CYTOTOXIC AND ANTI-MIGRATION PROPERTIES OF PROTEINS FROM *Pleurotus tuber-regium* (Fr.) Singer AND *Termitomyces heimii* Natarajan AGAINST HUMAN BREAST MDA-MB-231 AND COLON HCT-116 CANCER CELLS

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

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CYTOTOXIC AND ANTI-MIGRATION PROPERTIES OF PROTEINS FROM *Pleurotus tuber-regium* (Fr.) Singer AND *Termitomyces heimii* Natarajan AGAINST HUMAN BREAST MDA-MB-231 AND COLON HCT-116 CANCER CELLS

ABSTRACT

The discovery and identification of new safe drugs that are specifically cytotoxic against cancer cells becomes an important target in cancer research. Increasing number of cancer deaths around the world are associated with metastasis and lack of specificity and effectiveness of current treatment. In developed countries such as Korea, Japan and China, mushrooms have been used for centuries for medicinal purposes against cancer. Numerous studies have reported the vast medicinal values of proteins isolated from mushrooms including anti-tumour and anti-cancer effects. The present study aims to investigate the potential of protein extracts from two Basidiomycetes species which are *Pleurotus tuber-regium* (Fr.) Singer (sclerotium and fruiting bodies) and *Termitomyces heimii* Natarajan (fruiting bodies) for anti-tumour effects as well as the mechanism of action against targeted cancer cells. Protein extraction from *P. tuber-regium* sclerotium, *P. tuber-regium* fruiting bodies and *T. heimii* fruiting bodies were conducted using ammonium sulphate precipitation technique. The protein fractions were determined based on different salt/ammonium sulphate concentration at 30%, 60% and 90%. All protein fractions of were assessed for cytotoxicity towards breast cancer cell lines (MDA-MB-231), colorectal cancer cell lines (HCT-116) and normal lung cell lines (MRC-5) in MTT assay. Potential protein extracts were identified based on the IC_{50} values and selectivity index (SI) on both cancer cells and normal cells. IC_{50} values are the extract concentrations required to inhibit half of the cells growth and SI values are the measure of safety level of drugs/compounds/extracts against normal cells as referred to cancer cells. Extracts with IC_{50} values less than 20 µg/ml are considered to be actively cytotoxic against target cells

after 48-72 hours of incubation time, while SI value greater than 3 indicates potential selectivity of extracts or compounds towards cancer cells as compared to the normal cells. The most promising protein extract was PS60 of *P. tuber-regium* sclerotium with the lowest IC_{50} value (IC_{50}=0.75±0.57 µg/ml) against MDA-MB-231 cells and with minimum cytotoxicity towards the normal lung cells (MRC-5) at SI value of 14. Cellular migration through tissues is one of the important events in metastasis. Ability of the mushroom protein extracts to inhibit such migration was evaluated using the scratch wound assay. Cytotoxicity and anti-migration (MC_A) effects on cancer cells were best exhibited by PS60 of *P. tuber-regium* sclerotium with MC_A reading ranging between 5.6581±0.2015 nm/h and 5.4142±0.6916 nm/h. The purification of PS60 (*P. tuber-regium* sclerotium) using LCMS-Q-TOF MS, had successfully isolated several different proteins, two of which are identified as kinesin-like protein and keratin type 1, cytoskeletal 10. PS60 was then further evaluated for apoptotic effects. PS60 was shown to exert cytotoxic effects associated with the induction of apoptosis and cell cycle arrest in MDA-MB-231 cells at G1/G0 and S-phase. In conclusion, PS60 protein of *P. tuber-regium* sclerotium have good potential to be developed into novel anti-tumour drugs against breast cancer.

Keywords: anti-tumour, cancer, metastasis, *P. tuber-regium,* cytotoxic proteins

PROTEIN SITOTOKSIK DAN ANTI-MIGRASI DARIPADA *Pleurotus tuber-regium* (Fr.) Singer DAN *Termitomyces heimii* Natarajan TERHADAP SEL KANSER PAYUDARA MDA-MB-231 DAN SEL KANSER KOLOREKTAL HCT-116

ABSTRAK

Penyelidikan berkaitan penemuan sumber ubat-ubatan yang selamat dan mengandungi kesan toksik terhadap sel kanser menjadi sasaran utama dalam kajian terhadap kanser. Kes kematian yang semakin meningkat akibat kanser di seluruh dunia boleh dikaitkan dengan metastasis, kekurangan ubatan khusus, serta rawatan yang kurang berkesan. Di negara maju seperti Korea, Jepun dan China, cendawan telah digunakan bagi tujuan perubatan terhadap kanser sejak beratus tahun yang lalu. Banyak kajian mendapati protein yang dipencil dari cendawan mengandungi nilai perubatan yang tinggi termasuk kesan anti-tumor dan anti-kanser. Kajian ini dijalankan untuk menyiasat potensi ekstrak protein daripada dua spesies Basidiomycetes iaitu *Pleurotus tuber-regium* (Fr.) Singer (sklerotium dan jasad berbuah) dan *Termitomyces heimii* Natarajan (jasad berbuah), terhadap kesan anti-tumor dan mekanisme tindakannya ke atas sel kanser. Proses pengekstrakan protein daripada sklerotium *P. tuber-regium,* jasad berbuah *P. tuber-regium,* dan jasad berbuah *T. heimii* dilakukan dengan teknik pemendakan ammonium sulfat. Semua fraksi protein ditentukan berdasarkan kepekatan garam/ ammonium sulfat pada kadar 30%, 60% dan 90%. Fraksi protein tersebut seterusnya diuji untuk mengetahui tahap ketoksikan terhadap sel kanser payudara (MDA-MB-231), sel kanser kolorektal (HCT-116) dan sel normal paru-paru (MRC-5) melalui ujian MTT. Ekstrak protein yang berpotensi dikenalpasti melalui nilai purata IC_{50} dan indeks selektiviti (SI) terhadap kedua-dua sel kanser dan sel normal. Nilai IC_{50} ialah kepekatan ekstrak yang merencatkan 50 peratus daripada pertumbuhan sel. Manakala indeks selektiviti ialah pengiraan nilai purata sel normal terhadap sel kanser bagi penentuan kadar selamat sesuatu

ubatan/sebatian/ekstrak. Ekstrak dengan nilai IC_{50} kurang daripada 20 μg/ml dianggap aktif pada sifat sitotoksiknya terhadap sel yang disasar selepas inkubasi selama 48-72 jam. Manakala nilai SI melebihi 3 menunjukkan sesuatu ekstrak atau sebatian berpotensi selektif terhadap sel kanser berbanding sel normal. Ekstrak protein yang paling berpotensi ialah PS60, protein daripada sklerotium *P. tuber-regium* yang menunjukkan nilai terendah IC_{50} (IC_{50}=0.75±0.57 μg/ml) terhadap sel MDA-MB-231, dan aktiviti sitotoksik rendah terhadap sel normal paru-paru (MRC-5) dengan nilai SI bersamaan 14. Kebolehupayaan migrasi sel merupakan salah satu faktor penting dalam proses metastasis. Ujian luka calar dijalankan untuk menentukan aktiviti anti-migrasi oleh kesemua ekstrak protein cendawan. Kesan sitotoksik dan anti-migrasi (MC_A) telah ditunjukkan oleh PS60, sklerotium *P. tuber-regium*, dengan bacaan MC_A di antara 5.6581±0.2015 nm/j dan 5.4142±0.6916 nm/j. Proses penulenan PS60 (sklerotium *P. tuber-regium*) menggunakan LCMS-Q-TOF MS, telah berjaya mengasingkan beberapa protein yang dua daripadanya dikenalpasti sebagai protein bak kinesin dan keratin jenis 1, sitoskeletal 10. Kajian seterusnya telah dilakukan ke atas PS60 untuk melihat kesan ke atas apoptosis. Sifat sitotoksik pada PS60 adalah berpunca daripada faktor apoptosis dan perencatan kitaran sel MDA-MB-231 pada fasa G1/G0 and S. Kesimpulannya, kajian ini menunjukkan bahawa PS60, sklerotium *P. tuber-regium* berpotensi sebagai ubat anti-tumor yang novel terhadap kanser payudara.

Katakunci: anti-tumor, kanser, metastasis, *P. tuber-regium,* protein sitotoksik

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	Description
ACN	Aceonitrile
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CO_2	Carbon dioxide
CT	Computherised Tomography
Da	Dalton
DCM	Dicloromethane
ddH_2O	Deionised distilled water
DMSO	Dimethyl sulfoxide
DMEM	Dulbelco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
eg.	Example
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum

FIP	Fungal Immunomodulatory Proteins
FPLC	Fast Protein Liquid Chromatography
FU	Fluorouracil
g	Gram
h	hour
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HPV	Human papilloma virus
IC	Inhibitory Concentration
kDa	Kilodalton
L	Litre
LCMS	Liquid Chromatography Mass Spectrometry
MC_A	Migration Capability
mg	Miligram
ml	Mililitre
mm	Milimetre
mM	Micrometre
MTT	3-4(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

M_w	Molecular weight
NaCl	Sodium Chloride
nm	Nanometre
OP	Optical Density
PDA	Potato Dextrose Agar
PET	Positron Emission Tomography
PSP	Polysaccharopeptide
Q-TOF	Quadrupole Time-of-flight
RIP	Ribosome Inactivating Proteins
RNA	Ribonucleic acid
rpm	Revolution per minute
S.E.M	Standard error of mean
SI	Selectivity Index
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPSS	Statistical Package for the Social Sciences
TFA	Trifluoroacetic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
USA	United States of America

USD	United States Dollar
UV	Ultra violet
WHO	World Health Organization
µg	Microgram
$(NH_4)_2SO_4$	Ammonium sulphate
%	Percentage
α	Alpha
β	Beta
°C	Degree celcius

CHAPTER ONE

INTRODUCTION

Cancer is the second leading cause of death worldwide causing significant economic burden and social impact on the community. In 2018, the International Agency for Research on Cancer estimated that there were 18.1 million new cases and 9.6 million of death occurred due to cancer. The top 10 global cancer types among men and women were lung cancer (11.6%), breast cancer (11.6%), colorectal cancer (10.2%), and prostate cancer (7.1%), while the highest mortality were lung cancer (18.4%), followed by colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) (Bray *et al*., 2018).

According to The Malaysian Study on Cancer Survival (MyScan), cancer is the fourth largest cause of death in Malaysia which contributes to 12.6% of all deaths in government hospitals and 26.7% in private hospitals. Approximately 37,000 of newly diagnosed cancer cases were reported annually and it is estimated to rise to more than 55,000 cases by the year of 2030 (National Cancer Registry, 2018). There has been significant increase in the trend of newly diagnosed cancer cases in 2012 to 2016. Majority of cancer cases in Malaysia were detected at later stages with the overall proportion of cases diagnosed at advanced stages (stage III and IV) which increased from 58.7% (2007-2011) to 63.7% (2012-2016), thus reducing the chances of survival in patients. Besides, public awareness on cancer prevention, early detection and treatments were considered low among Malaysians that lead to various screening programmes, campaigns and seminar organised by the government and private sectors (Azizah *et al*., 2019).

Tremendous works have been done in cancer research treatments and clinical applications, but yet the incidence of cancer is still on the rise (Mou *et al*., 2011). To date, there is still lack of specificity and effectiveness of present treatment methods. Breast cancer and colorectal cancer are those which contributed to the largest percentage of cancer deaths worldwide (Ebrahimi Nigjeh *et al*., 2013; Siegel *et al*., 2017). In Malaysia, breast cancer incidence is the most common among female which accounted for 19.0 % of all cancers. In contrast, colorectal cancer is the highest (13.5%) among males and the second most common of all cancer incidence (Azizah *et al*., 2019). Both cancers are also leading in the percentage of patients who presented at late stage, thus increasing the risk of death.

The cancer treatments involve a multidisciplinary teamwork. Surgery remains the mainstay of treatments for breast cancer and colorectal cancer diseases. This is followed by chemotherapy, radiotherapy, biological therapy, hormone therapy, and palliative care (Friese *et al*., 2017; Hassan *et al*., 2017). Breast surgery has been reported to reduce the risk of death and chemotherapy has shown to improve patients' survival rate. However, those treatments are associated with side-effects such as nausea/vomiting, diarrhea, constipation, pain, and asthma (Friese *et al*., 2017).

Current cancer treatments are more challenging as the cancer cells are becoming more resistant to anti-cancer drugs. Scientists have put so much effort in research and clinical studies to find the treatment and remedies against cancer. Therefore, the best and ideal chemotherapeutic drugs are ones that are selective to only cancer cells preventing carcinogenesis without causing injury to normal cell. Also, prevention of migration of cancer cells by a drug is another important strategy to prevent the cancer cells from spreading.

Throughout the history of medicine, abundant of drugs from natural origins have proven to be effective in treating and controlling the risk of chronic diseases, including cancer, cardiovascular diseases, diabetes etc. (Morgan *et al*., 2008; Mou *et al*., 2011). According to WHO, about 30-50% of all cancers can be prevented by healthy lifestyle, dietary modification, and avoiding the risk factors such as radiations, infectious agents, carcinogens and environmental pollution (WHO, 2020). Therefore, nutritional practice plays a crucial role in cancer prevention and risk reduction by consumption of high nutrition foods containing elements such as antioxidants, vitamin A, C, E, selenium like folic acid, are believed to help in decreasing the risk of lung, breast, colorectal, prostate cancer (Mut-Salud *et al*., 2016; Pieroth *et al*., 2018). Advanced knowledge and informative technologies have also led to increasing demand on natural products.

In many parts of the world, mushrooms served as a functional food rich in dietary fibre, vitamins, minerals and phytochemicals. Its unique taste is considered as a delicacy and numerous researches have investigated various biologically active compounds extracted from mushrooms. In countries such as Korea, Japan and China, mushrooms have been used since hundreds of years ago for medicinal purposes against cancer (Daba & Ezeronye, 2003; Khan *et al*., 2010).

Mushrooms contain a huge number of biologically active compounds such as polysaccharides, dietary fibres, oligosaccharides, triterpenoids, peptides and proteins, alcohols and phenols, and minerals (Pardeshi & Pardeshi, 2009). The composition of mushrooms generally consists of 50-65% total carbohydrate, 19-35% proteins, and 2-6% fat content of dry matter (Rathore *et al*., 2017). Cytotoxic proteins such as lectins and glycan binding proteins are reported to be of potential significance in cell biology, especially tumour cell physiology, and as toxic components to fight against cancer cells (Tonevitsky *et al*., 1991; Barbieri *et al.,* 1993; Vallera, 1994; Zhang *et al*., 2017).

New safe drugs that are specifically cytotoxic against cancer cells become important targets of most researchers. In addition, mushrooms can be categorised as functional food and/or converted into supplement to maintain a good health and prevention of cancer. It is estimated that only 10% from 14,000 mushroom species are being explored in which 2,000 are safe for human consumption and about 650 possess medicinal properties (Thatoi & Singdevsachan, 2014). Approximately 200 species of higher Basidiomycetes were found to exhibit anti-tumour activity against different cell lines (Poucheret *et al*., 2006). This means that there is still a vast wealth of mushrooms that await exploration for medicinal values.

1.1 Research objectives

In this research, two mushroom species, *Pleurotus tuber-regium* (Fr.) Singer and *Termitomyces heimii* Natarajan selected, have been reported to have potential cytotoxic properties towards different cancer cells. Based on (Lau *et al*., 2013; Lau *et al*., 2014), sclerotial mushroom that formed sclerotia underground possess cytotoxic proteins as defence mechanism against other microorganisms. Hence, *P. tuber-regium* another sclerotial mushroom may possess cytotoxic proteins too and has not been investigated. This is an ideal candidate for cytotoxic protein compounds, specifically proteins responsible against both breast and colorectal cancers. *Termitomyces heimii* is an edible, seasonal and elusive mushroom which is familiar among Malaysian, nevertheless, there are also lack of study focusing on the cytotoxic proteins from this mushroom against both breast and colorectal cancers. Therefore, the present study aims to investigate the anti-tumour effects of selected Basidiomycetes, *P. tuber-regium* and *T. heimii* on breast and colorectal cancer cell lines.

The specific objectives of the study include:

i. to investigate the cytotoxicity of proteins from *Pleurotus tuber-regium* and *Termitomyces heimii* towards breast cancer cell lines (MDA-MB-231) and colorectal cancer cell lines (HCT-116).
ii. to evaluate the anti-migration activity of proteins towards the cancer cell lines.
iii. to separate and characterise the cytotoxic proteins from potential mushroom species.
iv. to investigate the mechanisms of cell death by the cytotoxic proteins.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cancer

Cancer is a disease in which a group of abnormal cells undergo uncontrollable growth. Regulation of cell proliferation and death are involved in the maintenance of homeostasis in normal cells but is most often disrupted in tumour cells (Youn *et al*., 2008). Genetic mutations or changes in the cellular genome prevent the old or damaged cells from undergoing apoptosis, a programmed cell death, resulting in the continuous multiplication of the cells which then eventually forms cancer (Hejmadi, 2010).

Tumours can be either benign or malignant, the former being non-cancerous as it is localised to one area of the body while the latter is considered life threating as it has gained the ability to invade far reaching regions of the body, primarily utilising the bloodstream and lymph nodes as a mode of transportation. Upon invasion to a distant site and developing a microenvironment catering to the cancerous cell needs a secondary cancer develops. Metastasis is known to be a key factor for over 90% of cancer-related deaths (Gupta & Massague, 2006).

Cancers are classified according to the type of cells from which they arise. Most cancers are categorised under three main groups either carcinomas, sarcomas, and leukaemias or lymphomas. Carcinomas are cancer of epithelial cells, making up to 90% of cancer cases; sarcomas on the other hand are solid tumours that originates from connective tissues such as muscle, bone, cartilage, and fibrous tissue. Lastly, leukaemias and lymphomas, also referred as liquid tumours make up to 8% of tumours. Leukaemias arise from blood forming cells, while lymphomas arise from cells of the immune system (T and B cells) (Cooper, 2000).

2.1.1 Characteristics of cancer cells

The most common feature of cancer cells is its ability to proliferate uncontrollably bypassing the internal check and balance system, eventually leading to the development of tumours or increase in the number of abnormal dispersed cells (Devi, 2004). Cancer cells vary from normal cells in several features as shown in Table 2.1.

Table 2.1: Characteristics of normal cells versus cancer cells (Adapted from Gopinadh, 2015; Eldridge, 2018).

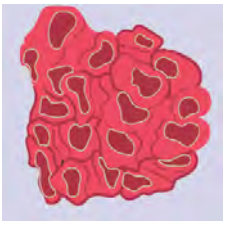
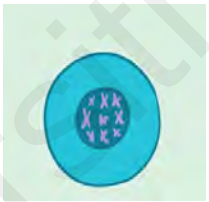
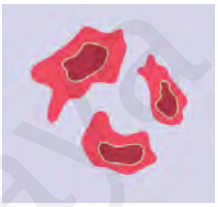
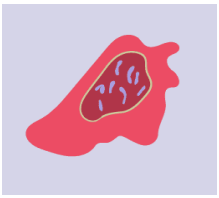
Cell characteristics	**Normal cells**	**Cancer cells**
Growth	• Cells stop growing when enough cells are present 	• Uncontrolled growth, thus resulting in the formation of tumour 
Communication	• Respond to signals sent from other nearby cells	• Do not interact with other cells and not respond to signals
Cell repair and death	• Damaged cells are either repaired or undergo apoptosis	• Cells not repaired or undergo apoptosis
Ability to metastasise	• Cells are localised in the organ where they develop	• Cells are able to spread to other parts of the body and begin to grow and form secondary tumour

Table 2.1, continued.

Morphology	• Uniform shape and size • Secrete cell surface adhesion molecules making them stick to each other  • Organised arrangement of chromosomes • Fixed number of chromosomes 	• Exhibit anaplasia • More variability in terms of cell size either larger or smaller than normal cells. • Larger and darker nuclei (contains excess DNA)  • Abnormal cellular and nucleus morphology • Abnormal chromosome number that are arranged in disorganised manner 
Maturity	• Well-differentiated	• Poorly differentiated, Indifferentiated
Blood supply	• Angiogenesis (a process where cells attract blood vessels to grow and feed the tissue) as part of normal growth when new tissue is needed to repair damaged tissue 	• Angiogenesis even when growth is not necessary 

2.2 Incidence of cancer

The International Agency for Research on Cancer (IARC), reported 17 million new cancer cases in 2018 worldwide (Bray *et al.*, 2018). The global burden is expected to grow to 27.5 million new cancer cases and 16.3 million cancer deaths by 2040 due to ageing and growth of the population (Ferlay *et al*., 2015). The trends of cancer incidence and mortality were more perturbing in economically developing countries than economically developed countries. This is true for cancers associated with lifestyles which are more typical in industrialised countries. The global population in Europe is only 9.0%, with cancer morbidities and mortalities accounting for 23.4% and 20.3%, respectively. In contrast, Asia region with nearly 60% of the global population, has recorded 57.3% and 48.4% of cancer mortalities and morbidities. This higher frequency can be related to poorer prognosis, and limited access to timely diagnosis and treatment in those countries (Bray, 2018).

Based on reports in Malaysian National Cancer Registry 2012-2016, Malaysia has recorded a total of 115,238 patients with cancer for the period of 2012-2016. The three most common cancer incidence among Malaysian were cancers of the breast (19.0%) followed by colorectal (13.5%) and lung (9.8%). Breast cancer incidence was reported as being the highest among Malaysian women while colorectal cancer was the most common among Malaysian men. The annual rate of cancer incidence in Malaysia has increased over the 10 years (2007-2016) with higher incidence among women (Azizah *et al*., 2019).

The age-specific incidence rate of cancer increased for both males and females after the age of 30 years. The incidence rate in males exceeded the incidence rate in females after the age of 60 years. In terms of ethnic variation in Malaysia, the statistics revealed that in males, the incidence of colorectal, lung, prostate, nasopharynx, and liver cancers were more common in the Chinese as compared to lymphoma, leukaemia, and bladder cancers which were more common among Malays. Among the females, the incidence

rates of breast, colorectal, cervix uteri and lung cancers were higher in the Chinese while ovarian, lymphoma, leukaemia, and thyroid cancer were higher among Malays (Azizah *et al*., 2019). The incidence rate of cancer can be attributed to several different factors which increase the risk (see subchapter 2.3).

2.2.1 Breast cancer

According to previous research, breast cancer occurs at earlier age in Malaysia as compared to in the Western countries. Approximately 50% of women in Malaysia were diagnosed before the age of 50, whereas in countries such as United Kingdom and Netherlands, 20% were diagnosed before age of 50. Also, numbers of Malaysian women who present at late stages were higher as compared to women in the Western countries (Yip *et al*., 2014). This is due to several factors such as relying on traditional medicine, negative perception of the disease, poverty, poor education, fear, and denial (Hisham & Yip, 2004).

Breast cancer refers to malignant cells that proliferate in the breast tissue, most commonly from the cells in the inner lining or lobules of milk ducts. Breast cancer can be categorised as non-invasive and invasive. Non-invasive breast cancer cells are localised to the ducts, while invasive breast cancer cells can break through the duct and lobular walls, and invade the surrounding and connective breast tissue leading to metastasis (Sharma *et al*., 2010). The metastatic dissemination has attributed to 90% of the breast cancer deaths worldwide where the most common metastatic sites were the lungs, bones, liver and lymph nodes (Cummings *et al*., 2014). Infiltrating ductal carcinoma (IDC) is one type of invasive breast cancer which accounts for 80% of breast cancer diagnosed. IDC begins in the milk ducts of the breast and penetrates the wall of the duct, invading the fatty breast tissue and other regions of the body (Figure 2.1) (Sharma *et al*., 2010; Bombonati & Sgroi, 2011).

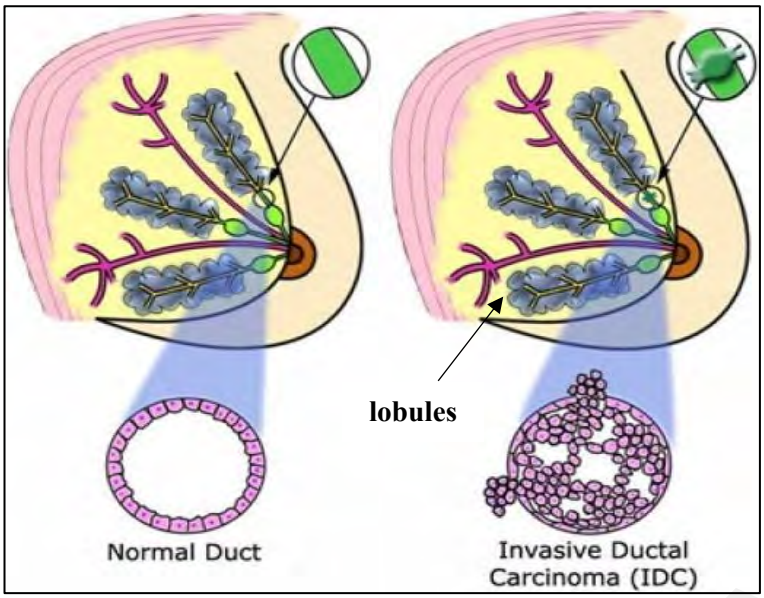


Figure 2.1: Structure associated with Invasive Ductal Carcinoma (IDC) (Adapted from Sharma *et al*., 2010).

2.2.2 Colorectal cancer

Colorectal cancer is life threatening and usually referred to as a silent killer. This is because the colon cancer progresses slowly and asymptomatic in the earlier stages, thus resulting in majority of diagnosis at a late stage. In Malaysia, the health burden also increases as 80% of colorectal cancer cases are diagnosed in people at ages above 50 years with comorbidities such as diabetes and hypertension (Veettil *et al*., 2017).

Most colorectal cancer begins from localised growth of small tumour called polyps that develop in the inner linings of the colon or rectum. The progression of polyps into cancers relatively takes years (Stracci *et al.,* 2014). Continual cellular proliferation increases the size of the polyps, while accumulating more genetic abnormalities by which they acquire the ability to invade nearby tissue and spread beyond the walls of the colon and rectum. The malignant growth may eventually enter the local lymph nodes and circulatory system and metastasise to distant sites (Figure 2.2) (Simon, 2016). Early screening for colorectal cancer risk is important to prevent the spread of colorectal cancer by appropriate treatment such as surgical removal of the polyps.

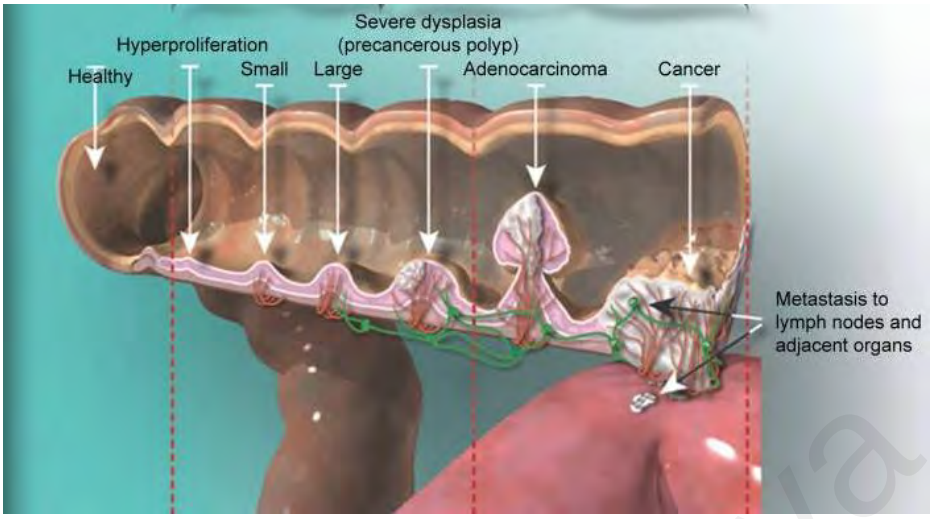


Figure 2.2: Stages of colorectal cancer progression (Adapted from Simon, 2016).

2.3 Risk factors and cancer prevention

2.3.1 Ageing

Ageing is one of the risk factors for various cancers. This is due to lower immunity, aged metabolic system, cumulative environmental exposure as well as hormonal and nutritional imbalance. Previous researchers proposed that aged people may accumulate the non-proliferating cells in the stroma which disrupts the local tissue integrity with factors secreted by these cells. This may create a pro-oncogenic tissue microenvironment which represent the combined pathogenic effects from mutation load, telomere dysfunction, and altered stromal milieu (Ukraintseva & Yashin, 2003). Ageing diminishes capacity of self-gene repairs resulting in a high number of acquired mutations, thus increasing the risk of an aged individual towards cancer.

2.3.2 Environmental factors

Environmental pollutants like chemicals, biological agents, industrial effluents, some therapeutic drugs, and mutagenic agents including ionising radiation, increase the risk of cancer incidence worldwide (Devi, 2004; Yari *et al*., 2018). Occupational factor

is the main cause of exposure to environmental carcinogens which include anilines, arsenic, asbestos, benzene, formaldehyde, radon gas, nickel compound, wood dust, and ultraviolet radiation (National Toxicology Program, 2011; Yari *et al.,* 2018). Lung cancer is one of the most common occupational related cancer which had caused up to 111,000 deaths (Espina *et al*., 2013; Yari *et al*., 2018).

Global population exposure to radiation is increasing annually where the largest exposure is due to radon, followed by medical imaging procedure (Shah *et al*., 2012; McColl *et al*., 2015). Natural radiation exposure from radon decay products, natural radionuclides in food and drinks, and naturally occurring radioactive substances existing in the earth, promote the increase of cancer risk. Radon inhalation is recognised as a cause of lung cancer. Radon is an inert gas with several radioactive isotopes present in most rocks and soil. Radon exposure arises from its presence and emanation from building materials and can deposit into the lungs through inhalation (McColl *et al*., 2015).

There is increasing evidence showing the association of medical imaging procedure with carcinogenicity. Medical imaging procedures such as CT scans, PET scans and mammograms have caused high exposure of ionising radiation to patients. The most common mechanism of radiation carcinogenesis is DNA damage, which results in the development of cancer through DNA misrepair (White *et al*., 2014; Friis *et al*., 2015). Research also found that radiotherapy for cancer treatment increases the risk of developing second cancers. For example, radiotherapy for benign breast were demonstrated to develop cancers of the thyroid, salivary gland, central nervous system, skin, and leukaemia (Ron, 2003; Hiatt & Brody, 2018).

