PURIFICATION AND CHARACTERIZATION OF GROWTH-INHIBITORY COMPOUNDS FROM 2 GENERA OF BRITISH COLUMBIA WILD MUSHROOMS: *FOMITOPSIS* AND *PHAEOLUS*

by

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Abstract

Modern science is currently working to characterize the beneficial compounds from mushrooms and their potential as pharmaceutical drugs. In this thesis, eight British Columbian wild mushroom collections (3 species), comprising *Fomitopsis pinicola, Phaeolus schweinitzii* and *Phaeolus* sp., were screened for growth-inhibitory potential. Out of 28 crude extracts, 15 exhibited strong growth-inhibitory activity. For the *Phaeolus* sp. collection, liquid-liquid extraction, size-exclusion chromatography and high-performance liquid chromatograph-mass spectrometry (HPLC-MS) were used to purify the growth-inhibitory compound of interest. NMR analyses confirmed its identity as hispidin, a known potent anti-cancer compound. Another compound with a mass to charge ratio of 283.2 in hexane extract of *Phaeolus* sp. was detected by HPLC-MS. This study provides the foundational work for further isolation of small molecule(s) from the three mushroom species and shed light on the profile of *Phaeolus* sp. as a source of growth-inhibitory compounds.

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Chapter 1 Introduction and literature review

1.1 Cancer and its properties

1.1.1 What is cancer

Cancer is a term used for specific diseases in which abnormal cells divide uncontrollably resulting in cell invasion and metastasis (Clark & Vignjevic, 2015). Cancer cells can spread to other parts of the body through the blood and lymphatic systems. The human body is made up of many different types of cells, such as skin, muscle, and blood cells. Many normal cells in our body are continually dividing to create new cells, and these healthy cells stop reproducing when enough cells are present (Mair, 2010). However, when an error occurs in the manufacturing of a new cell, that cell may become a cancer cell. In addition to an error in cell development, cancer cells do not have the internal system causing the cell to die after a certain time and this leads to the formation of abnormal cells. When this occurs, the cells can proliferate and build up a mass of tissue, called a tumor, or abnormal cells can crowd out the good, healthy cells, which occurs in leukemia or other cancers affecting the blood.

Cancer is a broad term that encompasses a complex group of more than 100 different types of cancerous diseases that can develop in the human body (Hanahan & Weinberg, 2000). Most of these can be considered as chronic diseases, representing one of the main health problems in the 21st century and the leading cause of death worldwide (Bray *et al*., 2018). It is reported that cancer is the second most common cause of death among children between the ages of 5 and 14 in the WHO European region (Kyu *et al*., 2016). As a chronic disease, cancer often leads to death or can have long-term devastating effects on an individual. Thus, finding effective cures and treatments for cancer is a major priority for the whole world in this century.

1.1.2 Conventional cancer treatment

Up to now, conventional cancer treatment mainly consists of surgery, chemotherapy and radiation therapy, depending on the type of cancer and the stage of tumor development inside the body (Gibbs, 2000). Surgery is the most common form of cancer treatment and can be used by itself or in combination with other treatments (Arruebo *et al.*, 2001). Common side effects from surgery are specific to the type of surgery done, which includes (but is not limited to) pain (often temporary), fatigue, risk of infection at the surgical site and organ dysfunction. Chemotherapy involves the use of drugs to destroy cancer cells; it is an effective and widely used treatment in most types of malignancies (Chabner & Roberts, 2005). The side effects of the chemotherapy will vary depending on the type of anti-cancer drug combination used, the dose of the anti-cancer drugs, and the frequency of treatments. Chemotherapy is a systemic treatment that results in the elimination of both healthy and cancerous cells (Aslam *et al*., 2014). Another treatment alternative is radiation therapy (or radiotherapy), which often uses X-rays to kill or damage cancer cells. Radiation is given before, during or after other treatments. The goal of radiation is to damage as many cancer cells as possible without harming healthy tissue (Baskar *et al*, 2014).

The major problem with these conventional therapies, especially radiotherapy and chemotherapy, is that they invariably result in damage or weakening of the patient's natural immunological defenses, and cause a number of side effects, with serious damage and suffering to the patient (Aslam *et al*., 2014, Nieder *et al*., 2000).

1.1.3 Cancer in Canada

Almost half of Canadians will suffer from cancer in their lifetime, and about one-quarter of Canadians are expected to die from the disease (Statistics Canada, 2014). Cancer is the leading cause of death in Canada (Figure 1), and is responsible for 30% of all deaths, followed by cardiovascular diseases (heart and cerebrovascular diseases), accidents, and chronic lower respiratory diseases. In 2019, an estimated 82,100 Canadians were expected to die from cancer. Such a large number of cancer deaths has huge implications on the demand of health care and support services (Canadian Cancer Statistics).

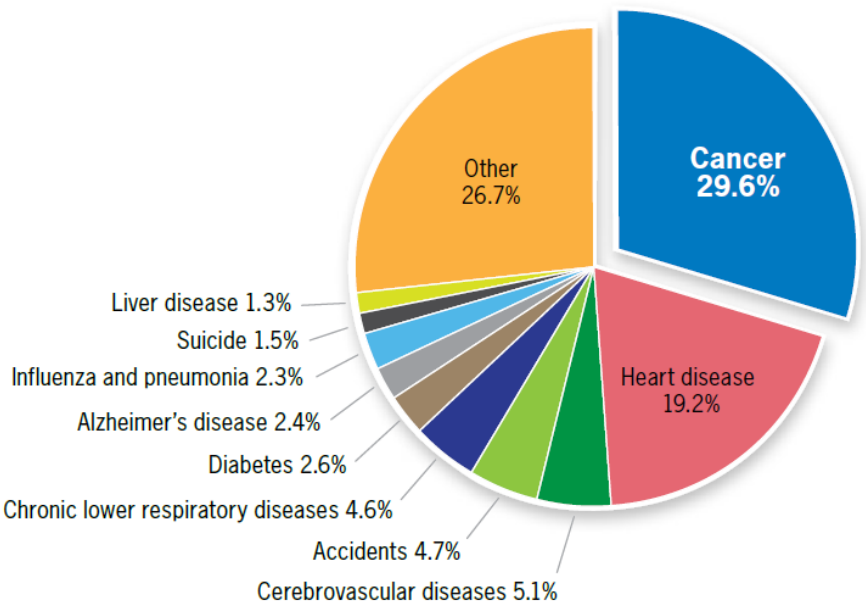


Figure 1 Proportion of deaths due to cancer and other causes in Canada, for 2016 (Statistics Canada, 2016).

Cancer is also a major cause of premature mortality, according to the data measured by potential years of life lost (PYLL) (Canadian Vital Statistics Death database, Statistics Canada, 2014-2016). PYLL is a summary measure of premature mortality that accounts for deaths

occurring at younger ages. As indicated, it tends to be more influenced by deaths from diseases and injuries affecting children and young adults, for whom the potential years of life are greater. During the period between 2014 and 2016, the PYLL for all cancers combined was almost 1,411,100. This is more than any of the other leading causes of premature death in Canada (Figure 2).

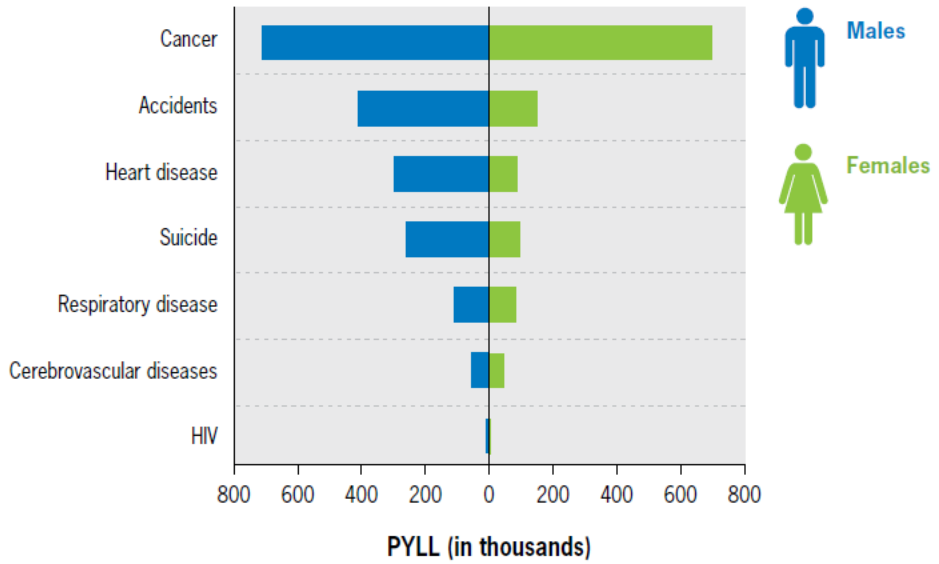


Figure 2 Selected causes of death and their associated potential years of life lost (PYLL), in Canada, for the period 2014–2016.

1.2 Natural products as a source for drug discovery

1.2.1 Natural products in history

Nature has been a source of medicinal agents for thousands of years. Plants, fungi and animals have been used as sources for the treatment of human diseases (Lahlou, 2007). The earliest records of natural products were written on clay tablets in cuneiform script, and are from Mesopotamia (2600 B.C.); they documented oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh), which are still in use today to treat coughs, colds and inflammation. The best known Egyptian pharmaceutical record is the Ebers Papyrus, dating

from 1500 B.C.; this documented some 700 drugs (mostly plants) including gargles, pills, infusions, and ointments (Cragg & Newman, 2005). The Chinese Materia Medica (1100 B.C.), Shennong Herbal (~100 B.C., 365 drugs) and the Tang Herbal (659 A.D., 850 drugs) also contain numerous records on the use of natural products (Cragg & Newman, 2005).

In the ancient western world, the Greeks contributed to the development of the use of herbal drugs. The natural scientist, Theophrastos (~300 B.C.) dealt with medicinal herbs, and the physician Dioscorides (100 A.D.), recorded the collection, storage and uses of medicinal herbs. During the Dark and Middle Ages (5th to 12th centuries), the monasteries in countries such as England, Ireland, France, and Germany preserved the remnants of this western knowledge. The Arabs were the first to establish privately owned drug stores in the eighth century, and the Persian pharmacist, physician and philosopher Avicenna, contributed much to the sciences of pharmacy and medicine through works such as *Canon Medicinae* (Cragg & Newman, 2005).

1.2.2 Why are natural products useful for drug discovery

Worldwide, it is estimated that less than 10% of all natural products have been evaluated for biological activity; many more useful novel natural compounds still await discovery (Cragg & Newman, 2005). Natural products have evolved and adapted over millions of years through defense against bacteria, insects and fungi, or interference with competing organisms, to become unique, structurally diverse secondary metabolites. Whatever their precise role, many natural products can reach human receptor sites on or within cells, just as a drug must do. The large number of pure natural products which have been found to interact with specific mammalian receptors testifies to the inherent bioactivity in natural products (Beutler, 2009).

The value of natural product chemistry is in providing novel molecular skeletons and functionalities, which may be used as templates for the development of new drugs. Recently, pharmaceutical scientists have experienced difficulty in identifying new lead structures, templates and scaffolds in the finite world of chemical diversity (Patwardhan *et al*., 2004). A number of synthetic drugs have adverse and unacceptable side effects (Karimi *et al*., 2015). There have been impressive successes with botanical medicines, most notably quinghaosu, artemisinin from Chinese medicine (Tu, 2011), its derivative was found to treat human cervical cancer in clinical trial (Jansen *et al*., 2011).

1.2.2.1 Natural products from plants

Plants have been known for their medicinal uses for thousands of years. About 80% of people in developing countries of the world still rely on plant-based medicines for primary health care (Farnsworth *et al*., 1985). Plant medicines have a long history of use for the treatment of cancer (Hartwell, 1982). The most widely used breast cancer drug is paclitaxel, which was originally isolated from the bark of *T. brevifolia* in 1962 by the United States Department of Agriculture (Cragg, 1998). Another example is tubocaurarine, originally isolated from the climbing plant, *Chondrodendron tomentosum*, and is one of the active constituents in a muscle relaxant used in surgical operations (Dewick, 2002).

1.2.2.2 Natural products from fungi

Like plants, fungi have been a part of human life for thousands of years. Fungi have been utilized in various aspects from food (mushrooms), alcoholic beverages (yeasts), traditional medicine (Hays & Watson, 2019), and to human rituals (Samorini, 1992).

Fungi have been used as medicine since the Neolithic and Paleolithic eras (Samorini, 2001). Mushrooms have been used in health care for treating simple and age-old common diseases like skin diseases, as well as present day health challenges including pandemic diseases like AIDS (Chang, 2006). They possess many biological activities including anti-bacterial, anti-inflammatory, anti-oxidant and anti-cancer properties. Compounds isolated from mushrooms such as *Ganoderma pfeifferi* Bres., *Lentinus edodes* (now *Lentinula edodes* (Berk.) Pegler) and *Cortinarius basirubescens* J.B. Cleland & J.R. Harris showed antimicrobial activity against pathogenic microorganisms like bacteria associated with nosocomial infections. (Alves *et al*., 2012). Fractions from the mushroom *Inonotus obliquus* (Ach. ex Pers.) Pilát were found to have anti-inflammatory effects on NO production and NF-κB luciferase activity in macrophage RAW 264.7 cells (Ma *et al*., 2013). A water extract from *L. edodes* showed potent anti-oxidant activity in three different assays (Cheung *et al*., 2003). Mushrooms are also one of the main sources for anticancer drug discovery (Newman & Cragg, 2012).

1.3 Mushrooms as a source of cancer treatment or prevention

1.3.1 Medicinal mushrooms and their anti-tumor activities

Conventional cancer therapies have side effects, as mentioned above; recently, some researchers have focused on treating cancers and improving the patient's quality of life by modifying the host's biological response against the malignant invasion. Immunotherapy is now gaining more attention, since it causes considerably fewer side effects and helps to overcome cancer growth (Farkona *et al*., 2016). Immunotherapy uses the body's immune system, either directly or indirectly, to fight cancer or to reduce the side effects that may be

caused by some severe cancer treatments (Kakimi *et al*., 2009). Figure 3 illustrates some medicinal mushrooms with anti-cancer potential properties.

Figure 3 Some medicinal mushrooms with anti-cancer potential (Modified from Patel, S., & Goyal, A., 2012)

There are many forms of biological immunotherapeutic agents, such as monoclonal antibodies, interferons, cancer vaccines and nonspecific immuno-modulating agents. Many mushroom-derived extracts are recognized as immuno-modulators or biological response modifiers (Lull *et al*., 2005, Novak & Vetvicka, 2009). Medicinal mushrooms, in particular, are not only hubs for a myriad of immuno-modulators, but also a source of anti-cancer agents, which could interfere with specific cellular signal transduction pathways linked to cancer development and progression (Kudugunti *et al*., 2010). Recently, more than 30 species of identified medicinal mushrooms (e.g, *Phellinus linteus* (Berk. & M.A. Curtis) Teng, *Grifola frondosa* (Dicks.) Gray, *Hypsizygus marmoreus* (Peck) H.E. Bigelow and *Flammulina*

velutipes (Curtis) Singer have showed antitumor activity in experimental studies (Wasser, 2011). Medicinal mushrooms show therapeutic action against the development of cancer cells, primarily because they contain a number of biologically active high and low molecular weight compounds (Lee & Hong, 2011, Li *et al*., 2015).

1.3.1.1 High molecular weight compounds

The high molecular weight compounds which have been found in medicinal mushrooms are mainly polysaccharides, proteins, and polysaccharide-protein complexes (Wasser, 2003). For example, lentinan, an anti-tumor polysaccharide produced by *L. edodes*, this mushroom is widely consumed as a nutritional health food throughout the world, especially in Asia, and is known to possess strong host-mediated anti-cancer activity by activating the immune system (Cheung, 2008). Mushrooms can also produce a large number of proteins with significant biological activities (Wong *et al*., 2010). Lectins, which are proteins that bind to cell surface carbohydrates, have the ability to produce cell agglutination and show several anti-proliferative and anti-tumor activities against cancer cell lines (Zhang *et al*., 2009). Lectins are abundant in many species of mushroom, including *Agaricus bisporus* (J.E. Lange) Imbach and *Pleurotus ostreatus* (Jacq.) P. Kumm. (Hassan *et al*., 2015). Medicinal mushrooms have immuno-stimulatory activity not only because of their bioactive polysaccharides, but also by having varying combinations of polysaccharide-protein complexes (Moradali *et al*., 2007). For instance, *Agaricus subrufescens* Peck is a well-known mushroom having several biologically active metabolites, including polysaccharides and glycoproteins that are thought to be responsible for its immuno-stimulant and anti-tumor properties (Wisitrassameewong *et al*., 2012). Table 1 shows a list of some biologically active large molecules from mushrooms.

Table 1 Large compounds from mushroom and their biological properties

Mushroom	Compounds	Biological activity	Reference
Polysaccharides:			
Paxillus involutus (Batsch) Fr.	GIP-inv polysaccharide	Anti-proliferative (apoptosis induction)	(Barad *et al*., 2018)
L. edodes	D-glucopyranose	Immunomodulatory	(Zheng *et al*., 2005)
Schizophyllum commune Fr.	Homopolysaccharide	Immunomodulatory	(Kumari *et al*. 2008)
Tricholoma crissum Sacc.	Hetero polysaccharide	Macrophage activations	(Patra *et al*., 2012)
P. ostreatus	Hetero polysaccharide	Stimulates macrophages	(Maity *et al*., 2011)
Lentinus squarrosulus Mont.	Heteroglycan	Immunoenhancing	(Bhunia *et al*., 2010)
Proteins/Enzymes:			
Russula delica Fr.	Lectin	Anti-proliferative	(Zhao *et al*., 2010)
H. marmoreus	(RIP) Ribosome inactivating protein	Anti-proliferative	(Wong *et al*., 2008)
Pleurotus citrinopileatus Singer	Glycoprotein	Immunomodulatory	(Chen *et al*., 2009)
Ganoderma lucidum (Curtis) P. Karst.	Glycoprotein	Antioxidant activity	(Du *et al*., 2007)
G. frondosa	(FIP) Fungal immunomodulatory protein	Enhances cytokine	(Kodama *et al*., 2010)

1.3.1.2 Low molecular weight compounds

Mushrooms produce a variety of complex low molecular weight compounds with diverse chemical compositions, such as phenolic compounds, polyketides and triterpenoids (Elisashvili, 2012). Ergosterol is a provitamin form of vitamin D_2 in human nutrition, it abundant in mushrooms such as *A. subrufescens* and *Lentinula edodes* (Wisitrassameewong *et al*, 2012, Jasinghe & Perera, 2005). Grifolin is a natural biologically active metabolite that was isolated from the mushroom *Albatrellus confluens* (Alb. & Schwein.) Kotl. & Pouzar; it was identified as a potential antitumor agent that can inhibit cancer cell growth by inducing apoptosis *in vitro* (Ye *et al*., 2005). Grifolin could also exert anti-oxidative activity and antimicrobial activity (Quang *et al*., 2006). Medicinal mushrooms have been reported to produce various polyphenolic pigments, known as the styrylpyrone class of phenols derived from polyketide pathways, which show significant biological effects including anti-cancer properties (Lee & Yun, 2011). For example, some early studies have shown that hispidin, is more cytotoxic toward cancerous cells (pancreatic duct and keratinocyte) than normal cells (Gonindard *et al*., 1997). Some potent biological small molecules purified from the fruiting bodies of mushrooms are shown in Table 2.

Table 2 Small compounds from mushrooms and their biological properties

Mushroom	Compounds	Biological activity	Reference
Agaricus blazei Murill	Ergosterol	Growth-inhibitory activities	(Takaku *et al*., 2001)
A. confluens	Grifolin	Growth-inhibitory activities	(Ye *et al*., 2005)

Suillus placidus (Bonord.) Singer	Irofulven	Immuno enhancing	(Fung & Tan, 2015)
G. lucidum	Ganoderic acid	Induces apoptosis	(Liu & Zhong, 2010)
I. obliquus	Ergosterol peroxide	Anti-inflammatory activities	(Ma *et al*., 2013)
Hericium erinaceum Burds., O.K. Mill. & Nishij.	Hericerin	Induction of apoptosis	(Li *et al*., 2015)
P. ostreatus	Concanavalin A	Anti-inflammatory activities	(Jedinak *et al*., 2011)
Phaeolus schweinitzii (Fr.) Pat.	Hispidin	Antioxidant activity	(Han *et al*., 2013)
Antrodia cinnamomea T.T. Chang & W.N. Chou	25R-antcin K & dehydrosulphuric acid	Growth-inhibitory effect	(Huang *et al*., 2018)
A. confluens	Conflamides D and E	Anti-proliferative	(Zhang *et al*., 2018)

1.3.2 Experimental studies with mushrooms

The mushroom *Agaricus blazei* is used as food, as natural remedy for cancer prevention, and as adjuvant in cancer treatment (Firenzuoli *et al*., 2008). There is also a similar study about adjuvant properties that investigated the combination of *A. blazei* extract with marine phospholipids on myeloma sp2 tumor suppression when administrated orally. Results of this *in vivo* study indicated that oral administration of *A. blazei* extract, with or without encapsulated liposomal form, suppressed myeloma in mice. Additionally, the study suggested

that the anti-tumor effect of marine phospholipid occurred without weight loss, which might indirectly affect the treatment of the disease (Murakawa *et al*., 2007).

The components of the fungus *Polyozellus multiplex* (Underw.) Murrill were tested *in vitro* against human gastric cancer cells as well as other cancer cells. The water fraction of *P. multiplex* dramatically increased glutathione S-transferase (GST) and superoxide dismutase activities, showing a tendency of increasing glutathione (GSH) levels, as compared to the N-methyl-N-nitro-N-nitrosoguanidine (MNNG) group alone (Lee & Nishikawa, 2003). The results demonstrated that proliferation of distinct types of cancer cells could be significantly inhibited by the *P. multiplex* water fraction, suggesting its possible use as a chemo-prevention agent in stomach cancer (Lee & Nishikawa, 2003).

1.3.3 Clinical studies with mushrooms

Some medicinal mushrooms with anti-cancer effects, subjected to human clinical trials, are indicated in Table 3.

Table 3 Summary of human clinical studies in anticancer effects of medicinal mushrooms

Mushroom	Type of study and number of participants	Type of cancer	Extracts or compounds or Active principle	Findings	Reference
A. bisporus	Observational study, 500 participants	Ovarian	Polysaccharides	Moderate inverse association between habitual mushroom intake and epithelial ovarian cancer	(Lee *et al*., 2013)
A. bisporus/ Amauroderma rude (Berk) Torrend	Randomized, clinical trial, 373 adults	Various types	Spore vs mycelium	Improve tumor response of lung cancer to conventional therapy. Enhance immunity	(Figueiredo & Régis, 2017)
Lentinula edodes (Berk.) Pegler	Open pilot study, 10 participants	Various types	*L. edodes* mycelia	Combine treatment of LEM and immunotherapy	(Tanigawa *et al*., 2016)

The first observational report associating mushrooms (*A. bisporus* and shiitake) intake and ovarian cancer was conducted in China; the participants were 500 patients diagnosed with ovarian cancer, along with a control group of 500 healthy participants. The patients with ovarian cancer consumed less mushrooms than the controls, and results showed that mushroom intake at increasing levels is associated with a decreasing risk of ovarian cancer (Lee *et al*., 2013). In another clinical trial, the effectiveness of the mushroom *Pleurotus cornucopiae* (Paulet) Rolland was evaluated and the mushroom extract was shown to exert immuno-potentiation for several diseases including cancer (Tanaka *et al*., 2016). *P. cornucopiae* has also been traditionally consumed by Japanese people due to its cancer preventative properties (Tanaka *et al*., 2016).

