INVESTIGATING BRITISH COLUMBIA WILD MUSHROOMS FOR GROWTH-INHIBITORY ACTIVITY

by

Aaron W. T. Smith

BSc. University of Northern British Columbia 2012

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOCHEMISTRY

UNIVERSITY OF NORTHERN BRITISH COLUMBIA

July 2017

Abstract

This thesis documents that crude extracts from B.C. wild mushrooms possess potent growth-inhibitory properties. In addition, this work shows, for the first time, that ethanol extracts from *Piptoporus betulinus* and *Ganoderma applanatum* have a strong growth-inhibitory activity and that methanol extracts of *Echinodontium tinctorium*, a previously untested fungus, exhibits growth-inhibitory activity as well. In addition to human cervical cancer HeLa cells, *E. tinctorium* extracts also exhibited a growth-inhibitory effect on human breast, ovarian, liver, lung, and colon cancer cell lines. Upon purification using Sephadex LH-20 gel filtration chromatography, two distinct growth-inhibitory compounds were documented: a higher molecular weight growth-inhibitory compound(s), likely to contain carbohydrate and a lower molecular weight growth-inhibitory compound(s), likely to be a small molecule. This thesis provides the foundation work to allow further purification and characterization of these potentially novel growth-inhibitory compound(s) from *E. tinctorium*, an essential step towards future therapeutic use.

Table of Contents

Abstract II
List of Tables V
List of Figures VI
List of Appendices IX
Acknowledgements X
Chapter 1 – Introduction 1
1.1 History of Medicinal Mushrooms 1
1.2 Anti-Cancer Compounds Isolated from Mushrooms 5
1.3 Project Goals 8
Chapter 2 – Collection, Identification, Extraction, Optimization, and Assessment of Anti-Proliferative Activity in B.C Wild Mushrooms 10
2.1 Methodology 10
2.1.1 Sample Collection and DNA / Morphological Identification 10
2.1.2 Chemical Extraction 16
2.1.3 Extraction Techniques 18
2.1.4 Cell Culture and Mushroom Extract Preparation 22
2.1.5 MTT Assay 24
2.2 Results 34
2.2.1 Species Identification 34
2.2.2 Dose and Time Dependent MTT Assays 44
2.3 Discussion 71
2.3.1 Morphological and DNA investigation 71
2.3.2 Growth-inhibitory Effect on HeLa Human Cervical Cancer Cells – Mushrooms Tested 71
Chapter 3 - Investigating the Growth-Inhibitory Activity of the Methanol Extract of *Echinodontium tinctorium* 76
3.1 Introduction 76
3.2 Methodology 76
3.2.1 Sephadex LH-20 Size Exclusion Purification 76
3.2.2 Total Carbohydrate Colorimetric Assay Kit (#K645-100) 77
3.2.3 Pierce Glycoprotein Carbohydrate Estimation Kit (#23260) 78
3.2.4 Pierce BCA Protein Assay Kit (#23225) 79
3.2.5 Phenol Chloroform Extraction (P.C.I) 80

3.3 Results 80
3.3.1 Large Scale Manual Extraction vs. Speed Extractor 80
3.3.2 Size Exclusion Chromatography and BCA Assay 82
3.3.3 Total Carbohydrate Assay (Hot H_2SO_4) 94
3.3.4 Glycoprotein Carbohydrate Estimation Kit 95
3.3.5 C16/70 LH-20 Column (100 mL) 97
3.3.6 Assessment of the Post-Sephadex LH-20 Fractions for Growth-Inhibitory Activity using Different Cancer Cell Lines. 101
3.4 Discussion 103
Chapter 4 - General Discussion 105
4.1 Screening BC wild mushrooms for growth-inhibitory activity 105
4.2 An attempt to purify the growth-inhibitory compound from *Echinodontium tinctorium* 106
4.3 Future Directions and Conclusions 108
References 109
Appendix 116
Sequences Derived from DNA Analysis 116
Piptoporus betulinus 116
Ganoderma applanatum 116
Fomes fomentarius 117
Echinodontium tinctorium 118

List of Tables

Table 1. Krestin (Polysaccharide K), isolated from *Trametes versicolor* has been shown to have anti-metastatic properties in a variety of different cell lines[34] 4

Table 2. A list of important HWM β-glucans that have been isolated from various mushroom species. The most famous compounds, Lentinan, Schizophyllan and PSK all belong to this group[47].....7

Table 3. Thermocycler protocol used for Fungal DNA amplification.....14

Table 4. BC Mushrooms: An Inventory List and Extraction Solvent Used 27

Table 5. MTT Assay (Extracts from *Fomitopsis pinicola*) – Visual Observations after 24 hours of treatment 46

Table 6. MTT Assay (Extracts from *Fomitopsis pinicola*) – Visual Observations after 48 hours of treatment 47

List of Figures

Figure 1. High and low molecular weight compounds isolated from medicinal mushrooms which have been shown to have anti-cancer properties[40]. 6

Figure 2. DNA Extraction flow chart provided from a PowerSoil DNA Isolation kit, purchased from MO BIO Laboratories.[55] 13

Figure 3. Confirmation of fungal DNA after DNA extraction. Samples were run on a 1% agarose gel, containing ethidium bromide, for approximately 1 hour. Sample A5 was a duplicate of *G. applanatum*. An NEB 1 kb DNA ladder was used. Values shown on the right are in kb....14

Figure 4. One percent agarose gel containing ethidium bromide in which 4 fungal DNA samples were run after PCR was performed. A band can be seen at 500 base pairs for each sample. Sample A5 was a duplicate of G. applanatum. An NEB 100 bp ladder was used for visualization. Values shown on the right are in bp 15

Figure 5. An example of a dried and powdered mushroom sample (Chaga). 16

Figure 6. A generalized flow/overview of Mizuno's mushroom extraction techniques[59]. The first step, with ethanol, is meant to separate out the low molecular weight compounds from the high molecular weight compounds. 17

Figure 7. An example of the final product of an MTT assay....24

Figure 8. Modified extraction technique to reflect the change in temperature and the addition of a 50% methanol extract. 29

Figure 9. The final extraction scheme adopted. This is the modified extraction technique to reflect the removal of the ammonium oxalate extract entirely 32

Figure 10. The results of a 72 hour MTT assay using four extracts obtained from the extraction of *F. pinicola*....48

Figure 11. The results of a time dependent assay using two concentrations of extract 2, which was extracted in 99% ethanol and then dissolved in 80% ethanol 49

Figure 12. The results of a time dependent assay using two concentrations of extract 2, which was extracted in 99% ethanol and then dissolved in 80% ethanol. 50

Figure 13. Seventy-two hour dose dependent MTT assay using extract 2 (99% ethanol) extracted from *F. pinicola*....51

Figure 14. Forty Eight Hour Dose dependent using Cis Platinum and Human HeLa cells.....51

Figure 15. MTT (48 hour) assay of *Lentinula edodes* 53

Figure 16. Dose dependent MTT assay using four extracts from *P. betulinus*....54

Figure 17. Seven day time dependent assay using E1 (80% ethanol) and E3 (water), extracted from *P. betulinus*....55

Figure 18. Dose dependent MTT assay using extracts from *F. fomentarius*....56

Figure 19. Seven Day MTT assay using extracts 1 (80% ethanol), extract 2 (50% methanol) and extract 3 (Water). 57

Figure 20. Dose dependent MTT assay using extracts extracted from *G. applanatum*.....58

Figure 21. Seven day time dependent MTT assay using extract 3 (water) extracted from *G. applanatum* 59

Figure 22. Dose dependent MTT assay using extracts from *E. tinctorium* 60

Figure 23. Seven day time dependent MTT assay using extract 2 (50% methanol) at two concentrations, 0.5 and 1 mg / mL.....61

Figure 24. Dose dependent MTT assay (72 hours) using extract 2 (80% ethanol) from *E. tinctorium*.....62

Figure 25. Dose dependent MTT assay using extracts from *I. obliquus*. 63

Figure 26. Seven day time dependent MTT assay using extracts obtained from *I. obliquus*. .64

Figure 27. Dose dependent MTT assay using extracts from *L. connata* 65

Figure 28. Seven day dose dependent MTT assay of extracts from *L. connata*. 66

Figure 29. Dose dependent MTT assay using extracts from *L. sulphureus*.....67

Figure 30. Seven day dose dependent MTT assay of extracts from *L. sulphureus* 68

Figure 31. Dose dependent MTT assay of extracts from *P. nigricans*. 69

Figure 32. Seven day time dependent MTT assay of extract 1 (80% ethanol), and extract 3 (water), obtained from *P. nigricans*.....70

Figure 33. Chemical structure of a furfural compound.....77

Figure 34. Growth-inhibitory effect of methanol extracts from *E. tinctorium*. 81

Figure 35. Trial 1 - Assessing growth-inhibitory activity of fractions collected from Sephadex LH-20. 83

Figure 36. Trial 2 - Assessing fractions collected from Sephadex LH20 for growth-inhibitory activity.....84

Figure 37. Trial 3 - Assessing fractions collected from Sephadex LH20 for growth-inhibitory activity.....85

Figure 38. Trial 4 - Assessing fractions collected from Sephadex LH20 for growth-inhibitory activity.....86

Figure 39. BCA assay using fractions collected from a 2 mL LH-20 column. 87

Figure 40. BCA assay and MTT assay using the same eluted fractions from a 2 mL LH-20 column.....88

Figure 41. Assessing fractions collected from Sephadex LH-20 for growth-inhibitory activity.....89

Figure 42. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity.....90

Figure 43. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. 91
Figure 44. BCA assay using fractions collected from an 18 mL LH-20 column. 92
Figure 45. BCA assay and MTT assay using eluted fractions from an 18 mL LH-20 column.....93
Figure 46. Linear trend line calculated from glucose standards created from Total Carbohydrate Kit.....94
Figure 47. Growth-inhibitory activity and carbohydrate content of fractions collected from an 18 mL Sephadex LH-20 column.....95
Figure 48. Linear trend line calculated from glycoprotein standards created from a glycoprotein estimation kit. 96
Figure 49. Growth-inhibitory activity and glycoprotein content of fractions collected from Sephadex LH-20 column. 97
Figure 50. 100 mL LH-20 column purifying crude E2 extract from *E. tinctorium*. The column is being run with water.....98
Figure 51. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. 99
Figure 52. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. 100
Figure 53. Assessing the post-Sephadex LH-20 Fraction 10 for growth-inhibitory activity, using various cancer cell lines, in a 48 h MTT assay. 101
Figure 54. Assessing the post-Sephadex LH-20 Fraction 10 for growth-inhibitory activity, using various cancer cell lines, in a 48 h MTT assay. 102

List of Appendices

Appendix 1. Sequences Derived from DNA Analysis…….116

Acknowledgements

Firstly I would like to thank Dr. Chow Lee for not only giving me the opportunity to pursue this Master's project, but to also work in his lab for back to back 499 and 460 undergraduate research projects. I would also like to thank my other co-supervisor, Dr. Kerry Reimer, whose assistance throughout but particularly at the beginning of this project, with respect to the physical mechanics of handling mushroom species and procuring equipment, was critical and instrumental. A huge thank you to my two other committee members, Dr. Hugues Massicotte and Dr. Keith Egger; this project would not have been possible without your bountiful fungi knowledge. I am also grateful to Dr. Maggie Li for lending me her technical expertise whenever it was required. Finally, I am grateful to Miss Vicky Myhre for giving me the final push of motivation to complete this project.

Chapter 1 – Introduction

1.1 History of Medicinal Mushrooms

Since the beginning of civilization, mankind has been fascinated with mushrooms and the mysticism surrounding them. Ancient Egyptian hieroglyphs, dating back more than 4500 years, illustrate that mushrooms were considered to be a plant of immortality and gifts from the gods. Only those closest to the deities were allowed to consume them, and no commoner could touch them[1,2]. This idea of mushrooms being of divine origin was also adopted by the Romans, who served them only during festivals, and later the ancient Greeks, who believed that mushrooms gave their warriors strength in battle[3]. Meso-Americans used mushrooms for their ancient religious ceremonies. They did not use mushrooms to give them strength but believed that the powerful psychotropic effects and hallucinations obtained by certain mushroom species allowed them to be closer to god[4]. Mushroom carvings left behind by the Mayans serve as evidence that mushrooms were used for the purposes as far back as 3000 BC. As more has become known about mushrooms, they have become prized for their antioxidant, anti-cancer, antiviral, and immune boosting properties[5].

Perhaps the first known use of mushrooms for medicinal purposes was by the ancient Chinese, who used mushrooms as a medical intervention for nearly two millennia[6]. Mushrooms such as *Ganoderma lucidum*, commonly referred to as Ling Zhi or Ling Chu by the Chinese, have been recorded in the oldest books on medicinal materials in China. “Shen Noug’s Herbel” contains references to many medicinal mushrooms, including *G. lucidum*, and is believed to have been written between 100 and 200 AD[7]. *Ganoderma lucidum* was prescribed to treat a wide variety of ailments ranging from arthritis and insomnia to

bronchitis and cancer[8,9]. Traditionally, *Ganoderma* compounds were ingested as hot water extracts due to their bitter taste. More recently, *G. lucidum* has been found to have many pharmacologically-active compounds including those involved in anti-viral defense[10], anti-inflammatory[11], and anti-cancer mechanisms[12].

Various extracts have been shown to have great potential in the treatment of a variety of cancers, such as: the induction of apoptosis in lung cancer cells in mice[13], anti-proliferative activity in human colorectal cancer cells[14], and anti-invasiveness in breast cancer models[15]. *Ganoderma lucidum* polysaccharide (GLPS) fraction, commonly known as Ganopoly®, was found to enhance the immune response in patients with advance-stage cancer through enhanced natural killer cell activity and phytohemagglutinin responses. Following 12 weeks of orally administered treatment, interleukin-2 (IL-2), IL-6, and interferon-γ (INF-γ) concentrations were increased in blood plasma, while tumor necrosis factor-α (TNF-α) and IL-1 concentrations were decreased[16].

The abundant use of medicinal mushrooms in ancient Asian medicine was not limited only to the Chinese. Mushrooms have long been used by the Japanese and Koreans to treat a plethora of illnesses. Species such as *Favolus alveolarius*, *Pluteus cervinus*, and *Ramaria formosa* have been under serious scientific investigation in Korea for the last 30 years. Their protein-bound polysaccharides have been shown to possess potent anti-cancer and immunomodulatory properties in sarcoma 180 cell lines[17–19].

Lentinula edodes (Berk.) Pegler, commonly referred to as Shiitake, has been used by the Japanese for hundreds of years to treat ailments such as the common cold and headaches, and was also used as a powerful aphrodisiac as well as for the beautification of the skin[20].

Several biologically active compounds have been isolated from Shiitake mushrooms, including those capable of inducing apoptosis in human cancer cells[21]. Lentinan, a beta-glucan isolated from Shiitake, has been available as an anti-cancer agent in Japan since 1985[22]. Administered at 0.5 – 1.0 mg / day, Lentinan has been used as an adjuvant in tumor therapy, and has been shown to help increase quality of life, increase survival time and help restore the functionality of the immune system, when coupled with other anti-cancer treatments such as radiotherapy and chemotherapy[23,24].

In addition to having anti-cancer properties, compounds isolated from Shiitake mushrooms have also been shown to have antibacterial[25] and antiviral[26] attributes. Furthermore, *L. edodes* has also been explored as an alternative to prevent tooth decay in countries where common dentistry is often not affordable to the general population[27].

Trametes versicolor is one of the more famous examples of mushrooms containing anti-cancer compounds. Polysaccharide K/PSK/Krestin, isolated from *T. versicolor*, has been available as an adjuvant to chemotherapy in Japan and other parts of Asia for over 40 years[28]. PSK is an orally active compound[29] and has been shown to have anti-cancer/anti-metastatic properties with a variety of different cell lines (Table 1). Although the exact mechanism of its anti-cancer properties is still unknown, it is believed to be a biological response modifier, which functions by increasing a host's ability to respond to an invasive tumor[28]. Its mechanism may involve Toll-Like receptor 4 and the activation of TNF-α, which helps mediate an immune response[30]. Immuno-modulation seems to be a common property among beta-glucan compounds isolated from medicinal mushrooms.

Inonotus obliquus, commonly known as Chaga, is another prime example of a mushroom that has been used as a treatment for a plethora of ailments dating back nearly half a century. Commonly found in northern climates such as those found in Russia, and northern British Columbia, Chaga can be found abundantly growing on birch (*Betula* spp.) trees. The bark from birch contains a chemical called betulin, which is not readily absorbed by humans. Chaga converts betulin to betulinic acid, a triterpene, which is water soluble, and is orally bioactive[31]. Both betulin and betulinic acid are being investigated for their anticancer, antiviral and anti-inflammatory properties[32,33].

Table 1. Krestin (Polysaccharide K), isolated from *Trametes versicolor* has been shown to have anti-metastatic properties in a variety of different cell lines[34]

Organ in which cancer is developed	**Cancer cell type/line**	**Result of PSK application**
Lung	autochthonous tumor	Increased survival time
Lung	human prostate cancer (DU-145M, PC-3M)	Increased survival time
Lung	mouse melanoma (B16-BL6)	Increased survival time
Lung	rat lung cancer	Increased survival time
Liver	human lung cancer (AOI)	Suppressed lesion growth
Lymph nodes	mouse leukemia (P388)	Increased survival time
Lymph nodes	mouse liver cancer (MH134)	Suppressed lesion growth
Liver	rat liver cancer (AH60C)	Suppressed lesion growth
Liver	mouse leukemia	Suppressed lesion growth
Liver	mouse colon cancer	Suppressed lesion growth

It is believed that, of the 140,000 estimated species of mushrooms on the planet, only 10% have been characterized[34,35]. Over 2000 mushroom species are non-toxic; there are over 700 from which anti-cancer compounds have been isolated. Many of those compounds have anti-proliferative, immuno-modulatory or pro-apoptotic properties[36–38]. British Columbia is estimated to be home to more than ~4600 mushroom species; of that number, only an estimated 1600 have been categorized[39]. Many Asiatic fungal species, which have established anti-cancer properties, along with many relatives belonging to the same genus, also exist in British Columbia and its surrounding area. This wealth of readily available specimens provides a huge sample source from which new anti-cancer compounds can potentially be discovered, isolated, purified and identified.

1.2 Anti-Cancer Compounds Isolated from Mushrooms

There is little doubt that the number and variety of compounds that can be isolated from mushrooms rivals the variety and abundance of mushroom species themselves. Of those known to have anti-cancer properties, there are two main groups: those of low molecular weight (LMW) and those of high molecular weight (HMW)[40]. LMW compounds include compounds such as quinones, isoflavones, amines and triacylglycerols. The high molecular weight group contains compounds such as polysaccharides, glycoproteins, glycopeptides and proteins (Figure 1). LMW compounds tend to have properties that allow them to inhibit a wide variety of enzymes such as DNA polymerase[8,41] and DNA topoisomerase[13] while HMW compounds tend to be immune-modulators[19,40,42,43].

Figure 1. High and low molecular weight compounds isolated from medicinal mushrooms which have been shown to have anti-cancer properties[40].

There are currently many high molecular weight compounds involved in clinical trials, including PSK, Lentinan, Grifolin and Schizophyllan. PSK is a glycopeptide while the other three are all (1->3) β-D-glucans with (1->6) β-D branches[44–46]. As a result, there is likely a higher probability of discovering high molecular weight compounds such as β-glucans, which seem to be commonly biologically active (Table 2). Crude extracts containing low molecular weight compounds are also of interest in respect to their anti-proliferative and pro-apoptotic abilities.

Table 2. A list of important HWM β-glucans that have been isolated from various mushroom species. The most famous compounds, Lentinan, Schizophyllan and PSK all belong to this group[47]

Herb	**Common Name**	**β-glucan structure**	**Type**
Lentinus edodes	Shiitake mushroom	β-1,3:1,6-glucan	Lentinan
Schizophyllum commune	Brazilian mushroom	β-1,3:1,6-glucan	Schizophyllan
Grifola frondosa	Maitake mushroom	β-1,3:1,6-glucan with xylose and mannose	Maitake D Fraction
Trametes versicolor	Yun Zhi	Protein bound β-1,3:1,6-glucan	PSP/PSK
Ganoderma lucidum	Lingzhi, Reishi	β-1,3:1,6-glucan	Ganoderma polysaccharides
Agaricus blazei	Brazilian sun mushroom	Protein bound β-1,6-glucan	Agaricus polysaccharides
Pleurotus ostreatus	Oyster mushroom	β-1,3-glucan with galactose and mannose	Pleuran
Coprinus comatus	Shaggy ink cap	β-1,3-glucan	Coprinus polysaccharides

Many bioactive polysaccharides isolated from mushrooms are water-soluble β-D-glucans, with or without heterosaccharide side chains, or β-D-glucan-protein complexes[48]. Activity has been linked to the 1→3 glycosidic chain that makes up the backbone of these polysaccharides, with differences primarily arising in the branch length and patterns between species[47]. Common branch patterns include 1→3, 1→4, and 1→6 linkages[49]. These β-glucans have been found to act on multiple immune receptors, including TLR-2 and 6,

Dectin-1, and complement receptor-3 (CR-3)[47,50,51]. This in turn triggers the activation of natural killer cells, macrophages, dendritic cells and neutrophils[47]. While not considered digestible, β-glucans have been effectively administered orally, where it is assumed that they are absorbed through fermentation by the gut flora, or by directly binding to receptor sites on macrophages. One theory suggests that, once internalized, they are fragmented and transported to the bone marrow, where they are released to be absorbed by other immune cells, which then elicit an immune response[47]. β-glucans have been linked to both innate and adaptive immune responses[47].

Schizophyllan, a polysaccharide extracted from *Schizophyllum commune* Fr., has been reported to have multiple activities, including antibacterial, anti-parasitic, anti-tumor and immuno-stimulatory effects. It was first recognized for its antitumor effects against sarcoma 180. This host-mediated activity was later found to be effective against multiple other cancers, including bladder, breast, lung, gastric, and leukemia. It is currently produced commercially by Japanese pharmaceutical companies as an immuno-potentiator, and often used as an adjuvant to chemotherapy to improve quality of life in patients[52].

1.3 Project Goals

This Thesis project project was broken down into two major components. The first being the design and optimization of extraction protocols and the extraction and screening of 9 different species of mushrooms obtained from the forests across Northern British Columbia. A tenth mushroom, Shiitake, was used as a proof of concept for a comparative analysis of extractions performed under different temperature variations. The second major component of the Thesis consisted of examining a methanol-based extract derived from *Echinodontium*

tinctorium (Ellis & Everh.) Ellis & Everh. in an attempt to purify the compounds(s) responsible for the growth-inhibitory effect on HeLa human cervical cancer cells.

Chapter 2 – Collection, Identification, Extraction, Optimization, and Assessment of Anti-Proliferative Activity in B.C Wild Mushrooms

Nine species of mushrooms were collected from Northern British Columbia, specifically from the areas surrounding Prince George, Terrace and McBride. A tenth sample, *Lentinula edodes*, was purchased from a grocery store and assayed, due to its known anti-cancer properties, as demonstrated in previously published papers[53,54]. Each sample was identified by either morphological characteristics or DNA analysis, or a combination of both. Then, each specimen was either pulverized with a hammer mill, frozen in liquid nitrogen and crushed with a pestle and mortar, or turned into a fine powder by use of a household blender. Different extraction techniques were explored while using a range of variables such as solvents, temperatures and time. Finally, extracted fractions from each sample were assessed for growth-inhibitory effects on HeLa human cervical cancer cells.

2.1 Methodology

2.1.1 Sample Collection and DNA / Morphological Identification

Nine mushroom samples had previously been obtained from Drs. Keith Egger and Hugues Massicotte. Each mushroom underwent chemical extraction and was tested for growth-inhibitory effect via MTT assay using the human cervical cancer HeLa cells. The four samples which showed the greatest growth-inhibitory activity were chosen for additional identification via DNA analysis.

DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA), following manufacturer protocols[55] (Figure 2). Two hundred and fifty

milligrams of mushroom sample was added to PowerBead tubes, which were included with the DNA isolation kit. The tubes were then gently vortexed. Solution C1 (included) was heated at 60°C to remove any precipitation. Sixty microliters of C1 were then added to the tubes containing mushroom sample and PowerBeads. The tubes were then briefly vortexed. The PowerBead tubes were then centrifuged at 10,000 x g for 30 seconds at room temperature, after which the supernatant was transferred to a clean 2 mL collection tube. Two hundred and fifty microliters of Solution C2 was then added and the combined mixture was then vortexed for 5 seconds and incubated for 5 minutes at 4 °C. The 2 mL collection tubes were then centrifuged for 1 minute at 10,000 x g. A maximum of 600 µL supernatant was then transferred to a new 2 mL collection tube followed by the addition of 200 µL of Solution C3 and a brief vortexed. The mixture was then incubated at 4 °C for 5 minutes and centrifuged for 1 minute at 10,000 x g. A maximum of 750 µL of supernatant was then transferred to a clean 2 mL collection tube. Solution C4 was shaken to mix, after which 1.2 mL was added to the supernatant, which was then vortexed for 5 seconds. Six hundred and seventy five microliters was then loaded onto a spin filter and centrifuged at 10,000 x g for 1 minute at room temperature. The flow through was discarded and an additional 675 µL of supernatant was added to the spin filter, which was then centrifuged at 10,000 x g for 1 minute at room temperature. Five hundred microliters of Solution C5 was then added and centrifuged at 10,000 x g for 30 seconds, at room temperature. The flow through was discarded and the mixture was centrifuged again at 10,000 x g for 1 minute, at room temperature. The spin filter was then carefully placed into a clean 2 mL collection tube as to avoid splashing any C5 solution onto the spin filter. One hundred microliters of Solution C6 was then added to the center of the white filter membrane after which the solution was

centrifuged at 10,000 x g for 30 seconds at room temperature. The spin filter was then discarded and the DNA was prepared for further analysis.

In order to confirm the presence of DNA, 5 μL of extracted sample was mixed with 2 μL of loading dye. The combined mixture from each sample was loaded onto a 1% agarose gel containing ethidium bromide. The gel was run for approximately 1 hour and then photographed under UV light (Figure 3). A PCR reaction was performed using 1 μL of template DNA and mastermix, containing 10x reaction buffer, 50 mM MgCl2, 2 mM dNTP's, fungal DNA primers (ITS3 and NLB4) and Taq polymerase. The thermocycler was programmed to perform 29 cycles of the PCR reaction using the protocol shown in Table 3. Next, amplified samples were loaded onto an agarose gel in order to determine the success of the PCR process by identifying an expected PCR product of about 500 base pair band, relative to the DNA ladder (Figure 4).

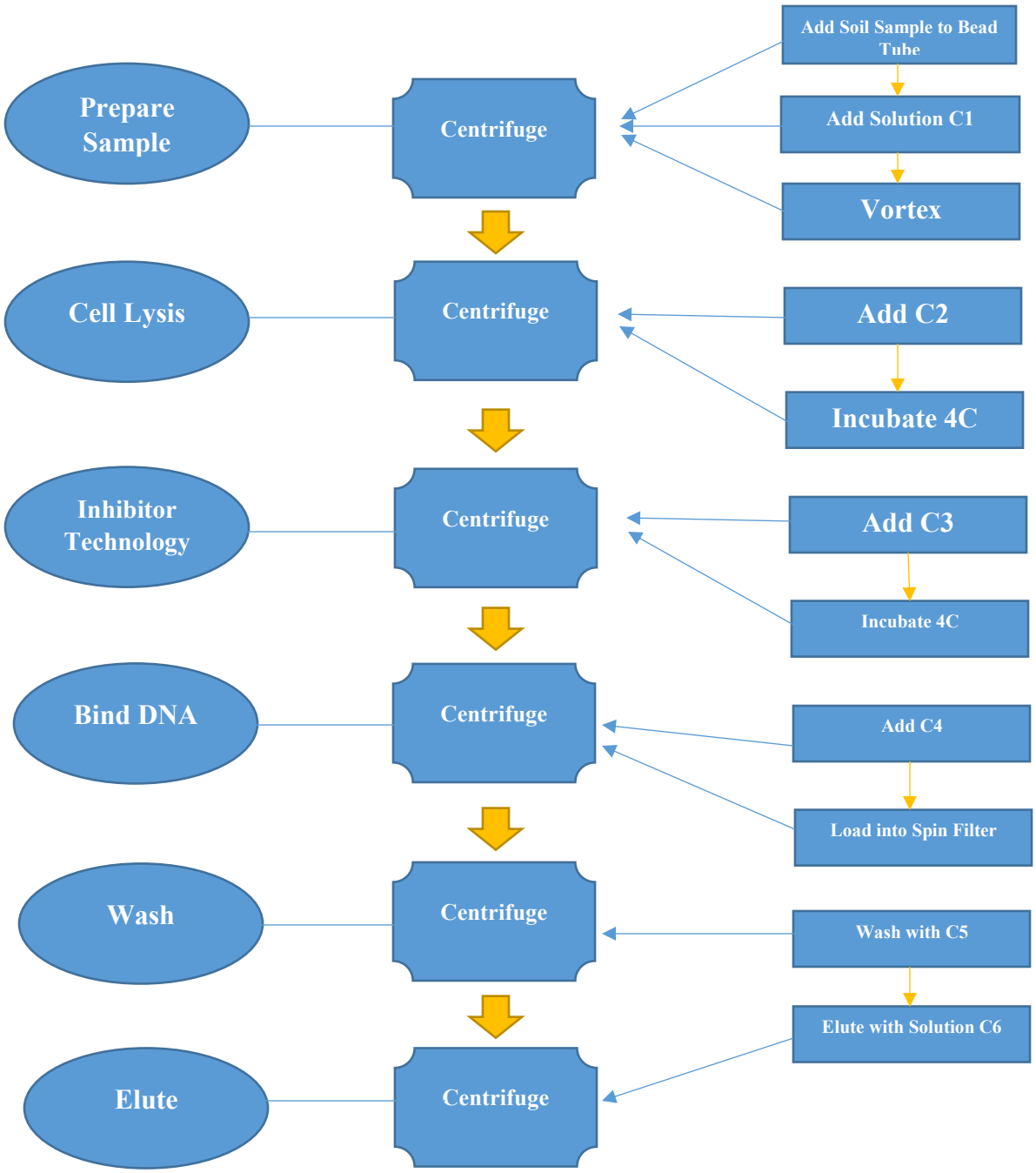


Figure 2. DNA Extraction flow chart provided from a PowerSoil DNA Isolation kit, purchased from MO BIO Laboratories.[55]

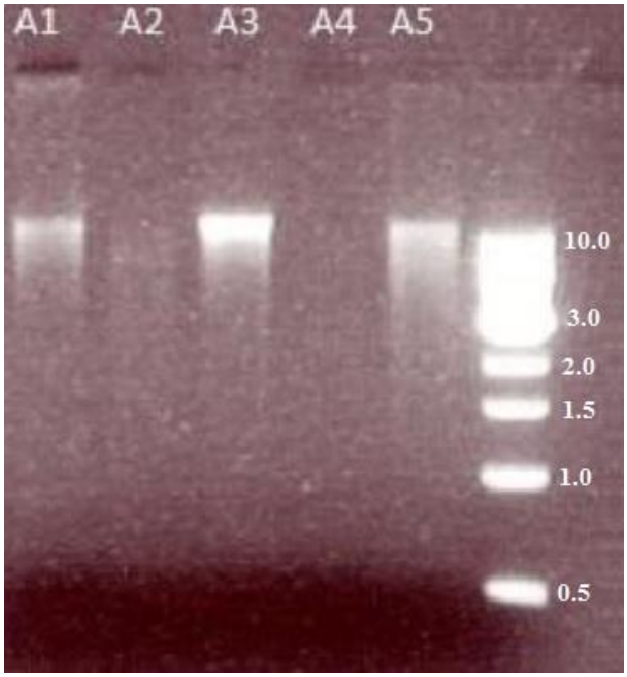


Figure 3. Confirmation of fungal DNA after DNA extraction. Samples were run on a 1% agarose gel, containing ethidium bromide, for approximately 1 hour. Sample A5 was a duplicate of *G. applanatum*. An NEB 1 kb DNA ladder was used. Values shown on the right are in kb.

