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Exploring the Antibacterial, Antioxidant, and AnticancerProperties of Lichen Metabolites

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ABSTRACT

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Natural products have been a significant source of new drugs, especially in treating cancer, infectious diseases, hypertension, and neurological disorders. Although many natural metabolites have been screened and yielded pharmaceutically important drugs, many potential sources of natural product drug therapies still need to be investigated, including lichens.

Lichens are symbiotic systems consisting of a filamentous fungus and a photosynthetic partner (an eukaryotic alga and/or cyanobacterium). Lichens produce an impressive variety of unique secondary compounds and have been used as ingredients in folk medicines for centuries. Demonstrated biological roles based on lichen chemistry include: antibiotics, anti-proliferative, antioxidants, anti-HIV, anti-cancer, immunomodulation, and anti-protozoans. Although North America is home to an impressive variety of lichen species, there is limited research to examine the biological potentials of these lichens. The core goal of this dissertation research has been to investigate some of the biological roles including, antibiotic, antioxidant, and anticancer potentials using lichen crude extracts and their metabolites collected from various locations in the United States.

Antibiotic screening of crude extracts of 36 lichen species demonstrated inhibitory effects against *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, and Methicillin-resistant *S. aureus* (MRSA). Generally, acetone extractions were found to be more effective than methanol extractions. It has also been shown that *L. vulpina* extract was bacteriocidal against MRSA with a relatively slow kill rate that disrupts cell membrane integrity and cell division as possible modes of action. Antioxidant screening of extracts from 11 lichen species, using the Oxygen Radical Absorbance Capacity (ORAC) assay, showed that lichen extracts inhibited the oxidative degradation of the fluorescent molecule (fluorescein-sodium salt) by the oxygen free radical initiator AAPH (2,2'-azobis(2-aminidopropane) dihydrochloride Acetone extracts as well as pure compounds from lichen species showed cytotoxic effects against Burkitt's lymphoma (Raji) cells and a colon cancer cell line (HT29 and SW620). They decreased proliferation, arrested cell cycle at various stages and force the cell to undergo apoptosis. The tested extracts or pure compounds were not toxic to normal cells. In colon cancer apoptosis took place independent of casapase-3.

The results of this dissertation showed that lichen compounds merits for further investigation.

Keywords: lichen, antibacterial, anticancer, antioxidant, immunomodulation, secondary metabolites, mode of action, cell cycle, apoptosis, MTT, bacteriolysis, transmission electron microscopy

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Chapter 1: Background

Lichens are obligate symbiotic systems consisting of a filamentous fungus and a photosynthetic partner (an eukaryotic alga and/or cyanobacterium), and in some cases non-photosynthetic bacteria (Hodkinson & Lutzoni, 2009; Selbmann et al., 2010). Lichens are ecologically diverse and are distributed from the tropics to the polar regions (Brodo et al., 2001). The worldwide lichen flora is estimated to include approximately 18,500 species (Boustie & Grube, 2005; Feuerer & Hawksworth, 2007) and cover about 8% of the earth's land surface (Ahmadjian, 1995). Lichens are one of the slowest growing symbiontic associations and according to Coley (1988) slow growing organisms occupying low-resource habitats produce higher levels of defense chemicals in order to defend themselves from various consumers. This is certainly true in the case of lichens since they are known to produce more than a 1000 different secondary metabolites. General functional categories of the various lichen compounds include: protection against a large spectrum of microbes, animal predators and plant competitors; defense against environmental stress like UV radiation and desiccation; physiological regulation of metabolism, such as the ability to increase algal cell membrane permeability in order to increase the flow of nutrients to the fungal component (Huneck & Yoshimura 1996). As lichens are capable of protecting themselves from various microbes including bacteria, non-lichenized fungi, and nematodes, the potential value of these metabolites for medicinal purposes is generating increasing research interest.

Lichens produce two different types of metabolites; primary and secondary. Primary lichen substances have structural functions and play essential roles in cellular metabolism, similar to those in other fungi. Primary metabolites are intracellular in origin and are synthesized independently by both symbionts. Primary compounds consists mainly of chitin (in hyphal

walls), lichenin, isolichenin, hemicellulose, pectins, disaccharides, polyalcohols, amino acids, enzymes, pigments like algal chromophores: chlorophyll and, β-carotenes, xanthophylls, etc. (Podterob, 2008). In contrast, secondary metabolites are produced exclusively by the fungal partner and are exported outside the fungal hyphae and deposited as extracellular crystals in different parts of the thallus, often in the upper cortex or in specialized structures such as fruiting bodies (Fahselt, 1994). Several studies have confirmed the exclusive fungal origin of secondary metabolites (Culberson & Armaleo, 1992; Hamada et al., 1996; Kon et al., 1997; Stocker-Wörgötter & Elix, 2002). Although lichen secondary metabolites are produced by the of fungal partner, the metabolic interaction between the mycobiont and photobiont is essential to the production of these secondary chemicals. This has been documented by studies where mycobionts grown without the photobiont do not produce the same metabolites as the intact lichen or produces a completely different suite of chemical products (Molina et al 2003; Fazio et al 2009). There are some situations where the photobiont, especially cyanobacteria, also produce some secondary metabolites (Cox et al., 2005; Yang et al., 1993).

Over 1050 secondary metabolites have been reported for lichens and aposymbiotically cultured mycobionts (Molnar & Farkas, 2010). Among them a relatively small number (50 – 60) are also produced by non-lichenized fungi or higher plants (Elix, 1996). One example is the anthraquinone parietin which is present in other fungi like *Aspergillus* and *Penicillium*, as well as in the vascular plant genera *Rheum, Rumex* and *Ventilago* (Romagni & Dayan, 2002). This metabolic diversity is due largely to the symbiotic relationship between the lichen partners (Lawrey, 1986). Lichen secondary products can comprise up to 20% of the thallus dry weight, but in most lichens the amount varies from 5–10%.

Natural products have been central in the discovery and development of various drug therapies. It was Friedrich Sertürner who for the first time isolated a pharmacologically active compound, morphine from opium produced by seed pods of the poppy plant, *Papaver somniferum* (Hamilton & Baskett, 2000). Since then several thousand compounds have been purified from various natural sources including plants, microorganisms, fungi etc. to treat various diseases. According to Newman (2008) about 60% of the drugs that are now available come either directly or indirectly from natural products. Natural products have been a significant source of new drugs, especially in the treatment of cancer, infections, hypertension, and immune and neurological disorders (Butler, 2004). Specifically in the development of anticancer drug therapies, a number of important new commercialized drugs have been obtained from natural sources. The search for improved cytotoxic agents continues to be an important endeavor in the discovery of modern anti-cancer drugs (Gordaliza, 2007). Although many natural metabolites have been screened and resulted in the production of many pharmaceutically important drugs (Saleem et al., 2010), many potential sources of drug therapies still need to be investigated. Among them are lichens.

North America is home to an impressive variety of lichen species. Many studies have been published examining the taxonomical (Barton et al., 2014; Hutten et al., 2013), phylogenetic (Leavitt et al., 2013), and ecological (Nelson et al., 2013) aspects of lichens in North America; however, the biological roles of lichens have been poorly studied.

The main goal of this dissertation research has been to investigate the biological roles of crude extracts and isolated metabolites of lichens collected from various regions of the United States. The specific aims of this dissertation are as follows:

1. Screen the acetone and methanol extracts of lichens against four pathogenic bacteria, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and methicillin-resistant *S. aureus*.

2. Study the mechanism of action of lichen crude extracts against methicillin-resistant *S. aureus*.
3. Investigate the antioxidant capacity of crude lichen extracts.
4. Screen and study the mode of action of acetone extracts and some purified lichen metabolites against Burkitt's lymphoma (Raji cells) and colon cancer cell lines (HT29 and SW620).

Chapter 2: Biological roles of lichen-derived compounds: A review

Introduction

Lichens have been used as ingredients in folk medicines for centuries and many cultures have used lichens to treat a variety of ailments as part of their traditional medicines (Dayan & Romagni, 2001). The medicinal properties of some lichens are mentioned in the Ayurvedic and Unani systems where they are used to treat a broad array of common ailments, including blood and heart diseases, bronchitis, scabies, leprosy, asthma inflammations, stomach disorders, etc. (Shukla et al., 2010). Recent advances in the medical field have resulted in exploration of the biological activity of a limited number of lichen products with some studies suggesting that some lichen chemicals could possible provide a promising source of future drug therapies (Shrestha and St. Clair, 2013).

Demonstrated biological roles based on lichen chemistry include: antibiotics, anti-proliferative, antioxidants, anti-HIV, anti-cancer, immunomodulation, and anti-protozoans. Lichens produce an abundance of secondary compounds, and more than 1000 secondary metabolites have been identified from intact lichens and aposymbiotically cultured mycobionts (Molnar & Farkas, 2010). Production and regulation of lichen compound is complex and variously influenced by environmental factors, including light, UV exposure, elevation, temperature fluctuations, and seasonality. A review paper detailing the effects of different environmental factors on lichen compounds have been published by Shrestha & St. Clair (2014).

As mentioned earlier, lichen compounds have multifaceted biological activities. This chapter provides a review of antibiotic, anticancer, immunomodulatory, and antioxidant activities of lichen compounds. The following tables will discuss the antibacterial, anticancer, immunostimulant and antioxidant properties of lichen extracts and their metabolites.

Table 2.1: A summary of the literature dealing with the antibiotic activity of lichen secondary chemistry.

Lichens/Lichen metabolites	Bacteria	Findings	References
Lobaric acid and lobastin isolated from *Stereocaulon alpinum*	*Bacillus subtilis* *Staphylococcus aureus* *Escherichia coli*	Both the isolated compounds were found to be active against *B. subtilis* and *S. aureus*. The MIC values of Lobaric acid and lobastin were 88 and 44 µM respectively against *B. subtilis* while 39.6 and 35.2 µM respectively against *S. aureus*. Both the compounds have no effect on *E. coli* at the tested concentrations.	Bhattarai et al., (2013)
Usnic acid, Atranorin, Fumarprotocetraric acid	Methicillin sensitive and methicillin resistant isolates of *S. aureus*	Usnic acid showed the highest activity against all the isolates. There as significantly higher (at least eightfold) activity of usnic acid as compared to atranorin. Fumarprotocetratic acid did not showed antibacterial activity in the tested concentrations. The killing quotient (MBC/MIC) suggested that usnic acid is bacteriostatic in nature. However, atranorin was found to be bactericidal in one of the strains.	Pompilio et al., (2013)
Combination of usnic acid and 5 different antibiotics namely- clindamycin, erythromycin, gentamicin, levofloxacin, and oxacillin	20 different methecillin resistant clinical isolates of *S. aureus*	Usnic acid inhibited 50% of growth of all *S.aureus* at 2 µg/ml while 90% growth inhibition was found at 4 µg/ml. Combination of usnic acid with gentamicin gave synergistic action, while antagonism was observed with levofloxin whereas there was no difference with erythromycin. However, variability was observed with clindamycin and oxacillin.	Segatore et al., (2012)

Crude extract of *Cladonia furcata, Hypogymnia physodes,* and *Umbilicaria polyphylla* and fumarprotocetraric acid, gyrophoric acid and physodic acid extract respectively from above mentioned lichens	*Bacillus mycoides* *Bacillus subtilis* *S. aureus* *Enterobacter cloaceae* *E. coli* *Klebsiella pneumoniae*	Both the crude extract of lichens and their components showed a relatively strong antimicrobial activity but comparatively the components were more active than crude extract. The acetone and methanolic extract showed relatively strong antimicrobial activity with MIC ranging from 0.78 to 6.25 mg/ml while the aqueous extract showed no activity against any bacteria. The lichen components showed very strong antimicrobial activity with MIC ranging from 0.031 to 1 mg/ml.	Kosanic and Rankovic (2011)
Crude extract of *Umbilicaria cylindrica*	*B. subtilis* *S.aureus* *E.coli* *Proteus vulgaris* *Proteus mirabilis* *K. pneumoniae*	Both methanol and ethyl acetate extracts exhibited significant inhibitory activity against all tested bacteria. The ethyl acetate extract was most potent against *E. coli* with MIC value of 15.62 μg/mL while methanolic extract was active against *B. subtilis* and *S. aureus* with the MIC value 15.62 μg/mL	Manojlovic et al., (2012a)
Various depsides, depsidones, xanthones, usnic acid, orsellinic acid esters, salazinic acid derivatives and lichexanthone derivatives extracted from *Parmotrema dilatatum* *Parmotrema tinctorum* *Pseudoparmelia sphaerospora* *Usnea subcavata*	*Mycobacterium tuberculosis*	Several compounds were found to inhibit growth of *M tuberculosis;* of which the most active is diffractaic acid (MIC = 15.6 μg/mL) followed by norstictic acid (MIC = 62.5μg/mL), usnic acid (MIC = 62.5μg/mL), hypostictic acid (MIC = 94.0 μg/mL) and protocetraric acid (MIC = 125 μg/mL).	Honda et al., (2010)

Usnic acid, norstictic acid, salazinic acid, stictic acid, diffractaic acid, barbatic acid, and galbinic acid from *Usnea baileyi, Ramalina dendriscoides, Stereocaulon massartianum,* and *Cladonia gracilis*	*B. subtilis* *S.aureus* *E. coli* *Pseudomonas aeruginosa*	Crude lichen extracts were very active against *Staphylococcus aureus* and *Bacillus subtilis* while moderately active against *E. coli* and *P. aeruginosa*. Among the different lichens, *R. dendriscoides* was the most active against *S. aureus* with MIC and MBC values of 156 µg/ml and 2500 µg/ml respectively.	Santiago et al., (2010)
Barbatic acid from *Cladia aggregata*	Four multi drug-resistant *S. aureus* strains	Barbatic acid either in crude organic form or in purified form inhibited the growth of *S. aureus.* The MIC values for purified barbatic acid against different strains of *Staphylococcus aureus* was 50µg/mL, except for the highly resistant IC 404, whose MIC was 100µg/mL.	Martins et al., (2010)
Crude extract and Usnic acid, Usimine A, Usimine B, Usimine C and Ramalin from *Ramalina terebrata*	*B. subtilis* *S.aureus* *E. coli* *P. aeruginosa*	Both crude extracts and individual fractions showed activity against *B. subtilis* with usnic acid having the highest MIC value of 1.2 µg/mL .*S. aureus* was inhibited by crude extracts and usnic acid only but not by other individual fractions. There was no effect of either crude extracts or individual fractions against *E. coli* and *P. aeruginosa*.	Paudel et al., (2010)
Crude extract and 6 isolates (+)-usnic acid, divaric acid, 2,4 –Di-O-methyldivaric acid, Divaricatinic acid, and 2-O-Methylnordivaricatic acid from *Evernia divaricata*	*B. subtilis* *S. aureus* *E. coli* *P. aeruginosa*	Crude extracts showed a significantly high inhibition zone against three species – *B. subtilis, S. aureus* and *P. aeruginosa*. Usnic acid and divaric acid showed very potent inhibitory activity against the above mentioned bacteria. This is first report concerning the anti-bacterial properties of divaric acid.	Yuan et al., (2010)

Crude extract of *Anaptychia ciliaris* *Cetrelia olivetorum* *Lecanora muralis* *Peltigera polydactyla Peltigera praetextata Ramalina farinacea* *Rhizoplaca melanophthalma* *Umbilicaria vellea* *Xanthoria elegans* *Xanthoria parietina* *Xanthoparmelia tinctina*	*B. subtilis* *S. aureus* *E. coli* *P. aeruginosa* *K. pneumonia* *Staphylococcus epidermidis*	Aqueous extracts of most lichen showed inhibition against *B. subtilis, S. aureus* and *E. coli* while ethanol extracts of some lichens were found to be active against *B. subtilis, S. aureus* and *S. epidermidis*. Ethanolic extracts of all tested lichens did not show any activity against *E. coli* while both extracts were not able to inhibit growth of *K. pneumonia* and *P. aeruginosa*.	Karagöz et al., (2009)
Physodic acid (*Hypogymnia physodes*) Usnic acid (*Parmelia caperata*) Atranorin (*Physcia aipolia*) Gyrophoric acid (*Umbilicaria polyphylla*)	*Bacillus mycoides* *B. subtilis* *Enterobacter cloacae E. coli* *K. pneumoniae* *S. aureus*	All the tested lichen substances inhibited growth of all microorganisms with the lowest recorded MIC (0.0037 mg/ml) against *K. pneumonia* with usnic acid. Physodic acid had the weakest activity level with an MIC value of 1mg/ml	Rankovic & Misic (2008)
Evernic acid (*Evernia prunastri*) Salazinic acid (*Flavoparmelia caperata)* Physodic acid (*Hypogymnia physodes)* 3- hydroxyphysodic acid (*Hypogymnia physodes*) Usnic acid (*Lecanora albescens*) Hybocarpone (*Lecanora conizaeoides*) Atranorin (*Lepraria lobificans*) Rhizocarpic acid (*Psilolechia lucida*)	Methicillin - and Multidrug-Resistant *S. aureus*(SA-1199B) Two hospital isolates of resistant *S. aureus* Norfloxacin -Susceptible strain of *S. aureus* Two epidemic MRSA strain in UK	Most of the lichen compounds showed consistent activity against various strains of *Staphylococcus aureus*. Among the tested extracts Hybocarpone was the most active compound against all strains with an MIC value of 4-8 ug/ml while Physcion and salazinic acid showed no activity against any of the strains at any concentration and evernic acid and atronorin showed activity against the *Staphylococcus aureus*-1199B strain only.	Kokubun et al., (2007)

Lobaric acid(*Sterocaulon dactylophyllum*) Physcion (*Xanthoria parietina*)			
Crude extract of *Rhizoplaca chrysoleuca Rhizoplaca melanophthalma Rhizoplaca peltata*	*E. coli* *Enterococcus faecalis* *Proteus mirabilis* *S.aureus* *B. subtilis* *B. megaterium* *P. aeruginosa*	Acetone extracts of *R. chrysoleuca* showed high activity against all bacteria except *P .aeruginosa*. While *R. melanophthalma* extracts showed activity against only 3 bacteria. The zone of inhibition of *R. chrysoleuca* was higher than antibiotic tetracyclin in the case of *E. coli, P. mirabilis* and *B. megaterium*.	Cansaran et al., (2006)
Crude extract of *Parmelia saxatilis Platismatia glauca* *Ramalina pollinaria* *Ramalina polymorpha* *Umbilicaria nylanderiana*	35 strains of bacteria	All extracts from five different lichens species demonstrated antibacterial activity against at least some of the bacterial species tested. *R. pollinaria*, inhibited the most (11) bacterial species. The maximum inhibition zone forbacterial strains based on all lichen extracts was<22 mm with MIC values ranging from 6 - 62.5 ul/ml.	Gulluce et al., (2006)
Usnic acid	*S. aureus* *P.aeruginosa*	The capacity of usnic acid to control bio-film formation by *S. aureus* or *P. aeruginosa* was tested by loading the acid into modified polyurethane. Usnic acid loaded polymers did not inhibit the initial attachment of *S. aureus* , but inhibited the formation of a bio-film by killing the attached cells where as in *P. aeruginosa* there was no inhibition of bio-film formation but the morphology of the biofilm was altered.	Francolini et al., (2004)

Usnic acid (*Cladonia arbuscula*) Atranorin (*Stereocaulon alpinum*) Lobaric acid (*S. alpinum*) Salazinic acid (*Parmelia saxatilis)* (+)-protolichesterinic acid (*Cetraria islandica*)	*Mycobacterium aurum*	Usnic acid is the most active compound with an MIC value of 32 μg/mL while others were inactive at the tested concentration.	Ingólfsdóttir et al., (1998)
(+)-Usnic acid (Commercial) (-)-Usnic acid (*Cladonia stellaris*) Vulpinic acid (*Letharia vulpina*)	10 different bacterial strains and some resistant strains of *S.aureus*	Lichen extracts inhibited the growth of *S. aureus, E. faecalis, E. faecium*, and some anaerobic species (*Bacteroides* and *Clostridium* species) at the concentrations tested. But gram negative bacteria were not susceptible. Vulpinic acid generally was less active than usnic acid. Susceptibility to usnic acid was not impaired in clinical isolates of *S. aureus* resistant to methicillin and/or mupirocin.	Lauterwein et al., (1995a)
Crude and(-)-Usnic acid, Physodic acid, 8'-O-Ethyl-p-alectoronic acid and Alectosarmentin from *Alectoria sarmentosa*	*E. coli* *S.aureus* *Salmonella gallinarum* *K. pneumoniae* *Mycobacterium smegmatis* *P. aeruginosa*	Crude extracts as well as all isolated acids were active against *S. aureus* and *M. semgmatis* but not *E. coli, S. gallinarum, K. pneumonia* and *P. aeruginosa*	Gollapudi et al., (1994)
17 species of lichens and fractions from two lichens	*Bacillus subtilis* *Staphylococcus aureus* *Escherichia coli* *Pseudomonas aeruginosa*	Extracts from the majority of the lichen species studied were active against gram-positive organisms and several against the gram-negative as well. Of the different fractions Methyl 1-orsellinate had the lowest MIC value against all microbes.	Ingolfsdottir et al. (1985)

Table 2.2: A summary of the literature dealing with the anti-cancer activity of lichen secondary chemistry

Lichen Acid/Lichen species	Cell lines tested	Major Finding	Reference
Parmelia sulcata	Human Breast cancer cell lines (MCF-7) Human Breast cancer cell lines (MDA-MB-231)	The methanolic extract of *P. sulcata* significantly reduced the cell viability in dose-dependent manner. The IC_{50} values for MCF-7 and MDA-MB-231 were 39.1 and 16.5 µg/ml respectively. They found increased in the expression of caspase-cleaved cytokeratin 18 (M30), a well-known marker of apoptosis, in MCF-7 but not in MDA-MB-231 showing that the extract of *P. sulcata* induced apoptosis. The induction of apoptosis was caspase-independent as the expression level of PARP and Caspase-3 remain unchanged.	Ari et al., (2014)
Parmotrema reticulatum	Human breast carcinoma (MCF-7) Human lung carcinoma (A549)	The study showed that the methanolic extract of *P. reticulatum* was cytotoxic against MCF-7 cells with an IC_{50} value 130.03 µg/ml while there was negligible cytotoxicity was observed on A549 and WI-38 (normal lung fibroblast)cells. It was further shown that the extracts arrested cell cycle at S and G2/M phases and induces apoptosis in dose as well as time dependent manner. There was down-regulation of cyclin B1, Cdk-2 and Cdc25C. There was increase Bax/Bcl-2 ratio confirming the apoptosis.	Ghate et al., (2013)
Hypogymnia physodes	Human Breast cancer cell lines (MCF-7) Human Breast cancer cell lines (MDA-MB-231)	The methanolic extract of *H. physodes* reduced cell viability in dose dependent manner. The IC_{50} values for MCF-7 and MDA-MB-231 were 50 and 44 µg/ml respectively. Apoptosis was observed only in MCF-7 but not in MDA-MB-	Ari et al., (2014b)

