CYTOTOXIC AND APOPTOTIC ACTIVITIES IN SELECTED PHYLLANTHACEAE SPECIES OF MALAYSIA

SUJATHA A/P RAMASAMY

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2012

CYTOTOXIC AND APOPTOTIC ACTIVITIES IN SELECTED PHYLLANTHACEAE SPECIES OF MALAYSIA

SUJATHA A/P RAMASAMY

THESIS SUBMITTED IN FULLFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2012

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate	:	Sujatha a/p Ramasamy
Registration/Matric No	:	SHC 070024
Name of Degree	:	Doctor of Philosophy (PhD.)
Title of Dissertation ("this Work)	:	

CYTOTOXIC AND APOPTOTIC ACTIVITIES IN SELECTED PHYLLANTHACEAE SPECIES OF MALAYSIA

Field of Study	:	Bioactivity of Natural Product

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;

(2) This Work is original;

(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledge in this Work;

(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;

(5) I hereby assign all and every right in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;

(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subjected to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

ABSTRACT

Phyllanthaceae species have been extensively used in folk medicine in most tropical and subtropical countries for thousand of years. However, there is a paucity of information on the cytotoxic properties of these plants. Therefore, the present study was undertaken to evaluate the cytotoxic and apoptotic activity of crude methanol (CME), hexane (CHE) and ethyl acetate (CEE) extracts of the selected Phyllanthaceae species collected from different parts of Peninsular Malaysia, namely *Phyllanthus niruri* (dukong anak), *P. pectinatus* (Pokok Melaka), *P. acidus* (cermai), *P. roseus* (Labu Kuncing), *P. watsonii* and *Baccaurea motleyana* (rambai). Cytotoxic activities were screened using an *in vitro* assay system of growth inhibition against four human cancer cell lines, namely breast cancer cells (MCF7), ovarian cancer cells (SKOV3), epidermal carcinoma of cervix cells (CaSki), colon cancer cells (HT29), and one normal lung fibroblast cells (MRC5). CME and CEE of *P. pectinatus* (leaves) exhibited potent cytotoxic activity against SKOV3 cells with IC_{50} values of 4.8 ± 1.04 and 5.8 ± 0.76 μg/ml, respectively. CEE of *P. pectinatus* (fruit) exhibited strong cytotoxicity on MCF7 and CaSki cells with IC_{50} values of 18.1 ± 0.66 and 19.4 ± 0.53 μg/ml, respectively. CHE of *P. watsonii* exhibited strong cytotoxicity with IC_{50} values of 7.9 ± 0.60, 5.8 ± 0.29, 6.9 ± 0.96 and 11.8 ± 1.61 μg/ml on MCF7, SKOV3, CaSki and HT29 cells, respectively. CEE of *P. watsonii* demonstrated the potent cytotoxicity with an IC_{50} value of 3.6 ± 1.01 μg/ml on CaSki cells, as well as on HT29 and SKOV3 cells with IC_{50} values of 5.1 ± 0.36 and 5.5 ± 0.50 μg/ml, respectively. CHE of *P. watsonii* was further subjected for bioassay-guided fractionation and yielded 10 fractions (PW1 – PW10). PW4 – PW8 portraying a stronger cytotoxic activity against MCF7, SKOV3, CaSki and HT29 cells and was further subjected for bioassay-guided fractionation and this

resulted in 8 fractions (PPW1 – PPW8). Cytotoxic activity of fraction PPW7 on MCF7, SKOV3, CaSki and HT29 cells were more active with IC_{50} values of 0.9 ± 0.06, 0.7 ± 0.06, 0.8 ± 0.00 and 0.8 ± 0.10 μg/ml. Cytotoxic activity of CME, CHE and CEE of *P. watsonii* and fraction PPW7 were shown to be quite selective for cancer cells with selectivity index ranging from 4.4 to 14.6. Fraction PPW7 was subjected to LC-MS/MS analysis and six main compounds were identified. The compounds detected were ellagic acid, geranic acid, glochidone, betulin, phyllanthin and sterol glucoside. Marked morphological changes, ladder-like appearance of DNA and increment in caspase-3 activity indicating of apoptosis were clearly observed in MCF7, SKOV3, CaSki and HT29 cells-treated with cytotoxically active crude extracts of *P. pectinatus* (leaves and fruits), and *P. watsonii*, and fractions PPW6 and PPW7. It was also observed that CHE of *P. watsonii* arrested MCF7, SKOV3 and CaSki cells at G_0/G_1- S, S-G_2/M and G_0/G_1- G_2/M phases, and fraction PPW7 arrested SKOV3 cells at S and G_2/M phases. Cytotoxic activity of endemic *P. watsonii* collected directly from Endau-Rompin Park, Johor against MCF7, SKOV3, CaSki and HT29 cells were investigated for the first time. These results demonstrated that *P. watsonii* has strong cytotoxic effect by inducing apoptotic cell death, increasing caspase-3 activity, and causing arrest of cancer cells at different growth phases. Hence, *P. watsonii* has the potential to be further exploited for the discovery and development for new anticancer pharmaceuticals.

ABSTRAK

Spesies Phyllanthaceae telah lama digunakan secara intensif dalam perubatan tradisional di kebanyakan negara tropika dan subtropika selama beribu-ribu tahun. Walaubagaimanapun, maklumat mengenai nilai sitotoksik spesies Phyllanthaceae ini masih kurang. Oleh sebab itu, kajian ini telah dijalankan untuk menilai aktiviti sitotoksik and apoptotik ekstrak mentah methanol (CME), heksane (CHE) dan etil asetat (CEE) bagi spesies Phyllanthaceae daripada enam spesies Phyllanthaceae yang dikutip dari kawasan-kawasan berlainan di Semenanjung Malaysia iaitu, *Phyllanthus niruri* (dukong anak), *P. pectinatus* (Pokok Melaka), *P. acidus* (cermai), *P. roseus* (Labu Kuncing), *P. watsonii* dan *Baccaurea motleyana* (rambai). Penyaringan aktiviti sitotoksik telah dijalankan menggunakan sistem asei perencatan pertumbuhan secara *in vitro* ke atas empat leluhur sel karsinoma manusia, iaitu sel kanser payudara (MCF7), sel kanser ovari (SKOV3), sel karsinoma epidermal serviks (CaSki), sel kanser kolon (HT29), dan sel normal fibroblas paru-paru (MRC5). CME dan CEE bagi spesies *P. pectinatus* (daun) mempamerkan kesan sitotoksik yang kuat ke atas SKOV3 dengan nilai IC_{50} sebanyak 4.8 ± 1.04 dan 5.8 ± 0.76 μg/ml, masing-masing. CEE dari *P. pectinatus* (buah) mempamerkan aktiviti sitotoksik yang kuat ke atas sel MCF7 dan CaSki dengan nilai IC_{50} sebanyak 18.1 ± 0.66 dan 19.4 ± 0.53 μg/ml. CHE dari *P. watsonii* mempamerkan sitotoksiti yang kuat dengan nilai IC_{50} sebanyak 7.9 ± 0.60, 5.8 ± 0.29, 6.9 ± 0.96 dan 11.8 ± 1.61 μg/ml ke atas sel MCF7, SKOV3, CaSki dan HT29, masing-masing. CEE dari *P. watsonii* menunjukkan aktiviti sitotoksik yang paling baik dengan nilai IC_{50} sebanyak 3.6 ± 1.01 μg/ml ke atas sel CaSki, begitu juga ke atas sel HT29 dan SKOV3 dengan nilai IC_{50} sebanyak 5.1 ± 0.36 and 5.5 ± 0.50 μg/ml, masing-masing. CHE dari *P. watsonii* disubjekkan ke atas pencerakinan bioasei secara pemanduan

dan menghasilkan 10 fraksi (PW1 – PW10). PW4 – PW8 mempamerkan aktiviti sitoktoksik yang paling kuat ke atas sel MCF7, SKOV3, CaSki and HT29 disubjekkan ke atas pencerakinan bioasei secara pemanduan dengan lebih lanjut dan menghasilkan pencerakinan sebanyak 8 fraksi (PPW1 – PPW8). Aktiviti sitoksoksik fraksi PPW7 ke atas sel MCF7, SKOV3, CaSki dan HT29 adalah paling mujarab dengan nilai IC_{50} sebanyak 0.9 ± 0.06, 0.7 ± 0.06, 0.8 ± 0.00 dan 0.8 ± 0.10 μg/ml. Ciri sitoktoksik bagi CME, CHE dan CEE bagi *P. watsonii* dan fraksi PPW7 menunjukkan selektiviti ke atas kanser sel dengan indeks selektiviti berjulat dari 4.4 ke 14.6. Fraksi PPW7 disubjekkan ke atas analisa LC-MS/MS dan enam sebatian utama dapat dikenalpasti. Sebatian-sebatian yang dikesan adalah asid ellagic, asid geraiinic, glochidone, betulin, phyllanthin dan sterol glukosida. Perubahan ciri-ciri morfologi, DNA kelihatan seperti tangga dan peningkatan dalam aktiviti caspase-3 yang menandakan apoptosis sel dapat dilihat dengan jelas di dalam sel MCF7, SKOV3, CaSki dan HT29 yang dirawat dengan ekstrak mentah yang aktif dalam sitotoksik dari *P. pectinatus* (daun dan buah) dan fraksi PPW6 dan PPW7. Ia juga didapati bahawa CHE dari *P. watsonii* merencat sel MCF7, SKOV3 dan CaSki pada fasa G_0/G_1- S, S-G_2/M dan G_0/G_1- G_2/M, dan fraksi PPW7 merencat sel SKOV3 pada fasa S dan G_2/M. Aktiviti sitotoksik bagi *P. watsonii* yang endemic dan dikutip secara terus dari Taman Negara Endau-Rompin, Johor ke atas sel MCF7, SKOV3, CaSki dan HT29 dikaji buat kali pertama. Kajian menunjukkan bahawa *P. watsonii* mempunyai kesan sitotoksik yang kuat dengan meningkatkan kematian sel secara apoptosis, meningkatkan pengaktifan caspase-3, dan menyebabkan perencatan sel kanser pada fasa berbeza. Dengan itu, *P. watsonii* berpotensi untuk dieksploitasikan selanjutnya dalam pencarian dan pembangunan antikanser farmaseutikal yang baru.

ACKNOWLEDGEMENTS

Firstly and foremost I would love to acknowledge my utmost appreciation to my supervisors Professor Datin Dr. Norhanom Abd. Wahab from the Institute of Graduate Studies and Associate Prof. Dr. Nurhayati Datuk Hj Zainal Abidin from the Institute of Biological Sciences for their guidance and constant support throughout the entire course of this study.

Special thanks to Dr. Sugumaran Manickam from Rimba Ilmu, University of Malaya for helping in the plant collection and guiding me in the identification of the Phyllanthacae species and to Prof. Zubaidah from Hematology Department, Institute of Medical Research (IMR) for allowing me to use the FACS Calibur unit. I am very grateful to the Johor National Parks Corporation for their permission on the collection of Phyllanthus watsonii.

I am grateful to my friends Syarifah Nur, Lai Li Kuan, Veronica Alicia Yap, Law Ing Kin, Sim Kae Shin, Rebecca Ng, Chew Ann Jee, Jaime Stella, Lee Guan Serm and Hong Sok Lai from Molecular Biology Lab (B503) for their invaluable assistance, patience, encouragements, and contributions towards this project and for making my years enjoyable and memorable. Thank you for the inspiration and for the lovely working environment in the lab.

I would also love to express my heartfelt gratitude to Dr Murugan Kalimutho, Mastura Malek, Wong Kah Hui, IPS Mycology Lab members and to all my friends who

took time out of their schedule to guide and share their views and comments on my research. To those whom I failed to mention but have helped in one way or another, May God blessed them.

I would like to express my special appreciation to my beloved parents, sisters and brothers in law, R.Latha - Loganathan, R.Kavitha - Kurunathan and R.Geetha for their love, support and constant encouragements that inspired me to accomplish this study. Last but not least, special thanks to my lovely husband, Vishnu Ramanathan for his patience and continued support.

R.Sujatha
umkl 2012

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
µl	microlitre
µm	micrometer
µg/ml	microgram per millilitre
µM	micromolar
ACE	angiotensin-converting enzyme
AFRO	World Health Organization Regional Office for Africa
AIDS	Acquired Immunodeficiency Syndrome
AO	acridine orange
Apaf-1	apoptotic protease activating factor 1
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
β	beta
bp	base pair
CDK	cyclin-dependent kinase
Cl^-	chlorine
cm	centimetre
CO_2	carbon dioxide
CoA	coenzyme A
CPP	cysteine protease
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DED	Death effectors domain
DFF	DNA fragmentation factor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EB	ethidium bromide
ED	effective dose
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISA	enzyme-linked immunosorbent assay
EMRO	European Media Research Organization
EndoG	endonuclease G
ER	endoplasmic reticulum
EtOAc	ethyl acetate
FADD	Fas-associated protein with death domain
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GAE	gallic acid equivalent
h	hour

HEPES	N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonoc
HIV	Human Immunodeficiency Virus
HMW	high molecular weight
HPV	Human Papillomavirus
IC	inhibition concentration
id	internal diameter
K^+	kalium
kbp	kilo-base pair
Kcal	kilocalorie
kg	kilogram
L	litre
LC-MS	liquid chromatography-mass spectrometry
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LMW	low molecular weight
LPO	lipid peroxidation
Me_2CO	acetone
MEM	Minimum Essential Medium Eagle
MeOH	methanol
mg	milligram
mg/ml	milligram per litre
min	minute
ml	mililitre
mm	millimetre
MS-MS	mass spectrometers
MTT	methyl tetrazolium
Na^+	sodium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
ND	not determined
nm	nanometer
NR	Neutral Red
^oC	degree Celsius
OD	optical density
PAP	Papanicolaou
PARP	Poly(ADP-ribose) polymerase
PBS	phosphate buffer saline
PDA	photodiode array
pNa	*p*-nitroanilide
ppm	parts per million
RNA	ribonucleic acid
rpm	rotation per minute
RPMI	Rosewell Park Memorial Institute
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SEARO	South East Asia Region
SI	selectivity index
spp	species
SRB	sulforhodamine B

t	time
TLC	thin layer chromatography
TNF	tumour necrosis factor
TUNEL	triphsphate-biotin nick end labelling assay
UV	ultraviolet
v	velocity
v/v	volume per volume
VLDL	very low-density lipoprotein
w/v	weight per volume
w/w	weight per weight
WHO	World Health Organization
WPRO	World Health Organization Western Pacific Region

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

There is a long history in the usage of medicinal plants in Southeast Asian countries, some of which have proven to be useful to human as pharmaceuticals drugs (Burkhill, 1966; Muhamad and Mustafa, 1998). The specific plants to be used and the methods of application for particular ailments were passed down through oral history. Many tropical plants have also been reported to possess interesting biological activities with potential therapeutic applications (Hu *et al*., 2009).

In the more recent history, the use of plants as medicine has involved the isolation and characterization of pharmacologically active compounds (Balunas and Kinghorn, 2005). Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs and active ingredients in the pharmaceutical industry (Prasanna *et al*., 2009). Plant-derived natural products still remain as an essential component in the search for medicines and continue to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain (Balunas and Kinghorn, 2005).

Cancer is one of the leading cause of death in both developed and under developed countries and is therefore of universal concern. Chemotherapy is an important option in modern cancer treatment, and many clinically available anticancer drugs, synthetic or of natural origin, is currently used to treat many types of cancer (Pan *et al*., 2010).

At present, the cancer treatment by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of most cancers (Moongkarndi *et al*., 2004). In recent years, considerable attention has been focused on

identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing the process of multistage carcinogenesis (Militão *et al*., 2006) with minimal side effects. The search for new anticancer agents from plant sources is one of the realistic and promising approaches in the field of cancer chemoprevention and this led to the discovery of many novel anticancer drugs, including taxol (Rowinsky *et al*., 1992), camptothecins (Potmesil, 1994), podophyllotoxins (Loike and Horwitz, 1976), vinblastine and vincristine (Mangeney *et al*., 1979).

Apoptosis is a highly regulated process that occurs in almost all living cells and plays an important function in removing unnecessary, aged, and damaged cells. It involves the activation of a series of molecular events that is characterized by cellular, morphological and biochemical changes ultimately leading to cell death. The changes include cell shrinkage, chromatin condensation and nuclear fragmentation, membrane blebbing, caspase activation, and the formation of membrane bound vesicles termed as apoptotic bodies (Wen *et al*., 2006). The apoptotic pathway has caught the interest of researchers worldwide because of its impairment which may result in cancer (Miller and Marx, 1998).

During the last few decades, it has been shown that the mechanism of action of many anticancer drugs is based on apoptosis induction, and thus opening a new strategy in the search of anticancer drugs (Kamesaki, 1998; Panchal, 1998). As induction of apoptosis is a highly desirable goal of preventive strategies for cancer control, it is therefore important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them (Taraphdar *et al*., 2001) by evaluating the cytotoxicity and apoptosis induction in cancer cell lines before whole animal studies or clinical trials begin.

Plant secondary metabolites such as terpenoids, carortenoids, flavonoids, phenolic compounds, and other groups of compounds have shown promise in suppressing

experimental carcinogenesis in various organs (Rabi and Bishayee, 2008) and are also good sources of well-characterized bioactive compounds (Le Marchand, 2002). Many chemopreventive agents derived from plants are believed to suppress the promotion and/or progressions of premalignant cells by modulating cell proliferation and/or differentiation (Wattenberg, 1995; Hong and Sporn, 1997). Considerable efforts are continuously underway for the production of many other higher value anticancer compounds.

Phyllanthaceae are a morphologically diverse pantropical family consisting of about 2000 species with 60 genera (Samuel *et al*., 2005) and have long been used in folk medicine in Malaysia to treat kidney and urinary bladder disturbances, intestinal infections, diabetes and hepatitis B (Burkill, 1966; Calixto *et al*., 1998; Kumaran and Karunakaran, 2007). Although Phyllanthaceae species have long been used as traditional medicines, very few authentic scientific studies in the field of cancer therapy have been carried out. Recent studies have shown that some of the constituents from Phyllanthaceae species have a wide range of biological activities. Jose *et al*., 2001 reported on the antitumour activity of the aqueous extract of *Emblica officinalis* where it was found to be cytotoxic to L929 cells in culture in a dose dependent manner and significantly reduced solid tumours in mice induced by Daltons lymphoma ascites cells (DLA) cells. Studies by Rajeshkumar *et al*., 2002 showed that the aqueous extract of *Phyllanthus amarus* exhibited potent anticarcinogenic activity against 20-methyl-cholanthrene (20-MC) induced sarcoma development and increased the survival of tumour harbouring mice. N-hexane extract of *Phyllanthus niruri* showed a good antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Gunawan *et al*., 2008) and extract of *Phyllanthus urinaria* exerted *in vivo* antiangiogenic effect in tumour developed in animal model, which implicated the potential antiangiogenic effect (Huang *et al*., 2006). Antioxidant effect of water extract of *Phyllanthus amarus* by healing of the indomethacin induced gastric ulcer in rat was

reported by Prakrashi *et al*., (2003). Phenolic compounds of *P. emblica* showed strongest antioxidant activities in scavenging DPPH radicals, superoxide anion radicals and reported to have highest reducing power capacity (Liu *et al*., 2008). Terpenes isolated from *P. oxyphyllus* root also exhibited strong radical scavenging activity towards DPPH radical (Sutthivaiyakit *et al*., 2003). Aqueous extract of *P. orbicularis* decreases the mutagenesis induced by H_2O_2 in bacterial cells, which indicated its antimutagenic activity (Ferrer *et al*., 2002). Flavonones isolated from the hexane extract of *P. niruri* exhibited nematicidal activity against *Meloidogyne incognita* and *Rotylenchulus reniformis* (Shakil *et al*., 2008).

Hence, the main objective of the present study is to evaluate the cytotoxic and apoptotic activity of selected Phyllanthaceae species grown in Malaysia and to characterize the signaling pathway associated with the cytotoxic effect in the active crude extract(s) and isolated fraction(s). Several reasons contribute to the selection of these plants in our studies, such as: (1) their greater distribution in many tropical and subtropical countries; (2) the great number of reports on the potential of the extracts and compounds derived from Phyllanthaceae species in suppressing experimental carcinogenesis in various organs and cells; (3) their broad therapeutic use in folk medicine; and (4) the greater diversity of organic compounds of medicinal interest isolated and characterized from Phyllanthaceae species, including alkaloids, flavonoids, lactones, steroids, terpenoids, lignans and tannins.

In our search for plant-derived natural products with cytotoxic and apoptotic activities six different species from the family of Phyllanthaceae, namely *Phyllanthus niruri*, *Phyllanthus pectinatus*, *Phylanthus acidus*, *Phyllanthus roseus*, *Phyllanthus watsonii* and *Baccaurea motleyana* were selected. Numerous pharmacological effects of *P. niruri*, such as antibacterial, anti-inflammatory, antihepatoxic, anticancer, antioxidant and antiviral activities have largely been documented. A lot of studies been carried out on phytochemical and pharmaceutical aspect of Pokok Melaka (local name referring to

P. emblica and *P. pectinatus*) in Malaysia, but this is the first study on the biological investigation reported with the taxonomy name of *P. pectinatus*. The leaves of *P. acidus* and *B. motleyana* have not been previously investigated for their cytotoxicity, although they have been numerous reports on their antioxidant and antimicrobial potency. A literature survey revealed that there had been no detailed biological pharmacological investigation described on *P. roseus* and there is limited published information on the bioactivity of *P. watsonii*, particularly on cytotoxicity.

The specific objectives of the present study are as follows:

1. to screen for the cytotoxic activity of the crude methanol, hexane and ethyl acetate extracts of six Phyllanthaceae species by an *in vitro* growth inhibition assay system against human breast cancer cells (MCF7), ovarian cancer cells (SKOV3), cervical cancer cells (CaSki) and colon cancer cells (HT29),
2. to evaluate the cytotoxocity of the fractions isolated from crude hexane extracts of *Phyllanthus watsonii* by bioassay guided fractionation approach,
3. to determine the major chemical constituents of the cytotoxically active and specific fraction from *Phyllanthus watsonii* in relation to their cytotoxic activity by LC-MS/MS system,
4. to analyze the potential apoptotic activity in cytotoxically active crude extract(s) and fraction(s) associated with their cytotoxic effect by various morphological and biochemical methods,
5. to investigate the possibility of the apoptotic cell death induction being mediated via cell cycle arrest at the G_0/G_1, S, or G_2/M phase by flow cytometry.

CHAPTER 2

LITERATURE REVIEW

2.1 Tropical Plants and Therapeutic Applications

Since the beginning of life, plants have served humankind as source of fuels, foods, clothing, shelter and medicines. Plants contain numerous chemicals as a result of their natural metabolic activities. These chemicals may be essential for plant growth or as by-products of its metabolism; they may also be potentially useful as food or as medicine (Jamia, 2006).

Until today, approximately 60 % of the world's population still relies almost entirely on plants for medication and they continue to provide mankind with new remedies (Harvey, 2000; Heinrich, 2010). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations. The specific plants to be used and the methods of application for particular ailments were passed down through oral history (Balunas and Kinghorn, 2005).

In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Balunas and Kinghorn, 2005). Due to their large biological activities, many modern drugs with plant origin have been discovered following folklore claims of their efficacy combined with the extensive ethno botanical knowledge of local peoples.

There are four basic ways in which plants that are used by tribal peoples are valuable for modern medicine: (1) plants from the tropics are sometimes used as sources of direct therapeutic agents (2) tropical plants are also used as sources of starting points for

the elaboration of semi-synthetic compounds (3) flora from the tropics can serve as sources of substances that can be used as models for new synthetic compounds (4) plants can also be used as taxanomic markers for the discovery of new compounds (Fakim, 2006).

Only a portion of the world's biodiversity has been explored and there are still many areas of the world and different habitats in which new and unusual biodiversity are yet to be discovered (Harvey, 2000). Scientific expedition have been carried out aggressively to explore rare environments, particularly diverse environment such as the tropical rainforests, where new species of higher plants are being encountered continually (McChesney *et al*., 2007).

There are approximately estimated to be 300,000 species of higher plants in the terrestrial habitats of the world (Samy *et al*., 2005; McChesney *et al*., 2007), with tropical rainforest housing the largest number of these plants (Samy *et al*., 2005). Approximately half (125,000) of the flowering plant species inhabit the tropical rainforest (Fakim, 2006). About 1 % of 3,000 of these higher plants have been utilized for food and out of this about 150 have been commercially cultivated throughout the world. On the other hand, approximately 10,000 of the plants have been documented for medicinal use (McChesney *et al*., 2007).

Southeast Asian tropical rainforests supported approximately 6,500 medicinal plant species with 1,800 plant species found in the Malaysian rainforests and 10 % of them reported to have some medicinal value (Samy *et al*., 2005; Fakim, 2006). As Malaysia is rich in natural resources, plants have played a major role in treating illness in Malaysian traditional medicines for many years (Muhamad and Mustafa, 1998). Some examples of popular Malaysian plants that been studied for its bioactive properties are shown in Table 2.1.

Isolation and characterization of pharmacologically active compounds from medicinal plants still continue until today. Drug discovery from medicinal plants has evolved to include numerous fields of inquiry, various methods of analysis and expertise such as botanist, ethnobotanist, ethnopharmacologist, or plant ecologist. Selection of plants may involves species with known biological activity for which active compound(s) have not been isolated (e.g., traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program (Baker *et al*., 1995).

Today, plants continue to retain their historical significance as important sources of medicinal agents (Balandrin *et al*., 1993) and will continue to be one of the best and most effective sources used to develop new plant-derived compounds as clinical candidates for new world-class medicines (Itokawa *et al*., 2008).

Table 2.1: Bioactivities of selected Malaysian plants

Plant species	Local name	Bioactivity
Alpinia galanga	Lengkuas	SRB cytotoxicity assay against two human cancer cell lines, COR L23 lung cancer cell line and MCF7 breast cancer cell line (Lee and Houghton, 2005).
Alpinia officinarum	Galangal	SRB cytotoxicity assay against two human cancer cell lines, COR L23 lung cancer cell line and MCF7 breast cancer cell line (Lee and Houghton, 2005).
Andrographis paniculata	Pokok cerita, hempedu bumi	*In vitro* and *in vivo* antimalarial activity on *Plasmodium falciparum* (Nik Najib *et al*., 1999). Antihyperglycaemic activity, particularly on reducing blood glucose level in hyperglycaemic rats (Husen *et al*., 2004).
Arecha catechu	Pokok pinang	Antinematodal activity against *Burshaphelenchus xylophilus* using a fungal-feeding assay (Mackeen *et al*., 1997).
Chasalia chartacea	Beras-beras	Nitric oxide inhibitory activity (Saha *et al*., 2004)
Curcuma domestica	Kunyit, temu kunyit	Antitumour activity using the short assay of inhibition of 12-*O*-hexadecanoylphorbol-13-acetate (HPA)-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells (Vimala *et al*., 1999).
Curcuma xanthorrhiza	Temulawak	Antitumour activity using the short assay of inhibition of 12-*O*-hexadecanoylphorbol-13-acetate (HPA)-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells (Vimala *et al*., 1999).
Eurycoma longifolia Jack	Tongkat Ali, bedara pahit, bedara putih; lempedu pahit, bidara laut	*In vitro* antitumour promoting and anti parasitic activity (Jiwajinda *et al*, 2002). *In vitro* cytotoxic activity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines (Ping *et al*., 2003). Antihyperglycaemic activity, particularly on reducing blood glucose level in hyperglycaemic rats (Husen *et al*., 2004). Antibacterial activity against Gram-positive and Gram-negative bacteria (Farouk and Benafri, 2007). Effects of a standardized methanol extract of *E. longifolia* Jack on the epidermal spermatozoa profile of normal rats and infertile rats (Chan *et al*., 2009).
Garcinia atroviridis	Asam gelugur, kayu gelugor	The leaves, fruits, roots, stem and trunk bark of *Garcinia atroviridis* were screened for antimicrobial, cytotoxic, brine shrimp toxic, antitumour-promoting and antioxidant activities (Mackeen *et al*., 2000).
Goniothalamus andersonii		Apoptosis induction in Jurkat T-cells assessed by the externalisation of phosphatidylserine (Inayat, 1999).

Table 2.1,continued

Hedyotis verticillata	Salasik lupa	nitric oxide inhibitory activity (Saha *et al*., 2004)
Kaempferia galanga	Cekur, cekur Jawa, Kencur	Antitumour activity using the short assay of inhibition of 12-*O*-hexadecanoylphorbol-13-acetate (HPA)-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells (Vimala *et al*., 1999).
Lasianthus oblongus	Koro, sekentut bulu	Nitric oxide inhibitory activity (Saha *et al*., 2004)
Leea indica	Memali, merbati padang	DPPH free radical scavenging activity (Saha *et al*., 2004)
Phyllantus niruri	Pokok dukung anak, amin buah, keman jolok	Antihyperglycaemic activity, particularly on reducing blood glucose level in hyperglycaemic rats (Husen *et al*., 2004).
Physalis minima	Pokok letup-letup, leletup, ubat pekong, shipluan	SRB cytotoxicity assay against two human cancer cell lines, COR L23 lung cancer cell line and MCF7 breast cancer cell line (Lee and Houghton, 2005).
Piper betle	Sirih carang, be, bed, siyeh, sirih Melayu, sirih kerakap, serasa, cabe, jambi, kerak	Antinematodal activity against *Burshaphelenchus xylophilus* using a fungal-feeding assay (Mackeen *et al*., 1997). Anti-tumor promoting activity toward tumor promoter 12-*O*-hexadecanoylphorbol-13-acetate (HPA)-induced Epstein-Barr virus (EBV) activation in Raji cells (Murakami *et al*., 2000).
Piper nigrum	Lada hitam, lada sulah	Antinematodal activity against *Burshaphelenchus xylophilus* using a fungal-feeding assay (Mackeen *et al*., 1997).
Piper sarmentosum	Daun kadok, sirih dukok,	*In vitro* and *in vivo* antimalarial activity on *Plasmodium falciparum* (Nik Najib *et al*., 1999).
Tabernaemontana divaricata	Susun kelapa, sisik kelah	SRB cytotoxicity assay against two human cancer cell lines, COR L23 lung cancer cell line and MCF7 breast cancer cell line (Lee and Houghton, 2005).
Tinospora crispa	Patawali	*In vitro* and *in vivo* antimalarial activity on *Plasmodium falciparum* (Nik Najib *et al*., 1999).
Zingiber zerumbet	Lempoyang	Anti-tumour promoter activity using the short assay of inhibition of 12-*O*-hexadecanoylphorbol-13-acetate (HPA)-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells (Vimala *et al*., 1999). Antihyperglycaemic activity, particularly on reducing blood glucose level in hyperglycaemic rats (Husen *et al*., 2004).

2.2 Phyllanthaceae

Phyllanthaceae are a morphologically diverse pantropical family consisting about 2,000 species with 60 genera. They have been segregated along with Pandaceae, Picrodendraceae, and Putranjivaceae from Euphorbiaceae sensu lato (s.l.) (Samuel *et al*., 2005).

Phyllanthaceae are probably best divided into two subforms:

i. Phyllanthoideae consisting of genus *Bridelia*, *Phyllanthus* and *Sauropus*.

ii. Antidesmotoideae consisting of genus *Antidesma*, *Aporosa*, *Baccaurea*, *Bischofia*, *Cleistanthus* and *Uapaca* (Mabberley, 2008)

2.3 *Phyllanthus* spp.

Phyllanthus, Linn, is the largest genus in the family Phyllanthaceae (Burkill, 1966; Jagessar *et al*., 2008) and Linnaeus described this genus (*Phyllanthus*) for the first time in 1737. It is a very large genus of approximately 550 to 800 species which subdivided into 10 to 11 subgenera, including *Isocladus*, *Kirganelia*, *Cicca*, *Emblica*, *Conani*, *Gomphidium*, *Phyllanthodendron*, *Xylophylla*, *Botryanthus*, *Ericocus* and *Phyllanthus* (Calixto *et al*., 1998; Silva, 2006).

Most of *Phyllanthus* plants are shrubs, trees and herbs (Burkill, 1966). A large majority are herbaceous, with phyllanthoid branching, staminate flower with a generally segmented glandular disk, pistillate flower with an entire glandular disk, and diversely sculptured seed coats (Silva, 2006). *Phyllanthus* has a remarkable diversity of growth forms including annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous, and phyllocladous (Jagessar *et al*., 2008).

Known as pantropical plant, the plants of the genus *Phyllanthus* are widely distributed in most tropical and subtropical countries (Calixto *et al*., 1998; Liu and McIntosh, 2001; Dhiman and Chawla, 2005; Silva, 2006). *Phyllanthus* have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes and hepatitis B (Calixto *et al*., 1998; Khatoon *et al*., 2006; Kumaran and Karunakaran, 2007), chiefly as external application (Burkill, 1966).

Decoctions of the leaves of *P. frondosus* are used to cure fevers, and be administered after childbirth. This species is also used as diuretic and diaphoretic in gonorrhoea. *P. gomphocarpus*, a shrub found in Thailand and Malay Peninsular which the sour fruits can be eaten (Burkill, 1966). *P. fraternus* is possess astringent, deobstruent, diuretic and antiseptic properties. *P. maderaspatensis* is used for headaches, as a laxative and diuretic. *P. amarus* has antiseptic properties, and is used to treat gonorrhoea, jaundice and mammary abscesses. *P. urinaria* is particularly used as a diuretic and for the treatment of dysentery (Basharat, 1998).

P. pulcher, an under shrub is much used medicinally by the Malays. Decoctions of *P. pulcher* are consumed for stomach-ache and the leaves applied to gums for toothache (Burkill, 1966).

In recent years, the interest in the plants of the genus *Phyllanthus* has increased considerably, especially regarding their therapeutic potential for the management of many diseases. Several reasons contribute to this; include their greater distribution in many tropical and subtropical countries, the great number of species in this genus, their broad therapeutic use in folk medicine and the greater diversity of secondary metabolites present in such plants (Kumaran and Karunakaran, 2007)

A great variety of plants belonging to the genus *Phyllanthus* have been phytochemically and pharmacologically investigated. Different classes of organic

compounds of medicinal interest have been isolated and characterized, including alkaloids, flavonoids, lactones, steroids, terpenoids, lignans and tannins (Liu and McIntosh, 2001; Calixto *et al*., 1998). Lignans, triterpenes, alkaloids and tannins are the most abundant compounds so far determined in this genus (Calixto *et al*., 1998). Table 2.2 shows the different chemical class of organic compounds isolated from plants of the genus *Phyllanthus.*

Table 2.2: Chemical constituents isolated from plants of the genus *Phyllanthus*

Species	Class	Compound(s)
P. acidus	Triterpene	lupeol
		β-amyrin
		phyllanthol
	Lignan	phyllanthoside
P. acuminatus		justicidin B
		phyllanthostatin 1
		phyllanthostatin 2
		phyllanthostatin 3
		phyllanthostatin 4
		phyllanthostatin A
P. amarus	Benzenoid	gallic acid
		corillagin
		4-O-galloylquimic acid
	Flavonoid	gallocatechin
		rutin
		quercetin-3-O-glucopyranoside
	Tannin	phyllanthusiin
		1,6-digalloylglucopyranose
		geraniin
		amariin
		furosin
		geraniinic acid
		amariinic acid
		elaeocarpusin
P. anisobulos	Lignan	justicidin B
		phyllanthostatin A
	Lactone	menisdaurilide
		aquilegiolide

Table 2.2, continued

P. caroliniensis	Benzenoid	ethyl gallate
	Flavonoid	quercetin
	Sterol	β-sitosterol
		stigmasterol
		campesterol
	Tannin	geraniin
P. corcovadensis	Benzenoid	salicylic acid methyl ester
	Monoterpene	cymene
		limonene (-)
	Sterol	β-sitosterol
		stigmasterol
		campesterol
P. discoideus	Alkaloid	phyllochristine
		phyllanthine
		phyllanthidine
		phyllalbine
		phyllantidine
		allosecurinine-15-β-ol, 14-15-dihydro
		securinine
		allo-securinine
		viro-allo-securinine
		nor-securinine
		dihydro-securinine
	Lignan	phyllanthin
	Triterpene	betulinic acid
P. emblica	Alkaloid	zeatin
		zeatin nucleotide
		zeatin riboside
	Benzenoid	chebulic acid
		chibulinic acid
		corilagin
		glucose-3-6-di-O-galloyl
		gallic acid
		gluco-gallin
		ethyl gallate
	Coumarin	ellagic acid
	Diterpene	giberellin A-1
		giberellin A-3
		giberellin A-4
		giberellin A-7
		giberellin A-9
	Flavonoid	leucodelphinidin

Table 2.2, continued

		rutin
	Sterol	β-sitosterol
P. engleri	Polyprenoid	rubber
	Triterpene	phyllanthol
P. flexuosus	Benzenoid	corilagin
		chebulagic acid
	Coumarin	brevifolin carboxylic acid
		bergenin
	Sterol	campesterol
		stigmasterol
		β-sitosterol
	Tannin	geraniin
		geraniic acid
		repandusinic acid
		phyllanthusiin A
		phyllanthusiin B
		phyllanthusiin C
		phyllanthusiin D
		phyllanthusiin E
	Triterpene	betulin
		olean-12-en-3-β-15-α-triol
		olean-12-en-3-β-15-α-diol
		lupeol
		glochidone
		friedelin
		β-amyrin
		ent-3-β-hydroxykaur-16-ene
		olean-12-en-3-β-24-diol
		oleana-11-13(18)-dien-3β-24-diol
P. maderaspatensis	Lipid	linoleic acid
		linolenic acid
		myristic acid
		oleic acid
		palmitic acid
		stearic acid
P. muellerianus	Sterol	β-sitosterol
	Triterpene	friedel-1-ene-22-β-ol
		friedellin-1-β-22-β-diol
P. myrtifolius	Lignan	phyllamicin
		retrojusticidin B

Table 2.2, continued

P. niruri	Alkaloid	4-methoxy-nor-securinine
		nirurine
		ent-norsecurinine
	Benzenoid	gallic acid
		corilagin
	Coumarin	ellagic acid
		ethyl brevifolin carboxylate
		quercetin
		rutin
		astragalin
		quercitrin
		isoquercitrin
		kaempferol-4'-rhamnopyranoside
		eridictyol-7-rhmnopyranoside
		fisetin-4-O-glucoside
		nirurin
	Lignan	phyllanthin
		hypophyllanthin
		niranthin
		nirtetralin
		phyltetralin
		hinokinin
		isolintetralin
	Lipid	ricinoleic acid
	Phytallate	phyllester
	Sterol	estradiol
		β-sitosterol
		isopropyl-24-chlosterol
	Tannin	geraniin
	Triterpene	lupeol acetate
		lupeol
		3,7,11,15,19,23-hexamethyl-2Z,6Z
		10Z,14E,18E,22E-tetracoshenen-1-ol
		phyllanthenol
		phyllanthenone
		phyllantheol
P. niruroidine	Alkaloid	Niruroidine
P. pectinatus	Monoterpene	
	Triterpene	
	Lignan	
	Flavonone	

Table 2.2, continued

P. orbiculatus	Flavonoid	astragalin quercetin quercitrin rutin isoquercitrin
P. reticulatus	Benzenoid Coumarin Sterol Triterpene	pyrogallic acid ellagic acid β-sitosterol friedelin friedelanol 21-α-hydroxy-friedelin friedel-4(23)-en-3-one,21-α-hydroxy betulinic acid glochidonol
P. sellowianus	Alkaloid Acetophenone Acid Benzenoid Coumarin Flavonoid Triterpene Sterol Sugar	phyllanthimide xanthoxyline caffeic acid chlorogenic acid ethyl gallate scopoletin isofraxidin 4'4'''-di-O-methylcupressuflavone rutin quercetin 7-hydroxyflavonone glochidone glochidiol glochidonol lup-20(29)-ene-1β,3β-diol phyllanthol β-sitosterol stigmasterol campesterol levulose glucose galactose saccharose
P. simplex	Alkaloid	phyllanthine simplexine
P. urinaria	Acid Alkanol	hexacosanoic acid triacontanol

Table 2.2, continued

	Benzenoid	gallic acid
	Coumarin	trimethylester dehydrochebulic acid
		methylbrevifolin carboxylate
		ellagic acid
	Ester	montanoic acid ethyl ester
	Flavonoid	astragalin
		quercetin
		quercitrin
		isoquercitrin
		rutin
		kaempferol
	Phytallate	phyllester
	Sterol	daucosterol
		β-sitosterol
	Tannin	geraniin
	Triterpene	lupeol acetate
		β-amyrin
P. verminatus	Sesquiterpene	phyllanthoside
		phyllanthostatin 1
		phyllanthostatin 2
		phyllanthostatin 3
P. virgatus	Lignan	hinokinin
		hypophyllanthin
		isolintetralin
		niranthin
		nirtetralin
		phyltetralin
		virgatusin
		(+)-8-(3,4-methylenedioxybenzyl-8'-(3',4'-dimethyoxybenzyl)-butyrolactone
		indole-3-carboxylic acid
P. watsonii	Triterpene	26-nor-D:A-friedoolean-14-en-3-one
		26-nor-D:A-friedoolean-14-en-3beta-ol

(Adapted from Calixto *et al*., 1998)

Among all, *P. niruri*, *P. urinaria*, *P. emblica*, *P. flexuosus*, *P. amarus* and *P. sellowianus* have been widely investigated for its phytochemical properties. Although

most of these compounds are chemically known, their complete pharmacological properties remain, in general, undetermined (Calixto *et al*., 1998).

Many studies suggest that most plants of the genus *Phyllanthus* have a beneficial effect against hepatitis B virus, possibly through inhibition of polymerase activity, mRNA transcription and replication (Liu and McIntosh, 2001). Studies by Huang *et al*. (2004) revealed the anticancer effect of aqueous extract of *P. urinaria*. The aqueous extract of *P. urinaria* could reduce the viability by inducing apoptosis in several human cancer cells as demonstrated by morphological changes and DNA fragmentation. *P .urinaria* extract however exhibited no cytotoxic effect on normal human cells such as vascular endothelial cells and liver cells (Huang *et al*., 2004).

P. fraternus and *P. maderaspatensis* reported to show hepatoprotective activity against carbon tetrachloride-induced mitochondrial dysfunction (Padma and Setty, 1999) and acetaminophen-induced hepatotoxicity (Asha *et al*., 2004), respectively. A new oxirano-furanocoumarin designated as debelalactone which was isolated from the whole plant of *Phyllanthus debilis* exhibited a significant antihepatotoxic activity against CCI_4-induced toxicity in Wistar rats in comparison with standard silymarin (Ahmed *et al*., 2009). Phyllanthin and hypophyllanthin present in *Phyllanthus amarus* are also reported as hepatoproctective agents and protect hepatocytes against carbon tetrachloride (CCl_4) and galactosamine induced cytotoxicity in rats (Syamsunder *et al*., 1985).

Studies by Kumaran & Karunakaran (2007) revealed that the five selected species of *Phyllanthus*, namely *P. debilis*, *P. urinaria*, *P. virgatus*, *P. maderaspatensis* and *P. amarus* have significant antioxidant activity. The antioxidant activity of methanol extracts of five plants from the genus *Phyllanthus* was evaluated by various antioxidant assays, which include total antioxidant, free radical scavenging assay, superoxide anion

radical scavenging assay, hydrogen peroxide scavenging assay, nitric oxide scavenging assay, reducing power assay and metal ion chelating assay.

Studies by Hossain *et al*., 2008 on the total phenolic content and antioxidative activity of the Bangladeshi fruits, revealed *Phyllanthus emblica* (emblic myrobalan) as had possessing the highest total polyphenol content (339 mg gallic acid equivalent , GAE/g). *P. emblica* also exhibited the most potent DPPH radical scavenging activity, (IC_{50} = 2.1 μg/ml) and promising reducing powers.

Ellagitannin and corilagin, a prolyl endopeptidase inhibitor isolated from the ethyl acetate soluble fraction of *Phyllanthus ussurensis* have been reported to have a potential to be developed as anti-dementia drugs (Chung *et al*., 2003). Methanol extracts of *Phyllanthus singampattiana* showed a remarkable antimicrobial activity against diarrhea and dysentery-causing organisms such as *A. hydrophilla*, *E. aerogenes*, *V. cholerae*, *P. vulgaris* and *K. pneumoniae* (Ramesh *et al*., 2004). Significant antimicrobial activity against *Shigella* spp., *E. coli*, *V. cholerae* and *S. aureus* of methanol extracts of *P. amarus* has also been reported (Mazumder *et al*., 2006). Phthalates with antimicrobial properties have been isolated from *Phyllanthus muellerianus* (Saleem *et al*., 2009). Table 2.3 shows pharmacological effect of isolated compounds of some species of *Phyllanthus*

.

Table 2.3: Pharmacological effect of compounds isolated from *Phyllanthus*

Species	Compound(s)	Pharmacological activity
P. corcovadensis *P. caroliniensis* *P. flexuosus* *P. sellowianus*	Stigmasterol	Analgesic
P. sellowianus *P. urinaria*		

Table 2.3, continued

P .emblica *P. orbiculatus* *P. amarus*	Rutin	Anti-inflammatory and analgesic
P. urinaria *P. acidus* *P. flexuosus*	β-amyrin	Analgesic, antilipoxigenase
P. sellowianus	Caffeic acid	Antiallergic, analgesic, nitrosamine blocker
P. niruri *P. urinaria* *P .emblica* *P. reticulatus*	Ellagic acid	Aldose reductase inhibitor
P. urinaria *P. niruri* *P. caroliniensis* *P. flexuosus*	Geraniin	ACE inhibitor, antiallergic, and analgesic
P. urinaria *P. niruri* *P. caroliniensis* *P. flexuosus*	Quercetin	Mitochondrial ATPase inhibitor, phosphodiesterase inhibitor, mutagenic effect in bacterial, cyclooxigenase inhibitor, analgesic, phosphorilase and tyrosine kinase inhibitor, phospholipase A_2 inhibitor
P. niruri	Niruside	HIV-1 Reverse transcriptase inhibitor
P. myrtifolius	Phyllamicin Retrojusticidin	HIV-1 Reverse transcriptase inhibitor
P. accuminatus *P. anisobulus* *P. verminatus*	Phyllantho-statins 1,2,3	Antineoplastic activity
P. flexuosus *P. niruri* *P. discoideus*	Repandusinic acid A, Phillanthin, Hypophoillanthin, Hirtetralin	HIV-1 Reverse transcriptase inhibitor, antihepatoxic, endothelin antagonists
P. corcovadensis *P. caroliniensis* *P. flexuosus* *P. sellowianus* *P. reticulates*	β-sitosterol	Analgesic and anti inflammatory

P. muellerianus
P .emblica
P. urinaria

(Adapted from Calixto *et al*., 1998)

2.3.1 *Phyllanthus niruri*

Figure 2.1: *Phyllanthus niruri*

Phyllanthus niruri Linn (Figure 2.1), locally known as Dukong anak (the child pick-a-back, from the position of the fruit on the back of the branches), dukong-dukong anak, Amin buah (fruit on the back), Rami buah, Turi hutan and Meniran in Java. It is an herb of small size and found very widely throughout the tropics country (Burkill, 1966).

Prior to 1985, most of the botanical, phytochemical and pharmacological works of "Bhuiamlki" was carried out in India. *P. niruri* is a perennial herb distributed throughout India. Whole plant, fresh leaves and fruits are used to treat various ailments particularly hepatitis (Harish and Shivanandappa, 2006), sores, jaundice, dysentery, dropsy, gonorrhoea, mennorrhagia, and mild fever (Burkill, 1966),

The pounded leaves and stems of *P. niruri* are used as poultices for skin complaints, including caterpillar itch. The Malays use *P. niruri* and *P. urinaria* vicariously, internally for diarrhoea, kidney problems, gonorrhoea, and syphilis; as an emmenagogue, and after a miscarriage and childbirth; they use the young leaves for coughs, especially in children (Burkill, 1966). The decoction of the roots is consumed after childbirth as a general tonic by the Malay people (Ong and Norzalina, 1999).

In Brazilian folk medicine, a tea made from the plant *P. niruri* (stone breaker or "quebra pedra") is used by patients with urolithiasis (Mello, 1980; Calixto *et al*., 1998). In tropical and subtropical regions of Nigeria, *P. niruri* is popular for the treatment of gallstone and kidney stone (Oparaocha and Okorie, 2009).

2.3.2 *Phyllanthus pectinatus*

Figure 2.2: *Phyllanthus pectinatus*

Locals treated *Phyllanthus pectinatus* (Figure 2.2) as a synonym of *Phyllanthus emblica* (both locally known as Pokok Melaka), which was scientifically proven to be incorrect. Until now, *P. pectinatus* still has no local name of its own to distinguish it from

P. emblica. *P. pectinatus* is a tall tree with the fruits dangling at the distal ends of the leafy twigs (Ng, 2000).

There is no any ethno botanical information on *P. pectinatus* as the identity of the *P. pectinatus* has been confused with *P. emblica. P. emblica* has always had a high reputation in Ayurvedic and Chinese traditional medicines and there numerous studies on its biological activities had been reported (Barthakur and Arnold, 1991; Suresh and Vasudevan, 1994; Bandyopadhyay *et al*., 2000; Pramyothin *et al*., 2006; Liu *et al*., 2008; Luo *et al*., 2009). Studies have also been carried out in Malaysia on phytochemical and pharmaceutical aspect, but these studies were often on the wrong tree, published under the wrong name (Ng, 2000).

2.3.3 *Phyllanthus acidus*

Figure 2.3: *Phyllanthus acidus*

Phyllanthus acidus (Figure 2.3) locally known as cermai (Engel and Phummai, 2000) or Malay Gooseberry (Polunin, 1988), is an annual erect little branched herb, 10 – 50 cm high. It is completely green including the flowers. Leaves are simple, oblong, acute or obtuse, slightly oblique to 14 mm long and 6 mm broad and bear the inconspicuous flowers

in pairs in their axils. Each pair of flowers comprises one male and one female. The capsule is a flattened globe about 2 mm in diameter (Jagessar *et al*., 2008).

It is believed that water boiled with the leaves of *P. acidus* helps to relief fever with skin manifestations (e.g. measles) (Engel and Phummai, 2000) and to reduce blood pressure (Rukayah, 2008). The decoction of the leaves and white pepper is taken as tonic to relief body ache (Kamarudin and Latiff, 2002).

The mixtures of *P. acidus* leaves with rice soaked overnight are crushed together with turmeric rhizome to form powder. This powder is use mainly to treat skin problems such as pimples and oily skin (Rukayah, 2008). The fruits are used for pickles and flavouring (Polunin, 1988).

2.3.4 *Phyllanthus roseus*

Figure 2.4: *Phyllanthus roseus*

Phyllanthus roseus (Figure 2.4) consists of shrubs or small trees to 5 m high. This plant is only distributed throughout Indochina and Malaysia (Chantharanothai, 2009). It is rarely found in the forest but can grow well in areas exposed to sunlight (Perbadanan Bioteknologi dan Biodiversiti Negeri Johor, 2009).

Phyllanthus roseus, are known as Labu Kuncing among the Jakun peoples. The root of this plant is eaten to increase the male sexual drive (Khazanah Endau Rompin Herba, 2007). There is no any biological and phytochemistry studies reported on this particular species.

2.3.5 *Phyllanthus watsonii*

Figure 2.5: *Phyllanthus watsonii*

Phyllanthus watsonii (Figure 2.5), a small shrub growing to about 1 m high, is one of the three Phyllanthaceae plants endemic to Peninsular Malaysia (Chopra *et al*., 1956; Burkill, 1966) and recorded only from north Johore and South Pahang on the banks of Endau River (Matsunaga *et al*., 1993) at Endau-Rompin (Darison, 1988). *P. watsonii* was listed as one of the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Kochummen, 1998).

Known as rheophytes shrub, *P. watsonii* can withstand strong water current and floods. Some of its characteristics enable this plant to adapt to the conditions of its habitat, along the edge of the river, where it is often rooted in the water. The plant root system is extensive and clings tenaciously to the substrate to prevent being dislodged by floods. (Darison, 1998; Polunin, 1998).

2.4 *Baccaurea* spp.

The genus *Baccaurea* has over 20 species growing wild in the tropical lowland forests. *Baccaurea* is found mainly in Malaysia and also around India and the Pacific. Most of the *Baccaurea* species are generally too sour but some selected species like *Baccaurea motleyana* are sweet enough to be eaten as fresh fruits and have thus become popular in Malaysia. The fruits of other species have been used in many other ways as drinks and liquor. The various parts of the *Baccaurea* trees can be used for things such as timber, dye and medicine (Chin, 1986).

Baccaureas are evergreen trees of average height from 10 to 18 m in the wild state. Those grown around the urban areas and in villages are not as tall and are normally about 10 m. The *Baccaurea* trees are easily recognizable by their shape, dense foliage and the spirally arranged leaves. The flowers are yellowish green arranged in string-like structures with scent. The fruits or berries are arranged in strings and hang down beautifully from the older woody branches of the tree. Flowering starts with the onset of dry weather. This followed by the fruiting season which comes once or twice a year (Chin, 1986).

2.4.1 *Baccaurea motleyana*

Figure 2.6: ***Baccaurea motleyana***

In Malaysia, rambai (*Baccaurea motleyana*) (Figure 2.6) or the golden berry is well known as a fruit tree (Chin, 1986). An evergreen tree growing to about 18 m in height, rambai has a low, round, bushy crown and large leaves, 15 – 30 cm long and 7 – 15 cm wide. Its leaf blade has a heart-shaped base with an uneven and wavy edge. Its flowers are borne on the larger branches of the tree, the male flowers being light yellow, fragrant and on strings 7 – 25 cm long while the female flowers hang on longer strings, 25 – 90 cm long. The fruits are buff in colour, oblong in shape with 1 – 3 seeds in them and the fruits skin is thin and velvety. The translucent white pulp is edible and its taste varies from sweet to sour (Chin, 1986).

The nutritional value based on 100 g edible portion of the fruit is 5 mg vitamin C, 2 mg calcium and 20 mg phosphorus. Its vitamin content is very low as compared to other fruits with vitamin B1 and B2 are totally absent. The fruits may also be fermented and made into liquor (Rukayah, 2008) and the timber can be used for making posts and the bark for fixing other dyes (Chin, 1986).

The bark of *B. motleyana* can be pounded with the leaves of *Adenostemma viscosum* and *Mimusops* elengi, and the liquid used for the treatment of sore eye (Werner, 2002; Rukayah, 2008). Pounded bark can also applied as facial powder (Rukayah, 2008).

2.5 Natural Products in Anticancer Drug Discovery

In the early 1900s, before the "Synthetic Era", 80 % of all medicines were obtained from roots, barks and leaves and the basis of its development remains rooted in traditional medicine and therapies (Patwardhan *et al*., 2004; McChesney *et al*., 2007). For many years, natural products continue to play an important role in the basis of treatment of human diseases and with an "unlimited" resource for future drug discovery (Li and Vederas, 2009), natural products have been one of the consistently successful sources of drug leads (Bindseil *et al*., 2001; Firn and Jones, 2003; Vuorelaa *et al*., 2004).

Natural products can be characterized as small-molecule secondary metabolites that originate from terrestrial and marine plants, microorganisms and animals. Natural products tend to present more structurally diverse "drug-like" and "biological friendly" molecular qualities compared to pure synthetic compounds at random. There are several theoretical advantages in screening natural products for drug discovery: (1) Natural products offer unmatched chemical diversity with structural complexicity and biological potency (2) Natural products occupy a complementary region of chemical space compared with synthetic compounds (3) The use of natural products as templates for combinatorial chemistry enables the generation of libraries of natural product analogs, which might have enhanced drug-like properties (e.g. pharmacokinetics, solubility) (4) Owing to our increased understanding of genetics and biosynthesis of natural products, the regulation of natural product biosynthesis can be optimized (5) Natural product compounds not only

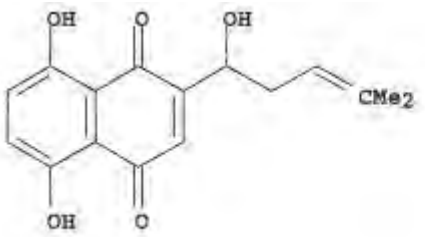
serve as drugs or templates for drugs, but in many instances lead to the discovery and better understanding of targets and pathways involved in the disease process (6) Whereas synthetic drugs are typically the results of numerous structural modifications over the course of an extensive drug discovery program, a natural product can go straight from 'hit' to drug (Lam, 2007).

Today, natural products play a key role in healthcare and pharmaceutical research, as about 40 % of all the drugs in clinical use in the world are either natural products originated from soil microorganisms, terrestrial fungi, and higher plants (Li *et al*., 2010) or semisynthetic derivatives (Newman and Cragg, 2007; John, 2010), with higher plants contributing no less than 25 % of the total drugs in clinical use (Balunas and Kinghorn, 2005; Fakim, 2006). Figure 2.1 shows some examples of secondary metabolites that had been successfully isolated from different plants and studied for its bioactivity.

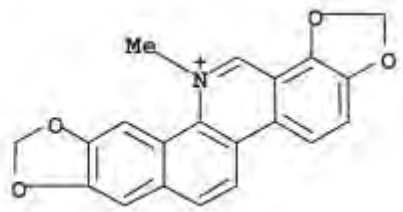
Drugs derived from natural product sources have progressed from the formulation of crude drugs to the isolation of active compound in drug discovery. Numerous methods have been utilized to acquire compounds for drug discovery including preparation of extract from the natural product materials, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation (Balunas and Kinghorn, 2005). In the sequence of their appearance, the scientific disciplines involved in natural product drug discovery were chemistry, pharmacology, physiology, microbiology, biochemistry and molecular biology (Patwardhan *et al*., 2004).

In recent years, the search for new anti cancer drug is one of the most prominent research areas of natural products (de Mesquita *et al*., 2009) and considerable attention has been focused on identifying naturally occurring substances capable of inhibiting, retarding, or revising the process of multistage carcinogenesis (Sharma *et al*., 2010). The search of

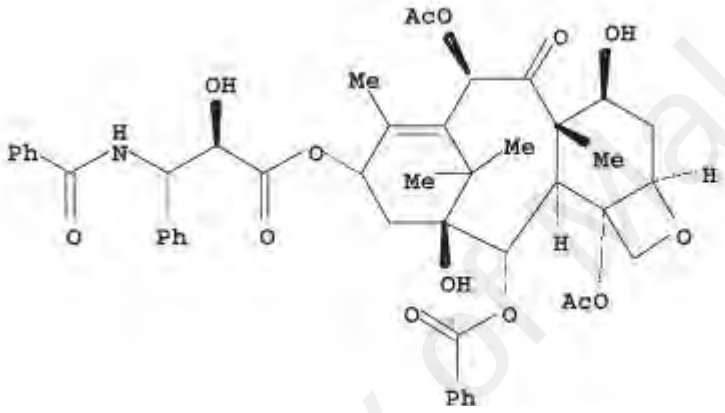
new anti cancer agents from microbes, marine and plant sources has provided many effective anticancer agents in current use, such as dactinomycin and doxorubicin derived from microorganisms, or from plants in the case of irinotecan, topotecan, paclitaxel and taxanes (Cragg and Newman, 1999; Ruffa *et al*., 2002; Patwardhan *et al*., 2004; John, 2010). An analysis of the anticancer drug market in North America, Europe, and Japan during the period 1981 – 2006 revealed that 47.1 % of a total of 155 clinically approved anticancer drugs were either unmodified natural products or their semi-synthetic derivatives, or synthesized molecules based on natural product compound pharmacophores (Newman and Cragg, 2007). Identification and development of new classes of anticancer drugs hold great promise in improving the efficacy of current clinical treatments by surgical resection, anti-hormonal therapy, radiation therapy, and/or chemotherapy (Mimeault and Batra, 2010).



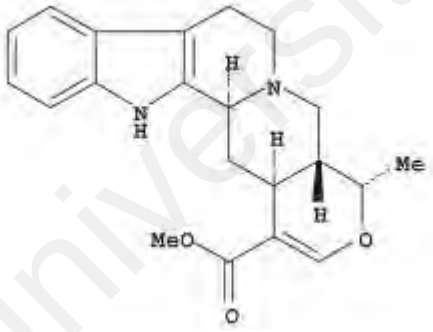
Shikonin , major component of the dried root of *Lithospermum erythrorhizon*, with various biological activities, including inhibition of human immunodeficiency virus (HIV) type 1 (HIV-1).