Table 3. Thermocycler protocol used for Fungal DNA amplification

Step	Temperature (°C)	Time (Minutes)
1	95	5
2	95	1
3	52	1
4	72	1
Steps 1-4 Repeated 29 Times		
5	72	5
6	4	Until Analysis

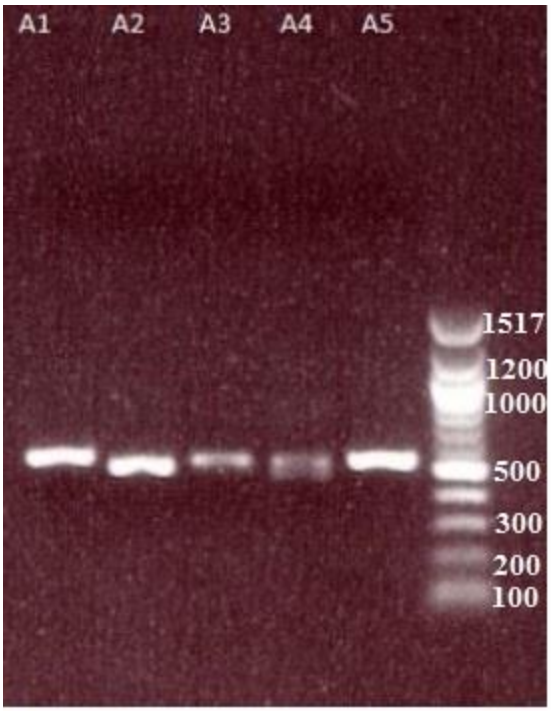


Figure 4. One percent agarose gel containing ethidium bromide in which 4 fungal DNA samples were run after PCR was performed. A band can be seen at 500 base pairs for each sample. Sample A5 was a duplicate of G. applanatum. An NEB 100 bp ladder was used for visualization. Values shown on the right are in bp.

Samples that were successfully extracted and amplified were cleaned via ethanol precipitation and centrifugation. After drying, samples were re-suspended in ddH_20 and were sent for sequencing at the DNA sequencing facility at UNBC. Finally the resulting chromatographs were edited using Chromas Lite[56] and were then queried against existing sequences for known fungal specimens in BLAST[57]. The closest match was then determined by evaluating Max Score and Percent Identification values.

2.1.2 Chemical Extraction

Mushroom species were either air or oven dried at low heat (50 °C) and were then powdered (Figure 5) using either a hammer mill, in the case of *F. pinicola*, or a regular household blender. Samples that were not extremely hard, such as Shiitake (*L. edodes*), were frozen in liquid nitrogen and were then crushed using a pestle and mortar. Initially, dried samples were extracted by closely following protocols outlined by Mizuno[59] (Figure 6), with the addition of an ammonium oxalate fraction. Protocols were later modified to remove the ammonium oxalate fraction and to change the extraction temperature.

Figure 5. An example of a dried and powdered mushroom sample (Chaga).

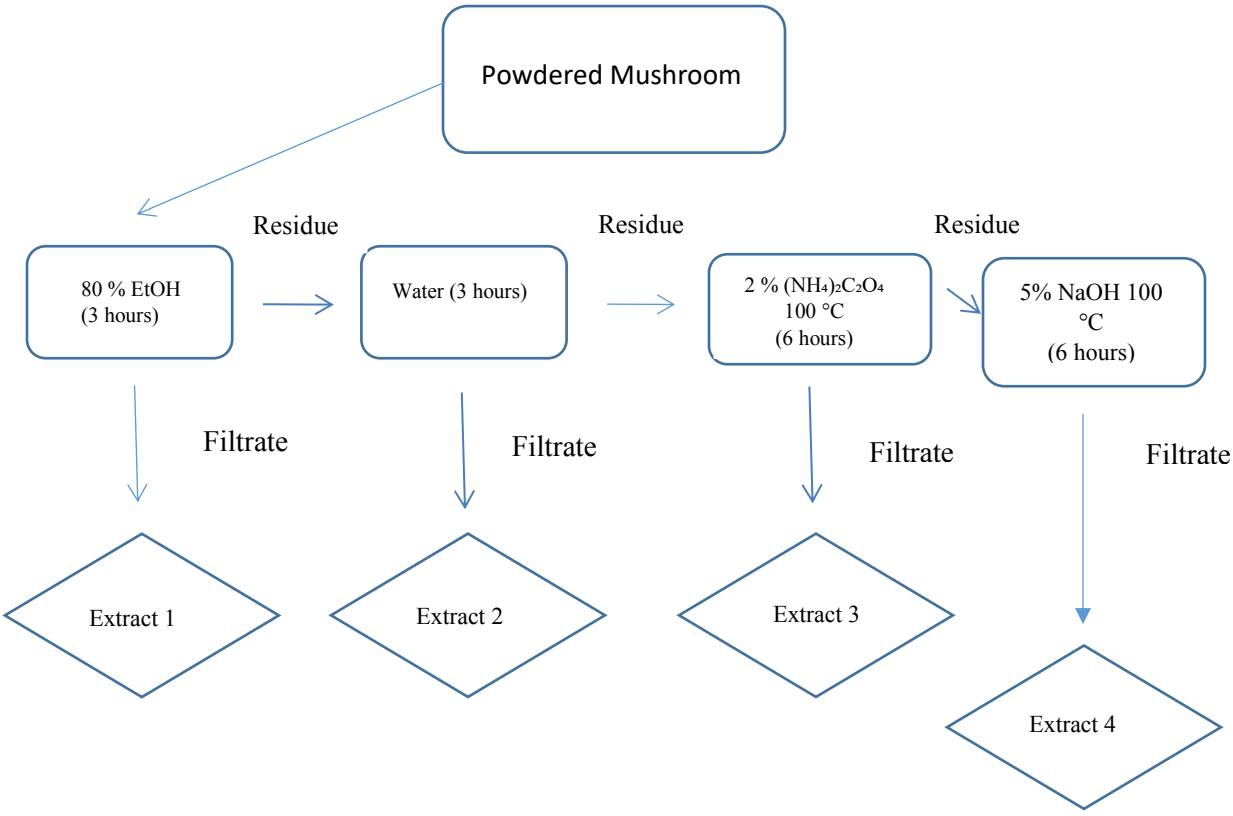


Figure 6. A generalized flow/overview of Mizuno's mushroom extraction techniques[59]. The first step, with ethanol, is meant to separate out the low molecular weight compounds from the high molecular weight compounds.

2.1.3 Extraction Techniques

Fomitopsis pinicola

A 250 gram conk was ground into fiber using a 240 v hammer mill. Thirty five grams was then combined with 750 mL 80% ethanol and refluxed for 3 hours in a round bottom flask with a condenser. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 60 °C until approximately 50 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -80 °C, for 3 days, until only a fine powder remained. The resulting powder was then dissolved in 100% ethanol and filtered using Whatman #3 paper. The residue was lyophilized and was labelled as fraction 1. The filtrate was evaporated to dryness and labelled as extract 2. This was a special method that was used during the very first mushroom extraction. Subsequent extractions followed protocols outlined in Figure 6 or Figure 8.

The residue from the first filtration was combined with 750 mL MilliQ and subjected to reflux for 6 hours, after which it was filtered, evaporated and lyophilized as per the above techniques. The resulting powder was labelled as extract 3. The process was then repeated using 750 mL 5% Sodium Hydroxide. All solutions were neutralized (pH 7) using either 1.25 M NaOH or 12 M HCl prior to lyophilisation.

Lentinula edodes

Four hundred grams of pre-packaged Shiitake mushrooms were purchased from Shoppers Wholesale Foods in Prince George, British Columbia. The sample was flash frozen in liquid nitrogen and then lyophilized at 0.02 Torr and -52 °C, for 2 days, until they were

completely dry. The dried sample was then crushed into a fine powder using a pestle and mortar.

Fifty grams of powdered Shiitake mushroom were combined with 1 L of 80% ethanol and was then refluxed for 3 hours in a round bottom flask with a condenser. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 60 °C until approximately 100 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 1. The remaining residue was combined with 1 L of 50% methanol and was heated to reflux for 3 hours, while being stirred in a 2 L round bottom flask with a condenser. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 65 °C until approximately 100 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 2. The remaining residue was combined with 1 L of distilled water and was then heated to reflux for 6 hours, while being stirred in a 2 L Erlenmeyer flask. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 65 C until approximately 100 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 3. The remaining residue was combined with 1 L of 1% ammonium oxalate and was then heated to reflux for 6 hours, while being stirred in a round bottom flask with a condenser. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 65 C until

approximately 100 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 4. The remaining residue was combined with 1 L of 5% Sodium Hydroxide and was then heated to reflux for 6 hours, while being stirred in a round bottom flask with a condenser. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 65 °C until approximately 100 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 5. All extracts were neutralized (pH 7) using either 1.25 M Sodium Hydroxide or 12 M Hydrochloric acid prior to lyophilisation.

The above extraction techniques were also used for *Piptoporus betulinus*, *Fomes fomentarius and Ganoderma applanatum*. The remaining four samples, *Inonotus obliquus*, *Leucocybe connata*, *Laetiporus sulphureus* and *Phellinus nigricans* all followed the same extraction procedure as outlined below with Chaga.

Echinodontium tinctorium

The manual extraction techniques were the same as those listed above. A separate extraction was also performed using a Buchi Speed Extractor using the following parameters; Samples cells could hold approximately 40 mL total volume. Approximately 20 mL of powdered *E. tinctorium* was loaded onto 0.7 mm of quartz sand. The sample was then extracted using 80% ethanol, at 65 °C for 1.5 hours followed by flushing for 15 minutes. This sequence was repeated for 3 iterations. Next, the sample was extracted using 50% methanol for 1.5 hours following by a flushing cycle for 15 minutes. Again, this cycle was repeated for 3 iterations.

Inonotus obliquus

Two hundred grams of *Inonotus obliquus* was ground in a blender at high speed. One hundred and fifty grams of raw material was then combined with 1.5 L of 80% ethanol and heated to 65 °C for 3 hours, while being stirred in a 2 L Erlenmeyer flask. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 65 °C until approximately 150 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 1. The remaining residue was combined with 1.5 L of 50% methanol and was heated to 65 °C for 3 hours, while being stirred in a 2 L Erlenmeyer flask. The resulting solution was filtered using a Buchner funnel and Whatman #3 paper. The filtrate was then condensed using a rotary-evaporator at 65 °C until approximately 150 mL remained. The remaining filtrate was flash

frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 2. The remaining residue was combined with 1.5 L of distilled water and was then heated to 65 °C for 6 hours, while being stirred in a 2L Erlenmeyer flask. The resulting solution was filtered using a Buchner funnel and cheese cloth. The filtrate was then condensed using a rotary-evaporator at 65 °C until approximately 150 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 3. The remaining residue was combined with 1.5 L of 5% Sodium Hydroxide and was then heated to 65 °C for 6 hours, while being stirred in a 2 L Erlenmeyer flask. The resulting solution was filtered using a Buchner funnel and cheese cloth. The filtrate was then condensed using a rotary-evaporator at 65 °C until approximately 150 mL remained. The remaining filtrate was flash frozen in liquid nitrogen and lyophilized at 0.02 Torr and -52 °C, for 3 days, until only a fine powder remained, and was labelled as extract 4. All extracts were neutralized (pH 7) using either 1.25 M Sodium Hydroxide or 12 M Hydrochloric acid prior to lyophilisation.

2.1.4 Cell Culture and Mushroom Extract Preparation

Human cervical cancer HeLa cells were grown to confluency using Thermo Fisher Scientific T25 Flasks. Once confluent, the supernatant was poured off and the adherent cells were then washed with 3 mL of Phosphate-buffered saline. Any remaining supernatant was removed prior to trypsinization. One mL of Trypsin, containing ethylenediamine tetra-acetic acid (EDTA), was then added to the T25 flask and slowly rocked in order to completely cover the bottom surface of the flask. The cells were then incubated at 37 °C, with 5% Carbon Dioxide, for a maximum of 5 minutes, after which 9 mL of Eagle's minimal essential

medium (containing 10% Fetal Bovine Serum) was added to suspend the cells. The solution was then gently mixed to ensure that the solution contained an even amount of cells per volume and was then transferred to a sterile 50 mL Falcon tube.

Cells counts were performed using a hemocytometer. Fifty microliters of stock cell solution was mixed with 50 µL of Trypan Blue dye. A cover slip was placed over the hemocytometer and a small drop of solution was added to the v-shaped groove. Cell counts were taken from 3-4 quadrants and a dilution calculation was performed. Blue cells were not included in the counts as they are dead.

Dried and powdered mushroom extracts were dissolved into appropriate solvents, using water whenever possible, ideally at a concentration of 40 mg / mL. Concentrated stock treatment solutions were then filter sterilized using a 0.2 µm filter. Working under sterile conditions, treatment solutions were prepared by diluting sterile stock solutions in EMEM+FBS to achieve desired concentrations, ranging from 0.01 mg / mL to 2 mg / mL, depending on the sample being assayed. The perimeter of a sterile, 96 well, flat bottomed plate, was filled with 200 µL of sterile water, leaving 60 wells for use with controls and various mushroom extract concentrations, coupled with applicable controls. In general, 4-5 different concentrations of mushroom extracts were used coupled with 4-5 controls, each using a set of 6 technical replicates.

Extracts of interest were also assayed in a time dependent manner. Samples were prepared using the same methods mentioned above, except that seven of the 96 well plates were used – 1 for each day. The wells in each plate were treated in the exact same manner and plates were scanned at 570 nm, daily. Results of each plate were then compared to the results of the plate from day 7.

2.1.5 MTT Assay

The MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a widely adopted method of testing for growth-inhibitory and cytotoxic activity in mammalian cells[60–62]. This method of analysis relies on the metabolism of living cells to produce a colorimetric change which can then be quantified via a plate reader at 570 nm[63]. This colour change occurs due to the reduction of the yellow tetrazolium MTT by mitochondrial dehydrogenase enzymes, among others, resulting in an intracellular soluble formazan, which is purple[64] (Figure 7). Mushroom extracts were assessed for possible growth-inhibitory effect on HeLa human cervical cancer cells. HeLa cells are commonly used to test anti-proliferative activity from substances derived from mushroom extract[65,66].

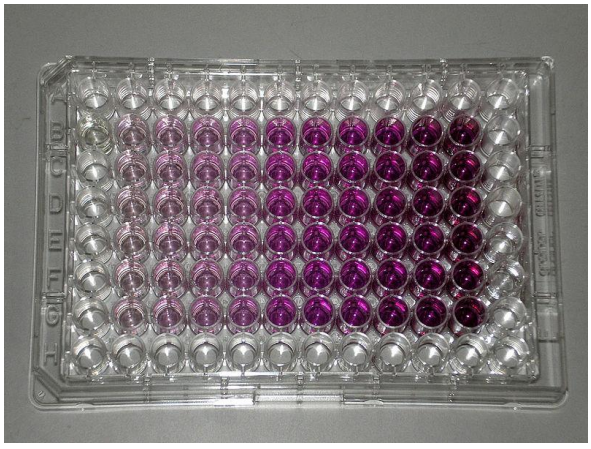


Figure 7. An example of the final product of an MTT assay[67]. More intense purple colouration is indicative of more living cells. If our mushroom extracts have anti-proliferative activity, we would expect to see a decrease in purple colour as the concentration of compound, and time increased.

HeLa cells, combined with mushroom extract, were incubated at 37 °C, with 5% CO_2, for a period of 48-72 hours. Next, stock MTT solution was diluted to 1/5th concentration, and 50 µL was added to each of the wells containing controls and mushroom extracts. The plate was then incubated for an additional 3-4 hours after which the supernatant was completely aspirated, leaving only the adherent HeLa cells. One hundred and fifty microliters of DMSO was then added to each well. The plate was then gently shaken for 5-10 minutes and then read at 570 nm on a Titertek Multiskan photometer, or a Synergy2 plate reader.

2.1.5.1 *Fomitopsis pinicola*

As this was the first sample extracted and assayed, it was necessary to attempt a variety of different concentrations of mushroom extract (in MTT assay), in order to ascertain potential concentrations to use in the future. This mushroom was extracted twice and 3 MTT assays were performed. In each of the assays, the addition of ammonium oxalate extract to cells in 96-well plates resulted in precipitation (further confirmed under a microscope). It is highly suspected that ammonium oxalate extract reacted with chemicals in the medium to generate the precipitate. Therefore, the ammonium oxalate extraction step was later removed.

Since the different extracts were all dissolved in different solvents (Table 4), it was necessary to also test the effect of the solvent itself in an MTT assay, in order to rule out false positive growth inhibitory effects. A basic MTT experiment was set up with all of the extracts except for ammonium oxalate and visual observations were taken under a microscope after 24 and 48 hours. The following concentrations were used: extract 1 (80% ethanol dissolved in water) at 0.5 mg / mL and 1 mg / mL. Extract 2 (99 % ethanol dissolved in 100% ethanol) at 0.1, 0.2, 0.4 and 1 mg / mL. Extract 3 (Water, dissolved in DMSO) at 0.01, 0.05, 0.2, and 0.5 mg / mL. Extract 4 (5% Sodium Hydroxide, dissolved in water) at 0.5

and 1 mg / mL. Additionally, Cis-Platinum, a known chemotherapeutic agent, was used as a positive control. The results of the visual assessments can be seen in Table 5 and 6.

After the initial MTT assay was completed, another dose dependent experiment was setup using the following concentrations: E1 (0.01, 0.05 and 0.1 mg / mL), E2 (0.02, 0.04, 0.1, 0.2, 0.4 mg / mL), E3 (0.01, 0.02, 0.05, 0.1, 0.2 mg / mL), E4 (0.005, 0.01, 0.05, 0.1, 0.5, 0.1 mg / mL). Extract E4 (5% Sodium Hydroxide extraction dissolved in water) was re-assayed an additional 3 times. A final dose dependent assay (72 hours) was setup using extract 2 at 0.02, 0.04, 0.1, 0.2 and 0.4 mg / mL. Two concentrations of interest were then chosen and a 7 day time dependent MTT assay performed using two different seed cell concentrations, 1500 and 3000 cells per well.

Table 4. BC Mushrooms: An Inventory List and Extraction Solvent Used

Mushroom	Processed Raw Weight	Used (g)	Extract #	Extraction Solvent
Fomitopsis pinicola	33	33	1	80% Ethanol
			2	99% Ethanol
			3	Water
			4	2 % Ammonium Oxalate
			5	5% Sodium Hydroxide
Lentinula edodes	50	50	1	80% Ethanol
			2	Water
			3	2 % Ammonium Oxalate
			4	5% Sodium Hydroxide
Piptoporus betulinus	128.5	35	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	2 % Ammonium Oxalate
			5	5% Sodium Hydroxide
Fomes fomentarius	126.45	40	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	2 % Ammonium Oxalate
			5	5% Sodium Hydroxide
Ganoderma carnosum	89.31	30	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	1 % Ammonium Oxalate
			5	5% Sodium Hydroxide
Echinodontium tinctorium	273.15	40	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	1 % Ammonium Oxalate
			5	5% Sodium Hydroxide
Inonotus obliquus	315	150	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	5% Sodium Hydroxide
Leucocybe connata	80	80	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	5% Sodium Hydroxide
Laetiporus sulphureus	77	77	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	5% Sodium Hydroxide
Phellinus nigricans	135.89	100	1	80% Ethanol
			2	50% Methanol
			3	Water
			4	5% Sodium Hydroxide

2.1.5.2 *Lentinula edodes*

Shiitake mushrooms have been ingested for their medicinal properties in countries such as Japan for nearly a century. They have also been shown to have cytotoxic properties when tested with HeLa cells using MTT assays[68]. In many cases, a hot water extract is performed and the extracted material is ingested as a tea. Because of this, it was decided to setup two side by side extractions, using two different temperatures. The first extraction was performed at reflux temperatures whereas the second was performed at 50 °C. Extracted compounds were dissolved in water at a concentration of 40 mg/mL. The following concentrations were used: E1 – 80% ethanol (0.1, 0.5, 1 mg / mL), E2 - Water (0.1, 0.5, 1 mg / mL), E3 – ammonium oxalate (0.1, 0.5, 1 mg / mL) and E4 – 5% Sodium Hydroxide (0.1, 0.5 and 1 mg / mL). A water control was used in equivalence for each extract. A second MTT assay was later performed using compounds from both the 50 °C and reflux extractions. This time the following concentrations were used 0.01, 0.05, 0.1, 0.5 and 1 mg / mL for extracts E1, E2 and E4. The ammonium oxalate extract was not tested again with this mushroom. Because of these results, it was determined that refluxed reactions were no longer required and the extraction protocols were modified as per Figure 8. It was also decided that a 50% methanol extract be included as per a suggestion from Dr. Karl Tsim from the Hong Kong University of Science and Technology. The addition of the methanol extract is proposed because methanol is more polar than ethanol, as such it may help to remove phenolic compounds. It was also decided to use 65 °C as this is between 50 °C and reflux temperature, as many mushrooms were extracted at reflux.

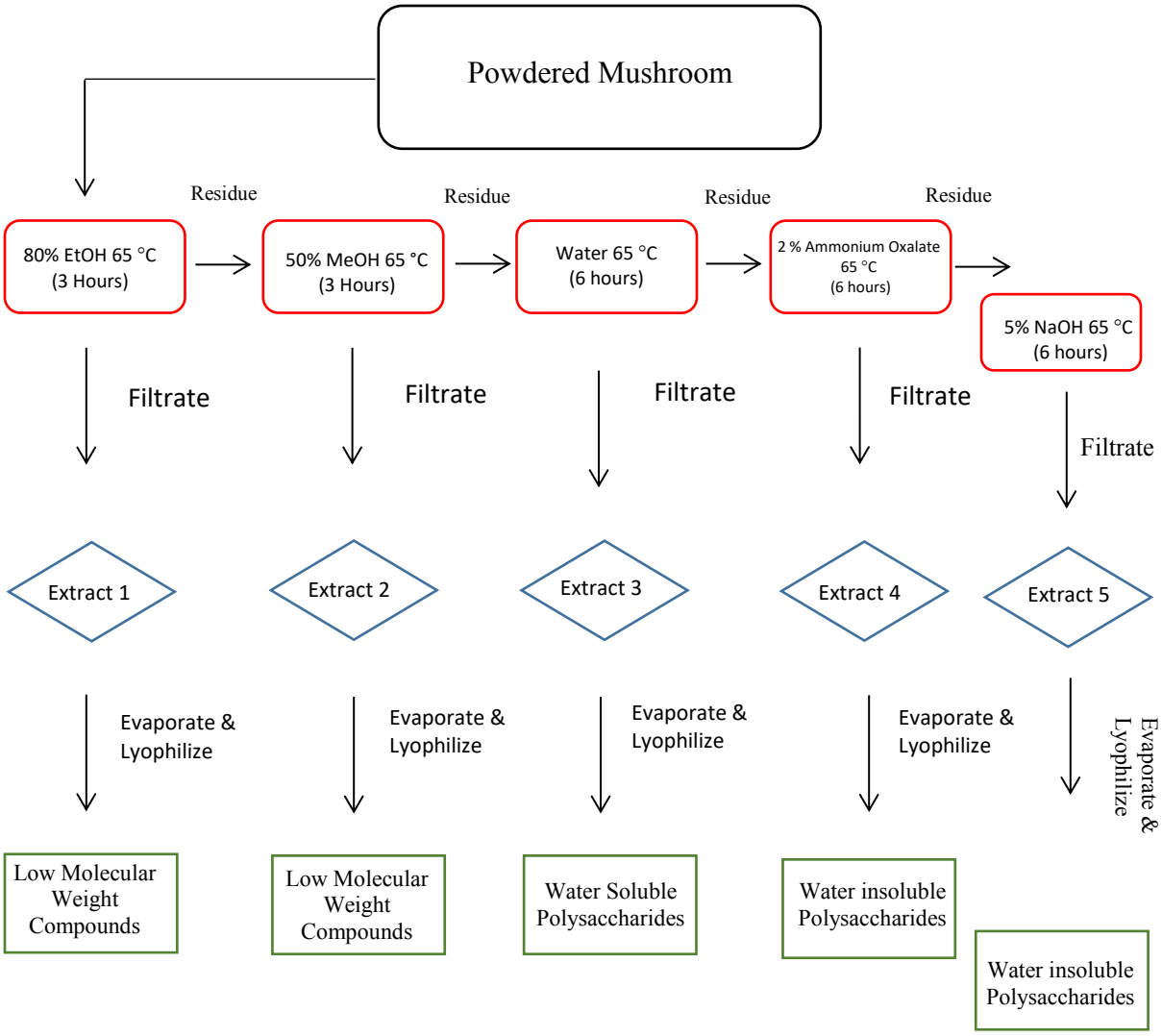


Figure 8. Modified extraction technique to reflect the change in temperature and the addition of a 50% methanol extract.

2.1.5.3 *Piptoporus betulinus*

This sample was extracted by following the procedures outlined in Figure 8. An MTT assay was setup using all 5 extracts. Extract 1 (80% ethanol) was dissolved in ethanol, while all other extracts were dissolved in water, at 20 mg / mL. The following concentrations were used for each of the 5 extracts: 0.01, 0.05, 0.1, 0.5 and 1 mg / mL. Even though an ammonium oxalate extract was extracted, it was decided to not use that particular extract in

further MTT assays for this mushroom sample. A 48 hour MTT assay was performed after which a time dependent assay was performed using E1 (80% ethanol) and E3 (Water).

2.1.5.4 *Fomes fomentarius*

This sample was also extracted using the procedures outlined in Figure 8. Extracts 1 and extract 2 (80% ethanol and 50% methanol, respectively) were dissolved in DMSO. Extracts 3 (Water) and 5 (5% NaOH) were dissolved in water. The following concentrations were used: 0.1, 0.05, 0.1, 0.5 and 1 mg / mL. The ammonium oxalate extract was omitted from MTT assay in this sample as well. Three extracts were selected for further investigation and a time dependent MTT assay was setup using extracts 1, 2 and 3.

2.1.5.5 *Ganoderma applanatum*

This sample was extracted using the procedures outlined in Figure 8. However, the 2% ammonium oxalate extract was modified to be 1%. Extracts 1 (80% ethanol) and extract 2 (50% methanol) were dissolved in DMSO. Extracts 3 (Water), 4 (1% ammonium oxalate) and extract 5 (5% NaOH) were all dissolved in water. Extract 4 was again omitted due to precipitation being seen under a microscope. An initial MTT assay was performed using E1, E2, E3 and F5, after which a seven day time dependent MTT was performed using only the water extract (extract 3). The following concentrations were used for the 48 hour MTT assay: 0.01, 0.05, 0.1, 0.5 and 1 mg / mL. The time dependent assay was run at 1 mg / ml.

2.1.5.6 *Echinodontium tinctorium*

This sample was extracted in parallel with *G. applanatum* and because of this, an ammonium oxalate extract was still extracted, but was omitted from assay. All 4 extracts were easily dissolved in water at a concentration of 40 mg / mL. Each extract was first tested

using a dose dependent 48 hour MTT assay at the following concentrations: 0.01, 0.05, 0.1, 0.5 and 1 mg / mL. The second extract (50% methanol) was selected for further investigating in a seven day time dependent MTT assay.

2.1.5.7 *Inonotus obliquus*

After the previous extractions, it was decided to omit the ammonium oxalate extract altogether, and move to extraction procedures as outlined in Figure 9. All samples were easily dissolved in water at a concentration of 40 mg / mL. An initial 48 hour MTT assay was set up in order to screen all 4 extractions for cell growth inhibition. The following concentrations were used for each extract: 0.01, 0.05, 0.1, 0.5 and 1 mg / mL. With the exception of extract 1 (80% ethanol), all extracts were then tested using a seven day time dependent MTT assay at a concentration of 0.5 mg / mL.

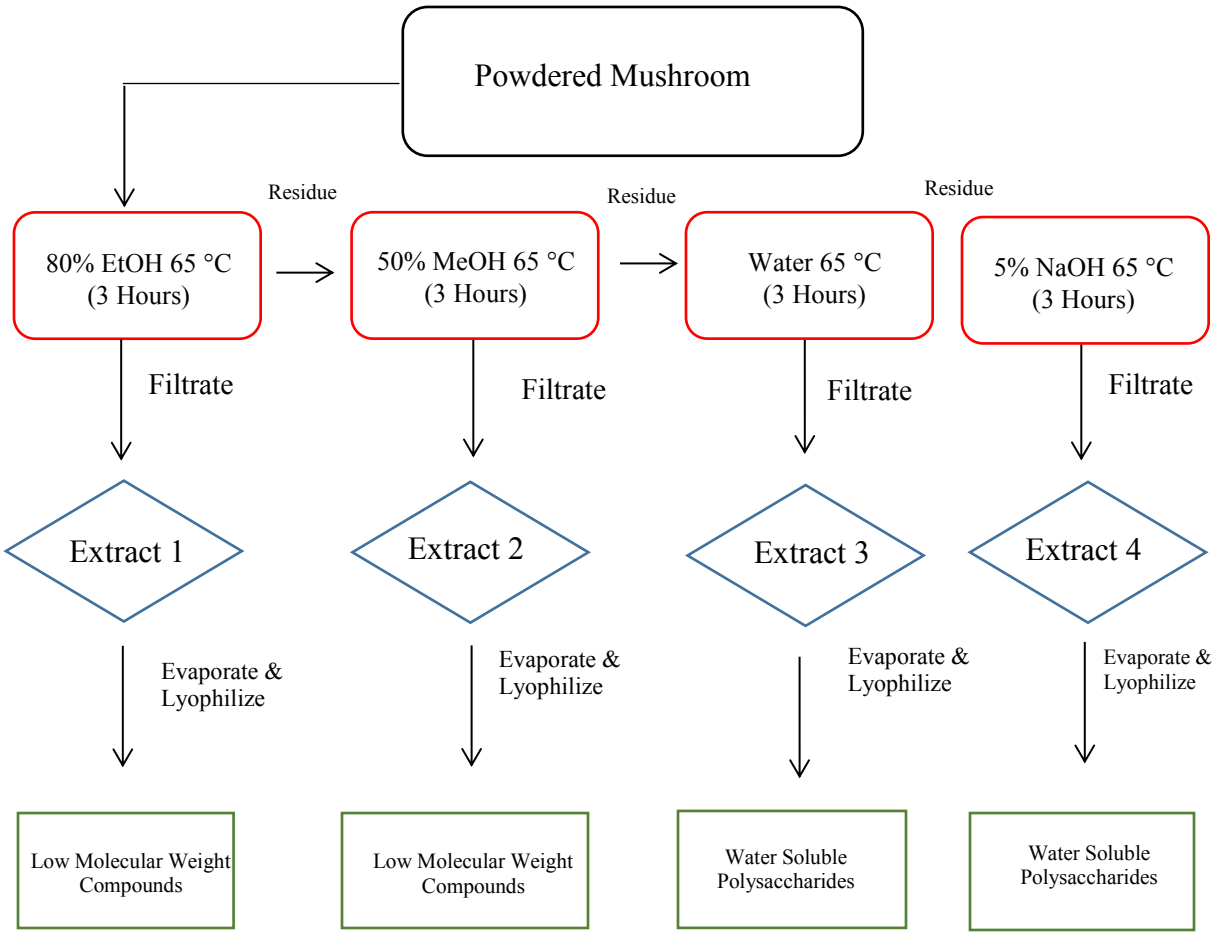


Figure 9. The final extraction scheme adopted. This is the modified extraction technique to reflect the removal of the ammonium oxalate extract entirely. A reduction from 2% to 1% of ammonium oxalate still caused visible precipitation when combined with HeLa cell / minimal essential medium (mem) solution.

2.1.5.8 *Leucocybe connata*, *Laetiporus sulphureus* and Phellinus *nigricans*

All three of these samples were extracted using the steps outlined in Figure 9. All extracts were easily dissolved in water at a concentration of 40 mg / mL. All extracts were assayed using the following concentrations: 0.01, 0.05, 0.1, 0.5 and 1 mg / mL. After an initial 48 hour MTT assay, time dependent assays were run using the following extracts and concentrations. *Leucocybe connata* was run using extract 1 (80% ethanol) and extract 4 (5% Sodium Hydroxide). *Laetiporus sulphureus* was run using extract 3 (water) at a concentration of 0.5 mg / mL. *Phellinus nigricans* was assayed using both extract 1 (80% ethanol) and E3 (water) at a concentration of 0.5 mg / mL.

