IN SEARCH OF NOVEL IMMUNO-MODULATORY COMPOUNDS FROM BRITISH COLUMBIA WILD MUSHROOMS AND THEIR EFFECTIVENESS IN INFLAMMATORY MICRO-CIRCULATION OF MICE

by

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Abstract

Natural products have been an integral component of people's health and health outcomes for thousands of years. In particular, several mushroom species have demonstrated beneficial therapeutic potential. The goals of this research are to explore the immuno-stimulatory and anti-inflammatory potential of wild mushrooms native to the North Central region of British Columbia. Out of 42 mushroom extracts examined, four exhibited strong immuno-stimulatory activity as assessed by induction of tumor-necrosis factor alpha (TNF-α) production in macrophage cells. Out of thirty-three extracts tested, nineteen demonstrated potent anti-inflammatory activity as determined by inhibition of lipopolysaccharide-induced TNF-α production in macrophage cells. Sodium hydroxide extract of *Echinodontium tinctorium* exhibited potent anti-inflammatory activity and was selected for further study. A small molecular weight (~5-25 kDa) carbohydrate was successfully purified using sequential size-exclusion and ion-exchange chromatography. GC-MS analysis showed that the polysaccharide has glucose (89.7%) as the major back-bone monosaccharide, and also the presence of other monosaccharides such as mannose (3.1%), galactose (2.8%), fucose (2.4%), and xylose (2.0%). The study also revealed the presence of 1,3-linked glucose, 1,6-linked glucose, 1,3-linked galactose and 1,3,6-linked glucose linkages. Both the semi-purified anti-inflammatory compound(s) from *E. tinctorium* and the methanol extract of *Inonotus obliquus* can ameliorate histamine-induced vasodilation in the 2A arterioles (*gluteus maximus* muscle) in mice. This is the first study to demonstrate the anti-inflammatory activity of purified compounds and extracts from mushroom in an animal microcirculation model using intravital microscopy.

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Chapter 1: Background and Objectives

This chapter highlights the research background, specifically providing some statistics of cancer prevalence and a brief introduction on the historical and traditional use of mushrooms, their medicinal properties, their role in immuno-modulation, and the topic of human inflammation. Finally, the chapter outlines my research objectives and the structure of this thesis.

1.1 The Havoc of Cancer

Cancer is a set of diseases characterized by uncontrolled cell division and their ability to metastasize (invade) other tissues by causing angiogenesis (vascularization) and tumor lumps, thus interfering with the normal functioning of the body (Vogelstein & Kinzler, 2004). According to the recent World Health Organization report (2017), cancer has been one of the leading causes of morbidity and mortality with approximately 14 million new cases in 2012 along with 8.8 million cancer-related deaths in 2015 (1 out of 6 of all global deaths). The coming two decades expect to see a rise in such cases by 70% (World Health Organization, 2017).

Canadian Cancer Statistics (2016) rates cancer as the leading cause of deaths in Canada, being responsible for around 30% of all the fatalities; moreover, 202,400 new cases of cancer and 78,800 cancer-associated deaths had been estimated to occur in 2016 in Canada alone. According to an estimation in 2009, almost 40% of all Canadians (i.e. 2 out of 5) are projected to develop cancer at least once in their lifetimes and 25% of these (i.e. 1 out of 4), are anticipated to die from this deadly disease (Canadian Cancer Statistics, 2016).

The alarming cancer death rates indicate the complexity of tumors, representing a combination of many diseases. Cancer cells are malignant neoplastic cells transformed by uncontrolled cell division, and expanding in the surrounding tissues and metastasizing to the important organs like the brain, lungs, and stomach. The transformation of normal cells into

cancerous cells can take place in 3 steps (Borchers et al., 2004; Oberstein & Olive, 2013). The first step is the binding of a mutagen to the cellular DNA, thus initiating the damage. This is not enough for triggering the entire tumor cascade and requires activation however; the second step is the activation of the tumor in which tumor-associated promoters are stimulated and benign tumors are formed. Finally, these benign tumors progress, take control of the cellular machinery, and cause unrestricted cellular proliferation (Borchers et al., 2004). Moreover, the transformed tumors are highly diverse and have many defective genes that regulate growth and division (Oberstein & Olive, 2013). These are not confined to the site of origin, and are marked by various epigenetic and genetic variations, post-transcriptional modifications, complex mechanisms, and various pathways alterations. Such varied alterations make it harder to target cancer cells.

Currently, chemotherapy is one of the major approaches (hormonal and radiotherapy being others) for treating cancer in which a cytotoxic agent is delivered to the cancerous cells. This approach decelerates the rate of constant division and restrains the growth and development of cancer cells (Miles & Chang, 2004). Chemotherapy, on the other hand, also damages healthy cells and causes serious side effects; it can decrease the number of leucocytes in the blood, increase risk of internal bleeding and brain hemorrhage, and suppress the immune system thus making the patient vulnerable to minor diseases that can eventually cause death (Miles & Chang, 2004, Kakde et al., 2011). Chemotherapy also causes other pharmacokinetic problems like altered bio-distribution, biotransformation, drug resistance, and drug clearance (Kakde et al., 2011). Despite these numerous side effects, chemo, hormonal, and radio therapy still remain among the possible options for the treatment of cancer patients worldwide. Therefore, there is a constant need for developing synergistic anti-tumor drugs or alternative therapies that have fewer

side effects. Due to the severe toxicity and aforementioned reasons, scientists are extensively studying various natural products from plants, marine organisms, animals and fungi as a possible substitute (Ali et al., 2012).

1.2 Natural Products to Treat Cancer

Natural products have been used and studied extensively for treating almost all kind of diseases known to man. Despite various advances in drug discovery techniques such as combinational chemistry and rational drug design, natural products remain the number one choice as sources of potential lead molecules for drug discovery and development (Cragg & Newman, 2013).

Similarly, the history of anticancer drug discovery also revolves around natural products. Scientists are trying to modify the cytotoxic and/or cytostatic mechanisms present in the defensive response of various plants, animals and other living creatures to achieve the desired anti-tumor response (Huryn & Wipf, 2014). Almost 80% of all marketed anticancer drugs are derived directly or indirectly from natural sources, indicating the efficacy and efficiency of natural compound (s) against cancer (Newman & Cragg, 2012). Out of 206 anti-cancer drugs, approved since the 1940s, 131 (64%) are either directly from natural sources or are inspired by them (75% without considering vaccines and biologics) (Newman & Cragg, 2012). Some well-known examples of naturally inspired anti-cancer drugs include vincristine, vinblastine, methotrexate, paclitaxel, and doxorubicin, among others (Huryn & Wipf, 2014). Among natural resources, fungi are one of the main sources of potential lead molecules for anticancer drug discovery (Newman & Cragg, 2012). The only requirement is to focus on primary screening from unexplored regions of the world and to combine high through-put and various other modern techniques in order to accelerate drug availability.

1.3 Mushrooms use-Historical Perspective

Fungi encompass eukaryotic organisms including both unicellular and multicellular species whose cell walls are made up of chitin and have an absorptive mode of nutrition. It is a hyper-diverse group but the estimation and collection of fungi is difficult because of their ephemeral and cryptic nature (Mueller et al., 2004). This kingdom is home to thousands of yeasts, rusts, smuts, lichens, and mushrooms which, due to the variations in their life cycles, are making undeniable contributions in numerous domains such as the environment (as decomposers, bioremediation, agriculture), industries (fermentation processes, biotechnology, fungal enzymes), pharmaceuticals (medically important species, holistic health benefits, biological drugs), foods (mushrooms, yeasts, prebiotics) and mycology research (better stains, more benefits, better solutions) (Lange, 2014; Lange & Grell, 2014). This makes fungi an important contributor in the sustainability and stability of human lives.

The term mushroom is not a taxonomic classification; rather, it is defined as macrofungi with distinct fruiting bodies that can be epigeous or hypogeous, large enough for the human eye to be seen and collected by hand (Chang & Miles, 1992). Epigeous mushrooms have their fruiting bodies above ground (mostly Basidiomycetes) while hypogeous mushrooms have their fruiting bodies below ground (Ascomycetes and Basidiomycetes, many of them living in symbiotic associations with plants roots as ectomycorrhizal associates) (Tang et al., 2015).

Of the 150,000-160,000-mushroom species that are present on Earth, hardly 10% are currently known to man (Hawksworth, 2012), and only 650 species have been assessed for their medicinal activity (Minato, 2010). Interestingly, there is an increase in the trend of discovering fungi, specifically mushrooms forming ectomycorrhizas with native trees, in different areas of Europe, North America and other tropical regions (Hawksworth, 2012). These vast and largely

untapped regions in the world are where novel mushrooms can be discovered and their medicinal value estimated.

Mushrooms have been in use since the earliest history of mankind. Ancient Greeks believed that mushrooms were "strength pills" that provided power to the warriors on the battlefield (Blanchette et al., 1992). Similarly, for thousands of years, Chinese culture has been endorsing mushrooms as the "elixir of life" (Valverde et al., 2015). Mushrooms are used extensively for nutritional and medicinal purposes in traditional medicines in Asia (specifically China and Japan), Canada, Mexico, Russia, USA, and Venezuela (Comandini et al., 2012; Garibay-Orijel et al., 2007).

Some mushroom species are used by human beings for both health and spiritual purposes. *Ganoderma lucidum* (Curtis) P. Karst is one of the oldest mushrooms used in traditional medicine as a tonic for fighting diseases, enhancing health, anti-aging, and life longevity (Thyagarajan-Sahu et al., 2011). Even in Europe, the traditional use of mushrooms can be witnessed after the discovery of the prehistoric mummy of the "Iceman" in the Tyrolean Alps in 1991. The mummified body had *Fomes fomentarius* (L.: Fr.) in a bag that was tied around his waist and it was probably used as a tinder for fire making (Peintner et al., 1998; Pöder & Peintner, 1999). Mushrooms were also part of the traditional diet of native North Americans because of the flavor and nutritional value they added to the food. Oyster mushroom, *Pleurotus ostreatus* (Jacq.) P. Kumm., is one of the common used mushroom by all four groups of Interior Salish peoples of British Columbia (i.e. the Okanagan, Nlaka'pamux, Lillooet, and Shuswap) (Turner & Szczawinski, 1991). Pine mushroom, *Tricholoma magnivelare* (Peck) Redhead, is also historically used by Lillooet, Nlaka'pamux and Lower Lillooet groups, specifically among the four groups of Interior Salish peoples of British Columbia (Turner 1975). Cottonwood

mushroom, *Tricholoma populinum* J.E. Lange, is another commonly food mushroom used by all four groups of Interior Salish peoples of British Columbia (Turner et al., 1980). Métis Nation of Ontario identified Chaga, *Inonotus obliquus* (Ach. ex Pers.) Pilát, and Turkey tail mushroom, *Trametes versicolor* (L.) Lloyd (=*Coriolus versicolor* (L.) Quél.), as strong therapeutic fungi and a cure for various diseases (Kuhnlein & Turner, 1991; Métis Nation of Ontario, 2010). Therefore, the medicinal potential of these mushrooms was not hidden from the first nation people as they used and applied these fungi for curing human diseases.

1.4 Mushrooms & Modern Medicines

In a western medicine context, mushrooms have been successful in making a profound impact since about half a century ago, after the publication of scientific articles on their various medicinal properties including anti-cancer effects (Espenshade & Griffith, 1966; Gregory et al., 1966). These studies have revolutionized the herbal perspective of mushrooms and introduced them into the pharmaceutical market (Wasser & Weis, 1999). Also, in recent times there has been a shift in the trend of consumption and research on wild mushrooms because of their pharmacological, nutritional, and prophylaxis characteristics (Günç et al., 2013).

Currently, mushrooms are treated as "mini-pharmaceutical biofactories", manufacturing compounds with important biological properties (Ferreira et al., 2010; Patel & Goyal, 2012). Hundreds of pharmacological benefits have been acquired from different mushrooms because of their widespread use as an antioxidant, antibacterial, antiallergic, antidiabetic, cardiovascular protector, anti-cholesterolemic, antiviral, antiparasitic, antifungal, detoxificant, hepatic-protector, anti-cancer, and immuno-modulator (Phan et al., 2015; Soccol et al., 2016). Mushrooms are also used as prophylactic (preventive) agents against inflammation development and tumorigenesis (Chang & Wasser, 2012). Thus, compounds from medicinal mushrooms can be a viable

alternative as they have the ability to activate the immune system against tumors while being cheaper and safer than conventional therapies (Miles & Chang, 2004). Therefore, the implication of this cumulative body of work is that mushrooms can potentially be used to treat various diseases, including many cancers.

1.5 British Columbia Fungi

Canada, and especially British Columbia, is host to various biogeoclimatic ecological zones, providing favorable habitats and ecosystems for growth of countless Basidiomycete and Ascomycete mushrooms, the majority of which have not been tested for their potential medicinal value. Extensive research has been conducted on mushrooms native to Asia; however, studies for mushrooms residing in North America, particularly British Columbia, are relatively minimal. It is likely that BC mushrooms contain biologically active compounds, some of which have unique medicinal properties. This thesis focuses on some of the wild mushrooms collected in forests of north central British Columbia in an effort to find and characterize novel immuno-modulatory compound(s).

1.6 Immunotherapy & Cancer

The defense mechanism in all living beings, especially humans, is a multiplex system that involves many immune cell types and pathways with unique yet interwoven responsibilities (Schepetkin & Quinn, 2006). The immune system, being the first barrier to pathogens, generates diverse immuno-modulators for the maintenance of the normal homeostasis of the human body (Fudenberg, 2012). Immunotherapy is based on the idea of using the immune system to treat the disease itself. This concept is a crucial factor in the prevention and eradication of many diseases including tumors. Major immune cells, present in the tumor vicinity, regulate the progression of the tumor. Cancer biologists are trying to manipulate these immune cells in the cancerous

microenvironment to enhance anti-tumor immunity while decreasing the power of cancer cells to surpass immune surveillance. This theory of treating cancer by modulating immune response is now becoming a clinical reality (Weiskopf & Weissman, 2015).

Immuno-modulation can be achieved by immuno-stimulation, immuno-suppression, and immuno-adjuvation. Immuno-stimulation is the activation of the immune system through induction of certain mediators or components of the immune system in order to enhance the defensive mechanism against diseases; immuno-suppression is inhibition of the immune system by shutting down different key mediators, thus helping the body fight against hypersensitive immune conditions, such as inflammation and autoimmune diseases; immuno-adjuvation is the enhancement of the efficacy of vaccines, and it may also act through a stimulation pathway (El Enshasy & Hatti-Kaul, 2013; Fudenberg, 2012). Immuno-modulators enhance the host's immune response to assist the efficient anti-tumor response. This occurs as a result of combined effort of both the innate and the adapted immune response. However, the impact of immuno-modulators (i.e. enhancement or suppression of immune responses) is dependent on a number of factors such as dosage, administrative route, and the timing and frequency of administration (Tzianabos, 2000).

The concept of immunotherapy is more than a century old, when William Coley (for the first time) tested bacterial derivatives for the treatment of solid tumors by manipulating the host's immune response (Coley, 1893). A few decades later, the role of cytokines in cancer was discovered with the initial trials of administrating IFN-α (Atanasiu, & Chany, 1960; Lampson et al., 1963) and later interleukin-2 (IL-2) in the hope of initiating a therapeutic revolution (Pizza et al., 1984). Recently, immunologists are focusing more on harnessing the immune system together with training it to recognize a patient's specific tumor.

Thus, immunotherapy empowers the patient's own immune system to distinguish the cancerous cells from normal body cells and equip it to combat them in a better way. This is also supported by the unprecedented advances in immunotherapy by the scientific community. There are also recent breakthroughs regarding getting the desired response by augmenting the action of T cells, including immune check point inhibitors and chimeric antigen receptor (CAR) T cells (Weiskopf & Weissman, 2015). Two main groups of checkpoint blockers include Programmed death ligand 1 (PD-1) blocker and cytotoxic T lymphocyte associated protein 4 (CTLA-4) blockers (Barrett et al., 2014). Recently, three main monoclonal antibody checkpoint blockers (ipilimumab, nivolumab and pembrolizumab) have been approved by the FDA for melanoma and are being tested for other types of cancers (Weiskopf & Weissman, 2015).

Macrophages, the major cells of innate immune response, are activated as a result of the first line defense brigade; these constitute the mononuclear phagocyte system with monocytes. Macrophage cells are involved in phagocytosis of invading pathogen; they have the ability to successfully eliminate mutated cells before the complete activation of humoral or cell mediated immune response (Borchers et al., 2008). Macrophages also behave as antigen-presenting cells and interact with T lymphocytes, thus acting as a mediator between innate and adaptive immune response. Moreover, these phagocytic cells are also involved in chemotaxis, wound healing, surveillance, tissue repair, and removal of cellular debris after apoptosis and hemolysis (Murray & Wynn, 2011; Steidl et al., 2010).

Macrophages are regulated by various chemical messengers called cytokines. Macrophages, once activated, release various pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukins (IL), and interferons (IFN) that help eradicate the tumor cells (Weiskopf and Weissman, 2015).

Thus, targeting the non-specific innate immune response by developing novel therapeutics could be one of the ideal approaches for killing tumor cells. This could overcome the resistance and serious side effects presented by chemotherapy. Therefore, targeting macrophages as key players in tumor immunity is a clever strategy for immuno-modulation.

1.7 Mushrooms with potential immuno-modulatory activity

Among many medicinal properties that mushrooms exhibit, immuno-modulation is the most prominent one. It is now widely accepted that compounds derived from mushrooms re-establish the normal homeostasis along with enhancing the resistance to various diseases (Borchers et al., 2008). This restoration of normal homeostasis is the central dogma of oriental medicine that relates the normal homeostasis within a healthy individual. Most mushrooms' immuno-modulators tend to stimulate both innate and adaptive immune responses (El Enshasy & Hatti-Kaul, 2013). For innate immunity, they have the ability to activate certain components like neutrophils, macrophages and natural killer (NK) cells. This stimulates the secretion and expression of different cytokines (Chen & Seviour, 2007), which in turn activate cellular immune response by either assisting the differentiation of T cells in the case of helper T cells (Th1 and Th2) or by producing antibodies after activating B cells. Therefore, cytokines mediate both cellular and humoral immunity (Borchers et al., 2008).

However, pharmacokinetically, the molecular weight of the immuno-modulatory polysaccharide also affects the bioavailability of the bioactive molecule. The higher the mushroom polysaccharide molecular weight, the more difficult it is for it to cross the plasma membrane and to activate immune cells directly. The activation occurs through different cellular receptors such as Complement receptor 3 (CR-3), Toll-like receptor 2 (TLR-2), Lactosylceramide (LacCer) and dectin 1 (reviewed in El Enshasy & Hatti-Kaul, 2013). Thus, the

higher the binding affinity of a polysaccharide to immune cell receptors, the higher the effectiveness of that polysaccharide in modulating the immune system (Chen & Seviour, 2007). This aspect will help pharmaceutical experts to develop an effective and efficacious dosage with maximum absorbance and distribution. Some well-known mushrooms bearing strong immuno-modulatory activities are as follows (modified from El Enshasy & Hatti-Kaul, 2013):

1.7.1 *Lentinula edodes* (Berk), Pegler

Shiitake (*Lentinula edodes),* is an edible mushroom in Japan and has been widely used for its immuno-modulatory activity, especially its polysaccharide Lentinan, which is a 1,3-β-glucan with 1,6-β-D-glucopyranoside. Lentinan has been used as an adjuvant therapy for cancer immuno-modulation or as an adjacent therapy in cancer chemo and/or radiotherapy. It enhances various cytokines levels and enhances cytotoxicity in macrophage cell lines (Bisen et al., 2010).

1.7.2 *Inonotus obliquus*

Chaga (*Inonotus obliquus*) is found widely across North America, Korea and Siberia along the trunk of birch trees. It has also been used as an immuno-modulator because of its high β-glucan content. This β-glucan has the ability to induce nitric oxide synthase (iNOS), interleukins (IL-1β & IL-6), and TNF-α for a strong immuno-stimulatory action in various *in vitro* and *in vivo* models (Won et al., 2011). Some extracts of Chaga are also very famous for potent anti-inflammatory potential.

1.7.3 *Ganoderma lucidum*

Ganoderma lucidum commonly known as Reishi, Lingzhi, and spiritual plant has been in use in China and Japan for a long time. It is documented to have almost 120 bio-active compounds (e.g. triterpenes) and more than fifty polysaccharides and peptide polysaccharide complexes. Some compounds in *G. lucidum* have immuno-stimulating effects. They can stimulate TNF-α, IL-1, IFN-γ, and NF- Kβ (Zhu et al., 2007). Some others also possess anti-

inflammatory potential (Taofiq et al., 2016). Moreover, the fungal immuno-modulatory protein isolated from *G. lucidum* also can enhance transcription of a variety of immuno-stimulatory cytokines including interferons (IFN-γ), TNF-α, and interleukins (IL-2, IL-3, & IL-4) among others for an enhanced immune response (Li et al., 2010b)

1.7.4 *Schizophyllum commune* Fr.

    Schizophyllum commune (Split gill) can activate macrophages (via binding to Dectin-1 receptor) and T cells, thereby increasing secretion of various interleukins and TNF-α (which in turn helps in reducing the size and growth of tumor cells) and also enhancing the survival rate of various tumor bearing test animals. The active β-glucan of this species, schizophyllan, is approved as an adjuvant in chemotherapy (Hobbs, 2005).

1.7.5 *Trametes versicolor*

    Commonly known as "Turkey tail" has been proven (with over 400 clinical trials) to have beneficial effects on the human immune system due to the presence of the polysaccharide Krestin (PSK) and the fungal immuno-modulatory protein, tvc (FIP tvc). Krestin, along with other polysaccharides, initiates the activation of T cells and enhances the production and gene expression of cytokines such as interleukin (IL-1, IL-2, IL-6 and IL-8) TNF-α, and IFN-γ (Cui & Chisti, 2003). Likewise, Luo et al. (2014) observed the effects of this species in metastatic breast cancer mice model and found remarkable immuno-stimulatory, anti-metastasis and anti-tumor action. It was concluded that the stimulatory effects are due to increasing concentrations of IFN-γ, TNF-α, and interleukins (IL-2, IL-6, IL-12) among others. This extract also demonstrated the prophylaxis ability against the breast cancer-induced bone destruction. FIP tvc has the tendency to increase human peripheral blood lymphocytes and enhance the production of TNF-α and nitric oxide (NO) (Li et al., 2011). Piotrowski and colleagues (2014) reviewed the anti-tumor activity of PSP (polysaccharide P) isolated from *T. versicolor* and concluded that PSP can restore

weakened immune response by: (i) activating various immune cells; and (ii) by inducing production of various eicosanoids (e.g. prostaglandin E2-PGE2), cytokines (e.g. IL-1β, IL-6 and TNF-α), reactive oxygen and nitrogen species, and histamine, among others.

1.7.6 Miscellaneous Mushrooms

In addition, there are some other mushrooms that can also modulate the immune system. Fu et al. (2015) found that the immuno-modulatory activity of the mushroom *Dictyophora indusiata* (Vent.) Desv., veiled lady mushroom, on macrophage cell line (RAW 264.7) is due to its induction of production of NO and cytokines such as interleukins (IL-1, IL-6, IL-12) and TNF-α. Similarly, Liu et al. (2015) studied that KOMAP (polysaccharide isolated from alkaline extract of *Pleurotus eryngii* (DC.) Quél demonstrated antitumor activity in Renca tumor-bearing mice (renal cancer model) via directly inhibiting the growth of implanted tumor; this inhibition was related to the improvement of the immune system function as various immuno-stimulatory mechanisms were observed such as activation of immune cells in the spleen (e.g. natural killer cells-NK and cytotoxic T lymphocytes), increase in splenocyte proliferation, and enhanced levels of various cytokines (e.g. IL-2, TNF-α, and IFN-γ).

Polysaccharide isolated from the medicinal fungus *Flammulina velutipes* (Curtis) Singer, exhibited antioxidant and immuno-modulatory activity via enhancing NO levels and augmenting cytokines such as IL-1β, IL-6, and TNF-α in RAW264.7 macrophage cell lines (Wu et al., 2014).

Therefore, an efficient immune system is important to recognize and eliminate tumor cells prior to the major damage to the surrounding microenvironment. Mushroom-derived compounds have a potential to modify the cellular machinery in favor of anti-tumor immunity by enhancing host recognition of tumor cells, targeting mutated pathways or signals and directly killing the cancerous cells. Consequently, the identification and isolation of immuno-stimulatory

compounds from mushrooms that can stimulate innate or acquired immune response might be useful in treating various tumors effectively with minimum side effects to the host system.

1.8 Mushrooms, Inflammation and Micro-circulation

Inflammation is a localized protective mechanism of the body in response to tissue injury or pathogen invasion. However, in chronic state, it can lead to serious diseases such as diabetes, and other cardiovascular issues. Previous literature has demonstrated that mushrooms, as mini pharmaceutical factories, are potential sources of natural anti-inflammatory metabolites tested in *in vitro* and *in vivo* settings (Elsayed et al., 2014). However, to our knowledge, this thesis will be the first of its kind to explore the effect of mushrooms in ameliorating the negative impact of inflammation at the level of animal microcirculation.

Microcirculation deals with the vasculature at the micro level. It constitutes arterioles, capillaries, venules, and vascular wall components (endothelium, pericytes, and vascular smooth muscles) that play a central role in delivering nutrients, removing wastes, transporting hormones, and providing defense against harmful agents.

Microcirculation holds importance in the initiation and perpetuation of inflammation (Libby et al., 2009). It is highly responsive to the inflammatory cascade and all of its vasculature components (arterioles, capillaries, venules, endothelial cells, vascular smooth muscles, and pericytes etc.) undergoing significant physiological changes during the inflammatory process (Libby et al., 2009; Medzhitov, 2008; Tuma, 2011). Rubor (redness), calor (heat), dolor (pain), and tumor (swelling) are the typical hallmarks of inflammation (Medzhitov, 2008; Serhan et al., 2010) and all these four manifestations predominantly encompass microcirculation. Heat and redness occur because of vasodilation (Medzhitov, 2008); pain is triggered because of nociceptors stimulation (Serhan et al., 2010), and microvascular leakage is clinically expressed

as swelling or edema (Granger & Senchenkova, 2010). Moreover, there is also a fifth symptom associated with inflammation, "functio laesa" (loss of function) which, at the microcirculation level, manifests as permeation of blood in arterioles that percolate the entire tissue (Granger & Senchenkova, 2010; Tuma, 2011). The microcirculation and its vasomotor control mechanisms play an integral role in engaging and resolving the inflammatory event through a variety of signaling mechanisms. One aspect of the inflammatory cascade is compromising cellular communication by increasing the vascular permeability of the vessels (Payne et al., 2004). Therefore, restoring vascular communication could be a pre-requisite for the anti-inflammatory response.

Thus, the fact that inflammation is a complex mechanism manifesting its true physiological form at the microcirculation level, coupled with the evidence that mushrooms are a potential source for treating inflammation, suggests that the successful anti-inflammatory extracts with the ability to reverse the induced inflammatory response in microcirculation will elucidate new mechanism of combating inflammation at the cellular level.

Mushrooms have an established reputation as a dietary supplement, owing to their nutritional benefits, supported both by traditional use and scientific literature. There is therefore, a strong potential for finding novel immuno-modulatory compounds (anti-cancer and anti-inflammatory) from BC wild mushrooms which have not been explored greatly for their therapeutic potential. Figure 1 illustrates the pharmacological basis of this thesis interconnecting role of medicinal mushrooms in both suppressed and hyper-active immune response.
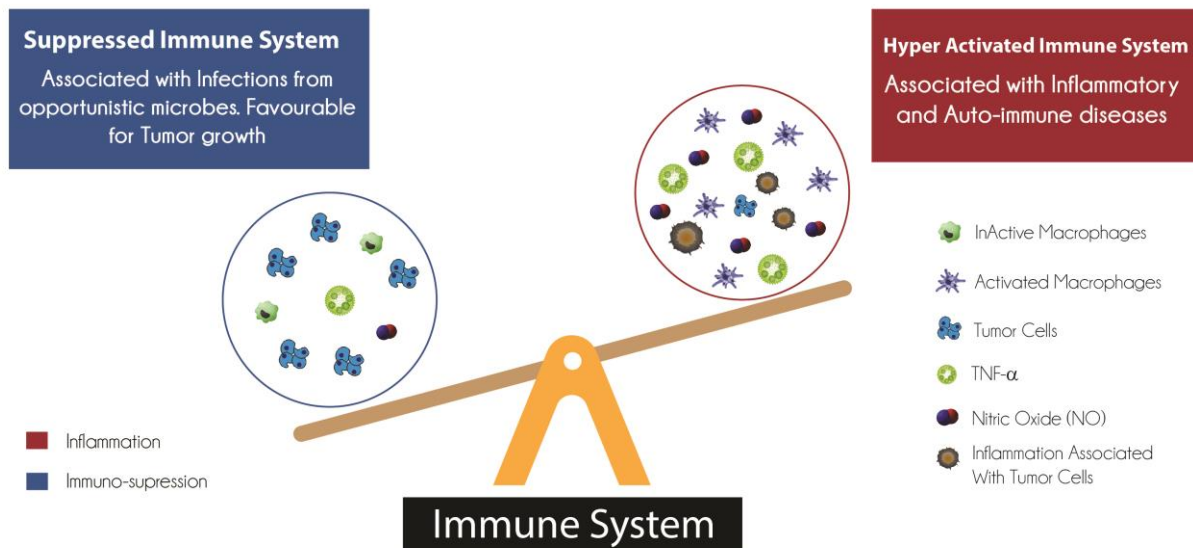
Figure 1.1 Illustration of an imbalanced immune system in pathology.

An under-reacted immune system is prone to various infections, including cancers whereas a hyper-activated immune system initiates inflammatory pathways causing allergic reactions and autoimmune disorders (e.g. arthritis, lupus, inflammatory bowel disease, inflammation dependent tumors). The immuno-modulatory compounds from mushrooms could potentially rescue both conditions by harnessing the depressed state (i.e. immuno-stimulation/immunotherapy) and by slowing down the hyper-activated one (i.e. anti-inflammation). (Graphics credit: Nosheen Javed).

1.9 Research Objectives

The major objective of this study is to discover novel immuno-modulatory compound(s) extracted from BC wild mushrooms.

1.9.1   Specific Aims

i.   To determine the immuno-stimulatory potential of 10 BC wild mushrooms (44 fractions) for their ability to induce TNF-α production in RAW 264.7 macrophages cells (Immuno-stimulation)

ii. To determine the anti-inflammatory activity of 14 BC wild mushrooms (inactive for

Immuno-stimulation) for their ability to inhibit Lipopolysaccharide (LPS) induced TNF-α

production in RAW 264.7 macrophages cells (Anti-Inflammation)

iii. To perform large-scale chemical extraction of selected mushroom species and establish a

protocol for purification and characterization of the compound(s) responsible for anti-

inflammatory activity.

iv. To understand the effects of the selected mushroom species in mice microcirculation for

their ability to improve the negative impact of histamine-induced inflammatory response (*in

vivo* model).

1.10   Thesis Structure

Chapter 1 contains the research background, specific aims and objectives of the research with an
outline of the thesis. **Chapter 2** deals with the connection of immuno-stimulation and cancer and
discusses the screening of mushrooms for immuno-stimulation (i.e. the production of TNF-α).
The second part of this chapter describes the implication of inflammation and explains the
screening of mushrooms for anti-inflammation (i.e. the inhibition of TNF-α) induced by LPS.
Chapter 3 presents the purification and characterization of the selected mushroom extract (i.e.
5% NaOH fraction of *Echinodontium tinctorium*). **Chapter 4** focuses on the impact of
inflammation at micro-circulation and discusses the *in vivo* model of testing the most potent
extracts of *Echinodontium tinctorium* (Fraction 4) and *Inonotus obliquus* (Fraction 2) in
inflammatory micro-circulation of mice. **Chapter 5** provides conclusions and future
recommendations.

Chapter 2: Immuno-modulatory Studies

This chapter is divided in two main parts. The first part encompasses the immuno-stimulatory assay while the second part is related to the anti-inflammatory analysis. The following flowchart describes the flow of the experimental steps.
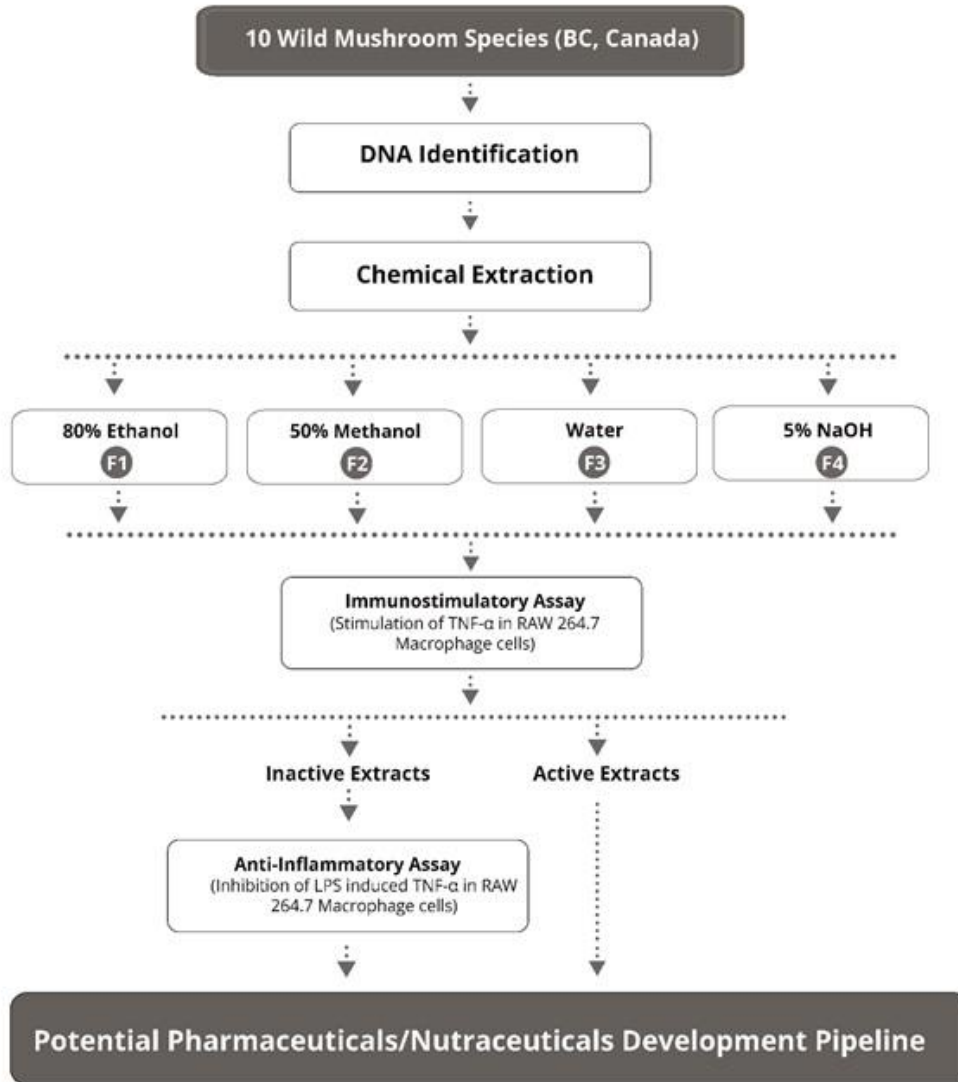
Figure 2.1. Flowchart demonstrating wild mushroom screening for Immuno-modulation. The chart also indicates the potential role of immuno-stimulatory and anti-inflammatory compound(s) towards pharmaceutical or nutraceutical development (Graphics Credit: Nosheen Javed).

Immuno-stimulatory Potential

This part of the chapter gives a brief introduction on the functioning of the immune system with a focus on macrophages as representative of the immune system and specifically on TNF-α as a potential target for immunotherapy. Some important immuno-modulatory compounds isolated from mushrooms will be discussed. The experimental design, methodology and the primary results on the screening of 10 wild mushrooms (44 extracts) for their immuno-stimulatory activity (i.e. stimulation of TNF-α in mice macrophage cells) are presented.

Introduction to Immuno-stimulation concepts

2.1 An overview of the immune system

A generalized overview of the immune system is presented for better understanding and appreciation of the mechanism of action of mushroom immuno-modulators and their role in anti-tumor immunity.

The immune system in animals, including humans, is extremely complex. We are constantly exposed to different microorganisms and infectious agents from our surroundings. The first line of defense, the external or internal epithelial surfaces, is always ready to combat these foreign invaders from any route of entry. The respiratory tract mucosa offers defense against airborne agents, the gastrointestinal mucosa combats infectious agents present in the food and water, and the skin provides a physical and chemical barrier against the outside world. Any damage to these barriers will allow micro-organisms to permeate the body (Janeway, 2001).

2.11 Innate immune response

In all vertebrates, invasions by nocuous agents are initially encountered by a pre-existing innate defense system of the body, the non-specific response, which is activated minutes after

exposure, providing the same non-specific response against any type of pathogen (Abbas et al., 2014; Janeway, 2001).

Macrophages, major cells of the innate immune response, are the first cells to encounter these infectious agents, but they are soon reinforced by numerous neutrophils recruiting at the site of infection (Abbas et al., 2014). Macrophages recognize these invading microorganisms immediately and engulf these agents by phagocytosis. These macrophages are the activated form of circulating monocytes that, once mature, reside in the tissues throughout the body (Abbas et al., 2014; Janeway, 2001). Neutrophils, or polymorpho-nuclear neutrophilic leukocytes, are the second major family of phagocytes that are activated following invasion of infectious agents (Janeway, 2001). Both macrophages and neutrophils are involved in recognition, ingestion, and destruction of many pathogens and are important components of the innate immune response (Abbas et al., 2014; Janeway, 2001). Later, certain dendritic cells and other lymphocytes also play their role in the recognition, and ultimately elimination, of the un-wanted foreign invaders.

This non-specific (innate) immunity is able to combat many invading foreigners without initiating the adaptive immune response (Janeway, 2001). Thus, innate immunity with the help of its effector mechanisms provides a front line of host defense that senses the pathogens immediately, combats them directly, and shows similar response irrespective of the invader type.

2.1.2 Adaptive Immune Response

Adaptive immune response is specific and selective in its target: it deals with the foreign or abnormal signal in a more directed manner and eliminates the offender selectively. Unlike innate immunity, this response is antigen-specific, diverse, possesses immunogenic memory, and can distinguish self from non-self molecules, thus minimizing the chances of auto-immune response.

This adaptive immune system is triggered because of the activating cascade of the innate immune system's phagocytes and natural killer cells' action. Interestingly, these adaptive

immune cells, once activated, further augment the action of innate immune cells, thus working in concert to provide an enhanced and stronger defense system. B and T lymphocytes (simply known as B and T cells), major components of adaptive immune response, are produced in bone marrow, circulated in blood and lymphatic system, and are concentrated in various lymphoid organs (Kuby, 1997).

B lymphocytes (B cells) mature in bone marrow and carry a distinct B cell (antigen-binding) receptor on the surface of their membrane. When these receptors are activated, B cells secrete specific antibodies that bind to the foreign antigen, producing antigen-antibody complexes. Such complexes are "destruction markers" for cytotoxic T lymphocytes in a process called antibody dependent cell cytotoxicity (ADCC). Moreover, antibodies also help in the direct neutralization of antigens by macrophages (phagocytosis) through opsonization (Kuby, 1997).

T lymphocytes (T cells), on the other hand, mature in the thymus gland and carry their unique antigen-binding receptor, T cell receptor (TCR). For identification and specificity, they can only recognize major histocompatibility complex (MHC) molecules (Kuby, 1997).

Naïve T cells (undifferentiated T cells), after encountering MHC associations, differentiates into memory T cells and various effectors T cells (Helper T cells, Suppressor T cells, and/or Cytotoxic T cells). In general, T cells displaying $CD4^+$ act as helper T cells, interacting with an antigen-MHC II molecule complex, and secrete various cytokines to help cytotoxic T cells. On the other hand, T cells displaying $CD8^+$ act as cytotoxic T cells, interacting with an antigen-MHC I molecule complex, and directly kill the affected cells (Kuby, 1997). These cytotoxic T cells are vital for destroying various infectious agents and tumor cells; these cells are also potential targets for immunotherapy, especially in recent scientific breakthroughs that augment the T cell response to achieve the anti-tumor activity. These involve immune checkpoint inhibitors, e.g. cytotoxic T

cell-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) antibodies. These antagonists along with engineered (chimeric) antigen receptor (CAR) T cells can improve the T cells action for enhanced host derived anti-cancer effect (Page et al., 2014; Weiskopf & Weissman, 2015).

Thus, both innate and adaptive components of the immune system are needed for the optimum working of the immune response. The suppression of the innate response also suppresses the adaptive response; this is the underlying mechanism of various diseases including cancer. Therefore, the main focus of immunotherapy is to re-activate this suppressed immune state so that it can regain the power to kill pathogens, including tumor cells.

2.2 Immunotherapy-Role of Macrophages

Macrophages, major cells of the innate system, are part of mononuclear phagocyte system derived from circulating monocytes; they act as first line defense and are involved in phagocytosis of the invading pathogen as well as in the pathology of many diseases such as infections, inflammation, wound healing, and tumor biology (Murray and Wynn, 2011).

Macrophages have two major phenotypes: M1 and M2. Inactive M1 macrophages are classically activated by various microbial products, including lipopolysaccharide (LPS: a wall component in gram negative bacteria), and IFN-γ etc. to secrete various pro-inflammatory cytokines (IL-1, IL-6, TNF-α etc.). The M1 phenotype is capable of phagocytosis and therefore kills various foreign invaders and tumors; they promote tumor destruction via antibody fragment crystallizable gamma receptors (Fcγ) that promotes ADCC response (Solinas et al., 2009). M1 phenotype is reduced in tumor micro-environment because of the increased level of the invading tumor and the side effects of chemotherapeutic drugs. M2 macrophages are the suppressed form of the macrophages that are involved in immuno-suppression and immuno-regulatory

mechanisms. The macrophages present in tumor stroma are similar to M2 phenotype and are called "Tumor associated Macrophages (TAMs)" (Mills et al., 2016). These tumor promoting macrophages suppress the immune system and enhance tumor angiogenesis and tumor infiltration capacity by compromising endothelium matrix integrity and therefore nourishing tumor development (Weiskopf & Weissman, 2015). TAMs also tilt the balance of the immune system to the suppressed state, thereby causing a decrease in M1 macrophages and pro-inflammatory cytokines.

Upon activation, M1 macrophages could eliminate cancer by initiating the pro-inflammatory response, secreting various cytokines and by enhancing the phagocytosis and direct killing of cancer cells (activating cytotoxic T cell response) (Solinas et al., 2009). Therefore, the aim behind the mechanism of action of anti-cancer immuno-modulatory drugs (targeting macrophages) is to increase the levels of pro-inflammatory macrophages (M1) that can exhibit a strong immune response while reducing the number of tumor associated macrophages (TAMs) that replenish the tumor microenvironment.

In early trials (1980's), monoclonal antibodies (mAbs) were used *in vitro* to stimulate macrophages against cancer cell lines. These mAbs enhance macrophages infiltration and ultimately enhance *in vivo* anti-tumor immunity by promoting macrophage-associated cancer cell destruction in mice models (Weiskopf & Weissman, 2015). Recently, studies are supporting the correlation of the efficiency of monoclonal antibodies and the presence of activated macrophages in the tumor micro-environment. For instance, the anti-CD20 antibody rituximab is dependent on the presence of macrophages for its effectual action both in *in vitro* (lymphoma and leukemia model) (Bologna et al., 2011) and in *in vivo* (CSF-1op mice – lacking normal number and development macrophage model) settings. Surprisingly these antibodies were not affected by B

cells, T cells, or natural killer cells depletion, thus highlighting the role of macrophages in the anti-tumor response (Uchida et al., 2004).

Tumor associated macrophages may enhance tumor progression in the absence of anti-tumor therapy. On the other hand, immuno-stimulatory and pro-inflammatory macrophages increase specific anti-tumor antibody level and are also involved in direct phagocytosis and ADCC anti-tumor response. Therefore, macrophages, being major immune effector cells, are one of the recent and main targets for cancer immunotherapy. However, it should also be kept in mind that only targeting macrophages may not demonstrate benefit in every tumor type, rather, the combinational therapy with other anti-neoplastic agents may be a more appropriate and effective approach for a strong and enhanced anti-cancer drug therapy response.