		231 as it was found M30-antigen level increased in MCF-7 but remained unchanged in MDA-MB-231. Nuclear fragmentation was also observed in MCF-7 further confirming apoptosis. It was further found that *H. physodes* was genotoxic at higher concentrations (250 and 500 µg/ml)	
Usnic acid (commercial) Atranorin (commercial) Parietin (*Xanthoria parietina*) Gyrophoric acid (*Umbilicaria hirsuta*)	Human ovarian carcinoma A2780 Human colon adenocarcinoma HT-29	Usnic acid and atranorin induced a massive loss in the mitochondrial membrane potential, along with caspase-3 activation in HT-29 cells but externalization of phosphatidylserine occurred in both tested cell lines. Cytotoxicity is mainly due to induction of both ROS and especially RNS. Usnic acid and atranorin activated programmed cell death in A2780 and HT-29, probably through the mitochondrial pathway.	Backorová et al. (2012)
Diffractaic acid (*Protousnea magellanica)* Vicanicin (*Psoroma pallidum*) Lobaric acid (*Stereocaulon alpium*) Variolaric acid (*Ochrolechia deceptionis*) Protolichesterinic acid (*Cornicularia aculeate*) Usnic acid (*Cladonia lepidophora*)	Human breast adenocarcinoma MCF-7 Human colon adenocarcinoma HCT-116 Human cervix adenocarcinoma HeLa	Lichen metabolites exhibited different cytotoxic effects with higher susceptibility in HCT-116 and less responsive in MCF-7. Among the 6 compounds, vicanicin did not showed any activity against any of the cell lines in tested concentration while only diffractaic acid and usnic acid were active against all three cancer cell lines. The apoptotic effect of protolichesterinic acid was further evaluated against HeLa cells by analyzing nuclear morphology and measuring the activity of caspase-3. 27% of apoptotic cells were found after 72 hours of treatment and a significant increase of caspase-3 activity was observed compared to control cell confirming apoptosis.	Brisdelli et al. (2012)

Atranorin (*Bacidia stipata*) Diffractaic acid (*Protousnea magellanica)* Divaricatic acid (*Protousnea malacea*) Vicanicin (*Psoroma dimorphum*) Protolichesterinic acid (*Rhizoplaca melanophthalma*)	Human prostate cancer androgen-responsive (LNCaP) Human prostate cancer androgen-non responsive DU-145	Atranorin, diffractaic acid and divaricatic acid were found to be active against prostate cancer cells only in high concentration while vicanicin and protolichesterinic acid showed dose – dependent response causing apoptosis in both types of cells. This study for the first time showed that apoptosis induced by the compounds appeared to be mediated, at least in part, via the inhibition of Hsp70 expression.	Russo et al. (2012)
Usnic acid (commercial) Atranorin (commercial) Parietin (*Xanthoria parietina*) Gyrophoric acid (*Umbilicaria hirsuta*)	Human ovarian carcinoma A2780 Human breast adenocarcinoma MCF-7 Human colon adenocarcinoma HT-29 Human promyelocytic leukemia Human T cells lymphocyte leukemia, Jurkat Human cervix adenocarcinoma HeLa Human breast adenocarcinoma SK-BR-3 Human colon carcinoma wild type p53 HCT-116 p53+/+ Human colon carcinoma p53 null HCT-116 p53-/-	Lichen compounds showed differential sensitivity to various cancer cells. All the tested compounds showed some cytotoxic activities but usnic acid and atranorin were highly effective against the whole spectrum of cell lines while the other two were less effective. Similar to cytotoxicity, usnic acid and atranorin also significantly inhibited the clonogenic ability of all the tested cell lines while the other two were effective in some cervix and breast tumor cells. All acids except parietin altered cell cycle distribution accumulating cells in S-phase in all tested cell lines, however, the efficiency and spectrum of the affected cells were different. Similarly, usnic acid and atranorin demonstrated strong pro-apoptotic action while in other two acids, the apoptotic index was less pronounced.	Backorová et al. (2010)

Lecanoric acid and it's orsellinate derivatives	Larynx carcinoma HEP-2 Breast carcinoma MCF-7 Kidney carcinoma 786-0 Murine melanoma cell B16-F-10 Vero cell	Structural modification of lecanoric acid increased the cytotoxicity of the compound as orsellinates derivatives have lower IC_{50} values than lecanoric acid. *n*-Butly orsellinate was the most active compound with IC_{50} values ranging from 7.2 – 14.0 µg/ml. The orsellinate activity has been found to increase with chain elongation (from methyl to *n*-butyl), a likely consequence of an increase in lipophilicity.	Bogo et al., (2010)
(+) Usnic acid (*Cladonia arbuscula*) (-) Usnic acid (*Alectoria ochroleuca*)	Breast cancer cell line T-47D Pancreatic cancer cell line Capan-2	Both (+) and (-) usnic acids are effective inhibitors of DNA synthesis, with IC_{50}values of 4.2 ug/ml and 4.0 µg/ml against T-47D and 5.3 µg/ml and 5.0 µg/ml against Capan-2. There wasa reduction in cell size and both acids inhibited cell entry into the S-phase. No apoptosis was observed but necrosis was seen in Capan-2. Usnic acid also caused loss of mitochondrial membrane potential.	Einarsdóttir et al., (2010)
Retigeric acid A and Retigeric acid B (*Lobaria kurokawae*)	Human Pca LNCaP PC-3 DU 145 Human epidermoid cancer KB and vincristine resistant KB (KB/VCR) Human ovarian cancer 3-AO and cisplatin-resistant 3-AO (3-AO/CDDP) Human benign prostate epithelial RWPE1 Human hTERT-RPE1 Human breast cancer	Both Retigeric acid A (RA) and Retigeric acid (RB) showed cytotoxicity at lower concentrations (>100 uM) but RB is more potent than RA. Structural analysis of RA and RB has shown that a methyl side chain of RA is substituted with –COOH in RB, suggesting a possible structure–activity relationship. Further investigation on the effect of RB on PC-3 cells showed that RB caused a dose-dependent accumulation of cells in the S phase accompanied with decreases in cyclin B, and increases in cyclin E and cyclin A. Both caspase dependent and caspase-independent pathways	Liu et al., (2010)

	MCF-7 Human osteosarcoma U2OS and Saos2	were responsible for apoptosis in PC-3 cells	
Olivetoric acid (*Pseudevernia furfuracea*)	Rat adipose tissue endothelial cells	Olivetoric acid displayed dose dependent anti-angiogenic activities, inhibited cell proliferation and disrupted endothelial tube formation in adipose tissue. Similarly, olivetoric acid also inhibited the formation of actin stress fibers in a dose dependent manner which may be due to the decrease in tube formation.	Koparal et al., (2010)
Usnic acid (Commercial)	Breast cancer cell lines MCF7 (estrogen-dependent, wild-type p53) Lung cancer cell line H1299 (null for p53)	No Morphological changes in microtubules or increase in the mitotic index was observed suggesting the antineoplastic activity of usnic acid is not related to alterations in the formation and/or stabilization of microtubules.	O'Neill et al., (2010)
(+) usnic acid (*Xanthoparmelia somloensis*) Salazinic acid (*Xanthoparmelia somloensis*) Vulpinic acid (*Letharia vulpina*) Gyrophoric acid (*Lasallia pustulata*) Evernic acid (*Evernia prunastri*)	Malignant mesothelioma cells MM98 Vulvar carcinoma cells A431 Keratinocytes HaCaT	Usnic acid showed high toxicity for all three cell lines while vulpinic acid was intermediately toxic and salzinic, gyrophoric and evernic acids showed low toxicity. The EC_{50} value of usnic acid was significantly lower than anionic surfactant SDS. Further, usnic acid and gyrophoric acid showed strong wound closure effects on HaCaT cells but the effect was less for the other three acids. A combination of usnic acid and gyrophoric acid further increased wound closure rates.	Burlando et al., (2009)

Lethariella zahlbruckneri	Human colon cancer cell (HT-29)	Both acetone and methanolic extracts of *L. zahlbruckneri* decreased viable cell number in both a time and dose dependent manner but an acetone extract showed higher toxicity than the methanolic extract. The acetone extract induced cell death by increasing cell population in the sub-G1 phase, as well as the formation of apoptotic bodies and nuclear condensation while such activities were not seen with the methanolic extract. Apoptosis by the acetone extract was induced both in a caspase-dependent and caspase-independent manner. Apoptosis was mitochondria mediated because there is increased level of Bax and decreased level of the Bcl-2 protein.	Ren et al., (2009)
16-O-Acetyl-leucotylic acid Leucotylic acid (Both from *Myelochroa aurulenta*)	Human leukemia cells HL-60	16-O-Acetly-leucotylic acid exhibited potent anti-proliferative activity against HL-60 with an EC50 value of 21μM while the Leucotylic acid, derivative of 16-O-Acetly-leucotylic acid has a higher EC50value (72 μM). The anti-proliferative properties of these two compounds were higher than other similar compounds (betulin and betulinic acid) derived from higher plants. Structural modification has also been found to affect the cytotoxicity of compounds like Leucotylic acid which was less toxic than16-O-Acetly-leucotylic acid.	Tokiwano et al. (2009)
Crude extract of *Evernia prunastri Xanthoria parietina*	Murine myeloma P3X63-Ag8.653	A crude extract of *Xanthoria parietina* significantly reduced cell proliferation in a dose-dependent manner while no such activities were seen with *Evernia prunastri*. The higher activity	Triggiani et al., (2009)

		of *X. parietina* may be due to the higher content of antioxidants such as peroxidases and superoxide dismutase.	
Usnic Acid and its 9 derivatives	Lymphocytic leukemia L1210 Murine Lewis lung carcinoma 3LL Chronic myeologenous leukemia K-562 Brain metastasis of prostate carcinoma DU 145 Breast adenocarcinoma MCF 7 Glioblastom U251 Hamster cell lines: CHO and CHO-MG	Usnic acid and its nine derivates were tested for cytotoxicity. Four polyamine derivates showed significant cytotoxicity on L1210 with an IC_{50} value significantly less than the parent compound usnic acid. The most active compound (N-tert-butoxycarbonyl-1,8-diaminooctane) had an IC_{50} value of 2.7 uM and induced a dose-dependent and time dependent apoptotic event in L1210. The cytotoxicity of usnic acid can be improved by its conjugation to a polyamine chain.	Bazin et al., (2008)
Sphaerophorin (*Sphaerophorus globosus*) Pannarin (*Psoroma* spp.)	Human melanoma cells M14	Both sphaerophorin and pannarin showed significant inhibitory effect on M14 cells at a concentration of 12.5-50 uM. They also induced apoptotic cell death substantiated by DNA fragmentation and increased caspase-3 activity.	Russo et al., (2008)
Crude extract of *Cetraria aculeata*	Human uterus carcinoma HeLa Human small lung carcinoma A549 Rrat embryonic fibroblast 5RP7 Normal rat embryonic fibroblasts F2408	Acrude extract of *C. aculeata*was found to be cytotoxic against HeLa and A549 with an IC_{50} value of 200 and 500ug/ml respectively. The extract also demonstrated higher cytotoxic activity against F2408 and 5RP7 with IC50 values of 80 and 280 ug/ml respectively.	Zeytinoglu et (al., 2008)

(+) Usnic acid (*Ramalina farinacea*) (-) Usnic acid (*Cladonia foliacea*)	Chinese Hasmster Lung fibroblast V79 Human lung carcinoma A549	Both types of usnic acid showed dose and time dependent cytotoxicity against V79 and A549 cell lines. Cytotoxicity was more pronounced in A549 than V79 with cell viability more diminished in A549 versus V79 after 2 days of treatment	Koparal et al., (2006)
Sphaerophorin (*Sphaerophorus globosus*) Pannarin (*Psoroma* spp.) Epiphorellic acid -1 (*Cornicularia epiphorella*)	Human prostrate carcinoma DU 145 Normal human prostatic epithelial cells	All compounds non-toxic to normal human prostatic epithelial cells at aconcentration of 6-50 umol/l showed significant inhibitory effects on DU-145 cells. Among the three compounds, Pannarin showed the most activity. At lower concentrations (12 and 25 μmol/l) all compounds induced apoptosis but when the concentration was increased to 50 μmol/l cell death was due to necrosis as documented by a significant release of lactic dehydrogenase. Apoptosis was further confirmed by the large amounts of DNA fragmentation in the 12 and 25 μmol/l concentration but not in 50 μmol/l.	Russo et al., (2006)
Usnic acid (Commercial)	Breast cancer cell lines MCF7 (estrogen-dependent, wild-type p53) Breast cancer cell lines MDA-MB-231 (estrogen independent, mutant p53)	All three cell lines were sensitive to usnic acid with IC_{50} values of 18.9 μM (MCF7) and 22.3 μM (MDA-MB-231 and H1299). There were elevated levels of the p53 and p21 proteins following treatment with usnic acid but there was no p53 transcriptional activity suggesting that the accumulation of p21 was not secondary to p53 transactivation.	Mayer et al., (2005)

(-) usnic acid Fumarprotocetraric acid 9'-(O-methly)protocetraric acid	Murine lymphocytic leukemia L1210 Murine Lewis lung carcinoma 3LL Human chronic myelogenous leukemia U251 Human brain metastasis of a prostate carcinoma DU145 Human breast adenocarcinoma MCF 7 Human glioblastoma RCB-0461	Of the three compound tested only usnic acid showed cytotoxic activity at IC_{50} values of 6, 12.1, 15.8, 17.8, 8.2 and 6.8 μg/ml for L1210, 3LL, DU145, MCF7, K-562 and U251 respectively. Usnic acid also induced apoptosis in L1210 in a dose- and time-dependent manner as fluorescence microscopy revealed condensation of nuclear chromatin, nuclear fragmentation, and formation of apoptotic bodies.	Bezivin et al., (2004)
Depsidones - Vicanicin, Pannarin, 1-chloropannarin, Salazinic acid, Stictic acid, variolaric acid, Psoromic acid, Fumarprotocetraric acid, Lobaric acid Depsides - Atranorin, Sphaerophorin, Divaricatic acid, diffractaic, gyrophoric and usnic acid	Hepatocytes from rat	IC_{10} and IC_{50} values for 15 different lichen compounds have been reported. Among them Usnic acid has the highest cytotoxicity with an IC_{50} value of 21 μg/ml after 24 hours which was measured using lactic acid dehydrogenase. Salazinic acid, stictic acid, and psoromic acid showed apoptosis of hepatocytes in a dose-dependent manner with stictic acid showing the strongest apoptotic activity.	Correche et al., (2004)
Cladonia convoluta *Cladonia rangiformis* *Evernia prunastri* *Parmelia caperata* *Parmelia perlata* *Platismatia glauca* *Ramalina cuspidata*	Murine lymphocytic leukaemia L1210 Murine Lewis lun carcinoma 3LL Human chronic myelogenous leukaemia Human brain metastasis	3 different extracts, n-hexane, diethyl ether, and methanol of 8 species were screened for cytotoxicity against 7 cell lines. Significant cytotoxicity ($IC_{50} \leq 20$μg/ml) was found on one of the tested cancer cell lines for at least one extract of each lichen species. Crude extracts of some of the lichens (*C. convoluta, C.*	Bézivin et al., (2003)

Usnea rubicunda	of a prostate carcinoma DU145 Human breast adenocarcinoma MCF 7 Human glioblastoma RCB-0461 African green monkey kidney cell (Vero)	*rangiformis, P. caperata, P. glauca,*and *R. cuspidata* had very high selectivity indices which suggests a potential role as anti-tumor agents.	
Pannarin 1'-chloro pannarin Salazinic acid Psoromic acid Fumarprotocetaric acid Lobaric acid Vicanicin Stictic acid Variolaric acid Atranorin Sphaerophorin Divaricatic acid Diffractaic acid Gyrophoric acid	Lymphocytes from ratspleens	The screening of depsides and depsidones revealed considerable cytotoxic effect for Pannarin, 1'-chloropannarin, and sphaerophorin with stronger effects than colchicine. Generally, depsidones showed stronger cytotoxic activity than depsides. The strong biological activity of some depsidones may be due to the strong hydrogen bond between the aldehyde group at C_3 and the hydroxyl group at C_4. Similarly, the cytotoxic activity of depsides may be in part due to the presence of a COOH group on C'_1 and an OH group on C'_2.	Correche et al., (2002)
(+)-usnic acid Methyl â-orcinolcarboxylate Ethyl hematommate Diffractaic acid Gyrophoric acid (+)-protolichesterinic acid	Human keratinocyte cell line HaCaT	Gyrophoric acid, usnic acid, and diffractaic acids were reported as potent anti-proliferative agents which inhibited cell growth at IC_{50} values of 1.7, 2.1, and 2.6 μM. However, the rest of the compounds did not affect cell growth at concentration of even 5μM. There was no release of lactate dehydrogenase in the culture medium suggesting no damage to the plasma membranes of keratinocyte cells by lichen acids. This further	Kumar KC & Müller (1999)

		document that the effects of gyrophoric acid, usnic acid, and diffractaic acid are cytostatic rather than cytotoxic.	
Lobaric acid (*Stereocaulon alpinum*) Protolichesterinic acid (*Cetraria islandica*)	Breast cancer cell T-47D and ZR-75-1 Erythro-leukemia K-563	Both test substances caused a significant reduction in DNA synthesis. Significant cell deaths in all three cell lines were observed at concentrations of 20 and 30 μg/ml of protolichesterinic acid and lobaric acid respectively. But DNA synthesis and proliferation and survival of normal skin fibroblasts were not affected at higher doses.	Ogmundsdottir et al. (1998)
Usnic acid derivatives	Lewis lung carcinoma L1210	Eleven usnic acid derivatives where evaluated for cytotoxicity against L1210 and seven derivatives almost completely inhibited cell growth at $1.4X10^{-7}$ mol/mL while other derivatives showed somewhat lesser cytotoxicity. The lipophilicity and the β-triketone moiety of usnic acid were found to be an important source of cytotoxicity.	Takai et al., (1979)

Table 2.3: A summary of the literature dealing with the immunomodulatory roles of lichen compounds

Lichen compound	Major Finding	Reference
Glucan from *Umbilicaria esculenta*	This paper studies the effect of glucan on the phenotype and functional maturation of dendritic cells. The authors found elevated expressions of CD40, CD80, CD86, and MHC class I/II molecules confirming phenotypic maturation of dendritic cells. They also discovered that there was increased production of cytokines like IL-12, IL-1β, TNF-α, and IFN-α/β, decreased endocytosis and enhanced proliferation of allogenic T cells suggesting functional activation of dendritic cells. Finally, they were able to show that glucan-induced dendritic cell maturation is related to MAPK and NF-κB activation.	Kim et al., (2010)

Methanolic extract from *Caloplaca regalis*	This study reports the tumorcidial activity of macrophages treated with methanolic extracts of *C. regalis*. It was observed that there was an increase in the tumorcidial activity of peritoneal macrophages against B16 tumor cells in mice when treated with crude extracts (CR-ME). The authors hypothesized that the increased tumorcidial activity of the macrophages was due to elevated levels of NO and TNF-α. They further speculated that the mechanism by which CR-ME activates macrophages might involve p38 MAPK because there was abrogation of tumoricidal effect of activated macrophages with a p38 MAPK inhibitor.	Choi et al., (2009)
Lichenan and isolichenan from *C. islandica,* as well as the secondary metabolites, protolichesterinic and fumarprotocetraric acids	This study reported the effect of lichen polysaccharides and secondary metabolites on the maturation of human monocyte-derived immature dendritic cells. Their effects on the maturation of dendritic cells. Lichenan up-regulated the secretion of both IL-10 and IL-12p40 whereas isolichenan and the two secondary metabolites showed no activity. The authors also reported higher secretion rates for IL-10 when compared to IL-12p40 which suggests that dendritic cells demonstrate tolerogenic effects. The maturation of dendritic cells by lichenan may be due to through DC-SIGN, a C-type lectin receptor.	Freysdottir et al. (2008)
Three heteroglycans Ths-4, Ths-5 and thamnolan and a β-glucan, Ths-2, isolated from *Thamnolia vermicularis* var. *subuliformis*	Ths-2, Ths-5, and thamnolan caused stimulation of rat spleen cell proliferation but not by Ths-4 which caused cell death early in the culture. Moreover, the secretion of IL-10 significantly higher when treated with Ths-2, Ths-4, and Ths-5. In addition, Ths-4 and Ths-5 stimulated significant TNF-a secretion by rat peritoneal macrophages. However, thamnolan did not induce the secretion of IL-10 by rat spleen cells or TNF-a secretion by peritoneal macrophages to significant levels.	Omarsdottir et al. (2007)
11 different polysaacharides isolated from different species of lichens	This study reports the effect of 11 different polysaccharides isolated from various lichen species on the maturation of dendritic cells. Out of 11 polysaccharides, 8 of them showed some tendency towards upregulation of IL-10 secretion by the dendritic cells and 4 of them, lichenan, Ths-2, Pc-4 and thamnolan had significantly higher level of IL-10 production.	Omarsdottir et al. (2006)

	Similarly, 7 polysaccharides upregulated with the production of IL-12p40. Further the maturation of dendritic cells was confirmed by the expression of CD86.	
Three homogenic heteroglycans, Pc-1, Pc-2, and Pc-3 and one galactoglucomannan, Pc-4 isolated from *Peltigera canina*	There was a dose-dependent increase in the proliferation of rat spleen cells when treated with different polysaccharides with Pc-1 having the highest stimulation index. All the polysaccharides also induced the production of IL-10 by spleen cells. However, they stimulated only the secretion of TNF-α but not IL-10 by peritoneal macrophages.	Omarsdottir et al. (2005)
Galactomannan isolated from *Ramalina celastri* (GMPOLY) and galactomannan in combination with the vanadyl ion (GMPOLY-VO)	This study reported that both GMPOLY and GMPOLY-VO were capable of diminishing the production of superoxide anions by macrophages when triggered with Phorbol 12-myristate 13-acetate (PMA). They also found that the diminution of superoxide anions production was lower in case of GMPOLY-VO than GMPOLY suggesting that the complexing with vanadyl ion led to the GMPOLY exacerbation the effects on the macrophages. The superoxide anion production is protein kinase C dependent; thus, the authors speculated that GMPOLY and GMPOLY-VO could interfere but they have not neglected the possibility of their scavenging properties. They also studied the effects of GMPOLY and GMPOLY-VO on nitric oxide production and found that GMPOLY enhanced nitric oxide (NO) production by 40% whereas no effect was observed with GMPOLY-VO. NO has cytotoxic properties and protect the host against potential pathogens. Hence this study suggests that lichen-derived polysaccharides can indirectly protect host by stimulating macrophages to produce nitric oxide.	Noleto et al., (2002)
α-D-glucan isolated from *Ramalina celastri*	This paper describe the phagocytotic activities of α-D-glucan. They injected a single intraperitoneal dose of α-D-glucan (200 mg/kg) to female albino Swiss mice; killed at 7, 15, and 30 days to isolate macrophages from peritoneal exudates and evaluated their phagocytotic activity. They found that that after 7 days of treatment, phagocytosis was increased by 70% and then started to decrease and finally leveled off	Stuelp-Campelo et al., (2002)

	with control by 30 days. This result indicates that the tested polysaccharides have time dependent effect on the macrophage phogocytotic activity.	
Ci-3, an (1→3)-(1→4)-α-D-Glucan extracted from *Cetraria. islandica*	This paper had examined the phagocytotic activity of granulocytes treated with Ci-3. The results have shown that there was increased in phagocytotic activity in both *in vitro* and *in vivo* setting. This study had also shown lichen polysaccharides can reduce the complement induced hemolysis The functions of the complement system include opsonization, chemotaxix and cell lysis; however, over-activation of the complement system can contribute to the etiology of many inflammatory and auto-immune diseases such as rheumatoid arthritis, asthma, adult respiratory distress syndrome, systemic lupus, and erythematosus	Olafsdottir et al. (1999)
Water soluble polysaccharides from *C. islandica*	This study found increased in the phagocytic activities of polysaccharide treated granulocytes both in *in vitro* and *in vivo* setting. The anti-complementary assay showed the reduction of complementary-induced hemolysis ranging from 49-85%.	Ingolfsdottir et al. (1998)
KI-M-7, a galactomannaon, isolated from *C. islandica*	This is the first study analyzing the immunostimulatory roles of polysaccharide isolated from lichen. The results showed that human granulocytes treated with KI-M-7, enhanced granulocytic phagocytosis by 68% as compared to the control. Similarly, the *in vivo* phagocytotic assay measured by calculating the rate of carbon clearance showed that when KI-M-7 was administered intraperitoneally there was an increase in the rate of carbon elimination by a mean ratio of 1.9 compared with the control.	Ingolfsdottir et al. (1994)