2.2 Results

2.2.1 Species Identification

The initial DNA extraction was fairly successful. Strong high molecular weight bands could be seen for samples A1, A3 and A5. A faint band could be seen for sample A2 and there was no band for sample A4 (Figure 3). All 5 samples were PCR amplified regardless of the results of the first DNA gel. The PCR reaction also appeared to be successful, with bands showing for all five samples. Samples A1 and A5 had the strongest bands, indicating that they underwent the most successful amplification. The majority of my samples had been previously identified via DNA analysis by Ms. Marta Zmudzinski.

2.2.1.1 Sample A1 – *Piptoporus betulinus* (Bull.) P. Karst.

Piptoporus (Polyporus) betulinus sporocarps, found on paper birch in a mixed-wood forest in Ste-Geneviève of Batiscan, Quebec (Chow Lee collection # 137, photo credit H. Massicotte)

This sample was obtained from Dr. Egger in the winter semester of 2013. The sample was ground to a fine powder and was stored at -4 °C. This sample was previously identified via DNA analysis as being *Piptoporus betulinus* by Ms. Zmudzinski. The sequences obtained using the ITS primer was edited to 498 bases, while the sequences obtained using the NLB primer was edited to 492 bases. A comparison of these two sequences to known fungal samples via BLAST yielded the sample result for both primers: *Piptoporus betulinus*. The ITS sequence had a max score of 890 and a 99% identification, while the NLB sequence had a max score of 915 and a 99% identification. Both sequences had *P. betulinus* as the first and second hit.

2.2.1.2 Sample A2 - *Ganoderma applanatum* (Pers.) Pat.

Ganoderma applanatum – sample harvested from Smithers, British Columbia, growing on a tree stump. (Photo credit V. Myhre)

This sample was obtained from Dr. Egger in the winter semester of 2013. The sample was ground to a fine powder and was stored at -4 °C. This sample was previously identified by Ms. Zmudzinski as being *Ganoderma carnosum*. The sequences obtained using the ITS primer was edited to 487 bases while the sequences obtained using the NLB primer was edited to 417 bases. Each sequence had multiple result possibilities; however, both had *Ganoderma applanatum* as the first hit and hence the most likely candidate. The ITS sequences yielded a max score of 927 and a % identification of 99% whereas the NLB derived sequences yielded a max score of 797 and a % identification of 99%. The next highest max score was 818 (*G. tsugae*) and 743 (*G. lipsiense*), for the ITS and NLB derived sequences.

2.1.1.3 Sample A3 - *Fomes fomentarius* (L.) Fr.

Image obtained from
https://upload.wikimedia.org/wikipedia/commons/7/7b/Fomes_fomentarius_2010_G2.jpg
(Accessed May 9, 2017)

This sample was obtained from Dr. Egger in the winter semester of 2013. The sample was ground to a fine powder and was stored at -4 °C. This sample was previously identified

by Ms. Zmudzinski as being *Fomes fomentarius.* The ITS sequences were edited to be 508 bases and the NLB sequences were edited to 374 bases. Both the ITS and NLB derived sequences showed that *Fomes fomentarius* was the most likely candidate. The sequence obtained from the ITS primer yielded a max score of 823 and a percent identification of 99% while the sequence obtained from the NLB primer had a max score of 658 and a percent identification of 98%. The next highest hit for both sequences was *Polyporales sp*. but the max score was far lower as was the percent identification (6% less in both cases).

2.2.1.4 Sample A4 – *Echinodontium tinctorium*

Echinodontium tinctorium, harvested from West Twins, near McBride, British Columbia. The specimen is easily identified by its tooth like spikes protruding from its top.

This sample was harvested from an old growth cedar-hemlock forest, West of McBride, and was found in a fairly shaded area, laying on moss. The outside of the specimen was dark on the outside and orange / red where the surface was cracked. Teeth could be seen on the lower surface of the specimen. This sample was previously identified to be *Echinodontium tinctorium* by using both MatchMaker (*www.matchmakermushrooms.com/)*

and David Arora's Mushrooms Demystified[58]. The sample was stored at -4 °C for approximately one month prior to performing DNA extraction / PCR. However, this sample also showed high levels of co-amplification or contamination for both the ITS3 and NLB4 derived sequences. The ITS derived sequence was edited to 498 bases and the NLB derived sequence was edited to 360 bases. The highest max score for the ITS sequence was 195 and had a hit for *E. tinctorium*. The highest max score value for the NLB sequences was 94.5, showing that the first hit was *E. tinctorium*.

2.2.1.5 *Lentinula edodes*

Lentinula edodes seen in quick freeze flasks, after being flash frozen in liquid nitrogen.

This sample was purchased from Shoppers Wholesale in Prince George, British Columbia (Avina Gourmet Shiitake Mushrooms 100 g packages). No morphological or DNA analysis was performed on this sample as it was purchased prepackaged and labelled.

2.2.1.6 *Fomitopsis pinicola* (Sw.) P.Karst.

Fomitopsis pinicola, found growing on a fallen log, in Twin Falls Provincial Park, near Smithers, B.C. (Photo credit V. Myhre)

This sample of red belted conk was obtained from the area surrounding Terrace during the summer of 2012. The vivid colour makes this species fairly easy to identify. The actual identity was first confirmed by Dr. Keith Egger and Dr. Hugues Massicotte and was later confirmed by Mushroom's Demystified and Match Maker software. When this project was initially being pioneered, I was enrolled in a Mycology course which had a DNA analysis section. As such, only a handful of samples were tested as there was limited access. Also, at that time, DNA analysis was not deemed critical as my samples had been morphologically identified by Drs. Keith Egger and Hugues Massicotte. Because of this, only

P. betulinus, *G. applanatum, F. fomentarius* and *E. tinctorium* were identified via DNA analysis.

2.2.1.7 *Inonotus obliquus* (Ach. ex Pers.) Pilát

Inonotus obliquus growing on a birch tree, collected from Forest for the World, an area surrounding UNBC. The second picture is the harvested sample.

This specimen was growing high up on paper birch and was collected during the spring of 2013, from the area surrounding UNBC. In fact, once the first one was located, it was easy to see many more far up on other birch trees. The sample weighed over 8 lbs and had a dry weight of about 5 lbs. The Chaga appears to burst out of the tree, almost in a tumor-like fashion, as can be seen in the picture above. It was extremely black, cracked, and dense and was nearly 25 cm across. Identification began with the Polypores section in MatchMaker and concluded that it was *Inonotus obliquus* – unmistakable from the pictures included with the software. The identity was also keyed out as per David Arora's Mushrooms

Demystified, beginning with the Polypore section. The spore bearing surface was not made of tubes and the pore surface was exposed and was growing on Birch but not 'curblike'. Additionally, the surface was not waxy and the flesh was red to orange, rusty-brown or dark brown when exposed to Potassium Hydroxide. This lead to the next section: *Phaeolus*, *Inonotus, Coltricia* and Allies. Because the fruiting body was a brown / dark black in colour and the surface was cracked and irregularly shaped, and also found on birch, it was determined that this specimen was *Inonotus obliquus*.

2.2.1.8 *Leucocybe connata* (Schumach.) Vizzini, P. Alvarado, G. Moreno & Consiglio

Sample of *Leucocybe connata* after being frozen in liquid nitrogen and lyophilized for 48 hours.

This sample was collected from the area surrounding Prince George by former students in Dr. Egger and Dr. Massicotte's Mycology course. The sample was provided and identified by Dr. Massicotte. Because this species is so moist, we had to go back and collect additional material in order to perform an adequate extraction.

2.2.1.9 *Laetiporus sulphureus* (Bull.) Murrill

Image obtained from
https://upload.wikimedia.org/wikipedia/commons/e/ec/Laetiporus_sulphureus_big.jpg
(Accessed May 9, 2017)

This sample was obtained from Northern British Columbia in the area surrounding UNBC, in the spring of 2013 and was identified by Dr. Egger and Dr. Massicotte. Since this species is so readily available (commonly referred to as Chicken of the Woods) and easily identified, no DNA analysis was performed on this sample.

2.2.1.10 *Phellinus nigricans* (Fr.) P. Karst

Oven dried sample of *Phellinus nigricans* obtained from the Greenway trail behind UNBC.

This specimen was collected behind UNBC in September 2013 and was located on a fallen birch towards the underside in a fairly shaded, undisturbed area. It is brown in colour and has a velvety underside. The shape was somewhat hoofed but it appears to have molded/formed around the tree. The top portion was fairly cracked. The tissue blackened when exposed to 10% Potassium Hydroxide solution. The measurements were 10 cm x 4 cm. Using MatchMaker, I got an 87% match to *Phellinus nigricans* (which happened to be the only match). The specimen was identified using David Arora's Mushrooms Demystified using the following criteria. The spore bearing surface was composed of tubes and the

fruiting body was hoof-life. Additionally, the pore surface was exposed and the fruiting body had a cap, was hard and a stalk was absent. The only match to this was *Phellinus nigricans*.

2.2.2 Dose and Time Dependent MTT Assays

2.2.2.1 *Fomitopsis pinicola*

Since this was the first mushroom that was processed, extracted and assayed, it was tested many times. Initially, only 2-3 concentrations were used and visual observations were taken after 24-48 hours (Tables 5-6) in order to assess the viability of the chosen concentrations. It was also necessary to examine whether or not the solvents, used to dissolve the dried mushroom samples, were able to cause cell growth inhibition in HeLa cells. After 24 hours, the cells treated with DMSO at 0.1% looked healthy while the cells treated with 5% DMSO were starting to round up and die. The cells treated with ethanol, both 0.25% and 2.5%, were all healthy. Cells treated with 25 μM and 50 μM of Cis-Platinum all appeared to be rounding up and slowly dying. The media in the wells treated with E1 (80% ethanol), appeared to yellow which is usually indicative of acidity. The cells were also completely dead. All wells treated with extract 2 (99% ethanol) appeared to be rounding up and dying at all concentrations, with the exception for at 1 mg / mL, where the cells were completely dead. The medium was not yellow in any of the wells treated with E2. Extract 3 also caused complete cell death at its highest concentration (1 mg / mL), but the medium also appeared to be yellowed. The cells in wells treated with E4 (5% NaOH) appeared to be healthy. Visual observations were also taken after 48 hours. The wells treated with E1 looked the same as they did after 24 hours, completely dead and yellow. Cells treated with E2 and E3 looked the same as they did after 24 hours. However, the wells treated with E4 now appeared to be

stimulatory. Further assays were performed using E4 and no stimulatory effect was observed, either visually, or after an MTT assay.

The results of a 72 hour MTT assay can be seen in Figure 10. Some growth inhibition can be seen in cells treated with E1, when compared to their respective controls. Cell growth inhibition, in cells treated with E2, was dramatic. All three concentrations were able to reduce HeLa cell growth to 20% or less. Extracts 3 and 4 had little effect.

Both time dependent assays had nearly the same result, at both 1500 and 3000 cells per well (Figures 11 and 12). Final concentrations as low as 0.1 mg / mL were able to completely obliterate cell growth. After this assay, it was determined that a cell density of 1500 cells per well would be sufficient for further 7 day time dependent assays.

Table 5. MTT Assay (Extracts from *Fomitopsis pinicola*) – Visual Observations after 24 hours of treatment

Treatment	Concentration	Medium looks yellow (acidic)	Cells look normal and healthy	Cells look round up/slowly dying	Complete death/toxic
DMSO	5%			x	
	0.1%		x		
Ethanol	2.5%		x		
	0.25%		x		
Cis-platinum	50 µM			x	
	25 µM			x	
Extract 1	1 mg/ml	x			x
	0.5 mg/ml	x			x
Extract 2	0.1 mg/ml (0.25% ethanol)			x	
	0.2 mg/ml			x	
	0.4 mg/ml			x	
	1 mg/ml (2.5% ethanol)	x			x
Extract 3	0.01 mg/ml (0.1% DMSO)		x		
	0.05 mg/ml		x		
	0.2 mg/ml			x	
	0.5 mg/ml (5% DMSO)	x			x
Extract 4	1 mg/ml		x		
	0.5 mg/ml		x		

Table 6. MTT Assay (Extracts from *Fomitopsis pinicola*) – Visual Observations after 48 hours of treatment

Treatment	**Concentration**	**Medium looks yellow (acidic)**	**Cells look normal and healthy**	**Cells look round up/slowly dying**	**Complete death/toxic**
DMSO	5%			x	
	0.1%		x		
Ethanol	2.5%		x		
	0.25%		x		
Cis-platinum	50 µM			x	
	25 µM			x	
Extract 1	1 mg/ml	x			x
	0.5 mg/ml	x			x
Extract 2	0.1 mg/ml (0.25% ethanol)			x	
	0.2 mg/ml			x	
	0.4 mg/ml			x	
	1 mg/ml (2.5% ethanol)	x			x
Extract 3	0.01 mg/ml (0.1% DMSO)		x		
	0.05 mg/ml		x		
	0.2 mg/ml			X (medium becoming yellow)	
	0.5 mg/ml (5% DMSO)	x			x
Extract 4	1 mg/ml		X (appeared stimulatory		
	0.5 mg/ml		X (appeared stimulatory)		

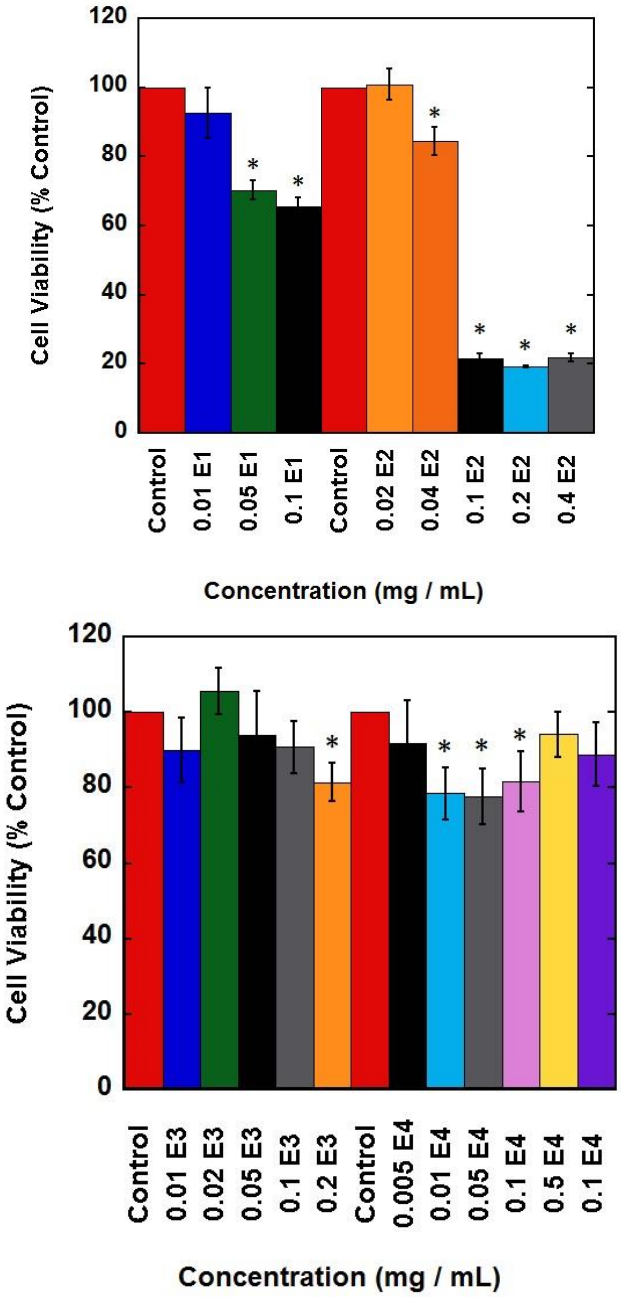


Figure 10. The results of a 72 hour MTT assay using four extracts obtained from the extraction of *F. pinicola*. The extracts were extracted in the following solvents. E1 (80% ethanol), E2 (99% ethanol), E3 (Water), E4 (5% Sodium Hydroxide). An ammonium oxalate extract was not tested. One biological replicate and six technical replicates were used for each extract and concentration. *P < 0.05 versus control; Student's t-test (one-tailed). Error bars are standard error of the mean (S.E.M).

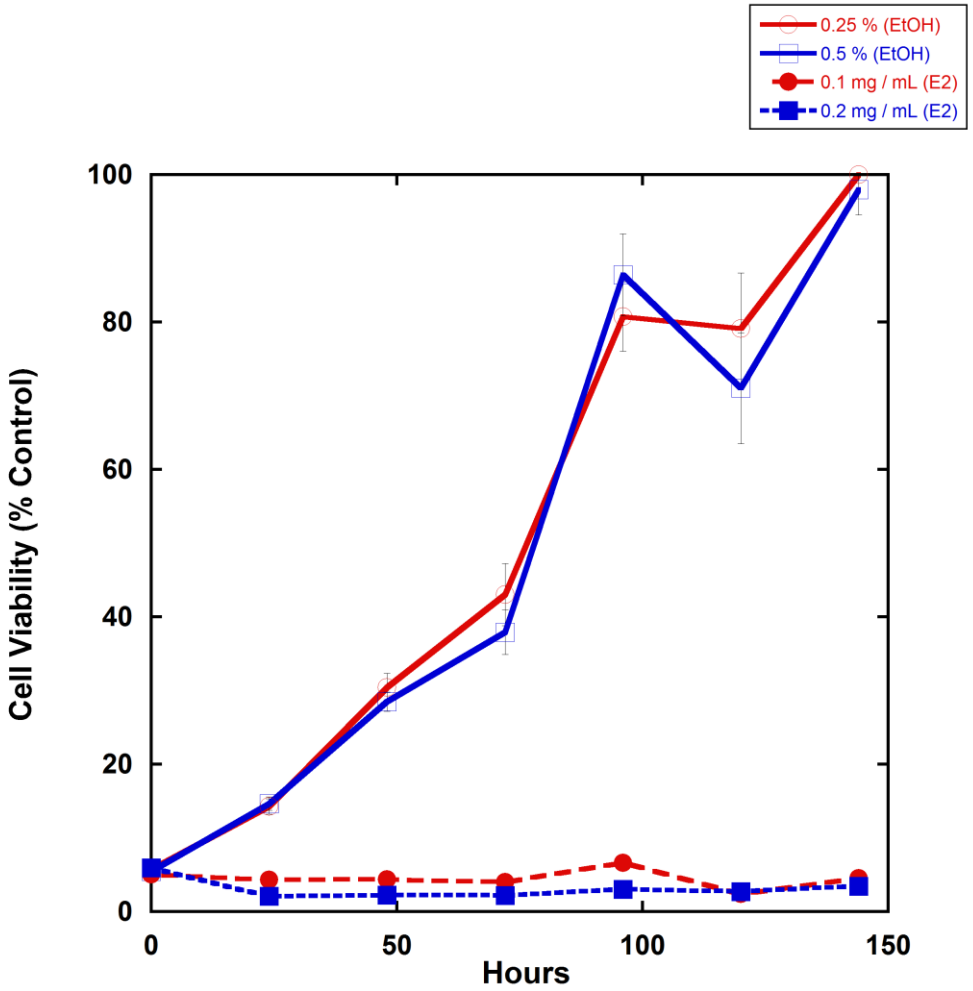
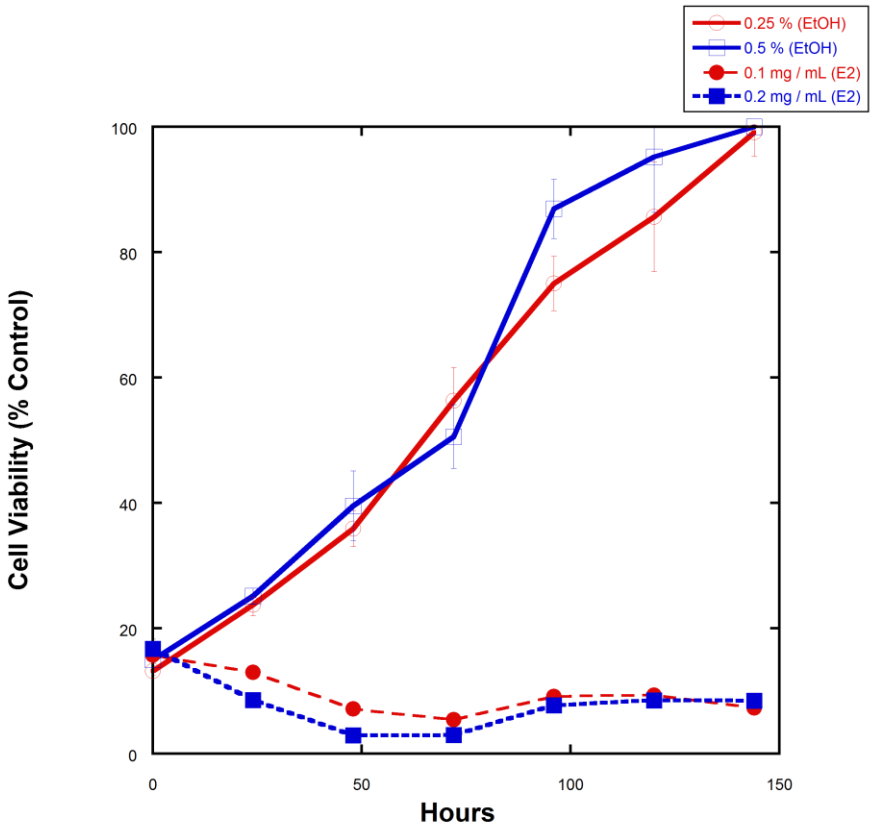


Figure 11. The results of a time dependent assay using two concentrations of extract 2, which was extracted in 99% ethanol and then dissolved in 80% ethanol. Two concentrations were used, 0.1 and 0.2 mg / mL. A cell density of 1500 cells were well was used. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.



***F. pinicola* E2 Time Dependent MTT Assay - 3000 Cells per Well**

Figure 12. The results of a time dependent assay using two concentrations of extract 2, which was extracted in 99% ethanol and then dissolved in 80% ethanol. Two concentrations were used, 0.1 and 0.2 mg / mL. A cell density of 3000 cells were well was used. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

Extract 2 appeared to be extremely promising, and the results of another MTT assay can be seen in Figure 13. Cell inhibition can be observed in a dose dependent manner, starting at 0.1 mg / mL. Cell death levels off at around 20% cell viability with all of the remaining concentrations. Cis-Platinum has a similar graph curvature to that of E2, and was shown to have potent cell kill capability even at concentrations of 10 uM (Figure 14).

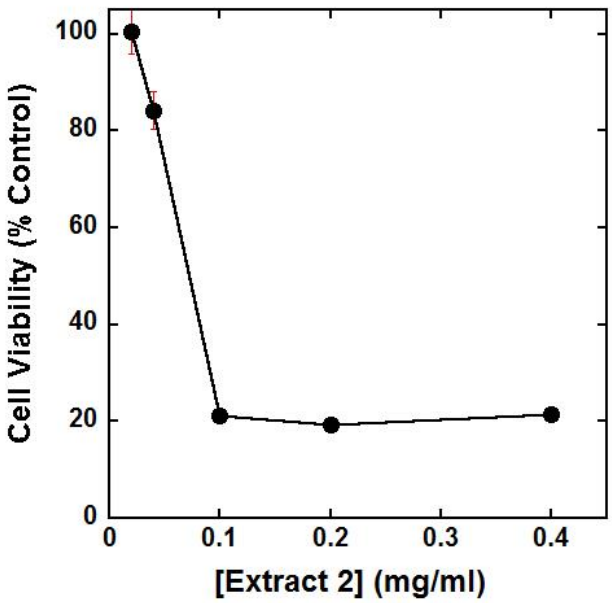


Figure 13. Seventy-two hour dose dependent MTT assay using extract 2 (99% ethanol) extracted from *F. pinicola*. One biological replicate and six technical replicates were used for each extract and concentration.

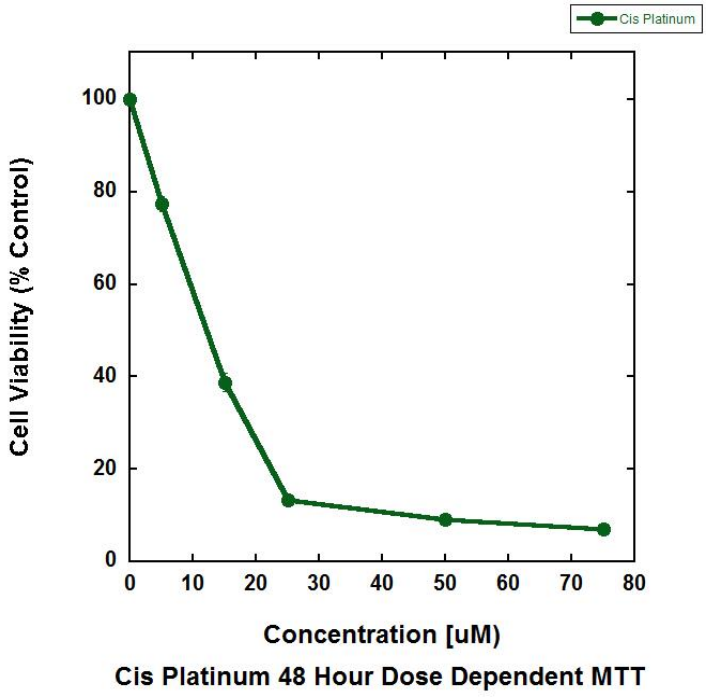


Figure 14. Forty Eight Hour Dose dependent using Cis Platinum and Human HeLa cells. Cell growth inhibition begins with concentrations of 5 uM. One biological replicate and six technical replicates were used for each extract and concentration.

2.2.2.2 *Lentinula edodes*

Because this mushroom has been so well characterized and investigated, we used a store bought sample as a test of our extraction protocols and MTT assay. There were some large differences (Figure 15). For example, E4 (5% NaOH) appeared to have little to no effect when extracted at reflux whereas cell viability was reduced to 50-60% when extracted at 50 °C. In both cases, E3 (water) had nearly the same result, around 60% cell viability when compared to their respective controls. Since this sample was strictly used as a test of protocol, a time dependent MTT assay was not performed.

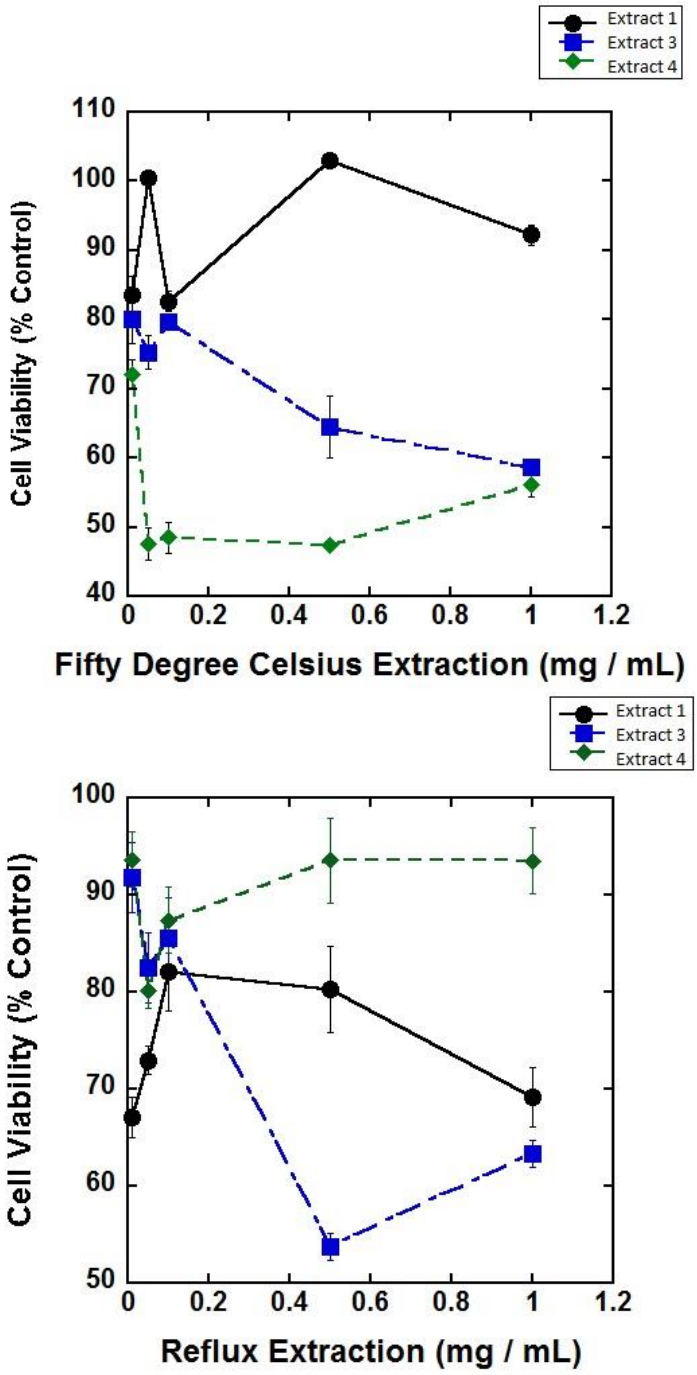


Figure 15. MTT (48 hour) assay of *Lentinula edodes*. Two separate extractions were performed. One at reflux and one at 50 °C. In both assays, the water extract E2, has similar growth inhibitory effect – around 60% of the control. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.3 *Piptoporus betulinus*

With the exception of extract 5 (5% NaOH), all of the extracts had a growth inhibitory effect as seen in a 48 hour MTT assay (Figure 16). The Water extract, E3, held steady at about 50% cell viability when used at the three highest concentrations. Extract 1 (80% ethanol) had the most dramatic effect and dipped below 20% cell viability (% control). Not surprisingly, extract 1 had potent cell kill capability as seen in a time- dependent assay (Figure 17). Although extract 3 reduced cell viability by over 40% in the dose dependent assay, this was not reflected in the time dependent assay, where cell viability was at 80% of the control.

