CYTOTOXIC AND ANTI-MIGRATION ACTIVITIES OF PROTEIN EXTRACTS FROM *Trametes scopulosa* (BERK.) BRES. MYCELIUM AGAINST HCT 116 COLON CANCER CELL LINE

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ABSTRACT

Colon cancer is one of the leading causes of cancer death. It is the third most common cancer in men and the second among women globally. Metastasis is a spread of cancer cells to distant locations in the body and usually occurs at stage IV of colon cancer. Distant metastasis is the principle cause of colon cancer deaths. It involves 6 main steps; local invasion, intravasation, circulation, extravasation, proliferation and angiogenesis. Polypore mushrooms have been investigated for its medicinal properties especially for anti-tumour activity. *Trametes scopulosa* is one of the polypore mushroom. However to date, no research concerning the anti-tumour effect of *Trametes scopulosa* has been published. This study is therefore aims to evaluate the anti-tumour potentials by looking at the cytotoxic and anti-migration activities of solvent and protein extracts from *T. scopulosa (*KUM70034) against colon cancer cell line (HCT 116). Pre-screening for cytotoxic activity against HCT 116 using MTT assay showed that all solvent extracts of *T. scopulosa* (methanol, hexane, dichloromethane and ethyl acetate) were deemed not actively cytotoxic against HCT 116 cells. However, all protein fractions obtained by ammonium sulphate precipitation at 30%, 60% and 90% (F30, F60 and F90) were actively cytotoxic against HCT 116 cells with fraction 60 exhibiting the most potent cytotoxicity (IC_{50} 0.84±0.05 µg/mL). However, the fractions were not cytotoxic against normal lung fibroblast cell line (MRC-5). F60 that contained the highest protein level of 459.745±17.55 µg/mL was separated into a few single bands corresponding to a molecular weight ranging from 12 kDa to 64 kDa. Subsequently, F60 was evaluated for cytotoxicity against HCT 116 and MRC-5 cells at 20 µg/mL protein concentration. Results showed that the F60 of *T. scopulosa* when tested by protein content was indeed cytotoxic against HCT 116 cells with IC_{50} value of 0.57±0.06 µg/mL protein but showed no cytotoxic effect against MRC-5 cells. The *in*

vitro scratch wound assay was used to measure inhibition of cellular migration activity. The HCT 116 cells treated with F60 slowed down the migration activity of the cells as compared to the control group (non-treated cell line). The fraction was further purified by anion exchange chromatography and collected fractions (P1, P2 and P3) were retested for cytotoxic and anti-migration activities. Results showed that P2 and P3 fractions had better cytotoxic and anti-migration effects against HCT 116 cell line as compared to P1. However, the cytotoxic effects of both fractions were lower than the partially purified protein (F60). The P2 and P3 fractions were then further evaluated for synergism study. Results showed that, the cytotoxic effect of the combine mixture at a ratio of 1:1 was greater than F60. This suggests that P2 and P3 fractions have synergistic cytotoxic effect. Both fractions were then subjected to protein analysis using LCMS Q-TOF. Two proteins were characterized as pyranose-2-oxidase and carboxylic ester hydrolase. Two uncharacterized proteins were also found. However, only pyranose-2-oxidase has potential as anti-tumour effect against HCT 116 cancer cells. As a conclusion, this study suggests that proteins from mycelial extract of *T. scopulosa* could potentially to be used as an anti-tumour agent against colon cancer cells.

ABSTRAK

Kanser kolon adalah salah satu punca utama kematian kanser. Ia adalah kanser ketiga paling biasa di kalangan lelaki dan yang kedua di kalangan wanita di seluruh dunia. Metastasis adalah penyebaran sel-sel kanser untuk lokasi yang berada jauh di dalam badan dan biasanya berlaku pada peringkat IV kanser kolon. Distant metastasis adalah penyebab utama kematian akibat kanser kolon. Ia melibatkan 6 langkah utama; tempatan serangan, intravasation, pengedaran, pengeluaran darah, percambahan dan angiogenesis. Cendawan polypore telah dikaji untuk ciri-ciri perubatannya terutamanya untuk aktiviti antitumor

. *Trametes scopulosa* adalah salah satu cendawan polypore. Oleh itu , tujuan kajian ini adalah untuk menilai sitotoksik dan aktiviti antimetastatik ekstrak-ekstrak pelarut dan protein dari *Trametes scopulosa* (KUM70034) terhadap kanser kolon (HCT 116). Pra-pemeriksaan untuk aktiviti sitotoksik terhadap sel-sel kanser kolon (HCT 116) menggunakan esei MTT menunjukkan kesemua ekstrak pelarut *T. Scopulosa* (metanol, hexana, diklorometana dan etil asetat) dianggap tidak aktif sitotoksik terhadap HCT 116 sel. Walaubagaimanapun, kesemua pecahan protein yang diperolehi daripada pemendakan ammonium sulfat pada 30%, 60% dan 90% (F30, F60 dan F90) adalah aktif sitotoksik terhadap HCT 116 sel dengan fraksi F60 mempamerkan aktiviti sitotosik yang terbaik (IC_{50} 0.84 ± 0.05 μg/mL). Walau bagaimanapun, kesemua fraksi tidak menunjukkan kesan sitotoksik terhadap sel normal peparu fibroblast (MRC-5). F60 yang mengandungi tahap protein tertinggi 459.745 ± 17.55 μg/mL telah dipisahkan kepada beberapa band tunggal yang sepadan dengan berat molekul yang terdiri daripada 12 kDa hingga 64 kDa. Oleh itu, F60 telah dinilai untuk aktiviti sitotoksik terhadap sel-sel HCT 116 dan MRC-5 pada kepekatan protein 20 μg/mL. Hasil kajian menunjukkan bahawa F60 *T. scopulosa* adalah sitotoksik terhadap sel HCT 116 dengan nilai IC_{50} 0.57

± 0.06 μg/mL protein dan tidak menunjukkan sebarang kesan sitotoksik terhadap sel-sel MRC-5. Esei 'scratch wound' secara *in vitro* digunakan untuk mengukur perencatan aktiviti migrasi selular. Sel HCT 116 dirawat dengan F60 menunjukkan aktiviti migrasi sel-sel yang perlahan berbanding dengan kumpulan kawalan (sel yang tidak dirawat). Fraksi tersebut ditulenkan dengan menggunakan pertukaran anion kromatografi dan fraksi-fraksi yang dikumpul (P1, P2 dan P3) telah diuji kembali untuk aktiviti-aktiviti sitotoksik dan antimigrasi. Hasil kajian menunjukkan bahawa fraksi-fraksi P2 dan P3 mempunyai kesan-kesan sitotoksik dan antimigrasi yang baik terhadap sel-sel HCT 116 berbanding dengan P1. Walau bagaimanapun, kesan sitotoksik kedua-dua pecahan ini adalah lebih rendah daripada protein separa purifikasi (F60). Fraksi-fraksi P2 dan P3 kemudiannya telah dinilai untuk kajian sinergi. Keputusan menunjukkan bahawa, kesan sitotoksik campuran gabungan pada kadar 1:1 lebih baik daripada F60. Ini telah mencadangkan bahawa fraksi-fraksi P2 dan P3 mempunyai kesan sitotoksik sinergi. Kedua-dua fraksi kemudiannya dipilih untuk analisis protein dengan menggunakan LCMS Q-TOF. Terdapat dua protein dicirikan sebagai piranosa-2-oksidase dan karboksilik ester hidrolase). Dua protein tidak dicirikan telah dikenal pasti. Walau bagaimanapun, hanya piranosa-2-oksidase mempunyai potensi kesan antitumor terhadap sel kanser HCT 116. Sebagai kesimpulan, kajian ini menunjukkan bahawa protein-protein daripada ekstrak miselium *T. scopulosa* berpotensi digunakan sebagai agen antitumor terhadap sel-sel kanser usus.

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May Allah S.W.T Bless.

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LIST OF SYMBOLS AND ABBREVIATIONS

	Description
5-FU	Fluorouracil
ACN	Acetonitrile
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BMI	Body Mass Index
BRM	Biological Response Modifiers
BSA	Bovine Serum Albumin
BSC	Best Supportive Care
CO_2	Carbon dioxide
Da	Dalton
DCA	Deoxycholic acid
DCM	Dichloromethane
ddH_2O	Deionized distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECIS	Electrical Cell-substrate Impendence
eg.	Example
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
EMR	Endoscopic Mucosal Resection
FBS	Fetal Bovine Serum
FIP	Fungal Immunomodulatory Protein

FD	Frequency Domain
FPLC	Fast Protein Liquid Chromatography
g	Gram
GYMP	Glucose-Yeast-Malt-Peptone
h	Hour
HCA	Heterocyclic amine
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
IC_{50}	Inhibition Concentration
kDa	Kilodalton
kpa	Kilopascal
L	Litre
LAC	Laparoscopy-Assisted Colectomy
LCA	Lithocholic acid
LCMS	Liquid Chromatography Mass Spectrometry
LDH	Lactate Dehydrogenase Leakage
MAPK	Mitogen-Activated Protein Kinase
MEA	Malt Extract Agar
mg	Miligram
mL	Mililitre
mM	Micrometer
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaCl	Sodium chloride
nm	Nanometer

NR	Neutral Red
OP	Optical Density
PAH	Polycyclic Aromatic Hyrocarbon
POI	Percentage of Inhibition
RIP	Ribosome Inactivationg Protein
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TFA	Trifluoroacetic acid
UV	Ultraviolet
WHO	World Health Organization
µg	Microgram
µL	Microlitre
µmol	Micromolar
%	Percentage
^{o}C	Degree Celcius

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CHAPTER 1

INTRODUCTION

Cancer is a class of diseases characterised by uncontrollable cell growth. It is one of the leading causes of death worldwide. A total of 1,658,370 new cancer cases have been reported in the United States with 589,430 cancer deaths in 2015 (Siegel, Miller, & Jemal, 2015). In Malaysia, 21,773 Malaysians are being diagnosed with cancer but approximates that nearly 10,000 cases are unregistered every year according to the National Cancer Registry of Malaysia (NCR). According to GLOBOCAN 2012 (Estimated Cancer Incidence, Mortality and Prevalence Worldwide), colorectal cancer was the third most common cancer in men and the second in women worldwide. In 2013, World Health Organization (WHO) stated that Europe had the highest incidence of colorectal cancer followed by the United State of America while Africa had the lowest incidence rate.

Cancer results from damage at the DNA level leading to genetic instability and subsequent cellular deterioration. Cancer becomes potentially fatal when cancer cells spread to the other part of the body. One of the ways of cancer spread is through the blood stream or lymph system, a process known as metastasis. Metastasis involves 6 main steps known as local invasion, intravasation, circulation, extravasation, proliferation and angiogenesis. If these steps are inhibited, metastasis can be blocked or slowed down. A tumour formed from this metastatic cancer cells is called a secondary tumour. It is approximated that 50% - 60% of patients diagnosed with colon and rectal cancer will develop metastases during the course of the disease (Van Cutsem *et al*., 2006).

The chance of survival of cancer depends on a few factors such as the type and stages of cancer, the age and lifestyle before cancer and how one respond to treatment.

The National Cancer Institute's SEER reported that 5 years relative survival rate for people diagnosed with stage I colon cancer between 2004-2010 is about 92% while stage IV colon cancer have a 5 years relative survival rate of about 11%. Chemotherapy is one of the standard approaches of treatment for cancer and many tumours have a satisfactory reaction when first exposed to the chemotherapeutic drugs, clinically (Liu, 2009). However, the toxicity of the drugs to normal tissues resulting in hostile side effects in various organ systems as well as drug resistance has remained the major problems for the outstanding clinical use (Sak, 2012). Based on these scenarios, many researchers are researching for new cancer therapeutic agents from natural products to replace the conventional anticancer drugs.

Polypore mushrooms have a great potential as natural source for the biologically active compounds. *In vitro* cytotoxic screenings on solvent extracts of polypore mushrooms against several cancer lines suggest that the mushroom extracts have potential anti-cancer compounds (Zjawiony, 2004). *Trametes* species is one of the genus under Polyporeceae family is known for medicinal properties especially *Trametes versicolor*. The polysaccharides of this species were reported to have cytotoxic effect against various cancer cell lines and have been used as a chemoimmunotherapy agent to treat cancer in Asia for over 30 years (Fisher and Yang, 2001).

Trametes scopulosa is a non-edible wild polypore mushroom because of its coarse texture and bitter taste. To date, there is no pharmacological report regarding *T. scopulosa*. This study therefore aims to evaluate *T. scapulosa* for its potential as anti-tumour effects. The potential anti-tumour compounds elucidated from this study can be further developed for the potential natural pharmaceutical interest. The specific objectives of this study are:

i. To evaluate the cytotoxic activity of crude and protein extracts of *T. scopulosa* mycelium against HCT 116 colon cancer cell line.

ii. To evaluate the anti-migration effect of active protein fractions of *T. scopulosa* mycelial extract against HCT 116 colon cancer cell line.

iii. To isolate and characterise the bioactive compound(s) from protein fractions of *T. scopulosa* mycelial extract.

CHAPTER 2
LITERATURE REVIEW

2.1 Cancer

Cancer is a major public health problem around the world and the number of cancer death has elevated each year. It is currently the second leading cause of death after heart disease. In 2012, an estimated 14.1 million new cases of cancer were diagnosed worldwide. According to the American Cancer Society, in 2016, more than a million new cancer cases are expected to be diagnosed. Thus, a large number of cancer researches range from epidemiology, molecular bioscience, biotechnology, cancer immunotherapy and gene therapy were carried out to evaluate and compare applications of the various cancer treatments.

Cancer is a class of diseases characterised by uncontrolled cell growth. It harms the body when damaged cells divide uncontrollably to form masses of tumours. Tumours that are localised and demonstrated limited growth are generally considered to be benign. A malignant tumour however, are those whose cells can spread to surrounding locations via blood stream or lymph systems in a process known as metastasis.

2.1.1 Mechanisms of Carcinogenesis

Cancer advancement is known to be a multi-step process. In 1948, Berenblum and Schubik first proposed the concept of multi-stage carcinogenesis (Devi, 2005). Carcinogenesis, oncogenesis or tumourigenesis is a process that converts normal cells from controllable to uncontrollable growth, whereby normal cells are transformed into cancer cells (Wang, 1999). The initiation of cancer formation can be divided into three stages; tumour initiation, tumour promotion and tumour progression. Tumour initiation is induced by exposure of carcinogenic agents on normal cells, which can modify the

molecular structure of DNA resulting in fixation of mutations within the genome of individual cells.

The initiators are often specific to particular tissue types or species because most of the initiator must be metabolized before becoming active (Pitot *et al*., 2004). However, not all initiated cells will become a tumour, as many of these cells may die by apoptosis (Tannock *et al*., 2013). When the initiator has mutated the cell, it is prone to affect the promoter. According to National Cancer Institute, tumour promotion is a process in which existing tumours are stimulated to grow. Promotion stages implicated interference of the cell-adhesion ability to other cells or to extracellular matrix. Meanwhile, tumour progression describes the stage whereby the tumours cells detach from the primary tumour mass and invade to adjacent tissue, which leads to a process called metastasis (Hayot *et al*., 2006).

Apoptosis is a programmed cell death by which unwanted or useless cells are removed during development and other normal biological processes. Apoptosis caused distinct morphologic changes, including cell shrinkage, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Wyllie, 1997). Inhibition of apoptosis can result in a number of cancers, autoimmune diseases, inflammatory diseases, and viral infections.

Cancer is a disease with an excessive cellular proliferation. Malignant cells of cancer experience an abnormal response to apoptosis induction. The tumour-suppressor protein (p53) and interferon-alpha and beta (IFN-alpha/beta) are essential for the induction of apoptosis in cancerous cells and in anti-viral immune responses (Takaoka, 2003). The p53 is a cell cycle regulator able to induce cell cycle arrest to allow DNA repair or apoptosis (Vogelstein & Kinzler, 1992). However, it will induce apoptosis if damage is pervasive and repair efforts fail. Any disturbance to the regulation of the p53

or interferon genes will result in defective apoptosis and the possible formation of tumours.

2.1.2 Mechanisms of Metastasis

Metastatic cancer is a secondary malignant tumour that has spread to the other part of body from the primary site. The cancer cells that break away from the primary tumour can travel to the other part of the body through two major routes, the blood vessels and/or lymphatic vessels. Metastasis is responsible for most cancer mortality, yet the process remains controversial (Talmadge & Fidler, 2010). More than 90% of the cancer mortality is not due to primary tumour but the spread of primary tumour to secondary site (Fidler, 1991; Liotta, Steeg, & Stetler-Stevenson, 1991; Sporn, 1996; Entschladen *et al*., 2004). Distant metastasis is the principle cause of colon cancer deaths. Metastasis is characterised by a series of steps such as local invasion, intravasation, circulation, extravasation, proliferation and angiogenesis (Fidler, 1999; Chambers *et al*., 2001). If these steps are inhibited, metastasis can be blocked/slowed down.

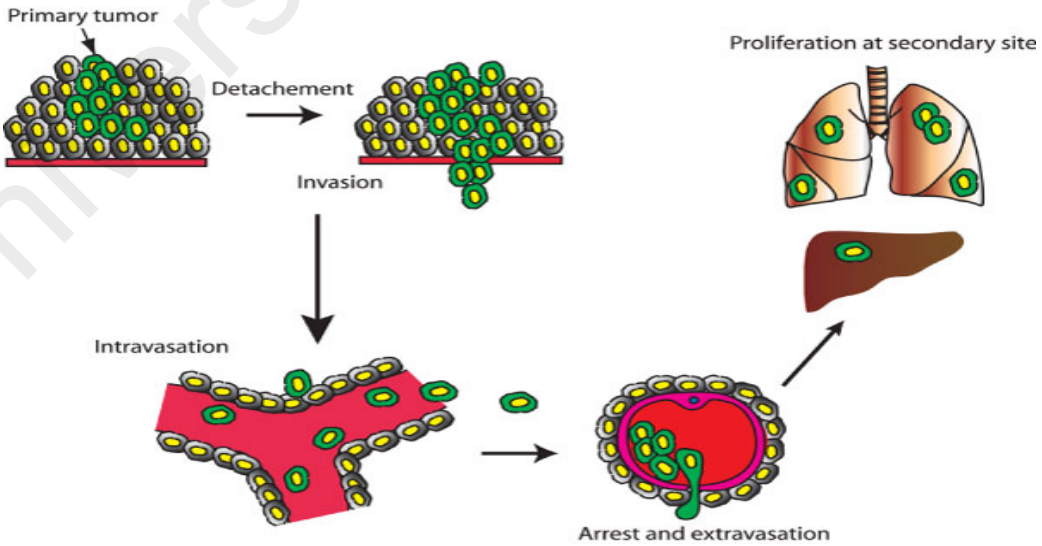

Figure 2.1: The metastatic process (Adapted from Hunter, Crowford, & Alsarraj, 2008).

According to National Cancer Institute, cancer cells invade adjacent normal tissue (invasion) and move through the walls of nearby blood vessels or lymph vessels (intravasation) and circulate to the other parts of the body. Then the cancerous cells will lodge in blood capillaries at a distant location. They then penetrate the walls of capillary and migrate into the surrounding tissue (extravasation) at the new location. Cancer cells proliferate at the distant location to form small tumours known as micro-metastases. To keep growing, the small tumours create their own new blood vessels to obtain blood supply. The blood supply contains the much needed oxygen, glucose, and other nutrients necessary for tumour growth and development. The process by which new blood vessels develop from pre-existing ones is known as angiogenesis. Bone is one of the most frequent sites of metastasis followed by liver and lung. Although most cancer has the ability to spread to many different parts of the body, they usually tend to occur in specific target organs (Table 2.1).

Table 2.1: Typical sites of metastasis of the common tumours.

Tumour Type	Principle Sites of Metastasis
Lung	Brain, bone, adrenal gland, and liver
Breast	Bone, lung, liver, and brain
Prostate	Bone
Colon	Liver and lung
Pancreas	Liver and lung
Skin	Lung, brain, skin, and liver
Sarcoma	Lung
Uveal (Eye)	Liver

Adapted from Tannock *et al*., 2013

2.1.3 Cytotoxic and Metastatic Drug

Cytotoxic drugs are a group of medicines that contains chemicals that can damage cells, prevent the cell proliferation which leading to cellular dysfunction. They are mainly used to treat cancer, commonly as part of a chemotherapy routine. However, these drugs also affect healthy cells that are actively dividing in the body among which are hair follicles and the lining of the digestive system. Subsequently, many normal cells are damaged along with the cancer cells.

National Cancer Institute has recorded more than 200 cancer drugs. There are many types of cancer drugs including drugs used in hormone therapies (eg: tamoxifen, anastrozole, and letrozole), biological therapies (eg: bevacizumab, cetuximab, and rituximab) chemotherapy and bisphosphonate (prevent bone damage). The typical combination of chemotherapy drugs is doxorubicin, asparanginase, methotrexate and cytarabine. Meanwhile the examples of bisphosphonate drugs are ibandronate, alendronate, zoledronic acid and risendronate. Most of the anti-cancer drugs are limited by their general toxicity to proliferating cells. Many researchers have developed novel cytotoxic agents with unique mechanism of action. However, a lot of this chemical compounds still lack tumour selectivity and have not been therapeutically useful (Chari, 2008).

