer that would be the focus of this study. The discovery of bioactive compound/s with the ability to modulate these 3 important hallmarks would be vital for the future of prostate cancer therapy.

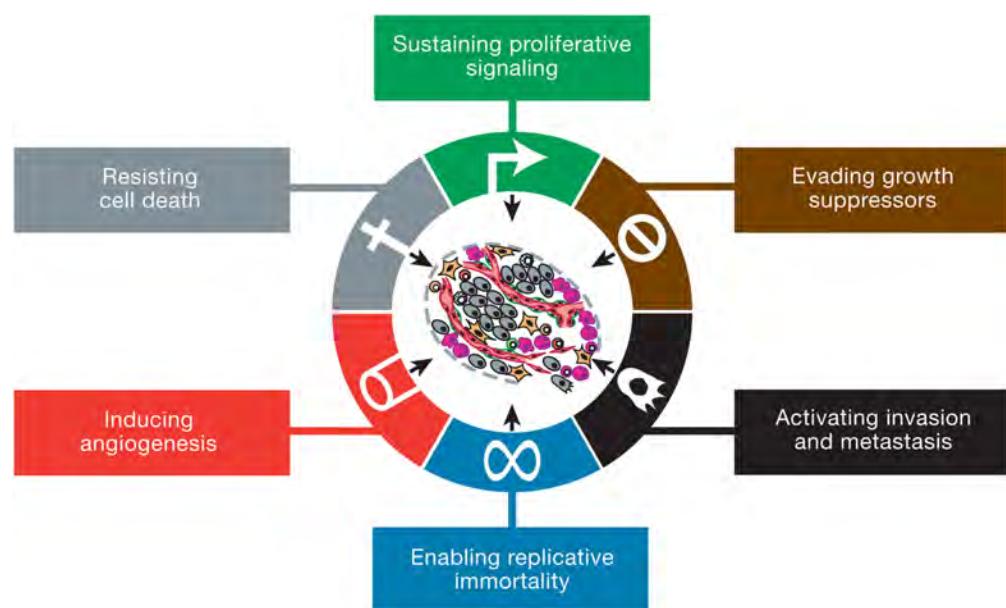


Figure 2.6 The Hallmarks of Cancer. Adapted from “Hallmarks of Cancer: The Next Generation” by D. Hanahan, and R. A. Weinberg, 2011, *Cell* (2011) 144 (5), 646-674. Copyright 2011 by Elsevier Inc. Adapted with permission.

2.2.1 Resisting cell death: Role of Smac/DIABLO in cancer progression

The ability of tumor cells to expand in numbers depends not only on the rate of cells proliferation but also on the rate of cells death, which occur normally through apoptosis. In normal cells, this process is tightly regulated in order to maintain cells population and immune system development (Vaux and Korsmeyer 1999). However, disruptions and deregulations of the process lead to uncontrolled cell growth, which is a characteristic of tumor cells. Therefore, inducing cancer cells apoptosis is important in any type of cancer therapy and in this case prostate cancer therapy.

Apoptosis can be activated through two distinct pathways (Figure 2.7): The extrinsic pathway, which is mediated by death receptors (CD95 and TRAIL) or the intrinsic pathway, which is mediated by the mitochondria (Martinez-Ruiz, Maldonado et al. 2008). Stimulation of death receptors such as CD95 or TNF-related apoptosis-inducing ligand (TRAIL) located on the plasma membrane result in the activation of the initiator caspase-8 which can cause direct cleavage of downstream effector caspases such as caspase-3 (Walczak and Krammer 2000). Effector caspases such as caspase-3 will cause irreversible damage to the nuclear lamina, cleave the proteins that normally hold a DNA degrading enzyme (DNase) in an inactive form, freeing the DNase to cut the DNA in the cell nucleus and ultimately causing the dismantling of the cells in a quick and neat way (Fulda and Debatin 2006).

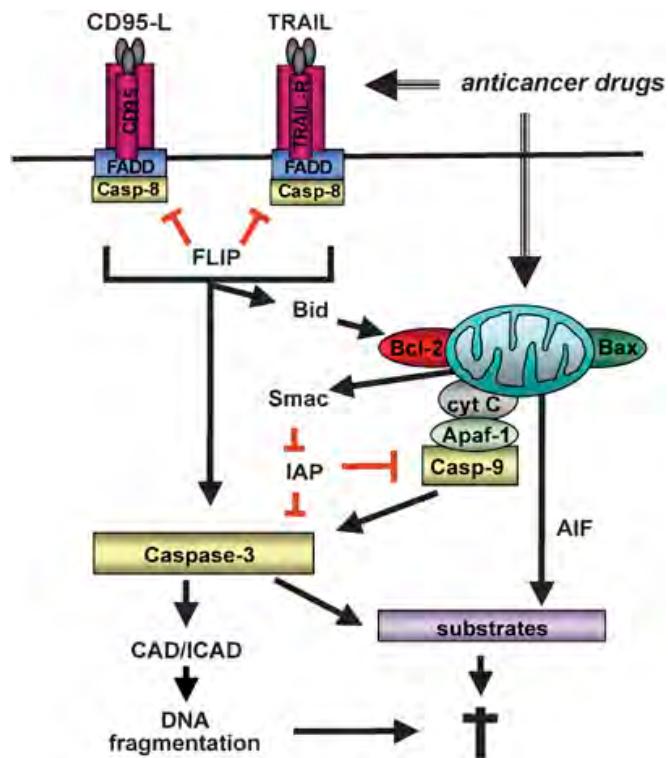


Figure 2.7 The Extrinsic and Intrinsic Apoptosis. Adapted from “Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy” by S. Fulda and K-M. Debatin, 2006. **Apoptosis signalling pathways.** Apoptosis pathways can be initiated through two main entry sites, at the plasma membrane by death receptor pathway or at the mitochondria by the mitochondrial-driven pathway. Stimulation of the death receptor pathway (Extrinsic Apoptosis) involves ligation to the tumor necrosis factor (TNF) receptor superfamily such as CD95 or TNF-related apoptosis-inducing ligand (TRAIL) receptors. Activation of the death receptors results in recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by cleaving and activating downstream effector caspases. The mitochondrial-driven pathway (Intrinsic Apoptosis) is initiated by stress signals through the release apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), or Smac/DIABLO from the mitochondrial intermembrane space. Cytochrome c forms an aggregate called apoptosome complex with apoptotic protease activating factor 1 (Apaf-1) and caspase-9. The formation of this complex will activate caspase-3 and other downstream effector caspases. Smac/DIABLO promotes caspase activation by neutralizing the inhibitory effects of IAPs, whereas IAF cause DNA condensation. *Oncogene* (2006) 25, 4798–4811. Copyright 2006 by Nature Publishing Group. Adapted with permission.

The mitochondrial-driven (intrinsic) pathway is initiated with the permeabilization of the outer mitochondrial membrane by pro-apoptotic members of the Bcl-2 family such as Bax and Bak (Green and Kroemer 2004). This process is regulated by proteins from the Bcl-2 family (e.g. Bcl-2, Bcl-xL, Bcl-w, Bax, Bak and Bok), mitochondrial lipids, proteins that regulate bioenergetics metabolic flux and components of the permeability transition pore (Green and Kroemer 2004). After the disruption of the outer mitochondrial membrane, apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), TG (Smac/DIABLO), Omi/HtrA2 or endonuclease G are released into the cytosol from the mitochondrial intermembrane space (Candé, Cecconi et al. 2002, Saelens, Festjens et al. 2004). The release of cytochrome c activates caspase-3 and eventually leads to the dismantling of the cells in a neat and quick way without causing any harm to the neighboring cells.

Smac/DIABLO is a novel mitochondria-derived pro-apoptotic protein that plays an important role in sensitizing tumor cells to die by apoptosis (Figure 2.8) (Du, Fang et al. 2000, Verhagen, Ekert et al. 2000). It has a pro-apoptotic effect that is mediated by its interaction with inhibitor of apoptosis proteins (IAPs) and the release of effector caspases from them. The IAP protein family that includes XIAP, c-IAP1 and c-IAP2 blocks both intrinsic and extrinsic apoptotic pathways by binding to and inhibiting active caspases, thus stopping the caspase cascade (Jia, Patwari et al. 2003). XIAP is the most potent among the IAPs due to the presence of three domains known as baculoviral IAP repeat (BIR) domains. BIR domains are very important in inhibiting the activity of active caspases (Deveraux, Roy et al. 1998). Smac/DIABLO functions by neutralizing the caspase-inhibitory properties of XIAP (Ekert, Silke et al. 2001). This is achieved by the displacement of XIAP from caspase 9 by Smac/DIABLO, thus overcoming the ability of XIAP to repress the activity of effector caspase, caspase-9, within the apoptosome complex (Srinivasula, Hegde et al. 2001). Several studies have shown that Smac/DIABLO is required for mitochondrial-

driven apoptosis in human multiple myeloma and prostate cancer cells (Carson, Behnam et al. 2002). Smac peptide was also reported to enhance apoptosis induced chemo or immunotherapeutic agents in the leukaemic Jurkat cell line (Guo, Nimmanapalli et al. 2002) and in malignant glioma cells *in vivo* (Fulda, Wick et al. 2002). These findings prompted scientists to develop peptides derived from NH2-terminal of Smac/DIABLO and small molecules that mimic the functions of Smac/DIABLO as therapeutic agents to induce cancer cells death or increase the apoptotic effects of the chemotherapeutic agents (Martinez-Ruiz, Maldonado et al. 2008). Currently small molecules such as AT-406, LCL-161 and Birinapant are all being in preclinical phase for their anticancer properties in combination with more specific chemotherapy drugs such as TRAIL and BRAF inhibitors (Perimenis, Galaris et al. 2016). In this study, we are interested to find bioactive compound/s from several species of Malaysian plant that could increase the expression of Smac/DIABLO protein, thus increasing the apoptotic effect.

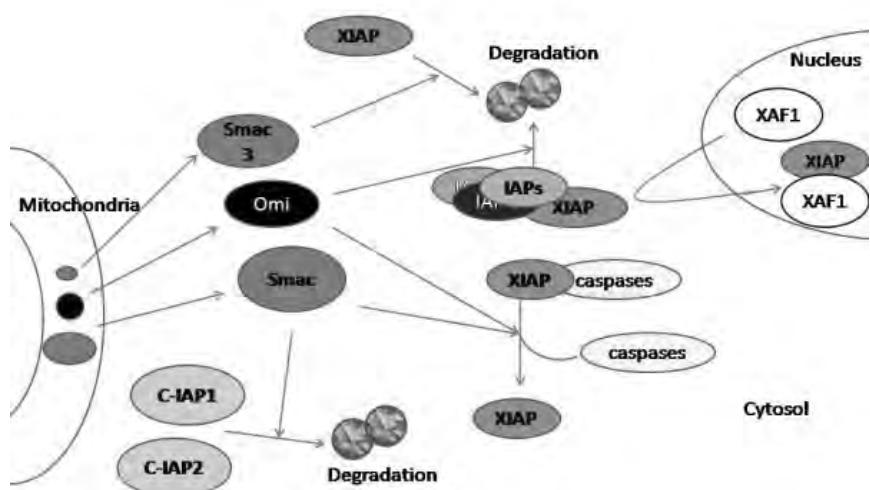


Figure 2.8 Role of Smac/DIABLO in apoptosis. Adapted from “Role of Smac/DIABLO in cancer progression” by G. M. Ruiz, V. Maldonado, G. Ceballos-Cancino, J. P. R. Grajeda, J. Melendez-Zajgla, 2008, *Journal of Experimental & Clinical Cancer Research* (2008) 27(1): 48. Open-access.

2.2.2 Activation of invasion and metastasis: Role of CXCL12/CXCR4 axis in cancer metastasis

Even though prostate cancer could spread to other distant sites including lymph nodes, lung, liver and brain, the spread to the bone marrow prove to be the most frequent as almost 90% of advanced prostate cancer patient suffer from pathologic fractures, spinal cord compression, and pain due in part of deregulated cycles of osteoblastic and osteolytic resorption/formation driven by the growing tumour mass (Rubens 1998). Several reports have shown that CXCL12/CXCR4 axis is involved in breast (Müller, Homey et al. 2001) and prostate (Taichman, Cooper et al. 2002, Chinni, Sivalogan et al. 2006) cancer cells metastasis to bone, where high expression of CXCR4, receptor for the CXCL12 chemokine, is detected (Conley-LaComb, Saliganan et al. 2013, Singareddy, Semaan et al. 2013).

Chemokines are a superfamily of cytokine-like proteins that activate chemokine receptors by binding to them (Sun, Cheng et al. 2010). To date, over 50 chemokines have been identified, and they are divided into 4 distinct families namely CXC, CX3C, CC and C. These divisions are made based on the positions of 4 conserved cysteine residues (Vindrieux, Escobar et al. 2009). Previously, chemokines are associated with their inflammatory responses achieved through the stimulation of leukocytes chemotaxis during inflammation (Thelen 2001), however new evidences suggest that they are major regulators of cells trafficking and adhesion (Kyriakou, Rabin et al. 2008, Gillette, Larochelle et al. 2009). CXCL12 or stromal-derived factor 1 (SDF-1), is a CXC chemokine, and its receptor CXCR4 are reported to play critical role in the spread of prostate cancer cells to the bone.

The binding of CXCL12 to CXCR4 is said to initiate divergent signalling pathways that lead to multiple responses including the phosphorylation of MEK/ERK signalling cascade and the activation of NF- κ B, which is important for tumor cells survival (Figure 2.9) (Sun, Cheng et al. 2010). Moreover, Sun, Schneider et al. (2005) has reported that the levels of CXCL12 in human and mouse tissues were higher at the preferable sites of metastasis for prostate cancer cells such as bone, liver and kidney when compared with tissues rarely affected by the tumor cells including lung, tongue and eye. Higher expression of CXCL12 leads to the increased adhesion of prostate cancer cells to an endothelial cell monolayer and to immobilized fibronectin, laminin, collagen (Engl, Relja et al. 2006), and to osteosarcoma cells (Taichman, Cooper et al. 2002) due to the up-regulation of α 5 and β 3 integrins.

Apart from that, overexpression of CXCR4 gene was documented in aggressive phenotypes of prostate tumor tissues and prostate cancer patient with this phenotypic characteristic often has poor survival rate (Lee, Kang et al. 2014). Sun, Schneider et al. (2005) has reported that in an *in vivo* study, the extent of bone metastasis in prostate cancer was limited by using neutralizing antibody against CXCR4. These data clearly show that CXCL12/CXCR4 axis plays a major role in prostate cancer cells progression. In our study, we investigate the potential of bioactive compound/s from plants to modulate this axis in order to reduce or prevent prostate cancer cells migration and invasion.

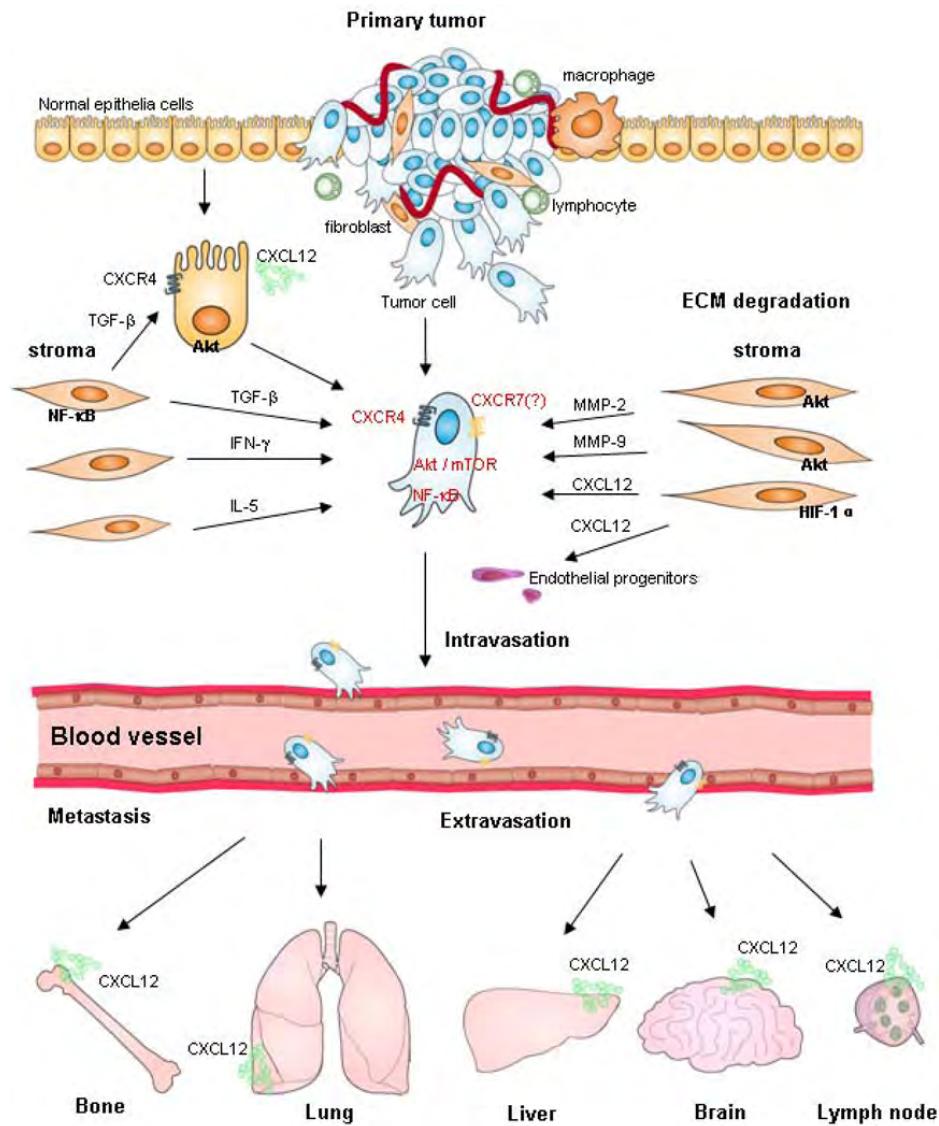


Figure 2.9 The role of CXCL12/CXCR4 axis and its microenvironment in tumor cells progression. Adapted from “CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression” by X. Sun, G. Cheng, M. Hao, J. Zheng, R. S. Taichman, K. J. Pienta, and J. Wang, 2010, *Cancer and Metastasis Reviews* (2010) 29 (4), 709-722. Copyright 2010 by Springer Science+Business Media, LLC. Adapted with permission.

2.2.3 Induction of Angiogenesis: Role of VEGF in angiogenesis

One of the most important phenotypic traits of malignant tumors is their ability to have sustained angiogenesis. Angiogenesis is a crucial process as it ensures continues supply of oxygen, nutrients, growth factors and hormones, and proteolytic enzymes (Folkman 1990). Without angiogenesis, the progression of malignant tumors to distant site will not be possible. Although, a report has been made a century ago regarding the observation that tumor growth can be accompanied by increased vascularity (Ferrara, Hillan et al. 2004), it was not until 1939 that the first postulate about the existence of a tumor-derived-blood-vessel-growth stimulating factor that serve to aid the formation of new blood supply to the tumor, was made by Ide and colleagues (Ide, Baker et al. 1939). 6 years later, Algire, Chalkley et al. (1945) made a proposal saying that the rapid growth of tumor transplant is dependent on the development of a rich vascular supply. This proposal was made based on the observation that tumor growth started after an increased in blood-vessel density using a transparent chamber technique to the mouse. (Algire 1943, Algire, Chalkley et al. 1945). In 1971, Folkman has proposed that finding an anti-angiogenesis factor could be an effective anticancer strategy (Folkman 1971), and this pioneering hypothesis has led to the understanding of several important angiogenic factors such as epidermal growth factor (EGF), transforming growth factor (TGF)- α , TGF- β , tumour-necrosis factor- α (TNF- α) and angiogenin (Folkman and Klagsbrun 1987). However, most of the research efforts initially put for these angiogenic factors were then discontinued as they were only shown to promote angiogenesis in bioassay models but not physiologically (Klagsbrun and D'Amore 1991).

Vascular Endothelial Growth Factor (VEGF), which is now known to be the main regulators of angiogenesis was first described by Senger, Galli et al. (1983) and then was successfully isolated and identified by Ferrara and

colleagues in 1989 (Ferrara and Henzel 1989). VEGF is a homodimeric glycoprotein with an approximate molecular weight of 45 kDa. It belongs to a family of structurally related mitogen known as platelet-derived growth factor (PDGF) (Carmeliet 2005). The VEGF family consists of six secreted glycoproteins known as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF-1 and PIGF-2) all of which are derived from distinct genes (Hicklin and Ellis 2005).

VEGF-A has been indicated to play the most critical role in the regulation of angiogenesis (Ferrara, Carver-Moore et al. 1996) whereas the vascular effect of the other VEGF in the family members remain to be fully characterized (Ferrara and Davis-Smyth 1997, Ferrara 1999, Ferrara 2001). Initially, two VEGF receptors were identified on endothelial cells and characterized as the specific tyrosine kinase receptors VEGFR-1 and VEGFR-2 (Shibuya, Yamaguchi et al. 1990, Terman, Dougher-Vermazen et al. 1992), and several years after that another tyrosine kinase receptors known as VEGFR-3 was identified and has been found to play major role in lymphangiogenesis (Kaipainen, Korhonen et al. 1995, Paavonen, Puolakkainen et al. 2000). All VEGF family members have different binding affinity towards these receptors, and this characteristic has helped to elucidate their specific functions. All of the isoforms of VEGF-A can bind to both VEGFR-1 and VEGFR-2, while the naturally occurring heterodimers of VEGF-A has been shown to have higher binding affinity towards VEGFR-2 (DiSalvo, Bayne et al. 1995, Cao, Chen et al. 1996).

Since VEGF-A has the ability to bind to both VEGFR-1 and VEGFR-2 receptors, questions has been asked about which receptor activation could lead to direct effect on tumor cells angiogenesis. A consensus has been reached on this matter as studies show that VEGFR-2 is the major mediator of angiogenesis

process (Ferrara 2004). Even though VEGFR-1 is said not to have direct effect on mitogenesis and angiogenesis, but it might act as a decoy receptor that sequesters VEGF and prevents its interaction with VEGFR-2 (Park, Chen et al. 1994).

Angiogenesis or also known as neovascularization, is very important in human cancers and it has been specifically linked to increased tumor growth and metastatic potential (Ferrara 2002). Figure 2.10 summarizes the role played by VEGF/VEGFR in tumor cells angiogenesis. Overexpression of VEGF mRNA has been observed in various human tumors including lung, breast, prostate, gastrointestinal tract, renal, and ovarian carcinomas through *in situ* hybridization studies (Yoshiji, Gomez et al. 1996, Ferrer, Miller et al. 1997, Sowter, Corps et al. 1997, Volm, Koomägi et al. 1997, Ellis, Takahashi et al. 1998, Tomisawa, Tokunaga et al. 1999). The expression of VEGF ligands in tumor cells supported the hypothesis that claimed that VEGF supports the growth of tumor cells not only by inducing angiogenesis but also through their direct action on VEGFRs that created a VEGF/VEGFR autocrine loop, which induces tumor growth arrest and apoptosis when disrupted (Hicklin and Ellis 2005). Studies conducted by Warren, Yuan et al. (1995) has demonstrated that in an *in vivo* mouse colon cancer model of liver metastasis, a significant decrease in the size of tumor was achieved after the treatment with anti-VEGF monoclonal antibodies. Another studies also show that a significant decreased in tumor size and prolonged survival were achieved in mice bearing human leukaemias xenograft after treatment with antibodies that function by blocking the VEGFR-2 receptor (Dias, Hattori et al. 2001). Therefore, the results from all these studies clearly demonstrate that angiogenesis is VEGF-dependent and finding bioactive compound/s that could block VEGFs and its receptors or both could reduce or prevent tumor cell angiogenesis.

Lymphatics (lymphangiogenesis)

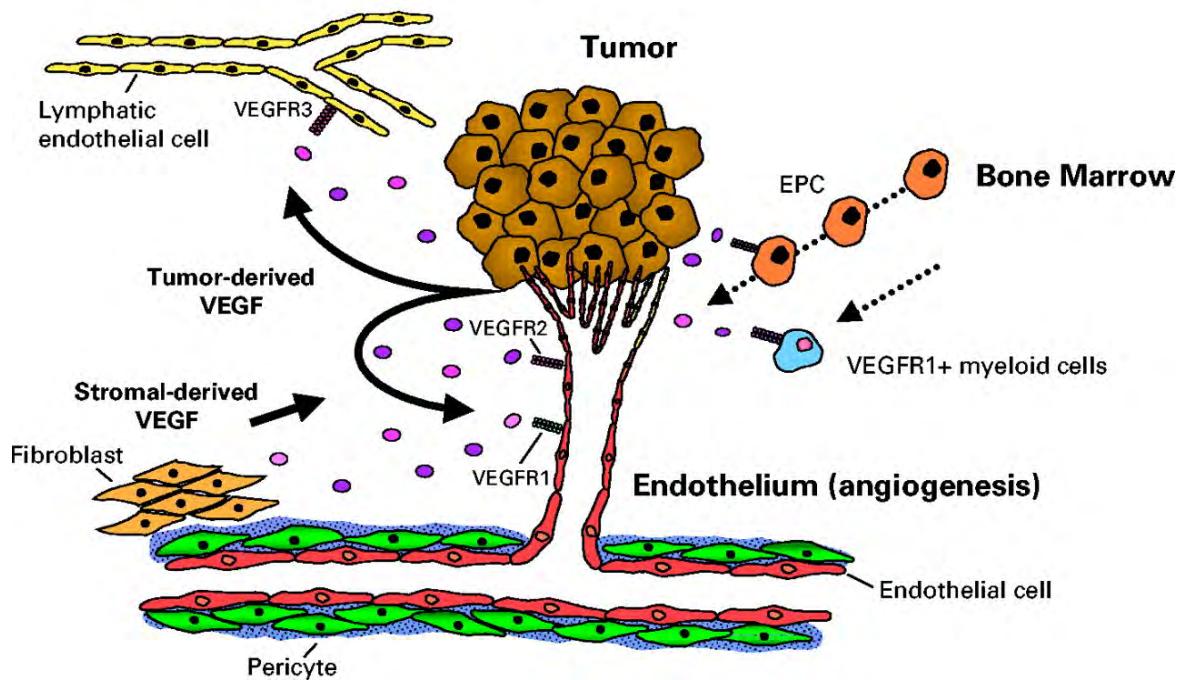


Figure 2.10 Role of VEGF/VEGFR in tumor cells angiogenesis. The expression of VEGF ligands on tumor cells or host stromal cells stimulate the VEGFR1 and VEGFR2 on endothelial cells and lead to the activation of tumor cells proliferation, migration, survival and vascular permeability. Adapted from “Role of the Vascular Endothelial Growth Factor Pathway in Tumor Growth and Angiogenesis” by D. J. Hicklin and L. M. Ellis, 2005, *Journal of Clinical Oncology* (2005) 23 (5), 1011-1027. Copyright 2005 by American Society of Clinical Oncology. Adapted with permission.

2.3 Modern trends in the use of natural products in cancer prevention and treatment

Chemical substances derived from natural products including plants, animals and microbes have played a vital role in cancer chemotherapy for the past 30 years (Mann 2002). Plants have been used by almost all cultures and communities in the world due to their continuous role as important source of bioactive compounds not just for cancer therapy but other diseases as well (Challand and Willcox 2009). Harwell (1982) has produced an extensive review that listed almost 3000 plants species that have been reported to be used in the treatment of cancer. However, in many instances, the term 'cancer' is undefined or reference is made to conditions such as abscesses, 'hard-swelling', calluses, wart, polyps or tumors. But many of the claims for efficacy should be viewed with extreme skepticism because cancer as a disease is likely to be poorly defined in terms of folklore and traditional medicine (Cragg and Newman 2005).

For example, the roots of mayapple, *Podophyllum peltatum* for example, have been used by the American Indians to treat skin cancer and venereal warts. The main chemical constituent in this plant is known as podophyllotoxin. This chemical belongs to the anticancer agent group known as podophyllins which includes other bioactive compounds such as etoposide and teniposide (Mann 2002). Similarly, a plant known as *Catharanthus roseus* has been used as hypoglycaemic agent in many parts of Asia, but it was not until 1958 that its two main constituents known as vinblastine and vincristine were discovered to have potent cytotoxic activities against cancer cells (Roussi, Guérin et al. 2012). These discoveries encouraged the National Cancer Institute (NCI) to launch a large-scale screening programme to look for antitumor agents (Zubrod 1984). 35,000 plant samples were evaluated primarily against the mouse leukaemia cell line, L1210 and P388, between 1960 and 1982 in this large-scale study. The most significant drug produced from this huge study was Taxol,

which was obtained from the bark of the Pacific yew *Taxus brevifolia*. (Kingston 2005). Positive outcomes from this massive screening programme have encouraged the National Cancer Institute to start a new programme in 1985 in which extracts from plants, animals and microorganisms were screened against a panel of 60 human cancer cells line including prostate, breast, lung, colon, kidney, skin, ovary and brain cancer. Due to this giant quest to find new anticancer drugs, almost 50% of the approved drugs during the last 30 years (1981-2010) are derived either directly or indirectly from natural products (Newman and Cragg 2012).

The above examples, although compelling for the case of natural products as effective sources of anticancer therapies; represent extremely expensive approaches. It also yields non-readily available therapies for much of the world populations. This makes equally compelling the case for high quality extracts standardized on proven anticancer phytochemicals, which may be put in the market at a reduced price with chemopreventives indications, thus making the final products more accessible to the public even in developing countries.

Saw palmetto berry is a perfect example for this as it is widely used for the treatment of benign prostatic hyperplasia (BPH), often as an alternative to pharmaceutical agents. Although pathologically, BPH is not considered as a precursor for prostate cancer, approximately 83% of prostate cancer incidence occur in men with BPH (Bostwick, Cooner et al. 1992, Chokkalingam, Nyrén et al. 2003) and both conditions share common risk factors and respond to anti-androgen therapy. Saw palmetto is a palm plant that is commonly found along the Atlantic and Gulf coast of the USA. The large berries produced by the plant are highly enriched in fatty acids, phytosterols, and flavonoids (Schantz, Bedner et al. 2008). Numerous clinical trials had been conducted to evaluate the effectiveness of the extract of the fruits in treating BPH (Bent and Ko 2004,

Gerber and Fitzpatrick 2004, Bonnar-Pizzorno, Littman et al. 2006), and based on the positive results of these clinical trials, saw palmetto was developed as a whole extract with no single active ingredient unlike any other conventional drug. It has been marketed as a dietary supplement to treat enlarged prostate. In continental Europe, saw palmetto is available as a Full Licensed Medicine (Permixon®) but also available as a Traditional Medicine (THMP) (Prostasan®) as well as food supplement without explicit indications but obviously transporting onto the health conscious consumer the hidden message of prostate health. Outside Europe, the market drastically splits into Food or Drug leaving Saw Palmetto manufacturers and consumers with the food supplement pathway as the sole way to bring this obviously efficacious natural product to the public

Approximately 2.5 millions adult in the United States were reported to use saw palmetto in a National Survey conducted in 2002 (Barnes, Powell-Griner et al. 2004). It is also very popular in Europe as half of German urologist prefers prescribing plant-based extracts to synthetic drugs (Lowe and Ku 1996, Bent , Kane et al. 2006). Saw palmetto is available in the market as encapsulated capsule, powdered berries and gel capsules containing liquid extract. This extract is thought to treat BPH by inhibiting the 5- α -reductase enzyme (Bayne, Donnelly et al. 1999, Habib, Ross et al. 2005), which is similar to finasteride, a popular chemopreventive used in prostate cancer therapy, as explained in section 2.1.5.1. Several studies were conducted to investigate whether saw palmetto is active against prostate cancer and these studies showed that the lipidosterolic extract of saw palmetto appears to inhibit LNCaP, PC3, 267B-1, and BRFF-41T prostate cancer cell line in-vitro when treated with physiologically plausible doses (Goldmann, Sharma et al. 2001, Iguchi, Okumura et al. 2001, Hill and Kyprianou 2004). The inhibition of the prostate cancer cell lines occurs through several different mechanism including apoptosis, necrosis, and growth inhibition (Goldmann, Sharma et al. 2001, Iguchi, Okumura et al. 2001). However, no studies have been conducted to examine the activity of saw

palmetto in any animal models. The development of saw palmetto as an agent to promote prostate health could be a good example on how the active extracts of both *Marantodes pumilum* and *Ficus deltoidea* plants can be introduced into the market in the near future.

2.3.1 R & D of Anticancer drugs from natural products

The process of developing a new drug is complex, time consuming and very expensive. Normally it takes around 12 years from the discovery of a new drug in laboratory settings to its application in human diseases. It also involves more than 1 billion USD of investment in today's context in order to develop a new drug from laboratories to clinics (Katiyar, Gupta et al. 2012). Drug development process in current pharmaceutical settings can be divided into 7 phases namely: 1) Preclinical research, 2) Investigational New Drug (IND) application, 3) Phase 1 trials, 4) Phase 2 trials, 5) Phase 3 trials, 6) New Drug Application (NDA), and 7) Approval.

In the case of natural products, drug discovery process started with rigorous screening procedures using different species of plants on a panel of different cancer cell lines (Monks, Scudiero et al. 1991). Different approaches can be used for screening of plants to be tested against cancer cell lines. These approaches include random approach, ethnopharmacology approach (Ganesan 2008), traditional system of medicine approach and zoo-pharmacognosy approach (Katiyar, Gupta et al. 2012). Once the task of screening potential candidates is over, the next steps include; 1) screening of biological activity using selective assays, 2) Bioguided fractionation of the identified plant, 3) Isolation and structure elucidation of the active compounds, 4) Evaluation of chemical do-ability, druggability, and patentability, 5) Decisions based on safety and biological activity screening(Katiyar, Gupta et al. 2012). This process is

considered as the preclinical research phase mentioned earlier. Once this phase is completed, the potential drug needs to go through 6 remaining phases for drug development before it can reach clinics for human application.

However, due to low success rate of finding new chemical entities (NCE) by following this conventional approach, maybe now is the right time for large-scale pharmaceutical organizations to open up the developmental strategies and adopt nutraceuticals approach (Section 2.3.2). This approach that allows the development of herbal extracts hitting multiple targets as new drugs should be given serious consideration. This strategy will not only reduce developmental cost, but would also enhance the chances of success in terms of providing effective and safe drugs as well as minimizing the risk of post-marketing withdrawal. In this study, the active extracts of the plants will be characterized and the bioactive compounds responsible for the inhibition of prostate cancer cells growth will be used as markers for future standardization of the extract. However, before the standardized active extracts could reach the market for human consumption, additional studies are required to be carried out to investigate the efficacy of the standardized extract on relevant animal models as well as following the regulations set for nutraceuticals development in the targeted market.

2.3.2 R & D of nutraceuticals for prostate health

The term 'nutraceuticals' was first introduced in 1989 by the founder and chairman of the Foundation for Innovation in Medicine (FIM), Dr Stephen DeFlie. It is a term derived from 'nutrition' and 'pharmaceuticals' and is defined as any substance that is food or part of a food that provide medical or health benefits, including prevention and/or treatment of a disease. Recently, the use of nutraceuticals in prostate disease is becoming more popular. Approximately

30% of men diagnosed with prostate disease in North America were reported to use some complementary and alternative medical (CAM) therapy or nutraceuticals products (Lee, Chang et al. 2002, Chan, Elkin et al. 2005). Prostate cancer develops over a long period of time thus making it suitable for primary, secondary and tertiary prevention strategies (Trottier, Bostrom et al. 2010). This is one of the reasons why CAM and nutraceuticals are becoming very popular to promote prostate health. Besides that, nutraceuticals are not regulated in the same way as drugs are, as they are classified according to different food categories. The regulations for nutraceuticals also vary from one country to another and not as stringent as medicines. This lowers their cost of production and development, so the population can use them in appreciable amounts to prevent diseases. Consequently, the global use and trade of nutraceuticals has boomed in recent years and investment in this sector is very attractive to pharmaceuticals companies, for example Glaxosmithkline (GSK) has Benecol® for lowering cholesterol and Bayer markets Iberogast® for functional dyspepsia.

The most popular dietary approaches to promote healthy prostate include popular nutraceuticals such as saw palmetto (Section 2.3), *Pygeum africanum*, phytosterols, rye pollen extract (eg, Cernilton®, Graminex LLC, Saginaw) and vitamins and minerals, such as vitamin E and selenium (Curtis Nickel, Shoskes et al. 2008). Despite the lack of stringent regulations associated with the production and development of nutraceuticals, sound research practices are required in order to provide high quality products backed by scientific evidence. Therefore, it is very common to see scientists involved in pre-clinical research as part of the development of nutraceuticals. Following this step, some might choose to continue testing the efficacy of the nutraceuticals on relevant animal models while the others could directly release the product into the market providing that the regulations for nutraceuticals development in that specific country or region are strictly followed. For example, in the US, nutraceuticals are

monitored as 'dietary supplements' according to the Dietary Supplement, Health and Education Act (DSHEA) of 1994 (Coppens, Da Silva et al. 2006). The DSHEA establishes that manufacturers are responsible for the safety evaluation of the product and if a new ingredient was to be introduced into the product, they must inform FDA that the new ingredient 'can reasonably be expected to be safe' at least 75 days before going to the market. In the European Union (EU), a specific law regulating nutraceuticals does not exist, however if a claim was made that implies medicinal benefit for a nutraceutical product, the product needs to comply with the regulatory requirements for medicinal products in respect of safety, efficacy, quality testing and marketing authorization procedures (Coppens, Da Silva et al. 2006). EU regulations also state that the beneficial effects of nutraceuticals can only be 'health claim' and not 'medicinal claim'. In Malaysia, no specific law on nutraceuticals exists, however they are monitored under the Malaysia's Food Act 1983 and the Food Regulations of 1985, which govern food safety and quality control, including food standards, hygiene, import and export activities, advertisement and accreditation of laboratories (Arora, Sharma et al. 2013). This law is enforced by the Food Safety and Quality Division (FSQD) of the Ministry of Health (MOH).

Despite the lack of stringent regulations for nutraceuticals development, this should not be an excuse for scientists and manufacturers to ignore good research practice to ensure production of high quality nutraceuticals backed by scientific evidence.

2.3.3 Development of Natural Anticancer Products from Malay Biodiversity

With the global resurgence of interest for natural remedies as a commodity, Malaysia is looking towards capitalizing on its megabiodiversity, which includes the oldest rain forest in the world and an estimated 1,200 medicinal plants. To harness this potential, a “Herbal Development Office” has been formed under the Ministry of Agriculture (MoA). It outlines the strategic direction, policies and regulation of research and development (R&D) clusters focusing on the discovery, crop production and agronomy, standardization and product development, toxicology/pre-clinical and clinical studies, and processing technology. Preference has been given to a subset of 11 traditional plant species with high economic potential. This list includes the two plants selected for this project namely *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var *pumila*) and *Ficus deltoidea* L. Given that Malaysia is mostly an agricultural country, sufficient supply of raw materials for research and development (R&D) is ensured. Moreover, its multi-ethnic population facilitates clinical trials which results can be extrapolated to many other markets. With this in mind, and to promote and protect the growth of a local herbal industry, endemic species and varieties of these medicinal plants are under active study to derive high value herbal supplements and remedies (Malaysian Ministry of Agriculture, 2011).

Phytochemical research previously conducted on both of the selected plant species showed that they contain phytochemicals linked to cancer prevention: flavonols such as epigallocatechins, phenolic acids such as gallic acids and phytoestrogens among others. Therefore, this project aims to investigate the effect of these phytochemicals on prostate cancer cells, isolate and identify the bioactive compound/s providing maximum efficacy and characterize the mechanism of actions that lead to prostate cancer cells growth inhibition.

2.4 *Marantodes pumilum* (Blume) Kuntze

2.4.1 Traditional Use

Marantodes pumilum (Blume) Kuntze (Figure 2.11 and Figure 2.12) (synonym *Labisia pumila* var *pumila*) belongs to Myrsinaceae family, locally known as Kacip Fatimah, is a herb that has been widely used in South East Asian communities for a variety of illnesses and also as health supplements (Jamal, Houghton et al. 2003). It is an indigenous medicinal herb of Malaysia and sometimes also referred locally as Akar Fatimah, Selusoh Fatimah, Tadah Matahari, Rumput Siti Fatimah, Bunga Belangkas Hutan and Pokok Pinggang. There are three varieties of *Marantodes pumilum*, i.e. *Marantodes pumilum* var. *alata*, *Marantodes pumilum* var. *pumila* and *Marantodes pumilum* var. *lanceolata*. Each variety has their respective use and local healers tend to use var *alata* and var *pumila* traditionally (Ibrahim and Jaafar 2011). This herb's extract is prepared by boiling the roots, leaves or the whole plant with water and the extract is taken orally (Jamal, Houghton et al. 2003, Ibrahim and Jaafar 2011). The decoction of the roots is also given to pregnant women between one or two months before delivery, as this is believed to induce and expedite labour. It has also been widely used with a long history by women in Malaysia to treat post-partum illnesses, to assist contraction of the birth channel, shrink the uterus, improve menstrual cycle, and weight loss (Jamal, Houghton et al. 2003). It was also reported that *Marantodes pumilum* can be used for delaying fertility and to regain body strength; while some other folkloric uses include treatment of flatulence, dysentery, dysmenorrhoea, gonorrhoea and “sickness in the bones”. Due to its various applications, hence *Marantodes pumilum* is known as “queen of plants” of all Malaysian herbs.



Figure 2.11 *Marantodes pumilum* (Blume) Kuntze plant



Figure 2.12 The farming of *Marantodes pumilum* (Blume) Kuntze plant in Malaysia

2.4.2 Anticancer Phytochemicals

Al-Mekhlafi, Shaari et al. (2012), described the anti-proliferative properties of *Marantodes pumilum* extracts against different types of cancer cells. In this study, 4 compounds isolated from the leaves of *Marantodes pumilum* namely 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol (1), labisiaquinone A (2), labisiaquinone B (3), and 1-O-methyl-6-acetoxy-5-pentadecylresorcinol (4) (Figure 2.13) showed strong cytotoxicity activity against 3 different cancer cell lines, PC3 (prostate), HCT116 (colon) and MCF-7 (breast) with very low IC₅₀ value (<10 µM). These 4 compounds also exhibited strong selectivity for PC3 and HCT116 relative to MCF-7. Moreover, the presence of certain phytochemicals such as favonols (catechin and epigallocatechin), phenolic acids (gallic acid, coumaric acid), flavanols (quercetin, myricetin) (Figure 2.14) and phytoestrogens indicate that the use of this plant could be expanded for other pharmacological applications (Chua, Lee et al. 2012). Other studies using plants containing similar phytochemical compounds have clearly demonstrated their anticancer properties by addressing some of the hallmarks of cancer. Gallic acid is identified as the major anticancer compound in *T. sinesis* leaf extract. Studies conducted using this extract have shown that gallic acid is cytotoxic against DU145 prostate cancer cells through generation of reactive oxygen species (ROS). It is also capable of blocking the growth of DU145 cells at G2/M phases by activating Chk1 and Chk2 and inhibiting Cdc25C and Cdc2 (Huei et al., 2009).

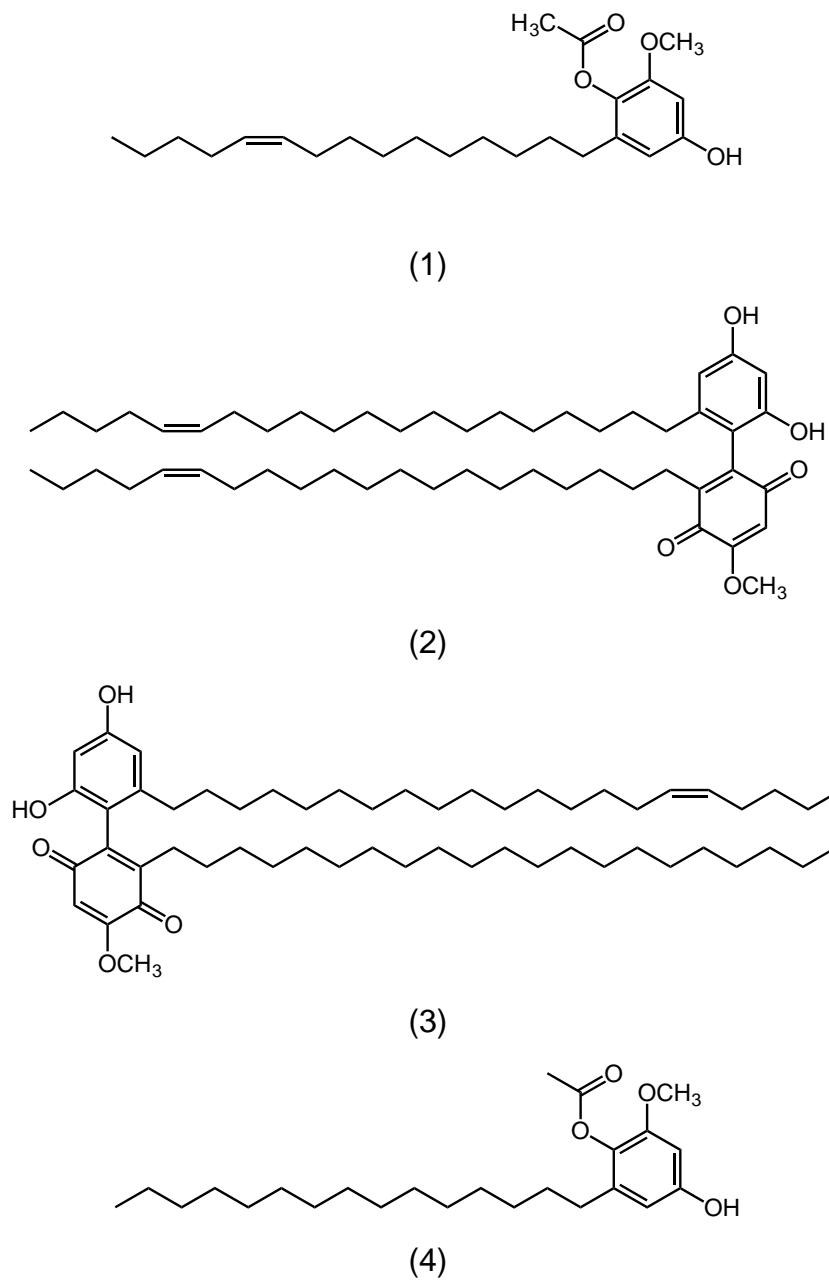
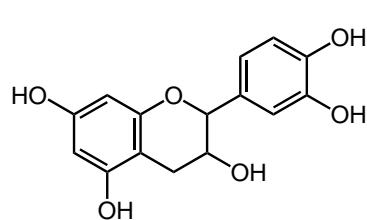
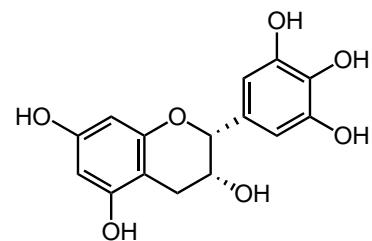


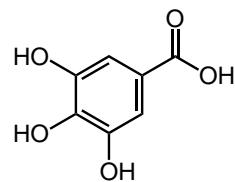
Figure 2.13 The chemical structure of the known phytochemicals in *Marantodes pumilum* with anticancer activity



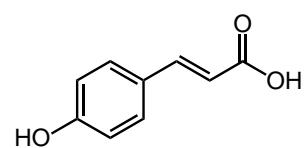
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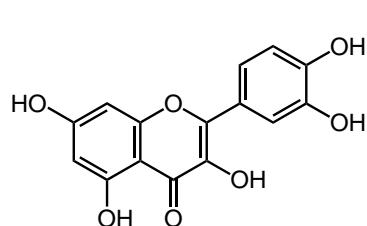
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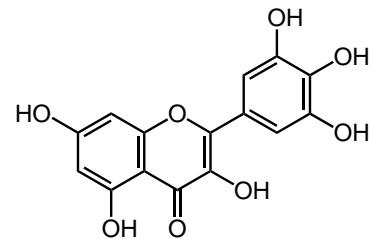
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(8)



(9)



(10)

Figure 2.14 The chemical structure of the known phytochemicals in *Marantodes pumilum*. (5) Catechin, (6) Epigallocatechin, (7) Gallic Acid, (8) Coumaric Acid, (9) Quercetin and (10) Myricetin

Another phytochemical compound available in *Marantodes pumilum*, epigallocatechin, has been shown to have anticancer properties on another prostate cancer cell line PC-3. This study has indicated that epigallocatechin was able to inhibit PC-3 prostate cancer cell proliferation via MEK-independent ERK1/2 activation (Albrecht, Clubbs et al. 2008).

The regulation of estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta) is very important in prostate cancer prevention (Pravettoni, Mornati et al. 2007). One of the phytochemical compounds that can mimic the action of estrogen in inhibiting the growth of prostate cancer cell line is phytoestrogens. Research has shown that one of the most important phytochemical compounds in *Marantodes pumilum* extract is the phytoestrogen (Adlercreutz 2002). Early biological studies on the activity of the phytoestrogen in soy (isoflavones) have demonstrated that phytoestrogen has the ability to upregulate estrogen receptor beta (ERbeta) and downregulate estrogen receptor alpha (ERalpha), which then lead to the inhibition of prostate cancer cell proliferation (Hussain, Banerjee et al. 2003). Therefore, it is believed that, the phytoestrogen content in *Marantodes pumilum* plant extract could also cause similar action, which might lead to the death of the cancer cells. With that in mind, this study aims to investigate the potential of *Marantodes pumilum* (Blume) Kuntze extract in prostate cancer prevention and characterize the mechanism involved in inhibiting prostate cancer cell proliferation leading to cancer cell death.

2.5 *Ficus deltoidea* L.

2.5.1 Traditional Use

Ficus deltoidea is a native shrub, which belongs to the family of Moracea (Figure 2.15). The plant is characterized by the evergreen small tree or shrub and in the wild the plant can reach around 5-7 meters tall. This species of plant can normally be found in south-east Asian countries including Malaysia, Indonesia and southern Phillipines. It is commonly known as “Mas Cotek” in the peninsular Malaysia and people in east Malaysia normally refer to this plant as “sempit-sempit” and “agolaran” (Berg 2003). This plant plays a vital role in traditional medicine, where different parts of the plant is used for the treatment of several ailments such as the relief of headache (fruit part), toothache (fruit part), and sores and wound (roots and leaves). Women consume the decoction of boiled leaves of *Ficus deltoidea* as an after-birth treatment to contract the uterus and vaginal muscles besides treating the disorders of the menstrual cycle and leucorrhoea (Burkill and Haniff 1930). Even though, the plant has a lot of important applications traditionally, only few studies have been conducted to explore its potential pharmacological properties. Abdullah, Karsani et al. (2008), reported that the fruit extract of *Ficus deltoidea* showed inhibitory effects against Angiotensin-I converting enzyme (ACE) enzyme. This finding suggested that the fruit extract has antihypertensive properties. Moreover, tea prepared from *Ficus deltoidea* showed high potential in reducing total cholesterol level, LDL-cholesterol and the risk of cardiovascular disease by lowering the antherogenic index (LDL/HDL ratio) and increasing the percentage of HDL/total cholesterol ratio (Hadijah, Normah et al. 2007). This study also finds no sign of cytotoxicity in rats based on a sub-acute toxicity study.

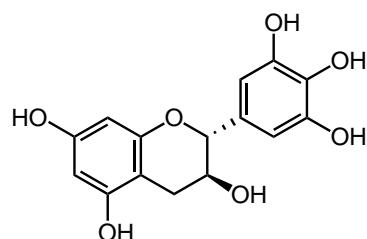


Figure 2.15 *Ficus deltoidea* plant

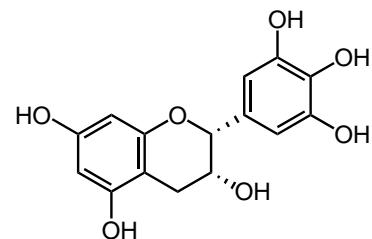
2.5.2 Anticancer Phytochemicals

Flavonoids is one of the phytochemical compounds that can be found in abundance in *Ficus deltoidea* which includes gallocatechin, epigallocatechin, catechin, luteolin-8-C-glucoside, 4-p-coumaroylquinic acid, orientin, vitexin, isovitexin, rutin, quercetin and naringenin (Figure 2.16 and Figure 2.17) (Bunawan, Amin et al. 2014). The presence of these phytochemicals gives its yellow pigmentation, and many studies confirmed that any herbs containing flavonoids could have the ability to acts as anti inflammatory, anti allergy, anti cancer and anti microbial agents, so this explain how the plant is able to protect itself from insects and microorganism (Abdullah, Hussain et al. 2009, Uyub, Nwachukwu et al. 2010, Akhir, Chua et al. 2011, Zakaria, Hussain et al. 2012). As mentioned earlier flavonoids such as epigallocatechin has the ability to inhibit PC3 prostate cancer cell line. Zhou, Liu et al. (2009) has reported that vitexin showed cytotoxic effect on breast, ovarian and prostate cancer cells by inducing apoptosis with the cleavage of PARP protein, up-regulation of Bax and

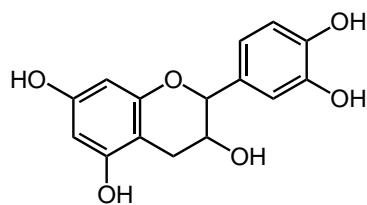
downregulation of Bcl-2. Rutin, quercetin and orientin have been reported to have anticancer properties by inducing apoptosis in murine leukemia WEHI-3 cells (rutin) (Lin, Yang et al. 2012), human lung cancer cell line A-549 (quercetin) (Zheng, Li et al. 2012), and human cervical carcinoma cells, HeLa (orientin) (Guo, Tian et al. 2014). *Ficus* species containing phenanthroindolizidine alkaloids and a series of triterpenoids with C-28 carboxylic acid functional groups are reported to be very strong cytotoxic compounds. Triterpenoids, isolated from the aerial roots of *Ficus microcarpa* demonstrated cytotoxicity in three human cancer cell lines with IC₅₀ values from 4.0 to 9.4 μ M, including HONE-1 nasopharyngeal carcinoma cells, KB oral epidermoid carcinoma cells, and HT29 colorectal carcinoma cells (Chiang and Kuo 2002, Chiang, Chang et al. 2005). In a study by Akhir, Chua et al. (2011), they found that both aqueous and ethanolic extracts of *Ficus deltoidea* extracts gave IC₅₀ value of $224.39 + 6.24 \mu\text{g/ml}$ and $143.03 \pm 20.21 \mu\text{g/ml}$ respectively. The aqueous extract caused the detachment of the cancer cell as observed by cell viability assay. While DNA fragmentation was not detectable after treatment with the aqueous extract, but at 1000 $\mu\text{g/ml}$ of the ethanolic extract DNA fragmentation occurred around 200 Kbp (Akhir, Chua et al. 2011). Therefore, this study aims to investigate the anticancer properties of *Ficus deltoidea* plant extracts and three other varieties of the plants on human prostate cancer cell lines.



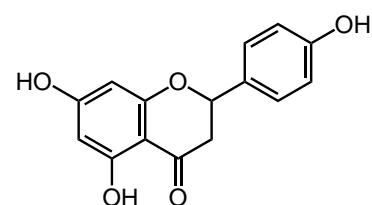
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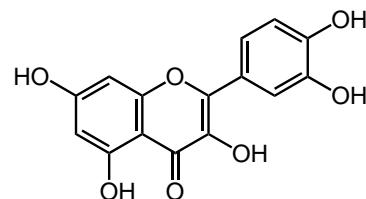
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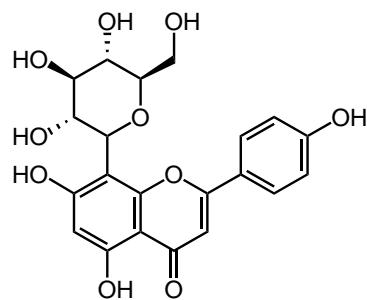


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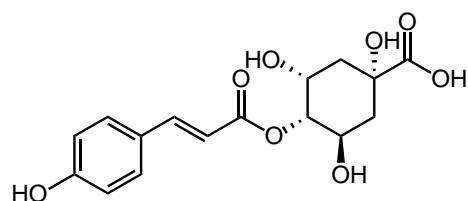


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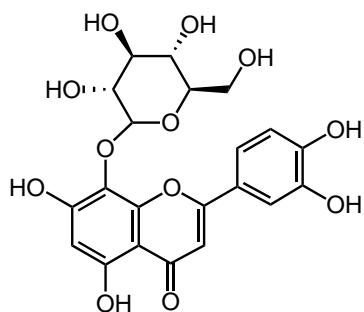
Figure 2.16 The chemical structure of the known phytochemicals in *Ficus deltoidea* (11) Gallocatechin, (12) Epigallocatechin, (13) Catechin, (14) Naringenin and (15) Quercetin.



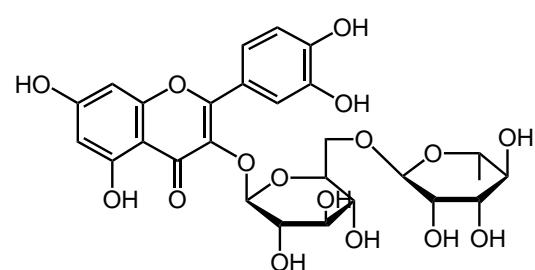
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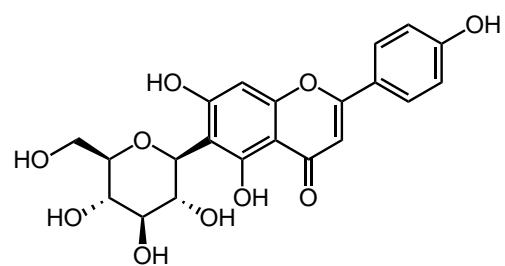
(17)



(18)



(19)



(20)

Figure 2.17 Chemical structure of known phytochemical in *Ficus deltoidea* (16) Vitexin, (17) 4-p-coumaroylquinic acid, (18) Orientin, (19) Rutin and (20) Isovitetin

Chapter 3

Materials and Methods

3 Materials and Methods

3.1 Plant Material

Plant materials used in this research were acquired by the Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), from a local farm (Johor, Malaysia) over the period of September 2013 to February 2014 (Table 3.1). A Pharmacognosist at Universiti Putra Malaysia (UPM), Malaysia, identified the plant materials and Vouchers were deposited at IBD.

Table 3.1 Plant Species, Voucher Number and Local names

Voucher Number	Code	Family Name	Scientific Name	Local Name	Parts
SK 2308/13	MP	Primulaceae	<i>Marantodes pumilum</i> (Blume) Kuntze (synonym) <i>Labisia pumila</i> var. <i>pumila</i>)	Kacip Fatimah	Aerial
SK 2309/13	FD1	Moraceae	<i>Ficus deltoidea</i> var. <i>angustifolia</i> (Miq.) Corner	Mas Cotek	Aerial
SK 2310/13	FD2	Moraceae	<i>Ficus deltoidea</i> Jack var. <i>deltoidea</i>	Mas Cotek	Aerial
SK 2311/13	FD3	Moraceae	<i>Ficus deltoidea</i> Jack var. <i>deltoidea</i>	Mas Cotek	Roots

3.1.1 Plant Extraction

Samples were processed using a laboratory scale mill until fine powder was produced at IBD. The air-dried and powdered parts of different amount of plants (\pm 50 g) were extracted at the UCL School of Pharmacy with methanol for 72 hours via a maceration process. The crude extracts were obtained after the evaporation of the methanol to complete dryness under reduced pressure at 40°C. The crude methanol extracts was re-dissolved in 90% methanol and subjected to liquid-liquid partition with n-hexane for three times (100 ml each time) to yield the n-hexane extracts (MPh, FD1h, FD2h). The aqueous phase was further partitioned with chloroform and ethyl acetate to give the chloroform (MPc, FD1c, FD2c) and aqueous extracts (MPa, FD1a, FD2a). This procedure was repeated three times. The n-hexane and chloroform extracts were collected and concentrated by using rotary evaporator under reduced pressure at 40°C. Water in the aqueous extracts was removed by freeze-drying overnight to produce dried extracts. These dried extracts were then stored at -20°C until required for further testing.

3.1.2 Fractionation of plant extracts

A sample (500 mg) of the crude chloroform extract was suspended in 2 mL of absolute methanol and applied onto a chromatographic column (2.3 x 40 cm) packed with Sephadex LH-20 (GE Healthcare, UK) and equilibrated with absolute methanol. The column was exhaustively washed with absolute methanol. The sample was eluted with absolute methanol at a flow rate of 4 mL/min and 6 mL fractions were collected using a Retriever® II fraction collector in soda glass test tubes. 140 fractions were collected for each plant extract. Eluates were then pooled into major fractions based on the TLC profiles. After evaporation of methanol, the samples were left to air-dried and residues weighed.

3.1.3 Thin Layer Chromatography (TLC)

All fractions were examined by a thin layer chromatography (TLC) methodology on silica gel plates (TLC Silica gel 60 F₂₅₄, Merck KGaG, 64271 Darmstadt) using (a) Chloroform-Methanol (80:20, v/v) as mobile phase. The bands were visualized at white light, 254 nm and 366 nm wavelengths before and after derivatization with anisaldehyde using CAMAG TLC Visualizer.

3.1.4 Preparation of stock solution of extracts and fractions

Stock solutions for each plant extracts and fractions were prepared by dissolving them in Dimethyl sulfoxide (DMSO) Cell culture grade (Sigma-Aldrich[®], UK) at a concentration of 50 mg/ml. All plant extracts and fractions were cold sterilized using a syringe-driven filter (pore size: 0.2 μ m) (Millex[®]) in a laminar flow cabinet before being used for cell-based assays.

3.2 Cell Lines

The following tumour cell lines were used: the PC3 cell line (ATCC Number: CRL-1435™) was kindly provided by Dr Cyril Bussy (Centre for Drug Delivery Research, UCL School of Pharmacy, UK), the DU145 cell line (ATCC Number: HTB-81™) was purchased from Sigma Aldrich UK, and was obtained from the American Type Culture Collection (ATCC) and the LNCaP clone FGC cell line (ATCC Number: CRL-1740™) was purchased from Sigma Aldrich UK, and was obtained from the American Type Culture Collection (ATCC). Human Dermal Fibroblasts, adult (HDFa) catalogue number C-013-5C was kindly provided by Dr George Pasparakis (Department of Pharmaceutics, UCL School of Pharmacy), this cell line has been used for specificity study as it is not a tumour cell line. All tumour cell lines were used for the cytotoxicity, and PC3 cell line was used for mechanistic studies including apoptosis, cell cycle analyses, migration and invasion studies. All cell lines are adherent cells that tend to grow as monolayer and are classified as Biosafety Level 1. Three cell lines were used because of the different characteristics of each cell lines, which are important in order to achieve the objectives and aims of this study. LNCaP cells have androgen receptors that are functional enabling them to be androgen sensitive and these cells also secrete prostate-specific antigen (PSA). Both PC3 and DU145 cells are androgen independent but PC3 cells are highly invasive with strong metastatic potential as compared to DU145 cells.

3.3 Cell Culture Protocols

3.3.1 Sub-culture and Routine Maintenance

Both PC3 and LNCaP cell lines were grown in a cell culture flask (Nunc), surface area 75 cm² and maintained in RPMI-1640 (Roswell Park Memorial Institute medium) (Lonza, BE12-702F) containing L-glutamine. The media was supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco®, 10500-064) and 1% penicillin-streptomycin antibiotics containing 10000 Units/ml of penicillin and 10000 µg/ml streptomycin (Gibco®, 15140-122) to prevent bacterial growth. DU145 cell line was grown in a cell culture flask (Nunc), surface area 75 cm² and maintained in EMEM (Eagle's Minimum Essential Medium) (Sigma, M4655) containing Earle's salt, L-glutamine and Sodium bicarbonate. The media was supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco®, 10500-064) and 1% penicillin-streptomycin antibiotics containing 10000 Units/ml of penicillin and 10000 µg/ml streptomycin (Gibco®, 15140-122) to prevent bacterial growth. HDFa cell line was grown in a cell culture flask (Nunc), surface area 75 cm² and maintained in DMEM (Dulbecco's Modified Eagle Medium) containing L-Glutamine and high glucose (Gibco® 11965092). The media was supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco®, 10500-064), 1% 100X NEAA (non-essential amino acids, Gibco® 11140035), and 0.1% of both 10 mg/mL Gentamicin solution 1000X (Gibco® 15710049) and 250 µg/mL Amphotericin B solution 1000X (Gibco® 15290018) to prevent bacterial growth. All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The prepared media was used to grow and seed the cells in a 96-well plate (Nunclon™) for cellular based assays and for plant extracts as well as fractions dilution.

