CYTOTOXIC AND APOPTOSIS-INDUCING ACTIVITIES OF THE EXTRACTS AND CHEMICAL CONSTITUENTS FROM THE TIGER'S MILK MUSHROOM, *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

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ABSTRACT

Lignosus rhinocerotis (Cooke) Ryvarden (tiger's milk mushroom) is regarded as a prized folk medicine by the indigenous communities in Peninsular Malaysia. Scientific validation of the medicinal properties of *L. rhinocerotis* sclerotium, particularly in treating cancer, is lacking partly due to its rarity. To overcome the problem of supply, artificial cultivation of *L. rhinocerotis* was attempted. Solid-substrate fermentation on agroresidues yielded the fruiting body and sclerotium while liquid fermentation under shaken and static conditions produced the mycelium and culture broth. Samples of *L. rhinocerotis* from different developmental stages were also explored as possible sources of cytotoxic compounds. Results from the MTT assay showed that cold aqueous extract of the sclerotium (LRSC-CAE) was active against MCF7 (breast adenocarcinoma) (IC_{50}: 36.7 µg/ml) and HCT 116 (colorectal carcinoma) (IC_{50}: 36.8 µg/ml) whereas for the organic solvent extracts, dichloromethane extract of the pileus (LRCP-DE) was most potent against MCF7 (IC_{50}: 3.8 µg/ml). Both extracts inhibited the growth of MCF7 and HCT 116 cells by inducing G1 cell cycle arrest and apoptotic cell death. Chemical investigations revealed that the cytotoxic activity of LRSC-CAE might be attributed to some heat-labile protein/peptide(s) that could be recovered from the aqueous fraction by ammonium sulfate precipitation. The ethyl acetate and butanol fractions derived from LRSC-CAE contained some low-molecular-weight compounds including palmitic acid (**1**) and oleamide (**2**) which demonstrated moderate cytotoxicity. On the other hand, ergosta-4,6,8(14),22-tetraen-3-one (ergone) (**3**), methyl palmitate (**4**), methyl linoleate (**5**), and methyl stearate (**6**) were identified in LRCP-DE using LC-MS and GC-MS. LRCP-DE was then subjected to bioassay-guided fractionation which lead to the isolation of 9,11-dehydroergosterol peroxide (**7**) and ergosterol peroxide (**8**) from *L. rhinocerotis* for the first time. Steroidal constituents (compounds **3**, **7**, and **8**)

inhibited the growth and proliferation of human colorectal cancer cell lines, including HCT 116, HT-29, and DLD-1, but exerted lesser damage on the non-cancerous counterpart, CCD-18Co. In addition, treatment with ergosterol peroxide (**8**) resulted in cell cycle arrest at G1 phase and apoptotic cell death in both HCT 116 and HT-29 cells that was associated with the collapse of mitochondria membrane potential, externalization of phosphatidylserine, activation of caspases, and DNA fragmentation in a time-dependent manner. Taken together, the nature of cytotoxic components from different developmental stages of *L. rhinocerotis*, including protein/peptide(s) from the sclerotium and lipophilic constituents, mainly steroids, from the pileus, was clarified. Findings revealed that the cytotoxicity of selected extracts and chemical constituents of *L. rhinocerotis* were attributed to their ability to induce cell cycle arrest and apoptosis.

ABSTRAK

Lignosus rhinocerotis (Cooke) Ryvarden (cendawan susu rimau) dianggap sebagai ubatan tradisional yang bernilai bagi masyarakat pribumi di Semenanjung Malaysia. Kajian saintifik untuk membuktikan keberkesanan sklerotium *L. rhinocerotis,* terutamanya untuk merawat kanser, masih terhad kerana kesukaran mendapatkan bekalan. Untuk mengatasi masalah tersebut, penanaman *L. rhinocerotis* telah dijalankan melalui teknik penapaian pepejal menggunakan sisa pertanian untuk menghasilkan jana buah dan sklerotium manakala penapaian cecair dilakukan dalam keadaan bergoncang dan statik untuk menghasilkan miselium dan kultur cecair. Sampel *L. rhinocerotis* daripada fasa pertumbuhan yang berbeza diterokai sebagai sumber sebatian sitotoksik. Keputusan daripada esei MTT menunjukkan bahawa ekstrak pelarut akueus sejuk daripada sklerotium (LRSC-CAE) adalah aktif terhadap MCF7 (kanser payudara) (IC_{50}: 36.7 µg/ml) dan HCT 116 (kanser kolon) (IC_{50}: 36.8 µg/ml) manakala dalam kategori ekstrak pelarut organik, ekstrak diklorometana daripada pileus (LRCP-DE) adalah paling aktif terhadap MCF7 (IC_{50}: 3.8 µg/ml). Kedua-dua ekstrak tersebut menghalang pertumbuhan sel kanser MCF7 dan HCT 116 melalui perencatan kitaran sel di fasa G1 dan induksi apoptosis. Berdasarkan hasil kajian pencerakinan kimia, didapati kesan sitotoksik LRSC-CAE kemungkinan disebabkan oleh protein/peptida sensitif haba yang boleh dimendapkan dengan menambah ammonium sulfat. Pecahan pelarut organik etil asetat dan butanol daripada LRSC-CAE pula mengandungi sebatian-sebatian seperti asid palmitik (**1**) dan oleamida (**2**) yang didapati menunjukkan kesan sitotoksik sederhana. Di samping itu, ergosta-4,6,8(14),22-tetraen-3-one (ergon) (**3**), metil palmitat (**4**), metil linoliat (**5**), dan metil stearat (**6**) dalam LRCP-DE berjaya dikenal pasti melalui teknik LC-MS dan GC-MS. Pencerakinan LRCP-DE dilakukan menggunakan pendekatan bioesei-petunjuk yang akhirnya membawa kepada

pengasingan 9,11-dihydroergosterol peroksida (**7**) dan ergosterol peroksida (**8**) daripada *L. rhinocerotis* buat pertama kalinya. Sebatian steroid daripada *L. rhinocerotis* (sebatian **3**, **7**, dan **8**) merencat pertumbuhan dan perkembangan sel-sel kanser kolon, termasuk HCT 116, HT 29, dan DLD-1, tetapi kerosakan pada sel bukan kanser seperti CCD-18Co adalah rendah. Ergosterol peroksida (**8**) mampu merencat kitaran sel di fasa G1 dan mengakibatkan apoptosis dalam sel-sel HCT 116 dan HT 29 yang berkait rapat dengan proses-proses seperti keruntuhan potensi membran mitokondria, pendedahan fosfatidilserina, pengaktifan enzim kaspas, dan pemecahan DNA. Secara keseluruhannya, sifat kimia komponen sitotoksik daripada *L. rhinocerotis* dari fasa perkembangan yang berbeza, iaitu protein/peptida dalam sklerotium dan juzuk lipofilik, terutamanya steroid, dalam pileus, berjaya dikenal pasti. Keputusan kajian menunjukkan bahawa kesan sitotoksik ekstrak dan sebatian daripada *L. rhinocerotis* bersandarkan pada kemampuannya untuk merencat kitaran sel dan mengakibatkan apoptosis.

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LIST OF SYMBOLS AND ABBREVIATIONS

AACC	:	American Association of Cereal Chemists
AIF	:	apoptosis-inducing factor
AOAC	:	Association of Official Agricultural Chemists
ANOVA	:	analysis of variance
Apaf-1	:	apoptosis protease-activating factor 1
ATCC	:	American type cell culture
BAD	:	Bcl-2-associated death promoter
Bak	:	Bcl-2 homologous antagonist/killer
Bax	:	Bcl-2-associated X protein
Bcl-2	:	B-cell leukemia/lymphoma 2
Bcl-$_X$L	:	B-cell lymphoma-extra large
BD	:	Becton Dickinson
Bid	:	BH-3 interacting domain death antagonist
Bim	:	pro-apoptotic BH3-only protein
Bik	:	Bcl-2 interacting killer
BCA	:	bicinchoninic acid
BrdU	:	bromo-deoxyuridine triphosphate
BSA	:	bovine serum albumin
Cdk	:	cyclin-dependent kinase
c-FLIP	:	cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CO_2	:	carbon dioxide
cm	:	centimeter
COSY	:	correlation spectroscopy

d	:	doublet
Da	:	Dalton
dATP	:	deoxyadenosine 5'-triphosphate
DISC	:	death-inducing signaling complex
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribose nucleic acid
DR5	:	death receptor 5
et al.	:	and others
etc	:	*et cetera* (and others of the same type)
EAM	:	energy-absorbing matrix
ELISA	:	enzyme-linked immunosorbent assay
ESI	:	electrospray ionization
FACS	:	fluorescence-activated cell sorting
FADD	:	Fas-associated death domain
FBS	:	fetal bovine serum
FITC	:	fluorescein isothiocyanate
g	:	gram
G1 phase	:	gap 1 phase
G2 phase	:	gap 2 phase
GC-MS	:	gas chromatography-mass spectrometry
h	:	hours
HCl	:	hydrochloric acid
HDMS	:	high-definition mass spectrometry
HPLC	:	high performance liquid chromatography
HMBC	:	heteronuclear multiple-bond correlation spectroscop

HSQC	:	heteronuclear single quantum coherence spectroscopy
Hz	:	Hertz
IGF-1/2	:	insulin growth factor-1/2
IFN-γ	:	interferon-gamma
IL-1β	:	interleukin-1beta
IL-18	:	interleukin-18
JC-1	:	5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide
kg	:	Kilogram
LC-MS	:	liquid chromatography-mass spectrometry
m	:	multiplet
m	:	meter
min	:	minute
mg	:	miligram
ml	:	mililitre
mm	:	milimeter
MS	:	mass spectrometry
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/*z*	:	mass to charge ratio
nm	:	nanometer
NADPH	:	nicotinamide adenine dinucleotide phosphate hydrogen.
NF-κB	:	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	:	nuclear magnetic resonance spectroscopy
oaTOF	:	orthogonal acceleration time-of-flight
p21	:	cyclin-dependent kinase inhibitor

PARP	:	poly(ADP-ribose) polymerase
PDA	:	photodiode array
PI	:	propidium iodide
ppm	:	parts per million
pRb	:	retinoblastoma protein
Puma	:	p53 upregulated modulator of apoptosis
RIP	:	receptor interacting protein
RNA	:	ribonucleic acid
RNase	:	ribonuclease
ROS	:	reactive oxygen species
rpm	:	revolutions per minute
s	:	singlet
s	:	second (time)
S phase	:	DNA synthesizing phase
SDS-PAGE	:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF	:	surface-enhanced laser desorption ionization-time of flight
S.D.	:	standard deviation
S.E.	:	standard error
t	:	triplet
TBE	:	trypan blue exclusion
TLC	:	thin layer chromatography
TFA	:	trifluoroacetic acid
TIC	:	total ion chromatogram
TMM	:	tiger's milk mushrooms
TMS	:	tetramethylsilane

TNF-α	:	tumour necrosis factor alpha
UPLC	:	ultra performance liquid chromatography
V	:	voltage
VEGF	:	vascular endothelial growth factor
v/v	:	volume per volume
w/v	:	weight per volume
µg	:	microgram
µl	:	microliter
µM	:	micromolar per litre
°C	:	degree Celsius
δ	:	NMR chemical shift in ppm
%	:	percentage

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

Cancer remains one of the major health problems and common cause of death worldwide (Kanavos, 2006; Ma & Yu, 2006). Prevailing treatment options seemed to have limited therapeutic success in cancer patients. Even with the advances in cancer therapies (e.g. surgery, chemotherapy, radiotherapy, hormonal therapy, immunotherapy, etc.), the mortality rate has not declined significantly over the years (Urruticoechea *et al*., 2010; Millimuono *et al*., 2014). In addition, the currently available anti-cancer drugs are mostly not target specific and even pose adverse side-effects that might be acute or chronic, mild or potentially life threatening (Patel & Goyal, 2012). This scenario highlights the urgent need for effective and less-toxic therapeutic approaches.

Chemoprevention (i.e. the potential of chemical intervention as a means of delaying carcinogenesis) involves the use of synthetic, natural or biological agents to inhibit, reverse or retard tumourigenesis (Bertram *et al*., 1987). In view of our improved understanding of the biology of carcinogenesis and the identification of potential molecular targets, chemoprevention is gaining attention as it is also regarded as cost-effective alternative for the management of various forms of cancer (Greenwald *et al*., 1995; Steward & Brown, 2013). Cancer chemoprevention using compounds derived from the nature has shown promising results against various malignancies (Surh, 2003; Naithani *et al*., 2008; William *et al*., 2009; Landis *et al*., 2014).

In the search for new chemopreventive and chemotherapeutic agents, some prized mushrooms with validated anti-cancers effect, such as *Ganoderma lucidum* (Curtis) P. Karst, *Lentinula edodes* (Berk.) Pegler, *Schizophyllum commune* Fr., *Trametes versicolor* (L.) Lloyd, *Grifola frondosa* (Dicks.) Gray, *Inonotus obliquus* (Ach. ex Pers.) Pilát, and others are of immense interest (Lindequist *et al*., 2005; Patel & Goyal,

2012). Studies have revealed that active components from mushrooms exist in various forms, some of which include triterpenes, phenolics, polysaccharides, polysaccharide-protein complexes, proteins, and others (Lindequist *et al*., 2005; Zhong & Xiao, 2009; Ferreira *et al*., 2010). Furthermore, clinical trials have been conducted to assess the benefits of using commercial preparations containing medicinal mushroom extracts in cancer therapy (Smith *et al*., 2002).

The ethnobotanical approach places emphasis on medicinal plants/mushrooms that are used as folk medicine. It has been proven to be one of the most effective ways to discover lead molecules for drug discovery (Fabricant & Farnsworth, 2001; Gu *et al*., 2014). In this context, wild mushrooms that are used by indigenous communities represent an untapped source of potential therapeutic drugs. The indigenous people of Peninsular Malaysia (Orang Asli) utilize a number of wild mushrooms in their traditional medicine practices (Lee *et al*., 2009a). Notable examples of these folk remedies are several members of the genus *Lignosus* that are locally known as *cendawan susu rimau* (in Malay) – literally "tiger's milk mushrooms" (Tan *et al*., 2010).

Findings from several ethnobotanical surveys revealed that the sclerotia of *Lignosus* spp. are purportedly effective in treating ailments such as cancer, cough, asthma, fever, and food poisoning (Lee *et al*., 2009a; Azliza *et al*., 2012; Mohammad *et al*., 2012). *Lignosus rhinocerotis* (Cooke) Ryvarden (synonym: *Polyporus rhinocerus* Cooke) is the most commonly encountered *Lignosus* spp. in Malaysia (Choong *et al*., 2014). Interestingly, *L. rhinocerotis* is also used by the Chinese physicians in China where it is called *hurulingzhi* (in Chinese) (Huang, 1999). While the use of *Lignosus* spp. as folk medicine has a long history, many of the claims regarding its beneficial medicinal effects have yet to be substantiated by scientific evidences with the exception of its

potential anti-cancer effect that received tremendous interest. Previous studies have demonstrated the cytotoxic and immunomodulatory activities of *L. rhinocerotis* sclerotial aqueous extracts (Lai *et al.,* 2008; Wong *et al*., 2011; Lee *et al*. 2012; Yap *et al*., 2013; Lau *et al*., 2014).

As the main supply of *L. rhinocerotis* is from the wild and the mushroom can only be noticed when the fruiting body sprouted from the sclerotium, collection of these mushrooms becomes difficult. In spite of the potential therapeutic values of *L. rhinocerotis*, it has not been extensively studied due to limited supply. In view of this, domestication of *L. rhinocerotis* is necessary so that it can be fully exploited for its medicinal properties. With that, however, additional questions popped up, some of which might include the effectiveness of *L. rhinocerotis* samples from different cultivation techniques and/or developmental stages (i.e. the fruiting body, sclerotium, mycelium, and culture broth). In the context of drug discovery, comparative analysis of the bioactivities of *L. rhinocerotis* samples is therefore necessary in order to identify the ones to be prioritized for further studies.

This scenario has led to our research hypothesis, i.e. the purported anti-cancer effect of *L. rhinocerotis*, as claimed in ethnobotanical records, is partly attributed to the presence of cytotoxic chemical component(s) with distinct molecular targets. This will lead to the first and second questions to be answered: (i) what is/are active cytotoxic component(s) in *L. rhinocerotis*, and (ii) do the samples of *L. rhinocerotis* from different developmental stages (e.g. fruiting body, sclerotium, mycelium, and culture broth) also contain cytotoxic compound(s)? To answer both questions, *L. rhinocerotis* crude extracts will be screened for cytotoxic activity against selected cancer and non-cancerous cell lines, and the cytotoxic component(s) of interest will be purified using chromatographic methods followed by chemical characterization using spectroscopic

and mass-spectrometric approaches. The third question will then be (iii) how do these extract/compound(s) induce cell death? To elucidate their mechanism of action, inhibition of cellular proliferation via cell cycle arrest and induction of apoptosis in selected cancer cell lines treated with extract/compound(s) will be studied.

The main objectives of this study are as follows:

1. To produce the fruiting body and sclerotium of *L. rhinocerotis* by solid substrate fermentation on agroresidues, as well as the mycelium and culture broth by liquid fermentation under shaken and static conditions, and to compare the chemical composition of *L. rhinocerotis* from different developmental stages
2. To evaluate the cytotoxic activity of *L. rhinocerotis* crude aqueous and organic solvent extracts on selected human cancer and non-cancerous cell lines
3. To determine the effect of selected *L. rhinocerotis* crude extracts on cell proliferation, cell cycle progression, and apoptosis induction
4. To deduce the nature of cytotoxic component(s) in selected *L. rhinocerotis* crude extracts by chemical fractionation and characterization
5. To identify, purify, and elucidate the structure of low-molecular-weight cytotoxic chemical constituent(s) from the active fractions of *L. rhinocerotis* using chromatographic, spectroscopic, and mass-spectrometric approaches
6. To investigate the cytotoxic and apoptosis-inducing activities of *L. rhinocerotis* steroidal constituents in selected human colorectal cancer and non-cancerous cell lines

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

2.1.1 The biology of cancer

Cancer is a group of diseases that are characterized by uncontrolled growth and spread of abnormal cells, and if the spread is not controlled, cancer can eventually result in death. According to Cancer Facts and Figures (2015), there are several external (e.g., tobacco, unhealthy diet, infectious organisms, etc.) and internal factors (e.g., genetic mutations, hormones, immune conditions, etc.) that may act together or in sequence to cause cancer. The early stage of cancer is referred to as a neoplasm (tumour), i.e. an abnormal mass of tissue that may be solid or filled with fluid, and appears as a lump or swelling. The process by which a normal cell becomes a tumour is referred to as tumourigenesis (Pardee *et al*., 2009). Most neoplasms develop following clonal expansion of a single cell that has undergone neoplastic transformation usually caused by factors that directly and irreversibly alter the cell genome.

Tumours can be categorized as benign, potentially malignant (pre-cancer) or malignant (cancer). Benign tumours pose little risk to the host because they are localized and of small size; however, these may have an impact on blood vessels or nerves, and possibly cause negative effects when their sheer bulk interferes with normal functions. On the other hand, malignant tumours tend to progress, invade surrounding tissues, and metastasize (i.e. the spread of cancer cells to tissues and organs beyond where the tumour originated and the formation of new tumours) at a high rate to other parts of the body and may eventually cause death (Yokota, 2000).

Almost every cell type in the body has the capacity to accumulate mutations and become a tumour. Approximately 85% percent of most reported cancer cases are

cancers of the epithelial cells and these are termed as carcinomas or adenocarcinomas. Cancers arising from cells in the blood are called leukemia. If they arise in the lymph nodes, they are known as lymphomas. Those that arise in the connective tissue are called sarcomas (Pienta, 2009).

While each type of cancer exhibits a unique set of behaviours and growth characteristics, they share some common characteristics or hallmarks (Figure 2.1). The hallmarks of cancer refer to the acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate. Most, if not all cancers have acquired these functional capabilities during the course of tumourigeneis, albeit through various mechanistic strategies (Hanahan & Weinberg, 2011). The hallmarks of cancer include:

- *Sustaining proliferative signaling.* While normal cells will cease to grow in the absent of the necessary growth factors, cancer cells, on the other hand, can continue to grow and divide even without external growth signals. Some oncogenes produce excessive or mutant version of proteins to enable cancer cells to self-sustain on prolonged stimulation (Goodsell, 2003; Witsch *et al*., 2010; Hanahan & Weinberg, 2011).

- *Evading growth suppressors.* The growth of normal cells is kept under control by growth inhibitors in the surrounding environment (e.g. extracellular matrix) and on the surfaces of neighbouring cells. These inhibitors might act on the cell cycle; for instance, the growth inhibitor signals are funnelled through the downstream retinoblastoma protein (pRb) which acts to prevent the inappropriate transition from G1 to S phase. If pRb is damaged through a mutation in its gene, or by interference from human papilloma virus, the cells will then divide uncontrollably (Giacinti & Giordano, 2006: Henley & Dick, 2012).

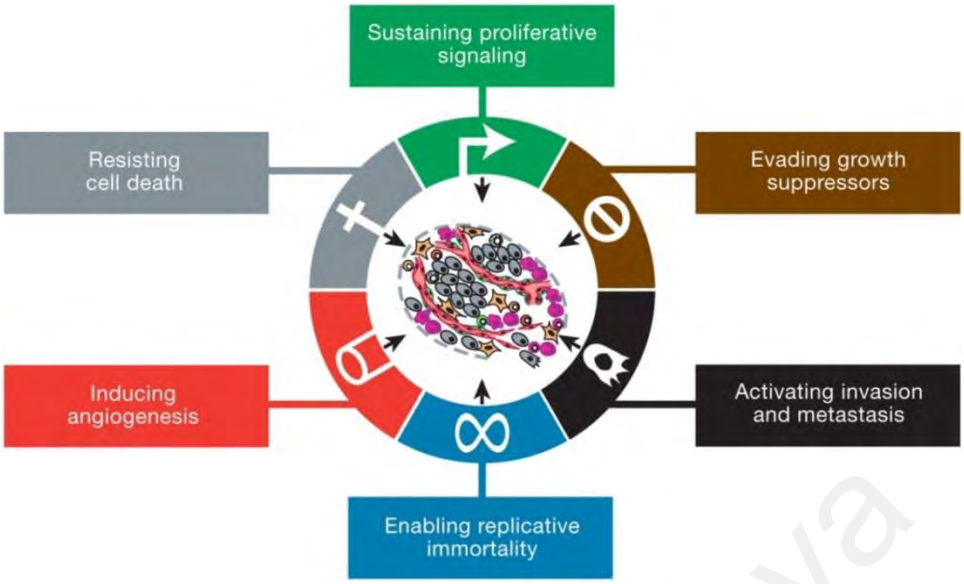


Figure 2.1: Hallmarks of cancer. Figure taken from Hanahan & Weinberg (2011).

- *Resisting cell death.* Cancer cells evolve a number of strategies to avoid apoptosis (a form of programmed cell death). An example is the loss of p53 tumour suppressor function which eliminates this critical damage sensor from the apoptosis-inducing circuitry (Fridman & Lowe, 2003). Alternatively, cancer cells may achieve this by increasing expression of anti-apoptotic regulators (e.g. Bcl-2, Bcl-x_L. etc.) or survival signals (e.g. IGF-1/2, etc.), downregulating pro-apoptotic factors (e.g. Bax, Bim, Puma, etc.), or by short-circuiting the extrinsic ligand-induced death pathway (Papaliagkas *et al*., 2007; Portt *et al*., 2011).

- *Enabling replicative immortality.* Normal cells usually die after a certain number of divisions; however, cancer cells can escape this limit and apparently are capable of indefinite growth and division (immortality). Telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation (Hahn, 2001; Blackburn, 2003).

- *Inducing angiogenesis*. Angiogenesis is the process by which new blood vessels are formed. During tumour progression, an "angiogenic switch" is almost always activated and remains switched on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths (Nishida *et al*., 2006; Hoff & Machado, 2012).

- *Activating invasion and metastasis*. The course of tumour metastasis (Figure 2.2) entails a series of stages that lead to the formation of secondary tumours in distant organs (Yokota, 2000). It is largely responsible for the mortality and morbidity of cancer. The process is noted to be largely dependent on the dissociation of the cell from the primary tumour due to the loss of adhesion between cells and followed by the ability of the cell to attain a motile phenotype through changes in cell to matrix interaction (Gupta & Massague, 2006; Chaffer & Weinberg, 2011).

As shown in Figure 2.2, there are other distinct attributes of cancer cells have been proposed to be functionally important for the development of cancer and therefore, considered to be emerging hallmarks of cancer (Hanahan & Weinberg, 2011). The first involves major reprogramming of cellular energy metabolism in order to support continuous cell growth and proliferation, replacing the metabolic program in most normal tissues (Costello & Franklin, 2012; Phan *et al*., 2014). The second involves active evasion by cancer cells from attack and elimination by the body's immune cells (Seliger, 2005; Finn, 2012).

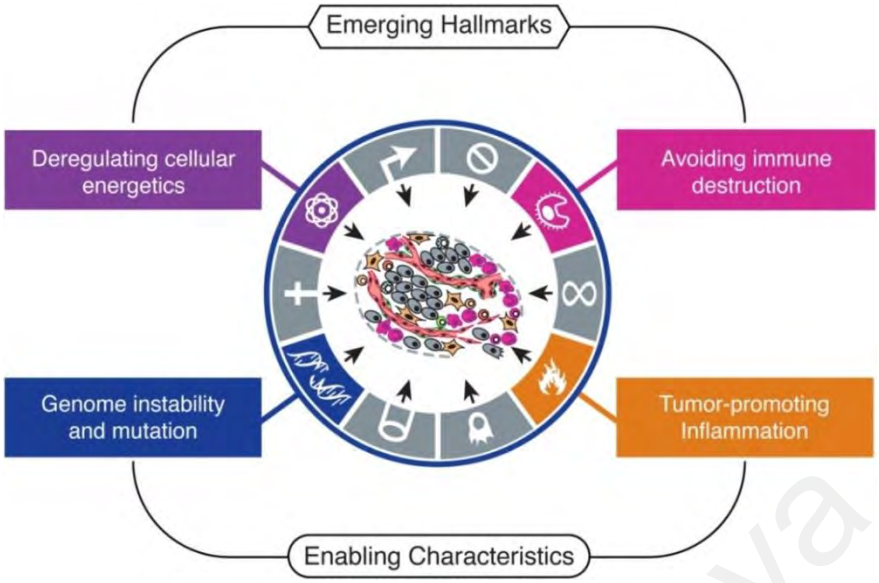


Figure 2.2: Emerging hallmarks and enabling characteristics of cancer cells. Figure taken from Hanahan & Weinberg (2011).

Further, their acquisition is made possible by two enabling characteristics. The first is the development of genomic instability in cancer cells that generates random mutations including chromosomal rearrangements (Abdel-Rahman, 2008). The second characteristic involves the inflammatory state of premalignant and malignant lesions that is driven by cells of the immune system, some of which serve to promote tumour progression (Grivennikov *et al*., 2010; Balkwill & Mantovani, 2012).

2.1.2 The burden of cancer

According to the Cancer Facts and Figures (2015), cancer is one of the major causes of mortality worldwide with an estimated 14.1 million new cancer cases in 2012 worldwide while the estimated cancer deaths in 2012 were 8.2 million, comprising of 2.9 million in economically developed countries and 5.3 million in economically developing countries. By 2030, the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths. Moreover, the estimated future cancer

burden will probably be considerably larger due to factors like the growth and aging of the population as well as adoption of lifestyles that are known to increase cancer risk (e.g. poor diet, smoking, physical inactivity, etc.) especially in economically developing countries (Kanavos, 2006).

While cancer affects all communities, there are marked differences in the prevalence and types of cancers. According to Cancer Facts and Figures (2015), the three most commonly diagnosed cancers in economically developed countries were prostate, lung, and colorectal among males, and breast, colorectal, and lung among females (Figure 2.3). On the other hand, the three most commonly diagnosed cancers were lung, liver, and stomach in males, and breast, cervix uteri, and lung in females for economically developing countries. In Malaysia, it was reported that the incidence of cancer has increased from 32 000 new cases in 2008 to 37 400 in 2012 and this number is expected to continue to rise to about 57 000 if no actions are taken (The Star, 2015).

The most common types of cancers worldwide also vary by geographic area (Figure 2.4). Lung and stomach cancer were the top cancers in Asia. In 2012, the most common cancer site among males in most economically developed countries was prostate with the exception of certain countries (e.g. stomach cancer in Japan). Among females, the most common cancer sites were either breast or cervical cancer, with some exceptions, such as China (lung) and South Korea (thyroid). These differences were attributed to a number of factors including the prevalence of risk factors, variations in the age structure of the population, the availability and use of diagnostic tests (e.g. for cancer screening) as well as the availability and quality of treatment (Kanavos, 2006).

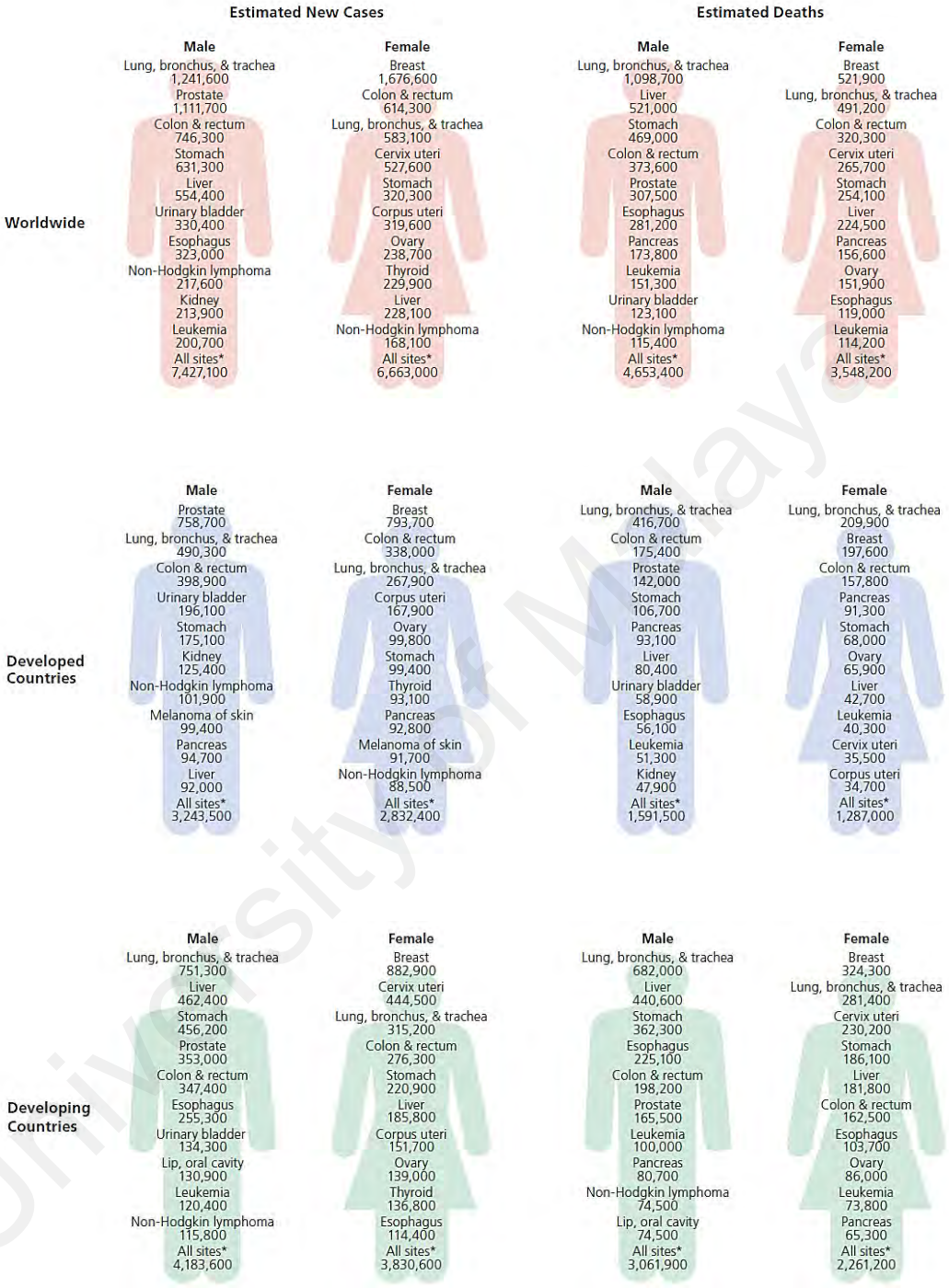


Figure 2.3: Estimated new cancer cases and deaths worldwide in 2012. Figure retrieved from the American Cancer Society: Global Cancer Facts & Figures (2015).

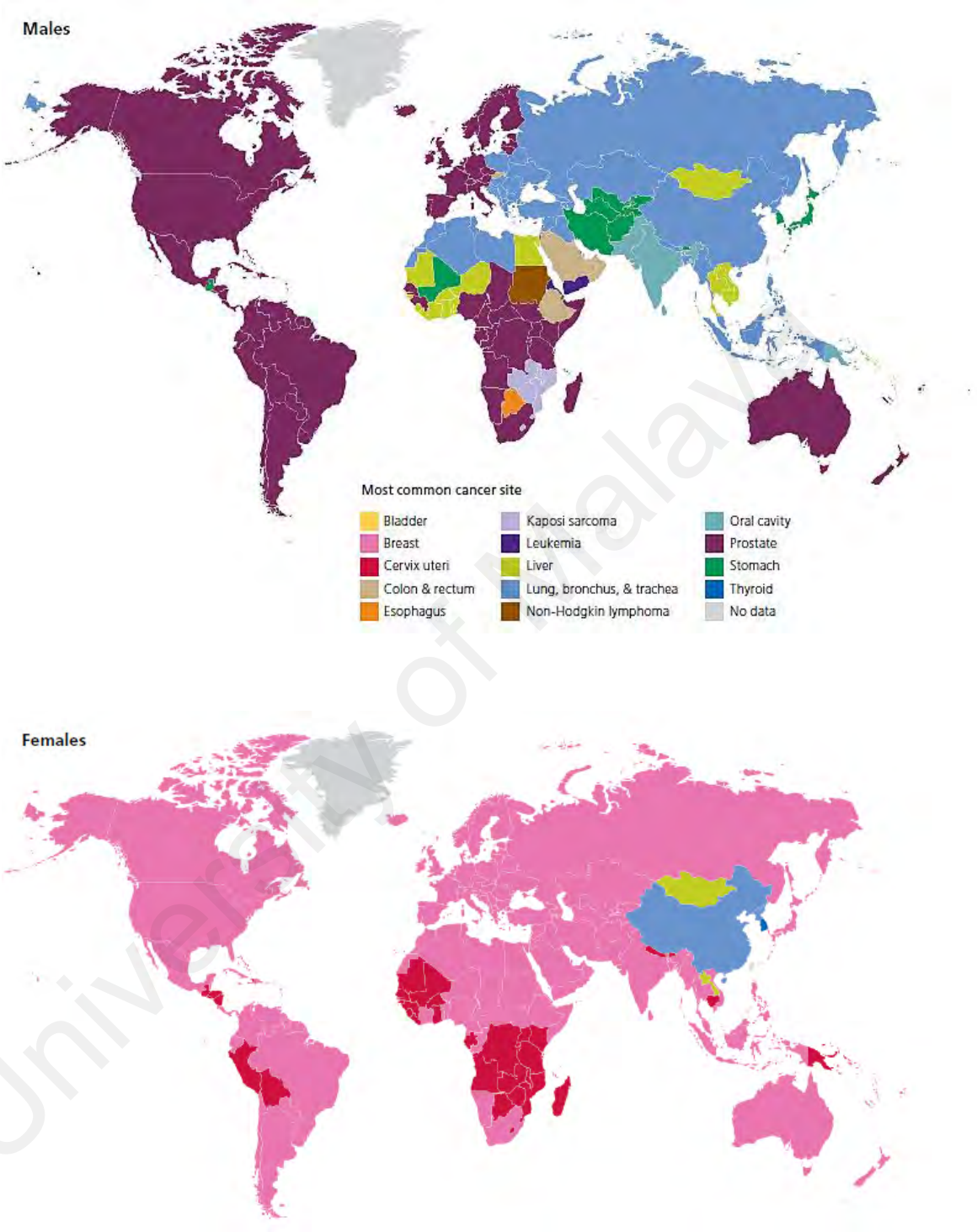


Figure 2.4: Most common cancer sites by sex in 2012**.** Figure retrieved from the American Cancer Society: Global Cancer Facts & Figures (2015).

The risk of being diagnosed with cancer increases substantially with age. In economically developed countries, almost 58% of all newly diagnosed cancer cases occur at 65 years of age and above as compared with 40% in developing countries. The incidence rate for all cancers combined was higher in more developed countries compared with less developed countries in both males (308.7 vs. 163.0, respectively) and females (240.6 vs. 135.8, respectively). On the other hand, the mortality rate for all cancers combined in developed and less developed countries was comparable. Furthermore, the risk is higher with a family history of the disease; however, it seemed that many familial cancers arise not exclusively from genetic makeup but also from the interplay between common gene variations and lifestyle and environmental risk factors (Jemal *et al*., 2010).

Studies have shown that survival statistics vary depending on cancer type and stage at diagnosis. Survival is expressed as the percentage of people who are alive in a certain period of time (usually 5 years) following a cancer diagnosis. According to the Cancer Facts and Figures (2015), cancer survival rates in a population are affected by a number of factors, mainly the types of cancer that occur, the stages at which cancers are diagnosed, and whether treatment is available. For cancers that are affected by screening and/or treatment (e.g. female breast, colorectal, and certain childhood cancers), there are large differences in the survival rate in economically developed and developing countries (McPherson *et al*., 2000; Haggar & Boushey, 2009). In contrast, for cancers without early detection or effective treatment (e.g. esophagus, liver, or pancreatic cancer), survival rates in either developing or developed countries show little variation (Bosch *et al*., 2005; Napier *et al*., 2014; Yadav & Lowenfels, 2013).

2.1.3 Cancer therapy

Conventional cancer therapy comprises of surgery, chemotherapy (i.e. treatment with cytotoxic drugs or a standardized treatment regimen consisting of a combination of these drugs), and radiation therapy (i.e. the medical use of ionizing radiation as part of cancer treatment to control malignant cells) depending on the type of cancer and the stage of tumour development inside the body. Cytotoxic drugs used in chemotherapy are classified according to their chemical structure and mechanisms of actions. These include alkylating agents, antimetabolites, topoisomerase inhibitors, anti-tumour antibiotics, mitotic inhibitors, and other miscellaneous agents (Neal, 2009).

The major problem arising from these treatments is the fact they invariably result in damage or weakening of the patient's natural immunological defenses (which may already have been damaged by the cancer itself). Treatments also may cause numerous side effects, serious damage, and suffering to the patient. Some of the side effects that are associated with these treatments include fatigue, nausea, lymphedema, effects on fertility and others. In some cases, treatments might increase a person's risk of developing different types of cancer later in life (Pardee *et al*., 2009).

Nowadays, cancer therapy also emphasizes on improving the patient's quality of life by modifying the host's biological response against the malignant invasion. Biological immunotherapy (also known as immunotherapy, biotherapy, or biological response modifier therapy) is now gaining more attention, since it considerably reduces the side effects and helps to overcome cancer growth (De Silva *et al*., 2012). It uses the body's immune system, either directly or indirectly, to fight cancer or to lessen the side effects that may be caused by some cancer treatments.

2.2 Programmed cell death

Programmed cell death serves to balance cell death with survival of normal cells. It plays important roles during preservation of tissue homeostasis and is involved in the elimination of damaged cells. Three main forms of programmed cell death are apoptosis, autophagy and necrosis (Figure 2.5) (Fink & Cooksoon, 2005; Duprez *et al*., 2009; Nikoletopoulou *et al*., 2013):

- Apoptosis is a pathway leading to cell death that features the activation of initiator caspases that activate effector caspases to cleave cellular substrates. The characteristics of apoptotic cells include cytoplasmic and nuclear condensation, DNA damage, maintenance of an intact plasma membrane, formation of apoptotic bodies, and exposure of surface molecules targeting apoptotic cells for phagocytosis (Elmore, 2007; Duprez *et al*., 2009; Hsiung & Kadir, 2011).

- Autophagy features the degradation of cellular components within the intact dying cell in autophagic vacuoles. Some of the morphological characteristics of autophagic cells are vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. Autophagic cells can also be taken up by phagocytosis (Mizushima, 2007; He & Klionsky, 2009).

- Necrosis is characterized as passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents. Necrosis causes cell swelling, organelle dysfunction, and cell lysis. In the absence of phagocytosis, apoptotic bodies may lose their integrity and proceed to secondary or apoptotic necrosis (dead cells that have reached this state via the apoptotic program) (Goldstein & Kroemer, 2007; Duprez *et al*., 2009; Chan *et al*., 2015).

Other modes of cell death include oncosis and pyroptosis. Oncosis refers to cell death that is accompanied by cellular and organelle swelling and membrane breakdown followed by the eventual release of inflammatory cellular contents (Fink & Cooksoon, 2005; Weerasinghe & Buja, 2012). On the other hand, pyroptosis is a type of cell death mediated by the activation of caspase-1, a protease that also activates the inflammatory cytokines, IL-1β, and IL-18 (Fink & Cooksoon, 2005; Bergsbaken *et al*., 2009).

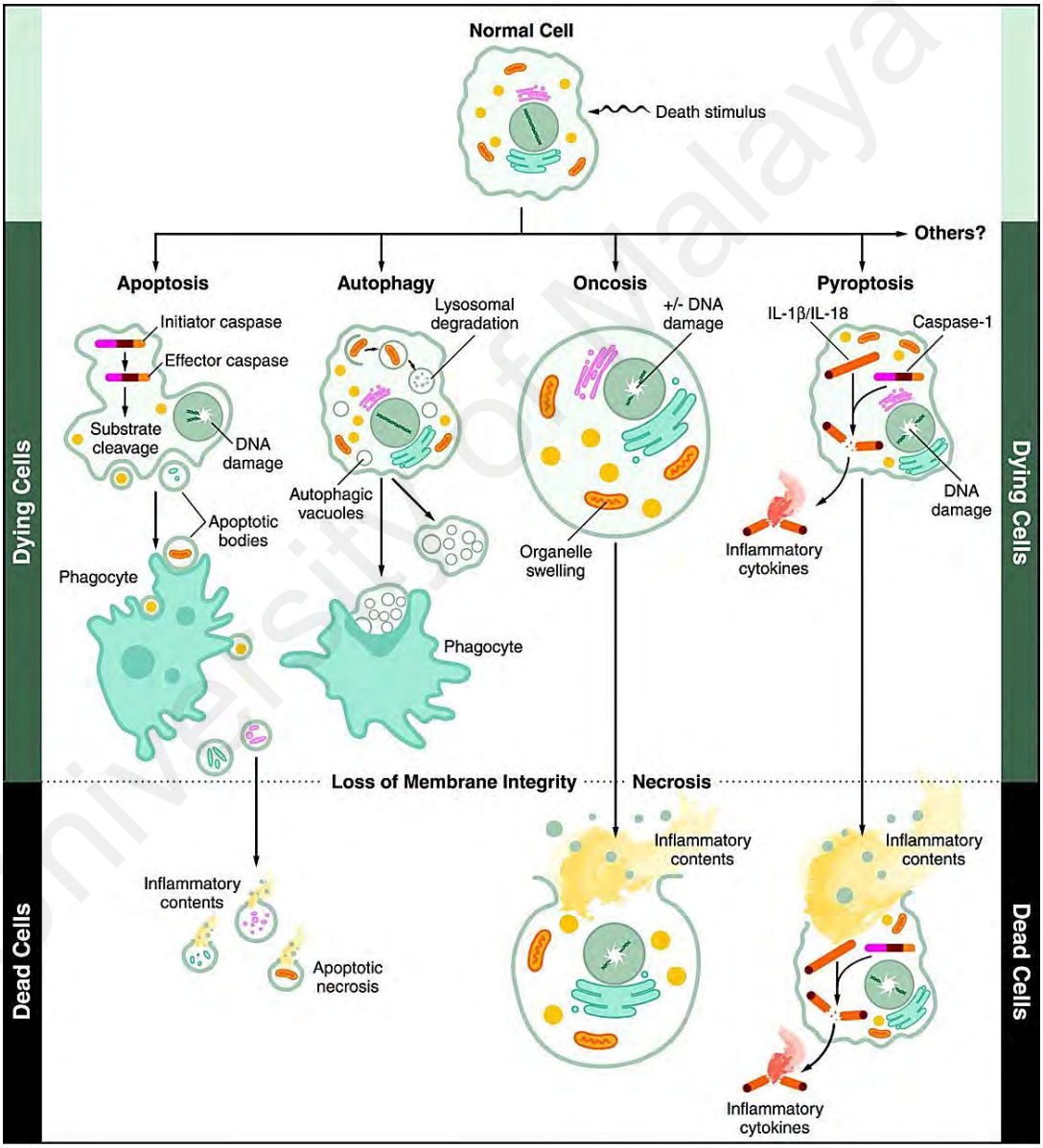


Figure 2.5: Pathways leading to cell death. When exposed to death-inducing stimuli, healthy cells respond by initiating a variety of molecular pathways leading to cell death. Figure taken from Fink & Cooksoon (2005).

2.2.1 Apoptosis

The typical features of apoptosis, irrespective of cell type and inducing stimulus, are largely morphological, and these include chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blebbing (Brown & Attardi, 2005; Elmore, 2007). The underlying mechanistic basis for the observed morphological changes is the activity of aspartate-specific cysteine proteases, i.e. the caspases (cysteinyl, aspartate-specific proteases). Caspases are synthesized as inactive zymogens, which must be proteolytically cleaved at two (or three in some cases) aspartate residues to generate the active mature enzyme (Earnshaw *et al*., 1999; Chang & Yang, 2000). The generation of active caspases forms a cascade in which "initiator" caspases interact with specific adapter molecules to facilitate their own auto-processing. These now active initiator caspases will cleave and activate the downstream "executioner" caspases, which, in turn, cleave their target substrates to initiate the proteolytic destruction of the cell. This sequence of events culminating in the activation of caspases has been broadly divided into two main pathways, i.e. the extrinsic and intrinsic pathways (Figure 2.6) that are characterized by the engagement of cell surface death receptors, and involvement of key mitochondrial events, respectively (Fulda & Debatin, 2005; Elmore, 2007). Both extrinsic and intrinsic pathways involve the self-activation of initiator caspase, via its interaction with a specialized adapter molecule, and subsequent activation of the downstream effector or "executioner" caspases (Kasibhatla & Tseng, 2003).

The extrinsic pathway is triggered by the binding of Fas (and other similar receptors, e.g. tumour necrosis receptor 1 and its relatives) plasma-membrane death receptor with its extracellular ligand, Fas-L. Briefly, Fas-L will combine with Fas to form a death complex that will recruit death domain-containing protein (FADD) and pro-caspase-8, and aggregate to become the death-inducing signaling complex (DISC). Consequently,

the protein complex activates its pro-caspase-8, triggering pro-caspase-3 that will execute apoptotic cell death (Kasibhatla & Tseng, 2003; Fulda & Debatin, 2005; Elmore, 2007).

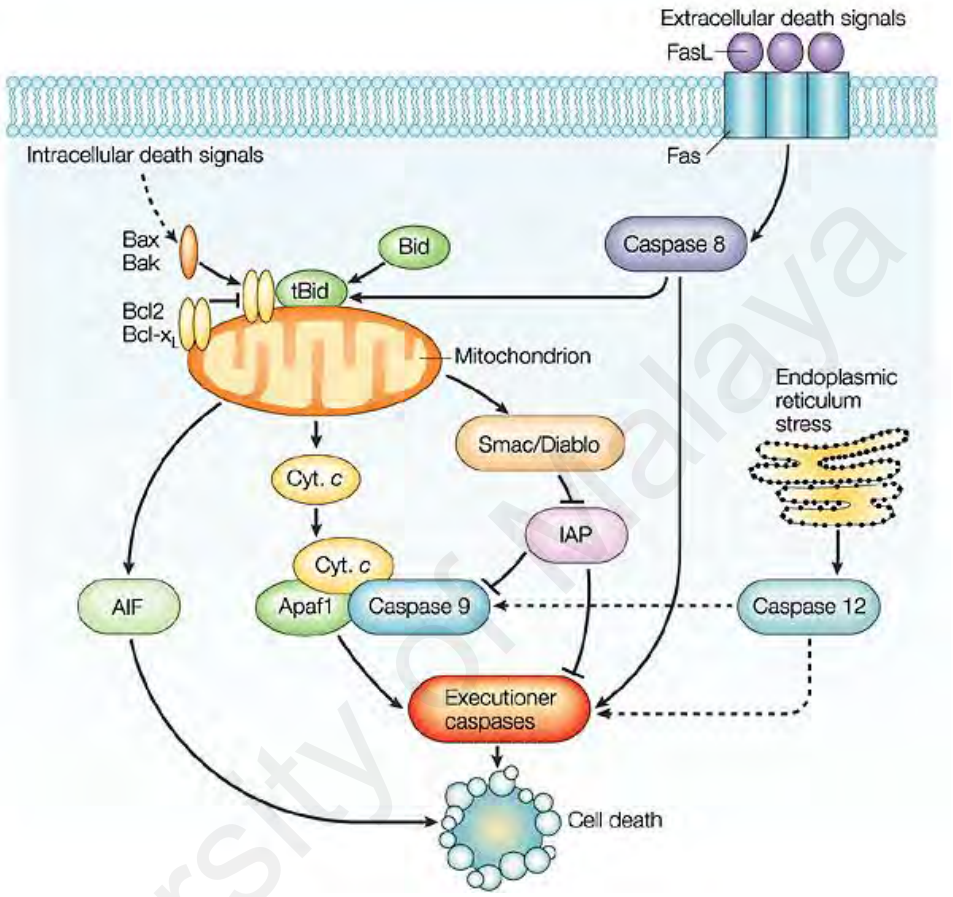


Figure 2.6: Extrinsic and intrinsic pathways of apoptosis. Figure taken from Vila & Przedborski (2003). AIF, apoptosis-inducing factor; Cyt., cytochrome; FasL, Fas ligand; IAP, inhibitor of apoptosis; tBid, truncated Bid.

On the other hand, the intrinsic pathway is under the control of mitochondrial pro-enzymes. When a cell becomes initiated by extracellular stimuli or intracellular signals, the outer mitochondrial membrane become permeable to internal cytochrome *c*, which will be released into the cytosol. Cytochrome *c* will then recruit Apaf–1 and pro-caspase-9 to form apoptosome, which triggers a caspase-9/3 signalling cascade, culminating in apoptosis (Kasibhatla & Tseng, 2003; Fulda & Debatin, 2005; Elmore,

2007). One of the primary regulators of the mitochondria-mediated pathway to apoptosis is the family of Bcl-2 proteins. These proteins are broadly characterized into pro-apoptotic members (e.g. Bax, Bak, Bik, Bad, and Bid) and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xL) based on their ability to either suppress or induce the release of cytochrome c (Papaliagkas et al., 2007; Portt et al., 2011).

2.2.2 Necrosis

Necrosis is typically described as a "nonspecific" form of cell death that is usually characterized by rupture of the plasma membrane with a consequent localized inflammatory response and damage to surrounding cells and tissues (Kasibhatla & Tseng, 2003). With the discovery of key mediators of necrotic death, the concept of programmed necrosis has recently been gaining ground. Receptor interacting protein (RIP) kinases, poly(ADP-ribose) polymerase-1(PARP1), NADPH oxidases, and calpains have been identified as potential signaling components of programmed necrosis (Golstein & Kroemer, 2007).

The differences between these two processes of cell death underscore the reason why apoptosis, and not necrosis, represents a more desirable target mechanism for the induction of cell death in cancer cells. Firstly, apoptosis is associated with the rapid engulfment and removal of cell corpses by phagocytic cells that recognize the signals displayed on the outer surface of the apoptotic cells. As apoptotic cell death is the consequence of a series of precisely regulated events that are frequently altered in cancer cells, it provides the opportunity for selective clinical intervention to bring about the death of the cancer cells without damage to normal cells (Kanduc *et al*., 2002; Kasibhatla & Tseng, 2003; Ouyang *et al*., 2012).

2.3 Cell cycle

2.3.1 Background

Genetic information is passed on to the next generation through cellular reproduction. As shown in Figure 2.7, during replication, chromatin (i.e. combination of DNA with additional proteins) packages itself into chromosomes to accommodate the increased volume of DNA. Replication of the chromosomes DNA molecules produces two sister chromatids whereby each chromatid is an identical copy of the chromosome DNA molecules. By faithfully distributing one complete chromatid to each daughter, cells are able to maintain entire DNA content.

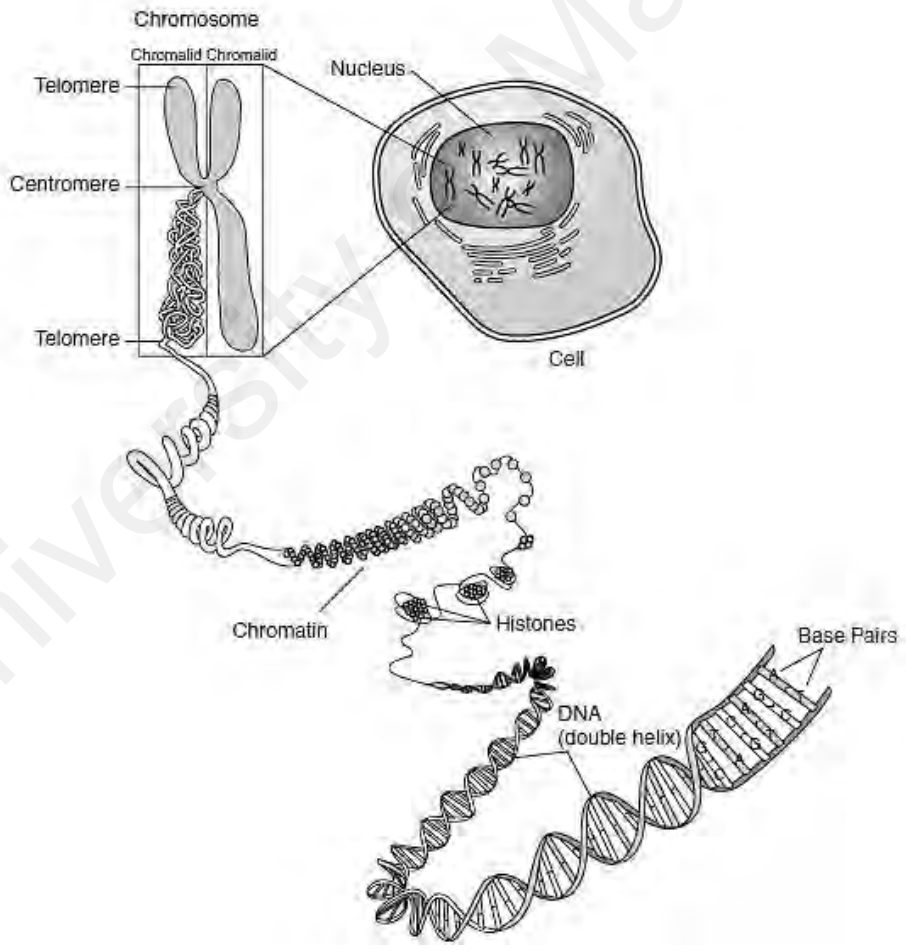


Figure 2.7: Structure of DNA during replication. Figure taken from Lambert *et al*. (2009).

The cell cycle is the orderly sequence of events by which cell replication and division are accomplished precisely (Figure 2.8). The two major divisions of cell cycle are interphase and mitosis. The cell spends most of its time in interphase, during which it grows, doubles its contents, and copies its DNA in preparation for dividing. Interphase, on the other hand, can be divided into several sub-phases: G1 (first gap in which the cell prepares DNA replication), S phase (period of DNA synthesis in which a second copy of the entire genome is generated), and G2 (second gap phase in which the cell prepares for division). The length of G1 depends on the types of the cells and growing conditions whereas the time a cells spends in S phase and G2 is relatively constant, i.e. approximately 8 and 2 hours, respectively (Hung *et al*., 1996; Schafer, 1998; Lambert *et al*., 2009).

Mitosis is the second major division of the cell cycle in which the two copies of DNA segregate and the cells divide into two genetically identical daughter cells. The five stages of mitosis are prophase (condensation of nuclear chromatin into chromosomes and formation of mitotic spindle), prometaphase (dissolution of nuclear envelope and the chromosomes interact with the mitotic spindle), metaphase (convene of chromosomes at the metaphase plate), anaphase (separation and migration of the sister chromatids towards spindle poles) and telophase (formation of daughter nuclei at the two poles of the cell and the chromosomes uncoil to form chromatin) (Lambert *et al*., 2009).

Entrance and exit out of the cell cycle occurs as a cell passes between active proliferation and a quiescent of G0 state in which the fundamental metabolism of the cell is depressed, including many of its usually active functions (e.g. transcription and protein synthesis). Once in G0, cells can either re-enter the cell cycle at an appropriate time or remain in G0 indefinitely (Hung *et al*., 1996).

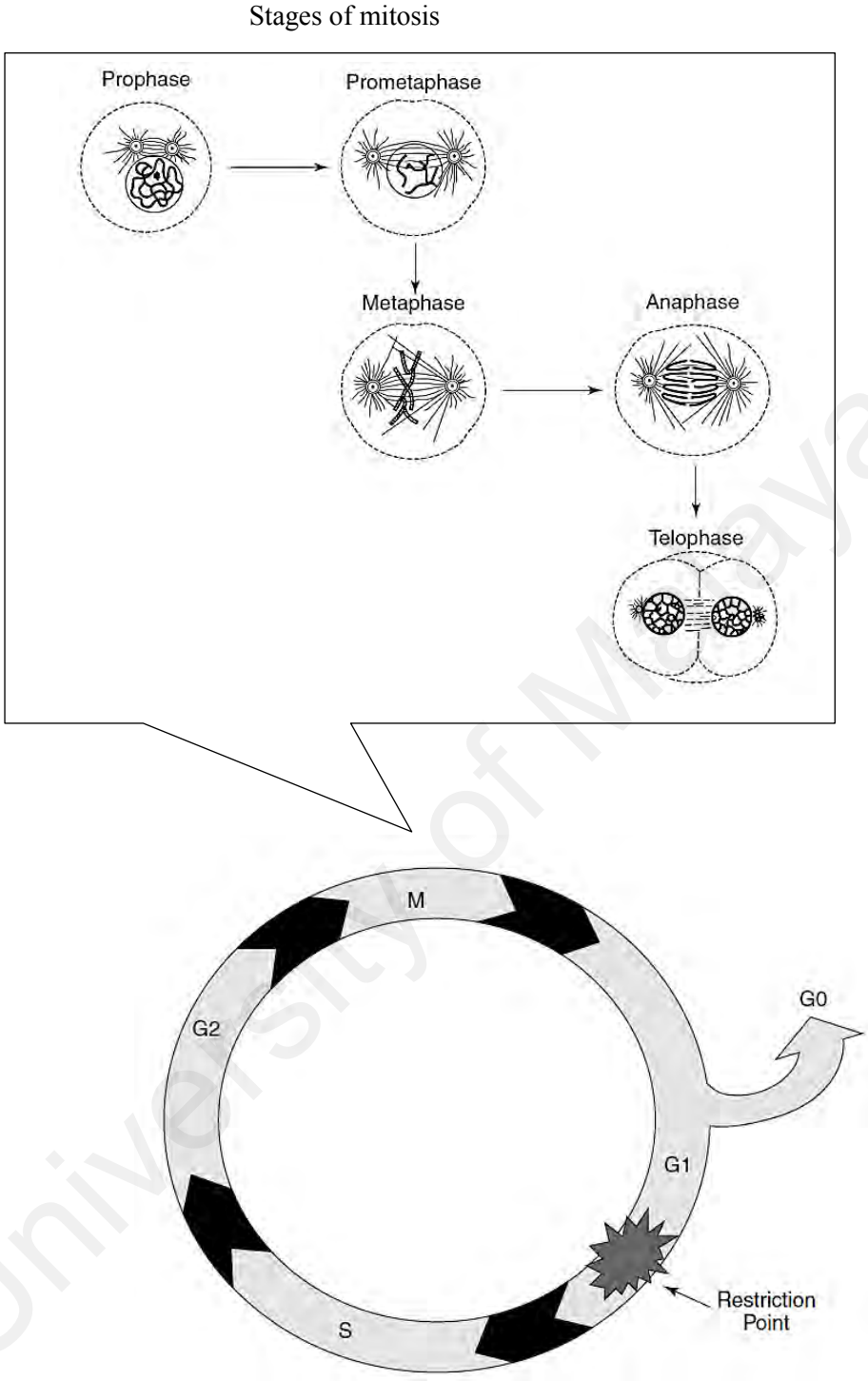


Figure 2.8: Cell cycle. Passage of the cell from G1 phase to the S phase is controlled by the restriction point. Once through the restriction point, the cell is committed to DNA replication, which occurs in the S phase. On the other hand, the G2 ensures that the newly replicated DNA is ready for segregation into daughter cells. In the M phase, the nucleus and then, the cytoplasm divides. Figures adapted from Lambert *et al*. (2009).

2.3.2 Regulation of cell cycle

The events of cell cycle are regulated by an independent biochemical control system that possesses a number of important features called checkpoints as high degree of precision required for faithful DNA replication and cell division. These checkpoints act as "clock or timer" turning on each event at a specific time and provide adequate time for their completion (Figure 2.9). Checkpoint signaling pathways exist in the cell cycle to maintain proper order and timing of events. These checkpoints function to allow time for a process (e.g. DNA replication or repair) to be completed after some types of DNA damage repair is also induced (Rhind & Russell, 2000).

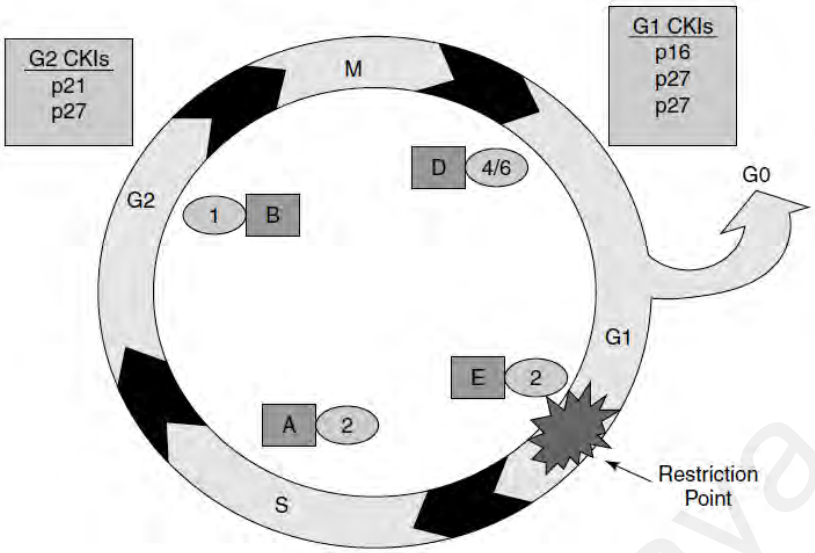
The elements responsible for driving the cell cycle from one phase to the next are a series of protein kinases and phosphatases that activate and deactivate each other. The cyclin-dependent kinases (Cdks) are responsible for phosphorylating various substrates critical to cell cycle progression. The levels of Cdks are varied throughout the cell cycle but their activities are modulated by their interaction with another set of proteins called cyclins, whose levels fluctuate. In general, the cyclin Ds are associated with G1, cyclin Es with the G1 to S transition, cyclin As with S phase as well as with the G2 to M transition, and cyclin Bs with the G2 to M transition. The presence of Cdk-cyclin complex does not ensure activity; however, as the Cdk-cyclin complexes can be inhibited by the Cdk inhibitors (Hung *et al*., 1996; Malumbres & Barbacid, 2009; Lim & Kaldis, 2013).

The G1 is a critical point at which the cell access whether it should enter another full round of division (Bertoli *et al*., 2013). The progression of G0 to G1 is triggered by the binding of extracellular growth factors to specific cell-surface receptors. Signal transduction cascades downstream of these receptors then activate various intracellular processes required for proliferation, including the transcription and translation of many

cell cycle-dependent factors. Among these are the G1 cyclins (predominantly the cyclin D family) that form active complexes with various Cdks. The Cdk-cyclin complexes release a brake on the cell cycle, allowing it to progress forward into S phase. Driven by the Cdk-G1 cyclin complexes, the cell progresses into S phase where a complicated assembly of DNA polymerase, primases, helicases, topoisomerases, and accessory factors duplicate the genome. Upon completion of DNA synthesis, the cell progresses through G2, a period of active protein synthesis in preparation for cell division. In mitosis, following activation of a Cdk-cyclin complex (Cdc2-cyclin B) and condensation of the chromosomes, the paired sister chromatids must be pulled apart by the spindle into two distinct daughter cells (Hung *et al*., 1996).

The cell's ability to complete a full cell cycle is confirmed at a critical point at the G1/S interphase prior to committing to the S phase. Besides, a distinct G2 checkpoint may be activated by inhibiting topoisomerase II. Selective inhibition of topoisomerase II, in the absence of DNA damage, results in G2 arrest at a checkpoint that monitors the ability to condense chromosomes (Hung *et al*., 1996; Larsen *et al*., 1996). Checkpoints are also intimately tied to the regulatory events that drive or halt the cycle; for example, p53 arrest seems to be mediated by a family of endogenous CDIs that negatively regulate or halt the cell cycle. p53 induces the transcription and subsequent expression of a 21 kDa protein (known as p21) in response to DNA damage (Shaw, 1996). Then, p21 interacts with Cdks in complex with different cyclins, preventing their ability to phosphorylate and activate substrates necessary for G1 progression. The related CD1, p27 is also capable of causing G1 arrest (Harada & Ogden, 2000; Besson *et al*., 2008). Mammalian cells also arrest in G2 in response either to DNA damage sustained during S phase, or incomplete DNA replication (Hung *et al*., 1996).

(A)



(B)

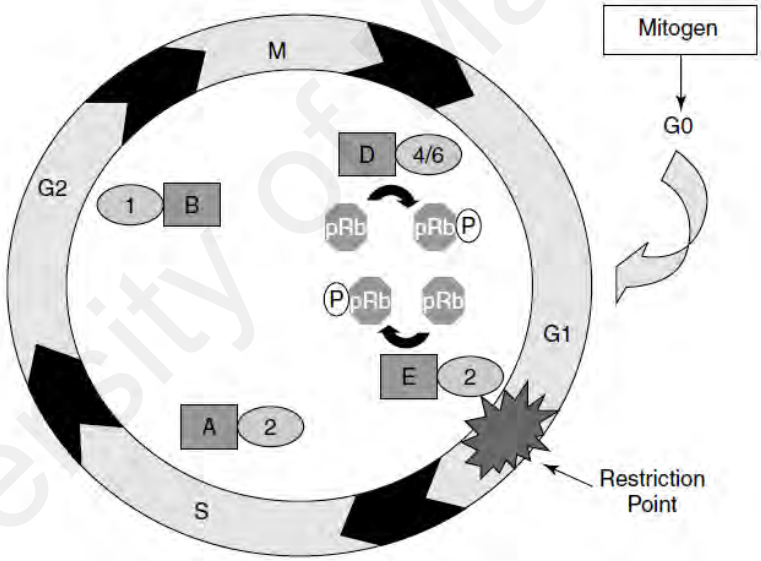


Figure 2.9: Regulation of cell cycle. (A) Progression through the cell cycle is dependent on the cyclin-dependent kinase (Cdk) proteins (ovals) that are active only when bound to a phase-specific protein (squares). The functions of the Cdks can be inhibited by the cyclin-dependent kinase inhibitors (CKIs). (B) Cell cycle and the restriction point. In response to stimulation of a G0 cell, cyclin D assembles with the G1-specific Cdks (i.e. Cdk4 and Cdk6). The cyclin D-Cdk complexes then phosphorylate and deactivate the retinoblastoma tumour suppressor protein (pRb). pRb deactivation allows the synthesis of cyclin E that will form complexes with Cdk2. Hence, the cell progresses through G1 into the S phase. These events form the basis of the restriction point. Figures taken from Lambert *et al*. (2009).

2.4 Medicinal mushrooms

2.4.1 Background

In many cultures worldwide, mushrooms are recognized to have profound health promoting effects. Some mushrooms have a long history of use in traditional oriental therapies especially in Japan, China, Korea and other Asian countries (Smith *et al*., 2002). Studies have shown that a number of mushrooms, such as *G. lucidum* (lingzhi mushroom) and *I. obliquus* (chaga mushroom), have been utilized traditionally in many different cultures for the maintenance of health, as well as prevention and treatment of diseases (Valverde *et al*., 2015). The use of wild mushrooms as folk medicine for treating cancer among the indigenous communities in Southeast Asia has been documented in various ethnomycological records (Chang & Lee, 2004; Lee *et al*., 2009a; Azliza *et al*., 2012; Mohammad *et al*., 2012).

Mushrooms are considered as untapped potential sources of therapeutic compounds for treatment and prevention of cancer. According to Wasser (2002), there are approximately 650 species of mushrooms that have been found to possess anti-tumour activity. There have been extensive *in vivo* studies demonstrating the anti-cancer effects of the mushroom active components, mainly in the form of polysaccharides and other high-molecular-weight components (Wasser & Weis, 1999). In fact, some of these polysaccharides have proceeded through Phase I, II, and III clinical trials in Japan and China. These polysaccharides are also being used as adjuvant treatments with conventional chemotherapy/radiotherapy where their intake have been shown to reduce the side-effects encountered by patients and hence, increase the quality of life of cancer patients and may even offer increased survival rates for some types of cancers (Kidd, 2000). Besides, there are also increasing experimental evidences that regular

incorporation of certain powdered medicinal mushrooms in the diets of animals or topical application of extracts can have chemopreventive effect (Smith *et al*., 2002).

2.4.2 Bioactive compounds and mechanisms of actions

Some of the bioactive compounds derived from medicinal mushrooms are considered as potential source of anti-cancer agents. Bioactive compounds from mushrooms which demonstrated *in vitro* and *in vivo* anti-cancer effects can be broadly divided into the low-molecular-weight and high-molecular-weight components. Both classes of chemical components have different characteristics and differ in their mechanism of action (Zaidman *et al*., 2005; Ferreira *et al*., 2010).

2.4.2.1 Low-molecular-weight compounds

Mushrooms are known to produce an array of low-molecular-weight compounds with anti-cancer potential. While most of the reported compounds are secondary metabolites, a few primary metabolites, e.g. fatty acids, have also been noted to exert anti-cancer effect. As primary metabolites are widely distributed in nature and can be found in most species, these are often not prioritized for further research. On the other hand, secondary metabolites are not involved in the central metabolic processes of the fungi (i.e. the generation of energy and the formation of the building blocks of proteins, nucleic acids, etc.) and most have no known physiological roles in the organism. Secondary metabolites are derived from intermediates from primary metabolism and these can be classified according to five main metabolic pathways, including (i) the shikimic acid pathway for biosynthesis of aromatic amino acids, (ii) the acetate-malonate pathway from acetyl coenzyme A, (iii) the mevalonic acid pathway, and (iv) the amino acids-derived pathways (Zaidman *et al*., 2005).

The commonly studied secondary metabolites from mushrooms with anti-cancer potential belonged to the classes of phenolics, polyketides, triterpenoids, steroids, and others. These secondary metabolites usually target processes such as apoptosis, cell cycle, metastasis, angiogenesis, and specific signal transduction cascades. Mechanistically, low molecular weight secondary metabolites from medicinal mushrooms can inhibit or trigger specific responses, i.e., activating or inhibiting the NF-κB, inhibiting proteins, especially tyrosine kinases, aromatase and sulfatase, matrix metalloproteinases, cyclooxygenases, DNA topoisomerases and DNA polymerase, anti-angiogenic substances, etc. (Zaidman *et al*., 2005; Ferreira *et al*., 2010; De Silva *et al*., 2012; Patel & Goyal, 2012).

Secondary metabolites isolated from mushrooms have been shown to exhibit cytotoxicity and induce apoptosis in human cancer cells. A notable example is ergone, a steroid isolated from the sclerotium of *Polyporus umbellatus* (Pers.) Fr. and displayed remarkable cytotoxic activity against selected cancer cell lines (e.g. Hep G2, Hep-2, and Hela) without affecting the normal cells (i.e. human umbilical vein endothelial cells, HUVEC). The effect was attributed to cell cycle arrest at the G_2/M stage and induction of apoptosis that was evidenced by chromatin condensation, nuclear fragmentation, and externalization of phosphatidylserine. Moreover, both intrinsic and extrinsic apoptotic pathways were involved based on the activation of caspase-3, -8, -9, up-regulation of Bax, down-regulation of Bcl-2, and the cleavage of PARP (Zhao *et al*., 2011a).

Other compounds were able to inhibit DNA topoisomerases or target angiogenesis. According to Mizushina *et al*. (2004), dehydroebriconic and dehydrotrametenoic acids isolated from *Wolfiporia cocos* (F.A. Wolf) Ryvarden & Gilb. strongly inhibited DNA topoisomerase II activity (IC_{50}: 4.6 μM). In addition, both compounds moderately inhibited the activities of mammalian DNA polymerases α, β, γ, δ, ε, η, κ and λ. On the

other hand, Lin *et al*. (2014) reported that the phenolics-rich, aqueous ethanol extract of *Lentinus tuber-regium* (Fr.) Fr. sclerotium (containing protocatechuic, chlorogenic, syringic, ferulic, and folic acids) inhibited the vascular endothelial growth factor (VEGF)-induced HUVEC migration and tube formation *in vitro*, as well as the formation of subintestinal vessel plexus in zebrafish embryos *in vivo*.

2.4.2.2 High-molecular-weight components

The high molecular weight compounds found in medicinal mushrooms are mainly polysaccharides, polysaccharide-protein complexes (e.g. glycoproteins, glycopeptides, and proteoglycans) and proteins. These are produced through primary metabolism and essential for their continuous growth and biomass production and regarded as integral constituents of the fungal fruiting body as well as the mycelium. Several polysaccharides from medicinal mushrooms that have shown clinically significant efficacy against human cancers are (i) lentinan from *L. edodes*, (ii) D-fraction from *G. frondosa*, (iii) schizophyllan from *S. commune*, (iv) PSK (krestin) and (v) PSP (polysaccharide peptide) from *T. versicolor* (Wasser, 2002; Zaidman *et al*., 2005, Ferreira *et al*., 2010; De Silva *et al*. 2012). The isolation process, structural characteristics, and anti-tumour activities of known mushroom polysaccharides have been reviewed (Daba & Ezeronye, 2003; Zhang *et al*., 2007).