Initiation and progression of cancer can also be associated with various infectious agents such as viruses, bacteria and parasites. Human papillomavirus (HPV), hepatitis B virus and human immunodeficiency virus (HIV) are some of the known oncogenic viruses associated with cervical cancer, nasopharyngeal carcinoma, and Kaposi sarcoma,

respectively, while *Helicobacter pylori* can cause stomach cancer. On the other hand, urinary bladder carcinoma can result from parasitic infection (Hejmadi, 2010; Smetana *et al*., 2016).

2.3.3 Lifestyle

Numerous researches have revealed compelling evidence linking a higher risk of developing cancer with dietary habits. For instance, high intakes of red and processed meat were found to increase the colorectal cancer risk by 20-30%. (Aykan, 2015; Key *et al*., 2020). Despite being considered safer than processed meat, fresh meat should be taken in moderation due to potential presence of carcinogens such as N-nitroso compounds, heterocyclic amines, or polycyclic aromatic hydrocarbons (Genkinger & Koushik, 2007).

Hot beverages and foods promote the risk of oral cavity, pharynx and oesophagus cancers (Key *et. al*., 2004; Key *et al.,* 2020). A number of studies revealed that the consumption of hot beverages and food are associated with a significantly increased risk of oesophageal cancer especially in Asia and South American populations (Chen *et al*., 2015). Hot beverages such as tea which are often consumed at boiling temperature practically in Japan and China has potential to cause chronic thermal injury to the upper digestive tract, and the long-term injury might increase the risk of chronic irritation and susceptible to carcinogenesis (Mirvish, 1995; Chen *et al*., 2015; Tai *et al*., 2017).

The management of healthy lifestyle, appropriate diet and nutritional intake are able to prevent almost 30-50% of cancer risks (WHO, 2020). The consumption of meals rich in fruits, vegetables, and spices are believed to provide protection for humans against various diseases and help to lower the risk of cancer in the stomach, oesophagus, lung, oral cavity and pharynx, endometrium, pancreas, as well as breast and colon (Block *et al*., 1992; Steinmetz & Potter, 1996; Reddy *et al.,* 2003; Anand *et al*., 2008). Table 2.2

summarises dietary components that contribute to the increase or decrease of cancer risks together with their level of evidence based on data and reports from literature.

Numerous compounds isolated from fruits and vegetables have shown significant positive effects in cancer therapy. These compounds include curcumin (turmeric), resveratrol (red grapes, peanuts, and berries), genistein (soybean), diallyl sulfide (allium), S-allyl cysteine (allium), allicin (garlic), lycopene (tomato), capsaicin (red chilli), diosgenin (fenugreek), 6-gingerol (ginger), ellagic acid (pomegranate), ursolic acid (apple, pears, prunes), silmarin (milk thistle), anethol (anise, camphor, and fennel), catechins (green tea), eugenol (cloves), indole-3-carbinol (cruciferous vegetables), limonene (citrus fruits), beta carotene (carrots), and dietary fibre (Bhanot *et al*., 2011). Thorough research has proven that bioactive compounds from fruits and vegetables are responsible for the inhibition of cancer progression by suppressing cell survival, proliferation, cell invasion, angiogenesis and metastasis (Nosrati *et al*., 2017).

Obesity has been associated with the lack of exercise or physical inactivity which may increase the risk of breast cancer through increase in serum concentration of estradiol, lower concentration of hormone-binding globulin, larger fat masses, and higher serum insulin levels. Less active persons are at high risk of developing colon cancer due to increase in GI transit time which in turns increase the contact time with potential carcinogens, increase the circulating levels of insulin (promote proliferation of colonic epithelial cells), alter prostaglandin levels, depress immune function, and modify bile acid metabolism (Anand *et al*., 2008). Previous study also revealed that those with highest level of physical activity have almost 50% of reduced risk from colon cancer incidence (Colditz *et al*., 1997; Lima & Gomes-da-Silva, 2005; Clinton *et al*., 2020).

Table 2.2: Diet, nutrition and cancer: levels of evidence (Key *et al*., 2004; Clinton *et al*., 2020**).**

Level of evidence	Decrease risk	Increase risk
Convincing	Physical activity (colon)	• Overweight and obesity (oesophagus, colorectum, breast in postmenopausal women, endometrium, kidney). • Alcohol (oral cavity, pharynx, larynx, oesophagus, liver, breast) Aflatoxin (liver). • Chinese-style salted fish (nasopharynx).
Probable	Fruits and vegetables (oral cavity, oesophagus, stomach) Whole grains Foods containing dietary fibre Physical activity (breast)	• Preserved meat and red meat (colorectum). • Salt preserved foods and salt (stomach). • Very hot (thermally) drinks and food (oral cavity, pharynx, oesophagus).
Insufficient	Fibre, soya, fish, *n*-3 fatty acids, carotenoids, vitamins B2, B6, folate, B12, C, D, E, calcium, zinc, selenium, non-nutrient plant constituents (e.g. flavanoids, isoflavones, lignans)	• Animal fats, heterocyclic amines, polycyclic aromatic hydrocarbons, nitrosamines. • Low intake of fruits. • Low intake of non-starchy vegetables.

2.4 Metastasis

Metastasis is one of the leading causes of death in patients suffering with cancer. For example, more than 30% of breast cancer patients who have no evidence of sentinel lymph nodes involvement at the time of primary breast tumour surgery will subsequently be at risk of developing secondary tumours (Heimann *et al*., 2000). Metastasis can be a crucial problem incorporated with the management of cancer diseases among medical practitioners and scientists.

Metastasis involves a complex process dealing with a series of sequential seps of cancer spread at secondary site before being clinically detectable. Those steps involve separation from the primary tumour, invasion through surrounding tissues and basement membranes, entry and survival in the circulation, lymphatics or peritoneal space and finally arrest at a distant target organ. These can further progress by extravasation into the surrounding tissue, survival in the foreign microenvironment, proliferation, and induction of angiogenesis, all the while evading apoptotic death or immunological response (Liotta & Stetler-Stevenson, 1993; Al-Mehdi *et al*., 2000).

The metastatic progression of cancer cells is extremely inefficient and not identical. In order to colonise and proliferate at the distant organ, a cancer cell must complete all of the steps of the cascade. Failure to complete any of the steps will result in millions of cells from tumours to spread into the bloodstream daily (Butler & Gullino, 1975; Martin *et al*., 2013). Many different theories and observations have been explored to describe the mechanisms by which a tumour cell may completely colonise at distant tissues. However, due to its complexity, none of those theories can explain and conclude all of the observed phenotypes (Hunter *et al*., 2008).

The potential of tumour cells to be at the metastatic state is closely related to the ability of the cells to migrate from the primary site to the other site/organ as well as within the circulation (Menezes *et al*., 2016). Tumour cell migration and metastasis involve both

functional and dynamic cytoskeleton to produce the necessary protrusions and forces that drive cells to other tissues (Webb *et al*., 2002; Grzanka *et al*., 2003). Actin cytoskeleton are important for cell migration where it plays a role in cell growth and differentiation as well as in the signalling pathway downstream of receptors that lead to the remodeling of the actin filaments (Grzanka *et al*., 2006). It is vital for tumour cells motility and chemotaxis which in turn influence the metastatic ability of tumour cells (Freitas *et al*., 2008). The motility of cells which lead to the cell movement is initiated by setting up a front-to-back polarisation, followed by a coordinated cycle of actin polymerisation-dependent protrusion, integrin/actin-mediated focal adhesion and cell body translocation resulting from actomyosin contractility (Vicente-Manzanares *et al*., 2009).

The intact of microtubule cytoskeleton is however required in the directional cell migration. An asymmetry of the microtubule network is initially established to generate the polarisation and directional movement of the cell (Vicente-Manzanares *et al*., 2009; Lyle *et al*., 2012; Ballestrem *et al*., 2000; Ganguly *et al*., 2012; Wittmann & Waterman-Storer, 2001). Microtubules are dynamic filamentous cytoskeletal proteins which is crucial as therapeutic target in tumour cells (Dumontet & Jordan, 2010). Microtubules binding agents are highly targeted for natural toxins in cancer research as their importance in different cellular processing including intracellular transport, cell division and migration (Yan *et al*., 2015; Wang *et al*., 2016; Jordan & Wilson, 2004; Kaur *et al*., 2014).

In vitro scratch wound assay or wound healing assay is a traditional and most commonly used method for investigating cell migration (Lampugnani, 1999). In this assay, a scratch is made on a confluent cells monolayer and the rate of the cells closing in the scratch or gap is determined over time. Transwell or modified Boyden chamber assay is another method used to examine the anti-migration activities of tumour cells. The migration activities of cells are estimated by measuring the ability of cells to migrate from the upper chamber towards a chemo-attractant lower chamber (Chen, 2005).

In vitro scratch wound assay is an economical and convenient assay to conduct in cell culture laboratory using available materials and equipment. In addition, it imitates the behaviour of cells migration *in vivo*. However, this assay requires longer time to perform and larger number of cells and chemicals to maintain in a culture dish. For example, it takes one or two days for cells to be confluent and another 8-18 hours for cells migration to close the scratch or gap (Liang *et al*., 2007). *In vitro* scratch wound assay has been applied in various types of cells for cell polarisation study, matrix remodeling, cell migration, and numerous other processes as the monolayers heal the wound in a characteristic manner (Lu *et al*., 2004; Herren *et al*., 2001; Huang *et al*., 2003).

2.5 Cell cycle and cell death

Apoptosis is a type of cell death associated with an intrinsic cell suicide programmed to ensure the maintenance of tissue homeostasis in an organism by getting rid of damaged cells that might interfere with normal cell function (Sharma *et al.*, 2014). Apoptosis is an active cellular signalling process triggered by a variety of stimuli such as growth deprivation factors, exposure to cytotoxic or DNA damaging agents, activation of death receptors and action of cytotoxic cells (Kumar & Dorstyn, 2009). The morphological characteristics of apoptotic cell death includes cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr *et al*., 1972; Wyllie *et al*., 1980; Kerr *et al*., 1994; Ziegler & Groscurth, 2004).

The apoptotic cell death is induced via several mechanisms including the down-regulation of telomerase activity and up-regulation of mRNA expression of the caspase-3 gene (Gao *et al*., 2007), regulation of Bcl-2 and caspase-3 (Jin *et al*., 2007), cleavage of poly (ADP-ribose) polymerase and pro-caspase 3 (Bae *et al*., 2009), caspase activation and mitochondrial membrane potential loss (Mizumoto *et al*., 2008), subsequent activation of caspase-9 and caspase-3 and release of mitochondrial cytochrome c (Hsu *et*

al., 2008), and signal transduction of kinases Akt and Erk (Calvino *et al*., 2010; Roupas *et al*., 2012).

Figure 2.3 shows the schematic representation of apoptosis. In general apoptosis divides into extrinsic and intrinsic pathway. Death receptors (like FAS) are involved in extrinsic pathway, which can later activate caspase-8. Caspase-8 activates caspase-3 in two separate ways (direct activation or activation via caspase-9). Stress signals and DNA damage triggers intrinsic apoptosis pathway via mitochondria. Intrinsic apoptosis (mitochondrial apoptosis) is divided to caspase-dependent or caspase-independent pathways (Marzban *et al*., 2015).

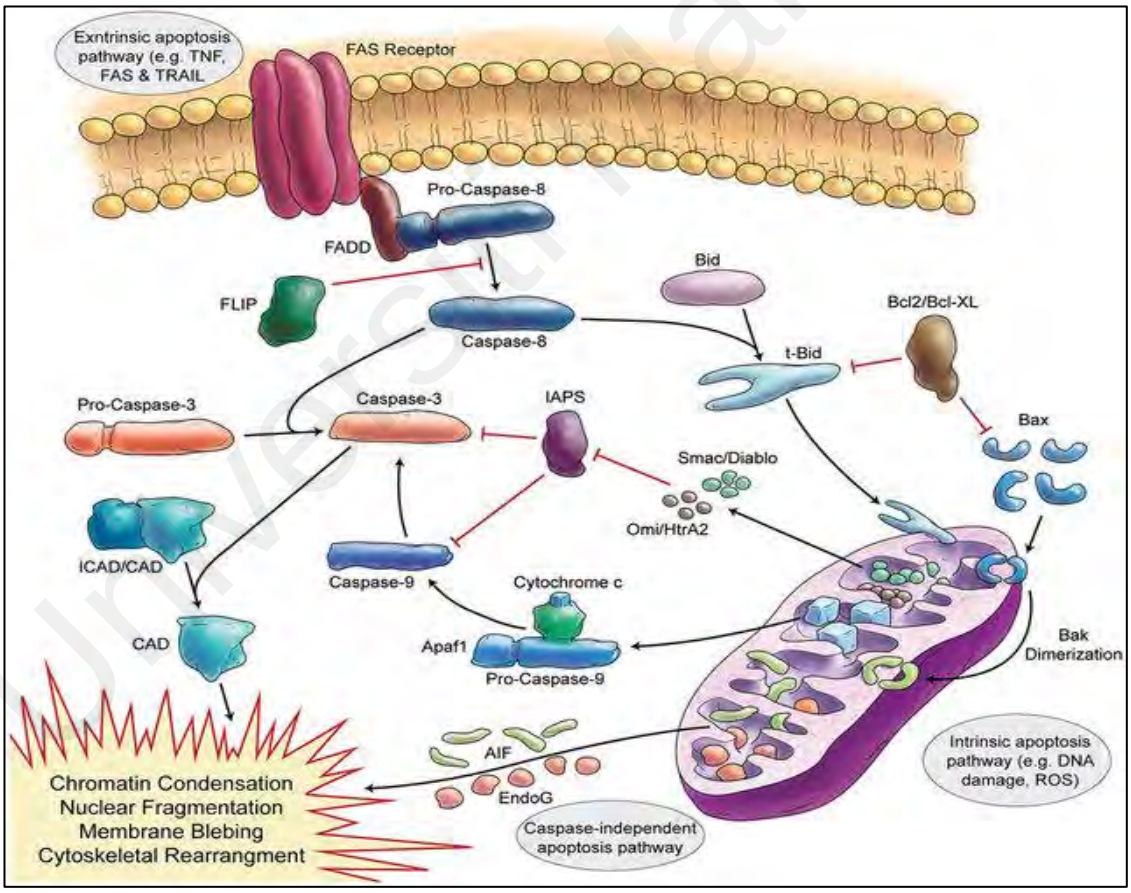


Figure 2.3: Schematic representation of apoptosis (Adapted from Marzban *et al*., 2015).

Inhibition of apoptosis can lead to cancer by providing higher survival rate of the cancer cells as well as giving longer time for the accumulation of mutations which

increase aggressiveness during tumour progression by stimulating angiogenesis, by dysregulating cell proliferation and interfering with cellular differentiation (Hassan *et al*., 2014). In cancer cells, the occurrence of apoptosis is too little which result to cell malignancy. Defects can happen along the apoptotic pathway leading to transformation of the affected cells, tumour metastasis and anti-cancer drug resistance. Therefore, targeting apoptosis is the most effective method and holds a good potential for non-surgical treatment in cancer therapy (Pfeffer & Singh, 2018).

Apoptosis, necrosis and senescence are cell death mechanisms involved in the tumour suppression and growth arrest by cellular damage depending on the magnitude of DNA damage as well as the concentrations of anti-cancer agents (Kim *et al*., 2006). A series of molecules involved in the execution and induction of apoptosis process including signal molecules, receptors, enzymes and gene regulating proteins. Molecules such as the inhibitor of apoptosis protein (IAP), Bcl-2 family proteins, and calpain, are regulatory factor for caspase-cascade signalling system which is vital in the apoptosis process (Launay *et al*., 2005).

The success of an apoptosis process is dependent on the balance between pro-apoptotic and anti-apoptotic protein regulators. Deregulation of apoptosis process is associated with unchecked cell proliferation, progression of cancer and lead to development of drug resistance (Plati *et al*., 2008; Fulda, 2009). Deregulation of apoptosis is considered as one of key factors in the hallmark of cancer (Hanahan & Weinberg, 2011). Therefore, an effective cancer treatment should target molecules involved in apoptotic resistance in order to restore the sensitivity of cancer cells to apoptosis (Giménez-Bonafé *et al*., 2009; Fulda, 2015).

Necrosis is a type of cell death which generally occurs accidentally, under unscheduled condition such as in physical-chemical stress, heat, osmotic shock, mechanical stress, freezing, thawing, and high concentration of hydrogen peroxide.

Cellular stress like hypoxia (low oxygen), cytokines, ischemia (lack of blood supply), heat irradiation, pathogens, and toxicity are all contributing factors of necrosis (Escobar *et al*., 2015). Anti-cancer drugs can directly induce necrosis by ATP depletion or impairment of the mitochondrial membrane pump (Bezabeh *et al*., 2001; Majno & Joris, 1995). Necrotic cells are characterised by osmotic swelling of the cell membrane and organelles, accompanied by chromatin condensation and irregular DNA degradation pattern. This is followed by plasma membrane leakage which releases the intracellular contents and affects neighbouring cells and consequently causes inflammation (Escobar *et al*., 2015).

The ultimate goal of cancer therapy is to promote the death of cancer cells by inducing apoptosis while at the same time limit concurrent death of normal cells (Gerl & Vaux, 2005). Some of the action of anti-tumour compounds are as reactive oxygen species inducer, mitotic kinase inhibitor, anti-mitotic, angiogenesis inhibitor, topoisomerase inhibitor, leading to apoptosis, and eventually checking cancer proliferation (Patel & Goyal, 2012). Flow cytometry is a proven useful method for the determination of apoptosis through the use of antibody labelled fluorescent substance by detection of expression of surface protein change (Ozdemir, 2011).

Cell cycle is a series of events which lead to division and multiplication of cells (Lewin *et al*., 2007; Alberts *et al*., 2008). A mechanism for the cytotoxic effects of anti-cancer drugs is the induction of apoptosis via an arrest or dysregulation of cell cycle in cancer cells (Juranek *et al*., 1993). Several methods of analysing cell cycle arrest were described previously in Pozarowski & Darzynkiewicz (2004). There are two methods that rely on the univariate analysis of cellular DNA content. The first method uses propidium iodide (PI) as the DNA fluorochrome which requires blue light as the excitation source (eg. 488 nm argon ion laser). The second method utilises UV-light-excitable 4',6'-diamidino-2-phenylindole (DAPI) as a DNA specific fluorochrome. The third method

involves the combined analysis of cellular DNA content with the expression of proliferation-associated proteins. The other method presented the immunocytochemical detection of incorporated thymidine analog 5'-bromo-2'-deoxyyuridine (BrdU), combined with DNA content analysis. This current study utilised the first method based on a single time-point measurement which reveals the percentage of cells in G1, S, and G2/M phase without cell cycle kinetics information.

2.6 Diagnosis and treatment of cancer

Modern chemotherapeutic drugs were first discovered in 1940 through research on nitrogen mustards which induced tumour regression (Chabner & Roberts, 2005). The combination of several drugs with different cytotoxic agents, as adjuvants, tend to be more effective in eradicating target tumours, typically through multiple mechanisms (Chabner & Roberts, 2005). Table 2.3 shows several drugs and their mode of action for treating cancer. Multidisciplinary effort and good teamwork between the surgeons, oncologists, radiologists, pathologists and other relevant disciplines improves the quality of cancer treatment and metastasis control for individual patients (Lim, 2002; Adam *et al*., 2012; Gholam *et al*., 2019).

2.6.1 Cisplatin

Cisplatin, cisplatinum or cis-diamminedichloroplatinum (II) is a metallic (platinum)-based compound, which is white or deep yellow to yellow-orange crystalline powder at room temperature (Dasari & Tchounwou, 2014). Cisplatin is a chemotherapeutic agent that has been extensively used in the treatment of various types of cancers including breast, testicular, ovarian, cervical, prostate, head and neck, bladder, lung, and non-Hodgkin's lymphomas (Tsimberidou *et al*., 2009; Dhar *et al*., 2011). Cisplatin is considered as cytotoxic drugs which kills cancer cells by damaging DNA,

inhibiting DNA synthesis and mitosis, and inducing apoptotic cell death (Dasari & Tchounwou, 2014).

Previous research has reported the apoptotic effects as well as anti-proliferating activities of cisplatin on breast cancer cells, MCF-7 and MDA-MB-231 (Liang *et al*., 2015; Jiang *et al*., 2017). The study revealed that cisplatin cytotoxicity was recorded at IC_{50}=6 µg/ml and IC_{50}=8 µg/ml towards MCF-7 and MDA-MB-231 cells, respectively (Jiang *et al*., 2017). In a different study, the MTT assay showed cisplatin inhibited the growth of MDA-MB-231 cells at IC_{50}=1.3±0.55 µg/ml and IC_{50}=0.9±0.31 µg/ml after 24 and 48 hours, respectively (Fani *et al*., 2016). Cisplatin is an ideal positive control to be employed in this present study because of its efficacy in inducing both cytotoxic and apoptotic effects especially towards rapidly dividing cancer cells.

Genomic DNA is the main target for cisplatin cytotoxicity. Cisplatin is activated once it enters the cells. Next, the chloride atoms on cisplatin are displaced by water molecules in the cytoplasm. The hydrolysed product is a potent electrophile that can react with nucleophile, including the sulfhydryl groups on proteins and nitrogen donor atoms on nucleic acids (Dasari & Tchounwou, 2014). Cisplatin covalently binds to the N-7 atoms of purines on DNA causing DNA damage in cancer cells which leads to cell cycle arrest and apoptosis through the activation of multiple signalling pathways (Miller *et al*., 2010). Several molecular mechanisms of cisplatin in cancer treatment include induction of oxidative stress as characterised by reactive oxygen species production and lipid peroxidation, induction of p53 signalling and cell cycle arrest, down-regulation of proto-oncogenes and anti-apoptotic proteins, and activation of both intrinsic and extrinsic pathways of apoptosis (Dasari & Tchounwou, 2014).

The administration of cisplatin is however associated with various side effects such as nausea, nephrotoxicity, cardiotoxicity, hepatotoxicity and neurotoxicity. Also, patients may relapse from the treatment due to cisplatin resistance that develops when tumour

cells fail to undergo apoptosis at particular drug concentrations (Siddik, 2003). Mechanisms of resistance include increased repair of damaged DNA, reduction in the accumulation of the drug intracellular and cytosolic inactivation (Aldossary, 2019). Hence, combination therapy of cisplatin with other cancer drugs have been identified to be an effective strategy in the treatment of various types of cancers with reduction of drug resistance and undesirable side effect (Aldossary, 2019; Dasari & Tchounwou, 2014).

Table 2.3: Major classes of chemotherapeutic drugs for cancer (Sloan & Gelband, 2007).

Drug Type	**Mode of Action and Examples**	**Drug Examples**
Alkylating agents	- Kill cells by directly damaging DNA - Used to treat chronic leukaemias, Hodgkin's disease, lymphomas, and certain carcinomas of the lung, breast, prostate, and ovary.	-Cyclophosphamide, Cisplatin
Nitrosoureas	- Act similarly to alkylating agents and also inhibit changes necessary for DNA repair. - Cross the blood-brain barrier and are therefore, used to treat brain tumours, lymphomas, multiple myeloma, and malignant melanoma	-Carmustine (BCNU) and lomustine (CCNU)
Hormonal agents	- Includes adrenocorticosteroids, estrogens, antiestrogens, progesterones, and androgens that modify the growth of certain hormone-dependent cancers	-Tamoxifen, used for estrogen-dependent breast cancer

Table 2.3, continued.

Plant (vinca) alkaloids	- Act by blocking cell division during mitosis Commonly used to treat acute lymphocytic leukaemia (ALL), Hodgkin's and non-Hodgkin's lymphomas, neuroblastomas, Wilms' tumour, and cancer of the lung, breast, and testes.	-Vincristine and Vinblastine
Anti-metabolites	- Block cell growth by interfering with certain activities, usually DNA synthesis, halting normal development and reproduction - Used to treat acute and chronic leukaemias, choriocarcinoma, and some tumours of the gastrointestinal tract, breast, and ovary	-6-mercaptopurine and 5-fluorouracil (5-FU)
Anti-tumour antibiotics	- Diverse group of compounds that generally act by binding with DNA and preventing RNA synthesis - Widely used to treat a variety of cancers	-Doxorubicin, adriamycin, mitomycin- C, bleomycin

2.7 Potential cytotoxic compounds / drugs of natural origins

The use of natural materials with therapeutic constituents had significant relevance to health from different perspective of religious belief and human culture since ancient time. For thousands of years, raw herbs have been used in traditional medicine for conditions such as cough relief, migraine, bronchial asthma, fever etc. Herbal medicines recognised by folklore are either directly consumed as raw or generally prepared using several methods such as soaking in cold water or grinding the plant into juices. On the other hand, some plants, flowers, stems or barks were soaked before using as concoction

for baths; boiling and consumed as tea, making the plant parts or powdered herbs as poultice for skin rashes or wounds. The use of natural products for medicinal applications is a result of experimentation through trial and error by mankind for centuries through palatability trials or untimely deaths, hunting for foods and searching of remedies for the treatment of various ailments (Hicks, 1971; Kinghorn *et al*., 2011).

Natural products have been a sustainable and effective source material for treating several diseases mainly due to its accessibility, being a safe avenue as well as cheap alternative. Because of these reasons researchers started to investigate their bioactive components for the treatments of ailments instead of merely considering the natural remedies as cultural or religious superstitious belief (Rates, 2001). The Traditional Medicine Division of WHO recognises the efficacy of centuries-old use of plant origins for therapeutic resources as proof in conducting pharmacological studies (Gilbert *et al*., 1997).

In Malaysia, the demand on herbal products is increasing tremendously due to public awareness on plant and/or natural based products in health care (Jantan, 2006; Alsarhan *et al*., 2014). Malaysia is also rich in medicinal plant species where more than 1,300 species and 7,411 species have been recorded in the Peninsular Malaysia and Sabah, respectively (Burkill, 1966; Kulip *et al*., 2010). Extensive studies have been conducted and more research are still ongoing in the quest of uncovering the potential medicinal properties from Malaysian plant species.

Numerous plants possess antioxidant activities which are effective in treating various diseases including cancer, Alzheimer, atherosclerosis, diabetes and other cardiovascular diseases (Karimi *et al*., 2015). Free radicals are associated with carcinogenesis. For example, peroxyl radicals and lipid peroxidation can lead to DNA mutation. Antioxidant phytochemicals on the other hand can protect the DNA from oxidative damage by simultaneously hindering carcinogenesis (Zhang *et al*., 2015). For

instance, the anti-tumour activities of the ethanolic extract of *Amaranthus paniculatus* is partly due to the free radical quenching property of phytochemicals it contains (Sreelatha *et al*., 2012). Polyphenols and bio-phenols are also phytochemicals essential for anti-cancer and anti-proliferating properties (Zhang *et al*., 2015).

The use of anti-cancer drugs from natural products is significant. According to a statistical data from 1946 to September 2019, it is estimated that there are 13.5% and 25.1% of approved anti-cancer drugs originating from natural products and natural product derivatives, respectively (Newman & Cragg, 2020). Potential anti-cancer agents with diverse chemical structure had been discovered from plants, animals, marine sources, as well as microorganisms (Newman *et al*., 2003; Butler, 2004). The selection of plant species for potential drug origins involves consideration of several criteria and strategies. Considering the plant habitat and their environment makes many plants ideal to be examined for their anti-bacterial, anti-oxidant and anti-cancer properties (Katiyar *et al*., 2012; Andriani *et al*., 2017). For instance, *Termitomyces heimii*, a fungus which grow on termite hills (Refer subsection 2.11), create symbiotic relationship that benefits plants in terms in terms of growth promotion and adapting to changing environmental conditions (Sawant *et al*., 2018).

The search for anti-cancer drugs often applies the pre-screening strategy of several plant species by targeting specific pharmacological activity, following which the active species is selected for further studies. Combination of several criteria together with other strategies such as databanks search, literature survey is a crucial step to selecting specific potent plant for a novel drug discovery (Pan *et al*., 2013). Table 2.4 shows different compounds that has been isolated from plant origin for cancer treatment.

Clinical trial phase indicates: - safety studies are evaluated during Phase I, efficacy during Phase II, and confirmation of safety and efficacy during Phase III. Sentry studies are done in Phase IV and comparative effectiveness research and community-based research in Phase V (Mahan, 2014).

Table 2.4: Plant based anti-cancer agents in clinical practices (Schwartsmann *et al.,* 2001; Bhanot *et al*., 2011; Sisodiya, 2013; Greenwell & Rahman, 2015).

Compound	Uses	Source	Clinical Trial Phase
Vincristine	Leukaemia, lymphoma, breast, lung, pediatric solid cancers and others	*Catharantus roseus*	Phase III/IV
Vinblastine	Breast, lymphoma, germ-cell and renal cancer	*Catharantus roseus*	Phase III/IV
Paclitaxel	Ovary, breast, lung, bladder and head and neck cancer	*Taxus brevifolia*	Phase II/IV
Docetaxel	Breast and lung cancer	*Taxus baccata*	Phase III
Topotecan	Ovarian, lung and pediatric cancer	*Camptotheca acuminata* derivative	Phase II/III
Irinotecan	Colorectal and lung cancer	*Camptotheca acuminata* derivative	Phase II/III

In the past decade scientists have revealed various unique toxins derived from marine life that have the potential to be applied as anti-cancer lead compounds. Marine microalgae, cyanobacteria, and heterotrophic bacteria living in association with invertebrates (eg. sponges, tunicates, and soft corals) have been identified and possess diverse group of bioactive constituents including cytotoxic compounds (Simmons *et al*., 2005; Lichota & Gwozdzinski, 2018). The production of high varieties of bioactive

compounds by marine organisms are associated with several roles, for example as a chemical defence against competing organisms, and as an adaptation to toxic environmental condition (Peterson *et al*., 2020).

Several promising marine derived compounds are bryostatin-1, didemnins, dolastatins, ectenaisdin 743 (ET-743) have shown different mechanisms of action against cancer cells (Amador *et al*., 2003; Lichota & Gwozdzinski, 2018). Nucleoside analogue cytarabine has been successfully isolated from the Caribbean sponge *Cryptotheca crypta*, and is currently used in the treatment of leukaemia and lymphoma. The cytarabine derivative known as gemcitabine has been recognised as one of the most promising cytotoxic agents against the cancer of pancreatic, breast, bladder, and non-small-cell lung cancer (Schwartsmann, 2000; Toschi *et al*., 2005; Dubey *et al*., 2016). Numerous researches which have isolated anti-cancer agent from marine sources show that our nature including marine ecosystem holds great potential awaiting more exploration.