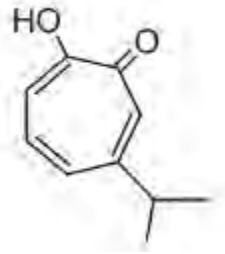
Sanguinarine, an alkaloid derived from the bloodroot plant *Sanguinaria canadensis*, possessed antimicrobial, anti-inflammatory, and antioxidant properties.



Taxol, a diterpene compound from *Taxus* spp. has very strong anticancer activity



Ajmalicine, a terpenoid indole from *Catharanthus roseus* with an antioxidant potential



β-Thujaplicin, an antifungal tropolone from *Cupressus lusitanica*

Figure 2.7: Several typical plant secondary metabolites with special interests in their bioactivities
(Modified from Zhao *et al*., 2005)

2.5.1 Plant as Anticancer Agents

The search for anticancer agents from plants sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins (Cragg and Newman, 2005). Today, drug discovery from plants still continues to play an important role in the treatment of cancer. Indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last century have been applied towards combating cancer and its continue to be one of the realistic and promising agents in cancer prevention (Newman *et al.,* 2000; Newman *et al*., 2003; Butler, 2004; Hu *et al*., 2009; Li *et al*., 2010). Table 2.4 shows several of plant-derived compounds that are currently successfully employed in cancer treatment.

Approaches been taken towards discovering anticancer agents from tropical plants (Balunas and Kinghorn, 2005) as alternative cancer remedies because of their low toxicity and costs (Hu *et al*., 2009). The potential of phytochemical derived from medicinal plants as anticancer agent had prompted a surge of *in vitro* studies of their biological effects in cultured human cells and the reduction of cancer is likely related to apoptosis pathways (Gosslau and Kuang, 2004).

Tamoxifen, a triphenylethylene derivative, acts predominantly as an antiestrogen. This drug has been in clinical use since the 1970s. *In vitro*, tamoxifen inhibits estrogen promotion of MCF7 cancer cell lines. Tamoxifen has also been shown to inhibit promotion and development of breast tumours in rats and mice (Jordan, 1976).

Paclitaxel was originally isolated from the bark of the yew tree *Taxus brevifolia* (Wani *et al*., 1971), a finite source of the compound. It took some years to develop a semi-

synthetic analog (docetaxel) which is derived from a renewable source, the leaves of *Taxus baccata* (Cortes and Pazdur, 1995).

Table 2.4: Plant-derived anticancer agents

Compound	Plant species	Cancer use
Vincristine	*Catharanthus roseus*	Leukaemia, lymphoma, breast, lung, paediatric solid cancers and others (De Vita *et al*., 1970; Noble, 1990)
Vinblastine	*Catharanthus roseus*	Breast, lymphoma, germ-cell and renal cancer (De Vita *et al*., 1970; Noble, 1990)
Paclitaxel	*Taxus brevifolia*	Ovary, breast, lung, bladder and head and neck cancer (Wani *et al*., 1971)
Docatexal	*Taxus baccata*	Breast and lung cancer (Cortes and Pazdur, 1995)
Topotecan	*Camptotheca accuminata*	Ovarian, lung and paediatric cancer (Creemers *et al*., 1996; Bertino, 1997; Liu *et al*., 2000)
Irinotecan	*Camptotheca accuminata*	Colorectal and lung cancer (Creemers *et al*., 1996; Bertino, 1997; Liu *et al*., 2000)

(Modified from da Rocha *et al*., 2001)

Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques (Balunas and Kinghorn, 2005). Probably less than 20 % of the known plants on earth have ever been subjected to laboratory investigation for potential therapeutic effects, and a smaller percentage still for possible anticancer activity (Cragg *et al*., 2009). This will help in continuously contributing source of highly effective conventional anticancer drugs from plant, and while the actual compounds isolated from the plant frequently may not serve as

the drugs, they provide leads for the development of potential novel agents (Cragg and Newman, 2005).

2.5.2 Plant Secondary Metabolites as Anticancer Agents

Plant species possessed a capacity to defend themselves from predators and to inhibit other plants competing for space that have been selected for ('natural selection') (Mans *et al*., 2000). In order to survive, plants have to developed sophisticated mechanisms including an elaborate chemical arsenal of secondary compounds or metabolites, such as terpenes, lectins, glycosides, saponins, tannins and alkaloids. These compounds are involved in defence against insect, herbivores and pathogens, or to survive other biotic and abiotic stresses, regulation of symbiosis, control of seed germination, and chemical inhibition of competing plant species (allelopathy) (da Rocha *et al*., 2001; Zhao *et al*., 2005; Harinder *et al*., 2007). Figure 2.2 shows some functions of secondary metabolites in plants.

Plant secondary metabolites are unique resources for pharmaceuticals, as food additives, cosmetics, nutraceuticals and fine chemicals (Bourgaud *et al*., 2001; Zhao *et al*., 2005) and have been used for centuries in traditional medicine. In fact, many secondary metabolites are highly toxic and are often stored in specific vesicles or in the vacuole. These secondary metabolites are produced from universally present precursors (most often acetyl-CoA, amino acids or shikimate) by specific enzymes that probably arise by the duplication and divergence of genes originally coding for primary metabolism (Pichersky and Gang, 2000). There are well over 24,000 structures, including many compounds that have antinutritional and toxic effects on mammals (Harinder *et al*., 2007).

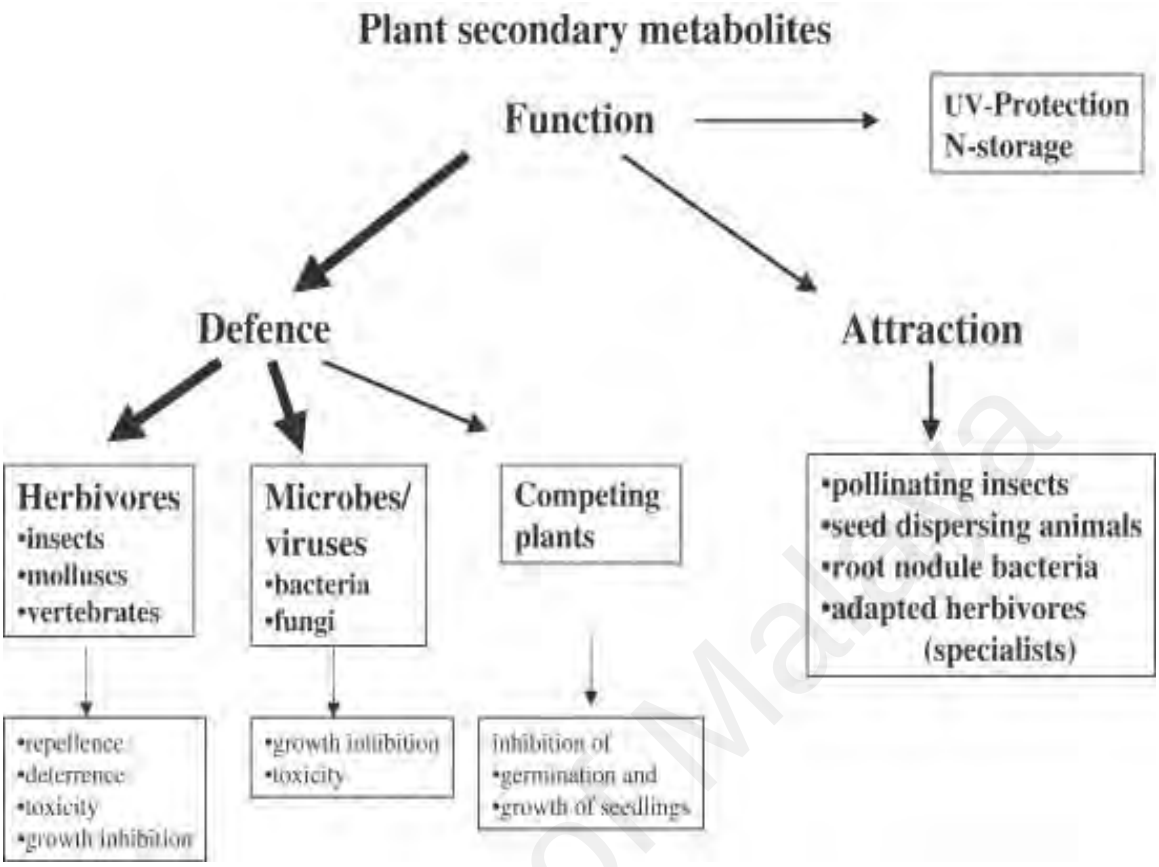


Figure 2.8: Function of secondary metabolites in plants (Wink, 2006)

Plant secondary compounds are a diverse group of molecules and usually classified according to their biosynthetic pathways. It can be classified into three major groups: nitrogen-containing compounds, terpenoids and phenolic compounds. The major class of nitrogen-containing compounds is represented by alkaloids, synthesized principally from aspartic acid, tryptophan, arginine and tyrosine (Lentini *et al*., 2010). Figure 2.3 shows examples of plant secondary metabolites arranged according to the major class.

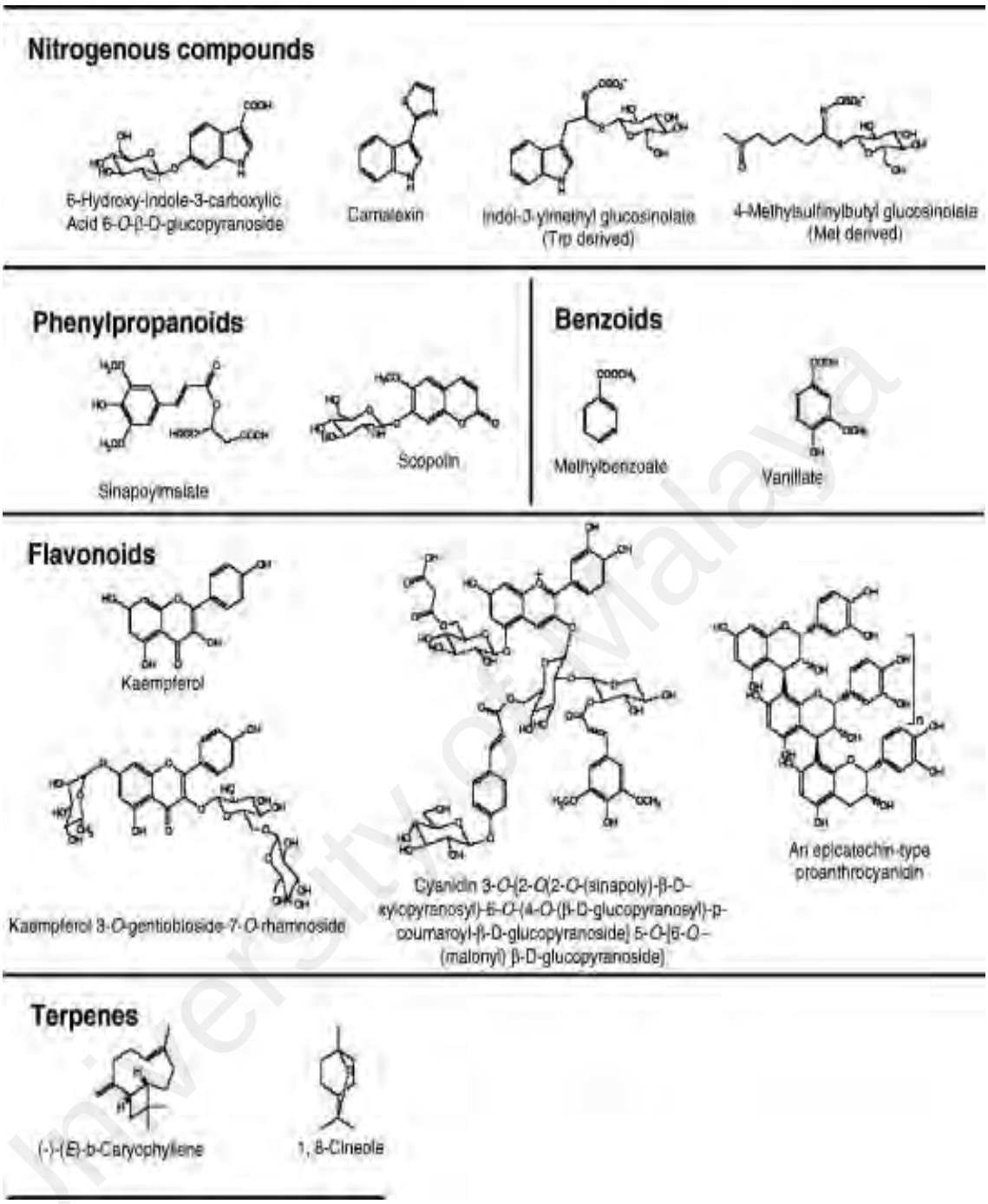


Figure 2.9: Examples of plant secondary metabolites arranged by major class (Fernie, 2007)

Phytochemicals obtained from plants such as terpenoinds, carortenoids, flavonoids, phenolic compounds, and other groups of compounds have shown promise in suppressing experimental carcinogenesis in various organs (Rabi and Bishayee, 2008) and also a good

source of well-characterized bioactive compounds (Le Marchand, 2002). Many chemo preventive agents derived from plants suppress the promotion and/or progressions of premalignant cells are believed to do so by modulating cell proliferation and/or differentiation (Wattenberg, 1995; Hong and Sporn, 1997). Considerable efforts are continuously being put in these studies for production of many other higher valuable anti cancer compounds. Figure 2.4 shows structures of some of pharmacologically active secondary plant metabolites which demonstrated anticancer properties.

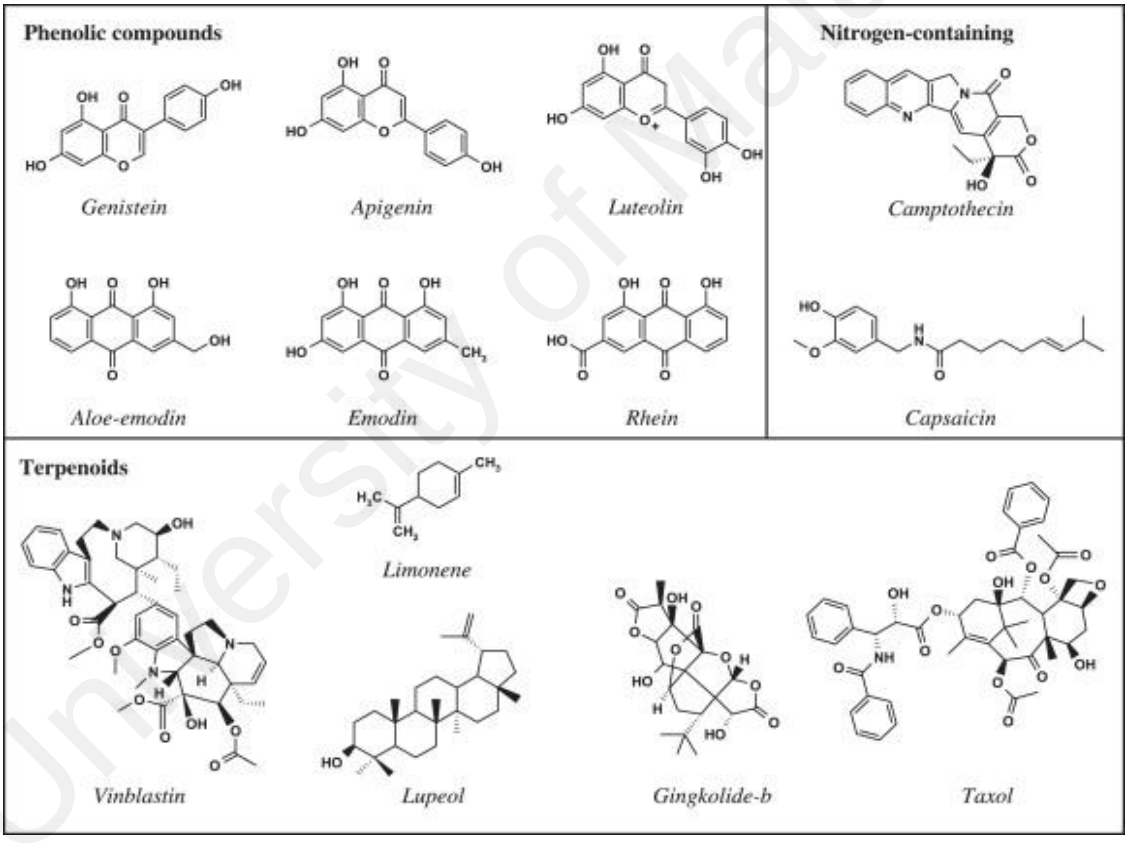


Figure 2.10: Structures of pharmacologically active secondary plant metabolites with demonstrated anticancer properties
(Lentini *et al*., 2010)

Alkaloids

Alkaloids are a diverse group of small, heterocyclic, nitrogen-containing molecules that are thought to be involved in defending plants against herbivores and pathogens (Gantet and Memelink, 2002). Alkaloid is one of the most important and largest groups of plant secondary metabolites (Suntornsuk, 2002). More than 10,000 different alkaloids have been discovered in species from over 300 plant families. These compounds protect plants from variety of herbivorous animals, and many possess pharmacologically important activities (Leete, 1969; Zenk and Juenger, 2007).

Alkaloids exert powerful physiological effects, particularly as directed against cancer. Some well-known examples of alkaloids are morphine, strychnine, quinine, ephedrine, nicotine and caffeine, used in medicines, potions, tea and poisons for thousands of years. Chemical structures of some common alkaloids are shown in Figure 2.11. Plant alkaloids are used as chemotherapeutic agents due to their capability to depolymerise the microtubules, inhibiting cell division. Vinca alkaloids (vinblastine, vincristine, vindesine) have been historically employed for their anticancer properties. They are isolated from *Vinca rosea* L. and are potent microtubule destabilizing agents first recognized for their myelosuppressive effects (Cutts *et al*., 1960). Another alkaloid used as chemotherapy drug for some types of cancer is taxol. It is mainly used to treat ovarian, breast and non small cell lung cancer (Wiseman and Spencer, 1998). Morever, capsaicin, the major pungent ingredients in red peppers, has profound antiproliferative effect on prostate cancer (Mori *et al*., 2006).

Wu and colleagues explored the mechanisms of tylophorine anticancer activity. Tylophorine down regulated cyclin A2, which plays an important role in G_1 arrest in carcinoma cells (Wu *et al*., 2009). In addition, camptothecin, a pentacyclic alkaloids

isolated from *Camptotheca acuminata* Decne, was reported to possess interesting antitumor activity. It is well-known that the cytotoxic activity of camptothecin was attributed to its ability to inhibit the nuclear enzyme DNA topoisomerase I (Staker *et al*., 2002).

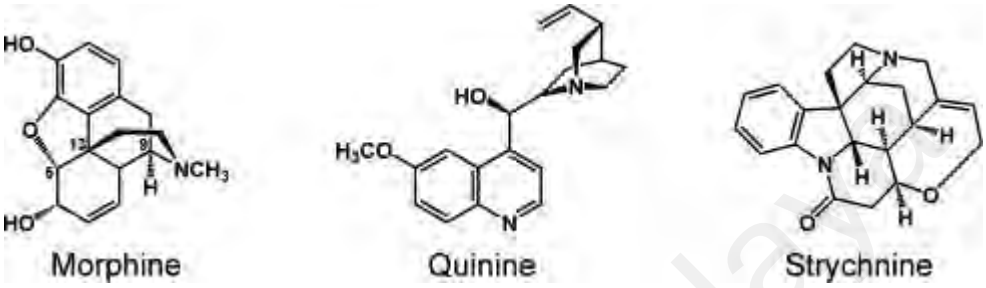


Figure 2.11: Chemical structure of three alkaloids (Zenk and Juenger, 2007)

Terpenoids

Terpenoids, also referred to as terpenes, are the largest group of natural compounds that play a variety of roles in many different plants. All terpenoids are synthesized from two five-carbon (C_5) building blocks called isoprene (Bakkali *et al*., 2008). Based on the number of building blocks, terpenoids are commonly classified as monoterpenes (C_{10}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes. Chemical structures of selected components of terpenes are shown in Figure 2.12. Terpenoids, also known as isoprenoids, are perhaps the most diverse family of natural products synthesized from plants, serving a range of important physiological functions. Over 40,000 different terpenoids have been isolated from plant, animal and microbial species (Bakkali *et al*., 2008; Rabi and Bishayee, 2008). Many plant terpenoids are toxins

and feeding deterrents to herbivores or are attractants of various sorts. Plant terpenoids are also used extensively for their aromatic qualities (Martin-Smith and Sneader, 1969).

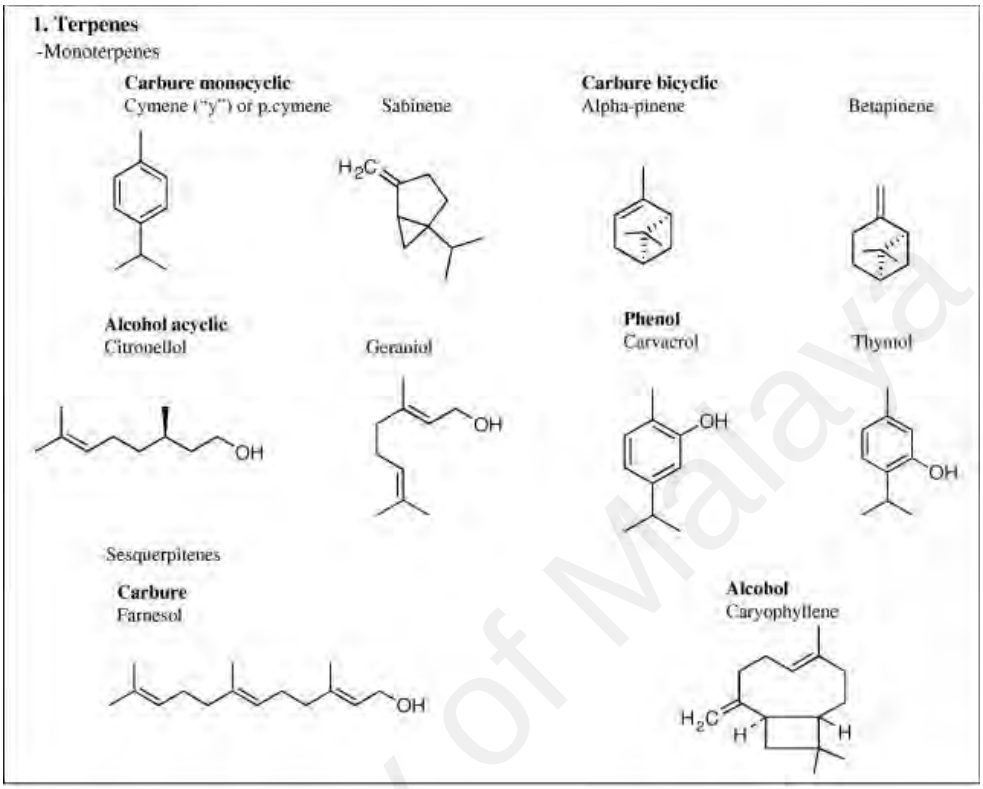


Figure 2.12: Chemical structures of selected components of terpenes. (Bakkali *et al*., 2008)

Monoterpenes are best known as secondary plant metabolites and constituents of essential oils, floral scents and defensive resins of aromatic plants (Loza-Tavera, 1999). A number of dietary monoterpenes have antitumor activity, exhibiting not only the ability to prevent the formation or progression of cancer, but the ability to regress existing malignant tumours (Crowell and Gould, 1994). *d*-Limonene is the most abundant monocyclic monoterpene found in nature, and it occurs in a variety of trees and herbs. It is major constituent of peel oil from oranges, citrus and lemons, and the essential oil of caraway. *d*-Limonene is a well established chemo preventive and therapeutic agent against many tumour cells (Bardon *et al*., 1998; Wagner and Elmadfa, 2003) and has chemo preventive

activity against rodent mammary cancer during the initiation phase as well as the promotion/progression phase (Elegbede *et al*., 1984).

The diterpenes represent a large group of terpenoids with a wide range of biological activities, isolated from a variety of organisms. Phytol is one of the simplest and most important acyclic diterpenes and vitamin A or retinol is the most important compound. Retinoids, a class of over 3,000 natural derivatives and synthetic analogs of vitamin A, are powerful modulators of epithelial carcinogenesis (Lippman *et al*., 1987; Tallman and Wiemik, 1992).

Triterpenoids represent a group of natural substances, which include steroids and consequently sterols (Beveridge *et al*., 2002). The large groups of steroids including sterols are present in very small amounts in bacteria but in large amounts in plants and animals (Kakuda *et al*., 2002). Triterpenoids have shown to possess antiinflammatory and anticarcinogenic properties (Manez *et al*., 1997). Phytosterols, especially sitosterol, are plant sterols that have been shown to exert protective effects against many types of cancer (Moghadasian, 2000).

Carotenoids belong to the category of tetraterpenoids, derived from a 40-carbon polyene chain. The hydrocarbon carotenoids are known as carotenes, while oxygenated derivatives of these hydrocarbons are known as xanthophylls. β-carotene is a tetraterpenoids distributed widely throughout the plant kingdom (Keijer *et al*., 2005). Carotenoids group include α-carotene, β-carotene, lycopene, lutein, astaxanthin, cryptoxanthin and zeaxanthin (Krinsky and Johnson, 2005). Of more than 700 carotenoids identified to date, about 40 carotenoids are found in our daily food. However, as a result of selective uptake in the digestive tract, less than 20 carotenoids with some of their metabolites have been detected in human plasma and tissues (Murakoshi *et al*., 1992).

Carotenoids have also received substantial attention because both their provitamin and antioxidant roles (Liu, 2004). Various carotenoids play an important role for the cancer prevention. Carotenoids likes α-carotene, lutein, lycopene, and β-carotene seem to be promising carotenoids, as these carotenoids showed strong anticarcinogenic activity in screening tests. Furthermore, these carotenoids may be more suitable in combinational use, rather than in single, since it has been found that the treatment with mixture of these carotenoids resulted in more effective inhibition than each carotenoid alone (Murakoshi *et al*., 1992).

α-Carotene showed higher activity than β-carotene to suppress the tumorigenesis in skin, lung, liver and colon (Nishino *et al*., 2008). Lutein is distributed among a large variety of vegetables, such as kale, spinach, and winter squash, and fruits, such as mango, papaya, peaches, prunes, and oranges (Nishino *et al*., 2008). An epidemiological study conducted in the Pacific Islands indicated that people with high intakes of the combination of three carotenoids (β-carotene, α-carotene, and lutein) had the lowest risk of lung cancer (Le Marchand *et al*., 1993).

Lycopene occurs in our diet, predominantly in tomatoes and tomato products (Nishino *et al*. 2008). An inverse association between high intake of tomato products and prostate cancer risk was reported (Giovannucci *et al*., 1995). In a case control study in Italy, high consumption of lycopene from tomatoes was shown to have a potential protective effect against cancers of digestive tract (Franceshi *et al*., 1994). An epidemiological study among elderly Americans indicated that high tomato intake was associated with a 50 % reduction of mortality from cancers at all sites (Colditz *et al*., 1985).

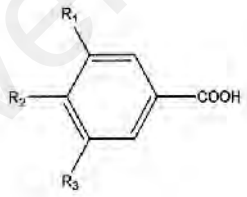
Zeaxanthin and fucoxanthin are also promising as cancer-preventive carotenoids. Other carotenoids, such as astaxanthin, neoxanthin, capsanthin, crocetin, and phytoene, also seem to be valuable in the field of cancer control (Nishino *et al*., 2008).

Phenolic compounds

Phenolic compounds constituents a group of substances that are widely present in the plant kingdom, where more than 8,000 are known, with different chemical structures and activities. They are found in fruits, vegetables, nuts and seeds, as well as in tea, red wine, citrus fruits and other food sources (Benavente-García *et al*., 1997; Middleton *et al*., 2000), which exist as ester or alcohol depending of the plant genera and parts used (Suntornsuk, 2002).

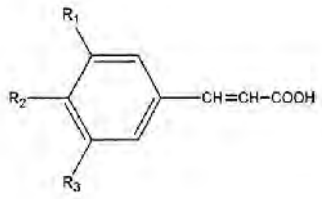
Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally categorized as phenolic acids, flavonoids, stilbenes, coumarins and tannins. Phenolics are the products of secondary metabolism in plants, providing essential functions in the reproduction and the growth of the plants; acting as defence mechanisms against pathogens, parasites, and predators, as well as contributing the colour of plants (Liu, 2004). Figure 2.13 illustrated the structures of common phenolic acids.

(a) Benzoic acid



Benzoic acid Derivatives	Substitutions		
	R_1	R_2	R_3
p-Hydroxybenzoic	H	OH	H
Protocatechuic	H	OH	OH
Vanillic	CH_3O	OH	H
Syringic	CH_3O	OH	CH_3O
Gallic	OH	OH	OH

(b) Cinnamic acid



Cinnamic acid Derivatives	Substitutions		
	R_1	R_2	R_3
p-Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	CH_3O	OH	H
Sinapic	CH_3O	OH	CH_3O

Figure 2.13: Structures of common phenolic acids: (*a*) benzoic acid and derivatives; (*b*) cinnamic acid and derivatives.
(Liu, 2004)

Flavonoids

Flavonoids are widely distributed group of plant phenolics and abundant in fruits, vegetables, nuts, flowers, seeds, and beverages (Wang *et al*., 1999). Flavonoids belong to the phenylpropanoid group of compounds, which are formed from the amino acid phenylalanine (Gantet and Memelink, 2002). Flavonoids are major metabolites of numerous plants and are also known as natural pigments in several fruits and vegetables (Suntornsuk, 2002). Flavonoids are a broad group of natural products found in all vascular plants, which are chemically defined as substances composed of a common 2-phenylchroman structure (C6-C3-C6), with one or more hydroxyl groups, including derivatives (Harbone and Williams, 2000; Birt *et al*., 2001).

Over 5,000 different flavonoids have been described to date and they are classified into at least 10 chemicals group. Among them, flavones (luteolin and apigenin), flavonols (quercetin, kaempferol and myricetin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate), flavanones (naringenin), anthocyanins and isoflavonoids (genistein) are particularly common in diet and most studied member of this group (Le Marchand, 2002; Liu, 2004). Figure 2.14 shows chemical structures of some common dietary flavonoids.

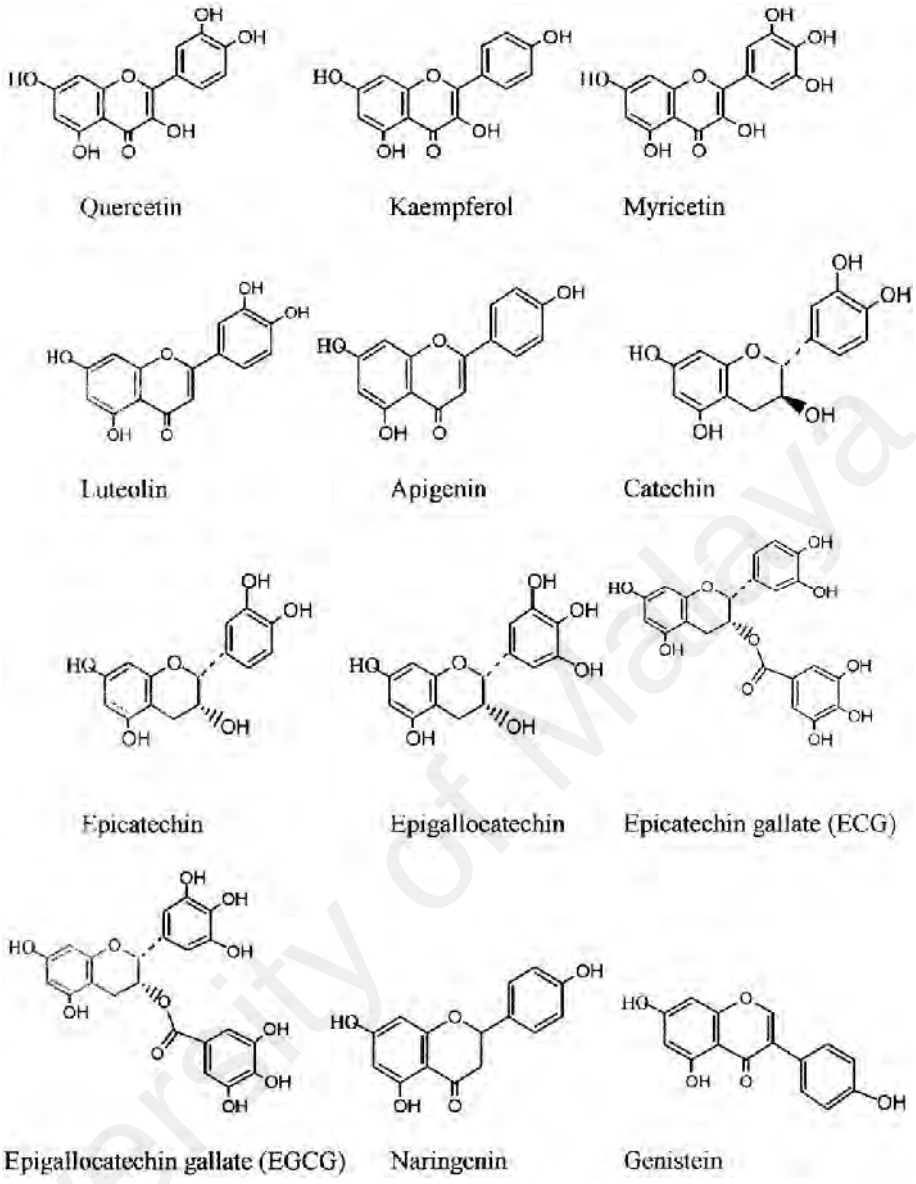


Figure 2.14: Chemical structures of common dietary flavonoids. (Liu, 2004)

Flavonoids in medicinal plants are complex and usually appear as mixtures (Suntornsuk, 2002). Flavonoids and isoflavonoids occur commonly as ester, ether, or glycoside derivatives or mixture thereof, and embrace over 4,000 compounds (Harborne, 1989).

In recent years, considerable attention has been given to their abilities to inhibit the cell cycle, cell proliferation, and oxidative stress, and to induce detoxification enzymes,

apoptosis, and the immune system (Birt *et al*., 2001). There have been a number of reports on flavonoids inducing apoptosis in cancer cells (Wang *et al*., 1999; Le Marchand, 2002; Park *et al*., 2008) and suppressing carcinogenesis in various animal models (Yang *et al*., 2001). Flavones and flavonols have been shown to possess cytotoxic effects on some cancer cells, such as colon cancer cells (Chen *et al*., 2004; Takagaki *et al*., 2005; Lim do *et al*., 2007), cervical cancer cells (Horinaka *et al*., 2005; Chiang *et al*., 2006), prostate cancer cells (Vijayababu *et al*., 2006), lung cancer cells (Leung *et al*., 2007) and esophageal cancer cells (Qiang *et al*., 2009).

There is currently considerable interest in these compounds as they appear to exert a beneficial effect on several key mechanisms involved in the pathogenesis of cancer. The antioxidant properties of flavonoids were the first mechanism of action studied, in particular with regards to their protective affect against cardiovascular diseases. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Bravo, 1998), which are possibly involved in DNA damage and tumour promotion (Cerrutti, 1985).

In estrogens-dependent tumour cells or animal models, this antiproliferative effect has been related to the antiestrogenic properties of certain flavonoids (e.g., isoflavonoids, quercetin) (Miodini *et al*., 1999). In other *in vitro* models, flavonoids have been shown to affect cell-signalling and cell cycle progression. Genistein and quercetin inhibit protein tyrosine kinase which is also involved in cell proliferation (Akiyama *et al*., 1987; So *et al*., 1996). Apigenin, luteolin and quercetin have been shown to cause cell cycle arrest and apoptosis by a p53-dependent mechanism (Plaumann *et al*., 1996).

2.6 Cancer

Cancer is one of the leading causes of death in both developed countries and developing countries and continues to be a major public health problem in many parts of the world (Jemal *et al*., 2010). Much effort has been made to develop various approaches to reduce the threat cause by cancer as only modest progress has been made in reducing the morbidity and mortality of this dreadful disease (Hail Jr. *et al*., 2008).

According to the world cancer report released by the International Agency for Research on Cancer, globally there were 12.4 million new cancer cases in 2008 (6,672,000 in men and 5,779,000 in women) and 7.6 million deaths from cancer (4,293,000 in men and 3,300,000 in women). Over half of the incident cases occurred in residents of four WHO regions with a large proportion of countries of low-and middle-income-AFRO, EMRO, SEARO and WPRO. Figure 2.5 shows the distribution of global cancer burden by WHO for 2008. It is estimated that in Southeast Asian Region (SEARO), there were 1,589,000 incident cases of cancer in 2008 (758,000 in men and 831,000 in women) and 1,072,000 deaths from cancer (approximately 557,000 in men and 515,000 in women). Figure 2.6 shows the cancer incidence and mortality in Southeast Asian Region (SEARO). In men, the commonest cancer was lung cancer, followed by oral cancer, pharyngeal cancer, oesophagus cancer, stomach cancer, colorectal cancer, liver cancer and cancer of the larynx and in women, cervical cancer and breast cancer were the commonest cancer (Boyle and Levin, 2008).

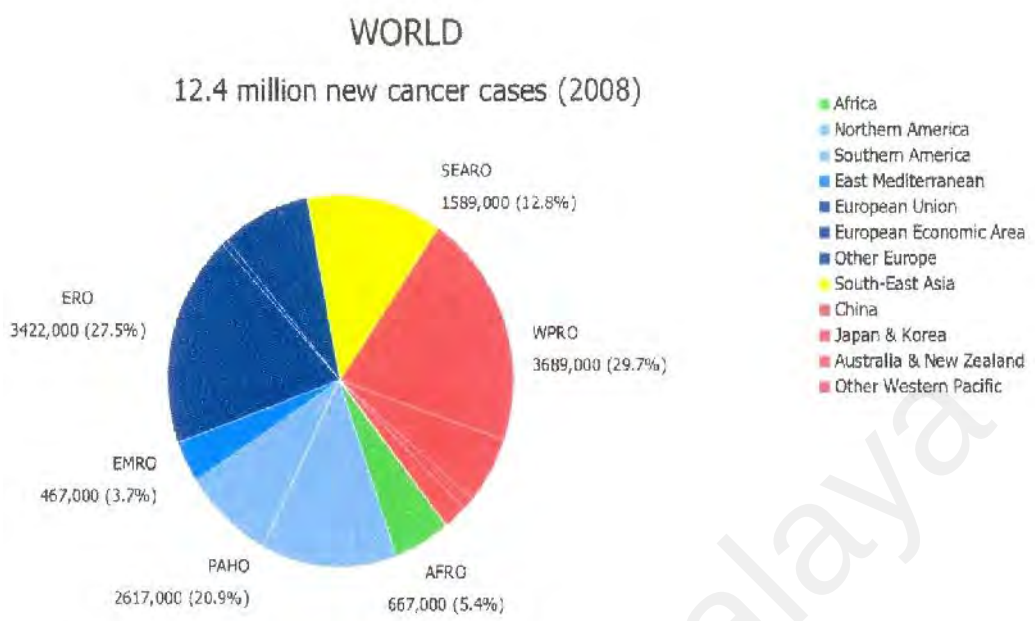


Figure 2.15: Distribution of Global Cancer burden by World Health Organization Region 2008
(Boyle and Levin, 2008)

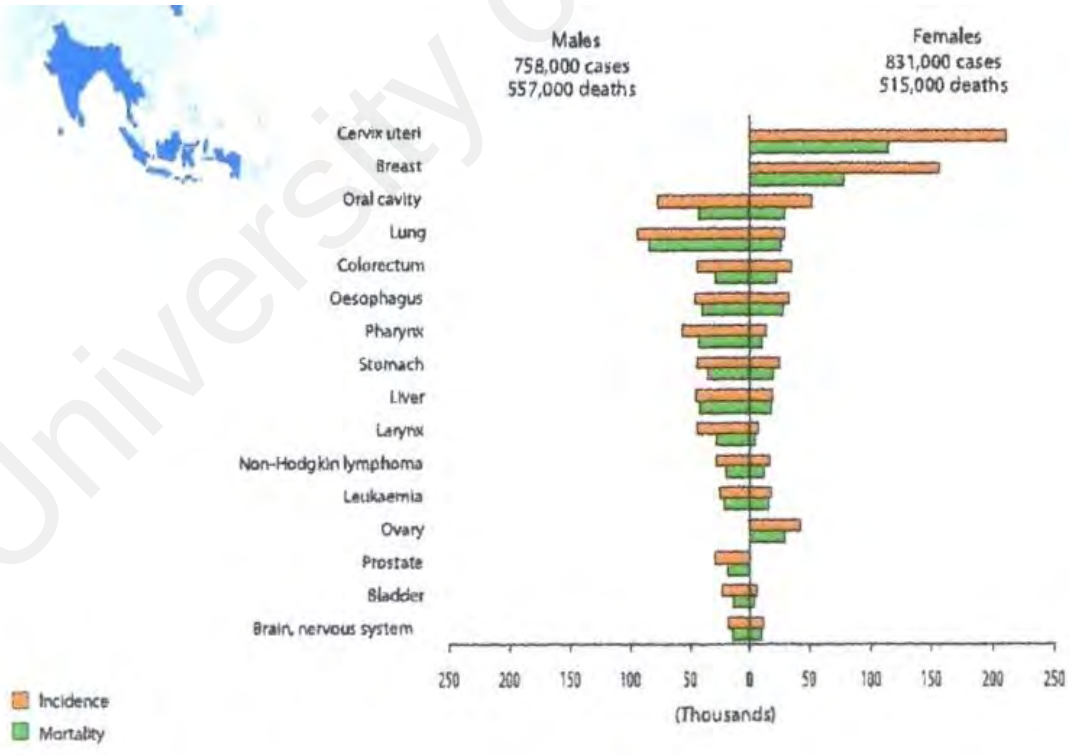


Figure 2.16: Cancer incidence and mortality in World Health Organization Southeast Asian Region (SEARO)
(Boyle and Levin, 2008)

Cancer is a group of disease characterized by uncontrolled growth and spread of abnormal cells (American Cancer Society, 2009). Cancer develops when the balance between cell proliferation and cell death is disturbed, and aberrant cell proliferation leads to tumour growth (Vaux and Korsmeyer, 1999). This neoplastic transformation occurs in three distinct steps; initiation, promotion and progression (Chemma *et al*., 2001). The "initiation" step involves interaction of carcinogens with DNA leading to transformation of normal cells to tumour cells. Since initiation is the result of permanent genetic change, any daughter cells produced from the division of the mutated cell will also carry the mutation. In the "promotion" step, the tumour promoter promotes the proliferation of the cell, giving rise to a large number of daughter cells containing the mutation created by the initiator. Promotion, resulting in the development of numerous benign tumours, is accomplished by the repeated application of a nonmutagenic tumour promoter. The conversion of numerous benign tumours to a neoplasm and to malignancy is known as progression step (Troll and Wiesner, 1985; Hennings *et al*., 1993; Aggarwal *et al*., 2009). Figure 2.7 shows various steps in the development of cancer in humans.

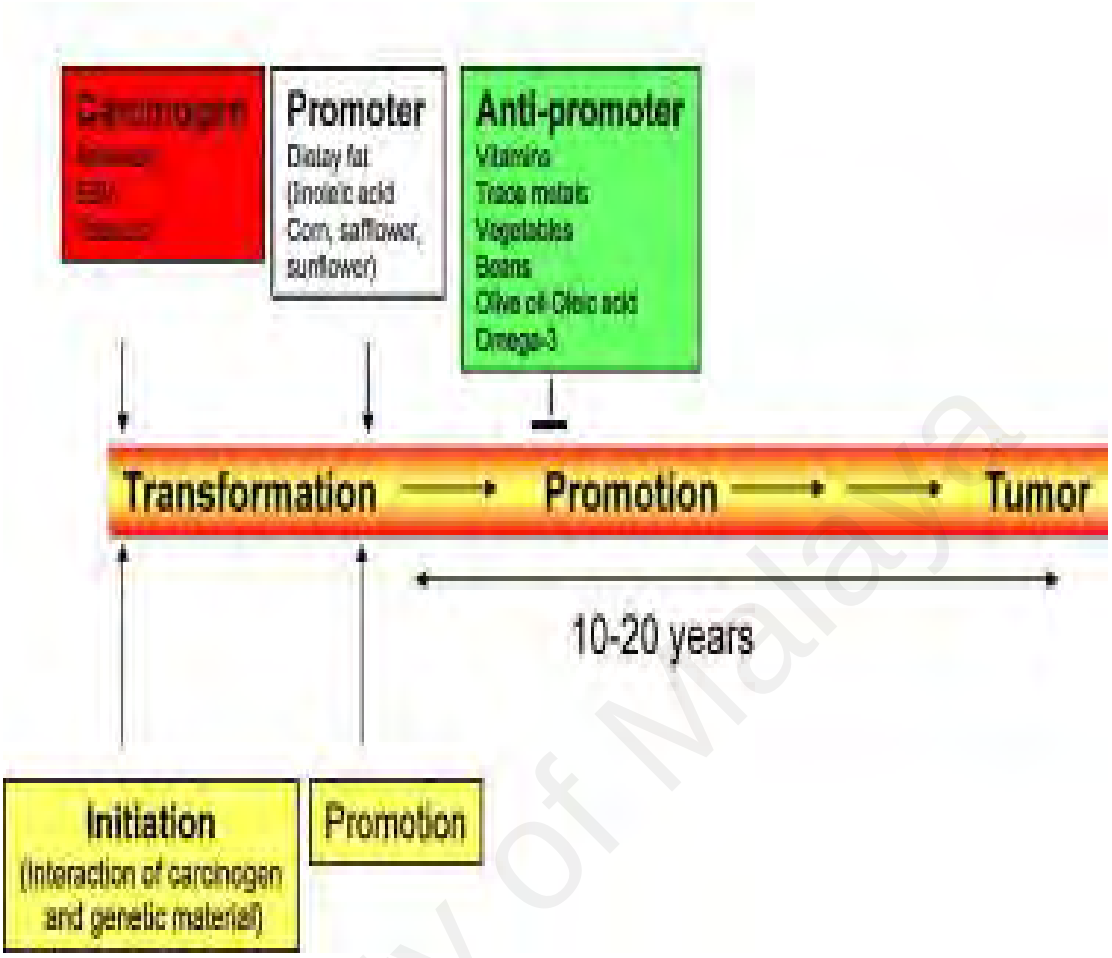


Figure 2.17: Carcinogenesis in humans (Aggarwal *et al*., 2009).

Development of cancer cells are trigger by both external (exogenous) factors (tobacco, infectious organisms, chemicals, and radiation) and internal (endogenous) factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). Both exogenous and endogenous factors may act together or in sequence to initiate DNA damage and promote carcinogenesis (American Cancer Society, 2009).

DNA damage is one of the underlying causes of mutations leading to cancer. Most mutations are associated with defective mismatch repairs resulted in inactivation of genes for DNA repair process (Friedberg *et al*., 2004). Deregulation in cell growth disrupts the

normal processes of cell division, which are controlled by the genetic material (DNA) of the cells (Reddy *et al*., 2003). During cell division, DNA opens and is replicated and at this stage various cancer-causing external stimuli (such as UV-radiation or exposure to carcinogens) all kinds of lesions and mutation can occur and seriously damage the DNA (Kroll *et al*., 2010). DNA damage can contribute to genomic instability and inducing mutations in critical DNA stability genes. Figure 2.8 illustrates the causes, cellular responses and consequences of DNA damage.

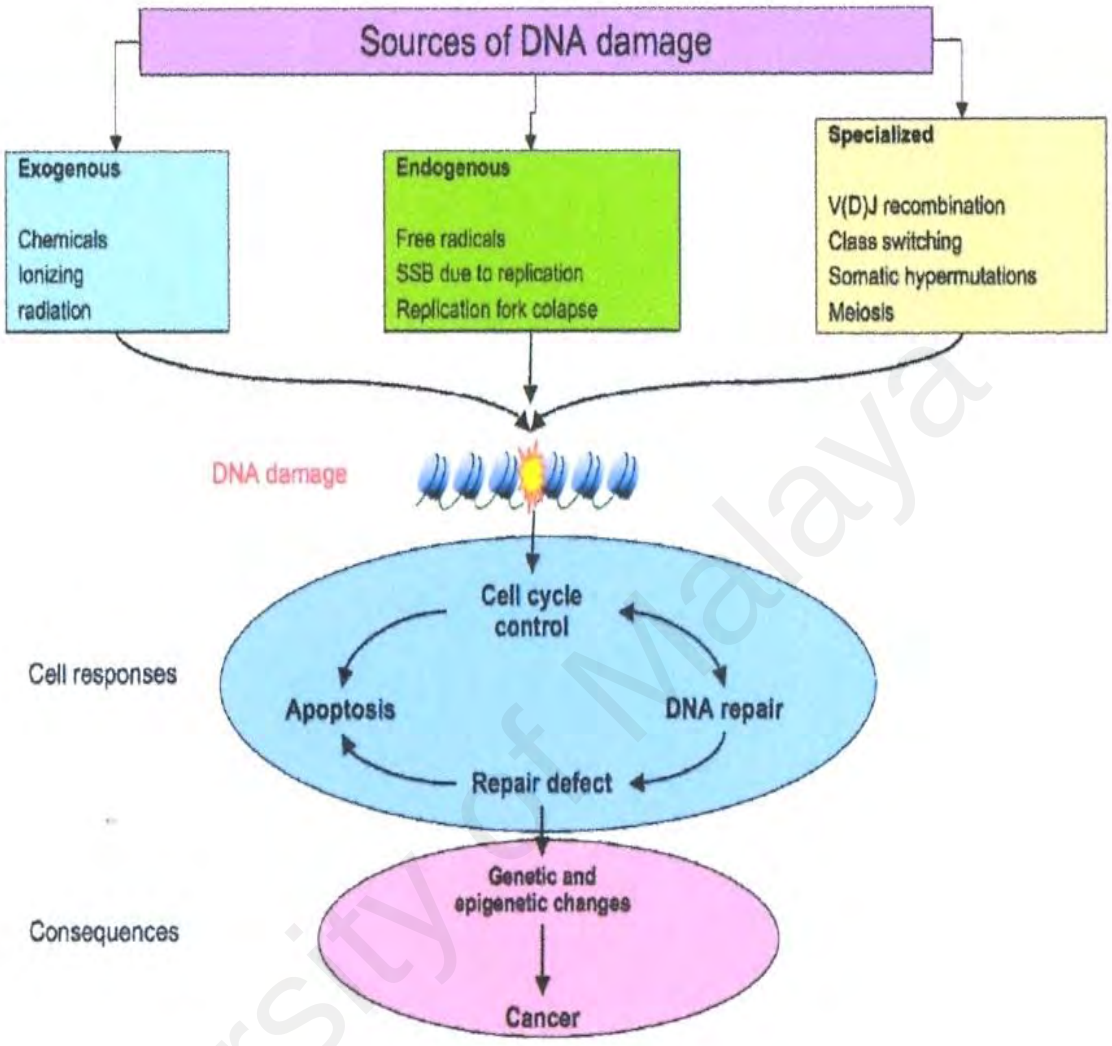


Figure 2.18: Causes, cellular responses and consequences of DNA damage (Boyle and Levin, 2008)

Breast cancer is the most frequently diagnosed cancers and the leading cause of cancer death in woman in both economically developed and developing countries. The number of newly diagnosed cases in the year 2008 was about 1.38 million, accounting for 23 % of the total new cancer cases and 458,000 (14 %) of the total cancer deaths world-wide. About half the breast cancer cases and 60 % of the deaths are estimated to occur in economically developing countries (Jemal *et al*., 2011). Even though the Asian region was

marked with the lowest geographical incidence (Parkin *et al*., 2005), data from the National Cancer Registry of Malaysia for 2004 showed that approximately 1 in 20 women in the country develop breast cancer in their lifetime (Yip *et al*., 2006). Breast cancer incidence increases with age, with about 80 % of all breast cancer arising in women over 50 years of age at diagnosis (Thomas and Leonard, 2009). There is epidemiological evidence that breast cancer is estrogenically dependent and early menarche, late menopause, delayed first pregnancy, use of hormone replacement therapy are risk factors for the disease (Chang, 1998).

Ovarian cancer is the most lethal gynaecologic cancer in the Western world (Peng *et al*., 2008), and the seventh most common cause of cancer death in women worldwide with 225,000 of new cases been diagnosed and 140,200 deaths from ovarian cancer in the year of 2008 (Jemal *et al*., 2011). While other gynaecological cancers can be diagnosed at an early stage due to effective screening (e.g. PAP smear in the case of cervical cancer) or symptoms (e.g. bleeding in the case of endometrial cancer), an early detection test for ovarian cancer does not exist and early disease is usually silent. Seventy percent of patients therefore present with advanced disease for which the 5-year survival rate is less than 30 %. Surgery and adjuvant chemotherapy with the two-drug combination carboplatin and paclitaxel is currently the standard treatment for advanced ovarian cancer. Despite an initial response rate of up to 80 % to first-line chemotherapy, most ovarian cancers will eventually recur. The underlying acquired resistance to chemotherapy is ultimately the reason for treatment failure and death of the patient (Ricciardelli and Oehler, 2009).

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide, accounting for 9 % (529,800) of the total new cancer cases and 8 % (275,100) of the total cancer deaths among females in 2008 (Au *et al*., 2007; Chung *et al*., 2009; Jemal *et al*., 2011) with more than 80 % of these cases and deaths

occur in low-resource countries (Parkin, 2001). Cervical cancer typically develops over many years after initial infection with the human papillomavirus (HPV), persistence and progression of this infection to high-grade intraepithelial neoplasia, and eventual invasion. The incidence of HPV infection characteristically peaks soon after initiation of sexual activity, usually in the late teens and early twenties, and is closely followed by a smaller peak in the incidence of HPV-induced, cytologically evident low-grade cervical lesions (Schiffman, 1992).

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 (Millan and Huerta, 2009; Jemal *et al*., 2011).The incidence rates substantially higher in males than in females (Jemal *et al*., 2011) and it becomes one of the most common malignant disease in both men and women in Asia (Sung, 2007). Colon cancer advances slowly, starting from hyperplastic lesions to early adenoma, to benign polyps, via localized tumours to invasive cancers and metastases (Vogelstein and Kinzler, 2004). Diets high in red and processed meat (Norat *et al*., 2002) and low in green vegetables are associated with increased colon cancer risk. This association might be partly due to the haem content (the iron porphyrin pigment) of red meat (Sesink *et al*., 1999; de Vogel *et al*., 2005). Most colorectal cancers take approximately 8 – 10 years to develop from an adenomatous polyp into an invasive cancer (Sung, 2007).

2.6.1 Cell Cycle

The cell cycle is an essential process in the development, differentiation, and proliferation of eukaryotic cells. Progression through the cell cycle is controlled by the appropriate and timely activation and inactivation of cell cycle-related proteins. Any

dysregulation of the events may lead to cellular dysfunction or even death. Dysregulation of the cell cycle machinery has been implicated in cancers (Wang *et al*., 2009).

In cells that are dividing, the nuclear DNA molecules must be duplicated and then distributed in a way that ensures that the two new cells each receive a complete set of genetic instructions. In preparing for and accomplishing these tasks, a cell passes through a series of discrete stages called G_1 phase, S phase, G_2 phase and M phase (Figure 2.9). The four phases are collectively referred to as the cell cycle (Bury and Cross, 2003; Kleinsmith, 2006).

G_1 (gap 1) phase, is the first phase to occur after a cell has just divided and varies the most in length. G_1 represents a critical period in which the decision is made either to proliferate or enter G_0. Growth occurs, and cellular metabolic requirements are at the highest. Since the duration of S, G_2 and M are more constant, the length of G_1 is the major determinant of the overall length of the cell cycle. A typical G_1 phase lasts about 8 to 10 hours in human cells, but rapidly dividing cells may spend only a few minutes or hours in G_1. Conversely, cells that divide very slowly may become arrested in G_1 and spend weeks, months, or even years in the offshoot of G_1 called the G_0 phase (G zero) (Bury and Cross, 2003; Kleinsmith, 2006).

After completing G_1, the cell enters S (synthesis) phase, a phase where the chromosomal DNA molecules are replicated. This typically takes roughly about 6 to 8 hours, although this time can be extended considerably in the presence of DNA damage or if replication is hindered in any way (Bury and Cross, 2003; Kleinsmith, 2006).

During the G_2 (gap 2) phase, 3 to 4 hours are spent making final preparations for cell division and to complete the DNA replication process by appropriately packaging chromosomes and sister chromatids. Like G_1, G_2 represents more than a passive ‘gap’. Cell

growth continues, and proteins required for mitosis are synthesized (Bury and Cross, 2003; Kleinsmith, 2006).

The cell then enters M phase, which lasts between 30 min and 1 h, time less than 5 % of the total duration of the cell cycle. The main events of M phase include division of the nucleus, or mitosis, followed by division of the cytoplasm. Strictly, mitosis refers only to nuclear division, with the subsequent formation of two daughter cells being termed *cytokinesis*. The two newly formed cells then enter G_1 phase and the cycle repeats (Bury and Cross, 2003; Kleinsmith, 2006).

The G_1, S, and G_2 phases are collectively referred to as interphase. Besides providing the time needed for a cell to make copies of its DNA molecules, interphase is also a period of cell growth. Interphase occupies about 95 % of a typical cell cycle; whereas the actual process of cell division (M phase) only takes about 5 % (Kleinsmith, 2006).

At the molecular level, this critical biological process is orchestrated by the expression and activation of cell cycle proteins, including the cyclin-dependent kinases (cdks) and their regulators, the cyclins and the cyclin-dependent-kinase inhibitors (CKIs) (Kohn, 1999; Bury and Cross, 2003). Cyclin D interacts with cdk4 or cdk6 to form a cdk4/6-cyclin D complex that prompts the cells to enter the G_1 phase. The cdk4/6-cyclin D complex phosphorylates Rb and releases E2F to activate DNA transcription. Progression through the cell cycle can be blocked by endogenous inhibitors, including the Cip/Kip family (p21, p27 and p57) and the INK4 family (p16, p15, p18 and p19) and exogenous inhibitors acting at various points of the cycle (Wang *et al*., 2009).

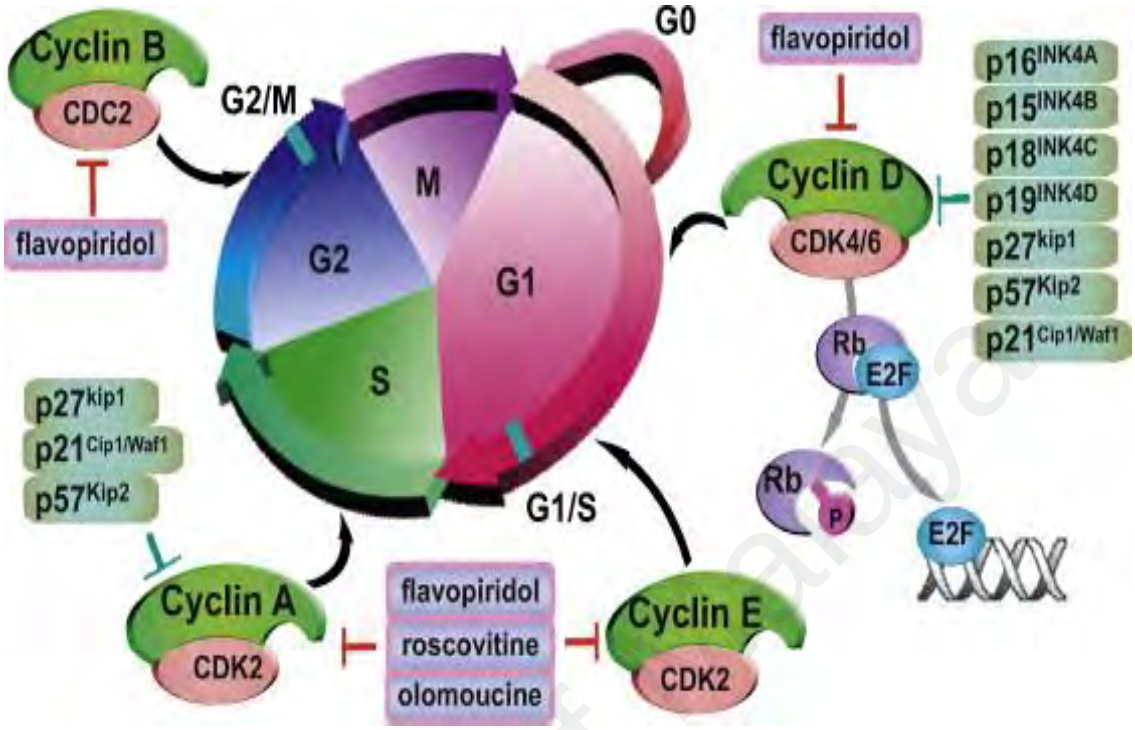


Figure 2.19: Cell cycle progression
(Adapted from Wang *et al*., 2009).