3.3.2 Cell passaging/sub-culturing

The growth media was changed every 2-3 days and all cell lines were sub-cultured every 3-4 days when the monolayer culture of the cells has reached almost 70-80% confluence. The cells were split into ratios of 1:3, 1:4 or 1:8. The media used in this monolayer culture was aspirated before the flasks were rinsed gently with 10 ml Phosphate buffered saline (PBS) pH 7.4 (Gibco, 10010-056). This procedure was done in order to remove traces of serum, which could cause inhibition to the action of trypsin. After that, the wash solution was removed and 1 ml of 0.25% trypsin EDTA (Gibco, 25200-056) was added. The 0.25% trypsin EDTA will act as a dissociation agent. The flask containing the cell lines was incubated for 5 minutes at 37°C in 5% CO₂. When the cells were ready, the cells began to change and transform into rounded shapes and detached from the surface of the flasks. These changes were observed by using an inverted microscope. After cell detachment, the proteolytic activity of trypsin was inhibited by the addition of 3 ml of growth media. A suitable amount of cell suspension, depending on the split ratio was transferred to a flask containing 20ml of pre-warmed media. The flask containing the cells was labelled with the cell name, passage number and date and was then carefully placed in an incubator (NuAire™ Autoflow CO₂ Air-Jacketed Incubator, NuAir Inc.) with humidified air of 5% CO₂ and atmosphere at 37°C (Wang 2006).

3.3.3 Cell Counting and Cell Viability

The dye exclusion test is normally used in cell culture to determine the number of viable cells present in a cell suspension. This method is based on the principle that live cells possess intact cell membrane, which will exclude certain dyes whereas dead cells do not. In this study, Trypan blue exclusion test using Neubauer improved bright-line haemocytometer (FORTUNA®, Germany) (Figure 3.1) was used for cell counting. Trypan blue (Gibco, 15250-061) is excluded by live cells but accumulates in dead cells. A clear coverslip was placed on a

haemocytometer slide. 50 μL of cell suspension was mixed with 50 μL trypan blue. The cell suspension mixture was carefully transferred to the edge of the coverslip. The number of stained (non-viable) cells and non-stained (viable) cells were counted under an inverted microscope. Concentration of viable cells and the percentage of viable cells are calculated using the following formula:

$$\text{Cells/ml} = \text{the average count per square} \times \text{dilution factor} \times 10^4$$

$$\text{Total cells} = \text{cell/ml} \times \text{original volume from which sample is removed}$$

The percentage viability is calculated as shown below:

$$\text{Percentage viability} = \frac{\text{Number of unstained cells} \times 100}{\text{Total number of stained and unstained}}$$

This test was used to count cells for experimental setup and also for the proliferation analysis of all cell lines in 75 cm^2 T- flask (Wang 2006).

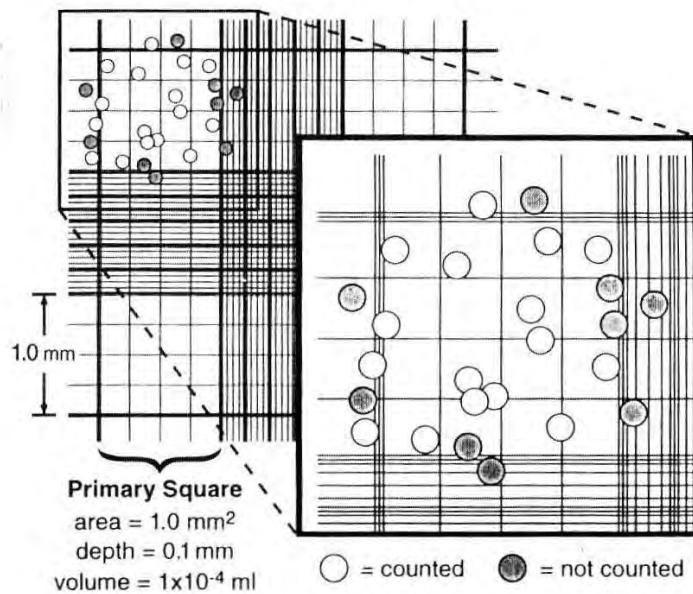


Figure 3.1 Haemocytometer (improved Neubauer), magnified view of the total area of the grid showing viable cells as unstained and clear, with a refractile ring around them and non-viable cells are dark and have no refractile ring (Freshney 2005).

3.3.4 Cell Cryopreservation

Healthy PC3, DU145, LNCaP, and HDFa cells at log phase (exponential phase) were used for the freezing procedure. The healthy cells needed to be trypsinized and centrifuged at 1000 rpm (Biofuge primo®) before the addition of the freeze medium. The freezing medium was prepared manually and contained 90% FBS and 10% DMSO or 90% complete media and 10% DMSO. The freezing medium was slowly added to the cell suspension containing approximately 10^6 – 10^7 cells/ml of cells concentration. Cells were then aliquoted at 1 ml and transferred into cryogenic vials. The cryogenic vials were labelled with the name of the cell lines, passage number and date. Before putting the cryogenic vials containing the cells into a liquid nitrogen tank, the vials were kept inside a freezing container (Nalgene Cryo™) containing 250 ml isopropanol to achieve a $-1^{\circ}\text{C}/\text{min}$ cooling rate and stored at -80°C overnight. On the next day the vials were then transferred into a liquid nitrogen Dewar with the temperature of -200°C and stored until needed (Wang 2006).

3.3.5 Cell Recovery

The cryopreserved cells are fragile and the thawing procedure is stressful to them, so careful handling is required to ensure a high proportion of cell survival. The cryogenic vial containing the cells was slowly and carefully removed from the liquid nitrogen storage. The cells were then thawed by gently agitating the vial in a water bath set at 37°C temperature until it was completely thawed. After thawing the content of the vials, the cells were transferred into a pre-warmed media and centrifuged at 1000 rpm. After that, the supernatant was discarded from the centrifuge tube leaving a cell pellet. The cell pellet was suspended in a culture flask containing 20 ml of growth media previously equilibrated at 37°C in 5% CO_2 and the flask was labelled with the cell name, split ratio, passage number, and date. The flask was placed back in an incubator

at 37°C in a humidified atmosphere with 5% CO₂ then the cell lines were subcultured as described earlier in section 2.4.2 (Wang 2006).

3.4 Proliferation and viability analysis

To measure the viability and/or proliferation of cultured cells, the Sulforhodamine B (SRB) and the (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays were chosen, respectively. Two different assays were chosen as both assays utilize different mechanisms in assessing cells proliferation and viability. The SRB assay determines cell's proliferation by staining the cellular protein contents whereas the MTT assay determine cell's viability by measuring the mitochondrial activity based on the conversion of MTT into formazan crystals by living cells. The total mitochondrial activity is related to the number of viable cells. Therefore, the use of these two assays was important for results validation.

PC3, DU145, LNCaP, and HDFa cells were seeded in 96-well flat-bottom microtiter plate at a density of 2×10^4 cells/well for proliferation and viability assays. The number of cells seeded in the 96-well flat-bottom microtiter plate for each cell lines were determined using an optimization procedure. This specific cells seeding was used because it will result in 80% of the space in each well to be occupied by each cancer cell lines. Therefore, it allows the cells especially in the negative control (untreated cells) to grow within the 72 hours incubation period without running out of space for growth. Lack of growing space will cause the cells to die either via apoptosis or necrosis thus influencing the results of the experiment. 72 hours of incubation time after the treatment with the active extracts or active fractions of the plants was chosen because at this period, the cells are at the exponential phase of growth for the specific cell lines used in this study (PC3, DU145 and LNCaP). Figure 3.2 shows the growth curve for cells,

which consist of lag phase, exponential phase, stationary phase and death phase. A complete understanding on the growth profile for each cell lines used in any scientific studies is important in order to produce reliable data. The plates were then incubated at 37°C in 5% CO₂ in order to allow the cells to grow and reach confluence. After 24 hours of incubation, the cells were treated with different concentration of extracts or fractions, vehicle control (DMSO) (Sigma, D2650), and Paclitaxel as positive control (Sigma, T7402-5MG). All the preparation was done in the culture media within a laminar flow cabinet.

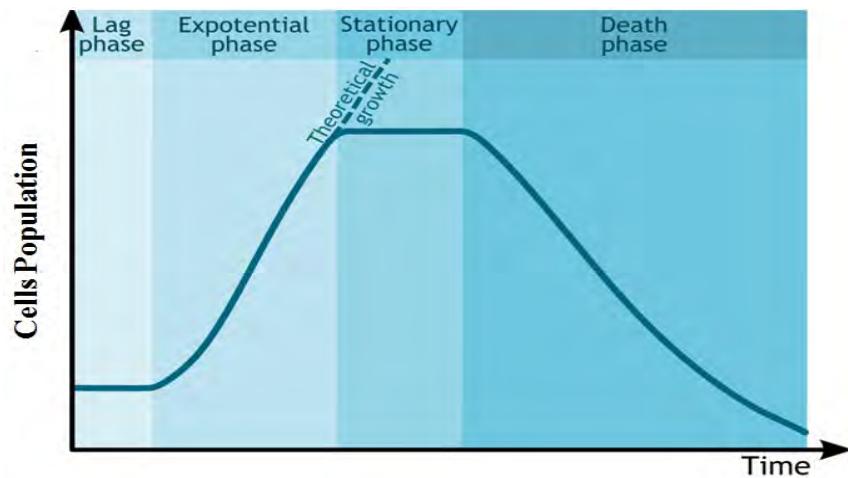


Figure 3.2 Phases of Cells growth (Freshney 2005)

3.4.1 Proliferation assay (SRB)

The sulforhodamine B assay (SRB) is a colorimetric assay characterized by simplicity with a high level of reproducibility (McCaffrey, Agarwal et al. 1988, Vichai and Kirtikara 2006). In this study, the SRB assay was performed according to a previously described method by Skehan, Storeng et al. (1990). In this method, cell number is estimated by the determination of cell density based on the staining of proteins with SRB. Under mild acidic condition (trichloroacetic

acid, TCA), the SRB has the ability to bind to the protein compounds of fixated cells providing a sensitive index of cellular protein content. As the binding is stoichiometric, an increase or decrease in the number of cells (total biomass) results in a corresponding change in the amount of dye extracted from stained cells (Skehan, Storeng et al. 1990, Vichai and Kirtikara 2006).

The cells were seeded in 96 well plates and plant extracts or fractions were added. After the incubation period of 24, 48, and 72 hours, 50 μ L of cold 40% w/v trichloroacetic acid (TCA) solution (Sigma, T6399-500G) in deionised water was added to the cell culture supernatant for cell fixation. The plates were incubated at 4°C for 1 hour and then rinsed four times with distilled water. The TCA-fixed cells were stained by adding 100 μ L of SRB solution (0.4% SRB (Sigma, S1402-5G0 in 0.1% acetic acid) and left at room temperature for 1 hour. After that, the plates were quickly rinsed four times with 1% acetic acid and flicked to remove unbound dye and then left to air-dry overnight.

After drying completely, 100 μ L of 10 mM Tris buffer solution (Sigma, T1503-25G) was added to each wells and the plates agitated in an orbital shaker for 5 minutes, to allow for the solubilisation of the SRB-protein complex. The optical density (OD) was measured at 510 nm by using a microtiter plate reader (Tecan Infinite® M200). The percentage of change in cell growth was calculated as follows:

$$\% \text{ Of } \text{ viable } \text{ cells } = [\text{OD } (\text{sample}) - \text{OD } (\text{Blank})] / [\text{OD } (\text{Control}) - \text{OD}(\text{Blank})] * 100.$$

This assay was performed independently in triplicate.

3.4.2 Mitochondrial viability Assay (MTT)

MTT is a colorimetric assay that can be used to measure cell viability. This assay assesses cell viability by measuring the activity of NAD(P)H-dependent cellular oxidoreductase enzymes which reflect the number of viable cells present. The yellow tetrazolium dye, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma, M5655-100MG) is reduced by metabolically active cells, in part by the action of mitochondrial dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be dissolved in acidified isopropanol and quantified by spectrophotometric means (microtiter plate reader). The amount of formazan formed is directly proportional to the number of metabolically active cells.

Normally, the MTT assay is used for cytotoxicity studies of a drug or a plant extract on certain types of cell and in this case, the PC3, DU145 and LNCaP cell lines. The cells were seeded in microplates and extracts were added as detailed earlier (section 3.1). The MTT assay was performed according to the original method (Mosmann 1983) with slight modifications.

After the 24, 48, and 72 hours incubation period, 10 μ L of the MTT solution (5 mg/ml dissolved in PBS) was added into all wells and incubated again for 4 hours in a humidified atmosphere at 37°C. After 4 hours, both the cell media and the MTT solution were removed from the wells and 200 μ l of DMSO was added in each well in order to allow dissolution of the purple MTT-formazan crystals.

The absorbancy (optical density, OD) was measured at wavelength of 570 nm and reference wavelength 630 nm with a microplate reader (Tecan Infinite® M200) within one hour after adding MTT solvent. The relative difference to control was determined by;

$$\text{Relative difference to control} = \frac{\text{OD (sample)} - \text{OD (Blank)}}{\text{OD (Control)} - \text{OD(Blank)}}$$

The assay was performed independently in triplicate.

3.4.3 Cells morphology

PC3 and LNCaP cells were seeded in 12 well plates and left to attach and proliferate for 48 hours of incubation time. Then the plant extracts were added, the morphology and the population of the cells were monitored using an EVOS® FL Imaging System at 24, 48 and 72 hours.

3.5 Apoptosis detection

The number of cells in a multicellular organism is highly regulated. This process does not only involve controlling the rate of cells division but also by controlling the rate of cell death. When cells are no longer needed or become a threat to the organism, these cells could be destroyed by a tightly regulated cell suicide process known as programmed cell death or apoptosis (Alberts, Johnson et al. 2002). Apoptosis is a complex process and proceed through at least two main pathways namely extrinsic and intrinsic (Elmore 2007). Both pathways are mediated by an intracellular proteolytic cascade involving a family of cysteine proteases known as Caspases (Alberts, Johnson et al. 2002). Apoptosis can be described by its morphological characteristics, which include cells shrinkage, membrane blebbing, nuclear fragmentation, chromatin

condensation, and global mRNA decay (Kerr, Winterford et al. 1994). In cancer study, the discovery of bioactive compound/s that could induce cancer cells death via apoptosis is more favorable than necrosis. Necrotic cell death leads to a spill of cells content all over their neighbors causing a potentially damaging inflammatory response whereas apoptotic cells shrink and condense to form a structure called apoptotic bodies which can be rapidly phagocytosed by macrophages preventing any leakage of the cells contents (Kerr, Winterford et al. 1994). Figure 3.3 shows the flowchart for the screening cascade used in determining prostate cancer cell death via apoptosis.

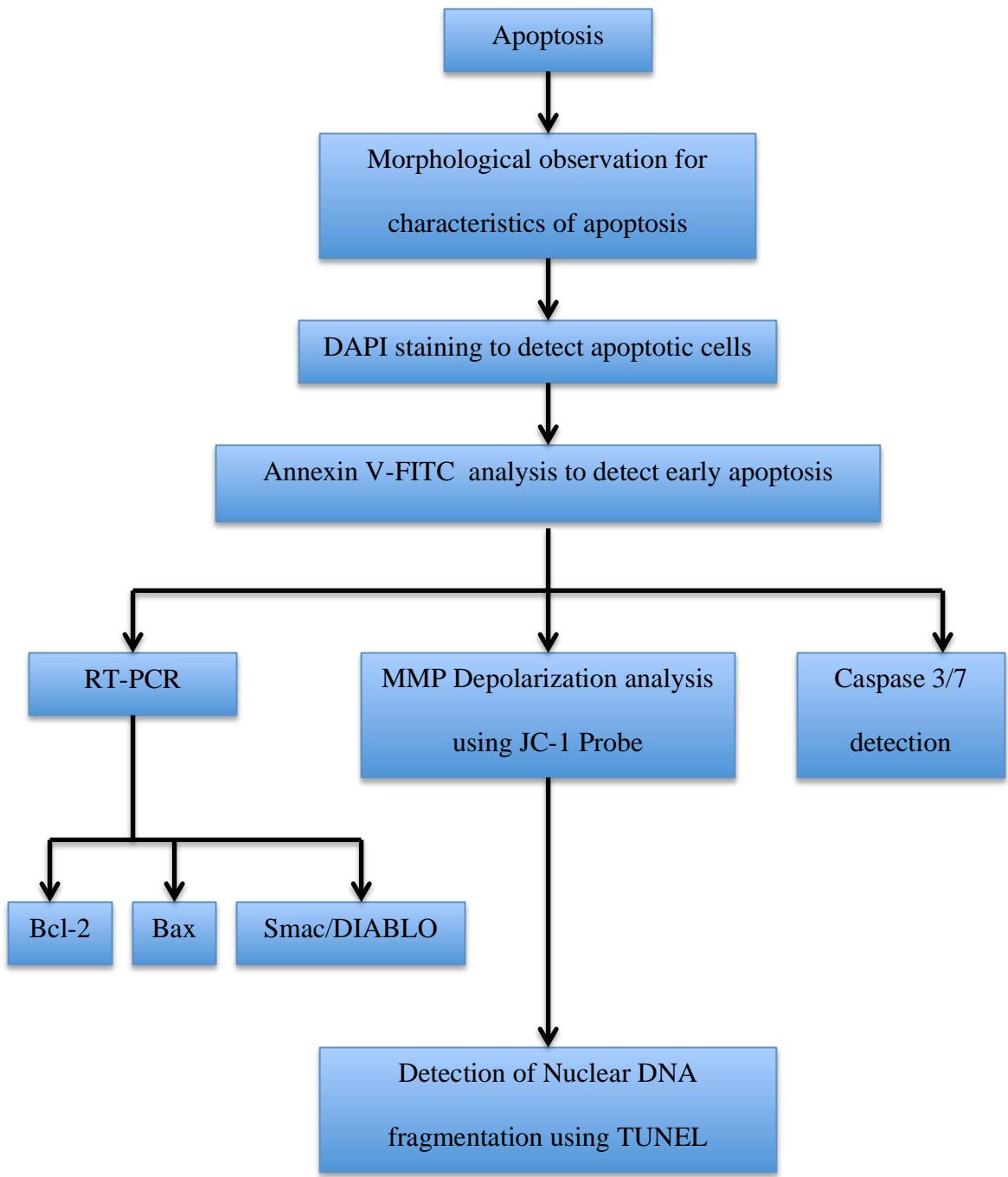


Figure 3.3 Screening cascade for Apoptosis

3.5.1.1 4'-6-Diamidino-2-phenylindole (DAPI) staining

This method was used to determine the presence of apoptotic cells. 1×10^5 cells/well of PC3 and LNCaP cells were seeded in 12 wells plates. After 48 hours, the cells were treated with plant extracts and positive control for 72 hours. After 72 hours the cells were rinsed with 1 mL of 1X PBS and fixed with 4% formaldehyde for 10 minutes. After 10 minutes, the cells were treated with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. After washing with PBS, the cells were stained with DAPI (1 μ g/mL) and incubated in the dark for 30 minutes. The cells were then viewed and photographed using the EVOS® FL Imaging System.

3.5.1.2 Annexin V-FITC and Propidium Iodide staining

PC3 and LNCaP cells were seeded in 12 well plates and incubated for 48 hours. After incubation, plant extracts and Paclitaxel (positive control) were added and further incubated for 6 hours at 37 °C in a 5% CO₂ atmosphere. The cells were then washed with PBS and detached with 0.25% Trypsin-EDTA solution. The cells were then re-suspended in 100 μ L of 1X binding buffer and 10 μ L of Annexin V-FITC were added. The mixture was mixed carefully and incubated for 15 minutes in the dark at room temperature. After 15 minutes, the cells were washed again with 1mL of 1X binding buffer and centrifuged at 300xg for 10 minutes. Subsequently the cells were re-suspended in 500 μ L of 1X binding buffer and Propidium Iodide (PI) solution were added prior to flowcytometry (MACSQuant® Analyzer 10) analysis.

3.5.1.3 Caspases 3/7 activity

In this study, the apoptosis induced by plant extracts was determined by measuring the activity of Caspases 3 and 7 using an apoptosis detection kit (Promega, G8091). The Caspase-Glo® 3/7 assay was performed according to the manufacturer's protocol. PC-3, DU145 and LNCaP cells were seeded in 96 well plate at 2.5×10^3 cells/well. The cells were left for 24 hours to allow cells attachment. After 24 hours the cells were treated with extracts, fractions, vehicle control (DMSO), Paclitaxel and a blank (cell-free medium) for 48 hours. 100 μ l of Caspase-Glo® 3/7 reagent was added to each wells after 48 hours incubation period and mixed gently for 30 seconds. Then, the plate was incubated for 1 hour at room temperature. After the completion of the incubation period, the luminescence of each plant extracts was measured using a plate-reading luminometer (Tecan Infinite® M200). The assay was performed independently in triplicate and the results were calculated using the following equation:

$$\text{RLU} = \text{Luminescence (samples)} - \text{Luminescence (blank)}$$

3.5.1.4 Determination of Mitochondrial Membrane potential (MMP)

The change in mitochondrial transmembrane potential ($\Delta\psi_m$) induced by the active extracts of the plants in prostate cancer cell line (PC3) was determined by using flowcytometry technique with the appropriate fluorescent probe known as JC-1 (Isenberg and Klaunig 2000). 1×10^6 cells/ml of PC3 was seeded into 6 well plate and incubated for 48 hours. After incubation, plant extracts and Paclitaxel (positive control) were added and further incubated for 6 hours at 37 °C in a 5% CO₂ atmosphere. The treated cells were then labeled with 2 μ M of JC-1 for 15 minutes at 37 °C and washed with warm phosphate-buffered saline (PBS). The cells were analyzed on a flowcytometer

(MACSQuant® Analyzer 10) with 488 nm with 530- and 585 nm pass emission filters. CCCP-treated cells (10 μ M) were taken as positive controls.

3.5.1.5 Terminal deoxynucleotidyl transferase-mediated biotin dUTP Nick End Labeling assay

To detect apoptotic cells, *in situ* end labelling of the 3'OH end of the DNA fragments generated by apoptosis-associated endonucleases was performed using the Dead End apoptosis detection kit (dUTP Nick End Labeling, TUNEL assay) from Promega (Madison, USA). Briefly, the cells were grown in LabTek II chamber slide and treated with plant extracts for 72 hours. The cells were washed in phosphate-buffered saline and fixed by immersing the slides in 4% paraformaldehyde for 25 min. All the steps were performed at room temperature, unless otherwise specified. They were then washed twice by immersing in fresh phosphate-buffered saline for 5 min. Cells were permeabilised with 0.1% Triton X-100 solution in phosphate-buffered saline for 5 min, washed twice in phosphate-buffered saline, and then covered with 100 μ L of equilibration buffer and kept for 5–10 min. The equilibrated areas were blotted around with tissue paper, and 50 μ L of terminal deoxynucleotidyl transferase (TdT) reaction mix was added to the sections on the slide and were then incubated at 37 °C for 60 minutes inside a humidified chamber for the end-labelling reaction to occur. Immersing the slides in 2X sodium chloride–sodium citrate buffer for 15 minutes terminated the reactions. The slides were washed three times by immersing them in fresh phosphate-buffered saline for 5 minutes to remove unincorporated biotinylated nucleotides. The slides were then immersed in a freshly prepared 1 μ g/mL propidium iodide solution for 15 minutes at room temperature in the dark. The washing procedure was repeated after 15 minutes and then the slides were immediately analyze under a fluorescence microscope (EVOS® FL Imaging System) using a standard

fluorescein filter set to view the green fluorescein at 520 ± 20 nm and the red fluorescence of propidium iodide at 620 nm.

3.6 Cell migration and Invasion study

The ability of cells to move or migrate is important for the development and maintenance of multicellular organisms. Processes including embryonic development, wound healing and immune responses require the orchestrated movement of cells in particular directions to specific locations. Cells migration is defined as the directed movement of cells within the body, which allow cells to change their position within tissues or different organs (Kramer, Walzl et al. 2013). Cells migration only occurs on 2D surfaces such as basal membranes, extracellular matrix (ECM) fibers or plastic plates without any obstructive fiber network (Kramer, Walzl et al. 2013). Cells invasion, which is defined as the ability of cells to penetrate through tissue barriers such as the basement membranes and the underlying interstitial tissues, occurs via a 3D matrix that requires restructuring and remodeling of the ECM (Friedl and Wolf 2010). Some malignant cancer cells have the ability to metastasize to other organs within the human body including advanced prostate cancer cells. Metastasis is a process, which involves infiltrative growth through the ECM, cells migration through blood and lymph vessels and rise of distant colonies (Gupta and Massagué 2006). Therefore, finding new strategies to control or inhibit cancer cells migration and invasion are important in cancer studies.

3.6.1 *In Vitro* cell migration assay

The *In Vitro* cell migration was determined by using the Oris™ 96-well cell migration assay kit (Platypus Technologies) (Figure 3.4) following the manufacturer's instruction. 5×10^4 of PC3 cells were seeded in each well and left for 24 hours. The stoppers that were used to create the migration zone were removed after 24 hours and the cells were washed with PBS to remove any unattached cells. 100 μ L of fresh media with or without the plant extracts were added to each well. The cells were allowed to migrate into the migration zone for 72 hours. Cells were fluorescently stained with CellTracker™ Green (Life Technologies). The seeded plate was incubated in a humidified chamber for 72 hours and at various time points (24h, 48h, 72h), the fluorescent signals in the detection zone were measured using a microplate reader (Synergy™ HT, BioTek) with 492 nm excitation and 517 nm emission filters.

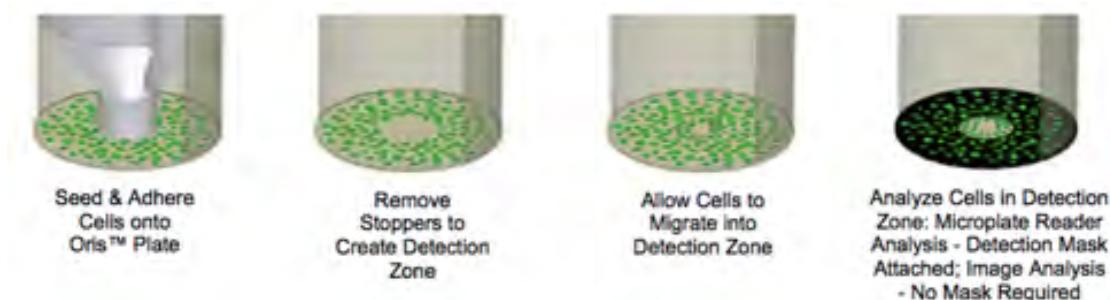


Figure 3.4 The schematic of Oris cell migration assay

3.6.1.2 Boyden chamber 3D migration assay

The cell migration study was also performed using the CytoSelect Cell Migration Assay kit (Catalogue number CBA-100-C purchased from Cell Biolab Inc.) (Ridley, Schwartz et al. 2003) (Figure 3.5). This kit contains polycarbonate membrane inserts (8 μ m pore size) in a 24 well plate. The membrane serves as a barrier to discriminate migratory cells from non-migratory cells. Migratory cells are able to extend protrusions towards chemoattractants (via actin cytoskeleton reorganization) and ultimately pass through the pores of the polycarbonate membrane. These migratory cells are then dissociated from the membrane and subsequently detected by a microtiter plate reader.

Under sterile conditions, the 24 well migration plate was allowed to warm up to room temperature for 10 min. Cell suspension containing 1.0×10^6 cells/ml in serum-free media was prepared. Fresh media (control) and media with respected plant extracts, were added directly to individual microwell inserts (8 μ m pore size) (Transwell[®], Corning[®], UK) with the cell suspension. Overnight serum starvation had been performed prior to running the assay. 500 μ L of media containing 10% fetal bovine serum was added to the lower well of the migration plate and 300 μ L of the cell suspension solution was added to the inside of each insert and then incubated for 24 hours in a cell culture incubator. After 24 hours incubation, the media were carefully aspirated from the inside of the microwell insert. The interior part of the inserts was washed with wet cotton swab in order to remove non-migratory cells. The inserts were transferred to a clean well containing 400 μ L of Cell Stain Solution (Crystal Violet dye) and incubated for 10 min at room temperature. Then, the inserts were gently washed in a beaker of water and left to air dry. Each inserts were transferred into wells containing 200 μ L of Extraction Solution (10% Acetic Acid), incubated for 10 minutes at room temperature on an orbital shaker. 100 μ L of each sample was

transferred to a 96 well plate and was measured at 560 nm by using a microtiter plate reader (Tecan Infinite® M200).

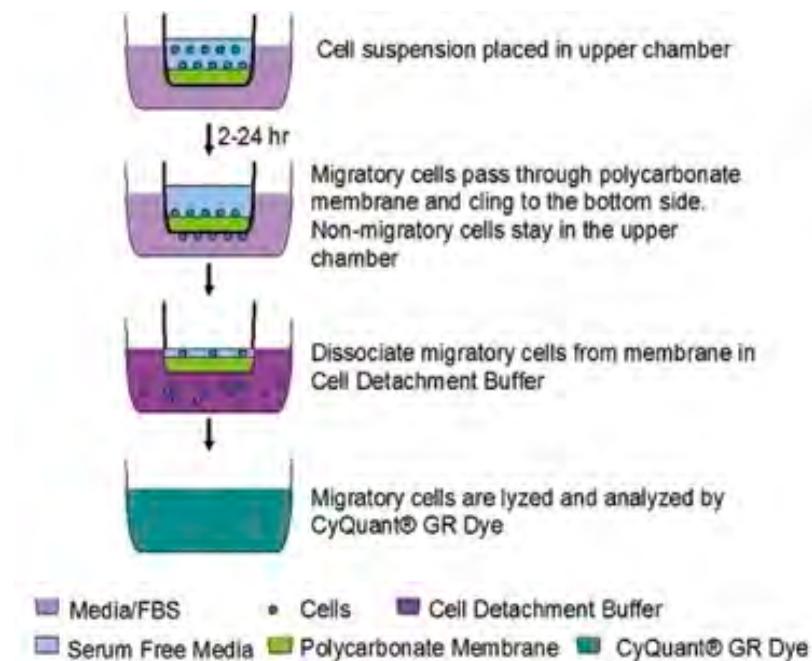


Figure 3.5 The schematic of Modified Boyden chamber 3D migration assay

3.6.2 In Vitro 3D cell invasion assay

The cell invasion was measured using a Cytoselect 24-well cell invasion assay kit (Catalogue number CBA-100-C purchased from Cell Biolabs, Inc.) (Figure 3.6). This kit included polycarbonate membrane inserts (8 μm pore size). The upper surface of the insert membrane was coated with a protein matrix isolated from Engelbreth-Holm-Swarm tumor cells. Basement membranes of Boyden chambers were rehydrated with 300 μL serum-free media, and 1×10^6 cells were then seeded into the upper area of the chamber in serum-free media (control) with or without the plant extracts. Overnight serum starvation had been performed prior to running the assay. 500 μL of media containing 10% fetal

bovine serum was added to the lower well of the migration plate. After incubation for 48 hours, the non-invading cells on the upper surface of the inserts were removed with a cotton swab and invading cells on the lower surface were stained with crystal violet Cell Stain Solution and incubated for 10 minutes at room temperature. Then, the inserts were gently washed in a beaker of water and left to air dry. Each inserts were transferred into wells containing 200 μ L of Extraction Solution (10% Acetic Acid), incubated for 10 minutes at room temperature on an orbital shaker. 100 μ L of each sample was transferred to a 96-well plate and was measured at 560 nm by using a microtiter plate reader (Tecan Infinite® M200).

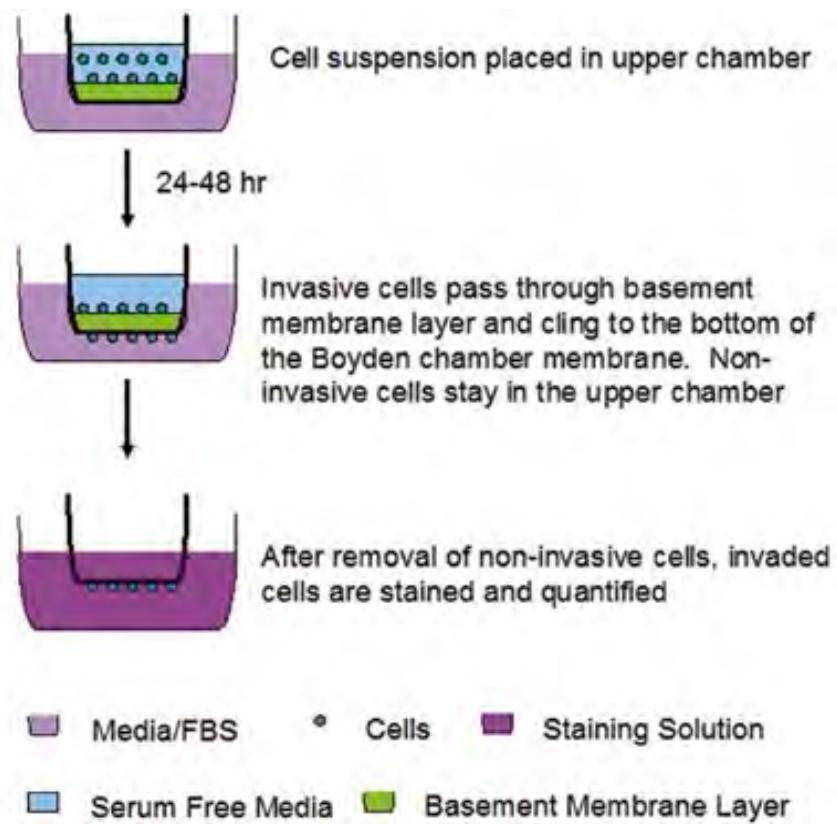


Figure 3.6 The schematic of Modified Boyden chamber 3D invasion assay

3.7 Cell cycle distribution study

PC3 and LNCaP cells were seeded in 6 well plates and incubated for 48 hours. Plant extracts and Paclitaxel (positive control) were added after 48 hours, and the cells were further incubated for 48 hours at 37 °C in a 5% CO₂ atmosphere. The cells were then washed and trypsinized with 0.25% Trypsin-EDTA solution for cells detachment. Cell pellet were re-suspended in 1 mL PBS and washed twice by adding 10 mL PBS. After that the cells were centrifuged at 300xg for 10 minutes at 4°C. Once the supernatant was removed, 1 mL of ice-cold 70% ethanol was slowly added drop by drop to the cell pellet. The cells were allowed to fix in ethanol for 18 hours. After 18 hours, the cells were centrifuged at 500xg for 10 minutes at 4°C. The supernatant was removed and 1 mL of staining solution containing 1 mg/mL Propidium Iodide and 100 Kunitz units/mL of RNase A were added to the cells and incubated for 30 minutes before being analysed using flowcytometry (MACSQuant® Analyzer 10).

3.8 Real-Time RT-qPCR Analysis

3.8.1 mRNA Extraction and cDNA Synthesis

After exposing PC3 cells (5×10^5 cells/well) to plant extracts, fractions and DMSO 1% for 96 hours, total RNA was extracted using RNeasy® Plus Mini (Qiagen) according to the manufacturer's protocol. Samples were treated with gDNA eliminator spin column (Qiagen) to avoid genomic DNA contamination. The quantity and quality of RNA was determined by differential readings at 260 and 280 nm wavelength using Nanodrop 2000 (Thermo Scientific). The integrity of total RNA from PC3 cells was assessed by visual inspection of the two rRNAs 28 and 18 s on agarose gels. cDNA was synthesized from 1 µg of total RNA by

using Omniscript® Reverse Transcription kit (Qiagen) as per the manufacturer's instructions in a final volume of 20 μ L.

3.8.2 RT-qPCR Conditions and Analysis

Sequence for the primers used in this study are listed in Table 3.2. All primers used in this study were designed and obtained from Primerdesign®. The RT-qPCR was carried out in 96-well plates using a PikoReal™ Real-Time PCR detection System (Thermo Fisher Scientific). Each well contained a final reaction volume of 20 μ L (10 μ L of *PrecisionFAST™* MasterMix with SYBR Green, 5 μ L of cDNA template diluted appropriately, 1 μ L of resuspended primer mix at a final concentration of 300nM and 4 μ L of RNase/DNase free distilled water). PCR reaction was performed using the following conditions: initial denaturation at 95°C for 2 minutes, then 40 cycles of denaturation at 95°C for 15 seconds, corresponding annealing temperature of each genes as listed in table 1 for 30 seconds and extension at 72°C for 30 seconds. At the end of the run, heating the amplicon from 60 to 95°C in order to confirm the specificity of the amplification for each primer pair generated a melting curve. All RT-qPCR were run in quadruplicates. Standard curves were produced to check the PCR efficiency using a fivefold dilution series of cDNA. Efficiency (E) of primer pairs was obtained from the slope of the calibration curve generated. The relative expression was calculated on the basis of 'delta delta Ct' ($\Delta\Delta Ct$) values. Normalization of the target genes was achieved by using GAPDH as a reference gene.

Table 3.2 Sequence of primers used in RT-qPCR analysis

Gene	Primer Sequences	Annealing temperature (°C)
GAPDH	Sense : 5'-ATGCTGGCGCTGAGTACGTC-3' Anti-sense : 5'-GGGCAGAGAGATGATGACCCTT-3'	55
BAX	Sense : 5'-ATGGAGCTGCAGAGGATGAT-3' Anti-sense : 5'-CAGTTGAAGTTGCCGTCAGA-3'	56.5
BCL-2	Sense : 5'-GAGGTCACGGGGGCTAATT-3' Anti-sense : 5'-GAGGCTGGGCACATTTACTG-3'	56.8
ALOX5	Sense : 5'-AAGCGATGGAGAACCTGTTCA-3' Anti-sense : 5'-GTCTTCCTGCCAGTGATTGATG-3'	56.8
VEGFA	Sense : 5'-TGCTCTACTTCCCCAAATCACT-3' Anti-sense : 5'-CTCTCTGACCCCGTCTCT-3'	57.6
Smac DIABLO	Sense : 5'-GCACAGAAATCAGAGCCTCATT-3' Anti-sense : 5'-TTCAATCAACGCATATGTGGTCT-3'	56.4
CXCR4	Sense: 5'-CCAAAGAAGGATATAATGAAGTCACT-3' Anti-sense : 5'-GGGCTAAGGGCACAAGAGA-3'	56.4
CXCL12	Sense : 5'-CTCCTCTTCAACCTCAGTGATT-3' Anti-sense : 5'-GAGAAGCAGAAGCAAGATTAAGC-3'	56.8

3.9 HPLC-DAD (High Performance liquid chromatography-diode array detector)

HPLC-DAD chromatograms were obtained on an Agilent 1200 series HPLC system (Agilent, USA). Fingerprint analysis of plant extracts was conducted as described by Springfield, Eagles et al. (2005). All samples were dissolved in methanol ($\geq 99.9\%$ for HPLC, Sigma-Aldrich) and mixed properly using an ultrasonic bath. The concentration of each extract was equivalent to 1 mg/mL. A volume of 5 mL of each sample was filtered through a $0.45\mu\text{m}$ filter before analysis. The rest were evaporated to dry and stored in a freezer. The filtered samples (10 μL) were injected for HPLC analysis. The stationary phase was Agilent C18 column (250mm x 4.6mm id, 5 μm). Samples were eluted with a mobile phase consisted of formic acid solution (A, 0.1 % v/v) and acetonitrile (B, $\geq 99.9\%$ for HPLC, Fisher Scientific) using a linear gradient program (5% B in 0–5 min, 5–15% B in 5–20 min, 15–20% B in 20–35 min, 20–35% B in 35–45 min, 35–100% B in 45–60 min). The flow rate was 1.0 mL/min and the chromatogram was detected at wavelengths of 210nm, 269 nm, 280nm, 365 nm. The data was collected and processed with the Agilent Chemstation[®] Edition software (Agilent).

3.10 Nuclear Magnetic Resonance (NMR)

Samples for NMR characterization were prepared by dissolving in 0.8 ml of appropriate deuterated solvents. All NMR spectra were obtained using Bruker Avance 500 MHz NMR spectrometer equipped with broadband and triple resonance (^1H , ^{13}C and ^{15}N) inverse probe in order to identify the correlation between protons and protons as well as protons and carbons. 2D experiments carried out include (i) COSY (Correlation Spectroscopy) that analyses direct correlation between a proton and its coupled partner, (ii) HMQC (Heteronuclear Multiple-Bond Correlation), shows correlation between carbons and protons, (iii)

HMBC (Heteronuclear Multiple-Bond Correlation), shows the present of long-range couplings between protons and carbons in the distance of two or three bonds, and (iv) NOESY (Nuclear Overhauser Effect Spectroscopy), which shows correlations between protons, through bonds and spaces.

3.11 Mass spectrometry

The mass spectrometric analyses were performed by the Research Services Unit of the UCL School of Pharmacy. The molecular weight of the isolated compounds was determined by electrospray ionisation mass spectrometry (ESI-MS), using both positive and negative mode on a LCQ Duo Ion-Trap mass spectrometer (Thermo Finnigan). The samples were run in either 50% methanol in water or a solvent it is best soluble in, as a mobile phase under the following conditions; sheath gas flow rate (20-100 units), auxiliary gas flow rate (0 - 60 units), ion spray voltage (1-8 KV), spray current (0.34uA), capillary temperature (100-220°C), capillary Voltage (38-100 V) and tube lens offset (0-40V).

3.12 Fourier Transformed Infrared spectroscopy (FT-IR)

The FT-IR spectra of the compounds were recorded on a Perkin Elmer precise spectrum 100 FT-IR spectrometer as a thin film.

3.13 Ultra High Performance Liquid Chromatography (UHPLC) Mass Spectrometry (MS)

This study was done in collaboration with Prof Jean-Luc Wolfender and funded by a Plants for Health Award (Appendix 1). The active fractions of FD1c and FD2c were dried and sent to Phytochimie et Produits Naturels Bioactifs, Université de Genève for analysis. The samples were analysed according to standard protocols previously published with slight modifications (Yang, Liang et al. 2014).

3.14 Statistical Analysis

Data collected and reported in this study is expressed as a mean \pm standard deviation. Statistical analysis of the data was carried out using the GraphPad software (GraphPad Software Inc, La Jolla, CA, USA). The values of $p < 0.05$ were considered to be statistically significant. All experiments were conducted three times independently in triplicate.

The Growth Inhibitory concentration 50 (GI50) values were taken from the minimal experimental concentration showing 50% cell death and calculated using GraphPad Prism 5. Each plant extracts was compared to the control group with GraphPad by using the student's t-test to determine any significant effect of the extract. Other statistical calculations (averages, standard deviation, etc.) in this study were calculated using Microsoft Excel (Microsoft, Redmont, USA)

Chapter 4

Results and Discussions

4 Results and Discussion

These experiments were conducted to study the *in vitro* effects of the plant extracts on three prostate cancer cell lines, LNCaP, DU145 and PC3. These cell lines represent two different stages of prostate cancer, LNCaP (Stage 1, hormone-dependent prostate cancer), DU145 and PC3 (Stage 2, hormone-independent prostate cancer). The cytotoxicity of the *n*-hexane, chloroform and aqueous extracts against LNCaP, DU145 and PC3 cell lines were measured by performing SRB and MTT assay after 72 hours of exposure to each respective extracts. Extracts exhibiting potential activity based on the results of the cytotoxicity assays were subjected to further mechanistic studies.

4.1 Results

4.1.1 Plant extractions

All plant materials were subjected to a solvent-solvent partitioning process with *n*-hexane, chloroform and ethyl acetate as described in the previous section (3.1.1). The yields of each fraction are presented in Table 4.1.

Table 4.1 The yields of *Marantodes pumilum*, *Ficus deltoidea* 1 (FD1), *Ficus deltoidea* 2 (FD2), and *Ficus deltoidea* 3 (FD3)

Plant	Extract	Plant Sample (g)	Yield (%)
<i>Marantodes pumilum</i>	Hexane	50	0.11
	Chloroform	50	0.42
	Aqueous	50	3.44
	Ethyl Acetate	50	0.49
<i>Ficus deltoidea</i> 1 (FD1)	Hexane	50	0.27
	Chloroform	50	0.96
	Aqueous	50	13.18
<i>Ficus deltoidea</i> 2 (FD2)	Hexane	50	0.22
	Chloroform	50	0.65
	Aqueous	50	13.10
	Ethyl Acetate	50	1.95
<i>Ficus deltoidea</i> 3 (FD3)	Hexane	50	0.24
	Chloroform	50	0.25
	Aqueous	50	6.92
	Ethyl Acetate	50	1.79

4.1.2 HPLC Fingerprint

4.1.2.1 HPLC Fingerprint analysis of *Marantodes pumilum* and *Ficus spp.*

The establishment of a standard fingerprint for various plant extracts is very important due to the high chemical complexity of the plant extracts. The fingerprint accounts for the variability in the chemistry of the plants depending on the variety, climates, and places. Chang, Ding et al. (2008) reported that chromatographic fingerprint is a powerful tool for the analysis of multi-component of herbal medicines and plant extracts. The Chinese State Food and Drug Administration (SFDA) and the World Health Organization (WHO) have accepted chromatographic fingerprint as an identification and quality evaluation technique for herbal medicines (Chang, Ding et al. 2008). The chromatographic fingerprints of the different plant extracts (hexane, chloroform and aqueous) are shown in Figure 4.1 to Figure 4.12. The acetonitrile: 0.1% formic acid solution was used as the solvent, and the peak distributions of various extracts were detected by using a reverse-phase column with ultraviolet (UV) detection at different wavelengths (210nm, 254nm, 269nm, 280nm and 365nm). Table 4.2 summarizes the distribution of the most characteristic peaks for *Marantodes pumilum* (MP) and three different varieties of *Ficus deltoidea* plant extracts as analysed by High Performance Liquid Chromatography (HPLC).

Table 4.2 The distribution of most characteristics peaks for *Marantodes pumilum* (MP) and three different varieties of *Ficus deltoidea* plant extracts as analysed by High Performance Liquid Chromatography (HPLC). Results shown are representative of three independent experiments.

Sample	Extract	Peak Region					
		t_R 0 to t_R 10	t_R 11 to t_R 20	t_R 21 to t_R 30	t_R 31 to t_R 40	t_R 41 to t_R 50	t_R 51 to t_R 60
<i>Marantodes pumilum</i> (MP)	n-Hexane		t_R 19	t_R 29	t_R 34, t_R 36, t_R 38		t_R 54, t_R 56
	Chloroform	t_R 10	t_R 16, t_R 19	t_R 22, t_R 27		t_R 47, t_R 50	t_R 54, t_R 56
	Aqueous	t_R 2, t_R 3	t_R 14, t_R 15				
<i>Ficus deltoidea</i> 1 (FD1)	n-Hexane	t_R 10	t_R 16	t_R 30			
	Chloroform		t_R 11, t_R 16				
	Aqueous	t_R 10	t_R 16				
<i>Ficus deltoidea</i> 2	n-Hexane		t_R 13, t_R 14, t_R 16, t_R 19	t_R 27, t_R 30	t_R 32, t_R 35, t_R 37		t_R 56

(FD2)	Chloroform		t_R 11, t_R 16	t_R 22, t_R 24, t_R 27			t_R 52, t_R 56
	Aqueous	t_R 12, t_R 16, t_R 17					
<i>Ficus</i> <i>deltoidea</i> 3 (FD3)	n-Hexane			t_R 30,	t_R 32, t_R 35		t_R 59
	Chloroform		t_R 11	t_R 21, t_R 22			
	Aqueous		t_R 15, t_R 16				

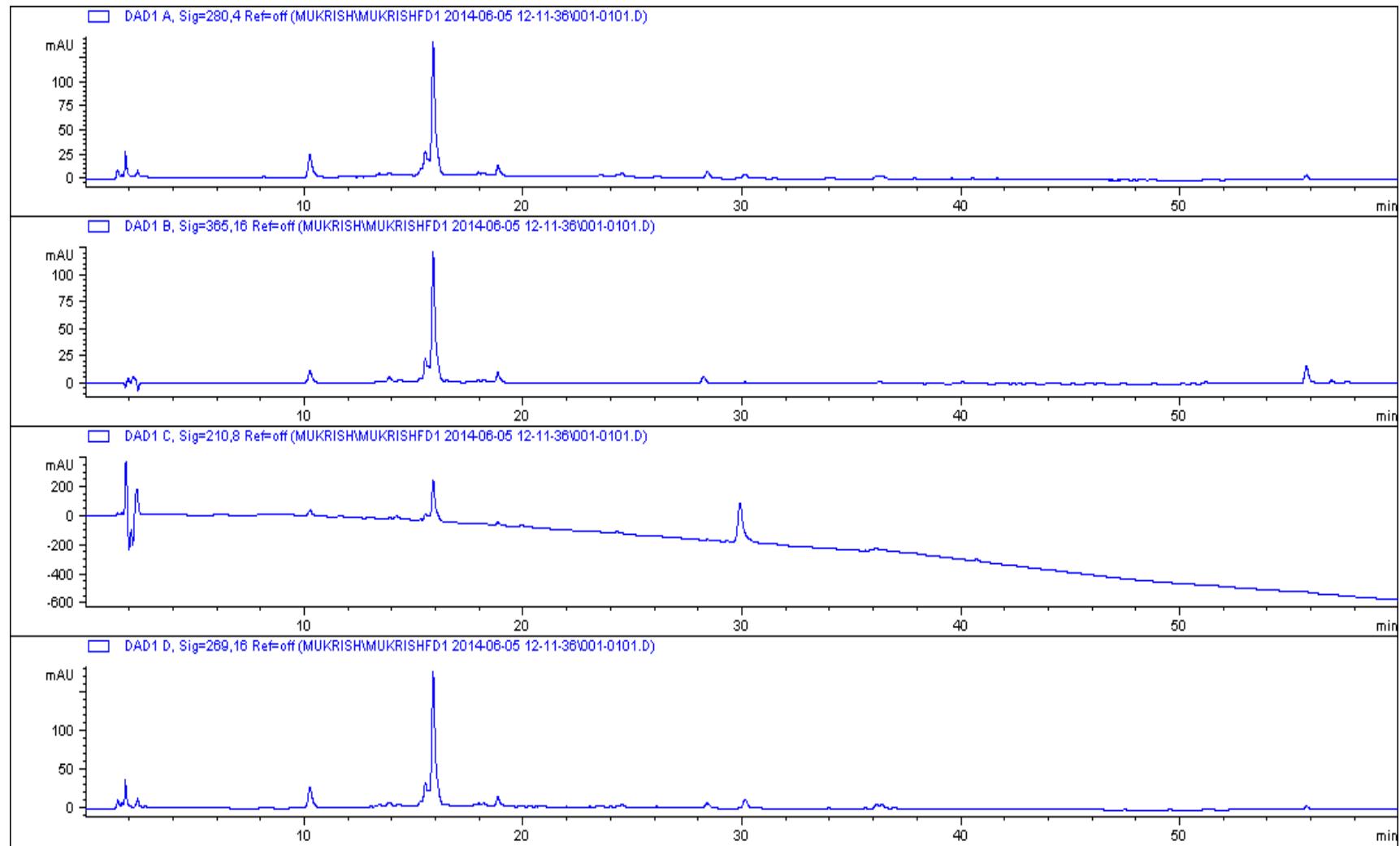


Figure 4.1 HPLC fingerprint of *Ficus deltoidea* 1 hexane extract.

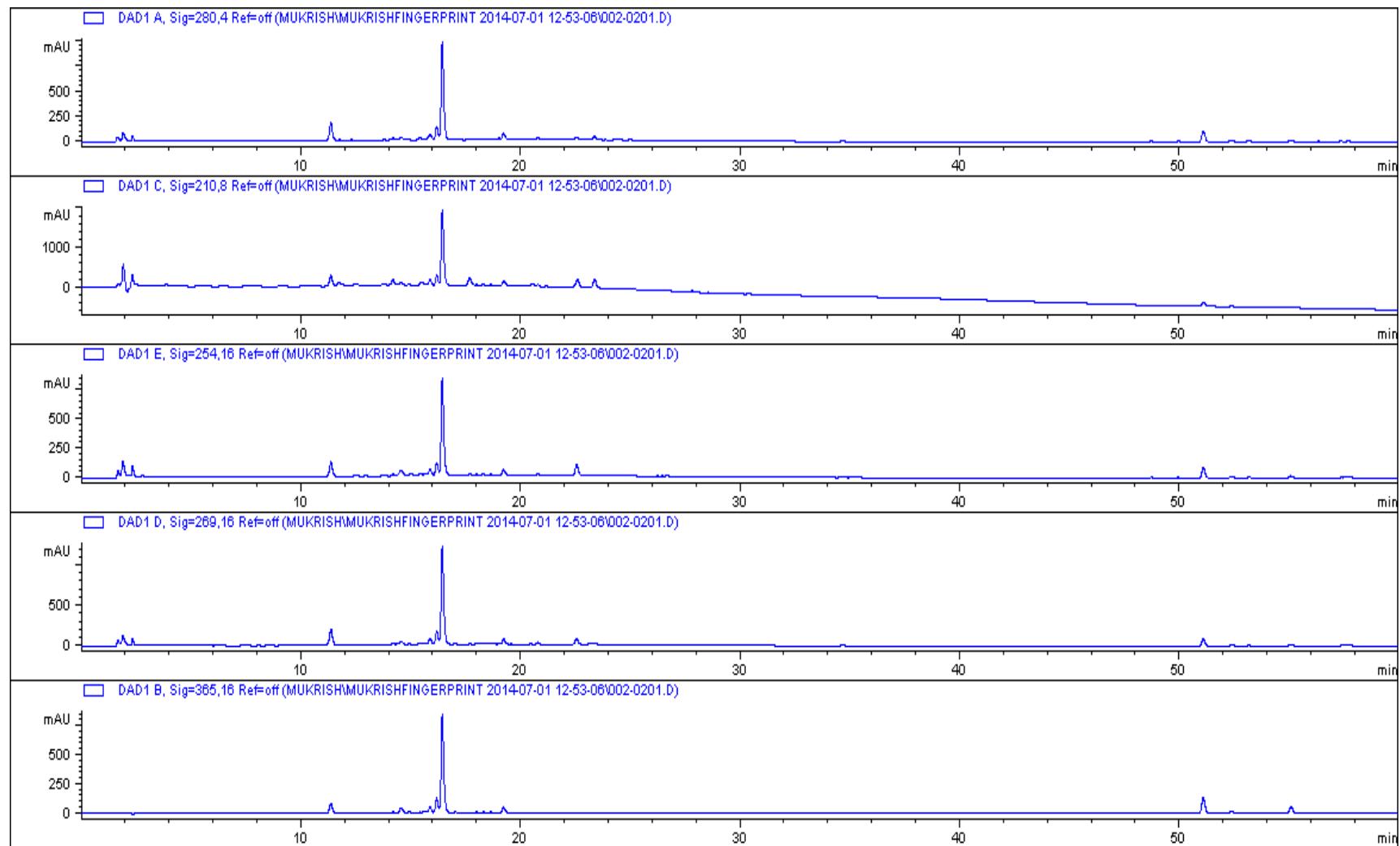


Figure 4.2 HPLC fingerprint of *Ficus deltoidea* 1 chloroform extract.

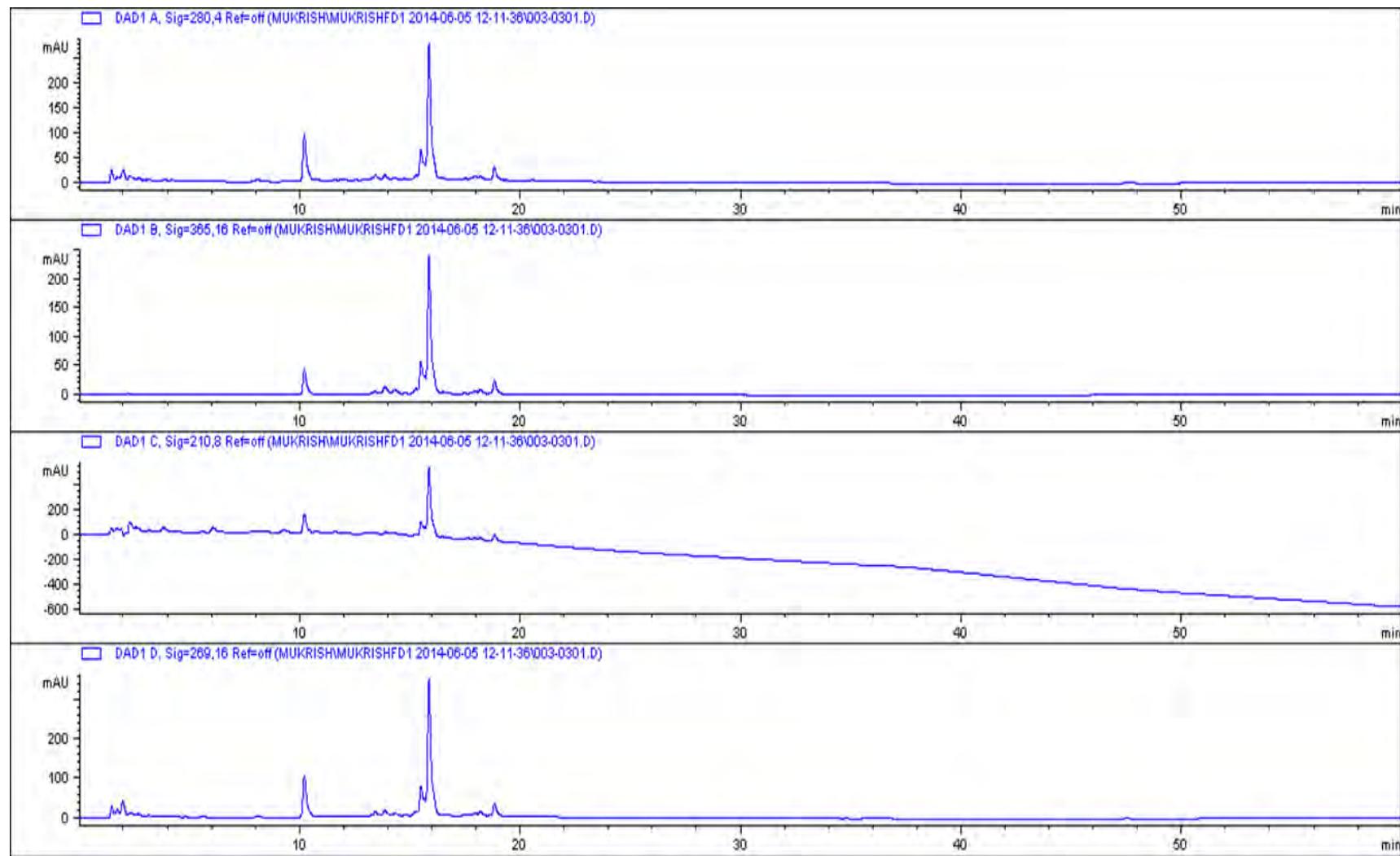


Figure 4.3 HPLC fingerprint of *Ficus deltoidea* 1 aqueous extract.

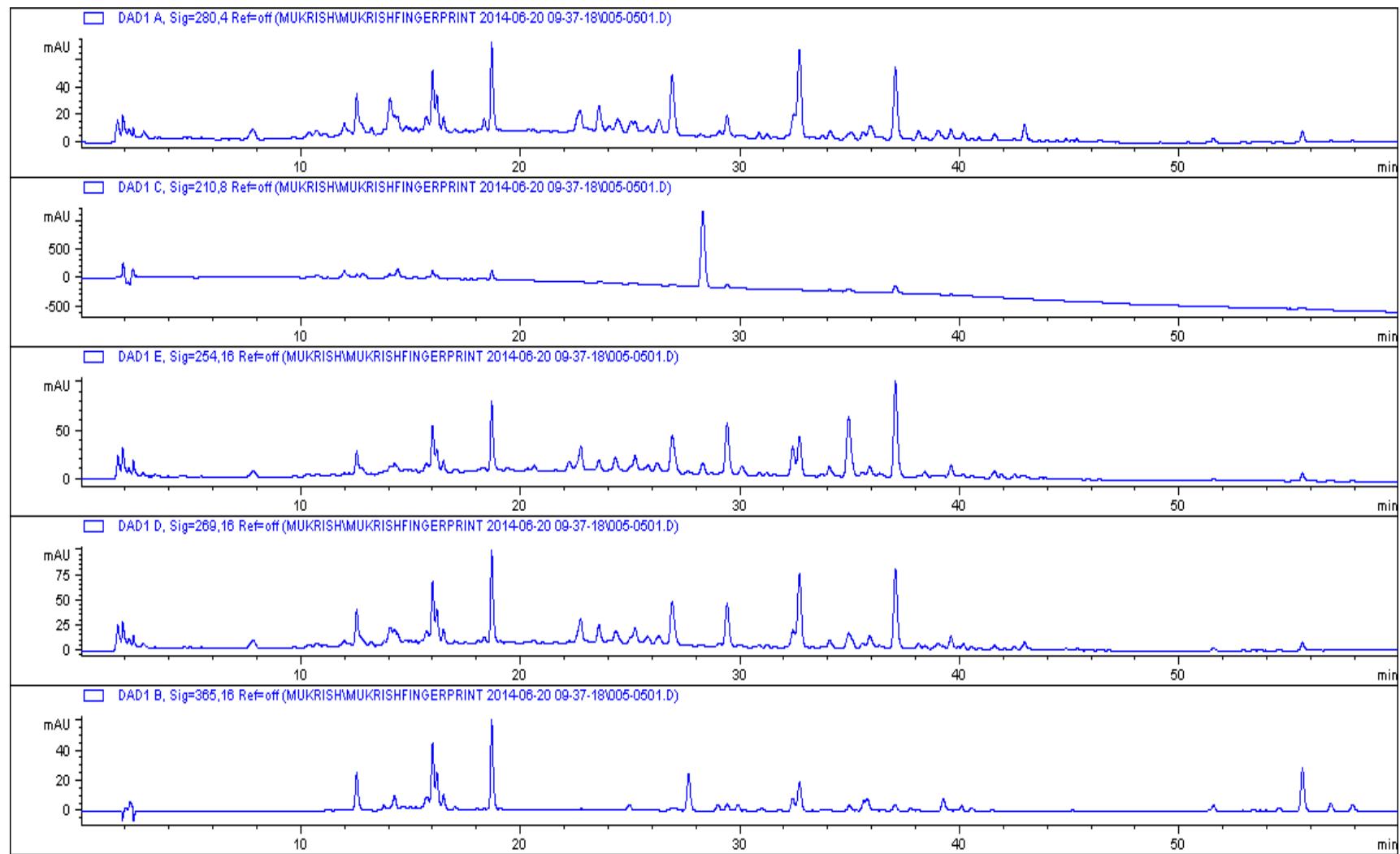


Figure 4.4 HPLC fingerprint of *Ficus deltoidea* 2 hexane extract.

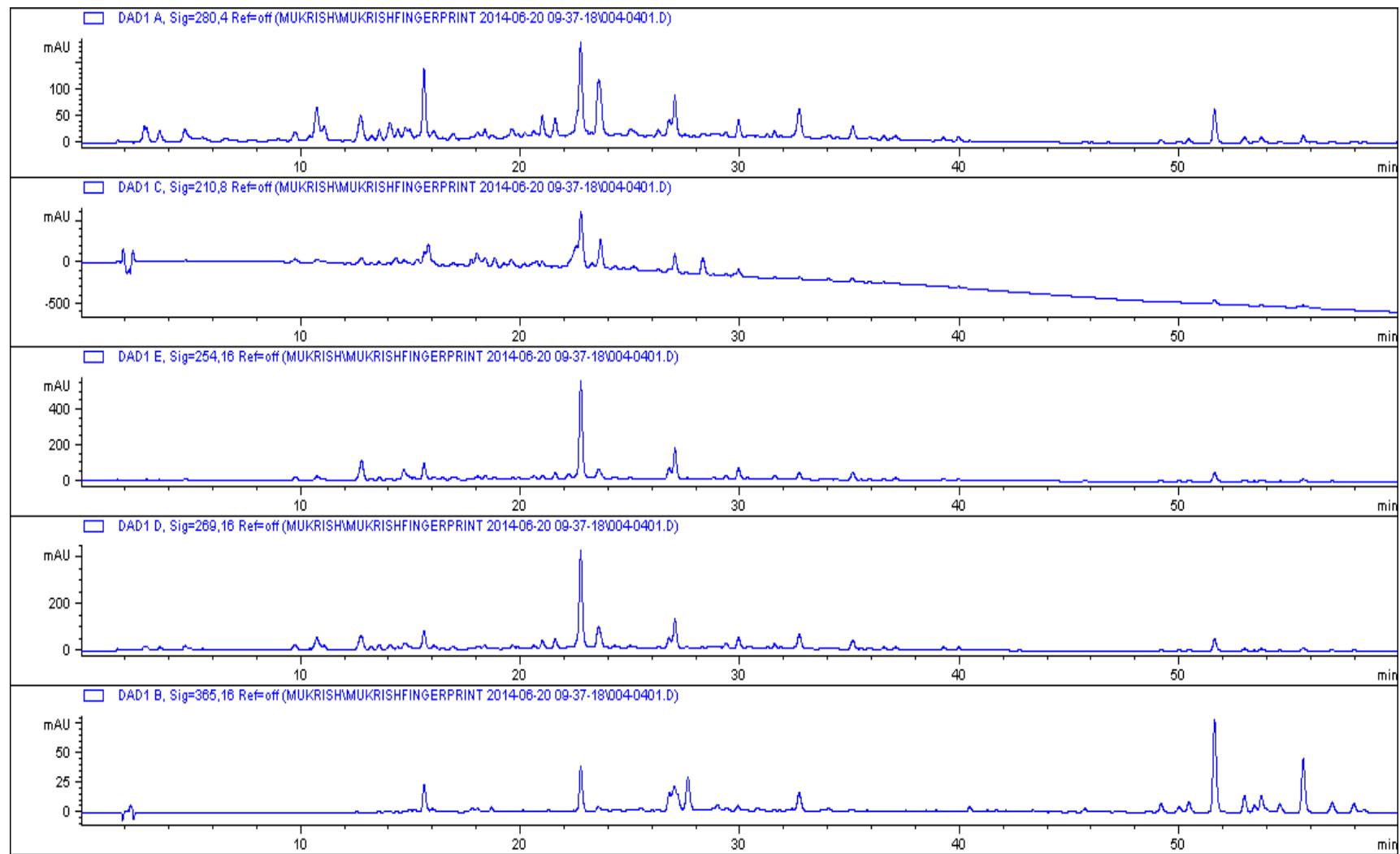


Figure 4.5 HPLC fingerprint of *Ficus deltoidea* 2 chloroform extract.

