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Biological activities of select North American lichens

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Biological Activities of select North American Lichens

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Abstract:

The search for novel, pharmacologically significant compounds is a perpetual quest on which biologists, chemists, and bioinformaticians incessantly embark. The sources of these compounds are often of natural origin, making bioprospecting an enticing area of research. One such source of these natural compounds are lichens, which can produce a plethora of bioactive compounds. Historically, lichens have been held as medicine in traditional healing, while in modern times, research has begun to lend validity to these claims. The aim of this thesis was to extract, separate, identify, and evaluate the anti-cancer and antibiotic activity of numerous bioactive compounds and extracts from North American lichens. Antibacterial efficacy was analyzed via resazurin reduction assay, while anticancer ability was evaluated using the MTT assay and propidium iodide flow cytometry. The lichens studied include *Xanthoparmelia mexicana, Lobothallia alphoplaca,* and *Umbilicaria muhlenbergii*. Results from the crude acetone maceration extracts of the lichen showed promise of antibacterial activity against gram-positive bacteria *(Xanthoparmelia mexicana,* MIC of 41.9 µg mL^{-1} and 20.9 µg mL^{-1} against *E. faecalis* and *S. aureus*, respectively) while other crude extracts showed promising anticancer activity (*Lobothallia alphoplaca,* IC_{50} of 87.0 µg mL^{-1} against MCF-7 cells, MTT assay). Furthermore, *Umbilicaria muhlenbergii* showed bioactive prowess against both gram-positive bacteria and cancerous cells, with an MIC of 22.5 µg mL^{-1} against *S. aureus* and an IC_{50} of 13.3 µg mL^{-1} against MCF-7 cells. Due to it's marked bioactivity, *Umbilicaria muhlenbergii* crude extract was further separated with silica gel 60 column chromatography, where multiple fractions were found to be bioactive against select bacteria and MCF-7 cells. Two of these silica gel 60 fractions were then further

separated with Sephadex LH-20, yielding multiple fractions which exhibited bioactivity against select bacteria, in addition to anti-cancer abilities and cell-cycle arrest abilities. Further studies will need to be undertaken to fully identify compounds from and evaluate the bioactivity of the lichen extracts investigated.

Lay Summary

The fight against common infections and cancers is an endless endeavour undertaken by researchers every day. To combat these ailments, novel treatments and pharmaceutical compounds must be discovered and tested. An incredibly important contributor to these new treatments are natural products, which are pharmaceutically relevant compounds that come from a natural source. The diverse range of organisms these compounds are obtained from is expansive, and often the organism's natural components are analyzed to determine if they'll be useful as drugs. In this particular thesis, an often overlooked area of natural products research, lichens, were investigated for anticancer and antibiotic properties. The extracted complex mixtures of these chemically uncharacterized North American lichens were also separated into individual components to identify the molecules responsible for the anticancer, antioxidant, and antibiotic response.

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1 Introduction

1.1 Thesis Objectives

The three main objectives of this thesis were to determine if the three lichen species evaluated contain bioactive molecules, to determine which bioactive qualities each of these lichens possess, and lastly to identify which compounds are responsible for this bioactivity.

To achieve these objectives, three chemically undescribed North American lichens were investigated for bioactivity against several bacteria and MCF-7 adenocarcinoma cells. The lichens were evaluated for bioactivity against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Enterococcus faecalis* bacteria, and a MCF-7 adenocarcinoma breast cancer cell line.

The three lichens were collected from three different locations (Figure 1-1). Two of the lichens, *Xanthoparmelia mexicana* (Figure 1-2) and *Lobothallia alphoplaca* (Figure 1-3), were collected by Mr. Kerry Knudsen of University of California Riverside Lichen Herbarium. *Xanthoparmelia mexicana* was collected from Catalina Island, California, USA, while *Lobothallia alphoplaca* was collected from Joshua Tree National Park, California, USA. The *Umbilicaria muhlenbergii* lichen (Figure 1-4) was collected from Thunder Bay, Ontario, Canada by Dr. Lada Malek of Lakehead University.

As the project progressed, a more refined and pointed investigation into the separation of *Umbilicaria muhlenbergii* extract into fractions, then evaluating and identifying the compounds present, evolved into the main objective.

Figure 1-1: Locations of lichens collected. *Xanthoparmelia mexicana* (triangle), *Lobothallia alphoplaca* (square), *Umbilicaria muhlenbergii* (star).

Figure 1-2: *Xanthoparmelia mexicana* lichen.

Figure 1-3: *Lobothallia alphoplaca* lichen.

Figure 1-4: *Umbilicaria muhlenbergii* lichen.

2 Literature Review

2.1 Natural Products

Since the beginning of mankind, botanical and natural sources have been utilized to treat ailments, whether through purposeful discovery or serendipitous ingestion[2]. Some of the earliest records of natural product use date to Mesopotamia in 2600 B.C., where the oils of myrrh and cypress were used to treat coughs and colds [3]. A few hundred years later, the ancient Egyptians created a pharmaceutical record known as the Ebers Papyrus. The Ebers Papyrus contains over 700 plant-based drugs and their doses for a plethora of ailments, in addition to numerous other medical observations and procedures [4]. These ancient texts were complimented by the likes of the Chinese *Materia medica* and Indian *Materia medica*, as the knowledge of these treatments began to travel the world. [5, 6]. The Greco-Roman empire, centuries later, built upon the findings of more ancient civilizations, with many Greek and Roman physicians and scientists contributing more knowledge, such as the *Historia plantarum* by Theophrastus, *De Materia Medica* by Pedanius Dioscorides, and volumes of records by Claudius Galenus [7]. There are countless other examples of pharmaceutical records as one searches nearer to the current millennia, making this list even more non-exhaustive. As world-wide literacy increased steadily throughout the centuries, more and more treatments can be found mentioned in the folklore of all cultures. One can trawl the history of any human culture to have lived, and will find some use of natural products for a medicinal and/or pharmaceutical purpose. This ubiquitous endeavour to utilize our surroundings has not been lost in the modern era of pharmaceutical bioprospecting.

These traditional oral and written pharmaceutical histories have been a foundation for modern pharmaceuticals, with many being further developed and verified by clinical, pharmacological, biological, and chemical studies [1, 8]. These include compounds which exhibit antibiotic, antifungal, antiviral, antiretroviral, anticancer, antioxidant, antiparastic, immunostimulating, pesticide, insecticide, and larvicidal bioactivities[9-17]. While those of ancient times sought relief from their ailments via observational analysis, brute force testing, or pure serendipity, our modern fields of medicine, biology, chemistry, and pharmacology have turned to more advanced methods of useful natural product identification. This modern approach to the age-old venture has proved quite fruitful, as many common pharmaceuticals in use today come from a naturally derived source [18].

Examples of these naturally-derived modern pharmaceuticals abound, with applications in everything from pain mitigation to cancer treatment[18]. Some well-known naturally derived pharmaceuticals include aspirin, digitoxin, morphine, quinine, pilocarpine, paclitaxel, artemisinin, reserpine, vinblastine, and atropine [8, 11, 18, 19]. Many of these compounds are produced by the organism for functions apart from those required to sustain life (primary metabolism), and are therefore known as secondary metabolites.

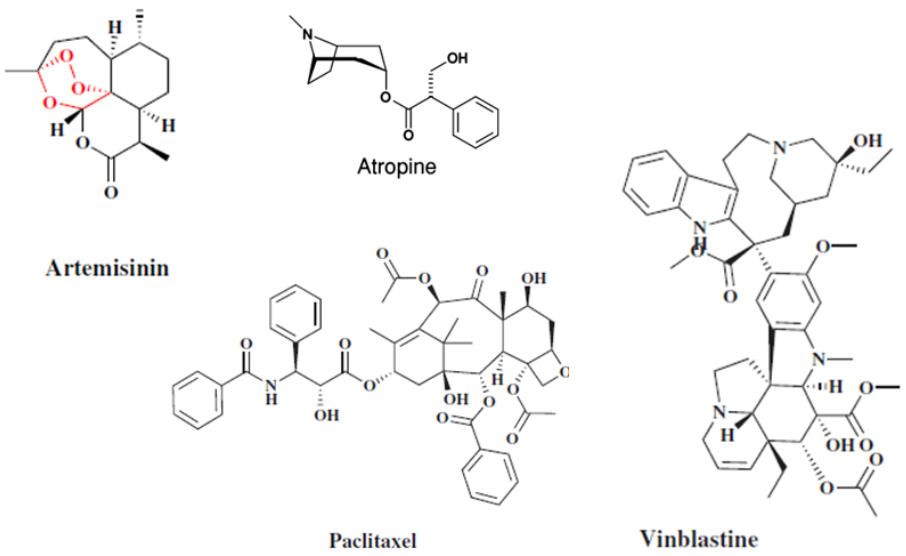


Figure 2-1: Structures of commonly used, naturally derived pharmaceuticals[21].

These naturally derived compounds make up a significant portion of novel pharmaceuticals, as evidenced by recent and regular investigations [7, 19-23]. The most recent of these reviews focused on specific anticancer drugs between 1981 and 2014, and drugs for all diseases between 1981 and 2014 [21]. Between the years 1981 and 2014, 1,562 new chemical entities were approved for pharmaceutical use, with a majority being from natural sources [21]. As shown in Figure 2-2, only 27% of all novel pharmaceuticals within the last 34 years have been purely synthetic in origin[21]. While completely chemically unaltered natural and botanical products make up only 20% of all novel approved drugs, natural products still play a significant role [21]. They provide researchers with molecules to mimic, model, and modify in order to produce naturally-derived, but more potent, synthetic drug compounds[21].

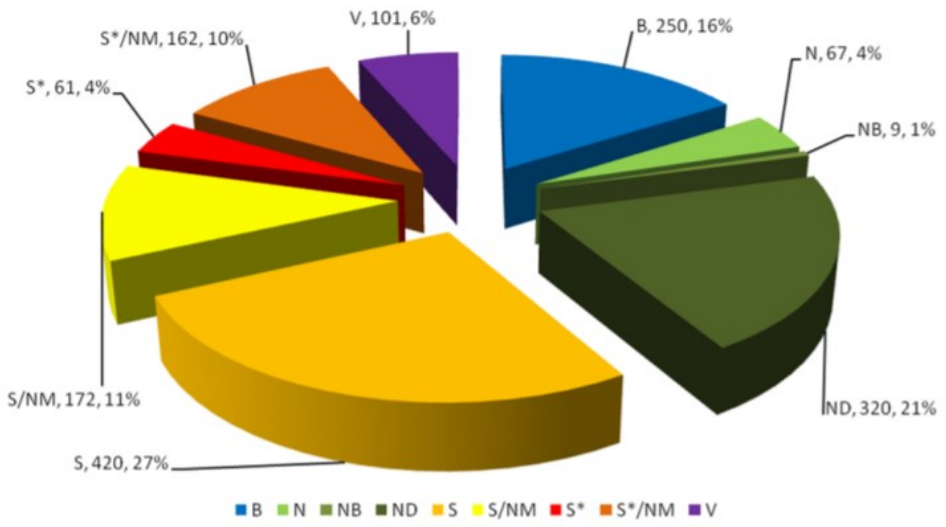
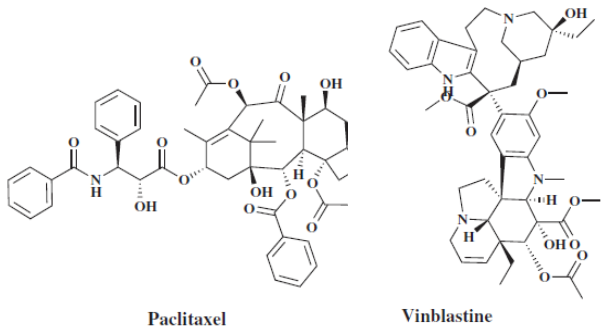


Figure 2-2: All new approved drugs, 1981-2014, n=1562[21].

B: biological macromolecule, N: Unaltered natural product, NB: Botanical drug of defined mixture, ND: natural product derivative, S: Synthetic drug, S*: Synthetic drug with a natural product pharmacophore, V: vaccine, /NM: mimic of a natural product.

This reliance on natural products becomes even more pronounced when only anti-cancer compounds are investigated. Between the 1940's and 2014, 175 small molecules were approved for anticancer use, with 131 (75%) of those compounds being of non-synthetic origin, and 85 (49%) of them being natural products or directly derived from natural products (Figure 2-4) [21].



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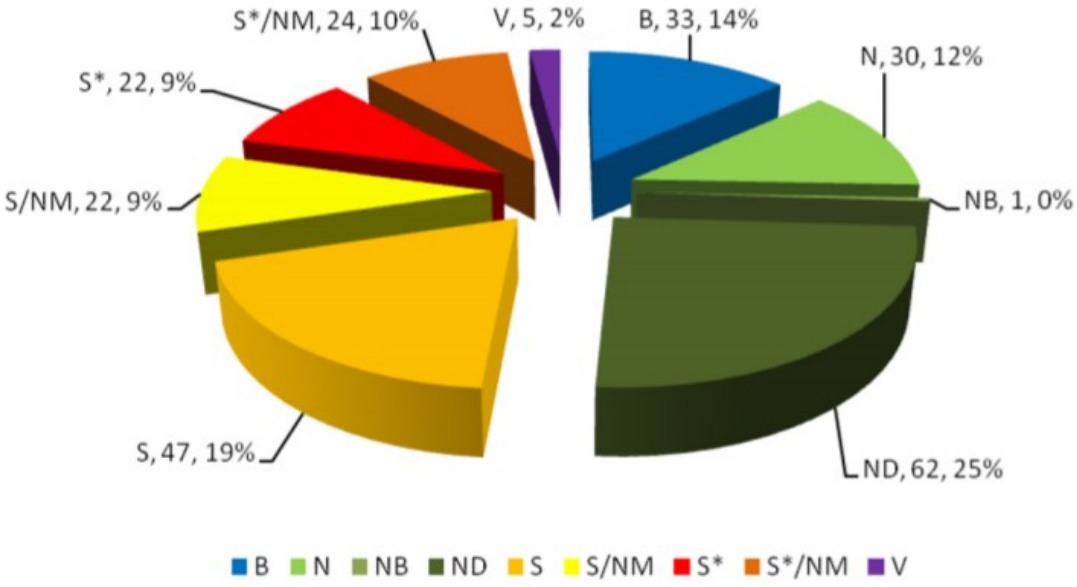

Figure 2-4: All anticancer drugs from 1940 to 2014, n=246[21].

The development of antibiotics is one research area which particularly blossomed due to natural products discovery. Following the 1928 discovery and subsequent synthetic production of penicillin in the 1940's, companies began to realize that collections of

organisms could be the key to finding new antibiotics [1]. These well-funded and well-supported ventures quickly churned out drugs which are now considered pillars of a modern healthcare system. These include antibiotics such as streptomycin, chloramphenicol, chlortetracycline, cephalosporin, erythromycin, and vancomycin (Figure 2-5) [19, 24, 25].

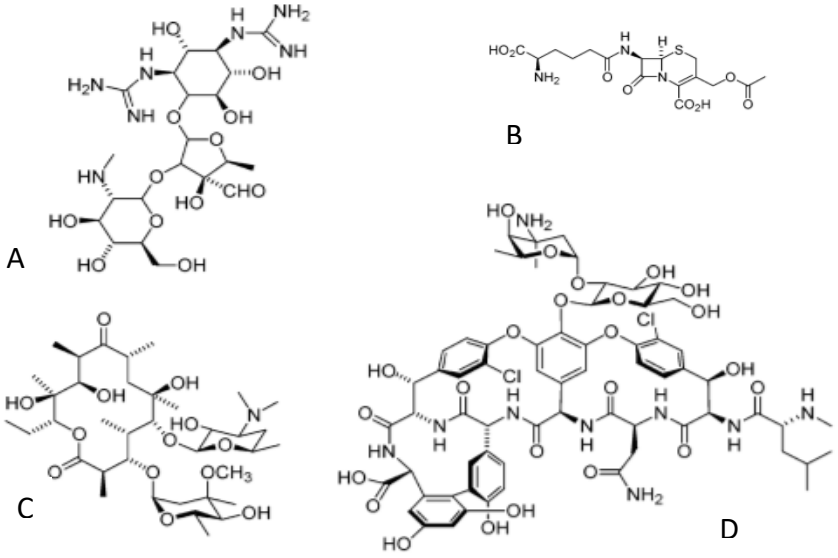

Figure 2-5: Naturally Derived Antibiotics[1].

A: Streptomycin, B: Cephalosporin, C: Erythromycinycin, D: Vancomycin

These naturally derived antibiotics have been in successful clinical use for decades, though the significance of some of them is beginning to wane [26]. New strains of antibiotic resistant bacteria, such as vancomycin-resistant *Enterococcus*, methicillin-resistant *Staphylococcus aureus*, and multiple-drug resistant *Mycobacterium tuberculosis*, are inciting a new push for novel antibiotics, through both synthetic and natural means.

Common sources of pharmaceutical compounds have been trees, flowers, herbs, roots, berries, and fungi. Of particular interest to this investigation are lichens, as they are a promising source of novel secondary metabolites which haven't yet been fully tapped [27].

2.2 What are lichens?

As renowned lichenologist Trevor Goward once humorously stated, "lichens are fungi that have discovered agriculture"[28]. He is not too far off, as lichens are a composite organism consisting of a fungus that can harness the primary metabolic production of a photosynthetic partner[29]. Lichens are ubiquitously found throughout the globe, with the number of extant species estimates ranging from 17,000 to 20,000 [29]. They are known as "pioneer organisms", as they can grow and flourish in incredibly harsh and inhospitable environments such as desert rocks, Antarctic wasteland, arctic tundra, tree bark, urban concrete, soils and countless other locations [30].