2.3 Immunotherapy-Role of TNF-α

TNF-α is a 157 amino-acid protein, first identified in 1975, and successfully cloned in 1984. It is one of the major immuno-modulatory cytokines released by macrophages and other immune cells including T cells, NK cells, and fibroblasts, among others (Rosenblum & Donato, 1988; Van Horssen et al., 2006). It is a multifunctional cytokine and is the main focus of the current study; TNF-α is involved in the inhibition of tumor proliferation and induction of tumor regression. It has two key TNF receptors (TNFRs): TNF-α receptor 1 (TNFR-1) and TNF-α receptor 2 (TNFR-2). Out of these two receptors, TNFR-2 is only confined to the immune cells. TNFR-1, on the other hand, is present on all cells and is the major initiator of all biological processes that comes under the tasks umbrella of TNF-α. This receptor has dual roles: it gives both cell apoptosis and cell survival signals which ultimately decide the cellular outcomes in terms of cell survival and proliferation or cell death (Bouwmeester et al., 2004; Wang & Lin,

2008). Thus, tumor death could be controlled by regulating the capability of TNF-α to generate apoptotic signaling.

The apoptotic or survival cascade of TNF-α is mainly dependent on the type of signal generated from the TNFR-1 receptor (Balkwill, 2006; Bouwmeester et al., 2004). When TNFR-1 signals apoptosis, FADD (Fas associated death domain) activates pro-caspase 8 that starts a protease cascade generating apoptotic signals and activating certain endonucleases like EndoG. This activation ultimately causes DNA fragmentation (Balkwill, 2006; Wang & Lin, 2008). However, when TNFR-1 signals survival, TRAF-2 (TNF-α associated factor 2) forms a complex and generates a cascade inhibiting apoptosis through cIAP (i.e. cytoplasmic inhibitor of apoptosis protein) (Wang & Lin, 2008). This triggers phosphorylation and activates cFOS / cJUN through stimulation of MAPK (mitogen activated protein kinase) and JNK (cJUN- N – terminal kinase) (Wang & Lin, 2008). TRAF-2 is also involved in the activation of NF-κB. These cFOS / cJUN and NF-κB are all the transcription factors that induce transcription for genes that are involved in anti-apoptosis cascades, proliferation, immunomodulation, and inflammation (Wang & Lin, 2008). The above pathway demonstrates that it is either the immuno-modulatory effect or the anti-tumor manifestation that is initiated with the activation of TNF-α. Detailed studies exploring the mechanism of action of TNF-α have revealed that the ability of TNF-α to cause apoptosis, could be applied to get a desired anti-tumor response. However, the signals that program TNF-α to opt for one of the aforementioned cascades still needs more exploration (Wang & Lin, 2008).

Previous studies have suggested that the stimulation of TNF-α could exert positive anti-cancer effects. In 1975, using *in-vivo* models, it was shown that TNF-α (produced by macrophages of endotoxins) could successfully induce hemorrhagic necrosis in Bacillus

Calmette Guerin (BCG, an attenuated strain of *Mycobacterium bovis*) infected mice (Carswell et al., 1975). Later, numerous recombinant TNF-α were applied both as a single agent (Braczkowski et al., 1998; Furman et al., 1993) as well as in combination with other cytokines and chemotherapeutic agents in various Phase I and Phase II clinical trials to explore their effectiveness against tumor in various *in vivo* and *in vitro* models (Roberts et al., 2011). *In vitro* studies encompassing TNF-α have shown inhibitory effects on SV-40-human mammary epithelial and human breast cancer cell lines. However, this is dependent on the type of cancer cell lines, pointing out to the specificity of the TNF-α mode of action (Dealtry et al., 1987). TNF-α has been shown to enhance the anti-cancer effects of various anti-neoplastic drugs e.g. etoposide, actinomycin D, DNA topoisomerase inhibitors, and Adriamycin in murine bladder tumor (MBT-2) cell lines (Roberts et al., 2011). However, sadly, injecting TNF-α (whether alone or in combination) is also associated with some serious side effects and toxicities, e.g. hypotension, neurotoxicity, hepatotoxicity, cardiotoxicity etc. Hence, keeping in view the problems with exogenous TNF-α, the emphasis of the current research is to stimulate the macrophages already present inside the body so that the TNF-α could be released in an indirect and safer mode to combat the tumor cells in a more effective way.

Therefore, the focus of this chapter will be on the induction of TNF-α as a major marker of immuno-stimulation. It will highlight the primary screening result of mushroom fractions for the stimulation of TNF-α (i.e. the stimulation of inactivated macrophage cells to release TNF-α) for the enhanced immuno-stimulatory effect.

2.4 Immuno-modulatory compounds from wild mushrooms

Mushrooms, as described earlier, act as dietary supplements because of their nutritional and medicinal values (Miles & Chang, 2004) and their medicinal potential has been confirmed by

extensive studies. There are four main classes of mushroom immuno-modulators depending upon their chemical nature: (i) immuno-modulatory polysaccharides, (ii) fungal immuno-modulatory proteins (FIPs), (iii) immuno-modulatory lectins and, (iv) immuno-modulatory terpenes and terpenoids (El Enshasy & Hatti-Kaul, 2013).

2.4.1 Immuno-modulatory polysaccharides

Mushrooms have various polysaccharides that have immuno-modulating properties. Krestin (PSK) from *T. versicolor* is a highly promising orally active polysaccharide that has a history of successful clinical trials in breast, colorectal, lung, stomach, and head and neck cancers. PSK is an approved adjuvant of chemotherapy in Japan since 1980 (Sun et al., 2012). In *in vitro* models, PSK increases human peripheral blood lymphocytes and enhance the production of TNF-α and NO (Li et al., 2011). Lentinan (a β-1, 3-D-glucan with β-1,6 branches) was the first reported polysaccharide with potential immuno-modulating and anticancer activity. It was extracted from fruiting bodies of *Lentinula edodes* in Japan in the 1960s (Bisen et al., 2010). Lentinan has also been approved for its adjuvant use with chemotherapy for treatment of stomach cancers in Japan since 1985 (Higashi et al., 2012)

Thus, immuno-modulatory polysaccharides have strong impact in the enhancement and/or restoration of immune responses in different cell and animal models.

2.4.2 Fungal immuno-modulatory proteins (FIPs)

Recently, a new family of proteins from mushrooms, the fungal immuno-modulatory proteins (FIPs), has been documented with strong immuno-modulating properties. The first FIP was characterized from *G. lucidum* in 1989 and named Ling-Zhi-8 (Tanaka et al., 1989). Other examples of FIP include FIP-fve from *F. velutipes* (Wang et al., 2004), FIP-gsi from *G. sinensis* (Li et al., 2010a), FIP-vvo and FIP-vvl from *Volvariella volvacea* (Bull.) Singer (Hsu et al.,

2008), FIP-pcp from *Poria cocos* F.A. Wolf (Chang & Sheu, 2007), FIP-gmi from *G. microsporum* R.S. Hseu (Li et al., 2010b), and FIP-tvc from *T. versicolor* (Li et al., 2011).

2.4.3 Immuno-modulatory lectins

Immuno-modulatory lectins constitute a diverse family of carbohydrate-binding proteins that have precise binding capabilities. Lectins isolated from mushrooms have well documented anti-tumor, anti-proliferative, and immuno-modulatory properties. TML-1 and TML-2, from *Tricholoma mongolicum* S. Imai, and Concanavalin A, from *V. volvacea* also exhibited anti-tumor and immuno-modulatory activities by activating the immune system rather than showing cytotoxicity effects (Wang et al., 1998). These lectins show their mechanism of action by inducing TNF-α and nitrite production and by producing macrophage-activating factors that inhibit the growth of mouse lymphoblast (p815) mastocytoma cells. Some of these macrophage-activating factors are IFN-γ and other cytokines and interleukins, triggered by enhanced expression of IL-1β, iNOS and transforming growth factor-β (TGF-β) (Liu et al., 2005). The lectins of *Grifola frondosa* (Dicks.) Gray, have stronger cytotoxic effects even at low doses and are thus more potent and effective against HeLa cells (El Enshasy & Hatti-Kaul, 2013).

2.4.4 Immuno-modulatory terpenes and terpenoids

Mushrooms also have a large number of immuno-modulatory terpenes showing anticancer and anti-infective properties. Terpenes constitute basic building blocks of isoprene unit which consists of five-carbon atom and have a molecular formula of $(C_5H_6)_n$. *G. lucidum* and *G. applanatum* (Pers.) Pat., have been documented to contain high terpene and tri-terpenoid concentrations. Lanostane (a triterpenoid) isolated from *G. lucidum* shows strong modulation activities against the immune system (Jeong et al., 2008); these terpenes from *G. lucidum* are even capable of preventing drug-induced nephrotoxicity and inflammation (Tanaka et al., 1989).

Some other examples of terpenes and terpenoids from *Ganoderma* species include ganodermanontriol, ganoderols, ganoderals, lucidone, ganodermanondiol, ganodermic and ganoderic acids that have strong roles in stimulating NF-k β and mitogen-activated protein kinases (MAP-K) pathways (Gao et al., 2002).

This literature indicates the role of the medicinal mushrooms in immunotherapy. The main principle of natural immuno-modulators is to employ bioactive metabolites from mushrooms to re-program the suppressed immune system for the eradication of tumor initiation and progression.

2.5 Materials & Methods

2.5.1   Mushroom Collection & Identification

At the outset of my research, the UNBC team comprising of Drs. Chow Lee, Hugues Massicotte and Keith Egger, collected several species of fungi from the North Central region of BC (e.g. Prince George, Prince Rupert, Terrace, McBride). These collected fungi belonged to one of three categories: (i) fungi found in BC and around the world, well known for their immuno-modulatory properties, (ii) fungi found only in BC that are sister species of others present around the world and are known to have immuno-modulatory properties, or (iii) fungi that are only found in BC and have not been previously explored for their therapeutic properties.

After collection, the specimens were dried and identified, first based on their morphology and later confirmed through DNA sequence analysis. Morphological identification involved measurements of height, cap diameter, stem length and width, as well as descriptions of habitat, color, odor, textures, and shape. Spore prints were also collected. Morphological identification was made using the identification software, "Matchmaker" (Gibson et al., 2010) and the dichotomous keys in the reference guide Mushrooms Demystified (Arora, 1986). Tissue samples from the internal flesh of the cap were stored at freezing temperature (-20°C) until DNA analysis

was performed. Specimens were preserved at 4°C so that deterioration could be minimized while handling was required.

To further confirm the identity of mushrooms, fungal specific primers for the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) were used; a successful protocol for the identification of a wide range of fungi (Schoch et al., 2012). Primers designed for the ITS region were also capable of amplifying fungal DNA from samples having other contaminating DNAs such as plant tissues or soil (Peay et al., 2008). Methods of identification in Dr. Lee's lab were similar to those of Rajaratnam & Thiagarajan (2012). Briefly, DNA was extracted from the samples and subjected to the polymerase chain reaction with the ITS3 (forward) and NLB4 (reverse) primers. The amplified DNA was checked for purity by agarose gel electrophoresis. The purified PCR products were then submitted to Macrogen Inc. (USA) for DNA sequencing and BLAST searches against the GenBank database were used to identify the fungal species. The analysis was based upon the sequence differences in the ITS2 spacer region in rDNA. This ITS2 region identification was consistent with the morphological identification with high certainty for all of the mushrooms tested (Figure 2.2.)
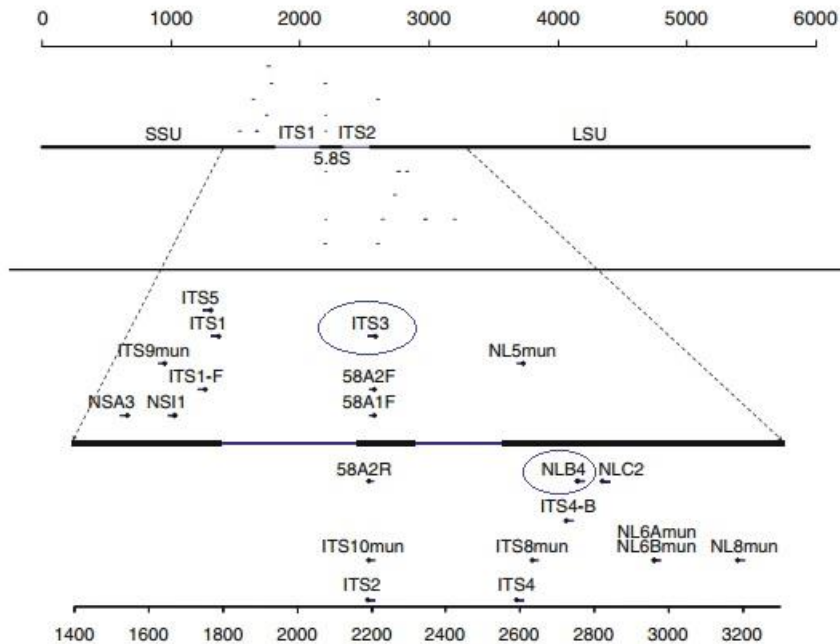
Figure 2.2 Illustration of ITS-1 and ITS-2 regions between conserved flanking regions with position of both forward (above) and reverse (below) primers. ITS3 is used as forward primer and NLB4 is used as reverse primer. Figure modified from Martin & Rygiewicz (2012).

2.5.2 Chemical extraction

After successful identification, fungal specimens were dehydrated at 50°C using a drying oven, and ground into a fine powder with a conventional household blender; for woody specimens (conks), a hammer mill was used for grinding. After this step, powdered fungal samples were kept at room temperature until chemical extraction was done.

The fungal extractions were performed by a colleague graduate student in Dr. Chow Lee's lab (Mr. Aaron Smith). For effective extraction of organic material from natural products, Mizuno (1999) had developed a basic four-step extraction that is widely accepted and practiced in the mycology world. This can be modified according to the specific extraction target (Mizuno, 1999, Zhang et al., 2007). Using the Mizuno method, samples were initially extracted with 80%

ethanol so that any low molecular weight compounds can be removed. Further samples were extracted with hot water (100°C, 3 hours), 2% ammonium oxalate (100°C, 6hrs), and 5% sodium hydroxide (80°C, 6hrs) (Mizuno, 1999). It is believed that the hot water fraction contains the water-soluble polysaccharides, and the alkali fractions contain the water-insoluble polysaccharides. The conditions of this extraction can be changed to suit the nature of any target molecules (Zhang et al., 2007).

The Mizuno's protocol was modified to fit the current fungal extraction process. The fungal samples were extracted using 80% ethanol (65°C, 3 hours) for the separation of soluble low molecular weight compounds. This solution, filtered through Whatman® paper No.3, is referred to as fraction 1, while the residue was subjected to the second step: 50% methanol extraction (65°C, 3 hours). Similarly, the filtrate (F2) was preserved and the residue was extracted with water, F3, (65°C, 6 hours). Finally, the residue was subjected to the last step, 5% NaOH, F4, (65°C, 6 hours). The flowchart of this extraction procedure is summarized in Figure 2.3. Initially, some samples were also extracted using 2% Ammonium hydroxide in the 4th step but it was omitted later because of its incompatibility with the anti-proliferative anti-tumor cell assay. However, for immuno-modulatory assay (both immuno-stimulatory and anti-inflammatory assay) of six fungal species (*Fomitopsis pinicola* (Sw.) P. Karst., *L. edodes*, *F. fomentarius*, *Piptoporus betulinus* (Bull.) P. Karst., *G. applanatum,* and *Echinodontium tinctorium* (Ellis & Everh.) Ellis & Everh. fraction 4 still represented the 2% ammonium oxalate extraction filtrate, while fraction 5 represented the 5% sodium hydroxide extraction filtrate. This extraction procedure allowed us to have low molecular weight compounds in the first two fractions, some water-soluble polysaccharides in the third fraction, and water insoluble polysaccharides in the fourth fraction. Collected fractions were subjected to rotor-evaporation for concentration. These

were later lyophilized and stored at 4ºC until assays were performed.
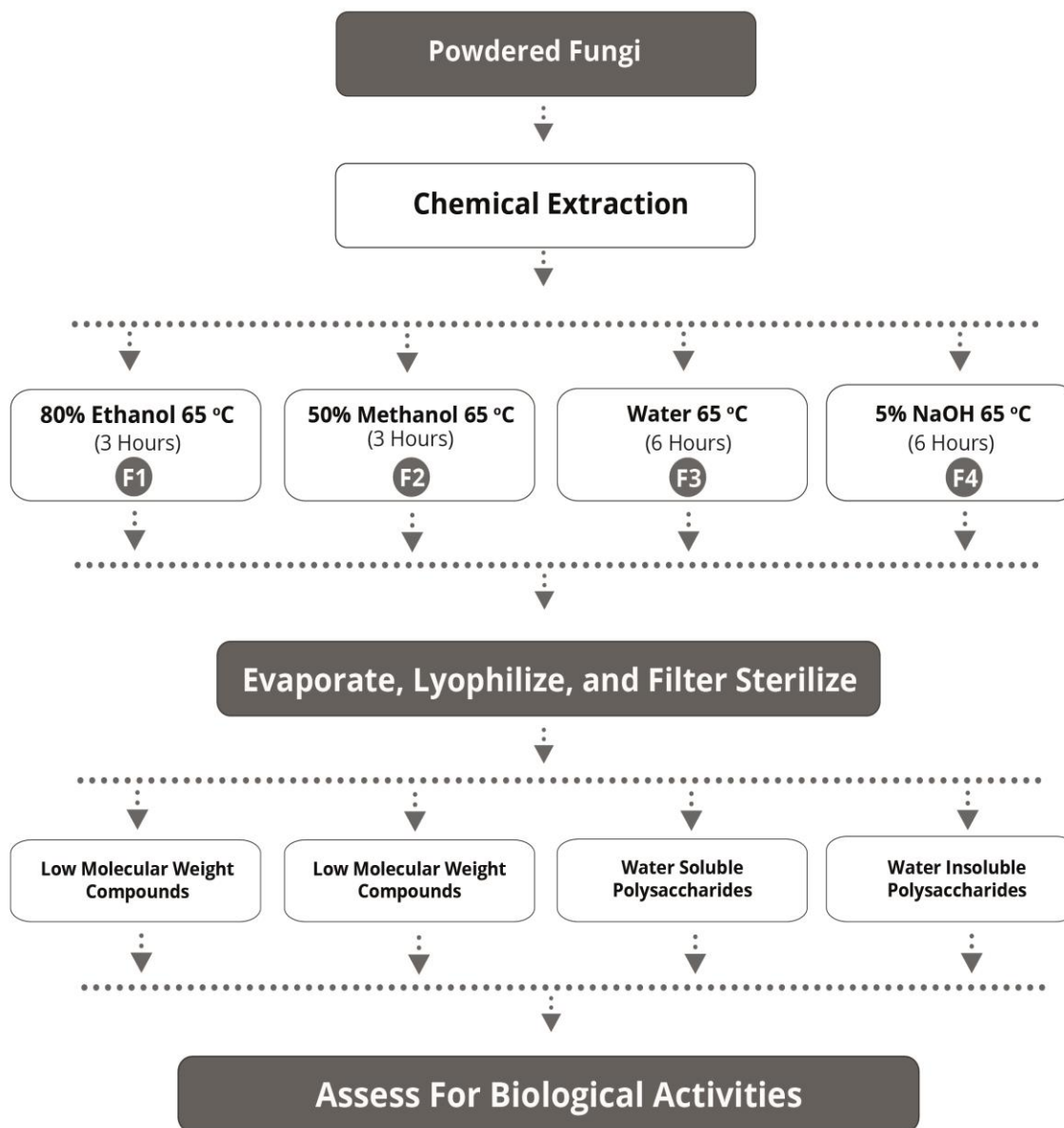
Figure 2.3. Flowchart showing the chemical extraction scheme adopted in the current study. This scheme is proposed to separate compounds on the basis of their molecular weights and solubility (Graphic credit: Nosheen Javed).

2.5.3 Assessing chemical fractions for immuno-stimulatory activity

Raw 264.7 mouse macrophage cell line for use in immuno-modulatory activities was purchased from the American Type Culture Collection (Rockville, Maryland). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with glutamine (LONZA, Walkersville, Maryland) and containing 10% v/v fetal bovine serum (FBS) in a humidified incubator at 37°C supplied with 5% carbon dioxide. A day following retrieval from the maintenance solution, Raw 264.7 cells were plated in a 96-well plate (100,000 cells/well) and were incubated overnight in 200 µL DMEM without FBS.

After an overnight incubation, the medium (200 uL DMEM without FBS) was removed. Cells were washed with 200 µL BioWhittaker® Phosphate Buffer Saline (PBS) (Lonza, Walkersville, Maryland) and then treated with 100 µL DMEM without FBS with: (i) various mushroom fractions (conc. 1 µg/uL), (ii) lipopolysaccharide (LPS) (500 ng/mL) for use as the positive control, or (iii) DMEM and respective solvents (i.e. water or methanol used for resuspension of lyophilized samples) for use as negative controls.

After a further 6 hours of incubation, supernatants (~100 µL) were collected, centrifuged (4°C, 200xg, 5 min using Allegra® X-15R, benchtop centrifuge, Beckman Coulter, CA, USA) and stored at -80ºC freezer until ELISA was performed for the quantification of TNF-α production.

2.5.4 Enzyme-Linked Immunosorbent Assay (ELISA)

For ELISA, BD OptEIA™ Mouse TNF (Mono/Mono) ELISA Set & Reagent Set A (BD Biosciences, CA, USA) were used according to the manufacturer's protocol to quantify the amount of TNF-α in the supernatant.

Briefly, a 96-well Microtiter™ polystyrene plate (Thermo Fisher Scientific, MA, USA) was coated overnight at 4°C with a capturing antibody (i.e. anti-mouse TNF-monoclonal antibody) diluted in coating buffer (1:500). The following day, the plate was washed with washing buffer (300 µL of wash buffer thrice) and blocked with assay diluent (200 µL) for 1 hour. After one hour, the plate was washed with washing buffer (300 µL of wash buffer thrice) and supernatants were added (diluted 1:10 in assay diluent) and incubated for 2 hours at room temperature. At this time, the TNF-α standard concentrations (1000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL) were prepared and added for the generation of standard curve. Subsequently, the wells were washed again (300 µL of wash buffer, five times), and the working detector (biotinylated mouse TNF-α monoclonal antibody + Streptavidin-horseradish peroxidase conjugate) was added (100 uL) for an hour. This was followed by an extensive washing (300 µL of wash buffer, five times), addition of substrate solutions (100 µL) and incubation for another 30 minutes. Finally, 50 µL of stop solution was added. The optical density was measured at 450 and 550 nm using a Synergy-2 (BioTek®, VT, USA) fluorescent polarizer. The concentration of TNF-α was calculated from the standard curve of mouse recombinant TNF-α via a log-log regression analysis using PRISM6. Figure 2.4 summarizes the protocol of immuno-modulation in RAW 264.7 cells from Day 1 to 5.

2.5.5 Data Analysis

Each experiment was repeated with at least one more biological replicate with 4 wells (quadruplicate) per treatment. Data was expressed as mean ± standard deviation. Error bars were incorporated as a representation of standard deviation. One-way ANOVA was performed for statistical analysis using PRISM.
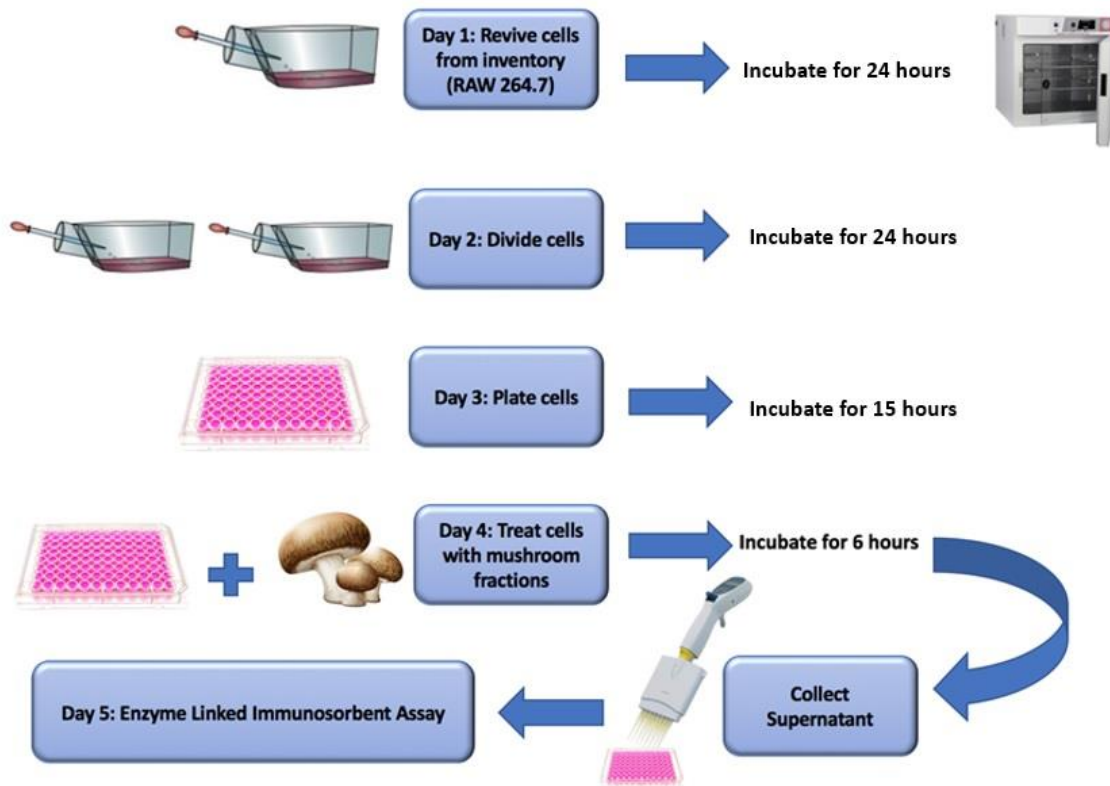
Figure 2.4. Schematic representation of immuno-modulation assay protocol using RAW 264.7 cells. The protocol is spread over a period of 5 days in the following sequence: cell reviving, cell dividing, cell plating, and cell treatment with mushroom extracts (Graphics credit: Sukhpreet Buttar).

2.6 Results

2.6.1 Mushroom Collection and Identification:

A total of 14 fungi were used for this study (10 species for immuno-stimulatory and 4 additional only for anti-inflammatory assays). These fungi were collected from 9 different locations of North Central BC between the 2012 and 2014. The fungal identification was performed by Ms. Sukhpreet Buttar and Mr. Jatinder Khatra (Undergraduate students in Dr. Lee lab) and the results presented confirmed the initial identification performed via morphological

analysis. Table 2.1 summarizes fungal samples collected across the BC wild forests with the

relevant details on collection and identification of fungal species.

Table 2.1: Summary of fungi collected from North Central forests of British Columbia

Identity	Collected By	Date of Collection	Place of Collection in BC	Best GenBank Match (%similarity/%Coverage)
Fomitopsis pinicola (Sw.: Fr.) P. Karst.	Drs. Keith Egger & Hugues Massicotte	Summer 2012	Terrace, BC	No sequencing data (verified via morphological analysis)
Lentinula edodes (Berk.) Pegler	Dr. Chow Lee	Jan 2013	Purchased at Shoppers Wholesale, Prince George	No sequencing data (verified via morphological analysis)
Piptoporus betulinus (Bull.) P. Karst.	Marta Zmudzinski	25 Oct 2012	Forests For The World (FFTW), Greenway Tr., Prince George	JX109856.1 (99%/100%)
Fomes fomentarius (L.) Fr.	Marta Zmudzinski	25 Oct 2012	FFTW, Greenway Trail, Prince George	JX109860.1 (98%/100%)
Ganoderma applanatum (Pers.) Pat.	Dr. Keith Egger	20 Aug 2012	Thornhill Mountain Trail, Terrace	KC581319.1 (99%/100%)
Echinodontium tinctorium (Ellis & Everh.) Ellis & Everh	Aaron Smith	Sep 2013	West Twin Prov Park	No sequencing data (verified via morphological analysis)
Inonotus obliquus (Ach. ex Pers.) Pilát	Marta Zmudzinski	April 2013	FFTW, Prince George	No sequencing data (verified via morphological analysis)
Leucocybe connata (Schumach.) Vizzini, P. Alvarado, G. Moreno, & Consiglio	Marta Zmudzinski	22 Sep 2012	Eskers Provincial Park, Prince George	KC581297.1 (99%/100%)
Laetiporus sulphureus (Bull.) Murrill	Drs. Keith Egger & Hugues Massicotte	Summer 2013	UNBC Campus, Prince George	No sequencing data (verified via morphological analysis)
Phellinus nigricans (Fr.) P. Karst	Keith	Aug 2012	Thornhill Mountain Trail, Terrace	AY558631.1 (98%/70%)
Phellinus igniarius (L.) Quel		Jul 2014	Kitselas Rd, Terrace Mountain, Terrace	AY558623.1 (99%/100%)
Trichaptum abietinum (Pers. Ex J.F. Gmel.) Ryvarden	Ankush	Oct 2013	FFTW, Prince George	KC581332.1 (99%/100%)
Hericium coralloides (Scop.) Pers.	Vicky Myhre	Sep 2013	Cottonwood Island Park, Prince George	AF506459.1 (99%/100%)
Letharia vulpina (L.) Hue	Vicky Myhre	June 2014	Beaverdam Lake, Clinton	75% (verified via morphological analysis)

*Immuno-stimulatory activity of extracts from these species was assessed by Mr. Ankush Barad and Ms. Vicky Myhre (Graduate students in Dr. Lee lab). Only inactive fractions of these fungi were assessed for anti-inflammatory activity and described in this thesis.

2.6.2 Mushroom Extraction

After the collection and identification of mushrooms, chemical extraction of mushrooms was performed using four successive solvent systems: 80% ethanol (F1), 50% methanol (F2), water (F3) and 5% NaOH (F4). Initially, 2% ammonium oxalate extraction was also performed at the fourth step, but it was later omitted because of the incompatibility of oxalate with the medium used for growing Raw264.7 cell lines. So, in these extracts, F4 represent 2% ammonium oxalate and F5 represents 5% NaOH fraction. This scheme is not followed by *F. pinicola* and *L. edodes* as these species were the very initial ones to be extracted before the whole protocol was developed. Therefore, in these two species, 50% methanol extraction was not performed at all. In *F. pinicola,* 99% ethanol (F2) was performed as a second step but in *L. edodes,* this step was omitted completely and the extraction scheme was as follows: 80% ethanol, water, 2% ammonium oxalate, and 5% NaOH. Table 2.2 summarizes the extraction of each species followed by percent yield. The solvent that was used for re-constituting the mushroom after lyophilization was also mentioned. It was observed that 5% NaOH extract had notably higher mass as compared to the other extracts; this could possibly be due to the presence of salt in these extracts.

Table 2.2: Representation of Extraction Solvent, yield and Re-suspension solvent for Mushroom Fractions

Mushroom Specimen	Processed Raw Weight (g)	Fraction No.	Extraction Solvent	Mass (g)	Percent Yield (%)	Resuspended Solvent
Fomitopsis pinicola	33	F1	80% Ethanol	0.16	0.50	H_2O
		F2	99% Ethanol	8.2	24.80	Methanol*
		F3	Water	0.65	1.96	H20**
		F4	2 % $(NH_4)_2C_2O_4$	N/D	N/D	N/D
		F5	5% NaOH	16.30	49.40	H_2O
Lentinula edodes	50	F1	80% Ethanol	13.90	27.70	H_2O
		F2	Water	6.64	13.30	H_2O
		F3	2 % $(NH_4)_2C_2O_4$	13.94	27.88	H_2O
		F4	5% NaOH	30.13	60.30	H_2O
Piptoporus betulinus	128.5	F1	80% Ethanol	3.77	2.93	Methanol*
		F2	50% Methanol	0.39	0.30	H_2O
		F3	Water	0.22	0.17	H_2O
		F4	2 % $(NH_4)_2C_2O_4$	4	3.1	H_2O
		F5	5% NaOH	22	17.1	H_2O
Fomes fomentarius	126.45	F1	80% Ethanol	2.21	1.75	Methanol**
		F2	50% Methanol	0.59	0.467	H20**
		F3	Water	0.37	0.30	H_2O
		F4	2 % $(NH_4)_2C_2O_4$	13.13	10.40	H_2O
		F5	5% NaOH	18.94	14.97	H_2O
Ganoderma applanatum	89.31	F1	80% Ethanol	1.56	1.75	Methanol**
		F2	50% Methanol	0.66	0.74	H20**
		F3	Water	0.51	0.57	H_2O
		F4	2 % $(NH_4)_2C_2O_4$	6.25	6.99	H_2O

		F5	5% NaOH	12.3	13.77	H_2O
Echinodontium tinctorium	273.15	F1	80% Ethanol	4.05	1.48	H_2O
		F2	50% Methanol	0.37	0.14	H_2O
		F3	Water	0.93	0.34	H_2O
		F4	2 % $(NH_4)_2C_2O_4$	6.15	2.25	H_2O
		F5	5% NaOH	14.7	5.38	H_2O
Inonotus obliquus	315	F1	80% Ethanol	5.5	1.75	H_2O
		F2	50% Methanol	11.8	3.75	H_2O
		F3	Water	7.6	2.41	H_2O
		F4	5% NaOH	64.8	20.57	H_2O
Leucocybe connata	80	F1	80% Ethanol	17.63	22.03	H_2O
		F2	50% Methanol	11.78	14.72	H_2O
		F3	Water	3.53	4.41	H_2O
		F4	5% NaOH	22.91	28.64	H_2O
Laetiporus sulphureus	77	F1	80% Ethanol	6.28	8.16	H_2O
		F2	50% Methanol	3.22	4.18	H_2O
		F3	Water	1.17	1.52	H_2O
		F4	5% NaOH	23.65	30.71	H_2O
Phellinus nigricans	135.89	F1	80% Ethanol	2.31	1.70	H_2O
		F2	50% Methanol	0.77	0.56	H_2O
		F3	Water	1.74	1.28	H_2O
		F4	5% Sodium Hydroxide	46.72	34.38	H_2O

*Previously re-suspended in ethanol, changed due to interference with anti-inflammatory assay

**Previously re-suspended in DMSO, changed due to interference with anti-inflammatory assay.
N/D is the fraction that was misplaced so this could not be assessed for biological activities.

2.6.3 Immuno-stimulatory Assay

Immuno-stimulatory activity of fungal fractions was assessed by measuring TNF-α

production in Raw 264.7 cells when treated with 1 μg/μL of the fungal fractions. LPS was used

as the positive control and DMEM, water and/or solvents (methanol and/or ethanol) were used as

negative controls.

The following fractions stimulated the production of TNF-α at or above 500 pg/mL: All

fractions of *L. edodes*; fraction 5 of *F. fomentarius*; fractions 3-5 of *G. applanatum*; fractions 3

and 4 of *E. tinctorium*, *L. connata*, *L. sulphureus* and *P. nigricans*. Fractions from three fungi (*F.

pinicola, P. betulinus,* and *I. obliquus*) stimulated the production of TNF-α at a level below 500

pg/mL or had no stimulatory activity at all. Results are presented in Figure 2.5.1, 2.5.2, and

2.5.3.
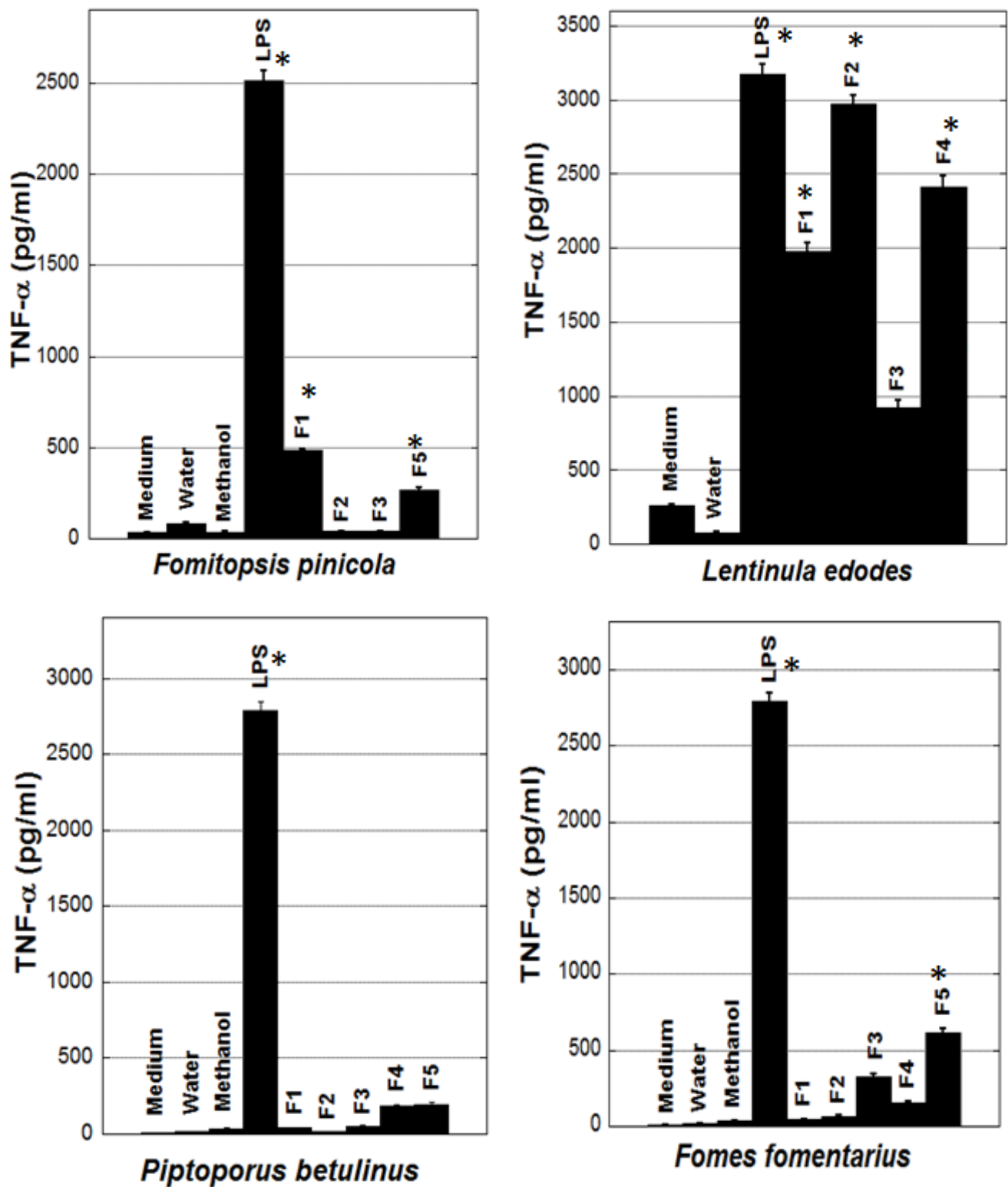
Figure 2.5.1. Assessing immuno-stimulatory potential of fungal extracts in RAW 264.7 macrophage cells. Fungal conc. 1 µg/µL; LPS (500 ng/mL) was used as positive control; solvents and media were used as negative controls. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).
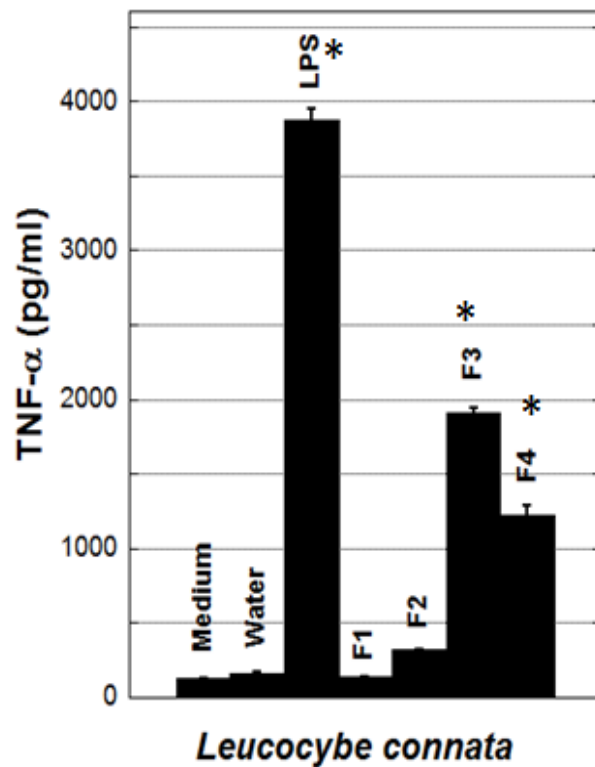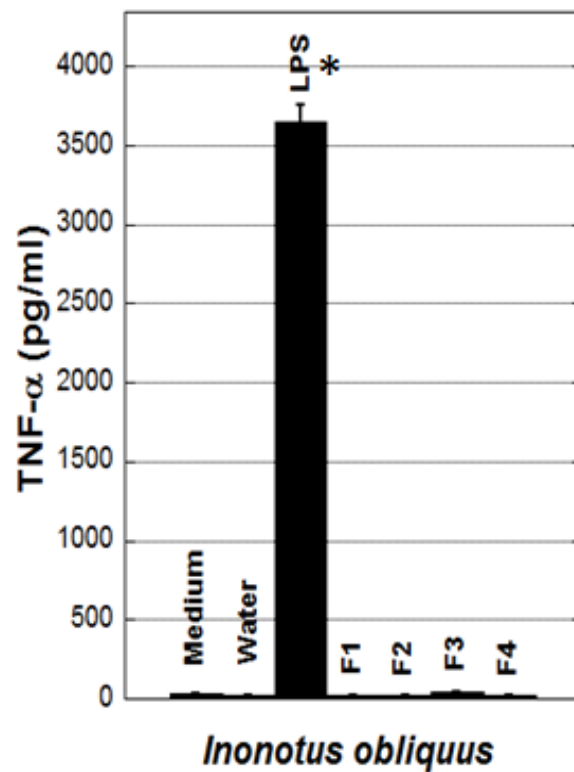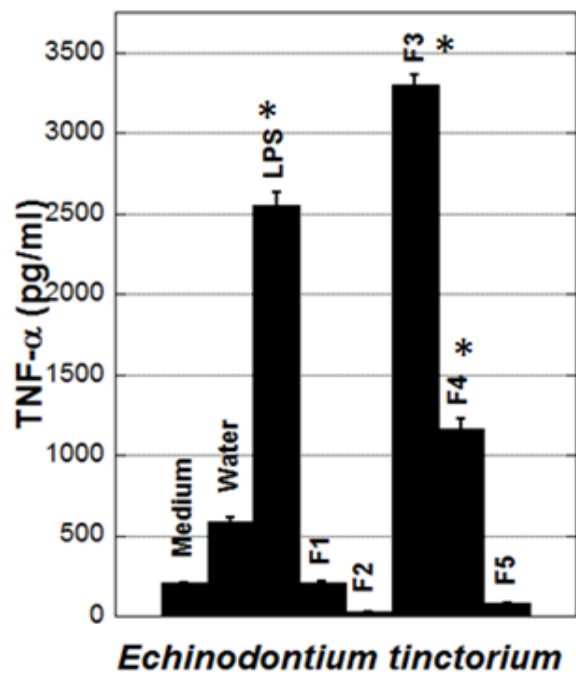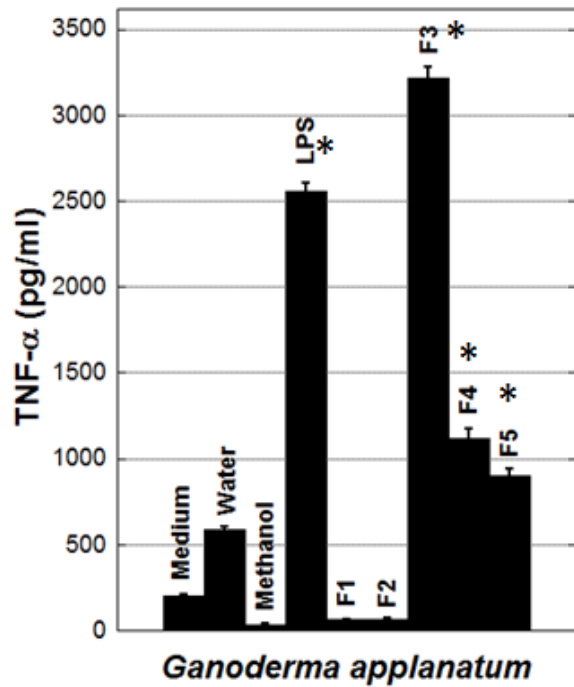
Figure 2.5.2. Assessing immuno-stimulatory potential of fungal extracts in RAW 264.7 macrophage cells. Fungal conc. 1 µg/µL; LPS (500 ng/mL) was used as positive control; solvents and media were used as negative controls. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).
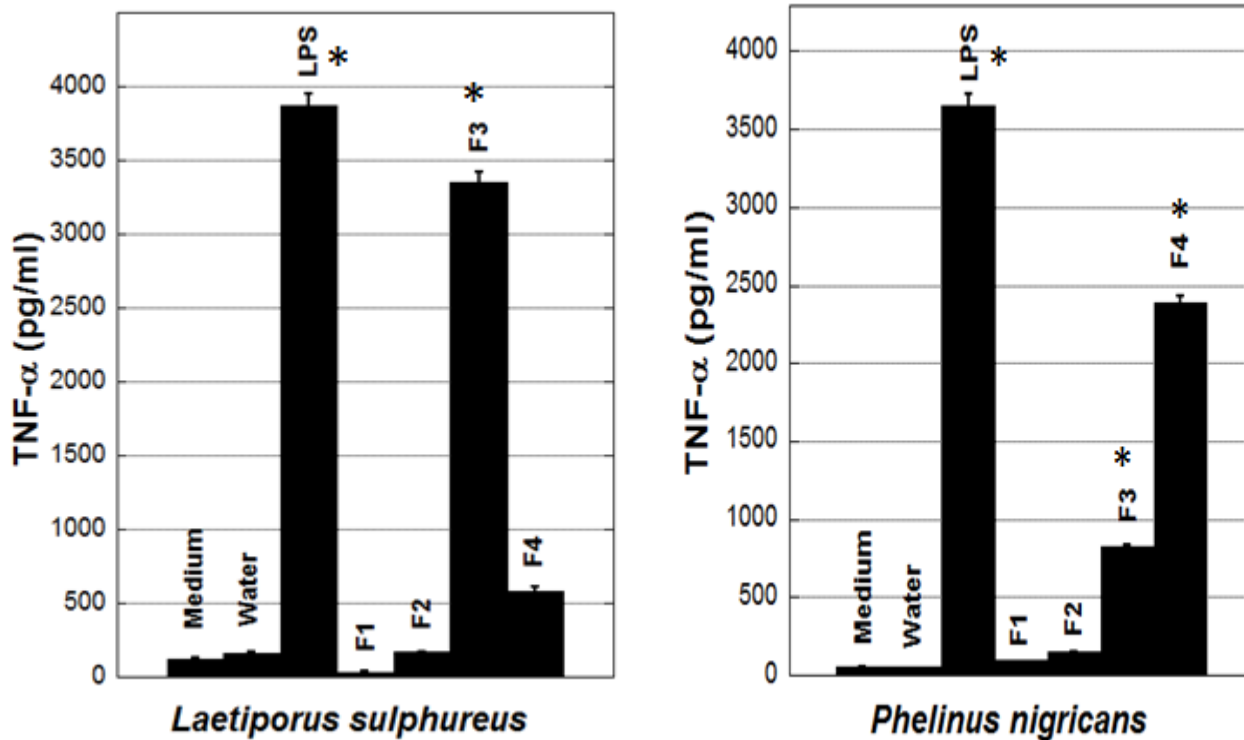
Figure 2.5.3. Assessing immuno-stimulatory potential of fungal extracts in RAW 264.7 macrophage cells. Fungal conc. 1 μg/μL, LPS (500 ng/mL) was used as positive control; solvents and media were used as negative controls. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).