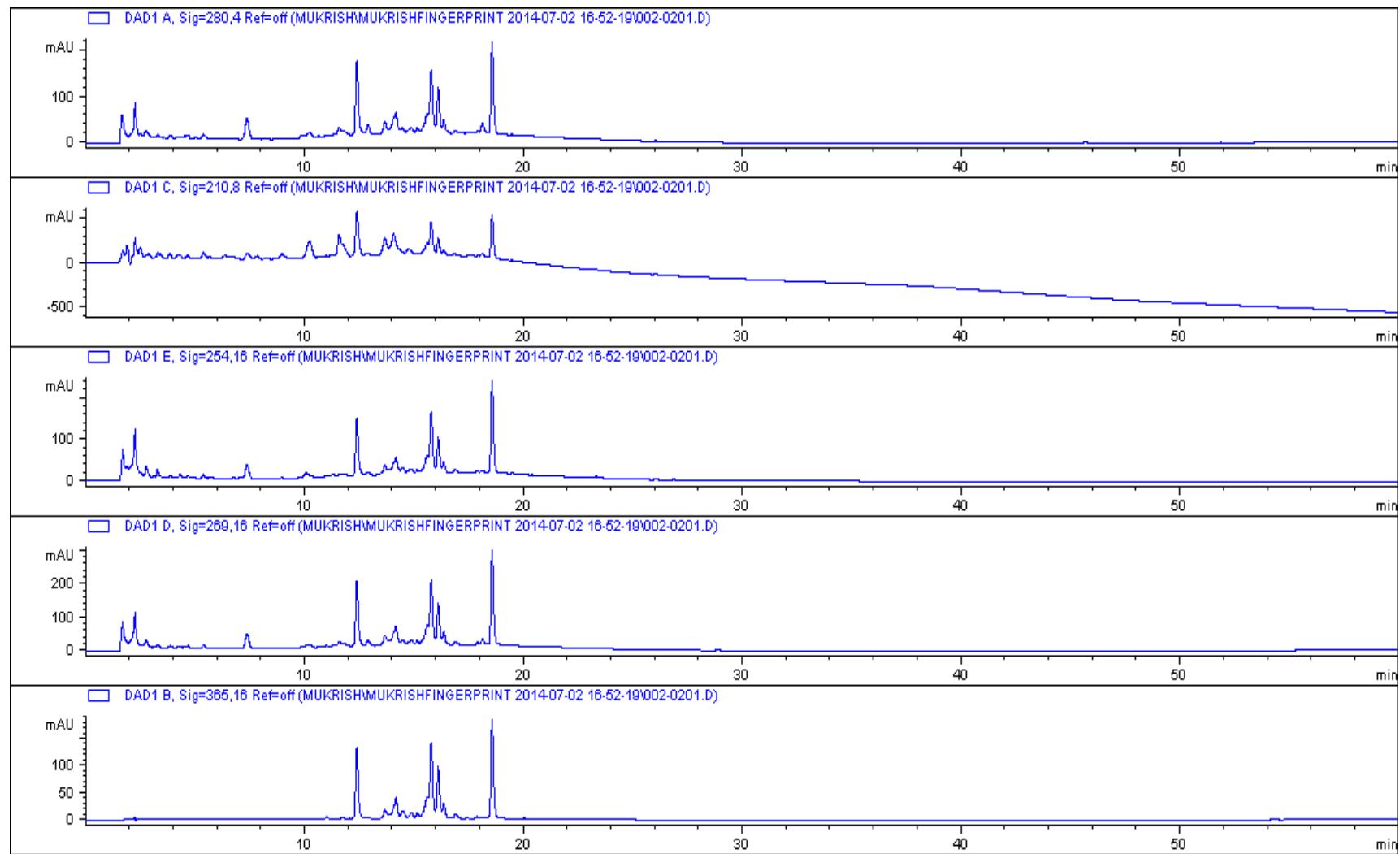


Figure 4.6 HPLC fingerprint of *Ficus deltoidea* 2 aqueous extract.

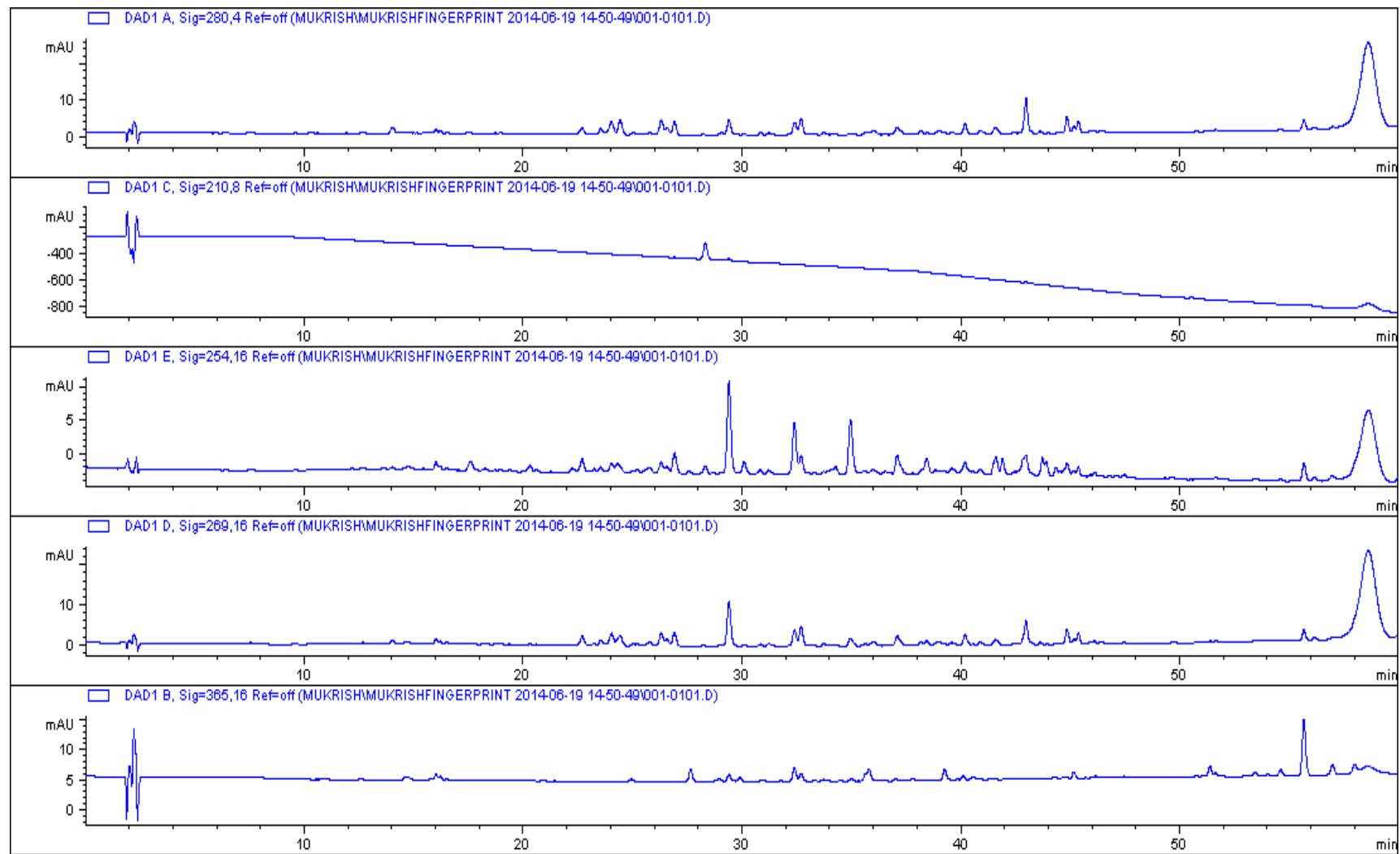


Figure 4.7 HPLC fingerprint of *Ficus deltoidea* 3 hexane extract.

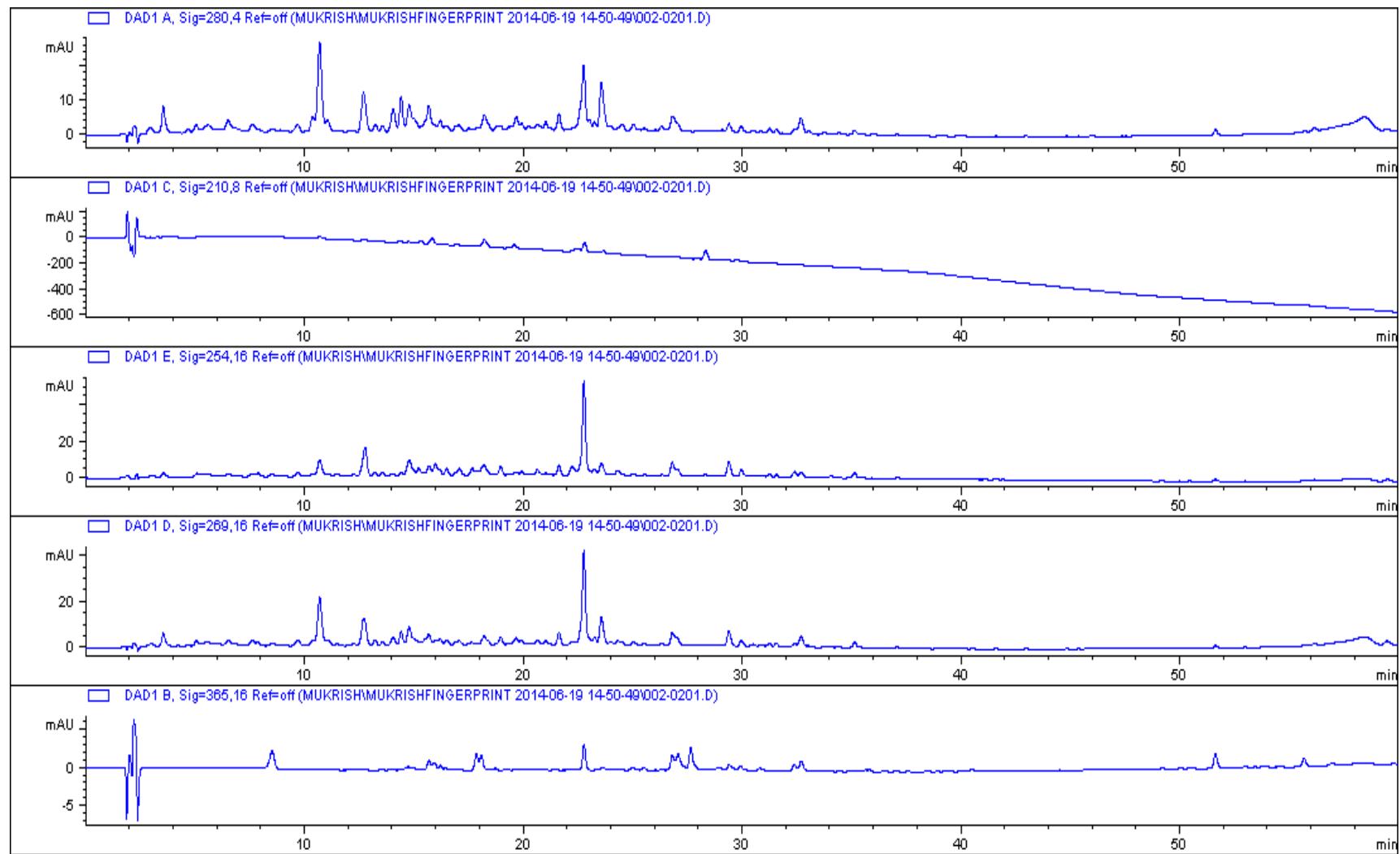


Figure 4.8 HPLC fingerprint of *Ficus deltoidea* 3 chloroform extract.

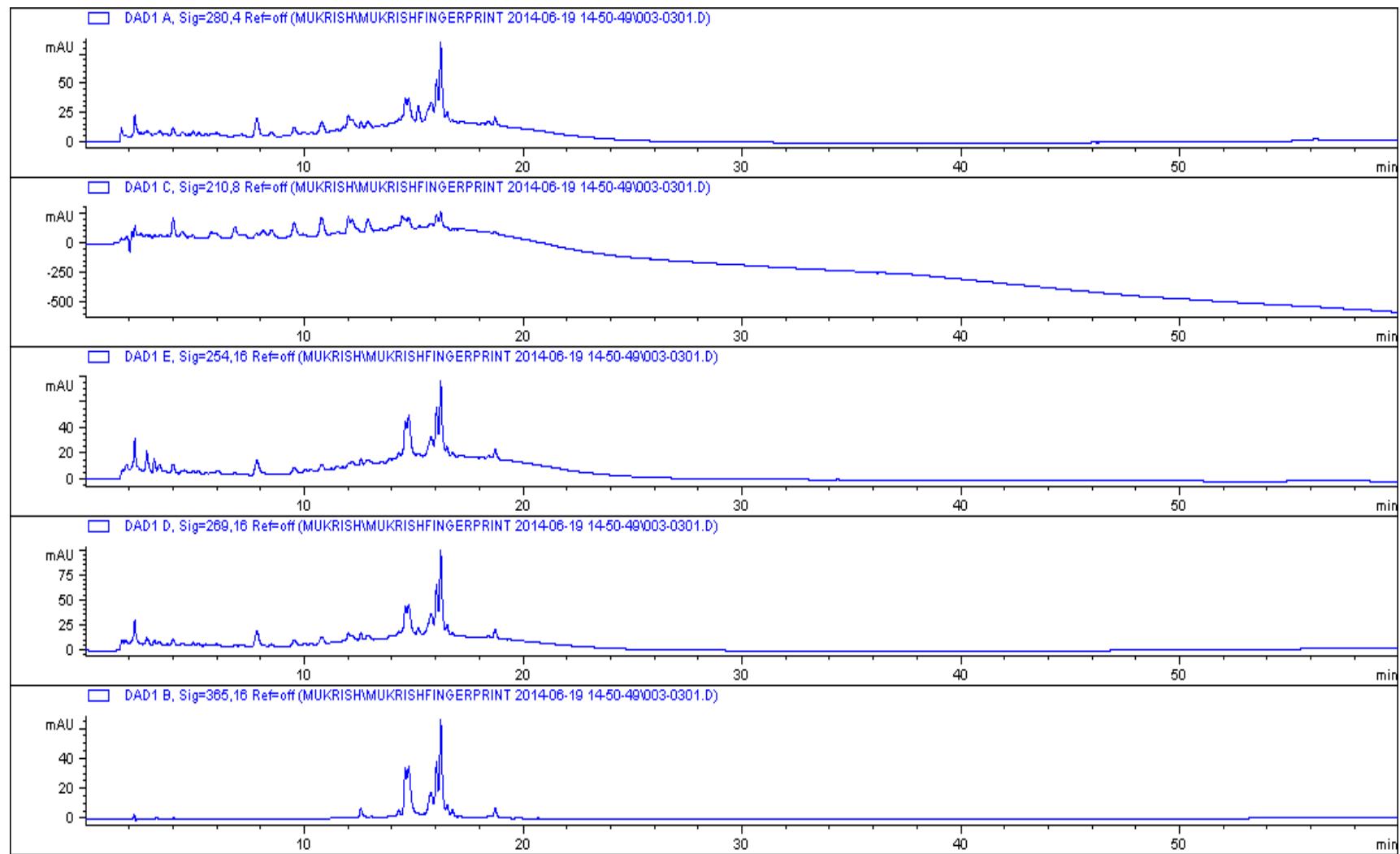


Figure 4.9 HPLC fingerprint of *Ficus deltoidea* 3 aqueous extract

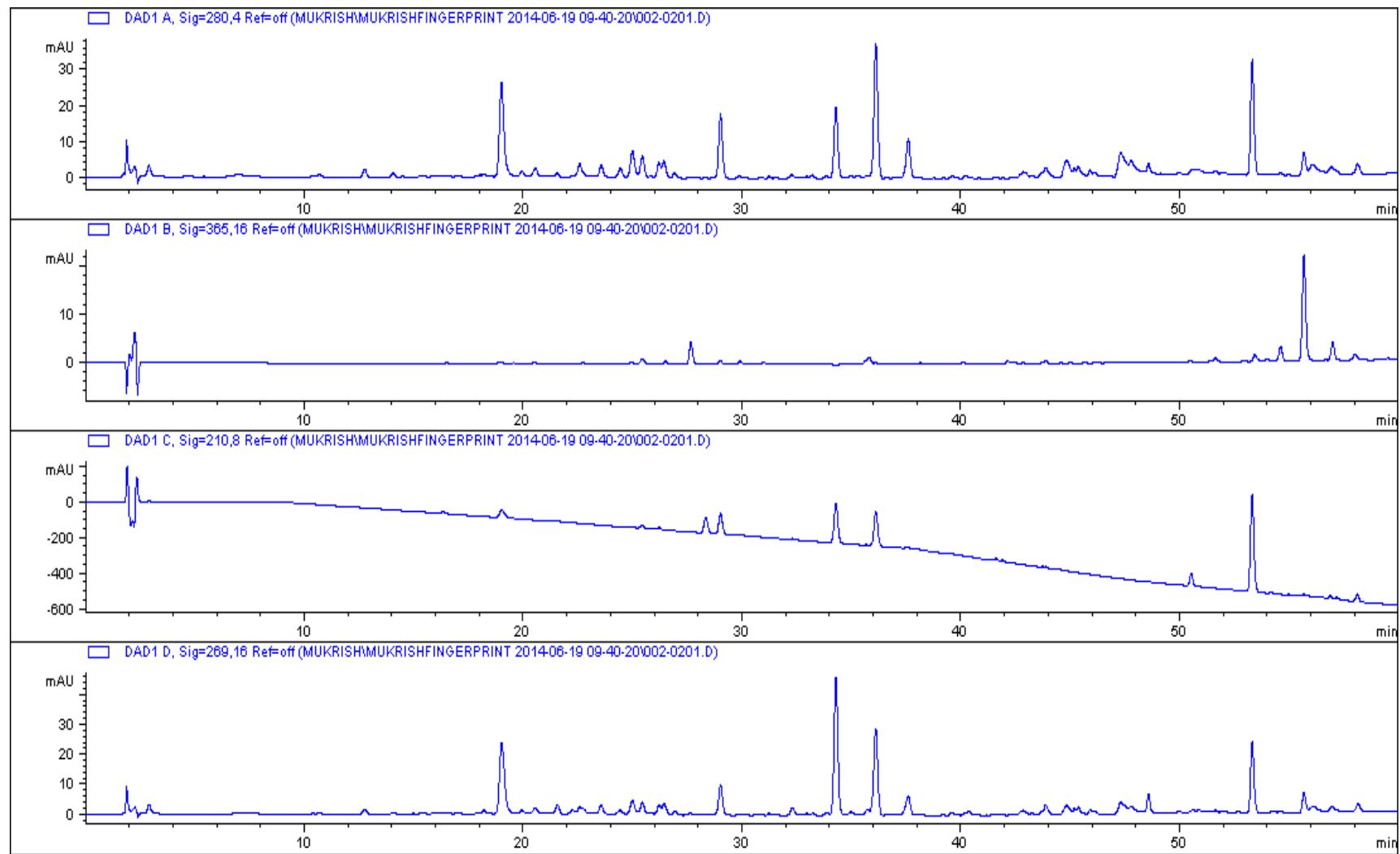


Figure 4.10 HPLC fingerprint of *Marantodes pumilum* hexane extract

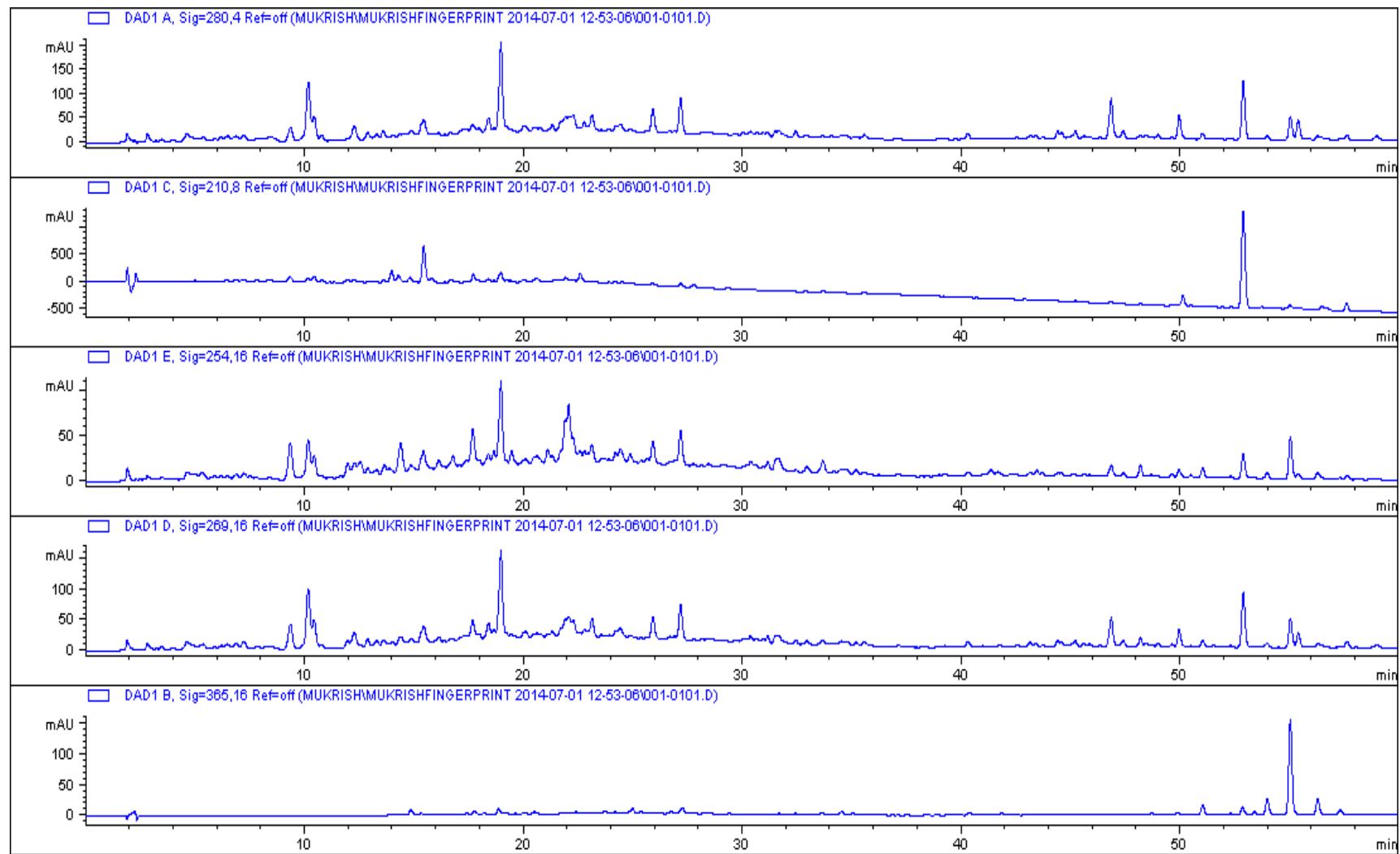


Figure 4.11 HPLC fingerprint of *Marantodes pumilum* chloroform extract

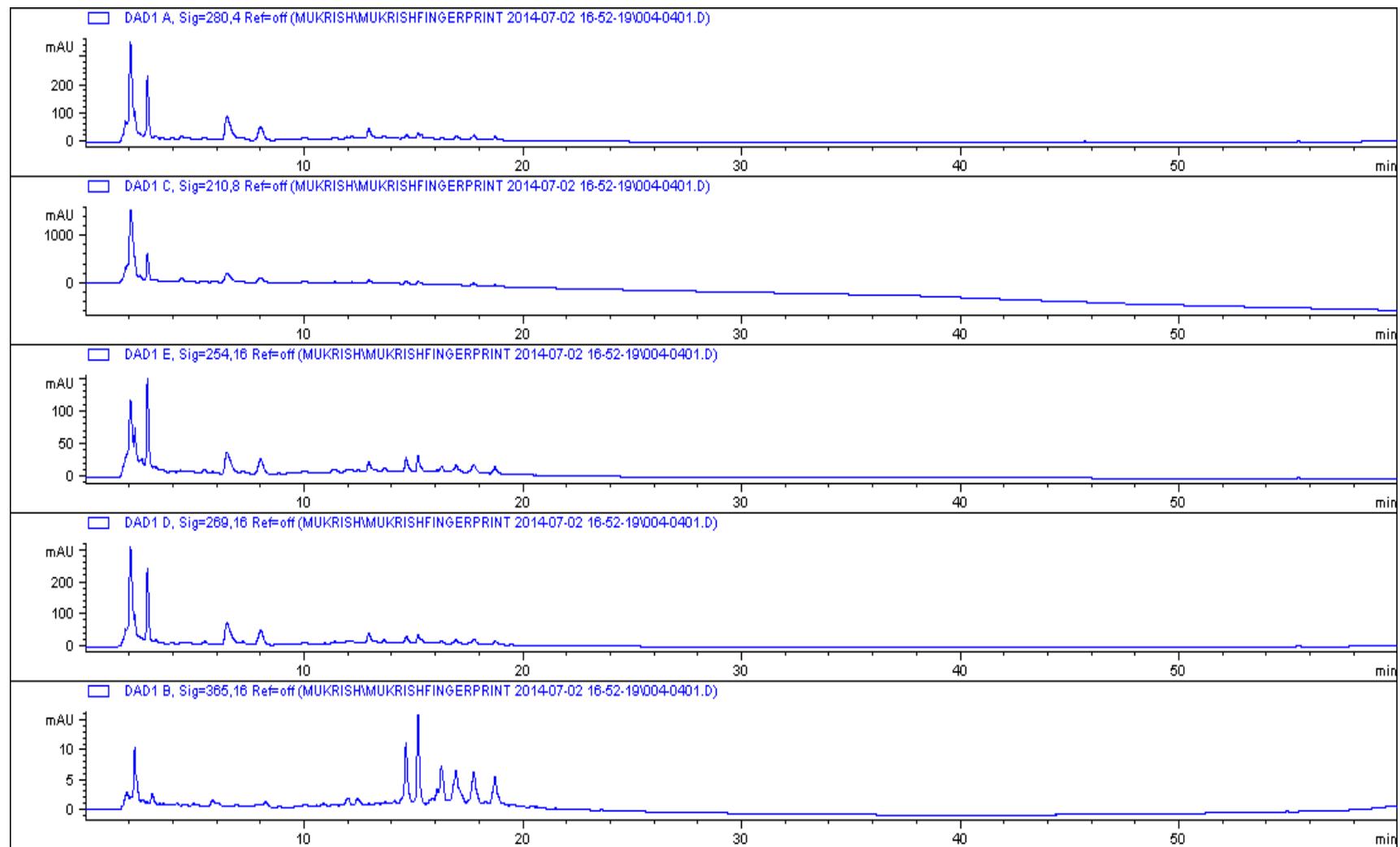


Figure 4.12 HPLC fingerprint of *Marantodes pumilum* aqueous extra

Chromatographic fingerprinting using HPLC mainly utilizes the ultraviolet (UV) detection system; therefore, different compounds had their maximum absorptions at specific wavelengths, which can affect the peak areas of the same compounds. More than one wavelength was used in this study as different compounds will be detected at different wavelengths and this will help in building the full fingerprints of the plant. Chua, Lee et al. (2012) and Bunawan, Amin et al. (2014) reported that the UV absorption for flavonoids and phenolic compounds vary greatly according to their structures. Kumar and Pandey (2013) and Barreca, Bellocchio et al. (2012) have further supported this finding by reporting that most flavones and flavonols exhibit two strong absorption bands; Band I (320-385nm) which is attributed to the styrene-type π -system represented the B ring absorption, while Band II (250-285nm) which is mostly attributed by the dihydroxybenzoyl portion of the conjugated π -system of ring A absorption. Apart from that, isoflavonoids also lack the structural presence of the styrene-type π -system (B ring) thus causing a significant difference in the UV-Vis absorption spectra when compared to flavones and flavonols. Therefore, by comparing chromatograms recorded simultaneously at various wavelengths, it will enable us to determine the proper wavelength for some analytes such as flavonoids and terpenes as well as building a full fingerprint of the plant.

From the chromatographs, we can see that different varieties of *Ficus deltoidea* extracts showed different phytochemical profiles at the same wavelength. This indicates extreme variability of chemistry in these extracts even though they are from the same species. Normally, we would expect plants from the same species to have close or similar phytochemical profiles. However, this is not the case for the 2 different varieties of *Ficus deltoidea* plants used in this study (FD1 and FD2). The chromatographs also showed that, different parts of *Ficus deltoidea* plant, aerial and roots, from the same species and variety (FD2 and FD3) have different phytochemical constituents. Figure 4.1 to Figure 4.9 showed that in FD1 most of the peaks could only be detected at the more

polar phase between retention times - t_R , 10 minute to 20 minute - and there was nearly no peaks detected in the last 30 minutes of the chromatographs in all three different extracts of FD1. However, FD2 showed different distribution of phytochemical constituents according to the chromatographs as more peaks are detected in both the polar phase and the non-polar phase in all three extracts of FD2. This could suggest that FD2 has more phytochemical constituents when compared to FD1. Figure 4.4 to Figure 4.6 also indicate that the distribution of peaks varies greatly depending on the type of extract being analysed. More peaks are detected between $t_R = 20$ to $t_R = 40$ in FD2 n-hexane extract, whereas more peaks were detected after $t_R = 40$ in FD2 chloroform extracts. The FD2 aqueous extract only showed peaks in the first 20 minutes of the retention time at which, more polar phytochemical constituents are normally detected. These results demonstrate that different extracts in different solvents could greatly influence the distribution of phytochemicals compounds in a plant. The chromatographs for FD3 (Figure 4.7 to Figure 4.9) also showed extreme variability in terms of the distribution of the phytochemical compounds depending on the type of extracts being analysed. More peaks were detected after $t_R = 20$ for the n-hexane extract of FD3, whereas for both aqueous and chloroform extracts of FD3, peaks were only detected during the first 30 minutes of the retention time with FD3 chloroform extracts showing more peak variability in this region.

Figure 4.10 to Figure 4.12 show the distribution of phytochemical constituents in *Marantodes pumilum* plant extracts. Similar to both FD2 and FD3, MP plant extracts also demonstrate great variability in phytochemical compound distribution, depending on the type of extracts being analysed. In MP n-hexane extract, even though several peaks were seen between $t_R = 20$ to $t_R = 30$, most peaks were detected after $t_R = 30$. Whereas peak distribution for MP chloroform extract could be detected in two separate regions - the first is between $t_R = 10$ to $t_R = 30$; and the second region is after $t_R = 40$. In the aqueous

extract of MP, peaks were only detected in the first 20 minutes of the retention time. Once again, these results demonstrate that the distribution of the phytochemical constituents in a plant could be influenced by the type of solvents used for extraction as different solvents have different polarity - therefore attracting phytochemical compounds based on their polarity. The HPLC chromatographs suggested that different varieties of a plant that belong in the same species may contain different chemical constituents, making HPLC fingerprinting to be one of the most important and acceptable approaches for quality evaluation of herbal preparation (Liang, Jin et al. 2009)

4.1.3 In vitro cytotoxic effects of plant extracts on different human Prostate Cancer cell lines

4.1.3.1 Effects of plant extracts on cells proliferation using sulphorhodamine staining assay (SRB)

In this study, SRB assay was used to determine the effect of the plant extracts on the proliferation of three prostate cancer cell lines, namely PC3, DU145 and LNCaP cells. All cell lines were treated with n-hexane, chloroform and aqueous extracts over a range of concentrations for 72 hours. The cytotoxic effects of all plant extracts were investigated and the GI50 values were determined.

From table 4.3, we can see that the most cytotoxic extract against all three prostate cancer cell lines (PC3, DU145 and LNCaP) was the chloroform extract of *Marantodes pumilum* with a GI50 value of less than 15 µg/mL. Both chloroform extracts of FD1 and FD2 also showed promising GI50 values (lower than 30 µg/mL) and therefore should also be considered for further mechanistic studies. The chloroform extract of FD3 will not be considered for further studies as the GI50 values for PC3 is more than 30 µg/mL.

4.1.3.2 Effects of plant extracts on cells viability using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay

In this study, the MTT assay was used to determine the effects of the plant extracts on the viability of two prostate cancer cell lines: DU145 and LNCaP cells. All cell lines were treated with n-hexane, chloroform and aqueous extracts over a range of concentrations for 72 hours. The cytotoxic effects of all plant extracts were investigated and their GI50 values were determined.

As shown in table 4.3, the most cytotoxic extract against the two prostate cancer cell lines (DU145 and LNCaP) was the chloroform extract of *Marantodes pumilum* with a GI50 value less than 15 µg/mL. The same pattern of cytotoxicity was observed with the MTT assay for the chloroform extracts of MP, FD1 and FD2 as their GI50 values were recorded to be below than 30 µg/mL when compared to the results collected from the SRB assay. Therefore, these results further validated the decision to choose the chloroform extracts of MP, FD1 and FD2 for further mechanistic studies

Table 4.3 GI50 concentrations (µg/mL) of the n-hexane, chloroform and aqueous plant extracts determined for PC3, DU145, and LNCaP cells as assessed by the MTT and SRB assays at 72 hours. Paclitaxel (0.01µM) was used as a reference drug. Each result was obtained in three independent experiments and run in triplicate. *(P<0.05) as determined by un-paired t-test.

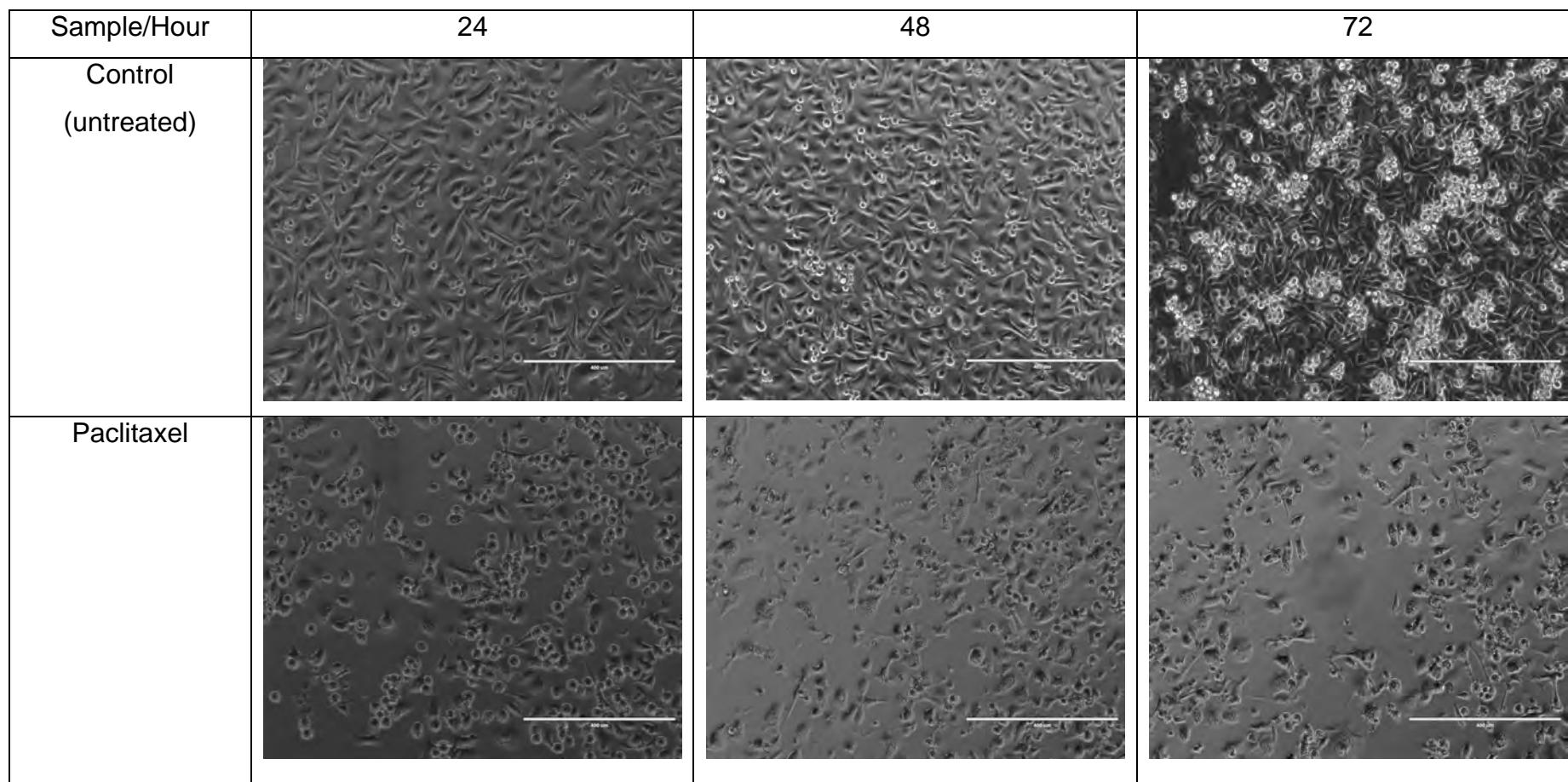
Plant Name and part	Extract	Sample Code	GI50 (µg/ml) based on SRB assay			GI50 (µg/ml) based on MTT assay	
			PC3	DU145	LNCaP	DU145	LNCaP
Paclitaxel		Pac	0.01*± 0.05 µM	0.01*± 0.06 µM	0.01*± 0.05 µM	0.01*± 0.04 µM	0.01*± 0.05 µM
Marantodes pumilum	n-hexane	MP (H)	20.2*± 0.5	25.3*± 0.6	26.7*± 0.8	24.3*± 0.5	23.8*± 0.7
(Aerial)	Chloroform	MP (C)	14.3*± 0.3	14.6*± 0.4	13.2*± 0.2	13.3*± 0.3	13.1*± 0.6
Ficus deltoidea	Aqueous	MP(A)	>200	>200	>200	150.2*± 0.3	135.8*± 0.6
1 (FD1)	n-hexane	FD1 (H)	>200	100*± 1.5	>200	96*± 1.3	95*± 2.1
(Aerial)	Chloroform	FD1 (C)	23.2*± 0.2	20.1*± 0.6	21.4*± 0.4	19.3*± 0.3	19.2*± 0.6
Ficus deltoidea	Aqueous	FD1 (A)	>200	>200	>200	>200	>200
2 (FD2)	n-hexane	FD2 (H)	58.4*± 1.2	74.3*± 2.1	>200	52.1*± 1.5	150.2*± 2.5
(Aerial)	Chloroform	FD2 (C)	29*± 0.3	15*± 0.4	27*± 0.5	14*± 0.6	23*± 0.3
	Aqueous	FD2 (A)	>200	>200	>200	>200	>200

Table 4.3 (Continues)

Plant Name	Extract	Sample Code	GI50 (µg/ml) based on SRB assay			GI50 (µg/ml) based on MTT assay	
			PC3	DU145	LNCaP	DU145	LNCaP
<i>Ficus deltoidea</i> 3 (FD3) (Root)	n-hexane	FD3 (H)	>200	>200	175 ± 6	>200	175.2 ± 7.4
	Chloroform	FD3 (C)	34.2 ± 3.4	30.3 ± 4.3	28.7 ± 5.2	28.3 ± 4.1	26.1 ± 4.1
	Aqueous	FD3 (A)	>200	>200	51.3 ± 4.2	>200	48.3 ± 3.9

4.1.4 Morphological changes of LNCaP and PC3 cell lines after treated with active extracts of the plant extracts

Cell death could occur via two distinct forms known as apoptosis and necrosis. Therefore, it is very important to determine the mode of cell death induced by the active extracts of the plants. Apoptosis is a programmed cell death characterized by the initial detachment and shrinkage of the cells, followed by the extensive plasma membrane blebbing, and the formation of apoptotic bodies that consist of cytoplasm with tightly packed cellular organelles with or without nuclear fragments (Elmore 2007). *In vivo*, these bodies will be subsequently phagocytosed by macrophages or adjacent cells such as parenchymal cells and neoplastic cells thus preventing them from going through lysis. However, when this process occurs in an *in vitro* environment, these apoptotic bodies will ultimately swell and lyses due to the absence of phagocytes and undergo secondary necrosis (Ho, Yazan et al. 2009). In this study, the morphology of the prostate cancer cell lines (LNCaP and PC3) untreated and treated with the active extracts of *Marantodes pumilum* and *Ficus deltoidea* was analysed using an EVOS® FL Imaging System at 24, 48 and 72 hours. The characteristics of apoptosis such as cells detachment from the substratum, cell shrinkage, nuclear condensation, membrane blebbing and the formation of apoptotic bodies were detected in the treated cells (Figure 4.19 to Figure 4.22). Reduction in the cell population was very obvious when comparing between the untreated and the treated cells (Figure 4.13 to Figure 4.18).



400 um

Figure 4.13. Morphological changes of PC3 cells treated with the GI50 of Paclitaxel (positive control) for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System (100x magnification). Steady decline of the cell population was noted at all endpoints as compared to the control (Untreated cells).length of the white scale is 400 um

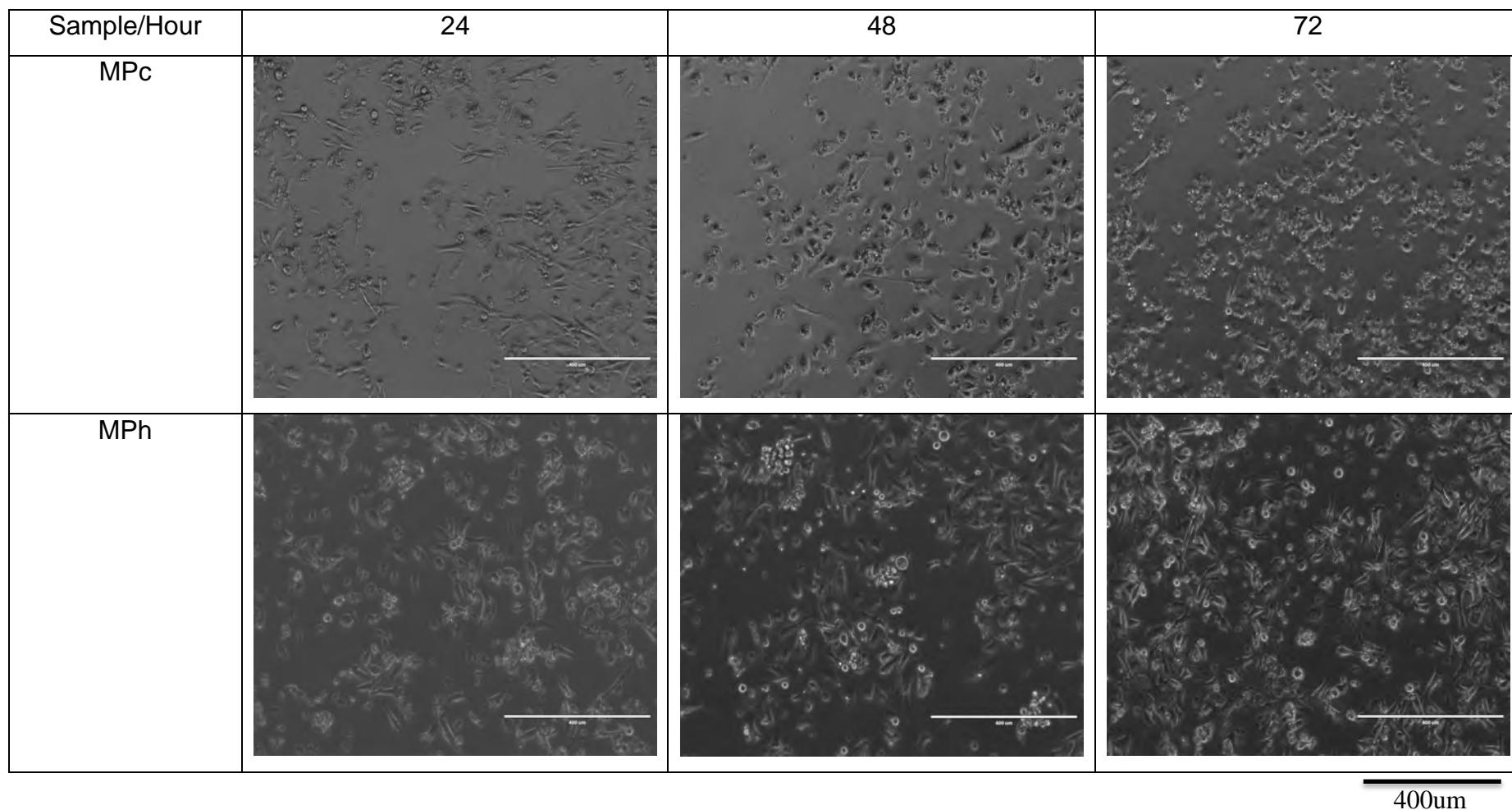


Figure 4.14. Morphological changes of PC3 cells treated with the GI50 of MPc and MPh for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System (100x magnification). Steady decline of the cell population was noted at all endpoints as compared to the control (Untreated cells).

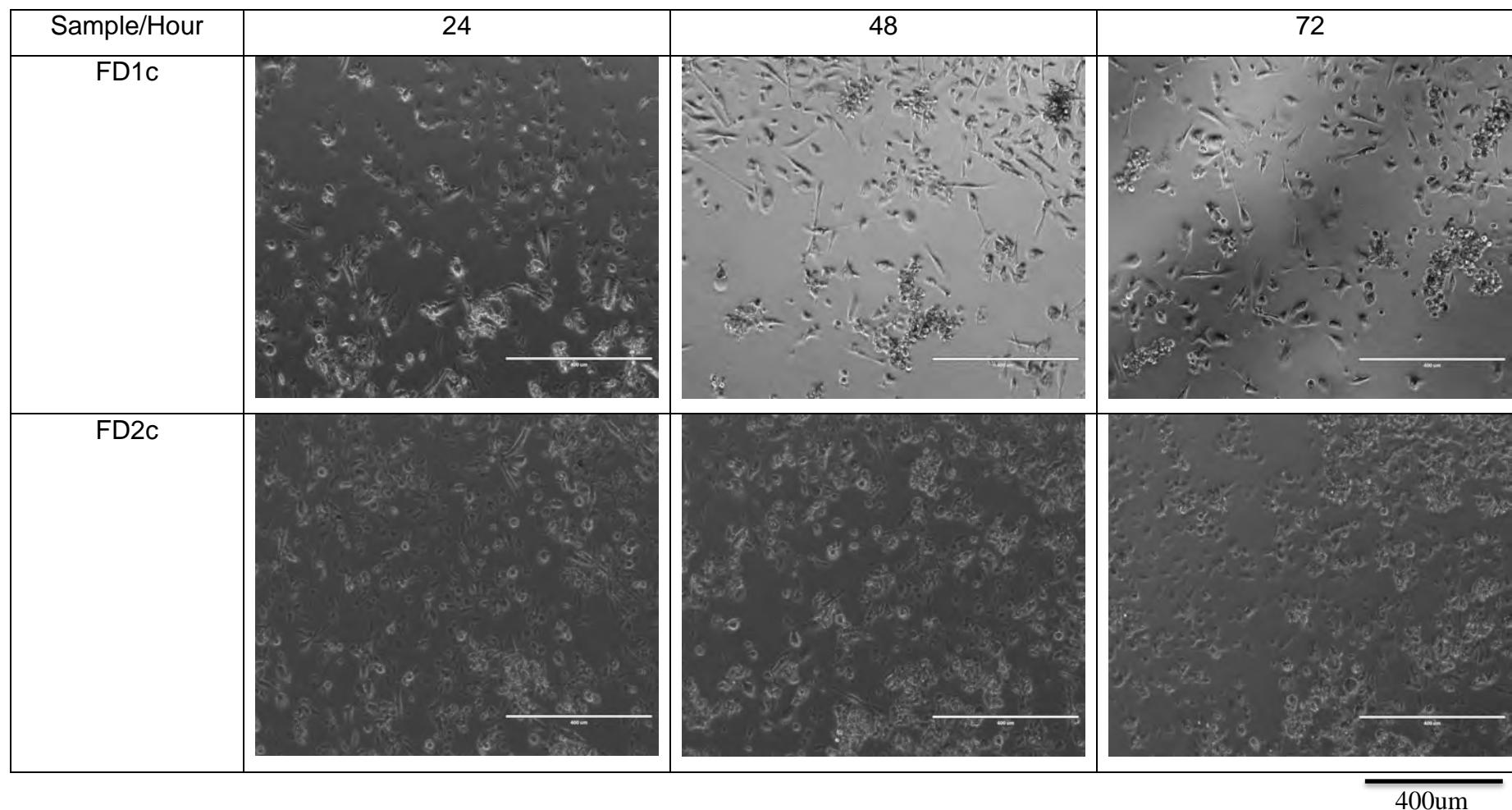


Figure 4.15. Morphological changes of PC3 cells treated with the GI50 of FD1c and FD2c for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System (100x magnification). Steady decline of the cell population was noted at all endpoints as compared to the control (Untreated cells).

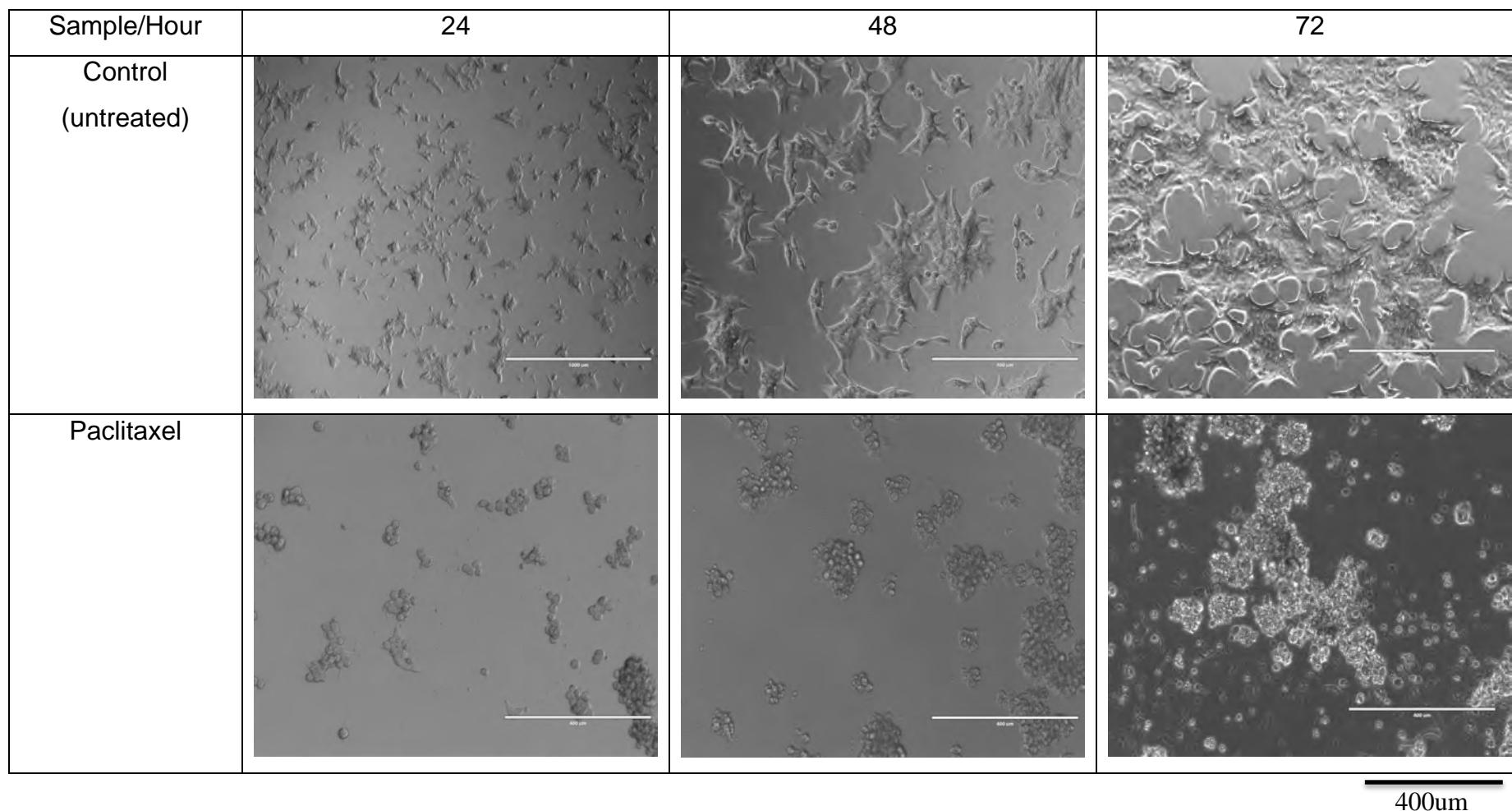


Figure 4.16. Morphological changes of LNCaP cells treated with the GI50 of Paclitaxel for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System (100x magnification). Steady decline of the cell population was noted at all endpoints as compared to the control (Untreated cells).

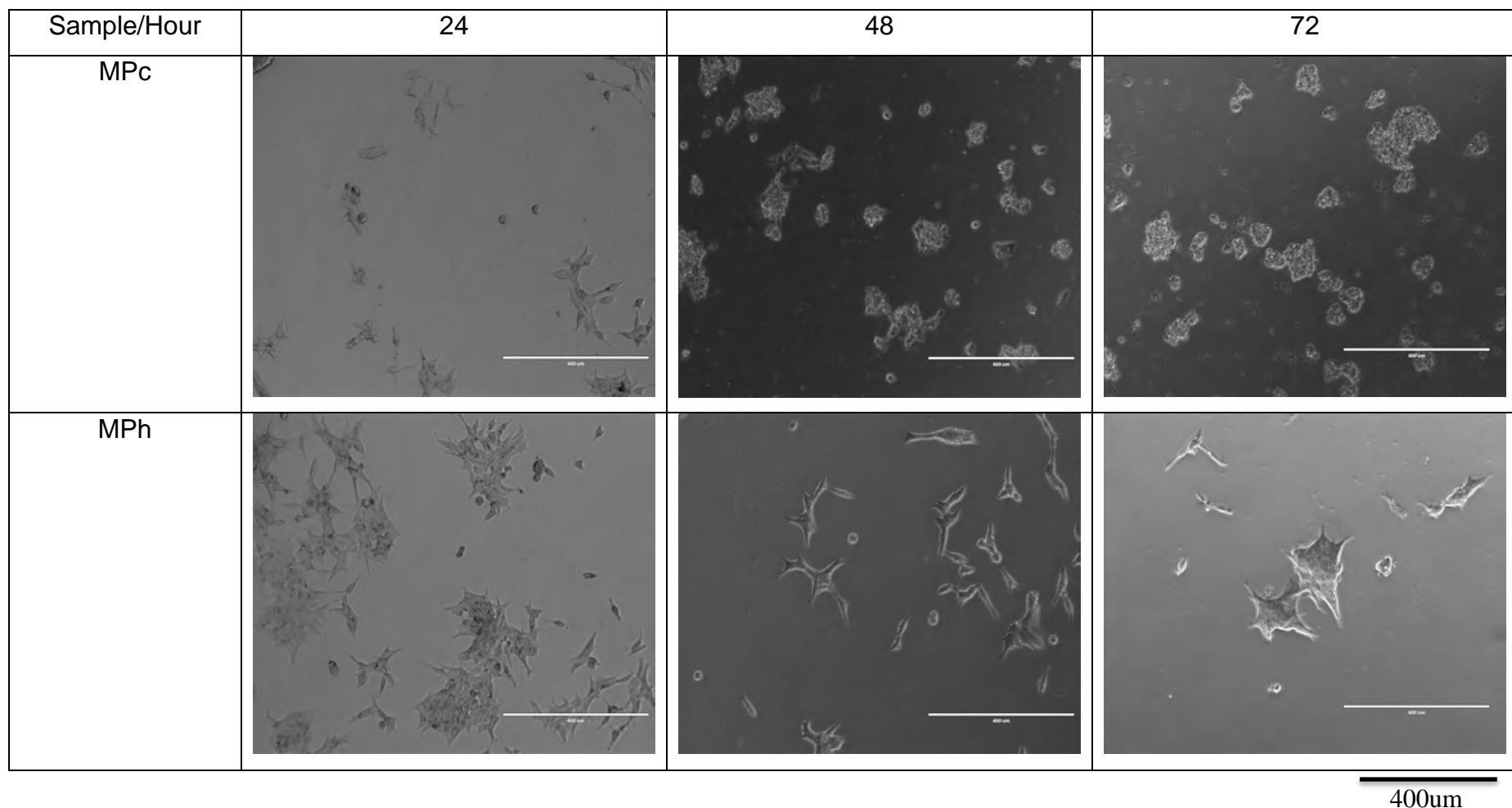


Figure 4.17. Morphological changes of LNCaP cells treated with the GI50 of MPc and MPh for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System (100x magnification). Steady decline of the cell population was noted at all endpoints as compared to the control (Untreated cells).

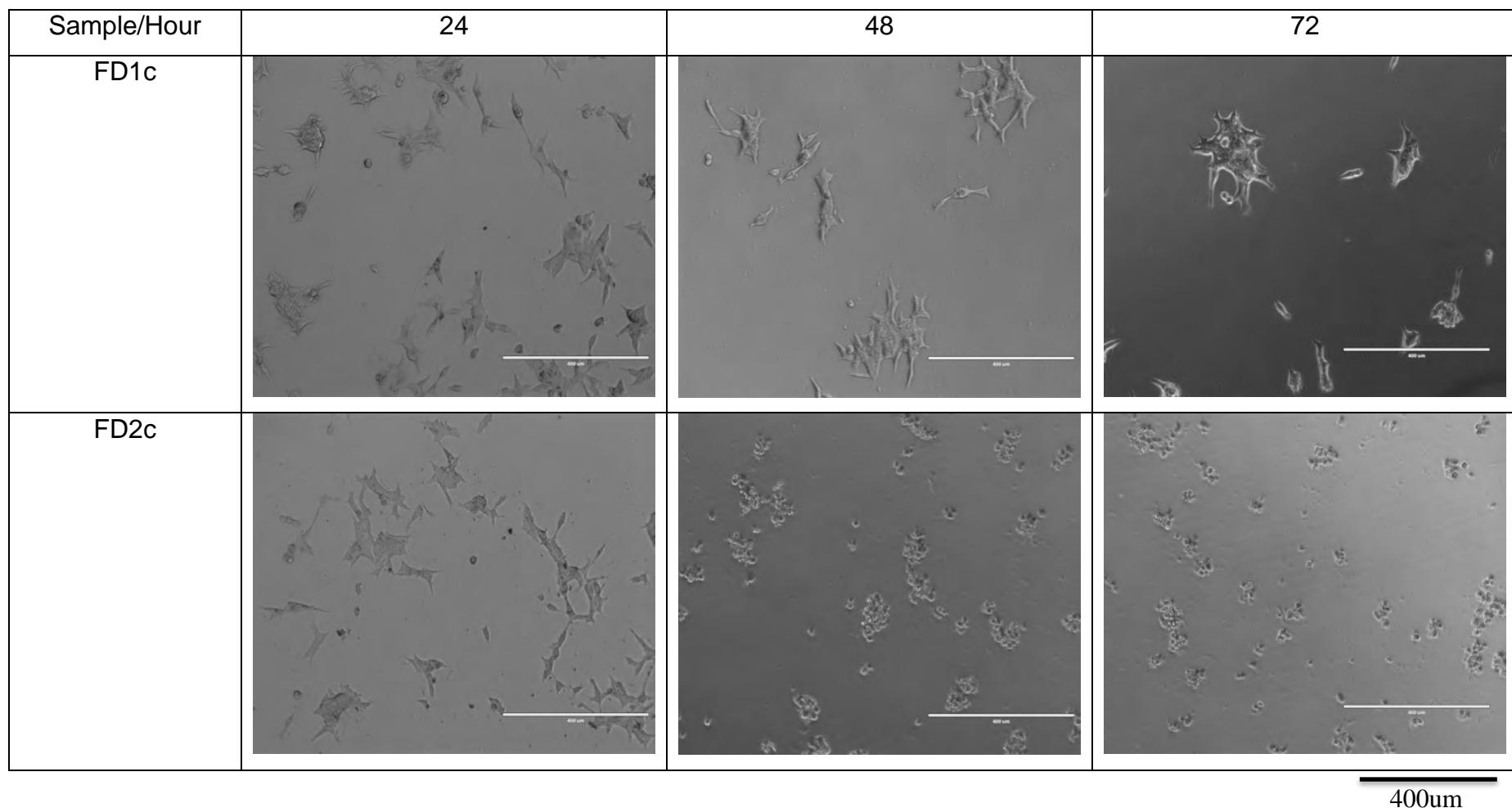


Figure 4.18. Morphological changes of LNCaP cells treated with the GI50 of FD1c and FD2c for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System (100x magnification). Steady decline of the cell population was noted at all endpoints as compared to the control (Untreated cells)

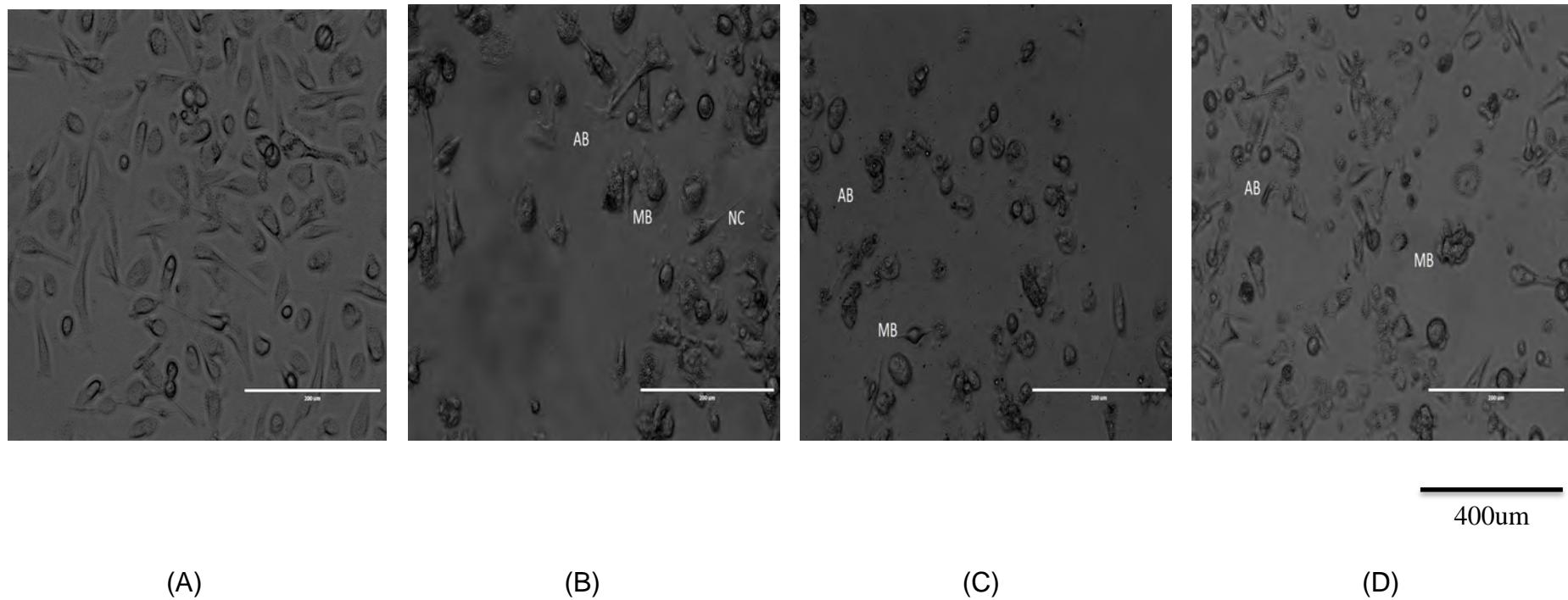


Figure 4.19. PC3 cells viewed under the EVOS® FL Imaging System (200x magnification) after 72 hours of treatment with the GI50 concentration of Paclitaxel (B), *Marantodes pumilum* (Chloroform) (C), and *Marantodes pumilum* (n-Hexane) (D) extracts. (A) represents the Control (Untreated PC3 cells). The cells showed characteristics of apoptosis such as the formation of apoptotic bodies (AB), membrane blebbing (MB) and nuclear compaction (NC).