Table 2.4: A summary of antioxidant properties of lichen

Lichens/Compounds	Main Findings	References
n-hexane, methanol and water extracts from fourteen saxicolous lichens	This study measured antioxidant capacity of 14 different lichen species by measuring the ferric reducing antioxidant power (FRAP), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) radical scavenging capacities and β-carotene-linoleic acid bleaching property. All the lichen extracts had a good amount of total proanthocyanidin, flavonoid and polyphenol. Methanolic extract of *Lobothallia alphoplaca* exhibited highest FRAP value; methanolic extract of *Xanthoparmelia stenophylla* had the highest ABTS radical scavenging capacity; n-hexane extract of *Rhizoplaca chrysoleuca* exhibited highest DPPH radical scavenging capacity; water extract of *Xanthoria elegans* had the highest antioxidant capacity in terms of β-carotene linoleic acid bleaching property; and water extract of *Melanelia disjuncta* showed highest NO scavenging capacity.	Kumar et al., (2014)
Lobaric acid and lobastin from *Stereocaulon alpinum*	This study reports the isolation of new pseudodespidone, lobastin, from *S. alpinum* collected from Antarctica. Antioxidant examination by DPPH radical scavenging activity showed that lobaric acid did not have scavenging activity whereas lobastin reduced DPPH free radicals in dose-dependent manner. The authors speculated that the antioxidant activity of lobastin might be due to the presence of double bond in between C-8 and C-9, and opening of heterocyclic ring.	Bhattarai et al., (2013)
Methanolic extract from *Parmotrema reticulatum* (PRME)	This study reports the antioxidant activities of PRME using different methods. Total antioxidant activity of PRME, calculated as a function of the trolox (standard) equivalent antioxidant capacity (TEAC), was 0.417±0.012. A dose dependent hydroxyl radical as well as hypochlorous radical scavenging activity was observed and both these activities were higher as compared to the respective standard	Ghate et al., (2013)

	compounds - Mannitol and ascorbic acid. It has also been shown that PRME moderately scavenged superoxide, singlet oxygen radicals and DPPH (2,2-diphenyl-1-picrylhydrazyl), a stable free radical.	
Hexane extract of *Ramalina roesleri* and nine isolated compounds - Atranorin, protolichesterinic acid, usnic acid, 2-hydroxy-4-methoxy-6-propyl benzoic acid, homosekikaic acid, sekikaic acid, benzoic acid, 2,4-dihydroxy-6-propyl and 2,4-dihydroxy-3,6-dimethyl benzoate	This study measured antioxidant capacity of hexane extract of *R. roesleri* and nine different isolated compounds. The DPPH radical scavenging activity of extract ranged from 29.42% to 87.90%. Among the isolated compounds activity sekikaic acid exhibited maximum DPPH radical scavenging followed by homosekikaic acid.	Sisodia et al., (2013)
Methanol and chloroform extracts of *Umbilicaria cylindrica*	This study demonstrated the antioxidant activities of methanolic and chloroform extracts of *U. cylindrical* as measured by DPPH and hydroxyl radical scavenging, chelating activity, and inhibitory activity towards lipid peroxidation. Both methanol and chloroform extracts had a good total phenolic contents as compared to Gallic acid (Standard). Similarly the methanolic and chloroform extracts possess antioxidant activity, with total antioxidant capacity of 74.65 ±0.75µg AA/g and 71.32±0.87 µg AA/g, respectively (AA= Ascorbic acid). The DPPH scavenging activity showed that both tested extracts were able to scavenge this radical but the chloroform extract displayed a higher activity than methanol extract.	Manojlovic et al. (2012b)
Methanol extracts of the lichen species *Parmelia sulcata, Flavoparmelia caperata, Evernia prunastri, Hypogymnia physodes* and *Cladonia foliacea*	This study evaluated the antioxidant activities of methanol extracts of five different lichen extract collected from southeast of Serbia by measuring their DPPH radical scavenging activity. The results showed that *Hypogymnia physodes* had the highest phenolic content and the strongest DPPH radical scavenging effect.	Mitrović et al., (2011)

Ramalin from *Ramalina terebrata*	This study compared antioxidant capacity of ramalin isolated from *R. terebrata* against various commercially available standards. The results showed that ramalin was five times more potent compared to butylated hydroxyanisole (BHA) in scavenging 1-diphenyl-2-picryl-hydazil (DPPH) free radicals, 27 times more potent in scavenging 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid free radicals ($ABTS^{\bullet+}$) than trolox, the vitamin E analogue used commercially for antioxidant, and 2.5 times more potent than BHT (Butylated hydroxytoluene) in reducing Fe3+ to Fe2+ ions. Similarly, ramalin was 1.2 times more potent than ascorbic acid in scavenging superoxide radicals. It was also shown that ramalin is non-toxic to human keratinocyte and fibroblast cells at its antioxidant concentration	Paudel et al., (2011)
Hot water extracts of *Lethariella sernanderi, L. sinensis* and *L. cashmeriana* and four isolated compounds	The antioxidant activities of hot water extracts and isolated compounds were evaluated on the basis of Cu(II) reducing activity and compared with quercetin (standard). Compound 3 (7-chlororubrocashmeriquinone) showed the strongest activity among the four isolated compounds suggesting that the 1,2-quinone and 7-Cl moieties are important for antioxidant activity. The hot water extracts of *L. sernanderi* had the highest antioxidant values among the crude extracts.	Kinoshita et al. (2010)
15 individual compounds extracted from *Usnea articulata*	This study reports the antioxidant activities of two new β-orcinol depsidones together with 13 known compounds were isolated from *U. articulata*. The newly reported β-orcinol depsidones along with fumarprotocetraric acid exhibited moderate antiradical activity DPPH assay. Two depsidones showed better superoxide anion scavenging activity compared to the standard quercetin.	Lohézic-Le Dévéhat et al., (2007)

Conclusion

Although lichens are one of the more promising reservoirs of low-molecular weight secondary compounds demonstrating some level of biological activity; a very limited number of compounds have been studied (Boustie & Grube, 2005). Hence, there is an urgent need for: 1) continued screening of lichen metabolites across their diversity, 2) more in-depth studies of those compounds that have already shown promising activity against pathogenic bacteria and/or various cancer cell lines, 3) clinical trials for those compounds that have shown significant activity, and finally 4) commercial production and implementation of effective drug lines.

One of the main issues related to the limited use of lichens compound in modern medicine is related to their slow growth rate and challenges with *in vitro* propagation. However, with recent advancements in technology, culturing lichens in the laboratory is achieving greater success (Behera et al., 2006; Stocker-Wörgötter, 2001; Stocker-Wörgötter & Elix, 2002; Yamamoto et al., 1993; Yamamoto et al., 1987; Yamamoto et al., 1985). Similarly, Miao et al. (2001) reviewed the possibilities of using molecular genetics techniques as an alternative approach for exploring the diversity of polyketide biosynthetic pathways in lichens. This approach can be extended to examine other pathways which can then be integrated with conventional culture methods. Also according to Miao et al. (2001) lichen genes can be introduced into a surrogate host with good fermentation characteristics and a well characterized endogenous chemical profile like *Aspergillus nudulans, Neurospora crassa, Saccharomyces cerevisiae, Escherichia coli, Streptomyces* spp. etc. to produce promising lichen metabolites in larger quantities. Furthermore, researchers have now been able to synthesize usnic acid in the laboratory from commercially available starting materials. The synthesis involves the methylation of phloracetophenone followed by oxidation with horseradish peroxidase (Hawranik et al., 2009). This work also provided the impetus for synthesizing other lichen metabolites. With

these recent advancements in technology, the development of cost effective options for growing and harvesting lichen metabolites commercially as a source of effective drugs against pathogenic bacteria and various forms of cancer show real promise.

Chapter 3: Screening antibiotic properties of acetone and methanol extracts of thirty-six lichen species

Introduction

The development of antibiotics in the 1940s provided a powerful tool for addressing bacterial infections. However, due to the extensive use and misuse of antibiotic drugs in the clinic, community, animal husbandry, and agriculture there is increased reemergence, diversification, and spread of various resistant pathogenic bacteria (Andersson & Hughes, 2012). Despite the need to effectively address antibiotic resistance, the discovery of new antibacterial drugs has declined in recent years. The main reason behind the decline is largely because before many drugs come to the market pathogens have already become resistant. The development of antibiotic resistant variants of bacterial strain has severely impacted a drug's long-term potential to return a profit (Leeb, 2004). These issues emphasize the increasing importance of investigating and developing new classes of antibiotics that are either opaque to pathogen resistance or express a new mode of action, hence increasing the potential lifetime of the antibacterial class (Kokubun et al., 2007).

Natural products continue to make a significant contribution to modern drug discovery efforts (Newman & Cragg, 2007). Although more than 300 natural metabolites with antimicrobial activities were reported between 2000 and 2008 (Saleem et al., 2010), many potential sources of drug therapies still need to be investigated. Among them lichens are.

In this chapter the inhibitory activities of acetone and methanol extracts of 36 species of lichens, collected from various locations in the United States, against four pathogenic bacterial strains have been reported.

Materials and methods

Collection of Lichen species

Thirty-six species of lichens were collected from various locations (June, 2010 to Aug, 2011) throughout the United States (Table 3.1). All voucher specimens have been deposited in the Herbarium of Nonvascular Cryptogams at Brigham Young University (BRY-C) in Provo, Utah, USA.

Extraction of lichen metabolites

Dried, cleaned lichen materials (4 g) were obtained from each of the 36 study species and ground in liquid nitrogen. The ground samples were then extracted sequentially with acetone and methanol. Sample extracts were dried under a stream of nitrogen gas, to reduce oxidation of metabolites, and then dissolved in DMSO to a final concentration of 16 mg/ml. The stock solution was stored at -20°C.

Identifying lichen metabolites

Lichen chemicals were identified using standard thin layer chromatography (TLC) techniques in solvent system G (Orange et al., 2001). Solvent system G includes 139 ml toluene, 83 ml ethyl acetate, and 8 ml formic acid and was used to process the acetone extracts from all lichen samples. The identity of individual lichen chemicals was inferred by comparison with the published Rf values of known lichen secondary metabolites. (Orange et al., 2001). Usnic and vulpinic acids were used as standards.

Microbial Cultures

In this study, lichen extracts were tested against four strains of bacteria, namely *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 6538P), and methicillin-resistant *Staphylococcus aureus* (S. aureus COL).

Micro-well Dilution assay

To quantify the biological activity of each lichen extract, minimum inhibitory concentration (MIC) values were determined against all four bacterial strains using the micro-well dilution assay method. Inocula were prepared by incubating a single colony of each bacterial strain in 10 ml of Muller Hinton Broth (Sigma Aldrich, St. Louis, MO) at 37°C for 24 h. Serial dilutions of each lichen extract were prepared using Muller Hinton Broth with extract concentrations ranging from 500-3.9 µg/ml in a 24-well plate. Four µl of an overnight culture of one of the 4 bacterial strains was then added to individual wells. Then 100 µl of each extract concentration was transferred to a 96-well plate in triplicate. Gentamycin and DMSO of equivalent concentrations were used as a positive and vehicle control respectively. The plate was then incubated for 24 h at 37°C. Following incubation 60 µl of p-iodonitrotetrazolium violet (INT; Sigma-Aldrich, St. Louis, MO) was added to each well. Living bacteria will reduce the INT dye and change the color of the solution from colorless to pink. The concentration at which there was no reduction of INT represents the MIC value (Mann & Markham, 1998).

Table 3.1: List of 36 Lichen species with their collection site information.

Lichens	Collection Site
Alectoria imshaugii Brodo & D. Hawksw.	Montana, Misoula Co., Flathead National Forest, vicinity of Smith creek trailhead, near Bob Marshall wilderness area boundary. 47° 33’ 11.18” N, 113° 40’ 2.64” W
Alectoria sarmentosa (Ach.) Ach.	Montana, Mission Mountain Wilderness Area, Cold lake trailhead, 47° 33’ 39.6” N, 113° 51’ 19.5” W

Bryoria fuscescens (Gyelnik) Brodo & D. Hawksw.	Washington, Yakima County, Wenatchee National Forest, Vicinity of Clear creek falls, in mixed conifer forest, 46° 39' 23.652" N, 121° 21' 7.524" W
Cladonia furcata (Hudson) Schrader	Tennessee, Carter County, Cherokee National Forest, Along Appalachian trail, 36° 10' 15.3" N, 82° 5' 57.78" W
Evernia prunastri (L.) Ach.	Washington, Klickitat County, North side of Columbia River Gorge, in mixed oak/ponderosa forest, along Courtney Road (off Highway 14, 2.6 miles East of Bingen, 45° 42' 0.2874" N, 121° 24' 27.18" W
Everniastrum catawbiense (Degel.) Hale ex Sipman	Tennessee, Carter County, Cherokee National Forest, Along Appalachian trail, 36° 6' 24.84" N, 82° 6' 32.46" W
Flavocetraria nivalis (L.) Kärnefelt & Thell	Montana, Granite Co., Anaconda Pintler Wilderness Area, Goat flat 46° 03’ 15.72” N 113° 16’ 38.22” W
Hypogymnia physodes (L.) Nyl.	Montana, Lewis and Clark Co., Lewis and Clark National Forest, vicinity of south fork of sun river trailhead 47° 30’ 9.84” N 112° 53’ 19.74” W
Letharia columbiana (Nutt.) J. W. Thomson	Idaho, Elmore Co., Sawtooth national recreational area, along Boise river, vicinity of Power plant recreational area (Near Atlanta) 43° 48’ 59.22” N 115° 05’ 57.18” W
Letharia vulpina (L.) Hue	Idaho, Blaine County, Sawtooth National Recreational Area, Vicinity of Alpine creek trailhead, 43° 53’ 53.04”N, 114° 54’25.62” W
Lobaria pulmonaria (L.) Hoffm.	Idaho, Idaho County, Clearwater National Forest, Along USFS RD #362, South of fish hatchery, 46° 30’ 23.46” N, 114° 40’ 52.08” W
Masonhalea richardsonii (Hooker) Kärnefelt	Alaska, Fairbanks North Star County, Alpine tundra at Eagle summit, north-west of parking area, Steese Highway, 65° 29’ 12.12” N, 145° 24’ 55.08” W
Parmelia sulcata Taylor	Montana, Misoula Co., along Holland falls trail, near boundary of Bob Marshall Wilderness Area 47° 27’ 10.98”N 113° 36’ 16.98” W
Parmotrema reticulatum (Taylor) M. Choisy	Montana, Mission Mountain Wilderness Area, Crystal lake trailhead, 47° 20’ 1.38” N, 112° 43’ 48.24”W
Peltigera aphthosa (L.) Willd.	Montana, Powell County, Near Bob Marshall Wilderness Area boundary, Vicinity of Pyramid Pass Trailhead and trail, 47° 15’ 34.98” N, 113° 25’ 29.28” W

Platismatia glauca (L.) W. L. Cul. & C. F. Culb.	Montana, Mission Mountain Wilderness Area, Crystal lake trailhead, 47° 20’ 1.38” N, 112° 43’ 48.24”W
Ramalina menziesii Taylor	California, Monterey County, Hasting Natural History Reservation, 38601 E. Carmel Valley Road, Carmel Valley, 36° 22' 45.47" N, 121° 33' 58.47" W
Ramalina sinensis Jatta	Utah, Utah Co., Uinta NF., Along filth water hotspring, about 0.3 miles from trailhead, 40° 4’ 55.49” N 111° 21’ 8.32” W
Rhizoplaca chrysoleuca (Sm.) Zopf	Utah, Daggett County, Ashley National Forest, Fleming Gorge National Recreational Area, Along Highway 191 at mile marker, 401, 40° 57' 53.24" N, 109° 32' 0.17" W
Rhizoplaca haydenii (Tuck.) W. A. Weber	Wyoming, Sweetwater County, Ashley National Forest, Fleming Gorge National Recreational Area, Along USFS Road # 146 (Lucerne valley Recreational Road), 41° 0' 14.616" N, 109° 32' 11.2914" W
Rhizoplaca idahoensis Rosentreter & McCune	Tennessee, Carter County, 9 miles south from Roan Mountain State Park (TN 143S/Roan Road), Near Tennessee and North Carolina boarder Along Appalachian trail, 36° 6' 28.38" N, 82° 6' 28.02" W
Rhizoplaca marginalis (Hasse) W. A. Weber	California, Inyo County: Death Valley National Monument, 5.64 km northeast of Scotty's Caste; northeast cliffs face.
Rhizoplaca melanophthalma (DC.) Leuckert & Poelt	Utah, Daggett County, Ashley National Forest, Fleming Gorge national recreational area, vicinity of Red Canyon visitor center 40° 53’ 31.32” N, 109° 33’ 37.68” W
Rhizoplaca peltata (Ramond) Leuckert & Poelt	Wyoming, Sweetwater County, Ashley National Forest, Fleming Gorge National Recreational Area, Along USFS Road # 146 (Lucerne valley Recreational Road), 41° 0' 14.616" N, 109° 32' 11.2914" W
Sphaerophorus globosus (Hudson) Vainio	Oregon, Hood Co., Mount Hood Nation Forest, vicinity of Tamanawas Falls Trailhead, on east fork of Hood River, in conifer forest 45° 23’ 49.34” N 121° 34’ 19.16” W
Thamnolia vermicularis (Sw.) Ach. ex Schaerer	Montana, Granite Co., Anaconda Pintler wilderness area, Goat flat, 46° 3' 15.72" N, 113° 16' 38.22" W
Tuckermanopsis ciliaris (Ach.) Gyelnik	Tennessee, Carter County, 9 miles south from Roan Mountain State Park (TN 143S/Roan Road), Near Tennessee and North Carolina boarder Along Appalachian trail, 36° 6' 24.84" N, 82° 6' 32.46" W
Umbilicaria americana Poelt & T. H. Nash	Idaho, Custer County, Sawtooth National Forest, Sawtooth National Recreational Area, Along USFS trail no. 101; at large granite boulders. 44° 4’.45.06” N, 114° 58’ 57.42”

Umbilicaria mammulata (Ach.) Tuck.	Tennessee, Carter County, Roan Mountain State Park, Raven Rock Trail, 36° 9' 57.54" N, 82° 5' 30.54" W
Usnea hirta (L.) F. H. Wigg.	Colorado, Garfield County, White River National Forest, Along Cline Top Road, Forest Service Road 603, 39°40' 23.30" N, 107° 33' 23.07" W
Usnea lapponica Vainio	Colorado, Garfield County, White River National Forest, Along Cline Top Road, Forest Service Road 603, 39°40' 23.30" N, 107° 33' 23.07" W
Usnea strigosa (Ach.) Eaton	Tennessee, Carter County., Near the vicinity of Roan Mountain State Park visitor center, 36° 10' 14.04" N, 82° 5' 53.3" W
Vulpicida canadensis (Räsänen) J.-E. Mattsson & M. J. Lai	Oregon, Clackamas County, In Ponderosa forest near small stream along Highway 26, about 20 miles North of Warm Springs 45° 0' 8.064" N, 121° 29' 32.3874" W
Xanthoparmelia chlorochroa (Tuck.) Hale	Colorado, Routt County, Way to Kremmling from Toponas, near the junction of Highway 134 and State Road # 206, 40° 4' 37.236" N, 106° 39' 43.776" W
Xanthoparmelia coloradoënsis (Gyelnik) Hale	Colorado, Rio Blanco County, White River National Forest, Along North Fork Road (205) Near the Trailhead Outlet, 40° 0' 9.0" N, 107° 13' 53.004" W
Xanthoparmelia wyomingica (Gyelnik) Hale	Montana, Granite Co., Anaconda Pintler wilderness area, Goat flat, 46° 3' 15.72" N, 113° 16' 38.22" W

Results

Identification of lichen metabolites

Major chemical compounds from lichen extracts are reported in Table 3.2.

Table 3.2: Lichen compounds identified using Thin Layer Chromatography

Lichens	Compounds
Alectoria imshaugii	Usnic acid, Squammatic acid, Thamnolic acid
Alectoria sarmentosa	Usnic acid, Barbatic acid, Squammatic acid, Thamnolic acid
Bryoria fuscescens	Fumarprotocetratic acid, Norstictic acid, Protocetraric acid
Cladonia furcata	Fumarprotocetraric acid, Protocetraric acid
Evernia prunastri	Atranorin, Evernic acid
Everniastrum catawbiense	Atranorin, Gyrophoric acid
Flavocetraria nivalis	Usnic acid
Hypogymnia physodes	Barbatic acid
Letharia columbiana	Vulpinic acid

Letharia vulpina	Vulpinic acid
Lobaria pulmonaria	Norstictic acid, Stictic acid Constictic acid, Cryptostictic acid, Confumarprotocetraric acid
Masonhalea richardsonii	Alectoronic acid
Parmelia sulcata	Atranorin, Salazinic acid, Consalazinic acid
Parmotrema reticulatum	Atranorin
Peltigera aphthosa	Teniorin, Triterpenes
Platismatia glauca	Atranorin, Caperatic acids
Ramalina menziesii	Usnic acid
Ramalina sinensis	Usnic acid
Rhizoplaca chrysoleuca	Usnic acid
Rhizoplaca haydenii	Usnic acid
Rhizoplaca idahoensis	Usnic acid
Rhizoplaca marginalis	Usnic acid
Rhizoplaca melanophthalma	Usnic acid, Psoromic acid, Constipatic acid, Dendroconstipatic acid, Subpsoromic acid
Rhizoplaca peltata	Usnic acid, Atranorin, Pannarin
Sphaerophorus globosus	Phaerophorin
Thamnolia vermicularis	Thamnolic acid, Baeomycesic acid
Tuckermanopsis ciliaris	Protolichesterinic acid
Umbilicaria americana	Gyrophoric acid
Umbilicaria mammulata	Gyrophoric acid
Usnea hirta	Usnic acid, Salazinic acid, Consalazinic acid
Usnea lapponica	Usnic acid
Usnea strigosa	Usnic acid, Norstictic acid, Gallbinic acids
Vulpicida canadensis	Usnic acid, Vulpinic acid, Zeorin
Xanthoparmelia chlorochroa	Usnic acid, Salazinic acid, Consalazinic acid, Norstictic acid
Xanthoparmelia coloradoënsis	Usnic acid, Norstictic acid, Stictic acid, Constictic acid
Xanthoparmelia wyomingica	Usnic acid, Salazinic acid, Consalazinic acid, Norstictic acid

Antimicrobial activities

MIC values for acetone and methanol extractions of the 36 lichen species against the four different bacterial strains are reported in Table 3.3. Most of the lichen extracts were found to be active against three of the four bacteria, *P. aeruginosa, S. aureus*, and *S. aureus* COL, while extracts from only three lichens, *Letharia columbiana*, *L. vulpina*, and *Vulpicida canadensis* were active against *E. coli* (Table 3.3). Acetone extractions were found to be more active than

methanol extractions. MIC values for gentamycin against *E. coli*, *S. aureus*, and *P. aeruginosa* were all 3.5 µg/ml and for methicillin-resistant *S. aureus* the MIC was 10 µg/ml. The vehicle control (DMSO) did not show any activity against the four bacterial strains.