These high molecular weight compounds are able to stimulate the non-specific immune system and exert anti-tumour activity through the stimulation of the host's defense mechanism. Mechanistically, they could activate effector cells, such as macrophages, T lymphocytes and NK cells to secrete cytokines like TNF-α, IFN-γ, IL-1β which exhibit anti-proliferative activity, induce apoptosis, and differentiation in cancer cells (Ooi & Liu, 2000). Since the biological activities of these high molecular

weight polysaccharides is related to their immunomodulating effect and enhancement of the person's defense system against various diseases, they are sometimes called "immunopotentiators" or "biological response modifiers" (Wasser, 2002; Zaidman *et al*., 2005). Moradali *et al*. (2007) summarized the anti-cancer activities of mushroom polysaccharides from immunological perspectives, mainly looking at their effects on innate and adaptive immunity as well as the mechanisms of activation of immune responses and signaling cascade.

The anti-cancer activities of mushroom polysaccharides are affected by several factors like degree of branching, molecular weight, number of substituents, presence of single and triple helices, and others. Studies have shown that higher anti-tumour potential seems to be correlated with high molecular weight, lower level of branching and greater solubility in water (Lemieszek & Rzeski, 2012; Ren *et al*., 2012). According to Zhang *et al*. (2007), chemical modification might enhance the anti-tumour properties of mushroom polysaccharides; for instance, Tao *et al*. (2009) extracted two water-soluble polysaccharide-protein complexes from the sclerotium of *L. tuber-regium* and chemically modified to obtain their sulfated and carboxymethylated derivatives which both showed good cytotoxicity against cancer cells. In another study by Wang *et al*. (2004), a water-insoluble (1→3)-β-glucan isolated from the sclerotium of *W. cocos* was sulfated, carboxymethylated, methylated, hydroxyethylated, and hydroxypropylated, respectively, to afford five water-soluble derivatives. They reported that the native β-glucan did not show anti-tumour activity, whereas the sulfated and carboxymethylated derivatives exhibit significant anti-tumour activities against S-180 and gastric carcinoma tumour cells.

2.5 Bioprospecting of medicinal mushrooms

The term "mushroom nutraceutical" was initially coined to include "refined or partially refined extract or dried biomass from either the mycelium or fruiting bodies, which is consumed in the form of capsules or tablets as dietary supplements (not food) and has potentially therapeutic applications" (Wasser & Akavia, 2008). Several types of mushroom products are available in the market today and these include (i) powdered fruiting bodies and the hot aqueous or alcoholic extracts of these, (ii) dried and powdered preparations of the combined mycelium, substrate, and mushroom primordia after inoculation of edible semisolid medium, usually grains, (iii) biomass or extracts from mycelium harvested from liquid fermentations, and (iv) naturally-growing, dried fruiting bodies in the forms of capsules and tables (Wasser, 2014). Taken into consideration their health-promoting effects and long history of use as traditional remedies for various physiological disorders, there is indeed a potential to develop mushrooms as dietary supplements or functional foods. Nevertheless, it should be noted that there are also some problems associated with the use of mushroom-based dietary supplements, such as chemical variability (e.g. potency of samples from different developmental stages and/or cultivation techniques and presence of bioactive compounds in different preparations) and the lack of standards for production and testing protocols necessary to guarantee product quality.

2.5.1 Mushroom samples from different developmental stages

In order to understand the relevance of samples from different developmental stages, an understanding of the life cycle of a mushroom (Figure 2.10) is essential. Briefly, it begins with the release and dispersal of spores. When the conditions are favourable, the spores germinate sending out tiny threads called hyphae (single, hypha). When two compatible hyphae meet, they fuse together to form a network or threads called a

mycelium. This mycelium eventually forms a hyphal knot which grows and develops into a pinhead. The pinhead, in turn, develops into a fruiting body. The formation of sclerotia is also observed in some species.

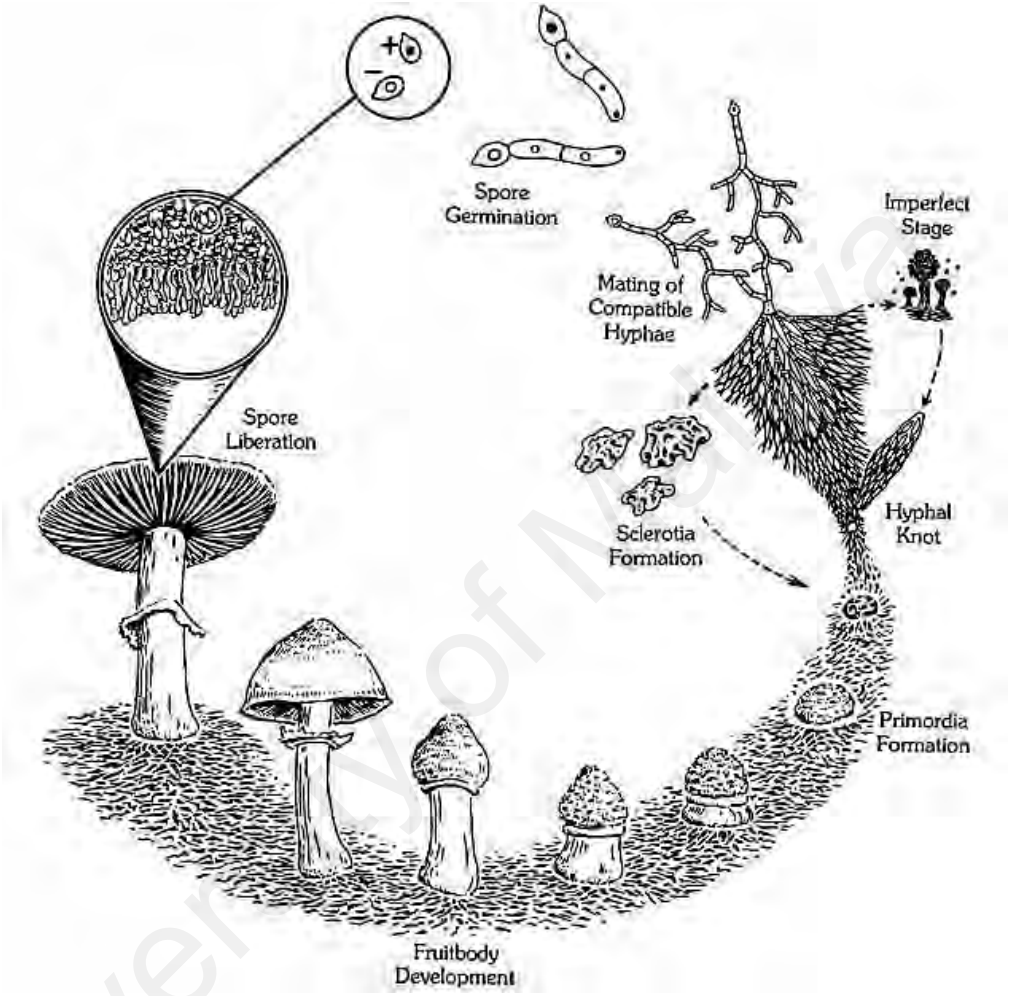


Figure 2.10: Mushroom life cycle. Figure taken from Stamets (2000).

Previous studies have revealed the differences in the bioactivities and chemical profiles of extracts prepared from various developmental stages and/or morphological forms of mushrooms, including fruiting bodies, sclerotia, mycelia, culture filtrate, and spores (Wong *et al*., 2009; Xu *et al*., 2011a; Carvajal *et al*., 2012; Heleno *et al*., 2012; Reis *et al*., 2012). The different physiological roles of these stages, e.g. mycelium (vegetative stage) and fruiting bodies (reproductive stage) might explain the differences

in the chemical profiles. Bioactive compounds produced in the fruiting bodies may not be produced in mycelial cultures and vice versa; therefore, for bioprospecting, all developmental stages of a mushroom species should be thoroughly investigated.

2.5.2 Mushroom samples from different origins and cultivation techniques

Mushroom fruiting bodies are either cultivated or collected from the wild. The overwhelming majorities of mushrooms used in the production of dietary supplements are cultivated commercially rather than collected from the wild. The main reason is that the quality of wild fruiting bodies is considerably diverse and could be affected by many factors (e.g. geographical conditions, substrates, humidity, etc.), whereas artificially cultivated mushrooms guarantees proper identification and pure, unaltered products (Wasser & Akavia, 2008).

Medicinal mushrooms can be cultivated through a variety of methods. Some methods are extremely simple and demand little or no technical expertise, while other cultivation techniques that require aspects of sterile handling technology are technically more demanding (Stamets, 2000). For the production of mushroom fruiting bodies, various forms of solid substrate or low moisture fermentations are employed whereas, for mycelial biomass production, liquid tank fermentations are now becoming increasingly important especially for the nutraceutical and pharmaceutical industries. As the cultivation of fruiting bodies by solid-substrate fermentation is a long process (e.g. usually up to several months), the growth of mycelium in liquid fermentation allows one to accelerate the speed of growth and reduces the duration to several days (Wasser & Akavia, 2008). The principles of mushroom cultivation *via* solid substrate and liquid fermentations are briefly discussed below.

2.5.2.1 Solid substrate fermentation

Since mushrooms are excellent degraders of lignocellulosic materials, they can use wood as substrates for mycelial growth and subsequent formation of fruiting bodies. In mushroom cultivation, substrates in the form of agroresidues supplemented with essential nutrients are packed in bags and autoclaved. On cooling, these are inoculated with mushroom spawn, usually in the form of young mycelium. Aseptic environment is required until the substrate is fully colonized by mycelium. After that, the bags opened and subjected to mild stress to induce the formation of fruiting bodies (Smith *et al*., 2002). For sclerotium-forming mushrooms, the mycelium-colonized substrates have to be buried for the sclerotium to form. The specific growth requirements and environmental conditions, such as temperature, relative humidity, light, and air flow have to be optimized for different species and cultivation stages, viz. spawn run, primordium initiation and fruiting body development. Almost all edible-medicinal mushroom fruiting bodies can be produced by modifications of this method. More details on the production of mushrooms using solid substrate fermentation are available elsewhere (Yamanaka, 1997; Chang, 2008).

2.5.2.2 Liquid fermentation

Liquid fermentation has been developed for most medicinal mushrooms. The underlying principle involves the use of mycelium in the active physiological state and of known purity. Liquid fermentation has been used in the propagation of mycelium for liquid spawn for solid substrate fermentation for production of fruiting bodies. The mycelium biomass and the extracellular products (e.g. secreted metabolites, exopolysaccharides, etc.) can be used in the nutraceutical and pharmaceutical industries. (Tang *et al*., 2007). Liquid fermentation can be carried out in flasks under shaken or static conditions, and bioreactor for small- and large-scale productions, respectively.

Some of the physical and chemical factors (e.g. inoculum size, pH, composition of nutrients, aeration and temperature) which can affect mycelial growth rate, biomass yield, and metabolite production have to be optimized (Tang *et al*., 2007; Elisashvili, 2012). The use of liquid fermentation for the production of mycelium biomass is particularly important for mushrooms species with limited availability and difficult to be cultivated, e.g. *Termitomyces* spp. (termite mushrooms). In addition, liquid fermentation enables the production of extracellular chemical components that are usually secreted into the culture broth; for instance, the two most widely used anti-cancer polysaccharides, PSK and PSP from *T. versicolor* produced respectively in Japan and China, have been produced by liquid fermentations (Smith *et al*., 2002). Submerged cultivation of mushrooms has significant industrial potential, but its success on a commercial scale relies on increasing product yields (Elisashvili, 2012).

2.5.3 Chemical constituents and mode of action

A central feature of the purported medicinal or chemopreventive role of crude mushroom extracts is the synergistic interaction of the multiple chemical constituents in the mixture. When such complex mixtures are submitted to fractionation the active ingredients are separated. Consequently, the bioactivity may be lost and such observation seemed to suggest that the activity results from the presence of multiple active principles which must be together to create the desirable response.

Besides, there are limited studies that address issues like the effectiveness of dried fruiting bodies and mycelium powders as compared to hot water, alcoholic or hydro-alcoholic extracts and fractions given the vast variety of mushroom-based products in the market. Although the use of mushrooms in anti-cancer therapies has promise, there are few conclusive mechanistic studies of their constituents and evidence for immuno-

stimulatory action is still considered to be hypothetical, especially where *in vivo* action is concerned. It has also been pointed out that some high-molecular-weight components (e.g. glucans) may not exhibit favorable pharmacological and pharmacokinetic properties (Wasser, 2014).

2.5.4 Safety consideration

The presence of therapeutic activities implies a lack of safety at sufficiently high dose. This means that mushroom preparations, like any pharmacodynamics agent, cannot have pharmacological action without toxicological effects. Conversely, a completely safe agent would be without any activity whatsoever (Wasser & Akavia, 2008). In addition to the direct toxicity of some chemical components, nutrient supplementation can cause problems related to nutrient imbalances or adverse interactions with medications. The absence of insufficiently elaborated standards for the recommended use of medicinal mushrooms and their products, including precise doses and duration of administration, require serious attention. There are some studies that showed too high a dose could lead to immune suppression but too low a dose might not trigger an immune response. To address the safety issues and enhance the quality of mushroom products, Chang (2006) has proposed the following guidelines – Good Laboratory Practice (GLP), Good Agriculture Practice (GAP), Good Manufacturing Practice (GMP), Good Production Practice (GPP), and Good Clinical Practice (GCP) and critically discussed the relevance of these practices in the mushroom industries.

2.6 *Lignosus* spp. (tiger's milk mushrooms)

2.6.1 Background

The sclerotia of mushrooms belonging to the genus *Lignosus* (Polyporaceae) are considered as valuable folk medicine by the local communities in Southeast Asia. In the Malay states (present-day Peninsular Malaysia) and Indonesia, they are known locally as *susu rimau* (in Malay/Indonesian) – literally "tiger's milk" (Burkill, 1966; Werner, 2002). As the name suggests, the mushroom is believed to grow from the spot where a prowling tigress dropped her milk during lactation; appearance-wise, the sclerotia are said to resemble congealed tiger's milk (Ridley, 1890; Corner, 1989; Chang, 2002). Based on earlier studies, *susu rimau* collected from different locations in Peninsular Malaysia were identified as *Lignosus rhinocerotis* (as '*rhinocerus*') (Cooke) Ryvarden based on morphological characteristics and molecular analysis (Tan *et al*. 2010). With the discovery of two new *Lignosus* spp. recently, i.e. *L. tigris* Chon S. Tan and *L. cameronensis* Chon S. Tan (Tan *et al*., 2013), it is suggested that *susu rimau* or tiger's milk mushrooms (TMM) might not refer to *L. rhinocerotis* exclusively, but *Lignosus* spp. collectively due to the gross similarity in the sclerotia and pilei. Nevertheless, *L. rhinocerotis* was reported to be the most commonly occurring member of *Lignosus* in Malaysia (Choong *et al*., 2014). Interestingly, it was also found in China where it is called *hurulingzhi* (in Chinese) – literally "tiger milk *Ganoderma*" (Huang, 1999).

2.6.2 Taxonomy

The genus *Lignosus* Lloyd ex Torrend comprised of members with centrally stipitate basidiocarps arising from subterranean sclerotia. These species have trimitic hyphal structure with clamped generative hyphae and hyaline, cylindrical to ellipsoid basidiospores (Ryvarden & Johansen, 1980). At the moment, eight *Lignosus* spp. have been discovered, and they are *L. dimiticus* Ryvarden, *L. ekombitii* Douanla-Meli, *L.*

goetzii (Henn.) Ryvarden, *L. sacer* (Afzel ex Fr.) Ryvarden, *L. rhinocerotis*, *L. hainanensis* B.K. Cui, *L. tigris*, and *L. cameronensis*. Morphological characteristics such as sizes of the pores and basidiospores are commonly used for species identification of TMM in Malaysia; for instance, *L. rhinocerotis* (7-8 pores/mm) is separated from *L. sacer* (1-3 pores/mm) by the smaller pores and spores. Detailed macroscopic (e.g. pileus, stipe, and sclerotium) and microscopic descriptions (e.g. hyphal system, cystidia, cystidioles, basidiospores, etc.) of *L. rhinocerotis* are available elsewhere (Corner, 1989; Ryvarden & Johansen, 1980). The taxonomical classification of *L. rhinocerotis* is as follows:

Kingdom	:	Fungi
Class	:	Basidiomycetes
Subclass	:	Agaricomycetidae
Order	:	Polyporales
Family	:	Polyporaceae
Genus	:	Lignosus
Species	:	*rhinocerotis* (as '*rhinocerus*')

Taxonomically, *L. rhinocerus* is considered to be an orthographic variant (spelling variant) for *L. rhinocerotis*. Synonyms for *L. rhinocerotis* include *Fomes rhinocerus* Cooke, *Microporus rhinocerus* (Cooke) Imazeki, *Polyporus rhinocerus* Cooke, *Polyporus sacer* var. *rhinocerotis* (Cooke) Lloyd, *Polystictus rhinocerus* (Cooke) Boedijn, and *Scindalma rhinocerus* (Cooke) Kuntze.

2.6.3 Biology, geographical distribution, and habitat

Structurally, *L. rhinocerotis* is characterized by a central pileus that is shallowly convex to flat, almost circular but sometimes lobed. The surface is velvety when young

but eventually becomes hard, often wrinkled, and dark brown. The pileus is supported by hard and woody stipe that emerged from a large, underground sclerotium (Figure 2.11). The sclerotium might be spherical, oval, or with irregular shape. It has a rough and wrinkly outer surface whereas the internal structure is white and powdery (Wong & Cheung, 2008a; Roberts & Evans, 2011).

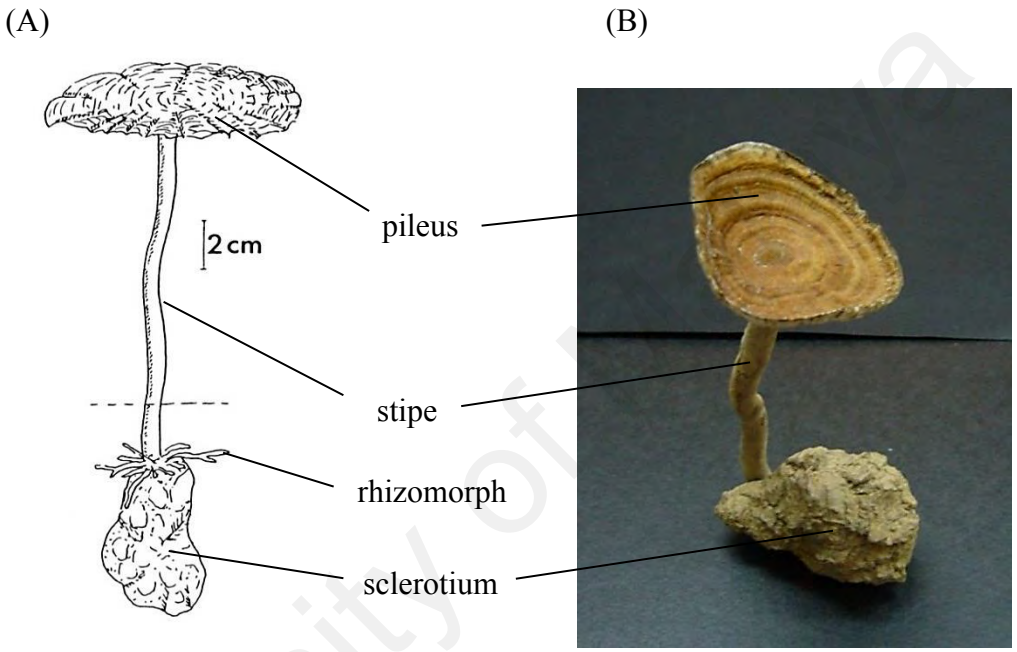


Figure 2.11: Morphological appearances of *L. rhinocerotis*. (A) The fruiting body (consisting of pileus and stipe) of a typical *Lignosus* sp. emerged from a sclerotium buried underground. Figure taken from Ryvarden & Johansen (1980). (B) Herbarium specimen of *L. rhinocerotis* (KUM61075) collected from the Kenaboi Forest Reserve, Negeri Sembilan, Malaysia.

In terms of geographical distribution (Figure 2.12), *L. rhinocerotis* has been reported to be found in in Peninsular Malaysia, Borneo, Thailand, the Philippines, China, Japan, Sri Lanka, India, New Guinea, Australia and East Africa (Bakshi, 1971; Huang, 1999; Wong & Cheung, 2008a; Yokota, 2011; Gani, 2013). In Malaysia, *L. rhinocerotis* was found in the Cameron Highlands, the Genting Highlands, and Kuala Lipis in Pahang (Tan *et al*., 2010; Wong, 2011).

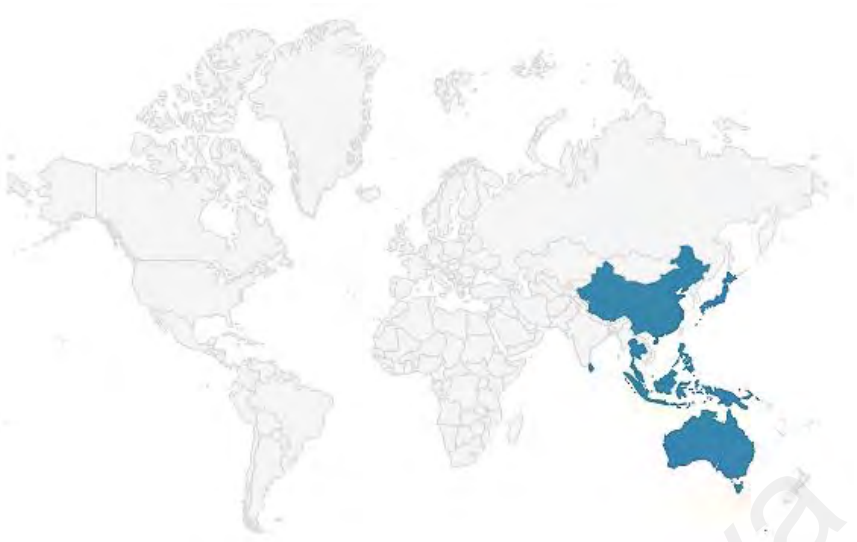


Figure 2.12: Geographical distribution of *L. rhinocerotis*. Figure was retrieved from http://iucn.ekoo.se/iucn/species_view/534962/.

The fruiting bodies of *Lignosus* spp. grow from sclerotium in the ground (Figure 2.13) rather than from wood as in the case with most polypores (Lepp, 2003). In its natural habitat, the sclerotium of *L. rhinocerotis* is formed within rotten wood of fallen-tree trunk and can continue to survive and produce fruiting body even long after the trunk has rotten away (Pegler, 1997).

Figure 2.13: *Lignosus rhinocerotis* in its natural habitat. It is difficult to locate the sclerotium until the formation of a fruiting body. Figure was retrieved from http://iucn.ekoo.se/iucn/species_view/534962/.

2.6.4 Ethnomedicinal uses

The use of TMM in the Malay states (present-day Peninsular Malaysia) as folk medicine date back in early 1900s whereby the sclerotia were valued by the Malays in the treatment of tuberculosis, asthma, cough, and chest complaints (Ridley, 1890; Gimlette & Thomson, 1939; Heyne, 1978; Ibrahim, 2002). Chee (1933) reported that TMM were also consumed as tonic to maintain health and to cool the body. The ethnomedicinal importance of *L. rhinocerotis* is clearly manifested by the findings by Lee *et al*. (2009a) which revealed that this mushroom could be recognized by more than 70% of the Orang Asli (indigenous people of Peninsular Malaysia) communities surveyed. There were about 15 uses of this mushroom according to different tribes of Orang Asli and these include to treat fever, cough, asthma, cancer, food poisoning, and to strengthen weak body (Burkill, 1966; Chang & Lee, 2004; Lee *et al*., 2009a; Chang & Lee, 2010). Noorlidah *et al*. (2009) noted that there are strong claims that TMM can be used in treating cervical and breast cancer. Hattori *et al*. (2007) mentioned that TMM are used as an energy food to revitalize the body and to stave off hunger. Sabaratnam *et al*. (2013a) pointed out that infusions prepared from TMM can improve vitality, increase alertness, energy, and overall wellness of the individual. It has to be mentioned that the use of TMM is not restricted to the indigenous communities but these are among the most popular wild mushrooms used for medicinal purposes among the urban population in Malaysia.

Findings from these ethnomycological surveys have provided useful information on the utilization of TMM for medicinal properties, including specific preparation methods which, most of the time, depend on the ailments intended to be treated (Chang & Lee, 2010; Azliza *et al*., 2012; Mohammad *et al*., 2012). Sclerotium is the part of the mushroom that is used as folk medicine. From the ethnomedicinal perspective, different

preparation methods of TMM have been documented and it appeared that TMM are usually consumed in the form of aqueous preparations (e.g. decoction) but non-aqueous preparations (e.g. tincture) have been reported too. One of the oldest records was provided by Chee (1933) who described a method to prepare a drink from TMM:

"Place a few drops of water on a *batu piring* (granite plate). Hold the ball of "congealed milk" with the thumb and two fingers. Press it on the plate and begin to rub with a forward and backward motion. Continue for about five minutes then drain off the water which now looks rather thick. This water or "syrup" is put in a glass, diluted with drinking water and is ready for use."

The use of decocted materials, often with other herbs, has been recorded in some ethnomycological surveys (Chang & Lee, 2004; Azliza *et al*., 2012). Further, there are records on specific preparation methods for communities from certain locations, for instance, TMM are chewed in betel-quid by the Malays in Pahang for the relief of cough (Gimlette & Thomson, 1939; Werner, 2002). In Kelantan, where TMM are given after childbirth, they are pounded with rice, infused, and drunk (Burkill, 1966). Although most reports pointed to the sclerotium as the only part with medicinal value, interestingly, Rolfe and Rolfe (1925) mentioned that the "whole plant" (presumably referred to the fruiting body and sclerotium) was utilized (consumed) for treating tuberculosis and colds.

2.6.5 Pharmacological activities

Over the last five years, there are numerous attempts to scientifically validate the ethnomedicinal uses of TMM; however, most studies were done on *L. rhinocerotis* possibly due to its availability as compared to other *Lignosus* spp. Specifically, the anti-

cancer, immunomodulatory, and anti-inflammatory effects of *L. rhinocerotis*, in accordance to its traditional uses, received the most attention.

The purported anti-cancer effect of *L. rhinocerotis*, *via* cytotoxicity and apoptosis-inducing activities as well as modulation of the immune system, has been investigated. The *in vitro* cytotoxic effect of *L. rhinocerotis* against panels of cells from leukemia and solid-tumours have been extensively studied (Lai *et al*., 2008; Abdullah *et al*., 2010; Lee *et al*., 2012; Suziana Zaila *et al*., 2013; Yap *et al*., 2013). In most cases, cell death was attributed to the cell cycle arrest and induction of apoptosis.

At *in vivo* level, Wong *et al*. (2011) examined the immunomodulatory effect of two *L. rhinocerotis* sclerotial polysaccharides, i.e. PRW (polysaccharide-protein complex) and PRS (β-glucan), in normal BALB/c and athymic nude mice. Their results demonstrated that PRS activated neutrophils whereas PRW stimulated a wide range of innate immune cells. The differences in the responses were attributed to structural differences between the two polysaccharides.

In addressing the anti-inflammatory activity, Lee *et al*. (2014) reported that the cold aqueous extract of the sclerotial powder of *L. rhinocerotis* cultivar TM02 possessed potent anti-acute inflammatory activity as measured by carrageenan-induced paw edema test. The activity was mainly contributed by the high molecular weight fraction of the extract. At the *in vitro* level, the anti-inflammatory activity of *L. rhinocerotis* cultivar TM02 was demonstrated by the inhibition of lipopolysaccharide induced TNF-α production in RAW 264.7 macrophages

Besides that, there are also reports on biological activities that are not directly related to their ethnomedicinal uses, such as anti-oxidant capacity (Yap *et al*., 2013; Lau *et al*., 2014), anti-microbial (Mohanarji *et al*., 2012), neurite-outgrowth stimulatory activity

(Eik *et al*., 2012; John *et al*., 2013), fibrinolytic, and anti-coagulant activities (Sabaratnam *et al*., 2013b). Taken together, their findings indicated that *L. rhinocerotis* indeed possesses a broad spectrum of medicinal properties.

Overall, findings of the pharmacological activities of *L. rhinocerotis* thus far are mostly preliminary in nature as the exact active compound(s) and plausible molecular mechanism of action has yet to be delineated. Regarding mushroom parts used, most studies utilized the sclerotium of *L. rhinocerotis* and very few reports dealt with the bioactivities of mycelium and culture broth from liquid fermentation, both of which might be alternatives to the sclerotium.

2.6.6 Toxicological studies

Current findings from several toxicity assessments revealed that cultivated *L. rhinocerotis*, in the forms of mycelium and sclerotium, are safe for consumption. According to Lee *et al*. (2013), the no-observed-adverse-effect level (NOAEL) dose of the sclerotial powder *of L. rhinocerus* cultivar TM02 in 180-day chronic toxicity study is more than 1000 mg/kg. Oral feeding of the sclerotial powder at 100 mg/kg did not induce adverse effect on rats' fertility nor causing teratogenic effect on their offspring. Similarly, Chen *et al*. (2013) demonstrated that *L. rhinocerotis* mycelium produced by liquid fermentation did not provoke mutagenicity and genotoxicity based on the results of Ames test, *in vitro* chromosome aberration test in Chinese Hamster Ovary (CHO-K1) cells and *in vivo* erythrocyte micronucleus test in ICR mice.

2.6.7 Chemical investigations

Earlier work mostly focused on the nutritional attributes of *L. rhinocerotis* sclerotium (Wong *et al*., 2003; Lai *et al*., 2013; Yap *et al*., 2013) as well as the

physicochemical and functionalities of the sclerotial dietary fibers (Wong & Cheung, 2005a; Wong & Cheung, 2005b; Wong *et al*., 2005; Wong *et al*., 2006). The medicinal properties of TMM are due to the presence of bioactive components which might range from low- to high-molecular-weight compounds. According to previous studies, the major components in *L. rhinocerotis* sclerotial aqueous extracts are polysaccharides (e.g. β-glucans), proteins/peptides, and/or polysaccharide-protein complexes; hence, these were suspected to be responsible for various pharmacological activities, such as anti-proliferative (Lai *et al*., 2008; Lee *et al*., 2012), immunomodulatory (Wong *et al*., 2011), and anti-inflammatory (Lee *et al*., 2014) activities. Reports on the low-molecular-weight compounds from *L. rhinocerotis* are still limited.

2.6.8 Artificial cultivation

TMM are not often readily available due to its growth characteristics, i.e. sclerotia might remain dormant for months or years until the fruiting bodies sprout out when conditions become favourable. To overcome the supply problem (mainly from the wild), it is of paramount importance to establish suitable cultivation methodology for the TMM. When comparison is made with other sclerotium-forming mushrooms, information on the cultivation aspects of *L. rhinocerotis* is limited. Huang (1999) demonstrated the first successful cultivation of *L. rhinocerotis* sclerotium. There are also attempts to grow *L. rhinocerotis* mycelium on agar plates (Lai *et al*., 2011) and in culture media using liquid fermentation (Lau *et al*., 2011; Rahman *et al*., 2012; Chen *et al*., 2013; Lai *et al*., 2014).

CHAPTER 3: CULTIVATION OF *LIGNOSUS RHINOCEROTIS* AND ANALYSIS OF ITS CHEMICAL COMPOSITION

3.1 Introduction

Although worldwide production of mushrooms has progressively improved during the last decade, cultivation of sclerotium-forming mushrooms has yet to reach commercially viable scale presumably due to limited understanding of their life cycle and ecological requirements. Artificial cultivation by solid substrate fermentation of agroresidues is routinely utilized to produce the fruiting bodies and sclerotia; however, the time required for this process might be relatively long (e.g. several months to a year) depending on the species and substrates used. Alternatively, liquid fermentation can be used to produce the mycelia and culture broth under controlled environment in a shorter period of time. For exploration of mushroom sclerotia for the nutraceutical and pharmaceutical industries, research on the cultivation aspects needs to be comprehensively conducted so that the scale and efficiency of production can be improved and their commercialization facilitated (Wong & Cheung, 2008a).

At present, the main source of *L. rhinocerotis* is still wild sclerotium collected by the indigenous communities in Peninsular Malaysia. The mushroom exists solitarily and often difficult to be encountered. As the supply is limited, it is highly priced, ranging from USD 15-25 per fruiting body inclusive of the sclerotium; however, most of the time, only the sclerotium (without the fruiting body) is available. To overcome these problems, domestication of *L. rhinocerotis* is warranted. Apart from an initial study by Huang (1999) who reported on the production of sclerotium using agroresidues, so far, there is little success in attempts to cultivate this mushroom, especially its fruiting body. Optimization of the cultivation techniques for *L. rhinocerotis* is of paramount

importance to ensure continuous supply, and at the same time, prevent depletion of the resources due to overharvesting.

Nutritional and medicinal attributes of mushrooms are related to their chemical composition but for *L. rhinocerotis*, this aspect has yet to be explored extensively presumably due to the limited and seasonal supply. Chemical composition of mushrooms is often affected by culture conditions, substrate composition, and properties of its ingredients (Wasser & Akavia, 2008). While there are numerous reports on the chemical composition of either wild type or cultivated mushrooms, very few actually focused on comparative analyses of mushroom samples from different developmental stages (Akindahunsi & Oyetayo, 2006; Apetorgbor *et al*., 2013). In this context, there are reports on the proximate composition and dietary fibres of *L. rhinocerotis* sclerotium (Wong & Cheung, 2008a; Lai *et al*., 2013; Yap *et al*., 2013); however, other mushroom parts have not been investigated. In view of that, it is necessary to expand the analysis of *L. rhinocerotis* chemical composition to include the fruiting body and mycelium.

The specific objectives of this study are:

1. To produce *L. rhinocerotis* fruiting body and sclerotium by solid substrate fermentation on optimized agroresidues formulation
2. To produce *L. rhinocerotis* mycelium by liquid fermentation under shaken condition
3. To determine the chemical composition of *L. rhinocerotis* samples including the fruiting body (separated into pileus and stipe), sclerotium, and mycelium

3.2 Materials and Methods

3.2.1 Chemicals

Agroresidues used in this study, such as rubberwood sawdust, paddy straw, and oil palm empty fruit bunch, are by-products of factories and plantations in Selangor, Malaysia. Brewery spent yeast was collected from a Carlsberg factory in Shah Alam, Selangor, Malaysia. Malt extract agar, yeast extract, malt extract, and peptone were obtained from Oxoid (Hampshire, UK). Glucose was purchased from Merck Millipore (Massachusetts, USA). Other chemicals used were of analytical grade.

3.2.2 Preparation and maintenance of mycelium culture

The axenic culture of *L. rhinocerotis* (KUM61075) was derived from tissue culture of a sclerotium collected from its natural habitat in the Kenaboi Forest Reserve, Negeri Sembilan, Malaysia in 2007. The cultures were maintained on malt extract agar (MEA, Oxoid) slants stored at 4 °C and deposited into the culture collections of Mushroom Research Centre, University of Malaya. Mycelial plugs used in solid substrate and liquid fermentations were taken from the periphery of mature colonies of approximately 12-14 days (Figure 3.1).

Figure 3.1: Mycelium culture of *L. rhinocerotis* maintained on MEA.

3.2.3 Solid-substrate fermentation

This technique was used for the production of *L. rhinocerotis* fruiting body and sclerotium. The formulation of agroresidues used as fruiting substrates was optimized based on mycelial growth rate. Based on our previous results, a combination of sawdust, paddy straw, and spent yeast at a ratio of 7.9:1:1 (w/w) gave the highest mycelial growth rate of 3.3 ± 0.1 mm/day in Petri dishes and 3.8 ± 0.1 mm for mycelium run in spawn bags (data not shown).

3.2.3.1 Preparation of spawn

The optimized formulation of agroresidues, as described above, was prepared. Moisture content was adjusted to 60% (v/w) and pH was adjusted to 6.0 using cooking vinegar. The substrates were filled into polypropylene bags (diameter: 90 mm) to a height of 80 mm, weighing around 200 g. The bags were covered with plastic caps and then autoclaved at 121 °C for 1 h. The bags were left to cool overnight before inoculated with three mycelial plugs (diameter: 9 mm). The plastic caps were replaced with sterile plastic caps plugged with cotton for aeration. The bags were then incubated at 28 °C for 3 weeks for full spawn development.

3.2.3.2 Preparation of fruiting substrates for mycelial run

The formulation of agroresidues was packed into polypropylene bags (diameter: 90 mm) to a height of 100 mm and sealed with plastic cap without holes. Fifty replicate bags were prepared and sterilized by autoclaving at 121 ℃ for 1 h. Upon cooling, the bags were inoculated with 3 weeks old *L. rhinocerotis* spawn, covered with plastic caps plugged with cotton, and incubated at room temperature in the dark until the mycelium completely colonized the substrates.

3.2.3.3 Development of sclerotium and fruiting body

After the incubation period, the plastic bags were removed and the mycelium-colonized substrate blocks were buried in loam soil (depth: 15 cm) for the development of sclerotium and fruiting body. The size of the beds was 1.00 m (length) and 0.15 m (width). The burial site was roofed but exposed to external environmental condition with the average temperature ranging from 28-32 °C. Throughout the cultivation period, the soil was watered every 2-3 days. After 8-12 months, mature fruiting body and sclerotium were harvested. The pileus, stipe, and sclerotium of *L. rhinocerotis* were separated, repeatedly washed with distilled water, sliced, and dried in the oven (40 °C).

3.2.4 Liquid fermentation

This technique was used for the production of *L. rhinocerotis* mycelium.

3.2.4.1 Preparation of medium

The basal fermentation medium consists of 15% (w/v) glucose, 0.8% (w/v) yeast extract, 0.8% (w/v) malt extract, and 0.8% (w/v) peptone (GYMP) fortified with the following salts: 1% (w/v) KH_2PO_4, 1% (w/v) K_2HPO_4, 1% (w/v) NH_4Cl, and 1% (w/v) $MgSO_4 \cdot 7H_2O$. GYMP medium was prepared in 500 ml Erlenmeyer flasks and pH was adjusted to 6.0-6.5. The medium was sterilized by autoclaving at 121 °C for 20 min. Upon cooling, each flask was inoculated with 10 mycelial plugs (diameter: 9 mm).

3.2.4.2 Shaken cultures

Inoculated flasks were then placed on a reciprocal shaker at 150 rpm at room temperature (25 ± 2 °C). After 15 days, the mycelial pellets of *L. rhinocerotis* were filtered off the culture broth, washed extensively with distilled water, and freeze-dried.

3.2.5 Chemical composition

3.2.5.1 Proximate composition

Samples of *L. rhinocerotis* (i.e. pileus, stipe, sclerotium, and freeze-dried mycelium from shaken cultures) were powdered in a Waring blender to pass a 40 mesh size and kept at 4 °C prior to analysis. Analysis of the proximate composition of the samples was carried out by Consolidated Laboratory (M) Sdn. Bhd. (Kuala Lumpur, Malaysia).

Crude protein was determined using the Kjeldahl method with boric acid modification (AACC 46-12). Total fat was determined based on the modified Mojonnier ether extraction method for milk (AOAC 989.05). Total ash was measured using the basic method (AACC 08-01). Moisture content was determined by the air-oven method (AACC 44-15A). Crude fibre was analysed according to AACC 32-10. Total carbohydrate and energy content were calculated by difference, whereby total carbohydrate (g) = 100 – (crude protein + total fat + ash + moisture) and energy content (kcal/ 100 g) = [(4× crude protein) + (9 × crude fat) + (4 × carbohydrates)]. Proximate composition was expressed on a dry weight basis. The energy content was expressed as kcal/100 g dry weight.

3.2.5.2 Determination of glucans

The levels of 1,3- and 1,6-β-D-glucans in the mushroom samples were measured using the Mushroom and Yeast β-glucan Assay Kit (Megazyme International Ireland) according to the recommended protocols. Total glucans and α-glucans were measured by enzymatic hydrolysis, and the β-glucan content was determined by difference.

An overview of the experimental design in this study is shown in Figure 3.2.

3.2.6 Statistical analysis

Results for the analysis of chemical composition were presented as means ± S.D. (n = 3). The data were statistically analysed using the IBM® SPSS® Statistics Version 19 (SPSS Inc., United States). All mean values were analysed by one-way analysis of variance (ANOVA) followed by Tukey-HSD ($p < 0.05$) to detect significance between groups.

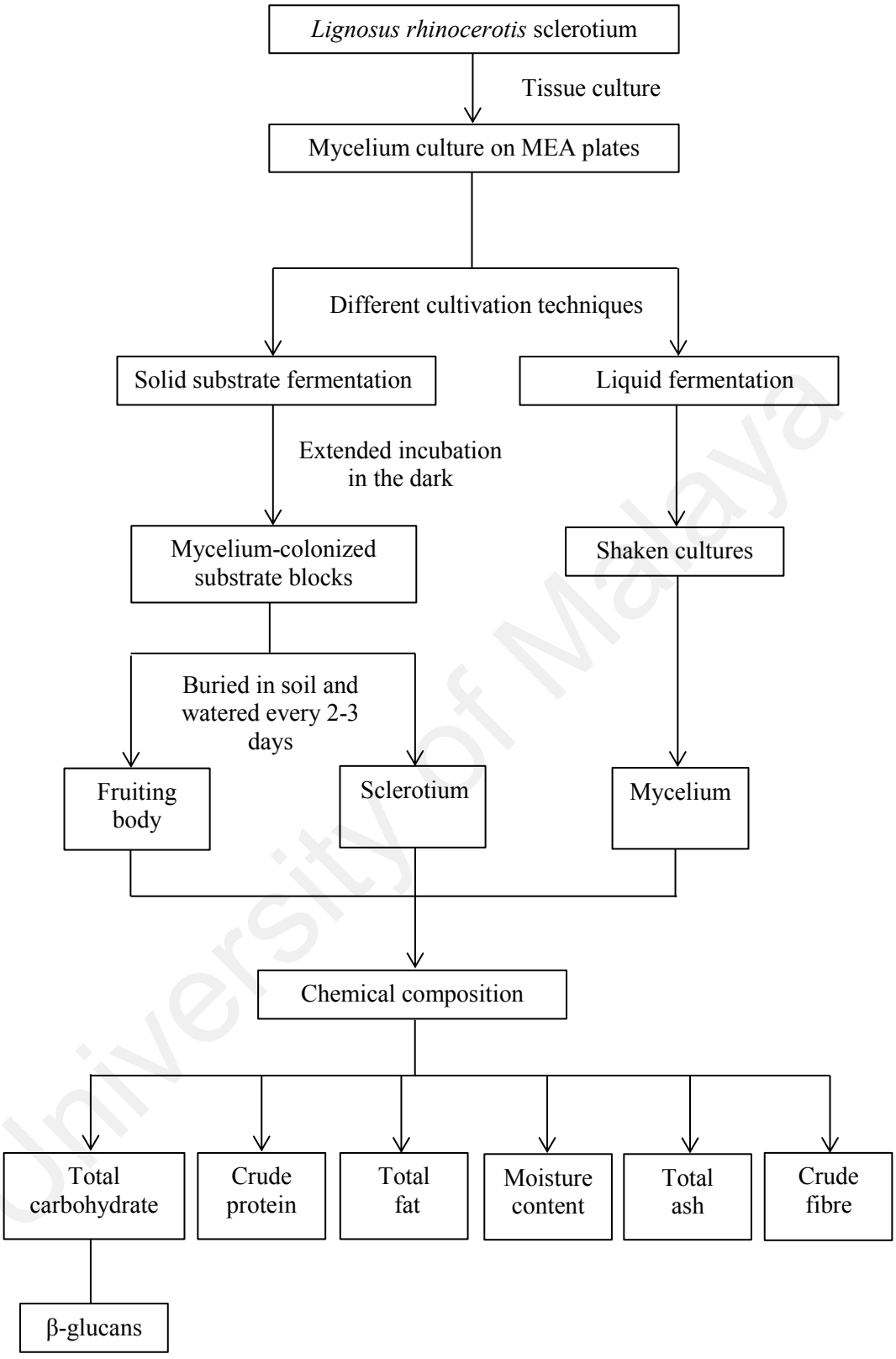


Figure 3.2: An overview of the experimental design for cultivation of *L. rhinocerotis* and analysis of its chemical composition. The fruiting body, sclerotium, mycelium, and culture broth, representing samples of *L. rhinocerotis* from different developmental stages, were produced by different technique, i.e. solid substrate and liquid fermentations.

3.3 Results

3.3.1 Production of sclerotium and fruiting body

Mycelial run of *L. rhinocerotis* on the substrates were completed after 40 days with mycelium fully colonized the substrate bags and having the appearance of brown patches as the mycelium aged under the extended incubation period of approximately 60 days (Figure 3.3). For approximately 10% of the bags, formation of sclerotium was observed during the extended incubation period but subsequent development of fruiting body from the sclerotium was not observed (Figure 3.4).

In an attempt to induce the formation of fruiting bodies, the mycelium-colonized substrate blocks (without plastic bags) were buried in soil and watered. Formation of sclerotium from the mycelium-colonized substrate bags was observed as early as after 3-4 weeks after burial (Figure 3.5). The development and maturation of sclerotium was accompanied by degradation of the substrate block (Figure 3.6). The mature sclerotium was white to light brown. At this stage, harvested sclerotium (diameter: 4-8 cm) weighed between 80-120 g on fresh weight basis.

Following burial and watering, emergence of primordium took place between 8-12 months (Figure 3.7). Elongation and expansion of the primordium was followed by formation, development, and thickening of the pileus. Depending on the environmental conditions, this process was observed to be completed between 10-14 days. Mature fruiting body was indicated by the brown edge of the pileus that was otherwise white during early developmental stages. The entire developmental process of the fruiting body is depicted in Figure 3.8. Each of the mature sclerotium gave rise to an individual fruiting body (Figure 3.9). The size and weight of sclerotium harvested was comparable to that of those harvested prior to formation of the fruiting body. Under current

experimental condition, yield of the sclerotium was determined to be 1.3-2.0 g of sclerotium (on fresh weight basis) per g of substrates used (on dry weight basis).

Figure 3.3: Mycelial run of *L. rhinocerotis* on optimized agroresidues. Substrate bags were incubated at room temperature. Entire substrate bag was colonized by the mycelium in about 40 days.

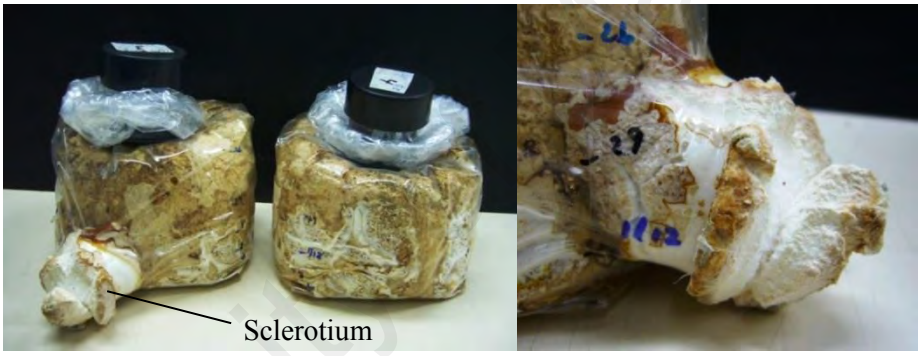


Figure 3.4: Formation of *L. rhinocerotis* sclerotium in the mycelium-colonized substrate bag during the extended incubation period. Formation of fruiting body was not observed.

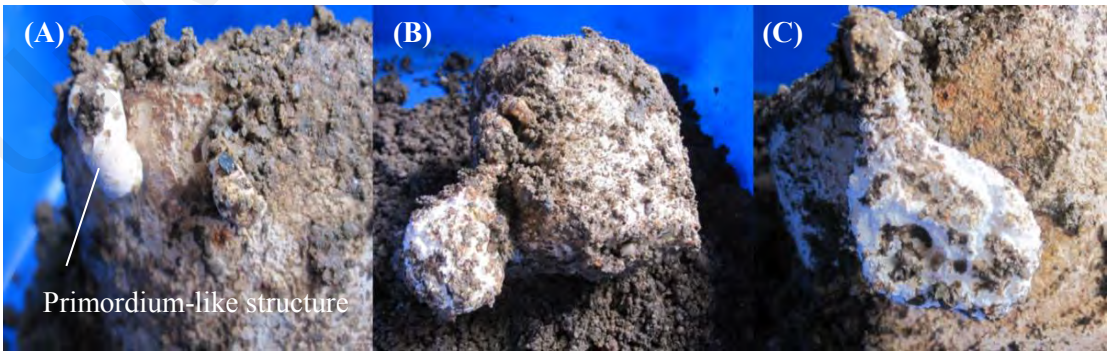


Figure 3.5: Initial stages in development of *L. rhinocerotis* sclerotium after burial in the soil. (A) Primordium-like structure formed at the edge of mycelium-colonized substrate block. (B) Maturation of sclerotium and gradual shrinkage of the substrate block. (C) The developing sclerotium was white with dark brown patches.

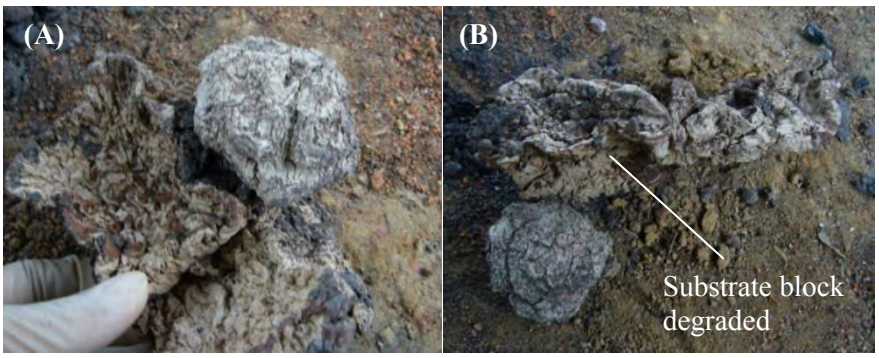


Figure 3.6: Development and maturation of *L. rhinocerotis* sclerotium. (A) Maturation of the sclerotium is indicated by the thickening and hardening of the outer skin. (B) This process is accompanied by shrinkage and degradation of the mycelium-colonized substrate block.

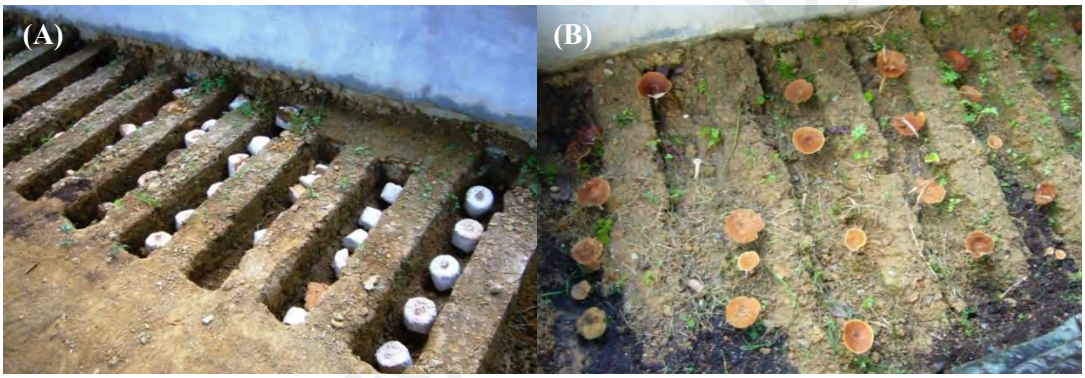


Figure 3.7: Pilot cultivation of *L. rhinocerotis*. (A) Mycelium-colonized substrate blocks were arranged in the cultivation plot, covered with soil, and watered. (B) Fruiting body of *L. rhinocerotis* developed after 8-12 months.

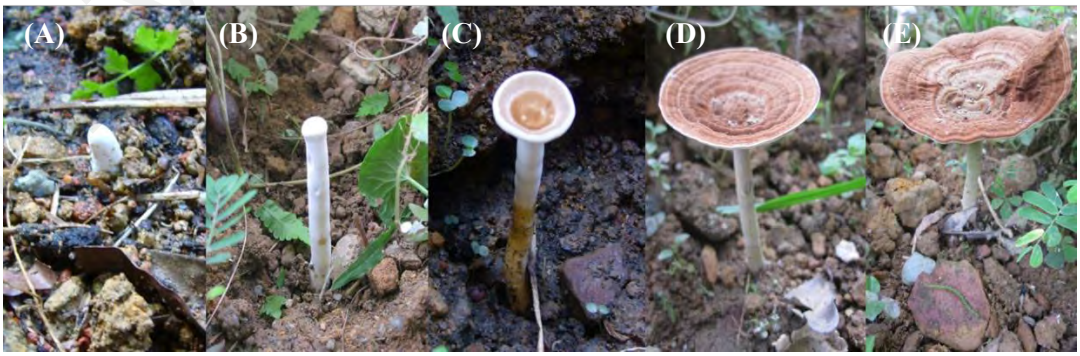


Figure 3.8: Major developmental stages of *L. rhinocerotis* fruiting body. (A) Emergence of primordium. (B) Elongation of primordium. (C) Formation of pileus. (D) Expansion and maturation of pileus. The pileus edge is white. (E) Mature fruiting body is indicated by the loss of white edge of the pileus.

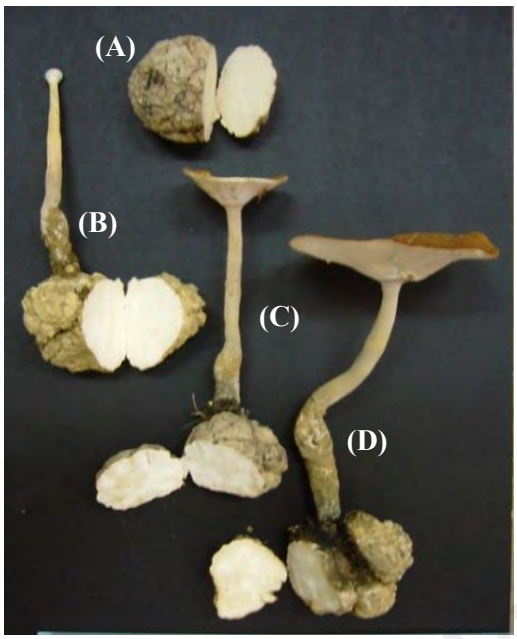


Figure 3.9: Morphology of *L. rhinocerotis* harvested at different growth stages. (A) *L. rhinocerotis* sclerotium formed prior to the formation of fruiting body. (B-D) Sclerotium attached to fruiting bodies that were harvested at different growth stages.

3.3.2 Production of mycelium

During liquid fermentation, growth in the form of mycelial pellets (sometimes clumped together) was observed in shaken cultures (Figure 3.10). The mycelium was filtered off from the culture broth, washed repeatedly with distilled water, and freeze-dried.

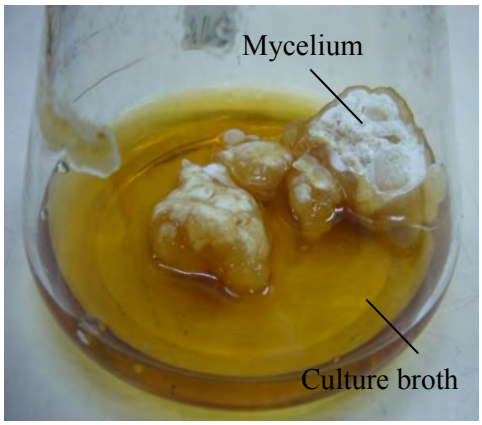


Figure 3.10: Morphology of *L. rhinocerotis* mycelium when cultured under shaken condition of liquid fermentation using GYMP medium.

The yields (g freeze-dried mycelium or culture broth/100 ml medium) of *L. rhinocerotis* mycelium and culture broth under shaken condition were determined to be 0.62 and 2.94, respectively.

3.3.3 Proximate composition

Table 3.1 shows the proximate composition, expressed on a dry weight (DW) basis, of *L. rhinocerotis*. Overall, *L. rhinocerotis* was found to be rich in carbohydrate (~80 g/100 g DW) but low in protein (< 10 g/100 g DW) and fat (< 2 g/100 g DW). The pileus, stipe, sclerotium, and mycelium showed comparable pattern of proximate composition though some nutrients were observed to be concentrated in different parts.

Table 3.1: Proximate composition and energy values of *L. rhinocerotis* from different developmental stages

Parameters	Fruiting body		Sclerotium	Mycelium
	Pileus	Stipe		
Total carbohydrate	81.03 ± 0.13^{a}	80.09 ± 0.69^{b}	82.60 ± 0.01^{c}	73.01 ± 0.04^{d}
Crude protein	9.85 ± 0.14^{a}	5.67 ± 0.22^{b}	7.02 ± 0.20^{c}	7.87 ± 0.01^{d}
Total fat	0.98 ± 0.02^{a}	0.42 ± 0.02^{b}	0.49 ± 0.00^{c}	2.30 ± 0.06^{d}
Moisture content	4.82 ± 0.06^{a}	7.22 ± 0.01^{b}	8.12 ± 0.10^{c}	9.93 ± 0.21^{d}
Total ash	3.33 ± 0.09^{a}	6.62 ± 0.46^{b}	1.79 ± 0.09^{c}	6.90 ± 0.11^{b}
Crude fibre	36.40 ± 0.36^{a}	36.71 ± 0.23^{a}	22.60 ± 0.35^{b}	7.43 ± 0.43^{c}
Energy value	372.28 ± 0.22^{a}	346.76 ± 1.70^{b}	362.83 ± 0.76^{c}	344.20 ± 0.71^{d}

Proximate composition (g/100 g DW) and energy values (kcal/100 g DW) of *L. rhinocerotis* were analysed according to standard methods. Results were expressed as mean ± S.D. (n = 3). Means with different letters (a-d) within the same row are significantly different ($p < 0.05$).

In terms of nutrient distribution in *L. rhinocerotis*, the fruiting body and sclerotium contained higher concentration of carbohydrates than the mycelium. The pileus

contained highest amount of proteins, followed by the mycelium, sclerotium, and stipe whereas the levels of fat decreased in the order of mycelium, pileus, sclerotium, and stipe. All samples of *L. rhinocerotis* possessed relatively low moisture content. Total ash in *L. rhinocerotis* varied with the mycelium and stipe having notably high amount of ash, followed by the pileus and sclerotium. The levels of crude fibres in the pileus and stipe of *L. rhinocerotis* were considered high when compared to the sclerotium and mycelium. The energy content of *L. rhinocerotis* samples was found to be moderate.

3.3.4 β-glucans

The levels of β-glucans in cultivated *L. rhinocerotis* ranged from 9.3-13.2 g/100 g DW (Figure 3.11). There was no significant difference ($p < 0.05$) in the levels of glucans in the pileus, stipe, sclerotium, and mycelium of *L. rhinocerotis.*

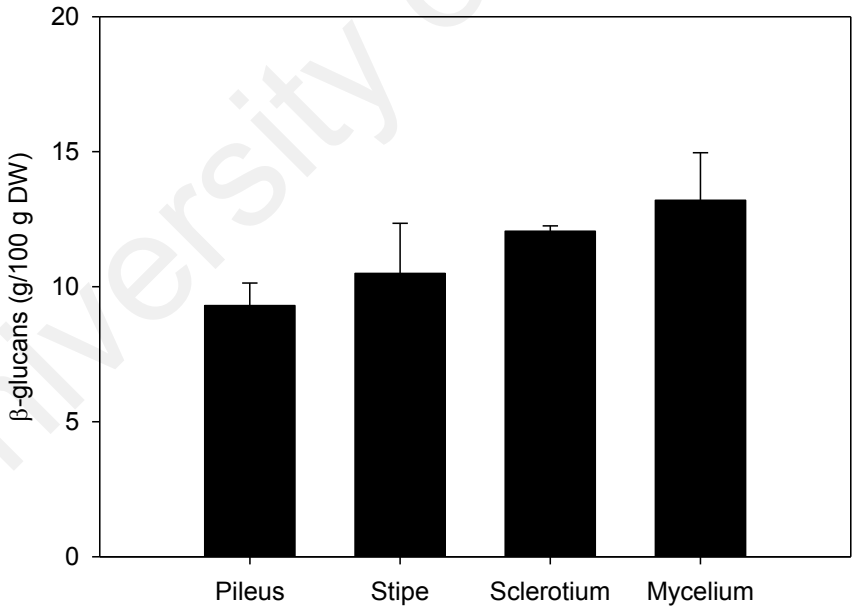


Figure 3.11: Levels of β-glucans in *L. rhinocerotis* from different developmental stages. Total glucans and α-glucans in the samples were measured by enzymatic hydrolysis, and the β-glucan content was determined by difference. Results were expressed as mean ± S.D. ($n = 3$).

3.4 Discussion

3.4.1 Cultivation of *L. rhinocerotis* by solid substrate and liquid fermentation

Cultivation of *L. rhinocerotis* by solid substrate fermentation in the present study was similar to that by Huang (1999) with reference to mycelial colonization of agroresidues and subsequent induction of sclerotium by the burial method; however, differences were noted in several aspects including mushroom strain, substrate utilization, and incubation conditions. These are some of the factors that might affect the yield of cultivated mushrooms.

Regarding mushroom strain, Huang (1999) used a strain that was originated from China whereas a local isolate was used in the present study. According to Isikhuemhen *et al*. (2000), mushrooms from different geographical locations varied in their genetic materials, physiological, and metabolic systems, and these are likely to affect their responses in different cultivation conditions. This is exemplified by the findings of Kobira *et al*. (2012) who observed differences (e.g. colour and sizes) between the cultivated sclerotium of the Japanese and Chinese strains of *W. cocos*. The differences between both strains were attributed to genomic polymorphism based on the rapid amplification of polymorphic DNA (RAPD) analysis.

For preparation of spawn, both studies utilized sawdust as the main carbon source but the choice of nitrogen source was different. Huang (1999) utilized wheat bran while spent yeast was used in our study. The spawn development in our study was completed in three weeks whereas Huang (1999) incubated the spawn for more than a month. This might be of importance as the age of spawn was found to influence mushroom yield based on previous studies (Pani, 2011). The formulation of substrates for mycelial run in both studies was also different. The formulation (w/w) used by Huang (1999) consisted of sawdust (80%), wheat bran (18%), sugar cane (1%), calcium carbonate

(1%), and water (1:1-1.4) whereas a combination of sawdust, paddy straw, and spent yeast was used in the present study. Our results indicated that *L. rhinocerotis* mycelium was capable of degrading and utilizing substrates containing lignocellulosic materials for growth. Incubation in the dark during mycelial run was necessary as studies have shown that light tend to inhibit the formation and development of both fruiting body and sclerotium; this might be related to the fact that mushroom sclerotia are usually covered by soil in its natural habitat. Similar observation was reported by Apetorgbor *et al*. (2013) who found that continuous darkness supported highest mycelial growth rate in *L. tuber-regium*.

According to Willets and Bullock (1992), the process of formation of sclerotium can be divided into three main stages, namely initiation, development, and maturation. In accordance with its role for survival during harsh conditions, production of sclerotium is initiated by the onset of starvation conditions or other circumstances that are unfavorable for continuous mycelial growth. When the substrates were fully colonized, formation of sclerotium might be initiated as a response to the cessation of nutrients. Therefore, for the cultivation of *L. rhinocerotis*, the choice of substrates is important as nutrient rich substrate might cause slower sclerotium initiation while a nutrient-deficient formulation might retard mycelial growth. In addition, numerous endogenous and exogenous factors, such as temperature, light, pH of substrate, humidity, osmotic potential, and contact with mechanical barrier, are reported to be involved in sclerotial initiation (Cheng *et al*., 2006; Wong & Cheung, 2008a). Further optimization has to be carried out to determine the best physicochemical factors favourable for sclerotium initiation in *L. rhinocerotis*.

Huang (1999) reported that the sclerotium could be left to grow and mature in the substrate bags. In our study, formation of sclerotium in the substrate was also observed

but since the production of fruiting body was desired, the substrate bags were buried in the soil. The absence of fruiting body from sclerotium that developed during extended incubation period could be explained by the fact that mycelium was still actively growing and utilizing the nutrients in the substrate bag. The burying depth is an important consideration as aerobic condition was found to be favourable for sclerotium formation in *P. umbellatus* (Choi *et al*., 2002). This is supported by the findings by Kubo *et al*. (2006) who found that aeration was an important abiotic factor for the cultivation of *W. cocos* sclerotium. Moreover, Singh *et al*. (2004) reported that sandy loam soils with humus and high aeration supported fruiting in morels.

The production of sclerotia has been successfully used for obtaining morels' fruiting bodies under controlled conditions (Volk & Leonard, 1990). The formation of sclerotium prior to development of fruiting body in the case of *L. rhinocerotis* was consistent with previous work on *Morchella rufobrunnea* Guzmán & F. Tapia (Masaphy, 2010) and *L. tuber-regium* (Apetorgbor *et al*., 2013). The nutrient supply for the production of fruiting bodies usually derived almost entirely from preexisting sclerotia (Apetorgbor *et al*., 2013). The second stage of mushroom cultivation dealt with induction and development of *L. rhinocerotis* fruiting body from the sclerotium by continuous watering. Previous researchers have demonstrated the importance of water as a factor which triggers the initiation of fruiting bodies (Ower *et al*., 1986). Masaphy (2010) reported that mature fruiting bodies of *M. rufobrunnea* developed within 2-3 weeks after primordia initiation.

The time taken for the production of *L. rhinocerotis* sclerotium and fruiting body is relatively long when compared to some commercially cultivated edible-medicinal mushrooms (Stamets, 2000), but comparable to other sclerotium-forming mushrooms (polypores); for instance, Choi *et al*. (2002) harvested the sclerotium of *P. umbellatus*

after 12 months of cultivation. Field cultivation of *W. cocos* inoculated onto tree logs required approximately eight months before the sclerotium (without fruiting body) could be harvested (Kobira *et al*., 2012). While some sclerotium-forming mushroom, such as *L. tuber-regium,* is relatively easy to be propagated, cultivation of other species, especially members of the Polyporaceae family, remained challenging with problems ranging from low and inconsistent yield to contamination of sclerotium with soil (Choi *et al*., 2002). In the context of *L. rhinocerotis*, current cultivation process can be further optimized by controlling the environmental conditions to enhance and fasten the yield of the sclerotium and fruiting body.

On the other hand, the production of *L. rhinocerotis* mycelium and culture broth using different liquid fermentation techniques, such as bioreactors as well as flasks under shaken and static conditions, have been reported (Lau *et al*., 2011; Rahman *et al*., 2012; Chen *et al*., 2013; Lai *et al*., 2014). Some workers attempted to optimize the optimal media composition for the production of mycelium. According to Lai *et al*. (2014), the optimal condition for production of mycelial biomass was 80 g/L glucose, 6 g/L potassium nitrate, 0.4 g/L $FeSO_4 \cdot 7H_2O$, and 0.1 g/L of $CaCl_2$. The maximum mycelial biomass production in a 1.5 L stirred tank bioreactor was 1.2 g/L that is comparatively lower than the yield of mycelium cultured in shaken condition of liquid fermentation in the present study. The differences in the mushroom strain and composition of media, for instance, higher concentration of glucose in the present study, might account for the differences. A large-scale fermentation of *L. rhinocerotis* using (w/w) glucose (2%), soya bean powder (0.5%), peptone (0.2%), yeast extract (0.1%), and magnesium sulfate (0.01%) at pH 4.0 was reported by Chen *et al*. (2013); however, the yield was not reported. Taken together, these studies have demonstrated that liquid fermentation is a viable method for the production of mycelium and culture broth of *L.*

rhinocerotis although the optimal media composition might differ depending on the techniques of liquid fermentation and mushroom strain used.

3.4.2 Comparative chemical composition of *L. rhinocerotis*

The sclerotium of *L. rhinocerotis* was found to follow the typical patterns of carbohydrate-rich sclerotial proximate composition with substantial amount of carbohydrates and extremely low lipid content (Wong *et al*., 2003). Our results were, in general, consistent with previous findings on either cultivated (Wong *et al*., 2003; Lee *et al*., 2011; Yap *et al*., 2013) or wild type (Lai *et al*., 2013; Yap *et al*., 2013) sclerotium of *L. rhinocerotis*, although some variations were noted (Table 3.2).

While the cultivated sclerotium in the present study has higher crude protein and fat contents than that of *P. rhinocerus* (synonym of *L. rhinocerotis*) sclerotium (Wong *et al*., 2003), these are relatively low when compared to the values obtained for *L. rhinocerus* (orthographic variant of *L. rhinocerotis*) cultivar TM02 (Lee *et al*., 2011; Yap *et al*., 2013). Carbohydrates in the cultivated sclerotium, on the other hand, were higher than that of *L. rhinocerus* cultivar TM02. In addition, the levels of carbohydrate, crude protein and total fat for the cultivated sclerotium in the present study were either higher or comparable to the wild type samples previously reported (Lai *et al*., 2013; Yap *et al*., 2013).

Table 3.2: Comparative chemical composition of *L. rhinocerotis* cultivated and wild type sclerotium

[a]Mushroom samples		Proximate composition (g/100 g DW)						Energy value (kcal/100 g DW)	References
		Carbohydrate	Crude protein	Crude fat	Moisture content	Total ash	Fibre		
[b]Cultivated sclerotium	*L. rhinocerotis* (KUM61075)	82.60	7.02	0.49	8.12	1.79	22.60	362.83	Current study
	P. rhinocerus	95.70	2.75	0.02	15.30	1.52	N.D.	N.D.	Wong *et al*. (2013)
	L. rhinocerus (cultivar TM02)	60.55	16.95	1.86	N. D.	N.D.	N.D.	326.76	Lee *et al*. (2011)
	L. rhinocerus (cultivar TM02)	77.60	13.80	0.80	N. D.	N.D.	N.D.	321.90	Yap *et al*. (2013)
[c]Wild type sclerotium	*L. rhinocerus* (Cameron Highlands)	88.40	3.80	0.30	N. D.	N.D.	N.D.	218.00	Yap *et al*. (2013)
	L. rhinocerus (Kuala Lipis)	51.30	3.04	0.46	39.82	4.38	N.D.	N.D.	Lai *et al*. (2013)

[a]Scientific names used in the original reports, including *P. rhinocerus* (synonym of *L. rhinocerotis*) (Wong *et al*., 2003) and *L. rhinocerus* (orthographic variant of *L. rhinocerotis*) (Lee *et al*., 2011; Yap *et al*., 2013) were retained.

[b]Cultivated sclerotium of *L. rhinocerotis* (KUM61075) and *L. rhinocerus* (cultivar TM02) were originated from Malaysia but *P. rhinocerus* was cultivated in Sanming Mycological Institute, China. Details on the cultivation conditions for *P. rhinocerus* and *L. rhinocerus* (cultivar TM02) were not available.

[c]Wild type sclerotium of *L. rhinocerus* used by Yap *et al*. (2013) and Lai *et al*. (2013) was collected from different locations in the state of Pahang, Malaysia. The sclerotium analysed by Lai *et al*. (2013) was attached to fruiting body. The sclerotium used by Yap *et al*. (2013) was harvested before the development of fruiting body. N.D, not determined.

The differences between the cultivated sclerotium of *L. rhinocerotis* (KUM61075) with that of *P. rhinocerus* (Wong *et al*., 2003) and *L. rhinocerus* cultivar TM02 (Lee *et al*., 2011; Yap *et al*., 2013) might be attributed to several factors such as dissimilar cultivation techniques, media composition, culture conditions, pre- and post-harvest processing and others (Manzi *et al*., 2001; Harada *et al*., 2004; Dundar *et al*., 2009). On the other hand, it will be more difficult to explain the differences between the cultivated and wild type sclerotium as mushrooms that grow in natural habitat are affected by multiple biotic and abiotic factors in comparison to cultivation in controlled conditions. Besides, the cultivated sclerotium (with attached fruiting body) analysed in the present study might differ, in terms of chemical composition, from those of different physiological ages or harvested at different growth stages. These reasons might, in fact, account for the differences in the sclerotial composition (e.g. crude protein and fat) between *L. rhinocerotis* in the present study and *L. rhinocerus* cultivar TM02 as the latter was harvested prior to the formation of fruiting body and described as "at the earlier stage of maturation" (Yap *et al*., 2013).

The abundance of nutrients in the different parts of *L. rhinocerotis* (i.e. pileus, stipe, and sclerotium) was compared to the findings by Lai *et al*. (2013) who analyzed wild type *L. rhinocerus*. Some inconsistencies were observed; for instance, the cultivated fruiting bodies and sclerotium contained comparable levels of carbohydrates (80.09-82.60 g/100 g DW) whereas wild type *L. rhinocerus* has uneven distribution of carbohydrates in the pileus, stipe, and sclerotium (38.47-51.30 g/100 g DW). Total fat in the pileus also varied significantly for the cultivated (0.98 g/100 g DW) and wild type (0.21 g/100 g DW) samples. The level of proteins in both cultivated and wild type samples, nevertheless, followed a similar trend in the order of pileus, sclerotium and stipe. The differences in the distribution of the nutrients might also be linked to specific

functional and/or physiological roles in fungal development; for instance, the stipe was rich in fibres as these might provide structural role for the pileus (Fasidi & Kadiri, 1993).

The chemical composition of *L. rhinocerotis* mycelium was determined for the first time. Nutrients in the mycelium were generally comparable or lower than other samples; for instance, crude fibres in the mycelium were approximately 3- and 5-times lower than those of the fruiting bodies and sclerotium, respectively. Nevertheless, the mycelium contained highest levels of crude fat and ash (minerals) in all samples. As the chemical composition of mushroom mycelia is noted to be greatly influenced by media composition and culture conditions, only the mycelium of *L. rhinocerotis* from shaken flasks was analysed because the growth in the form of pellets, rather than pellicles in static cultures, might be of relevance for scale-up studies (e.g. cultivation in bioreactors). The variation in the chemical composition of mycelium might be explained by the fact that the mycelium (vegetative state), fruiting bodies (reproductive stage) and sclerotium (dormant stage) represent distinct developmental stages in the life cycle of *L. rhinocerotis* and these might differ in their nutritional requirement and chemical components. Variation in the nutritional composition between fruiting bodies and mycelia of other edible-medicinal mushrooms has been extensively documented (Cheung, 1996; Huang *et al*., 2006; Ulziijargal & Mau, 2011).