2.7 Discussion

The current study explores the immuno-modulatory activity of wild fungi collected from the forests of North Central BC and the overall results show that immuno-modulation potential is not uncommon in BC wild mushrooms as 9 out of forty-four extracts tested have shown at least moderate (≥40% stimulation w.r.t LPS) and 4 have shown strong (≥90% stimulation w.r.t LPS) immuno-stimulation potential. The following information encompasses the immuno-modulatory literature of the fungi that exhibited positive immuno-stimulatory activity in the present study.

In this thesis, we determined that all 4 extracts of *Lentinula edodes* presented strong immuno-stimulatory activity with high stimulatory concentrations of TNF-α, making the results consistent with previous studies. *Lentinula edodes* is a well-known biological response modifier, especially its active polysaccharide "Lentinan" which is a cell wall β-glucan. Lentinan is not cytotoxic, rather it exerts its effect via immuno-modulation. Lentinan is being manufactured by various pharmaceutical companies and is approved for clinical use in Japan; it is used as an adjuvant in anti-cancer therapy because of its ability to augment helper T cells' and natural killer cells' activity and to enhance various cytokine and interferon levels (Sunagawa et al., 2014). Consumption of *L. edodes* improves human immune function by increasing T cell and natural killer cell function (Dai et al., 2015). In human cell line models, Lentinan has shown synergistic effects with various anticancer drugs when it is used with docetaxel, cisplatin (Zhao et al., 2013) and paclitaxel (Liu et al., 2015). In human case studies, Lentinan has demonstrated enhanced effect when used with gemcitabine (Sun et al., 2015). In mouse models, Lentinan has augmented the effects of anti-cancer monoclonal antibodies (Allendorf et al., 2005) and, as an adjuvant with chemotherapy, it has improved survival rate in patients with gastric (Oba et al., 2009) and hepatocellular (Yang et al., 2008) carcinomas. Also, gastric (Yoshino et al., 2010), hepatocellular (Isoda et al., 2009), pancreatic (Shimizu et al., 2009) and colorectal (Hazama et al., 2010) cancer

patients were reported to have improvement in their quality of life and outcomes with oral doses of lentinan.

There is also a report of improved quality of life in esophageal carcinoma cases (Wang et al., 2012a). As described earlier, since 1985, lentinan is an approved adjuvant of chemotherapy for stomach cancers in Japan (Higashi et al., 2012). In RAW 264.7 cell lines, *L. edodes*'s polysaccharides are reported to have immuno-stimulatory effects on various cytokine levels both at the innate and adaptive immune response level (Bisen et al., 2010; Xu et al., 2011). Lentinan is also known to enhance ROS, NO, TNF-α, IL1, and phagocytosis in various murine peritoneal macrophages, C4M macrophages, and human monocytes (reviewed in Schepetkin and Quinn, 2006). In the current study, all 4 extracts of *L. edodes* presented strong immuno-stimulatory activity with high stimulatory concentrations of TNF-α making the results consistent with the previous studies.

Fomes fomentarius, the tinder fungus, is a well-known ancient mushroom with documented use for more than 5000 years. The iceman called Oetzi used this mushroom for preserving fire, as an insect repellant, and for medical and spiritual therapy (Pöder & Peintner, 1999). In the current research, the 5% NaOH soluble of *F. fomentarius* extract stimulates TNF-α in RAW 264.7 cells, which is consistent with the previous research findings. In 5th century BC, *F. fomentarius* was commonly used for cauterization. Surprisingly, the same treatment of using *F. fomentarius* for cauterization was practiced by Okanagan-Colville natives of BC for curing rheumatic and inflammatory diseases (Hobbs, 1995). It is commonly known as "Mudi" in Chinese culture and is used traditionally for the treatment of gastro-enteric disorders, gastric and uterine carcinoma, inflammation, and various other cancers. Positive anti-proliferative, anti-cancer effects of *F. fomentarius* were reported on SGC-7901 and MKN-45 human gastric cancer

cell lines (Chen et al. 2008, Chen et al. 2011). Immuno-stimulatory effects of *F. fomentarius* were also reported by Kim et al. (2014): they observed 80% methanol extract to stimulate macrophages and to augment the levels of NO, TNF-α, IL-1β, IL-6, IκBα and MAP kinases (p38, ERK, and JNK) levels. Moreover, the polysaccharides of *F. fomentarius* enhance humoral immunity, improve phagocytosis in macrophages, and induce TNF-α, IFN-γ, and IL-2 level in mouse immunocytes (Gao et al. 2009).

Ganoderma applanatum, commonly known as "Artist's Conk", is a member of the *Ganoderma* genus whose members are reputed for their promising therapeutic potential in China, for at least 2000 years. We have observed strong immuno-stimulatory activity of 3 fractions (water, 2% ammonium oxalate and 5% NaOH) of *G. applanatum,* making it a promising species for future studies. Fruiting bodies of *G. applanatum* are documented to have anti-viral, anti-cancer and immuno-modulatory potential (Lee et al., 2007). Its β-D-glucan has shown anti-tumor activity against Sarcoma-180 that was transplanted in mice (Sasaki et al., 1971). Later, it was found that this anti-tumor activity is related to immuno-modulation as natural killer cell activity was enhanced in a similar study (Jeong et al., 2008). Recently, a new exopolysaccharide isolated from this fungus has shown anti-tumor and immuno-stimulatory properties *in vitro*. The exopolysaccharide demonstrated cytotoxic effects against SiHa tumor cell lines and stimulated production of macrophages derived from TNF-*α* and THP-1 (human leukemia monocytic cell line) (Osińska-Jaroszuk, 2014).

Strong stimulating activity was exhibited by water and 2% ammonium oxalate extract of *E. tinctorium,* which is the first report of immuno-stimulatory activity of this wild mushroom. This represents another potentially novel source of immuno-modulatory anti-cancer lead candidate(s) for drug development. *Echinodontium tinctorium* (toothed conk) is a wood decaying fungus

commonly known as "Indian paint fungus". It is only found in western North America, and it is not related to the other toothed fungi so it is sequestered in a family of its own. No literature on the therapeutic activity of this fungi is available. It owes its common name "Indian Paint Fungus" to its use by Native Americans in a preparation of war paint (Arora 1986; Gibson 2014). Also, natives from Oregon used this mushroom as an anti-bacterial agent (Ye et al., 1996).

In the present study, the water and 5% NaOH extracts of *Leucocybe connata* were found to stimulate macrophages for the release of TNF-α, which is a novel finding. *Leucocybe connata* is a wide spread edible basidiomycete. This species has limited scientific literature in regards to the therapeutic potential. There has been one study on the anti-oxidant potential of ergothioneines derived from *L. connata* that could scavenge free radicals in *in vitro* assay (Kimura et al., 2005) but there has been no literature, to date, regarding immuno-modulatory effects of this species.

Laetiporus sulphureus, commonly known as chicken of the woods, is an edible bracket fungus widely found in Europe and North America. We observed that water and 5% NaOH soluble extracts of *L. sulphureus* bear immuno-stimulatory potential and therefore have stimulated RAW 264.7 macrophage cells to release TNF-α. Lanostanoid triterpenes isolated from this species shows cytotoxic effects against human promyelocytic leukemia cell lines (HL-60) by activating caspase-3, an apoptotic inducer (León et al., 2004; Rios et al., 2012). Moreover, exopolysaccharide isolated from *L. sulphureus* demonstrates immuno-modulation on U937 human leukemia cell lines by activating genes and proteins (Bax and Bad) of the B cell lymphoma-2 (Bcl-2) family that regulates cell death (Seo et al., 2011). However, little or no literature is available on the immuno-stimulatory activity with regards to stimulation of TNF-α in RAW-264.7 macrophage cells.

Phellinus nigricans is a wood decay fungus. In our study, the primary screening also showed positive immuno-stimulatory activity of water and 5% NaOH soluble extracts of *P. nigricans* which is consistent with previously reported studies. Two proteoglycans isolated from *P. nigricans* (PNW1 and PNM1) demonstrated anti-tumor activity *in vivo* in mice transplanted with Sarcoma 180 model; however, this anti-tumor effect could not be associated with direct tumoricidal activity, rather detailed experiments deduce the possibility of immuno-modulation. These results indicated that the anti-tumor effect could be because of the stimulation of lymphocytes and activation of macrophages as increased concentrations of TNF-α and NO production were observed (Li, 2008). More recently, researchers from China have demonstrated immuno-modulatory activity of three polysaccharides (PNMP1, PNMP2, and PNMP3) isolated from *P. nigricans* that stimulate lymphocyte proliferation proving their immuno-stimulatory potential (Wang et al., 2014).

These primary screening results show the immense potential of BC wild mushrooms to stimulate the immune system, as an important source for drug discovery.

Anti-inflammatory Studies

This section provides first a brief overview of inflammation and discusses some important anti-inflammatory compounds isolated from mushrooms. It then explores the experimental design, methodology and primary results on the screening of 13 wild fungi (31 extracts) for their potential anti-inflammatory activity (i.e. inhibition of LPS-induced TNF-α production in mouse Raw264.7 macrophage cells).

2.8 Introduction

2.8.1 Inflammation

Inflammation is the localized protective mechanism of the body in response to tissue injury or pathogen invasion. During inflammation, an immune system stimulant will enhance the production of pro-inflammatory immune cells e.g. macrophages and monocytes to release inflammatory mediators like NO, ROS, IL1, IL6, TNF-α, COX-2, PGE2, and NF-κβ etc. (Balkwill & Mantovani, 2001).

Innate inflammation is usually self-limiting, and resolves through apoptosis, scarring, and clearing of inflammatory debris (Wu & Zhou, 2010). The uncontrolled release of inflammatory mediators causes cellular damage and initiates the chronic inflammatory cascade. This unrestrained, persistent, and unresolved condition contributes to the pathophysiology of various inflammatory mediated diseases like diabetes, hepatitis, arthritis, and other cardiovascular complications. There is also a strong correlation between inflammation and the development of cancer (Balkwill & Mantovani, 2001).

Macrophages are major immune cells present in the inflammatory microenvironment. These cells are activated by various pro-inflammatory cytokines such as LPS, TNF-α, IFN-γ to name a few. These phagocytic cells are one of the most important cells of the inflammatory environment that are involved throughout the process of inflammation from initiation to resolution and even in

case of its exacerbation into the chronic stage (Ostuni et al., 2015). While macrophages in major tumor micro-environments are in suppressive or inactivated forms, macrophages in the inflammatory environment are highly activated; their stimulated form release various cytokines (including TNF-α) and other biological mediators that control the inflammatory cascade. Because of their major role in inflammation, macrophages could be a lucrative target for the anti-inflammatory response (Weiskopf & Weissman, 2015)

TNF-α is one of the important pro-inflammatory cytokines released during inflammation. As described earlier, it is mainly secreted by activated macrophages but may also be secreted by monocytes, natural killer cells, and other inflammatory cells (Chu, 2013). Once released, TNF-α is involved in the secretion of other inflammatory cytokines and chemokines for an enhanced inflammatory response. Endothelial TNF-α enhances endothelial cell permeability, thereby enhancing inflammatory cells infiltration (Waters et al., 2013). It also increases vascular endothelial growth factor (VEGF) therefore, supporting angiogenesis. This mechanism is one of the major pieces of evidence relating excess TNF-α with tumor growth and progression. Enhanced levels of this cytokine are observed in cellular damage, chronic inflammatory mediated diseases, and apoptotic cell death (O'shea et al., 2002). Thus, the suppression of this crucial pro-inflammatory mediator could be a pre-requisite for an anti-inflammatory response.

2.8.2 Anti-Inflammatory Activity/Compounds from Mushrooms
Like immuno-stimulation, natural products (specifically mushrooms) are also being screened and used as anti-inflammatory agents because they have fewer or no side effects.

The general trend of research studies on the therapeutic potential of mushrooms involve the ability of mushroom extracts and/or active constituents to reduce the levels of pro-inflammatory cytokines (i.e. NO, TNF-α, IL-1, IL-6) as an indicator of decreasing inflammation. The RAW 264.7 macrophage cell model is a common *in vitro* cell model for exploring the anti-

inflammatory potential of mushrooms based on their ability to inhibit the induced inflammatory mediators/cytokines (Elsayed et al., 2014; Taofiq et al., 2016). Some of the common mushrooms recognized for their anti-inflammatory potential described briefly here are *Cordyceps sinensis* (Berk.) Saac., *I. obliquus* and *G. lucidum*.

Cordyceps sinensis has a long history of use as a folk and herbal medicine. The major anti-inflammatory ability of this species is exhibited by its purified peptide "cordymin" that inhibited IL-1β and TNF-α serum levels in a carrageenan-induced inflammatory mice model. It also inhibited, in a dose dependent manner, acetic acid induced abdominal constrictions in mice, reflecting the anti-inflammatory potential of this peptide (Qian & Guo, 2012). Cordymin, due to its anti-inflammatory and anti-oxidant activity, was also found useful in treating ischemia in rat brain (Wang et al., 2012b). The methanol extract of *C. sinensis* from Taiwan has also exhibited anti-inflammatory effects by inhibiting TNF-α and NO in LPS/IFN-γ induced murine peritoneal macrophage cell lines (Rao et al., 2010).

Inonotus obliquus (Chaga) is an important folk medicine in Russia and Western Siberia. Sterols isolated from this species reduced the NO concentration by 50% and inhibited the levels of IL-1β, IL-6, and TNFα in LPS induced RAW 164.7 cells in a dose dependent manner (Van et al., 2009). Trametenolic acid, ergosterol peroxide, 3β-hydroxy-8, 24-dien-21-al, ergosterol, trametenolic acid and inotodiol isolated from the petroleum ether and ethyl acetate fractions of Chaga inhibited NO and NF-κβ in RAW 264.7 macrophage cells (Ma et al., 2013).

Methanolic extract of *I. obliquus* inhibited production of NO, prostaglandin E2, and TNF-α; it also inhibited mRNA expression of iNOS and COX-2 in RAW264.7 macrophage cells (*in vitro*) and reduced inflammation in a carrageenan-induced paw edema mice (*in vivo*) model (Park et al., 2005). Ethanol extract of *I. obliquus* grown on germinated brown rice decreased levels of

TNF-α, COX-2, IL-4, IFN-γ, signal transducers and activators of transcription (STAT) 1, and STAT6. The levels of IgE and IgA in the spleen and mesenteric lymph node were also reduced significantly in Colitis associated inflammatory mice model (Debnath et al., 2012).

Ganoderma lucidum, as mentioned earlier, is a well-known traditional mushroom that has also demonstrated potent anti-inflammatory activity. It is widely used as an anti-allergic, anti-aging, anti-inflammatory and longevity supplement. Active immuno-modulatory protein (FIP-LZ8), isolated from *G. lucidum,* has immunosuppressive action as it can inhibit autoimmune reactions in diabetic animal models with lesser toxic effects on kidneys as compared to cyclosporine A, an immunosuppressive drug with strong nephrotoxic side effects (Van der Hem et al., 1995). Both ethanolic and methanolic extracts have also shown anti-inflammatory potential in *in vitro* settings (Chu et al., 2015; Yoon et al., 2013). Ethanolic extracts have shown to inhibit IL-1β, NO, TNF-α, and PGE$_2$ in LPS-stimulated murine BV2 (mouse brain, microglial) cell lines while methanolic extract has shown inhibition of LPS-induced NO production in RAW264.7 cell lines (Chu et al., 2015).

The anti-inflammatory metabolites of medicinal mushrooms belong to a chemically diverse group. This section of my thesis will highlight some of the major anti-inflammatory classes of medicinal mushrooms based on their chemical structures (i.e. anti-inflammatory polysaccharides, glyco-proteins, steroids, terpenoids, lectins, lipids etc).

Anti-inflammatory polysaccharides represent the major class of medicinal mushroom compounds and have been shown to be effective in many *in vitro* and *in vivo* settings. Polysaccharide isolated from the chloroform extract of *Agaricus blazei* Murrill, inhibited IL-6 production in mouse bone marrow derived mast cells; these mast cells were stimulated with phorbol myristate acetate and calcium ionophore A23187. This purified polysaccharide also

suppressed the production of prostaglandin D2 and leukotriene C4 and downregulated the phosphorylation of the serine/threonine kinase AKT (Song et al., 2012). In 2009, a Phase 1 clinical trial on healthy individuals demonstrated that a regular oral dose (60 mL for 12 days) of a blend of mushroom extracts (82% *A. blazei* with 15% *Hericium erinaceum* (Bull.) Pers. and 3% *Grifola frondosa* (Dicks.) Gray) reduces the level of certain pro-inflammatory cytokines (IL-1β, Il-2, Il-6, Il-7 and TNF-α) in the body, thus acting as prophylactic agents against inflammation. This aqueous extract is named AndoSan. Although not completely purified, this extract has been found to be rich in proteoglucan and β-glucan (Johnson et al., 2009); in 2011, the same AndoSan extract with the same dose (60 mL for 12 days) was administered to 21 patients suffering from chronic inflammatory diseases e.g. ulcerative colitis, inflammatory bowel disease and Crohn's disease and results showed suppressed levels of pro-inflammatory cytokines, indicating the extract potential against inflammatory diseases (Førland et al., 2011).

Terpenoids are another important class of anti-inflammatory compounds from medicinal mushrooms. Out of five novel di-terpenes isolated from *Cyathus africanus* H.J. Brodie, three (Cyathins D-H 3 and 5, 11-O-acetylcyatha-triol, and neosarcodonin) significantly inhibited NO production in LPS-induced macrophage cells (Han et al., 2013). Triterpenes isolated from *G. lucidum* also significantly inhibited IL-6, TNF-α, NO, PGE-2 and NF-κβ in an LPS-induced RAW 264.7 macrophage cell model (Dudhgaonkar et al., 2009). Moreover, six Lanostane type triterpenes isolated from *P. betulinus* are active anti-inflammatory candidates in *in vivo* mouse ear edema model (Kamo et al., 2003)

Mushroom anti-inflammatory compounds include the "Sterols" family of compounds. Chaga, as described earlier, is an important source of anti-inflammatory sterols isolated on the basis of their exclusive ability to suppress inflammatory cytokines in *in vitro* cell model (RAW 164.7)

(Van et al., 2009). Six other sterols (Trametenolic acid, ergosterol peroxide, 3β-hydroxy-8,24-dien-21-al, ergosterol, trametenolic acid and inotodiol) from the same species were also isolated on the basis of their anti-inflammatory potential in RAW 264.7 cells.

Peptides are a small family of anti-inflammatory metabolites from mushrooms. Cordymin, as described earlier, is a reputed peptide that shows documented action against inflammation in various *in vitro* and *in vivo* models. Phenolics (especially hispolon) isolated from *Phellinus linteus* (Berk. & M.A. Curtis) Teng, Zhong Guo De Zhen Jun are active against inflammation as they have significantly suppressed NO production in LPS-induced RAW 264.7 macrophage cells (Lin et al., 2014).

Thus, the anti-inflammatory metabolites from medicinal mushrooms have immense diversity in terms of their chemical structure and characterization. There is a lot more literature on the chemical extracts of mushrooms showing anti-inflammatory activity but in most cases, the active molecules were not isolated nor identified. The active metabolites that have already being isolated, combined with ones that are yet to be purified, serve as a library of potential lead molecules for the development of mushroom-based nutraceuticals or drugs that are effective against inflammation.

2.9 Material & Methods

2.9.1   Tumor Necrosis Factor Alpha Inhibition after LPS treatment
For the anti-inflammatory assay, RAW264.7 cells were used. The maintenance and plating of RAW264.7 cells was essentially as previously described in the first part of this chapter. The difference is on the day of treatment after overnight incubation. The medium in cells (200 µL DMEM without FBS) was removed and the cells were washed with 200 µL BioWhittaker® Phosphate Buffer Saline (PBS) (Lonza, Walkersville, Maryland) followed by treatment with 100 uL DMEM (added LPS so final conc. of LPS is 250 ng/ml) without FBS with: (i) various

mushroom fractions (conc. 1 µg/µL), (ii) Polymyxin B (PMB; Sigma 100 units) as the positive control, or (iii) DMEM and respective solvents (i.e. water or methanol, used for re-suspension of lyophilized samples) for use as negative controls.

After a further 6 hours of incubation, supernatants (~100 µL) were collected, centrifuged (4°C, 200xg, 5 min using Allegra® X-15R, benchtop centrifuge; Beckman Coulter, CA, USA) and stored at -80ºC freezer until ELISA was performed for the quantification of TNF-α.

2.9.2 Tumor Necrosis Factor Alpha Inhibition after Histamine treatment

In the *in-vivo* mouse model described in Chapter 4, the effect of fungal extracts in response to histamine-induced inflammatory was evaluated. So, in order to mimic similar conditions, histamine-induced inflammation was also assessed in the RAW264.7 cell-based assay. LPS is replaced by histamine to assess the effect of various histamine concentrations in RAW 264.7 macrophage cells. For this assay, the maintenance and plating of cells was as previously described.

The difference is on the treatment day, cells were treated with: i) various concentrations of histamine (1 M-1 µM), ii) *I. obliquus* (F2) and *E. tinctorium* (F5) (1 µg/µL) plus histamine, iii) PMB as the positive control, and iv) DMEM and/or solvents as negative controls. The time of treatment and the collection protocol was also as previously described.

2.9.3 Chemical Extracts-Treatment

Fungal extracts that do not show any stimulatory activity were selected for the anti-inflammatory assay. These are from 10 species used for immuno-stimulatory assay and 4 more species assessed for stimulation by 2 other graduate students in Dr. Lee's lab (Mr. Ankush Barad and Ms. Vicky Myhre).

2.9.4   Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA following fungal treatment was also as previoule described, except that, the inhibition of TNF-α induced by LPS indicated the anti-inflammatory potential.

2.10   Results

2.10.1 Tumor Necrosis Factor Alpha Inhibition after LPS treatment

To assess anti-inflammatory activity, cells were treated with 250ng/mL of LPS plus fungal extracts. The concentration of each extract was 1 µg/µL, except for *Inonotus obliquus* which was applied with 3 concentrations. Polymyxin B (PMB: active ingredient of reputed anti-inflammatory ointment-Polysporin®) was used as positive control for the estimation of relative anti-inflammatory potential of wild fungi. PMB consistently inhibited TNF-α induced by LPS. Only fungal extracts that did not demonstrate any stimulatory activity in Figure 2.4 were selected for the anti-inflammatory assay.

Results are presented in Figure 2.6.1, 2.6.2, 2.6.3, and 2.6.4; they shows that all four extracts of *I. obliquus* have potent anti-inflammatory potential, at concentration as low as 0.25µg/µL. Other active extracts that were strongly effective against inflammation included fractions of *F. pinicola* (F1 & F3), *P. betulinus* (F1, F2 & F3), *F. fomentarius* (F1 & F2), *G. applanatum* (F1 & F2), *E. tinctorium* (F5), *L. connata* (F2), *L. sulphureus* (F1), *P. nigricans* (F2), *P. igniarius* (F1), and *T. abietinum* (F1). Moderate activity was demonstrated by *E. tinctorium* (F1 & F2), *L. sulphureus* (F2), *P. nigricans* (F1), *Phellinus igniarius* (F4), *H. coralloides* (F2), and *L. vulpina* (F2).

2.10.2 Tumor Necrosis Factor Alpha Inhibition after Histamine treatment

As the *in vivo* model involved the induction of inflammatory events in the microcirculation, therefore the *in vivo* anti-inflammatory conditions were simulated in RAW 264.7 macrophage cells. This was done by stimulating RAW cells with histamine, instead of

LPS. Initially, cells were treated with different concentrations of histamine to explore the dose at which the maximum stimulation of TNF-α is observed. Figure 2.7.1 demonstrates the effect of histamine concentration on RAW 264.7 cells. The extent of stimulation in the presence of histamine is less compared to LPS; 1μM triggered the activation of macrophages to its maximum so this concentration was selected as the optimum dose for the anti-inflammatory assay. Later, potent extracts of *I. obliquus* (F2) and *E. tinctorium* (F5) (1μg/μL) were treated in the presence of histamine to observe the inhibitory tendency of these extracts in the presence of an inflammatory mediator. Both extracts inhibited histamine induced TNF-α > 90% indicating their strong potential in treating inflammation (Figure 2.7.2.).
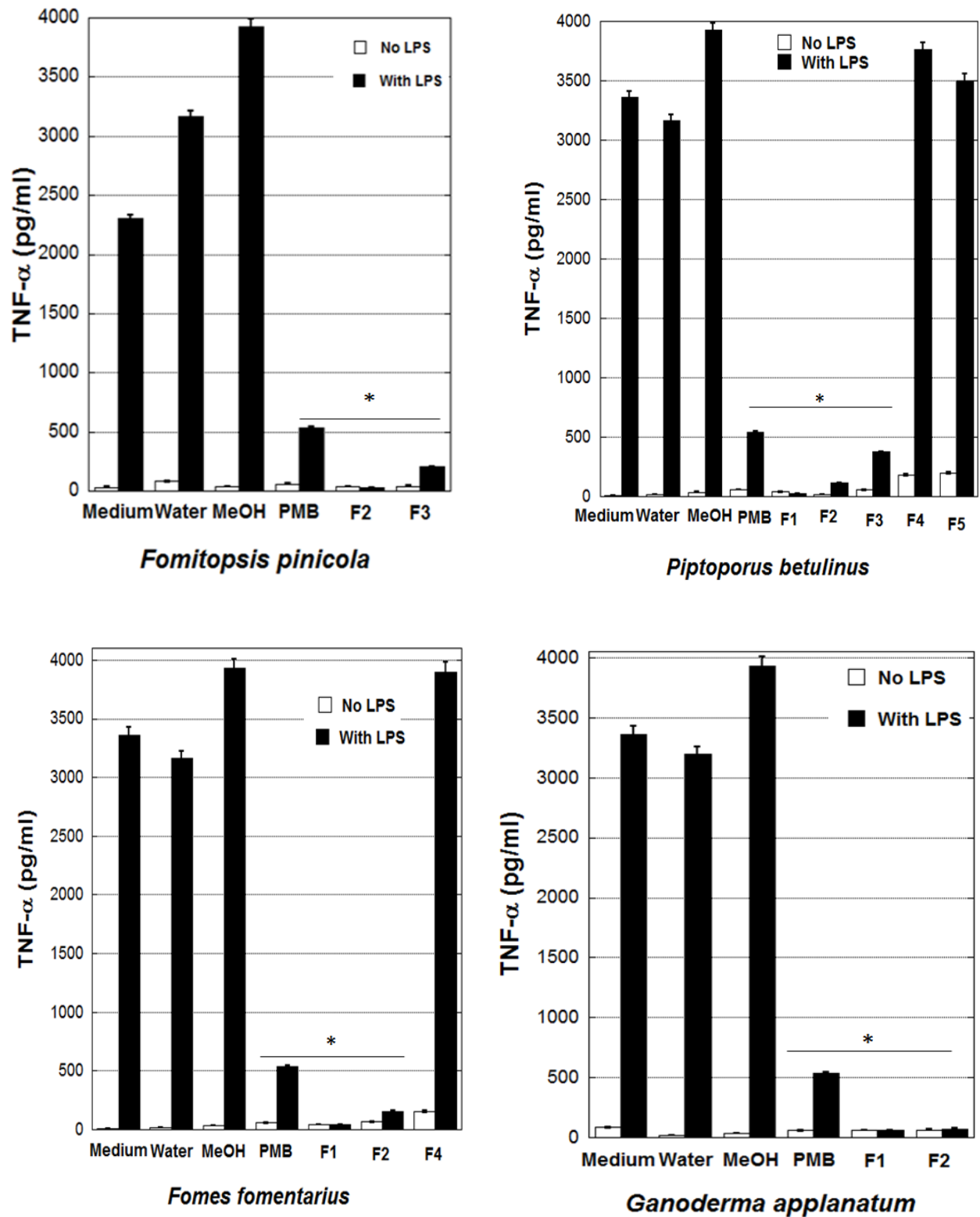
Figure 2.6.1. Assessing anti-inflammatory potential of fungal extracts in LPS (250ng/mL) induced RAW 264.7 macrophage cells. Fungal conc. 1µg/1µL, PMB (100 units) is used as positive control, solvents and media as negative control. Error bars are standard deviations and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).
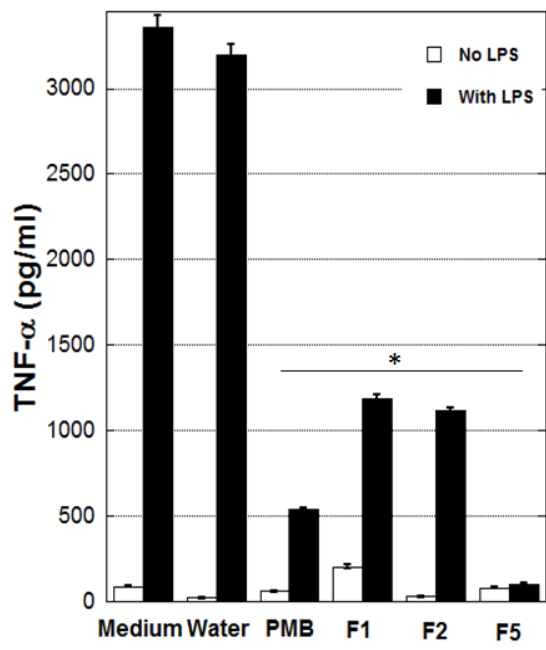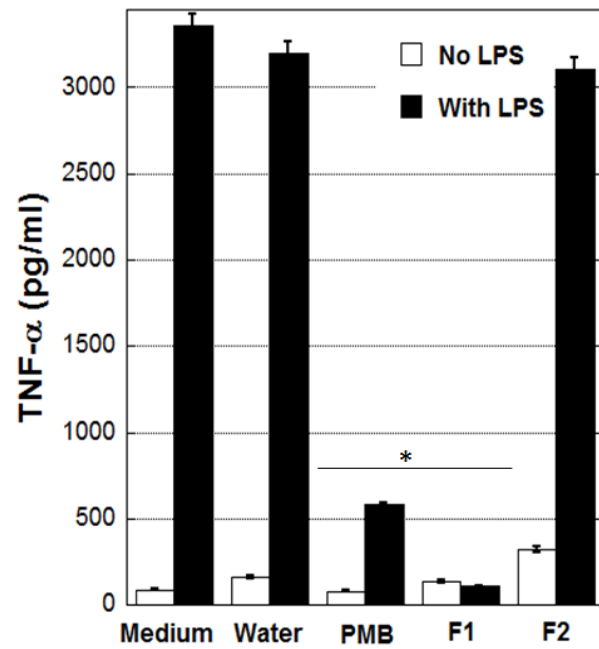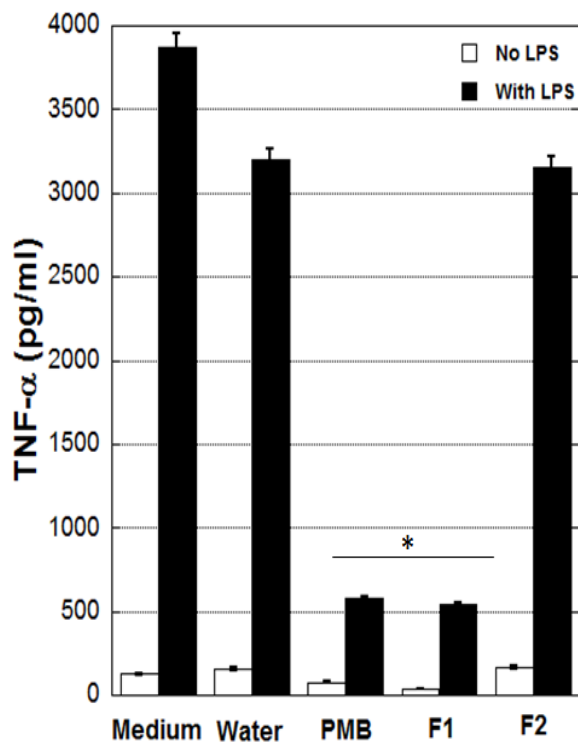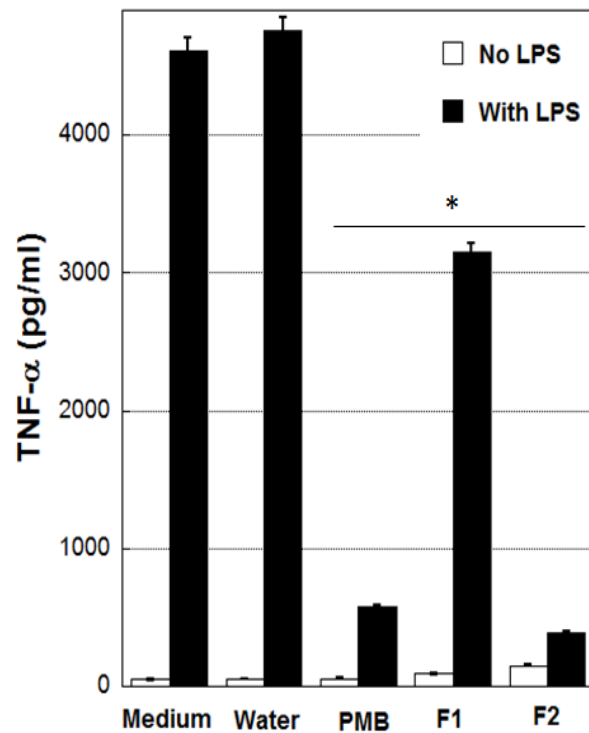
Figure 2.6.2. Assessing anti-inflammatory potential of fungal extracts in LPS (250ng/mL) induced RAW 264.7 macrophage cells. Fungal conc. 1 µg/1µL; PMB (100 units) was used as positive control; solvents and media were used as negative controls. Error bars are standard deviations and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).
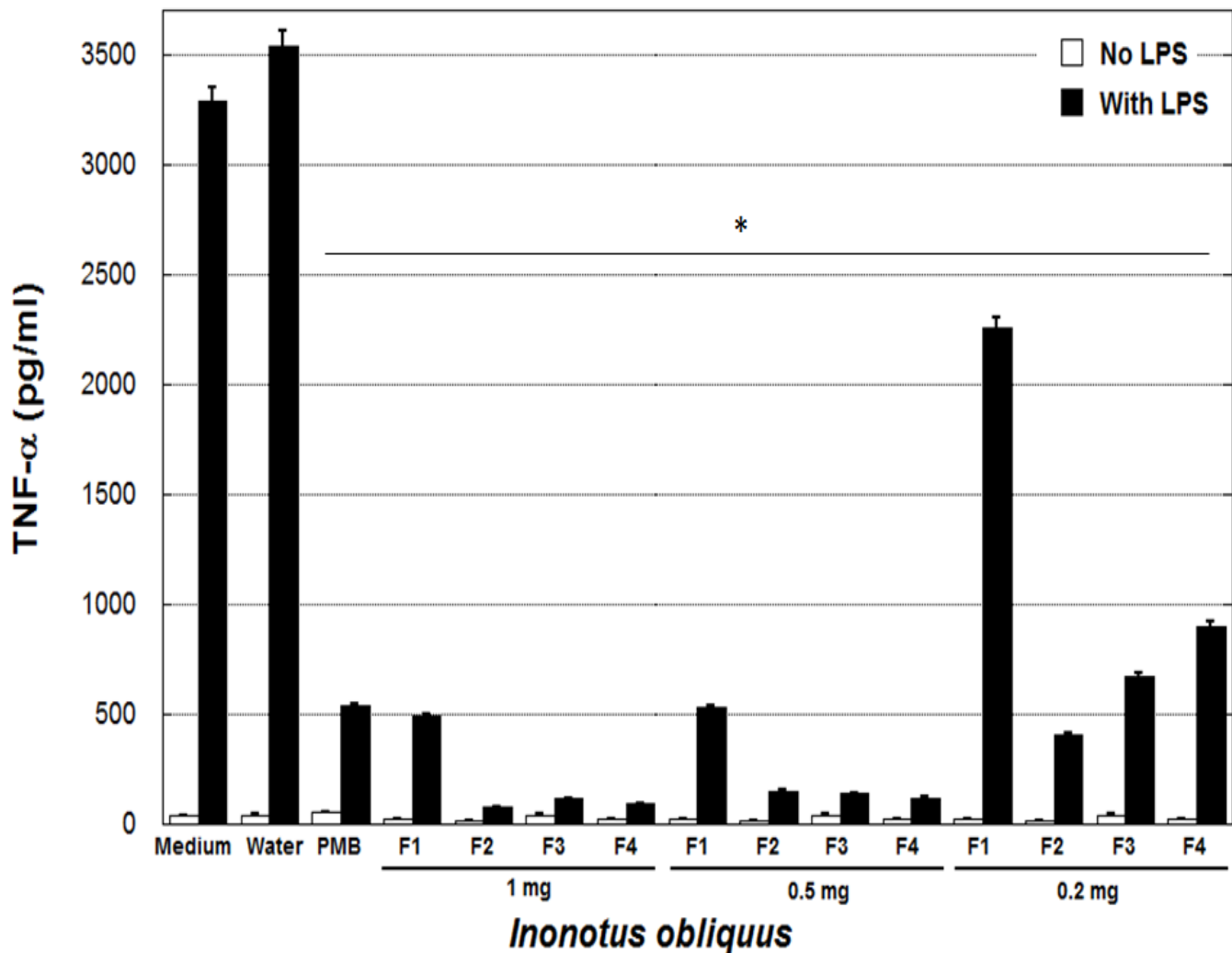
Figure 2.6.3. Assessing anti-inflammatory potential of *I. obliquus* in LPS (250ng/mL) induced RAW 264.7 macrophage cells. Fungal conc. 1 µg, 0.5µg; 0.25 µg/µL, PMB (100 units) was used as positive control; solvents and media were used as negative controls. Error bars are standard deviations and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).
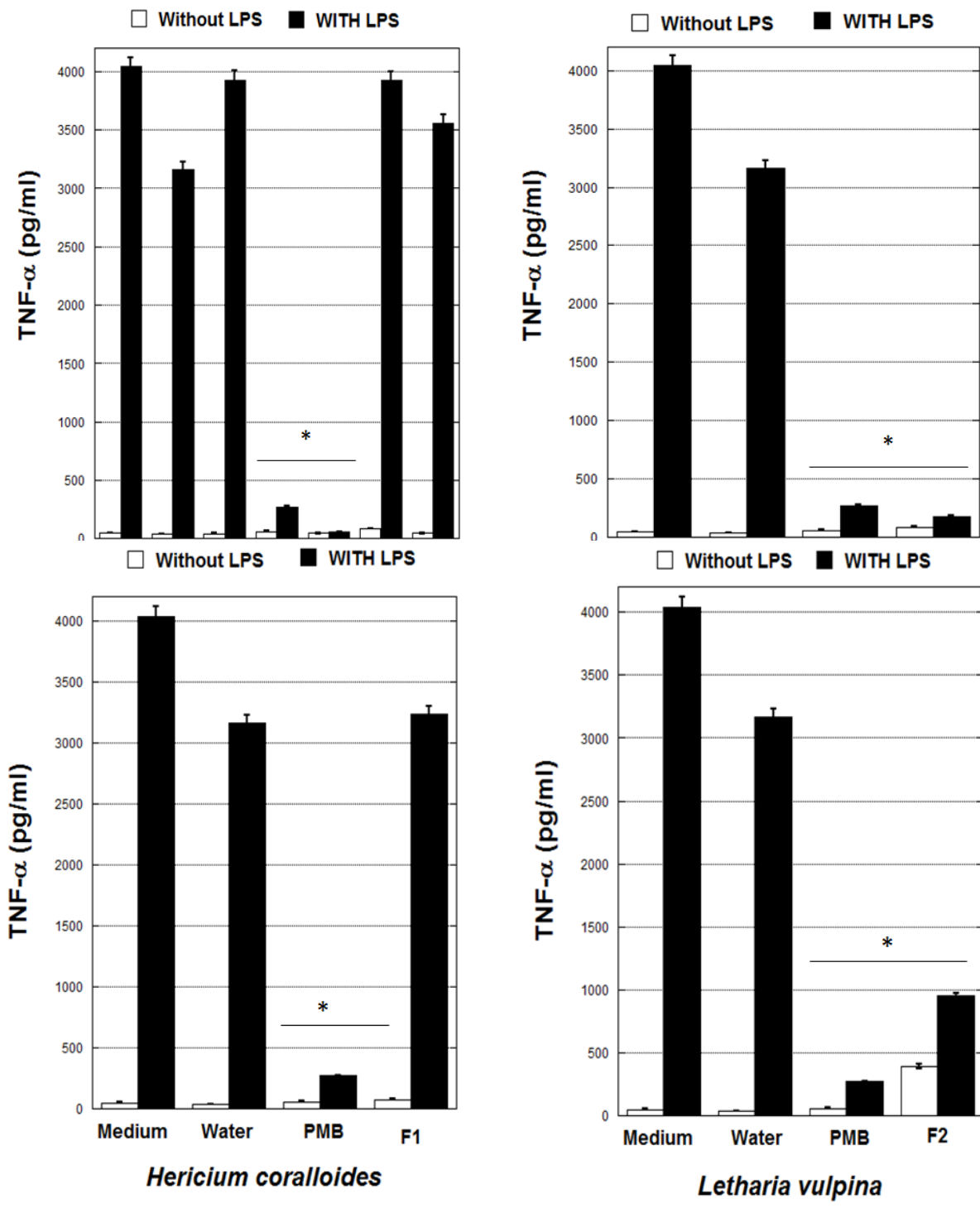
Figure 2.6.4. Assessing anti-inflammatory potential of fungal extracts in LPS (250ng/mL) induced RAW 264.7 macrophage cells. Fungal conc. 1 μg/μL; PMB (100 units) was used as positive control; solvents and media were used as negative controls. Error bars are standard deviations and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).
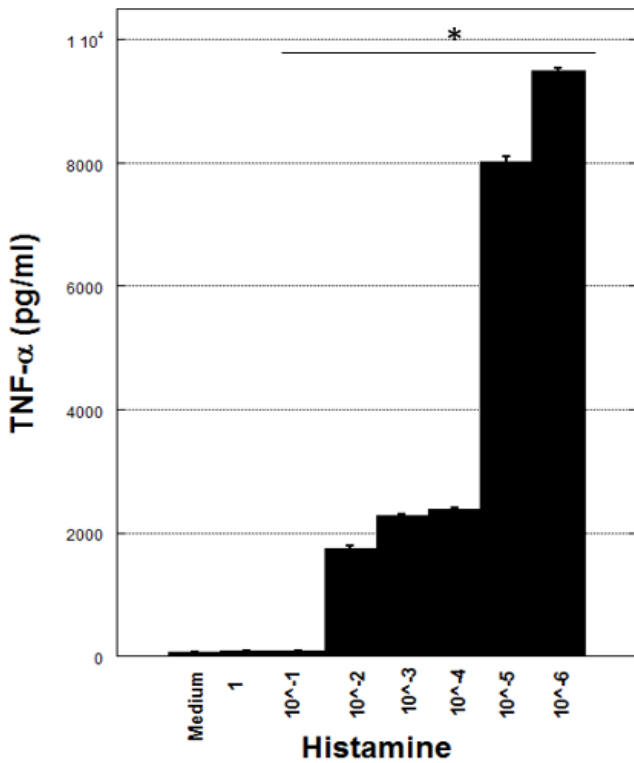
Figure 2.7.1. Histamine dose response curve for inducing TNF-α in RAW 264.7 macrophage cells. The histamine conc. was 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. The maximum induction occurs at 10^{-6} M. Error bars represent standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).
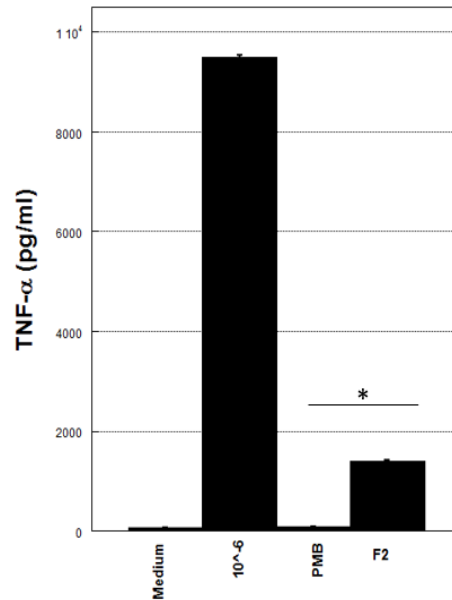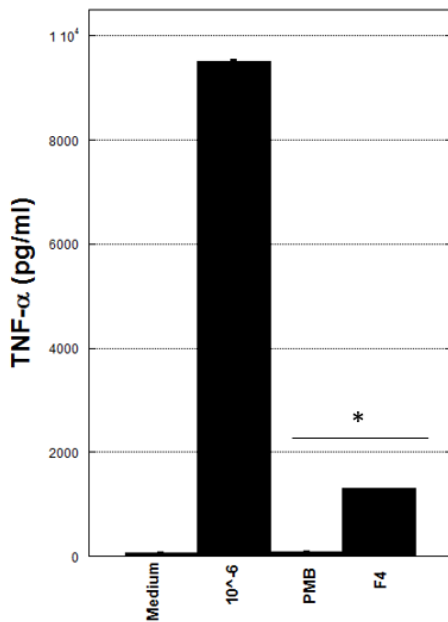
Figure 2.7.2. Inhibition of histamine induced TNF-α in RAW 264.7 macrophage cells by potent mushroom extracts *E. tinctorium* F4 (left) and *I. obliquus* (right). The histamine conc. used is the dose for inducing maximum TNF-α- 10^{-6}M. Error bars represent standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).

