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MICROBIOLOGY

Antibacterial potencial of 12 Lichen species

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Abstract: Resistant bacterial infections are a major public health problem worldwide, which entails the need to search for new therapeutic agents. In this context, lichens stand out, provided that they are producers of structurally diverse compounds that have attractive biological properties, including antimicrobial activity. Thus, extracts of 12 lichen species were prepared and their potential to inhibit the growth of 5 bacterial strains was evaluated in this work. The chemical compositions of these extracts were examined using TLC and microcrystallization, being the identity of the active compounds in each extract attributed based on the bioautography technique. The most active extracts (and their identified active compounds) were from *Cladonia borealis* (usnic, barbatic and 4-*O*-demethylbarbatic acids), *Cladina confusa* (usnic and perlatolic acids), *Stereocaulom ramulosum* (atranorin, perlatolic and anziaic acids) and *Canoparmelia cryptochlorophaea* (cryptochlorophaeic and caperatic acids), with MICs ranging from 7.8 to 31.25 µg/mL, including for resistant clinical strains. MIC values were also obtained for substances isolated from lichens for comparison purposes. A group of four extracts containing usnic acid was analyzed by ^{1}H NMR in order to correlate relative proportion of major metabolites and extracts activity. The less active extracts in this group, in fact, presented low proportion of usnic acid.

Key words: Antimicrobial, bioautography, lichen, microdilution.

INTRODUCTION

Antibiotic resistance has drawn the attention of public agencies all over the world. The cost of treatment for patients infected with resistant microorganisms is high, as well as the risks in surgical procedures and infections (Picconi et al. 2017, WHO 2018). According to Rai et al. (2017) there are about 2 million cases of resistant infections per year in the United States, with 23,000 deaths, and in Europe, the death toll reaches 25,000 per annum. The situation in Asia and developing countries is even more worrying and considering the increase in antibiotic resistance to several pathogens, infections with multi-resistant microorganisms are estimated to account for 10 million deaths per year by 2050, surpassing other diseases such as cancer (Rai et al. 2017).

Taking this into account, the search for new active compounds should be frequent to keep up with the adaptability of bacteria. Consequently, processes in drug discovery, activity optimization and characterization of selectivity and toxicity of compounds are of utmost importance.

Nature is an important source of chemical compounds for the search for unknown therapeutic agents (Owen & Laird 2018, Rondevaldova et al. 2018), and is a great ally in the urgent search for new antimicrobial agents. In this context, lichens have a vital role because they produce a number of unique compounds (e.g., depsides, depsidones, dapsones, dibenzofurans, anthraquinones, xanthones),

with a wide range of biological activities (Calcott et al. 2018, Galanty et al. 2019, Reddy et al. 2019), among them, antibiotic activity. According to Kosanić & Ranković (2015), more than 50% of the lichens studied for their antibiotic potential were active. A recent review by Basnet et al. (2018) compiles the antimicrobial activity information of lichen compounds described in the literature between 1985 and 2017, showing the wide structural variety and biological potential, with quantitative and qualitative data. Among the compounds described, some prominent examples are physodic, lobaric and rhizocarpic acids, active on multiresistant strains of *S. aureus*; gyrophoric acid, with MICs up to 0.125 µg/mL for various bacterial strains; the sulfur compounds coniothiepinol A and coniothienol A, active on *Enterococcus* strains.

Given the importance of lichen as a source of biologically active substances and in order to contribute to the search for strategies to constrain bacterial infections, 12 species of lichen were selected to evaluate their antibacterial potential, together with the analysis of their chemical composition as well as to investigate the substances responsible for the activity presented by the extracts.

MATERIALS AND METHODS

Lichens and isolated compounds

The species selected for the study were: *Cladonia borealis* Stenroos, *Cladonia confusa*, R. Santesson, *Cladonia crispatula* (Nyl.) Ahti, *Cladonia furcata* (Hudson) Schrader, *Punctelia canaliculata* (Lynge) Krog, *Parmotrema lichexanthonicum* Eliasaro & Adler, *Pseudoparmelia sphaerospora* (Nyl.) Hale, *Ramalina anceps* Nyl., *Stereocaulon ramulosum* (Sw.) Räusch, *Usnea jamaicensis* Ach., *Canoparmelia cryptochlorophaea* (Hale) Elix and *Concamerella pachyderma* (Hue) W.L. Culb. & C.F. Culb. Exsiccates are deposited in the herbarium of the Federal University of Mato Grosso do Sul, in Campo Grande/ MS (CGMS 52970, CGMS 40953, CGMS 39230, CGMS 39229, CGMS 40952, CGMS 52969, CGMS 49837, CGMS 49839, CGMS 40957, CGMS 49838, CGMS 37949 and CGMS 52968, respectively). All species studied are registered at SisGen platform (entry ABAE41C).