2.8 Mushrooms

Macrofungi or mushrooms have been recognised as nutritious foods in many parts of the world. Mushrooms are searched and consumed not only for their delectableness in terms of texture and flavour but also for their biochemical compounds and medicinal values (Manzi *et al*., 1999; Pinho *et al*., 2008). The advancement in microbiology has extended the usage of mushrooms including enzyme technologies, biological controls, antibiotics, pharmacology as well as agricultural industries. Mushrooms are categorised into three different groups which are edible, medicinal and poisonous (Cheung, 2010). In general, there are less than 100 species of edible mushrooms being cultivated out of 2,000 varieties all over the world (Amin *et al*., 2014). Edible mushrooms are usually fruiting bodies that can be consumed either fresh or in dried form, such as abalone (*Pleurotus cystidiosus* O.K. Mill.), rice straw (*Volvariella volvacea* (Bull.) Singer), black jelly

(*Auricularia polytricha* (Mont.) Sacc.), grey oyster (*Pleurotus pulmonarius* (Fries) Quél.), white oyster (*Pleurotus floridanus* Singer), red oyster (*Pleurotus flabellatus* Sacc.), ganoderma (*Ganoderma lucidum* (Curtis) P. Karst.) and shiitake (*Lentinula edodes* (Berk.) Pegler), those have been cultivated commercially in Malaysia (Amin *et al*., 2014; Amirullah *et al*., 2018; Samsudin & Abdullah, 2019). Edible mushroom in Malaysia is growing in demand because of their great taste and nutritional values such as vitamins and minerals (Amin *et al*., 2014). Mushrooms also have the ability to grow in agro-industrial waste in a short period of time making them as one of the fundamental horticultural crops that create huge economic impact in many developed countries through the commercialisation of its protein-source product (Chang, 1996; Chang, 2002; González *et al*., 2020).

Medicinal mushrooms are those proven to have bioactive constituents for improving health and used to treat various diseases. Medicinal functions of mushrooms including anti-tumour, immunomodulating, anti-oxidant, radical scavenging, cardiovascular, anti-hypercholesterolemia, anti-viral, anti-bacterial, detoxification, hepatoprotective, and anti-diabetic effects (Wasser & Didukh, 2003; Gao *et al.*, 2003; Rowan *et al*., 2003; Gao *et al*., 2004a; Gao *et al*., 2004b; Masuko *et al.,* 2005; Sullivan *et al*., 2006; Zhang *et al*., 2007; Dai *et al*., 2009; Ichinohe *et al*., 2010; Wasser, 2010). It is estimated that only 10% of mushroom species were identified over the 140,000 on earth. Assuming that 5% of undiscovered and unexamined species are useful to mankind that would indicate that 7,000 species of mushroom have yet to be explored and may possess medicinal applications (Hawksworth, 2001). There are about 10,000 species of higher Basidiomycetes from 550 genera and 80 families with macroscopic fruiting bodies and approximately 700 species of higher Basidiomycetes have been found to possess potential pharmacological properties (Mizuno, 1995; Wasser, 1995; Wasser & Weis, 1999). Medicinal mushrooms usually have bitter taste and tough texture, and they are consumed

in the form of powdered concentrates, liquid extracts or pills (Paterson & Lima, 2014; Amirullah *et al*., 2018). For example, sclerotium of the *Lignosus rhinocerotis* (tiger's milk mushroom) was traditionally prepared by either boiling or maceration and used as tonic for the treatment of cancer, fever, cough and asthma by indigenous communities in Malaysia (Lau *et al*., 2013). Particularly, about 200 species of mushrooms have been found to inhibit different kind of tumours and being potent against cancers of stomach, oesophagus, lungs, etc. in several countries including China, Russia, Japan, Korea, United States of America and Canada. However, most of anti-tumour compounds from mushroom origins have not been clearly defined (Wasser & Weis, 1999).

Poisonous mushrooms are the ones that have been confirmed to be or suspected of being toxic if ingested. The level of toxicity varies among different species of poisonous mushroom where in some cases the extremely powerful toxins contain can be a real hazard to health even when ingested in small doses and might cause fatality (Lima *et al*., 2012). For instance, amatoxin present in *Amanita phalloides* (Vaill. ex Fr.) Link is known as cytotoxic towards kidney and causes necrosis of liver cells, and this species is responsible for most cases of fatalities by mushroom poisoning (Karlson-Stiber & Persson, 2003). Mild intoxication has been reported from *Amanita muscaria* (L.) Lam. ingestion such as motor depression, ataxia, changes in mood, perception and feelings, dizziness, euphoria, drowsiness, gastrointestinal disturbances and muscle twitches (Stormer *et al*., 2004; Tsujikawa *et al*., 2006; Tsujikawa *et al*., 2007). The main reason for mushroom intoxication is mis-identification between the edible and poisonous one and the other reason is confusion with species that are consumed frequently. Therefore, proper identification and further studies on cytotoxicity of hazardous toxins present in mushrooms is very important to avoid intoxications (Lima *et al*., 2012).

2.9 Mushrooms as source of anti-tumour agents

Mushrooms have long been studied for their chemical properties responsible to prevent and cure a variety of diseases, including cancer. It has been reported that at least 651 species of mushroom classes, Hetero- and Homobasidiomycetes, possess anti-tumour or immune-stimulatory effects (Wasser, 2002). The most extensively studied species is *Ganoderma lucidum* that possesses anti-tumour and immuno-modulatory compounds through several mechanisms involving the inhibition of activities of replicative DNA polymerases (Mizushina *et al.*, 1998), the stimulation of cytokine release and apoptosis (Wang *et al.*, 1997) as well as the inhibition of angiogenesis and invasion (Lin & Zhang, 2004). Another species of *Ganoderma*, *G. applanatum* (Pers.) Pat. have also been documented for the anti-tumour and immuno-modulatory effects in mice (Jeong *et al*., 2008).

Potential anti-tumour effect against gastrointestinal, lung, liver and breast cancer cells had been exhibited by Grifolan from *Grifola frondosa* (Dicks.) (Poucheret *et al*., 2006). The Grifolan compound is a macrophage activator that augments cytokine production independently from endotoxins. It also enhances the mRNA level of IL-6, IL-1 and TNF (tumour necrosis factor)-α of macrophages (Ferreira *et al*., 2010). Ooi & Fang (2000) have listed mushrooms along with their specific bioactive polysaccharide or polysaccharide protein complexes responsible for inhibiting tumour growth. For instance, lentinan have been isolated from *Lentinus edodes*, schizophyllan from *Schizophyllum commune*, Maitake-D fraction from *Grifola frondosa* and protein-bound polysaccharide (PSK, Krestin) from *Coriolus versicolor*, have been widely used as anti-cancer agent in China and Japan (Kurashige *et al*., 1997; Daba & Ezeronye, 2003).

A number of studies have revealed the anti-cancer properties of mushrooms exerted by polysaccharide-derived compounds, mainly β-glucans, by the extension of cellular immune response. Previous studies have suggested that most of the mushroom

polysaccharides do not exert direct cytotoxic effect against tumour cell lines because of their high molecular weight (Wasser & Weis, 1999). The average molecular weight, M_w of mushroom polysaccharides ranges from 5 kDa to 2,000 kDa, while its β-glucans possesses a mean M_w of 500-2,000 kDa (Bohn & BeMiller, 1995). For example, β-glucan isolated from *Lentinula edodes* have molecular mass between 400 kDa to 1,000 kDa, while Crestin isolated from *Coriolus versicolor* showed molecular mass of 100 kDa Rather than having a direct effect on the tumour cells, polysaccharides utilise the immune system to induce death in these cancerous cells (Sobieralski *et al*., 2012).

Beta-D-glucans (β-glucans) is claimed as the best-known polysaccharides derivatives with a broad spectrum of biomedical implementations specifically to boost up the immune response via activation of cellular, and humoral components and increasing the macrophages functional activity, thus improving the body system and defence against cancers (Daba & Ezeronye, 2003; Carbonero *et al*., 2012; Patel & Goyal, 2012). The biological activities of β-glucans are highly depending on their degree of branching, structure, molecular weight as well as several other factors such as the location of β-glucans on mushrooms cell walls (Dyson & Rutter, 2012). The main chain of glucans with β-1,3-linkages having β-1,6-linkages are known as branches with the most effective anti-tumour properties. Generally, β-glucans with more 1,6-linkages would have lower anti-tumour effects (Franz, 1989).

Previous study suggested that the mechanisms of immune-modulatory and anti-tumour effect of sclerotial β-glucans might be due to the surface receptor interactions between immune cells and β-glucans. β-glucans receptor known as dectin-1 is able to recognise β-glucans from yeast to trigger immune-modulatory activities in both human and mice (Goodridge *et al*., 2007; Olsson & Sundler, 2007). Dectin-1 is a lectin that consists of four components which are an extracellular carbohydrate-recognition domain (CRD), a stalk, a transmembrane region, and an intracellular cytoplasmic tail (Schwartz

& Hadar, 2014). It can be found on the surface of a number of innate immune cells including monocytes, macrophages, NK cells and dendritic cells in human and mice (Brown & Gordon, 2001; Taylor *et al*., 2002). Several signalling pathways involved in the activation of immunological responses due to the interaction between β-glucans and Dectin-1, including phagocytosis activation, production of ROS, and induction of inflammatory cytokines (Willment *et al*., 2001).

Generally, the mushroom polysaccharides do not directly kill tumour cells growth (Zhang *et al*., 2007; Wong *et al*., 2011). In other words, they are regarded as biological response modifiers that support the host to adapt to various biological stresses and exert a nonspecific action on the host, improving a part of, or entire major systems (Zhang *et al*., 2007). Clinical trials have also proved that β-glucans linked with proteins showed stronger anti-tumour activity as compared to free β-glucans (Jeurink *et al*., 2008).

2.10 *Pleurotus tuber-regium* (Fr.) Singer

However, recent studies have shown numbers of mushroom compounds including *P. tuber-regium* polysaccharides have been shown to exert direct cytotoxic effect on cancer cells (Table 2.5). *P. tuber-regium*, a Basidiomycete belonging to the Pleurotaceae family and commonly known as the king tuber mushroom. It was first collected in Africa by Elias Magnus Fries and later reclassified by Rolf Singer in 1951 (Huang *et al*., 2012). The taxonomic synonym of *P. tuber-regium* is listed on the Species Fungorum Record Details page as follows: -

Current Name:

Pleurotus tuber-regium (Fr.) Singer, *Lilloa* 22: 271 (1951) [1949]

Synonymy:

Agaricus tuber-regium Fr., Syst. mycol. (Lundae) 1: 174 (1821)

Lentinus cameroensis Cooke & Massee, in Cooke, Grevillea 16(no. 77): 15 (1887)

Lentinus cyathus Berk. & Broome, Trans. Linn. Soc. London, Bot. 1(6): 399 (1879)

Lentinus descendens Afzel. ex Fr., Adami Afzelii fungi Guineenses ...: 8 (1837)

Lentinus dybowskii Pat., Bull. Soc. mycol. Fr. 11(2): [85] (1895)

Lentinus flavidus Massee, Bull. Misc. Inf., Kew: 163 (1901)

Lentinus kaernbachii Henn. [as 'kärnbachii'], in Saccardo, Syll. fung. (Abellini) 9: 72 (1891)

Lentinus pachymae Fr., Syst. orb. veg. (Lundae) 1: 77 (1825)

Lentinus scleroticola G. Murray, Trans. Linn. Soc. London, Bot. 2(10): 229 (1887)

Lentinus stenophyllus Berk., London J. Bot. 6: 495 (1847)

Lentinus taylorii G. Murray, Trans. Linn. Soc. London, Bot. 2(10): 232 (1887)

Lentinus tuber-regium (Fr.) Fr., Syn. generis Lentinorum: 3 (1836)

Pachyma tuber-regium Fr., Syst. mycol. (Lundae) 2(1): 243 (1822)

Panus tuber-regium (Fr.) Corner, Beih. Nova Hedwigia 69: 98 (1981)

Pocillaria cyathus (Berk. & Broome) Kuntze, Revis. gen. pl. (Leipzig) 2: 866 (1891)

Pocillaria descendens (Afzel. ex Fr.) Kuntze, Revis. gen. pl. (Leipzig) 2: 866 (1891)

Pocillaria stenophylla (Berk.) Kuntze, Revis. gen. pl. (Leipzig) 2: 866 (1891)

Pocillaria tuber-regium (Fr.) Kuntze, Revis. gen. pl. (Leipzig) 2: 866 (1891)

P. tuber-regium or *Lentinus tuber-regium* is one of the most common edible mushrooms that can be found in both tropical and subtropical regions of the world including Malaysia, India and Africa in general (Isikhuemhen & LeBauer, 2004; Kumar & Kaviyarasan, 2012). This mushroom can be found growing on many species of hard and soft woods like *Mangifera indica, Daniellia oliveri* and *Treculia africana* (Apetorgbor *et al*., 2013).

The mushroom looks almost similar to an oyster mushroom except that, the cup curves upward to form a cup-like shape when mature. The sclerotium is produced when

the fungus infects dry wood which, is usually buried within the wood tissues but are also found between the wood and the bark (Ikewuchi & Ikewuchi, 2008). Generally, they are of various sizes, spherical to oval shaped, dark brown on the outside and whitish on the inside (Okhuoya & Okogbo, 1991) (Figure 2.4 (a) & (b)).

Extensive studies on the genus *Pleurotus* have been carried out all over the world as most species are edible, fast growing, and high degradation of wood (Lambert, 1938; Block *et al.,* 1959; Zakia & Srivastava, 1962; Jandaik & Kapoor, 1974; Bisht & Harsh, 1983; Gregori *et al*., 2007; Sánchez, 2010). As *Pleurotus* spp. grows and fruits easily, numerous studies have been conducted to investigate their growth rate on different media and substrates, as well as studies into their physiological aspects such as its response to light, temperature, gravity and allergenic properties of its spores (Okhuoya & Etugo, 1993; Onuoha, 2007).

Figure 2.4 (a): *Pleurotus tuber-regium* **(b):** Sclerotium of *Pleurotus tuber-regium*

The study by Wu *et al.* (2004), on submerged fermentation of *P. tuber-regium* found that the appropriate harvest time, nitrogen and carbon sources as well as aeration and mass transfer are among the crucial factors for optimising the mushroom mycelia yield. Furthermore, their continuing study observed that *P. tuber-regium* mycelium grew best at a carbon nitrogen ratio ranging from 18:1 to 36:1 (Wu *et al*., 2004).

The extracts of *Pleurotus* species have been found to contain several medicinal properties including anti-tumour, antigenotoxic, bioantimutagenic, anti-inflammatory, anti-lipidaemic, antihypertensive, anti-hyperglycaemic, antibacterial and antifungal (Ijeh *et al.*, 2009). In many parts of the world, *P. tuber-regium* have been consumed as a natural ingredient in preparation of soup and utilised as traditional herbs for the treatment of various ailments. Indigenous people from the Brong Ahafo region in Ghana suggested that breast cancer disease can be treated by smearing the powdered form of *P. tuber-regium* all over the breast. In combination with other herbs, such as honey and water, the formulation can be used to overcome several other health problems like stomach ulser, headaches, hypertension, skin infection, rheumatic pain, heart pains, asthma etc (Oso, 1977; Fasidi & Olorunmaye, 1994; Dzomeku *et al.,* 2014).

In India especially from different parts of the country, *P. tuber-regium* mushroom is only being consumed as food rather than apply as ailment for various diseases (Kumar & Kaviyarasan, 2012). Likewise, in eastern peninsular Malaysia reports have mentioned its effective application against diarrhea, coughs, indigestion and dysentery (Burkill, 1966; Sumaiyah *et al*., 2007; Kumar & Kaviyarasan, 2012). In Nigeria, the sclerotia are prepared in soup for consumption as well as applied in traditional medicine for the treatment of various ailments (Zoberi, 1973; Oso, 1977). The sclerotia and fruiting bodies of this mushroom have demonstrated effectiveness in treating several diseases including constipation, stomachache, fever and colds (Oso, 1977). It also plays a role as a functional food due to being a great source of fibre, where the sclerotia contribute over 80% of dietary fibre content (Cheung & Lee, 1998; Blackwood *et al*., 2000).

Besides the medicinal importance of its active compounds, *P. tuber-regium* has also been documented to be used in a number of other biotechnological applications including food industry, enzyme production and effluent treatment (Manjunathan & Kaviyarasan, 2010). For instance, the enzyme produced by *P. tuber-regium* can act in lignocellulosic

substrates which has the potential to be used in the paper industry as biopulping, residues treatment and improvement in the animal ration digestibility (Shashirekha *et al*., 2005).

Table 2.5: Anti-cancer effects of *Pleurotus tuber-regium.*

Part used	Extract/s Compound/s	Type of cancer	Model	Mechanism of anti-cancer effect
Fruiting body and mycelium	Polysaccharides	Leukaemia	HL-60	Anti-proliferation and apoptosis against HL-60 cell lines (Wong *et al*., 2007; Patel & Goyal, 2012).
Mycelium and sclerotium	β-glucans	Leukaemia	HL-60	Apoptosis of acute promyelocytic leukaemia cells (Zhang *et al*., 2004; Wong *et al*., 2007).
Sclerotium	Protein-bound polysaccharides, β-glucans	Mice-tumour	Sarcoma 180	Anti-proliferative activities (Zhang *et al*.,2001; Isikhuemhen *et al*., 2005).

Table 2.5, continued.

Sclerotium	Novel Carboxy-methylated β-glucans	Breast	MCF-7	Cell cycle arrest and apoptosis of MCF-7 mediated by down-regulation of cyclin D1 and cyclin E expression at the G1 phase as well as the up-regulation of the expression of Bax/Bcl-2 ratio (Zhang *et al.,* 2006).
Fruiting body	LT-1 and LT-2	Ovarian and Rhabdomyo-sarcoma	SK-OV-03 and A673	Antiproliferative activity by growth inhibition against SK-OV-03 and A673. (Kaviyarasan & Shenbagaraman, 2014).
Sclerotium	Alcoholic and water extract (organic compounds)	Colon and Cervical	HCT-116 and HeLa	Anti-proliferative effect against HCT-116 and HeLa cell lines (Maness *et al*., 2011).
Sclerotium	β-glucan, Novel Carboxy-methylated β-glucans	Hepatic	HepG2	Anti-proliferative effect against HepG2 cells (Zhang *et al.,* 2001; Zhang *et al.,* 2004).

2.11 *Termitomyces heimii* Natarajan

One of the edible mushrooms that can be found in Peninsular Malaysia is *Termitomyces heimii* Natarajan (Figure 2.5). They belong to the class of Basidiomycota, order Agaricales and family Agaricacea which grow wildly and symbiotically in association with termite (insects) especially during rainy season (Batra & Batra, 1979). Literature reports on biochemical compositions including proteins from *T. heimii* are limited because they are difficult to obtain in the wild and considered as seasonal mushrooms. *T. heimii* is also difficult to cultivate making this species as rare and highly valuable due to its nutritional values as well as delicateness (Abd Malek *et al*., 2012).

Figure 2.5: *Termitomyces heimii*

A former study has shown *Termitomyces* is a potential source of sugar, protein, fibre, lipid, vitamin, and mineral. In addition, it has therapeutic uses in reducing blood pressure, immune response, anti-oxidant activity, apoptosis and infection (Apetorgbor *et al.,* 2005; Abd Malek *et al*., 2012; Mitra *et al*., 2016). Lai *et al*. (2009) reported that *Termitomyces* spp. possess high protein and low-fat contents. *Termitomyces microcarpus* and *Termitomyces heimii*, which are consumed by indigenous people in Peninsular

Malaysia, were identified to be high in protein content with 39.7% (*T. microcarpus*) and 29.1% (*T. heimii*). Research also found that *T. heimii* may have chemopreventive effect towards selected human diseases. Ergosterol is a major compound successfully isolated from *T. heimii* has been shown to act as biological precursor of vitamin D2, which is responsible for protection against colon and prostate cancer (Jasinghe *et al*., 2007; Guyton *et al*., 2003). Linoleic acid is another major component from *T. heimii* important for anti-tumour activity which is proven to reduce the tumour size through apoptotic effect (Dox *et al*., 1993).

2.12 Bioactive proteins identified from natural sources

Human body requires proteins for building and repairing cells and tissues. Protein sources are plants, animals and microorganisms. Plant proteins contribute as substantial food source because they contain essential amino acids necessary for human physiological and they cannot be produced by human body (Millward, 1999; Nehete *et al*., 2013). Amino acids are the building blocks of proteins linked together in varying sequence to form a vast variety of proteins and defined by its unique sequence of amino acid residues (Pauling *et al*., 1951; Chaing *et al*., 2007). Essential amino acids are the main tissue protein assembling units which come only from the protein in food sources as it cannot be synthesised by the organism (Ghaly & Alkoaik, 2010). Essential amino acids consist of isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and lucine (Young, 1994).

Protein can be categorised into complete protein and incomplete protein. Animals are good source of complete protein consists of all nine essential amino acids in the correct proportion for protein building in human bodies, especially. However, animals and animal products such as meat, milk, milk products, egg, poultry and fish are generally high in fat content which leads to high risks of cardiovascular diseases. In contrasts,

incomplete protein mostly derived from plants such as vegetables, legumes and fruits, contain all essential amino acids but not in correct proportion, also known as limiting amino acids (McArdle *et al*., 1991; Nehete *et al*., 2013).

2.13 Protein purification techniques for natural products

Several techniques have been implemented to determine the protein content in plant sources or food extracts. There are nine different techniques of protein quantification described by Walker in The Protein Protocols Handbook (Walker, 2002). The most commonly used method is Lowry, bicinchoninic acid (BCA) assay, as well as Bradford. Bradford method has been implemented by many scientists in laboratories for quantifying proteins. The method is recognised as simple, fast and more sensitive as compared to Lowry method. The protein content in this assay relies on the binding of the dye Coomassie Blue G250 to protein. The quantity of protein is usually estimated at maximum absorbance of 595 nm by determining the amount of dye in the blue ionic form (Kruger, 2009).

Protein purification is important in the large-scale production of purified protein for pharmaceutical products like Insulin and food supplements. A number of purified proteins have been produced in large amount using the novel techniques of recombinant DNA technology and tissue culture. Purified proteins are essential for research and analytical purposes such as identification, function characterisation, structure determination and interaction of specific protein (Kumar & Sharma, 2015). An ideal protein purification strategy is one in which the highest purification level is achieved in the fewest steps as each process of purification will results in product loss. Method selection is dependent on the size, charge, solubility and other properties of target protein (GE Healthcare handbook).

The first step involves in protein purification is the isolation process where a complex mixture of protein samples is being concentrated or precipitated (to increase the percentage of target protein in total protein) and separated (removal of unwanted contaminants) (Kumar & Sharma, 2015). Precipitation of protein is normally conducted by the salting out technique using salt such as ammonium sulphate at specific concentration. This process is also an advantage where the desired proteins can be separated from other proteins and the precipitated protein is very stable and high shelf life (Lee, 2017).

The next step for protein purification is using chromatographic techniques. Chromatography is the most commonly used method for detection and purification of biological compounds including proteins. The principle of separation in chromatography is based on mobile phase (normally liquid or gas) and stationary phase (composed of solid or a layer of a liquid adsorbed on the surface of solid support) to fractionate or separate the compounds/molecules (Coskun, 2016). Selection of chromatographic technique is dependent on several criteria such as the physical shape of stationary phase, the nature of mobile phase and/or stationary phase, the mechanisms of separation or the other properties of chromatographic systems (Sattayasai, 2012). Different chromatographic techniques are briefly described in Kumar & Sharma (2015). The purification of proteins/enzymes have utilised modern or combined chromatography techniques as shown in Table 2.6.

The purified protein should be free from contaminants such as nucleic acids, viruses, pyrogens, residual host cell proteins, cell culture media, and leachates from media, as well as various isoforms originating from posttranslational modifications (Kalyanpur, 2002). Identification of purified protein can be performed using LC-MS/MS technique where mass spectrometry and LC are combined.

This method is proven reliable and accurate since sequence information can be obtained from trace amounts of target proteins (Lee, 2017).

Table 2.6: Chromatographic techniques for protein purification (Kumar & Sharma, 2015).

No.	Technique	Protein purified
1	Radial flow chromatography	Endonuclease
2	Ion exchange chromatography	Vibronectin-binding surface protein, tubulin
3	Hydrophobic interaction chromatography	α-N-acetyl-galactosamidase
4	Reversed phase liquid chromatography	Sanguinarine
5	Perfusion chromatography	Early light-inducible proteins, BRCA 1 protein
6	Membrane affinity chromatography	Polyclonal antibodies, Human chorionic gonadotrophin
7	Electrokinetic chromatography	Tylosin
8	Capillary electro-chromatography	Basic proteins
9	Displacement chromatography	Dairy whey protein

2.14 Cytotoxic proteins isolated from mushrooms

The first lectin discovered was ricin, a very toxic protein from castor bean (*Ricinus communis*), which was extracted by Stillmark in 1888 (Podjana, 2004). Previous research had revealed the isolation of parasporin, a new category of protein known as bacterial parasporal proteins, from a gram-positive bacterium *Bacillus thuringiensis* which has the potential to kill human cancer cells (Ho¨fte & Whiteley, 1989; Mizuki *et al.,* 2000). In a different study, a novel cytotoxic protein from *B*. *thuringiensis* recognised as Cry45Aa had selective cytotoxic activity against various mammalian cells with markedly divergent target specificity, preferentially killing colonic, uterine and blood cancer cells (Okumura *et al.,* 2005).

Ribosome inactivating proteins (RIPs) have been isolated from several mushrooms such as *Calvatia caelata, Falmmulina velutipes, Hypsizigus marmoreus* and *P. tuber-regium*. This protein exhibited promising anti-proliferative activity towards hepatoma Hep G2 cell and breast cancer MCF-7 cells (Attarat & Phermthai, 2015). Pleuturegin is a designated RIP that has been successfully isolated from the fresh sclerotium of *P. tuber-regium*. Previous studies have highlighted pleuturegin's ability to inhibit the translation of a cell-free rabbit reticulocyte lysate system with an IC_{50} of 0.02 µg/ml. The N-terminal amino acid sequence of pleuturegin was different from RIPs isolated from other mushrooms such as *Flammulina velutipes* (flammulin and velutin), *Hypsizygus marmoreus* (hypsin), and *Lyophyllum shimeji* (lyophyllin). The molecular weight of pleuturegin was compatible to flammulin with 38 kDa and 40 kDa, respectively. However other RIPs were considerably smaller with 13.8 kDa (velutin) and 20 kDa (hypsin and lyophyllin). Ribonucleases (RNases) and *N*-acetylglucosamine-binding lectin were also isolated from the fresh sclerotia of *P. tuber-regium* with a molecular weight of 29 kDa and 32 kDa, respectively (Wang & Ng, 2001a; Wang & Ng, 2001b).

Lectins isolated from different mushrooms have been elucidated with promising anti-tumour properties towards different types of cancer cell lines including colon cancer

cell lines, HT29, and Caco-2, MCF-7, HeLa cells, mouse mastocytoma P815 cells, and sarcoma S-180 cells. Previous studies have reported the release of tumour necrosis factor-α (TNF-α) from mononuclear cell cultures by induction of lectins from *Boletus satana* (Wang & Ng, 2003). Additionally, purified peptide from fruiting bodies of the puffball mushroom, *Calvatia caelata*, was found to inhibit the growth of breast carcinoma cell line, MDA-MB-231, at IC_{50} of 0.1 μM. The purified peptide is 8 kDa with an N-terminal sequence which is close to ubiquitin (Grube *et al*., 2001; Lam *et al*., 2001; Maness *et al*., 2011).

Protein-bound polysaccharide or PSP is reported to enhance immune status in 70-97% of patients suffering from cancers of the stomach, oesophagus, liver, lung, ovaries, and cervix, thus significantly improving the life quality as well as providing substantial pain relief (Parris, 2000). PSP extracted from mycelium or fruiting body of *Coriolus versicolor*, is commercially established, known to have the ability to distinguish cancerous cells from normal cells by its immunoreactive protein characteristics as well as anti-proliferative functions (Attaraj & Permthai, 2015). The cytotoxic effect of certain S-phase targeted-drugs on human cancer is enhanced by PSP (Sekete *et al*., 2012). A chemopreventive effect on prostate cancer is also revealed from PSP by targeting of prostate cancer stem cell-like populations (Liu *et al*., 2006). In addition, anti-proliferation activity of PSP on human breast cancer cells (T-47D) is discovered through upregulation of the p53 protein expression and down regulation of Bcl-2 protein expression, but in MCF-7 it was by means of down-regulation Bcl-2 protein expression only (Jeon *et al*., 2013). Thus, PSP significantly being a potential candidate for development of drug in the treatment and prevention of human cancer.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Source and preparation of *Pleurotus tuber-regium* and *Termitomyces heimii*

Pleurotus tuber-regium was cultivated at Glami Lemi Biotechnology Research Centre, Jelebu, Negeri Sembilan, Malaysia as described below. Both sclerotia and fruiting bodies of *P. tuber-regium* were used in this study. The fruiting bodies of *Termitomyces heimii* were supplied by a mushroom collector from Palm Estate in Trolak, Perak, Malaysia. All samples were washed with running tap water and sliced into small pieces before being freeze-dried using freeze dryer (LabConco, Kansas City, Missouri) for 4-5 days. The samples were ground into powder form and kept at 4°C until use.

3.1.1 Cultivation of *Pleurotus tuber-regium*

The cultivation of *P. tuber-regium* was employed as referred to Abdullah *et al*. (2013), with some modifications. Mycelia culture of *P. tuber-regium* was obtained from Mushroom Research Centre, Faculty of Science, Universiti Malaya. Mycelium was grown onto malt extract agar (MEA) media on petri dishes. After 7-8 days, the mycelial discs were cut using a 9-mm diameter cork borer before inoculation into sterile sawdust formulation in plastic bags. Inoculated bags were incubated at 28°C for 3 weeks until full mycelium penetration throughout the sawdust. After that, the mycelial-colonised sawdust blocks were removed from the plastic and the substrate blocks were buried in loam soil for sclerotia and fruiting bodies development. The sclerotia and fruiting bodies were harvested after 3-4 months of burial of substrate blocks.