2.2.1 Lichen Structure

Lichens are not one single organism, but an obligate mutualistic association between two partners: a photobiont and a mycobiont. The photobiont is an alga or cyanobacterium, while the mycobiont is a species of fungus[29]. Once the two organisms are living harmoniously, the lichen will have its own morphology and physiology that will differ from that of its individual parts [31].

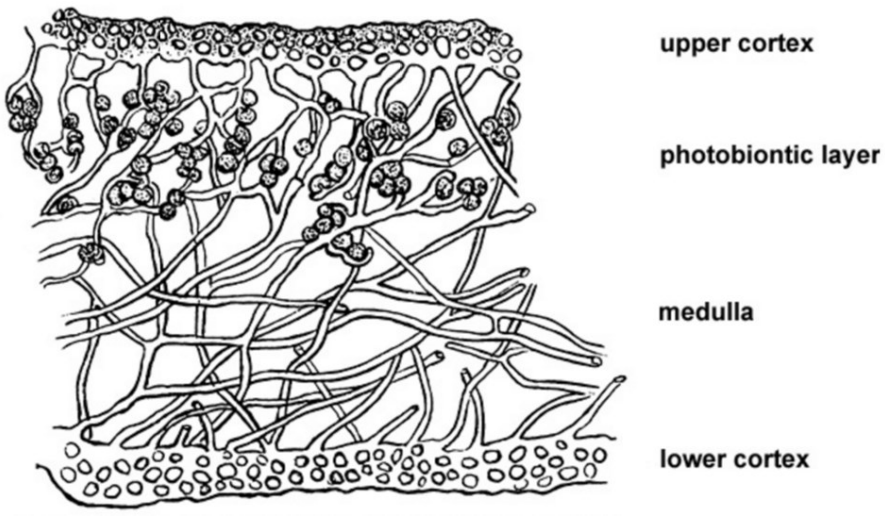

Figure 2-6: Four main parts of a lichen.

The part of the lichen which attaches to the substrate is known as the lower cortex (Figure 2-6) [32]. The lower cortex will often become so strongly attached to the substrate that any attempt at removal of the lichen will result in the removal of substrate along with the lichen [32]. This attachment often occurs through the use of rhizines, which penetrate into the substrate[33]. Other types of lichen may use attachment methods such as plate-like thalli [29]. Distal to the cortex, the mycobiont forms the medulla, where primary metabolites from the photobiont are stored by the mycobiont [29]. The photobiont layer can be found within this area of the medulla (Fig 2-6), where the mycobiont can protect the photobiont from overexposure to sun, heat, pollution, and excess water, while simultaneously giving the photobiont ideal amounts of light and water to grow [29]. In exchange for this ideal growth condition, the mycobiont uses the photobiont's photosynthetically produced metabolites to fuel its metabolic processes [29]. The final major structural component of the lichen, the upper

cortex, is the part of the lichen which is visible to a passer-by[29]. It can vary quite drastically in shape, size, colour, and configuration, and therefore is often used as a means of classifying types of lichens [34].

The overall lichen structure is provided by a mycobiont component known as the thallus (or lichen body) [29, 35, 36]. The differences in thallus structure can be classified into crustose, foliose, and fruticose types [29]. These different thallus structures arise due to different photobionts and mycobionts interacting in different environments, resulting in variances of morphology. The thallus often has a thorough intermingling of the photobiont and mycobiont, giving them the appearance of one individual which is markedly different than its individual parts. The thallus is also markedly important to the search for natural products, as the thallus is where secondary metabolites, produced by the mycobiont, are always found[29].

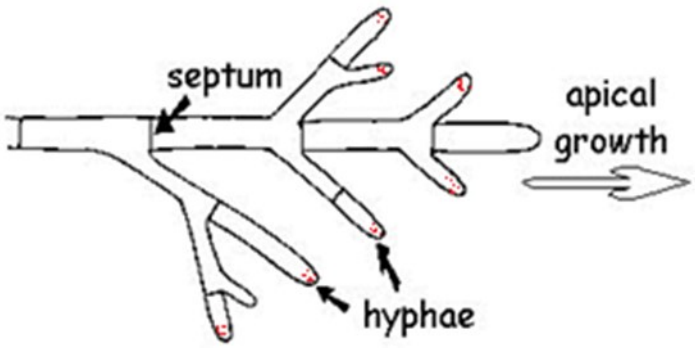

Figure 2-7: Drawing of lichen thallus showing fungal hyphae and septum.

The thallus of the lichen is composed of small structures, strands of fungal cells known as hyphae (Figure 2-7), which extend both apically toward the outer cortex, and inwards into the photobiont[29, 36]. The hyphae extract nutrients from the photobiont on the inside, while also being a location of secondary metabolite production and deposition on the upper cortex [29, 35, 36]. These secondary metabolites are often deposited onto the ends of the hyphae, where they can be found as either crystals or amorphous deposits (indicated by red dots in Figure 2-7).

2.3 Metabolites in Lichens

The metabolites found in these lichens are a part of the overall symbiotic metabolism, which is composed of primary metabolites and secondary metabolites, with each serving very different purposes. The primary metabolites (Figure 2-8) are those which are necessary for the growth of the lichen, and include organic compounds such as proteins, lipids, and carbohydrates [37]. These are produced by both the photobiont and the mycobiont [29, 37]. Secondary metabolites (Figure 2-8) are compounds which are metabolically non-essential to the growth of the organism, but serve a function more suited to the evolutionary goals of long term survival and reduction of competition [34]. These secondary metabolites are produced only by the mycobiont[38].

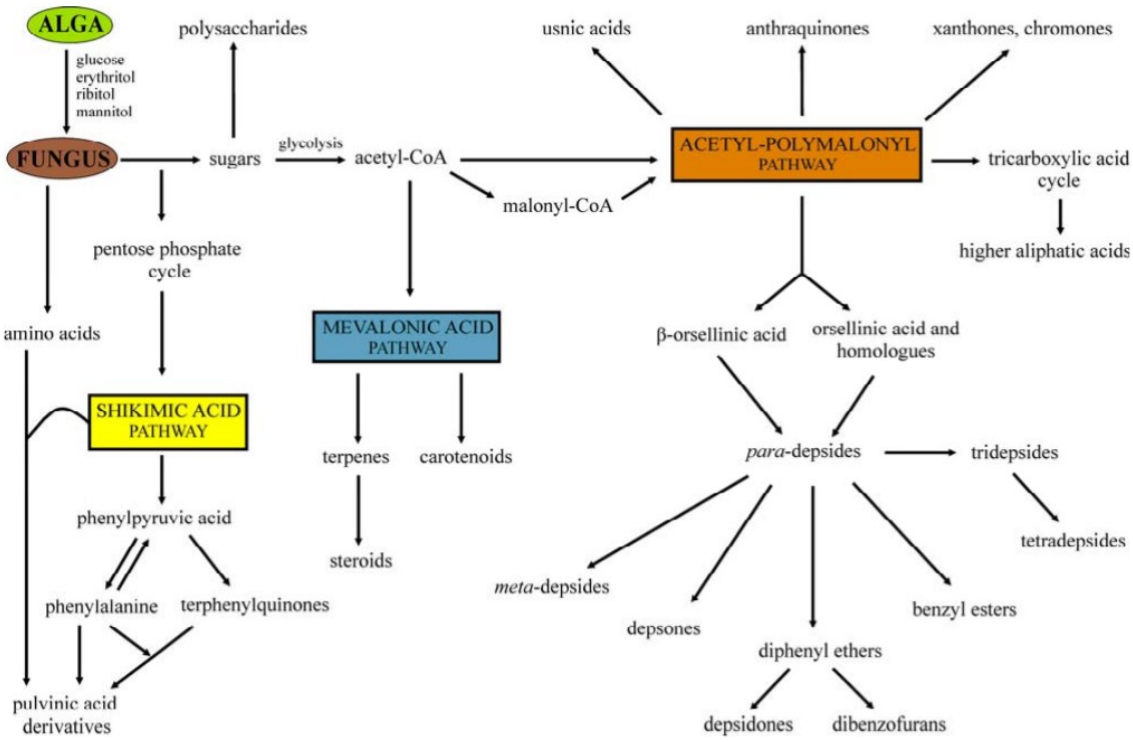

Figure 2-8: Lichen Metabolic Pathway Leading to Lichen Secondary Metabolites[39].

Lichen secondary metabolites are formed through either the acetyl polymalonyl pathway, the shikimic acid pathway, or the mevalonic acid pathway (Figure 2-8). Though the mechanistic intricacies of these pathways are beyond the context of this review, it is important to note the vast array of compounds which can be synthesized by lichens through these secondary metabolite pathways.

2.3.1 Secondary Metabolite Variation

The plethora of combinations between primary and secondary metabolism within lichens result in a staggering number of compounds being produced. Interestingly, a significant number of these compounds are entirely unique to lichens, and cannot be found

in any other fungi, algae, or higher plants[40-42]. There is a vast amount of interspecies and intraspecies variation due to these complex pathways. The intraspecies variation is quite significant, as the aforementioned pathways result in the lichen incorporating compounds from the substrate into the secondary metabolic profile. The variances in the substrate can lead to two different lichens of the same species growing on different substrates to produce an entirely different cassette of different compounds in different concentrations [43-45]. Currently, it is reported that there are approximately 1,050 known lichen secondary metabolites [40, 46]. Commonly found compounds include phenolic compounds (e.g. orcinol and β-orcinol, anthraquinones (parietin), dibenzofurans (usnic acid), depsides (gyrophoric acid), depsidones (norstictic acid), depsones (picrolichenic acid), γ-lactones (protolichesterinic acid), and pulvinic acid derivatives (vulpinic acid) [34].

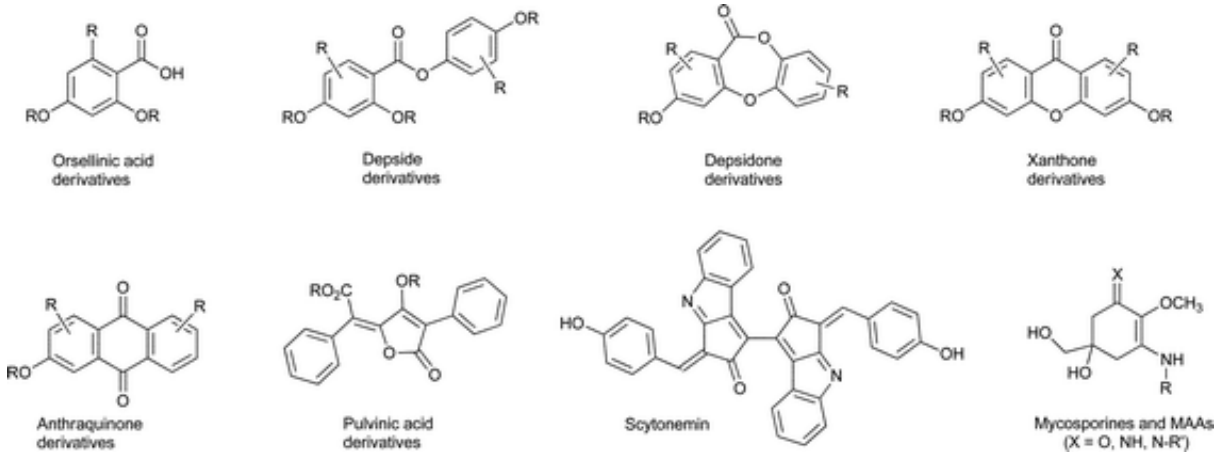

Figure 2-9: Common groups of lichen secondary metabolites[34].

A majority of these compounds are produced by the mycobiont, though a small percentage can be produced via the combined metabolism of the photobiont and

mycobiont[47]. It is these complex arrangements of chemicals that contribute to the ultimate bioactivity of lichens.

2.4 Lichen Constituent Bioactivities

2.4.1 Historical and ethnobotanical significance of lichens

Lichens have been utilized for a variety of uses by humankind since prehistory, including dyes, perfumes, pH indicators, emergency food sources, and traditional medical remedies [48].

A significant barrier to lichen metabolites being incorporated into pharmaceutical applications is the inherent toxicity of many secondary metabolites. This has been exemplified by the toxicity of usnic acid, which was included in a weight-loss drug called Lipokinetix®. It was intended to cause an increase in metabolic rate, thereby increasing caloric usage and inducing weight loss [49]. Clinical trials in which the drug was taken for 3 months resulted in all test subjects developing acute severe hepatotoxicity, with one death being attributed to the drug [50, 51]. After discontinuing ingestion of the drug, patients made full recoveries [49]. Though the mechanism of hepatotoxicity was never fully resolved, usnic acid has been strongly linked to be the culprit. This lichen metabolite toxicity then brought into question the validity and safety of all lichenic substances used as drugs.

These toxic qualities of many of these lichen metabolites are not entirely prohibiting, as *Umbilicaria muhlenbergii* has been known to be used as an emergency food source and traditional medicine [52]. The lichen is first boiled, presumably to extract any readily soluble secondary metabolites, and then eaten without any ill effects. Further research into toxicity

of lichen substances will need to be undertaken as they run the gamut of *in vitro* and *in vivo* evaluation.

2.4.2 Antioxidant

Free radicals and reactive oxygen species can cause oxidative stress within the human body, resulting in harmful effects [53]. It is often postulated that oxidative stress contributes to aging and an increased chance of disease and infection [53]. One particular culprit is the presence of free radicals, which are known to cause a multitude of disorders in humans [34]. Free radicals are molecular fragments which contain one or more unpaired electrons [53]. They are incredibly reactive with metabolites involved in biochemical pathways, and attract electrons from other cellular components. This can lead to cellular damage, such as cancerous unchecked cell proliferation, and cell death. These free radicals and reactive oxygen species are normally neutralized within the body by naturally produced anti-oxidant compounds, though recent research has proposed that supplementary antioxidants could be beneficial [54]. This has led to a body of research into supplemental antioxidants that can mitigate these effects. Natural products are being looked towards as a means of discovering novel antioxidant compounds, as many organisms, lichens included, produce many antioxidant compounds. In lichens, a significant number of the metabolites produced exhibit antioxidant and photoprotective abilities, making them an attractive target for investigation.

Previous investigations into lichen antioxidants have yielded positive results [34]. For example, species such as *Peltigera rufescens* had methanolic extracts exhibiting strong antioxidant potential. Other species have also followed suit, such as *Acarospora socialis*, *Usnea ghattensisin, Cetraria islandica*, *Parmotrema stuppeum*, *Usnea longissimia,* and

Lobaria pulmonaria [55-59]. In many of these instances, a correlation between phenolic content and antioxidant abilities has been noted[55-57, 60].

One compound, lecanoric acid, is often found in *Umbilicaria* species. It has prominent antioxidant potential, indicating that the *Umbilicaria muhlenbergii* lichen used in this investigation could be analyzed for antioxidant activity as well. Another lichen investigated, *Xanthoparmelia mexicana*, is noted to contain usnic acid, salazinic acid, consalazinic acid, and norstictic acid, all of which have exhibited *in vitro* antioxidant abilities to some degree[54, 58, 59, 61, 62].

In vivo research into lichen metabolite antioxidant effects has also been undertaken, though the field has not yet resulted in an abundance of findings.

The literature on lichen antioxidants stretches both wide and deep, but some variance in lichen species and compounds is present. These variances in the literature could be due to the antioxidant activity not being the result of single metabolites, but due to synergistic effects of multiple metabolites. This is also exemplified by crude extract evaluations showing more favourable antioxidant results than those of separated extracts and identified, purified compounds.

2.4.3 Anticancer

The realm of anticancer research is an expansive one, with many different target approaches to stopping the disease. Lichen metabolites are one small part of this large research endeavour, as their metabolites have begun to show some promise as potential anticancer agents. The ultimate goal of lichen-derived anticancer research is to discover a

compound which presents differential effects against cancerous cells, inhibiting their growth, while not affecting the growth of normal, non-cancerous cells. The target of these compounds is also a mechanism where apoptosis is induced, rather than necrosis of the cell. Selective apoptotic potential would lead to the compounds having an effect on cancerous cells, but not affecting regular cell growth.

This research into lichens has ramped up within the last 15 years, leading to a plethora of literature reports available on the anticancer effects of crude extracts [27, 34, 46, 63-66]. A review on specific lichen species to have been evaluated is not the focus of this thesis, so an exhaustive investigation will not be completed, though a multitude of publications are available which cover countless species. Further background research on the anticancer effects of the individual lichens investigated can be found below, in section 2.5, Lichen Species Investigated.

Of more interest are the specific compounds from various lichens which have been assessed for anticancer activity. Particularly, the methods used for evaluation and the types of compounds which have bioactivity are of interest. This includes compounds such as usnic acid, protolichesterinic acid, atranorin, and physodic acid[67, 68].

Further investigation into lichen metabolites has not been extended greatly into the realm of *in vivo* work, though some experiments have been performed. One particular investigation found that lichen extracts did not cause toxic effects in mice, and did exhibit the ability to reduce tumour sizes in mice [69].

2.4.4 Antibacterial

Antibacterial activity is often cited as one of the most thoroughly investigated lichen bioactivities, owing to the large number of compounds, which appear to have antibacterial qualities. These compounds have been found to be effective against both gram-positive (G+) and gram-negative (G-) bacteria. The current spread of bacterial resistance, combined with the decreased funding towards novel antibiotic discovery, has led to a void in effective antibiotics coming in to play in modern healthcare.

The most prominent and thoroughly researched antibacterial is usnic acid [38, 70-72]. The compound's efficacy against G+ bacteria has been thoroughly investigated, and it has been incorporated as a natural product topical antibiotic agent in some countries [73].