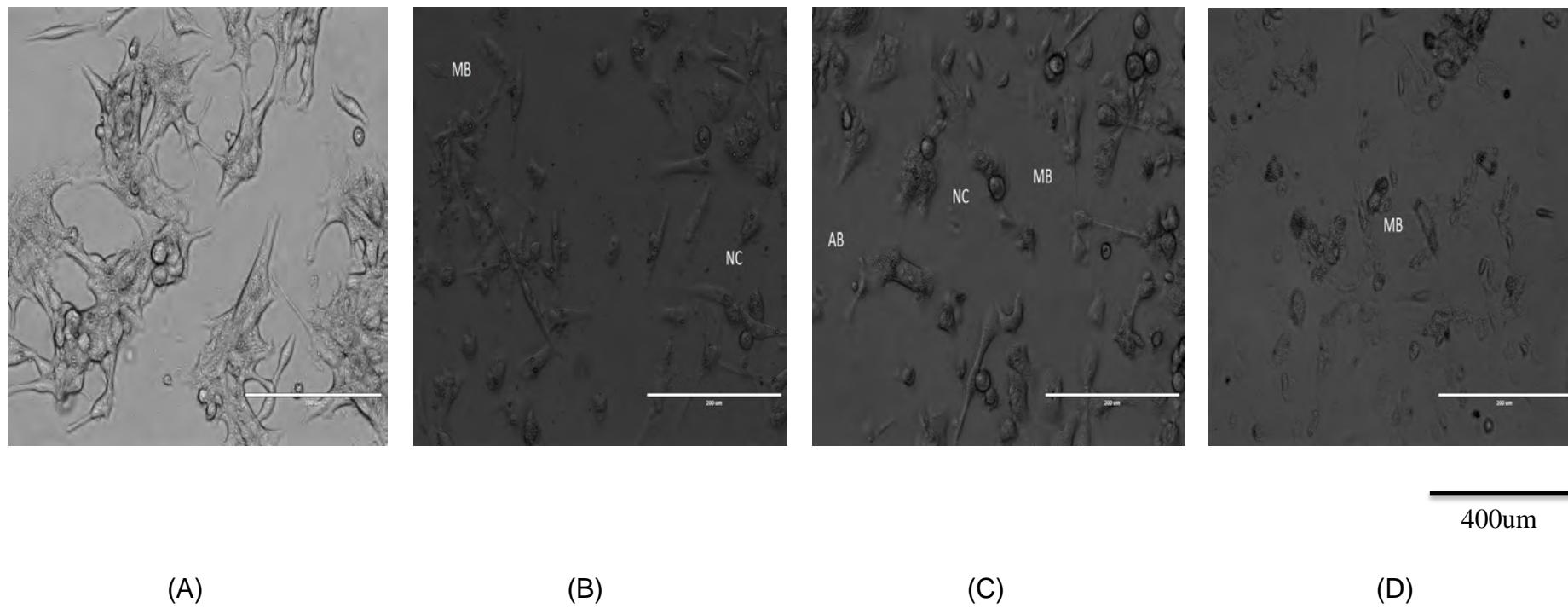


Figure 4.20. LNCaP cells viewed under the EVOS® FL Imaging System (200x magnification) after 72 hours of treatment with the GI50 concentration of Paclitaxel (B), *Marantodes pumilum* (Chloroform) (C), and *Marantodes pumilum* (n-Hexane) (D) extracts. (A) represented the Control (Untreated LNCaP cells). The cells showed characteristics of apoptosis such as the formation of apoptotic bodies (AB), membrane blebbing (MB) and nuclear compaction (NC).

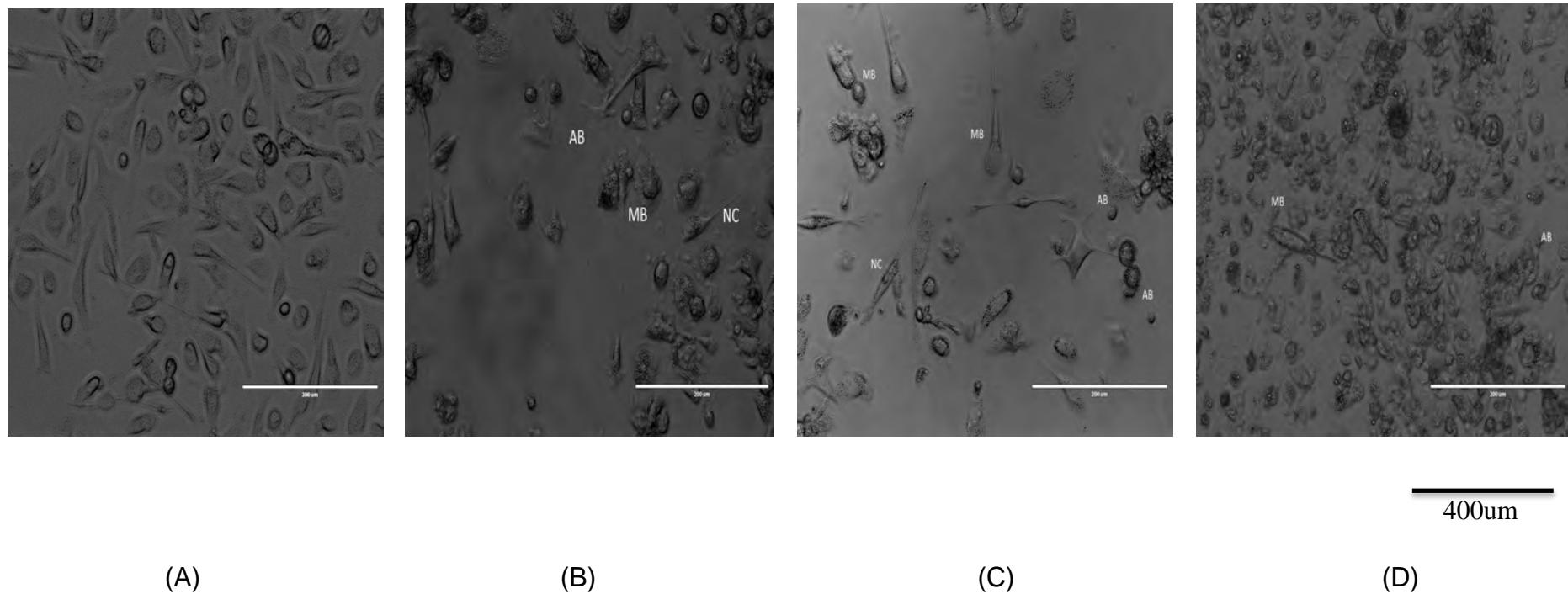


Figure 4.21. PC3 cells viewed under the EVOS® FL Imaging System (200x magnification) after 72 hours of treatment with the GI50 concentration of Paclitaxel (B), FD1 (Chloroform) (C), and FD2 (Chloroform) (D) extracts. (A) represents the Control (Untreated PC3 cells). The cells showed characteristics of apoptosis such as the formation of apoptotic bodies (AB), membrane blebbing (MB) and nuclear compaction (NC).

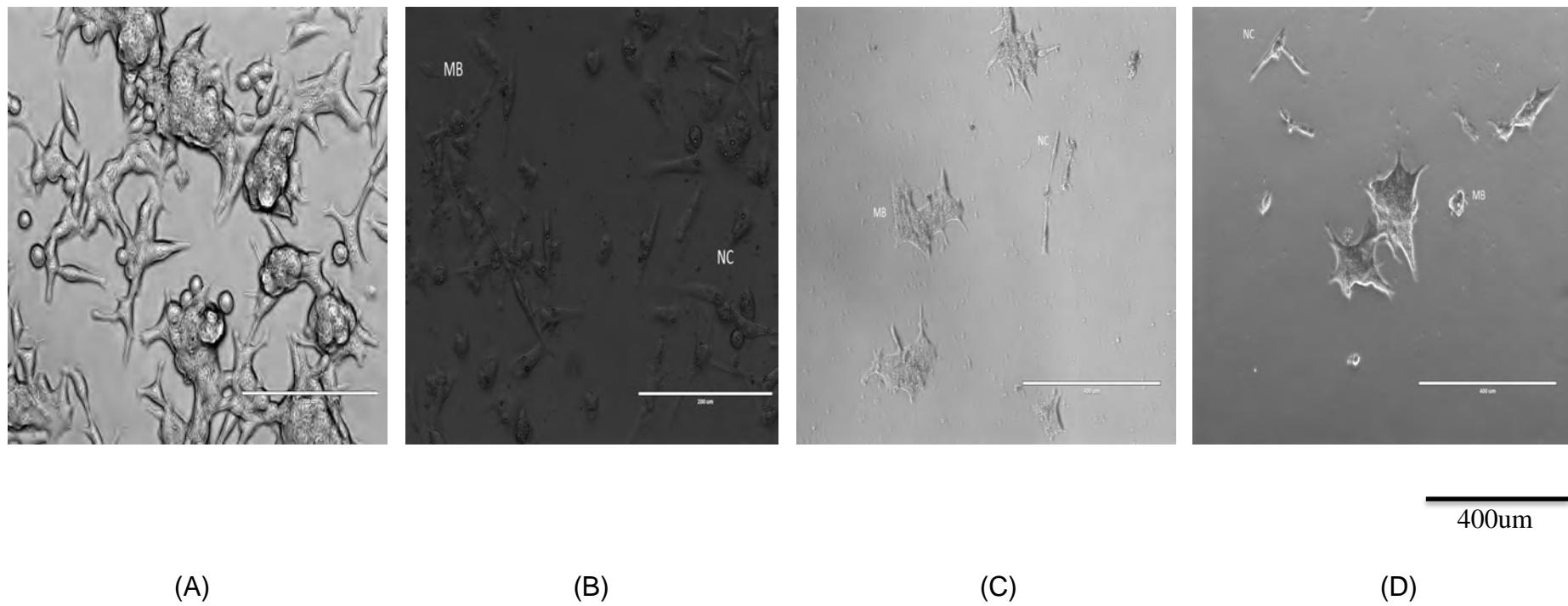


Figure 4.22. LNCaP cells viewed under the EVOS® FL Imaging System (200x magnification) after 72 hours of treatment with the GI50 concentration of Paclitaxel (B), FD1 (Chloroform) (C), and FD2 (Chloroform) (D) extracts. (A) represents the Control (Untreated PC3 cells). The cells showed characteristics of apoptosis such as the formation of apoptotic bodies (AB), membrane blebbing (MB) and nuclear compaction (NC).

4.1.5 Effect of the active extracts of the plants on apoptosis of prostate cancer cell lines

4.1.5.1 DAPI staining

4'-6-Diamidino-2-phenylindole or DAPI is a dye that can be used as a tool to visualize the nuclear changes that occur during apoptosis (Wang, Martindale et al. 2000). During apoptosis, the condensation and fragmentation of nuclei allow the apoptotic cells to be visualized using the fluorescence microscopy technique - as DAPI is able to bind strongly and selectively to the minor groove of adenine-thymine regions of the DNA, producing a fluorescent blue colour that is directly proportional to the amount of DNA present. The compromised apoptotic cell membrane will allow more DAPI to enter the cells and stains a stronger blue colour. This study indicates that the number of apoptotic cells were higher in the treated cells than the untreated cells (Figure 4.23 to Figure 4.26).

Samples	Images		Samples	Images	
Control (untreated)			MPc		
Paclitaxel			MPh		

400 um

Figure 4.23. Fluorescence image of PC3 cells stained with DAPI after 72 hours incubation with the active extracts of *Marantodes pumilum* and Paclitaxel (Positive control). Apoptotic cells were represented by the blue fluorescence colour seen in the images. More apoptotic cells can be seen in the cells treated with Paclitaxel and the active extracts of *Marantodes pumilum*.

Samples	Images		Samples	Images	
Control (untreated)			MPc		
Paclitaxel			MPh		

400um

Figure 4.24. Fluorescence image of LNCaP cells stained with DAPI after 72 hours incubation with the active extracts of *Marantodes pumilum* and Paclitaxel (Positive control). Apoptotic cells were represented by the blue fluorescence colour seen in the images. More apoptotic cells can be seen in the cells treated with Paclitaxel and the active extracts of *Marantodes pumilum*.

Samples	Images		Samples	Images	
Control (untreated)			FD1c		
Paclitaxel			FD2c		

400um

Figure 4.25. Fluorescence image of PC3 cells stained with DAPI after 72 hours incubation with the active extracts of *Ficus deltoidea* and Paclitaxel (Positive control). Apoptotic cells were represented by the blue fluorescence colour seen in the images. More apoptotic cells can be seen in the cells treated with Paclitaxel and the active extracts of *Ficus deltoidea*.

Samples	Images		Samples	Images	
Control (untreated)			FD1c		
Paclitaxel			FD2c		

400μm

Figure 4.26. Fluorescence image of LNCaP cells stained with DAPI after 72 hours incubation with the active extracts of *Ficus deltoidea* and Paclitaxel (Positive control). Apoptotic cells were represented by the blue fluorescence colour seen in the images. More apoptotic cells can be seen in the cells treated with Paclitaxel and the active extracts of *Ficus deltoidea*.

4.1.5.2 Annexin V-FITC and Propidium Iodide staining

Up-regulation of anti-apoptotic protein expression and the inactivation of pro-apoptotic proteins in most type of cancers have led to the unchecked cellular growth of tumor, inability to respond to cellular stress, harmful mutations and DNA damage. These cellular irregularities enable cells to evade programmed cell death or commonly known as apoptosis and this has been recognized as one of the six essentials alterations in cell physiology that dictate the growth of tumor cells (Fesik 2005). Therefore, in cancer research it is a favorable characteristic to have plant extracts or compound/s that could induce cancer cell death through apoptosis rather than necrosis due to the inflammatory response induced by the necrotic cells leading to the release of chemoattractants to the surrounding tissues (Nicholson 2000).

In this study, the Annexin V-FITC Apoptosis detection kit is used to evaluate the mode of cell death induced by the active plant extracts. The kit is a combination of conjugated fluorescein isothiocyanate (FITC) to Annexin V and Propidium Iodide that can detect and discriminate apoptotic, necrotic and dead cells. Annexin V has a high affinity to phosphatidylserine (PS) which is a negatively charged phospholipid normally located in the cytosolic leaflet of the plasma membrane lipid bilayer (Koopman, Reutelingsperger et al. 1994). During early apoptosis, the redistribution of PS from the inner leaflet to the outer leaflet will enable Annexin V to bind to it in the presence of physiological concentration of Calcium ions (Ca^{2+}) whereas the dead cells will be detected by the binding of Propidium Iodide to the cellular DNA inside the cell (Moss, Edwards et al. 1991).

The results showed that there was a significant increase ($p<0.05$) in the population of LNCaP cells in the early apoptosis phase after the treatment with *Marantodes pumilum* (27%) (Figure 4.27 & Figure 4.28) and *Ficus deltoidea* (Figure 4.31 & Figure 4.32) (FD1: 35%, FD2: 16%) chloroform extract when compared to the untreated cells (11%). LNCaP cells treated with *Marantodes pumilum* n-Hexane extract also demonstrated a significant increase ($p<0.05$) in the early apoptosis (20%), late apoptosis (or secondary necrosis) and necrosis phases when compared to the untreated cells.

PC3 cells treated with *Marantodes pumilum* (Figure 4.29 & Figure 4.30) (20%) and *Ficus deltoidea* (Figure 4.33 & Figure 4.34) (FD1: 29%, FD2: 13%) chloroform extract show a similar increasing trend ($p<0.05$) in the cell's population in the early apoptosis phase when compared to the untreated cells (7%). Treatment with *Marantodes pumilum* n-Hexane (21%) extract caused a significant increase ($p<0.05$) of the cell population in the early apoptosis phase when compared to the untreated cells (7%). This shows an evasion of apoptosis and is recognized to be one of the six hallmarks of cancer, failure to activate apoptosis is regarded as one of the major obstacles to the successful treatment of cancers with drugs (Kaufmann and Vaux 2003, Cummings, Ward et al. 2004).

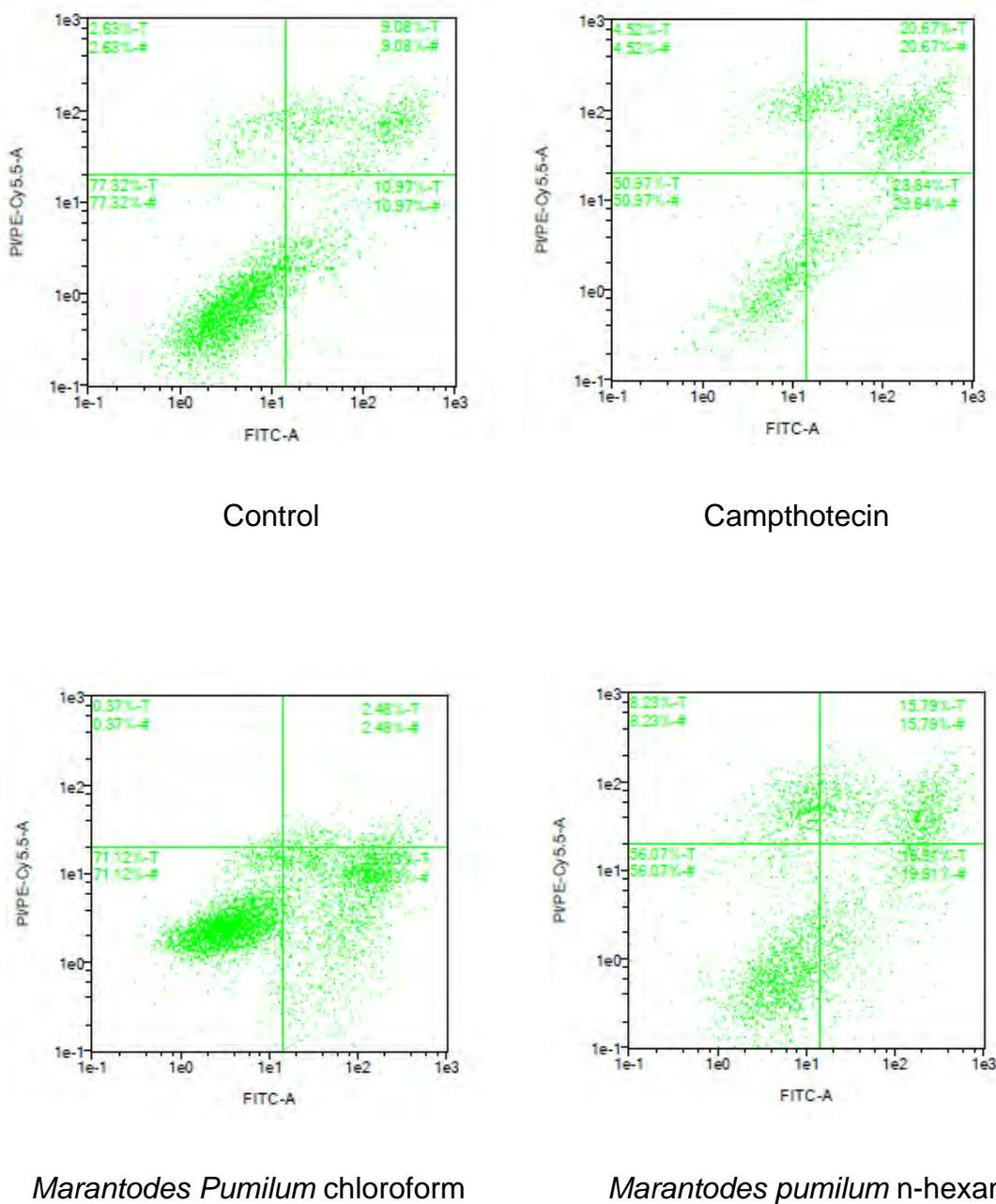


Figure 4.27. The dot plots of LNCaP cells after 6 hours treatment with the active extracts of *Marantodes pumilum* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. Results shown are representative of three independent experiments.

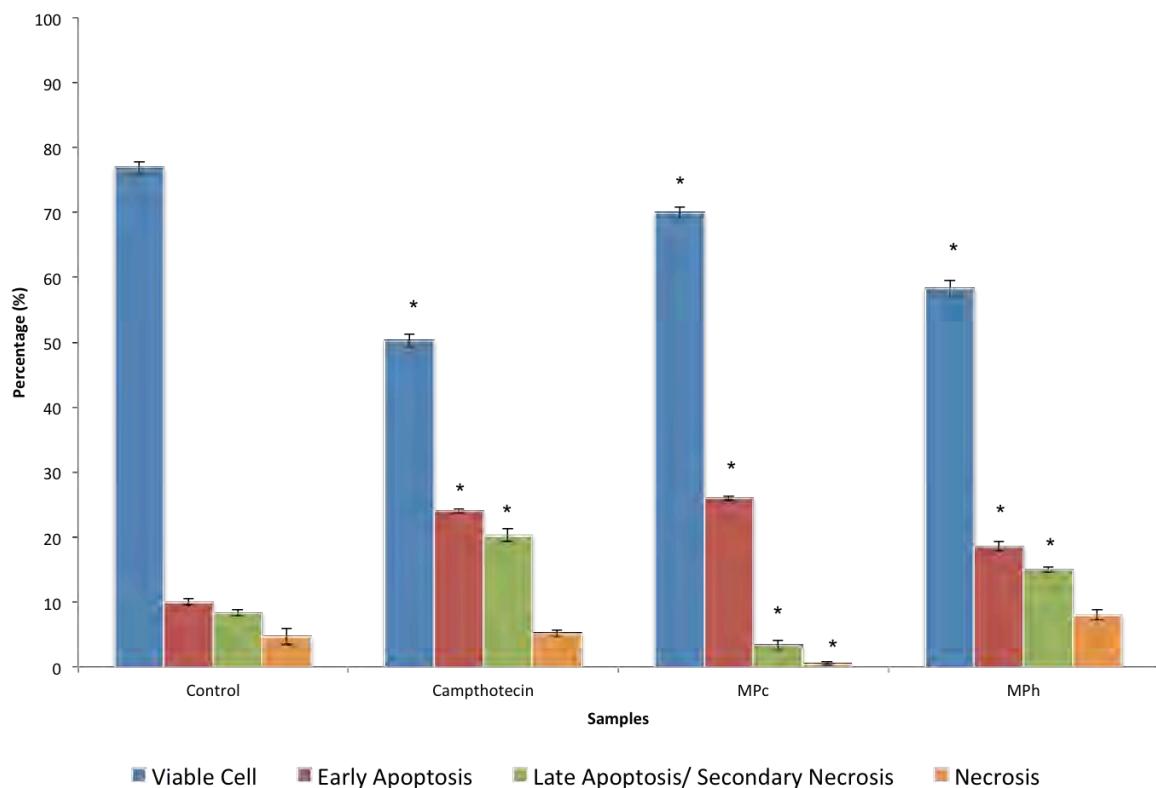


Figure 4.28. The percentage (%) of cell distribution of LNCaP cells after 6 hours treatment with the active extracts of *Marantodes pumilum* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. * $p < 0.05$ as compared to the untreated cells. Results shown are representative of three independent experiments.

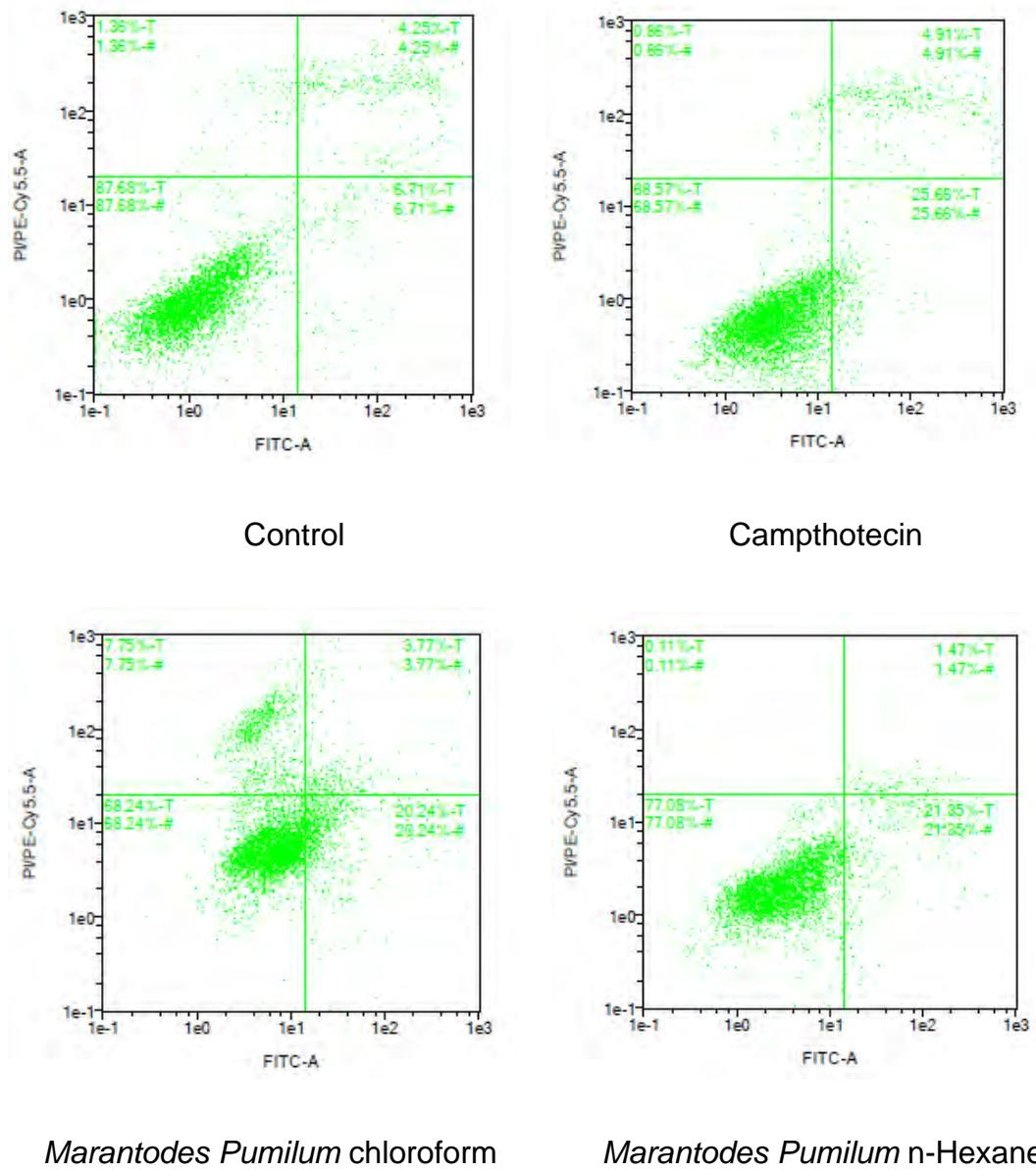


Figure 4.29. The dot plots of PC3 cells after 6 hours treatment with the active extracts of *Marantodes pumilum* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. Results shown are representative of three independent experiments.

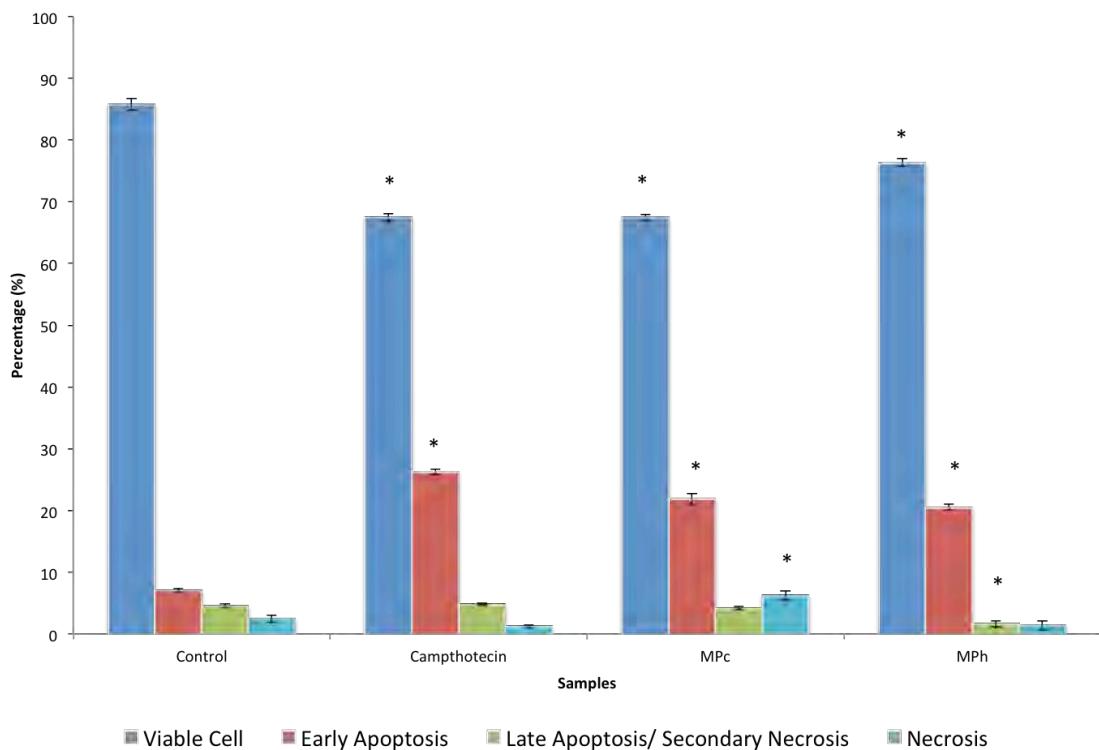


Figure 4.30. The percentage (%) of cell distribution of PC3 cells after 6 hours treatment with the active extracts of *Marantodes pumilum* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. * $p < 0.05$ as compared to the untreated cells. Results shown are representative of three independent experiments.

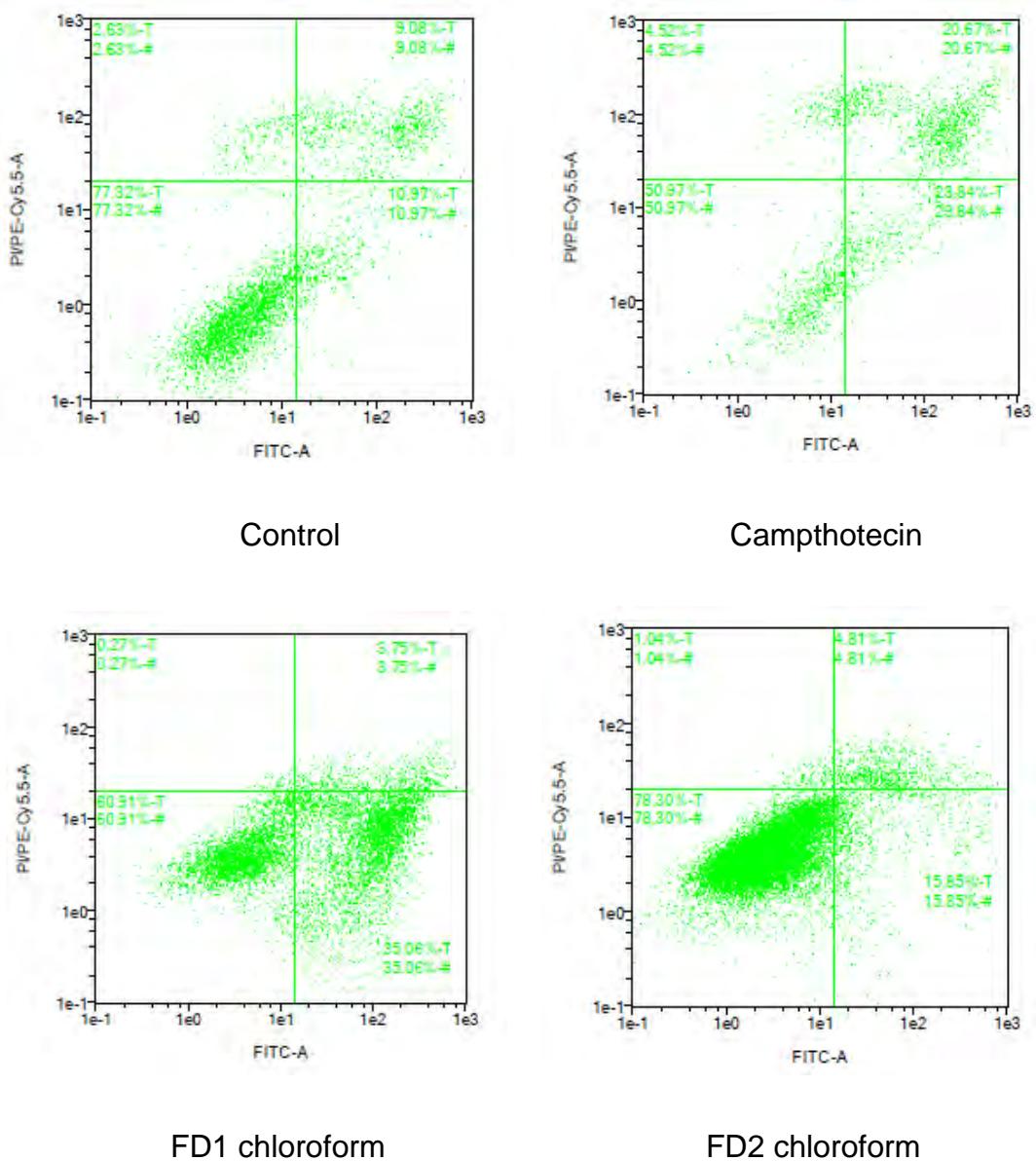


Figure 4.31. The dot plots of LNCaP cells after 6 hours treatment with the active extracts of *Ficus deltoidea* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. Results shown are representative of three independent experiments.

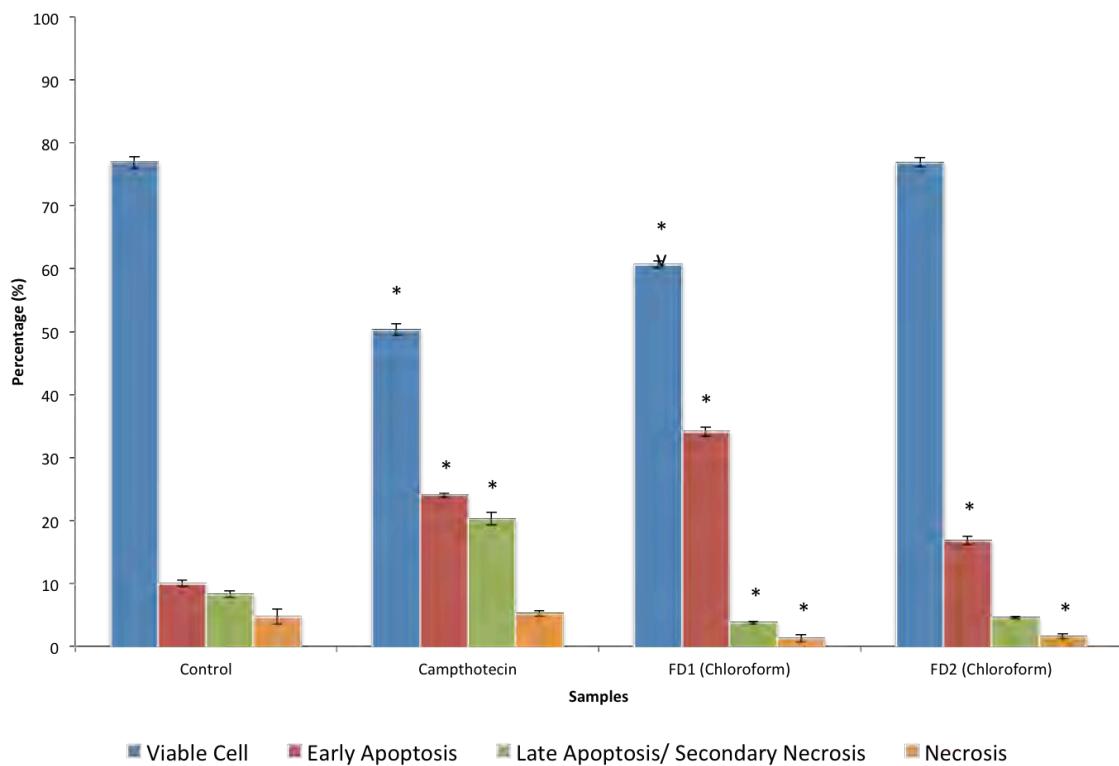


Figure 4.32. The percentage (%) of cell distribution of LNCaP cells after 6 hours treatment with the active extracts of *Ficus deltoidea* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. * p<0.05 as compared to the untreated cells. Results shown are representative of three independent experiments.

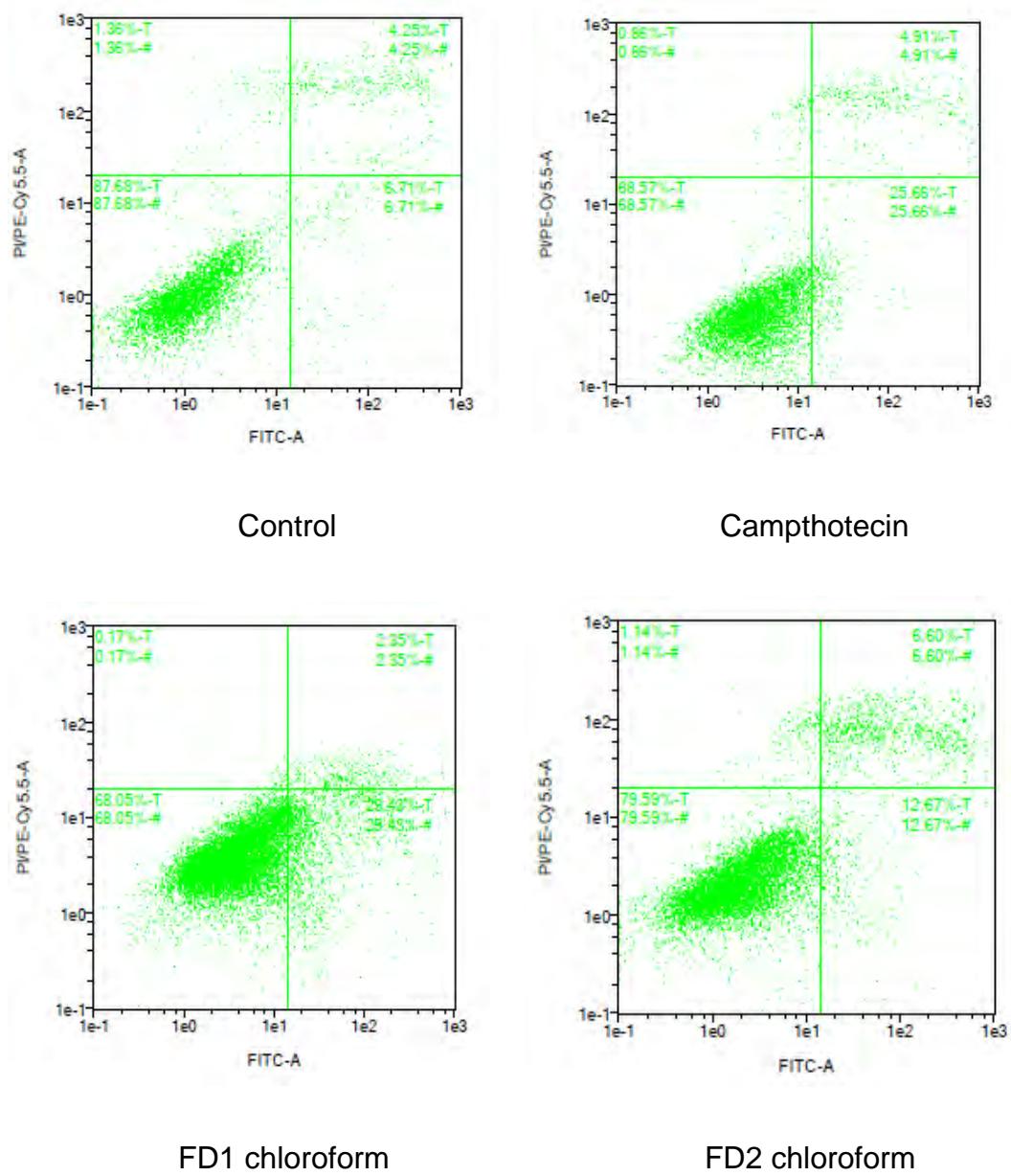


Figure 4.33. The dot plots of PC3 cells after 6 hours treatment with the active extracts of *Ficus deltoidea* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. Results shown are representative of three independent experiments.

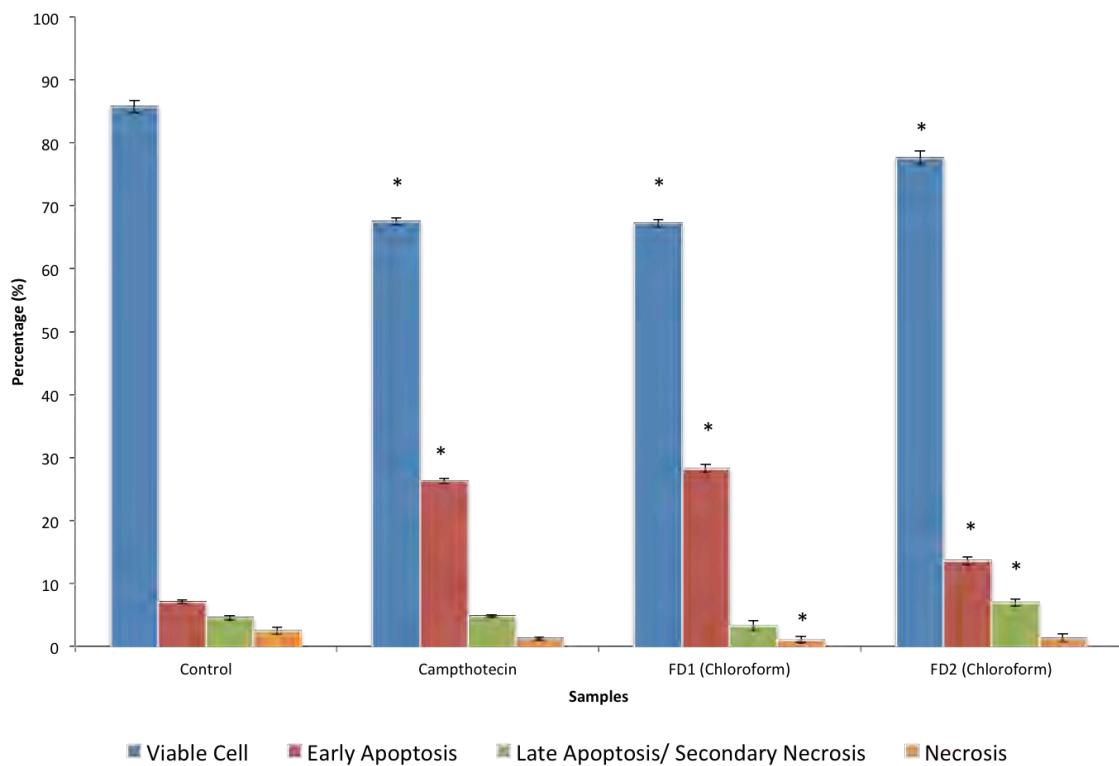


Figure 4.34. The percentage (%) of cell distribution of PC3 cells after 6 hours treatment with the active extracts of *Ficus deltoidea* as determined by Annexin V-FITC and PI staining. The cells were treated with different active extracts for 6 hours. * $p < 0.05$ as compared to the untreated cells. Results shown are representative of three independent experiments.

4.1.5.3 Caspase 3 and 7 activity

Apoptosis is a programmed cell death coordinated by members of the caspase family (Walsh, Cullen et al. 2008). These caspases can be divided into two distinct types, the initiator caspases (caspase-8, caspase-9, and caspase-10). Initiator caspases are highly specific proteases that cleave and activate proteins including the downstream caspases also known as the effector caspases (caspase-3, caspase-6, and caspase-7). Effector caspases are responsible for most of the proteolysis taking place during apoptosis causing the cleavage of Poly ADP Ribose Polymerase 1 (PARP-1) and other cellular proteins (Slee, Harte et al. 1999). In normal conditions, the primary function of PARP-1 is to detect and repair DNA damage. However, in caspase-mediated apoptotic cell death, the cleavage of several key proteins that are required for cellular functioning and survival - including PARP-1 - is very important. In fact, the cleavage of PARP-1 by caspases especially caspase-3 is considered to be one of the important hallmarks of apoptosis (Kaufmann, Desnoyers et al. 1993). The cleavage of PARP-1 by the caspases will result in the formation of two specific fragments; an 89-kD catalytic fragment and a 24-kD DBD (Lazebnik, Kaufmann et al. 1994). The formation of these two fragments will irreversibly inhibit its function to detect and repair DNA damage (Chaitanya, Alexander et al. 2010).

Both LNCaP (Figure 4.35 & 4.37) and PC3 (Figure 4.36 & 4.38) cells treated with *Marantodes pumilum* and *Ficus deltoidea* chloroform extract displayed a significant increase ($p<0.01$) in the activity of caspases 3 and 7 when compared to the untreated cells. LNCaP and PC3 cells treated with *Marantodes pumilum* n-Hexane extract also showed a similar effect ($p<0.05$). These findings further indicate that both plant extracts stimulate prostate cancer cell death via apoptosis and that this process is mediated, at least in part of the activation of caspases 3 and 7.

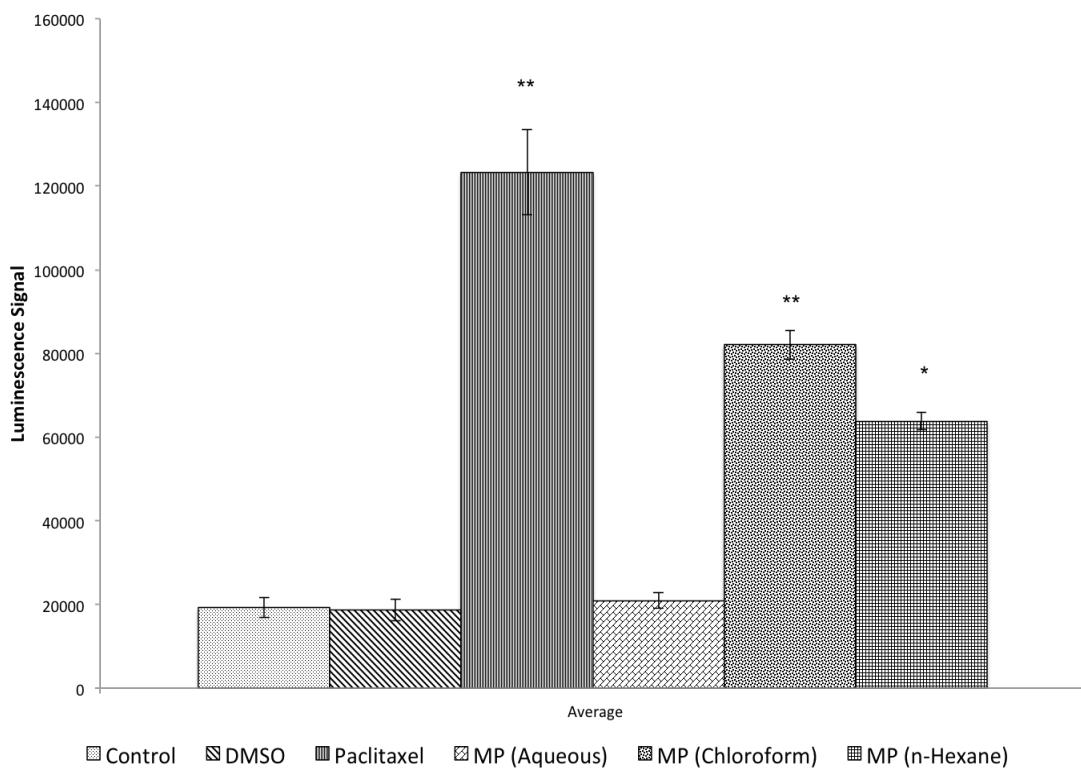


Figure 4.35. Caspase 3/7 activity in LNCaP cells treated with the active extracts of *Marantodes pumilum* for 72 hours. The y-axis shows the luminescent signal subtracted from a blank, which is proportional to the caspase activity. The error bars display the standard error of mean (SEM) obtained from 3 independent experiments. Significance compared to control, *(P<0.05), **(P<0.01) as determined by un-paired t-test.

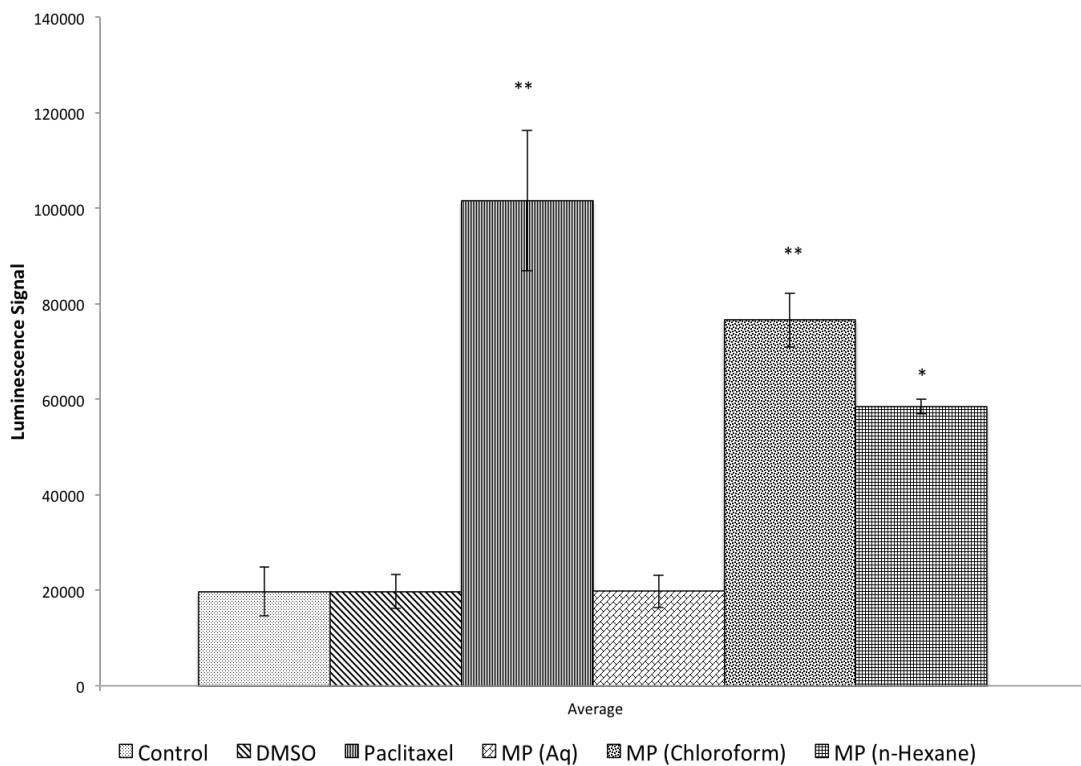


Figure 4.36. Caspase 3/7 activity in PC3 cells treated with the active extracts of *Marantodes pumilum* for 72 hours. The y-axis shows the luminescent signal subtracted from a blank, which is proportional to the caspase activity. The error bars display the standard error of mean (SEM) obtained from 3 independent experiments. Significance compared to control, *($P<0.05$), **($P<0.01$) as determined by un-paired t-test.

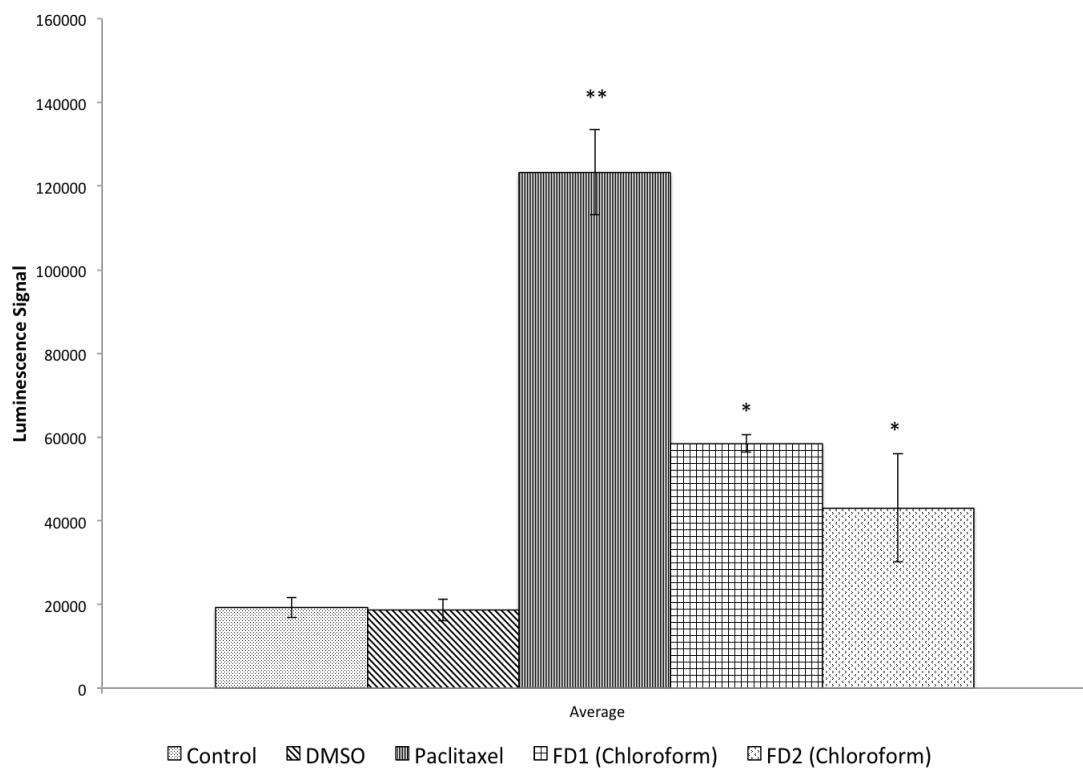


Figure 4.37. Caspase 3/7 activity in LNCaP cells treated with the active extracts of *Ficus deltoidea* for 72 hours. The y-axis shows the luminescent signal subtracted from a blank, which is proportional to the caspase activity. The error bars display the standard error of mean (SEM) obtained from 3 independent experiments. Significance compared to control, *(P<0.05), **(P<0.01) as determined by un-paired t-test.

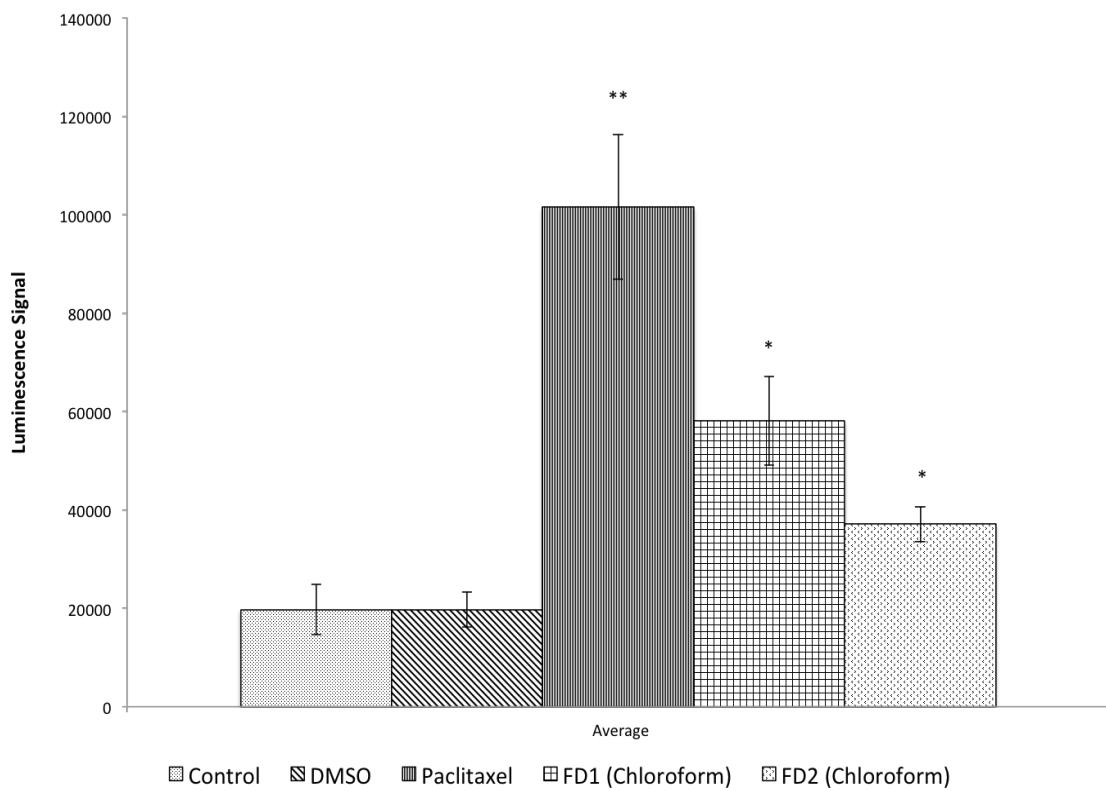


Figure 4.38. Caspase 3/7 activity in PC3 cells treated with the active extracts of *Ficus deltoidea* for 72 hours. The y-axis shows the luminescent signal subtracted from a blank, which is proportional to the caspase activity. The error bars display the standard error of mean (SEM) obtained from 3 independent experiments. Significance compared to control, *(P<0.05), **(P<0.01) as determined by un-paired t-test.

4.1.5.4 MMP depolarization in active plant extracts treated PC3 cell line

In addition to its metabolic functions, mitochondria play a major role in the management of other cellular functions like cell survival and death (Galluzzi, Kepp et al. 2012). Mitochondrial changes, including variations in mitochondrial membrane potential ($\Delta\psi_m$), are the key events during drug-mediated apoptosis (Zamzami, Marchetti et al. 1995). One of the distinctive features of the early stage of apoptosis is the disruption of active mitochondria, which includes changes in the mitochondrial membrane potential and alterations to the oxidation-reduction potential of the mitochondria. These changes are believed to be caused by the opening of the mitochondrial permeability transition pore (PTP) that leads to the passage of ions and small molecules (Ly, Grubb et al. 2003). Hence, to assess the effect of the active plant extracts on mitochondrial activity, the mitochondrial membrane potential (MMP) was studied using the cationic fluorescent probe JC-1. This compound can selectively enter into the mitochondrion of cells, and reversibly changes from red to green fluorescence as the mitochondrial membrane potential decreases. By measuring such a shift in the fluorescence emission by flow cytometry, MMP can be readily detected. As shown in Figure 4.39 to Figure 4.41, the active extracts of the plants increased the percentage of mitochondrial membrane potential (MMP) depolarization significantly (* $p<0.05$ and ** $p<0.01$) in PC3 cell lines which indicate that the active extracts of the plants induce cell death in human prostate cancer cells with the involvement of mitochondrial membrane depolarization. These findings further support previous results shown in section 4.1.5.3, which showed the activation of caspases 3 and 7.

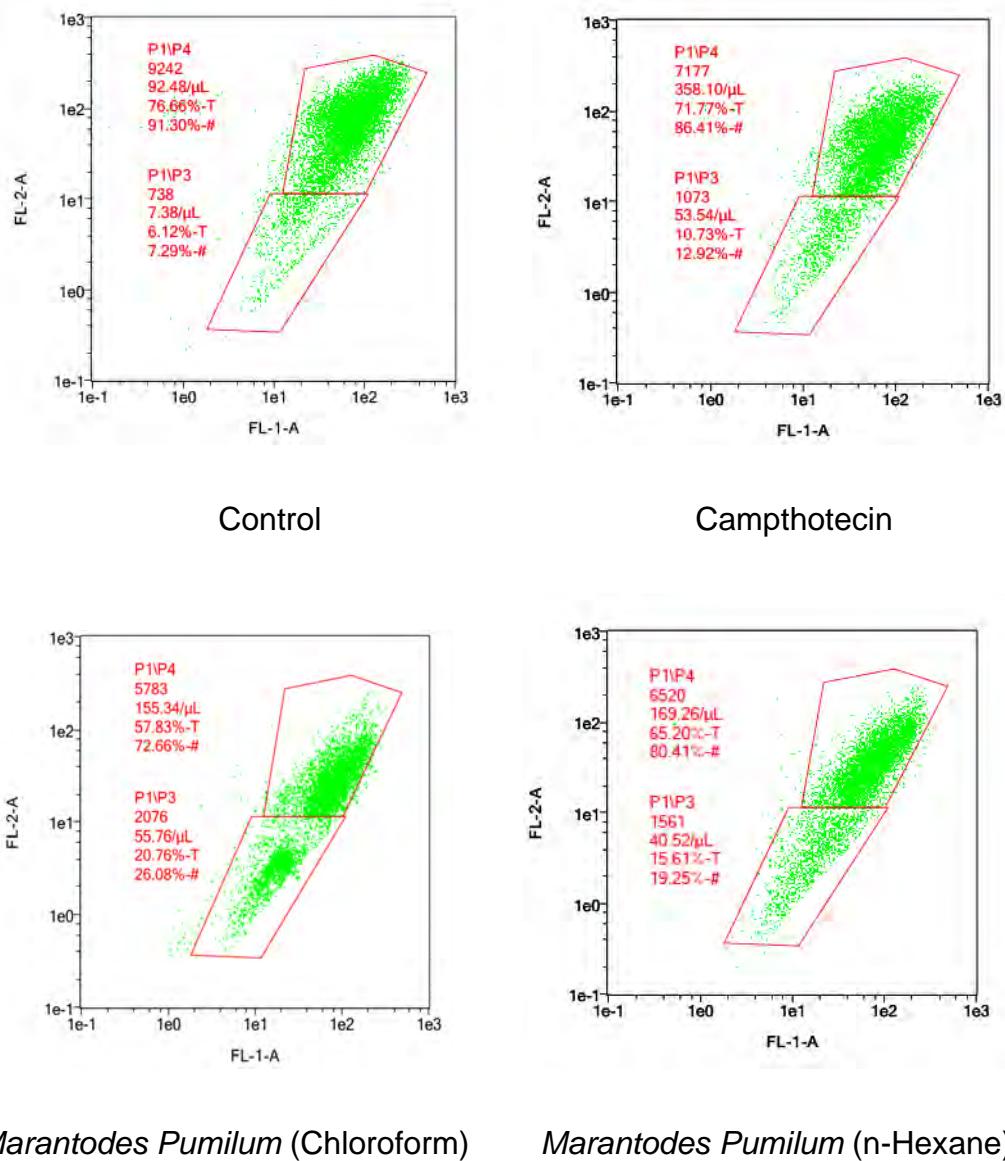


Figure 4.39. Representative MMP profiles of flow cytometry for active plant extracts-treated PC3 cells. Depolarization of mitochondrial membrane potential (MMP) of PC3 prostate cancer cell lines was induced by the active extracts of *Marantodes pumilum*. PC3 cell lines were treated with the GI50 of the plant extracts for 6 hours. Results shown are representative of three independent experiments.

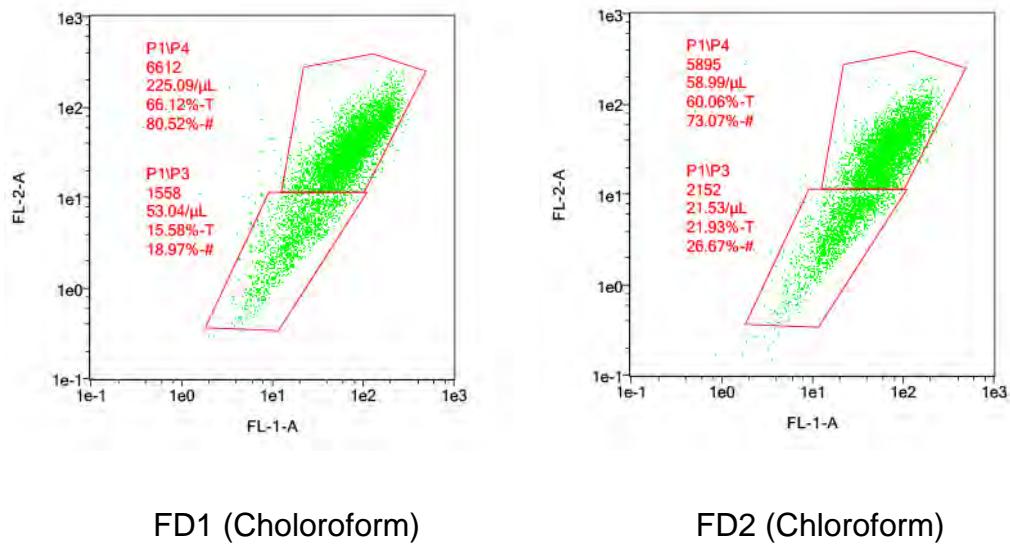


Figure 4.40. Representative MMP profiles of flow cytometry for active plant extracts-treated PC3 cells. Depolarization of mitochondrial membrane potential (MMP) of PC3 prostate cancer cell lines was induced by the active extracts of *Ficus deltoidea*. PC3 cell lines were treated with the GI50 of the plant extracts for 6 hours. Results shown are representative of three independent experiments.