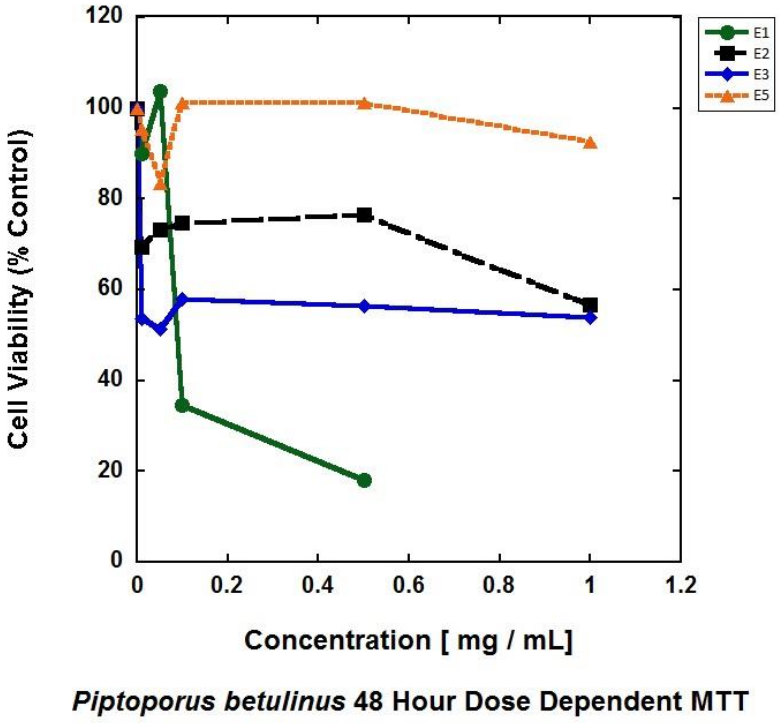


Figure 16. Dose dependent MTT assay using four extracts from *P. betulinus*. The ammonium oxalate extract was omitted. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

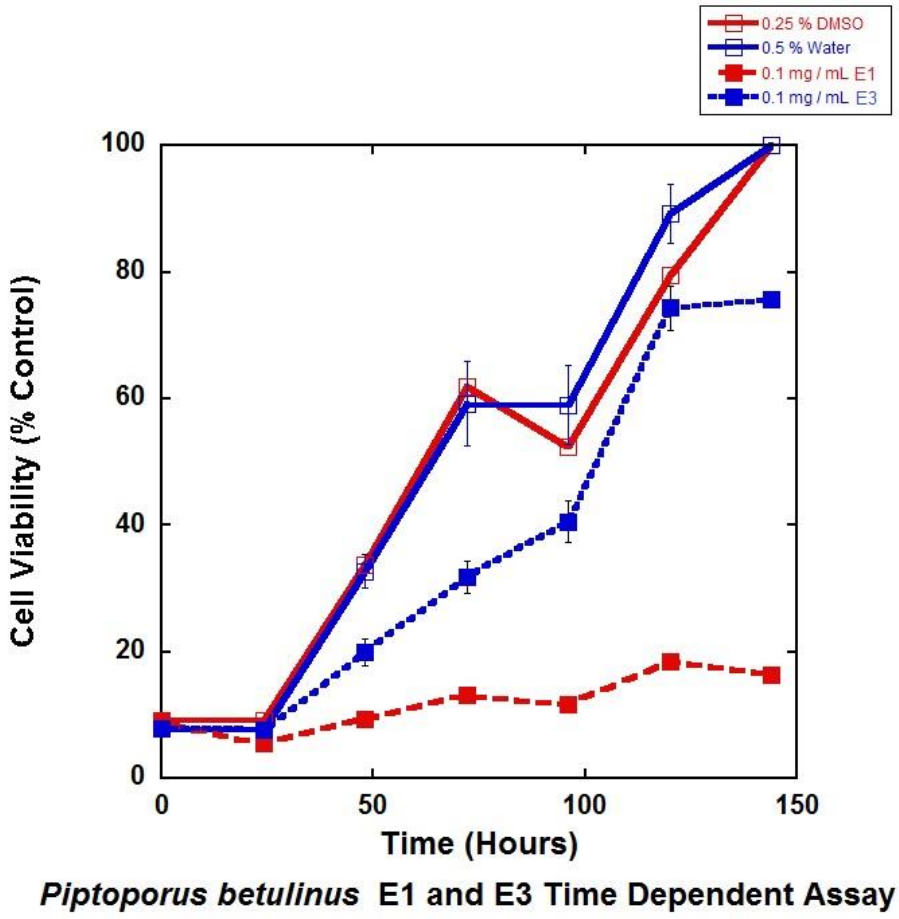


Figure 17. Seven day time dependent assay using E1 (80% ethanol) and E3 (water), extracted from *P. betulinus*. The water extract had little effect as compared to the control whereas the 80% ethanol extract appeared to have potent growth inhibitory effect on HeLa cells. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.4 *Fomes fomentarius*

Extract 5 had little to no effect on cell growth, as seen in a 48 hour dose dependent assay (Figure 18). Extracts 1 and extracts 2 (80% ethanol and 50% methanol), had extremely similar levels in the reduction of cell viability, as compared to their controls, and had their greatest effect at 0.5 mg / mL. The third extract (water) demonstrated a near linear curve in the reduction of cell viability as the concentration of extract increased. At 1 mg / mL, E3

reduced cell viability by nearly 80%. Extract 3 was also the most potent when used in a time dependent MTT assay (Figure 19), further reinforcing the data collected during the dose dependent assay. Extract 1 decreased cell viability by 20% by the 7th day, while extract 2 ended up with nearly no visible inhibitory effect; by the 7th day cell viability was close to that of its control.

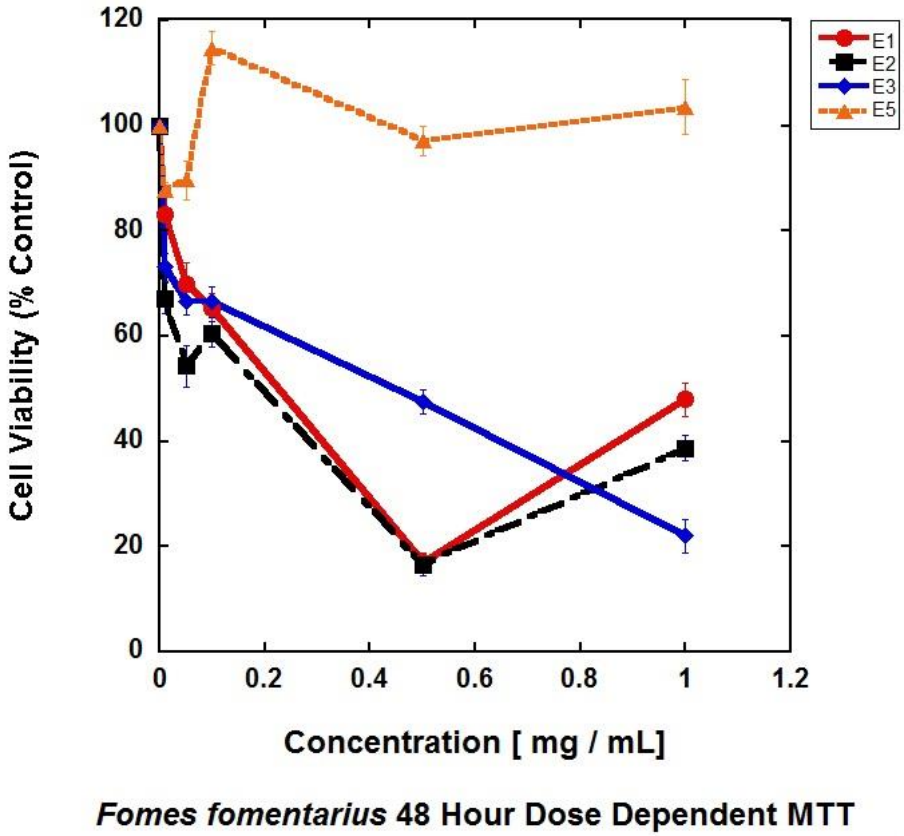


Figure 18. Dose dependent MTT assay using extracts from *F. fomentarius*. Extract 4 (ammonium oxalate) was omitted from this assay. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

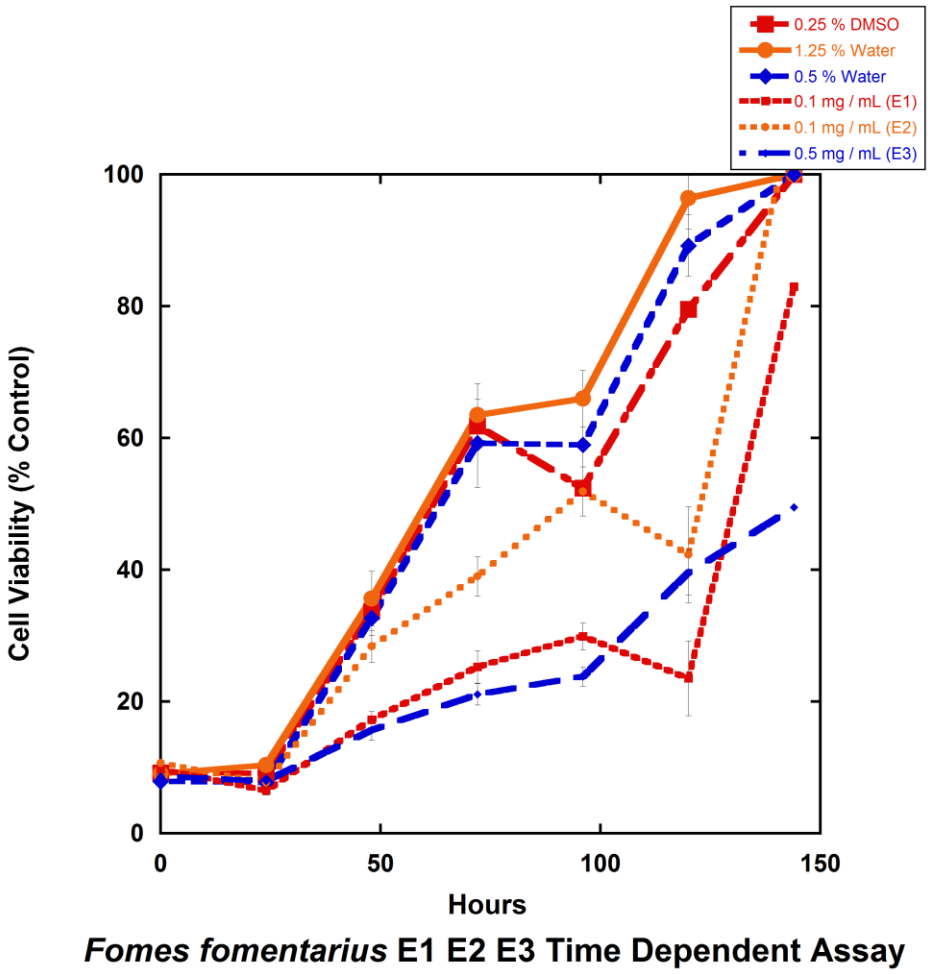


Figure 19. Seven Day MTT assay using extracts 1 (80% ethanol), extract 2 (50% methanol) and extract 3 (Water). The water extract produced the largest growth inhibitory effect. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.5 *Ganoderma applanatum*

Extract 2 (50% methanol) and extract 5 (NaOH) had little to no effect with respect to cell viability (Figure 20). Extract 1 (80% ethanol) had greatly decreased cell viability at 0.5 mg / mL. However, at a higher dose (1 mg / mL), cell viability increased by 40%. Extract 3 (Water) decreased cell viability in a dose dependent manner and decreased cell growth by 55% at its highest concentration – 1 mg / mL. Extract 3 was selected for further investigation

in a seven day time dependent MTT assay (Figure 21). When compared to a water control, extract 3 drastically reduced cell viability. After 7 days, cell viability was deceased by 50%.

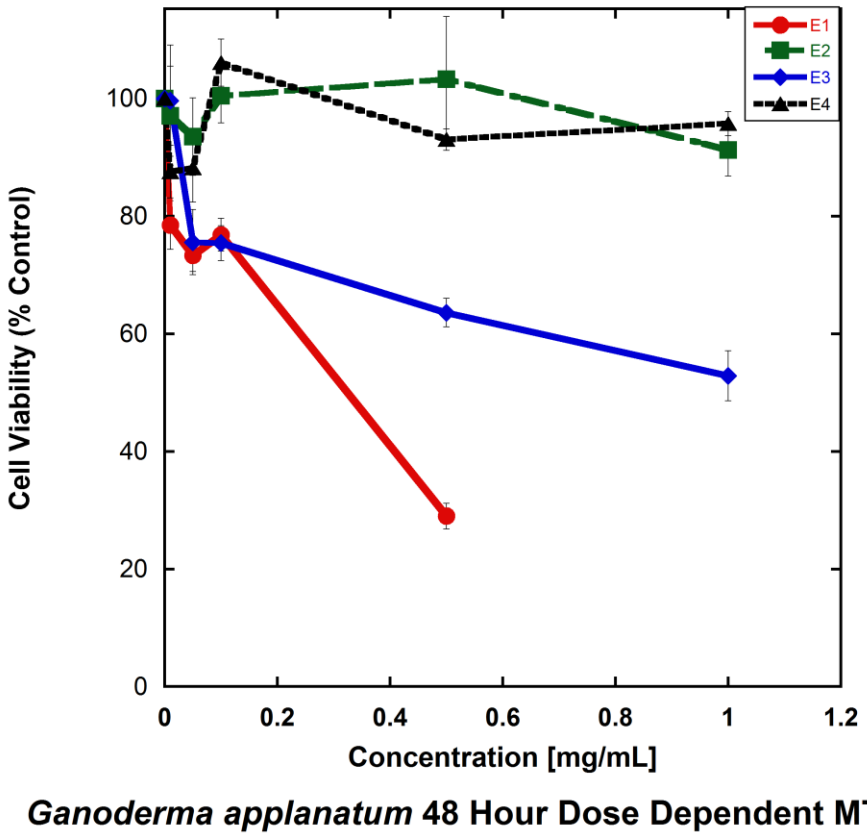


Figure 20. Dose dependent MTT assay using extracts extracted from *G. applanatum*. Extract 4 (ammonium oxalate) was omitted from this assay. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

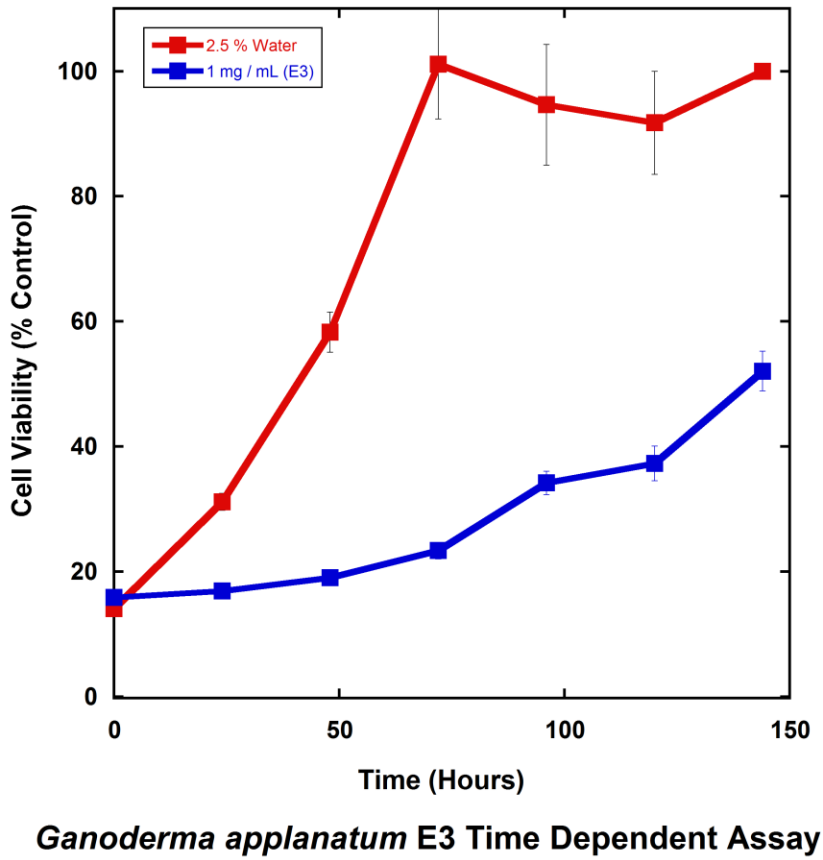


Figure 21. Seven day time dependent MTT assay using extract 3 (water) extracted from *G. applanatum*. Extract 3 potently reduced cell viability during each day and reduced cell viability by 55% on day seven, when compared to its control. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.6 *Echinodontium tinctorium*

Extract 1 (80% ethanol) and extract 5 (5% NaOH) had little to no effect inhibiting cell growth in HeLa cells (Figure 22). Extract 3 (Water) decreased cell viability by 30% at its highest dose of 1 mg / mL. Extract 2 (50% methanol) decreased cell viability in a dose dependent manner, in a near linear fashion. At 0.5 mg / mL, growth was inhibited by 50%. At 1 mg / mL, growth was inhibited by over 80%. Because of this, extract 2 was selected for further investigation in a time dependent MTT assay (Figure 23). While a dose of 0.5 mg / mL did not have a dramatic effect on cell viability, a dose of 1 mg / mL wiped out nearly all

the cells, and decreased cell viability to well under 10%, as compared to its respective control.

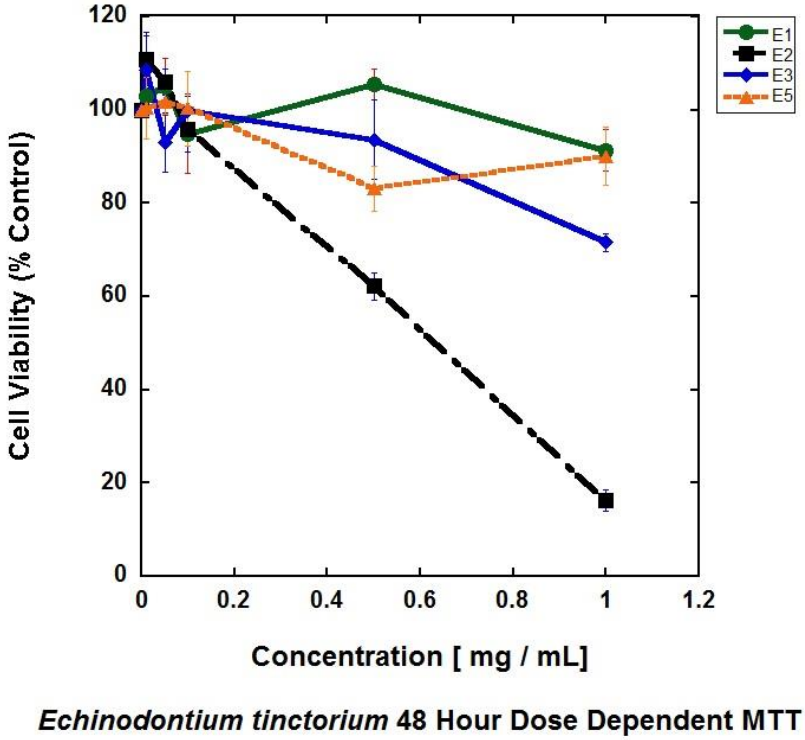


Figure 22. Dose dependent MTT assay using extracts from *E. tinctorium.* Extract 4 (ammonium oxalate) was omitted from this assay. Extract 2 (50% methanol) reduced cell viability in a dose dependent manner. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

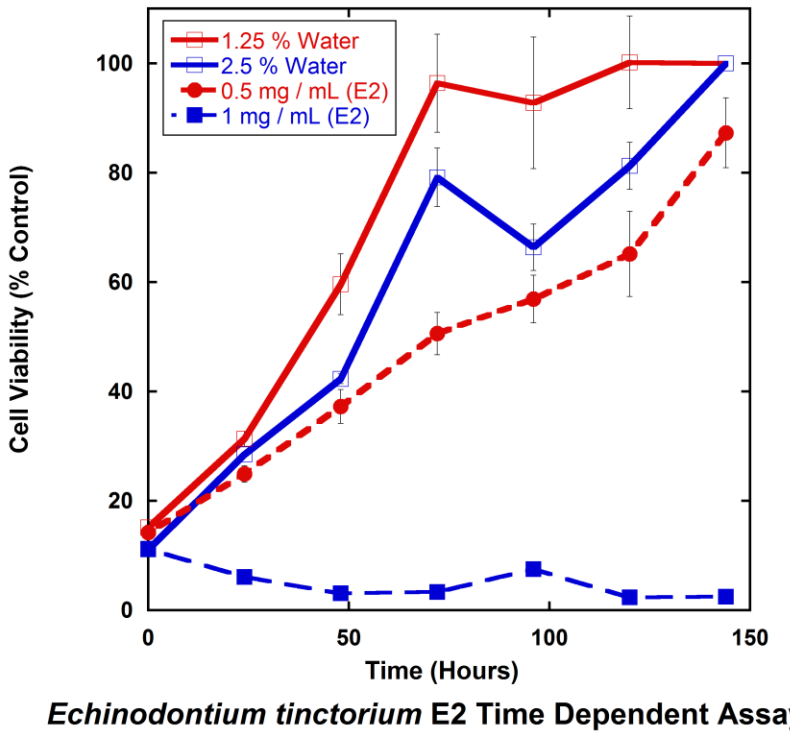


Figure 23. Seven day time dependent MTT assay using extract 2 (50% methanol) at two concentrations, 0.5 and 1 mg / mL. While 0.5 mg / mL had little effect over a 7 day period, 0.1 mg / mL dramatically reduced cell viability over the entire range of the assay. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

The results of two separate extraction methods can be seen in Figure 24. Both the manual/traditional and speed extracted samples had very similar effect on cell viability of HeLa cells. However, the speed extracted sample decreased cell viability by an additional 10% over that of the manually extracted sample. Both methods extracted approximately the same ratio of E2 from the sample, 1%.

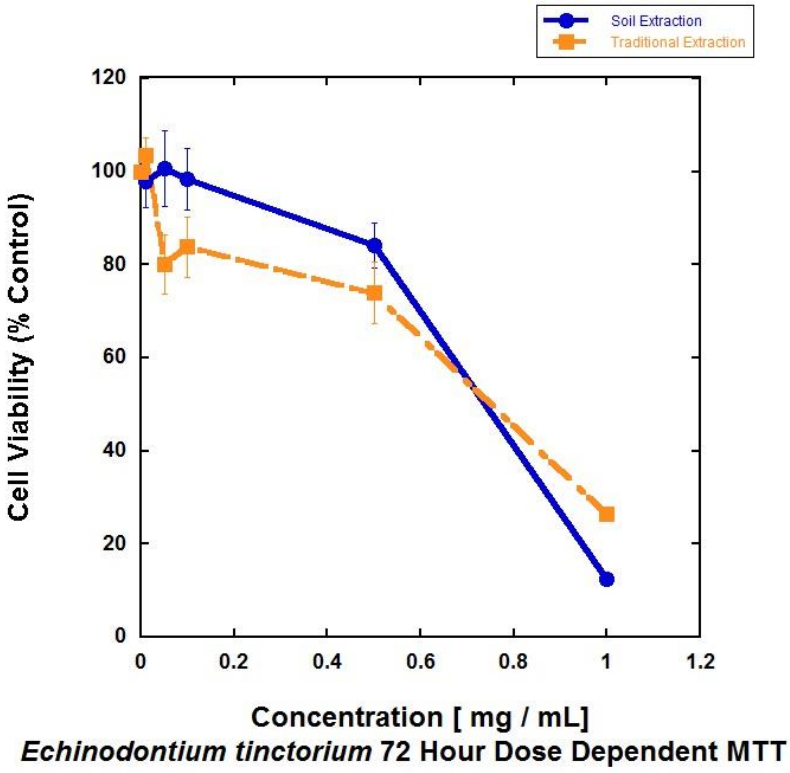
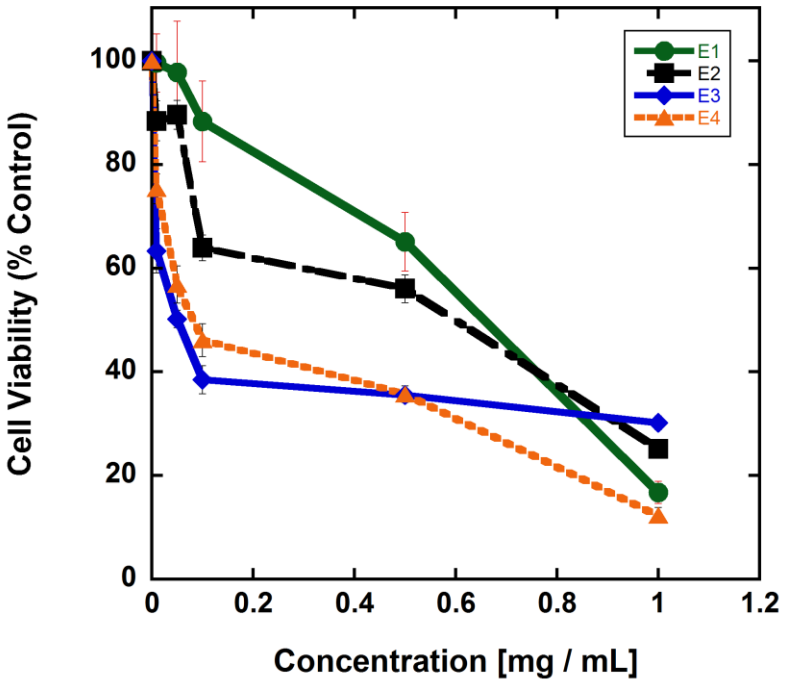


Figure 24. Dose dependent MTT assay (72 hours) using extract 2 (80% ethanol) from *E. tinctorium*. Two separate extractions were performed. The first using traditional extraction methods as outlined in Figure 8, and the second using a Buchi Speed Extractor. Both methods yielded similar results. However, the speed extractor reduced cell viability by 10% more than tradition methods. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.7 *Inonotus obliquus*

All extracts decreased cell viability by over 70%. Extract 1 (80% ethanol) and extract 4 (5% NaOH) both decreased cell viability by 80%. The three highest doses of extract 3 (Water) decreased cell viability in a linear manner, and inhibited HeLa cell growth by 35-40% (Figure 25). Extracts 2, 3 and 4 were selected for further investigation in a time-dependent assay (Figure 26). All three extracts had very similar curves and decreased cell

viability in a similar fashion. However, extract 3 (Water) inhibited HeLa cell growth by the largest amount after 7 days, 65%.



***Inonotus obliquus* 48 Hour Dose Dependent MTT**

Figure 25. Dose dependent MTT assay using extracts from *I. obliquus*. All extracts demonstrated a major reduction in cell viability, in a dose dependent manner. One biological replicate and six replicates were used for each extract and concentration. Error bars are S.E.M.

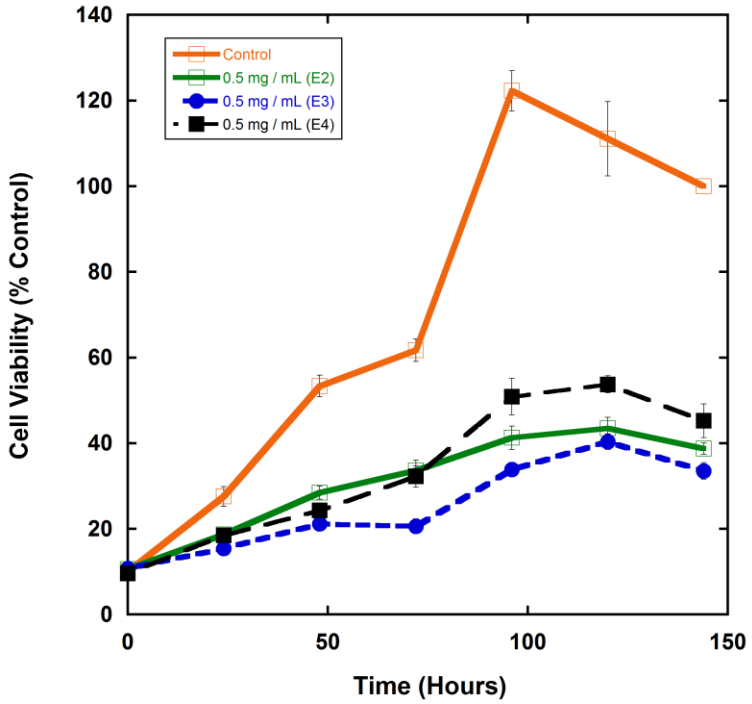


Figure 26. Seven day time dependent MTT assay using extracts obtained from *I. obliquus*. All three extracts had a very similar cell growth inhibitory effect. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.8 *Leucocybe connate*

Extracts 1-3 all decreased cell viability by virtually the same amount, 40%, at the highest dose (1 mg / mL). Extract 4 (5% NaOH) decreased cell viability by 70% at 1 mg / mL (Figure 27). Extract 1 and extract 4 were further investigated in a time dependent MTT assay (Figure 28). At 0.5 mg / mL, both extracts inhibited cell growth with similar strength, decreasing cell viability by 15-20%, after seven days.

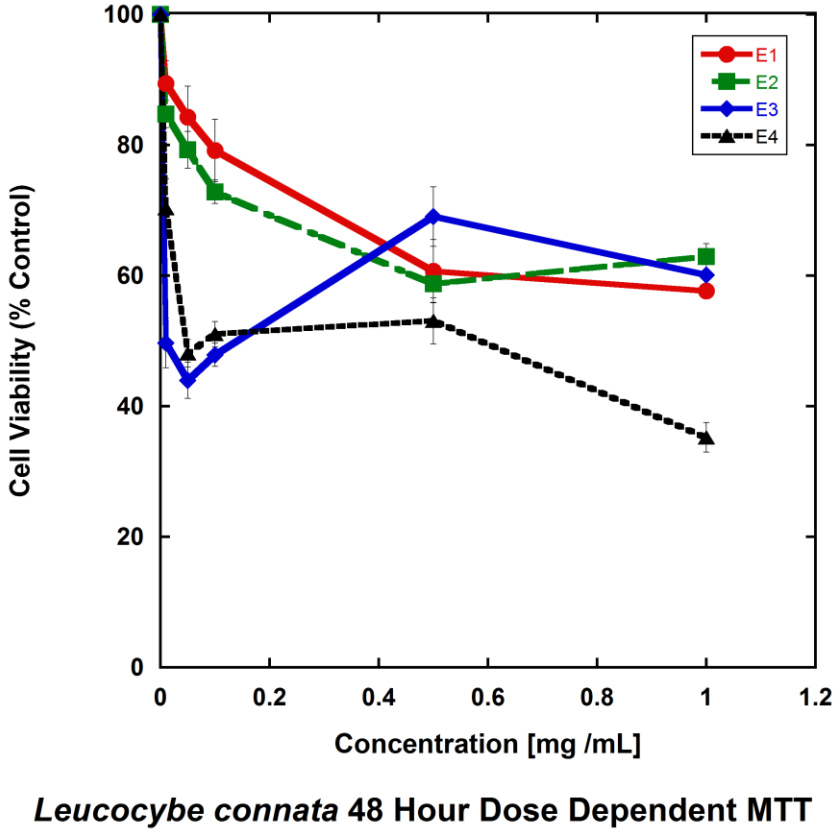


Figure 27. Dose dependent MTT assay using extracts from *L. connata.* Extracts 1-3 had a very similar effect while Extract 4 reduced cell viability to 35%. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

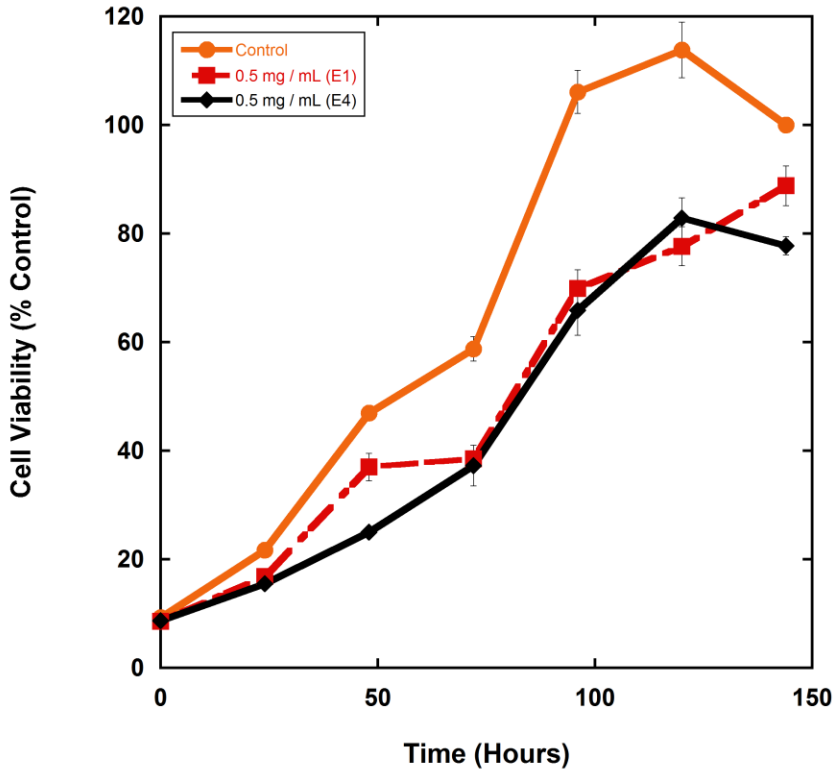


Figure 28. Seven day dose dependent MTT assay of extracts from *L. connata*. Extract 1 (80% ethanol) and extract 4 (5% NaOH) reduced cell growth by very similar amounts. The NaOH extract reduced cell viability by more than 20% when assayed in day 7 whereas E1 reduced cell viability by 25%. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.9 *Laetiporus sulphureus*

In a 48-hour dose dependent MTT assay (Figure 29), extract 1 appeared to be stimulatory while extract 4 appeared to have no effect. The methanol extract (E2) reduced cell viability by 20%. Extract 3 (water) had the largest growth inhibitory effect and reduced cell viability by 30%. When subjected to a time dependent assay, extract 3 reduced cell viability by 40% after the week-long treatment (Figure 30).