Ganoderma lucidum, also known as *Reishi* mushroom, is widely believed to be responsible for longevity and general well-being; recently, it has also been used as an alternative medicine in cancer treatment. A review has evaluated the clinical activities of *G. lucidum* to prolong survival time, tumor response, and the quality of life in cancer patients (Jin *et al*., 2016). This study demonstrated that the incorporation of *G. lucidum* extract treatment can enhance tumor response of lung cancer in conventional therapy, and is believed to improve immunity by stimulating T-lymphocyte proliferation, suggesting that it is a promising adjuvant to restrain the undesired immunosuppressive effect of many chemotherapeutic drugs. Furthermore, this study reported an improvement in the quality of life of cancer patients who were treated with *G. lucidum*, and concluded the absence of serious toxicity through the clinical trial as it was well tolerated by most participants.

1.4 Mushrooms in British Columbia

There is general acceptance among mycologists that the number of global fungal species is between 1 to 5 million (Blackwell, 2010). It is believed that there are about 150,000-160,000 species of mushrooms in the world, and approximately 10% have been explored (Wasser, 2014). This large diversity provides a huge sample source from which new anti-cancer compounds can potentially be discovered, purified, identified, and further studied for pharmaceutical use. Besides the work from Dr. Lee's lab at UNBC, only two other studies have been conducted on the bioactivity of mushrooms in Canada. The first one was the discovery of a novel compound, ganoderma aldehyde, derived from the mushroom *Ganoderma applanatum* (Pers.) Pat. which was collected in the forest of British Columbia (BC). However, the bioactivity of the compound isolated was not described in detail (Ming *et al*., 2002). Another study involved the mushroom *I. obliquus*, collected from Manitoba and was shown to exert anti-inflammatory activity on Raw 264.7 macrophage cells. However, no further studies were done to identify and purify the active compound (Van *et al*., 2009).

Currently, there are several ongoing research projects being carried out in Dr. Lee's lab, focusing on screening a variety of mushrooms collected from British Columbia for their anti-cancer properties (anti-proliferative, immuno-stimulatory and anti-inflammatory) (Smith *et al*., 2017; Deo *et al*., 2019). In one study, 17 different fungal species were collected from multiple locations in Haida Gwaii and were screened for anti-cancer properties (Deo *et al*., 2019); among these mushrooms, *Amanita augusta* Bojantchev & R.M. Davis, *Phellodon atratus* K.A. Harrison, *Guepinia helvelloides* (DC.) Fr., *Chroogomphus tomentosus* (Murrill) O.K. Mill.,

Laetiporus conifericola Burds. & Banik, and *Inocybe sp*. were shown to have strong anti-proliferative and anti-inflammatory activities. In another study, a polysaccharide extracted from the fungus *P. involutus*, collected from Prince George, BC, was shown to possess growth-inhibitory activity against several cancer cell lines and could also trigger apoptosis of cancer cells (Barad *et al*., 2018). Yet another study also revealed that an anti-inflammatory polysaccharide, successfully isolated from *Echinodontium tinctorium* (Ellis & Everh) Ellis & Everh. exhibited activity both *in vitro* and *in vivo* (Javed *et al*., 2019a). A companion study, testing the methanol extract from *I. obliquus,* showed similar anti-inflammatory activities (Javed *et al*., 2019b). A recent study, characterizing 3 compounds from the terricolous polypore *Albatrellus flettii* Morse ex Pouzar, documented the suppression of KRAS expression in human colon cancer cells (Yaqoob *et al*., 2020). Many other studies are ongoing in the lab, including my project. Therefore, taking into account the limited research conducted on the bioactivity of BC wild mushrooms, it seemed logical to further study the potential therapeutics of Canadian mushrooms in the field of cancer.

1.5 Future prospects of drug discovery from mushrooms

Today, some medicinal mushrooms are identified as sources for biologically active metabolites with potential anti-cancer properties. The bioactive metabolites from medicinal mushrooms could offer beneficial effects, not only as pharmaceutical drugs but also as a novel class of supportive products (Jiang & Silva, 2010). Examples of the available drug products and supplementary foods developed from medicinal mushrooms claiming to provide beneficial effects on immune stimulation and help in cancer prevention are shown in Figure 4.

Figure 4 Examples of products marketed with claimed immune stimulatory and anti-cancer properties containing mushrooms or their extracts 1. Dr Myko San—Health from Mushrooms 2. *Ganoderma lucidum* Spore 3. Grifron Maitake Mushrooms 4. I'm-Yunity® 5. MC-S (Metabolic Cell Support) 6. MycoPhyto® Complex 7. Red Reishi 8. Super Royal *Agaricus* Mushroom. *Figure modified from De Silva et al. (2012)*

Medicinal mushrooms contain many active metabolites that can be used as support remedies for cancer treatments (Evidente *et al*., 2014). The above examples just describe a few studies about anti-cancer properties of mushrooms, and the numbers will keep increasing as more mushrooms are tested. With articles citing their medicinal potential, it is clear that medicinal mushrooms would serve as ideal candidates for the further discovery of a potential anti-cancer drug.

1.6 Research goals

Based on the fact that relatively limited studies have been done on bioprospecting wild mushrooms native to Canada, I set out to explore several wild mushrooms found in BC. The specific research objectives of my MSc. thesis are:

(i) To chemically extract and biologically assess eight Canadian mushroom collections (comprising 3 species in two genera, *Fomitopsis* and *Phaeolus*) for anti-cell viability activity.

(ii) To purify and characterize the small molecule growth-inhibitors from the unique species of *Phaeolus*.

Chapter 2 Evaluation of wild mushrooms collected in British Columbia for anti-cell viability activity

This chapter describes studies investigating eight mushroom collections from BC (including the 2 genera *Fomitopsis* and *Phaeolus*). Each sample was identified by morphological characteristics and DNA analysis. Samples were pulverized and two extraction techniques were employed using various parameters such as solvents, temperatures and time. Finally, extracts from each sample were screened for anti-cell viability activity against HeLa human cervical cancer cells.

About 40 species have been recorded in *Fomitopsis* genus worldwide (Li *et al*., 2013). Most species are parasitic on woody plants, and tend to cause brown rots (Cannon & Kirk, 2007). *Fomitopsis pinicola* (Sw.) P. Karst. is a brown-rot fungus, which grows on coniferous and broad-leaved trees and is widespread in north America, in addition to Asia and Europe (Rösecke *et al*., 2000). This mushroom belongs to the family Polyporaceae, the dried fruiting bodies of different *Fomitopsis* species have been used in folk medicine as haemostatics and anti-inflammation agents (Keller *et al*., 1996). Japanese people have widely cultivated this mushroom to be used as a health food source for growth regulation and diabetes (Högberg *et al*., 1999). In addition, *F. pinicola* has also been used as an anti-diabetic material in Korea as a traditional folk medicine (Lee *et al*., 2008). In Europe, *F. pinicola* is considered to be a nontoxic mushroom (Keller *et al*., 1996).

The genus *Phaeolus* is distributed in the northern hemisphere and Australia (Sinclair & Lyon, 1987 & Simpson & May, 2002), and the name is derived from the Ancient Greek word, meaning "dark" or "obscure" (Donk, 1960). The genus *Phaeolus* comprises 27 species

(Patouillard, 1900) and the most important one, from a forestry standpoint, is *schweitnitzii*. *P. schweinitzii* is a common root and butt pathogen of conifers in North America and Eurasia (Sinclair & Lyon, 1987). *Schweinitzii* root and butt rot is a major disease of older trees; it causes decay of the roots and lower stem and the resulting strength loss predisposes trees to windthrow and breakage (Fairweather, 2006).

2.1 Materials and methods

2.1.1 Collection and identification of wild mushrooms

All wild mushrooms were collected from Haida Gwaii, British Columbia in August 2015. A pocketknife or a hatchet was used to separate the mushroom from the tree substrate to which it was attached. The morphological descriptions such as shape, size and color were recorded, as well as location, habitat and surrounding vegetation to be used in mushroom identification. Paper bags were used for temporary storage of samples. After that, mushrooms were dried for further analysis. Two different methods were used to identify the mushrooms: macro-morphological analysis and DNA analysis.

2.1.1.1 Macro-morphological identification

This type of identification is based on morphological details and characteristics of the mushrooms, as different mushrooms will exhibit different morphological appearances such as cap, spore-bearing surface, stipe, veils and odor, which could help identify the samples preliminarily. I used the reference Mushrooms Demystified by David Aurora (1986) as an identification guide, as it is effective to determine the mushroom or narrow down the identity of the mushroom samples to family, genus and often species level. In addition, Matchmaker Mushroom ID Software (Gibson *et al*., 2010) was used for further identification of mushroom

samples. This software describes the details of over 4000 gilled and non-gilled mushroom species from the Pacific Northwest, and can help to identify the mushroom species with more detailed information.

2.1.1.2 Identification of mushrooms using DNA analysis

Identification of mushroom based on morphology is simple and efficient, but it can be misleading due to several factors, like hybridization (Olson & Stenlid, 2002) and convergent evolution (Brun & Silar, 2010). DNA-based identification methods have emerged as an efficient way to distinguish species among diverse fungi (Raja *et al*., 2017).

Genomic DNA was extracted from collected mushrooms using a MoBio DNA PowerSoil Kit. After successful DNA extraction, ITS3 and NLB4 primers were used to amplify the sequences of the samples (Smith *et al.,* 2017). The genomic DNA (gDNA) of mushroom samples were subjected to PCR using the conditions indicated in Tables 4 and 5.

Table 4 PCR reaction protocols required to amplify the extracted DNA from the mushroom species.

Component	Volume used (µL)
H_2O	16
10x Thermo Pol Buffer	2.5
dNTPs (2.5 mM)	2.5
ITS3 (10 µM)	1
NLB4 (10 µM)	1
DNA template	1.5
TAQ polymerase	0.5

Table 5 PCR conditions for DNA amplification.

Step	Temp	Time
1	95 °C	5min
2	95 °C	30sec
3	52 °C	30sec
4	72 °C	30sec
5	72 °C	10min
6	4 °C	10min

The exponentially amplified DNA samples were further subjected to gel electrophoresis to confirm the success of DNA amplification and ensure sufficient quantity for DNA sequencing. The post-PCR amplified DNA products needed to be cleaned using the QIAquick PCR kit.

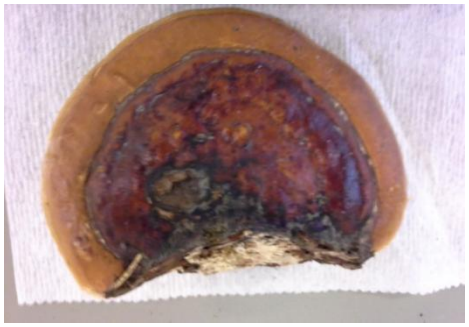
The sequences obtained from Macrogen Corporation were compared with sequences available in the National Center for Biotechnology Information (NCBI) internet database, GenBank, using the Basic Local Alignment Search Tool (BLAST) program. The best Genbank match of mushroom species is shown in Table 6. The photographs and data on the mushrooms are shown in Table 7.

Table 6 Identification of mushroom species using BLAST searches, along with the percent of matching bases between the unknown sample and the closest match.

Identity	**Collected By**	**Best GenBank Match (% similarity/ % coverage)**
Fomitopsis pinicola (50)	Drs. Chow Lee, Wai Ming Li, Hugues Massicotte, Keith Egger, and Ms. Linda Tackaberry	KC595922.1 (98% / 100%)
Fomitopsis pinicola (53)		EF530947.1 (100% / 100%)
Fomitopsis pinicola (65)		KC595922.1 (98% / 100%)
Fomitopsis pinicola (77)		EF530947.1 (100% / 100%)
Phaeolus schweinitzii (58)		KC581334.1 (99% / 100%)

Phaeolus schweinitzii (60)		KC581354.1 (99% / 100%)
Phaeolus sp. (71)		MH277962.1 (100% / 71%)

Table 7 Collection location and photographs of the mushrooms collected from different locations in British Columbia.

Collection Number	Site of collection	Date of collection	Photographs
Fomitopsis pinicola			
Collection Number # 50	Cape Fife Trail, Naikoon Park, Haida Gwaii, BC	August 24, 2015	
Collection Number # 53	Cape Fife Trail, Naikoon Park, Haida Gwaii, BC	August 24, 2015	
Collection Number # 65	Pesuta Shipwreck Trail, Naikoon Park, Haida Gwaii, BC	August 25, 2015	
Collection Number # 77	Copper Rd-Sandspit area (towards Gray Bay, Haida Gwaii), BC	August 26, 2015	

Phaeolus schweinitzii			
Collection Number # 57	Tlell, Crow's Nest Cafe, Haida Gwaii, BC	August 24, 2015	
Collection Number # 58	Anvil Interpretive Trail, Tlell, Haida Gwaii, BC	August 25, 2015	
Collection Number # 60	Anvil Interpretive Trail, Tlell, Haida Gwaii, BC	August 25, 2015	
Phaeolus sp.			
Collection Number # 71	Pesuta Shipwreck Trail, Naikoon Park, Haida Gwaii, BC	August 25, 2015	

2.1.2 Chemical extraction of mushrooms

After collection, the mushroom specimens were dried at low heat (50 °C) and then powdered (Figure 5) using either a regular household blender or a hammer mill.

Figure 5 An example of a dried and powdered mushroom sample

The chemical extraction protocol has been established in Dr. Chow Lee's lab: dried samples were extracted with four different solvents, firstly with 80% ethanol and 50% methanol, which were expected to obtain low molecular weight compounds. After this, the remaining mushroom powder was extracted using water, and then 5% sodium hydroxide. The water and 5% NaOH extract were expected to contain water-soluble polysaccharides. The first three solvent extractions were performed using the Dionex Accelerated Solvent Extractor (ASE) 350 Speed Extractor (Thermo Fisher) at 65°C and at a pressure of 1500 psi. The Dionex ASE 350 is an automated, easy and effective tool for sequential extraction (Richter *et al*., 1996). The 5% NaOH extract was done manually at 65°C as it will corrode the walls of the machine. The outline of the extraction process using the Speed Extractor is shown in Table 8:

Table 8 Outline of the extraction process

Dionex ASE 350 Extraction Process	Duration
Static extraction with rinse (80% ethanol)	4 cycles, 15 minutes per cycle
Static extraction with rinse (80% ethanol)	4 cycles, 15 minutes per cycle

Static extraction with rinse (80% ethanol)	4 cycles, 15 minutes per cycle
Static extraction with rinse (80% ethanol)	4 cycles, 15 minutes per cycle
Manual Extraction	**Duration**
5% sodium hydroxide extraction	6 hours with constant stirring

The obtained mushroom extracts were labeled as E1, E2, E3 and E4 for 80% ethanol, 50% methanol, water, and 5% NaOH extracts respectively.

2.1.3 Rotary evaporation, pH neutralization, dialysis, and lyophilization of the extracts

All extracts were further concentrated by eliminating the redundant solvent volume. This process was achieved by using a rotary evaporator. Once the volume of the extractions was reduced to a required amount, the extracts were subjected to pH neutralization. The pH range used as reference to neutralize the mushroom extracts was 6.90 to 7.10. For pH neutralization, either 2 M of NaOH or 2 M of HCl was added. After this, the extracts were dialyzed using Thermo Scientific SnakeSkin Dialysis Tubing (3.5K MWCO) for 24 hours in order to remove salts. Finally, extracts were lyophilized to become dried: the extracts were firstly frozen in -80 ºC freezer and then lyophilized by using Labconco Freeze dryer (0.008 bar vacuum pressure and -80 ºC). Freeze-dried samples were stored at 4°C for subsequent experiments. For long-term storage, dried samples were kept at -80°C.

2.1.4 Preparation of stock solution and filter sterilization

To prepare stock solution, the solubility needs to be taken into account when making concentrated extracts. The extracts were analyzed first for their maximum solubility in an

appropriate solvent. After obtaining the stock solution of the extracts, the solutions were filter sterilized with 0.2 µm filters (Sarstedt, Nümbrecht, Germany). Finally, the filtered solutions were assessed for anti-cell viability activity.

Figure 6 outlines the chemical extraction and the downstream steps taken.

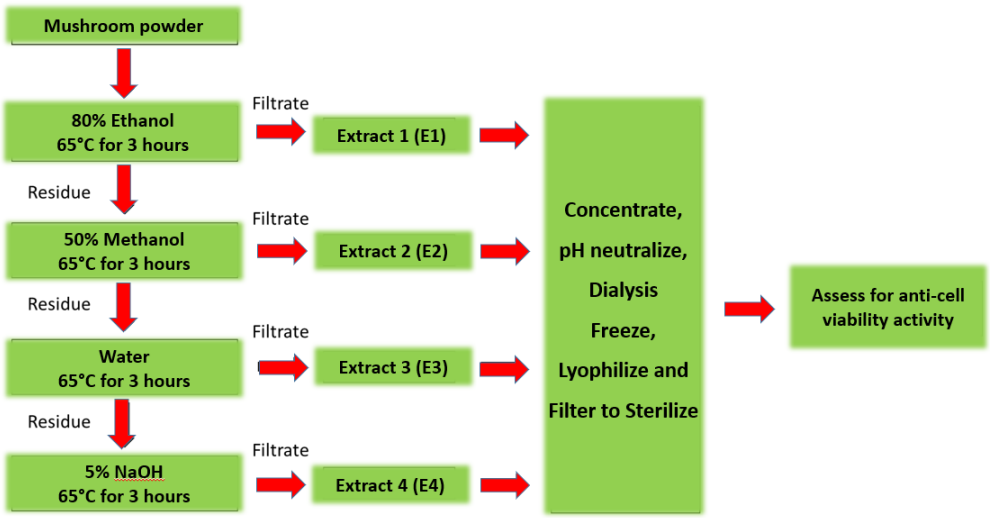


Figure 6 Sequential chemical extraction

2.1.5 Assessment for anti-cell viability activity

2.1.5.1 MTT assay

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] is a water-soluble yellow dye and can be reduced to water-insoluble purple formazan crystals by the dehydrogenase system of active cells (Carmichael *et al*., 1987). The MTT tetrazolium assay is a popular tool in assessing cytotoxicity and cell viability (Van Meerloo *et al*., 2011). The test is based on enzymatic reduction of the lightly colored tetrazolium salt to its formazan of intense

purple-blue color; the concentration of the purple formazan makes it possible to estimate the number of viable cells, which can be quantified spectrophotometrically.

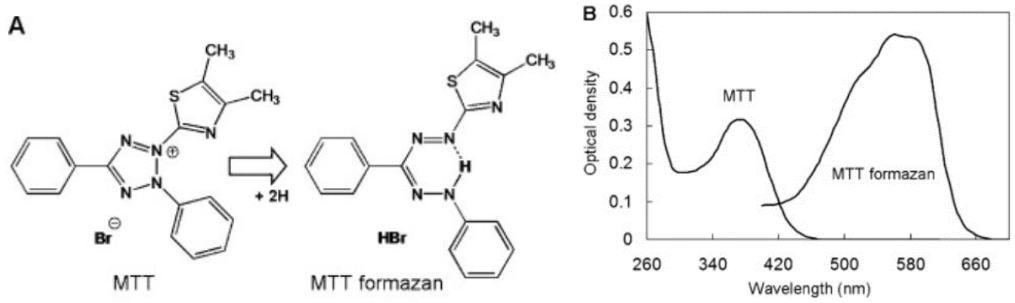


Figure 7 (A) Chemical structure of MTT and its reduced formazan product; (B) absorption spectra of MTT

Dimethyl sulfoxide (DMSO) was used to dissolve formazan crystals. The absorbance at 570 nm was measured on a spectrophotometer Synergy 2 multi-plate reader (BioTek®, USA) as formazan has the highest optical density at 570 nm (refer to Figure 7 B).

2.1.5.2 Dose-dependent MTT assay to determine anti-cell viability activity

Dried mushroom extracts in powder were reconstituted in their respective suitable solvents, shown in Table 11. For extracts which were dissolved in water, the stock concentration was prepared at 20 mg/mL. As for methanol soluble extracts, the stock concentration was prepared at 40 mg/mL. The stock solutions were then filter sterilized using 0.2 μm filter (Sarstedt, Nümbrecht, Germany).

For the dose-dependent MTT assay, the stock solution was further diluted by adding EMEM (Eagle's minimum essential media) supplemented with 10% fetal bovine serum (FBS) to make different doses of extracts. HeLa cells (ATCC®, Maryland US) cells were first subjected to

treatment with different concentrations (from 0.1 to 1 mg/mL) of the extracts for 48 hours and the MTT assay was performed.

The dose-dependent MTT assay was used to investigate the percent viability of HeLa cells after treating with different concentrations of mushroom extracts. HeLa cells were maintained in a T-25 flask with around 10 mL of Lonza Eagle's Minimum Essential Medium (EMEM by VWR, ON, CA) supplemented with 10% v/v FBS. When reaching 80% of confluency, the growth media (EMEM+ 10% FBS) was removed from the flask and cells were washed with 4 mL of Dulbecco's Phosphate Buffer Saline (DPBS) (Lonza, Maryland), followed by 1 mL of Trypsin EDTA to remove cells which adhere to the bottom of T-25 flask surface.

Cells were plated into a 96-well plate. The plated cells at (1500 cells/100 µL/well) were incubated for 24 hours at 37°C in 5% CO_2. After incubating for 24 hours, different concentrations of mushroom extracts were prepared with EMEM (containing10% FBS and the respective solvents) as shown in Table 9.

Table 9 Representation of the different doses of mushroom extracts used for MTT assay.

		For One Well			**For 6 Wells**		
Final conc. (mg/mL) (in plate)	**Stock (mg/mL) (in tube)**	**Diluted water in MEM (µL)**	**Fraction at 4 mg/mL**	**EMEM (µL)**	**Diluted water in MEM (µL)**	**Fraction at 4 mg/mL**	**EMEM (µL)**
1	2	0	50	50	0	300	300
0.75	1.5	12.5	37.5	50	75	225	300
0.5	1	25	25	50	150	150	300
0.375	0.75	31.2	18.75	50	187.5	112.5	300
0.25	0.5	37.5	12.5	50	225	75	300
0.1	0.2	45	5	50	270	30	300
0	0	50	0	50	300	0	300
		Total Volume of 100 µL			Total Volume of 600 µL		

Finally, the different doses of 100 µL mushroom extracts as well as the negative control (water or methanol) were added to the cells. Five replicates of each sample and control (same solvent in which the fractions were reconstituted) were included. The plate pattern is shown in Table 10.

