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ORIGINAL ARTICLE

In vitro evaluation of the antibacterial activity of extracts from 34 species of North American lichens

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Abstract

Context: The emergence of antibiotic resistant pathogens is a serious global health threat. Hence, the search for new antibiotic drugs from various natural sources should be given high priority. Lichens produce a variety of low molecular weight metabolic compounds and many cultures have utilized these compounds in traditional medicine for centuries.
Objective: Report the antibiotic properties of extracts from 34 North American lichens screened against four pathogenic bacteria.
Materials and methods: The micro-well dilution method was used to determine the minimum inhibitory concentration (MIC) of acetone and methanol extracts of 34 lichen species against four bacterial strains. Major chemical compounds in each species were identified using thin layer chromatography (TLC).
Results: Most of the lichen extracts demonstrated inhibitory effects against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and methicillin-resistant *S. aureus* (MRSA) with MIC values ranging from 3.9 to 500 μg/ml. In addition, extracts from three species, *Letharia columbiana* (Nutt.) J. W. Thomson (Parmeliaceae), *Letharia vulpina* (L.) Hue (Parmeliaceae), and *Vulpicida canadensis* (Räsänen) J.-E. Mattsson & M. J. Lai (Parmeliaceae) (MIC = 125–500 μg/ml) were also effective against *Escherichia coli*. Generally, acetone extractions were found to be more effective than methanol extractions.
Discussion and conclusion: Results of this study show that lichen extracts provide significant antimicrobial activity against both Gram-positive and Gram-negative bacteria. These results suggest that lichens may be an important potential source of antibacterial drugs.

Keywords

Antibiotic, MIC, micro-broth dilution, natural products, TLC

History

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Introduction

The extensive use and misuse of antibiotic drugs in the clinic, community, animal husbandry, and agriculture have resulted in the re-emergence, diversification, and spread of various resistant pathogenic bacteria (Andersson & Hughes, 2012). The recurrence of high-profile pathogens is a growing concern as global mortality rates from drug-resistant bacterial infections continue to increase (Boucher et al., 2009). Despite the need to effectively address antibiotic resistance, the discovery of new antibacterial drugs has declined in recent years. This is largely because before many drugs come to the market pathogens have already become resistant. Furthermore, microbial resistance 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. Lichens are symbiotic systems consisting of a fungus and an alga and/or a cyanobacterium. Lichens represent one of the more promising potential reservoirs for low molecular weight secondary compounds with more than 1000 different secondary chemicals reported for lichens and their cultured mycobionts (Molnar & Farkas, 2010). Various lichen-derived compounds have been shown to have antimicrobial activities (Boustie et al., 2011; Podterob, 2008; Shrestha & St. Clair, 2013a; Shukla et al., 2010). Activity of lichen compounds has also been demonstrated against a limited number of drug-resistant pathogenic microbes including vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*

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(Elo et al., 2007; Kokubun et al., 2007; Lauterwein et al., 1995). However, due to the diversity of lichen species and compounds, more aggressive efforts to identify and test lichen secondary metabolites for potential antibiotic properties seem to be logical.

North America is a home to a diverse assemblage of lichen species (Esslinger, 2012). Recently, there has been a general increase in taxonomical, ecological, and phylogenetic studies of North American lichens; however, studies exploring the pharmaceutical properties of lichens and/or their secondary metabolites remain largely understudied.

In this paper, we report the inhibitory activities of acetone and methanol extracts of 34 species of lichens, collected from various locations in the United States, against four pathogenic bacterial strains.

Materials and methods

Collection of lichen species

Thirty-four lichen species were collected from various locations (June 2010–August 2011) throughout the United States (Table 1). Species identifications were made by Prof. Dr. Larry L. St. Clair, Brigham Young University. All voucher specimens have been deposited in the Herbarium of Nonvascular Cryptogams at Brigham Young University (BRY-C) in Provo, UT.

Extraction of lichen metabolites

Dried, cleaned lichen materials (4 g) were obtained from each of the 34 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 a 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. Relative R_f (rate of flow) values for the chemicals extracted from each species were compared with the published literature (Orange et al., 2001) to identify the specific compounds in each extract. 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), *S. aureus* (ATCC

Table 1. Lichen compounds identified using thin layer chromatography.