3.2 Preparation of crude aqueous extract (CAE) and protein fractions

Sixty grams of powdered samples were soaked in 1,200 ml of distilled water and stirred using magnetic stirrer for overnight at 4°C. Then, the mixture was subjected to centrifugation (Hermle Laboratechnik GmbH, Germany) at 10,000 rpm for 15 minutes at 4±2°C to separate the insoluble and supernatant. The supernatant was collected and freeze-dried as crude aqueous extract (CAE). The sample was kept in schott bottles at -20°C before use.

To prepare the protein fractions, the freeze-dried CAE was weighed and dissolved in distilled water at a ratio of 1:20 (g/ml). Protein was fractionated by ammonium sulphate precipitation (salting out) at 30%, 60% and 90% salt saturation to partially fractionate the proteins (Lau *et al*., 2012). Proteins were precipitated out from aqueous solutions by slowly dissolving ammonium sulphate into the CAE extract solution based on the percentage saturation values described by Dixon (1952) (Appendix A1.0). Ammonium sulphate solution starting at 30% saturation was poured gradually into the CAE solution and stirred constantly using a magnetic stirrer for 30 minutes on ice bath to avoid possible protein denaturation. The mixture was centrifuged at 10,000 rpm and 4±2°C for 15 minutes. The residual supernatant was used for subsequent salt saturations (60% and 90%) and the precipitated protein yield was then dissolved in distilled water.

The precipitated protein fractions were then dialysed using SnakeSkin pleated dialysis (Thermo Fisher Scientific, USA) tubing with 3,500 Da molecular weight cut off. This retained all 3.5 kDa and higher size of proteins in the sample solution while smaller molecules, such as salt and small peptides were dialysed out into the buffer solution exhibiting low concentration of salt. The dialysis tubing filled with protein sample was placed in a beaker containing 500 ml of distilled water. The tubing was left stirring at 4±2°C for 48 hours with 4 times buffer changes. After dialysis, the liquid protein fractions were freeze-dried and stored at -20°C prior to use. The protein fractions were labelled

according to the concentration of the salt saturation at which they were formed as shown in Figure 3.1. The flow chart of experiments conducted in this study is shown in Figure 3.2.

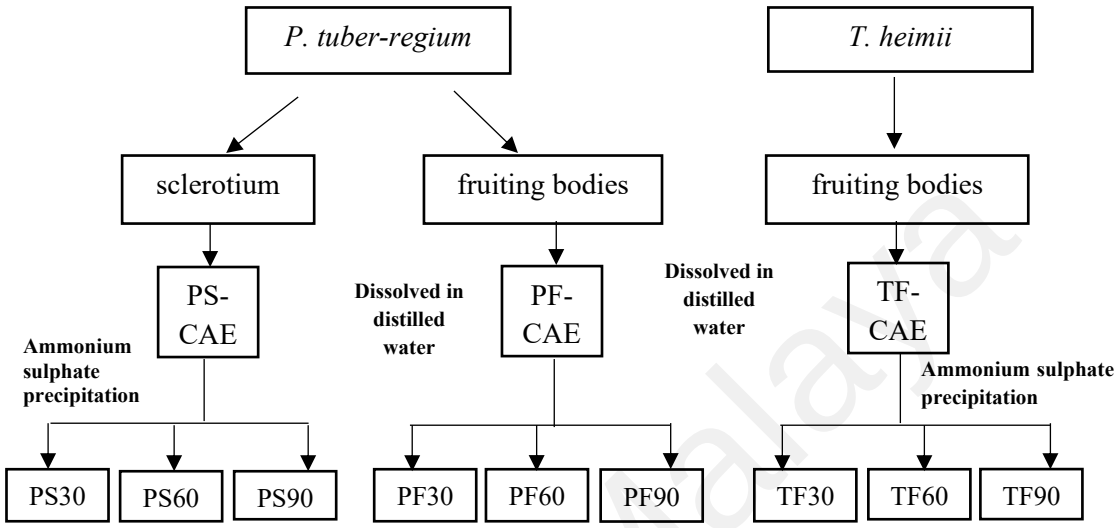


Figure 3.1: The flow chart of extraction process to produce crude aqueous extracts and protein fractions from both *Pleurotus tuber-regium* and *Termitomyces heimii.*

3.3 Protein estimation of CAE and protein fractions

Protein content of CAE from both mushroom species and their protein fractions (F30, F60, and F90) were estimated using Bradford Protein Assay method following the instruction of Pierce™ Coomassie Plus (Bradford) Protein Assay Kit (Thermo Fisher Scientific, USA). The protein concentrations were determined with reference to a standard protein curve, bovine serum albumin, (BSA) (Appendix B1.0). In order to develop the standard protein curve, BSA was prepared in a series of dilution range from 2,000 µg/ml to 0 µg/ml and assayed together with protein samples. Preparation of the BSA dilutions is shown in Table A2 (Appendix A2.0). Ten microlitres (10 µl) of each standard protein, CAE, and protein fractions were pipetted into a microplate well. Each of the well was added with 300 µl of Coomassie Plus Reagent. The plate was covered and agitated

for about 30 seconds to mix the content, and incubated for 10 minutes at room temperature. After that, the absorbance values were determined at wavelength 595 nm using microplate reader (Thermo scientific, Multiskan Go).

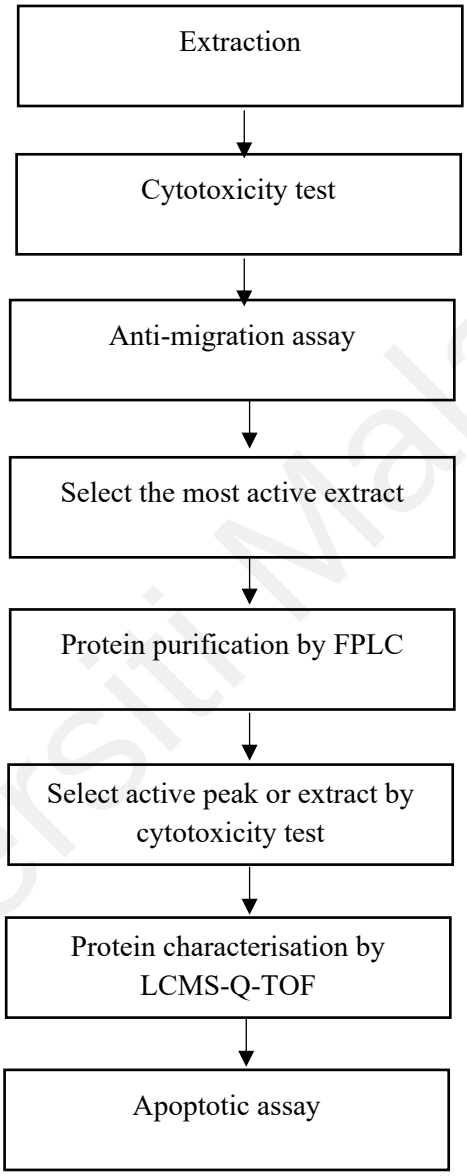


Figure 3.2: The schematic diagram of extraction process, cytotoxic and anti-migration assay, FPLC, LCMS-Q-TOF analysis, and apoptotic assay.

3.4 Cytotoxic activity of CAE and protein fractions against cancer and normal cells

3.4.1 Cell culture

Breast cancer cell lines (MDA-MB-231), colorectal cancer cell lines (HCT-116) and normal lung fibroblast cell line (MRC-5) were purchased from the American Type Culture Collection (Rockville, USA). Cells were grown in Dulbelco's Modified Eagle's Medium (DMEM) (Sigma, USA), McCoy's 5A (modified) and Essential Minimum Eagle's Medium (EMEM) (Sigma, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories GmbH, Australia), 2% penicillin/streptomycin (PAA Laboratories GmbH, Australia) and 1% amphotericin B (PAA Laboratories GmbH, Australia), respectively. Cells were incubated and maintained in a humidified incubator (ESCO) at 37°C with 5% carbon dioxide (CO_2). All other chemicals used were of analytical grade. The maintenance and sub-cultivation steps of these cell lines are shown in subsection 3.4.2 and 3.4.3.

3.4.2 Reviving MDA-MB-231, HCT-116 and MRC-5 cell lines and maintaining the culture

The cells were kept in liquid nitrogen tanks and stored at -196°C. The cryovial containing cell line was removed from the liquid nitrogen tank and transferred into the water bath at 37°C for quick thawing. Then, the cells were transferred into the centrifuge tube containing 20% media and centrifuged at room temperature and 1,000 rpm for 5 minutes. The supernatant was discarded. 1 mililitre of 20% media was added to the cells pellet and mixed using a micropipette. 9 mililitres of 20% media was placed into the culture flask and cells from the centrifuge tube were transferred into the tissue culture flask. The cells were incubated in a 5% carbon dioxide (CO_2) incubator at 37°C. The growth of the cells was observed every 2 days. The culture media was changed every 2

days. The cell culture was maintained until the cells were 90% confluent.

3.4.3 Sub-cultivation of cell lines

Sub-cultivation was carried out once the cell growth was fully confluent. The confluence of the cells was determined by the observation of elongated monolayer of cells at the bottom surface of the tissue culture flask. The old media was discarded. The cells were then washed with 10 ml of sterile PBS. The PBS was discarded and 3 ml of accutase was added into the flask. The flask was incubated for 3-5 minutes in 5% CO_2 incubator at 37°C.

The cells were detached from the bottom of the flask by accutase. The flask was gently tapped to detach the cells from the surface. Cells that are detached are observed as floating pieces through the inverted microscope. After the cells have detached from the flask, the cell suspension was transferred into a centrifuge tube and centrifuged at room temperature and 1,000 rpm for 5 minutes. The supernatant was discarded and 3 ml of 10% supplemented media were added into the pellet and mixed well with the micropipettes. Meanwhile, 9 ml of 10% supplemented media was added into the tissue culture flask, followed by 1 ml of mixed cell suspension into the flask. The flasks were incubated once again until used. The medium was changed every 2 days to maintain the cells.

3.4.4 Cell proliferation and viability assay

The CAE and protein fractions were prepared at 200 µg protein per ml in distilled water as stock solution for cell proliferation and viability assay. The activities of all extracts against MDA-MB-231, HCT-116 and MRC-5 cells growth were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay based on Mosmann (1983), with some modifications. Cells were seeded into a 96-well plate at a density of 10^4 cells per well, in 100 µl medium containing 10% FBS

and 2% penicillin/streptomycin and 1% amphotericin B.

The seeding of cells was carried out by firstly, detachment of once fully confluent tissue culture flask. The detachment protocol was performed as described in subsection 3.4.3. The detached cells were centrifuged at 1,000 rpm for 5 minutes at room temperature, and the supernatant was discarded. The cell pellets were dissolved in 1 ml of 10% supplemented medium (DMEM for MDA-MB-231, Mc Coy's for HCT-116, and EMEM for MRC-5) as cell suspension stock. For enumeration, 10 µl of cells suspension and 900 µl of tryphan blue were added into a micro-centrifuged tube and mixed well. Haemacytometer covered with a glass cover slip was prepared. Twenty µl of cell suspension and tryphan blue mixture were pulled together into the haemacytometer chamber by capillary action. The number of colourless cells in each chamber was counted through the microscope.

The total number of cells was recorded in sections marked 1-4 and divided by 4 (Figure 3.3) to get an average number of cells (N) (Morten *et al*., 2016). The concentration of stock cell suspension was calculated using the formula as follows:

$$M_1V_1=M_2V_2$$

Where M_1 = Number of cells projected in tissue culture flask (N x 10^4 x 10)

V_1 = Volume of cell suspension in centrifuge tube (990 µl)

M_2 = Cell number per ml

V_2 = Volume of cell suspension to use for seeding

Ten percent of supplemented media (V2) was added into a beaker and cell suspension from centrifuge tube (V1) was added and shaken well. The mixture was

transferred into reagent reservoir. The cell suspension (100 µl) was transferred into each well in 96 well plate and incubated for 24 hours in a CO_2 incubator at 37 °C with 5% humidified air, to allow cells attachment.

1		2
3		4

Figure 3.3: Placement of cells in Haemacytometer

After 24 hours of incubation, the cells were observed for growth and attachment before treatment with CAE and protein extracts of *P. tuber-regium* and *T. heimii*. The old media of each well was discarded and replaced with new 10% of supplemented media for negative control, while others were treated with samples diluted with 10% supplemented media. The cells were then treated with serially diluted CAE and protein fractions of *P. tuber-regium* and *T. heimii* at concentration of 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25.00, 50.00, and 100.00 µg/ml, and then incubated in CO_2 incubator for 72 hours. After 72 hours of incubation, 20 µl of 5 mg/ml MTT in phosphate buffer saline (PBS) pH 7.2 were added in each well of treated and untreated cells. The plates were incubated for another 4 hours before replacing the medium with 100 µl of DMSO to dissolve the formazan products formed at the bottom of each well. The plates were agitated for 10 minutes and the optical density (OD) of the viable cells was measured at 570 nm with reference wavelength at 690 nm using a microplate spectrophotometer (Thermo scientific, Multiskan Go).

3.4.5 Calculation of cytotoxic activity

The half-maximal inhibitory concentration (IC_{50}) value was determined using Microsoft Excel by plotting a logarithmic best curve and extrapolated at percentage of inhibition of 50%. Each measurement was performed in triplicates. The percentage of inhibition (POI) of the cells was calculated using the optical density of the sample extracts and controls using a formula (Cory *et al*., 1991) given below: -

$$\text{Percentage of Inhibition (\%)} = \frac{\text{(OD control)} - \text{(OD sample)}}{\text{OD control}} \times 100\ \%$$

OD = optical density of the cells

The POI obtained was plotted into a dose-response curve. The IC_{50} values refer to the effective dose (µg/ml) required to inhibit the growth of the cells by 50%.

3.4.6 Statistical analysis for MTT assay

All experimental results were calculated as mean of 3 individual readings (n=3) with ± standard error of mean (S.E.M). The 50% inhibitory concentrations of the MTT results were estimated by extrapolation of the data using nonlinear regression analysis.

3.5 Evaluation of anti-migration activity of protein fractions from *Pleurotus tuber-regium* and *Termitomyces heimii* fruiting bodies

3.5.1 Anti-migration assay

Protein fractions of *P. tuber-regium* sclerotia and *T. heimii* fruiting bodies showed potent cytotoxicity, hence, further analysis was done. Anti-migration assay was carried out to investigate the potential of CAE and protein fractions in inhibiting migration of MDA-MB-231 cells and HCT-116 cells. Both cancer cell lines were seeded into 6-well

plates at a density of 10^6 cells per well in 2 ml supplemented media (DMEM for MDA-MB-231 and Mc Coy's for HCT-116) containing 10% FBS for 24 hours. The confluent monolayer of cells was scratched using a sterile 200 µl pipette tip along the diameter of the well. The cell debris was removed and discarded by washing with PBS (refer subsection 3.4.3). DMEM medium containing 1% FBS and 2% penicillin/streptomycin and 1% amphotericin B, was used to maintain the treated and untreated cells with final volume of 4 ml in each well. After that, the cells were treated with non-toxic concentrations of protein extracts (at both IC_{50} and IC_{25} concentrations) for 24 hours and 48 hours in CO_2 incubator. The wound area was photographed at 0 hour, 24 hours and 48 hours with an inverted microscope to observe the extent of wound closure. Three spots were identified, where gap distance was measured and mean values were calculated (Liang *et al*., 2007; Chew *et al*., 2012). Migration capabilities is defined as absolute migration capability (MC_A) which were measured using a formula below (Peng *et al*., 2007): -

$$MC_A \text{ (mm/h)} = \frac{G_0 - G_t}{2t}$$

Where G_0 is the initial gap for the cell line at 0 h (mm)

Gt is the final gap for the cell line treated with extracts at certain concentration for a time period of *t* h (mm)

t is the overall time period for incubation (h)

After that, the percentage of migration of the treated to the untreated cells was evaluated using the formula below: -

$$\frac{MC_A \text{ of treated cells}}{MC_A \text{ of untreated cells}} \times 100\%$$

3.5.2 Statistical analysis for anti-migration assay

The anti-migration assay was analysed using Statistical Package for the Social Sciences (SPSS) version 24.0 program. The mean differences of the results were compared by one-way ANOVA using Duncan test where p-values ($p \leq 0.05$) indicate a significance difference between the data and control.

3.6 Separation and characterisation of cytotoxic proteins from *Pleurotus tuber-regium* extracts

3.6.1 Protein profiling by Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein extracts from *P. tuber-regium* sclerotia were separated and characterised using SDS-PAGE method where the proteins were separated according to their molecular size, shape and charge. The sodium dodecyl sulphate (SDS) binds to most proteins in an amount approximately proportional to the molecular weight of the proteins. The SDS-PAGE consists of stacking gel and separating gel that act as a molecular sieve to separate different sizes of proteins. Larger proteins will migrate slower as it become highly entangled in the gel. In addition, proteins with compact globular shape move faster than elongated fibrous proteins of comparable molecular mass. The applied current through the gel matrix is also a factor that influence the movement of proteins where molecules

with high charge density (charge per unit of mass) migrate rapidly through the gel system (Sattayasai, 2012; Kumar & Sharma, 2015).

In the present study, all extracts of *P. tuber-regium* sclerotia (PS-CAE, PS30, PS60, PS90) were incorporated onto SDS-PAGE for separation verification to profile protein. The separating gel of 16% and stacking gel of 4% were prepared (Appendix A4.1) and developed into a mini vertical system. Extract stock solution of 100 µg/ml was mixed with sample buffer containing bromophenol blue as tracking dye with ratio of 4:1. Proteins in the sample solutions were denatured by incubation at 95°C for 5 minutes. After that, 15 µl of the sample solution were loaded into the wells of polyacrylamide gel. Protein ladder (Thermo Fisher scientific, Rockford, IL, USA) of 5 µl was also introduced into the gel as a biomarker for the determination of molecular weight bands (Saini & Sarin, 2012).

Polyacrylamide gel electrophoresis was performed in an electric field at 100 V and 60 mA with running buffer of 0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) SDS (pH8.3). After electrophoresis, the gel was fixed with fixing solution consisting of 40% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes. The polyacrylamide gel was then silver stained using silver stain kit as described in manufacturing safety data sheet provided (Thermo Fisher Scientific, Rockford, IL, USA). Molecular weights of proteins separated were compared with the standard molecular mass marker proteins. Samples and reagents preparation are shown in Appendix A4.0.

3.6.2 Fast Protein Lipid Chromatography (FPLC)

The most cytotoxic protein fraction, PS60 (fraction of *P. tuber-regium* sclerotia) was further purified using anion-exchange column packed with DEAE (label) attached to an FPLC instrument (AKTA Avant 25). It is a preparative chromatography system designed for fast and secure development of scalable methods and processes. AKTA

Avant 25 is specialised for media screening and method optimisation using small columns. The column used in the study was HiTrap 1 ml Capto Q (Strong anion exchanger) column. The FPLC protocol was optimised several times before final protocol was achieved. Pump wash was performed for the necessary pumps and column with water followed by buffer (Appendix A5.0).

The start or binding buffer (20 mM Tris-HCl; pH 8.0) and elution buffer (20 mM Tris-HCl, 1 M NaCL; pH 8.0) were used for the separation of fractions (Appendix A5.0). The protein peak fractions were filtered through 0.22 µm syringe filters (Sartorius) before injected into the column. Three millilitre of 1 mg/ml PS60 protein sample was injected into the injector valve ports and allowed to run for 70 minutes. The elution of peak fractions was attained by the use of step gradient from 0-60%, 60-90%, and 90-100% salt at the constant flow rate 1.0 ml/min. Peaks were detected at 280 nm wavelength. Every single peak shown by the chromatogram was automatically collected by the machine and labeled. Fractions collected were pooled and desalted by dialysis using SnakeSkin pleated dialysis tubing (membrane cut off, 3.5 kDa) against distilled water for at least 48 hours at 4±2°C, lyophilized and diluted in medium to a concentration of 1 mg/ml. The fractions were then freeze dried and stored at -20°C.

3.6.3 Cytotoxicity test of FPLC peak fractions

Each of the peak fractions collected from FPLC was reevaluated for cytotoxic activity against cancer cell lines using MTT assay as described in subsection 3.4.2 and 3.4.3. The IC_{50} values of peak fractions were then compared with cisplatin, the FDA approved drug for the treatment of breast cancer diseases.

3.6.4 Identification of protein by LCMS-Q-TOF MS

The most active cytotoxic fraction, PS60 was further subjected to LCMS-Q-TOF MS analysis. Digestion process and desalting procedure as described in subsection 3.6.4.1 and 3.6.4.2 were conducted before proceeding with the analysis.

3.6.4.1 In-solution tryptic digestion for LC separation

In-solution digestion protocol was applied for this analysis and the protocol was divided into 3 steps; reduction and alkylation, digestion and extraction.

In reduction and alkylation steps, digestion buffer (ammonium bicarbonate) and reduction buffer (DTT) were prepared (Appendix A6.0). Fifteen ml of 50 mM ammonium bicarbonate and 1.5 µl of 100 mM DTT were mixed in microcentrifuge tube. Ten microlitre of protein solution (sample of PS60) was added into the tube and the final volume was adjusted to 27 µl with ddH_2O. The solution was incubated at 95°C in the water bath. The sample was then left to cool at room temperature. The alkylation buffer (100 mM Iodoacetamide) was freshly prepared and 3 µl of this buffer was added into the microcentrifuge tube. Incubation was carried out in the dark at room temperature for 20 minutes.

For the digestion steps, 0.1 µg/µl trypsin was prepared (Appendix 6.4) and 1 µl of trypsin was added into the reaction tube. The mixture was incubated at 37 °C for 3 hours. After 3 hours, an additional trypsin was added into the tube and incubation was carried out at 30°C for overnight. The mixture was then vortex and spun down briefly at 1000 rpm for 1 minute.

In the extraction steps, 50 µl of 50% acetonitrile was added into the mixture and was shaken for 15 minutes. All the mixtures were transferred into fresh tubes and subsequently mixed with another 50 µl of 100% acetonitrile for 15 minutes. The mixture was transferred back to the previous tube. The digested sample was then completely dried

using speed vacuum at low speed for 2 hours. The samples were further to zip-tip desalting protocols.

3.6.4.2 Zip tip protocol for protein samples cleaning

The zip tip procedure was used to clean up protein samples from any chemicals that were used during protein digestion. This protocol was divided into 5 steps; wetting, equilibration, binding, washing, and elution. All the materials used in this protocol was prepared prior used (Appendix A7.0). The protein samples in digestion protocol were reconstituted in 10 µl of 0.1% FA (formic acid). The elution buffer (1.5 µl) was added into the new tube. During zip tip procedure, the zip tip resin should not be completely dry (except for final elution step) after aspiration to avoid resin damage and alteration of binding capacity. The aspiration steps must be done carefully to avoid any bubbles formation within the zip tip column.

Wetting : 10 µl of wetting solution (100% acetonitrile) was aspirated into zip tip and dispensed. The steps were repeated 3 times.

Equilibration : 10 µl of equilibration solution (0.1% FA) was aspirated into zip tip and dispensed. The steps were repeated 3 times.

Binding : 10 µl of samples was aspirated into zip tip for 10 times and dispensed.

Washing : 10 µl of washing solution (0.1% FA) was aspirated into zip tip and dispensed. The steps were repeated 3 times.

Elution zip tip : 1-4 µl elution solution (0.1% FA in 50% acetonitrile) was aspirated into and dispensed for 3 times.

All of the elution solution was fully dispensed in the sample solution. The solution was subjected to speed vacuum at 1,000 rpm until the solution was completely dried.

3.6.5 LCMS-QTOF MS Analysis

Liquid chromatography mass spectrometry was used to separate and identify protein compounds from the protein fraction of *P. tuber-regium* sclerotia (PS60). The analysis was performed using Agilent 6530 Q-TOF LC/MS equipped with auto sampler. The Agilent 6530 Accurate Mass Q-TOF was used to obtain the MS and MS/MS data. The separation was carried out using 1269 Infinity Nanoflow LC System (Agilent, Santa Clara, CA, USA) directly connected to Accurate Mass Q-TOF 6550 with nano electrospray ionisation source for MS analysis. There were two mobile phases used in this analysis, mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in ACN). The column used was HPLC Large Capacity Chip Column (Zorbax 300SB-C18, 160 nL enrichment column, and 75 µm x 100 mm analytical column, and 5 µm particles, Agilent, USA). The flow rate for capillary pump was 4.0 µl/min while for nano pump was 0.4 µl/min. The total run time for this analysis was 25 minutes. The injection sample volume was 2 µl.

The sample was run at 5-70% linear gradient, mobile phase B. Iodoacetamide was used for alkylation during sample preparation. Therefore, carbamidomethylation was specified as a fixed medication and oxidised methionine as a variable modification. In addition, the precursor mass shift was set between -18 Da to 177 Da to take into consideration of variable modifications such as presence of sodium and potassium. The mass spectra acquired using Mass Hunter software (Agilent, Santa, Clara, CA, USA) with acquisition rate of 8 spectra per second from 200 to 3,000 m/z and was followed by collision-induced dissociation of the twenty most intensive ions. MS/MS data were obtained in the range of 50-3,200 m/z (san rate= 4 spectra/sec).

Spectrum Mill software (Agilent, Santa Clara, CA, USA) was used for MS/MS search. The database used in this analysis was Swiss-Prot (Homo sapiens) with 168,628 entry sequences. In this analysis, proteins and peptides were validated using Spectrum

Mill software, based on the software default settings. The inclusion criteria were protein score > 20, peptide score > 10, and Scored Peak Intensity (%SPI) > 70%. Proteins that shared at least one peptide were grouped together and the identified proteins were then filtered to achieve a false discovery rate (FDR) of < 1% for the peptide-spectrum matches.

3.7 Apoptotic effect of PS60 against MDA-MB-231 cells

3.7.1 Annexin V/ propidium iodide (PI) assay for determination of mode of cell death

Annexin V/PI assay was utilised to investigate the effect of potential cytotoxic protein fraction (PS60) in inducing cell death through apoptosis. The MDA-MB-231 cells were seeded at a concentration of 500,000 cells per plate in a 60 mm culture dish and incubated for 24 hours at 37°C. Next, the culture medium was changed to fresh medium before being treated with different concentrations of PS60 and cisplatin. The cells were then harvested at 24, 48, and 72 hours, washed with phosphate buffered saline (PBS) and stained with Annexin V-FITC conjugates and propidium iodide (PI) following the protocols of FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA). A minimum of 10,000 events were collected and analysed using a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

3.7.2 Cell cycle assay for analysing the disruption of cells

The Cycletest plus DNA reagent kit (BD Biosciences, San Jose, CA, USA) was used to determine the presence of cell cycle disruption caused by the cytotoxic protein fraction, PS60 against MDA-MB-231 cells. The cells were seeded at a concentration of 500,000 cells per dish on to a 60 mm culture dish and left for 24 hours at 37°C before treated with positive control, cisplatin and PS60 at different concentrations. The cells were then incubated for another 48 hours before being harvested and further assessment

of cell cycle arrest following the steps of the Cycletest plus DNA reagent kit. A minimum of 10,000 events were collected and analysed using the BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

3.7.3 Statistical analysis for apoptotic assay

The apoptotic assay results were analysed using Statistical Package for the Social Sciences (SPSS) version 24.0 program. The mean differences of the results were compared by one-way ANOVA using Duncan test where p-values ($p \leq 0.05$) indicate a significant difference between the data and negative control (untreated cells).

CHAPTER FOUR

RESULTS

In this chapter, the anti-tumour activities of proteins extracted out from *P. tuber-regium* and *T. heimii* towards breast cancer (MDA-MB-231) and colorectal cancer cell lines (HCT-116) were evaluated. Potential cytotoxic proteins, PS60 fraction (*P. tuber-regium* sclerotia) was separated and characterised using SDS-PAGE, FPLC and LCMS-Q-TOF MS. Finally, the cell death mechanisms exerted by the cytotoxic proteins (PS60) towards breast cancer cell lines (MDA-MB-231) were analysed using Annexin V-FITC/ propidium iodide (PI) assay and cell cycle arrest.

4.1 Protein yield of CAE and protein fractions from *Pleurotus tuber-regium* and *Termitomyces heimii*

The yield of protein quantitated using Bradford Protein Assay method (Bradford, 1976) is shown in Table 4.1. Protein was analysed in both sclerotia (PS) and fruiting bodies of *P. tuber-regium* crude aqueous extract (CAE) and protein fractions precipitated by 30% (P30), 60% (P60), and 90% (P90) ammonium sulphate. Similarly, protein content in fruiting bodies *of T. heimii* crude aqueous extract (TF-CAE) and protein fractions (TF30, TF60, and TF90) were also analysed. The highest protein yield in *P. tuber-regium* fruiting body extracts were from PF60 (1.830±0.080 mg/ml), followed by PF90 (1.785±0.099 mg/ml), PF30 (1.636±0.135 mg/ml) and PF-CAE (1.430±0.134 mg/ml). While in the sclerotia extracts, the highest protein content was found in PS30 (1.421±0.052 mg/ml), followed by PS60 (1.310±0.058 mg/ml), PS90 (0.671±0.057 mg/ml), and the lowest was in PS-CAE (0.480±0.007 mg/ml).

For *T. heimii* fruiting body extracts, TF60 exhibited the highest protein yield of 1.462±0.191 mg/ml followed by TF90 (1.258±0.150 mg/ml), TF30 (0.737±0.029 mg/ml),

and the lowest was in TF-CAE with a value of 0.500±0.003 mg/ml. For *P. tuber-regium*, fruiting bodies yielded higher protein compared to the sclerotia structure. Also, in general, protein yield from fruiting bodies of *P. tuber-regium* was higher than that in *T. heimii*.

Table 4.1: Protein concentration (mg/ml) from different extracts of *Pleurotus tuber-regium* sclerotium, *Pleurotus tuber-regium* fruiting body and *Termitomyces heimii* fruiting body obtained through ammonium sulphate precipitation method.