Table 10 Plate pattern (96-well) followed to screen different doses of extracts from selected mushroom samples, for their potential to exhibit anti-proliferative activity based on the MTT assay.

	1	2	3	4	5	6	7	8	9
A	water	water	water	water	water	water	water	water	water
B	water	1 mg/mL	0.75 mg/mL	0.50 mg/mL	0.375 mg/mL	0.25 mg/mL	0.1 mg/mL	Control	water
C	water	1 mg/mL	0.75 mg/mL	0.50 mg/mL	0.375 mg/mL	0.25 mg/mL	0.1 mg/mL	Control	water
D	water	1 mg/mL	0.75 mg/mL	0.50 mg/mL	0.375 mg/mL	0.25 mg/mL	0.1 mg/mL	Control	water
E	water	1 mg/mL	0.75 mg/mL	0.50 mg/mL	0.375 mg/mL	0.25 mg/mL	0.1 mg/mL	Control	water
F	water	1 mg/mL	0.75 mg/mL	0.50 mg/mL	0.375 mg/mL	0.25 mg/mL	0.1 mg/mL	Control	water
G	water	water	water	water	water	water	water	water	water

On day four, 50 µL of MTT diluted with EMEM solution (1 mg/mL) was added to the cells. The cells were incubated for three hours allowing the live cells to react with the MTT dye, and converting it to formazan crystals by mitochondrial reductase. Finally, the supernatant was removed and 150 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was read using Synergy 2 multi-plate reader. The activity of various fractions was measured in percent cell viability compared to that of the control. Standard deviation was the measure of error for the MTT bioassay. Kaleidagraph© version 4.03 (Synergy Software) was used to generate the graph for the acquired data. The workflow is shown in Figure 8.

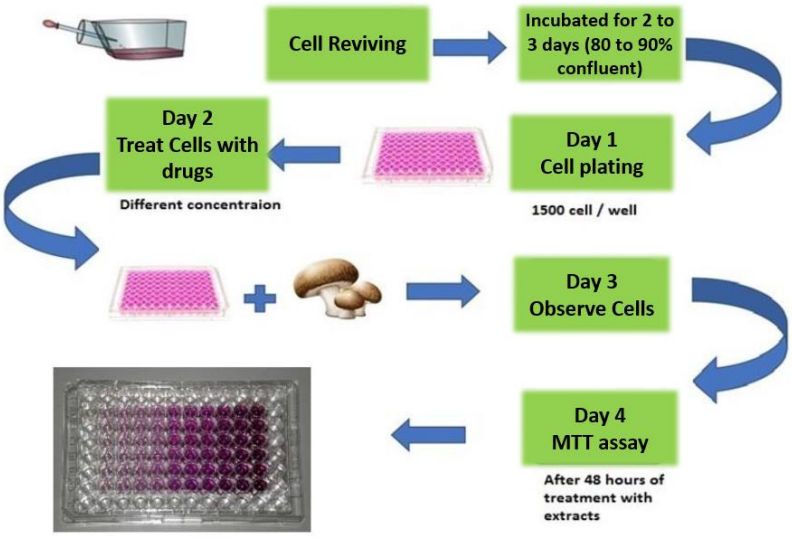


Figure 8 Schematic representation of dose-dependent MTT assay protocol using HeLa Cells.

2.2 Results and discussion

2.2.1 Chemical extraction of mushrooms

The Dionex ASE 350 speed extractor was used to obtain E1, E2 and E3 crude extracts while the E4 5% sodium hydroxide extract was done manually using a hotplate. The summary of extraction results is shown in Table 11.

Table 11 Summary of the fungal extracts obtained through successive extraction process.

Extraction sample (g)	Extract type	Solvent used for extraction	Extract weight (gm)	Yield (%)	Physical appearance	Solvent used for reconstitution
Fomitopsis pinicola (#50)						
5.403	E1	80% Ethanol	0.174	3.22	Coarse powder	Methanol
	E2	50% Methanol	0.093	1.72	Coarse powder	Methanol
	E3	Water	0.046	0.85	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.724	13.39	Fluffy (Cotton like)	Water
Fomitopsis pinicola (#53)						
4.831	E1	80% Ethanol	0.469	9.71	Coarse powder	Methanol
	E2	50% Methanol	0.186	3.85	Coarse powder	Methanol

	E3	Water	0.045	0.93	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.401	8.30	Fluffy (Cotton like)	Water
Fomitopsis pinicola (#65)						
5.057	E1	80% Ethanol	0.054	1.06	Coarse powder	Methanol
	E2	50% Methanol	0.078	1.54	Coarse powder	Methanol
	E3	Water	0.067	1.32	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.508	10.05	Fluffy (Cotton like)	Water
Fomitopsis pinicola (#77)						
4.987	E1	80% Ethanol	0.322	6.46	Coarse powder	Methanol
	E2	50% Methanol	0.113	2.27	Coarse powder	Methanol
	E3	Water	0.148	2.97	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.249	4.99	Fluffy (Cotton like)	Water
Phaeolus schweinitzii (#57)						
5.316	E1	80% Ethanol	0.406	7.68	Coarse powder	Methanol
	E2	50% Methanol	0.415	7.81	Coarse powder	Methanol
	E3	Water	0.304	5.72	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.652	12.26	Fluffy (Cotton like)	Water
Phaeolus schweinitzii (#58)						
5.325	E1	80% Ethanol	0.210	3.94	Coarse powder	Methanol
	E2	50% Methanol	0.216	4.06	Coarse powder	Water
	E3	Water	0.177	3.32	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.873	16.39	Fluffy (Cotton like)	Water
Phaeolus schweinitzii (#60)						
5.345	E1	80% Ethanol	0.189	3.53	Coarse powder	Methanol
	E2	50% Methanol	0.292	5.46	Coarse powder	Water
	E3	Water	0.156	2.91	Fluffy (Cotton like)	Water
	E4	5% NaOH	0.640	11.97	Fluffy (Cotton like)	Water

Generally, the extract E4 has the highest yield amongst all extracts except for mushroom sample #53 and #77. The lowest yields were obtained mostly for E3 water extracts. The highest

yield (16.39%) amongst all extracts was exhibited by E4 of *P. schweinitzii* (#58), while the lowest yield (0.85%) was exhibited by E3 of *F. pinicola* (#50). The physical appearance of these powder extracts were quite distinct. All E1 and E2 extracts were coarse powder. E3 and E4 extracts were generally fluffy in nature. All E1 and E2 extracts were dissolved in methanol except for E2 of *P. schweinitzii* #58 and #60 which was reconstituted in water. All E3 and E4 extracts were soluble in water.

2.2.2 Dose-dependent cell viability assessment of mushroom extracts using HeLa cells

2.2.2.1 *Fomitopsis pinicola* (#50)

Results of the dose-dependent MTT assay of extracts 1-4 from *Fomitopsis pinicola* (#50) are shown in Figure 9. Extracts 1 and 2 significantly reduced cell viability to 40% at the lowest concentration of 0.1 mg/mL. Extract 3 also reduced the cell viability to around 20% at the doses of 0.75 and 1 mg/mL. In contrast, extract 4 did not appear to have any significant effect on cell viability, decreasing viability to only 80% at the highest concentration of 1 mg/mL.

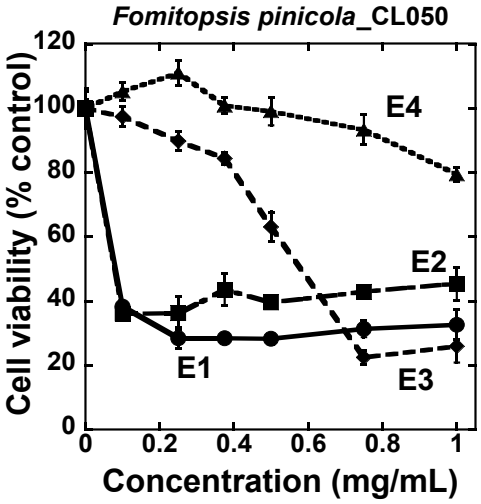


Figure 9 Dose-dependent assessment of cell viability by extracts from *F. pinicola* (#50) Different concentrations of crude extracts E1 (80% ethanol), E2 (50% methanol), E3 (water) and E4 (5% NaOH) were added to HeLa for 48 hours followed by assessment of cell viability

using MTT assay. The graph shows results from one biological replicate (n=1). Error bars are standard deviation.

2.2.2.2 *Fomitopsis pinicola* (#53)

Results in Figure 10 show that extract 3 from *Fomitopsis pinicola* (#53) reduced the cell viability to around 20% at the doses of 0.75 and 1 mg/mL. Extracts 1 and 2 inhibited the cell viability to around 40% at the lowest concentration of 0.1 mg/mL. At and above 0.5 mg/mL concentration, both E1 and E2 extracts caused an unexpected increase in cell viability. Such false negative effect had been observed with crude extracts from some mushroom species (Smith *et al*., 2017; Deo *et al*., 2019) and is most likely due to chemical(s) in extracts interacting with MTT leading to colored solution. In comparison, extract 4 only showed little effect on cell viability, decreasing viability to only 60% at the highest concentration of 1 mg/mL.

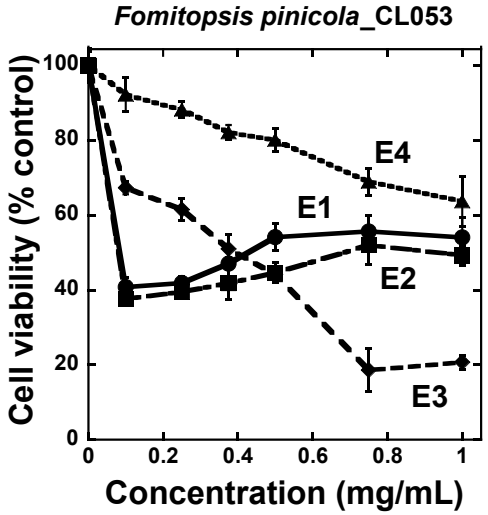


Figure 10 Dose-dependent assessment of cell viability by crude extracts isolated from *F. pinicola.* (#53) Error bars are standard deviation.

2.2.2.3 *Fomitopsis pinicola* (#65)

The results of dose-dependent MTT assays performed using extracts from *Fomitopsis pinicola* (#65) are shown in Figure 11. Extracts 1 and 2 dramatically reduced cell viability to approximately 20% by 0.1 mg/mL. Extract 3 reduced cell viability to 60% at the highest dose of 1 mg/mL. Extract 4 did not show any significant effect on cell viability even at 1 mg/mL.

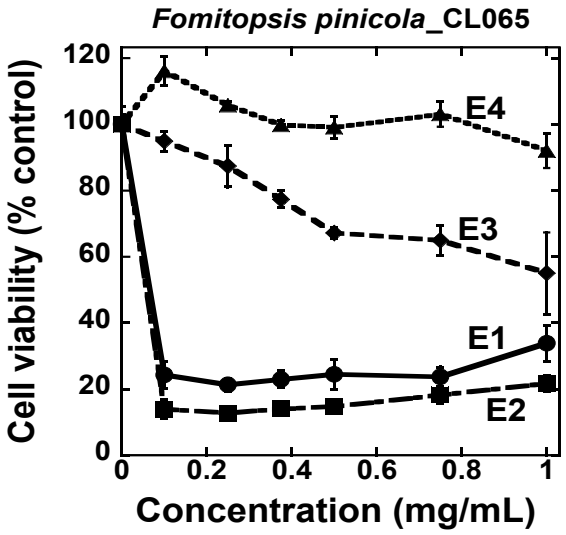


Figure 11 Dose-dependent assessment of cell viability by crude extracts isolated from *F. pinicola.* (#65) Error bars are standard deviation.

2.2.2.4 *Fomitopsis pinicola* (#77)

Results of dose-dependent MTT assays of extracts 1-4 from *Fomitopsis pinicola* (#77) are shown in Figure 12. The results show that extracts 1 and 2 reduced cell viability to 20% at the lowest concentration of 0.1 mg/mL. Extract 3 exerted dose-dependent inhibitory effect leading to 20% cell viability at the highest concentration of 1 mg/mL. In contrast, extract 4 had no effect on cell viability.

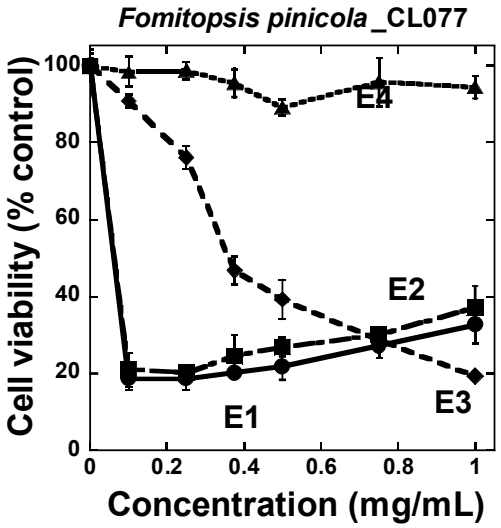


Figure 12 Dose-dependent assessment of cell viability by crude extracts isolated from *F. pinicola.* (#77) Error bars are standard deviation.

2.2.2.5 *Phaeolus schweinitzii* (#57)

Results of dose-dependent MTT assay of extracts 1-4 from *P. schweinitzii* (#57) are shown in Figure 13. Extracts 1 and 2 reduced cell viability to 20% and 40% at 0.25 mg/mL respectively. At higher concentrations, especially for E2, dark brown colored solution appeared upon addition of MTT. This could interfere with reading plate process and gave rise to the false negative results as shown in Figure 13. Extracts 3 and 4 also reduced cell viability to 20% but at higher concentration of 1 mg/mL.

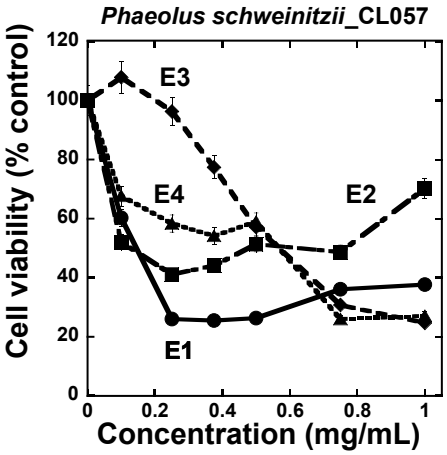


Figure 13 Dose-dependent assessment of cell viability by crude extracts isolated from *P. schweinitzii.* (#57) Error bars are standard deviation.

2.2.2.6 *Phaeolus schweinitzii* (#58)

The dose-dependent MTT assays on fractions from *Phaeolus schweinitzii* (#58) showed that extract 1 exhibited growth-inhibitory activity; it reduced cell viability to under 20% at 0.1 mg/mL, and the effect increased with the increasing dose, which might be the color of the extraction interfering with the MTT reagent, resulting in false absorbance. Extracts 3 and 4 reduced cell viability to about 30% at 1 mg/mL while extract 2 did not inhibit the cells.

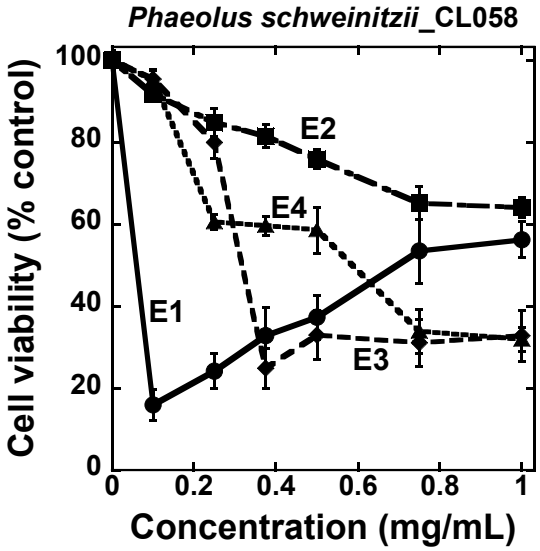


Figure 14 Dose-dependent assessment of cell viability by crude extracts isolated from *P. schweinitzii.* (#58) Error bars are standard deviation.

2.2.2.7 *Phaeolus schweinitzii* (#60)

Results of dose-dependent assay of *Phaeolus schweinitzii* are shown in Figure 15. The results show potent effect by extract 3, reducing cell viability to 20% at 0.25 mg/mL. Extract 4 reduced cell viability to 40% at 1 mg/mL. Extract 1 is the most potent, reducing cell viability to 20% at the lowest concentration of 0.1 mg/mL At and above 0.25 mg/mL, E1 exhibited false negative effect as mentioned above. Extract 2 only had modest effect, reducing cell viability to 80% at the highest concentration of 1 mg/mL.

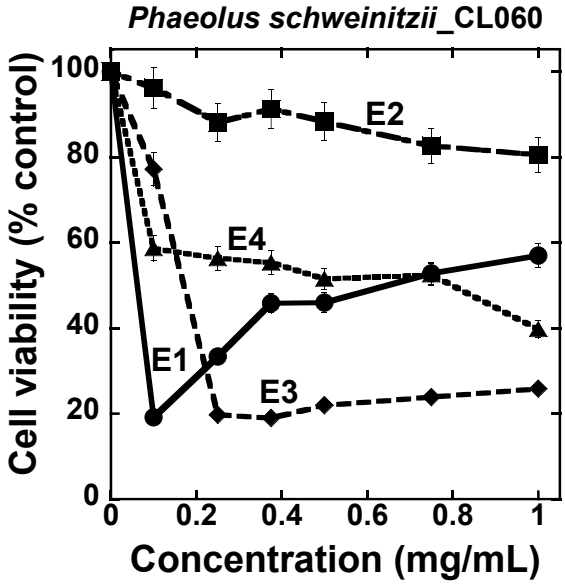


Figure 15 Dose-dependent assessment of cell viability by crude extracts isolated from *P. schweinitzii.* (#60) Error bars are standard deviation.

2.2.2.8 *Phaeolus* sp. (#71)

Results of dose-dependent assay of *Phaeolus* sp. are shown in Figure 16. The results demonstrate potent effect by extract 1, reducing cell viability to 10% at 0.1 mg/mL. Both Extracts 2 and 3 reduced cell viability to 30% at 1 mg/mL. Extract 4 only had modest effect, reducing cell viability to 60% at the highest concentration of 1 mg/mL.

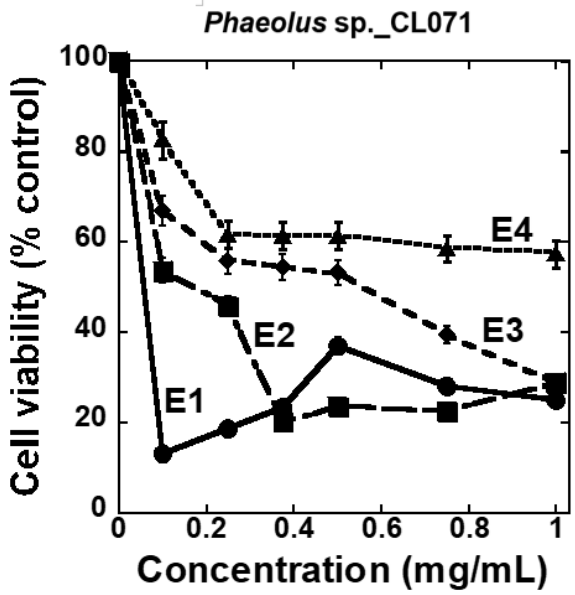


Figure 16 Dose-dependent assessment of cell viability by crude extracts isolated from *Phaeolus* sp. (#71) Error bars are standard deviation.

In this study, extracts E1, E2 and E3 from *F. pinicola* inhibited the growth of cancer cells dramatically, which were confirmed by previous studies (Wu *et al*., 2014 & Choi *et al.,* 2007). Extract E4 of *F. pinicola* had no significant inhibitory effect on HeLa cells which is in agreement with the existing literature (Smith, 2017). Extracts E1 and E3 from *P. schweinitzii* showed strong growth-inhibitory on HeLa cells, while other two extracts did not exhibit potent inhibitory activity.

The primary screening method demonstrated results regarding the therapeutic potentials of extracts from the eight BC mushrooms studied here, summary of the results are shown in Table 12. The main challenge of this project is the purification and structural elucidation of the compounds from mushrooms. The reason for selecting *Phaeolus* sp. for further studies is that not many researches have been reported on the anti-cancer activity of this mushroom specie,

chapter 3 of this thesis will investigate in detail of the purification and structural elucidation of the small molecules from E1 extract of *Phaeolus* sp.; more information about this mushroom is shown in chapter 3.

Table 12 Summary of twenty-eight crude extracts isolated from seven BC mushroom specimens on HeLa cell viability

Mushroom specimen	Extract	Anti-proliferative activity[1] (% viability at 1 mg/mL)
***Fomitopsis pinicola* (50)**	E1 (80% ethanol)	38.63
	E2 (50% methanol)	55.39
	E3 (water)	25.92
	E4 (5% NaOH)	79.46
***Fomitopsis pinicola* (53)**	E1 (80% ethanol)	54.07
	E2 (50% methanol)	49.25
	E3 (water)	20.72
	E4 (5% NaOH)	63.69
***Fomitopsis pinicola* (65)**	E1 (80% ethanol)	33.81
	E2 (50% methanol)	21.73
	E3 (water)	54.99
	E4 (5% NaOH)	92.03
***Fomitopsis pinicola* (77)**	E1 (80% ethanol)	32.68
	E2 (50% methanol)	37.21
	E3 (water)	19.25
	E4 (5% NaOH)	94.38
***Phaeolus schweinitzii* (57)**	E1 (80% ethanol)	37.70
	E2 (50% methanol)	70.16
	E3 (water)	24.66
	E4 (5% NaOH)	27.13
***Phaeolus schweinitzii* (58)**	E1 (80% ethanol)	56.15
	E2 (50% methanol)	64.02
	E3 (water)	32.77
	E4 (5% NaOH)	31.89
***Phaeolus schweinitzii* (60)**	E1 (80% ethanol)	56.98
	E2 (50% methanol)	80.48
	E3 (water)	25.84

	E4 (5% NaOH)	39.74

[1]Percent viability of HeLa cells as compared to control.

Chapter 3 Purification, identification and characterization of a growth-inhibitory compound from *Phaeolus* sp.

This chapter focuses on *Phaeolus* sp. (#71) which was collected from Pesuta Shipwreck Trail in Naikoon Provincial Park, Haida Gwaii, BC. The samples were morphologically and genetically identified as a member of the genus *Phaeolus*. Using the BLAST program, the sequence obtained from our collection matched 100% in the GenBank database to a voucher specimen identified as *Phaeolus* sp. by Jonathan Frank at Southern Oregon University, who believes it may be an undescribed species (Frank, personal communication); further research will need to be done to determine its identity. This mushroom was selected for purification and characterization to potentially discover novel compounds. Part A of this chapter focuses on the purification, characterization and structural elucidation of a growth-inhibitory compound from the E1 (80% ethanol) extract of *Phaeolus* sp. Part B focuses on isolating other small molecules from *Phaeolus* sp.