Table 3.3: Minimum Inhibitory Concentration (MIC) values (µg/ml) for different lichens against four bacteria (A = Acetone; M = Methanol)

Lichens	*E. coli*		*P. aeruginosa*		*S. aureus*		*MRSA*	
	A	M	A	M	A	M	A	M
A. imshaugii	-	-	31.25	15.6	62.5	62.5	250	125
A. sarmentosa	-	-	31.25	15.6	62.5	31.25	125	62.5
B. fuscescens	-	-	125	-	125	-	500	-
C. furcata	-	-	250	-	500	-	500	-
E. prunastri	-	-	31.25	125	62.5	250	125	500
E. catawbiense	-	-	250	-	125	-	500	-
F. nivalis	-	-	31.25	15.6	62.5	31.25	500	62.5
H. physodes	-	-	62.5	250	62.5	62.5	62.5	250
L. columbiana	50	-	125	-	125	500	31.25	125
L. vulpina	125	500	125	500	3.9	15.6	31.25	125
L. pulmonaria	-	-	-	-	-	-	-	-
M. richardsonii	-	-	250	500	125	125	125	250
P. sulcata	-	-	125	-	250	-	125	-
P. reticulatum	-	-	250	-	250	-	250	-
P. aphthosa	-	-	250	-	-	-	-	-
P. glauca	-	-	250	-	500	-	500	-
R. menziesii	-	-	15.6	62.5	15.6	125	250	500
R. sinensis	-	-	15.6	250	15.6	-	62.5	-
R. chrysoleuca	-	-	7.8	125	7.8	250	15.6	500
R. haydenii	-	-	3.9	31.25	15.6	31.25	15.6	62.5
R. idahoensis	-	-	7.8	31.25	15.6	125	15.6	125
R. marginalis	-	-	7.8	125	7.8	250	7.8	500
R. melanophthalma	-	-	15.6	62.5	15.6	125	31.25	250
R. peltata	-	-	15.6	62.5	31.25	250	31.25	250
S. globosus	-	-	7.8	31.25	62.5	500	62.5	500
T. vermicularis	-	-	31.25	125	125	500	500	500
T. ciliaris	-	-	125	-	62.5	250	250	500
U. americana	-	-	500	-	500	-	-	-
U. mammulata	-	-	500	-	-	-	-	-
U. hirta	-	-	3.9	15.6	7.8	31.25	7.8	62.5
U. lapponica	-	-	15.6	15.6	15.6	31.25	15.6	62.5

U. strigosa	-	-	3.9	31.25	7.8	62.5	15.6	250
V. canadensis	250	-	15.6	15.6	15.6	62.5	31.25	125
X. chlorochroa	-	-	3.9	62.5	7.8	62.5	31.25	-
X. coloradoënsis	-	-	7.8	62.5	7.8	250	15.6	500
X. wyomingica	-	-	15.6	15.6	15.6	62.5	62.5	500

Discussion

This study provides data based on the first broad-scale screening of lichens collected from different parts of the United States against four different pathogenic bacteria.

Since Burkholder et al. (1944) first reported on the antibiotic properties of lichens, a number of studies have investigated the antimicrobial activities of several lichen species against various Gram positive, Gram negative and mycobacteria (Burkholder & Evans, 1945; Hobbs, 1986; Ingólfsdóttir et al., 1985; Kokubun et al., 2007a; Lauterwein et al., 1995c; Lawrey, 1989; Manojlovic et al., 2011; Vartia, 1950). More specifically, studies have shown that lichen compounds either in crude extract or as purified compounds are not active against gram negative bacteria such as *E. coli* and *P. aeruginosa* (Francolini et al., 2004; Ingólfsdóttir, 2002; Lauterwein et al., 1995; Melgarejo et al., 2008; Paudel et al., 2012; Yilmaz et al., 2005). In contrast, there are other studies that show that lichen extracts are active against *E. coli* (Hoskeri et al., 2010; Manojlovic et al., 2011; Rankovič et al., 2010) and *P. aeruginosa* (Hoskeri et al., 2010; Ingólfsdóttir et al., 1985; Rankovič et al., 2010; Srivastava et al., 2013). In this study we found most of our lichen extracts inhibited the growth of the gram negative bacterium *P. aeruginosa* and extracts from three lichen species, *L. columbiana, L. vulpina*, and *V. canadensis*, were also effective against *E. coli*. Each of these three lichen species produce vulpinic acid as a principal secondary metabolite, suggesting that vulpinic acid may be active against E. coli. To our knowledge there are no data available regarding the antibiotic effects of crude extracts of Letharia or Vulpicida species or purified vulpinic acid against E. coli except (Lauterwein et al.,

1995b). However, they were not able to document the antibiotic effects of vulpinic acid against *E. coli* at their highest concentration of 32μg/ml. In contrast, in our study, using higher concentrations of lichen extracts containing vulpinic acid (MIC = 125-250 μg/ml), the growth of *E. coli* was inhibited. This variation in the results among different studies may be due to a combination of factors, including extraction of different lichen species, the solvent used for extraction, and the specific bacterial strain. Additional research is required to determine the specific factors influencing antimicrobial properties of lichen extracts.

Our data show that the vast majority of lichen extracts inhibited the drug sensitive strains of the gram positive bacterium *S. aureus* in at least one of the tested concentrations. These results against *S. aureus* are similar to many other studies (Gulluce et al., 2006; Paudel et al., 2008; Paudel et al., 2012; Srivastava et al., 2013). We found that all crude extracts of the tested lichen species except for *L. pulmonaria* and *U. mammulata* were found to be active against S. aureus. Out of the 32 active extracts against *S. aureus*, acetone extracts of 13 species were generally more active with MIC values lower than 16 μg/ml, with *L. vulpina* having the lowest MIC value at 3.9 μg/ml (Table 3.3). All sampled lichens that were found to be more active against *S. aureus* contained usnic acid as the major compound, with the exception of *L. vulpina* and *V. canadensis* which contain vulpinic acid as the major metabolite. A number of previous studies have also produced results similar to ours, i.e. lichens producing usnic acid demonstrate higher inhibition against *S. aureus* (Cocchietto et al., 2002b; Ingólfsdóttir, 2002; Lauterwein et al., 1995; Ranković et al., 2008).

We also found that most of our lichen extracts were not only capable of inhibiting the growth of sensitive strains of *S. aureus*, but also a methicillin-resistant strain of *S. aureus*. Various studies have shown similar results (Kokubun et al., 2007; Pompilio et al., 2013).

Extracts from 9 of our tested lichen species showed relatively low MIC values (<16 μg/ml) against the methicillin resistant *S. aureus* and extracts from *R. marginalis* and *U. hirta* had the lowest values at 7.8 μg/ml. Similarly 16 of our lichen extracts showed lower MIC values (<16 μg/ml) against *P. aeruginosa* while extracts from *U. hirta*, *U. strigosa*, *R. haydenii*, and *X. chlorochroa* were most effective with MIC values of 3.8 μg/ml (Table 3.3). Lichen extracts of species with usnic acid as a major chemical component consistently showed significant higher activity against *S. aureus, P. aeruginosa*, as well as the methicillin-resistance *S. aureus*. Although both the acetone and methanol extractions demonstrated activity against all of the bacterial strains except *E. coli*, the acetone extraction was more active than the methanol extraction. Several studies (Ranković et al., 2007; Turk et al., 2003; Yilmaz et al., 2005) have reported the same pattern. Hence it can be concluded that the solvent used in the extraction process has an effect on the inhibitory strength of the lichen compounds. This phenomenon may be due to the extraction efficiency of the solvent.

Conclusions

Our study provides evidence that North American lichens represent a potentially important source of future antibiotic drugs. Our research provides specific evidence indicating that lichen extracts are effective against both drug sensitive and drug resistant strains of *S. aureus*. In particular, extracts from *Letharia vulpina*, *Letharia columbiana*, and *Vulpicida canadensis* were effective against all bacterial strains tested in this study. These three species clearly merit further investigation in order to determine their mode of action against bacterial pathogens and also their levels of cytotoxicity against normal cells.

Chapter 4: Lichen compound targets cell membrane and cell division processes in methicillin-resistant *Staphylococcus aureus*

Introduction

Antibiotics are naturally-occurring compounds that act specifically to disable vital cellular functions like cell wall synthesis, cell membrane integrity, and protein synthesis (Mobashery & Azucena, 2002; Wilson et al., 2011). However, the overuse and frequent misuse of these 'miracle drugs' have strongly selected for drug-resistant strains, as well as given place to greater incidence of opportunistic and nosocomial infections. One common and frightening example of an antibiotic-resistant human pathogen is Methicillin-resistant *Staphylococcus aureus* (MRSA).

The discovery of new antibacterial drugs has diminished in recent years. The development of drug-resistant isolates has severely impacted a drug's long-term potential to return a profit (Leeb, 2004). Hence, it is necessary to investigate and develop new classes of antimicrobials that are either opaque to pathogen resistance or express a new mode of action, thereby increasing the potential lifetime of the antibacterial agent (Kokubun et al., 2007). Natural products derived from plants, fungi, marine organisms, and microorganism have served as a rich source of novel biologically active compounds. According to Saleem et al. (2010), more than 300 new natural metabolites with antimicrobial activities were reported between 2000 and 2008, but there are still many compounds that deserve thorough investigation for their medicinal properties. Hence, there is a growing interest in phytomedicine, and many medicinally important plants are being investigated for new antimicrobial agents (Gupta et al., 2012).

Although the antimicrobial properties of lichen compounds have been well documented (Kantheti et al., 2012; Pompilio et al., 2013; Shrestha & St. Clair, 2013b; Stojanovic et al.,

2012). However, information regarding the mode of action of these compounds against pathogenic bacteria is very limited. To our knowledge, there is only one published report on the mechanism of action of lichen-derived compounds (Gupta et al., 2012). This study reports the membrane-damaging potential of usnic acid in MRSA.

In this study the mode of action of the crude extracts of *L. vulpina* against MRSA has been examined by testing bacteriolytic, bactericidal, and membrane damaging potential of the extracts.

Materials and methods

Bacterial strain.

MRSA COL used in this study was kindly provided by Dr. Bryan Wilkinson, University of Illinois.

Lichen materials.

Acetone extract of *L. vulpina* was used in this study. The extraction and identification of lichen compounds have been described in chapter 2.

Minimum inhibitory concentration (MIC) assays.

The MIC for *L. vulpina* extracts was 31.25 µg/ml. The results have been published in (Shrestha et al., 2014). In this study, we tested three different concentrations (1x MIC, 5x MIC, and 10x MIC) in an effort to determine the mode of action of the crude extract.

Bacteriolysis.

The effect of *L. vulpina* extract on cell lysis was evaluated by measuring the OD_{660} of treated bacteria at various time points, in accordance with the method described by (Isnansetyo & Kamei, 2003) with slight modification. Briefly, overnight cultures of MRSA were prepared by

inoculating 10 ml of Müller Hinton Broth (Sigma Aldrich, St. Louis, MO) with a single MRSA colony, and incubating at 37 °C with shaking (200 rpm). Then, the culture was diluted with Müller Hinton broth to an OD_{660} of approximately 0.1. The bacterial suspensions were treated with three different concentrations (1x MIC, 5x MIC, and 10x MIC) of *L. vulpina* extract. Immediately, samples were taken and the OD_{660} was measured. Additional samples were taken and the ODs were measured at 4, 8, 12, 16, and 24 h. Corresponding dilutions of lichen extract were used as blanks.

Time kill assay

The antimicrobial activities of *L. vulpina* crude extract against MRSA were evaluated by measuring the reduction in the numbers of colony forming unit (CFU) over 24 h. Cell suspensions of MRSA were prepared and treated with lichen extracts as described above. Viability of bacteria was assessed at various time points (0, 8, 16, and 24 h) by spread-plating diluted bacterial suspensions onto Müller Hinton agar plate. After 24 h of incubation at 37 °C, the colonies were counted and data were presented as CFU (% of control) = (number of CFU lichen extracts/number of CFU control) ×100 (Andra et al., 2008).

Propidium iodide uptake assay

The effect of crude extract of *L. vulpina* on MRSA membrane permeability was studied by measuring propidium iodide uptake by bacterial cells. Cell suspensions of MRSA, prepared as described above, were treated for 24 hours with various concentrations of the extract. The protocols of (Nobles et al., 2013) were followed with slight modification. Briefly, after 24 h of treatment, the cells were pelleted by centrifugation and washed once with 1x phosphate buffer solution (PBS). Ten-fold dilutions of the cell suspensions were made in PBS, and propidium iodide (50 µg/ml) was added to the cells. A 200 µl aliquot of the cell suspension was transferred

in triplicate to a 96 well plate, which was incubated at room temperature for 15 min in the dark. Florescence was measured at excitation and emission wavelengths of 535 and 625 nm, respectively, using a Synergy HT Multi-Mode Microplate reader (BioTek, Winooski, VT, USA).

Transmission electron microscopy.

In an effort to better understand the membrane damaging properties of these lichen extract, transmission electron microscopy (TEM) was performed. MRSA suspensions were treated with the extract of *L. vulpina* at 10 times the MIC for 24 h at 37°C. This concentration was chosen in an effort to see the maximum effect on a large percentage of cells. After treatment, the bacterial cells were pelleted and embedded in 1% low melting point agarose. The cells were then fixed with 2.5% buffered glutaraldehyde for 1 h, washed with washing buffer, and then post-fixed in osmium tetroxide for 1 h before final staining en bloc with 1% uranyl acetate overnight. The buffer used was 0.1M sodium cacodylate, pH 7.4. After overnight staining, cells were dehydrated in a graded series of acetone, and embedded in epoxy resin. Ultra-thin sections were prepared and stained with 1% uranyl acetate and lead citrate. Microscopy was performed with a Tecnai F30 microscope, under standard operating conditions.

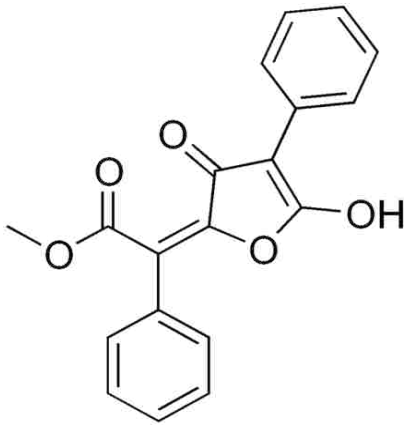
Statistical analysis.

All experiments were repeated three times with at least two replicates for each experiment. The results are reported as mean values ± SD of the three independent runs. One-way analysis of variance and Dunnett's multiple comparison was applied to determine the significance of differences in all measured parameters. Statistical analyses were performed using GraphPad Prism 6 software.

Results

Extrolites of L. vulpina extract:

To identify compounds present in the extract of *L. vulpina* thin layer chromatography of the acetone extracts was performed. Results showed that *L. vulpina* contained exclusively vulpinic acid (Fig 4.1).



Vulpinic acid

Figure 4.1: Molecular structure of vulpinic acid identified with thin layer chromatography, from acetone extracts of *L. vulpina*

Minimum inhibitory concentration

This study is a follow up study of two previously published studies (Shrestha et al., 2014; Shrestha & St. Clair, 2013a), wherein acetone and methanol extracts of 36 different lichen species were screened for activity against four different pathogenic bacteria. In this study we selected acetone extract of *L. vulpina* to study their mode of action against MRSA. The MIC value reported for the extract was 31.25µg/ml. We used three different concentrations: 31.25, 156.25, and 312.5 µg/ml of lichen extracts which represented 1x, 5x, and 10x the MIC, respectively.

Bacteriolysis assay

To assess whether the tested lichen extracts have bacteriolytic effect or not, we measured the OD_{660} of the treated bacterial culture at various time points. The results obtained from measuring OD_{660} revealed a dose and time dependent response by MRSA (Fig 4.2). When MRSA was treated with 1x MIC and 5x MIC concentrations of *L. vulpina* extract, there was an increase in the OD_{660}. However, these increases in the OD_{660} were still lower than the controls. The OD_{660} remained unchanged throughout the experiment when using the 10x MIC of either lichen extract.

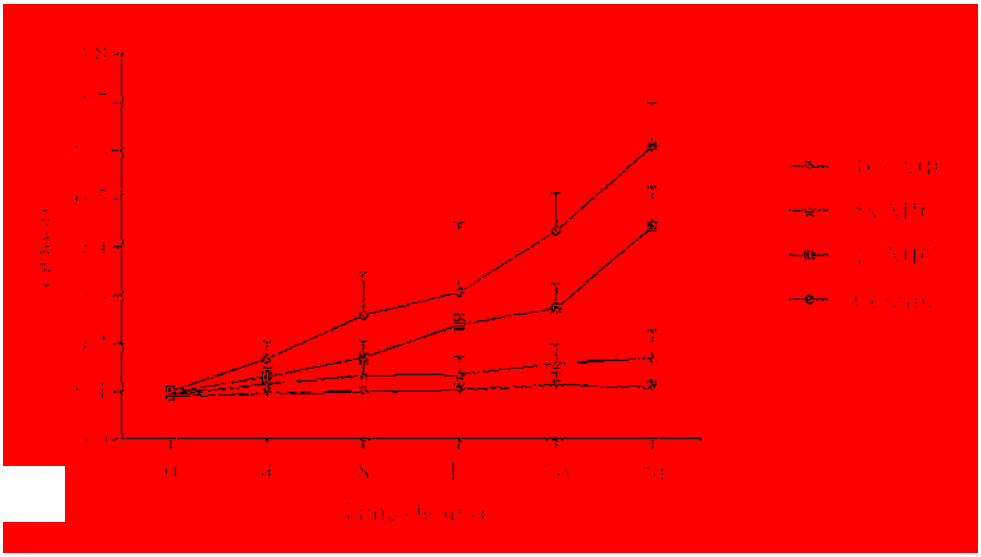


Figure 4.2: Bacteriolytic activity of *L. vulpina* extract using MRSA.

Bacteriocidal assay

Bacterial cell killing kinetics by lichen extracts were assessed by plating out treated bacterial suspensions and counting the number of viable cells at different time points. The treatment of MRSA with the extract showed a reduction in CFU when compared to controls (Fig 4.3). However, the bacterial cell killing kinetics was relatively slow. We observed that there was

34%, 77%, and 91% reduction in the CFU when treated with 1x MIC, 5x MIC, and 10x MIC of *L. vulpina* extract, respectively.

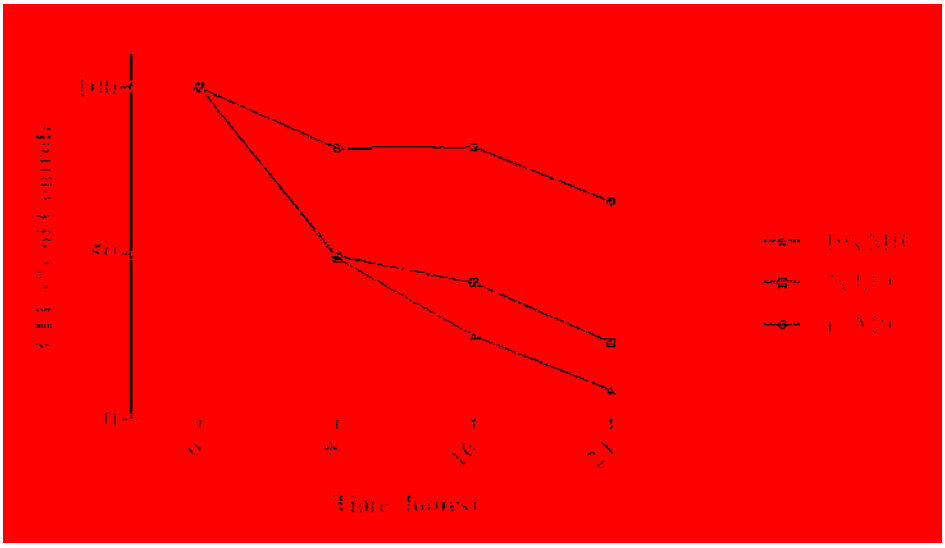


Figure 4.3: Kill kinetics of lichen extracts on MRSA.

Propidium Iodide (PI) uptake assay

To test whether lichen extracts cause membrane instability, we added PI after 24 h of treatment. Propidium iodide, when associated with DNA, fluoresces intensely and its use in this context would indicate if the membrane became permeable after lichen extract exposure (Arndt-Jovin & Jovin, 1989). Our results showed that there was a significant increase ($p < 0.05$) in the florescence value when MRSA were treated with 1x MIC, 5x MIC, and 10x MIC of the lichen extracts (Fig 4.4).

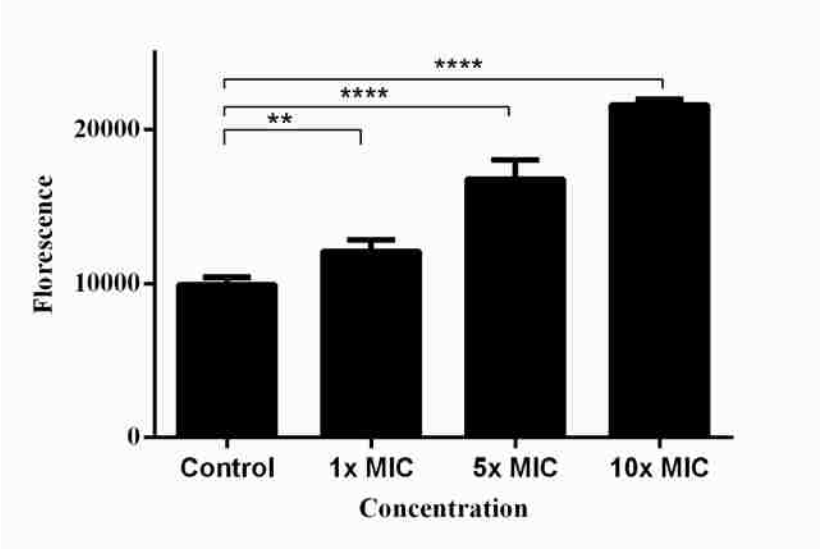


Figure 4.4: Propidium Iodide uptake by MRSA treated with increasing concentrations of lichen extracts.

Transmission Electron Microscopy

To further investigate the suspected membrane damaging activities of the lichen extract, and also to search for evidence of possible additional mechanisms of action, we performed transmission electron microscopy (TEM) on thin sections of MRSA that had been treated with 10x MIC concentrations of both lichen extracts for 24 h. Untreated cells are shown in Fig 4.5. Extract of *L. vulpina* was found to adversely affect the membrane of MRSA causing abnormal morphology (Fig 4.6). In addition, the extract from *L. vulpina* also interrupted the normal cell division process (Fig 4.6 C and D).

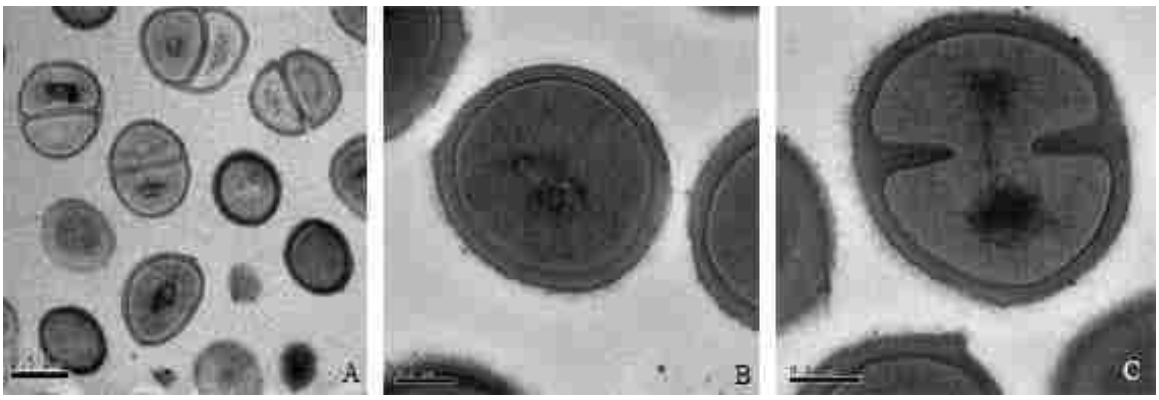


Figure 4.5: Transmission electron micrograph of untreated MRSA.