2.11 Discussion

Natural products extracted from fungi also have impressive anti-inflammatory properties. The following literature brings forward the existing knowledge regarding anti-inflammatory fungal extracts that have exhibited positive activity in this thesis chapter.

In the current study, strong anti-inflammatory activity (strong inhibition of TNF-α induced with LPS) by 99% ethanol and water fractions of *F. pinicola* was observed which, to our knowledge, is the first report in RAW 264.7 cells but also consistent with previous anti-inflammatory activity in other *in vitro* and *in vivo* models. *Fomitopsis pinicola* is a brown rot fungus present widely in Asia and Europe. In traditional medicines, this mushroom is used for treating nausea, headaches and hepatic complications. Other traditional uses of *F. pinicola* include haemostatics (as astringent) and as anti-inflammatory agent (Grienke et al., 2014). *Fomitopsis pinicola* from Taiwan has demonstrated strong anti-angiogenic and anti-inflammatory activity *in vitro*; a strong inhibitory effect was also observed by extracted polysaccharides on VEGF-induced tube formation in endothelial cells while the ethanolic extract of the same mushroom showed inhibition of IFN-γ induced IP-10 protein (a marker for inflammation) (Cheng et al., 2008). Lanostane, triterpenoids and triterpene glycosides isolated from *F. pinicola* also showed anti-inflammatory activity by inhibiting cylooxygenase-1 and cylooxygenase-2 in *in vitro* setting (Yoshikawa et al., 2005).

In the current study, the first three extracts (80% ethanol, 50% methanol and water extracts) of *P. betulinus* exhibited strong anti-inflammatory activity by inhibiting LPS-induced TNF-α in mouse macrophage cells. *Piptoporus betulinus* is a birch polypore and its tea is well known for soothing and anti-fatigue effects among traditional users (Peintner & Pöder, 2000). Also, in Finland and Siberia *P. betulinus* tea from young fruit bodies is known to have anti-cancer effects (Lucas 1960). The fungal exacts from *P. betulinus* were also effective in treating vaginal cancer

in female dogs where tumors disappeared in 5 weeks upon treatment with this wild fungus (Grienke et al., 2014). Scientists from Japan have isolated 6 lanostane type triterpenes from *P. betulinus* and found all of them to be active in *in vivo* mouse ear edema model (Kamo et al., 2003). This indicates the potential of finding new anti-inflammatory molecules from this wild mushroom.

In the present study both ethanolic and methanolic extracts of *F. fomentarius* have demonstrated strong anti-inflammatory activity which is consistent with the literature. Similar to its immuno-stimulatory activity, *F. fomentarius* has also been explored for its anti-inflammatory effects. Methanolic extract of Korean *F. fomentarius* have shown positive anti-inflammatory activity both in *in vivo* (reduced carrageenan induced acute paw edema in rats) and *in vitro* (inhibition of LPS induced NO, PGE2 and TNF-α in RAW 264.7 macrophage cells) settings (Park et al., 2004).

The ethanolic and methanolic fractions of *G. applanatum* have demonstrated strong anti-inflammatory activities, which is supported by the previous literature. Ganodermycin isolated from *G. applanatum* by German scientists has demonstrated inhibition of CXCL-10 (inducible protein-10), an inflammatory chemoattractant marker, in LPS + IL induced CXCL-10 promoter in acute monocytic leukemia derived cell lines (MonoMac-6) (Jung et al., 2011).

The current study has shown a novel anti-inflammatory potential of previously un-studied *E. tinctorium* extracts. Both ethanolic and methanolic extracts have demonstrated moderate anti-inflammatory activity but the 5% NaOH extract showed strong anti-inflammatory potential. This extract has also shown inhibition of TNF-α induced by histamine in RAW264.7 cells. Because of this new finding and its abundance in Northern British Columbian forests, the 5% NaOH extract

of *E. tinctorium* was selected for further study on purification, identification and characterization of anti-inflammatory compound(s) (Chapter 3) and on mice microcirculation (Chapter 4).

Inonotus obliquus, as explained above, is a well-known and well characterized mushroom since the time of Avicenna. It is one of the most abundant wild mushrooms present in forests of Northern BC, Canada. All four fractions of this species showed strong anti-inflammatory activity which supports the previously published research. The methanol extract (F2) showed the most potent activity. It has also inhibited histamine-induced TNF-α in RAW 264.7 cells. Owing to this powerful therapeutic effect, this extract was used as a positive control for the *in vivo* mouse model described in Chapter 4. Methanolic extract of *L. connata* showed strong inhibition on LPS-induced TNF-α production, which is a new finding.

In the current study, the 80% ethanol fraction of *L. sulphureus* exhibited strong anti-inflammatory activity while the 50% methanol extract showed a milder inhibition of LPS-induced TNF-α in RAW 264.7 cells, thus supporting the previous findings. Korean researchers have isolated an exopolysaccharide from *L. sulphureus* that suppressed LPS induced pro-inflammatory mediators like NO, PEG2, and TNF-α. The down regulation of protein and mRNA levels of iNOS, COX-2 and TNF-α in LPS induced BV2 microglia cells (brain and spinal cord resident macrophage) were observed. Moreover, this anti-inflammatory activity is via inhibiting the NF-$\kappa\beta$ pathway (Jayasooriya et al., 2011). Recently, Korean scientists have documented the anti-inflammatory activity of a triterpene isolated from *L. sulphureus* that inhibits LPS-induced NO production in RAW 264.7 cells. They further observed the suppression of various pro-inflammatory cytokines like iNOS, COX-2, IL-1β, IL-6 and TNF-α levels, suggesting a possible explanation for the mechanism of action (Saba et al., 2015)

Contrary to the immuno-stimulatory study, there is no literature available for anti-inflammatory activity of *P. nigricans*. The methanol extract of this BC wild mushroom showed a strong effect while the 80% ethanol fraction showed mild anti-inflammatory activity in *in vitro* model.

In the current primary screening, ethanolic extract of *P. igniarius* showed very strong anti-inflammatory activity while the 5% NaOH exhibited mild inhibition of TNF-α production. *Phellinus igniarius* is a well-characterized Chinese traditional mushroom that has been studied for its anti-tumor and anti-inflammatory properties. Recently, Suabjakyong et al. (2015) have shown that a β-D-Polysaccharide isolated from *P. igniarius* has the potential to inhibit expression of TNF-α and IL-6 and stimulate the expression of IL-10 (anti-inflammatory cytokine) in LPS induced RAW 264.7 macrophage model (Suabjakyong et al., 2015).

Trichaptum abietinum is a wood decaying polypore fungus present widely in boreal coniferous forests across the northern hemisphere (Kauserud & Schumacher, 2003). It has not been studied previously for its medicinal values. The 80% ethanolic extract of *T. abietinum* has demonstrated potent anti-inflammatory activity.

In this study, the 50% methanol extract of *H. coralloides* has also shown moderate anti-inflammatory activity which is a novel finding. The genus *Hericium* is a traditional mushroom genus well-known for its therapeutic potential, especially in Asia. *Hericium erinaceus* (Bull.) Pers. has been a major constituent of health supplements in China for a long time. However, *H. coralloides* has not been explored for its immuno-modulatory or anti-inflammatory potential.

The 50% methanol (F2) extract of *L. vulpina* has shown moderate anti-inflammatory potential, which is a novel finding. *Letharia vulpina* is a relatively abundant lichen present in North America but is nearly extinct species in Europe (Hogberg et al., 2002). This lichen is a rich

source of vulpinic acid. It has been studied for its anti-bacterial effect but the current study is the first to explore its anti-inflammatory potential.

2.12   Conclusion

The primary screening results highlight the therapeutic importance of BC wild mushroom extracts as potential sources of anti-inflammatory compounds. The challenge is to purify the bioactive metabolites so that they can be applied in pharmaceutical and biotechnological industries to determine the optimum dosage and to design compounds for maximum efficacy and effectiveness. The next chapter of this thesis will highlight the purification and characterization of the 5% NaOH fraction of *E. tinctorium*.

The summarized results from the primary screening of the wild mushrooms used in the current study (immuno-stimulatory and anti-inflammatory potential) are presented in Tables 2.3 and 2.4. Table 2.3 also summarizes the current literature available regarding the specific species used in the present research.

Table 2.3 Immuno-stimulatory and anti-inflammatory activities of BC wild mushrooms and lichen

Species	Fractions	Immuno-stimulation[1]	Anti-inflammatory[2]	Known Immuno-stimulatory and Anti-inflammatory activity	References
Fomitopsis pinicola	F1	19%	-	No immuno-stimulatory study Anti-inflammatory	Cheng et al, 2008 Yoshikawa et al, 2004
	F2	1.7%	99%		
	F3	1.2%	94%		
	F4	ND	ND		
	F5	10.5%	-		
Lentinula edodes	F1	62%	-	Immuno-stimulatory (RAW 264.7 cells)	Bisen et al., 2014 Xu et al., 2011
	F2	94%	-		
	F3	29%	-		
	F4	76%	-		
Piptoporus betulinus	F1	1.17%	99%	Anti-Inflammatory (6 Lanostanes)	Kamo et al., 2003
	F2	0.5%	96.5%		
	F3	2%	89%		
	F4	6.5%	0%		
	F5	7%	0%		
Fomes fomentarius	F1	1.2%	99%	Immuno-stimulation (Isolated Polysaccharide) Anti-Inflammation (methanolic extract)	Kim et al., 2014, Gao 2009 Park et al., 2004
	F2	2.0%	95%		
	F3	12%	-		
	F4	5.5%	0%		
	F5	22%	-		
Ganoderma applanatum	F1	1.8%	98%	Immuno-stimulation (GpEPS) Anti-inflammation (Ganodermycin)	Osińska-Jaroszuk, 2014 Jung et al., 2011
	F2	1.9%	98%		
	F3	126%	-		
	F4	44%	-		
	F5	36%	-		
Echinodontium tinctorium	F1	8%	65%	Not studied	-
	F2	1%	67%		
	F3	129%	-		
	F4	46%	-		
	F5	3%	97%		
Inonotus obliquus	F1	0.6%	92%	Anti-inflammatory (Sterols) Anti-inflammatory	Van et al., 2009
	F2	0.5%	99%		
	F3	1.2%	98%		

	F4	46%	98%	(Ergosterols derivatives)	Ma et al., 2013
Leucocybe connata	F1	3.5%	97%	Not studied	-
	F2	8%	7.5%		
	F3	49%	-		
	F4	31.5%	-		
Laetiporus sulphureus	F1	0.9%s	86%	Immuno-stimulation Anti-inflammatory (exopolysaccharide) Anti-inflammatory (triterpenoid)	Seo et al., 2011 Jayasooriya et al., 2011 Saba et al., 2015
	F2	4%	18%		
	F3	86.5%	-		
	F4	15%	-		
Phellinus nigricans	F1	2.5%	32%	Immuno-stimulatory (Isolated PNMP1, PNMP2, PNMP3 immuno-stimulatory) No anti-inflammatory Study	Li, 2008 Wang et al., 2014
	F2	4%	91.5%		
	F3	22.5%	-		
	F4	65%	-		
Phellinus igniarius	F1	AB*	98%	Anti-inflammatory (B-D-Polysaccharide)	Suabjakyong et al, 2015
	F2	AB*	3%		
	F4	AB*	12%		
Trichaptum abietinum	F1	AB*	96%	Not studied	-
Hericium coralloides	F1	VM*	20%	Not studied	-
Letharia vulpina	F2	VM*	76%	Not studied	-

Summary of data taken from Figures 2.4, 2.5 & 2.6. F1, F2, F3, F4 correspond to the 80% ethanol (F1), 50% methanol (F2), H_2O (F3) and 5% sodium hydroxide (F4). In cases where there is F5 *(F. pinicola, P. betulinus, F. fomentarius, G. applanatum and E. tinctorium)* F4 represents 2% Ammonium oxalate extract and F5 represents 5% sodium hydroxide extract.

The 2% Ammonium oxalate extract was omitted later as this was incompatible with the medium used. *F. pinicola* and *L. edodes* follow a different extraction pattern as stated in Table 2.2.

[1]Percent stimulation of TNF-α relative to LPS.

[2]Percent inhibition of LPS induced TNF-α.

Table 2.4. Summary of the biological activity (immuno-stimulation and anti-inflammation) of the mushroom species of BC

Names of Species	Biological Activity	
	Immuno-stimulation[1]	Anti-inflammatory[2]
Fomitopsis pinicola	+ (F1, F5)	+++ (F2, F3)
Lentinula edodes	+++(F2), ++ (F1, F4), + (F3),	ND
Piptoporus betulinus	-	+++ (F1, F2, F3), - (F4, F5)
Fomes fomentarius	+ (F3, F5)	+++ (F1, F2), - (F4)
Ganoderma applanatum	+++ (F3) ++ (F4) + (F5)	+++ (F1, F2)
Echinodontium tinctorium	+++ (F3) ++ (F4)	+++ (F5), ++(F1, F2)
Inonotus obliquus	-	+++ (F1, F2, F3, F4)
Leucocybe connata	++ (F3) + (F4)	+++ (F1), + (F2)
Laetiporus sulphureus	+++(F3), +(F4)	+++ (F1), +(F2)
Phellinus nigricans	++ (F4), +(F3)	+++ (F2), +(F1)
Phellinus igniarius	AB*	+++ (F1), +(F4), - (F2)
Trichaptum abietinum	AB*	+++ (F1)
Hericium coralloides	VM*	+ (F1)
Letharia vulpina	VM*	++ (F2)

Summary of data taken from Figures. 2.4, 2.5 & 2.6. F1, F2, F3, F4 correspond to the 80% ethanol (F1), 50% methanol (F2), H_2O (F3) and 5% sodium hydroxide (F4). In cases where there is F5 *(F. pinicola, P. betulinus, F. fomentarius, G. applanatum and E. tinctorium)* F4 represents 2% Ammonium oxalate extract and F5 represents 5% sodium hydroxide extract. *F. pinicola* and *L. edodes* follow a different extraction pattern as stated in Table 2.2.

[1]Percent stimulation of TNF-α relative to LPS. [2]Percent inhibition of LPS induced TNF-α.

+++; greater than 80% stimulation[1]; greater than 80% inhibition or complete inhibition[2]
++; 40-80% stimulation[1] ; 40-80% inhibition[2]
+; 10%- 40% stimulation[1] ; 10%- 40% inhibition[2], - less than 10%[1,2] or no stimulation[1]; no inhibition[2]

-AB* immune-stimulatory assay performed by Ankush Barad (a former graduate student in Dr. Lee lab). Fractions less than 10% stimulatory or no stimulatory were selected for anti-inflammation assay.

-VM* immune-stimulatory assay performed by Vicky Myhre (a graduate student in Dr. Lee lab). Fractions less than 10% stimulatory or no stimulatory were selected for anti-inflammation assay. -ND denotes not determine

Chapter 3: Purification and Characterization of Anti-Inflammatory Compound(s) from *Echinodontium tinctorium*

This chapter focuses on the purification and characterization of anti-inflammatory compound(s) from the 5% NaOH extract of *E. tinctorium*.

3.1 Introduction

3.1.1 Approaches in isolating active therapeutic compounds from medicinal mushrooms

Extracts of natural products have an established history for their applications in many industries around the globe (e.g. food, pharmaceuticals, cosmetics, additives etc.). Since their use is mainly associated with the mechanism of action of their active constituent(s), the desire to purify the active compound responsible for the main activity is a central goal. Moreover, the isolation and purification of the active constituent is also associated with the following advantages: understanding the chemical structure of the active component, obtaining potent biological activity, quantifying the active constituent for quality assurance purposes, exploring the structure activity relationship (SAR) for synthetic and semi-synthetic drug development, and industrial scale production. Extensive studies on the isolation of the active ingredients along with the development of newer approaches have made analytical chemistry much easier in recent years. Over the past few decades, techniques like high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), fast protein liquid chromatography (FPLC), and membrane filtration have revolutionized the field of natural products chemistry.

Medicinal mushrooms have a variety of active chemical constituents possessing a plethora of biological activities. As the fungal extract contains both therapeutic and non-therapeutic constituents, it is essential to purify the active agent responsible for the respective biological activity. As described earlier, Mizuno (1996) successfully developed a reliable method for the extraction of various chemical constituents from mushrooms. He further suggested the possibility of water insoluble polysaccharide in 5% NaOH extract, which is the major focus of the current study.

The fungal cell wall, a complex structure, is rich in two types of polysaccharides: chitin and glucans. Both these polysaccharides cross-link firmly to provide strength and integrity to the cell wall. Chitin is an abundant polysaccharide present around the outer core of the cell wall whereas glucans are present in the central core. A typical structure is a β-1,3 1,6 glucans connected with the outer core, chitin, via β-1,4 linkages. Mushroom bioactive polysaccharides belong to the glucan family of the fungal cell wall. β-1,3 and -1,6 are mainly alkali insoluble polysaccharides while α-1,3 glucans are alkali soluble polysaccharides (Latgé, 2007). Once the polysaccharides (alkali soluble or insoluble) are extracted, these can be purified by techniques such as ethanol precipitation, ion-exchange chromatography, and gel permeation chromatography, etc, however, it is worth mentioning that a single technique is often not sufficient and a combination of techniques may be applied simultaneously for an effective purification. Therefore, an individualized protocol is needed for each extract's purification depending on the type of polysaccharide, its solubility characteristics, branching patterns, molecular weights, linkages, and various other physical and chemical factors.

For the purification of initial crude extract, chromatography is the major technique that fractionates compounds based on various physical and chemical properties e.g. size (size

exclusion), ionic charge (ion exchange), and respective affinity (affinity chromatography) (Ganetsos & Barker, 1992). Chromatography, derived from the Greek language, means "color writing". It is a technique in which the components are separated based on their relative affinities to the stationary and mobile phase. The adsorbent or stationary phase may be paper, glass, gels (aluminium or silica) and combination of various solid resins packed in columns. The mobile phase may be either liquid or gas (Weston & Brown, 1998). In principle, the mobile phase is allowed to run over the stationary phase (with sample) and the mixture is separated, owing to its relative affinity with the respective phase. The resolving fraction is then separated and analysed using various sophisticated techniques such as mass spectroscopy (MS), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR). In 1906, the importance of chromatography was first demonstrated by Tswett when he separated various green and yellow pigments from plants using a calcium carbonate column (Ganetsos & Barker, 1992).

Based on the mobile phase, chromatography can be divided in two groups: Gas Chromatography (GC) and Liquid Chromatography (LC). GC is the form of chromatography in which the mobile phase is a gas while LC is the form of chromatography in which the mobile phase is liquid. The separation in LC is due to the interaction and relative affinity of various molecules between the solid (stationary) and the liquid (mobile) phase (Weston & Brown, 1998).

Moreover, based on the nature of stationary and mobile phase, LC can be both normal and reverse phased. In normal phase, the solvent applied is less polar and organic than the stationary phase. The stationary phase is usually silica or alumina. In this mode, neutral compounds are eluted according to the degree of their polarity; the higher the polarity, the greater will be the retention of the active molecule in the column and vice versa. In contrast, reverse phase separates the compounds based on their respective hydrophobicity; the stationary phase

used is the adsorption of hydrophobic groups (e.g. methyl, butyl, phenyl, and amino etc.) on silica plate whereas the solvent system applied is more polar and aqueous e.g. water, acetonitrile, methanol etc. In this case, the more polar compounds are separated faster as compared to the less polar ones. Reverse phase chromatography is economical as compared to the normal phase, due to its regeneration ability. For natural products purification, specifically for the preparative scale, reverse phase is one of the methods of choice for the majority of natural products chemists (McChesney & Rodenburg, 2014). This technique was applied to the analysis of polysaccharides from the medicinal mushroom, *Pleurotus abalonus* Y.H. Han, K.M. Chen & S. Cheng, which was completed using HPLC, NMR, and FTIR (Wang et al., 2011).

Size exclusion chromatography is usually one of the initial purification techniques applied in the isolation of the molecule of interest from the crude extract. As indicated by the name, the compounds are separated based on the difference in their relative molecular sizes. The column has the ability to sieve the sample through the pores, thus allowing molecules of higher molecular weight compounds to be eluted in earlier fractions as compared to the lower molecular weights. In Gel Filtration (GF) size exclusion columns, the aqueous mobile phase is passed over a hydrophilic resin (e.g. silica gel) packed in a glass column. In Gel Permeation (GP) columns, the organic mobile phase is eluted through a hydrophobic column bed (e.g. polystyrene divinylbenzene) (Weston & Brown, 1998). Figure 3.1 represents the basic principle of a size exclusion chromatography.

A. Sample Loading   B. Sample Sieving   C. Elution HMW   D. Elution LMW
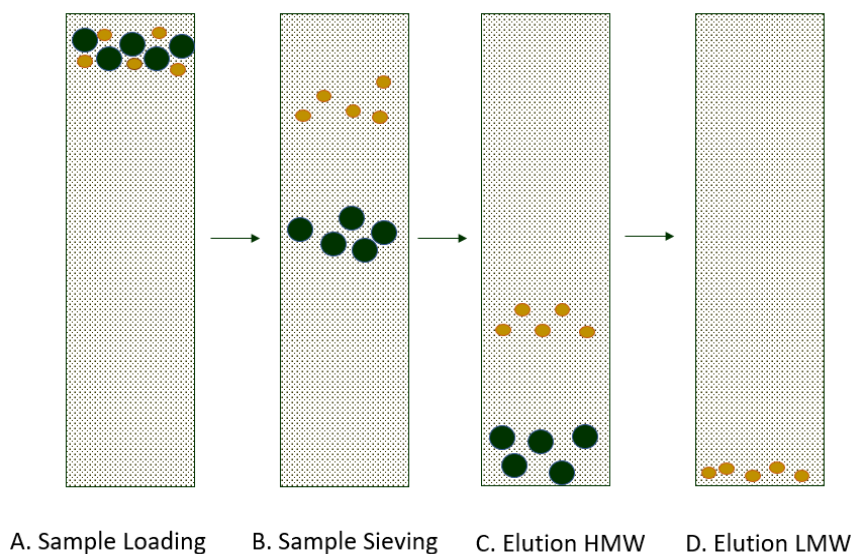
Figure. 3.1. Illustration of Size Exclusion Chromatography's principle. In this technique, the sample is separated based on molecular sizes; after loading the sample, the interaction with the resin occurs in such a way that higher molecular weight compounds (HMW) are eluted first as compared to the lower molecular weight compounds (LMW).

Ion exchange chromatography separates the molecules based on the net charge present on the molecule. Like size exclusion column, ion exchange is also performed in a glass column packed with solid ion-exchange resin. A typical anion exchange column is used for the purification of biomolecules; it has a positively charged resin (cations) packed in the glass container. The principle is to allow the resin to bind and retain the anions from the sample which is later eluted with the help of a salt or a pH gradient. Figure 3.2 demonstrates the basic principle of an anion exchange chromatography.

Ion-exchange chromatography
(anion exchange)

Elution With NaCl

A. Mushroom Sample Loading

B. Adhesion of negatively charged particles from sample on solid cation surface

C. Elution of mushroom bioactive after competitive binding of anion from salt with the resin.
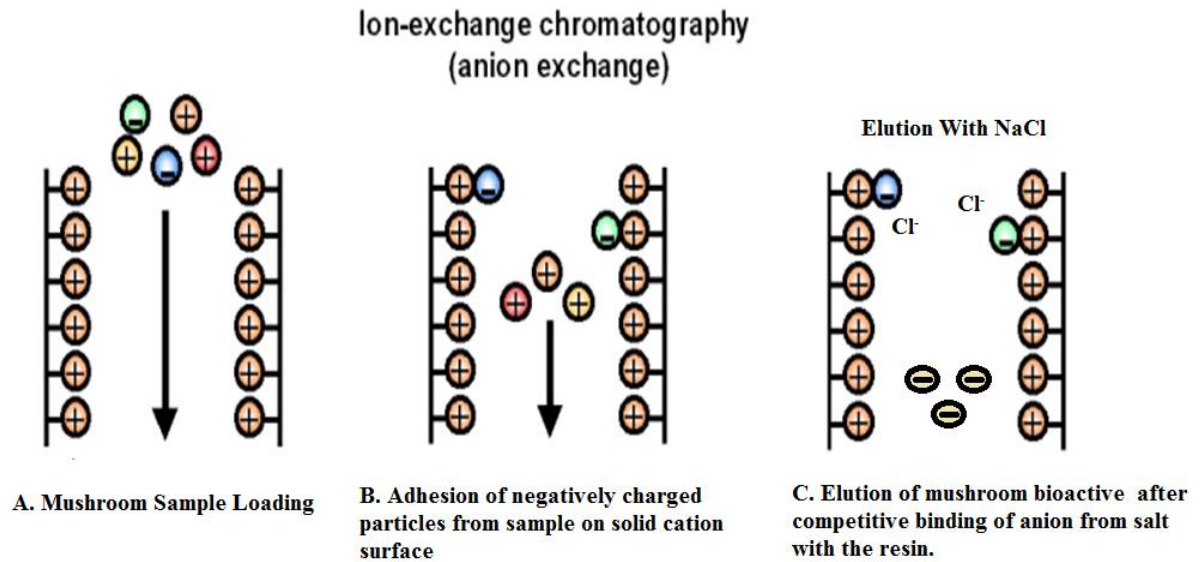
Figure. 3.2. Illustration of Ion Exchange Chromatography's principle. In this technique, the sample is separated based on molecular charges. DEAE Sephadex® A-50 resin is an anion exchange resin (i.e. the resin has positively charged cations that binds the negatively-charged active moiety from sample and allow the impurities to pass through). The sample is later eluted with the help of a salt or a pH gradient (modified from Blaber & Harvey, 2016).

This chapter describes the steps used to attempt the purification of anti-inflammatory compound(s) from the 5% NaOH extracts of *E. tinctorium*; both size exclusion and ion exchange chromatography were used in the purification step.

3.1.2   *Echinodontium tinctorium*

Echinodontium tinctorium (toothed conk), the Indian paint fungus, is a wood decaying fungus found only in western North America. It owes its common name to its distinctive red color that was used by Native Americans in preparation of war and body paints (Arora 1986; Gibson 2014). It also has a history of use as anti-bacterial agent by native Americans (Ye et al., 1996). Information is limited regarding its potential bioactivity, as most studies pertaining to the fungus relate to the taxonomy and host-fungus relationships (Aho et al., 1987; Gross, 1964). Because of its native origin from North America and lack of study for therapeutic purposes, the

5% NaOH extract of *E. tinctorium* which contains strong anti-inflammatory activity, was selected for purification studies.

3.2 Materials & Methods

3.2.1 Bulk Extraction & Confirmation of Activity

The fresh sample of *E. tinctorium* was collected in August 20, 2015 by Drs. Chow Lee, Hugues Massicotte, and Maggie Li from hemlock trees in Twin Falls Trail, Smithers. The sample was identified using the same protocol as described earlier (refer 2.2.1-chapter 2). Then parts of the conk were powdered using a hammer mill, and bulk extraction of *E. tinctorium* was performed manually according to the protocol described earlier (refer 2.2.2 and figure 2.3 in chapter 2). The first two extractions (i.e. 80% ethanol and 50% methanol) were performed by Mr. Faran Rashid, an undergraduate student in Dr. Lee's lab. For the current study, the extraction began from the residue of Fraction 2 (i.e. 50% methanol) and was processed for water and 5% NaOH extractions. In short, the dried mushroom powder was added in the respective solvent, using 1.5 L Erlenmeyer flask, in a ratio of 10 mL/g with constant stirring. After each extraction, the filtrate was vacuum filtered, pH neutralized, concentrated and lyophilized. The residue was then used for the next extraction step, the 5% NaOH extraction. The NaOH extracts were then used for further biological cell assays and purification studies.

5% NaOH extracts from new batches of *E. tinctorium* were also tested on RAW 264.7 macrophage cells for the reproducibility of the activity. The protocol used was previously described (refer section 2.6.1.1-chapter 2), and the anti-inflammatory activity was then quantified using the ELISA protocol also as described earlier (refer section 2.2.4-chapter 2).

3.2.2 Size Exclusion Chromatography- Sephadex™ LH-20 Column
3.2.2.1 Size Exclusion Column Sizes

Sephadex™ LH-20 (GE Healthcare, Quebec) resin was used as the stationary phase for all size exclusion chromatography experiments. Initially, the small-scale column was set up at 22 mL, and later it was scaled up to 56 mL and finally to 450 mL. The 22 mL column was a gravity drip column made from a 25 mL serological pipette (Sarstedt, Nümbrecht, Germany). In order to get higher yield of the semi-purified sample, the 22 mL column was increased to 56 mL using a C16/70 column (GE Healthcare, Quebec), which was finally scaled up to 450 mL using a larger C26/100 column (GE Healthcare, Quebec). Both the 56 mL and 450 mL columns were operated with the help of a solvent pump maintained at a flow rate of 1 mL/min. A three-way stopper allowed the smooth switch between the solvent system and the loading sample. The fractions were collected via an automated fraction collector.

3.2.2.2 Column Set-up

For the size exclusion column, Sephadex™ LH-20 (GE Healthcare, Quebec) resin was soaked in degassed water for 3-5 hours at room temperature. For each gram of dry resin, 4 mL water was required for the resin to swell. After the resin was swollen, the excess water was discarded so that the final slurry to water ratio was 75%-80%. To make a 22 mL sized column, wet glass wool was introduced at the bottom of the serological pipette before pouring the resin. The resin was then packed swiftly in the column to avoid air bubbles. For the 56 mL and 450 mL columns, after pouring the resin, the adapter was loaded and the column was packed at flow rate of 2 mL/min. All columns were equilibrated with water in start for around 2-4 bed volumes of the column size.

3.2.2.3 Sample Preparation, Loading and Fractions collection

For a 22 mL column, the loading sample was prepared by dissolving 20 mg/mL of 5%

NaOH extract of *E. tinctorium* filtered via a 0.2 μm filter (Sarstedt, Nümbrecht, Germany). For

larger scale columns, 70 mg/mL sample was prepared. The sample was centrifuged at 100 x g for

5 minutes prior to loading onto the column. In each scenario, 2% (i.e. 400 μL for 22 mL, 1120

uL for 56 mL & 9 mL for 450 mL column) of this sample was carefully loaded onto the column

using a Pasteur pipette (22 mL) and a sample injector (larger columns). After loading, the

samples were collected in 20 fractions (1 mL each) were collected for 22 mL size column, 35

fractions (2 mL each) for 56 ml size column and 45 fractions (10 mL each) for 450 mL size

column using manual (22 mL) and automated fractions collector (larger columns). The fractions

were stored at 4ºC until I was ready to assess them on Raw 264.7 macrophage cell lines for

biological activity.

3.2.2.4 Potency Estimation (Crude F4 versus Post Sephadex™ LH-20)

After testing and eventually pooling fractions from the larger Sephadex™ LH-20 column,

a dose-dependent assay was performed to determine the potency of the Crude sample (c) vs. Post

Sephadex™ LH-20 sample (LH). Various doses (1 ug/uL, 0.5 ug/uL, 0.25 ug/uL and 0.1 ug/μL)

were assessed for their ability to inhibit LPS -induced TNF-α production.

3.2.2.5 Sephadex™ LH-20 Column Clean-up & Regeneration

After each use, the column was washed with 2-bed volumes of water, followed by 1-bed

volume of 0.1 M NaOH, and again by 2-bed volumes of water, at a flow rate of 1 mL/min. This

protocol ensured that the column was reusable and cross contamination avoided.

3.2.3   Carbohydrate & Protein Assay

Post Sephadex™ LH-20 fractions (i.e. fractions collected from Sephadex™ LH-20) from

the 56 mL column were tested for their carbohydrate and protein content using commercially

available Total Carbohydrate Assay Kit (Sigma-Aldrich®, St. Louis, MO, USA) and Pierce™

BCA Protein Assay Kit (Thermo Scientific®, Waltham, MA, USA) respectively.

For carbohydrate estimation, the protocol followed the instructions of the manufacturer. Briefly, both Post Sephadex™ LH-20 test fractions and standards (30 uL each) were analyzed in a 96-well plate. These fractions were tested against the already provided standard concentrations of glucose at 0, 4, 8, 12, 16, 18 and 20 mg/mL. After adding the required volume of sample and standards, 150 uL of concentrated sulphuric acid (12 M) was added in each well and incubated for 15 minutes at 90°C in Haratherm™ General Oven (Thermo Fischer, Waltham, MA, USA). After 15 minutes, 30 μL of the developer solution was added to each well to complete the reaction. The plate was mixed for 5 minutes using a horizontal shaker and read at 490 nm using SYNERGY 2 (BioTek®, VT, USA).

For protein estimation, the protocol followed the instructions of the manufacturer. In-short, both the Post Sephadex™ LH-20 test fractions and standards (25 uL each) were added in a 96-well plate. Post LH-20 fractions were tested against the already provided standard concentrations of albumin at 0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 μg/mL. Later, 200 uL of the working reagent was added in each well and the plate was shaken for 5 minutes. After shaking, the plate was incubated at 37ºC for 30 minutes and read at 562 nm using SYNERGY 2 (BioTek®, VT, USA).

3.2.4   Ion-Exchange Chromatography

The post Sephadex™ LH-20 fractions which exhibited anti-inflammatory activity were pooled and subjected to a second step purification using ion exchange chromatography. DEAE Sephadex™ A-50 (GE Healthcare, Quebec), a weak anion exchange resin, was used. For initial trials, a variety of DEAE buffers and pH conditions were used in order to determine the optimum

conditions for the specific purification. The detailed protocol was followed as indicated by the manufacturer. Briefly, the DEAE Sephadex™ was allowed to swell in respective buffer (tabulated in Table 3.1) for at least 48 hours. Initially, Poly-Prep® chromatography columns (max. 12 mL) were used. The columns were loaded with 9 ml slurry (75% resin:25% buffer) for an initial bed volume and then equilibrated with 3-5 bed volumes of respective buffers. After equilibration, 500 uL of sample (i.e. Post Sephadex LH-20 of 5% NaOH *E. tinctorium*) was loaded-10 mg/mL).

The column was then subjected to three bed volumes of the initial buffer to collect "flow-through" followed by three bed volumes of the same buffer containing 1 M NaCl to collect "eluents". Both the flow through and eluent were collected manually and lyophilized using a FREEZONE freeze dryer (Labconco, MO, USA) at -52°C and 0.02 Torr, for 24 hours. The freeze-dried samples were re-constituted in water and then dialyzed using Slide-A-Lyzer™ dialysis cassettes (Thermo Fischer Scientific, MA, USA) with molecular size cut-off (MWCO) of 2000 Da to remove the salt. The post-dialyzed fractions were again subjected to lyophilization followed by filter sterilization using 0.2 μm filters (Sarstedt, Nümbrecht, Germany) and stored at 4°C until bioassays were performed.

Table 3.1: Buffers with respective pH and concentration used in Ion-Exchange Chromatography

S.no	Buffer	pH Range	pH Used in Initial Screening	Concentration
1.	Methyl-piperazine	4.3-5.3	4.5	20 mM
2.	Piperazine	4.8-5.8	5.5	20 mM
3.	L-Histidine	5.5-6.5	6.1	20 mM
4.	TRIS	7.6-8.6	7.7	20 mM
5.	Diethanolamine	8.4-9.4	8.7	20 mM

After the initial trials, the optimum buffer (i.e. Piperazine) and pH conditions (i.e. pH= 5.5) were selected for scaling up the ion-exchange column. A large scale XK-50 Column (GE Healthcare) was used to increase the column setup to 1000 mL. Eighteen grams of DEAE Sephadex® A-50 was swollen in about 1 L of 20 mM Piperazine buffer (pH= 5.5). The same buffer was used as flow through and with added 1 M NaCl used for eluting the column.

The eluent was collected to two bed volumes (i.e. around 2000 mL), lyophilized, re-constituted in water, dialyzed, re-lyophilized and re-constituted in water for the cell assay. The anti-inflammatory assay was performed in a similar way to evaluate the reproducibility of the activity (refer section 2.6.1.1 and 2.2.4, chapter 2). After the biological assay, this purified sample was then assessed for carbohydrate and protein content (along with crude and semi-purified sample) and was then compared to the respective carbohydrate and protein content in Polysaccharide-K (PSK) as a reference sample (3.2.3-Chapter 3). The two-step purified sample was sent to Dr. Peter Cheung's lab at the Chinese University of Hong Kong for further chemical characterization using GC-MS. The GC-MS analysis was performed by Dr. Maggie Li. The percent content of carbohydrate was calculated by the following formula: (amount of carbohydrate in mg/mL / stock concentration in mg/mL x 100). The same formula was used for percent protein estimation.

3.2.5   Characterization of Carbohydrate Content Using GC-MS

For the characterization of carbohydrate content, GC-MS technique was applied in Dr. Peter Cheung's lab (Hong-Kong) by Dr. Maggie Li. After the evaluation of monosaccharide content in the purified *E. tinctorium* sample, further experiments were performed for the estimation of the respective linkages present in the purer sample.

3.2.6   Heat Denaturation Experiment

To assess whether proteins are important in the anti-inflammatory activity, both semi purified (Sephadex™ Post LH-20) and purified (Sephadex™ Post LH-20+Post DEAE) samples were subjected to heat denaturation. Both samples were heated to 100ºC for 5 minutes and then were evaluated on Raw 264.7 cells for anti-inflammatory activity as previously described (refer to section 2.6.1.1 and 2.2.4, Chapter 2).

3.2.7   Anti-inflammatory activity of purified samples from *E. tinctorium* in comparison with Acetaminophen

For comparing the potency of the crude, semi purified (Post Sephadex™ LH-20), and purified (Post Sephadex™ LH-20+Post DEAE) moieties, all samples were evaluated in a dose dependent manner (0.1, 0.25, 0.5, 1 µg/mL) in RAW 264.7 cell lines and compared with the respective dose of acetaminophen as a reference (refer section 2.6.1.1 and 2.2.4, Chapter 2).

3.2.8   Fast protein liquid chromatography (FPLC) Superdex™ 200- *Ä*KTA Pure

After getting the separation based on sizes (Sephadex™ LH-20) and charges (Sephadex™ DEAE), the semi-purified (Post Sephadex™ LH-20) and purified (Post Sephadex™ LH-20 + Post Sephadex™ DEAE) samples were subjected to FPLC analysis via Superdex™ 200 (GE Healthcare, Quebec) for a higher resolution separation and an estimation of the size of the bio-active compound(s). This column was connected with an *Ä*KTA pure chromatographic purification system (GE Healthcare, Quebec). *Ä*KTA pure purification is a systemic combination of UV multiple wavelength detection, UV fixed wavelength detection, conductivity monitoring and automated sample collector. For running the samples, 20 mg/mL of the stock solution was prepared and 2% of a 20 mL column (i.e. 500 uL) was loaded onto the column with a flowrate of 1 mL/min. Twenty fractions were collected with a volume of 1 mL

each. These fractions were then subjected to anti-inflammatory assay, protein and carbohydrate analysis (Refer section 2.6.1.1 and 2.2.4, Chapter 2 and Section 3.2.3-Chapter 3).

3.2.9   Nitrite Determination Assay

As nitric oxide (NO) is a short lived chemical in *in vitro* settings, for the estimation of inhibition of NO, the levels of nitrite were measured as representative of NO. It is important to point out that this assay absolutely requires the use of Raw macrophage cells that have not been passaged (divided) extensively. The same numbers of RAW 264.7 cells (i.e., 100,000 cells/well) as described previously were plated. After an overnight incubation, cells were treated with: DMEM without FBS (control), 1 µg of LPS (negative Control), 1 µg of LPS + PMB (100 units, positive control), 1 µg of LPS + Sephadex Post LH-20 + Post DEAE (1 mg, test sample). After treatment, cells were incubated for 24 hours. After 24 hours, supernatants were collected and tested using Promega^{TM} Griess Reagent System. The protocol was followed as mentioned by the supplier with a little modification. Briefly, NO standards were prepared via serial dilution from stock (0.1 M Sodium Nitrite). One hundred µL of samples were added in each well followed by 50 µL of Sulphanilamide solution and 50 µL of N-1-napthylethylenediamine dihydrochloride (NED) solution. The plates were read at 540 nm and the amount of NO was quantified using by interpolating the serial dilution of nitrite (NaNO$_2$) levels using PRISM. The experiment was repeated twice with triplicate wells each time.

3.3 Results

3.3.1   Bulk Extraction & Confirmation of Anti-inflammatory Activity

A new batch of *E. tinctorium* was collected from Twin Falls Trail, Smithers in August 2015. The conk was subjected to grinding and the first 2 steps of the extraction (80% ethanol and 50% methanol) were performed by Mr. Faran Rashid, a former undergraduate student in Dr. Lee's lab. Three hundred grams of initial sample was extracted as mentioned previously (refer to

section 2.2.2- Chapter 2). The 80% ethanol extract was discarded while the 50% Methanol fraction was used by the undergraduate student for his research project. The residue of Fraction 2 was subjected to sequential extraction with water (F3) and 5% NaOH (F4). Note that at this stage, the ammonium oxalate extraction that was performed in preliminary screening in the initial phase of the project was omitted, and F4 from this point forward represents the 5% NaOH extract. The filtrate of 5% NaOH was concentrated, pH neutralized, lyophilized and stored in 4ºC until cell assays were performed. The activity of this new batch was confirmed (as shown in Figure 3.3).