Part A: Purification and characterization of hispidin from *Phaeolus* sp.

3.1 Purification and chemical characterization strategies

Numerous studies describing the therapeutic compounds medicinal fungi possess have been published (Pennerman *et al*., 2015). To understand these compounds better, one must undertake their purifications. Generally, the purification strategies for bioactive compounds is dependent on the nature of compounds. Liquid-liquid extraction is first employed to purify the compounds based on the polarity. After this, techniques like size-exclusion chromatography (e.g., Sephadex-LH-20), ion-exchange chromatography (e.g., DEAE Sephadex), and high-

performance liquid chromatography (HPLC) are used for further purification. When the purification process is finished, analytical instruments like LC-MS (Liquid Chromatography-Mass Spectrometry), GC-MS (Gas Chromatography-Mass Spectrometry), Fourier transform infrared spectroscopy (FTIR) and NMR (Nuclear Magnetic Resonance) are used to characterize the target compounds further.

3.2 Materials and methods

3.2.1 Large scale chemical extraction of *Phaeolus* sp.

The *Phaeolus* collection (#71) was finely powdered using a hammer mill, and large-scale extraction of extract E1 (80% ethanol) was performed manually using a hot plate. After that, the extracts were concentrated, pH neutralized and lyophilized using a Labconco freeze dryer and finally stored at -80°C.

3.2.2 Liquid-liquid extraction (phase separation)

Liquid–liquid extraction or phase separation is a method to separate compounds based on relative solubility in two different immiscible liquids, usually water (polar) and an organic solvent (non-polar). To carry out liquid-liquid extraction, an extraction flask was used (Figure 17).

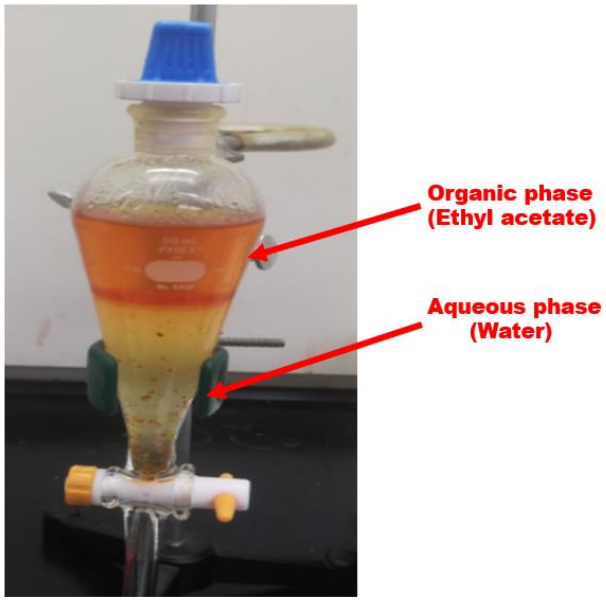


Figure 17 Illustration of the liquid-liquid extraction technique

At first, 100 mg of E1 extract (80% ethanol) powder was added in the extraction flask. Then, 40 mL of water was added to the flask as a primary solvent. Later, an equal volume (40 mL) of ethyl acetate was added and the sample was dissolved in two layers. The flask was shaken to ensure that the separation of compounds between the two layers was maximized. After five minutes (or until the layers separated) the layers were observed (Figure 17). Each layer was collected into a different beaker. The upper layer (ethyl acetate layer) was dried by heating on a hot plate at 65 °C. The lower layer (water layer) was lyophilized using Labconco Freeze dryer. After obtaining the two dried samples, the yield was calculated and the two layers were further screened for their growth-inhibitory activity using the MTT assay as described in section 2.1.5.1.

3.2.3 Sephadex™ LH-20 (size exclusion chromatography)

Size exclusion chromatography was also performed for better separation of the compounds in the crude ethyl acetate extract. During this purification process, the molecules were separated by their size as they passed through the stationary and mobile phase (100% Methanol) packed in the column. The larger molecules cannot fit in the pores of the stationary media and they flow more quickly through the stationary phase, which causes them to be eluted first. The smaller molecules easily penetrate the pores of the medium, causing them to flow slowly and elute out of the column later. Due to this physico-chemical property, Sephadex™ was selected to do the next step of purification.

Sephadex™ LH-20 is a liquid chromatography medium designed for natural product isolation (Datta *et al*., 2013). Since the medium is Sephadex LH-20, the method is also termed as Sephadex LH-20 column chromatography. Sephadex™ LH-20 is prepared by hydroxypropylation of cross-linked Sephadex G-25 dextran and an organic solvent is used as a mobile phase. This LH-20 resin has both hydrophilic and hydrophobic characteristics, which make it swell in aqueous solution and organic solvents. In my study, the mobile phase used was 100% methanol as the mobile phase is based on the solubility of active compounds (ethyl acetate layer). Two types of columns, including a 22 mL column (micropipette) and 400 mL (C26/100 column), were used for packing the resin.

3.2.3.1 Sephadex™ LH-20 resin swelling and column packing

The swelling ratio of Sephadex™ LH-20 resin to methanol was 1:4. Firstly, Sephadex™ LH-20 dry powder was transferred into a beaker and soaked in excess methanol to swell

overnight. After the resin was completely soaked with 100% methanol, the excess methanol was removed from the slurry. Methanol was degassed for 2-3 hours to ensure that the mobile phase had no dissolved gases. Otherwise, the gas would be pumped into column and crack the resin.

Two different types of columns were used for the purification. To pack a small sized column (22 mL), a 25 mL serological pipette (Sarstedt, Germany) was used as a column, and a cotton plug was used to hold the resin above the tip of the pipette and also ensure the mobile phase (methanol) could flow out of the column; the column was first packed with a total of 22 mL of resin under the influence of gravity. As for 400 mL (C26/100) (GE Healthcare) glass columns, the swollen resin was transferred to the columns first. The top and lower adapters were used to support the resin in the glass column, and the top of the column was linked to the solvent reservoir. The Pump P-50 (Pharmacia Biotech) was used to pump the methanol solvent to flow through the column for packing and washing at a flow rate of 2 mL/min. The column was required to equilibrate with degassed methanol for at least two column volumes between every run.

3.2.3.2 Sample loading and fractions collection

The sample was prepared using the active extract from the upper layer (ethyl acetate layer) of the liquid-liquid extraction and methanol (100%) was used to reconstitute the sample. The final concentration of loading sample was 40 mg/mL. The sample was centrifuged before loading onto the column to remove the impurities which could be trapped in the resin. For a 22 mL column, the sample was loaded onto the column using a micropipette (Thermo Fisher

Scientific, USA). The volume of the sample (ethyl acetate layer) loaded was 2% (440 µL) of the packed column volume. Fraction collection was based on gravity. The sample (8 mL) was introduced into the large (400 mL) (GE Healthcare) columns with the help of a syringe in a three-way switch (Figure 18).

Figure 18 Photograph of the Sephadex™ LH-20 size exclusion technique using a large C26/100 column (GE Healthcare) and the mobile phase (degassed methanol)

The flow rate of the mobile phase for C26/100 columns was 2 mL/min. An automatic fraction collector (Pharmacia Biotech) was used to collect fractions, and the volume of the collected fractions was 14 mL. After collection, the fractions were stored in a cold room (4°C) before assessment for growth-inhibitory activity.

3.2.3.3 Sephadex™ LH-20 column clean-up, unpacking and storage

After each run is finished, the columns were washed to ensure no sample remained in the resin. The original degassed mobile phase (100% methanol) was used to wash the resin at 1.5 times the actual flow rate and with mobile phase volume twice the bed volume. However, if the sample was sticky and oily, the resin must be washed with NaOH (5%), at 1 mL/min flow

rate with half the column bed volume, then twofold of the column bed volume of water was used to wash the resin, at 1.5 mL/min flow rate to elute both the remnant loading sample and NaOH residue. Finally, two-column bed volume of degassed mobile phase were used to run through the column to ensure the long shelf life and reusability of the resin. After the column was washed, the resin was removed from the column and stored in an Erlenmeyer flask in degassed 100% methanol at 4°C to prevent any microbial contamination of the resin.

3.2.4 UV absorbance analysis

A nanodrop spectrophotometer (Thermo Fisher Scientific, SA) was used to determine the maximum UV absorbance (λmax) of bioactive fractions 39 to 42 from the Sephadex LH-20 (400 mL). After calibration with water, the spectrophotometer was blanked with HPLC grade methanol. The sample peddle was rinsed with autoclaved milli-Q water and wiped with Kimwipe paper before analyzing the sample. A 2 µL sample was used to determine the λmax of the fractions. The λmax for fractions was read at around 270 nm, which was later used in HPLC analysis of the active fractions.

3.2.5 High performance liquid chromatography (HPLC-UV) analysis

HPLC is a powerful method for separation and purification of natural products (Adams & Nakanishi, 1979), which yields an excellent separation in a short time. Due to this, it was chosen to further purify the bioactive compounds collected from the Sephadex LH-20 fractions. HPLC operates under high pressure to push the mobile phase and the sample through the stationary phase. The Agilent 1260 Infinity Systems (HPLC) was used to purify the growth-

inhibitory compound. The HPLC system was equipped with solvent systems, a sample injection valve, detector, controller, and computer to control the system and display results.

Reverse-phase HPLC is the most common type of HPLC used (Robards *et al*., 1994). In this study, RP-HPLC was used to isolate the growth-inhibitory compound. RP-HPLC has a nonpolar hydrophobic stationary phase and a moderately polar solvent as a mobile phase. Sample elution is based on polarity; the highly polar compounds elute first, and the non-polar compounds elute later (Sundaram *et al*., 2009). As a result, the non-polar (hydrophobic) molecules in the mobile phase tend to interact with the non-polar stationary phase more, while the polar molecules in the mobile phase pass through faster and elute from the column first.

Water is the most polar solvent so it can repel the hydrophobic molecules into the stationary phase more than other solvents, therefore, it causes longer retention times (Realini, 1981). When adding another organic solvent to the mobile phase system, the polarity could be modified, this causes the solvent system to no longer retard the sample in the stationary phase and thus spend less time in the column and elutes earlier.

The solvents used as the HPLC mobile phase were chosen based on their appropriateness to the RP-HPLC. Water and methanol were selected to consist the solvent system. Water was used because it is miscible, chemically non-reactive, has low viscosity, low UV absorbance, and high solubility, while the HPLC-grade methanol was selected based on good solubility of the sample.

A gradient elution method was used during all HPLC purifications to provide separations of mixture. The gradient elution method contains two or more mobile phases which differ in

polarity. The solvent system started with 30/70% water/methanol and ended at 40/60% water to methanol over nine minutes. The mobile phase was automatically degassed through the degasser system of the HPLC before entering the quaternary pump. The quaternary pump controls the flow rate as well as the gradient of the solvent system. The flow rate for the gradient mobile phase was set at 1.0 mL/min.

Samples (bioactive LH-20 fractions) were filtered using a 0.2 μm filter (Sarstedt, Germany) before injecting into the system. The wavelength (nm) used to visualize UV signal of bioactive compounds was 270 nm (based on reading from nanodrop spectrophotometer). The needle of the auto-sampler was programmed to wash every time before injection to prevent any cross contamination. The column was washed with the solvent system for at least 10 column volumes. The sample volume loaded onto the column was 10 μL (10 mg/mL). Each run took 9 minutes to separate and elute all the peaks out of the column. The fraction collection was done based on the HPLC-UV. The fractions were collected into 8.0 mL glass vials using a fraction collector (Agilent infinity Lab 1260).

3.2.6 LC-MS analysis

LC-MS is a technique, which combines the separating power of HPLC, with the detection power of mass spectrometry (MS). Liquid chromatography separates mixtures into multiple components, and mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity (McMaster, 2005). This technique can be used to analyze organic compounds efficiently (De Hoffmann, 2000).

All LC-MS analyses were performed using Agilent 1260 Infinity Systems HPLC and Agilent 6120 quadrupole mass spectrophotometer. Mass spectrometry was performed using an active splitter, and a split ratio of 66:1. After separation of the sample components in the HPLC system, an Electrospray ionization (ESI) was used to convert separate sample species into ions in the gas phase, and the mass analyser was used to sort ions according to their mass to charge ratio (m/z).

3.2.7 Nuclear magnetic resonance (NMR) analysis

Nuclear magnetic resonance spectroscopy, commonly referred to as NMR, has become the preeminent technique for determining the structure of organic compounds (Pretsch *et al*., 2000). The structural elucidation of the compound was achieved using a Bruker 300 MHz NMR spectrophotometer. And 1D and 2D NMR spectra were recorded on a Bruker 300 spectrometer with a 5 mm Fourier easy probe.

One-dimension NMR is a technique used to probe the chemical environments of nuclei in molecules and recorded on a frequency axis while the intensity is represented as the 1D information of the molecules. This technique is highly automatable, reliable, and fast (Emwas *et al*., 2019). Although the 1D NMR spectrum of a natural product can be acquired rapidly, they are often complicated by peak overlap that can significantly impede the identification of a sample (Viant, 2003). Such an issue can be addressed by using two-dimensional (2D) NMR techniques by spreading out cross-peaks of resonances along the indirect dimensions that overlap in a 1D NMR spectrum, which can reduce the likelihood of peak overlap (Bingol *et*

al., 2014). The 2D NMR spectra can provide more information about a molecule than 1D NMR spectra, and are especially useful in determining the structure of a molecule.

One-dimension ^{1}H NMR and ^{13}C-NMR were performed to identify the proton and carbon atoms in our purified sample. The 2D-NMR spectroscopy correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) were employed to further provide data about the sample. COSY is a 2D NMR method used to identify pairs of spin–spin coupled protons. In the spectrum, 1D NMR spectra of chemical shifts is indicated along a diagonal ridge running from the lower left to upper right corner of the plot. Cross peaks are produced by spin–spin coupling of two protons; most commonly, cross peaks are produced by adjacent protons. COSY is the most common form of 2D NMR spectroscopy and is a routine and highly useful method for identifying molecules through their spin–spin couplings (Xi *et al*., 2006). HSQC spectra are 2D data matrices consisting of cross peaks of protons and carbons. It is a proton-detecting experiment, and it is more sensitive and less time-consuming to acquire. Also, it is richer in information as it simultaneously allows the list of directly bound ^{1}H-^{13}C pairs to be assembled (Szakács & Sánta. 2015). The HMBC NMR experiment is probably the most useful and the most widely used 2D heteronuclear NMR method in the structural elucidation of organic molecular structures. (Furrer, 2012). It is a popular technique for multiple-bond heteronuclear chemical shift correlation (Martin & Zektzer, 1988). (^{1}H-^{13}C)-HMBC can provide correlations between protons and carbons that are two or three bonds apart from each other. The HMBC spectra is

important for the detection of quaternary carbons as it links separate structural fragments obtained from analysis of COSY and HSQC (Edison & Schroeder, 2010).

The magnets of the Bruker 300 MHz NMR spectrophotometer were shimmed properly before analysis of samples (Topspin 4.0.7). Approximately 7 mg of the sample was dissolved in deuterated acetone $(CD_3)_2CO$ (99.96 % acetone-d and 0.3% Tetramethylsilane(TMS)) (Sigma Aldrich) and transferred directly into a 5 mm NMR tube (Kimble, USA). The final volume of the sample in NMR tube was approximately 1 mL. After performing the different types of NMR mentioned above, 1D and 2D NMR spectrums integrated together to provide clear information.

3.2.8 Melting point

The melting point of a substance is the temperature at which the material changes from a solid to a liquid state, and it is one of the most widely used fundamental physical properties (Jain *et al*., 2004). Pure crystalline substances have a clearly defined melting point. Determining the melting point is a simple and fast method used to obtain a first impression of the purity of a substance, as even small quantities of impurities could change the melting point or expand its melting range.

The capillary method was used to determine the melting point of the purified compound using the MPA160 DigiMelt Student Melting Point System. A thin glass capillary tube containing little sample is placed into a heated stand in close proximity to a high accuracy thermometer. The temperature in the heating stand is increased, at a fixed rate, until the sample in the tube becomes liquid. Visual observations were taken to observe changes during the

melting point determination, including color change, loss of solvent and change in crystallization state. The onset point, when liquid clearly appears for the first time, is generally considered the start of the melting. The clear point corresponds to the stage of melting at which the substance becomes completely liquid. The melting point range is defined as the interval between the onset and clear points, which is an indicator of purity of a solid compound.

3.3 Results

3.3.1 Large scale chemical extraction of *Phaeolus* sp.

Large scale chemical extraction to isolate E1 was performed on the collection 71 of *Phaeolus* sp. The percent yield for E1 (80% ethanol) was 16.80% (Table 13). The extract can be partially dissolved in 100% methanol.

Table 13 Percent yield of E1 extract from *Phaeolus* sp. (#71)

Mushroom No.	Mass of sample (g)	Mass of E1 extract (g)	Yield (%)
71	50.217	8.441	16.80

3.3.2 Liquid-liquid extraction (phase separation)

Liquid-liquid extraction performed on E1 of *Phaeolus* sp. resulted in two distinct layers. The lower layer was water and the top layer was ethyl acetate. The color of the aqueous layer was yellow, and it contained approximately 45% yield of product. The color of the upper organic layer was brown; The percent yield from the top ethyl acetate layer was 33.50% (Table 14). Furthermore, solubility of both layers was determined in order to perform growth-inhibitory assays; both extract samples were soluble in methanol.

Table 14 Percent yield of three layers based on liquid-liquid extraction (Phase separation) of E1 (80% ethanol) extract of *Phaeolus* sp.

Layer of Liquid-Liquid extraction	Color of the layer	Mass (mg) out of 20mg	Yield (%)	Growth-inhibitory Activity	Solubility
Organic (Ethyl acetate)	Brown	6.70	33.50%	Yes	100% Methanol
Aqueous (Water)	Cream	8.90	44.50%	Yes	100% Methanol

The dose-dependent MTT assay was performed on lyophilized samples from the two layers of the liquid-liquid extraction; HeLa cell plating and treatment was described in section 2.1.5. The final concentration of the methanol was less than 1% in each well to prevent non-specific toxicity to cells. The doses tested on HeLa cells were 0.05 and 0.1 mg/mL.

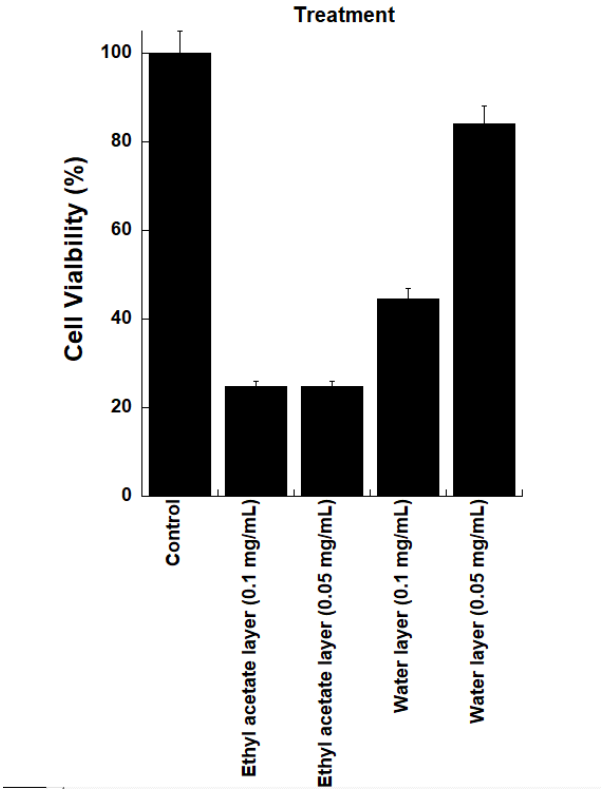


Figure 19 Assessing the water and ethyl acetate layers from E1 extract for anti-proliferative activity. Two different concentrations of water and ethyl acetate layers were added to HeLa for 48 hours followed by assessment of cell viability using MTT assay. The graph shows results from one biological replicate (n=1). Error bars are standard deviation.

As shown in Figure 19, strong growth-inhibitory activity was exhibited by the upper ethyl acetate layer. The cell viability was around 20% at both 0.1 and 0.05 mg/mL and results were consistent. At 0.1 mg/mL, the water layer also exhibited potent growth-inhibitory activity. However, in general, as compared to the ethyl acetate layer, the water layer had less potent activity against HeLa cells. Based on the MTT results, it was decided to proceed with purification of bioactive compounds from the upper ethyl acetate layer.

3.3.3 Assessing post-Sephadex™ LH-20 fractions collected from a 22 mL column for bioactivity

Fractions from the 22 mL sized Sephadex LH-20 column were collected in 1.5 mL Eppendorf tubes and the final volume of each fraction was around 1.3 mL. Fractions 25-35 were pale yellow in color, suggesting most compounds were present in these fractions. After collection, these tubes were put in racks and left in a fume hood for three days to evaporate the excess solvent. The dose-dependent MTT assay was performed to test the activity of these fractions, HeLa cells (1500 cells/well) were plated and after 24 hours of incubation, the cells were treated with 2 µL of the fraction and 98 µL of EMEM with 10% FBS. The final concentration of methanol in each well was maintained at 1% which was non-toxic to the cells.

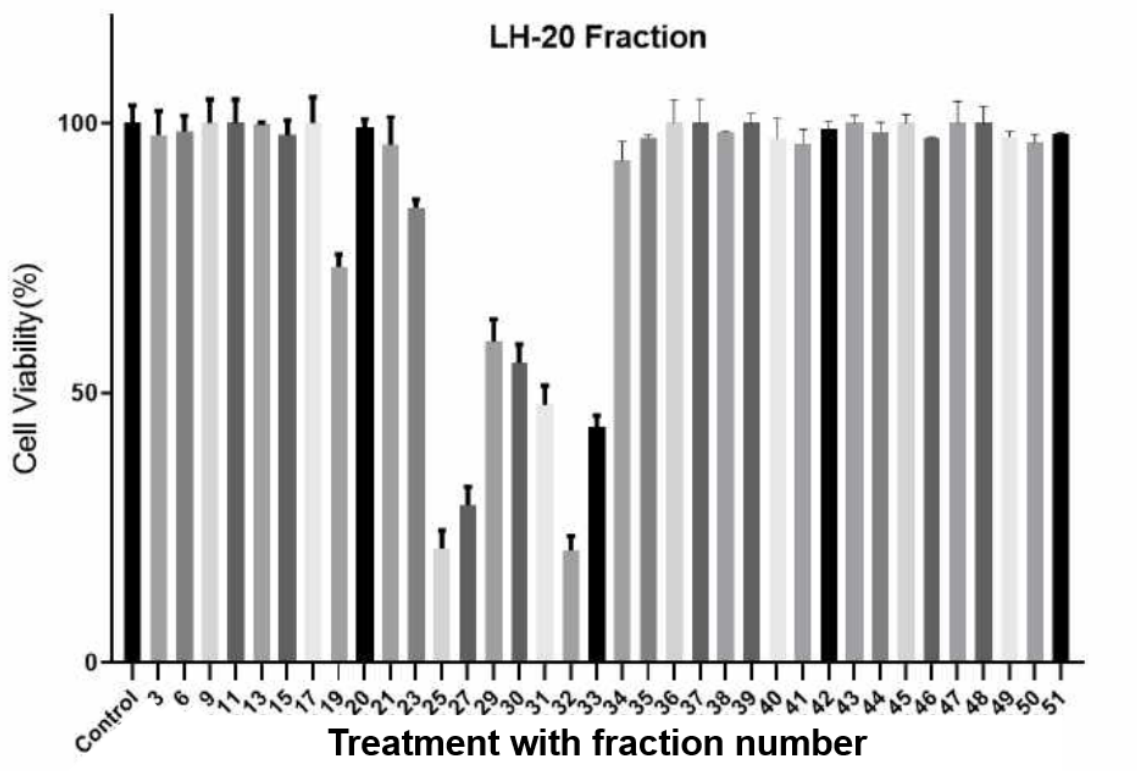


Figure 20 Assessing anti-proliferative activity of fractions collected from 22 mL size Sephadex™ LH-20 column. Effect of 2 µL from 22 mL size post-Sephadex™ LH-20 fractions on HeLa cell viability. The graph shows results from one biological replicate (n=1). Error bars are standard deviation.