2.5 Lichen Species Investigated

2.5.1 Xanthoparmelia mexicana

Xanthoparmelia mexicana is a foliose lichen which is commonly found throughout the western United States of America, Canada, and Mexico. It has been previously evaluated for some bioactivity, with data showing that it contains a large concentration of usnic acid[34].

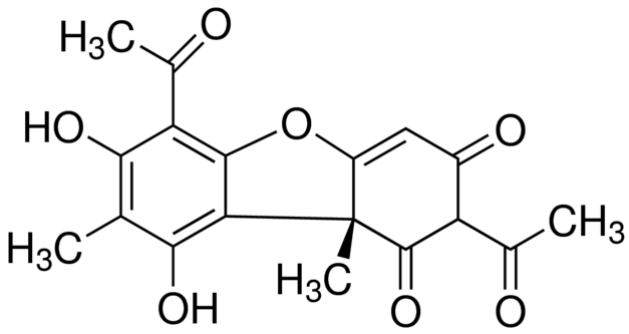

Figure 2-10: Structure of usnic acid[74].

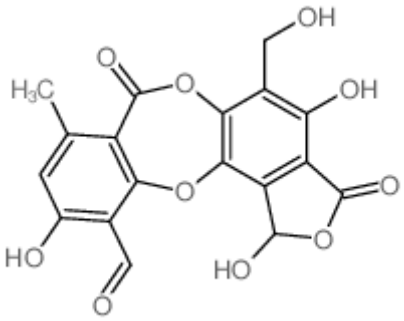

Figure 2-11: Structure of salazinic acid[75].

Usnic acid has been shown to possess anticancer abilities and antibacterial abilities[74]. Another abundant *Xanthoparmelia mexicana* metabolite, a depside called salazinic acid, has also shown antibacterial activity against both gram-positive and gram-negative bacteria[34, 75].

2.5.2 Lobothallia alphoplaca

Lobothallia alphoplaca, a placodioid, areolate lichen species found throughout the USA and Canada, has three main metabolic constituents: salazinic acid, norstictic acid and constictic acid. Norstictic acid has previously been identified as possessing antimicrobial activity when extracted from *Ramalina farinacea, Toninia candida,* and *Usnea barbata* lichens [76-78]. Constictic acid has also exhibited antimicrobial and anticancer abilities, as has

salazinic acid [75]. The activities of these compounds, and the hopes for similar compounds within *Lobothallia alphoplaca* encouraged investigation into it's bioactivities.

2.5.3 Umbilicaria muhlenbergii

Umbilicaria muhlenbergii, a foliose lichen common throughout central and Eastern North America, is the third lichen subject of investigation. It is abundant throughout North America, and has been noted as a source of food throughout history, by both Europeans and Aboriginals in North America. It has been noted that the Cree of Manitoba prepared it by mixing flakes of lichen into fish broth, and eating the soup was considered a good meal for one that had a sick stomach [52]. In Chipewyan of Saskatchewan culture, the flakes were burned slightly in a frying pan, mashed, then boiled down to make a syrup which was used to expel tapeworms from the body [79]. The Thcho of the Northwest Territories used the lichen to produce a soup, which was then eaten as a tonic and as a treatment for breathing problems [80].

Other species within the genus *Umbilicaria* were also used for traditional purposes. The Dena'ina of Alaska and the Inuit of Northern Quebec boiled *Umbilicaria* lichen and used it as a drink to treat tuberculosis and prolonged bleeding, while the Ekuanitshit of Quebec made a tea with *Umbilicaria* sp. and used it to treat urinary proplems [80] It is eaten around the world as well, with some cultures treating it as a delicacy.

Though quite common, easy to collect, and noted for medicinal and food uses, *Umbilicaria muhlenbergii* lacks a comprehensive bioactivity assessment. It has been studied for decades, but with an emphasis on other aspects of its biology. Its most-studied attribute

pertains to its ability to bind heavy metal compounds, in addition to studies on lichen primary metabolism [81-84]. This predictable heavy metal incorporation behaviour has resulted in *Umbilicaria muhlenbergii* being utilized as a bioindicator organism of sorts, especially in regions where mining is prevalent [83, 85-88]. Common metals investigated include mercury, nickel, cesium, copper, silver, and lead [83, 85, 86, 89, 90]. It has also been used as a target organism in lichen species distribution studies, specifically those where the distribution of multiple lichen species in one area is of interest [91]. These investigations were undertaken in the late 1970's to late 1990's, with little research being put into *Umbilicaria muhlenbergii* since that time.

The precedent set by its ethnobotanical uses, in addition to the lack of assessment into its bioactivities, make *Umbilicaria muhlenbergii* an enticing and promising prospect for study. Its anticancer and antimicrobial properties will be investigated, filling in a gap in the our knowledge.

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Previous investigation into North American lichens used for this publication had already begun by Erik Yeash, a graduate student under the supervision of Dr. Lew P. Christopher at the North Dakota School of Mines and Technology. Upon starting as the director of the Biorefining Research Institute in Thunder Bay, ON, Dr. Christopher resumed investigation of the bioactivities of North American lichens. For this publication, I was responsible for the acetone extraction of the lichens, MTT testing of the lichen crude extracts against MCF-7 breast cancer cells, and the MIC experiments where the lichen extracts were used against several species of bacteria.

3 Biological activities of undescribed North American lichen species

3.1 Running Title: *Biological Activities of Lichen Extracts*

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3.4 Abstract

BACKGROUND: Lichens provide a large array of compounds with the potential for pharmaceutical development. In the present study, extracts from three previously undescribed North American lichen species were examined for antioxidant, antibacterial and anticancer activities.

RESULTS: The results from this study demonstrated the following: 1) *Acarospora socialis* ethanol extract exhibited significant DPPH antioxidant scavenging activities of up to 88.6%; the antioxidant activities were concentration-dependent; 2) the acetone and ethyl acetate extracts of *Xanthoparmelia mexicana* inhibited Gram-positive bacteria with a zone of inhibition of up to 21 mm; the *X. mexicana* acetone extract yielded a minimum inhibitory concentration (MIC) of 20.9 ug mL^{-1} against *Staphylococcus aureus*, and 41.9 ug mL^{-1} against *Enterococcus faecalis*; 3) the acetone extract of *Lobothallia alphoplaca* inhibited growth of cultured breast cancer MCF-7 cells at a relatively low effective concentration (EC_{50}) of 87 µg mL^{-1}; the MCF-7 cell cycle appears arrested in the G2 phase whereas the DNA synthesis cell cycle (S) may be inhibited.

CONCLUSION: New lichen species that possess strong biological activities have been characterized. Potential exists to develop new, potent and non-toxic antioxidant, antimicrobial and anticancer agents from naturally-occurring lichen metabolites that can substitute for their synthetic counterparts.

3.5 Keywords: Lichens, North America, biological activities, *Acarospora socialis, Xanthoparmelia mexicana, Lobothallia alphoplaca*

3.6 Introduction

Lichens and their diverse metabolic products provide a large array of compounds with the potential for pharmaceutical development.[1] General characteristics of lichens and their historical ethnobotanical uses have been described previously.[2] However, the search for novel antibiotic compounds and for anti-cancer compounds is particularly relevant in view of the spread of antibiotic resistant bacteria and the growing need for better and improved cancer drugs, respectively.

The antioxidant, antibacterial and anti-proliferative activities of lichens have been the focus of several reports. Generally, antioxidant activity may be relevant as a mechanism for reversing cellular damage induced by metabolic dysfunction or by external factors. Radical scavenging capacity by several lichen extracts was reported recently[3, 4], as well as in older work using extracts from cultured lichen material.[5] Reports by Turkez and co-workers[6] suggest protective role of lichen extracts against specific types of oxidative cell damage.

Search for lichen-derived antibiotic compounds started in the post-WWII era[7] and continues until the present. Shrestha and St. Clair[8] tabulated some of the more recent work and additional reports have appeared since.[9-11] Unfortunately, of the hundreds of natural products investigated, only a few have reached clinical trials and none of these originated from lichens.[12] We are not aware of any current clinical work with lichen-derived

compounds. Commercial natural health products tend to be used topically, and internally consumed lichen extracts have proven toxic.[13]

Nevertheless, the toxicity of lichen extractives may yet turn out to be beneficial in relation to their potential use as anticancer drugs. Usnic acid, a common lichenic acid, is reported to have some effect against lung carcinoma in mice.[14] Much of the work on cultured neoplastic cells performed between 1979 and 2012 has been summarized.[8, 11] More recent investigations report anti-proliferative activity against a variety of cultured cell lines.[8, 10, 11, 15] To our knowledge, only two investigations included non-neoplastic cells as controls and showed a degree of selectivity (SI) of the lichen compounds against the transformed cell as compared to normal cells.[16, 17] Studies on living model animals are rarely performed or reported, but of note is the report of inhibition of tumor angiogenesis by olivetoric acid.[18]

The objective of this study was to investigate the *in vitro* biological effects of lichens, with the expectation that further work will be possible to characterize the chemical nature of the active constituents and elucidate in some detail their mechanisms of action. Three lichen species occurring in Western United States, that have not been studied to date, were selected for investigation: *Acarospora socialis, Xanthoparmelia mexicana* and *Lobothallia alphoplaca*. The biological activity of these lichens was assessed by determining their antioxidant, antibacterial and anti-proliferative activities.

3.7 Experimental

Lichens

Two of the lichens, *Acarospora socialis* and *Lobothallia alphoplaca*, were obtained from Joshua Tree National Park in California whereas *Xanthoparmelia mexicana* was collected on Catalina Island, 22 miles south-west of Los Angeles, California. The images of the lichen species are depicted in Fig. 1. These species were collected and identified by lichenologist Kerry Knudsen, and voucher specimens deposited in the herbarium of University of California at Riverside, under accession numbers 15106.1, 15119.1, and 15436, respectively. The collected material was kept until needed in sealable plastic bags at room temperature.

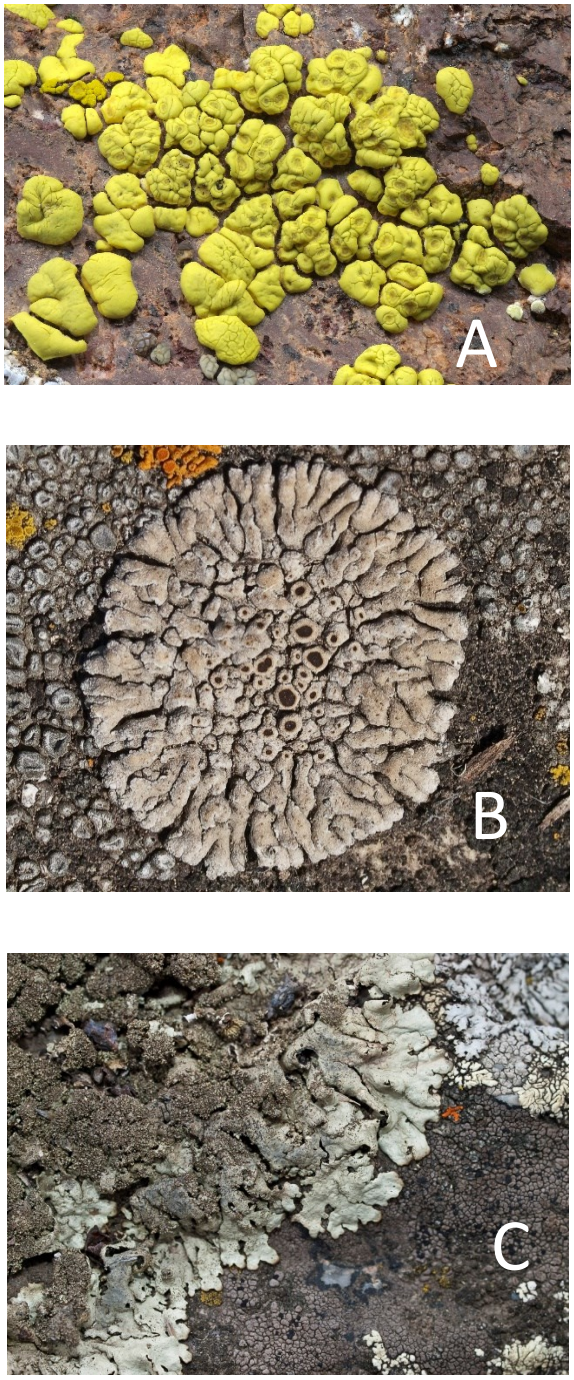

Figure 3-1: Lichens investigated: (A) Acarospora socialis (Tim Wheeler); (B) Lobothallia alphoplaca (Doug Waylett), (C) Xanthoparmelia mexicana (Doug Waylett).

Chemicals and instruments

Extraction solvents, bacterial media, dimethyl sulfoxide (DMSO), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), and CellTiter-Blue® were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Materials for cell viability staining including 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO, USA). The instruments utilized in this study included a Biotek PowerWaveXS microplate spectrophotometer (Biotek Instruments Inc., Winooski VT, USA), a BD ACCURI C6 flow cytometer (BD Biosciences, San Jose, CA, USA), and a BMG FLUOstar Optima Microplate Reader (BMG Labtech, Offenburg, Germany).

Preparation of lichen extracts

Lichens were pulverized in mortar and pestle, the lichen powder was stirred with 100 mg mL^{-1} solvent (99.5% ethanol, acetone or ethyl acetate) at 120 rpm and 37^{o}C for 24 h in an Innova© R42 incubator shaker (New Brunswick, Connecticut, USA), and thereafter filtered through Whatman #1 filter paper. The extraction yield was calculated by subtracting the mass of the filtered solids from the original mass of ground lichen.

Antioxidant activity

The procedures of Sharma and Bhat (2009) and Rankovic *et al.* (2011) were followed [19, 20] to determine the free radical scavenging activity of lichen extracts. A 1 mM solution of

DPPH was prepared (in the same solvent as extract), 1 mL of the DPPH solution was mixed with 25 uL of solvent or extract and absorbency at 517 nm (A_{517}) read in Shimadzu UV-2450 spectrophotometer (Shimadzu Scientific, Maryland, USA) at 30 and 60 min. Results from three independent and parallel experiments were expressed as % absorbance compared to solvent only. The DPPH radical concentration was calculated using the following equation:

$$\text{Antioxidant (DPPH-scavenging) activity (\%)} = [(A0 - A1)/A0] \times 100$$

where $A0$ is the absorbance of the negative control and $A1$ is the absorbance of the reaction mixture or standard.

Anticancer activity

Cell culture

Human breast cancer MCF-7 cells (ATCC HTB-22) were purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA) and maintained in supplemented Dulbecco's Modified Eagle Medium (DMEM). The DMEM was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cell line was incubated at 37°C with a humidified 5% CO_2 atmosphere.

Anticancer activity assay

MCF-7 cells were seeded in a 96-well tissue culture microplate at a density of 10,000 cells per well, then incubated at 37°C under 5% CO_2 for 24 h. Next, the lichen extract was concentrated 5 times to a final metabolite concentration of 17.25 mg mL^{-1} in order to

reduce the amount of acetone used in the treatment. Upon the MCF-7 cells reaching confluency, the medium was removed from the wells and 200 µL of lichen extract diluted in non-supplemented DMEM was added in triplicate. The concentration of lichen metabolites in the treatments ranged from 5.39 µg mL^{-1} to 517.5 µg mL^{-1}. Note that the lichen metabolite concentration must be expressed as a value of µg mL^{-1}, as the active compounds have not yet been isolated and identified. For clarity, the concentration of the positive control, cis-diammineplatinum(II) dichloride (cisplatin) is given in both molar concentration and mass per volume concentration. Appropriate negatives and controls were also added. The positive control, cis-diammineplatium(II) dichloride (cisplatin), was prepared as a 198 ug mL^{-1} (0.66 mM) stock solution in sterile 0.9% NaCl solution. It was serially diluted from 0.6 µg mL^{-1} (2 µM) to 24 µg mL^{-1} (80 µM) for treatment conditions. The negative control was an untreated control group of MCF-7 cells which were grown in non-supplemented DMEM. A 12 mM solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) in phosphate buffered saline was prepared, filter sterilized, and stored in the dark at 4°C. The plate was incubated at 37°C and 5% CO_2 atmosphere for 44 h, followed by the addition of 20 µL of MTT solution to each well. Next, the plates were incubated at 37°C and 5% CO_2 for 4 h. The MTT solution was then removed from each well and replaced with 50 µL of dimethyl sulfoxide (DMSO). The plates were covered in foil and mixed on a Belly Dancer for 10 min. The absorbance was then measured at 490 nm and 650 nm with a BioTek Powerwave XS spectrophotometer. To determine cell survival, the absorbance of the samples, controls, and negatives were measured and compared to baseline and negative control. All experiments were run in triplicate.

Cell culture treatment and analysis

Cell cycle analysis was performed in triplicate by treating MCF-7 cells with 5 µL of extract per 200 µL media, incubating for 48 h, trypsinizing and collecting them, then fixing the cells with 70% ethanol. The fixed cells were then stained with propidium iodide (50 µg mL^{-1}) for 30 min at room temperature. The cell cycle analysis was performed on a BD ACCURI C6 flow cytometer and was used to determine the frequency of cells which had their cell cycles in the G1, S and G2 phases.

Antibacterial activity

Microorganisms and media

Bacteria used as test organisms in this study included both Gram-positive (*Streptococcus pyogenes, Staphylococcus aureus, Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis*). All bacteria were acquired from ATCC and stored at -80^{o}C as a solution comprised of 50% glycerol and 50% cell suspension in Luria-Bertani (LB) broth.

When taken out of cryostorage, one loop of bacteria was streak plated on Mueller Hinton II (MHII) agar and incubated at 37^{o}C until individual colonies were sighted. Next, one colony was taken from the plate and used to inoculate 10 mL of MHII broth. The broth culture was incubated at 37^{o}C until it reached a turbidity level which was equal to that of a 0.5 McFarland Turbidity standard (approximately 1.5 x 10^{8} CFU mL^{-1}).