Cell division is tightly regulated and is dependent upon growth factors and other external stimuli. In the absence of such stimuli, cells in G_0 will not enter the cell cycle and will instead remain quiescent. Similarly, the daughter cells resultant from a completed cycle will, in the absence of continuing external stimulation, pass to G_0 rather than undergoing further division. However, once triggered, the cycle is completed in full even if those factors necessary for its initiation are removed. This helps ensure that changes in the external environment do not result in the premature cessation of the cycle before its proper completion, thus avoiding undesirable outcomes such as the single, tetraploid cell which would result from a cell leaving the cycle in G_2, for example (Blagosklonny and Pardee, 2002).

The time-point at which a cell becomes committed to division independent of further stimulation falls in late G_1, 2 – 3 h before the G_1/S phase transition, and is termed the *restriction point* (Pardee, 1974). The restriction point is a major determinant of the fate of the daughter cells resultant from mitosis, and represents a crucial mechanism in controlling the balance between differentiation and proliferation (Bury and Cross, 2003).

Once the restriction point has been passed and S phase initiated, the ordered progression of cell-cycle events is achieved in two ways. Intrinsic links between processes exist in some cases, whereby one event is necessarily dependent on the result of some previous step. *Checkpoints* pathways, on the other hand, provide molecular mechanisms for establishing dependency between events where no such dependency would otherwise exist. These signalling pathways operate at key time points during the cell cycle to arrest further progression until appropriate conditions have been achieved, or to direct the cell towards fates other than division – i.e. apoptosis. The most important checkpoint pathways are those concerned with sensing and responding to DNA damage, and sensing and responding to failure appropriately to complete the key cell-cycle activities of DNA replication and spindle formation (Samuel *et al*., 2002). Progression through the cell cycle is thus not dependent upon the passage of time, but upon the establishment of an appropriate molecular milieu by previous stages of the cycle (Bury and Cross, 2003).

External stimuli are the major regulators of initiation of the cell cycle through the restriction point, whilst checkpoint pathways monitor the *intracellular* state as it changes throughout the cycle. Physiologically passage through the G_1 checkpoint is controlled by the RB protein and its phosphorylation state. DNA damage or similar stimuli activate p53 and, subsequently, p53 activation induces cell cycle arrest at the G_1 or G_2 checkpoint (Strahm and Chapra, 2005; Jaroudi and SenGupta, 2007). Figure 2.10 shows the different phases of the cell cycle with the two distinct checkpoints, the G_1 and G_2 checkpoints.

Cell cycle arrest at G_1/S or G_2/M checkpoints allows DNA repair to take place before commencing DNA synthesis or committing to cell division (Aquilina *et al*., 1999).

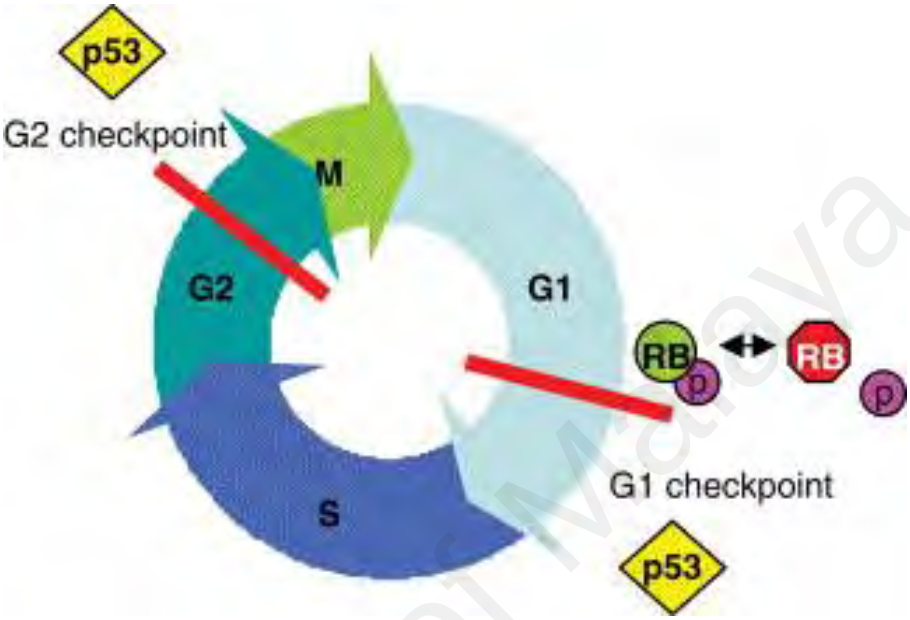


Figure 2.20: Progression through the four phases of the cell cycle, G_1 (gap 1), S (DNA synthesis), G_2 (gap2) and M (mitosis) (Strahm and Capra, 2005).

2.6.2 Apoptosis

Definition

Apoptosis is derived from Greek word and refers to the dropping or falling of leaves from a tree (Kiechle and Zhang, 2002). The term "apoptosis" was first coined in 1972 to describe a series of morphologic alterations associated with cell death in experimental model of ischemia and hormonal withdrawal (Kerr *et al*., 1972).

Apoptosis, or programmed cell death is a highly regulated process that occurs in almost all living cells that eliminates no longer wanted cells. It occurs in a systematic fashion and is characterized by a sequence of morphological alterations and biochemical changes upon activation of a series of molecular events (Allen *et al*, 1997; Hu and Kavanagh, 2003; Wang *et al*., 2005; Millan and Huerta, 2009).

These morphologic alterations include cell shrinkage due to loss of cell volume, chromatin and cytoplasmic condensation, organelle relocalization and compaction, production of numerous cell surface protuberances or "blebs" at the plasma membrane (membrane blebbing) and production of membrane-enclosed particles containing intracellular material known as 'apoptotic bodies' (Kerr *et al*., 1972; Bortner *et al*., 1995; Ioannou and Chen, 1996; Bold *et al*., 1997; Gosslau and Kuang, 2004; Wang *et al*., 2005).

These events are often accompanied by separation of the nucleus into several discrete fragments, and finally, fragmentation of the cell into numerous membrane-bounded vesicles, which are phagocytosed and digested by nearby resident cells (Kerr *et al*., 1994; Martin and Green, 1995) due to the exposure of phosphatidylserine and changes in surface sugars (Gosslau and Kuang, 2004). There is no associated inflammation (Kerr *et al*., 1994).

A characteristic biochemical feature of the process is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the DNA dreakdown into multiples of ~200 bp oligonucleosomal size fragments (Kerr *et al*., 1994; Ioannou and Chen, 1996). At the molecular level, apoptosis represents a collection of intricate pathways with more than 100 different proteins actively participating in activities from signal transduction, zymogen-type cascade, to precision surgical execution of key cytoskeletal structures and command centre DNA within marked cell which lead to DNA fragmentation, blebbing, the formation of apoptotic bodies and ultimately to cell death (Gosslau and Kuang, 2004).

These properties differ significantly from characteristics of necrosis or accidental cell death, where pronounced swelling of the cell, as well as the nucleus and organelles within, is observed (Martin and Green, 1995). Necrosis is accompanied by membrane rupture and leakage of cellular contents, organelle dysfunction, mitochondrial collapse and ultimately cellular disintegration and it thus often causes tissue inflammation (Bortner *et al*., 1995; Bold *et al*., 1997; Zhang and Xu, 2000). The comparison of the apoptosis and necrosis features is shown in Table 2.5 and the morphological difference of the two phenomena is illustrated in Figure 2.11.

Apoptosis is involved in a whole array of normal physiologic processes that plays an important role in many normal functions in multicellular organisms by allowing the organism to tightly control cell numbers and tissue size in many developmental and physiological settings (Hengartner, 2000; Hu and Kavanagh, 2003). The ability to regulate cell death with a similar efficiency to cell growth and differentiation and to protect itself from rogue cells that threaten homeostasis is vital (Martin and Green, 1995; Hengartner, 2000).

Apoptosis is known to serve many critical function, such as cell deletion during embryonic development, balancing cell number in continuously renewing tissues, hormone-dependent involution in the adult, immune defence, selective immune cell deletion, and many other physiologically processes (Allen *et al*, 1997; Zhang and Xu, 2000).

Table 2.5: Comparison of the features of apoptosis and necrosis

	Apoptosis	Necrosis
Occurrence	Scattered, single cell	Massive, tissue injury
Cytoplasm	Shrinkage, condensed and dehydrated; normal organelles; later fragments	Swelling, ER and mitochondria swell
Nucleus	Chromatin condensed into crescentic masses adjacent to nuclear envelope; later fragments ('DNA ladders')	Ill-defined, randomly dispersed, smaller chromatin masses, later lyses ('DNA' smears')
Plasma and nuclear membrane	Intense convolutions (blebbing); apoptotic bodies containing normal cytoplasmic organelles; nuclear chromatin	Membrane injury/lyses; leakage of intracellular contents; dysfunction and destruction of organelles
DNA breakdown	Internucleosomal; gene activation; endonuclease	Random degradation including histones; diffuse
Tissue response	No inflammation; phagocytosis of apoptotic bodies	Inflammation

(Adapted from Allen *et al*., 1997; Bold *et al*., 1997)

As apoptosis play an essential role in immune defence, tissue homeostasis, and development, and any tilt of the balance between life and death within an organism can lead to disease. Thus, the loss essential cells of post mitotic tissues due to enhanced cell death may lead to a number of functional deficiencies and degenerative disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, myocardial infarction, arteriosclerosis, chronic inflammation, rheumatoid arthritis, sterility, or cataract. However, apoptosis can be considered a proactive self-defence mechanism of a living organism to weed out dysfunctional cells such as the precursors of metastatic cancer

cells without creating secondary oxidative stress due to inflammation (Gosslau and Kuang, 2004).

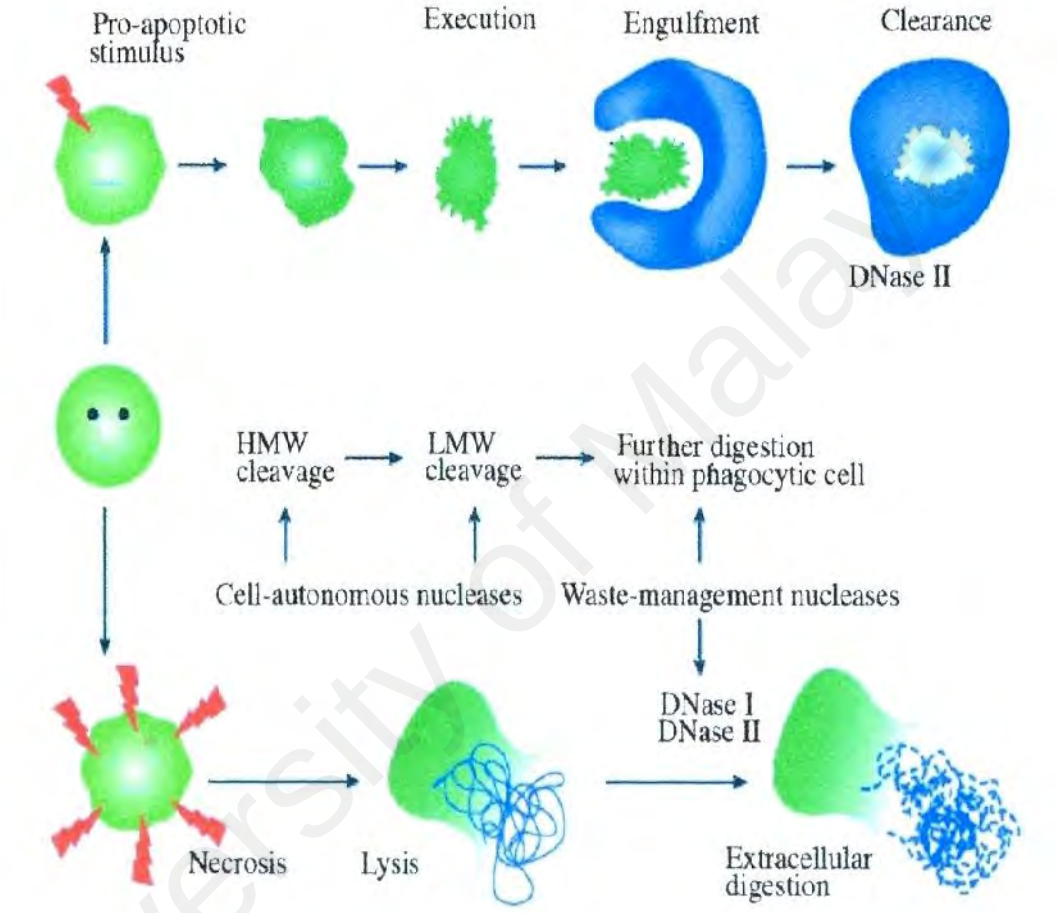


Figure 2.21: Characteristic of morphological changes and molecular machineries involved in apoptosis and necrosis (Boyle and Levin, 2008)

As apoptosis has become central to many basic and clinically oriented investigations, various methods had been established for detection of apoptotic cells, including the study of morphology, analysis of DNA degradation, DNA end labelling techniques, flow cytometric analysis, and nuclease assays (Sgonc and Wick, 1994). Table 2.6 shows some methods that been using in lab for detection of apoptosis in cell study.

Table 2.6: Methods for the detection of apoptosis

Methods	
Microscopic techniques	Cellular features by light microscopy Fluorescence DNA stains for nuclear morphologic analysis Confocal laser microscopy Electron microscopy
Assessment of DNA fragmentation	Enzyme-linked immunosorbent assay (ELISA) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling assay (TUNEL) Immunohistochemistry for single-stranded DNA Comet method DNA diffusion Gel electrophoresis
Flow cytometry and laser scanning cytometry	Cell cycle DNA content Phosphatidyserine translocation Inner mitochondrial transmembrane potential Caspase activity
Gene expression analysis	RT-PCR Northern blot RNA protection assay Immunohistochemistry
Measurements of apoptosis-associated protein	ELISA Western blot Electrophoretic mobility shift assay

(Kiechle and Zhang, 2002)

Mechanisms of Apoptosis

Apoptosis is a genetically regulated biological process, guided by the ratio of pro-apoptotic and anti-apoptotic proteins. Regulation of these proapoptotic and antiapoptotic proteins is triggered by the cascade of cell signalling and caspase-mediated events upon activation of two major signalling pathways (Hu and Kavanagh, 2003).

Apoptosis occurs through two main pathways, namely extrinsic and intrinsic apoptosis signalling pathway (Chen and Wang, 2003; Twomey and McCarthy, 2005). The process involves a carefully orchestrated sequence of intracellular events that systematically dismantle the cell. Both of these pathways activate a cascade of proteolytic enzymes system involving a family of proteases called caspases (Hu and Kavanagh, 2003; Gosslau and Kuang, 2004) (Figure 2.12).

Caspases, also known as cysteine aspartate-specific proteases, are a family of intracellular proteins and are synthesized as inactive zymogens (proenzymes) that are cleaved at the internal proteolytic site to form an active enzyme involved in the initiation and execution of apoptosis. Initiator caspases are able of activating effector caspases or amplifing the caspase cascades by increased activation of initiator caspases (Walker *et al*., 1994; Thornberry and Lazebnik, 1998).

Caspases -2, -3, -6, -7, -8, -9, and -10 are sequentially activated during apoptosis via a zymogen-type cascade. Caspases -8, -9, and -10 are considered initiator caspases responsible for activating the downstream effector caspases (-3, -6, and -7). Procaspases represent the inactive forms (zymogens) of caspases, which results in their activation (Gosslau and Kuang, 2004). Caspases are all expressed as single-chain proenzymes (30 – 50 kDa) that contain three domains: an NH2-terminal domain, a large subunit (about 20 kDa) and a small subunit (about 10 kDa) (Nicholson and Thornberry, 1997; Earnshaw *et al*., 1999; Creagh and Martin, 2001). Before, during, and after caspase activation, many important proteins are released from the mitochondria or cleaved by caspases and these proteins participate in key processes in apoptosis. There are cytochrome *c*, Apaf-1 and Poly (ADP-ribose) polymerase (PARP) (Zi *et al*., 2005).

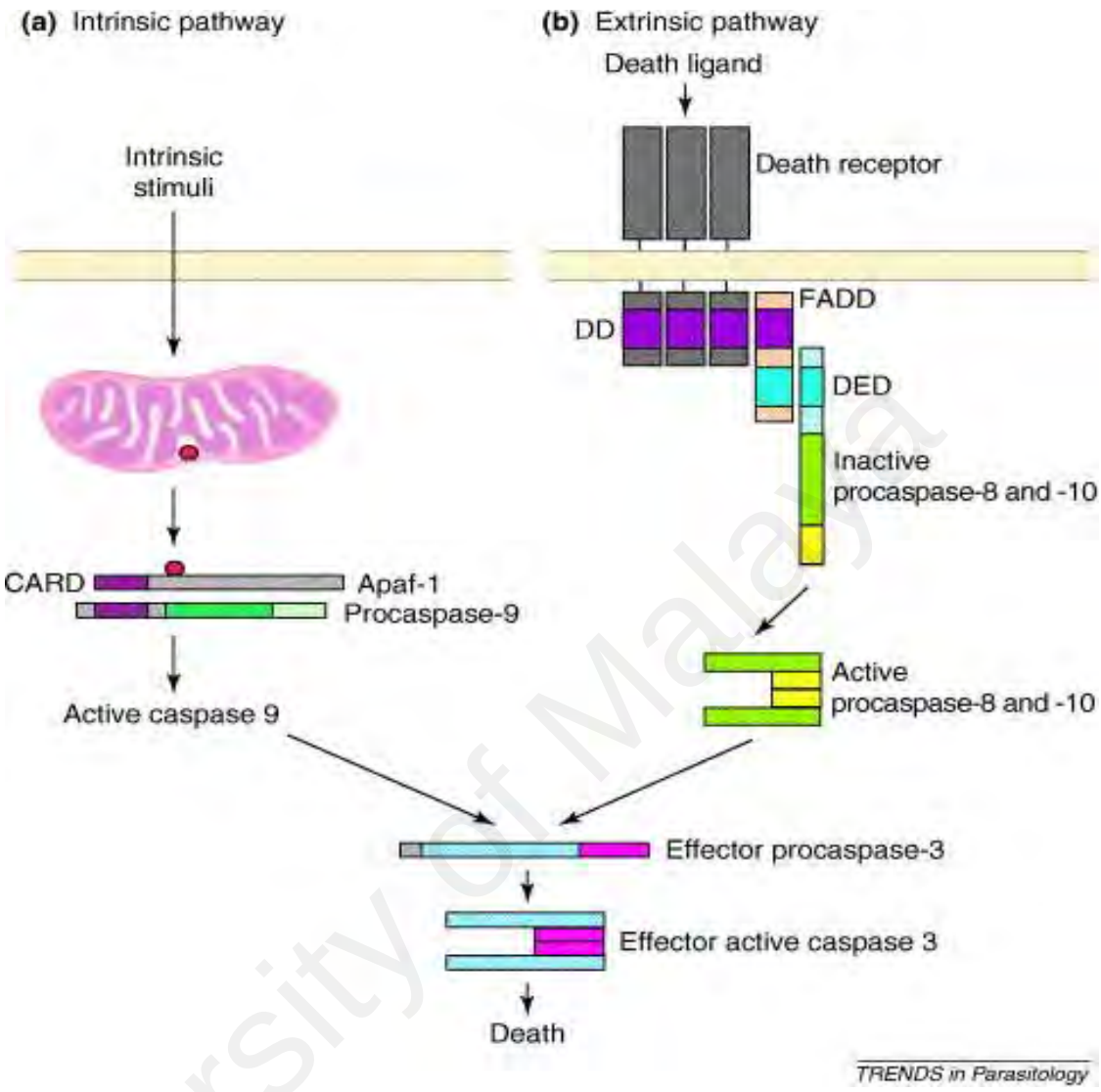


Figure 2.22: Extrinsic and intrinsic pathways of apoptosis (Dessauge *et al*., 2005)

i . Intrinsic/Mitochondrial Pathway

The intrinsic or mitochondria-mediated apoptosis pathway, is a signal transduction pathway regulated by the Bcl-2 family of proteins (Gosslau and Kuang, 2004), which involved in positive and negative regulation of apoptotic cell death (Vander Heiden and Thompson, 1999) (Figure 2.13). Among these antiapoptotic members, Bcl-2, Bcl-XL, Bcl-w, Bfl-1, Bag-1, Mcl-1 and A1 play an important role in protecting against cell death,

whereas others, such as Bax, Bak, Blk, Bad, and Bid will promote or accelerate cell death (Wolter *et al*., 1997). Antiapoptotic Bcl-2 and proapoptotic Bax are two of the major members of the Bcl-2 family. An elevated intracellular Bax/Bcl-2 ratio occurs during increased apoptotic cell death (Zha and Reed, 1997).

Bcl-2 protein which is located primarily in the outer mitochondrial membrane (Reed, 1998), blocks apoptosis by preventing cytochrome *c* release from mitochondria (Yang *et al*., 1997; Kluck *et al*., 1997) as well as inhibiting caspase-3 activity. Bax resides in the cytoplasm or is loosely attached to cellular membranes. Upon receipt of an apoptotic stimulus it inserts into the mitochondrial outer membrane and interacts with outer membrane proteins to form pores, thereby releasing cytochrome *c* (Schendel *et al*., 1998; Müllauer *et al*., 2001; Prasanna *et al*., 2009). Cytochrome *c* binds to the cytoplasmic adaptor molecule Apaf-1 (Green and Reed, 1998; Bossy-Wetzel and Green, 1999; Kroemer and Reed, 2000). The Apaf-1 then recruits pro-caspase 9, which becomes activated by autoprocessing and triggers a cascade of downstream caspase reactions (Adams and Cory, 1998; Reed, 1998; Gross *et al*., 1999) and trigger cell death.

Caspases are activated in a cascade-like fashion. Initiator or upstream caspases (caspases-8, -9 and -10) can activate effectors or downstream caspases, including caspases-3, -6 and -7, which leads to induction of apoptosis (Hu and Kavanagh, 2003).

This mitochondria-apoptosome-mediated pathway includes apoptotic stimuli induced by radiation therapy and chemotherapy, mitochondria, apoptosome, and key effector caspases (Hu and Kavanagh, 2003). The mitochondrial pathway is used extensively in response to extracellular cues and internal insults such as DNA damage (Herr and Debatin, 2001; Fan *et al*., 2005).

ii. Extrinsic/Death Receptor Pathway

The extrinsic pathway or death receptor pathway is triggered through the Fas death receptor, a member of the tumour necrosis factor (TNF) receptor superfamily (Vander Heiden and Thompson, 1999; Gosslau and Kuang, 2004) (Figure 2.13). Tumour necrosis factor (TNF) is a cytokine with pleiotropic biological activities. It stimulates immune cells to secrete cytokines, causes endothelial cells to express adhesion molecules for leukocyte binding and exerts a pyrogenic effect. The TNF induces also apoptosis in some cell types but usually only when new protein synthesis is blocked. It is produced predominantly by activated macrophages and in response to infection (Baker and Reddy, 1998; Ledgerwood *et al*., 1999).

TNF receptors are the largest known family of death receptors. The best characterized members of TNF-receptors are TNF-R1 (also called p55, CD120a), TNF-R2 (p75, CD120b), Fas (CD95, Apo-1), death receptor 3 (DR3), death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2) (Ashkenazi and Dixit, 1998; Hu and Kavanagh, 2003). The ligands that activate these receptors are structurally related molecules with homologies to tumour necrosis factor α (TNFα) (Ashkenazi and Dixit, 1998; Griffith and Lynch, 1998; Ledgerwood *et al*., 1999; Nagata, 1999; Pinkoski and Green, 1999). Upon ligand binding an intracellular 'death domain' of the receptor interacts with homologous domain in an adaptor protein, which recruits specific proteases, caspases. Caspases reside as inactive pro-forms within the cell and become activated by autocleavage when recruited to a death receptor signalling complex. Activated upstream caspases subsequently initiate a cascade of downstream effectors caspases which cleave a plethora of cellular proteins and thereby ultimately cause cell death (Thornberry and Lazebnik, 1998; Kumar, 1999; Slee *et al*., 1999). The death receptor pathway plays a fundamental role in

the maintenance of tissue homeostasis, especially in the immune system (Herr and Debatin, 2001; Fan *et al*., 2005).

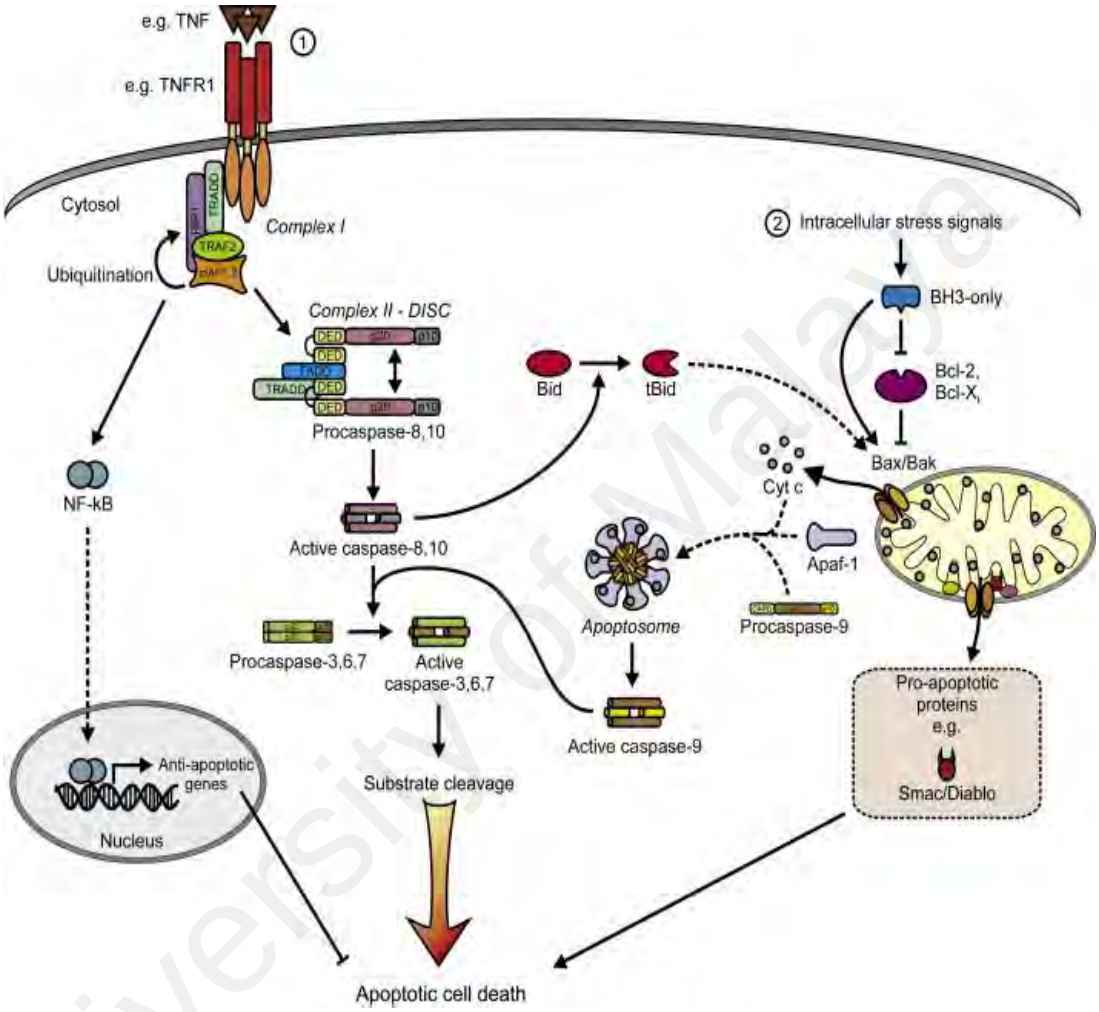


Figure 2.23: Schematic representation of extrinsic and intrinsic apoptotic signalling. (Adapted from Duprez *et al*., 2009).

Apoptosis and Cancer

Disruption in apoptosis mechanism which lead to deregulation of cell proliferation and suppression of apoptotic signalling is recognized as one of the important causes that

contributes substantially to the transformation of a normal cell to cancerous cell (Igney and Krammer, 2002; Reed, 2003; Hu and Kavanagh, 2003; Qi *et al*., 2010).

As disruption in apoptosis is critically involved in the entire progression of neoplastic transformation and metastases, activation of the apoptotic pathway in tumour cells is considered to be a protective mechanism against the development and progression of cancer. An understanding of the mechanisms involved in apoptosis may lead to potential targets for one of the newest approaches for cancer treatment (Bold *et al*., 1997).

Recently, inducer of apoptosis such as chemotherapeutic agents has been used in cancer therapy and become a rationale for therapeutic approaches and entertains the possibility that apoptosis may be enhanced in tumour for therapeutic benefit (Gibb *et al*., 1997). Chemopreventive agents refer as the compound of natural or synthetic agents that reserve, suppress or arrest carcinogenic and/or malignant phenotype progression towards invasive cancer (Rabi and Bishayee, 2008).

Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally, cures. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis (Taraphdar *et al*., 2001).

The list of chemotherapeutic agents that have been tested on variety of tumour types is lengthy and it is clearly demonstrated that most, if not all, chemotherapeutic agents ultimately induce cell death by triggering the apoptotic pathway (Bold *et al*., 1997).

Table 2.7 shows the list of chemotherapeutic agents documented to induce apoptosis and the tumour type and in which demonstrated its mechanism of action.

Modulation of apoptosis by targeting proapoptotic and antiapoptotic proteins may be an important way of treating cancer. Alternatively, tumour resistance to apoptosis is due

to inactivation of proapoptotic genes. Enhanced mutation rates of Bax, Apaf-1, and caspase-8 have been found in various types of cancer. Moreover, death receptors are down-regulated, mutated, or inactivated in many tumours (Igney and Krammer, 2002). Strategies to overcome apoptosis resistance in cancer cells may include treatment with or up-regulation of proapoptotic factors (e.g., caspases, apoptosis-inducing factor, endonucleases, Apaf-1, cytochrome *c*, Bax, Bid, p53, death receptors and/or its ligands, apoptosis signal-regulating kinase-1, second mitochondria-derived activator of caspase) and/or inhibition or downregulation of antiapoptotic factors (e.g., Bcl-2, Bcl-xL, Bfl-1, IAPs, Akt, NF-κB) (Igney and Krammer, 2002; Reed, 2003; Hu and Kavanagh, 2003).

Table 2.7: List of chemotherapeutic agents documented to induce apoptosis and tumour type in which demonstrated its mechanism of action

Chemotherapy action	Cell types tested
Dexamethasone	B-lymphocytes
Cisplatin	Myeloid, neuroblastoma
Etoposide	Neuroblastoma, lymphoma (B-cell), leukemia
Taxol	Breast carcinoma, gastric carcinoma
5-Fluorouracil	Colon carcinoma
Cytosine arabinoside	Leukemia
Doxorubicin	Breast carcinoma, leukemia
Topotecan	Leukemia, ovarian carcinoma
Bleomycin	Testicular, leukemia

(Adapted from Bold *et al*., 1997)

2.6.3 Plants as Apoptotic Inducers

Up to early 1990s, the discovery of novel antitumor agents from natural sources was largely based on testing for cytotoxic activity against cancer cell lines grown either *in vitro* or *in vivo* models (Cragg and Newman, 2005). Recently interest has been focused on the manipulation of apoptotic processes in the treatment and prevention of cancer as a new target for innovative mechanism-based anticancer drug discovery (Fisher, 1994; Sun *et al*., 2004; Hail Jr, 2005).

A vast amount of work on synthesis or modification of known drugs has contributed relatively small improvements over the prototype drugs. There is a continued need for new prototypes-new templates to use in the design of potential chemotherapeutic agents. Significantly, natural products especially plant-derived natural products are providing such templates. It is thus considered important to screen for apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them (Taraphdar *et al*., 2001).

Evidence has emerged from various studies that suggest that products derived from plants are potential apoptotic inducers and have great potentials and provide useful information for their possible application in cancer prevention (Powell *et al*., 2003; Moongkarndi *et al*., 2004; Kwon *et al*., 2006; Liu *et al*., 2006; Hu *et al*., 2009; Park *et al*., 2009; Prasanna *et al*., 2009; Radhika *et al*., 2010; Sharma *et al*., 2010; Mohd Fazley *et al*., 2010; Choedon *et al*., 2010). Figure 2.14 shows the schematic overview of the cellular events involved in the apoptotic pathway from the trigger by chemotherapeutic agent. The cytotoxic process following exposure to chemotherapeutic agents can be broken down into four separate stages. In the first stage, the chemotherapeutic agents disrupt cellular homeostasis through a specific interaction with an intra cellular target, such as RNA, DNA

or microtubules. This interaction results in dysfunction of the target structure. The second stage is the recognition by the cell of the disruption of homeostasis, which involves p53, bcl-2 and other proteins and later activates caspases, which belong to a family of cysteine proteases. Finally, the fourth stage is the initiation of apoptosis with the sequential activation of the cellular machinery leading to cell death (Bold *et al*., 1997).

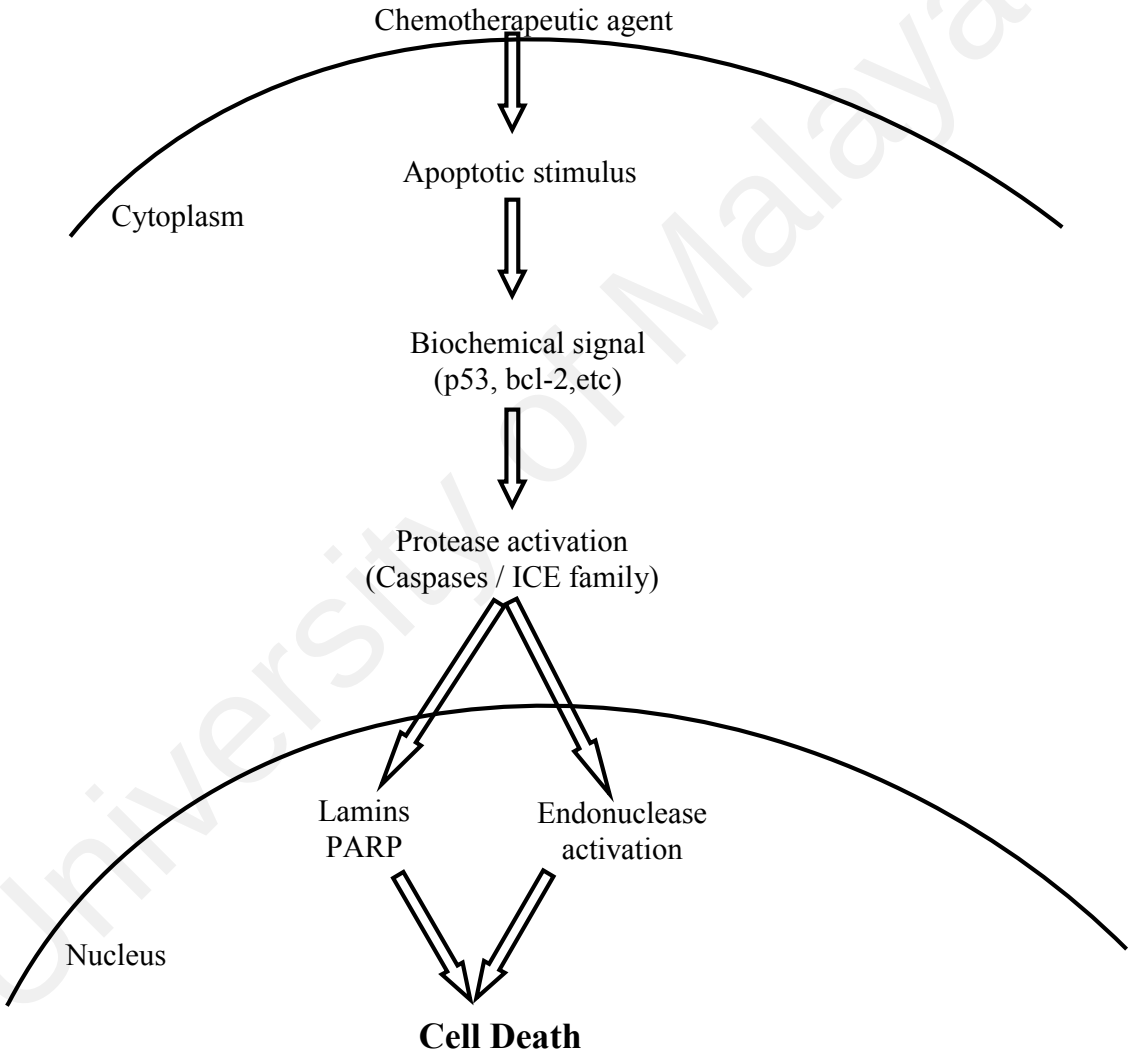


Figure 2.24: Schematic overview of the cellular events involved in the apoptotic pathway from the triggering event by chemotherapeutic agent (Modified from Bold *et al*., 1997).

CHAPTER 3

MATERIALS & METHODS

3.1 Preparation of Crude Extract of Selected Phyllanthaceae Species

3.1.1 Solvents

Organic solvents with analytical grade were used for the extraction and fractionation process on the plant samples. The organic solvents such as hexane, ethyl acetate (EtOAc), acetone (Me_2CO) and methanol (MeOH) were purchased from Fisher Scientific, UK.

3.1.2 Phyllanthaceae Samples

Phyllanthus niruri (aerial part), *Phyllanthus acidus* (leaves) and *Baccaurea motleyana* (leaves) were collected from a home garden at Port Dickson, *Phyllanthus pectinatus* (leaves and fruits) was collected from Rimba Ilmu, University of Malaya, *Phyllanthus roseus* (leaves) was collected from Gunung Machincang, Langkawi Island and *Phyllantus watsonii* (leaves) was collected from Endau Rompin Park, Johor during the month October 2007 and June 2008. Authentication of *Phyllanthus niruri* (KLU42610) *Phyllanthus pectinatus* (KLU47659), *Phyllathus acidus* (KLU47679), *Phyllanthus roseus* (KLU47660), *Phyllanthus watsonii* (KLU46068) and *Baccaurea motleyana* (KLU30038)

was carried out in the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya by Dr Sugumaran Manickam.

3.1.3 Extraction of Plant Materials

Figure 3.1 shows the schematic extraction of the plant material. The leaves, aerial parts and fruits of selected Phyllanthaceae species were cleaned immediately to remove any extraneous material, sliced and dried in a hot-air oven (Memmert) at 40 – 50 °C. The dried materials were grounded into powder and soaked in methanol with ratio 1:10 for 3 days at room temperature with shaking. The solvent-containing extract was then decanted and filtered. The extraction of the ground sample was further repeated (3 ×) with methanol with the ratio of 1:10 each time. The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) yielding a dark-greenish extract. The remaining portion of methanol extract was further shaken vigorously with hexane. The hexane soluble obtained was poured into a clean flask and this step was repeated with fresh hexane until the resultant hexane added remains almost colourless after shaking. Remaining hexane insoluble was subjected to solvent-solvent extraction with a mixture of ethyl acetate and distilled water (1:1) followed by fairly vigorous mixing to allow hexane insoluble to go into solution. This mixture was then successively fractionated using separating funnel in which two distinct layers were formed. The bottom layer, which is the water residue is released and discarded while the ethyl acetate phase (top layer) is released into a clean beaker. Resultant filtrate was concentrated under reduced pressure using a rotary evaporator to yield crude hexane and ethyl acetate extract of each plant. All the crude extracts were weighed and dissolved in

dimethylsulfoxide (DMSO), to form stock solutions of 20 mg/ml and kept in desiccators prior to use in bioassays.

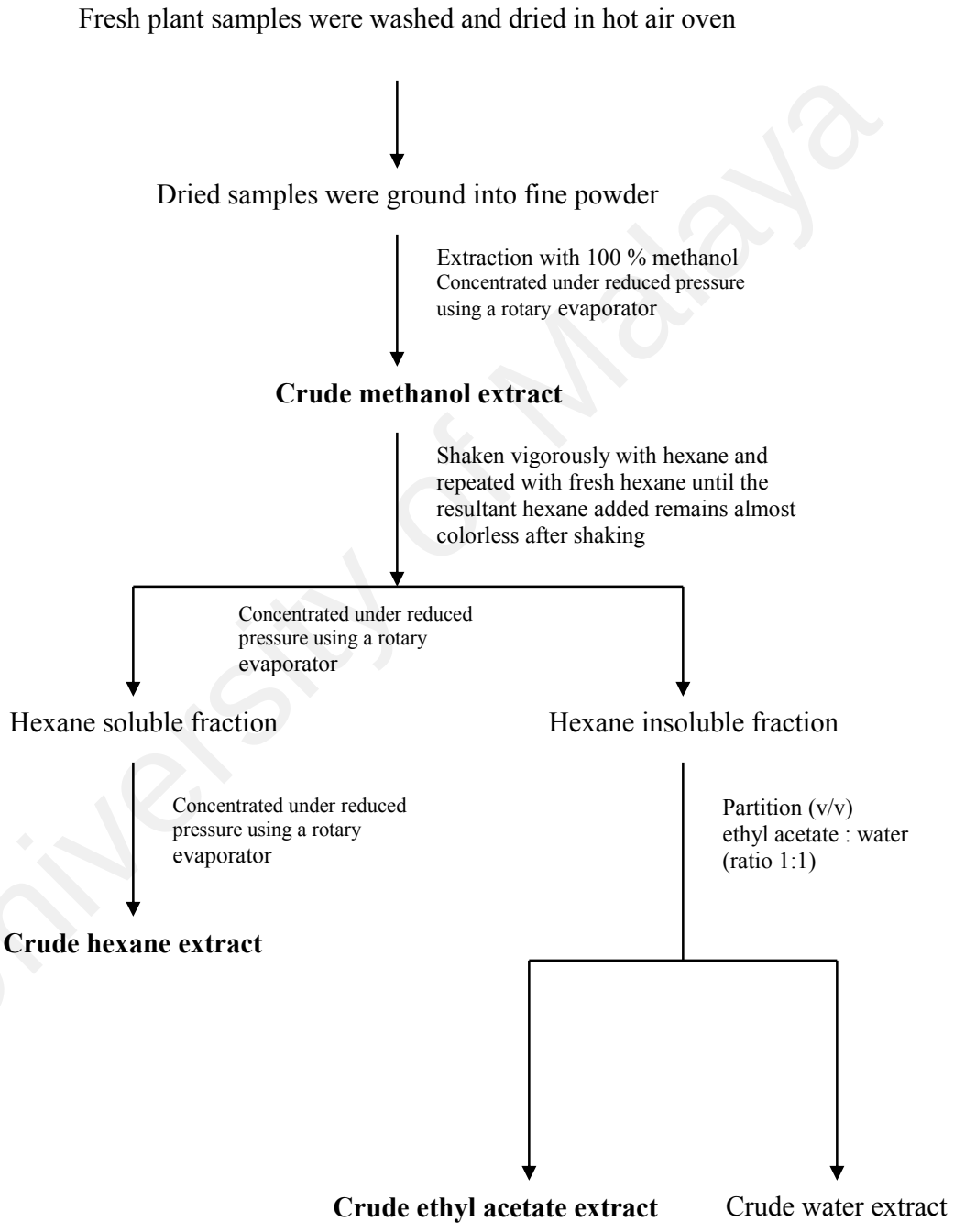


Figure 3.1: Schematic extraction of Phyllanthaceae species

3.2 Cytotoxicity Screening of Phyllanthaceae Crude Extracts

3.2.1 Chemicals

RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), MEM medium were purchased from Sigma. Foetal bovine serum, penicillin-streptomycin (100 ×) and amphotericin B (250 μg/ml) were obtained from PAA Lab., Austria. Accutase in DPBS, 0.5 mM EDTA was purchased from iCT, CA. HEPES was purchased from Molekula, UK. Neutral Red was purchased from ICN, Ohio. All other chemicals and solvents used were of the highest purity grade available purchased from BDH AnalaR, UK and Sigma-Aldrich, USA. Cell culture plastic ware was from Nunc (Denmark).

3.2.2 Cell Lines

Human breast cancer cell line (MCF7), ovarian cancer cell line (SKOV3), epidermal carcinoma of cervix cell line (CaSki), colon cancer cell line (HT29), and normal lung fibroblast cell (MRC5) were purchased from American Type Culture Collection (ATCC, USA). MCF7, CaSki and HT29 cells were cultured in RPMI 1640 media; SKOV3 in DMEM media and MRC5 in MEM media supplemented with 10 % v/v foetal bovine serum as a complete growth media. Cells were maintained in 25 cm^3 flasks with 10 ml of media and were incubated at 37 °C in an incubator with 5 % CO_2 in a humidified atmosphere (Shel Lab.). The culture was sub cultured every 2 or 3 days and routinely checked under an inverted microscope (Leica DMI 3000B) for any contamination.

3.2.3 Cytotoxic Assay

All the extracts in methanol, hexane and ethyl acetate were further subjected to Neutral Red Cytotoxic Assay in order to determine theirs cytotoxic activity on the selected human cancer cell lines.

3.2.4 Neutral Red Cytotoxic Assay

The neutral red cytotoxicity assay is based on the initial protocol described by Borenfreund and Puerner, (1984). Cells were detached from the flask with 1.0 ml solution of accutase in phosphate buffer solution (PBS) pH 7.4. The cell pellet was obtained by centrifugation at 1000 rpm for 5 min (Kubota 2420, Tokyo) and the density of the viable cells was counted by 0.4 % of tryphan blue exclusion method using a haemocytometer (Marienfeld, Germany) as described by Freshney, (1994). Cells were then plated in 96-well microtiter plate (Nunc), at a concentration of 30,000 cells/ml and then incubated in a CO_2 incubator at 37 °C for 3 h to allow the cells to adhere before addition of the test agents. After 3 h, the crude extracts were then added to the wells at six different concentrations of 1, 10, 25, 50, 75 and 100 μg/ml. Incubation with the extracts were carried out for 72 h. Negative control comprised of cells not treated with any extract. Doxorubicin was used as a positive control in the experiments.

At the end of the incubation period, the medium was replaced with a medium containing 50 μg/ml Neutral Red. The plates were incubated for another 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the medium was removed and cells were washed with the Neutral Red washing solution. The dye was eluted from the cells by incubation with 200 μl of Neutral

Red resorb solution for 30 min at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). The optical density (OD) was measured at 540 nm using microplate reader (Emax, Molecular Devices). Three replicate plates were used to determine the cytotoxic activity of each extract.

The percentage inhibition of each of the test samples was calculated according to the following equation: % of inhibition = ($OD_{control}$ - OD_{sample}) / ($OD_{control}$) x 100 %. The IC_{50} is the concentration of extract that causes 50 % inhibition or cell death (Chiang *et al*., 2003) and obtained by plotting the percentage of inhibition against respective concentration used in the assay using MsExcel. In the US National Cancer Institute Plant Screening Program, a crude extract is generally considered to have active cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 h, is equal or less than 20 μg/ml, while it is equal or less than 4 μg/ml for pure compounds (Geran *et al*., 1972; Boik, 2001).

The selectivity index (SI) was also calculated from the IC_{50} ratio of normal lung fibroblast and cancer cells (MCF7, SKOV3, CaSki and HT29). SI value indicates selectivity of the sample to the cell lines tested. Samples with SI greater than 3 were considered to have high selectivity (Mahavorasirikul *et al*., 2010).

3.3 Bioassay Guided-Fractionation of Crude Hexane Extract of *Phyllanthus watsonii*

Crude hexane extract of *P. watsonii* were found to be very active in the cytotoxicity screening assay and were further subjected to bioassay guided-fractionation methods. The fractionation was done using standard silica gel chromatography with solvent combinations of increasing polarity gradient (hexane, acetone and ethyl acetate).

3.3.1 Chemicals

Pre-coated TLC plates of silica gel 60 F254, 0.25 mm thickness (Merck) were used for analytical thin layer chromatography (TLC). Silica gel 60 (70 – 230 mesh) was purchased from Merck.

3.3.2 Thin Layer Chromatography

TLC of the active crude extracts was performed on pre-coated TLC plates of silica gel 60 F254, 0.25 mm thickness (Merck). Spot of each sample were applied using disposable capillaries. The solvent system used to separate the extract was n-hexane/acetone in the ratio 20:60 as developing solvent. The chromatographic plates were developed at room temperature in a saturated chamber. Plates were removed from the chamber after 30 – 40 min and examined both in daylight and in UV light (254 and/or 365 nm) for fluorescence and any additional spots and observations were recorded. The plate was then sprayed with *p*-anisaldehyde reagent (Sigma-Aldrich, USA) and heated in an oven at 105 °C for 5 – 10 min and spots were detected under UV light (254 and/or 365 nm).

3.3.3 Fractionation of Crude Hexane Extract of *P.watsonii* by Column Chromatography

Crude hexane extract (6.1 g) of *Phyllanthus watsonii* was chromatographed on a column (4 cm id × 40 cm length) packed with silica gel (160 g). Elution began with 100 % hexane and polarity of eluting solvent was gradually increased using acetone and methanol. Fractions of 25 ml volume were collected in numbered vials. The separations were

monitored using TLC. Zones were detected under UV light (254 and/or 365 nm) after spraying with *p*-anisaldehyde reagent and heating at 105 °C for 5 – 10 min. The fractions were pooled according to the similarity of the spots detected on TLC on pre-coated TLC silica gel 60 F254 plate using n-hexane/acetone (20:80) as developing solvent. The excess solvent was evaporated under reduced pressure using a rotary evaporator and obtained fractions were transferred into 50 ml vials.

3.3.4 Fractionation of Active Fractions of *P. watsonii* by Column Chromatography

The most active fractions (581.0 mg) obtained from the first step of bioassay-guided fractionation process were pooled and further subjected to bioassay-guided fractionation using silica column chromatography (2.6 cm id × 60 cm length) packed with silica gel (70 – 230 mesh; 160 g). Elution began with 100 % hexane and polarity of eluting solvent was gradually increased using acetone and methanol. Fractions of 25 ml volume were collected in numbered vials. The separations were monitored using TLC. Zones were detected under UV light (254 and/or 365 nm) after spraying with *p*-anisaldehyde reagent and heating at 105 °C for 5 – 10 min. The fractions were pooled according to the spots detected on TLC. The excess solvent was evaporated under reduced pressure using a rotary evaporator and the fractions were transferred into 50 ml vials.

3.3.5 Neutral Red Cytotoxic Assay

Fractions collected from the chromatography methods were subjected to Neutral Red Cytotoxicity assay as described previously. All the fractions were tested on MCF7, SKOV3, CaSki and HT29 cell lines and the IC_{50} values were determined for each fraction.

3.4 LC-MS/MS Analysis of Cytotoxically Active Fraction PPW7

The most active fraction, PPW7 was analyzed by LC-MS/MS system equipped with the 3200 QTrap mass spectrometer (Applied Biosystem, Darmstadt, Germany) and Shidmazu UHPLC system. The chromatographic separation was performed on a 50 mm × 2.0 mm × 5 μM Aqua C18 column (Phenomenex, Torrance, CA), eluted with a mobile phase consisting of water (A) and acetonitrile (B) containing 0.2 % formic acid and 2 mM ammonium formate. A gradient elution (starting from 10 % of A to 90 % of B, from 0.01 min to 10.0 min, hold for 2 min and back to 10 % of A in 0.1 min and re-equilibrated for 5 min) was used to separate the compounds of interest prior to mass spectral analysis. The mass spectrometer analysis was performed in a positive ion mode (*m/z* $M + H^+$) for detection of secondary compounds. Identities of the compounds were obtained by matching their molecular ions (*m/z*) obtained by LC-MS/MS with reference standards where available and by correlation with previous published data on chemical constituents from *Phyllanthus*.

3.5 Assessment of Apoptosis of Cytotoxically Active Crude Extracts and Fractions

According to US National Cancer Institute Plant Screening Program, a crude extract is generally considered to have active cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 h, is equal or less than 20 μg/ml, while it is equal or less than 4 μg/ml for pure compounds (Geran *et al*., 1972; Boik, 2001). Based on these criteria, only the crude extract and fraction of Phyllanthaceae species studied with IC_{50} value equal to or less than 20 μg/ml is considered cytotoxically active crude extract or

fraction and were selected for further assessment of their apoptotic activities. In order to evaluate whether the cytotoxic effects of these cytotoxically active crude extracts nd faractions upon MCF7, SKOV3, CaSki and HT29 cells were related with the apoptotic processes, we investigated the effects of these test agents on the induction of apoptotic cell death by various morphological and biochemical methods.

3.5.1 Chemicals

Acridine orange hemi(zinc chloride) salt, ethidium bromide, Tergitol® solution Type NP-40 were purchased from Sigma-Aldrich, USA. RNAse (10 mg/ml) and Proteinase K (895 U/ml) and 6 × DNA loading dye were obtained from Fermentas. Trizma-Base and Sodium acetate anhydrous were purchased from Fluka Analytical, US. Phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v/v/v) solution was obtained from Invitrogen. All other chemicals were of the molecular grade purity commercially supplied by Sigma.

3.5.2 Analysis of Cell Morphological Changes by Phase Contrast Microscope

Analysis of cell morphological changes by phase contrast microscope was carried out according to the method described by Moongkarndi *et al*., (2004). Cells (3×10^4) were plated onto 24-well tissue culture plate and incubated in a CO_2 incubator at 37 °C for 3 h to allow the cells to adhere before addition of the test agents. After 3 h, the cytotoxically active crude extracts and fractions of selected Phyllanthaceae species were then added to the wells at concentrations of 10.0, 25.0 and 50.0 µg/ml. The cells were incubated with the extracts for 24 h. The negative control is the well containing the untreated cells (without

addition of any extract). At the end of the incubation period, the medium was removed and cells were washed once with phosphate buffer saline (PBS pH 7.4). The plates were observed by phase contrast inverted microscope (Leica DMI 3000B) at 200 × magnification.

3.5.3 Analysis of Cell Morphological by Acridine Orange/Ethidium Bromide (AO/EB) Fluorescence Staining

Analysis of cell morphology by acridine orange/ethidium bromide (AO/EB) fluorescence staining was carried out according to the method described by Jimenez *et al*., (2008) with slight modifications. Cells (3×10^6) were plated onto 24-well tissue culture plate and incubated in a CO_2 incubator at 37 °C for 3 h to allow the cells to adhere before the addition of the test agents. After 3 h, the cytotoxically active crude extracts and fractions of selected Phyllanthaceae were then added to the wells at concentrations of 10.0 µg/ml. The cells were incubated with the extracts for 24 h. The negative control is the well containing the untreated cells (without addition of any test agent).

At the end of the incubation period, the cells were detached from the plate with 1.0 ml accutase in PBS pH 7.4. The cell pellet was obtained by centrifugation at 1000 rpm for 5 min and resuspended in 25 µl of PBS pH 7.4. To each sample, 1 µl of AO/EB solution (1 part of 100 µg/ml of AO in PBS; 1 part of 100 µg/ml of EB in PBS) was added just prior to microscopical examination. The cell suspension was placed on a 3-well teflon coated microscopic slide, covered with a glass coverslip and was examined with a Nikon Eclipse 80i microscope under fluorescence illumination using FITC, Cy3 and DAPI filters and images were analyzed by Nikon's Imaging Software, NIS-Elements.

3.5.4 Detection of DNA Fragmentation by Agarose Electrophoresis

The DNA fragmentation assay was carried out according to the method as described by Jin *et al*. (2006) and Matassov *et al*. (2004) with slight modifications. Cells (3×10^6) were plated onto 6-well tissue culture plate and incubated in a CO_2 incubator at 37 °C for 3 h to allow the cells to adhere before addition of the test agents. After 3 h, the cytotoxically active crude extracts and fractions of selected Phyllanthacae species were then added to the wells at concentrations of 1.0, 10.0 and 100.0 µg/ml. The cells were incubated with the extracts for 48 h. Negative control comprised of cells not treated with any extract.

At the end of the incubation period, the cells were detached from the plate with 1.0 ml accutase in PBS pH 7.4. The cells were harvested by centrifugation at 2000 × g for 5 min (Kubota 2420, Tokyo) and washed once with ice cold PBS pH 7.4. From then on, all operations were carried out on ice.

Cells pellets were gently resuspended in 500 µl lysis buffer and incubated in a dual-hybridization oven (Hybaid, UK) at 65 °C for 30 min. Then, 100 µl of 8 M potassium acetate was added to the suspended mixtures and incubated on ice bath for 1 h. At the end of incubation period, the lysates were centrifuged at 10 000 × g for 10 min (Thermo Scientific, Heraeus PICO 17) and the supernatant were transferred to a new microcentrifuge tube. DNA was extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, v/v/v) to the supernatant. The mixtures were vortexed with vortex mixer (Stuart, UK) for a few seconds to properly mix the solution and centrifuged at 12 000 × g for 3 min at room temperature. The top aqueous layer was carefully removed and placed in a new microcentrifuge tube. The DNA was

precipitated by adding 2 × volume of ice-cold absolute ethanol and leaving it overnight at -20 °C.

The sample was centrifuged at 12 000 × g for 30 min at room temperature. The ethanol was aspirated carefully and the DNA pellet was washed with 1 ml of 70 % ethanol. The pellet was dislodged by inverting the tube gently several times so that ethanol can remove the excess salt. The sample was centrifuged at 12 000 × g for 20 min at room temperature. After centrifugation, the ethanol wash was removed carefully and left over the DNA pellet.

The DNA pellet was spooled with glass hook and left at room temperature to dry. The dried DNA pellet was resuspended by adding 20 µl of TE buffer together with 1 µl of RNAse (10 µg/ml) and 1 µl of Proteinase K (100 µg/ml). The tube was flicked few times and mixture incubated at 37 °C for 30 min. At the end of incubation period, the DNA sample was placed on ice and 2 µl of 10 × loading dye was added. The tube was gently flicked to pool all the liquid at the bottom of the tube.

30 ml of 1.5 % agarose was melted in microwave (National NN-7806) and poured into the gel-casting tray. The 17-wells comb was placed until its base is 1 mm from the base of the gel. After the gel has hardened (20 – 30 min.), the gel comb was removed and the gel was placed into an electrophoresis tank (Gel X_L Plus, Labnet) containing sufficient 1 × TAE running buffer to cover the gel approximately 1 mm. Individual DNA samples were gently added into each well. The power supply was turned off when the bromophenol blue dye has migrated two thirds of the way down the gel. The gel was transferred to a container contain ethidium bromide solution, placed on a mini rocker (Biosan MR-1) and stain for 20 min. The gel was destained with distilled water for 10 min. The stained gel was observed and photographed using gel documentation system (Gene Flash, Syngene Bioimaging).

3.5.5 Determination of Caspase-3 Activation

The activity of caspase-3 was determined using the Caspase-3/CPP32 colorimetric assay kit (Biovision) according to manufacturer's protocol. The assay involved spectrophotometric detection of the chromophore, *p*-nitroanilide (*p*NA), after its cleavage from the labelled substrate DEVD-*p*NA. All the experiments were carried out in triplicates.

After treatment of the cells (2×10^6 cells/ml) with 10 µg/ml of cytotoxically active crude extracts and fractions of Phyllanthaceae species for 48 h, cells were centrifuged at 200 × g for 5 min. (Kubota 2420, Tokyo) and pelleted. Cell pellets were lysed with 50 µl of chilled cell lyses buffer and incubated on ice for 20 min. At the end of incubation period, cell suspension was centrifuged for 10 min at 12 000 × g. The supernatant (cytosolic extract) was transferred to a fresh test tube and placed on ice prior to use in caspase-3 detection assay. The protein concentration was measured using Biovision Protein Quantitation Kit. Protein extract (100 µg), 50 µl of 2 × reaction buffer supplemented with 10 mM DTT, and substrates of 4 mM DEVD-pNA in a volume total of 100 µl were added to each tube. After being incubated for 1.5 h at 37 °C, formation of *p*-nitroanilide in samples was measured with an ELISA microplate reader (Emax, Molecular Devices) at 405 nm. The activity of caspase-3 in the treated cells was determined by comparing the results with controls (untreated cells).