Fractions 25 to 32 inhibited the HeLa cell viability compared to the control (Figure 20). Weak growth-inhibitory activity was observed for other fractions except for fractions 25 to 32. Given that the inhibitory activity was observed in later eluting fractions, it is likely that the bioactive compounds are small in size.

3.3.4 Assessing post-Sephadex™ LH-20 fractions collected from a 400 mL column for growth-inhibitory activity

After bioactivity was detected in specific fractions collected from the small Sephadex column (22 mL), the technique was scaled up to 400 mL using a C26/100 (GE Healthcare, Quebec) column. Fractions were collected in 15 mL tubes (Sarstedt) and the final volume of each fraction was around 5 mL. After collection, these tubes were left in the fume hood to

evaporate excess solvent. The MTT assay (1500 cells/well) was performed using fractions (14 mL each) obtained by running a total 320 mg of the E1 (80% ethanol extract) ethyl acetate layer of *Phaeolus* sp. through a 400 mL Sephadex™ LH-20 column. The results of the MTT assay using the fractions are shown in Figure 21.

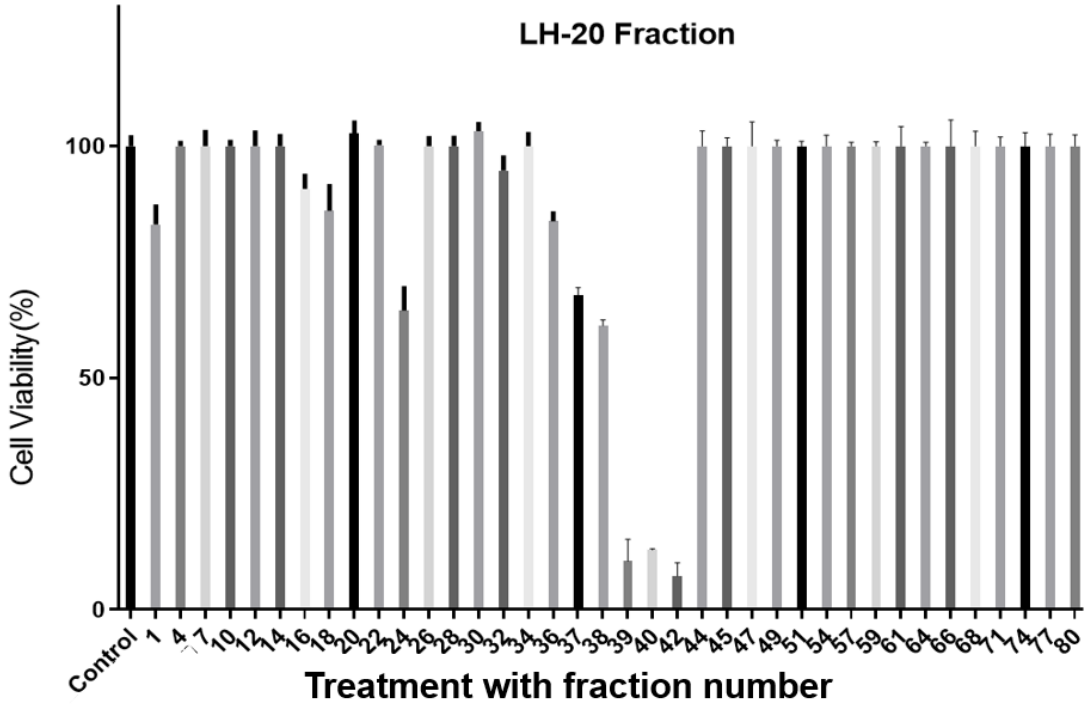


Figure 21 Assessing anti-proliferative activity of fractions collected from a 400 mL Sephadex™ LH-20 column. Effect of 2 µL from 400 mL size post-Sephadex™ LH-20 fractions on HeLa cell viability. The graph shows results from one biological replicate (n=1). Error bars are standard deviation.

Fractions 39 to 42 reduced the HeLa cell viability dramatically as compared to the control, suggesting the presence of bioactive compounds in these fractions. The results were consistent with the results from the 22 mL size column, the color of bioactive fractions were same, and the cell viabilities were similar, which inhibited at around 20%. The active fractions from the 400 mL column were further analyzed using analytical HPLC.

3.3.5 HPLC results

3.3.5.1 Analytical

The purification process is depicted in Figure 22.

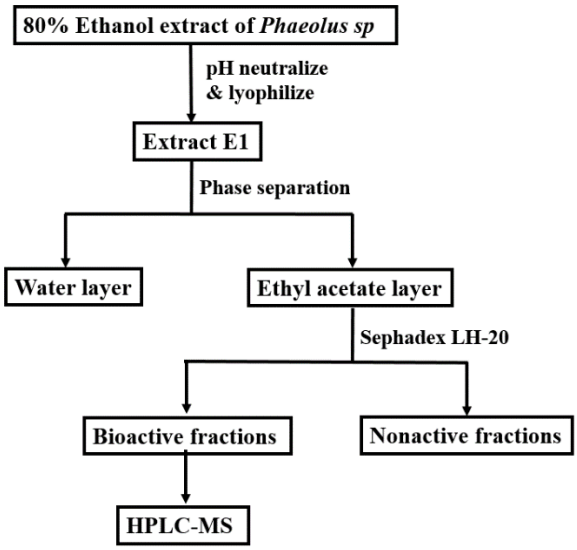


Figure 22 Flow chart diagram representing the method of compound purification from E1 (80% ethanol) extract of *Phaeolus* sp.

The resolution based on the analytical HPLC conditions was excellent: each peak was distinct and well separated (Figure 23). The retention time for the peak was 4.312 min (later confirmed to be hispidin). The percent abundance based on UV spectra for the peak was 95.57%, and this peak was further aggregated and characterized.

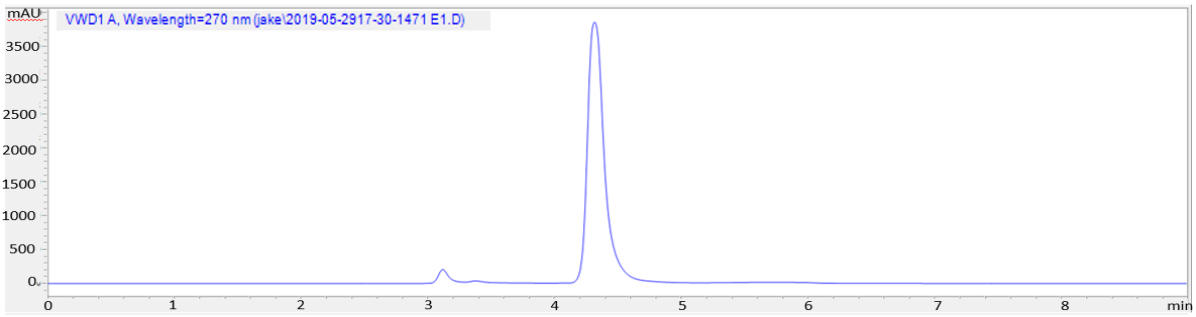


Figure 23 UV spectrum showing the relative abundance and retention time of peak at 4.312 min at λmax 270 nm (using Agilent C-18 analytical column and Sephadex LH-20 active fraction sample).

3.3.5.2 HPLC-Mass spectroscopy (MS) analysis

The HPLC-purified compounds were protonated with the aid of 0.1% formic acid isocratic solution. Figure 24 shows the UV spectrum for confirmation of the purified compound. The percent abundance based on UV spectra for the peak was 99.33%. Spectra shown in Figures 25 and 26 represent the MS SIM and scan signal of the peak respectively..

The mass to charge ratio (m/z) of the protonated purified peak was 247.1 (Figure 27) which confirmed the presence of the target masses. The abundance of the targeted masses was also found to be higher in SIM mode than in the scan mode, confirming the presence of the compounds in the sample.

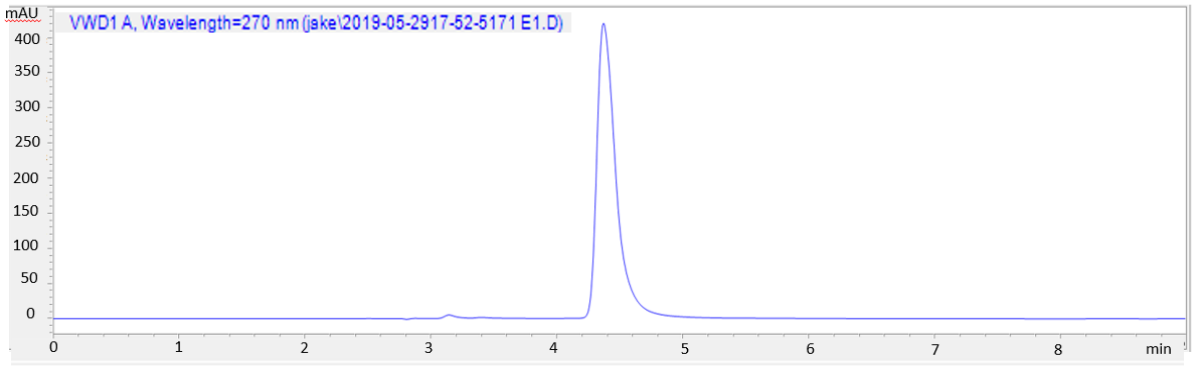


Figure 24 UV spectrum confirming the purity of the compound $[M+H]^+$ m/z 247.1 detected at 4.312 min through ESI (Electrospray ionization) chamber.

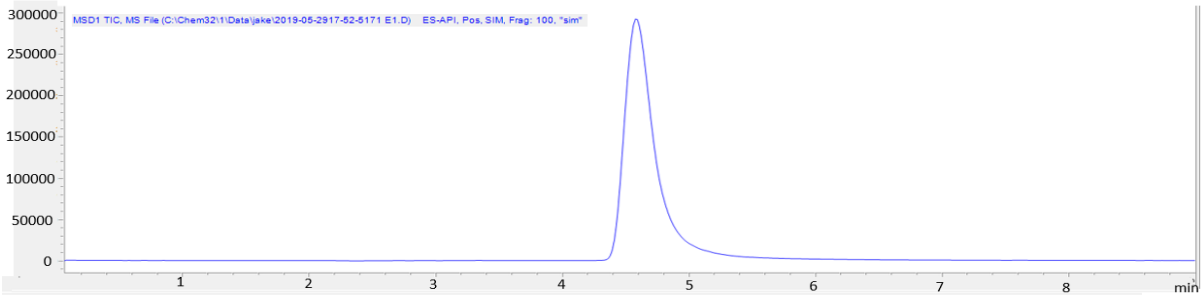


Figure 25 MS SIM spectrum showing the relative abundance and retention time of the compound with [M+H]+ m/z 247.1 detected at 4.312 min through ESI (Electrospray ionization) chamber.

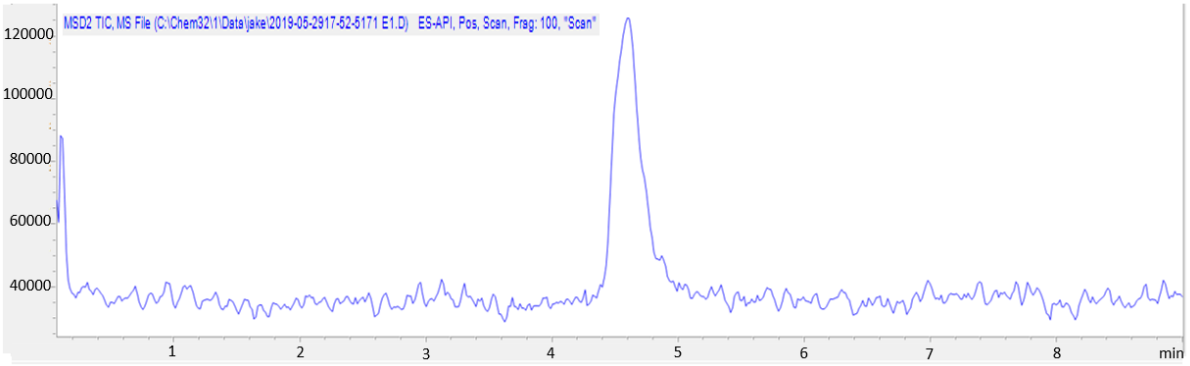


Figure 26 MS scan spectrum showing the relative abundance and retention time of the compound with $[M+H]^+$ m/z 247.1 detected at 4.312 min through ESI (Electrospray ionization) chamber.

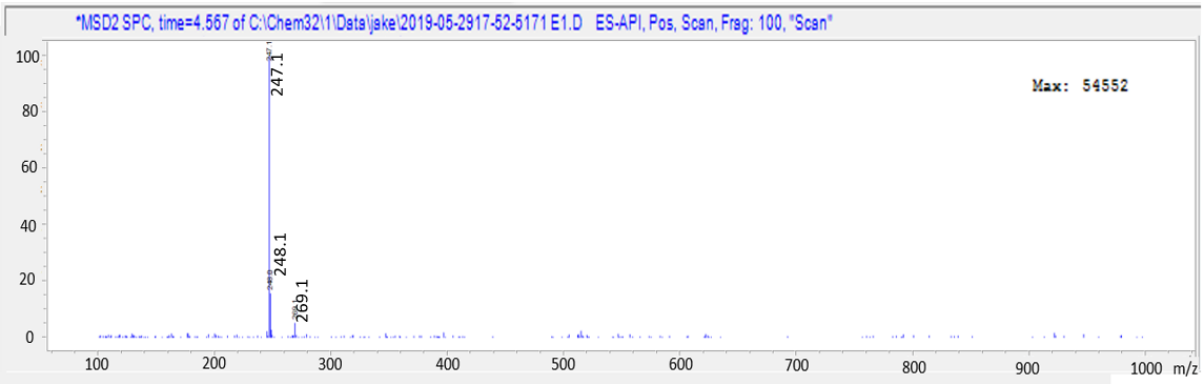


Figure 27 Integration of MS scan spectrum indicating the presence and abundance of compound with $[M+H]^+$ m/z 247.1 visible at 4.567 min

In the MS scan spectrum, the ion peaks at m/z 247.1 $[M+H]^+$ and m/z 269.1 $[M+Na]^+$ in the positive mode, suggests the molecular weight of the purified compound to be 246.1 g/mol.

3.3.6 Nuclear magnetic resonance (NMR) analyses

Comparing the NMR spectra found here (Tables 15-16) with previously published NMR data revealed that the growth-inhibitory compound in *Phaeolus* sp. is hispidin, which had been previously isolated and structurally elucidated (Park *et al*., 2004).

Table 15 ^{1}H NMR spectral data of purified compound (hispidin) obtained using Bruker Fourier A-300 NMR

Hydrogen	Hispidin	Coupling constant value (J)	Hispidin[1]
H-3	5.36	J3,5=1.86 Hz	5.28 (d)
H-5	6.11	J5,3=2.01 Hz	6.06 (d)
H-7	6.65	J7,8=15.96 Hz	6.55 (d)
H-8	7.24	J8,7=15.96 Hz	7.16 (d)
H-10	7.15	J10,14=1.95 Hz	7.01 (s)
H-13	6.84	J13,14=8.19 Hz	6.77 (d)
H-14	7.01	J=6.21 Hz	6.88 (d)

[1]Published NMR data of hispidin (Park *et al*., 2004) compared to this study.

The ^{1}H NMR spectrum of hispidin (Figure 29) exhibited two doublets at 5.36 (d, 1H, H-3, J3,5=1.86 Hz) and 6.11 (d, 1H, H-5, J5,3=2.01 Hz), suggesting a long-range coupling of ^{4}J in a lactone ring system. Two olefinic methine peaks attributable to a trans-1, 2-disubstituted double bond at the signals 6.65 (d, 2H, H-7, J7,8=15.96 Hz) and 7.24 (d, 2H, H-8, J8,7=15.96 Hz). Three aromatic protons, which could be assigned to the protons of 1,3,4-tri-substituted benzene, can be observed at 7.15 (d, 1H, H-10, J10,14=1.95 Hz), 6.84 (d, 2H, H-13, J13,14=8.19 Hz), and 7.01 (dd, 2H, H-14, J=6.21 Hz).

Table 16 ^{13}C NMR spectral data of purified compound (hispidin) obtained using Bruker Fourier A-300 NMR.

Carbon	Hispidin	Hispidin[1]
C-2	162.9	171.0 (s)
C-3	89.8	89.4 (d)
C-4	169.9	163.6 (s)
C-5	100.1	101.5 (d)
C-6	160.3	159.9 (s)
C-7	116.6	116.1 (d)
C-8	134.9	134.7 (d)
C-9	127.8	127.2 (s)
C-10	113.8	114.4 (d)

C-11	147.0	147.6 (s)
C-12	145.5	145.9 (s)
C-13	115.5	115.9 (d)
C-14	120.7	120.3 (d)

[1]Published NMR data of hispidin (Park *et al*., 2004) compared to this study.

The signals observed for the ^{13}C NMR spectrum (Figure 30) of hispidin indicate the presence of 13 carbons in this structure. The signals for non-proton bearing carbons of hispidin (C-4, C-2, C-6, C-11, C-12 and C-9) were observed downfield at 169.9, 162.9, 160.3, 147.0, 145.5 and 127.8 ppm respectively. The signals for aromatic carbons (C-10, C-13 and C-14) were observed at 113.8 ppm, 115.5 ppm and 120.7 ppm. Two olefinic methine carbons C-7 and C-8 were observed at 116.6 ppm and 134.9 ppm respectively. The signals at 89.8 ppm and 100.1 ppm are due to two methine carbons C-3 and C-5 on the lactone ring.

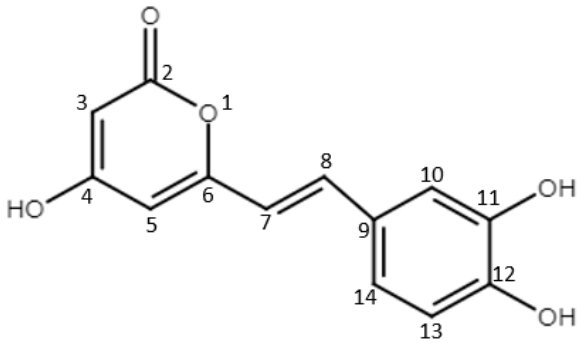


Figure 28 Chemical structure of hispidin

^{1}H-^{1}H COSY is a 2D method used for determining the signals arising from neighboring protons. The COSY spectrum as shown in Figure 31, the peaks show the coupled signals of neighboring protons. Cross peaks at 6.84 and 7.01 ppm are due to two neighboring aromatic

protons (H-13 and H-14). Two olefinic methine protons at position (H-7) display a cross peak at 6.65 ppm and 7.24 ppm with the proton at position H-8, suggesting they are neighbors.

Heteronuclear single quantum correlation (HSQC) is a two-dimensional spectrum which has two axes, one for protons (^{1}H NMR) and the other for carbons (^{13}C spectrum). HSQC provides detailed information about correlations between a carbon and its attached protons. The HSQC spectrum (Figure 32) of hispidin showed resonances for three aromatic protons 7.15 ppm (H-10), 6.84 ppm (H-13) and 7.01 ppm (H-14), two olefinic methine protons 6.65 ppm (H-7) and 7.24 ppm (H-8), and two methine protons 5.36 ppm (H-3) and 6.11 ppm (H-5) on the lactone rings. The carbons at position C-2, C-4, C-6, C-9, C-11 and C-12, displayed no resonance signals with the ^{1}H spectrum, indicating the carbons are quaternary.

The ^{13}C - ^{1}H NMR heteronuclear multiple bond correlation (HMBC) 2D spectrum (Figure 33) of hispidin showed long-range correlations from one singlet methine proton δ 5.36 to C-3, another proton δ 6.11 to C-3, C-6 and C-7, from two olefinic methine protons at δ 6.65 and 7.24 to C-5, C-6, C-9, and C-10, from methine protons at δ 7.15 to C-9 and C-11, from methine protons at δ 6.84 to C-8, C-12, and C-14, and from methine protons at δ 7.01 to C-8, C-10, and C-12.

Based on the above described NMR data obtained from my experiments, the structure of the purified compound was determined to be hispidin (Figure 28), which has been previously isolated from the fungi *P. linteus* (Jang *et al*., 2010) and *Inonotus hispidus* (Bull.) P. Karst. (Benarous *et al*., 2015).