While *L. rhinocerotis* is presumably not an important source of food due to its woody texture, the fruiting body, sclerotium, and mycelium, either in the form of powdered materials or processed state, might be potential sources of functional ingredients. Based on its proximate composition, *L. rhinocerotis* is aptly utilized as a dietary supplement fitting the nutritional requirements of various individuals; for instance, a high level of carbohydrate, a moderate level of protein and a very low level

of lipid is suitable for those under diet restriction such as for diabetes patients, whereas a high ratio of potassium to sodium renders *L. rhinocerotis* suitable for hypertension and heart diseases patients (Liu *et al*., 2012). Moreover, being rich in dietary fibres, *L. rhinocerotis* could be beneficial to intestinal function by increasing fecal bulk and enhancing peristalsis (Alobo, 2003). To date, the physicochemical, functional, and biopharmacological properties of *L. rhinocerotis* sclerotial dietary fibres have been extensively studied (Wong & Cheung, 2005a; Wong & Cheung, 2005b; Wong *et al*., 2005; Wong *et al*., 2006).

Our results revealed that the level of β-glucans in *L. rhinocerotis* from different developmental stages were comparable in the range of 9.3-13.2% (w/w); however, wild type *L. rhinocerotis* was reported to contain higher amount of β-glucans, ranging from 33.95-70.80% (w/w) (Lai *et al*., 2010; Mohd Jamil *et al*., 2013). Differences in the levels of β-glucans between the cultivated and wild type samples might have been attributed to a number of factors such as the differences in the habitat and growth conditions (e.g. substrate degraded by the mushroom mycelium) as well as maturity stage when harvested. Based on previous findings, it has been suggested that some of the medicinal properties of *L. rhinocerotis* were attributed to the sclerotial polysaccharides such as β-glucans that are known for their role as immunomodulators (Lai *et al*., 2008; Wong *et al*., 2011). Should β-glucans in *L. rhinocerotis* account for its medicinal properties, our results herein seemed to indicate that the sclerotium, valued from the ethnomedicinal perspective, was not superior to the underutilized fruiting body as well as the mycelium as these also contained fair amount of β-glucans. Nevertheless, chemical characterization of the polysaccharides in samples other than the sclerotium is necessary. Our findings from the present study, including (i) successful cultivation of the fruiting body and sclerotium by solid-substrate fermentation, (ii) the ease of

producing mycelium and culture broth by liquid fermentation, and (iii) comparative analysis of chemical composition, have provided a rationale for extending the bioactivities evaluation on mushroom samples other than the sclerotium.

3.5 Conclusion

Successful domestication of *L. rhinocerotis* under controlled conditions has been achieved. Firstly, solid substrate fermentation of optimized agroresidues consisting of sawdust, paddy straw, and brewery spent yeast produced the sclerotium and fruiting body, and at the same, provided insights into the developmental stages of *L. rhinocerotis* for the first time. Secondly, mycelium and culture broth were produced using liquid fermentation under shaken condition. Proximate composition and levels of β-glucans in the fruiting body, sclerotium, and mycelium of *L. rhinocerotis* were found to be comparable, suggesting that samples other than the sclerotium might emerge as alternative source of active ingredients. This will be substantiated by further bioactivity evaluation and chemical profiling.

CHAPTER 4: CYTOTOXIC ACTIVITY OF *LIGNOSUS RHINOCEROTIS* CRUDE AQUEOUS AND ORGANIC SOLVENT EXTRACTS

4.1 Introduction

In Southeast Asia, *Lignosus* spp. (known locally as tiger's milk mushrooms) are regarded as indispensable medicinal mushrooms with the ability to cure numerous ailments such as cancer, cough, asthma, fever, food poisoning, and others (Lee *et al*., 2009a). The use of *Lignosus* spp. particularly for treating cancer of the breast and cervix by the indigenous people of Peninsular Malaysia has been reported (Noorlidah *et al*., 2009; Mohammad *et al*., 2012). Based on ethnomedicinal records, the sclerotium, rather than the fruiting body, is the part with medicinal value (Tan *et al*., 2010; Azliza *et al*., 2012; Mohammad *et al*., 2012).

Herbal preparation is given emphasis as the choice of processing methods (e.g. boiling, maceration, preparing tincture, etc.) might enhance the efficacy and/or reduce the toxicity of resulting preparations (Zhao *et al*., 2010). Traditionally, aqueous preparations of the sclerotium, either in the form of decoction or macerated-materials, are consumed for medicinal benefits. Of interest would be a method that mimics cold aqueous extraction as described by Chee (1933). It involves grating the sclerotium on a hard surface with some water, and the resulting mixture is further diluted with water before it is drunk. In many ethnobotanical surveys, the sclerotia of *Lignosus* spp. were consumed in the form of decoction, either alone or in combination with other herbs (Chang and Lee, 2004; Azliza *et al*., 2012; Mohammad *et al*., 2012). Besides that, there are reports on preparation of *L. rhinocerotis* sclerotium in the form of tincture (crushed/powdered mushrooms soaked in a mixture of alcohol and water); however, in most cases, the tinctures were purportedly not for treating cancer (Azliza *et al*. 2012).

Previous attempts to validate the anti-cancer effect of *L. rhinocerotis* focused mainly on immunopotentiation (Wong *et al*., 2011) and cytotoxicity against several cancer cells (Lai *et al*., 2008; Abdullah *et al*., 2010; Lee *et al*., 2012, Suziana Zaila *et al*., 2013; Yap *et al*., 2013). Even though reports on the cytotoxicity of *L. rhinocerotis* sclerotium exist in the literature, the present study focused on a number of aspects which have not been systematically and thoroughly studied prior to this. These include comparisons of (i) extracts that are prepared from different methods, such as hot aqueous and cold aqueous extracts, and organic solvent (e.g. dichloromethane and methanol) extracts; (ii) extracts of mushroom samples from different developmental stages (e.g. fruiting body, sclerotium, and mycelium), different parts of the fruiting body (e.g. pileus and stipe), and different cultivation techniques (e.g., shaken and static conditions of liquid fermentation for the production of mycelium); and (iii) extracts of the wild type, laboratory-scale cultivated, and commercially-produced mushroom samples.

Further, the cytotoxic potential of *L. rhinocerotis* extracts will be compared against selected edible-medicinal mushrooms. These include *Ganoderma lucidum* (lingzhi mushroom) that has been previously shown to demonstrate anti-cancer effects (Paterson, 2006), *Lentinus tuber-regium* (king tuber oyster mushroom) that forms sclerotium in its life cycle (similar to *L. rhinocerotis*) (Wong & Cheung, 2008b), and *Termitomyces heimii* Natarajan (termite mushroom) that grows symbiotically with termite in the soil (Mohd Jamil *et al*., 2013). Besides that, the cytotoxic activity of *L. rhinocerotis* was previously examined only on a limited number of cell lines and by different researchers, and hence, a comparative study to assess their efficacy against different cell lines might be useful in order to assess their potential anti-cancer effects. Therefore, in the present study, cytotoxicity evaluation will be expanded to include a large panel of cancer and

non-cancerous human cell lines so that the selectivity of the extracts and their respective chemical components can be determined.

In accordance with the traditional practices, hot aqueous and cold aqueous extracts, which mimics the consumption method as recorded in various ethnobotanical surveys, and organic solvent (i.e. dichloromethane and methanol) extracts of *L. rhinocerotis* will be prepared, chemically profiled, and screened for cytotoxic activity.

The specific objectives of the present study are:

1. To perform chemical characterization and profiling on *L. rhinocerotis* crude aqueous and organic solvent extracts
2. To evaluate and compare the cytotoxic activity of the crude aqueous and organic solvent extracts of *L. rhinocerotis* from different developmental stages, parts, cultivation techniques and origin across a panel of cancer and non-cancerous human cell lines using the MTT assay
3. To compare the cytotoxic activity of *L. rhinocerotis* extracts with those of selected edible-medicinal mushrooms

4.2 Materials and Methods

4.2.1 Mushroom samples

4.2.1.1 *Lignosus rhinocerotis* (tiger's milk mushroom)

Three types of mushrooms (i.e. laboratory-scale cultivated, wild type, and commercially-produced samples) were used in the present study.

- Solid substrate fermentation was used to produce the fruiting body and sclerotium as described in Chapter 3. Mature fruiting body with sclerotium (Figure 4.1) was harvested after 12-15 months. Sclerotium that was formed after burial of mycelium-colonized substrate blocks but prior to the formation of fruiting body were also harvested.
- Mycelium and culture broth under shaken and static conditions of liquid fermentation were produced (Figure 4.2). Flasks were inoculated with mycelial plugs and placed on orbital shaker rotating at 150 rpm (shaken condition) or in the incubator (static condition) for 15 days.
- Wild type fruiting body and sclerotium of *L. rhinocerotis* were obtained from local herbalists at Lata Iskandar, Perak, Malaysia in 2012. Most of the wild type samples consist of sclerotium attached to the fruiting body and only a small number of sclerotium that was without fruiting body (usually detached during the harvesting and sorting processes).
- Commercially-produced *L. rhinocerotis* was obtained from a retailer in Selangor, Malaysia. Each capsule contains powdered form of the sclerotium (250 mg) (Figure 4.3A).

Species identification of the cultivated and wild type *L. rhinocerotis* was carried out based on morphological characteristics and their internal transcribed spacer (ITS)

regions of the ribosomal RNA. Voucher specimens (KLU-M1238) were kept at the Mushroom Research Centre, University of Malaya.

4.2.1.2 *Ganoderma lucidum* (lingzhi mushroom)

The sliced fruiting body of *G. lucidum* (Figure 4.3B) was purchased from a commercial mushroom grower in Tanjung Sepat, Selangor, Malaysia. Validated voucher specimens (KLU-M1233) were kept at Mushroom Research Centre, University of Malaya.

4.2.1.3 *Lentinus tuber-regium* (king tuber oyster mushroom)

The axenic culture of *L. tuber-regium* was obtained from China. The fruiting body and sclerotium of *L. tuber-regium* (Figure 4.3C) were produced by solid substrate fermentation using methodology similar to that for *L. rhinocerotis*; however, the mushrooms were harvested earlier (i.e. approximately six months after the burial of mycelium-colonized substrate blocks). Validated voucher specimens (KLU-M1236) were kept at Mushroom Research Centre, University of Malaya.

4.2.1.4 *Termitomyces heimii* (termite mushroom)

The fruiting body of *T. heimii* (Figure 4.3D) was collected by Noorlidah Abdullah in Kepong, Selangor. These species grow out from the termite mounds. Validated voucher specimens (KLU-M1237) were kept at Mushroom Research Centre, University of Malaya.

4.2.1.5 Processing of mushroom samples

Fruiting bodies and sclerotia of the mushrooms (with the exception for *G. lucidum* and the commercially-produced sclerotium of *L. rhinocerotis*) were cleaned with

distilled water, air-dried at room temperature, sliced or cut into smaller pieces before these are dried in the oven (50 ± 2 °C) for 3-5 days. The fruiting body of *L. rhinocerotis* was further separated into pileus and stipe. For liquid fermentation, the mycelium were filtered off the culture broth, washed extensively with distilled water, and freeze-dried. Powdered sclerotium of commercially-produced *L. rhinocerotis* was carefully removed from the capsules. Other mushroom samples were powdered using a Waring blender and kept at room temperature prior to extraction. Figure 4.4 depicts the different samples of edible-medicinal mushrooms used in the present study.

Figure 4.1: *Lignosus rhinocerotis* fruiting body and sclerotium from solid substrate fermentation. (A) The fruiting body consists of pileus (cap) and stipe (stalk), and is attached to a sclerotium. Sliced samples of the (B) pileus, (C) stipe, and (D) sclerotium were dried in the oven and then, powdered using a Waring blender.

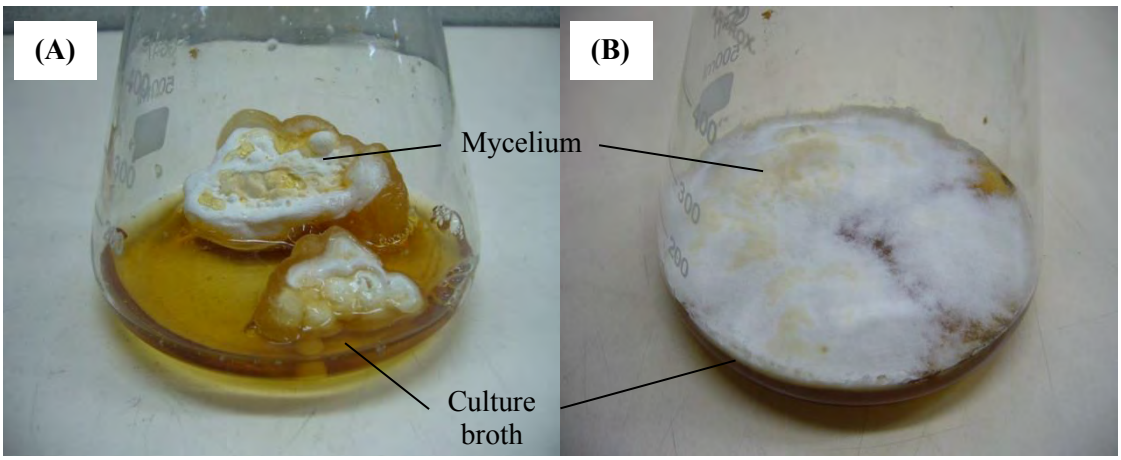


Figure 4.2: *Lignosus rhinocerotis* mycelium and culture broth from liquid fermentation. Morphological differences of the mycelium from (A) shaken (mycelial pellets) and (B) static (mycelial mat) conditions of liquid fermentation were observed.

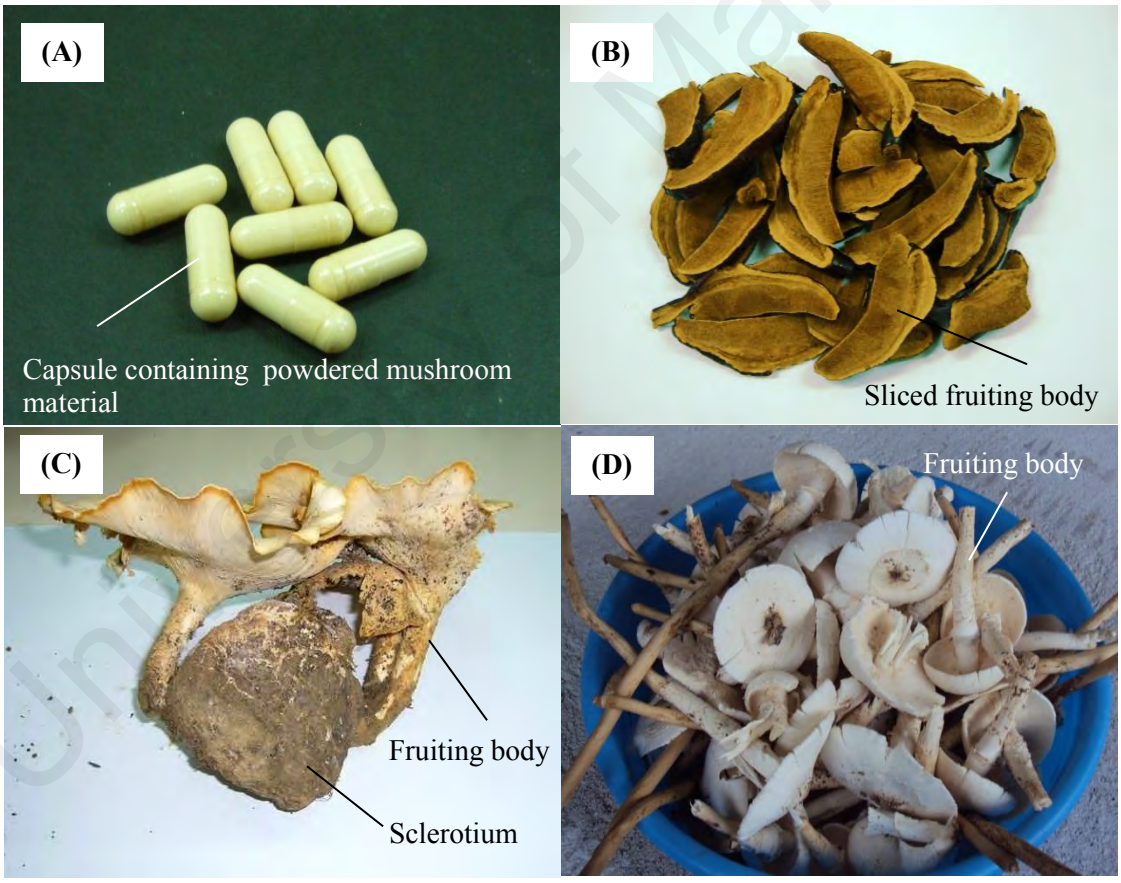


Figure 4.3: Commercially-produced *L. rhinocerotis* and selected edible-medicinal mushrooms. (A) The commercially-produced sclerotial powder of *L. rhinocerotis*. (B) Sliced *G. lucidum* fruiting body was obtained from commercial mushroom grower. (C) The fruiting body and sclerotium of *L. tuber-regium* cultivated by solid substrate fermentation. (D) The fruiting body of *T. heimii* collected from the wild. The image of *T. heimii* was taken from http://suriaamanda.blogspot.com.

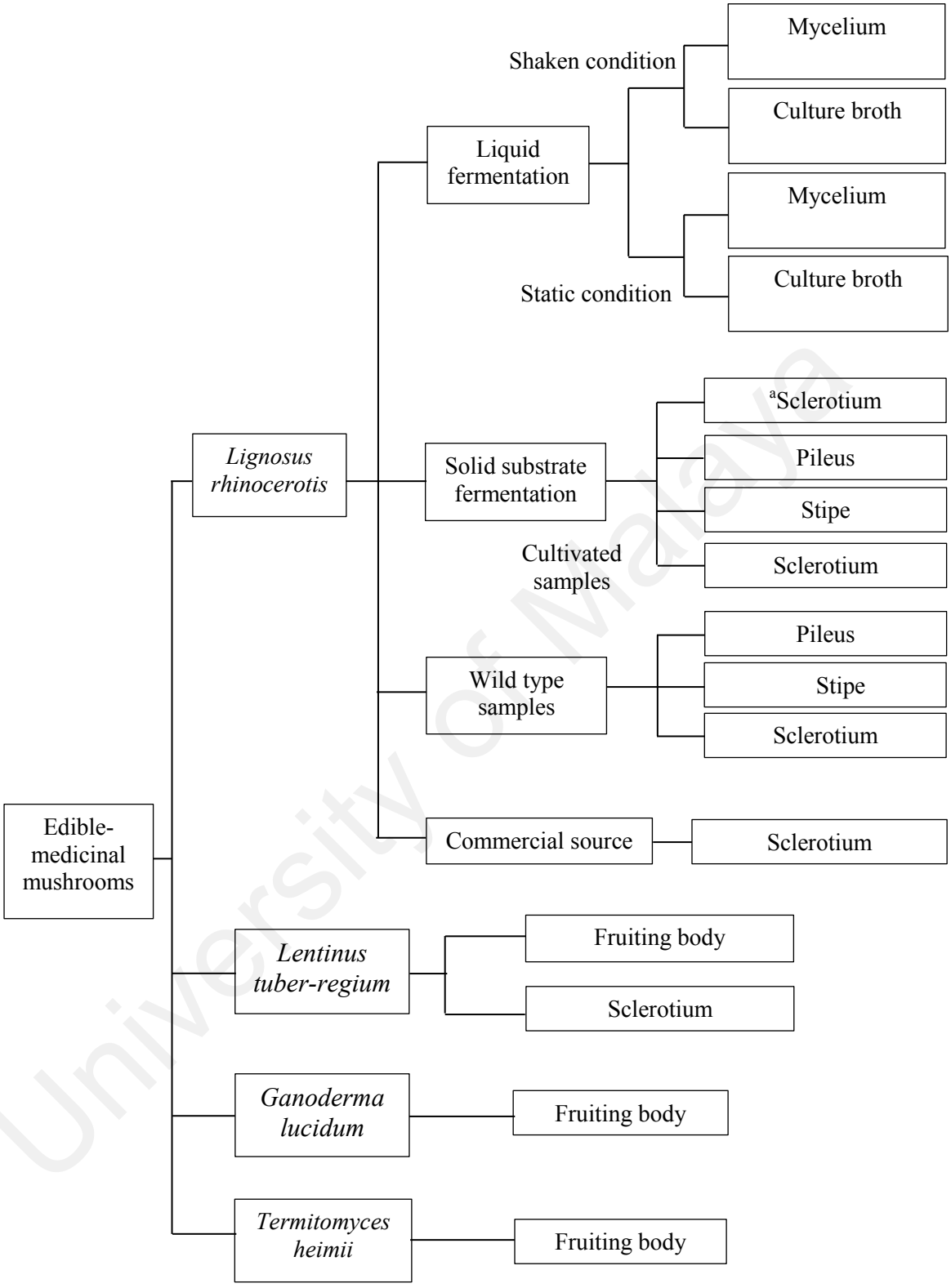


Figure 4.4: Samples of edible-medicinal mushrooms used in the present study. These are *L. rhinocerotis*, *L. tuber-regium*, *G. lucidum*, and *T. heimii*. [a]The sclerotium of *L. rhinocerotis* that was harvested prior to the formation of fruiting body.

4.2.2 Preparation of crude extracts

The crude aqueous and organic solvent extracts of *L. rhinocerotis*, *G. lucidum*, *L. tuber-regium*, and *T. heimii* were prepared as described below.

4.2.2.1 Aqueous extracts

For hot aqueous extraction, powdered mushroom samples were boiled in distilled water at the ratio of 1: 20 (w/v) at 90-95 °C for 60 min, whereas the mixture at similar ratio were subjected to continuous stirring at 4 °C for 24 h for the cold aqueous extraction. Resulting mixtures from the hot aqueous and cold aqueous extraction were filtered with Whatman No. 1 filter paper and residues were re-extracted twice. Combined mixtures from three rounds of the hot aqueous and cold aqueous extraction were separately centrifuged at 10 000 rpm and freeze-dried. The aqueous extracts were kept at -20 °C prior to analysis. The freeze-dried extracts are mostly light-brown to dark brown and fluffy.

4.2.2.2 Organic solvent extracts

Powdered mushroom samples were extracted successively using dichloromethane and methanol (analytical grade). Samples were soaked in each solvent at a ratio of 1:20 (w/v) for 3 days at room temperature under shaking conditions. Solvents were decanted and the extraction procedures were repeated twice. The filtrates were combined and excess solvents were removed using a rotary evaporator. The dried extracts were kept at -20 °C.

4.2.3 Chemical characterization

The crude aqueous extracts and fractions of *L. rhinocerotis* were chemical characterized and profiled using liquid chromatography-mass spectrometry (LC-MS).

4.2.3.1 Determination of carbohydrate and protein contents

The aqueous extracts of *L. rhinocerotis* were dissolved in distilled water and further diluted to desired concentrations for the following assays:

The phenol-sulfuric acid assay in microplate format as described by Masuko *et al*. (2005) was used for estimating total carbohydrate content. Briefly, concentrated sulfuric acid (50 µl) was added to the aqueous extracts (150 µl), followed by the addition of 5% (v/v) phenol in water (30 µl). The mixture was heated at 90 °C for 5 min in a water bath. After cooling to room temperature, absorbance was taken at 490 nm. D-glucose (0-2 mg/ml) (Sigma) was used as a standard.

Protein content was determined using the Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) according to recommended protocols. A working reagent was prepared by mixing BCA Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and B (4% cupric sulfate in water) at a ratio of 50:1 (v/v). To perform the assay, working reagent (200 µl) was added to aqueous extracts (25 µl). The mixture was incubated at 37 °C for 30 min. After cooling to room temperature, absorbance was taken at 562 nm. Protein content was estimated from the standard curve of bovine serum albumin (BSA) (0-2 mg/ml).

4.2.3.2 Liquid chromatography-mass spectrometry (LC-MS)

The crude extracts of *L. rhinocerotis* were dissolved in 50% (v/v) aqueous methanol at a final concentration of 20 mg/ml and filtered through a membrane filter (0.45 µm). Analysis was carried out using the Acquity™ Ultra Performance Liquid Chromatography (UPLC)-Photodiode Array detector (PDA) system coupled to Synapt High Definition Mass Spectrometry (HDMS) quadrupole-orthogonal acceleration time-of-flight (oaTOF) detector (Waters Corporation, USA) equipped with an electrospray ionization source (ESI). Sample (3 µl) was injected into the system and separation was achieved on Acquity™ UPLC BEH C18 (1.7 µm, 2.1 × 50 mm) column at room temperature. Mobile phase A and B consisted of water containing 0.1% (v/v) formic acid and acetonitrile (ACN) containing 0.1% (v/v) formic acid, respectively. Elution was performed at a constant rate of 0.5 ml/min in a gradient of 0 to 100% of acetonitrile (0-7 min) and held at 100% ACN for another 3 min. PDA was set at several wavelengths (210-360 nm) for simultaneous monitoring of different group of compounds. The ESI-MS was acquired in negative ionization mode. Total ion chromatogram (TIC) with mass-to-charge ratio (*m*/*z*) ranging from 100 to 1000 was obtained.

4.2.4 Cell culture

HL-60 (ATCC® CCL-240™, human acute promyelocytic leukaemia), MCF7 (ATCC® HTB-22™), MDA-MB-231 (ATCC® HTB-26™) (human breast adenocarcinoma), HCT 116 (ATCC® CCL-247™, human colorectal carcinoma), PC-3 (ATCC® CRL-1435™, human prostate adenocarcinoma cells), MRC-5 (ATCC® CCL-171™, human lung fibroblast), Hep G2 (ATCC® HB-8065™, human hepatocellular carcinoma) and WRL 68 (ATCC® CL-48™, human embryonic liver, HeLa derivative) cell lines were obtained from the American Type Culture Collection (ATCC, USA).

HSC2 (human squamous carcinoma cells) were obtained from the Human Science Research Resources Bank (Osaka, Japan) while HK1 (human nasopharyngeal carcinoma) was a kind gift from Professor Tsao at University of Hong Kong.

HL-60, MCF7, HCT 116, A549 and PC-3 were cultured in RPMI-1640 (Roswell Park Memorial Institute) media (Gibco). MDA-MB-231, Hep G2 and WRL 68 were maintained in DMEM (Dulbecco's Modified Eagle Medium) while HSC2, HK1 and MRC-5 were grown in MEM (Minimum Essential Medium) (Gibco). All media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 100 unit/ml penicillin/streptomycin (Gibco). Cells were cultured in 5% CO_2 incubator at 37 °C in a humidified atmosphere. Cells were sub-cultured every 2 or 3 days and routinely checked under an inverted microscope. For solid tumours, cells were harvested using 0.25% (v/v) trypsin in PBS when 80% confluence was reached. Only cells at exponential growth were used throughout the experiments.

4.2.5 Cell viability assay

4.2.5.1 Preparation of samples

Mushroom aqueous extracts were dissolved in 50% (v/v) aqueous dimethyl sulfoxide (DMSO) whereas organic solvent extracts were dissolved in DMSO. Cisplatin [*cis*-diamineplatinum (II) dichloride] (Sigma-Aldrich, USA) was dissolved in sterile phosphate buffered saline (PBS). Stock of extracts (20-50 mg/ml) and cisplatin (10 mM) were prepared and further diluted with culture media to achieve desired final concentrations. The effect of various mushroom extracts and cisplatin on cell viability was determined using the MTT assay.

4.2.5.2 MTT assay

The *in vitro* cytotoxicity of the aqueous extracts of *L. rhinocerotis* was evaluated using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the method of Mosmann (1983) with some modifications. This assay relies on the reduction of yellow tetrazolium MTT to a purple formazan dye by mitochondrial dehydrogenase enzyme; hence, the amount of formazan produced reflects the number of metabolically active viable cells.

Briefly, viable cells were seeded at optimal cell density (3-5 × 10^3 cells/well) into 96-well flat bottom culture plates and incubated overnight for attachment. Following that, the cells were treated with the extracts or cisplatin. For the untreated cells (negative control), DMSO or PBS were added. After 72 h of incubation, 20 µl of MTT (5 mg/ml in PBS pH 7.4, Sigma-Aldrich, USA) was added to each well and the plate was further incubated for another 4 h at 37 °C. Following incubation, media were removed by gentle aspiration and replaced with 100 µl of DMSO to dissolve the formazan crystals. The amount of formazan product was measured at 570 nm using a microplate reader (Synergy H1 Multi-mode Reader, BioTek Instruments, Inc.). The percentage of cell viability was determined by the following equation:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \times 100$$

The IC_{50} values (concentration of extracts required to reduce cell viability to 50%) were determined from dose-response curves using SigmaPlot for Windows Version 11.0 (Systat Software, Inc.).

4.2.6 Statistical analysis

Each analysis was performed in triplicates and results were expressed as mean ± S.D. or S.E. (n = 3). The data were statistically analysed using the IBM® SPSS® Statistics Version 19 (SPSS Inc., United States). All mean values were analysed by one-way analysis of variance (ANOVA) followed by Tukey-HSD ($p < 0.05$) to detect significance between groups.

4.3 Results

4.3.1 Extraction yield

The mycelium, culture broth, fruiting body (separated into pileus and stipe), and sclerotium of *L. rhinocerotis* were subjected to extraction procedure using different solvents such as water, dichloromethane, and methanol. Table 4.1 shows the yield (on a dry weight basis) of the crude hot aqueous, cold aqueous, dichloromethane, and methanol extracts of *L. rhinocerotis*.

In general, the yield (w/w) of crude aqueous extracts (3.6-13.8%) was higher than organic solvent extracts (0.1-10.2%). Between the two types of aqueous extracts, it was apparent that the yield of hot aqueous extracts (7.0-13.6%) was higher than cold aqueous extracts (3.6-7.9%) for all samples of *L. rhinocerotis* except in the case of the commercially-produced sclerotium. On the other hand, when organic solvents were used, the yield of extracts prepared from dichloromethane (0.1-1.0%) was consistently lower than those prepared from methanol (1.4-10.2%).

Our results indicated that efficiency of the extraction procedure using water (with or without heating), dichloromethane, and methanol in the present study differed depending on the samples of *L. rhinocerotis*. For instance, when dichloromethane was used as extraction solvent, the yield increased in the order of culture broth < sclerotium < pileus < stipe < mycelium whereas a different trend was observed with extraction using methanol whereby sclerotium < stipe < mycelium < pileus < culture broth. For aqueous extracts, there seemed to be no consistent pattern; however, it was noted that, amongst the samples of *L. rhinocerotis* from different developmental stages, extracts of the stipe seemed to have the lowest yield irrespective of the choice of hot or cold aqueous extractions.

Table 4.1: Yield of *L. rhinocerotis* crude aqueous and organic solvent extracts on a dry weight basis

Mushroom samples	Yield (%, w/w)			
	Hot aqueous extracts	**Cold aqueous extracts**	**Dichloromethane extracts**	**Methanol extracts**
Mycelium				
Shaken condition	10.2	7.8	0.92	3.42
Static condition	11.8	5.6	0.68	4.16
Culture broth				
Shaken condition	N.A.	N.A.	0.07	10.21
Static condition	N.A.	N.A.	0.06	8.91
Pileus				
Cultivated	13.8	7.6	0.98	6.85
Wild type	10.7	7.4	0.81	4.50
Stipe				
Cultivated	8.7	4.7	0.29	3.40
Wild type	8.5	3.6	0.42	3.20
Sclerotium				
Cultivated (without fruiting body)	9.8	6.5	0.12	2.92
Cultivated (with fruiting body)	13.6	6.8	0.14	2.54
Wild type	11.3	5.5	0.09	1.44
Commercial source	7.0	7.9	0.11	3.75

N.A., not available.

4.3.2 Chemical composition

The proportion of carbohydrates and proteins in the aqueous extracts of *L. rhinocerotis* were determined using the phenol-sulfuric and BCA assays, respectively. Table 4.2 shows the carbohydrate and protein contents (on a dry weight basis) of *L. rhinocerotis* hot aqueous and cold aqueous extracts.

Carbohydrates (w/w) were generally higher in hot aqueous (30.5-53.7%) than cold aqueous (21.9-45.3%) extracts of *L. rhinocerotis*. Amongst the hot aqueous extracts, extracts of the mycelium from shaken (56.2%) and static (53.7%) conditions have the highest carbohydrate contents. For the cold aqueous extracts, relatively high carbohydrate contents were observed in extracts of the commercially-produced sclerotium (45.3%), wild type sclerotium (39.2%), and wild type pileus (39.2%).

Proteins also made up a substantial amount of the chemical components in *L. rhinocerotis* aqueous extracts with protein contents in hot aqueous extracts (22.2-51.8%) being overall higher than those in cold aqueous extracts (11.9-39.6%). The hot aqueous extracts of *L. rhinocerotis* sclerotium (regardless of origin) and wild type fruiting body have higher protein content (>37.0%) than other samples prepared in similar manner. Amongst the cold aqueous extracts, extracts of the wild type sclerotium, fruiting body, and cultivated sclerotium contained relatively high (> 30%) protein content when compared to other samples.

Table 4.2: Chemical composition of *L. rhinocerotis* aqueous extracts

Mushroom samples	Chemical content (%, w/w)			
	Hot aqueous extracts		Cold aqueous extracts	
	Carbohydrates	Proteins	Carbohydrates	Proteins
Mycelium				
Shaken condition	56.2 ± 2.11^{a}	23.7 ± 0.50^{b}	27.6 ± 1.91^{a}	18.3 ± 0.67^{b}
Static condition	$53.7 \pm 3.23^{a,b}$	22.2 ± 0.32^{a}	24.4 ± 1.00^{a}	19.7 ± 0.60^{b}
Pileus				
Cultivated	33.6 ± 1.78^{e}	28.2 ± 0.13^{c}	34.5 ± 2.58^{b}	25.8 ± 0.60^{c}
Wild type	$30.5 \pm 1.29_{e}$	40.3 ± 0.45^{e}	$39.2 \pm 2.85^{b,c}$	$36.1 \pm 2.12^{d,e}$
Stipe				
Cultivated	$41.1 \pm 2.20^{c,d}$	27.9 ± 0.14^{c}	26.8 ± 3.51^{a}	26.5 ± 1.26^{c}
Wild type	45.8 ± 2.55^{c}	40.4 ± 0.76^{e}	26.5 ± 1.15^{a}	35.1 ± 0.83^{d}
Sclerotium				
Cultivated (without fruiting body)	$46.6 \pm 3.75^{b,c}$	39.2 ± 0.31^{e}	21.9 ± 2.08^{a}	19.7 ± 1.39^{b}
Cultivated (with fruiting body)	$48.2 \pm 2.78^{b,c}$	37.0 ± 0.43^{d}	36.4 ± 1.69^{b}	33.1 ± 1.95^{d}
Wild type	$43.1 \pm 2.55^{c,d}$	$37.0 \pm .019^{d}$	$39.2 \pm 0.75^{b,c}$	39.6 ± 2.05^{e}
Commercial source	$36.6 \pm 2.85^{d,e}$	51.8 ± 0.30^{f}	45.3 ± 2.61^{c}	11.9 ± 0.47^{a}

Carbohydrate and protein contents were determined using the phenol-sulfuric and BCA assays, respectively. Results were expressed as mean ± S.D. (n = 3). Means with different letters (a-f) within the same column are significantly different ($p < 0.05$).

In addition, protein contents in *L. rhinocerotis* aqueous extracts were either comparable or lower than carbohydrates (irrespective of the choice of hot or cold aqueous extraction procedure) except in the case of the cold aqueous extract of commercially-produced sclerotium that contained higher proteins (51.1%) than carbohydrates (36.6%). In some cases, hot aqueous and cold aqueous extraction of the same mushroom sample yielded extracts with comparable amount of proteins as exemplified by the case of mycelium from shaken and static conditions (hot aqueous extract: 22.2-23.7%, cold aqueous extract: 18.3-19.7%), cultivated pileus (hot aqueous extract: 28.2%, cold aqueous extract 25.8%), and cultivated stipe (hot aqueous extract: 27.9%, cold aqueous extract, 26.5%).

4.3.3 Chemical profiling

The crude aqueous and organic solvent extracts of *L. rhinocerotis* were profiled using UPLC-ESI-MS. TIC was collected in the negative ionization mode. For the hot aqueous (Appendix A, pp. 307-310) and cold aqueous (Appendix B, pp. 311-314) extracts, chemical profiles of the crude aqueous extracts from the following groups (i.e. mycelium from shaken and static conditions; cultivated and wild type fruiting body; as well as sclerotium that was cultivated, collected from the wild, and commercially-produced) were fairly comparable; however, some differences were observed when extracts of the same mushroom sample were prepared by different methods (i.e. hot or cold aqueous extraction). Elution of most peaks at lower organic strength seemed to suggest that most of the components in the aqueous extracts were those of higher polarity.

For the dichloromethane extracts of *L. rhinocerotis* (Appendix C, pp. 315-319), chemical profiles of the mycelium and culture broth produced under shaken or static

conditions of liquid fermentation did not differ much. The dichloromethane extracts of the mycelium showed similarity to those of the culture broth at lower organic strength but there were multiple peaks eluted at higher organic strength that were specific to the mycelium. Chemical profiles of the cultivated and wild type pileus and stipe were overall comparable although the intensity of some peaks varied.

The methanol extracts of *L. rhinocerotis* (Appendix D, pp. 320-324) generally have lesser peaks compared to the dichloromethane extracts with most peaks eluted at higher organic strength. Similar to the trends observed for the dichloromethane extracts, chemical profiles of the methanol extracts of mycelium and culture broth from shaken and static conditions as well as the pileus and stipe from different origins were comparable. Only the sclerotial methanol extracts were characterized with peaks eluted at lower organic strength.

Based on our results, differences in the chemical composition of *L. rhinocerotis* from different developmental stages (e.g. fruiting body vs. sclerotium vs. mycelium) was apparent based on LC-MS profiling; however, variation in chemical profiles of the cultivated and wild type samples (different origin), as well as mycelium and culture broth from shaken and static conditions of liquid fermentation (different cultivation condition), was lesser. For instance, depending on the solvent used in the extraction procedure, chemical profiles of the cultivated sclerotium mostly resembled that of the wild type sclerotium in terms of the characteristic major peaks in the PDA and TIC but both showed some differences when compared to the commercially-produced sclerotium.

4.3.4 Cytotoxic activity of the aqueous extracts of mushrooms

The hot aqueous and cold aqueous extracts of *L. rhinocerotis* (final concentration: 15.63-500.00 μg/ml) were screened for cytotoxic activity against a panel of human cancer (i.e. HL-60, MCF7, MDA-MB-231, HCT 116, HSC2, HK1, PC3, Hep G2, and WRL 68) and non-cancerous (i.e. MRC-5) cell lines using the MTT assay. Cytotoxic activity of the extracts, after an incubation period of 72 h, was expressed in the form of IC_{50} values that were calculated from dose-response curves.

4.3.4.1 Mycelium

Table 4.3 shows the cytotoxic activity of the aqueous extracts of *L. rhinocerotis* mycelium from liquid fermentation. In general, the mycelial extracts, irrespective of extraction methods and culture conditions, exhibited moderate to weak cytotoxicity against the cell lines (IC_{50} > 250 μg/ml). When comparing the mycelium from shaken and static conditions, lower IC_{50} values were noted for the latter in most cases. For instance, hot aqueous (IC_{50}: 264.6 μg/ml) and cold aqueous (IC_{50}: 403.1 μg/ml) extracts of the mycelium from static condition inhibited the growth of PC-3 but both hot and cold aqueous extracts of the mycelium from shaken conditions were inactive (IC_{50} > 500 μg/ml). In fact, apart from demonstrating very weak cytotoxic activity against MCF7 (IC_{50}: 387.4-404.3 μg/ml), the mycelial aqueous extracts from shaken condition were inactive (IC_{50} > 500 μg/ml) against other cell lines tested in the present study.

When comparing the effect of extraction methods, our results indicated that mycelial hot aqueous extracts, particularly that from static condition, showed stronger cytotoxic activity against several cell lines (e.g. MCF7, HCT 116, PC-3 and Hep G2) than the cold aqueous extract. The leukemic cell line, HL-60, was not affected by any of the mycelial aqueous extracts (IC_{50} > 500 µg/ml). All *L. rhinocerotis* mycelial extracts were also non-cytotoxic against the non-cancerous cell line, MRC-5 (IC_{50} > 500 µg/ml).

Table 4.3: Cytotoxic activity of the aqueous extracts of *L. rhinocerotis* mycelium

Cell lines	IC_{50} (µg/ml)			
	Mycelium (shaken condition)		Mycelium (static condition)	
	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous
HL-60	> 500	> 500	> 500	> 500
MCF7	404.3 ± 27.39	387.4 ± 64.69	289.9 ± 30.85	435.9 ± 15.93
MDA-MB-231	> 500	> 500	> 500	> 500
HCT 116	> 500	> 500	400.0 ± 18.68	> 500
HSC2	> 500	> 500	> 500	> 500
HK1	> 500	> 500	> 500	> 500
PC-3	> 500	> 500	264.6 ± 26.00	403.1 ± 19.19
Hep G2	> 500	> 500	431.8 ± 3.68	> 500
WRL 68	> 500	> 500	> 500	> 500
MRC-5	> 500	> 500	> 500	> 500

Cell viability post treatment with the mushroom hot and cold aqueous extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.4.2 Fruiting body

Table 4.4 shows the cytotoxic activity of aqueous extracts of the cultivated and wild type fruiting body of *L. rhinocerotis*. Results showed that aqueous extracts of the wild type pileus of demonstrated stronger cytotoxicity than the cultivated pileus. Disregarding the origin of the samples, cold aqueous extracts of the pileus were active against MCF7 (IC_{50}: 24.1-37.2 μg/ml) and HSC2 (IC_{50}: 45.6-98.6 μg/ml). On the other hand, hot aqueous extracts of the pileus showed cytotoxicity against MCF7 (IC_{50}: 36.5-60.6 μg/ml), PC3 (IC_{50}: 36.5-55.6 μg/ml), and HCT 116 (IC_{50}: 58.8 μg/ml).

Aqueous extracts of the stipe of *L. rhinocerotis* showed overall moderate to weak activity. For instance, hot aqueous extract of the cultivated stipe moderately inhibited the growth of PC-3 (IC_{50}: 147.6 μg/ml) but its cytotoxicity against HCT 116 (IC_{50}: 308.0 μg/ml) and MDA-MB-231 (IC_{50}: 342.6 μg/ml) was considered weak. Although hot aqueous extract of the wild type stipe (IC_{50}> 500 μg/ml) was weaker than that of the cultivated samples in all cases, extracts prepared by cold aqueous extraction exhibited stronger cytotoxicity against HSC2 (IC_{50}: 115.4 μg/ml) and MDA-MB-231 (IC_{50}: 258.7 μg/ml) compared to the same extract of the cultivated samples which demonstrated weaker activity against the same cell lines (HSC2, IC_{50}: 347.8 μg/ml; MDA-MB-231, IC_{50}> 500 μg/ml).

All extracts, except for the cold aqueous extract of wild pileus (IC_{50}: 393.4 μg/ml), showed no effect on HL-60 (IC_{50}> 500 μg/ml). The aqueous extracts of *L. rhinocerotis* fruiting body, either cultivated or wild type, also showed no cytotoxicity against MRC-5 (IC_{50} > 500 μg/ml).

Table 4.4: Cytotoxic activity of the aqueous extracts of *L. rhinocerotis* cultivated and wild type fruiting body

Cell lines	IC_{50} (µg/ml)							
	Cultivated pileus		Wild type pileus		Cultivated stipe		Wild type stipe	
	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous
HL-60	> 500	393.4 ± 20.44	> 500	> 500	> 500	> 500	> 500	> 500
MCF7	60.6 ± 4.47	37.2 ± 2.12	36.5 ± 3.04	24.1 ± 1.76	> 500	> 500	> 500	> 500
MDA-MB-231	> 500	307.4 ± 4.19	332.6 ± 41.44	260.2 ± 18.91	342.6 ± 10.78	> 500	> 500	258.7 ± 3.42
HCT 116	205.5 ± 6.92	> 500	58.8 ± 0.80	> 500	308.0 ± 9.97	> 500	399.9 ± 8.31	> 500
HSC2	208.5 ± 5.39	45.6 ± 2.35	138.1 ± 4.80	98.6 ± 5.14	> 500	347.8 ± 9.25	> 500	115.4 ± 1.07
HK1	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
PC-3	56.0 ± 6.42	> 500	36.5 ± 3.04	> 500	147.6 ± 8.68	> 500	> 500	425.7 ± 21.89
Hep G2	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
WRL 68	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
MRC-5	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500

Cell viability post treatment with the mushroom hot and cold aqueous extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.4.3 Sclerotium

Table 4.5 shows the cytotoxic activity of the cultivated, wild type, and commercially-produced sclerotium of *L. rhinocerotis*. The effect of extraction methods (hot or cold aqueous extraction) on the cytotoxic activity of *L. rhinocerotis* sclerotium is clear. Contrary to some of the hot aqueous extracts of the mycelium and fruiting body which demonstrated moderate to weak cytotoxicity, *L. rhinocerotis* sclerotial hot aqueous extracts from different sources (i.e. cultivated, wild-type, and commercially-produced) were inactive (IC_{50} > 500 μg/ml) against all cell lines.

Lignosus rhinocerotis sclerotial cold aqueous extracts, on the other hand, exhibited different degree of cytotoxicity against the cells lines and results were influenced by the origin of the samples. It was observed that cold aqueous extract of the sclerotium that was harvested prior to formation of fruiting body exhibited moderate cytotoxicity against most cell lines, including MCF7 (IC_{50}: 36.7 μg/ml), HCT 116 (IC_{50}: 36.8 μg/ml), PC3 (IC_{50}: 43.1 μg/ml), HSC2 (IC_{50}: 56.8 μg/ml), MDA-MB-231 (IC_{50}: 79.7 μg/ml), WRL 68 (IC_{50}: 84.5 μg/ml), and HK1 (IC_{50}: 88.1 μg/ml). Similarly, cold aqueous extract of the cultivated sclerotium attached to fruiting body was cytotoxic against the aforementioned cell lines but the IC_{50} values were generally higher, except for HSC2 (IC_{50}: 19.1 μg/ml) and WRL 68 (IC_{50}: 81.4 μg/ml).

Further, cytotoxicity of the wild type sclerotium of *L. rhinocerotis* was also fairly comparable to the cultivated sclerotium harvested at different stages. In some cases, cold aqueous extract of the wild type sclerotium inhibited the growth of cancer cell lines such as MDA-MB-231 (IC_{50}: 82.2 μg/ml), HSC2 (IC_{50}: 51.9 μg/ml), and WRL 68 (IC_{50}: 81.6 μg/ml) with potency considered comparable to the cultivated sclerotium (refer above). However, its cytotoxic activity against other cell lines, for example, MCF7 (IC_{50}: 67.2 μg/ml), HCT 116 (IC_{50}: 105.7 μg/ml) and HK1 (IC_{50}: 172.9 μg/ml), was

approximately 2-3 folds lower than those observed for the cultivated sclerotium (refer above).

Cold aqueous extract of the commercially-produced sclerotium of *L. rhinocerotis* showed only mild cytotoxicity (IC_{50}: 154.7-456.3 μg/ml) against cancer cell lines that were previously observed to be sensitive to the cultivated and wild type sclerotial aqueous extracts. By comparison, the IC_{50} values of the commercially-produced sclerotial extracts were approximately 5-10 times higher than those observed for the cultivated and wild type samples.

While the sclerotial cold aqueous extracts showed inhibition against all solid tumours tested in the present study, interestingly, only cold aqueous extracts of the sclerotium with fruiting body were able to inhibit the leukemic cell line, HL-60 with extract of the wild type sclerotium (IC_{50}: 128.7 μg/ml) being more potent than the cultivated sclerotium (IC_{50}: 288.8 μg/ml).

The non-cancerous cell line, MRC-5, was not affected by *L. rhinocerotis* sclerotial hot aqueous extracts (IC_{50} > 500 μg/ml). On the contrary, it was susceptible to the aqueous extracts of sclerotium harvested before and after the formation of fruiting body (IC_{50}: 52.5-68.5 μg/ml) and wild type samples (IC_{50}: 63.4 μg/ml) sclerotial cold aqueous extract but surprisingly, not for the commercially-produced sclerotium of *L. rhinocerotis* (IC_{50} > 500 μg/ml).

Table 4.5: Cytotoxic activity of the aqueous extracts of *L. rhinocerotis* cultivated, wild type, and commercially-produced sclerotium

Cell lines	IC_{50} (µg/ml)							
	Cultivated sclerotium (without fruiting body)		Cultivated sclerotium (with fruiting body)		Wild type sclerotium		Commercially-produced sclerotium	
	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous	Hot aqueous	Cold aqueous
HL-60	> 500	> 500	> 500	288.8 ± 10.90	> 500	128.7 ± 10.55	> 500	> 500
MCF7	> 500	36.7 ± 3.46	> 500	44.01 ± 0.14	> 500	67.2 ± 1.49	> 500	154.7 ± 6.77
MDA-MB-231	> 500	79.7 ± 3.57	> 500	151.8 ± 8.90	> 500	82.2 ± 5.41	> 500	325.6 ± 32.07
HCT 116	> 500	36.8 ± 0.87	> 500	58.4 ± 0.15	> 500	105.7 ± 10.31	> 500	209.5 ± 9.12
HSC2	> 500	56.8 ± 6.20	> 500	19.1 ± 0.85	> 500	51.9 ± 1.97	> 500	319.7 ± 20.7
HK1	> 500	88.1 ± 10.88	> 500	143.2 ± 3.53	> 500	172.9 ± 21.88	> 500	456.3 ± 12.59
PC-3	> 500	43.1 ± 4.63	> 500	57.9 ± 0.45	> 500	59.5 ± 0.54	> 500	213.2 ± 12.90
Hep G2	> 500	120.0 ± 1.95	> 500	105.7 ± 6.65	> 500	97.8 ± 3.74	> 500	369.8 ± 14.41
WRL 68	> 500	84.5 ± 2.00	> 500	81.4 ± 1.49	> 500	81.6 ± 0.76	> 500	421.6 ± 8.92
MRC-5	> 500	52.5 ± 0.70	> 500	68.5 ± 0.45	> 500	63.4 ± 0.73	>500	> 500

Cell viability post treatment with the mushroom hot and cold aqueous extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.4.4 Edible-medicinal mushrooms

Cytotoxic activity of selected edible-medicinal mushrooms against MCF7, HCT 116, and MRC-5 was also tested for comparison purposes. The results are shown in Table 4.6. The cold aqueous extract of *L. tuber-regium* fruiting body (IC_{50}: 11.4 μg/ml) showed remarkable cytotoxicity against MCF7 whereas the hot aqueous extract (IC_{50}: 135.2 μg/ml) was approximately 10 times weaker. On the other hand, the cold aqueous extract of *L. tuber-regium* sclerotium showed moderate cytotoxicity against MCF7 (IC_{50}: 77.4 μg/ml) but the sclerotial hot aqueous extract was inactive (IC_{50} > 500 μg/ml). Cytotoxicity of the hot aqueous extracts of *G. lucidum* (IC_{50}: 309.6 μg/ml) and *T. heimii* (: 324.5 μg/ml) fruiting bodies against MCF7 was rather weak in comparison to the aqueous extracts of *L. tuber-regium*. All mushroom extracts were only marginally cytotoxic (IC_{50}: 328.8-453.3 μg/ml) against HCT 116. The hot aqueous and cold aqueous extracts of *G. lucidum*, *L. tuber-regium*, and *T. heimii* were shown to be non-cytotoxic against MRC-5 (IC_{50} > 500 μg/ml).

Table 4.6: Cytotoxic activity of the aqueous extracts of selected edible-medicinal mushrooms

Cell lines	IC_{50} (µg/ml)							
	G. lucidum		***L. tuber-regium* fruiting body**		***L. tuber-regium* sclerotium**		***T. heimii***	
	Hot aqueous	**Cold aqueous**	**Hot aqueous**	**Cold aqueous**	**Hot aqueous**	**Cold aqueous**	**Hot aqeuous**	**Cold aqueous**
MCF7	309.6 ± 9.95	> 500	135.2 ± 19.42	11.41 ± 1.00	> 500	77.4 ± 7.50	324.5 ± 33.18	440.4 ± 32.87
HCT 116	> 500	> 500	> 500	> 500	> 500	328.8 ± 8.21	453.3 ± 18.38	377.8 ± 37.00
MRC-5	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500

Cell viability post treatment with the mushroom hot and cold aqueous extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.4.5 Comparison of cytotoxic activity of the aqueous extracts of *L. rhinocerotis* with selected edible-medicinal mushrooms

Lignosus rhinocerotis sclerotial cold aqueous extracts exhibited broad-spectrum cytotoxic activity and hence, it is of interest to compare its activity with other mushrooms under similar experimental condition. By comparison, it is apparent that the cold aqueous extract of both cultivated and wild type *L. rhinocerotis* sclerotium demonstrated higher cytotoxic activity against MCF7 (IC_{50}: 36.7-67.2 µg/ml) and HCT 116 (IC_{50}: 36.8-105.7 µg/ml) than the extracts of *L. tuber-regium* sclerotium (prepared in similar manner) which showed weaker inhibition against the cell lines (MCF7, IC_{50}: 77.4 µg/ml; HCT 116, IC_{50}: 328.8 µg/ml). The sclerotial hot aqueous extracts of both mushrooms were inactive (IC_{50}> 500 µg/ml) against both cell lines.

Amongst the fruiting bodies, the hot aqueous (IC_{50}: 36.5-60.6 µg/ml) and cold aqueous extracts (IC_{50}: 24.1-36.5 µg/ml) of *L. rhinocerotis* pileus also showed comparable or stronger cytotoxicity against MCF7 than *G. lucidum* hot aqueous (IC_{50}: 309.6 µg/ml) and cold aqueous (IC_{50}> 500 µg/ml) extracts, and *L. tuber-regium* hot aqueous extract (IC_{50}: 135.2 µg/ml). Cold aqueous extract of *L. tuber-regium* fruiting body, however, was more potent (IC_{50}: 11.4 µg/ml) than that of *L. rhinocerotis*. For HCT 116, the hot aqueous extract of *L. rhinocerotis* pileus showed moderate cytotoxicity (IC_{50}: 58.8-205.5 µg/ml) whereas those of the fruiting bodies of *L. tuber-regium* and *G. lucidum* were inactive (IC_{50} > 500 µg/ml). Similarly, the cold aqueous extracts of *L. rhinocerotis* pileus, and *L. tuber-regium* and *G. lucidum* fruiting bodies were inactive against HCT 116 (IC_{50} > 500 µg/ml).

Based on our results, it can be deduced that cytotoxic potential of the aqueous extracts of *L. rhinocerotis*, especially the pileus and sclerotium, was indeed comparable to that of *L. tuber-regium* and *G. lucidum*.

4.3.5 Cytotoxic activity of the organic solvent extracts of mushrooms

In a similar manner, the dichloromethane and methanol extracts of *L. rhinocerotis* (final concentration: 6.25-200.00 µg/ml) were screened for cytotoxic activity against similar panel of cancer and non-cancerous cell lines using the MTT assay. Results, in the form of IC_{50} values, were tabulated.

4.3.5.1 Mycelium and culture broth

Table 4.7 shows the cytotoxic activity of the organic solvent extracts of *L. rhinocerotis* mycelium and culture broth. Dichloromethane extracts of the mycelium from shaken (IC_{50}: 57.6-187.6 µg/ml) and static (IC_{50}: 39.4-151.4 µg/ml) conditions showed moderate cytotoxicity against the cancer cells whereas both mycelial methanol extracts were inactive (IC_{50} > 200 µg/ml). Between the two different conditions of liquid fermentation, it was noted that extracts of the mycelium from static condition exhibited stronger cytotoxicity against most cell lines than that of the shaken cultures. For instance, the dichloromethane extract of mycelium from static condition inhibited the growth of HCT 116, PC3, Hep G2, and WRL 68 with the IC_{50} values of 50.9, 81.2, 128.6, and 39.4 µg/ml, respectively. By comparison, cytotoxic activity of the dichloromethane extract of mycelium from shaken condition against similar cell lines was weaker with IC_{50} values of 90.3, 104.1, 187.6, and 76.8 µg/ml, respectively.

On the other hand, both dichloromethane and methanol extracts of the culture broth of *L. rhinocerotis*, irrespective of shaken or static conditions, were observed to be inactive (IC_{50} > 200 µg/ml) against all cell lines.

Table 4.7: Cytotoxic activity of the organic solvent extracts of *L. rhinocerotis* mycelium and culture broth

Cell lines	IC_{50} (µg/ml)							
	Mycelium (shaken condition)		Mycelium (static condition)		Culture broth (shaken condition)		Culture broth (static condition)	
	DCM	MeOH	DCM	MeOH	DCM	MeOH	DCM	MeOH
HL-60	80.6 ± 2.78	> 200	73.8 ± 3.14	> 200	> 200	> 200	> 200	> 200
MCF7	135.1 ± 5.99	> 200	128.7 ± 2.18	> 200	> 200	> 200	> 200	> 200
MDA-MB-231	57.6 ± 1.79	> 200	151.4 ± 9.38	> 200	> 200	> 200	> 200	> 200
HCT 116	90.3 ± 4.84	> 200	50.9 ± 0.37	> 200	> 200	> 200	> 200	> 200
HSC2	139.0 ± 2.38	> 200	124.1 ± 2.50	> 200	> 200	> 200	> 200	> 200
HK1	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200
PC-3	104.1 ± 4.21	> 200	81.2 ± 4.22	> 200	> 200	> 200	> 200	> 200
Hep G2	187.6 ± 5.83	> 200	128.6 ± 3.30	> 200	> 200	> 200	> 200	> 200
WRL 68	76.8 ± 9.99	> 200	39.4 ± 1.55	> 200	> 200	> 200	> 200	> 200
MRC-5	71.1 ± 5.29	> 200	> 200	> 200	> 200	> 200	>200	> 200

Cell viability post treatment with the mushroom dichloromethane (DCM) and methanol (MeOH) extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

The dichloromethane extract of mycelium from shaken condition inhibited the growth of MRC-5 (IC_{50}: 71.1 µg/ml) but surprisingly, the mycelial extract from static condition was non-cytotoxic (IC_{50} > 200 µg/ml) within the tested concentrations. This observation implied the differences in the cytotoxic potential of the chemical components of mycelial extracts from different conditions of liquid fermentation. All extracts of the culture broth, on the other hand, showed no cytotoxicity against MRC-5 (IC_{50} > 200 µg/ml).

4.3.5.2 Fruiting body

Table 4.8 shows the cytotoxic activity of the cultivated and wild type fruiting body of *L. rhinocerotis*. Similar to previous observation on the mycelial extracts, the dichloromethane extracts, in general, showed stronger cytotoxicity than the methanol extracts. Dichloromethane extracts of the cultivated (IC_{50}: 3.5-23.9 µg/ml) and wild type (IC_{50}: 4.2-56.2 µg/ml) pileus exhibited potent cytotoxic activity. Similar trends were observed for the extracts of the stipe in which the extracts of the cultivated (IC_{50}: 4.8-46.2 µg/ml) and wild type (IC_{50}: 6.18-62.9 µg/ml) stipe showed strong to moderate cytotoxicity against the cells. The potency of dichloromethane extracts of the pileus and stipe of *L. rhinocerotis* seemed comparable in most cases, but it was also dependent on the susceptibility of the individual cell line. For instance, MCF7 was noted to be sensitive to the dichloromethane extracts of the pileus (IC_{50}: 3.5-4.2 µg/ml) and stipe (IC_{50}: 4.2- 4.8 µg/ml).

Methanol extracts of the pileus of *L. rhinocerotis* were largely inactive (IC_{50} > 200 µg/ml) although these were shown to exhibit cytotoxicity against some cell lines, such as MDA-MB-231 (IC_{50}: 22.4-167.9 µg/ml), MCF7 (IC_{50}: 88.8 µg/ml), and HCT 116 (IC_{50}: 128.5 µg/ml). Methanol extracts of the stipe, on the other hand, showed different degree of cytotoxicity against the cell lines with MDA-MB-231 (IC_{50}: 30.3 µg/ml), WRL 68 (IC_{50}: 57.5 µg/ml), and HK1 (IC_{50}: 86.7 µg/ml) being susceptible to extracts of wild type stipe (LRWS-ME). Other cell lines, such as PC-3 and HSC2, were not affected by the methanol extracts of the stipe (IC_{50} > 200 µg/ml).

Cytotoxic activity of extracts of the cultivated and wild type samples extracted using dichloromethane was fairly comparable in most cases; however, bigger differences were observed for the methanol extracts. In most cases, methanol extracts of the wild type samples were more cytotoxic than the cultivated counterparts. For instance, methanol extracts of the wild type pileus (IC_{50}: 88.8 µg/ml) and stipe (IC_{50}: 165.1 µg/ml) inhibited the growth of MCF7 whereas similar extracts of the cultivated samples were inactive (IC_{50} > 200 µg/ml).

Consistent with the trend observed for the mycelial extracts, dichloromethane extracts of the pileus (IC_{50}: 26.9-27.8 µg/ml) and stipe (IC_{50}: 10.8-49.2 µg/ml) were observed to be cytotoxic against MRC-5, the non-cancerous cell line. The methanol extracts were inactive (IC_{50} > 200 µg/ml).

Table 4.8: Cytotoxic activity of the organic solvent extracts of *L. rhinocerotis* cultivated and wild type fruiting body

Cell lines	IC_{50} (µg/ml)							
	Cultivated pileus		Wild type pileus		Cultivated stipe		Wild type stipe	
	DCM	MeOH	DCM	MeOH	DCM	MeOH	DCM	MeOH
HL-60	9.9 ± 0.33	> 200	11.7 ± 0.15	> 200	13.5 ± 0.36	114.0 ± 10.42	16.0 ± 0.49	121.4 ± 8.32
MCF7	3.8 ± 0.77	> 200	4.2 ± 0.25	88.8 ± 5.33	4.8 ± 0.26	> 200	6.1 ± 0.10	165.1 ± 5.50
MDA-MB-231	11.9 ± 0.60	167.9 ± 16.67	6.9 ± 0.66	22.4 ± 1.92	22.02 ± 0.83	82.9 ± 9.53	8.83 ± 1.31	30.3 ± 1.88
HCT 116	23.9 ± 5.58	> 200	56.2 ± 0.63	128.5 ± 2.96	40.3 ± 7.00	132.7 ± 6.58	62.9 ± 0.55	135.2 ± 2.50
HSC2	18.2 ± 0.49	> 200	11.0 ± 0.85	> 200	23.0 ± 0.35	> 200	23.8 ± 2.04	> 200
HK1	14.4 ± 0.39	> 200	11.4 ± 1.32	> 200	46.2 ± 4.42	> 200	24.0 ± 3.13	86.7 ± 4.13
PC-3	13.1 ± 0.39	> 200	14.5 ± 0.71	> 200	14.7 ± 0.46	> 200	24.9 ± 1.49	> 200
Hep G2	19.3 ± 0.72	> 200	11.9 ± 1.18	> 200	23.1 ± 1.47	164.7 ± 9.94	25.9 ± 2.35	> 200
WRL 68	11.0 ± 0.68	> 200	7.78 ± 0.69	> 200	23.6 ± 0.17	131.4 ± 1.44	10.3 ± 1.25	57.5 ± 4.46
MRC-5	26.9 ± 0.79	> 200	27.8 ± 2.15	> 200	10.8 ± 0.61	> 200	49.2 ± 3.88	> 200

Cell viability post treatment with the mushroom dichloromethane (DCM) and methanol (MeOH) extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.5.3 Sclerotium

Cytotoxic activity of the cultivated, wild type, and commercially-produced sclerotium of *L. rhinocerotis* is presented in Table 4.9. Dichloromethane extracts of the sclerotium harvested before and after the formation of fruiting body showed overall comparable cytotoxic activity; nevertheless, they are considered to be weaker than extracts of the wild type sclerotium. For instance, cytotoxicity of sclerotial extracts against HL-60 decreased in the order of wild type sclerotium (IC_{50}: 18.4 µg/ml) > cultivated sclerotium with fruiting body (IC_{50}: 30.1 µg/ml) > cultivated sclerotium without fruiting body (IC_{50}: 45.2 µg/ml) and similar trends were also observed for other cell lines, such as MCF7, MDA-MB-231, HK1, PC3, Hep G2, and WRL 68. On the other hand, methanol extracts of both cultivated and wild type sclerotium were inactive against most cell lines (IC_{50}> 200 µg/ml) except that for wild type sclerotium (IC_{50}: 82.9 µg/ml) and cultivated sclerotium with fruiting body (IC_{50}: 142.8 µg/ml) which demonstrated moderate cytotoxicity against MDA-MB-231.

Cytotoxicity of extracts prepared from the commercially-produced sclerotium did not follow similar trend observed for the cultivated and wild type sclerotium with regard to the relative potency of the extracts and susceptibility of cell lines. For instance, the growth of HCT 116 was inhibited by dichloromethane extracts (IC_{50}: 64.9-133.5 µg/ml) of the cultivated and wild type sclerotium, but not for the sclerotial methanol extracts (IC_{50}> 200 µg/ml). For the commercially-produced sclerotium, cytotoxic activity of the dichloromethane (IC_{50}: 58.1 µg/ml) and methanol (IC_{50}: 67.6 µg/ml) extracts against HCT 116 turned out to be fairly comparable. Besides, dichloromethane extract of commercially-produced sclerotium (IC_{50}: 34.5 µg/ml) also showed stronger cytotoxicity against MCF7 than cultivated sclerotium (IC_{50}: 65.7- 85.1 µg/ml).

In terms of susceptibility, some of the cell lines (e.g. Hep G2, MDA-MB-231, and WRL 68) were not affected by the dichloromethane extract of the commercially-produced sclerotium as opposed to their sensitivity towards the dichloromethane extracts of the cultivated and wild type sclerotium. Similarly, methanol extract of the commercially-produced sclerotium were able to inhibit the growth of PC-3 (IC_{50}: 132.1 µg/ml) and Hep G2 (IC_{50}: 125.6 µg/ml) that were otherwise not affected by the other sclerotial methanol extracts (IC_{50}> 200 µg/ml).

Dichloromethane extracts of the cultivated and wild type sclerotium exhibited marked cytotoxic activity against MRC-5 (IC_{50}: 20.5-95.0 µg/ml) whereas that of the commercially-produced sclerotium was non-cytotoxic (IC_{50} > 200 µg/ml). The sclerotial methanol extracts were also inactive against MRC-5 (IC_{50} > 200 µg/ml).

Table 4.9: Cytotoxic activity of the organic solvent extracts of *L. rhinocerotis* cultivated, wild type, and commercially-produced sclerotium

Cell lines	IC_{50} (µg/ml)							
	Cultivated sclerotium (without fruiting body)		Cultivated sclerotium (with fruiting body)		Wild type sclerotium		Commercially-produced sclerotium	
	DCM	MeOH	DCM	MeOH	DCM	MeOH	DCM	MeOH
HL-60	45.2 ± 1.30	> 200	30.1 ± 0.33	> 200	18.4 ± 0.25	> 200	57.3 ± 0.56	> 200
MCF7	85.1 ± 4.10	> 200	65.7 ± 1.54	> 200	11.3 ± 0.75	> 200	34.5 ± 7.51	> 200
MDA-MB-231	81.2 ± 5.04	> 200	41.3 ± 1.21	82.9 ± 9.53	19.9 ±1.68	142.8 ± 1.47	> 200	> 200
HCT 116	64.9 ± 3.07	> 200	133.5 ± 11.65	> 200	65.2 ± 1.41	> 200	58.1 ± 0.83	67.6 ± 4.93
HSC2	85.8 ± 5.64	> 200	92.1 ± 1.52	> 200	35.0 ± 2.49	> 200	93.2 ± 1.07	> 200
HK1	124.0 ± 6.98	> 200	70.0 ± 1.72	> 200	58.9 ± 2.23	> 200	> 200	> 200
PC-3	39.0 ± 2.12	> 200	55.6 ± 9.86	> 200	34.1 ± 1.30	> 200	33.4 ± 0.49	132.1 ± 1.34
Hep G2	109.9 ± 2.04	> 200	47.3 ± 1.67	> 200	18.9 ± 0.97	> 200	> 200	125.6 ± 4.79
WRL 68	27.8 ± 1.35	> 200	11.0 ± 0.68	> 200	9.1 ± 0.93	> 200	> 200	> 200
MRC-5	95.0 ±5.60	> 200	20.5 ± 1.72	> 200	53.7 ± 4.03	> 200	> 200	> 200

Cell viability post treatment with the mushroom dichloromethane (DCM) and methanol (MeOH) extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.5.4 Edible-medicinal mushrooms

For comparison purposes, organic solvent extracts of selected edible-medicinal mushrooms were tested for cytotoxicity against MCF7, HCT 116, and MRC-5. As shown in Table 4.10, similar trends were noted for the organic solvent extracts of *G. lucidum*, *P. tuber-regium*, and *T. heimii* in which the dichloromethane extracts showed stronger cytotoxicity than the methanol extracts. In particular, dichloromethane extracts of the fruiting bodies of *G. lucidum* (IC_{50}: 17.0 µg/ml) and *L. tuber-regium* (IC_{50}: 8.45 µg/ml), and sclerotium of *L. tuber-regium* (IC_{50}: 6.2 µg/ml) demonstrated strong cytotoxicity against MCF7. These extracts were also active against HCT 116 (IC_{50}: 8.3-24.3 µg/ml). Extracts of the fruiting body of *T. heimii*, however, showed only marginal cytotoxicity (IC_{50} > 120 µg/ml). Similarly, the methanol extract of *G. lucidum* fruiting body exhibited weak cytotoxicity against MCF7 (IC_{50}: 184.0 µg/ml) and HCT 116 (IC_{50}: 151.4 µg/ml) and others were inactive

In addition, the dichloromethane extract of *G. lucidum* fruiting body (IC_{50}: 50.8 µg/ml) and the methanol extract of *L. tuber-regium* sclerotium (IC_{50}: 142.8 µg/ml) demonstrated cytotoxicity against MRC-5. Other extracts did not show pronounced cytotoxicity against MRC-5 (IC_{50} > 200 µg/ml) within the tested concentrations.

Table 4.10: Cytotoxic activity of the organic solvent extracts of selected edible-medicinal mushrooms

Cell lines	IC_{50} (µg/ml)							
	G. lucidum		***L. tuber-regium* fruiting body**		***L. tuber-regium* sclerotium**		***T. heimii***	
	DCM	**MeOH**	**DCM**	**MeOH**	**DCM**	**MeOH**	**DCM**	**MeOH**
MCF7	17.0 ± 2.13	184.0 ± 4.15	8.45 ± 0.13	> 200	6.16 ± 0.14	> 200	128.9 ± 1.93	> 200
HCT 116	8.3 ± 0.651	151.4 ± 3.30	16.9 ± 3.36	> 200	24.3 ± 2.18	> 200	181.3 ± 4.31	> 200
MRC-5	50.8 ± 1.82	> 200	> 200	> 200	> 200	142.8 ± 1.47	> 200	> 200

Cell viability post treatment with the mushroom dichloromethane (DCM) and methanol (MeOH) extracts for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.3.5.5 Comparison of cytotoxic activity of the organic solvent extracts of *L. rhinocerotis* with selected edible-medicinal mushrooms

The sclerotial cold aqueous extract of *L. rhinocerotis* demonstrated stronger cytotoxicity than that of *L. tuber-regium* sclerotium; however, a different trend was observed for the mushroom organic solvent extracts. By comparing the cytotoxic activity against MCF7 and HCT 116, it was apparent that the dichloromethane extract of *L. tuber-regium* sclerotium (MCF7, IC_{50}: 6.16 µg/ml; HCT 116, IC_{50}: 24.3 µg/ml) was more potent than *L. rhinocerotis* sclerotial extracts (MCF7, IC_{50}: 11.3-85.1 µg/ml; HCT 116, IC_{50}: 64.9-133.5 µg/ml). The sclerotial methanol extracts of both mushroom species were inactive against HCT 116 (IC_{50}> 200 µg/ml)

On the other hand, dichloromethane extracts of the pileus (IC_{50}: 3.8-4.2 µg/ml) and stipe (IC_{50}: 4.8-6.1 µg/ml) of *L. rhinocerotis* exhibited stronger cytotoxicity against MCF7 than the extracts of *G. lucidum* (IC_{50}: 17.0 µg/ml) and *L. tuber-regium* (IC_{50}: 8.45 µg/ml) fruiting bodies. It is to be noted that a different trend was observed for HCT 116 whereby *L. rhinocerotis* pileus extracts (IC_{50}: 23.9-56.2 µg/ml) were weaker than those of *G. lucidum* (IC_{50}: 8.45 µg/ml) and *L. tuber-regium* (IC_{50}: 8.45 µg/ml) fruiting bodies. Methanol extracts of *L. rhinocerotis* pileus and stipe, in general, showed comparable cytotoxicity to that of the fruiting bodies of *G. lucidum*.

Our results from the cytotoxicity evaluation of *L. rhinocerotis* organic solvent extravts revealed that dichloromethane extract of the pileus was most potent (MCF7, IC_{50}: 3.8 µg/ml). Cytotoxic potential of dichloromethane extract of the pileus was found to be comparable to the extracts of *G. lucidum* and *L. tuber-regium* fruiting bodies prepared in a similar manner.

4.3.6 Cytotoxic activity of cisplatin

Cisplatin, a chemotherapy drug, was used as a positive control for the MTT assay. Results are shown in Table 4.11. It was showed that cisplatin was highly cytotoxic against all cell lines with HL-60 (IC_{50}: 3.2 μM) being more sensitive than the other solid-tumours (IC_{50}: 7.2-28.7 μM). In addition, MRC-5 (IC_{50}: 11.2 μM) was observed to be more sensitive to cisplatin compared to some cancer cell lines such as MCF7 (IC_{50}: 23.3 μM) and HCT 116 (IC_{50}: 16.0 μM).

Table 4.11: Cytotoxic activity of cisplatin

Cell lines	IC_{50} (μM)	
HL-60	3.3 ± 0.17	(1.0 ± 0.05 μg/ml)
MCF7	23.3 ± 1.62	(7.0 ± 0.48 μg/ml)
MDA-MB-231	10.2 ± 0.42	(3.1 ± 0.13 μg/ml)
HCT 116	16.0 ± 1.73	(4.8 ± 0.52 μg/ml)
HSC2	17.5 ± 2.17	(5.3 ± 0.65 μg/ml)
HK1	22.8 ± 5.82	(6.9 ± 1.75 μg/ml)
PC-3	8.7 ± 0.28	(2.6 ± 0.08 μg/ml)
Hep G2	12.7 ± 1.03	(3.8 ± 0.31 μg/ml)
WRL 68	7.6 ± 0.22	(2.3 ± 0.07 μg/ml)
MRC-5	11.2 ± 0.74	(3.4 ± 0.22 μg/ml)

Cell viability post treatment with cisplatin for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

4.4 Discussion

4.4.1 Relevance of extraction methods

In the present study, mushroom hot aqueous and cold aqueous extracts were prepared as these mimic the traditional preparation methods. Chemistry-wise, hot aqueous extraction facilitated the breakdown of the fungal chitinous cell wall, releasing polysaccharides, proteins, and some low-molecular-weight compounds with increased solubility at high temperature. For instance, Choi *et al*. (2006) reported an increase in free polyphenolics after heat treatment of mushrooms and postulated that high heating temperature brought about disruption of cell wall and liberated the polyphenolics that are otherwise covalently bound to these insoluble components of the cell wall. Aqueous extraction performed at low temperature (4 °C), on the other hand, prevented excessive degradation of thermo-labile components such as proteins/peptides.