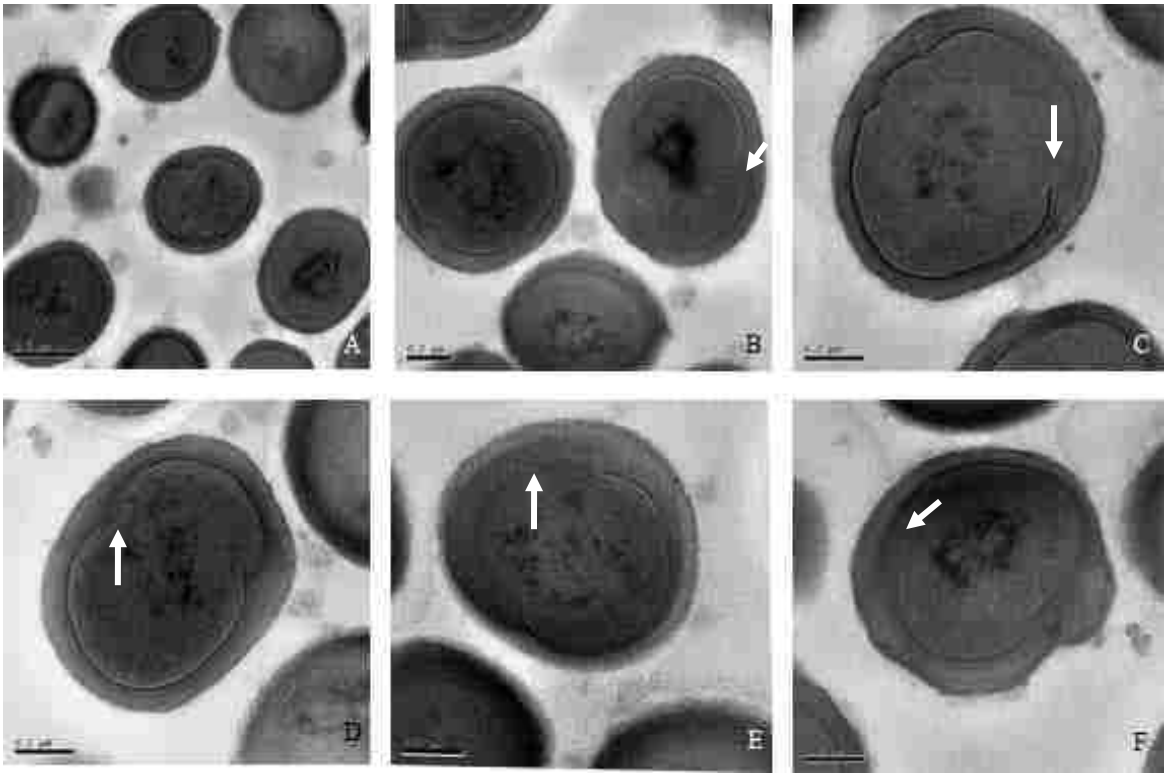


Figure 4.6: Transmission electron micrograph of MRSA treated with10x MIC concentrations of L. vulpina. Extract of *L. vulpina* was found to affect both cell membrane integrity (Fig 4.6 E and F) and cell division processes (Fig 4.6 B, C, and D) shown by arrows

Discussion

MRSA isolates have evolved resistance to a range of β-lactam antibiotics, thus limiting effective treatment options. This condition has resulted in a concerted effort to identify and develop new potential therapies for treatment of these strains (Stapleton & Taylor, 2002). The

antimicrobial activity of some lichen compounds indicates promise as a potential solution to this problem. Since the first report of lichen antimicrobial properties (Burkholder et al., 1944), several studies have documented the activities of various lichen extracts/chemicals against different pathogenic bacterial strains. Research (Boustie et al., 2011; Cocchietto et al., 2002a; Francolini et al., 2004a; Lauterwein et al., 1995c; Molnar & Farkas, 2010b; Pompilio et al., 2013; Ramos et al., 2014; Ranković et al., 2014; Shrestha & St. Clair, 2013a; Shrestha & St. Clair, 2013b) has shown that lichen-derived compounds show promise as potentially effective antibacterial agents. Most of these studies have focused on screening various crude extracts or isolated lichen compounds against different bacterial strains. However, our research provides some of the first data describing possible mechanisms of action for lichen extracts against pathogenic bacterial strains. Specifically, this study provides mechanism of action-related data for a crude extract from *L. vulpina* against a MRSA isolate.

Many antimicrobial agents containing polyphenolics cause gross membrane damage and whole-cell lysis (Sikkema et al., 1995). Our study showed that there was no significant reduction in the OD_{660}, even at the highest concentration (10x MIC) of the extract after 24 h. This suggests that the primary mode of action of the lichen-derived polyphenolic compound is not gross cell membrane damage. We also found steady increases in the absorbance at OD_{660} with 1x MIC and 5x MIC, however, the absorbance was still lower than the control. (Gupta et al., 2012) also found increases in the absorbance after a 24 h treatment of MRSA cultures with various concentrations of usnic acid.

Sub-lethal injury of microbes may alter the permeability of cell membranes and affect the membrane's capacity to osmoregulate or to exclude toxic materials (Gilbert, 1984). It appears that treatment of MRSA cells with this *L. vulpina* extract was fatal, but the killing kinetics were

slow. The time kill assay showed that there was a significant reduction in the number of CFUs after 24 h of exposure. The propidium iodide uptake assay also revealed that there was a dose-dependent increase in the uptake of PI by treated cells. Many polyphenolic compounds, derived either from lichens or higher plants, have given similar results (Alwash et al., 2013; Carson et al., 2002; Gupta et al., 2012). We found viable bacterial cells after 24 h of treatment, even at the highest extract concentrations (10x MIC), indicating a slow kill rate. Similar results were also reported by (Gupta et al., 2012).

Examination of lichen extract-treated MRSA cells using electron microscopy further documented the inability of this extract to lyse MRSA cells, in spite of clear evidence of damage to cell membranes. The membrane damaging activity of usnic acid has also been previously demonstrated (Gupta et al., 2012). Our data also provide preliminary documentation suggesting that the *L. vulpina* extract may also affect cell division processes. Many phenolic compounds have been shown to affect cell division processes in different strains of pathogenic bacteria. FtsZ, a tubulin-like GTPase, plays a central role in bacterial cell division. Inhibition of FtsZ polymerization prevents cells from dividing, leading ultimately to cell death (Wang et al., 2003). Crysophaentin A, a natural phenolic compound isolated from a marine alga, has been shown to inhibit FtsZ (Plaza et al., 2010). Similarly, another polyphenolic compound, Viriditoxin, produced by a species of the fungus *Aspergillus,* also inhibits FtsZ. It is important to mention here that lichens are symbiotic associations between a fungus and an alga, and they produce an impressive variety of polyphenolic compounds. Therefore, the results of this study demonstrate the merits of further biochemical investigations of lichen extracts, as a possible source for new antibacterial compounds that specifically target cell division processes in MRSA.

Thin layer chromatography result has shown that *L. vulpina* contain only vulpinic acid as its secondary compound. Other published reports also suggest that vulpinic acid is the major compound present in *L. vulpina* (Abo-Khatwa et al., 1996; Lawrey, 1983). So, it can be further inferred that the mode of action we observed in to the crude extract of *L. vulpina* was due to vulpinic acid.

Conclusion

A study by Gupta et al. (2012) demonstrated that usnic acid, a commonly occurring polyphenolic compound in many species of lichens, can destabilize the membrane integrity of MRSA. Similar to the Gupta study, our results also show that *L. vulpina* extracts disrupt the integrity of MRSA membranes. In addition, our data document that *L. vulpina* extracts also impact MRSA cell division processes. However, we cannot eliminate the possibility of other modes of action in addition to destabilizing membranes and disrupting cell division processes, mediated by this *L. vulpina* extract. As indicated in the introduction section, there are many studies showing promising results for the antimicrobial properties of lichen metabolites, but studies exploring modes of action are very limited. Our study opens the door for future mechanistic research, and *L. vulpina* merits further investigation.

Chapter 5: Examination of the Antioxidant capacity of acetone extracts of North American lichens using the Oxygen Radical Absorbance Capacity (ORAC) assay

Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and the superoxide anion (O_2^-) cause oxidative stress. Oxidation reactions, although crucial to life, can sometimes have damaging effects on cells often leading to the development of various chronic diseases like arthritis, cancer, and immunodeficiency syndromes (Kosanić et al., 2011). Hence a balance between oxidative stress and antioxidants is necessary in living systems. To effectively deal with the damaging effects of ROS, living organisms maintain a complex system of antioxidants. Research has shown that antioxidants, obtained either from natural or synthetic sources, are effective in reducing oxidative damage from ROS in the human body (Karihtala and Soini, 2007). However, there are reports showing that synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are toxic and can have carcinogenic effects (Zhang et al., 2009). For these reasons, recent studies have focused on the screening of natural products for their antioxidant properties (Naveena et al., 2008). According to Saha et al. (2008), some phenolic compounds have demonstrated antioxidant properties in biological systems. Lichens produce an impressive variety of polyphenolic compounds (Shrestha & St. Clair, 2014) therefore, extending our search for new potentially viable, natural antioxidants to lichens seems prudent.

Lichens have been used for various medicinal purposes since ancient times (Huneck, 1999; Vartia, 1973). For example, *Lobaria pulmonaria, Parmelia sulcata,* and *Letharia vulpina* have been used to treat pulmonary, cranial, and stomach disorders respectively (Huneck, 1999; Kirmizigul et al., 2003; Malhotra et al., 2008). More recently research has shown that

lichen metabolites demonstrate significant biological activity, including antiviral, antibiotic, antitumor, antioxidant, anti-herbivory, anti-HIV etc. (Shrestha & St. Clair, 2013b). Of their various biological properties, their antibiotic and antioxidant potentials have been most commonly studied.

North America is home to an impressive variety of lichen species. Many studies have been published examining the taxonomical (Barton et al., 2014; Hutten et al., 2013), phylogenetic (Leavitt et al., 2013), and ecological (Nelson et al., 2013) aspects of lichens in North America; however, the biological roles of lichens have been poorly studied.

This study reports on the antioxidant properties of acetone extracts of twelve different lichen species collected from various parts of the United States. We have used the Oxygen Radical Absorbance Capacity (ORAC) assay to assess the antioxidant capacity of each lichen extract.

Materials and Methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was obtained from Enzo Life Sciences, Inc. (Plymouth, PA). The 2,2'-azobis(2-aminidopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA), and the Fluorescein-sodium salt, was purchased from Sigma-Aldrich, Inc. (Milwaukee, WI).

Lichen materials

Samples of twelve species of lichens were collected from various parts of the United States. Collection information of each lichen species is given in Chapter 2.

Extraction of lichen compounds

Extraction of lichen compounds is described in Chapter 2.

Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay was performed following the protocol of (Garrett et al., 2010). Five different concentrations of each lichen extract (50 µg/ml to 3.125 µg/ml) were prepared from the stock solution (16 mg/ml). Twenty µL of each concentration was then combined with 200 µl of a 0.1061 µM fluorescein solution. A BMG Fluostar Optima plate reader was used to measure fluorescence at 37 °C over three - 2 minute cycles. Initial fluorescence was determined using 485 nm excitation and 590 nm emission filters. After the first 3 cycles, 20 µL of 26.55mM AAPH was added to the test solutions. The final concentration of each lichen extract ranged from 4.2 to 0.26 µg/ml after dilution with fluorescein and AAPH. Fluorescence was then measured for an additional 57 cycles. All fluorescent measurements were expressed relative to the initial gain adjustment reading. Control samples contained only fluorescein and fluorescein with AAPH. Trolox is a water-soluble analogue of vitamin E which is used as a standard for antioxidant experiments. Five different concentrations of trolox standards - 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.12 µg/ml, and 1.56 µg/ml were used in this study. Fluorescence curves were generated for all extracts and standards and the area under the curve was calculated to determine the Trolox Equivalents per microgram (TE/µg) of each lichen extract. The area under the curve (AUC) was calculated by determining the difference between the area under the fluorescein decay curves and the area under the sample curve and the fluorescein + AAPH curve. The AUC value indicates the total antioxidant activity of the sample minus the area under the fluorescein + AAPH antioxidant curve. Sample fluorescence decay curves of trolox and lichen extracts are shown in Fig 5.1. Each value is the mean of at least 3 experimental runs each conducted in triplicate.

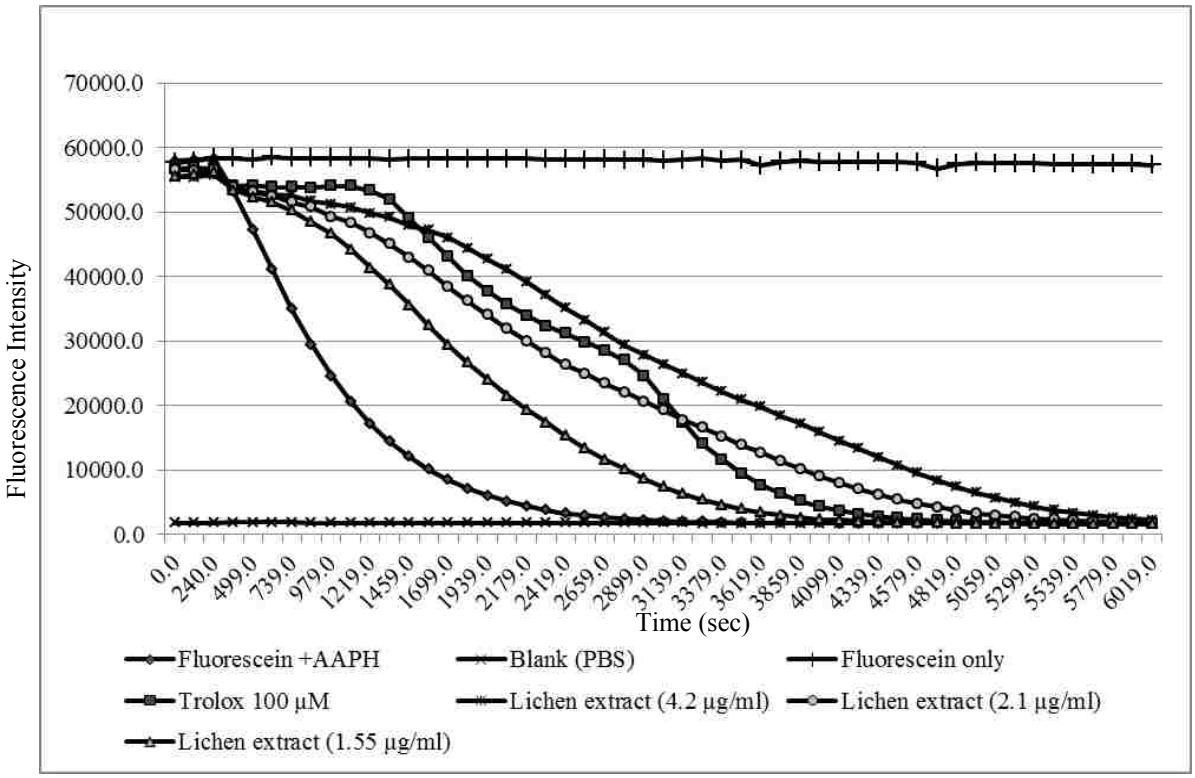


Figure 5.1: Sample fluorescence decay curves for controls and different concentrations of the *Parmotrema reticulatum* extract.

Results and Discussion

This study reports on the antioxidant capacity of acetone extracts of twelve lichens species collected from different parts of the United States (Table 3.1). We determined the antioxidant properties of each lichen extract using the Oxygen Radical Absorbance Capacity (ORAC) assay. The ORAC assay is a commonly used tool to measure the antioxidant activity of vitamins, phytochemicals, and other organic and inorganic compounds (Huang et al., 2002). This assay measures the oxidative degradation of a fluorescent molecule (usually fluorescein-sodium salt or beta-phycoerythrin) by an oxygen radical initiator such as AAPH (Huang et al., 2005). With the addition of the oxygen radical initiator, the intensity of the fluorescent molecules begins to decrease; however, by adding an antioxidant compound you can significantly reduce the rate at which the fluorescent molecule degrades. Thus, the longer a fluorescent molecule maintains its

intensity, the more effective the antioxidant compound (Garrett et al., 2010). One important aspect of the ORAC assay is calculating Trolox Equivalents (TE). Trolox, a water-soluble vitamin E analogue, is a standard used in antioxidant experiments and its capacity for preventing oxidation-related damage has been well documented (Hamad et al., 2010). In this study we used Trolox as our standard for evaluating the antioxidant capacity of lichen extracts.

Our results showed that except for *T. vermicularis,* all other lichen extracts demonstrated antioxidant properties (Table 5.1). We also show that out of the twelve lichen extracts, six species namely, *P. reticulatum, M. richardsonii, U. mammulata, R. peltata, E. catawbiense,* and *L. pulmonaria* reduced the oxidative degradation of the fluorescent molecule by AAPH even at the lowest extract concentration (0.26 µg/ml). Several reports have already documented the antioxidant properties of various lichen extracts. Specifically, Ranković et al. (2011) studied the antioxidant properties of acetone extracts of three lichens – *Cladonia furcata, Lecanora atra,* and *Lecanora muralis.* Results showed that all of the extracts demonstrated antioxidant activity with *Lecanora atra* having the highest free radical scavenging capacity. Buçukoglu et al. (2013) researched the antioxidant activity of methanolic extracts and various purified lichen metabolites obtained from six *Umbilicaria* species. All extracts demonstrated moderate DPPH radical-scavenging activity. They also showed that umbilicaric acid had the highest antioxidant activity. Kumar et al. (2014) evaluated the antioxidant properties of n-hexane, methanol, and water extracts of fourteen saxicolous (rock) lichens from the trans-Himalayan Ladakh region and documented that the species tested showed a broad spectrum of free radical scavenging and high antioxidant capacity. Most studies evaluating the antioxidant properties of lichen compounds have demonstrated a strong correlation between antioxidant activity and phenolic content (Brisdelli et al., 2013; Buçukoglu et al., 2013; Ghate et al., 2013; Kosanić et al., 2011).

Documentation of the major secondary chemicals in the 12 species tested in our study has been previously published (Shrestha et al., 2014); and all 12 species contained various phenolic compounds. Phenolic compounds demonstrate strong antioxidant qualities because of their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Saha et al., 2008). Phenolic compounds transfer hydrogen to free radicals to break the chain reaction of lipid oxidation at the first initiation step (Ranković et al., 2011). Hence our results provide strong evidence that the acetone extracts from the lichen species included in this study have promising antioxidant properties.

Table 5.1: TE/µg values at different concentrations (µg/ml) of lichen extract

Species	Antioxidant Value (TE/µg)				
	4.2 µg/ml	2.1 µg/ml	1.05 µg/ml	0.525 µg/ml	0.26 µg/ml
P. reticulatum	993.4 ± 54.5	536.3 ± 37.5	392.7 ± 11.9	278.4 ± 41.7	130.8 ± 18.1
M. richardsonii	829.1 ± 99.4	462.6 ± 89	243.8 ± 35.2	110 ± 24.2	23.5 ± 12.5
U. mammulata	794.6 ± 79.2	561.2 ± 12.6	326.2 ± 43.2	179.8 ± 24.1	116.2 ± 46.2
R. peltata	740.8 ± 54.4	400.7 ± 70.8	242.1 ± 30.6	115.7 ± 14.8	98.7 ± 13.4
E. catawbiense	681.5 ± 25.9	438.8 ± 51.3	253.8 ± 21.8	181.6 ± 10.9	68.2 ± 23.3
L. pulmonaria	529.4 ± 69.5	333.7 ± 68.9	114 ± 25.7	67.4 ± 25.3	12.4 ± 6.9
A. sarmentosa	279.9 ± 39.1	158.8 ± 21.1	71.1 ± 12.7	77.8 ± 27.1	-
X. wyomingica	194.5 ± 15.8	121.4 ± 19.9	65.8 ± 20.6	39.5 ± 7.4	-
C. furcata	160.5 ± 17.6	99.4 ± 13	53.9 ± 18.2	-	-
L. columbiana	132.9 ± 18.0	-	-	-	-
R. sinensis	109.6 ± 11.6	58.07 ± 6.26	28.5 ± 17	-	-
T. vermicularis	-	-	-	-	-

We also ranked our 12 lichen species on the basis of TE/µg at 4.2 µg/ml concentration of the lichen extracts (Fig. 5.2). The three species with the highest antioxidant capacity were *P. reticulatum, M. richardsonii,* and *U. mammulata* with TE/µg, values of 993.4 ± 54.5, 829.1 ± 99.4, and 794.6 ± 79.2 respectively. These three lichen species contained atranorin, alectoronic acid, and gyrophoric acid, respectively, as major secondary metabolites (Shrestha et al., 2014). Many lichen species containing atranorin, alectoronic acid, and gyrophoric acid have already

been screened for their antioxidant properties and have shown positive results (Buçukoglu et al., 2013; Kosanić et al., 2011; Manojlovic et al., 2012b; Melo et al., 2011; Ravaglia et al., 2014). The phenolic groups typically associated with lichen metabolites are considered to be key elements in determining their antioxidative capacity (Marković & Manojlović, 2010). The above mentioned lichen acids have two or more phenolic groups which likely explains their antioxidant activity.

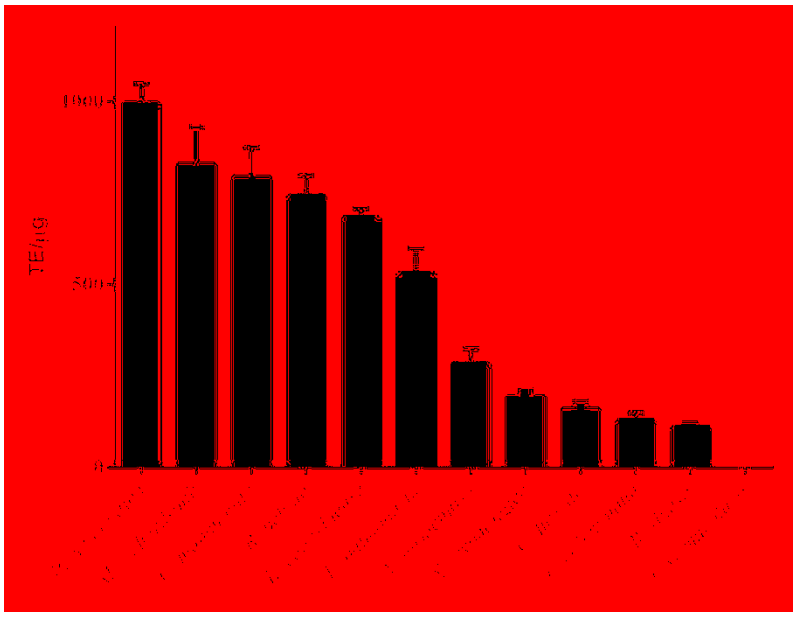


Figure 5.2: Comparison of antioxidant capacity (TE/µg) of extracts from twelve lichen species at 4.2 µg/ml concentration.

In our study the *T. vermicularis* extract, at the tested concentrations, did not show antioxidant capacity. However Luo et al. (2006) reported antioxidant activity in methanol extracts of the same species. These conflicting results might be due to differences in the chemical extraction process and different assay method used for measuring antioxidant activity.

Based on the current literature, of the twelve species examined in this study six species, *A. sarmentosa, E. catawbiense, M. richardsonii, R. sinensis, R. peltata,* and *X. wyomingica* have not been previously surveyed for their antioxidant properties.

In conclusion, of the 12 lichen species considered in this study 11 contain strong antioxidant compounds; the only exception being *T. vermicularis.* Considering that antioxidants are effective therapeutic agents against various serious health issues, our results suggest that lichen extracts merit further investigation.