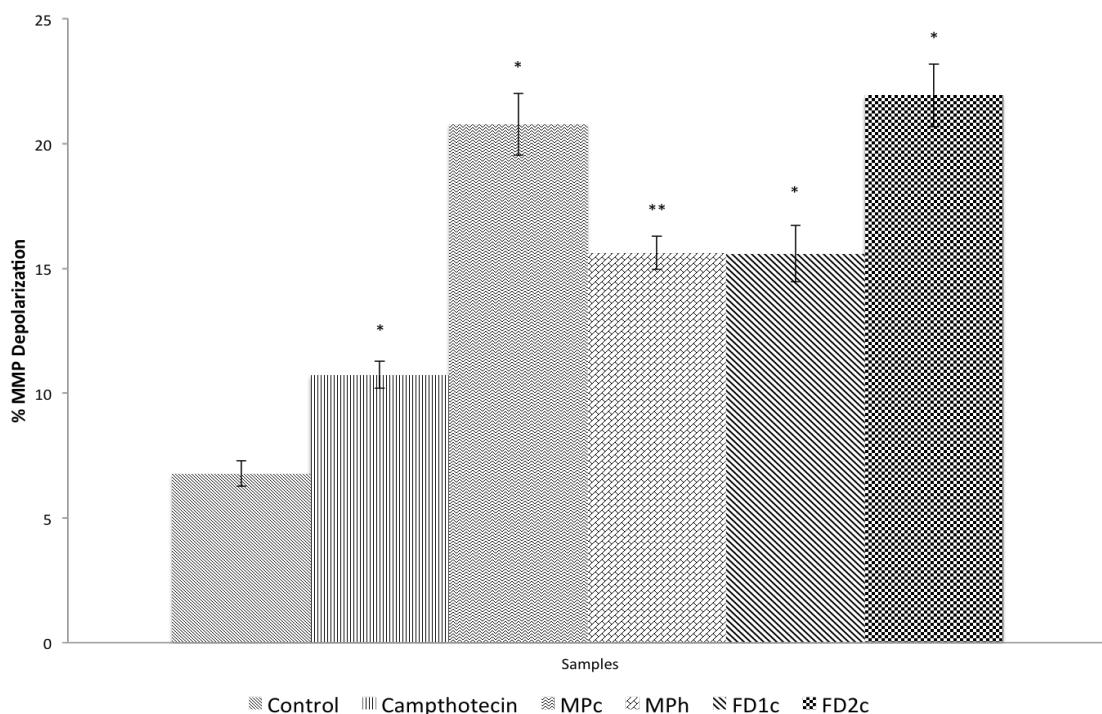
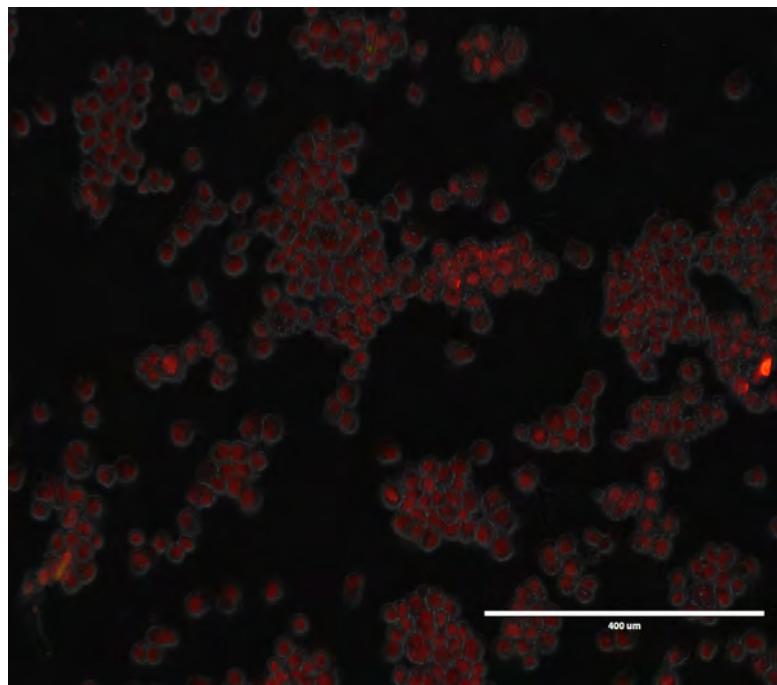


Figure 4.41. Quantification analysis of percentage MMP intensity in Figure 4.39 & Figure 4.40. Data represented as means \pm SEMs (n=3). *p<0.05 and **p<0.01 and against control.

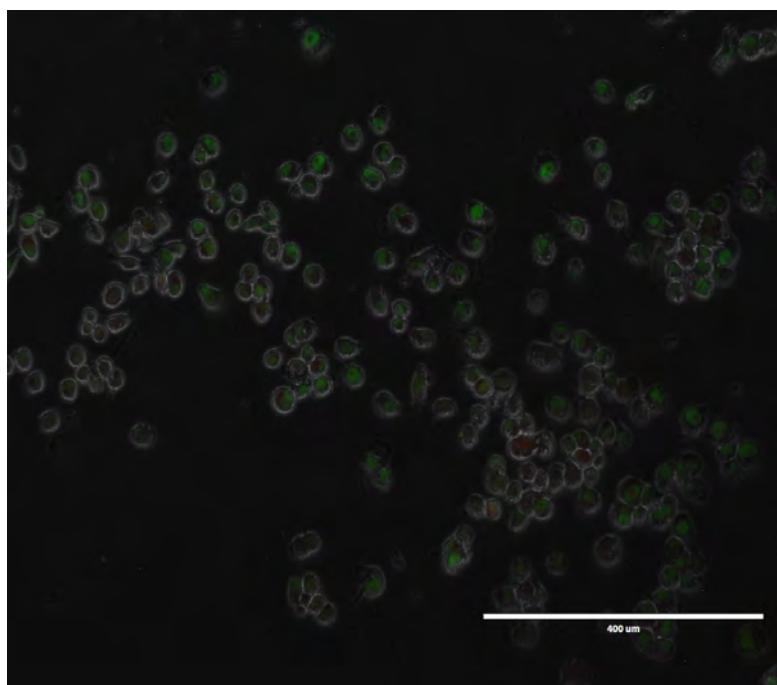
4.1.5.5 Nuclear DNA fragmentation

Nuclear DNA fragmentation is one of the important hallmarks of apoptosis. This process involves the double-strand cleavage of nuclear DNA at the linker regions between nucleosomes, which leads to the production of oligonucleosomal fragments (Kerr, Winterford et al. 1994). The degradation of the double-stranded nuclear DNA into oligonucleosomal fragments or nucleosomal units is one of the best characterized biochemical features of apoptotic cell death (Nagata 2000), as it enables the detection of apoptotic cells by using the in-situ assays of DNA fragmentation called the Terminal deoxynucleotidyl transferase mediated biotin dUTP Nick End Labelling assay (TUNEL). However, the interpretation of this assay must be carefully assessed together with other morphological features of apoptotic cells because single-strand DNA ends in cell nuclei are also present in necrotic cells (Collins, Schandl et al. 1997).

In order to prevent the possible experimental bias, the results from this study must be supported by other hallmarks of apoptosis such as the activation of caspases and the up-regulation of pro-apoptotic proteins as well as the down-regulation of anti-apoptotic proteins. In this study the TUNEL assay was used to assess the effects of the active plant extracts on prostate cancer cells' nuclear DNA fragmentation. Figure 4.42 to Figure 4.44 show that after 72 hours of treatment with active plant extracts, a more localized green fluorescent (fluorescein-12-dUTP) colour can be detected, in the treated cells as compared to the control. The localized green fluorescent label can only be detected in apoptotic cells while the red Propidium Iodide stain will label both apoptotic and non-apoptotic cells. These findings provide more evidence that both *Marantodes pumilum* and *Ficus deltoidea* plant extracts induce prostate cancer cell death via apoptosis.

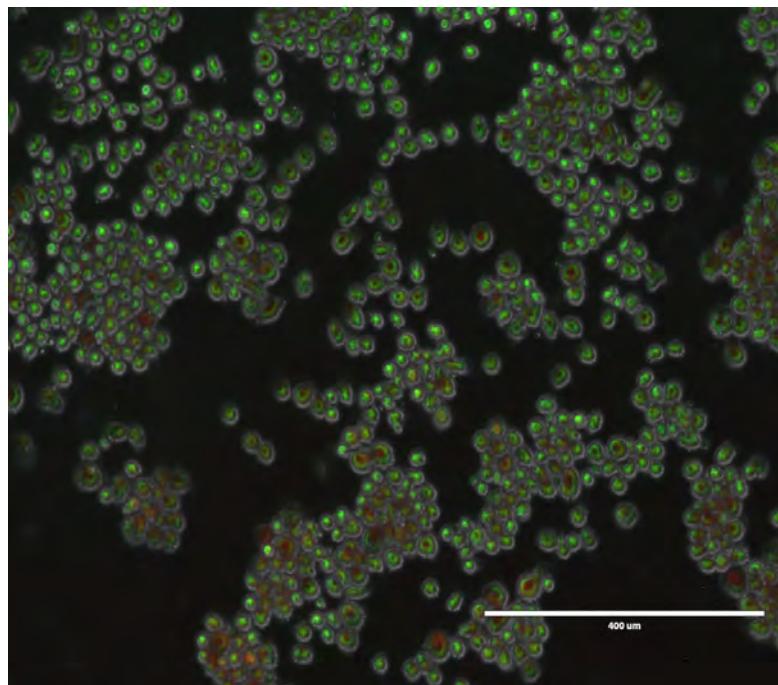


Control

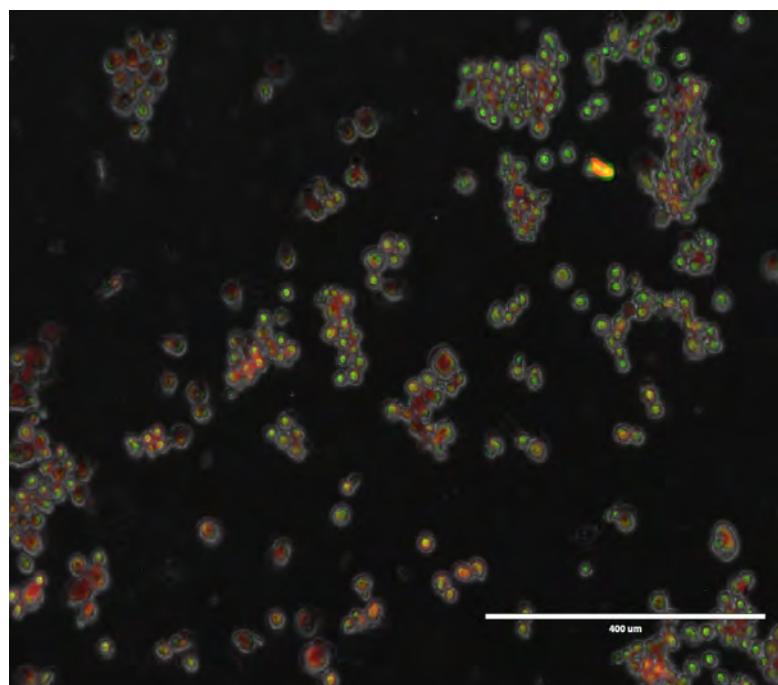


DNAse (Positive Control)

Figure 4.42. PC3 cells treated DNAse (positive control) and stained with DeadEndTM Flurometric TUNEL system after 72 hours. Red fluorescence indicates healthy cells while green fluorescence shows fragmented nuclear DNA. Results shown are representative of three independent experiments.

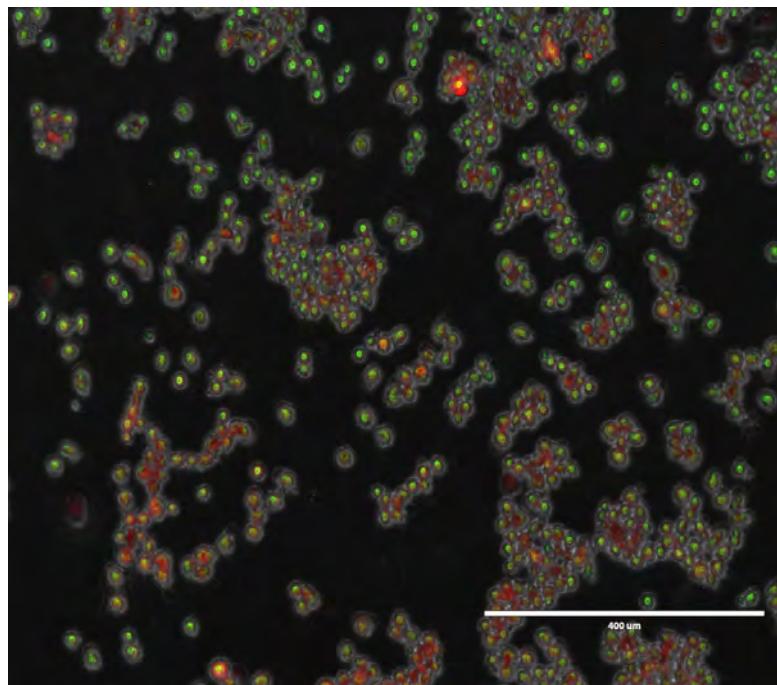


MPc

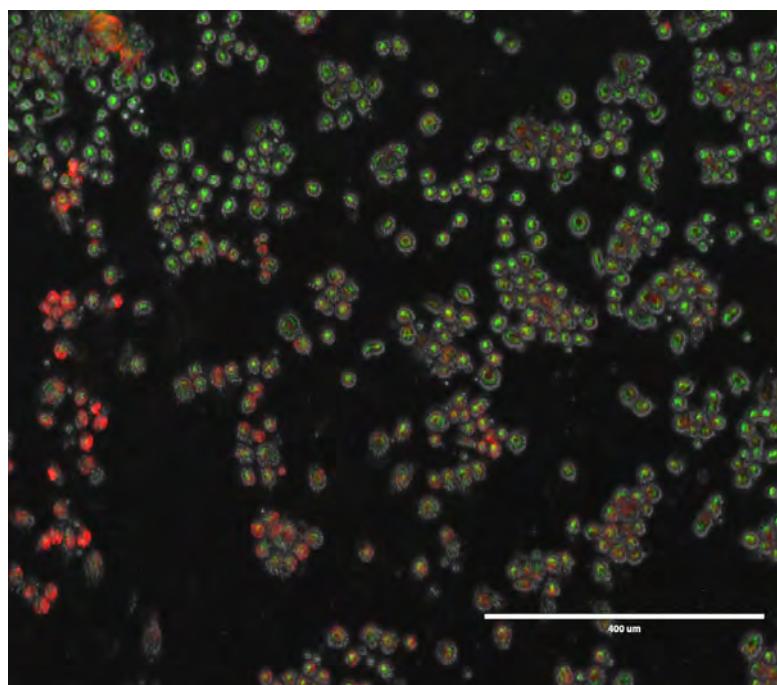


MPh

Figure 4.43. PC3 cells treated with *Marantodes pumilum* plant extracts and stained with DeadEnd™ Fluorometric TUNEL system after 72 hours. Red fluorescence indicates healthy cells while green fluorescence shows fragmented nuclear DNA.



FD1c



FD2c

Figure 4.44. PC3 cells treated with *Ficus deltoidea* plant extracts and stained with DeadEnd™ Fluorometric TUNEL system after 72 hours. Red fluorescence indicates healthy cells while green fluorescence shows fragmented nuclear DNA.

4.1.5.6 PC3 cell death via apoptosis (intrinsic pathway) based on Bax, Bcl-2 and Smac/DIABLO mRNA gene expression

Apoptosis is a programmed cell death that occurs through a highly complex and sophisticated mechanism involving an energy dependent cascade of molecular event. Research has indicated that there are two major pathways for apoptosis: (1) extrinsic, also called 'the death receptor pathway'; and (2) intrinsic, otherwise known as the 'mitochondrial-driven pathway'. However, new findings by Igney and Krammer (2002) suggest that the two pathways are linked and molecules in one pathway can influence the other. In addition, new evidence suggests that there is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cells (Martinvalet, Zhu et al. 2005). However all of these pathways show important similarities in which they converge into the same terminal (or execution) pathways and are initiated by the activation of caspase-3. This results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cells receptors and finally uptake by phagocytic cells (Hengartner 2000).

The intrinsic signaling pathways involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals, which act directly on targets within the cells and are driven by mitochondria. These stimuli may act in either a positive or negative fashion; where negative signals involve the absence of certain growth factors, hormones and cytokines without which will trigger apoptosis and on the other hand positive signals include exposure to radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals (Elmore 2007). All of these stimuli will cause disruption to the inner mitochondrial membrane that lead to the opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of pro-apoptotic proteins from the intermembrane space to the cytosol (Saelens, Festjens et al. 2004). The first group - which consists of cytochrome c, Smac/DIABLO and the serine

protease HtrA2/OMI - are responsible in the activation of the caspase-dependent mitochondrial pathway (Du, Fang et al. 2000, Garrido, Galluzzi et al. 2006), while the second group - consisting of the apoptosis-inducing factor (AIF), endonuclease G and caspase-activated DNase (CAD) - are responsible for DNA fragmentation and condensation of peripheral nuclear chromatin, which is a late event that occurs after the cell has committed to die (Joza, Susin et al. 2001).

All these apoptotic mitochondrial events are controlled and regulated by members of the Bcl-2 family of proteins (Cory and Adams 2002). The Bcl-2 family of proteins is responsible in maintaining the mitochondrial membrane permeability and can either be pro-apoptotic (Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk) or anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG). These proteins are very important and their balance (usually measured as the ratio Bax/Bcl-2) will determine if the cell commits to apoptosis or aborts this process. Therefore, in this study, we will look at the effects of the active plant extracts on the expression of 3 different genes (Bax, Bcl-2, and Smac/DIABLO), which play vital roles in the production of proteins that are actively involved in the intrinsic pathway of apoptosis.

Figure 4.45 (summary in Table 4.4) shows that MPc is able to increase the expression of Bax and Smac/DIABLO by 9-fold and 4-fold respectively, while reducing the expression of Bcl-2 by more than 20-fold in a significant manner. MPh also significantly stimulates the expression of both Bax and Smac/DIABLO by 12-fold and 3-fold respectively, while inhibiting Bcl-2 expression by 7-fold. A similar trend was observed for both FD1c and FD2c extracts as they significantly up-regulate the expression of Bax (FD1c: 2-fold, FD2c: 2-fold) and Smac/Diablo (FD1c: 4-fold, FD2c: 2-fold) even though the extent of gene expression stimulation was not as much as both MPc and MPh. Both FD1c and FD2c also significantly down-regulated the expression of Bcl-2 by 7 and 12-fold respectively. These findings suggest that all active

extracts of the plants increase the expression of pro-apoptotic genes and reduce the expression of anti-apoptotic genes, which commit PC3 cells into apoptosis by an increased production of pro-apoptotic proteins and reduced production of anti-apoptotic proteins. Table 4.5 shows the summary of all the investigations being carried out in order to determine the mode of cells death induced by the active extracts of both *Marantodes pumilum* and *Ficus deltoidea* plants and their respective results. Evidences from these studies suggest that all active plant extracts induce prostate cancer cell death via apoptosis.

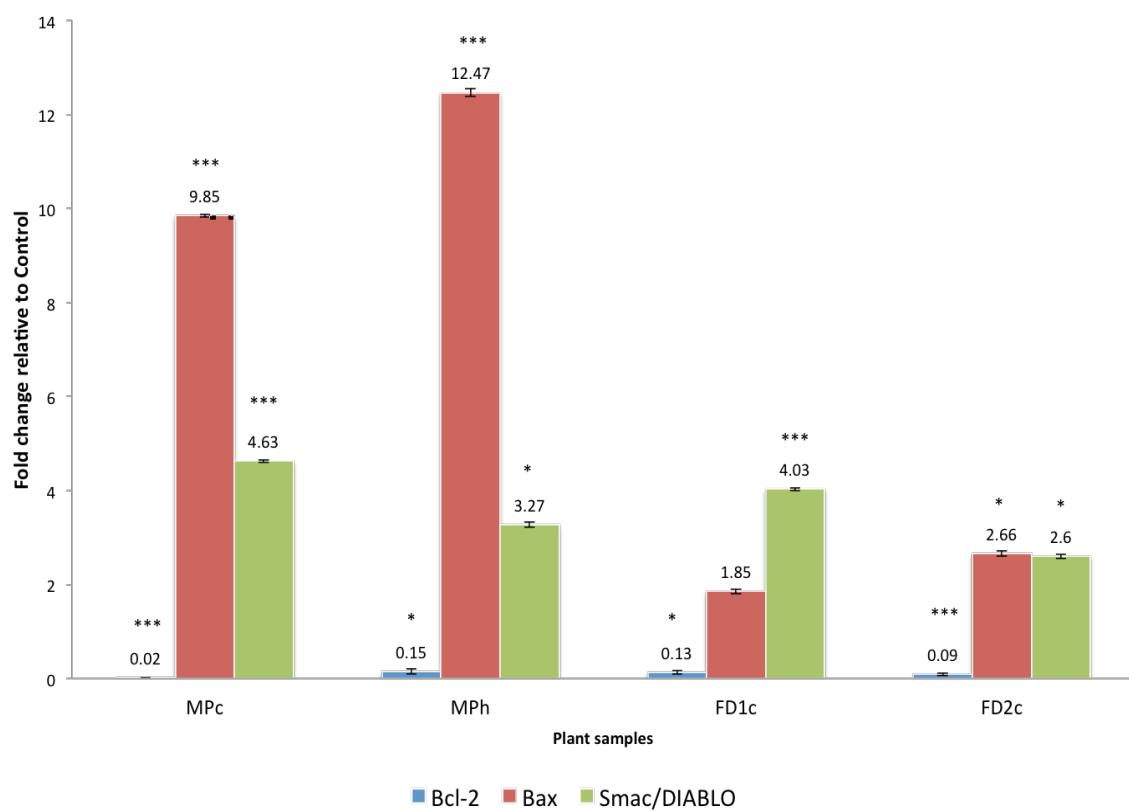


Figure 4.45. Bcl-2, Bax and Smac/DIABLO mRNA gene expressions in PC3 cells treated with MNTC of the active plant extracts of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours. The genes expressions were determined as described in RT-qPCR Conditions and Analysis. Data are mean \pm SD; $n=4$ experiments. * $P < 0.05$, * $P < 0.001$.**

Table 4.4 mRNA gene expression analysis (Bax, Bcl-2 & Smac/DIABLO) in PC3 cells treated with MNTC of the active plant extracts of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours. The genes expressions were determined as described in RT-qPCR Conditions and Analysis where Control = 1. Data are mean \pm SD; n=4 experiments. * P < 0.05, * P < 0.001.**

Sample	Gene	Fold expressions relative to Control in PC3 cells
MPc	Bax	9***
	Bcl-2	0.02***(> 20-Fold)
	Smac/DIABLO	4***
MPh	Bax	12***
	Bcl-2	0.15*(7-Fold)
	Smac/DIABLO	3*
FD1c	Bax	2
	Bcl-2	0.13*(7-Fold)
	Smac/DIABLO	4***
FD2c	Bax	2*
	Bcl-2	0.09***(> 12-Fold)
	Smac/DIABLO	2*

Table 4.5 Summary list of all the investigation carried out to determine the mode of death induced by the active extracts of both *Marantodes pumilum* and *Ficus deltoidea* plants and their respective results.

Assay/Sample	MPc	MPh	FD1c	FD2c
Morphological observation (Characteristics of apoptosis or necrosis)	Apoptosis (+) Necrosis (-)	Apoptosis (+) Necrosis (-)	Apoptosis (+) Necrosis (-)	Apoptosis (+) Necrosis (-)
DAPI staining	Apoptotic cells visible	Apoptotic cells visible	Apoptotic cells visible	Apoptotic cells visible
Annexin V-FITC fluorocytometric analysis	↑ Number of cells in early apoptosis compared to control (untreated cells)	↑ Number of cells in early apoptosis compared to control (untreated cells)	↑ Number of cells in early apoptosis compared to control (untreated cells)	↑ Number of cells in early apoptosis compared to control (untreated cells)

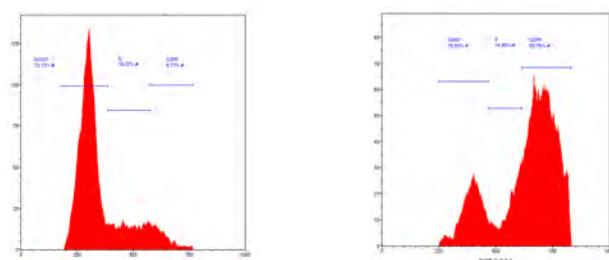
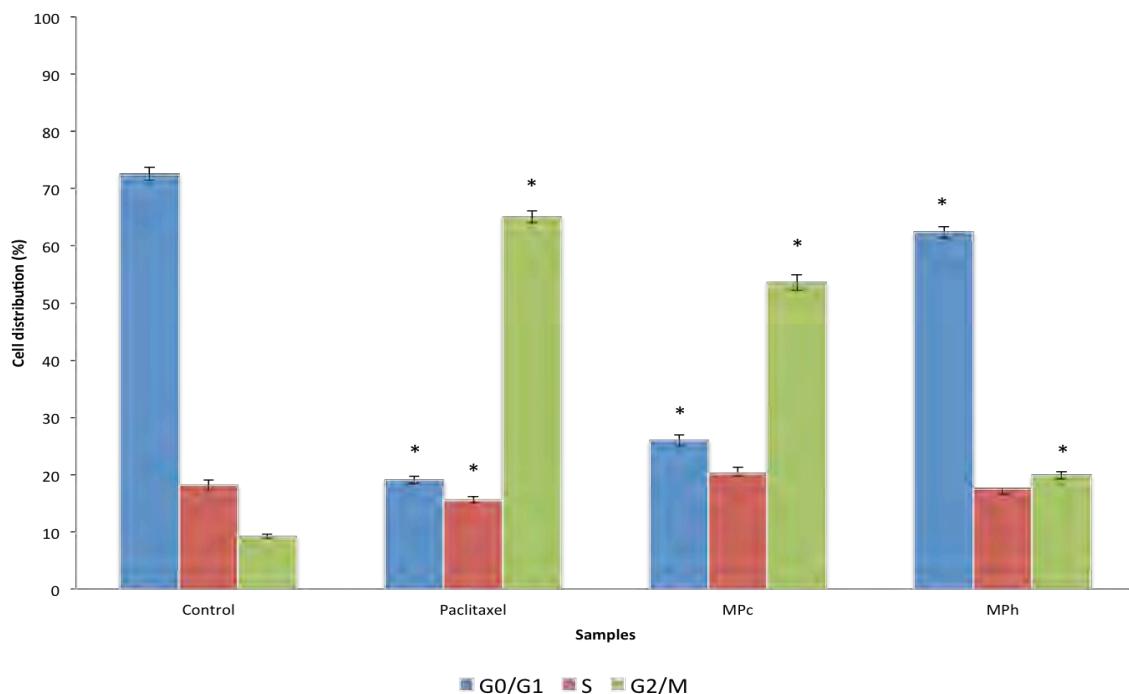
Table 4.5 (Continues)

Assay/Sample	MPc	MPh	FD1c	FD2c
Mitochondrial membrane potential (MMP) depolarization analysis	↑ MMP depolarization	↑ MMP depolarization	↑ MMP depolarization	↑ MMP depolarization
Detection of Caspase 3/7	↑ Caspases 3/7 production			
Nuclear DNA fragmentation analysis	Nuclear DNA fragmentation (+)			
mRNA gene expression analysis	↑ Pro-apoptotic genes ↓ Anti-apoptotic genes			

4.1.6 Effect of the active extracts of the plants on the cell cycle of prostate cancer cell lines

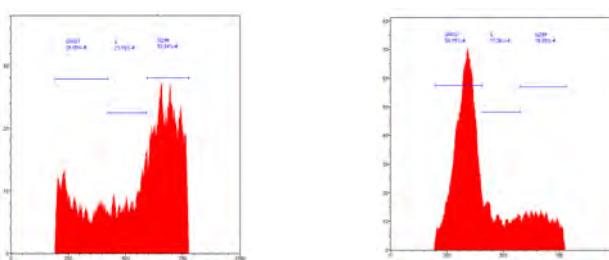
The effects of the active extracts of *Marantodes pumilum* and *Ficus deltoidea* on the cell cycle of LNCaP and PC3 cell lines were evaluated using a flow cytometer. As shown in Figure 4.46 & Figure 4.48, there was a significant increase ($p<0.05$) in the population of LNCaP cells at the G2M phase as compared to the untreated cells when treated with the GI50 of both *Marantodes pumilum* chloroform and n-Hexane extracts and *Ficus deltoidea* extracts. The same trend was observed (Figure 4.47 & Figure 4.49) when the PC3 cells were treated with the GI50 of both plant extracts. In addition, treatment with the GI50 of both plant extracts also caused a significant decrease in the population of both LNCaP and PC3 cells at G0/G1 phase ($p<0.05$) while the percentages of cell population at the S phase remains almost the same for both cell lines.

Cell cycle arrest enables DNA repair to occur thus preventing replication of the damaged templates (Murray 2004). Cell cycle arrests specifically at the G2M phase and it serves to prevent the cells from entering the mitosis (M) phase with damaged genomic DNA. The transition of G2-phase into M-phase is regulated by the activity of Cyclin B-cdc2 (CDK1) complex (Al-Ejeh, Kumar et al. 2010). Cell cycle arrest at G2M phase ultimately inactivates the Cyclin B-cdc2 complex via two parallel cascades. The first cascade involves the phosphorylation of Chk kinases that inactivates cdc25 - which prevents the activation of cdc2 - while the second cascade involves the up-regulation of p53 proteins - which binds to and dissociates the Cyclin B-cdc2 complex (Lukas, Lukas et al. 2004, Foster 2008). Nevertheless, further studies need to be carried out in order to understand the actual mechanisms on how the active extracts of *Marantodes pumilum* and *Ficus deltoidea* induces cell cycle arrest at G2M phase. Apart from that, further studies should be done in order to identify the phytochemicals responsible for the activity.



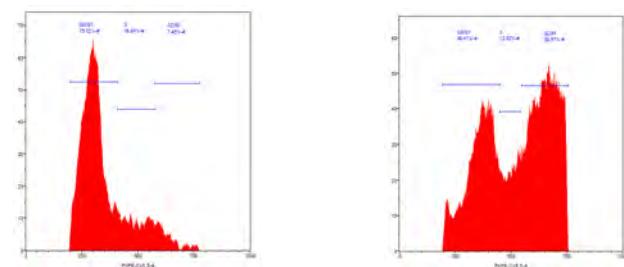
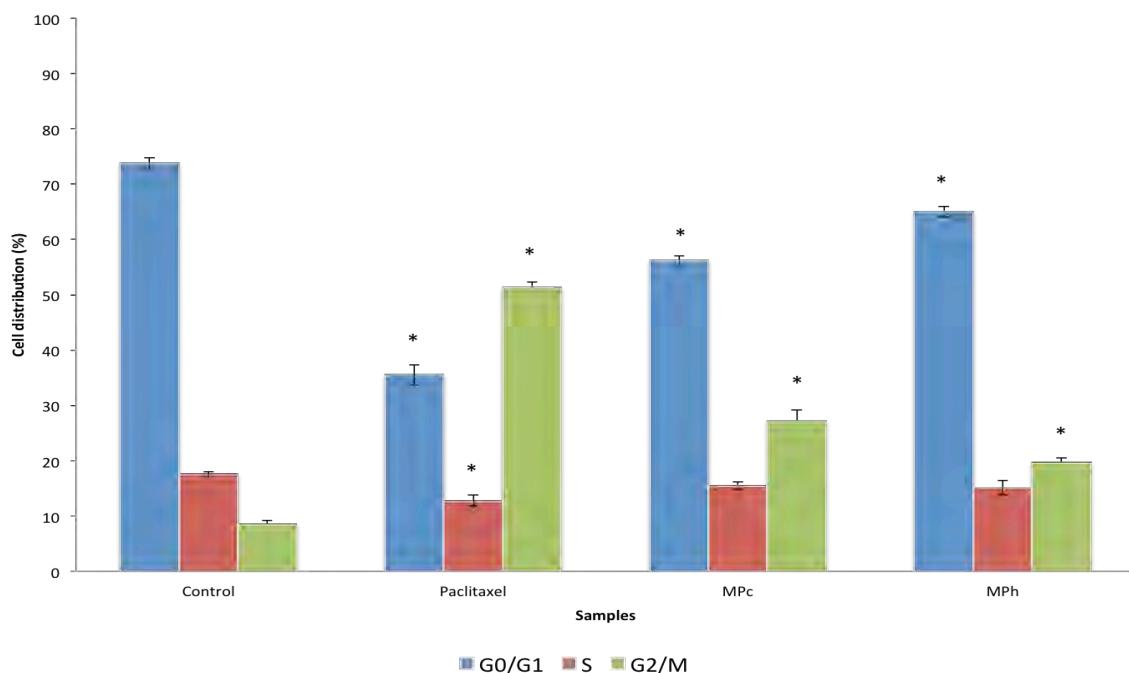
Control

Camptothecin



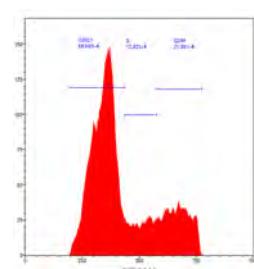
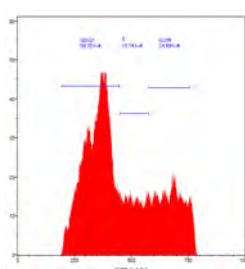
Marantodes Pumilum chloroform *Marantodes Pumilum* n-Hexane

Figure 4.46. Effect of the active extracts of *Marantodes pumilum* on the cell cycle of LNCaP cells analysed by measuring DNA content using flow cytometer. The cells were treated with the GI50 of the active extracts. *p<0.05 as compared to the untreated cells. Paclitaxel treatment was used as positive control. Results shown are representatives of three independent experiments.



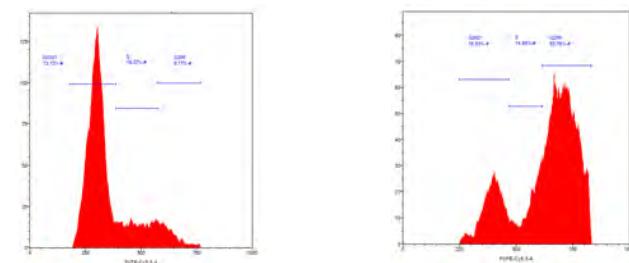
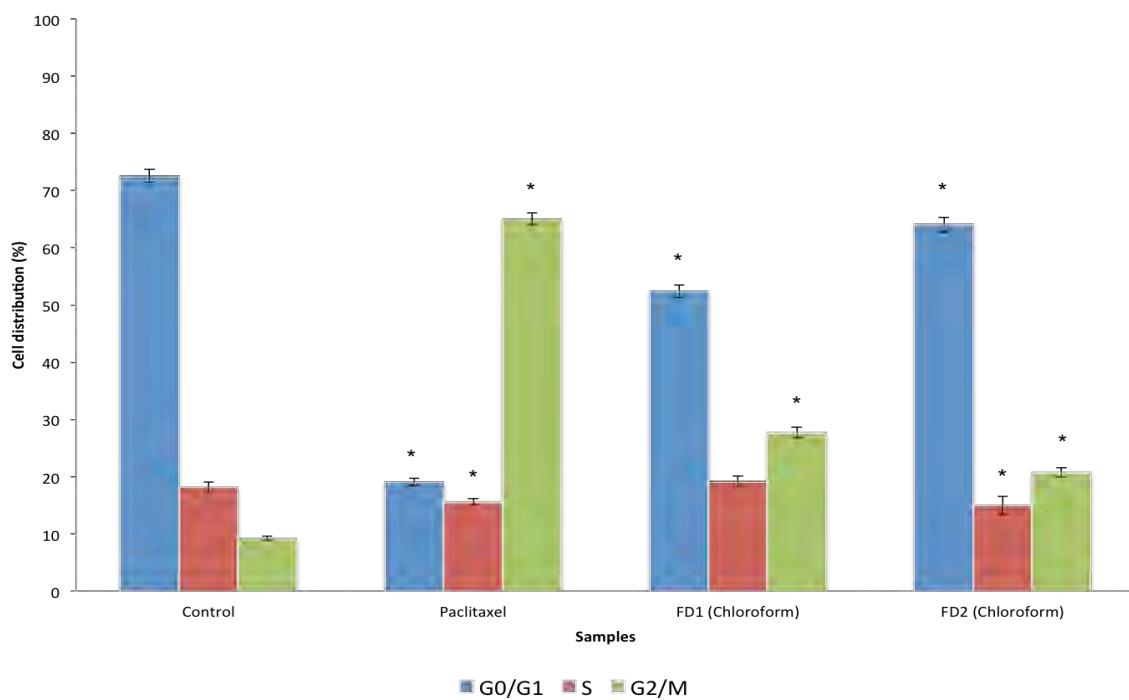
Control

Camphotecin



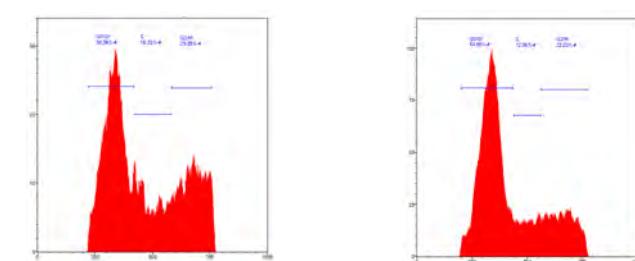
Marantodes Pumilum chloroform *Marantodes Pumilum* n-Hexane

Figure 4.47. Effect of the active extracts of *Marantodes pumilum* on the cell cycle of PC3 cells analysed by measuring DNA content using flow cytometer. The cells were treated with the GI50 of the active extracts. *p<0.05 as compared to the untreated cells. Paclitaxel treatment was used as positive control. Results shown are representatives of three independent experiments.



Control

Camphotecin



FD1 chloroform

FD2 chloroform

Figure 4.48. Effect of the active extracts of *Ficus deltoidea* on the cell cycle of LNCaP cells analysed by measuring DNA content using flow cytometer. The cells were treated with the GI50 of the active extracts. *p<0.05 as compared to the untreated cells. Paclitaxel treatment was used as positive control. Results shown are representatives of three independent experiments.

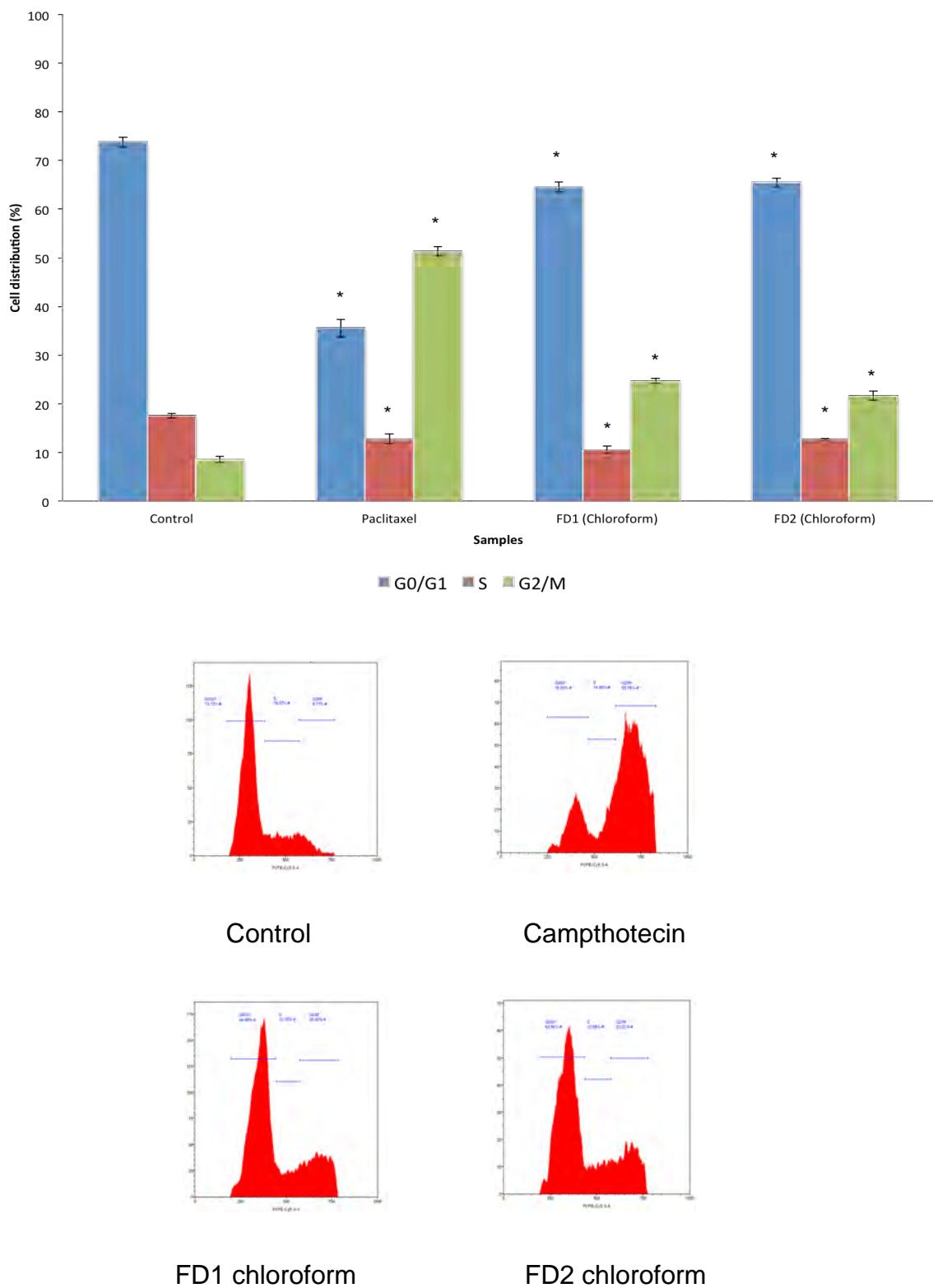


Figure 4.49. Effect of the active extracts of *Ficus deltoidea* on the cell cycle of PC3 cells analysed by measuring DNA content using flow cytometer. The cells were treated with the GI50 of the active extracts. *p<0.05 as compared to the untreated cells. Paclitaxel treatment was used as positive control. Results shown are representatives of three independent experiments.

4.1.7 Effect of the plant extracts on Arachidonic metabolism (ALOX-5)

As mentioned in section 2.1.4.4, the production of the 5-LOX enzyme could also contribute to the growth of prostate cancer cells. This enzyme is known to be encoded by the ALOX-5 gene. Ghosh and Myers (1997) reported that the inhibition of 5-LOX would block the production of its metabolites and triggers apoptosis in prostate cancer cells. This study was conducted to investigate the effects of the active plant extracts of the mRNA gene expression of the ALOX-5 gene. Figure 4.50 shows that only MPc was able to inhibit the expression of ALOX-5 gene in a significant manner. The other 3 plant extracts were not able to have any significant effect on the expression of the gene. This data is very interesting as it suggests that MPc was also able to trigger PC3 cell death via apoptosis by inhibiting ALOX-5 gene expression apart from activating the intrinsic pathway of apoptosis.

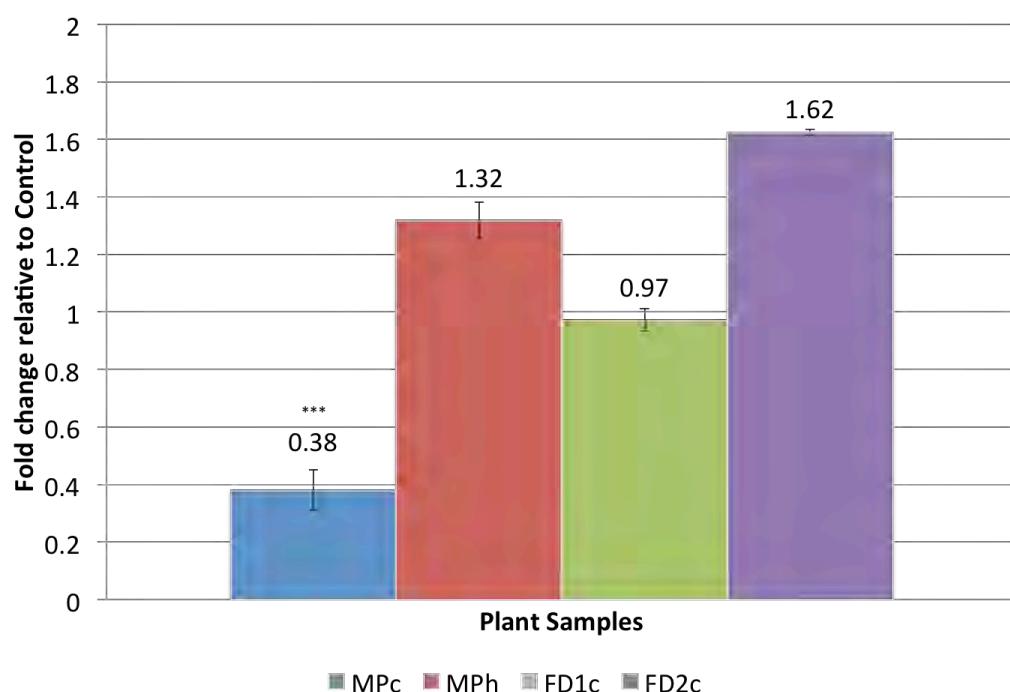


Figure 4.50. ALOX-5 mRNA gene expressions in PC3 cells treated with MNTC of the active plant extracts of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours. The genes expressions were determined as described in RT-qPCR Conditions and Analysis. Data are mean \pm SD; $n=4$ experiments. * $P < 0.05$, *** $P < 0.001$.

4.1.8 Inhibition of PC3 cells migration and invasion

Metastasis is responsible for 90% of cancer mortality rates. It represents the major problem in the treatment of cancer, is indicative for poor prognosis, and has dramatic effects on the survival of patients. Metastasis involves multiple processes such as infiltrative growth through the extracellular matrix (ECM), cell migration through blood or lymph vessels and rise of distant colonies (Gupta and Massagué 2006) Migration is described as any directed cell movement in the body. The ability to migrate allows cells to change their position within tissues or different organs. Invasion on the other hand, is defined as the penetration of tissue barriers, such as passing through the basement membrane and infiltration into the underlying interstitial tissues by malignant tumor cells.

Migration and invasion are clearly separated terms in experimental biology as migration only involves the directed movement of cells on a substrate such as basal membranes, ECM fibers, or plastic plates (2D surfaces); whereas, invasion involves cell movement through a 3D matrix, which is accompanied by a restructuring of the 3D environment (Friedl and Wolf 2010). Despite the difficulties in defining different modes of migration, it is a fact that the ability to migrate is a prerequisite to invade; a cell cannot invade without migration but can move without invasion (Fackler and Grosse 2008).

4.1.8.1 Active extracts of the plants suppressed Migration of PC3 cells in Vitro

To investigate the effects of the active extracts of the plants on the migration of PC3 cells, cells were treated with the maximum non-toxic concentration (MNTC) for 72 hours and seeded in the 96 well plate (cell exclusion zone assay). After 24, 48 and 72 hours of incubation, the migratory activity of the cells were examined and the results are shown in Figure 4.36. Throughout this study, the morphology of PC3 cells were monitored frequently in order to make sure that the cells did not show any significant signs of cell death as these will influence the outcome of this study. If a drug or an extract has the ability to inhibit cancer cells migration, it could be detected by using MNTC.

Figure 4.51 to Figure 4.53 show the inhibitory activity of the active extracts of *Marantodes pumilum*, *Ficus deltoidea* plants and Paclitaxel (positive control) on the migration of PC3 cells as viewed under EVOS® FL Imaging System. Figure 4.54 indicates that, the active extracts of both plants - *Marantodes pumilum* and *Ficus deltoidea* - at MNTC significantly inhibited the migration of PC3 cells. This observation also indicates that the active extracts of the plant suppressed the migration of PC3 cells in a time-dependent manner. Both MPc and FD1c reduced the number of migrated cells by 50% at every time point. To further validate the results of this study, another migration study was conducted by using the transwell migration assay method (Boyden Chamber). Results obtained from this experiment are shown in Figure 4.55.

After 24 hours of treatment with the active extracts of the plant, we can clearly see that all active extracts of both plants showed significant inhibition to the migration of the PC3 cells with MPc and FD1c reducing the number of migrated cells by almost 60%. These results are in agreement with the previous data obtained previously when conducting the cell migration study by

using the cell exclusion zone assay method. Thus, it seems quite likely that MPc, MPh, FD1c and FD2c could inhibit PC3 cell migration.

In section 2.2.2, we have mentioned that chemokines such as CXCL12 can act as major regulators of cells trafficking and adhesion (Kyriakou, Rabin et al. 2008). The binding of CXCL12 to its receptor, CXCR4, is said to initiate divergent signalling pathways that lead to multiple responses including the phosphorylation of MEK/ERK signalling cascade and the activation of NF- κ B, which is important for tumor cell progression and survival. Therefore, further study needs to be conducted to get a better understanding on how the active plant extracts suppress the migration of PC3 cells.

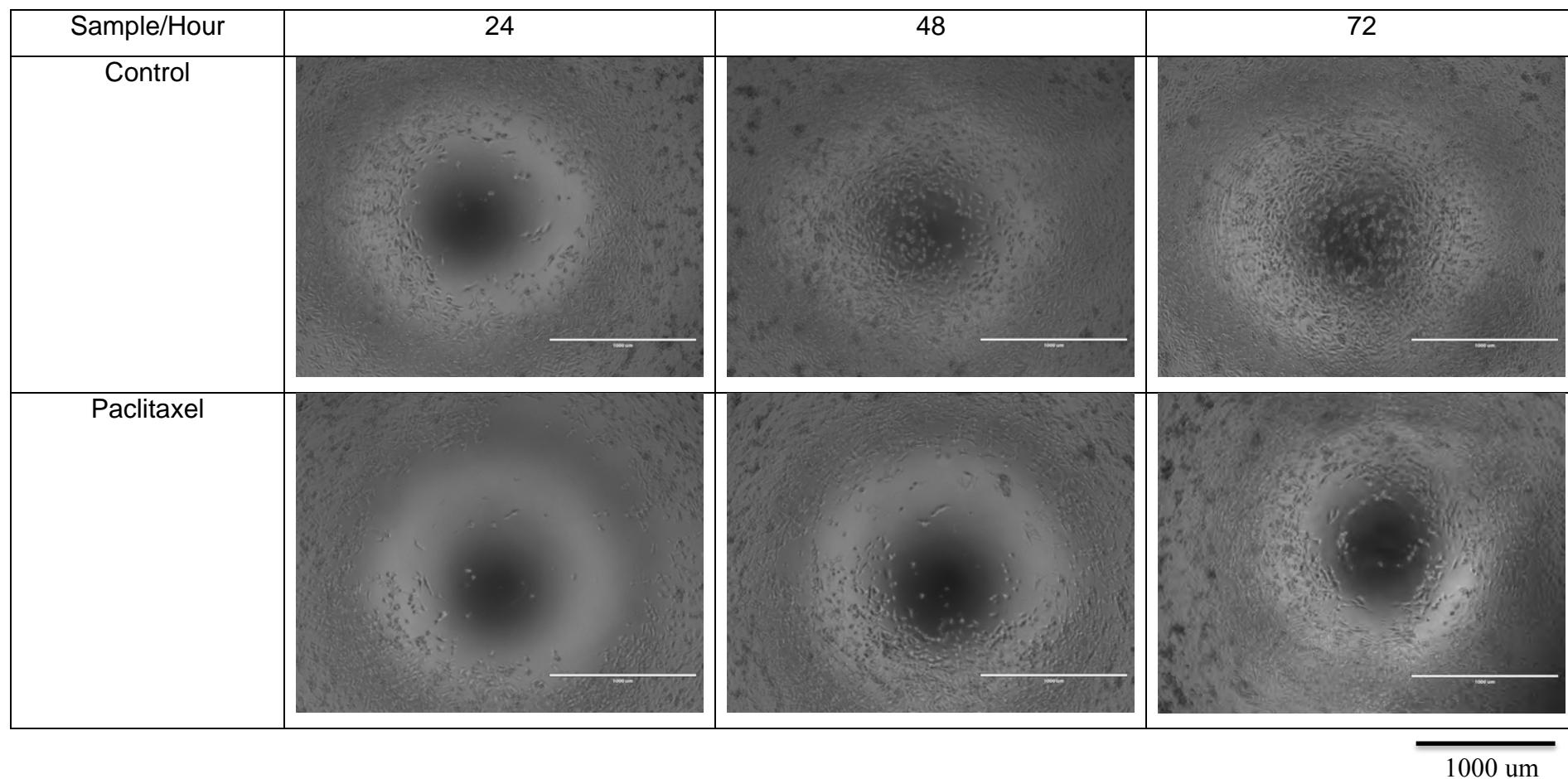


Figure 4.51. Migration of PC3 cells treated with the MNTC of Paclitaxel for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System. Inhibition of migration can be clearly seen after treatment with Paclitaxel compared to the untreated cells. Results shown are representative of three independent experiments.

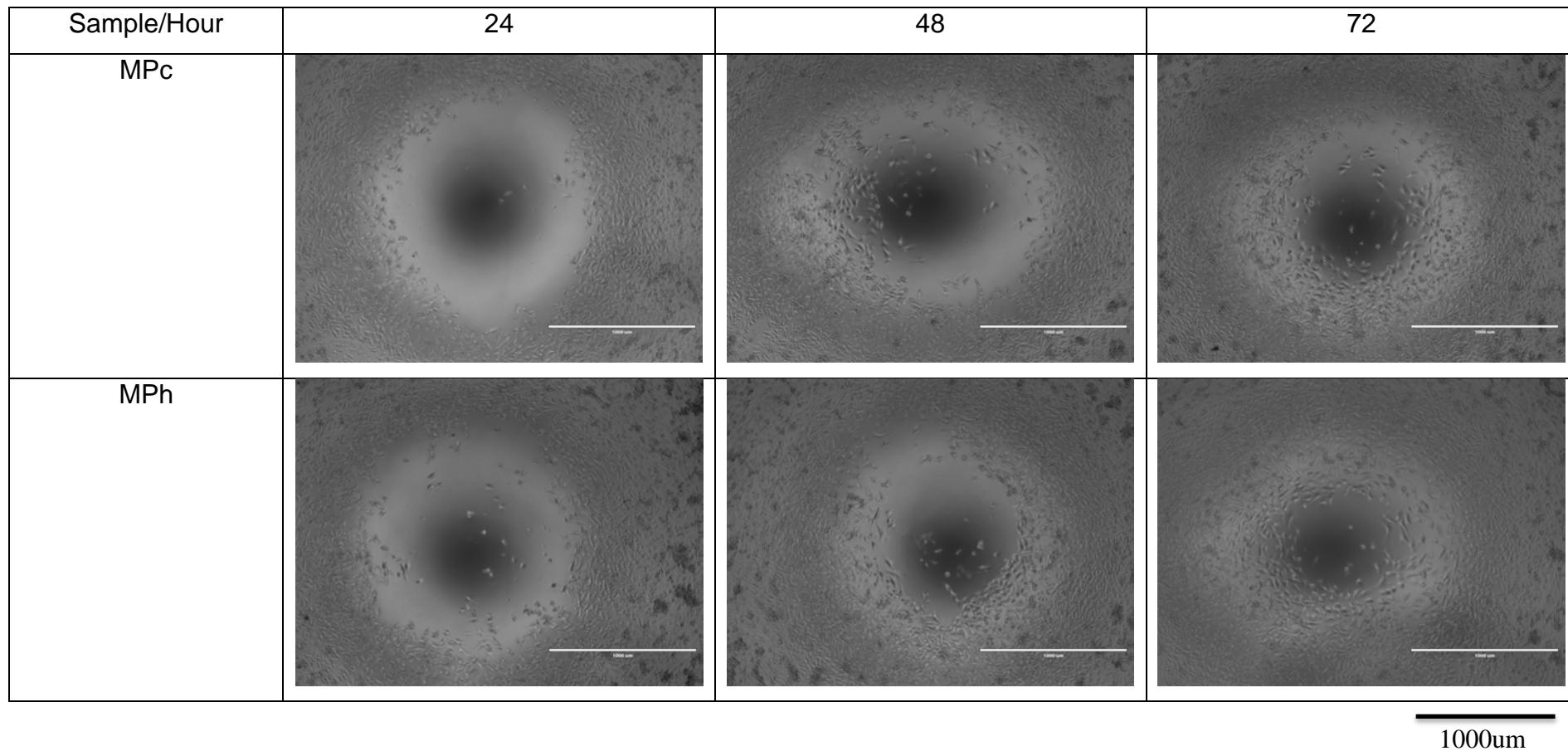


Figure 4.52. Migration of PC3 cells treated with the MNTC of the active extracts of *Marantodes pumilum* for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System. Inhibition of migration can be clearly seen after treatment with MPc and MPh compared to the untreated cells. Results shown are representative of three independent experiments.

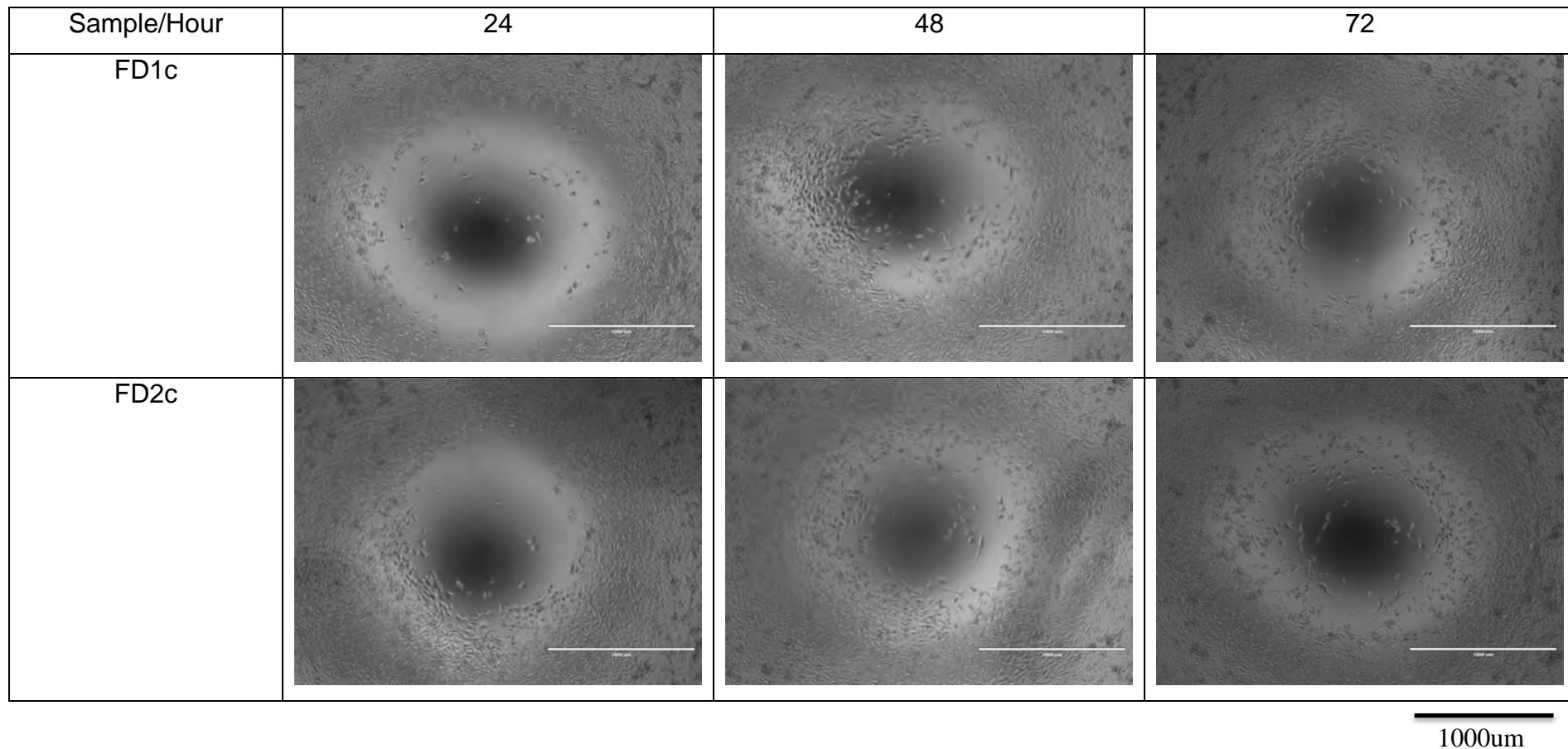


Figure 4.53. Migration of PC3 cells treated with the MNTC of the active extracts of *Ficus deltoidea* for 24, 48, and 72 hours viewed under the EVOS® FL Imaging System. Inhibition of migration can be clearly seen after treatment with FD1c and FD2c compared to the untreated cells. Results shown are representative of three independent experiments.

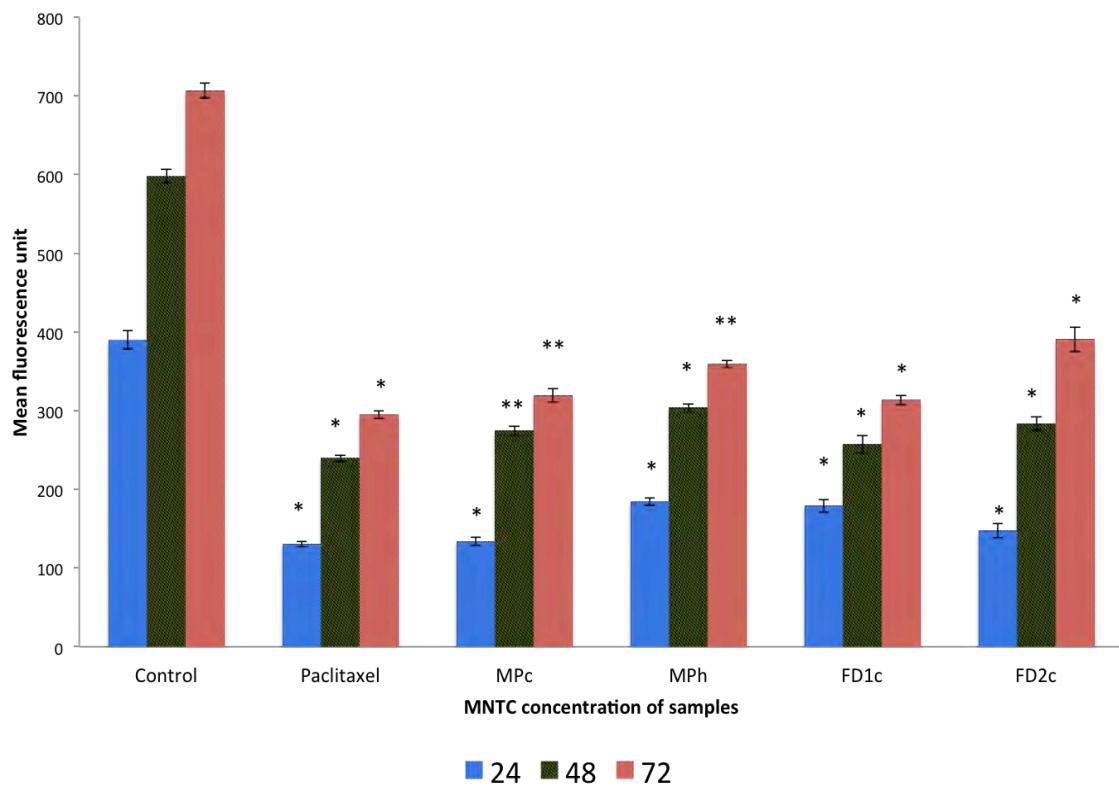


Figure 4.54. Oris™ Cell Migration analysis: 5×10^4 of PC3 cells were seeded per well and allowed to adhere. Stoppers were then removed and the active extracts of both *Marantodes pumilum* (MPc and MPh) and *Ficus deltoidea* (FD1c and FD2c) were added to the wells, and the plate was incubated to permit cell migration. The cells were labelled with CellTracker™ Green and the fluorescence quantified in the detection zone using a Synergy HT BioTek plate reader. Results shown are representatives of three independent experiments, * $p < 0.05$ and ** $p < 0.01$ and against control as analysed by the Student's *t* test.