Cytotoxic drugs can be divided into several groups based on factors such as mechanisms, chemical structure, and their relationship to another drug (Table 2.2). According to American Cancer Society, knowing how the drug works is important in predicting side effects. In recent times, new type of anti-cancer drugs have been discovered for example monoclonal antibodies that target cell-surface receptors (eg: rituximab, trastuzumab and bevacizumab) and small molecules that interact with varies cell signaling pathways (eg: imatinib) (Tannock *et al.*, 2013). This newer drugs target specific metabolic pathways that intervene with various functions of the cells, including

those that promote cell division (trastuzumab and imatinib) or contribute to immune-mediated cellular damage (rixutimab). Meanwhile, bevacizumab can suppress angiogenesis, which indirectly inhibits tumour growth. This new finding is in contrast to conventional cytotoxic drugs that usually target proliferating cells and interact with DNA.

Table 2.2: Different groups of cancer drugs.

Agents	Classes	Drugs
Alkylating	Nitrogen mustards	mechlorethamine, chlorambucil, cyclophosphamide (Cytoxan®) , ifosfamide, and melphalan
	Nitrosources	streptozocin, carmustine (BCNU), and lomustine
	Alkylating sulforates	busulfan
	Triazines	dacarbazine (DTIC) and temozolomide (Temodar®)
	Ethylenimines	thiotepa and altretamine (hexamethylmelamine)
Anti-metabolites		5-fluorouracil (5-FU),6-mercaptopurine (6-MP), Capecitabine (Xeloda®), Cytarabine (Ara-C®), Floxuridine, Fludarabine, Gemcitabine (Gemzar®), Hydroxyurea, Methotrexate, Pemetrexed (Alimta®)
Anti-tumour antibiotics	Antracyclines	Daunorubicin, Doxorubicin (Adriamycin®), Epirubicin, Idarubicin
	Non- antracyclines	Actinomycin-D, Bleomycin, Mitomycin-C, Mitoxantrone
Topoisomerase	Topoisomerase I	Topotecan, Irinotecan
	Topoisomerase II	Etoposide (VP-16), Teniposide, Mitoxantrone
Mitotic Inhibitor	Taxanes	paclitaxel (Taxol®) and docetaxel (Taxotere®)
	Epothilones	ixabepilone (Ixempra®)

Table 2.2, continued

	Vinca alkaloids	**vinblastine (Velban®), vincristine (Oncovin®), and vinorelbine (Navelbine®)**
		Estramustine (Emcyt®)
Cortesteroids		Prednisone, Methylprednisolone (Solumedrol®), Dexamethasone (Decadron®)

Adapted from American Cancer Society, 2016

Because most of the existing cancer drugs inhibit only proliferation of cancer cells, the suppression of cancer metastasis is an urgent therapeutic need (Weber, 2013). More than 90% of cancer deaths are due to metastasis formation and yet there is no anti-metastatic drug on the market (Stock *et al*., 2013). According to National Foundation for Cancer Research, to improve the survival rate of cancer is by understanding how metastasis occurs. In spite of this, metastasis is such a complicated and multi-step processes that requires multiple approaches including biochemistry, molecular biology, cell biology and genetics. Thus, anti-metastatic drugs or treatments should be based on the stage of a metastasis in patients.

Tumour metastases travelled to different body organs and different anatomic organ may possibly trigger different pathways linking neoplasm metastases (Lu *et al*., 2013). This results in different types of drugs affecting or inhibiting different stages of metastatic processes. Thus, different anti-cancer drugs will certainly not act in the same way in all metastatic organs. Previous studies hypothesized that anti-cancer and anti-metastatic drugs might act inversely in various courses of sub-stages and could be applied according to metastatic cascade (Lu *et al.*, 2010; Lu & Xi, 2012).

The new type of anti-cancer drug, bevacizumab with combination of interferon alfa was reported to result in significant improvement in progression-free survival when treated on patients with metastatic renal cell carcinoma, compared with interferon alfa alone (Escudier *et al*., 2007). Sternberg *et al.* (2010) evaluated efficacy and safety of randomized placebo-controlled phase III and pazopanib monotherapy in treatment-naive and cytokine-pretreated patients with advanced metastatic renal carcinoma. Pazopanib showed significant enhancement in progression-free survival and tumour response compared with the placebo.

A research by Van Cutsem *et al.* (2007) had compared the activity of panitumumab with best supportive care (BSC) or BSC alone on patient with metastatic colorectal cancer. BSC is the treatment of choice when cure is not achievable with anti-cancer treatments and involves management of disease-related symptoms (Numico, Russi, & Merlano, 2011). Result showed that panitumumab significantly prolonged the progression-free survival with controllable toxicity in patients with colorectal cancer. Based on all the previous studies, it was shown that different types of cancer drugs could be used to treat the metastatic tumour depending on the tumour type and the combination of the drugs.

2.2 Colon cancer

Colon cancer is the second commonest form of cancer death worldwide and usually common in Western societies (Jemal *et al*., 2011). It affects men and women almost equally. Colon cancer is the third most commonly diagnosed cancer and the average lifetime risk of developing colon cancer is about 1 in 20 (5%). About 72% of cases emerge in the colon while 20% in the rectum. An estimated number of 135,430 individuals for new cases are diagnosed with colorectal cancer and 50,260 for mortality cases in the United State in 2017. The majority of new cases occur in people aged 65 years old or older (58%) (Siegel *et al*., 2017). Colon and rectum (colorectal) cancer is

also the second most common cancer in Malaysia (Magaji *et al*., 2010). A review by Ghee (2014) stated that colorectal cancer is the most frequent cancer among men and the third most common among women in Peninsular Malaysia.

Colon cancer progresses slowly and unnoticeable. Hence, early screening and medication for this cancer type is very important. Most colorectal cancer takes relatively 8-10 years to develop from adenomatous polyp into an invasive cancer (Sung, 2007). Nearly all colon cancer begins from small, benign (non-cancerous) tumour called polyps. It develops in the epithelial lining inside of the colon or rectum (Figure 2.2). Some of these polyps may grow into malignant (cancerous) tumour if they are not removed during colonoscopy. The progression of a polyp which leads to a colorectal cancer was first describe by scientists at John Hopkins. Dr. Burt Vogelstein discovered that genetic mutations or other chemical modification were the factors of colorectal polyps and colorectal cancer development. It causes activation in or promotion of specific genes known as tumour suppressor and tumour promoter genes.

Malignant tumour can spread and destroy surrounding tissue and cause other tumour to develop. The malignant tumour that does not spread to the surrounding tissue is called carcinoma *in situ*. Malignant tumour stimulates formation of new blood vessels in a process known as angiogenesis. Invasive or metastatic cancer invades and spread to the other organs via blood stream and lymphatic system.

More-advanced tumours usually require surgical removal, which in many cases is followed by chemotherapy. Early screening can prevent colon cancer by finding and removing polyps before they become malignant. According to a data released by American Cancer Society in 2014, colon cancer incidence rate have dropped 30% in the last 10 years among adults ages 50 and older due to the widespread used of colonoscopy.

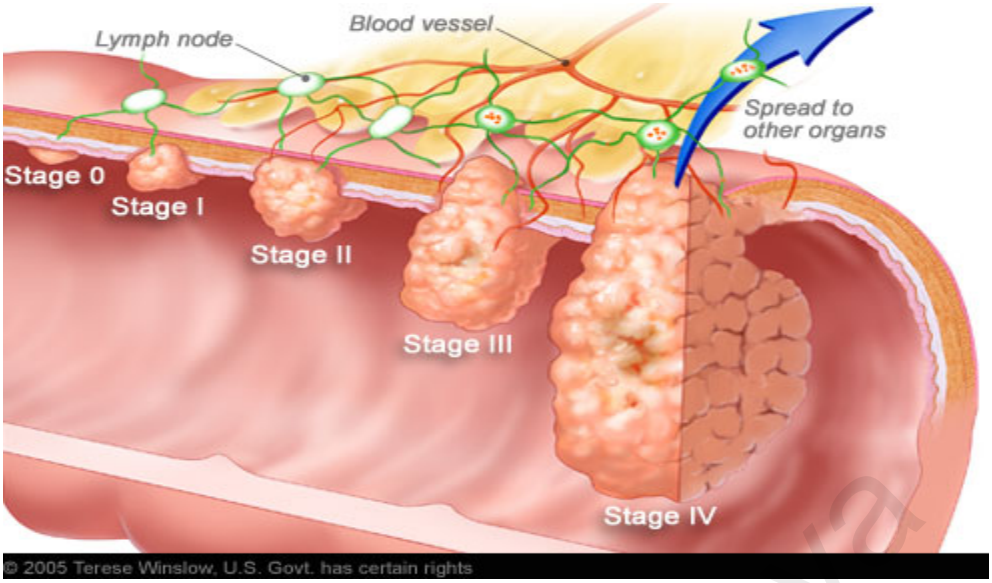

Figure 2.2: The polyp on the inner lining of the colon (Adapted from Terese Winslow Medical and Scientific Illustration, 2005)

2.2.1 Risk Factors of Colon Cancer

i. Diet

Diet is considered a factor of increased risk in the development of colon cancer. However, it is difficult to comprehend which components of diet are most crucial in increasing the risk of colon cancer. Diet high in fat and red meat are believed to have strong association with colon cancer. The higher digestion of fat and meat in the gastrointestinal tract increased secondary bile acid formation. Secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid (LCA) are carcinogens (Bernstein *et al*., 2009; Reddy *et al*., 1980). An epidemiologic studies done by several researchers have also found that population with high prevalence of colorectal cancer would have high concentration of fecal bile acids. Higher cooking temperatures such as grilling meats can create chemical reactions that lead to the formation of many potent carcinogens, for examples heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs). HCAs are formed when creatine, amino acids and sugar (found in meat) react with heat during high-temperature cooking (Cross & Sinha, 2004; Knize *et al.*, 2005; Felton *et al.*, 2007). When meats are cooked for longer period of time at higher temperature, the

production of HCA is increased. PAH is common contaminant of processed food but not all PAH is carcinogenic. It is the product of combustion and pyrolysis that is existent in petroleum or coal (Lijinsky, 1991). The formation of PAH is influenced by temperature of cooking, duration of cooking, type of the fuel used in heating and fat content of the food. Meat that is cooked above an open flame by using high temperature method such as grilling and barbequing contains the highest level of PAHs because it is exposed to smoke formed from the pyrolysis of fatty juices that dripping onto the fire (Cross & Sinha, 2004).

ii. Cigarette smoke

Cigarette smoke can be divided into gas-phase smoke and particulate matter (or tar). Gas phase smoke passes through a filter while tar retained on the filter. The tar phase contains several stable free radicals (Church & Pryor, 1985). Both of these phases are easy to oxidize (high oxidizing). These can cause oxidative stress especially on the lungs. Research has shown 20 that cigarette smoke can initiate lipid peroxidation in rat tracheal explants as well as in other systems (Churg & Cherukupalli, 1993). Smoking causes many type of cancer including colon cancer. According to American Cancer Society, long-term cigarette smoking is correlated with an increased risk of colorectal cancer. Giovannucci and Martinez (1996) stated that tobacco smoking would attribute 20% of colorectal cancer cases in the United States and it has been perpetually correlated with a higher risk of colorectal adenoma, precursor of cancer. The study also hypothesized that carcinogens in cigarette smoke might be an initiator for colorectal carcinogenesis.

iii. Family History

The association between family history of colon cancer in the family first-degree (patents, brother, sister, children) or many other family members has been well defined. Cancers within the same family may result from diet, lifestyle factor or a shared exposure to an environmental carcinogen such as cigarette smoke or sunlight. These gene changes by carcinogen do not affect all body cells and are not inherited and are called sporadic cancer. According to National Cancer Institute, sporadic cancer occur in people who do not have a family history of that cancer or an inherited change in their DNA that would increase their risk for that cancer. A study done by Slattery *et al.* (2003) reported that family history of colorectal cancer was related with the utmost risk among those diagnosed at age 50 or younger. This confirmed the remarks proclaimed by other researchers that a family history of colorectal cancer increases risk of cancer among those diagnosed at a younger age. The risk of having this cancer can be reduced through sigmoidscopy screening, healthy diet intake, no smoking cigarette and maintaining a BMI (body mass index) below 25 since certain diet and lifestyle factors could effect the development of cancer among those with a family history of colon cancer.

iv. Aging

A review by Kennedy (2000) reported that the incidence of cancer specifically breast, lung, prostate, and colon increases with age. Cancer is well- known as a disease of older person. In United States, patients with cancer are usually more than 70 years old (Eshler & Longo, 1997). It can occur in younger people, but the occurrence is much less frequent. However in recent years, the increasing of colorectal cancer is rising among young adults. According to the American Cancer Society (2015), researchers from The University of Texas MD Anderson Cancer Center predicted that by 2030, the incidence rate of colon cancer among young people ages 20-34 years would increase by

90%. Colon cancer does not discriminate and can happen to men and women at any age according to individual risk factors. More than 60% of all cases are diagnosed after age 65 years, with 67% of cancer mortality arising in this older group (Kennedy, 1997). Older persons have less resistance and longer exposure to carcinogenesis. This biologic and clinical change is the major influence of carcinogenesis that can lead to cancer growth among older age groups (Balducci & Eshler, 2005).

2.2.2 Diagnosis of Colon Cancer

Colon cancer is generally discovered after symptoms appear but most people with early colon or rectal cancer do not have symptoms of the disease. Thus, it is recommended to undergo screening test. Early detection and treatment of colon cancer is always encouraged for better disease prognosis. Early diagnosis of the malignant tumour at early stage increases the chance for successful treatment. A research done by Dinish *et al*. (2007) reported that a sensitive frequency domain (FD) fluorescence imaging technique could be used to detect colon cancer at early stage. From the result obtained, several stages of cancer growth can be strongly simulated and imagined using the FD technique.

A few factors may be considered when choosing a diagnostic test. For example, age and medical condition, type of cancer suspected, signs and symptoms, and previous test results. Fecal occult blood test is one of colon cancer diagnosis to detect the presence of blood in stool (Rex *et al.*, 1993). Bloody stool is an early sign of colon cancer. However, gastric disorder or wound might also cause blood in the stool. Therefore, this technique is not confirmatory. If the result of the physical exams or blood test suggests colon cancer might be present, a thorough and accurate cancer diagnosis test is recommended.

Endoscopic tests such as sigmoidoscopy, colonoscopy or endoscopic ultrasound may be used as one of the diagnostic tests for colon cancer. Flexible plastic tubing with

tiny camera (sigmoidoscope) is inserted into the rectum to give a view of the rectum and lower colon. Meanwhile, colonoscopy is a longer version of sigmoidoscope and the test allows visual examination inside the entire rectum and colon. If any suspicious areas are found, biopsy will be taken and sent for analysis. Biopsies can give a definite diagnosis of colon cancer. An endoscope is inserted into the rectum in endoscopic ultrasound (endosonography) test. A probe at the end of the endoscope rebounds high-energy sound waves (ultrasound) off internal organ resulting in an image (sonogram). A large colorectal polyp can also be removed by endoscopy technique (Doniec *et al*., 2003).

2.2.3 Colon Cancer Treatment and Drugs

The types of treatment for colon cancer patients are largely dependent on the stage of the cancer. There are three primary treatment options for colon cancer; surgery, chemotherapy, and radiotherapy. Colon cancer may be treated with surgery alone, surgery and chemotherapy, and/or other treatments. Surgery can be divided into three types; for pre-cancer stage, for invasive cancer and for advanced cancer. The types of surgery depend on the stage and location of the tumour. If the polyp is very small, doctor may recommend a minimally invasive approach to surgery such as removing polyps (polypectomy) during colonoscopy. Endoscopic mucosal resection (EMR) may be used when the cancer is small and only on the lining of the colon. Laparosic surgery (minimally invasive surgery) is when polyps cannot be removed during colonoscopy. Laparoscopy-assisted colectomy (LAC) in colon cancer patient was claimed to reduce perioperatic morbidity. However, its influence on long-term result is still unknown. A study by Lacy *et al.* (2002) stated that LAC was more efficient for treatment of non-metastatic colon cancer in terms of tumour recurrence and survival as compared to open colectomy.

After surgery, chemotherapy is usually given if the cancer has spread to the lymph nodes and it may help reduce the risk of cancer recurrence. However, chemotherapy may be used before surgery to shrink the tumour before the operation. Fluorouracil (5-FU) and leucovorin, or capecitabine are usually the main drugs for chemotherapy. A research by André *et al.* (2004) revealed that adding oxaliplatin with fluorourocil and leucovorin improves the adjuvant treatment of colon cancer. Adjuvant chemotherapy is common treatment for colon cancer patients who are at high risk for cancer recurrence. In 2001, Sargent *et al.* has reported the pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients. The result showed that fluoroucil-based adjuvant therapy could be beneficial to elderly patients with colon cancer, without a significant increase in toxic effects.

According to National Cancer Institute, cetuximab is one of chemotherapy drugs that have been approved to be used alone or with other drugs to treat colorectal cancer that has metastasized. Cetuximab has activity against colorectal cancer that expresses epidermal growth factor receptor (EGFR). A research by Jonker *et al.* (2007) studied the effect of cetuximab on patients with colorectal cancer expressing immunohistochemically detectable EGFR and who beforehand treated with other cancer drugs such as fluoropyrimidine, irinotecan and oxiplatin. Results showed that cetuximab increase overall survival and progression-free survival and conserves quality-of-life measures in patients with colorectal cancer, which other treatments have failed.

Radiotherapy is a treatment using high-energy rays such as x-rays or particles to kill cancer cells and is commonly given in combination with chemotherapy. It is not a common way to treat colon cancer. Radiation therapy is often given in advanced stages of colon cancer instead of surgery when an operation cannot be performed. Randomized trials have shown that adjuvant chemotherapy enhances survival for patients with stage III colon cancer and that chemotherapy combined with radiotherapy improves survival

for patients with stage II or III of rectal cancer. Thus, Ayanian *et al.* (2003) had designed a population-based study to evaluate use of these treatments in clinical practice. Chemotherapy rates varied widely by age from < 55 years (88%) to age ≥ 85 years (11%) and radiotherapy varied similarly.

Chemotherapy was used less often among older and unmarried patients while radiotherapy was used less often among older patients, black patients and those initially treated in low-volume hospitals. In this study, the physicians' reason for not administering adjuvant therapy included patients' refusal (30% for chemotherapy and 20% for radiotherapy), comorbid illness (22% and 14%, respectively) or lack of clinical indication (22% and 45%, respectively). This study revealed that the use of adjuvant therapy for colorectal cancer differs extensively by age, race, marital status, hospital volume and individual hospital indicating opportunities to develop better care.

Cancer treatments may cause side effects. This problem occurs because treatment affects healthy tissues or organs. The side effects usually vary from person to person depending on the type of treatments. National Cancer Institute have listed down common side effects caused by cancer treatments such as anaemia, fatigues, edema, hair loss (alopecia), nausea and vomiting, nerve problems, appetites loss, sleep problems, bleeding and bruising (thrombocytopenia), urinary and bladder problem. There is also a possibility that cancer will recur or metastasizes. Some patients also may develop secondary cancers, some of which may be a result of treatments used for the original cancer. There is a few evidence of risk of second cancers after adjuvant chemotherapy in women who received cyclophosphamide, methotrexate, and fluorouracil for the treatments (Shapiro & Recht, 2001). It is important to have an understanding of the potential side effects because of the high incidence rate of cancer in the world (Palumbo *et al*., 2013). Because of these overwhelming effects, many cancer patients do not want

to continuously administer cancer drugs and are looking for alternative natural medicines that are safe and health promoting solution.

2.3 Discovery of Natural Anti-cancer Drug

Natural product is a substance or chemical compound produced by a living organism, which can be found in nature. Screening natural products from plants, animals, marine organisms and microorganism has leads to the discovery of numerous pharmaceutical agents. Due to serious side effects of the current cancer drugs, researchers are widely searching to develop anti-cancer agents from natural sources.

For a thousand years, plants have been employed as medicines (Samuelsson, 1999). Plant-derived natural products have been an important source of anti-cancer drugs (Grothaus, Cragg, & Newman, 2010). The examples of plant-derived compound used in cancer treatment are vincristine, irinotecan, etoposide and paclitaxel (da Rocha, Lopes, & Schwartsmann, 2010). Compounds have been successfully used in cancer treatment as shown in Table 2.3. These natural drugs are usually used during chemotherapy either to protect patients or to enhance the effectiveness of chemotherapy. For example, ironotecan (plant alkaloid) combined with fluorouracil and calcium folinate could increase response rate, time to progression and survival of the patients with metastatic colorectal cancer (Douillard *et al*., 2000).