Lichens	Compounds
Alectoria imshaugii Brodo & D. Hawksw.	Usnic acid, squammatic acid, thamnolic acid
Alectoria sarmentosa (Ach.) Ach.	Usnic acid, barbatic acid, squammatic acid, thamnolic acid
Bryoria fuscescens (Gyelnik) Brodo & D. Hawksw.	Fumarprotocetratic acid, norstictic acid, protocetraric acid
Cladonia furcata (Hudson) Schrader	Fumarprotocetraric acid, protocetraric acid
Evernia prunastri (L.) Ach.	Atranorin, evernic acid
Everniastrum catawbiense (Degel.) Hale ex Sipman	Atranorin, gyrophoric acid
Flavocetraria nivalis (L.) Kärnefelt & Thell	Usnic acid
Hypogymnia physodes (L.) Nyl.	Barbatic acid
Letharia columbiana (Nutt.) J. W. Thomson	Vulpinic acid
Letharia vulpina (L.) Hue	Vulpinic acid
Lobaria pulmonaria (L.) Hoffm.	Norstictic acid, stictic acid constictic acid, cryptostictic acid, confumarprotocetraric acid
Masonhalea richardsonii (Hooker) Kärnefelt	Alectoronic acid
Parmelia sulcata Taylor	Atranorin, salazinic acid, consalazinic acid
Parmotrema reticulatum (Taylor) M. Choisy	Atranorin
Peltigera aphthosa (L.) Willd.	Teniorin, triterpenes
Platismatia glauca (L.) W. L. Cul. & C. F. Culb.	Atranorin, caperatic acids
Ramalina sinensis Jatta	Usnic acid
Rhizoplaca chrysoleuca (Sm.) Zopf	Usnic acid
Rhizoplaca haydenii (Tuck.) W. A. Weber	Usnic acid
Rhizoplaca idahoensis Rosentreter & McCune	Usnic acid
Rhizoplaca marginalis (Hasse) W. A. Weber	Usnic acid
Rhizoplaca melanophthalma (DC.) Leuckert & Poelt	Usnic acid, psoromic acid, constipatic acid, dendroconstipatic acid, subpsoromic acid
Rhizoplaca peltata (Ramond) Leuckert & Poelt	Usnic acid, atranorin, pannarin
Sphaerophorus globosus (Hudson) Vainio	Phaerophorin
Thamnolia vermicularis (Sw.) Ach. ex Schaerer	Thamnolic acid, baeomycesic acid
Tuckermannopsis ciliaris (Ach.) Gyelnik	Protolichesterinic acid
Umbilicaria americana Poelt & T. H. Nash	Gyrophoric acid
Umbilicaria mammulata (Ach.) Tuck.	Gyrophoric acid
Usnea hirta (L.) F. H. Wigg.	Usnic acid, salazinic acid, consalazinic acid
Usnea strigosa (Ach.) Eaton	Usnic acid, norstictic acid, gallbinic acids
Vulpicida canadensis (Räsänen) J.-E. Mattsson & M. J. Lai	Usnic acid, vulpinic acid, zeorin
Xanthoparmelia chlorochroa (Tuck.) Hale	Usnic acid, salazinic acid, consalazinic acid, norstictic acid
Xanthoparmelia coloradoënsis (Gyelnik) Hale	Usnic acid, norstictic acid, stictic acid, constictic acid
Xanthoparmelia wyomingica (Gyelnik) Hale	Usnic acid, salazinic acid, consalazinic acid, norstictic acid

6538P), and methicillin-resistant *S. aureus* (*S. aureus* COL). Cultures of *S. aureus*, *E. coli*, and *P. aeruginosa* were provided by Dr. Rex G. Cates, Department of Biology, Brigham Young University and the *S. aureus* COL culture was obtained from Dr. Bryan Wilkinson, at the University of Illinois.

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 (Shrestha & St. Clair, 2013b). 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 (Sigma-Aldrich, St. Louis, MO) with extract concentrations ranging from 500 to 3.9 μg/ml in a 24-well plate. Four microliter of an overnight culture of one of the four 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).

Results

Identification of lichen metabolites

Major chemical compounds from lichen extracts are reported in Table 1.

Antimicrobial activities

MIC values for acetone and methanol extractions of the 34 lichen species against the four different bacterial strains are reported in Table 2. 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, Letharia vulpina,* and *Vulpicida canadensis* were active against *E. coli* (Table 2). 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.