3.5.6 Cell Cycle Analysis by Flow Cytometry

The cell cycle analysis was determined using the CycleTEST^{TM} PLUS DNA Reagent Kit (Becton Dickinson, USA) according to manufacturer's protocol.

i. Sample collection and preparation

MCF7, SKOV3, CaSki and HT29 cells (1×10^6 cells/ml) were seeded onto 6 well plates (Nunc) and treated with 10 µg/ml of crude hexane extract of *P. watsonii* and fraction PPW7 and incubated for in a CO_2 incubator at 37 °C for 24 h. At the end of incubation period, cells were detached from the plated and placed into a 17 × 100 mm tube and centrifuged for 5 min at 300 × g at room temperature. The supernatant was aspirated leaving approximately 50 µl of residual in the tube to avoid disturbing the pellet. These washing steps were repeated twice with the addition of 1 ml buffer solution and the cells were resuspended by gently vortexing at low speed before centrifuging it for 5 min at 300 × g at room temperature. At the end of the washing step, the cells pellet was resuspended in 1 ml buffer solution. The cells were counted by standard laboratory methods using a haemacytometer and the concentration of the cells were adjusted to 1.0×10^6 cells/ml with buffer solution. Cells were frozen in -80 °C for later flow cytometric analysis.

ii. Freezing procedure

Cells suspended in buffer solution were transferred to a freezer-safe screw-capped polypropylene tube (Becton Dickinson, San Joce, CA) and frozen rapidly in a mixture of dry ice and 99 % ethanol. The frozen cells were then stored at -80 °C.

iii. Staining procedure

The frozen samples were thawed rapidly in a water bath at 37 °C, without allowing the sample material to reach 37 °C. The cells were counted by standard laboratory methods using a haemacytometer and the concentration of the cells were adjusted to 5.0×10^5 cells/ml with buffer solution.

The cell suspension was centrifuged at 400 × g for 5 min at room temperature. The supernatant was carefully decanted and the last drop was tap onto a tissue. 250 µl of Solution A (trypsin buffer) was added to each tube and gently mixed. Solution A was allowed to react for 10 min. at room temperature. 200 µl of Solution B (trypsin inhibitor and RNase buffer) was then added to each tube without removing solution A. The mixture was gently mixed by tapping the tube by hand and incubating for 10 min at room temperature. 200 µl of cold Solution C (propidium iodide stain solution) was added to each tube without removing Solution A and B. Mixtures was gently mixed and incubated for 10 min. in the dark at 2 ° to 8 °C.

Cells were filtered through 35 µm cell strainer cap into 12 × 75 mm tube and analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Joce, CA) with at least 10,000 cells per sample were analyzed. The percentage of cells in G_1, S and G_2-M phases was analyzed by ModFit LT software (Verity Software House, Topsham, ME). All the results were expressed as mean ± SD for three replicates.

3.5.7 Statistical Analysis

The data presented was as mean ± S.D were from three separate experiments. Statistical analysis of the data was performed by using the Students *t*-test. $p < 0.05$ denotes a statistically significant difference.

CHAPTER 4

RESULTS

4.1 Extraction from the Aerial Part, Leaves and Fruits of Selected Phyllanthaceae Species

In the search for natural products with cytotoxic activity, crude methanol extract (CME), crude hexane extract (CHE) and crude ethyl acetate extract (CEE) were prepared from six different species of Phyllanthaceae species collected from different parts of Peninsular Malaysia. The selection of the plants is based on the ethno medical data which state that the plants are used in the Malaysian traditional medicine for treatment various diseases. Plant species and the parts used for extract preparation, as well as the collection site are shown in Table 4.1.

Table 4.1: Plant and parts of plant used in this study

Plant species	Parts of plant tested	Voucher number	Collection site
Phyllanthus niruri	Aerial parts	KLU 42610	Home garden, Port Dickson
Phyllanthus pectinatus	Leaves	KLU 47659	Rimba Ilmu Botanical Garden
Phyllanthus pectinatus	Fruits	KLU 47659	Rimba Ilmu Botanical Garden
Phyllanthus acidus	Leaves	KLU 47679	Home garden, Port Dickson
Phyllanthus roseus	Leaves	KLU 47660	Gunung Machincang, Langkawi Island
Phyllanthus watsonii	Leaves	KLU 46068	Endau Rompin Park
Baccaurea motleyana	Leaves	KLU 30038	Home garden, Port Dickson

Yields from methanol, hexane and ethyl acetate extraction are shown in Table 4.2. The extraction of Phyllanthaceae species with ethyl acetate provided higher yield (0.36 – 5.49 % yield) compared to the extraction with hexane which gave lower yield (0.06 – 1.64 % yield). Percentage yield for crude methanol extract was not recorded as obtained crude methanol extract was shaken immediately with hexane for further extraction process.

Table 4.2: Yield (%, w/w) of crude extracts of selected Phyllanthaceae spp. obtained from methanol, hexane and ethyl acetate extraction

Phyllanthaceae spp.	Plant parts	Solvent	Yield (%, w/w) of crude extract
P. niruri	Aerial part	Methanol	NR
	Aerial part	Hexane	0.46
	Aerial part	Ethyl acetate	3.14
P. pectinatus	Leaves	Methanol	NR
	Leaves	Hexane	0.27
	Leaves	Ethyl acetate	5.49
P. pectinatus	Fruits	Methanol	NR
	Fruits	Hexane	0.10
	Fruits	Ethyl acetate	1.51
P. acidus	Leaves	Methanol	NR
	Leaves	Hexane	0.49
	Leaves	Ethyl acetate	0.9
P. roseus	Leaves	Methanol	NR
	Leaves	Hexane	0.06
	Leaves	Ethyl acetate	0.36
P. watsonii	Leaves	Methanol	NR
	Leaves	Hexane	1.64
	Leaves	Ethyl acetate	2.95

Table 4.2, continued

B. motleyana	Leaves	Methanol	NR
	Leaves	Hexane	0.70
	Leaves	Ethyl acetate	0.88

NR= not recorded

4.2 Cytotoxicity Screening of Phyllanthaceae Crude Extracts

In the present study, the cytotoxic activities of a total 21 crude extracts of selected Phyllanthaceae species were screened by an *in vitro* assay system for growth inhibition against four human cancer cell lines, namely breast cancer cell (MCF7), ovarian cancer cell (SKOV3), epidermal carcinoma of cervix cell (CaSki) and colon cancer cell (HT29). In addition, the cytotoxicity of the crude extracts against normal human lung fibroblast cell (MRC5) was also examined.

Complete dose-response curves were generated and the IC_{50} values (μg/ml) of crude methanol (CME), crude hexane (CHE) and crude ethyl acetate (CEE) extracts of selected Phyllanthaceae spp. on four human cancer cell lines were determined and summarized in Table 4.3. Typical complete dose-response curves are shown in Figure 4.1. In the US National Cancer Institute Plant Screening Program, a crude extract is generally considered to have active cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 h, is equal or less than 20 μg/ml, while it is equal or less than 4 μg/ml for pure compounds (Geran *et al*., 1972; Boik, 2001).

In the present study, the stock materials of the Phyllanthaceae extracts (20 mg/ml) were dissolved in DMSO 100 % and stock materials were further subjected to 10-folds serial dilution with DMSO 10 %. The highest final concentration of the DMSO used in the present study was 0.5 % (v/v), and this concentration was found to be non-toxic to the cells.

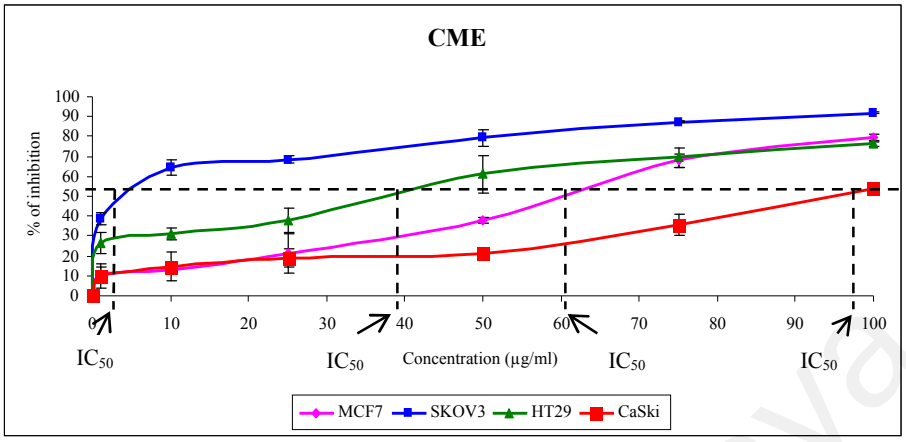


Figure 4.1: The *in vitro* growth inhibitions of various human cancer cell lines by crude methanol extract (CME) of *P. pectinatus* (leaves) determined by using the Neutral Red Cytotoxicity Assay. Each value is the mean ± standard deviation (S.D) consisting of three replicates.

All the crude extracts of the Phyllanthaceae species studied exhibited a significant concentration-dependent increase in growth inhibition at concentration of 1 – 100 µg/ml after 72 h incubation period. The IC_{50} values shown in Table 4.3 clearly indicated that the cytotoxic potency of the Phyllanthaceae species in descending order was as follows: *P. watsonii* > *P. pectinatus* (leaves) > *P. niruri* > *P. roseus* > *P. pectinatus* (fruits) > *P. acidus* > *B. motleyana*. The cytotoxic effect of the Phyllanthaceae crude extract was more pronounced towards MCF7 cells followed by CaSki, SKOV3 and HT29 cells.

Crude methanol, hexane and ethyl acetate extracts of *P. niruri* possessed IC_{50} values more than 20 µg/ml when tested on MCF7, SKOV3, CaSki and HT29 cells and considered cytotoxically not active. Crude ethyl acetate extract of *P. niruri* exhibited IC_{50} values of 31.1 ± 2.72, 33.3 ± 1.61 and 52.8 ± 0.58 µg/ml when tested on MCF7, SKOV3 and CaSki cells, respectively. SKOV3 cells-treated with crude methanol and hexane extracts of *P. niruri* exhibited IC_{50} values of 40.8 ± 8.29 and 50.5 ± 5.63 µg/ml, respectively. On

HT29 cells-treated with *P. niruri* extract, the IC_{50} values obtained were higher, ranged from 79 – 98 μg/ml. Viability of normal MRC5 cells is not affected by crude extracts of *P. niruri* treatment.

Crude methanol and ethyl acetate extract of *P. pectinatus* (leaves) exhibited strong cytotoxic activity against SKOV3 human ovarian cancer cells with IC_{50} values of 4.8 ± 1.04 and 5.8 ± 0.76 μg/ml, respectively. On MCF7 cells, crude methanol and ethyl acetate extracts of *P. pectinatus* (leaves) exhibited IC_{50} values of 60.2 ± 1.76 and 50.5 ± 4.09 μg/ml, respectively and considered not active in cytotoxic activity. On HT29 cells, crude methanol and ethyl acetate extracts of *P. pectinatus* (leaves) were not active with IC_{50} values of 39.1 ± 8.75 and 28.2 ± 3.21 μg/ml, respectively. Crude hexane extract possessing IC_{50} value more than 100 μg/ml when tested on MCF7, CaSki and HT29, where it does not inhibit the growth of those cells. All the crude extracts of *P. pectinatus* (leaves) were found to be not toxic to normal MRC5 lung fibroblast cell which IC_{50} values obtained were greater than 100 μg/ml.

Crude ethyl acetate extract of *P. pectinatus* (fruit) exhibited the most potent cytotoxicity with IC_{50} values of 18.1 ± 0.66 μg/ml and of 19.4 ± 0.53 μg/ml, when tested against MCF7 and CaSki cells, respectively. Crude methanol was not cytotoxically active on MCF7 and CaSki cells with IC_{50} values obtained were 51.0 ± 2.65 and 73.3 ± 2.84 μg/ml, respectively. Among all the crude extracts of *P. pectinatus* (fruits), extracts in hexane does not affect the viability of the MCF7, SKOV3, CaSki and HT29 cells where IC_{50} values obtained were more than 100 μg/ml. Crude methanol, hexane and ethyl acetate extract of *P. pectinatus* (fruits) were found not toxic to normal MRC5 cells where all the extracts possessed IC_{50} values more than 90.0 μg/ml on normal MRC5 cells.

Crude methanol, hexane and ethyl acetate extracts of *P. acidus* exhibited IC_{50} values more than 20 μg/ml when tested on MCF7, SKOV3, CaSki and HT29 cells and considered not actively cytotoxic. Crude methanol extract of *P. acidus* produced IC_{50} values more than 100 μg/ml toward all the cancer cell lines tested (except in SKOV3 where the IC_{50} value was 88.0 ± 5.97 μg/ml). Crude hexane extracts exhibited IC_{50} values of 96.8 ± 7.11, 80.8 ± 8.33, 83.3 ± 8.25 and > 100 μg/ml, when tested on MCF7, SKOV3, CaSki and HT29 cells, respectively. Crude ethyl acetate extracts exhibited IC_{50} values of 44.2 ± 7.82, 67.8 ± 3.55, 55.5 ± 14.76 and 66.5 ± 7.05 μg/ml, when tested on MCF7, SKOV3, CaSki and HT29 cells, respectively. With IC_{50} values > 100 μg/ml on normal MRC5 cells, crude extracts of *P. acidus* were found not deleterious to normal MRC5 cells.

P. roseus crude extracts in methanol, hexane and ethyl acetate were found to not active in cytotoxic activity where IC_{50} values obtained were more than 20 μg/ml when tested on MCF7, SKOV3, CaSki and HT29 cells. IC_{50} values of these extracts on MCF7 cells were 40.0 ± 5.22, 54.3 ± 8.25 and 24.7 ± 5.58 μg/ml, respectively. Extracts of *P. roseus* in all the three solvents possessed IC_{50} more than 100 μg/ml when tested on SKOV3 cells. On CaSki and HT29 cells-treated with crude hexane extracts of *P. roseus* with IC_{50} values obtained were 59.2 ± 12.29 and 58.7 ± 8.39 μg/ml, respectively, and crude ethyl acetate extracts of *P. roseus* IC_{50} values obtained were 83.0 ± 11.26 and 89.0 ± 10.15 μg/ml, respectively. No toxicity was detected on normal MRC5-treated with crude methanol, hexane and ethyl acetate of *P. roseus*.

Crude methanol, crude hexane and crude ethyl acetate extracts of *P. watsonii* were cytotoxically active on all four cancer cell lines tested with an IC_{50} values less than 20.0 μg/ml. Crude ethyl acetate extracts possesses the strong cytotoxicity with an IC_{50} value of 3.6 ± 1.01 μg/ml on CaSki cell, as well as on HT29 and SKOV3 cells with IC_{50}

values of 5.1 ± 0.36 and 5.5 ± 0.50 μg/ml, respectively. Extracts in hexane also exhibited strong cytotoxicity with IC_{50} values of 7.9 ± 0.60, 5.8 ± 0.29, 6.9 ± 0.96 and 11.8 ± 1.61 μg/ml on MCF7, SKOV3, CaSki and HT29 cell lines, respectively. Methanol crude extracts possessed slightly higher IC_{50} values compared to the other two crude extracts. IC_{50} values range from 8 – 18 μg/ml. However, extracts in all three solvents were found not to be toxic to normal MRC5 cell with IC_{50} values ranging from 33 – 57 μg/ml.

Crude extracts of *B. motleyana* did not show any cytotoxic activity when tested on all the human cancer cell lines with IC_{50} values more than 100 μg/ml. MCF7 cells treated with crude methanol, hexane and ethyl acetate of *B. motleyana* exhibited IC_{50} values of 60.6 ± 2.80, 93.5 ± 5.27 and 89.0 ± 3.12 μg/ml, respectively. All the three crude extracts of *B. motleyana* exhibited IC_{50} values more than 100 μg/ml on normal MRC5 cells and consider not toxic to the normal cells.

The results showed that 69 % of the crude extracts of Phyllanthaceae species tested possessed IC_{50} values less than100 μg/ml in all four human cancer cells. Out of this, 19 % of the extracts were considered as cytotoxically active extracts with IC_{50} values < 20 μg/ml. It was shown that all the crude extracts *P. watsonii* in three different solvent of were able to induce cytotoxicity in all four human cancer cells in dose-dependent manner, and the cytotoxic potency of the *P. watsonii* extracts was in order of crude ethyl acetate > crude hexane extract > crude methanol extract.

Table 4.3: IC_{50} values of crude extracts of selected Phyllanthaceae species on human cell lines

Plant species	Part of plant tested	Extraction solvent	IC_{50} values[a] (µg/ml) in different cell lines[b]				
			MCF7	SKOV3	CaSki	HT29	MRC5
Phyllanthus niruri	Aerial part	MeOH	61.7 ± 3.94	40.8 ± 8.29	84.0 ± 4.77	95.5 ± 2.65	98.8 ± 3.33
		Hexane	75.0 ± 1.73	50.5 ±5.63	>100	98.2 ± 17.96	>100
		EtOAc	31.1 ± 2.72	33.3 ±1.61	52.8 ± 0.58	79.7 ± 3.75	>100
Phyllanthus pectinatus	Leaves	MeOH	60.2 ± 1.76	**4.8 ± 1.04**	95.0 ± 2.00	39.1 ± 8.75	>100
		Hexane	>100c	52.3 ± 8.84	>100	>100	>100
		EtOAc	50.5 ± 4.09	**5.8 ± 0.76**	43.8 ± 3.21	28.2 ± 3.21	>100
Phyllanthus pectinatus	Fruits	MeOH	51.0 ± 2.65	>100	73.3 ± 2.84	>100	>100
		Hexane	>100	>100	>100	>100	90.5 ± 13.26
		EtOAc	**18.1 ± 0.66**	69.0 ± 15.76	**19.4 ± 0.53**	61.5 ± 5.77	>100
Phyllanthus acidus	Leaves	MeOH	>100	88.0 ± 5.97	>100	>100	>100
		Hexane	96.8 ± 7.11	80.8 ± 8.33	83.3 ± 8.25	>100	>100
		EtOAc	44.2 ± 7.82	67.8 ± 3.55	55.5 ± 14.76	66.5 ± 7.05	>100
Phyllanthus roseus	Leaves	MeOH	40.0 ± 5.22	>100	>100	60.0 ± 6.54	>100
		Hexane	54.3 ± 8.25	>100	59.2 ± 12.29	58.7 ± 8.39	63.0 ± 4.36
		EtOAc	24.7 ± 5.58	>100	83.0 ± 11.26	89.0 ± 10.15	>100
Phyllanthus watsonii	Leaves	MeOH	**12.7 ± 4.65**	**8.5 ± 0.50**	**8.0 ± 0.87**	**18.3 ± 1.53**	49.3 ± 5.80
		Hexane	**7.9 ± 0.60**	**5.8 ± 0.29**	**6.9 ± 0.96**	**11.8 ± 1.61**	57.3 ± 2.57
		EtOAc	**7.7 ± 0.29**	**5.5 ± 0.50**	**3.6 ± 1.01**	**5.1 ± 0.36**	33.8 ± 2.57
Baccaurea motleyana	Leaves	MeOH	60.6 ± 2.80	>100	>100	60.3 ± 5.20	>100
		Hexane	93.5 ± 5.27	>100	>100	>100	>100
		EtOAc	89.0 ± 3.12	>100	>100	>100	>100
Doxorubicin	Positive Control	DMSO	0.72 ± 0.03	0.42 ± 0.24	0.68 ± 0.08	0.63 ± 0.03	1.72 ± 0.08

[a] Crude extracts with IC_{50} value ≤ 20 µg/ml considered active (Geran *et al*, 1972; Boik, 2001). Values in bold characters are considered to have cytotoxic activity.
[b] MCF7 (breast cancer); SKOV3 (ovarian cancer); CaSki (cervical cancer); HT29 (colon cancer) and MRC5 (normal lung fibroblast)

4.3 Bioassay-guided Fractionation of Crude Hexane Extract of *Phyllanthus watsonii*

Crude hexane extract (CHE) of *P. watsonii* exhibited strongest cytotoxicity on all four cancer cells tested compared to other extracts of *P. watsonii* and was selected for bioassay-guided fractionation. CHE of *P. watsonii* was also not toxic to MRC5 cells. 6.1 g of CHE of *P. watsonii* was subjected to column chromatography to fractionate the compounds. Fractionation process yielded 10 fractions which were coded as PW1 – PW10. Yields obtained for each fraction and the solvent system used in the fractionation process are shown in Table 4.4 and the thin layer chromatography (TLC) profile of collected fractions (PW2–PW9) are shown in Figure 4.2.

Cytotoxic activities of the fractions (PW1 – PW10) were screened by an *in vitro* assay system of growth inhibition against four cancer cell lines, namely human breast cancer cell line (MCF7), human ovarian cancer cell line (SKOV3), human epidermal carcinoma of cervix cell line (CaSki) and human colon cancer cell line (HT29). Cytotoxicity (IC_{50} value in μg/ml) of isolated fractions (PW1 – PW10) towards MCF7, SKOV3, CaSki and HT29 cells is summarized in Table 4.5.

4.3.1 Fractionation of Crude Hexane Extract of *Phyllanthus watsonii* by Column Chromatography

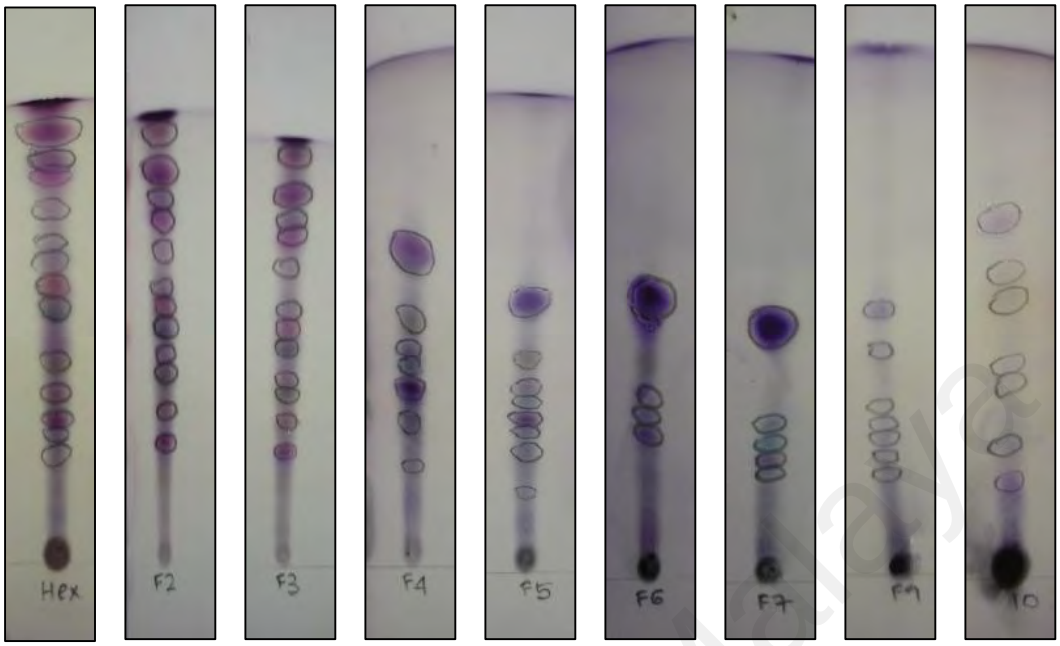
Crude hexane extract of *P. watsonii* were found to be very active in the cytotoxicity screening assay and were further fractionated. The fractionation was done using standard silica gel chromatography with solvent combinations of increasing polarity gradient (hexane, acetone and ethyl acetate). The fractions were collected based on visual bands. These fractions were organized by similarity of spots detected in TLC on pre-coated silica

gel 60 F254 plate using n-hexane/acetone (20:80) as developing solvent and *p*-anisaldehyde in H_2SO_4 as detection system.

Silica column chromatography of the crude hexane extract of *P. watsonii* yielded a total of 10 fractions. One fraction was obtained from hexane 100 %, six fractions were obtained from n-hexane/acetone (40:60) solvent system; one fraction from n-hexane/acetone (40:60 and 50:50) solvent system; one fraction from combination of n-hexane/acetone (50:50) and acetone/methanol (40:60) solvent system; and the last fraction obtained from acetone/methanol (40:60 and 90:10) solvent system.

Table 4.4: Fractions (PW1–PW10) collected from different solvents system in column chromatography

Fraction no.	Fraction code	Solvent system	Ratio	Yield (%, w/w)
1	PW1	n-hexane 100 %	100	5.49
2	PW2	n-hexane /acetone	40 : 60	4.67
3	PW3	n-hexane/acetone	40 : 60	60.64
4	PW4	n-hexane/acetone	40 : 60	5.13
5	PW5	n-hexane/acetone	40 : 60	0.30
6	PW6	n-hexane/acetone	40 : 60	3.10
7	PW7	n-hexane/acetone	40 : 60	0.83
8	PW8	n-hexane/acetone n-hexane/acetone	40 : 60 50 : 50	0.16
9	PW9	n-hexane/acetone Acetone/methanol	50 : 50 40 : 60	8.83
10	PW10	Acetone/methanol Acetone/methanol	40 : 60 90 : 10	41.71



CHE PW2 PW3 PW4 PW5 PW6 PW7 PW8 PW9

Figure 4.2: Thin layer chromatography (TLC) profile of collected fractions: PW2 – PW8 in n-hexane/acetone and PW9 in mixture of n-hexane/acetone and acetone/methanol solvent system

Based on literature, the nature of the compounds forming zones on the TLC plates were determined. Terpenoid gives purple, blue or red spots on the TLC plates when sprayed with *p*-anisaldehyde reagents followed by heating up the plate at 105 °C for 5 – 10 min. Other compounds that can be detected by *p*-anisaldehyde spraying reagents are lignans, sugars and flavonoids (Houghton and Raman, 1998).

As shown in Figure 4.2, it is clear that all of the TLC plates developed with n-hexane/acetone (20:80) yielded a majority of purple and red spots with different intensities. Plates PW4, PW5, PW6 and PW7 developed clear and well-separated purplish spots after spraying with *p*-anisaldehyde reagents followed by heating on a hot plate. It can be concluded that terpenoids are present in the isolated fractions of PW2 – PW9.

4.3.2 Cytotoxic Activity of Isolated Fractions (PW1 – PW10) of *Phyllanthus watsonii* on Human Cancer Cell Lines

Fractions PW1 – PW10 were evaluated for cytotoxicity by NR assay on MCF7, SKOV3, CaSki, HT29 and MRC5 cell at concentrations of 1,10, 25, 50, 75 and 100 µg/ml. The fractions of *P. watsonii* demonstrated stronger cytotoxic activity compared to the crude hexane extracts particularly when tested on SKOV3 cells. On SKOV3 cells, fractions PW4, PW5, PW6, PW7 and PW 8 exhibited IC_{50} values of 0.3 ± 0.06, 0.2 ± 0.06, 0.4 ± 0.12, 0.8 ± 0.29 and 0.4 ± 0.12 µg/ml, respectively. These IC_{50} values were lower when compared to crude hexane extract of *P. watsonii* (IC_{50} = 5.8 ± 0.29 µg/ml) on the same cell line. IC_{50} values range obtained when MCF7, CaSki and HT29 cells were treated with fractions PW4 – PW8 were 2.0 – 7.3, 2.8 – 13.2 and 1.2 – 10.8 µg/ml, respectively. PW8 was the most cytotoxically active fraction on all the cell lines with IC_{50} value of 2.0 ± 1.50 µg/ml on MCF7 cells, 0.4 ± 0.12 µg/ml on SKOV3 cells, 2.8 ± 0.76 µg/ml on CaSki cells and 1.2 ± 0.29 µg/ml on HT29 cells. Fractions PW4 – PW8 was also found to be toxic towards normal MRC5 cell with IC_{50} values ranges from 7.8 – 16.3 µg/ml.

The bioassay-guided fractionation of crude hexane extract of *P. watsonii* revealed presence of highly cytotoxic compounds at intermediate polarity phase. Fractions PW4, PW5, PW6, PW7 and PW8 were obtained from the mid portion of the silica column with the ratio of the solvent (n-hexane/acetone) used was 40:60 and 50:50. These fractions were further pooled and subjected to the second step of bioassay-guided fractionation.

Table 4.5: IC_{50} values of PW1–PW10 fractions from crude hexane extracts of *P. watsonii* on human cell lines

Fractions	IC_{50} values[a] (µg/ml) in different cell lines[b]				
	MCF7	**SKOV3**	**CaSki**	**HT29**	**MRC5**
PW1	>100	89.8 ± 1.25	>100	>100	>100
PW2	>100	**6.8 ± 0.29**	>100	>100	>100
PW3	90.5 ± 6.26	**5.3 ± 0.76**	85.5 ± 3.04	66.3 ± 2.02	>100
PW4	**7.7 ± 0.29**	**0.3 ± 0.06**	**13.2 ± 3.25**	**10.8 ± 0.76**	16.3 ± 1.76
PW5	**7.7 ± 0.29**	**0.2 ± 0.06**	**7.2 ± 1.15**	**5.2 ± 0.76**	12.3 ± 2.52
PW6	**7.3 ± 0.29**	**0.4 ± 0.12**	**5.5 ± 0.50**	**5.7 ± 0.76**	7.8 ± 1.04
PW7	**7.3 ± 1.04**	**0.8 ± 0.29**	**8.8 ± 0.58**	**1.2 ± 1.04**	8.0 ± 1.80
PW8	**2.0 ± 1.50**	**0.4 ± 0.12**	**2.8 ± 0.76**	**1.2 ± 0.29**	8.3 ± 1.04
PW9	>100	70.7 ± 2.89	>100	>100	>100
PW10	>100	>100	>100	>100	>100
CHE	**7.9 ± 0.60**	**5.8 ± 0.29**	**6.9 ± 0.96**	**11.8 ± 1.61**	57.3 ± 2.57
Doxorubicin	0.72 ± 0.03	0.42 ± 0.24	0.68 ± 0.08	0.63 ± 0.03	1.72 ± 0.08

[a] Crude extract with IC_{50} value ≤ 20 µg/ml considered active (Geran *et al*, 1972; Boik, 2001). Values in bold characters are considered to have cytotoxic activity.
[b] MCF7 (breast cancer); SKOV3 (ovarian cancer); CaSki (cervical cancer); HT29 (colon cancer) and MRC5 (normal lung fibroblast)

4.3.3 Fractionation of Combined Cytotoxically Active Fractions (PW4 – PW8) of *Phyllanthus watsonii* by Column Chromatography

PW4 – PW8 was revealed as most active fractions for cytotoxic activity on MCF7, SKOV3, CaSki and HT29 cell lines. The TLC profile of these fractions was similar and they were therefore pooled and subjected for further bioassay-guided fractionation. Pooled fractions were chromatographed on silica gel column (2.6 cm id × 60 cm length; 70 – 230 mesh; 160 g) and eluted in a gradient manner with hexane-acetone-methanol. The fractions obtained were collected based on the visual bands. These fractions were organized by the similarity of spots detected in TLC on pre-coated silica gel 60 F254 plate using n-hexane/acetone (20:80) as developing solvent and *p*-anisaldehyde in H_2SO_4 as detection system.

Silica column chromatography of the pooled active fractions of *P. watsonii* resulted in fractionation into 8 fractions. Yields obtained for each fraction and the solvent system used in the fractionation process are shown in Table 4.6 and the TLC profile of collected fractions (PPW1 – PPW8) are shown in Figure 4.3.

One fraction was obtained from n-hexane 100 %, three fractions were obtained from n-hexane/acetone (90:10) solvent system; one fraction from n-hexane/acetone (90:10 and 60:40) solvent system; one fraction from n-hexane/acetone (60:40) solvent system; one fraction from n-hexane/acetone (50:50) solvent system and the last fraction obtained from acetone/methanol (50:50 and 40:60) solvent system.

All the fractions were subjected again on NR assay to evaluate the cytotoxicity of the fractions on MCF7, SKOV3, CaSki, HT29 and MRC5 cell lines.

Table 4.6: Fractions (PPW1 – PPW8) collected from different solvents system in column chromatography

Fraction no.	Fraction code	Solvent system	Ratio	Yield (%, w/w)
1	PPW1	n-hexane 100%	100	10.26
2	PPW2	n-hexane/acetone	90 : 10	14.56
3	PPW3	n-hexane/acetone	90 : 10	3.34
4	PPW4	n-hexane/acetone	90 : 10	2.79
5	PPW5	n-hexane/acetone n-hexane/acetone	90 : 10 60 : 40	0.91
6	PPW6	n-hexane/acetone	60 : 40	20.71
7	PPW7	n-hexane/acetone	50 : 50	10.61
8	PPW8	n-hexane/acetone n-hexane/acetone	50 : 50 40 : 60	1.27

As terpenoids had been reported as one of the most abundant compound determined in the genus of *Phyllanthus*, detection of terpenoids was carried on the obtained fractions PPW1 – PPW8 by using the *p*-anisaldehyde spraying reagents followed by heating the plate at 105 °C for 5 – 10 min. As mentioned above, terpenoid gives purple, blue or red spots on the TLC plates when sprayed with *p*-anisaldehyde reagents.

It is clearly shown in Figure 4.3 that the intense purple spot was detected on TLC plate of fractions PPW3 and PPW4. Plate of fractions PPW5, PPW6, PPW7 and PPW8 produced more reddish spot. High intensity red spots were seen in plate with fractions PPW5 and PPW7. PPW7, which possessed the strongest cytotoxicity on all the human cancer cell lines tested (Section 4.3.4), was found to produce four clear overlapping red spots developed with n-hexane/acetone (20:80). The presence of purples and red spots indicated that the isolated fractions PPW1–PPW8 contained terpenoids.

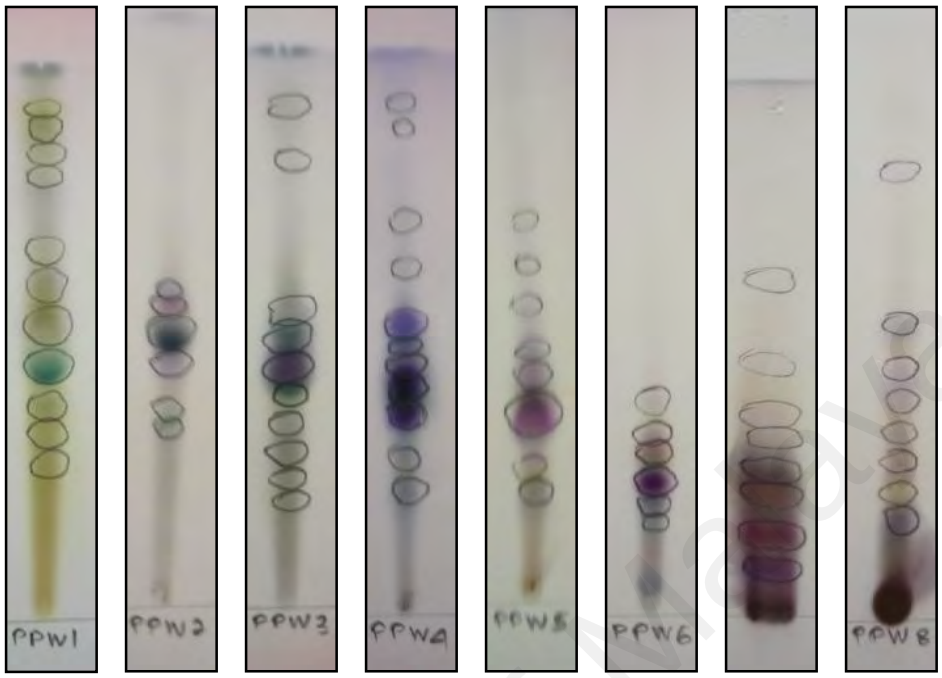


Figure 4.3: Thin layer chromatography (TLC) profile of collected fractions: PPW1 in n-hexane 100% and PPW2 – PPW8 in n-hexane/acetone solvent system

4.3.4 Cytotoxic Activity of Isolated Fractions (PPW1 – PPW8) from Combined Active Fractions (PW4 – PW8) on Human Cancer Cell Lines

Cytotoxic activities of the fractions (PPW1 – PPW8) were screened by an *in vitro* assay system of growth inhibition against four cancer cell lines, namely human breast cancer cell line (MCF7), human ovarian cancer cell line (SKOV3), human epidermal carcinoma of cervix cell line (CaSki) and human colon cancer cell line (HT29). IC_{50} values of fractions PPW1 – PPW8 against MCF7, SKOV3, CaSki and HT29 cell lines are summarized in Table 4.7.

Cytotoxicity testing of each fraction showed that the fraction PPW7 was most active. The study also shows that, the cytotoxic activity of fraction PPW7 were more potent with IC_{50} values of 0.9 ± 0.06, 0.7 ± 0.06, 0.8 ± 0.00 and 0.8 ± 0.10 μg/ml, than that of its crude hexane extract with IC_{50} values of 07.9 ± 0.60, 5.8 ± 0.29, 6.9 ± 0.96 and 11.8 ± 1.61 μg/ml on MCF7, SKOV3, CaSki and HT29 cells, respectively. Fractions PPW6 and PPW8 were also actively cytotoxic on all the cancer cell lines tested with IC_{50} values ranges from 8.83 – 14.8 and 0.9 – 18.3 μg/ml, respectively. Fraction PPW7 showed similar cytotoxic activity as the standard anticancer drug, doxorubicin which was used as a positive control in the present study. The IC_{50} values of doxorubicin were found to be 0.72 ± 0.03, 0.42 ± 0.24, 0.68 ± 0.08 and 0.63 ± 0.03 μg/ml against MCF7, SKOV3, CaSki and HT29 cell lines, respectively. Fractions PPW6, PPW7 and PPW8 were also observed to be cytotoxic towards normal MRC5 cells with the IC_{50} values of 14.5 ± 1.80, 10.2 ± 1.26 and 16.8 ± 1.26 μg/ml, respectively. Doxorubicin was also found to be toxic to normal MRC5 cell with IC_{50} value of 1.72 ± 0.08.

Table 4.7: IC_{50} values of fractions PPW1 – PPW8 from fractions PW4 – PW8 of *P. watsonii* on human cell lines

Fractions	IC_{50} values[a] (µg/ml) in different cell lines [b]				
	MCF7	**SKOV3**	**CaSki**	**HT29**	**MRC5**
PPW1	>100	100.0 ± 2.89	>100	72.8 ± 6.66	>100
PPW2	>100	>100	>100	>100	>100
PPW3	>100	74.0 ± 8.53	98.8 ± 2.02	>100	>100
PPW4	61.3 ± 3.62	37.0 ± 0.50	60.0 ± 2.60	40.8 ± 2.08	>100
PPW5	55.7 ± 3.01	**4.8 ± 2.57**	45.2 ± 3.18	63.8 ± 2.08	>100
PPW6	**8.83 ± 0.29**	**9.5 ± 1.80**	**12.0 ± 3.50**	**14.8 ± 4.19**	14.5 ± 1.80
PPW7	**0.9 ± 0.06**	**0.7 ± 0.06**	**0.8 ± 0.00**	**0.8 ± 0.10**	10.2 ± 1.26
PPW8	**13.8 ± 1.04**	**0.9 ± 0.10**	**9.2 ± 0.58**	**18.3 ± 2.75**	16.8 ± 1.26
CHE	**7.9 ± 0.60**	**5.8 ± 0.29**	**6.9 ± 0.96**	**11.8 ± 1.61**	57.3 ± 2.57
Doxorubicin	0.72 ± 0.03	0.42 ± 0.24	0.68 ± 0.08	0.63 ± 0.03	1.72 ± 0.08

[a] Fractions with IC_{50} value ≤ 20 µg/ml considered active (Geran *et al*, 1972; Boik, 2001). Values in bold characters are considered to have cytotoxic activity.
[b] MCF7 (breast cancer); SKOV3 (ovarian cancer); CaSki (cervical cancer); HT29 (colon cancer) and MRC5 (normal lung fibroblast)

Selectivity of the cytotoxically active crude extracts and fractions of *P. watsonii* was determined by comparing the cytotoxic activity (IC_{50}) of each crude extract and fraction against each cancerous cell with that of the normal human lung fibroblast cell (MRC) and shown in Table 4.8. Results were expressed as selectivity index (SI). SI of greater than 3 was considered as highly selective.

Fraction PPW7 appears to be the most potent and most selective against MCF7 (IC_{50} = 0.9 ± 0.06 µg/ml, SI 11.3); CaSki (IC_{50} = 0.8 ± 0.00 µg/ml, SI 12.8) and HT29

(IC_{50} = 0.8 ± 0.10 µg/ml, SI 12.8); whereas fraction PPW8 appears to be most selective against SKOV3 (SI 18.7). Among all the *P. watsonii* crude extracts, crude hexane extract exhibited the most promising and most selective cytotoxic activity against MCF 7 cells (IC_{50} = 7.9 ± 0.60 µg/ml, SI 7.3) and SKOV3 cell (IC_{50} = 5.8 ± 0.29 µg/ml, SI 9.9); whereas crude ethyl acetate extract exhibited the most promising and most selective cytotoxic activity against CaSki cells (IC_{50} = 3.6 ± 1.01 µg/ml, SI 9.4) and HT29 cells (IC_{50} = 5.1 ± 0.36 µg/ml, SI 6.6).

Fraction PPW8 possessed a potent cytotoxic activity against all the four human cancer cell lines studied, but the selectivity index is lower than 3 and considered as low selectivity on MCF7, CaSki and HT29 cells. For SKOV3 cell, fraction PPW8 exhibited promising cytotoxicity with higher selectivity index (IC_{50} = 0.9 ± 0.10 µg/ml, SI 18.7) when compared to fractions PPW6 and PPW7. Overall, the cytotoxic properties of crude methanol, crude hexane and crude ethyl acetate extracts of *P. watsonii* and fraction PPW7 were shown to be selective for cancer cells even though it possessed a higher cytotoxicity on normal lung fibroblast, MRC5.

Table 4.8: Selectivity of the cytotoxically active crude extracts and fractions of *P. watsonii* in comparison with MRC5 cells

Crude extracts/fractions	Selectivity index [a]			
	MCF7	SKOV3	CaSki	HT29
CME [x]	3.9	5.8	6.2	2.7
CHE [y]	7.3	9.9	8.3	4.9
CEE [z]	4.4	6.1	9.4	6.6
PPW6	1.6	1.5	1.2	1.0
PPW7	11.3	14.6	12.8	12.8
PPW8	1.2	18.7	1.8	0.9
Doxorubicin	2.4	4.1	2.5	2.7

[a] The selectivity index is the ratio of the IC_{50} values of extract/fraction on MRC5 cells to those in the cancer cell lines. Samples with SI greater than 3 were considered to have high selectivity (Mahavorasirikul *et al*., 2010).

[x] crude methanol extract; [y] crude hexane extract and [z] crude ethyl acetate extract

4.4 LC-MS/MS Analysis of Cytotoxically Active Fraction PPW7

Fractions PPW7 fractionated from the crude hexane extract of *P. watsonii* by column chromatographic methods were analyzed by LC-MS/MS system allowing the detection of major compound(s) and the data obtained were compared with the previous published data of chemical constituents of *P. watsonii*.

The LC-MS/MS-TIC profiles of major compounds in fraction PPW7 obtained using Aqua C18 column (50 mm × 2.0 mm × 5 μM) was shown in Figure 4.4. Based on mass spectrometric analysis, major compounds were tentatively identified and were listed in Table 4.9.

Results of the LC-MS/MS analysis showed that at least 15 compounds were present in fraction PPW7 of which 7 compounds with retention times of major peaks were identified through mass spectrometry. The major peak with a retention time of 13.99 was identified as methyl ester of geraiinic acid (MS *m/z* = 397), a monoterpene and potassium phyllanthin (MS *m/z* = 457), a lignan. Phyllanthin also was identified as sodium phyllanthin (MS *m/z* = 441) at a retention time of 11.66. Another major peak was identified as sterol glucoside (MS *m/z* = 718), an isoprenoids at retention time of 9.39. Two triterpene were eluted at retention time of 10.20 and 13.45 and were identified as glochidone (MS *m/z* = 423) and betulin (MS *m/z* = 443), respectively. A polyphenolic compound identifies as trimethyl ether of ellagic acid (MS *m/z* = 345) was eluted at retention time of 7.77.

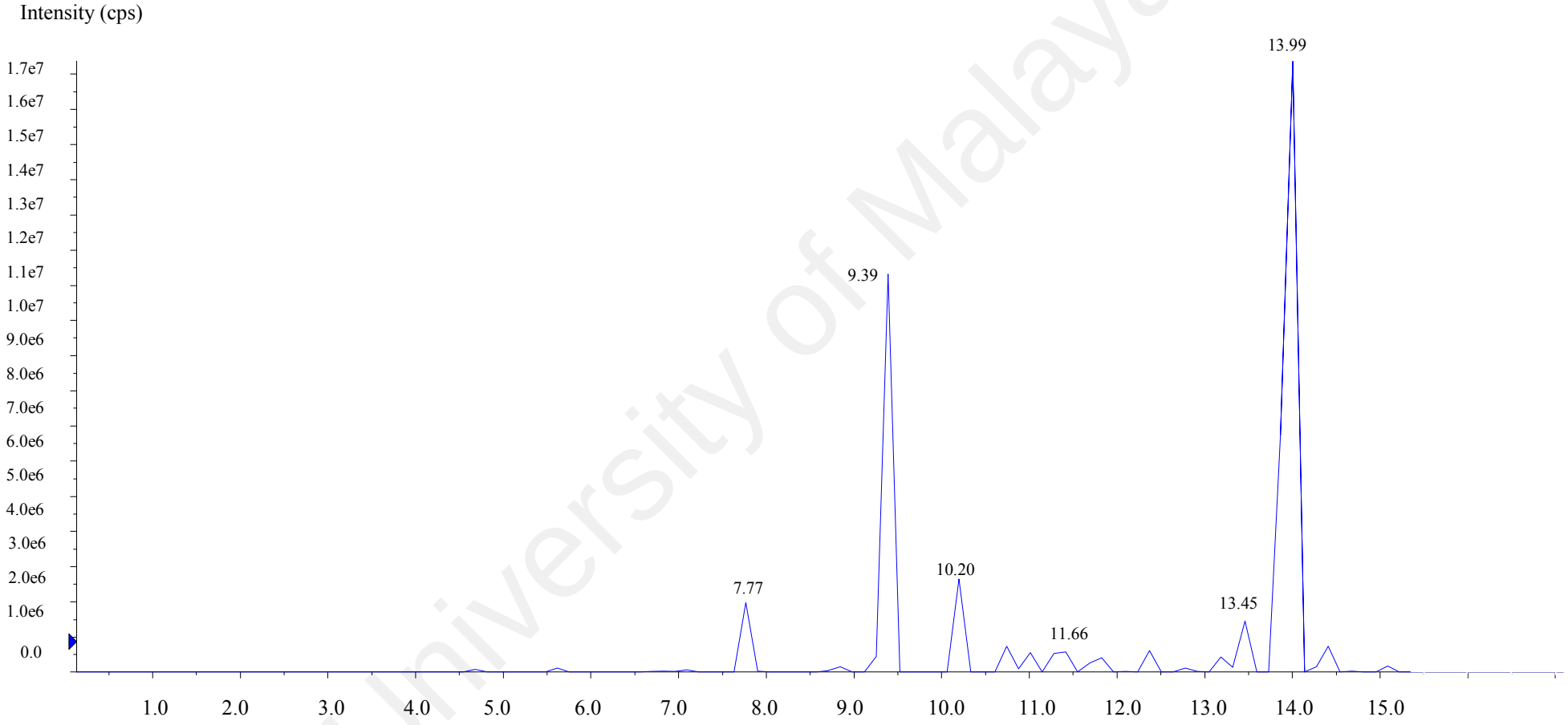
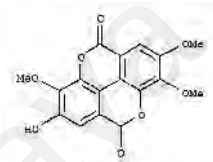
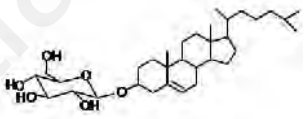
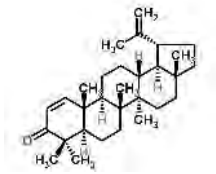
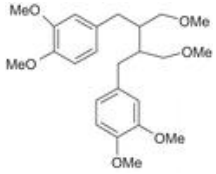
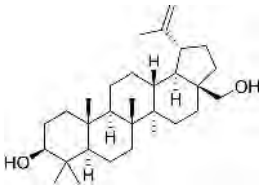
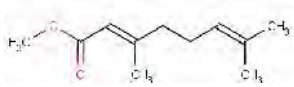
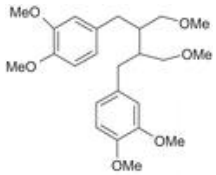


Figure 4.4: LCMS-TIC profiles of secondary compounds in the fraction PPW7

Table 4.9: Identification of compounds in fraction PPW7 by using LC-MS/MS data

Retention time (min)	MW (*m/z*)	MS/MS	Tentative ID*	Chemical structure of proposed compounds
7.77	345	330, 315	trimethyl ether of ellagic acid	
9.39	718	701, 641, 613, 581	sterol glucoside	
10.20	423	405, 381, 363	glochidone	
11.66	441	423, 405, 395, 339, 315	sodium phyllanthin	
13.45	443	425, 369	betulin	
13.99	397	379, 353, 339, 327	methyl ester of geraiinic acid	
	457	439, 397, 379, 369, 353	Potassium phyllanthin	

*Identification were aided by comparison with reference standards where available and by correlation with previous literature reports

4.5 Assessment of Apoptosis in Cell Treated with Cytotoxically Active Crude Extracts of Phyllanthaceae and Fractions of *Phyllanthus watsonii*

4.5.1 Analysis of Cell Morphological Changes by Phase Contrast Microscope

In the present study, exposure of all the MCF7, SKOV3, CaSki and HT29 cell lines to the cytotoxically active extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions of *P. watsonii* (PPW6 and PPW7) at 10, 25 and 50 µg/ml for 24 h, led to some morphological alteration of the cells which suggest that the cells were undergoing apoptosis stage. The morphological changes were compared with the untreated or control cells which showed cuboids and polygonal in cell shapes as shown in Figures 4.5a – 4.5d.

The most recognizable morphological feature of an apoptotic cell observed in this study by inverted light microscopy is the cytoplasmic condensation, resulting in cell shrinkage, production of numerous cell surface protuberances or "blebs" at the plasma membrane and condensation as well as aggregation of the nuclear chromatin into dense masses beneath the nuclear membrane. Cells undergoing apoptosis also showed characteristics such as rounding up of cells, shrunken cells, loss of contact with neighbouring cells and in some sensitive cells, detachment from the surface of the well plates. Separation of the nucleus into several discrete fragments, resulting in the fragmentation of the cell into numerous membrane-bounded vesicles that are recognized as apoptotic bodies had also been observed.

These results suggest that the cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 were able to induce marked apoptotic morphology in MCF7, SKOV3, CaSki and HT29 cell lines.

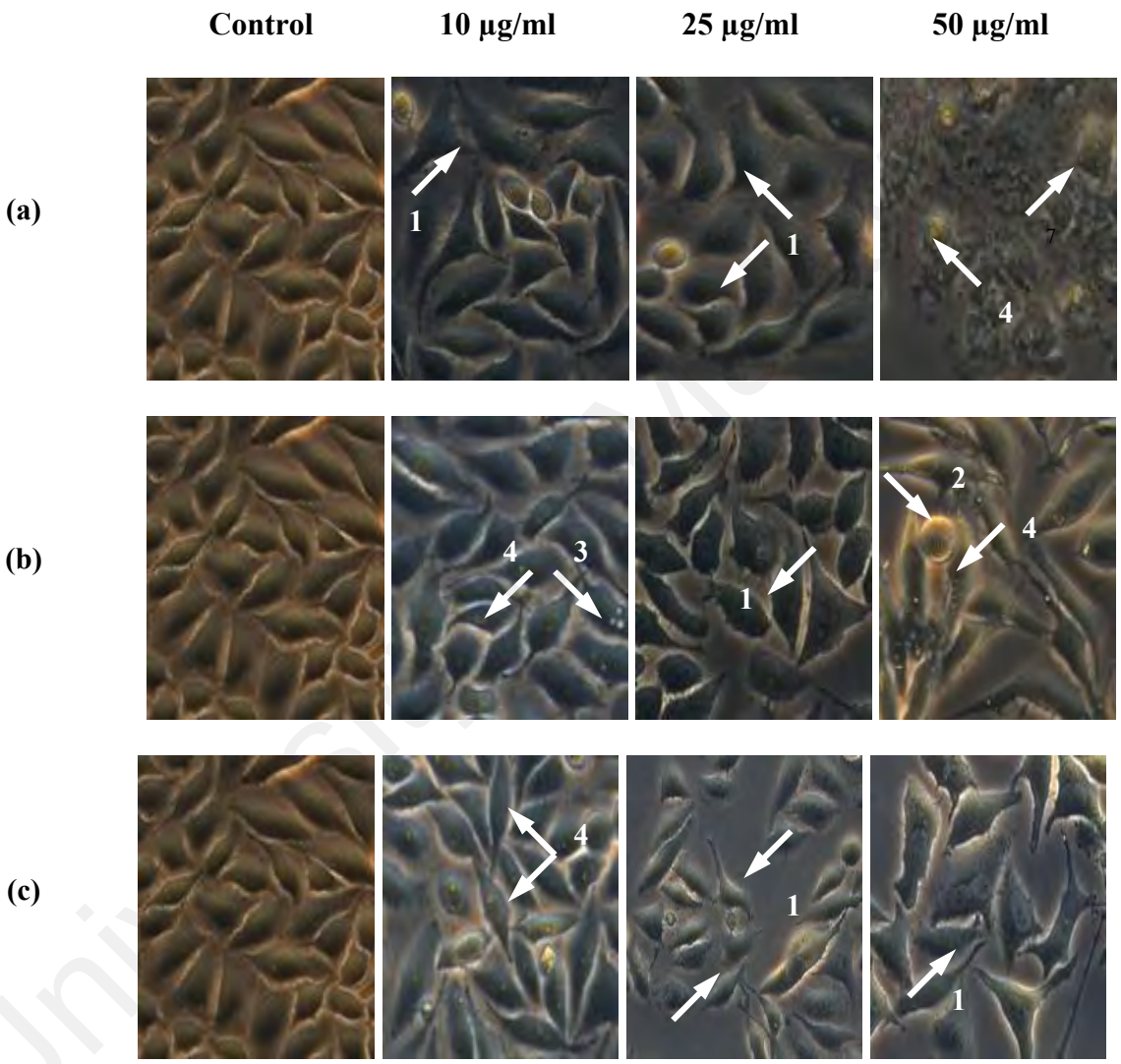
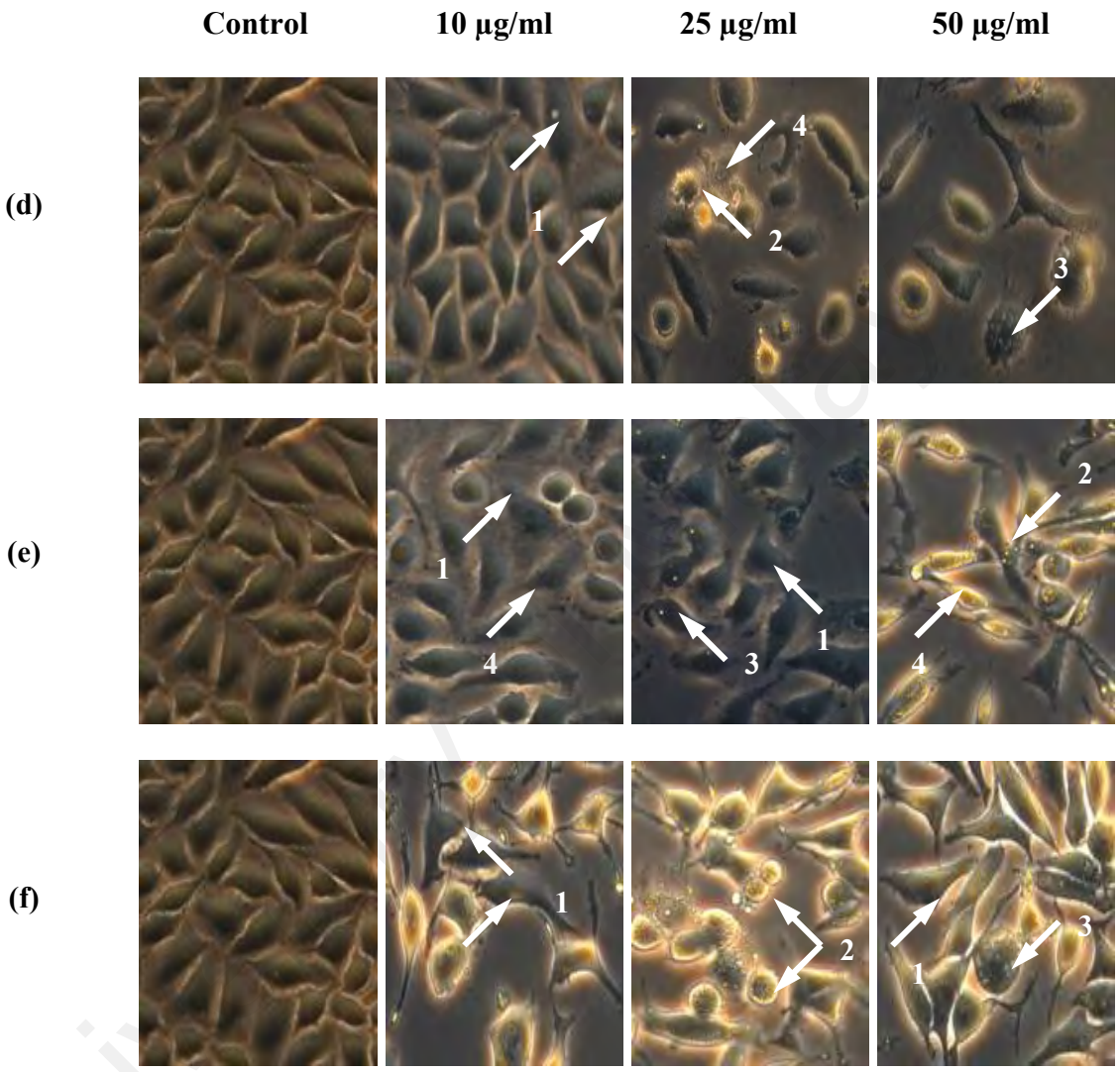


Figure 4.5(a): Morphological changes of MCF7 cells treated with (a) crude methanol extract of *P. watsonii*, (b) crude hexane extract of *P. watsonii*, (c) crude ethyl acetate extract of *P. watsonii*, (d) crude ethyl acetate extract of *P. pectinatus* (fruit), (e) fraction PPW6 and (f) fraction PPW7. Cells were treated at 10, 25 and 50 µg/ml for 24 h. The cells morphology was observed under the phase-contrast inverted microscope (magnification 200 ×). Data are representative of one of three independent experiments
1 ► membrane blebbing; 2 ► apoptotic body; 3 ► vacuolization; 4 ► fragmented nucleus