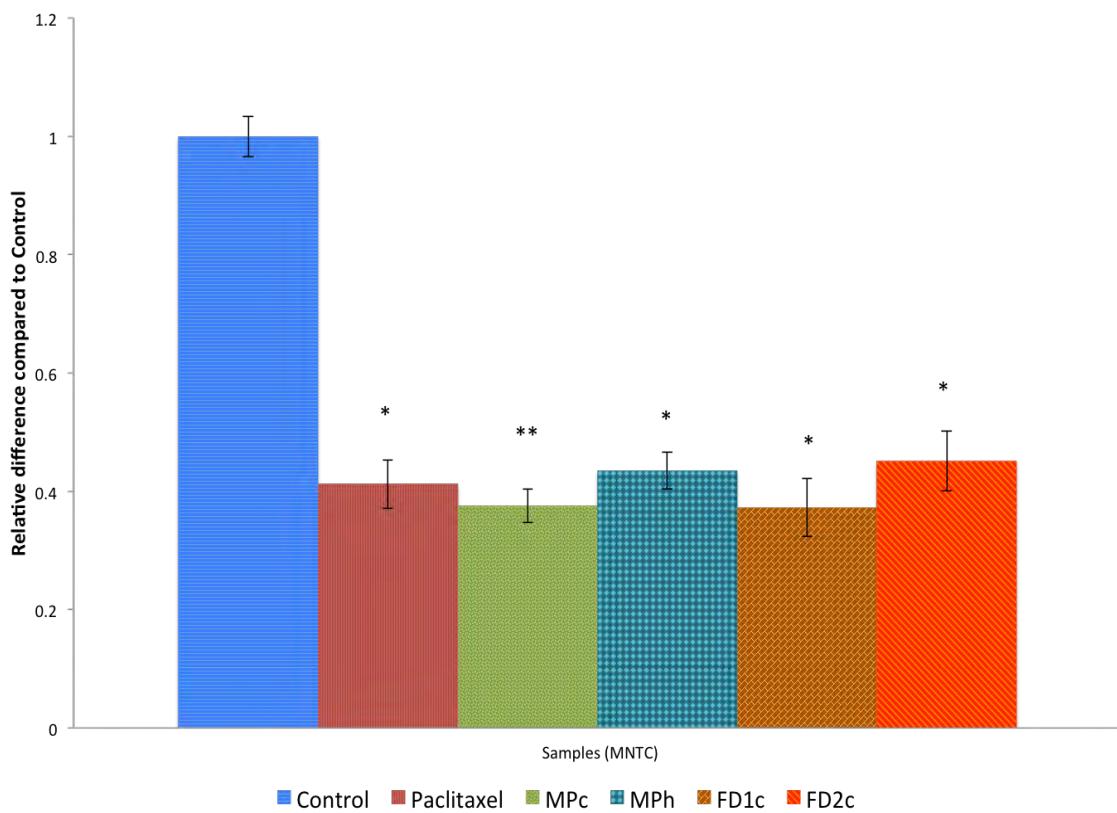


Figure 4.55. CytoSelect Cell Migration analysis: Effects of the active extract of both *Marantodes pumilum* (MPC and MPh) and *Ficus deltoidea* (FD1c and FD2c) plants on the migration of the PC3 cells. PC3 cells were treated with the MNTC concentration of MPC, MPh, FD1c and FD2c for 24 hours. Results shown are representatives of three independent experiments, * $p<0.05$ and ** $p<0.01$ and against control as analysed by the Student's *t* test.

4.1.8.2 Active extracts of the plants suppressed invasion of PC3 cells in vitro

Figure 4.56 shows the effect of the active extracts of the plants on PC3 cell invasion after 48 hours of treatment. All active extracts exhibited significant inhibition on the invasion of the prostate cancer cells after 48 hours of treatment. Both MPc and FD1c extracts reduced the number of invading cells by more than two-fold when compared to control. These results indicate that the active extracts of both plants are not only capable of inhibiting or delaying PC3 cell migration but could also prevent cell invasion in a significant way. As mentioned previously, the inhibition of both cell migration and invasion could occur through MMP modulation. It is well known that: (1) the derangement of ECM by MMPs play an initial key role in cell movement via the alteration of cell-ECM interactions; and (2) MMPs breakdown the basement membrane allowing cancer cells to migrate and invade.

Among MMPs, MMP-2 and MMP-9 play the most important role in basement membrane type IV collagen degradation (Stetler-Stevenson 1990, Giancotti and Ruoslahti 1999, Zeng, Cohen et al. 1999). In particular, MMP-2 expression is associated with tumor invasion, angiogenesis, metastasis and recurrence (Wang, Wang et al. 2003, Komatsu, Nakanishi et al. 2004). It was reported that MMP-2 and MMP-9 are correlated with an aggressive, invasive or metastatic tumor phenotype (Cockett, Murphy et al. 1997, Papathoma, Zoumpourlis et al. 2001). It is well known that MMP inhibitors block endothelial cell activities which are essential for new vessel development leading to proliferation and invasion (Benelli, Adatia et al. 1993, Murphy, Unsworth et al. 1993). Therefore, MMP-2 and MMP-9 are thought to be therapeutic targets of anticancer drugs based on the degrading action of both enzymes on gelatins, which are major components of the basement membrane. As this is the first time that these active extracts are reported to inhibit both the migration and invasion of prostate cancer cells (PC3), more studies should be conducted in order to understand the actual mechanism that led to this activity.

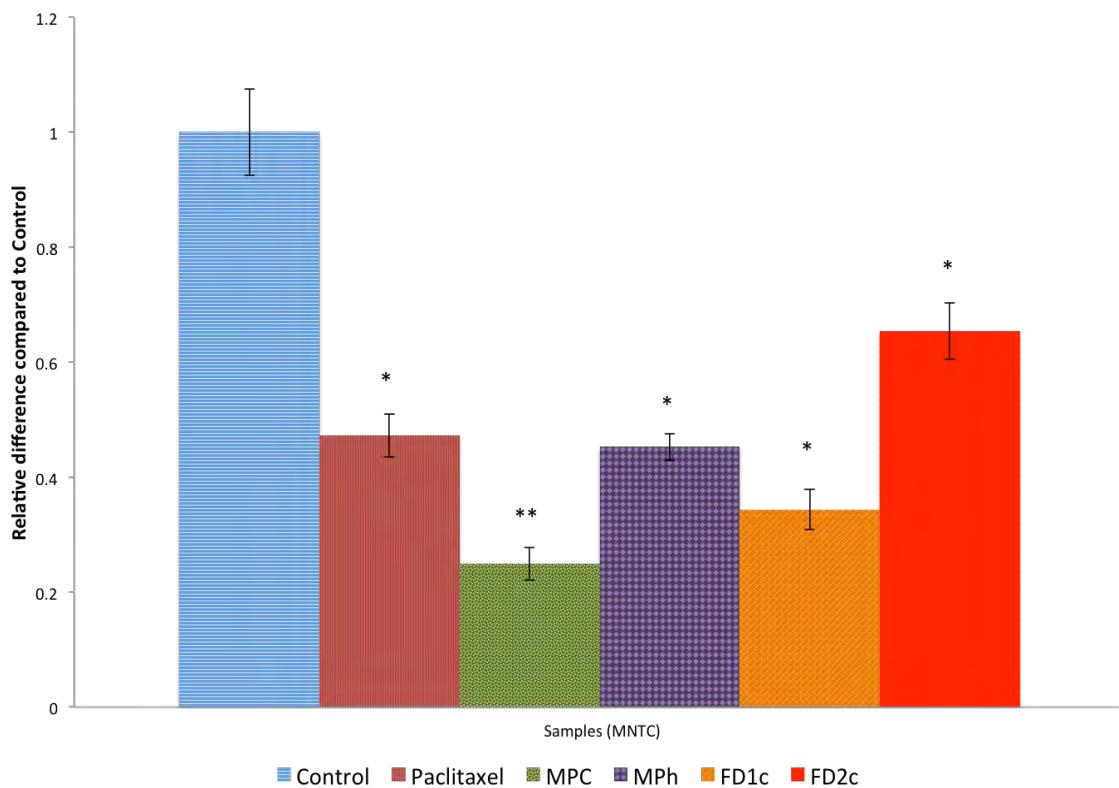


Figure 4.56. CytoSelect Cell Invasion analysis: Effects of the active extract of both *Marantodes pumilum* (MPC and MPh) and *Ficus deltoidea* (FD1c and FD2c) plants on the invasion of the PC3 cells. PC3 cells were treated with the MNTC concentration of MPC, MPh, FD1c and FD2c for 48 hours. Results shown are representatives of three independent experiments, * $p<0.05$ and ** $p<0.01$ and against control as analysed by the Student's *t* test.

4.1.8.3 VEGF-A, CXCR4 and CXCL12 mRNA Gene expression

This study was conducted to investigate the effects of the active plant extracts on the mRNA gene expression of VEGF-A, CXCL12 and CXCR4. Figure 4.57 shows that only FD1c was able to increase the expression of CXCR4 by 2-fold significantly. However, for CXCL12 mRNA gene expression, all plant extracts inhibit its expression in a significant manner. MPh reduced the expression of CXCL12 by 16-fold while MPc, FD1c and FD2c reduced the expression of the gene by more than 20-fold. Figure 4.57 also show that MPh and FD1c were able to down-regulate the expression of VEGF-A by 7-fold and 9-fold respectively in a significant manner. MPc inhibited the expression of VEGF-A significantly by 20-fold. However, FD2c did not show any significant effect on the mRNA gene expression. The significant inhibition of the mRNA gene expression of both CXCL12 and VEGF-A by the active plant extracts suggests an explanation on how these plant extracts were able to suppress both migration and invasion as reported earlier. Table 4.6 summarizes the activity of the active extracts of both *Marantodes pumilum* and *Ficus deltoidea* plant on the mRNA gene expression of CXCR4, CXCL12 and VEGF-A

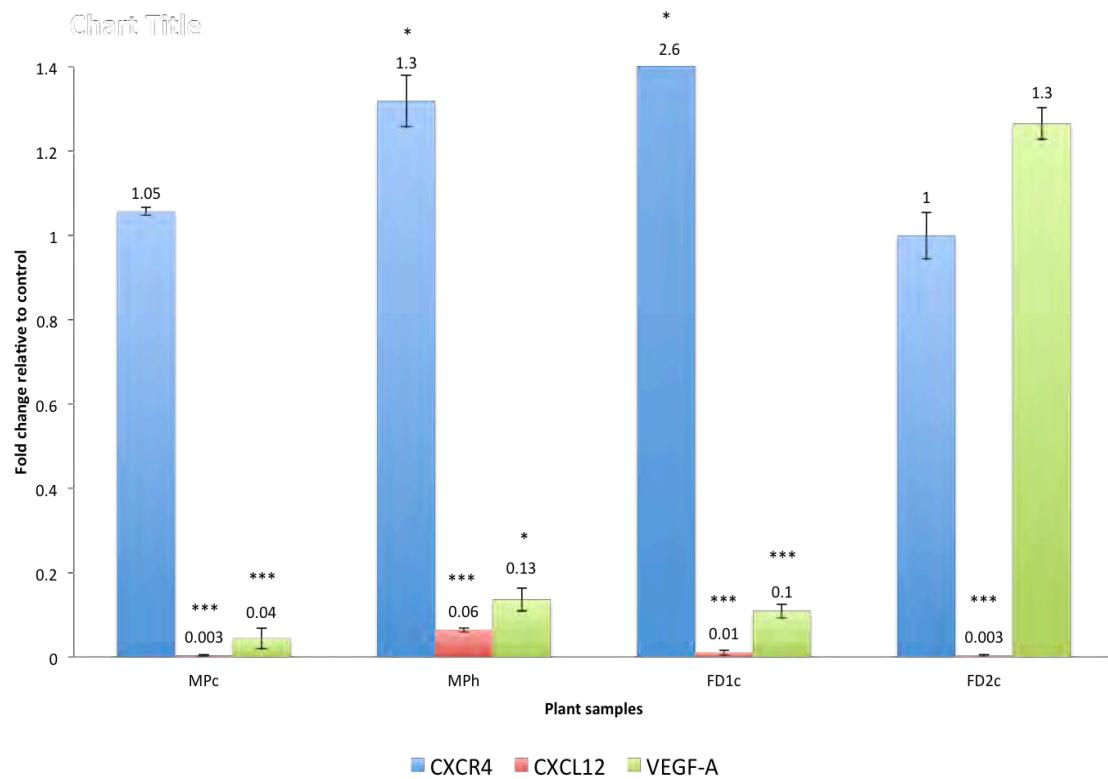


Figure 4.57. CXCR4, CXCL12 and VEGF-A mRNA genes expression in PC3 cells treated with MNTC of the active plant extracts of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours. The genes expressions were determined as described in RT-qPCR Conditions and Analysis. Data are mean \pm SD; $n=4$ experiments. * $P < 0.05$, * $P < 0.001$**

Table 4.6 mRNA gene expression analysis (CXCR4, CXCL12 & VEGF-A) in PC3 cells treated with MNTC of the active plant extracts of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours. The genes expressions were determined as described in RT-qPCR Conditions and Analysis where Control = 1. Data are mean \pm SD; n=4 experiments. * P < 0.05, * P < 0.001.**

Sample	Gene	Fold expressions relative to Control in PC3 cells
MPc	CXCR4	1
	CXCL12	0.003***(>20-Fold)
	VEGF	0.05***(2-Fold)
MPh	CXCR4	1
	CXCL12	0.07*** (16-Fold)
	VEGF	0.1*(7-Fold)
FD1c	CXCR4	2*
	CXCL12	0.01*** (>20-Fold)
	VEGF	0.1*** (9-Fold)
FD2c	CXCR4	1
	CXCL12	0.003***(>20-Fold)
	VEGF	1

4.1.9 Identification of the active fractions from the plant extracts

4.1.9.1 Fractionation of the plant extracts

Three plant extracts namely *Marantodes pumilum* choloroform extract (MPC), *Ficus deltoidea* var. *angustifolia* (Miq) corner chloroform extract (FD1c), and *Ficus deltoidea* Jack var. *deltoidea* chloroform extract (FD2c) were chosen to be further fractionated using the method explained in 3.1.2 based on the GI50 values of each plant extracts obtained from cytotoxicity studies with all 3 prostate cancer cell lines (PC3, DU145 and LNCaP). 140 fractions were collected for each plant extract and each fraction was examined by a thin layer chromatography (TLC) (section 3.1.3). TLC is a common analytical chromatography technique used to separate non-volatile mixtures. In this study, this method is used to ascertain the presence of different materials/compounds in fractions from column chromatography

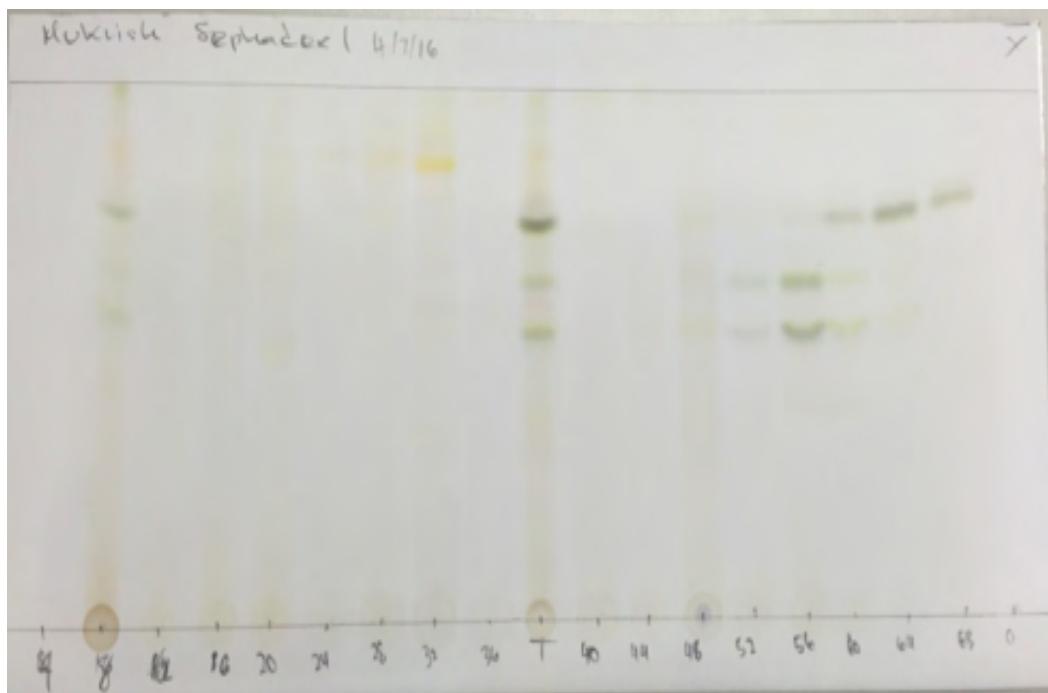


Figure 4.58 TLC analysis of SEPHADEX of the MPc extract (fraction 4 to 68) viewed under white light. T stands for total crude extract (crude extract of MPc).

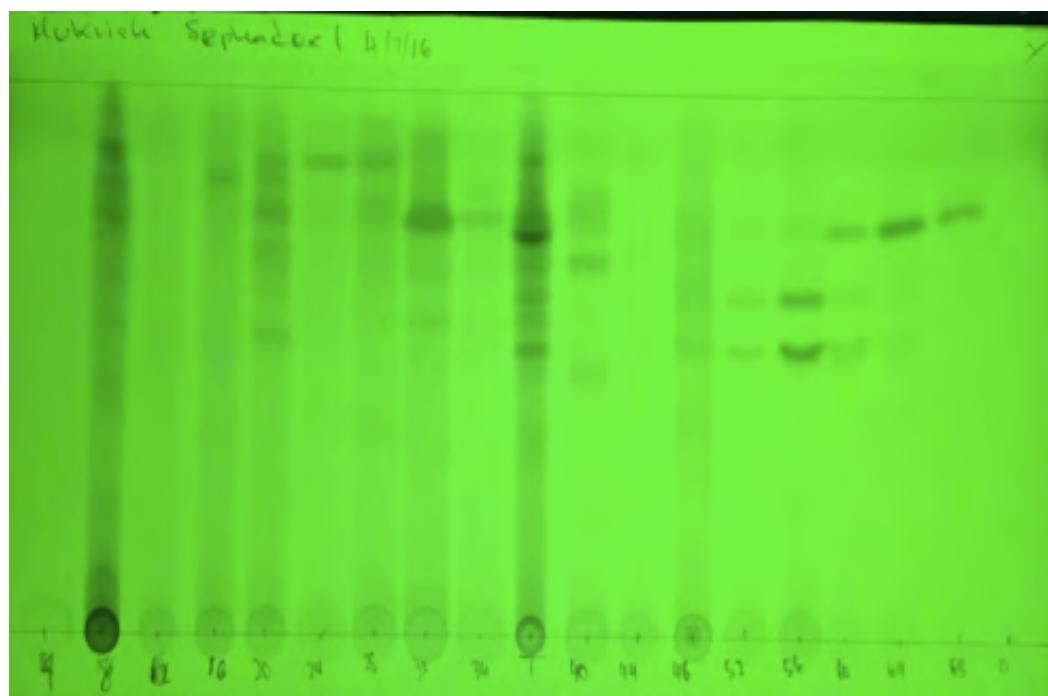


Figure 4.59 TLC analysis of SEPHADEX of the MPc extract (fraction 4 to 68), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of MPc).

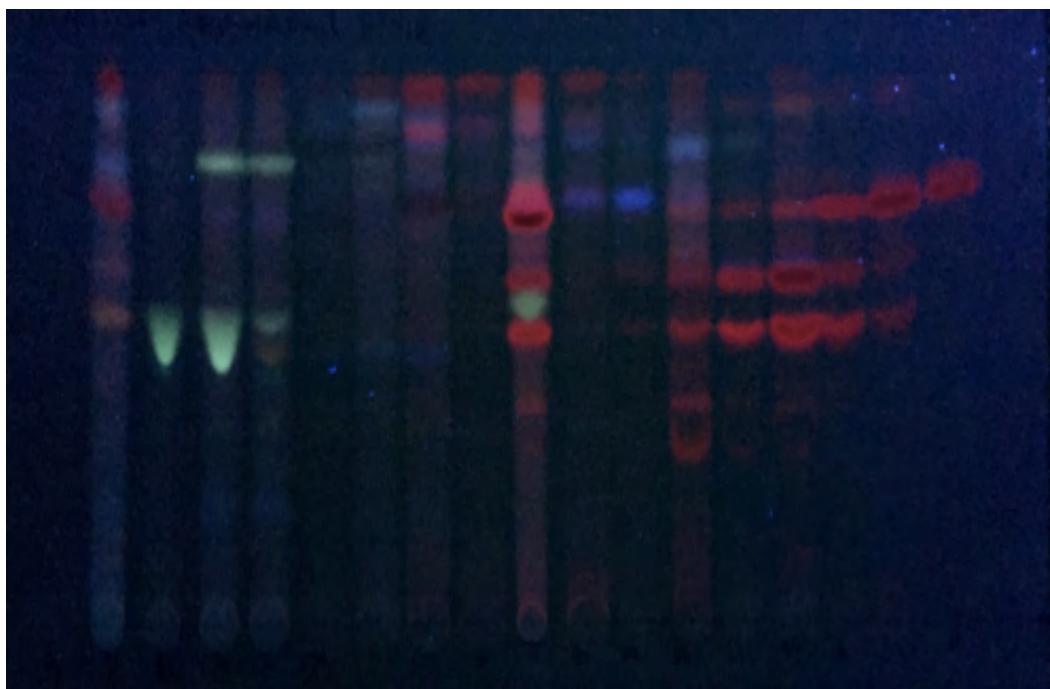


Figure 4.60 TLC analysis of SEPHADEX of the MPc extract (fraction 4 to 68), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of MPc).

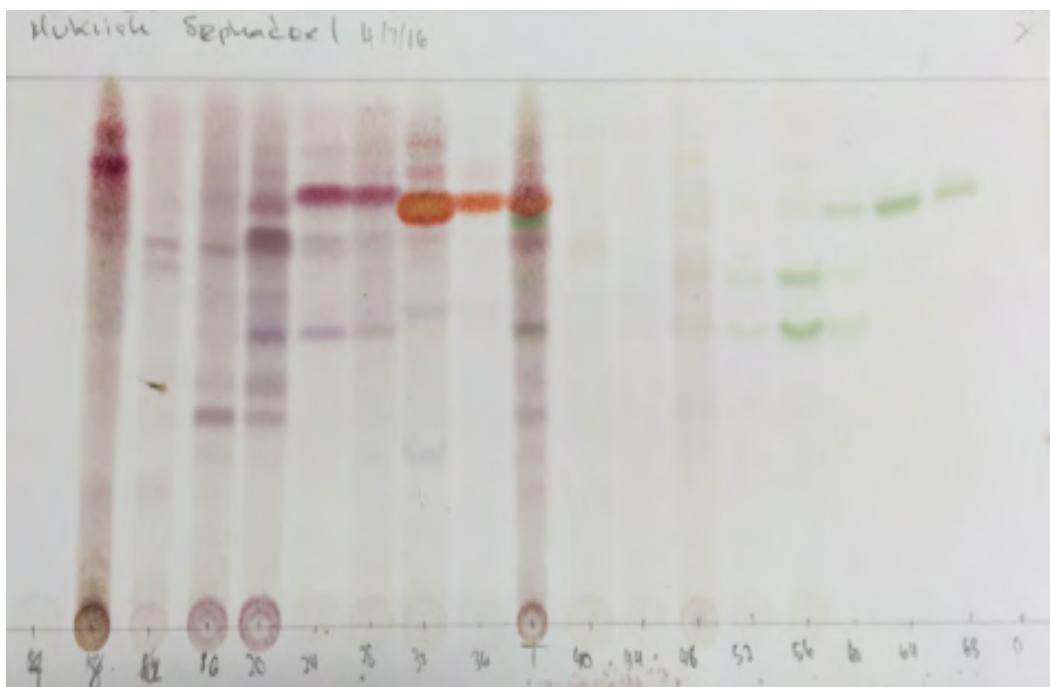


Figure 4.61 TLC analysis of SEPHADEX of the MPc extract after derivatisation with anisaldehyde (fraction 4 to 68), viewed under white light. T stands for total crude extract (crude extract of MPc).

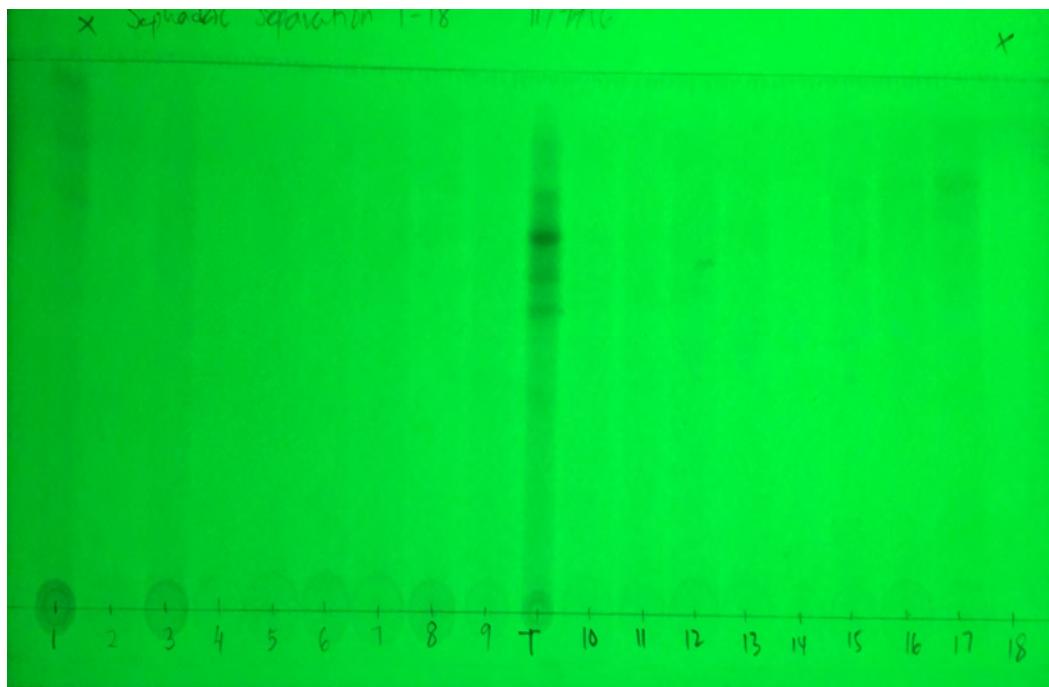


Figure 4.62 TLC analysis of SEPHADEX of the MPC extract (fraction 1 to 18), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of MPC).

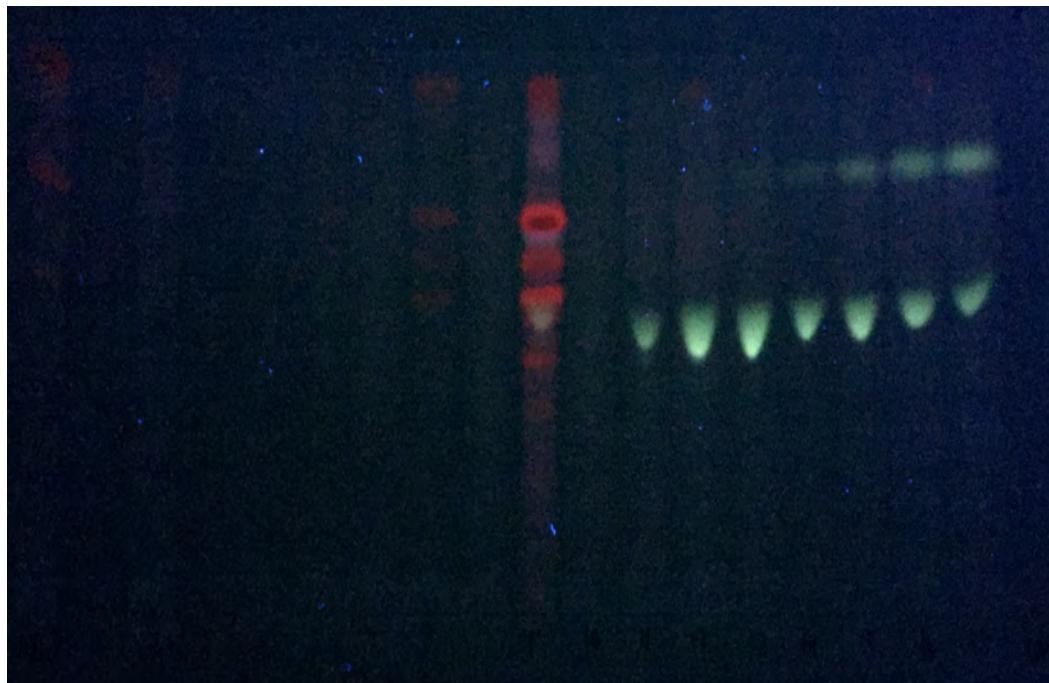


Figure 4.63 TLC analysis of SEPHADEX of the MPC extract (fraction 1 to 18), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of MPC).

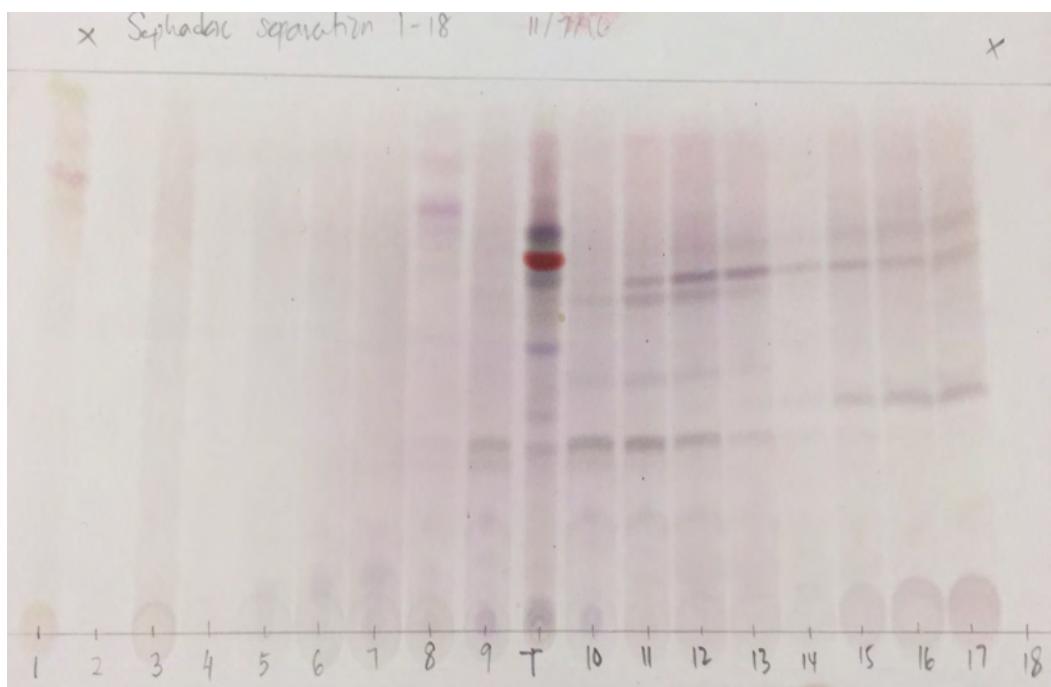


Figure 4.64 TLC analysis of SEPHADEX of the MPc extract after derivatisation with anisaldehyde (fraction 1 to 18), viewed under white light. T stands for total crude extract (crude extract of MPc).

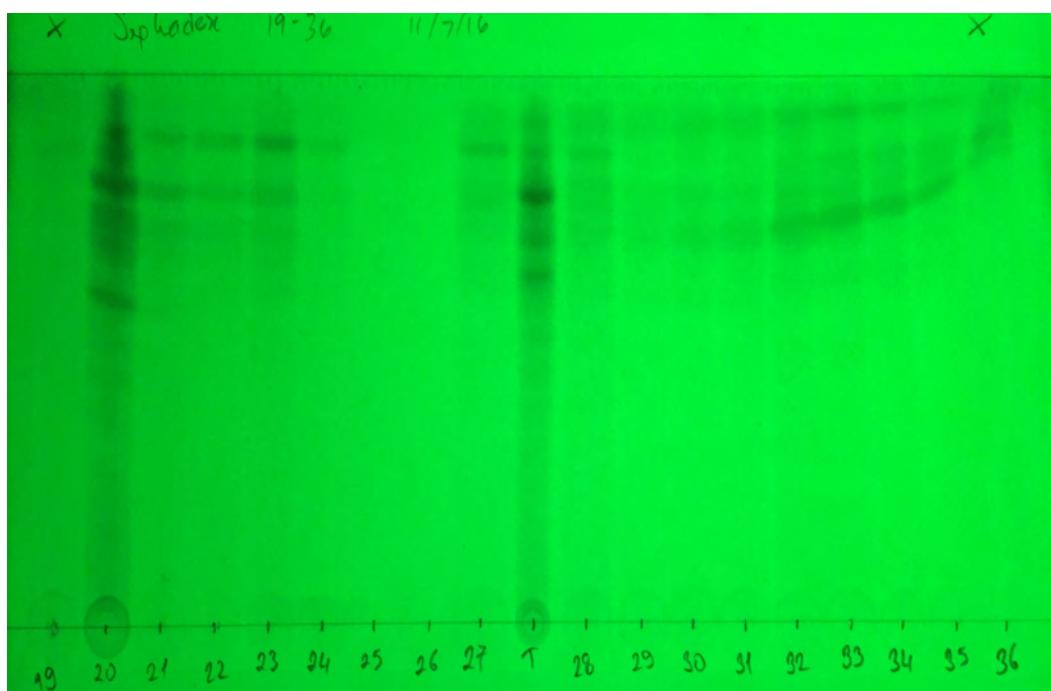


Figure 4.65 TLC analysis of SEPHADEX of the MPc extract (fraction 19 to 36), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of MPc).

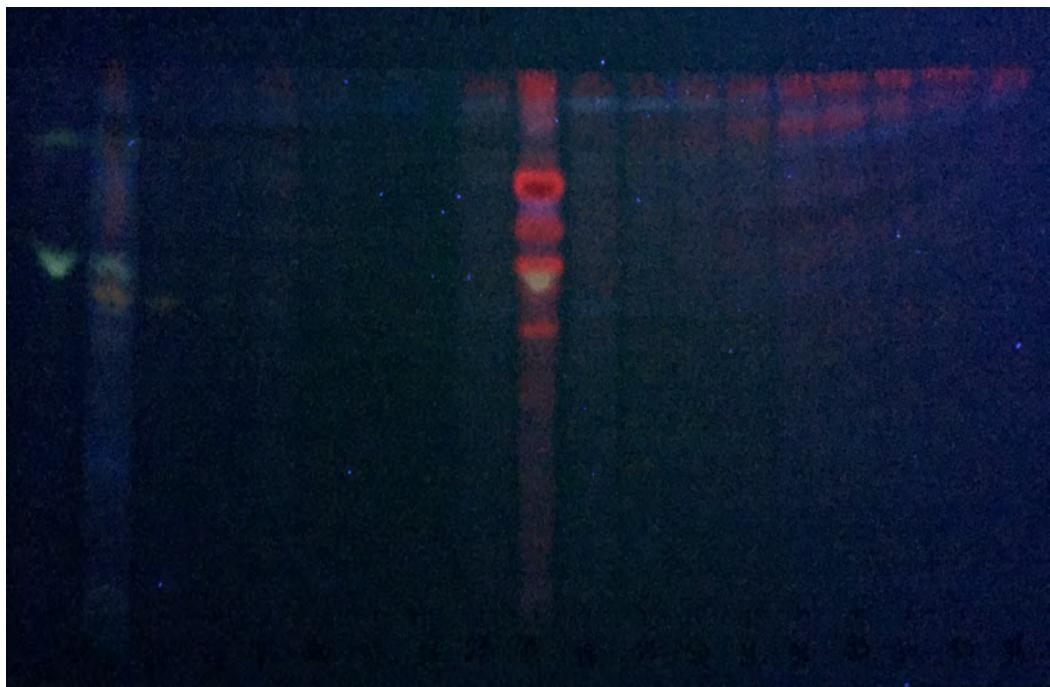


Figure 4.66 TLC analysis of SEPHADEX of the MPc extract (fraction 19 to 36), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of MPc).

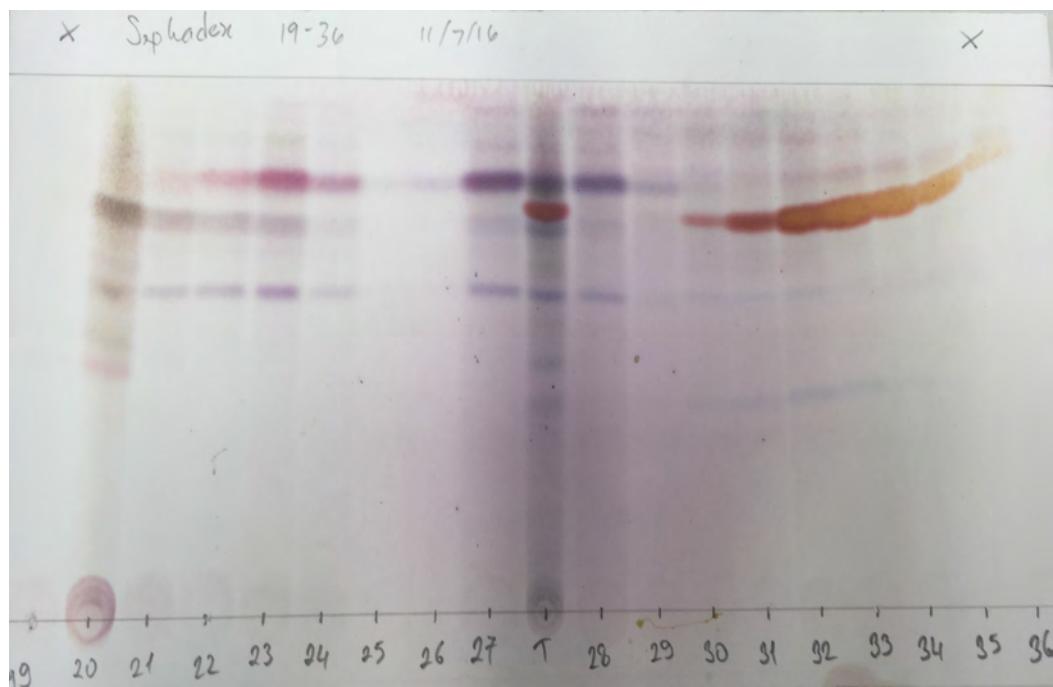


Figure 4.67 TLC analysis of SEPHADEX of the MPc extract after derivatisation with anisaldehyde (fraction 19 to 36), viewed under white light. T stands for total crude extract (crude extract of MPc).

Based on the TLC profiles of MPc shown in Figures 4.58 to 4.67 the eluates were then pooled into 7 major fractions (fraction 1, fraction 2-8, fraction 10-13, fraction 15-17, fraction 21-24, fraction 27-29, fraction 30-33). These fractions were subjected to another cytotoxicity study with PC3 cell lines to determine their activity.

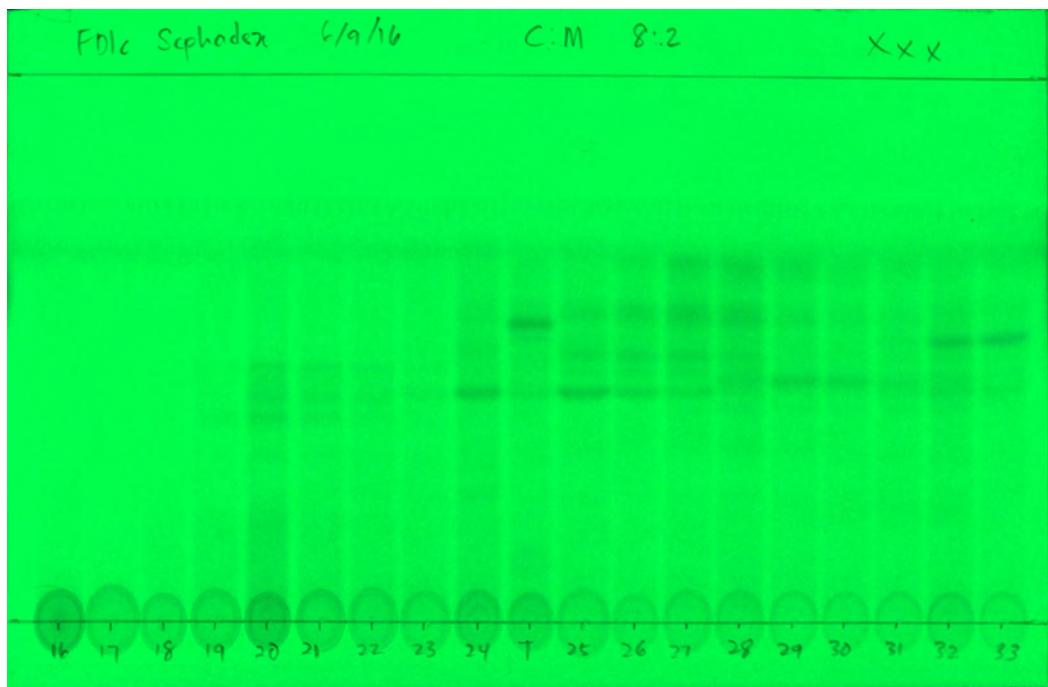


Figure 4.68 TLC analysis of SEPHADEX of the FD1c extract (fraction 16 to 33), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of FD1c).

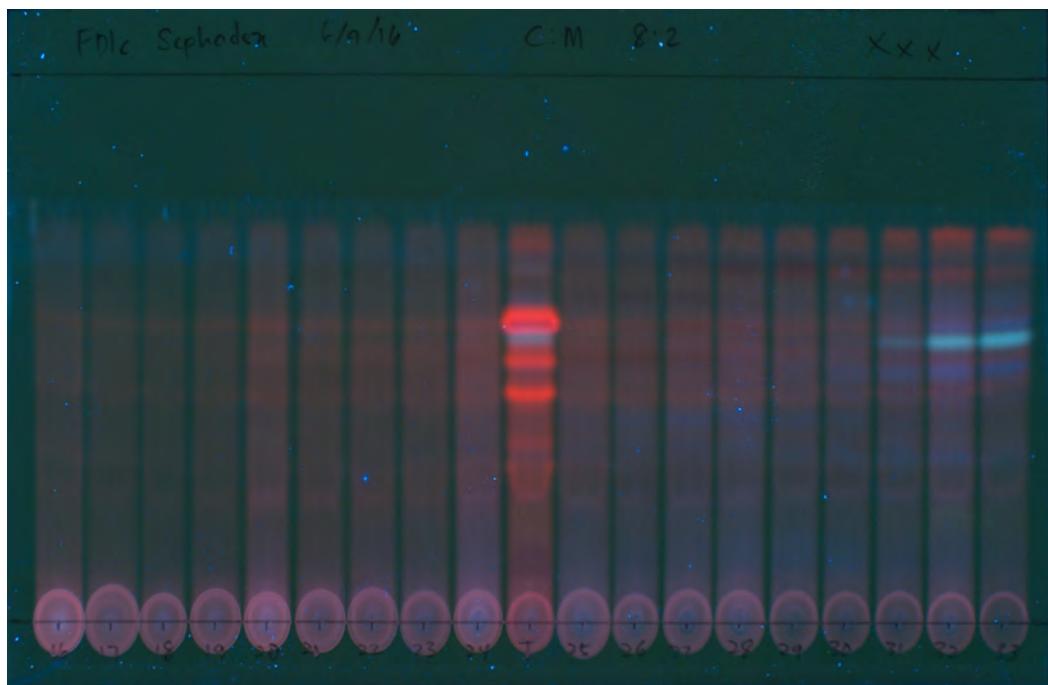


Figure 4.69 TLC analysis of SEPHADEX of the FD1c extract (fraction 16 to 33), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of FD1c).

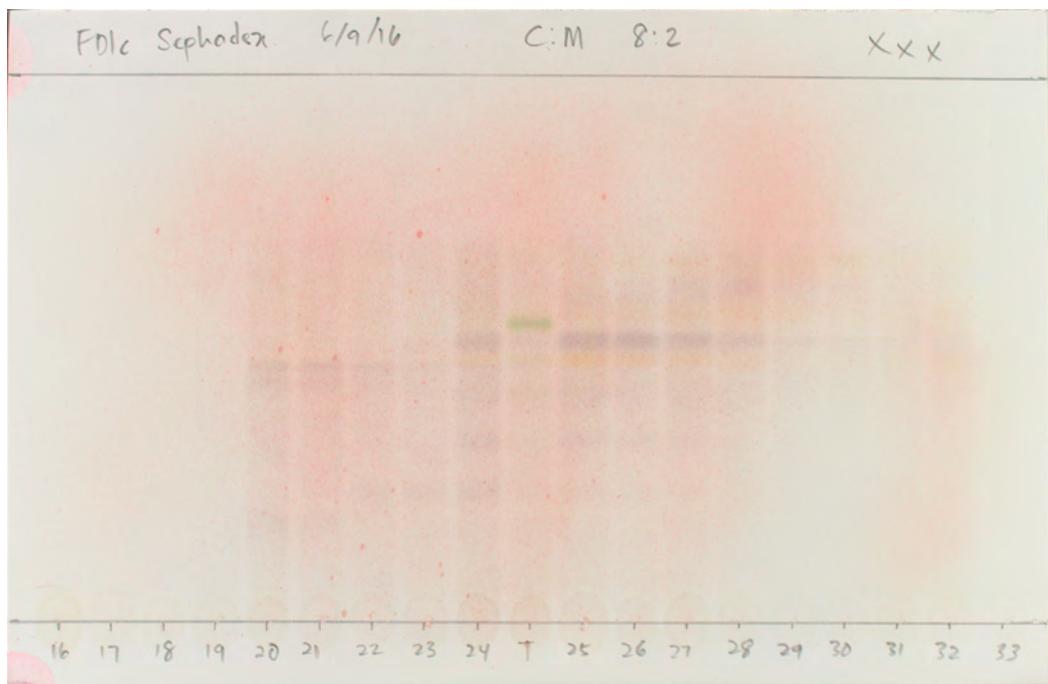


Figure 4.70 TLC analysis of SEPHADEX of the FD1c extract after derivatisation with anisaldehyde (fraction 16 to 33), viewed under white light. T stands for total crude extract (crude extract of FD1c).

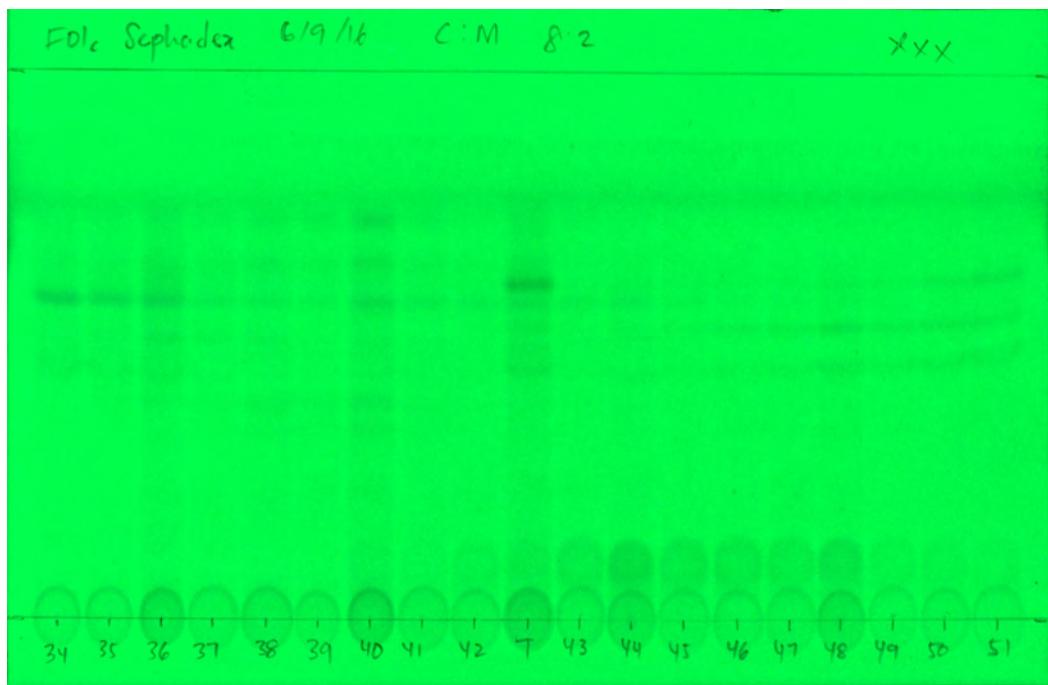


Figure 4.71 TLC analysis of SEPHADEX of the FD1c extract (fraction 34 to 51), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of FD1c).

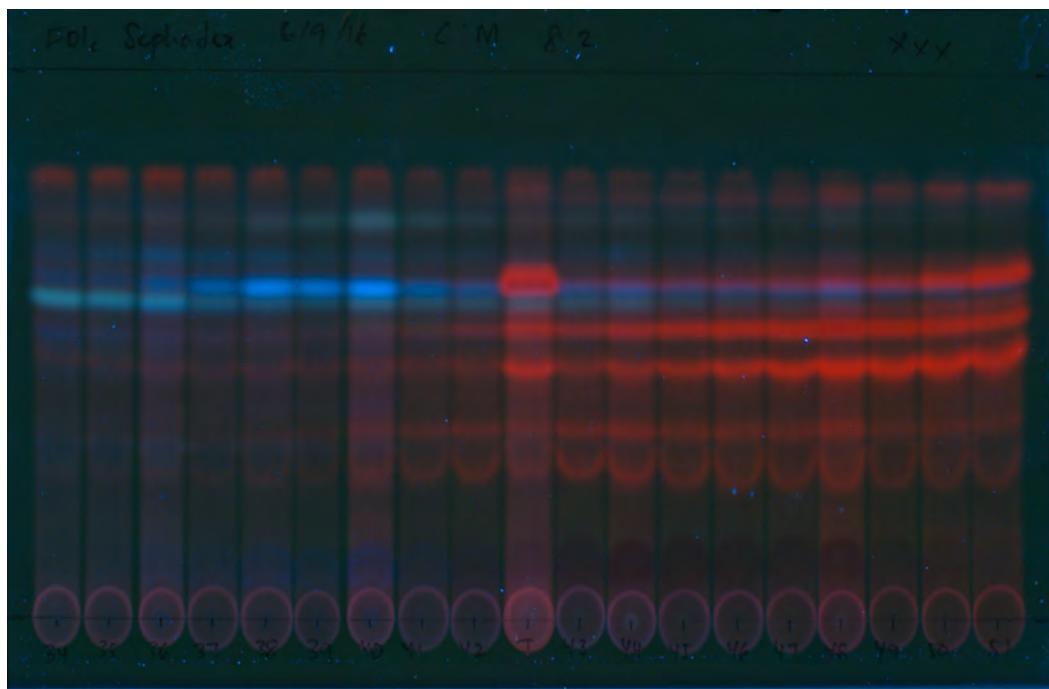


Figure 4.72 TLC analysis of SEPHADEX of the FD1c extract (fraction 34 to 51), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of FD1c).

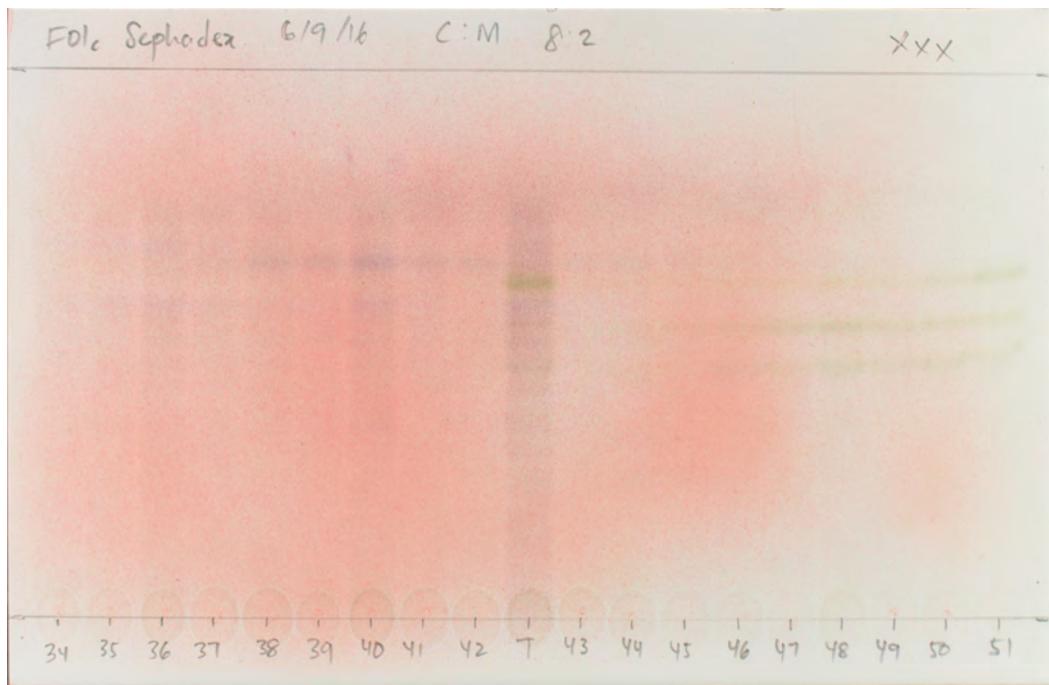


Figure 4.73 TLC analysis of SEPHADEX of the FD1c extract after derivatisation with anisaldehyde (fraction 34 to 51), viewed under white light. T stands for total crude extract (crude extract of FD1c).

Based on the TLC profiles of FD1c shown in Figures 4.68 to 4.73 the eluates were then pooled into 5 major fractions (fraction 20-23, fraction 24-30, fraction 31-36, fraction 37-42, fraction 43-51). These fractions were subjected to another cytotoxicity study with PC3 cell lines to determine their activity.

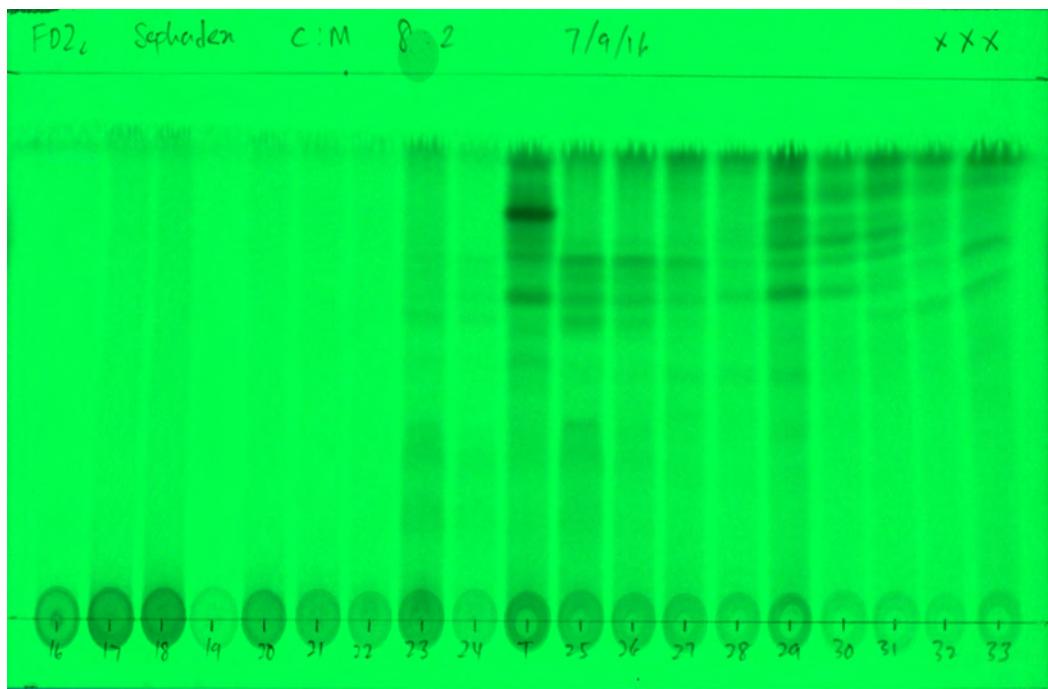


Figure 4.74 TLC analysis of SEPHADEX of the FD2c extract (fraction 16 to 33), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of FD2c).

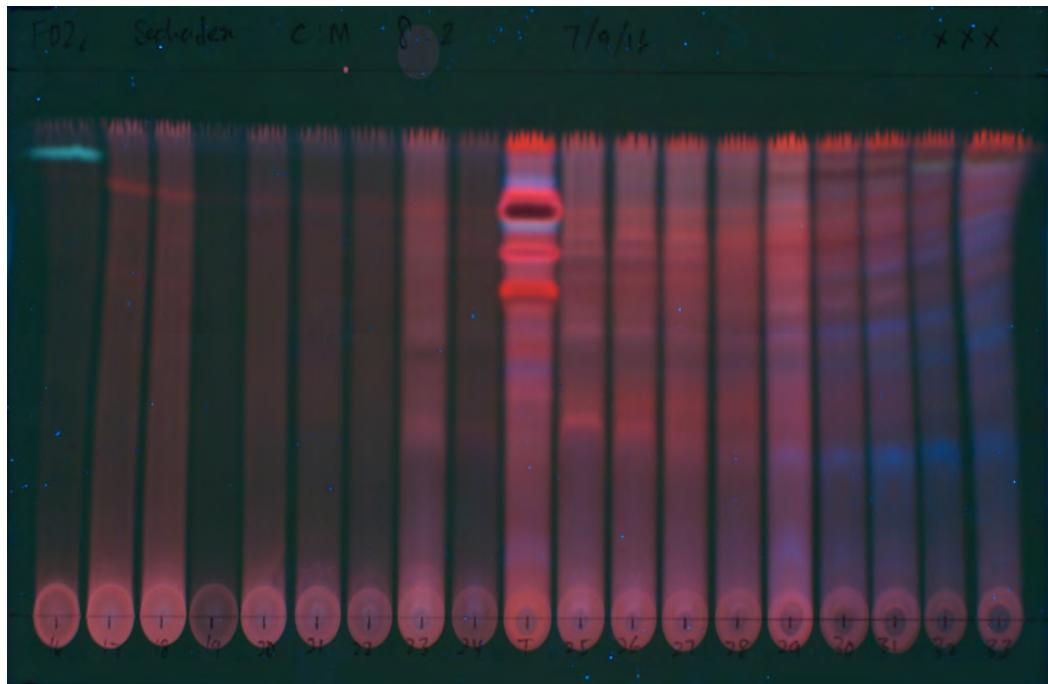


Figure 4.75 TLC analysis of SEPHADEX of the FD2c extract (fraction 16 to 33), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of FD2c).

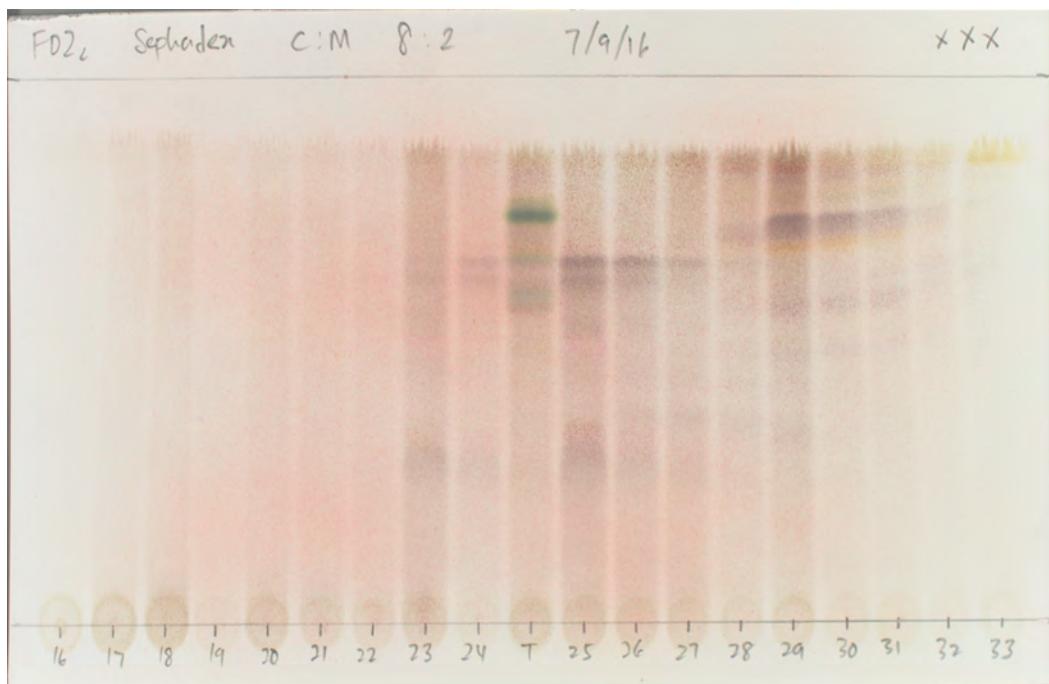


Figure 4.76 TLC analysis of SEPHADEX of the FD2c extract after derivatisation with anisaldehyde (fraction 16 to 33), viewed under white light. T stands for total crude extract (crude extract of FD2c).

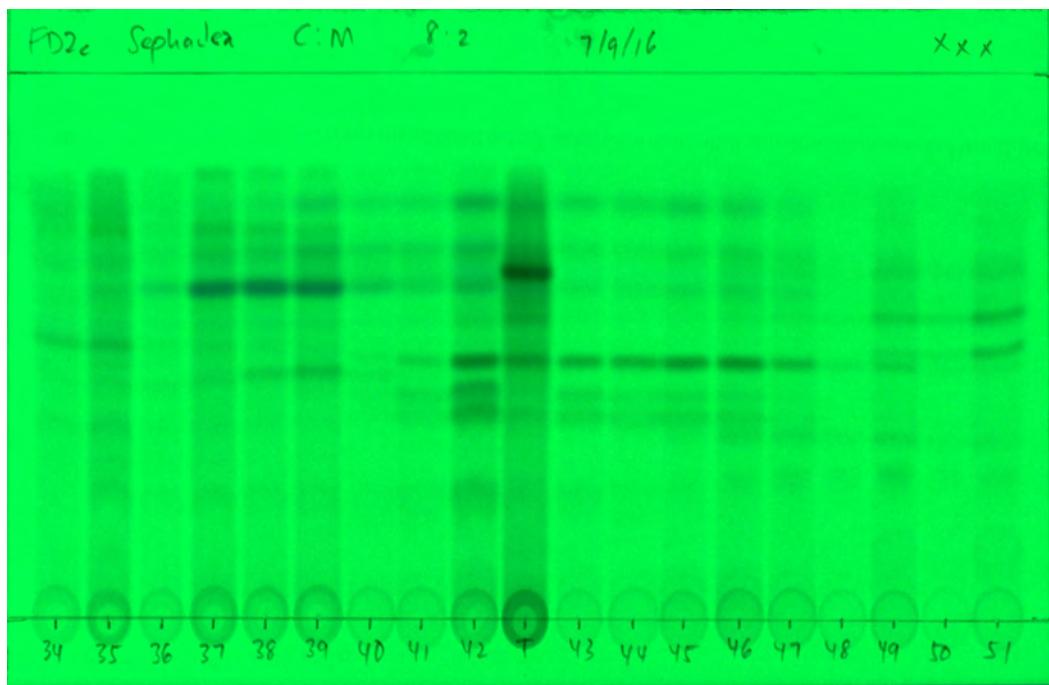


Figure 4.77 TLC analysis of SEPHADEX of the FD2c extract (fraction 34 to 51), viewed under 254 nm wavelength. T stands for total crude extract (crude extract of FD2c).

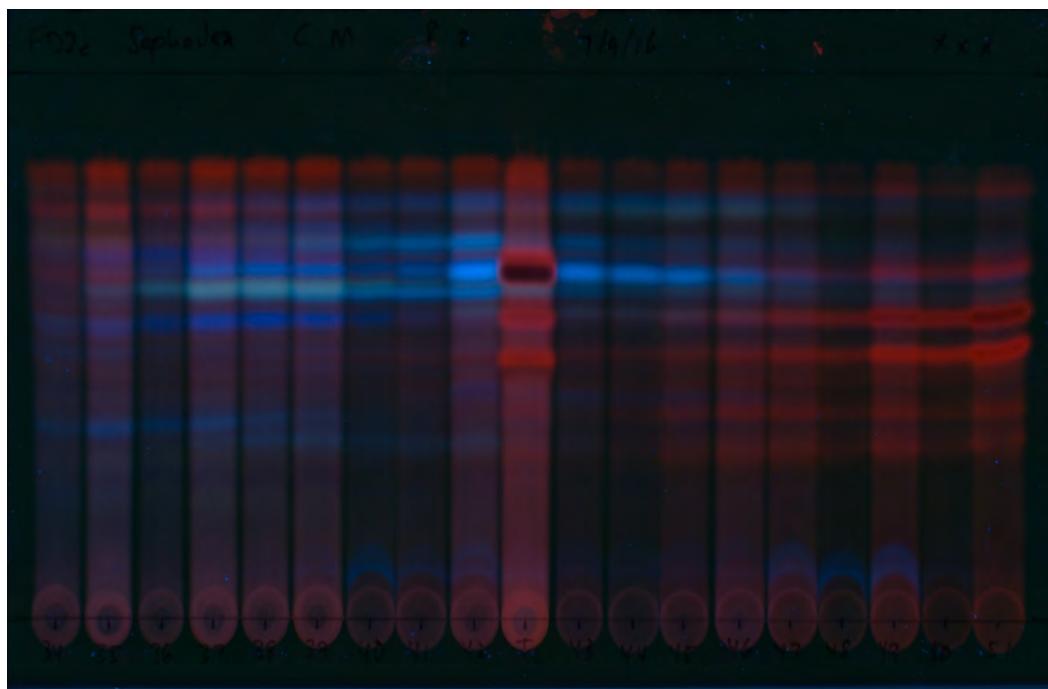


Figure 4.78 TLC analysis of SEPHADEX of the FD2c extract (fraction 34 to 51), viewed under 366 nm wavelength. T stands for total crude extract (crude extract of FD2c).