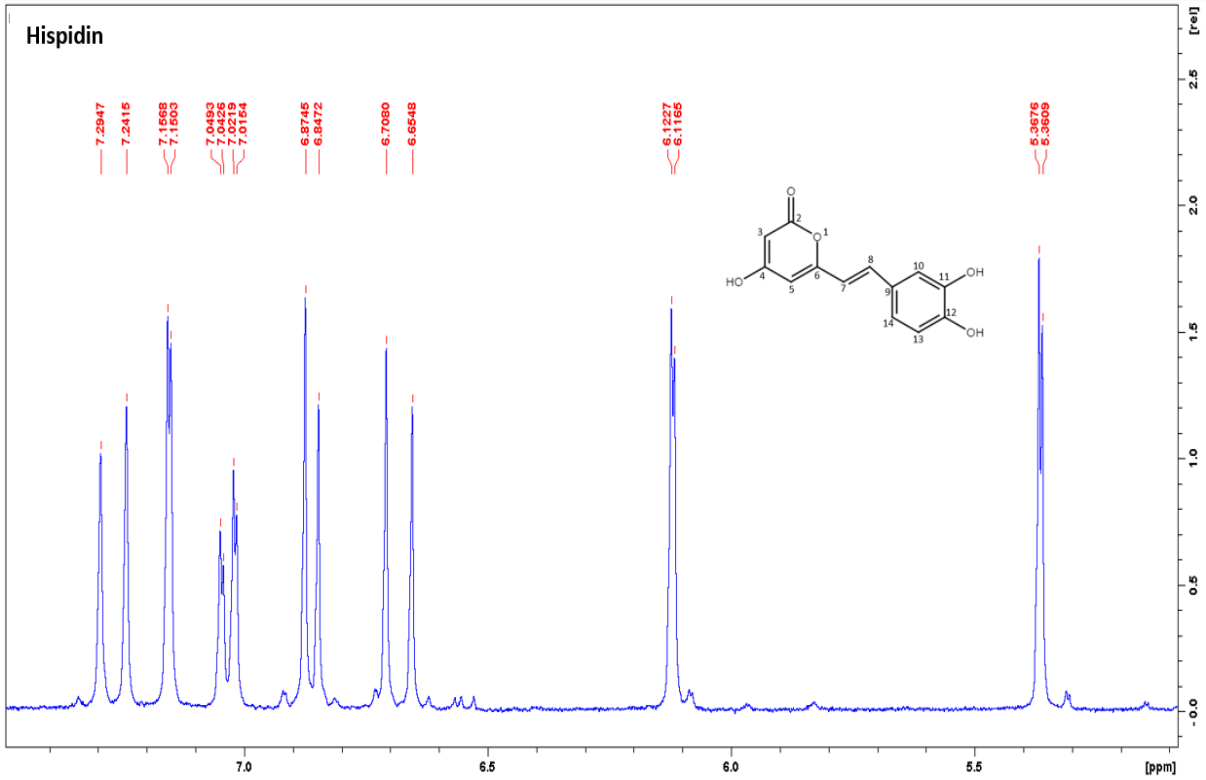


Figure 29 ^{1}H -NMR spectrum of hispidin in $(CD_3)_2CO$ containing 0.3% TMS

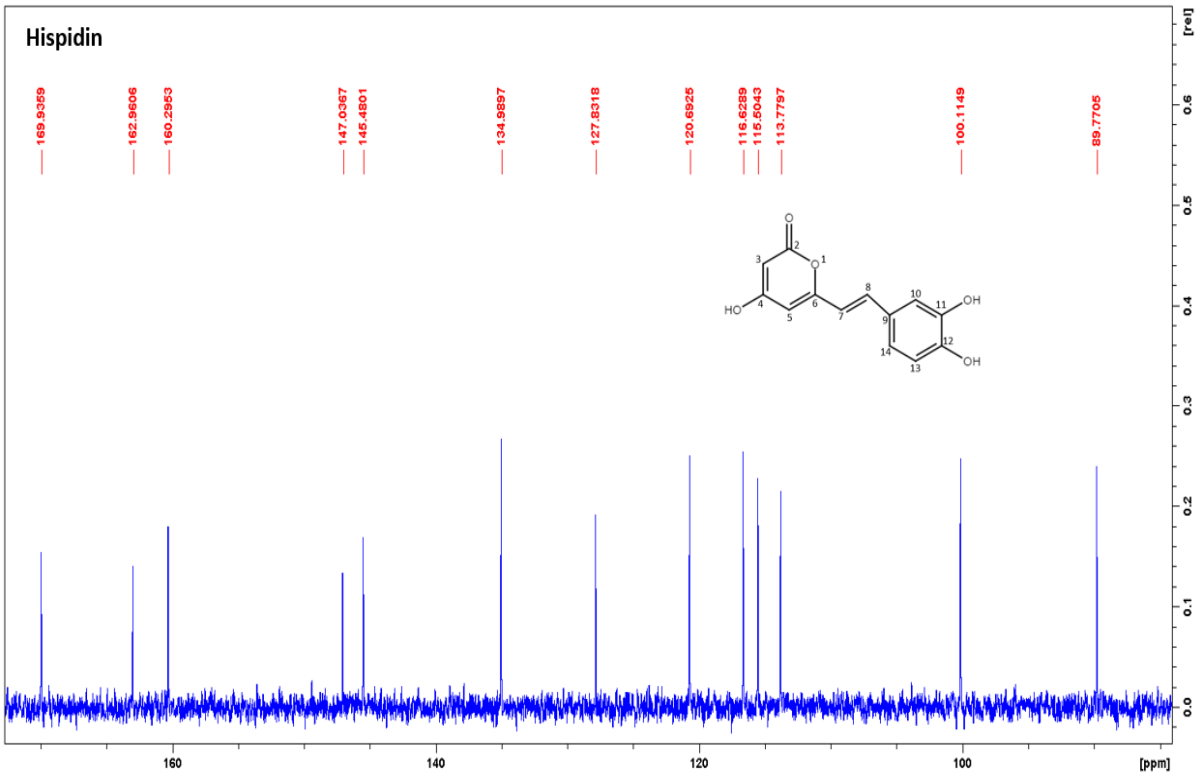


Figure 30 ^{13}C-NMR spectrum of hispidin in $(CD_3)_2CO$ containing 0.3% TMS

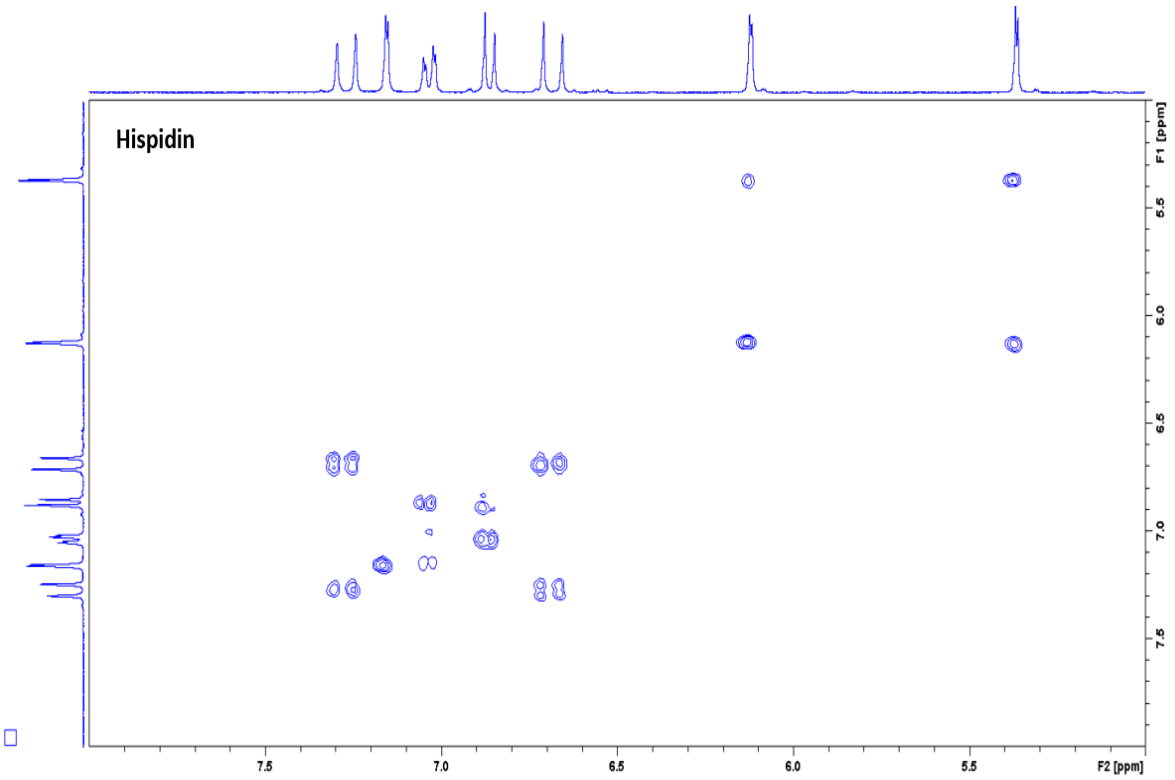


Figure 31 ^{1}H - ^{1}H NMR correlation spectroscopy (COSY) 2D spectrum of hispidin in $(CD_3)_2CO$ containing 0.3% TMS.

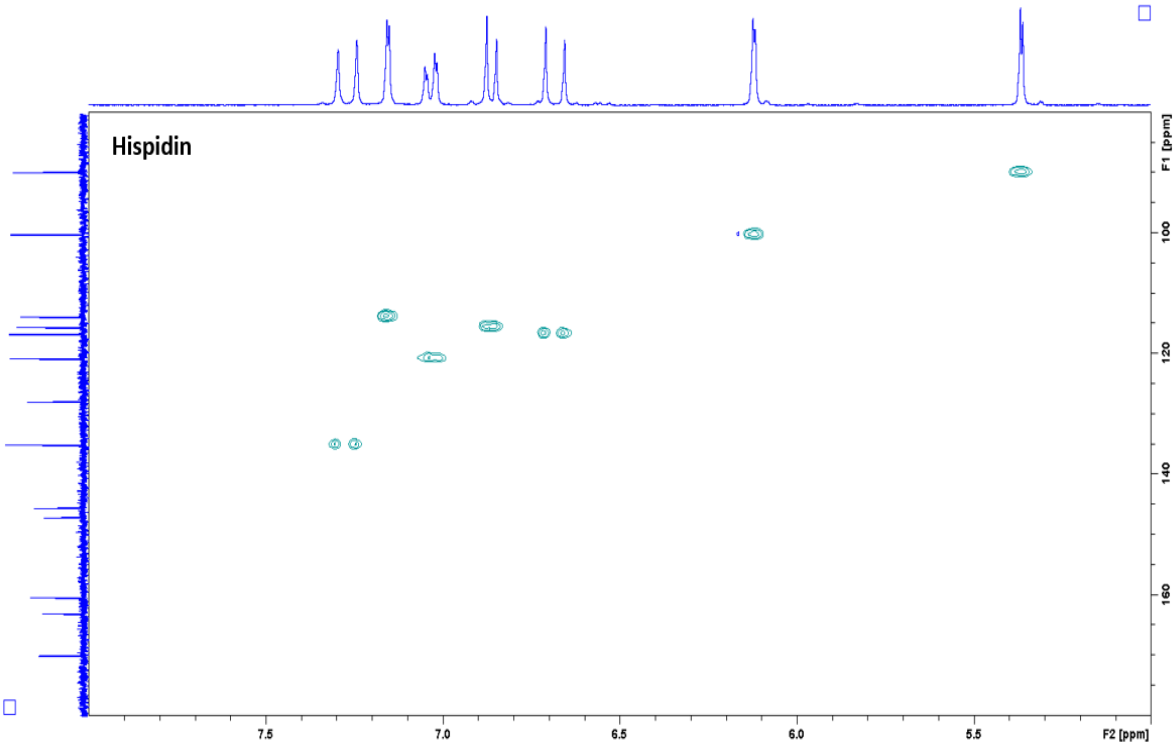


Figure 32 ^{13}C - ^{1}H NMR heteronuclear single quantum coherence (HSQC) 2D spectrum of hispidin in $(CD_3)_2CO$ containing 0.3% TMS.

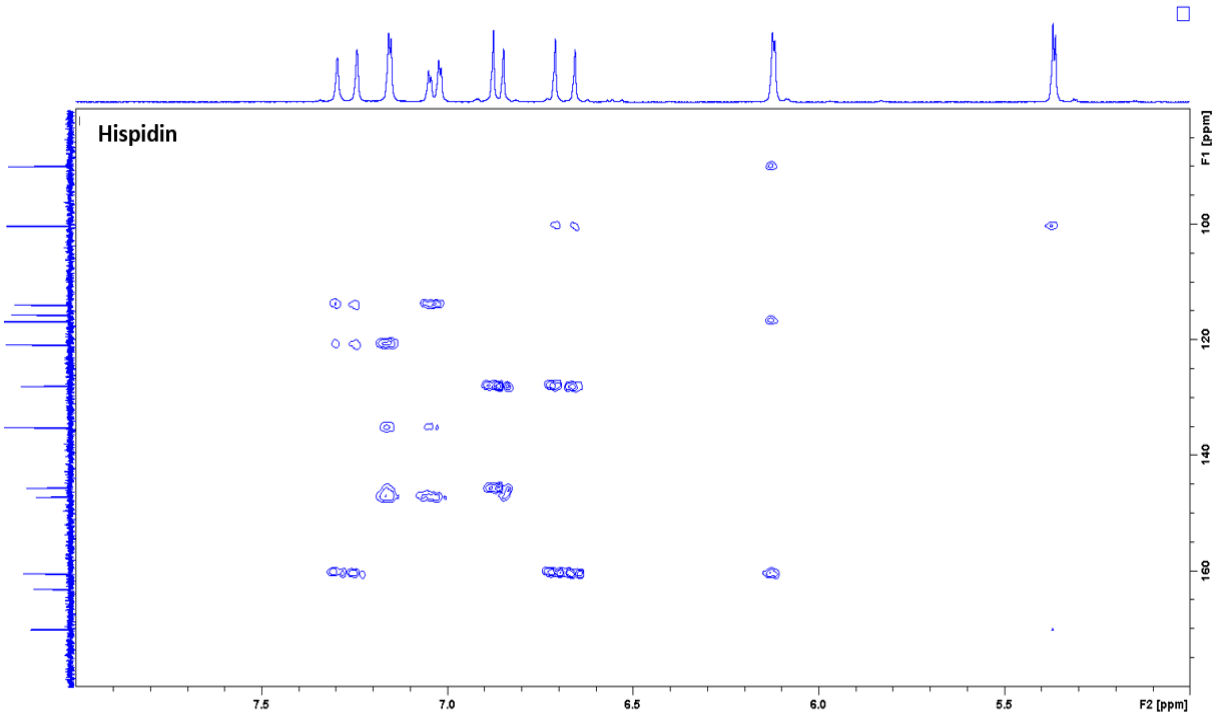


Figure 33 ^{13}C - ^{1}H NMR heteronuclear multiple bond correlation (HMBC) 2D spectrum of hispidin in $(CD_3)_2CO$ containing 0.3% TMS.

3.3.7 Physical properties of hispidin from *Phaeolus* sp.

The compound hispidin, purified using HPLC, was dried: it is yellow and has produced a fluffy needle-like powder (Figure 34).

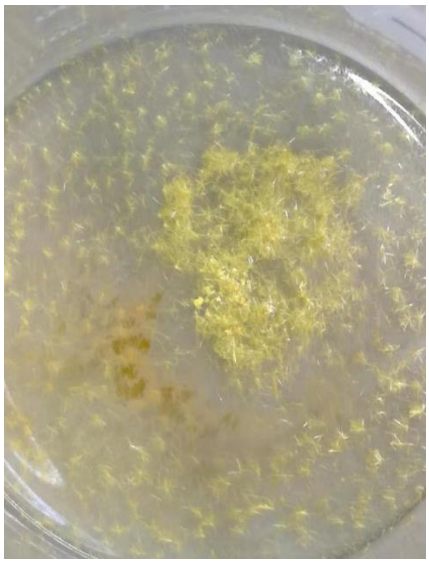


Figure 34 Hispidin from *Phaeolus* sp.

3.3.8 Melting point

About 1 mg of hispidin was loaded into a capillary and placed into the chassis hole. A quick trial run was completed with a starting temperature of 70 °C and ramp rate of 2 ºC per minute. The trial run provided an estimation of the sample's melting point. After this, a slow run was done to provide an accurate or true melting point range for the sample. The starting temperature was set at 210 °C, and the ramp rate was 0.5 ºC/minute. At 232.0 °C, a little liquid appeared on the sample, and at 233.0 °C, the powder sample turned into liquid. Meantime, another capillary with the same sample was also loaded into an oven and melted at the same time to improve the repeatability and accuracy of the melting point range determination. Table 17 shows the melting point range. In this study, the purity of hispidin was greater than 99%, and operation process was completed twice which can be considered reliable.

Table 17 Melting point range of hispidin.

Ramp Rate [°C/min]	Onset Point - Clear Point [°C]	Temp. Range [°C]
0.5	232.0-233.0	1.0

The reported melting point of hispidin was different from the previously published melting points: the CAS DataBase List showed the melting point range of hispidin to be from 312 to 315℃, while the Sigma-Aldrich Corporation database states the melting point as 237.5 to 238.5 °C. Gonindard *et al*. (1997) indicated the melting point is 259 °C. Such differences could result from differences in the purity of hispidin or distinct melting point measuring systems and experimental operations.

3.3.9 Percent yield of each step of the purification of *Phaeolus* sp.

Table 18 shows the percent yield at each step of the purification, the yield was calculated based on previous step of purification technique.

Table 18 Techniques followed and percent yield of compounds from E1 (80% ethanol) extract of *Phaeolus* sp.

Technique	Yield (in mass)	Yield based on technique (%)
Powdered mushroom sample # 71	50.217 g	-
E1 (80% Ethanol) Fraction weight	8.476 g	16.88
Liquid-liquid extraction yield.	3.012 g	35.53
Loaded on Sephadex™ LH-20 (400 mL)	= 320 mg/run	-
Active fractions obtained from Sephadex™ LH-20 (400 mL)	~40.80 mg/run	12.75
Sample purified by HPLC-UV	10 mg/run	-
Hispidin	1.5 mg	15.00

Part B: In search of other small molecules from *Phaeolus* sp.

3.4 Purification and chemical characterization strategies

Hispidin is a known compound, and its therapeutic properties and underlying mechanisms have been investigated as described in section 4.2.1. Some other purification techniques were used in this part to discover potential novel compounds other than hispidin in the mushroom *Phaeolus* sp.

3.5 Materials and methods

3.5.1 Silica gel chromatography

Normal-phase liquid chromatography is an important complement to more frequently used reversed-phase liquid chromatography. It can be performed in organic mobile phases (adsorption liquid chromatography), where it can offer advantages for separation of low molecular weight, moderately polar molecules, and allow variability in selectivity by using different mobile phases (Soukup *et al*., 2013).

Silica gel is a typical stationary phase used in normal phase liquid chromatography; it consists of a three-dimensional network of thousands of alternating silicon and oxygen bonds, with surface hydroxyl groups whose number and geometric arrangement determine the retention of the adsorbent. The slightly acidic silanol groups (Si-OH) in silica gel exist at the surface and extend into the internal channels of the pore structure. These hydroxyl groups interact with polar moieties by hydrogen bonding.

An organic solvent or a mixture of solvents will flow down through the column of silica gel. Components of the sample separate from each other by partitioning between the silica gel and the mobile phase. Molecules with different polarities will partition to different extents, and then flow through the column at different rates.

3.5.1.1 Solvent selection

Solvent selection for achieving a good separation was important in this technique. A thin layer chromatography (TLC) plate sheet was used in this process to determine the suitable solvent system. TLC is a method for analyzing mixtures; it is a sensitive technique and takes little time for analysis (Touchstone, 1992). Some solvents were tested by TLC, as no more fractions except hispidin were observed on the TLC plates in a single solvent system (Figure 39). A single gradient solvent system with a slow change of solvent polarities was used as the mobile phase to flush the column, which provides a distinct adsorption strength (Snyder, 1978). During the gradient-elution process, mixtures of analytes whose polarities span over a large polarity range can be separated (Soukup *et al*., 2013).

Elution of compounds in the column started with the first solvent as a mobile phase that has the lowest polarity, followed with other solvents as given in Table 19 (Snyder, 1978). Such a gradient solvent system was deemed to elute substances with different polarities out of the column gradually. Karami *et al*. (2010) have used a similar solvent system to successfully separate effective compounds from the plant *Thymus vulgaris* using silica column.

Table 19 The strength of mobile phases for elution.

Mobile Phase	Solvent Strength (Snyder, 1978).
80% Toluene+20% Ethyl acetate	0.28
40% Toluene+60% Ethyl acetate	0.34
Ethyl acetate	0.38
80% Ethyl acetate+20% Ethanol	0.48
Methanol	0.73

3.5.1.2 Silica gel column packing

The normal phase column was 30 cm × 1.5 cm, manually packed with silica gel. Silica gel (F60) particle size is between 40 to 63 μm, which is suitable for compounds with a molecular weight less than 1000. The FMI Lab pump (Fluid Metering, Inc) was used to pump out the solvents to flow through the column for packing. The column was first moistened and balanced by a solvent mixture of toluene and ethyl acetate (4:1).

3.5.1.3 Sample loading and fraction collection

The sample was prepared using the E1 extract (80% ethanol) powder and methanol (100%) was used to reconstitute the sample. The final concentration of loading sample was 30 mg/mL. The sample was centrifuged before loading onto the column to remove the impurities. The sample (2 mL) was introduced into the column with the help of a syringe (Figure 35).

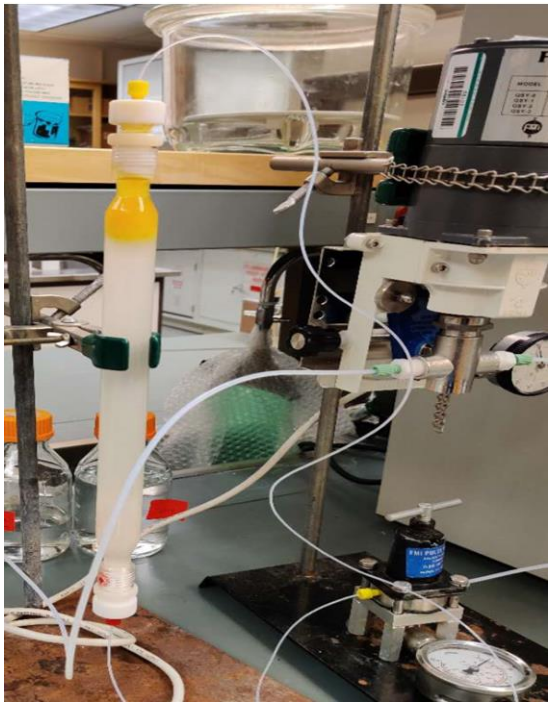


Figure 35 Photograph of the silica gel chromatography using a 30 cm × 1.5 cm size column and the mobile phase.

The flow rate of the mobile phase was 6 mL/min. The volume of the collected fractions was 6 mL. Five solvents with increasing solvent strength were used in this process as shown in Table 15. About 60 mL of each solvent was run through the column, which is almost double the column volume. After collection, the fractions were left in a fume hood and dried for further analysis.

3.5.2 Small scale chemical extraction using hexane

As 80% ethanol (E1) is a moderately polar solvent, then a non-polar solvent hexane was used to perform extraction of *Phaeolus* sp. in order to isolate other small molecules. A compound with an m/z of 283.2 was detected in the hexane extract.

This extraction was performed manually, as hexane is highly flammable, and it corrodes plastics, rubber, and coatings. Therefore, a hot plate and magnetic stir bar cannot be used in the

extraction process. Firstly, 500 mg of *Phaeolus* sp. mushroom powder was added to 40 mL of hexane and stirred gently with a glass rod. The beaker was then covered with foil and left in the fume hood overnight. The following day, the hexane layer was collected into another beaker using a Pasteur pipette and dried in a fume hood. After obtaining the dried sample, the yield was calculated and further analyzed using LC-MS.