Zone of inhibition

Initial tests as reported in Table 1 were carried out by agar disk diffusion tests, also commonly referred to as the Kirby-Bauer susceptibility procedure.[21] The plates used were LB agar plates inoculated with bacterial isolates via cotton swab, which covered the entire agar surface with bacteria. The disks were sterile 6 mm sizes punches of Whatman® No. 1 filter paper, which was then loaded with lichen sample. Dry disks were prepared by pipetting 5 µL of 100 mg mL^{-1} extract (500 µg of lichen metabolites dissolved in 5 µL solvent) onto each disk, then the disks were dried until all the solvent evaporated, then they were placed on LB agar plates. The wet disks were prepared by placing sterile disks on the agar plates, then 5 µL of 100 mg mL^{-1} extract (500 µg of lichen metabolites dissolved in 5 µL solvent) was pipetted onto the disk. The plates were then immediately incubated at 37°C for 24 h. After incubation, the zone of inhibition was determined by measuring the diameter of the growth-inhibited area with a ruler. Appropriate solvent control disks were evaluated as well. All experiments were performed in triplicate. The sensitivity of microorganisms to the acetone extracts of the investigated lichen species was tested by determining the minimal inhibitory concentration (MIC).

Minimum Inhibitory Concentration (MIC)

The MIC was determined in triplicate as follows: an 18-h broth culture (37°C and 150 rpm) was diluted to 10 x 10^6 CFU mL^{-1} and 100 µL added to microplate, together with lichen extract dilutions (from .86 µg/mL to 860 µg mL^{-1}) and appropriate solvent and negative controls. Baseline absorbances at 600 nm were measured using BioTek PowerwaveXS

spectrophotometer, followed by measuring the same plates incubated at 37°C for 24 h. At 24 h, 40 µL of CellTiter-Blue® was added to each well of the microplate. The plates were incubated for 4 h at 37°C, then fluorescence was measured at 544 nm excitation/590 nm emission. To ensure accuracy of the spectrophotometer absorbance and fluorescence readings, 10 µL of each treatment condition were streaked onto LB agar plates, then incubated at 37°C for 24 h. After 24 h, the growth on each plate was noted.

Statistical analyses

Statistical analyses were performed with the EXCEL, GraphPad Prism 6, and FlowJo software packages. To determine the statistical significance of antioxidant activity, Student's *t* test was used. All values were expressed as mean ± standard deviation (SD) of three parallel measurements. To determine statistical significance of cell death, a two-sample t-test assuming unequal variance was used.

3.8 Results and Discussion

Antioxidant activity

The antioxidant properties of both *A. socialis* ethanolic and methanolic extracts were more effective than any other extracts examined in this study (Fig. 2). Furthermore, the *A. socialis* ethanolic and methanolic extracts showed higher DPPH free radical scavenging activities (88.6% and 80.6%, respectively) than the 1 mM trolox positive control (58.1%).

There was a statistically significant difference between these extracts and the trolox control ($P < 0.05$). The *X. mexicana* acetone extract exhibited half the DPPH free radical scavenging activity of 1 mM trolox treatment, while the *L. alphoplaca* extracts did not show any significant antioxidant effects (Fig. 3-2). The antioxidant activities were concentration-dependent and generally increased with the increase in the extract concentrations applied, although the increase did not follow any particular pattern and was dependent on the type of solvent and lichen used (data not shown).

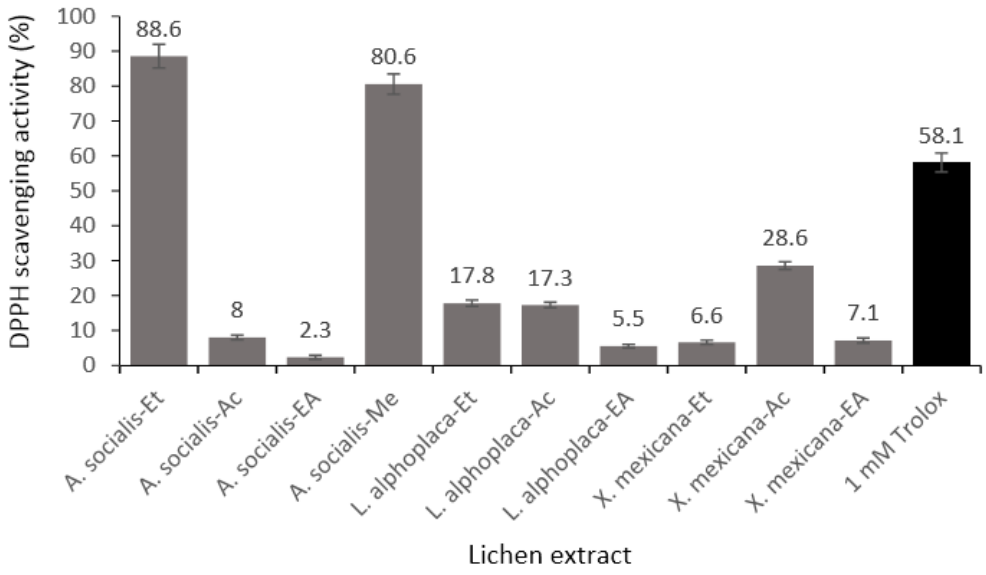

Figure 3-2: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability of lichen extracts. Et, ethanol; Ac, acetone; EA- ethyl acetate; Met- methanol; Trolox, positive control.

Many compounds with marked antioxidant capabilities have been isolated from lichen species. The activities of these compounds have proven to be comparable to those of common antioxidant controls. Some examples of such compounds are sekikaic acid

(depside), lobaric acid (depsidone) and lecanoric acid (depside)[22]. These compounds exhibit superoxide radical scavenging abilities very similar to that of a common antioxidant food additive, propyl gallate.[22] Another study found that 8'-methylmenegazziaic acid and antranorin, both lichen-derived compounds, have antioxidant activities which are comparable in activity to a Trolox® standard.[23] A lichen-derived bisnaphthoquinone, cuculloquinone, was found by another investigation to have 80% DPPH inactivation, which was twofold more effective than the butylated hydroxytoluene (BHT) standard it was compared to.[24] As evident from Fig. 1, the *Acarospora* ethanolic extracts exhibited high antioxidant potential. The other two lichen extracts tested, *Lobothallia alphoplaca* and *Xanthoparmelia mexicana*, did not exhibit statistically significant antioxidant abilities (data not shown). The results from our study and those of others[25] have shown that naturally-occurring lichen metabolites produce *in vitro* antioxidant effects similar to or exceeding those of common synthetic antioxidant compounds. The impetus of natural antioxidant research is to find novel and effective antioxidant compounds which do not exhibit carcinogenic effects, as some of the synthetic antioxidants do[25].

Antibacterial activity

The antibacterial activity of the lichen extracts against Gram-negative and Gram-positive bacteria was evaluated using the disk diffusion method. None of the lichen extracts were active against Gram-negative bacteria (data not shown). In addition, the acetone and ethyl acetate extracts of *X. mexicana* were the only two extracts which showed antibiotic activity on Gram-positive bacteria (Table 1). All *Lobothallia alphoplaca* and *Acarospora socialis* extracts did not have significant antibacterial activity (data not shown). Moreover,

the solvent control disks did not show any significant zones of inhibition (data not shown). Hence, there was a statistically significant difference between the extracts and the solvent control ($P < 0.05$). Further investigation into the *X. mexicana* acetone extract using the microdilution assay yielded a MIC of 20.9 ug mL^{-1} against *S. aureus*, and 41.9 ug mL^{-1} against *E. faecalis* (data not shown). MIC tests performed with acetone controls showed no statistically significant effect on bacterial viability ($P < 0.05$). The fact that the *X. mexicana* extract lacked any activity against Gram-negative bacteria points towards a possible antibiotic mechanism involving the peptidoglycan cell wall of Gram-positive bacteria.

Table 1: Zone of Inhibition (mm) by *X. mexicana* extracts

Solvent	**Method[a]**	***S. aureus* (G+)**	***S. pyogenes* (G+)**	***E. faecalis* (G+)**
Acetone	Dry	11 ± 0.5	18 ± 1.0	11 ± 0.5
Acetone	Wet	15 ± 1.0	20 ± 1.5	13 ± 1.0
Ethyl acetate	Dry	12 ± 0.5	19 ± 2.0	11 ± 1.0
Ethyl acetate	Wet	15 ± 1.0	21 ± 1.5	14 ± 1.5
[a]Wet, disk placed on agar before extract treatment; Dry, disk soaked in lichen extract, dried, then placed on agar.				

The search for novel antibiotic compounds is critical[26], given the spread of antibiotic resistance, particularly among bacteria acquired in hospitals.[27] Screening for natural compounds in unusual sources such as lichens may provide new leads. *Xanthoparmelia mexicana* showed promise for further investigation of specific components of the crude extract. Examination of related species (*Xanthoparmelia conspersa* and *Xanthoparmelia stenophylla*) was not found to inhibit growth of pathogenic fungi.[28] However, usnic acid extracted from *Xanthoparmelia farinosa* appeared to have some biological activity.[29] While numerous reports of potential antibiotic activities from lichens abound[9, 10], the general

paucity of lichen antibiotic therapeutics may be related to lichen compound toxicities[13] and this may argue for only topical use of lichen preparations.

Anticancer activity

The anticancer activity of acetone extract from *L. alphoplaca* against MCF-7 cells showed promising results (Fig. 3), though the extracts of *Acarospora socialis* and *Xanthoparmelia mexicana* did not show significant anticancer activity (data not shown). The effective concentration where 50% of cells are killed (EC_{50}) for *Lobothallia alphoplaca* was recorded at a lichen metabolite concentration of 87 µg mL^{-1},while the cisplatin positive control had an EC_{50} of 5 µg mL^{-1} (Fig. 3). This led to the conclusions that there is a statistically significant difference between the lichen treatment and the cisplatin control ($P < 0.05$). Furthermore, the acetone solvent alone caused a 5% decrease in viability at the maximum concentration used (data not shown). Although the effective concentration of the acetone lichen extract was significantly higher than cisplatin, the similarities in the kinetics are evidence to suggest that the lichen extract may contain constituent(s) that acts via a similar mechanism of cisplatin. Currently, we have undertaken experiments to isolate the active compound(s) from the lichen acetone extract which might provide us with a single compound of marked potency comparable to that of cisplatin. In addition, the observation of anti-proliferative activity of the *L. alphoplaca* extract (Fig. 3) encouraged us to undertake further investigations, currently in progress, on the toxicity of fractionated material of known concentration, not only against MCF-7 cells, but other commonly used transformed

cell lines, as well as against primary cells. The latter approach is essential for the ultimate development of any lichen-derived compound to be used therapeutically. Toxin differential sensitivity between primary and neoplastic cells is paramount, yet the degree of selectivity is rarely investigated with lichen extracts.[16, 17] Even if the *L. alphoplaca* active ingredients do not show a high degree of selectivity in future work, topical applications against epithelial cancers may prove useful, since general toxicity at the body surface may not be as critical. Preliminary evidence showed that MCF-7 cell cycle is arrested in the G2 phase, and that DNA synthesis may also be inhibited by the acetone extracts of *L. alphoplaca* (Table 2). Investigations are underway to identify specific active compounds in the *L. alphoplaca* extracts which inhibited neoplastic MCF-7 cells and determine the selectivity range against primary cells and a wider variety of cultured cancer cells that could lead to new and improved anti-cancer compounds.

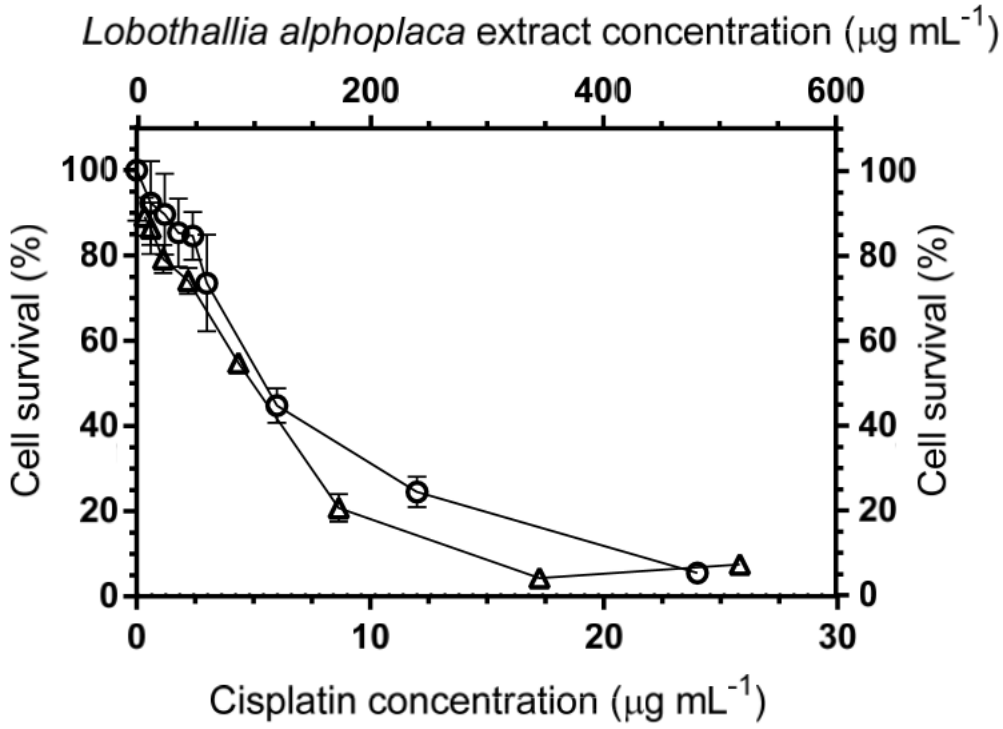

Figure 3-3: MCF-7 cell sensitivity to cisplatin (open circles) and L. alphoplaca acetone extract (open triangles).

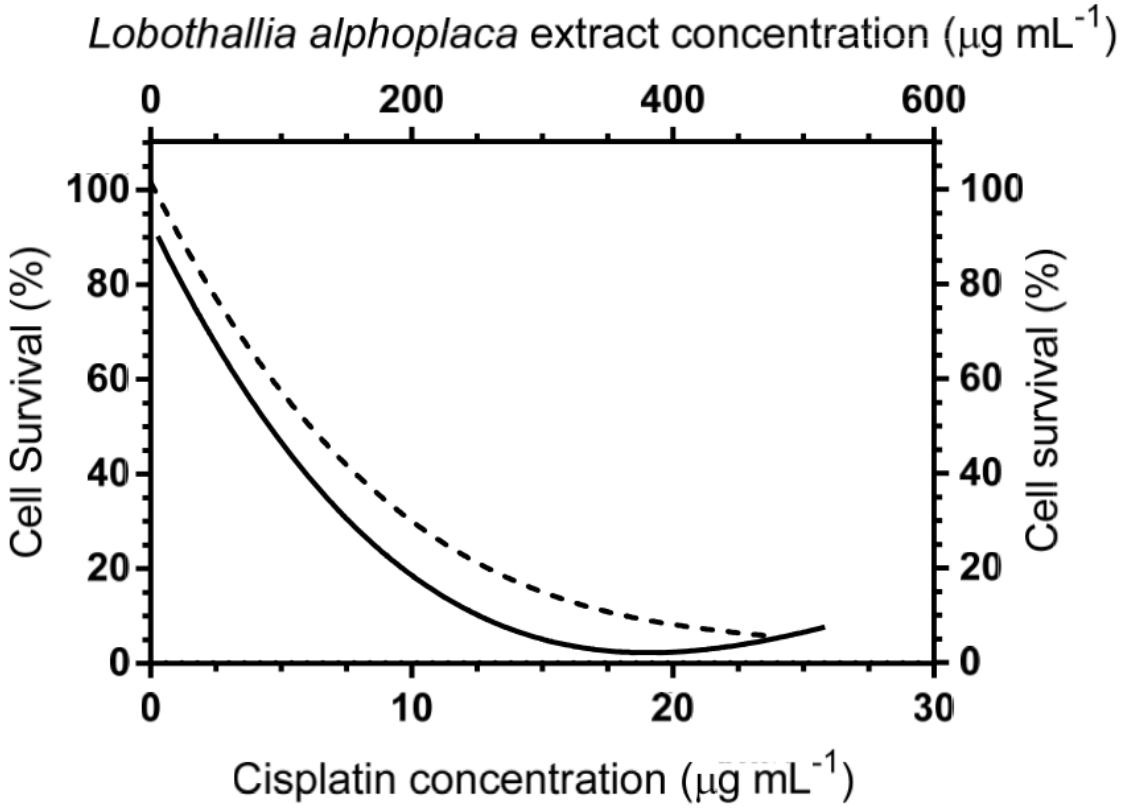

Figure 3-4: Concentration-effect curve representing MCF-7 cell sensitivity to cisplatin (dashed line) and L. alphoplaca acetone extract (solid line).

Table 2: Flow Cytometry Data for MCF-7 cells treated with *L. alphoplaca* crude acetone extracts.

Phase	Acetone control	Acetone extract
G1	65.72 ± 1.07	52.51 ± 0.96
S	14.01 ± 0.51	5.40 ± 0.34
G2	22.33 ± 1.95	48.58 ± 2.33
[a]G1, cells in Gap 1 phase of the cell cycle; S, cells in the Synthesis phase of the cell cycle; G2, cells in the Gap 2 phase of the cell cycle.		