Chapter 6: Anticancer activities of selected species of North American lichen extract against Burkitt's lymphoma (Raji cell)

Introduction

Cancer is a disease that circumvents the human immune system, migrates, evolves, invades organs, destroys tissues, and resists drugs (Mukherjee, 2010). It is the second leading cause of death in the United States with an estimated 600,000 deaths in 2013. Efforts to combat various types of cancers have not achieved the expected results in light of the fact that cancer mortality rates have remained essentially unchanged for the last several decades (Lambert et al., 2011). Some standard cancer treatments such as chemotherapy, although effective in some cancers, are inherently problematic with challenging side effects as well as resistance issues. The need for novel chemotherapeutics has promoted a search for alternative cancer treatments which are more effective but have fewer side effects.

Natural products, derived from plants, microbes, fungi, and marine have proven to be promising sources of anticancer drugs (Mondal et al., 2012). Over 60% of the current anticancer drugs have been developed from natural sources (Cragg & Newman, 2009). However, there are still many unexamined natural sources that merit further investigation. Among them are lichens. Lichen chemicals demonstrate various types of biological activity including, antibiotic, anticancer, antifungal, antioxidant, anti-HIV etc. (Molnár & Farkas, 2010; Shrestha & St. Clair, 2013b; Shukla et al., 2010). Various lichens compounds, either as crude extracts or in purified form, have been screened against various solid cancer cell lines (Ari et al., 2014; Kumar KC & Müller, 1999; Mayer et al., 2005; Munzi et al., 2013; Perry et al., 1999; Russo et al., 2012; Takai et al., 1979; Triggiani et al., 2009). However, studies examining the effectiveness of lichen compounds against lymphoma have been limited. In this investigation we selected Burkitt's

lymphoma (Raji cells) as a model cell line to study the effects of crude lichen extracts and their mechanism of action against a lymphoma type cancer. Burkitt's lymphoma is a fast growing non-Hodgkin's cancer of the lymphatic system (B lymphocytes). It is a highly aggressive cancer and often life threatening.

The primary goals of this research are to 1) screen a large number of lichen extracts to determine their cytotoxic effects against Raji cells and normal lymphocytes and 2) understand the related mechanism of cell death. Cytotoxicity of lichen extracts was measured using the trypan blue exclusion method. In order to understand the mechanism of cell death, we studied cell cycle kinetics, apoptosis, and expression patterns of two genes associated with cancer – the tumor suppressor gene (p53) and the DNA synthesis and repair gene (TK1).

Materials and Methods

Lichen samples

Seventeen species of lichens were collected from various parts of the United States. Collection site details are given in chapter 2.

Chemicals

Acetone, dimethyl sulfoxide (DMSO), toluene, ethyl acetate, trypan blue (0.4%), propidium iodide, and ethanol were purchased from Sigma Aldrich, MO, USA. Formic acid and L-glutamine were from Fischer Scientific, NJ, USA; Sodium bicarbonate was from Mallinckrodt chemicals; RNAase from Thermoscientific; and Acridine orange from Life technology, NY, USA. The cell culture media RPMI-1640, fetal bovine serum (FBS), and phosphate buffer solution (PBS) were from Hyclone, UT, USA. Thymidine [methyl-3H] was from ViTrax, CA, USA. RNA extraction kit (RNAqueous, catalog # AM1912) was from Ambion, NY, USA.

qScript™ One-Step qRT-PCR was used for RT-PCR and purchased from Quanta Bioscience, MD, USA. Primer for p53 (Hs99999147_m1) and TK1 (Hs00177406_m1) and phytohaemagglutanin (PHA) was from Invitrogen, NY, USA). Lymphocyte separation media was bought from Corning, Cellgro, VA, USA. Annexin V-FITC kit (# 4830-01-k) was from Trevigen, MD, USA. Thin layer chromatographic plates (Silica gel 60 F254) were purchased from Merck, NJ, USA.

Extraction of lichen compounds

Procedure for extracting of lichen compounds has been described in Chapter 2.

Thin Layer Chromatography:

Thin layer chromatography (TLC) was performed using standard extraction protocol (Orange et al., 2001).

Cell culture:

Burkitt's Lymphoma (Raji) cell was used for this study. Raji cells were grown in RPMI-1640 supplemented with 10% FBS, L-glutamine (0.2995 mg/L) and Sodium Bicarbonate (1.5 gm/L). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Normal lymphocytes were obtained from healthy donors with prior consent. Lymphocytes were separated using lymphocytes separation media (LSM). To separate lymphocytes, blood was first mixed with PBS in equal ratio and layered on top of LSM in a 50 ml conical vial. The vial was then centrifuged (400 g, 20 minutes). The buffy layer separated after centrifugation was collected, washed once with PBS and cultured in RPMI-1640, and phytohaemagglutanin (PHA) was used to stimulate cell division. Lymphocytes were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay

Cytotoxicity was determined using the trypan blue exclusion assay. Raji cells were seeded at a density of 1 X 10^5 /ml in a 12 well plate and treated for 48 h at 37 °C with the various lichen extracts in final concentrations of 0, 6.25, 12.5, 25, and 50 μg/ml. All tests included a vehicle control with an equivalent concentration of DMSO and doxorubicin, an established chemotherapeutic drug, was used as a positive control. The final concentration of DMSO did not exceed 0.32%. After 48 h, cells were counted and viability was assessed using the trypan blue exclusion assay. Living and dead cells were counted under microscope at 100x magnification. Cells stained blue with the dye were considered dead and cells not stained with dye were considered live. IC_{50} values were calculated by plotting a cell growth curve. Based on the IC_{50} values, extracts from two lichen species were selected to determine their mechanism of action against the Raji cell line. To determine the effects of lichen extracts in a broad range, we selected three different concentrations 15, 25, and 40 μg/ml representing a low to high range of IC_{50} values and were used in all subsequent experiments. We also tested the cytotoxicity of the two lichen extracts against normal, dividing human lymphocytes following the same procedures as described above.

Cell Proliferation

Raji cells were treated with three different concentrations of the two lichen extracts – 15, 25, and 40 μg/ml. After 48 h of treatment, 1 μl/ml of [methyl-^{3}H] thymidine (tritium) with a specific activity of 185 Gbq/mmol, 5.0 Ci/mmol was added and the culture was incubated for an additional 4 h at 37°C. An INOTECH cell harvester was then used to extract the radioactively labeled DNA on glass fiber filter paper and incorporation was determined using the scintillation counter.

Cell Cycle Arrest

Raji cells were treated with the various concentrations of each lichen extract. After 48 h, cells were harvested, washed in cold PBS and fixed in cold 70% ethanol and kept at 4°C overnight. Fixed cells were then washed again with cold PBS, centrifuged, and incubated for 2 h at room temperature with RNAse (200 μg/ml) and Propidium Iodide (50 μg/ml). After incubation, various stages of the cell cycle were analyzed using a FACS Canto flow cytometer (BD-Biosciences, San Jose, CA) equipped with the CellQuest version 3.3 software. The ModFit LT version 3.1 program was then used to determine the percentage of cells in the different phases of the cell cycle.

Biochemical Detection of Apoptosis

Biochemical detection of apoptosis was performed using an Annexin V-FITC kit. Raji cells were treated for 48 h with various concentrations of each lichen extract. The manufacturer's protocol was followed for the detection of apoptosis. After 48 h cells were washed with PBS and incubated with annexin V-FITC and PI for 15 minutes at room temperature. Samples were then analyzed using a FACS Canto flow cytometer.

Morphological detection of apoptosis

Morphological detection of apoptosis was accomplished by double staining treated Raji cells with Acridine orange (100 μg/ml) and Propidium Iodide (100 μg/ml). Pictures were then taken using Zeiss Imager A.1 florescence microscope. Green cells were considered to be alive, whereas orange and red cell were identified as apoptotic and necrotic respectively.

Gene Expression pattern of p53 and TK1

The effects of lichen extracts on the gene expression pattern of the tumor suppressor gene (p53) and DNA repair gene (TK1) were studied with Real-time quantitative PCR using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, CA, USA). Initially, total RNA from treated and untreated cells was isolated using an RNAqueous kit. RT-PCR was then performed using a one-step kit. Amplifications were performed in triplicate for each sample and PCR conditions were as follows: Initial incubation at 50°C for 10 min, denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 10 sec, and 60°C for 1 min. We then analyzed the relative quantitation of gene expression using the comparative C_T ($\Delta\Delta C_T$) method (Denman & McSweeney, 2005). The threshold cycle (C_T) was identified as the fractional cycle number at which the amount of amplified target reached a fix threshold. We then subtracted the C_T value of the ß-actin from the target gene for data normalization. The $\Delta\Delta C_T$ was then calculated as the difference between the normalized C_T value (ΔC_T) of the treatment and control samples. The comparative expression levels of the target genes was equal to $2^{-\Delta\Delta CT}$.

Statistical Analysis

All experiments were repeated at least three times using separate cell preparations. The results were reported as mean values ± SD of the three independent runs. One-way analysis of variance and Dunnett's multiple comparison was applied to determine the significance of differences in all measured parameters. Statistical analyses were performed using GraphPad Prism 6 software.

Results

Cytotoxicity

To determine the potential role of lichen extracts as anti-cancer agents, we treated Raji cells with acetone extracts of 17 different lichens at the various concentrations. After 48 h, extracts from 14 of the 17 lichens species, all except *B. fuscescens, U. mammulata,* and *C. furcata,* effectively reduced the viability of Raji cells at the concentrations tested in a dose-dependent manner. The IC_{50} values for the lichen extracts as well as the positive (Doxorubicin) and negative (DMSO) controls are reported in Table 6.1. Out of the 14 lichen extracts, – *X. chlorochroa* and *T. ciliaris* had the lowest IC_{50} values at 28.3±1.4 and 28.4±2.7 µg/ml respectively. Consequently, we used extracts from these two species to examine the mechanism behind Raji cell death. We also tested the cytotoxicity of these two lichen extracts against normal human lymphocytes and found that the viability of the lymphocytes was not affected (viability >90%) by the treatments.

Extrolites of X. chlorochroa and T. ciliaris

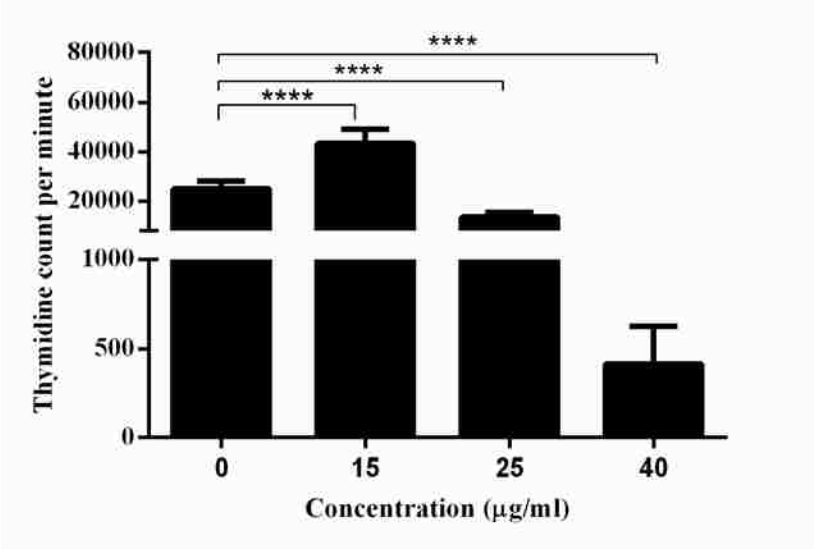
Thin layer chromatography was used to extract and identify the secondary compounds present in *T. ciliaris* and *X. chlorochroa*. Protolichesterinic acid was found in *T. ciliaris* while *X. chlorochroa* contained a mixture of usnic, salazinic, norstictic, and constictic acids.

Table 6.1: IC50 values (μg/ml) of extracts from 17 species of lichens assessed using the Trypan Blue assay after 48 h of treatment.

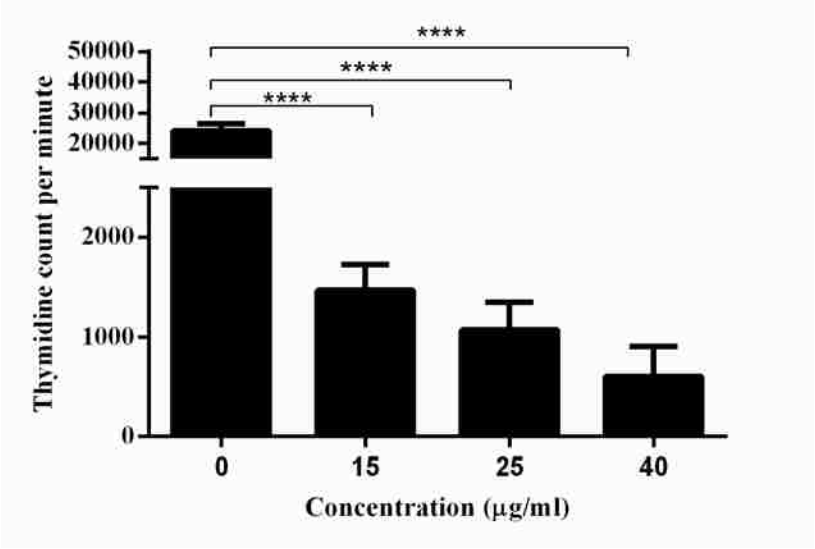
Species	IC_{50}
Xanthoparmelia chlorochroa	28.3±1.4
Tuckermannopsis ciliaris	28.4±2.7
Rhizoplaca chrysoleuca	29.2±1.0
Evernastrum catawbiensis	29.3±1.6
Ramalina menziesii	29.0±2.3
Alectoria sarmentosa	32.4±0.2
Peltigera aphthosa	33.0±3.4
Usnea strigosa	34.7±2.8
Evernia prunastri	35.8±4.7
Rhizoplaca peltata	36.7±6.8
Lobaria pulmonaria	37.3±3.5
Parmotrema reticulata	38.2±3.8
Letharia vulpina	43.3±12
Vulpicida canadensis	43.3±16.6
Bryoria fuscescens	-
Umbilicaria mammulata	-
Cladonia furcata	-
Doxorubicin	7.15±1.5
DMSO	-

Effects on cell proliferation

The effects of the crude extracts of *X. chlorochroa* and *T. ciliaris* on proliferation of Raji cells were examined. In the case of the *T. ciliaris* extract only the higher concentrations (25 and 40 μg/ml) significantly ($p < 0.001$) reduced the Raji cell proliferation rate but we observed a significant ($p < 0.0010$ increase with the lowest concentration (15 μg/ml). A dose dependent response was observed for the *X. chlorochroa* extract at all concentrations and there was a significant reduction ($p < 0.001$) in the uptake of thymdine by treatment groups when compared with control (Fig 6.1).



A



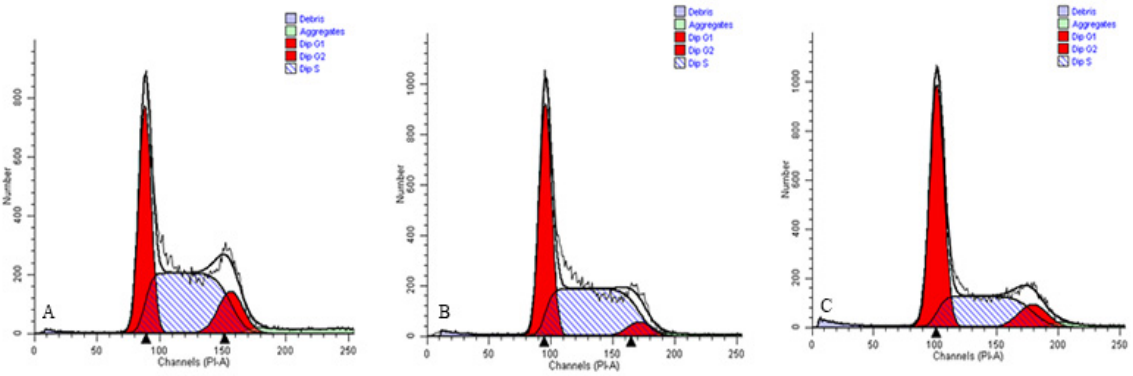
B

Figure 6.1: Effects of crude extracts of T. ciliaris (A) and X. chlorochroa (B) on proliferation of Raji cells assessed by the thymidine incorporation assay. * = $p < 0.05$, ** = $p < 0.01$, and *** and **** = $p < 0.001$

Cell cycle arrest

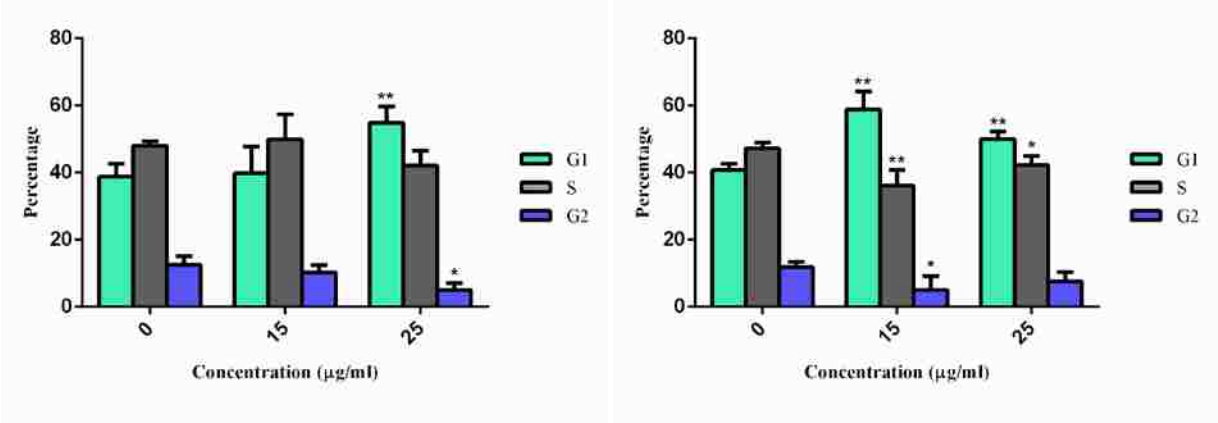
In addition to cell viability and proliferation, we also studied the effects of the two lichen extracts on the Raji cell cycle. Results of the cell cycle study are shown in Fig 6.2 and 6.3. The *T. ciliaris* extract at 25 µg/ml arrested cell growth ($p < 0.05$) at the G_0/G_1 stage but at the lower

concentration (15 µg/ml) cell growth did not vary significantly from the control. However, the *X. chlorochroa* extract arrested cell growth at G_0/G_1 stage at both (15 and 25 µg/ml) concentrations ($p < 0.01$).



A B C

Figure 6.2: Results of flow cytometer study showing the number of Raji cells in the various stages of the cell cycle after treating with *T. ciliaris*. A = control, B = treated with 15 µg/ml, and C= treated with 25 µg/ml.



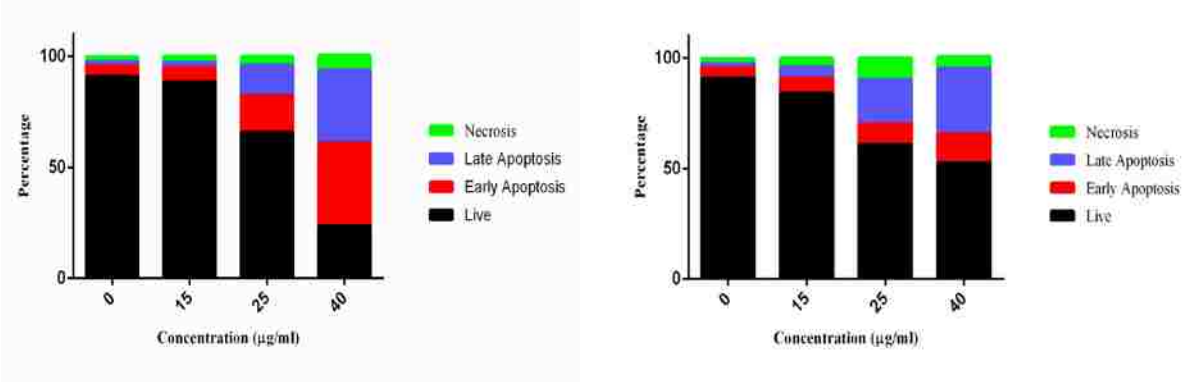
A B

Figure 6 3: Effects of crude extracts of *T. ciliaris* (A) and *X. chlorochroa* (B) on cell cycle dynamics. * = $p < 0.05$ and ** = $p < 0.01$

Apoptosis

To test whether the decrease in cell viability after treatment with the lichen extracts was due to apoptosis, we used both biochemical and morphological methods for detecting apoptosis. Our results after 48 h of treatment showed that there was a dose-dependent effect with both

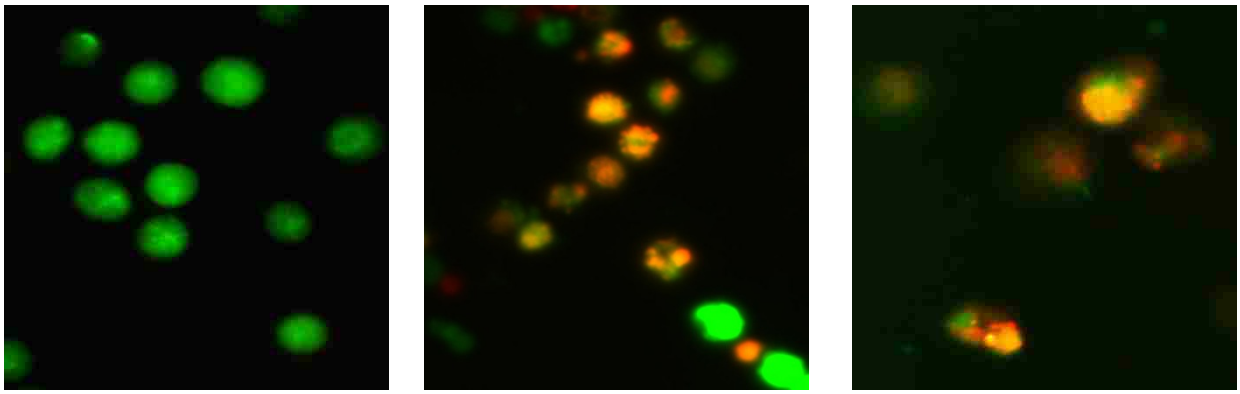
lichen extracts (Fig 6.4). We found that the percentage of apoptotic cells was 5.2±2.1 in the control sample while at the highest treatment concentration with the *T. ciliaris* extract (40 µg/ml) the percentage of apoptotic cells was 37.2±2.2 for early apoptotic cells and 32.8±5.1 for late apoptotic cells. Similarly, in the case of the *X. chlorochroa* extract at the highest treatment concentration (40 µg/ml) the percentages were 12.9±0.9 and 29.9±0.9 at the early and late apoptotic stages respectively.



A B

Figure 6.4: Biochemical detection of apoptosis in Raji cells treated with crude extracts of *T. ciliaris* (A) and *X. chlorochroa* (B).

We also used morphological changes in the Raji cells to detect apoptosis. Our results showed that treatment with increasing concentrations of either lichen extract resulted in more orange colored cells indicating apoptosis (Fig 6.5 B and C). Cells treated with the higher extract concentrations also showed membrane "blebs" which is a hallmark of apoptosis. Control cells were predominantly green without membrane blebs (Fig 6.5 A) indicating that the cells in the control samples were alive with no evidence of apoptosis.



A B C

Figure 6.5: Morphological detection of apoptosis in Raji cells treated with *T. ciliaris* and *X. chlorochroa* (both 40 µg/ml) extracts. A – Control, B - *T. ciliaris* extract and C – *X. chlorochroa* extract

Gene expression pattern of p53 and TK1

We also measured the relative expression of the p53 and TK1 genes in the Raji cells treated with the *X. chlorochroa* and *T. ciliaris* extracts as compared to untreated (control) cells (Table 2). Our results showed that when Raji cells were treated with the *T. ciliaris* extract, there was a dose dependent expression of p53 across all extract concentrations. There was also a two fold increase in the TK1 expression for all *T. ciliaris* extract concentrations. Similarly, the expression of p53 was also dose dependent when treated with the *X. chlorochroa* extract but the lowest concentration resulted in the highest expression. Expression of TK1 was not affected by the *X. chlorochroa* extract treatment.