Alkaloids, polysaccharides and proteins from plants and fungi are common compounds that have been used in cancer therapy research for the past decades. Alkaloids are important and well-known chemical compounds for drug discovery. Numerous alkaloids extracted from natural herbs have been proven to have anti-proliferation and anti-metastatic effects on several types of cancers both *in vitro* and *in vivo* (Lu *et al*., 2012).

Secondary metabolites from mushrooms have received an increasing public interest and research in food and pharmaceuticals are discovering new drugs or lead

compound. Polysaccharide and protein in mushrooms are widely investigated and proven to have good anti-cancer and other biological properties. Mushroom polysaccharides are considered to be biological response modifiers (BRM), or immunopotentiators, because of their action mechanism (Mizuno, 1999). Meanwhile, bioactive proteins from mushrooms have become popular source of natural pharmacological agents. Polysaccharides from mushroom are the most widely investigated compounds as compared to proteins. However, bioactive proteins are also substantial part of functional components in mushroom that also have increasing interest due to their pharmaceutical potential (Wong *et al*., 2010).

Table 2.3: Several plant-derived compounds used in cancer treatment

Compound	Cancer use	Status
Vincristine	Leukemia, lymphoma, breast, lung, pediatric solid cancers and others	Phase III/IV
Vinblastine	Breast, lymphoma, germ-cell and renal cancer	Phase III/IV
Paclitaxel	Ovary, breast, lung, bladder, and head and neck cancer	Phase III/IV
Docetaxel	Breast and lung cancer	Phase III
Topotecan	Ovarian, lung and pediatric cancer	Phase II/III
Irinotecan	Colorectal and lung cancer	Phase II/III
Flavopiridol	Experimental	Phase I/II
Acronyciline	Experimental	Phase II/III
Bruceantin	Experimental	Preclinical/phase I
Thalicarpin	Experimental	Preclinical/phase I

Adapted from da Rocha, Lopes, & Schwartsmann, 201

2.3.1 Mushroom proteins

Proteins are complex three-dimensional structure with a wide variety of functions in nature. Like polysaccharides, the structures of proteins correspond to their functions. Amino acid is a simple biologically organic compound containing amine and carboxyl group with a side chain specific to each amino acid. One or more long chains of amino acid were combined to form protein. There are 20 different amino acids generally found in proteins. The types of amino acids produced depend on the conditions that existed at the time of synthesis (Cleaves Ii, 2009). However, it remains controversial. Human being can only synthesize some amino acids but need to obtain the other amino acids from the diet. However, most microorganisms such as *E. coli* can synthesize the whole basic set of 20 amino acids by themselves (Berg, Tymoczko & Stryer, 2002). The amino acids that can be produced by human body are termed non-essential amino acids (eg: alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine) meanwhile the amino acids that must be provided in the diet are called essential amino acids (eg: histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan and valine). In 1967, Fred Richards, David Harker, Richard Dickerson, Joe Kraut, and William Lipscomb are the first U.S. scientists that published about protein structures such as ribonucleases A and S, carboxypeptidase, cytochrome c, and chymotrypsin.

There are few level of proteins structure, namely primary, secondary, tertiary and quaternary structures (Price & Nairn, 2009). Primary protein structure refers to the sequence of amino acid present in the polypeptide chain, which are covalently linked by peptide bonds. A segment of polypeptide chain may form helicase, strands of sheet or turns are refers as secondary structure meanwhile, a tertiary structure is the long-range folding of the polypeptide chains. This structure refers to the three-dimensional

structure of the entire polypeptide chain. The quaternary structure is the association of the protein molecule (polypeptide chain) in a multi-subunit complex.

Mushrooms have very little carbohydrate or fat, making them a preferred meal for anyone inspecting their daily diet while the types of protein found in mushrooms are unique and have a positive effect on human health. About 200 mushroom species are consumed as a delicacy throughout the world (Kalač, 2013). Thus, the knowledge of nutritional value, health- promoting effect and chemical composition of mushrooms have developed during the last few years. Estimated analyses of mushroom protein in *Pleurotus* sp. discovered that it contains 2.78% protein and 0.14% non-protein nitrogen on a fresh-weight basis (Bano, 1963). A research by Matilla in 2002 reported that protein content (based on amino acid analyses) of cultivated *Agaricus bisporus* (white and brown), *Lentinula edodes* and *Pleurotus ostreatus* varied 2.09, 2.13, 1.88 and 1.87 g/100 g fresh weight, respectively.

Mushroom proved to be good sources of almost all essential amino acid when compared with common vegetables such as potatoes, carrots or cauliflower. A review by Xu *et al*. (2011) reported that lectins, fungal immunomodulatory protein (FIP), ribosome inactivating protein (RIP), ribonucleses, laccase, and other proteins are the examples of proteins with interesting biological activities in various mushrooms. These proteins are believed to have medicinal effects such as anti-tumour, anti-microbial, anti-viral, antioxidative and immunomodulatory agents (Table 2.4).

Table 2.4: Isolated proteins from various mushrooms

Lectins	Characteristics (Molecular weight and N-Sequence terminal)	Immuno-modulation	Anti virus	Anti fungal	References
Pholiata adiposa	MW: 16 kDA N-Seq: DILMGTYGML		+		Zhang *et al*. (2009)
Hericium erinaceum	MW: 51 kDa N-Seq: AFGQLSFANLAAADF		+		Li *et al.* (2010b)
Russula delica	MW: 60 kDa N-Seq: GLKLAKQFAL		+		Zhao *et al.* (2010)
RIPs					
Hypsizigus marmoreus	MW: 9,567 Da N-Seq: AEGTLLGSRA TCESGNSMY		+		Wong *et al.* (2008)
Laccase					
Pleurotus eryngii	MW: 34 kDa N-Seq: AVGPVLGPDA		+		Wang and Ng (2006b)
Tricholoma mongolicum	MW: 66 kDa N-Seq: GIGPVADLYVGNRIL		+		Li *et al.* (2010a)
Clitocybe maxima	MW: 62 kDa N-Seq: DIGPVTPLAI		+		Zhang *et al.* (2010)
FIPs					
Antrodia camphorate	MW: 66 kDa N-Seq: VVTYDPFFDNPPNNL LYYAASSDDTN	+			Sheu *et al.* (2009)

Table 2.4, continued

Other protein and peptide			
Agrocybe cylindracea	MW: 9 kDa N-Seq: ANDPQCLYGNVAAKF	+	Ngai *et al.* (2005)
Trichiloma giganteum	MW: 27 kDa N-Seq: QVHWPMF	+	Guo *et al.* (2005)
Ganoderma lucidum	MW: 15 kDa N-Seq: AGETHTVMINHAGRGAPKLVVGGKKLS.	+	Wang and Ng (2006a)
Clitocybe sinopica	MW: 44 kDa N-Seq: SVQATVNGDKML	+	Zheng *et al.* (2010)

Adapted from Xu *et al*., 2011

2.3.2 Anti-tumour mushroom proteins

Lectin is usually the most widely investigated protein and have been discovered during the past few years (Singh *et al*., 2010). Lectins are carbohydrate-binding proteins that allow molecules to stick together without getting the immune system involved (non-immune proteins or glycoproteins). Xylose-specific lectin (28.8 kDa) was isolated for the first time from fresh fruiting bodies of the wild ascomycete mushroom (*Xylaria hypoxylon*) by Liu *et al*. (2006). The lectin had a potent anti-mitogenic activity in mouse splenocytes and exhibited highly strong anti-proliferative activity against tumour cell lines. A study by Zhang *et al*. (2009) also showed lectin with molecular weight of 16 kDa from *P. adiposa* possesses anti-proliferative activity towards breast cancer MCF-7 cells and hepatoma HEP G2 cells.

According to a review from Ng (2003), only several proteins have been isolated from fungi but a large of number of RIPs and peptides have been isolated from flowery plants. RIPs, mostly in plants, are enzymes that inactivate ribosome by eliminating one or more adenosine residues from rRNA. Several mushroom species such as *Calvatia caelata*, *Flammulina velutipes*, *H. marmoreus*, *Lyophyllum shimeji* and *Pleurotus tuber-regium* were proclaimed to have RIPs (Wang & Ng, 2000; Lam & Ng, 2001; Ng *et al*., 2003). These RIPs were proven to exhibit various bioactivities like anti-fungal, anti-proliferative and HIV-1 reverse transcriptase inhibitory. A new RIP marmorin with 9 kDa molecular weight (MW) from mushroom *H. marmoreus* inhibited proliferation of hepatoma cells (HEP G2) and breast cancer cells (MCF-7), and HIV-1 reverse transcriptase activity with IC_{50} values lower than 30 μM (Wong *et al*., 2008).

Laccase is also a protein that possesses enzymatic activity like RIPs that have been reported to have medicinal properties. Laccase occur widely in many plants, fungi and microorganisms. They are belonging to multi-copper oxidases enzymes. The anti-proliferative activity of laccase was first suggested in 2016. The aqueous extracts of *Trametes versicolor* and *Trametes trogii* were tested against HeLa cancer cell lines. Both extracts were cytotoxic to the cell line. Laccase and peroxidase enzyme in *T. trogii* extract were involved in the inhibitory effect on the cancer cell line (Ünyayar *et al*., 2006). The laccase of fruiting body of the *P. eryngii* mushroom was reported to have an inhibitory activity on HIV-1 reverse transcriptase with an IC_{50} of 2.2 μM (Wang and Ng, 2006). Anti-viral laccase (58 kDa) purified by M El Fakharany *et al*. (2010) from *P. ostreus* (oyster mushroom). Besides that, laccase from *T. mongolicum* also have anti-proliferative effect against hepatoma HEP G2 cells and breast cancer cells (MCF-7) (Li *et al*., 2010).

Fungal immunomodulatory proteins (FIPs) are proteins that target immune cells. Kino *et al*. (1989) isolated immunomodulating proteins from *G. lucidium* and the

biochemical and immunological properties were evaluated. Ko *et al*. (1995) also purified FIP-fve from *F. velutipes* which is commonly known as edible golden needle mushroom. Its immunomodulatory activity was demonstrated as stimulating activity toward human peripheral blood lymphocytes. A new immunomodulating protein GMI was isolated from *Ganoderma microsporum* by Lin *et al*. (2010) and its activity in suppressing tumour invasion and metastasis was investigated. The results showed that GMI inhibited epidermal growth factor mediated migration and invasion in A549 lung cancer cells by several pathway such as inhibiting EGF-induced phosphorylation, EGF-induced activation of Cdc42 GTPase, activation of EGFR and Ala pathway kinases

Mushroom produces a large number of proteins and polysaccharides. However, due to time consuming, high cost and low yield, it is difficult to isolate proteins from mushroom. Thus, it is important to develop a new method like genetic engineering for producing mass yield of bioactive proteins. Besides that, there are increasing number of research focusing on the isolation, purification and functions of mushrooms protein but the mechanisms of their actions such as immunomodulation, anti-microbes, anti-proliferation, anti-migration, etc. are still poorly understood (Xu *et al*., 2011). Hence, more research on relationship between protein structure and bioactivity are highly required, which may result in production of new natural medicinal drugs to human diseases.

Table 2.5: Isolated proteins with anti-tumour activity from various mushrooms

Lectins	Characteristics (Molecular weight and N-Sequence terminal)	Reference
X. hypoxylon	MW: 14.4 kDa N-Seq: SSAHNT·LGNGEWL··LVGQQCLF	Liu *et al.* (2006)
Clitocybe nebularis	MW: 15.9 kDa N-Seq: 149 amino acid-long unprocessed protein	Pohleven *et al.* (2009)
P. adiposa	MW: 16 kDa N-Seq: DILMGTYGML	Zhang *et al.* (2009)
Russula lepida	MW: 32 kDa N-Seq: VWYIVAIKTDVPRTT	Zhang *et al.* (2010a)
R. delica	MW: 60 kDa N-Seq: GLKLAKQFAL	Zhao *et al.* (2010)
H. erinaceus	MW: 51 kDa N-Seq: AFGQLSFANLAAADF	Li *et al.* (2010a)
RIPs		
H. marmoreus	MW: 9,567 Da N-Seq: AEGTLLGSRATCESGNSMY	Wong *et al.* (2008)
Laccase		
T. mongolicum	MW: 66 kDa N-Seq: GIGPVADLYVGNRIL	Li *et al.* (2010b)
C. maxima	MW: 62 kDa N-Seq: DIGPVTPLAI	Zhang *et al.* (2010b)

Table 2.5, continued

FIPs		
G. lucidum	MW: 36.6 kDa N-Seq: DINGGGATLPQKLYLTPDVL	Du *et al.* (2007)
Pleurotus citrinopileatus	MW: 45 kDa N-Seq: No amino acid sequence reported	Chen *et al.* (2009)

Adapted from Xu *et al*., 2011

2.4 Polypore mushrooms

Polypore mushrooms (Family: Polyporaceae) are a group of fungi with pores on the fruiting bodies. Murill first surveyed the genera in Polyporaceae in 1903 and tremendous contribution towards the stabilization of the nomenclature and typification was done throughout the decades. Besides plant and animals, polypores are also important members in the biodiversity of forest. Comprehensive studies of polypores have been published in Europe and North America, East Asia, subtropical and tropical Asia Pacific by various mycologists. However, only a few numbers of species were listed in Malaysia.

There are many tiny holes or pores on the underside of mushroom's cap. The mushrooms are among the most common, widespread and easily identifiable groups of wild mushrooms. They are a morphological group of basidiomycetes characterised by basidiocarps (fruit bodies) with hymenophore and basidiopores. These types of mushrooms mostly grow on logs, branches and other woody substrata or pathogens on living trees. Polypore mushrooms are sometimes called bracket fungi because of the shelf-like shapes and their woody fruiting bodies are called conks.

Polypore mushrooms are commonly used in traditional medicine and have a great potential as source for the biologically active compounds (Zjawiony, 2004). Due to health risk and toxicity, antioxidant compounds from various mushrooms have been broadly used in natural products to replace synthetic antioxidant (Stone *et al*., 2003). Antioxidant potentials of medicinal polypore mushrooms such as *T. versicolor* (Wasser, 2002), *G. lucidum* and *G. tsugae* (Mau, Lin, & Chen, 2002) have been studied and proven to have good antioxidant properties.

Ikekawa *et al.* (1969) published the first scientific report on anti-tumour activities obtained from fruiting bodies of polypore mushrooms (Aphyllophoromycetideae) and a few other families. *Fomitopsis betulina* or also known as brown rot macrofungus is one of the polypore mushrooms that has been widely investigated for its pharmacological studies such anti-cancer, anti-microbial and anti-inflammatory agent (Pleszczyńska *et al.*, 2017).

Biologically active higher Basidiomycetes mushroom compounds such as polysaccharides and peptidoglycan continue to be the subject of most research. However, mushroom also produce a large number of proteins and peptides with interesting biological activities. Bioactive proteins constitute another important part of functional components in mushrooms that also have rising interests due to their pharmaceutical potential (Wong *et al*., 2010).

2.4.1 *Trametes* spp.

Trametes is one of the most familiar genus in the family Polyporaceae that is distinguished by a pileate basidiocarp, di-trimitic hyphal system and generative hyphae with clamps (Ryvarden, 1991). To date, there are about 846 *Trametes* species are recorded on Index Fungorum. Although this genus is very familiar, its species-level taxonomy is unresolved. According to Carlson, Justo and Hibbett (2014), the Internal Transcribed Spacer (ITS) region is frequently used molecular marker for species

delimitation in fungi, but it has been shown to have a low molecular variation in *Trametes* resulting in poorly resolved phylogenies and unclear species boundaries, especially in the *T. versicolor* species complex (*T. versicolor* sensu stricto, *T. ochracea*, *T. pubescens*, *T. ectypa*). *Trametes* spp. such as *T. feei, T. menziezii* and *T. pocas* were commonly found in the Peninsular Malaysia while *T. hirsuta* and *T. lactinea* were rarely found (Mohd Nor Rashid, 2010). *T. scopulosa* were reported to have been found in Batu Caves, Selangor by Corner in 1991.

Trametes species are always known for their medicinal properties especially *T. versicolor* or also known as Turkey tail mushroom (Hobbs, 2004). The Chinese and Japanese have used this mushroom for a very long time. Polysaccharide-K (PSK) and Polysaccharopeptide (PSP) are an example of cancer drugs derived from *T. versicolor* which have invaluable benefits. PSK and PSP have similar physiological activities but are different in structure. They are produced from CM-101 and COV-1 strains of *T. versicolor*, respectively (Cui & Chisti, 2003). They have been used as a chemoimmunotherapy agent to treat cancer in Asia for over 30 years. In spite of this, PSK has been studied longer than PSP and therefore has undergone more thorough laboratory, animal and clinical testing. Although the mechanism of their anti-tumour effect is still uncertain, these polysaccharides and polysaccharide-protein complexes are suggested to enhance cell-mediated immune responses *in vivo* and *in vitro* and act as biological response modifiers (Ooi & Liu, 2000). Some clinical trials have showed that PSK has great potential as an adjuvant cancer therapy for gastric, esophageal, colorectal, breast and lung cancers. These studies have implied the efficacy of PSK as an immunotherapy or biological response modifier (BRM). BRMs conceivably have the ability to enhance the "host versus tumour response" thus increasing the ability of the host to defend itself from tumour progression (Fisher & Yang, 2001). The Health and Welfare Ministry of Japan also has approved the drugs in the 1980s. Besides that, a

laccase from *Trametes trogii* or also known as *Funalia trogii* was reported to have anti-cancer activity when tested on several cancer cell lines such as breast (MCF-7 and MDA-MB 231), colon (HT 29), prostate (PC3 and LNC) cancer cell lines (Rashid e*t al*., 2011; Guest & Rashid, 2016).

Lee *et al*. (2009) published a report on anti-tumour effect of *Inonotus obliquus* mushroom. The water extract of *I. obliquus* was proved to have cytotoxic effect against HT-29 human colon cancer cells. The result also suggests that the mushroom as an anti-tumour agent via the induction of apoptosis and inhibition of the growth of cancer cells. Protein-bound polysaccharides from oriental medicinal mushroom, *Phellinus linteus* was reported to have the ability to inhibit the proliferation and colony formation of SW480 human colon cancer cells. The flow cytometry analysis of the extracts showed that the polysaccharides induced G2/M phase arrest and apoptosis. Studies to date have identified a number of mushroom extracts with anti-tumour effects against various cancer cells. However, only several researches concerning the effect of *Trametes* species on colon cancer cells have been published. Thus, this study focuses more on the cytotoxic effect of *Trametes* sp. against colon cancer cells.

2.4.2 *Trametes scopulosa* (Berk.) Bres.

Trametes scopulosa (Family: Polyporaceae) is a group of Basidiomycota. *T. scopulosa* is a non-edible wild polypore mushroom because of its coarse texture and bitter taste. The pileus of *T. scopulosa* is around 8 cm in radius, 12.5 cm wide, semicircular, horizontal and often imbricates. It is neither tuberculate nor padded at the base, faintly sulcate, pallid tan white or dingy buff, becoming dingy ochraceous tan to fawn brown and rather darker at the base. The spore of this species is 6-8 x 2.5-3 μm, white, smooth, subcylindric, thin-walled, aguttate and inamyloid (Bresadola, 1912). Based on Index Fungorum, the current name is *Amauroderma scopulosum* (Berk.)

(Imazeki, 1952). Figures 2.3 shows (A) a mature *T. scopulosa* fruiting body cultured on sawdust substrate and (B) cultivated fruiting body of *T. scopulosa*.

Trametes sp. and *Ganoderma* sp. are usually known only for their medicinal properties as compared to other edible mushroom such as *Lentinula*, *Flammulina* and *Pleurotus* (Smith, Rowan, & Sullivan, 2002). According to the American Cancer Society, available scientific evidence does not support claims that the raw mushroom itself is an effective anti-cancer agent in humans. But there is some scientific evidence that substances derived from parts of the mushroom may be useful against cancer. To date, there is no pharmacological report regarding *T. scopulosa*. However, *T. versicolor* is one of the species in the genus *Trametes* proven to possess anti-cancer properties (Wasser & Weis, 1999).