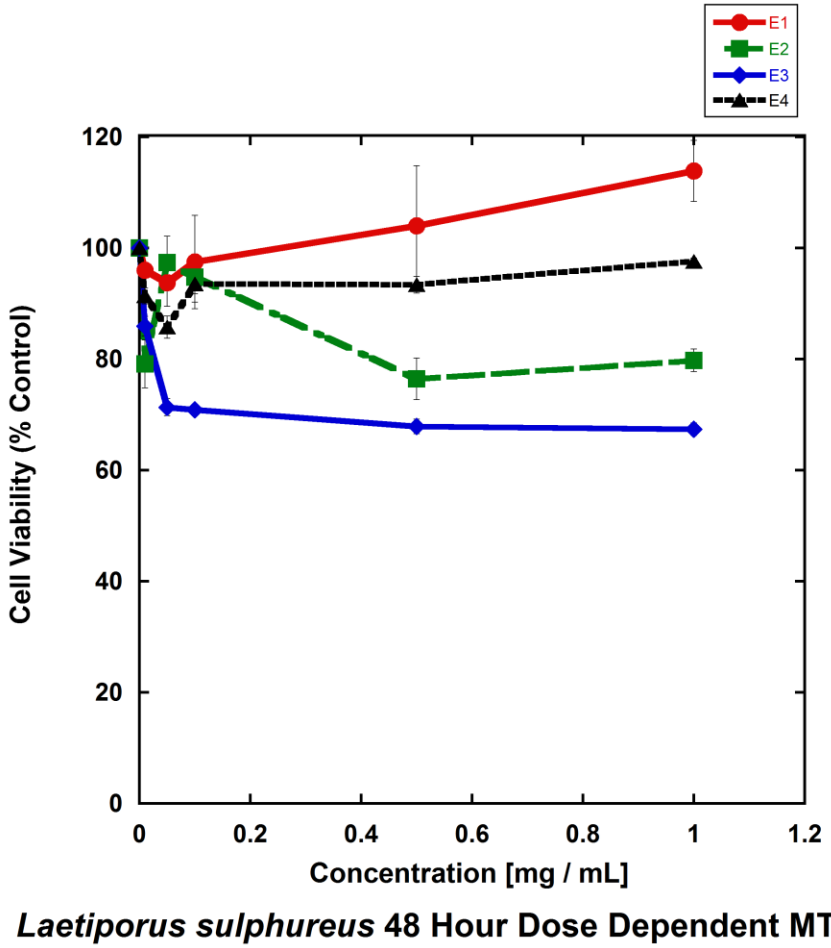


Figure 29. Dose dependent MTT assay using extracts from *L. sulphureus*. Extract 1 appeared to be stimulatory while extract 4 appeared to have no effect. The methanol extract (E2) reduced cell viability by 20%. Extract 3 (water) had the largest growth inhibitory effect and reduced cell viability by 30%. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

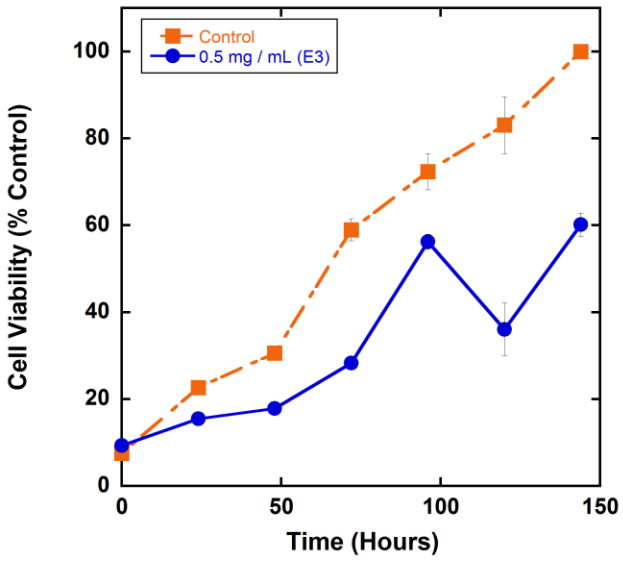


Figure 30. Seven day dose dependent MTT assay of extracts from *L. sulphureus*. After seven days, extract 3 (water) reduced cell viability by 40 %, when compared to controls. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.2.2.10 *Phellinus nigricans*

At a dose of 0.5 mg / mL, extract 4 (5% NaOH) appeared to be stimulatory but decreased cell viability at 1.0 mg / mL. At a very low dose, 0.1 mg / mL, extract 2 (50% methanol) decreased cell viability by 20% but that was reduced to 10% at a dose of 0.5 mg / mL. Both extract 1 and extract 2 decreased cell viability by 50% at 1 mg / mL (Figure 31). Because of this, both E1 and E2 were selected for use in a time dependent MTT assay (Figure 32). Extract 1 had little effect on growth inhibition while extract 3 decreased cell viability (% control) by more than 40% after the seventh day of incubation with HeLa cells.

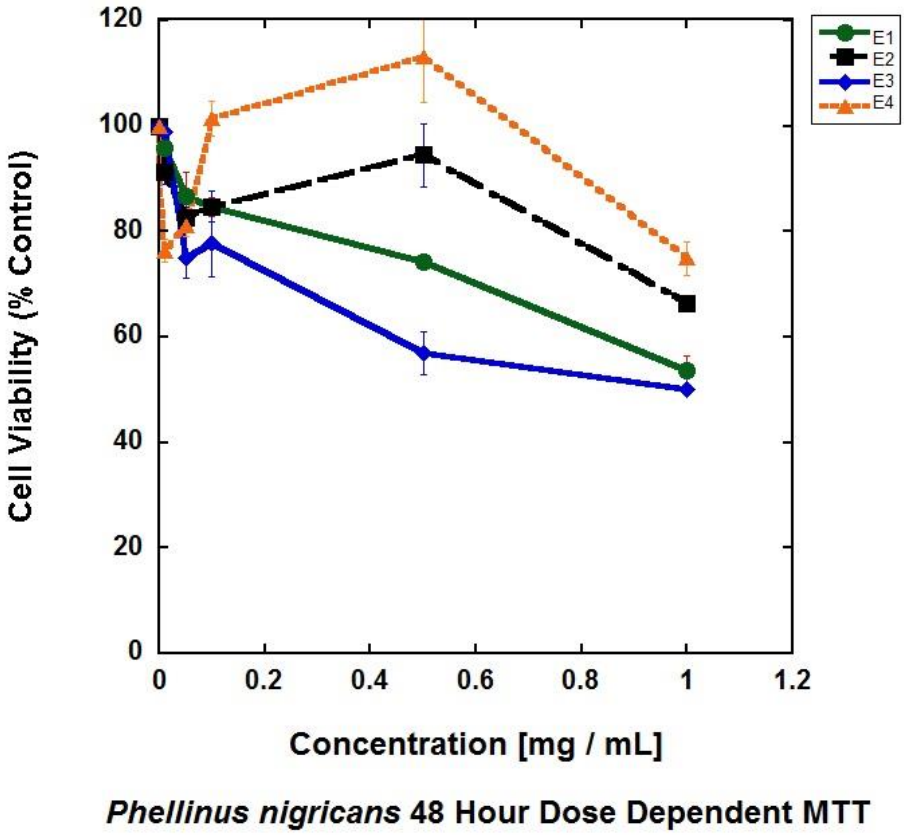


Figure 31. Dose dependent MTT assay of extracts from *P. nigricans*. Extract 4 appeared to be stimulatory at 0.5 mg / mL but reduced cell viability at a higher dose. Extract 1 and extract 3 both reduced cell viability by 50%. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

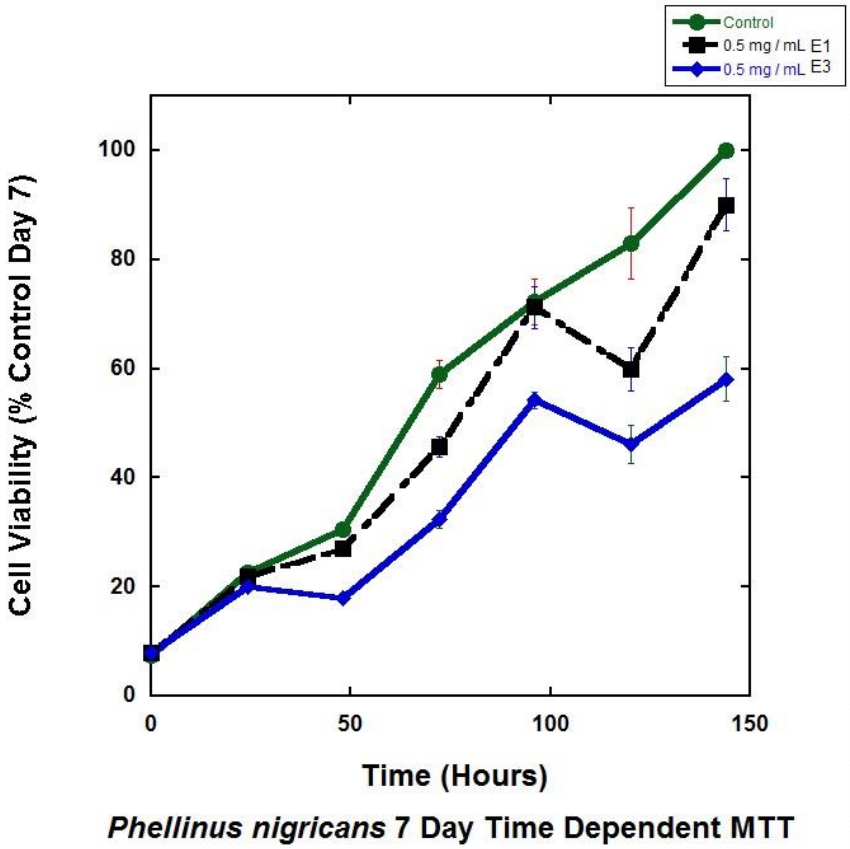


Figure 32. Seven day time dependent MTT assay of extract 1 (80% ethanol), and extract 3 (water), obtained from *P. nigricans*. Extract 1 had little to no effect by the seventh day whereas extract 3 reduced cell viability by 50%. One biological replicate and six technical replicates were used for each extract and concentration. Error bars are S.E.M.

2.3 Discussion

2.3.1 Morphological and DNA investigation

Each sample, with the exception of A4, was positively identified using DNA analysis. Sample A2 may have been misidentified by Marta Zmudzinski as it was previously identified as being *Ganoderma carnosum*, which is not found in B.C, according to MatchMaker. *Ganoderma applanatum* is however found in the province. Also, when comparing my sample to pictures of *G. applanatum*, the two are found to be highly similar in appearance. Samples A1 and A3, which were identified as being *Piptoporus betulinus* and *Fomes fomentarius*, agreed with Ms. Zmudzinski's previous DNA analysis. Sample A4 had high levels of contamination and as such the sequences had to be highly edited. However, based on previous identification using MatchMaker and Mushroom's Demystified, it is fairly certain that this sample is *Echinodontium tinctorium.* Overall, the DNA analysis yielded expected results. However, DNA analysis coupled with morphological identification may provide for the highest degree of certainty when positively identifying fungal species.

2.3.2 Growth-inhibitory Effect on HeLa Human Cervical Cancer Cells – Mushrooms Tested

Fomitopsis pinicola is widely used as a medicinal mushroom in Asia[69], due to its strong antimicrobial[70], antioxidant and antitumor activitites[71]. Previous research has shown that ethanol extracted polysaccharides from *F. pinicola* have a large inhibitory effect on vascular endothelial growth factor (VEGF) mediated tubule formation in endothelial cells[72]. VEGF plays a key role in the mediation of angiogenesis[73]. It is really no surprise that the

ethanolic-based extraction (extract 2) performed here on *F. pinicola* had a very strong inhibitory effect on the growth of HeLa cells, in both dose and time dependent MTT assays.

*Lentinula edodes (*Shiitake*)* is another mushroom species that is commonly used for its medicinal properties in Asiatic countries – especially in Japan. Previous research has shown that ethyl acetate extracts can induce apoptosis in breast cancer cell line MCF-7[21]. *L. edodes* has also been shown to have antioxidant and antimicrobial properties[74], as well as antiviral activity[75]. *Lentinula edodes* is often ingested as a tea (hot water extract) for the treatment of a plethora of illnesses, including cancer[76]. Since the polysaccharides extracted from Shiitake are water soluble, this made it an ideal candidate to test my initial extraction protocols. The data collected from both the reflux and 50 degree extraction correlated well with previous research in that the water extract (E3) had a high growth inhibitory effect on HeLa cells.

Piptoporus betulinus is a birch polypore that was used extensively as a medicinal treatment for a plethora of ailments, in ancient Europe. It has been shown to possess anti-inflammatory properties[77] which are caused by lanostane-type triterpene acids, which were measured by inhibition on a 3α-hydroxysteroid dehydrogenase enzyme[77]. Furthermore, researcher in Poland have shown that a carboxymethylated α-(1→3)-D-glucan, extracted using an alkali solution, was able to inhibit growth of HeLa cells by 50% in an MTT assay[78]. To our knowledge, this is the first time that ethanol based extractions (E1) have been shown to possess potent growth-inhibitory effects, and are capable of reducing cell viability to approximately 30%, at doses as low as 0.1 mg / mL (Figures 16-17). Sodium Hydroxide extracts (5%) had no growth-inhibitory effect (Figure 16). This data correlates well with the above research into Alpha-D-Glucans isolated from *P. betulinus* in Poland[78].

Fomes fomentarius, the tinder polypore and a pathogenic saprotroph, has been shown to have anti-tumour[79] and anti-inflammatory activities[80]. An ergostane-type sterol obtained from the ethanol extract of *F. fomentarius,* from northwestern China was documented to have moderate cytotoxic effects on a plethora of human cancer cell lines, with an IC_{50} from 24 to 30 uM[79]. Results here show that *F. fomentarius* has strong growth inhibitory effect in every extract, with the exception of extract 5 (Figures 18-19).

Abundantly found growing throughout Northern British Columbia, *Ganoderma applanatum*, or artist's conk, has been found to possess anti-tumor[81,82], immuno-modulatory and anti-inflammatory properties[81]. Water extracts from mycelial cultures of *G. applanatum* from Korea[81] and China[82], were found to reduce solid tumor growth in mice. It is thought that the observed anti-tumor effect is caused by immuno-modulation since water extracts also significantly activated natural killer cells (NK) in mice[81]. A water based extraction (E3) was able to greatly reduce HeLa cell viability in both dose dependent and time dependent MTT assays (Figure 20-21), which is consistent with previous research. Additionally, cell growth inhibition by an ethanol based extraction was demonstrated for the first time (Figure 20).

Commonly referred to as Indian paint fungus, *Echinodontium tinctorium* can be found growing in many locations in Northern B.C. To my knowledge, this is the first time that *E. tinctorium* has ever been investigated for any type of growth inhibitory properties with respect to cancer cells. We show for the first time, that ethanol extractions from *E. tinctorium* possess potent growth inhibitory properties in HeLa cells, in a dose dependent manner (Figure 22). This data is further demonstrated in a time dependent MTT assay (Figure 23). The strong cell growth inhibitory effect and the lack of research and publication, make *E. tinctorium* a novel candidate for further investigation as described in Chapter 3.

Despite its abundant usage as a folk remedy, little research has been performed on Chaga (*Inonotus obliquus*) of Canadian origin. *Inonotus obliquus* has traditionally be used as a treatment for a number of illnesses, including cancer, in Russia and several Baltic countries[83]. Compounds such as triterpenoids and ergosterol peroxide, extracted using ethanol, from Russian samples of *I. obliquus*, have been shown to have anti-tumor effects in mice and growth inhibitory effects in cancer cell lines[84,85]. Additionally, large molecules such as lignin and polysaccharides, extracted with water from *I. obliquus*, have been shown to have anti-tumor activity in mice[86]. Growth inhibitory effect and anti-invasion activity have also been shown in cancer cell lines[87]. Anti-tumor effect of polysaccharides, observed in mice, are thought to be immuno-modulatory since they concomitantly active lymphocyte and splenocyte proliferation[86]. In this project, I have shown that all four extracts, from *I. obliquus*, have strong growth inhibitory effects (Figure 25). This data is further strengthened by a dose dependent MTT assay (Figure 26), in which the water extract has the most potent effect on cell viability. This research is consistent with papers published from Russia and China[84,85,87].

Leucocybe connata is a white capped mushroom found growing in clusters on disturbed ground, and can be commonly harvested from areas surrounding UNBC. Very little research / investigation have been performed on *L. connata*, especially with respect to growth inhibition of cancer cells. E4 (5% NaOH) was shown to have potent growth inhibitory reduction of 65% (Figure 27). Extracts 1-3 all reduced cell viability by approximately 40%. This may be the first time any type of growth inhibitory effect has been demonstrated from extractions obtained from *L. connata*.

Laetiporus sulphureus, or chicken of the woods, is commonly found throughout North America. Previous research has shown *L. sulphureus* to possess antimicrobial and anti-oxidant properties[88]. Furthermore, methanol and dichloromethane extracts, obtained from *L. sulphureus*, have been shown to inhibit HIV-1 reverse transcriptase[89]. *Laetiporus sulphureus* has also been show to possess anti-parasitic and anti-tumor properties, on T_4 leukemic cancer cells[90] as well as inducing apoptosis on HL-60 human myeloid leukemia cells[91]. The mechanism of activation is thought to be intrinsic as the release of cytochrome c was detected[91]. In this project, it was shown that extract 1 appeared to be stimulatory (Figure 29), while extract 4 appeared to have little to no effect. Extract 3 had potent cell growth inhibition and reduced cell viability by 30%.

The last species examined in this study was *Phellinus nigricans*. Due to difficulties arising from cultivation in Asia, there has been little research with respect to anti-tumor properties. However, polysaccharides, isolated using water extractions in China, have been found to have anti-tumor activity against mice inoculated with Sarcoma 180 cells[92]. Additionally, water extracted polysaccharides from *P. nigricans* have been found to have antioxidant and immunological activity[93]. In this project, it was shown that the water extract (extract 3) has potent grown inhibitory properties at 1 mg / mL (Figure 31).

To my knowledge, this may be the first research project to investigate B.C wild mushrooms for anti-cancer properties. A few species have never been examined in this manner, such as *L. connata* and *E. tinctorium*. The potent growth inhibitory properties of ethanol extracts obtained from *E. tinctorium* are novel and lay a foundation for further investigation.

Chapter 3 - Investigating the Growth-Inhibitory Activity of the Methanol Extract of *Echinodontium tinctorium*

3.1 Introduction

Due to the potent growth-inhibitory effect demonstrated by the methanol extract of *E. tinctorium* in Chapter 2, and the fact that there had been virtually no research or publications on *E. tinctorium*, it was deemed that further analysis was warranted. Extract 2 (50% methanol) was first tested using a variety of different cancer cells lines in MTT assays. Afterwards, purification was attempted using Sephadex LH-20 (size exclusion chromatography). Purified samples were then assessed for the presence of protein using a BCA kit, and total carbohydrate content was determined using a hot sulphuric acid assay and glycoprotein content using a total glycoprotein estimation kit.

3.2 Methodology

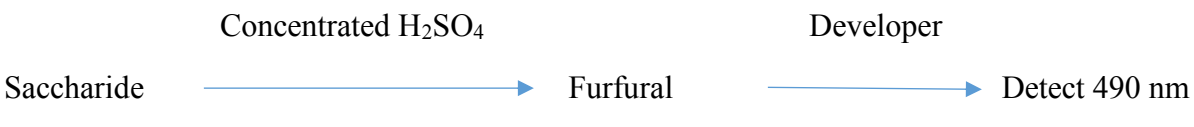
3.2.1 Sephadex LH-20 Size Exclusion Purification

Sephadex LH-20 is a cross-linked, beaded dextran commonly used to separate compounds and molecules based on size. The dextran is cross linked and is pH stable from 2-13 and has a standard operating temperature of 4 °C to 40 °C[94]. A variety of solvents can be used with LH-20, making it ideal for use in purifying crude mushroom extracts. For this study, LH-20 was swollen over night with water and excess liquid was carefully decanted the next day. Swollen LH-20 was then poured into an appropriate sized column in one continuous motion. In later assays, a C16/70 column was packed using an Akta-Prime Plus system at 2 mL/min, with a final bed volume of 100 mL. Two mL of sample was then loaded using a 5 mL loop and sample was eluted using 50% methanol at a rate of 1 mL/min.

Fractions were collected at a volume of 10 mL. Once the column run was complete, the column was flushed with 3 bed volumes of 50% methanol.

3.2.2 Total Carbohydrate Colorimetric Assay Kit (#K645-100)

A total carbohydrate estimation colorimetric assay kit was purchased from BioVision (U.S.A). In this type of phenol-sulfuric acid assay, polysaccharides and their derivatives are hydrolyzed to monomers in the presence of sulfuric acid and are converted to furfural or hydroxyfurfural, which then reacts with the included developer to form a chromogen, which can then be quantified by measuring the absorbance on a plate reader at 490 nm. This kit can detect most carbohydrates, both simple and complex saccharides, glycans, glycoproteins and glycolipids.



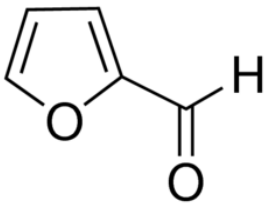


Figure 33. Chemical structure of a furfural compound.

Firstly, 0, 2, 4, 6, 8 and 10 µL of provided glucose standard are added into a series of wells of a 96-well flat bottom plate. These are used to generate standards at the following concentrations: 0, 4, 8, 12, 16 and 20 µg. Volumes are then adjusted to 30 µL with dH_2O. Samples are prepared by adding a maximum of 30 µL per well, in triplicate, and volumes are

adjusted with dH_2O. In this study, 30 µL of LH-20 purified fraction was used. Next, 150 µL of technical grade Sulphuric Acid is added to each standard and sample well and the plate is then gently shaken by hand to mix. The plate is then incubated for 15 min at 90 °C, after which 30 µL of Developer is added, which is provided with the kit. Samples and standards are then gently mixed by hand for 3-5 min at room temperature. Finally, the optical density is read on a Synergy2 Plate Reader at 490 nm. A standard curve is then plotted based on OD readings of the standards and their known concentration of glucose. Sample concentrations can then be extrapolated from the equation of the curve[95].

3.2.3 Pierce Glycoprotein Carbohydrate Estimation Kit (#23260)

A Pierce glycoprotein carbohydrate estimation kit (purchased from Thermo Scientific in the U.S.A) was used to measure the total amount of protein glycosylation as a percent of the total protein mass. Glycoproteins are oxidized using sodium *meta*-periodate to form aldehydes, which then react with the included glycoprotein reaction agent. The resulting reaction causes a purple colour, quantified at 550 nm.

The kit includes the following materials: 500 mg sodium *meta*-periodate, 500 mg of glycoprotein detection reagent and 250 mL glycoprotein assay buffer (containing 1% sodium azide). The kit also includes 2 negative controls: 2.5 mg lysozyme and 2.5 mg bovine serum albumin, and 4 positive controls: 2.5 mg ovalbumin, 2.5 mg apotransferrin, 0.25 mg fetuin and 0.25 mg α_1-acid glycoprotein.

Materials are prepared in the following manner: a 10 mM solution of *meta*-periodate is made by dissolving 21.4 mg using 10 mL of glycoprotein assay buffer. Next, 50 mg of

glycoprotein detection buffer is dissolved in 10 mL of 1M Sodium Hydroxide. Samples are dissolved in glycoprotein assay buffer at 0.25 and 2.5 mg/mL.

Using a sterile 96-well, flat-bottomed plate, 50 µL of each sample and standard were added to each well, in triplicate. A blank containing 50 µL of glycoprotein assay buffer was also added. Next, 25 µL of sodium *meta*-periodate was added to each of the sample and standard wells. The plate was then mixed for 30 sec by hand, after which it was incubated at room temperature for 10 min. One hundred and fifty microliters of glycoprotein detection reagent was then added to each well and the plate was mixed for an additional 30 sec by hand. Lastly, the plate was covered and incubated at room temperature for 1 h. Absorbance was then measured at 550 nm using a Synergy2 plate reader and a standard curve was plotted. Because both Fetuin and α_1-acid glycoprotein have carbohydrate content greater than 10 %, the 2.5 mg/mL standard values must be calculated by multiplying the absorbance values obtained from their respected 0.25 mg/mL by a factor of 10. Glycoprotein concentration is then extrapolated from the standard curve[96].

3.2.4 Pierce BCA Protein Assay Kit (#23225)

This kit was purchased from Thermo Scientific (U.S.A) and was used to measure the total protein content of LH-20 purified samples, obtained from the ethanol extraction of *E. tinctorium*. Proteins reduce Cu^{+2} to Cu^{+1}, in alkaline solutions, resulting in a purple colour, which can then be quantified on a plate reader at 562 nm. The colouration is caused by the chelation of one molecule of BCA with 2 molecules of Cu^{+1}.

Standards are prepared by diluting stock BSA with ddH_2O to nine different concentrations. In this case the following concentrations were used: 0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 1.5 and 2 mg/mL. Next, 50 parts BCA reagent A are combined with 1 part BCA

reagent B, to form a working reagent. Ten microliters of each standard and sample are then added to the wells of a 96-well flat-bottomed plate, in triplicate. Next, 200 µL of working reagent is added to each well and the plate is mixed by hand for 30 seconds, after which the plate is covered and incubated at 37 °C for 30 min. The plate is then cooled to room temperature and quantified on a plate reader at 562 nm. Finally, a standard curve is plotted, after subtracting the 562 nm absorbance reading from the blank wells. The linear trend line is then used to calculate protein concentration in each sample. In this study, standards of 0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 1.5 and 2 mg/mL were used and 10 µL of LH-20 purified fractions were assayed. The values of the standards were then used to create a standard curve.

3.2.5 Phenol Chloroform Extraction (P.C.I)

A phenol chloroform extraction was attempted in order to remove protein from then 50% methanol extracted sample from *E. tinctorium*. Two hundred microliters of phenol was combined with 200 µL of Chloroform:isoamyl alcohol and was then added to 400 µL of crude E2 fraction, in a 1.5 mL microcentrifuge tube. The tube was then inverted to mix and spun at 12,000 rpm for 5 min. The aqueous phase was then collected and used in a 48 h MTT assay.

3.3 Results

3.3.1 Large Scale Manual Extraction vs. Speed Extractor

In order to proceed with further investigation into the potent growth inhibitory effect shown by E2 (50% methanol) in Chapter 2, it was first necessary to perform a large-scale extraction of the crude material. Seven hundred and fifty grams of powdered *E. tinctorium*

was extracted manually, as per the protocols in Table 9 – Chapter 2. A second extraction was also performed using a BÜCHI Speed Extractor® E916 and the protocols are laid out in Chapter 2. An MTT assay was then setup using E2 from both the large-scale manual extraction, as well as E2 obtained from the speed extractor. The results can be seen in Figure 35. Both methods showed that E2, extracted using both methods, possess potent growth inhibitory effect in human HeLa cells. Because the speed extractor has very limited capacity, it is ideal for initial mushroom screening. However, when a large amount of material is required, a manual extraction is necessary and was used here.

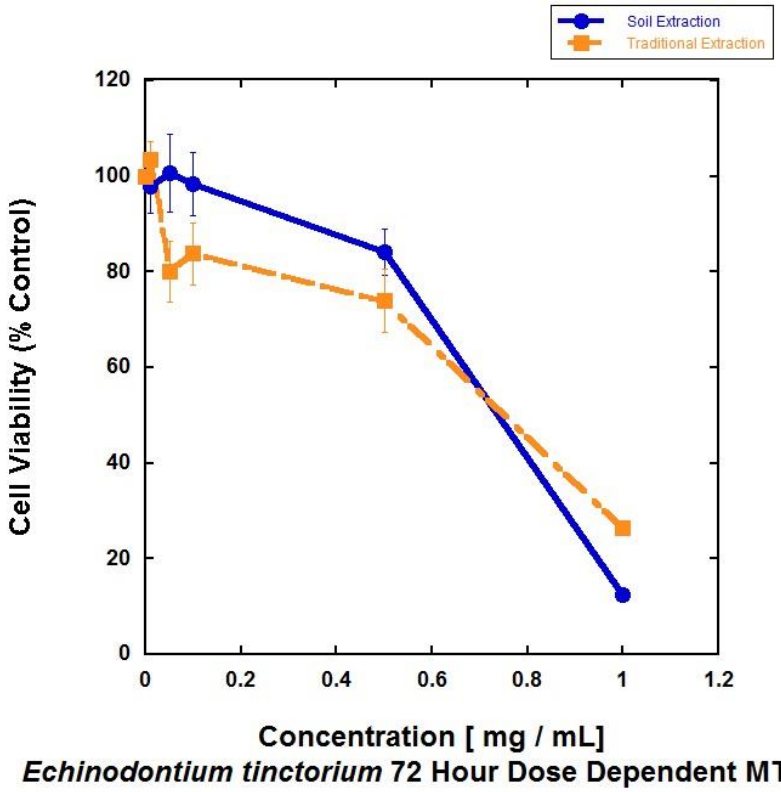


Figure 34. Growth-inhibitory effect of methanol extracts from *E. tinctorium*. Powdered *E. tinctorium* was extracted using the manual and speed extraction methods in methanol. Both methods had relatively similar effect on cell growth inhibition at 1 mg/mL. Errors bars are S.E.M.

3.3.2 Size Exclusion Chromatography and BCA Assay

Sephadex LH-20 was mixed with ddH_2O and left to swell overnight. It was then poured into a 2 mL column, packed using gravity for several hours. Fraction 2 (50% methanol) was dissolved in water at a concentration of 40 mg/mL. Forty microliters of solution was added to the column. The column was eluted using ddH_2O and 10 fractions were collected at 600 µL each. Fractions were sterilized using 0.2 µm filters. A 48 h MTT was setup in which 100 µL of each collection fraction was added to 100 µL of HeLa cells, at a density of 1500 cells per well. Fractions 2 and 3 had extremely potent growth-inhibitory effect on HeLa cells (Figure 35). The remaining fractions were still able to reduce cell viability by 20% as compared to their controls.

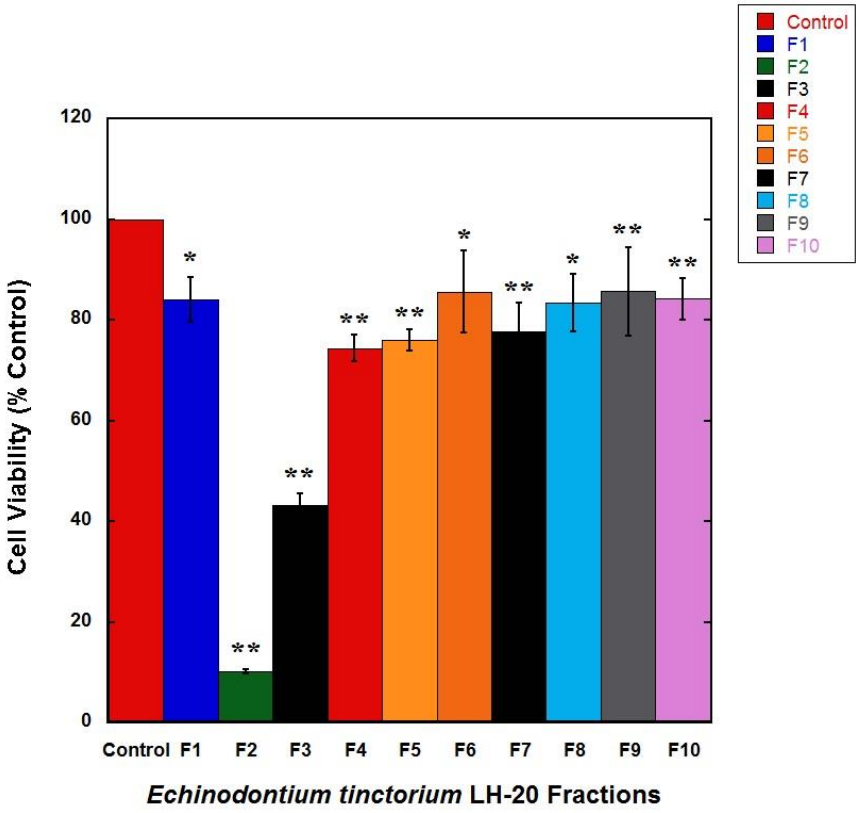


Figure 35. Trial 1 - Assessing growth-inhibitory activity of fractions collected from Sephadex LH-20. Methanol extracts from *E. tinctorium* were dissolved in water at 40 mg/ml and run on a 2 ml gravity Sephadex LH-20 column. Fractions collected were assessed for growth-inhibitory activity using the MTT assay. *P < 0.05, **P < 0.01 versus control. Error bars are S.E.M.