Table 6.2: Relative expression of p53 and TK1 gene in treated samples as compared to control samples

	Fold increase compared to control	
Concentrations	p53	TK1
TC40	8.3	2.2
TC 25	3.5	1.6
TC 15	2.3	2.0
XC 40	1.3	1.2
XC 25	5.0	0.5
XC 15	11.0	0.8

Discussion

Recently, in the United States there have been extensive research efforts focused on the ecology, taxonomy, and phylogentics of lichens (Geiser et al., 2010; Leavitt et al., 2012; Leavitt & St. Clair, 2011; Lendemer & Hodkinson, 2013; McMurray et al., 2013; Shrestha et al., 2012). However, research reports examining the anticancer properties of North American lichens are limited. This study presents data examining the cytotoxicity of chemical extracts from 17 different lichen species, collected from different parts of the United States, against Burkitt's lymphoma. To the best of our knowledge, none of these lichen species have ever been tested against any cancer cell lines in crude extract form except for *E. prunastri* (Triggiani *et al*., 2009). We also report the mechanism of action for two lichen extracts against Burkitt's lymphoma cells. Our results showed that out of the 17 lichen species tested, extracts of 14 species indicated activity against Burkitt's lymphoma at the tested concentrations with IC_{50} values ranging from 28.3±1.4 to 43.3±16.6 μg/ml. We further demonstrated that lichen extracts have differential cytotoxicity levels and according to (Bézivin et al., 2003), this may be a useful attribute for potential anti-tumor agents. Variance in the cytotoxic dose of lichen extracts may in part be due to the specific combinations of chemicals characteristic of a given species. Similar results

showing different IC_{50} values for lichens have been published (Bézivin et al., 2003; Perry et al., 1999).

Although several attempts have been made to elucidate the mechanism of cell death caused by lichen metabolites in different cancer cell lines, no reports for Burkitt's lymphoma have been published. Therefore, in order to better understand specifically how lichen extracts impact Burkitt's lymphoma cells we selected the most potent extracts from two lichens (*T. ciliaris* and *X. chlorochroa*) to examine their mechanism of action.

In this study we found a dose dependent response on the proliferation of Raji cells after treatment with the different concentrations of the *X. chlorochroa* extract. The proliferation rate was significantly lower at all three concentrations when compared with the control. However, only two concentrations (25 and 40 μg/ml) of the *T. ciliaris* extract reduced proliferation of Raji cells significantly when compared to the control. It seems that *T. ciliaris* at its lower concentration favors the growth of Raji cell. The difference in the effect of these two lichens extract can most likely be attributed to differences in the chemical composition of the two species. For example, *X. chlorochroa* contains usnic, salazinic, norstitic and constitic acids as its major compounds while *T. ciliaris* produces protolichesterinic acid. Various lichen compounds either in crude or purified forms have been found to reduce the proliferation of various cancer cell lines (Ari et al., 2012; Liu et al., 2010; Ren et al., 2009; Triggiani et al., 2009). Einarsdottir et al. (2010) also demonstrated that when compared to untreated cells, treatment with usnic acid can significantly decrease thymidine uptake in breast (T-47D) and pancreatic (Capan-2) cancer cells. Similarly Ogmundsdottir et al., (1998) found that protolichesterinic acid also has capacity to decrease thymidine uptake in erythro-leukemia (K-562). These results suggest that lichen compounds either as a crude extract or in purified form can act as anti-proliferative agents.

Our study further documented that the decrease in the proliferation rate of Raji cells after treatment with both lichen extracts was due to the arrest of cell cycle progression at the G_0/G_1 phase. Except for treatment with the lowest concentration of the *T. ciliaris* extract (15 μg/ml), all other concentrations of both extracts arrested the cell cycle at the G_0/G_1 phase. Failure to arrest the cells by *T. ciliaris* at the lowest concentration seems obvious because at this concentration proliferation of Raji cells was not affected. There are few published reports which suggest that lichen compounds are able to arrest the cell cycle at various stages (Backorova et al., 2011; Liu et al., 2010; Singh et al., 2013). Although the compounds tested in these studies are different from our studies, the results from these studies shows that lichen compounds have potential to arrest the cell cycle.

Many studies have shown that lichen compounds induce apoptosis in various cancer cell lines. For example, Backorova et al., (2011) reported that usnic acid induced apoptosis in human ovarian carcinoma (A2780), human cervix adenocarcinoma (HeLa), and human colon carcinoma (HCT-116); while data based on research by (Bezivin et al., 2004; Russo et al., 2012) showed a dose and time dependent apoptotic response to usnic acid in murine lymphocytic leukemia (L1210). Protolichesterinic acid has also been found to induce apoptosis in human prostate cancer cell lines LNCaP and DU-145 (Russo et al., 2012) and HeLa cells (Brisdelli et al., 2013). In our study we demonstrated that both of our lichen extracts induced apoptosis in Raji cells in a dose dependent manner. Results of our study are similar to previously published results because thin layer chromatography showed that usnic acid and protolichesterinic acid are also consistently present in *X. chlorochroa* and *T. ciliaris* respectively.

We also studied the effects of lichen extracts on the expression pattern of two cancer-related genes – p53 and TK1. Our results showed that both lichen extracts affected expression of

the p53 gene in a dose dependent manner (Table 2). In addition, when Raji cells were treated with the *T. ciliaris* extract, expression of TK1 gene was double the expression rate in the control cells. This pattern suggests that the *T. ciliaris* extract may actually stimulate expression of the TK1 gene. This might be the reason why we found higher thymidine count in Raji cells treated with the extract of *T. ciliaris* as compared to the extract of *X. chlorochroa.* With reference to the expression of p53, it could be suggested that the apoptotic effect seen in the Raji cells after treatment with the *X. chlorochroa* extract was independent of general p53 expression while p53 dependent in the case of the *T. ciliaris* extract.

Conclusion

In conclusion, we have provided evidences that the crude extracts of two lichen species *X. chlorochroa* and *T. ciliaris* have anti-proliferative effect against Raji cells. Both the extracts are able to stop the cell cycle at G_1 stage and force the cells to undergo apoptosis in p53 dependent and independent manner. Although studies regarding effect of lichen secondary compounds have been study before; this is the first attempt to study effectiveness of lichen compounds against lymphoma. This study examined only a small fraction of the potential anticancer compounds available from the more than 15,000 species of lichens. Considering the fact that lichens produce a broad spectrum of chemical compounds many of which show promising levels of biological activity, lichen secondary compounds can realistically be considered a promising source of future anticancer drugs. However, more detailed *in vitro* as well as *in vivo* studies are necessary to establish the potential anticancer properties of lichen compounds.

Chapter 7: Usnic and Vulpinic acid induces caspase-3 independent apoptosis in two colon cancer cell lines (HT29 and Sw620)

Introduction

According to the American Cancer Society report (2014-16), colorectal cancer is the third most commonly diagnosed cancer in the United States and the third leading cause of cancer-related death in both men and women. The report also estimates that there will 136,830 new cases of colorectal cancer and 50,310 people will die. Modern diets and lifestyles such as high meat consumption, alcohol use, and limited physical activity can be blamed in part for the increased mortality rates associated with colon cancer (Kuppusamy *et al.*, 2014). Epidemiological studies show that there is small, but significant association between fruit and vegetable intake and a reduction in colorectal cancer risk (Ricciardiello *et al.*, 2011). Research also shows that diets rich in phenolic compounds may also result in a lower risk of colon cancer (Yi *et al.*, 2005; Khanal *et al.*, 2011) as well as other types of cancers (Soobrattee *et al.*, 2006; Huang *et al.*, 2010). In light of these patterns, increased efforts in screening natural products containing polyphenolic compounds as potential sources for future anticancer drugs should be a priority.

Lichens produce a diverse and unique group of secondary metabolites including more than 1050 compounds (Molnar and Farkas, 2010). Polyphenolic compounds comprise one of the major groups of lichen secondary metabolites. Although initial studies of lichen compounds as anticancer agents began in the 1960s (Fukuoka and et al., 1968; Nishikawa et al., 1969), only a limited number have thoroughly examined the potential capacity of lichen compounds as a viable source of anticancer drugs. As mentioned earlier, natural products containing phenolic

compounds are considered to be possible sources of anticancer drugs. Therefore, researching lichen phenolics as a potential source of new anticancer drug therapies seems logical.

In this study we report data based on the screening of acetone extracts of 17 lichen species against human colon cancer cell line (HT-29). Based on our preliminary data we selected four lichen compounds, lecanoric, usnic acid, stictic acid, and vulpinic acid, to study their effects on cell proliferation, cell cycle dynamics, and mode of cellular death in two different colon cancer cell lines (HT-29 and SW620).

Materials and Methods

Chemicals

Usnic acid, dimethyl sulfoxide (DMSO), and Propidium iodide were purchased from Sigma Aldrich, MO, USA and stictic, lecanoric and vulpinic acid were purchased from Gaia Chemical Corporation, CT, USA. The cell culture media DMEM (high glucose) and fetal bovine serum (FBS) were obtained from Hyclone, UT, USA. RNase was purchased from Thermoscientific, Waltham, MA; MTT (4890-025-K) and an Annexin V-FITC kit (4830-01-k) was obtained from Trevigen, MD, USA. Antibodies caspase-3, cleaved caspase-3, and actin were purchased from Cell Signaling Technology, MA, USA and AIF was obtained from Bioss Inc. MA, USA.

Lichen samples

Seventeen species of lichens were collected from various parts of the United States (Table 7.1). Voucher collections of each species have been deposited in the Herbarium of Non-vascular Cryptogams at Brigham Young University, Provo, UT, USA.

Table 7.1: Lichens used in this study

Lichen species	
Alectoria sarmentosa (Ach.) Ach.	*Ramalina menziesii* Taylor
Bryoria fuscescens (Gyelnik) Brodo & D. Hawksw.	*Rhizoplaca chrysoleuca* (Sm.) Zopf
Cladonia furcata (Hudson) Schrader	*Rhizoplaca peltata* (Ramond) Leuckert & Poelt
Evernia prunastri (L.) Ach.	*Tuckermannopsis ciliaris* (Ach.) Gyelnik
Everniastrum catawbiense (Degel.) Hale ex Sipman	*Umbilicaria mammulata* (Ach.) Tuck.
Letharia vulpina (L.) Hue	*Usnea strigosa* (Ach.) Eaton
Lobaria pulmonaria (L.) Hoffm.	*Vulpicida canadensis* (Räsänen) J.-E. Mattsson & M. J. Lai
Parmotrema reticulatum (Taylor) M. Choisy	*Xanthoparmelia chlorochroa* (Tuck.) Hale
Peltigera aphthosa (L.) Willd.	

Cell culture

Two different colon cancer cell lines- HT29 and SW620 and a normal cell line –Vero (kidney epithelial cells from an African green monkey) were used in connection with this experiment. The colon cancer cell lines (HT29 and SW620) and the Vero cell line were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37 °C in a humidified 5% CO2 incubator.

MTT Assay

HT29, SW620, and Vero cells were seeded into 96-well cell culture plates at initial densities of 3 x 10^4 cells/well in 100 µl of media. Only the HT29 cells were treated with the crude lichen extracts for 48 h. While the purified lichen compounds (lecanoric, stictic, usnic, and vulpinic acids) were used to treat cells (HT29, SW620, and Vero) for 24, 48 and 72 h. Final concentrations of crude lichen extracts ranged from 6.25 µg/ml to 100 µg/ml while the purified lichen chemicals (usnic, stictic, lecanoric, and vulpinic acids) were tested at concentrations of 25, 50, 100, and150 µM. DMSO of equivalent concentrations was used as a vehicle control. After treating all cell lines for the defined time frames, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-

diphenyl tetrazolium bromide (MTT) was added to each cell type and the plates were incubated for 2 h at 37 °C. After incubation 100 µl of detergent was added to each well and plates were then incubated for 1 more h at 37 °C. Absorbance (at 570 nm) was then measured.

Cell cycle Analysis

HT29 and SW620 cells were treated with the various concentrations of usnic and vulpinic acids for 48h. Cells were then harvested, washed in cold PBS and fixed in cold 70% ethanol and then kept at 4°C overnight. Fixed cells were then washed again with cold PBS, centrifuged, and incubated for 20 minutes at 37 °C with RNAse (200 µg/ml) and Propidium Iodide (50 µg/ml). Stages of the cell cycle were then analyzed using an Attune flow cytometer. FlowJO_X (v10) was used to determine the percentage of cells in the different phases of the cell cycle.

Biochemical Detection of Apoptosis

Annexin V-FITC was used to detect apoptosis after the cells (HT29 and SW620) had been treated with the various concentrations of usnic and vulpinic acid. The manufacturer's protocol was followed for the detection of apoptosis. Briefly, cells were harvest, washed with PBS, and then incubated with annexin V-FITC and PI for 15 minutes at room temperature. Samples were then analyzed using an Attune flow cytometer.

Western Blotting

The Western blotting method was used to measure expression of the proteins involved in apoptosis. The Western blotting technique was performed following the methods of Arroyo et al. (2009). Both colon cancer cell lines were treated with different concentrations of usnic and vulpinic acid for various time periods. Cell lysates (50 µg) were separated on a 4–12% Bis-Tris gel SDS-PAGE and then transferred to a nitrocellulose membrane. The membranes were then

incubated with antibodies against total Caspase-3 (1: 200), cleaved caspase-3 (1: 200), and the apoptosis inducing factor (1: 300). Following incubation overnight with primary antibodies, the membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The membranes were then incubated with an ECL substrate, and emitted light was detected using x-ray film. To determine loading consistencies, each membrane was stripped and re-probed with an antibody against rabbit β-actin (dilution 1:4000). Expression levels of the proteins were quantified using densitometry normalized to β-actin expression and changes in expression were then compared to untreated controls.

Statistical Analysis

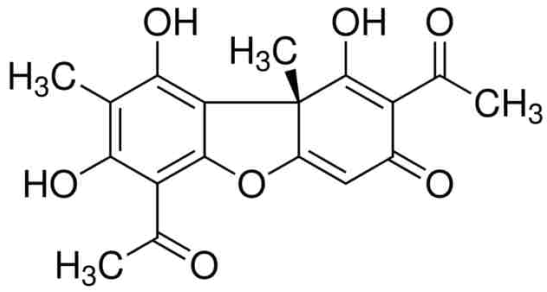
All experiments were repeated at least three times using separate cell preparations. Results were reported as mean values ± SD of the three independent runs. One-way analysis of variance and Dunnett's multiple comparison were used to determine the significance of differences in all measured parameters. Statistical analyses were performed using GraphPad Prism 6 software.

Results

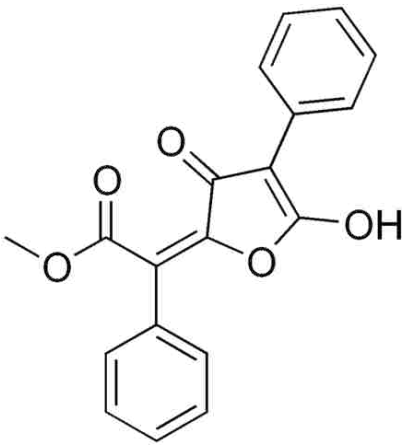
MTT Assay

The cytotoxicity of acetone extracts obtained from seventeen species of lichens against HT29 cells were screened using the MTT assay. Among the seventeen species, extracts from fourteen were found to inhibit the growth of HT29 cells in the tested concentrations. The IC_{50} values for all the species are reported in Table 7.2. We used thin layer chromatography techniques (Orange et al., 2001) to identify the major compounds present in the acetone extracts of all of seventeen lichen species (Table 3.2). Usnic acid was the most common metabolite found

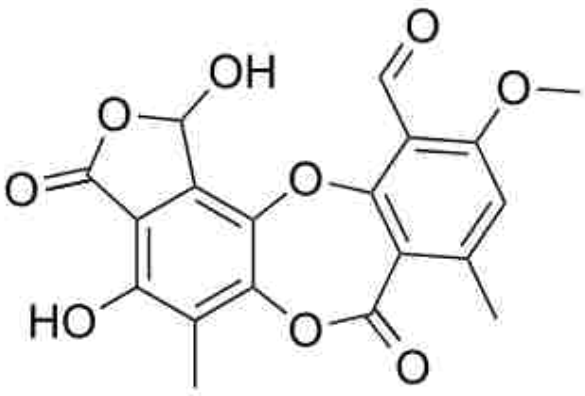
in seven of the extracts. Therefore, we selected usnic acid to study its mechanism of action against cancer cell lines. In order to develop a broader understanding about the effects of lichen compounds on cancer cells we also selected three additional lichen compounds - vulpinic, stictic, and lecanoric acid. We also added an additional colon cancer cell line (SW680) for our expanded study.



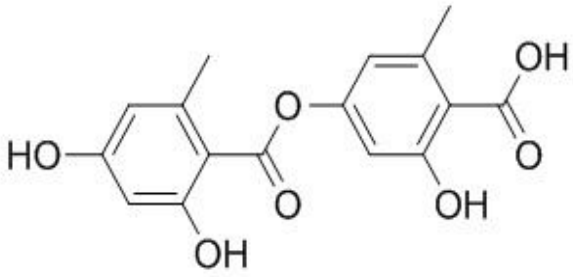
Usnic acid



Vulpinic acid



Stictic acid



Lecanoric acid

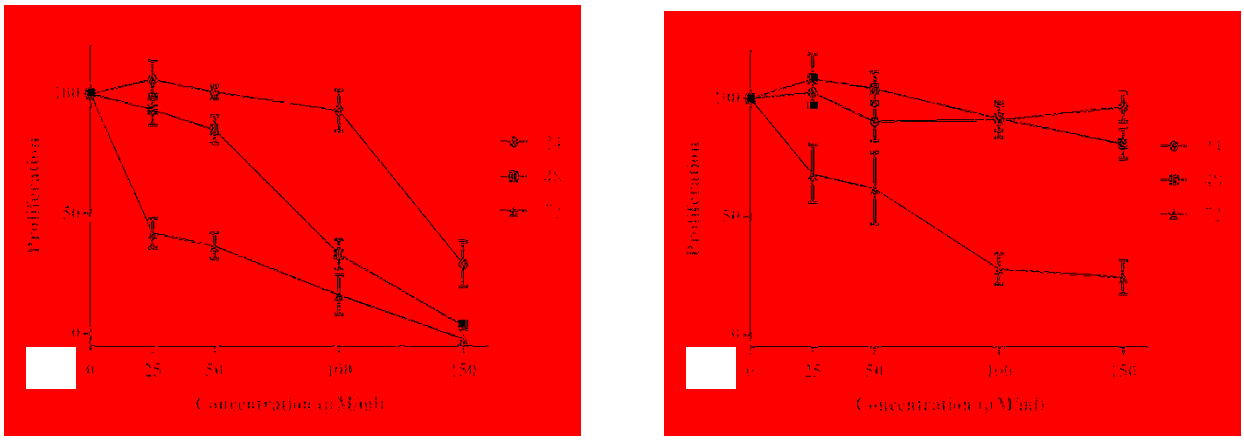
Figure 7.1: Chemical structures of four lichen metabolites used in the experiment.

Table 7.2: IC50 values (μg/ml) for acetone extracts of the 17 lichen species against HT-29 cells

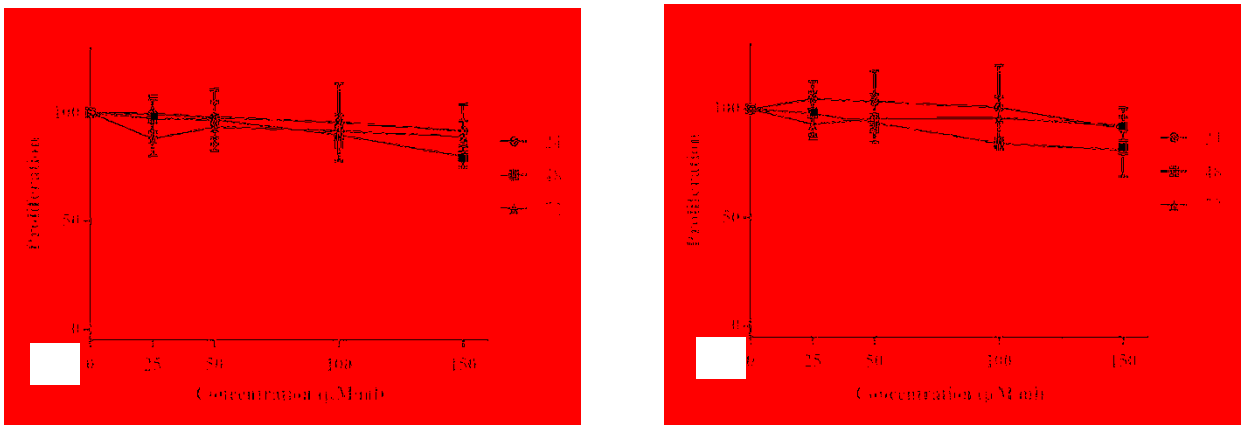
Species	IC_{50} Values
Cladonia furcata	59.9 ± 3
Ramalina menziesii	60.5 ± 6
Rhizoplaca peltata	60 ± 12
Rhizoplaca chrysoleuca	61.2 ± 1
Xanthoparmelia chlorochroa	64.5 ± 5
Tuckermannopsis ciliaris	68.2 ± 6
Evernastrum catawbiense	69.5 ± 3
Umbilicaria mammulata	75 ± 6
Alectoria sarmentosa	75.2 ± 12
Letharia vulpina	79.1 ± 4
Parmotrema reticulatum	79.8 ± 14
Bryoria fuscescens	85.9 ± 9
Usnea strigosa	87.5 ± 14
Vulpicida canadensis	91 ± 20
Evernia prunastri	-
Lobaria pulmonaria	-
Peltigera aphthosa	-

Both usnic and vulpinic acids showed time and dose-dependent anti-proliferative activity against both cell lines whereas stictic and lecanoric acids demonstrated no anti-proliferative activity (Fig 7.2 and 7.3).

We also tested the cytotoxicity of usnic and vulpinic acid against normal cells (Vero). Our results showed that vulpinic acid was not able to reduce proliferation of Vero cells significantly until 72 h at all tested concentrations while usnic acid, at its highest concentration (150 uM/ml), significantly reduced the proliferation of vero cells after 72 h. Hence, the highest concentration of usnic acid was not used for further analyses.