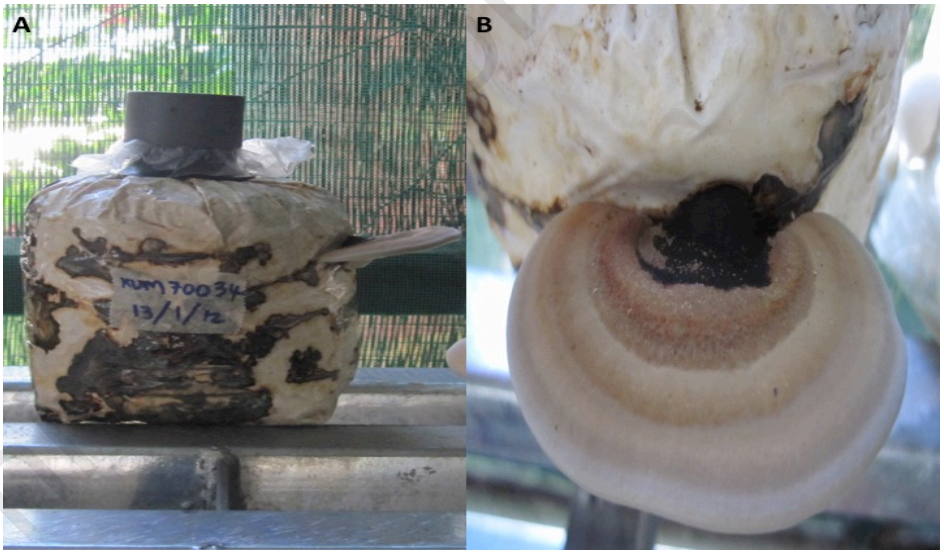

Figure 2.3: *Trametes scopulosa*. **A)** A mature fruiting body cultured on sawdust substrate. **B)** Cultivated fruiting body of *T. scopulosa.*

CHAPTER 3
MATERIALS AND METHODS

3.1 Fungal materials

Trametes scopulosa (KUM70034) was analyzed for cytotoxic and anti-migration activities. Mycelium of *T. scopulosa* was obtained from the Mycology Laboratory, Faculty of Science, University of Malaya previously verified by Mohamad Hasnul Bolhassan (2012).

3.1.1 Cultivation of *T. scopulosa* mycelium

Mycelium of *T. scopulosa* was cultured and maintained on Malt Extract Agar (MEA) plate (Appendix A (1.0), page 100) under sterile condition. Mycelial plugs were taken from the colony by using a loop and was placed on the center of MEA plate and incubated for 7 days at 25 ± 2^{o}C. The plate was maintained and sub-cultured routinely. After 7 days, ten plugs were cut from periphery of mycelial colony and transferred into 100 mL Glucose-Yeast-Malt-Peptone (GYMP) sterile media (Appendix A (2.0), page 100) in 250 mL conical flasks. The flasks were incubated at room temperature for 14 days under static conditions to produce mycelial biomass. The mycelial biomass was then freeze-dried and the product was stored at 4 ± 2^{o}C.

3.1.2 Preparation of crude extracts from *T. scopulosa* mycelial

Harvested mycelium was extracted with organic solvents such as methanol, dichloromethane (DCM), ethyl acetate and water fraction to produce crude organic solvent extracts. One gram of the freeze-dried mycelial biomass was ground into powder and soaked with a mixture of methanol and DCM (2:1) at a ratio of 1:20 (w/v) at room temperature. This step was repeated for 3 times and the solvent was evaporated using rotary evaporator to produce crude extract. The crude solvent extract was

dissolved in 90% aqueous methanol (and partitioned with 3 x 100 mL hexane. The extract was rotary evaporated to produce hexane fraction and the residual aqueous methanol was evaporated under pressure to produce a semisolid mass. This semisolid mass was then dissolved in 200 mL of distilled water. Next, the solution was further partitioned with 3 x 100 mL of DCM and 3 x 100 mL ethyl acetate. Finally, the extracts were evaporated to produce DCM, ethyl acetate and water fractions as shown in the flow chart in Figure 3.1. The flow chart of the experiment is shown in Figure 3.2.

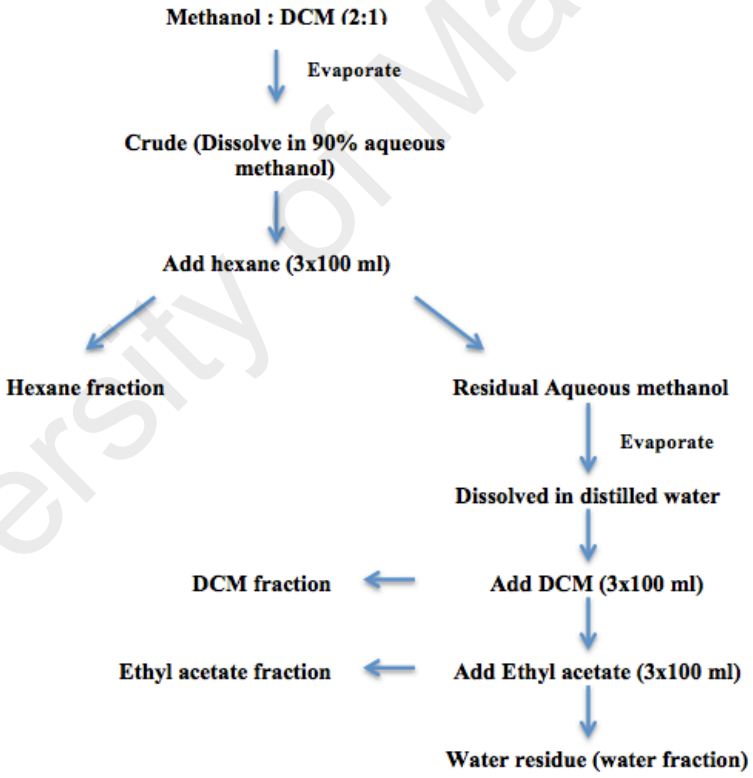

Figure 3.1: The flow chart of solvent extraction of *T. scopulosa* mycelium extract.

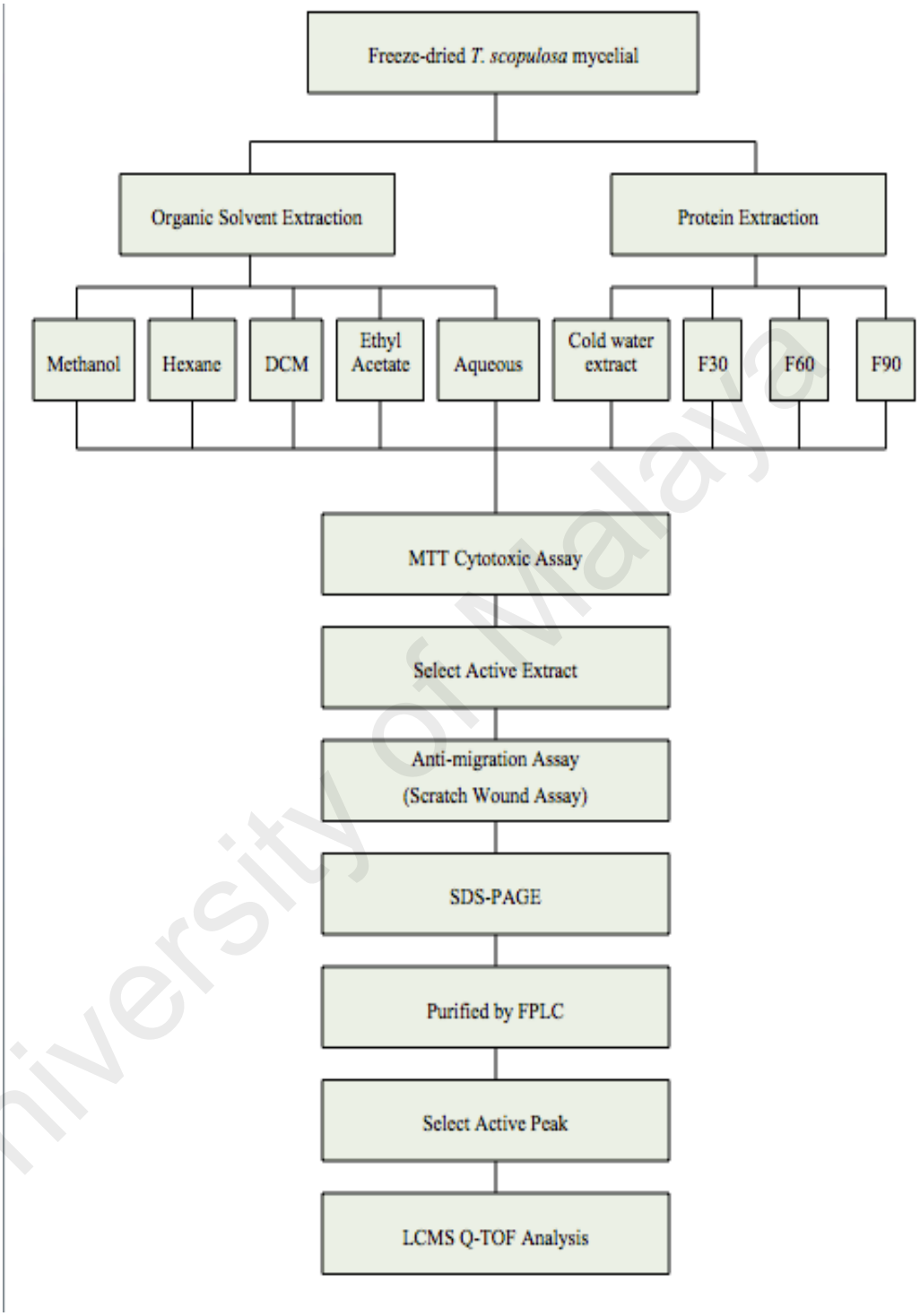

Figure 3.2: The schematic diagram of *T. scopulosa* mycelium extraction procedures, cytotoxic and anti-migration assays, SDS-PAGE, FPLC and LCMS Q-TOF analysis.

3.1.3 Extraction of Protein

Extraction of proteins was carried out by ammonium sulphate precipitation at 30%, 60% and 90% ammonium sulphate concentration. Proteins were precipitated from aqueous solutions by slowly dissolving ammonium sulphate into the protein solution. The mycelial aqueous solution (cold water extract) was also used to analyse for protein content estimation as shown in section 3.4. The ammonium sulphate precipitation was conventionally carried out at 0°C - 4°C to avoid possible denaturation of proteins. Protein solution was prepared by soaking dried mycelial biomass in distilled water at a ratio 1:20 and was placed on magnetic stirrer overnight in a 4°C incubator. The mixture was filtered using vacuum filter and then centrifuged at 10,000 rpm for 10 to 15 minutes at 4 ± 2°C to remove impurities.

Ammonium sulphate was added onto the filtrate and percentage saturation values were based on the table described by Dixon, 1952 (Appendix A (3.0), page 101). The ammonium sulphate was poured gradually into the extract solutions and was constantly stirred for 30 minutes on ice bath. The solutions were centrifuged at 10,000 rpm for 15 minutes at 4 ± 2°C. The supernatant was then used for the subsequent salt saturation and the precipitated proteins yield was dissolved in 4 mL distilled water. The precipitated protein was dialysed using SnakeSkin pleated dialysis tubing with 3,500 Da molecular weight cut off (Thermo Fisher Scientific, USA) for 48 hours at 4 ± 2°C. Finally, the dialysed proteins were freeze dried and stored at -20°C prior to use. The proteins obtained were categorized according to the concentration of the salt saturation at which they were formed, and the fractions were labeled as F30, F60, and F90.

3.2 Cytotoxicity Assay (*In vitro* MTT Assay)

The cytotoxic activities of the crude and protein extracts of *T. scopulosa* mycelial were evaluated using MTT assay. A total weight of 0.04 g of crude and protein

extract were dissolved in 200 µL of sterile distilled water and was kept in the -20^{o}C freezer. HCT 116 colon cancer cell line and MRC-5 lung normal cell line (ATCC) were used in this present study. Mc Coy's 5A (modified) medium and Eagle's Minimum Essential Medium (EMEM) from Sigma Aldrich were used to culture HCT 116 and MRC-5 cells, respectively. The maintenance and sub-cultivation steps of these cell lines were shown in Appendix A (4.0), page 101.

The cytotoxic effect of *T. scopulosa* was determined by *in vitro* MTT cell viability assay. The MTT assay was conducted based on the method developed by Mosmann, 1983 with some modifications. This assay is based on the conversion of MTT to MTT-formazan by mithochondrial enzymes. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. Cytotoxicity is expressed based on the concentration of the purple formazan. Therefore, it is possible to distinguish between viable and dead cells via spectrophotometric measurements.

3.2.1 Seeding of cells in microtitre plates

Once fully confluent tissue culture flask was used in this assay. The cells detachment protocol was performed as described in Appendix A (4.0), page 101. After centrifugation, the supernatant was discarded. 1 mL of 10% supplemented medium was added into the pellet and mixed well to get a stock cell suspension. For cell enumeration, 10 µL of cells suspension and 900 µL of tryphan blue were added into a micro-centrifuge tube and mixed well. Hematocytometer covered with a glass cover slip was prepared. The mixture (20 µL) was pulled into the hematocytometer chamber by capillary action. The amount of colourless cells in each chamber was counted through the microscope.

The total number of cells was recorded and divided the number by four (Figure 3.3) to get average (N) and concentration of stock cell suspension needed to prepare a

cell suspension 80,000 cells/mL (HCT 116) and 100,000 cells/mL (MRC 5) was calculated. The calculation is as follows.

Equation 1:

$$M_1 V_1 = M_2 V_2$$

Whereby

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 (V_2) was added into a beaker and cell suspension from centrifuge tube (V_1) was added and shaken well. The mixture was transferred into reagent reservoir. The cell suspension (200 µL) was transferred into each reaction well (96 wells plate) of plate and incubated (24 hours, 37^{o}C, 5% CO_2, humidified air).

1		2
3		4

Figure 3.3: Chambers of hematocytometer.

3.2.2 Treatment of HCT 116 and MRC-5 cells with extracts

After 24 hours of incubation, the HCT 116 and MRC-5 cells were observed for growth and attachment at the bottom of 96 wells plate. A total number of 10µL of stock was diluted with 90 µL of 10% media. The stock extracts were diluted serially (100, 50.0, 25.0, 12.5, 6.25, 3.13, and 1.56 µg/mL). An empty microtitre plate (mock plate) was used for serial dilution. A total volume of 150 µL of 10% media was added into wells of row B to G. Substock (300 µL) was added into wells of row A. The substock in row A (150 µL) was aspirated and put into B. The substock in row B (150 µL) was then aspirated and placed into C. This serial dilution was continued until well G, whereby after mixing, half the content in well G (150 µL) was discarded. Every well (A to G) should have solutions of same volume (150 µL). Seeded plate from incubator was taken out and the old media was decanted. 10% supplemented media (100 µL) into each well with cells. The contents in the A to G well (100 µL) of mock plate was transferred to seeded plate in exact replica. 200 µL of 10% media without any extracts was added into wells H (control) of seeded plate. All the treatments were carried out in triplicates and incubated for 72 hours at 37^{o}C in 5% CO_2.

3.2.3 Measurement of cell viability

After 72 hours of incubation, 20 µL of MTT reagent was added into the 96 wells plate. The cells were viewed under the microscope to detect any contaminations. Contaminated plate (if any) was immediately discarded. The cells were incubated at 37^{o}C in a 5% CO_2 incubator for 2 to 4 hours to allow the maximum uptake of the dye. The plate was viewed periodically with the inverted microscope for the presence of intracellular punctate purple precipitate after 4 hours of incubation. When the purple precipitates were clearly visible under the microscope, 100 µL of detergent reagent (DMSO) was added to all wells including controls. The plates were then incubated for

30 minutes at room temperature in a microtitre plate shaker. Optical density (OD) reading was taken at 570 nm with reference wavelength at 690 nm using a microplate ELISA spectrophotometer.

3.2.4 Calculations of cytotoxic activity of solvent and protein extracts of *T. scopulosa*.

The optical density of the samples and controls were used to calculate the killing percentage or the percentage of inhibition (POI) of the cells using the formula given below.

Equation 2:

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

*OD = optical density of the cells

The POI obtained was plotted into dose-response curve. The IC_{50} values were determined by plotting a logarithmic best curve and extrapolated at percentage of inhibition of 50%. The IC_{50} values refer to the effective dose (µg/mL) required to inhibit the growth of the cell by 50%.

3.3 Anti-migration Assay (*In vitro* Scratch Wound Assay)

The partially purified protein (F60) of *T. scopulosa* was evaluated for its ability to inhibit the migration of colon cancer cell lines (HCT 116) using *in vitro* scratch wound assay. This assay was conducted based on the method by Liang, Park and Guan (2007) with some modifications. This assay is a straightforward, low-cost and well-developed method to study cell migration *in vitro*.

3.3.1. Cells plating (seeding of cells in 6 wells plates)

The procedure of cell plating and the calculation of the number of cells for seeding were described in section 3.2.1. However, in this study, $1x10^6$ cells per well were seeded into 6 wells plate. The cells were incubated at 37^oC in a 5% CO_2 incubator for 24 hours.

3.3.2. Treatment of HCT 116 with extracts

In this study, anti-migration activities of the extracts were examined at concentrations based on the IC_{50} and IC_{25} values. One percent of PAA Foetal Bovine Serum (FBS) supplemented media was prepared. The extract was prepared using calculation as follows.

Equation 3:

$$M_1 V_1 = M_2 V_2$$

Whereby

M_1 = Value of protein content of the extract or fraction

V_1 = Volume of extract or fraction

M_2 = IC_{50} or IC_{25} value

V_2 = Volume of 1% FBS supplemented media (n wells x 4 mL media)

The cell-seeded plate was taken out from the incubator after 24 hours incubation. A wound along the diameter of each cell monolayer was scratched using a sterile yellow pipette. The old media was discarded and the cells were washed with 3 mL of PBS. Two milliliter of 1% supplemented media and 2 mL of media containing extract was added into each reaction wells. Meanwhile, 4 mL of 1% supplemented media (without extract) was added into control wells. The photographs of the wound at 0 hour were captured using inverted microscope. The gap distance (μm) was recorded at

3 different points for each well. The treated seeded plate was incubated for 48 hours at 37^{o}C in 5% CO_2.

3.3.3 Measurement of Cell Migration Capability

Photographs of the wound closure were captured immediately after the treatment (0 hour), at 24 hours and 48 hours of incubation under the inverted microscope at 50x magnification. Three fields of each wound were captured and the mean values of gap distance (μm) were analyzed for the selected fields.

The absolute migration capability (MC_A) (Peng *et al*., 2007) was calculated using the equation

Equation 4:

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

Where, G_0 was the initial gap for the cell line at 0 h (μm)

G_t was the final gap for the cell line treated with extract at certain concentration or vehicle alone for a time period of t (h) (μm)

t was the overall time period for incubation (h)

3.4 Protein content estimation by BCA protein assay

BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for determining the total concentration of protein in a solution. This assay is ideal for measuring detergent-solubilized membrane proteins and can be used to measure protein on solid surfaces such as affinity supports, plastics microplate and membranes. In this study, Pierce BCA Protein assay kit (#23225 #23227) from Thermo Scientific was used to measure the protein content of the *T. scopulosa* protein fractions.

3.4.1 Microplate procedure

Twenty-five microlitre of each standard or sample replicate was pipetted into microplate well (working range = 20-2000 µg/mL). 200 µL of the WR (Appendix A (5.0), page 103) was added to each well and the plate was mixed thoroughly on the plate shaker for 30 seconds. The plate was covered with lid and incubated at 37^{o}C for 30 minutes. Next, the plate was cooled at room temperature. The absorbance at or near 562 nm was measured on a plate reader. Protein content was determined by comparing the absorbance value of the samples with the standard curve graph of bovine serum albumin (BSA) (Appendix B (Figure 1), page 114).

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

SDS-PAGE (Bio-Rad) was used to pool the protein based on molecular weight of partially purified protein extracts. The SDS-PAGE gel in a single electrophoresis run can be divided into stacking gel and separating gel. Stacking gel (acrylamide 5%) is poured on top of the separating gel (after solidification) and a gel comb is inserted in the stacking gel. The acrylamide percentage in SDS-PAGE gel depends on the size of the target protein in the sample.

3.5.1 Gel casting

A comb was completely placed into an assembled gel cassette. The glass plate was marked 1 cm below the comb teeth. This was the level to which the separating gel is poured. The comb was removed. The separating gel was prepared (Appendix A (6.0), page 104) by combining all solutions. The separating solution was poured slowly to the mark using disposable plastic pipette. Slowly and evenly the separating solution was overlaid with water. The gel was allowed to polymerize for 45 minutes to 1 hour. Next, the stacking gel was prepared according to Table 3.2. The top of separating gel was

dried using filter paper and the stacking gel was poured onto the separating gel until the top of the short plate is reached. The desired comb was inserted between the spacers starting at the top of the spacer plate. The stacking gel was allowed to polymerize for 30-45 minutes. The comb was removed gently and the wells were rinsed thoroughly with distilled water.

3.5.2 Electrophoresis

Sample buffer and protein extracts were mixed at a ratio of 1:3 (v/v). The samples (15 µL) and broad range SDS-PAGE standard marker (10 µL) from Bio-Rad were then loaded into the wells. Electrophoresis was conducted at a constant current 100V for separating gel (16% polyacrylamide) for 1 hour. After electrophoresis, the gel was fixed with fixing solution consisting of 40% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes and the protein bands were then stained by silver staining (Pierce™ Silver Stain Kit by Thermo Fisher).