***P. tuber-regium* extracts (sclerotium)**	**Protein content (mg/ml)**	***P. tuber-regium* extracts (fruiting body)**	**Protein content (mg/ml)**	***T. heimii* Extracts (fruiting body)**	**Protein content (mg/ml)**
PS-CAE	0.480 ±0.006	**PF-CAE**	1.430 ±0.135	**TF-CAE**	0.500 ±0.003
PS30	1.421 ±0.052	**PF30**	1.636 ±0.135	**TF30**	0.737 ±0.029
PS60	1.310 ±0.058	**PF60**	1.830 ±0.080	**TF60**	1.462 ±0.192
PS90	0.671 ±0.056	**PF90**	1.785 ±0.099	**TF90**	1.259 ±0.150

Results were expressed as the mean ± standard deviation of triplicates values (n=3).

4.2 Cytotoxic activity of protein extracts against MDA-MB-231 cells, HCT-116 cells and MRC-5 cells

Table 4.2 presents the concentration (IC_{50}) of *P. tuber-regium* sclerotia (PS-CAE, PS30, PS60, and PS90), *P. tuber-regium* fruiting bodies (PF-CAE, PF30, PF60, PF90) and *T. heimii* fruiting bodies (TF-CAE, TF30, TF60, TF90) extracts which inhibits the growth of breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines by 50%. The 50% inhibitory concentration (IC_{50}) values indicate the dosage required to inhibit or kill half of the cell population. According to the U.S National Cancer Institute, extracts with IC_{50} values less than 20 µg/ml are considered to be actively cytotoxic against target cells after 48-72 hours of incubation time (Abdel-Hameed *et al*., 2012).

The protein of fraction 60 from *P. tuber-regium* sclerotium (PS60) showed the most prominent cytotoxic activity against breast cancer cell lines (MDA-MB-231) with an IC_{50} value of 0.75±0.57 µg/ml. From the result obtained, it is proven that the sclerotium of *P. tuber-regium* exhibited higher cytotoxic activity compared to its fruiting body, with IC_{50} values 3 times lower than that of PF60 (IC_{50}=2.15±0.07 µg/ml) against MDA-MB-231 cells. Therefore, *P. tuber-regium* sclerotium extracts were selected over *P. tuber-regium* fruiting body for further study. Table 4.3 presents the IC_{50} values of protein fractions as well as the aqueous extracts of *P. tuber-regium* sclerotium and *T. heimii* (fruiting body) against the growth of normal lung cell lines (MRC-5).

The potential extract to be selected for anti-cancer therapies should have no effect or exert at least minimum cytotoxic effect towards normal cell lines. Thus, selectivity index (SI) was calculated as a reference selectivity indicator by evaluating the ratio of IC_{50} values between non-tumoural lung carcinoma cells (MRC-5) and cancer cells (Popiolkiewicz *et al*., 2005). Selectivity index (SI) greater than 3 indicates potential selectivity of extracts or compounds towards cancer cells as compared to the normal cells (Bézivin *et al*., 2003). Table 4.4 shows the ratio between normal cells and cancer cells, or selectivity index (SI) (Peña-Morán *et al*., 2016).

$$SI = \frac{IC_{50}\ (\text{normal cells})}{IC_{50}\ (\text{cancer cells})}$$

Table 4.2: Cytotoxicity (**IC_{50}**, µg/ml) of *Pleurotus tuber-regium* (sclerotium and fruiting body) and *Termitomyces heimii* (fruiting body) extracts against breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines in MTT assay. Results were expressed as the mean of triplicates values (n=3). The cells were incubated for 72 hours.

Cell lines	***P. tuber-regium* sclerotium (µg/ml)**				***P. tuber-regium* fruiting body (µg/ml)**				***T. heimii* fruiting body (µg/ml)**			
	PS-CAE	PS30	PS60	PS90	PF-CAE	PF30	PF60	PF90	TF-CAE	TF30	TF60	TF90
MDA-MB-231	**8.00 ±0.00**	**7.50 ±0.71**	**0.75 ±0.57**	**8.50 ±0.00**	**3.05 ±0.21**	**7.10 ±0.14**	**2.15 ±0.07**	51.85 ±14.63	**7.50 ±0.71**	**12.50 ±0.00**	**2.95 ±0.78**	**14.50 ±2.12**
HCT-116	**12.00 ±2.83**	**14.25 ±5.30**	**3.75 ±2.47**	20.75 ±1.06	**4.35 ±0.21**	**4.18 ±0.04**	**2.20 ±0.14**	**19.50 ±2.83**	56.50 ±15.20	**13.05 ±2.12**	20.25 ±6.72	36.50 ±7.78

$IC_{50} \leq 20$ µg/ml, considered highly cytotoxic against breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines.

Table 4.3: Cytotoxicity (**$IC_{50,}$** µg/ml) of *Pleurotus tuber-regium* (sclerotium) and *Termitomyces heimii* (fruiting body) extracts against normal lung fibroblast (MRC-5) cell line in MTT assay. Results were expressed as the mean of triplicates values (n=3). The cells were incubated for 72 hours.

Cell line	***Pleurotus tuber-regium* (sclerotium) (µg/ml)**				***Termitomyces heimii* (fruiting body) (µg/ml)**			
	PS-CAE	PS30	PS60	PS90	TF-CAE	TF30	TF60	TF90
MRC-5	**8.13 ±2.65**	**11.25 ±0.35**	**10.50 ±0.00**	74.00 ±1.40	23.00 ±7.07	**12.50 ±2.83**	**1.90 ±1.27**	33.25 ±4.60

$IC_{50} \leq 20$ µg/ml, considered highly cytotoxic against normal lung fibroblast (MRC-5) cell line.

Table 4.4: Selectivity index (SI) of *Pleurotus tuber-regium* (sclerotium) and *Termitomyces heimii* (fruiting body) extracts on breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines as compared to normal lung cell lines (MRC-5).

Cell lines	***Pleurotus tuber-regium* (sclerotium)**				***Termitomyces heimii* (fruiting body)**			
	PS-CAE	PS30	PS60	PS90	TF-CAE	TF30	TF60	TF90
MDA-MB-231	1.02	1.50	**14.00**	**8.70**	**3.07**	1.00	0.64	2.29
HCT-116	0.68	0.79	2.80	**3.57**	0.41	0.96	0.09	0.91

SI > 3 indicate that the particular protein extract produces selective toxicity towards breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines.

From the results obtained, all extracts from *P. tuber-regium* sclerotium have shown active cytotoxic activities ($IC_{50} \leq 20$ µg/ml) against both breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines. Fraction 60 of *P. tuber-regium* sclerotium (PS60) showed the most promising anti-tumour cytotoxic effect towards the growth of both cancer cell lines, MDA-MB-231 and HCT-116 with IC_{50}=0.75±0.57 µg/ml, and IC_{50}=3.75±2.47 µg/ml, respectively. PS60 is actively cytotoxic towards normal cell lines, MRC-5, with IC_{50} value of 10.50±0.00 µg/ml. SI values of PS60 is higher for MDA-MB-231 (SI=14.00) compared to HCT-116 (SI=2.80). IC_{50} value of PS90 (74.00±1.40 µg/ml) towards MRC-5 shows that it is non-cytotoxic. The SI value of 8.70 shows that it is more selective towards MDA-MB-231 cells (IC_{50}=8.50±0.00 µg/ml). Clearly, PS90 holds less potential to be selected as cytotoxic agent against MDA-MB-231 cells. However, PS90 is deemed as the best protein fraction among the other extracts of *P. tuber-regium* sclerotium for the inhibition of HCT-116 cell lines. Both aqueous (PS-CAE) and PS30 extracts exhibited potential cytotoxic effect against HCT-116 cell line, however they also showed high cytotoxicity towards normal cell line MRC-5, with low selectivity index, SI=0.68 and 0.79, respectively.

Protein fraction of *T. heimii* fruiting body, TF60 was highly cytotoxic towards MDA-MB-231 growth with IC_{50} of 2.95±0.78 µg/ml, however its toxicity towards MRC-5 is greater (IC_{50}=1.90±1.27 µg/ml), resulting a low SI value, 0.64. Thus, this fraction is considered to be non-selectively cytotoxic (SI < 3) towards the breast cancer cell lines, MDA-MB-231. The other protein fractions such as TF90 (SI=2.29) and TF30 (SI=1.00), they were all of which non-selectively cytotoxic extracts against MDA-MB-231. The aqueous extract of *T. heimii* (TF-CAE) revealed a potential selectivity towards MDA-MB-231 cell line with SI value of 3.07. All extracts from *T. heimii* species have lower selectivity index towards colon cancer (HCT-116) cell lines with SI value less than 1.00.

Even though the aqueous extract (TF-CAE) and TF90 demonstrated less toxic effect towards MRC-5 (IC_{50}=23.00±7.07 µg/ml, 33.25±4.60 µg/ml), cytotoxic activities of both extracts towards HCT-116 were also low with IC_{50} =56.50±15.20 µg/ml and 36.50±7.78 µg/ml, respectively. Thus, among all extracts from *T. heimii* fruiting body, only TF-CAE showed interesting selectivity index towards MDA-MB-231 cell line, with SI values greater than 3, which indicates that the particular protein extract produces selective toxicity towards breast cancer (MDA-MB-231) cell lines (Bézivin *et al*., 2003).

4.3 Anti-migration effect of protein extracts towards cancer cells growth

The aqueous extracts and protein fractions from both *P. tuber-regium* sclerotia and *T. heimii* fruiting bodies were further evaluated for anti-migration activities against MDA-MB-231 and HCT-116 cell lines. The *in vitro* scratch wound method was carried out. A scratch was made on a confluent cell's monolayer. The cells were treated with IC_{50} concentrations of extracts (obtained from the MTT assay) and IC_{25} concentrations of protein extracts for 48 hours (Tables 4.5- 4.6). Here the potency of all extracts at 50% of cells inhibitory concentration in inhibiting the migration of cells were investigated. The cells migration activities were also examined at lower cytotoxicity concentration, which is half of the IC_{50} values (IC_{25}), thus providing dose-dependent treatment.

The width of the scratch (gaps) measured at 0 hour, 24 hours and 48 hours indicated cells migration activity as photographed by inverted microscope and shown in Figures 4.1 and 4.2. The anti-migration activity of cells was measured by observing the rate of cells migrate towards filling the artificial gap (scratch). The most potential anti-migration effect is demonstrated by the largest remaining gap after 48 hours of incubation with the extract. The average gaps measured at different time duration were utilised to calculate the absolute migration capability (MC_A) of treated and untreated cells (negative control) (Figures 4.3-4.6). The untreated cells (negative control) act as a reference for cell

migration in normal (unaffected) environment. Smaller MC_A value indicates lower migration capability thus reflecting higher anti-migration activity.

Table 4.5: Two different protein concentrations (µg/ml) of *Pleurotus tuber-regium* (sclerotium) extracts used in the anti-migration assay against breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines.

Cell lines	PS-CAE IC_{50}	PS-CAE IC_{25}	PS30 IC_{50}	PS30 IC_{25}	PS60 IC_{50}	PS60 IC_{25}	PS90 IC_{50}	PS90 IC_{25}
MDA-MB-231	8.00	4.00	7.50	3.75	0.75	0.38	8.50	4.25
HCT-116	12.00	6.00	14.25	7.13	3.75	1.88	20.75	10.38

IC_{50} is the protein concentration (µg/ml) at IC_{50} values, while IC_{25} is the protein concentration (µg/ml) at half of IC_{50} values.

Table 4.6: Two different protein concentrations (µg/ml) of *Termitomyces heimii* (fruiting body) extracts used in the anti-migration assay against breast cancer (MDA-MB-231) and colorectal cancer (HCT-116) cell lines.

Cell lines	TF-CAE IC_{50}	TF-CAE IC_{25}	TF30 IC_{50}	TF30 IC_{25}	TF60 IC_{50}	TF60 IC_{25}	TF90 IC_{50}	TF90 IC_{25}
MDA-MB-231	7.50	3.75	12.50	6.25	2.95	1.44	14.50	7.25
HCT-116	56.50	28.25	13.05	6.53	20.25	10.13	36.50	18.25

IC_{50} is the protein concentration (µg/ml) at IC_{50} values, while IC_{25} is the protein concentration (µg/ml) at half of IC_{50} values.

Figures 4.1 and 4.2 show the phase contrast micrograph of MDA-MB-231 cells and HCT-116 cells upon treatment with *P. tuber-regium* sclerotia and *T. heimii* fruiting bodies extracts. Based on Figures 4.1 and 4.2, the gap diameter (gap closure) and the absolute migration capabilities (MC_A) of MDA-MB-231 cells and HCT-116 cells upon treatment with *P. tuber-regium* (sclerotia) and *T. heimii* (fruiting bodies) were measured. Both cell lines showed complete closure of the gap after 48 hours of incubation. Cells treated with PS60 have exhibited low migration abilities towards filling the gap after 24 hours to 48 hours. The quantitative results are presented in Figures 4.3- 4.6.

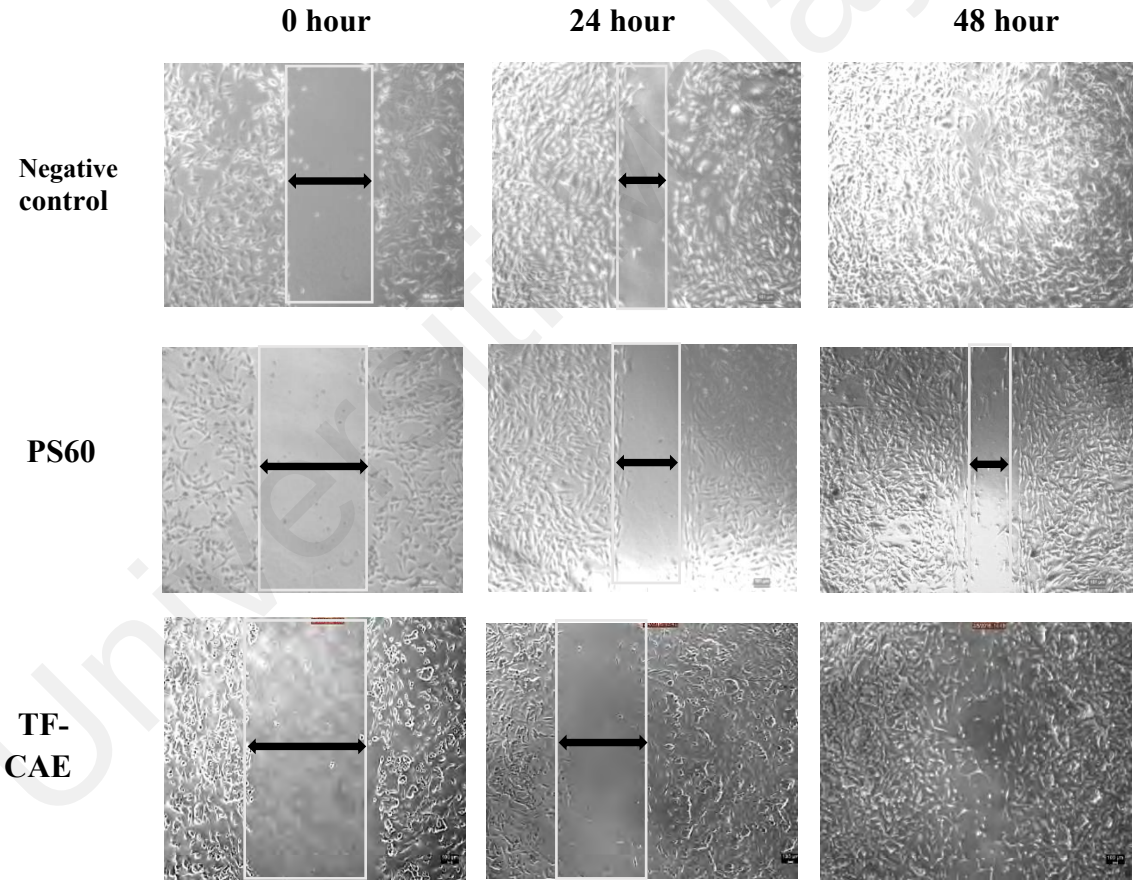


Figure 4.1: The anti-migration effect in the anti-migration assay from the most active protein fraction, *Pleurotus tuber-regium* sclerotium (PS60 at IC_{50}) and the aqueous extract of *Termitomyces heimii* (TF-CAE at IC_{50}) against MDA-MB-231 cells. The untreated cells act as negative control. The arrow shows the gap diameter of MDA-MB-231 cells inhibited before (0 hour) and after treatment (24 hour and 48 hour).

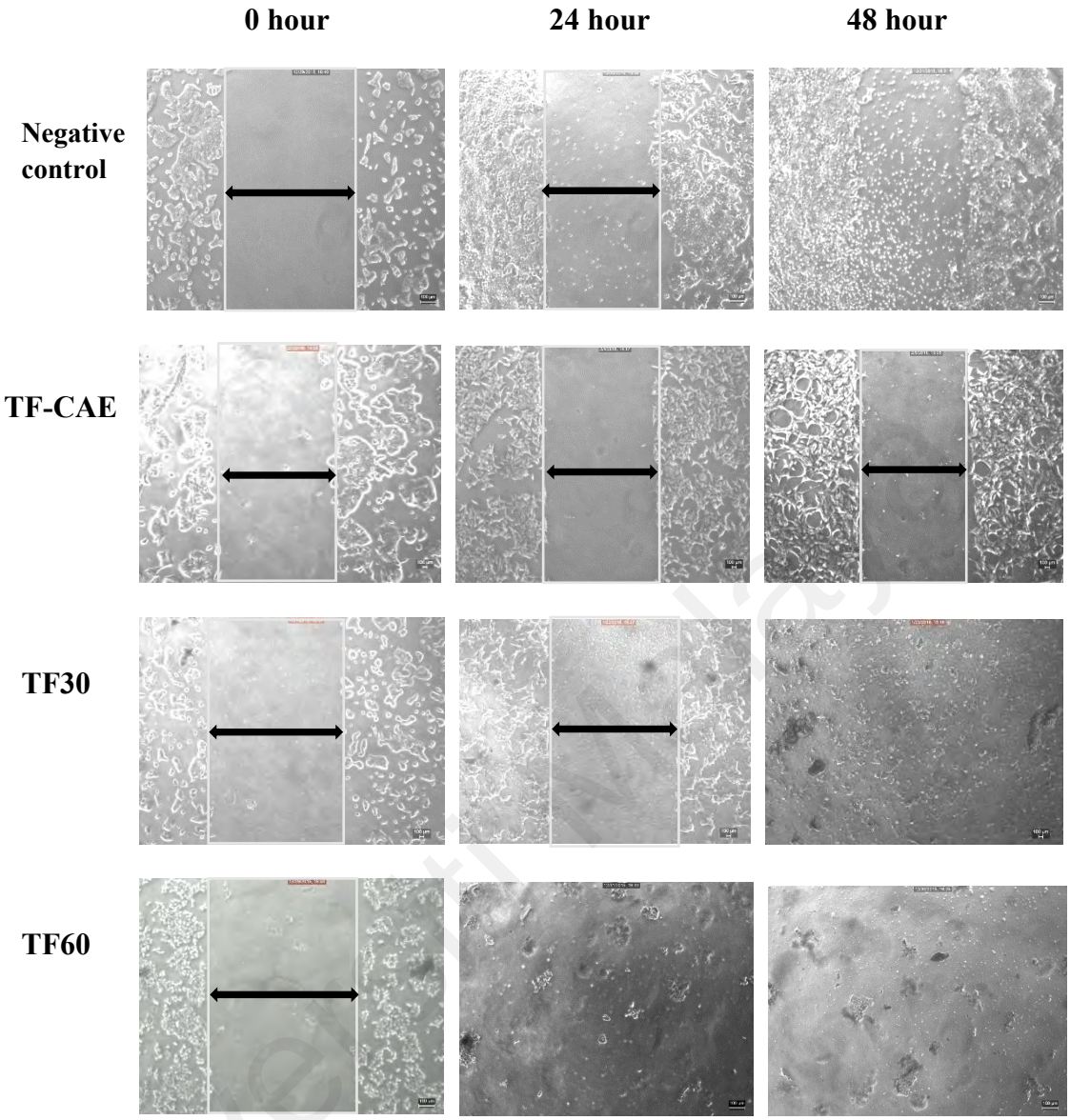


Figure 4.2: The different anti-migration effect in the anti-migration assay of HCT-116 cells. The aqueous extract of *Termitomyces heimii* (TF-CAE at IC_{25}) had inhibited the HCT-116 cells migration showed by the cells gap after 48 hours of incubation. While, for protein fraction of *Termitomyces heimii* (TF30 at IC_{25} and TF60 at IC_{50}), cells gaps were not detected (cells detached because of toxicity) after 24 hours and 48 hours, respectively. The untreated cells act as negative control. The arrow shows the gap diameter of HCT-116 cells inhibited before (0 hour) and after treatment (24 hour and 48 hour).

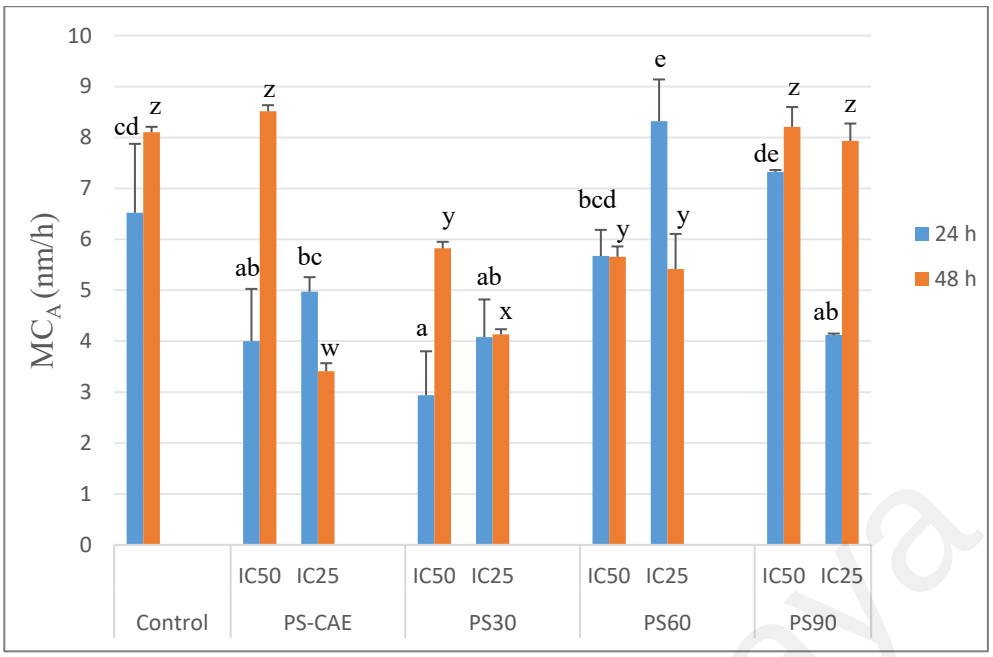


Figure 4.3: The absolute migration capabilities (MC_A) of MDA-MB-231cells upon treatment with *Pleurotus tuber-regium* sclerotium extracts at two different concentrations. The cells were incubated for 48 hours. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control (untreated) group. Alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p>0.05$).

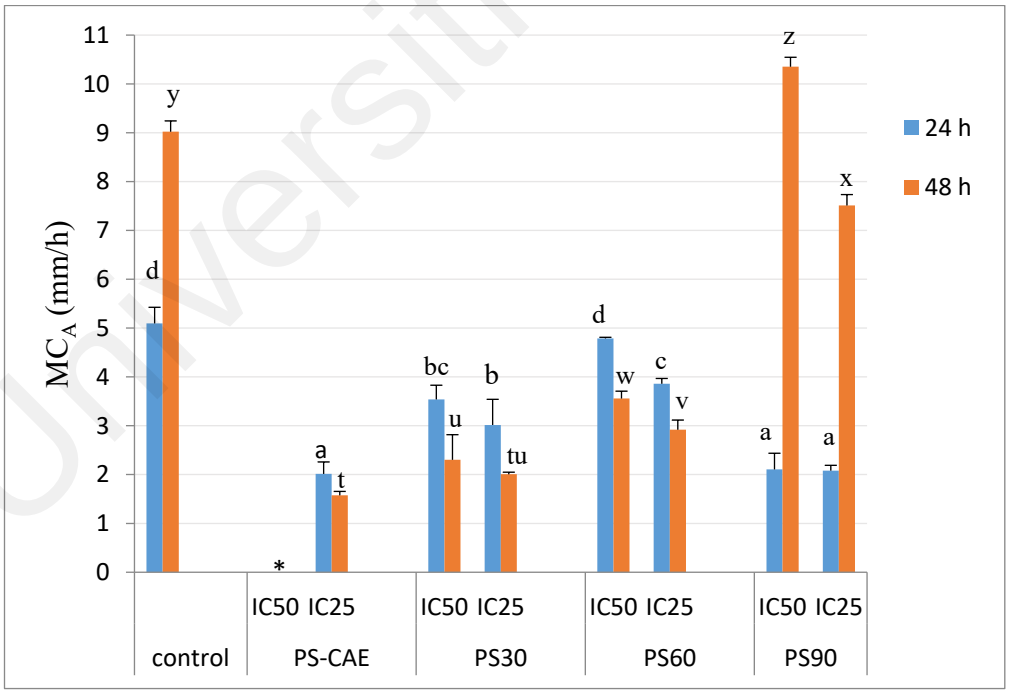


Figure 4.4: The absolute migration capabilities (MC_A) of HCT-116 cells upon treatment with *Pleurotus tuber-regium* sclerotium extracts at two different concentrations. The cells were incubated for 48 hours. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control (untreated) group. Alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p>0.05$). (*not determined).

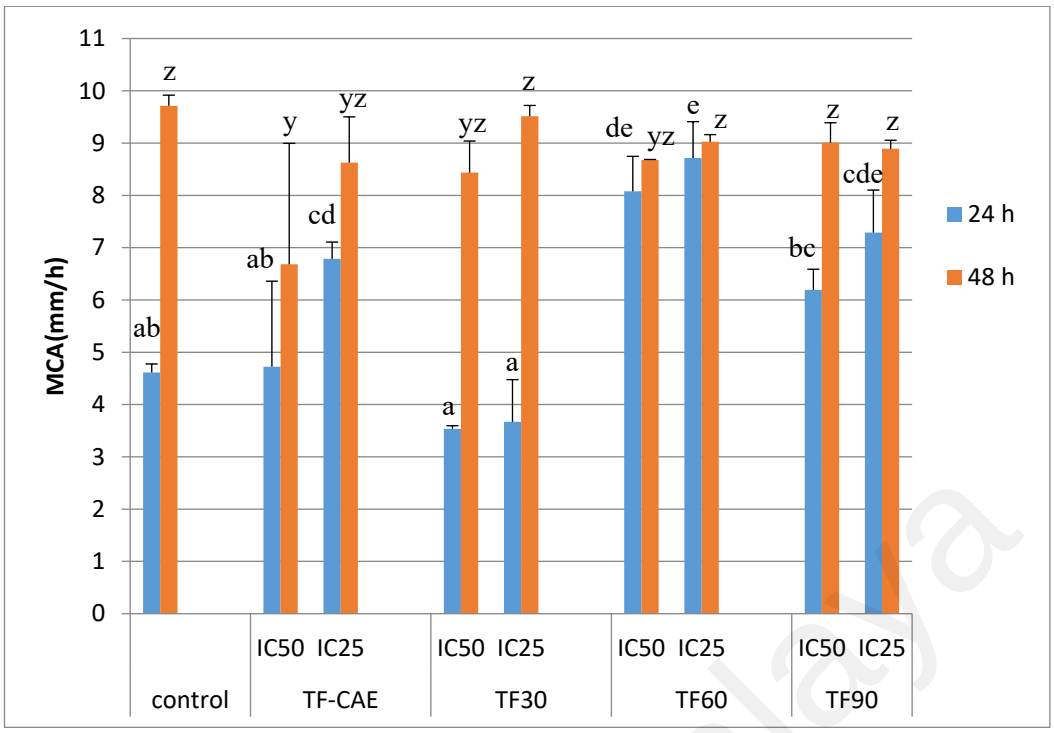


Figure 4.5: The absolute migration capabilities (MC_A) of MDA-MB-231 cells upon treatment with *Termitomyces heimii* extracts at two different concentrations. The cells were incubated for 48 hours. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control (untreated) group. Alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p>0.05$).