Figure 4.5(a), continued



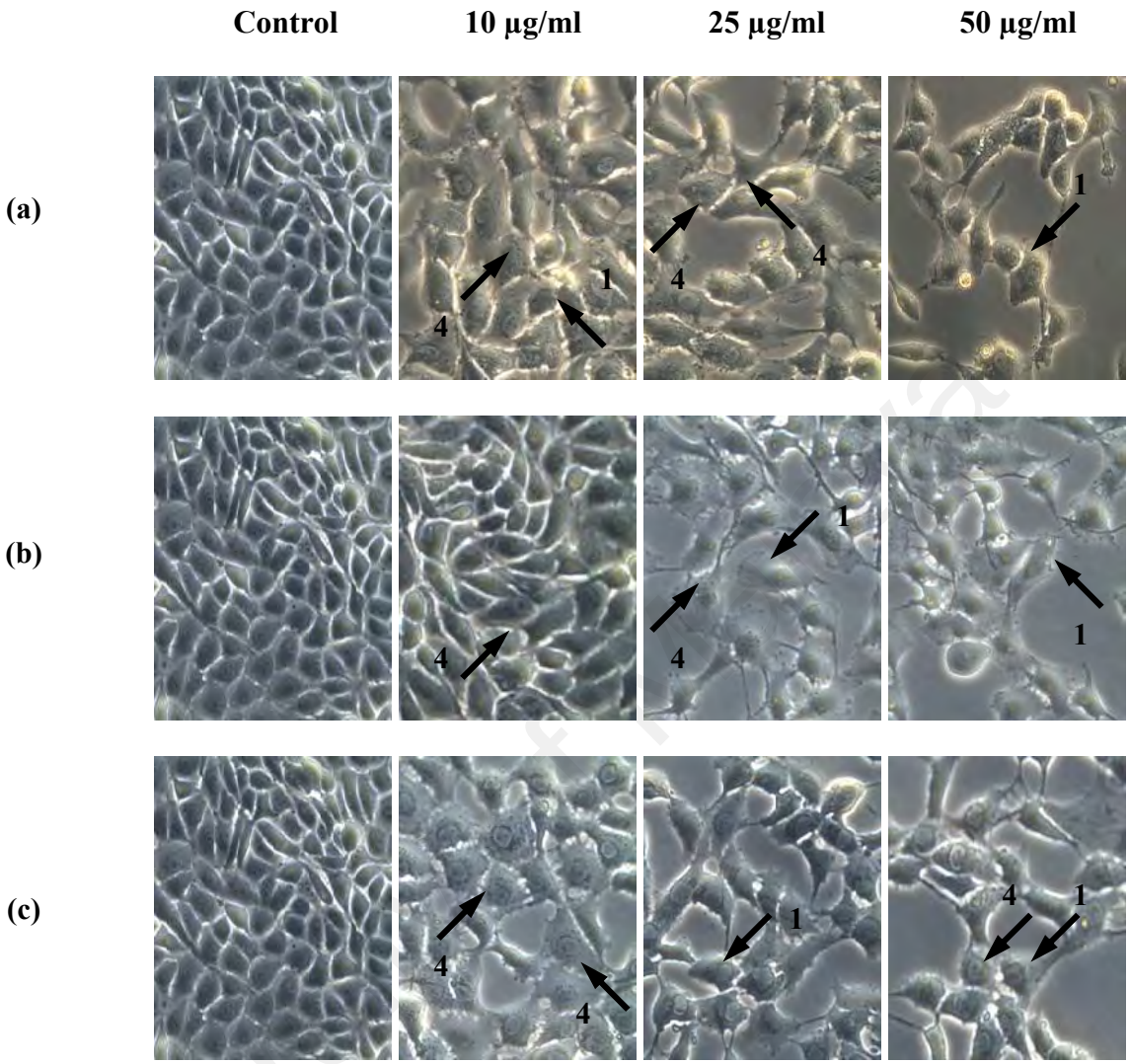
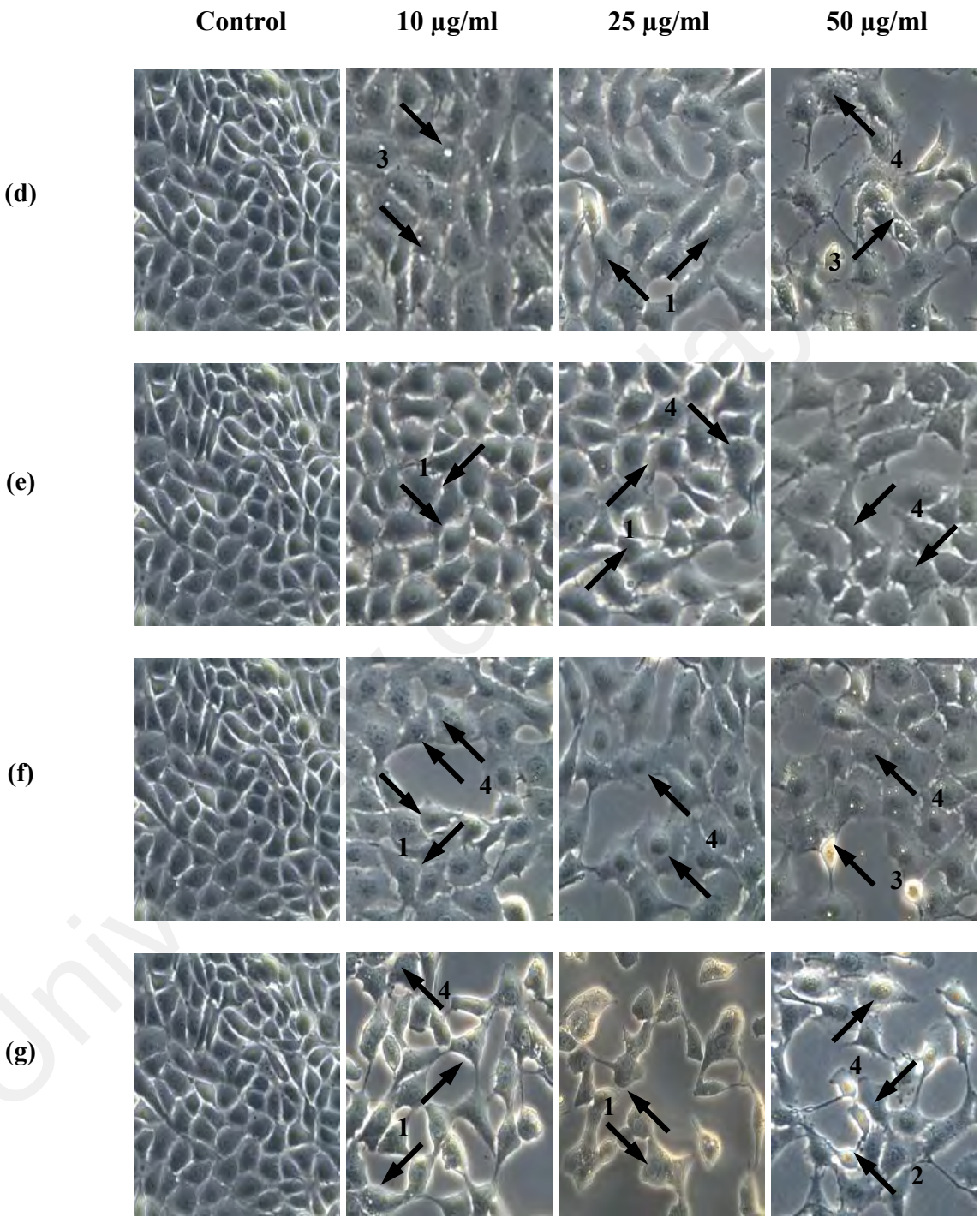


Figure 4.5(b): Morphological changes of SKOV3 cells treated with (a) crude methanol extract of *P. watsonii*, (b) crude hexane extract of *P. watsonii*, (c) crude ethyl acetate extract of *P. watsonii*, (d) crude methanol extract of *P. pectinatus* (leaves), (e) crude ethyl acetate extract of *P. pectinatus* (leaves), (f) fraction PPW6 and (g) fraction PPW7. Cells were treated at 10, 25 and 50 µg/ml for 24 h. The cells morphology was observed under the phase-contrast inverted microscope (magnification 200 ×). Data are representative of one of three independent experiments

1 ► membrane blebbing; 2 ► apoptotic body; 3 ► vacuolization; 4 ► fragmented nucleus

Figure 4.5(b), continued



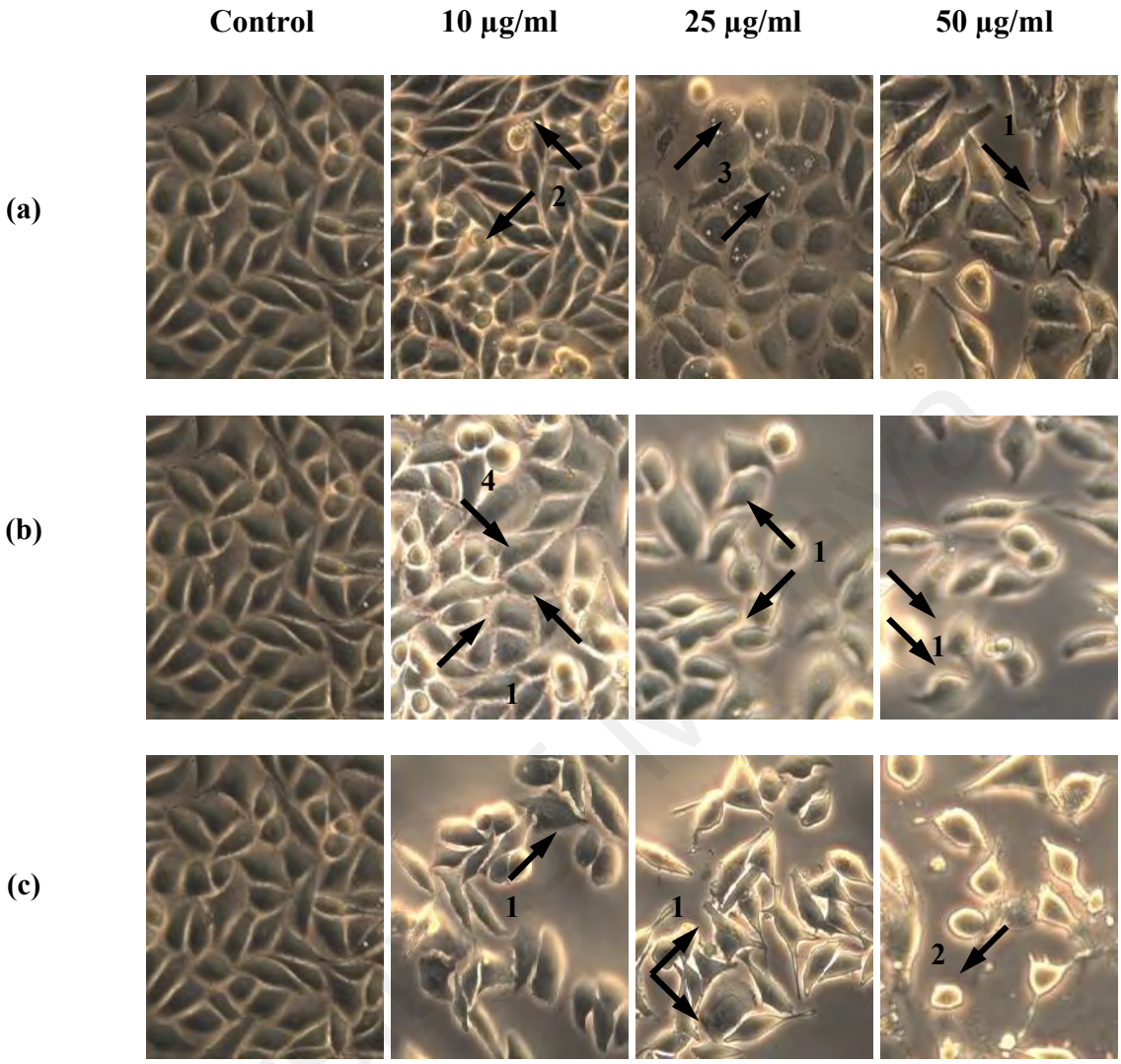
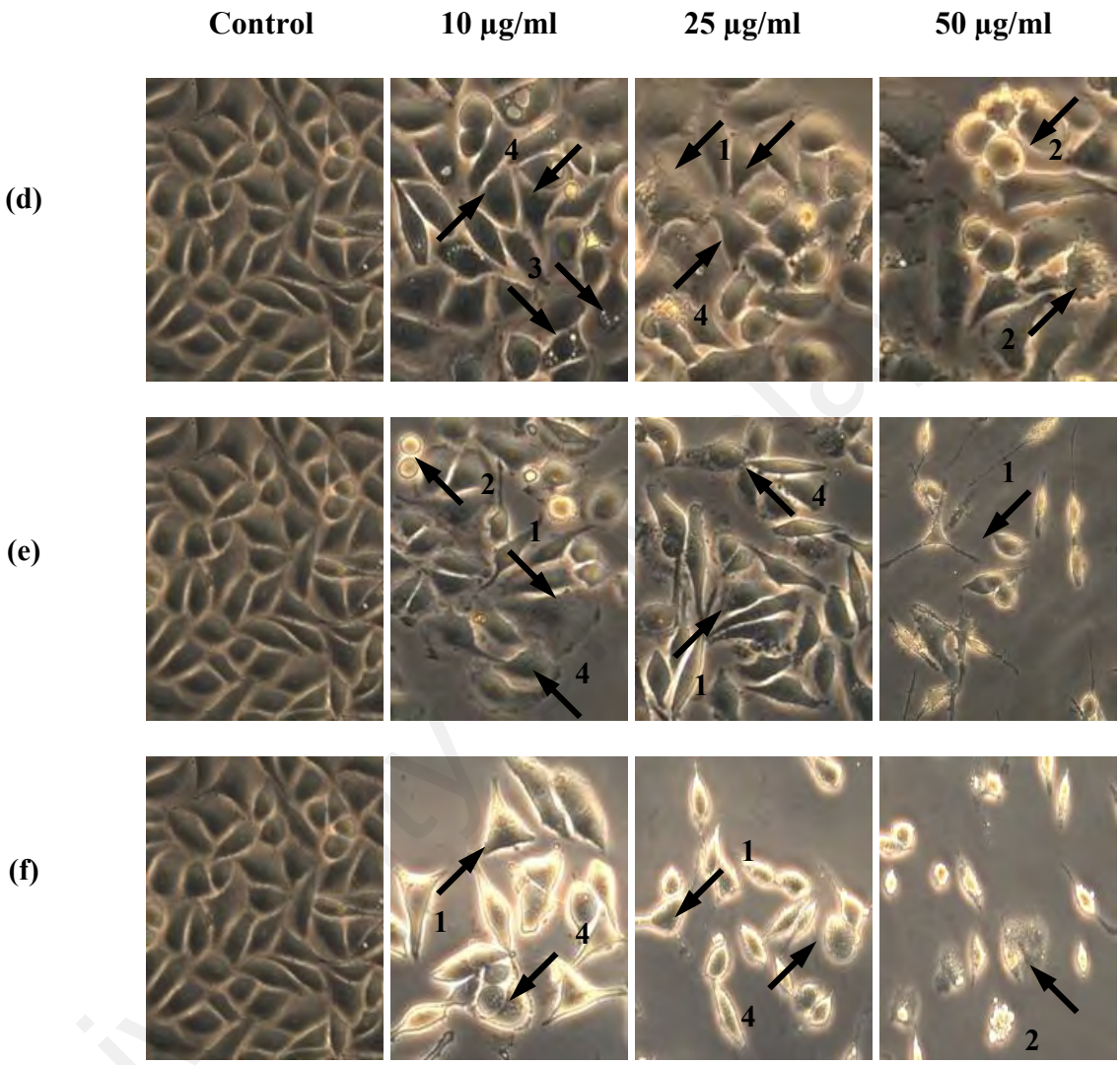


Figure 4.5(c): Morphological changes of CaSki cells treated with (a) crude methanol extract of *P. watsonii*, (b) crude hexane extract of *P. watsonii*, (c) crude ethyl acetate extract of *P. watsonii*, (d) crude ethyl acetate extract of *P. pectinatus* (fruit), (e) fraction PPW6 and (f) fraction PPW7. Cells were treated at 10, 25 and 50 µg/ml for 24 h. The cells morphology was observed under the phase-contrast inverted microscope (magnification 200 ×). Data are representative of one of three independent experiments

1 ► membrane blebbing; 2 ► apoptotic body; 3 ► vacuolization; 4 ► fragmented nucleus

Figure 4.5(c), continued



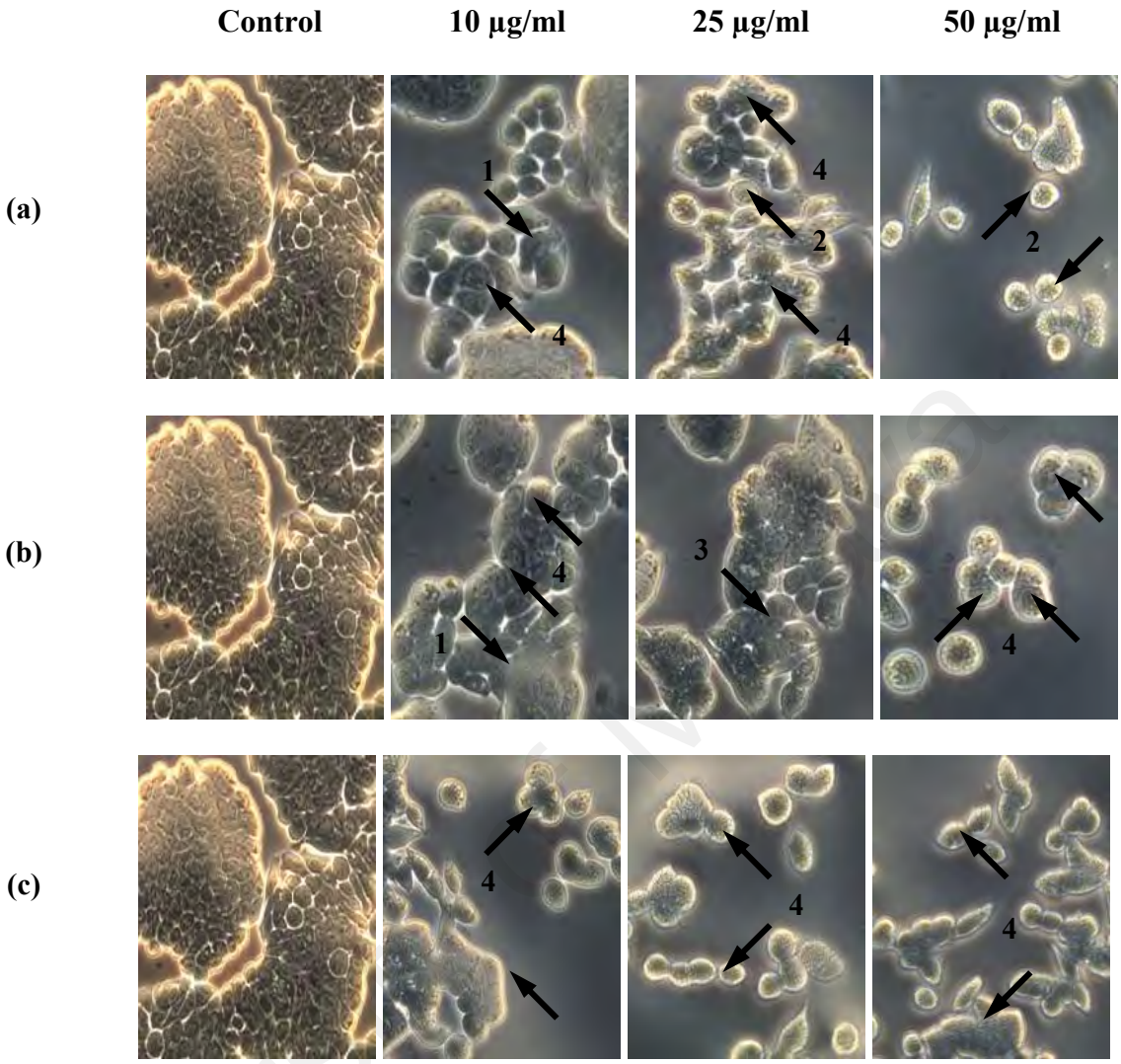
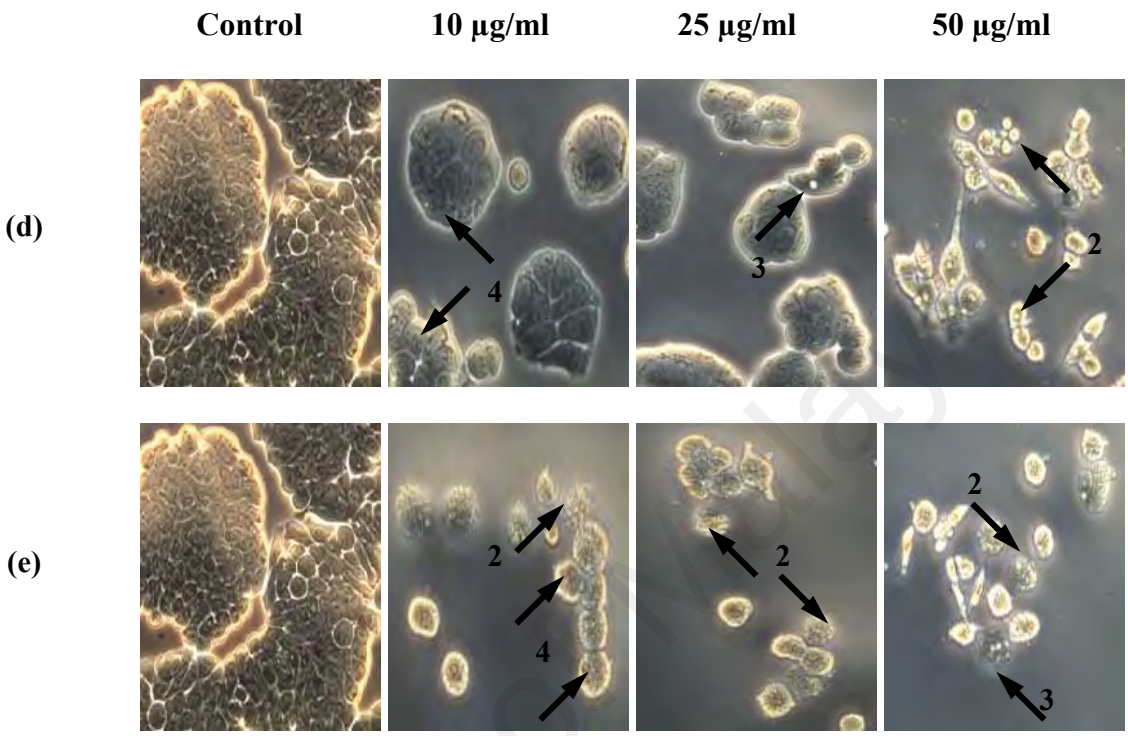


Figure 4.5(d): Morphological changes of HT29 cells treated with (a) crude methanol extract of *P. watsonii*, (b) crude hexane extract of *P. watsonii*, (c) crude ethyl acetate extract of *P. watsonii*, (d) fraction PPW6 and (e) fraction PPW7. Cells were treated at 10, 25 and 50 µg/ml for 24 h. The cells morphology was observed under the phase-contrast inverted microscope (magnification 200 ×). Data are representative of one of three independent experiments

1 ► membrane blebbing; 2 ► apoptotic body; 3 ► vacuolization; 4 ► fragmented nucleus

Figure 4.5(d), continued



4.5.2 Analysis of Cell Morphological by Acridine Orange/Ethidium Bromide (AO/EB) Fluorescence Staining

The morphological changes of the MCF7, SKOV3, CaSki and HT29 cells-treated with 10.0 µg/ml of cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 for 24 h were also been observed by acridine orange/ethdium bromide (AO/EB) fluorescence staining and the significant morphological apoptotic changes compared to untreated cells (control) were captured and illustrated in Figures 4.6a – 4.6d. In order to distinguish between viable, apoptotic and necrotic cells, the cells morphological criteria was based on reports by Xiao *et al*., (2007). Xiao *et al*., (2007) reported that in AO/EB staining, the viable cell will possess uniform bright green nucleus, early apoptotic cells will have bright green areas of condensed or fragmented chromatin in the nucleus, and necrotic cells will have uniform bright orange nucleus.

The control cells showed uniform bright green nuclei and cytoplasm. Cells treated with extracts or fractions exhibited the characteristic changes indicative of apoptosis, with cell shrinkage, nuclear condensation and fragmentation and formation of apoptotic bodies.

In Figures 4.6(a) – 4.6(d), it is clearly evident that the apoptotic nuclei have highly condensed chromatin that is uniformly fluorescent. The condensed chromatin can be observed in the form of crescents around the periphery of the nucleus, or the entire chromatin present as one or group of bright spherical beads. The nuclei were bright green and the cell profile dark green, suggesting that the treatment of 10.0 µg/ml of cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 indeed induced apoptosis.

In the control cells, the nuclei were bigger and look smoother when compared to the cells treated with the crude extracts or fractions of Phyllanthaceae species. Many "apoptotic bodies" were observed in SKOV3 cells-treated with crude hexane extract of *P. watsonii*, crude methanol and ethyl acetate extract of *P. pectinatus* (leaves). The formation of the "apoptotic bodies" was also observed in HT29 cells-treated with crude ethyl acetate of *P. watsonii* and fraction PPW7; and in MCF7 cells treated with fraction PPW7.

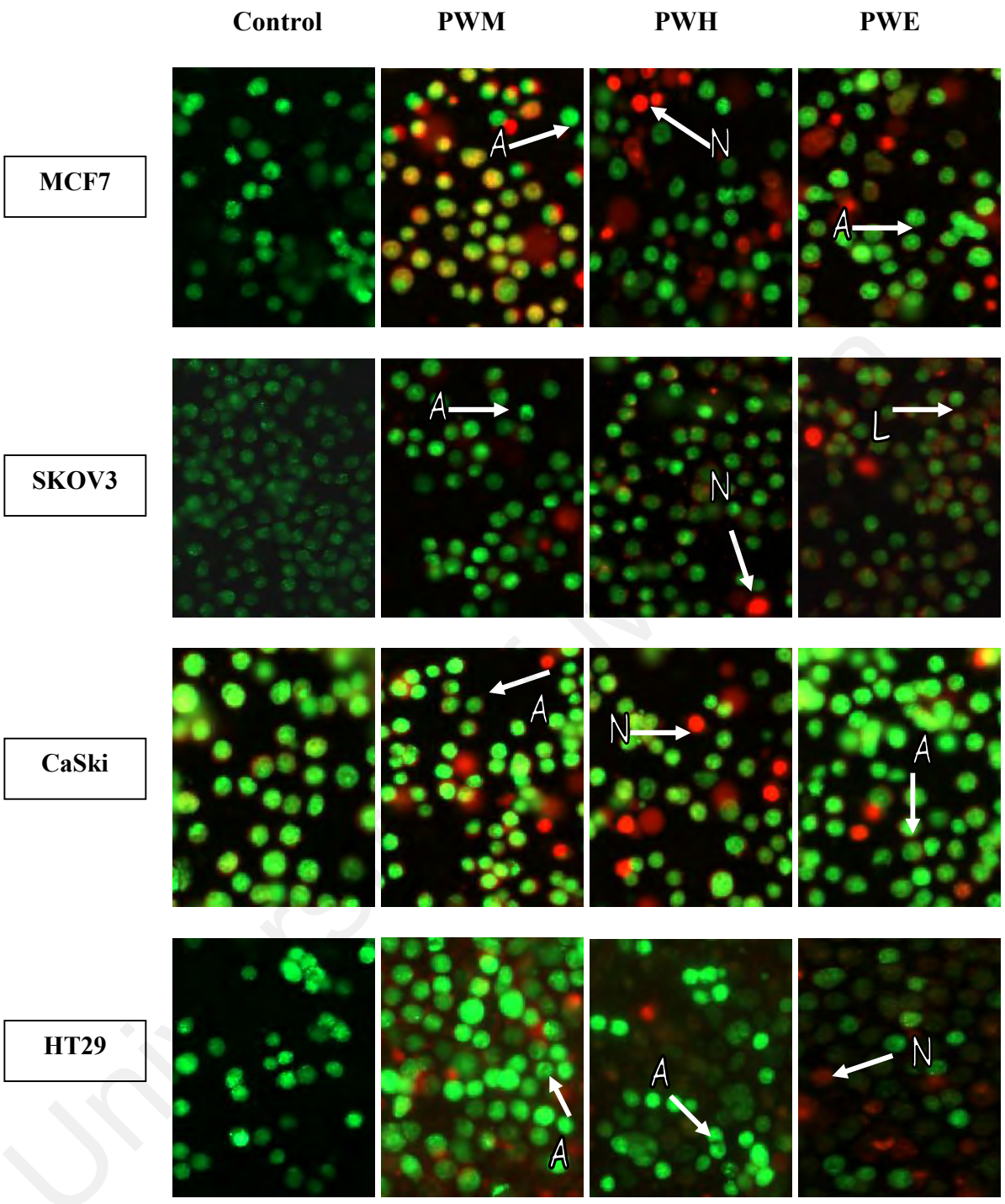


Figure 4.6(a): Apoptotic morphological changes of MCF7, SKOV3, CaSki and HT29 cells induced by cytotoxically active crude extracts of *Phyllanthus watsonii*. Cellular morphology was observed under fluorescence microscope by staining with AO/EB (magnification 200 ×). Data are representative of one of three independent experiments. (PWM= crude methanol extract of *P. watsonii*; PWH= crude hexane extract of *P. watsonii* and PWE= crude ethyl acetate extract of *P. watsonii*).
L ► Live cells; A ► Apoptotic cells; and N ► necrotic cells

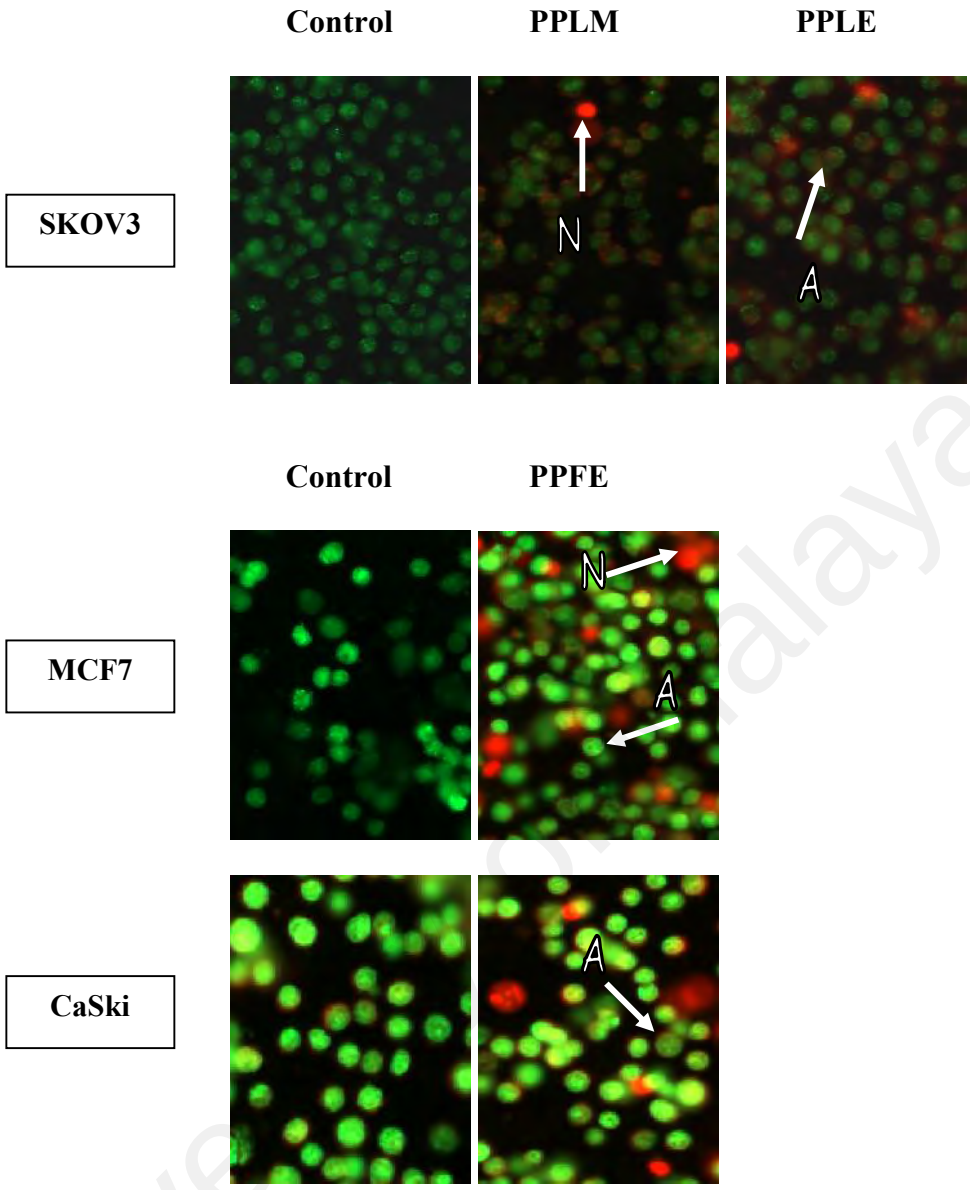


Figure 4.6(b): Apoptotic morphological changes of MCF7, SKOV3, CaSki and HT29 cells induced by cytotoxically active crude extracts of *Phyllanthus pectinatus*. Cellular morphology was observed under fluorescence microscope by staining with AO/EB (magnification 200 ×). Data are representative of one of three independent experiments. (PPLM= crude methanol extract of *P. pectinatus* leaves; PPLE= crude ethyl acetate extract of *P. pectinatus* leaves and PPFE= crude ethyl acetate extract of *P. pectinatus* fruits).
L ► Live cells; A ► Apoptotic cells; and N ► necrotic cells

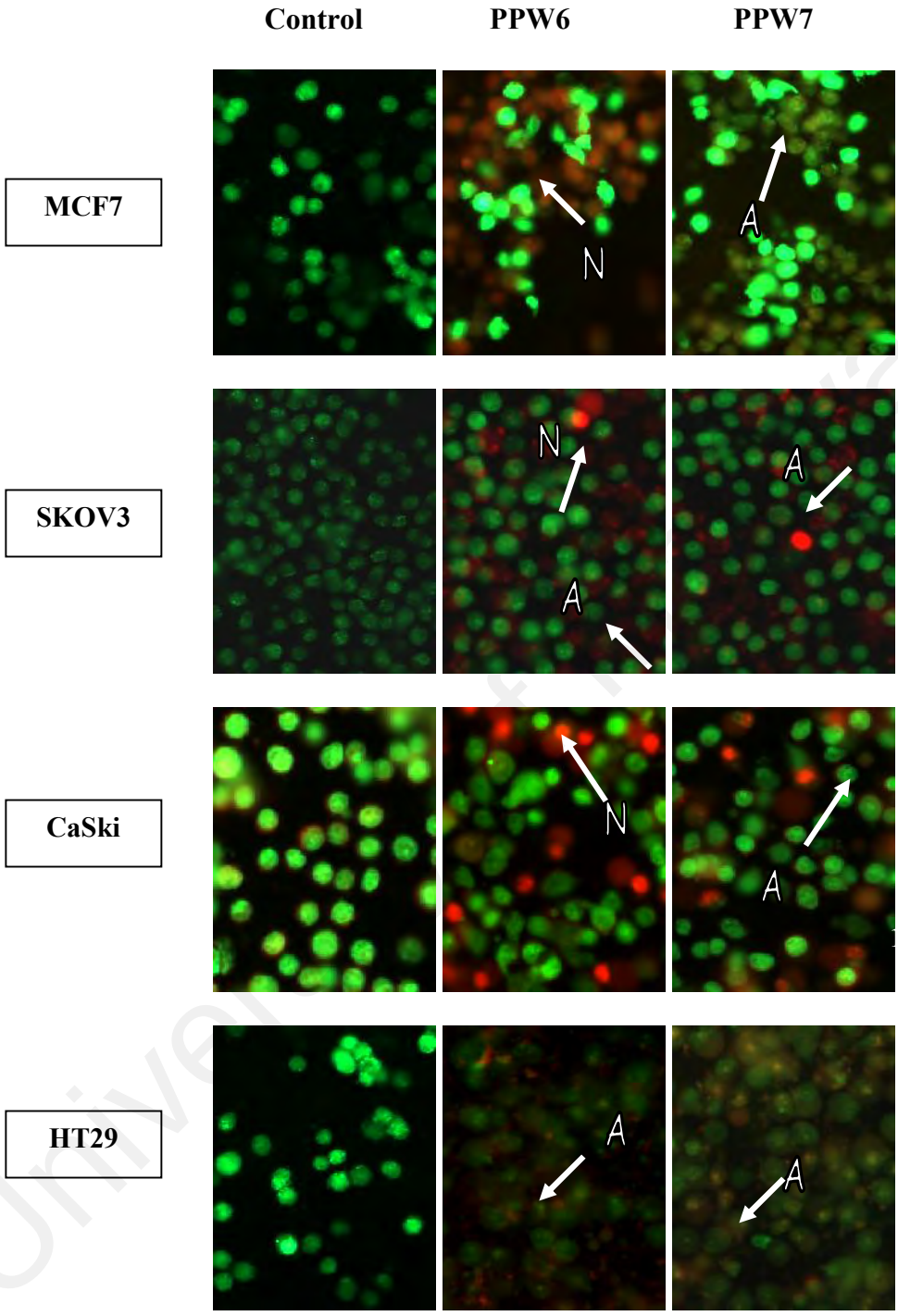


Figure 4.6(c): Apoptotic morphological changes of MCF7, SKOV3, CaSki and HT29 cells induced by cytotoxically active fractions of *Phyllanthus watsonii*. Cellular morphology was observed under fluorescence microscope by staining with AO/EB (magnification 200 ×). Data are representative of one of three independent experiments.
L ► Live cells; A ► Apoptotic cells; and N ► necrotic cells

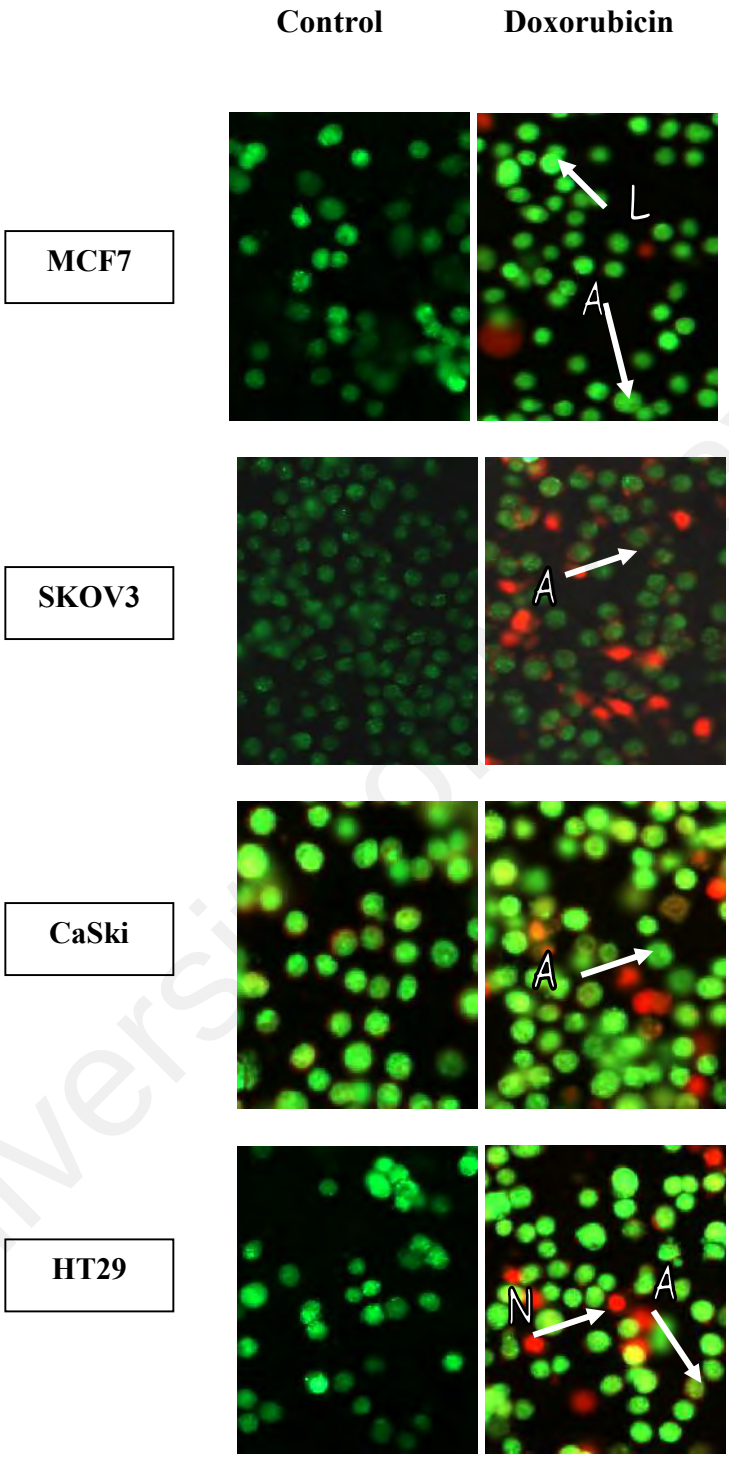


Figure 4.6(d): Apoptotic morphological changes of MCF7, SKOV3, CaSki and HT29 cells induced by doxorubicin (positive control). Cellular morphology was observed under fluorescence microscope by staining with AO/EB (magnification 200 ×). Data are representative of one of three independent experiments.
L ► Live cells; A ► Apoptotic cells; and N ► necrotic cells

4.5.3 Detection of DNA Fragmentation by Agarose Electrophoresis

An important feature of cell apoptosis is the fragmentation of genomic DNA into integer multiples of 180 – 200 base pairs (bp) units producing a characteristic ladder on agarose gel electrophoresis. In contrast, random cleavage of DNA in necrotic cells will produce a diffuse smear upon electrophoresis of DNA. To elucidate whether cytotoxically active extracts and fractions of Phyllanthaceae decrease cell survival by the induction of DNA fragmentation, genomic DNA was isolated from MCF7, SKOV3, CaSki and HT29 cells exposed to different concentrations, 100, 10 and 1 µg/ml of crude extracts or fractions of Phyllanthaceae species and electrophoresed.

DNA fragmentation was observed in a dose-dependant manner. The electrophoresis results are shown in Figure 4.7(a) – 4.7(d). Characteristic oligonucleosomal DNA fragments (ladders) of MCF7, SKOV3, CaSki and HT29 cells treated with cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 at 100, 10 and 1 µg/ml for 48 h was observed on a 1.5 % agarose gel. The efficient induction of apoptosis was observed in MCF7, SKOV3, CaSki and HT29 cells treated with 100 µg/ml crude extracts or fractions of Phyllanthaceae species for 48 h by formation of distinct ladders. DNA isolated from untreated cells (control) did not show any ladder formation.

The presence of DNA ladder (fragmented DNA) at all the concentrations (100, 10 and 1 µg/ml) can be clearly observed in SKOV3 cells treated with crude hexane and ethyl acetate extract of *P. watsonii*, crude ethyl acetate extract of *P. pectinatus* (leaves). Even at lower concentration (1 µg/ml), DNA ladder was clearly visible and this became increasingly prominent in SKOV3 cells treated with higher concentration (100 µg/ml) of the crude extracts. The similar pattern of ladder formation was also observed in CaSki cells

treated with fraction PPW7. In MCF7 cells-treated with crude hexane and ethyl acetate extract of *P. watsonii* and in CaSki cells-treated with crude methanol and ethyl acetate extract of *P. watsonii* and crude ethyl acetate extract of *P. pectinatus* (fruits), fragmented DNA (DNA ladder) was clearly observed in two higher concentrations, 100 and 10 µg/ml. Treatment of MCF7 cells with crude methanol of *P. watsonii* produced DNA ladder at only highest concentration (100 µg/ml). Interestingly, in MCF7 cells-treated with fraction PPW7, formation of DNA ladder can only be observed clearly at lower concentrations (10 and 1 µg/ml).

In HT29 cells-treated with cytotoxically active crude extracts and fractions of Phyllanthaceae species, the formation of DNA ladder was diffused interspersed with smear which indicating the presence of some post-apoptotic necrosis cells. This pattern can be clearly observed in HT29 cells-treated with crude methanol, hexane and ethyl acetate extract of *P. watsonii* and fractions PPW6 and PPW7 at concentration of 100, 10 and 1 µg/ml. This similar pattern also can be observed in MCF7, SKOV3 and CaSki cells treated with cytotoxically active crude extracts and fractions of Phyllanthaceae species. The presence of DNA ladder which was diffused interspersed with smear at all the concentrations (100, 10 and 1 µg/ml) can be clearly observed in MCF7 cells treated with crude ethyl acetate extract of *P. watsonii* and fraction PPW6, whereas in SKOV3 cells-treated with crude methanol extract of *P. watsonii*, crude methanol extract of *P. pectinatus* (leaves), fractions PPW6 and PPW7 and in CaSki cells-treated with crude hexane extract of *P. watsonii* and fraction PPW6.

The formation of DNA ladder which was diffused interspersed with smear in MCF7 cells-treated with fraction PPW7 can only be observed at the highest concentrations (100 µg/ml). Interestingly, the formation of DNA ladder which was diffused interspersed with smear can also be observed in MCF7 cells-treated with extracts or fractions at lower

concentration. This DNA pattern was observed in MCF7 cell-treated with crude methanol extract of *P. watsonii* at concentrations of 10 and 1 µg/ml and with crude hexane extract of *P. watsonii* and crude ethyl acetate extracts of *P. pectinatus* (fruits) concentrations of 1 µg/ml. Caski cells-treated with crude methanol and ethyl acetate extract of *P. watsonii* and crude ethyl acetate extract of *P. pectinatus* (fruits) at 1 µg/ml produced this DNA ladder pattern which was diffused interspersed with smear.

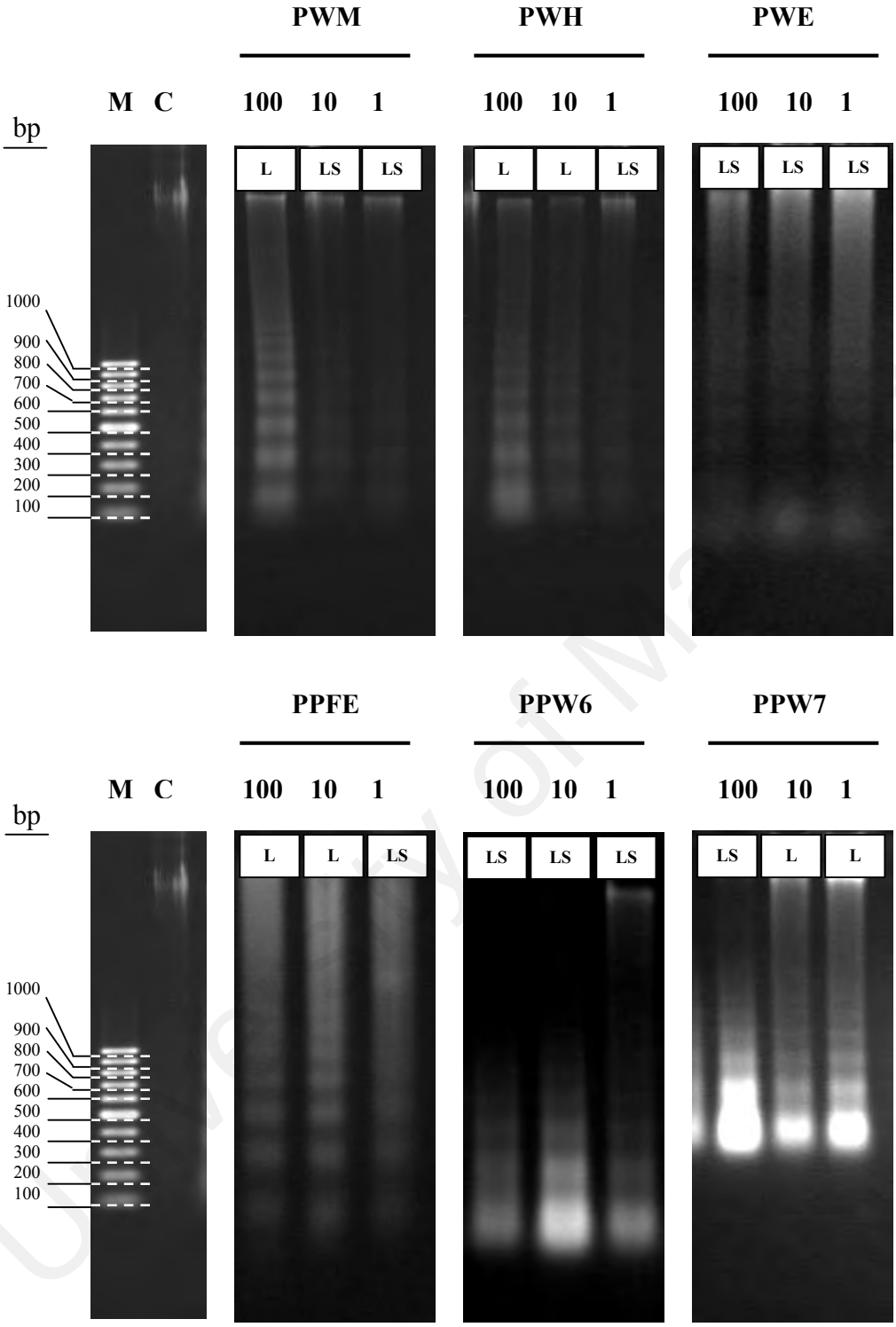


Figure 4.7(a): DNA fragmentation of MCF7 cells after incubation with 100, 10 and 1 µg/ml of cytotoxically active extracts and fractions of Phyllanthaceae species for 48 h. DNA ladders reflecting the presence of DNA fragments were viewed on ethidium bromide-stained gel. Typical result from three independent experiments is shown. M: 100 bp DNA markers; C: control (untreated cells); PWM: crude methanol extract of *P. watsonii*; PWH: crude hexane extract of *P. watsonii*; PWE: crude ethyl acetate extract of *P. watsonii*; and PPFE: crude ethyl acetate extract of *P. pectinatus* fruits. L=DNA ladder and LS=DNA ladder which diffused interspersed with smear.

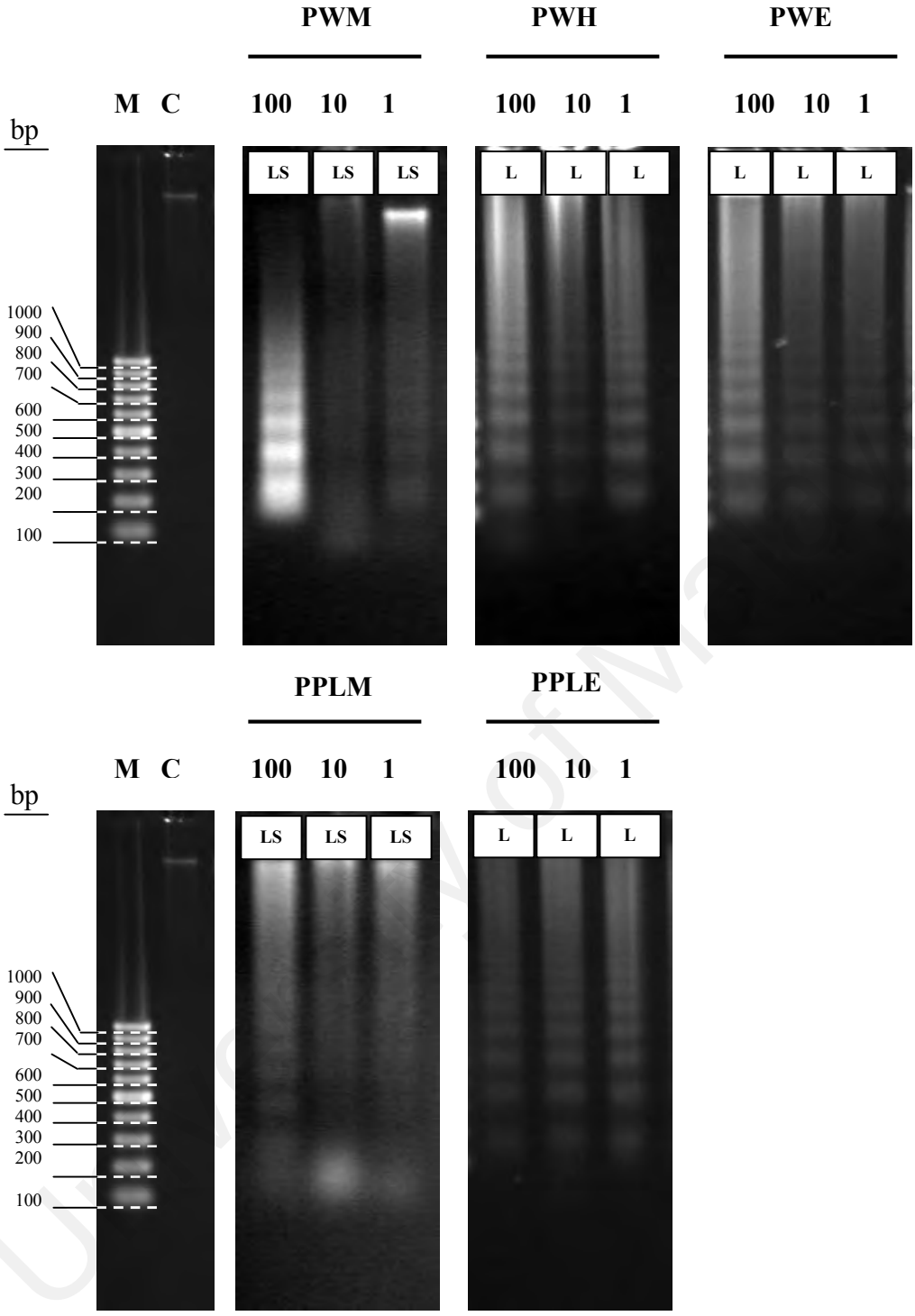
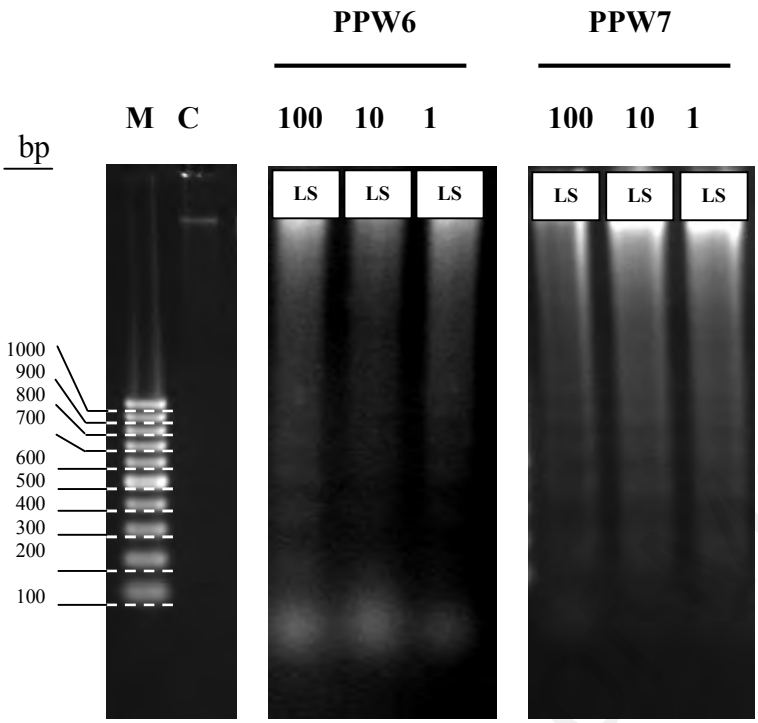


Figure 4.7(b): DNA fragmentation of SKOV3 cells after incubation with 100, 10 and 1 µg/ml of cytotoxically active extracts and fractions of Phyllanthaceae species for 48 h. DNA ladders reflecting the presence of DNA fragments were viewed on ethidium bromide-stained gel. Typical result from three independent experiments is shown. M: 100 bp DNA markers; C: control (untreated cells); PWM: crude methanol extract of *P. watsonii*; PWH: crude hexane extract of *P. watsonii*; PWE: crude ethyl acetate extract of *P. watsonii*; PPLM: crude methanol extract of *P. pectinatus* leaves and PPLE: crude ethyl acetate extract of *P. pectinatus* leaves. L=DNA ladder and LS=DNA ladder which diffused interspersed with smear.

Figure 4.7 (b), continued



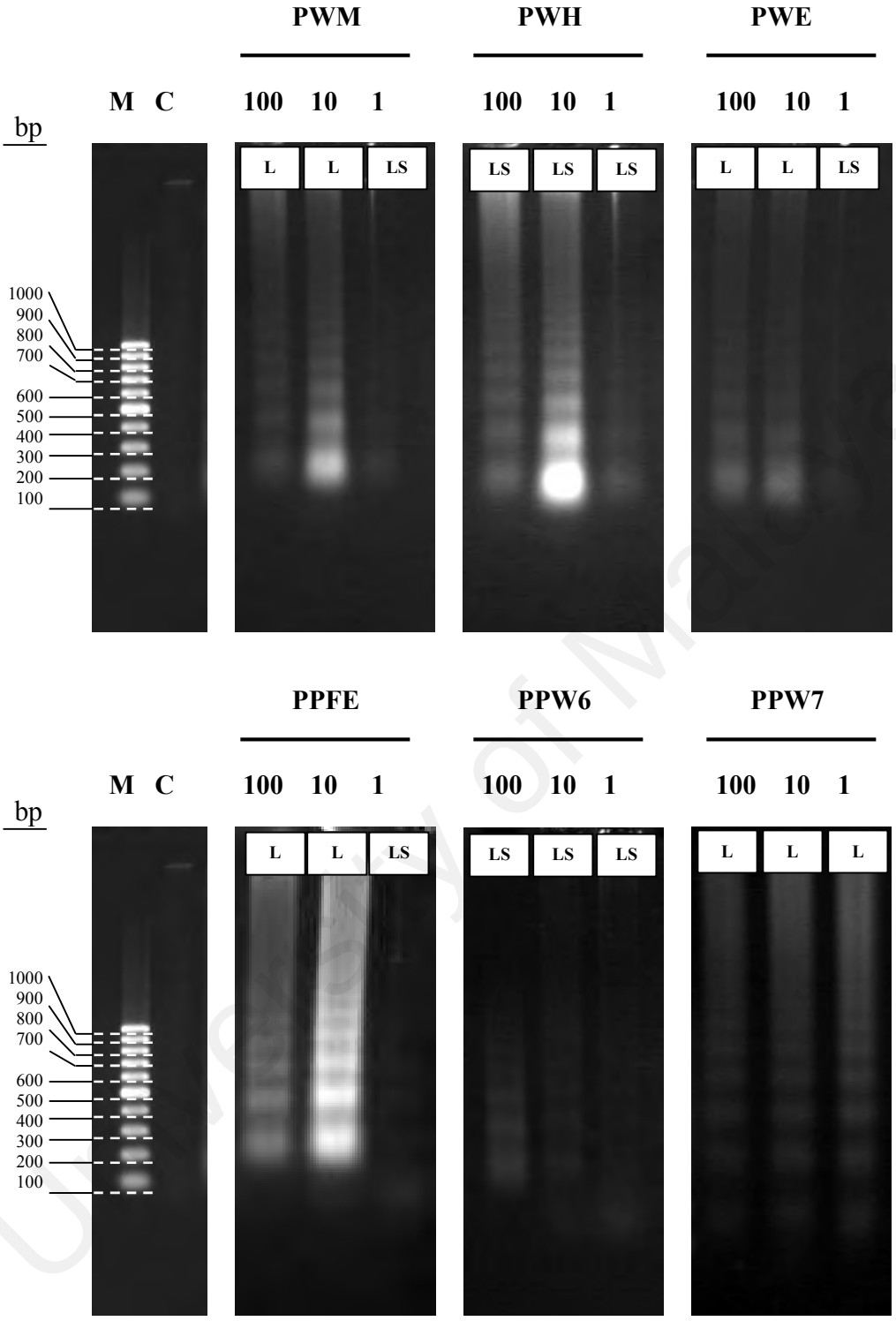


Figure 4.7(c): DNA fragmentation of CaSki cells after incubation with 100, 10 and 1 µg/ml of cytotoxically active extracts and fractions of Phyllanthaceae species for 48 h. DNA ladders reflecting the presence of DNA fragments were viewed on ethidium bromide-stained gel. Typical result from three independent experiments is shown. M: 100 bp DNA markers; C: control (untreated cells); PWM: crude methanol extract of *P. watsonii*; PWH: crude hexane extract of *P. watsonii*; PWE: crude ethyl acetate extract of *P. watsonii*; and PPFE: crude ethyl acetate extract of *P. pectinatus* fruits. L=DNA ladder and LS=DNA ladder which diffused interspersed with smear.