Discussion

Although ecological, taxonomical, and phylogenetic studies of North American lichens have been active study areas in lichenology (Geiser et al., 2010; Leavitt et al., 2012; Leavitt & St. Clair, 2011; Lendemer & Hodkinson, 2013; McMurray et al., 2013; Shrestha et al., 2012), relatively few studies have explored the biological roles of lichens (He et al., 2005; Lawrey, 1989; Shrestha & St. Clair, 2013b). 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 (Hobbs, 1986; Ingólfsdóttir et al., 1985; Kokubun et al., 2007; Lauterwein et al., 1995; Lawrey, 1989; Manojlovic et al., 2011). 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 produces 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., 1995). However, they were not able to document the antibiotic effects of vulpinic acid against *E. coli* at their highest concentration of 32 μg/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, 2012; Srivastava et al., 2013). We found that all crude extracts of the tested lichen species except for *Lobaria pulmonaria* and *Umbilicaria 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 2). 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*

Table 2. Minimum Inhibitory Concentration (MIC) values (μg/ml) for different lichens against four bacteria.

	E. coli		*P. aeruginosa*		*S. aureus*		*MRSA*	
	A	M	A	M	A	M	A	M
Alectoria imshaugii	–	–	31.25	15.6	62.5	62.5	250	125
Alectoria sarmentosa	–	–	31.25	15.6	62.5	31.25	125	62.5
Bryoria fuscescens	–	–	125	–	125	–	500	–
Cladonia furcata	–	–	250	–	500	–	500	–
Evernia prunastri	–	–	31.25	125	62.5	250	125	500
Everniastrum catawbiense	–	–	250	–	125	–	500	–
Flavocetraria nivalis	–	–	31.25	15.6	62.5	31.25	500	62.5
Hypogymnia physodes	–	–	62.5	250	62.5	62.5	62.5	250
Letharia columbiana	250	–	125	–	125	500	31.25	125
Letharia vulpina	125	500	125	500	3.9	15.6	31.25	125
Lobaria pulmonaria	–	–	–	–	–	–	–	–
Masonhalea richardsonii	–	–	250	500	125	125	125	250
Parmelia sulcata	–	–	125	–	250	–	125	–
Parmotrema reticulatum	–	–	250	–	250	–	250	–
Peltigera aphthosa	–	–	250	–	–	–	–	–
Platismatia glauca	–	–	250	–	500	–	500	–
Ramalina sinensis	–	–	15.6	250	15.6	–	62.5	–
Rhizoplaca chrysoleuca	–	–	7.8	125	7.8	250	15.6	500
Rhizoplaca haydenii	–	–	3.9	31.25	15.6	31.25	15.6	62.5
Rhizoplaca idahoensis	–	–	7.8	31.25	15.6	125	15.6	125
Rhizoplaca marginalis	–	–	7.8	125	7.8	250	7.8	500
Rhizoplaca melanophthalma	–	–	15.6	62.5	15.6	125	31.25	250
Rhizoplaca peltata	–	–	15.6	62.5	31.25	250	31.25	250
Sphaerophorus globosus	–	–	7.8	31.25	62.5	500	62.5	500
Thamnolia vermicularis	–	–	31.25	125	125	500	500	500
Tuckermannopsis ciliaris	–	–	125	–	62.5	250	250	500
Umbilicaria americana	–	–	500	–	500	–	–	–
Umbilicaria mammulata	–	–	500	–	–	–	–	–
Usnea hirta	–	–	3.9	15.6	7.8	31.25	7.8	62.5
Usnea strigosa	–	–	3.9	31.25	7.8	62.5	15.6	250
Vulpicida canadensis	250	–	15.6	15.6	15.6	62.5	31.25	125
Xanthoparmelia chlorochroa	–	–	3.9	62.5	7.8	62.5	31.25	–
Xanthoparmelia coloradoënsis	–	–	7.8	62.5	7.8	250	15.6	500
Xanthoparmelia wyomingica	–	–	15.6	15.6	15.6	62.5	62.5	500

A, acetone extract; M, methanol extract.

(Cocchietto et al., 2002; 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 nine of our tested lichen species showed relatively low MIC values (<16 μg/ml) against the methicillin-resistant *S. aureus* and extracts from *Rhizoplaca marginalis* and *Usnea 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*, *Usnea strigosa, Rhizoplaca haydenii*, and *Xanthoparmelia chlorochroa* were the most effective with MIC values of 3.8 μg/ml (Table 2). 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 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.

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Declaration of interest

There is no conflict of interest in any form between the authors.

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