In conclusion, significant biological activity was noted as follows: (1) ethanol (or methanol) extracts of *A. socialis* were effective in scavenging free radicals; (2) acetone or ethyl acetate extracts of *X. mexicana* inhibited growth of three species of Gram-positive bacteria; and (3) acetone extracts of *L. alphoplaca* were effective in inhibiting the growth of cancerous MCF-7 cells. Our work extends the list of lichen species with known biological activities, and we are encouraged by the fact that lichens are progressively found to be an effective source of biochemical entities useful in therapeutics and as food additives.

3.9 Acknowledgements

We wish to acknowledge Drs. Keith Miskimins and Yongzian "Cathy" Zhuang from the Sanford Cancer Biology Research Center, Sioux Falls, SD, for their assistance with some of the initial anti-cancer research experiments; the assistance of Ms. Dan Liu from Lakehead University, Thunder Bay, ON with the anti-oxidant assays; the Center for Bioprocessing Research and Development and Biomedical Engineering Program at the South Dakota School of Mines and Technology, Rapid City, SD as well as the Biorefining Research Institute and Northern Ontario School of Medicine at Lakehead University, for the financial support. The work of Kerry Knudsen was financially supported by grant 42900/1312/3166 ''Environmental aspects of sustainable development of society'' from the Faculty of Environmental Sciences, Czech University of Life Sciences, Prague.

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The investigation into the three North American lichens encouraged the investigator to delve into the metabolite profile and bioactivity of other lichens, notably those of Northern Ontario. This led to the collection and analysis of a Northwestern Ontario lichen, *Umbilicaria muhlenbergii*, which has not yet been fully chemically characterized nor had a bioactivity evaluation completed. Initial investigation yielded promising results, leading to a broader investigation into its metabolites' bioactivities and identities. The following manuscript, intended for publication within the Journal of the Science of Food and Agriculture, outlines the process and results of the *Umbilicaria muhlenbergii* bioactivity investigation.

4 Extraction, separation, and identification of bioactive compounds from a *Umbilicaria muhlenbergii*, a Northern Ontario lichen

4.1 Running Title: *Bioactivity of Umbilicaria muhlenbergii*

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4.3 Abstract:

BACKGROUND: The world of bioprospecting is constantly searching for novel sources of bioactive compounds. Lichens are emerging as a promising source of these compounds. In the present study, metabolites from a chemically uncharacterized Northern Ontario lichen were extracted, separated via silica gel column chromatography and Sephadex LH-20 column chromatography, tested for anticancer and antibiotic activity, and identified.

RESULTS: This study found significant results in the areas of anti-cancer and antibiotic activities of *Umbilicaria muhlenbergii*. The crude extract was evaluated for bioactivity, then separated via silica gel 60 column chromatography. Two of these fractions were then separated further by Sephadex LH-20 column chromatography. The anticancer results are as follows: 1) *Umbilicaria muhlenbergii* acetone crude lichen extract exhibited an IC_{50} point of 13.3 µg mL^{-1}, silica gel 60 separated fractions #1-6 of 8 exhibited anticancer activity against MCF-7 cells, and Sephadex LH-20 separated fractions produced multiple bioactive fractions. Two of the silica gel 60 (SG 60) fractions cause apparent cell cycle arrest in MCF-7 cells at the G2 phase. As for the

antibiotic activity of *Umbilicaria muhlenbergii,* the crude extract exhibited an MIC of 22.5 µg/mL against *Staphylococcus aureus*, SG 60 fractions did not have statistically significant results, and Sephadex LH-20 separated fractions exhibited MIC points against *Staphylococcus aureus*. No statistically significant results were found with MICs against *E. faecalis* and *P. aeruginosa*.

CONCLUSION: *Umbilicaria muhlenbergii*, has shown marked bioactivities after acetone extraction, silica gel 60, and Sephadex LH-20 separation of constituent compounds. The potential now exists to further investigate the identity of these compounds and further study their anticancer and antibiotic effects.

4.4 Keywords: *Umbilicaria muhlenbergii*, anticancer, antibiotic, separation, extract

4.5 Introduction

The research area of bioprospecting has always had the goal of sustainably and responsibly discovering useful and novel compounds from natural sources [1, 2]. One area of research interest is that of lichens, organisms which produce a staggering array of potentially bioactive compounds [3]. There have been approximately 1050 lichen secondary metabolites identified, with over 350 of them exhibiting bioactivity [4, 5]. Interestingly, out of these 650 compounds produced by the fungal component of lichens, only approximately 60 of them are also produced by free living fungi and higher plants [6]. This indicates an ability of lichens to dedicate a greater amount of energy and resources towards secondary metabolite production[5, 6].

The historical and ethnobotanical significance, physiology, and ecological characteristics of lichens have been previously investigated, but the search for bioactive compounds in lichens has not yet resulted in the identification of useful pharmaceuticals [7-9]. The ever-pressing need for new solutions to the problem of antibiotic resistance, in addition to the persistent search for effective anticancer compounds, has led to the investigation of these abundant lichen secondary metabolites for pharmaceutically relevant compounds.

The lichen species *Umbilicaria muhlenbergii (*Ach.) Tuck (formerly *Actinogyra muhlenbergii*), also known as lesser rocktripe or plated rocktripe, is a species of foliose lichen native to much of the Great Lakes region and the East Coast of North America (Figure 4-1). Preliminary work has shown promising results, warranting further inquiry. Previous work researching the bioactivity of several species within the genus *Umbilicaria* has been completed*,* with a handful of compounds being identified as responsible for bioactivity [10-12]. These include umbilicaric acid, gyrophoric acid, and lecanoric acid [13]. Though these lichenic acids tend to be ubiquitously present in *Umbilicaria* species, their exact concentrations and functional groups vary [13]. The species of focus in this investigation, *Umbilicaria muhlenbergii*, has been analyzed as a bio-indicator species quite adept at uptake of heavy metals, but has not been the target of investigation for bioactivity nor specific identification of its secondary metabolites [14-18]. This gap in the literature the species and geographical level leaves a niche to be investigated.

Figure 4-1: *Umbilicaria muhlenbergii* lichen.

The objective of this study was to probe into the *in vitro* bioactivity of *Umbilicaria muhlenbergii* extracts, with the intent of separating and possibly identifying the compound(s) responsible for *Umbilicaria muhlenbergii*'s anticancer and antibiotic properties. It is the authors' expectation that the results garnered by this investigation could lead to further inquiry into the specific compound's mechanisms of action and further development into useful therapeutic compounds.

4.6 Experimental

4.6.1 Lichen Investigated

In this study, the North American lichen *Umbilicaria muhlenbergii* (Ach) Tuck was the target organism (Figure 4-1). It was obtained from Onion Lake Dam Road, within the municipal jurisdiction of Thunder Bay, Ontario, Canada. The lichen was collected and

identified by Dr. Lada Malek of Lakehead University, and a voucher specimen was deposited into the Claude Garton Herbarium at Lakehead University. The collected lichen was stored in sealed 1 L jars at room temperature, in a cabinet without exposure to light, until the time of extraction.

4.6.2 Chemicals and Instruments

Acetone, bacterial media, McFarland turbidity standards, mammalian cell culture media, fetal bovine serum (FBS), antibiotic-antimycotic (anti-anti), dimethyl sulfoxide (DMSO), silica gel 60 (SG60) and resazurin CellTiter-Blue® were purchased from Thermo Fischer Scientific Canada (Whitby, ON, Canada). Sephadex LH-20, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), propidium iodide, hexane, ethyl acetate, methanol, and acetonitrile were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). The instruments utilized in this investigation included a Biotek PowerWave XS microplate spectrophotometer (Biotek Instruments, Winooski VT, USA), a BD FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA), a BioLogic LP chromatography system (Bio-Rad Laboratories INC, Hercules, CA, USA), a BioFrac Fraction Collector (Bio-Rad Laboratories INC, Hercules, CA, USA) and a BMG FLUOstar Optima Microplate Reader (BMG Labtech, Offenburg, Germany).

4.6.3 Lichen metabolite extraction

The lichen was pulverized in mortar and pestle, the lichen powder was shaken with 10 g of lichen powder in 100 mL of solvent (acetone) at 120 rpm and 37^{o}C for 24 h in an Innova© R42 incubator shaker (New Brunswick, Connecticut, USA), and thereafter filtered through Whatman #1 filter paper. The extraction yield was calculated by subtracting the

mass of the filtered solids from the original mass of ground lichen. After yield calculation, the extract was concentrated until it reached a set concentration of 10 mg mL^{-1}.

4.6.4 Silica gel 60 separation

4.6.4.1 Column chromatography separation

A 600 mL, large-diameter glass column was chosen for the crude separation. First, 250 mL of silica gel 60 (SG60), (Thermo Fischer Scientific Canda) was measured and poured into a 1 L beaker. Next, 1.5x volume of acetone (375 mL) was added to the 250 mL of SG60. This was thoroughly mixed via gentle stirring with a glass rod. Next, 100 mL of acetone was added to the column with the petcock closed. The mixed SG60 slurry was then carefully poured down a glass rod into the column. A further 200 mL of acetone was then added to the column, the petcock was opened, allowing the acetone to flow through the column to pack it. Once the acetone level reached 1 cm from the top of the column, the petcock was closed. The crude extract, at a concentration of 10 mg mL^{-1}, was mixed with 50 mL of SG60 in a mortar, and mixed with a pestle until well mixed and uniform in colour. Next, the *Umbilicaria muhlenbergii* crude extract slurry was added to the column, again by gently pouring along a glass rod into the column. Once the column was loaded, solvents of increasing polarity were poured through the column and allowed to elute via force of gravity. The solvents, in order of elution, are found in Table 3: Fraction number and solvents used for SG60 column elution..

Table 3: Fraction number and solvents used for SG60 column elution.

Fraction Number	Solvent
1	100% Hexane
2	50% hexane, 50% ethyl acetate
3	100% ethyl acetate
4	100% acetone
5	50% ethyl acetate, 50% methanol
6	100% methanol
7	90% methanol, 10% dH_2O
8	100% dH_2O

First, 200 mL of hexane was measured and gently poured into the column. The petcock was opened, allowing the eluent to flow through the column via force of gravity. A labelled screw-top flask was placed under the petcock to collect the eluent. Once the solvent level reached 1 cm above the silica gel, a new screw-top flask was placed under the petcock and 200 mL of 50% hexane, 50% ethyl acetate was added to the column. The same procedure was repeated for all eight of the solvent mixtures used.

4.6.4.2 Concentration of silica gel 60 fractions

After collection, all eight collected silica gel 60 fractions were concentrated via rotary evaporator. First, eight scintillation vials were labelled, weighed, and had their weights recorded. Next, the volume of fraction 1 was loaded into a round bottom flask and attached to the rotary evaporator (BUCHI Corp, New Castle, DE, USA). The vacuum was turned, the flask was set to spin at a medium speed, and the flask was lowered into a water bath set at 45 C. The fraction was evaporated until it had a volume of 5-10 mL, after which the

concentrated fraction was removed from the flask and pipetted into a scintillation vial via Pasteur pipette. The scintillation vial was opened and placed in a fume hood to allow the fraction to fully dry. The round bottom flask was then cleaned with the appropriate solvents, and the process was repeated with the next fraction.

4.6.4.3 Resuspension of SG60 fractions at set concentration

After all fractions had been dried fully, each scintillation vial was weighed and the mass of metabolites present calculated. Each vial was then resuspended to give a concentration of 10 mg mL^{-1}. For fractions 1 and 2, DMSO was used for resuspension. For samples 3 and 4, acetonitrile was used. For fractions 5, 6, 7, and 8, methanol was used for resuspension.

4.6.5 Sephadex LH-20 separation of fractions

SG60 fractions 5 and 6 both displayed prominent bioactivity against MCF-7 cells, along with a TLC result showing fewer compounds present (see Results), making the two fractions suitable candidates for further separation/purification. This further separation was accomplished with the use of Sephadex LH-20 column chromatography.

The column was prepared according to parameters set by the manufacturer, GE Healthcare [19]. The 34 cm long, 1.5 cm diameter chromatography column filled with 60 mL of Sephadex LH-20 swelled in MeOH was connected to a BioLogic LP solvent pump (Bio-Rad) running at a solvent flow rate of 2 mL $minute^{-1}$. The column outflow was managed by a BioFrac fraction collector (Bio-Rad) set to collect 2 mL fractions. Each fraction tube had been labelled and pre-weighed. Once the column stabilized and MeOH was flowing steadily, 5 mL

of 0.22 µm filtered SG60 fraction 5 was loaded into the column via syringe. The fraction was run until the column was clear. The column was flushed with 10% ethanol and 100% MeOH, then all steps were repeated for SG60 fraction 6.

After collection, all fractions were dried and weighed to determine the mass of metabolites present in each fraction. Fractions were then resuspended at a set concentration of 10 mg mL^{-1}, if possible. All others had 100 µL of solvent added. Fractions were then transferred to 2.0 mL microcentrifuge tubes for sealed storage at 4 C in darkness until needed for bioactivity evaluation.

4.6.6 Anticancer activity

4.6.6.1 Maintenance of cell line

Human breast cancer MCF-7 cells (ATCC HTB-22) were purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA) and maintained in supplemented Dulbecco's Modified Eagle Medium (DMEM). The DMEM was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cell line was incubated at 37°C with a humidified 5% CO_2 atmosphere.

4.6.6.2 Anticancer activity assay

MCF-7 cells were seeded in a 96-well tissue culture microplate at a density of 10,000 cells per well, then incubated at 37°C under 5% CO_2 for 24 h. Next, the lichen extract or separated fraction was concentrated to a final metabolite concentration of 10.00 mg mL^{-1} to reduce the amount of acetone used in the treatment while simultaneously standardizing the experiments. Upon the MCF-7 cells reaching confluency, the medium was removed from the

wells and 150 µL of DMEM was added to each well. Next, 150 µL of lichen extract diluted in non-supplemented DMEM was added, in triplicate, to the first column of wells. Next, a serial dilution was performed (with non-supplemented DMEM) to dilute the metabolites. The concentration of lichen metabolites in the treatments ranged from 1000 µg mL^{-1} to 3.91 µg mL^{-1}. Note that the lichen metabolite concentration must be expressed as a value of µg mL^{-1}, as the active compounds have not yet been isolated and identified, thus negating the convention of molarity.

For clarity, the concentration of the positive control, cis-diammineplatinum(II) dichloride (cisplatin) is given in both molar concentration and mass per volume concentration. The positive control, cis-diammineplatium(II) dichloride (cisplatin), was prepared as a 198 ug mL^{-1} (0.66 mM) stock solution in sterile 0.9% NaCl solution. It was serially diluted from 0.6 µg mL^{-1} (2 µM) to 24 µg mL^{-1} (80 µM) for treatment conditions. The negative control was an untreated control group of MCF-7 cells which were grown in non-supplemented DMEM. A 12 mM solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) in phosphate buffered saline was prepared, filter sterilized, and stored in the dark at 4°C. The plate was incubated at 37°C and 5% CO_2 atmosphere for 44 h, followed by the addition of 20 µL of MTT solution to each well. Next, the plates were incubated at 37°C and 5% CO_2 for 4 h. The MTT solution was then removed from each well and replaced with 50 µL of dimethyl sulfoxide (DMSO). The plates were covered in foil and mixed on a Belly Dancer for 10 min. The absorbance was then measured at 490 nm and 650 nm with a BioTek Powerwave XS spectrophotometer. To determine cell survival, the

absorbance of the samples, controls, and negatives were measured and compared to baseline and negative control. All experiments were run in triplicate.

4.6.6.3 Propidium Iodide flow cytometry

Cells were grown to confluency in a Corning T-75 cell culture flask, then 300,000 cells were seeded into a Corning Biolite 6-well multidisc tissue culture plate and allowed to grow for 24 hours. After 24 hours, cells were treated with increasing concentrations of methanol, acetone, *Umbilicaria muhlenbergii* crude extract, SG60 fractions 5 and 6, and Sephadex LH-20 separated fractions. Overall, 48 samples were analyzed. The cells were treated for 48 hours, afterwhich the following ethanol fixation and propidium iodide staining protocol was followed.

4.6.6.4 Ethanol Fixation

After 48 hours of treatment time had passed, the media was removed from each well of the 6 well treatment plates and pipetted into labelled 15 mL centrifuge tubes. Next, 500 µL of 0.1 % trypsin-0.02% EDTA solution (Sigma) was added to each well of the plates, which were then incubated for 5 minutes at 37 C. After incubation, 1.5 mL of SDMEM (Fischer Scientific) was added to each well to quench the trypsin-EDTA. The mixture of SDMEM, cells, and trypsin was then pipetted from each well into the appropriate 15 mL centrifuge tube which contained each specific wells' earlier removed media. The 15 mL centrifuge tubes were then centrifuged at 500 RPM for 5 minutes at 4 C. After centrifugation, the supernatant was aspirated from each tube and the pellet resuspended in 5 mL of PBS. This mixture was lightly vortexed, then again centrifuged with aforementioned parameters. The PBS was then aspirated, followed by the addition of 5 mL of PBS and a third

centrifuge spin. After this final PBS wash, the supernatant was removed and 5 mL of cold, -20 C ethanol was added drop-wise to each tube while the tube was being lightly vortexed. The tubes were then placed on ice for 10 minutes, then centrifuged at 500 RPM for 5 minutes at 4 C. The ethanol was then removed from each tube, and 1 mL of propidium iodide solution (Sigma) was added to each tube. The tube was then lightly mixed, then incubated in a dark cabinet for 30 minutes at room temperature. Upon flow cytometry evaluation, each tube was lightly vortexed to suspend cells, then the suspension was pipetted into a flow cytometry tube (Fischer Scientific) and analyzed.

4.6.6.5 Propidium Iodide Flow Cytometry Analysis

A BD FACScalibur system was utilized for flow cytometry analysis of the *Umbilicaria muhlenbergii* crude extract, silica gel 60 separated extract fractions, and Sephadex LH-20 separated silica gel 60 fractions. The machine was first calibrated with non-treated MCF-7 P12 cells. After calibration, each sample was run until 10,000 events were collected.