Figure 3.3 shows that the new batch 5% NaOH (F4) demonstrated an activity similar to the old batch of 5% NaOH (F5), indicating the potency and reproducibility of the anti-inflammatory activity of this extract.
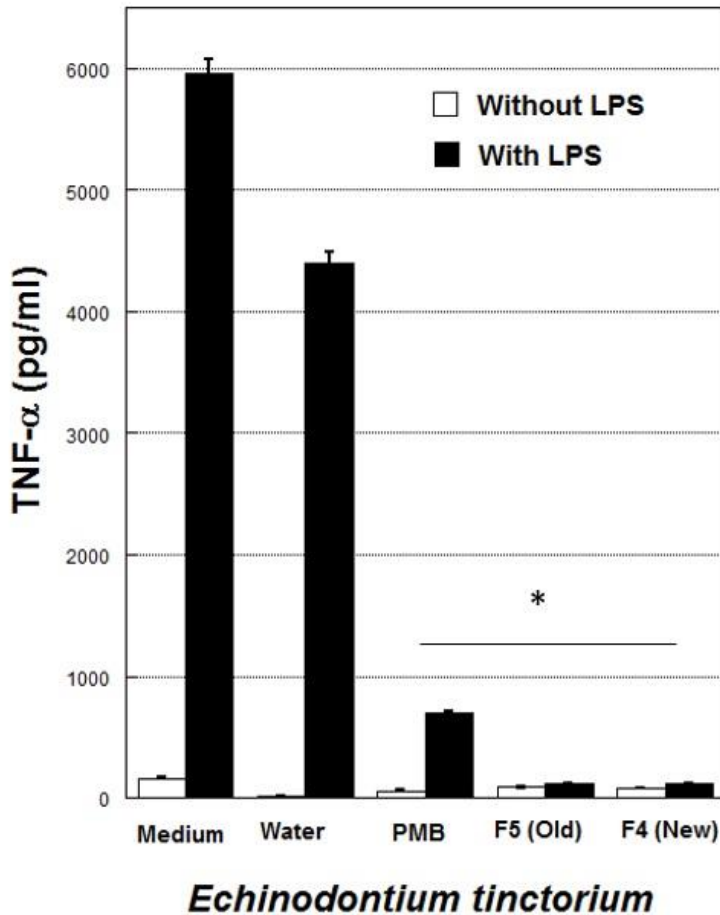
Figure 3.3. Anti-inflammatory activity of NaOH extracts from *E. tinctorium*. Assessing anti-inflammatory activity of two different batches of NaOH extracts of *E. tinctorium.* The newly extracted batch fraction (F4) and the older batch (F5) of NaOH extracts of *E. tinctorium* was assessed for their ability to inhibit LPS (250 ng/mL)-induced TNF-α production in RAW 264.7 macrophage cells. The concentration of NaOH extracts from *E. tinctorium* used was 1 μg/1μL. PMB (100 units) was used as the positive control and solvents and media were used as negative controls. The assay was performed in triplicates. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).

3.3.2 Size Exclusion Chromatography- Sephadex™ LH-20 Column

After confirming the reproducibility of the activity, the F4 NaOH extract was subjected to

a 22 ml Sephadex™ LH-20 column: 400 uL of 20 mg/mL sample was loaded and 20 fractions (1

mL each) were collected for further analysis. Figure 3.4 illustrates the results from the 22 ml

manual column: it demonstrates that fractions 7-10 and fraction 15 had strong anti-inflammatory

activity as these inhibited the LPS induced TNF-α ≥90%.
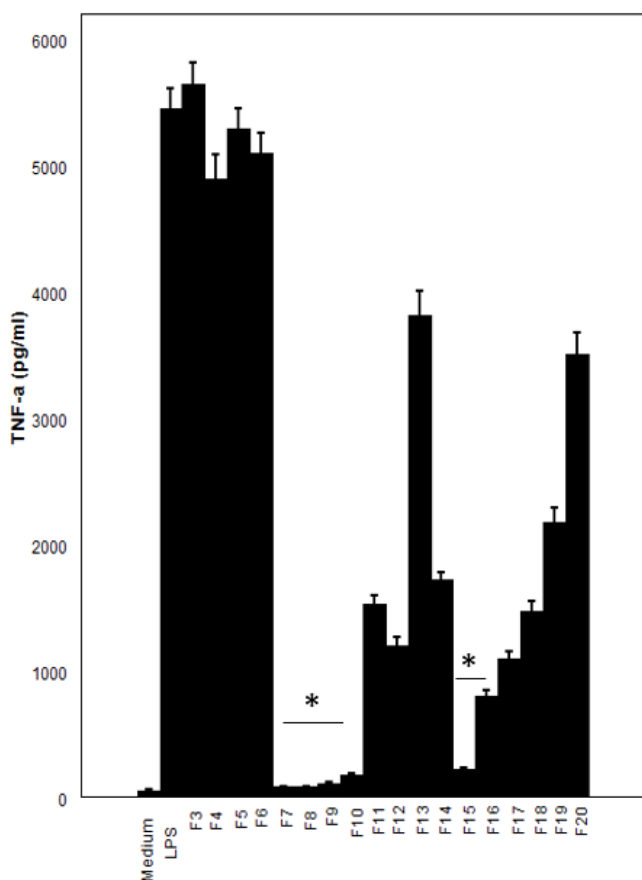
Figure 3.4. Assessing anti-inflammatory activity of fractions collected from 22-ml size Sephadex™ LH-20 column. The loading sample was 400 uL of NaOH extract from *E. tinctorium* (20 mg/mL) and 1 mL fractions were collected. One hundred uL from each fraction was tested for activity. LPS treatment alone was used as a negative control. ≥90% inhibition of LPS was a positive activity. The assay was performed in triplicates. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).

After the anti-inflammatory activity was detected in fractions collected from the 22 mL

size Sephadex™ LH-20 column, the column was scaled up to a 56 ml using a C 16/70 (GE

Healthcare, Quebec) column. Crude F4 NaOH extracts (70 mg/mL) at 1120 uL was loaded and

35 fractions of 2 mL each were collected and then assessed for anti-inflammatory activity. Figure

3.5 shows the results from a 56 ml Sephadex™ LH-20 column: it demonstrates that fractions 10-

20 inhibited the LPS-induced TNF-α production by ≥90%. These fractions were considered

active and thus indicated the reproducibility of Sephadex™ LH-20 column at the higher scale.
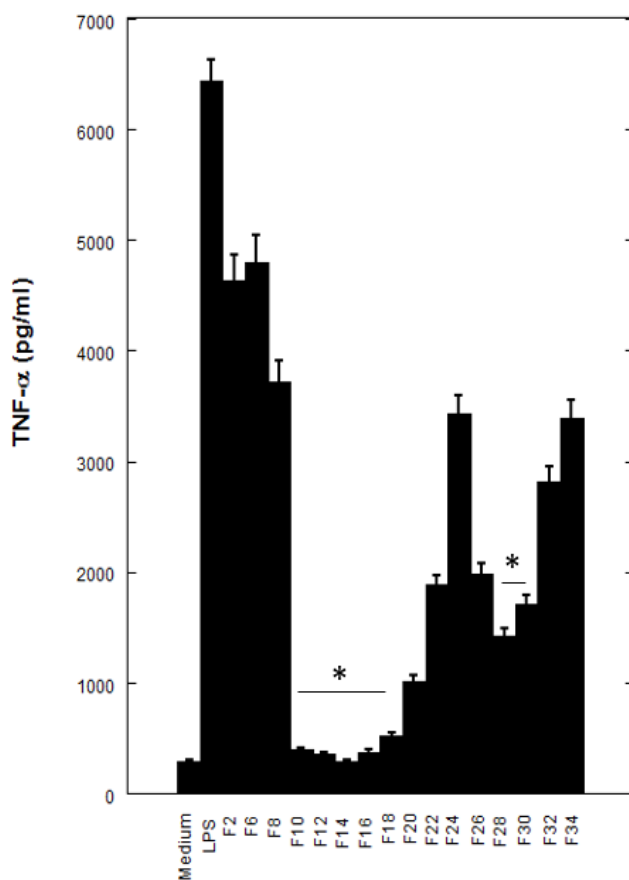
Figure 3.5. Assessing anti-inflammatory activity of fractions collected from 56 mL size Sephadex™ LH-20 column. The loading sample was 1120 uL at 70 mg/mL. Thirty five fractions of 2 mL each were collected and alternate fractions were tested. LPS was used as a negative control. ≥90% inhibition of LPS was a positive activity. The assay was performed in triplicates. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).

After reproducible results were obtained from 56 mL Sephadex™ LH-20 column, the protocol was finally scaled up to a 450 ml using C 26/100 (GE Healthcare, Quebec) column. Nine mL of 70 mg/mL crude F4 NaOH extracts were loaded and 45 fractions of 10 mL each were collected and assessed for anti-inflammatory activity. Figure. 3.6 shows that fractions 16-24 inhibited the LPS-induced TNF-α production at ≥90% which correlates with the activity obtained from the 56 mL Sephadex™ LH-20 column. These fractions are considered active and further testify to the reproducibility of the Sephadex™ LH-20 column at the higher scale.
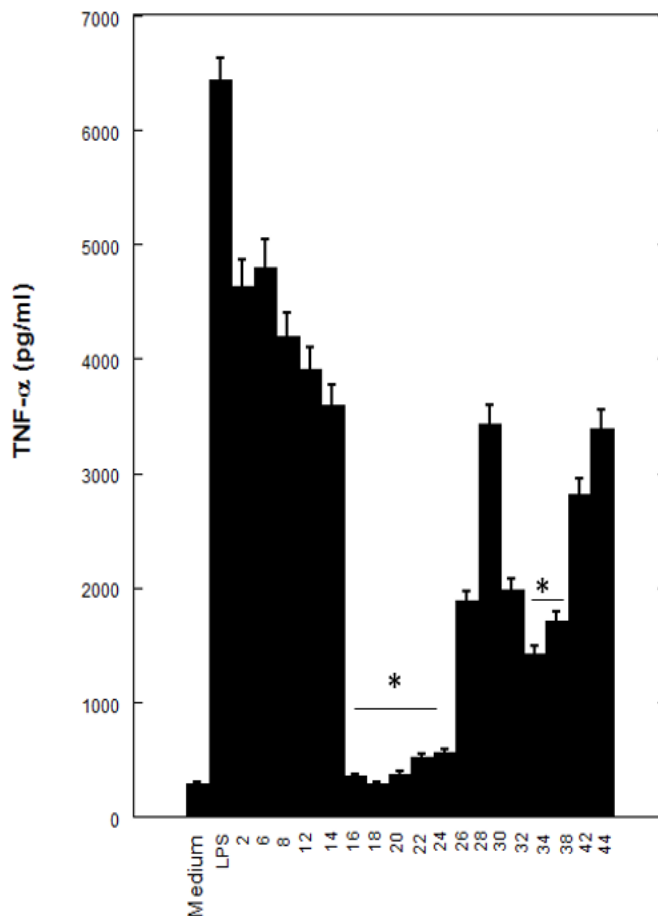
Figure 3.6. Assessing anti-inflammatory activity of fractions collected from a 450 mL Sephadex™ LH-20 column. The loading sample was 9 mL at 70 mg/mL and 45 fractions of 10 mL each were collected. Alternate fractions were tested for activity. LPS was used as negative control. ≥90% inhibition of LPS was a positive activity. The assay was performed in triplicates. Error bars were standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).

Following establishement of the reproducibility of the 450 mL size Sephadex™ LH-20

column, another 9 runs were performed at this scale in to generate sufficient quantity for the

second purification step. After each run, the active initial elution volume fractions were pooled

and lyophilized. Subsequently, the potency of this semi-purified, Post Sephadex™ LH-20

samples, was determined. This was achieved by performing and comparing the dose-dependent

effect of the crude sample vs. the semi-purified sample from the size exclusion chromatography.

Figure 3.7 shows that the post LH-20 sample has higher potency as compared to the crude

sample. The sample was not only more potent at 0.2-1 µg/µL but it appeared to be still active at

and below 0.1 µg/µL. These results demonstrated the efficiency of Sephadex™ LH-20 in

purifying the anti-inflammatory compound(s) from *E. tinctorium*.
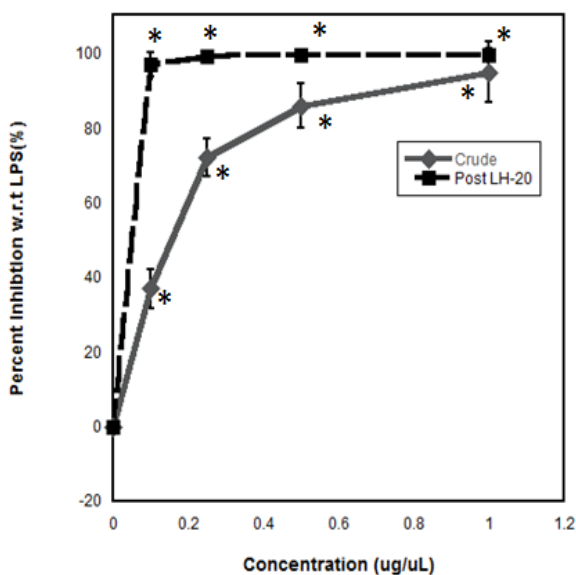
Figure. 3.7. Comparing the anti-inflammatory activity of the crude F4 NaOH extract with the semi-purified post-Sephadex LH20 sample. The following concentrations of crude NaOH extracts and post-Sephadex LH20 sample were used: 0.1, 0.25, 0.5, and 1 ug/uL. Results were plotted as percent of TNF-α inhibition relative to LPS treatment alone. Assay was performed in triplicates. Error bars are standard deviation from mean and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).

3.3.3 Carbohydrate & Protein Assay

In addition to assessing the anti-inflammatory activity, the fractions collected from 56 mL

Sephadex™ LH-20, using the C 16/70 column, were assessed for the possible presence of

carbohydrate. This was performed using the Total Carbohydrate Assay Kit (Sigma-Aldrich®, St.

Louis, MO, USA). The results in Figure 3.8 show that there are two major anti-inflammatory

activities: one at an elution volume 20-35 mL and one at around 55 mL. Both activities appear to

correlate with the carbohydrate content, suggesting the bio-active compound(s) contains

carbohydrate.
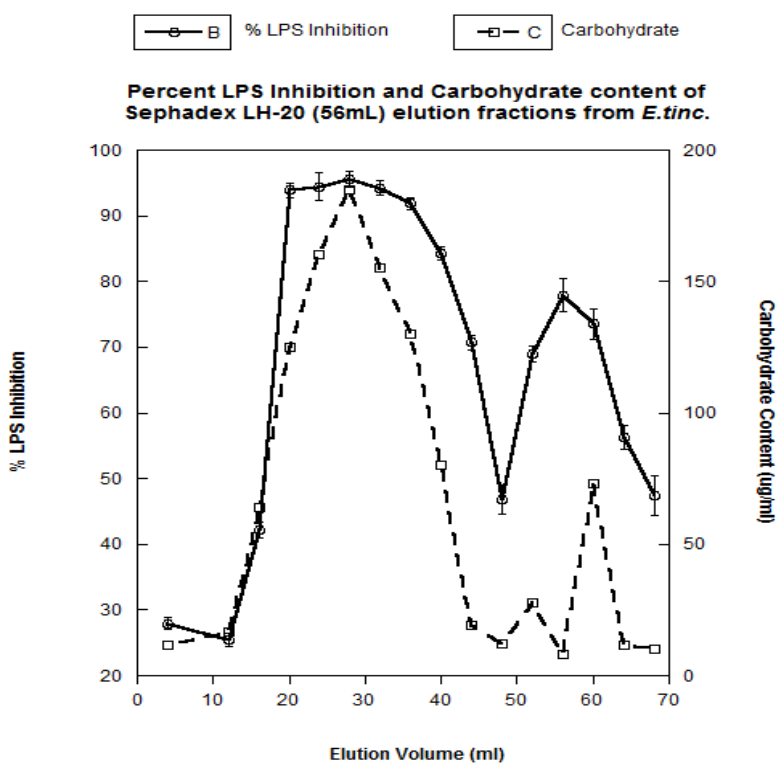
Figure 3.8. Carbohydrate Content-Post Sephadex™ LH-20 (56mL). Anti-inflammatory activity and carbohydrate content of fractions, collected from a 56 mL size Sephadex™ LH-20 C 16/70 column. Results show a positive correlation between the carbohydrate content and the bio-activity. Assay was performed in triplicates and error bars represented standard deviation from the mean.

The post LH-20 fractions were also assessed for the possible presence of protein. This was performed using a Pierce™ BCA Protein Assay Kit (Thermo Scientific®, Waltham, MA, USA). The results in Figure 3.9 show that the anti-inflammatory activity at elution volume 20-35 mL correlates with protein content, suggesting the presence of protein in the bio-active compound. In contrast, the anti-inflammatory activity at elution volume 55 mL did not appear to correlate with the protein content, suggesting that this anti-inflammatory compound may not contain protein.
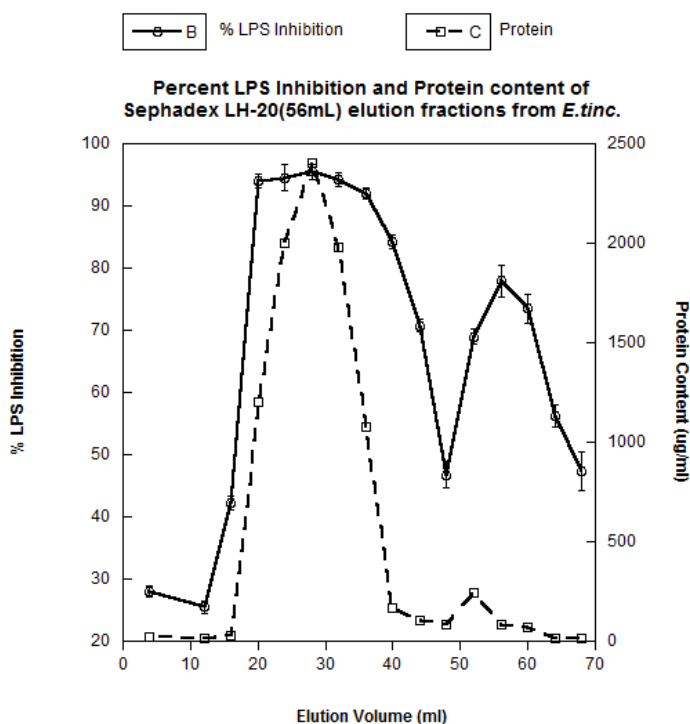
Figure 3.9. Protein Content-Post Sephadex™ LH-20 (56mL). Anti-inflammatory activity and total protein content of fractions collected from a 56 mL size Sephadex™ LH-20 C 16/70 column. Results show a positive correlation between the protein content and the earlier eluted bio-activity. Assay was performed in triplicates and error bars represents standard deviation from the mean.

3.3.4 Ion-Exchange Chromatography

DEAE Sephadex™ A-50 (GE Healthcare, Quebec) was used as a weak anion exchanger resin. The initial trials involved 5 buffers (Methyl-piperazine, piperazine, L-Histidine, TRIS & Diethanolamine) and 5 different pH conditions (4.5, 5.5, 6.1, 7.7 & 8.7) for the selection of the optimum condition(s) for use as the second purification step of the bioactive molecule). Figures 3.10 and 3.11 illustrate the results from the mini Poly-Prep® ion-exchange column. "Flow-through" represents the sample when subjected to the same buffer, and "eluent" represents the sample eluted using the same buffer containing 1 M NaCl. All the collected flow-through and eluent were lyophilized, re-constituted, dialyzed, and re-lyophilized. The post-lyophilized sample was re-constituted in water so that the final concentration for the cell treatment was 1 µg/µL. For buffers (methyl-piperazine, pH=4.5 and piperazine, pH=5.5), there was not enough sample for the flow-through test, so only the eluents were tested. As shown by Figures 3.10 and 3.11, all the buffer conditions applied showed bioactivity in eluents, indicating that ion-exchange column was successful in retaining the bio-active compound and consequently eluting in out, in the presence of salt. The results, however, indicate that piperazine at pH=5.5 yielded the most potent activity (i.e. >98%). Based on this observation, the piperazine buffer at pH 5.5 was chosen as the buffer for use in the ion-exchange column.
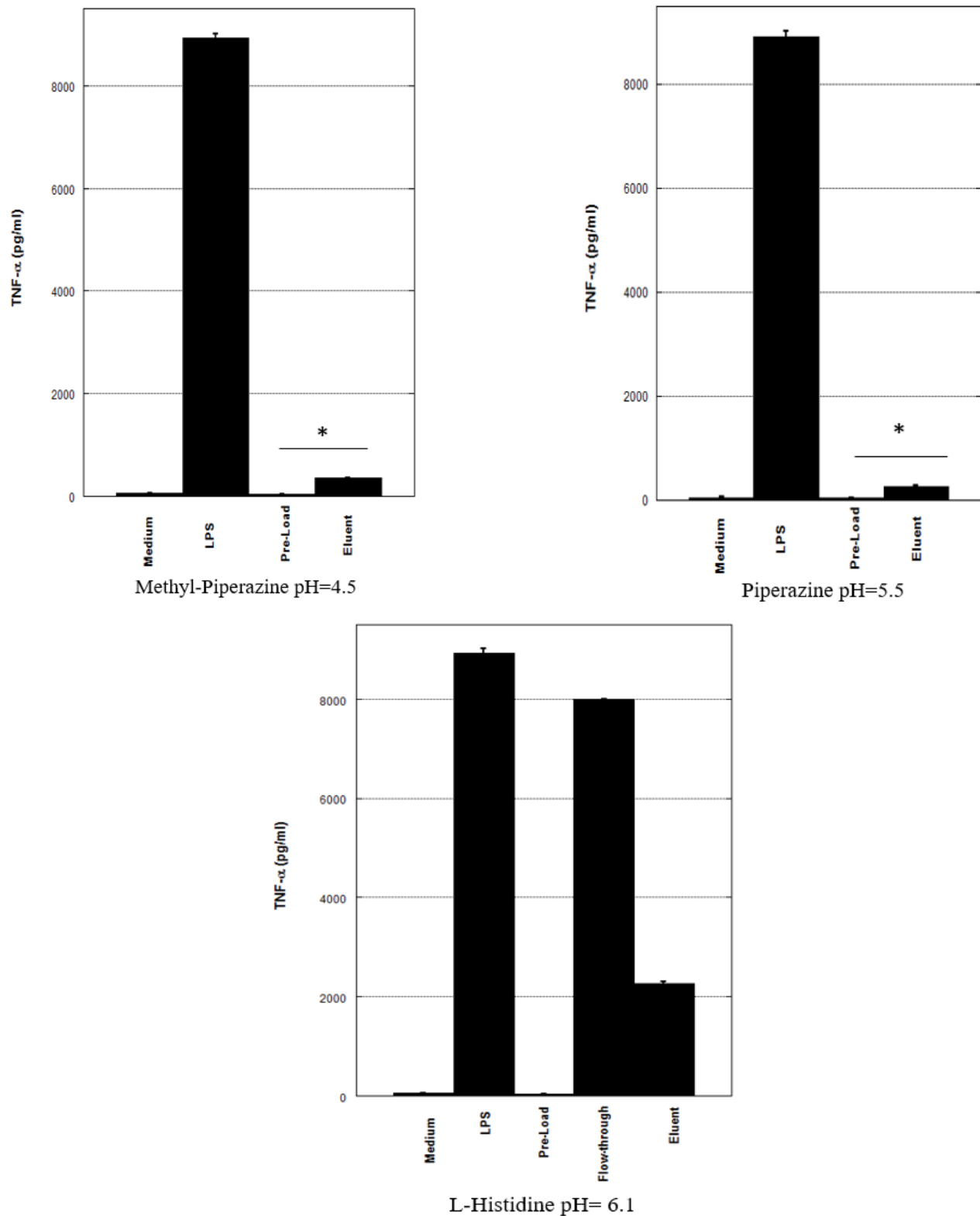
Figure.3.10 Assessing different pH buffers for possible use in purifying the anti-inflammatory compound(s) from *E. tinctorium*. Raw 264.7 cells were induced with LPS (250 ng/mL) in absence or presence of various buffers and pH conditions (Methyl-piperazine pH=4.5, Piperazine pH=5.5, and L. Histidine pH= 6.1). LPS was used as negative control. Pre-load (Post LH-20: 1 µg/µL) was used as the positive control. Assay is performed in triplicates. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).
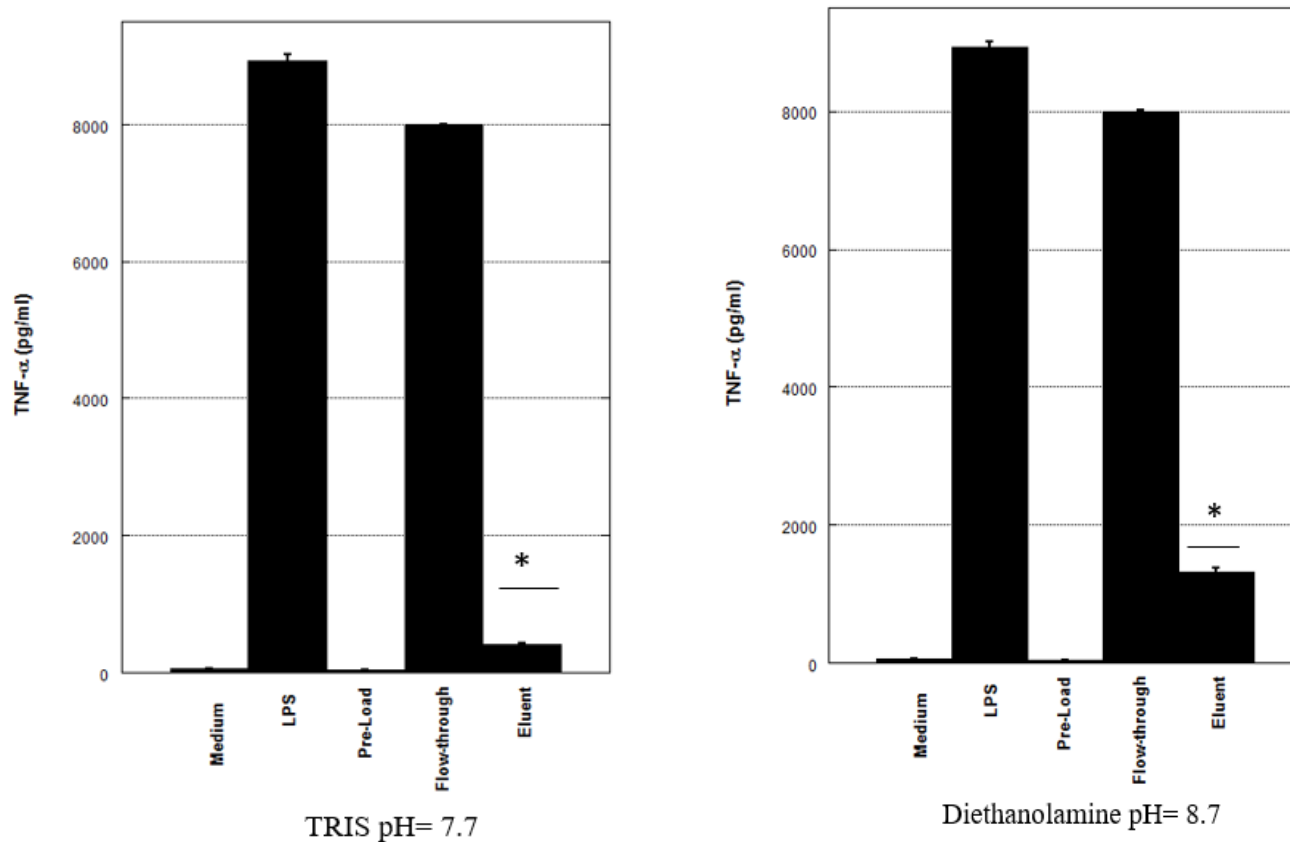
Figure.3.11. Assessing different pH buffers for possible use in purifying the anti-inflammatory compound(s) from *E. tinctorium*. Raw 264.7 cells were induced with LPS (250 ng/mL) in the presence or absence of various buffers and pH conditions (Tris pH=7.7, Diethanolamine pH=8.7). LPS was used as negative control. Pre-load (Post LH-20: 1 µg/µL) was used as positive control. Assay was performed in triplicates. Error bars were standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).

Based on the above results, I proceeded to use a large-scale ion exchange column (XK-50/100; DEAE Sephadex® A-50) to purify the anti-inflammatory compound (s). Figure 3.12 shows that indeed the salt eluted fractions from the large scale DEAE column still possess anti-inflammatory activity.
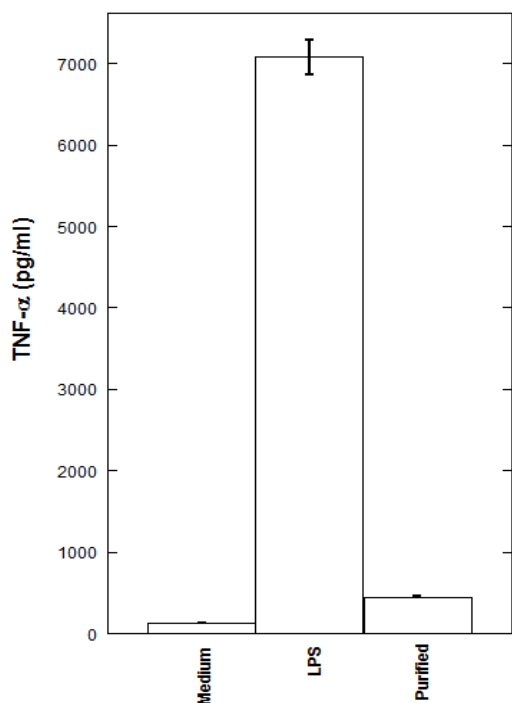
Figure 3.12. Confirmation of anti-inflammatory activity from DEAE-purified sample. An XK-50 1000 mL DEAE-Sephadex was used for purifying the bioactive compound collected from Sephadex LH20. Eluent collected after the column was washed with 1 M NaCl in Piperazine, pH5.5 was dialyzed and lyophilized. DEAE-Sephadex-purified sample (1 µg/µL) was assessed for anti-inflammatory activity in Raw 264.7 cells. LPS was used as a negative control. Assay was performed in triplicates. Error bars are standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).

After obtaining the relatively pure compound, the percentage of carbohydrate and protein content was estimated. Table 3.2 shows the respective carbohydrate and protein content in crude,

semi-purified and purified molecules. The content of carbohydrate and protein in Polysaccharide-K (PSK) was also determined for comparison. It can be seen that, as the degree of purification increased, the percentages of carbohydrate content increased while the protein content was more in semi-purified compound(s) as compared to the purer ones. The results also revealed that the purified anti-inflammatory compound(s) from *E. tinctorium* has less carbohydrate but more proteins as compared to PSK.

Table 3.2: Percentages of Carbohydrate and Protein Content in Crude and Purified Anti-Inflammatory Sample from *E. tinctorium*

Extract Characterization*	Carbohydrate Content (%)	Protein Content (%)
Crude (F4)	31	9.9
Semi-purified (Sephadex Post LH-20)	53	26.5
Purified (Sephadex Post LH-20 + Post DEAE)	76.5	21.3
Polysaccharide K (PSK)	87	12.4

*1 mg/ml of each sample was analyzed

This assay confirmed the presence of carbohydrate in the purified material. Next, the two-step (LH20 followed by DEAE) purified sample was shipped to Dr. Peter Cheung's lab at the Chinese University of Hong-Kong for detailed chemical characterization of the carbohydrate content using GC-MS that was performed by Dr. Maggie Li.

3.3.5 Chemical Characterization Using GC-MS

According to the preliminary GC-MS analysis on the carbohydrate content of the Sephadex Post LH-20 + Post DEAE purified sample, glucose is the major component. Other monosaccharide contents along with their percentages as determined by Dr. Maggie Li are summarized in Table 3.3.

Table 3.3 Monosaccharide type & percent content in purified sample-determined using GC-MS

S. No.	Monosaccharide	Percent Content
1.	Xylose	2.0%
2.	Fucose	2.4%
3.	Galactose	2.8%
4.	Mannose	3.1%
5.	Glucose	89.7%

Furthermore, the linkage analysis shows that the active moiety has 1,3-linked glucose, 1,6-linked glucose, 1,3-linked galactose and 1,3,6-linked glucose present within the complex.

3.3.6 Heat Denaturation

To assess if the protein present in the active sample was associated with bioactivity, both pure and relatively semi-pure samples (1 µg/uL) were subjected to heat treatment at 100ºC for 5 minutes. The approach is to denature the protein secondary structure at high temperature and correlate the change in anti-inflammatory activity with respect to the presence or absence of protein content in the compound. Figure 3.13 shows that the activity in the heat treatment experiment was retained (i.e. there was no change in the activity before and after heating). This indicates one of two possibilities: the proteins are denatured by heating and are not important for the biological activity, or the proteins are significant for the activity but destroying its secondary structure did not affect the activity as secondary structure of the protein may not be important in producing the bio-activity. With these two possibilities, the role of protein component with respect to the biological activity remained inconclusive and future experiments are needed to better answer this important question.
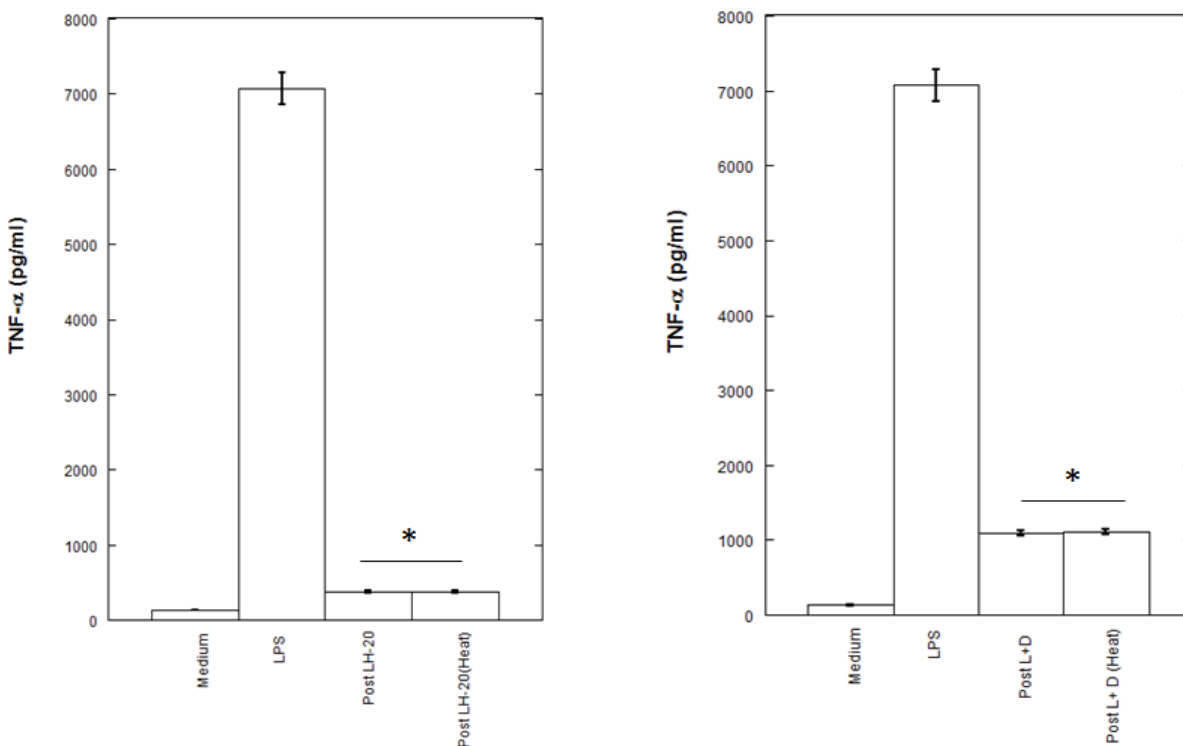
Figure 3.13. Heat denaturation had no effect on the anti-inflammatory activity of bio-active compound(s) isolated from *E. tinctorium*. Assessing anti-inflammatory potential of semi-purified (Post LH-20, left) and purified (Post LH-20 + DEAE, right) samples on LPS-induced RAW 264.7 macrophage cells. Assay was performed in triplicates. Error bars were standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).

3.3.7 Anti-inflammatory activity of purified samples from *E. tinctorium* in comparison with Acetaminophen

With respect to the potency of purified and semi-purified sample, it was important to compare the activity to a known anti-inflammatory agent (e.g. acetaminophen). The dose-dependent assay was performed to compare the relative potency (i.e. response per milligrams) of crude F4, post-Sephadex LH-20, post SephadexLH-20 + Post DEAE and acetaminophen. Figure 3.14 shows the dose-dependent response (i.e. 1-, 0.5 -, 0.25 -, and 0.1 µg/µL). It was observed that the acetaminophen inhibited 99% of LPS-induced TNF-α at 1 µg/µL. This response

decreased with the decrease in dose (i.e. 97% at 0.5, 77% at 0.25 and 0% at 0.1 µg/µL). Crude mushroom sample demonstrated a slightly lesser response at the initial dose as compared with acetaminophen (i.e. 95% at 1 µg/µL), the response also decreased with the decrease in dose but there was still some anti-inflammatory response at lowest dose in contrast to acetaminophen (i.e. 86% at 0.5, 72% at 0.25 and 37% at 0.1 µg/µL). The purified sample from *E.tinctorium* showed a similar response to the crude extract, also being active at the lowest dose (i.e. 89% at 1, 77% at 0.5, 62% at 0.25 and 35% at 0.1 µg/µL). The semi-purified sample (i.e. Sephadex^{TM} Post LH-20) exhibited the same response as acetaminophen at 1 µg/µL (i.e. 99.5% inhibition), and surprisingly, it showed a more potent response at lower dose as compared to other samples including the pure sample and acetaminophen (i.e. 99.5% at 0.5, 99.3% at 0.25 and 97% at 0.1 µg/µL). The run using Superdex200 column (see Section 3.3.8) helped clarify why the semi-purified sample is more potent than the more pure sample.
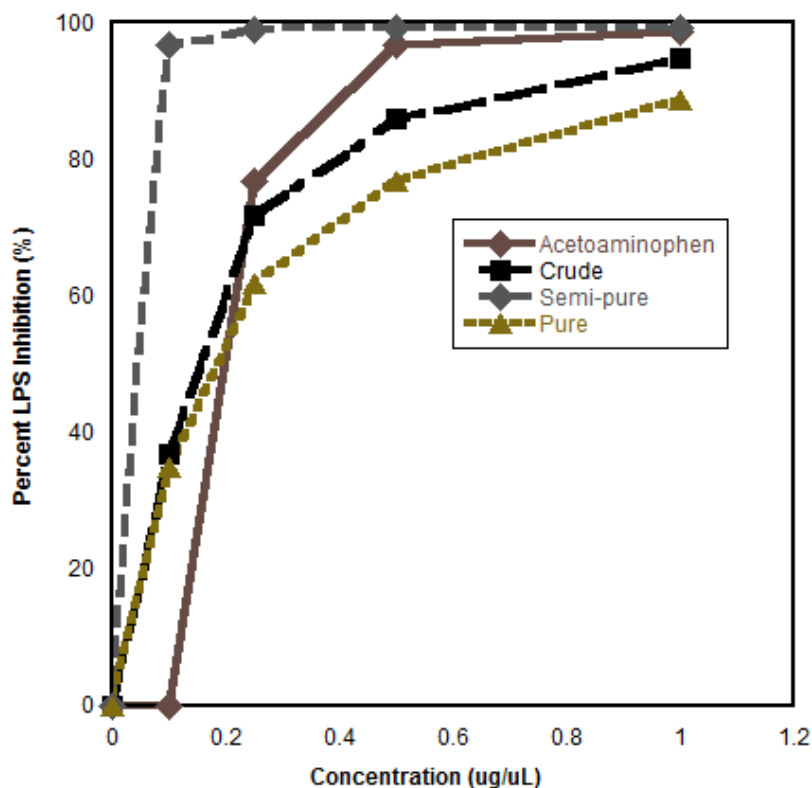
Figure 3.14. Assessing the anti-inflammatory potency of purified compound(s) from *E. tinctorium*. Dose-dependent anti-inflammatory activity on LPS (250 ng/mL)-induced RAW 264.7 macrophage cells. Acetaminophen, crude F4, Post Sephadex™ LH-20, and Post Sephadex™ LH-20 + DEAE were used at the following concentrations: 0.1, 0.25, 0.5, and 1 ug/uL. Results were plotted as percent of TNF-α inhibition relative to LPS treatment alone. Assay was performed in triplicate. Error bars are standard deviation from mean and One-way ANOVA analysis was performed as described in Materials and Methods (*, P < 0.0001).

3.3.7   Fast protein liquid chromatography (FPLC) Superdex™ 200- *Ä*KTA Pure

FPLC analysis was performed on both semi-purified and purified samples for improved resolution and for estimating the size of the bio-active compound(s). The Superdex200 column was run by Mr. Sebastian Mackedenski, a Research Associate in Dr. Lee's lab. Figures 3.15 and 3.16 show the approximate size ranges for the semi-purified (Sephadex™ Post LH-20) and the purified (Post Sephadex™ LH-20 + Post Sephadex™ DEAE) samples respectively when plotted against the elution volume and the percent LPS inhibition. Figure 3.15 shows two peaks of

activity; the earlier peak was relatively more potent than the later peak (i.e. it exhibited greater

percent inhibition as compared to the other one). The larger molecule peak is around 50-200 kDa

in size, while the smaller molecule peak is around 5-25 kDa in size.
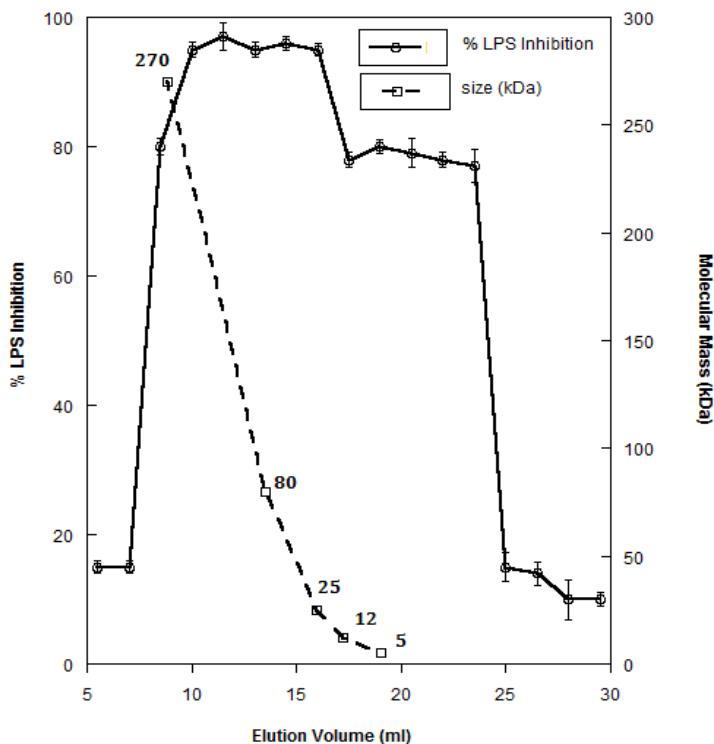
Figure 3.15. Estimating the size of the semi-purified bio-active compound using Superdex-200. Assessing approximate sizes of the bio-active compound(s) purified from SephadexLH-20. The results show two bio-activities: a higher molecular weight compound at around 50-200 kDa in size and a smaller molecular weight compound at around 5-25 kDa in size. The anti-inflammatory activity assay (% LPS inhibition) was performed in triplicate and error bars represented standard deviation.