A second experiment was performed in order to verify the results shown in Figure 35. Forty microliters of crude extract were loaded onto the LH-20 column and fractions were collected at 600 µL. However, in this MTT experiment, 0.5 and 1.0 mg/mL of 50% methanol (crude) extracted material was also assayed, in order to compare the growth inhibitor effect of LH-20 purified sample versus crude extract. The results can be seen in Figure 36. While the second fraction still maintained a very high ability to inhibit HeLa cell growth, it was nowhere near as powerful as an effect as seen from the initial purification. Unpurified material had a much greater effect on cell growth (Figure 36).

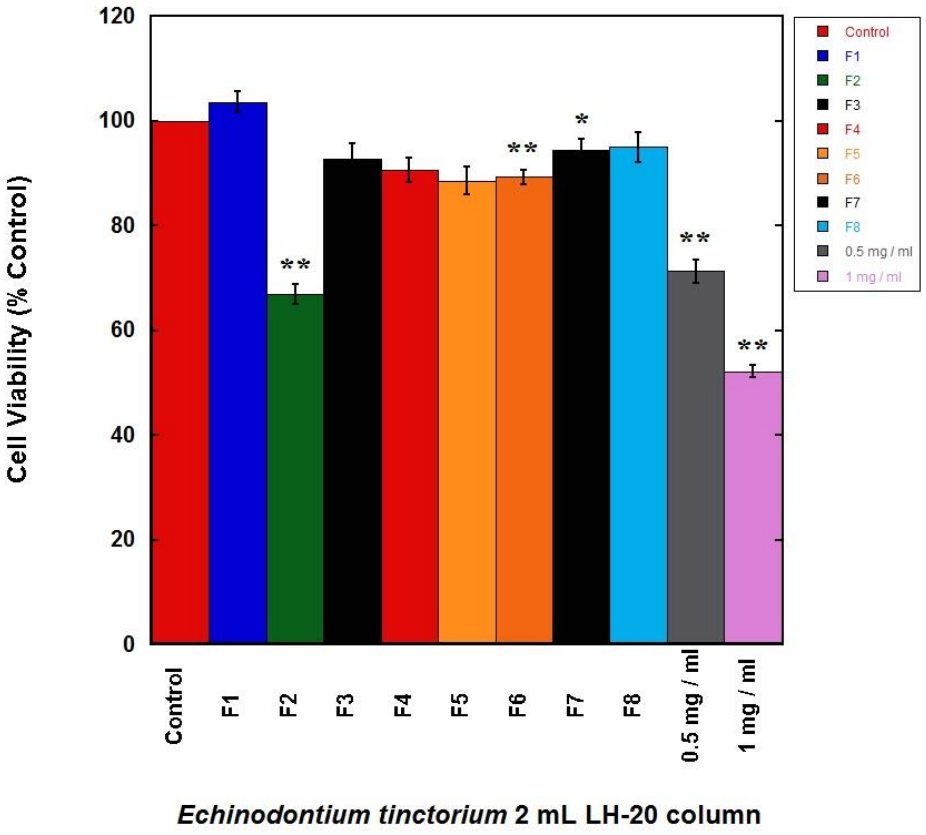


Figure 36. Trial 2 - Assessing fractions collected from Sephadex LH20 for growth-inhibitory activity. Methanol extracts from *E, tinctorium* was loaded onto Sephadex LH20 column. Samples were collected at 600 µL volumes and were then subjected to a 48 h MTT assay. Crude extract, E2, was also assayed in order to compare the effectiveness of the purification process. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

Since the results of the two experiments were quite different, with respect to the potency of the E2 fraction, a third LH-20 column was run. Crude extract was added to the column at a 3% load (60 µL). A 48 h MTT assay was run using all 10 collected fractions (Figure 37). The results were very similar to those of Figure 36, with F2 reducing cell viability approximately 40-45 % in both assays.

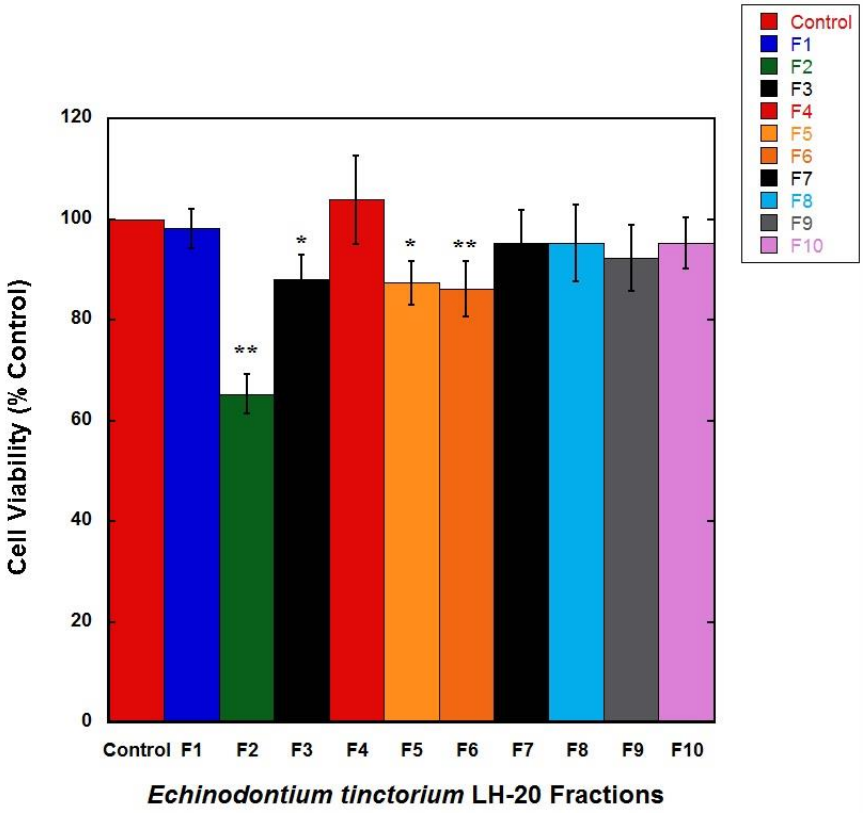


Figure 37. Trial 3 - Assessing fractions collected from Sephadex LH20 for growth-inhibitory activity. Methanol extracts from *E. tinctorium* was loaded onto Sephadex LH20 column and fractions collected at 600 µL volumes were subjected to a 48 h MTT assay. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

A fourth and final 2 mL LH-20 column was run, again using a 3% load (60 µL). Ten fractions were collected, filter sterilized and analyzed with a 48 h MTT assay (Figure 38). Fraction 2 had very similar results to those shown in Figure 36 and in Figure 37. In all three cases, Fraction 2 was able to reduce HeLa cell viability by approximately 40% in MTT assays. In all 2 mL LH-20 purifications, Fraction 3 also had significant (Student t test) growth inhibitory effects, except for in Figure 36.

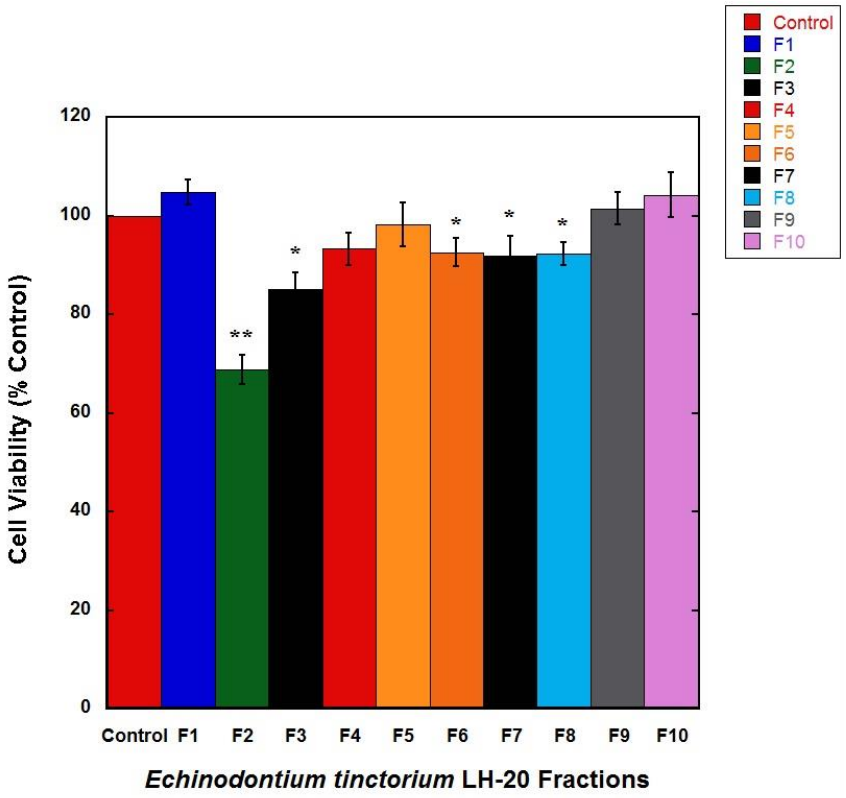


Figure 38. Trial 4 - Assessing fractions collected from Sephadex LH20 for growth-inhibitory activity. Methanol extracts from *E. tinctorium* was loaded onto Sephadex LH20 column and fractions collected at 600 µL volumes were subjected to a 48 h MTT assay. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

A BCA assay was then performed using the fractions collected in Figure 38 as per the protocols outlined in section 3.2.4. A standard curve was then constructed using the values from the BCA assay (Figure 39). Extrapolated data was then graphed alongside the MTT data from Figure 38 (Figure 40) below. A strong regression value of 0.9966 was obtained. Fraction 2 had the highest growth inhibitory effect but did not have the highest protein content.

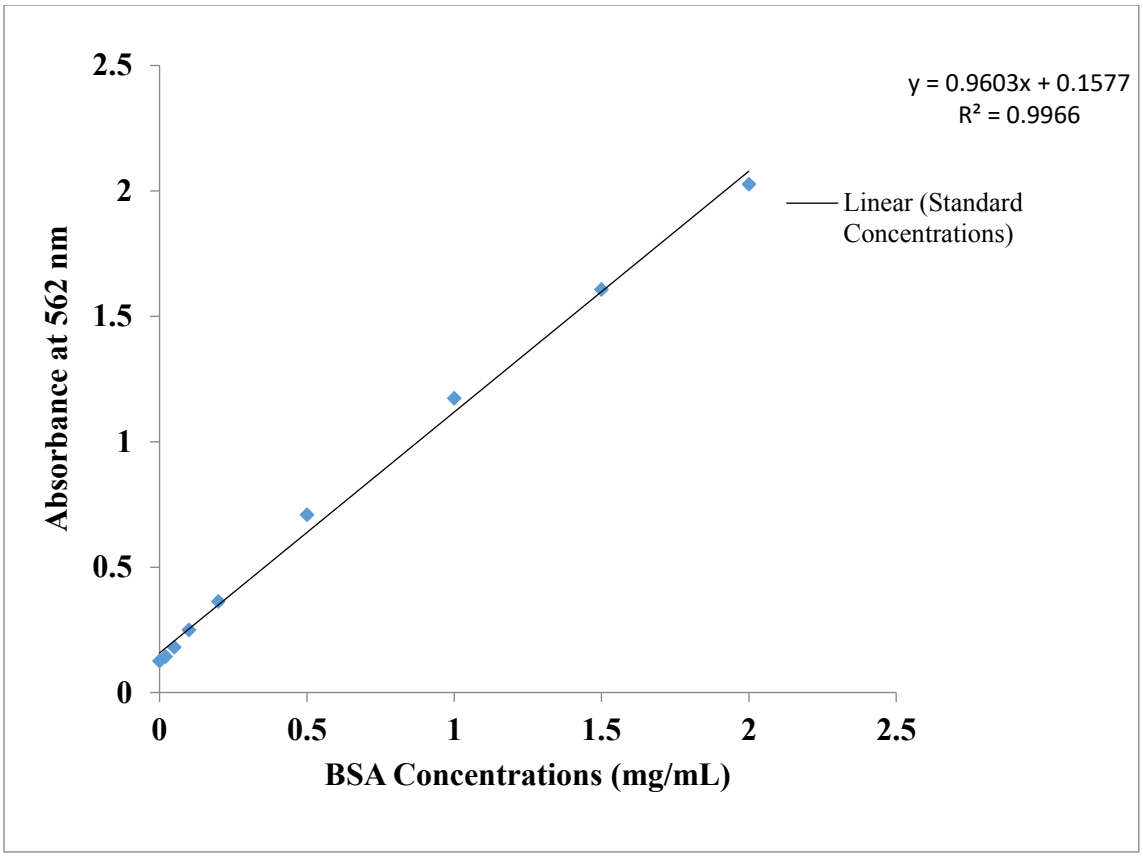


Figure 39. BCA assay using fractions collected from a 2 mL LH-20 column. Standards and samples were run in triplicate. A strong regression value of 0.9966 was obtained.

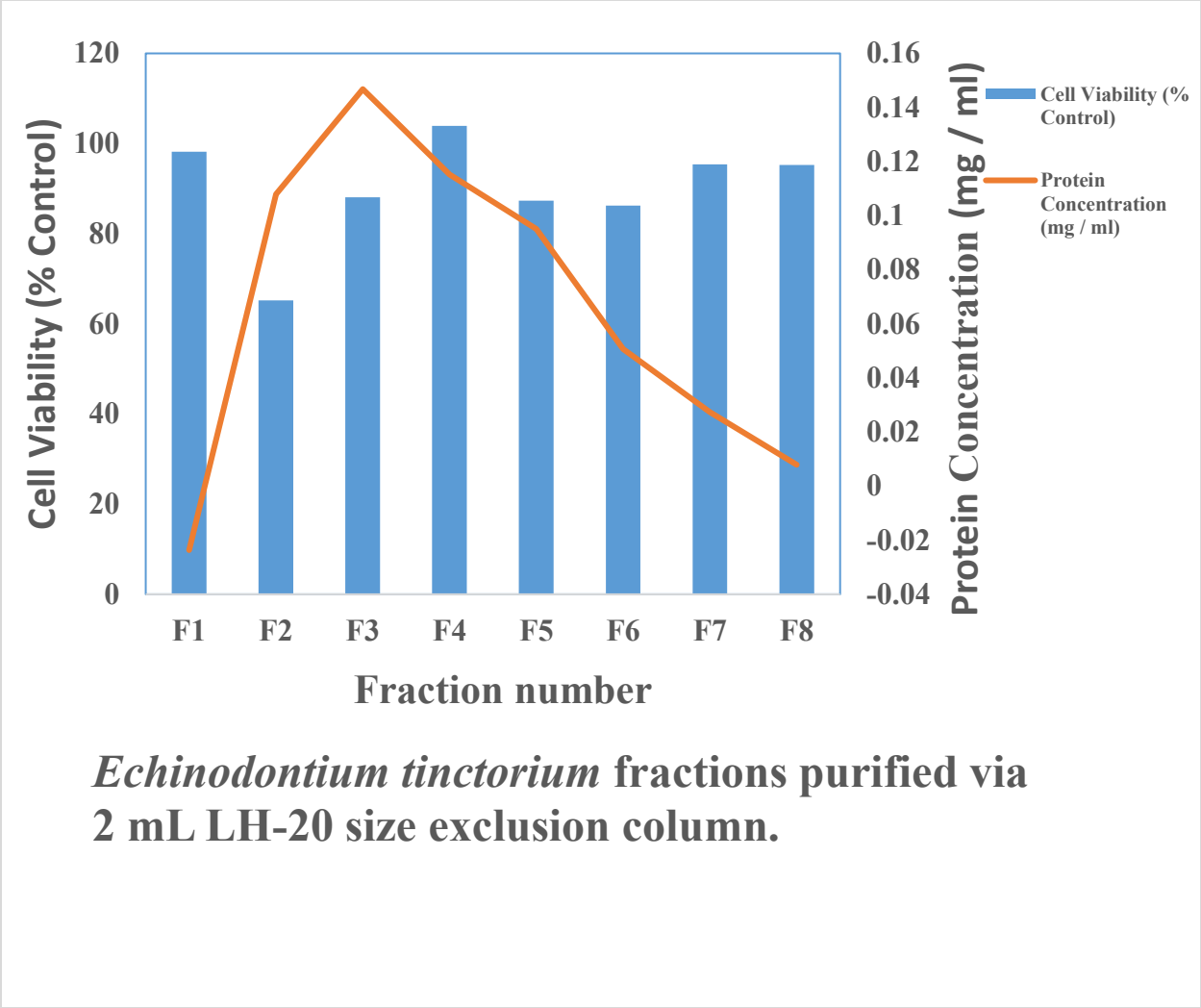


Figure 40. BCA assay and MTT assay using the same eluted fractions from a 2 mL LH-20 column. Fraction 2 had the highest cell growth inhibition activity while fraction 3 had the highest protein content.

Next, a 25 mL serological pipette was cut down, packed with cotton, and used to fashion a 10 mL LH-20 column. Two hundred microliters of crude E2 (50% methanol) fraction were added to the column and fractions were eluted at a volume of 1 mL. Fractions were then assayed for growth inhibitory properties using a 48 h MTT assay. Additionally, a P.C.I. extraction was also performed in an attempt to remove protein from crude fraction of

E2. Unfortunately, there were small amounts of phenol remaining in the aqueous layer and it caused toxicity, killing all of the cells in both the phenol extraction and the phenol control. Crude E2 methanol extract was also assayed and retained its potent cell growth inhibitory effect. In this assay, F4-F6 collected from the Sephadex LH-20 column have a significant reduction in cell viability. Fractions 9-11 also showed a significant reduction in cell viability (Figure 41).

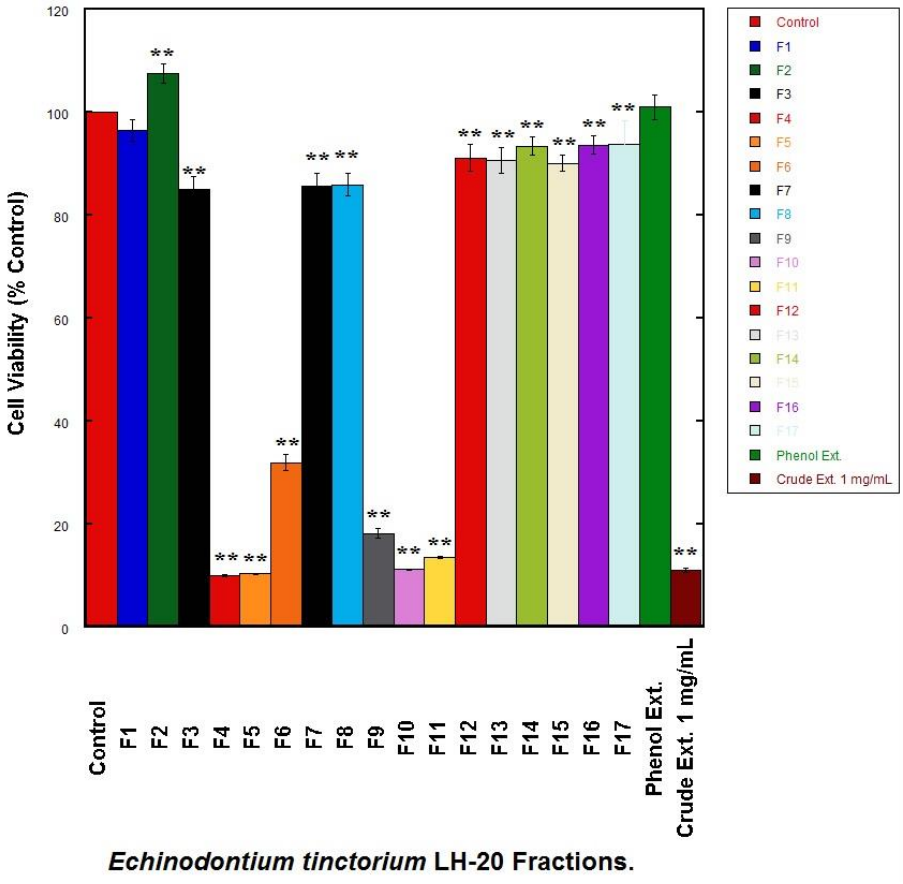


Figure 41. Assessing fractions collected from Sephadex LH-20 for growth-inhibitory activity. Methanol extracts from *E. tinctorium* were loaded onto a 10 mL Sephadex LH-20 column. Fractions collected at 1 mL volumes were subjected to a 48 h MTT assay. One biological replicate and 6 technical replicates were used. A 2% load was added to the LH-20 column. A phenol extraction was also performed to remove protein. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

After suspected bimodal activity was seen using a 10 mL column, another 25 mL serological pipette was cut down, packed with cotton, and used to fashion a 18 mL LH-20 column. Three hundred and sixty microliters of crude fraction 2 was loaded onto the column and fractions were collected at a volume of 1.5 mL. Fractions were then analyzed using a 48h MTT assay, in which 100 µL of LH-20 purified fraction was added to 100 µL of cells. Fractions 5-6 had very potent cell growth inhibitory effects as did F11. To assess the possible role of proteins, the crude E2 methanol fraction (0.5 mg/mL) was heated to 95 °C for 1 h in an attempt to denature protein. As shown in Figure 42, both the heated and unheated samples had a very similar effect on cell viability.

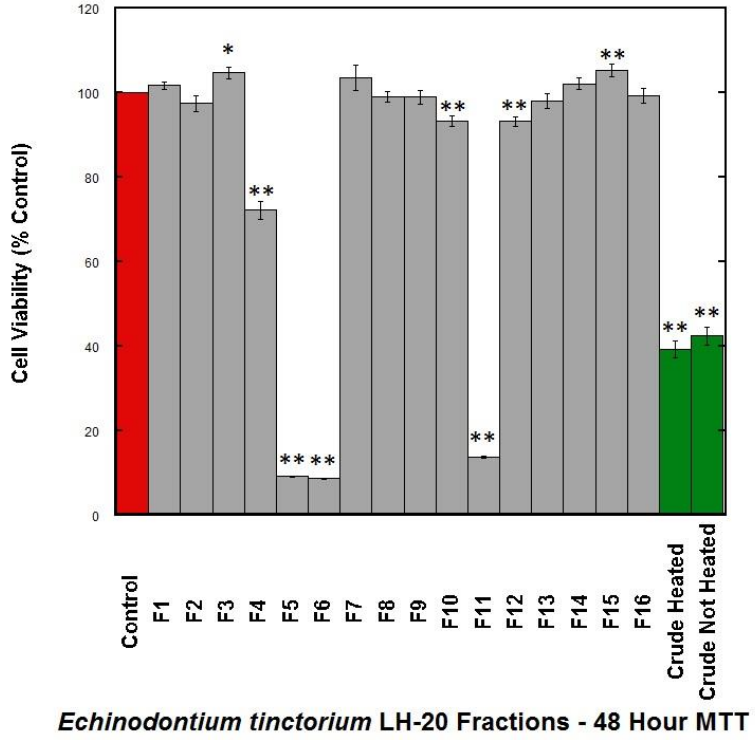


Figure 42. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. Methanol extracts from *E. tinctorium* was loaded at 2% onto an 18 mL Sephadex LH-20 column. Fractions collected at 1.5 mL volumes were subjected to a 48 h MTT assay. One biological replicate and 6 technical replicates were used. Crude extract was heated at 95 °C for 1 h and tested alongside a non-heated sample. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

A second 18 mL LH-20 column was run, again using 360 µL of crude fraction 2 (50% methanol). Fractions were collected at 1.5 mL and then assayed using a 48 h MTT. This time, F5 did not have near the same effect on cell growth (Figure 43). However, fraction 11 retained very similar activity as that seen in the previous MTT assay.

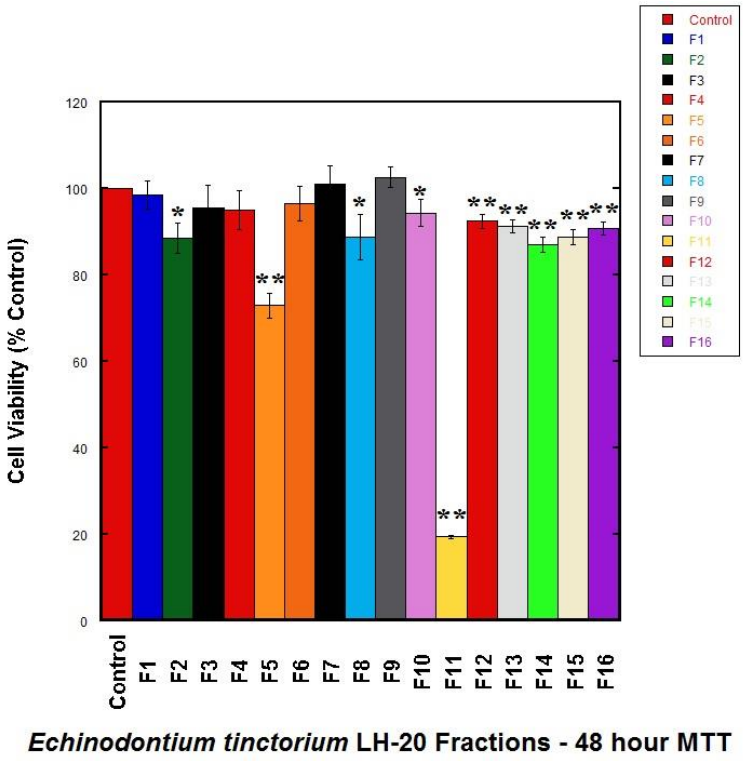


Figure 43. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. Methanol extracts from *E. tinctorium* was loaded at 2% onto an 18 mL Sephadex LH-20 column. Fractions collected at 1.5 mL volumes were subjected to a 48 h MTT assay. One biological replicate and 6 technical replicates were used. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

Next, another BCA assay was performed using fractions collected from the MTT assay shown in Figure 42. The standard curve can be seen in Figure 44. A strong regression value of 0.9952 was obtained. Extrapolated data was then graphed against the MTT data from Figure 42. The highest protein content was seen in fractions 7 and 10 whereas the highest cell growth inhibitory effect was seen in fractions 5, 6 and 11 (Figure 45).

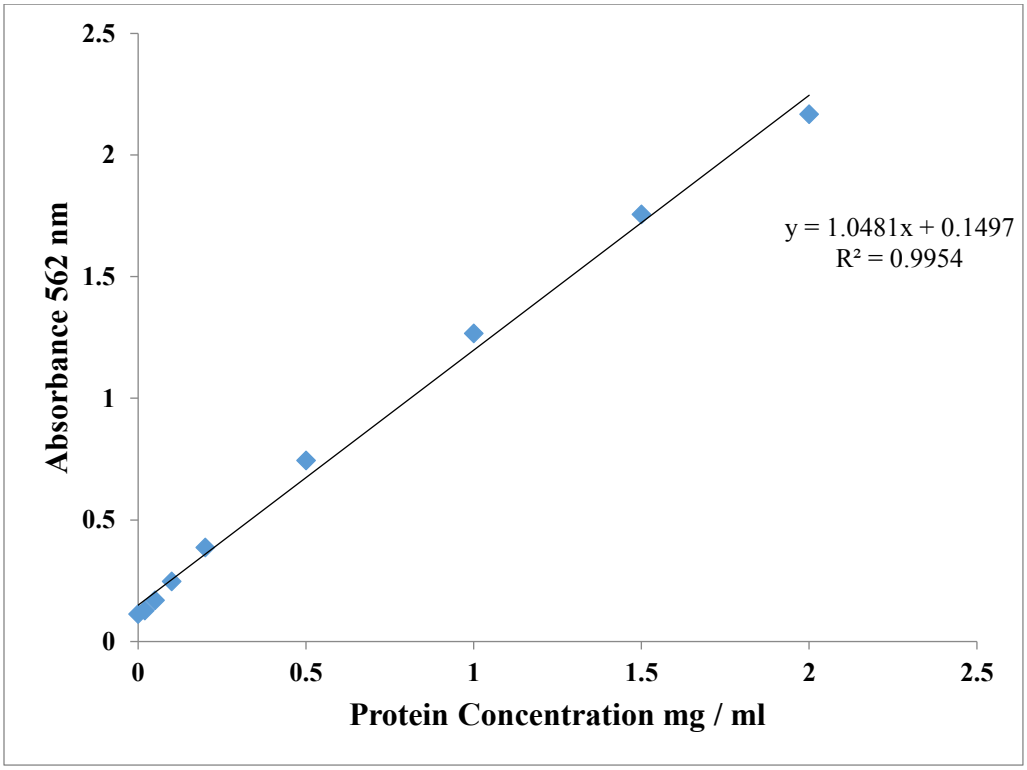


Figure 44. BCA assay using fractions collected from an 18 mL LH-20 column. Standards and samples were run in triplicate. A strong regression value of 0.9954 was obtained.

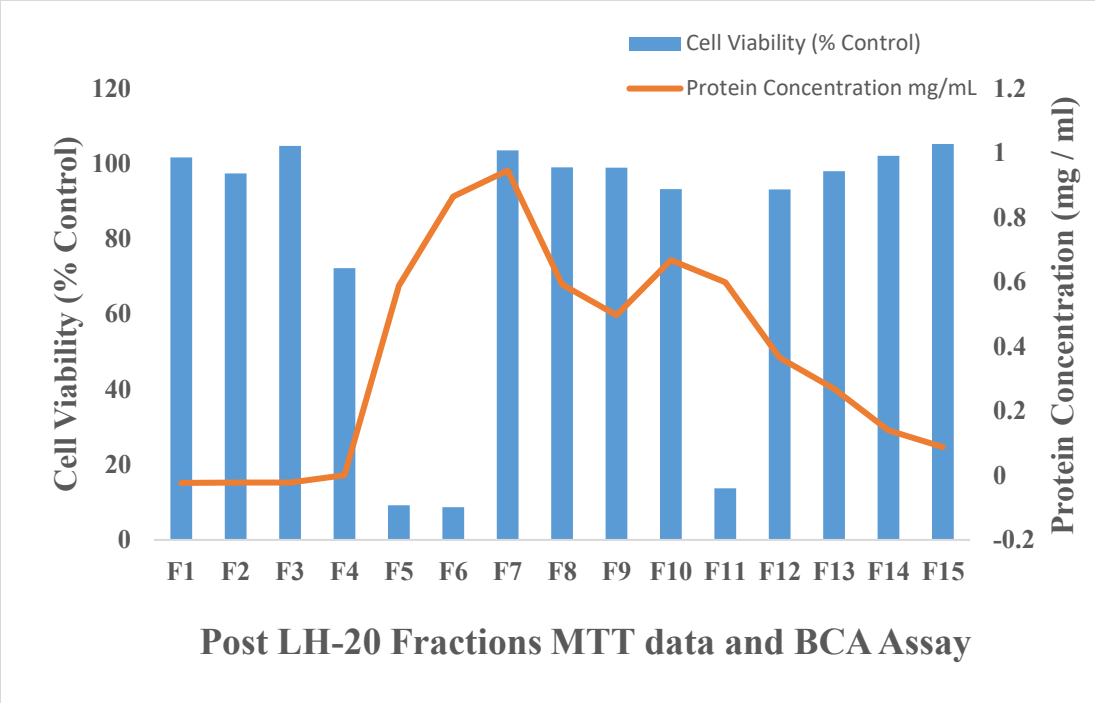


Figure 45. BCA assay and MTT assay using eluted fractions from an 18 mL LH-20 column. Fractions 5, 6 and 11 had the highest cell growth inhibition activity while fractions 7 and 10 had the highest protein content. This assay was run twice.