For the organic solvent extracts, mushroom samples were first soaked in dichloromethane to extract the lipophilic (nonpolar) compounds and sequentially with methanol to extract the remaining compounds that mostly polar in nature (Athanasakis *et al*., 2013). Using dichloromethane as extraction solvent successfully yielded extracts that, based on GC-MS analysis, contained mainly aliphatic hydrocarbons, fatty acid methyl esters, fatty acid amides, and steroids, as well as other unidentified lipophilic constituents (data not shown). Methanol extracts of mushrooms usually contain primary metabolites (e.g. sugars, amino acids, etc.) and secondary metabolites (e.g. phenolic compounds) as shown in previous studies (Palacios *et al*., 2011; Kalogeropoulos *et al*., 2013; Lau *et al*., 2014; Woldegiorgis *et al*., 2014).

Extraction yield provide an indication on the effectiveness of the choice of solvent used in the extraction procedure and this can be related to the chemical characteristic of the major components in *L. rhinocerotis* from different developmental stages.

Regarding the differences in the yield, our results were in accordance with previous studies which found that higher extraction yield was recorded with solvents of higher polarity (Makropoulou *et al*., 2012; Athanasakis *et al*., 2013). The higher yield of methanol extracts for all samples (Table 4.1) suggested that polar, rather than nonpolar low-molecular-weight compounds made up a bulk portion of the mycelium, culture broth, fruiting body, and sclerotium of *L. rhinocerotis*. The yield of the organic solvent extracts was lower than those prepared from either hot aqueous or cold aqueous extraction. For instance, the yield of the dichloromethane and methanol extracts of *L. rhinocerotis* sclerotium was lower than those of the aqueous extracts as well as the organic solvent extracts of other mushroom samples. This could be explained by the fact that sclerotium made up of predominantly polysaccharides that are not likely to be soluble in either dichloromethane or methanol. As for the aqueous extracts, the yield of hot aqueous extracts was consistently higher than that of the cold aqueous extracts indicating that boiling was more efficient to extract the water-soluble components that might possibly be in the form of polysaccharides, proteins, and/or polysaccharide-protein complexes based on the results of chemical composition analysis. The abundance of proteins in some hot aqueous extracts might be due to some cellular and storage proteins being solubilized at high temperature as hypothesized by Lee et al. (2012).

4.4.2 Comparative cytotoxic activity of *L. rhinocerotis* extracts

Our results demonstrated that the use of different extraction methods produced extracts with varying cytotoxic activity that might be attributed to differences in their chemical composition (based on LC-MS profiling). Moreover, the differences in the cytotoxic activity of extracts prepared from different mushroom samples has to be

highlighted because comparative studies of mushroom samples from different cultivation techniques (e.g. solid substrate vs liquid fermentation), culture conditions (e.g. shaken and static conditions of liquid fermentation), different parts (e.g. pileus and stipe of fruiting body), and developmental/morphological stages (e.g., mycelium, fruiting bodies, and sclerotium) are still scarce even though these factors are usually taken into consideration for further large scale chemical and pharmacological studies or commercialization as sources of functional ingredients.

4.4.2.1 Extraction methods

Lignosus rhinocerotis aqueous extracts exhibited different degree of cytotoxicity against the cell lines but in general, the cold aqueous extracts were more potent than the hot aqueous extracts, especially in the case of the sclerotial aqueous extracts. This is likely to suggest that the major water-soluble, cytotoxic components in the sclerotium are heat-labile. We hypothesize that these chemical components might be deactivated or degraded during the boiling process; hence, the sclerotial hot aqueous extracts were devoid of activity. On the other hand, the presence of other chemical components that are not heat-labile in *L. rhinocerotis* was evidenced by our observation that hot aqueous extracts of the mycelium and fruiting body demonstrated moderate to weak cytotoxicity. Our results seemed to suggest that that heat affects the extraction of water-soluble cytotoxic components from *L. rhinocerotis*.

Hot aqueous extracts of *L. rhinocerotis* and selected edible-medicinal mushrooms showed mostly weak cytotoxic activity against most cell lines. This is consistent with some previous work on the cytotoxicity of hot aqueous extracts of other mushrooms (Zhou *et al*., 2007; Lee *et al*., 2009b). In most cases, the hot aqueous extracts were reported to be rich in high-molecular-weight polysaccharides and/or polysaccharide-

protein complexes, which act by modulating immune system rather than targeting cells directly (Zhang *et al*., 2007). Nevertheless, some mushroom-derived polysaccharides exerted cytotoxicity and induced apoptosis in cancer cells (Lavi *et al*., 2006; Wang *et al*., 2012). Previously, Lai *et al*. (2008) reported that the hot aqueous extract of *P. rhinocerus* sclerotium exhibited moderate cytotoxicity against HL-60 (IC_{50}: approximately 100 µg/ml); however, in our investigation, the sclerotial hot aqueous extracts (either cultivated, wild type, or commercially-produced) failed to reduce the viability of the cells to 50% after 72 h, even at the highest concentration (500 µg/ml). The observation could be attributed to differences in the extraction techniques. The hot aqueous extract used by Lai *et al*. (2008) has been subjected to ultrafiltration to retain high molecular weight components (> 10 kDa) whereas the sclerotial hot aqueous extracts in the present study might contain a mixture of various low- and high-molecular-weight components. Cytotoxic activity of the sclerotial cold aqueous extracts was compared to the work of Lee *et al*. (2012) who reported that the cold aqueous extract (IC_{50}: 96.7 µg/ml) of *L. rhinocerus* cultivar TM02 cultivar as well as the high-molecular-weight fraction (IC_{50}: 70.0 µg/ml) inhibited the growth of MCF7. In the present study, the sclerotial cold aqueous extracts also exerted cytotoxicity against MCF7 (IC_{50}: 36.7-67.2 µg/ml) and another breast cancer cell line, MDA-MB-231 (IC_{50}: 79.7-151.8 µg/ml). Taken together, these findings provide scientific validation for the traditional usage of *L. rhinocerotis*, in the form of decoction and macerated materials, for the treatment of breast cancer with cytotoxicity as one of the possible anti-cancer mechanism.

For the organic solvent extracts, the extracts of *L. rhinocerotis* prepared using dichloromethane were able to inhibit the growth of cancer cells to a different extent depending on the mushroom samples and cell lines. Methanol extracts at similar

concentration, on the other hand, were non-cytotoxic against most cell lines. This implied that amongst the low-molecular-weight metabolites, the nonpolar constituents of *L. rhinocerotis* have stronger cytotoxic effects than the polar constituents. This observation was consistent with previous findings whereby crude extracts and fractions prepared using solvent of lower polarity (e.g. hexane, dichloromethane, and ethyl acetate) generally exhibited higher cytotoxicity although results might also vary depending on mushroom species (Younis *et al*., 2014; Elbatrawy *et al*., 2015).

Regarding the cytotoxicity of the extracts of *L. rhinocerotis* sclerotium, we also compared our results with that by other researchers (Yap *et al*. 2013; Suziana Zaila *et al*. 2014). Previous studies have shown that *L. rhinocerotis* alcoholic sclerotial extracts, in general, were non-cytotoxic; for instance, Yap *et al*. (2013) reported that the crude methanol extracts of the cultivated sclerotium of *L. rhinocerus* cultivar TM02 and a wild type sclerotium were inactive (IC_{50} > 1000 µg/ml) against MCF7 after 72 h of incubation. Similarly, Suziana Zaila *et al.* (2014) demonstrated that a sclerotial methanol extract prepared by pressurized liquid extraction were non-cytotoxic (IC_{50} > 1000 µg/ml) against HCT 116. It is important to note that both studies performed direct extraction whereas the methanol extracts in the current study was prepared after dichloromethane extraction.

The choice of successive extraction technique was rather important in view of previous findings by Yap *et al*. (2013) and Suziana Zaila *et al*. (2013). While it is agreed that exhaustive extraction using methanol could extract a wide range of compounds, it might have captured lower proportion of the nonpolar compounds as such the cytotoxicity exerted by these nonpolar compounds might have been masked by the bulk of polar compounds as in the observation by Yap *et al*. (2013) and Suziana Zaila *et al*. (2014). Hence, our findings proved that the sclerotium of *L. rhinocerotis*

also contain cytotoxic compounds, albeit in lower abundance than water-soluble components, and this is similar to the case of other sclerotium-forming mushrooms such as *L. tuber-regium*.

4.4.2.2 Mushroom developmental/morphological stages

Amongst the different developmental/morphological stages in the life cycle of *L. rhinocerotis*, only the sclerotium that was claimed to have medicinal values based on traditional knowledge, and it naturally became the central focus of previous workers (Lee *et al*., 2012; Suziana Zaila *et al*., 2013; Yap *et al*., 2013). While the cytotoxic potential of the sclerotial extracts have been established previously and in the present study, there are limited work done on the mycelium, and the fruiting body has not been studied before. Therefore, we feel that it might be of interest to evaluate the cytotoxicity of *L. rhinocerotis* from different developmental/morphological stages in a comparative manner.

The dichloromethane extracts of *L. rhinocerotis* exhibited moderate to strong cytotoxicity with the extracts of the pileus being most potent (IC_{50} < 20 µg/ml in most cases), followed closely by the extracts of the stipe, sclerotium, and mycelium. Similar trend regarding the stronger cytotoxicity of the hot aqueous and cold aqueous extracts of pileus than stipe was also observed. Moreover, in some cases, potency of cold aqueous extracts of the cultivated and wild type pileus were shown to be comparable to that of the sclerotium; for instance, the susceptibility of MCF7 to the cold aqueous extracts of the pileus (IC_{50}: 24.1-37.2 µg/ml) and sclerotium IC_{50}: 36.7-67.2 µg/ml). In other words, the fruiting body of *L. rhinocerotis,* that was thought to have no medicinal values based on ethnomedicinal claims, are the best sources of cytotoxic ingredients in the form of lipophilic low-molecular-weight compounds (with reference to the

dichloromethane extracts). The fruiting body of *L. rhinocerotis* also contained some water-soluble components that are cytotoxic. Despite the lack of ethnomedicinal claims, such observation is not entirely surprising as it is not uncommon to discover cytotoxic metabolites from fruiting bodies. In fact, a multitude number of cytotoxic components, ranging from low- to high-molecular-weight constituents, have been isolated from the fruiting bodies of various edible-medicinal mushrooms (Lindequist *et al*., 2005; Ferreira *et al*., 2010; Patel & Goyal, 2012).

In addressing the developmental stages in relation to bioactivity, no consistent pattern in the potency of the cultivated sclerotium that was harvested before and after the formation of fruiting body was observed as the genetic material of the cell lines might affect the cells' susceptibility or response to treatment, as in the case of MCF7 and MDA-MB-231. In an attempt to explain the differences in nutrient composition and bioactivity between the wild type and *L. rhinocerus* cultivar TM02 sclerotium, Yap *et al*. (2013) hypothesized that their observation (cultivar being more potent than wild type) was due to the cultivated sclerotium harvested just before the formation of fruiting body and hence, was at the earlier stage of maturation. In the present study, cytotoxicity of the sclerotium harvested before and after the formation of fruiting body was compared. Our results with the cold aqueous extracts seemed to follow similar pattern whereby in some cell lines (e.g. MCF7, MDA-MB-231, HCT 116, HK1, PC3, and MRC-5) sclerotium harvested before the formation of fruiting body showed slightly stronger cytotoxicity than those with fruiting body or wild type samples. On the other hand, extracts of sclerotium with fruiting body was more potent when other lines are considered (e.g. HL-60, HSC2, Hep G2, and WRL 68). Consequently, no conclusion can be drawn regarding potency of sclerotial aqueous extracts based on the current study because only two distinct developmental stages were compared. On the other

hand, our results from the cytotoxicity studies of organic solvent extracts did not seem to support that hypothesis. This is further supported by our observation with dichloromethane extracts of the cultivated sclerotium with fruiting body showed stronger cytotoxicity than those without fruiting body and wild type sclerotium with fruiting body is approximately 2-4 folds more potent than cultivated samples.

On the other hand, mycelium was found to be weaker than either the fruiting body or sclerotium, irrespective of the extraction method. This observation was similar to previous studies where mycelium and culture broth were shown to be either non-cytotoxic or weaker than the fruiting bodies of selected edible-medicinal mushrooms (Younis *et al*. 2014). Such observation could be explained from a physiological point of view whereby the mycelium, fruiting body, and sclerotium represent the vegetative, reproductive, and dormant/survival stages; therefore, their chemical composition was altered to suit their physiological roles. This is backed up by the differences in the UPLC-ESI-MS and GC-MS profiles of *L. rhinocerotis* from different developmental stages. In fact, some previous findings, such as one on the comparative proteomic analysis *L. tuber-regium* from different developmental stages also revealed the differences in protein composition of the mycelium, fruiting body, and sclerotium (Chen *et al*. 2012).

4.4.2.3 Cultivation techniques and culture conditions

As mycelium of *L. rhinocerotis* was cultured only by liquid fermentation in the present study, it can be deduced that mushroom samples from solid substrate fermentation (i.e. fruiting body and sclerotium) are better sources than those from liquid fermentation (i.e. mycelium and culture broth). Even though the process in obtaining the fruiting body and sclerotium by solid substrate fermentation is more tedious and

time-consuming, overall, both developmental stages appeared to be better sources of cytotoxic compounds than the mycelium. Nevertheless, liquid fermentation is still a faster and easier way to obtain fungal biomass, and should the active components responsible for the potent cytotoxicity of the pileus and sclerotium also present in the mycelium, then the mycelium also merit further consideration, taking into account the yield of active components from mycelium.

In the present study, *L. rhinocerotis* was cultured under shaken and static conditions of liquid fermentation as our previous experience (Abdullah *et al*., 2010; Lau *et al*., 2011; Lau *et al*., 2014) and that by other researchers showed that chemical profiles varied depending on the conditions of liquid fermentation. In order to assess the cytotoxic potential of intracellular and extracellular components, the mycelium was separated from culture broth during harvesting. While this step might appear to be tedious, our results have demonstrated its usefulness as the mycelium and culture broth of *L. rhinocerotis* was shown to be distinctively different with regard to cytotoxicity of low-molecular-weight constituents (based on the results of the dichloromethane and methanol extracts). For the organic solvent extracts, the mycelium from liquid fermentation showed low cytotoxicity compared to other samples while the culture broth was inactive. Our results indicate that different culture conditions (i.e. shaken and static conditions) employed in the current study did not have significant effect on the biosynthesis of cytotoxic compounds in the mycelium. Based on our findings, cytotoxic compounds were presumably retained only in the mycelium (intracellular) and not secreted out during the course of the experiment (15 days). Although culture broth are not considered to be good sources of cytotoxic compounds, there are attempts to manipulate their culture conditions to accumulate certain metabolites of interest; for instance, the production of ganoderic acids (previously shown to have cytotoxic activity)

under various conditions of liquid fermentation (Fang & Zhong, 2002; Tang & Zhong, 2003; Zhang & Tang, 2008; Zhao *et al*., 2011b; Xu *et al*., 2013).

On the other hand, aqueous extracts of the mycelium from static conditions generally showed stronger cytotoxicity than its counterpart from shaken condition, a trend that was not observed in the organic solvent extracts. These seemed to indicate that varying the culture condition of liquid fermentation has effect on the biosynthesis of water-soluble chemical components with cytotoxic effect in the mycelium. The chemical basis for this observation has yet to be elucidated, but the lack of aeration and spatial homogeneity as well as the merging of growth phases in static cultures might be responsible in the difference in mycelial chemical composition. Technically, the mycelium mat in static condition was exposed to different microenvironment with surplus of oxygen but nutrient deficient in upper portion and nutrient abundance but restricted air flow at the bottom (Rhodes & Fletcher, 1966). The low availability of oxygen at the bottom might also affect the synthesis of fungal proteins. The assumption that proteins might be responsible for cytotoxicity is partly supported by the findings of Abdullah *et al*. (2010) that mycelial protein extracts inhibited the growth of Ca Ski cells.

4.4.2.4 Cultivated vs. wild type vs. commercially-produced samples

Comparative analysis in the present study also pointed out the differences in the activity of the cultivated, wild type, and commercially-produced sclerotium. Amongst the solvent extracts, while the extracts of the sclerotium were slightly weaker than those of the fruiting body, we observed that the breast cancer cells, namely MCF7 (IC_{50}: 11.3 µg/ml) and MDA-MB-231 (IC_{50}: 19.9 µg/ml) were highly sensitive towards extracts of the wild sclerotium; however, both cell lines were relatively less susceptible towards extracts prepared from cultivated sclerotium. For instance, the cultivated sclerotium

harvested prior to the fruiting body were less effective compared those harvested after formation of fruiting body with IC_{50} (μg/ml) values of 85.1 and 65.1 for MCF7 as well as 81.2 and 41.3 for MDA-MB-231, respectively.

The stronger cytotoxicity of the extracts of wild type sclerotium (and to a lesser extent, the fruiting bodies) might be attributed to biotic (e.g. strain) and abiotic factors (e.g. environmental conditions such as humidity, temperature, etc.) (Mattila *et al*., 2002; Cheung, 2008). There are many possible reasons. Firstly, owing to its role for survival, the sclerotium might accumulate chemical constituents for defense purposes (usually with cytotoxic activity) against antagonistic microorganisms (Gloer, 2007; Smith *et al.* 2015), and the differences in the soil conditions (e.g. pH, microbiota, etc.) where the wild type sclerotium collected and cultivated sclerotium grown might affect the sclerotial chemical composition. Secondly, cultivated samples were harvested at similar developmental and maturity stages whereas we were not able to control this parameter for the wild type samples. There are evidences presented earlier that mushrooms at different developmental stages contained varied amount of active constituents resulting in different levels of bioactivities (Barros *et al*., 2007a; Barros *et al*., 2007b; Tsai *et al*., 2008; Cui *et al*., 2014). As such, the stronger cytotoxicity of the wild type sclerotium compared to the cultivated samples might be due to this reason.

Interestingly, both the dichloromethane and methanol extracts of the commercially-produced sclerotium showed cytotoxicity against the cancer cells. Our results also indicate that the commercially-produced sclerotium (rather than cultivated or wild type samples) contained polar cytotoxic compounds (captured in the methanol extracts) in abundance. Our observation on the differences in cytotoxicity most probably attributed to the differences in the chemical profiles as shown by UPLC-ESI-MS and GC-MS analyses (data not shown). The differences in the chemical profile, in turn, might be due

to the composition of substrate and other factors such as genetic/cultivar, harvesting stages, post-harvest processing and others (Cheung, 2008).

In addressing the same issue, Yap *et al*. (2013) reported that the wild type sclerotial cold aqueous extract (IC_{50}: 206.3 μg/ml) has lower cytotoxicity against MCF7 than their cultivated strain (IC_{50}: 90.0 μg/ml). In our study, the activity of cultivated sclerotium in the present study was found to be higher than the wild type sample with regard to cytotoxicity against MCF7. In other cases, the cultivated sclerotium was either comparable or weaker than the wild type samples depending on the cell lines being studied. Hence, it is difficult to arrive at a conclusion regarding the potency of cultivated and wild type samples based on only limited number of cell line as our results with a panel of 10 cell lines have clearly demonstrated that responses might vary depending on the cell lines. The same applies for the extracts of the pileus and stipe as well. For the comparison to be more accurate, other factors such as mushroom cultivars, geographical origin, physiological stage and condition, post-collection processing, and other factors need to be taken into consideration. Moreover, such comparative analysis might benefit from a larger number of wild type samples collected from various geographical locations.

The cytotoxic components in the sclerotial cold aqueous extract of *L. rhinocerotis* were hypothesized to be protein-carbohydrate complex (Lee *et al*., 2012; Yap *et al*., 2013). Formation of fruiting body from the sclerotium might be accompanied by a change in the chemical composition of the sclerotium possibly involving the metabolism of carbohydrates and proteins. Accordingly, this scenario might explain the stronger cytotoxicity of the sclerotium harvested prior to the formation of fruiting body. On the other hand, it is unlikely that sclerotial secondary metabolites were also involved or utilized in the morphogenesis, and this is reflected in our results whereby the

dichloromethane extracts of sclerotium harvested before and after the formation of fruiting body showed comparable activity. As our understanding on this aspect is still limited and no such work has been done on *L. rhinocerotis*, comparative chemical profiling is suggested to provide an explanation which might further aid in *L. rhinocerotis* bioprospecting.

4.4.2.5 Effect on non-cancerous cells

Since *L. rhinocerotis* has been traditionally used for treatment of cancer, it would be desirable to exploit the cytotoxic component(s) as potential anti-cancer agents; however, a good candidate for anti-cancer agent should be selective with regards to its cytotoxicity. Although noted for strong cytotoxicity, it appeared that the cold aqueous extracts of the sclerotium, irrespective of cultivated or wild type samples, lacks selectivity as it was found to be cytotoxic also to MRC-5 with differences in their IC_{50} values were less than 2-fold. This is contradictory to the findings by Lee *et al*. (2012) who reported that the cold aqueous extract of *L. rhinocerus* cultivar TM02 was not cytotoxic to both human breast (184B5) and lung (NL20) normal cells (IC_{50} > 1000 µg/ml) after 72 h of incubation. Regarding the organic solvent extracts, the potent dichloromethane extract of the pileus (LRCP-DE), for instance, was also cytotoxic against MRC-5; however, the fold differences for selected extracts against certain cell lines were higher. For instance, by a comparison of their IC_{50} values, LRCP-DE was approximately 7 times more cytotoxic to MCF7 than to MRC-5. This observation indicates that LRCP-DE demonstrated some degree of selectivity in its cytotoxic activity.

The variation in the response of the non-cancerous cell lines to mushroom extracts might be attributed to several factors with chemical composition of the extracts having a

bigger role. We hypothesized that the composition of cytotoxic components in sclerotium of *L. rhinocerotis* was greatly influenced by cultivation techniques. In the literature, solid-substrate fermentation has been routinely used to produce sclerotia-forming mushrooms (Okhuoya & Okogbo, 1991; Olufokunbi & Chiejina, 2010); hence, the key differences would lie on the type of substrates used (agroresidues or food-based material, e.g. grains) and culture conditions. In addition, sclerotia formed under different environment (e.g. flasks, bags, buried in the field, etc.) may have significant difference in their chemical composition. It might be logical to postulate that production of cytotoxic components (with possible role in fungal defense) in sclerotia buried in the field is higher than those cultivated under controlled environment and conditions without antagonistic microorganisms. As the wild type sclerotium also showed high cytotoxicity against MRC-5, it seemed that toxicity of the cultivated sclerotium was not a consequence of the cultivation techniques and growth conditions specific to the present study. The similarity in their response to the wild type samples further suggest that the cultivated samples are fair representatives, in the context of cytotoxic constituents, of the naturally-growing *L. rhinocerotis* sclerotium.

Furthermore, it is to be noted that, in the context of *in vitro* cytotoxicity, selectivity is usually expressed in the form of numerical value obtained by comparing the IC_{50} values obtained from non-cancerous to cancer cell lines (selectivity index). The value might vary depending on the non-cancerous cell line used as comparison because cellular response might differ depending on the genetic make-up of the cells, physiological age (i.e. immortalized cells are known to enter senescence at earlier passage), seeding density, and others. Hence, apart from the variation in chemical composition of the extracts, these factors might explain the differences in results obtained by different workers. In assessing the potential cytotoxicity against normal cells, further testing

using primary cell lines might be a better choice as these resemble more closely to the characteristics of cell/tissues studied.

4.4.3 Considerations for bioprospecting of cytotoxic compounds from *L. rhinocerotis*

Chemical profiles obtained from LC-MS and GC-MS analyses (data not shown) revealed that the cultivated and wild type *L. rhinocerotis*, in general, were comparable in terms of identity of the major constituents (based on retention time, UV absorption profile, molecular ion, mass fragmentation, etc.) although their quantity showed some variations. This observation implied that cultivation of *L. rhinocerotis* by optimized solid substrate fermentation technique developed in this investigation (as described in Chapter 3) produced fruiting body and sclerotium that are comparable to the wild type samples used by the indigenous communities for medicinal benefits. As the chemical and bioactivity (e.g. cytotoxicity) profiles of the cultivated samples are comparable to those of the wild type samples, it is feasible to continue to use these cultivated samples for further chemical and pharmacological investigations.

On the other hand, the differences in the chemical and bioactivity profiles of the commercially-produced sclerotium with those of the cultivated sclerotium and wild type samples might indicate that it might not be produced by similar technique used in the present study (i.e. solid substrate fermentation of agroresidues followed by burial of mycelium-colonized substrate blocks). The bioactivity of mushrooms depends on the composition of active components that are largely dependent on the cultivation techniques, culture conditions, substrates used, and other factors (Wasser & Akavia, 2008). In contrast to the potent dichloromethane extracts and inactive methanol extracts of the cultivated and wild type sclerotium, both dichloromethane and methanol extracts

of the commercially-produced sclerotium showed cytotoxicity against the cell lines. In terms of potency, both extracts were still weaker than, for instance, dichloromethane extracts of the pileus. Moreover, the use of the commercially-produced sclerotium for further chemical investigations, e.g. isolation of compounds, is not feasible due to the high cost (approximately USD375/100 g sclerotial powder) to purchase samples in bulk compared to the wild type (approximately USD30 for fruiting body and sclerotium) and cultivated samples where minimal cost is involved for production as mostly agroresidues were utilized.

In selecting extracts to be subjected for further chemical investigation, potency, yield, and availability of mushroom samples must be considered. Although the wild type fruiting body in the present study showed stronger cytotoxicity, its availability is a major constraint as these are usually discarded by the indigenous people who collect *L. rhinocerotis* in the jungle. In fact, the supply of *L. rhinocerotis* fruiting body (not used in traditional medicine) is even lower than that of the sclerotium. On the other hand, the cultivated samples are better alternative as these can be continuously produced by solid substrate fermentation. Moreover, it was observed that more than one batch of fruiting bodies could be harvested from the same sclerotium if the sclerotium were left undisturbed in the soil (data not shown). The use of cultivated samples is viewed as more sustainable manner of bioprospecting of cytotoxic compounds from *L. rhinocerotis*. Based on the IC_{50} values, amongst the organic solvent extracts, dichloromethane extract of the pileus (MCF7, IC_{50}: 3.8 µg/ml) was chosen for further study.

For the aqueous extracts, it was obvious that the cold aqueous extraction was the preferable method for extracting water-soluble, cytotoxic components from *L. rhinocerotis*. Results from the cytotoxicity studies revealed that cold aqueous extracts of

the cultivated and wild type pileus and sclerotium showed high potential for bioprospecting. In view of the use of cultivated samples being more sustainable and the fact that wild type samples might varied in their activity depending on geographical origin, we focused on the activity of extracts of the cultivated samples although wild type samples were shown to be more potent in some cases. Cold aqueous extract of the cultivated pileus showed high cytotoxicity against MCF7 (IC_{50}: 37.2 μg/ml) comparable to that of the cultivated sclerotium (36.7-44.1 μg/ml); however, strong cytotoxic effect of the former was restricted only to two cell lines, MCF7 and HSC2 (IC_{50}: 45.6 μg/ml), and its effect on other cell lines were almost non-existence (IC_{50} > 500 μg/ml). The sclerotial extract, on the other hand, was cytotoxic to most of the tested cell lines (IC_{50} < 150 μg/ml), particularly extracts of the sclerotium that was harvested prior to the formation of fruiting body. Although sclerotium that was harvested after the formation of fruiting body gave results comparable to that of the sclerotium without fruiting body, it takes a longer time before the former can be harvested and this translates into lower productivity. Therefore, cold aqueous extract of the sclerotium harvested prior to the formation of fruiting body was selected for further study. The decision to select the sclerotium, rather than the pileus, based on cytotoxicity also coincided with the fact that the sclerotium of *L. rhinocerotis* is used in ethnomedicine; hence, further work on the sclerotial extract might reinforce our understanding on the potential use of aqueous preparations of *L. rhinocerotis* in treating cancer.

Another factor to be considered is the potency of the extracts when compared with other known cytotoxic agents. The dichloromethane extract of the pileus (MCF7, IC_{50}: 3.8 μg/ml), surprisingly, exhibited stronger cytotoxicity than cisplatin (MCF7, IC_{50}: 7.0 μg/ml) and higher selectivity with regard to cytotoxicity on MRC-5, a non-cancerous cell line. It was also more potent than the extracts of *G. lucidum* (IC_{50}: 17.0 μg/ml) and

L. tuber-regium (IC_{50}: 8.45 μg/ml) fruiting bodies prepared in the similar manner. Lanostane-type triterpenoids (e.g. ganoderic acids) have been documented to contribute to the cytotoxicity of *G. lucidum* (Paterson, 2006; Boh *et al*., 2007; Wu *et al*., 2013) but the identity of secondary metabolites with cytotoxic activity from *L. tuber-regium* received little attention. The sclerotial cold aqueous extract, on the other hand, was weaker than cisplatin but stronger than the aqueous extract of *L. tuber-regium* sclerotium that has been documented to contain cytotoxic high-molecular-weight hydrophilic components in the form of polysaccharides (Zhang *et al*., 2001; Wong *et al*., 2007).

Ethnomedicinal records might provide useful clues in drug discovery as exemplified by the presence of cytotoxic compounds (water-soluble components) in cold aqueous extracts of the sclerotium. On the other hand, organic solvent extracts (containing low-molecular-weight compounds) prepared from other parts of the mushroom, such as the fruiting body, showed stronger cytotoxicity than the sclerotium. The fact that mushrooms' fruiting bodies have been documented to be an importance source of bioactive secondary metabolites should not be neglected (Lindequist *et al*., 2005; Ferreira *et al*., 2010; Patel & Goyal, 2012). When comes to bioprospecting, the importance of a thorough investigation, rather than to rely only on ethnomedicinal claims, is further emphasized.

4.5 Conclusion

Our results have demonstrated the variation in the cytotoxic activity of the mycelium, culture broth, pileus, stipe, and sclerotium of *L. rhinocerotis* when subjected to extraction procedure using hot water, cold water, dichloromethane, and methanol. Chemical profiling confirmed that cultivated samples were comparable to the wild type samples. The relationship between different mushroom samples (i.e. from different developmental/morphological stages, parts, cultivation techniques, culture conditions, and geographical origin) and cytotoxicity was also compared and critically discussed. Overall, results showed that the sclerotial cold aqueous extract and dichloromethane extract of the pileus were the most potent extracts amongst the aqueous and organic solvent extracts, respectively, and both were comparable to other edible-medicinal mushrooms such as *G. lucidum* and *L. tuber-regium*. Therefore, both extracts were selected for further studies in order to elucidate their mechanism of action and the nature of cytotoxic compounds.

CHAPTER 5: CELL CYCLE ARREST AND APOPTOSIS-INDUCING ACTIVITIES OF *L. RHINOCEROTIS* CRUDE EXTRACTS AND THE NATURE OF CYTOTOXIC COMPONENTS

5.1 Introduction

The long history of medicinal mushrooms across diverse cultures is now backed up by extensive scientific evidences, mainly pertaining to the plethora of bioactive compounds and mechanism of actions. In the search for potential new resources of bioactive compounds, our attention shifted towards wild mushrooms that are used as folk medicine by the indigenous community. In our endeavor to validate the claimed anti-cancer effect of *L. rhinocerotis* based on traditional knowledge, a comprehensive and systematic investigation on the cytotoxic activity of the extracts of *L. rhinocerotis* mycelium, culture broth, fruiting body, and sclerotium was carried out as described in Chapter 4.

Based on our findings, cold aqueous extract of the sclerotium (LRSC-CAE) and dichloromethane extract of the pileus (LRCP-DE) of *L. rhinocerotis* were identified as the most potent extract within the aqueous and organic solvent extracts, respectively. While the cytotoxic potential of the extracts of *L. rhinocerotis* against various cell lines have been shown earlier, the underlying mechanism of action remain unclear. Based on previous studies, sclerotial hot aqueous extract was reported to induce cell cycle arrest in HL-60 (Lai *et al*., 2008) whereas the sclerotial cold aqueous extract induced apoptotic cell death in MCF7 and A549 based on the results of DNA fragmentation assay (Lee *et al*., 2012). While some preliminary information regarding the mode of cell death of the sclerotial cold aqueous extract is available, there is no work on any organic solvent extracts of *L. rhinocerotis*, such as LRCP-DE thus far.

In addition, the nature of active components in *L. rhinocerotis* has not been thoroughly studied. According to Lee *et al*. (2012), cytotoxicity of the sclerotial cold aqueous extract of *L. rhinocerus* cultivar TM02 was attributed to the high-molecular-weight fraction which comprised of mainly polysaccharides (68.7%, w/w) and a small amount of proteins (3.6%, w/w) but the exact nature of active compound and the possible roles of other chemical components (e.g. secondary metabolites) are still not known. LRCP-DE, on the other hand, might contain different array of chemical components than those in the aqueous extracts. In the absence of chemical investigation on the pileus, chemical constituents that are relatively non-polar in nature (e.g. fatty acids, triterpenoids, steroids, etc.) are suspected to be the active ingredients based on the information in the literature (Lindequist *et al*., 2005; Fereira *et al*., 2010).

Herein, we further evaluate the potential of selected *L. rhinocerotis* crude extracts as possible source of cytotoxic compounds by comparing their mechanism of action and identifying the nature of the active component(s).

The specific objectives of the present study include:

1. To investigate the effect of selected *L. rhinocerotis* extracts on cell proliferation, cell cycle progression, and apoptosis induction
2. To deduce the nature of cytotoxic chemical components in *L. rhinocerotis* sclerotial cold aqueous extract based chemical fractionation coupled to the MTT assay
3. To identify the chemical constituents in the dichloromethane extract of *L. rhinocerotis* pileus by GC-MS and LC-MS, and to evaluate the cytotoxic activity of selected compounds

5.2 Materials and Methods

5.2.1 Chemicals

Methyl palmitate, methyl linoleate, methyl stearate, and oleamide were obtained from Sigma-Aldrich (USA). Ergone was obtained from BioBioPha Co., Ltd. (Yunnan, China). Purity of the compounds was ≥ 98%. Compounds were dissolved in DMSO to produce stock solutions.

5.2.2 Preparation of extracts

The cold aqueous extract of *L. rhinocerotis* sclerotium (LRSC-CAE) was prepared according to the method described in Section 4.2.2.1. The dichloromethane extract of *L. rhinocerotis* pileus (LRCP-DE) was prepared according to the method described in Section 4.2.2.2. LRSC-CAE was dissolved in 50% (v/v) aqueous DMSO whereas LRCP-DE was dissolved in dimethyl sulfoxide. Stock (20-50 mg/ml) of extracts and fractions were prepared and further diluted with culture media to achieve desired final concentrations.

5.2.3 Cell culture

The following human cell lines were obtained from the American Type Culture Collection (ATCC): MCF7 (ATCC® HTB-22™, breast adenocarcinoma); HCT 116 (ATCC® CCL-247™, colorectal carcinoma); MRC-5 (ATCC® CCL-171™, lung fibroblast). MCF7 and HCT 116 were cultured in RPMI whereas MRC-5 was grown in MEM. All media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 100 unit/ml penicillin/streptomycin (Gibco). Cells were cultured in 5% CO_2 incubator at 37 °C in a humidified atmosphere. Cells were sub-cultured every 2 or 3 days and routinely checked under an inverted microscope. For solid tumours, cells

were harvested using 0.25% (v/v) trypsin in PBS when 80% confluence was reached. Only cells at exponential growth were used throughout the experiments.

5.2.4 MTT assay

The effect of *L. rhinocerotis* extracts and fractions on cell viability was determined using the MTT assay as described in Section 4.2.5.2. Cells were treated with the compounds and results were taken after 72 h of incubation. Results were expressed in the form of IC_{50} values.

5.2.5 Sulforhodamine B (SRB) assay

The SRB assay is based on the measurement of cellular protein content. Briefly, cells (3×10^3 cells/well) were seeded and treated with mushroom extracts for desired incubation period. Following that, cells were fixed with 50% (w/v) trichloroacetic acid at 4 °C and then, stained with 0.4% (w/v) SRB (Sigma-Aldrich, USA) for 30 min at room temperature. Excess dye was removed by repeated washing with 1% (v/v) acetic acid in water, followed by water, and then, air-dried. The protein-bound dye was dissolved in 10 mM Tris base solutions before absorbance was taken at 565 nm. The percentage of cell viability was calculated based on the equation stated in Section 4.2.6.2.

5.2.6 Trypan blue exclusion (TBE) assay

The TBE assay measures the number of viable cells; viable cells with intact membrane will exclude the dye. Cells (1.5×10^5 cells) were cultured in 40 mm culture dishes and allowed to attach overnight. Then, cells were treated with extracts for desired incubation period. Following that, media were removed; cells were washed with PBS

before harvested. Cell pellets were suspended in media and mixed with equal volume of 0.4% (w/v) trypan blue in PBS. Viable cells were counted using a haemocytometer.

5.2.7 Cell cycle analysis

The effect of *L. rhinocerotis* extracts on cell cycle distribution was evaluated using propidium iodine (PI) staining based on the method of Lim *et al*. (2011) with modifications. After the desired treatment period, cells were collected, washed with PBS, and fixed in 70% (v/v) ethanol at -20 °C overnight. Fixed cells were washed and re-suspended in staining solution containing PI (10 µg/ml) and RNase (20 µg/ml) at room temperature in darkness for 30 min before analysed using a flow cytometer (BD FACSCanto II, Becton Dickenson, CA, USA). At least 10 000 events were collected for each sample. The cell cycle distribution was analysed using ModFit LT^{TM} (Verity Software House) that uses mathematical estimates of the G1, S, and G2/M populations that contribute to the shape of the curve in histograms.

5.2.8 Assessment of apoptosis

5.2.8.1 Annexin V-FITC/PI analysis

This assay relies on the binding of annexin V to the exposed phosphatidylserine in early and late apoptotic cells and PI is excluded from cells with intact and uncompromised cell membrane, such as the viable and early apoptotic cells. The assay was performed using the annexin V-FITC/PI apoptosis detection kit I (BD Biosciences, USA) according to the manufacturer's instructions. Cells (1.5×10^5 cells) were cultured in 40 mm culture dishes, allowed to attach overnight and treatment were added the following day. After appropriate treatment period, the cells were harvested, washed with PBS, resuspended in 1× annexin V binding buffer, and stained with annexin V and PI for 15 min at room temperature in the dark. Apoptosis was detected using a flow

cytometer (BD FACSCanto II, Becton Dickenson, CA, USA). At least 10 000 events were collected for each sample. Distribution of cell population in different quadrants was analyzed with quadrant statistics.

5.2.8.2 Cell death detection ELISA

The assay is based on the quantitative "sandwich enzyme immunoassay" principle using mouse monoclonal antibodies directed against DNA and histones, and hence, allowing the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Assay was performed using the cell death detection ELISA kit (Roche Dignostics, Germany). Cells (3×10^3 cells/well) were seeded into the wells of 96-well plates and allowed to attach overnight before treatments were added. Following the incubation period, cell lysates were collected and analysed according to the manufacturer's instruction. Absorbance was taken at 405 nm. The enrichment factor (i.e. specific enrichment of mono- and oligonucleosomes released into the cytoplasm) was calculated using the following equation:

$$\text{Enrichment factor (apoptotic index)} = \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}}$$

5.2.9 Chemical fractionation

LRSC-CAE was fractionated using various methods in order to identify the nature of cytotoxic components (Figure 5.1). All chemicals and organic solvents used in this section were of analytical grade, unless otherwise stated.

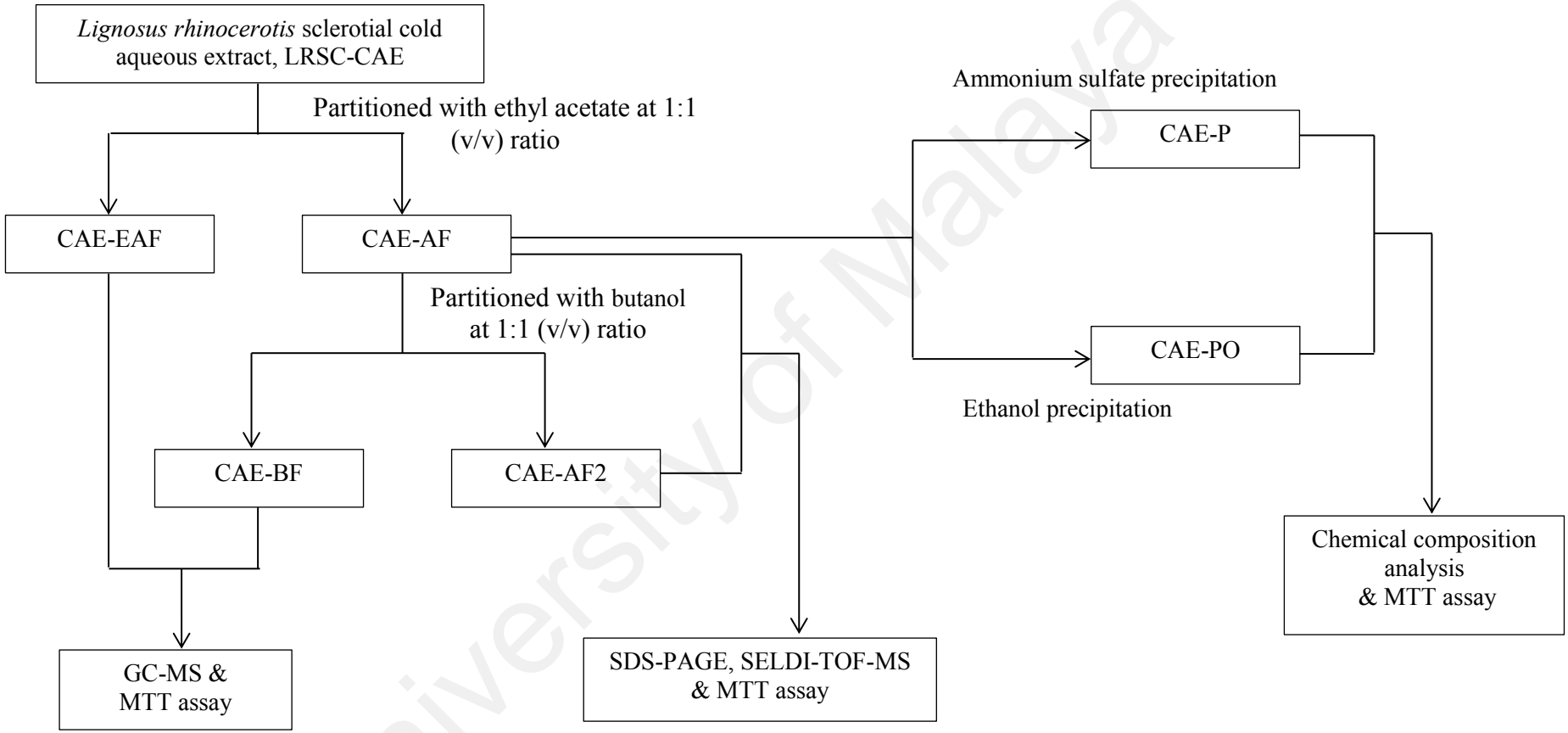


Figure 5.1: Chemical fractionation and characterization of LRSC-CAE. CAE-EAF, CAE-BF, CAE-AF, and CAE-AF2 are fractions derived from LRSC-CAE after liquid-liquid partition; CAE-EAF, ethyl acetate fraction; CAE-BF, butanol fraction; CAE-AF, aqueous fraction from liquid-liquid partitioning between LRSC-CAE and ethyl acetate; CAE-AF2, aqueous fraction from liquid-liquid partitioning between CAE-AF1 and butanol; CAE-P and CAE-PO were obtained by salting-out using ammonium sulfate and ethanol precipitations, respectively; CAE-P, crude proteins; CAE-PO, crude polysaccharides.

5.2.9.1 Liquid-liquid partition

LRSC-CAE was prepared and partitioned with ethyl acetate (1:1, v/v) to give ethyl acetate-soluble (CAE-EAF) and aqueous (CAE-AF) fractions. CAE-AF was further extracted with butanol (1:1, v/v), yielding the butanol-soluble (CAE-BF) and aqueous (CAE-AF2) fractions. Temperature was carefully maintained below 15 °C during the partitioning process. Excess solvents in the organic and aqueous fractions were removed using a rotary-evaporator. Both aqueous fractions were then freeze-dried.

5.2.9.2 Ammonium sulfate precipitation

CAE-AF was dissolved in distilled water at a ratio of 1:20 (w/v) and centrifuged at 15 000 rpm for 15 min. The supernatant was collected. The water-soluble components in the supernatant were precipitated using two different methods. In the first method, powdered ammonium sulfate was gradually added into CAE-AF until 90% (v/v) saturation was achieved and the mixture was left overnight with continuous stirring at 4 °C. Then, the mixture was centrifuged and the pellet was re-dissolved in distilled water. The fraction was extensively dialyzed (SnakeSkin™ Dialysis Tubing 5 kDa MWCO, Thermo Scientific, USA) against distilled water at 4 °C for 3 days, with the water being changed every 12 h, before being freeze-dried. This fraction was termed as CAE-P (comprised mainly of proteins) and was kept at -20 °C prior to analysis.

5.2.9.3 Ethanol precipitation

In the second method, ethanol was added to CAE-AF until the final concentration of ethanol was 80% (v/v). The mixture was left overnight at 4 °C without stirring. Following that, the mixture was centrifuged and the pellet was washed with acetone and ethanol, successively, before re-dissolved in water. The fraction was also subjected to dialysis against distilled water at 4 °C for 3 days before being freeze-dried. This fraction

was termed as CAE-PO (comprised mainly of polysaccharides) and was kept at -20 °C prior to analysis.

5.2.10 Chemical characterization

Protein components in LRSC-CAE were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS). Organic solvent fractions and LRCP-DE were subjected to gas chromatography-mass spectrometry (GC-MS) and/or liquid-chromatography-mass spectrometry (LC-MS) analyses.

5.2.10.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The aqueous extracts of *L. rhinocerotis* containing approximately 20 μg protein were mixed with equal volume of sample buffer containing 2% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl, 1% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol blue, and heated for 5 min at 90 °C. SDS-PAGE analysis of the aqueous extracts was performed as described by Laemmli (1970) using 16% (w/v) separating and 5% (w/v) stacking gels in a vertical slab gel apparatus (C.B.S. Scientific Company, Inc.). Bands were visualized by Coomassie Brilliant Blue R250 (Sigma-Aldrich, USA) and silver staining.

5.2.10.2 Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS)

The aqueous extracts of *L. rhinocerotis* (10 mg/ml) were analysed using the reversed-phase or hydrophobic H50 ProteinChip® arrays (Bio-Rad Laboratories, Inc.). Solvents of HPLC grade were used for this analysis. Sample preparation on protein arrays were done according to Lau *et al*. (2011) with minor modifications. The arrays were equilibrated in binding buffer consisting of 0.5% (v/v) TFA in 50% (v/v) ACN prior to spotting of samples (5 µl). Arrays then were washed twice with binding buffer followed by deionised water once before they were air-dried. Then, saturated energy-absorbing matrix (EAM) solution, α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, USA) in binding buffer (2 µl), was added before the arrays were allowed to air-dry again. Analysis was performed using the ProteinChip SELDI System (PSC 4000) (Bio-Rad Laboratories, Inc.). Data collection was carried out in positive mode. Spectra were calibrated using external calibration against a mixture of standards consisting of somatostatin (1.64 kDa), arg-insulin (5.96 kDa) and cytochrome c (bovine) (12.23 kDa).

5.2.10.3 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis of the extracts and solvent fractions of *L. rhinocerotis* (i.e. CAE-EAF and CAE-BF) was performed using 6890 N gas chromatography (Agilent Technologies, Inc., USA) equipped with 5975 Mass Selective Detector. The HP-5 MS (5% phenylmethylsiloxane) capillary column (30.0 m × 25 mm × 25 µm) was initially set at 70 °C, increased to 300 °C, and then held for 30 minutes. Helium (flow rate: 1 ml/min) was used as the carrier gas. The total ion chromatogram (TIC) was auto-integrated by ChemStation and the chemical constituents were identified by comparison with the accompanying spectral database (NIST 2011, Mass Spectral Library, USA).

5.2.10.4 Liquid chromatography-mass spectrometry (LC-MS)

LRCP-DE (10 mg/ml in methanol) were profiled using Acquity™ UPLC-PDA system coupled to Synapt HDMS oaTOF (Waters Corporation, USA) equipped with an electrospray ionization (ESI) source as previously described in Section 4.2.3.2. Full scan mass spectra (*m*/*z* 100-1000) were acquired in positive and negative ion modes.

5.2.11 Statistical analysis

Each analysis was performed in triplicates and results were expressed as mean ± S.D. (n = 3). The data were statistically analysed using the IBM® SPSS® Statistics Version 19 (SPSS Inc., United States). Student's *t*-test was used for comparison of means between control and treated samples.

5.3 Results

5.3.1 Growth-inhibitory activity

In view of the strong cytotoxicity of LRSC-CAE (cold aqueous extract of sclerotium) and LRCP-DE (dichloromethane extract of pileus) based on the MTT assay, we investigated their growth-inhibitory activities against MCF7 and HCT 116 using two additional cell viability assays that are based on principles different from the MTT assay, including the SRB assay (which is based on the measurement of cellular protein content) and the TBE assay (which is based on membrane integrity). LRSC-HAE (hot aqueous extract of sclerotium) and LRCP-ME (methanol extract of pileus) were included for comparison purposes (where necessary) for LRSC-CAE and LRCP-DE, respectively.

5.3.1.1 Effect of LRSC-CAE on the growth of MCF7 and HCT 116

The growth-inhibitory effect of LRSC-HAE and LRSC-CAE on MCF7 and HCT 116 was determined using the SRB assay. As shown in Figure 5.2, treatment with both extracts for 72 h caused a dose-dependent decrease in the viability of MCF7 and HCT 116 with LRSC-CAE exhibited stronger cytotoxicity against than LRSC-HAE particularly from 25-200 μg/ml.

Our findings from the SRB assay were supported by results from the TBE assay. As shown in Figure 5.3, treatment with LRSC-CAE at 50 and 100 μg/ml significantly reduced the percentage of viable cells (relative to control) to 60.5 and 16.4% for MCF7, and 56.4 and 13.3% for HCT 116. The percentage of viable cells when treated with LRSC-HAE (100 μg/ml) for 72 h, however, was maintained above 90%.

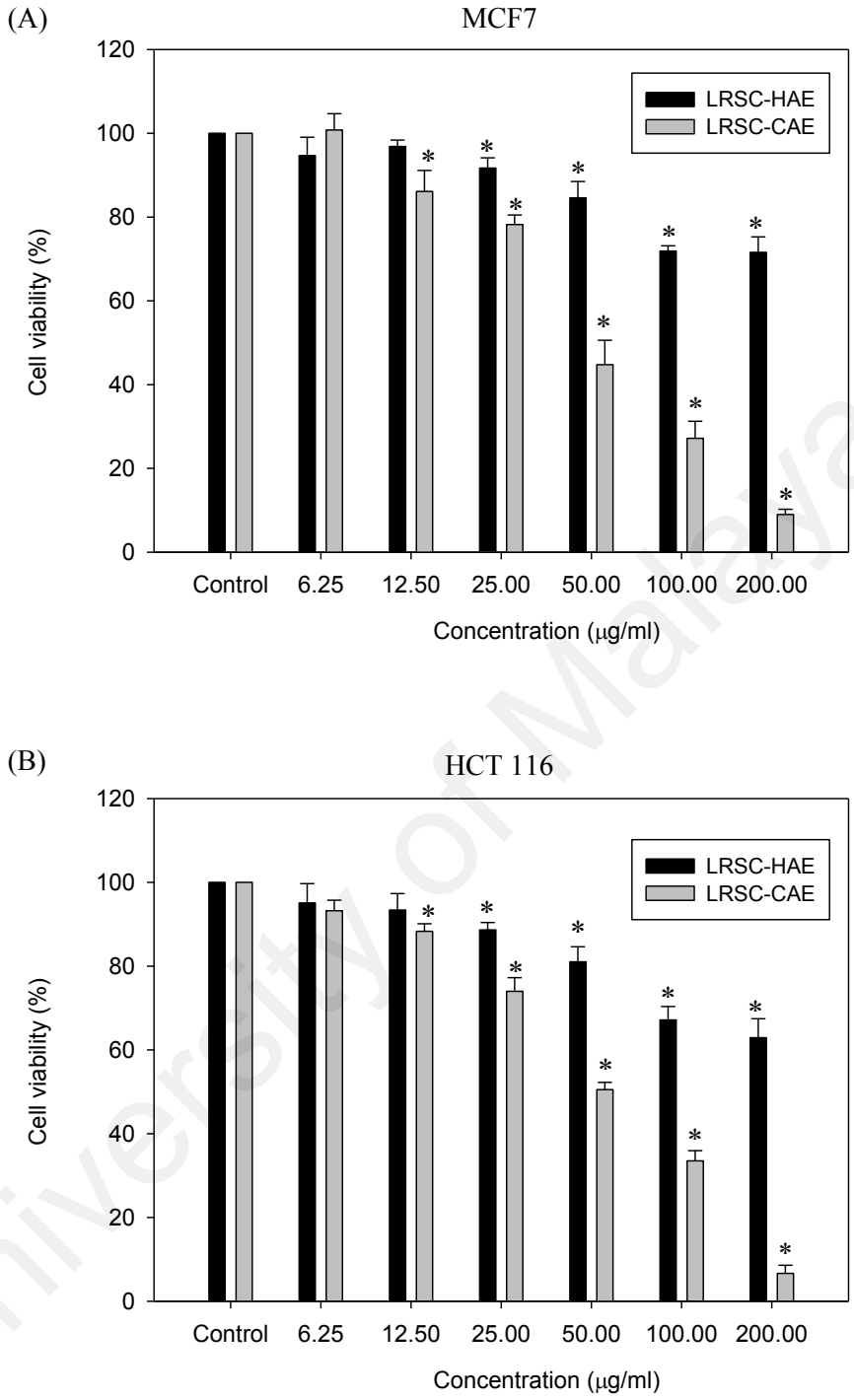


Figure 5.2: Effect of *L. rhinocerotis* aqueous extracts on cell viability (based on cellular protein content). (A) MCF7 and (B) HCT 116 cells were treated with LRSC-HAE and LRSC-CAE (6.25-200.00 μg/ml). Cell viability was determined using the SRB assay after 72 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells (p < 0.05).

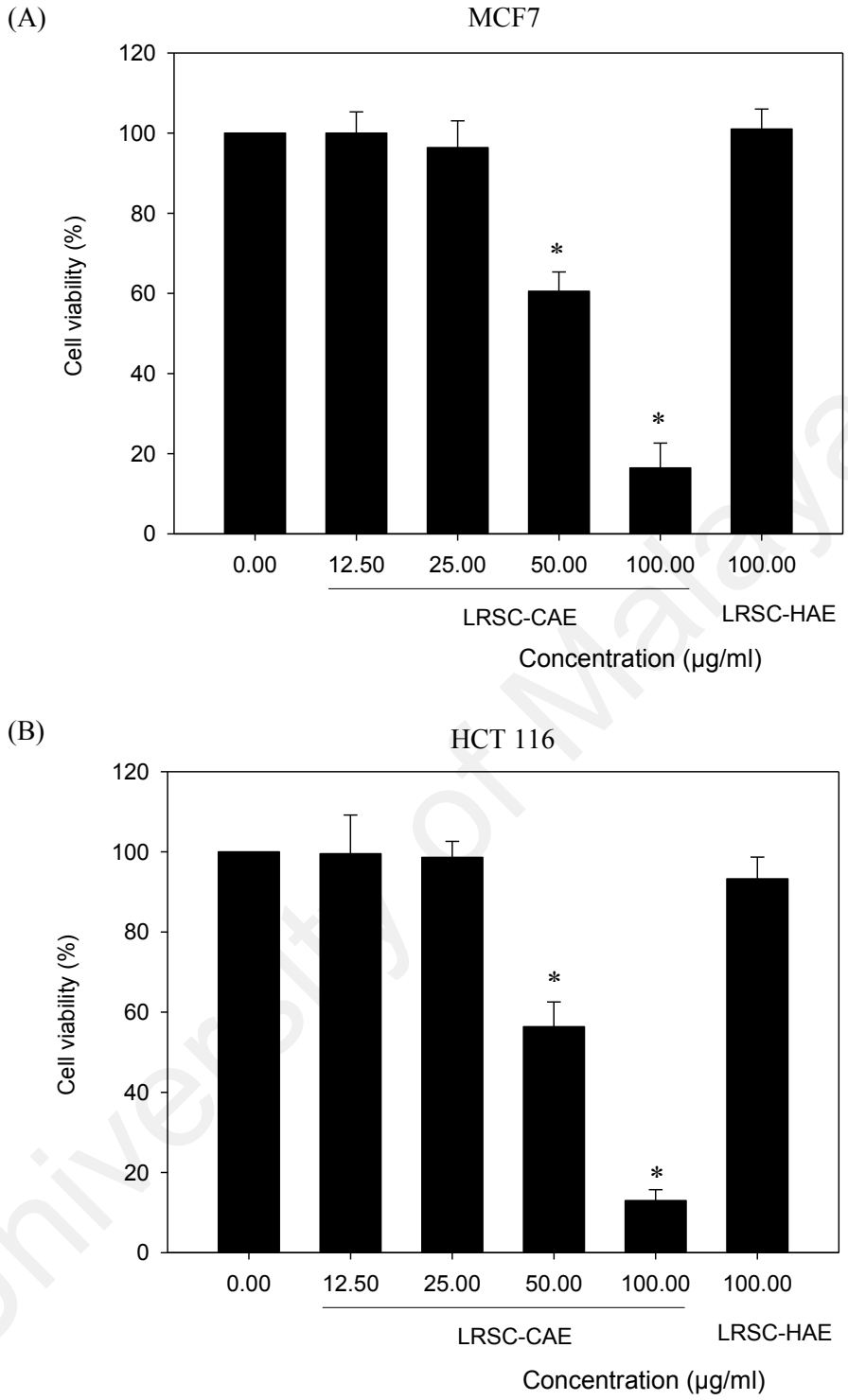


Figure 5.3: Effect of *L. rhinocerotis* aqueous extracts on cell viability (based on dye exclusion). (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (12.50-100.00 µg/ml) and LRSC-HAE (100.00 µg/ml). Cell viability was determined using the TBE assay after 72 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells (p < 0.05).

5.3.1.2 Effect of LRCP-DE on the growth of MCF7 and HCT 116

We also investigated the growth-inhibitory effect of LRCP-DE and LRCP-ME on MCF7 and HCT 116. As shown in Figure 5.4, treatment with LRCP-DE resulted in a dose-dependent decreased in cell viability of MCF7 and HCT 116. LRCP-ME at similar concentration range has lower growth-inhibitory effect on both cell lines.

In the TBE assay, treatment with LRCP-DE for 72 h resulted in a dose-dependent decrease in the number of viable cells as depicted in Figure 5.5. At the highest concentration of 50 μg/ml, cell viability of MCF7 and HCT 116 was reduced to 18.4 and 27.8% (relative to untreated controls), respectively. LRCP-ME at similar concentration exerted no effect on cell viability.

Taken together, the growth-inhibitory effect of the *L. rhinocerotis* extracts against MCF7 and HCT 116 was validated using the SRB and TBE assays. Consistent with the MTT results, the stronger potency of LRSC-CAE compared to LRSC-HAE and LRCP-DE as compared to LRCP-ME, was observed.

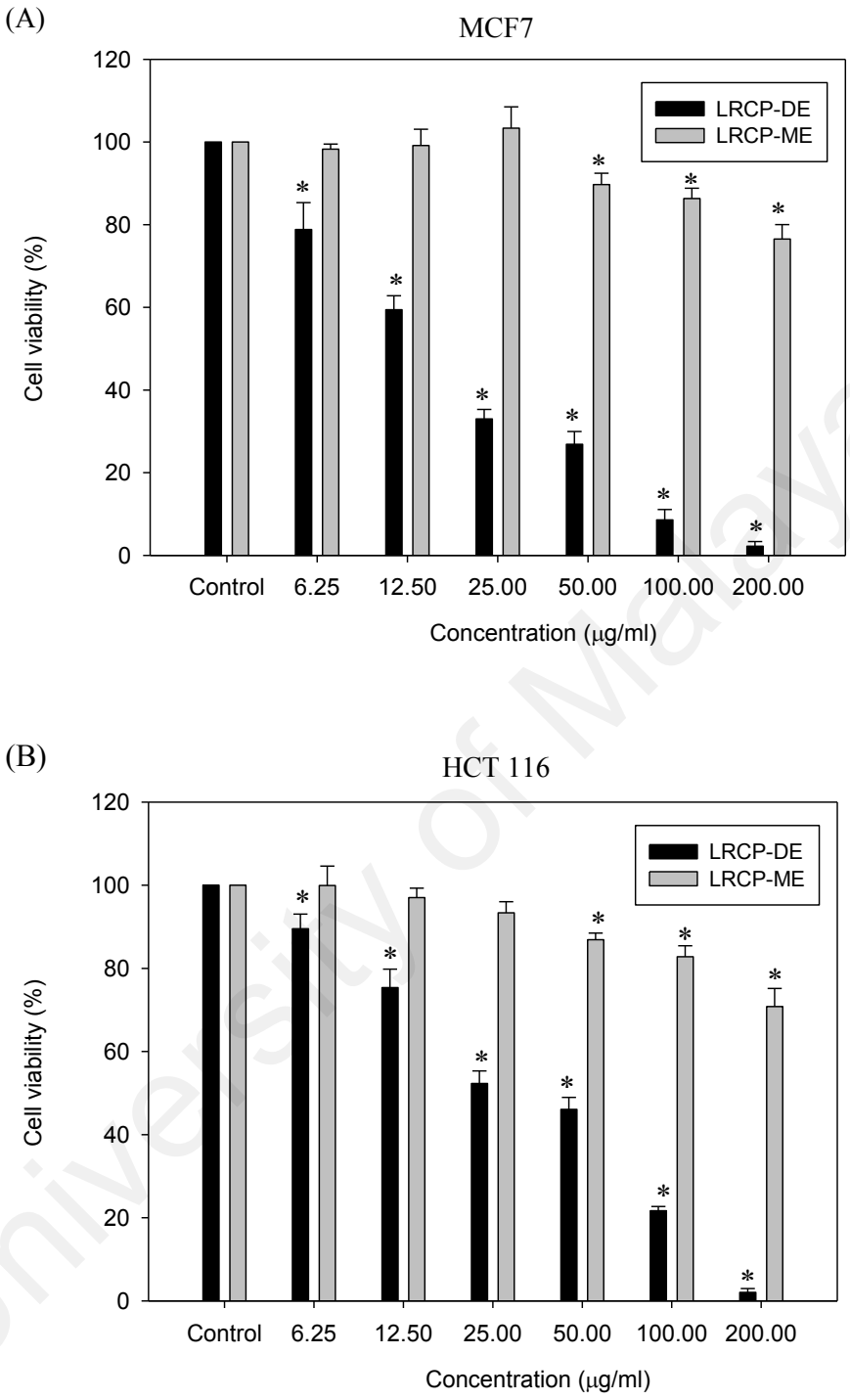


Figure 5.4: Effect of *L. rhinocerotis* organic solvent extracts on cell viability (based on cellular protein content). (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE and LRCP-ME (6.25-200.00 μg/ml). Cell viability was determined using the SRB assay after 72 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

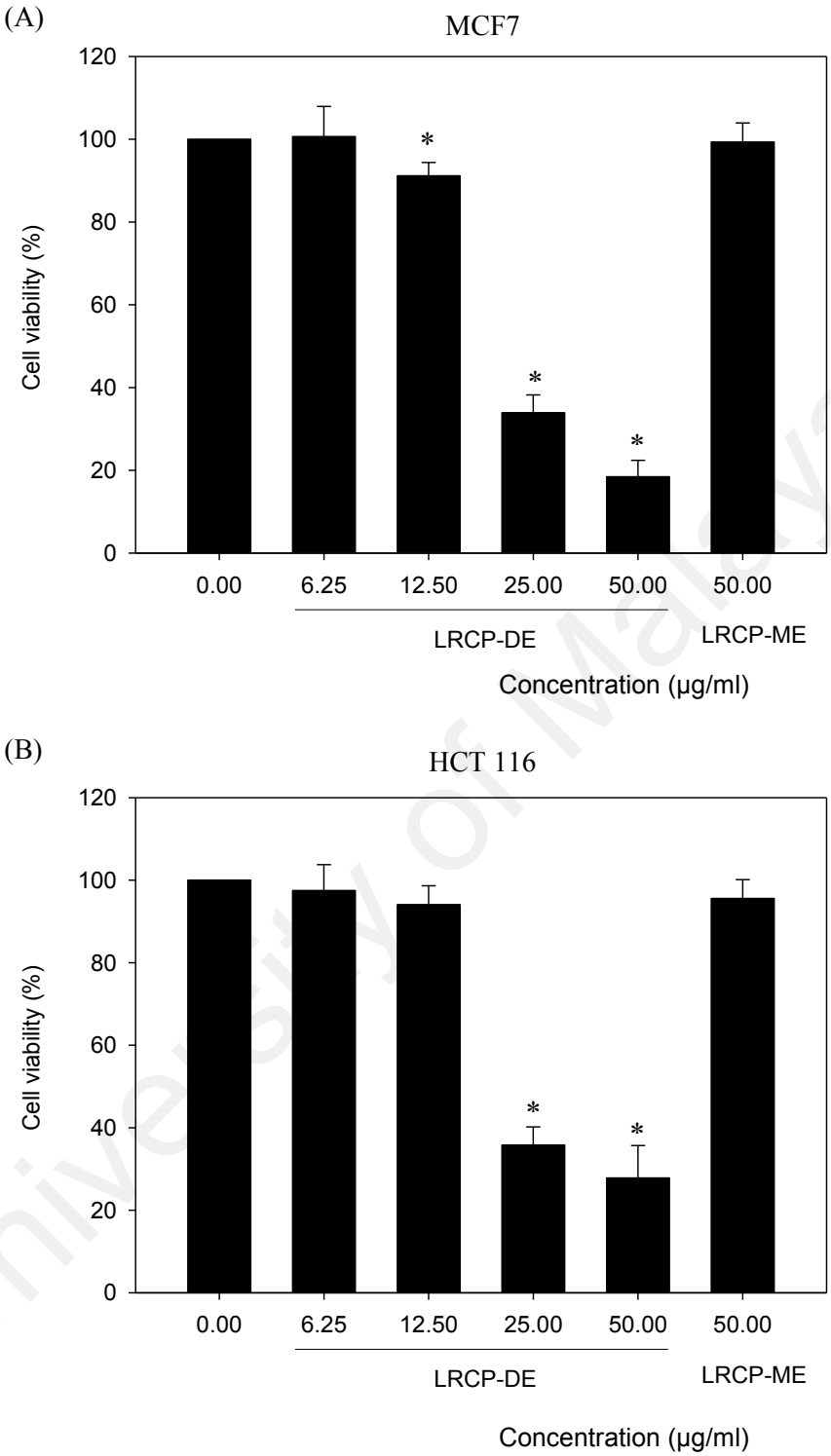


Figure 5.5: Effect of *L. rhinocerotis* organic solvent extracts on cell viability (based on dye exclusion). (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (6.25-50.00 µg/ml) and LRCP-ME (50.00 µg/ml). Cell viability was determined using the TBE assay after 72 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells (p < 0.05).

5.3.2 Cell cycle distribution

To investigate if the growth inhibitory effect of the extracts was due to cell cycle arrest, their effect on cell cycle progression was determined using the flow cytometer.

5.3.2.1 Effect of LRSC-CAE on MCF7 and HCT 116 cell cycle progression

Figure 5.6 shows the effect of LRSC-CAE and LRSC-HAE on cell cycle distribution in MCF7 and HCT 116. During 24 h treatment, LRSC-CAE at lower concentrations (25-50 μg/ml) exerted no significant changes to the cell cycle distribution in MCF7; however, at 100 μg/ml, the population of cells in the G1 (49.5 vs. 37.8%) and G2/M phase (32.7 vs. 16.7%) was significantly higher than the untreated control (Figure 5.7). In HCT 116, there was a gradual increase in the G1 population (40.9-46.6%) when treated with LRSC-CAE for 24 h. Additionally, there was a time-dependent accumulation of cells in G1 for both MCF7 (43.3-56.5%) and HCT 116 (34.1-81.1%) when treated with LRSC-CAE (50 μg/ml), suggesting that the extract induced cell cycle arrest at G1 phase in both cell lines (Figure 5.8 and 5.9). When treated with LRSC-HAE (100 μg/ml), a higher population of MCF7 cells in G1 phase (56.2 vs. 37.8%), compared to the untreated control, was observed. LRSC-HAE at similar concentration, on the other hand, caused no significant changes in the cell cycle distribution in HCT 116.

The sub-G1 population, representing cells with decreased PI staining and possess sub-diploid DNA content, is an indicative of apoptotic cells. When treated with LRSC-CAE for 24 h, the sub-G1 populations were 37.0 and 38.5% for MCF7 and HCT 116, respectively. In the time-dependent studies, treatment with LRSC-CAE increased the sub-G1 population from 1.7% in the untreated control to 67.8% in HCT 116 after 72 h of incubation, whereas the sub-G1 population in MCF7 remained unchanged.

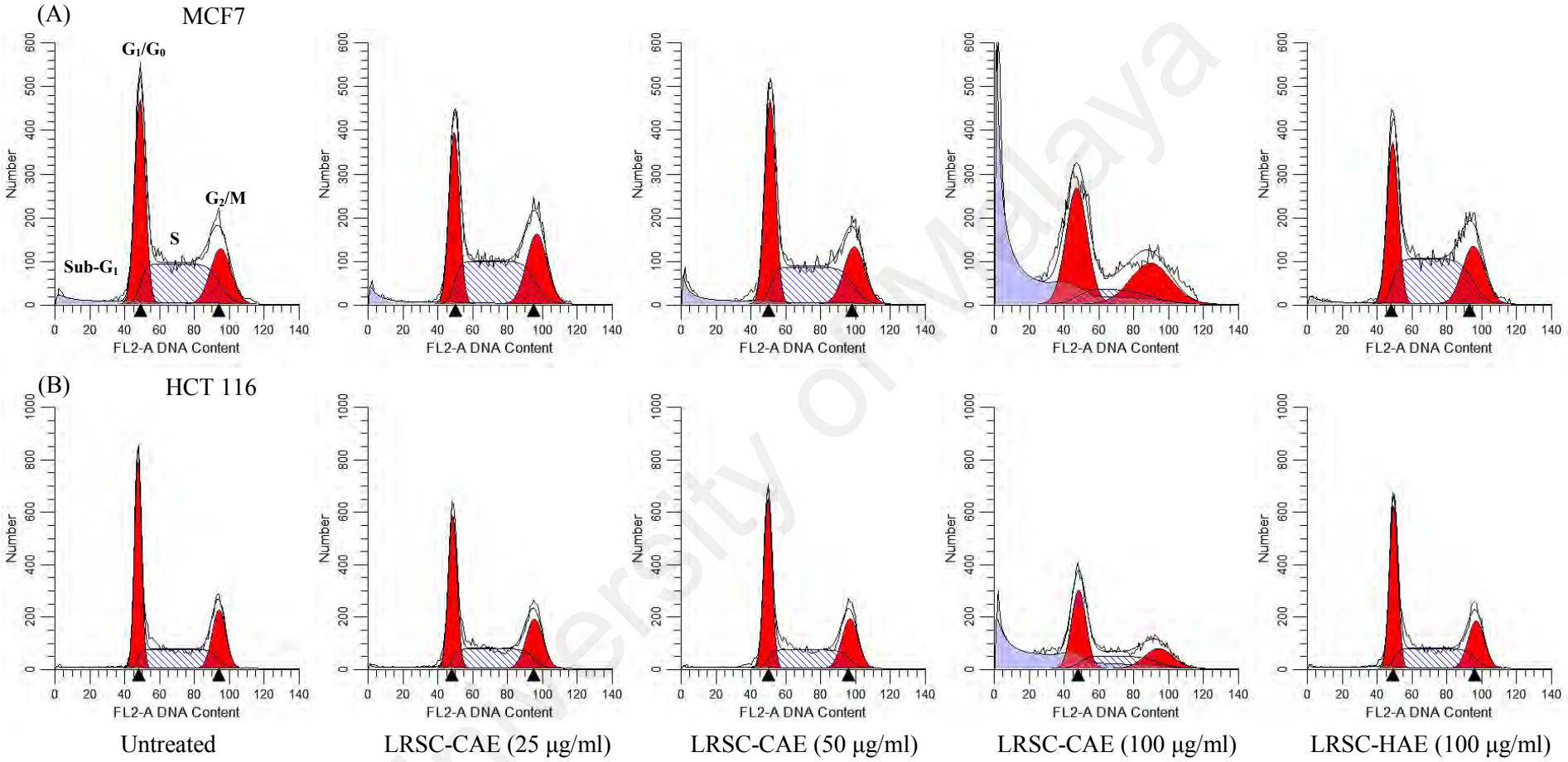


Figure 5.6: Representative histograms showing the effect of *L. rhinocerotis* aqueous extracts on cell cycle distribution (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (25-100 µg/ml) and LRSC-HAE (100 µg/ml), and after 24 h of incubation, the cells were stained with PI and analysed using the flow cytometer. The position of sub-G1, G1, S, and G2/M phases were shown in the untreated control of MCF7.