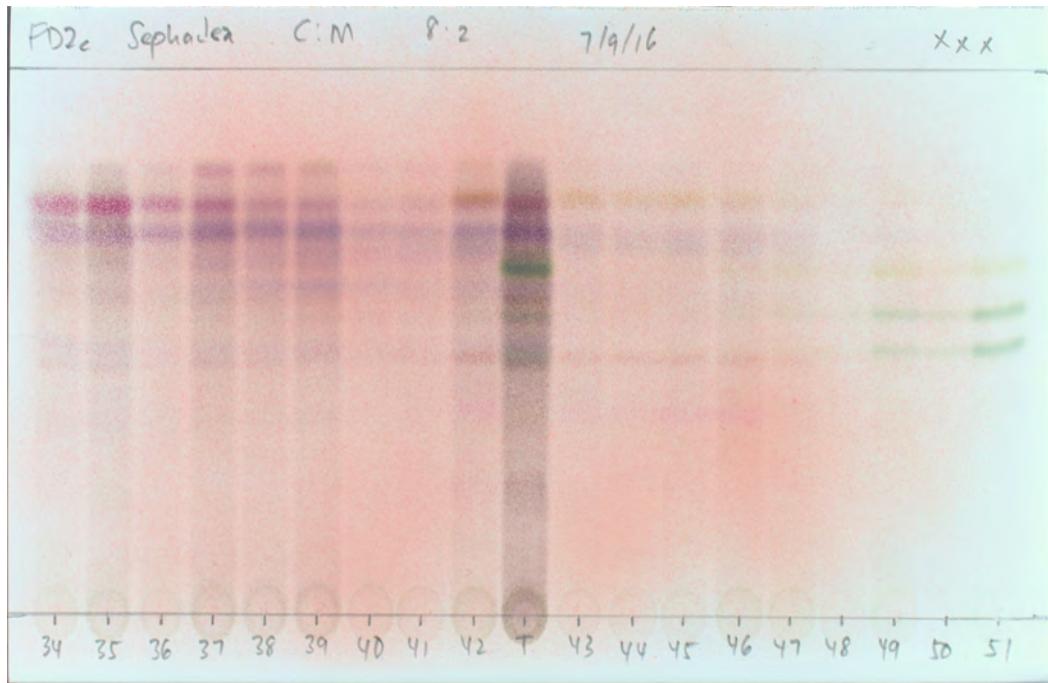


Figure 4.79 TLC analysis of SEPHADEX of the FD2c extract after derivatisation with anisaldehyde (fraction 34 to 51), viewed under white light. T stands for total crude extract (crude extract of FD2c).

Based on the TLC profiles of FD2c shown in Figures 4.74 to 4.79 the eluates were then pooled into 8 major fractions (fraction 16, fraction 17-22, fraction 23-26, fraction 27-28, fraction 29-33, fraction 34-36, fraction 37-39, fraction 40-45). These fractions were subjected to another cytotoxicity study with PC3 cell lines to determine their activity.

4.1.9.2 Identification of the active fractions using SRB staining assay

Even though the main objective of this research is to produce standardized extracts with chemopreventive properties, further extraction and fractionation of the plant extracts is important in order to identify active compounds that could be used for their standardization.

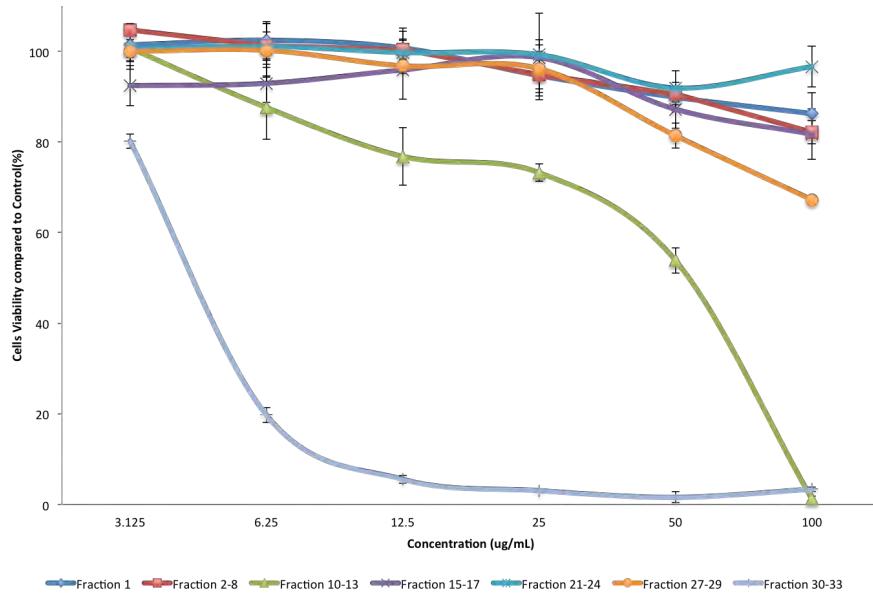
Size exclusion chromatography of the active plant extracts yielded 140 fractions. Based on the TLC profiles, the eluates were then pooled into 20 major fractions. These fractions were then tested for their cytotoxic activity (SRB staining assay on PC3 cells) and their GI50 determined.

Figure 4.80 shows that out of the 20 major fractions being tested, only 4 fractions were observed to have better efficacy than their respective plant extracts. These fractions were FD1c F30-33, FD1c F43-51, FD2c F29-33 and FD2c F34-36. Table 4.7 shows the GI50 of each active fraction. The GI50 values for each fraction were significantly lower than their respective plant extracts before further fractionation process was carried out ($P < 0.05$). Therefore, these findings suggest that further extraction and fractionation processes implied on the active plant extracts were able to increase their efficacy.

Table 4.7 GI50 concentrations (µg/mL) of the active fractions from *Marantodes pumilum* and *Ficus deltoidea* plant extracts determined for PC3 cells as assessed by SRB assays after 72 hours. Each result was obtained in three independent experiments, which was run in triplicate.

Plant name	Fraction (Chloroform)	Sample Code	GI50 (µg/ml) with PC3
<i>Marantodes pumilum</i> (Blume) Kuntze (synonym <i>Labisia pumila</i> var. <i>pumila</i>)	Fraction 30-33	MPc F30-33	4.1 ± 0.3
<i>Ficus deltoidea</i> var. <i>angustifolia</i> (Miq.) Corner	Fraction 43-51	FD1c F43-51	12.3 ± 0.4
<i>Ficus deltoidea</i> Jack var. <i>deltoidea</i>	Fraction 29-33	FD2c F29-33	28.2 ± 0.2
	Fraction 34-36	FD2c F34-36	24.2 ± 0.1

a)



b)

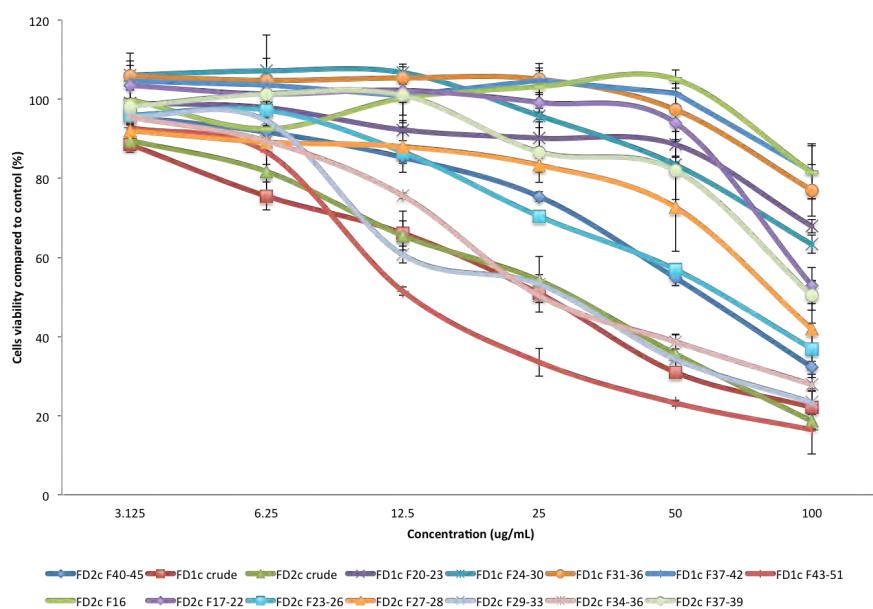


Figure 4.80. Dose-response curves of the effect of the extracts against PC3 cell line. a) Effect of MPC fractions, b) Effect of FD1c and FD2c fractions on the viability of PC3 cell line after 72 hours of exposure. All data represent the mean values and standard error of mean (SEM) for the cytotoxic extracts after 72 hours of exposure. Each point was obtained from three independent experiments, which was run in triplicate.

4.1.10 Effects of the active fractions of the plant extracts on mRNA genes expression

4.1.10.1 Apoptosis-related Gene expression (Bcl-2, Bax, Smac-Diablo, ALOX-5)

The 4 active fractions were further tested to investigate their mode of action on PC3 prostate cancer cell lines. Table 4.8 shows that MPc F30-33 has significantly inhibited both Bcl-2 and ALOX-5 mRNA gene expression by more than 20-fold and 3-fold respectively. MPc F30-33 also increased the expression of Bax by 20-fold in a significant manner. Smac/DIABLO mRNA gene expression was significantly stimulated by MPc F30-33 (50-fold), FD2c F29-33 (5-fold), and FD2c F34-36 (4-fold). However, FD1c F43-51 did not show any significant effect on the expression of Smac/DIABLO. This is contradictory to the previous data in section 4.1.5.6, which show that the active extract of FD1c significantly increased the expression of the gene by 4-fold. This could happen due to the loss of active metabolite/s during the fractionation process. Even though FD1c F43-51 showed better efficacy than its active extracts, the loss of some bioactive component/s might lead to PC3 cell death through a different mode of action. Other active fractions showed a similar trend in activity when compared to their active extracts but with better efficacy. Therefore, these data suggest that the active fraction of the plant extracts (MPc F30-33, FD2c F29-33, and FD2c F34-36) also induced PC3 prostate cancer cells death via apoptosis as observed with their active extracts.

Table 4.8 mRNA gene expression analysis (Bax, Bcl-2 & Smac/DIABLO) in PC3 cells treated with MNTC of the active fractions of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours (Apoptosis-related genes). The genes expressions were determined as described in RT-qPCR Conditions and Analysis where Control = 1. Data are mean ± SD; n=4 experiments. * P < 0.05, * P < 0.001.**

Sample	Gene	Fold expressions relative to Control in PC3 cells
MPc F30-33	Bax	20***
	Bcl-2	0.02*** (20-Fold)
	ALOX-5	0.3* (3-Fold)
	Smac/DIABLO	54***
FD1c F43-51	Smac/DIABLO	1
FD2c F29-33	Smac/DIABLO	5***
FD2c F34-36	Smac/DIABLO	4*

4.1.10.2 Migration-related Gene expression (VEGF, CXCR4, CXCL12)

In this study we will investigate the effect of the active fractions of mRNA gene expression related to cell migration and invasion. Table 4.9 shows that both MPc F30-33 and FD1c F43-51 have significantly down-regulated the mRNA gene expression of VEGF by 22-fold and 4-fold respectively, whereas both active fractions of the FD2c extract did not cause any significant changes to the expression of VEGF. Even though none of the active fractions significantly caused any changes to the expression of CXCR4, all 4 of them had caused significant inhibition to the expression of CXCL12 by more than 20-fold. These findings are similar to the previous results observed with the active extracts of the plants but with greater efficacy. Therefore, the active fractions of the plant extracts also inhibit PC3 prostate cancer cells migration and invasion through similar mode of action.

Table 4.9 mRNA gene expression analysis (VEGF-A, CXCR4 & CXCL12) in PC3 cells treated with MNTC of the active fractions of *Marantodes pumilum* and *Ficus deltoidea* after 96 hours (Migration-related genes). The genes expressions were determined as described in RT-qPCR Conditions and Analysis where Control = 1. Data are mean ± SD; n=4 experiments. * P < 0.05, * P < 0.001.**

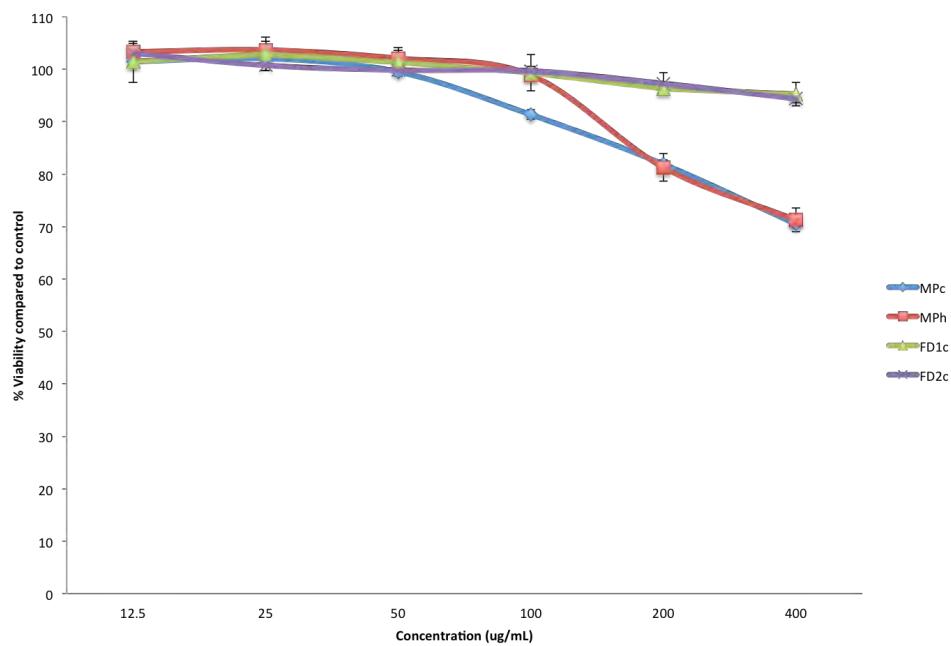
Sample	Gene	Fold expressions relative to Control in PC3 cells
MPc F30-33	VEGF-A	0.04*** (22-Fold)
	CXCR4	1
	CXCL12	0.009*** (>20-Fold)
FD1c F43-51	VEGF-A	0.3*** (4-Fold)
	CXCR4	1
	CXCL12	0.02* (>20-Fold)
FD2c F29-33	VEGF-A	1
	CXCR4	1
	CXCL12	0.005*** (>20-Fold)
FD2c F34-36	VEGF-A	1
	CXCR4	2
	CXCL12	0.005*** (>20-Fold)

4.1.11 Specificity study using normal Human Dermal Fibroblast cells

This study is carried out in order to investigate the effect of the active plant extracts on normal human cells. The results presented in this thesis indicate that the active extracts of both *Marantodes pumilum* (MPc and MPh) and *Ficus deltoidea* (FD1c and FD2c) and their active fractions (MPc F30-33, FD1c F43-51, FD2c F29-33, and FD2c F34-36) show great potential in prostate cancer cell inhibition by inducing cell death via apoptosis, causing cellular arrest at G2M phase and significantly inhibiting both prostate cancer cells migration and invasion.

However, all these activities would be useless if the active extracts also exert toxic effect on normal human cells. Normal human dermal fibroblast cells (HDFa) were used to ascertain the differential toxicity of the active extracts. The results of this study are shown in Figure 4.81. We established the Maximum Non-Toxic Concentrations of the extracts (MNTC) as the concentration allowing 80% of the cell population to grow. The MNTC of both MPc and MPh are about 200 $\mu\text{g}/\text{mL}$ and the MNTC values for both FD1c and FD2c are more than 200 $\mu\text{g}/\text{mL}$. All fractions but MPc F30-33 ($\geq 100 \mu\text{g}/\text{mL}$) were observed to have MNTC values of more than 200 $\mu\text{g}/\text{mL}$. However, considering the very low GI50 value of MPc F30-33 (4 $\mu\text{g}/\text{mL}$), this fraction is still considered to show selective cytotoxicity. The remaining 3 active fractions, FD1c F43-51, FD2c F29-33, and FD2c F34-36 were observed to have MNTC value of more than 200 $\mu\text{g}/\text{mL}$. This information indicates that all the active extracts of the plant as well as the active fractions show selectivity and is specifically inhibiting PC3 cells line growth.

a)



b)

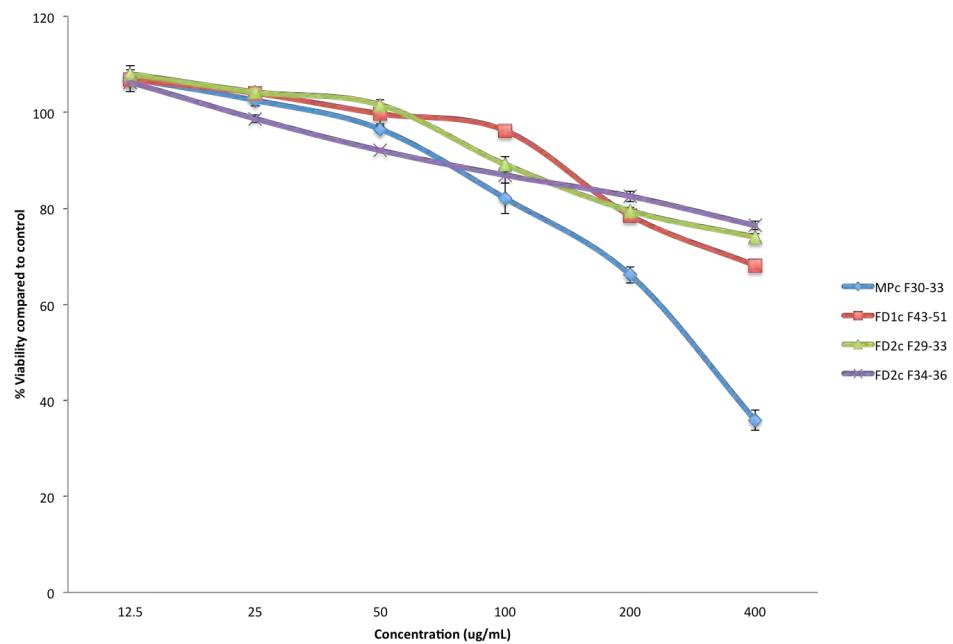


Figure 4.81. Effects of a) MPc, MPh, FD1c and FD2c, and b) MPc F30-33, FD1c F43-51, FD2c F29-33, and FD2c F34-36 plants on the growth of HDFa cells as assessed by SRB assays after 72 hours. Each result was obtained in three independent experiments, which was run in triplicate.

4.1.12 Identification of the active principles from the plant extracts

4.1.12.1 Bioguided isolation of monounsaturated Alkyl Resorcinol from MPc F30-33 fraction

4.1.12.1.1 HPLC microfractionation of MPc F30-33

A high performance liquid chromatography (HPLC) microfractionation was carried out for MPc F30-33 in order to investigate the separation of the compounds and identify which fraction/s are responsible for the cytotoxic activity against PC3 prostate cancer cell lines. Figure 4.82 shows HPLC chromatogram of MPc F30-33. 4 different wavelengths were used in this study including 254, 210, 270 and 330nm. The peaks distribution looks almost similar in all investigated wavelengths, however the intensity of each peak is different. Therefore, the scale on the Y-axis is different for each wavelength due to different signal intensities. Based on the chromatograph shown in Figure 4.82, 8 microfractions were collected from MPC F30-33 and all of these microfractions were subjected to a cytotoxicity assay on PC3 cells using SRB. The GI50 of each microfractions were calculated and Figure 4.83 shows that only microfraction 5 was significantly cytotoxic ($P < 0.05$) against PC3 cells with a GI50 value of 4 $\mu\text{g/mL}$. Therefore, microfraction 5 was analyzed further in order to identify its bioactive compound that might be responsible for the cytotoxic activity against prostate cancer cell lines.

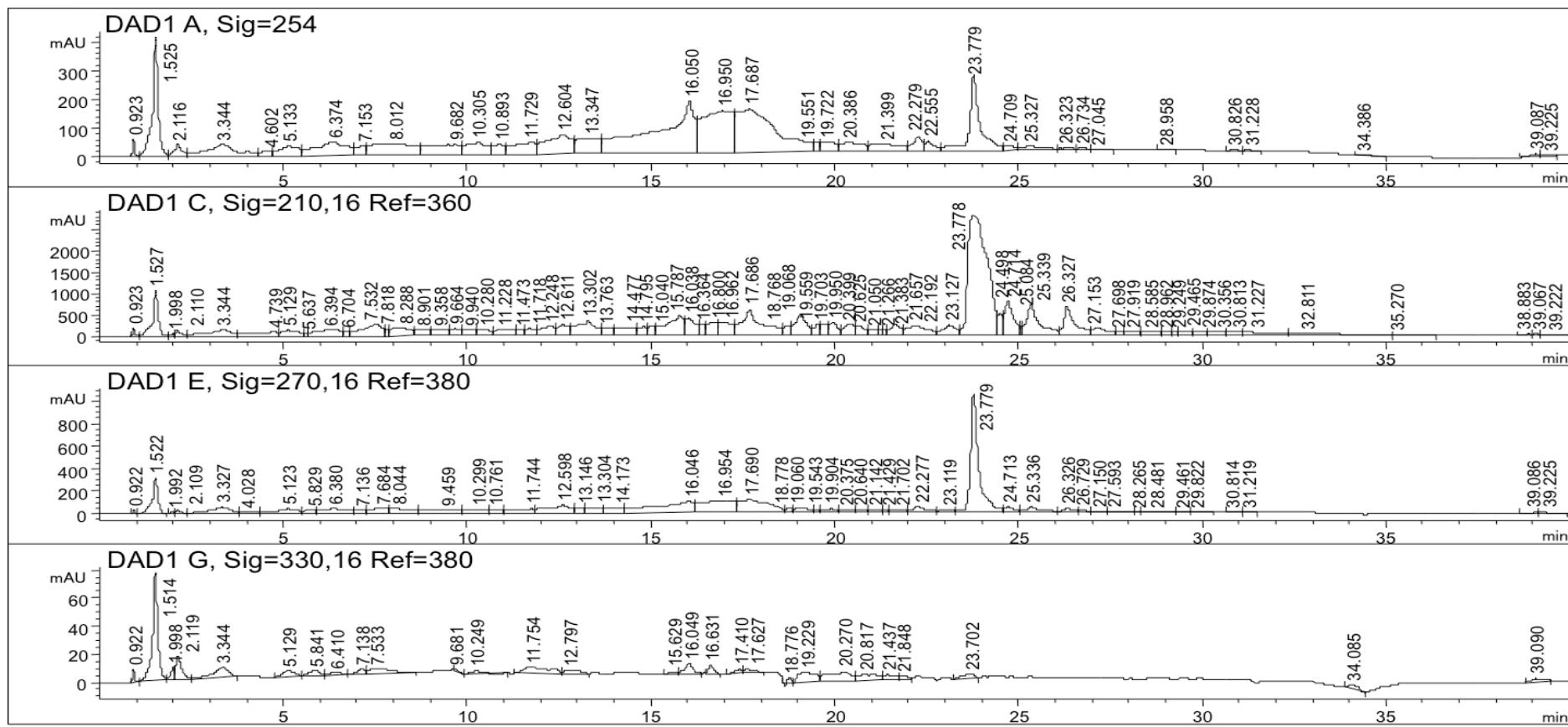


Figure 4.82. HPLC chromatogram of MPc F30-33. 4 different wavelengths were used in this study including 254, 210, 270 and 330nm. The peaks distribution looks almost similar in all investigated wavelengths, however the intensity of each peak is different. Therefore, the scale on the Y-axis is different for each wavelength due to different signal intensities.

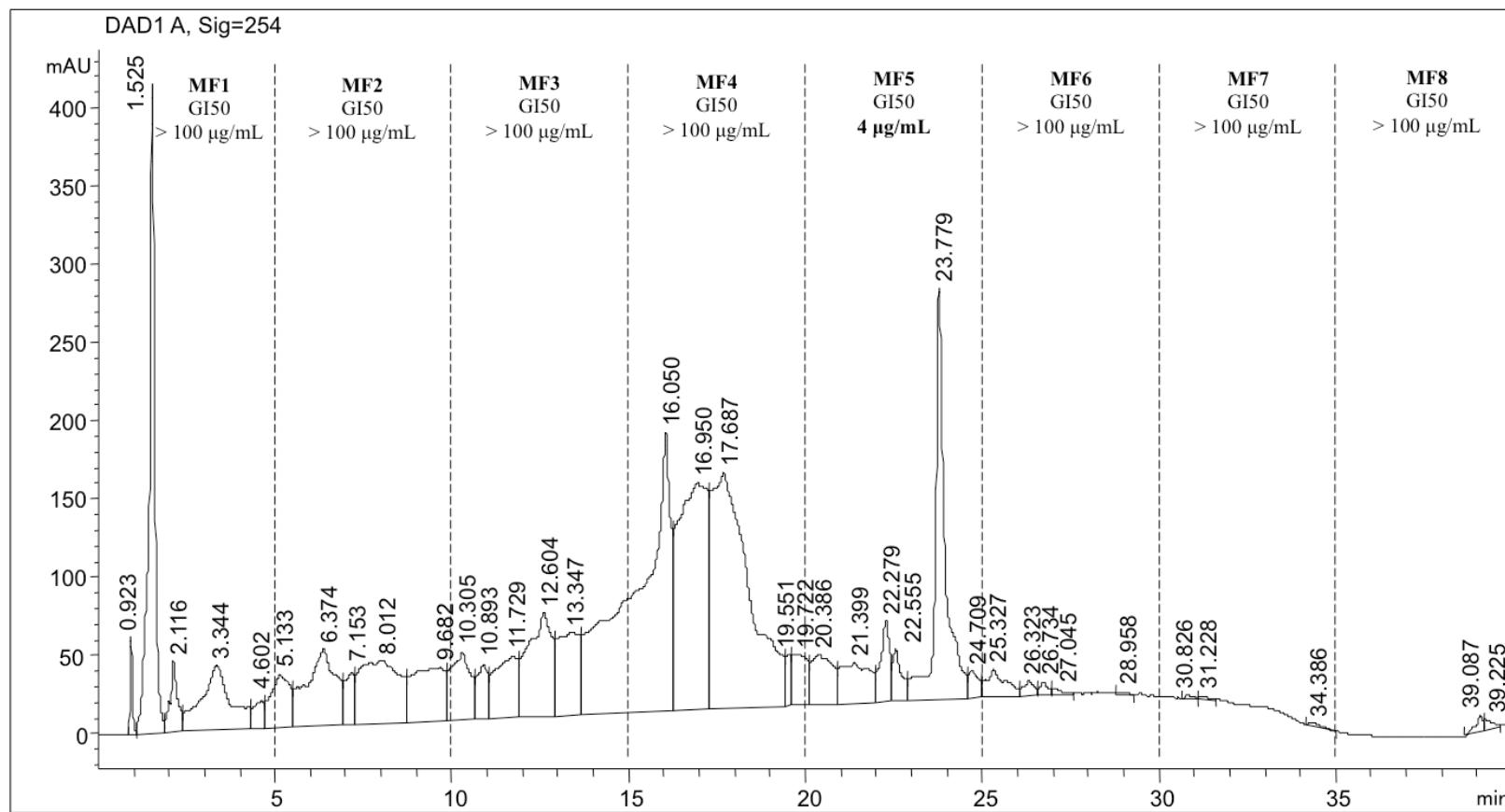
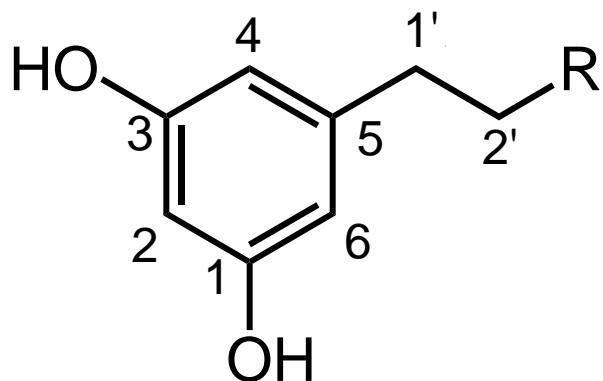


Figure 4.83. Overlay of the HPLC chromatogram of MPc F30-33 with their respective GI50. The GI50 concentrations ($\mu\text{g/mL}$) of the microfractions determined for PC3 cells as assessed by the SRB assays at 72 hours. Paclitaxel ($0.01\mu\text{M}$) was used as a reference drug. Each result was obtained in three independent experiments and run in triplicate.

4.1.12.1.2 Elucidation of microfraction no. 5

Compound **MP-1**, as shown in Figure 4.84, was isolated as a colorless oil. In the IR spectrum shown in Figure 4.94, absorption bands for hydroxyl was observed at 3380.75 cm^{-1} , and for alkenyl at 1600.16 cm^{-1} . The HRESI-MS (Figure 4.86) of compound **MP-1** exhibited a protonated molecular ion $[\text{M}+\text{H}]^+$ peak at m/z 402.2 (calcd. for $\text{C}_{27}\text{H}_{45}\text{O}_2+\text{H}$, 401) which corresponded to the molecular formula $\text{C}_{27}\text{H}_{45}\text{O}_2$. The ^1H NMR (Figure 4.87) spectrum gave signals that are characteristic of resorcinol with unsaturated alkyl substituents at the 5th position, 5-alkylresorcinol (AR). A singlet at δ 5.87 ppm and overlapped signals at δ 6.02 ppm, form the aromatic part of the compound. The ^1H NMR spectrum exhibited resonances for the alkenyl side chain at δ 2.37 ppm (m), δ 1.53 ppm (m), δ 2.07 ppm (q, $J=26.5, 13.5, 7$), and δ 5.45 ppm (m), overlapped signals from δ 1.21 ppm to δ 2.37 ppm, and at δ 0.87 ppm (m). The ^{13}C NMR spectrum (Figure 4.88) showed 6 resonances in the aromatic region (δ 100.8- δ 157.9ppm). Inspection of key HMQC (Figure 4.90) correlations showed two bond correlation between H at position 2 to C-1 and C-3, H at position 4 to C-3, H at position 6 to C-1 and H at position 1' to C-2 and C-5. HMBC (Figure 4.91) data showed three bond correlation between H at position 4 to C-2, H at position 6 to C-2, and H at position 1' to C-4; and four bond correlation between H at position 4 to C-1', H at position 6 to C-1', and H at position 1' to C-6. The aliphatic chain has a double bond due to the signals at δ 5.45- δ 5.53 ppm, which is a multiplet and integrated for 2H revealed the presence of a double bond in the aliphatic chain. We did not determine the position of the double bond in the aliphatic chain. Since the structure of **MP-1** is not yet fully elucidated, in an effort to validate the structure of **MP-1**, a comparison is made to another 5-alkylresorcinol compound namely 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol (Figure 4.85) isolated by Al-Mekhlafi, Shaari et al. (2012). Table 4.10 shows ^1H and ^{13}C NMR spectroscopic data for 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol measured in CDCl_3 . The spectroscopic data in Table 4.10 display similar peaks positioning for both C and H spectra especially in the aromatic region and the location of the alkenyl side chain when compared

to the spectroscopic data of **MP-1** in the same table with slight differences in chemical shifts. The slight difference in chemical shift is expected as different solvents were used for NMR analysis. However, the C at position 6 in 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol corresponds to δ 131.7 ppm whereas the same C in **MP-1** corresponds to δ 108.4 indicating a huge difference in the chemical shift. This phenomenon can be explained due to the fact that C at position 6 in 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol is attached to a stronger electron-withdrawing group thus making it more deshielded. This comparison has further validated the elucidated structure of **MP-1**.



$R=19:1$

Figure 4.84 Structure of MP-1

Table 4.10 ^1H and ^{13}C NMR spectroscopic data for compound MP-1 measured in benzene- d_6 and ^1H and ^{13}C NMR spectroscopic data for 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol measured in CDCL_3 . The spectroscopic data is put side by side so that the structure can be easily compared, thus validating the structure of MP-1. The spectroscopic data for 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol is adapted from “Alkylresorcinols and cytotoxic activity of the constituents isolated from *Labisia pumila*” by N. A. Al-Mekhlafi, K. Shaari, F. Abas, R. Kneer, E. J. Jeyaraj, J. Stanslas, N. Yamamoto, T. Honda, and N. H. Lajis, 2012, *Phytochemistry* (2012) 80, 42-49. Copyright 2012 by Elsevier. Adapted with permission.

MP-1					1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol		
Position	^1H	^{13}C	2J	$^3J / ^4J$	Position	δ_{H} (J in Hz)	δ_{C}
1		157.9			1		151.8
2	5.87 (s)	100.8	C-3, C-1		2	6.24 (d, $J = 2.5$ Hz)	98.4
3		157.9			3		154.3
4	6.02 (overlapped)	108.4	C-3	C-1', C-2	4	6.18 (d, $J = 2.5$ Hz)	107.9
5		146.1			5		136.7
6	6.02 (overlapped)	108.4	C-1	C-1', C-2	6		131.7
1'	2.37 (m)	36.6	C-5, C-2'	C-4, C-6	1'	2.39 (t, $J = 7.5$ Hz)	32.2
2'	1.53 (m)	31.9			2'	150 (m)	30.3
	2.07 (q, $J = 26.5, 13.5, 7$)	28.0			3'-8'	1.32-1.27 (m)	29.6-30.2
	5.45 (m)	130.6			9'	2.01 (m)	27.2
	5.45 (m)	130.6			10'	5.35 (m)	130.0

Table 4.10 (Continues)

MP-1					1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol		
Position	¹ H	¹³ C	² J	³ J / ⁴ J	Position	δ_H (J in Hz)	δ_C
	2.07 (q, $J = 26.5, 13.5, 7$)	27.7			11'	5.35 (m)	130.0
	1.21-2.37 (overlapped)	23.1- 30.6			12'	2.01 (m)	27.2
21	0.87 (m)	14.6			13'-14'	1.32-1.27 (m)	22.6
					15'	0.89 (<i>t</i> , $J = 7.0$ Hz)	14.3
					2-OCH ₃	3.71 (s)	56.1
					1-O <u>COCH</u> ₃	2.32 (s)	170.3
					1-O <u>COCH</u> ₃		20.7

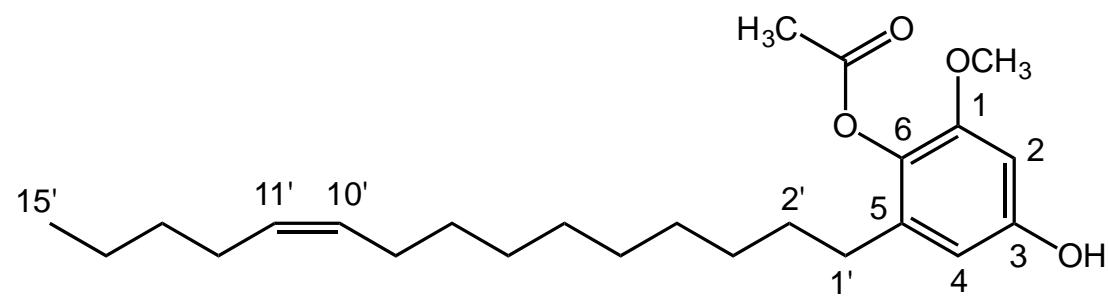


Figure 4.85 Structure of 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol

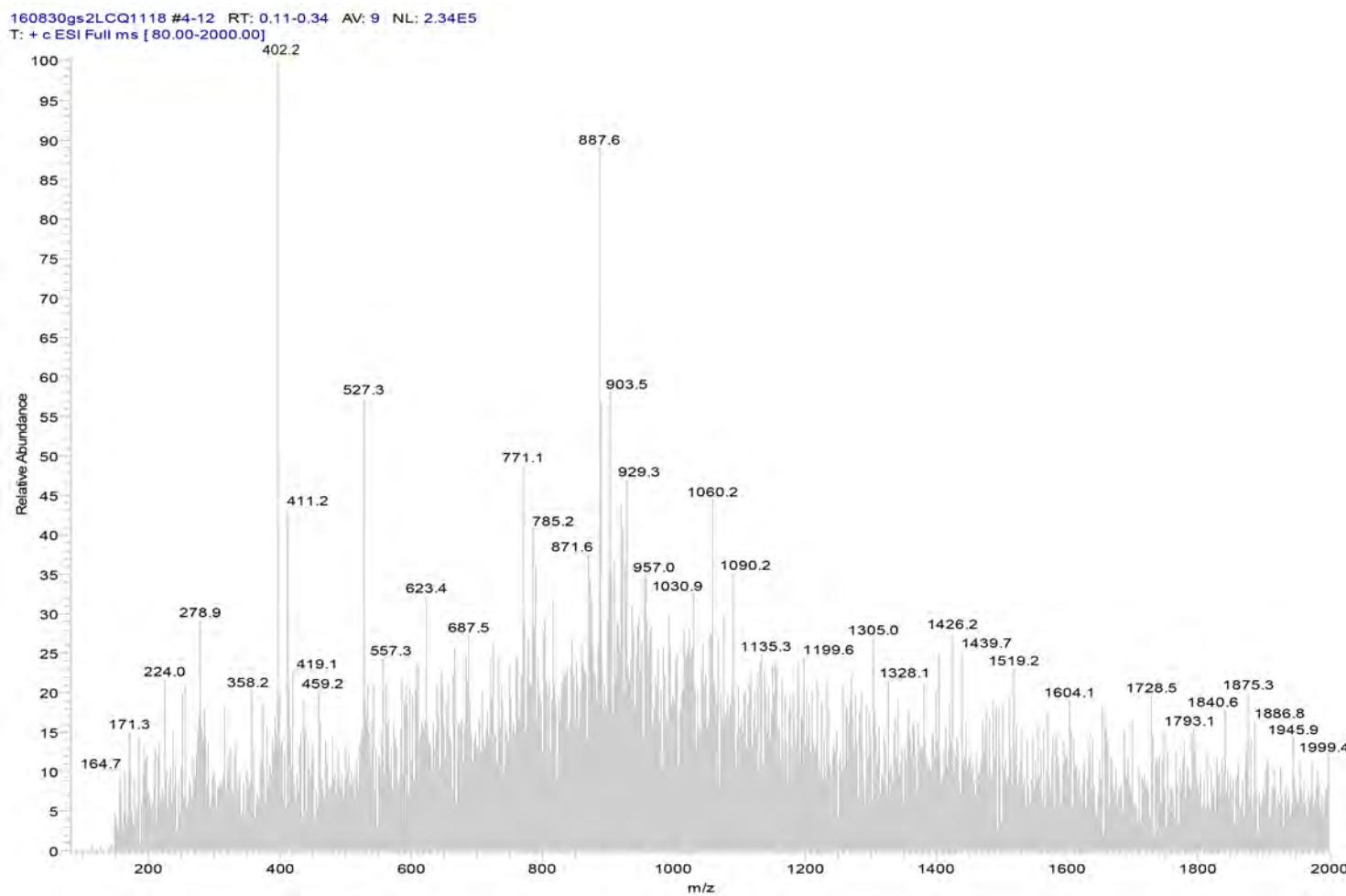


Figure 4.86 MP-1 ESI-MS result

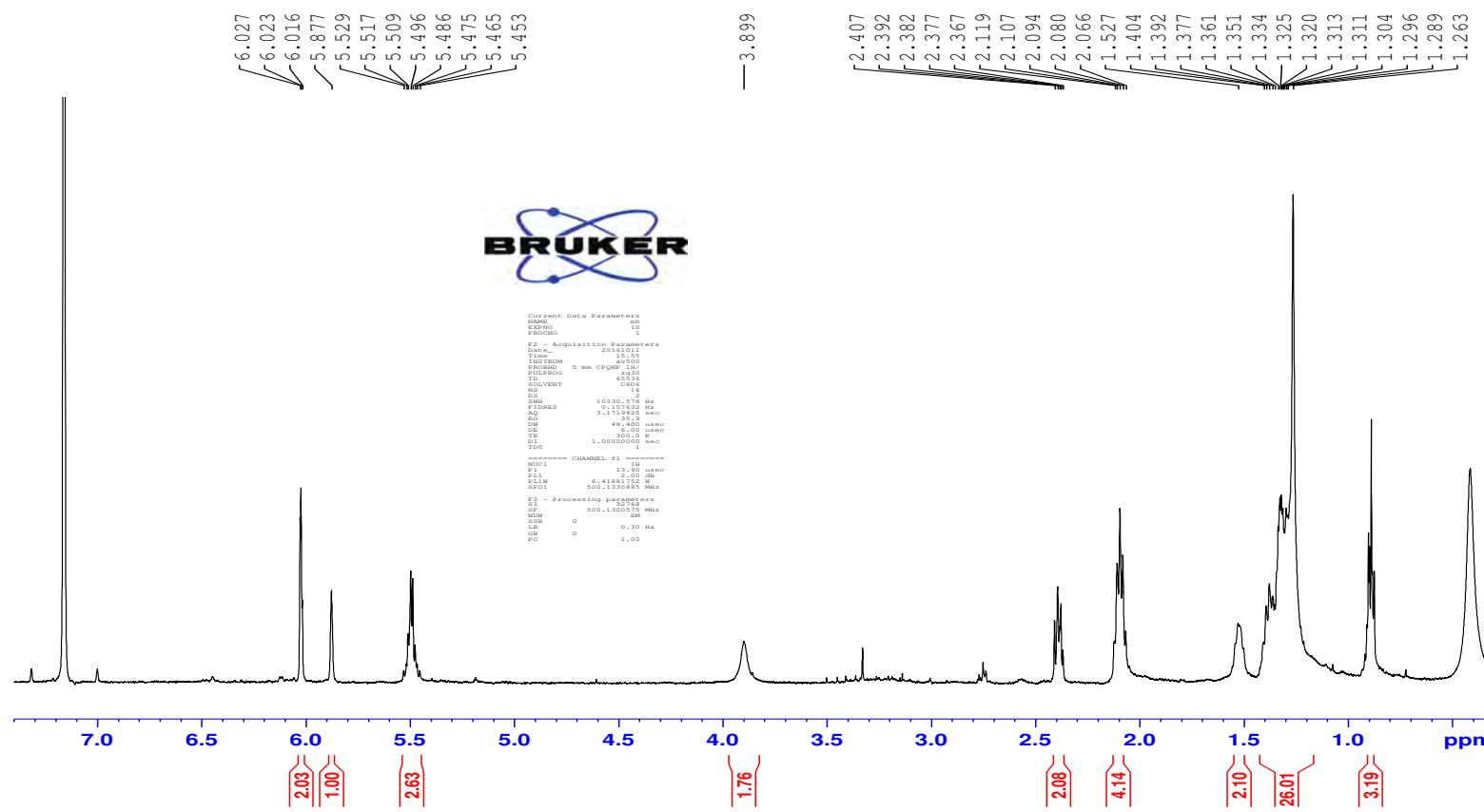


Figure 4.87 ¹HNMR of MP-1 dissolved in benzene-*d*₆ at 500MHz. The ¹HNMR spectrum gave signals that are characteristic of resorcinol with unsaturated alkyl substituents at the 5th position, 5-alkylresorcinol (AR). A singlet at δ 5.87 ppm and overlapped signals at δ 6.02 ppm, form the aromatic part of the compound. The alkenyl side chain is identified at δ 2.37 ppm (m), δ 1.53 ppm (m), δ 2.07 ppm (q, J =26.5, 13.5, 7), and δ 5.45 ppm (m), overlapped signals from δ 1.21 ppm to δ 2.37 ppm, and at δ 0.87 ppm (m).

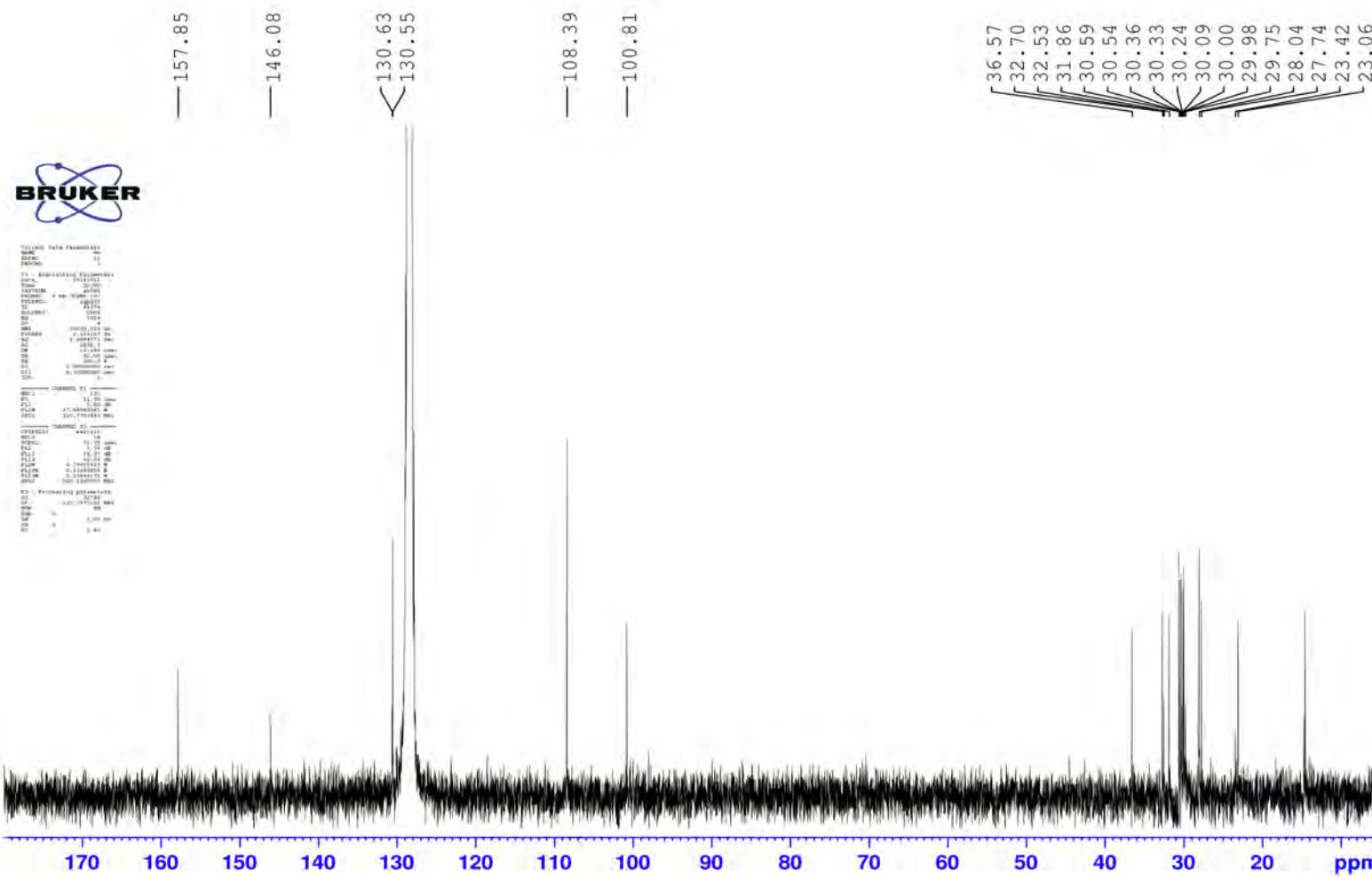


Figure 4.88 ^{13}C NMR of MP-1 dissolved in benzene- d_6 at 500MHz

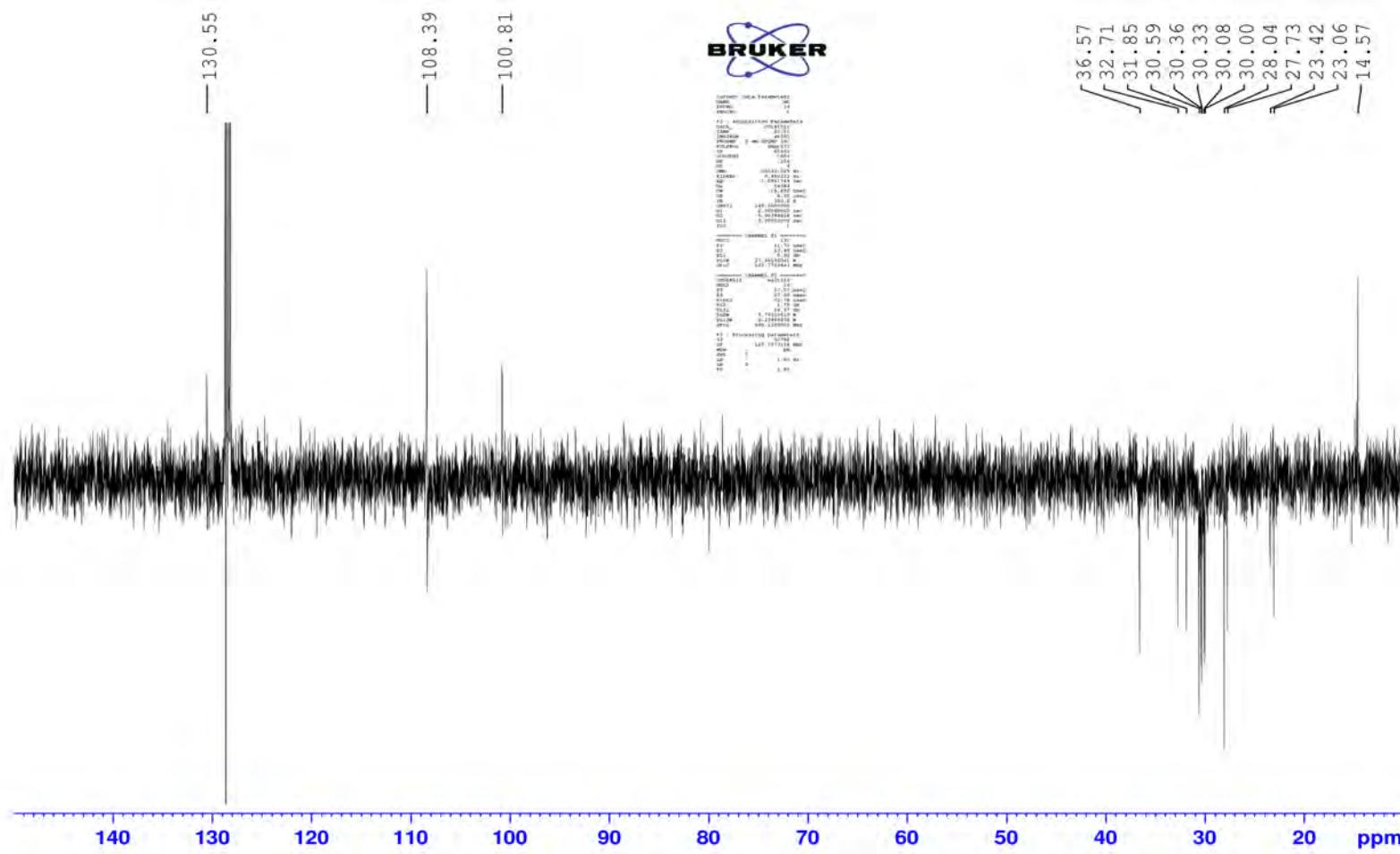


Figure 4.89 DEPT of MP-1 dissolved in benzene- d_6 at 500MHz

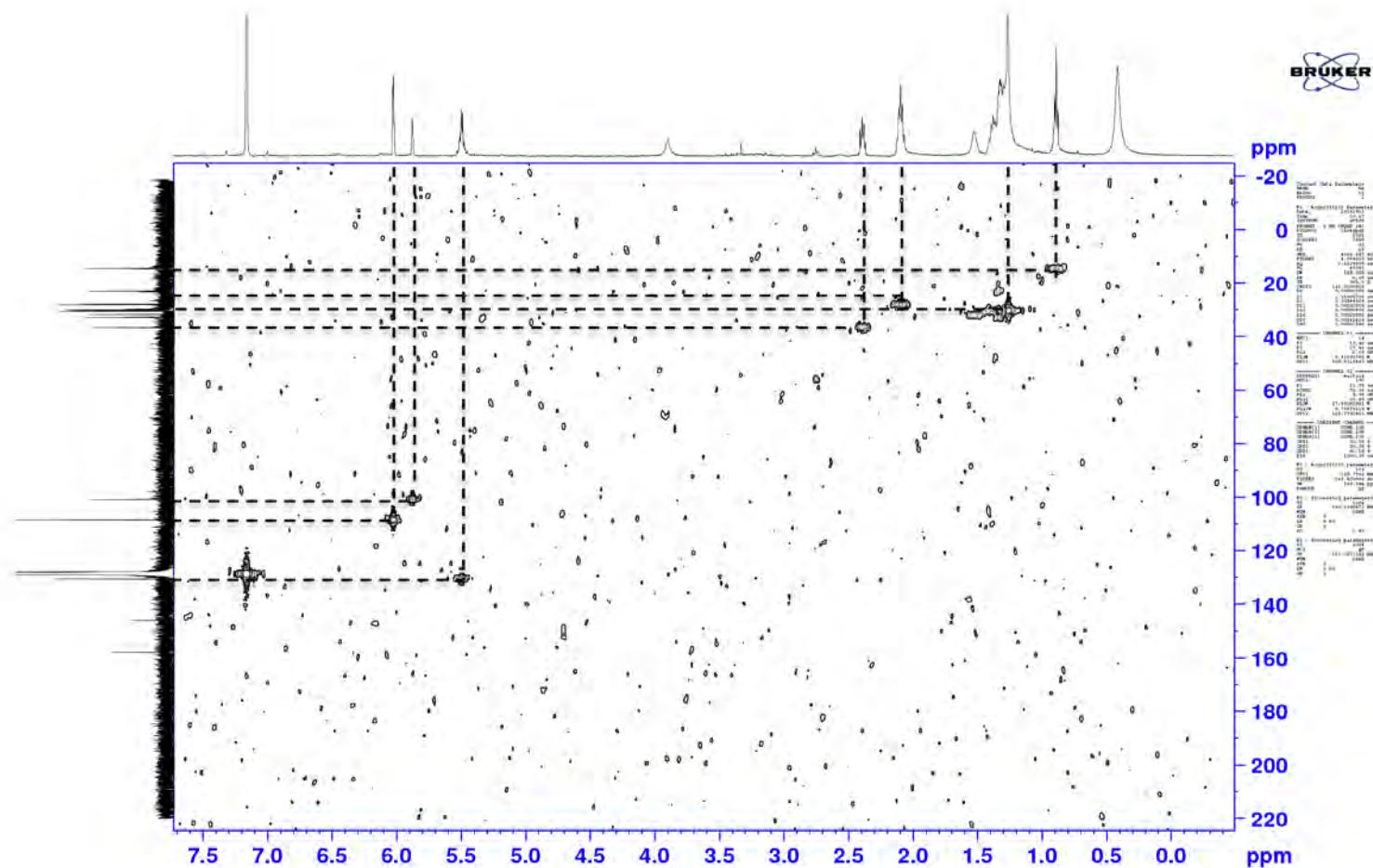


Figure 4.90 HMQC correlations of MP-1 dissolved in benzene-*d*₆ at 500MHz. Correlations between H and C are shown in the figure

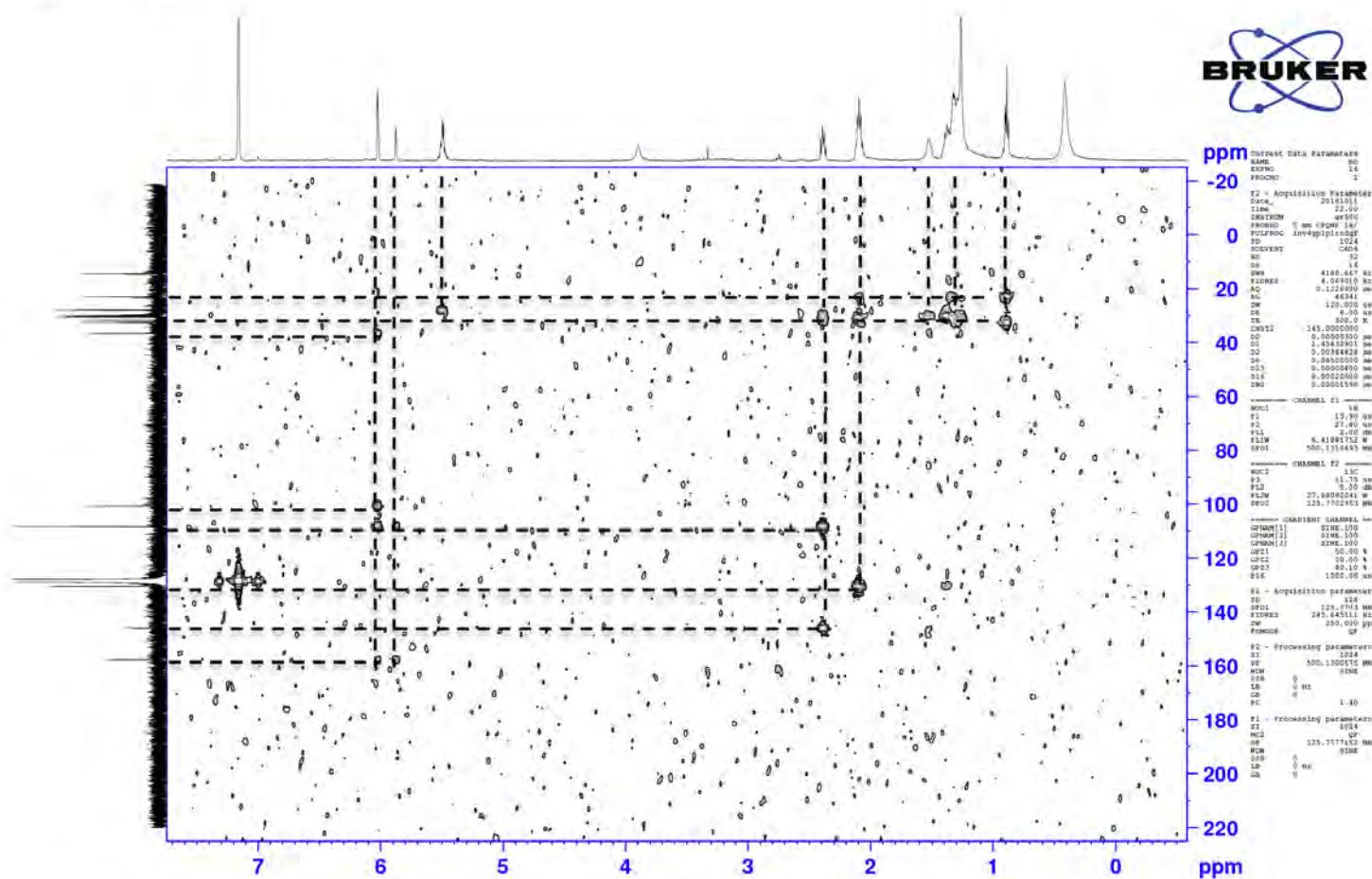


Figure 4.91 HMBC correlations of MP-1 dissolved in benzene- d_6 at 500MHz. Correlations between H and C are shown in the figure.