3.5.3 HPLC-MS analysis

3.5.3.1 Silica gel column fraction

The HPLC-MS analysis of the silica gel fraction was performed using a Phenomenex Aqua® 5 µm C18 125 Å analytical column and isocratic solvent system of 70 % acetonitrile and 30 % water. The flow rate for the mobile phase was set at 1 mL/min. The sample (silica gel fraction 42) was filtered using a 0.2 µm filter (Sarstedt, Germany) before injecting 5 µL - MS system.

Mass spectroscopy was performed using an active splitter. The active splitter ratio used was 66:1. Scan mode was performed to determine the mass of ionized compounds in the peaks. A formic acid (0.1%) isocratic solution was used to ionize the sample compounds.

3.5.3.2 Hexane extraction

HPLC-MS analysis of the hexane extract was performed using a Phenomenex Aqua® 5 µm C18 125 Å analytical column and isocratic solvent system (two solvent system). The Sephadex LH-20 size based and silica gel polarity-based chromatography separations were not performed on this extraction. The composition of the isocratic mobile phase was based on 10

% water and 90% acetonitrile. The flow rate was set at 1 mL/min. The sample was filtered and 10 µL injected into the HPLC-MS system. Mass spectroscopy was performed using an active splitter, and the split ratio used was 66:1. Scan and SIM modes were performed to determine the mass of ionized compounds in the peaks. A formic acid (0.1%) isocratic solution was used to ionize the molecules in the sample.

3.6 Results

3.6.1 Silica gel column fraction

To determine the appropriate solvent system for the E1 extract of *Phaeolus* sp., a series of solvents with different polarities were used during TLC spot test. The control for the TLC plates was hispidin. Figure 36 shows TLC plate spots test eluting with different solvents. No separation was observed, suggesting that a single solvent system may not be suitable for the separation.

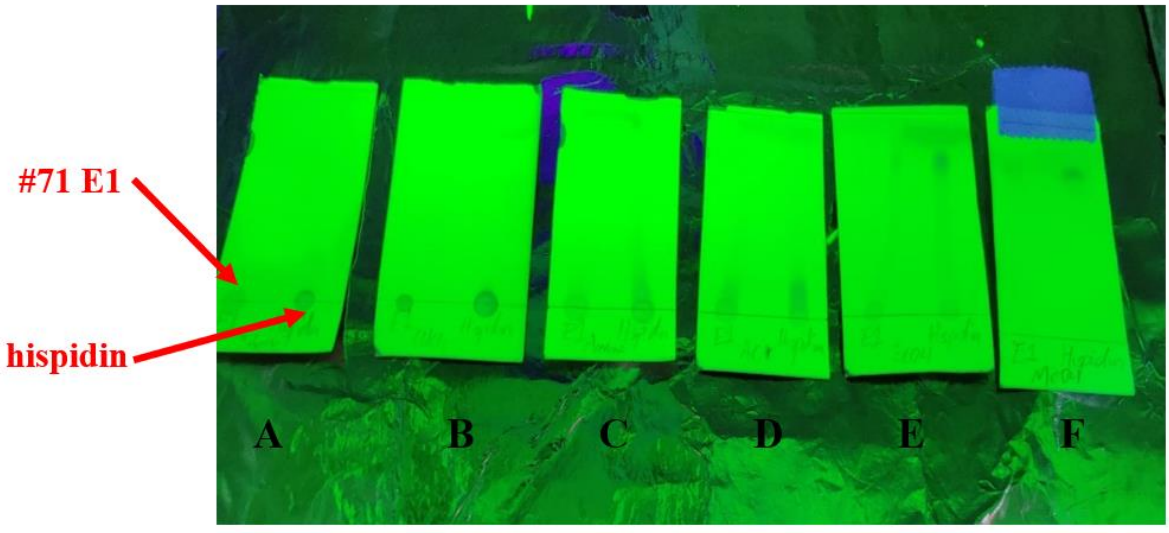


Figure 36 TLC of E1 of #71 Phaeolus sp. (lane 1), and hispidin (lane 2). The plates were eluted using toluene (A), chloroform (B), acetone (C), acetonitrile (D), ethanol (E) and methanol (F). Plates were visualized with UV light.

The solvent system as shown in Table 15 was used to run through the column and in total 53 fractions were collected from the silica gel column (Table 20). All the fractions were collected in glass tubes and the final volume of each fraction was around 6 mL. Fractions 42-44 were yellow in color, suggesting that most compounds were present in these fractions. After collection, these fractions were left in a fume hood to evaporate the excess solvent, and finally analyzed using LC-MS.

Table 20 Fractions collected from silica gel chromatography.

Mobile Phase	Number of fractions
80% Toluene+20% Ethyl acetate	1-10
40% Toluene+60% Ethyl acetate	11-20
Ethyl acetate	21-30
80% Ethyl acetate+20% Ethanol	31-40
Methanol	41-53

3.6.2 Hexane extraction

A small scale chemical extraction with hexane was performed on mushroom sample #71 *Phaeolus* sp. The percent yield for the hexane extract was 0.42% (Table 21). The extract was dissolved in 100% methanol and further analyzed by LC-MS.

Table 21 Percent yield of hexane extract from *Phaeolus* sp. (#71)

Mushroom No.	Mass of mushroom (mg)	Mass of hexane extract E1 (mg)	Yield (%)
71	500	2.1	0.42

3.6.3 HPLC-MS analysis

3.6.3.1 Silica gel column fraction

The most abundant peak on the UV spectrum was observed at 3.301 min containing 90.62% of the sample (Figure 37). Figure 38 represents the MS scan spectrum, consistent with the UV spectrum. The mass to charge ratio (m/z) of the protonated peak at 3.301 min was 247.0 (Figure 39), which is most likely to be hispidin, suggesting that hispidin is the dominant compound in E1 extract of *Phaeolus* sp., and the quantities of other potential constituents produced in this extract were possibly too small to be observed and detected.

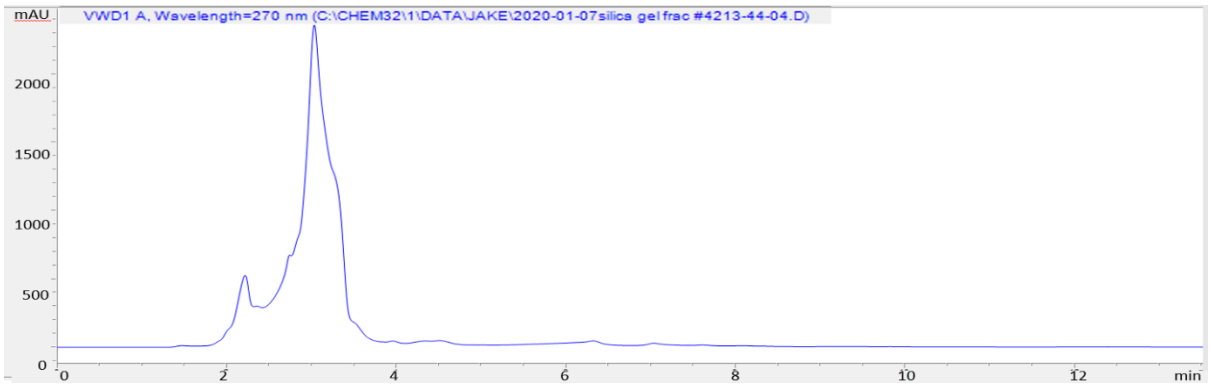


Figure 37 UV spectrum showing the relative abundance and retention time of at 3.031 min at λmax 270 nm (using Agilent C-18 analytical column and silica gel fraction sample).

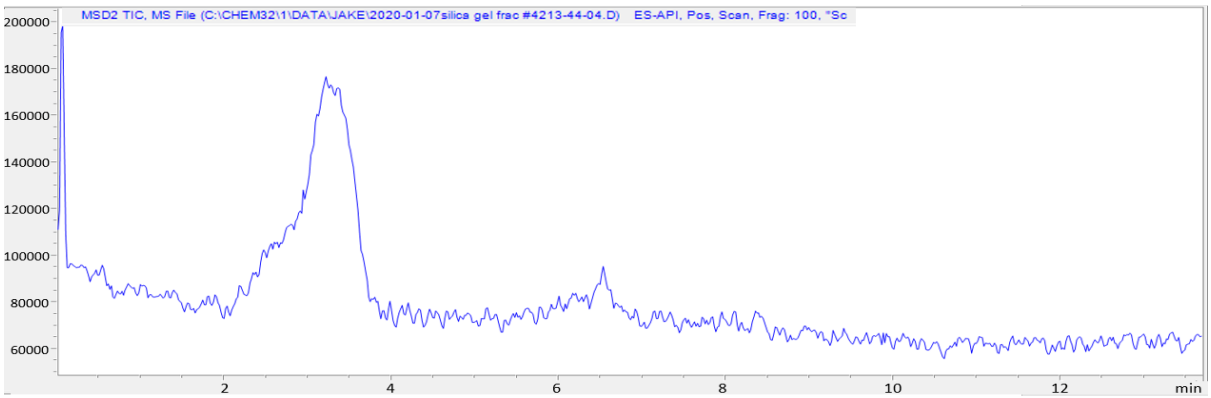


Figure 38 MS scan spectrum representing the relative abundance and retention time of the compounds detected through ESI (Electrospray ionization) chamber.

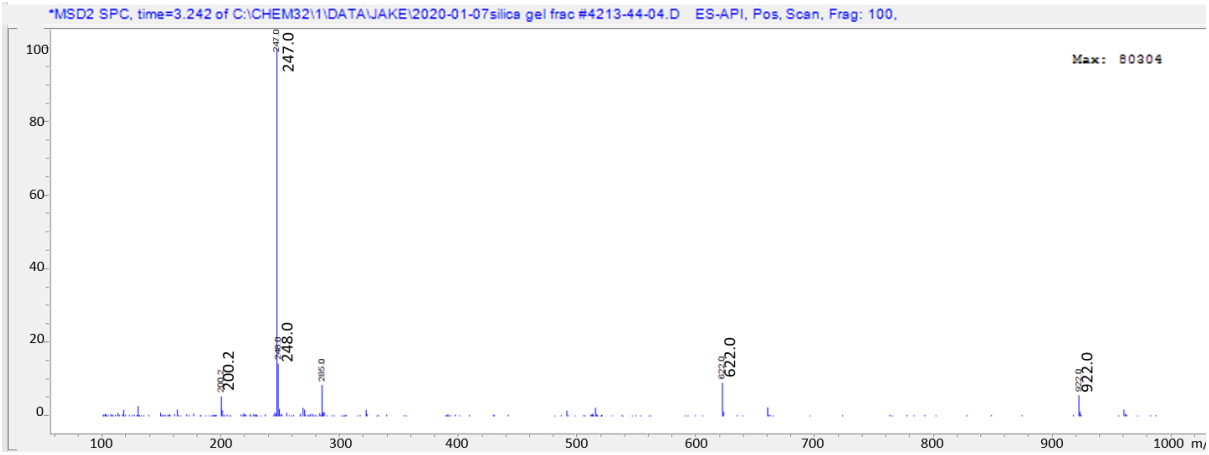


Figure 39 Integration of MS scan spectrum indicating the presence and abundance of compound with $[M+H]^+$ m/z 247.0 visible at 3.242 min.

3.6.3.2 Hexane extraction

The most abundant peak on the UV spectrum was observed at 3.114 min, containing 35.44% of the sample mixture (Figure 40). The percent abundance of the UV peak visible at 8.856 min was 26.82%, making it the second most abundant peak on the spectrum. The third abundant peak at 7.092 min was 21.79%. Figure 41 represents the MS scan signal; a major peak with m/z values of 283.2 can be observed at 9.214 min, which is consistent with the UV spectrum peak at 8.856 min.

The SIM mode and the m/z value of 283.2 were utilized to confirm the presence of this molecule. The m/z value of 247.0 was also set to identify the presence of hispidin in the sample. Figure 42 represents the SIM signal of the peaks. The abundance of the targeted mass 283.2 was found to be much higher in the SIM mode than in the scan mode (Figure 45), confirming the presence of a compound in the sample. Figure 44 shows the m/z value of 247.0 at 3.295 min, indicating this peak may be hispidin. The MS signals are visible with a slight time delay

because of the travel time through the 24-inch line from the UV spectrophotometer to mass spectrophotometer.

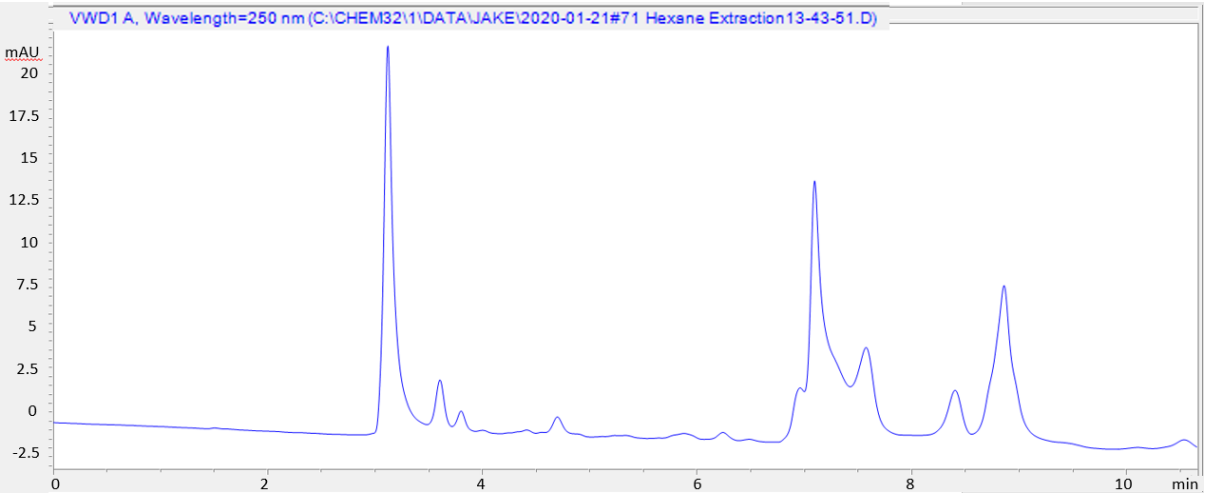


Figure 40 UV spectrum showing the relative abundance and retention time of peaks at λmax 250 nm (using Agilent C-18 analytical column and Sephadex LH-20 active fraction sample).

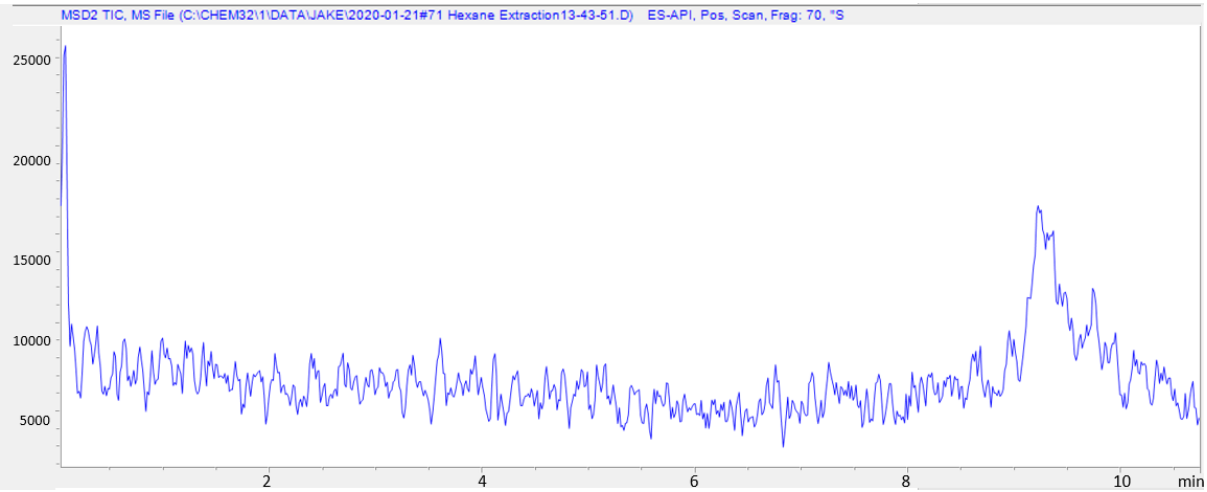


Figure 41 MS scan spectrum representing the relative abundance and retention time of the compounds detected through ESI (Electrospray ionization) chamber.

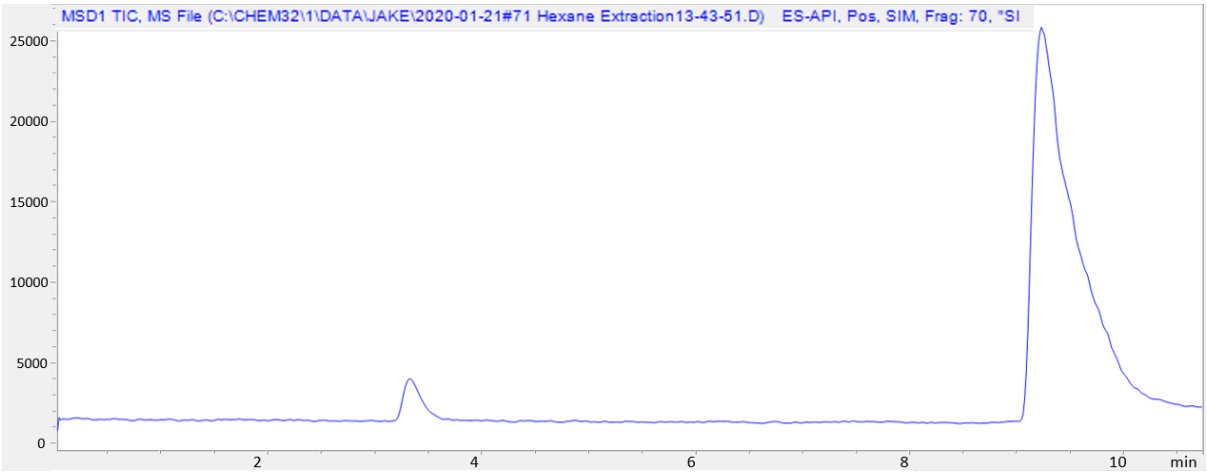


Figure 42 MS SIM spectrum representing the relative abundance and retention time of the targeted masses, detected through ESI (Electrospray ionization) chamber.

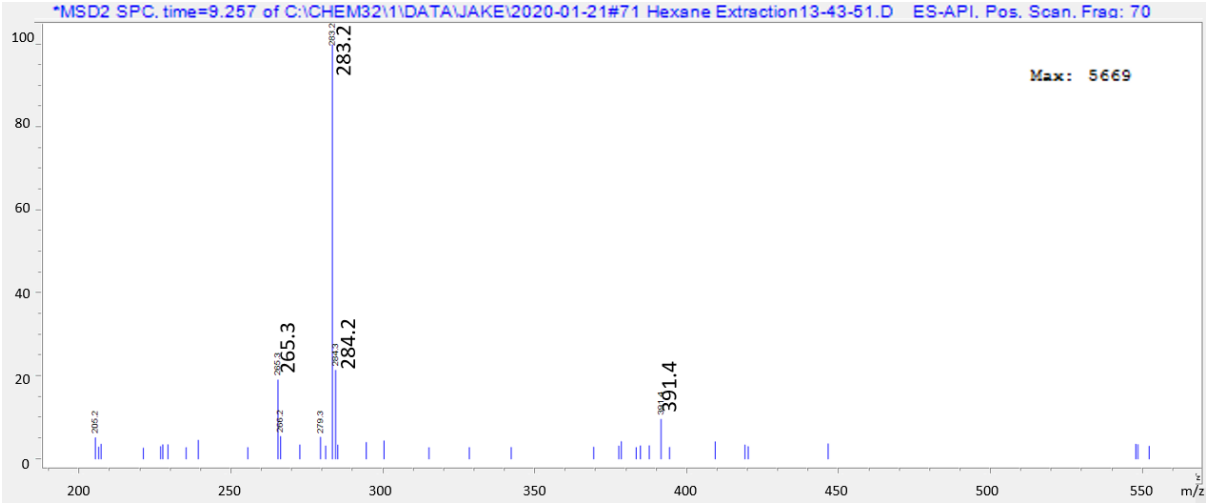


Figure 43 Integration of MS scan spectrum indicating the presence and abundance of compound with $[M+H]^+$ m/z 283.2 visible at 9.257 min

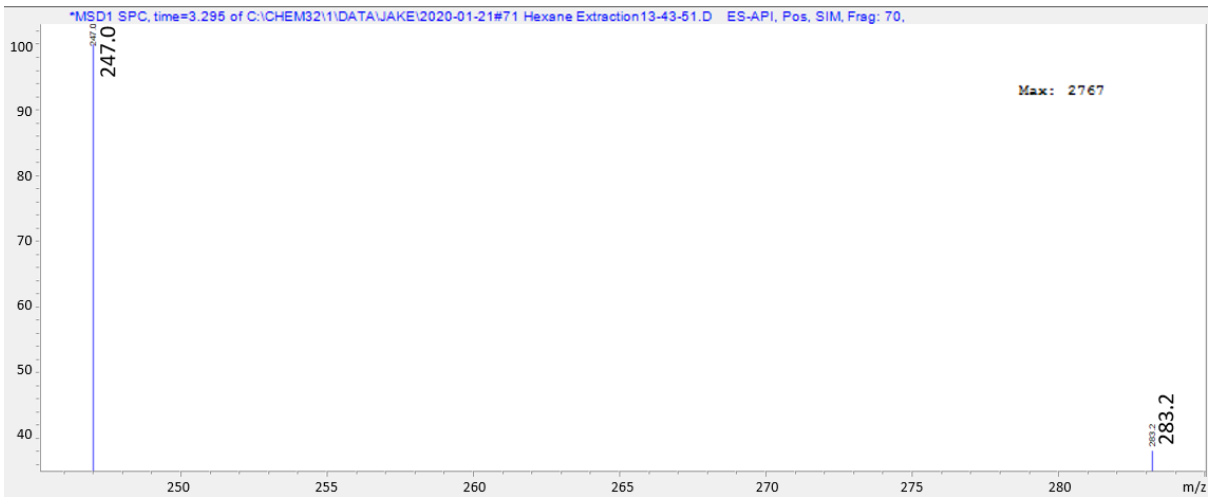


Figure 44 Integration of MS SIM spectrum indicating the presence and abundance of compound with $[M+H]^+$ m/z 247.0 visible at 3.295 min

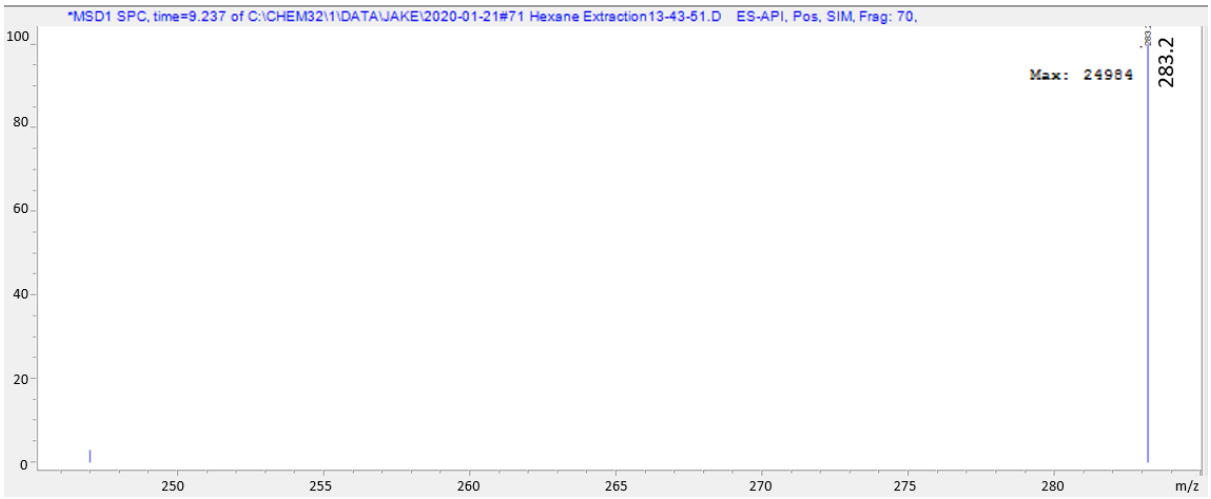


Figure 45 Integration of MS SIM spectrum indicating the presence and abundance of compound with $[M+H]^+$ m/z 283.2 visible at 9.237 min

3.6.3.3 Possibility of isolating novel compounds from *Phaeolus* sp.

HPLC-MS analyses of the hexane extraction have provided an insight into the possibility of isolating compounds other than hispidin from *Phaeolus* sp. The m/z value of the compound present in the hexane extract was 283.2, which could possibly be lactapiperanol A or

lactapiperanol C (purified from *Lactarius piperatus* (L.) Pers. (Yaoita *et al*., 1999). Figure 46 shows the structures of compounds lactapiperanol A and lactapiperanol C.