Some isolated compounds were selected for evaluation of their antimicrobial activity. Usnic (from *Usnea subcavata*), perlatolic (from *C. confusa*), psoromic (from *U. jamaicensis*), hypostictic, secalonic (both from *P. sphaerospora*), salazinic (from P. *lichexanthonicum*), norstictic (from *Ramalina* sp.), barbatic (from *C. borealis*) and protocetraric acids and atranorin (both from *Parmotrema dilatatum*) were isolated according to the procedure described in the literature (Honda et al. 2010, Brandão et al. 2013, Guterres et al. 2017).

Chemical composition of the extracts

The composition analysis of each extract was performed by thin layer chromatography (TLC) and also by making use of the microcrystallization (MC) technique. Portions of the thalli were cleaned, fragmented and extracted twice with acetone at room temperature. Silica gel 60 GF_{254} precoated TLC plates (Merck) were used for TLC by applying the following eluents: toluene: ethyl acetate: formic acid (139: 83: 8, v/ v/ v); toluene: acetic acid (85: 15, v/ v). The spots were visualized under UV light (254 nm) and then chemically revealed by nebulization with methanol / H_2SO_4 solution (10%) followed by heating, and after that, with *p*-anisaldehyde / H_2SO_4 solution, which was also followed by heating.

For MC technique, the following solutions were used: glycerin: acetic acid (GE 1: 3 and 3: 1 v/ v), glycerin: ethanol: water (GAW 1: 1: 1 v/ v/ v) and glycerin: ethanol: *O*-toluidine (GAoT 2: 2: 1 v/ v/ v). The produced crystalline forms were

observed under a microscope (Nikkon Eclipse E 220) with 10x magnification. Purity grade P.A solvents were employed at all stages of the work.

NMR spectroscopy was used for composition analysis of some extracts of interest in order to obtain the relative proportion of their major constituents (*C. confusa, U. jamaicensis, C. borealis and R. anceps*). The dried extracts were resuspended using DMSO-d_6 (Sigma-Aldrich), at a concentration of 15 mg/mL. ^{1}H NMR spectra were acquired on a Bruker DPX-300 spectrometer (operating at 300.13 MHz for ^{1}H) using one single pulse sequence (90°x) with 8 transient scans. The spectra were acquired and processed with 64k points. Manual phase, baseline corrections and exponential multiplication of 0.3 Hz were applied. The chemical shifts were calibrated using the residual solvent signal as a reference. Signals unambiguously attributed to the analyzed substances were used to assess the relative proportion. In *C. confusa* extract, the signals at 2.0 ppm (methyl-16 of usnic acid) and 3.74 ppm (methoxyl group of perlatolic acid) were analyzed and in *U. jamaicensis*, the signals in ppm 6.26 (H-4 of usnic acid) and 3.83 ppm (psoromic acid methoxyl) were used. For *C. borealis* extract, the signals analyzed were that at 6.18 ppm (H-4 of usnic acid) and 3.82 ppm (barbatic acid methoxyl). *R. ance*ps extract showed ^{1}H NMR signals for only one major component.

Antibacterial assays

Sigma-Aldrich culture media and reagents were used for the evaluation of the biological activity. Commercial Newprov bacterial strains *Staphylococcus aureus* (NEWP0023), *Enterococcus faecalis* (NEWP0012), *Escherichia coli* (NEWP0022), and clinical strains which were provided by UFMS University Hospital were used (*S. aureus* resistant to clindamycin, erythromycin and penicillin G, and *Enterococcus faecium* resistant to vancomycin, SisGen entries ACF89BA and A38E2AF).

Microdilution and bioautography assays were conducted as described by Honda et al. 2016a. Briefly, for microdilution test, the samples were serially diluted in 96-well plates prepared with Mueller Hinton broth, with final concentrations ranging from 1000 to 0.98 µg/mL. For positive control (gentamicin), the final concentrations ranged from 60 to 0.5 µg/mL. A 5 µL aliquot of the bacterial inoculum was added to each well (24h culture in Mueller-Hinton agar suspended in 0.45% sterile saline solution at 10^8 CFU/mL, diluted 1:10 in saline solution). Assays were performed in triplicate and the microdilution plates were incubated at 36°C for 18 h. After this time, 20 µL triphenyltetrazolium chloride aqueous solution (0.5%) (TTC) was added to each well and the plates were re-incubated at 36°C for 2 h. In wells where microbial metabolism remained active, it went from colorless to red. The minimum inhibitory concentration (MIC), was defined as the lowest concentration of each sample in which no color change occurred.