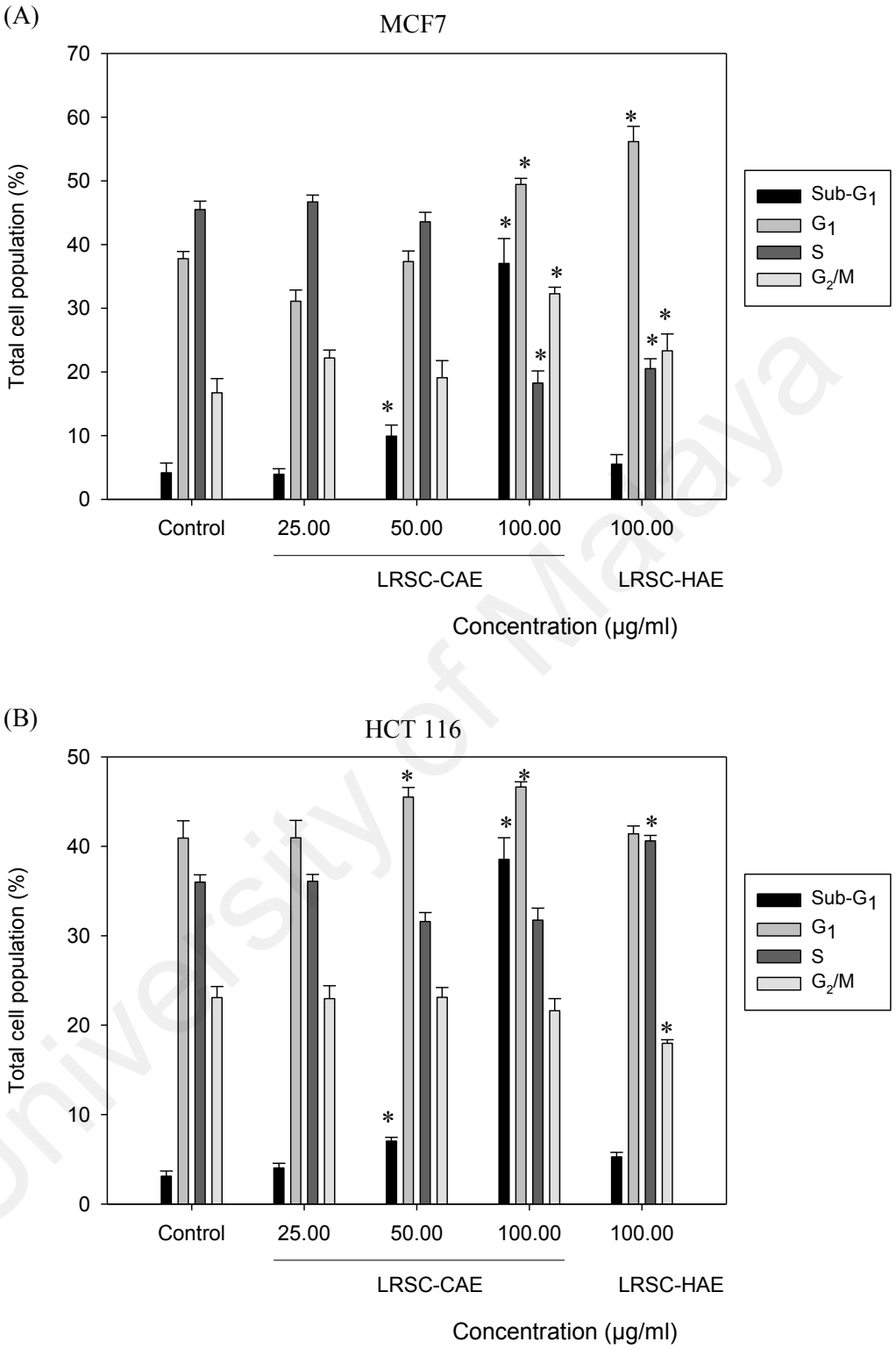


Figure 5.7: Effect of *L. rhinocerotis* aqueous extracts on the distribution of cell cycle phases after treatment for 24 h. (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (25-100 µg/ml) and LRSC-HAE (100 µg/ml) for 24 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

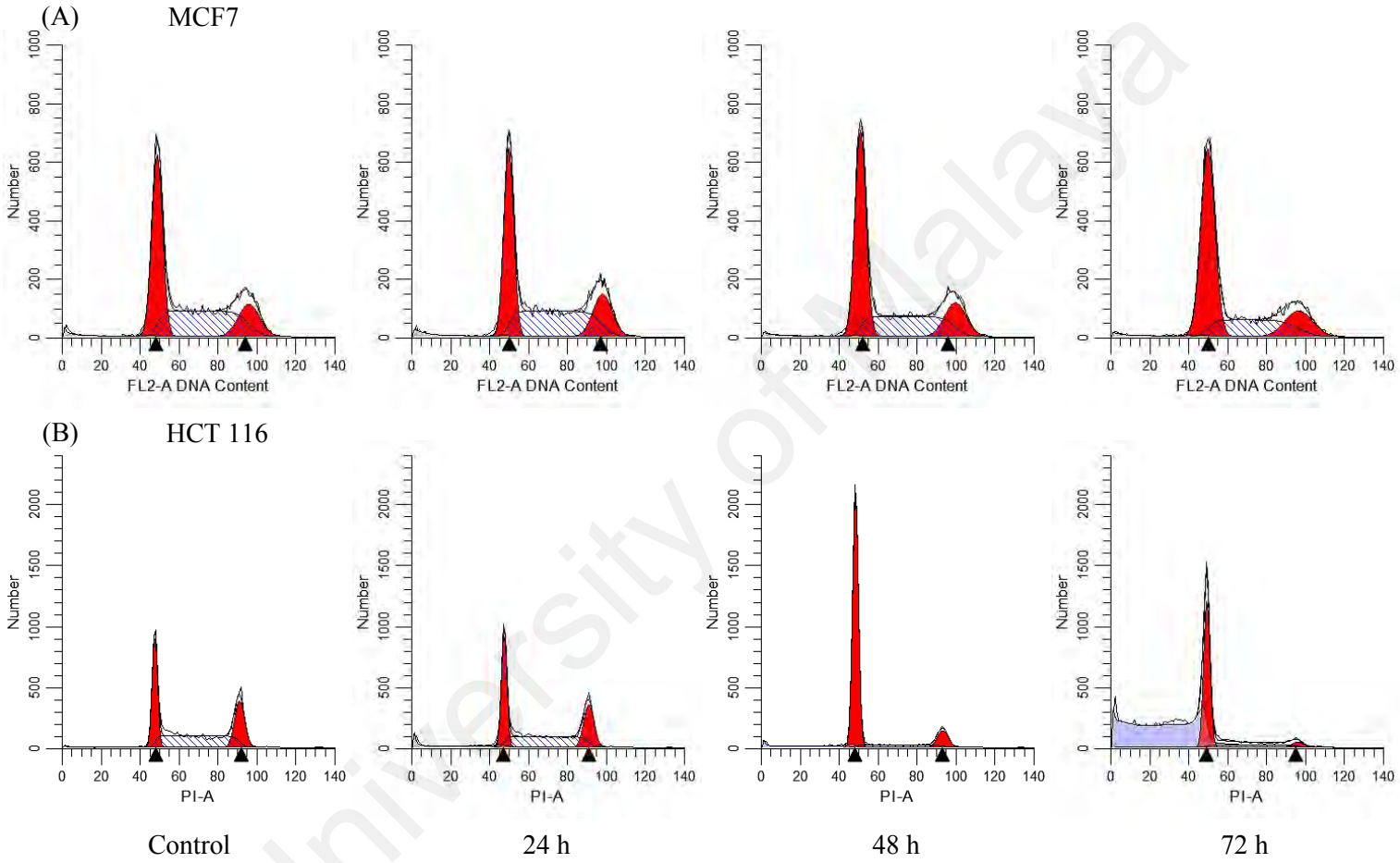


Figure 5.8: Representative histograms showing the time-dependent effect of LRSC-CAE on cell cycle phase distribution. (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (50 µg/ml) and after different incubation periods (24-72 h), the cells were stained with PI and analysed using the flow cytometer.

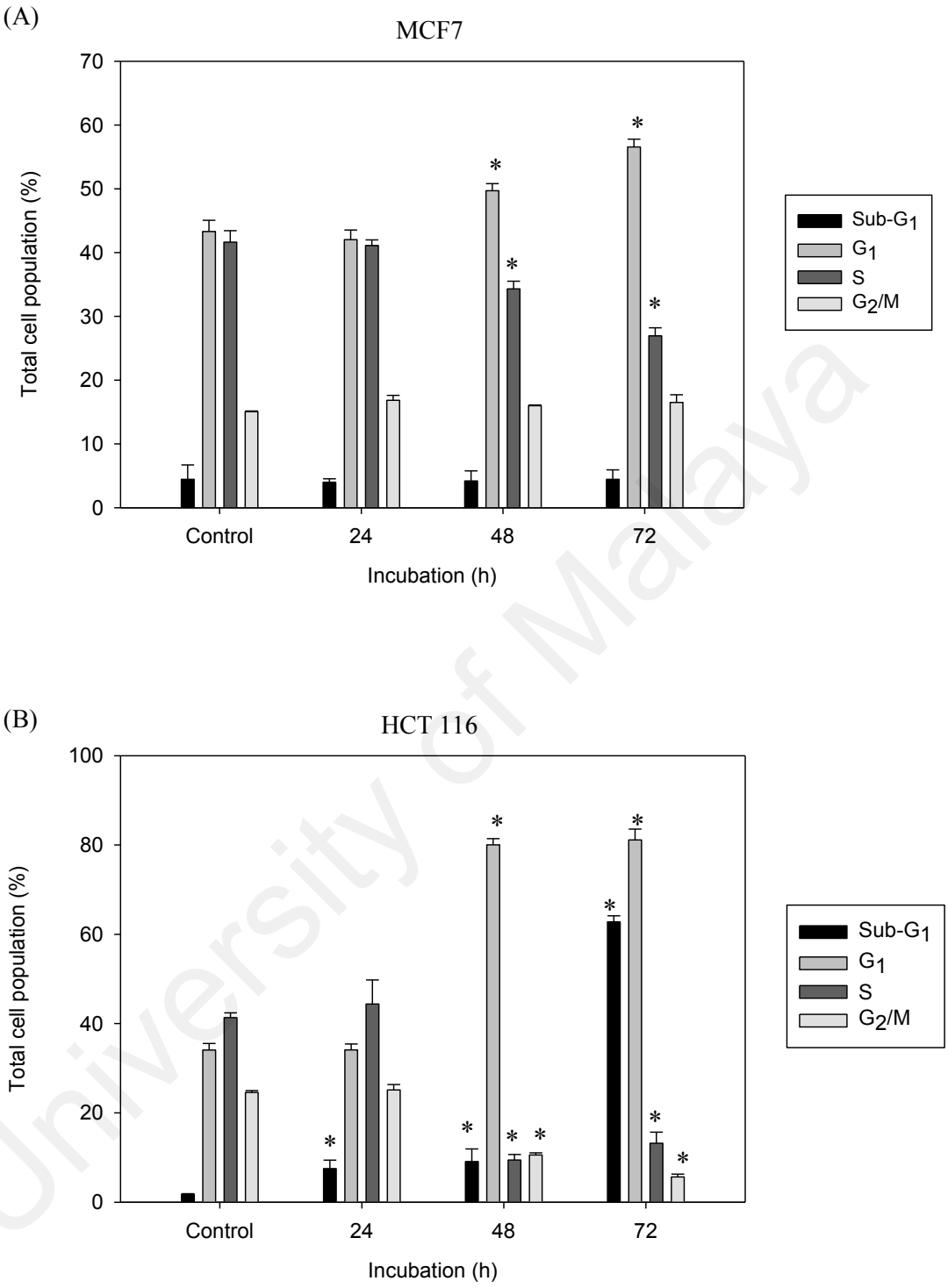


Figure 5.9: The distribution of cell cycle phases after treatment with LRSC-CAE at different incubation periods (24-72 h). (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (50 µg/ml) for 24-72 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells (p < 0.05).

5.3.2.2 Effect of LRCP-DE on MCF7 and HCT 116 cell cycle progression

Figure 5.10 depicts the effect of LRCP-DE and LRCP-ME on the cell cycle phase distribution in MCF7 and HCT 116 cells after 24 h of incubation. In MCF7, LRCP-DE caused a dose-dependent increase in the population of G1 cells and a concurrent decrease of S-phase cells. The percentage of cells in G1 increased from 49.9% in control to 55.6, 58.8, and 79.3% when treated with 12.5, 25.0, and 50 μg/ml of LRCP-DE, respectively (Figure 5.11). In HCT 116, the population of cells in G1 also increased gradually from 34.3% in untreated control to 44.6% in cells treated with 50 μg/ml of LRCP-DE. LRCP-ME (25 μg/ml) has little effect on cell cycle distribution. The cell cycle arrest in G1 phase post treatment with LRCP-DE (12.5 μg/ml) was also observed in time-dependent studies as shown in Figure 5.12. Treatment with LRCP-DE led to time-dependent increase of cells in G1 whereby the increase was 55.6-77.3% for MFC7, and 33.2-83.6% for HCT 116 (Figure 5.13).

Treatment with LRCP-DE caused a dose- and time-dependent increase in sub-G1 cells that is considered as apoptotic population. In MCF7, the population of sub-G1 increased gradually from 3.8 to 11.1% when treated with LRCP-DE (6.25-25.00 μg/ml). For HCT 116, the increase was 3.8 to 15.7% under similar experimental condition. Taken together, cell cycle analysis revealed that LRCP-DE triggered a dose- and time-dependent G1 phase arrest in both cell lines.

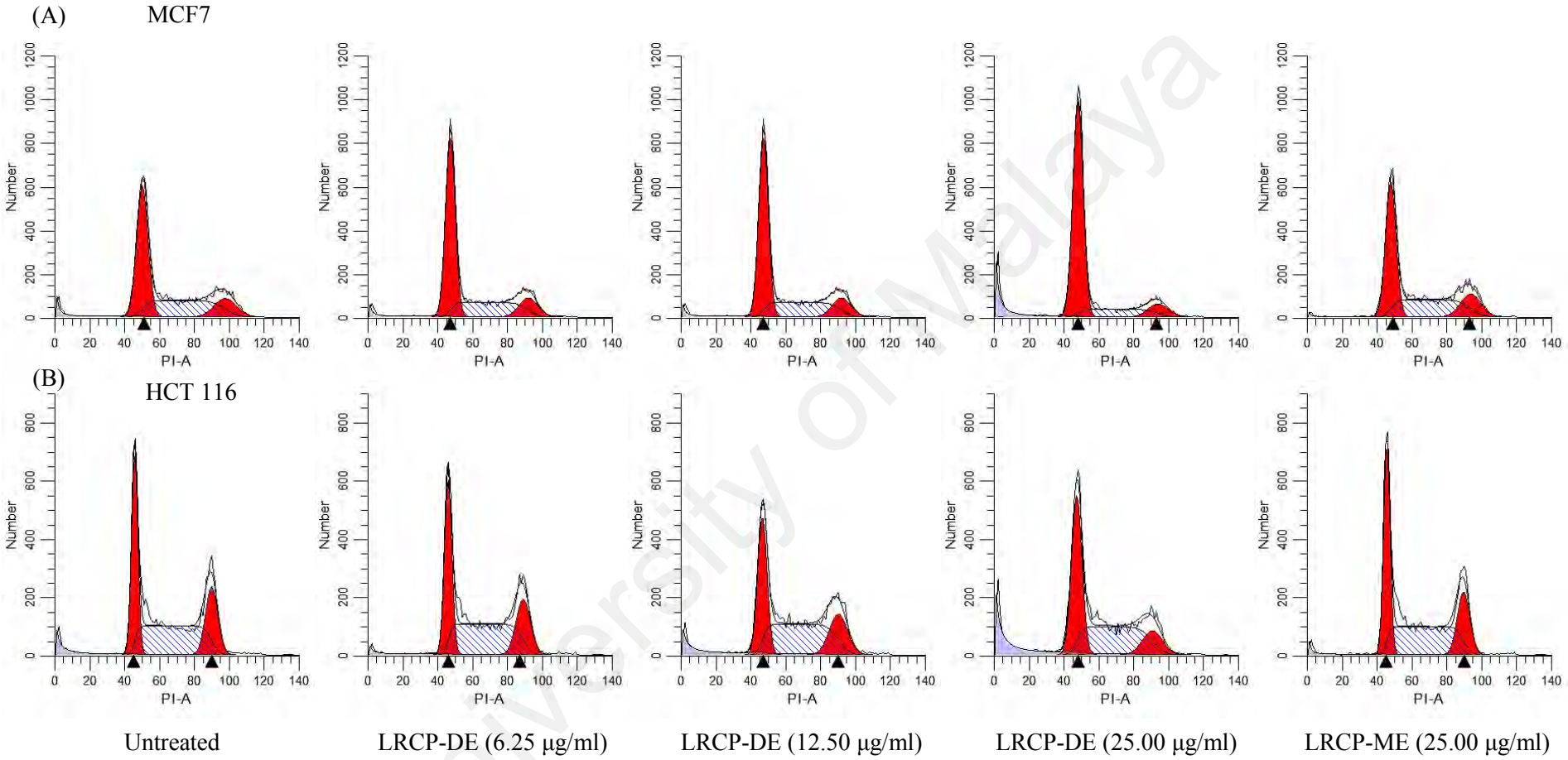


Figure 5.10: Representative histograms showing the effect of *L. rhinocerotis* organic solvent extracts on cell cycle phase distribution. (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (6.25-25.00 µg/ml) and LRCP-ME (25.00 µg/ml), and after 24 h of incubation, the cells were stained with PI and analysed using the flow cytometer.

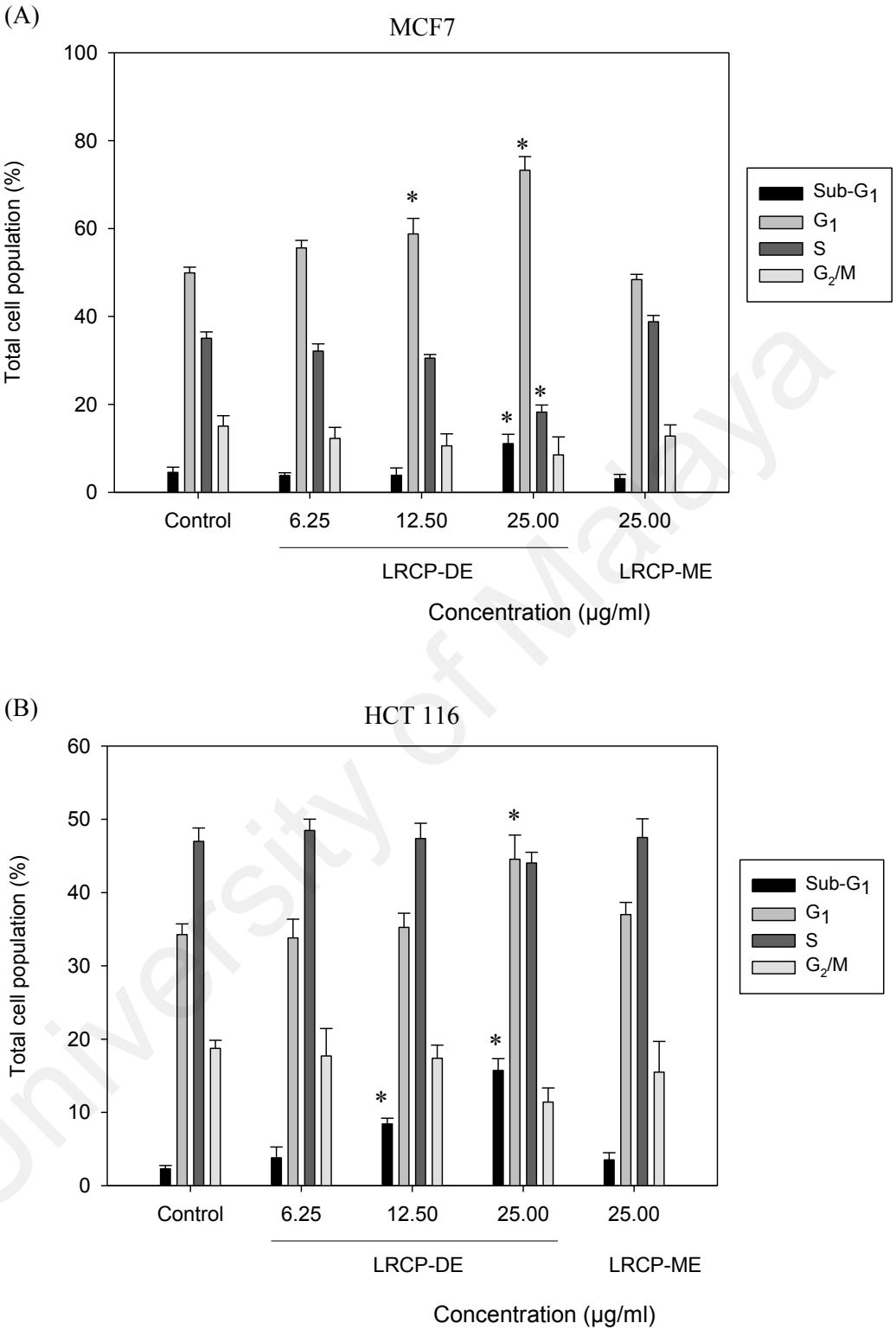


Figure 5.11: Effect of *L. rhinocerotis* organic solvent extracts on the distribution of cell cycle phases after treatment for 24 h. (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (25-100 µg/ml) and LRCP-ME (25 µg/ml) for 24 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

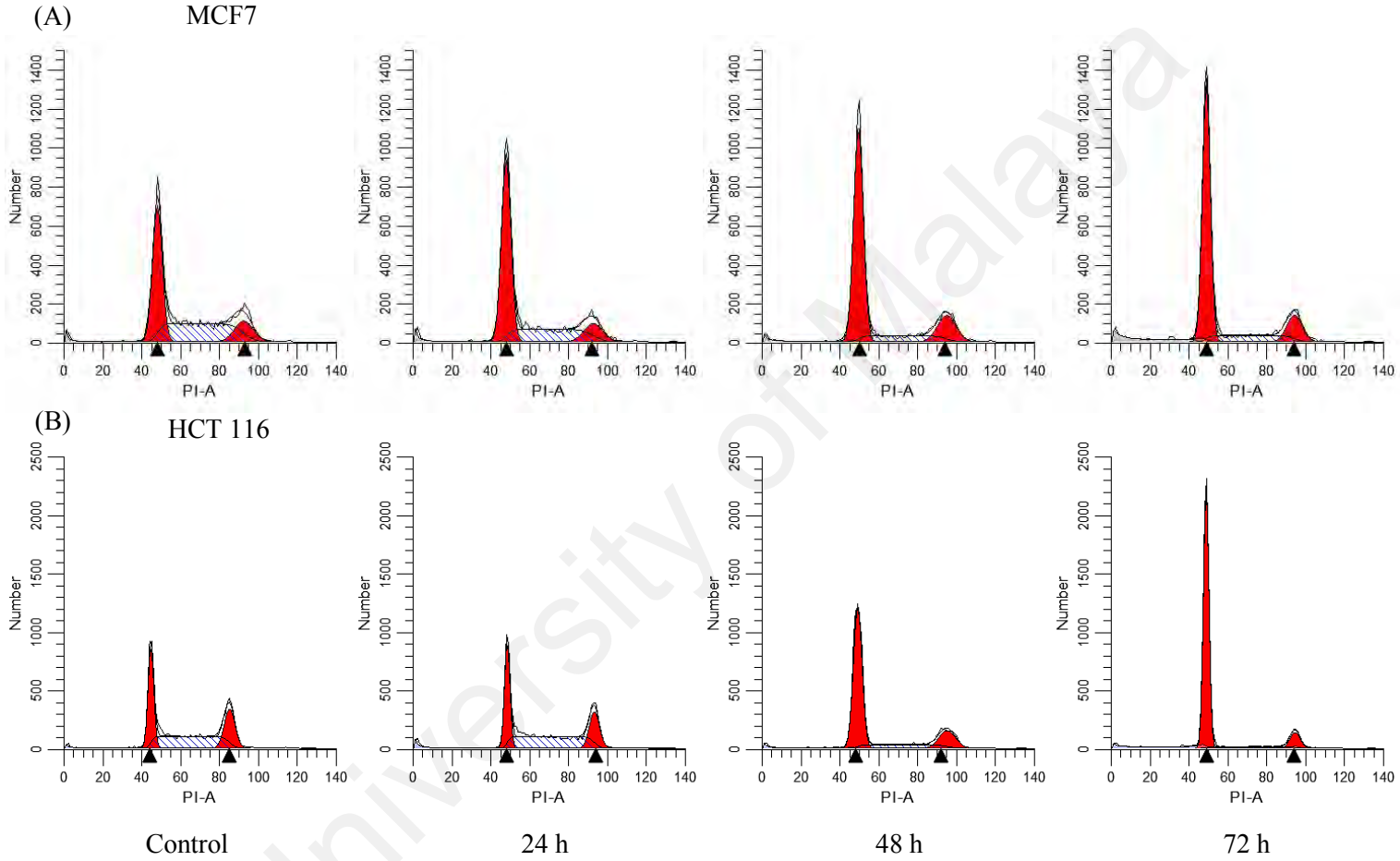


Figure 5.12: Representative histograms showing the time-dependent effect of LRCP-DE on cell cycle phase distribution. (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (12.50 µg/ml) and after different incubation periods (24-72 h), the cells were stained with PI and analysed using the flow cytometer.

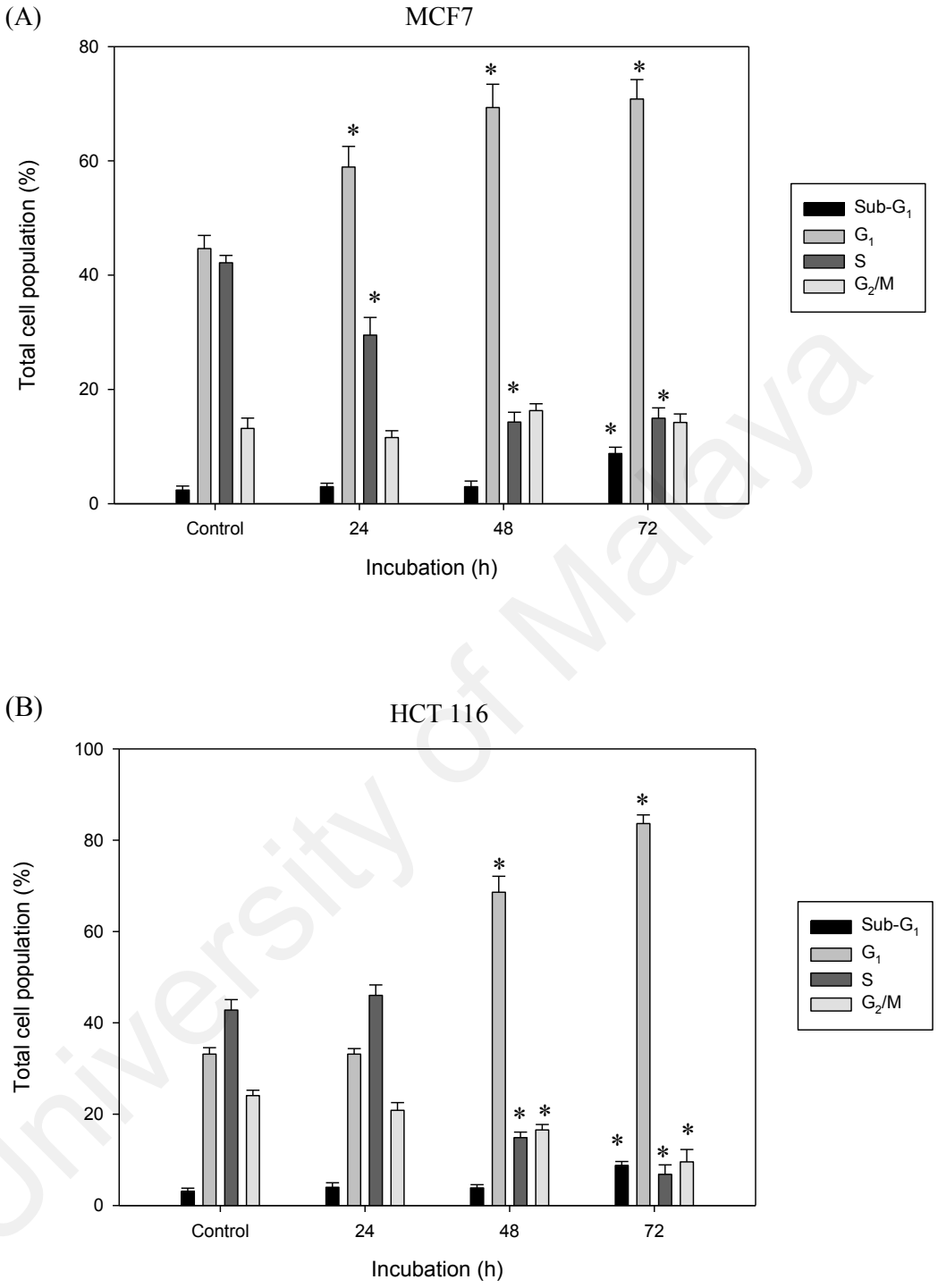


Figure 5.13: The distribution of cell cycle phases after treatment with LRCP-DE at different incubation periods (24-72 h). (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (12.50 µg/ml) for 24-72 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells (p < 0.05).

5.3.3 Induction of apoptosis

One of the characteristics of cells undergoing apoptosis is the loss of phospholipid symmetry of the plasma membrane which involves the translocation of the phosphatidylserine from the inner to outer plasma membrane. To detect the exposed phosphatidylserine; the annexin V-FITC/PI assay was employed in which the phosphatidylserine is recognized and bound by FITC-conjugated annexin V and PI will stain nuclear DNA. Therefore, the amount of viable cells (with no stain), early apoptotic cells (with annexin V stain only), late apoptotic cells (with both annexin V and PI stain) or necrotic or dead cells (with PI only) in a population of cells can be quantitated. Apoptotic cells were counted as early and late apoptotic cells.

5.3.3.1 Induction of apoptosis in MCF7 and HCT 116 by LRSC-CAE

As depicted in Figure 5.14, there was a dose-dependent decrease in the percentage of viable cells that was accompanied by an increase in the percentage of apoptotic cells. The percentage of apoptotic cells in untreated controls was 4.6 and 6.4% for MCF7 and HCT 116, respectively (Figure 5.15). Treatment with LRSC-CAE (25-100 µg/ml) for 24 h resulted in an increase in the population of apoptotic cells of 8.7-21.5% for MCF7 and 8.8-33.2% for HCT 116. LRSC-HAE, on the other hand, caused only a slight increase in apoptotic cells for MCF7. The percentage of apoptotic cells in HCT 116 treated with LRSC-HAE was comparable to the control. The pro-apoptotic potential of LRSC-CAE was further checked with the cell death detection by ELISA method. It measures the levels of nucleosomes in cytoplasmic fraction of treated cells that is taken as indication of DNA fragmentation. As shown in Figure 5.16, LRSC-CAE (50 µg/ml) caused an increase in the apoptotic index in a time-dependent manner in both MCF7 and HCT 116, suggesting DNA fragmentation occurred post treatment with the extracts.

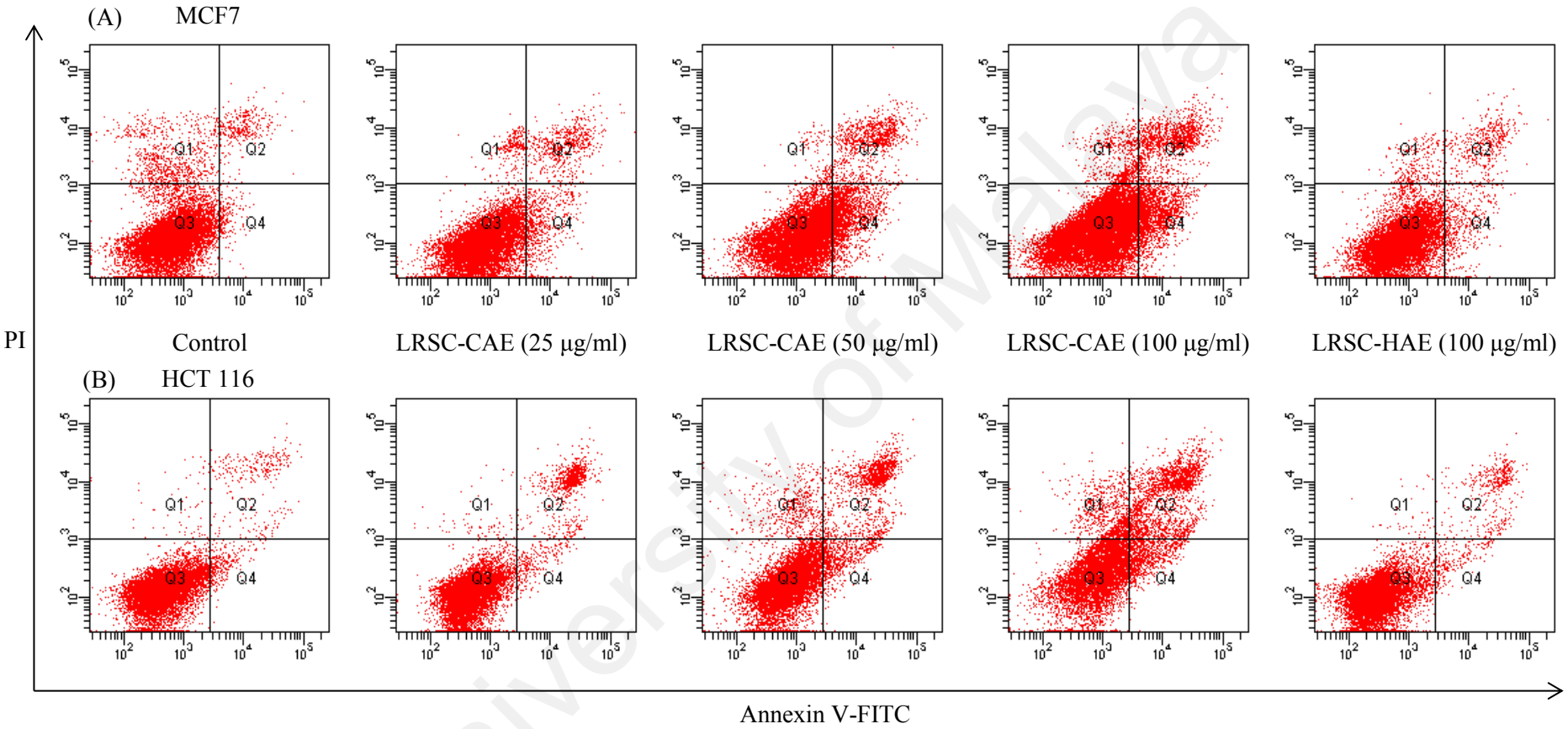


Figure 5.14: Representative histograms showing the effect of *L. rhinocerotis* aqueous extracts on apoptosis induction. (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (25-100 µg/ml) and LRSC-HAE (100 µg/ml), and after 24 h of incubation, the cells were stained with annexin V-FITC and PI, and analysed using the flow cytometer. Q1, necrotic cells; Q2, late apoptotic cels; Q3, viable cells; Q4, early apoptotic cells.

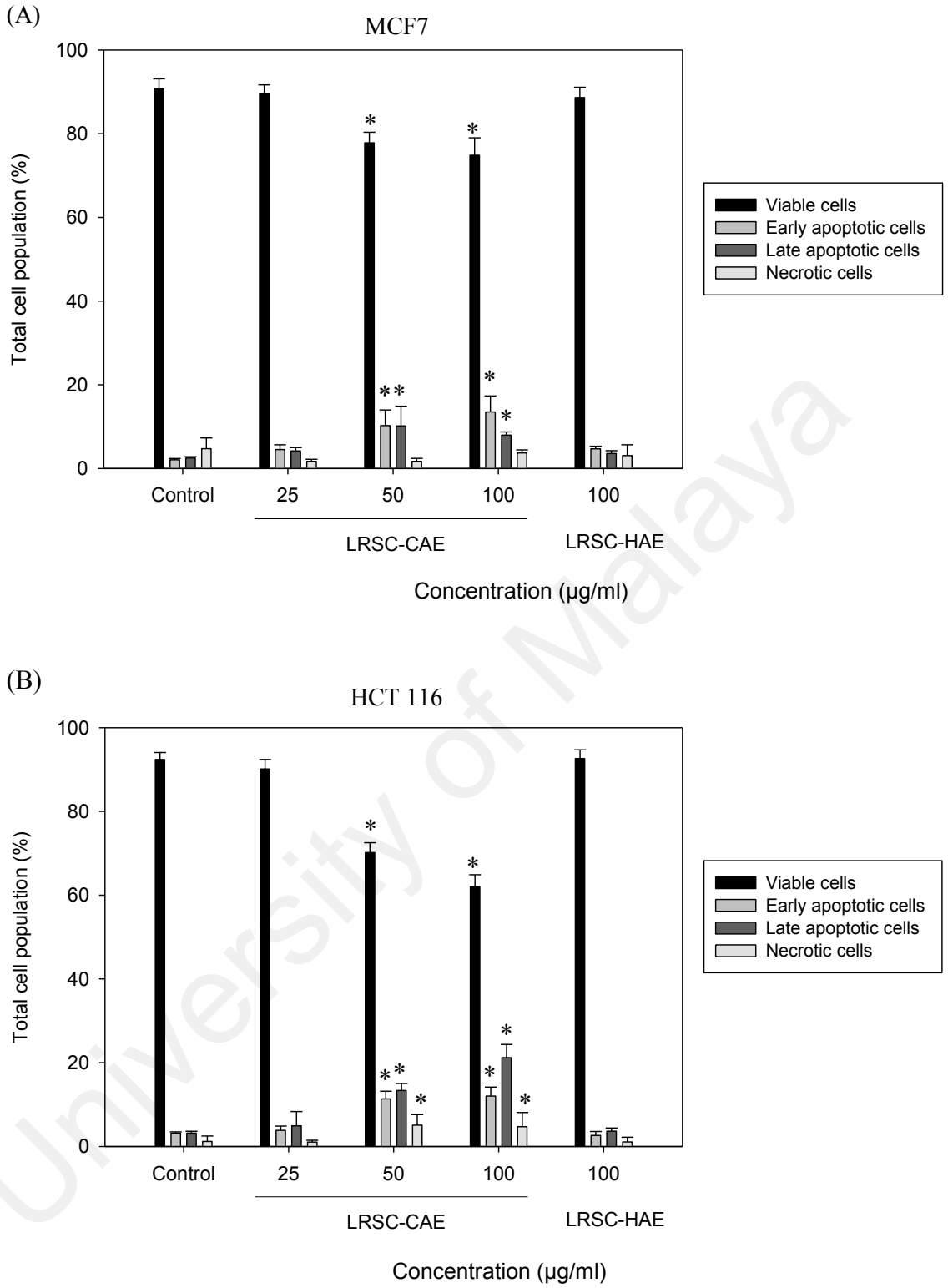


Figure 5.15: Effect of *L. rhinocerotis* aqueous extracts on induction of apoptosis. (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (25-100 µg/ml) and LRSC-HAE (100 µg/ml) for 24 h. Cells were stained with annexin V-FITC and PI, and then, analysed using the flow cytometer. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

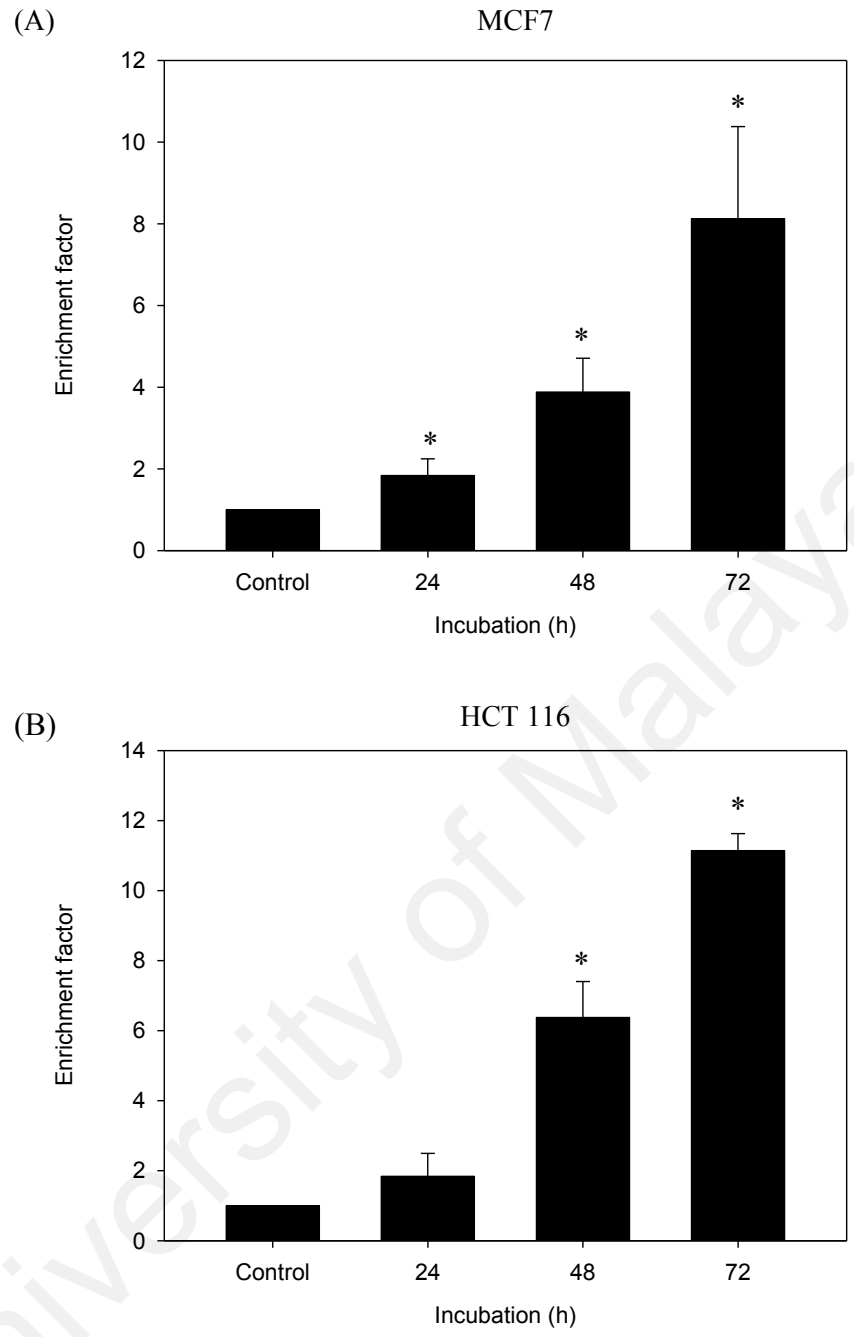


Figure 5.16: Detection of nucleosomes in cell cytoplasmic fractions after treatment with LRSC-CAE. (A) MCF7 and (B) HCT 116 cells were treated with LRSC-CAE (50 µg/ml) for 24-72 h. Cells were harvested and the cytoplasmic fraction was analysed using the cell death detection ELISA kit. The enrichment factor (apoptotic index) is a measure of the levels of histone-associated DNA fragments in the lysates of the treated cells compared to the untreated control. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

In summary, results from the cell cycle analysis, annexin V/PI, and DNA fragmentation assays suggested that the cell cycle arrest and the apoptosis-inducing effect of LRSC-CAE was partly responsible for growth inhibition of MCF7 and HCT 116.

5.3.3.2 Induction of apoptosis in MCF7 and HCT 116 by LRCP-DE

The emergence of sub-G1 population in cell cycle analysis suggests that apoptosis occurred when the cells were treated with LRCP-DE. To evaluate if the cell death was due to apoptosis, the annexin-V/PI assay was performed to assess the effect of LRCP-DE on early and late apoptosis. Figure 5.17 shows the effects of LRCP-DE and LRCP-ME on the externalization of phosphatidylserine in MFC7 and HCT 116 cells. In both cell lines, treatment with LRCP-DE caused a dose-dependent decrease in the number of viable cells and concurrent increase in the early and late apoptotic cells. For MCF7, LRCP-DE caused an increase in the percentage of cells from 6.7% (in controls) to 14.8, 19.3, and 27.4% when treated with 6.25, 12.50, and 25.0 µg/ml LRCP-DE. In HCT 116, the percentage of apoptotic cells ranged from 12.7-16.5% when treated with similar concentrations of LRCP-DE (Figure 5.18). On the other hand, LRCP-ME (25.00 µg/ml) exerted no effect on the externalization of phosphatidylserine in MCF7 and HCT 116 cells.

To further confirm the apoptotic cell death, the cell death detection ELISA analysis was carried out. As shown in Figure 5.19, treatment with LRCP-DE (12.50 µg/ml) caused a time-dependent increase in the apoptotic index in both MCF7 and HCT 116. There was approximately 8-fold increase in the apoptotic index after 72 h of treatment.

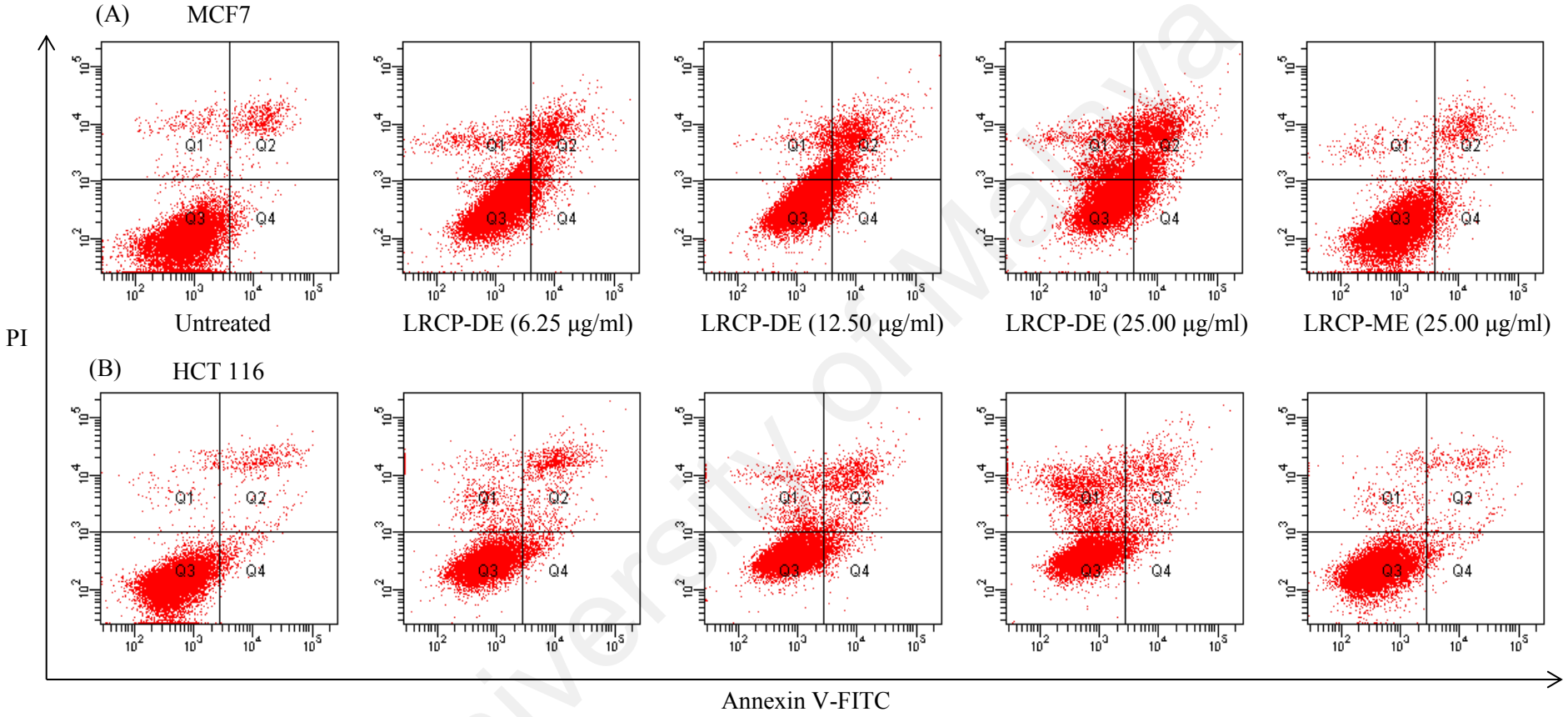


Figure 5.17: Representative histograms showing the effect of *L. rhinocerotis* organic solvent extracts on apoptosis induction. (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (6.25-25.00 µg/ml) and LRCP-ME (25.00 µg/ml), and after 24 h of incubation, the cells were stained with annexin V-FITC and PI and analysed using the flow cytometer. Q1, necrotic cells; Q2, late apoptotic cels; Q3, viable cells; Q4, early apoptotic cells.

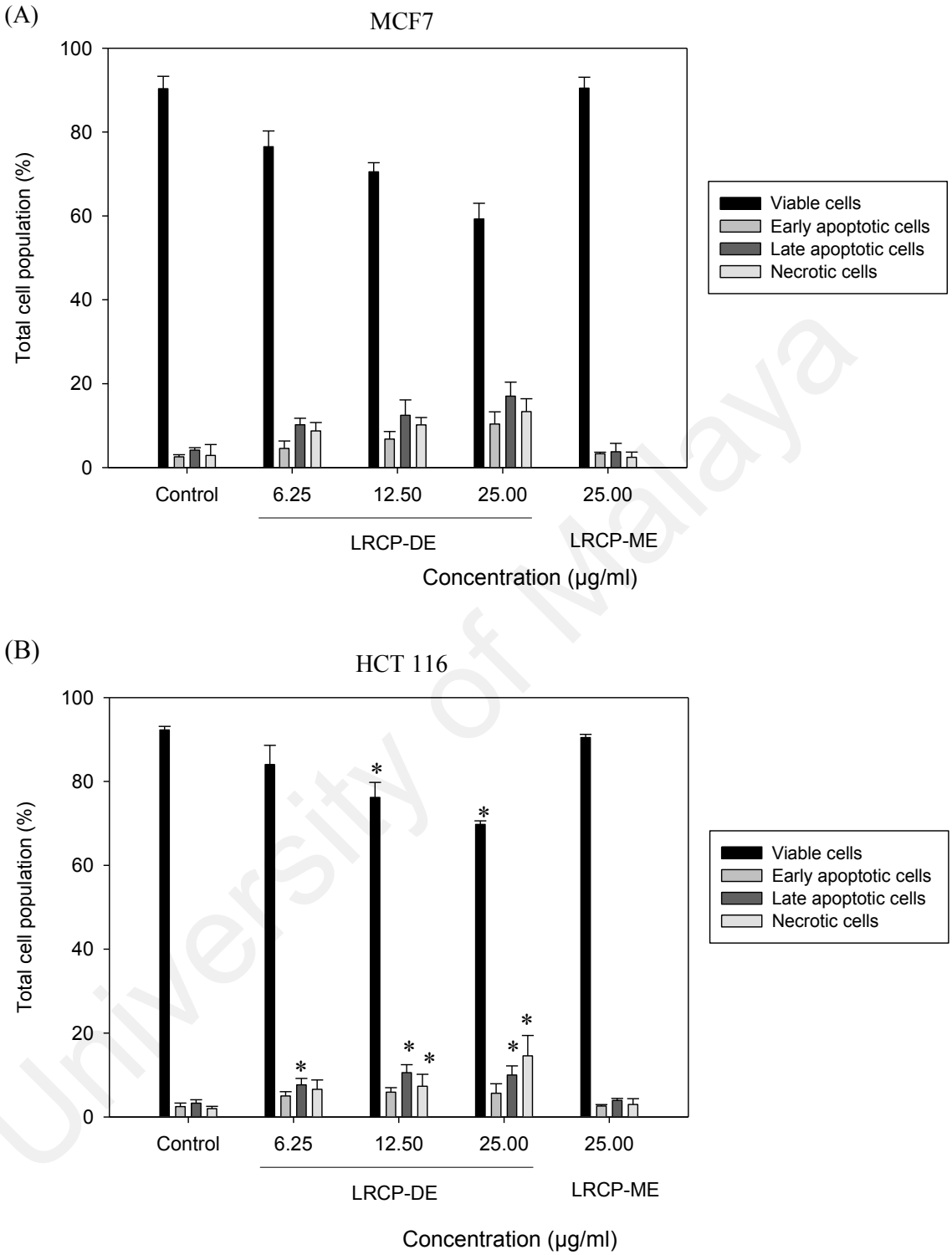


Figure 5.18: Effect of *L. rhinocerotis* organic solvent extracts on induction of apoptosis. (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (6.25-25.00 µg/ml) and LRCP-ME (25.00 µg/ml) for 24 h. Cells were stained with annexin V-FITC and PI, and then, analysed using the flow cytometer. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

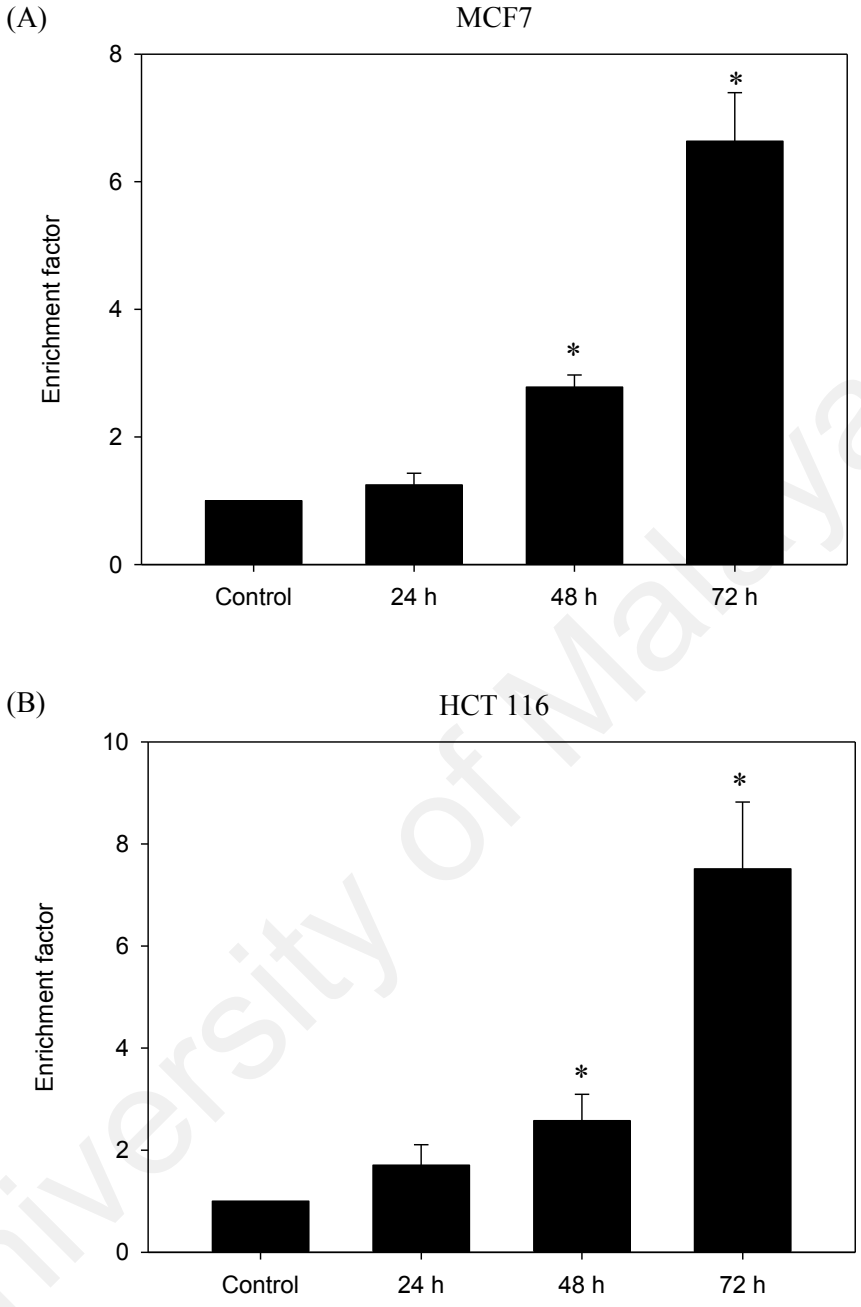


Figure 5.19: Detection of nucleosomes in cell cytoplasmic fractions after treatment with LRCP-DE. (A) MCF7 and (B) HCT 116 cells were treated with LRCP-DE (12.50 µg/ml) for 24-72 h. Cells were harvested and the cytoplasmic fraction was analysed using the Cell Death ELISA kit. The enrichment factor (apoptotic index) is a measure of the levels of histone-associated DNA fragments in the lysates of the treated cells compared to the untreated control. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

Our results from the cell cycle analysis, annexin V/PI, and cell death detection ELISA assays suggest that cell cycle arrest and apoptosis were involved in the growth inhibition and cell death mediated by LRCP-DE, similar to that of LRSC-CAE.

5.3.4 Chemical nature of cytotoxic component(s) in LRSC-CAE

Chemical fractionation of LRSC-CAE was carried out to identify the nature of the sclerotial cytotoxic component(s). Chemical profiling of the fractions was carried out using electrophoretic, chromatographic, and mass-spectrometric approaches (where necessary).

5.3.4.1 Comparison of protein profiles of LRSC-HAE and LRSC-CAE

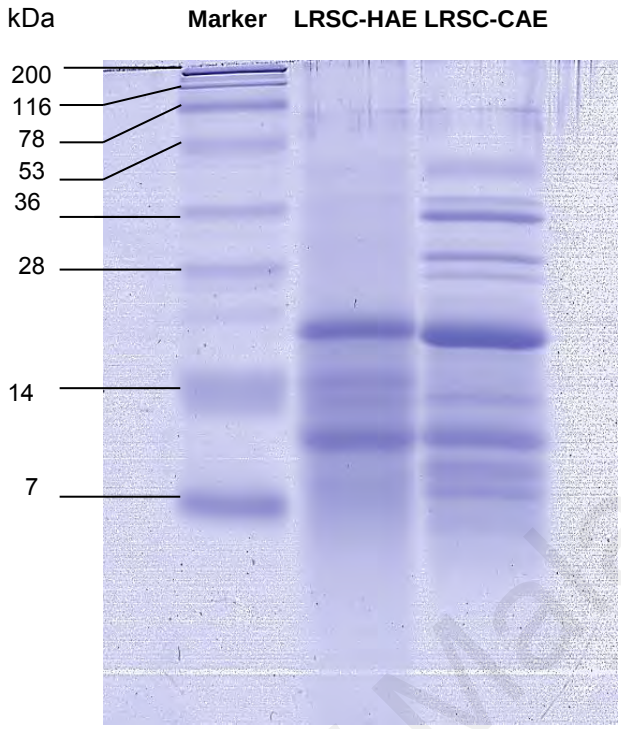
In view of the differential cytotoxic activity of LRSC-HAE (sclerotial hot aqueous extract) and LRSC-CAE (sclerotial cold aqueous extract), comparison of their chemical profiles was necessary. Electrophoretic and protein chip array-based approaches were used to profile the high- and low-molecular-weight proteins, respectively.

Figure 5.20A shows the SDS-PAGE profile of the extracts. There were more bands in the SDS-PAGE profile of LRSC-CAE than LRSC-HAE notably in the high-molecular-weight region with several bands (28.9, 32.5, 41.1, 46.3, and 56.4 kDa) were observed to be specific only to LRSC-CAE. Two bands of 20.3 and 10.4 kDa were present in both extracts. In addition, LRSC-HAE also contained other bands of 14.8, 12.6 and 7.9 kDa whereas LRSC-CAE was characterised by bands of 13.7, 10.4, 8.5 and 7.3 kDa.

Further characterisation of the extracts using SELDI-TOF-MS revealed that LRSC-HAE and LRSC-CAE also contained fairly similar proteins in the low-molecular-weight region (2-15 kDa). As shown in Figure 5.20B, several major peaks in the region of 5–15 kDa were found to be present in both LRSC-HAE and LRSC-CAE with different intensities. There were multiple peaks (2-3.5kDa) that were present only in LRSC-HAE.

Results from chemical profiling clearly demonstrated the differences in the protein profiles of LRSC-HAE and LRSC-CAE. This observation showed that heat influences the extraction of water-soluble components, such as proteins, from the sclerotium of *L. rhinocerotis*.

(A)



(B)

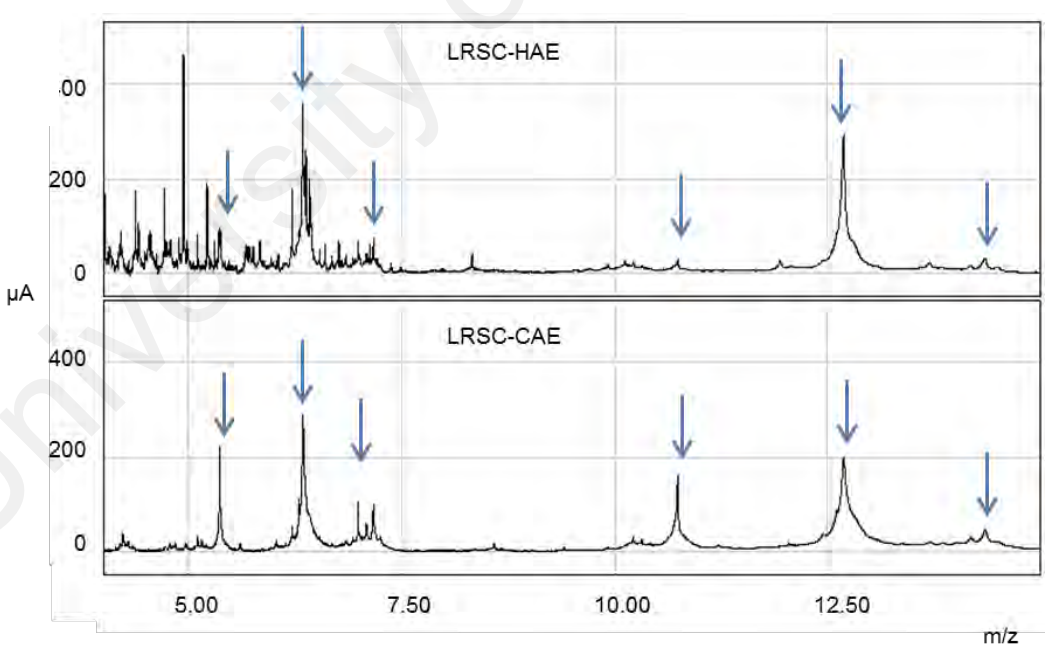


Figure 5.20: Protein profiles of *L. rhinocerotis* sclerotial aqueous extracts. (A) SDS-PAGE profiles of LRSC-HAE and LRSC-CAE. Gel was visualized with Coomassie Brilliant Blue R-250 staining. (B) Representative SELDI-TOF-MS spectra of LRSC-HAE and LRSC-CAE (2-15 kDa). Arrows indicate similar protein peaks in both extracts.

5.3.4.2 Heat-sensitivity of cytotoxic components in LRSC-CAE

Results from cell viability assays (i.e. MTT, SRB, and TBE) consistently showed that LRSC-CAE was more potent than LRSC-HAE. Contrary to LRSC-HAE that was prepared by boiling, the preparation of LRSC-CAE did not involve heating; hence, we hypothesized that the cytotoxic components in LRSC-CAE were heat-labile.

To confirm our hypothesis, LRSC-CAE was subjected to incubation at 40, 60, 80 and 100 °C for 20 min., and the heat-treated extracts were tested for cytotoxicity against MCF7 and HCT 116 at 50 and 100 µg/ml. As shown in Figure 5.21, the cell viability of both cells was not affected by LRSC-CAE that were heated at 40 and 60 °C; however, there was a significant increase in number of viable cells upon treatment with LR-CA that has been incubated at 80 °C. Cytotoxicity of LRSC-CAE was completely lost upon exposure to heat treatment between 80-100 °C.

Our results have shown that heating LRSC-CAE at high temperature (between 60-100 °C) resulted in the loss of cytotoxic activity against MFC7 and HCT 116. This seemed to suggest that the cytotoxic components in LRSC-CAE were heat-labile.

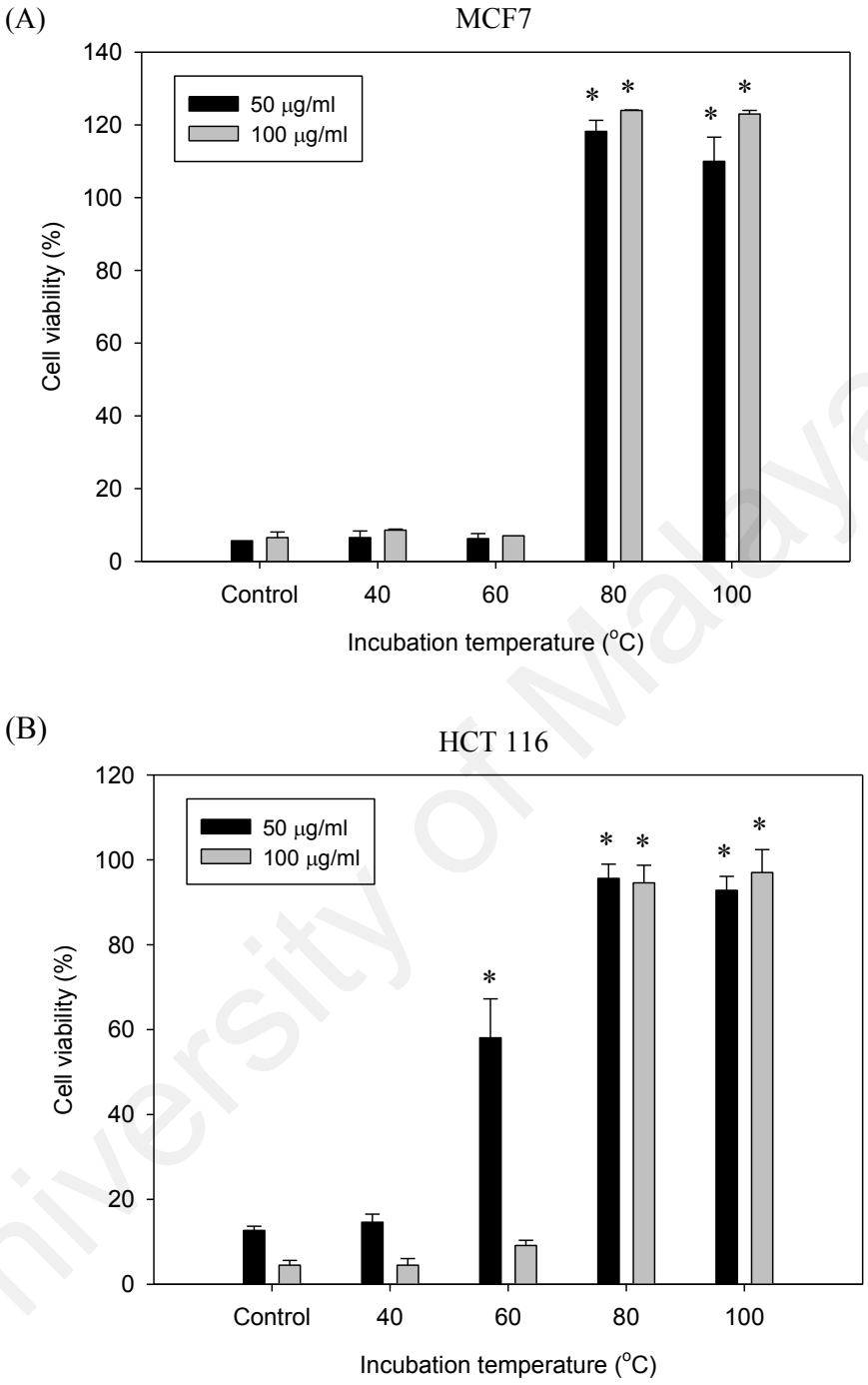


Figure 5.21: Cytotoxic activity of heat-treated LRSC-CAE. LRSC-CAE was subjected to heat-treatment between 40-100 °C for 20 min and their cytotoxic activity against (A) MCF7 and (B) HCT 116 cells was determined using the MTT assay after 72 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and cells treated with extracts subjected to heat-treatment ($p < 0.05$).

5.3.4.3 Changes in protein profiles of heat-treated LRSC-CAE

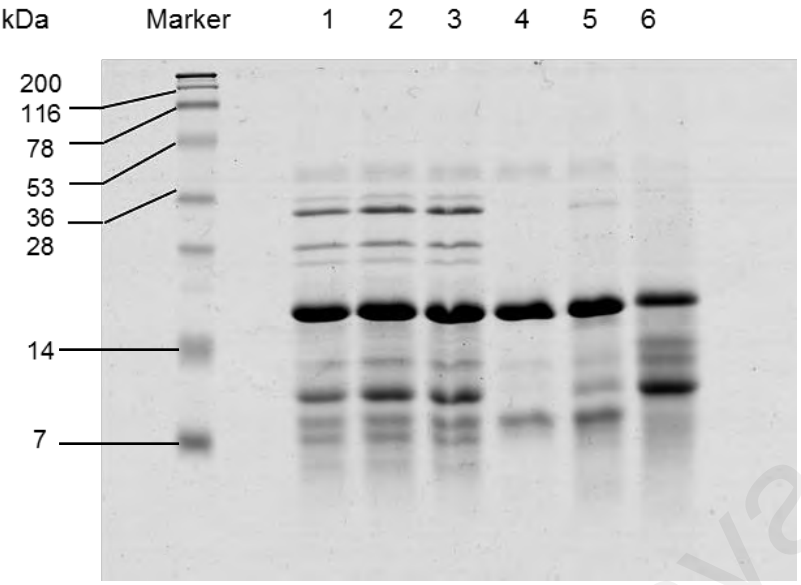
The heat-treated extracts were also subjected to protein profiling using SDS-PAGE and SELDI-TOF-MS. The loss of cytotoxic activity of heat-treated extracts was found to be accompanied by changes in the protein profiles.

The SDS-PAGE profile of LRSC-CAE that has been subjected to heat-treatment was shown in Figure 5.22A. After incubation at 40 and 60 °C, we observed several bands (> 20 kDa) specific to LRSC-CAE were still present after but these eventually disappeared when the temperature was raised to 80 and 100 °C. The 10.4 kDa band also followed similar trend. On the other hand, some bands (e.g. 8.5 and 20.3 kDa) remain unchanged regardless of the temperature of heat treatment. It is to be noted that the profile of LRSC-CAE treated at 100 °C for 20 min was, however, not identical to LRSC-HAE that was prepared using boiling method (hot aqueous extraction).

Similar changes were also observed in the SELDI-TOF-MS profiles of heat-treated extracts as shown in Figure 5.22B. Overall, the intensity of 10 and 12 kDa peaks showed gradual decrease upon heat-treatment, and became almost undetectable at 80 and 100 °C. Protein peaks in the region 5.0-7.5 kDa remained stable up to 60 °C. Following that, several new peaks between 6-7 kDa were detected. When the temperature was increased from 80-100 °C, the intensity of the 3 kDa peak was almost doubled. The SELDI-TOF-MS profile of LRSC-CAE treated at 100 °C was also dissimilar to that of LRSC-HAE.

Taken together, our results demonstrated that the alteration of protein profiles of the heat-treated extracts correlated with the loss of cytotoxicity. This suggested that proteins might be indeed involved in the cytotoxic action of LRSC-CAE.

(A)



(B)

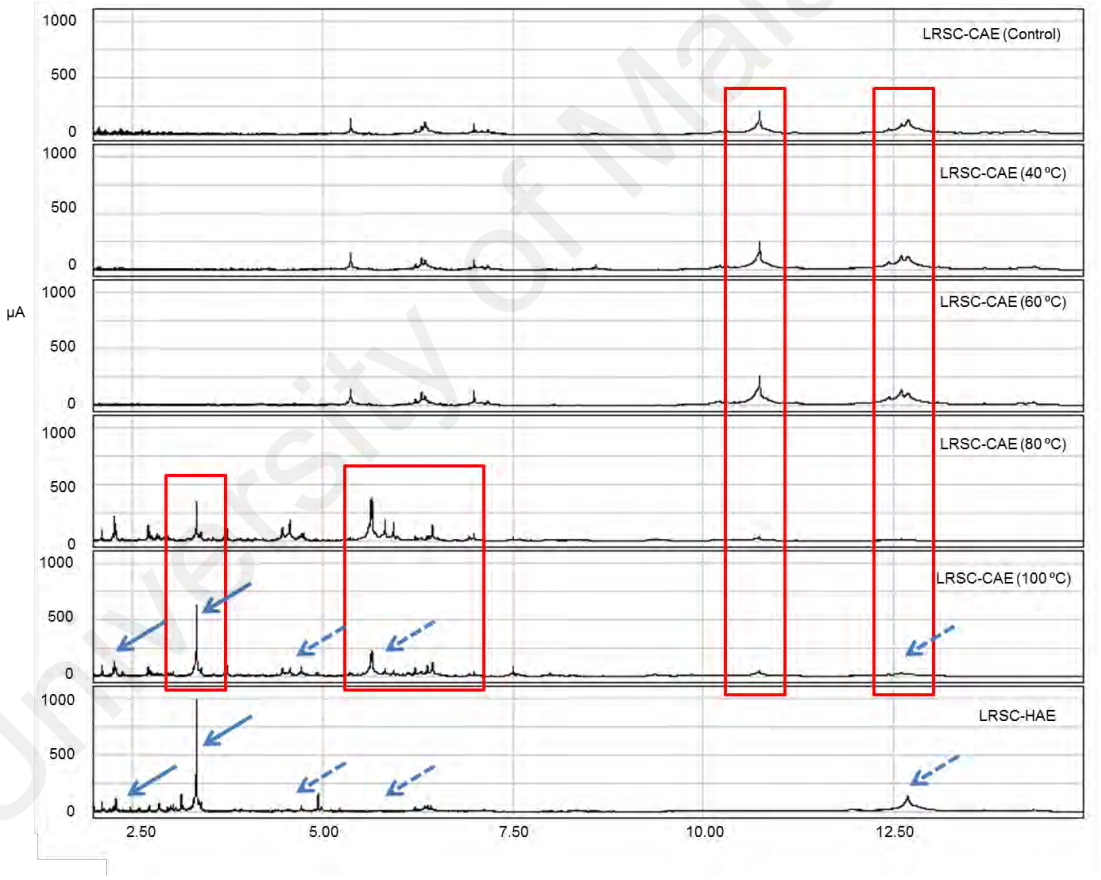


Figure 5.22: Alteration in the protein profiles of LRSC-CAE subjected to heat-treatment. (A) SDS-PAGE profiles of heat-treated LRSC-CAE. Lane 1 = untreated LRSC-CAE (control), lane 2-5 = LRSC-CAE treated at 40, 60, 80, 100 °C for 20 min, lane 6 = LRSC-HAE. (B) Representative SELDI-TOF-MS spectra of heat-treated LRSC-CAE (2-15 kDa). Changes in the intensities of several peaks (boxes) following heat-treatment of LRSC-CAE were observed. Similar (bold arrows) and dissimilar (dashed arrows) protein peaks in LRSC-CAE treated at 100 °C and LRSC-HAE.

5.3.4.4 Chemical profiles and cytotoxic activity of LRSC-CAE fractions

Previous results have confirmed the heat sensitivity of the cytotoxic components in LRSC-CAE. To further explore the chemical nature of the cytotoxic components, LRSC-CAE was chemically fractionated by liquid-liquid partition followed by precipitation methods (Figure 5.1).