FPLC analysis of the purer sample (i.e. Sephadex Post LH-20 + Post DEAE)

demonstrated only one activity at the approximate size of 5-25 kDa. This activity corresponded

with the second activity exhibited by the semi-purified sample shown in Figure 3.15. Based on

this observation, I propose that the ion-exchange DEAE column was effective in purifying only

the smaller molecular weight activity. This may partly explain why there is lower potency of the

relatively purer sample as the higher molecular weight compound (i.e. relatively more potent) had been removed in the second purification step using DEAE-Sephadex (Figure 3.14). Figure 3.16 shows the results of the purified sample with respect to the percent inhibition and elution volume.
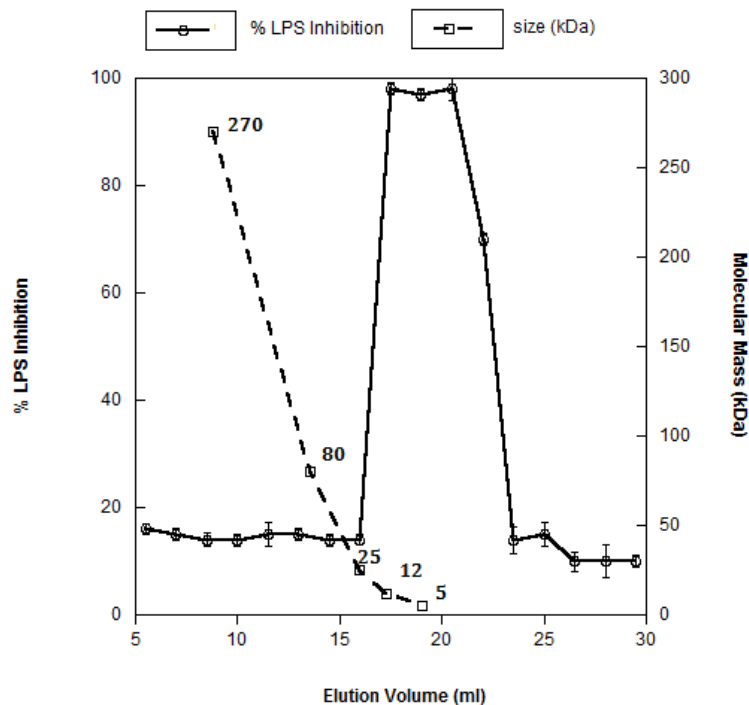
Figure 3.16. Estimating the size of the purified bio-active compound using Superdex-200. Assessing the approximate size of the bio-active compound(s) purified sequentially from Sephadex LH20 and Sephadex DEAE columns. The results show a single activity at around 5-25 kDa size. The anti-inflammatory activity assay (% LPS inhibition) was performed in triplicates and error bars represented standard deviation from the mean.

After the size estimation, the possible presence of carbohydrate and protein content was determined on the fractions collected from the Superdex-200 column. Figures 3.17 and 3.18 show the carbohydrate and protein content in the semi-purified and purified samples after passing through the Superdex-200 column. Figure 3.17 shows a correlation between the carbohydrate and protein content with the bioactivity while Figure 3.18 show a positive

correlation between the carbohydrate content, but not the protein content, with the bioactivity. This indicates that the larger molecular weight compound could be a combination of both carbohydrate and protein, while the smaller molecular weight compound could only be a carbohydrate. This was also observed earlier in Figure 3.9, when the anti-inflammatory activity at elution volume 55 mL did not appear to correlate with the protein content, suggesting that the 5-25 kDa anti-inflammatory compound may not contain protein.
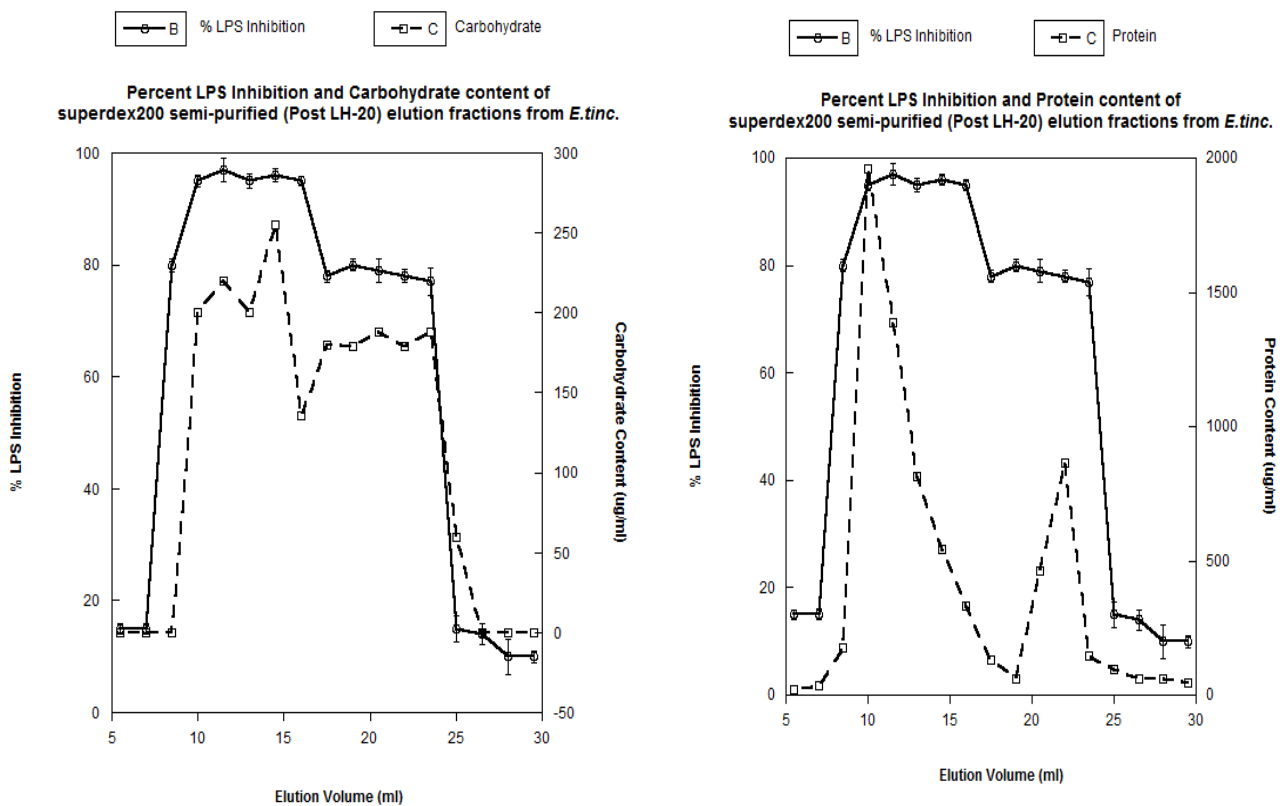
Figure 3.17. Assessing the presence of carbohydrate and protein in fractions collected from the Superdex-200 column. Semi-purified post-LH20 sample (500 μL at 20 mg/mL) was loaded onto the Superdex-200 column. Fractions collected were assessed for total carbohydrate (μg/mL, left) and protein content (μg/mL, right). Results show a positive correlation between the carbohydrate/protein content with the bioactivity. Assay was performed in triplicates and error bars represented standard deviation from the mean.
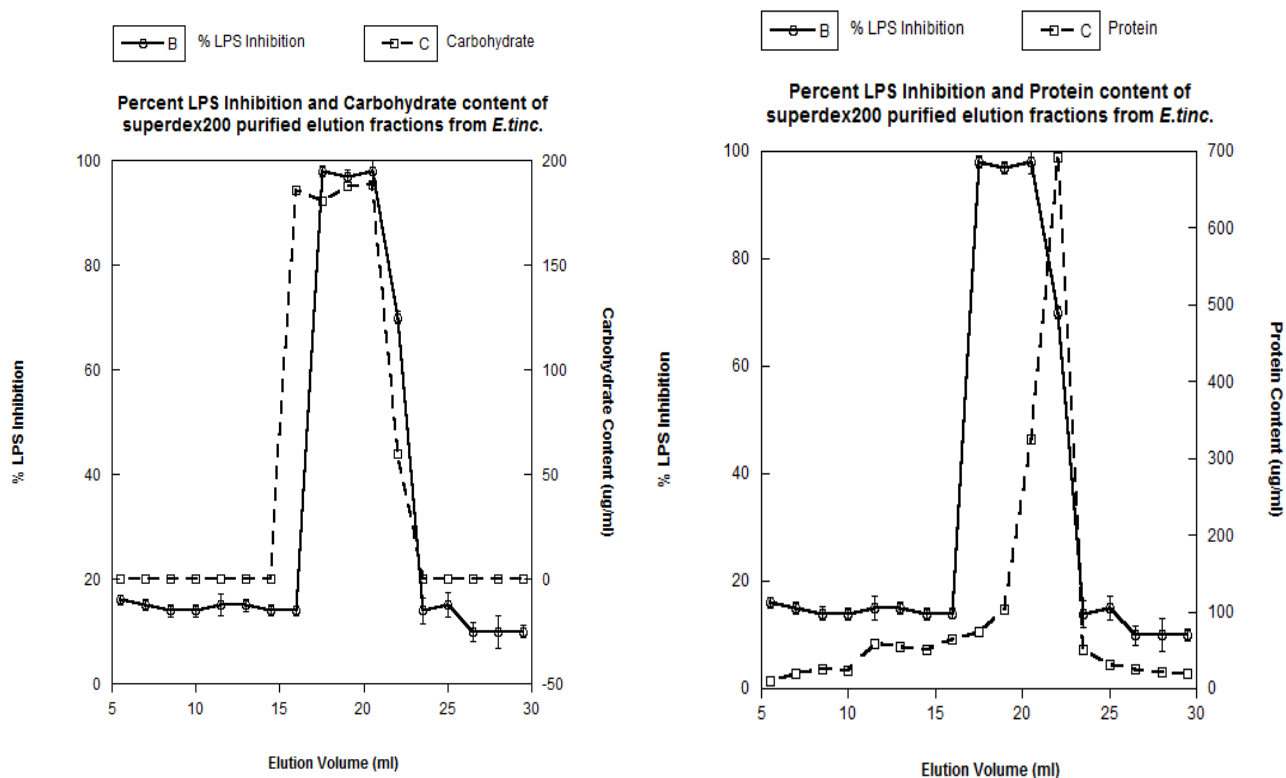
Figure 3.18. Assessing the presence of carbohydrate and protein in fractions collected from the Superdex-200 column. Sample purified from sequential Sephadex LH20 and DEAE-Sephadex columns (500 μL at 20 mg/mL) was loaded onto the Superdex-200 column. Fractions collected were assessed for total carbohydrate (μg/mL, left) and protein content (μg/mL, right). Results show a positive correlation between the carbohydrate content, but not the protein content, with the bioactivity. Assay was performed in triplicates and error bars represented standard deviation from the mean.

3.3.8 Nitrite Determination Assay

After obtaining the relatively purified compound, the sample was tested for NO inhibition potential by measuring nitrite levels. NO assay was also performed in RAW 264.7 cells. Figure 3.19 shows that, at 1 μg/μL, the purified sample was able to inhibit LPS-induced NO production by greater than 98%.
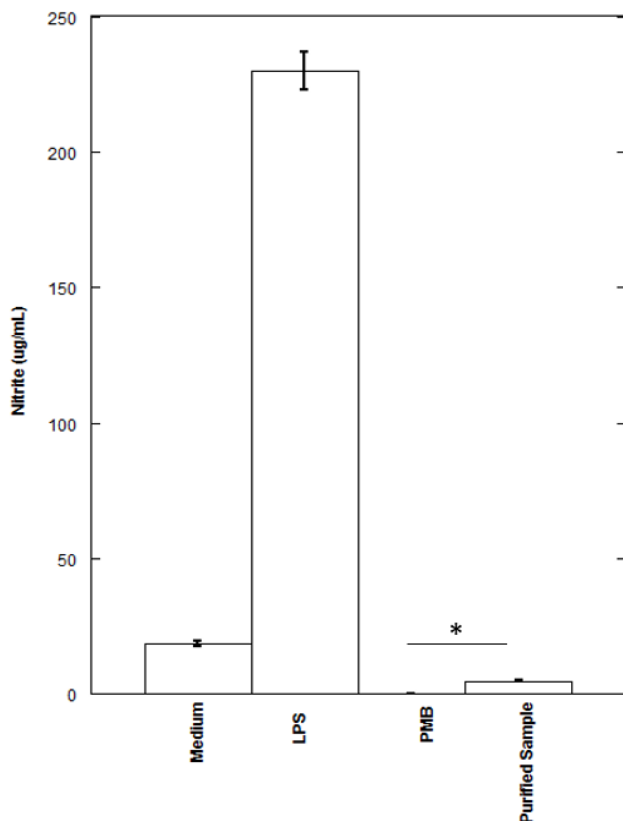
Figure 3.19. Inhibition of LPS-induced niric oxide production by anti-inflammatory compound from *E. tinctorium*. Purified sample (Post Sephadex™ LH-20 and Post DEAE Sephadex) (1µg/µL) was added to LPS (1 µg/mL) induced RAW 264.7 macrophage cells. LPS was used as a negative control and PMB was used as a positive control. Purified sample inhibited ≥ 98% of stimulated NO. Assay was performed in triplicates. Error bars were standard deviation and One-way ANOVA analysis was performed as described in Materials and Methods (*, $P < 0.0001$).

3.3.9 Yield of Purified Sample

As the extraction and purification was carried out, it was essential to keep a record of the respective yield at each step so that a relative concentration of the product could be estimated. Table 3.4 summarizes the yield of the mushroom sample applied and purified at each step (i.e. from raw to two-step purification stage).

Table 3.4: Summary of the Yield of 5% NaOH Extract after Each Extraction/Purification Step.

Technique	Yield
Powdered Crude Conk weight	300 g
F4 (5% NaOH) Fraction weight	16 g
Loaded on Sephadex™ LH-20 (450 mL)	= 630 mg/run (9 ml of 70 mg/mL each).
Purified on Sephadex™ LH-20 (450 mL)	~100 mg/run.
Loaded on Sephadex™ DEAE A-50 (1000 mL)	~800 mg from 8 runs (Post LH-20 Sephadex®)
Purified on Sephadex™ DEAE A-50 (1000 mL)	~60 mg
Post Sephadex™ LH-20 and Sephadex™ DEAE shipped to Dr. Maggie Li for further characterization.	~32 mg

3.4 Discussion

As this thesis was aimed at highlighting the special position of North American wild mushrooms for their novel therapeutic potential, this chapter focused on the successful purification and characterization of an anti-inflammatory compound(s) from the Indian paint fungus, *E. tinctorium* from northern BC forests. In Dr. Lee's research group, the 50% methanol extract (F2) of this fungus has shown potent anti-proliferative activity, which was purified and studied by an undergraduate student Mr. Faran Rashid. The water extract of the same species (as shown in Figure 2.5.2) has exhibited strong immuno-stimulatory potential and is in the process of purification by Ms. Mehreen Zeb, a PhD Candidate in Dr. Lee's lab. Similarly, the 5% NaOH extract which is the main focus of this chapter, has shown strong anti-inflammatory activity in RAW 264.7 cells against both LPS- and Histamine-induced TNF-α production and thus was purified using size exclusion (1st step) and ion-exchange chromatography (2nd-step), and further characterized using FLPC and GC-MS.

The 5% NaOH extract was first subjected to a size exclusion chromatographic purification. Sephadex™ LH-20 is derived from a dextran bead resin, Sephadex G-25, after the introduction of a hydroxy-propyl group and has been developed mainly for the separation and purification of the natural products. The resin has the tendency to separate molecules of less than 5000 Da in molecular weight. Using this size exclusion resin, it was observed that the bio-active fractions of *E. tinctorium* elutes at around 32-45% of the eluting volume. This volume was close to the void volume of the Sephadex LH-20, which is 30% of the column bed volume. Void volume is the elution volume for the larger molecular weights compounds in the sample that are excluded from a particular column as their larger size would not allow them to interact with the resin so they will pass straight through the column.

Exclusion at this volume indicates that the targeted molecule (s) was likely to have higher molecular weight as it was eluting out in the earlier fractions. The experiment in determining the potency of samples demonstrated the successful purification using Sephadex™ LH-20 because the post Sephadex LH-20 sample was not only more effective at the same dosage but was also effective at a much lower dose of 0.1 µg/µL. The active fractions collected from this size exclusion column correlated well with the respective carbohydrate/protein content in the higher molecular weight compound, suggesting the higher molecular weight bio-active compound(s) is made up of carbohydrate and protein, while the smaller molecular weight anti-inflammatory compound(s) consists mainly of carbohydrate. It was observed that, for a typical 450 mL column on which ~630 mg of the crude F4 extract was loaded, active fractions were pooled, lyophilized and ~100 mg of the sample was obtained at each run. This column was run at least 9 times and ~800 mg of this product was saved for a large-scale ion-exchange column. The remaining

product was utilized in initial screening and other different protocol development trials in an effort to develop a reliable second purification step.

After the first step of purification, the post Sephadex^{TM} LH-20 sample was subjected to a second step purification through ion exchange column chromatography. Sephadex^{TM} DEAE A-50 is an anion exchange column in which the column was likely to bind the active compound with the resin in the start of the run and was expected to elute when subjected to a lower pH or a salt gradient. Initially, small columns were used to optimize the conditions. It was observed that Piperazine buffer at pH 5.5 produces potent activity in the "eluent", so this condition was scaled up to get the higher yield of relatively purified bioactive compound(s). This ion-exchange column allowed further separation of non-bioactive molecules from the bioactive ones. Later, 1000 mL column was adopted as the final purification step.

The sample purified through sequential Sephadex^{TM} LH-20 and Post Sephadex^{TM} DEAE (~32 mg) was shipped to Dr. Peter Cheung's Lab at the Chinese University of Hong Kong for further chemical characterization by Dr. Maggie Li. GC-MS analysis was conducted to determine the percentages of monosaccharides and the types of carbohydrate linkages present in the bio-active sample. The preliminary results show the presence of glucose, mannose, galactose, fucose and xylose. It also revealed the presence of 1,3-linked glucose, 1,6-linked glucose, 1,3-linked galactose and 1,3,6-linked glucose linkages in the carbohydrate moiety.

The heat denaturation experiment did not provide any conclusive findings and therefore future experiments are needed to explore the role of the protein component for the anti-inflammatory activity. This could be done by treating the sample with proteases, separate the components through Superdex200 column and then re-assessing the collected fractions for

bioactivity. If the proteins have an important role in the bioactivity, the protein could be isolated from the complex and its amino-acid sequence determined.

Experiments aimed at determining the potency of purified samples showed that the semi-purified sample (bio-active sample from Sephadex LH20) is more potent than the purer sample (bio-active sample from sequential purification through Sephadex LH20 and DEAE-Sephadex). This was also confirmed by the FPLC experiments. The FPLC experiments also allowed determination of the size of the anti-inflammatory compound(s). The semi-purified sample revealed presence of two anti-inflammatory compounds with different molecular weight (higher molecular weight and lower molecular weight), while the purified sample only revealed the retention of lower molecular weight compound. Future studies could attempt to purify the larger molecular weight compound using a larger scale Superdex-200 column.

Like TNF-α, Nitric oxide (NO) is also an important inflammatory biomarker, which is released from LPS-induced macrophage cells. A moderate level of NO is associated with anti-inflammatory and anti-tumor response (Bogdan, 2015). However, it is known that NO over-production causes cellular damage. Hyper-secreted NO reacts with super-oxides in the inflammatory micro-environment and plays a major role in chronic inflammation and carcinogenesis (Bogdan, 2015; Taofiq et al., 2016). Therefore, inhibition of NO could further support the anti-inflammatory potential of the compound. In the current study, the pure sample (i.e. Post LH-20 + Post DEAE) successfully inhibited LPS-induced NO release. Moreover, TNF-α in the downstream pathway activates NF-κβ, which is the transcription factor for stimulating inducible nitric oxide synthase (iNOS). This iNOS then initiates the production of nitric oxide. This inhibition of LPS-induced NO assay confirmed that the inhibition of TNF-α production can lead to the inhibition of down-stream mechanisms for producing anti-inflammatory response.

Future studies could study the expression of various inflammatory genes and proteins in response to treatment of the anti-inflammatory compound purified from *E. tinctorium*.

In summary, the lower molecular weight anti-inflammatory polysaccharide was successfully purified and partially characterized from 5% NaOH extract of *E. tinctorium*. Preliminary results show the presence of glucose as the major skeleton of the bio-molecule. However, the higher molecular weight anti-inflammatory compound, which is likely made up of polysaccharide and protein still needs to be separated and characterized. The roles of the protein and carbohydrate components are yet to be confirmed and need further studies. The semi-purified sample (Sephadex Post-LH 20) was more potent than acetaminophen and was further tested on inflammatory animal model. The purified anti-inflammatory sample successfully inhibited LPS-induced NO production. To my knowledge, this is the first study reporting the anti-inflammatory compound(s) from *E. tinctorium*. However, the particular mechanism of action of this purified sample is yet to be investigated.

Chapter 4: Mushrooms in Microcirculation

This chapter focuses on an *in-vivo* model for assessing the anti-inflammatory potential of selected mushroom extracts. A brief description of micro-circulation in skeletal muscles and the impact of inflammation on compromising the cellular communication are provided. The effect of histamine in inducing inflammatory events in mice gluteus maximus muscle was first established followed by investigation into the role of *I. obliquus* (Chaga-F2) extract and semi-purified *E. tinctorium* (F4-Post LH-20: *E. tinc.* PL20) in ameliorating histamine-induced inflammation.

Introduction

4.1 Role of Micro-circulation in Skeletal Muscles

Skeletal muscles are voluntary controlled and present at the effector ends of the locomotory signaling pathway; they account for 40% of the whole muscle mass and are controlled via nerve stimulation (Hopkins, 2006). The muscle demands for oxygen depend greatly on the metabolic activity, which varies considerably during rest (blood flow: 5 mL/min/100g) and active state (blood flow: 250-400 mL/min/100g) of the body (Segal, 2015). The blood supply in skeletal muscles is regulated by a coordinated interaction of the skeletal muscle fibers, endothelial, and smooth muscle cells, along with the local microcirculation (Segal et al, 2005). This vasculature is crucial for the optimum skeletal muscle output, transporting oxygen and essential nutrients while removing wastes. Interestingly, during intense physical activity, the increased oxygen demand does not increase the cardiac output significantly, highlighting the role of resistance vasculature that regulates and satisfies this intensified variation of metabolic and energy need (Segal, 2015). Miniature resistance arteries (arterioles) are responsible for supplying oxygen directly to the muscles at the micro-circulation level. At this stage, a coordinated vasodilation is crucial to meet the enhanced demand of the muscle by

releasing vasodilatory mediators or by spreading hyperpolarized signals between the endothelium and smooth muscles (Payne, 2006). These vasodilatory stimuli are generated locally and then are propagated upstream in the form of hyper-polarized electrical signals through gap junctions (present between the endothelium of the arteriolar walls) and eventually meet the enhanced demand by increasing the blood flow locally (Payne, 2006; Segal, 2015).

This conducted vasodilation is manifested as a result of cell to cell coupling via gap junctions (Segal & Duling, 1986). Moreover, the intact endothelium is crucial for the un-interrupted transmission of the hyperactive signal upstream, as it is now also established that endothelium, and not the surrounding smooth muscle layer, acts as a cellular medium for this cell to cell transmission of acetylcholine for the muscle contraction signals (Looft-Wilson et al., 2004). Therefore, the endothelium integrity plays an important role in the maintenance of normal physiology and thus, any damage to endothelium will affect the coordinated communication between the endothelium (lining the interior of the arterioles) and adjacent smooth muscle cells in response to a neuronal impulse for the maintenance of the desired vascular tone. Given the importance of this coordinated cross-talk during conducted vasodilation, scientists have evaluated the impact of inflammation on this cellular communication.

4.2 Impact of Inflammation on Micro-circulation

Broadly stated, microcirculation has a major role to play in the development and transduction of the inflammatory response. Microvasculature undergoes several structural and functional changes during the inflammatory process (Granger & Senchenkova, 2010; Libby et al., 2009). Some of these manifestations are: the inability of the arterioles to dilate, a decline in the perfusion of capillaries, the activation of the endothelial cells lining the blood vessel to accommodate adhesion molecules stimulating the extravasation (escape) of leukocytes, and

finally enhanced vascular permeability (Granger & Senchenkova, 2010; Tuma, 2011). The primary goals of these stimulated mechanisms are: to promote the spontaneous delivery of inflammatory cells at the site of original insult, to counter-act the effect of hyper-stimulated inflammatory agents, to separate the infected region from the healthy one, and to finally start the process of tissue regeneration and re-modelling as soon as possible.

Different cells that either circulate in the blood in normal conditions (i.e. platelets, leucocytes) or remain in the perivascular space (i.e. mast cells, macrophages); combined with those present in vessel wall (i.e. endothelial cells or pericytes) get activated because of the inflammatory response (Ley, 2001). These inflammatory cells, along with different chemical mediators and signaling molecules, induce phenotypic changes in the normal microvasculature of the body (Granger & Senchenkova, 2010; Ley, 2001). Although the roles of different chemical mediators and signaling mechanisms differ widely between the acute and chronic stage of inflammation, the role of microcirculation in sustaining and mounting the inflammatory response remains the same under both conditions (Granger & Senchenkova, 2010; Harlan, 1992).

Inflammation activates almost every cell present in the vicinity of the affected area, and therefore, allows meaningful contribution from each cell in the outcome of the provoked response. Even the highly-specialized cells respond in a similar way, by releasing for instance certain cytokines and chemokines and by stimulating certain reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). These similarities in a combined response produce an altogether intense and effective defense for the rapid eradication of the intruding pathogen. Inflammation negatively impacts the vascular tree and all segments of microvasculature (i.e. arterioles, venules, capillaries, endothelial cells, pericytes, and vascular smooth muscles) respond to the inflammatory signal (Granger & Senchenkova, 2010; Kvietys & Granger, 2012; Payne,

2006). In arterioles, inflammatory mediators enhance superoxide (O_2^-) formation, halt nitric

oxide (NO) formation, and diminish vascular smooth muscle relaxation; these changes cause

oxidative stress (due to O_2^-), impair vasomotor function (due to inhibition of NO), and enhance

thrombosis in arterioles (due to hemostasis) (Granger & Senchenkova, 2010; Sumagin &

Sarelius, 2013). In the venules, inflammatory mediators enhance oxidative stress and leucocyte

adhesion, and also favor platelets adhesion and enhance vascular permeability, finally promoting

thrombosis. In the capillaries, inflammatory cascade reduces perfusion, supports the coagulation

of leucocyte, and finally promotes angiogenesis (Granger & Senchenkova, 2010; Kvietys &

Granger, 2012; Ley, 2001; Tuma, 2011). Consequently, the overall physiological changes in the

micro-vasculature is summarized as: diminished capillary perfusion, vasomotor dysfunction,

leukocyte adhesion and trans-endothelial migration, endothelial barrier deterioration (enhanced

vascular permeability), increased proliferation rate of blood (i.e. angiogenesis) and lymphatic

vessels, activation of coagulation cascade, and finally enhanced thrombosis (Granger &

Senchenkova, 2010; Kvietys & Granger, 2012; Sumagin & Sarelius, 2013; Tuma, 2011). Thus,

micro-circulation acts as a first aid responder in any inflammatory-associated event and is critical

for any therapeutic target to maximize the targeted efficacy of any anti-inflammatory drug.

4.3 Impact of Inflammation on Endothelium Integrity-Cellular Communication

The endothelium, lining the inner wall of arterioles, acts as a semi-permeable membrane

and is involved in creating a selective barrier to the transport of fluids, solutes and oxygen

through the blood. This layer also mediates vasomotor tone and plays a role in the adhesion of

cells (normal as well as inflammatory) and proteins within the vasculature (Granger &

Senchenkova, 2010). Under normal conditions, the endothelium provides a non-adhesive surface

and ensures consistent and uninterrupted hemostasis. In contrast, endothelial impairment is

associated with providing the ideal surfaces for the adhesion of platelets, leucocytes and other inflammatory cells and is ultimately associated with various pathological states, including inflammation (Payne, 2006). The loss of endothelial integrity during inflammation also causes the loss in vascular permeability and impairs cell to cell communication; thus, in a disease situation the upstream signal conduction, including conducted vasodilation, is compromised. This makes it difficult to meet the enhanced oxygen demand of the muscle in an hyper-active state. Further research is needed for the elucidation of the proper pathophysiology of blood flow in inflammatory endothelium.

Histamine, a common inflammatory mediator, enhances the vascular permeability and is implied in various *in-vitro* and *in-vivo* models to simulate the inflammatory event (Majno & Palade, 1961). In one of the pioneering studies exploring the effect of histamine in compromising cellular communication in inflammatory micro-circulation, Payne et al. (2004) have demonstrated that the transduction of upstream signal in second order arterioles (2A) in mice cremaster muscle is reduced by half when the arterioles are subjected to histamine. However, the exact mechanism of histamine in compromising the communication remains unknown. It was proposed that the inhibition of the response could be attributed to a NO-dependent interaction via gap junctions and intracellular adhesion molecules. Moreover, histamine could alter gap junction configuration by either phosphorylating the connexin proteins present in the junctions or by dis-assembling the junctional proteins isoforms (i.e. connexin Cx37, Cx40, and Cx43) (Payne, 2004). Thus, this thesis chapter focuses on the 2A arterioles of mice gluteus maximus muscle as a representative of the skeletal muscle: histamine was applied to simulate the *in-vivo* inflammatory events, observe the effect of histamine in reducing vasodilation and explore the role of potent mushroom extracts in this diminished communication state.

4.4 Studies of Mushrooms on *In vivo* Anti-inflammatory Model

There have been a number of studies exploring the impact of mushroom extracts and purified compounds in various anti-inflammatory animal models. The common model includes the induction of inflammation, using a chemical agent (e.g. carrageenan, acetic acid, and formalin etc.) and then applying mushroom extracts and/or isolated purified compounds to test the anti-inflammatory potential (Park et al., 2005, Qian et al., 2012, Ruthes et al., 2013). Park et al. (2004) showed that methanolic extract of *F. fomentarius* had a positive anti-inflammatory activity in rats as it successfully reduced carrageenan-induced acute paw edema. Qian et al. (2012) isolated lower molecular weight peptide (10,906 Da), Cordymin, from *C. sinensis* and successfully inhibited acetic-acid-induced inflammation in mice by decreasing TNF-α and IL-1β levels. The fucogalactan extract from *A. bisporus* also inhibited formalin-induced licking by decreasing iNOS and COX-2 expression (Ruthes et al., 2013). Our study using intra-vital microscopy technique to monitor the effects of mushroom extracts and/or purified compound(s) from mushrooms on the microcirculation is novel and has not been previously described. This model involves the live imaging of the inflammatory event and observation on the improvement of the inflammatory event in real time. We have applied methanolic extract of *I. obliquus* (F2) as a reference and then tested semi-purified (Sephadex Post LH-20) sample of *E. tinctorium* in replenishing the inflammatory event.

4.5 Use of Methanolic Extract of *I. obliquus* as a reference for *in vivo* model

Inonotus obliquus is a well-known traditional mushroom and the literature supports its anti-inflammatory therapeutic activity (please refer to chapter 2, section 2.8.2). Various ethanolic and methanolic extracts of *I. obliquus,* along with isolated sterols and terpenoids have demonstrated positive anti-inflammatory effects in both cell cultures and animal studies. In *in vitro* model, *I. obliquus* inhibited LPS-induced macrophages by decreasing hyperactive release of

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inflammatory cytokines such as: TNF-α, NO, COX-2 etc. whereas, in *in vivo* model, *I. obliquus* was successful in treating carrageenan-induced edema in rats and in decreasing colitis-associated inflammation in mice models (Debnath et al., 2012, Ma et al., 2013, Park *et al*., 2005, Van *et al*., 2009). The following table summarizes the reported anti-inflammatory activity of *I. obliquus* in various *in vivo* and *in vitro* settings, making it a suitable candidate for our model development studies. Moreover, we have selected methanolic extract based on its maximum inhibitory effects on LPS-induced RAW 264.7 cells in our *in vitro* screening assay where it demonstrated potent anti-inflammatory activity (please refer to chapter 2, figure 2.6.3). This extract, along with the crude extract of *E. tinctorium,* also inhibited histamine-induced TNF-α in RAW 264.7 cells (please refer to Chapter 2, Figure 2.6.2).

Table 4.1 A Summary of *In Vivo* & *In vitro* Anti-Inflammatory Activities of *Inonotus obliquus*.

Model	Activity	Findings	Active Structure	References
LPS stimulated RAW164.7 cells	Anti-inflammatory (in-vitro)	50% reduction in nitrate conc. Dose-dependent inhibition of IL-1β, IL-6, and TNFα in LPS Induced RAW 164.7 cells	Sterols	Van et al., 2009
i. LPS Stimulated RAW 264.7 cells. ii. Paw edema in rats.	Anti-inflammatory and anti-nociceptive effects	i. Methanolic extract inhibited production of NO, prostaglandin E2, and TNF-α, mRNA expression of iNOS and COX-2 was also inhibited. ii. Inhibitory effects on carrageenan induced edema	Structure analysis was not done in this study	Park et al., 2005
RAW 264.7 macrophage cells	Anti-inflammatory and anti-cancer activity	NO production, and NF-κB luciferase activity inhibition	Trametenolic acid, ergosterol peroxide, 3β-hydroxy-8,24-dien-21-al, ergosterol, trametenolic acid and inotodiol	Ma et al., 2013
Colitis-induced Mice model	Colitis associated Inflammation	Decrease levels of TNF-α, COX-2, IL-4, IFN-γ, signal transducers and activators of transcription (STAT) 1, and STAT6. Lower levels of IgE and IgA in the spleen and mesenteric lymph node	Structure analysis was not done in this study	Debnath et al., 2012

Based on our available expertise and the novelty of the technique in the field of medicinal mushrooms, we proceeded to investigate the feasibility of using intravital microscopy to study the potential anti-inflammatory effect of mushroom extracts/compounds on mice microcirculation.

4.6 Material & Methods

4.6.1 Animal and Chemicals

Male black mice C57BL6 were purchased from Jackson Laboratories, BarHarbor, ME, USA. All chemicals and reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) or J. T. Baker (Phillipsburg, NJ, USA).

4.6.2 Animal Care

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Northern British Columbia. The procedures used were an extension of Dr. Payne established protocols (Bearden et al., 2004; Looft-Wilson et al., 2004; Payne et al., 2004). Mice used for this study were male C57BL6 (22-30g, n=20) and were between 8–44 weeks old. Mice were housed at ~24°C and maintained on a 12:12-h light-dark cycle with free access to food and water *ad libitum*. Body mass was measured before each experiment. At the end of each experiment, mice were euthanized using intraperitoneal injection of the anesthetic (Euthanyl-120-150mg/kg).

4.6.3 Surgery and Glute Muscle preparation

Each mouse was anesthetized with isoflurane with an induction rate of 3-4% and supplemented with a maintenance rate of 1-1.5% to maintain a surgical plane. During both surgical and experimental protocols, toe pinch was monitored to ensure a constant aesthetic state.

Temperature of the animal was maintained using a heat lamp. The surgical protocol for the left gluteus maximus muscle was followed as described by Bearden et al. (2004). In short, the

left area covering glute was shaved in order to expose the muscle and was disinfected with alcohol. The mouse was placed in prone position on a clear surgical (acrylic) plate. While observing through a stereomicroscope, the skin and connective tissue layer over the muscle was removed. The vasculature was exposed by carefully dissecting the proximal edge of the muscle away from its origin along the spine and pinning it on transparent sylgard platform. To maintain the internal physiological state of the muscle, the exposed tissue was constantly super fused with a bicarbonate-buffered physiological saline solution (PSS). The PSS used was equilibrated at 34°C, has pH= 7.4, and has the following composition: NaCl (137 mM), KCl (4.7 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2 mM) and $NaHCO_3$ (18 mM). Careful skills were practiced to preserve the muscle architecture and to mimic the *in-situ* condition of the muscle so that a perfect *in vivo* model could be established.

4.6.4   Intravital Microscopy

After surgery, the muscle preparation was transferred to the stage of an intra-vital microscope (modified model 20T, Zeiss). The preparation was left to equilibrate for around 45-60 mins while being super-fused with PSS to recover from the impact of surgery. Meanwhile, any signs of inflammatory event in the muscles were also observed (e.g. excessive rolling leukocytes) to ensure that there was no induction of inflammation by the surgery. If there is any, enough time was allowed to ensure the health of the preparation and vessel before recording any data. At that stage, PSS was equilibrated with 5% CO_2 and its temperature was maintained inside a Radnoti perfusion reservoir. The flow rate of PSS was maintained at approximately 3mL/min with the help of a drip and suction line. The preparation was then observed with bright field illumination (Zeiss ACH/APL condenser, numerical aperture 0.32). Then, a second order arteriole (2A) was selected from the central region of the tissue preparation by viewing it via a

Zeiss UD 40 objective (numerical aperture 0.41) coupled to a video camera (model no. C2400, Hamamatsu), whereby the total magnification on the video monitor (Sony model PVM-132) was 950x. The vessel diameter was determined from the outer coat edges of the lumen with the help of a video caliper (modified model 321, Colorado Video, Boulder, CO) with spatial resolution of $\leq 2\mu m$. The data was obtained using a PowerLab system (model 8S, ADI Instruments, Castle Hill, Australia) at 40Hz connected to a personal computer. Only one arteriole was studied in each mouse.

4.6.5 Vasoactive Substances & Dose Response Curves:

The vessel tone is crucial for the optimum experimental results. Therefore, various concentrations of phenylephrine (PE, a selective α1-adrenergic receptor agonist-vasoconstrictor) and acetylcholine (ACh, a muscarinic receptor agonist-endothelium dependent vasodilator) were used. The stocks were prepared such that, after a 10-fold dilution with the fresh PSS, the concentration range was from 10^{-9} to 10^{-4} M of each reagent. Sodium nitroprusside (SNP., a source of NO, 10^{-2} M) was used in the end of each experiment to make sure that the endothelium was intact and healthy in all muscle preparations.

After equilibration with PSS for 45-60 mins (as described in section 4.3.3), the resting diameter of vessels was first measured and then followed by ACh and PE responses to measure vessel tone. The dose of each vasoactive reagent was evaluated by the cumulative addition (10^{-9} to 10^{-4}M) to the super-fused solution in the Radnoti perfusion reservoir. The choice of adding PE or ACh first was dependent on the vessel diameter at rest. If the resting diameter was larger than ~30μm, PE was used first as to allow the maximum diameter change from the baseline. As the addition of a dilator at this increased diameter will not make significant dilation, there will be little or no change at all from the reading at rest. If the initial diameter is smaller than ~30 μm,

ACh was added first. At each agonist concentration, the preparation was allowed to stabilize at the respective concentration for at least 2 mins before the next increment is being made. The preparation was subsequently washed with PSS for 30 mins in between ACh and PE applications to allow the return of the vessel diameter to the resting state. The addition of these vasoactive chemicals was in accordance to the protocol mentioned by Bearden et al. (2004). At the end of each experiment, maximum dilation was induced and measured by running SNP (10^{-2} M) over the preparation for 10 minutes.

Histamine dose response was also estimated (10^{-9} to 10^{-4} M) to evaluate the response of histamine in 2A arterioles of mouse gluteus maximus muscle (n=5). This effect of changing histamine concentration was determined in the similar manner (i.e. by the cumulative addition to the super-fused solution). Arteriolar diameter was allowed to stabilize for 2 mins at each concentration and then the reading was taken, before the next increase. After the highest concentration, maximal dilation was induced and recorded through super-fusion of SNP.

4.6.6 Conducted Vasodilation (CVD)

To measure conducted responses, pre-pulled micropipettes (TIP2TW1, World Precision Instrument) with an inner tip diameter of 2μm were backfilled with 1 M ACh. This pipette was then adjusted in a micro-manipulator connected to a pressure pulse injector. The tip of the micropipette was then positioned adjacent to the downstream part of a 2A arteriole in the muscle preparation with the help of a remote-controlled micro-manipulator. This micro-manipulator allowed the smooth adjustment of the tip of the micro-pipette with respect to the 2A arteriole location. The tip was pre-equilibrated in PSS to ensure that the ACh inside is balanced and there was no leak on the muscle preparation. After equilibrating the muscles for 45 mins with PSS, a brief pulse of ACh (1 psi, 500 ms) was delivered to the selected 2A arteriole (n=10). The change

in diameter at the site where the micropipette was placed was recorded as "local response", while the conducted vasodilation was measured 500µm upstream of the micropipette injection site and was recorded as "conducted response".

After measuring the local and conducted response under controlled conditions (i.e. PSS), the preparation was equilibrated with histamine (10^{-5} M) for 30 mins. This concentration was selected as it has negligible effects on the diameter of 2A arteriole under rest condition (Figure 4.2). Then, the effect of histamine on local and conducted vasodilation was evaluated in the same manner as described previously at 10 mins intervals while the histamine was still super-fused on the glute preparation.

After recording the effect of histamine, the preparation was super-fused separately with either Chaga-F2 (n=5) or *E. tinctorium* (refer to as *E. tinc.*)-PL20 (n=5) and the added histamine so that the final concentration of the mushroom extract is 12.5 µg/mL. The concentration of the mushroom extracts was selected on the same criteria (i.e. the dose at which there is negligible effects on the diameter of 2A arteriole under rest conditions) (Figure 4.3). This mixture (histamine + mushroom extract) was allowed to run on the preparation for 30 mins and the local and conducted response was measured in the same way as described previously while the solution was still running on the muscle preparation.

4.6.7 Data Analysis

The vasomotor response to ACh, PE, SNP and histamine were calculated using the following formula at the respective site of observation:

$$\% \text{ Difference} = \frac{\text{response diameter} - \text{resting diameter}}{\text{resting diameter}} \times 100\%$$

For the conducted vasodilation experiment, at each site of arteriole, the response to ACh was calculated as the diameter change (i.e. peak response diameters minus the resting diameters).

For all experiments, data were analyzed using One-way repeated-measures ANOVA (PRISM). Summary data is represented as means ± S.E. Value of n refers to the number of arterioles studied in as many mice. Difference among group is statistically significant for $P < 0.05$.

4.7 Results

4.7.1 Vascular Integrity

For determining the baseline and maximum diameters, second-order arterioles (2A) were studied in male C57B76 mice. Minimal variation was observed in the resting diameter of 2A arterioles of the mice. The average resting diameter was calculated as 19 ± 2 µm (n=10). The maximum dilation of the test vessel in response to ACh (10^{-4} M) equals 38 ± 2 µm (n=5) and that of SNP (10^{-2} M) equals 37 ± 3 µm (n=10). There is approximately a 151% change between the resting and the maximum diameter. Table 4.1 displays the graphical representation of minimum (resting) and maximum (ACh and SNP) diameters changes.

Table 4.2: Baseline (Resting) and Maximum diameters (ACh and SNP) in 2A Arteriole

Parameters	Vessel Diameter (µm)	n
Baseline (Resting)	19 ± 2	10
Acetylcholine (ACh) 10^{-4} M	38 ± 2	5
Sodium Nitroprusside (SNP) 10^{-2} M	37 ± 3	10

For evaluation of the vascular tone, gluteus maximus muscle was subjected to different doses of ACh and PE (10^{-9} to 10^{-4} M). Figure 4.1 demonstrates the successful induction of vasodilation and vasoconstriction in the arterioles with the help of ACh and PE respectively in a dose-dependent manner. The arteriole increased steadily by raising the dose of ACh. On the other hand, the percent change of diameter from resting diameter decreased gradually by increasing the dose of PE in the super-fused solution. These results indicate that the endothelium and smooth

muscles in the arterioles are intact and reactive to the added molecules and the doses of vasoactive chemicals were sufficient to elicit a response.
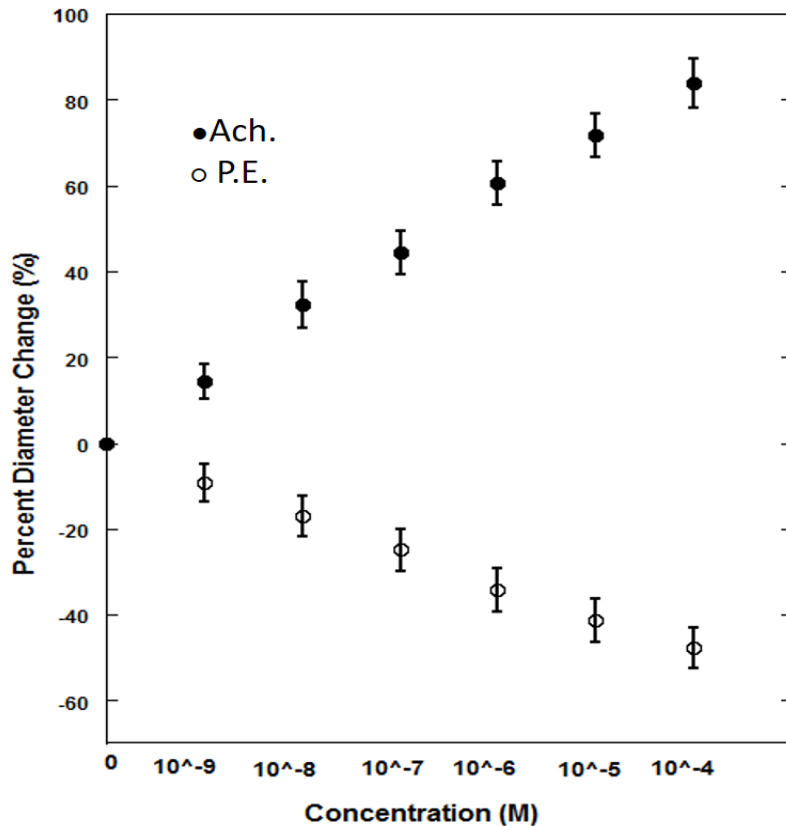
Figure 4.1. Vaso-activity of ACh and PE. The percent change in diameter of the 2A arteriole of male C57BL/6 mouse gluteus maximus preparation (n = 5) with the addition of vasoactive compounds. The vasoactive compounds, phenylephrine (PE) and acetylcholine (ACh) were diluted in super-fused solution so that the final concentrations were 10^{-9} to 10^{-4} M using PSS. The percent change was measured as the (baseline diameter-recorded diameter)/baseline diameter multiplied by 100%. Error bars were determined using standard error of the mean.