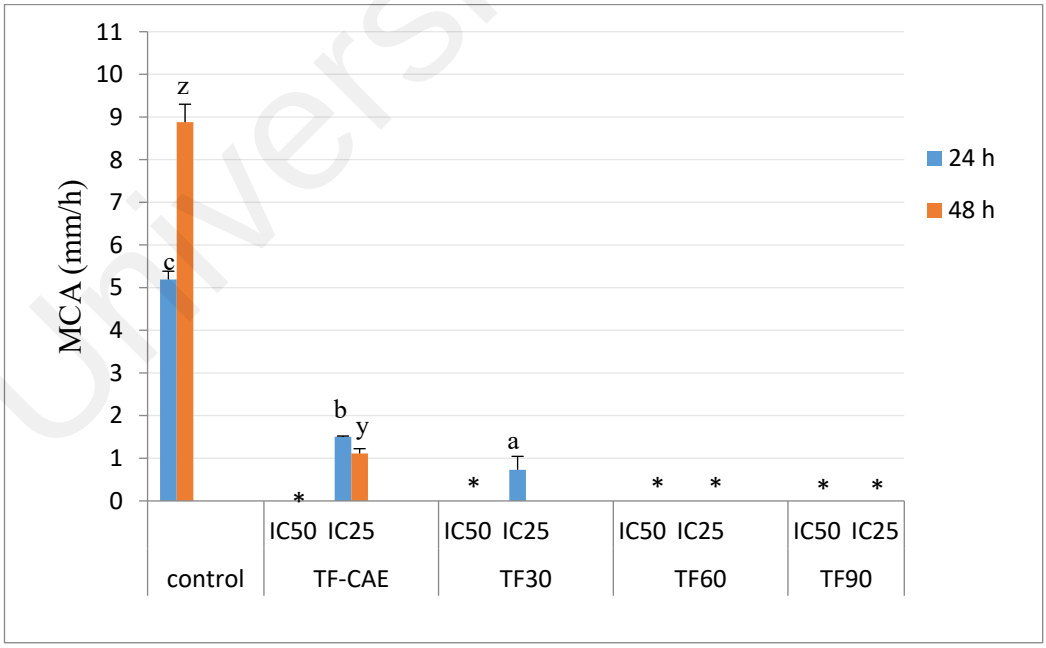


Figure 4.6: The absolute migration capabilities (MC_A) of HCT-116 cells upon treatment with *Termitomyces heimii* extracts at two different concentrations. The cells were incubated for 48 hours. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control (untreated) group. Alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p>0.05$). **(***not determined**).**

Tables 4.5 and 4.6 show protein concentrations of both *P. tuber-regium* (sclerotia) and *T. heimii* (fruiting bodies) extracts tested in the anti-migration assay at IC_{50} and IC_{25} concentrations. The untreated cells act as negative control. The slower the rate of cells migration the higher the anti-migratory activity of extracts as compared to the negative control. For *P. tuber-regium* sclerotium, the migration capability (MC_A) of untreated cells (MDA-MB-231) after 24 hours and 48 hours is recorded at 6.52±1.35 nm/h and 8.10±0.10 nm/h, respectively. The PS-CAE at IC_{25} (4.00±0.00 µg/ml) and PS30 at both IC_{50} (7.50± 0.71 µg/ml) and IC_{25} (3.75±0.36 µg/ml) showed the most promising anti-migratory effect against MDA-MB-231 cells after 48 hours of experimental study, with MC_A=3.41±0.16 nm/h, 5.82±0.13 nm/h, and 4.13±0.10 nm/h, respectively. While the protein of fraction 60 from *P. tuber-regium* sclerotium (PS60) demonstrated good anti-migratory effect at IC_{50} (0.75±0.57 µg/ml) and IC_{25} (0.38±0.29 µg/ml) with MC_A =5.66±0.20 nm/h and 5.41±0.69 nm/h as compared to untreated cells (control, MC_A = 8.1042±0.1042 nm/h) after 48 hours of incubation. The protein extracts of PS90 (at both IC_{50} (8.50±0.00 µg/ml) and IC_{25} (4.25±0.00 µg/ml)), however did not slow the migration rate of MDA-MB-231 cells as compared to untreated cells, with MC_A=8.21±0.39 nm/h and 7.93±0.34 nm/h. At shorter incubation time (24 hours), most of the extracts exhibited positive anti-migratory response towards the MDA-MB-231 cells with MC_A ranging between 2.94±0.86 nm/h to 5.67±0.51 nm/h except for PS60 at IC_{25} and PS90 at IC_{50} concentration, with MC_A=8.32±0.82 nm/h and 7.32±0.04 nm/h, respectively.

For *T. heimii* (fruiting bodies), the untreated MDA-MB-231 cells migrate at MC_A=4.61±0.16 nm/h (24 hours) and MC_A=9.71±0.20 nm/h (48 hours). Only the aqueous extract (TF-CAE at IC_{50}=7.50±0.71 µg/ml) showed some anti-migratory effect against MDA-MB-231 cells growth after 48 hours of incubation period causing reduced migration rate, MC_A=6.68±2.31 nm/h. Cells treated with other protein fractions had higher migration rate at 48 hours with MC_A=8.44±0.60 nm/h (TF30 at IC_{50}=12.50±0.00

μg/ml), 8.63±0.88 nm/h (TF-CAE at IC_{25}=3.75±0.36 μg/ml), 8.68±0.01 nm/h (TF60 at IC_{50}=2.95±0.78 μg/ml), 8.90±0.16 nm/h (TF90 at IC_{25}=7.25±1.06 μg/ml), 9.01±0.38 nm/h (TF90 at IC_{50}=14.50±2.12 μg/ml), 9.03±0.14 nm/h (TF60 a IC_{25}=1.44±0.39 μg/ml), and 9.52±0.21 nm/h (TF30 at IC_{25}=6.25±0.00 μg/ml). At 24 hours of treatment, TF30 at both IC_{50} and IC_{25} concentrations caused slower rate of cells migration (MCA=3.54±0.06 nm/h and 3.67±0.81 nm/h) as compared to negative control. While cells treated with the others (TF-CAE at IC_{50} and IC_{25}, TF90 at IC_{50} and IC_{25}, TF60 at IC_{50} and IC_{25}) demonstrated higher migration rate (4.72±1.64 nm/h, 6.79±0.32 nm/h, 6.20±0.40 nm/h, 7.29 ±0.81 nm/h, 8.08±0.67 nm/h, 8.71±0.70 nm/h), than the negative control.

Both *P. tuber-regium* (sclerotia) and *T. heimii* (fruiting bodies) extracts were further examined for anti-migration activities towards HCT-116 cell lines. The untreated cells (negative control) migrate at 5.10±0.33 nm/h and 9.02±0.22 nm/h after 24 hours and 48 hours of incubation time. The aqueous extract of *P. tuber-regium* (PS-CAE) exerted the highest anti-migration effect towards HCT-116 at IC_{25}=6.00±1.42 μg/ml resulting in MC_A= 1.58±0.08 nm/h after 48 hours of incubation. However, at IC_{50}=12.00±2.83 μg/ml, the PS-CAE showed the lowest anti-migration activity which resulted in MC_A=8.61±0.73 nm/h. Strong anti-migration effects towards HCT-116 cell lines were also demonstrated by PS30 at IC_{50}=14.25±5.30 μg/ml and IC_{25}=7.13±2.65 μg/ml which resulted in MC_A=2.30±0.51 nm/h and 2.01±0.04 nm/h, and PS60 at both IC_{50}=3.75±2.47 μg/ml and IC_{25}=1.88±1.24 μg/ml resulted in MC_A=3.56±0.15 nm/h and 2.92±0.20 nm/h. PS90 showed slight anti-migration effect at IC_{25}=10.38±0.53 μg/ml resulting in MC_A=7.51±0.22 nm/h, while at IC_{50}=20.75±1.06 μg/ml, HCT-116 cell lines demonstrated higher migration rate ($MC_{A=}$10.35±0.19 nm/h) as compared to the untreated cells ($MC_{A=}$9.02±0.22 nm/h). At 24 hours of incubation time, all extracts except PS-CAE at IC_{50}=12.00±2.83 μg/ml resulted in slower migration of cells (MC_A=17.12±1.60 nm/h) than the control. The anti-migration activities of *P. tuber-regium* extracts against HCT-

116 cell lines at 24 hours in increasing order were as follows, PS60 at IC_{50} and IC_{25} (MC_A=4.79±0.02 nm/h, 3.86±0.11 nm/h), PS30 at IC_{50} and IC_{25} (MC_A=3.54±0.29 nm/h, 3.02±0.53 nm/h), PS90 at IC_{50} and IC_{25} (MC_A=2.11±0.33 nm/h, 2.10±0.11 nm/h), and PS-CAE at IC_{25} (MC_A=2.01±0.24 nm/h).

For *T. heimii* extracts, the untreated HCT-116 cells migrate at MC_A=5.19±0.19 nm/h, and 8.88 ±0.42 nm/h, after 24 hours and 48 hours of incubation. The aqueous extract of *T. heimii* (TF-CAE) at IC_{25}=28.25±7.60 µg/ml showed positive anti-migratory effect towards HCT-116 cells after 24 hours and 48 hours of treatment resulting in MC_A=1.50±0.02 nm/h and 1.11±0.11 nm/h, respectively. Fraction 30 of *T. heimii* extracts (TF30) showed interesting anti-migration activity at IC_{25}=6.53±1.06 µg/ml which resulted in MC_A=0.73±0.32 nm/h after 24 hours, but cannot be determined after 48 hours of treatment because the gap was not observed. Also, other protein extracts from *T. heimii* at both IC_{50} and IC_{25}, the anti-migration activity towards HCT-116 cell lines cannot be determined as the cells had already detached from the flasks at 24 hours of incubation time.

In order to select the best extract or protein fraction to be further purified, both anti-migration activities and 50% inhibitory concentration (IC_{50}) with selectivity index (SI) of each extract towards breast cancer cell lines (MDA-MB-231) was considered. *P. tuber-regium* extracts (PS-CAE, PS30 and PS60) showed high anti-migration effects towards MDA-MB-231 cells at the end of treatment (48 hours of incubation), while most of *T. heimii* extracts were less effective in lowering the rate of cell migration. Among the extracts with best anti-migration effects (PS30 and PS60), PS60 was selected to be further studied after considering that PS60 had before resulted the highest cytotoxic effect with SI=14.00. Based on our study, the most promising extract to inhibit both the growth and migration of MDA-MB-231 cells is PS60. Therefore, it was selected to be further purified and investigated for apoptotic effect towards breast cancer cell lines (MDA-MB-231).

4.4 Separation and characterisation of cytotoxic protein compounds from *Pleurotus tuber-regium* sclerotia

4.4.1 Protein profile analysis by SDS-PAGE

P. tuber-regium sclerotium, PS-CAE and protein fractions were further separated based on its molecular mass and compared to protein ladder (Spectra Multicolour Broad Range Protein Ladder) as a marker. Figure 4.7 shows the profile of different fractions from *P. tuber-regium* sclerotia (PS-CAE, PS30, PS60, PS90) which revealed more than 50 distinct protein bands on the polyacrylamide gel. The crude aqueous extract (PS-CAE) showed several protein bands within the range of 10 kDa to 140 kDa as compared to standard markers. PS60 consists of 20 protein bands with sizes ranging from ~8.7 kDa to ~139.2 kDa while PS30 revealed fewer protein bands with sizes ranging from ~8.7 kDa to ~131.2 kDa. On the other hand, fraction 90 exhibited no protein profiles within 11 kDa to 50 kDa. Only few bands within ~8.7 kDa to ~11 kDa and one band with protein size ~56.23 kDa were observed.

4.4.2 Purification of proteins in PS60 fraction of *Pleurotus tuber-regium* sclerotia by fast protein liquid chromatography (FPLC)

Purification was done on the most cytotoxic fraction, PS60 against MDA-MB-231 cells using FPLC system (AKTA avant 25). In this study, three distinct peaks (Q1, Q2, and Q3) were collected from the chromatography as revealed in Figure 4.8. Q1 was eluted at 60% concentration of NaCl, while Q2 at 90% and Q3 at 100% NaCl. Protein peaks (Q1, Q2, and Q3) from fraction 60 of *P. tuber-regium* sclerotia (PS60) were tested for cytotoxic activity against MDA-MB-231 cells using MTT assay. FDA drug approved for breast cancer diseases; cisplatin was used as a positive control.

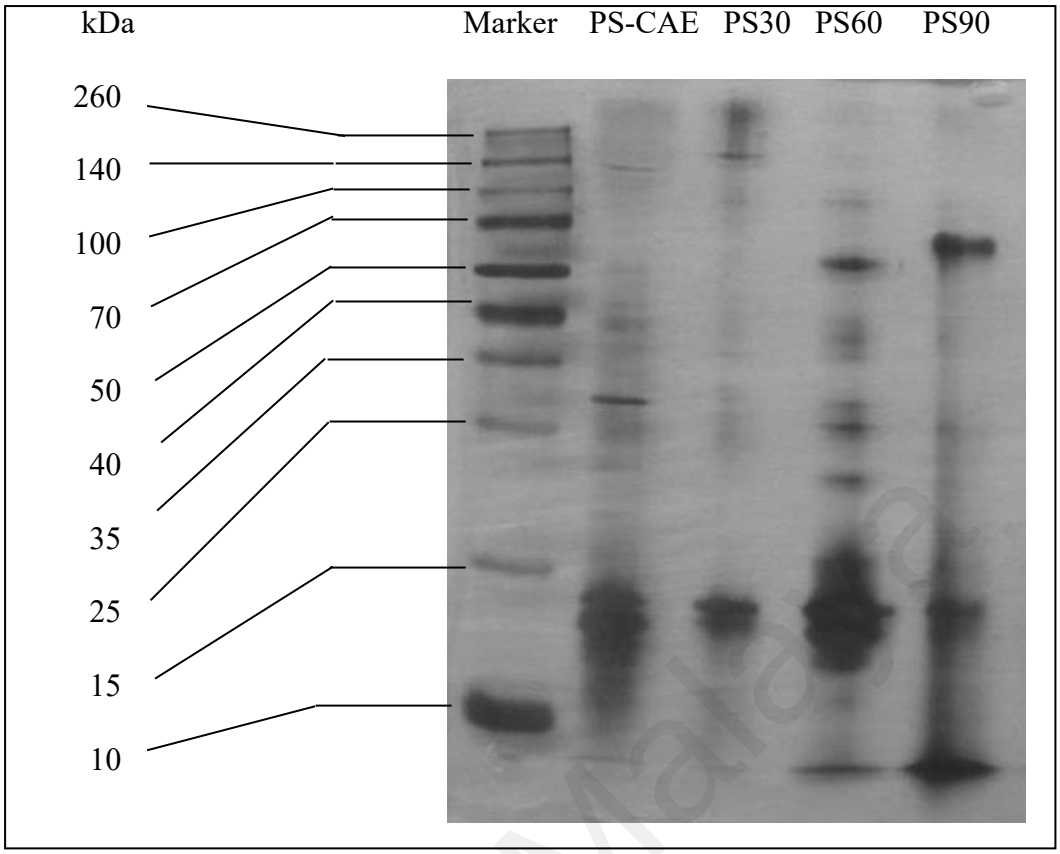


Figure 4.7: SDS-PAGE of *Pleurotus tuber-regium* sclerotium extracts (PS-CAE: crude aqueous extract of *Pleurotus tuber-regium*; PS30: fraction 30 of *Pleurotus tuber-regium*; PS60: fraction 60 of *Pleurotus tuber-regium*; PS90: fraction 90 of *Pleurotus tuber-regium*).

Table 4.7 shows the results of 50% inhibitory concentration (IC_{50}) of protein peaks from PS60 fraction against MDA-MB-231 cell lines. The lowest IC_{50} values is shown by Q3 (0.60±0.02 µg/ml), followed by Q1 (0.65±0.03 µg/ml), Q2 (0.75±0.03 µg/ml), and PS60 (0.75±0.57 µg/ml). From the result obtained, all peak fractions were highly cytotoxic towards the breast cancer cell lines (MDA-MB-231). Referring to Boik (2001), a pure compound is considered as significantly cytotoxic against the cancer cell lines when the IC_{50} value is less than 4 µg/ml. All peaks and fraction (PS60) showed high cytotoxic effect with IC_{50} values of 0.60±0.02 µg/ml, 0.65±0.03 µg/ml, 0.75±0.03 to 0.75±0.57 µg/ml for Q3, Q1, Q2, and PS60, respectively. Unseparated fraction (PS60) and its separated components (Q1, Q2, and Q3) had shown comparable cytotoxic activity

to cisplatin (IC_{50}=0.56±0.09 µg/ml). There was no improvement upon further purification in terms of cytotoxicity based on IC_{50} values revealed by PS60 and its separated components (Q1, Q2, and Q3). Therefore, Q1, Q2, and Q3 were selected to be further analysed for protein identification using LCMS Q-TOF MS, and due to insufficient sample of Q1, Q2 and Q3 the apoptotic effect was evaluated using only PS60.

Table 4.7: Cytotoxicity **(**$IC_{50,}$ µg/ml) of the FPLC protein fractions (Q1, Q2, Q3) of *Pleurotus tuber-regium* sclerotium (PS60) against MDA-MB-231 cell line in MTT assay. Results were expressed as the mean of triplicates values (n=3). The cells were incubated for 72 hours.

Cell line	Cisplatin	PS60	Q1	Q2	Q3
MDA-MB-231	0.56±0.09	0.75±0.57	0.65±0.03	0.75±0.03	0.60±0.02

$IC_{50} \leq 20$ µg/ml, considered highly cytotoxic against normal lung fibroblast (MRC-5) cell line.

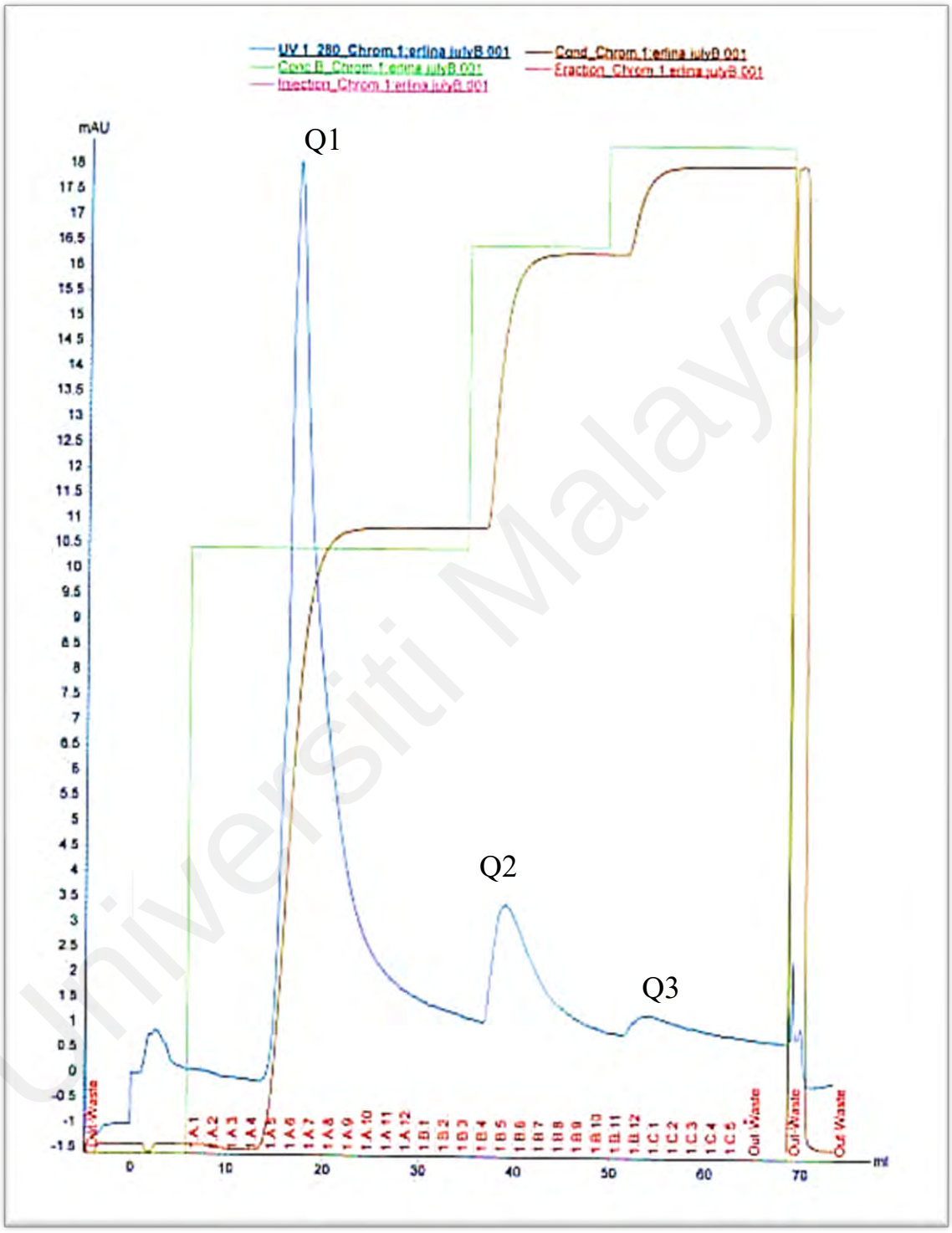


Figure 4.8: Elution profile of strong anion exchanger Hi-Trap Capto-Q (Absorbance at 280 nm versus elution volume). The light green represents the gradient of NaCl (0-100% buffer B) where buffer A is 10mM Tris-HCl at pH 8.0 and buffer B is buffer A containing 1.0 M NaCl. All bound protein were fractionated into three peaks (Q1, Q2, Q3).

4.4.3 LCMS-Q-TOF MS analysis of PS60 FPLC fractions

All peaks from PS60 fractions (Q1, Q2, and Q3) were subjected to LCMS-Q-TOF MS analysis for the identification of proteins isolated. Data was obtained using Spectrum Mill software (Agilent, Santa Clara, CA, USA). The proteins and peptides were validated using the same software, based on the software default settings. Table 4.8 and 4.9 listed the details of proteins identified from the analysis. There were two proteins identified from Q1 and Q3 peaks known as kinesin-like protein and keratin, type 1 cytoskeletal 10, respectively, while, four uncharacterised proteins labelled as UP1, UP2, UP3 and UP4, were isolated from all peaks (Q1, Q2, and Q3) as shown in Table 4.9. Uncharacterised proteins refer to protein sequence identified matching in the sequence database, but the protein name and/or its function is still unknown. Database accession number is the entry name of a protein listed in the database. Distinct summed MS/MS search score means score of the overall proteins, which is the summation of all peptide scores for the protein. Peptide score is the score of an individual peptide which reflects the information on amount of useful fragmentation in the MS/MS spectrum. The higher the score the higher the peptides sequences matching in the database list. Peptide score greater than 15 for Agilent Q-TOF and ion trap instruments represent an outstanding valid result with thorough peptide fragmentation (Agilent Technologies, 2012).

Kinesin like protein hits one distinct peptide from the database with MS/MS score of 14.58. While Keratin type 1, cytoskeletal 10, hits 3 distinct peptides with higher MS/MS score, 39.26. Both proteins have almost similar size /molecular weight, with 56.6562 kDa (kinesin-like protein) and 59.0543 kDa (keratin type 1, cytoskeletal 10). For the uncharacterised protein, UP1 hits one peptide with score equals to 13.46 and molecular weight, 84.2029 kDa. While UP2 and UP4, both hit two peptides with score 26.70 and 24.31, respectively, and both with similar molecular weight, 67.6041 kDa. For

UP3, two peptides were hits in the database with 32.04 score and molecular weight of 26.5795 kDa.

Percentage of amino acid coverage out of total number of amino acids in the entire protein sequence, and protein isoelectric point (pI) of all proteins were shown in Table 4.8 and Table 4.9. The protein sequences of all identified proteins were presented in Table 4.10. UP2 and UP4 revealed two protein sequences each, with one of them was identical sequence which is –AEAESLYQSK-. Both proteins (UP2 and UP4) therefore, provide similar accession number, and molecular weight, however their score, amino acid coverage, isoelectric point (pI) as well as their protein sequences was slightly differed.

The Gene Ontology (GO) analysis of identified proteins and their molecular functions was presented in Table 4.11. The two characterised proteins and all uncharacterised proteins identified were investigated for their correlation with anti-tumour activities as well as their mechanism of anti-tumour effects based on previous reports.

Table 4.8: List of characterised proteins identified by LCMS-Q-TOF MS Database search. Proteins and peptides were validated using Spectrum Mill software, based on the software default settings.

Peak	Protein Name	Database accession	Distinct Summed MS/MS Score	Amino Acid Coverage (%)	Mw (Da)	Protein pI	Distinct peptides
Q1	Kinesin-like protein	A0A098VS72	14.58	1.9	56656.2	6.52	1
Q3	Keratin, type 1 cytoskeletal 10	P13645	39.26	4.7	59054.3	5.14	3

Table 4.9: List of uncharacterised proteins identified by LCMS-Q-TOF MS Database search. Proteins and peptides were validated using Spectrum Mill software, based on the software default settings.

Peak	Protein Name	Database accession	Distinct Summed MS/MS Score	Amino Acid Coverage (%)	Mw (Da)	Protein pI	Distinct peptide
Q1	UP1	A0A0C2WZP4	13.46	2.4	84202.9	8.00	1
Q2	UP2	A0A0D9R013	26.70	3.6	67604.1	8.59	2
Q3	UP3	F1SRS2	32.04	7.3	26579.5	6.9	2
Q3	UP4	A0A0D9R013	24.31	2.8	67604.1	8.59	2

Table 4.10: The protein sequence fragments of identified proteins.

Protein Name	Protein sequence (fragments)
Kinesin-like protein	• LSSSGATGDR
Keratin, type 1 cytoskeletal 10	• VTMQNLNDR • LAADDFR • ALEESNYELEGK
UP1	• ECEQDIARYLEDRFDAIR
UP2	• GSGGGSSGGSIGGR • AEAESLYQSK
UP3	• LSSPATLNSR • VATVSLPR
UP4	• AEAESLYQSK • TLLEGEESR

Table 4.11: Gene Ontology (GO) analysis and the functions of the identified proteins.

Protein name[1]	Submit-ted name[2]	Organism[3]		GO[4]	Source[5]
Kinesin-like protein	Kinesin motor domain contain-ing protein	*Mitospori-dium daphniae*	Molecular function	- ATP binding	UniProt-KB
				- microtubule binding - microtubule motor activity	InterPro
			Biological process	- microtubule-based movement	InterPro
Keratin, type 1 cytoske-letal 10	NIL	*Homo sapiens (Human)*	Molecular function	- protein heterodimerisation activity	UniProt-KB
				- structural constituent of skin epidermis	CAFA
			Biological process	- cornification - keratinisation	Reactome
				- keratinocyte differentiation - positive regulation of epidermis development - protein Heterotetramerisa-tion	UniProt-KB
				- peptide cross-linking	CAFA

Table 4.11, continued.

UP1	Uncha-racte-rised protein	*Amanita muscaria Koide BX008*	Cellular component	- integral component of membrane	UniProtKB-KW
UP2 **UP4**	Keratin 1	*Chlorocebus sabaeus (Green monkey) (Cercopith-ecus sabaeus)*	Molecular function	- carbohydrate binding - protein heterodimerisation activity - structural constituent of skin epidermis	Ensembl
			Biological process	- complement activation, lectin pathway - establishment of skin barrier - negative regulation of inflammatory response - peptide cross-linking - protein Heterotetramerisa-tion	Ensembl
UP3	NIL	NIL	NIL	NIL	NIL

[1] Name of identified protein or uncharacterised protein; [2] Protein name submitted in database; [3] Organism from which protein identification originates; [4] Gene ontology resulted from database; [5] Source of databases, NIL=Not in list.

4.5 Apoptotic effects of PS60 on MDA-MB-231 cells

4.5.1 Induction of apoptosis by PS60 fraction using Annexin V-FITC/ Propodium Iodide (PI) assay

The induction of apoptosis in MDA-MB-231 cells by PS60 fraction was examined using the Annexin V-FITC/ PI assay to differentiate between early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) apoptotic, necrotic and live cells as shown in Figure 4.10. With reference to untreated cells and cisplatin as positive control, the apoptotic effect over time (24 hours, 48 hours and 72 hours) was conducted at concentrations of 50% and 75% of cells inhibition (IC_{50} and IC_{75}). The induction of apoptosis is more prominent at 24 hours-48 hours of incubation as shown in Figure 4.9. Flow cytometric analysis showed an increase of MDA-MB-231 cells population in the early apoptotic and late apoptotic after 48 hours of exposure to PS60 (at IC_{50} and IC_{75}) and cisplatin, indicating that the treated cells have undergone apoptosis. The percentage of early apoptotic cells have increased from 10.17±0.61% (untreated) to 16.03±0.12% and 17.07±0.45% at IC_{50} and IC_{75} concentration, respectively. For late apoptotic cells the percentage of events increases from 4.07±0.75% (untreated) to 6.70 ±0.40 % (at IC_{50}) and 7.50±0.26% (at IC_{75}). There was no improvement on cells population in both early and late apoptotic stage after 72 hours incubation time. Based on results presented, PS60 promoted induction of MDA-MB-231 cell apoptosis to a greater extent than necrosis. Moreover, the percentage of cell population treated with PS60 were of similar trend to cisplatin treated cells (22.03±0.61% in early apoptosis, 6.07±0.16% in late apoptosis, and 1.77±0.06% in necrosis) (Appendix B, Table B9.0) validating the potential of PS60 in inducing the apoptotic effect.

4.5.2 Disruptive effect of PS60 on the cell cycle

The effect of PS60 treatment on MDA-MB-231 cell cycle disruption was performed by staining fixed and permeabilised cell with PI dye (Figure 4.12). Figure 4.11 showed

the effect of PS60 exposure on MDA-MB-231 cell cycle distribution at concentration of 50% and 75% of cells inhibition (IC_{50} and IC_{75}). The number of MDA-MB-231 cells exposed to PS60 was initially decreased in the G0/G1 phase (from 74.66±0.00% (untreated) to 66.13±7.01% (at IC_{50}) and 63.63±4.65% (at IC_{75}).), then followed by significant increase ($p=0.037$) in the population of cells in S phase from 19.63±0.00% (untreated) to 29.14±5.82% (at IC_{50}) and 28.78±3.01% (at IC_{75}). However, no significant ($p=0.183$) differences of cell events in G2/M phase population between untreated and treated cells was observed (control=5.71±0.00%, PS60 at IC_{50}=4.74±1.19% and PS60 at IC_{75}=7.5±1.77%. It was clearly observed that the trend of cell cycle disruption by PS60 revealed a similar effect as cisplatin treated cells (57.6±0.00%, at G0/G1 phase, 35.5±0.00% at S phase and 6.9±0.00% at G2/M phase), in which the DNA content was slightly depleted in G0/G1 phase and increased in S phase population.