4.6.7 Antibacterial activity

4.6.7.1 Microorganisms and media

Bacteria used as test organisms in this study included both Gram-positive (*Streptococcus pyogenes, Staphylococcus aureus, Enterococcus faecalis*) and Gram-negative bacteria (*Pseudomonas aeruginosa)*. All bacteria were acquired from ATCC and stored at -80^{o}C as a solution comprised of 50% glycerol and 50% cell suspension in Luria-Bertani (LB) broth.

When taken out of cryostorage, one loop of bacteria was streak plated on Mueller Hinton II (MHII) agar and incubated at 37°C until individual colonies were sighted. Next, one colony was taken from the plate and used to inoculate 10 mL of MHII broth. The broth culture was incubated at 37°C until it reached a turbidity level which was equal to that of a 0.5 McFarland Turbidity standard (approximately 1.5 x 10^8 CFU mL^{-1}).

4.6.7.2 Minimum Inhibitory Concentration (MIC)

The MIC was determined in triplicate as follows: an 18-h broth culture (37°C and 150 rpm) was diluted to 10 x 10^6 CFU mL^{-1} and 100 µL added to microplate, together with lichen extract dilutions (from .86 µg/mL to 860 µg mL^{-1}) and appropriate solvent and negative controls. Baseline absorbances at 600 nm were measured using BioTek PowerwaveXS spectrophotometer, followed by measuring the same plates incubated at 37°C for 24 h. At 24 h, 40 µL of CellTiter-Blue® was added to each well of the microplate. The plates were incubated for 4 h at 37°C, then fluorescence was measured at 544 nm excitation/590 nm emission. To ensure accuracy of the spectrophotometer absorbance and fluorescence readings, 10 µL of each treatment condition was streaked onto LB agar plates, then incubated at 37°C for 24 h. After 24 h, the growth on each plate was noted.

4.6.8 Statistical analyses

Statistical analyses were performed with the EXCEL, GraphPad Prism 6, and BD CellQuest Pro packages. All values were expressed as mean ± standard deviation (SD) of three parallel measurements. To determine statistical significance of cell death, a two-sample t-test assuming unequal variance was used.

4.7 Results and Discussion

4.7.1 Chemical Separation

4.7.1.1 Crude extract

The multiple maceration extractions performed in this investigation proved to be both consistent and effective. Previous investigations, preliminary results, cost, and ease of handling considerations made acetone the solvent of choice for the extractions [20]. The extraction consistently yielded an extract with a concentration of 3.40 ± 0.16 mg mL^{-1}. The crude extract was then analyzed with thin layer chromatography, as shown in Figure 4-2.

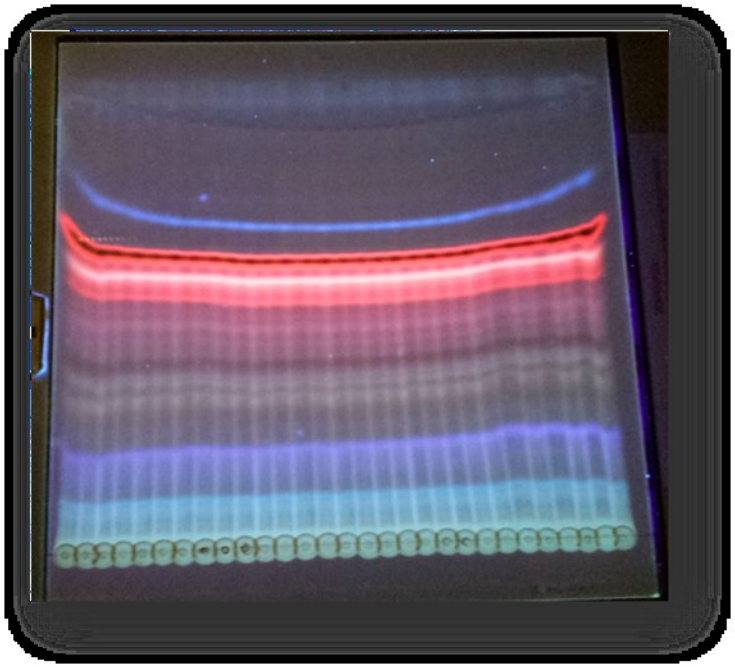

Figure 4-2: Thin layer chromatography of *Umbilicaria muhlenbergii* crude extract.

Silica gel 60 plate, with 40 µL of crude extract loaded per spot. Solvent system used was 170:30 toluene:acetic acid, which was then dried and illuminated under 365 nm UV light.

The thin layer chromatography was analyzed and input into Wintabolites, a lichen metabolite database, but the large bands proved too mixed and ambiguous to return a specific compound or family [21]. After crude extract bioactivity analysis was complete, the crude extract was separated via column chromatography. These separated fractions could then be visualized by TLC and prepared for further separation and chemical identification.

4.7.1.2 Silica gel 60 separation

The silica gel 60 separation yielded eight fractions, all of which were resuspended in solvents according to the elution solvent used and the mass of metabolites present.

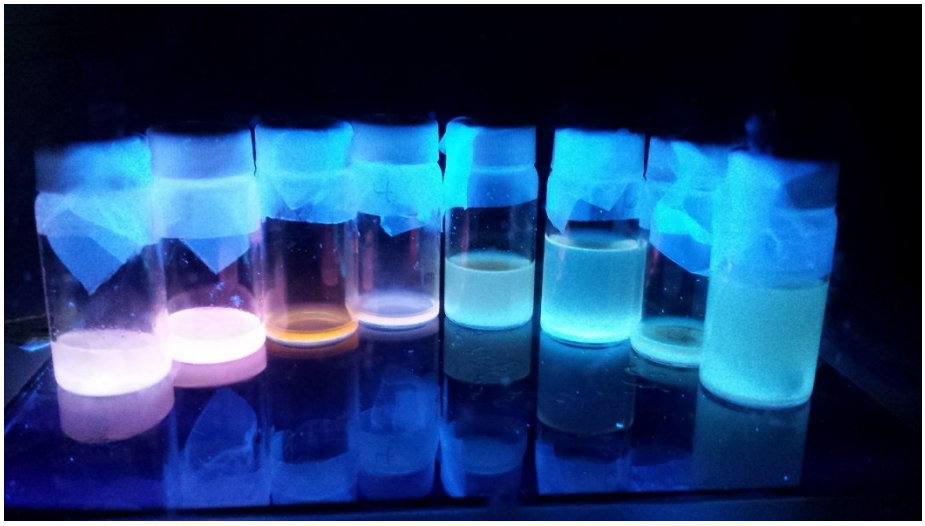

Figure 4-3: Resulting 8 fractions from the silica gel 60 separation of *Umbilicaria muhlenbergii* crude extract.

These eight fractions were analyzed with thin layer chromatography, and again the identities of the compounds present were searched for.

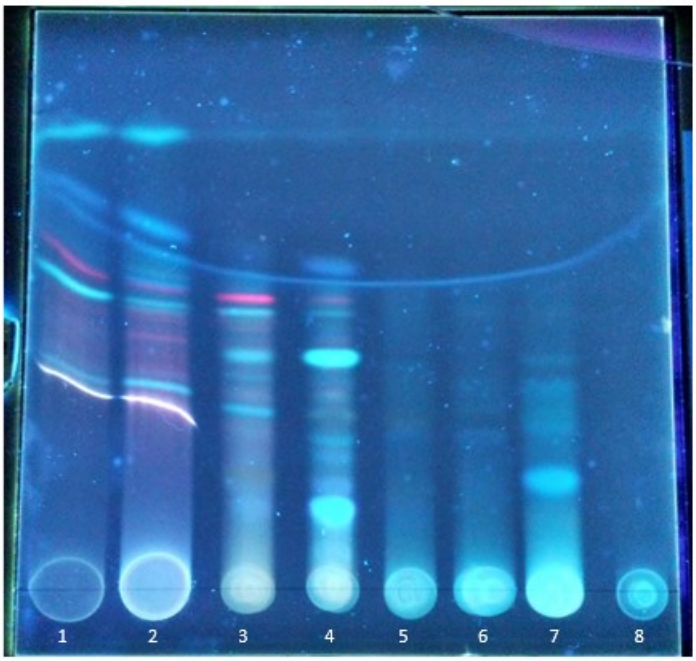

Figure 4-4: Thin layer chromatography of *Umbilicaria muhlenbergii* silica gel 60 separated fractions 1-8. 20 x 20 cm glass backed silica gel 60 plate (Merck), solvent used was 170:30 toluene:acetic acid. Image taken with 365 nm UV light illumination.

Anticancer activity testing showed that fractions 1-7 all exhibited some level of bioactivity (see Anticancer Activity). Analysis of the TLC plate in Figure 4-4 showed that SG60 fractions 5 and 6 had the fewest number of compounds apparently present, while also showing promising anticancer activity. Due to the inherent complexity that comes with separating and identifying natural compound mixtures, these two fractions, having the fewest compounds, were the most logical choice for further investigation and separation. Fractions 5 and 6 were again separated with TLC and analyzed.

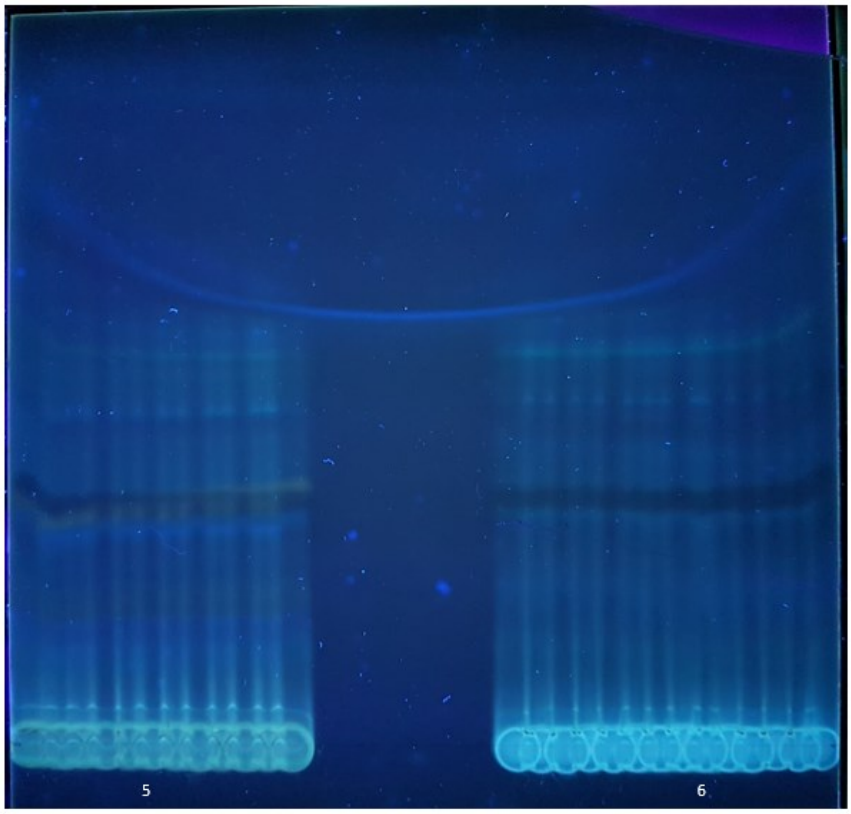

Figure 4-5: TLC of *Umbilicaria muhlenbergii* SG60 separated fractions 5 and 6.

The bands apparent in Figure 4-5 were input into Wintabolites, resulting in the identification of the main chemical families present in the mixtures. Fractions 5 and 6 contained depsides, depsidones, anthraquinones, and xanthones. Though TLC analysis produced many indicated chemical identities, the mixtures were still too complex to discern any concrete single compounds. This led to the use of Sephadex LH-20 as a separation media which was used to fractionate the SG60 fractions.

4.7.1.3 Sephadex LH-20 separation

Due to the SG60 fractions being a muddled mixture of compounds, further separation was deemed necessary. Sephadex LH-20 (GE Healthcare) was chosen as the separation media due to performance, ease of use, and previous work with other lichen

substances [22-26]. The Sephadex LH-20 also has proven to be an effective means of separation for despides, depsidones, and Xanthones, all of which are compounds of interest that can be found in *Umbilicaria* species [27-30].

After separation and resuspension, the separated Sephadex LH-20 fractions were visualized via TLC. The SG60 fraction #6 was separated into 22 fractions via the Sephadex, resulting in a collection of 9 fractions which had enough mass to be resuspended and analyzed further. Other fractions not shown in Figure 4-6 either did not have any metabolites present, or did not contain sufficient metabolite mass to resuspend at a useful concentration and analyze.

Figure 4-6: TLC of SG60 Fraction #6 Sephadex LH-20 Separated Fractions. Solvent used was 170:30 toluene:acetic acid, plate used was silica gel 60 glass backed TLC plate, plate is illuminated with 365 nm UV light.

This separation of SG60 fraction #6 on Sephadex LH-20 yielded fractions with significantly fewer compounds present, making them ideal for further analysis. Wintabolites analysis again confirmed the presence of depsides, depsidones, xanthones, and anthraquinones. The same results were found for the 46 fractions of SG60 fraction #5 being separated by Sephadex LH-20 (see Figure 4-7). Again, only fractions which had sufficient mass for resuspension and bioactivity analysis were included in the TLC experiments.

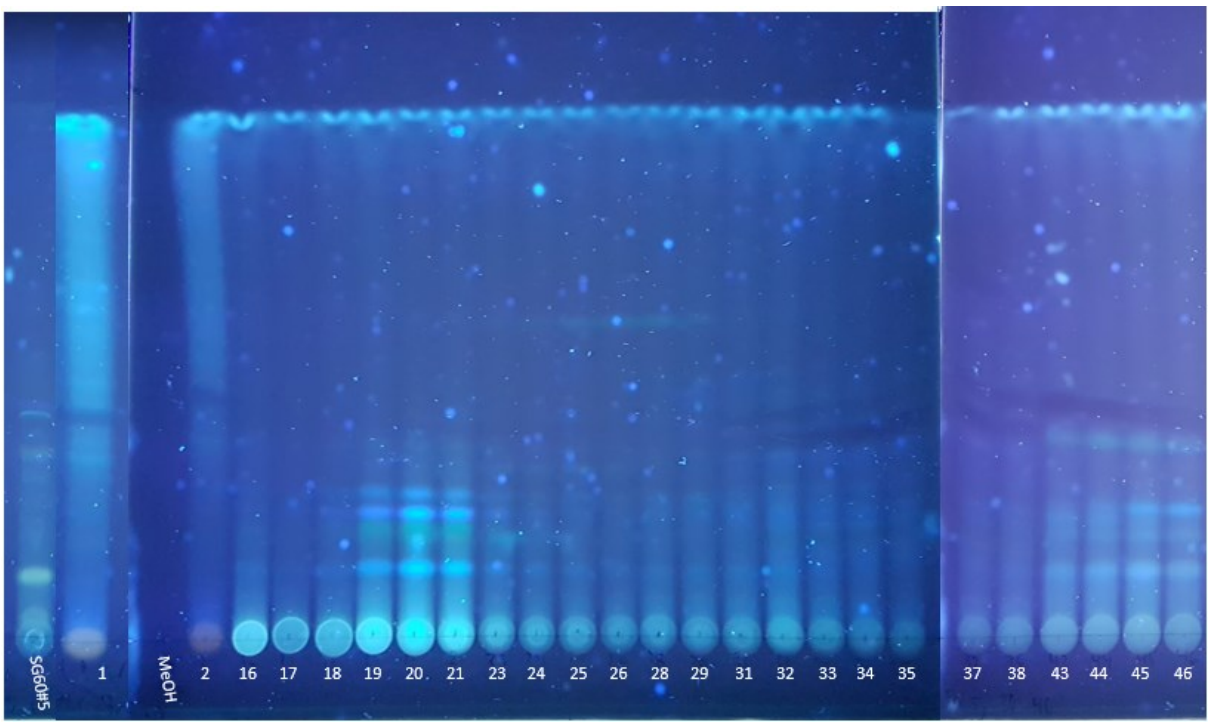

Figure 4-7: TLC of Sephadex LH-20 separated SG50 fraction #5.

The separation achieved by Sephadex LH-20 was enough to achieve some individual bands, while also ensuring that mixed bands did not have such a high number of compounds present. At this stage, the Sephadex LH-20 fractions shown in Figure 4-7 were utilitzed for

anticancer and antibiotic activity evaluation. For future investigations into *Umbilicaria muhlenbergii*, these fractions could again be separated to attain the least complicated mixture possible. From there, positive identification of the compounds could be made via analytical chemistry methods.

4.7.2 Antibacterial activity

The antibacterial activity of the *U muh* crude acetone extract was evaluated using the minimum inhibitory concentration (MIC) broth microdilution method [31]. This investigation revealed a MIC of 22.5 µg mL^{-1} against *S. aureus* bacteria. Tests against *E. faecalis, S. pyogenes,* and *P. aeruginosa* did not yield any statistically significant results.

The SG60 separated fractions did not fare as well, with a lack of statistically relevant results against any of the bacteria tested (data not shown). Further separation of the SG60 fractions with Sephadex LH-20 did yield statistically significant results though. Out of SG50 fraction #5's 46 Sephadex LH-20 separated fractions, five exhibited antibacterial activity against *S. aureus* (see Table 4).

Table 4: MIC of Sephadex LH-20 Separated SG60 Fraction #5 against *S. aureus*.