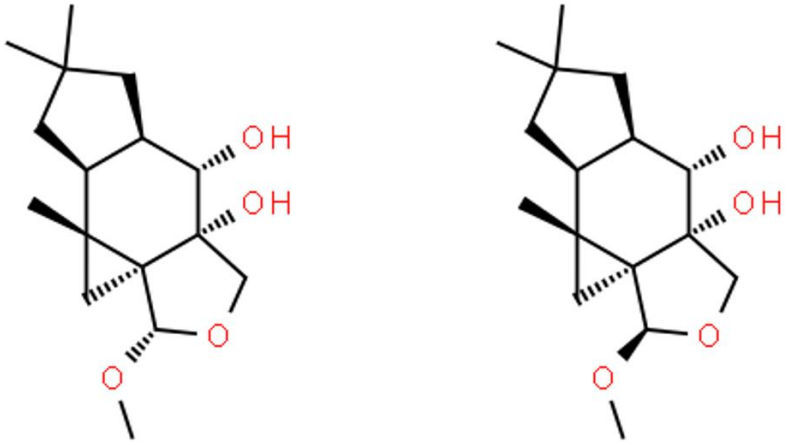


Figure 46 The structures of lactapiperanol A and lactapiperanol C.

However, the LC-MS spectra were not provided by Yaoita *et al*. (1999), so a comparison cannot be made. Figure 47 shows one predicted MS scan spectrum of lactapiperanol C from FooDB database which is similar to the spectra obtained from *Phaeolus* sp. (Figure 48).

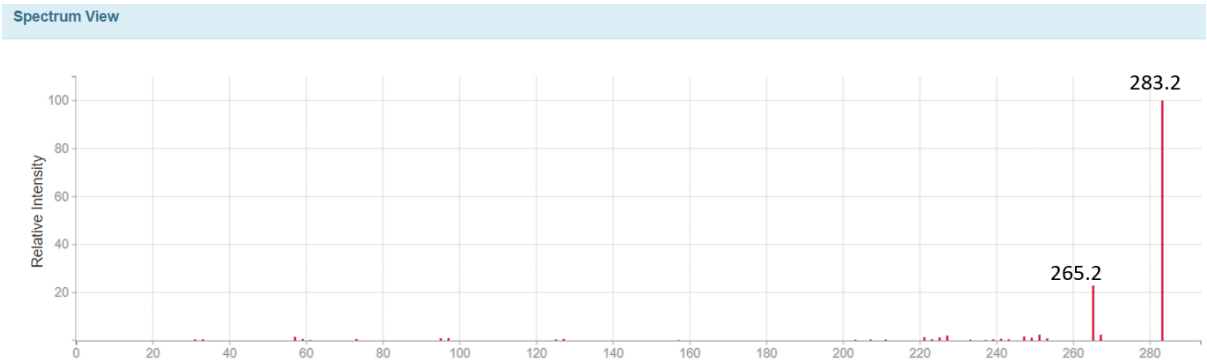


Figure 47 The predicted MS spectrum of lactapiperanol C (spectrum from FooDB database).

Chapter 4: General Discussion

4.1 Screening BC wild mushrooms for growth-inhibitory activities

Most wild mushrooms in British Columbia remain unexplored, and their medicinal properties remain unknown. Therefore, one of the major objectives of this thesis was to screen several mushrooms collected from British Columbia forests, for their growth-inhibitory potential. This primary screening method using MTT assay provides a relatively fast procedure to find potential anticancer agents in mushroom extracts and justify progression into the next stage of exploration.

In this thesis, eight mushroom collections belonging to three species: *Fomitopsis pinicola, Phaeolus schweinitzii* and *Phaeolus* sp, were collected from Haida Gwaii in British Columbia and chosen for the investigation of their growth-inhibitory potential.

4.1.1 *Fomitopsis pinicola*

In this study, extracts E1 and E2 from all *F. pinicola* specimens showed strong anti-cell viability activity, which is consistent with previous studies. Wu *et al*. (2014) revealed that the *F. pinicola* ethanol extract possesses anticancer activity both *in vitro* and *in vivo*, whereas the inhibitory effect of the water extract was less pronounced. Choi *et al*. (2007) demonstrated that the *F. pinicola* methanol extract had strong antitumor activity. Interestingly, 5% NaOH extract of *F. pinicola* had no significant inhibitory effect on HeLa cells (Smith, 2017), which is consistent with the results obtained in our study.

In recent years, many studies have reported on the biological function and chemical analysis of *F. pinicola* extracts. Wang *et al*. (2014) indicated that *F. pinicola* chloroform extract

could exert anticancer activity by inhibiting human colorectal cancer SW-480 cells viability with the IC_{50} of 190.28 µg/mL. Elsewhere, a chloroform extract of *F. pinicola* also demonstrated potent cell-growth inhibition activity, not only on *in vitro* murine sarcoma cancer S180 cells but also in mouse models by prolonging their lifespan (Gao *et al*., 2017). Wu *et al*. (2014) also documented *F. pinicola* ethanol extract significantly decreased tumor size with growth inhibitory ratio at 54% and increased the lifespan in mice bearing sarcoma-180 tumors. Furthermore, ethyl acetate and petrol ether extracts of *F. pinicola* were found to exhibit cytotoxicity against HeLa cell lines and hepatocellular cancer SMMC-7721 cell lines (Ren *et al*., 2006). One compound (3-acetoxylanosta-8,24-dien-21-oic acid), isolated from chloroform extract of *F. pinicola*, showed promising effect on human liver cancer SMMC-7721 cells and human breast cancer MCF-7 cells with inhibition rate at 77.63% and 90.29%, respectively (Sun *et al*., 2012). Shi *et al*. (2017) reported four lanostane triterpenes isolated from *F. pinicola* could inhibit breast cancer MCF-7 cell lines significantly. Table 22 provides a literature list of the extracts and anticancer agents found in *F. pinicola*.

Table 22 The agents found in *Fomitopsis pinicola* and their therapeutic potential against cancer

Mushroom specimen	Compound/ extract	Targets/ mechanisms of action	Cancer types affected	Experimental models	References
Fomitopsis pinicola	Chloroform extract	Cytotoxicity	Colorectal cancer	SW-480 cell lines	(Wang *et al*., 2014)
	Methanol extract	Cytotoxicity	Hepatocarcinoma, cervical cancer	HeLa and Hep3B cell lines	(Choi *et al*., 2007)
	Ethyl acetate and petrol ether extracts	Cytotoxicity	Cervical cancer	HeLa and SMMC-7721 cell lines	(Ren *et al*., 2006)
	Ethanol extract	Tumor growth arrest	Sarcoma	Mouse xenograft tumors	(Wu *et al*., 2014)
	Chloroform extract	Tumor growth arrest	Sarcoma	Mouse xenograft tumors	(Gao *et al*., 2017)
	3-acetoxylano-sta-8,24-dien-21-oic acid	Cytotoxicity	Liver cancer, breast cancer	SMMC-7721 and MCF-7 cell lines	(Sun *et al*., 2012)
	Four lanostane triterpenes	Cytotoxicity	Breast cancer	MCF-7 cell lines	(Shi *et al*., 2017)

Because extensive research had been conducted on *F. pinicola*, this mushroom was not investigated further in this thesis.

4.1.2 *Phaeolus schweinitzii*

In our study, extracts E1 and E3 of *P. schweinitzii* were found to possess the most potent growth-inhibitory effects. Hispidin and five new hispidin derivatives with antioxidant activity were isolated from *P. schweinitzii* (Han *et al*., 2013). *P. schweinitzii* is used for arsenic and

antimony volatilization in relation to sudden infant death syndrome (Pearce *et al*., 1998). Based on these results, it can be suggested that these extracts of *P. schweinitzii* are good candidates for future potential discovery of novel anti-cancer compounds.

4.2 Purification, identification and characterization of a growth-inhibitory compound from *Phaeolus* sp.

Phaeolus sp. was the mushroom selected for this study because limited studies have been performed on this mushroom regarding its anti-cancer activity, and preliminary work (as described in section 2.2.2.8) suggested that the ethanol extract of the mushroom was able to inhibit cancer cells in vitro. *Phaeolus* sp. (#71) sample was also more readily available and of substantial quantity compared to other *P. schweinitzii* collections.

4.2.1 Purification and characterization of compound hispidin from *Phaeolus* sp.

In this part of my MSc thesis, I have successfully developed a purification method to isolate one growth-inhibitory compound, hispidin, from *Phaeolus* sp.

The first purification step is liquid-liquid extraction with ethyl acetate and water. The cytotoxic MTT assay was performed to assess the inhibitory activity. Growth-inhibitory compounds were found in the ethyl acetate layer, suggesting that they are more hydrophobic. Further purification was achieved using Sephadex LH-20 size exclusion chromatography which could provide information about the size of the bioactive molecules, as smaller molecules will elute at later fractions when using a Sephadex LH-20 column. The last purification step was HPLC to isolate the major growth-inhibitory small molecule present in the bioactive fractions from Sephadex LH-20. After collection of the bioactive small molecule from HPLC, followed by 1D and 2D NMR analyses, the obtained spectra were compared with

previously published NMR data (Park *et al*., 2004, Lee *et al*., 2008) and finally, the compound's identity was confirmed to be hispidin. The final percent yield of hispidin was 0.115%.

Hispidin was first discovered from *Inonotus hispidus* (Bu'Lock *et al*., 1962). It is a phenolic compound, which has various biological functions. It can be synthesized (Gonindrd *et al*., 1997) and purified from different mushrooms, using a variety of purification strategies. Benarous *et al.* (2015) used silica gel column chromatography and gradient eluent to purify hispidin from the mushroom *I. hispidus*, however, the extraction process and data were not reported in detail. The compound was also extracted from the mushroom *P. linteus* using only silica gel column chromatography, and the purity was greater than 90%, as assessed by HPLC-UV (Jang *et al*., 2010). In our study, after size-exclusion chromatography, the purity was greater than 95% as assessed by HPLC-UV, and the purity was greater 99% after HPLC purification.

The previous studies which isolated hispidin from mushrooms only used the silica gel polarity-based chromatography to do the separation, which is not efficient, as some other components with similar polarities can also be eluted with hispidin simultaneously, resulting in an impure product. Thus, Sephadex LH-20 size-based chromatography can be employed as an intermediate step, as it is an effective method to purify small molecules (Nandi, 1976). Finally, HPLC can be utilized for the separation of molecules by their polarities, as a crucial step to obtain a higher purity of the final product. By comparison with those purifying methods of hispidin from other mushrooms, this purification strategy of phase separation, size-exclusion chromatography and HPLC can be considered an efficient way to isolate small molecules.

Hispidin was found to be bioactive and effective in many manners. It exerts antitumor effects against human pancreatic BxPC-3 cancer cells and cancer stem cells while also being capable in sensitizing pancreatic cancer stem cells from chemotherapy medication (gemcitabine) (Chandimali et al., 2018). Hispidin also induces intrinsic and extrinsic apoptotic pathways mediated by reactive oxidative species (ROS) in both BxPC-3 cancer cells and cancer stem cells (Chandimali et al., 2018). In addition, hispidin is known to be more cytotoxic towards certain cancer cell lines and cancer cells (human cancerous keratinocytes SCL-1 cell line, human cancerous pancreatic duct Capan-1 cell line, adenocarcinomic human alveolar basal epithelial cells A549, hepatocellular carcinoma cells (Bel7402), and human pancreatic ductal adenocarcinoma cells (Capan-1)) than normal human MRC-5 cells because it inhibits protein kinase enzymes (Gonindard et al., 1997). The compound was found to induce autophagic and necrotic death of SGC-7901 gastric cancer cells via lysosomal membrane permeabilization through inhibiting tubulin polymerization (Lv et al., 2017). Furthermore, Lim et al. (2014) found that hispidin could significantly decrease cell viability in both mouse colon cancer CMT-93 cells and human colon cancer HCT 116 cells.

Based on the above description, it is clear that hispidin had been widely studied for its medicinal properties and underlying mechanisms. Therefore, the biological activities were not further pursued in this project.

4.2.2 In search of other small molecules from *Phaeolus* sp.

As hispidin is a well-studied compound, other purification methods were used to explore other potential small molecules from *Phaeolus* sp. Silica gel chromatography was first

performed to separate potential compounds in the E1 extract of *Phaeolus* sp. The collected silica gel fraction was analyzed by HPLC-MS, and it was concluded that hispidin was the major compound present in the E1 extract of *Phaeolus* sp. A 80% ethanol is a moderately polar solvent, and is likely to extract hispidin.

The non-polar solvent hexane was therefore used to perform an extraction of *Phaeolus* sp., aiming to isolate other small molecules. The hexane extract was subjected to HPLC-MS analysis, and the m/z of the most abundant compound present was 283.2, which could be compounds lactapiperanol A or lactapiperanol C (purified from *L. piperatus*) (Yaoita *et al*.,1999), or a novel compound. Both lactapiperanol A and C were not found to possess medicinal activity. However, due to the low yield of hexane extract and insufficient *Phaeolus* sp. mushroom powder, not enough sample could be collected for further investigation. Supplies of material and low yield of natural compound are often a challenge for studies of natural products (Beutler, 2009).

4.3 Conclusion

Previous studies have indicated that British Columbian wild mushroom species possess immuno-modulatory and growth-inhibitory activities. This is the second study in Dr. Lee's lab describing purification and characterization of small molecules from Canadian wild mushrooms. This MSc thesis sheds further light on the anti-cancer and therapeutic potential of BC mushrooms and the purification of their small molecules. The results obtained from this study are providing basic knowledge for future investigations into discovering potential novel compounds from BC wild mushrooms. For example, the crude water extracts from mushroom

Phaeolus sp. that contain growth-inhibitory activity, were not studied here, and might serve as a potential avenue for new compound discovery. This study has successfully led to the purification of hispidin from *Phaeolus* sp. using phase separation, size-exclusion chromatography and HPLC. A compound with an m/z of 283.2 was identified in the hexane extract of *Phaeolus* sp. and will be subjected to further chemical analysis in the future.

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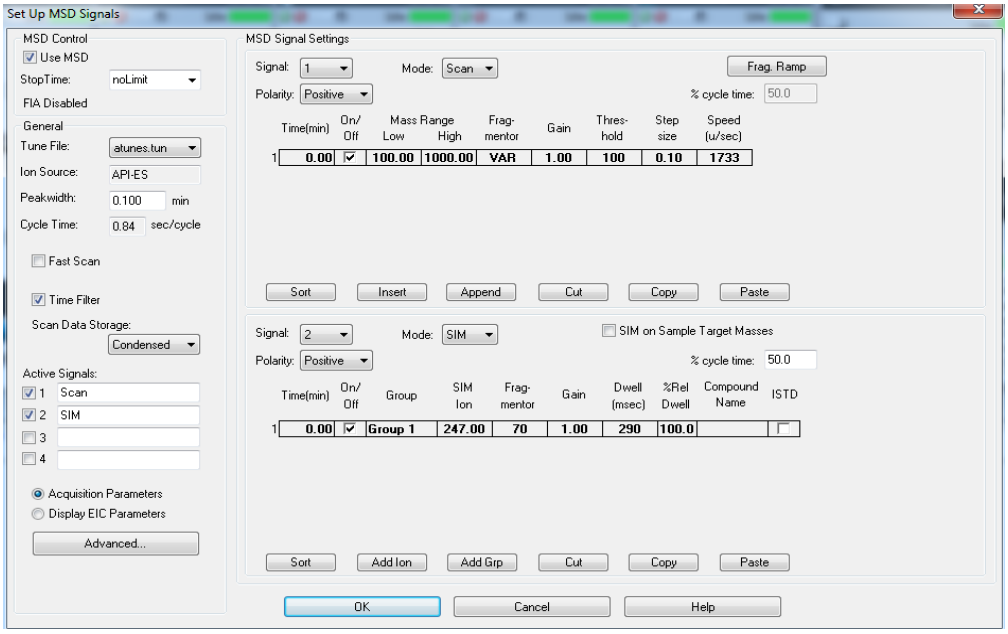
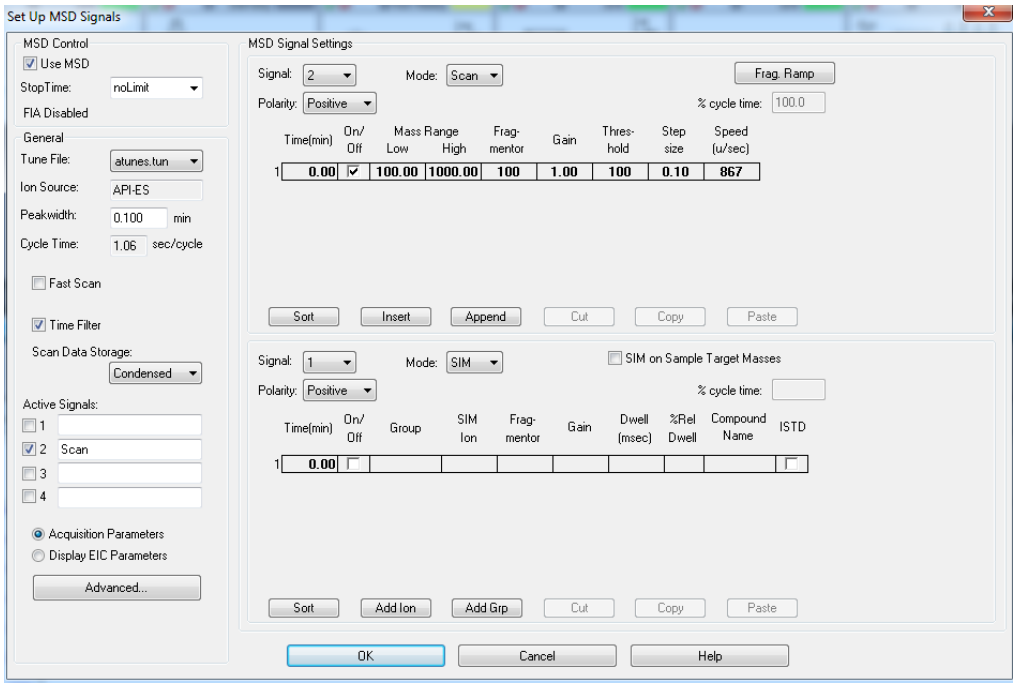
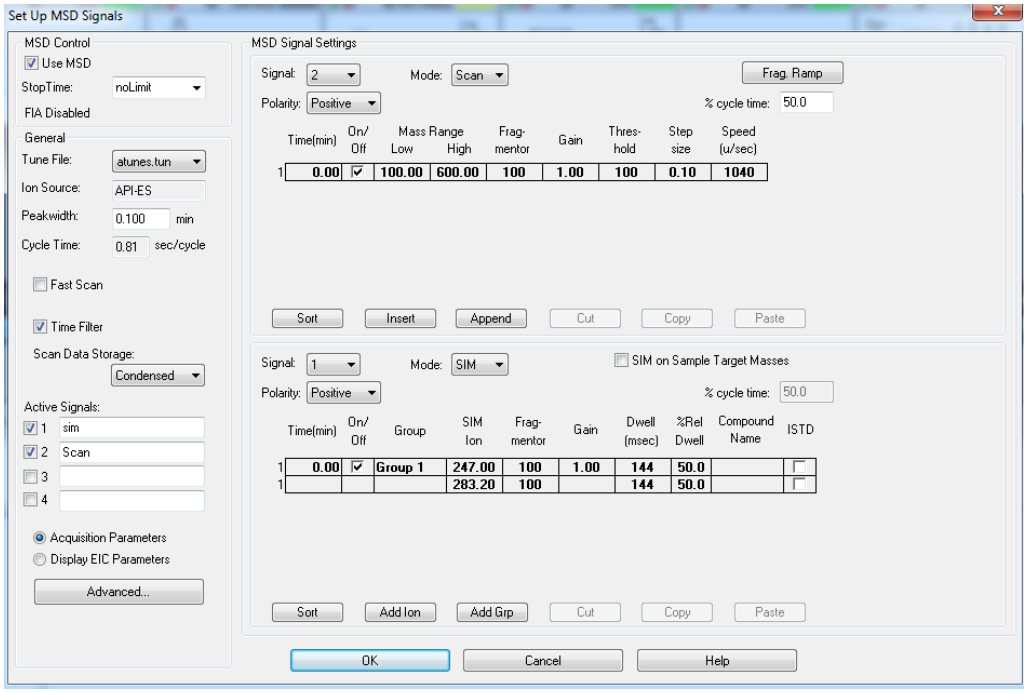
Appendix



SIM and scan mode targeted masses and the fragmented value as well as % relative dwell time for the targeted masses set up for LH-20 active fraction from *Phaeolus* sp.



Scan mode targeted masses and the fragmented value as well as % relative dwell time for the targeted masses set up for silica gel fractions from *Phaeolus* sp.



SIM and scan mode targeted masses and the fragmented value as well as % relative dwell time for the targeted masses set up for hexane fraction from *Phaeolus* sp.