3.6 Fast protein liquid chromatography (FPLC)

Purification of the protein fractions with active cytotoxic activity was carried out using FPLC system (AKTA Avant 25). It is a preparative chromatography system designed for fast and secure development of scalable methods and processes. AKTA Avant 25 is specialized for media screening and method optimization using small columns. The column used in the study was HiTrap 1 mL Capto Q (strong anion exchanger) column. The FPLC protocol was optimized several times before the final protocol was achieved. Pump wash was performed for the necessary pumps and column with water followed by buffer (Appendix).

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 fractions separation (Appendix A (7.0), page 106). The protein fractions were filtered through 0.22 µm syringe filters

(Sartorius) before injected into the column. Three milliliter of 1 mg/mL F60 protein sample was injected into the injector valve ports and allowed to run for 70 minutes. The elution of 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. Fraction peaks were detected at 280 nm wavelength. Every single peak shown by the chromatogram was automatically collected by the machine and labeled. The collected fractions were then neutralized with 1 M sodium hydroxide (1:5) and dialysed using SnakeSkin pleated dialysis tubing with 3,500 Da molecular weight cut off (Thermo Fisher Scientific, Rockford, IL, USA) for 48 hours at 4 ± 2^{o}C. The fractions were then freeze dried and stored at -20^{o}C.

3.7 Protein Identification by LCMS-QTOF

The active cytotoxic fractions from FPLC were further subjected by LCMS-QTOF analysis. Before the analysis, the protein samples underwent digestion and desalting procedures as described in 3.7.1 and 3.7.2.

3.7.1 In-solution tryptic digestion for LC separation

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

In the reduction and alkylation steps, digestion buffer (ammonium bicarbonate) and reducing buffer (DTT) was prepared (Appendix A (8.0), page 107). 15 mL of 50 mM ammonium bicarbonate and 1.5 μL of 100 mM DTT were mixed in microcentrifuge tube. Ten microliter of protein solution (sample) was added into the tube and the final volume was adjusted to 27 μL with ddH_2O. The solution was incubated at 95^{o}C for 5 minutes 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 A (8.4), page 107) and 1 μL of trypsin was added into the reaction tube. The mixture was incubated at 37^{o}C for 3 hours. After 3 hours, an additional 1μL trypsin was added into the tube and incubation was carried out at 30^{o}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 the completely dried using speed vacuum at low speed for 2 hours. The samples were furthered to zip tip-desalting protocols.

3.7.2 Zip tip protocol

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 A (9.0), page 108). The protein samples in digestion protocol was reconstituted in 10 μL of 0.1% TFA. 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 of solution 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 for 3 times.

Equilibration: 10 μL of equilibration solution (0.1% TFA) was aspirated into zip-tip and dispensed. The steps were repeated for 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% TFA) was aspirated into zip tip and dispensed. This step was repeated for 3 times.

Elution: 1-4 μL elution solution (0.1% TFA in 50% acetonitrile) was aspirated into zip tip 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 1000 rpm until the solution was completely dried.

3.7.3 LCMS Q-TOF Analysis

Liquid chromatography mass spectrometry was used to separate and identify protein compounds from the protein fraction of *T. scopulosa*. The analysis was performed using Agilent 6530 Q-TOF LC/MS equipped with autosampler. 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 ionization source for MS analysis. There are 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 150 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 volume of sample was 2 μL.

The samples were 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 oxidized 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 acquisition software (Agilent, Santa Clara, CA, USA) with an acquisition rate of 8 spectra per second from 200 to 3000 m/z and were followed by collision-induced dissociation of the twenty most intensive ions. MS/MS data were obtained in the range of 50-3200 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), updated on 22/7/2015 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.

3.8 Statistical Analysis

Statistical analysis was performed using SPSS V. Results are presented as mean ± standard deviation (mean ± SD). One-way ANOVA was used to determine the significant difference in multiple comparisons.

CHAPTER 4

RESULTS

4.1 Yield of *T. scopulosa* mycelial biomass and extracts

The cultivation of *T. scopulosa* mycelium was performed on the MEA plate at 25^{o}C. The mycelium took around 7 days to completely colonize the surface of the agar plate. The structure of mycelium was whitish, cottony, and grew longitudinally radial on the MEA agar (Figure 4.1). After the cultivation on agar, the mycelia were transferred into GYMP media for submerged fermentation under static condition. Ten mycelial plugs were punctured from the periphery of the culture using the sterile cork borer and inoculated into conical flask containing 100 mL of GYMP media. The flask was kept in the incubator at 25^{o}C for 14 days. Based on the observation, the mycelium grew and dispersed according to the shape of conical flask. The mycelial yield and the whole broth were freeze dried, then stored at 4^{o}C prior to use. The yield of dried mycelial biomass of *T. scopulosa* obtained from the submerged cultivation was 5.296 ± 0.142 g/L.

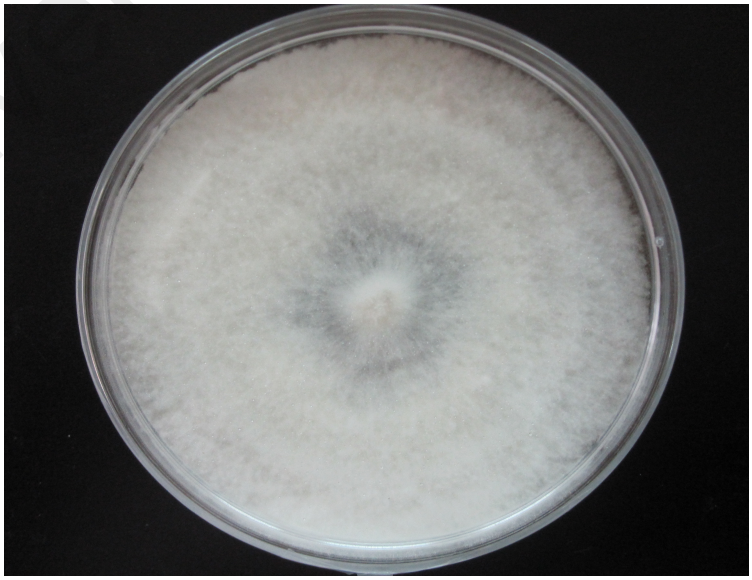

Figure 4.1: *Trametes scopulosa* mycelia on MEA plate

4.1.1 Percentage yield of crude and protein extracts.

The percentage yield of crude and protein extracts obtained from the extraction of *T. scopulosa* mycelial was presented in Table 4.1. The freeze-dried mycelial was extracted with organic solvents such as methanol, dichloromethane (DCM), ethyl acetate and water fraction to produce crude organic solvent extracts. Meanwhile, extraction of proteins from *T. scopulosa* mycelial was carried out by ammonium sulphate precipitation.

Table 4.1: The percentage yield of crude and protein extracts of *T. scopulosa* mycelial. Results were expressed as mean ± SD of triplicates values (n=3).

Extracts		Percentage of yield (%)
Crude	Methanol	19.01 ± 0.5
	Hexane	14.5 ± 0.25
	Ethyl acetate	12.5 ± 0.32
	DCM	9.52 ± 0.20
	Aqueous	21.15 ± 0.14
Protein	Cold water	18.5 ± 0.05
	F30	14.58 ± 0.17
	F60	11.0 ± 0.25
	F90	7.0 ± 0.15

4.2 Pre-screening of solvent and protein extracts for cytotoxicity against HCT 116

The mycelial crude (methanol, hexane, DCM, ethyl acetate and aqueous) and protein extracts (cold water extract, F30, F60, F90) of *T. scopulosa* were evaluated for cytotoxic activities using *in vitro* MTT assay. The crude extracts and protein extracts were prescreened for cytotoxic activity against colon cancer HCT 116 cell line at 50.0 µg/mL as presented in Figure 4.2.

As seen in Figure 4.2, crude extracts of *T. scopulosa* mycelium exerted inhibition of 18.457 ± 0.032% (methanol), 12.366 ± 0.35% (hexane), 14.464 ± 0.341% (DCM), 6.864 ± 0.102% (ethyl acetate) and 19.048 ± 0.438% (aqueous) against HCT 116 cell line at 50.0 µg/mL. Meanwhile, crude protein extract and all protein fractions exerted higher inhibition of more than 80%, which are higher than inhibition exerted by solvent extracts. Pre-screening for cytotoxic activity against colon cancer cells (HCT 116) showed that all solvent extracts of *T. scopulosa* showed less inhibitory effects against HCT 116 cells with percentage of inhibiton lower than 20% even at 50 µg/mL. However, all protein fractions obtained by ammonium sulphate precipitation (F30, F60 and F90) showed good inhibitory effects against HCT 116 cells with percentage of inhibition were higher than 50%. Thus, protein extracts of *T. scopulosa* were selected for further research.

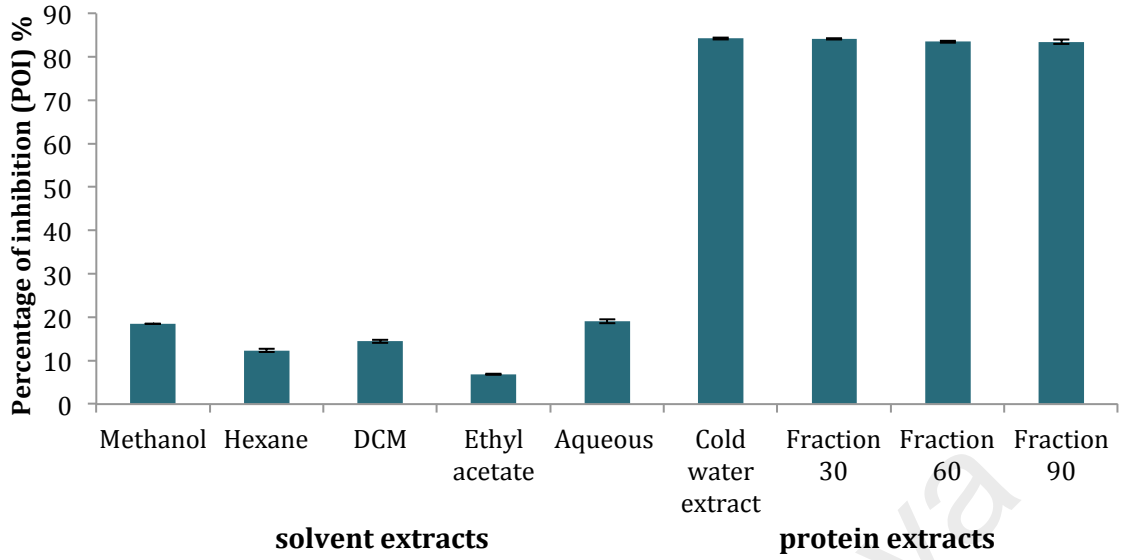

Figure 4.2: Percentage inhibitions of HCT 116 cell lines treated with solvent and protein extracts of *T. scopulosa* at 50.0 µg/mL in the *in vitro* MTT assay. Results were expressed as mean ± SD of triplicates values (n=3). The cells were incubated for 72 hours.

4.3 Protein Quantitation

In this experiment, protein content was estimated using Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit. Absorbance of the samples was measured with microplate reader at 562 nm. Protein content was determined by comparing the absorbance value of the samples with the standard curve of bovine serum albumin, BSA (Appendix B, page 114).

Determination of protein content in all fractions revealed that yield of proteins was highest in F60 containing 459.745 ± 17.55 µg/mL as compared to cold water extract (403.334 ± 16.72 µg/mL), F30 (86.154 ± 33.99 µg/mL) and F90 (173.333 ± 5.56 µg/mL) (Figure 4.3).

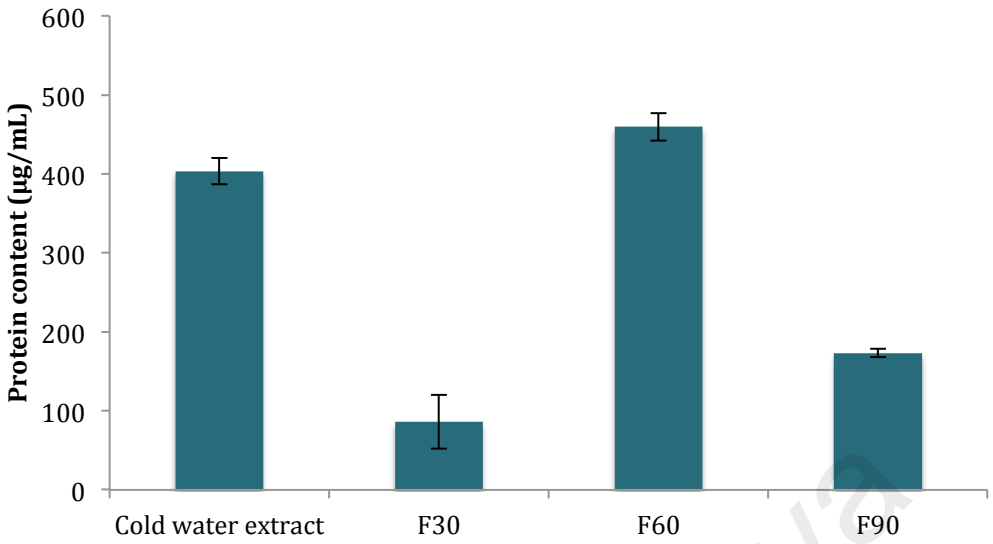

Figure 4.3: Protein concentration (µg/mL) of the *T. scopulosa* protein fractions obtained through ammonium sulfate precipitation method. Results were expressed as mean ± standard deviation of triplicates values (n=3).

4.4 Cytotoxicity of protein fractions against HCT 116 and MRC-5 cells

The percentage of inhibition of HCT 116 and MRC-5 cells by protein extracts of *T. scopulosa* at different concentrations of 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100 µg/mL were presented in Figures 4.4 and 4.5. Figure 4.4 illustrates that the inhibition activities of all protein extracts against HCT 116 cells increased with increasing concentrations of the protein extracts. Protein extracts showed good inhibitory effects against HCT 116 cells even at lower concentrations. F60 exerted inhibition of more than 80% even at lowest concentration of protein extract.

The IC_{50} value denotes the concentration of an extract (µg/mL) that exhibited 50% inhibition of cancer cells. The IC_{50} value for F30 in this experiment was 17.23 ± 0.25 µg/mL while crude extracts and F90 were 4.56 ± 0.05 and 4.47 ± 0.12 µg/mL, respectively. According to Geran *et al*. (1972), an extract with IC_{50} 20 µg/mL or less is considered active for the cytotoxicity assay. Thus, all protein fractions obtained by ammonium sulphate precipitation at 30%, 60% and 90% (F30, F60 and F90) were

actively cytotoxic against HCT 116 cancer cells line with F60 exhibiting most potent cytotoxicity with IC_{50} value at 0.84 ± 0.05 µg/mL. F60 had even a good cytotoxic activity as compared to the positive control (Cisplatin). This study indeed shows that protein extracts of *T. scopulosa* have good cytotoxic effect against colon cancer cells.

In the present study, protein extracts of *T. scopulosa* was evaluated for cytotoxicity against normal lung fibroblast cells (MRC-5) to determine whether or not these protein extracts will employ widespread cytotoxicity in the body, affecting even the normal cells. Figure 4.5 showed the inhibition activities of all protein extracts against MRC-5 cells increased with increasing concentrations of the protein extracts. The figure showed that at lower concentrations of the protein extracts (0.56, 3.13, 6.20, 12.5 and 25.0 µg/mL), the percentage of inhibitions were less than 50%. Crude extract once again exhibited the highest percentage inhibition (82.78 ± 0.614%) as compared to other protein fractions. F30 and F90 did not exhibit cytotoxic effects against the normal cells with IC_{50} values of 38.5 ± 8.5 and 70.75 ± 2.47 µg/mL, respectively. Meanwhile, F60 of *T. scopulosa* was deemed not actively cytotoxic against MRC-5 cells with IC_{50} value of 84.5 ± 4.95 µg/mL. This results show that F60 of *T. scopulosa* is cytotoxic against colon cancer cells while not harming the normal cells. F60 was therefore selected for cytotoxicity assay against HCT 116 and MRC-5 (based on protein content) to confirm whether proteins are the cytotoxic compounds responsible for cytotoxicity.

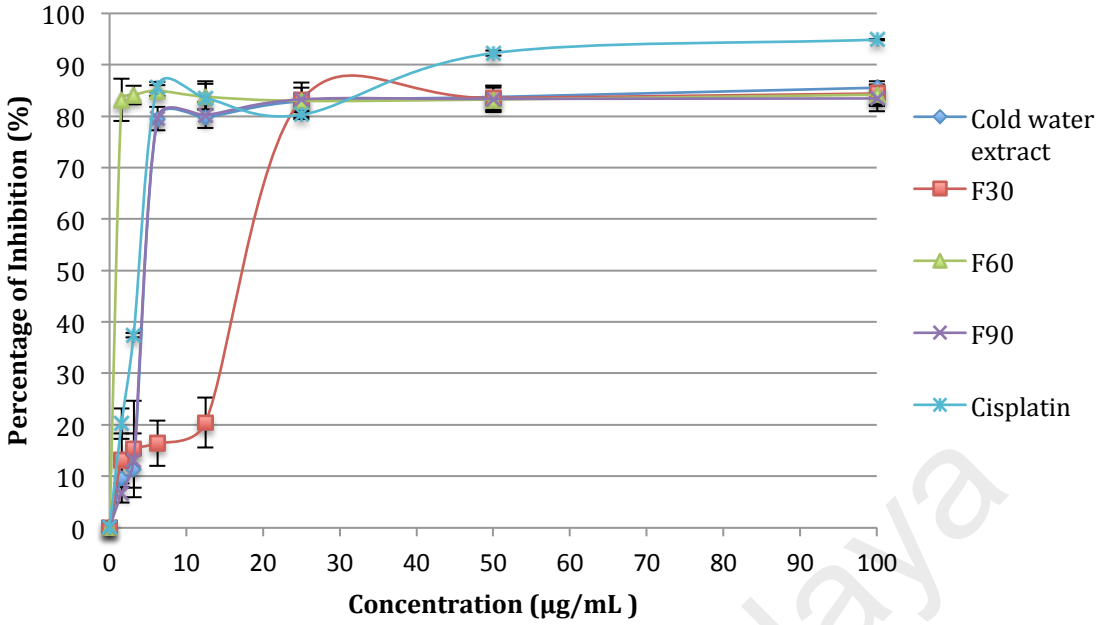

Figure 4.4: Dose-response curves for the inhibition of HCT 116 cell lines by protein extracts of *T. scopulosa* in the *in vitro* MTT assay. Results were expressed as the mean of triplicates values (n=3). Cisplatin was used as a positive control. The cells were incubated for 72 hours.

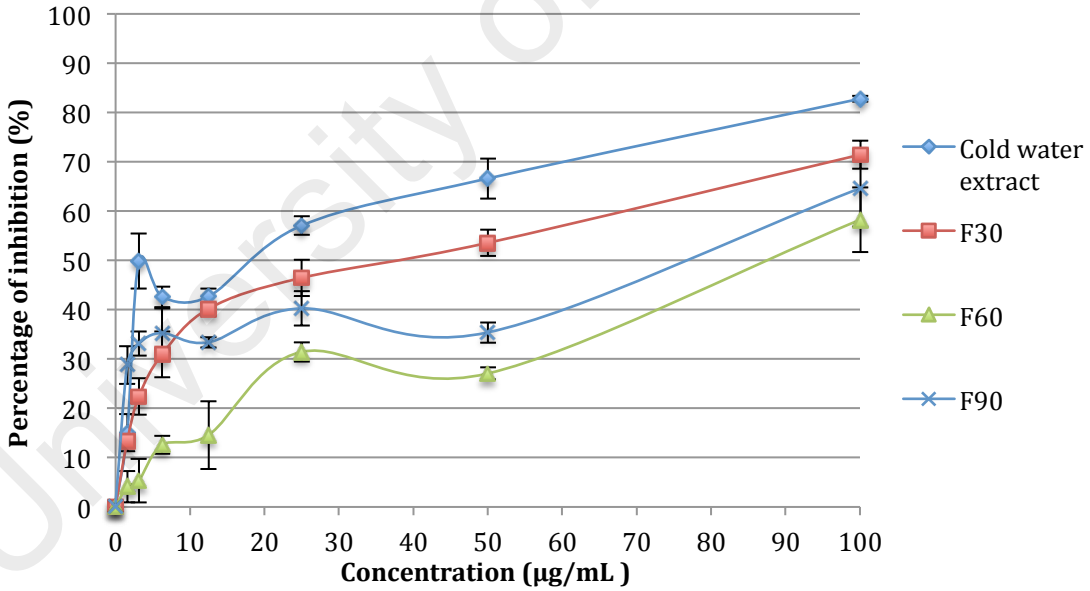

Figure 4.5: Dose-response curves for the inhibition of MRC-5 cell lines by protein extracts of *T. scopulosa* in the *in vitro* MTT assay. Results were expressed as the mean of triplicates values (n=3). The cells were incubated for 72 hours.