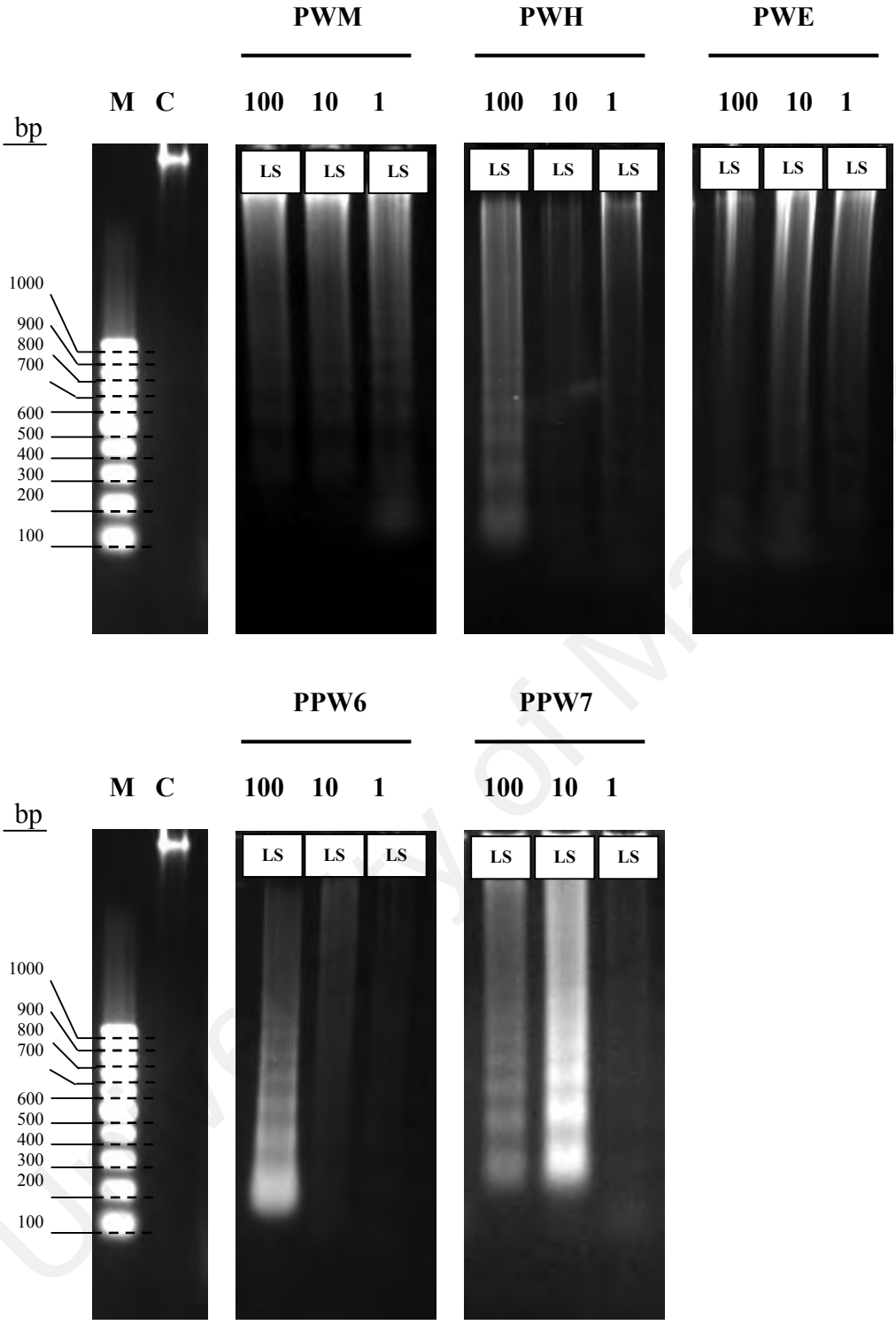


Figure 4.7(d): DNA fragmentation of HT29 cells after incubation with 100, 10 and 1 µg/ml of cytotoxically active extracts and fractions of Phyllanthaceae species for 48 h. DNA ladders reflecting the presence of DNA fragments were viewed on ethidium bromide-stained gel. Typical result from three independent experiments is shown. M: 100 bp DNA markers; C: control (untreated cells); PWM: crude methanol extract of *P. watsonii*; PWH: crude hexane extract of *P. watsonii* and PWE: crude ethyl acetate extract of *P. watsonii*. L=DNA ladder and LS=DNA ladder which diffused interspersed with smear.

4.5.4 Determination of Caspase-3 Activation

The cellular pathway of Phyllanthaceae-induced cell death was examined by assessing caspase-3 activity, which plays a central role in mediating apoptotic responses (Nieves-Neira and Pommier, 1999). Following a 48 h treatment of MCF7, SKOV3, CaSki and HT29 cells with 10 µg/ml of cytotoxically active crude extracts Phyllanthaceae species and fractions of *P. watsonii*, caspase-3 activities were measured and compared with control (untreated) cells (Figure 4.8). Doxorubicin was used as positive control in the assay. As illustrated in Figure 4.8, caspase-3 specific activity was detected after treatment with cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7).

The caspase-3 activities in MCF7 cells were increased 1.5 to 2.4-folds after treatment with 10 µg/ml of cytotoxically active crude extracts and fractions of Phyllanthaceae species for 48 h. The highest increment of the caspase-3 activity was observed in MCF7 cells-treated with crude methanol extracts of *P. watsonii* (2.4-folds increment), followed by MCF7 cells- treated with fraction PPW6 (2.2-folds increment) and MCF7 cells-treated with fraction PPW7 (1.9-folds increment). However, the increment of caspase-3 activity due to addition of standard anticancer drug, doxorubicin was the highest with increment of 4.4-folds.

SKOV3 cells-treated with the cytotoxically active crude extracts and fractions exhibited a dramatic increment in caspase-3 activity with range from 1.9 to 3.7-folds higher compared to untreated cells. The caspase activity detected in SKOV3 cells-treated with cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 were higher or similar to the SKOV3 cells-treated with doxorubicin, which exhibited increment of 2.5-fold. Caspase-3 activity was

increased 3.7-fold, 3.5-fold and 3.2-fold after treatment with crude ethyl acetate extract of *P. pectinatus* leaves, crude methanol extract of *P. watsonii* and crude methanol extract of *P. pectinatus* leaves, respectively.

The caspase-3 activity increased slightly, 1.7-folds in all CaSki cells-treated with cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7. The caspase-3 activity observed in CaSki cells after addition of the extracts and fractions was lower compared to cells treated with doxorubicin, which showed increment 3.1-fold in caspase-3 activity.

After addition of cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 in HT29 cells, increment in caspase-3 activities observed ranged from 1.8 to 2.3-folds. Crude ethyl acetate of *P. watsonii* caused the highest increment in caspase-3 activity in HT29 followed by crude hexane extract of *P. watsonii* and fraction PPW6. The increments were 2.3-fold, 2.0-fold and 1.8-fold respectively compared to untreated cells. These results suggest that cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7-induced apoptosis through the activation of caspase-3, common executors of apoptosis.

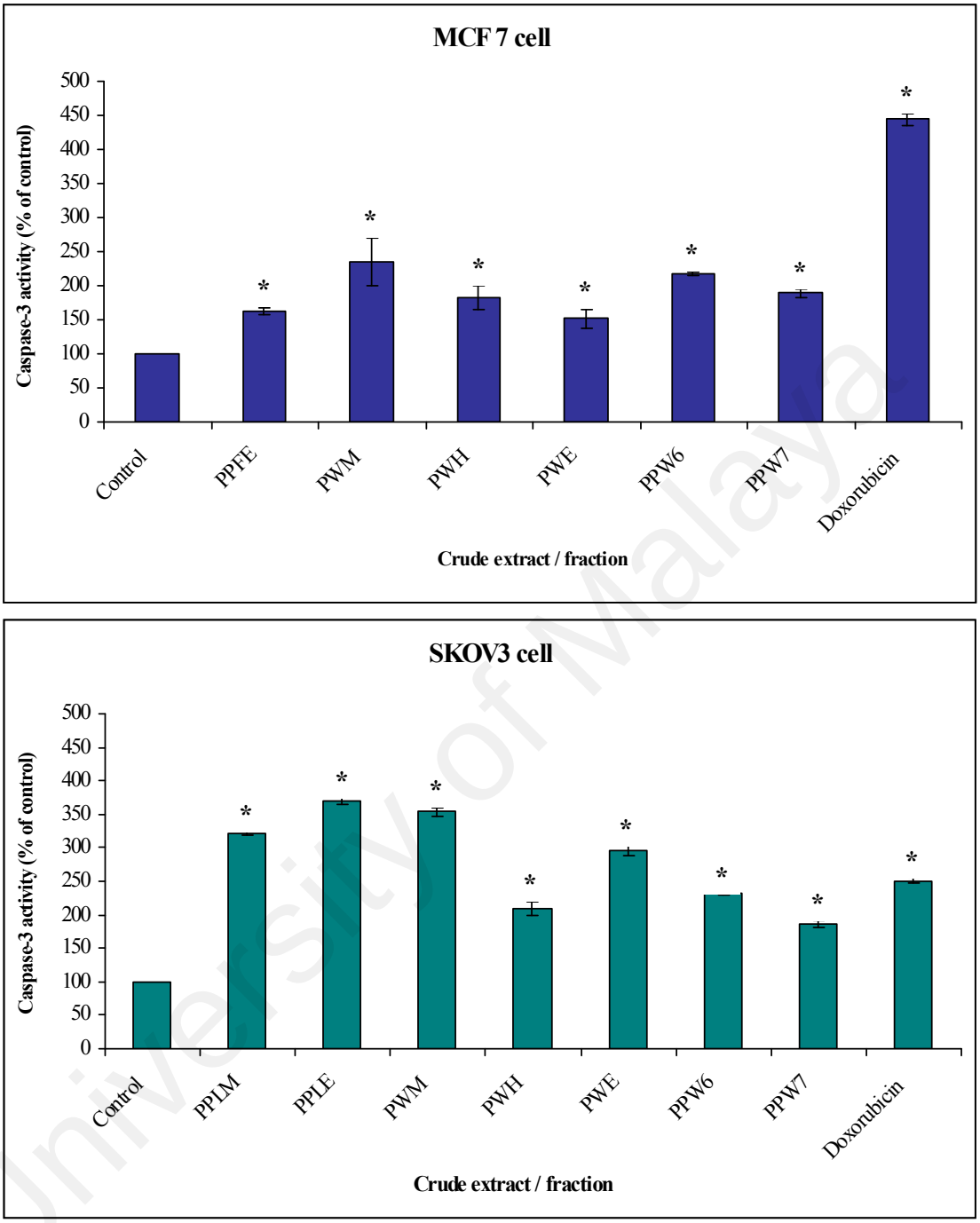
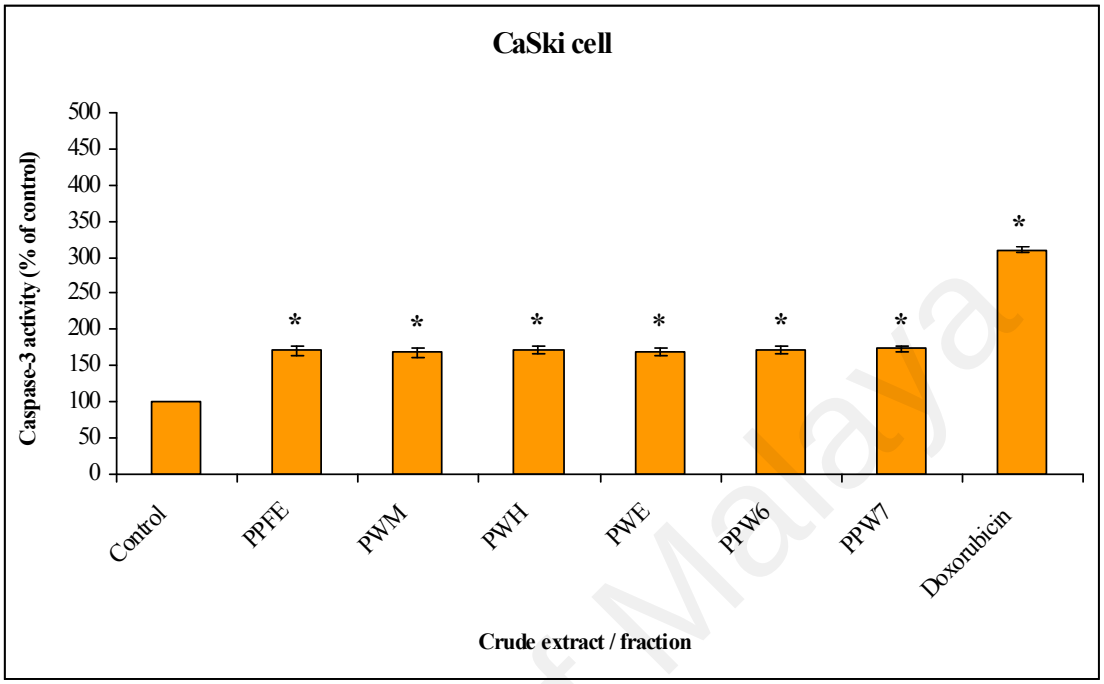
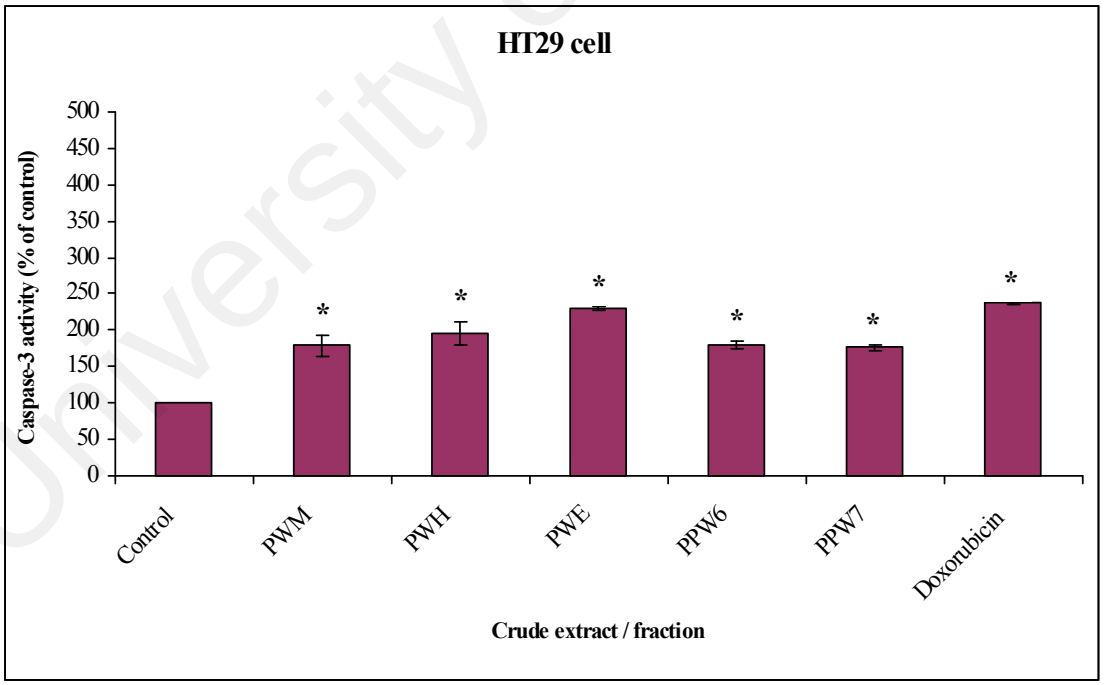


Figure 4.8: Caspase-3 activity in cells treated with cytotoxically active crude extracts and fractions of Phyllanthaceae species. Caspase-3 activity was analyzed using Caspase-3/CPP32 Colorimetric Assay Kit after treatment with 10 µg/ml of extracts/fraction for 48 h. Data are mean ± S.D. Three separate experiments were performed and the caspase activities are expressed as percentage of enzyme activity compared with control. (PPFE= crude ethyl acetate extract of *P. pectinatus* fruits; PPLM= crude methanol extract of *P. pectinatus* leaves; PPLE= crude ethyl acetate extract of *P. pectinatus* leaves; PWM= crude methanol extract of *P. watsonii*; PWH= crude hexane extract of *P. watsonii*; PWE= crude ethyl acetate extract of *P. watsonii*). * indicates a significant difference from the control (p<0.05).

Figure 4.8, continued





4.5.5 Cell Cycle Analysis by Flow Cytometry

In order to determine whether the crude hexane extract of *P. watsonii* and fraction PPW7 affects cell growth by blocking the cell cycle in human MCF7 breast cancer, SKOV3 ovarian cancer, CaSki cervical cancer and HT29 colon cancer cell lines, the cell distribution in the different phases of the cell cycle was studied by flow cytometry following the extract and fraction treatment. Figure 4.9 (a) – 4.9(d) shows the effect of crude hexane extract of *P. watsonii* and fraction PPW7 on the cell cycle of MCF7, SKOV3, CaSki and HT29 cells.

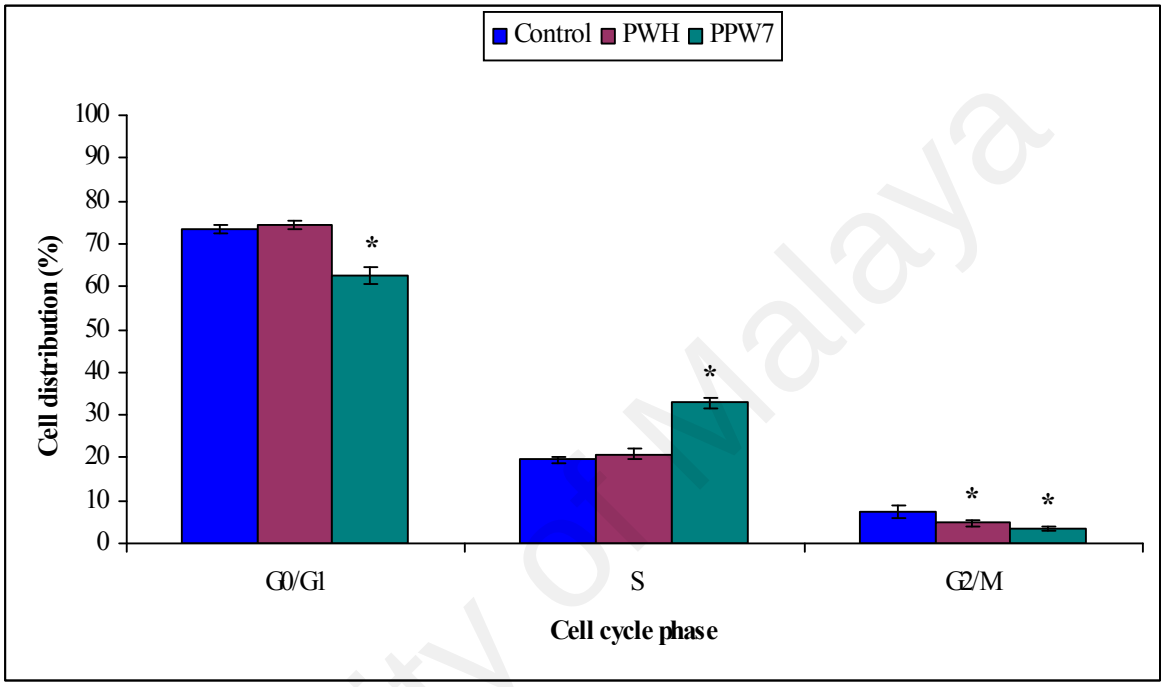
The stained DNA of MCF7 cells after treatment with 10 µg/ml crude hexane extract of *P. watsonii* demonstrated that 74.3 ± 0.99 % of the cells were in the G_0/G_1 phase, 20.9 ± 1.21 % in the S phase, and 4.7 ± 0.76 % in the G_2/M phase. Stained DNA of MCF7 cells after treatment with 10 µg/ml fraction PPW7 demonstrated that 62.7 ± 1.99 % of the cells were in the G_0/G_1 phase, 32.9 ± 1.26 % in the S phase, and 3.5 ± 0.50% in the G_2/M phase. These results suggested that 10 µg/ml crude hexane extract of *P. watsonii* caused the G_0/G_1 and S phase arrest of MCF7 cells and those 10 µg/ml fraction PPW7 significantly induced the S phase arrest of MCF7 cells ($p<0.05$).

Interestingly, SKOV3 cell when treated with crude hexane extract of *P. watsonii* and fraction PPW7 exhibited a dramatic accumulation of cells in S and G_2/M-phases and a concomitant decrease of the percentage of cells in G_0/G_1 phase, suggesting that both crude extract and fraction induced SKOV3 cell cycle arrest at S and G_2/M-phases. Thus, as shown in Figure 3.7(b), the percentage of treated cells in G_0/G_1 phase diminished from 93.4 ± 0.78 % (control) to 61.6 ± 1.05 % after 24 h of incubation with crude hexane extract of *P. watsonii*, whereas a significant increment from 5.5 ± 0.50 % (control) to 26.4 ± 0.87 % and 0.8 ± 0.20 % (control) to 12.3 ± 0.64 % was observed in S and G_2/M–

phases, respectively (p<0.05). In addition, the percentage of treated cells in G_0/G_1 phase diminished from 93.4 ± 0.78% (control) to 58.4 ± 1.11 % after 24 h of incubation with fraction PPW7, whereas a significant increment from 5.5 ± 0.50 % (control) to 25.3 ± 1.16 % and 0.8 ± 0.20 % (control) to 16.5 ± 0.60 % was observed in S and G_2/M–phases, respectively (p<0.05)..

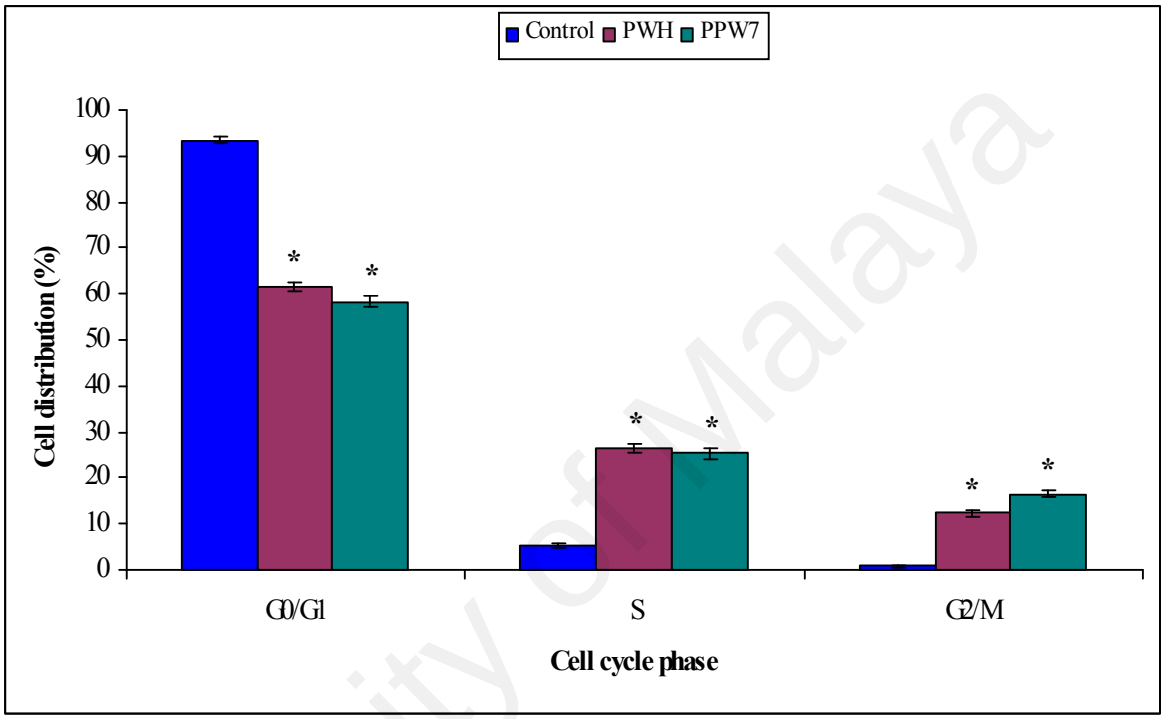
After treatment with crude hexane extract of *P. watsonii*, a drastic increase in the number of cells in G_0/G_1 phase compared to control was observed. In the CaSki-treated cells, the number of cells in G_0/G_1 phase was significantly increased to 93.0 ± 1.11 % compared to control cells (76.3 ± 0.81 %) (p<0.05).Treatment with 10 µg/ml of fraction PPW7 for 24 h significantly increased the proportions of cells in S phase from 13.6 ± 0.60 % (control) to 29.6 ± 1.27 % (p<0.05). These results provide evidence that the observed apoptotic cell death was partly due to the cell cycle arrest in S phase induced by fraction PPW7.

As shown in Figure 3.7(d), after 24 h treatment, crude hexane extract of *P. watsonii* increased the percentage of HT29 cells in G_0/G_1 phase (74.5 ± 1.26 %), while concomitantly decreasing the percentage of cells in S phase (20.5 ± 0.64 %) and G_2/M phase (4.5 ± 0.50 %) as compared with the control cells (72.6 ± 1.60 %, 20.7 ± 0.49 % and 6.78 ± 0.80 %, respectively). Growth arrest after exposure to 10 µg/ml of fraction PPW7 for 24 h occurred mainly in the G_0/G_1 phase, significantly increasing the HT29 cells population from 72.6 ± 1.60 % to 88.5 ± 0.57 % (p<0.05) while significantly depleting cells in the S and G_2/M phase from 20.7 ± 0.49 % to 11.2 ± 0.75 % and from 6.78 ± 0.80 % to 0.6 ± 0.20 %, respectively (p<0.05). It is clearly evident that the crude hexane extract of *P. watsonii* and fraction PPW7 induced HT29 cell cycle arrest at G_0/G_1 phase.



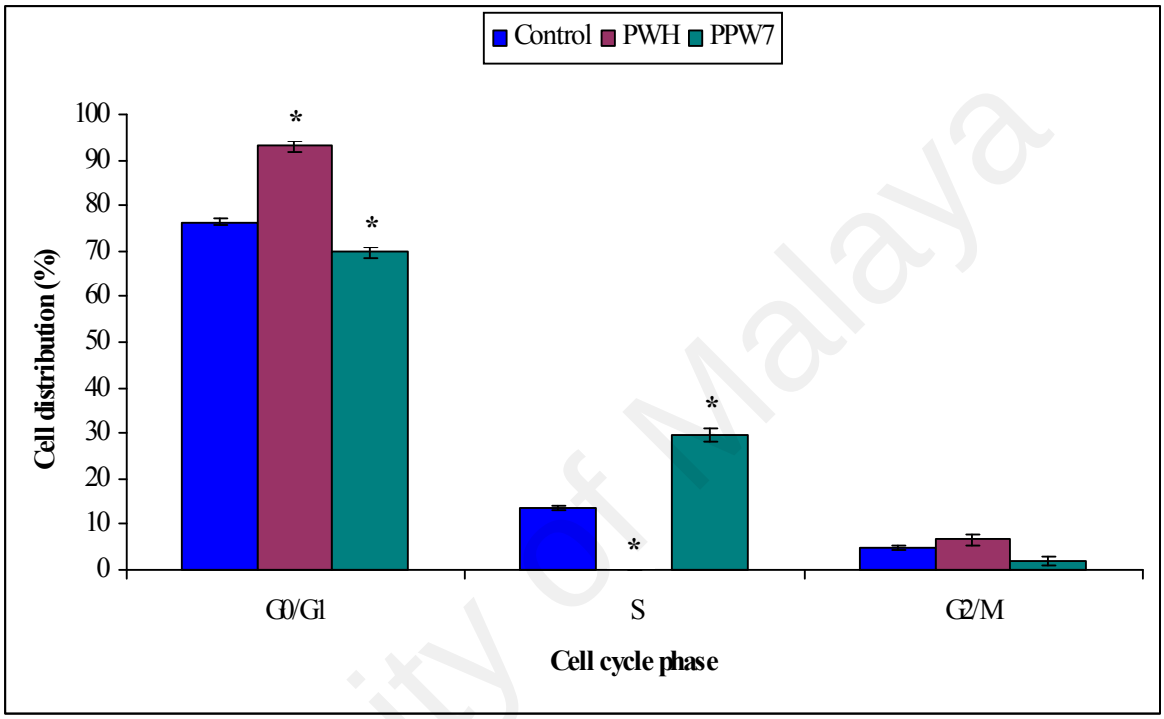
Cell-cycle	Distribution of cell-cycle (%)		
	Control	PWH	PPW7
G_0/G_1	73.5 ± 0.89	74.3 ± 0.99	62.7 ± 1.99
S	19.5 ± 0.81	20.9 ± 1.21	32.9 ± 1.26
G_2/M	7.6 ± 1.59	4.7 ± 0.76	3.5 ± 0.50

Figure 4.9(a): Effect of crude hexane extract of *P. watsonii* (PWH) and fraction PPW7 on cell cycle phase distribution in MCF7 cell. Cells (1×10^6 cells/ml) were incubated in the absence (control) or presence of PWH and PPW7 for 24 h and then analyzed by flow cytometry. * indicates a significant difference from the control ($p<0.05$).



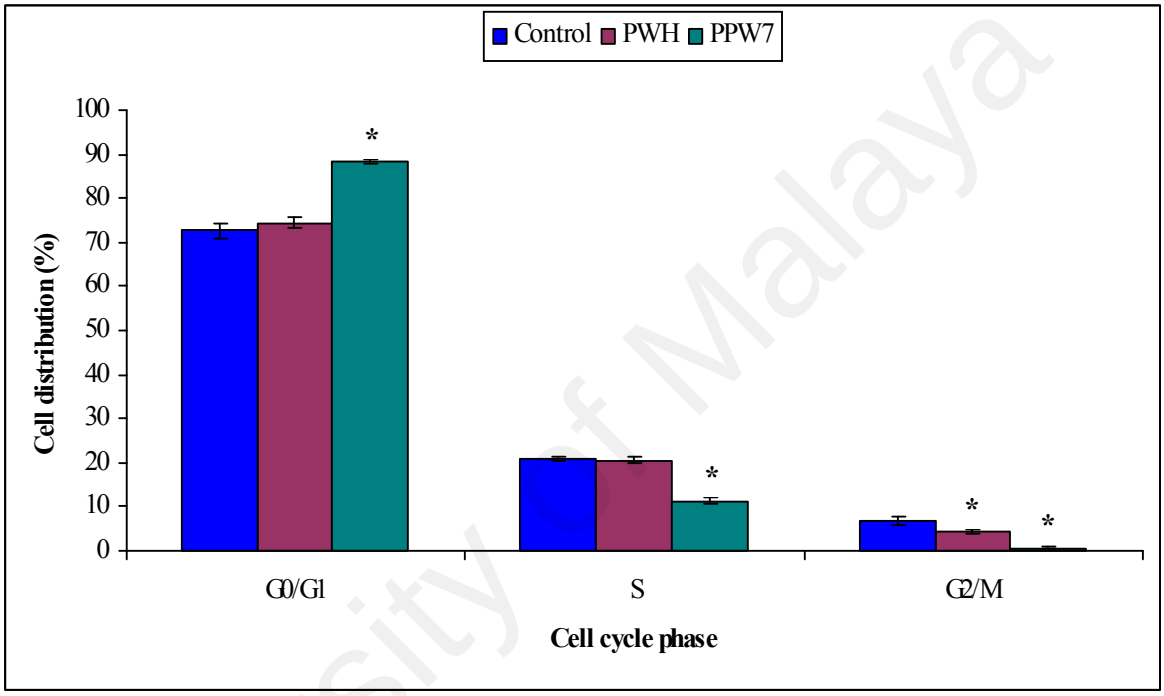
Cell-cycle	Distribution of cell-cycle (%)		
	Control	PWH	PPW7
G_0/G_1	93.4 ± 0.78	61.6 ± 1.05	58.4 ± 1.11
S	5.5 ± 0.50	26.4 ± 0.87	25.3 ± 1.16
G_2/M	0.8 ± 0.20	12.3 ± 0.64	16.5 ± 0.60

Figure 4.9(b): Effect of crude hexane extract of *P. watsonii* (PWH) and fraction PPW7 on cell cycle phase distribution in SKOV3 cell. Cells (1×10^6 cells/ml) were incubated in the absence (control) or presence of PWH and PPW7 for 24 h and then analyzed by flow cytometry. * indicates a significant difference from the control ($p<0.05$).



Cell-cycle	Distribution of cell-cycle (%)		
	Control	PWH	PPW7
G_0/G_1	76.3 ± 0.81	93.0 ± 1.11	69.7 ± 1.08
S	13.6 ± 0.60	0.08 ± 0.08	29.6 ± 1.27
G_2/M	5.0 ± 0.50	6.67 ± 1.21	1.9 ± 0.90

Figure 4.9(c): Effect of crude hexane extract of *P. watsonii* (PWH) and fraction PPW7 on cell cycle phase distribution in CaSki cell. Cells (1×10^6 cells/ml) were incubated in the absence (control) or presence of PWH and PPW7 for 24 h and then analyzed by flow cytometry. * indicates a significant difference from the control ($p<0.05$).



Cell-cycle	Distribution of cell-cycle (%)		
	Control	PWH	PPW7
G_0/G_1	72.6 ± 1.60	74.5 ± 1.26	88.5 ± 0.57
S	20.7 ± 0.49	20.5 ± 0.64	11.2 ± 0.75
G_2/M	6.78 ± 0.80	4.5 ± 0.50	0.6 ± 0.20

Figure 4.9(d): Effect of crude hexane extract of *P. watsonii* (PWH) and fraction PPW7 on cell cycle phase distribution in HT29 cell. Cells (1×10^6 cells/ml) were incubated in the absence (control) or presence of PWH and PPW7 for 24 h and then analyzed by flow cytometry. * indicates a significant difference from the control ($p<0.05$).

CHAPTER 5

DISCUSSIONS

Many clinically used cancer chemotherapeutic agents are derived from natural products or semi-synthetic compounds of natural origins include topotecan, irinotecan, etoposide, teniposide, vinblastine, vincristine, docetaxel, and paclitaxel. Studies therefore, on the evaluation of anticancer properties of natural products have been considered to play an important role in the development of anticancer agents (Mann, 2002). The search for anticancer agents from natural sources has been successful worldwide, active constituents have been isolated and are used to treat human tumour (Ruffa *et al*., 2002).

Higher plants continue to be an important and valuable resource for anticancer drug discovery, especially since 300,000–500,000 such species are known, and these represent about 15 % of the taxonomically authenticated global organism biodiversity (Tan *et al*., 2006). A wide variety of biological activities from medicinal plants have recently been reported, in addition to their traditional medicinal effects. Herbal medicines have attracted considerable interest as alternative cancer remedies because of their low toxicity and costs (Hu *et al*., 2009). In the present study, selected Phyllanthaceae species grown in Malaysia were evaluated for their cytotoxic and apoptotic activity and the signalling pathway associated with the cytotoxic effect in the cytotoxically active crude extract(s) and isolated fraction(s) were characterized.

5.6 Preparation of Crude Extract from the Aerial Part, Leaves and Fruits of Selected Phyllanthaceae Species

In the present study, six different species from the family of Phyllanthaceae, *Phyllanthus niruri*, *Phyllanthus pectinatus*, *Phyllanthus acidus*, *Phyllanthus roseus*, *Phyllanthus watsonii* and *Baccaurea motleyana* were collected, dried, extracted using cold extraction method in three different solvents (methanol, hexane and ethyl acetate) with increasing polarity and obtained crude extracts were subjected to cytotoxicity screening on four human cancer cells, namely breast cancer cell (MCF7), ovarian cancer cell (SKOV3), epidermal carcinoma of cervix cell (CaSki) and colon cancer cell (HT29) and one human normal cell, human lung fibroblast cell (MRC5). Different types of phytochemical can be extracted by using solvents with different polarity. The main groups of compounds can be extracted by using hexane were waxes, fats and volatile oils. Alkaloids, aglycones and glycosides can be extracted by using ethyl acetate and chemical class extracted in methanol extraction were sugars, amino acids and glycosides (Houghton and Raman, 1998).

Extraction of chemical compound from plants found to be more efficient by using organic solvent due to efficiency of organic solvent to degrade pant cell wall and therefore able to extract a greater amount of endocellular materials compared to water (Lim & Murtijaya, 2007). Ethyl acetate extraction was found to yielded higher extract compared to the hexane extraction. The differences could be the presence of certain middle polarity compound(s) in the dried plant material, which can only be extracted with middle polarity solvent, such as ethyl acetate.

Plant samples were dried in low temperature at 40 – 50 °C for two days. It is important to employ quick drying methods to avoid degradation of the components by air or by microbes. Improper extraction methods may result in the degradation or loss of

access of the active component(s). Cold extraction method was used in the present study as it allows for maximum extraction of most components from plant samples (Fakim, 2006) compared to hot extraction method. In hot extraction method which uses soxhlet extractor, extract may suffer thermal degradation reactions as the extracts that been collected in the lower container will continuously being heated.

5.7 Cytotoxicity Screening of Crude Extracts of Selected Phyllanthaceae Species on Various Human Cancer Cell Lines

Cultured mammalian cells provide an important tool for determining the cytotoxicity of compounds with therapeutic activity (Pailard *et al*., 1999). Established human cancer cell lines are routinely used as experimental models for human cancers. They have retained hallmarks of cancer cells such as cell sufficiency in growth control, insensitivity to antigrowth signals, and escape from checkpoints, immortality and genetic instability. They are pure, easily propagated and genetically manipulated, and provide reproducible results when used with the same protocol and the same stage, or after numerous passages. Human cell lines are preferred because the same oncogene may give different phenotypes in human and in transgenic mice. Oncogenic mechanisms however may differ between mouse and human (Rangarajan and Weinberg, 2003; Vargo-Gogola and Rosen, 2007). In this study, four human cancer cells, namely breast cancer cell (MCF7), ovarian cancer cell (SKOV3), epidermal carcinoma of cervix cell (CaSki) and colon cancer cell (HT29) were used to evaluate the cytotoxic potential of crude methanol, hexane and ethyl acetate extract of *Phyllanthus niruri*, *Phyllanthus pectinatus*, *Phyllanthus acidus*, *Phyllanthus roseus*, *Phyllanthus watsonii* and *Baccaurea motleyana*.

In the past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). The most convenient, modern assays have been optimized for the use of microtiter plates (96-well format). This miniaturization allows many samples to be analyzed rapidly and simultaneously. Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) has been used previously for the identification of vital cells in cultures and recommended as one of the inexpensive possibility to measure cell death (Weyermann *et al*., 2005). This assay quantifies the number of viable, uninjured cells being exposed to toxicants. It is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red. Quantification of the dye extracted from the cells have been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Borenfreud and Puerner, 1985).

Experimental agents derived from natural products offers great opportunity to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of action (da Rocha *et al*., 2001). These non-toxic "natural products" could be useful in combination with conventional chemotherapeutic agents for the treatment of cancer and such strategies are expected to have lower toxicity but higher efficacy (Sarkar and Li, 2009). Additionally, natural products that are biologically active in assays are generally small molecules with drug-like properties. These small molecules are capable of being absorbed and metabolized by the body (Harvey, 2000).

With the difficulty of gaining access to large tracts of biodiversity in natural habitats, several techniques have been developed to produce natural products in non-natural ways. These range from plant tissue culture to combinatorial genetics. In addition to offering a secure supply of naturally occurring metabolites, such technologies can be used to produce more diverse chemicals (Harvey, 2000).

In this present study, a convenient short-term *in vitro* assay was conducted to screen for the cytotoxicity potentials of selected locally grown Phyllanthaceae species against several human cancer cell lines, namely MCF7 breast cancer, SKOV3 ovarian cancer, CaSki cervical cancer and HT29 colon cancer cells. Investigation of comparative cytotoxic activities of the crude extracts of the six plants of Phyllanthaceae and the standard drug doxorubicin against MCF7, SKOV3, CaSki and HT29 cell lines indicate differences in degrees of responsiveness and sensitivity of different cancerous cells to these plant extracts. Cytotoxically active crude extracts of Phyllanthaceae species and fractions of *P. watsonii* were found to be most active on SKOV3 cells compared to other cell lines studied. Differences in responsiveness and sensitivity of different cancerous cells to different chemotherapeutic agents were observed in various studies (Manthey and Guthrie, 2002; Lee and Houghton, 2005; Jansen *et al*., 2006; Yanez *et al*., 2009; Mena-Rejon *et al*., 2009). This variation may be related to the molecular characteristic of the cell lines (Verma *et al*., 2008), in which the patterns of chemosensitivity and gene expression profiles are differ from cell line to cell line (Szakacs and Gottesman, 2004).

The inhibition of proliferation of normal MRC5 lung fibroblast cells induced by the crude extracts of Phyllanthaceae after 72 h were low, indicating that the crude extracts were not deleterious to non-cancerous cells. Crude methanol, hexane and crude ethyl acetate of *P. watsonii* showed some inhibitory activity on normal MRC5 cells with the IC_{50} values ranging from 33.8 to 49.3 µg/ml but the crude extracts consider not active on normal MRC5 cells. Studies by Tang *et al*., (2010), showed that the aqueous and methanolic extract of *P. watsonii* did not cause any significant changes on cell viability of both normal human skin (CCD-1127Sk) and prostate (RWPE-1) cell lines with the IC_{50} values obtained were greater than 500 µg/ml. Numerous reports have been documented on the use of normal MRC5 lung fibroblast cells as a control in cytotoxicity studies. *Monascus*-

fermented red rice was found to be cytotoxic on human cancer cell lines HepG2 and A549 but possessed no significant toxicity on normal MRC5 lung fibroblast cells at concentration of 15 µg/ml (Su *et al*., 2005). Goto *et al*., (2007) reported that the cytotoxically active taxoid conjugate exhibited lower inhibitory effects on normal MRC5 lung fibroblast cell and Jaiaree *et al*., (2010) reported that ethanolic extract of *Dioscorea birmanica* Prain & Burkill exhibited IC_{50} value of 37.09 ± 0.67 µg/ml on normal MRC5 lung fibroblast cell. Both crude hexane and butanol extract prepared from *Dactyloctenium aegyptium* and *Eleusine indica* were found not toxic to normal MRC5 lung fibroblast cells with IC_{50} values ranging from 648.1–1603.2 µg/ml (Hansakul *et al*., 2009). Hexane and ethyl acetate fractions of *Pereskia bleo* were found not toxic on normal MRC5 lung fibroblast cells with an IC_{50} value more than 100 µg/ml (Sri Nurestri *et al*., 2008). Sri Nurestri *et al*., (2009) also reported that the compounds isolated from *Pereskia bleo*, namely dihydroactinidiolode, β-sitosterol, α-tocopherol and phytol were not toxic towards normal MRC5 lung fibroblast cells with IC_{50} values of 91.3, >100.0, 30.5 and 74.3 µg/ml, respectively. It is important for an anticancer agent to exhibit cytotoxicity but such activity should be specific for cancer cells only (Lai *et al*., 2008). Toxicity of the crude extracts or fractions of Phyllanthaceae species on normal MRC5 cells can be reduced by understanding the molecular action of each extract on cells and testing the possibility of synergistic effects on cells with combination of two or more extracts or derivatives.

Doxorubicin (Adriamycin) has been used in oncologic practice since the late 1960s. It has been used clinically as a powerful drug in the treatment of cancer (Singal and Iliskovic, 1998) and one of the most potent chemotherapeutic drugs for the treatment of acute leukemia, lymphomas and different types of solid tumours such as breast, liver and lung cancers (Tokarska-Schlattner *et al*., 2006). In this study, doxoribucin was used as positive control agent in the *in vitro* cytotoxicity assay. The results of this study indicate

that crude extracts and fractions of *P. watsonii* had lower toxicity effect on normal MRC5 lung fibroblast cell compared to doxorubicin. This result are in good agreement with those previously reported by Tang *et al*., (2010) concerning the cytotoxic activity of anticancer drug doxorubicin against normal cells. On normal human skin cell lines (CCD-1127 Sk), doxorubicin possessed IC_{50} values of 1.0 ± 0.2 µg/ml, and 1.0 ± 0.1 µg/ml on normal prostate (RWPE-1) cells.

The current study found that the ability of selected Phyllanthaceae species crude extract in inhibiting cancer cell proliferation was in a concentration-dependent manner. These results support the traditional use of these medicinal plants by local people in treating kidney and urinary bladder disturbances, sexual diseases, skin manifestation, wound healing, inflammation, diabetes and hepatitis B (Burkill, 1966; Calixto *et al*., 1998; Khatoon *et al*., 2006; Kumaran and Karunakaran, 2007). Inflammation (Coussens and Werb, 2002); hepatitis B (Perz *et al*., 2006), skin manifestation (Braverman, 2002); gonorrhoea and syphilis (Hayes *et al*., 2000) have been reported to be associated with several cancer and this clearly indicate the chemopreventive potential of *Phyllanthus* species. There have been a number of reports on the potential of the extracts and compounds derived from various *Phyllanthus* species in suppressing experimental carcinogenesis in various organs and cells such as inhibition of hepatocarcinogenesis induced by *N*-nitrosodiethylamine by extract of *P. emblica* (Jeena *et al*., 1999), inhibition of LPS-induced production of NO in rat Kupffer cells and in RAW264.7 macrophages cells by ethanol/water and hexane extracts of *P. amarus* (Kiemer *et al*., 2003), inhibition of murine melanoma B16F10 cells, human gastric adenocarcinoma MK-1 cells and human uterine carcinoma HeLa cells by phenolic compounds isolated from *P. emblica* (Zhang *et al*., 2004), suppression of tumour developed in mice with implantation of Lewis lung carcinoma cells by extract of *P. urinaria* (Huang *et al*., 2006), inhibition of human breast

cancer MCF7 cells, human colon cancer HT29 cells and human liver cancer HepG2 cells by methanol extract of *P. polyphyllus* (Rajkapoor *et al*., 2007); and inhibition of human stomach cancer MKN28 cell by ethanol extract *P. urinaria* (Ji *et al*., 2010)

The ethyl acetate extract of *P. pectinatus* and *P. watsonii* tend to be more active than the methanol and hexane extract and the data obtained suggest that the extract of *Phyllanthus* in ethyl acetate seemed to have more pronounced cytotoxic effect than extract in methanol and hexane. Different classes of organic compounds of medicinal interest have been isolated and characterized from various *Phyllanthus* species, including alkaloids, flavonoids, lactones, steroids, terpenoids, lignans, tannins (Liu and McIntosh, 2001; Calixto *et al*., 1998) and fatty acids with the mixtures of linolenic, linoleic, oleic and saturated acids (Ahmad *et al*., 1981). These compounds might be responsible for the cytotoxic actions reported in this present study in relation to these plants extract.

In this study, crude extracts of *P. niruri* were found to be not cytotoxically active on cancer cell lines tested but numerous pharmacological effects of *P. niruri*, such as antibacterial, anti-inflammatory, antihepatoxic, anticancer, antioxidant and antiviral activities have largely been documented. It has been described to exhibit several biological activities such as *in vitro* antiplasmodial activity of the aqueous, methanol and chloroform extract on two strains of *P. falciparum* , chloroquine-resistant (FCR-3) and chloroquine-sensitive (D10) strains (Sholikhah *et al*., 2004), inhibition of membrane lipid peroxidation (LPO), scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, inhibition of reactive oxygen species (ROS) *in vitro* by the methanolic and aqueous extract of leaves and fruits (Harish and Shivanandappa, 2006) and antiplasmodial activity in mice at a low dose of 30 mg/kg body weight (Oparaocha and Okorie, 2009). Clinical studies by Nishiuara *et al*. (2004) demonstrated that *P. niruri* can promote stone elimination in stone-forming patients, as well as the normalization of calcium levels in hypercalciuric patients. Barros *et al*.

(2006) reported the potential prophylactic effect of *P. niruri* on the growth of calculi as it caused a significant inhibition in the growth of calculi by interfering with the crystal deposition and substantially modified stone shape and texture. According to Calixto *et al*. (1998), alkaloids from plants of the genus *Phyllanthus* present an antispasmodic activity leading to smooth muscle relaxation, mostly evidenced in the urinary tract, which would facilitate the elimination of urinary calculi. Previous phytochemical studies of *P. niruri* extracts revealed the presence of acyclic triterpene (Singh *et al*., 1989); phenolic compounds (De Souza *et al*., 2002); flavonoids, glycosides and tannins (Rajeshkumar *et al*., 2002); and lignans (phyllanthin, hypophylanthin, phyltetralin and niranthin) (Murugaiyah and Chan, 2007). Previous studies showed that 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan from *P. urinaria* was capable of inhibiting telomerase activity and inhibit activities of bcl2 and activate caspase 3 and caspase 8 significant in the induction of apoptosis (Giridharan *et al*., 2002). Studies have also shown that lignan-rich fraction and the lignans nirtetralin, niranthin or phyllanthin isolated from *P. amarus* exerted cytotoxic effects on K-562 leukaemia cells (Leite *et al*., 2006).

In this present study, crude methanol and ethyl acetate extracts of *P. pectinatus* (leaves) were found to be cytotoxically active on SKOV3 and crude ethyl acetate extracts of *P. pectinatus* (fruits) were found to be cytotoxically active on MCF7 and CaSki cells. The observed cytotoxic activity of this *P. pectinatus* might be related with the presence of chemical compounds reported in these plants. Phytochemical report revealed the presence of monoterpene, triterpene, lignan and flavonone in *P. pectinatus* (Ong *et al*., 2009). A number of dietary monoterpenes have been reported to possess antitumour activity, exhibiting not only the ability to prevent the formation or progression of cancer, but the ability to regress existing malignant tumours (Crowell and Gould, 1994). Triterpenoids have been shown to possess antiinflammatory and anticarcinogenic properties (Manez *et*

al., 1997). Several types of triterpenoids were isolated from different part of *Phyllanthus* species such as lupane and oleanane from the bark of *P. flexuosus* (Wada *et al*., 2001) and dichapetalin from the aerial parts of *P. acutissima* (Tuchinda *et al*., 2008) which showed remarkable cytotoxic activity. A lot of studies been carried out on phytochemical and pharmaceutical aspect of Pokok Melaka (local name referring to *P. emblica* and *P. pectinatus*) in Malaysia, but their studies are often on the wrong tree, published under the wrong name (Ng, 2000). To our knowledge, this is the first study on the biological investigation reported with the taxonomy name of *P. pectinatus*. Cytotoxic activity of *P. emblica* had been reported by El-Desouky *et al*., (2008). A new acylated apigenin glucoside (apigenin-7-O-(6"-butyryl-β-glucopyranoside) isolated from the methanolic extract of the leaves of *P. emblica* has been described to exhibit strong cytotoxicity on A549 non-small cell lung, SKOV3 ovarian cancer cell, SK-MEL-2 skin melanoma cell and HCT-15 colon cancer cells with IC_{50} values range from 2.25 – 7.22 μM (El-Desouky *et al*., 2008). Phytochemical investigation done by Ur-Rehman *et al*. (2007) on *P. emblica* have resulted in the isolation of the two new flavonoids, kaempferol-3-*O*-α-L-(6”-methyl)-rhamnopyranoside and kaempferol-3-*O*- α-L-(6”-ethyl)-rhamnopyranoside.

In this present study, crude methanol, hexane and ethyl acetate extracts of *P. acidus* were found to be not active in cytotoxic activity but Vongvanich *et al*., (2000) reported on the cytotoxicity of isolated compounds from *P. acidus* Phyllanthusol A and B glycosides, isolated from the root of *P. acidus* were found to exhibit cytotoxic activity on KB nasopharyngeal cell lines (Vongvanich *et al*., 2000). Studies by Jagesar *et al*., (2008) showed that the ethanol extracts of *P. acidus* exhibited potent antimicrobial inhibitory activity at 0.18 mg/10 ml plate of medium. This study suggests that the ethanol extracts of *P. acidus* can be used as herbal medicines in control of *E. coli* and *S. aureus* following clinical trials. Sousa *et al.,* 2007, identified bioactive components in herbal extracts of

P. acidus that have a potential in cystic fibrosis treatment by inducing airway chloride secretion. Leeya *et al*., (2010) isolated adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid and kaempferol from the n-butanol extract from leaves of *P. acidus*. Numerous documented reports shown that gallic acid, a highly water soluble compound is also one of the active components in *Phyllanthus* plants. Gallic acid is the most commonly used component, which has pharmacological properties as an antimicrobial, astringent and obstruent agent (Lee *et al*., 2005).

A literature survey revealed that there had been no detailed biological pharmacological investigation described on *P. roseus*, although several of these genuses are described to have medicinal value for the treatment of jaundice, dropsy and genitourinary infection (Chopra *et al*., 1956; Burkill, 1966). In this study, *P. roseus* crude extract did not produce cytotoxic effect towards MCF7, SKOV3, CaSki and HT29 cells, suggesting that these extracts not cytotoxically active. This plant has not yet been assessed for *in vitro* cytotoxicity against cancer cells and the phytochemistry of *P. roseus* is completely unexplored.

In the present work, the strongest cytotoxic activities were detected from the crude extract of *P. watsonii* on all the cancer cell lines tested with IC_{50} values ranging from 3.6 ± 1.01 to 18.3 ± 1.53 µg/ml. Literature survey revealed that there is limited published information on the bioactivity of *P. watsonii*, particularly on cytotoxicity. Tang *et al*. (2010) reported on cytotoxic activities of aqueous and methanolic extract of *P. watsonii* (grown in plant house) against skin melanoma MeWo cell and prostate cancer PC-3 cells that were determined by using the MTS reduction assay. On skin melanoma MeWo cell, aqueous and methanolic extract of *P. watsonii* possessed IC_{50} values of 160.0 ± 3.2 and 100.7 ± 2.0 µg/ml, and 156.7 ± 2.4 and 100.5 ± 1.2 µg/ml on prostate cancer PC-3 cells. These effects are less marked and considered not active than that obtained in the present

study. Differences in results obtained in both studies could be ascribed to differences in extraction procedures and the natural variability in plants. The chemical composition and characteristics of plant constituents depend on nature origin, ecological conditions, harvesting period and seasonal variations. Age of the plant and parts of the plant may also influence bioactivity of the plant constituents (Unander *et al*., 1993; Ramesh *et al*., 2004).

Based on the phytochemistry report done by Tang *et al*. (2010), the potent cytotoxicity activity of the crude methanol extract of the *P. watsonii* could be due to the polyphenols content of the plant. Tang *et al*., (2010) identified various polyphenols compounds from methanolic and water soluble extract of *P. watsonii* by High Performance Liquid Chromatography (HPLC) coupled with photodiode array (PDA) and MS-MS detection system. The compounds detected were gallic acid, galloylglucopyronoside, digalloylglucopyronoside, corilagen geraniin, rutin, quercetin glucoside, quercetin rhamnoside and caffeolquinic acid. Polyphenols have a wide range of action, which includes antitumour, antiviral, antibacterial and antimutagenic activities. They may act against different stages of the development of malignant tumours by protecting the DNA from oxidative damage. The protection of DNA may achieve by inactivating carcinogens through the inhibition of the expressions of mutagenic genes. They also inactivate the enzymes charged with activating procarcinogens and activate the systems responsible for detoxification of xenobiotics (Bravo, 1998). The correlation between the reported cytotoxic activity and the previously isolated compounds from *P. watsonii* will be discussed further in Section 5.4.

Baccaurea motleyana has not been previously investigated for cytotoxicity and the crude methanol, hexane and ethyl acetate extracts of *B. motleyana* did not possessed cytotoxic activity in this study. Extracts of *B. motleyana* has been studied for its antioxidant activity. Emmy *et al*. (2009) reported on the antioxidant capacity and total phenolic content

of *B. motleyana*. Studies showed that the methanolic extract of the *B. motleyana* exhibited a good antioxidant capacity, 71.17 ± 5.63 % (based on β-carotene bleaching assay) with higher total phenolic content of 1160.14 ± 20.56 mg GAE/100 g edible portion.

5.8 Cytotoxicity Screening of Isolated Fractions of *Phyllanthus watsonii* on Various Human Cancer Cell Lines

Crude hexane extract (CHE) of *P. watsonii* exhibited strongest cytotoxicity on all four cancer cells tested compared to other extracts of *P. watsonii* and was selected for bioassay-guided fractionation. Bioassay-guided method was used to isolate the most effective constituent of *Phyllanthus watsonii* in inhibiting the growth of the human cancer cells. Bioassay-guided fractionation of medicinal plants is a feature of routine in the attempt to isolate bioactive components from natural sources. Most of the plant compounds that have been found to be medicinally useful and interesting tend to be secondary metabolites (Fakim, 2006).

Fractionation process of CHE of *P. watsonii* yielded 10 fractions (PW1 – PW10) which were organized by similarity of spots detected in TLC on pre-coated silica gel 60 F254 plate using n-hexane/acetone (20:80) as developing solvent. TLC profiles of PW4–PW8 fractions, suggest a strong similarity of these cytotoxically active fractions. The bioassay-guided fractionation of crude hexane extract derived from *P. watsonii* revealed that cytotoxic compounds were found mainly in the middle polarity phase of the solvent system used (n-hexane/acetone phase). It can be concluded that the non-polar fractions of *P. watsonii* potently inhibited the *in vitro* cell proliferation of MCF7 human breast cancer, SKOV3 human ovarian cancer, CaSki human cervical cancer and HT29 human colon cancer cells.

PW4 – PW8 were pooled and subjected for further bioassay-guided fractionation and resulted in fractionation of 8 fractions (PPW1 – PPW8). PPW7 was the most cytotoxically active fraction and showed similar cytotoxic activity as the standard anticancer drug, doxorubicin which was used as a positive control in the present study. In the US National Cancer Institute Plant Screening Program, a pure compound considered to have active cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 h, is equal or less than 4 μg/ml for (Geran *et al*., 1972; Boik, 2001). IC_{50} value established for fraction PPW7 ranged from 0.7 – 0.9 μg/ml, a highly cytotoxic activity when considering a pure compound and this findings suggesting the potential of *P. watsonii* to be developed as anticancer drug.

5.9 LC-MS/MS Analysis of Fraction PPW7 of *P. watsonii*

At present, LC-MS is one of the most sensitive analytical methods and with the high power of mass separation of MS detector, very good selectivity can be obtained. Moreover, this techniques has the potential to yield information about the molecular weight as well as the structure of the analysts, using the MS/MS or MS^n capabilities of the analyzer available (Marston, 2007). In the present study, the analysis of the active fraction PPW7 using LC-MS/MS indicates the presence of trimethyl ether of ellagic acid, methyl ester of geraiinic acid, glochidone, betulin, Phyllanthin sodium salt, Phyllanthin potassium salt and sterol glucoside which may be responsible for the observed cytotoxic activity of *P. watsonii*.

These compounds have previously been detected in other Phyllanthaceae species and some of these compounds have been shown to have anticarcinogenic activity. Ellagic acid has been isolated from *P. emblica*, *P. niruri*, *P. reticulatus* and *P. urinaria*. Geraniin

was reported to be present in *P. amarus*, *P. caroliniensis*, *P. niruri*, *P. urinaria*, and *P. flexuosus*, and geraniinic acid were reported in *P. amarus* and *P. flexuosus*. Glochidonol was isolated from *P. reticulates* and *P. sellowianus*. Betulin was reported in *P. discoideus* and *P. flexuosus*. Phyllanthin had been found in *P. discoideus* and *P. simplex* (Calixto *et al*., 1998).

Plant-derived ellagic acid, a polyphenolic compound present abundantly in fruits and nuts (Narayanan *et al*., 1999; da Silva Pinto *et al*., 2008), has previously been identified as a potent anticarcinogenic agent (Das *et al*., 1985; Teel, 1986; Perchellet *et al*., 1992 and Stoner and Morse, 1997). Various studies have been shown that ellagic acid possessed growth-inhibiting and apoptosis promoting activities in cancer cell lines *in vitro*. Narayanan *et al*. (1999) reported the effects of ellagic acid on cell cycle events and apoptosis in cervical carcinoma (CaSki) cells. In the study, ellagic acid at a concentration of 10^{-5}M induced G1 arrest within 48 h, inhibited overall cell growth and induced apoptosis in CaSki cells after 72 h of treatment. Studies by Losso *et al*., (2004) found that ellagic acid showed strong antiproliferative activity against the human Caco-2 colon, MCF7 breast, Hs 578T breast and DU 145 prostatic cancer cells and induced the cancer cell death by apoptosis and decrement in ATP levels of the cancer cells studied. Ellagic acid also has been reported to demonstrate a very potent inhibitor of the SH-SY5Y neuroblastoma cell line and induced apoptosis as measured by DNA strand breaks and showed G_0/ G_1 arrest (Fjaeraa and Nanberg, 2009). All these are in good agreement with reported data in these current studies. Among the suggested molecular targets for ellagic acid effects are NF-κB, cyclin D1, $p21^{cip1/waf1}$ and p53 (Aggarwal and Shishoida, 2006).