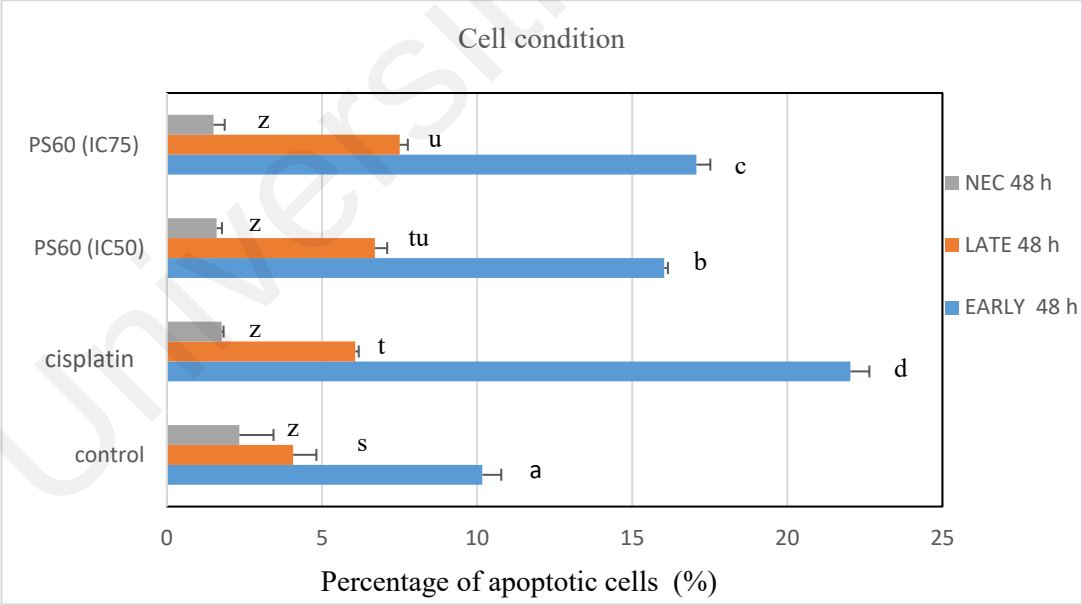
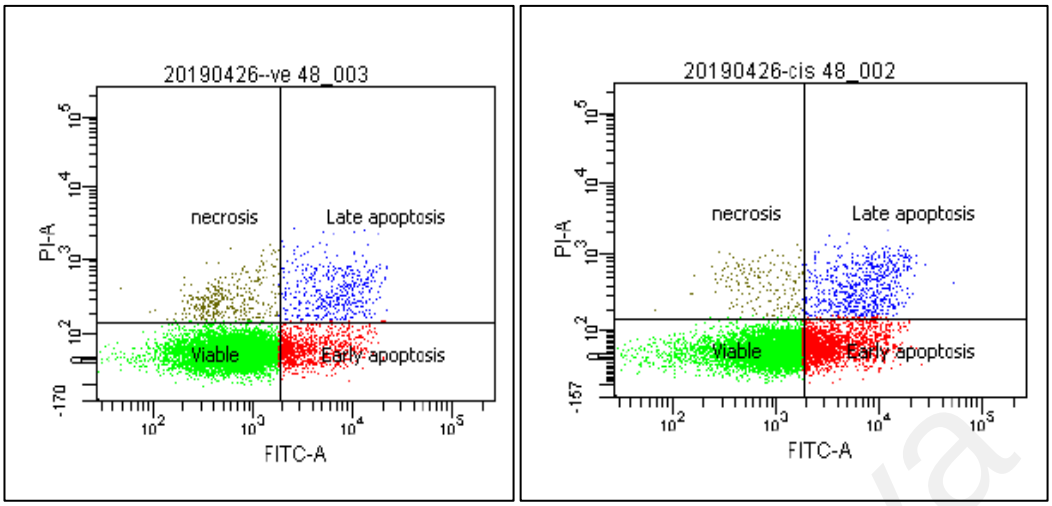
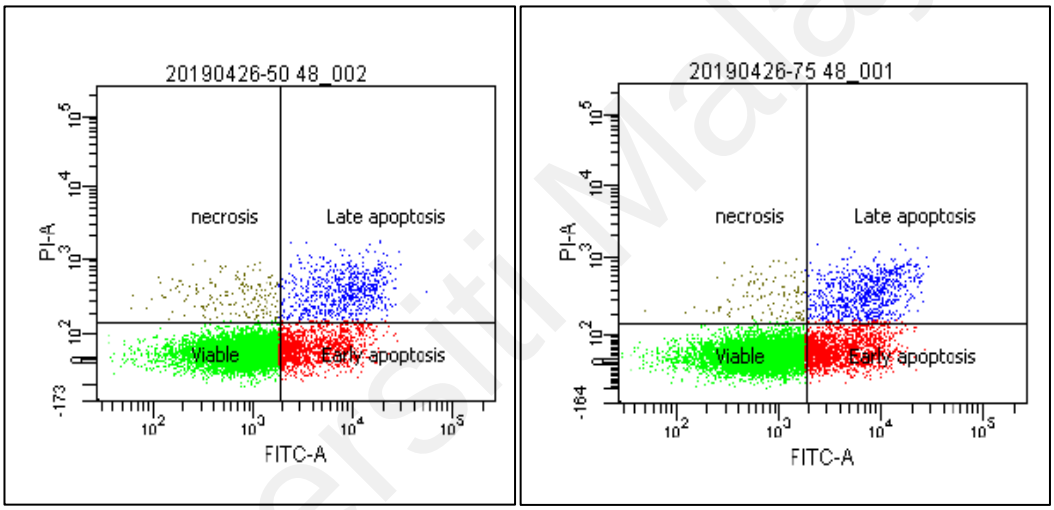


Figure 4.9: MDA-MB-231 cells population on PS60 treatment at different apoptotic phase. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control (untreated) group. Alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p>0.05$).

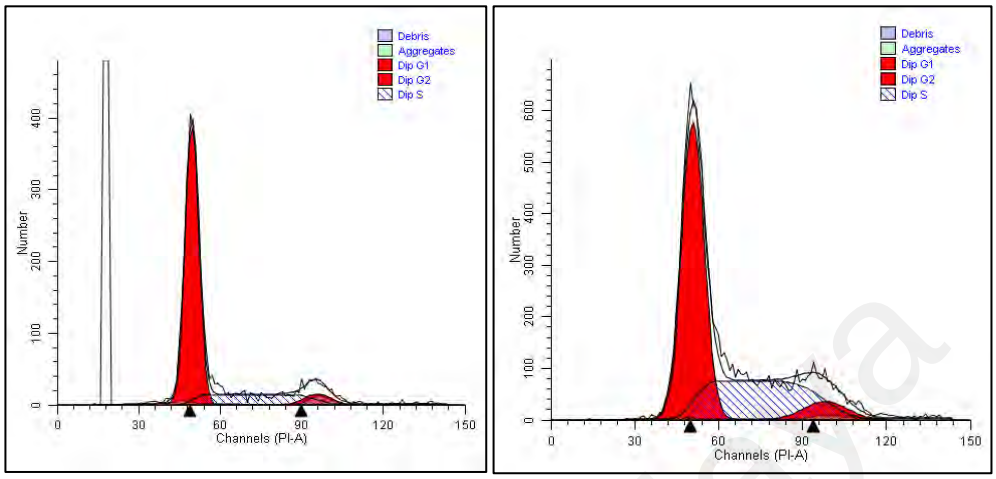


i) Untreated cells ii) Cisplatin treated cells

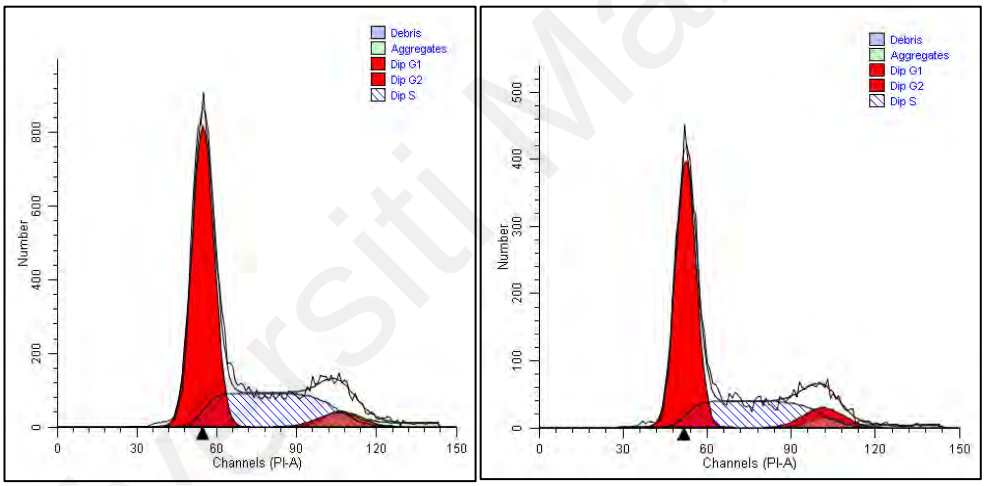


iii) PS60 (at IC_{50}) treated cells iv) PS60 (at IC_{75}) treated cells

Figure 4.10: Expression of MDA-MB-231 treated cells at different apoptotic phase.



i) Untreated cells ii) Cisplatin treated cells



ii) PS60 (at IC_{50}) treated cells iv) PS60 (at IC_{75}) treated cells

Figure 4.11: Effect of PS60 on cell cycle distribution on MDA-MB-231 cells**.**

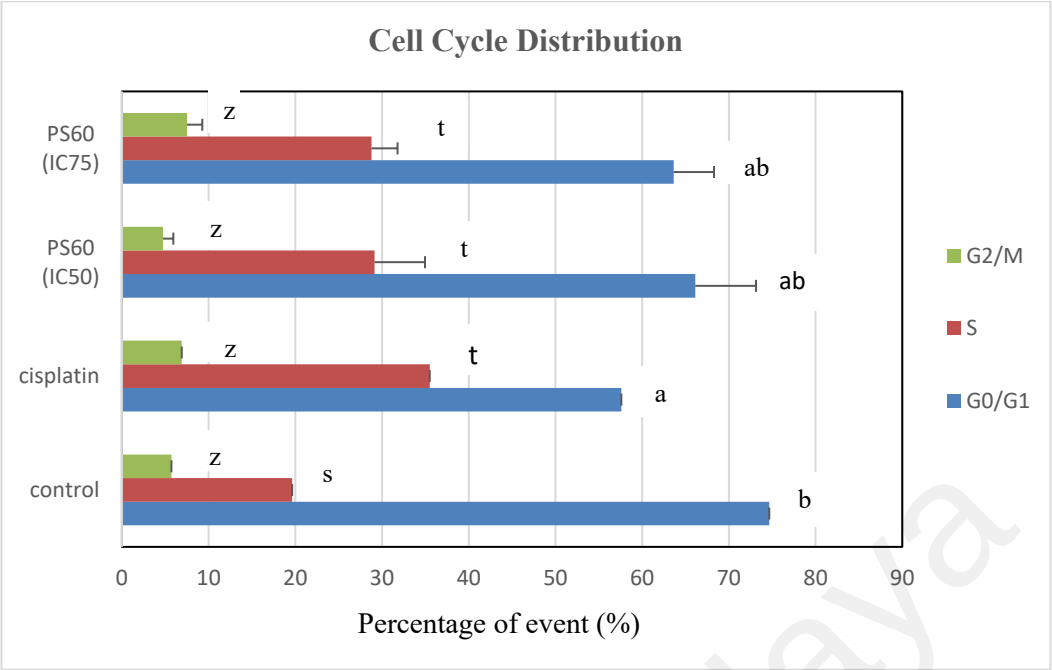


Figure 4.12: Effect of PS60 treatment on MDA-MB-231 cell cycle distribution. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control (untreated) group. Alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p>0.05$).

CHAPTER FIVE

DISCUSSION

A pre-screening test on the cytotoxic activities of protein extracts from *P. tuber-regium* sclerotium, *P. tuber-regium* fruiting body and *T. heimii* fruiting body on both cancer cells, MDA-MB-231 and HCT-116 were done. The most active protein fraction was identified as PS60. The PS60 was highly cytotoxic towards MDA-MB-231 cells but less cytotoxic against normal lung cells, MRC-5. The proteins present in PS60 were known as kinesin-like protein and keratin type 1, cytoskeletal 10. PS60 was shown to exert cytotoxic effects associated with the induction of apoptosis and cell cycle arrest in MDA-MB-231 cells at G1/G0 and S-phase. Factors that might contribute to the cytotoxic activities of the protein fractions and the mechanism of cell death were discussed in this chapter.

5.1 Cytotoxicity of protein extracts from *Pleurotus tuber-regium* and *Termitomyces heimii* towards breast cancer cell lines (MDA-MB-231) and colorectal cancer cell lines (HCT-116)

The protein contents of different extracts of *P. tuber-regium* sclerotium, *P. tuber-regium* fruiting body and *T. heimii* fruiting body were determined. The cytotoxicity of *P. tuber-regium* sclerotium towards MDA-MB-231 was better (IC_{50} ranging from 0.75 µg/ml to 8.50 µg/ml) than its fruiting body (IC_{50} ranging from 2.15 µg/ml to 51.85 µg/ml). While the cytotoxic effects of *P. tuber-regium* sclerotium towards HCT-116 cells are comparable to its fruiting body with IC_{50} ranging from 3.75 µg/ml to 20.75 µg/ml and 2.20 µg/ml to 19.50 µg/ml, respectively. It is on this basis that the sclerotium of *P. tuber-regium* was further selected and compared with *T. heimii* fruiting body for determination of cytotoxicity towards MRC-5 and selectivity index (SI).

The cytotoxicity of *P. tuber-regium* sclerotium is observed to be three times greater than its fruiting body. This could be due to the production of toxin which is higher in the sclerotium compared to other parts of the mushroom. Numerous studies had revealed that mushroom sclerotia produced toxins as one of chemical defence strategy to protect themselves from antagonistic microorganisms, animals and extreme environment conditions (Thorn *et al.,* 2000; Oranusi *et al*., 2014; Künzler, 2018). The toxins class include secondary metabolites, peptides and proteins and they act against the antagonists by binding to their specific target molecules (Künzler, 2018). For example, the cytotoxic, ribosomally synthesised octapeptide α-amanitin produced by some *Amanita*, *Galerina*, *Conocybe*, and *Lepiota* species act against insects or nematodes by targeting RNA polymerase II/III of the organism (Hallen *et al*., 2007).

Literature surveys showed that mushroom sclerotia are the part which contain the most medicinal properties including anti-proliferative, anti-tumour, immunomodulatory and useful to treat cancer such as liver cancer (Lee *et al*., 2017). Some examples include lanostane-type triterpenoids and polysaccharides which are chemical constituents from the sclerotium of *Poria cocos* Wolf that have been found to possess anti-cancer activity (Rios, 2011). *Innotus obliquus* is another mushroom species abundant with bioactive metabolites identified from its sclerotia as revealed in Song *et al.* (2013). Also, several extracts/compounds from *P. tuber-regium* sclerotium with anti-tumour and/ or anti-cancer activities have been reported in different studies as presented in Chapter 2. For instance, N-acetylglucosamine-specific lectin isolated from the sclerotia of *P. tuber-regium* have a molecular weight of 32 kDa. The N-terminal amino acid sequences (DRXAGYVLYXXVPY) of lectin from *P. tuber-regium* showed some similarity to that of *Agaricus bisporus* (Wang & Ng, 2003). The cytotoxic effect of lectins was due to their specific binding capacity to diverse sugar structures without altering the covalent structure of glycosyl ligands, cells agglutinating effects, and their ability to differentiate

between normal and malignant cells (Valadez-Vega *et al*., 2014). *Pleurotus* sp. was identified to possess the largest number of lectins besides other species such as *Schizophyllum commune*, *Laccaria amethystina, Agrocybe cylindracea, Agrocybe aegerita* and *Coprinus cinereus* (Hassan *et al*., 2015).

Studies on bioactive compound and anti-tumour effect of cytotoxic proteins extracted from *P. tuber-regium* and *T. heimii* are still lacking. To the best of our knowledge, this research is the first to report on the anti-tumour effect of cytotoxic proteins extracted from *P. tuber-regium* and *T. heimii* against breast cancer cells (MDA-MB-231) and colon cancer cells (HCT-116). In this study, the sclerotium of *P. tuber-regium* which were extracted using ammonium sulphate precipitation technique had resulted in promising cytotoxic effect against both cancer cell lines (MDA-MB-231 and HCT-116), where PS60 (moderately hydrophobic proteins) was the most active in combating the MDA-MB-231 cells proliferation. Ammonium sulphate, $(NH_4)_2SO_4$, is commonly used to salting out the proteins due to its high solubility that allows solutions of very high ionic strength, and its stabilising effect on the protein structure (Burgess, 2009). In this research, the highest protein content isolated from both *P. tuber-regium* (sclerotia and fruiting bodies) were precipitated out at 30% and 60% of ammonium sulphate concentration, respectively, while for *T. heimii* (fruiting bodies) at 60% ammonium sulphate concentration.

The protein concentrations of both mushroom species can be associated with the degree of protein-water interaction during the extraction process. Three main categories of protein-water interactions including hydrophobic hydration, hydrogen bonding between polar groups and water, and ion hydration between charged side chain (Wingfield, 2001). Hydrophobic groups interaction particularly within the molecules 'core' or hydrophobic region are important to sustain the tertiary structure of proteins. Generally, the most nonpolar amino acid side chains are buried inside a protein molecule

in water forming hydrophobic core(s), and polar amino acids are distributed in hydrophilic regions on the protein surface (Vakhtang *et al*., 2002). Hydrophobic interaction between protein and water increased as the surface tension of water increases due to salt addition to the solution. In response to this situation, the protein decreases its surface area in an attempt to minimise contact with the solvent (Wingfield, 2001).

The importance of hydrophobic interactions are for protein functions, stability and conformation, and the hydrophobic surface affects the interaction between different protein molecules. Several analyses have shown that some hydrophobic groups may also be located on the protein surface and affect the surface hydrophobicity of the protein. The surface hydrophobicity could be more important for protein functioning rather than overall hydrophobicity, thus can be used as an indicator of the ability of different molecules interaction (Shuryo, 1983; Jiang *et al*., 2015).

Our findings are in contrast with previous research on other mushroom sclerotium, *Lignosus rhinocerotis,* which Lau *et al*. (2014) used 80% methanol, Lee *et al*. (2012) used cold water extract, Lau *et al*. (2013) used hot & cold aqueous extracts, all of those resulted with non-cytotoxic (IC_{50} >20µg/ml) effect against different cancer cells tested. The chemical composition extracted out from both *P. tuber-regium* and *T. heimii* samples might vary depending on the method and culture condition of the extraction process. In Lau *et al*. (2014), the aqueous methanol (80%) may not be able to extract the cytotoxic proteins from *L. rhinocerotis* against MDA-MB-231 and HCT-116 cells inhibition. The extract however, might consist of secondary metabolites with lower proportion of low molecular weight and cytotoxic compounds. This could be due to solubility factor where polysaccharides and proteins have been reported to be less soluble in aqueous methanol extracts, while simple compounds like sugars, amino acids and peptides can easily be dissolved (Carvajal *et al*., 2012).

In a different study, Lau *et al.* (2013), had revealed that the active cytotoxic compound in the sclerotium of *L. rhinocerotis* could be from the protein/peptide(s). The protein profiling showed more protein bands presents (>20 kDa) in the active cold aqueous extracts as compared to inactive hot aqueous extracts. Protein denaturation could occur at higher temperature of sample extraction such as boiling, or pressurised liquid extraction at 100°C, thus limiting the presence of protein bands. It can be suggested that the extraction of proteins using salt saturation (ammonium sulphate) had successfully isolated maximum amounts of cytotoxic compounds (hydrophobic proteins) from *P. tuber-regium* sclerotium, thus proven to be an effective method for optimum extraction of the cytotoxic proteins.

The cytotoxic activities of *P. tuber-regium* sclerotium was then compared to another Basidiomycetes which is *T. heimii* fruiting bodies. It can be observed that almost all *T. heimii* extracts have lower cytotoxic activities against breast cancer cell lines (MDA-MB-231) and colon cancer cell lines (HCT-116). Both mushrooms were also tested against the normal lung cell lines for cytotoxicity to determine their selectivity index (SI). The SI values were calculated to measure the safety level of drugs/ compounds/extracts against normal cells as compared to cancer cells. The higher the SI values, the greater the selectivity of the extracts/compounds to be promoted as safe drugs (Rashidi *et al.*, 2017). Previous report stated a compound with SI value more than 3 means the compound is three times more cytotoxic towards tumour cell lines than to the normal cell lines (Bézivin *et al*., 2003). A compound with SI value less than 2 is generally cytotoxic (Koch *et al*., 2005).The treatment of *T. heimii* fruiting body extracts (both crude aqueous and protein fractions) on HCT-116 resulted in SI value less than 1, indicating its general toxicity towards the MRC-5. With the SI value of 14.00, PS60 is the best candidate to be selected for cytotoxic protein fraction towards the breast cancer, MDA-

MB-231 cells inhibition with minimal cytotoxicity effect towards the normal lung fibroblast cells, MRC-5.

5.2 Anti-migration effect of protein extracts on MDA-MB-231 and HCT-116 cells

All extracts from both *P. tuber-regium* sclerotia and *T. heimii* fruiting bodies were examined for anti-migration activities on MDA-MB-231 and HCT-116 cell lines. Anti-migration activities were determined by measuring the rate of cellular migration (MC_A) upon exposure with different concentration of extracts and compared with untreated cells (control). Extracts which reduced/slowed down the rate of cellular migration towards filling the gap made on a confluent monolayer of cells were considered to have anti-migration activity. Both untreated MDA-MB-231 and HCT-116 cells showed complete closure of the gap after 48 hours of incubation indicating normal cellular migration rate, morphology and synchronisation (Justus *et al*., 2014).

From the results obtained, it can be concluded that *P. tuber-regium* sclerotia extracts is more potent than *T. heimii* fruiting bodies extracts in inhibiting the migration of both cancer cell lines (MDA-MB-231) and (HCT-116). This is due to retardation effects that can be observed on both cells migration upon treatment with all *P. tuber-regium* sclerotia extracts, except PS90 (less anti-migration effects). Moreover, the migration of cells were highly inhibited by the extracts at lower concentration (IC_{25}) which is 2-fold diluted from its IC_{50} values towards both cancer cell lines. For example, most of the extracts (PS30, PS60 and PS90) demonstrated better anti-migration activities at IC_{25} rather than its IC_{50} concentration, and some extracts (PS-CAE) were not effective in inhibiting the cell migration at IC_{50}. This could be recommended that anti-migration activities can be achieved or even better performed at lower concentrations than that required to inhibit the 50% of cells growth. It is a good sign where those extracts have the

ability to control the cancer cells migration at non-cytotoxic concentrations (IC_{25}).

In this study, it can be suggested too that the anti-migration effects were inversely proportional to the concentration of the extracts, thus not dose-dependent. In previous findings, evodiamine, a natural compound has been identified to possess strong anti-migration activities at a concentration of 2-fold lower than the dose required to inhibit the proliferation of tested cells. The results reported that 70% of colon cancer (26-L5) cells migration were inhibited by evodiamine at a concentration of 10 µg/ml while the IC_{50} value was recorded at 25.10 µg/ml. At very low concentration (1.25 µg/ml), evodiamine has been observed to inhibit 50% of the colon cancer cells migration (Ogasawara *et al.,* 2001). Other researchers also reported findings comparable to that of this present study where the salinomycin treatment against breast cancer had reduced the migration capability of the cells to 50-55% at lower toxic doses (Kopp *et al*., 2014). Selecting anti-migration agents able to inhibit migration of cancer cells at low concentration is good because this will also probably result in less cytotoxicity towards normal cells, thus increasing the chances of reducing the side effects of common clinical therapy against cancer patients.

In terms of time dependency, 24 hours to 48 hours of incubation is considered suitable to complete this assay. At 24 hours, the untreated cells showed some movements and migration towards filling the gap, while the treated cells exhibited slowing down in migration into the gap. As the untreated cells showed complete closure of the gap at 48 hours, the anti-migration activities is best seen in the treated cells with the lowest MC_A after 48 hours of incubation. Previous study have shown an effective anti-migration activities of compounds from *Alpinia conchigera* (Zingiberaceae) on MDA-MB-231 cells only after 24 hours of treatment (Liew *et al*., 2017). In different work, smaller range (12 hour to 24 hour) of incubation time was considered for anti-migration study of totonacin, a protein isolated from snake venom against MDA-MB-231 cells. It was observed that

the totonacin had significantly inhibited the MDA-MB-231 migration by 41.4% after 24 hours (Mercado *et al*., 2020). An optimisation study using Design Expert Software can be done in future to further identify the maximum migration capability, MC_A of *P. tuber-regium* extracts on MDA-MB-231 cell line by considering other parameters such as extract concentration and time.

This study is the first report on the strong anti-migration activity of cytotoxic proteins from *P. tuber-regium* extracts against breast cancer cells (MDA-MB-231). Results showed that MDA-MB-231 cell lines were more susceptible upon treatment with both *P. tuber-regium* and *T. heimii* extracts compared to HCT-116 cell lines. In MDA-MB-231 cells, all extracts from both species showed different rate of anti-migration activities while maintaining the cell growth at maximum concentration of their IC_{50} values at 48 hours of incubation. Further incubation will lead to cell death and toxicity as previous data showed that 50% of cell death occurred at 72 hours. The HCT-116 cell lines were seen to be more sensitive towards the exposure of most extracts, where either *P. tuber-regium* extracts exerted lower migration effect than that of MDA-MB-231 cells and/or *T. heimii* extracts caused toxic environment making the HCT-116 cells detached from the plates. MDA-MB-231 cells are characterised as triple negative breast cancer (TNBC) cells which is identified to be most aggressive, highly metastatic and difficult to treat (Peña-Morán *et al*., 2016). Current findings provide an insight data on potential extracts/ compounds in the formulation of highly metastatic breast cancer diseases.

Tumour cell migration and metastasis comprises of both functional and dynamic cytoskeleton to produce the necessary protrusions and forces that drive the cell to other tissues (Webb *et al*., 2002; Grzanka *et al*., 2003). Actin cytoskeleton are important for cell migration where it plays role in the process of cell growth and differentiation as well as involved in the signalling pathway downstream of receptors that lead to the remodelling of the actin filaments (Grzanka *et al*., 2006). It is vital for tumour cells

motility and chemotaxis which in turn influence the metastatic ability of tumour cells (Freitas *et al*., 2008). The motility of cells which lead to the cell movement is initiated by setting up a front-to-back polarisation, followed by a coordinated cycle of actin polymerization-dependent protrusion, integrin/actin-mediated focal adhesion and cell body translocation resulting from actomyosin contractility (Vicente-Manzanares *et al*., 2009). The intact of microtubule cytoskeleton is however, required in the directional cell migration. An asymmetry of the microtubule network is initially established to generate the polarisation and directional movement of the cell (Vicente-Manzanares *et al*., 2009; Lyle *et al*., 2012; Ballestrem *et al*., 2000; Ganguly *et al*., 2012; Wittmann & Waterman-Storer, 2001).

Microtubules are dynamic filamentous cytoskeletal proteins which is crucial as therapeutic target in tumour cells (Dumontet & Jordan, 2010). Microtubules binding agents are highly targeted for natural toxins in cancer research as their importance in different cellular processing including intracellular transport, cell division and migration (Yan *et al*., 2015; Wang *et al*., 2016; Jordan & Wilson, 2004; Kaur *et al*., 2014). The ability of cytotoxic proteins PS60 to inhibit the MDA-MB-231 cells might be associated to the presence of microtubule binding agent (MBA) which have been reported as one of the most important components widely used in clinical chemotherapy to treat different kind of cancers (Field *et al*., 2014). Natural MBAs such as paclitaxel, vinblastine, colchicine and podophyllotoxin have been known to act as microtubule stabiliser or destabiliser. For example, the microtubule stabiliser paclitaxel-a-diterpene alkaloid from *Taxus* were clinically used to treat Kaposi's sarcoma, lung, ovarian and breast cancer, while the microtubule destabiliser vinblastine-a-vinca alkaloid from *Catharanthus roseus* have been used to treat bladder, lung and breast cancer, Hodgkin's disease, solid tumours, leukaemia and lymphomas (Dumontet & Jordan, 2010; Stanton *et al*., 2011).

The present observations are in accordance with previous data which demonstrated that those natural MBAs also inhibited the cell migration at the concentrations lower than those causing cytotoxicity (Wang *et al*., 2019). Therefore, the partially purified cytotoxic proteins from *P. tuber-regium* sclerotia (PS60) could be a potential candidate as microtubule binding agent for anti-tumour therapy targeting the inhibition and suppression of migration of highly metastatic breast cancer cells, MDA-MB-231. Galmarini *et al*. (2018), in their work have revealed that Plocabulin (a novel tubulin-binding agent), presents inhibition of angiogenesis vascular-disrupting activities by modulation of microtubule dynamics in human umbilical vein endothelial cells (HUVECs). The alteration of microtubule dynamics in endothelial cells by plocabulin inhibits the *in vitro* migration and invasion of endothelial HUVEC cells as well as interferes their abilities to induce the formation of 3D capillary-like networks and disrupts pre-existed vessels. This rapid collapse of endothelial tubular networks in vitro is observed at concentrations lower than that inhibiting cells growth. Finally, the in vitro anti-angiogenic activity of plocabulin was confirmed via evaluation in nude mice bearing MDA-MB-231 breast cancer xenografs, in which the treatment of plocabulin (16 mg/kg) had induced the reduction in the number of vessels after 24 hours.

Moreover, several studies also showed the microtubule targeting agents inhibit endothelial cell proliferation, migration, and tube formation and alteration of endothelial cell morphology which associated with rapid vascular collapse in vivo by affecting the microtubule network (Hotchkiss *et al*., 2002; Belotti *et al*., 1996; Dark *et al*., 1997; Klauber *et al*., 1997). Future work should be carried out for PS60 in order to confirm its potency as microtubule binding agent by induction of angiogenesis and/or inhibition of vascular activities as demonstrated in various studies (Hotchkiss *et al*., 2002; Stafford *et al*., 2005; Vacca *et al*., 2005; Belotti *et al*., 1996; Dark *et al*., 1997; Klauber *et al*., 1997).

5.3 Proteins separation and characterisation from *Pleurotus tuber-regium* sclerotia

Based on cytotoxicity test (MTT assay) and anti-migration study, *P. tuber-regium* sclerotia extracts were selected to be further assessed on their protein separation and characterisation. The profiling of proteins from *P. tuber-regium* sclerotia extracts were carried out by SDS-PAGE for protein size/ molecular weight determination, isolation and characterisation by FPLC and LCMS-Q-TOF MS analysis.

The present findings showed the protein profiling by SDS-PAGE of all extracts exhibited several different protein sizes less than 10 kDa which might be overlapping. It can be expected that there are still few protein bands which are not completely separated at low molecular weights as compared with the standard markers. This observation suggested that the sclerotium of *P. tuber-regium* contains a wide range of protein sizes including few small sized proteins (< 10 kDa). The fraction 60 (PS60) which previously exerted the highest cytotoxic effect towards breast cancer cell lines (MDA-MB-231) with IC_{50}=0.75±0.57 μg/ml, had revealed majority of protein bands as compared to other fractions.

The principle of protein solubility effect termed as salting-in and salting out mentioned that, few proteins are soluble in water while most are affected by ions and require salt at certain concentration to remain stable and folded (Duong-Ly & Gabelli, 2014). The addition of salt (<0.15M), termed as salting in, increases the solubility of proteins. At higher salt concentrations, the solubility of proteins decreases thus precipitation or salting out take place (Green & Hughes, 1955). This can be observed in our study, where the present of protein bands from the aqueous extract of *P. tuber-regium* sclerotia (PS-CAE) were initially detected based on the proteins which were soluble in water. The addition of salt (ammonium sulphate) at 30% concentration (PS30) showed few numbers of distinct protein bands including larger protein sizes (>140 kDa). At higher

salt concentration (60% and 90% ammonium sulphate), (PS60 and PS90), abundance of protein bands was presented especially at low molecular weight/sizes. Our findings clearly showed that, the present of smaller protein sizes were more distinctive at 60-90% salt saturation.