4.4.1 MTT assay of fraction F60 based on protein content

The percentage of inhibition of HCT 116 and MRC-5 cell lines by protein extract (F60) of *T. scopulosa* at different concentrations of protein content; 0.313, 0.625, 1.25, 2.5, 5.0, 10.0 and 20.0 µg/mL is presented in Figure 4.6. As illustrated in Figure 4.6, F60 protein fraction showed good inhibitory effect against HCT 116 cell line with percentage of inhibition 90.465±0.1 % at 20 µg/mL protein. Meanwhile, F60 extract exhibited low inhibitory effect towards MRC-5 cell line at 20 µg/mL with percentage of inhibition 49.135 ± 1.269 %.

In the present study, the percentage of inhibition of HCT 116 and MRC-5 cell lines treated with extract increased with increasing concentrations of the F60. IC_{50} value obtained suggested that F60 of *T. scopulosa* protein was actively cytotoxic against HCT 116 cells. The IC_{50} value for F60 against HCT 116 cancer cells was 0.57 ± 0.06 µg/mL while the IC_{50} value for MRC-5 cell could not be determined since the percentage of inhibition did not reach 50% at the concentrations evaluated. F60 protein fraction of *T. scopulosa* was therefore deemed not actively cytotoxic against normal human lung-fibroblast MRC-5 but actively cytotoxic against HCT 116 colon cancer cells.

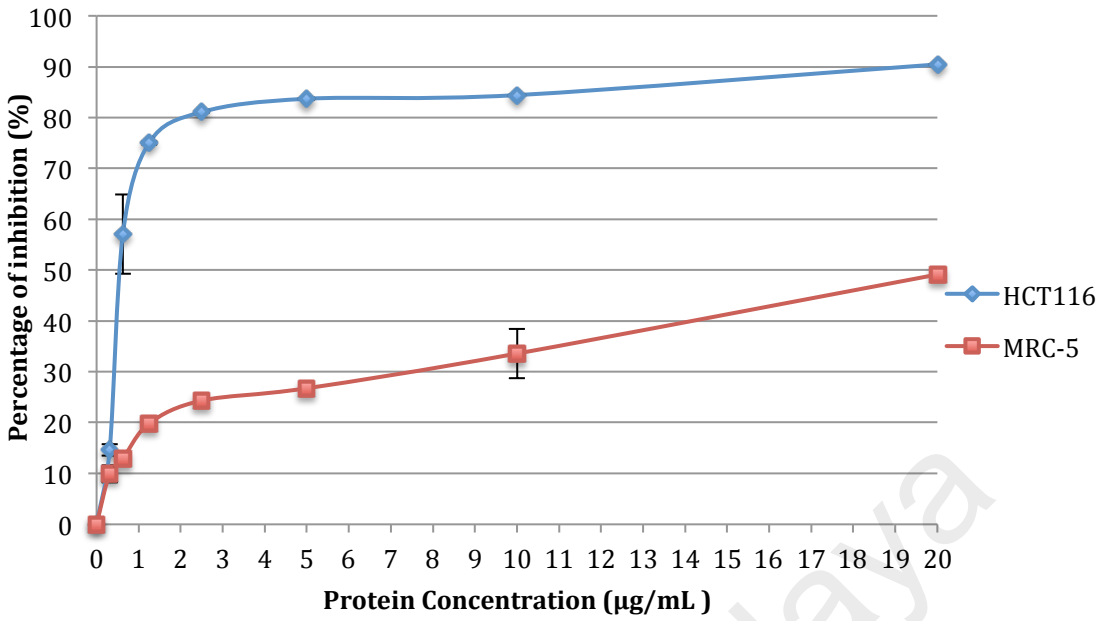

Figure 4.6: Dose-response curves for the inhibition of HCT 116 and MRC-5 cell lines by F60 based on protein content of *T. scopulosa* in the *in vitro* MTT assay. Results were expressed as mean ± standard deviations of triplicates values (n=3). The cells were incubated for 72 hours.

4.5 Scratch wound assay of fraction F60 of *T. scopulosa*

The *in vitro* scratch wound assay was used to measure inhibition of cellular migration activity by observing the artificial gap (scratch) on a confluent cell monolayer. The scratch was observed using inverted microscope at the beginning until the cells fully recolonised. The most effective anti-migration agent showed the largest gaps after treated with extracts. In this study, a scratch was made on the confluent cells and treatment was carried out with 0.39 µg/mL (IC_{25}) and 0.57 µg/mL (IC_{50}) of F60 protein extracts for 48 hours. The width of the gaps was measured at 0, 24 and 48 hours (Figure 4.7). From the gap distance of the wound, the absolute migration capability of HCT 116 cells was calculated. The data obtained was compared with the untreated cells (negative control). The migration capability of HCT 116 cells after treatment with F60 fraction was evaluated using absolute migration capability (MC_A) formula.

As shown in Figure 4.7, MC_A value for negative control (untreated cells) was 8.131±0.163 µm/h at 24 h of incubation time, which showed migration capability of

HCT 116 cells in normal condition. The MC_A for colon cancer cell lines treated with F60 at 24 hours were 6.07±0.852 µm/h for HCT 116 treated with 0.39 µg/mL (IC_{25}) while 5.517±0.225 µm/h for HCT 116 treated with 0.57 µg/mL (IC_{50}). At 48 hours, the MCA were 4.556±0.504 µm/h and 4.591±0.493 µm/h for HCT 116 treated with 0.39 µg/mL and 0.57 µg/mL respectively. Meanwhile, the MC_A value for untreated cells (control) was 5.68±0.392 µm/h. The smaller the MC_A value indicates the lower migration capability and as shown in Figure 4.8, significant anti-migration effect could be seen for both concentrations of F60 as compared to the untreated cells.

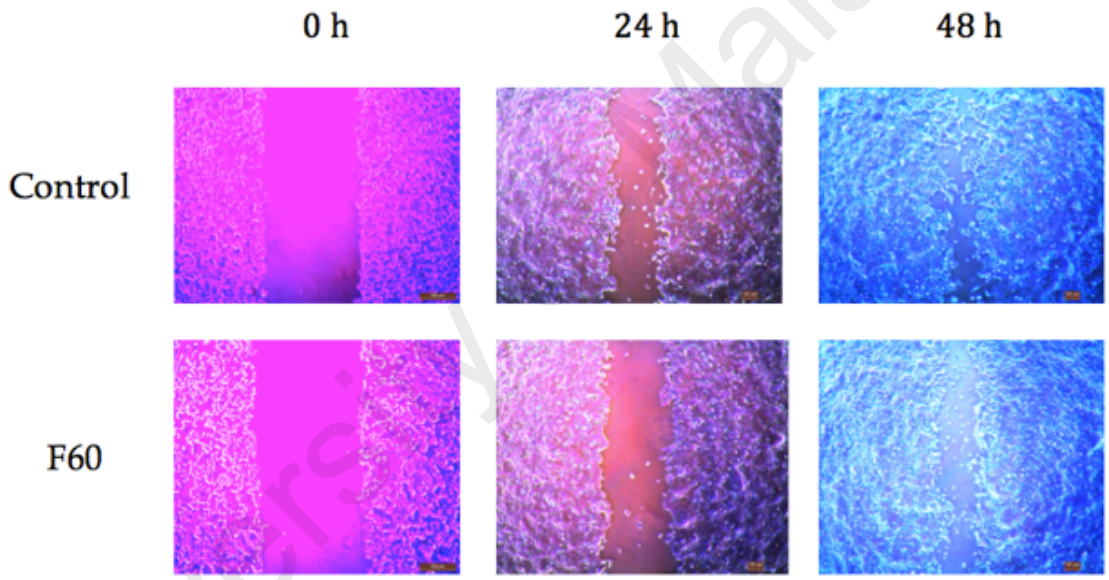

Figure 4.7: The *in vitro* scratch wound assay of F60 of *T. scopulosa* against HCT 116 cells. The migration was observed using inverted microscope (50X magnification) at 0, 24 and 48 h.

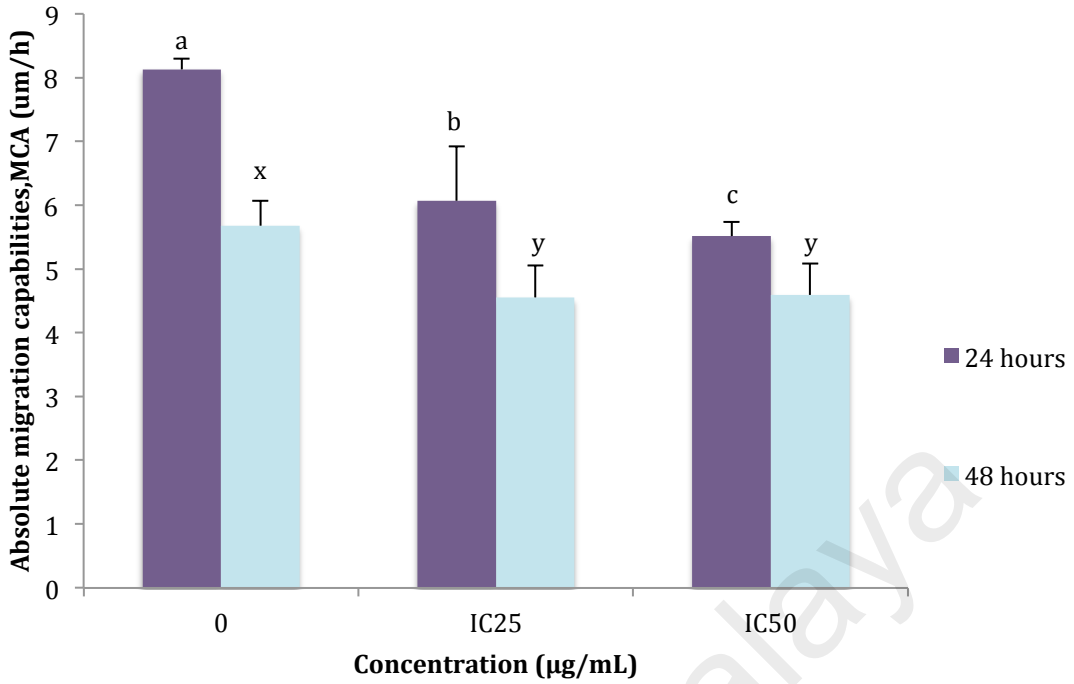

Figure 4.8: The absolute migration capability of HCT 116 cells treated with F60 of *T. scopulosa* at 2 different concentrations. Results were expressed as mean ± standard deviation of triplicates values (n=3). The letters represent significant difference in comparison to control group ($p<0.05$). The cells were incubated for 48 hours.

4.6 Protein Profile of Selected Active Fraction by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In this study, SDS-PAGE was performed to pool the F60 protein fraction showing most active cytotoxic activity. This fraction was screened to determine and separate proteins based on its molecular mass. Spectra Multicolor Broad Range Protein Ladder was used as a marker. This protein ladder was used to monitor protein migration during SDS-PAGE and determine size of the protein separated on the gel. Based on Figure 4.9, SDS-PAGE analysis on 16% gel of F60 revealed a few distinct protein bands corresponding to a molecular mass of 12 kDa, as well as 16 kDA, 21 kDa, 27 kDa, 33 kDa, 37 kDa, 57 kDa and 64 kDa. This fraction was furthered purified by using Fast protein liquid chromatography (FPLC).

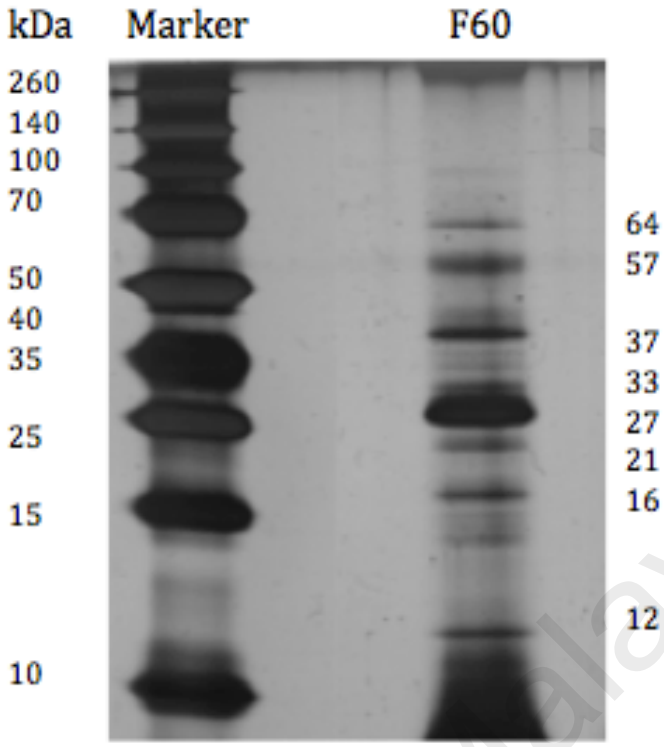

Figure 4.9: Proteomic analysis of F60 by SDS-PAGE using Spectra Multicolour Broad Range Protein Ladder as a marker.

4.7 Purification of proteins by Fast Protein Liquid Chromatography (FPLC)

Purification of the protein fraction with active cytotoxic activity was carried out using FPLC system (AKTA avant 25). F60 fraction that showed the greatest cytotoxic effect was selected to be further purified by FPLC. The column used in the current study was HiTrap 1 mL Capto Q anion exchange column. The protein fraction was eluted using elution buffer 1M (pH 8.0) for 80 minutes with the flow rate 1.0 mL/min. All the protein fractions were sub-fractioned according to the peaks obtained.

In the present study, 3 distinct peaks were collected from the chromatography (P1, P2 and P3) as shown in Figure 4.10. P1 was eluted at 60% concentration of NaCl, while P2 at 90% and P3 at 100% NaCl. All the bound proteins were subjected to MTT and scratch wound assay to determine the activity of each peak.

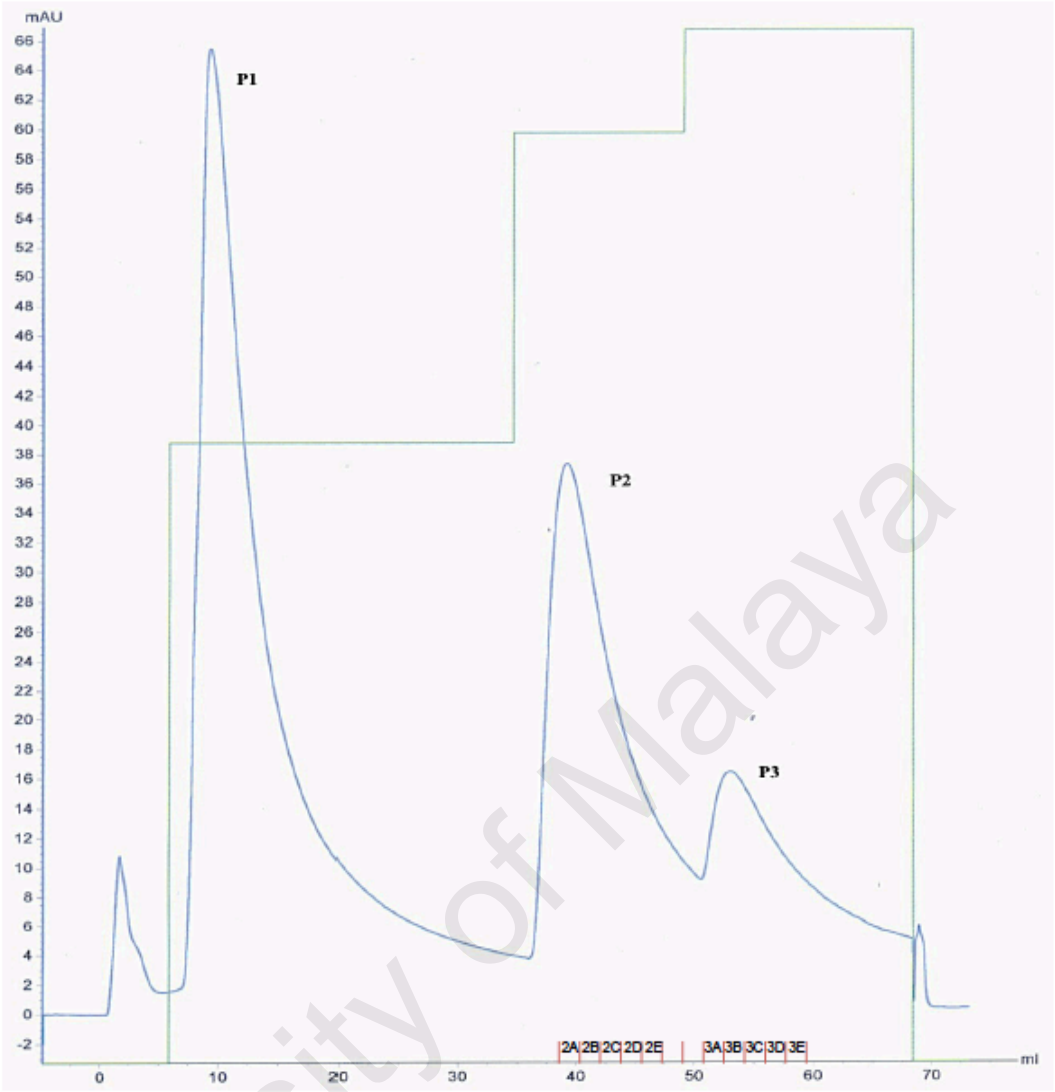

Figure 4.10: Elution profile of strong anion exchanger Hi-Trap Capto Q (Absorbance at 280 nm versus elution volume). The light green line represents the gradient of NaCl (0–100% of buffer B) where buffer A is 10 mM Tris–HCl at pH 8.0 and buffer B is buffer A containing 1.0 M NaCl. All bound proteins were fractionated into three peaks (P1-P3). Protein fragments of interests that were selected for further identification of protein analysis by LCMS Q-TOF were labeled as 2A-2B and 3A-3B.

4.7.1 MTT assay against HCT 116 and MRC-5 cells

Proteins (P1, P2, and P3) from fraction F60 of *T. scopulosa* were tested for cytotoxic assay individually. The IC_{50} values of the proteins against HCT 116 and MRC-5 cells are presented in Table 4.2. A pure compound was considered cytotoxic against cancer cell line if the IC_{50} value is less than 4 µg/mL (Boik, 2001). According to the results shown in Table 4.2, P2 and P3 showed strong cytotoxic effect against HCT 116 cells. The IC_{50} value for P2 was 0.83 ± 0.03 µg/mL while P3, 0.78 ± 0.03 µg/mL. However, P1 was deemed not cytotoxic against HCT 116 since the IC_{50} value could not be determined because the percentage of inhibition did not reach even 50% at the highest protein concentration evaluated (20 µg/mL). Nevertheless, the cytotoxic effect of P2 and P3 were lower than the partially purified protein (F60) as shown in Table 4.2. In addition, the IC_{50} value of P2 when tested on MRC-5 was slightly cytotoxic as compared to the other fractions. This showed that, both fractions could bind together (synergism) to gain higher cytotoxic effect against the colon cancer cell line. Thus, the samples were subjected for synergistic mechanism study.

Table 4.2: The IC_{50} values of the FPLC protein fractions of *T. scopulosa* against colon cancer (HCT116) and human lung fibroblast (MRC-5) cells. Results were expressed as mean ± standard deviations of triplicates values (n=3). The cells were incubated for 72 hours.

Cell lines	**IC_{50} (µg/mL)**				
	F60	P1	P2	P3	P2+P3
HCT 116	0.57 ± 0.06	NA	0.83 ± 0.03	0.78 ± 0.03	0.41 ± 0.02
MRC-5	NA	NA	1.1 ± 0.06	NA	NA

*NA: Not Available

4.7.2 Evaluation of synergism of P2 and P3 from F60 fraction for cytotoxic activity

The active protein peaks (P2, and P3) from fraction F60 of *T. scopulosa* were tested for cytotoxic assay at concentration ratio 1:1 and the dose-response curves were shown in Figure 4.11. The IC_{50} value of the combined mixture when tested on HCT 116 cells was 0.41 ± 0.02 µg/mL, meanwhile the IC_{50} value when treated on MRC-5 was not available because the percentage of inhibition did not reach even 50% at the concentrations evaluated. This showed that the cytotoxic effect of the combined mixture was greater than the cytotoxic effect of partially purified protein, F60. Based on the result, it could be suggested that the combination of proteins from P2 and P3 from mushroom *T. scopulosa* might result for possible synergistic cytotoxic effect between proteins in F60.