4.7.2 Histamine dose response

Histamine was used to induce inflammation in the 2A arteriole in a dose-dependent manner. Figure 4.2 illustrates the effect of various doses (10^{-9} to 10^{-4} M) of histamine on the arteriolar reactivity. The dose response curve demonstrates a bi-phasic reaction in which the

vessels demonstrate vasodilation when subjected to 10^{-9} to 10^{-6} M histamine concentration, whereas it demonstrates vasoconstriction when subjected to 10^{-5} to 10^{-4} M concentration of histamine. Maximum dilation was observed at 10^{-6} M that shifts to vasoconstriction at lower dose of 10^{-5} to 10^{-4} M (Figure 4.2).
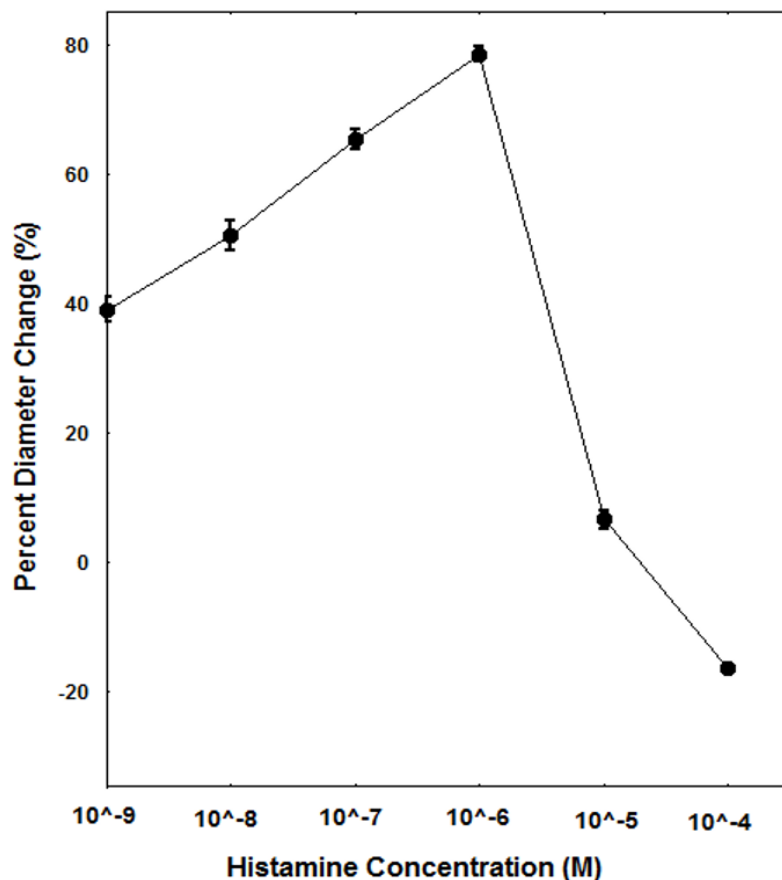
Figure 4.2. Bi-phasic response of Histamine. The percent change in diameter of the 2A arteriole of male C57BL/6 mouse gluteus maximus preparation (n = 5) with the addition of histamine. The histamine is diluted in super-fused solution so that the final concentration is 10^{-9} to 10^{-4} M using PSS. The percent change was measured as the (baseline diameter-recorded diameter)/baseline diameter multiplied by 100%. Error bars were determined using standard error of the mean.

4.7.3 Conducted Vasodilation

For employing mushroom extract in CVD, a dose response curve for the extract was generated using Chaga-F2 as a reference extract. Figure 4.3 represents the dose response curve for Chaga-F2, indicating the decreasing tendency of the extract to change vascular diameter with the decrease in dose. The dose selected for the CVD experiment was 12.5 µg/mL because, at this dose, the vessels were minimally active. The same dose was used for the other mushroom extract (*E. tinc.* -PL20) as the mushroom has almost the same potency and dose-dependent response in *in vitro* settings (Refer to Chapter 2, Figure 2.6.2, 2.6.3, 2.7.2).
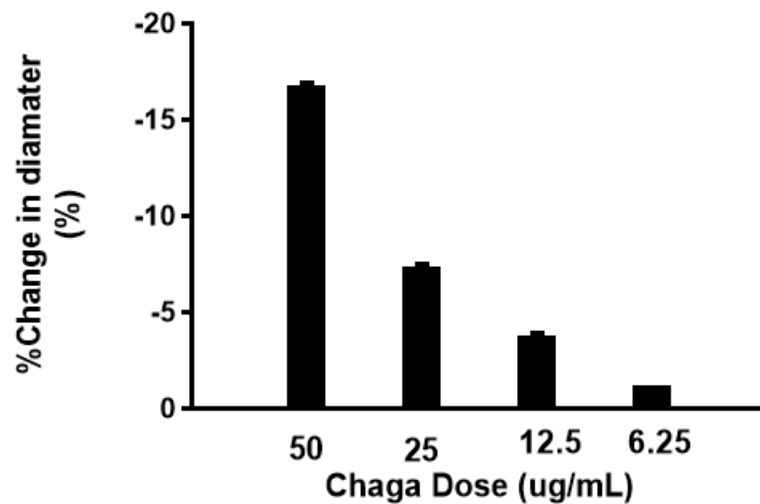
Figure 4.3. Dose response curve of Chaga (*Inonotus obliquus*, F2). The percent change in diameter of the 2A arteriole of male C57BL/6 mouse gluteus maximus preparation (n = 7) with the addition of Chaga. The histamine is diluted in super-fused solution so that the final concentration is 50, 25, 12.5 and 6.25 µg/mL using PSS. The percent change was measured as the (baseline diameter-recorded diameter)/baseline diameter multiplied by 100%.

For the CVD experiments, the same local response was observed among diverse treatments (Figure 4.4) as the baseline diameter change was almost the same among different control groups (15±0.2), Chaga-F2 (13.5±0.5), histamine (13±0.3), histamine + Chaga-F2 (13±0.3), *E. tinc.* -PL20 (13.5±0.3) and histamine + *E. tinc.* -PL20 (14.8±0.16).
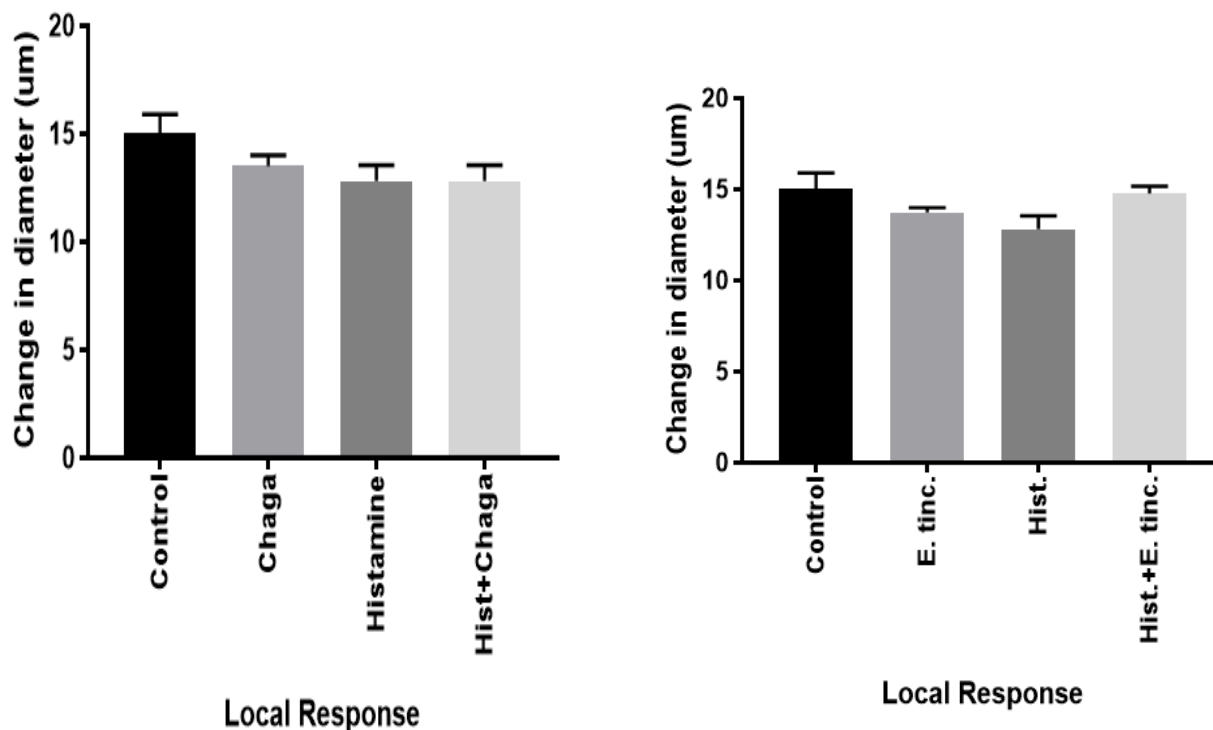
Figure 4.4. Local response to conducted vasodilation in arterioles of C57BL6 mice (n=10). Responses to ACh (1psi, 500 ms) were recorded at the site of stimulation (local: distance=0). At each site, "diameter change" was calculated as peak response diameter – resting diameter. There was no significant difference in the local response in arterioles of C57BL6 mice between control, Chaga (left), histamine, histamine + Chaga (left), *E. tinc.* (right) and histamine + *E. tinc*. -PL20 (right). One-way ANOVA is applied as described in methodology section.

For the conducted response, histamine decreased the conducted vasodilation at 500 µm upstream as predicted. The change in diameter dropped from 10±0.3 µm in controlled state to 6±0.06 µm in inflammatory state induced by histamine indicating the decrease in the cellular communication. There is no significant change in response in the case of Chaga (9.5 ± 0.5 µm) and *E. tinctorium* (10 ± 0.5 µm) alone. Figure 4.5 (left) shows the CVD of Chaga in response to histamine-induced response. Chaga restored the decreased conducted vasodilation induced by histamine to 8.8 ±0.2 µm from 6±0.06 µm. Figure 4.5 (right) shows the CVD of *E. tinc.* -PL20 in

response to histamine-induced response. In this case, the semi-purified mushroom extract restored the conducted back to 10 ± 0.2 µm.
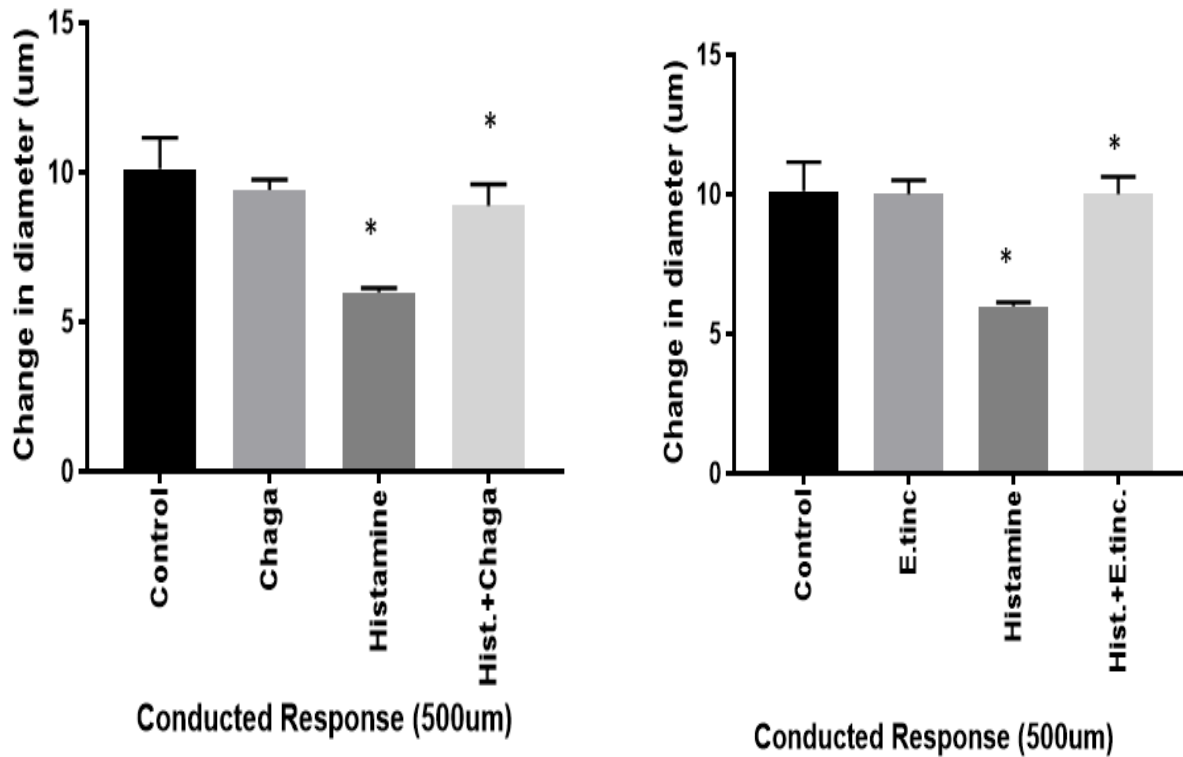
Figure 4.5. Conducted vasodilation in arterioles of C57BL6 mice (n=10). Responses to ACh (1psi, 500 ms) were recorded at the remote site upstream (with respect to blood flow) along the arteriole through a distance of 500 µm (conducted response). At each site, "diameter change" was calculated as peak response diameter – resting diameter. There is significant difference between conducted response of histamine vs. Chaga-F2 and E. tinc. -PL20, indicating the anti-inflammatory potential of mushroom extracts. One-way ANOVA was applied as described in methodology section *P <0.001.

4.8 Discussion

This study determined the reactivity of 2A arterioles in mice gluteus maximus muscle under both normal and pathological state. The dose response curve with the ACh and PE (Figure 4.2) demonstrated that the vessels possess a healthy vascular tone as they showed desired response upon treatment with a vasoconstrictor and/or a vasodilator. It further supports the skills of the experimenter to perform a reliable and healthy muscle preparation each time in which both

smooth muscles and endothelium are intact and the capacity of this preparation to produce powerful and consistent response without eliciting inflammatory events or compromising vascular tone.

The histamine dose response curve showed a bi-phasic response and helped in choosing 10^{-5} M dose for the conducted vasodilation (CVD) experiments. The biphasic histamine response is in accordance with the previous work done by Payne et al. (2003) in which 2A arteriole of cremaster muscle showed the similar trend. Furthermore, normal and pathological responses to the conduction of signal was observed using CVD technique. There was no significant difference between local response under different stages, but the conducted response (500 μm upstream n=5) was significantly less in the presence of histamine (inflammatory state). As suggested by our results, this reduced signal transmittance can be re-established by running mushroom extracts: F2 Chaga along with the histamine (n=5) and F4 Post LH-20 *E. tinctorium* with histamine (n=5). To our knowledge, the current study is the first of its kind to explore the role of wild mushrooms in micro-circulation.

Intravital microscopy, an imaging technique, enables viewing of live vessels in the tissue at the micro-circulation level. Tissues are mainly prepared from live anesthetized animals, and are viewed using a set of high resolution lenses that enables a clear visual of the physiological events happening in the vessels. A physiological saline solution (PSS) replicates the internal physiological environment and as the muscle is still intact with the animal, an *in vivo* condition is simulated. The live event can also be seen with the help of a video camera and computer attached with the preparation. There is also an attached video caliper that measures the vessel diameter, red blood cells velocity etc. from which major qualitative and quantitative conclusions can be drawn in real time (Gavins & Chatterjee, 2004).

The mice surgical preparation using gluteus maximus and its response to vasoactive reagents were previously developed and applied in Dr. Payne's lab. The same response can be observed in mice cremaster muscles but the gluteus maximus muscle preparation provides several strengths over the cremaster muscle. Some of these advantages are: intact vasculature, being present in all mammals, common to both sexes, and a strong organ of locomotion. The benefit of using glute muscle is the opportunity to study the intact microvasculature in its native environment (Payne, 2006; Payne & Bearden, 2006)

Previous studies have demonstrated that the gluteus maximus arteriolar network architecture is highly conserved across different age groups (young, adult, old) in C57BL/6 mice during their lifetime (Payne & Bearden, 2006). It further explains that the tortuosity and branching angle of the smallest arterioles is affected with aging but there is no age-associated differences in arteriolar segment numbers, lengths or surface areas in these mice (Payne & Bearden, 2006). The baseline and maximum diameter and the responsiveness of the vessel to the vasoactive reagents are also similar among different age groups, indicating the intactness of endothelium and smooth muscle in the vessel. However, the study does show a decrease in vascular communication (i.e. conducted vasodilation) with the process of aging. Therefore, the arteriolar network architecture, endothelium-dependent and independent vasodilation, and smooth muscle contraction to adrenoreceptor activation are highly conserved in various age groups but the cellular communication is compromised in older age (Payne & Bearden, 2006). These reasons make the gluteus maximus muscle an ideal model system to use for intravital microscopy and explore the relationship of various physiological and pathological states. It is also worth mentioning that 2A arterioles were selected as the vessels of the choice of study as these are the key distributing vessels in the mouse gluteus maximus muscle and these are also

positioned ideally to control the flow of the blood (Payne 2006). Inducing histamine in the microcirculation and observing it directly via intravital microscopy (IVM) also allows the observer to examine the inflammatory changes at the same time as they happen.

The current *in vivo* study highlights the importance and complexity of the inflammatory response in the microvasculature of a human body. The diverse and constantly expanding research on microcirculation and its role in inflammation highlights the contribution of all its segments (i.e. arterioles, capillaries, venules, endothelial cells, pericytes and vascular smooth muscles etc.) in the progression of the inflammatory micro-environment. Typical phenotypic changes demonstrated by the microvasculature during the inflammatory cascade include vasomotor dysfunction, diminished capillary perfusion, leukocytes and platelets adhesion, coagulation cascade and thrombosis initiation, vascular impermeability, and blood and lymphatic vessels hyper-proliferation. All these changes work towards fast delivery of leukocytes at infected sites, transportation of inflammatory cells to affected areas, isolation of damaged site from systemic circulation and healthy tissues, dilution of harmful agents in affected areas, and regulation of the regeneration and repair processes. Since inflammation is a predominant cause of many pathological conditions, exploring the impact of inflammation directly on the microcirculation clarifies the pathophysiology and the effect of inflammation as it takes place. To our knowledge, wild mushroom extracts being tested in mice micro-circulation is also a novel study.

Conducted vasodilation experiments were also consistent with the previous studies in which the local response was consistently less than the conducted response (500 μm). The histamine super-perfusion consistently impaired the coordinated vasodilation, indicating the impact of inflammation in decreasing the coordinated response. These findings are also

consistent with the previous work on the cremaster muscles of mice, indicating the

reproducibility of the data in gluteus maximus muscle (Payne et al., 2004; Payne, 2006). One of

the reasons for the diminished response could be attributed to the fact that the presence of the

inflammatory mediators may disrupt the cellular signaling transmission by causing structural

alterations in the endothelial layer (e.g. causing hyper-permeability or changes in gap junction

assembly) or by enhancing the production of harmful cytokines (such as, ROS or NO), therefore

promoting the hyper-activated state and causing imbalance.

Moreover, the current study builds upon the findings from the previous studies and

explores the role of novel wild mushrooms extracts that have demonstrated potent anti-

inflammatory potential in *in-vitro* models. The methanolic extract (Chaga-F2) was applied in

crude form while the 5% NaOH extract (*E.tinc.* -PL20) was applied after the first purification

step (i.e. after size exclusion chromatography-Post LH-20; Chapter 3). This post LH-20 extract

has already demonstrated a higher potency in the *in-vitro* settings as compared to the crude

extract (Chapter 3). In the *in vivo* settings, this semi-purified extract has demonstrated a slightly

higher response at the same dose as compared to the crude (Chaga-F2) extract but as the

mushrooms species are different, these two responses are not comparable with one another.

The fact that the respective fungal extracts consistently and dose-dependently inhibited

LPS- and histamine-induced TNF-α production in RAW 264.7 cells (please refer to Chapter 2)

and that these anti-inflammatory activities were also confirmed in mice microcirculation model,

shows that indeed the mouse macrophage cell line is a useful model as a first step for screening

for anti-inflammatory compounds. Our study confirms the anti-inflammatory activity of *I.*

obliquus which is consistent with previous studies (Debnath et al., 2012, Ma et al., 2013, Park *et*

al., 2005; Van *et al*., 2009). This study shows for the first time the novel and previously unknown

anti-inflammatory potential of 5% NaOH extract of *E. tinctorium* (*E.tinc.* -PL20) in ameliorating the negative impact of inflammation in both *in vitro* and *in vivo* settings.

This study provides a framework for exploring mushroom extracts in mice gluteus maximus micro-circulation model. Future studies could explore the specific mechanism of action of these extracts in perfecting the diminished conducted response. Following the model study by Looft-Wilson et al. (2004), future studies could also look into the role of special gap junctions' protein, Connexin, and its three isoforms (i.e. Cx37, Cx40, an Cx43) in diminished (i.e. inflammation) and re-established (i.e. anti-inflammation) conducted response. As the semi-purified mushroom extract (*E.tinc*. -PL20) has two anti-inflammatory compounds (the larger molecular weight (~50-200 kDa) and the smaller molecular weight (~5-25 kDa) compound), future studies can establish their respective roles in micro-circulation and anti-inflammatory potential. More extensive studies could also involve the use of inflammation-induced mouse models (e.g. colorectal cancer mouse model) to study the effect of potent extracts in treating inflammation-associated tumors which will be helpful in the prevention and treatment of chronic inflammatory states and the tumors associated with these hyper-active immune conditions.

Chapter 5: General Discussion & Future Directions

5.1 General Discussion

5.1.1 Screening BC wild mushrooms for Immuno-stimulatory and Anti-inflammatory activities

One of the major aims of this MSc thesis was to study some of the wild mushrooms collected from Northern BC forests for their immuno-modulatory potential. Forty-four mushroom extracts were tested for immuno-stimulatory activity and thirty-one mushroom extracts were evaluated for their anti-inflammatory ability. Crude mushrooms extracts were screened on macrophage (RAW 264.7) cells for both immuno-stimulatory and anti-inflammatory potential where the activation of cells and production of TNF-α was observed in the former (immuno-stimulatory) and the inhibition of previously activated cells and suppression of excessively stimulated TNF-α was quantified in latter (anti-inflammatory) assays. After successful screening, the potent anti-inflammatory NaOH extract from *E. tinctorium* was selected for further purification and characterization. In addition, the semi-purified extract of *E. tinctorium* was further studied for anti-inflammatory activity *in vivo* using a mouse microcirculation model.

Recent studies have applied various *in vitro* techniques to explore novel bioactive compounds from medicinal mushrooms. Thus, the objective of the present study was to reveal the immuno-stimulatory potential of ten wild mushroom species collected from across the forests of Northern British Columbia: *F. pinicola, L. edodes, P. betulinus, F. fomentarius, G. applanatum, E. tinctorium, I. obliquus, L. connata, L. sulphureus,* and *P. nigricans.* The extracts that failed to exhibit immuno-stimulatory potential, which include extracts from 4 other species extracted by fellow graduate students, *P. igniarius, T. abietinum, H. coralloides, L. vulpina,* were further studied for anti-inflammatory potential. This study was carried out to demonstrate the

potential of wild mushrooms from a unique and largely untapped region of the world, British Columbia (BC), for discovering novel mushroom extracts that could contain immuno-modulatory compound(s) for natural product drug discovery.

The results from the screening study on mushroom extracts demonstrated that indeed Northern BC's wild mushrooms have enormous potential for the discovery of immuno-modulatory compounds. Out of forty-four extracts tested, nine have shown at least moderate (≥40% stimulation) immuno-stimulation potential. Among them, four extracts, *L. edodes* (F2), *G. applanatum* (F3), *E. tinctorium* (F3) and *L. sulphureus* (F3), have shown very strong (≥90%) immuno-stimulatory activity. Out of thirty-one extracts tested, twenty-two extracts have shown at least moderate (≥40% inhibition) anti-inflammatory activity. Among them, nineteen extracts from 11 species, *F. pinicola* (F2, F3), *P. betulinus* (F1, F2, F3), *F. fomentarius* (F1, F2), *G. applanatum* (F1, F2), *E. tinctorium* (F5), *I. obliquus* (F1-F4), *L. connata* (F1), *L. sulphureus* (F1), *P. nigricans* (F2), *P. igniarius* (F1), and *T. abietinum* (F1), have demonstrated very strong (≥90%) anti-inflammatory activity. The anti-inflammatory activity was mostly confined to the ethanolic and methanolic extracts of the mushrooms. The fact that there were more extracts active for the anti-inflammatory assay than immuno-stimulatory activity could indicate that Northern BC wild mushrooms might have greater potential for the anti-inflammatory compound(s) than immuno-stimulatory ones. However, the reasons for this remain unknown. The screening results have implications for future study of mushroom extracts and their potential bioactive compounds that can be used to aid both suppressive and hyper-active states of the immune system.

Bernard Shaw, in "The Doctor's Dilemma", said: "*Nature has provided, in the white corpuscles as you call them, in the phagocytes as we call them, a natural means of devouring*

and destroying all disease germs. There is at bottom, only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes". In conformity with this quote and previous studies, the current study established the application of phagocytes (macrophages-RAW 264.7 cells) in testing *in vitro* immuno-modulatory potential.

The present study also reinforced the theory of stimulating macrophages in response to fungal extracts to stimulate TNF-α production as the quantification marker of the extract's immuno-stimulatory potential. Furthermore, lipopolysaccharide (LPS) successfully established the positive reference scale for comparing the immuno-stimulatory potential of the extracts. LPS is present in the gram-negative bacterial cell wall. It binds to the Toll Like Receptor (TLR-4) on the CD-14 receptor of macrophages. This binding is aided by 2 proteins CD14 and MD-2. This complex triggers the downstream transcription factor, nuclear factor kappa-β (NF-κβ) that regulates the expression of target genes that encodes and activates the release of various pro-inflammatory cytokines including TNF-α (Solov'eva et al., 2013).

The current project also showed that, when macrophage RAW 264.7 cells were stimulated with LPS, the enhanced release of pro-inflammatory cytokines (such as TNF-α) could be inhibited by the mushroom extracts as a measure of their anti-inflammatory potential. Polymyxin B (PMB) resulted in a reduction of TNF-α production in LPS-stimulated macrophages, therefore providing a scale for equating anti-inflammatory potential of the extracts. Conceptually, PMB inhibits LPS-induced TLR-4 activation by forming a LPS-PMB complex, which disrupts the bacterial cell wall. This ultimately inhibits the LPS-induced cytokines release in downstream pathway (Yu et al., 2015). The current study adopted the use of PMB as a negative control from these prior studies (Lei et al., 2015).

For the *in vitro* anti-inflammatory experiments exploring the potential of *I. obliquus* (F2) and *E. tinctorium* (F4) in inhibiting histamine-induced TNF-α, both extracts successfully inhibited the induced TNF-α production and therefore have shown similar results as were shown in experiments with LPS-induced TNF-α production. This experiment was used to simulate *in vivo* model conditions (i.e. histamine-induced inflammatory event as opposed to LPS-induced inflammatory response). The results with the histamine dose response in RAW 264.7 cells were surprising, as histamine did not stimulate the release of TNF-α at higher doses (1-10^{-4} M) and only stimulated the release at lower doses (10^{-5} and 10^{-6} M). This experiment was repeated four times and similar results were obtained. There was no cytotoxicity in higher doses so the inability of the histamine to stimulate macrophages cannot be attributed to the cell death. However, to our knowledge, this study is the first of its kind to explore the role of histamine in mouse macrophages RAW 264.7 cells. There is one similar study in human liver macrophages in which histamine was used to induce exocytosis and stimulate the release of pro-inflammatory cytokines (IL-6). Interestingly, in that study, 10^{-6} M histamine stimulated the maximum release of the cytokine, which is in agreement with the present study (Triggiani et al., 2001).

Moreover, this research provides pioneering protocols in assessing anti-inflammatory potential of wild mushrooms in an *in vitro* setting and both anti-inflammatory models (i.e. LPS induced TNF-α production and histamine induced TNF-α production) are the first ones used in Dr. Lee's lab. This provides a framework for the screening of other wild fungi for their anti-inflammatory ability. Another important finding that arose during the development of the protocol was the observation that ethanol, DMEM and Sodium Chloride (NaCl) could inhibit the LPS-induced TNF-α. Thus, these solvents (ethanol and DMEM) should be avoided for re-suspending the mushroom extracts, and the 5% NaOH extract and the NaCl eluents from the ion-

exchange column should be thoroughly dialysed as to avoid any salt in the sample, thereby, circumventing any negative hits.

5.1.2 Purification and characterization of anti-inflammatory compounds from *Echinodontium tinctorium*

The results of the purification study presented a successful method for isolating polysaccharide(s) from a potent extract of *E. tinctorium* (F4). The purification study after the size exclusion and Superdex-200 (Post LH-20 + Post Superdex-200) revealed that there were 2 bioactive anti-inflammatory compounds present in the semi-purified extract as 2 peaks were observed. The higher molecular weight compound (~50-200kDa) was more potent than the lower molecular weight compound (~5-25kDa). The results from carbohydrate assays on these fractions suggested that both compounds have carbohydrate content. The results from protein assays on these fractions suggested that only the larger molecular weight compound has protein, hence, the higher molecular weight compound is a potential protein-bound polysaccharide, while the smaller molecular weight compound is only a carbohydrate.

The results from size exclusion, ion exchange and Superdex-200 (Post LH-20 + Post DEAE + Post Superdex-200) demonstrated a single peak suggesting that the ion exchange column (DEAE) was able to purify the smaller molecular weight anti-inflammatory molecule (~5-25 kDa). The characterization of this smaller molecular weight compound was performed by Dr. Maggie Li in Dr. Peter Cheung's lab (Hong-Kong). Preliminary results showed the presence of glucose and other mono-saccharides with 1-3, 1-6, and 1-3-6 linkages. However, there is still a need to devise a method to verify the purity of this purified anti-inflammatry sample using High Performance Gel Permeation Chromatography (HPGPC). Moreover, the configuration of this purified glucan (i.e. alpha or beta) also needs to be determined using FTIR analysis. There is also a need to develop the method to isolate the more potent and larger molecular weight

polysaccharide from the semi-purified sample. To the best of our knowledge, this is the first study to reveal anti-inflammatory compound(s) from *E. tinctorium*.

The ion exchange and size-exclusion columns used to purify the potential anti-inflammatory compound(s) had limited capacity. Only a few milligrams (e.g. 630 mg in case of 450 mL size exclusion column) of crude or semi-purified material could be loaded on the column each time as overloading would halt the column ability to cause effective separation. This would cause a low yield of the active compound(s) and therefore multiple runs of the same column were needed. These cycles were repeated several times to attain the adequate amount of the product, which is indeed laborious and time consuming.

Echinodontium tinctorium is commonly known as Indian paint fungus and, living up to its name, produces a color pigment that created one of the biggest challenges in this project: chromatographic column cleaning. The mushroom stained the column and it did not become clean even with multiple washes of ethanol, methanol, acetone and water. The pre-loaded mushroom fraction was also de-colorized with charcoal treatment, but only a very small change in the color was observed and the original dark reddish color of the extract remained. When the column was cleaned with 1% bleach solution, the color on the column washed away quickly. The activity from the column remained reproducible (i.e. the activity peak remained same) but when the active fractions were pooled and lyophilized, there was a significant decrease in the percent yield of the final product. Finally, washing the column with 0.1 M NaOH was attempted which left a pink stain on the column that was easily washed away with water.

5.1.3 An Intravital Microscopy method to study anti-inflammatory activity of Chaga extracts and purified compound from *E. tinctorium*

Results of the experiments in the *in vivo* model reiterated the strong *in vitro* anti-inflammatory potential of both *I. obliquus* and *E. tinctorium* as both of these extracts

successfully and reproducibly reversed the histamine-induced inflammatory event in mice micro-circulation. One of the major challenges in the intravital microscopy was the generation of intact muscle preparation without inducing inflammatory events from the outside environment (i.e. surgicals). The results indicate that, as the cellular communication was blocked up-stream by histamine-induced inflammatory events, both mushroom extracts were able to normalize the abated communication separately when tested with histamine. Chaga (*I. obliquus)* was used as a reference in this study because of existing literature in support of its anti-inflammatory potential. Also, this F2 extract of *I. obliquus* was most potent in inhibiting LPS -induced TNF-α production in our *in vitro* LPS model. This was also active in our *in vitro* histamine experiments. This study is the first of its kind in studying mushroom extracts for their anti-inflammatory activity in mice micro-circulation.

5.1.4 Conclusions

One of the limitations associated with this study could be the route of administration of the active polysaccharide. Normally, polysaccharides are hypothesized to be mal-absorbed by the gut, therefore leading to a potential problem in the pharmacokinetics studies during pharmaceutical dosage form development. There is, however, some literature on the efficacy of the active polysaccharide after oral administration. Liu et al. (1993) observed that when PSP was given in drinking water to C57BL/6 mice, their peritoneal macrophages showed an enhanced level of RNI, RON, TNFα, and other transcription factors, suggesting the enhanced immuno-stimulatory response of the absorbed PSP. Similarly, Harada et al. (1997) observed the effect of oral administration of PSK on the anti-tumor T cell response in gut-associated lymphoid tissue (GALT). They observed the impaired anti-tumor CD4 [+] T cell response in GALT to produce Th1-type cytokines. The results reinforced the efficacy of PSK upon oral administration. Likewise, Deng et al. (2009) observed the immunological effects of a polysaccharide extract from *G.*

frondosa in breast cancer patients in phase 1 and 2 pre-clinical trials. The post-menopausal women were given various doses of 0.1, 0.5, 1.5, 3, or 5 mg/kg of extract twice a day for 3 weeks and their peripheral blood samples were collected. The results suggested the association of the extract with various immunological effects on the patients, highlighting the effect of oral polysaccharide in the cancer patients.

This research project on the screening of BC wild mushrooms, their purification and chemical characterization, and their elucidation in an *in vivo* model is an extensive project that demands multi-disciplinary expertise from mycologists, biochemists, chemists, physiologists and pharmaceutical science experts. The present study has successfully screened BC mushrooms for their immuno-modulatory abilities, re-extracted the potent extract, purified and separated the potent anti-inflammatory compound(s) and has tested the potent extracts *in vivo*.

5.2 Future Directions

Future directions for this extensive research project require further investigations into the mechanism of action of the purified anti-inflammatory compound. The role of the active compound in inhibiting TNF-α should be explored for a profound understanding of the anti-inflammatory response (summarized in Figure 5.1). TNF-α exerts its effect by acting on TNF receptor 1 or 2 (TNFR-1 or TNFR-2). This complex activates either receptor interacting protein (RIP) or mitogen-activated protein kinase- extracellular signal-regulated kinase kinase (MEKK). RIP in-turn activates IkB kinase (IKK) that ultimately activates transcription factor NF-κβ. RIP also activates other MEKK and activates mitogen activated protein kinase (MAPK). Similarly, MEKK initiates the c-Jun N-terminal protein kinase cascade that activates AP-1 and plays a role in inflammation (Wu & Zhou, 2010).

Extensive research has proven that the major inflammatory cascade after activation of TNF-α is the activation of transcription factor NF-κβ that enhances the expression of pro-inflammatory cytokines genes expression such as; inducible nitric oxide synthase (iNOS), Vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) (Chen & Goeddel, 2002; D'Acquisto et al., 2002; Tak & Firestein, 2001; Wu & Zhou, 2010). Thus, inhibiting TNF-α could demonstrate anti-inflammatory responses by inhibiting NF- κβ that could halt the expression of pro-inflammatory cytokines release and ameliorate the induced inflammation. Figure 5.1 demonstrates the summary of some of the convincing TNF-α pathway studies in initiating inflammation.
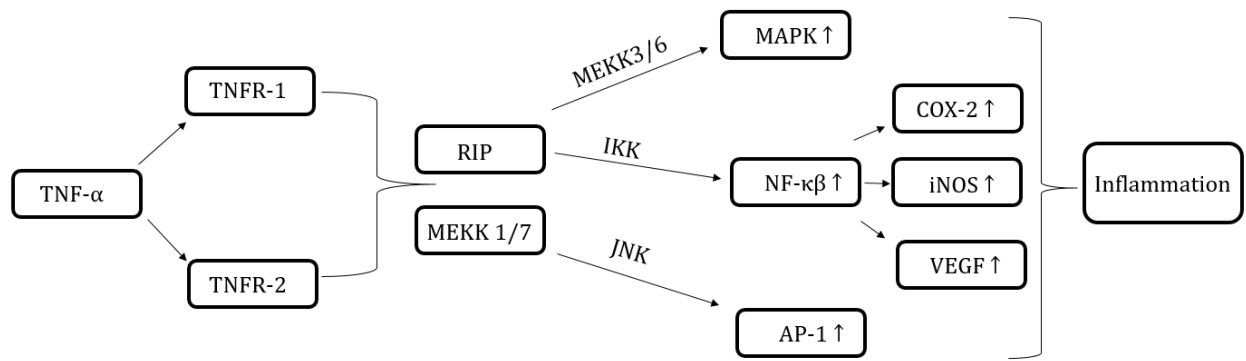
Figure 5.1. Summary of the TNF-α activation pathways and its role in inflammation. The above flow-chart is a representation adopted from the research work of Chen & Goeddel (2002); Tak & Firestein (2001); Wu & Zhou, (2010).

Mushroom researchers have explored the NF-κβ pathway in LPS-induced RAW 264.7 cells for the demonstration of anti-inflammatory pathways (Kim et al., 2007; Shao et al., 2015). Western blotting could be used in determining the expression of MAPK, IKK, AP-1 or NF-κβ to have a deeper understanding (Jedinak et al., 2011; Kim et al., 2007; Shao et al., 2015). The expression of iNOS can also be observed as an indicator of the anti-inflammatory action of TNF-α in reducing NO levels (an important inflammatory agent). In the current study, the NO

inhibition assay showed that the purified lower molecular weight compound from *E. tinctorium* successfully inhibited LPS-induced NO, which supports the involvement of the NF-κβ pathway in the inhibition of TNF-α. However, further studies are needed to understand the exact mechanism of action of both higher and lower molecular weight anti-inflammatory compounds identified in the current research project. There is also a dire need to determine the exact chemical structure of the responsible polysaccharide so that the mechanism of action could be justified. This will also be useful for the structure activity relationships and developing the anti-inflammatory drugs with maximum efficacy and minimum toxicity. Therefore, a thorough and extensive chemical characterization is required for both bioactive compound(s). The exact chemical structure will be useful in exploring both *in vitro* as well as *in vivo* drug response.

As the *E. tinctorium* extract used in *in vivo* studies has both larger (50-200kDa) and smaller molecular weight compound(s) (5-25kDa) (demonstrated by Post LH-20 + Post Superdex-200), it would be interesting to apply the large and small molecular weight compounds in their pure state to see whether the responsible compound can generate the *in-vivo* response in mice micro-circulation and other inflammatory animal model.

Ultimately, after completing these preliminary studies, chemically characterizing the polysaccharide and determining its respective mechanism of action, as well as incorporating other animal studies will allow for further investigations into possible clinical applications of the mushroom bioactive compounds in treating inflammation. It is only possible when every aspect of the inflammatory pathophysiology is kept in focus to target various down-stream mechanisms. These reflections will also be important in designing the preventive and prophylaxis approaches (using mushroom extracts) against inflammation and applying this concept to a larger population. Indeed, the take-home message from this effort of bridging the practices against

treating chronic inflammatory conditions would be the simple fact that "Inflammatory conditions are preventable". Preventing inflammation will help with the prophylaxis of certain chronic disease states like arthritis, chronic heart diseases, diabetes etc. (Esser et al., 2014). Therefore, treating inflammation at the right time with the right pharmacological interventions may potentially inhibit pathways that can lead to development of these serious pathologies.

Future research efforts should continue to focus on identifying new bioactive molecules from natural products research and in discovering molecules from wild mushrooms to target specific inflammatory events for the therapeutic purposes. The focus of the studies should also be on the possibility of studying single chemical target/mediator intervention vs. multiple targets/mediators for better synergistic outcomes in clinical settings. This is just the beginning of research on the immuno-modulatory compound(s) from Northern British Columbian wild mushrooms and the future shows a lot of promise. The current need is to carefully process the promising extracts for purification and other molecular studies so that the distance from bench to bedside could be reduced significantly, as Isaac Newton said:

"I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me".

References

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2014). Cellular and Molecular Immunology: with Student Consult Online Access. *Elsevier Health Sciences*.

Aho, P. E., Filip, G. M., & Lombard, F. F. (1987). Decay fungi and wounding in advance grand and white fir regeneration. *Forest Science*, 33(2), 347-355.

Ali, R., Mirza, Z., Ashraf, G. M., Kamal, M. A., Ansari, S. A., Damanhouri, G. A., Abuzenadah, A.M., Chaudhary, A.G. and & Sheikh, I. A. (2012). New anticancer agents: recent developments in tumor therapy. *Anticancer Research*, 32(7), 2999-3005.

Allendorf, D. J., Yan, J., Ross, G. D., Hansen, R. D., Baran, J. T., Subbarao, K., ... & Haribabu, B. (2005). C5a-mediated leukotriene B4-amplified neutrophil chemotaxis is essential in tumor immunotherapy facilitated by anti-tumor monoclonal antibody and β-glucan. *The Journal of Immunology*, 174(11), 7050-7056.

Arora, D. (1986). Mushrooms demystified. A comprehensive guide to the fleshy fungi. Berkeley, California: Ten Speed Press.

Atanasiu, P., & Chany, C. (1960) Action d'un interféron provenant de cellules malignes sur l'infection expérimentale du hamster nouveau-né par le virus du polyome, *C. R. Acad. Sci.* 251(16), 1687–1689.

Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *The Lancet*, 357(9255), 539-545.

Balkwill, F. (2006) TNF-α in promotion and progression of cancer. *Cancer and Metastasis Reviews,* 25(3), 409-416.

Barrett, D. M., Singh, N., Porter, D. L., Grupp, S. A., & June, C. H. (2014). Chimeric antigen receptor therapy for cancer. *Annual Review of Medicine*, 65, 333-347.

Bearden, S.E., & Segal, S.S. (2004). Microvessels promote motor nerve survival and regeneration through local VEGF release following ectopic attachment. *Microcirculation*, 11(8), 633-644.

Bisen, P. S., Baghel, R. K., Sanodiya, B. S., Thakur, G. S., & Prasad, G. B. K. S. (2010). *Lentinus edodes*: a macrofungus with pharmacological activities. *Current Medicinal Chemistry*, 17(22), 2419-2430.

Blaber, M., & Harvey, D. (2016). *Ion Exchange Chromatography*. *Chemistry LibreTexts*. Retrieved 21 May 2017, from https://chem.libretexts.org/Core/Analytical_Chemistry/Instrumental_Analysis/Chromatography/Liquid_Chromatography/Ion_Exchange_Chromatography

Blanchette, R. A., Compton, B. D., Turner, N. J., & Gilbertson, R. L. (1992). Nineteenth century shaman grave guardians are carved *Fomitopsis officinalis* sporophores. *Mycologia*, 84(1), 119-124.

Bogdan, C. (2015). Nitric oxide synthase in innate and adaptive immunity: an update. *Trends in Immunology*, 36(3), 161-178.

Bologna, L., Gotti, E., Manganini, M., Rambaldi, A., Intermesoli, T., Introna, M., & Golay, J. (2011). Mechanism of action of type II, glycoengineered, anti-CD20 monoclonal antibody GA101 in B chronic lymphocytic leukemia whole blood assays in comparison with rituximab and alemtuzumab. *The Journal of Immunology*, 186(6), 3762-3769.