Previous study had revealed that a low molecular weight protein like interleukin 1β was effectively fractionated between 50-77% saturation, while large multi-protein complexes can be salted out with less than 20% saturation (Wingfield, 2001). Maximum number of protein bands were observed in PS60 fraction which denotes that the optimal percentage of ammonium sulphate or salt concentration for precipitation of proteins is at 60%. Similarly, a previous proteomic study on *Trametes versicolor* protein extracts showed that at lower salt concentration (75% ammonium sulphate), *T. versicolor* protein fraction were found to yield wider range and maximum number of protein bands rather than (90% ammonium sulphate) (Parroni *et al.,* 2019).

The PS60 fraction was further separated to purify the proteins using FPLC. There were three different peaks (Q1, Q2, Q3) identified resulted from FPLC purification, with IC_{50} values of 0.65±0.03 µg/ml, 0.75±0.03 µg/ml, and 0.60±0.02 µg/ml, respectively, towards the breast cancer (MDA-MB-231) cells growth. All peaks denoted as Q1, Q2, Q3, and the unseparated protein of fraction 60 (PS60) (IC_{50}=0.75±0.03 µg/ml) showed almost similar cytotoxic effect towards the breast cancer (MDA-MB-231) cell line. It can be concluded here that the purified protein peaks (Q1, Q2, Q3) have not performed better cytotoxic activity on the breast cancer (MDA-MB-231) cells proliferation. Therefore, the purification step on PS60 that isolate Q1, Q2, and Q3, have no improvement on the cytotoxic effects against MDA-MB-231 cell line.

Further research work can be conducted to investigate the potential of PS60 (*P. tuber-regium* protein fraction 60) in combination with cisplatin and/ or other anti-cancer drugs for their synergistic effects to increase the cytotoxic activities and apoptotic effects

against cancer cells. Several studies have shown the increased in cytotoxic activities of combined cisplatin with chemical extracts on different cancer cell lines. For example, an essential oil derivative, β-elemene was reported to inhibit cell growth and proliferation as well as increases cisplatin cytotoxicity in human bladder cancer 5637 and T-24 cells. It also amplified cisplatin cytotoxicity and sensitivity towards cell models (such as brain, cervix, breast, colorectal, ovary, and small-cell lung cancer) *in-vitro* (Zou *et al.,* 2013; Liu *et al.,* 2017). In addition, the combination of cisplatin and methyl eugenol (essential oil) significantly enhanced its anti-cancer activity against HeLa cells, via induction of apoptotic effect, caspase-3 activity, cell cycle arrest and mitochondrial potential loss (Yi *et al.,* 2015).

Characterisation of responsible protein fractions, PS60, for anti-tumour effects was carried out by collecting the peaks from FPLC (Q1, Q2, Q3) and subjected to LCMS-Q-TOF MS. Proteins/peptides were then identified by Spectrum Mill software searching against the Swiss-Prot protein database. Protein from the samples (Q1, Q2, Q3) were identified by matching the observed spectral measurements to the previously observed measurements in the database. The steps involved in protein identification are identification of the constituent peptides, followed by comparing the observed features to entries in the database of previously identified peptides. Next, in tandem mass spectrometry (MS/MS), a parent ion corresponding to a peptide is selected in MS^1 for further fragmentation in MS^2. Finally, the resulting fragmentation spectra are compared to the one in the Swiss-Prot database (Karpievitch *et al*., 2010).

Swiss-Prot is a protein sequence database which provide a high level of annotation such as the protein name, protein function, taxonomic data, enzyme-specific information, biologically relevant domains and sites, post-translational modification(s), protein molecular weight, subcellular location(s) of the protein, tissue-specific expression, developmentally-specific expression of the protein, secondary structure, quaternary

structure, similarities to other proteins, biotechnological and pharmaceutical application, diseases associated with protein deficiency, sequence conflicts etc. (Bairoch & Apweiler, 2000; Boeckmann *et al*., 2003)

Two types of proteins were identified, known as kinesin-like protein, from Q1, and keratin, type 1 cytoskeletal, from Q3. Four uncharacterised proteins labelled as UP1, UP2, UP3 and UP4, were isolated from all peaks (Q1, Q2, and Q3). Among all uncharacterised proteins, UP2 and UP4 shared one common amino acid sequence which is (AEAESLYQSK), with similar accession number, molecular weight, and pI, whereas both are different in terms of MS/MS score and amino acid coverage. UP1 and UP3 were totally different in terms of their amino acid sequences which indicate that they might be of either different or similar types of protein.

Keratin type 1, cytoskeletal 10, reveals 3 distinct peptides from the database which result to the highest distinct summed MS/MS score, 39.26. The MS/MS score was less (14.58) in kinesin-like protein as it hits only one peptide. An individual peptide with score value greater than 15 represents a valid results with excellent database match. The kinesin-like protein provided with score lower than 15, however can be considered a good match (score >10) and almost a valid results of its peptide fragmentation. The same goes to UP1, with one distinct peptide contributes to MS/MS score of 13.46. All other uncharacterised protein (UP2, UP3, UP4) showed distinct summed MS/MS score of higher than 15, with 2 distinct peptides.

Gene ontology revealed that kinesin-like protein is associated with ATP binding and microtubule binding activity. This finding proves our hypothesis that microtubule binding agent/compound might be present in PS60 fractions for the anti-migration effects as discussed in 5.2. Through literature surveys, several kinesin-like protein group as identified from our study has also marked a potential target for breast cancer diseases. The first kinesin was isolated from squid nervous tissue in 1985 (Vale *et al.,* 1985). To

date, it was reported that more than 40 kinesin proteins have been identified from eukaryotes and humans and being classified into 14 families (Lawrence *et al.,* 2004; Miki *et al.,* 2001). Kinesins are a family of microtubule dependent motor proteins which utilising the chemical energy from ATP breakdown by ATPase activity for molecules transportation. Kinesins are depending on a number of additional proteins to efficiently transporting various cargos such as organelles, nucleic acids and proteins (Hirokawa & Noda, 2008; Daire & Poüs, 2011). Kinesin-like protein has been reported as a new target for anti-cancer therapy including breast cancer disease (Li *et al.,* 2015; Zhang *et al.,* 2016). Former study revealed kinesin associated protein 3 (*KIFAP3*) gene was identified to be upregulated at mRNA and protein level in breast tumours, indicating its potential role in breast cancer diseases (Telikicherla *et al.,* 2012).

A plus-end directed microtubule depolymerase kinesin (Kif18A) was reported to be overexpressed in breast cancer cells which resulted to cell multinucleation, while the suppression of Kif18A significantly inhibits the proliferation of breast cancer cells *in vitro* and *in vivo*. The inhibition of Kif18A also perturbs the critical mitotic function and the migration of cancer cells as well (Zhang *et al.,* 2010). Mitotic kinesins are responsible for several mitosis process such as bipolar spindle assembly, chromosome alignment, chromosome segregation and cytokinesis (Huszar *et al.,* 2009). For instance, MCAK (a kinesin-13 family member) involvement in mitosis is essential, and it was reported that MCAK is one of the multiple upregulated genes in a genome-wide expression analysis of 81 breast cancer tissues (Maney *et al.,* 1998; Nishidate *et al.,* 2004). The specific function of mitotic kinesins during mitosis will affect the proliferation of cells only, thus could be promising for new chemotherapeutic agents targeting inhibition of cell mitosis (Sarli & Giannis, 2006; Nagle *et al.,* 2006). Previous study had revealed that RNA interfering against KIF2C/MCAK (kinesin family member 2C (KIF2C)/mitotic centromere-associated kinesin (MCAK) had significantly suppressed the expression of

KIF2C/MCAK and further inhibit the growth of breast cancer cell lines T47D and HBC5. Also, the ectopic introduction of p53 had revealed the suppression of KIFC/MCAK expression (Shimo *et al.,* 2008). As numerous research had demonstrated that most kinesins are upregulated in tumour cells compared to normal cells, hence targeting inhibitors to suppress the kinesin expression is highly relevant. Monastrol is found to be the first identified Eg5 (Kinesin-5 subfamily) inhibitor and several new potent Eg5 inhibitors for cancer treatment are still under investigation (Mayer *et al.,* 1999; Huszar *et al.,* 2009). Inhibitors such as kinesin spindle protein (KSP/Eg5) and kinesins other than Eg5 with various promising anti-cancer effects are also constantly being studied and discovered (Liu *et al.,* 2013).

Microtubules targeted drugs have been reported as a successful anti-cancer agents. They are generally bind to one of three main classes of sites on tubulin, the paclitaxel site, the *Vinca* domain and the colchicine domain. Although, changes in microtubules resulting from altered expression of tubulin isotypes, tubulin mutations, and altered expression or binding of microtubule-regulatory proteins lead to the development of microtubule-targeted drug resistance (Jordan & Wilson, 2004; Kanakkanthara *et al.,* 2013). Multidrug resistance where cancer cells become resistant towards different type of cytotoxic drugs is a major problem to the treatment of cancer diseases (Kathawala *et al.,* 2015). Some kinesins responsible for the chemotherapeutic resistance including P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), breast cancer resistance protein (BCRP) and lung resistance-related protein (LRP) (Scheper *et al.,* 1993; Germann, 1996; Gottesman *et al.,* 2002). Motor protein kinesin (KIF4A), one of the Kinesin-4-family, is a microtubule-based plus-end directed motor protein has been reported to be involved in a variety of cell processes such as intracellular trafficking of HIV gag and integrin-β (Martinez *et al.,* 2008; Willemsen *et al.,* 2014), mitotic progression (Zhu & Jiang, 2005; Zhu *et al.,* 2005; Wandke *et al*., 2012), DNA damage response (Wu *et al.,* 2008) and brain

neuron development (Midorikawa *et al.,* 2006). The KIF4A was upregulated in lung cancer and has been observed to be overexpressed in cisplatin (DDP)-resistant human lung adenocarcinoma cells (A549/DDP cells) compared with A549 cells. The expression of KIF4A in A549 or A549/DDP cells had significantly affects cisplatin resistance. Therefore, KIF4A might be a cisplatin resistance-associated protein and serves as a potential target for chemotherapeutic drug resistance in lung cancer (Taniwaki *et al.,* 2007; Zhu *et al.,* 2015; Pan *et al.,* 2017).

We hypothesise that kinesin-like protein identified could be an inhibitor to suppress the overexpression of kinesins in tumour cells particularly the breast cancer cells as small molecules that block kinesin activity can lead to mitotic arrest (Sarli, & Giannis, 2006). Additionally, kinesin inhibitors might be ideal for microtubule targeting drug resistance (Huszar *et al.,* 2009). Further exploration on the molecular network of kinesin regulation and its combined treatments with other anti-cancer drugs is necessary for the development of specific kinesin inhibitiors (Liu *et al.,* 2013).

Keratin 1 and keratin type 1, cytoskeletal 10 are other proteins identified from our study. Keratin 1 was recognised based on the characteristics revealed from both UP2 and UP4. The organism responsible for the submitted protein (keratin 1) is known as *Chlorocebus sabaeus (Green monkey) (Cercopithecus sabaeus).* In terms of protein size, keratin 1 isolated from UP2 and UP4 have molecular weight of 67.6041 kDa which is in accordance with previous report with molecular weight of approximately 65 kDa (Srikanth *et al*., 2015). The role of keratin in cancer cell invasion and metastasis has been proven and further to be explored on its multifunctional regulators of epithelial tumoregenesis (Karantza, 2011). The keratin, type 1 cytoskeletal 10, also might be one of important protein from PS60 responsible for the cytotoxicity and inhibition of MDA-MB-231 cells migration in our study. Keratin is a protein product/compound of numerous nonfood source such as wool, feathers, hairs, horns, and nails (Aluigi *et al.,* 2016).

Keratins are the intermediate filament-forming proteins expressed in epithelial cells, and recognised as one part of cytoskeletal components, which found to be expressed in several types of tumour cells that reflect their morphological and functional differentiation. Cytoskeleton plays role in determining cell shape, cell motility, anchorage-dependent growth, distribution of cell surface proteins, and the localisation of organelles (Bernal *et al.,* 1985). In cancer research, keratins are recognised as diagnostic tumour markers which involve in cell invasion and metastasis, treatment responsiveness and may act as multifunctional regulators of epithelial tumoregenesis (Karantza, 2011). Moreover, particular tripeptidic sequences present on keratin backbone bind specifically to the overexpressed integrin receptors of several cancer cells (Yu *et al.,* 2010). This is interesting to be inter-related with earlier study where keratin, type 1 cytoskeletal 10, and keratin 1 have been identified as *Sclerotium rolfsii* lectin (SRL) interacting membrane protein in colon cancer HT29 cells. It was believed that the apoptotic effects exerted by the proteins might be by binding to and altering the actin filament integrity in HT29 cells (Srikanth *et al*., 2015).

Keratin type I cytoskeletal 10, keratin type II cytoskeletal 7, N-myc downstream-regulated gene 1 (NDRG1) are identified among of the upregulated proteins expressed in *Cordyceps cicadae*-treated MHCC97H cells (Wang *et al.,* 2014). The water extracts of *Cordyceps cicadae* had revealed apoptotic effect against human hepatocellular carcinoma cells (MHCC97H) via G_2/M cell cycle arrest (Wang *et al.,* 2014). NDRG1 from NDRG gene family, had revealed anti-cancer and anti-metastatic activities in different types of cancer cells including pancreatic, colon, cervical, prostate, as well as breast cancer, therefore relates the involvement of keratin type 1 cytoskeletal 10 in the apoptotic activity of the treated cells (Angst *et al.,* 2011; Bandyopadhyay *et al.,* 2004; Li & Chen, 2011; Zhao *et al.,* 2011a).

Paclitaxel is one of the most effective cytotoxic drugs for pancreatic, ovarian, lung, as well as breast cancer treatment (Horwitz, 1992; Nicolaou *et al.,* 1994; Rivera, & Cianfrocca, 2015). However, the use of paclitaxel is associated with adverse effects such as hypersensitivity reactions and peripheral neuropathy (Holmes *et al.,* 1991), extremely less soluble in water that require the use of toxic solvents, and in need of relatively high doses for therapeutic effects (Holmes *et al.,* 1991; Meng *et al.,* 2016). Proteins from natural polymers such as keratins have been proposed as nanocarriers to improve the effectiveness of paclitaxel treatment and its water solubility (Gagliardi *et al.,* 2010; Lohcharoenkal *et al.,* 2014) as they are less toxic, biodegradable, and readily available. In previous research, keratin extracted from Australian Merino wool was combined with paclitaxel to investigate its anti-cancer activity against breast cancer cells. Results demonstrated that only paclitaxel loaded in keratin nanoparticle (KER-NPs-PTX) was able to induce apoptotic effects upon 24 h treatment for MCF-7 and 48 h treatment for MDA-MB-231 cells, as compared to paclitaxel loaded in albumin nanoparticle (HSA-NPs-PTX) and paclitaxel (PTX) alone (Foglietta *et al.,* 2018).

In a different study, maslinic acid (MA) have shown anti-tumour activity towards HT29 colon-cancer cells through G1 cell-cycle arrest. The exposure of MA against HT29 had revealed the expression of fourteen differential cytoskeletal proteins including keratin 2, keratin 8, keratin type II cytoskeletal 8, keratin type I cytoskeletal 9, keratin type I cytoskeletal 18, cytokeratins 18 and 19, β-actin (one group-which exert a structural function), lamin B1, glesolin 1, septin 2, villin 1, actin-related protein 2, and moesin (another group- which related to the nucleation of actin and cytoskeletal formation (Rufino-Palomares *et al.,* 2013). Various studies mentioned before, provided evident on keratin functions as anti-tumour agent by inducing apoptosis on different cancer cells.

The uncharacterised protein, UP1 was identified to match the protein sequence of *Amanita muscaria Koide BX008*. However the biological and molecular function of the

protein, UP1 have not been reported. UP1 might be suggested as a novel protein with similar identity found in *Amanita muscaria* species. *A. muscaria* is categorised under poisonous mushrooms where lectin (carbohydrate-binding protein) activity was reported to be responsible for its toxicity effect (Singh *et al.,* 2015). Lectins from diverse mushroom species are widely reported, however, the cytotoxic activity of lectin from *A. muscaria* against cancer cells was not found via literature surveys. Among all uncharacterised proteins, UP3 protein sequences are still unreported in the database.

5.4 Apoptotic effects exerted by cytotoxic proteins, PS60

Based on cytotoxicity of Q1, Q2, Q3 (PS60 FPLC peaks), which had no improvement upon further purification, and due to sample limit, the apoptotic study was conducted on the unseparated PS60 fraction. Apoptosis is a type of cell death associated with an intrinsic cell suicide programme to ensure the maintenance of tissue homeostasis in an organism by getting rid of damaged cells that might interfere with normal cells function (Sharma *et al.*, 2014). In cancer cells, the occurrence of apoptosis is reduced and hence result in accumulation of cells leading to tumour. Defects can happen along the pathway of apoptosis process that led to transformation of the affected cells, tumour metastasis and anti-cancer drug resistance. Hence, targeting apoptosis is one of the key strategies in the development of cancer treatments (Wong, 2011).

Cisplatin is a chemotherapeutic agent used for various cancer diseases including lung, ovary and breast. It covalently binds to the N-7 atoms of purines on DNA to form DNA adducts, causing the conformation of DNA which led to cell cycle arrest and apoptosis through the activation of multiple signalling pathways (Miller *et al*., 2010). Previous research has reported the apoptotic effects as well as anti-proliferating activities of cisplatin on breast cancer cells, MCF-7 and MDA-MB-231 (Liang *et al*., 2015; Jiang *et al*., 2017). The study revealed that cisplatin cytotoxicity was recorded at IC_{50}=6 µg/ml

and IC_{50} =8 µg/ml towards MCF-7 and MDA-MB-231 cells, respectively (Jiang *et al*., 2017). In a different study, the MTT assay showed cisplatin inhibited the growth of MDA-MB-231 cells at IC_{50}=1.3±0.55 µg/ml and IC_{50}=0.9±0.31 µg/ml after 24 and 48 hours, respectively (Fani *et al*., 2016). The cytotoxic effect of cisplatin treated MDA-MB-231 resulted from the present experiment was more promising as compared to both previous findings, with IC_{50}=0.56±0.09 µg/ml. Therefore, the apoptotic activities of cisplatin on MDA-MB-231 is best to be employed as positive control and compared with the tested extract, PS60.

The induction of apoptosis by PS60 is MDA-MB-231 cells was confirmed by annexin V/propidium iodide assay. The percentage of cells stained by Annexin V and PI analysed by flow cytometry can be classified into four categories according to quadrants. The population of cells residing in the Annexin V−/PI – were determined as living cells and the necrotic cells in Annexin V−/PI+ quadrants. While Annexin V+/PI- and Annexin V+/PI+ quadrants determined the early and late apoptotic cells, respectively (Pumiputavon *et al*., 2017). The exposure of PS60 on MDA-MB-231 cells had demonstrated significant increase of apoptosis (early and late stage) in dose-dependent manner at incubation time, 48 hours. The percentage of life cells also decreased at this point of time. It can be suggested that the optimum time of incubation for induction of apoptosis is 48 hours. PS60 at IC_{50} (0.75 µg/ml) and PS60 at IC_{75} (1.50 µg/ml) indicate different concentration of *P. tuber-regium* sclerotia (PS60) being exposed to MDA-MB-231 cells growth. Based on results obtained, higher concentration of protein extracts exerted much higher percentage of apoptotic cells at 48 hours. The percentage of necrotic cells is shown to be increased slightly in PS60 at IC_{75} only after 72 hours of treatment.

The induction of apoptosis is determined in this study which indicates that the annexin V-FITC/PI staining had detected the translocation of phosphatidylserine in MDA-MB-231 cells that were exposed to PS60. Phosphatidylserine translocation to the

outer leaflet of the lipid bilayer which occurs in the very early apoptosis is a sign of phagocytosis (or characterised as "eat-me" process) that result from the expression at the surface of apoptosing cells of apoptotic cell-associated molecular patterns (ACAMPs) that phagocytes the multicellular organisms (Silva, 2010; Franc *et al*., 1999; Krysko *et al*., 2006; Erwig & Henson, 2008). Elimination of cells in the early apoptosis by scavenger cells is a deletion mechanism of cells populations and tissues to regulate cell numbers (Kerr *et al.,* 1972). The apoptotic cells were further progressed to late apoptosis and necrosis in the absence of phagocytes to ingest the apoptotic bodies (Silva, 2010).

One of the important hallmarks of apoptosis is the activation of Bax proteins (a family of pro-apoptotic Bcl2 members) by DNA damage which caused the penetrability of the mitochondrial membrane and subsequent mitochondrial *c* release. Cytochrome *c* triggers the activity of caspase 9, and caspase 3, which eventually activate the other members of the caspase cascade, resulting in the appearance of cells apoptotic characters such as DNA condensation and fragmentation, and membrane blabbing (Mohan *et al*., 2012; Sayer, 2011). The fraction of Bcl2/Bax proteins in the mitochondria employs the initiation of apoptosis by anti-cancer drugs (Korsmeyer *et al*., 2007; Green & Reed, 1998). For instance, previous study had demonstrated the apoptotic effect of peptide extracted from *Lentinus squarrosulus* in human lung cancer, H460, by increasing BAX (pro-apoptotic protein) while decreasing Bcl-2 protein (anti-apoptotic protein) (Prateep *et al*., 2017).

Novel anti-cancer drug/s targeting to increase the rate of apoptosis is useful and of importance in the management of cancer research (Gerl & Vaux, 2005) as it will not cause inflammation reaction to the surrounded cells. This is because the apoptotic cells are self-destructive that involve only individual cells and they do not release their cellular constituents into the surrounding interstitial tissue and quickly phagocytosed by macrophage or adjacent normal cells (Savill & Fadok, 2000; Kurosaka *et al*., 2003). It is

in contradiction to necrosis that results to inflammatory response which promote the progression of tumour, by induction of angiogenesis, cancer cell proliferation and invasiveness (Jin & White, 2007; Vakkila & Lotze, 2004; Degenhardt *et al*., 2006; Lotze & Tracey, 2005; Schlueter *et al*., 2005; Taguchi *et al*., 2000; Kang *et al*., 2013). For instance, breast tumour necrosis is associated with high-grade disease, tumour size increased, negative status of receptor, high microvessel density, and macrophage infiltration (Fisher *et al*., 1993; Jimenez *et al*., 2001; Leek *et al*., 1999).

Numerous studies reported that anti-cancer agents had induced apoptotic cell death via cell cycle arrest at G0/G1, S, or G2/M phase (Harada *et al*., 2002; Hu *et al*., 2010). PS60 at low dose (0.50 μg/ml and 0.75 μg/ml) demonstrated an increase in the number of cells in the S-phase with a corresponding decrease in the proportion of G0/G1 population. Cell cycle arrest at S-phase is a mechanism by which PS60 may act to inhibit the MDA-MB-231 cells proliferation and progression. In cell cycle division, S-phase is the period in which cells replicate their DNA. The inhibition of MDA-MB-231 cells proliferation by PS60 represents that the DNA replication was interrupted and result to the accumulation of cells whose progress is arrested in the S-phase, and therefore were unable to progress to the G2/M phase (Xu *et al*., 2001).

Cell cycle arrest in response to DNA damage is highly correlated with the degree of damage occurs at checkpoints enabling DNA repair prior to cell cycle progress. However, excessive DNA damage led to cells senescence (non-proliferating or growth resistant state) or apoptosis, therefore, the concentrations of drugs used are of highly importance. At low concentrations, the anti-cancer drugs increase the extrinsic senescence rate which is associated with G1-S phase arrest and stabilization of p53 gene leading to p21 (also known as p21 (WAF1/Cip1)) transcription increases and growth arrest (Mansilla *et al*., 2003). Activation of p53 expression that induced the p21 up-regulation are essential in the control of the cancer cell cycle progression. Cyclin-

dependent kinase (CDK) inhibitor p21 is one of important factors that inhibit the cell cycle progression (Karimian *et al*., 2016). There are two mechanisms in which p21 inhibit the cell cycle progression, by targeting the cyclin-CDK complexes that control cell cycle transitions and the DNA polymerase processivity factor PCNA, which is essential for DNA replication (Cayrol *et al*., 1998). Xie *et al*. (2017) suggested in their study that the inhibition of MDA-MB-231 cell cycle at S-phase was due to decrease in CyclinD1 and CyclinE by p21 and the cell cycle arrest at G1 and G2 were resulted from PCNA inhibition.

Findings of this study are in agreement with previous study where the mushroom extracts of *Phellinus linteus* had demonstrated the induction of cell cycle arrest of MDA-MB-231 cells at S-phase. The mechanism responsible for cell cycle arrest at S-phase were conducted through western blot analysis to identify the regulatory proteins (cyclin) and cdks involved in the progression from G1-phase to S-phase (Sherr & Roberts, 1999; Ekholm & Reed, 2000). The MDA-MB-231 treated cell extracts showed the expression of cyclin-dependent kinase inhibitor p27 indicating the cell cycle arrest in S-phase (Sliva *et al*., 2008). Therefore, as in our study, the cyclin-dependent kinase inhibitor p27 is expected to be upregulated in the MDA-MB-231 extracts treated with PS60 that result to cell cycle arrest at S-phase checkpoint. Further research can confirm this hypothesis by conducting the western blot analysis with respective antibody. For example, changes in the expression level of G1-phase regulatory such as cyclin-D1, cdk4 or p21, or G1-late phase or G1/S-phase regulatory proteins such as cyclin E, A and cdk2 and the S-phase regulatory protein which is cyclin dependent-kinase inhibitor p27.

Numerous evidences have revealed that the exposure of different extracts on MDA-MB-231 cells which induce the cell cycle arrest at S-phase. The methanolic extracts of *Dicranopteris linearis* have demonstrated the inhibition cell cycle progression of MDA-MB-231 via induction in S-phase arrest after 72 hours of incubation (Baharuddin *et al*.,

2018). In different study, the water extract of Pu-erh tea (*Cammelia assamica* (Mas.) Chang) had significantly increased the proportion of MDA-MB-231 cells in S-phase, suggesting the S-phase arrest on the cells (Xie *et al*., 2017). Other study showed the Pu-erh tea water extracts selectively inhibited tumour cell progression by down-regulated S phase and arrest at G1 or G2 phase (Zhao *et al*., 2011b), and the ethyl acetate extract of Pu-erh tea have blocked the HepG2 cell cycle progression at G1 phase via induction of p53 and up-regulation of p21 (Way *et al*., 2009). It can be observed that different cell lines result to different checkpoint at which the cell cycle arrest, as this might be due to different mechanisms addressed by cells.

The present study provides an insight into the mechanisms of cytotoxic proteins from PS60 in inducing apoptosis in breast cancer cells (MDA-MB-231). The proteins involved such as kinesin-like protein, keratin 1, and keratin type 1, cytoskeletal 10, might be acting to MDA-MB-231 cells by binding to or altering its microtubule sites. Further explorations and investigations of the specific relationship between identified proteins and their mechanisms of action against cancer cells are of importance.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

In the present study, the anti-tumour activities of cytotoxic proteins from *Pleurotus tuber-regium* (sclerotium and fruiting bodies) and *Termitomyces heimii* (fruiting bodies) were investigated against breast cancer cells (MDA-MB-231) and colorectal cancer cells (HCT-116). Potential protein extracts were identified by considering the half-maximal inhibitory concentration (IC_{50}), selectivity index (SI) and migration capabilities (MC_A). The most promising protein extract is PS60 against MDA-MB-231 cells while exerting minimum cytotoxicity towards the normal lung cells, MRC-5.

The ability of cytotoxic proteins PS60 to inhibit the MDA-MB-231 cells might be associated to the presence of microtubule binding agent (MBA) which have been reported as one of the most important components widely used in clinical chemotherapy to treat different kind of cancers. Several different proteins were identified, 2 of which are known as kinesin-like protein and keratin type 1, cytoskeletal 10. Kinesin-like protein identified could be an inhibitor for the overexpression of kinesins in tumour cells particularly the breast cancer cells as small molecules that block kinesin activity leading to mitotic arrest. Additionally, kinesin inhibitors might be ideal for microtubule targeting drug resistance. The keratin, type 1 cytoskeletal 10, also might be one of important protein from PS60 responsible for the cytotoxicity and inhibition of MDA-MB-231 cells migration in this study. In cancer research, keratins are recognised as diagnostic tumour markers involved in cell invasion and metastasis, treatment responsiveness and may act as multifunctional regulators of epithelial tumourigenesis.

The exposure of PS60 on MDA-MB-231 cells had demonstrated significant increase of apoptosis (early and late stage) in a dose-dependent manner at 48 hours of incubation. Cell cycle arrest at S-phase is one of mechanisms by which PS60 may act to

inhibit the MDA-MB-231 cells proliferation and progression. The accumulation of MDA-MB-231 cells in the S-phase and were unable to progress to the G2/M phase, suggesting that the DNA synthesis were interrupted. This indicates that the MDA-MB-231 cells growth ceased to progress through the cell cycle. The potential of PS60 in blocking the division of cancer cells was proven. The present study provides an insight into the mechanisms of cytotoxic proteins from PS60 in inducing apoptosis in breast cancer cells (MDA-MB-231). The proteins involved such as kinesin-like protein, keratin 1, and keratin type 1, cytoskeletal 10, might be acting to MDA-MB-231 cells by binding to or altering its microtubule sites.

Identification of those protein compounds validates *P. tuber-regium* as a potential source for anti-tumour drug development towards breast cancer. Further, the findings of this study also could be useful for the community to maintain a good healthcare and well-being by the consumption of both mushrooms as foods in their daily meals. However, further explorations and investigations of the specific relationship between identified proteins and their mechanisms of action against cancer cells are of importance. The mechanisms of MDA-MB-231 cell death exposed to PS60 should be further investigated through several other experiments such as western blot analysis (to investigate the protein expression in MDA-MB-231 treated cells) and TUNEL assay (for detection of the apoptotic cells).

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