3.3.3 Total Carbohydrate Assay (Hot H_2SO_4)

A total carbohydrate assay was run using fractions collected from an 18 mL LH-20 column, shown in Figure 42. This assay had to be run twice as initially the carbohydrate content in the stock LH-20 fractions was far too high to be read on the Synergy2 plate reader. Samples were diluted by 50% (15 µL of LH-20 purified fraction) and the assay was run again. The trend line can be seen in Figure 46.

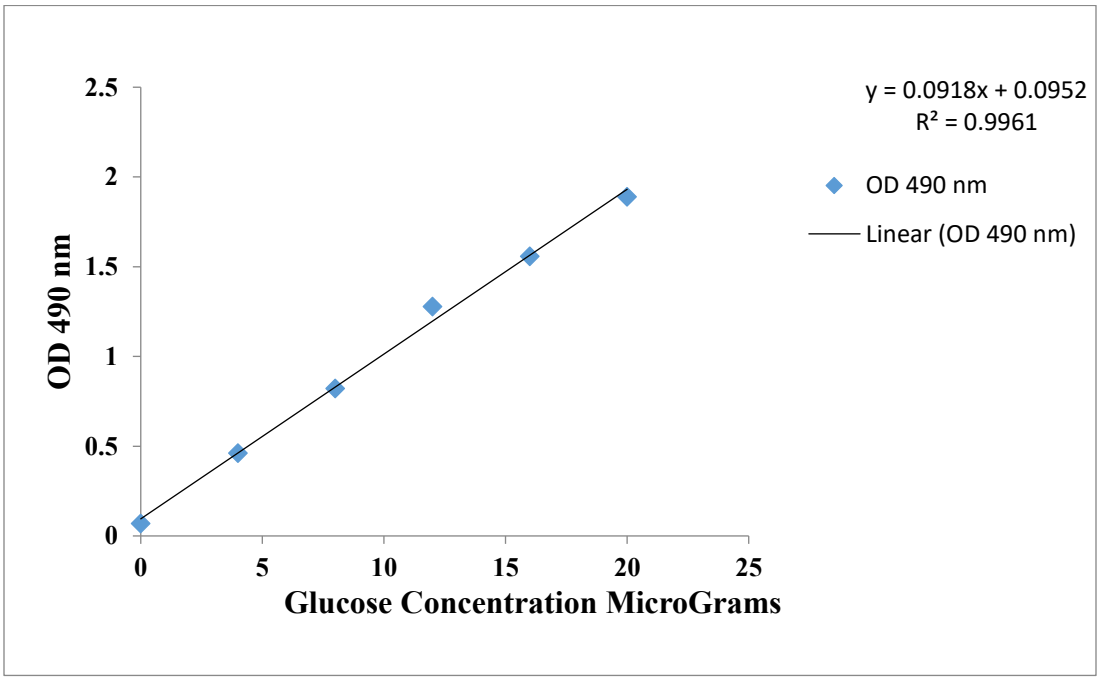


Figure 46. Linear trend line calculated from glucose standards created from Total Carbohydrate Kit. A strong regression value of 0.9961 was achieved.

The total carbohydate results were then graphed alongside the MTT data from Figure 42 (18 mL LH-20 Column). Fraction 5 had the highest total carbohydrate content and fraction 11 had next to no carbohydrate content – even though it had strong cell growth inhibitory effects as shown in an MTT assay (Figure 47). These results were consistent with

the first total carbohydrate assay performed, other than the first peak, over fraction 5, was out of bounds.

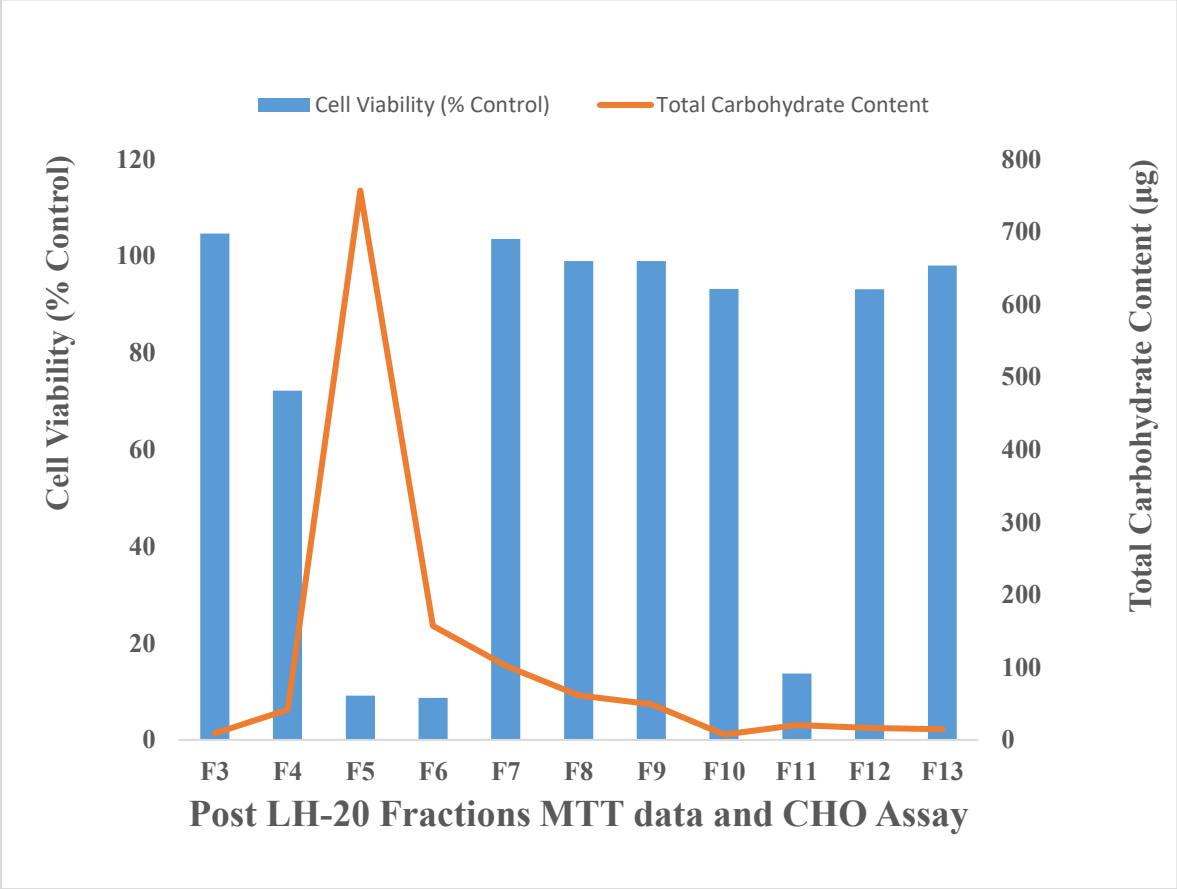


Figure 47. Growth-inhibitory activity and carbohydrate content of fractions collected from an 18 mL Sephadex LH-20 column. Fractions were analyzed using MTT assay and were also assayed with a total carbohydrate kit. Fraction 5 had the highest carbohydrate content while fraction 11 had next to none. The carbohydrate kit was run twice and samples / standards were run in triplicate. MTT samples were run with one biological replicate and 6 technical replicates.

3.3.4 Glycoprotein Carbohydrate Estimation Kit

This kit was originally run using the maximum volume of LH-20 purified fraction, 50 µL. This turned out to be far too much as many of the fractions had a greater glycoprotein concentration than the Synergy2 plate reader could read. The assay was run a second time,

with a 4x sample dilution (12.5 µL per fraction) and the trend line can be seen in Figure 48. Samples and standards were run in triplicate.

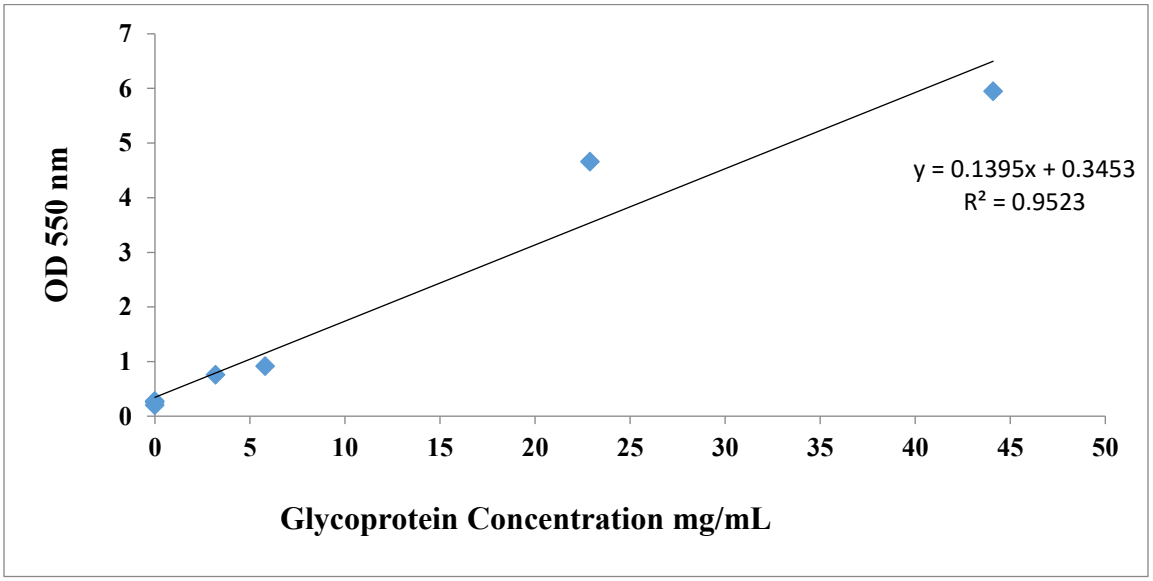


Figure 48. Linear trend line calculated from glycoprotein standards created from a glycoprotein estimation kit. A regression value of 0.9523 was achieved.

Data extrapolated from the trend line was used to calculate glycoprotein content in mg/mL and was then graphed alongside the MTT data from Figure 42 – an 18 mL LH-20 column. Fractions 5 and 8 had the highest glycoprotein content, while fraction 11 had only 4.2 mg/mL. Fractions 5 and 11 had the highest growth inhibitory effect in the corresponding MTT assay (Figure 49).

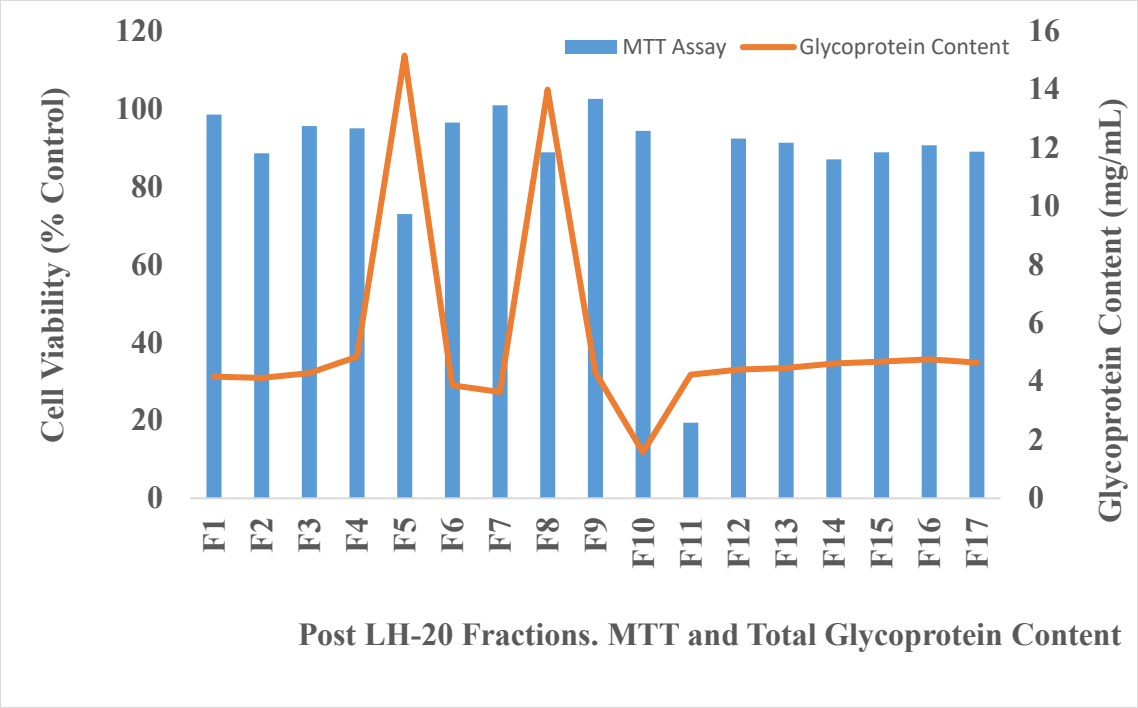


Figure 49. Growth-inhibitory activity and glycoprotein content of fractions collected from Sephadex LH-20 column. Fractions were analyzed using MTT assay and were also assayed with a glycoprotein estimation kit. Fraction 5 and fraction 8 had the highest glycoprotein content while fraction 11 had very little. The glycoprotein assay was run twice and samples / standards were run in triplicate. MTT samples were run with one biological replicate and 6 technical replicates.

3.3.5 C16/70 LH-20 Column (100 mL)

In order to collect enough materials for further investigation, possibly by an undergraduate research student, Mr Faran Rashid, a larger column was required. A C16/70 column was therefore packed to a bed volume of 100 mL, at 2 m /minute. Crude fraction 2, obtained from *E. tinctorium*, was dissolved in water at a concentration of 50 mg/mL. Two milliliters of sample was then loaded onto the column and eluted at 1 mL per minute and collected in 10 mL fractions. After some time, two bands could be seen moving down the column (Figure 50).

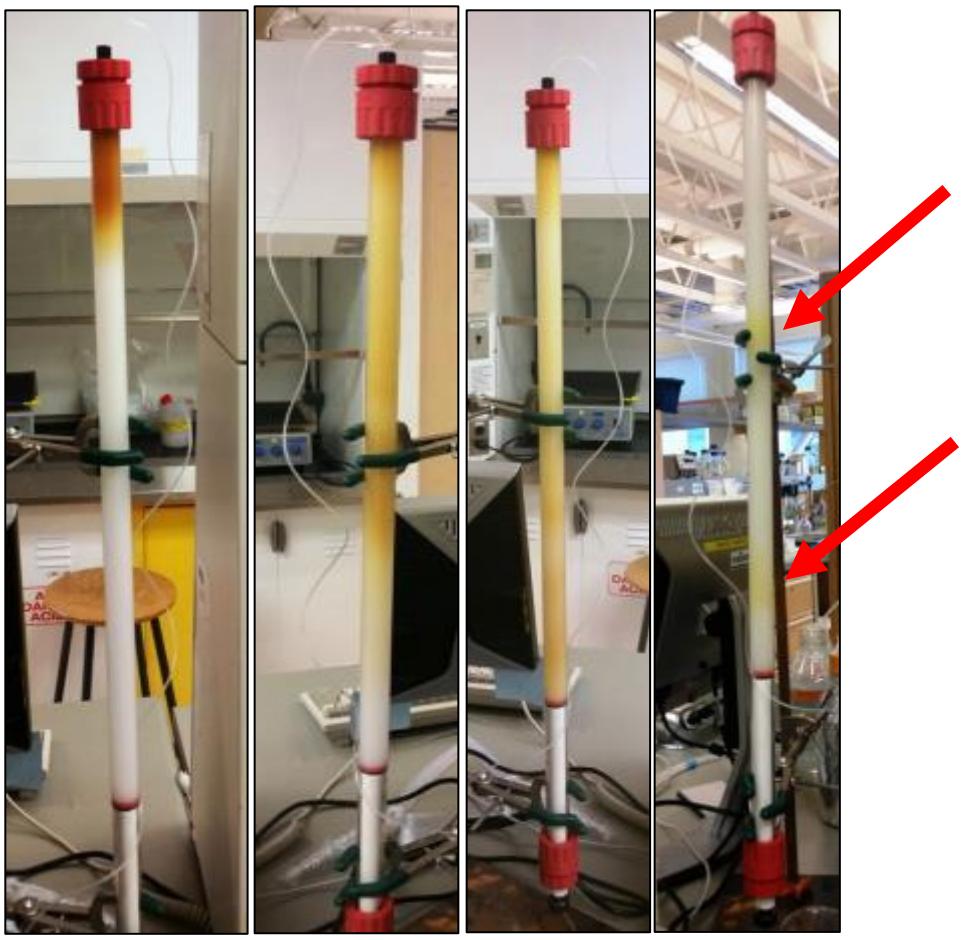


Figure 50. 100 mL LH-20 column purifying crude E2 extract from *E. tinctorium*. The column is being run with water. Two bands can be seen in the 4th picture (see arrows).

The C16/70 column was used to purify crude Fraction 2 for two runs, at 2 mL each (Figure 51). The first attempt had high cell growth inhibitory effect on fractions 4, 5 and fraction 10. As indicated earlier, the second run was performed in order to provide more sample to an undergraduate research student to further analyze fraction 10 (Figure 52).

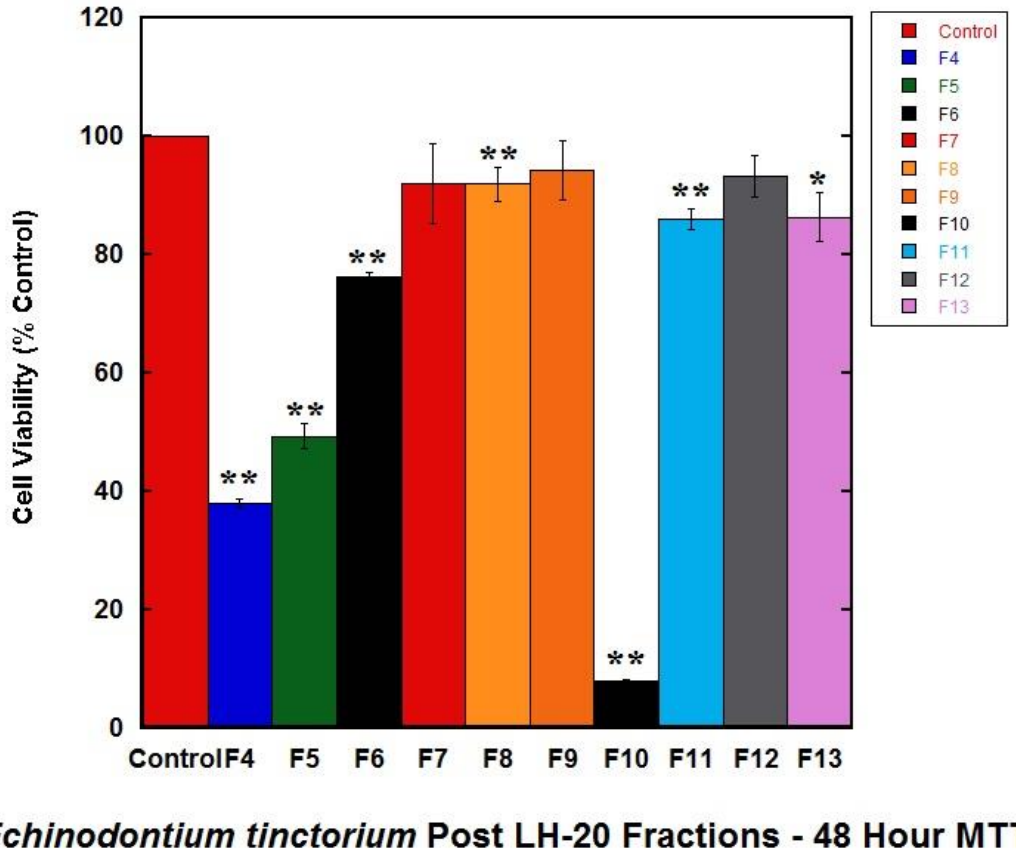


Figure 51. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. Methanol extracts from *E. tinctorium* was loaded at 2% onto a 100 mL Sephadex LH-20 column. Fractions collected at 10 mL volumes were subjected to a 48 h MTT assay. One biological replicate and 4 technical replicates were used. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

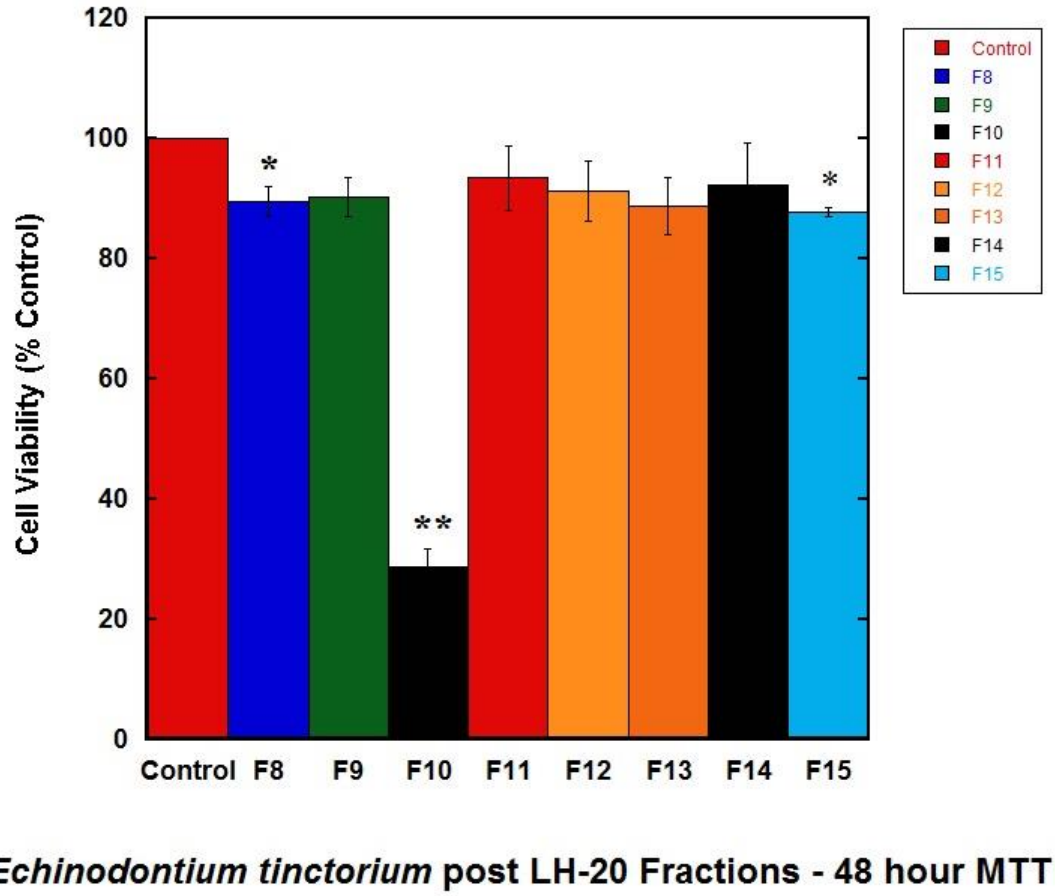


Figure 52. Assessing the post-Sephadex LH-20 fractions for growth-inhibitory activity. Methanol extracts from *E. tinctorium* was loaded at 2% onto a 100 mL Sephadex LH-20 column. Fractions collected at 10 mL volumes were subjected to a 48 h MTT assay. One biological replicate and 4 technical replicates were used. $P < 0.05$, $^{**}P < 0.01$ versus control. Error bars are S.E.M.

3.3.6 Assessment of the Post-Sephadex LH-20 Fractions for Growth-Inhibitory Activity using Different Cancer Cell Lines.

Post-Sephadex LH-20 fraction 10, purified from crude methanol extract from *E. tinctorium*, was assessed for growth inhibitory effect using variety of cancer cell lines. The following cell lines were used in a 48 hour MTT assay: HCT116 – Human Colon Cancer, H441 – Human Lung Cancer, HeLa – Human Cervical Cancer, HepG2 – Human Liver Cancer, MCF-7 – Human Breast Cancer and SKOV3 – Human Ovarian Cancer. The results can be seen in Figures 53 and 54.

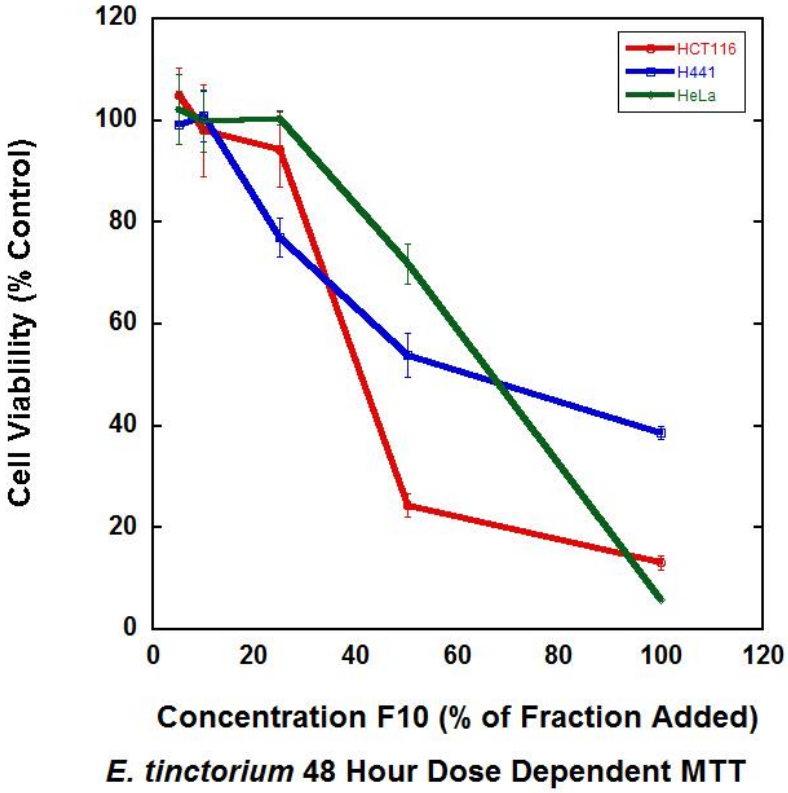


Figure 53. Assessing the post-Sephadex LH-20 Fraction 10 for growth-inhibitory activity, using various cancer cell lines, in a 48 h MTT assay. Fraction 10 had the most potent effect on HeLa and HCT116. Fraction 10 reduced cell viability of H441 by over 60%. One biological replicate and 3 technical replicates were used.

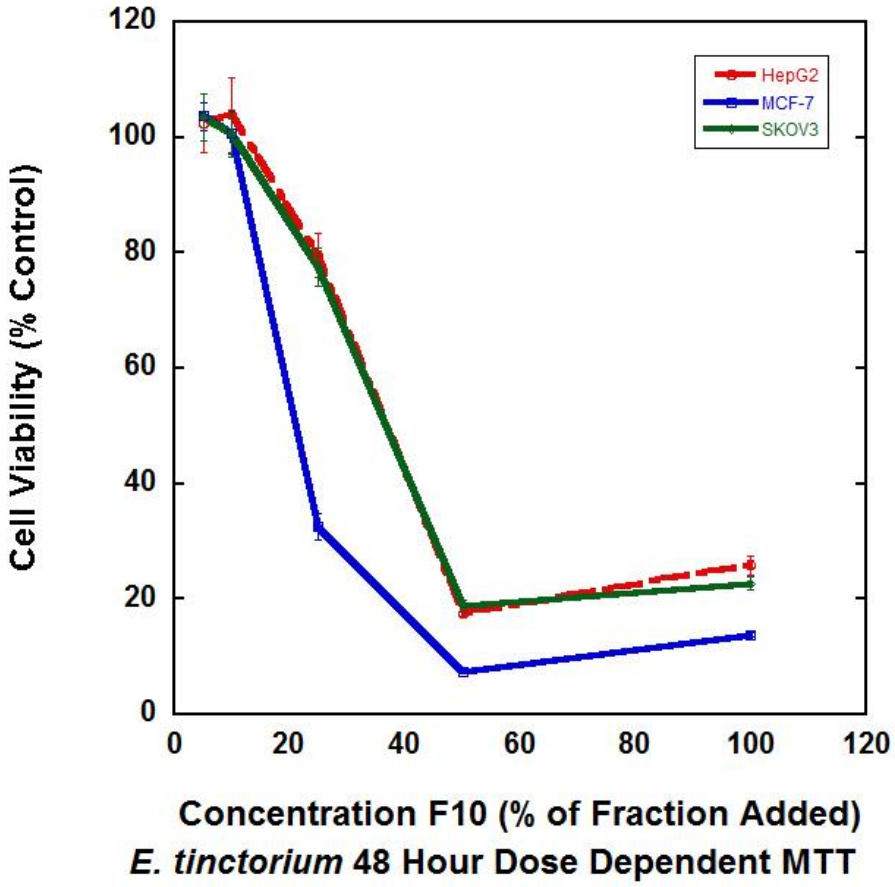


Figure 54. Assessing the post-Sephadex LH-20 Fraction 10 for growth-inhibitory activity, using various cancer cell lines, in a 48 h MTT assay. Fraction 10 had the post potent effect on MCF-7. However, fraction 10 reduced cell viability of both SKOV3 and MCF-7 by approximately 70%. One biological replicate and 3 technical replicates were used.

Fraction 10, purified using a C16/70 Column, had an extremely potent effect on all of the cell lines assayed. The most potent cell inhibitory effect can be seen with HeLa cells, however, in all cases, cell viability is reduced by a minimum of 60% to a maximum of 90% with Hela cells. All cell lines assayed in Figure 54 had very similar dose dependent graph line.

3.4 Discussion

With every size exclusion column run, the first active compound had a very high growth inhibitory effect during the first run of a freshly packed column, then the next run would have a highly reduced effect on cell viability (Figures 35-38). It appeared as though something was clogging up the column. After the initial run, the column was flushed with 50% methanol, which cleared out the pigmentation. However, that had little effect on clearing out the column. Once the purification process was moved to a larger column, 10 mL and higher, it was found that there appeared to be two active compounds. The first activity eluted in the void volume, which would put it larger than 4-5 kDa, and another activity being eluted in fractions 10 or 11. The second activity had potent cell growth inhibitory effect on HeLa cells and had no issues being eluted on a column after the first run (Figures 41-43). This was also the case when we moved to a larger C16/70 column, with a 100 mL Sephadex LH-20 bed volume (Figure 51-52). For this reason, it was decided to pursue the compound(s) in fraction 10 as they were less difficult to purify using size exclusion chromatography.

Protein concentration did not necessarily correlate with the largest growth inhibitory effect. For example, fraction 2 had the highest effect on cell viability but fraction 7 had the highest protein content (Figure 40). A BCA assay run using fractions eluted from a larger column (18 mL) showed a similar trend. Fraction 5 had the highest growth inhibitory effect while fraction 7 had the largest protein content. Additionally, when the crude methanol fraction was heated at 95 °C for 1 h, it had little effect on growth inhibitory effects (Figure 41). Heating the sample at that temperature for that amount of time should have denatured most proteins, and as such, it is unlikely that the active compound(s) contain protein component.

Assessment of the total carbohydrate content revealed interesting insights. Fractions 5, 6 and 11 had the highest growth inhibitory effect while fraction 5 had the highest carbohydrate content, suggesting that the growth-inhibitory activity in fraction 5 may contain carbohydrate component. Fraction 11 had next to no carbohydrate content but had a potent effect on cell viability as shown in an MTT assay (Figure 47), suggesting that the growth-inhibitory compound(s) in fraction 11 does not contain carbohydrate. The results in estimating the glycoprotein content of post-Sephadex LH-20 fractions supported the above findings: that is, fractions 5 and 7 had the highest glycoprotein content, while fraction 5 and 10 had the highest cell inhibitory effect on HeLa cells. This provided further support that the growth-inhibitory compound(s) in fraction 5, but not fraction 10, contains carbohydrate component.