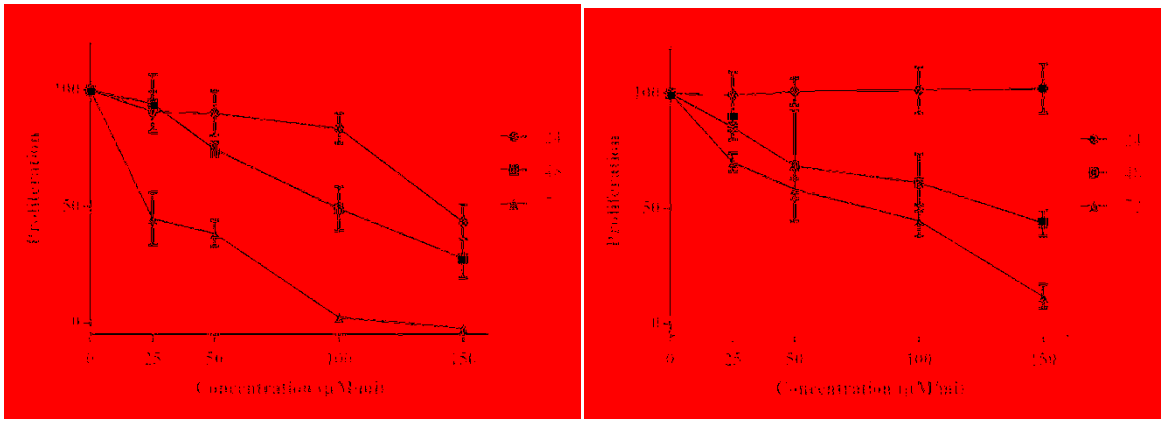


A B

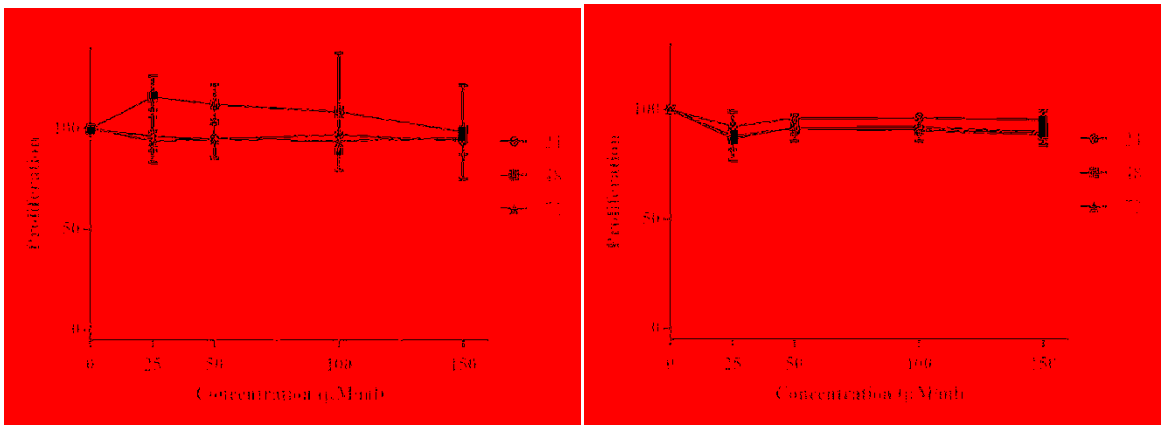


C D

Figure 7.2: Antiproliferative activity of Usnic (A), Vulpinic (B), Stictic (C), and Lecanoric (D) acids against HT29 as measured using the MTT assay.



A B



C D

Figure 7.3: Antiproliferative activity of Usnic (A), Vulpinic (B), Stictic (C), and Lecanoric (D) acids against SW620 as measured using the MTT assay.

Cell cycle

To understand the effects of usnic acid and vulpinic acids on cell cycle dynamics, we used flow cytometric analysis to measure the percentage of cancer cells at the various stages of the cell cycle. Cells were treated with usnic and vulpinic acid at different concentrations for 48 h followed by PI staining prior to flow cytometric analysis (Fig 7.4). Usnic and vulpinic acids had different effects on the two cancer cell lines. When HT29 cells were treated with usnic acid at 100 and 150 μM/ml concentrations, there was a significant ($p < 0.05$) increase in the cell population at the G_2/M stage and a significant decrease in the cell population at the G_0/G_1 stage when compared to the control. This result indicates that the usnic acid treated cancer cells were arrested at the G_2/M cell cycle stage (Fig 7.4A). In contrast, when HT29 cells were treated with vulpinic acid there was an increase in the cell population at the G_0/G_1 stage but the difference was not significantly different from the control (Fig 7.4 B). It can be inferred from these results that vulpinic acid arrests the HT29 cancer cells at the G_0/G_1 stage. In case of the SW680 cell line, treatment with usnic acid caused increased accumulation of cells at the G_0/G_1 stage when

compared to the control but the increase was not significant (Fig 7.4 C). However, treatment of the SW620 with vulpinic acid caused the cells to arrest at the S-phase (Fig 7.4 D).

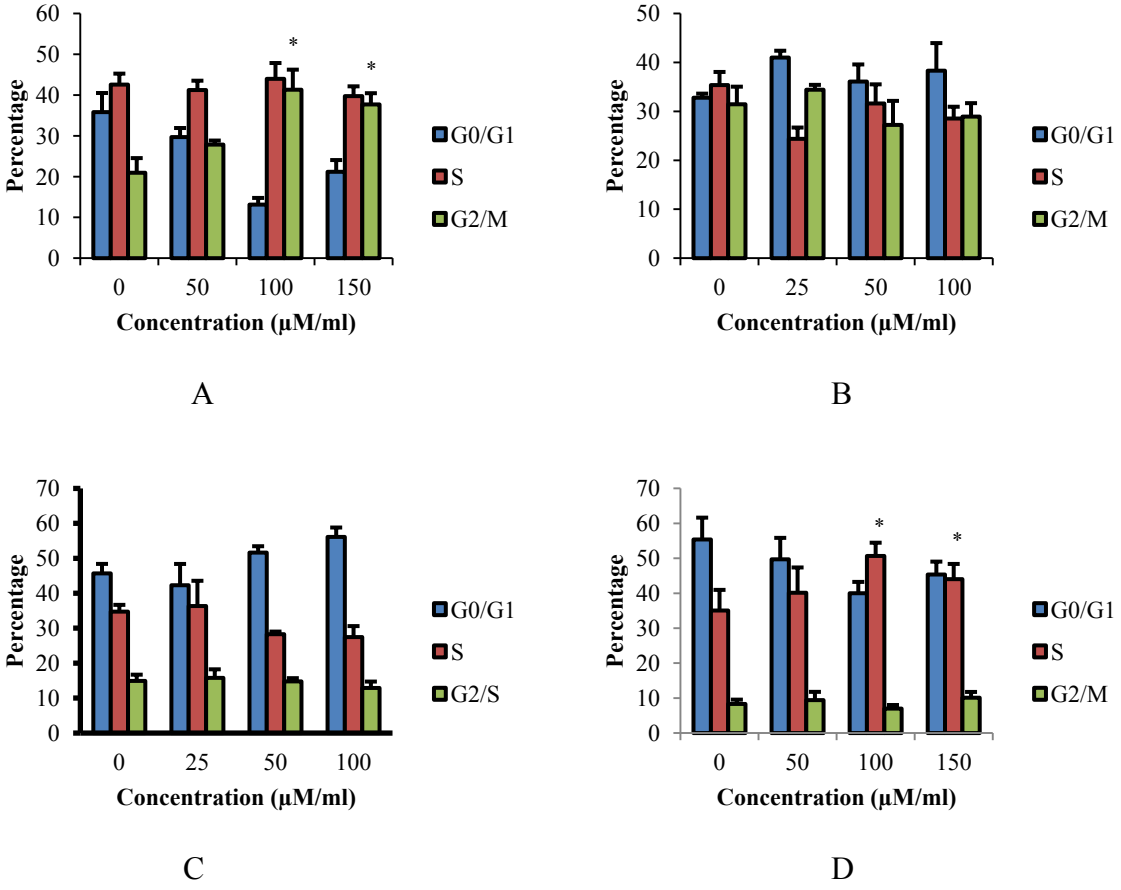


Figure 7.4: Cell cycle analysis in HT29 (A and B) and SW620 (C and D). Cells were treated with various concentrations of usnic acid (A and C) vulpinic acid (B and D)

Apoptosis

In order to test whether the decrease in cell proliferation and arrest of the cell cycle resulted in cell death, we stained the treated cells with Annexin-V and Propidium Iodide and examined their status using flow cytometry. Results of this experiment showed that there is both a dose- and time-dependent increase in the apoptotic cell population for both cancer cell lines (Table 7.3 - 7.6, Fig 7.5 - 7.8). However, in the case of the HT29 cell line, the highest concentration of usnic acid seemed to cause cell necrosis (Table 7.3 and Fig 7.5).

Table 7.3: Measuring mode of cell death in HT29 cells treated with usnic acid.

Conc (µM/ml) ↓	Live		Early Apoptosis		Late Apoptosis		Necrotic	
	24h	48h	24h	48h	24h	48h	24h	48h
0	91.2±4.6	79.6±4.6	1.8±1.1	1.2±0.1	6.0±4.5	10.9±1.7	1.2±1.0	8.4±5.2
25	89.0±5.5	79.4±1.6	4.7±1.7	4.4±0.3	6.1±3.0	12.1±1.3	0.7±0.4	4.3±1.7
50	57.1±13.9	50.4±2.7	21.1±7.9	9.6±1.3	18.2±5.2	19.5±3.2	3.6±2.1	20.9±5.0
100	38.4±2.7	17.7±0.9	7.0±3.0	3.5±1.8	11.1±3.3	26.0±12.9	43.6±8.5	52.9±13.8

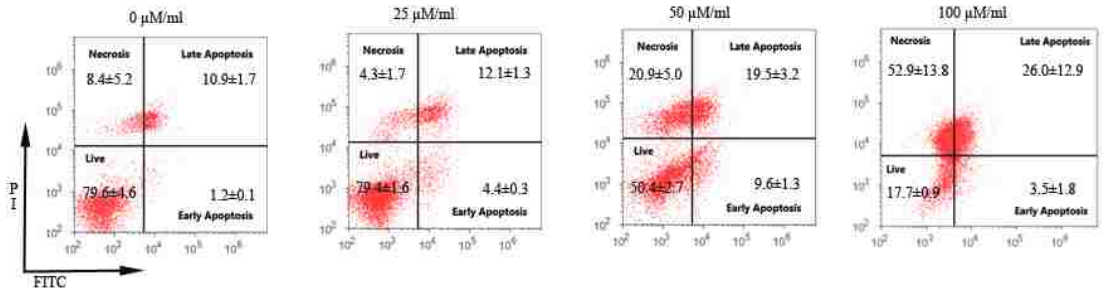


Figure 7.5: Flow cytometry analysis of HT29 cells treated with various concentration of usnic acid.

Table 7.4: Measuring mode of cell death in HT29 cells treated with vulpinc acid.

Conc (µM/ml) ↓	Live		Early Apoptosis		Late Apoptosis		Necrotic	
	48h	72h	48h	72h	48h	72h	48h	72h
0	88.9±0.6	82.9±4.2	1.1±0.3	1.2±0.1	4.3±0.5	10.9±1.7	5.6±0.9	5.0±2.5
50	85.7±0.5	74.7±1.6	1.2±0.1	1.6±0.1	4.8±0.2	11.4±0.5	8.3±0.5	13.0±2.1
100	86.0±1.4	68.1±4.1	1.1±0.2	13.2±2.8	5.4±1.5	11.9±4.1	7.4±0.8	6.8±1.0
150	71.7±8.1	59.7±4.1	1.7±0.8	17.4±7.5	16.9±2.1	17.7±4.1	9.6±5.5	5.2±2.2

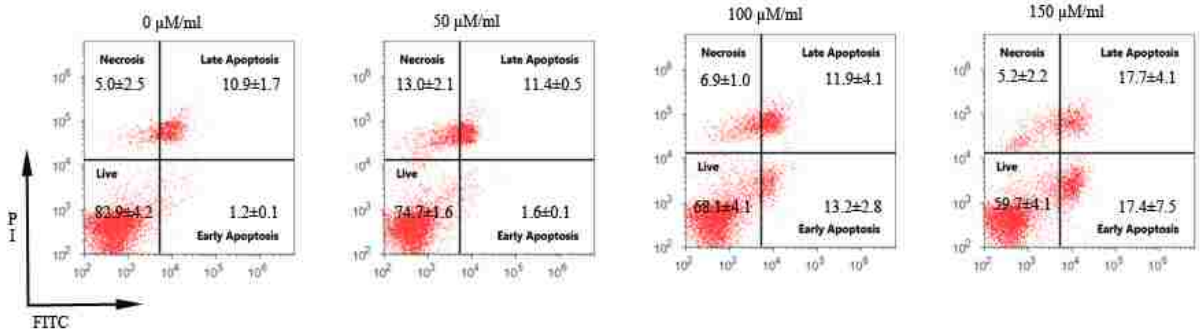


Figure 7.6: Flow cytometry analysis of HT29 cells treated with various concentration of vulpinic acid.

Table 7.5: Measuring mode of cell death in SW620 cells treated with usnic acid

Conc^{n}	Live		Early apoptosis		Late Apoptosis		Necrotic	
(µM/ml)↓	24h	48h	24h	48h	24h	48h	24h	48h
0	92.4±1.1	91.7±1.7	2.1±0.7	1.8±1.1	2.5±0.6	2.5±0.2	2.9±0.2	4.0±0.9
25	72.0±5.0	84.9±5.1	2.6±0.2	5.4±3.6	18.6±4.8	7.4±2.4	6.7±2.5	2.4±2.3
50	68.5±1.1	56.9±1.4	2.5±0.2	11.7±5.7	22.2±1.9	22.4±1.5	6.7±1.7	9.1±5.7
100	65.3±6.7	17.8±3.2	4.8±1.4	38.5±4.5	25.5±8.0	39.4±2.0	6.1±2.7	4.2±2.5

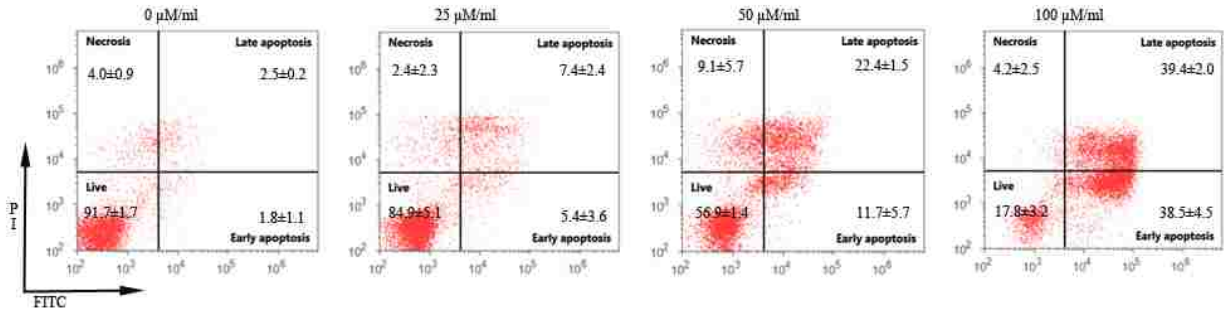


Figure 7.7: Flow cytometry analysis of SW620 cells treated with various concentrations of usnic acid.

Table 7.6: Measuring mode of cell death in SW620 cells treated with vulpinic acid

Conc^{n}	Live		Early apoptosis		Late Apoptosis		Necrotic	
(µM/ml)↓	48h	72h	48h	72h	48h	72h	48h	72h
0	92.6±2.0	85.5±3.8	0.7±0.6	3.2±0.5	1.9±1.7	7.8±1.4	4.8±0.4	3.2±2.0
50	92.4±1.4	79.0±1.8	0.6±0.6	3.3±0.3	1.7±1.7	14.7±1.5	5.5±1.0	2.9±0.6
100	90.1±0.8	68.6±7.0	1.4±0.7	5.2±0.5	3.8±1.2	22.3±5.5	4.9±2.9	3.4±1.6
150	89.3±0.4	44.2±4.4	1.6±1.1	9.8±2.0	4.1±2.5	39.3±3.7	5.0±3.2	6.8±2.2

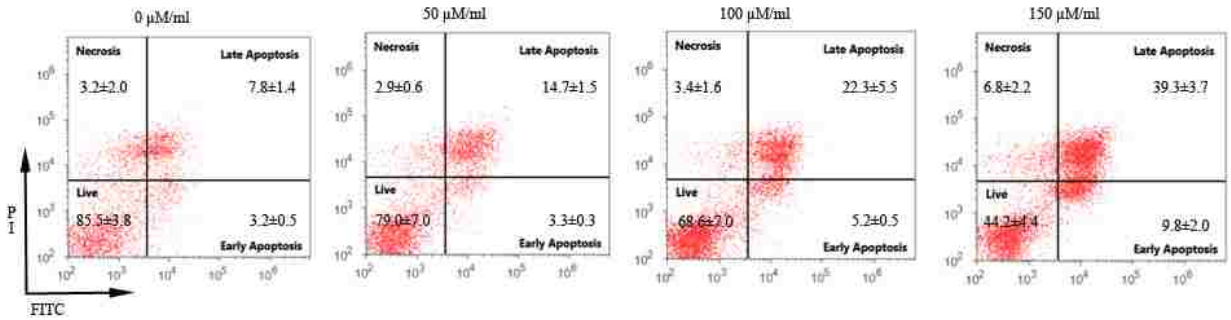


Figure 7.8: Flow cytometry analysis of SW620 cells treated with various concentrations of vulpinic acid.

Western Blotting

Caspases are key mediators of programmed cell death (apoptosis). Caspase-3 is an effector caspase that is activated through cleavage by caspase-9. We analyzed cell extracts for activation of caspase-3 and cleaved-caspase-3 after treatment with usnic acid for 48 h and vulpinic acid for 72 h. We did not include the 100 μM/ml concentration of usnic acid in HT29 cell treatment because this concentration appeared to be toxic. Caspase-3 was present in all samples including the control (Fig 7.9 and 7.10) but there was no expression of cleaved-caspase-3 suggesting that apoptosis had occurred independent of caspase-3. Apoptosis can occur independent of casapase-3 activation and it has been shown that apoptosis inducing factor (AIF) is involved in the induction of apoptotic cell death via a caspase-independent pathway (Ren et al. 2009). When cells receive a death stimulus, Mitochondrial AIF is release and translocated into the nucleus causing nuclear condensation. Therefore, we analyzed changes in AIF levels using the Western blotting method to determine whether or not AIF played a role in apoptotic cell death. When HT29 cells were treated with usnic acid, AIF was stimulated by a 2.2 and 3.2-fold at 50 and 100 μM/ml concentrations respectively compared to the control. Similarly, treatment of HT29 with vulpinic acid stimulated AIF by a 1.9, 2.2, and 2.3- fold at 50, 100, and 150 μM/ml concentrations respectively compared to the control. In SW620 cell lines there was also a marked increase in the expression of AIF in treated cells when compared to the control. When SW620 cells were treated with usnic acid at 25, 50, and 100 μM/ml concentrations there was a 1.3, 1.7, and 2.3-fold increase in the expression of AIF respectively and similarly with vulpinic acid there was a 1.3, 1.3, and 1.5-fold increases in the expression of AIF with concentrations of 50, 100, and 150 μM/ml of vulpinic acid respectively.

Figure 7.9: Expression levels of AIF, Caspase 3, and β-actin proteins in usnic and vulpinic acid treated HT29 cells using Western blot analysis.

Figure 7.10: Expression levels of AIF, Caspase 3, and β-actin proteins in usnic and vulpinic acid treated SW620 cells using Western blot analysis.

Discussion

Secondary compounds from various natural sources, including lichens, represent a promising reservoir of various kinds of therapeutic drugs. For example, several lichen secondary metabolites have demonstrated potential as antibiotics and anticancer drugs. (Shrestha & Clair, 2013). In this study we screened acetone extracts of 17 different lichen species against the HT29

colon cancer cell line. For most of the lichen species screened this represents the first time these extracts have been tested against this cancer cell line. TLC analysis of the extract guided us to examine the potential effects of four different purified lichen compounds namely, lecanoric, stictic, vulpinic, and usnic acids against two colon cancer cell lines - HT29 and SW620. This report represents the initial attempt to determine the effects of the above lichen compounds on the SW620 cancer cell line.

Our data show that both leconaric and stictic acids had no significant effect on the proliferation of either cancer cell lines while both usnic and vulpinic acids show both a dose- and time-dependent response against both cancer cell lines. The data clearly show that usnic acid is more effective in reducing proliferation of both types of cancer cells than vulpinic acid. Our data also show that both usnic and vulpinic acids are relatively non-toxic to normal cells (Vero). Usnic acid is one of the most extensively studied lichen metabolites and a number of studies have shown that usnic acid is effective as an anti-cancer agent against an impressive range of cancer cell lines (Backorova et al., 2012). Compared to usnic acid, vulpinic acid has been poorly studied. To date, research has shown that vulpinic acid expresses intermediate cytotoxicity against MM98 malignant mesothelioma cells, A431 vulvar carcinoma cells, and HaCaT keratinocytes (Burlando et al., 2009).

More specifically, our data show that the decrease in the proliferation rate of cancer cells after treatment with usnic and vulpinic acids is due to cell cycle arrest. However, usnic and vulpinic acids exhibited variable responses in terms of cell cycle arrest as shown by accumulated cell population at different cell cycle stages for both cancer cell lines. Usnic acid arrests the cell cycle at the G_2/M stage in the HT29 cell line resulting in the accumulation of the cancer cell population at the G_0/G_1stage. Whereas, vulpinic acid arrests HT29 cells at the S stage and

SW620 cells at G_0/G_1 stage. Usnic acid is known to arrest the cell cycle at various stages in different cancer cell lines. For example, Singh *et al.* (2013) reported that usnic acid arrests A549 cancer cells at the G_0/G_1 stage and as reported by Backorova *et al.* (2011) usnic acid also arrests cells at the S phase in several different cancer cell lines.

One of the hallmarks of apoptosis is the externalization of phosphatidylserine as a consequence of loss in cell membrane asymmetry, a condition which can be measured before the loss of membrane integrity (Mikes et al., 2009). The externalization of phosphatidylserine can be determined using flow cytometry by measuring the quantity of the protein, Annexin V which binds to the membrane. Our results show that there is an increase in the percentage of Annexin V bound cells with both increasing concentrations of lichen metabolites as well as exposure time. The data also indicate that the higher concentration of usnic acid (100 μM/ml) was toxic to the HT29 cell line causing necrosis instead of apoptosis. Whereas with the SW620 cell line the higher concentrations of usnic acid increased the percentage of apoptotic cells without causing necrosis. Similarly vulpinic acid also resulted in apoptosis in both a dose and time dependent manner for both cancer cell lines. Other researchers have also shown that crude lichen extracts as well as purified lichen metabolites will induce apoptosis in various cancer cell lines. For example, Nguyen et al. (2014) showed that crude acetone extracts of *Flavocetraria cucullata* and its isolated compound, usnic acid, induced apoptosis in colon cancer (HT29), gastric cancer (AGS), lung cancer (A549), and prostate cancer (CWR22Rv-1) cell lines. They also reported increasing levels of apoptotic proteins like Bax, cleaved PARP, and cleaved caspase-3 in all tested cancer cell lines as well as a significant decrease in the anti-apoptotic protein Bcl-xL. Induction of apoptosis by acetone extracts from the lichen *Lethariella zahlbruckneri* was reported by Ren et al. (2009). The secondary compounds of *L. zahlbruckneri* include vulpinic

acid as well as other depsides and aliphatic acids (Obermayer, 1997). Ren et al. (2009) also found that the induction of apoptosis was associated with the activation of initiator caspases-8 and -9, as well as the effector caspase-3, an increase in the expression of the pro-apoptotic protein Bax, and a decrease in the expression of the anti-apoptotic protein Bcl-2.

Caspases are a group of intracellular proteases which are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis (Thornberry & Lazebnik, 1998). Caspases are present as inactive pro-enzymes that are activated by proteolytic cleavage. In our study we were unable to detect expression of cleaved caspase-3 however we did find expression of caspase-3 in all treatment as well as controls. This result suggests that in some cases apoptosis can occur independent of caspase-3. In our study we found that the treatment of HT29 and SW620 cancer cell lines with usnic and vulpinic acid increased the expression of the AIF protein when compared to the control sample. AIF is a mitochondrial apoptosis inducing factor which translocates to the nucleus, in response to apototic stimuli, where it causes partial DNA fragmentation and chromatin condensation (Ren et al., 2009).

In summary, both usnic and vulpinic acids show promise as potential anti-cancer compounds while lecanoric and stictic acids showed no anti-proliferative action at the tested concentrations. We found that usnic and vulpinic acids arrested the cell cycle in both of the tested cancer cell lines but at different cell cycle stages while externalizing the phosphatidylserine protein and inducing apoptosis independent of caspase-3.

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