Fraction Number	MIC against *S. aureus* (ug mL^{-1})
21	31.25
23	62.5
24	125
25	250
38	500

Though these five fractions showed activity against *S. aureus,* the full utility of the metabolites comes into question once concentrations are considered. Fractions 21 and 23

could be considered of useful pharmaceutical interest, as they are within the concentration range of existing antibiotics, though fractions 24, 25, and 38 are of concentrations which are much too high to be pharmaceutically relevant. As a result, most of these results are more valuable in an academic context than a medical one. Again, a lack of statistically significant results of MIC against *E. faecalis, S. pyogenes,* and *P. aeruginosa* were found (data not shown).

These findings are in line with previous lichen bioactivity investigations, where a lack of activity against gram-negative bacteria was noted [20]. This lack of activity against gram-negative bacteria could indicate that the compounds are bioactive in a way that somehow affects the peptidoglycan cell wall of gram-positive bacteria. A compound or mixture of compounds with this specificity would not affect gram-negative bacteria.

The presence and absence of antibacterial activity at different stages of fractionation and separation could also be indicative of a synergistic effect[32, 33]. The presence of a complex mixture of metabolites could be the key to the bioactivity of the extract, as the individual compounds present within the extract may not be effective as standalone treatments, but when combined, can have a potent effect [32, 34, 35]. The number of compounds involved in this mixture could also greatly affect the potency, as evidenced by the crude extract and Sephadex LH-20 separated fractions exhibiting bioactivity, but the SG60 fractions being statistically insignificant.

These antibacterial findings are promising in an era where the search for novel and effective antibiotic compounds is paramount[36, 37]. Unexplored natural product sources, such

as the *Umbilicaria muhlenbergii* lichen investigated here, are poised to become valuable troves of bioactive compounds, which are needed evermore as traditional antibiotics begin to confer resistance[36, 37]. The promising results yielded by this study indicate that *Umbilicaria muhlenbergii*, and other species within the genus, could be useful as a source of antibiotics. Previous investigations into other *Umbilicaria* species have exhibited antibiotic effects, such as *Umbilicaria virginis* inhibiting *B. cereus, B. subtilis, S. aureus, K. pneumoniae, E. coli, P. aeruginosa, M. morganii,* and *Y. enterocolitica*[10]. The activity against gram-negative bacteria is of note, as the differences in metabolites produced between *Umbilicaria* species could be used to determine which compounds are bioactive against which type of bacteria.

Other *Umbilicaria* species have shown antifungal, antioxidant, anticancer, and antibacterial effects [10, 12, 38-40]. The major bioactive secondary metabolites known to be found in the genus; gyrophoric acid, lecanoric acid, and umbilicaric acid, have also been evaluated for antibacterial activity [10, 11, 40]. The activity of other species within the genus, coupled with the preliminary results of *Umbilicaria muhlenbergii*'s antibacterial abilities, warrant further investigation. Though outside of the scope of this study, a more systematic approach could be taken through analytical chemistry means, such as mass spectroscopy, to positively identify these antibacterial compounds. Once identified, the compounds could be synthesized, thereby opening the door to further research.

One hurdle facing these lichen-originated compounds is that of lichen toxicity, which needs further investigation. Though promising, natural products from the genus *Umbilicaria* have not yet been moved out of the *in vitro* investigation stage, leaving open opportunity for more in-depth investigation into these antibacterial compounds.

4.7.3 Anticancer Activity

4.7.3.1 Crude extract anticancer activity

The crude acetone extract of *Umbilicaria muhlenbergii* exhibited marked anticancer ability, having an effective concentration when 50% of the cells are killed (EC_{50} point) of 13.3 µg mL^{-1} (Figure 4-8), while the cisplatin positive control had an EC_{50} of 5.8 µg mL^{-1} (Figure 4-9). This shows a statistically significant difference between the lichen treatment and the cisplatin positive control ($P<0.05$). This compares favourably with previous lichen metabolite investigations undertaken, but with *Umbilicaria muhlenbergii* being effective at lower doses than other lichens investigated[20]. Furthermore, solvent death due to acetone caused a 5% decrease in cell viability at the highest concentration, and negligible cell death at lower concentrations (data not shown).

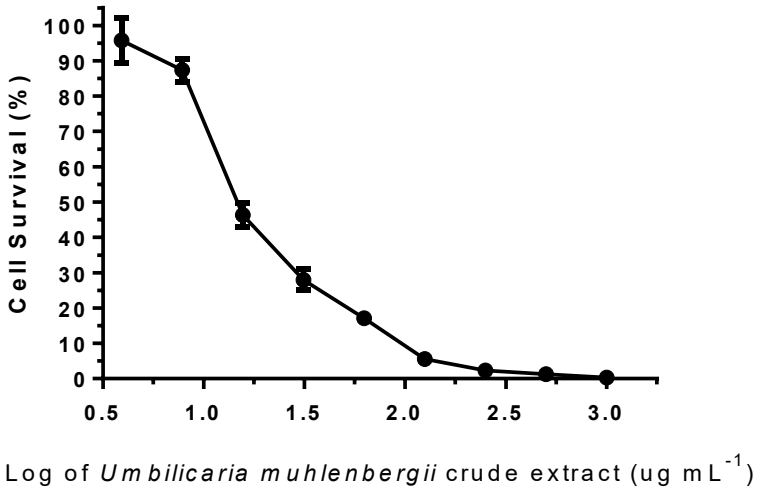

Figure 4-8: Survival of MCF-7 cells after 48-hour treatment with crude U. muhlenbergii extract. Cell survival analyzed via MTT assay.

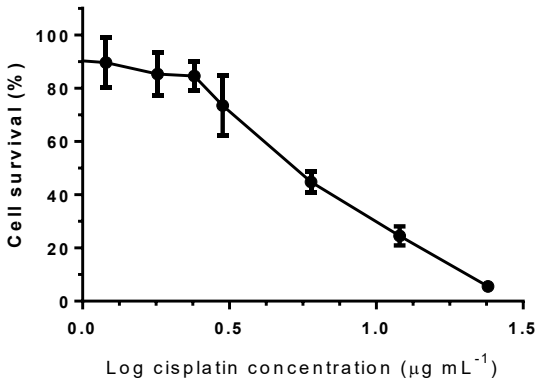

Figure 4-9: Survival of MCF-7 cells after 48-hour treatment with cisplatin chemotherapy drug. Cell survival analyzed via MTT assay.

Though the *Umbilicaria muhlenbergii* crude extract needs a higher dose than the cisplatin for an equivalent level of cell death, the more aggressive dose response curve could suggest that the compounds found within the lichen extract may elicit an inhibitory effect similar to that of known chemotherapeutics such as cisplatin.

The response of MCF-7 cells to the crude extract encouraged further investigation, which required the separation of the compounds to more concisely assess the bioactivity of the secondary metabolites found within the extract. This began with chemical separation of the crude extract via silica gel 60 (SG60) column chromatography.

4.7.3.2 Silica gel 60 separated fractions anticancer activity

All eight of the SG60 fractions were evaluated for anticancer activity against an MCF-7 breast adenocarcinoma cell line, and all 8 fractions were found to be bioactive (Figure 4-10 and Figure 4-11). All eight fractions had a significant effect on the cell viability, though their dose response curves differed. The more prominent changes in cell viability due to

treatment with SG60 fractions 5 and 6 were noted, as their dose response curves differ from many of the others. This observation, in addition to the TLC data indicating fewer compounds present, encouraged further investigation into these fractions.

Flow cytometry was utilized to gain insight into how these SG60 fractions and their metabolites were affecting the cells. Investigation of the cell cycle in cells treated with SG60 fraction 5 yielded a result which was not statistically significant different from the untreated control group of cells (data not shown). The same was found when crude extract was tested; there was a negligible effect on the cell cycle (data not shown). Solvent control treated cells did not exhibit any significant effects (data not shown).

The cell cycle differs significantly from the untreated control group for fraction 6 though, showing promising results (Figure 4-12 and Figure 4-13).

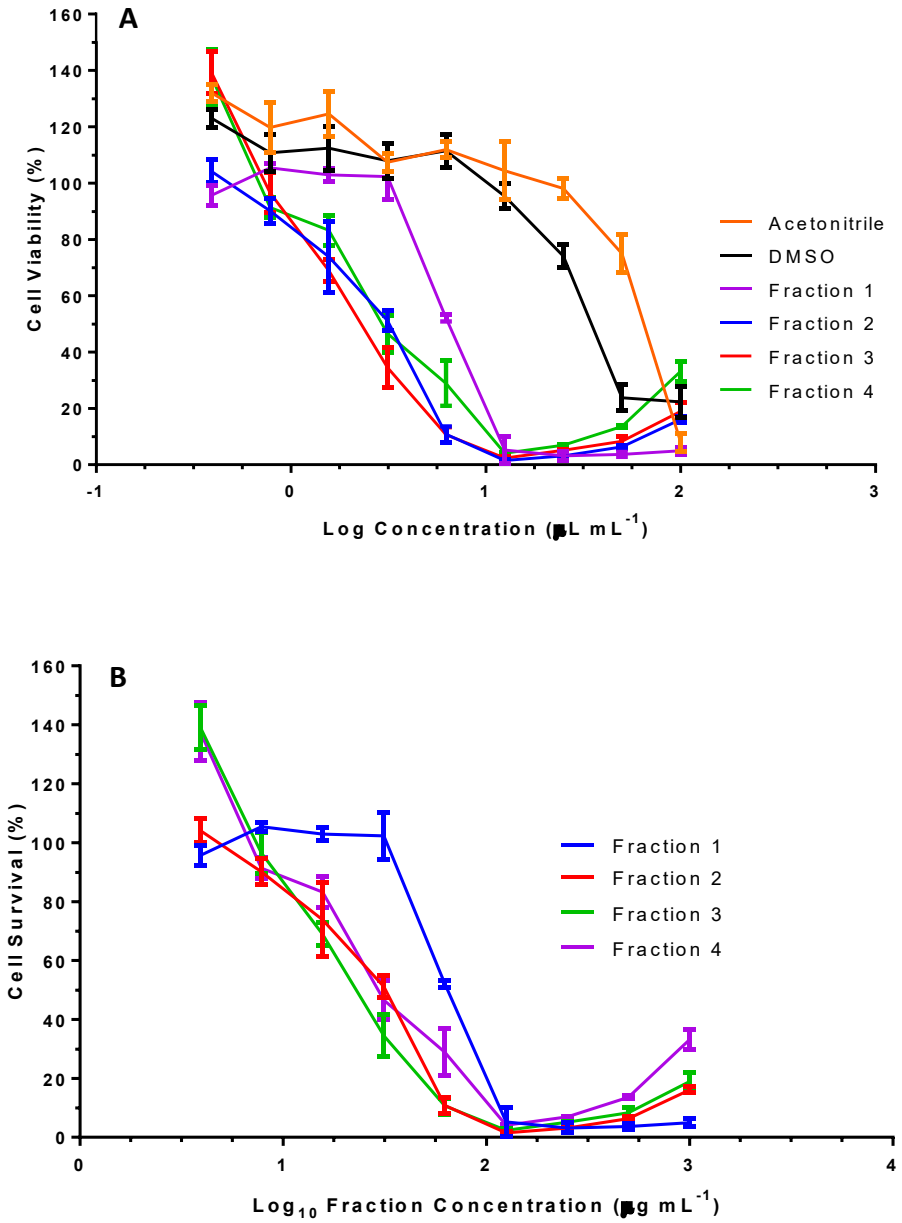

Figure 4-10: Dose response curve of MCF-7 cells exposed to increasing concentrations of solvents and silica gel 60 separated Umbilicaria muhlenbergii fractions 1-4. Cells were exposed to treatments for 48 hours, then viability was analyzed via MTT assay. Concentrations of treatment are log transformed. A is given in µL mL^{-1} for comparison to solvent control, B is given as µg mL^{-1}.

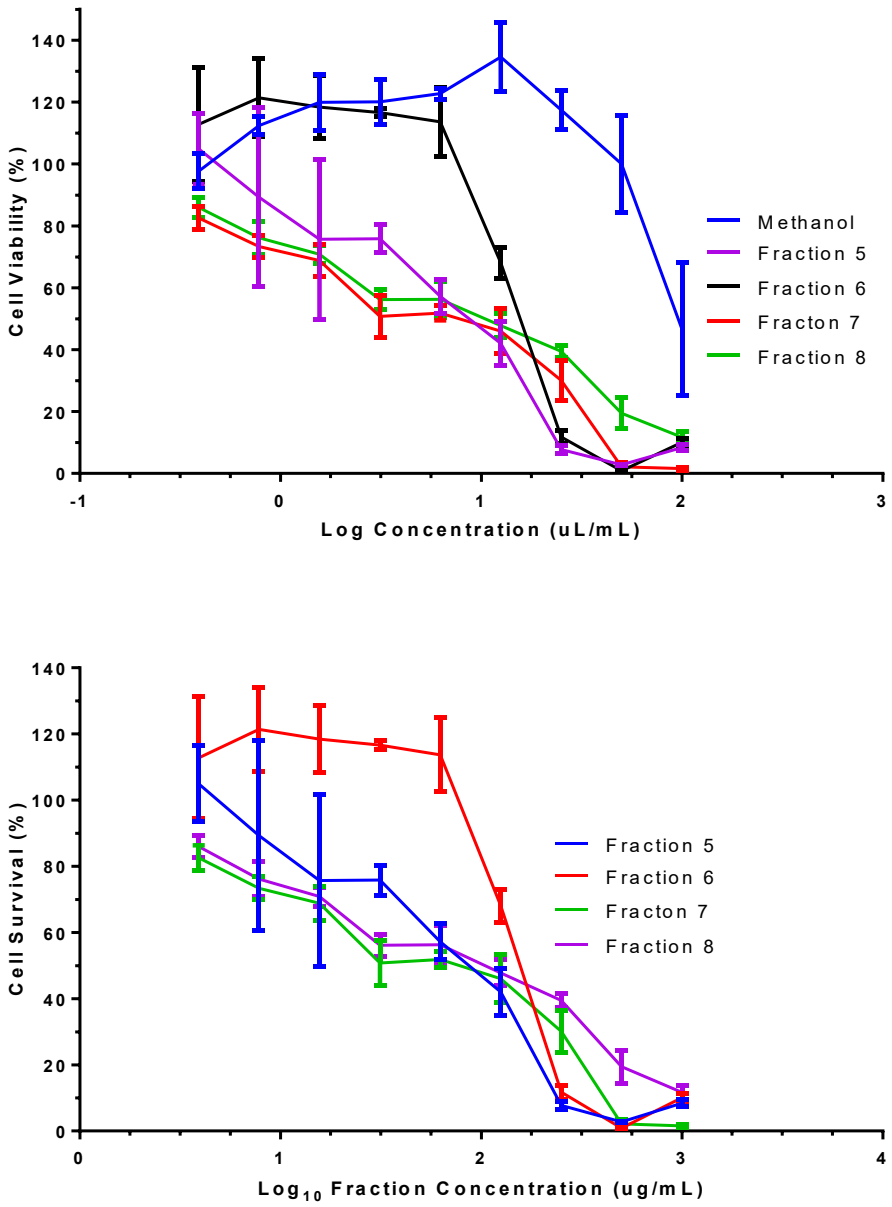

Figure 4-11: Dose response curve of MCF-7 cells exposed to increasing concentrations of solvents and silica gel 60 separated Umbilicaria muhlenbergii fractions 5-8. Cells were exposed to treatments for 48 hours, then viability was analyzed via MTT assay. Concentrations of treatment are log transformed. A is given in µL mL^{-1} for comparison to solvent control.

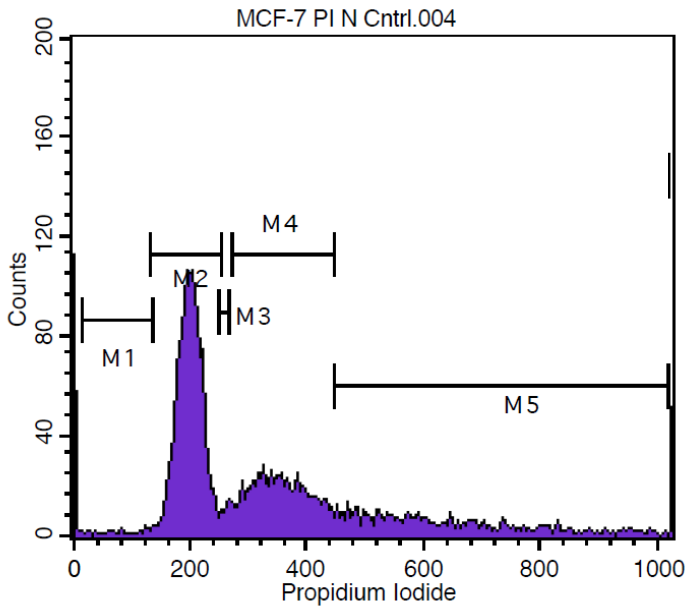

Figure 4-12: Propidium iodide stained MCF-7 cells, untreated growth control in TDMEM. Go/G1 phase= 52.21% (M1 +M2+M3), S phase= 30.80% (M4), G2/M=16.99% (M5)

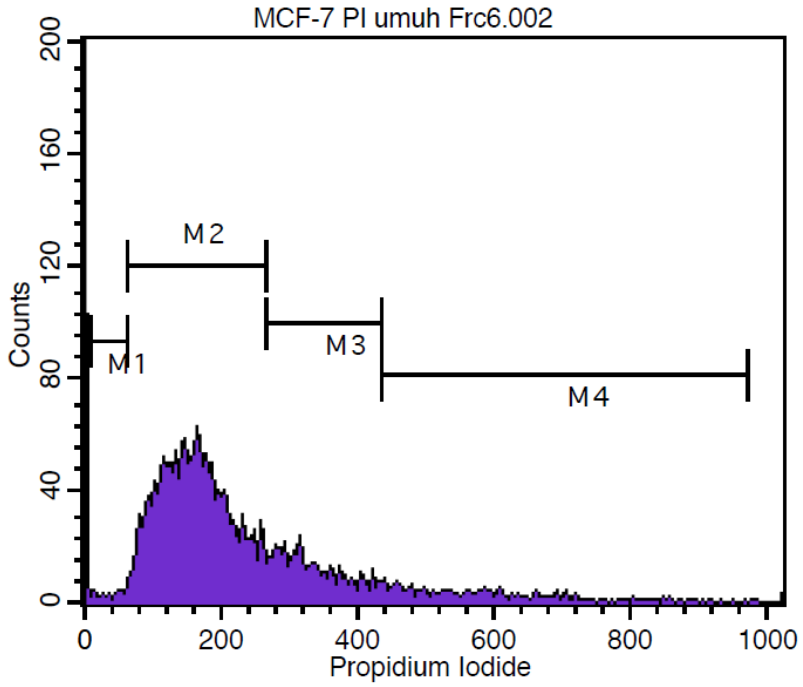

Figure 4-13: Propidium iodide stained MCF-7 cells, treated with 253.3 µg mL^{-1} SG60 Fraction 6. Go/G1 phase= 73.14% (M1 + M2), S phase= 17.27% (M3), G2/M=7% (M4)

Figure 4-13 shows a buildup of cells in the G_o/G_1 phase (73.14% of cells), which is indicative of cell cycle arrest. A pre-G_1 accumulation is also suggestive of apoptotic cells, rather than necrotic cells.