Although this study has not led to the complete purification and identification of the growth-inhibitory compound(s), I have shown for the first time that *Echinodontium tinctorium* has potent growth-inhibitory compounds. I have also shown for the first time that Sephadex LH-20 purified fraction 10 has a potent growth inhibitory effect on other cell lines, such as H441, HCT116, HepG2, MCF-7 and SKOV3. The larger molecular weight growth-inhibitory compound(s) collected in fraction 5 mostly likely contains carbohydrate. In contrast, the smaller molecular weight growth-inhibitory compound(s) collected in fraction 10 is likely a small molecule because it does not contain protein or carbohydrate. Given these exciting findings, it is clear that there remains much work and investigation to be completed for this particular project but we hope that these insights but provide momentum for the next group of investigators.

Chapter 4 - General Discussion

At the outset of this research, it was hypothesized that wild mushrooms in Northern British Columbia contain compounds with potent bio-activities. The results from this study showed for the first time that indeed Northern BC is home to a large variety of mushrooms containing potent growth inhibitory compounds[97]. The extraction methods developed and adapted (using both the manual and speed extractor) during this study have laid the critical foundation for future mushroom research studies at UNBC and elsewhere. As the pioneer graduate student of this project, I had the opportunity to explore mushrooms and optimize the processing (from collection to powder) and extraction techniques, ranging from small scale soxhlet extractions using a few grams of material, to large scale extractions using close to 1 kilogram of starting materials. Furthermore, specifications for the growth-inhibitory MTT assays using different concentration mushroom extracts and the incubation time, for both dose- and time dependent experiments, have also been essential for the progression of this project. The crude fractions extracted from 9 samples of B.C wild mushrooms will also continue to be further investigated for immuno-stimulatory and anti-inflammatory properties by other research students at UNBC[97].

4.1 Screening BC wild mushrooms for growth-inhibitory activity

Fomitopsis pinicola, perhaps the most potent mushroom investigated, can be found growing abundantly throughout area surrounding UNBC. Because of the extensive research previously conducted on this specimen, it was not investigated further. However, its potent

growth inhibitory effect on HeLa cell viability, was used as a positive control in optimizing MTT assay and hence in further advancing this project.

Due to its copious use in herbal medicine, it was not surprising that compound(s) extracted from *Lentinula edodes* (Shiitake) had potent growth-inhibitory effect on HeLa cells. Since extractions conducted at both reflux and 50 °C temperatures had a similar effect on HeLa cell viability, it was decided to use 65 °C for future extractions. The temperature of 65°C was chosen because: (i) it is between 50°C and reflux temperature, (ii) many mushrooms were extracted at reflux temperature , and (iii) it is a convenient and manageable temperature for use during manual extraction and with the speed extractor.

Some mushrooms species, such as *Piptoporus betulinus*, were shown to have activity for the first time. In the case of *P. betulinus*, E1 (ethanol) extract was demonstrated to have potent cell growth inhibitory properties at doses as little as 0.1 mg/mL. Similarly, compounds extracted using ethanol from *Ganoderma applanatum,* were shown for the first time to have cell growth inhibitory effects; additionally, water extracted compounds were also shown to have an effect on cell viability. This is corroborated by previous research.

4.2 An attempt to purify the growth-inhibitory compound from *Echinodontium tinctorium*

Undoudtedly, the most exciting species investigated was *Echinodontium tinctorium*. We were able to show for the first time, that ethanol extractions from *E. tinctorium* possess potent growth inhibitory properties in HeLa cells, in a dose dependent and time dependent manner. Furthermore, since virtually no previous research has been conducted on this species, it was determined to be a novel species for further investigation.

Size exclusion chromatography by use of Sephadex LH-20 initially indicated that one fraction contained the majority of the growth inhibitory compounds. After a column was used once, it seemed to clog up, and the growth inhibitory effect of fractions collected from the second run was greatly reduced. Methanol was used to rinse the column, which cleared the staining caused by crude E2 (50% methanol) fraction, but this had no consequence on capturing the same level of growth inhibitory effect as seen on compounds obtained from a 'first run' on a fresh column.

Once the column size was increased from 2 mL to 10 mL, growth-inhibitory activity could clearly be seen in two different fractions (bimodal graphs); the first presumably eluting in the void volume and a second eluting in later fractions, with fractions in between having little to no activity as shown in MTT assays. This was also the case when the column size was increased to 18 mL and eventually 100 mL – two discrete fractions were shown to possess potent growth inhibitory compound(s).

Post-Sephadex LH-20 fractions were assayed for protein, total carbohydrate and glycoprotein contents. Fraction 5 had the highest effect on cell viability and also contained a large protein component. The growth-inhibitory effect of fraction 5 correlated strongly with both carbohydrate and glycoprotein content, suggesting that growth-inhibitory compound(s) in fraction 5 has a carbohydrate component. Fraction 10 also had high cell growth inhibitory effect. Given that the growth-inhibitory effect of fraction 10 did not correlate with either protein, carbohydrate or glycoprotein contents, suggest that it is likely to be a small molecule. Additionally, crude fraction 2 (50% methanol) was heated to high temperatures for over an hour and still retained potent cell growth inhibitory properties.

4.3 Future Directions and Conclusions

Based on the growth-inhibitory screening studies that I have performed on BC wild mushrooms, one can now proceed in choosing a mushroom fraction which is novel for further purification and identification of the responsible growth-inhibitory compound(s). Although I was unable to further purify and identify the small molecule growth-inhibitory compound(s) from *E. tinctorium*, I was able to show for the first time that extracts from *E. tinctorium* are also capable of drastically reducing cell viability in other human cancer lines in addition to human cervical cancer HeLa cells. Protocols and assay optimizations solidified by this project has also laid the foundation for future investigation into the assessment of B.C wild mushrooms for new and natural anti-cancer compounds.

References

1. Halpern G. Healing mushrooms. Effective treatments for today's illnesses. 2007:192.

2. Dias ES, Abe C, Schwan RF. Truths and myths about the mushroom *Agaricus blazei*. Scientia Agricola. 2004;61(5):545–549.

3. Chang ST (Shu-ting), Quimio TH, Regional Workshop on the Cultivation of Edible Mushrooms in the Tropics (1980 : Manila P. Tropical mushrooms : biological nature and cultivation methods. Chinese University Press; 1982. 493 p.

4. Wasson G. The Wondrous Mushroom: Mycolatry in Mesoamerica. McGraw-Hill; 1980. 248 p.

5. Akgul NB, Basaran P, Sidhu JS. Mushrooms in the Middle Eastern Diet. In: Hui YH, editor. Handbook of Fruit and Vegetable Flavors. John Wiley & Sons, Inc.; 2010. p. 889–908.

6. Mushrooms M. An Exploration of Tradition, Healing and Culture. 7th ed. Botanica Press; 1995. 264 p.

7. Smith J, Rowen N, Sullivan R. Medicinal Mushrooms: Their therapeutic properties and current medical usage with special emphasis on cancer treatments. Cancer Research UK. 2002;(May):4–33, 47–51.

8. Paterson RRM. *Ganoderma* - A therapeutic fungal biofactory. Phytochemistry. 2006;67(18):1985–2001.

9. Zhu M, Chang Q, Wong LK, Chong FS, Li RC. Triterpene antioxidants from *Ganoderma lucidum*. Phytotherapy Research. 1999;13(6):529–531.

10. Eo S-K, Kim Y-S, Lee C-K, Han S-S. Antiviral activities of various water and methanol soluble substances isolated from *Ganoderma lucidum*. Journal of Ethnopharmacology. 1999;68(1–3):129–136.

11. Lakshmi B, Ajith TA, Sheena N, Gunapalan N, Janardhanan KK. Antiperoxidative, anti-inflammatory, and antimutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* occurring in South India. Teratogenesis, Carcinogenesis, and Mutagenesis. 2003;23(S1):85–97.

12. Hong KJ, Dunn DM, Shen CL, Pence BC. Effects of *Ganoderma lucidum* on apoptotic and anti-inflammatory function in HT-29 human colonic carcinoma cells. Phytotherapy Research. 2004;18(9):768–770.

13. Tang W, Liu JW, Zhao WM, Wei DZ, Zhong JJ. Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. Life Sciences. 2006;80(3):205–211.

14. Xie JT, Wang CZ, Wicks S, Yin JJ, Kong J, Li J, Li YC, Yuan CS. *Ganoderma lucidum* extract inhibits proliferation of SW 480 human colorectal cancer cells. Experimental Oncology. 2006;28(1):25–29.

15. Slivova V, Valachovicova T, Jiang J, Sliva D. *Ganoderma lucidum* inhibits invasiveness of breast cancer cell. Journal of Cancer Integrative Medicine. 2004;2:8.

16. Gao Y, Zhou S, Jiang W, Huang M, Dai X. Effects of Ganopoly ® (a *Ganoderma lucidum* polysaccharide extract) on the immune functions in advanced-stage cancer patients. Immunological Investigations. 2003;32(3):201–215.

17. Chang J, Park W, Choi E, Kim B. Studies on constituents of the higher fungi of Korea (LIV) antitumor components of *Favolus alveolarius*. Archives of Pharmacal Research. 1988.

18. Chung KS, Choi EC, Kim BK, Kim YS, Park YH. Studies on the constituents and culture of Korean basidiomycetes - Antitumor polysaccharides from the cultured mycelia of some basidiomycetes. Archives of Pharmacal Research. 1982;5(1):17–19.

19. Moradali MF, Mostafavi H, Ghods S, Hedjaroude GA. Immunomodulating and anticancer agents in the realm of macromycetes fungi (macrofungi). International Immunopharmacology. 2007;7(6):701–724.

20. K. J. Shittake the healing mushroom. 1st ed. Healing Arts Press; 2000. 128 p.

21. Fang N, Li Q, Yu S, Zhang J, He L, Ronis MJJ, Badger TM. Inhibition of growth and induction of apoptosis in human cancer cell lines by an ethyl acetate fraction from shiitake mushrooms. The Journal of Alternative and Complementary Medicine. 2006;12(2):125–132.

22. Sia GM, Candlish JK. Effects of shiitake (*Lentinus edodes*) extract on human neutrophils and the U937 monocytic cell line. Phytotherapy Research. 1999;13(2):133–137.

23. Avinash J, Vinay S, Jha K, Das D, Goutham B, Kumar G. The unexplored anticaries potential of shiitake mushroom. Pharmacognosy Reviews. 2016;10(20):100.

24. Ina K, Kataoka T, Ando T. The use of lentinan for treating gastric cancer. Anti-cancer agents in medicinal chemistry. 2013;13(5):681–8.

25. Hirasawa M, Shouji N, Neta T, Fukushima K, Takada K. Three kinds of antibacterial substances from *Lentinus edodes* (Berk.) Sing. (Shiitake, an edible mushroom). International Journal of Antimicrobial Agents. 1999;11(2):151–157.

26. Bisen P, Baghel R, Sanodiya B, Thakur GS, Prasad GBKS. *Lentinus edodes*: A Macrofungus with Pharmacological Activities. Current Medicinal Chemistry. 2010;17(22):2419–2430.

27. Ng J, Yang C. Effect of Mushrooms on Dental Caries. J. Pharm. Sci. & Res. 2013;5(12):284–286.

28. Fisher M, Yang L-X. Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. Anticancer research. 2002;22(3):1737–1754.

29. Nio Y, Tsubono M, Tseng C-C, Morimoto H, Kawabata K, Masai Y, Shiraishi T, Imai S, Ohgaki K, Tobe T. Immunomodulation by orally administered protein-bound polysaccharide PSK in patients with gastrointestinal cancer. Biotherapy. 1992;4(2):117–128.

30. Price LA, Wenner CA, Sloper DT, Slaton JW, Novack JP. Role for toll-like receptor 4 in TNF-alpha secretion by murine macrophages in response to polysaccharide Krestin, a

Trametes versicolor mushroom extract. Fitoterapia. 2010;81(7):914–919.

31. Hyun KW, Jeong SC, Lee DH, Park JS, Lee JS. Isolation and characterization of a novel platelet aggregation inhibitory peptide from the medicinal mushroom, *Inonotus obliquus*. Peptides. 2006;27(6):1173–1178.

32. Van Q, Nayak BN, Reimer M, Jones PJH, Fulcher RG, Rempel CB. Anti-inflammatory effect of *Inonotus obliquus*, Polygala senega L., and Viburnum trilobum in a cell screening assay. Journal of Ethnopharmacology. 2009;125(3):487–493.

33. Mullauer FB, Kessler JH, Medema JP. Betulinic acid, a natural compound with potent anticancer effects. Anti-cancer drugs. 2010;21(3):215–227.

34. Reshetnikov S V., Tan K-K. Higher Basidiomycota as a Source of Antitumor and Immunostimulating Polysaccharides (Review). International Journal of Medicinal Mushrooms. 2001;3(4):361–394.

35. Wasser S. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology. 2003;60(3):258–274.

36. Ooi VEC, Liu F. Immunomodulation and Anti-Cancer Activity of Polysaccharide-Protein Complexes. Current Medicinal Chemistry. 2000;7:715–729.

37. Ng M-L, Yap A-T. Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes*). Journal of alternative and complementary medicine (New York, N.Y.). 2002;8(5):581–589.

38. Lavi I, Friesem D, Geresh S, Hadar Y, Schwartz B. An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces anti-proliferative and pro-apoptotic effects on HT-29 colon cancer cells. Cancer Letters. 2006;244(1):61–70.

39. The macrofungi of British Columbia. 2012 [accessed 2012 Jan 1]. http://www.geog.ubc.ca/biodiversity/eflora/fungi.html.

40. Ferreira IC, Vaz J. Compounds from wild mushrooms with antitumor potential. Anti-Cancer Agents in. 2010:424–36.

41. Zaidman B-Z, Yassin M, Mahajna J, Wasser SP. Medicinal mushroom modulators of molecular targets as cancer therapeutics. Applied Microbiology and Biotechnology. 2005;67(4):453–468.

42. Patrick Poucheret. Biological and Pharmacological Activity of Higher Fungi: 20-Year Retrospective Analysis. Cryptogamie, Mycologie,. 2006;27(4):311–333.

43. Lindequist U, Niedermeyer THJ, Jülich WD. The pharmacological potential of mushrooms. Evidence-based Complementary and Alternative Medicine. 2005;2(3):285–299.

44. Adachi, Yoshiyuki; Okazaki, Mitsuhiro; Ohno, Naohito; Yadomae T. Enhancement of Cytokine Production by Macrophages Stimulated with (1-3)-B-D-Glucan, Grifolan (GRN), Isolated from *Grifola frondosa*. Pharmaceutical Society of Japan. 1994;17(12):1554–1560.

45. Matsuo T, Arika T, Mitani M, Komatsu N. Pharmacological and toxicological studies of a new antitumor polysaccharide, schizophyllan. Arzneimittel-Forschung. 1982;32(6):647–56.

46. Muto S, Kobayashi A, Ohara M, Matsunaga K. Structure and anti-tumor effect of PSK (krestin): Mechanistic aspects of the anti-tumor activity. International Journal of. 1982;4(4):308.

47. Chan GC-F, Chan WK, Sze DM-Y. The effects of beta-glucan on human immune and cancer cells. Journal of hematology & oncology. 2009;2(1):25.

48. Smith JE, Rowan NJ, Sullivan R. Medicinal mushrooms: A rapidly developing area of biotechnology for cancer therapy and other bioactivities. Biotechnology Letters. 2002;24(22):1839–1845.

49. Manzi P, Pizzoferrato L. Beta-glucans in edible mushrooms. Food Chemistry. 2000;68(3):315–318.

50. Brown GD, Gordon S. Immune recognition: A new receptor for β-glucans. Nature. 2001;413(6851):36–37.

51. Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L, Wong SYC, Gordon S. Dectin-1 Is A Major β-Glucan Receptor On Macrophages. The Journal of Experimental Medicine. 2002;196(3):407–412.

52. Zhang Y, Kong H, Fang Y, Nishinari K, Phillips GO. Schizophyllan: A review on its structure, properties, bioactivities and recent developments. Bioactive Carbohydrates and Dietary Fibre. 2013;1(1):53–71.

53. Yang G. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis. 1998;19(4):611–616.

54. Han B, Toyomasu T, Shinozawa T. Induction of apoptosis in cultured cells by extracts from shiitake (*Lentinula edodes*) mycelial culture broth. Mycoscience. 2000;41(6):623–631.

55. PowerSoil DNA Isolation Kit. 2017 [accessed 2017 May 9]. https://mobio.com/media/wysiwyg/pdfs/sds/12888-1.pdf

56. Technelysium - DNA Sequencing Software. 2017 [accessed 2017 May 9]. http://technelysium.com.au/wp/

57. Basic Local Alignment Search Tool. 2017 [accessed 2014 Feb 4]. https://blast.ncbi.nlm.nih.gov/Blast.cgi

58. Arora D. Mushrooms demystified : a comprehensive guide to the fleshy fungi. Ten Speed Press; 1986. 959 p.

59. Mizuno T. The Extraction and Development of Antitumor-Active Polysaccharides from Medicinal Mushrooms in Japan (Review). International Journal of Medicinal Mushrooms. 1999;1(1):9–29.

60. Lee ML, Tan NH, Fung SY, Tan CS, Ng ST. The antiproliferative activity of sclerotia of *Lignosus rhinocerus* (tiger milk mushroom). Evidence-based Complementary and Alternative Medicine. 2012;2012:1–5.

61. Jedinak A, Dudhgaonkar S, Wu Q-L, Simon J, Sliva D. Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-κB and AP-1 signaling.

Nutrition journal. 2011;10(1):52.

62. Phan CW, Wong WL, David P, Naidu M, Sabaratnam V. *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde: Nutritional value and in vitro neurite outgrowth activity in rat pheochromocytoma cells. BMC Complement Altern Med. 2012;12(1):102.

63. Mosmann T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of lmmunological Methods. 1983;65:55–63.

64. Berridge M V., Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnology Annual Review. 2005;11:127–152.

65. L. Maness L, Sneed N, Hardy B, Yu J, Ahmedna M, Goktepe I. Anti-proliferative effect of *Pleurotus tuberregium* against colon and cervical cancer cells. Journal of Medicinal Plants Research. 2011;5(30):6650–6655.

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

67. MTT Assay. [accessed 2017 May 12]. http://en.wikipedia.org/wiki/File:MTT_Plate.jpg.

68. Finimundy TC, Gambato G, Fontana R, Camassola M, Salvador M, Moura S, Hess J, Henriques JAP, Dillon AJP, Roesch-Ely M. Aqueous extracts of *Lentinula edodes* and *Pleurotus sajor-caju* exhibit high antioxidant capability and promising in vitro antitumor activity. Nutrition Research. 2013;33(1):76–84.

69. Kaya M, Akata I, Baran T, Mentes A. Physicochemical Properties of Chitin and Chitosan Produced from Medicinal Fungus (*Fomitopsis pinicola*). Food Biophysics. 2014;10(2):162–168.

70. Keller AC, Maillard MP, Hostettmann K. Antimicrobial steroids from the fungus *Fomitopsis pinicola*. Phytochemistry. 1996;41(4):1041–1046.

71. Choi D, Park S-S, Ding J-L, Cha W-S. Effects of *Fomitopsis pinicola* extracts on antioxidant and antitumor activities. Biotechnology and Bioprocess Engineering. 2007;12(5):516–524.

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

73. Carmeliet P. VEGF as a Key Mediator of Angiogenesis in Cancer. Oncology. 2005;69(3):4–10.

74. Kitzberger CSG, Smânia A, Pedrosa RC, Ferreira SRS. Antioxidant and antimicrobial activities of shiitake (*Lentinula edodes*) extracts obtained by organic solvents and supercritical fluids. Journal of Food Engineering. 2007;80(2):631–638.

75. Takehara M, Kuida K, Mori K. Antiviral activity of virus-like particles from *Lentinus edodes* (Shiitake). Archives of Virology. 1979;59(3):269–274.

76. Nanba H. Antitumor Mechanisms of Orally Administered Shiitake Fruit Bodies. Chemical & Pharmaceutical Bulletin. 1987;35(6):2459–2464.

77. Wangun HVK, Berg A, Hertel W, Nkengfack AE, Hertweck C. Anti-inflammatory and anti-hyaluronate lyase activities of lanostanoids from *Piptoporus betulinus*. The Journal of antibiotics. 2004;57(11):755–8.

78. Wiater A, Paduch R, Pleszczyńska M, Próchniak K, Choma A, Kandefer-Szerszeń M, Szczodrak J. α-(1 → 3)-d-Glucans from fruiting bodies of selected macromycetes fungi and the biological activity of their carboxymethylated products. Biotechnology Letters. 2011;33(4):787–795.

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

80. Choe J-H, Yi Y-J, Lee M-S, Seo D-W, Yun B-S, Lee S-M. Methyl 9-Oxo-(10E,12E)-octadecadienoate isolated from *Fomes fomentarius* attenuates lipopolysaccharide-induced inflammatory response by blocking phosphorylation of STAT3 in murine macrophages. Mycobiology. 2015;43(3):319–326.

81. Jeong YT, Yang BK, Jeong SC, Kim SM, Song CH. *Ganoderma applanatum*: a promising mushroom for antitumor and immunomodulating activity. Phytotherapy Research. 2008;22(5):614–619.

82. Sun X, Zhao C, Pan W, Wang J, Wang W. Carboxylate groups play a major role in antitumor activity of *Ganoderma applanatum* polysaccharide. Carbohydrate Polymers. 2015;123:283–287.

83. Balandaykin ME, Zmitrovich I V. Review on Chaga Medicinal Mushroom, *Inonotus obliquus* (Higher Basidiomycetes): Realm of Medicinal Applications and Approaches on Estimating its Resource Potential. International Journal of Medicinal Mushrooms. 2015;17(2):95–104.

84. Kang J-H, Jang J-E, Mishra SK, Lee H-J, Nho CW, Shin D, Jin M, Kim MK, Choi C, Oh SH. Ergosterol peroxide from Chaga mushroom (*Inonotus obliquus*) exhibits anti-cancer activity by down-regulation of the β-catenin pathway in colorectal cancer. Journal of Ethnopharmacology. 2015;173:303–312.

85. Liu C, Zhao C, Pan HH, Kang J, Yu XT, Wang HQ, Li BM, Xie YZ, Chen RY. Chemical constituents from *Inonotus obliquus* and their biological activities. Journal of Natural Products. 2014;77(1):35–41.

86. Fan L, Ding S, Ai L, Deng K. Antitumor and immunomodulatory activity of water-soluble polysaccharide from *Inonotus obliquus*. Carbohydrate Polymers. 2012;90(2):870–874.

87. Wang Q, Mu H, Zhang L, Dong D, Zhang W, Duan J. Characterization of two water-soluble lignin metabolites with antiproliferative activities from *Inonotus obliquus*. International Journal of Biological Macromolecules. 2015;74:507–514.

88. Turkoglu A, Duru ME, Mercan N, Kivrak I, Gezer K. Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. Food Chemistry. 2006;101(1):267–273.

89. Mlinaric A, Kac J, Pohleven F. Screening of selected wood-damaging fungi for the HIV-

1 reverse transcriptase inhibitors. Acta pharmaceutica (Zagreb, Croatia). 2005;55(1):69–79.

90. Lovy A, Knowles B, Labbe R, Nolan L. Activity of edible mushrooms against the growth of human T4 leukemic cancer cells, HeLa cervical cancer cells, and Plasmodium falciparum. Journal of Herbs, Spices and Medicinal Plants. 1999;6(4):49–57.

91. Léon F, Quintana J, Rivera A, Estévez F, Bermejo J. Lanostanoid triterpenes from *Laetiporus sulphureus* and apoptosis induction on HL-60 human myeloid leukemia cells. Journal of Natural Products. 2004;67(12):2008–2011.

92. Li X, Jiao LL, Zhang X, Tian WM, Chen S, Zhang LP. Anti-tumor and immunomodulating activities of proteoglycans from mycelium of *Phellinus nigricans* and culture medium. International Immunopharmacology. 2008;8(6):909–915.

93. Wang Z, Zhou F, Quan Y. Antioxidant and immunological activity in vitro of polysaccharides from *Phellinus nigricans* mycelia. International Journal of Biological Macromolecules. 2014;64:139–143.

94. Sephadex LH-20. [accessed 2017 May 23]. http://www.sigmaaldrich.com/catalog/product/sigma/lh20100?lang=en&region=CA

95. Total Carbohydrate Colorimetric Assay Kit. [accessed 2017 May 23]. http://www.biovision.com/documentation/datasheets/K645.pdf

96. Glycoprotein Estimation Kit. [accessed 2017 May 23]. https://tools.thermofisher.com/content/sfs/manuals/MAN0011353_Glycoprotein_Carb_Est_UG.pdf

97. Smith A, Javed S, Barad A, Myhre V, Li WM, Reimer K, Massicotte H, Takaberry L, Payne G, Egger K, et al. Growth-Inhibitory and Immuno-Modulatory Activities of Fungi from North Central British Columbia. International Journal of Medicinal Mushrooms. 2017;19(6).

Appendix

Sequences Derived from DNA Analysis

Piptoporus betulinus

ITS Sequence:

TGCAGATTCAGTGATCATCGATCTTTGACGCACCTTGCGCTCCTTGGTATTCCGA
GGAGCATGCCTGTTTGAGTGTCATGGAATCATCAACTCTATTTACTTTTGTGAAT
AGGGCTTGGACTTGGAGGTTTTGCCGGTACTTGTGATCGGCTCCTCTTGAATGCA
TTAGCTCGAACCTTTGTGGATCAGCTTATCGGTGTGATAATTGTCTACGCCGTTA
CTGTGAAGCATATATTAAAGGGCTCGGCTTCTAATCGTCCTTCACAGGACAATAA
CTTTGACCTTTGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCA
ATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAG
CGGGAAAAGCTCAAATTTAAAATCTGGCGGTCTTATGGCCGTCCGAGTTGTAGTC
TGGAGAAGTGCTTTCCGCGCTGGACCGTGTACAAGTCTCTTGGAACAGAGCGTC
ATAGAG

NLB Sequence:

CGCGGAAGCACTTCTCCAGACTACAACTCGGACGGCCATAAGACCGCCAGATTT
TAAATTTGAGCTTTTCCCGCTTCACTCGCAGTTACTAGGGGAATCCTTGTTAGTTT
CTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAATCCTACCTGATTTGA
GGTCAAAGGTCAAAGTTATTGTCCTGTGAAGGACGATTAGAAGCCGAGCCCTTT
AATATATGCTTCACAGTAACGGCGTAGACAATTATCACACCGATAAGCTGATCC
ACAAAGGTTCGAGCTAATGCATTCAAGAGGAGCCGATCACAAGTACCGGCAAAA
CCTCCAAGTCCAAGCCCTATTCACAAAAGTAAATAGAGTTGATGATTCCATGACA
CTCAAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGAT
TCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTCT

Ganoderma applanatum

ITS Sequence:

TCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGASGAG
CATGCCTGTTTGAGTGTCATGAAATCTTCAACCTATAASCTTTTGTGGTTTGTAGG
CTTGGACTTGGAGGCTTGTCGGCCTTGATCGGTCGGCTCCTCTTAAATGYATTAG
CTTGATTCCTTGCGGATCGGCTCTCGGTGTGATAATATCTACGCCGCGACCGTGA
AGCGTTTGGCGAGCTTCTAAYCGTCTCACTTGAGAGACAACTTTATGACCTCTGA
CCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
AAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCA
AATTTAAAATCTGGCGGTCTTTGGCCGTCCGAGTTGTAGTCTGGAGAAGTGCTTT
CCGCGCTGGACCGTGTATAAGTCTCTTGGAACAGAGCGTCATAGAGGG

NLB Sequence:

GACTACAACTCGGACGGCCAAAGACCGCCAGATTTTAAATTTGAGCTTTTCCCGC
TTCACTCGCAGTTACTAGGGGAATCCTTGTTAGTTTCTTTTCCTCCGCTTATTGAT
ATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAGAGGTCATAAAGTT
GTCTCTCAAGTGAACGRTTAGAAGCTCGCCAAACGCTTCACGGTCGCGGCGTAG
ATATTATCACACCGAGAGCCGATCCGCAAGGAATCAAGCTAATRCATTTAAGAG
GAGCCGACCGATCAAGGCCGACAAGCCTCCAAGTCCAAGCCTACAAACCACAAA
AGCTTATAGGTTGAAGATTTCATGACACTCAAACAGGCATGCTCCTCGGAATACC
AAGGAGCGCAAGGTGCGTTCAAAGATTCGATGA

Fomes fomentarius

ITS Sequence:

GTGATTGCAGATTCAGTGATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGT
ATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTATAAACCTTT
GCGGGTTTGTAGCGTTGGATGTTGGAGGCTTTTTGCTGGCCCAGTCAGCTCCTCT
TAAATGCATTAGCTTGGTTCCTTGTGGATCGGCTGTCGGTGTGATAATGTCTACG
CCGCGACCGTGAAGCGTTTGGAGAGCTTCTAATGGTCTCGTCAGAGACAGCTTTT
ATGAACTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAT
AAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCG
GGAAAAGCTCAAATTTAAAATCTGGCGGTCTTTGGCCGTCCGAGTTGTAGTCTGG
AGAAGTGCTTTCCGCGCTGGACCGTGTACAAGTCTCTTGGAACAGAGCGTCATA
GAGGGTGGAGAATCCA

NLB Sequence:

ATTTTAAATTTGAGCTTTTCCCGCTTCACTCGCAGTTACTAGGGGAATCCTTGTTA
GTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGTCCTACCTGAT
TTGAGGTCAGAGTTCATAAAAGCTGTCTCTGACGAGACCATTAGAAGCTCTCCAA
ACGCTTCACGGTCGCGGCGTAGACATTATCACACCGACAGCCGATCCACAAGGA
ACCAAGCTAATGCATTTAAGAGGAGCTGACTGGGCCAGCAAAAAGCCTCCAACA
TCCAACGCTACAAACCCGCAAAGGTTTATAGGTTGAGAATTTCATGACACTCAA
ACAGGCATGCTCCTCGGAATACCAAGGAGCGGCAAGGTGCGTTCA

Echinodontium tinctorium

ITS Sequence:

GATTGCAGATTCAGTGATCATCGAATCTTTGAACGCACMTTGCGCCCCTTGGTAT
TCCGRGGGGCAYGCCTGTTYGAGTGTCRTKWMTTYCYCCACTTCCCKCCYGCCY
TTGGGGTMTSSTGGGACTTGSATGTGTAAGTTTTTGCGGGGGTGCSTCYSCTCCTC
TACMAMTGMATTACTGAAACCCCYGGTGGTGCCTCCTTGGTGGGAWGTGGCTA
CGTCTAGGTTWTGTCCYACTGGGTTCTTCGCTACCAAGCATCCACACCGGATGAT
TYTTCGCATCTTGACCTCCGAACGAGAGGAACTACCCGCTGAATTCCTCCATATC
AGTAAGCGAAGGAAAAAAAACTCACATTGAATCCCCGACTWCCTGCCAATGART
MRTTAAAAGMWCATRTTTCAAGTGTGGCTCCCGTGGACGTCCCATGTAAAATTA
AGACAAACACTTTNCAGAACCCAACCGTGTACAAGTCTCCTGGAACGGAGCGTC
ATAGAGGG

NLB Sequence:

CACTCGACGCCCGMGGATTTCAGATTTGAATTGTTGCTTTTTCCGCTTCCTCACTG
ATGCTATGGGAGTCGGTGTCATTTCCTTTTCCTCCGGTTATGGATATGTTTAAGGT
CAATGGGTACCCGCTCCTGAGCCGAGGTCAAAWTACTATGAGTTGCCGCTTGRG
GACCGTTGGACTCRATCAACCAGGGTATTGTACAAAACCTAGCACGTNAACTATT
ATGGCGCCGAGGCCCACCTTGGGGCTTCACTAATGCATTTGAGAGGAGCGACAC
GCCGCCCGCAGAAGCCTCAAATGACGATYCCCAAGCTCCCCCCCAGGAAGTACG
GAAGTTGACAAAGTCCCKACACWCAAWCAAG