Geraiinic acid is a compound under the chemical class of monoterpene (Avato and Tava, 1995). Anticancer potential specifically on geraiinic acid has not been reported but there a numerous studies on cytotoxicity of monoterpenes and its derivatives (Mills *et al*.,

1995; Stark *et al*., 1995; Reddy *et al*., 1997; Bardon *et al*., 2002; Chien *et al*., 2007). The monoterpene perillyl alcohol has been reported to possess anticancer activities which include both prevention and treatment of a wide variety of cancers in animal models (Ren *et al*., 1997). Geraniol also exhibited antitumor activity against various cancer cell and enhances sensitivities of 5-fluorouracil towards colon cancer cells (Yamamoto *et al*., 2008). Mansoor *et al*., (2009) isolated monoterpene indole alkaloids from the methanol extract of leaves of *Tabernaemontana elegans* and found that the isolated compounds showed promising apoptosis induction activity in human hepatoma HuH-7 cells. Sumadain C, a novel monoterpene-chalcone conjugate was isolated from the seeds of *Alpinia katsumadai* and showed potent cytotoxic activity against human HepG2 liver, MCF7 breast and MDA-MB-435 breast cancer cell lines with IC_{50} values of 13.00, 15.93 and 12.78 µg/ml, respectively (Hua *et al*., 2009).

Betulin, lup-20(29)-ene-3β,28-diol, also known as betulinol, betuline and betulinic alcohol, is pentacyclic triterpene alcohol with a lupine skeleton of plant origin that is widely distributed in the plant kingdom throughout the world. Betulin can be easily converted to betulinic acid, known to possess a wide spectrum of biological and pharmacological activities (Fulda, 2008). The antitumour activity of betulin and its derivatives could be therapeutically important, because these compounds exhibit high cytotoxic activity against several tumours and cancer cells. Betulin was found to be cytotoxic against all nine tested neuroblastoma cell lines (Schmidt *et al*., 1997), triggered apoptotic cascade in human malignant glioma cells (Wick *et al*., 1999) and inhibited the growth of neoplastic cell lines, such as ovarian carcinoma (A2780, OVCAR-5 and IGROV-1), lung carcinoma (H460 and POGB) and cervix carcinoma (A431) (Zuco *et al*., 2002). Fulda *et al*. (1999) found that betulinic acid exerted cytotoxic activity against primary tumor cells and primary glioblastoma tumor cells by apoptosis induction involving

mitochondrial perturbations, triggering DNA fragmentation, releasing cytochrome c and activating caspase 3. In *Phyllanthus* species, betulin has been isolated from the stem bark of *P. flexuosus* (Tanaka *et al*., 1988).

Phyllanthin, a lignan compound, is a known principal constituent of *P. niruri*. There are few studies examining the pharmacological action of phyllanthin and most of the studies investigated its hepatoprotective effect. Phyllanthin was reported to reduce hepototoxicity induced by carbon tetrachloride and galactosamine in rats and may be used as a marker for hepatoprotection of *P. niruri* (Syamasundar *et al*., 1985 and Khatoon *et al*., 2006). Phyllanthin may have exhibited its hepatoprotection *in vitro* through a mechanism independent of β-glucuronidase inhibition (Joshi and Priya, 2007). Murugaiyah and Chan, (2006) reported the potential of phyllanthin in lowering the plasma uric acid level in hyperuricemic rats to its normal level. Study by Chirdchupunseree and Pramyothin, (2010) demonstrated the hepatoprotective effect of phyllanthin against ethanol-induced oxidative stress causing rat liver damage and suggested that the activity is due to its antioxidant capability.

Sterols are part of the vast family of isoprenoids. In plants, sterols are substrates for the synthesis of a wide range of secondary metabolites such as cardenolides, glycoalkaloids, pregnane derivatives and saponins (Hartman, 1998). Sterols isolated from various marine organisms and alga was found to possess cytotoxic activity (Sheu *et al*., 1997; Duh *et al*., 1998; Maia *et al*., 2000; Sheu *et al*., 2000 and Santafe *et al*., 2002).

Generally, the observed activity of the PPW7 fractions of the *P. watsonii* may be due to the presence of terpenoid content of the plant since earlier study done by Matsunaga *et al.* (1993) obtained terpenoid from these plant. Chemical investigation done by Matsunaga *et al.* (1993) isolated two new unsaturated nor-triterpenes together with lupenyl palmitate, friedelin, epifriedelanol, glochidone, glochidonol, lupeol, lup-20(29)-en-1 beta,3

beta-diol, sitosterol and sitosterol-beta-(D)-glucoside from the aerial parts of *Phyllanthus watsonii*. Their structures were established to be 26-nor-D:A-friedoolean-14-en-3-one and 26-nor-D:A-friedoolean-14-en-3beta-ol, respectively. Glochidone was also detected in this present study.

5.10 Detection of Apoptosis in Cells Treated with Cytotoxically Active Crude Extracts of Selected Phyllanthaceae Species and Fractions of *Phyllanthus watsonii*

A wide variety of plant-derived compounds appear to possess significant cytotoxic as well as chemopreventive activity and many of these agents were reported to act via apoptosis, a programmed cell death. Many researchers considered induction of apoptosis in cancer cells or malignant tissues as one of the key mechanisms for the targeted therapy of various cancers and has also been recognized as an efficient strategy in cancer chemotherapy (Constantini *et al*., 2000; Taraphdar *et al*., 2001).

Apoptosis also seems to be a reliable marker for the evaluation of potential agents for cancer prevention and numerous biochemical works have been carried out in identifying the molecules involved in this cell death process (Taraphdar *et al*., 2001). Elegant genetic and biochemical work has identified several families of proteins such as Bcl-2 family, which regulate apoptosis together with caspases, proteins that mediate apoptosis by downstream molecules (Zhang and Xu, 2002).

To distinguish apoptosis from necrosis, both morphological and biochemical characteristics need to be examined collectively and this will help in determining that cell death has occurred via apoptosis. Adequate morphological studies are necessary to ascertain that apoptosis has occurred, particularly when exceptions to DNA degradation are observed (Bortner *et al*., 1995). After receiving an apoptotic death stimulus, cells first enter

a signalling phase followed by the final degradation phase, in which apoptosis is identifiable by chromatin condensation, cell shrinkage, caspase activation, membrane lipid rearrangement, DNA fragmentation, and cell fragmentation, through the formation of 'apoptotic bodies' (Jacobson *et al*., 1997).

In the present study, crude extracts and fractions of Phyllanthaceae species studied with IC_{50} value equal to or less than 20 μg/ml are considered cytotoxically active and were selected for further assessment of their apoptotic activities by various morphological and biochemical methods. Cytotoxically active crude extracts from *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 were analyzed for their potential apoptotic activity on MCF7, SKOV3, CaSki and HT29 cell lines by using phase contrast and fluorescence microscopy method, detection of DNA fragmentation by agarose electrophoresis, determination of caspase-3 activity and cell cycle analysis by flow cytometry.

Analysis of Cell Morphological Changes by Phase Contrast Microscope

Apoptotic cells were first recognized from the characteristic changes in their morphology, particularly changes in the nucleus. Before commencing any assay, the presence of apoptotic cells should be confirmed by observing the nuclear morphology; the simplest method being to use inverted phase contrast or fluorescence microscopy (Ormerod, 2002). The morphological changes of apoptosis can either be observed in fixed and stained tissue or in cell grown in culture (Kiechle and Zhang, 2002).

In the present study, marked morphological changes indicative of apoptosis were clearly observed in MCF7, SKOV3, CaSki and HT29 cells-treated with cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii*, and

cytotoxically active fractions of *P. watsonii* after 24 h, particularly at the concentration of 0.05 mg/ml. Cells showed some apoptotic cell morphological characteristics such as rounding up cells, membrane blebbing, chromatin condensation and formation of apoptotic bodies. Similar characteristic features of a apoptosis including cell volume shrinkage, chromatin condensation, presence of membrane-bound apoptotic bodies, plasma membrane blebbing and irregular cell morphological changes were also been observed in human hepatocellullar carcinoma BEL-7404 cells-induced by progallin, which was isolated from the acetic ether part of the leaves of *P. emblica* (Zhong *et al*., 2011). Harikumar *et al*., (2009) also reported on apoptotic morphological changes such as formation of apoptotic bodies, fragmentation and chromatin condensation in Dalton's lymphoma ascites (DLA) cells in culture when treated with extracts of *P. amarus*. Moongkarndi *et al*., (2004) reported that exposure of human breast cancer SKBR3 cells to crude methanol extract of *Garcinia mangostana* (mangosteen) for 48 h led to retraction, rounding up cells, detachment of cells from the surface of well and membrane blebbing which were observed under phase contrast microscope.

Once the cell has committed to die, the earliest observable ultra structural event is the compaction and segregation of the nuclear chromatin, with the formation of sharply delineated, uniformly finely granular masses that become marginated against the nuclear envelope, and condensation of the cytoplasm. Progression of the condensation is accompanied by convolution of the nuclear and cell outlines, and this is followed by breaking up of the nucleus into discrete fragments that are surrounded by a double-layered envelope and by budding of the cell as a whole to produce membrane-bounded apoptotic bodies. The size and composition of the latter vary considerably; many contain several nuclear fragments whereas others lack a nuclear component. The cytoplasmic organelles of

newly formed apoptotic bodies remain well preserved (Wyllie, 1987; Kerr *et al*., 1994; Martin and Green, 1995).

Abrupt cell shrinkage is one of the earliest light microscopic features, occurring almost simultaneously to nuclear changes and is evidenced as cells in culture losing contact with adjacent cells, during which time any specialized surface structures disappear and convolutions begin to form (Allen *et al.*, 1997). Shrinkage occurs due to a net outward fluid movement, likely due to the inhibition of the Na^+ -K^+ -Cl^- co transporter system (Wilcock and Hickman, 1988). The cell becomes smaller and denser at this time. The light microscopic appearance of a shrunken cell with condensed chromatin masses appears intensely eosinophilic with a pyknotic nucleus. Pyknosis, however, is a relatively early event in apoptosis as compared to necrosis, and the shape and outcome of structural changes in chromatin are distinctly different in these two types of cell death. Convolutions of nuclear and plasma membranes occur progressively with chromatin condensation, giving the cell a bubbling or tremendously "blebbed" surface (also called *outward blebbing*). It is more common in cells with abundant cytoplasm. Observation of the intense surface blebs is restricted almost entirely to cells in culture and is rarely seen *in vivo*, apparently because the duration of blebbing is too short to be found in tissue sections (Allen *et al.*, 1997).

Shrunken and convoluted cells pinch together at points where membrane valleys are close, sealing the broken portion behind itself. The nucleus often breaks apart to form discrete nuclear fragments enclosed within a double-layered nuclear envelope, usually containing condensed chromatin which has segregated during blebbing. Since cytoplasmic blebbing occurs simultaneously, these nuclear fragments are found within larger, oval, or round-shape cytoplasmic fragments called *apoptotic bodies* (Kerr *et al*., 1972; Allen *et al*, 1997). *In vitro*, however, apoptotic bodies which remain suspended in culture media mostly escape phagocytosis and undergo degenerative changes, such as organelle disruption and

membrane rupture, usually within an hour (Kerr *et al*., 1994) and this is referred to as *secondary necrosis*. Apoptotic bodies are initially intact structures with the ability to exclude vital dye, but lose this ability after secondary necrosis has occurred (Sheridan *et al*., 1981; Wyllie, 1985). Some studies show that apoptotic structures can persist for up to an hour (Wylie, 1992). Thus, histological quantification can be misinterpreted if the timing parameters during experimentation are not clear (Allen *et al*, 1997).

Analysis of Cell Morphological by Acridine Orange/Ethidium Bromide (AO/EB) Fluorescence Staining

In the present studies, cells morphological changes were also evaluated through acridine orange/ethidium bromide (AO/EB) staining. By using AO/EB technique, morphological changes of the MCF7, SKOV3, CaSki and HT29 cells-treated with 10.0 µg/ml of cytotoxically active crude extracts of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii* and fractions PPW6 and PPW7 for 24 h that were observed in this study were cytoplasmic vacuolization, chromatin condensation and nuclear fragmentation. This observation helped in deducing that the cell death observed occurred not due to necrosis, but due to apoptosis.

These results are in good agreement with previous studies reported on the cell death morphological changes induced by various plants species assessed by using the AO/EB double staining method. Detection of apoptotic-like characteristics with AO/EB staining was also observed by Nagamine *et al*., 2009. Treatment of human breast cancer MCF7 cell with 1000 µg/ml of butanolic extract of the roots of *Ptaffia paniculata* lead to some morphological alterations of the cells such as contraction of nucleus, formation of blebs and high intensity staining. Peng *et al*., (2008) had also reported on the morphological changes

of the human ovarian cancer SKOV3 cells when treated with 40 – 160 µg/ml of *Duchesnea* phenolic fraction for 36 h which was observed by acridine orange/ethdium bromide staining. The control cells had uniform bright green nuclei, whereas cells treated with *Duchesnea* phenolic fraction exhibited the characteristic changes of apoptosis, with cell shrinkage, nuclear condensation and fragmentation and formation of apoptotic bodies.

In this protocol, fluorescent DNA-binding dyes were added to a mixture of effectors and target cells and examined by fluorescence microscopy to visualize and enumerate its aberrant chromatin distribution. A mixture of acridine orange and ethidium bromide were used to differentiate between intact (viable) and lysed (nonviable) cells. Both live and dead cells take up AO, whereas only dead cells take up EB. AO intercalates into DNA, making it appear bright green, and binds to RNA in the cytoplasm, staining it red. EB intercalates into DNA, making it appear orange, but bind weakly to RNA, which may appear slightly red. Thus a viable cell stained with mixture of AO/EB will have bright green chromatin and red cytoplasm, whereas a dead cell will have bright orange chromatin (EB overwhelms AO) and its cytoplasm, if it is any RNA remaining, will appear dark red (Duke, 2004).

Detection of DNA Fragmentation by Agarose Electrophoresis

Formation of DNA fragmentation is one of the characteristic features observed in apoptotic cells and it is generally considered as the biochemical hallmark of apoptosis (Zhang and Xu, 2002). The formation of this DNA ladder correlates with the early morphological signs of apoptosis (Wylie *et al*., 1984), and has been widely used as a distinctive marker of the apoptosis process.

In the present study, the ladder-like appearance of DNA observed in the cells-treated with active crude extract of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and

P. watsonii, and in the cells treated with active fractions of *P. watsonii*, reconfirmed the apoptosis-inducing capability of the both extracts and fractions. Conventional agarose gel electrophoresis method was used in the present study to analyze fragmented nuclei in studied cells. Conventional agarose gel electrophoresis was first described for cell death by Wyllie, (1980) and allows the detection of low-molecular weight (LMW) DNA ladders (Karasavvas *et al*., 1996). This method involved three major steps: (1) the fragmented DNA is extracted from the induced cells, (2) the DNA is purified to remove protein and RNA contaminants that could interfere with the resolution of the DNA ladder and (3) the DNA is resolved by electrophoresis and is photographed to record the results (Matassov *et al*., 2004).

There been a numerous report on internucleosomal DNA cleavage during apoptosis in a wide variety of cells and tissue under many conditions (Lee *et al*., 2003; Son *et al*., 2003; Kuo *et al*., 2005; Yang *et al*., 2006; Kim *et al*., 2008; Liu *et al*., 2009). Formation of 180-200 bp ladder of the DNA extracted from cancer cell lines treated with cytotoxically active extracts and compounds from various plants species including Phyllanthaceae species have been widely reported. 2 mg/ ml of water extract of *P. urinaria* showed to induce the typical pattern of DNA ladder in Lewis lung carcinoma cells in a time-dependent manner as revealed by DNA gel electrophoresis (Huang *et al*., 2003). DNA extracted from human hepatocarcinoma SMMC-7721 cells treated with flavonoids from *Astragalus complanatus* at concentrations of 200 and 400 µg/ml for 48 h revealed a progressive increase in the ladder fragments and control cells did not exhibit any such DNA fragmentation (Hu *et al*., 2009). Kim *et al*., (2008) observed a typical ladder formation in the human colon cancer HT29 cells treated for 24 h with 60 and 80 µg/ml of crude saponin isolated from the roots of *Platycodon grandiflorum*. Treatment of human colon cancer COLO205 cells with essential oil from *Ocimum viride* indicated internucleosomal DNA

breakdown which leading to DNA fragmentation as expected for apoptotic cells (Sharma *et al*., 2010). Jin *et al*., (2006) observed typical ladder profile of oligonucleosomal fragments ranging from 200 to 1400 bp in glioblastoma U87 cells treated with methanol fraction of *Ulmus davidiana*, whereas DNA fragments were absent in control cells.

This DNA ladder feature was firstly reported by Wylie, (1980) in the experiment which showed that glucocorticoid-induced death of thymocytes, which was known to display the typical ultrastructural features of apoptosis, is associated with the unique change in the nuclear DNA. When endonuclease attacks the linker regions between nucleosomes, this resulted in the internucleosomal cleavage and this leading to the formation of fragments that are multiples of units comprising 180 – 200 bp. These fragments are detected readily by agarose gel electrophoresis and a characteristic ladder being evident when ethidium bromide-stained gels are viewed in ultraviolet light (Allen *et al.*, 1997).

Three types of DNA fragmentation occurring during apoptosis can thus be distinguished: (1) internucleosomal DNA cleavage, (2) fragmentation into large 50 – 300 kbp lengths; and (3) single-stranded cleavage events. In almost all circumstances of morphologically well-characterized apoptosis, internucleosomal DNA cleavage has been the biochemical event used as the definitive apoptotic marker. This pattern of DNA degradation occurs by activation of an endogenous endonuclease that cleaves the DNA in the linker region between histones on the chromosomes. Since the DNA wrapped around the histones comprises ~180 – 200 bp, multiples of these intervals are characteristically observed and are commonly referred to as "apoptotic" ladder. Once these cells have fragmented their DNA they are committed to die and cannot be rescued by removal of the apoptotic signal. This DNA degradation remains a good indicator of apoptosis, particularly when combined with morphological characterization (Bortner *et al*., 1995).

In contrast, DNA laddering is not seen in cells undergone necrosis, which shows a random fragmentation and histone degradation pattern leading to diffuse smears on DNA agarose electrophoresis (Wylie, A.H. 1980; Afanas'ev *et al*., 1986; Duvall and Wyllie, 1986). The present study clearly shown that the DNA isolated from untreated cells (control) did not show any ladder formation and smears formation was observed. The necrotic DNA fragments are also larger, and therefore significantly less in number, than apoptotic DNA (Allen *et al.*, 1997).

Apoptotic endonucleases play a major role in degrading chromosomal DNA during programmed cell death. There appear to be at least two apoptotic endonucleases with different enzymatic properties, and they use different mechanisms to ensure removal of unwanted DNA and timely completion of cell-autonomous apoptosis. DNA fragmentation factor (DFF) exists in the nucleus in normal cells and is a major apoptotic endonuclease for DNA fragmentation *in vitro*. DFF is a heterodimeric protein composed of DNA fragmentation factors 45 and 40. Upon activation of apoptosis, cleavage of DFF45 by activated caspase 3 resulted in the activation of DFF40 endonuclease activity and DNA fragmentation in dying cells (Zhang and Xu, 2000). Endonuclease G (EndoG) resides in mitochondria in normal cells and travels to the nucleus, where it fragments chromosomal DNA after apoptotic stimulation. Once released from the mitochondrial intermembrane space, EndoG activity is caspase-independent. Both endonucleases can affect the timely completion of apoptosis *in vivo*. DNase II resides in macrophages and cleaves phagocytosed DNA whereas, DNase I might be involved in degrading DNA from necrotic cells (Zhang and Xu, 2002). The exact timing of DNA fragmentation is likely relative to when the phosphatidylserine exposure occurs prior to DNA cleavage. It appears, there is an initial cleavage of DNA into 300-kilobase-pair fragments and/or 50-kbp fragments

(Oberhammer *et al*., 1993), most commonly followed by oligonucleosomal-sized fragments due to double-stranded cleavage of DNA at linker regions between nucleosomes.

In some cases, DNA fragmentation appears to be delayed (Zakeri *et al*., 1993), partial, or absent in cells that otherwise meet the morphologic criteria for apoptosis (Cohen *et al*., 1992; Collins *et al*., 1992) and may show more limited DNA degradation with the formation of 300- or 50-kb fragments (Walker *et al*., 1991). It is therefore, not an absolute requirement for all forms of apoptosis (Allen *et al.*, 1997); importantly, mitochondrial DNA does not appear to be cleaved (Murgia *et al*., 1992).

Determination of Caspase-3 Activation

The mitochondrial pathway is an important mechanism of apoptosis. The key element in mitochondria pathway is the efflux of cytochrome *c* from mitochondria to cytosol, where it subsequently forms a complex with Apaf-1 and caspase-9, leading to the activation of the caspase-3 (Mehmet, 2002). Since they bring about most of the visible changes that characterize apoptotic cell death, caspases can be thought of as the central executioners of the apoptotic pathway (Earnshaw *et al*., 1999). It is well established that activation of caspases lead to degradation of cellular proteins, cell shrinkage, DNA fragmentation, loss of plasma membrane potential and membrane blebbing (Nicholson, 1999).

Data in this present study indicated that following the addition of cytotoxically active crude extract of *P. pectinatus* (leaves), *P. pectinatus* (fruits) and *P. watsonii*, and cytotoxically active fractions of *P. watsonii*, MCF7 breast cancer and SKOV3 ovarian cancer cells displayed increment in the activity of caspase-3. In the present study, the Caspase-3/CPP32 Colorimetric Assay Kit was used to measure the increased enzymatic

activity of caspase-3 in cells treated with the active crude extracts and fractions. Cells that have been induced to undergo apoptosis are first lysed to collect their intracellular contents. The cell lysate was then tested for protease activity by the addition of caspase-specific peptide that is conjugated to the colour reporter molecule *p*-nitroaniline (*p*NA), which was quantified based on spectrophotometric detection of the *p*NA after cleavage from labelled substrate DEVD- *p*NA. The *p*NA light emission was quantified using microtiter plate reader at 405-nm.

In agreement with our findings, many of plant extracts as well as Phyllanthaceae species extracts have been demonstrated to induce apoptosis in various cell types by inducing the activity of caspase-3 protein. Huang *et al.,* (2003) reported that the enzymatic activity of caspase-3 in Lewis lung carcinoma cells increased after treated with 2 mg/ml of water extract of *P. urinaria* for 24 h. Studies by Mohd Fadzelly *et al*., (2010) showed that the *Mangifera pajang* kernel extract significantly increase the activity of caspase-3 in human breast cancer MCF7 and MDA-MB-231 cells following exposure to the extract for 48 and 72 h. Liu *et al*., (2006) reported the increment of caspase-3 activity after being induced with 5.0 µg/ml of extracts from *Narcissus tazetta* var. chinensis. The caspase-3 activity peaked at 16 h.

Over a dozen caspases have been identified in humans, and two-thirds of these have been suggested to function in apoptosis (Earnshaw *et al*., 1999; Thornberry and Lazebnik, 1998). Caspases, the cytoplasmic aspartate-specific cysteine protease (Thornberry and Lazebnik, 1998; Nhan *et al*., 2006) can be further divided into two functional subgroups based on their known or hypothetical roles in the process: initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6 and -7). Initiator caspases possess long N-terminal prodomains that contain recognizable protein-protein interaction motifs while

effector caspase have short or no prodomain. Usually, initiator caspases, once activated, will activate the downstream effector caspases in a cascade-like pattern (Slee *et al*., 1999).

The functions of caspases can be summarized as to: (1) arrest the cell cycle and inactive DNA repair; (2) inactivate the inhibitor of apoptosis (XIAP); and (3) dismantle the cellular cytoskeleton (Zi *et al*., 2005). Four pathways to caspase activation during apoptosis can be categorized as: (1) the mitochondria-mediated pathway; (2) the death receptor-mediated pathway; (3) the granzyme B-mediated pathway; and (4) the endoplasmic reticulum (ER)-mediated pathway (Zi *et al*., 2005).

Mitochondria undergo two major changes during apoptosis induced by variety of apoptosis-inducing agents such as neoplastic agents, UV radiation, growth factor withdrawal, and DNA damage (Yang *et al*., 1997; Kluck *et al*., 1997). First, the mitochondrial outer membrane becomes permeable to proteins, resulting in the release of proteins normally found in the space between the inner and outer membrane (including cytochrome *c*, apoptosis inducing factor, and others). Second, the inner mitochondrial membrane's transmembrane potential is reduced (Zi *et al*., 2005). The release of cytochrome *c* from mitochondria to cytosol (Yang *et al*., 1997; Kluck *et al*., 1997) leads to the formation of a heptameric wheel-like caspase-activating complex, which has been termed as apoptosome. This is a high molecular weight complex composed of cytochrome *c*, apoptosis protease activating factor-1 (Apaf-1), deoxyadenosine triphosphate (dATP), and procaspase-9, which forms a platform for the efficient processing and activation of caspase-9 (Cain *et al*., 1999; Zi *et al*., 2005). Caspase-9 has a caspase-associated recruitment domain (CARD) in the N-terminus, and this is a key site when it associates with Apaf-1 and cytochrome *c*. Activation caspase-9, in turn, cleaves effector caspases such as caspase-3, -6, and -7 (Liu *et al*., 1996; Li *et al*., 1997; Zi *et al*., 2005). Caspase-3 activates other caspases, cleaves cytoskeletal proteins, or activates the

caspase-activated DNase. In particular, caspase-3 cleaves an inhibitor of caspase-activated DNase that allows caspase-activated DNase to enter the nucleus and to fragment nuclear DNA and culminate in the orderly demise of the cell (Gosslau and Kuang, 2004).

In this pathway of apptosis, caspase-3 and -9 may be the most important as their activities influence the process of apoptosis as well as the type of cell death. Deregulation of the apoptotic process can lead to many diseases and pathological conditions including cancer, autoimmunity, and neurodegeneration. To cure them, caspases are attractively used as potent targets because of their requisite role in apoptosis and the appealing prospect of small-molecules inhibitor therapy (Zi *et al*., 2005).

Induction of apoptosis is almost always associated with the activation of caspases; therefore, measurement of caspase activity is a convenient way to assess whether the cells are undergoing apoptosis. There are several ways to measure caspase activation. Most common ones involve use of chromegenic or fluorogenic peptide substrates that release the chromogen or fluorescent tag upon cleavage by a caspase (Kumar, 2004).

Cell Cycle Analysis by Flow Cytometry

It is well known that the cell growth and proliferation of mammalian cells are mediated via cell cycle progression (Schwartz and Shah, 2005). Cell cycle progression and apoptosis are two pivotal signalling mechanisms used to maintain homeostasis in healthy tissues (Hu *et al*., 2009). The inhibition of the cell cycle has become an appreciated target for management and treatment of tumour cells with cytotoxic agents (Shih and Stutman, 1996; Schwartz and Shah, 2005).

To determine whether the growth inhibition of the human MCF7 breast cancer, SKOV3 ovarian cancer, CaSki cervical cancer and HT29 colon cancer cell by crude

hexane extract of *P. watsonii* and fraction PPW7 may be the result of cell-cycle arrest, the DNA content was analyzed by propidium iodide staining followed by flow cytometry. In the present study, it was found that crude hexane extract of *P. watsonii* arrested MCF7, SKOV3 and CaSki cells at two different degree (combination of G_0/G_1- S, S-G_2/M, and G_0/G_1- G_2/M phases), and fraction PPW7 arrested SKOV3 cells at two different degree (S-G_2/M phases).

Many anti cancer agents and DNA-damaging agents arrest the cell cycle at the G_0/G_1, S, or G_2/M phase an then induce apoptotic cell death (Kessel and Luo, 2000; Purohit *et al*., 2000; Harada *et al*., 2002; Cheng *et al*., 2004; Sun and Liu, 2006; Hu *et al*., 2010). The percentage of Lewis lung carcinoma cells in sub-G1 phase after treatment with the water extract of *P. urinaria* was significantly increased from 3.71 % to 54.25 % (Huang *et al*., 2003). Progallin A, a compound isolated from the acetic ether part of the leaves of *P. emblica* induced significant G_0/G_1 and G_2/M arrest of human hepatocarcinoma BEL-7404 cells. The percentage of BEL-7404 cells treated with 20 µg/ml of Progallin A in G_0/G_1 phase increased from 56.0 % in control to 76.3 % and in G_2/M phase increased from 13.3 % in control to 21.7 % after 72 h incubation (Zhong *et al*. 2011). Treatment of Chinese hamster ovary (CHO) cells with 750 µg/ml of aqueous extract of *P. orbicularis* increased the proportions of cells in G_2/M phase from 15.3 % (control) to 38.5 % which indicated that the observed apoptotic cell death in CHO cells due to the cell cycle arrest in G_2/M phase (Sanchez-Lamar *et al*., 1999).

Flow cytometric analysis of cellular DNA content has become an increasingly important research tool for measuring and identifying abnormal cell populations. Examinations of cellular DNA content can provide information for use in cell-cycle analysis, as well as for establishing a DNA index (Hiddermann *et al*., 1984) for diploid and aneuploid cells in cancer studies (Ronot *et al*., 1986; Frankfurt *et al*., 1984; Barlogie *et al*.,

1983). Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescence signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell (Nunez, 2001).

Cell cycle progression is regulated by the activity of cyclins, a family of proteins that activate the cell-cycle-dependent kinases (Cdks) (Sherr, 2000). Cyclin A is required for the S phase and passage through G_2 phase and cyclin E activates cdk2 protein near the start of the S phase (Le and Richardson, 2002). Cyclin D1 is known to bind to and activate cdk4, which is largely involved in controlling the G_1/S restricting point and the G_2/M transition is positively regulated by the cdk2 and cyclin B complex (Coqueret, 2002; Popov *et al*., 2005). Cdk-cyclin complexes are negatively controlled by the Kip/Cip family of cyclin-dependent kinase inhibitors (CDKIs), namely $p27^{Kip1}$ and $p21^{Cip1}$ (Besson *et al*., 2008). Uncontrolled expressions of cyclins and/or CdksA, for example, over-activation of cdk-cyclin, often leads to uncontrolled cell division and malignancy (Le and Richardson, 2002; Hu *et al*., 2009). Arrest in the G_1 phase resulted in an accumulation in the sub-G_1 phase, which has been identified as a hallmark of apoptosis (Hu *et al*., 2009).

Numerous of studies had been carried out in order to elucidate the mechanism of action of extract on cell cycle and the changes of multiple regulatory molecules in the cell cycle. Studies by Park *et al*., (2009) showed a well correlation between honikiol-mediated cell cycle arrests at G_0/G_1 phase with the up regulation of one of cdk inhibitor $p27^{Kip1}$ and the down regulation of cdk4, cyclin D1, cdk2, cyclin A and cyclin E. Honikiol is naturally occurring neolignan mainly found in Magnoliae Cortex, the bark of *Magnolia obovata* Thurnberg (Park *et al*., 2009). Liu *et al*., (2009) reported that the synthetic phenylacetate

derivatives (4-fluoro-*N*-butylphenylacetamides)–mediated G_2/M-phase arrest of the cell cycle in CaSki and HeLa cells is associated with the down-regulation of cdk1, cdk2, cyclin A and cyclin B proteins. These data implied that 4-fluoro-*N*-butylphenylacetamides-induced cell cycle arrest at the G_2/M-phase most likely occurs through the reduction of these cyclin proteins. Hu *et al*., (2010) suggested that the G_0/G_1 arrest induced by 20 μM salidroside (isolated from plants of the *Rhodiola* genus) in human breast cancer MCF7 cells may be related to the downregulation of cyclin B1 and Cdk2. Studies by Sun and Liu, (2006) showed that the cell cycle related protein cyclin D1 and Cdk4 were down-regulated by cranberry extract, thus providing a possible explanation for the observed G_1 arrest induced by cranberry extract treatment.

The G_0/G_1 arrest induced by crude hexane extract of *P. watsonii* in MCF7, CaSki and HT29 cells and fractions PPW7 in HT29 cells may be related to the down-regulation of cdk4 and cyclin D1. In addition, the accumulation of MCF7 and SKOV3 cells in S phase induced by crude hexane extract of *P. watsonii* and accumulation of MCF7, SKOV3 and CaSki cells in S phase induced by fraction PPW7 may be related to the down regulation of cyclin A and cdk2. Crude hexane extract of *P. watsonii* and fraction PPW7 might induce G_2/M arrest in SKOV 3 cells through the regulation of cyclin B1 and cdk2.

CHAPTER 6

CONCLUSIONS

6.1 Conclusions

Crude methanol, hexane and ethyl acetate extracts of selected Phyllanthaceae studied were found to exhibit potent cytotoxic activity against several human cancer cell lines tested with greatest cytotoxicity was observed in *Phyllanthus watsonii* and *Phyllanthus pectinatus*. Although the plants from the Phyllanthaceae species were randomly selected based on their documented medicinal values and reported phytochemical and biological activities, 19% of these plant extracts were able to induce cytotoxicity in all four human cancer cells tested in dose-dependent manner. Based on this data, it appears that unrelated medicinal use of the source plants may serve as an initial guide in selection of plants for cytotoxicity screening.

Plants derived natural products hold great promise for discovery and development of new pharmaceuticals. The potential growth inhibitory activity of the Phyllanthaceae extracts and fractions should be taken into account when considering the further development and prioritization as a cancer chemotherapeutic agent in either alone or combination with other anticancer agents.

The potential *in vitro* cytotoxic activity of original *P. watsonii* collected directly from Endau-Rompin against MCF7, SKOV3, CaSki and HT29 were investigated for the first time. The results demonstrated that *P. watsonii* has strong cytotoxic effect by inducing apoptotic cell death, increase caspase-3 activation, and causing G_0/G_1 phase arrest in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of

apoptosis, Thus, modulating apoptosis may be useful in the management and therapy or prevention of cancer. Understanding the modes of action of these compounds will help in providing useful information for their possible application in cancer prevention and perhaps also in cancer therapy. Because of its relative selective cytotoxicity against cancer compared to normal cells, compounds from *P. watsonii* are promising new experimental anticancer agents for the treatment of human cancers.

6.2 Future Study

Some constituents from *Phyllanthus pectinatus* and *Phyllanthus watsonii* might serve as a novel and potent anticancer agents. As current studies were focused on *in vitro* investigation of cytotoxic effects crude extracts and fractions on human cancer cell lines, further research might include exploration of its effects in animal tumour models to confirm its anticancer activity *in vivo*. Further studies on the isolation of pure compound(s) and chemical characterization of the cytotoxically active compounds of *P. watsonii* are necessary. The present evidence suggests the cytotoxic effect of the cytotoxically active extracts and fractions of selected Phyllanthaceae species is mediated by induction of apoptosis, but the exact pathway involved in the caspase-3 activation is still unclear. Further investigations are necessary in order to understand the interaction mechanism involved in the cytotoxic and apoptotic effect of these extracts and fractions.

REFERENCES

Adams, J.M., & Cory, S. (1998). The bcl-2 protein family: arbiters of cell survival. *Science, 281*, 1322–1326.

Afanas'ev, V.N., Korol', B.A., Mantsygin, Y.A., Nelipovich, P.A., Pechatnikov, V.A., & Umansky, S.R. (1986). Flow cytometry and biochemical analysis of DNA degradation characteristics of two types of cell death. *FEBS Letter, 194*, 347–350.

Aggarwal, B.B., & Shishoida, S. (2006). Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical Pharmacology, 71*, 1397–1421.

Aggarwal, B.B., Danda, D., Gupta, S., & Gehlot, P. (2009). Models for prevention and treatment of cancer: Problems vs promises. *Biochemical Pharmacology, 78(9)*, 1083–1094.

Ahmad, M.U., Husain, S.K., & Osman, S.M. (1981). Ricinoleic acid in *Phyllanthus niruri* seed oil. *Journal of the American Oil Chemists' Society, June 1981*, 673–674.

Ahmed, B., Khan, S., Verma, A., & Habibullah. (2009). Antihepatotoxic activity of debalalactone, a new oxirano-furanocoumarin from *Phyllanthus debilis*. *Journal of Asian Natural Products Research, 11(8)*, 687–692.

Akiyama, T., Ishida, J., Nikagawa, H., Watanabe, S., Itoh, N., Shibuy, A.M., et al. (1987). Genistein. A specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry, 262*, 5592–5595.

Allen, R.T., Hunter III, W.J., & Agarwal, D.K. (1997). Morphological and biochemical characterization and analysis of apoptosis. *Journal of Pharmacological and Toxicological Methods*, *37*, 215–228.

American Cancer Society. (2009). Cancer Facts & Figure 2009. Atlanta: American Cancer Society. Retrieved 10 July 2010, from http://www.cancer.org/acs/groups/content/@nho.../cffaa20092010pdf.pdf.

Aquilina, G., Crescenzi, M., & Bignami, M. (1999). Mismatch repair, G(2)/M cell cycle arrest and lethality after DNA damage. *Carcinogenesis*, *20*, 2317–2326.

Asha, V.V, Akhila, W.P.J., & Subramaniam, A. (2004). Further studies on the antihepatotoxic activity of *Phyllanthus maderaspatensis* Linn. *Journal of Ethnopharmacology, 92,* 67–70.

Ashkenazi, A., & Dixit, V.M. (1998). Death receptors: signaling and modulation. *Science, 281,* 1305–1308.

Au, W.W., Abdou-Salama, S., Sierra-Torres, C.H., & Al-Hendy, A. (2007). Environment risk factors for prevention and molecular intervention of cervical cancer. *International Journal of Hygiene and Environmental Health, 210(6),* 671–678.

Avato, P., & Tava, A. (1995). Acetylenes and terpenoids of *Bellis perennis*. *Phytochemistry, 40(1),* 141–147.

Baker, J.T., Borris, R.P., Carte, B., Cordell, G.A., Soejarto, D.D., Cragg, G.M., et al. (1995). Natural products drug discovery and development: new perspectives on international collaboration. *Journal of Natural Products, 58(9),* 1325–1357.

Baker, S.J., & Reddy, E.P. (1998). Modulation of life and death by the TNF receptor superfamily. *Oncogene, 17,* 3261–3270.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils-A review. *Food and Chemical Toxicology, 46,* 446–475.

Balandrin, M.F., Kinghorn, A.D., & Farnsworth, N.R. (1993). Plant-derived natural products in drug discovery and development. In *Human Medicinal Agents from Plants*. (p.2). Washington DC: American Chemical Society.

Balunas, M.J., & Kinghorn, A.D. (2005). Drug discovery from medicinal plants. *Life Sciences, 78,* 431–441.

Bandyopadhyay, S.K., Pakrashi, S.C., & Pakrashi, A. (2000). The role of antioxidant activity of *Phyllanthus emblica* fruits on prevention from indomethacin induced gastric ulcer. *Journal of Ethnopharmacology, 70(2),* 171–176

Bardon, S., Foussard, V., Fournel, S., & Loubat, A. (2002). Monoterpenes inhibit proliferation of human colon cancer cells by modulating cell cycle-related protein expression. *Cancer Letters, 181,* 187–194.

Bardon, S., Picard, K., & Martel, P. (1998). Monoterpenes inhibit cell growth, cell cycle progression, and cyclin D1 gene expression in human breast cancer cell lines. *Nutrition and Cancer, 32,* 1–7.

Barlogie, B., Raber, M.N., Schumann, J., Johnson, T.S., Drewinko, B., Swartzendruber, D.E., et al. (1983). Flow cytometry in clinical cancer research. *Cancer Letters, 43,* 3982–3997.

Barros, M.E., Lima, R., Mercuri, L.P., Matos, J.R., Schor, N., & Boim, M.A. (2006). Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis. *Urology Research, 34,* 351–357.

Barthakur, N.N., & Arnold, N.P. (1991). Chemical analysis of the emblic (*Phyllanthus emblica* L.) and its potential as a food source. *Scientia Horticulturae, 47(1-2*), 99–105.

Basharat, A. (1998). Medicinal properties of various species of *Phyllanthus*. *Hamdard-Medicus, 41,* 109–110.

Benavente-García, O., Castillo, J., Marín, F.R., Ortuño, A., & Del Rio, J.A. (1997). Uses and properties of citrus flavonoids. *Journal of Agricultural and Food Chemistry, 45,* 4505–4515.

Bertino, J.R. (1997). Irinotecan for colorectal cancer. *Seminars in Oncology, 24,* S18–S23.

Besson, A., Dowdy, S.F., & Roberts, J.M. (2008). CDK inhibitors: cell cycle regulators and beyond. *Developmental Cell, 14,* 159–169.

Beveridge, T.H., Li, T.S., & Drover, J.C. (2002). Phytosterol content in American ginseng seed oil. *Journal of Agricultural and Food Chemistry, 50,* 744–750.

Bindseil, K.U., Jakupovic, J., Wolf, D., Lavayre, J., Leboul, J., & van der Pyl, D. (2001). Pure compounds libraries: a new perspectives for natural product based drug discovery. *Drug Discovery Today, 6,* 840–847.

Birt, D.F., Hendrich, S., & Wang, W. (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacology & Therapeutics, 90,* 157–177.

Blagosklonny, M.V., & Pardee, A.B. (2002). The restriction point of the cell cycle. *Cell Cycle, 1,* 103–110.

Boik, J. (2001). *Natural Compounds in Cancer Therapy*. Minnesota, USA: Oregon Medical Press.

Bold, R.J., Termuhlen, P.M., & McConkey, D.J. (1997). Apoptosis, cancer and cancer therapy. *Surgical Oncology. 6,* 133–142.

Borenfreund, E., & Puerner, J.A. (1984). A simple quantitative procedure using monolayer culture for toxicity assays. *Journal of Tissue Culture Methods*. 9: 7–9.

Borenfreund, E., & Puerner, J.A. (1985). Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters*. 24: 119–124.

Bortner, C.D. Nicklas, B., Oldenburg, E. & Cidlowski, J.A. (1995). The role of DNA fragmentation in apoptosis. *TRENDS in Cell Biology, 5,* 21–26.

Bossy-Wetzel, E. and Green, D.R. (1999). Apoptosis: checkpoint at the mitochondrial frontier. *Mutation Research*. 434: 243–251.

Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science, 161,* 839–851.

Boyle, P., & Levin, B. (2008). World Cancer Report 2008. *International Agency for Research on Cancer*. 1–260.

Braverman, I.M. (2002). Skin manifestations of internal malignanacy. *Clinics in Geriatric Medicine, 18(1),* 1–19.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutrition Reviews, 11,* 317–333.

Burkill, I.H. (1966). *A Dictionary of the Economic Products of the Malay Peninsula*. London: Crown Agents for the Colonies.

Bury, J., & Cross, S. (2003). Molecular biology in diagnostic histopathology: Part 1-The cell cycle. *Current Diagnostic Pathology, 9(4),* 266–275.

Butler, M.S. (2004). The role of natural products chemistry in drug discovery. *Journal of Natural Products, 67(12),* 2141–2153.

Cain, K., Brown, D.G., Langlais, C., & Cohen, G.M. (1999). Caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex. *Journal of Biological Chemistry, 274,* 22686–22692.

Calixto, J.B., Santos, A.R.S., Filho, V.C., & Yunes, R.A. (1998). A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. *Medicinal Research Reviews, 18(4),* 225–258.

Cerutti, P.A. (1985). Prooxidant states and tumor promotion. *Science, 227,* 375–381.

Chan, K.L., Low, B.S., Teh, C.H., & Das, P.K. (2009). The effect of *Eurycoma longifolia* on sperm quality of male rats. *Natural Product Communications, 4(10),* 1331–1336.

Chang, J.C.N. (1998). A review of breast cancer chemoprevention. *Biomedicine and Pharmacotherapy, 52*, 133–136.

Chantharanothai, P. (2009). Flora of Thailand. Retrieved 23 September 2010, from http://www.nationaalherbarium.nl/thaieuph/ThPspecies/ThPhyllanthus.htm#Phyllanthus_roseus.

Chemma, M., Cody, R., & Krishnan, K. (2001). Cancer chemoprevention. *Federal Practitioner, 18(10),* 48–53.

Chen, M., & Wang, J. (2003). Initiator caspases in apoptosis signaling pathways. *Apoptosis, 7,* 313–319.

Chen, Y.C., Shen, S.C., Chow, J.M.. Ko, C.H., & Tseng, S.W. (2004). Flavone inhibition of tumor growth via apoptosis *in vitro* and *in vivo*. *International Journal of Oncology, 25,* 661–670.

Cheng, Y.L., Chang, W.L., Lee, S.C., Liu, Y.G., Chen, C.J., Lin, S.Z., et al. (2004). Acetone extract of *Angelica sinensis* inhibits proliferation of human cancer cells via inducing cell cycle arrest and apoptosis. *Life Sciences, 75,* 1579–1594.

Chiang, L.C., Ng, L.T., Lin, I.C., Kuo, P.I., & Lin C.C. (2006). Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. *Cancer Letters, 237,* 207–214.

Chiang,L.C., Chiang,W., Chang, M.Y., Ng, L.T., & Lin, C.C. (2003). Antileukimic activity of selected natural products in Taiwan. *The American Journal of Chinese Medicine, 31*, 37–46.

Chien, S.c., Chang, J.y., Kuo, C.C., Hsieh, C.C., Yang, N.S. & Kuo, Y.H. (2007). Cytotoxic and novel skeleton compounds from the heartwood of *Chamaecyparis obtuse* var. *formosana*. *Tetrahedron Letters, 48,* 1567–1569.

Chin, H.F. (1986). Rambai and tampoi in Malaysia. *Yearbook-West Australian Nut and Tree Crops Association, 11,* 72–73.

Chirdchupunseree, H. & Pramyothin, P. (2010). Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes. *Journal of Ethnopharmacology. 128,* 172–176.

Choedon, T., Shukla, S.K. & Kumar, V. (2010). Chemopreventive and anti-cancer properties of the aqueous extract of flowers of *Butea monosperma*. *Journal of Ethnopharmacology, 129,* 208–213.

Chopra, R.N., Nayer, S.L., & Chopra, I.C. (1956). Glosarry of Indian Medicinal Plants. (p.191). CSIR, New Delhi.

Chung, H.L., Li, J.H., Sheng, C.K., Tsung, H.L., Kan, J.T., & Hsu, C.C. (2009). 4-Fluoro-*N*-butylphenylacetamide (H6) inhibits cell growth via cell-cycle arrest and apoptosis in human cervical cancer cells. *Biorganic & Medicinal Chemistry, 17,* 42–48.

Chung, S.K., Nam, J.A., Jeon, S.Y., Kim, S.I., Lee, H.J., Chung, T.H., et al. (2003). A prolyl endopeptidase-inhibiting antioxidant from *Phyllanthus ussurensis*. *Archives of Pharmal Research, 26(12),* 1024–1028.

Cohen, G.M., Sun, X.M., Snowden, R.T., Dinsdale, D., & Skilleter, D.N. (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochemical Journal, 286,* 331–334.

Colditz, G.A., Branch, L.G., Lipnick, R.J., Willett, W.C., Rosner, B., & Hennekens, C.H. (1985). *American Journal of Clinical Nutrition, 41,* 32–26.

Collins, R.J., Harmon, B.V., Gobe, G.C., & Kerr, J.F. (1992). Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Journal of Radiation Biology, 61,* 451–453.

Constantini, P., Jocotot, E., Decaudin, D. & Kroemer, G. (2000). Mitochondrion as a novel target of anticancer chemotherapy. *Journal of National Cancer Institute, 92,* 1042–1053.

Cook, J.A., & Mitchell, J.B. (1989). Viability measurements in mammalian cell systems. *Analytical Biochemistry. 179,* 1–7.

Coqueret, O. (2002). Linking cyclins to transcriptional control. *Gene, 299,* 35–55.

Cortes, J.E., & Pazdur, R. (1995). Docetaxel. *Journal of Clinical Oncology, 13,* 2643–2655.

Coussens, L.M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420(6917),* 19–26.

Cragg, G.M., & Newman, D. (1999). Discovery and development of antineoplastic agents from natural sources. *Cancer Investigation, 17,* 153–163.

Cragg, G.M., & Newman, D.J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100,* 62–79.

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

Creagh, E.M., & Martin, S.J. (2001). Caspases: cellular demolition experts. *Biochemical Society Transactions, 29,* 696–702.

Creemers, G.J., Bolis, G., Gore, M., Scarfone, G., Lacave, A.J., Guastalla, J.P., et al. (1996). Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer. *Journal of Clinical Oncology, 14,* 3056–3061.

Crowell PL, & Gould MN (1994). Chemoprevention and therapy of cancer by *d*-limonene. *CRC Critical Reviews in. Oncology, 5,* 1–22.

Crowell, P.L., & Gould, M.N. (1994). Chemoprevention and therapy of cancer by *d*-limonene. *CRC Critical Review in Oncology, 5,* 1–22.

Cutts, J.H., Beer, C.T., & Noble, R.L. (1960). Biological properties of vincaleukoblastine, an alkaloid in *Vinca rosea* Linn, with reference to its antitumor action. *Cancer Research, 20,* 1023–1031.

da Rocha, A.B., Lopes, R.M., & Schwartsmann, G. (2001). Natural products in anticancer theraphy. *Current Opinion in Pharmacology, 1,* 364–369.

da Silva pinto, M., Lajolo, F.M., & Genovese, M.I. (2008). Bioactive compounds and quantification of total ellagic acid in strawberries (*Fragaria x ananassa* Duch.). *Food Chemistry, 107,* 1629–1635.

Darison, G.W.H. (1988). *Endau Rompin: A Malaysian Heritage*. (p.112). Kuala Lumpur: Malaya Nature Society.

Das, M., Bickers, D.R., & Mukhtar, H. (1985). Effects of ellagic acid on hepatic and pulmonary xenobiotic metabolism in mice: studies on the mechanism of its anticarcinogenic action. *Carcinogenesis, 6,* 1409–1413.

de Mesquita, M.L., de Paula, J.E., Pessoa, C., de Moraes, M.O., Lotufo, L.V.C., Grougnet, R., et al. (2009). Cytotoxic activity of Brazilian Cerrado plants used in traditional medicine against cancer cell lines. *Journal of Ethnopharmacology, 123,* 439–445.

De Souza, T.P., Holzschuh, M.H., Lionço, M.I., Ortega, G.G., & Petrovick, P.R. (2002). Validation of LC methods for the analysis of phenolic compounds from aqueous extract of *Phyllanthus niruri* aerial parts. *Journal of Pharmaceutical and Biomedical Analysis, 30(2),* 351–356.

De Vita, V.T Jr., Serpick, A.A., & Carbone, P.O. (1970). Combination chemotherapy in the treatment of advanced Hodgkin's disease. *Annals of Internal Medicines, 73,* 881–895.

de Vogel, J., Jonker-Termont, D.S,M.L., van Lieshout, E.M.M., Katan, M.B., & van deer Meer, R. (2005). Green vegentables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis, 26(2),* 387–393.

Dessauge, F., Lizundia, R., Baumgartner, M., Chaussepied, M., & Langsley, G. (2005). Taking the Myc is bad for *Theileria*. *Trends in Parasitology, 21(8),* 377–385.

Dhiman, R.K., & Chawla, Y.K. (2005). Herbal medicines for liver diseases. *Digestive Diseases and Sciences, 50(10),* 1807–1812.

Duh, C.Y., Wang, S.K., Chu, M.J., & Sheu, J.H. (1998). Cytotoxic sterols from the soft coral *Nephthea erecta*. *Journal of Natural Products, 61(8),* 1022–1024.

Duke, R.C. (2004). Methods of analyzing chromatin changes accompanying apoptosis of target cells in killer cell assays. In Brady, H.J.M. (ed), *Methods in Molecular Biology: Apoptosis Methods and Protocols*. (pp.47-48). Humana Press Inc.

Duprez, L., Wirawan, E., Berghe, T.V., & Vandenabeele, P. (2009). Major cell death pathways at a glance. *Microbes and Infection, 11(13),* 1050–1062.

Duvall, E., & Wyllie, A.H. (1986). Death and the cell. *Immunology Today, 7,* 115–119.

Earnshaw, W.C., Martins, L.M., & Kaufmann, S.H. (1999). Mammalian caspases: structure, activation, substrate, and functions during apoptosis. *Annual Review of Biochemistry, 68,* 383–424.

El-Desouky, S.K., Ryu, S.H., & Kim, Y.K. (2008). A new cytotoxic acylated apigenin glucoside from *Phylanthus emblica* L. *Natural Product Research, 22(1),* 91–95.

Elegbede, J.A., Elson, C.E., Qureshi, A., Tanner, M.A., & Gould, M.N. (1984). Inhibition of DMBA-induced mammary cancer by the monoterpene *d*-limonene. *Carcinogenesis, 5,* 661–664.

Emmy, H.K.I., Khoo, H.E., Abbe Maleyki, M.J., Amin, I., Salma, I., Azrina, A., et al. (2009). Antioxidant capacity and total phenolic content of Malaysian underutilized fruits. *Journal of Food Composition and Analysis, 22,* 388–393.

Engel, D.H., & Phummai, S. (2000). *A Field Guide to Tropical Plants of Asia*. (p.110). Times Edition.

Fakim, A.G. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine, 27,* 1–93.

Fan, T.J., Han, L.H., Cong, R.S., & Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica and Biophysica Sinica, 37,* 719–727.

Farouk, A.A., & Benafri, A. (2007). Antibacterial activity of *Eurycoma longifolia* Jack: A Malaysian medicinal plant. *Saudi Medical Journal, 28(9),* 1422–1424.

Fernie, A. (2007). The future of metabolic phytochemistry: Larger numbers of metabolites, higher resolution, greater understanding. *Phytochemistry, 68 (22–24),* 2861–2880.

Ferrer, M., Sanchez-Lamar, A., Fuentes, J.L., Barbe, J., & Llagostera, M. (2002). Antimutagenic mechanisms of *Phyllanthus orbicularis* when hydrogen peroxide is tested using *Salmonella* assay. *Mutation Research, 517,* 251–254.

Firn, R.D., & Jones, C.G. (2003). Natural products-a simple model to explain chemical diversity. *Natural Product Reports Articles, 20,* 382–391.

Fisher, D.E. (1994). Apoptosis in cancer therapy: crossing the threshold. *Cell, 78,* 539–542.

Fjaeraa, C., & Nanberg, E. (2009). Effect of ellagic acid on proliferation, cell adhesion and apoptosis in SH-SY5Y human neuroblastoma cells. *Biomedicine & Pharmacotheraphy, 63,* 254–261.

Franceschi, S., Bidoli, E., La Veccia, C., Talamini, R., D'Avanzo, B., & Negri, E. (1994). Tomatoes and risk of digestive-tract cancers. *International Journal of Cancer, 59,* 181–184.

Frankfurt, O.S., Greco, W.R., Slocum, H.K., Arbuck, S.G., Gamarra, Z., Pavelic, P., et al. (1984). Proliferative characteristics of primary and metastatic human solid tumors by DNA flow cytometry. *Cytometry, 5(6),* 629–635.

Freshney, R.I. (1994). *Culture of Animal Cells: A Manual of Basic Technique*. (pp.287–296). New York: Wiley-Liss.

Friedberg, E.C., McDaniel, L.D., & Schultz, R.A. (2004). The role of endogenous and exogenous DNA damage and mutagenesis. *Current Opinion in Genetics & Development, 14,* 5–10.

Fulda, S. (2008). Betulinic acid for cancer treatment and prevention. *International Journal of Molecular Sciences, 9,* 1096–1107.

Fulda, S., Jeremias, I., Steiner, H.H., Pietsch, T., & Debatin, K.M. (1999). Betulinic acid: A new cytotoxic agent against malignant brain-tumor cells. *International Journal of Cancer, 82,* 435–441.

Gantet, P., & Memelink, J. (2002). Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. *Trends in Pharmacological Sciences, 23(12),* 563–569.

Geran, R.I., Greenberg, N.H., McDonald, M.M., Schumacher, A.M., & Abbott, B.J. (1972). Protocols for screening chemical agents and natural products against animal tumor and other biological systems. *Cancer Chemotherapy Reports*, *3(2)*, 17–19.

Gibb, R.K., Taylor, D.D., Wan, T., O'Connor, D.M., Doering, D.L., & Taylor, C.G. (1997). Apoptosis as a measure of chemosensitivity to cisplatin and taxol therapy in ovarian cancer cell lines. *Gynecologic Oncology, 65,* 13–22.

Giovannucci, E., Ascherio, A., Rimm, E.B., Stampfer, M.J., Colditz, G.A., & Willet, W.C. (1995). Intake of carotenoids and retinol in relation to risk of prostate cancer. *Journal of the National Cancer Institute, 87,* 1767–1776.

Giridharan, P., Somasundaram, S., Perumal, K., Vishwakarma, R.A., Karthikeyan, N.P., Velmurugan, R., et al. (2002). Novel substituted methylenedioxy lignan suppress proliferation of cancer cells by inhibiting telomerase and activation of *c*-myc and caspases leading apoptosis. *British Journal of Cancer, 87,* 98–105.

Gosslau, A., & Kuang, Y.C. (2004). Nutraceuticals, apoptosis, and disease prevention. *Nutrition, 20,* 95–102.

Goto, K.N., Nakamura, S., Bastow, K.F., Nyarko, A., Peng, C.Y., Lee, F.C., et al. (2007). Antitumor agents. 256. Conjugation of paclitaxel with other antitumor agents: evaluation of novel conjugates as cytotoxic agents. *Bioorganic & Medicinal Chemistry Letters, 17(10)*, 2894–2898.

Green, D.R., & Reed,J.C. (1998). Mitochondria and apoptosis. *Science, 281,* 1308–1312.

Griffith, T.S., & Lynch, D.H. (1998). TRAIL: a molecule with multiple receptors and control mechanisms. *Current Opinion in Immunology, 10,* 559–563.

Gross, A., McDonnell, J.M. & Korsmeyer, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes & Development, 13,* 1899–1911.

Gunawan, I.W.G., Gede Bawa, I.G.A., & Sutrisnayanti, N.L. (2008). Isolasi dan identifikasi senyawa terpenoids yang aktif antibakteri pada herba meniran (*Phyllanthus niruri* Linn). *Jurnal Kimia, 2(1),* 31–39.

Hail Jr, N. (2005). Mitochondria: a novel target for the chemoprevention of cancer. *Apoptosis, 10,* 687–705.

Hail Jr, N., Cortes, M., Drake, E.N., & Spallholz, J.E. (2008). Cancer chemoprevention: A radical perspective. *Free Radical Biology & Medicine, 45,* 97–110.

Hansakul, P., Ngamkitidechakul, C., Ingkaninan, K., Sireeratawong, S., & Panunto, W. (2009). Apoptotic induction activity of *Dactyloctenium aegyptium* (L.) P.B. and *Eleusine indica* (L.) Gaerth. Extracts on human lung and cervical cancer cell lines. *Songklanakarin Journal of Science and Technology, 31(3)*, 273–279.

Harada, H., Yamashita, U., Kurihara, H., Fukushi, E., Kawabata, J., & Kamei, Y. (2002). Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. *Anticancer Research, 22,* 2587–2590.

Harbone, J.B. & Williams, C.A. (2000). Advances in flavonoid research since 1992. *Phytochemistry, 55,* 481–504.

Harikumar, K.B., Kuttan., G.K., & Kuttan, R. (2009). *Phyllanthus amarus* inhibit cell growth and induces apoptosis in Dalton's Lymphoma Ascites cells through activation of caspase-3 and downregulation of Bcl-2. *Integrative Cancer Therapies, 8(2)*, 190–194.

Harinder, P., Makkar, S., Siddhuraju, S., Sidduraju, P., & Becker, K. (2007). *Plant secondary metabolites*. (p.5). New Jersey: Humana Press Inc.

Harish, R., & Shivanandappa, T. (2006). Antioxidant activity and hepatoproctective potential of *Phyllanthus niruri*. *Food Chemistry, 93,* 180–185.

Hartmann, M.A. (1998). Plant sterols and the membrane environment. *Trends in Plant Science, 3(5),* 170–175.

Harvey, A.L. (2000). Strategies for discovering drugs from previously unexplored natural products. *Drug Discovery Today, 5(7),* 294–300.

Hasenah, A., & Houghton, P.J. (2006). α-amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacology, 107(3),* 449–455.

Hayes, R.B., Pottern, L.M., Strickler, H., Rabkin, C., Pope, V., & Swanson, G.M. (2000). Sexual behaviour, STDs and risks for prostate cancer. *British Journal of Cancer, 82(3),* 718–725.

Heinrich, M. (2010). Ethnopharmacology and Drug Discovery. In Mander, L. and Liu, H.W (ed), *Comprehensive Natural Products II: Chemistry and Biology vol. 3*. (pp. 351–381). Elsevier Science.

Hengartner, M.O. (2000). The biochemistry of apoptosis. *Nature, 407,* 770–776.