Liquid-liquid partition of LRSC-CAE with ethyl acetate yielded the ethyl acetate-soluble (CAE-EAF) and aqueous (CAE-AF) fractions with yields (expressed on dry weight basis) of about 1.5 and 93.5% of LRSC-CAE, respectively. Further, extraction of CAE-AF with butanol yielded the butanol-soluble (CAE-BF) and aqueous (CAE-AF2) fractions with yields of about 2.2 and 90.1%, respectively. The organic solvent fractions were further analysed using GC-MS and the results (TIC) were presented in Appendix E (p. 325). Regarding their chemical composition, CAE-EAF contained mainly of long-chain hydrocarbons and fatty acids (Table 5.1) whereas CAE-BF was abundant with butyl esters of fatty acids (Table 5.2). Compounds detected by GC-MS analysis (volatile chemical constituents), however, accounted for only 20% of each fraction.

The aqueous fractions, CAE-AF and CAE-AF2, were profiled using SDS-PAGE and SELDI-TOF. Interestingly, the protein profiles of both fractions, in general, were found to be similar to that of the crude extract, LRSC-CAE (Figure 5.23A) suggesting that proteins were not affected during the liquid-liquid partition step. Nevertheless, there were slight differences in the intensity of some peaks in SELDI-TOF-MS profiles of the fractions (Figure 5.23B).

Table 5.1: Chemical constituents in CAE-EAF

Rt (min)	Compounds	Molecular formula	Molecular weight	Area (%)
6.24	2-Dodecene, (Z)-	$C_{12}H_{24}$	168.3190	0.45
8.85	2-Tetradecene	$C_{14}H_{28}$	196.3721	0.75
10.19	Pentadecane	$C_{15}H_{32}$	212.4146	0.30
11.30	Cetene	$C_{16}H_{32}$	224.4253	1.56
11.90	Benzophenone	$C_{13}H_{10}O$	182.2179	1.23
13.22	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.3709	0.85
13.53	1-Octadecene	$C_{18}H_{36}$	252.4784	1.30
15.30	*n*-Hexadecanoic acid (palmitic acid)	$C_{16}H_{32}O_2$	256.4241	4.30
15.49	1-Nonadecene	$C_{19}H_{38}$	266.5050	2.74
16.96	Cis-Vaccenic acid	$C_{18}H_{34}O_2$	282.4614	3.95

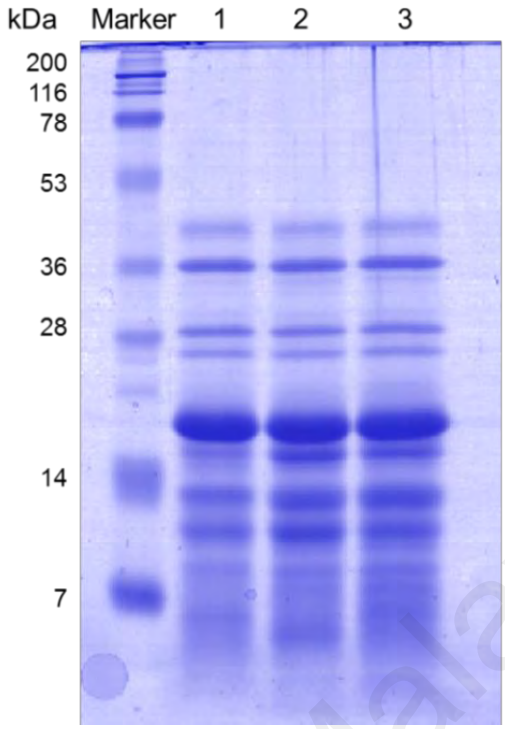
Area (%) was determined based on the TIC of CAE-EAF (Appendix E, p. 324). Identification of the compounds was based on mass spectral analysis. CAE-EAF, ethyl acetate fraction of LRSC-CAE; Rt, retention time; ND, not detected.

Table 5.2: Chemical constituents in CAE-BF

Rt (min)	Compounds	Molecular formula	Molecular weight	Area (%)
4.53	4-Heptanone, 3-methyl-	$C_8H_{16}O$	128.2120	6.15
4.88	Butanoic acid, butyl ester	$C_8H_{16}O_2$	144.2114	13.90
17.34	Hexadecanoic acid, butyl ester	$C_{20}H_{40}O_2$	312.5304	1.05
18.91	9-Octadecenamide, (Z)- (oleamide)	$C_{18}H_{35}NO$	281.4766	0.74
19.04	Octadecanoic acid, butyl ester	$C_{22}H_{44}O_2$	340.5836	1.18

Area (%) was determined based on the TIC of CAE-BF (Appendix E, p. 324). Identification of the compounds was based on mass spectral analysis. CAE-BF, butanol fraction of LRSC-CAE; Rt, retention time; ND, not detected.

(A)



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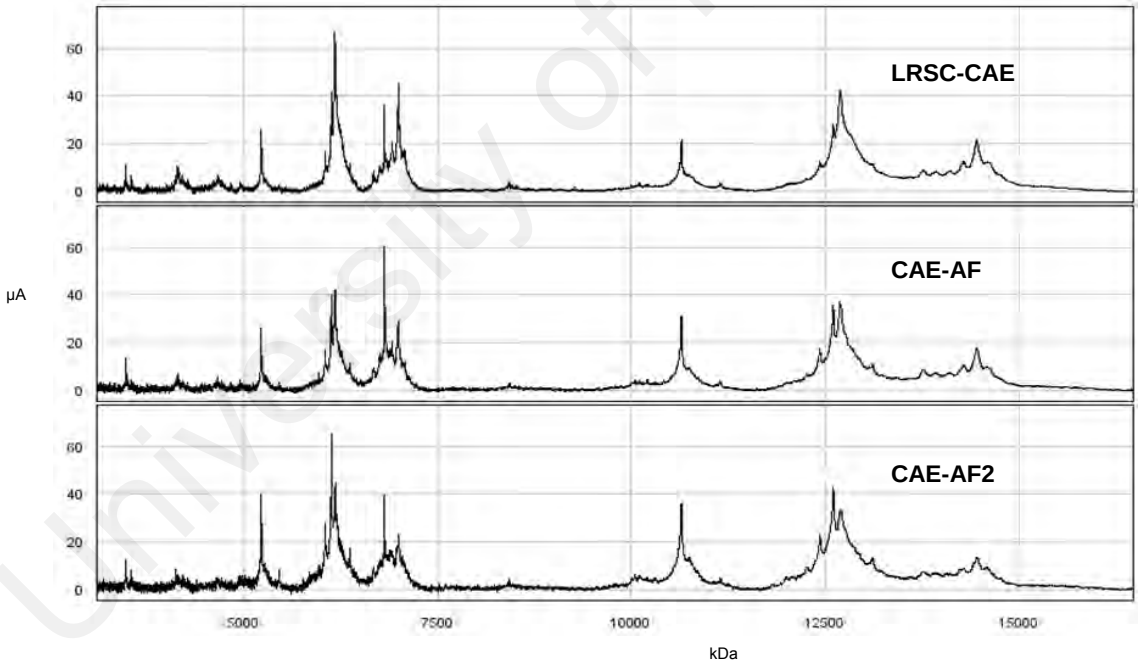


Figure 5.23: Protein profiles of LRSC-CAE and aqueous fractions. Both aqueous fractions, CAE-AF and CAE-AF2, were derived from liquid-liquid partition of LRSC-CAE with ethyl acetate and butanol, respectively. (A) SDS-PAGE profiles of LRSC-CAE and the aqueous fractions. Lane 1 = LRSC-CAE, lane 2 = CAE-AF, lane 3 = CAE-AF2. (B) Representative SELDI-TOF-MS spectra of LRSC-CAE and the aqueous fractions (2-18 kDa). Protein profiles of the all samples were found to be comparable.

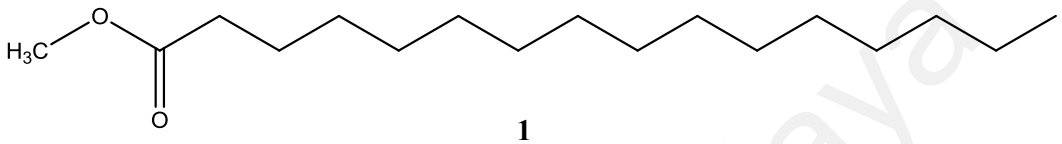
The fractions derived from liquid-liquid partition of LRSC-CAE with organic solvents were screened for cytotoxic activity against MCF7 and HCT 116 using the MTT assay. Results of the MTT assay were summarized in Table 5.3. For MCF7, we observed that both aqueous fractions (CAE-AF, IC_{50}: 33.3 µg/ml; CAE-AF2, IC_{50}: 33.4 µg/ml) demonstrated cytotoxicity that was somewhat comparable to that of the crude extract (LRSC-CAE, IC_{50}: 34.3 µg/ml). Similar trend was observed when HCT 116 was treated with LRSC-CAE (IC_{50}: 26.2 µg/ml) and its fractions, CAE-AF (IC_{50}: 26.4 µg/ml) and CAE-AF2 (IC_{50}: 25.4 µg/ml). Amongst, the organic solvent fractions, CAE-EAF displayed moderate cytotoxicity against MFC7 (IC_{50}: 58.7 µg/ml) and HCT 116 (IC_{50}: 80.1 µg/ml). CAE-BF, on the other hand, was inactive (IC_{50} > 200 µg/ml).

Table 5.3: Cytotoxic activity of LRSC-CAE and its fractions

Extract and fractions	IC_{50} (µg/ml)		
	MCF7	**HCT 116**	**MRC-5**
LRSC-CAE	34.3 ± 0.78	26.2 ± 1.22	53.5 ± 4.30
CAE-EAF	58.7 ± 3.73	80.1 ± 5.80	72.0 ± 9.13
CAE-BF	> 200	> 200	> 200
CAE-AF	33.3 ± 0.84	26.4 ± 1.17	48.6 ± 3.85
CAE-AF2	33.4 ± 1.90	25.4 ± 1.52	44.0 ± 1.82

Cell viability post treatment with the mushroom extracts and fractions for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3). LRSC-CAE, *L. rhinocerotis* sclerotial cold aqueous extract; CAE-EAF, CAE-BF, CAE-AF, and CAE-AF2 are fractions derived from LRSC-CAE after liquid-liquid partition; CAE-EAF, ethyl acetate fraction; CAE-BF, butanol fraction; CAE-AF, aqueous fraction from liquid-liquid partitioning between LRSC-CAE and ethyl acetate; CAE-AF2, aqueous fraction from liquid-liquid partitioning between CAE-AF and butanol.

In addition, palmitic acid (**1**) and oleamide (**2**) (Figure 5.24) identified from the GC-MS analysis of the organic solvent fractions were selected (based on their abundancy in the extracts and availability from commercial suppliers) and tested for cytotoxicity. Table 5.4 shows the results of the MTT assay. Both compounds, in general, exhibited weak cytotoxic activity (IC_{50} > 100 μM) against MCF7 and HCT 116.



1

2

Figure 5.24: Chemical structures of compounds **1** and **2**

Table 5.4: Cytotoxic activity of chemical constituents in CAE-EAF and CAE-BF

Compounds	IC_{50} (μM)		
	MCF7	**HCT 116**	**MRC-5**
Palmitic acid (**1**)	260.1 ± 30.0 (66.7 ± 7.68 μg/ml)	102.1 ± 2.93 (26.2 ± 0.75 μg/ml)	125.9 ± 6.32 (32.3 ± 1.62 μg/ml)
Oleamide (**2**)	120.7 ± 3.28 (34.0 ± 0.94 μg/ml)	113.7 ± 4.14 (32.0 ±1.16 μg/ml)	135.5 ± 8.29 (38.2 ± 2.33 μg/ml)

Cell viability post treatment with the compounds for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

Cytotoxicity was retained in the aqueous fractions (rather than the organic fractions), and the potency of CAE-AF was comparable to that of CAE-AF2; hence, CAE-AF was subjected to ammonium sulfate and ethanol precipitations separately. CAE-P was obtained from CAE-AF by salting out using ammonium sulfate whereas ethanol precipitation of CAE-AF gave CAE-PO. Total carbohydrate and protein contents of both fractions were determined. CAE-CP comprised of approximately 11% (w/w) carbohydrates and 58% (w/w) proteins. CAE-CPO contained about 60% (w/w) carbohydrates and 15% (w/w) proteins.

Cytotoxicity of the fractions was then examined using the MTT assay. As shown in Table 5.5, only CAE-P exhibited cytotoxicity against MCF7, HCT 116, and MRC-5. This indicated that the cytotoxic components in CAE-AF were retained in CAE-CP rather than CAE-PO, and ammonium sulfate precipitation was more effective in recovering the cytotoxic components in CAE-AF that was very likely to be proteins.

Table 5.5: Cytotoxic activity of CAE-P and CAE-PO

Fractions	IC_{50} (μg/ml)		
	MCF7	**HCT 116**	**MRC-5**
CAE-P	14.9 ± 1.37	19.6 ± 1.36	29.5 ± 1.75
CAE-PO	> 100	> 100	> 100

Cell viability post treatment with the fractions for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3). CAE-P and CAE-PO were obtained from CAE-AF (aqueous fraction) by salting out using ammonium sulfate and ethanol precipitation, respectively.

In summary, chemical fractionation coupled the MTT assay demonstrated that the major cytotoxic components in LRSC-CAE were heat-labile and proteinaceous in nature. Additionally, LRSC-CAE also contained very small amount of cytotoxic low-

molecular-weight compounds that were separated into the organic solvent fractions during liquid-liquid partition.

5.3.5 Chemical nature of cytotoxic component(s) in LRCP-DE

To explore the nature of the cytotoxic compounds present in the dichloromethane extracts of *L. rhinocerotis*, GC-MS and LC-MS profiling of LRCP-DE (dichloromethane extract of cultivated pileus) and LRWP-DE (dichloromethane extract of wild type pileus) were performed. The inclusion of LRWP-DE allows a comparative analysis of the chemical profiles of cultivated and wild type samples of *L. rhinocerotis.*

5.3.5.1 Identification of chemical constituents by GC-MS

LRCP-DE and LRWP-DE were subjected to GC-MS. The chemical profiles, in the form of TIC, are shown in Appendix F (p. 326). The profiles of both extracts did not differ much from one another. Table 5.6 shows the volatiles chemical constituents in LRCP-DE and LRWP-DE identified using GC-MS. Lipophilic constituents in both extracts belonged to the classes of aliphatic hydrocarbons, fatty acid methyl esters, and steroids.

5.3.5.2 Identification of chemical constituents by LC-MS

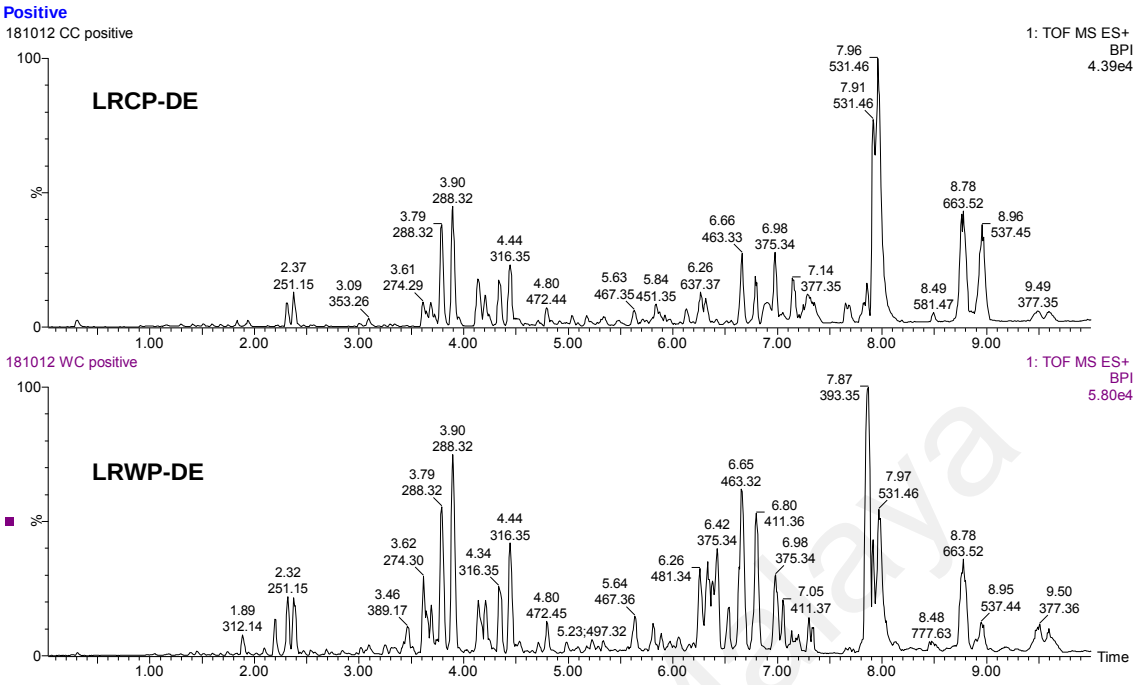
In the course of LC-MS profiling, a molecular ion, $[M+H]^+$ at *m*/*z* 393, consistent with the molecular formula of $C_{28}H_{40}O$, was observed in the TIC (positive ion mode) of LRCP-DE and LRWP-DE (Figure 5.25). It produced major MS/MS fragments at *m*/*z* 268 $[M+H\text{-}C_9H_{17}]^+$, 253 $[M+H\text{-}C_9H_{17}\text{-}CH_3]^+$, 224, and 209. On the basis of its mass fragmentation patterns, it was identified as ergosta-4,6,8(14),22-tetraen-3-one or ergone (**3**), which was later confirmed with an authentic standard (Figure 5.26 and 5.27).

Table 5.6: Chemical constituents in LRCP-DE and LRWP-DE

Rt (min)	Compounds	Molecular formula	Molecular weight	Area (%)	
				LRCP-DE	LRWP-DE
12.47	Heptadecane	$C_{17}H_{36}$	240.4677	0.42	0.56
12.76	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242.3975	0.41	N.D.
13.83	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256.4241	0.88	0.53
14.64	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	268.4348	0.68	0.57
14.85	Hexadecanoic acid, methyl ester (methyl palmitate)	$C_{17}H_{34}O_2$	270.4507	14.39	7.97
15.82	Hexadecanoid acid, 14-methyl-, methyl ester	$C_{18}H_{36}O_2$	284.4772	0.42	0.22
16.50	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (methyl linoleate)	$C_{19}H_{34}O_2$	294.4721	19.11	13.84
16.75	Octadecanoic acid, methyl ester (methyl stearate)	$C_{19}H_{38}O_2$	298.5038	3.63	2.72
18.86	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281.4766	0.49	N.D.
20.12	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354.6101	0.92	1.11
20.89	Tricosanoic acid, methyl ester	$C_{24}H_{48}O_2$	368.6367	0.32	0.51
21.62	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382.6633	2.28	2.42
25.71	5,6-Dihydroergosterol	$C_{28}H_{46}O$	398.6642	36.94	36.94
26.04	7,22-Ergostadienone	$C_{28}H_{44}O$	396.6481	8.71	8.45

Area (%) was determined based on the TIC of LRCP-DE and LRWP-DE (Appendix F, p. 325). Identification of the compounds was based on mass spectral analysis. LRCP-DE, dichloromethane extracts of cultivated pileus; LRWP-DE, dichloromethane extract of wild type pileus; Rt, retention time; N.D., not detected.

(A)



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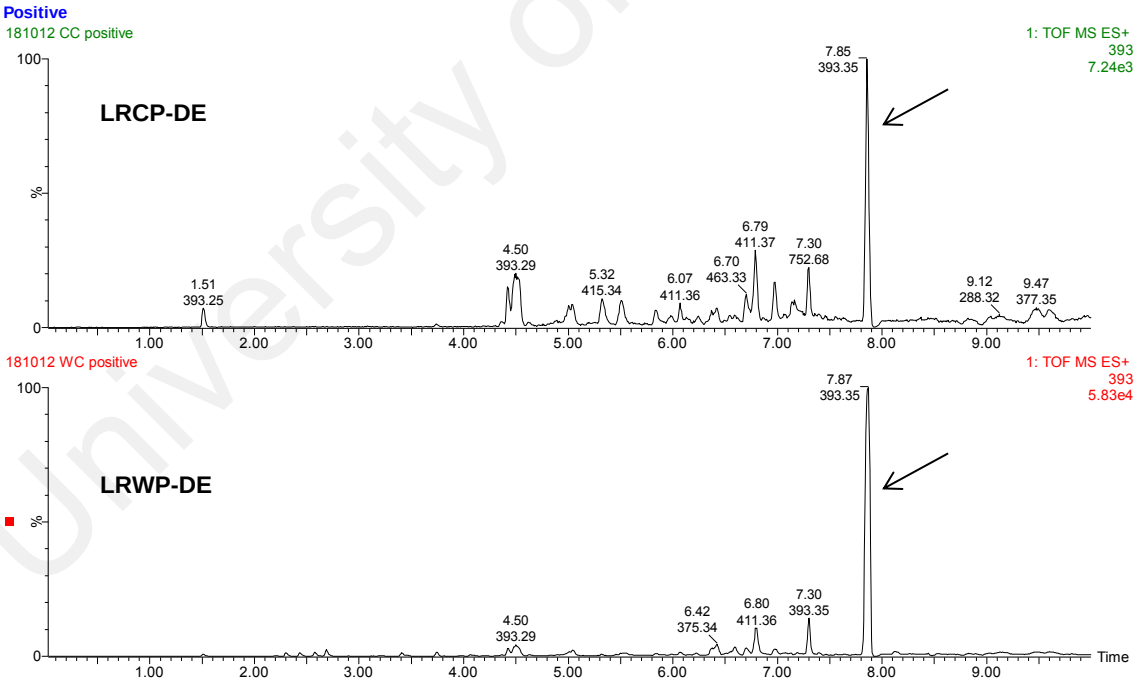


Figure 5.25: MS chromatogram of LRCP-DE and LRWP-DE. (A) TIC profiles of the extracts recorded in positive ionization mode. Ergone (**3**) (Rt = 7.8 min) produced molecular ion at *m*/*z* 393. (B) Selective ion monitoring of the molecular ion at *m*/*z* 393 (indicated by arrow) in both extracts. LRCP-DE, dichloromethane extracts of cultivated pileus; LRWP-DE, dichloromethane extract of wild type pileus.

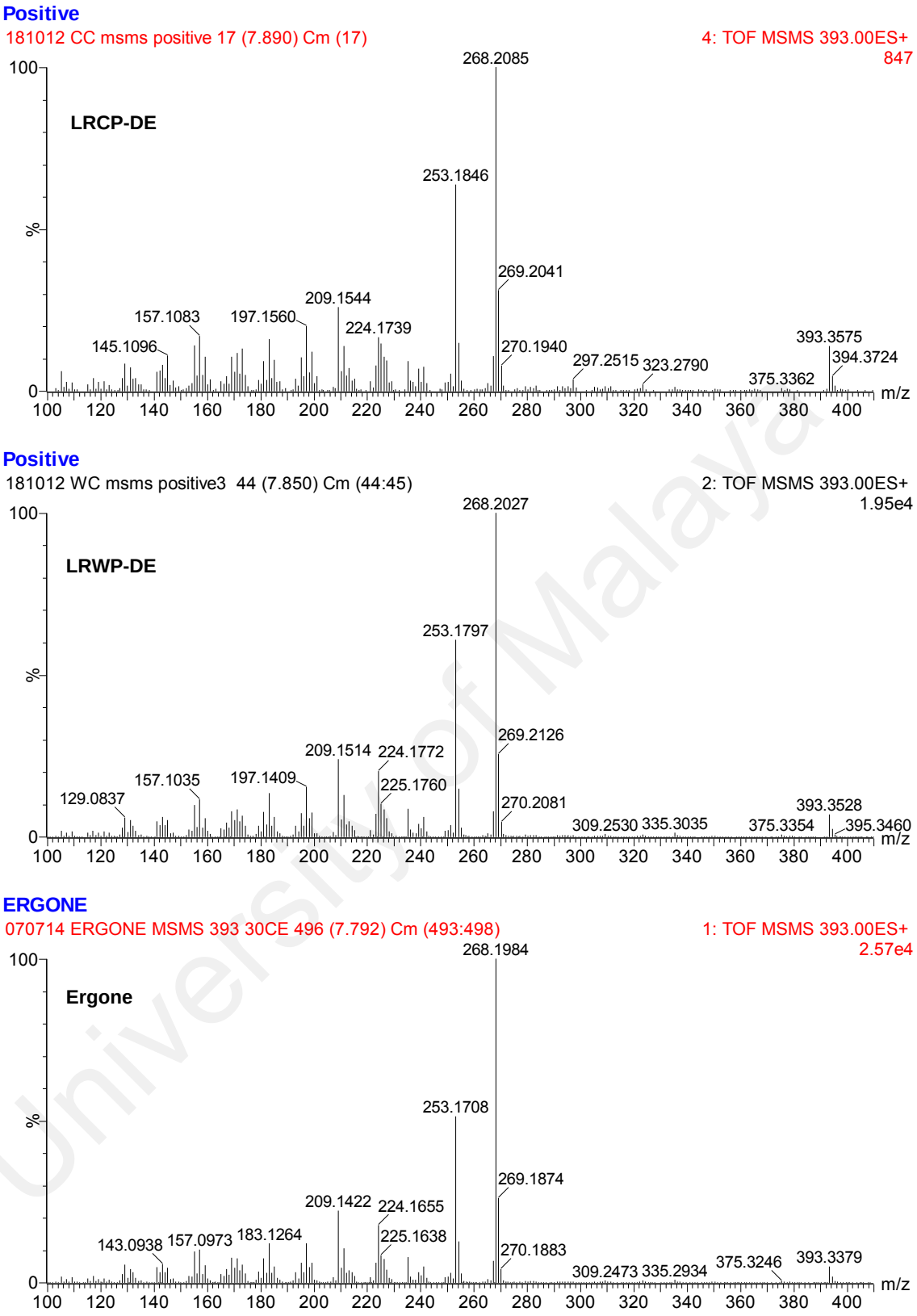
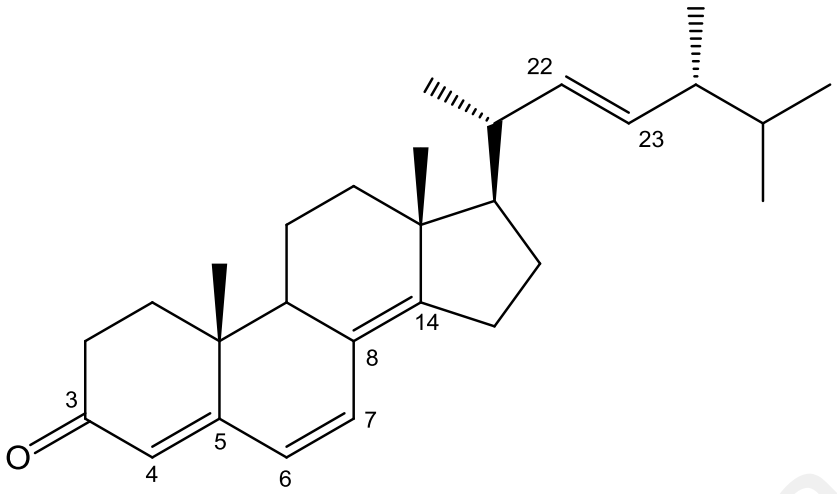


Figure 5.26: Comparison of MS/MS fragmentation patterns of ergone (**3**) (authentic compound) and the molecular ion at *m/z* 393 that were present in LRCP-DE and LRWP-DE. The MS/MS fragmentations of the molecular ion at m/z 393 in the extracts were observed to identical to the authentic standard, confirming the presence of ergone (**3**) in both extracts.



3

Figure 5.27: Chemical structure of compound **3**

5.3.5.3 Cytotoxic activity of selected chemical constituents

To ascertain the cytotoxic activity of the lipophilic constituents, selected chemical constituents identified from GC-MS and UPLC-ESI-MS analysis were obtained from commercial sources. Methyl esters, including methyl palmitate (**4**) (14.4%), methyl linoleate (**5**) (19.1%), and methyl stearate (**6**) (3.6%) were identified to be the major volatile chemical constituents in LRCP-DE. Ergone (**3**) was previously reported to be cytotoxic. The chemical structures of the fatty acid methyl esters are shown in Figure 5.28.

Cytotoxic activity of ergone (**3**), methyl palmitate (**4**), methyl linoleate (**5**), and methyl stearate (**6**) against MCF7, HCT 116, and MRC-5 was evaluated using the MTT assay. Results of the cytotoxicity evaluation of the compounds were presented in Table 5.7. Our results demonstrated that amongst the tested compounds, only ergone (**3**) exhibited cytotoxicity with IC_{50} (μM) values of 51.6, 23.1 and 49.5 against MCF7, HCT 116, and MRC-5, respectively. Methyl esters of fatty acids (**4**-**6**) were non-cytotoxic within the tested concentrations (IC_{50} > 350 μM).

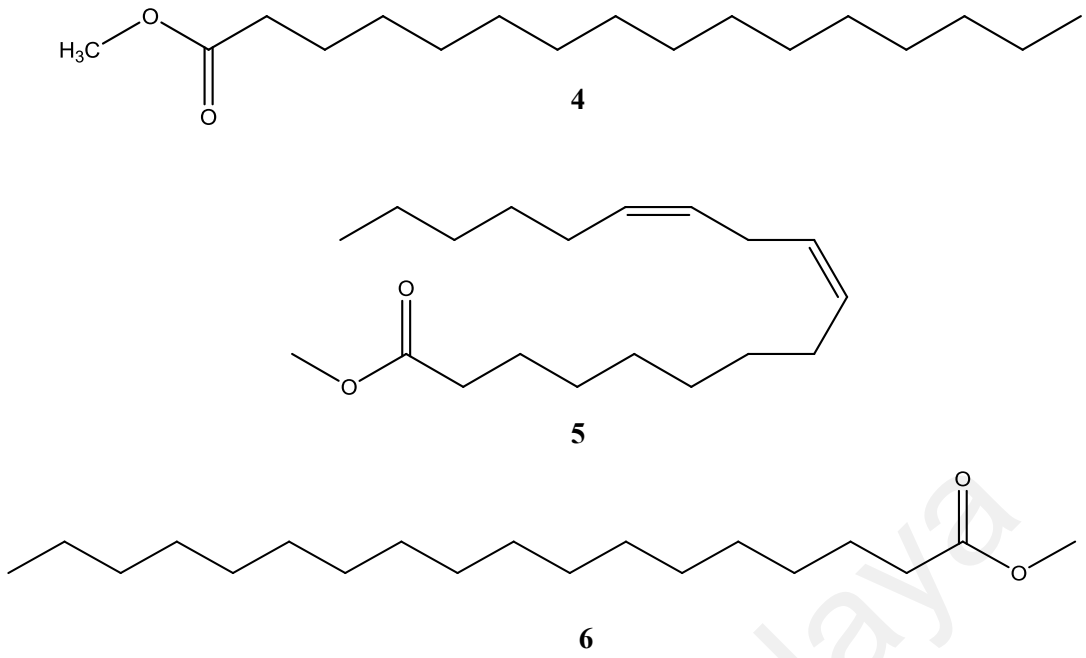


Figure 5.28: Chemical structures of compounds **4**-**6**.

Table 5.7: Cytotoxic activity of chemical constituents in LRCP-DE

Compounds	IC_{50} (μM)		
	MCF7	**HCT 116**	**MRC-5**
Ergone (**3**)	51.6 ± 2.45 (15.4 ±0.73 μg/ml)	23.1 ± 0.54 (6.9 ± 0.16 μg/ml)	49.5 ± 2.20 (14.8 ± 0.66 μg/ml)
Methyl palmitate (**4**)	> 350	> 350	> 350
Methyl linoleate (**5**)	> 350	> 350	> 350
Methyl stearate (**6**)	> 350	> 350	> 350

Cell viability post treatment with the compounds for 72 h was determined using the MTT assay. Results were expressed as mean ± S.E. (n = 3).

Chemical profiling showed that the cultivated and wild type pileus of *L. rhinocerotis* have comparable lipophilic chemical constituents based on LC-MS (Chapter 4) and GC-MS analyses. In addressing the bioactivity of LRCP-DE, our results seemed to indicate that ergone (**3**) and other unidentified chemical constituents, rather of the abundant methyl esters, were the responsible for the potent cytotoxic effect of LRCP-DE.

5.4 Discussion

5.4.1 *Lignosus rhinocerotis* extracts inhibited growth, induced cell cycle arrest, and apoptotic cell death in MCF7 and HCT 116

The balance between cell proliferation and death are important in the maintenance of homeostasis in normal cells; however, homeostasis is often disrupted in cancer cells due to the deregulation of cell cycle mechanisms. It is generally accepted that induction of cell cycle arrest and apoptosis are good strategies in treating cancer; hence, studies were carried to identify extracts and compounds from mushrooms that target both processes. Indeed, many mushrooms have been reported to kill cancer cells by inducing cell cycle arrest and apoptotic cell death (as reviewed by Fereira *et al*., 2010; Patel & Goyal, 2012).

The degree of cytotoxicity of a particular extract or compound can vary depending on the assays used to estimate it; therefore, the choice of appropriate cell viability assay is important (Sumantran, 2011). The MTT assay, which measures the mitochondrial function, was used in large-scale cytotoxicity screening (Chapter 4). In the present study, two additional assays were employed to assess the growth-inhibitory effect of two active crude extracts, namely LRSC-CAE (cold aqueous extract of sclerotium) and LRCP-DE (dichloromethane extract of pileus). The SRB assay measures the total cell protein based on the formation of electrostatic complex between SRB (an anionic aminoxanthene dye) with basic amino acid residues of proteins. Cell counting by the trypan blue exclusion (TBE) method is still being used as a confirmatory test for quantitating changes in the number of viable cells. Our results from the SRB and TBE assay further substantiated results from the MTT assay as reported earlier (Chapter 4). The stronger growth inhibitory effect of LRSC-CAE and LRCP-DE, as compared to

LRSC-HAE (hot aqueous extract of sclerotium) and LRCP-ME (methanol extract of pileus), respectively, was confirmed.

To investigate if the growth inhibitory effect of LRSC-CAE and LRCP-DE was related to cell cycle arrest and/or apoptosis, the cell cycle distribution post treatment with the extracts were analysed using flow cytometry. Our results showed that there was an increase in the percentage of G1 phase in both MCF7 and HCT 116 following the treatment with LRSC-CAE and LRCP-DE, suggesting that the cells were arrested at G1/S transition. These observations implied that LRSC-CAE and LRCP-DE induced G1 arrest that will decelerate the cell cycle and prevent the cells from entering the S phase and proliferating as reported by Wang *et al*. (2014a). There was also a concurrent increase in the sub-G1 population during the treatment period. As the sub-G1 peak represented cells with hypodiploid DNA content and regarded as the apoptotic population, it can be inferred that apoptosis occurred through G1 arrest in both MCF7 and HCT 116. The higher percentage of sub-G1 cells indicates a greater number of apoptotic cells.

Thus far, only the sclerotial hot aqueous extract of *L. rhinocerotis* has been reported to induce cell cycle arrest in HL-60 and K562 leukemic cell lines. According to Lai *et al*. (2008), treatment with the sclerotial hot aqueous extract (200 μg/ml) caused an increase in the percentage of cells in sub-G1 (33.9-73.8%) and G1 (53.9-89.3%) phases in HL-60 when incubation was prolonged from 24-72 h. On the other hand, the extract did not affect the cell cycle progression in K562 cells during the incubation period (24-72 h) but the population of sub-G1 cells was observed to be consistently higher than that of untreated cells. While LRSC-CAE (cold aqueous extract) and LRCP-DE (dichloromethane extract) also induced cell cycle arrest in MCF7 and HCT 116 at G1 phase, the active components in both extracts are likely to be different than that of the

extract used by Lai *et al*. (2008). Reports on similar mechanism of action (with regard to cell cycle arrest) by different extracts of one mushroom are available; for instance, it was documented that the aqueous and ethanol extracts of *Inonotus obliquus* were reported to induce G0/G1 arrest in Hep G2 (hepatoma) (Youn *et al*., 2008), B16-F10 (melanoma) (Youn *et al*., 2009), and HT-29 (colorectal) (Lee *et al*., 2015) but the underlying molecular mechanisms of action were not entirely identical.

To ascertain that treatment with LRSC-CAE and LRCP-DE indeed caused apoptosis, an alternative was adopted whereby MCF7 and HCT 116 cells were stained with annexin V and PI followed by flow cytometric analysis. The use of annexin V/PI dual staining method allows analysis of the externalization of phosphatidylserine from the inner to the outer leaflet of membranes during the early phase of apoptosis and hence, this can be used to quantitate apoptosis. Our results demonstrated that LRCP-DE induced apoptosis in MCF7 and HCT 116. This was inferred based on the increment of early and late apoptotic cells compared to the untreated controls. Furthermore, during the late stage of apoptosis, endonucleases will cleave the double-stranded DNA at the accessible internucleosomal linker region and thus, generates nucleosomes, resulting in fragments of DNA strand breaks. DNA fragmentation is considered to be another hallmark of apoptosis. The apoptosis-inducing activity of LRSC-CAE and LRCP-DE was further analysed in another assay with different readouts whereby the lysates of MCF7 and HCT 116 cells treated with the extracts were evaluated for the content of apoptotic oligonucleosomes by a colourimetric ELISA. Our results showed a time-dependent increase of DNA fragmentation (represented by apoptotic index) in both cells lines after treatment with the extracts. The accumulation of nucleosomes in the cytoplasm of the cells following treatment with the extracts suggested that apoptotic cell death took place. This observation was in accordance with the findings of Lee *et al*.

(2012) who observed the presence of DNA ladder fragments on the MCF7 cells treated with *L. rhinocerus* cultivar TM02 sclerotial cold aqueous extract, indicating that cell death was mediated by apoptosis.

Taken together, our findings from the flow cytometric analysis were in agreement with results from different cell viability assays (i.e. MTT, SRB, and TBE). The growth-inhibitory effect of LRSC-CAE and LRCP-DE appeared to be explained in part by the induction of cell cycle arrest and apoptosis.

5.4.2 Chemical nature of cytotoxic components in LRSC-CAE

The differential cytotoxic activity of LRSC-HAE (hot aqueous extract of sclerotium) and LRSC-CAE (cold aqueous extract of sclerotium) was suspected to be attributed to certain water-soluble chemical components that are heat-labile. These chemical components might have been degraded or heat-inactivated during the boiling process and hence, LRSC-HAE was inactive. Loss of cytotoxicity of the heat-treated LRSC-CAE seemed to suggest that some heat-labile components were indeed responsible for the observed cytotoxic effect. At the same time, depletion and/or disappearance of protein bands and protein peaks in SDS-PAGE and SELDI-TOF-MS, respectively, were also observed when LRSC-CAE was heated. This is more likely to indicate proteins as the cytotoxic components since heat denaturation of proteins typically begin at temperature higher than 60 °C. Based on the results of the MTT assay, the cytotoxic component(s) in *L. rhinocerotis* sclerotium were stable up to 60 °C. Based on the combined results of cytotoxicity evaluation and protein profiling, it is feasible to speculate that some heat-labile protein/peptide(s) were the active cytotoxic component(s) in LRSC-CAE. Differences in protein profiles of LRSC-HAE and LRSC-CAE, especially the lack of bands in higher-molecular-mass region (> 20 kDa) of the SDS-

PAGE profile of LRSC-HAE, further strengthen our hypothesis that proteins might play a major role in the cytotoxic effect of LRSC-CAE.

Chemical profiling of LRSC-HAE, LRSC-CAE, and heat-treated LRSC-CAE has provided insights into the nature of potential cytotoxic proteins/peptides from the sclerotia of *L. rhinocerotis*, which could be either of high-molecular-weight, low-molecular-weight or combination of both. Firstly, LRSC-CAE was cytotoxic but LRSC-HAE was not. In the SDS-PAGE profiles, there were several high-molecular-weight proteins (> 20 kDa) that were present only in LRSC-CAE; hence, cytotoxicity of LRSC-CAE might be attributed to the aforementioned proteins. On the other hand, loss of cytotoxicity of the heat-treated LRSC-CAE was also accompanied by changes in some low-molecular-weight proteins as evidenced by the SDS-PAGE and SELDI-TOF-MS profiles. As a result, the possibility of low-molecular-weight proteins exhibiting cytotoxicity cannot be excluded. At the moment, it is still unclear if the cytotoxicity was contributed by a single of mixture of both high- and low-molecular-weight compounds.

Results from chemical fractionation of LRSC-CAE have shown that the cytotoxic components remained in the aqueous fractions (i.e. CAE-AF and CAE-AF2) following successive partition with ethyl acetate and butanol. During liquid-liquid partition, some of the low-molecular-weight compounds were extracted into the ethyl acetate and butanol fractions. Between both fractions, only the ethyl acetate fraction demonstrated moderate cytotoxicity against MCF7 (IC_{50}: 58.7 μg/ml) and HCT 116 (IC_{50}: 80.1 μg/ml), whereas the butanol fraction was inactive (IC_{50} > 200 μg/ml) against both cell lines.

Further, two different approaches (i.e. adjusting salt saturation and concentration of alcohol) were used to precipitate the water-soluble components in CAE-AF. Both precipitation techniques are commonly used to recover mainly proteins and

polysaccharides. In the case of *T. versicolor*, two different high-molecular-weight components were recovered from the hot aqueous extract using the above methods. From the hot aqueous extract of the mycelium, a form of polysaccharide-peptide known as krestin (PSK) was obtained by salting out with ammonium sulfate whereas the polysaccharopeptide (polysaccharide-peptide) (PSP) was extracted by alcoholic precipitation. Although their chemical composition varied, both PSK and PSP were demonstrated to have anti-tumour effect (Cheng & Leung, 2008; Fereira *et al*., 2010). In our study, CAE-P and CAE-PO were produced by salting out using ammonium sulfate and ethanol precipitation, respectively. In terms of chemical composition, both fractions have different proportions of carbohydrates and proteins but only CAE-P exhibited cytotoxicity against MCF7 (IC_{50}: 14.9 μg/ml) and HCT 116 (IC_{50}: 19.6 μg/ml). The fact that the IC_{50} values of CAE-P were approximately 1-fold lower than that of the crude extract, LRSC-CAE (MCF7, IC_{50}: 34.3 μg/ml; HCT 116, IC_{50}: 26.2 μg/ml), seemed to suggest that proteins were likely the active components in the sclerotium of *L. rhinocerotis*.

Our postulation regarding the presence of cytotoxic proteins in *L. rhinocerotis* is supported by a previous report by Abdullah *et al*. (2010) who found that mycelial protein fractions of *L. rhinocerotis* exerted cytotoxicity against cervical cancer carcinoma cells (Ca Ski) and inhibitory effect on human papillomavirus (anti-HPV). Furthermore, several types of proteins from other medicinal mushrooms, mainly lectins, have been shown to exert anti-cancer effects (Wang *et al*., 1998; Hassan *et al*., 2015; Singh *et al*., 2015). In a report by Rouf *et al*. (2014), a lectin purified from the fruiting body of *Psathyrella asperospora* (Cleland) Guzmán, Bandala & Montoya (termed PAL, 41.8 kDa) exhibited anti-proliferative activity and induced cell cycle arrest at G2/M phase in HT-29. PAL was reported to be stable at temperatures up to 55 °C. Another

lectin isolated from *Pholiota nameko* (T. Ito) S. Ito & S. Imai (termed PNAP, 18.5 kDa) was also thermal stable up to 60 °C and it induced apoptotic cell death in MCF7 that was associated with the accumulation of sub-G1 cells (Zhang *et al*., 2014a). In a recent genome-based proteomic analysis of the sclerotium of *L. rhinocerotis,* 16 putative lectins were identified and these were the major protein components in the sclerotium (Yap *et al*., 2015). Therefore, it is feasible to suggest that sclerotial proteins (presumably lectins due to their abundance), at least to some extent, contribute to the cytotoxic and apoptosis-inducing activities of LRSC-CAE. Further investigation is needed to identify, isolate, and characterize the cytotoxic proteinaceous component in the sclerotium.

5.4.3 Chemical nature of cytotoxic components in LRCP-DE

While mushroom aqueous extracts contain mostly mainly high-molecular-weight, hydrophilic components, such as polysaccharides and proteins, organic solvent extracts are likely to be made up of a vast range of low-molecular-weight compounds with varying polarity, belonging to the either primary or secondary metabolites. As only dichloromethane extracts were found to be active in the assays, this observation seemed to suggest that the cytotoxicity of *L. rhinocerotis* were mainly attributed to the lipophilic constituents. On the other hand, LRCP-ME failed to evoke any biological responses and this implied that the relatively polar chemical constituents in LRCP-ME were devoid of cytotoxicity and hence, it will not be investigated further in the present study.

From GC-MS and LC-MS profiling, we identified some of the chemical constituents in LRCP-DE. Selected compounds were further tested for cytotoxicity. Ergone (**3**), methyl palmitate (**4**), methyl linoleate (**5**), and methyl stearate (**6**), have been previously

identified in other edible-medicinal mushrooms. Ergone (**3**) is a metabolite that is derived from ergosterol and has been isolated from several mushrooms (Lee *et al*., 2005; Lee *et al*. 2007; Shen *et al*., 2008). Fatty acid methyl esters (**4**-**6**), on the other hand, made up a substantial amount of the extracts of *L. rhinocerotis* were also typically identified in other mushrooms (Abdul Malek *et al*., 2010; Kanagasabapathy *et al*., 2011; Mohamed, 2012). Selection of compounds (**3**-**6**) was due to their abundance in LRCP-DE and availability from commercial sources.

Amongst all compounds, only ergone (**3**) demonstrated cytotoxicity against MCF7 (IC_{50}: 51.6 μM) and HCT 116 (IC_{50}: 23.1 μM). Our results was in accordance with previous studies whereby it was reported to be cytotoxic against several cell lines, such as Hep 3B (liver cancer, IC_{50}: 12.7 μM), HT-29 (colon cancer, IC_{50}: 18.4 μM), Hela 229 (cervical cancer, IC_{50}: 67.0 μM), and AGS (stomach cancer, IC_{50}: 56.1 μM) (Lee *et al*. 2005). Mechanistic studies demonstrated that ergone induced cell cycle arrest and apoptosis in Hep G2 (liver) cancer cells (Zhao *et al*., 2011a). Hence, ergone is postulated to contribute, at least to some extent, to the growth-inhibitory effect of LRCP-DE on cancer cell lines.

Regarding the cytotoxicity of methyl esters of fatty acids, Takeara *et al*. (2008) found that methyl palmitate exhibited cytotoxicity against T-cell leukemia cells (Molt-4, IC_{50}: 22.8 μg/ml). In addition, methyl stearate also inhibited the growth of HL-60 (IC_{50}: 3.1 μg/ml) and Molt-4 (IC_{50}: 4.7 μg/ml). The difference results obtained in the present study might be due to variation in the cellular response of MCF7 and HCT 116 (solid tumours) compared to the leukemic cell lines used in the previous study by Takeara *et al*. (2008).

5.5 Conclusion

The nature of cytotoxic compounds in the extracts of *L. rhinocerotis* from different developmental stages (pileus vs. sclerotium) and prepared using different methods (cold aqueous vs. dichloromethane extractions) was investigated. Cytotoxic components in LRSC-CAE (cold aqueous extract of sclerotium) might include thermo-labile, water-soluble, cytotoxic protein/peptide(s). On the other hand, LRCP-DE (dichloromethane extract of pileus) contained low-molecular-weight compounds belonging to the groups of hydrocarbons, fatty acid methyl esters, and steroids but thus far, only ergone (**3**) was found to be cytotoxic. Despite the differences in chemical composition, both LRSC-CAE and LRCP-DE inhibited cancer cell growth by inducing cell cycle arrest at G1 phase and triggered apoptotic cell death. In view of the higher potency of LRCP-DE (lower IC_{50} values) than LRSC-CAE and the absence of detailed chemical studies on the secondary metabolites of *L. rhinocerotis*, LRCP-DE was prioritized for further investigation with the aims to identify, isolate, and characterize the cytotoxic compounds.

CHAPTER 6: ISOLATION AND CHARACTERIZATION OF CYTOTOXIC COMPOUNDS FROM *LIGNOSUS RHINOCEROTIS*

6.1 Introduction

Natural products represent an essential pool of unexplored chemical diversity for drug discovery as these are considered as evolved, privileged structures with greater likelihood to exhibit specific bioactivities of interest than many synthetic compounds (Crawford *et al*., 2011). It is estimated that approximately 50% of cancer therapeutics approved to date are either natural products or derivatives thereof (Newman & Cragg, 2012). Notable examples include taxol (microtubule inhibitor) from the bark of *Taxus brevifolia* Nutt. (Pacific yew tree) and mitomycin C (alkylating agent) from the culture filtrate of *Streptomyces caespitosus* (Newmann & Cragg, 2007; Molinari, 2009; Kinghorn *et al*., 2012).

In the context of drug discovery, mushrooms represent a remarkable, yet largely untapped source of natural products. Mushrooms, like other saprophytic or soil-inhabiting microorganisms, are prolific producers of secondary metabolites as their mycelia, fruiting bodies, and sclerotia are exposed to a number of antagonistic microorganism and predators which necessitates the production of defensive chemical components. These secondary metabolites in mushrooms are often reported to have a wide range of biological activities including anti-cancer effects. The common classes of secondary metabolites found in mushrooms with reported anti-cancer effects are phenolics, isoflavones, quinones, catechols, amines, cerebrosides, sesquiterpenes, triterpenoids, steroids, and others (Ferreira *et al*., 2010).

Based on our previous results, the dichloromethane extracts of *L. rhinocerotis*, particularly that of the pileus (LRCP-DE), exhibited pronounced cytotoxic activity against a panel of cancer cell lines (i.e. HL-60, MCF7, MDA-MB-231, HCT 116, HSC2, HK1, PC3, Hep G2, and WRL 68) (Chapter 4). Further, LRCP-DE was demonstrated to inhibit the growth of MCF7 and HCT 116 by inducing cell cycle arrest at G1 phase and apoptotic cell death (Chapter 5). GC-MS analysis revealed that fatty acid methyl esters were the major volatile chemical components of LRCP-DE but these compounds showed weak cytotoxic activity (IC_{50} > 350 μM). To identity the cytotoxic compounds that are responsible for the growth-inhibitory effect of LRCP-DE, it was subjected to bioassay-guided fractionation.

The specific objectives of this study are:

1. To isolate the low-molecular-weight cytotoxic compound(s) from LRCP-DE using various chromatographic techniques
2. To elucidate the structure(s) of the isolated compound(s) using mass-spectrometric and spectroscopic techniques
3. To evaluate the cytotoxic activity of the isolated compound(s) against selected cancer and non-cancerous cell lines using the MTT assay

6.2 Materials and Methods

6.2.1 Preparation of extract

The powdered pileus of cultivated *L. rhinocerotis* was soaked in dichloromethane at a ratio of 1:20 (w/v) for 3 days. The extract was filtered and the residue was re-extracted with dichloromethane twice. Combined filtrate was dried under reduced pressure to give a brown extract (LRCP-DE).

6.2.2 Fractionation of LRCP-DE and compounds isolation

6.2.2.1 Liquid-liquid partition

LRCP-DE was dissolved in a mixture of methanol and water (1:2, v/v) and partitioned with hexane (1:1, v/v) three times to yield the hexane-soluble (LRCP-HF) and hexane-insoluble or aqueous (LRCP-AF) fractions. Excess solvents in both fractions were evaporated off under reduced pressure and LRCP-AF was freeze-dried.

6.2.2.2 Column chromatography

Silica gel of varying particle sizes (i.e. 70-230 and 230-400 mesh) was obtained from Jones Chromatography Ltd (Hengoed, UK). Solvents used were of analytical grade. Briefly, the silica gel was made into slurry with solvent before it was packed into a glass column and allowed to equilibrate for approximately an hour before use. Elution was performed using a combination of different organic solvents (e.g. hexane and ethyl acetate) with stepwise increase in the solvent polarity. Fractions were collected and monitored by thin layer chromatography (TLC). All of the collected fractions that give similar TLC profiles were pooled and subjected to further chromatographic separations, where necessary. All fractions were dried *in vacuo* and stored at 4 °C for further analysis.

6.2.2.3 Thin layer chromatography (TLC)

TLC analysis of the fractions obtained from column chromatography was carried out using pre-coated TLC plates 60 F_{254} (20 × 20 cm) (Merck, Darmstadt, Germany). The fractions were introduced on the TLC plate with a piece of fine capillary tube. The plates were then developed in a glass tank filled with solvent systems at room temperature. The TLC profiles were visualized under UV light (254 and 365 nm) prior to exposure to the visualizing agent, sulfuric acid 1 N solution.

6.2.2.4 High performance liquid chromatography (HPLC)

The analysis was performed using Waters HPLC system equipped with Waters 2998 Photodiode Array Detector, Waters 600 Controller, Waters Delta 600 Pumps, and Waters 2707 Autosampler. Separation was achieved using Merck Chromolith® RP-18e (4.6 × 100.0 mm) HPLC column. The mobile phase used was water (A) and HPLC grade acetonitrile (B) with a flow rate of 1 ml/min. Samples were dissolved in methanol, filtered, and injected into the HPLC system. Observation of compound peaks was done at the wavelength of 210 nm.

6.2.2.5 Liquid chromatography-mass spectrometry (LC-MS)

The extracts and fractions of *L. rhinocerotis* (10 mg/ml in methanol) were profiled using Acquity™ UPLC-PDA system coupled to Synapt HDMS oaTOF (Waters Corporation, USA) equipped with an electrospray ionization (ESI) source as previously described in Section 4.2.3.2. Full scan mass spectra (*m*/*z* 100-1000) were acquired in positive and/or negative ESI mode.

6.2.3 Characterization of isolated compounds

6.2.3.1 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed to determine the molecular weight of the isolated compounds. Analysis was carried out using GCMS-QP 2010 Plus (Shimadzu Corporation, Tokyo, Japan). The TG-5MS VB-5 BPX5 column (30.0 m × 25 mm × 25 μm) was initially set at 70°C, increased to 300°C, and then held for 10 min. Helium (flow rate: 1 ml/min) was used as the carrier gas. The total ion chromatogram (TIC) was auto-integrated and chemical constituents were identified by comparison with the accompanying spectral database (NIST 2011, Mass Spectral Library, USA) and literature, where applicable.

6.2.3.2 Nuclear magnetic resonance (NMR)

NMR spectroscopy was used to elucidate the structures of isolated compounds. Analysis was performed by the Department of Chemistry, Faculty of Science, University of Malaya. Briefly, the isolated compounds were dissolved in deuterated chlroroform ($CDCl_3$) and subjected to proton (^1H), carbon-13 (^{13}C), heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and correlation spectroscopy (COSY) NMR analysis. The NMR spectra were recorded from a JEOL FT NMR spectrometer 400 MHz. The internal standard used in ^1H NMR and ^{13}C NMR spectra was tetramethylsilane (TMS, δ: 0.00 ppm).

6.2.4 Cell viability analysis

The following cell lines of human origin were used: MCF7, MDA-MB-231 (breast adenocarcinoma); HCT 116 (colorectal carcinoma); HT-29 (colorectal adenocarcinoma); PC-3 (prostrate adenocarcinoma); A549 (lung carcinoma); HSC2 (oral squamous carcinoma); HK1 (nasopharyngeal carcinoma); MRC-5 (lung fibroblast). Details on the culture media used and culture conditions have been provided in Section 4.2.4.

The effect of *L. rhinocerotis* extracts, fractions, and isolated compounds on cell viability after 72 h of incubation was determined using the MTT assay as described in Section 4.2.6.2. The results were expressed as percentage of viable cells compared to DMSO treated control cells set at 100%. For pure compounds, IC_{50} values (concentration of compounds required to reduce cell viability to 50% under experimental condition) were determined from dose-dependent curves.

6.2.5 Statistical analysis

Each analysis was performed in triplicates and results were expressed as mean ± S.D. (n = 3). The data were statistically analysed using the IBM® SPSS® Statistics Version 19 (SPSS Inc., United States).

6.3 Results

6.3.1 Bioassay-guided fractionation of LRCP-DE

The dichloromethane extract of *L. rhinocerotis* pileus (LRCP-DE) demonstrated strong cytotoxic activity (IC_{50} < 20 µg/ml) against selected cancer cell lines after 72 h of incubation and amongst the cell lines, MCF7 (IC_{50}: 3.8 µg/ml) was observed to be most susceptible to LRCP-DE (Chapter 4). Therefore, LRCP-DE was selected for further chemical investigations in order to isolate the cytotoxic compounds.

The crude extract, LRCP-DE (1.62 g) was fractionated into two fractions with different polarity, namely LRCP-HF (1.10 g, 67.9%, w/w) and LRCP-AF (0.45 g, 27.8%, w/w) by liquid-liquid partition. Results from the MTT assay showed that cell viability of MCF7 was reduced to 27.5%, 25.0% and 50.7% when treated with LRCP-DE, LRCP-HF, and LRCP-AF, respectively (Figure 6.1). Between the two fractions, the non-polar fraction of LRCP-DE, LRCP-HF was more potent than LRCP-AF.

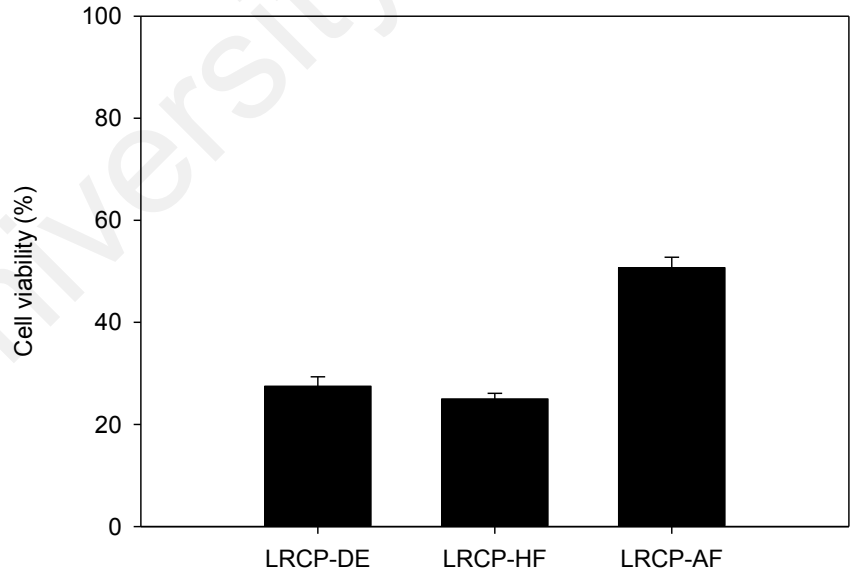


Figure 6.1: Cytotoxic activity of *L. rhinocerotis* crude extract (LRCP-DE) and fractions (LRCP-HF and LRCP-AF) against MCF7. Cells were treated with extracts and fractions (20 µg/ml) and incubated for 72 h. Cell viability was measured using the MTT assay. Results were expressed as mean ± S.D. of two independent experiments.

On the basis of higher potency, LRCP-HF was chosen for further fractionation. LRCP-HF (approximately 1.0 g) was subjected onto silica gel (70-230 mesh) column chromatography eluted with hexane and ethyl acetate in increasing manner to yield nine fractions, namely CP1 (3.3 mg), CP2 (205.5 mg), CP3 (29.2 mg), CP4 (149.6 mg), CP5 (161.4 mg), CP6 (115.mg), CP7 (50.4 mg), CP8 (81.5 mg), and CP9 (29.2 mg).

From the cell viability analysis, four fractions (CP4-CP7) at 20 µg/ml demonstrated strong cytotoxicity against MCF7 (cell viability < 50%) (Figure 6.2). The most active fraction was CP6 (cell viability: 12.2%), followed by CP7 (24.1%), CP5 (26.0%), and CP4 (35.5%). Other fractions were considered to be non-cytotoxic against MCF7.

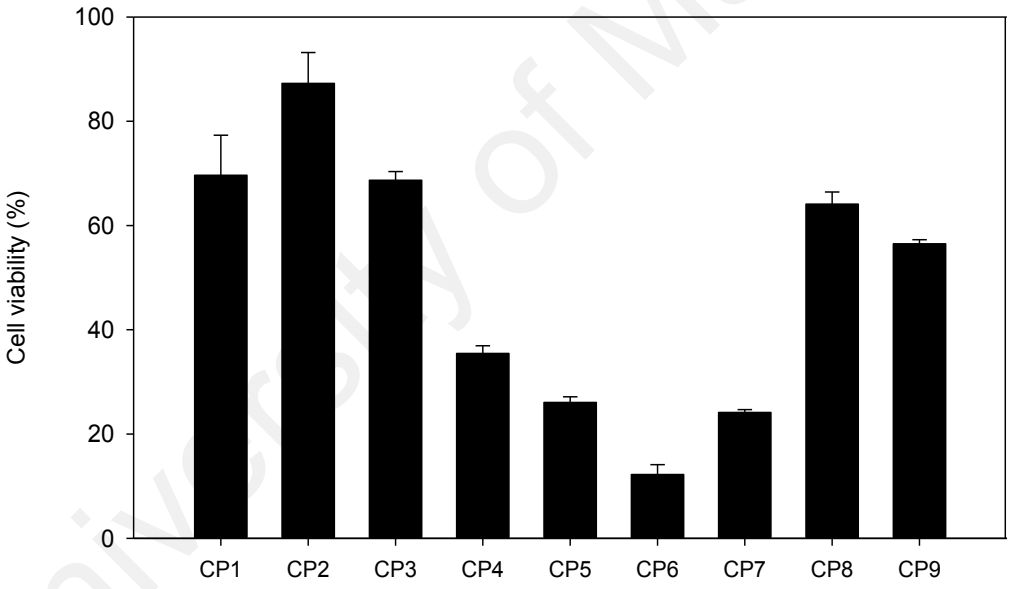


Figure 6.2: Cytotoxicity of *L. rhinocerotis* fractions (CP1-CP9) against MCF7. Cells were treated with the fractions (20 µg/ml) and incubated for 72 h. Cell viability was measured using the MTT assay. Results were expressed as mean ± S.D. of two independent experiments.

On the basis of cytotoxicity (cell viability < 50% at 20 µg/ml) and chemical profiles similarity of CP4-CP7 based on TLC analysis (data not shown), these fractions were pooled and re-labeled as CP4 (439.7 mg), and further chromatographed over silica gel

(230-400 mesh) column chromatography. Elution was performed using hexane and ethyl acetate at ratio of 80:20 (v/v), followed by hexane: ethyl acetate (50:50), ethyl acetate, and finally by methanol. A total of twelve fractions were obtained, CP4F1 (10.3 mg), CP4F2 (5.8 mg), CP4F3 (112.1 mg), CP4F4 (22.1 mg), CP4F5 (10.0 mg), CP4F6 (3.5 mg), CP4F7 (79.9 mg), CP4F8 (2.5 mg), CP4F9 (58.4 mg), CP4F10 (36.1 mg), CP4F11 (11.9 mg), and CP4F12 (78.2 mg) in the order of increasing polarity.

The effect of the fractions on MCF7 cell viability is depicted in Figure 6.3. Three fractions, namely CP4F7, CP4F8, and CP4F11 (20 µg/ml) showed strong cytotoxicity against MCF7 with cell viability reduced to 22.9%, 1.1%, and 8.6%, respectively. Some fractions displayed moderate cytotoxicity; for instance, treatment with CP4F3, CP4F4, CP4F9 and CP4F10 reduced to cell viability to 51.7%, 55.9%, 60.1% and 51.6%, respectively. Other fractions were non-cytotoxic (cell viability > 80%).

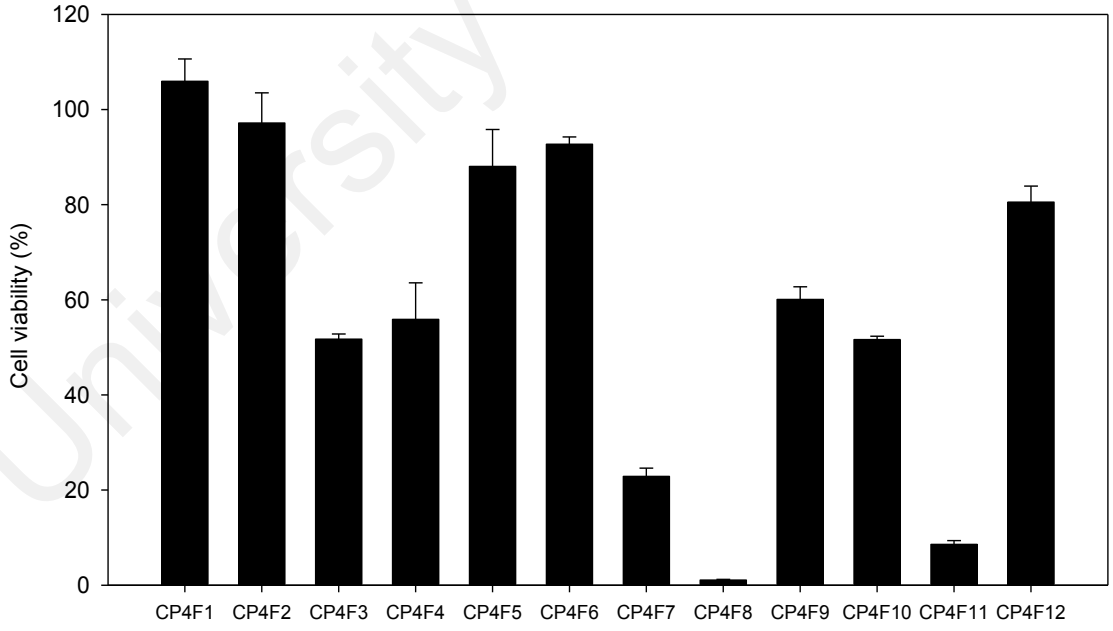


Figure 6.3: Cytotoxicity of *L. rhinocerotis* fractions (CP4F1-CP4F12) against MCF7. Cells were treated with the fractions (20 µg/ml) and incubated for 72 h. Cell viability was measured using the MTT assay. Results were expressed as mean ± S.D. of two independent experiments.

From TLC analysis (data not shown), CP4F7 and CP4F8 were found to have similar chemical profiles and hence, were combined for further purification. The combined fraction (approximately 75.0 mg) was subjected to silica gel (230-400 mesh) column chromatography with isocratic elution of hexane and ethyl acetate at a ratio of 80:20. Nine sub-fractions were obtained and labeled as CP4F71 (1.8 mg), CP4F72 (0.9 mg), CP4F73 (0.8 mg), CP4F74 (0.6 mg), CP4F75 (7.5 mg), CP4F76 (42.2 mg), CP4F77 (7.3 mg), CP4F78 (1.7 mg), and CP4F79 (4.1 mg). Based on the results of MTT assay only three fractions were found to be cytotoxic against MCF7 (Figure 6.4). Treatment with CP4F76 and CP4F77 reduced cell viability to 15.7% and 5.6%, respectively. This is followed by CP4F75 with cell viability of 61.0%. Other fractions were inactive (cell viability > 90%).

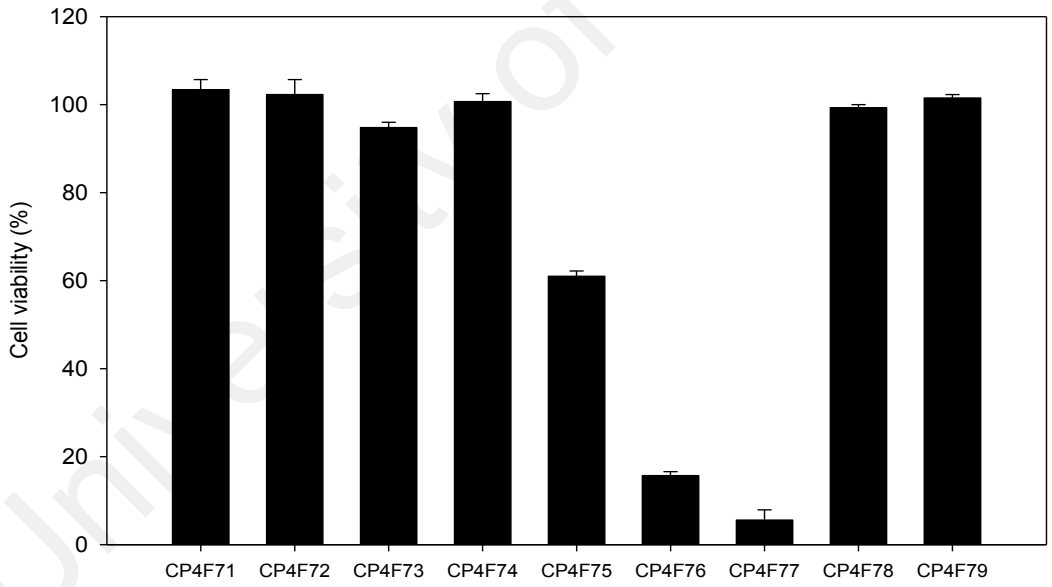


Figure 6.4: Cytotoxicity of *L. rhinocerotis* fractions (CP4F71-CP4F79) against MCF7. Cells were treated with the fractions (20 µg/ml) and incubated for 72 h. Cell viability was measured using the MTT assay. Results were expressed as mean ± S.D. of two independent experiments.

TLC analysis of these three fractions revealed only a single spot after visualization with sulpfuric acid; however, UPLC-ESI-MS analysis indicated the presence of two

closely eluted peaks in the fractions (Appendix G1-G3, pp. 326-327). To further purify the compounds, the fractions (CP4F75-CP4F77) were combined and subjected to reverse-phase HPLC. Separation of the pooled fraction was performed in a gradient manner with 0 to 100% acetonitrile (0-5 min) and held at 100% acetonitrile for 6 min. This has afforded compounds **7** (4.1 mg) and **8** (10.0 mg) at the retention times of 12.4 and 13.5 min, respectively (Figure 6.5).

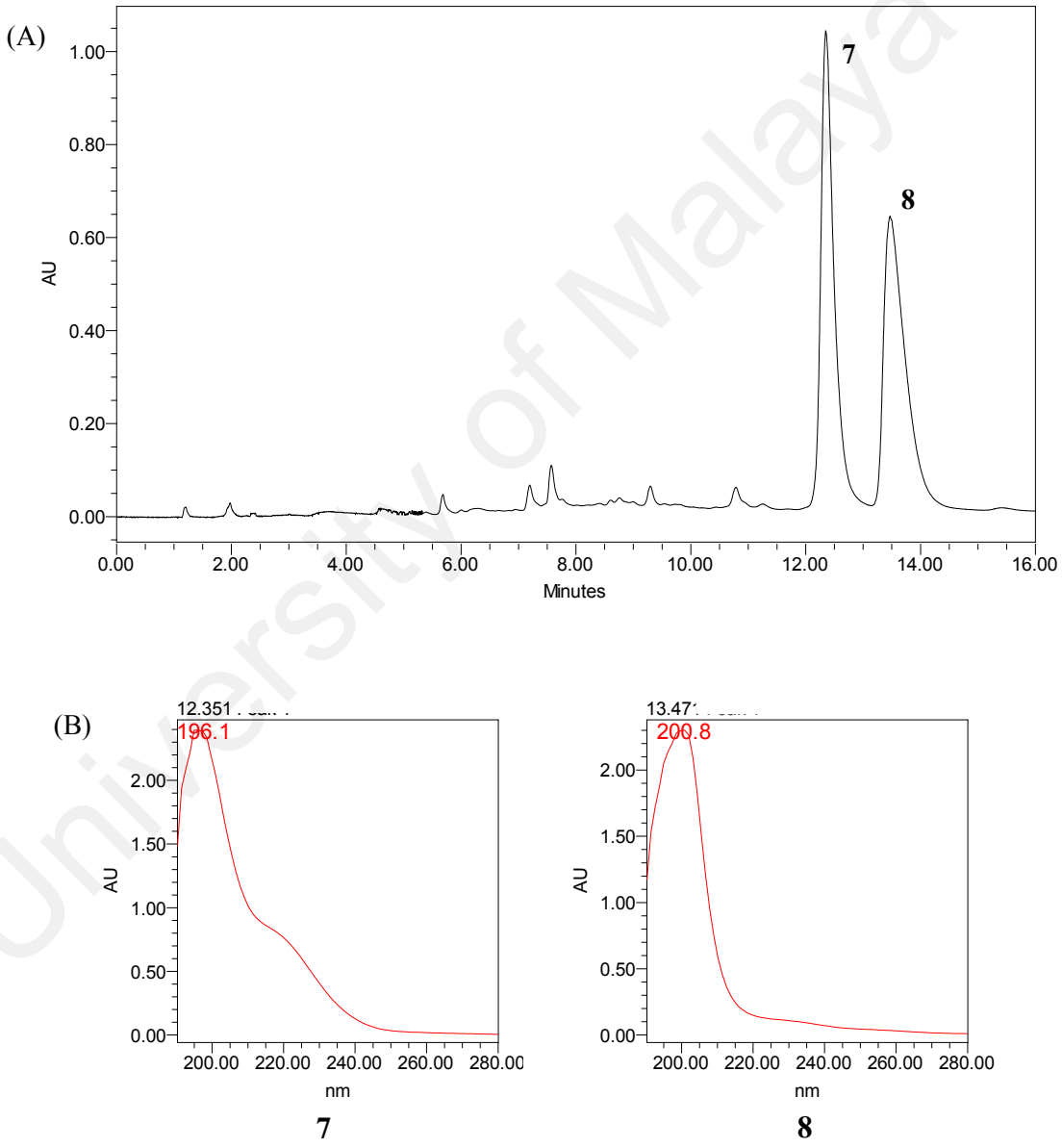


Figure 6.5: HPLC analysis of *L. rhinocerotis* fractions. (A) Representative PDA chromatogram of the fractions (CP4F75-CP4F77) monitored at 210 nm. (B) UV spectra of the major components in the fractions, labeled as compounds **7** (Rt = 12.4 min) and **8** (Rt = 13.5 min).

On the other hand, the remaining two fractions with moderate cytotoxicity, namely CP4F3 and CP4F4 were combined and re-labeled as CP4F3 (113.1 mg) and further separated by silica gel (230-400 mesh) column chromatography eluted with hexane and ethyl acetate at a ratio of 80:20 and 100% ethyl acetate to yield seven fractions labeled as CP4F31 (2.0 mg), CP4F32 (0.4 mg), CP4F33 (15.2 mg), CP4F34 (76.7 mg), CP4F35 (7.3 mg), CP4F36 (4.1 mg), and CP4F37 (3.4 mg).

Results from the MTT assay revealed that CP4F8 demonstrated strong cytotoxicity with cell viability reduced to 8.0% but the yield was low for further purification. Other fractions, including CP4F33, CP4F34, CP4F35, and CP4F37 exhibited moderate cytotoxicity with cell viability ranging from 36.1% to 55.3% after treatment with the fractions. As these fractions are only marginally cytotoxic, these were not purified further in the present study.