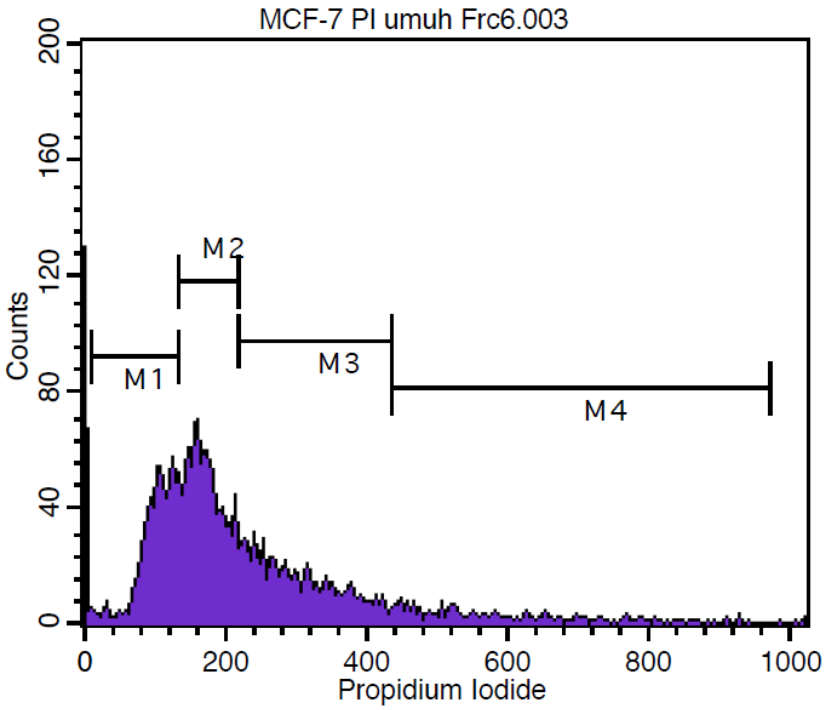

Figure 4-14: Propidium iodide stained MCF-7 cells, treated with 126.6 µg mL^{-1} SG60 Fraction 6. Go/G1 phase= 66.3% (M1+M2), S phase= 27.18% (M3), G2/M=5.56% (M4)

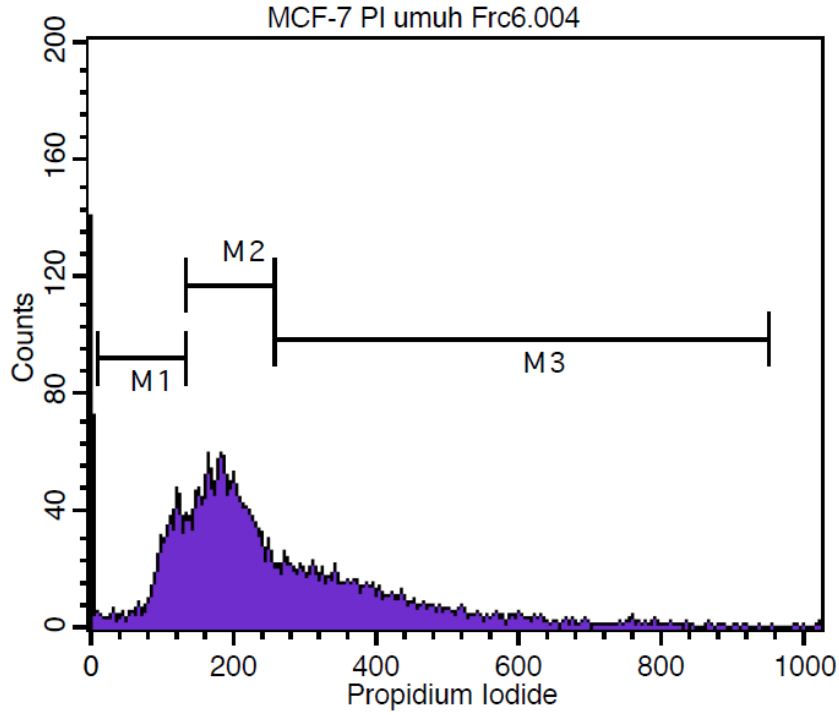

Figure 4-15: Propidium iodide stained MCF-7 cells, treated with 66.6 µg mL^{-1} SG60 Fraction 6. Go/G1 phase= 66.17% (M1), G2/M: 32.1%

The ability to elicit an arrest of the cell cycle was noted as the treatment concentrations were decreased (Figure 4-14 and Figure 4-15). The ability to affect the cell cycle at lower doses is paramount, as many natural products, including lichen metabolites, are toxic upon ingestion [41-44]. Reducing the concentration to a level low enough that does not cause negative health effects would thereby make the compounds viable candidates as anticancer compounds.

The induction of apoptosis via chemical means would be ideal, as apoptotic pathways are acted upon by currently utilized chemotherapeutic agents [45]. Though a promising result, the simple analysis with propidium iodide falls short of the mark for definite apoptotic detection, as more sensitive methods are required to determine the mechanism at play.

Other lichen compounds have been found to induce apoptosis in human cancer cell lines [46-49]. These include compounds such as salazinic acid, stictic acid, protolichesterinic acid, usnic acid, and sporomoric acid [46-49]. This inhibition of cellular proliferation and induction of apoptosis could make these lichen compounds a valuable source of anticancer compounds. This apoptotic evaluation of isolated and purified *Umbilicaria muhlenbergii* metabolites has yet to be completed, leaving room for further investigation. If purified compounds from *Umbilicaria muhlenbergii* do exhibit apoptotic effects as other lichen metabolites have, this would follow the suit of existing anticancer drugs, which are adept at causing apoptosis and arresting the normal cell cycle.

4.7.3.3 Sephadex LH-20 separated fractions anticancer activity

Once the bioactivity of the silica gel fractions had been investigated, it was apparent that further separation was necessary to reduce the number of compounds being used to treat the cells. It was the investigator's goal to achieve as few compounds as possible per fraction in order to identify individual or small groups of compounds.

SG60 fraction 5 was separated into 22 fractions with Sephadex LH-20, while SG60 fraction #6 was separated into 46 fractions. Each of these fractions was evaluated for bioactivity against MCF-7 cells (Figure 4-16 and Figure 4-17).

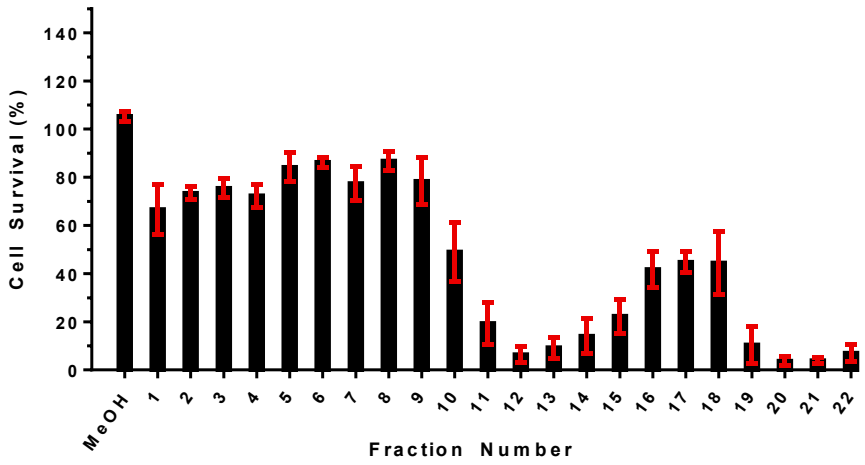

Figure 4-16: Survival of MCF-7 cells treated with 22 fractions from Sephadex LH-20 separated silica gel 60 fraction #6. Silica gel 60 fraction #6 obtained from separation of Umbilicaria muhlenbergii crude extract. Treatment time was 48 hours. Each treatment well contained 50 µg of metabolites.

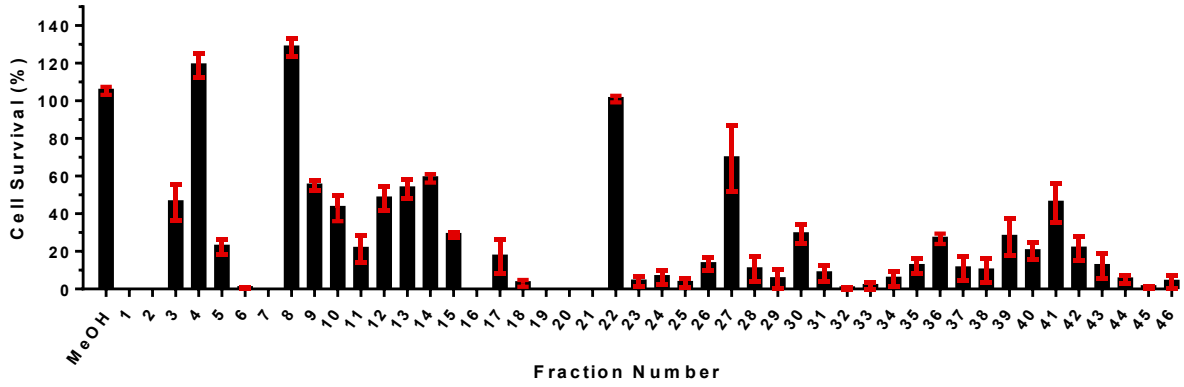

Figure 4-17: Survival of MCF-7 cells treated with 46 fractions from Sephadex LH-20 separated silica gel 60 fraction #5. Silica gel 60 fraction #5 obtained from separation of Umbilicaria muhlenbergii crude extract. Treatment time was 48 hours. Each treatment well contained 50 µg of metabolites.

SG60 fraction 5's Sephadex fractions showed potent bioactivity from fractions 10 through 22, with the most bioactive fractions being 11, 12, 13, 14, 15, 19, 20, 21, and 22 (Figure 4-16). For SG60 fraction 6, various areas of bioactivity were noted (Figure 4-17). Note that these bioactive fractions correspond with areas on the TLC plates where bands, consisting of similar compounds, are spread out laterally between several lanes on the plate. This indicates that a compound is shared between these bands, and that the compound in question is responsible for the MCF-7 cell death.

Propidium iodide flow cytometry analysis of the Sephadex LH-20 fraction treated cells did not produce any results of interest, with none of the fractions indicating cell cycle arrest (data not shown). This could be due to the cells not possessing apoptotic effects, but could also be attributed to the lack of time course investigation into the metabolites. It

could be possible that at the time of analysis, further necrosis of the cells and cellular breakdown could have occurred, leading to the results shown.

In conclusion, it was noted that *Umbilicaria muhlenbergii:* (1) acetone crude extract exhibits potent MCF-7 anticancer activity and antibacterial activity against select gram-positive bacteria, (2) silica gel 60 separated fractions exhibit MCF-7 anticancer activity and the ability to arrest the cell cycle, and (3) Sephadex LH-20 separated fractions show MCF-7 anticancer activity and antibacterial activity against select gram-positive bacteria. This work extends the evaluation of lichen metabolites to the species *Umbilicaria muhlenbergii*, and shows encouraging results which set up lichens as being an effective source of new chemical entities for eventual therapeutic use.

4.8 Acknowledgements

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5 Thesis Conclusions and Future Directions

Overall, lichen secondary metabolites present an interesting source of bioactive compounds which could prove useful for pharmaceutical means. The extracts and subsequent fractions have proved to be potent inhibitors of cancerous cells, while simultaneously being an effective antibacterial compound against gram-positive bacteria and exhibiting antioxidant potential. *Lobothallia alphoplaca* exhibited anticancer activity, with an IC_{50} of 87 µg mL^{-1}. *Xanthoparmelia mexicana* exhibited potent antibacterial properties, with a MIC of 20.9 µg mL^{-1} against *S. aureus* and 41.9 µg mL^{-1} against *E. faecalis.* Further investigation into the antibiotic effects of *Xanthoparmelia mexicana* need to be pursued, as further studies of their metabolites could yield valuable antibiotics. A more mechanistic or molecular approach to the antimicrobial experiments could now be pursued as well, allowing future researchers to determine exactly how the bacteria are responding to the metabolites.

Of particular interest to the author are the auspicious results of the *Umbilicaria muhlenbergii* evaluation. The *in vitro* bioactivities presented in this investigation pose a starting point for further research into *Umbilicaria muhlenbergii*. Antibacterial activity was noted with an MIC of 22.5 µg mL^{-1} against *S. aureus*, proving to be similar to that of *Xanthoparmelia mexicana*. It also showed a very strong anticancer tendency, with an IC_{50} of 13.3 µg mL^{-1}. Even more interesting are the apparent abilities to arrest the cell cycle in MCF-7 cells, as shown in Figure 4-15. Further investigation into the remaining SG60 fractions could also result in valuable results, as they all exhibited anticancer activity but were not separated further. All of these results could be taken as a starting point for further

mechanistic investigation into *Umbilicaria muhlenbergii*'s anticancer activities. While cisplatin, a common chemotherapeutic, was found to have an EC_{50} of 5.8 µg mL^{-1}, *Umbilicaria muhlenbergii* crude extract was not too far off the mark of clinical relevance, especially being an unprocessed crude extract. Perhaps with further refinement and identification of particular bioactive compounds or combinations, a treatment of therapeutic value within a useful range of concentration could be discovered.

This proven anticancer and antibiotic ability can lead one to question whether other bioactivities will be present as well, such as antifungal or antioxidant. Some of the compounds could be pluripotent, making their discovery even more valuable.

Though promising, challenges must be overcome before further results can be gathered. One area of improvement would be the increase in sample collection. The lichen metabolites make up a very small fraction of the lichens mass, thereby causing diminishing returns as the metabolites are extracted and separated. With each separation, the metabolites are separated into smaller groups and lost through experimental processes, meaning that every subsequent step has a smaller and smaller concentration of metabolites to investigate. This leads to concentrations where the metabolites are un-testable, as they are in such low quantities that they cannot be resuspended in an appropriate solvent at a useful concentration. The difficulty faced in separating the compounds will have to be overcome as well. Many of the compounds are of very similar structure and/or molecular weight, so the separation becomes difficult. A lack of standards for these compounds also exacerbates the issue, as lichen metabolite standards are incredibly expensive. One approach to this could be do use general molecular weight standards with the Sephadex LH-

20 column, thereby giving the ability to discern the unidentified compounds' molecular weights. More advanced methods of separation will have to be employed as well.

Achieving better separation and higher concentrations of lichen metabolites would make identifying the compounds through HPLC, LC-MS, GC-MS, NMR, or a plethora of other analytical chemistry methods easier as well. Further work could increase the mass of lichens extracted, increasing throughput and increasing metabolite mass present.

Further inquiry through processes of flow cytometry could be pursued, as the mechanisms at play could be discerned using techniques such as caspase tagging, annexin-PI, APO BrdU apoptosis assays, and more. Furthermore, other cell lines other than MCF-7 could be treated with the metabolites to determine if they are effective against other cell types. Though *in vivo* anticancer work has not been a very prominent point in lichen bioactivity analysis, it could be pursued once compounds are positively identified, evaluated for *in vitro* toxicity, and either isolated or synthesized in sufficient quantities.

The results of the antibacterial assays indicate an antibiotic effect which is on the higher end, but still within, the range of useful concentrations for modern, in-use antibiotics. The separated fractions from *Umbilicaria muhlenbergii* could be applied towards a more broad-spectrum test of many different types of bacteria, thereby determining if the lichen's metabolites have any specificity towards different bacteria. The small set of bacteria tested here was sufficient for a surface level evaluation of whether antibacterial activity was present, but it is non-exhaustive in terms of discerning the selectivity and overall effectiveness of the compounds present. The mechanism of action could also be elucidated,

which could lead to further studies of *Umbilicaria*'s antibiotic abilities. Few lichen metabolites have had their antibacterial mechanisms clarified, making investigation into mechanisms an attractive, novel, and productive area of future research.

In conclusion, lichens posses the ability to produce hundreds of different compounds, making them an attractive target for bioprospecting. After bioactivity analysis of *Xanthoparmelia mexicana*, *Lobothallia alphoplaca,* and *Umbilicaria muhlenbergii*, it is apparent that all three could contain valuable chemical entities that could be used for antibacterial and anticancer purposes. Further research into the topic will be required, but with these results, a precedence for further inquiry has been established.