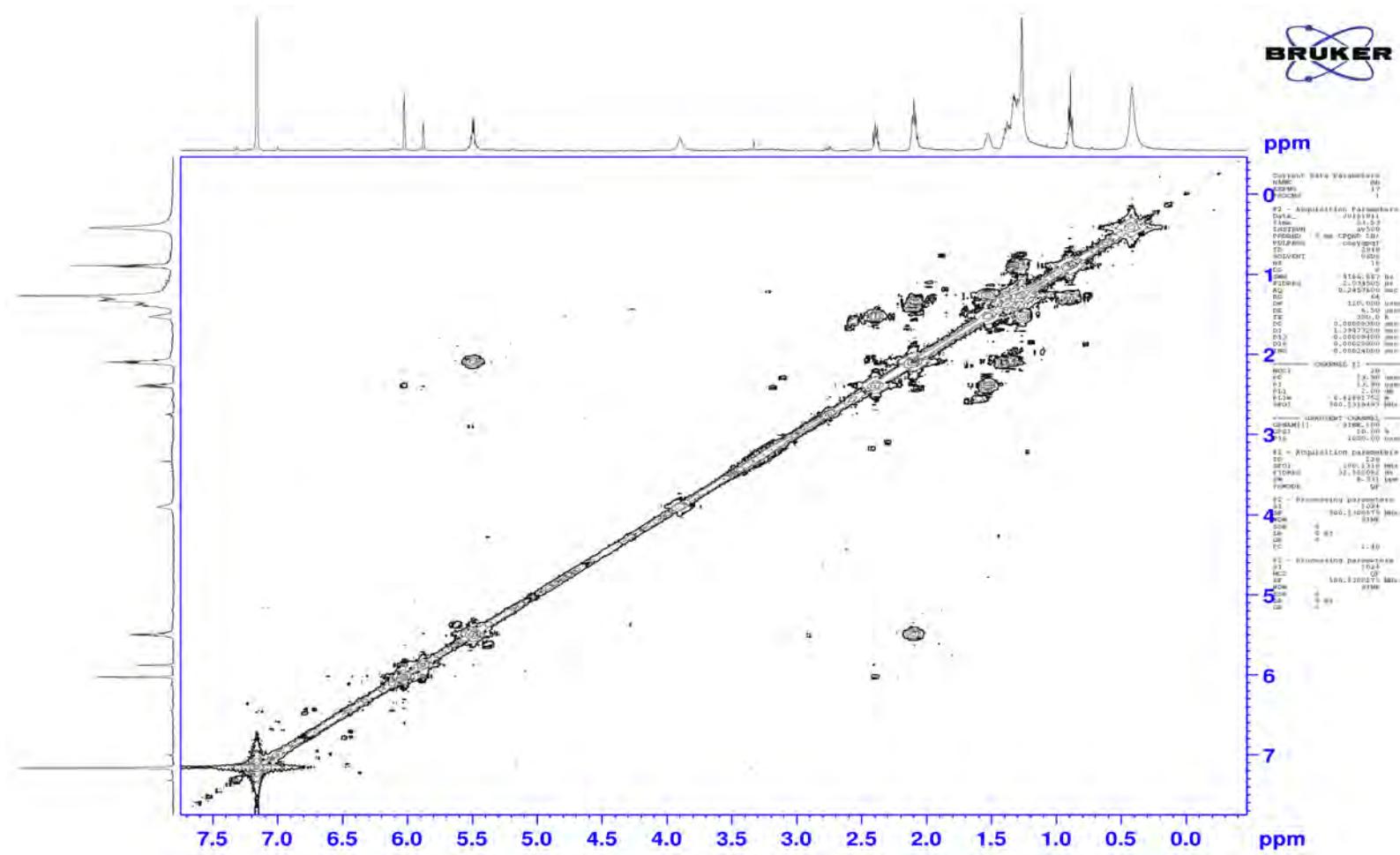


Figure 4.92 COSY of MP-1 dissolved in benzene- d_6 at 500MHz

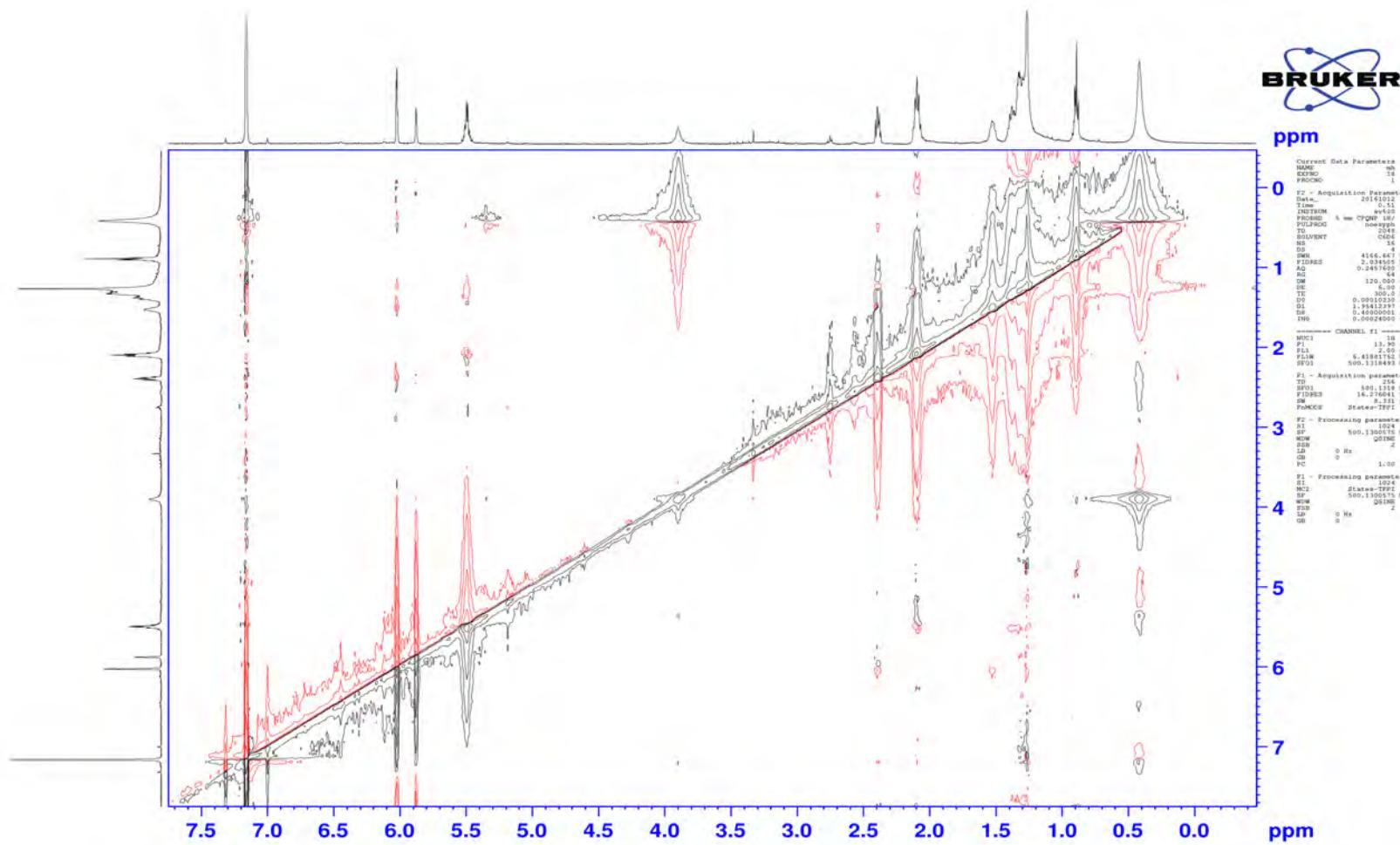


Figure 4.93 NOESY of MP-1 dissolved in benzene- d_6 at 500MHz

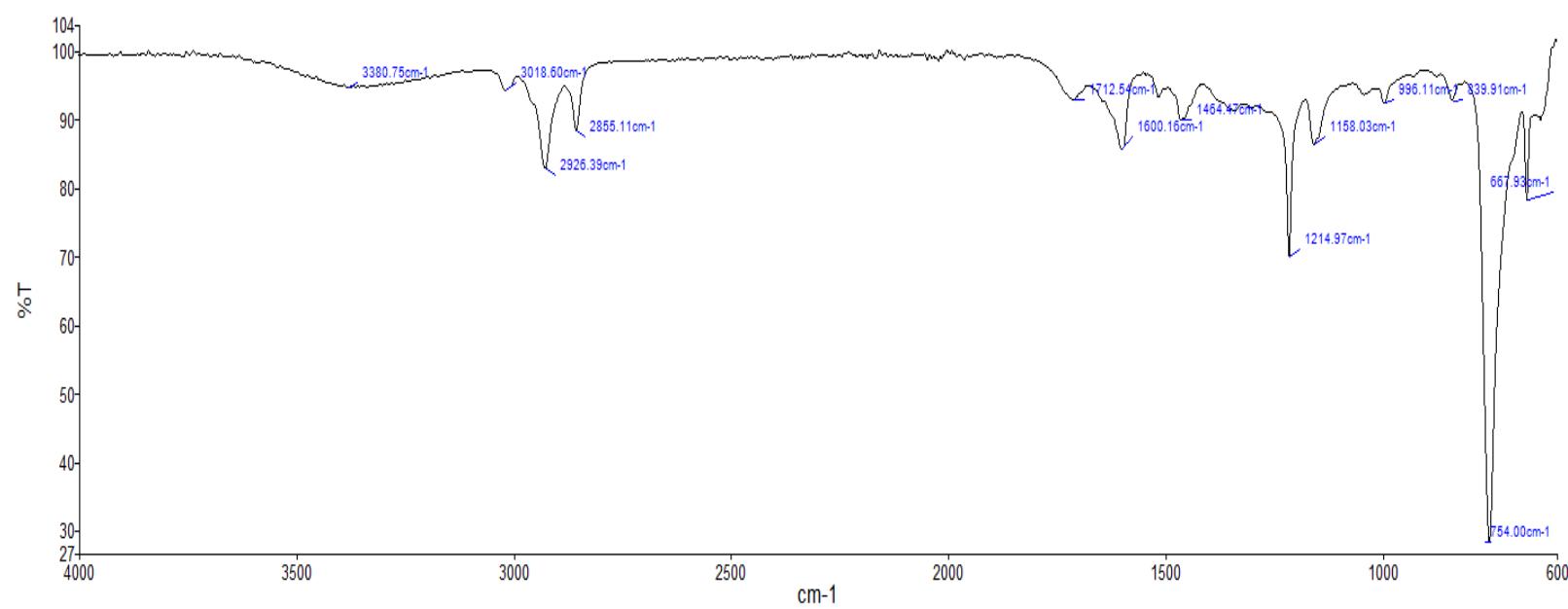


Figure 4.94 Infrared spectra for MP-1

4.1.12.2 Dereplication of Isovitetoxin from FD1c F43-51, Lupeol, Moretenol from FD2c F29-33 and Oleanolic acid from FD2c F34-36 fractions

Previously, researchers have used the conventional methods, which rely heavily on bioactivity-directed fractionation methodology in order to identify, isolate and elucidate the bioactive lead compound from the crude extracts. This process, which depends on the results of the bioassay to guide the isolation, is often tedious, expensive, time consuming - and quite frequently we may end up with disappointing outputs when isolating a previously known and well characterized compound (Ghisalberti 1993, Alali and Tawaha 2009). In order to tackle these issues, a procedure known as dereplication has been used in natural products and phytochemistry research. This procedure is able to discriminate between previously known compounds and potential novel entities in a more efficient and rapid way while only working at the level of crude extracts (Alali and Tawaha 2009).

Dereplication will prevent us from wasting important resources, as this procedure will be oriented towards targeted isolation of compounds presenting novel spectroscopic features. Dereplication could also be a very useful procedure that can be used to investigate the presence of known compounds in a crude extracts or fractions in a quick and efficient way, thus making it essential for natural product research (Constant and Beecher 1995, Ackermann, Regg et al. 1996). In the dereplication method, hyphenated techniques using UV spectroscopy and mass spectrometry coupled with the use of natural products database are very important in order to determine the identity of the active compound at the earliest possible stage of the discovery process (Sedlock, Sun et al. 1992, Su, Rowley et al. 2002, Sarker and Nahar 2012). In this study, the Ultra High Performance Liquid Chromatography-Electro Spray Ionization-Mass Spectrometry (UHPLC-ESI-MS) and Liquid Chromatography-Photodiode Array Detection (LC-PDA) was used in order to analyse the secondary metabolite constituents of the active fractions from both FD1 and FD2 plants.

4.1.12.2.1 UHPLC-MS analysis of FD1c F43-51

FD1c F43-51 was active against PC3 cancer cell lines with a GI₅₀ value of 12 µg/mL. The UHPLC-MS-ELSD-PDA profiles are shown in Figure 4.95. The shifts for the retention time (t_R) of the 12 peaks in this chromatogram (Figure 4.95) are due to the sequential four-channel detection system. The universal ELSD detection method provides accurate data in accessing the relative content of individual compounds in a mixture that may not be UV active (Yang, Liang et al. 2014). Peaks 3a and 3b with t_R 5.72 min were present in the highest concentration, which accounts for 75.27% of the total mass. The positive and negative-ion ESIMS detection procedures showed different sensitivities (Table 4.11), with the positive mode producing a strong total ion chromatogram (TIC) for all the compounds. Therefore, the positive ESIMS and UV data were used to determine the structural information of the compounds present. Peaks 3a and 3b gave the same accurate mass of 433 and were dereplicated as Vitexin and Isovitexin respectively (Figure 4.96). These two compounds are known constituents of *Ficus deltoidea* (Bunawan, Amin et al. 2014). Peak 5 with t_R 9.46 min was also detected with a good concentration with 8.22% of total mass and it was dereplicated as Brosimacutin (Figure 4.96). The other 10 compounds (Compounds 1,2,4 and 6-12) were present in a very small concentration and thus not considered to play a major role in the cytotoxic activity of FD1c F43-51 fraction.

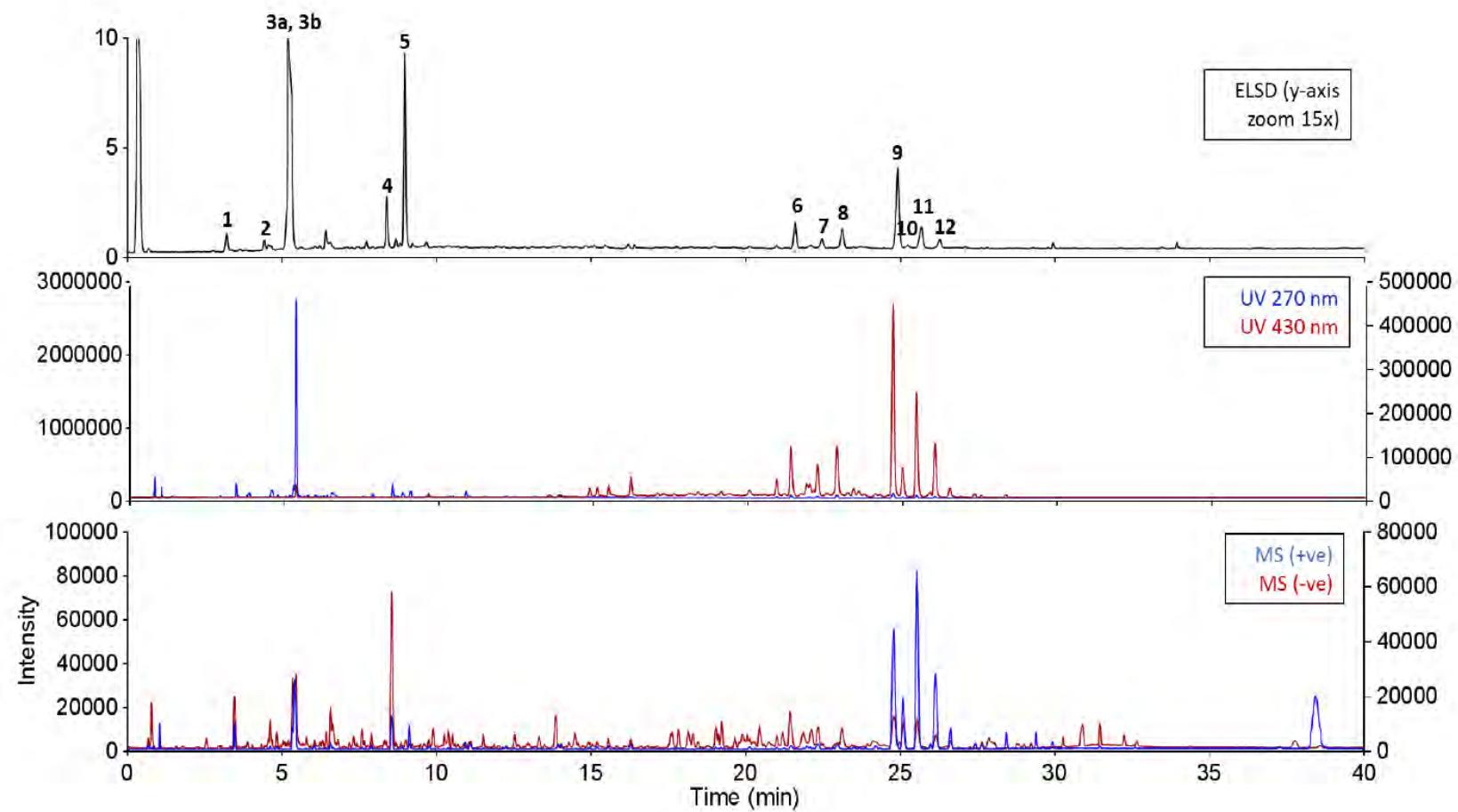
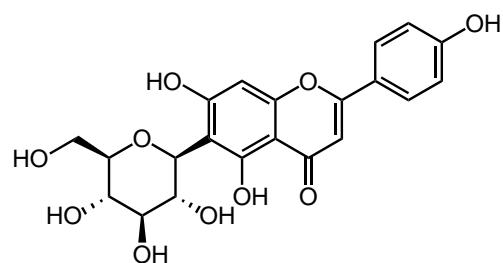


Figure 4.95 Overlay of ELSD, UV and MS (base peak monitoring) chromatograms for sample FD1c F43-51.

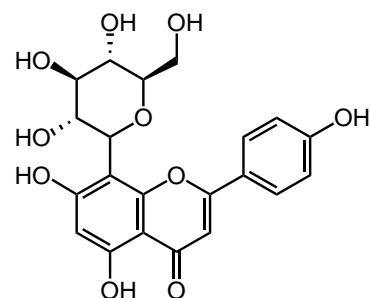
Table 4.11 MSI dereplication of FD1c F43-51 sample.

Peak no	UHPLC-DAD-ELSD			UHPLC-DAD-Orbitrap MS				Identification	Method of identification*	
	Time (min)	% Area	PDA (UV _{max})	Neutral Molecular formula	[M+H] ⁺		[M-H] ⁻			
					Accurate mass	Absolute Error (ppm)	Accurate mass	Absolute Error (ppm)		
1	3.71	0.91	271, 325	C27H30O15	595.1660	C27H31O15 (0.41)	593.1518	C27H29O15 (2.92)	Saponarin (Isovitexin-7-O-beta-D-glucopyranoside)	Match with DNP (Dicot), ubiquitous
									4',5,6,7-Tetrahydroxyflavone; 6-O-[α -L-Rhamnopyranosyl-(1 \rightarrow 2)- β -D-galactopyranoside]	Match with DNP (Ficus)
									4',5,6,7-Tetrahydroxyflavone; 7-Me ether, 6-O-[α -L-arabinopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside]	Match with DNP (Ficus)
2	4.92	0.42	270, 349	C21H20O11	449.1080	C21H21O11 (0.33)	447.0935	C21H19O11 (2.95)	Isoorientin	Match with isolated compound
3a	5.72	75.27	268, 351	C21H20O10	433.1132	C21H21O10 (0.50)	431.0983	C21H19O10 (2.41)	Vitexin	Match with isolated compound
3b				C21H20O10	433.1132	C21H21O10 (0.78)	431.0982	C21H19O10 (0.33)	Isovitetin	Match with isolated compound
4	8.88	2.20	250, 312	C16H16O6	305.1016	C16H17O6 (1.28)	349.0931 [M-H+FA]-	C17H17O8 (3.67)	Aviprin	Match with isolated compound
5	9.46	8.22	231, 280	C20H22O6	359.1483	C20H23O6 (1.58)	No signal		2',4'-Dihydroxy-7-methoxyflavan-8-butanoic acid.	Match with DNP (Moraceae)
									Brosimacutin B	Match with DNP (Moraceae)
									Brosimacutin A	Match with DNP (Moraceae)
									Brosimacutin M	Match with DNP (Moraceae)
									2,2',4,4'-Tetrahydroxy-3'-(4-hydroxy-3-methyl-2-butenyl)dihydrochalcone	Match with DNP (Moraceae)
6	22.08	1.66	296, 369, 436	C34H38O11	623.2503	C34H39O11(1.69)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)
7	22.95	0.64	295, 368, 435	C34H38O11	623.2507	C34H39O11(2.33)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)
8	23.62	1.20	297, 331, 370, 436	C34H38O10	607.2552	C34H39O10 (1.59)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)
9	25.40	6.58	230, 277, 410	C34H40O10	609.2717	C34H41O10 (2.95)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)
10	25.73	0.27	226, 277, 401	C34H40O11	625.2658	C34H41O11 (1.44)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)
11	26.16	1.88	230, 277, 409	C34H40O10	609.2718	C34H41O10 (3.09)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)
12	26.77	0.76	229, 327, 410	C34H40O9	593.2762	C34H41O9 (1.92)	No signal		Unknown (Aurone flavonoids)	No match with DNP (Aurone flavonoids)

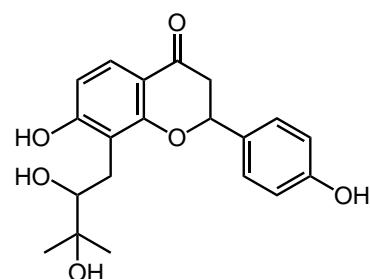
* Database generated from Dictionary of Natural Products (DNP) according to family (Moraceae), genus (Ficus), Type of Organism (Dicot) Type of Compound (Aurone flavonoids).



Isovitexin



Vitexin



Brosimacutin A

Figure 4.96 The chemical structure of isovitexin, vitexin and brosimacutin A.

4.1.12.2.2 UHPLC-MS analysis of FD2c F29-33 and FD2c F34-36 fractions

FD2c F29-33 and FD2c F34-36 were active against PC3 cancer cell lines with GI₅₀ values of 28 µg/mL and 24 µg/mL respectively. The UHPLC-MS-ELSD-PDA chromatogram in Figure 4.97 shows 6 peaks. For FD2c F29-33, 4 major peaks were detected - peak 1 is similar to peak 3 - at t_R 24.15 (peak 2), t_R 24.42 (peak 3), t_R 25.87 (peak 4) and t_R 31.59 (peak 6). These peaks correspond to 21.07% (peak 3 + peak 1), 20.09% (peak 2), 34.40% (peak 4), and 31.59% (peak 6) of total mass respectively. ESIMS data shows that these peaks gave accurate masses of 457, 425, 427 and 443 respectively and were dereplicated as Oleanolic acid, Lupenone, Lupeol, Moretenol, and Betulin (Figure 4.98).

For FD2c F34-36 fraction, 2 major compounds were dereplicated as Oleanolic acid - at t_R 24.42, which correspond to 73.33% (peak 1 + peak 3) of total mass - and Lupenone at t_R 24.15, and t_R 27.77 - which correspond to 18.65% of total mass. Both Oleanolic acid and Lupenone gave accurate masses of 457 and 425 respectively according to the ESIMS data (Table 4.12).

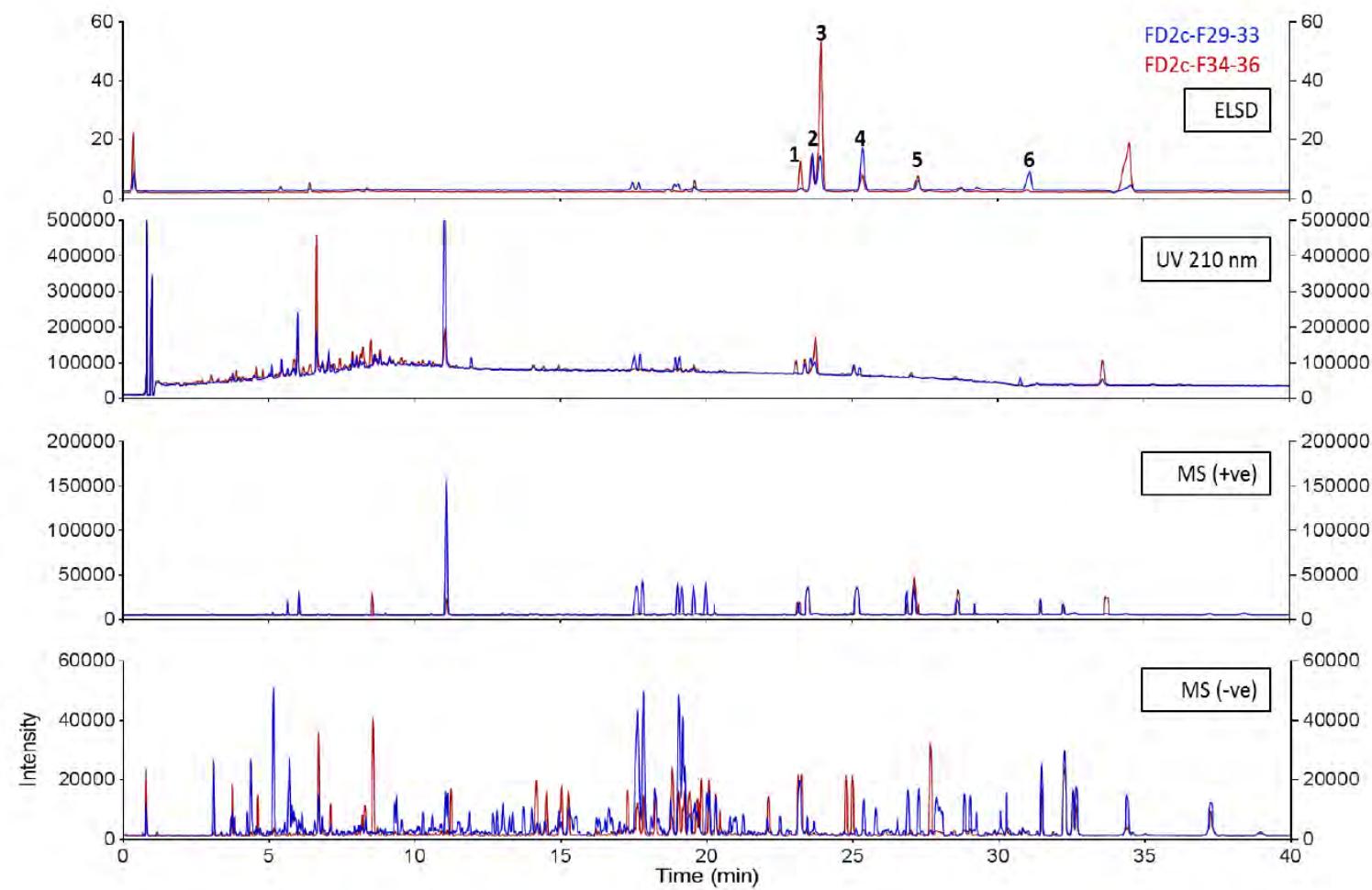
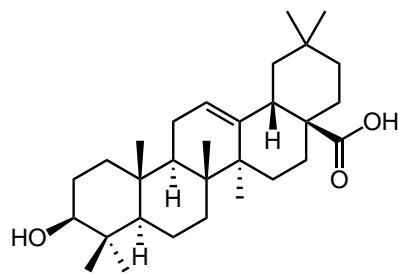


Figure 4.97 Overlay of ELSD, UV and MS (base peaks monitoring) chromatograms for samples FD2c F29-33 and FD2c F34-36.

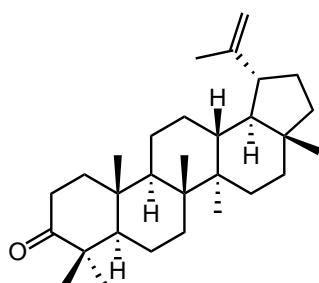
Table 4.12 MSI dereplication of FD2c F29-33 and FD2c F34-36 samples.

Peak no	UHPLC-DAD-ELSD			UHPLC-DAD-TOF MS (Positive ionization)			Identification	Method of identification*
	Time (min)	% Area (F29-33)	% Area (F34-36)	Neutral Molecular formula	Accurate mass (M+H) ⁺	Molecular formula (error in ppm)		
1	23.75	1.18	10.81	Not determined	439.3576 (M+H-H ₂ O) 501.3599 (M-H+formate ion)	Not determined	Same as Peak no. 3	Same as Peak no. 3
2	24.15	20.09	12.72	C ₃₀ H ₄₈ O	425.3784	C ₃₀ H ₄₉ O (1.7)	Cycloartenone	Match with DNP (Moraceae)
							Lupenone	Match with DNP (Dicot), ubiquitous
3	24.42	19.89	62.52	C ₃₀ H ₄₈ O ₃	457.3678	C ₃₀ H ₄₉ O ₃ (0.3)	3 α -Hydroxy-22(29)-isohopen-24-oic acid	Match with DNP (Ficus)
							19,20-Seco-3,19,20-ursanetrione	Match with DNP (Ficus)
							Moruslupenoic acid A	Match with DNP (Moraceae)
							Oleanolic acid	Match with DNP (Dicot), ubiquitous
4	25.87	34.40	8.01	C ₃₀ H ₅₀ O ₂	443.3872	C ₃₀ H ₅₁ O ₂ (2.7)	13(18)-Neohopene-3,24-diol; 3 β -form	Match with DNP (Ficus)
							Gmeliniin A, 21 α -Hydroxytaraxasterol	Match with DNP (Ficus)
							20-Taraxastene-3,22-diol; (3 β ,22 β)-form	Match with DNP (Ficus)
							20,21-Epoxy-3-taraxastanol	Match with DNP (Ficus)
							20-Taraxastene-3,22-diol; (3 β ,22 α)-form	Match with DNP (Ficus)
							29(20 \rightarrow 19)-Abeo-3-hydroxy-20-lupanone; (3 β ,19 β Me)-form	Match with DNP (Ficus)
							Betulin	Match with DNP (Dicot), ubiquitous
5	27.77	6.50	5.93	C ₃₀ H ₄₈ O	425.3766	C ₃₀ H ₄₉ O (2.9)	Cycloartenone	Match with DNP (Moraceae)
							Lupenone	Match with DNP (Dicot), ubiquitous
6	31.59	17.94	Not determined	C ₃₀ H ₅₀ O	427.3924	C ₃₀ H ₅₁ O (2.5)	Moretenol	Reported in <i>F. deltoidea</i> (literature), Match with DNP (Ficus)
							Lupeol	Match with DNP (Ficus), ubiquitous
							Rhoiptelenol	Match with DNP (Ficus)
				C ₃₀ H ₄₈ O	425.3760	C ₃₀ H ₄₉ O (4.3)	3-Serratanone	Match with DNP (Ficus)
							Cycloartenone	Match with DNP (Moraceae)
							Lupenone	Match with DNP (Dicot), ubiquitous

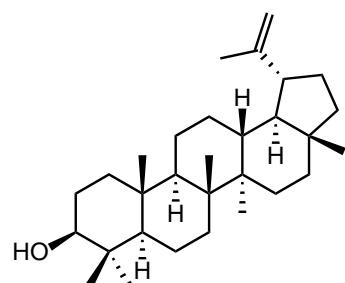
* Database generated from Dictionary of Natural Products (DNP) according to Family (Moraceae), Genus (Ficus) or Type of Organism (Dicot).



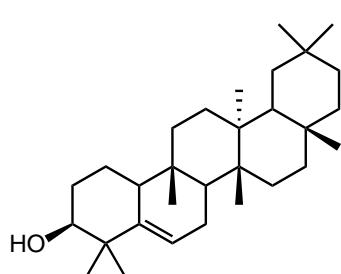
Oleanolic acid



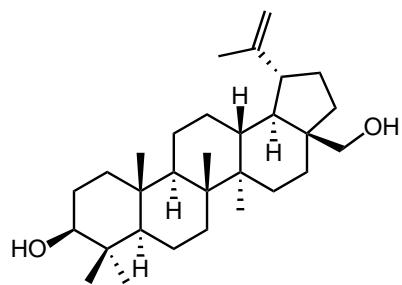
Lupenone



Lupeol



Moretenol



Betulin

Figure 4.98 The chemical structure of Oleanolic acid, Lupenone, Lupeol, Moretenol and Betulin

4.1.12.3 Anti-proliferative activity of dereplicated compounds

Previously, LC-MS dereplication has identified isovitexin in the active fraction of FD1c and oleanolic acid, betulin, and lupeol in the active fractions of FD2c. This study was conducted to evaluate the cytotoxic activity of the individual compounds identified through LC-MS dereplication method. SRB staining assay was used for the purpose of this study.

Table 4.13 shows the GI50 of each compound. LC-MS dereplication has identified isovitexin as one of the major compound in the active fraction of FD1c, however the GI50 value for isovitexin is significantly ($P < 0.05$) higher than its respective active fraction ($GI50 = 12 \mu\text{g/ml}$). Similar findings were observed with the GI50 values for oleanolic acid, betulin and lupeol, which were identified through LC-MS dereplication of the active fractions of FD2c. The GI50 values for each of these compounds were significantly ($P < 0.05$) higher than their respective active fractions (FD2c F29-33 = $28 \mu\text{g/ml}$, FD2c F34-36 = $24 \mu\text{g/ml}$). These results indicated that the active fractions of both FD1c and FD2c were more cytotoxic against PC3 cells than each of these individual compounds.

In the case of FD1c, even though isovitexin was identified as the major compounds in the active fraction, lower cytotoxic activity of the compound against PC3 cells might suggest that the other minor compounds such as brosimacutin and aurone flavonoids might have contributed to the more potent cytotoxic activity of the active fraction. In the active fractions of FD2c, oleanolic acid, betulin, and lupeol were dereplicated as the major compounds in the active fraction. However, as mentioned earlier, none of these individual compounds were shown to have better cytotoxic activity than their respective active fractions. Once again, this could suggest that the more potent cytotoxic activity of the active fractions of FD2c might be due to the additives or synergistic effects of the compounds identified in the plant.

Table 4.13 GI50 concentrations (µg/mL) of the compounds identified through LC-MS dereplication determined for PC3 cells as assessed by SRB assays after 72 hours. Each result was obtained in three independent experiments, which was run in triplicate.

Compound	Source of Identification	GI50 (µg/ml) with PC3 cells
Isovitetoxin	FD1c	43
Oleanolic Acid	FD2c	34
Betulin	FD2c	47
Lupeol	FD2c	36

Table 4.14 and Table 4.15 below summarize all the investigations that have been carried out for both the active extracts and the active fractions of both *Marantodes pumilum* and *Ficus deltoidea* plants in this project.

Table 4.14 Summary of all the investigations carried out using the active extracts of *Marantodes pumilum* (n-hexane and chloroform) and *Ficus deltoidea* (chloroform) plants. '↑' Increased, '↓' Decreased '+' characteristic detected, and '−' characteristic not detected

Assay/Sample		MPc	MPh	FD1c	FD2c
Apoptosis	Morphological observation (Characteristics of apoptosis or necrosis)	Apoptosis (+) Necrosis (−)	Apoptosis (+) Necrosis (−)	Apoptosis (+) Necrosis (−)	Apoptosis (+) Necrosis (−)
	DAPI staining	Apoptotic cells visible	Apoptotic cells visible	Apoptotic cells visible	Apoptotic cells visible
	Annexin V-FITC fluorocytometric analysis	↑ Number of cells in early apoptosis			
	Mitochondrial membrane potential (MMP) depolarization analysis	↑ MMP depolarization	↑ MMP depolarization	↑ MMP depolarization	↑ MMP depolarization
	Detection of Caspase 3/7	↑ production of Caspases 3/7	↑ production of Caspases 3/7	↑ production of Caspases 3/7	↑ production of Caspases 3/7
	Nuclear DNA fragmentation analysis	+	+	+	+

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Table 4.14 (Continues)

Assay/Sample		MPc	MPh	FD1c	FD2c
mRNA gene expression analysis	Bcl-2	↓ Expression	↓ Expression	↓ Expression	↓ Expression
	Bax	↑ Expression	↑ Expression	↑ Expression	↑ Expression
	Smac/DIABLO	↑ Expression	↑ Expression	↑ Expression	↑ Expression
Cell cycle analysis		Cell cycle arrest at G2M phase			
mRNA gene expression (ALOX-5)		↓ Expression	No Changes	No changes	No changes
Migration & Invasion	2D Migration study using Platypus cells migration kit		↓ Number of migrated cells	↓ Number of migrated cells	↓ Number of migrated cells
	3D Migration study using modified Boyden Chamber		↓ Number of migrated cells	↓ Number of migrated cells	↓ Number of migrated cells
	3D Invasion study using modified Boyden Chamber		↓ Number of invading cells	↓ Number of invading cells	↓ Number of invading cells
	mRNA gene expression analysis	CXCR4	No Changes	↑ Expression	No Changes
		CXCL12	↓ Expression	↓ Expression	↓ Expression
		VEGF-A	↓ Expression	↓ Expression	↓ Expression
Specificity study using HDFa		G150 >200µg/ml	G150 >200µg/ml	G150 >200µg/ml	G150 >200µg/ml

Table 4.15 Summary of all the investigations carried out using the active fractions of *Marantodes pumilum* and *Ficus deltoidea* plants. '↑' Increased, '↓' Decreased and 'NA' Not Available

Assay/Samples			MPc F30-33	FD1c F43-51	FD2c F29-33	FD2c F34-36
Apoptosis	mRNA gene expression analysis	Bcl-2	↓ Expression	NA	NA	NA
		Bax	↑ Expression	NA	NA	NA
		Smac/DIABLO	↑ Expression	↑ Expression	↑ Expression	↑ Expression
		ALOX-5	↓ Expression			
Migration & Invasion	mRNA gene expression analysis	CXCR4	No Changes	No Changes	No Changes	No Changes
		CXCL12	↓ Expression	↓ Expression	↓ Expression	↓ Expression
		VEGF-A	↓ Expression	↓ Expression	No Changes	No Changes
Specificity study using HDFa			G150 >100µg/ml	G150 >200µg/ml	G150 >200µg/ml	G150 >200µg/ml

4.2 Discussions

Approximately 90% of androgen-dependent prostate cancer is detected in the local and regional stages, therefore the cure rate is very high as nearly 100% of men diagnosed and treated at this stage will be disease free after 5 years. However, many men die as therapy eventually fails when the disease progresses to androgen-independent stage (Feldman and Feldman 2001). This stage of prostate cancer is lethal and has the ability to progress and metastasize. At present, there is no effective therapy that can be used for men diagnosed with androgen-independent prostate cancer. There is a need for chemopreventive products targeting middle-aged men in my country. This is because treatment results are not sustainable for the local health system, which does not cover all the population. Developing such a product from traditional Malay medicinal plants would facilitate better compliance among Malaysian patients as the use of traditional plants and herbs are culturally embedded into the local lifestyle.

The three plants chosen for this study - namely *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var. *pumila*), *Ficus deltoidea* var. *angustifolia* (Miq.) Corner and *Ficus deltoidea* Jack var. *deltoidea* - have shown promising cytotoxicity in a preliminary screening conducted at the start of this study. In natural product research, crude extracts showing a GI50 value of less than 100 µg/mL can be considered to be cytotoxic and selected for further studies, with the most promising ones are those with a GI50 lower than 30 µg/mL (Ab Shukor, Merrina et al. 2008). In the preliminary screenings, three different cell lines have been used (PC3, DU145 and LNCaP) - each representing a different stage of prostate cancer: LNCaP cancer cell line represents the androgen-dependent prostate cancer whereas PC3 and DU145 cancer cell lines represent the androgen-independent prostate cancer (Shafi, Yen et al. 2013). For mechanistic studies, our main focus turns onto PC3 cells, which is a type of androgen-independent prostate cancer cell overexpressing ALOX-5, thus allowing for the measurement of a wider array of potential targets.

From my investigations, the data collected has shown that all of the active plant extracts and their respective fractions were cytotoxic against all three types of prostate cancer cell lines - including PC3 with a very low GI50 value. Al-Mekhlafi, Shaari et al. (2012) described the anti-proliferative properties of *Marantodes pumilum* extracts against prostate cancer cells lines. Here, the mechanistic details of such property are characterised in detail: the active plant extracts induce apoptosis in PC3 cells via the intrinsic pathway, as evidenced by the significant activation of caspases 3 and 7. This activation is mediated –at least in part- by their ability to affect the mRNA gene expression of Bax, Bcl-2, and Smac/DIABLO. These genes are responsible for the production of their respective proteins, which play an important role in the intrinsic pathway of apoptosis as depicted in Figure 4.99.

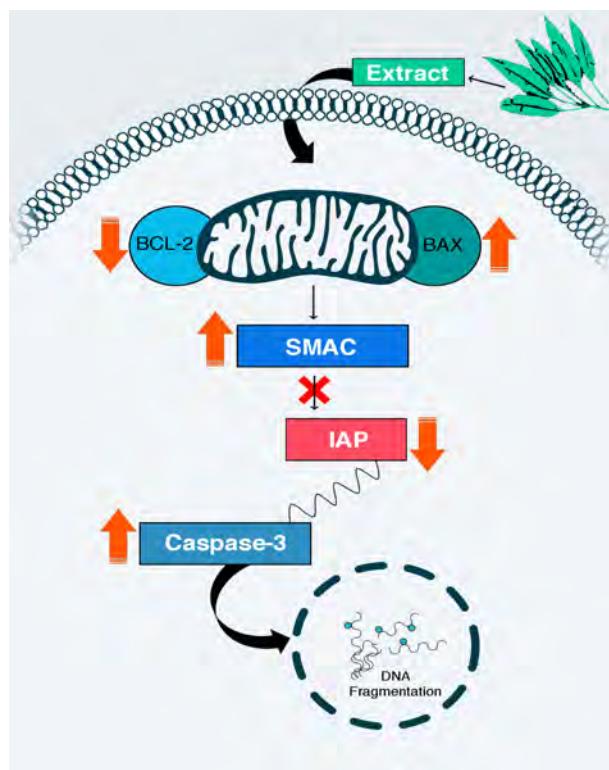


Figure 4.99 Effects of active plant extracts on the intrinsic pathway of apoptosis

However, MPc is also able to induce PC3 cell death via apoptosis in an alternative way, namely the inhibition of mRNA gene expression of ALOX-5, which is responsible for the production of LOX-5 enzyme. According to Ghosh and Myers (1997), the inhibition of 5-LOX would block the production of its metabolites triggering apoptosis in this prostate cancer cell line. In PC3 cells treated with the active extracts of the plants, the cell population in the G0/G1 phase was reduced, whereas cell population in the G2/M phase was increased significantly when compared to the untreated PC3 cells. These data implied that the active plant extracts induced cell cycle arrest at G2/M phase. Many other studies using compounds isolated from plants have reported a similar activity in other types of cancer cell lines including prostate cancer, where they exhibited an anti-proliferative activity such as: 8-O- β -glucopyranoside isolated from *Rumex japonicus* Houtt against A549, (human lung cancer cells) (Xie and Yang 2014); *Scrophularia striata* against Jurkat tumor cells (T cell leukaemia) (Azadmehr, Hajiaghaei et al. 2013); and *Solanum nigrum* against PC3 (prostate cancer cells) (Nawab, Thakur et al. 2012). Cell cycle arrest enables DNA repair to occur, thus preventing replication of the damaged templates (Murray 2004) and cell cycle arrest specifically at the G2M phase serves to prevent the cells from entering the mitosis (M) phase with damaged genomic DNA.

In this study, all anti-proliferative plant extracts (MPc, FD1c and FD2c) were observed to have significant inhibitory effect on the migration and invasion of PC3 cancer cell lines. Further mechanistic studies showed that the active plant extracts and their respective fractions (MPc F30-33, FD1c F43-51, FD2c F29-33 and FD2c F34-36) were able to inhibit both PC3 cells migration and invasion by modulating the CXCR4, CXCL12 and VEGF mRNA gene expression. Recently, increasing evidence indicate that tumor microenvironment including tumor-stromal cells interactions have a crucial role in tumor initiation and progression (Guo, Wang et al. 2015). Singh, Singh et al. (2004) reported that CXCL12-CXCR4 interactions were able to modulate prostate cancer cell migration and invasion. CXCL12 is a type of chemokines that bind to and activate a family of chemokine receptors (Vindrieux, Escobar

et al. 2009). CXCR4 is a type of chemokine receptor that consists of seven-transmembrane receptors coupled to G protein (Ransohoff 2009). CXCL12 and chemokine receptors CXCR4 and CXCR7, are the key factors that link between cancer cells and their microenvironment. Functional CXCR4 was reported to be expressed by prostate cancer cell lines PC3 and LNCaP, as well as normal prostate epithelial cells (PrEC). However, significantly higher levels of CXCR4 were observed in the malignant cell lines such as PC3 and LNCaP compared to the normal prostatic epithelial cells. Previous studies conducted by Singh, Singh et al. (2004) shows that the number of malignant prostate cancer cell lines that migrated in response to CXCL12 was significantly higher than for cells not exposed to CXCL12 as chemoattractant. Therefore, interruption to the level of CXCL12 and disruption to CXCL12-CXCR4 litigation could possibly lead to the inhibition of prostate cancer cells migration and invasion. All the active extract of the plants and their respective fractions were able to down-regulate the CXCL12 mRNA gene expression significantly. This might lead to the fall of the production of CXCL12 chemokines and thus inhibiting both PC3 cells migration and inhibition. However, none of them were able to have any significant effect on the expression of CXCR4.

Even though not all of the active extracts of the plants show any significant effects on the mRNA gene expression of VEGF, two of the active fractions namely MPc F30-33 and FD1c F43-51 had shown significant inhibition to the expression of the gene. Vascular endothelial growth factor (VEGF) is known to be a critical regulator of endothelial cell migration by increasing endothelial cell permeability, stimulating proliferation, and promoting migration of phosphatidylinositol-3-kinase and the small GTPase Rac-1 (Soga, Connolly et al. 2009). Apart from that, VEGF also play a very important role in the formation of new blood vessels from pre-existing capillaries and venules - a process called angiogenesis. Overexpression of VEGF is normally seen in cancer cells, as it is vital to help create the microenvironment that would promote the growth of the cells. Therefore, by

reducing the expression of VEGF, the production of its protein will be decreased and thus creating a microenvironment that is not suitable for the cancer cells to migrate or invade.

Despite the cytotoxic effects of all the active plant extracts and their respective fractions on prostate cancer cell lines, they showed specific selectivity when tested with normal human dermal fibroblast cells (HDFa). Extracts from these plants have been reported to be cytotoxic towards other cancer cell lines but we cannot surmise from the available reports if these extracts show any specific potency to one of them.

Bioguided isolation of MPc F30-33 revealed that the 5-alkylresorcinol **MP-1** was responsible for the cytotoxicity against PC3 prostate cancer cell lines. Al-Mekhlafi, Shaari et al. (2012) isolated two similar cytotoxic compounds against the same cell lines. They were identified as 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl)resorcinol and 1-O-methyl-6-acetoxy-5-pentadecylresorcinol (see Figure 2). However, they did not study their mechanism of action (Al-Mekhlafi, Shaari et al. (2012). Thus, this is the first time a report on the mechanisms of the cytotoxic activity against prostate cancer cell line (PC3) of an alkenylresorcinol isolated from *Marantodes pumilum*. The activity of alkylresorcinol was first described by Anderson, David et al. (1931), after extracting and isolating them from *Ginkgo biloba*. More alkylresorcinols were subsequently isolated from higher plants and also from bacteria, fungi, algae and mosses (Kozubek and Tyman 1999, Zarnowski, Suzuki et al. 2000).

Alkylresorcinol compounds have been reported to show anti-parasitic, anti-fungal, anti-microbial, anti-oxidants, and anti-cancer effects. Their various biological activities have been reviewed comprehensively by Kozubek and

Tyman (1999). The cytotoxic activity of 5-alkylresorcinol compounds on cancer cell lines have been reported on several studies previously such as breast (Chuang and Wu 2007), colon (Al-Mekhlafi, Shaari et al. 2012), leukemia (Arisawa, Ohmura et al. 1989), prostate (Chuang and Wu 2007, Al-Mekhlafi, Shaari et al. 2012), liver (Filip, Anke et al. 2002) and human squamous carcinoma (Buonanno, Quassinti et al. 2008). In this study we have shown that **MP-1** induced prostate cancer cell death via apoptosis instead of necrosis.

In 2008, a resorcinolic lipid known as Climacostol, which is a natural toxin isolated from the freshwater ciliated protozoan *Climacostomum virens*. It effectively inhibits the growth of two tumor cell lines, human squamous carcinoma (A431 cells) and human promyelocytic leukaemia (HL60 cells), in a dose-dependent manner by inducing apoptosis (Buonanno, Quassinti et al. 2008). Another study conducted by Barbini, Lopez et al. (2006) showed that after 24 hours of treatment with 5-alkylresorcinol, the hepatocellular carcinoma cell lines showed the typical morphological characteristics of apoptosis, DNA fragmentation and condensed and fragmented nuclei. Arisawa, Ohmura et al. (1989) has reported the relationship between the cytotoxicity to KB cells and the number of carbon of the side chain of an 5-alkylresorcinol and according to this report, the cytotoxic activity was positively correlated with the number of carbons of the side chain - increasing from 5 to 13; longer side chains becoming detrimental to the effect. The same report also concluded that hydroxylation at C1 and C3 carbons was a structural requirement to enhance the cytotoxicity towards cancer cell lines. Both C1 and C3 in **MP-1** are hydroxylated. Of note, we have also demonstrated that **MP-1** inhibits both prostate cancer cell migration and invasion. No similar report has been found after extensive literature search. Therefore, this is the first time such activity has been documented for this class of compound. **MP-1** maybe used as marker for future standardization of the extract and developed as nutraceuticals. However, before the standardized active extracts could reach the market for human consumption,

they will have to go through different phases of development depending on the regulatory requirements as mentioned in section 2.3.2. The development of MP-1 as a chemoteraphy drug for prostate cancer would have to follow the multiple phases set for drug development.

The LC-MS dereplication of active fractions of *Ficus* spp. identified isovitexin in FD1c F43-51; oleanolic acid, moretenol, betulin, lupenone, and lupeol in FD2c F29-33; and oleanolic acid, and lupenone in FD2c F34-36. Both isovitexin and moretenol have been previously reported to be isolated from *Ficus deltoidea* (Bunawan, Amin et al. 2014). However, this is the first time that oleanolic acid, betulin, lupenone, and lupeol are identified in this species. Oleanolic acid has been identified in *Ficus microcarpa* (Ao, Li et al. 2008), *Ficus hispida* Linn (Joseph and Raj 2011), and *Ficus nervosa* (Chen, Cheng et al. 2010). Betulin was identified in *Ficus foveolata* (Somwong, Suttisri et al. 2013), *Ficus racemosa* (Bopage), and *Ficus carica* L. (Oliveira, Baptista et al. 2012). Lupenone was identified in *Ficus pseudopalma* (Ragasa, Tsai et al. 2009), *Ficus microcarpa* (Kuo and Lin 2004), and *Ficus sycomorus* (Ahmadu, Agunu et al. 2007). While, lupeol has been previously identified in *Ficus pseudopalma* Blanco (Santiago and Mayor 2014), *Ficus odorata* (Tsai, De Castro-Cruz et al. 2012), and *Ficus polita* Vahl (Kuete, Kamga et al. 2011).

All compounds have been reported to show cytotoxic activity against cancer cell lines (Hwang, Chai et al. 2003, Peng, Fan et al. 2006, Jung, Kim et al. 2007, Chaturvedi, Bhui et al. 2008, Yan, Huang et al. 2010). Despite this, only lupeol (Saleem 2009), oleanolic acid (Deeb, Gao et al. 2010) and betulin (Chinthaрапalli, Papineni et al. 2007) were reported to have cytotoxic activity against prostate cancer cell lines. We added to this list for the first time, isovitexin, as one of the compound that play an important role in the cytotoxic activity of *Ficus deltoidea* var. *augustifolia* against prostate cancer cell line. This study also suggests that, moretenol and lupenone showed cytotoxic activity against prostate cancer cell lines. Deeb, Gao et al. (2010)

also claimed that oleanolic acid was able to inhibit growth and induce apoptosis in prostate cancer cells. Apart from that, another report by Saleem, Kweon et al. (2005) stated that lupeol induced cell death via apoptosis in androgen-sensitive prostate cancer cells. A study conducted by Liu, Bi et al. (2016) demonstrated that lupeol inhibited gallbladder carcinoma cell invasion by suppressing the EGFR/MMP-9 signaling pathway. However this study showed that FD1c and FD2c were able to inhibit both prostate cancer cell migration and invasion by modulating the VEGF-CXCR4-CXCL12 axis. None of the compounds identified through the dereplication of FD1c and FD2c were reported previously to show similar activity. However in this study only differential mRNA gene expression of CXCR4, CXCL12 and VEGF-A was carried out. Further investigations are needed to validate the findings. These include western blotting and proteomic studies. Western blot study for example will give information about the expression of the proteins linked with CXCR4, CXCL12 and VEGF-A genes (Mahmood and Yang 2012).

The findings reported in this study establish a pharmacological rationale and inform standardization methods to develop active extracts from the two selected Malaysian *Ficus deltoidea* varieties into nutraceutical applications that may be used as chemopreventives for prostate cancer. Here the complexity and lack of novelty in chemical terms preclude any drug development efforts.

Chapter 5

Conclusions and Future Work

5 Conclusions and Future Work

5.1 Conclusion

This study was conducted to investigate the potential of two species of plants from Malaysia namely *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var. *pumila*) and *Ficus deltoidea* L. against the growth of prostate cancer cells. In order to achieve this objective, this study has been divided into 3 major parts; 1) *In vitro* mechanistic studies using PC3 prostate cancer cell lines, 2) Characterisation of the active extracts, bioguided isolation and identification of bioactive compounds from the plants and 3) Effects of cytotoxic fractions and principles on 2D migration and 3D invasion.

Firstly, this work has thoroughly characterized the *in vitro* mechanisms by which the selected plants have a potential chemopreventative effect against prostate cancer. The *in vitro* cytotoxic activity against both androgen dependent (LNCaP), and non-androgen dependent (DU145 and PC3) human prostate cancer cell lines. This activity is concentrated in the chloroform extracts. The cytotoxic activity was 5 to 10 times more selective towards prostate cancer cells when compared to normal human fibroblasts. Because the non-androgen dependent prostate cancer is nowadays lacking efficient clinical management we decided to carry out mechanistic studies using the highly malignant PC3 cell line. It is now evident that all act by inducing mitochondria-driven (intrinsic pathway) apoptosis characterized by Annexin V expression, MMP depolarization, activation of caspases 3 and 7, and cell cycle arrest at G2/M phase, thus leading to nuclear DNA fragmentation and cell death. The extracts specifically modulate the expression of Bax, Bcl-2, and Smac/Diablo genes, which encode the respective proteins, which together will cause mitochondrial membrane permeabilization, thus compromising cell viability. Additionally, MPc is able to down-regulate the

Alox-5 mRNA gene expression. It is known that PC3 cells are highly dependent on Alox-5 activity (biosynthesis of leukotrienes) for survival.

Secondly, this study has revealed that the PC3 prostate cancer cells inhibitory activity of *Marantodes pumilum* (Blume) Kuntze (MP) is mainly due to the presence of a 5-alkenyl resorcinol. Therefore, this is the first time a report on the mechanism of the cytotoxic activity against prostate cancer cell lines (PC3) of an alkenylresorcinol isolated from *Marantodes pumilum*.has been produced.

In the case of *Ficus deltoidea* var. *angustifolia* (FD1) the active fraction is rich in flavonoids such as isovitexin. In the case of *Ficus deltoidea* var. *deltoidea* (FD2), the active fraction consists of a mixture of terpenes including oleanolic acid, moretenol, betulin, lupenone and lupeol. This is the first time that oleanolic acid, betulin, lupenone, and lupeol were identified in this plant species. In addition, no other report has demonstrated the important role played by isovitexin in the cytotoxic activity of *Ficus deltoidea* var. *angustifolia* against prostate cancer cell line. Regardless the phytochemical differences, both active fractions are able to modulate the expression of Bax, Bcl-2, and Smac/Diablo genes, thus mirroring the crude extracts mechanism of action. However, we cannot rule out that other fractions may also contribute to the overall pro-apoptotic effect of the crude and/or chloroform extract without being endowed with significant direct cytotoxic activity.

In the last part of this study, the effects of cytotoxic extracts on 2D migration and 3D invasion were tested by exclusion assays and modified Boyden chamber, respectively. All active plant extracts inhibited both migration and invasion of PC3 cells (MPc; $P<0.01$, FD1c and FD2c; $P<0.05$). These effects were accompanied by a very significant down-regulation of both

VEGF-A and CXCL-12 gene expressions but not CXCR4 by 2 of the active plant extracts (MPc, and FD1c) and all of their active fractions (MPc F30-33 and FD1c F43-51) respectively. These results, for the first time, have demonstrated that **MP-1**, FD1c and FD2c were able to inhibit both prostate cancer cells migration and invasion. For the case of **MP-1**, this is the first time such activity has been documented for this class of compound. The modulation of the CXCL12-CXCR4 axis plays important role in cancer cells metastasis whilst inhibition of the VEGF-A gene expression could eventually impair cancer cells angiogenesis. However, since only differential mRNA genes expression study was conducted that lead to these findings, more investigations including western blotting and proteomic studies need to be carried out in order to validate and confirm the findings.

In summary, evidence gathered in this study suggests a role for interaction of MPc, FD1c and FD2c in three of the Hallmarks of Cancer in PC3 cells; apoptosis by activating of the intrinsic pathway, inhibition of both migration and invasion by modulating the CXCL12-CXCR4 axis, and inhibiting angiogenesis by modulating VEGF-A expression. The promising results seen in this study warrant further investigation of these plants for a role in the treatment of prostate cancer.

5.2 Future Work

The findings from this study shows promising potential for the development of herbal preparations for prostate health. To this end no further work would be needed because food supplements are not regulated as medicines and only reasonable safety data needs to be provided. However from a manufacturing point of view more work needs to be done to understand if the plants are consistently synthesizing the phytochemicals profile that is involved in the cytotoxic activity year after year and if their geographical precedence is influencing it.

Second, from an academic point of view, it would be highly interesting to see if our *in vitro* data would translate into *in vivo* effects as it is by no means a good representative of how complex the cells in the human body worked. *In vitro* models do not take into account the interactions that occur when cells go through the oncogenic and metastatic process. Therefore, the use of *in vivo* model is very important as cancer cell lines do not and cannot recapitulate the disease processes that occur in living tissues. Mouse and mice models are often used for prostate cancer research. Significant reduction of the tumor size is expected after the treatment with the bioactive compounds from *Marantodes pumilum* (Blume) Kuntze (synonym *Labisia pumila* var. *pumila*) and *Ficus deltoidea* L. plants.

Third, the use of Next Generation Sequencing-based approaches have become very popular recently in the field of medicine. NGS-based approaches have been used in genetic diagnosis, building disease network, pharmacogenomics and drug discovery. One of the predominant use of NGS is by using its RNA-sequencing platform to profile gene expression levels in normal or mutant cells and identify differentially expressed transcripts among group of samples. NGS could also be used to investigate the effect of plant extracts or their active principle on the gene expression profile in specific cells or tissues.

Lastly, it is not yet known whether the identified/dereplicated active principles do directly interact with the therapeutic targets described in this thesis as being the basis of the pro-apoptotic effects of the extracts and fractions.

5.2.1 Future Phytochemical studies of the plants

The plant materials used in this study were mostly collected from farms in southern Malaysia. Since these plants also grow wildly in different parts of the country, it is also interesting to conduct a study, which involves the collection of similar plant materials that grow in the wild or in farms from different part of Malaysia. This study is important as we can develop complete phytochemical profiles, make comparison of these profiles and gain better understanding on the distribution of the phytochemicals available in these plants and whether they are affected by external factors such as locations, weather conditions or the way they are planted.

In Malaysia, *Marantodes pumilum* and *Ficus deltoidea* plants can be found growing wildly in the lowland and hill forests at an altitude of 300 to 700 meters in several states such as Kedah, Perak, Negeri Sembilan, Johor, Pahang, Terengganu and Kelantan. In the last 5 years, some local biotechnology companies have started to do contract farming for several well-known plant species in Malaysia including *Marantodes pumilum* and *Ficus deltoidea*. These agricultural lands or farms are located in different part of peninsular Malaysia including Desaru, Kota Tinggi, Parit Raja, and Dungun. In this study, plant samples will be collected from different part of Malaysia for both farmed and wildly growing plants. These plant samples will be dried and extracted using the same method before phytochemical profiles analysis is carried out using HPLC and mass spectrometry. This analysis will provide vital information regarding the distribution of phytochemicals available in the plant and whether the ways they are planted or geographical location affect the profiles. In addition, this study would also tell us whether the plants are consistently synthesizing the phytochemicals involved in the cytotoxic activity year after year and if their geographical precedence is influencing it.

5.2.2 In vivo study using Prostate cancer xenograft model

As mentioned earlier, *in vivo* study is a very important step in drug discovery. Data from this thesis shows the promising *in vitro* activity of the plant extracts, therefore *in vivo* study using a suitable model must be carried out to see the effect of the active plant extracts on a more complicated organism model such as mice. Non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice (males; 6–8 weeks old; body weight, 23–25 g) will be chosen and are bred in the animal facility at Universiti Sains Malaysia, housed in sterile micro-isolator cages under specific pathogen-free conditions with controlled temperature (20–21 °C) and humidity (50–60%). Daily light cycles will be regulated with 12 hours light and 12 hours dark. Cages will be completely changed once or twice a week. Animals are handled under sterile conditions. The maximum tolerated dose of the plant extracts will be determined using conventional methodology. Animal care and experiments will be carried out in accordance with the guidelines of the Universiti Sains Malaysia ethical and animal care council.

The mice used in this experiment will be transplanted with the LTL-313H, PTEN-deficient, metastatic and PSA-secreting, patient-derived prostate cancer tissue line (Choi, Gout et al. 2012) and maintained as grafts under renal capsules of male NOD-SCID mice supplemented with testosterone (Watahiki, Wang et al. 2011). Tumors will be harvested 10 weeks after grafting and pieces of tumor tissue ($2.5 \times 2.5 \times 1.25 \text{ mm}^3$) are grafted under the renal capsules of 36 testosterone-supplemented male mice (6 groups; 6 mice/group; 4 grafts/mouse). The grafts are expected to have 100% engraftment rate with an average tumor volume doubling time of 13–15 days. The rise in the plasma PSA levels of the mice will be used as an indicator for tumor growth. When the level of plasma PSA reaches 12 ng PSA/ml, i.e. equivalent to tumor volumes of 30–50 mm^3 , the mice will be randomly distributed into 6 groups and treated with the active plant extracts orally. The mice will be euthanized after 3 weeks and tumor volumes will be measured using callipers and tissue sections prepared for histopathological analysis.

Tumor growth of treated animals relative to untreated animals will be used as a measure of antitumor activity using the following formula;

$T/C = (treated\ tumor\ volume_{3wks} - treated\ tumor\ volume_{0wks}) / (control\ tumor\ volume_{3wks} - control\ tumor\ volume_{0wks}) \times 100\%$, with $T/C > 0$ indicating tumor growth and $T/C < 0$ indicating tumor shrinkage. Tumor growth inhibition = $100\% - T/C$.

5.2.3 Transcriptomics using Next Generation Sequencing (NGS)

Next generation sequencing-based transcriptome analysis (RNA sequencing) has enabled the profiling of global patterns of expression of distinct RNA species including mRNA, miRNA, lncRNA and tRNA. These RNA species are important as they perform unique functions in a cell or tissue during the development of disease or pathogenesis (Chaitankar, Karakülah et al. 2016). A process called temporal transcriptome, which involved the profiling of normal and mutant cells, could help in identifying genetic changes that lead to cellular dysfunction and elucidate gene networks associated with homeostasis and disease (Lander 2011). Dramatic reduction in cost and increased throughput of screening has led to the widespread use of NGS in genomic analysis.

The use of NGS technology to profile gene expression level and identify differentially expressed transcripts among group of samples could provide important information about the effect of a certain drug or plant extracts on the gene expression in normal or mutant cells. The genomic data collected from NGS will lead to systemic level approaches for quantitative analysis of the dynamic interplay of molecules within a specific cells or tissues. With this in mind, we have collaborated with UCL Genomic to use the Illumina MiSeq facility available at their disposal to investigate the effect of the active plant extracts on the gene expression profile in PC3 cells. Table 5.2

below showed the results for the differential gene expression in PC3 cells when cells treated with MPc was compared with untreated cells (Control). Figure 5.1 showed the MA plot where Robust Multiarray Average (RMA) normalized data of all the possible comparisons between the pairwise log2 intensities of the samples. This study will be completed once the effect of the active principles, which are isolated and identified in the plant, on the gene expression profile in PC3 has been done in the future. This study will be carried out in collaboration with Malaysia Genome Institute and once the study is completed the results will be published as soon as possible.

Table 5.1 Differential gene expression in PC3 cells analysed using Illumina MiSeq RNA sequencing

Annotation Gene Count	26,364
Assessed Gene Count	12,710
Differentially Expressed Gene Count	9,238

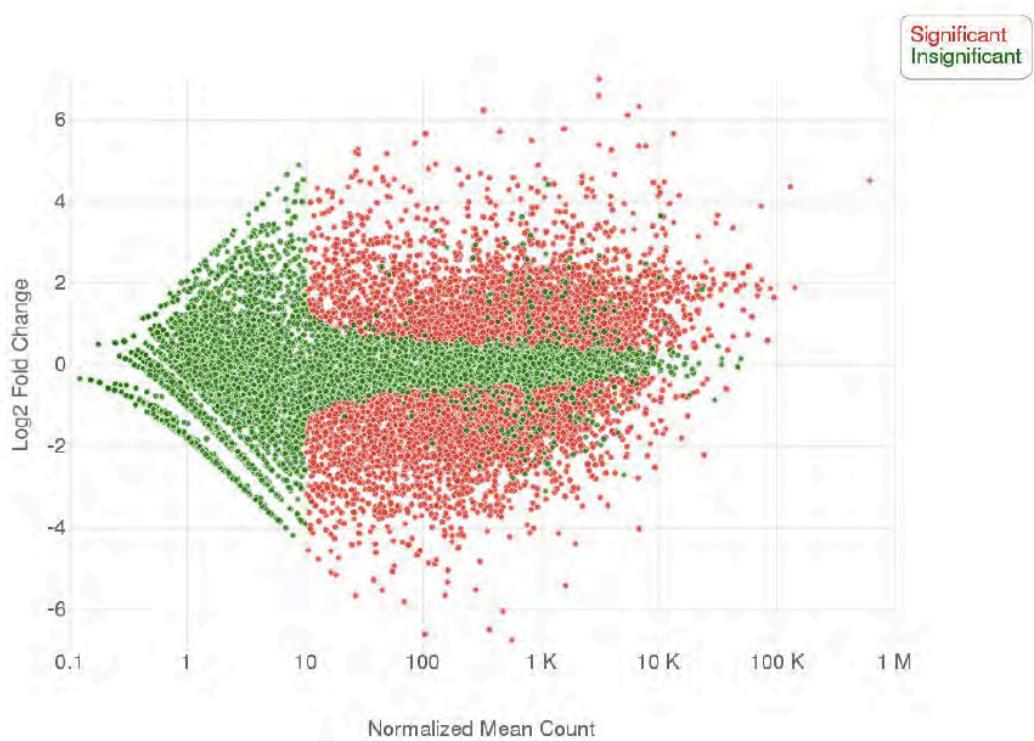


Figure 5.1 MA plot showing the RMA normalised data of all the possible comparisons between the pairwise log2 intensities of the samples

5.2.4 In silico studies of selected phytochemicals

We have characterised for the first time, the mechanism of actions that lead to PC3 cancer cells death and inhibition of PC3 cells migration, invasion and angiogenesis by the bioactive compounds isolated and identified from MP, FD1 and FD2 plants through *in vitro* mechanistic studies. However, we have yet to characterize the interactions between the active compounds with the therapeutic targets. The effects of these compounds are normally initiated by direct interactions with target proteins. *In silico* docking techniques can be used to generate a three-dimensional (3D) structure of the protein and investigate their interactions at molecular level. Docking software such as Autodock, GOLD and Glide can be used for this study (Verdonk, Cole et al. 2003, Friesner, Murphy et al. 2006, Morris, Huey et al. 2009). This will enable us to understand the complementarity at the molecular level of a ligand (bioactive compound) and a protein target.

These are some of the studies that need to be conducted before the output of this study can be used for human application. Data collected from these studies will provide more information and contribute towards future development of these active plant extracts.

Chapter 6

References

6 References

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Chapter 7

Appendix

7 Appendix

Appendix 1



A Foundation of the Society for Medicinal Plant and Natural Product Research (GA)
Eine Stiftung der Gesellschaft für Arzneipflanzen und Naturstoffforschung (GA)

Mr. Mohd Mukrish bin Mohd Hanafi
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Chairman:
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1st Research Award of the Foundation Plants for Health

13.09.2016

Dear Mr. Mohd Mukrish bin Mohd Hanafi,

Thank you very much for your application for the 1st Research Award of the Foundation Plants for Health.

We are happy to inform you, that among several excellent applications, your and one other proposal have been selected by the Board of the Foundation Plants for Health for the **Research Award of the Foundation Plants for Health** in 2016.

By the award, the foundation **Plants for Health** wants to provide start-up funds, which help to develop projects leading to promising developments in the area of medicinal plant and natural product research including the search for innovative lead compounds, exciting product ideas and novel pharmacological, clinical and other approaches in medicinal plant and natural product research. The grant is intended to support visits of collaborative labs for performing experiments or learning innovative techniques.

The foundation **Plants for Health** will provide you a research subsidy of 1.500 € to initiate a research collaboration with Prof Jean-Luc Wolfender of University of Geneva in order to gain more understanding and learn new methods and techniques in plant metabolomics research.

Please contact our treasurer Dr. Bernd Röther at bernd@roether.org to arrange transfer of the money.

We are expecting you to report to the foundation about the outcome of your collaborative research until 31st of August 2017, and to present results at the GA meeting in Basel.

Wishing you a successful project!

Best regards,

A handwritten signature in blue ink, appearing to read "R. Bauer".

Prof. Dr. Rudolf Bauer
Chairman of the Foundation Plants for Health