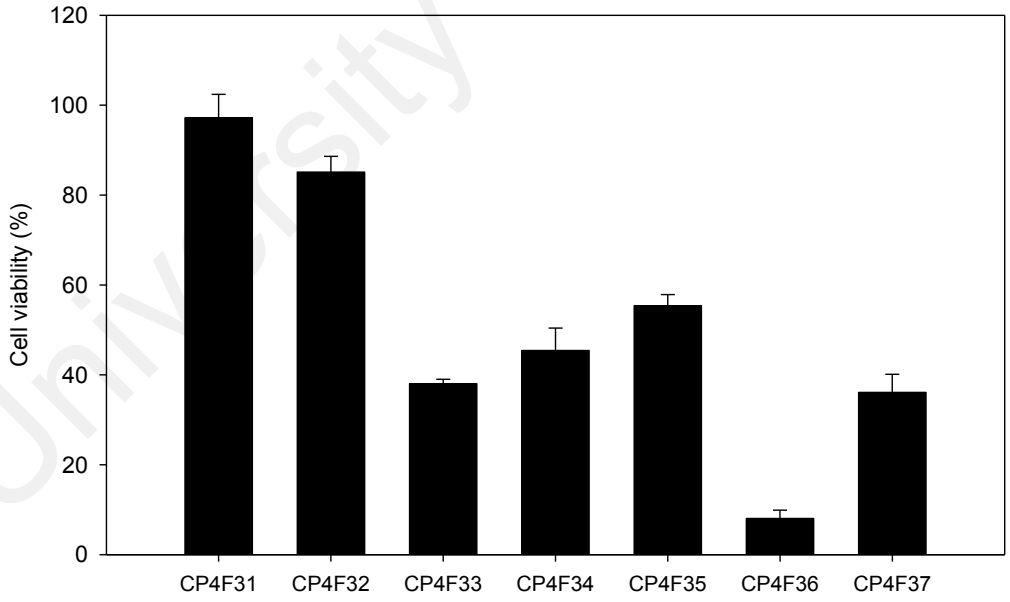


Figure 6.6: Cytotoxic activity of *L. rhinocerotis* fractions (CP4F31-CP4F38) against MCF7. Cells were treated with the fractions (20 µg/ml) and incubated for 72 h. Cell viability was measured using the MTT assay. Results were expressed as mean ± S.D. of two independent experiments.

Another fraction derived from the separation of combined CP4-CP7 was CP4F11 (20 µg/ml) that showed cytotoxicity against MCF7 (cell viability: 8.6%). Although CP4F11 was active, it was chemically complex based on TLC (data not shown) and LC-MS analysis (Appendix G4, p. 327) and the yield was low (11.9 mg); hence, it was not further analysed.

An overview of the chemical investigation of LRCP-DE that resulted in the isolation of compounds **7** and **8** is depicted in Figure 6.7.

6.3.2 Structural characterization of isolated compounds

Chemical structures of the isolated compounds were elucidated using spectroscopic and mass-spectrometric analyses. They were identified as 5*α*,8*α*-epidioxy-22*E*-ergosta-6,9(11),22-trien-3*β*-ol (9,11-dehydroergosterol peroxide) (**7**) and 5*α*,8*α*-epidioxy-22*E*-ergosta-6,22-dien-3*β*-ol (ergosterol peroxide) (**8**), respectively. The physical and spectroscopic features of compounds **7** and **8** were listed below and their chemical structures were presented in Figure 6.8. Identification of compounds **7** and **8** was based on comparison of the spectroscopic data (^{1}H, ^{13}C, HSQC, and HMBC) with literature data (Table 6.1-6.2). The relevant NMR spectra of the compounds were included in Appendix H (pp. 329-332) and Appendix I (pp. 333-337) for compounds **7** and **8**, respectively.

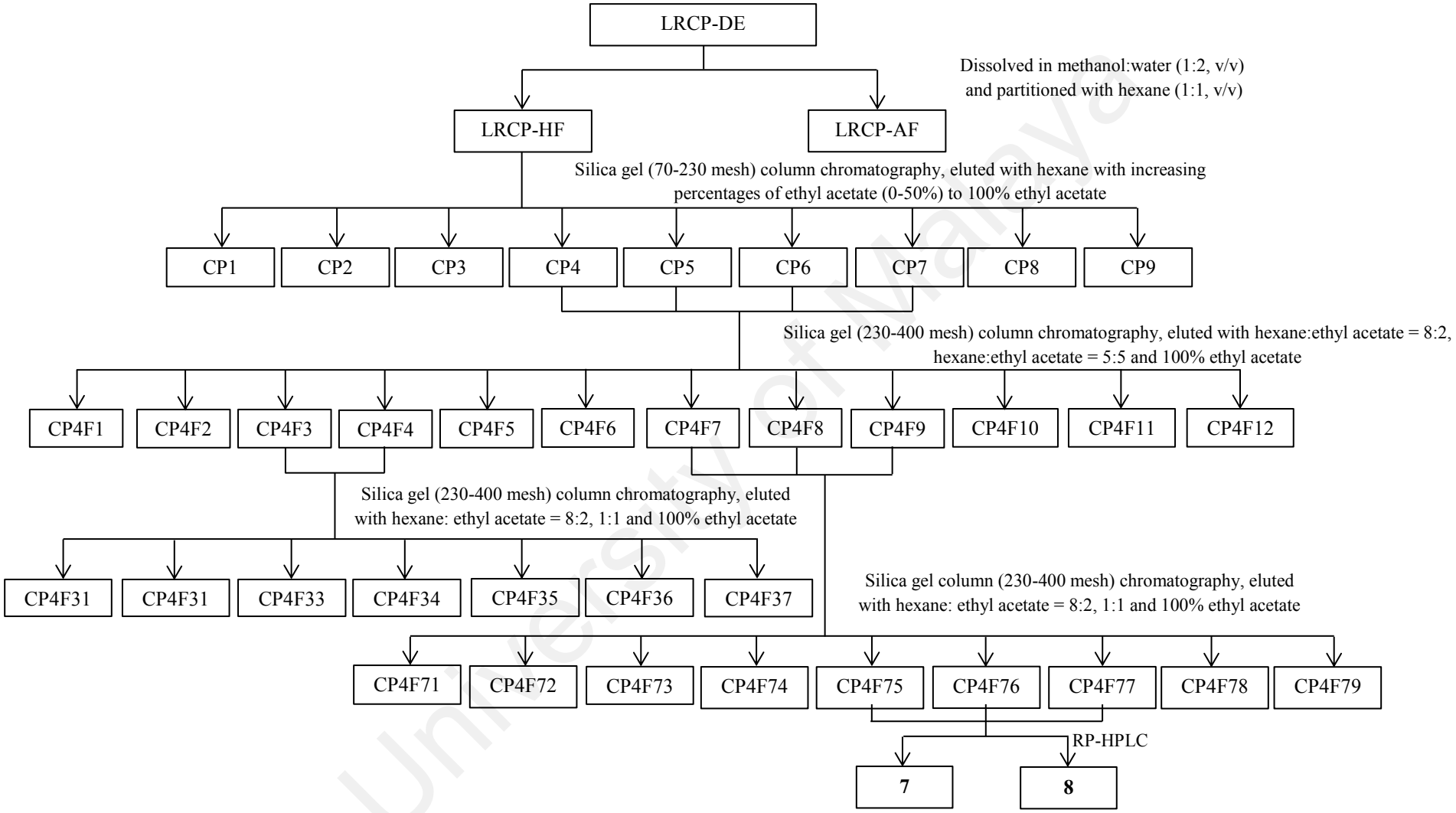
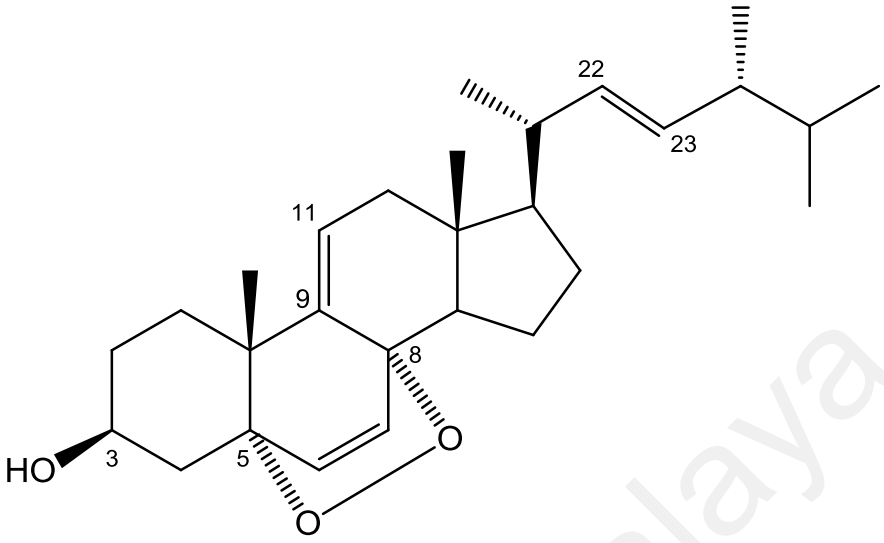


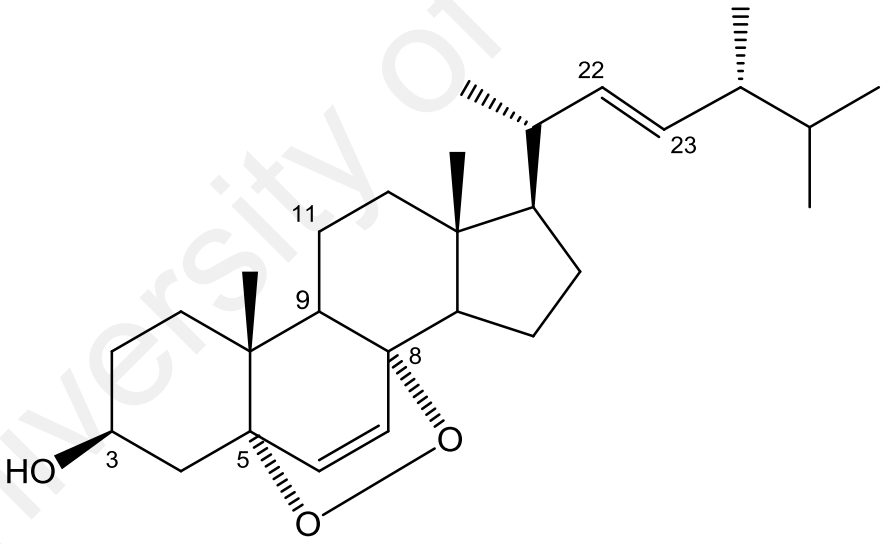
Figure 6.7: Bioassay-guided isolation of compounds **7** and **8** from the dichloromethane extract of *L. rhinocerotis* pileus (LRCP-DE)

9,11-dehydroergosterol peroxide (**7**): colourless needles. $C_{28}H_{42}O_3$. EI-MS *m*/*z*: 426 [M^+]; 1H-NMR (400 MHz, $CDCl_3$) δ_H: 0.73 (3H, s, H18), 0.81 (3H, d, *J* = 6.88 Hz, H-27), 0.83 (3H, d, *J* = 6.40 Hz, H-26), 0.90 (3H, d, *J* = 8.40 Hz, H-28), 0.99 (3H, d, *J* = 6.87 Hz, H-21), 1.08 (3H, *s*, H-19), 3.99 (1H, m, H-3), 5.15 (1H, dd, *J* = 15.56, 8.68 Hz, H-22), 5.23 (1H, dd, *J* = 15.08, 7.32 Hz, H-23), 5.42 (1H, dd, *J* = 1.84, 1.80 Hz, H-11), 6.28 (1H, d, *J* = 8.68 Hz, H-6), 6.57 (1H, d, *J* = 8.24 Hz, H-7); ^{13}C NMR (400 MHz, $CDCl_3$); Table 6.1.

Ergosterol peroxide (**8**): colourless needles. $C_{28}H_{44}O_3$. EI-MS *m*/*z*: 428 [M^+]; ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 0.80 (3H, s, H-18), 0.80 (3H, d, *J* = 6.40 Hz, H-26), 0.82 (3H, d, *J* = 6.44 Hz, H-27), 0.87 (3H, s, H-19), 0.89 (3H, d, *J* = 7.32 Hz, H-28), 0.98 (3H, d, *J* = 6.84 Hz, H-21), 3.94 (1H, m, H-3), 5.13 (1H, dd, *J* = 15.08, 8.24 Hz, H-22), 5.20 (1H, dd, *J* = 15.08, 7.80 Hz, H-23), 6.23 (1H, d, *J* = 8.72 Hz, H-6), 6.49 (1H, d, *J* = 8.72 Hz, H-7); ^{13}C NMR (400 MHz, $CDCl_3$); Table 6.2.



7



8

Figure 6.8: Chemical structures of compounds **7** and **8**

Table 6.1: ^{1}H and ^{13}C NMR spectroscopic data for compound **7**

Position	δ_H (*J* in Hz) (in $CDCl_3$)	δ_C	δ_H (*J* in Hz) (in pyridine-d_6) Kobori *et al*. (2006)	δ_C
1		32.6		33.3
2		30.7		31.7
3	3.99 (m)	66.4	4.42 (m)	65.7
4		36.1		37.4
5		82.8		83.0
6	6.28 (d) *J* = 8.68	135.5	6.40 (d) *J* = 8.4	136.4
7	6.57 (d) *J* = 8.24	130.8	6.70 (d) *J* = 8.4	131.0
8		78.4		78.4
9		142.6		144.0
10		38.0		38.6
11	5.42 (dd) *J* = 5.96, 180	119.8	5.49 (dd) *J* = 6.1, 1.8	119.2
12		41.3		41.4
13		43.7		43.8
14		48.2		48.7
15		29.8		29.0
16		21.0		21.3
17		55.9		55.9
18	0.73 (s)	13.0	0.76 (s)	13.1
19	1.08 (s)	25.6	1.17 (s)	25.7
20		40.0		40.2
21	0.99 (d) *J* = 6.88	20.8	1.00 (d) *J* = 6.6	20.9
22	5.15 (dd) *J* = 15.56, 8.68	135.2	5.16 (dd) *J* = 15.2, 8.9	136.0
23	5.23 (dd) *J* = 15.08, 7.32	132.5	5.26 (dd) *J* = 15.2, 7.8	132.4
24		43.8		43.0
25		33.1		33.3
26*	0.83 (d) *J* = 6.40	20.0	0.86 (d) *J* = 6.8	19.8
27*	0.81 (d) *J* = 6.88	19.7	0.85 (d) *J* = 6.8	20.1
28	0.90 (d) *J* = 6.84	17.6	0.95 (d) *J* = 6.8	17.8

^{1}H NMR spectrum measured at 400 MHz (current study) and 800 MHz (Kobori *et al*., 2006). ^{13}C NMR spectrum measured at 400 MHz (current study) or 125 MHz (Kobori *et al*., 2006). Assignments were supported with HSQC, HMBC, and COSY. *Assignments are interchangeable.

Table 6.2: ^{1}H and ^{13}C NMR spectroscopic data for compound **8**

Position	δ_H (*J* in Hz) (in $CDCl_3$)	δ_C	δ_H (*J* in Hz) (in $CDCl_3$) Kobori *et al*. (2006)	δ_C
1		34.8		34.7
2		30.2		30.1
3	3.94 (m)	66.6	3.97 (m)	66.5
4		37.0		37.0
5		82.2		82.2
6	6.23 (d) *J* = 8.72	135.5	6.24 (d) *J* = 8.5	135.4
7	6.49 (d) *J* = 8.72	130.8	6.51 (d) *J* = 8.5	130.8
8		79.5		79.4
9		51.2		51.1
10		37.0		37.0
11		23.5		23.4
12		39.4		39.4
13		44.6		44.6
14		51.8		51.7
15		20.7		20.6
16		28.7		28.7
17		56.3		56.2
18	0.80 (s)	13.0	0.82 (s)	12.9
19	0.87 (s)	18.3	0.89 (s)	18.2
20		39.8		39.7
21	0.98 (d) *J* = 6.84	21.0	1.00 (d) *J* = 6.6	20.9
22	5.13 (dd) *J* = 15.08, 8.24	135.3	5.14 (dd) *J* = 15.2, 8.6	135.2
23	5.20 (dd) *J* = 15.08, 7.80	132.4	5.22 (dd) *J* = 15.2, 7.8	132.3
24		42.8		42.8
25		33.1		33.1
26*	0.80 (d) *J* = 6.40	19.7	0.82 (d) *J* = 6.8	19.7
27*	0.82 (d) *J* = 6.44	20.0	0.83 (d) *J* = 6.8	20.0
28	0.89 (d) *J* = 7.32	17.6	0.91 (d) *J* = 6.8	17.6

^{1}H NMR spectra were measured at 400 MHz (current study) or 800 MHz (Kobori *et al*. 2006). ^{13}C NMR spectrum measured at 400 MHz (current study) or 150 MHz (Kobori *et al*. 2006). Assignments were supported with HSQC, HMBC, and COSY. *Assignments are interchangeable.

6.3.3 Cytotoxic activity of isolated compounds

The isolated compounds were tested for cytotoxic activity against selected cancer (i.e. MCF7, MDA-MB-231, HCT 116, HT-29, PC3, A549, HSC2, and HK1) and non-cancerous (i.e. MRC-5) cells using the MTT assay. As shown in Table 6.3, both compounds exhibited cytotoxicity against all cancer cells (with the exception for compound **8** on HK1) after 72 h of incubation with IC_{50} in the range of 11.8-31.9 μM and 4.7-40.5 μM for compounds **7** and **8**, respectively. Amongst the cell lines of different origins, the colorectal cells (HCT 116 and HT-29) were found to be sensitive to both compounds with HT-29 being most susceptible to compound **8** (IC_{50}: 4.7 μM). MRC-5 was used as a model of non-cancerous cell line for comparison purposes. Under the current experimental condition, it was observed that compound **8** (IC_{50}: 40.5 μM) exerted lesser damage on MRC-5 than compound **7** (IC_{50}: 19.5 μM).

Table 6.3: Cytotoxic activity of compounds isolated from LRCP-DE

Cell lines	IC_{50} (μM)	
	9,11-dehydroergosterol peroxide (7)	**Ergosterol peroxide (8)**
MCF7	29.0 ± 1.41	29.1 ± 6.43
MDA-MB-231	14.2 ± 1.02	28.4 ± 3.86
HCT 116	20.6 ± 0.71	16.0 ± 3.45
HT-29	11.8 ± 0.25	4.7 ± 1.28
PC3	18.6 ± 1.16	34.5 ± 0.98
A549	31.9 ± 1.16	40.3 ± 2.48
HSC2	21.5 ± 1.62	33.8 ± 3.49
HK1	18.9 ± 0.98	> 45
MRC-5	19.5 ± 1.85	40.5 ± 0.55

Cell viability post treatment with the compounds for 72 h was determined using the MTT assay. Results were expressed as mean ± S.D. (n = 3) of three independent experiments.

6.4 Discussion

Based on previous cytotoxicity screening results, LRCP-DE exhibited strongest cytotoxicity against MCF7 (IC_{50}: 3.8 µg/ml) after 72 h of treatment; hence, it was subjected to bioassay-guided fractionation for the identification of cytotoxic compounds. Repeated column chromatography and RP-HPLC afforded 9,11-dehydroergosterol peroxide (**7**) and ergosterol peroxide (**8**) with the yield (w/w) of approximately 0.25% and 0.60% of LRCP-DE, respectively. To the best of our knowledge, both compounds were isolated for the first time from *L. rhinocerotis*. Both compounds belong to the group of oxidized sterol (steroid having a hydroxyl group attached to C-3) derivatives with the 5*α*,8*α*-endoperoxide skeleton that was formed as a result of oxidation on the a 5,7-diene system in the molecule of initial sterols, i.e. 9,11-dehydroergosterol and ergosterol for compounds **7** and **8**, respectively (Bu *et al*., 2014).

Compound **7** showed a molecular ion peak at *m*/*z* 426 that was in accordance with the molecular formula $C_{28}H_{42}O_3$. Compound **8** gave a molecular ion at *m*/*z* 428 that was consistent with the molecular formula $C_{28}H_{44}O_3$. It is noted that the difference in the molecular weight of both compounds was two a.m.u. which indicated the possibility of the presence of a double bond system in compound **7**.

The ^{1}H and ^{13}NMR spectra of both compounds **7** and **8** revealed the characteristic of a steroidal skeleton. The presence of three methyl doublets and two methyl singlets in the up-field region of ^{1}H NMR spectrum of compound **7** were reminiscent of a steroidal skeleton. Consistent with Kasal *et al*. (2010), there was a region of overlapping signals appearing as a characteristic hump in the region between 0.5 to 2.5 ppm that was difficult to be analysed. These region corresponded to the methylene ($-CH_2$) and methine (-CH) groups. The signal at δ 3.99 was assigned to the proton at C-3. The signals at δ 5.15 (1H, *dd*, J = 15.56, 8.68 Hz, H-22), 5.23 (1H, *dd*, *J* = 15.08, 7.32 Hz,

H-23), 6.28 (1H, *d*, *J* = 8.68 Hz, H-6), and 6.57 (1H, *d*, *J* = 8.24, H-7) were assigned to the olefinic protons. The large ^{1}H-^{1}H coupling constant (*J* = 15.08 and 15.56 Hz) between H-22 and H-23 suggested a *trans* configuration. The ^{13}C NMR spectrum of compound **7** revealed a total of 28 signals, consistent with its molecular formula. The presence of peaks at *δ* 66.4, 78.4, and 82.8 were due to the oxygenated carbons at C-3, C-8, and C-5, respectively. The chemical shift at *δ* 135.5, 130.8, 135.2, and 132.5 were assigned for C-6, C-7, C-22, and C-23, respectively. An additional signal at *δ* 119.8 that was present only in compound **7** was assigned to C-11.

The ^{1}H NMR spectrum of compound **8** was similar to that of compound **7** with the absence of a signal at *δ* 5.42 ppm that was present only in compound **7**. Similarly, the ^{13}C NMR spectrum of compound **8** lacked a signal at 119.8 that was also attributed to the double bond of C-11 of compound **7**. This observation was well supported by the mass difference observed from mass-spectrometric analysis whereby compound **7** is 2 a.m.u. lesser than compound **8** in their molecular weight.

Steroids (including sterols) are commonly isolated from various edible-medicinal mushrooms. Compound **8** is rather ubiquitous in mushrooms whereby it was previously identified and/or isolated from a number of edible-medicinal mushrooms such as *Hypsizigus marmoreus* (Peck) H.E. Bigelow (Yasukawa *et al*., 1994), *Isaria cicadae* Miq. (synonym: *Cordyceps cicadae*) (Kuo *et al*., 2003), *Lactarius hatsudake* Nobuj. Tanaka (Gao *et al*., 2007), *Laetiporus sulfureus* (Bull.) Murrill*, Morchella esculenta* (L.) Pers.*, Boletus edulis* Bull.*, B. badius* Pers.*, Suillus bovinus* (L.) Roussel (Krzyczkowski *et al*., 2009), *Sarcodon aspratus* (Berk.) S. Ito (Takei *et al*., 2005), *Hericium erinaceus* (Bull.) Pers. (Noh *et al*., 2015), and *S. commune* (Liu *et al*., 2015). On the other hand, compound **7** is always isolated together with compound **8**. The simultaneous isolation of both from *L. rhinocerotis* in the present study is not surprising as similar

observations have been reported by researchers working on *Scleroderma aurantium* (L.) Pers. (Vrkoc *et al*., 1976), *Pisolithus tinctorius* (Pers.) Coker & Couch, *Microporus flabelliformis* (Klotzsch) Kuntze, and *Lenzites betulina* (L.) Fr. (Fujimoto *et al*., 1994), *S. aspratus* (Takei *et al*., 2005; Kobori *et al*., 2006), *G. lucidum* mycelium (Chen *et al*., 2009; Zheng *et al*., 2009), and *Penicillium roqueforti* Thom (Mioso *et al*., 2014).

Compounds **7** and **8** were demonstrated to be cytotoxic against a panel of cancer cell lines, consistent with previous studies (Chen *et al*., 2009; Makropoulou *et al*., 2012). This observation indicated that both compounds might, at least partially, contribute to the overall cytotoxicity of LRCP-DE. The cytotoxicity of compound **8** against several cell lines, for instance, PC-3 (IC_{50}: 30.6 µM), MCF7 (IC_{50}: 35.8 µM), and Hep 3B (IC_{50}: 45.3 µM) have been reported (Chen *et al*., 2009; Makropoulou *et al*., 2012). While the cytotoxicity of compound **8** was extensively studied, compound **7**, thus far, was reported to inhibit the growth of HL60 and HT-29 (Kobori *et al*., 2006), MCF7 (Zheng *et al*., 2009) and Hep 3B (IC_{50}: 39.2 µM) (Chen *et al*., 2009). Therefore, our results represent the first cytotoxicity evaluation of compound **7** on MDA-MB-231, PC-3, A549, HSC2, and HK1. Direct comparison of the IC_{50} values in the current study and those previously reported is impossible due to the differences in the methodology (e.g., seeding density, treatment period, end-point measurement, etc.). From another perspective, our results were in accordance with previous findings in which both compounds indeed have promising value to be developed as cancer therapeutics. Moreover, apart from their potential anti-cancer effect, both compounds exhibited also broad spectrum of pharmacological activities. There are previous reports on the anti-inflammatory (Akihisa *et al*., 2007; Kobori *et al*. 2007), immunosuppressive (Fujimoto *et al*., 1994), anti-oxidative (Kim *et al*., 1999), anti-bacterial (Duarte *et al*., 2007) and potential anti-aging activities (Noh *et al*., 2015) of the compounds.

The IC_{50} values of the compounds were relatively high when compared to that of the crude extract, LRCP-DE. One of the possible explanations is that in the crude extract, multiple chemical constituents may act in a synergistic manner to give the desirable biological activity and in many cases; synergistic effect could result in stronger biological effects (Mertens-Talcott *et al*., 2003; Zhang *et al*., 2014b; Liu & Xu, 2015). It is to be noted that the isolation of compounds **7** and **8** did not rule out the possibility of other cytotoxic constituents present in LRCP-DE. Further chemical investigations into the polar fraction derived from liquid-liquid extraction which also demonstrated cytotoxicity (LRCP-AF) as well as the sub-fractions CP4F33 and CP4F34 might potentially yield other compounds that might have contributed to the potent activity of LRCP-DE.

One of the major goals in the search for potential new chemotherapeutic drugs is the ability of target specifically at cancer cells but innocuous to normal cells; however, most anti-cancer drugs failed to meet this criterion due to the fact they cannot discriminate between cancer and normal cells. In the present study, compound **8** was noted to be highly cytotoxic against colorectal cell lines, i.e. HCT 116 (IC_{50}: 16.0 μM) and HT-29 (IC_{50}: 4.7 μM) but MRC-5 (IC_{50}: 40.5 μM) was less susceptible. On the other hand, under similar experimental condition, compound **7** was found to be cytotoxic across cancer and non-cancerous cell lines on the basis on the IC_{50} values. Taken together, compound **8** seemed to be a more promising candidate for cancer therapeutics than compound **7** in view of its selectivity (comparison with the IC_{50} value for MRC-5), i.e. approximately 2- and 8-fold differences for HCT 116 and HT-29, respectively. Previous studies have reported that both compounds exerted less damage on the selected non-cancerous cells, such as INT 407 (human embryonic intestinal epithelial cells) (Chen *et al*., 2009) and WI-38 (human embryonic fibroblasts) (Kobori

et al., 2006). The differences in our observation, i.e. the low selectivity of compound **7**, might also result from variation in methodology and characteristic of cells used as models of non-cancerous cell lines. Further toxicity assessment of both compounds in primary cell lines and *in vivo* models are necessary to clarify the toxicity of the compounds.

6.5 Conclusion

Two sterol epoxides, namely 9,11-dehydroergosterol peroxide (**7**) and ergosterol peroxide (**8**) were successfully isolated from the dichloromethane extract of *L. rhinocerotis* pileus for the first time through bioassay-guided fractionation. Both compounds exhibited strong cytotoxicity against a panel of human cancer cell lines. Further investigations into the mechanism of cytotoxicity of the compounds are warranted.

CHAPTER 7: CYTOTOXIC AND APOPTOSIS-INDUCING ACTIVITIES OF *LIGNOSUS RHINOCEROTIS* STEROIDAL CONSTITUENTS

7.1 Introduction

The current anti-cancer drugs that are available in market are not target specific and have been demonstrated to pose several side-effects and complications. This highlights the urgent need for effective and less-toxic agents (Patel & Goyal, 2012). In search for potential candidates for the use in treatment and prevention of cancer, mushrooms, either in the form of extracts or pure compounds, might be good candidates. An array of low molecular weight compounds (e.g. phenolic acids, triterpenoids, steroids, fatty acids, etc.) and high-molecular-weight components (e.g. polysaccharides, polysaccharide-protein complexes, proteins, lectins, etc.) from mushrooms have been shown to exhibit anti-cancer effects by interfering with specific transduction pathways, particularly that of apoptosis and those related to cell cycles (Lindequist *et al*., 2005; Ferreira *et al*., 2010; Patel & Goyal, 2012; Ivanova *et al*., 2014)

Apoptosis is a process that is controlled by a sequence of regulated events that eventually resulted in cell death. Regulating the balance between cell proliferation and cell death is the fundamental but crucial role of apoptosis in maintaining tissue homeostasis. Apoptotic cells can be defined by several morphological and biochemical changes occurring in the nucleus, plasma membrane, and mitochondria such as nuclear condensation and fragmentation, membrane blebbing, externalization of phosphatidylserine, collapse of mitochondrial membrane potential, activation of caspases, and DNA fragmentation (Fink & Cooksoon, 2005; Elmore, 2007; Hsiung & Abdul Kadir, 2011). The induction of apoptosis has been suggested to be an efficient and promising strategy in treating cancer (Bold *et al*., 1997; Fesik, 2005).

In the current study, some steroidal constituents have been identified and isolated from *L. rhinocerotis*, including ergone (**3**), 9,11-dehydroergosterol peroxide (**7**), and ergosterol peroxide (**8**), and these were shown to be highly cytotoxic (Chapter 5 & 6). The cytotoxicity of these compounds has been investigated previously (Lee *et al*., 2005; Takei *et al*. 2005; Kobori *et al*., 2007; Cui *et al*., 2010); nonetheless, our understanding on their mechanism of action in human colorectal cancer cells is still lacking. In order to evaluate their potential as cancer therapeutics, we investigated the possible mechanism of cytotoxicity of the compounds in selected human colorectal cancer cells

The specific objectives of this study include:

1. To evaluate the cytotoxic activity of the steroidal constituents against a panel of colorectal cancer (i.e. HCT 116, HT-29, and DLD-1) and non-cancerous (i.e. CCD-18Co) cell lines using the MTT and SRB assays
2. To assess the anti-proliferative activity of the steroidal constituents using the DNA synthesis assay
3. To investigate the effect of selected compounds on cell cycle progression and the expression of cell cycle regulatory proteins
4. To investigate the apoptosis-inducing activities of the selected isolated compounds by flow cytometry (i.e. annexin V-FITC/PI and JC-1 analyses) and colorimetric (i.e. DNA fragmentation and caspase -3/7, -8 and -9) assays

7.2 Materials and Methods

7.2.1 Compounds

Compound **3** (ergone) was purchased from BioBioPha Co., Ltd. (Yunnan, China) while compounds **7** (9,11-dehydroergosterol peroxide) and **8** (ergosterol peroxide) were isolated from the dichloromethane extract of *L. rhinocerotis* pileus (cultivated samples).

7.2.2 Cell culture

The following cell lines were obtained from the American Type Culture Collection (ATCC): HCT 116 (ATCC® CCL-247™, human colorectal carcinoma); HT-29 (ATCC® HTB-38™) DLD-1 (ATCC® CCL-221™) (human colorectal adenocarcinoma). CCD-18Co (ATCC® CRL-1459™, normal human colon fibroblast) was a gift from Dr. Sim Kae Shin from the Institute of Biological Sciences, Faculty of Science, University of Malaya.

HCT 116, HT-29, and DLD-1 were cultured in RPMI while CCD-18Co was grown in MEM. All media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 100 unit/ml penicillin/streptomycin (Gibco). Cells were cultured in 5% CO_2 incubator at 37 °C in a humidified atmosphere. Cells were sub-cultured every 2 or 3 days and routinely checked under an inverted microscope. For solid tumours, cells were harvested using 0.25% (v/v) trypsin in PBS when 80% confluence was reached. Only cells at exponential growth were used throughout the experiments.

7.2.3 Cell viability assays

The effect of the compounds on cell viability was determined using the MTT and SRB assays as described in Section 4.2.5.2 and Section 5.2.5, respectively. Cells were treated with the compounds and results were taken after 24 h of incubation.

7.2.4 Cell proliferation assay

The effect of the compounds on cell proliferation was measured using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, USA) according to the manufacturer's instruction. This assay quantitates cell proliferation based on the measurement of BrdU incorporation during DNA synthesis in replicating cells. Cells were treated with the compounds and results were taken after 24 h of incubation. Absorbance was taken at 405 nm. Results were expressed as percentage of proliferating cells (relative to control).

7.2.5 Cell cycle analysis

The effect of compound **8** on cell cycle progression was determined as described in Section 5.2.7 except that the cells were treated with compound **8** (30 μM) for 12-72 h.

7.2.6 Annexin V-FITC/PI staining

The effect of compound **8** on apoptosis induction was determined as described in Section 5.2.8.1 except that the cells were treated with compound **8** (30 μM) for 24-72 h.

7.2.7 Mitochondria membrane potential analysis

The change in mitochondria membrane potential (MMP) was assessed using the MitoScreen (JC-1) kit (BD Biosciences, USA) according to the manufacturer's instructions. Cells were seeded and treated with compound 8 (30 μM) for 24-72 h. Following that, cells were harvested, washed, and re-suspended in medium containing JC-1 before incubated at 37 °C for 15 min. Then, the cells were washed twice and re-suspended in the buffer provided. MMP of the cells was analysed using a flow cytometer by detecting the green and red fluorescence signals.

7.2.8 Activities of caspase-3/7, -8, and -9

The effect of compound **8** on the activities of caspase-3/7, -8, and -9 was evaluated using Caspase-Glo® luminenscence assay kits (Promega, Madison, Wisconsin, USA) according to manufacturer's instructions. Briefly, cells (10 000 cells/well) were seeded and allowed to attach overnight before treated with compound **8** (30 μM) for 24-72 h. Following that, Caspase-Glo® reagent (100 μl) was added to each well, gently mixed, and incubated at room temperature for 60 min. Luminenscence of the samples was taken using a luminometer (Synergy H1 Multi-mode Reader, BioTek Instruments, Inc.).

7.2.9 Cell death detection ELISA

The effect of compound **8** on apoptosis induction was determined as described in Section 5.2.8.2 except that the cells were treated with compound **8** (30 μM) for 24-72 h.

7.2.10 Western blot

7.2.10.1 Reagents

Antibodies specific for $p21^{Waf1/Cip1}$, p53, and cleaved PARP as well as goat anti-rabbit IgG-conjugated to horseradish peroxidase (HRP), and goat anti-mouse IgG-conjugated to HRP were purchased from Cell Signaling Technology (MA, USA).

7.2.10.2 Preparation of cell lysates

Cells were cultured in 60 mm petri dishes and allowed to attach overnight before compound **8** (30 μM) was added. Following the desired treatment period, medium containing detached cells was collected and attached cells were rinsed with PBS. Both attached and floating cells were pooled by centrifugation. Lysis buffer was then added to the cell pellet. The lysis buffer composed of 50 mM Tris-HCl pH 7.4, 1% (v/v) Triton-X, 0.5% (w/v) Na-deoxycholate, 0.1% (w/w) SDS, 150 mM NaCl, 2 mM EDTA,

and 1% (v/v) protease and phosphatase inhibitors cocktail (Pierce). After a brief sonication on ice, cell lysates were centrifuged at 13 000 rpm for 15 min at 4 °C to remove cell debris. The supernatant was collected and protein content was quantified using the bicinchoninic acid (BCA) protein assay kit as described in Section 4.2.3.1. The protein content of cell lysates was calculated from a BSA standard curve.

7.2.10.3 Western blot analysis

Protein mixture in cell lysates was subjected to SDS-PAGE for separation according to molecular size. Briefly, cell lysates containing equal amount of proteins (approximately 20 µg) were diluted with Laemmli (1970) sample buffer, and boiled at 95 °C for 10 min. The samples were then loaded into wells in the stacking gel. The proteins were separated by 10% (w/v) resolving gel under constant voltage of 80 V using runner buffer containing 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS. After the SDS-PAGE separation, the proteins were transferred to Immobilon-P Transfer Membranes (Merck-Milipore, MA, USA) under constant voltage of 100 V at 4 °C for 60 min using transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol. Then, the transblotted membrane was blocked with 5% (w/v) skim milk in TBS-T (113 mM NaCl, 25 mM Tris-HCl with 0.05% (v/v) Tween 20) at room temperature for 1 h. The membrane was washed with TBS-T for 3 times before incubated with the primary antibody overnight at 4 °C. After washing the membrane with TBS-T for 3 times, appropriate secondary antibody was added onto the membrane and it was further incubated for 1 h at room temperature. The membrane was then rinsed in TBS-T for three times to remove excess secondary antibody. The antibody-protein complexes were visualized by enhanced chemiluminescence (ECL Western Blotting Substrate, Pierce) and images were captured directly using the FluorChem™ HD2 system (ProteinSimple, Californa, USA).

7.2.11 Statistical analysis

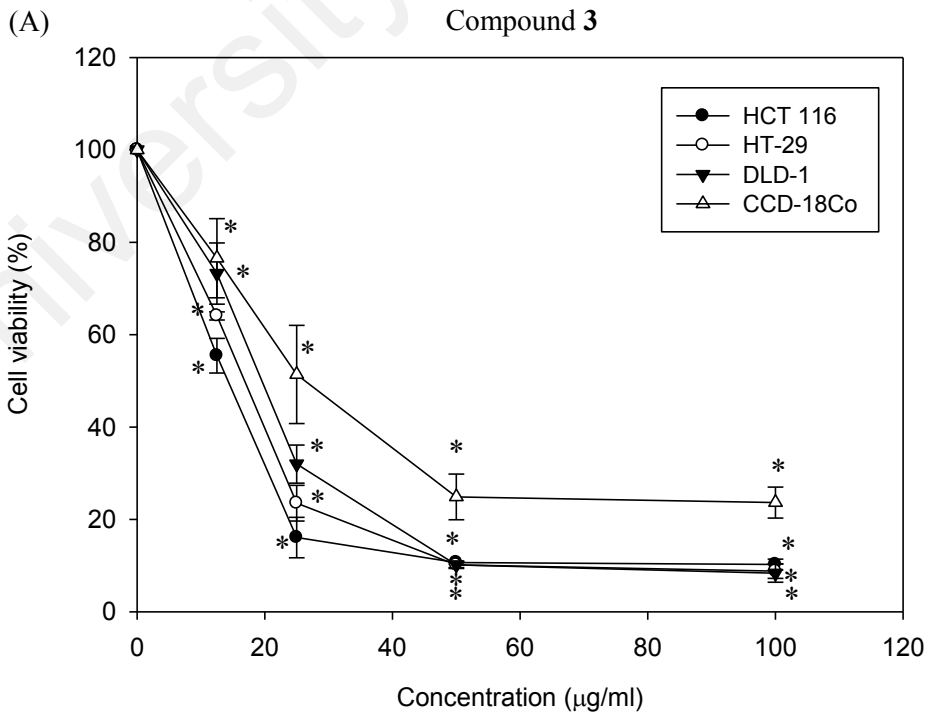
Each analysis was performed in triplicates and results were expressed as mean ± S.D. or S.E. (n = 3). The data were statistically analysed using the IBM® SPSS® Statistics Version 19 (SPSS Inc., United States). Student's t-test was used for comparison of means between control and treated samples.

7.3 Results

7.3.1 Effect of *L. rhinocerotis* steroidal constituents on cell viability

We evaluated the cytotoxic activity of *L. rhinocerotis* steroidal constituents, including ergone (**3**), 9,11-dehydroergosterol peroxide (**7**), and ergosterol peroxide (**8**), against a panel of human colorectal cell lines after 24 h of incubation. Cell viability post treatment with the compounds was measured using two different assays, namely the MTT (based on mitochondria status or metabolic activity) and SRB (based on cellular protein content) assays.

The effect of the compounds on cell viability as measured by the MTT assay is presented in Figure 7.1. A dose-dependent reduction of cellular viability of the cancerous (i.e. HCT 116, HT-29, and DLD-1) and non-cancerous (i.e. CCD-18Co) cell lines after 24 h of treatment was observed.



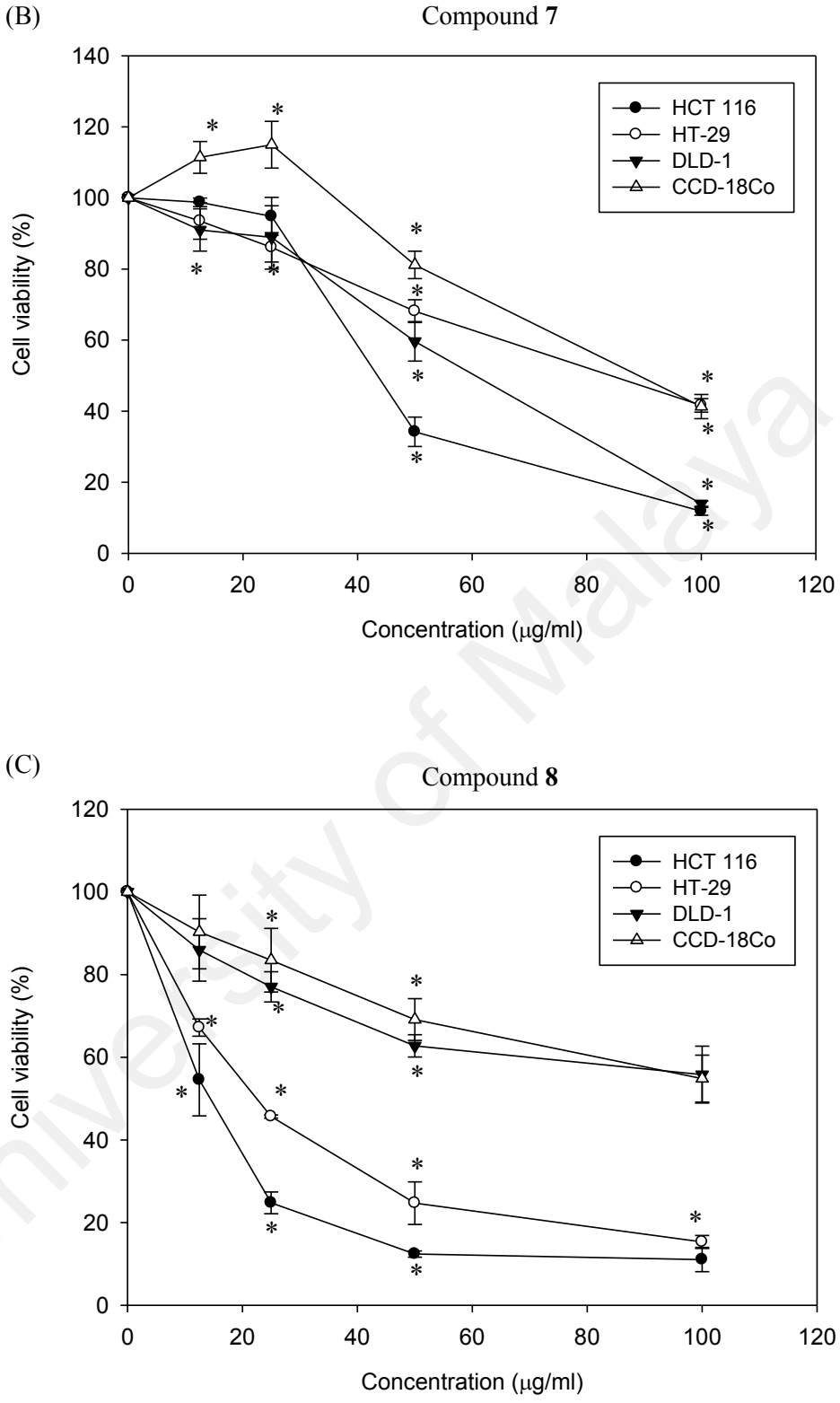


Figure 7.1: Effect of *L. rhinocerotis* steroidal constituents on the viability (metabolic activity) of human colorectal cell lines. Cell viability was determined using the MTT assay after 24 h of incubation. Results were expressed as mean ± S.E. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

Cytotoxicity of the compounds was expressed in the forms of IC_{50} values (Table 7.1). It was apparent that compound **3** was highly potent against both the cancer (IC_{50}: 33.8-46.6 μM) and non-cancerous (IC_{50}: 62.7 μM) cell lines. By comparison, compound **8** exhibited cytotoxicity against HCT 116 (IC_{50}: 31.6 μM) and HT-29 (IC_{50}: 50.0 μM) but not for DLD-1 and CCD-18Co (IC_{50} > 230 μM) whereas compound **7** demonstrated overall weak cytotoxicity (IC_{50}: 103.7-208.0 μM) against all cell lines.

Table 7.1: Cytotoxic activity of *L. rhinocerotis* steroidal constituents

Cell lines	IC_{50} (μM)		
	Compound 3	**Compound 7**	**Compound 8**
HCT 116	33.8 ± 1.35	103.7 ± 9.49	31.6 ± 4.85
	(13.3 ± 0.53 μg/ml)	(44.4 ± 4.07 μg/ml)	(13.5 ± 2.08 μg/ml)
HT-29	39.3 ± 1.32	189.5 ± 16.59	50.0 ± 1.14
	(15.4 ± 0.52 μg/ml)	(81.2 ± 7.11 μg/ml)	(21.5 ± 0.49 μg/ml)
DLD-1	46.6 ± 4.77	208.0 ± 8.33	> 230
	(18.3 ± 1.87 μg/ml)	(89.1 ± 3.57 μg/ml)	(> 100 μg/ml)
CCD-18Co	62.7 ± 15.38	127.1 ± 2.05	> 230
	(24.6 ± 6.04 μg/ml)	(54.5 ± 0.88 μg/ml)	(> 100 μg/ml)

Cell viability post treatment with the compounds for 24 h was determined using the MTT assay. IC_{50} values were determined from dose-response curves. Results were presented as mean ± S.D. (n = 3).

On the basis of IC_{50} values (Table 7.1), the potency of the compounds, in general, decreased in the order of compounds **3** > **8** > **7**. Nevertheless, compound **8** differed from compound **3** as the former demonstrated selectivity in killing the cancer cells with lesser effect on the non-cancerous cells; for instance, in the case of HCT 116 and HT-29, cytotoxicity of compounds **3** and **8** was comparable; however, compound **3** inhibited the

growth of CCD-18Co (IC_{50}: 62.7 μM) but compound **8** exerted no effect on the viability of CCD-18Co even at the highest concentration of 100 μg/ml (~230 μM) (Figure 7.1C).

Selectivity index of the compounds is calculated by dividing the IC_{50} values of CCD-18Co by the IC_{50} values of the cancer cell lines (i.e. HCT 116, HT-29, and DLD-1). Higher SI values indicate selectivity or specificity towards cancer cells. As shown in Table 7.2, SI values of compound **8** were approximately 7.3 and 4.6 against HCT 116 and HT-29, respectively. On the other hand, SI values of compound **3** were lower with 1.9 and 1.6 for HCT 116 and HT-29, respectively.

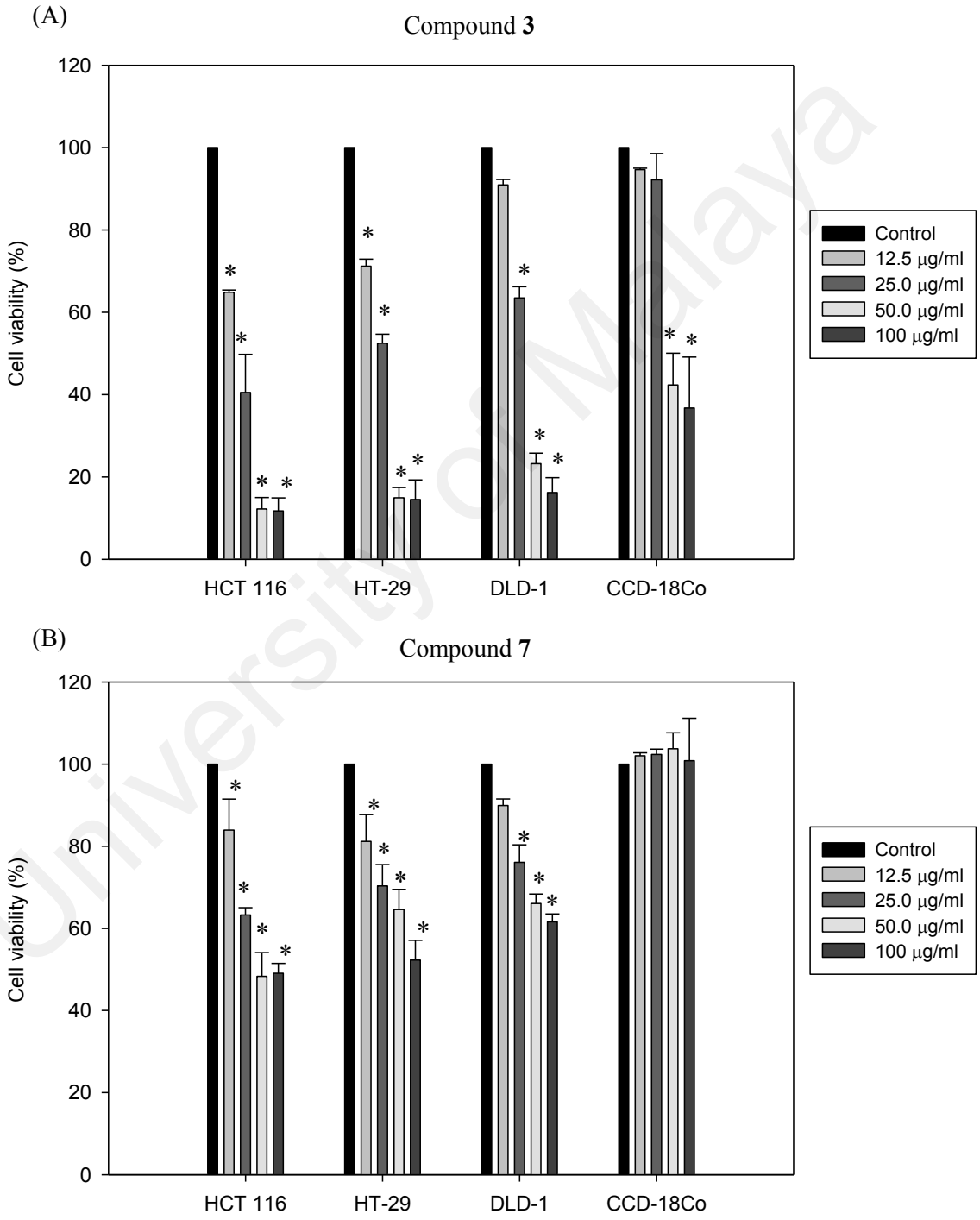
Table 7.2: SI (selectivity index) values of *L. rhinocerotis* steroidal constituents

Cell lines	SI		
	Compound 3	**Compound 7**	**Compound 8**
HCT 116	1.9	1.2	~7.3
HT-29	1.6	0.7	~4.6
DLD-1	1.3	0.9	N.A.

SI values were determined by dividing the IC_{50} value of CCD-18Co with the IC_{50} value of HCT 116, HT-29, and DLD-1. N.A., not available.

Results from the MTT assay was further substantiated with findings from the SRB assay. The growth inhibitory effect of the compounds towards the cell lines, as measured by the SRB assay, is shown in Figure 7.2. Treatment with the compounds resulted in a dose-dependent decrease in the percentage of viable cells with the cancer cells (i.e. HCT 16, HT-29, and DLD-1), in general, being more sensitive than CCD-18Co, the non-cancerous cell line. Amongst the steroidal constituents, compound **3** inhibited the growth of all colorectal cell lines but CCD-18Co observed to be less susceptible when compared with HCT 116, HT-29, and DLD-1. This observation was

similar to the trend observed in the MTT assays. On the other hand, compounds **7** and **8** demonstrated selective growth inhibition against the cancer cell lines with CCD-18Co (non-cancerous) not affected (viability > 80%) even at highest concentration. Results from the SRB assay indicated that HCT 116 was the most sensitive cell line.



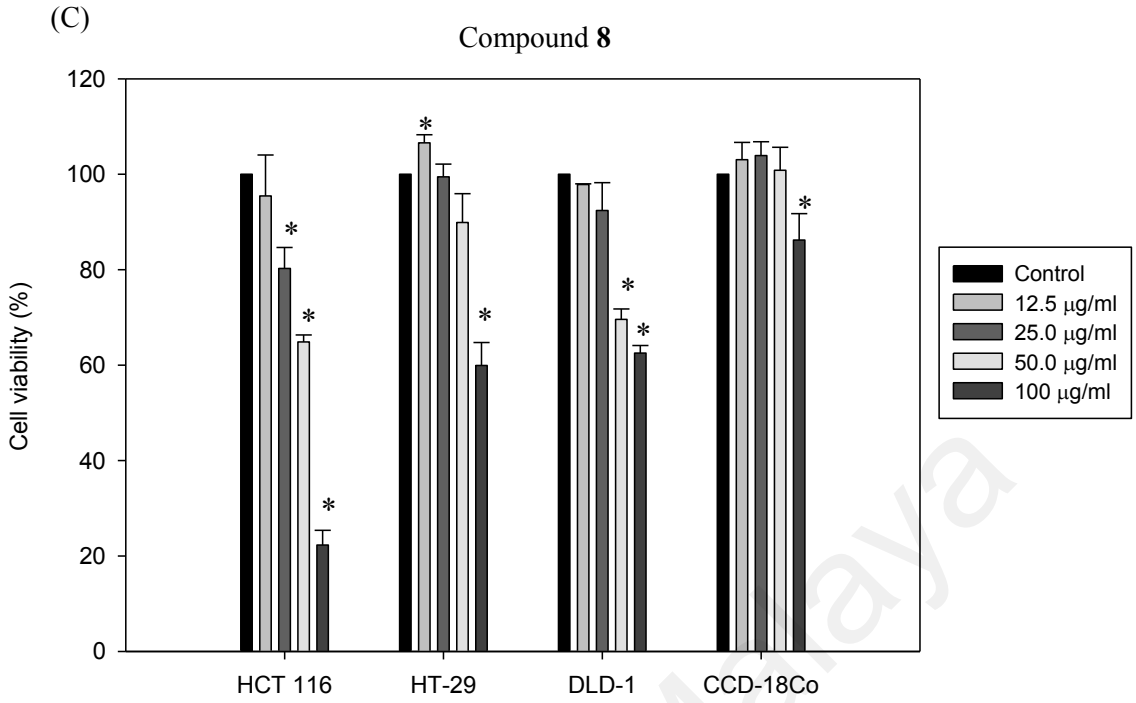
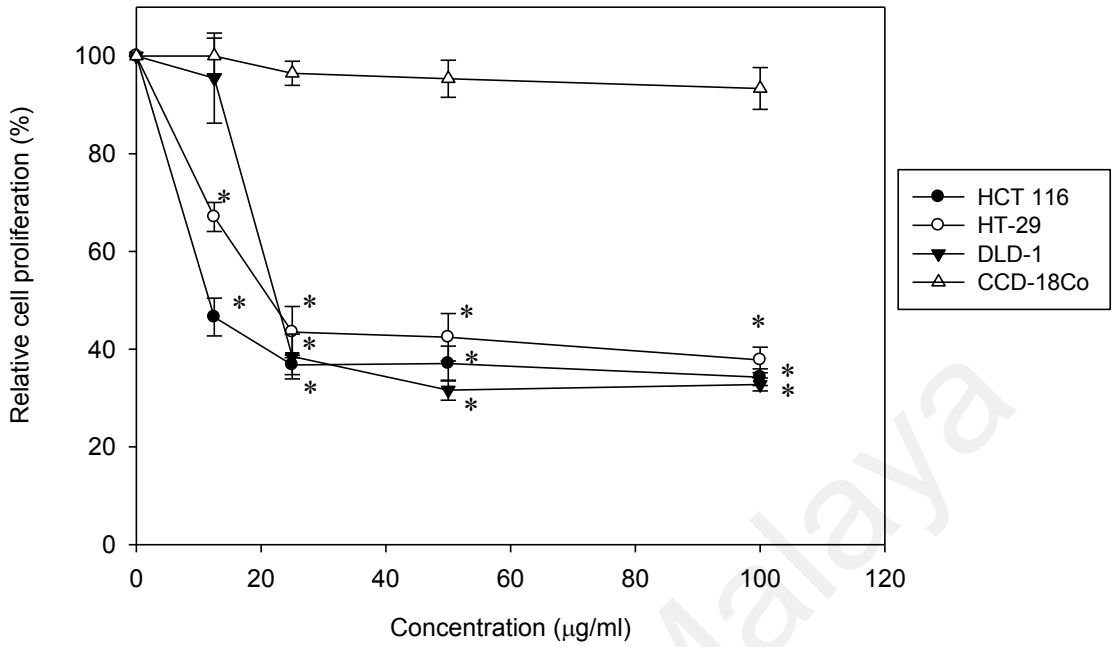


Figure 7.2: Effect of *L. rhinocerotis* steroidal constituents on the viability (cellular protein content) of human colorectal cell lines. Cell viability was determined using the SRB assay after 24 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

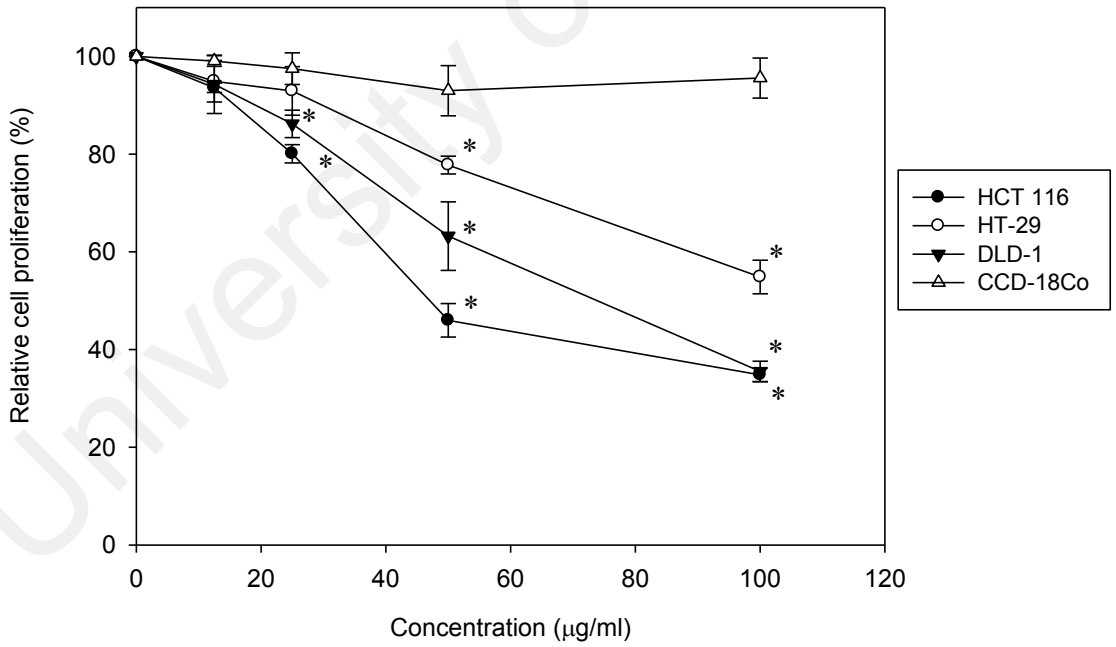
7.3.2 Effect of *L. rhinocerotis* steroidal constituents on cell proliferation

To investigate if the growth-inhibitory effect of the compounds was due to suppression of cell proliferation, we monitored cellular DNA synthesis based on incorporation of BrdU by actively growing cells. As shown in Figure 7.3, treatment with the steroidal constituents for 24 h effectively decreased the number of proliferating cancer cells (i.e. HCT 116, HT-29, and DLD-1) in a dose-dependent manner; however, CCD-18Co, the non-cancerous cell line seemed to be not affected. The compounds demonstrated different degree of anti-proliferative activity with compound **3** (25 μg/ml) strongly inhibited the growth cancer cell lines with percentage of proliferating cells reduced to 36.8-43.5%. At similar concentrations, percentage of proliferating cells decreased to 80.1-92.9% and 38.5-75.2% for compounds **7** and **8**, respectively.

(A) Compound **3**



(B) Compound **7**



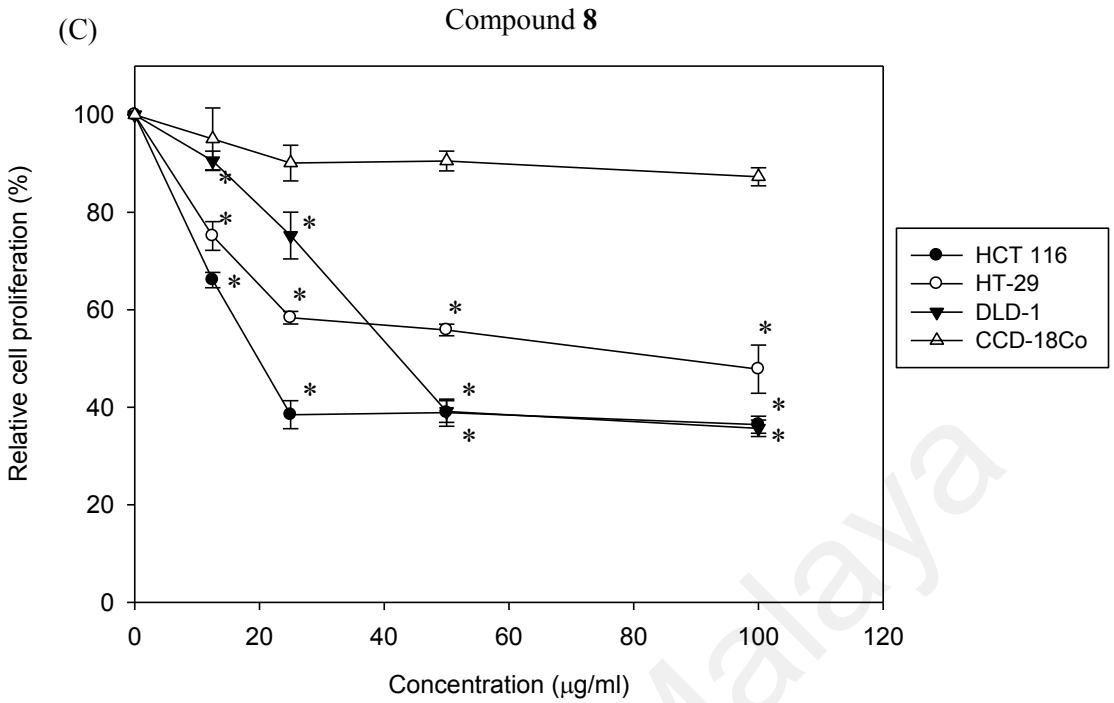


Figure 7.3: Effect of *L. rhinocerotis* steroidal constituents on the proliferation of human colorectal cell lines. Cell proliferation was quantitated based on the measurement of BrdU incorporation during DNA synthesis in replicating cells after 24 h of incubation. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

As demonstrated by the MTT and SRB assays, the compounds were able to inhibit the growth of cancer cells with a lesser extent on the non-cancerous cells. The compounds also suppressed the proliferation of the cancer cell lines. As the cytotoxic action of compounds **8** was selective towards the cancer cell lines, further investigations were carried out only on this compound. The mechanism of action of compound **8** (30 μM, approximate IC_{50} concentration) on two colorectal cell lines, i.e. HCT 116 and HT-29 (previously shown to be fairly comparable in terms of sensitivity to compound **8**), was compared.

7.3.3 Effect of compound 8 on cell cycle progression

To investigate if the anti-proliferative activity of compound **8** was associated with arrest in any stages of the cell cycle, flow cytometry analysis was carried out after the cells were treated with compound **8** for 12-72 h. The effect of compound **8** on cell cycle progression in HCT 116 and HT-29 is shown in Figure 7.4 and 7.5.

Treatment with compound **8** (30 μM) resulted in a time-dependent increase in the percentage of cells in G1 phase as well as concomitant decrease in the percentage of cells in G2/M and S phases in HCT 116 and HT-29. For HCT 116, the percentage of cells in G1 phase was 33.9% for control, and it increased to 75.3% when treated with compound **8** for 12-72 h. On the other hand, the percentage of cells in G1 phase for HT-29 increased sharply from 38.1% in control to 51.8, 65.5, 76.7, and 85.5% after 12, 24, 48, and 72 h of treatments, respectively. We also noted that there was a gradual, time-dependent increase in the percentage of sub-G1 cells from 2.6-14.8% for HCT 116. On the other hand, the percentages of sub-G1 cells in HT-29 maintained between 1.2-1.5% under similar incubation periods.

In summary, cell cycle analysis revealed that compound **8** induced arrest at G1 phase in both HCT 116 and HT-29. However, significant population of sub-G1 cells, which might indicate apoptotic cells, was observed only in HCT 116 under the current experimental condition.

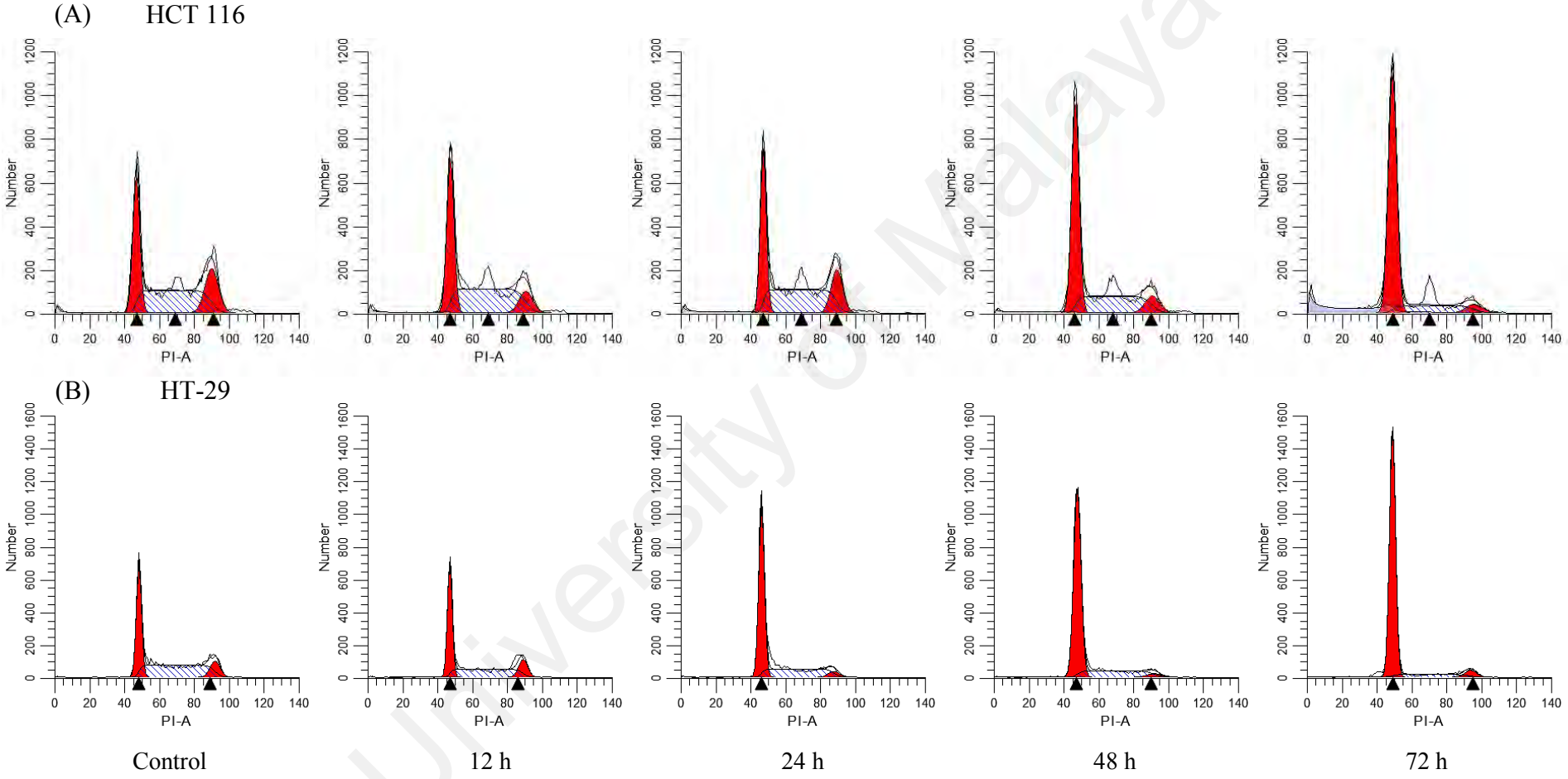


Figure 7.4: Representative histograms showing the effect compound **8** on cell cycle phase distribution. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for different treatment periods (12-72 h).

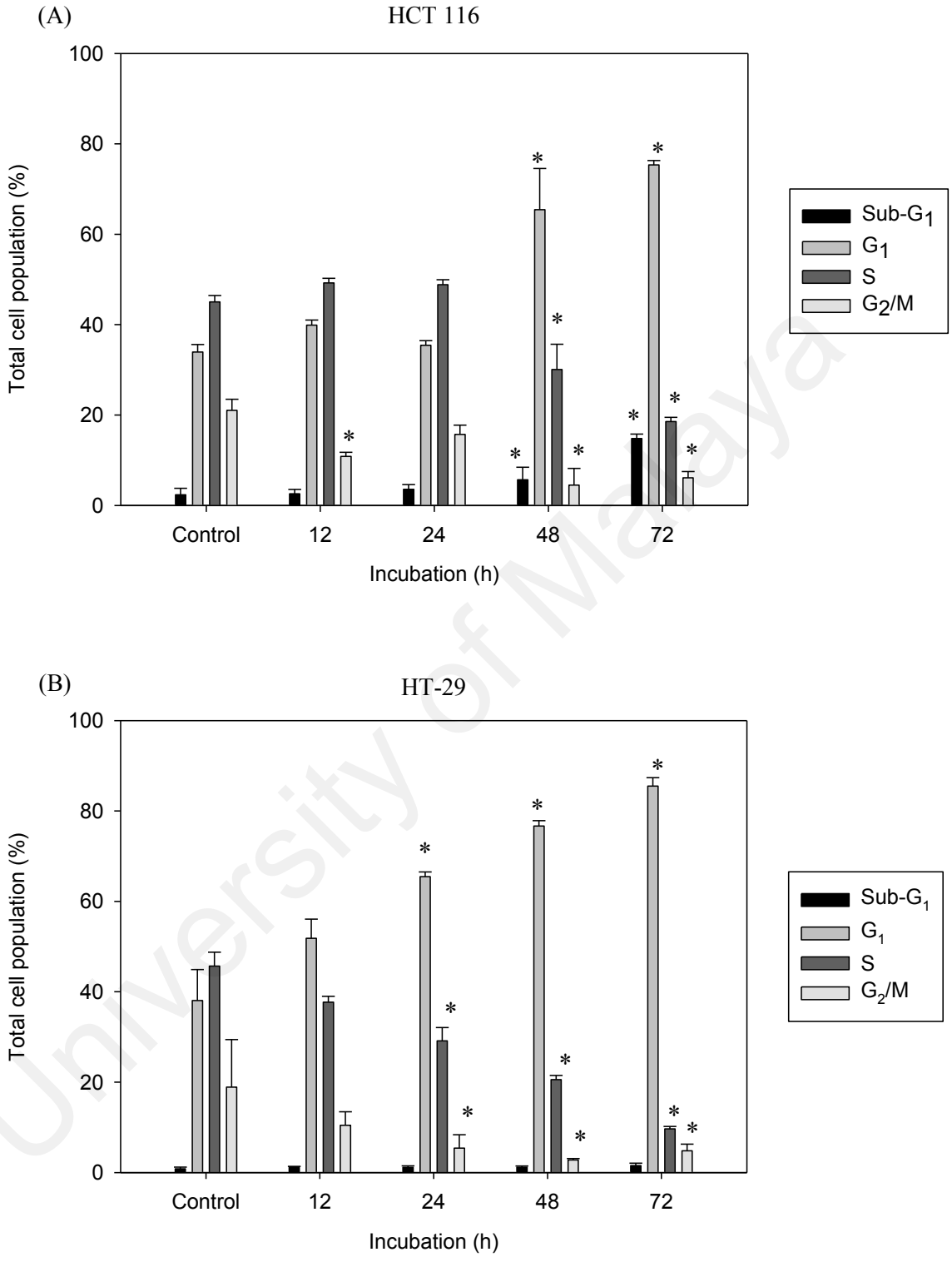


Figure 7.5: Effect of compound **8** on distribution of cell cycle phases. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for 12-72 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

7.3.4 Effect of compound 8 on cell cycle regulatory proteins

Proteins p21 and p53 have important roles in the regulation of cell cycle progression; hence, the effect of compound **8** (30 μM) on both proteins was examined by Western blotting. As shown in Figure 7.6, treatment with compound **8** caused a time-dependent increase in the expression of p21 in HCT 116. The expression of p21 was found to be very low in HT-29. Similarly, the expression of p53 in HCT 116 cells also increased after treatment with compound **8**. On the other hand, the level of p53 in HT-29 cells was observed to be comparable to control after treatment with compound **8**.