Hennings, H., Glick, A.B., Greenhalgh, D.A., Morgan, D.L., Strickland, J.E., Tennenbaum, T. & Yuspa, S.H. (1993). Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogensis. *Proceedings of the Society for Experimental Biology and Medicine, 202(1),* 1–8.

Herr, I., & Debatin, K.M. (2001). Cellular stress response and apoptosis in cancer therapy. *Blood, 98,* 2603–2614.

Hiddemann, W., Schumann, J., Andreef, M., Barlogie, B., Herman, C.J., Leif, R.C., et al. (1984). Convention on nomenclature for DNA cytometry. *Cancer Genetics and Cytogenetics, 13(2),* 181–183.

Hong, W.K., & Sporn, M.B. (1997). Recent advances in chemoprevention of cancer. *Science, 278,* 1073–1077.

Horinaka, M., Yoshida, T., Shiraishi, T., Nakata, S., Wakada, M., Nakanishi, R., et al. (2005). Luteolin induces apoptosis via death receptor 5 upregulation in human malignant tumor cells. *Oncogene, 24,* 7180–7189.

Hossain, S.J., Tsujiyama, I., Takasugi, M., Islam, M.A., Biswas, R.S., & Aoshimka, H. (2008). Total phenolic content, antioxidative, anti-amylase, anti-glucosidase, and antihistamine release activities of Bangladeshi fruits. *Food Science Technology Research, 14(3),* 261–268.

Houghton, P.J., & Raman, A. (1998). *Laboratory Handbook for the Fractionation of Natural Extracts*. London: Chapman & Hall.

Hu, W., & Kavanagh, J.J. (2003). Anticancer therapy targeting the apoptotic pathway. *The Lancet Oncology, 4,* 721–729.

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

Hu, Y.W., Liu, C.Y., Du, C.M., Zhang, J., Wu, W.Q., & Gu, Z.L. (2009). Induction of apoptosis in human hepatocarcinoma SMMC-7721 cells *in vitro* by flavonoids from *Astragalus complanatus*. *Journal of Ethnopharmacology, 123,* 293–301.

Hua, S.Z., Luo, J.G., Wang, X.B., Wang, J.S., & Kong, L.Y. (2009). Two novel monoterpene-chalcone conjugates isolated from the seeds of *Alpinia katsumadai*. *Bioorganic & Medicinal Chemistry Letters, 19,* 2728–2730.

Huang, S.T., Yang, R.C., & Pang, J.H. (2004). Aqueous extract of *Phyllanthus urinaria* induces apoptosis in human cancer cells. *American Journal of Chinese Medicine, 32(2),* 175–183.

Huang, S.T., Yang, R.C., Lee, P.N., Yang, S.H., Liao, S.K., Chen, T.Y., et al. (2006). Anti-tumor and anti-angiogenic effects of *Phyllanthus urinaria* in mice bearing Lewis lung carcinoma. *International Immunopharmacology, 6,* 870–879.

Huang, S.T., Yang, R.C., Yang, L.J., Lee, P.N., & Pang, J.W.S. (2003). *Phyllanthus urinaria* triggers the apoptosis and Bcl-2 down-regulation in Lewis lung carcinoma cells. *Life Sciences, 72*, 1705–1716.

Husen, R., Azimahtol, H.L.P., & Meenakshi, N. (2004). Screening for antihyperglcaemic activity in several local herbs of Malaysia. *Journal of Ethnopharmacology, 95(2-3),* 205–208.

Igney, F., & Krammer, P. (2002). Death and anti-death: tumour resistance to apoptosis. *Nature Reviews Cancer, 2,* 277–288.

Inayat, S.H. (1999). Caspases-3 and -7 are activated in goniothalamin-induced apoptosis in human Jurkat T-cells. *FEBS Letters, 456(3),* 379–383.

Ioannou, Y., & Chen, F.W. (1996). Quantification of DNA fragmentation in apoptosis. *Nuclei Acids Research, 24(5),* 992–993.

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

Jacobson, M.D., Weil, M., & Raff, M.C. (1997). Programmed cell death in animal development. *Cell, 88,* 347–354.

Jagessar, R.C., Mars, A., & Gomes, G. (2008). Selective antimicrobial properties of *Phyllanthus acidus* leaf extract against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* using stokes disc diffusion, well diffusion, streak plate and a dilution method. *Nature and Science, 6 (2),* 24–38.

Jaiaree, N., Itharat, A., & Kumapava, K. (2010). Cytotoxic saponin against lung cancer cells from *Dioscorea birmanica* Prain & Burkill. *Journal of Medical Association of Thailand, 93(7)*, 192–197.

Jamia, A.J. (2006). Malay traditional medicine: An overview of scientific and technological progress. *Tech Monitor, Nov-Dec 2006,* 37–49

Jansen, O., Akhmedjanova, V., Angenot, L., Balansard, G., Chariot, A., Ollivier, E., et al. (2006). Screening of 14 alkaloids isolated from *Haplophyllum* A. Juss. for their cytotoxic properties. *Journal of Ethnopharmacology, 105,* 241–245.

Jaroudi, S., & Sen Gupta, S. (2007). DNA repair in mammalian embryos. *Mutation Research/Reviews in Mutation Research, 635 (1),* 53–77.

Jeena, K.J., Joy, K.L., & Kuttan, R. (1999). Effect of *Emblica officinalis*, *Phyllanthus amarus* and *Picrorrhiza kurroa* on *N*-nitrosodiethylamine induced hepatocarcinogenesis. *Cancer Letters, 136(1),* 11–16.

Jemal, A., Bray, F., Center, M.M., Ferley, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA Cancer Journal for Clinicians,* doi: 10.3322/caac.20107.

Jemal, A., Siegel, R., Xu, J., & Ward. (2010). Cancer Statistics, 2010. *CA Cancer Journal for Clinicians, 60,* 277–300.

Ji, J.M., Hu, W.J., Yu, L.X., Qian, X.P., & Liu, B.R. (2010). Inhibiting effect of *Phyllanthus urinaria* alcohol extract on human stomach cancer cell MKN28. *Journal of Nanjing University of Traditional Chinese Medicine, 01-008*.

Jimenez, P.C., Wilke, D.V., Takeara, R., Lotufo, T.M.C., Pessoa, C., de Moraes, M.O., et al. (2008). Cytotoxic activity of a dichloromethane extract and fractions obtained from *Eudistoma vannamei* (Tunicata: Ascidiacea). *Comparative Biochemistry and Physiology, Part A. 151,* 391–398.

Jin, U.H., Lee, D.Y., Kim, D.S., Lee, I.S., & Kim, C.H. (2006). Induction of mitochondria-mediated apoptosis by methanol fraction of *Ulmus davidiana* Planch (Ulmaceae) in U87 glioblastoma cells. *Environmental Toxicology and Pharmacology, 22,* 136–141.

Jiwajinda, S., Santisopasri, V., Murakami, A., Kawanaka, M., Kawanaka, H., Gasquet, M., et al. (2002). *In vitro* anti-tumor promoting and anti-parasitic activities of the quassinoids from *Eurycoma longifolia*, a medicinal plant in Southeast Asia. *Journal of Ethnopharmacology, 82(1),* 55–58.

John, J.E. (2010). Natural products as lead-structures: a role for biotechnology. *Drug Discovery Today, 15 (11/12),* 409–410.

Jordan, V.C. (1976). Effects of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *European Journal of Cancer, 12,* 419–424.

Jose, J.K., Kuttan, G., & Kuttan, R. (2001). Antitumour activity of *Emblica officinalis*. *Journal of Ethnopharmacology, 75,* 65–69.

Joshi, C.S., & Priya, E.S. (2007). B-Glucuronidase inhibitory effect of phenolic constituents from *Phyllanthus amarus*. *Pharmaceutical Biology, 45,* 363–365.

Kakuda, R., Iijima, T., & Yaolta, Y. (2002). Triterpenoids from *Gentiana scabra*. *Phytochemistry, 59,* 791–794.

Kamaruddin, M.S., & Latiff, A. (2002). *Malaysian Medicinal Plants*. (p.343). University Kebangsaan Malaysia.

Kamesaki, H. (1998). Mechanisms involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. *International Journal of Hematology, 68,* 29–43.

Karasavvas, N., Erukulla, R.K., Bittman, R., Lockshin, R., & Zakeri, Z. (1996). Stereospecific induction of apoptosis in U937 cells by N-octanoyl-spingosine stereoisomers and N-octyl-spingosine. *European Journal of Biochemistry, 236,* 729–737.

Keijer, J., Bunschoten, a., Palou, A., & Franssen-van Hal, L.W. (2005). Beta-carotene and the application of transcriptomics in risk-benefit evaluation of natural dietary component. *Biochima et Biophysica Acta, 1740,* 139–146.

Kerr, J.F.R., Winterford, C.M., & Harmon, B.V. (1994). Apoptosis: Its significance in cancer and cancer therapy. *Cancer, 73,* 2013–2026.

Kerr, J.F.R., Wylie, A.H., & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implication in tissue kinetics. *British Journal of Cancer, 26,* 239–257.

Kessel, D., & Luo, Y. (2000). Cells in cryptophycin-induced cell-cycle arrest are susceptible to apoptosis. *Cancer Letters, 151,* 25–29.

Khatoon, S., Rai, V., Rawat, A.K.S., & Mehrota, S. (2006). Comparative pharmacognostic studies of three *Phyllanthus* species. *Journal of Ethnopharmacology, 104,* 79–86.

Khazanah Endau Rompin Herba. (2007). (pp.66–68). Utusan Publications and Distributions.

Kiechle, F.L., & Zhang, X. (2002). Apoptosis: biochemical aspects and clinical implications. *Clinica Chimica Acta, 326,* 27–45.

Kiemer, A.K., Hartung, T., Huber, C., & Vollmar, A.M. (2003). *Phyllanthus amarus* has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via NF-κB pathway. *Journal of Hepatology, 38(3),* 289–297.

Kim, J.Y., Park, K.W., Moon, K.D., Lee, M.K., Choi, J., Yee, S.T., et al. (2008). Induction of apoptosis in HT-29 colon cancer cells by crude saponin from Platycodi Radix. *Food and Chemical Toxicology, 46,* 3753–3758.

Kleinsmith, L.J. (2006). Principles of Cell Biology. (pp.22–29). Pearson Benjamin Cummings, CA.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., & Newmeyer, D.D. (1997). The release of cytochrome *c* from mitochondria blocked. *Science, 275,* 1129–1132.

Kochummen, K.M. (1998). *Phyllanthus watsonii.* In: IUCN 2008. 2008 IUCN Red List of Threatened Species. Retrieved 22 February 2009, from http://www.iucnredlist.org.

Kohn, K.W. (1999). Molecular interaction map of the mammalian cell cycle control and DNA repair systems. *Molecular Biology of the Cell, 10,* 2703–2734

Krinsky, I. & Johnson, E.J. (2005). Carotenoid action and their relation to health and disease. *Molecular Aspects of Medicine, 26,* 459–516.

Kroemer, G., & Reed, J.C. (2000). Mitochondrial control of cell death. *Nature Medicine, 6,* 513–519.

Kroll, K.M., Ferrantini, A., & Domany, E. (2010). Introduction to biology and chromosomal instabilities in cancer. *Physica A, 389,* 4374–4388.

Kumar, S. (1999). Mechanisms mediating caspase activation in cell death. *Cell Death and Differentiation, 6,* 1060–1066.

Kumar, S. (2004). Measurement of caspase activity in cells undergoing apoptosis. In Brady, H.J.M (ed), *Methods in Molecular Biology: Apoptosis Methods and Protocols*. (pp.19-20). Humana Press Inc.

Kumaran, A., & Karunakaran, R.J. (2007). *In vitro* antioxidant activities of methanol extracts of five *Phyllanthus* species from India. *LWT, 40,* 344–352.

Kuo, P.L., Hsu, Y.L., Chang, C.H., & Lin, C.C. (2005). The mechanism of elliptine-induced apoptosis and cell cycle arrest in human breast MCF-7 cancer cells. *Cancer Letters, 223,* 293–301.

Kwon, H.J., Hong, Y.K., Kim, K.H., Han, C.H., Cho, S.H., Choi, J.S., et al. (2006). Methanolic extracts of *Pterocarpus santalinus* induces apoptosis in HeLa cells. *Journal of Ethnopharmacology, 105,* 229–234.

Lai, C.S., Rosemal, H.M.H.M., Nair, N.K., Majid, M.I.A., Mansor, S.M., & Navaratnam, V. (2008). *Typhonium flagelliforme* inhibits cancer cell growth *in vitro* and induces apoptosis: An evaluation by the bioactivity guided approach. *Journal of Ethnopharmacology, 118,* 14–20.

Lam, K.S. (2007). New aspects of natural products in drug discovery. *Trends in Microbiology, 15(6),* 279–289.

Le Marchand, L. (2002). Cancer preventive effects of flavonoids-a review. *Biomedicine and Pharmacotherapy*. 56: 296–301.

Le, N.T.V., & Richardson, D.R. (2002). The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochimica et Biophysica Acta, 1603,* 31–46.

Ledgerwood, E.C., Pober, J.S., & Bradley, J.R. (1999). Recent advances in the molecular basis of TNF signal transduction. *Laboratory Investigation, 79,* 1041–1050.

Lee, C.C., & Houghton, P. (2005). Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. *Journal of Ethnopharmacology, 100(3),* 237–243.

Lee, C.Y., Chiu, T.H., & Tsai, S.W. (2005). Quantitative HPLC methods for gallic acids of *Phyllanthus* (Euphorbiaceae). *Journal of Liquid Chromatography & Related Technologies, 28,* 2965–2977.

Lee, Y.M., Wu, T.H., Chen, S.F., & Chung, J.G. (2003). Effect of 5-methoxypsoralen (5-MOP) on cell apoptosis and cell cycle in human hepatocellular carcinoma cell line. *Toxicology In Vitro, 17,* 279–287.

Leete, E. (1969). Alkaloid biosynthesis. *Advances in Enzymology & Related Areas in Molecular Biology, 32,* 373–422.

Leeya, Y., Mulvany, M.J., Queiroz, E.F., Marston, A., Hostettmann, & Jansakul, C. (2010). Hypotensive activity of an n-butanol extract and their purified compounds from leaves of *Phyllanthus acidus* (L.) Skeels in rats. *European Journal of Pharmacology, 649 (1-3),* 301–313.

Leite, D.F.P., Kassuya, C.A.L., Mazzuco, T.L., Silvestre, A., de Melo, L.V., Rehder, V.L.G., et al. (2006). The cytotoxic effect and the multidrug resistance reversing action of lignans from *Phyllanthus amarus*. *Planta Medica, 72(15),* 1353–1358.

Lentini, A., Tabolacci, C., Provenzano, B., Rossi, S., & Beninati, S. (2010). Phytochemicals and protein–polyamine conjugates by transglutaminase as chemopreventive and chemotherapeutic tools in cancer. *Plant Physiology and Biochemistry, 48(7),* 627–633.

Leung, H.W., Lin, C.J., Hour, M.J., Yang, W.H., Wang, M.Y., & Lee, H.Z. (2007). Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes. *Food and Chemical Toxicology, 45,* 2005–2013.

Li, J.H.W., & Vederas, J.G. (2009). Drug discovery and natural products: end of an era or and endless frontier? *Science, 325,* 161–165.

Li, P., Chai, H., & Kinghorn, A.D. (2010). The continuing search for antitumor agents from higher plants. *Phytochemistry Letters, 3,* 1–8.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., et al. (1997). Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell, 91,* 479–489.

Lim do, Y., Jeong, Y., Tyner, A.I., & Park, J.H. (2007). Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin. *American Journal of Physiology, Gastrointestinal and Liver Physiology, 292,* G66–G75.

Lim, Y.Y., & Murtijaya, J. (2007). Antioxidant properties of *Phyllanthus amarus* extracts as affected by different drying methods. *LWT, 40,* 1664–1667.

Lippman, S.M., Kessler, J.F., & Meyskens, F.L. (1987). Retinoids as preventive and therapeutic anticancer agents (part1). *Cancer Treatment Reports, 71,* 391–405.

Liu, C.H., Huang, L.J., Kuo, S.C., Lee, T.H., Tsai, K.J., & Chan, H.C. (2009). 4-Fluoro-*N*-butylphenylacetamide (H6) inhibits cell growth via cell-cycle arrest and apoptosis in human cervical cancer cells. *Bioorganic & Medicinal Chemistry, 17,* 42–48.

Liu, J., Lin, H., & McIntosh, H. (2001). Genus *Phyllanthus* for chronic hepatitis B virus infection: a systemic review. *Journal of Viral Hepatitis, 8,* 358–366.

Liu, Jing., Li, Yan., Ren, W., & Hu, W.X. (2006). Apoptosis of HL-60 cells induced by extracts from *Narcissus tazetta* var. chinensis. *Cancer Letters, 242,* 133–140.

Liu, L.F., Desai, S.D., Li, T.K., Mao, Y., Sun, M., & Sim, S.P. (2000). Mechanism of action of camphotechin. *Annals of the New York Academy of Sciences, 922,* 1–10.

Liu, R.H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *Journal of Nutrition, 134,* 3479S–3485S.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell, 86,* 147–157.

Liu, X., Zhao, M., Wang, J., Yang, B., & Jiang, Y. (2008). Antioxidant activity of methanolic extract of emblica fruit (*Phyllanthus emblica* L.) from six regions in China. *Journal of Food Composition and Analysis, 21,* 219–228.

Loike, J.D., & Horwitz, S.B. (1976). Effects of podophyllotoxin and VP-16-213 0n microtubule assembly *in vitro* and nucleoside transport in HeLa cells. *Biochemistry, 15(25)*, 5435–5443.

Losso, J.N., Bansode, R.R., Trappey, A., Bawadi, H.A., & Truax, R. (2004). In vitro anti-proliferative activities of ellagic acid. *Journal of Nutritional Biochemistry, 15,* 672–678.

Loza-Tavera, H. (1999). Monoterpenes in essential oils: biosynthesis and properties. *Advances in Experimental Medicine and Biology, 464,* 49–62.

Luo,W., Zhao, M., Yang, B., Shen, G. & Rao, G. (2009). Identification of bioactive compounds in *Phyllanthus emblica* L. fruit and their free radical scavenging activities. *Food Chemistry, 114 (2),* 499–504.

Mabberley, D.J. (2008). Mabberley's Plant Book- A Portable Dictionary of Plants, Their Classification and Uses. (pp.659-660). Cambridge University Press.

Mackeen, M.M., Ali, A. M., Lajis, N.H., Kawazu, K., Hassan, Z., Amran, M., et al. (2000). Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. ex T. Anders. *Journal of Ethnopharmacology, 72(3),* 395–402.

Mackeen, M.M., Ali, A.M., Abdullah, M.A., Nasir, R.M., Mat, N.B., Razak, A.R., et al. (1997). Antinematodal activity of some Malaysian plant extracts against the pine wood nematode, *Bursaphelenchus xylophilus*. *Pesticide Science, 51(2),* 165–170.

Mahavorasirikul, W., Viyanant, V., Chaijoroenkul, W., Itharat, A., & Na-Bangchang, K. (2010). Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*. *BMC Complementary & Alternative Medicine, 10(55),* 1–8.

Maia, L.F., de Epifanio, R.A., & Fenical, W. (2000). New cytotoxic sterol glycosides from the Octocoal *Carijoa* (*Telesto*) *riisei*. *Journal of Natural Products, 63(10),* 1427–1430.

Manez, S., Recio, M.C., Giner, R.M., & Luis Rios, J. (1997). Effect of selected triterpenoids on chronic dermal inflammation. *European Journal of Pharmacology, 334,* 103–105.

Mangeney, R., Andriamialisoa, Z. Langlois, N., Langlois, Y., & Potier, P. (1979). Preparation of vinblastine, vincristine, and leurosidine, antitumor alkaloids from *Catharanthus* species (Apocynaceae). *Journal of the American Chemical Society, 101(8)*, 2243–2245.

Mann, J. (2002). Natural products in cancer chemotherapy: past, present and future. *Nature Reviews Cancer, 2,* 143–148.

Mans, D.R.A., Rocha, A.B., & Schwartsmann, G. (2000). Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. *Oncologist, 5,* 185–199.

Mansoor, T.A., Ramalho, R.M., Mulhovo, S., Rodrigues, C.M.P., & Ferreira, M.J.U. (2009). Induction of apoptosis in HuH-7 cancer cells by monoterpene and β-carboline indole alkaloids isolated from the leaves of *Tabernaemontana elegans*. *Bioorganic & Medicinal Chemistry Letters, 19,* 4255–4258.

Manthey, J.A., & Guthrie, N. (2002). Antiproliferative activities of citrus flavanoids against six human cancer cell lines. *Journal of Agricultural and Food Chemistry, 50,* 5837–5843.

Marston, A. (2007). Role of advances in chromatographic techniques in phytochemistry. *Phytochemistry, 68,* 2785–2797.

Martin, S.J., & Green, D.R. (1995). Apoptosis and cancer: the failure of controls on cell death and cell survival. *Critical Reviews in Oncology & Hematology, 18,* 137–153.

Martin-Smith, M., & Sneader, W.E. (1969). Biological activity of the terpenoids and their derivatives-recent advances. *Progress in Drug Research, 13,* 11–100.

Matassov, D., Kagan, T., Leblanc, J., Sikorska, M., & Zakeri, Z. (2004). Measurement of Apoptosis by DNA Fragmentation . In Brady, H.J.M. (ed), *Methods in Molecular Biology: Apoptosis Methods and Protocols.* (pp. 1-17). Humana Press Inc.

Matsunaga, S., Tanaka, R., Takaoka, Y., In, Y., Ishida, T., Rahmani, M., et al (1993). 26-Nor-D:A-friedoolean Triterpenes from *Phyllanthus watsonii. Phytochemistry, 32(1),* 165–170.

Mazumder, A., Mahato, A., & Mazumder, R. (2006). Antimicrobial potentiality of *Phyllanthus amarus* against drug resistant pathogens. *Natural Product Research, 20(4),* 323–326.

McChesney, J.D., Venkataraman, S.K., & Henri, J.T. (2007). Plant natural products: Back to the future or into extinction? *Phytochemistry, 68,* 2015–2022.

Mehmet, H. (2002). Apoptosis: caspase find a new place to hide. *Nature, 403,* 29–30.

Mello, J.F. (1980). Plants in traditional medicine in Brazil. *Journal of Ethnopharmacology, 2(1),* 49–55.

Mena-Rejon, G., Caamal-Fuentes, E., Cantillo-Ciau, Z., Cedillo-Rivera, R., Flore-Guido, J., & Moo-Puc, R. (2009). *In vitro* cytotoxic activity of nine plants used in Mayan traditional medicine. *Journal of Ethnopharmacology, 121,* 462–465.

Middleton, E., Kandaswami, C., & Theoharides, T.C. (2000). The effectes of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Review, 52,* 673–751.

Militão, G.C.G., Damtas, I.N.F., Pessoa, C., Falcão, M.J.C., Silveira, E.R., Lima, M.A.S., et al. (2006). Induction of apoptosis by pterocarpans from *Platymiscium floribundum* in HL-60 human leukemia cells. *Life Sciences, 78,* 2409–2417.

Millan, A., & Huerta, M.D. (2009). Apoptosis-inducing factor and colon cancer. *Journal of Surgical Research, 151,* 163–170.

Miller,L.J., & Marx,J. (1998). Apoptosis. *Science, 281,* 1301.

Mills, J.J., Chari, R.S., Boyer, I.J., Gould, M.N., & Jirtle, R.L. (1995). Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. *Cancer Research, 55,* 979–983.

Mimeault, M., & Batra, S.K. (2010). New promising drug targets in cancer-and metastasis-initiating cells. *Drug Discovery Today, 15(9/10),* 354–362.

Miodini, P., Fioravantio, L., Di Fronzo, G., & Cappelletti, V. (1999). The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *British Journal of Cancer, 80,* 1150–1155.

Moghadasian, M.H. (2000). Pharmacological properties of plant sterols: in vivo and in vitro observations. *Life Science, 67,* 605–615.

Mohd Fazley, A.B., Maryati, M., Asmah, R., Burr, S.A., & Fry, J.R. (2010). Cytotoxicity, cell cycle arrest, and apoptosis in breast cancer cell lines exposed to an extract of the seed kernel of *Mangifera pajang* (bambangan). *Food and Chemical Toxicology, 48,* 1688–1697.

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N., & Neungton, N. (2004). Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostona* (mangosteen) on SKBR3 human breast cancer cell line. *Journal of Ethnopharmacology, 90,* 161–166.

Mori, A., Lehmann, S., O'Kelly, J., Kumagai, T., Desmond, J.C., Pervan, M., et al. (2006). Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Research, 66,* 3222–3229.

Muhamad, Z., & Mustafa, A.M., (1998). *Traditional Malay Medicinal Plants*. (pp.3–5). Kuala Lumpur: Fajar Bakti Publication.

Müllauer, L., Gruber, P., Sebinger, D., Buch, J., Wohlfart, S., & Chott, A. (2001). Mutations in apoptosis genes: a pathogenetic factor for human disease. *Mutation Research, 488,* 211–231.

Murakami, A., Ali, A.A., Mat-Salleh, K., Koshimizu, K., & Ohigashi, H. (2000). Screening for the *In Vitro* Anti-tumor-promoting Activities of Edible Plants from Malaysia. *Bioscience, Biotechnology, and Biochemistry, 64(1)*, 9–16.

Murakoshi, M., Nishino, H., Satomi, Y., Takayusu, J., Hasegawa, T., Tokuda, H., et al. (1992). Potent preventive action of α-carotene against carcinogenesis: Spontaneous liver carcinogenesis and promoting stage of lung and skin carcinogenesis in mice are suppressed more effectively by α-carotene than by β-carotene. *Cancer Research, 52,* 6583–6587.

Murgia, M., Pizzo, P., Sandona', D., Zanovello, P., Rizzuto, R., & Di Virgilio, F. (1992). Mitochondrial DNA is not fragmented during apoptosis. *Journal of Biological Chemistry, 267,* 10939–10941.

Murugaiyah, V., & Chan, K.L. (2006). Antihyperuricemic lignans from the leaves of *Phyllanthus niruri*. *Planta Medica, 72,* 1262–1267.

Nagamine, M.K., da Silva, T.C., Matsuzaki, P., Pinello, K.C., Cogliati, B., Pizzo, C.R., et al. (2009). Cytotoxic activity of butanolic extract from *Pfaffia paniculata* (Brazilian Ginseng) on cultured human breast cancer cell line MCF-7. *Experimental and Toxicologic Pathology, 61*, 75–82.

Nagata, S. (1999). Fas ligand-induced apoptosis. *Annual Reviews of Genetics, 33,* 29–55.

Narayanan, B.A., Geoffroy, O., Willingham, M.C., Re, G.G., & Nixon, D.W. (1999). p53/p21 (WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. *Cancer Letters, 136,* 215–221.

Newman, D.J., & Cragg, G.M. (2007). Natural products as sources of new drug over the last 25 years. *Journal of Natural Products, 70,* 461–477.

Newman, D.J., Cragg, G.M., & Snader, K.M. (2000). The influence of natural products upon drug discovery. *Natural Products Reports, 17(3),* 215–234.

Newman, D.J., Cragg, G.M., & Snader, K.M. (2003). Natural products as sources of new drug over the period 1981-2002. *Journal of Natural Products, 66(7),* 1022–1037.

Ng, F.S.P. (2000). The identity of the Melaka. *Malaysian Naturalist, 53(3),* 32–35.

Nhan, T.Q., Liles,W.C., & Schwartz, S.M. (2006). Physiological Functions of Caspases Beyond Cell Death. *American Journal of Pathology, 169,* 729

Nicholson, D.W. (1999). Caspase structure, proteolytic substrates, and functioning during apoptotic cell death. *Cell Death and Differentation, 6,* 1028–1042.

Nicholson, D.W., & Thornberry, N.A. (1997). Caspases: killer proteases. *Trends in Biochemical Sciences, 22,* 299–306.

Nik Najib, N.A.R., Furuta, T., Kojima, S., Takane, K., & Mustafa, A.M. (1999). Antimalarial activity of extracts of Malaysian medicinal plants. *Journal of Ethnopharmacology, 64(3),* 249–254.

Nishino, H., Muakoshi, M., Tokuda, H., & Satomi, Y. (2008). Cancer prevention by carotenoids. *Archives of Biochemistry and Biophysics.*

Nishiura, J.L., Campos, A.H., Boim, M.A., Heilberg, I.P., & Schor, N. (2004). *Phyllanthus niruri* normalizes elevated urinary calcium levels in calcium stone forming (CSF) patients. *Urology Research, 32(5),* 362–366.

Noble, R.L. (1990). The discovery of the vinca alkaloids-chemotherapeutic agents against cancer. *Biochemistry and Cell Biology, 68,* 1344–1351.

Norat, T., Lukanova, A., Ferrari, P., & Riboli, E. (2002). Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. *International Journal of Cancer, 98,* 241–256.

Nunez, R. (2001). DNA measurement and cell cycle analysis by flow cytometry. *Current Issues in Molecular Biology, 3(3),* 67–70.

Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., et al. (1993). Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *The EMBO Journal, 12,* 3679–3684.

Ong, C.Y., Ling, S.K., Rasadah, M.A., Chee, C.F., Zainon, A.S., Ho, A.S.H., et al. (2009). Systematic analysis of in vitro photo-cytotoxic activity in extracts from terrestrial plants in Peninsula Malaysia for photodynamic therapy. *Journal of Photochemistryt and Photobiology B*: *Biology, 96,* 216–222.

Ong, H.C., & Norzalina, J. (1999). Malay herbal medicine in Gemencheh, Negri Sembilan, Malaysia. *Fitoterapia, 70,* 10–14.

Oparaocha, E.T., & Okorie, C. (2009). In vivo evaluation of anti-malarial activity of three medicinal plants used in South Eastern Nigeria. *Journal of Herbs, Spices & Medicinal Plants, 15,* 121–128.

Ormerod, M.G. (2002). Investigating the relationship between the cell cycle and apoptosis using flow cytometry. *Journal of Immunological Methods, 265,* 73–80.

Padma, P., & Setty, O.H. (1999). Protective effect of *Phyllanthus fraternus* against carbon tetrachloride-induced mitochondrial dysfunction. *Life Sciences, 64,* 2411–2417.

Pailard, F., Finot, F., Mouche, I., Prenez, A., & Vericat, J.A. (1999). Use of primary cultures of rat hepatocytes to predict toxicity in the early development of new entities. *Toxicology in Vitro, 1,* 693–700.

Pan, L., Chai, H., & Kinghorn, A.D. (2010). The continuing search for antitumor agents from higher plants. *Phytochemistry Letters, 3,* 1–8.

Panchal, R.G. (1998). Novel therapeutics strategies to selectively kill cancer cells. *Biochemical Pharmacology, 55,* 247–252.

Pardee, A.B. (1974). A restriction point for control of normal animal cell proliferation. *Proceedings of the National Academy of Science U.S.A., 71,* 1286–1290.

Park, E.J., Min, H.Y., Chung, H.J., Hong, J.Y., Kang, Y.J., Hung, T.M., et al. (2009). Down-regulation of c-Src/EGFR-mediated signaling activation is involved in the honokiol-induced cell cycle arrest and apoptosis in MDA-MB-231 human breast cancer cells. *Cancer Letters, 277,* 133–140.

Park, H.J., Kim, M.J., Ha, E., & Chung, J.H. (2008). Apoptotic effect of hesperidin through caspase-3 activation in human colon cancer cells, SNU-C4. *Phytomedicine, 15,* 147–151.

Parkin, D.M. (2001). Global statistics in the year 2000. *Lancet Oncology, 2,* 533–543.

Parkin, D.M., Bray, F., Ferlay, J. & Pisani, P. (2005). Global cancer statistics 2002. *A Cancer Journal for Clinicans, 55,* 74–108.

Patwardhan, B., Vaidya, A.D.B. & Chorghade, M. (2004). Ayurveda and natural products drug discovery. *Current Science, 86(6),* 789–799.

Peng, B., Chang, Q., Wang, L., Hu, Q., Wang, Y., Tang, J., et al. (2008). Suppression of human ovarian SKOV-3 cancer cell growth by *Duchesnea* phenolic fraction is associated with cell cycle arrest and apoptosis. *Gynecologic Oncology, 108,* 173–181.

Perbadanan Bioteknologi dan Biodiversiti Negeri Johor. (2009). Johor Biodiversiti Database. Retrieved 27 February 2010, from http://iwana.com.my/jbiotech/index.php.

Perchellet, J.P., Gali, H.U., Perchellet, E.M., Klish, D.S., & Armbrust, A.D. (1992). Antitumor promoting activities of tannin acid, ellagic acid, and several gallic acid derivatives in mouse skin. *Basic Life Science, 59,* 73–801.

Perz, J.F., Armstrong, G.L., Farrington, L.A., Hutin, Y.J.F., & Bell, B.P. (2006). The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *Journal of Hepatology, 45(4),* 529–538.

Pichersky, E., & Gang, D.R. (2000). Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. *Trends in Plant Science, 5(10),* 439–445.

Ping, C.K., Li, S.S., Damu, A.G., Chung, R.S., Chieh, H.H., Chih, H.K., et al. (2003). Cytotoxic and antimalarial β-Carboline alkaloids from the roots of *Eurycoma longifolia*. *Journal of Natural Products, 66(10)*, 1324–1327.

Pinkoski, M.J., & Green, D.R. (1999). Fas ligand, death gene. *Cell Death and Differentiation, 6,* 1174–1181.

Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., & Hess, R.D. (1996). Flavonoids activate wild-type p53. *Oncogene, 13,* 1605–1614.

Polunin, I. (1988). *Plants and flowers of Malaysia*. (pp.25–148). Times edition.

Popov, B., Chang, L.S., & Serikov, V. (2005). Cell cycle-related transformation of the E2F4-p130 repressor complex. *Biochemical and Biophysical, Research Communication, 336,* 762–769.

Potmesil, M. (1994). Camptothecins: From bench research to hospital wards. *Perspectives in Cancer Research, 54*, 1431–1439.

Powell, C.B., Fung, P., Jackson, J., Dall' Era, J., Lewkowicz, D., Cohen, I., et al. (2003). Aqueous extract of herb *Scutellaria barbatae*, a chinese herb used for ovarian cancer, induces apoptosis of ovarian cancer cell lines. *Gynecologic Oncology, 91,* 332–340.

Prakrashi, A., Pandit, S., Bandyopadhyay, S.K., & Prakrashi, S.C. (2003). Antioxidant effect of *Phyllanthus emblica* fruit on healing of indomethacin induced gastric ulcer in rats. *Indian Journal of Clinical Biochemistry, 18(1),* 15–21.

Pramyothin,P., Samosorn, P., Poungshompoo, S., & Chaichantipyuth, C. (2006). The protective effects of *Phyllanthus emblica* Linn. extract on ethanol induced rat hepatic injury. *Journal of Ethnopharmacology, 107(3),* 361–364.

Prasanna, R., Harish, C.C., Pichai, R., Sakthisekaran, D., & Gunasekaran, P. (2009). Anti-cancer effect of *Cassia auriculata* leaf extract *in vitro* through cell cycle arrest and induction of apoptosis in human breast and larynx cancer cell lines. *Cell Biology International, 33,* 127–134.

Purohit, A., Hejaz, H.A., Walden, L., MacCarthy-Morrogh, L., Packam, G., Potter, B.V., et al. (2000). The effect of 2-methoxyoestrone-3-O-sulphamate on the growth of breast cancer cells and induced mammary tumours. *International Journal of Cancer, 85,* 584–589.

Qi, F., Li, A., Zhao, L., Xu, H., Inagaki, Y., Wang, D., et al. (2010). Cinobufacini, an aqueous extract from *Bufo bufo gargarizans* Cantor, induces apoptosis through a mitochondria-mediated pathway in human hepatocellular carcinoma cells. *Journal of Ethnopharmacology, 128,* 654–661.

Qiang, Z., Xin, H.Z., & Zhu, J.W. (2009). Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G_2/M arrest and apoptosis. *Toxicology In Vitro, 23,* 797–807.

Rabi, T., & Bishayee, A. (2008). Terpenoids and breast cancer chemoprevention. *Breast Cancer Research and Treatment, 115(2),* 223–239.

Radhika, N.K., Sreejith, P.S., & Asha, V.V. (2010). Cytotoxic and apoptotic activity of *Cheilanthes farinose* (Forsk.) Kaulf. against human hepatoma, Hep3B cells. *Journal of Ethnopharmacology, 128,* 166–171.

Rajeshkumar, N.V., Joy, K.L., Girija, K., Ramsewak, R.S., Nair, M.G., & Ramadasan, K. (2002). Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. *Journal of Ethnopharmacology, 81,* 17–22.

Rajkapoor, B., Sankari, M., Sumithra, M., Anbu, J., Harikirishnan, N., Gobinath, et al. (2007). Antitumor and cytotoxic effects of *Phyllanthus polyphyllus* on Ehrlich ascites carcinoma and human cancer cell lines. *Bioscience, Biotechnology and Biochemistry, 71(9),* 2177–2183.

Ramesh, N., Viswanathan, M.B., Tamil Selvi, V., & Lakshmanaperumalsamy, P. (2004). Antimicrobial and phytochemical studies on the leaves of *Phyllanthus Singampattiana* (Sebastine & A.N. Henry) Kumari & Chandrabose from India. *Medicinal Chemistry Research, 13 (6/7),* 348–360.

Rangarajan, A., & Weinberg, R.A. (2003). Opinion: comparative biology of mouse versus human cells: modeling human cancer in mice. *Nature Reviews of Cancer, 3,* 952–959.

Reddy, B.S., Wang, C.X., Samaha, H., Lubet, R., Steele, V.E., Kelloff, G.J., et al. (1997). Chemoprevention of colon carcinogenesis by dietary perillyl alcohol. *Cancer Research, 57,* 420–425.

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

Reed, J.C (2003). Apoptosis-targeted therapies for cancer. *Cancer Cell, 3,* 17.

Reed, J.C. (1998). bcl-2 family proteins. *Oncogene, 17,* 3225–3236.

Ren Z., Elson, C.E., & Gould, M.N. (1997). Inhibition of type I and type II Geranylgeranyl protein transferase by the monoterpene perillyl alcohol in NIH3T3 cells. *Biochemical Pharmacology, 54,* 113–120.

Ricciardelli, C., & Oehler, M.K. (2009). Diverse molecular pathways in ovarian cancer and their clinical significance. *Maturitas, 62(3),* 270–275.

Ronot, X., Hecquet, C., Larno, S., Hainque, B., & Adolphe, M. (1986). G_2 Arrest, binucleation, and single parameter DNA flow cytometric analysis. *Cytometry, 7(3),* 286–290.

Rowinsky, E.K., Onetto, N., Canetta, R.M., & Arbuck, S.G. (1992). Taxol: the first of the taxanes, an important new class of antitumour agents. *Seminars in Oncology, 19(6)*, 646-662.

Ruffa, M.J., Ferraro, G., Wagner, M.L., Calcagno, M.L., Campos, R.H., & Cavallaro, L. (2002). Cytotoxic effect of Argentine medicinal plant extracts on human hepatocellular carcinoma cell line. *Journal of Ethnopharmacology, 79,* 335–339.

Rukayah, A. (2008). *Tanaman berkhasiat ubatan*. (pp.27–109). Kuala Lumpur: Dewan Bahasa dan Pustaka.

Saha, K., Lajis, N.H., Israf, D.A., Hamzah, A.S., Khozirah, S., Khamis, S., et al. (2004). Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *Journal of Ethnopharmacology, 92 (2–3),* 263–267.

Saleem, M., Nazir, M., Akhtar, N., Onocha, P.A., Riaz, N., Jabbar, A., et al. (2009). New phthalates from *Phyllanthus muellerianus* (Euphorbiaceae). *Journal of Asian Natural Products Research, 11(11),* 974–977.

Samuel, R., Kathriarachchi, H., Hoffmann, P., Barfuss, M.H.J., Wurdack, K.J., Davis, C.C., et al. (2005). Molecular phylogenetics of Phyllanthaceae: Evidence from plastid MATK and nuclear PHYC sequences. *American Journal of Botany, 92(1),* 132–141.

Samuel, T., Weber, H.O., & Funk, J.O. (2002). Linking DNA damage to cell cycle checkpoints. *Cell Cycle, 1,* 162–168.

Samy, J., Sugumaran, M., & Lee, K.L.W. (2005). *Herbs of Malaysia: An introduction to the medicinal, culinary, aromatic and cosmetic use of herbs*. (p.11). Times Edition.

Sanchez-Lamar, A., Fiore, M., Cundari, E., Rocordy, R., Cozzi, R., & De Salvia, R. (1999). *Phyllanthus orbicularis* aqueous extract: Cytotoxic, genotoxic, and antimutagenic effects in the CHO cell line. *Toxicology and Applied Pharmacology, 161,* 231–239.

Santafe, G., Paz, V., Rodriguez, J., & Jimenez, C. (2002). New cytotoxic oxygenated C29 sterols from the Colombian marine sponge *Polymastia tenax*. *Journal of Natural Products, 65(8),* 1161–1164.

Sarkar, F.H., & Li, Y. (2009). Harnessing the fruits of nature for the development of multi-targeted cancer therapeutics. *Cancer Treatment Reviews, 35,* 597–607.

Schendel, S.L., Montal, M., & Reed, J.C. (1998). bcl-2 family proteins as ion-channels. *Cell Death and Differentiation, 5,* 372–380.

Schiffman, M.H. (1992). Recent progress in defining the epidemiology of human papillomavirus infection and cervical neoplasia. *Journal of the National Cancer Institute, 84,* 394–398.

Schmidt, M.L., Kuzmanoff, K.L., Ling-Indeck, L., & Pezzuto, J.M. (1997). Betulinic acid induces apoptosis in human neuroblastoma cell lines. *European Journal of Cancer, 33,* 2007–2010.

Schwartz, G.K., & Shah, M.A. (2005). Targeting the cell cycle: a new approach to cancer therapy. *Journal of Clinical Oncology, 23,* 9408–9421.

Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H., & van der Meer, R. (1999). Read meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Research, 59,* 5704–5709.

Sgonc, R., & Wick, G. (1994). Methods for the detection of apoptosis. *International Archives of Allergy and Immunology, 105(4),* 327–332.

Shakil, N.A., Pankaj, Kumar, J., Pandey, R.K., & Saxena, D.B. (2008). Nematicidal prenylated flavones from *Phyllanthus niruri*. *Phytochemistry, 69,* 759–764.

Sharma, M., Agrawal, S.K., Sharma, P.R., Chadha, B.S., Khosla, M.K., & Saxena, A.K. (2010). Cytotoxic and apoptotic activity of essential oil from *Ocimum viride* towards COLO 205 cells. *Food and Chemical Toxicology, 48,* 336–344.

Sheridan, J.W., Bishop, C.J., & Simmons, R.J. (1981). Biophysical and morphological correlates of kinetic change and death in a starved human melanoma cell line. *Journal of Cell Science, 49,* 119–137.

Sherr, C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research, 60,* 3689–3695.

Sheu, J.H., Chang, K.C., & Duh, C.Y. (2000). A cytotoxic 5a,8a-Epidioxysterol from soft coral *Sinularia* species. *Journal of Natural Products, 63(1),* 149–151.

Sheu, J.H., Wang, G.H., Sung, P.J., Chiu, Y.H., & Duh, C.Y. (1997). Cytotoxic sterols from the Formosan brown alga *Turbinaria ornate*. *Planta Medica, 63(6),* 571–572.

Shih, S.C., & Stutman, O. (1996). Cell cycle-dependent tumor necrosis factor apoptosis. *Cancer Research, 56,* 1591–1598.

Sholikhah, E.N., Mustofa & Wahyuono, S. (2004). Antiplasmodial and cytotoxic activities of extracts of meniran (*Phyllanthus niruri*) traditionally used to treat malaria in Indonesia. *Clinical and Experimental Pharmacology and Physiology, 31(1),* A168–A169.

Silva, M.J.D. (2006). A new species of *Phyllanthus* (Phyllanthaceae) from North Eastern Brazil. *Novon, 16,* 421–423.

Singal, P.W., & Iliskovic, N. (1998). Doxorubicin-Induced Cardiomyopathy. *The New England Journal of Medicine, 339,* 900–905.

Singh, B., Agarwal, P.K., & Thakur, R.S. (1989). An acyclic triterpene from *Phyllanthus niruri*. *Phytochemistry, 28(7),* 1980–1981.

Slee, E.A., & Adrian, C. (1999). Serial killers: ordering caspase activation events in apoptosis. *Cell Death and Differentiation, 6,* 1067–1074.

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., & Newmeyer, D.D. (1999). Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8 and -10 in a caspase-9 dependent manner. *The Journal of Cell Biology, 144,* 281–292.

So, F.V., Guthrie, N., Chambers, A.F., Moussa, M., & Carroll, K.K. (1996). Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis of flavonoids and citrus juices. *Nutrition and Cancer, 26,* 167–81.

Son, Y.O., Kim, J., Lim, J.C., Chung, G.H., & Lee, J.C. (2003). Ripe fruits of *Solanum nigrum* L. inhibits cell growth and induces apoptosis in MCF-7 cells. *Food and Chemical Toxicology, 41,* 1421–1428.

Sousa, M., Ousingsawat, J., Seitz, R., Puntheeranurak, S., Regalado, A., Schmidt, A., et al. (2007). An extract from medicinal plant *Phyllanthus acidus* and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis. *Molecular Pharmacology, 71(1),* 366–376.

Sri Nurestri, A.M., Norhanom, A.W., Hashim, Y., Sim, K.S., Hong, S.K., Lee, G.S., et al. (2008). Cytotoxic activity of *Pereskia bleo* (Cactaceae) against selected human cell lines. *International Journal of Cancer Research, 4(1)*, 20–27.

Sri Nurestri, A.M., Sim, K.S.,Norhanom, A.W., & Hashim, Y. (2009). Cytotoxic components of *Pereskia bleo* (Kunth) DC. (Cactaceae) Leaves. *Molecules, 14*, 1713–1724.

Staker, B.L., Hjerrild, K., Feese, M.D., Behnke, C.A., Burgin Jr, A.B., & Stewart, L. (2002). The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proceedings of the National Academy of Sciences U.S.A., 99,* 5387–5392.

Stark, M.J., Burke, Y.D., McKinzie, J.H., Ayoubi, A.S., & Crowell, P.L. (1995). Chemotherapy of pancreatic cancer with the monoterpene perillyl alcohol. *Cancer Letters, 96,* 15–21.

Stoner, G.D., & Morse, A.M. (1997). Isothiocyanates and plant polyphenols as inhibitors of lung and esophageal cancer. *Cancer Letters, 114,* 113–119.

Strahm, B., & Capra, M. (2005). Insights into the molecular basis of cancer development. *Current Pediatrics, 15(4),* 333–338.

Su, N.W., Lin, Y.L., Lee, M.H., & Ho, C.Y. (2005). Ankaflavin from *Monascus*-fermented red rice exhibits selectivite cytotoxic effect and induces cell death on HepG2 cells. *Journal of Agricultural and Food Chemistry, 53(6)*, 1949–1954.

Sun, J., & Liu, R.H. (2006). Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Letters, 241,* 124–134.

Sun, S.Y., Hail Jr, N., & Lotan, R. (2004). Apoptosis as a novel target for cancer chemprevention. *Journal of National Cancer Institute, 96,* 662–672.

Sung, J. (2007). Colorectal cancer screening: its time for action in Asia. *Cancer Detection and Prevention, 31,* 1–2.

Suntornsuk, L. (2002). Capillary electrophoresis of phytochemical substances. *Journal of Pharmaceutical and Biomedical Analysis, 27,* 679–698.

Suresh, K., & Vasudevan, D.M. (1994). Augmentation of murine natural killer cell and antibody dependent cellular cytotoxicity activities by *Phyllanthus emblica*, a new immunomodulator. *Journal of Ethnopharmacology, 44(1),* 55–60.

Sutthivaiyakit, S., Nakorn, N.N., Kraus, W., & Sutthivaiyaki, P. (2003). A novel 29-*nor*-3,4-*seco*-friedelane triterpene and a new guaiane sesquiterpene from the roots of *Phyllanthus oxyphyllus*. *Tetrahedron, 59,* 9991–9995.

Syamsundar, K.V., Singh, B., Thakur, R.S., Hussain, A., Kiso, Y., & Hikino, H. (1985). Antihepatotoxic principles of *Phyllanthus niruri* herb. *Journal of Ethnopharmacology, 14,* 41–44.

Szakacs G & Gottesman, M.M. (2004). Comparing solid tumor with cell lines: Implications for identifying drug resistance genes in cancers. *Molecular Interventions, 4(6)*, 323–325.

Takagaki, N., Sowa, Y., Oki, T., Nakanishi, R., Yogosawa, S., & Sakai, T. (2005). Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. *International Journal of Oncology, 26,* 185–189.

Tallman, M.S., & Wiemik, P.H. (1992). Retinoids in cancer treatment. *Journal of Clinical Pharmacology, 32,* 868–888.

Tan, G.T., Gylenhaal, C., & Soejarto, D.D. (2006). Biodiversity as a source of anticancer drugs. *Current Drug Targets, 7,* 265–277.

Tanaka, R., Tabuse, M., & Matsunaga, S. (1988). Triterpenoids from the stem bark of *Phyllanthus flexuosus*. *Phytochemistry, 27(11),* 3563–3567.

Tang, Y.Q., Jaganath, I.B., & Sekaran, S.D. (2010). *Phyllanthus* spp. induces selective growth inhibition of PC-3 and MeWo human cancer cells through modulation of cell cycle and induction of apoptosis. *Plos One, 5(9),* 1–11.

Taraphdar, A.K., Roy, M., & Bhattarcharya, R.K. (2001). Natural products as inducers of apoptosis: implication for cancer therapy and prevention. *Current Science, 80,* 10–11.

Teel, R.W. (1986). Ellagic acid binding to DNA as a possible mechanism for its antimutagenic and anticarcinogenic action. *Cancer Letters, 30,* 329–336.

Thomas, G.A., & Leonard, R.C.F. (2009). How age affects the biology of breast cancer. *Clinical Oncology, 21,* 81–85.

Thornberry, N.A., & Lazebnik, Y. (1998). Caspases: enemies within. *Science, 281,* 1312–1316.

Tokarska-Schlattner, M., Wallimann, T., & Schlattner, U. (2006). Alterations in myocardial energy metabolism induced by the anti-cancer drug doxorubicin. *Comptes Rendus Biologies, 329(9),* 657–668.

Troll, W., & Wiesner, R. (1985). The role of oxygen radicals as a possible mechanism of tumor promotion. *Annual Review of Pharmacology and Toxicology, 25,* 509–528.

Tuchinda, P., Kornsakulkarn, J., Pohmakotr, M., Kongsaeree, P., Prabpai, S., Yoosook, C., et al. (2008). Dichapetalin-type triterpenoids and lignana from the aerial parts of *Phyllanthus acutissima*. *Journal of Natural Products, 71(4),* 655–663.

Twomey, C., & McCarthy, J.V. (2005). Pathways of apoptosis and importance in development. *Journal of Cellular and Molecular Medicine, 9,* 345–359.

Unander, D.W., Herbert, H.B., Lance, C.J., & McMillan Jr, T. (1993). Cultivation of *Phyllanthus amarus* and evaluation of variables potentially affecting yield and the inhibition of viral DNA polymerase. *Economic Botany, 47(1),* 79–88.

Ur-Rehman, H., Yasin, K.A., Choudhary, M.A., Khaliq, N., Ur-Rahman, A., Choudhary, M.I., et al. (2007). Studies on the chemical constituents of *Phyllanthus emblica*. *Natural Product Research, 21(9),* 775–781.

Vander Heiden, M.G., & Thompson, C.B. (1999). Bcl 2 proteins: regulators of apoptosis or of mitochondrial homeostasis. *Nature Cell Biology, 1,* 209–216.

Vargo-Gogola, T., & Rosen, J.M. (2007). Modeling breast cancer: one size does not fit all. *Nature Reviews Cancer, 7,* 659–672.

Vaux, D.I. , & Korsmeyer, S.J. (1999). Cell death in development. *Cell, 96,* 245–254.

Verma, M., Singh, S.K., Bhushan, S., Sharma, V.K., Dalt, P., Kapachi, B.K., et al. (2008). *In vitro* potential of *Polyalthia longifolia* on human cancer cell lines and induction of apoptosis through mitochondrial-dependent pathway in HL-60 cells. *Chemico-Biological Interactions, 171,* 45–56.

Vijayababu, M.R., Kanagaraj, P., Arunkumar, A., Ilangovan, R., Dharmarajan, A., & Arunakaran, J. (2006). Quercetin induces p53-independent apoptosis in human prostate cancer cells by modulating Bcl-2 related proteins: a possible mediation by IGFBP-3. *Oncology Research, 16,* 67–74.

Vimala, S., Norhanom, A.W., & Yadav. (1999). Anti-tumour promoter activity in Malaysian ginger rhizobia used in traditional medicine. *British Journal of Cancer, 80(1/2),* 110–116.

Vogelstein, B., & Kinzler, K.W. (2004). Cancer genes and the pathways they control. *Nature Medicine, 10(8),* 789–799.

Vongvanich, N., Kittakoop, P., Kramyu, J., Tantichareon, M., & Thebtaranonth, M. (2000). Phyllanthusols A and B, cytotoxic norbisabolane glycosides from *Phyllanthus acidus* Skeels. *Journal of Organic Chemistry, 65,* 5420–5423.

Vuorelaa, P., Leinonenb, M., Saikkuc, P., Tammelaa, P., Rauhad, J.P., Wennberg, T., et al. (2004). Natural products in the process of finding new drug candidates. *Current Medicinal Chemistry, 11,* 1375–1389.

Wada, S.I., Iida, A., & Tanaka, R. (2001). Screening of triterpenoids isolated from *Phyllanthus flexuosus* for DNA topoisomerase inhibitory activity. *Journal of Natural Products, 64(12),* 1545–1547.

Wagner, K.H., & Elmadfa, I. (2003). Biological relevance of terpenoids. *Annals of Nutrition and Metabolism, 47,* 95–106.

Walker, N.P., Talanian, R.V., Brady, K.D., Dang, L.C., Bump, N.J., & Ferenz, C.R. (1994). Crystal structure of the cysteine protease interleukin-1 beta converting enzyme: a (p20/p10)2 homodimer. *Cell, 78,* 343–352.

Walker, P.R., Smith, C., Youndale, T., Leblanc, J., Whitfield, J.F., & Sikorska, M. (1991). Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Research, 51,* 1078–1085.

Wang, I.K., Lin-Shiau, S.Y., & Lin, J.K. (1999). Induction of apoptosis by apigenin and related flavonoids through cytochrome *c* release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *European Journal of Cancer, 35,* 1517–1525.

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

Wang, Z.B., Liu, Y.Q., & Cui, Y.F. (2005). Pathways to caspase activation. *Cell Biology International, 29,* 489–496.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., & McPhail, A.T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of the American Chemical Society, 93,* 2325–2327.

Wattenberg, L.W. (1995). What are the critical attributes for cancer chemopreventive agents? *Annals of the New York Academy of Science, 768,* 73–81.

Wen, T.H., Kuan,Y.H., Hui, Y.L., & Jing, G.C. (2006). *Physalis angulata* induced G2/M phase arrest in human breast cancer cells. *Food and Chemical Toxicology, 44,* 974–983.

Werner, R. (2002). *Medicines in Malay Village*. (p.90). Kuala Lumpur: University Malaya Press.

Weyermann, J., Lochmann, D., & Zimmer, A. (2005). A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics, 288,* 369–376.

Wick, W., Grimmel, C., Wagenknecht, B., Dichgans, J., & Weller, M. (1999). Betulinic acid-induced apoptosis in glioma cells: a sequential requirement for new protein synthesis,

formation of reactive oxygen species, and caspase processing. *Journal of Pharmacology and Experimental Therapeutics, 289,* 1306–1312.

Wilcock, C., & Hickman, J.A. (1988). Characterization of a Na^+ /K^+ /Cl^- cotransporter in alkylating agent-sensitive L1210 murine leukemia cells. *Biochimica et Biophysica Acta. 946,* 359–369.

Wink, M. (2006). Importance of plant secondary metabolites for protection against insects and microbial infections. *Advances in Phytomedicine, 3,* 251–268.

Wiseman, L.R., & Spencer, C.M. (1998). Paclitaxel. An update of its use in the treatment of metastatic breast cancer and ovarian and other gynaecological cancers. *Drugs Aging, 12,* 305–334.

Wolter, K.G., Hsu, Y.T., Smith, C.I., Nechushtan, A., Xi, X.G., & Youle, R.J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *Journal of Cell Biology, 139,* 1281–1292.

Wu, C.M., Yang, C.W., Lee, Y.Z., Chuang, T.H., Wu, P.L., Chao, Y.S., et al (2009). Tylophorine arrests carcinoma cells at G1 phase by downregulating cyclin A2 expression. *Biochemical and Biophysical Research Communication, 386,* 140–145.

Wylie, A.H., Morris, R.G., Smith, A.L., & Dunlop, D. (1984). Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *Journal of Pathology, 142,* 67–77.

Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature, 284,* 555–556.

Wyllie, A.H. (1985). The biology of cell death in tumors. *Anticancer Research, 5,* 131–136.

Wyllie, A.H. (1987). Cell death. *International Review of Cytology*. 17: 755–785.

Wyllie, A.H. (1992). Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer Metastasis Reviews, 11,* 93–103.

Xiao, J.X., Huang, G.Q., Zhu, C.P., Ren, D.D., & Zhang, S.H. (2007). Morphological study on apoptosis Hela cells induced by soyasaponins. *Toxicology In Vitro, 21*, 820–826.

Yamamoto, Y., Hosokawa, M., Kurihara, H., Maoka, T., & Miyashita, K. (2008). Synthesis of phosphatidylated-monoterpene alcohols catalyzed by phospholipase D and their antiproliferative effects on human cancer cells. *Bioorganic & Medicinal Chemistry Letters, 18,* 4044–4046.

Yanez, J., Vicente, V., Alcara, M., Castilo, J., Benavente-Garcia, O., Canteras, M., et al. (2009). Cytotoxocity and antiproliferative activities of several phenolic compounds against three melanocytes cell lines: Relationship between structure and activity. *Nutrition and Cancer, 49(2),* 191–199.

Yang, C.S., Landau, J.M., Huang, M.T., & Newmark, H.L. (2001). Inhibition of carcinogenesis by dietary polyphenolics compounds. *Annual Review of Nutrition, 21:* 381–406.

Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., et al. (2006). Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorate*. *Cancer Letters, 231,* 215–227.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., et al. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science, 275,* 1129–1132.

Yip, C.H., Nur Aishah, M.T., & Ibraham, M. (2006). Epidemiology of breast cancer in Malaysia. *Asian Pacific Journal of Cancer Prevention, 7,* 369–374.

Zakeri, Z.F., Quaglino, D., Latham, T., & Lockshin, R.A. (1993). Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB Journal, 7,* 470–478.

Zenk, M.H., & Juenger, M. (2007). Evolution and current status of the phytochemistry of nitrogenous compounds. *Phytochemistry. 68:* 2757–2772.

Zha, H., & Reed, J.C. (1997). Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. *Journal of Biological Chemistry, 272,* 31482–31488.

Zhang, J., & Xu, M. (2002). Apoptotic DNA fragmentation and tissue homeostasis. *TRENDS in Cell Biology, 12(2),* 84–89.
Zhang, J.H., & Xu, M. (2000). DNA fragmentation in apoptosis. *Cell Research, 10,* 205–211.

Zhang, Y.J., Nagao, T., Tanaka, T., Yang, C.R., Okabe, H., & Kouno, I. (2004). Antiproliferative activity of the main constituents from *Phyllanthus emblica*. *Biological & Pharmaceutical Bulletin, 27 (2),* 251–255.

Zhao, J., Davis, L.C., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances, 23(4),* 283–333.

Zhong, Z.G., Wu, D.P., Huang, J.L., Liang, H., Pan, Z.H., Zhang, W.Y., & Lu, H.M. (2011). Progallin A isolated from the acetic ether part of the leaves of *Phyllanthus emblica* L. induces apoptosis of human hepatocellular carcinoma BEL-7404 cells by up-regulation of Bax expression and down-regulation of Bcl-2 expression. *Journal of Ethnopharmacology, 133*, 765–772.

Zi, B.W., Yu, Q.L., & Yu, F.C. (2005). Pathways to caspase activation. *Cell Biology International, 29,* 489–496.

Zuco, V., Supino, R., Righetti, S.C., Cleris, L., Marchesi, E., Gambacorti-Passerini, C., et al. (2002). Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Letters, 175,* 17–25.