Borchers, A. T., Keen, C. L., & Gershwin, M. E. (2004). Mushrooms, tumors, and immunity: an update. *Experimental Biology and Medicine*, 229(5), 393-406.

Borchers, A. T., Krishnamurthy, A., Keen, C. L., Meyers, F. J., & Gershwin, M. E. (2008). The immunobiology of mushrooms. *Experimental Biology and Medicin*e, 233(3), 259-276.

Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P. O., Bergamini, G., Croughton, K., ... & Hopf, C. (2004). A physical and functional map of the human TNF-α/NF-κB signal transduction pathway. *Nature Cell Biology*, 6(2), 97-105.

Braczkowski, R., Zubelewicz, B., Romanowski, W., Lissoni, P., & Brivio, F. (1998). Possible biological indicators of the response to recombinant tumour necrosis factor alpha in patients with advanced neoplasms. *Journal of Experimental & Clinical Cancer Research*, 17(3), 349-354.

Canadian Cancer Statistics (2016) Canadian Cancer Society's Advisory Committee on Cancer Statistics. Toronto, ON: Canadian Cancer Society; 2016.

Carswell, E. A., Old, L. J., Kassel, R., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences*, 72(9), 3666-3670.

Chang ST, & Miles PG 1992. Mushrooms biology -a new discipline. *Mycologist* 6(2), 64-65.

Chang, H. H., & Sheu, F. (2007). A novel fungal immuno-modulatory protein (PCP) isolated from *Poria cocos* activates mouse peritoneal macrophage involved in toll-like receptor 4. *The Faseb Journal*, 21(6), A738-A738.

Chang, S. T., & Wasser, S. P. (2012). The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. *International journal of Medicinal Mushrooms*, 14(2) 95-134.

Chen, G., & Goeddel, D.V. (2002). TNF-R1 signaling: a beautiful pathway. *Science*, 296(5573), 1634-1635.

Chen, J., & Seviour, R. (2007). Medicinal importance of fungal β-(1→ 3), (1→ 6)-glucans. *Mycological Research*, 111(6), 635-652

Chen, W., Zhao, Z., & Li, Y. (2011). Simultaneous increase of mycelial biomass and intracellular polysaccharide from *Fomes fomentarius* and its biological function of gastric cancer intervention. *Carbohydrate Polymers*, 85(2), 369-375.

Chen, W., Zhao, Z., Chen, S. F., & Li, Y. Q. (2008). Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect in vitro. *Bioresource Technology*, 99(8), 3187-3194.

Cheng, J. J., Lin, C. Y., Lur, H. S., Chen, H. P., & Lu, M. K. (2008). Properties and biological functions of polysaccharides and ethanolic extracts isolated from medicinal fungus, *Fomitopsis pinicola*. *Process Biochemistry*, *43*(8), 829-834.

Chu, C. H., Ye, S. P., Wu, H. C., & Chiu, C. M. (2015). The *Ganoderma* extracts from tein-shan *Ganoderma* capsule suppressed lps-induced nitric oxide production in RAW 264. 7 cells. *MC-Transaction on Biotechnology*, 7(2015), 1-11.

Chu, W. M. (2013). Tumor necrosis factor. *Cancer Letters*, *328*(2), 222-225.

Coley, W. B. (1893). The treatment of malignant tumors by repeated inoculations of erysipelas: with a report of ten original cases. *The American Journal of the Medical Sciences*, 105(5), 487-510.

Comandini, O., Erős-Honti, Z., Jakucs, E., Arzú, R. F., Leonardi, M., & Rinaldi, A. C. (2012). Molecular and morpho-anatomical description of mycorrhizas of *Lactarius rimosellus* on *Quercus* sp., with ethnomycological notes on *Lactarius* in Guatemala. *Mycorrhiza*, 22(4), 279-287.

Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1830(6), 3670-3695.

Cui, J., & Chisti, Y. (2003). Polysaccharopeptides of *Coriolus versicolor*: physiological activity, uses, and production. *Biotechnology Advances*, 21(2), 109-122.

D'Acquisto, F., May, M. J., & Ghosh, S. (2002). Inhibition of nuclear factor kappa B (NF-B). *Molecular Interventions*, 2(1), 22-35.

Dai, X., Stanilka, J. M., Rowe, C. A., Esteves, E. A., Nieves Jr, C., Spaiser, S. J., ... & Percival, S. S. (2015). Consuming *Lentinula edodes* (Shiitake) mushrooms daily improves human immunity: A randomized dietary intervention in healthy young adults. *Journal of the American College of Nutrition*, 34(6), 478-487.

Dealtry, G. B., Naylor, M. S., Fiers, W., & Balkwill, F. R. (1987). The effect of recombinant human tumour necrosis factor on growth and macromolecular synthesis of human epithelial

cells. *Experimental Cell Research*, 170(2), 428-438.

Debnath, T., Hasnat, M. A., Pervin, M., Lee, S. Y., Park, S. R., Kim, D. H., ... & Lim, B. O. (2012). Chaga mushroom (*Inonotus obliquus*) grown on germinated brown rice suppresses inflammation associated with colitis in mice. *Food Science and Biotechnology*, 21(5), 1235-1241.

Deng, G., Lin, H., Seidman, A., Fornier, M., D'Andrea, G., Wesa, K., ... & Cassileth, B. (2009). A phase I/II trial of a polysaccharide extract from *Grifola frondosa* (Maitake mushroom) in breast cancer patients: immunological effects. *Journal of Cancer Research and clinical oncology*, 135(9), 1215-1221.

Dudhgaonkar, S., Thyagarajan, A., & Sliva, D. (2009). Suppression of the inflammatory response by triterpenes isolated from the mushroom *Ganoderma lucidum*. *International Immunopharmacology*, 9(11), 1272-1280.

El Enshasy, H. A., & Hatti-Kaul, R. (2013). Mushroom immunomodulators: unique molecules with unlimited applications. *Trends in Biotechnology*, 31(12), 668-677.

Elsayed, E. A., El Enshasy, H., Wadaan, M. A., & Aziz, R. (2014). Mushrooms: a potential natural source of anti-inflammatory compounds for medical applications. *Mediators of Inflammation* 2014 http://dx.doi.org/10.1155/2014/805841

Espenshade, M. A., & Griffith, E. W. (1966). Tumor-inhibiting basidiomycetes. Isolation and cultivation in the laboratory. *Mycologia*, 58(4) 511-517.

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A. J., & Paquot, N. (2014). Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Research and Clinical Practice*, 105(2), 141-150.

Ferreira, I., A Vaz, J., Vasconcelos, M. H., & Martins, A. (2010). Compounds from wild mushrooms with antitumor potential. *Anti-Cancer Agents in Medicinal Chemistry,* 10(5), 424-436.

Førland, D. T., Johnson, E., Saetre, L., Lyberg, T., Lygren, I., & Hetland, G. (2011). Effect of an extract based on the medicinal mushroom *Agaricus blazei* Murill on expression of cytokines and calprotectin in patients with ulcerative colitis and Crohn's disease. *Scandinavian Journal of Immunology*, 73(1), 66-75.

Fu, H., Deng, C., Teng, L., Yu, L., Su, T., Xu, X., ... & Yang, C. (2015). Immunomodulatory activities on RAW 264.7 macrophages of a polysaccharide from veiled lady mushroom, *Dictyophora indusiata* (Higher basidiomycetes). *International journal of Medicinal Mushrooms*, 17(2) 151-160.

Fudenberg, H. H. (Ed.). (2012). Immunomodulation: New Frontiers and Advances. *Springer Science & Business Media*.

Furman, W. L., Strother, D., McClain, K., Bell, B., Leventhal, B., & Pratt, C. B. (1993). Phase I clinical trial of recombinant human tumor necrosis factor in children with refractory solid tumors: a Pediatric Oncology Group study. *Journal of Clinical Oncology*, 11(11), 2205-2210.

Ganetsos, G., & Barker, P.E.(Eds.) (1992). Preparative and production scale chromatography (Vol. 61). CRC Press.

Gao, H. L., Lei, L. S., Yu, C. L., Zhu, Z. G., Chen, N. N., & Wu, S. G. (2009). Immunomodulatory effects of *Fomes fomentarius* polysaccharides: an experimental study in mice. *Nan fang yi ke da xue xue bao= Journal of Southern Medical University*, 29(3), 458-461.

Gao, J. J., Min, B. S., Ahn, E. M., Nakamura, N., Lee, H. K., & Hattori, M. (2002). New triterpene aldehydes, lucialdehydes A-C, from *Ganoderma lucidum* and their cytotoxicity against murine and human tumor cells. *Chemical and Pharmaceutical Bulletin*, 50(6), 837-840.

Garibay-Orijel, R., Caballero, J., Estrada-Torres, A., & Cifuentes, J. (2007). Understanding cultural significance, the edible mushrooms case. *Journal of Ethnobiology and Ethnomedicine*, 3(1), 4. **DOI:** 10.1186/1746-4269-3-4

Gavins, F. N., & Chatterjee, B. E. (2004). Intravital microscopy for the study of mouse microcirculation in anti-inflammatory drug research: focus on the mesentery and cremaster preparations. *Journal of Pharmacological and Toxicological Methods*, 49(1), 1-14.

Gibson, I., Gibson, E., & Kendrick, B. (2010). MatchMaker: mushrooms of the Pacific Northwest.

Granger, D. N., & Senchenkova, E. (2010). Inflammation and the Microcirculation. *In* Colloquium Series on Integrated Systems Physiology: From Molecule to Function (Vol. 2, No. 1, pp. 1-87). Morgan & Claypool Life Sciences.

Gregory, F. J., Healy, E. M., Agersborg, H. P. K., & Warren, G. H. (1966). Studies on antitumor substances produced by Basidiomycetes. *Mycologia*, 58(1) 80-90.

Grienke, U., Zöll, M., Peintner, U., & Rollinger, J. M. (2014). European medicinal polypores–A modern view on traditional uses. *Journal of Ethnopharmacology*, 154(3), 564-583.

Gross, H. L. (1964). The Echinodontiaceae. *Mycopathologia et Mycologia Applicata*, 24(1), 1-26.

Günç Ergönül, P., Akata, I., Kalyoncu, F., & Ergönül, B. (2013). Fatty acid compositions of six wild edible mushroom species. *The Scientific World Journal* (2013).

Han, J., Chen, Y., Bao, L., Yang, X., Liu, D., Li, S., et al. (2013). Anti-inflammatory and cytotoxic cyathane diterpenoids from the medicinal fungus *Cyathus africanus*. *Fitoterapia*, 84, 22-31.

Harada, M., Matsunaga, K., Oguchi, Y., Iijima, H., Tamada, K., Abe, K., ... & Nomoto, K. (1997). Oral administration of psk can improve the impaired anti-tumor CD4+ T-cell response in gut-associated lymphoid tissue (GALT) of specific-pathogen-free mice. *International Journal of Cancer*, 70(3), 362-372.

Harlan J, Liu DY. (1992) *In vivo* models of leukocyte adherence to endothelium. In: Adhesion: Its role in inflammatory disease. Chapt. 6, pp. 117–76, W.H. Freeman & Co., New York.

Hawksworth, D. L. (2012). Global species numbers of fungi: are tropical studies and molecular approaches contributing to a more robust estimate? *Biodiversity and Conservation*, 21(9), 2425-2433.

Hazama, S., Watanabe, S., Ohashi, M., Yagi, M., Suzuki, M., Matsuda, K., ... & Oka, M. (2009). Efficacy of orally administered superfine dispersed lentinan (β-1, 3-glucan) for the treatment of advanced colorectal cancer. *Anticancer Research*, 29(7), 2611-2617.

Higashi, D., Seki, K., Ishibashi, Y., Egawa, Y., Koga, M., Sasaki, T., ... & Sudo, M. (2012). The effect of lentinan combination therapy for unresectable advanced gastric cancer. *Anticancer Research*, 32(6), 2365-2368.

Hobbs, C. (1995). Medicinal Mushrooms: An Exploration of Tradition, Healing and Culture. Botanica Press, Santa Cruz.

Hobbs, C. (2005). The chemistry, nutritional value, immunopharmacology, and safety of the traditional food of medicinal split-gill fungus *Schizophyllum commune* Fr.: Fr. (Schizophyllaceae). A literature review. *International Journal of Medicinal Mushrooms*, 7(1&2), 127-140.

Högberg, N., Kroken, S., Thor, G., & Taylor, J. W. (2002). Reproductive mode and genetic variation suggest a North American origin of European *Letharia vulpina*. *Molecular Ecology*, *11*(7), 1191-1196.

Hopkins, P. M. (2006). Skeletal muscle physiology. *Continuing Education in Anaesthesia, Critical Care & Pain*, *6*(1), 1-6.

Hsu, H. Y., Hua, K. F., Wu, W. C., Hsu, J., Weng, S. T., Lin, T. L., ... & Huang, C. T. (2008). Reishi immuno-modulation protein induces interleukin-2 expression via protein kinase-dependent signaling pathways within human T cells. *Journal of Cellular Physiology*, 215(1), 15-26.

Huryn, D. M., & Wipf, P. (2014). Natural Product Chemistry and Cancer Drug Discovery In

S. Neidle (Ed.) Cancer Drug Design and Discovery (pp. 91-120) San Diego SD: Academic Press

Isoda, N., Eguchi, Y., Nukaya, H., Hosho, K., Suga, Y., Suga, T., ... & Sugano, K. (2008). Clinical efficacy of superfine dispersed lentinan (beta-1, 3-glucan) in patients with hepatocellular carcinoma. *Hepato-gastroenterology*, 56(90), 437-441.

Janeway, C. A., Travers, P., Walport, M., & Shlomchik, M. J. (1997). *Immunobiology: the Immune System in Health and Disease* (Vol. 1, p. 11). Singapore: Current Biology.

Jayasooriya, R. G. P. T., Kang, C. H., Seo, M. J., Choi, Y. H., Jeong, Y. K., & Kim, G. Y. (2011). Exopolysaccharide of Laetiporus sulphureus var. miniatus downregulates LPS-induced production of NO, PGE 2, and TNF-α in BV2 microglia cells via suppression of the NF-κB pathway. *Food and Chemical Toxicology*, 49(11), 2758-2764.

Jedinak, A., Dudhgaonkar, S., Wu, Q. L., Simon, J., & Sliva, D. (2011). Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-κB and AP-1 signaling. *Nutrition Journal*, 10(1), 52.

Jeong, Y. T., Yang, B. K., Jeong, S. C., Kim, S. M., & Song, C. H. (2008). *Ganoderma applanatum*: a promising mushroom for antitumor and immuno-modulating activity. *Phytotherapy Research*, 22(5), 614-619.

Johnson, E., Førland, D. T., Saetre, L., Bernardshaw, S. V., Lyberg, T., & Hetland, G. (2009). Effect of an extract based on the medicinal mushroom *Agaricus blazei* Murill on release of cytokines, chemokines and leukocyte growth factors in human blood *ex vivo* and *in vivo*. *Scandinavian Journal of Immunology*, 69(3), 242-250.

Jung, M., Liermann, J. C., Opatz, T., & Erkel, G. (2011). Ganodermycin, a novel inhibitor of CXCL10 expression from *Ganoderma applanatum*. *The Journal of Antibiotics*, 64(10), 683-686.

Kakde, D., Jain, D., Shrivastava, V., Kakde, R., & Patil, A. T. (2011). Cancer therapeutics-opportunities, challenges and advances in drug delivery. *Journal of Applied Pharmaceutical Science*, 1(9), 1-10.

Kamo, T., Asanoma, M., Shibata, H., & Hirota, M. (2003). Anti-inflammatory Lanostane-Type Triterpene Acids from *Piptoporus betulinus. Journal of Natural Products,* 66(8), 1104-1106.

Kauserud, H., & Schumacher, T. (2003). Ribosomal DNA variation, recombination and inheritance in the basidiomycete *Trichaptum abietinum*: implications for reticulate evolution. *Heredity*, 91(2), 163-172.

Kim, H. G., Yoon, D. H., Lee, W. H., Han, S. K., Shrestha, B., Kim, C. H., Lim, M.H., Chang, W., Lim, S., Choi, S. & Song, W. O. (2007). *Phellinus linteus* inhibits inflammatory

mediators by suppressing redox-based NF-κB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage. *Journal of Ethnopharmacology*, 114(3), 307-315.

Kim, Y. H., Park, E. G., Batsuren, D., Ganbaatar, J., Nho, C. W., Pan, C. H., & Lee, J. K. (2014). Immune-stimulatory Effects of *Fomes fomentarius* Extract in Murine Macrophages. *Journal of Applied Biological Chemistry*, 57(4), 373-377.

Kimura, C., Nukina, M., Igarashi, K., & Sugawara, Y. (2005). β-Hydroxyergothioneine, a new ergothioneine derivative from the mushroom *Lyophyllum connatum*, and its protective activity against carbon tetrachloride-induced injury in primary culture hepatocytes. *Bioscience, Biotechnology, and Biochemistry*, 69(2), 357-363.

Kuby, J. (1997). Overview of the immune system. *Immunology*, *3*, 3-24.

Kuhnlein, H. V., & Turner, N. J. (1991). Traditional plant foods of Canadian indigenous peoples: nutrition, botany, and use (Vol. 8). Taylor & Francis.

Kvietys, P. R., & Granger, D. N. (2012). Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. *Free Radical Biology and Medicine*, 52(3), 556592.

Lampson, G. P., Tytell, A. A., Nemes, M. M., & Hilleman, M. R. (1963). Purification and characterization of chick embryo interferon. *Proceedings of the Society for Experimental Biology and Medicine*, 112(2), 468-478.

Lange L, Grell MN (2014) The prominent role of fungi and fungal enzymes in the ant-fungus biomass conversion symbiosis. *Applied Microbiology and Biotechnology* 98(11), 4839-4851.

Lange, L. (2014). The importance of fungi and mycology for addressing major global challenges. *IMA Fungus*, 5(2), 463.

Latgé, J. P. (2007). The cell wall: a carbohydrate armour for the fungal cell. *Molecular Microbiology*, 66(2), 279-290.

Lee, W. Y., Park, Y., Ahn, J. K., Ka, K. H., & Park, S. Y. (2007). Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. *Enzyme and Microbial Technology*, 40(2), 249-254.

Lei, W., Browning, J. D., Eichen, P. A., Lu, C. H., Mossine, V. V., Rottinghaus, G. E., Folk, W.R., Sun, G.Y., Lubahn, D.B. & Fritsche, K. L. (2015). Immuno-stimulatory activity of a polysaccharide-enriched fraction of Sutherlandia frutescens occurs by the toll-like receptor-4 signaling pathway. *Journal of Ethnopharmacology*, 172, 247-253.

León, F., Quintana, J., Rivera, A., Estévez, F., & Bermejo, J. (2004). Lanostanoid Triterpenes from *Laetiporus sulphureus* and apoptosis Induction on HL-60 Human Myeloid Leukemia Cells. *Journal of Natural Products*, 67(12), 2008-2011.

Ley K. (2001). History of inflammation research. In: Physiology of Inflammation (Ley K,

ed.), Chapt. 1, pp. 1–10, Oxford Univ Press.

Li, F., Wen, H., Zhang, Y., Aa, M., & Liu, X. (2011). Purification and characterization of a novel immuno-modulatory protein from the medicinal mushroom *Trametes versicolor*. *Science China Life Sciences*, 54(4), 379-385.

Li, Q. Z., Wang, X. F., Bao, T. W., Ran, L., Lin, J., & Zhou, X. (2010b). In vitro synthesis of a recombinant fungal immunomodulatory protein from Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae) and analysis of its immunomodulatory activity. *International Journal of Medicinal Mushrooms*, 12(4), 347-358.

Li, Q., Wang, X., Chen, Y., Lin, J., & Zhou, X. (2010a). Cytokines expression induced by *Ganoderma sinensis* fungal immunomodulatory proteins (FIP-gsi) in mouse spleen cells. *Applied Biochemistry and Biotechnology*, 162(5), 1403-1413.

Li, X., Jiao, L. L., Zhang, X., Tian, W. M., Chen, S., & Zhang, L. P. (2008). Anti-tumor and immunomodulating activities of proteoglycans from mycelium of *Phellinus nigricans* and culture medium. *International Immunopharmacology*, 8(6), 909-915.

Libby, P., Ridker, P. M., Hansson, G. K., & on Atherothrombosis, L. T. N. (2009). Inflammation in atherosclerosis: from pathophysiology to practice. *Journal of the American College of Cardiology*, 54(23), 2129-2138.

Lin, C. J., Lien, H. M., Chang, H. Y., Huang, C. L., Liu, J. J., Chang, Y. C., ... & Lai, C. H. (2014). Biological evaluation of *Phellinus linteus*-fermented broths as anti-inflammatory agents. *Journal of Bioscience and Bioengineering*, *118*(1), 88-93.

Liu, F., Ng, T. B., Wang, H., Fung, M. C., & Ooi, V. E. C. (2005). Lectin from *Tricholoma mongolicum* S. Imai (Agaricomycetideae) mycelia stimulates gene expression of immuno-modulating cytokines in mouse peritoneal macrophages and splenocytes. *International Journal of Medicinal Mushrooms*, 7(1&2), 243-248.

Liu, W. K., Ng, T. B., Sze, S. F., & Tsui, K. W. (1993). Activation of peritoneal macrophages by polysaccharopeptide from the mushroom, Coriolus versicolor. *Immunopharmacology*, 26(2), 139-146.

Liu, W., Gu, J., Qi, J., Zeng, X. N., Ji, J., Chen, Z. Z., & Sun, X. L. (2015). Lentinan exerts synergistic apoptotic effects with paclitaxel in A549 cells via activating ROS-TXNIP-NLRP3 inflammasome. *Journal of Cellular and Molecular Medicine*, 19(8), 1949-1955.

Liu, X., Wang, L., Zhang, C., Wang, H., Zhang, X., & Li, Y. (2015). Structure characterization and antitumor activity of a polysaccharide from the alkaline extract of king oyster mushroom. *Carbohydrate Polymers*, 118(2015), 101-106.

Looft-Wilson, R. C., Payne, G. W., & Segal, S. S. (2004). Connexin expression and

conducted vasodilation along arteriolar endothelium in mouse skeletal muscle. *Journal of Applied Physiology*, 97(3), 1152-1158.

Lucas, E. H. (1960). Folklore and plant drugs. *Michigan Academy of Science, Arts, and Letters*, 45, 127-136.

Luo, K. W., Yue, G. G. L., Ko, C. H., Lee, J. K. M., Gao, S., Li, L. F., ... & Bik-San Lau, C. (2014). *In vivo* and *in vitro* anti-tumor and anti-metastasis effects of *Coriolus versicolor* aqueous extract on mouse mammary 4T1 carcinoma. *Phytomedicine*, 21(8), 1078-1087.

Ma, L., Chen, H., Dong, P., & Lu, X. (2013). Anti-inflammatory and anticancer activities of extracts and compounds from the mushroom *Inonotus obliquus*. *Food Chemistry*, 139(1), 503-508.

Luo, K. W., Yue, G. G. L., Ko, C. H., Lee, J. K. M., Gao, S., Li, L. F., ... & Bik-San Lau, C. (2014). *In vivo* and *in vitro* anti-tumor and anti-metastasis effects of *Coriolus versicolor* aqueous extract on mouse mammary 4T1 carcinoma. *Phytomedicine*, 21(8), 1078-1087.

Ma, L., Chen, H., Dong, P., & Lu, X. (2013). Anti-inflammatory and anticancer activities of extracts and compounds from the mushroom *Inonotus obliquus*. *Food Chemistry*, 139(1), 503-508.

Majno, G., & Palade, G. E. (1961). Studies on inflammation: I. The effect of histamine and serotonin on vascular permeability: An electron microscopic study. *The Journal of Biophysical and Biochemical Cytology*, 11(3), 571.

Medzhitov R. (2008). Origin and physiological roles of inflammation. *Nature*. 454: 428–435. doi:10.1038/nature07201.

Métis Nation of Ontario, M-étis Complete Traditional Plant Use Study for Southern Ontario. (2010). Retrieved February 15, 2017, from http://www.metisnation.org/programs/lands-resources-consultations/traditional-knowledge.aspx

Miles, P. G., & Chang, S. T. (2004). Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact. CRC press.

Mills, C. D., Lenz, L. L., & Harris, R. A. (2016). A breakthrough: macrophage-directed cancer immunotherapy. *Cancer Research*, 76(3), 513-516.

Minato, K. I. (2010). Mushrooms: immunomodulating activity and role in health promotion. In R. R Watson, S. Zibadi, & V. R Preedy (Eds.), *Dietary components and immune function* (pp. 529-539). New York, NY: Humana Press.

Mizuno, T. (1999). The extraction and development of antitumor-active polysaccharides from medicinal mushrooms in Japan (review). *International Journal of Medicinal Mushrooms*, 1(1), 9-29.

Mueller GM, Bills GF, Foster MS (2004) Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic Press, San Diego.

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology*, 11(11), 723-737.

Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products*, 75(3), 311-335.

Oba, K., Kobayashi, M., Matsui, T., Kodera, Y., & Sakamoto, J. (2009). Individual patient based meta-analysis of lentinan for unresectable/recurrent gastric cancer. *Anticancer Research*, 29(7), 2739-2745.

Oberstein, P. E., & Olive, K. P. (2013). Pancreatic cancer: why is it so hard to treat? *Therapeutic Advances in Gastroenterology*, 6(4), 321-337.

O'Shea, J. J., Ma, A., & Lipsky, P. (2002). Cytokines and autoimmunity. *Nature Reviews Immunology*, 2(1), 37-45.

Osińska-Jaroszuk, M., Jaszek, M., Mizerska-Dudka, M., Błachowicz, A., Rejczak, T. P., Janusz, G., ... & Kandefer-Szerszeń, M. (2014). Exopolysaccharide from *Ganoderma applanatum* as a promising bioactive compound with cytostatic and antibacterial properties. *BioMed Research International*, 2014, 1–10.

Ostuni, R., Kratochvill, F., Murray, P. J., & Natoli, G. (2015). Macrophages and cancer: from mechanisms to therapeutic implications. *Trends in Immunology*, 36(4), 229-239.

Page, D. B., Postow, M. A., Callahan, M. K., Allison, J. P., & Wolchok, J. D. (2014). Immune modulation in cancer with antibodies. *Annual Review of Medicine*, 65, 185-202.

Park, Y. M., Kim, I. T., Park, H. J., Choi, J. W., Park, K. Y., Lee, J. D. et al., (2004). Anti-inflammatory and anti-nociceptive effects of the methanol extract of *Fomes fomentarius*. *Biological and Pharmaceutical Bulletin*, 27(10), 1588-1593.

Park, Y. M., Won, J. H., Kim, Y. H., Choi, J. W., Park, H. J., & Lee, K. T. (2005). In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of *Inonotus obliquus*. *Journal of Ethnopharmacology,* 101(1), 120-128.

Patel, S., & Goyal, A. (2012). Recent developments in mushrooms as anti-cancer therapeutics: a review. *3 Biotech*, 2(1), 1-15.

Payne, G. W. (2006). Effect of inflammation on the aging microcirculation: impact on skeletal muscle blood flow control. *Microcirculation*, 13(4), 343-352.

Payne, G. W., & Bearden, S. E. (2006). The microcirculation of skeletal muscle in aging. *Microcirculation,* 13, 275-277.

Payne, G. W., Madri, J. A., Sessa, W. C., & Segal, S. S. (2003). Abolition of arteriolar dilation but not constriction to histamine in cremaster muscle of eNOS–/–mice. *American Journal of Physiology-Heart and Circulatory Physiology*, 285(2), H493-H498.

Payne, G. W., Madri, J. A., Sessa, W. C., & Segal, S. S. (2004). Histamine inhibits conducted vasodilation through endothelium-derived NO production in arterioles of mouse skeletal muscle. *The FASEB Journal*, 18(2), 280-286.

Peay, K. G., Kennedy, P. G., & Bruns, T. D. (2008). Fungal community ecology: a hybrid beast with a molecular master. *Bioscience*, 58(9), 799-810.

Peintner, U., & Pöder, R. (2000). Ethnomycological remarks on the Iceman's fungi. *The Iceman and his Natural Environment: Palaeobotanical Results*, 4, 143-150.

Peintner, U., Pöder, R., & Pümpel, T. (1998). The Iceman's fungi. *Mycological Research*, 102(10), 1153-1162.

Phan, C. W., David, P., Naidu, M., Wong, K. H., & Sabaratnam, V. (2015). Therapeutic potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases: diversity, metabolite, and mechanism. *Critical Reviews in Biotechnology*, 35(3), 355-368.

Piotrowski, J., Jędrzejewski, T., & Kozak, W. (2014). Immunomodulatory and antitumor properties of polysaccharide peptide (PSP). *Postepy higieny i medycyny doswiadczalnej (Online)*, 69, 91-97.

Pizza, G., Severini, G., Menniti, D., De Vinci, C., & Corrado, F. (1984). Tumor regression after intralesional injection of interleukin 2 (IL-2) in bladder cancer. Preliminary report. *International Journal of Cancer*, 34(3), 359-367.

Pöder, R., & Peintner, U. (1999). Laxatives and the Ice Man. *The Lancet*, 353(9156), 926-927.

Qian, G. M., Pan, G. F., & Guo, J. Y. (2012). Anti-inflammatory and antinociceptive effects of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*. *Natural Product Research*, 26(24), 2358-2362.

Rajaratnam, S., & Thiagarajan, T. (2012). Molecular characterization of wild mushroom. *European Journal of Experimental Biology*, 2(2), 369-373.

Rao, Y. K., Fang, S. H., Wu, W. S., & Tzeng, Y. M. (2010). Constituents isolated from *Cordyceps militaris* suppress enhanced inflammatory mediator's production and human cancer cell proliferation. *Journal of Ethnopharmacology*, 131(2), 363-367.

Ríos, J. L., Andújar, I., Recio, M. C., & Giner, R. M. (2012). Lanostanoids from fungi: a

group of potential anticancer compounds. *Journal of Natural Products*, 75(11), 2016-2044.

Roberts, N. J., Zhou, S., Diaz, L. A., & Holdhoff, M. (2011). Systemic use of tumor necrosis factor alpha as an anticancer agent. *Oncotarget*, 2(10), 739-751.

Rosenblum, M. G., & Donato, N. J. (1988). Tumor necrosis factor alpha: a multifaceted peptide hormone. *Critical Reviews in Immunology*, 9(1), 21-44.

Ruthes, A. C., Rattmann, Y. D., Malquevicz-Paiva, S. M., Carbonero, E. R., Córdova, M. M., Baggio, C. H., ... & Iacomini, M. (2013). *Agaricus bisporus* fucogalactan: structural characterization and pharmacological approaches. *Carbohydrate Polymers*, 92(1), 184-191

Saba, E., Son, Y., Jeon, B. R., Kim, S. E., Lee, I. K., Yun, B. S., & Rhee, M. H. (2015). Acetyl Eburicoic Acid from *Laetiporus sulphureus* var. *miniatus* Suppresses Inflammation in Murine Macrophage RAW 264.7 Cells. *Mycobiology*, 43(2), 131-136.

Sasaki, T., Arai, Y., Ikekawa, T., Chihara, G., Fukuoka, F., 1971. Antitumor polysaccharides from some polyporaceae, *Ganoderma applanatum* (Pers.) Pat and *Phellinus linteus* (Berk. et Curt) Aoshima. *Chem. Pharm. Bull*. (Tokyo) 19, 821–826.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, 6(3), 317-333.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., & Chen, W. (2012). Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List: Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proc. Natl. Acad. Sci*. USA, 109, 6241-6246.

Segal, S. S. (2005). Regulation of blood flow in the microcirculation. *Microcirculation*, 12(1), 33-45.

Segal, S. S. (2015). Integration and modulation of intercellular signaling underlying blood flow control. *Journal of Vascular Research*, 52(2), 136-157.

Segal, S. S., & Duling, B. R. (1986). Flow control among microvessels coordinated by intercellular conduction. *Science*, 234, 868-871.

Seo, M. J., Park, J. U., Lee, H. H., & Jeong, Y. K. (2011). Biochemical characterization of the exopolysaccharide purified from *Laetiporus sulphureus* mycelia. *Journal of Microbiology and Biotechnology*, 21(12), 1287-1293.

Serhan, C.N., P.A., & Gilroy, D.W. (2010). Fundamentals of Inflammation. Cambridge University Press.

Shao, H. J., Jeong, J. B., Kim, K. J., & Lee, S. H. (2015). Anti-inflammatory activity of

mushroom-derived hispidin through blocking of NF-κB activation. *Journal of the Science of Food and Agriculture*, 95(12), 2482-2486.

Shimizu, K., Watanabe, S., Matsuda, K., Suga, T., Nakazawa, S., & Shiratori, K. (2008). Efficacy of oral administered superfine dispersed lentinan for advanced pancreatic cancer. *Hepato-gastroenterology*, 56(89), 240-244.

Soccol, C. R., Bissoqui, L. Y., Rodrigues, C., Rubel, R., Sella, S. R., Leifa, F., ... & Soccol, V. T. (2016). Pharmacological properties of biocompounds from spores of the Lingzhi or Reishi medicinal mushroom *Ganoderma lucidum* (Agaricomycetes): A Review. *International Journal of Medicinal Mushrooms*, 18(9), 757-767.

Solinas, G., Germano, G., Mantovani, A., & Allavena, P. (2009). Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *Journal of Leukocyte Biology*, 86(5), 1065-1073.

Solov'eva, T., Davydova, V., Krasikova, I., & Yermak, I. (2013). Marine compounds with therapeutic potential in gram-negative sepsis. *Marine Drugs*, 11(6), 2216-2229.

Song, H. H., Chae, H. S., Oh, S. R., Lee, H. K., & Chin, Y. W. (2012). Anti-inflammatory and anti-allergic effect of *Agaricus blazei* extract in bone marrow-derived mast cells. *The American Journal of Chinese Medicine*, 40(05), 1073-1084.

Steidl, C., Lee, T., Shah, S. P., Farinha, P., Han, G., & Nayar, T et al. (2010). Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *New England Journal of Medicine*, 362(10), 875-885.

Suabjakyong, P., Nishimura, K., Toida, T., & Van Griensven, L. J. (2015). Structural characterization and immunomodulatory effects of polysaccharides from *Phellinus linteus* and *Phellinus igniarius* on the IL-6/IL-10 cytokine balance of the mouse macrophage cell lines (RAW 264.7). *Food & Function*, 6(8), 2834-2844.

Sumagin, R., & Sarelius, I. H. (2013). Emerging understanding of roles for arterioles in inflammation. *Microcirculation*, 20(8), 679-692.

Sun, C., Rosendahl, A. H., Wang, X. D., Wu, D. Q., & Andersson, R. (2012). Polysaccharide-K (PSK) in cancer-old story, new possibilities? *Current Medicinal Chemistry*, 19(5), 757-762.

Sun, M., Zhao, W., Xie, Q., Zhan, Y., & Wu, B. (2015). Lentinan reduces tumor progression by enhancing gemcitabine chemotherapy in urothelial bladder cancer. *Surgical Oncology*, 24(1), 28-34.

Sunagawa, M., Isogai, M., Harada, T., Kaneoka, Y., Kamei, K., Maeda, A., & Takayama, Y. (2014). A giant Krukenberg tumor from a perforated gastric cancer that was successfully removed after multidisciplinary therapy: report of a case. *Surgery Today*, 44(1), 171-174.

Tak, P. P., & Firestein, G. S. (2001). NF-κB: a key role in inflammatory diseases. *The Journal of Clinical Investigation*, 107(1), 7-11.

Tanaka, S., Ko, K., Kino, K., Tsuchiya, K., Yamashita, A., Murasugi, A., ... & Tsunoo, H. (1989). Complete amino acid sequence of an immunomodulatory protein, ling zhi-8 (LZ-8). An immunomodulator from a fungus, Ganoderma lucidium, having similarity to immunoglobulin variable regions. *Journal of Biological Chemistry*, 264(28), 16372-16377.

Tang, Y. J., Liu, R. S., & Li, H. M. (2015). Current progress on truffle submerged fermentation: a promising alternative to its fruiting bodies. *Applied Microbiology and Biotechnology*, 99(5), 2041-2053.

Taofiq, O., Martins, A., Barreiro, M. F., & Ferreira, I. C. (2016). Anti-inflammatory potential of mushroom extracts and isolated metabolites. *Trends in Food Science & Technology*, *50*, 193-210.

Thyagarajan-Sahu, A., Lane, B., & Sliva, D. (2011). ReishiMax, mushroom-based dietary supplement, inhibits adipocyte differentiation, stimulates glucose uptake and activates AMPK. *BMC Complementary and Alternative Medicine*, 11(1), 74.

Triggiani, M., Gentile, M., Secondo, A., Granata, F., Taglialatela, M., Annunziato, L., & Marone, G. (2001). Histamine induces exocytosis and IL-6 production from human lung macrophages through interaction with H1 receptors. *The Journal of Immunology*, *166*(6), 4083-4091.

Tuma, R. F., Durán, W. N., & Ley, K. (Eds.). (2011). Microcirculation. Academic Press.

Turner, N. J. (1975). *Food plants of British Columbia Indians* (1st ed.). Victoria: British Columbia Provincial Museum handbook no. 34.

Turner, N. J., & Szczawinski, A. F. (1991). Common poisonous plants and mushrooms of North America. Timber Press Inc.

Turner, N. J., Bouchard, R., & Kennedy, D. I. (1980). Ethnobotany of the Okanagan-Colville Indians of British Columbia and Washington. British Columbia Provincial Museum handbook no. 34.

Tzianabos, A. 2000. Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. *Clinical Microbiology Reviews*, 13, 523-533.

Uchida, J., Hamaguchi, Y., Oliver, J. A., Ravetch, J. V., Poe, J. C., Haas, K. M., & Tedder, T. F. (2004). The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor–dependent mechanisms during anti-CD20 antibody immunotherapy. *Journal of Experimental Medicine*, 199(12), 1659-1669.

Valverde, M. E., Hernández-Pérez, T., & Paredes-López, O. (2015). Edible Mushrooms: Improving Human Health and Promoting Quality Life. *International Journal of Microbiology*, 2015.

Van Der Hem, L. G., Van Der Vliet, J. A., Bocken, C. F. M., Kino, K., Hoitsma, A. J., & Tax, W. J. (1995). Ling Zhi-8: studies of a new immunomodulating agent. *Transplantation*, 60(5), 438-443.

Van Horssen, R., ten Hagen, T. L., & Eggermont, A. M. (2006). TNF-α in cancer treatment: molecular insights, antitumor effects, and clinical utility. *The Oncologist*, 11(4), 397-408.

Van, Q., Nayak, B. N., Reimer, M., Jones, P. J. H., Fulcher, R. G., & Rempel, C. B. (2009). Anti-inflammatory effect of *Inonotus obliquus, Polygala senega* L., and *Viburnum trilobum* in a cell screening assay. *Journal of Ethnopharmacology*, 125(3), 487-493.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine*, 10(8), 789-799.

Wang, C. R., Ng, T. B., Li, L., Fang, J. C., Jiang, Y., Wen, T. Y., ... & Liu, F. (2011). Isolation of a polysaccharide with antiproliferative, hypoglycemic, antioxidant and HIV-1 reverse transcriptase inhibitory activities from the fruiting bodies of the abalone mushroom *Pleurotus abalonus*. *Journal of Pharmacy and Pharmacology*, 63(6), 825-832.

Wang, H., Ng, T. B., & Ooi, V. E. (1998). Lectins from mushrooms. *Mycological Research*, 102(8), 897-906.

Wang, J. L., Bi, Z., Zou, J. W., & Gu, X. M. (2012a). Combination therapy with lentinan improves outcomes in patients with esophageal carcinoma. *Molecular Medicine Report*, 5(3), 745-8.

Wang, J., Liu, Y. M., Cao, W., Yao, K. W., Liu, Z. Q., & Guo, J. Y. (2012b). Anti-inflammation and antioxidant effect of Cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*, in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. *Metabolic Brain Disease*, 27(2), 159-165.

Wang, P. H., Hsu, C. I., Tang, S. C., Huang, Y. L., Lin, J. Y., & Ko, J. L. (2004). Fungal immuno-modulatory protein from *Flammulina velutipes* induces interferon-γ production through p38 mitogen-activated protein kinase signaling pathway. *Journal of Agricultural and Food Chemistry*, 52(9), 2721-2725.

Wang, X., & Lin, Y. (2008). Tumor necrosis factor and cancer, buddies or foes? *Acta pharmacologica Sinica*, 29(11), 1275-1288.

Wang, Z., Zhou, F., & Quan, Y. (2014). Antioxidant and immunological activity in vitro of polysaccharides from *Phellinus nigricans* mycelia. *International Journal of Biological Macromolecules*, 64, 139-143.

Wasser, S. P., & Weis, A. L. (1999). Medicinal properties of substances occurring in higher basidiomycetes mushrooms: current perspectives (review). *International Journal of Medicinal Mushrooms*, 1(1), 31-62.

Waters, J. P., Pober, J. S., & Bradley, J. R. (2013). Tumour necrosis factor and cancer. *The Journal of Pathology*, 230(3), 241-248.

Weiskopf, K., & Weissman, I. L. (2015). Macrophages are critical effectors of antibody therapies for cancer. *MAbs*, 7(2), 303-310.

Weston, A., Brown, P. R., & Foret, F. (1998). HPLC and CE, Principles and Practice. *Analytical Biochemistry*, 255(1), 164.

Won, D. P., Lee, J. S., Kwon, D. S., Lee, K. E., Shin, W. C., & Hong, E. K. (2011). Immunostimulating activity by polysaccharides isolated from fruiting body of *Inonotus obliquus*. *Molecules and Cells*, 31(2), 165-173.

World Health Organization. (2017). Cancer (fact sheet). Retrieved from http://www.who.int/mediacentre/factsheets/fs297/en/

Wu, M., Luo, X., Xu, X., Wei, W., Yu, M., Jiang, N., ... & Fei, X. (2014). Antioxidant and immunomodulatory activities of a polysaccharide from *Flammulina velutipes*. *Journal of Traditional Chinese Medicine*, 34(6), 733-740.

Wu, Y. D., & Zhou, B. P. (2010). TNF-α/NF-κB/Snail pathway in cancer cell migration and invasion. *British Journal of Cancer*, 102(4), 639-644.

Xu, X., Pan, C., Zhang, L., & Ashida, H. (2011). Immunomodulatory β-glucan from *Lentinus edodes* activates mitogen-activated protein kinases and nuclear factor-κB in murine RAW 264.7 macrophages. *Journal of Biological Chemistry*, 286(36), 31194-31198.

Yang, P., Liang, M., Zhang, Y., & Shen, B. (2008). Clinical application of a combination therapy of lentinan, multi-electrode RFA and TACE in HCC. *Advances in Therapy*, 25(8), 787.

Ye, Y., Josten, I., Arnold, N., Steffan, B., & Steglich, W. (1996). Isolation of a fluorone pigment from the Indian paint fungus *Echinodontium tinctorium* and *Pyrofomes albomarginatus*. *Tetrahedron*, 52(16), 5793-5798.

Yoon, H. M., Jang, K. J., Han, M. S., Jeong, J. W., Kim, G. Y., Lee, J. H., & Choi, Y. H. (2013). *Ganoderma lucidum* ethanol extract inhibits the inflammatory response by suppressing the NF-κB and toll-like receptor pathways in lipopolysaccharide-stimulated BV2 microglial cells. *Experimental and Therapeutic Medicine*, 5(3), 957-963.

Yoshikawa, K., Inoue, M., Matsumoto, Y., Sakakibara, C., Miyataka, H., Matsumoto, H., &

Arihara, S. (2005). Lanostane Triterpenoids and Triterpene Glycosides from the Fruit Body of Fomitopsis p inicola and Their Inhibitory Activity against COX-1 and COX-2. *Journal of Natural Products*, 68(1), 69-73.

Yoshino, S., Watanabe, S., Imano, M., Suga, T., Nakazawa, S., Hazama, S., & Oka, M. (2009). Improvement of QOL and prognosis by treatment of superfine dispersed lentinan in patients with advanced gastric cancer. *Hepato-gastroenterology*, 57(97), 172-177.

Yu, Z., Qin, W., Lin, J., Fang, S., & Qiu, J. (2015). Antibacterial mechanisms of polymyxin and bacterial resistance. *BioMed Research International*, 2015.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, 18(1), 4-19.

Zhao, L., Xiao, Y., & Xiao, N. (2013). Effect of lentinan combined with docetaxel and cisplatin on the proliferation and apoptosis of BGC823 cells. *Tumor Biology*, 34(3), 1531-1536.

Zhu, X. L., Chen, A. F., & Lin, Z. B. (2007). *Ganoderma lucidum* polysaccharides enhance the function of immunological effector cells in immunosuppressed mice. *Journal of Ethnopharmacology*, 111(2), 219-226.