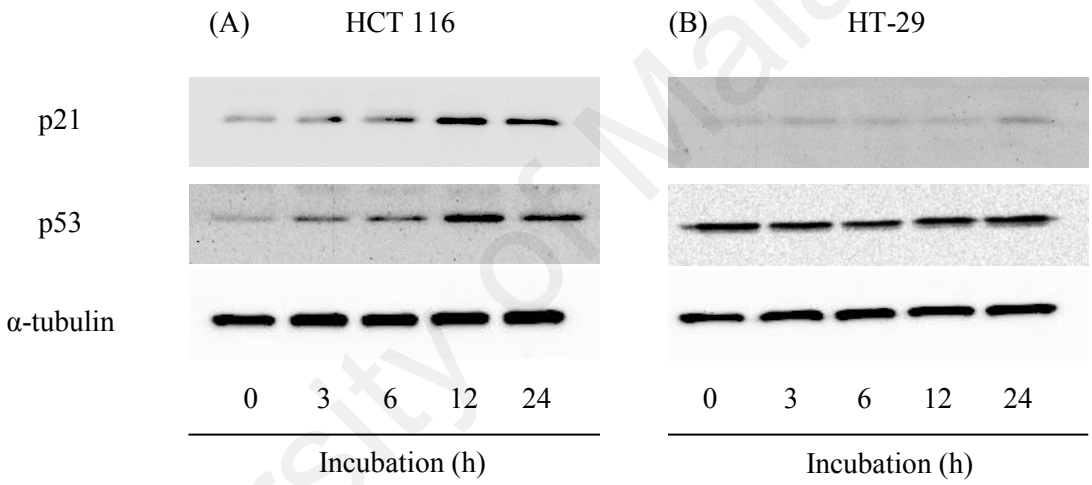


Figure 7.6: Effect of compound **8** on expression of cell cycle regulatory proteins. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for different incubation period. Total cellular proteins was extracted, resolved using SDS-PAGE, and immunoblotted with the indicated antibodies. The analysis was repeated twice with similar results and a representative immunoblot is shown.

Our results indicated that treatment with compound **8** caused an increase in the protein expression levels of p21 and p53 in HCT 116 cells. These, in turn, suggest the possible involvement of p53-p21 pathway in the cell cycle arrest induced by compound **8**.

7.3.5 Effect of compound 8 on externalization of phosphatidylserine

To check if apoptosis was induced in the cells following the treatment with compound **8**, we conducted the annexin V-FITC/PI assay. The results of the annexin V-FITC/PI analysis are shown in Figure 7.7 and 7.8.

When the cells were treated with compound **8**, there was a decrease in the percentage of viable cells that was accompanied by a marginal increase in percentage of apoptotic and necrotic cells. In HCT 116, the percentage of apoptotic cells was 5.6, 8.3, 12.9, and 14.7% for control, 12 h, 48 h, and 72 h, respectively. The apoptosis-inducing effect of compound **8** in HT-29 was marginal with percentage of apoptotic cells of 4.5% for control and 6.2-8.5% for cells treated for 24-72 h.

Overall, results from the annexin V-FITC/PI analysis demonstrated that compound **8** induced apoptosis in both cell lines. As the treatment period with compound **8** was prolonged from 24-72 h, the percentage of apoptotic cells increased.

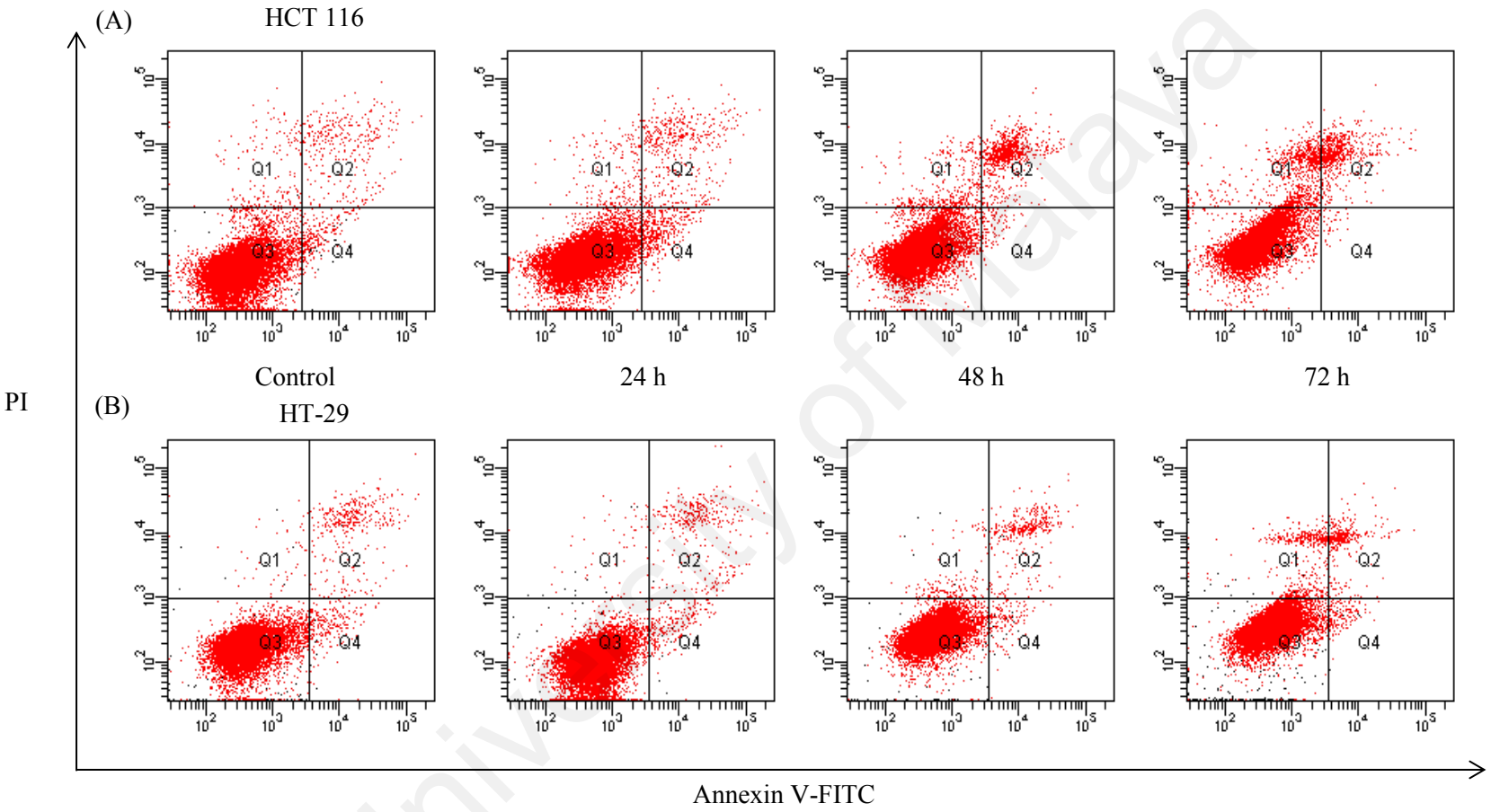


Figure 7.7: Representative histograms showing the effect of compound **8** on apoptosis induction. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 µM) for different treatment periods (12-72 h).

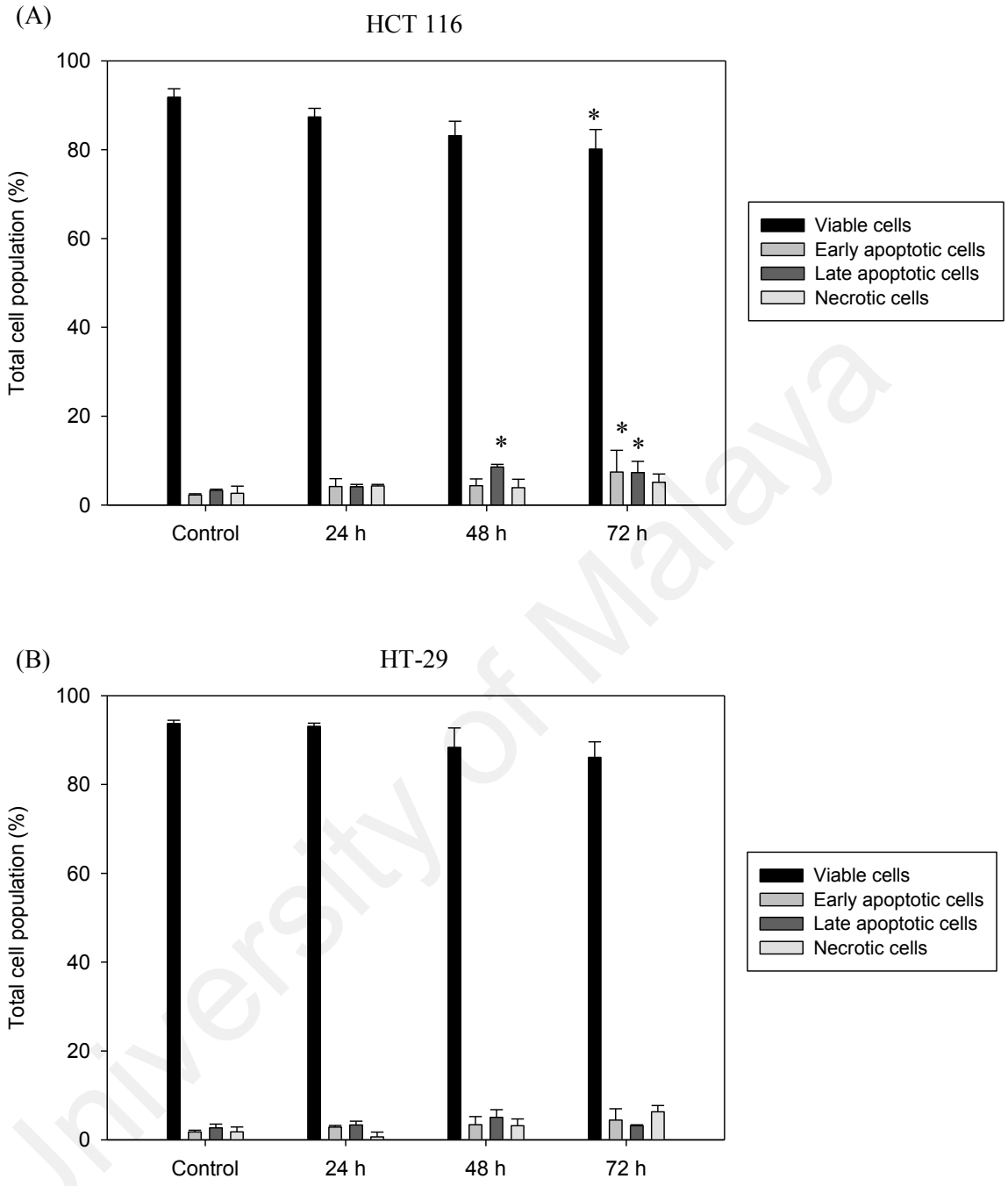


Figure 7.8: Effect of compound **8** on apoptosis induction. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for 24-72 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

7.3.6 Effect of compound 8 on mitochondria membrane potential

As dysfunctional mitochondria are usually implicated in apoptotic cell death, we measured the change in mitochondrial membrane potential using the fluorescent probe, JC-1. In viable cells, JC-1 monomers combine as aggregates that emit an intense red fluorescence signal that is detectable by FL2-A channel. On the other hand, in apoptotic cells, JC-1 monomers emit intense green fluorescence that can be detected in FL1-A channel. The effect of compound **8** on the mitochondria membrane potential of HCT 116 and HT-29 is shown in Figure 7.9 and 7.10.

For the control, most of the JC-1 fluorescence appeared in the upper quadrant, representing polarized cells. Following the treatment with compound **8**, there was a shift of fluorescence signal resulting in the treated cells having lower red fluorescence as compared to the untreated cells. Compound **8** was found to cause a substantial time-dependent increased of depolarized cells in HCT 116 but not in HT-29 (Figure 7.10). Under similar treatment periods, the increase in percentage of depolarized cells for HCT 116 (2.9-25.9%) was higher compared to that of HT-29 (2.6-8.1%).

Results from this analysis demonstrated that compound **8** evoked the loss of mitochondria membrane potential in both HCT 116 and HT-29 cells. This is evidenced by the time-dependent increase in the percentage of depolarized cells.

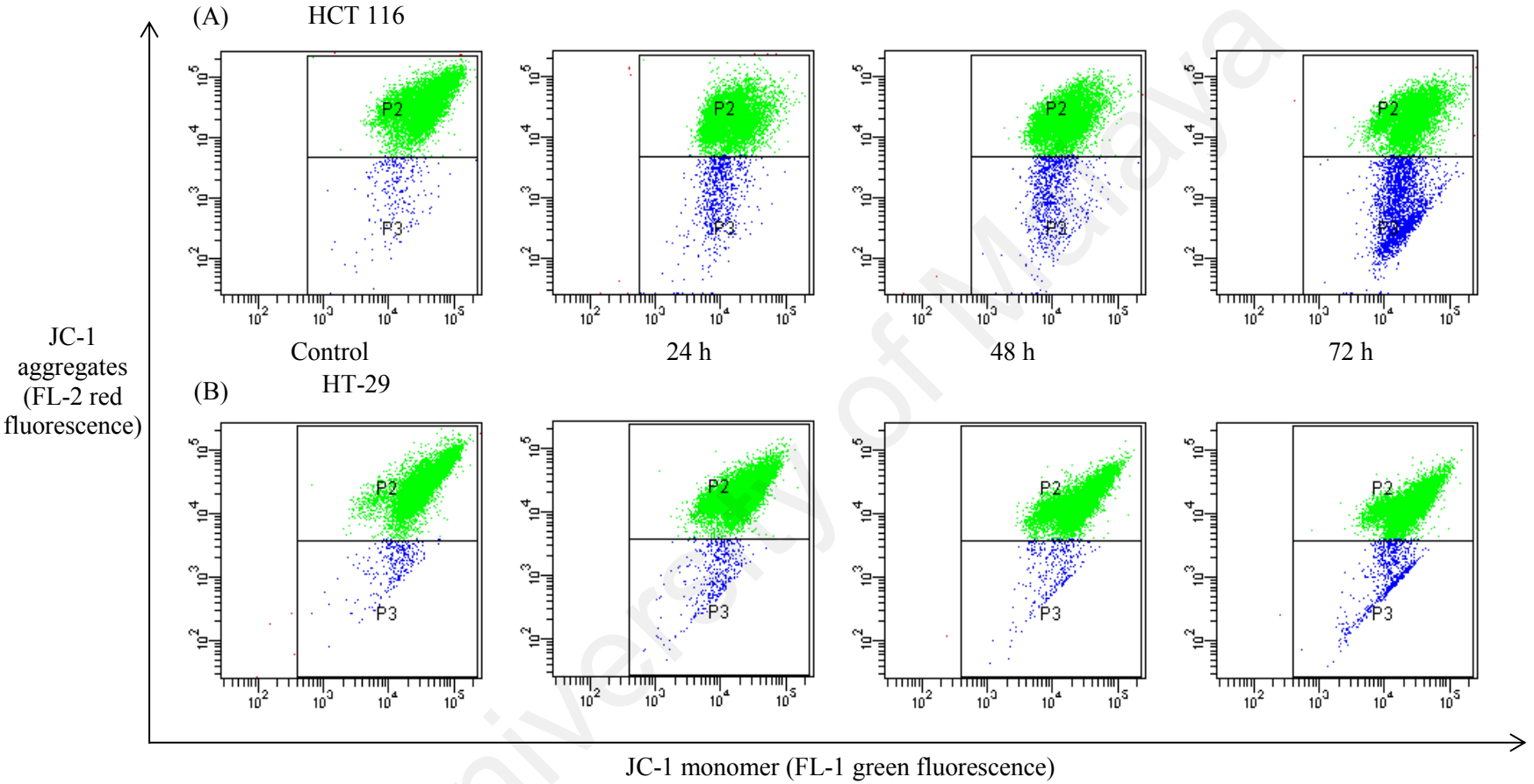


Figure 7.9: Representative dot plots of JC-1 aggregates (FL-2 red fluorescence) versus JC-1 monomers (FL-1 green fluorescence) for cells treated with compound **8**. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for different treatment periods (24-72 h). P2 represents polarized (healthy) cells whereas P3 represents depolarized (apoptotic) cells.

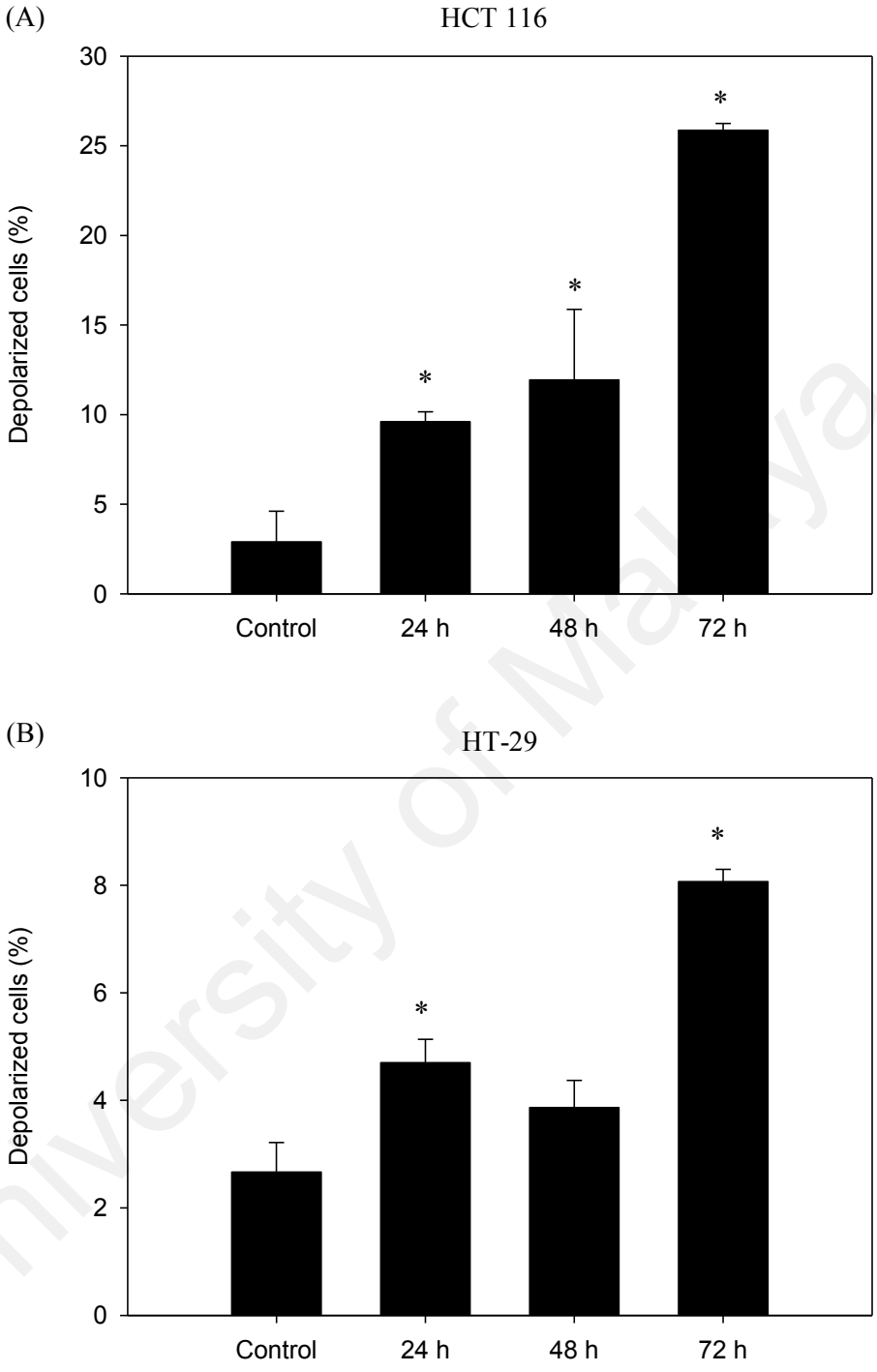


Figure 7.10: Effect of compound **8** on mitochondria membrane potential. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 µM) for 24-72 h. Depolarized cells were considered to as apoptotic cells. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

7.3.7 Effect of compound 8 on caspase-3/7, -8, and -9 activities

Next, we investigated the involvement of initiator capsase-8 and -9 as well as executioner caspase-3/7 in the cell death induced by compound **8**. The ability of compound **8** to activate the caspases in HCT 116 and HT-29 was studied using luminescence assays. The effects of compound **8** on the caspase-3/7, -8, and -9 activities are presented in Figure 7.11, 7.12, and 7.13, respectively.

We found that compound **8** increased the activities of caspase-3/7 and -9 in both cell lines. On the other hand, the activity of caspase-8 in treated and untreated HCT 116 cells was comparable whereas there was a slight increase when HT-29 was treated with compound **8**. Our results, henceforth, indicated that compound **8** was able to induce apoptosis through execution by caspase cascade based as evidenced by the increased activities of caspase-3/7 and -9 in a time-dependent manner.

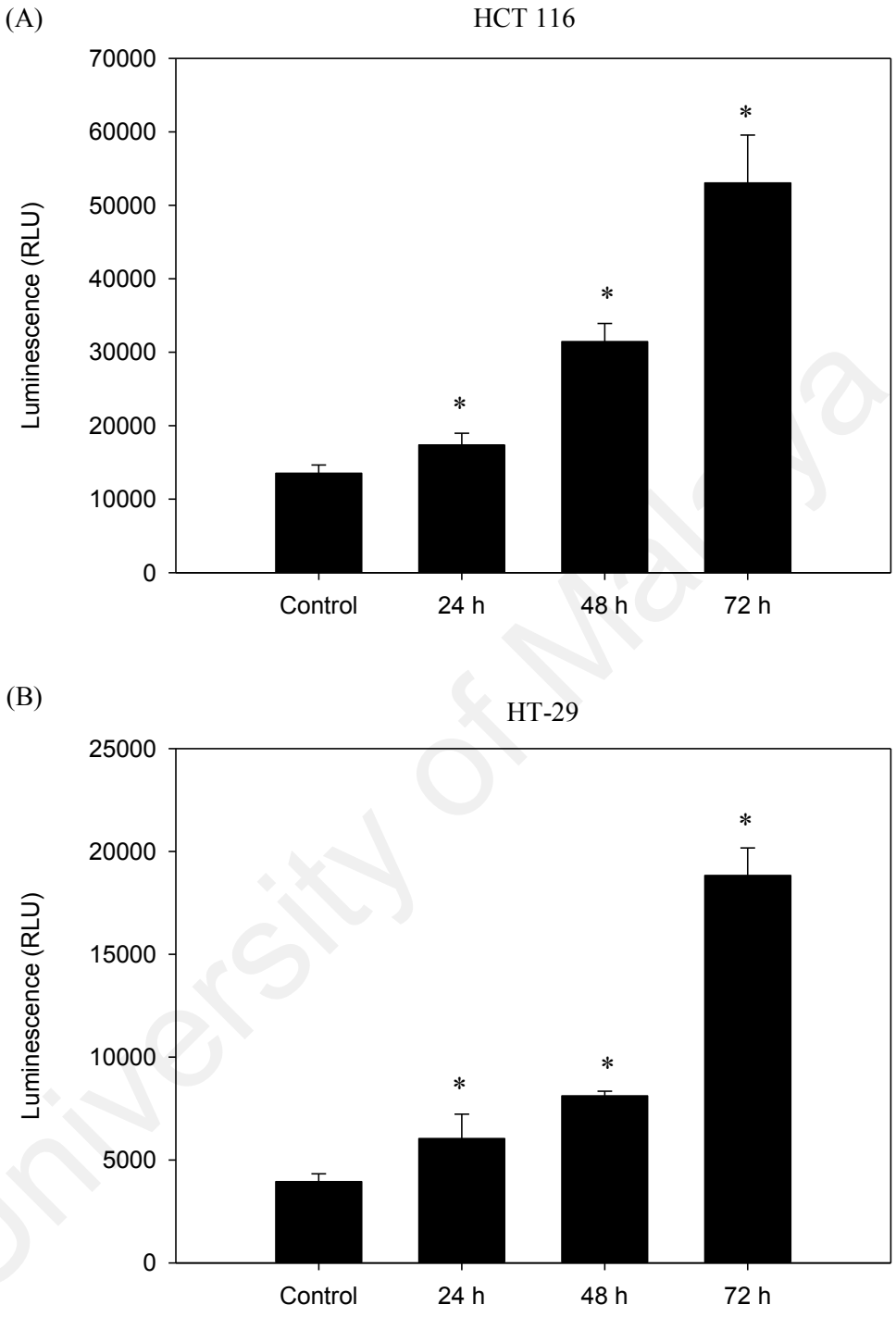


Figure 7.11: Effect of compound **8** on caspase-3/7 activity. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for 24-72 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

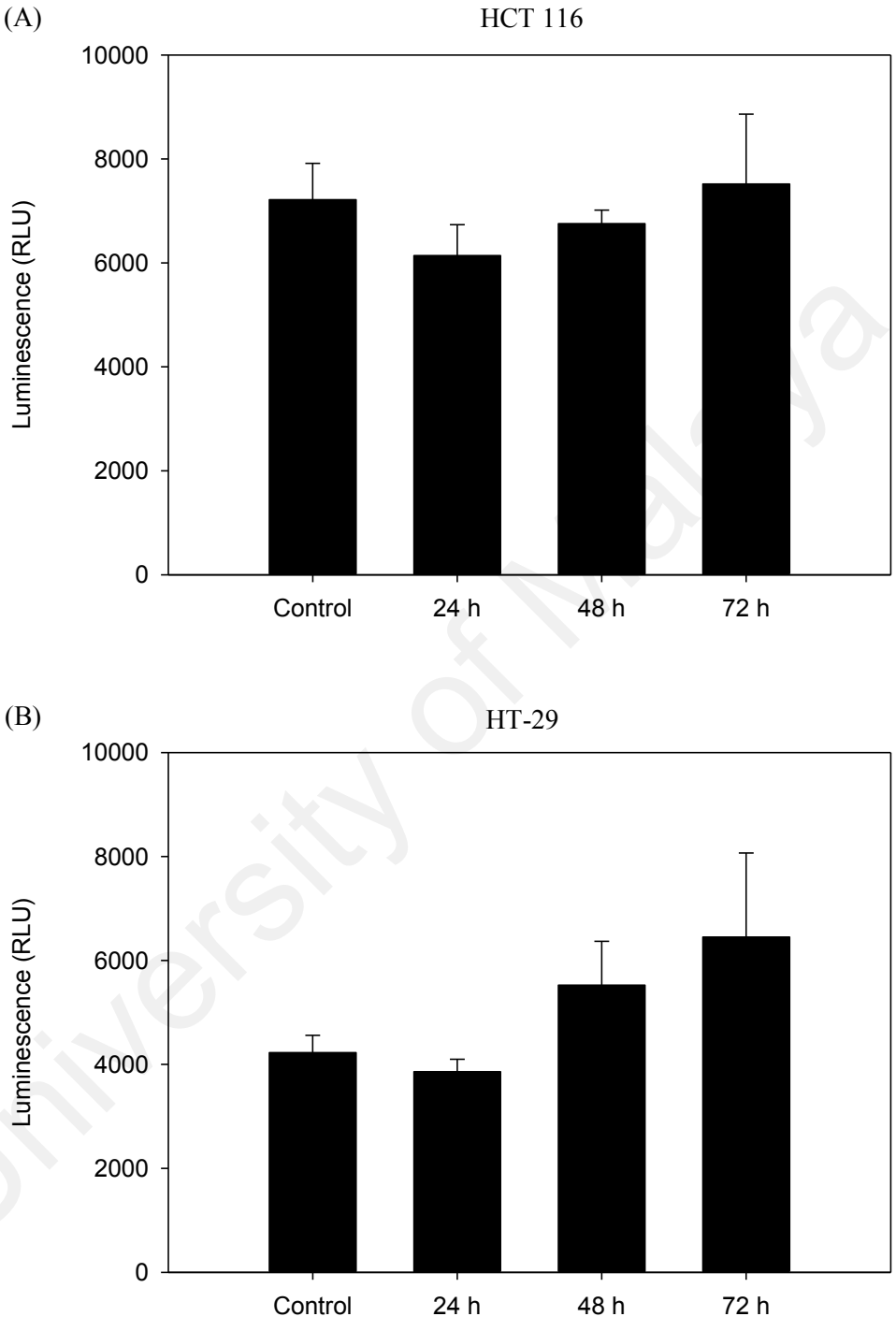


Figure 7.12: Effect of compound **8** on caspase-8 activity. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for 24-72 h. Results were expressed as mean ± S.D. (n = 3).

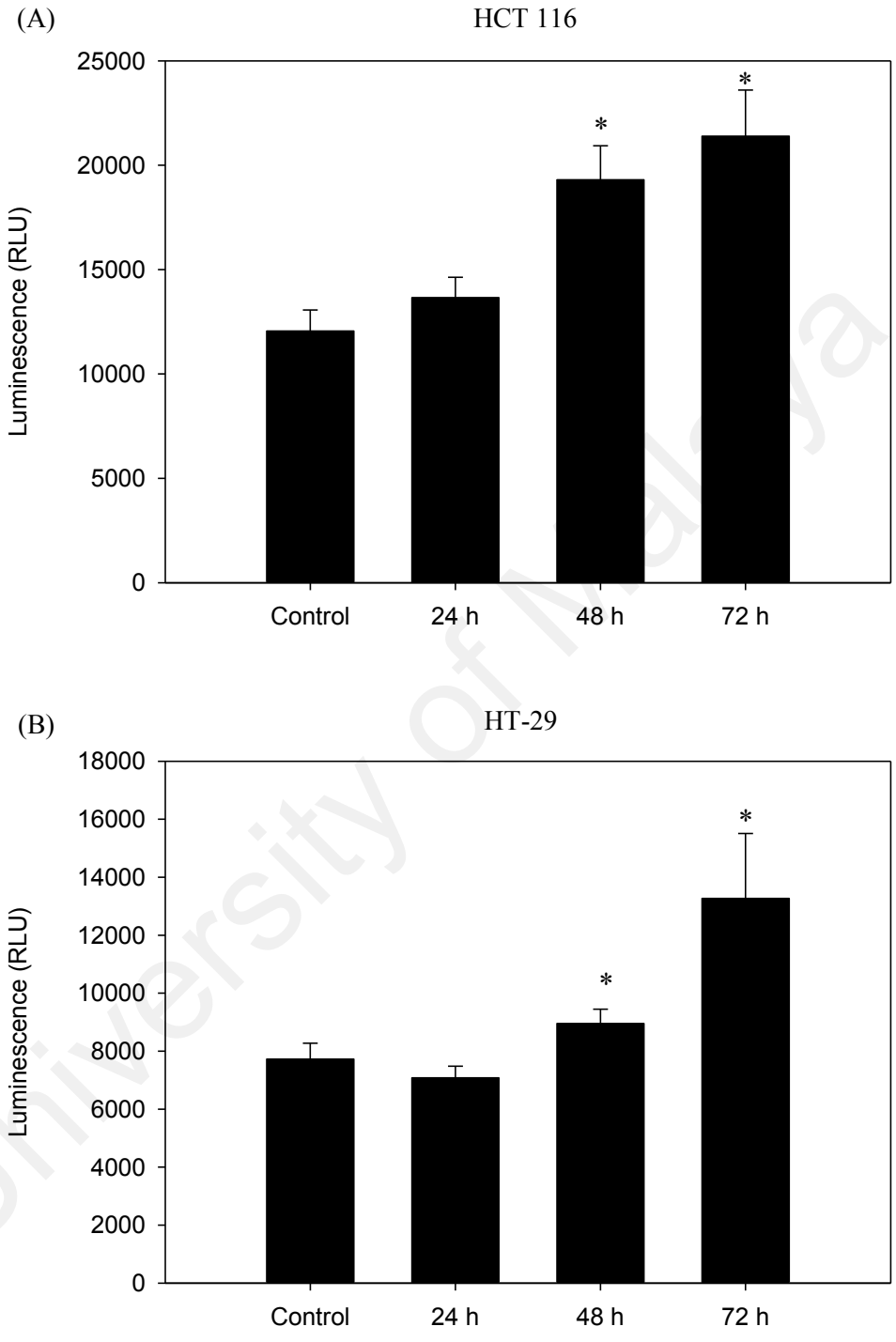


Figure 7.13: Effect of compound **8** on caspase-9 activity. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 µM) for 24-72 h. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

7.3.8 Effect of compound 8 on DNA fragmentation

Caspase-3 cleaves an inhibitor of DNase to activate the enzyme. Upon activation, the caspase-activated DNase will enter the nucleus, resulting in fragmentation of nuclear DNA and the orderly demise of the cells. As the treatment with compound **8** was shown to cause an increase in the activity of caspase-3/7 in HCT 116 and HT-29 cells, we investigated if DNA fragmentation was observed following treatment with compound **8** using the cell death detection ELISA kit. Apoptosis was assayed through the detection of histone-associated DNA fragments that are present in cell lysates.

As shown in Figure 7.14, treatment of the cells with compound **8** caused a time-dependent increase in DNA fragmentation (as reflected by the value enrichment factor which is used as a parameter of apoptosis). Besides, the highest signal was observed after treatment period of 72 h in both HCT 116 and HT-29, which is consistent with the fact that DNA fragmentation is considered to be an event of late apoptosis.

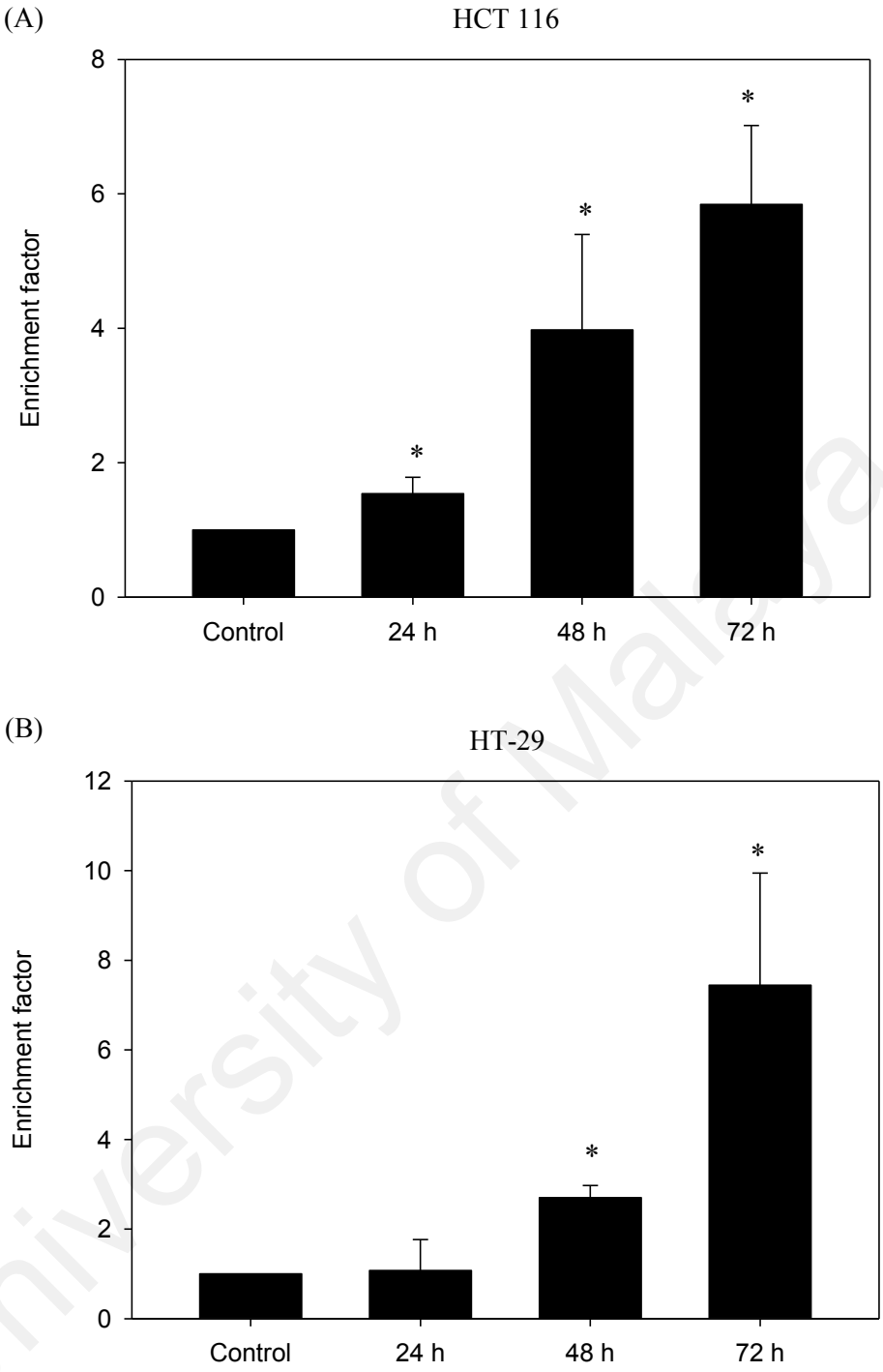


Figure 7.14: Detection of nucleosomes in cytoplasmic fractions of cells treated with compound **8**. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for 24-72 h. Histone-associated DNA fragments were detected using the cell death detection ELISA kit. The enrichment factor (apoptotic index) is a measure of the levels of histone-associated DNA fragments in the lysates of the treated cells compared to the untreated control. Results were expressed as mean ± S.D. (n = 3). Asterisks (*) indicate significant differences between control and treated cells ($p < 0.05$).

7.3.9 Effect of compound 8 on apoptosis-related protein

During apoptosis, PARP, a 116 kDa nuclear poly (ADP-ribose) polymerase, is cleaved by caspase-3 to yield fragments of 89 and 24 kDa. Previously, compound **8** was found to activate caspase-3/7 in HCT 116 and HT-29; hence, we examined the effect of compound **8** on the level of PARP, the substrate for caspase-3. As shown in Figure 7.1, cleavage of PARP was observed when both HCT 116 and HT-29 cells were treated with compound **8** (30 μM) for 72 h.

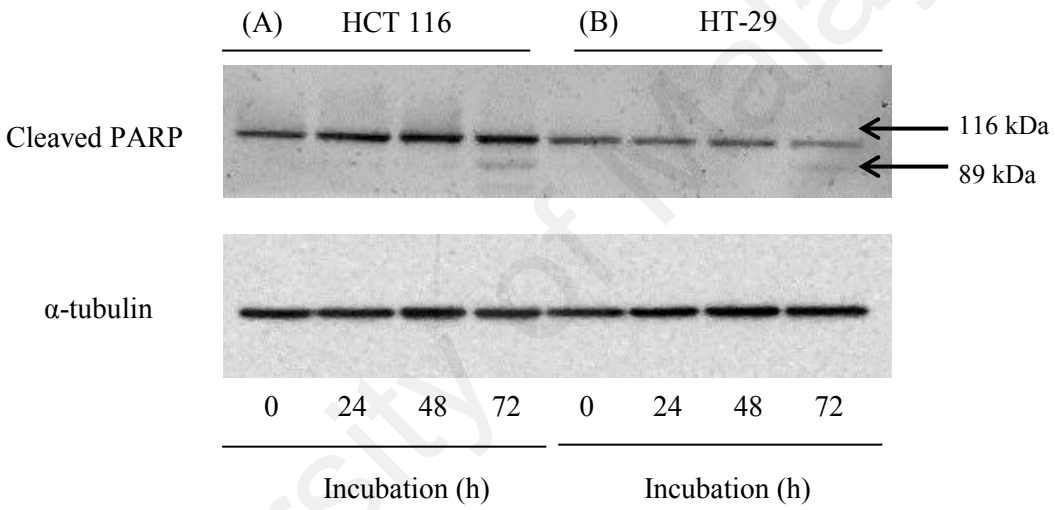


Figure 7.15: Effect of compound **8** on PARP cleavage. (A) HCT 116 and (B) HT-29 cells were treated with compound **8** (30 μM) for different incubation period. Total cellular proteins was extracted, resolved using SDS-PAGE, and immunoblotted with the indicated antibodies. The analysis was repeated twice with similar results and a representative immunoblot is shown.

Proteolytic cleavage of PARP by caspases is considered to be a hallmark of apoptosis. Therefore, our findings thus far indicated that compound **8** induced apoptosis in the both HCT 116 and HT-29 through the activation of caspases.

7.4 Discussion

7.4.1 *Lignosus rhinocerotis* steroidal constituents selectively inhibited the growth of colorectal cancer cell lines

Cytotoxicity screening of *L. rhinocerotis* steroidal constituents against human colorectal cell lines (i.e., HCT 116, HT-29, DLD-1, and CCD-18Co) was carried out. Results from the MTT assay, which measured metabolic activity, and SRB assay, which quantitated cellular proteins, was in agreement with one another and clearly demonstrated the growth inhibitory effect of the compounds on the cell lines. Moreover, suppression of cell proliferation based on the DNA synthesis assay, particularly for the cancer cell lines, correlated with the results of the MTT and SRB assays.

Cytotoxic potential of the compounds after 24 h of incubation, as determined from their IC_{50} values, varied with compound **3** showed strong cytotoxicity (IC_{50}: 33.8-62.7 μM) whereas compound **7** exhibited overall weak cytotoxicity (IC_{50} > 100 μM) against all cell lines. On the contrary, compound **8** was selectively cytotoxic against HCT 116 (IC_{50}: 31.6 μM) and HT-29 (IC_{50}: 50.0 μM) cells with lower cytotoxicity against CCD-18Co (IC_{50} > 230 μM) cells that served as the model of non-cancerous cell line in the present study. When a comparison is made with CCD-18Co, it is to be noted that, under the current experimental condition, compound **8** was approximately 4- and 7-times more cytotoxic to HT-29 and HCT 116 cells, respectively.

The anti-cancer effects and possible mode of actions of compounds **3** and **8** have been extensively investigated (Lee *et al*., 2005; Zhao *et al*., 2011a; Wu *et al*., 2012; Han *et al*., 2014) but the chemotherapeutic potential of compound **7** has not been fully explored. Previously, cytotoxicity of compound **3** against HT-29 (IC_{50}: 18.4 μM, 24 h) (Lee *et al*., 2005) and compound **8** against HCT 116 (IC_{50}: 80 μM, 72 h) (Han *et al*., 2014) were reported. Compound **7** was reported to inhibit the growth of HT-29 cells

after 5 days of incubation but the IC_{50} value was not determined (Kobori *et al*., 2006). Our findings on the cytotoxicity of the compounds are consistent with previous findings but a direct comparison of the values obtained in the present study with those previously reported is not possible due to the differences in the methodology employed (e.g. seeding density, incubation periods, and end-point measurement). Similar to our work, the compounds were also reported in some studies to be more cytotoxic against cancer than non-cancerous counterparts (Chen *et al*., 2009; Kobori *et al*., 2007). One plausible explanation is that the compounds were better at targeting rapidly dividing cancer (e.g. doubling time of HCT 116 and HT-29 cells are < 30 h) while non-cancerous cells were less susceptible because they are usually slow-growing (Mitchison, 2012). Other possible reasons for the selective cytotoxicity of the compounds might be differences in the genetic make-up of the cells and the characteristics of the compounds which may influence the overall effect of the compounds on the cells.

According to Boik (2001), any cytotoxic natural product is considered active against cancer cells *in vitro* when the IC_{50} value is within the range of 1-50 μM. By this criterion, both compounds **3** and **8** were regarded as active against HCT 116 and HT-29 cells; however, after taking selectivity into consideration, only compound **8** was chosen for further studies. Next, we attempted to compare the differences (if any) in the mechanism of action underlying the cytotoxic effect of compound **8** in two colorectal cell lines (i.e. HCT 116 and HT-29). It is to be noted that genetically, HCT 116 is a p53 wild-type line with a defect in DNA mismatch repair machinery but no chromosomal instability whereas HT-29, on the contrary, is a p53 mutant with intact mismatch repair, and chromosomal instability phenotype (Yao *et al*., 2005).

7.4.2 Compound 8 caused cell cycle arrest at G1 phase in colorectal cancer cells

Treatment with compound **8** (30 μM) effectively reduced the percentage of proliferating cells to 58.4 and 38.5% for HT-29 and HCT 116, respectively. As compound **8** was shown to exhibit anti-proliferative activity against HCT 116 and HT-29 cells, we investigated its effect on cell cycle progression. Our results showed that treatment with compound **8** (30 μM) for 12-72 h markedly increased the percentage of cells in the G1 phase for both cell lines with almost 2-fold increase compared to the controls. Our results for HT-29 were compared to the work by Kobori *et al*. (2007) who also worked with HT-29 and reported that compound **8** (7 μM) caused an increase in the percentage of G1 cells (71.1-76.8%) and a reduction in S phase cells (21.9-16.6) after 5 days of treatment. We deduced that a significant increase in the percentage of G1 cells (38.1-85.5% of total cell population) was observed after only 72 h of treatment in the present study presumably due to higher concentration of compound **8** (30 μM) was used. Apart from that, compound **8** was also reported to trigger G1 arrest in phytohemagglutinin-activated T lymphocytes (Kuo *et al*., 2003).

At the molecular level, Kobori *et al*. (2007) in HT-29 found that the cell cycle arrest was associated with an increase in the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A). This gene encodes for protein p21 ($^{CIP1/WAF1}$), a potent cyclin-dependent kinase inhibitor. p21 regulates the cell cycle progression at G1 and S phase by inhibiting the activities of cyclin-CDK2, -CDK1, and CDK4/6 complexes (Shaw, 1996). As an increase in the expression of p21 was found to be responsible for cell cycle arrest at G1 phase based on previous studies, we deduced that the G1 arrest in HT-29 cells treated with compound **8** in the present study was also attributed to the upregulation of p21 that was consistent with the results of Western blot. Moreover, the expression of p53 and p21 proteins were concurrently elevated suggesting that the

compound **8** induced G1 phase cell cycle arrest in HCT 116 through the activation of p53-dependent upregulation of p21.

Another observation in the cell cycle analysis was an increase of hypodiploid cells (sub-G1) in a time-dependent manner from 2.6-14.8% when HCT 116 was treated with compound **8** for 12-72 h. The percentage of sub-G1 cells for HT-29 was maintained at approximately 1.5% under similar conditions. These hypodiploid cells are indicative of apoptotic cells and the differences in the percentage of sub-G1 cells between HCT 116 and HT-29 might be due to former being more sensitive towards compound **8** and apoptosis was triggered as a consequence of the G1 phase arrest. In the case of HT-29, the lack of sub-G1 cells was contrary to the findings of Kobori *et al*. (2006) who reported that treatment with compound **8** (7 μM) caused an increase of sub-G1 cells from 0.7-16.8% after 5 days of treatment. It seemed that longer exposure period, albeit at a lower concentration, was more effective in inducing apoptosis in HT-29.

7.4.3 Compound 8 induced apoptosis in colorectal cancer cells *via* the activation of caspases

The perturbation of cell cycle progression in the cells due to G1 arrest might be responsible for the anti-proliferative activity of compound **8**. The observation of sub-G1 population indicated that apoptotic cell death was induced by compound **8** particularly in HCT 116. Additional assays were carried out to assess the apoptosis-inducing activity of compound **8**. During the early event of apoptosis, the asymmetry of phospholipid is broken whereby the phosphatidylserine, usually present in the inner leaflet of plasma membrane, is translocated to the outer leaflet of the plasma membrane. Detection of apoptotic cells was performed with the annexin V/PI analysis whereby annexin V will bind to the externalized phosphatidylserine in early and late apoptotic

cells. Our results revealed a gradual increase in the number of early and late apoptotic cells in HCT 116 and HT-29 cells and this suggest that apoptosis indeed occurred, albeit at a fairly low rate, when the cells were treated with compound **8** (7 μM) for 24-72 h. Judging from a greater proportion of apoptotic cells, HCT 116 was more sensitive to compound **8** than HT-29 at all time-points investigated. Moreover, the proportion of apoptotic cells observed here are similar to those in sub-G1 peak of the cell cycle progression profiles.

Mitochondria dysfunctional have been reported to be involved in the induction of apoptosis whereby changes in the mitochondrial structure and the collapse in mitochondrial membrane potential were observed during apoptosis (Ly *et al*. 2003). Following the collapse of mitochondria membrane potential, the cells become depolarized. As a consequence, the amount of JC-1 aggregates decreases and the monomeric dye molecules move out from the mitochondria resulting in a drop in the red fluorescence. In our study, treatment with compound **8** in HCT 116 and HT-29 resulted in the increase of depolarized cells in a time-dependent manner. This implied that compound **8** evoked the loss of mitochondria membrane potential in both cell lines but the increase in the percentage of depolarized cells was higher in HCT 116 than HT-29.

Apoptotic cell death can be broadly divided into two pathways, i.e. the extrinsic and intrinsic pathways which involve caspases that are constitutively expressed during the process. Intrinsic (mitochondria-mediated) pathway involves the permeabilisation of the mitochondria resulting in the release of cytochrome *c* into the cytoplasm. This is followed by the formation of the apoptosome complex and activation of the caspase cascade through caspase-9. On the other hand, the extrinsic pathway is activated by death receptors on the plasma membrane. Binding of ligands to these receptors form the death inducing signaling complex that leads to the initiation of the caspase cascade

through caspase-8. Regardless of which pathway is initiated, both will eventually converge and activate caspase-3 and -7 that are regarded as the execution caspase (Fan *et al*., 2005; Elmore, 2007).

Our results showed that, in HCT 116 and HT-29 cells treated with compound **8**, the activity of caspase-3/7 increased in a time-dependent manner. Further, we found that compound **8** also activated caspase-9 but not caspase-8 in both cell lines. This might serve as an indication that the apoptotic cell death triggered by compound **8** is mitochondria-mediated. Activation of caspase-3 triggers the proteolytic cleavage of PARP. As PARP is involved in DNA repair and helps cells to maintain their viability, cleavage of PARP facilitates cellular disassembly and at the same time, serves as a marker of cells undergoing apoptosis. In the present study, PARP cleavage was observed when HCT 116 and HT-29 cells were treated with compound **8** for 72 h, and this correlated with the activation of caspase-3/7 in which the activity was highest at that time point.

The apoptosis-inducing effect of compound **8** in HCT 116 and HT-29 is in accordance with previous findings on other cell lines. For instance, compound **8** (25 μM) induced apoptosis in HL-60 after 24 h of treatment (Takei *et al*., 2005). Chen *et al*. (2009) reported that compound **8** caused morphological changes typical of cells undergoing apoptosis (e.g. condensed and fragmented chromatin) and induced DNA fragmentation in Hep 3B (human hepatocellular carcinoma) cells. In these studies, apoptotic cell death was usually confirmed based on the evidence from gel-based DNA fragmentation assay; however, there is lack of in-depth studies on its mode of action. Previously, Kobori *et al*. (2007) postulated that apoptosis induced by compound **8** in HT-29 cells was caused by an increase in the intracellular reactive oxygen species (oxidative stress). In a more recent work, Han *et al*. (2014) reported that compound **8**

induced cell death in DU 145 cells (human prostate carcinoma) via the death receptor-dependent pathway. Specifically, compound **8** activated caspase-3, caspase-8 without affecting caspase-9, cleaved PARP, attenuated the expression of anti-apoptotic proteins (e.g. Bcl-xL, Bcl-2, and C-FLIP), and activated the expression of pro-apoptotic proteins (e.g. Bax, FADD, and DR5) based on their Western blot results.

On the other hand, our findings seemed to suggest that the cell death induced by compound **8** in HCT 116 and HT-29 was partly mediated by the intrinsic pathway. This postulation is based on our results on the collapse of mitochondria membrane potential following treatment with compound **8**. The loss of mitochondria membrane potential is often associated with the release of cytochrome *c* that would bind with dATP to form apoptosome which in turn binds and activates the procaspase-9 and subsequently, caspase-9 and caspase-3/7 will be activated (Jiang & Wang, 2004). The activation of the caspase-3/7 and -9 following treatments with compound **8** and the subsequent cleavage of PARP by caspase-3 were also observed in the present study. These clearly indicated that compound **8** induced apoptosis in HCT 116 and HT-29 through the activation of caspase-3/7. Nevertheless, the expression of mitochondrial-related pro-apoptotic and anti-apoptotic molecules have to be investigated in order to confirm the involvement of intrinsic pathway. Besides that, the possible role for the involvement of the extrinsic pathway in HT-29 cannot be ruled out at the moment as treatment with compound **8** was shown to have some effect on the activity of caspase-8. Further studies are needed to clarify this.

Our results complement the current understanding on the mechanism of action underlying the *in vitro* cytotoxicity and apoptosis-inducing activities of compound **8,** specifically in colorectal cell lines in which it has not been thoroughly investigated when the study was initiated. Moreover, the broad cytotoxicity of compound **8** against

various cancer cell lines merits further investigation into its anti-cancer potential. A recent study on the *in vivo* anti-tumour effect of compound **8** in mouse xenograft tumour model was reported by Han *et al*. (2014). Treatment with compound **8** significantly inhibited U266 (human myeloma) tumour growth in female BALB/c athymic nude mice. Additional studies, especially on pharmacokinetics and pharmacodynamics, are necessary to develop compound **8** as a potential anti-cancer drug.

7.5 Conclusion

Amongst the selected steroidal constituents, only compound **8** showed selective cytotoxicity against colorectal cancer cell lines. In addressing its mechanism of action, we found that compound **8** induced cell cycle arrest at G1 phase and apoptosis in HCT 116 and HT-29. The induction of apoptosis was associated with the collapse in mitochondria membrane potential, activation of caspase-3/7 and caspase-9, and DNA fragmentation.

CHAPTER 8: GENERAL DISCUSSION

Lignosus rhinocerotis, also known as the tiger's milk mushroom, is a wild medicinal mushroom that is previously available only by collection from the wild; hence, the lack of supply becomes a limiting factor in attempts to investigate its purported medicinal properties. While the methodology for production of mushroom mycelia *via* liquid fermentation is generally well-established (Smith *et al*., 2002; Tang *et al*., 2007; Elisashvili, 2012), cultivation of sclerotium-forming mushrooms *via* solid substrate fermentation is challenging due to the lack of understanding of their life cycle and ecological requirements. All stages of the mushroom's life cycle (i.e. fruiting bodies, sclerotia, mycelia, etc.) are not permanently observable in nature and in fact, most of the time fungi exist as delicate mycelium embedded in the substrates such as rotten woods. The emergence of fruiting bodies, without which the sclerotium cannot be discovered, is dependent on environmental factors such as temperature, humidity, levels of carbon dioxides, and others (Kues & Liu, 2000; Wosten & Wessels, 2006).

Successful domestication of *L. rhinocerotis via* solid substrate and liquid fermentations (as described in Chapter 3) yielded the fruiting body, sclerotium, mycelium, and culture broth that can be used for chemical and pharmacological studies. It was noted that the production of fruiting body relied mainly on the production of correctly formed sclerotium (which acts as a nutrient sink source) under suitable conditions (i.e. buried in the soil and watered). When these criteria are not met (e.g. mycelium-colonized bags that were not buried), fruiting will not occur even though sclerotium was formed. The methodology developed for *L. rhinocerotis* can be optimized to produce other *Lignosus* spp. on a large scale not only for the purposes of commercialization, but also to better understand their ecological roles.

Drug discovery from natural sources (e.g. medicinal plants and mushrooms) has benefited from the incorporation of ethnomedicinal knowledge of traditional practices into the pipeline. The value of ethnobotanical information in drug development is based on our ability to correlate the traditional knowledge (e.g. parts eaten, preparation methods, ailments to be treated, etc.) with corresponding scientific data (e.g. results from biological assays and chemical investigation). In the present study, the idea to screen *L. rhinocerotis* for potential cytotoxic activity derived from its purported use as a folk remedy to treat cancer by the indigenous communities of Peninsular Malaysia (Lee *et al*., 2009a; Noorlidah *et al*. 2009). The use of both aqueous and organic extracts was based on its traditional preparation methods, which were reported to be in the form of decoction, macerated materials, and tincture – representing the main methods for consuming mushrooms for medicinal purposes by the Asian communities (Chee, 1933; Chang & Lee, 2004; Azliza *et al*., 2012).

Prior to these, there are several studies on the cytotoxic activity of *L. rhinocerotis* aqueous extracts but only the sclerotium was utilized (Lai *et al*., 2008; Lee *et al*., 2012; Yap *et al*., 2013). In view of that, the present study was undertaken to expand the currently limited understanding of the anti-cancer effects of *L. rhinocerotis* with regard to active compounds and their possible mode of actions. Our results from the cytotoxicity screening might have provided some scientific evidence to support the traditional use of *L. rhinocerotis* sclerotium in the form of aqueous preparations in treating cancer *via* cytotoxicity, cell cycle arrest, and induction of apoptosis. Specifically, only the sclerotial cold aqueous extracts (i.e. cultivated and wild type samples) consistently exhibited strong cytotoxicity (IC_{50} < 50 μg/ml, 72 h) against most cell lines. Aqueous extracts of other mushroom parts were either weak or inactive (IC_{50} > 100 μg/ml, 72 h) and this seem to correlate with ethnomedicinal records which

mentioned that the sclerotium is the only part used for medicinal purposes. Cytotoxicity screening of *L. rhinocerotis* dichloromethane extracts revealed that, while the extracts prepared from sclerotium also demonstrated cytotoxicity, these were not the most potent. Extracts of the fruiting body, particularly the pileus, showed marked cytotoxicity against most cell lines. Overall, the most potent organic solvent extract, which was the dichloromethane extract of the pileus (LRCP-DE, IC_{50}: 3.8 µg/ml) exhibited approximately 10-fold higher cytotoxicity than the most potent aqueous extract (LRSC-CAE, IC_{50}: 36.8 µg/ml) against MCF7 which was observed to be one of the most susceptible cell lines.

Our findings also yielded information useful for *L. rhinocerotis* bioprospecting. On the basis of chemical profiles and bioactivities, the cultivated samples appeared to be good substitutes for the wild type samples. We observed that, in general, the chemical constituents were comparable between both groups although slight variations can be found. We also noted that some of the wild type samples showed greater cytotoxicity but the exact reason for this observation is still unclear. It could be due to factors such as geographical locations, stage of maturity, and others. One of the limitations in the current study is that the exact developmental stages and geographical origin of the wild type samples were not known so it is more difficult for us to hypothesize the exact reasons for the differences (as discussed in Chapter 5). Secondly, wild type samples are not uniform in terms of the chemical components and biological activities (based on a comparison of our results and previously reported findings). Looking at this factor, the cultivated samples obviously offer more advantages in terms of uniformity of genetic materials and chemical profiles. One of the ways to tackle the problem of difference in potency (e.g. cultivated mushrooms are less potent than wild type samples in most cases) would be to profile the active components from various developmental ages in the

maturation of the fruiting body and sclerotium of *L. rhinocerotis*. By identifying the chemical components to be used as markers, the cultivation process could be improved so that harvesting could be done at a time point when the bioactive components are most abundant.

It has been shown that *L. rhinocerotis* from different cultivation techniques and developmental stages did not demonstrate the same bioactivity and this was explained by the dissimilarity in their chemical profiles. Focusing on cytotoxic compounds, the mycelium and culture broth appeared to be inferior to the fruiting body and sclerotium, regardless of low- or high-molecular-weight compounds. Nevertheless, as the condition of liquid fermentation could be further optimized to enhance the production of metabolite of interest within a short time, the possibility of using mycelium as biomass is still promising. While the chemical components in the mycelium are likely to be different than that of the sclerotium, some of the cytotoxic steroids identified in this study could also be produced by the mycelium as exemplified by the isolation of 9,11-dehydroergosterol peroxide (**7**) and ergosterol peroxide (**8**) from liquid cultures of *G. lucidum* (Chen *et al*., 2009). The biosynthetic pathways for the production of steroids are present at all developmental stages as these also produce other compounds that might be needed for fungal growth.

From the chemical-ecological perspective, the production of cytotoxic components is usually for defense against predators and other microorganisms. The cultivated sclerotium retained the cytotoxic proteins that were found in wild type samples this is likely to suggest that the cultivation method *via* solid-substrate fermentation (as described in Chapter 3) were comparable to the growing condition in the wild as the mycelium-colonized substrate bags were buried in the soil, exposing them to antagonistic microorganisms. Similarly, this might also explain the lack of cytotoxic

proteins from other samples such as fruiting body and mycelium where antagonistic microorganisms were absent.

Chemistry-wise, the nature of active components in *L. rhinocerotis* aqueous and organic solvent extracts differed significantly. Cytotoxic activity of the sclerotial cold aqueous extracts was attributed to some water-soluble, heat-labile protein/peptide(s) although the possibility of other components, such as polysaccharides, polysaccharide-protein complexes, and glycoproteins could not be totally ruled out at this point. The fact that the cold aqueous extracts retained its cytotoxicity even after heat treatment up to 60 °C seemed to suggest that these components were relatively stable and could be extracted at room temperature as well. This corresponded well to the aqueous preparations based on the grinding method described by Chee (1933). On the other hand, the hot aqueous extract, which mimic the traditional preparation method in the form of decoction, were not cytotoxic. Based on the previous findings, the hot water extract contained high-molecular-weight components, possibly polysaccharides which could act as anti-cancer agents by modulating the immune system (Wong *et al*., 2011).

For therapeutic purposes, *L. rhinocerotis* sclerotial cold aqueous extract (LRSC-CAE) is comparable to the protein fraction (CAE-P) in terms of cytotoxicity, and this might indicate that proteins were likely to be the major cytotoxic component in the sclerotium. LRSC-CAE even showed higher cytotoxicity compared to *G. lucidum* and *L. tuber-regium* in which both have been reported to contain cytotoxic polysaccharide and polysaccharide-protein components with immunomodulatory effects (as reviewed by Lin, 2005; Zhang *et al*., 2007; Wong & Cheung, 2008b; Xu *et al*., 2011b; Ren *et al*., 2012; Ferreira *et al*., 2015). The dichloromethane extracts of *L. rhinocerotis* which demonstrated cytotoxicity contained mainly lipophilic low-molecular-weight compounds, including ergone (**3**) that has been shown to induce apoptosis in other cell

lines (Zhao *et al*., 2011a). The higher cytotoxic potency (lower IC_{50} values) of the dichloromethane extract of *L. rhinocerotis* pileus (LRCP-DE) than that of *G. lucidum* fruiting body (known to contain cytotoxic lanostane-type triterpenoids, e.g. ganoderic acids) formed the basis for further chemical investigations to isolate the active compounds in LRCP-DE.

In the current study, the stronger cytotoxicity of LRCP-DE (dichloromethane extract of pileus, IC_{50}: 3.8 µg/ml) than LRSC-CAE (cold aqueous extract of sclerotium, IC_{50}: 36.8 µg/ml) has prompted us to prioritize the former for purification work. Chemical investigation directed to LRCP-DE resulted in the identification of ergone (**3**) and methyl ester of fatty acids (**4-6**), and the purification of two sterol peroxides, i.e. 9,11-dehydroergosterol peroxide (**7**) and ergsoterol peroxide (**8**). We believe that these represent only some of the cytotoxic compounds from LRCP-DE as the chemical constituents in some other fractions were not isolated due to lower potency (relative to the fraction containing 9,11-dehydroergosterol peroxide and ergosterol peroxide), time constraint and/or low yield. With the advances in chromatographic techniques, it is plausible that more chemical constituents, including those with novel structures, can be isolated from *L. rhinocerotis*. The protein fraction derived from LRSC-CAE, termed as CAE-P (IC_{50}: 14.9-19.6 µg/ml) showed slightly higher cytotoxicity against cancer cell lines than the crude extract (IC_{50}: 26.2-34.3 µg/ml) and this seemed to indicate that that chemical fractionation and precipitation techniques have minimal effect on the purification of active proteins. This necessitates the use of other chromatographic separations, such as ion-exchange and gel filtration, to isolate the cytotoxic protein/peptide(s). The comparable bioactivity and low yield are some of the reasons why the mechanistic study for aqueous components (e.g. cell cycle and apoptosis

analysis) was carried on the crude sclerotial cold aqueous extract, rather than the fractions.

Bioassay-guided isolation approach resulted in the purification of known compounds with known activities for most of the time, including in our study. As such, alternatives, such as de-replication approaches might be employed to eliminate fractions with known compounds in the earlier part of the isolation work (Cordell & Shin, 1999; Gaudencio & Pereira, 2015). Alternatively, fractions containing compounds without known activity can be emphasized in the chemical studies. While the pharmaceutical industries require pure compounds for drug developments, some also begin to look into the advantages of using extracts which were regarded as "wholesome" form. Proponents of this view put forward several points, such as shorter time and lower cost of production. Moreover, the use of extracts directly mimics the traditional practices of indigenous people of Peninsular Malaysia in the case for *L. rhinocerotis*. The effectiveness of mushroom crude extracts has been demonstrated in many studies; for instance, the ethanol extracts of *Pleurotus ferulae* (Wang *et al*., 2014b) and *Fomitopsis pinicola* (Sw.) P. Karst. (Wu *et al*., 2014) were recently reported to inhibit cancer cell growth *in vitro* and reduced tumour growth *in vivo*. In many cases, the observation was attributed to the presence of multiple bioactive compounds and the additive and synergistic effects of chemicals might be responsible for potent effects (Liu, 2004). In addition, synergism purportedly increases the therapeutic effect by blocking targets in the signal transduction pathway, increasing the bioavailability of the other drug or stabilizing the other drugs (Hemalswarya & Doble, 2006). Nevertheless, it has to be acknowledged that our understanding on synergisms still remain far from complete.

On the other hand, the use of crude extracts also possessed some limitation, mainly pertaining to the reproducibility of the results. This is exemplified by the contrasting

findings on the cytotoxicity of the sclerotial cold aqueous extract against selected non-cancerous cells (e.g. NL20, 184B5, and MRC-5, as discussed in Chapter 4). While identifying the possible reasons that could explain the differences is crucial, we feel that the inclusion of a chemical fingerprint of the extracts to be of utmost importance as chemical composition is usually responsible for differences in the observed bioactivity. Chemical fingerprints revealed information such as differences in major components and levels that aid in correlating the activities with chemical composition for extracts from different studies.

Cancer cells are characterized by defects that render them insensitive to apoptotic signals whereby dysregulation of apoptosis causes excessive cell growth and little cell death, leading to unlimited growth of cancer cells. Apparently, apoptosis is one of the most important targets for cancer treatment (Kasibhatla & Tseng, 2003; Brown *et al*., 2005; Gerl & Vaux, 2005; Hassan *et al*., 2014). Therefore, restoration of the apoptosis machinery in cancer cells can be a useful strategy in chemotherapeutics. Indeed, many of the conventionally used chemotherapeutic agents including natural products exert their anti-cancer effects largely through the induction of apoptosis in cancer cells (Debatin, 2000; Millimouno *et al*., 2014). Our findings have clearly shown that the extracts and chemical constituents of *L. rhinocerotis* induced apoptosis in a number of cell lines. Interestingly, the sclerotial cold aqueous extract and dichloromethane extract of the pileus, although contained active components of different chemical characteristics, induced cell cycle arrest and apoptosis. Treatment with the extracts and compounds caused a conspicuous increase in G1 phase and followed by sub-G1 percentage. These results seem to suggest that the cells were arrested in G1 phase and followed by the accumulation of fragmented cells with reduced DNA content. The

apoptotic cell death was also confirmed by evidence from other analyses, such as annexin V/PI and DNA fragmentation assays.

The low-molecular-weight compounds attract more interest as these are known to act on multiple pathways related to carcinogenesis (Ferreira *et al*., 2010; Patel & Goyal, 2012). The broad spectrum of the potential anti-cancer effects of the steroidal constituents (e.g. pro-apoptotic, anti-angiogenesis, immuno-stimulatory) has been previously documented. Although the mechanism of action of the steroidal constituents in other cell lines have been reported, findings from the current study improved our understanding on the mode of action of these compounds in colorectal cell lines which, to the best of our knowledge, have not been reported. Ergosterol peroxide (**8**) was shown to induce cell cycle arrest at G1 phase and apoptosis in HCT 116 and HT-29. The activation of caspase family of proteolytic enzymes plays an important role in apoptosis triggered by pro-apoptotic stimuli. Owing to its role as a critical executioner of apoptosis, caspase-3 is responsible for the proteolytic cleavage of many key proteins such as PARP. The observation of a typical 89 kDa PARP cleavage product, a hallmark of apoptosis further confirmed the proteolytic activity of caspase-3. Activation of the apoptotic signaling pathways by mushroom extracts and chemical constituents is not uncommon. For instance, ergone (**3**) was previously reported to induce apoptosis through the intrinsic and extrinsic pathways (Zhao *et al*., 2011a) whereas ergosterol peroxide (**8**) induced cell death in DU 145 cells via the extrinsic pathway (Han *et al*., 2014). Based on our results, the apoptosis-inducing activity of ergosterol peroxide (**8**) in human colorectal cell lines was suggested to be mediated by mainly the intrinsic pathway of apoptosis but further exploration of the relevant molecular targets in both pathways will confirm this.

CHAPTER 9: CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

Successful domestication of *L. rhinocerotis via* optimized solid substrate and liquid fermentations yielded the fruiting body, sclerotium, mycelium, and culture broth to be used in bioprospecting for potential cytotoxic compounds in the present study. Overall, our findings have provided insights into the cytotoxic and apoptosis-inducing activities of the aqueous and organic solvent extracts, and chemical constituents of *L. rhinocerotis*. From more than 40 extracts screened for cytotoxic activity against a panel of cancer and non-cancerous cell lines, cold aqueous extract of the sclerotium (LRSC-CAE) and dichloromethane extract of the pileus (LRCP-DE) were identified as the most potent extract from the aqueous and organic solvent extracts, respectively. Both extracts inhibited the growth of MCF7 (breast adenocarcinoma) and HCT 116 (colorectal carcinoma) *via* G1 phase cell cycle arrest and induction of apoptosis. The nature of cytotoxic compounds in both extracts, however, differed with LRSC-CAE contained heat-labile, protein/peptide(s) while LRCP-DE comprised of mainly low-molecular-weight, lipophilic constituents. Further chemical investigation directed to LRCP-DE resulted in the identification of ergone (**3**) as well as the isolation of 9,11-dehydroergosterol peroxide (**7**) and ergosterol peroxide (**8**). Ergosterol peroxide (**8**) exhibited selectivity in its cytotoxic action against cancer colorectal cell lines (IC50: 30.0-50.0 μM) than the non-cancerous counterpart, CCD-18Co (IC_{50} > 230 μM). Mechanistically, ergosterol peroxide (**8**) induced cell cycle arrest at G1 phase and triggered apoptotic cell death in HCT 116 and HT-29 that was associated with the collapse of mitochondria membrane potential, activation of caspase -3/7 and -9, and DNA fragmentation. In conclusion, the nature of cytotoxic components in the sclerotium (protein/peptides) differed from that of the pileus (sterol epoxides), and

selected extracts and chemical constituents of *L. rhinocerotis* inhibited cancer growth by causing apoptotic cell death. From the ethnomedicinal point of view, our results validated the traditional uses of *L. rhinocerotis* for treating cancer but at the same time, possible uses of mushroom parts other than the sclerotium as sources of cytotoxic compounds with potential application as chemotherapeutics was highlighted. Furthermore, our findings also provided insights into the selection of appropriate samples of *L. rhinocerotis* for the development and evaluation of potential therapeutics for cancer therapy.

Some recommendations for future work are as follows:

In depth chemical investigations should be carried out to identify additional cytotoxic components from *L. rhinocerotis*. The specific protein/peptide(s) responsible for the cytotoxic and apoptosis-inducing activities of the sclerotial cold aqueous extract should be isolated using chromatographic techniques and characterized using mass-spectrometry. The mechanism of action should be elucidated and compared to that of the steroidal constituents investigated in the present study. Besides that, exhaustive isolation of secondary metabolites from *L. rhinocerotis* should be carried on, for instance, the aqueous fraction of the dichloromethane extract of the pileus that was not investigated further in the present study.

Our results have shown that the extracts and steroidal constituents of *L. rhinocerotis* were able to inhibit the growth and induce apoptosis in selected human cancer cells, such as MCF7, HCT 116, and HT-29; however, it is not known whether the concentrations effective for these biological effects can be achieved *in vivo*. Therefore, further *in vivo* evaluation of these extracts and chemical constituents on xenograft tumour models is warranted.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of Publications

Lau, B. F., Abdullah, N., Aminudin, N., Lee, H. B., and Tan, P. J. (2015). Ethnomedicinal uses, pharmacological activities, and cultivation of *Lignosus* spp. (tiger's milk mushoroms) in Malaysia – a review. *Journal of Ethnopharmacology, 169*: 441-458.

Lau, B. F., Abdullah, N., Aminudin, N., Lee, H. B., Yap, K. C., and Sabaratnam, V. (2014). The potential of mycelium and culture broth of *Lignosus rhinocerotis* as substitutes for the naturally occurring sclerotium with regard to antioxidant capacity, cytotoxic effect, and low-molecular-weight chemical constituents. *PLoS ONE, 9*(7): e102509. doi:10.1371/journal.pone.0102509.

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Lau, B. F., Abdullah, N., Aminudin, N., and Tan, A. B. B. (2013). Proteomic approaches for mushroom protein profiling: ProteinChip and SELDI-TOF complement electrophoretic analysis. *Journal of Health and Translational Medicine, 16*(3): 63.

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List of Oral Presentations

Lau, B. F., Abdullah, N., Aminudin, N., Patel, V., Tan, P. J., and Samat, N. Evaluation of *Lignosus rhinocerotis* (tiger's milk mushroom) sclerotial extracts for anticancer properties using HCT 116 human colorectal carcinoma cells. International Anatomical and Biomedical Scientific Conference (IABS 2015), 18th-20th August 2015, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia.

Lau, B. F., Abdullah, N., Aminudin, N., Lee, H. B., Tan, P. J., and Lim, S. H. The dichloromethane fractions of *Lignosus rhinocerotis* (Cooke) Ryvarden from submerged fermentation suppress proliferation and induce apoptosis in cancer cells. International Congress of the Malaysian Society for Microbiology (ICMSM 2013), 12th-15th December 2013, Langkawi Lagoon Resort, Kedah, Malaysia.

Lau, B. F., Abdullah, N., Aminudin, N., and Lee, H. B. Potential of mycelia and culture broth of the tiger's milk mushroom as source of nutraceuticals and substitute for the naturally occurring sclerotia. International Conference on Natural Products (ICNP 2013), 4th-6th March 2013, Shah Alam Convention Centre, Selangor, Malaysia.

Lau, B. F., Abdullah, N., Aminudin, N., and Lee, H. B. The nature of cytotoxic components from aqueous extracts of sclerotia and mycelia biomass of an indigenous macrofungi, *Lignosus rhinocerotis* (Cooke) Ryvarden. National Postgraduate Seminar (NPS 2015), 11th July 2012, Research Management and Innovation Complex, University of Malaya, Kuala Lumpur.

List of Poster Presentations

Lau, B. F., Abdullah, N., Aminudin, N., Tan, P. J., Lee, H. B., and Patel, V. Evaluation of the antiproliferative effect of *Lignosus rhinocerotis* sclerotial extracts against the human breast adenocarcinoma, MCF7. Joint Malaysia-UK Symposium on Natural Product Chemistry and Drug Discovery, 13th December 2014, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia.

Lau, B. F., Abdullah, N., Aminudin, N., and Lee, H. B. Application of SELDI-TOF-MS for profiling and characterization of proteins in aqueous extracts of *Lignosus rhinocerus* in relation to their *in vitro* cytotoxic effect against several cancer cells. 37th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology, 18th-19th July 2012, Sime Darby Convention Centre, Kuala Lumpur, Malaysia.