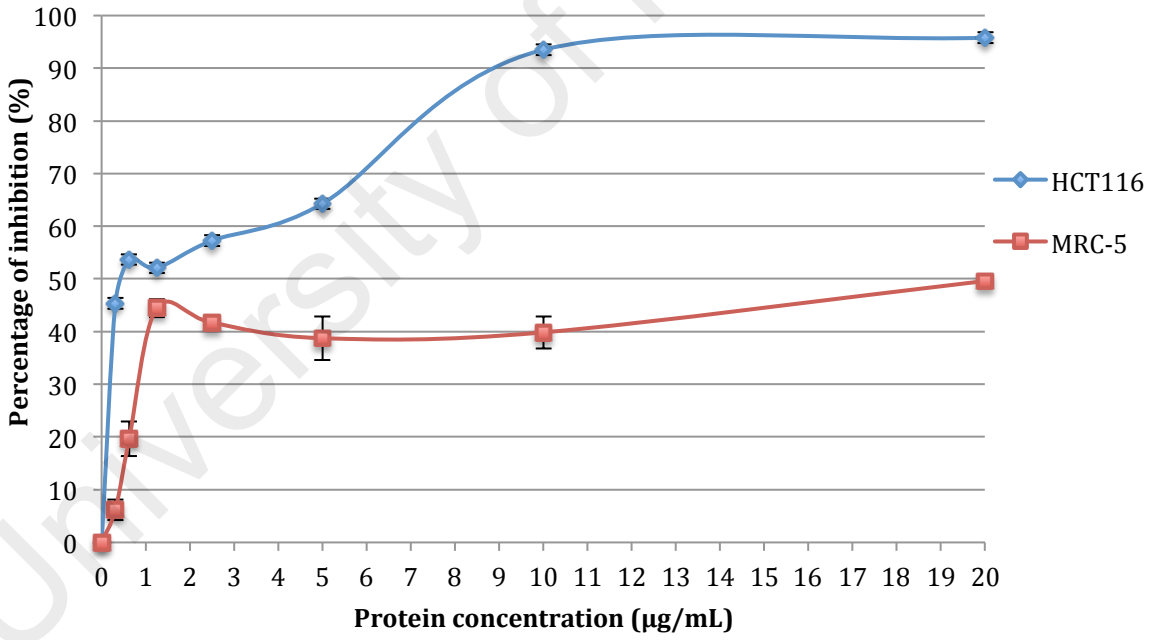

Figure 4.11: Dose-response curves for the inhibition of HCT 116 and MRC-5 cell lines by combined mixture (1:1) of P2 and P3 of *T. scopulosa* in the *in vitro* MTT assay. Results were expressed as mean ± standard deviations of triplicates values (n=3). The cells were incubated for 72 hours.

4.7.3 Scratch wound assay

In the present study, protein fractions (P1, P2 and P3) were tested for anti-migration activity. A scratch was made on the confluent cells and treatment was carried out based at IC_{50} value (P2: 0.83 µg/mL; P3: 0.78 µg/mL) and IC_{25} (P2: 0.69 µg/mL ; P3: 0.32 µg/mL). However for P1, the concentrations used were 20 µg/mL and 10 µg/mL. The width of the gaps was measured at 0, 24 and 48 hours. From the gap distance of the wound, the absolute migration capability of HCT 116 cells was calculated. MC_A value for the untreated cells (negative control) was 5.567±0.378 µm/h and 4.865±0.009 µm/h at 24 and 48 hours of incubation time respectively which showed migration capability of HCT 116 cells in normal condition.

According to Figure 4.12, the MC_A value for colon cancer cell lines treated with P1 at 24 hours was 2.252 ± 0.465 µm/h for HCT 116 treated with 20 µg/mL meanwhile 1.573 ± 0.22 µm/h for HCT 116 treated with P2. P3 showed MC_A value of 2.648 ± 0.498 µm/h. P2 showed lower MC_A value as compared to untreated cells and other fractions (P1 and P3). At 48 hours, the MC_A was 2.061 ± 0.311 µm/h and 1.0.35 ± 0.304 µm/h for HCT 116 treated with P1 and P2, respectively. While, the MC_A value for P3 was 2.143 ± 0.278 µm/h. The smaller the MC_A value indicates the lower migration capability and as shown in Figure 4.12, cell treated with all fractions showed lower migration activities when compared to the untreated cells. However, P2 showed significant high anti-migrative effect after 48 hours as compared to untreated cells and other fractions.

Figure 4.13 showed the absolute migration capability when treated with P1-P3 at three different concentrations of IC_{25} values. The untreated cells showed MC_A values of 6.095 ± 0.156 µm/h at 24 hours and 4.824 ± 0.123 µm/h at 48 hours. P1 showed MCA value of 4.567 ± 0.463 µm/h at 24 hours while at 48 hours, the reading was 4.485±0.181 µm/h. The MC_A values for P2 and P3 at 24 hours were 2.02 ± 0.234 µm/h and 2.382 ±

0.328 µm/h, respectively. Meanwhile the MC_A values at 48 hours for both extracts were 2.917 ± 0.128 µm/h and 1.674 ± 0.315 µm/h, respectively. P1 showed less anti-migrative effect when compared to the other fractions. Meanwhile, P2 and P3 displayed better anti-migrative effect as compared to untreated cells and the other fractions. Based on the results, P2 and P3 fractions were selected for further identification of protein analysis by LCMS Q-TOF.

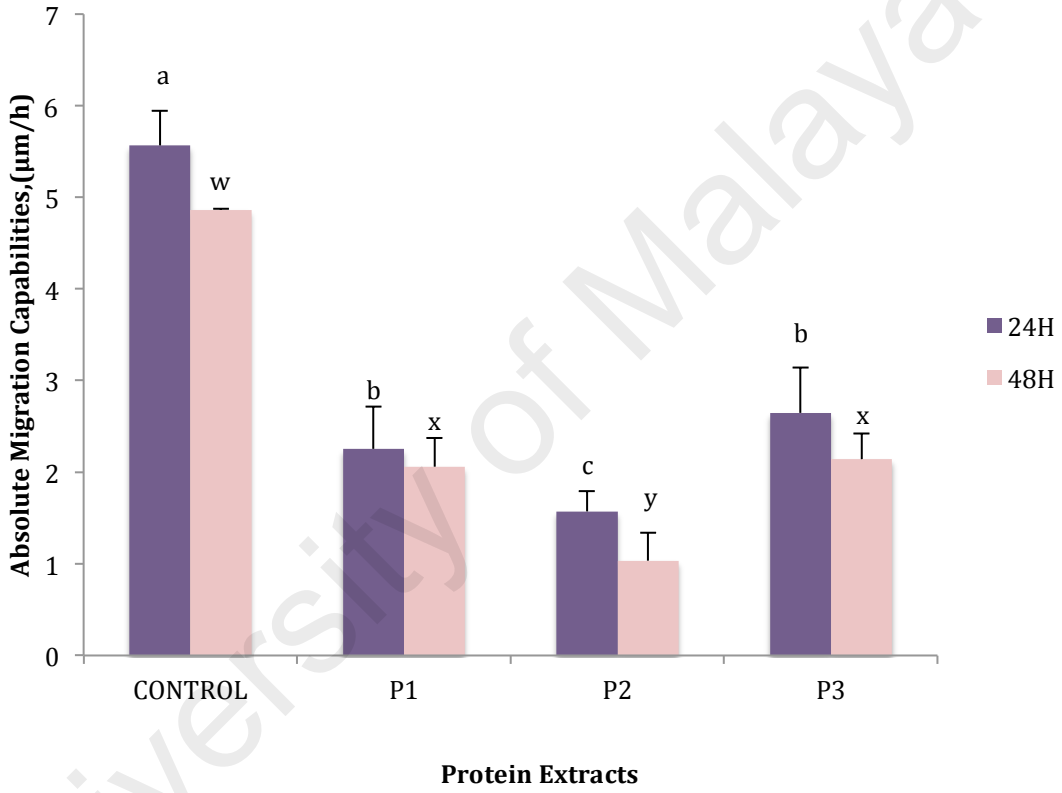

Figure 4.12: The absolute migration capability of HCT 116 cells treated with P1 - P3 of fraction F60 of *T. scopulosa*. The fractions were tested at IC_{50}. Results were expressed as mean ± standard deviation of triplicates values (n=3). Different letters represent significant difference in comparison to control group ($p<0.05$). The cells were incubated for 48 hours.

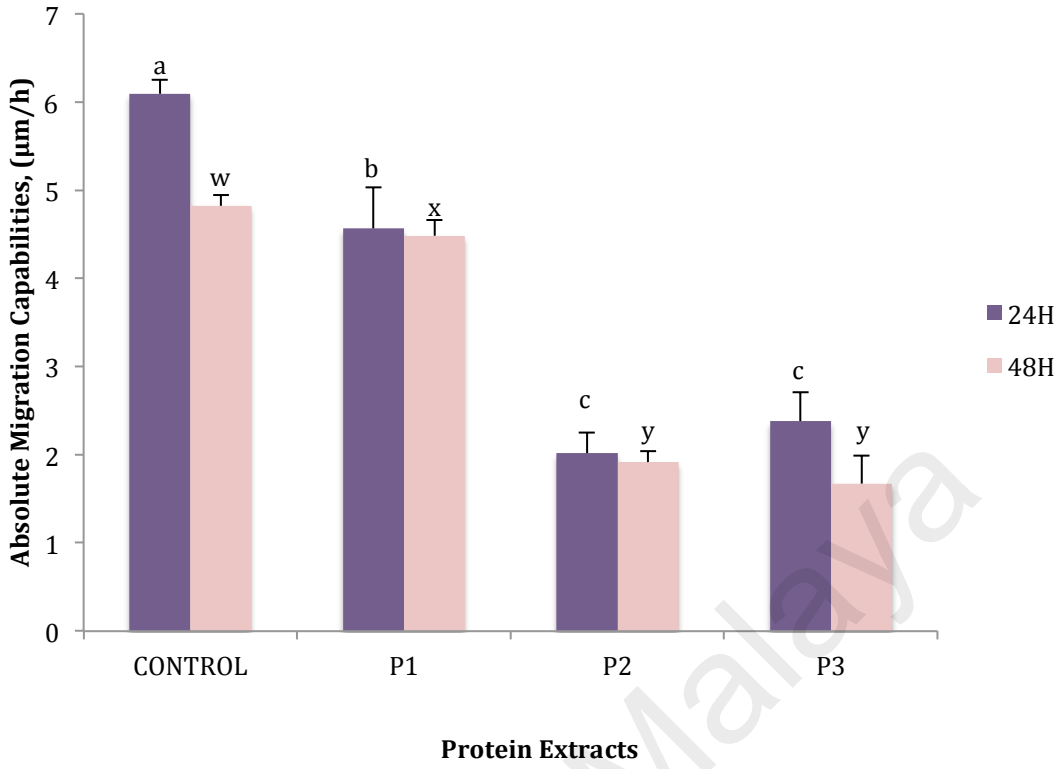

Figure 4.13: The absolute migration capability of HCT 116 cells treated with P1-P3 of fraction F60 of *T. scopulosa*. The fractions were tested at IC_{25}. Results were expressed as mean ± standard deviation of triplicates values (n=3). Different letters represent significant difference in comparison to control group ($p<0.05$). The cells were incubated for 48 hours.

4.8 LCMS Q-TOF Analysis of P2 and P3 by using in-solution digestion protocol

The selected proteins, P2 and P3 that showed good cytotoxic effect against colon cancer cell line was chosen for further characterization using LCMS Q-TOF MS analysis. Details of proteins obtained are listed on Table 4.3 and 4.4. There are two characterized proteins named pyranose-2-oxidase and carboxylic ester hydrolase. In addition, there are also two uncharacterized proteins, namely UP1 and UP2 identified from the analysis. The protein sequences of the identified proteins are shown in Table 4.5. Both of the uncharacterized proteins might have a high chance to be a different protein since the sequences were totally dissimilar. All the proteins were identified from P2 fraction (sample 2B; as shown in Figure 4.10) meanwhile there was no protein

identified in P3 fraction. It was suggested that the protein concentration in P3 was very low. Thus it was beyond detection limit.

The 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. The Gene Ontology (GO) analysis of the identified proteins and their molecular functions were presented in Table 4.6. However, the functions of the uncharacterized proteins are still unreported. The two characterized proteins identified by the database were investigated for their correlation with anti-tumour mechanism based on the previous reported studies and only pyranose-2-oxidase was found to be having indirect relationship with anti-tumour effect because of the flavin adenine dinucleotide binding and pyranose oxidase activity in the pyranose-2-oxidase.

Table 4.3: List of characterized proteins identified by LCMS-QTOF MS Database search. Proteins and peptides were validated using Spectrum Mill software, based on the software default settings.

Sample ID	Protein Name	Database accession	Score	AA Coverage (%)	Mw (Da)	pI	Distinct Peptide
2B	Pyranose-2-oxidase	R7RZB9	34.03	4.1	67343.9	6.27	2
2B	Carboxylic ester hydrolase	M2RL22	20.21	2.5	59857.4	4.42	1

* Sample 2B was collected from P2 fraction

Table 4.4: List of uncharacterized proteins identified by LCMS-QTOF MS Database search. Proteins and peptides were validated using Spectrum Mill software, based on the software default settings.

Sample ID	Uncharacter-ized Protein	Database accession	Score	AA Coverage (%)	Mw (Da)	pI	Distinct Peptide
2B	UP 1	A0A0C3RS55	24.42	2.7	69071.7	5.58	2
2B	UP 2	A0A0C3NJT6	20.21	2.5	59821.6	9.43	1

Table 4.5: The protein sequence fragments of four identified proteins.

Protein Name	Protein sequence (fragments)
Pyranose-2-oxidase	• DIDRFVNVIK • DAFSYGAVAETIDTR
Carboxylic ester hydrolase	• LAALQGDLVFQAPR
UP 1	• NEIEYQKDIDR • DIDRFVNVIK
UP 2	• LAALQGDLVFQAPR

Table 4.6: Gene Ontology (GO) analysis and the functions of the identified proteins.

Protein Name	GO	Functions	Source
Pyranose-2-oxidase	Molecular function	- Flavin adenine dinucleotide binding - Pyranose oxidase activity	InterPro
Carboxylic ester hydrolase	Molecular function	- hydrolase activity	UniProtKB-KW

CHAPTER 5

DISCUSSION

5.1 Cultivation of *T. scopulosa* mycelium using liquid submerged fermentation

Liquid submerged fermentation is a method of developing biomolecules in which enzymes and other reactive compounds are submerged in a liquid that contains a nutrient needed for growth. This type of fermentation is among the most common technique that has been used particularly for large-scale production of mycelial biomass. Various medicinal mushrooms are capable of growing in the form of mycelial biomass in submerged cultures for the extraction of bioactive compounds. Besides that, mycelial biomass powder can be used to formulate numerous types of health medicines (Suberu *et al.*, 2013).

In this study, the *T. scopulosa* mycelium was cultivated using liquid submerged fermentation. The mycelial biomass yield that was grown on GYMP media containing glucose, yeast extract, malt extract and peptone was 5.296 ± 0.142 g/L. It was slightly higher compared to the previous study done by Wang *et al.* in 2013, which the production of mycelial biomass of *T. versicolor* was 5.18 g/L. In that cultivation, the medium used consisted only of glucose and yeast extract, which was slightly different with our study. In the present study, malt extract and peptone were also added in the media. Malt extract and peptone are important substrates for fungal cultivation. This formulation containing carbon, nitrogen and minerals were essential for the mycelial growth. Glucose is a source of carbon meanwhile the nitrogenous sources utilized by the mycelia were peptone and yeast extract (Miles & Chang, 2004). Malt extract was also the best nitrogen source for the growth (Choi *et al*., 2007). Based on the result, it was proved that the addition of other substrates such as malt and peptone could increase the production of mycelia biomass in liquid submerged cultivation.

5.2 Screening for cytotoxicity of *Trametes scopulosa* protein and crude extracts.

Trametes scopulosa mycelium was extracted using solvent and protein extraction method. In this study, the solvent extracts were obtained using the liquid-liquid solvent fractionation. The compounds were separated based on their relative solubility in two different immiscible solvents. The basis of solvent extraction is manipulating polarity of organic solvents used during extraction. According to Nyiredy (2004), organic solvents of different polarities will extract chemicals of specific characteristics, which mean non-polar solvent will extract non-polar substances while polar compounds will be extracted by polar solvents. From the fractionation, 5 solvent extracts were obtained namely as aqueous, ethyl acetate, DCM, hexane and methanol. Meanwhile to obtain protein extracts, the ammonium sulfate precipitation method was used. Ammonium sulfate is commonly used in the purification of proteins due to its high solubility that allows salt solutions with high ionic strength and generally has a stabilizing effect on proteins. In this study, protein fractions were isolated by ammonium sulphate precipitation at concentration of 30%, 60% and 90% from the water extract of *T. scopulosa.*

Several selected mushrooms-extracted compounds of higher Basidiomycetes have been proven to be effective for the prevention and treatment of cancer (Wasser, 2002). Thus in this study, both solvent and protein extracts from *T. scopulosa* were analyzed for potential cytotoxic activities using the *in vitro* MTT assay. Cancer is a disease with an excessive cellular proliferation. MTT cell proliferation assay measures cell proliferation rate, the reduction in cell viability happened when metabolic events lead to apoptosis or necrosis. The cytotoxic activities of the extracts were evaluated using MTT assay against colon cancer cell line (HCT 116) and lung normal fibroblast cell line (MRC-5). The *in vitro* MTT assays of the solvent and protein extract is based on the formation of intracellular purple formazan precipitate in viable cells. Low

intensity of the dye shows lower number of viable cells, reflecting high cytotoxic effect towards colon cancer cells HCT 116 and normal lung MRC-5 cells. There are other several common methods to evaluate cytotoxic activity such as lactate dehydrogenase leakage assay (LDH), neutral red assay (NR) and protein assay (Fotakis & Timbrell, 2006).

The pre-screening for cytotoxic activity against colon cancer cells (HCT 116) using MTT assay showed that all solvent extracts of *T. scopulosa* i.e methanol, hexane, DCM, ethyl acetate and aqueous showed less inhibitory effects against HCT 116 cells with percentage of inhibiton lower than 20% even at 50 µg/mL. Methanol exctract showed higher percentage of inhbition as compared to hexane, DCM and ethyl acetate as shown in Figure 4.1. According to Melappa *et al*. (2015), methanol extract of *Trametes ochracea* yielded potent phytochemicals as compared to hexane extract. Some phytochemicals such as saponins, flavanoids, alkaloids, steroids, phenols and tannis were present at higher concentration of methanol extracts as compared to hexane. The methanol extract of the wood rooting fungi was also reported to have strong antioxidant, anti-microbial, anti-inflammatory and cytotoxic activities.

Younis *et al*. (2014) has reported that the solvent extracts (water, methanol, ether, and ethyl acetate) of fresh and freeze-dried fruiting bodies of three edible mushrooms such as *F. velutipes, G. lucidum* and *P. eryngii* had a stronger inhibition effect against the human liver carcinoma (Hep G2), human colonic epithelial carcinoma (HCT 116) and human cervical cancer cells (HeLa) cell lines as compared to mycelial and broth extracts. This showed that, fruiting bodies of solvent extracts of *T. scopulosa* might have active compounds to cause cytotoxicity towards the cells as compared to the mycelia extract.

However in this study, all protein fractions obtained by ammonium sulfate precipitation (F30, F60 and F90) were actively cytotoxic against HCT 116 cells with

percentage of inhibition were higher than 50%. To date, there is no work that has been done on cytotoxic protein of *Trametes* species. Thus, protein extracts of *T. scopulosa* were chosen for further research.

5.3 Cytotoxic activity of Protein Fractions Against HCT 116 and MRC-5 Cell Lines

Cytotoxicity test against cancer cell lines is the most prevalent screening methods utilized in the search for new anti-cancer drugs. In the discovery of new anti-cancer drugs, thousands of substances are being tested and the most active compound is chosen. *In vivo* toxicity tests are usually conducted before clinical test. However, the *in vivo* study is expensive, time consuming and requires large number of animal to be tested (Popiołkiewicz *et al*., 2005). Thus, *in vitro* studies are mostly chosen for the cytotoxicity test because of the low cost and time consuming. The cell lines can be obtained easily which could reduce the number of animal needed for experiment.

In the present study, *in vitro* MTT assay was used to determine the cytotoxic activity of the protein extracts of the *T. scopulosa*. All protein fractions obtained by ammonium sulphate precipitation at 30 %, 60 % and 90 % (F30, F60 and F90) showed reading of IC_{50} that are less than 20 µg/mL. According to the National Cancer Institute, extract with IC_{50} 20 µg/mL or less is considered active for the cytotoxicity assay (Geran *et al*., 1972). This showed that, all the fractions were actively cytotoxic against HCT 116 cancer cells line with F60 exhibiting most potent cytotoxicity with the IC_{50} value at 0.84 ± 0.05 µg/mL as compared to the other fractions. A research by Maiti *et al.* (2008) found that protein fraction (10-100 µg/mL) of edible mushrooms such as *Termitomyces clypeatus, Pleurotus florida, Calocybe indica, Astraeus hygrometricus,* and *Volvariella volvacea* have mediated anti-proliferative activity towards several tumour cell line and also exhibited stimulatory effects on splenocytes, thymocytes and bone marrow cells. However, the IC_{50} values were higher than 10 µg/mL, which was less cytotoxic when

compared to this study. This showed that the protein fraction from *T. scopulosa* has good cytotoxic effect against cancer cell line as compared to other species.

Protein extracts of *T. scopulosa* was also evaluated for cytotoxicity against normal lung fibroblast cells to verify whether or not these protein extracts will exert widespread cytotoxicity in the body affecting even normal cells. A good cytotoxic drug for cancer therapy should be selective towards just cancer cells while not harming normal cells. In this study, the IC_{50} value obtained showed that F60 of *T. scopulosa* is not actively cytotoxic against MRC-5 cells with IC_{50} higher than 20 μg/mL as compared to F30 and F90. This indicated that the composition of cytotoxic compound in *T. scopulosa* was greatly influenced by the protein in the F60. This active protein might contribute to the medicinal effects of the fungus and has value in treating various diseases especially cancer. Hence, it is possible to suggest that F60 protein fraction of *T. scopulosa* protein holds potential as a therapeutic agent with cytotoxic activity against colon cancer.

The estimation of protein content also showed that fraction F60 contains higher protein content as compared to the other fractions. Thus, the fraction was subjected to SDS-PAGE for protein separation. F60 was separated into a few single bands corresponding to a high molecular mass between 12 to 64 kDa. A novel lectin with molecular mass 16 kDa was isolated from *Pholiota adiposa* by Zhang *et al.* (2009) showed anti-proliferative activity toward hepatoma Hep G2 cells and breast cancer (MCF7 cells) with IC_{50} of 2.1 μM and 3.2 μM, respectively. This suggested that the protein with molecular weight of 16 kDA in this study might be the active compound that has the cytotoxic effect against the colon cancer cell lines. Besides that there are also some previous studies that isolated proteins with anti-tumour activity for various mushroom with different molecular weight that was similar to this study. For example, there were lectins and fungal immunomodulatory protein (FIP) with molecular weight

of 32 kDa and 36.6 kDa were isolated from *Russula leptida* and *Ganoderma lucidum*, respectively (Du *et al*., 2007; Zhang *et al*., 2010a). Besides that, there were also anti-tumour proteins with molecular weight of 60 kDa, 62 kDa and 66 kDa were found in *Russula delica* (Zhao *et al*., 2010), *Tricholoma mongolicum* (Li *et al*., 2010b), and *Clitocybe maxima* (Zhang *et al*., 2010b), respectively.

In this study, F60 was tested on both cell lines (HCT 116 and MRC-5) based on the protein content. Results showed that the IC_{50} value of F60 (based on protein content) was even smaller than the IC_{50} value of F60 by weight, which proved better cytotoxic effects against the colon cancer cell line. There are a few studies work on cytotoxic effect of polysaccharides from *Trametes* species. However, no study has been reported on cytotoxic protein of the same genus.

A research done by Mizuno *et al.* (1999) found three different bioactive polysaccharides anti-tumour agents from mushroom fruit body, mycelium and cultured medium of three polypore mushroom species. A polysaccharide of *Trametes versicolor* (CVP) has been proven to inhibit the proliferation of cancer cells *in vitro* or *in vivo*. Besides that, a research by Cai *et al.* (2010) showed that CVP inhibited the proliferation of human hepatoma cancer (QGY) cell lines with IC_{50} value was 4.25 mg/L. Interestingly, the active cytotoxic protein fraction (F60) based on protein content in this study showed even higher cytotoxic effect (IC_{50} 0.57 ± 0.06 µg/mL protein) compared to this polysaccharide. Moreover, the F60 protein also showed good cytotoxic effect as compared to the positive control (Cisplatin).

A new immunomodulatory protein was discovered on *T. versicolor,* named TVC has significant mitogenic activity for lymphocytes, splenocytes and exerted the activity of machrophage (Li *et al*., 2011). However, to date, there is no specific validation regarding the medicinal values of *T. scopulosa* proteins. This study indeed showed that protein extracts of *T. scopulosa* have good cytotoxic effect against colon cancer cell.

5.4 Migration Effects of The F60 Fraction on HCT 116 Cells

Metastasis is a spread of cancer cells to distant locations in the body. Distant metastasis is the principle cause of colon cancer deaths. Metastasis occurs through a complex multistep, it involves 6 main steps; local invasion, intravasation, circulation, extravasation, proliferation and angiogenesis. If these steps are inhibited, metastasis can be blocked/ slowed down. This is an important approach for anti-metastatic therapy.

In vitro scratch wound, ECIS wound healing, Transwell or Boyden chamber assay, and microfluidics-based system are the examples of assays that can measure cell migration activity. The scratch wound method was used in this study because the ability of tumour cells to migrate is closely associated to their metastatic potential. This assay is a well-developed and inexpensive method to measure cell migration activity. There is no extra cost than routine tissue culture is required for this assay. One of the advantages of this method is that it imitates the behavior of cell during *in vivo* migration (Liang, Park, & Guan, 2007). However, this assay takes a relatively longer time to perform than some other methods. Five days are usually needed with the first two days are required for the formation of cell monolayer and then 24 - 48 hours for cell migration to close the scratch. The migration effect can be measured by using anti-proliferation agents or 0-1 % serum media since cell filling the wounded area may be due to both migration and proliferation (Goodwin, 2007; Choi *et al*., 2009 and Sathya *et al*., 2010).

In the present study, the *in vitro* scratch wound assay showed the HCT 116 cells treated with F60 (at 0.57 µg/mL protein) significantly slowed down cellular migration activity as compared to control (untreated cells). Cells treated with 0.39 µg/mL protein showed lower migration capability as compared to higher concentration (IC_{50}). However, F60 still showed good anti-migration effect even at low (IC_{25}) or high (IC_{50}) concentration. Growing evidence showed that polysaccharide-K (PSK) isolated from *T. versicolor* interrupted distant metastases and improves survival rates in colorectal

cancer (Yoshikawa *et al*., 2005). Yang *et al.* (2010) has reported that fermented culture broth of *Antrodia camphorata* (Family: Polypocerea) could also enhanced anti-metastatic effect against MDA-MB-231 breast cancer cells by inhibiting several essential steps of metastasis, including migration and invasion of the cells through the suppression of the MAPK (mitogen-activated protein kinases) signaling pathway. In recent years, mushroom polysaccharides have been known due to its anti-tumour and immunostimulating properties. However, no research has been done to evaluate the anti-migration activity of *T. scopulosa* proteins. Thus, this experiment may suggest that the protein of *T. scopulosa* could inhibit the migration activity of HCT 116 cells. Nevertheless, further research is needed to determine the mechanism responsible for the *in vitro* migration effect of the protein against the cell lines.

5.5 Purification by Fast Protein Liquid Chromatography and Protein Identification by LCMS-QTOF

Chromatography refers to a group of separation techniques that can be used to isolate individual compound or to analyze their structural properties. Ion exchange chromatography is usually the most frequently used for the separation and purification of proteins. This type of chromatography relies on electrostatic interaction between protein and matrix (Price & Nairn, 2009). Besides that, ion exchange chromatography can offer high absorption capacity and excellent resolution properties. Anion exchange matrices are positively charged and interact with negatively charged protein, meanwhile cation exchangers are negatively charged and interact with positively charged protein.

The pre-packed HiTrap column is most to develop the basic separation method. This approach is particularly helpful if the properties of the target protein(s) are unknown. In the present study, HiTrap strong anion exchanger quaternary ammonium (Q) column was used to separate and purify the F60 fraction. Strong anion exchanger

contained a charged group that was a strong base. This exchanger was able to maintain the positive charges across a variable pH range while weak anion exchanger tend to lose their charges as the pH increases. Typically, increasing the ionic strength of the elution buffer can elute protein like in this study; the buffer with 1 M NaCl was used. The salt ions compete with the protein in binding to the ion exchanger, thus releasing the protein from its bound state (Price & Nairn, 2009).

In this study, fraction F60 that has a good cytotoxic and metastatic effect was further purified by anion exchange chromatography. Three fractions (P1, P2, and P3) have been isolated from the fraction F60 of the *T. scopulosa* and were tested back for cytotoxic and anti-migration activities. The results from both assays showed that, P2 and P3 of the fraction have good cytotoxic effect against the colon cancer cell line. Both of the fractions also showed best anti-metastatic effect as compared to P1 when tested for anti-migration activities. Nevertheless, the cytotoxic effects of these fractions were slightly lower than the partially purified protein, F60. Thus, both of the fractions were combined with ratio 1:1 and tested back on the colon cancer and normal lung fibroblast cell lines to determine whether the fractions have synergistic interaction. Result indicated a possible synergistic cytotoxic effect with IC_{50} reading much lower than F60. They effectively inhibited the cell proliferation and turn out to be very cytotoxic when present in mixture but the cytotoxic effect became less effective when they were separated through FPLC. A possible synergism between eluted peaks from RP-HPLC of protein fraction of *Pleurotus pulmonarius* (grey oyster mushroom) when tested for anti-diabetic effect was also reported back in 2014. The peak showed tremendous decrease in percentage of inhibition towards α-amylase as compared to the percentage of inhibition before purification by RP-HPLC when tested for α –amylase inhibition assay. However, the combined peaks showed an increase to 12% of inhibition (Abdul Wahab, Abdullah, & Aminudin, 2014). This showed that some of the proteins in mushroom

might have even good bioactivity and synergistic effect when the compounds were combined.

Several bioactive proteins from mushroom have been found via a series of chromatographic analysis including anion exchange. Li *et al.* (2010) isolated a laccase from the edible wild mushroom *Tricholoma mongolicum*. It significantly inhibited proliferation of hepatoma HepG2 cells and breast cancer MCF7 cells. The polypore mushroom, *G. lucidum* was also reported to have a novel water-soluble Selenium-containing protein Se-GL-P with molecular mass of 36 kDa (Du *et al*., 2006). The selenium was combined into the proteins in the form of selenocysteine and selenomethionine and this protein boosts its activity in preventing multiplication of tumour cells. Beside that, a monomeric protein enzyme with molecular mass of 67 kDa was purified by ion-exchange chromatography from *Trametes versicolor*. The purified laccase inhibited proliferation of MCF7 and HepG2 cancer cells with IC_{50} value of 2.3 μmol/L and 4.4 μmol/L, respectively (QianQian, Miao, & HeXiang, 2010).

Based from these previous studies, it was implied that the proteins in F60 that had been purified (P2 and P3) using anion exchange chromatography might be the contributor for cytotoxic and metastatic effect on the colon cancer cells. With regard to the results, P2 and P3 of fraction F60 were subjected to LCMS-QTOF for further analysis and to validate the protein extracted from the anion exchange chromatography. From the study, there were 4 proteins identified. Two of the proteins were characterized as pyranose-2-oxidase and carboxylic ester hydrolase and the other two were uncharacterized proteins. All the proteins were identified from P2 fraction meanwhile there was no protein identified in P3 fraction. It was suggested that the protein concentration in P3 was very low (123.077 ± 2.308 μg/mL) as compared to P2 (213.846 ± 7.338 μg/mL); hence, it was beyond detection limit. Although the amount of protein in P3 was lesser than in P2, the fraction still have good cytotoxic effect as compared to

P2. In addition, the combination of these fractions (synergism) showed even good cytotoxic effect as compared to F60.

Pyranose-2-oxidase (P2O) is the most abundant protein found in this fraction. This protein was also found in other *Trametes* species such as *T. versicolor* (Machida and Nakanishi, 1984), *T. multicolor* (Leitner, Volc & Haltrich, 2001; Halada *et al.,* 2003) and *T. pubescens* (Marešová *et al*., 2005). Thus, it was suggested that pyranose-2-oxidase is a common protein in genus *Trametes* based on this study. Besides that, it was also has been found in other mushrooms such as *Phanerochaete chrysosporium* (Volc & Eriksson, 1988; Artolozaga *et al*., 1997)*, Phlebiopsis gigantean* (Schafer *et al*., 1996)*, P. ostreatus* (Shin *et al*., 1993) and *Polyporus obtusus* (Janssen & Ruelius, 1975).

Pyranose-2-oxidase is a homotetrameric protein and contained flavin adenine dinucleotide (FAD) covalently bound to the polypeptide chain. This protein could interact with FAD, the coenzyme or the prosthetic group of various flavoprotein oxidoreductase enzymes in either the oxidized form, FAD or reduced form, $FADH_2$. Halada *et al*. reported a first research on characterization of the covalent flavinylation site in flavoprotein pyranose-2-oxidase in 2003. No study has been reported on the cytotoxic activity of P2O. However, there is a possibility that P2O could promote apoptosis because of the existing FAD or flavoprotein. Halada *et al.,* (2003) also stated that no flavin was found after protein denaturation, signifying covalent attachment of the cofactor. Flavoprotein or also known as flavin enzyme contains a nucleic acid derivative of riboflavin such as FAD and flavin mononucleotide (FMN).

Flavin adenine dinucleotide is a redox cofactor involved in several important reactions in metabolism. Flavin adenine dinucleotide–binding oxidoreductase, an apoptosis-inducing factor (AIF) is the first mitochondrial protein that involved in apoptosis through a caspase-independent mechanism (Susin *et al*., 1999). Caspase-independent cell death is a death that occurs when a signal that usually induces

apoptosis fails to activate caspases. Apoptosis is an organized and often energy-dependent process that involves the activation of a group of caspases and a complex cascade of events that connect the initiating stimuli to the final death of the cell (Elmore, 2007). Based on this study, it was suggested that FAD that bound to pyranose-2-oxidase might be the inducer for apoptosis in the colon cancer cell line.

Pyranose-2-oxidase is also involved in pyranose oxidase activity where the catalysis of D-glucose and oxygen happened as follows:

$$\text{D-glucose} + O_2 = \text{2-dehydro-D-glucose} + H_2O_2$$

In this catalysis, it showed that the presence of P2O would promotes catalysis of D-glucose, which then produces hydrogen peroxide (H_2O_2). The production of this peroxidase would increase chances of oxidative reaction because it is the most powerful oxidizing agent.

Hydrogen peroxide is naturally produced in organisms as a by-product of oxidative metabolism. It is not a free radical but involves in the production of many reactive oxygen species (ROS). Although hydrogen peroxides can cause a potent oxidative stress, it is also has been demonstrated to be a potent apoptosis inducer in many kind of cells. A report by López-Lázaro (2007) suggested that an increase in the cellular levels of H_2O_2 may play, directly or indirectly, a key role in malignant transformation, but can also stimulate cancer cells to H_2O_2-induced cell death. Several researches have demonstrated that H_2O_2 could induce cell proliferation (Burdon, 1995; Zanetti, Katusic, and O'Brien, 2002; Polytarchou, Hatziapostolou, and Papadimitriou, 2005), cells invasion and metastasis (Zanetti,Katusic & O'Brien, 2002; Nelson *et al*., 2003; Nishikawa *et al*., 2004). Burdon in 1995 stated that specific receptors or oxidations of growth signal transduction molecules such as protein kinases, protein

phosphatases, transcription factors or transcription factor inhibitor might be involved in the mechanism that related to proliferative/growth responses.

There were several studies suggested that the key role of H_2O_2 in carcinogenesis was because of the increased levels of H_2O_2 in cancer cells (Szatrowski & Nathan, 1991; Burdon, 1995; Lim *et al*., 2005). This high amount of H_2O_2 produced by cancer cells and high cellular level of ROS was believed to have direct or indirect correlation with cancer. However, as stated above, there is also evidence that H_2O_2 can induce cell death in cancer cells. There were a few studies reported that significant increase in the production of cellular H_2O_2 and acidification provides an environment favorable for apoptotic cell death in tumour cells (Clement, Hirpara, & Pervaiz, 2003; Ahmad *et al*., 2004). Hydrogen peroxide mediated cytosolic acidification was reported as an effector mechanism during drug-induced apoptosis of several tumour cells such as colorectal carcinoma (HCT 116) and leukemia cells (HL 60 and CEM) (Ahmad *et al*., 2004). The production of H_2O_2 by pyranose-2-oxidase might be the main factor on how this protein could inhibit the proliferation of colon cancer cell line in the present study.

In addition, there was also evidence that cancer cells were more susceptible to H_2O_2-induced cell death than normal cells. A study by Chen *et al.* (2005) showed that ascorbic acid or ascorbate in pharmacological concentration intervened by H_2O_2 could selectively kill cancer cell lines. Ten cancer and 4 normal cell lines were used in this experiment. A low concentration (50 μm) of H_2O_2 induced more percentage on cell death in Burkitt's lymphoma cells as compared to high concentration of H_2O_2 (250 μm) induced in normal lymphocytes and monocytes. In this study, the percentage of colon cancer cell death was also higher as compared to the normal cell line. This showed that, the presence of H_2O_2 could not effect the normal cells, which made pyranose-2-oxidase would be a good cancer therapeutic agent.

Besides that the pyranose oxidase activity of the P2O which is also known as H_20_2-generating enzyme, also plays an important role in lignin degradation of wood rot fungi by producing hydrogen peroxide for the ligninolytic peroxidases, lignin peroxidase and manganese-dependent peroxidase in white rot fungus *Phanerochaete chrysosporium* (Volc *et al.*, 1996). The preferred substrate is D-glucose which is converted to 2-dehydro-D-glucose, an intermediate of a secondary metabolic pathway leading to the antibiotic cortalcerone (Volc *et al*., 1991). This enzyme acts also on D-xylose, together with D-glucose the major sugars derived from wood, on L-sorbose, D-galactose and 1,5-anhydroglucitol, a diagnostic marker of diabetes mellitus (Database Source: UniProtKB - P79076).

Looking at the different functions of pyranose-2-oxidase, it was suggested that the possible mechanism of cell killing in this study might be via both flavine adenine dinucteotide binding and the presence of peroxidase. That is why the IC_{50} value in this study was very low as compared to other chemopreventive drug such as Cisplatin because there are two ways to kill the cancer cell lines. However, further study must be done to determine the exact mechanism of anti-tumour effect of pyranose-2-oxidase against cancer cell line.

The second protein that has been found in this study is carboxylic ester hydrolase. It is involved in the catalysis of the hydrolysis of various bonds, e.g. C-O, C-N, C-C, phosphoric anhydride bonds, etc. Hydrolase is the systematic name for any enzyme of EC class 3 (Database Source: UniProtKB - M2RL22). However through literature search, this protein was not found to be having direct or indirect relationship with cytotoxic activity.

5.6 Limitations of study and future work

This study has achieved important findings in the field of mycology especially in the study of a polypore mushrooms, *Trametes scopulosa.* However, there are some limitations of this study that need to be considered. In comparison with other polypore mushrooms, the research on *T. scopulosa* has been less extensively studied in recent years. Hence, less information regarding its qualities and properties has been published.

Another important limitation of this study is the availability of protein samples collected from FPLC analysis. The present study was not conducted to investigate the synergism effect between proteins of mycelial extract. However, results indicated a possible synergistic cytotoxic effect between 2 protein fractions of *T. scopulosa*. Due to the limited sample, only one combination of concentration of protein fractions (P2 and P3) was used in this study. Thus, the synergy effect could not be calculated using isobologram curve to investigate whether the protein fractions are synergistic, additive or antagonistic.

This study forms a basis for further work for example; the verified cytotoxic proteins identified in this study should be further investigated to obtain a drug against colon cancer and to investigate synergistic effect between protein fractions in *T. scopulosa* mycelial extract.

CHAPTER 6

CONCLUSIONS

Proteins of *T. scopulosa* derived from mycelial biomass showed potential *in vitro* cytotoxic and anti-migration effects against colon cancer cell line. Proteins can be extracted from mycelium produced in high amounts by liquid fermentation. In the present study, the cytotoxic and anti-migration activities of solvent and protein extracts of *T. scopulosa* were successfully evaluated using *in vitro* MTT and scratch wound assay on colon cancer cell line, respectively. Protein extracts of this species showed higher percentage of cell death as compared to the solvent extracts and were chosen for further research.

Among the proteins eluted using ammonium sulphate precipitation, the fraction F60 exhibited the best cytotoxic effect as compared to the other fractions (F30 and F90) with IC_{50} value of 0.57 ± 0.06 µg/ml. The fractions were further purified by FPLC. Three fractions were collected from the purification but only two fractions (P2 and P3) showed good cytotoxic and anti-migration effect against cancer cell line. However, the cytotoxic effects of these fractions were slightly lower than the partially purified protein (F60). Hence, both of the fractions were combined and tested back on the colon cancer and normal lung fibroblast cell lines to determine whether the fractions have synergistic interaction. Result indicated a possible synergistic cytotoxic effect with IC_{50} reading much lower than F60 (IC_{50}: 0.41 ± 0.02 µg/ml).

Protein identification by LCMS-QTOF has revealed four identified proteins. Two of the proteins were characterised as pyranose-2-oxidase and carboxylic ester hydrolase while another two were uncharacterised protein. Of the two, only pyranose-2-oxidase seemed to be the potential anti-tumour protein. Looking at the different functions of pyranose-2-oxidase, the possible mechanism of the cytotoxicity is via

flavine adenine dinucleotide binding and the production of peroxidase by pyranose oxidase activity.

Based on this research, the findings of this study suggested that *T. scopulosa* is a potential source of natural anti-tumour drug. Besides that *in vivo* study could be proposed to add assurance regarding the safety and efficiency of the new drug. However, further investigation is needed to identify the exact mechanism on how the protein can kill and inhibit the migration activity of the colon cancer cell line.

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