For bioautography assays chromatograms of each extract were placed on a Petri dish containing Mueller-Hinton agar and covered with an approximately 2 mm thick agar layer. The plates were then seeded with bacterial inocula and incubated at 36°C for 24 h. An aqueous solution (0.5%) of TTC was nebulized over the plates and the absence of color change indicated regions of the chromatograms containing active substances.

RESULTS AND DISCUSSION

After TLC and MC analyses (figure S1 – see Supplementary Material), with support from the literature (Culberson 1972, Culberson et al.

1981, Huneck & Yoshimura 1996) and using some isolated substances as standards, it was possible to access the main chemical constituents of each extract, as shown in Table I.

The extracts and some isolated compounds were evaluated for their antibiotic activity against Gram-positive bacteria *S. aureus* (NEWP0023 and clinical strain resistant to clindamycin, erythromycin and penicillin G), *E. faecalis* (NEWP0012) and *E. faeci*um (clinical strain resistant to vancomycin) and Gram-negative *Escherichia coli* (NEWP0022) by the broth microdilution assay (Honda et al. 2016a). The results, expressed as the minimum inhibitory concentration (MIC) in µg/mL, are shown in Table II. Gentamicin was used as a positive control.

Among the 12 assessed extracts, the antimicrobial activity of 9 of them is being described for the first time. For *C. furcata*, *S. ramulosum* and *C. confusa* extracts, antibacterial activity data are described in the literature, however, the evaluations were not performed with the same bacteria and, for the last two, the assay method was also different (Perry et al. 1999, Ranković et al. 2011, Kosanić et al. 2014).

Some extracts showed significant activity on the evaluated bacteria, with MIC values up to 7.8 µg/mL. According to Kuete, MICs up to

Table I. Studied species and their chemical composition.

Species	Chemical constituents	references
Cladonia borealis Stenroos (CGMS 52970)	usnic, barbatic and 4-*O*-demethylbarbatic acids	Osyczka 2006
Cladonia confusa R. Santesson (CGMS 40953)	usnic and perlatolic acids	Honda et al. 2016b
Cladonia crispatula (Nyl.) Ahti (CGMS 39230)	thamnolic acid	Honda et al. 2016b
Cladonia furcata (Hudson) Schrader (CGMS 39229)	atranorin, fumarprotocetraric acid	Honda et al. 2016b
Punctelia canaliculata (Lynge) Krog (CGMS 40952)	atranorin, protolichesterinic and caperatic acids	Honda et al. 2016b
Parmotrema lichexanthonicum Eliasaro e Adler (CGMS 52969)	atranorin, lichexanthone, salazinic and consalazinic acids	Micheletti et al. 2009
Pseudoparmelia sphaerospora (Nyl.) Hale (CGMS 49837)	atranorin, hypostictic and secalonic acids	Honda et al. 2010, Guterres et al. 2017
Ramalina anceps Nyl. (CGMS 49839)	usnic and stictic acids	Honda et al. 2015
Stereocaulon ramulosum (Sw.) Räusch (CGMS 40957)	atranorin, perlatolic and anziaic acids	Honda et al. 2016b
Usnea jamaicensis Ach. (CGMS 49838)	usnic, psoromic and 2'-*O*-demethylpsoromic acids	Guterres et al. 2017
Canoparmelia cryptochlorophaea (Hale) Elix (CGMS 37949)	atranorin, cryptochlorophaeic and caperatic acids	Fleig et al. 2008, Ravaglia et al. 2014
Concamerella pachyderma (Hue) W.L. Culb. & C.F. Culb. (CGMS 52968)	atranorin and protocetraric acid	Fleig et al. 2008

10 µg/mL are considered as expressive activity for pure compounds, and for the extracts, this limit would be up to 100 µg/mL (Kuete 2010). Also noteworthy is the fact that several extracts showed good activity on multi-resistant clinical strains. It can be highlighted the activity of *C. borealis*, *C. confusa*, *S. ramulo*sum and *C. cryptochlorophaea* extracts, which presented the lowest values of MIC. *C. crispatula, C. furcata* and *S. sphaerospora* extracts were moderately active (100 <CMI ≤ 625 µg/mL) for both *S. aureus* strains, while extracts from *P. canaliculata*, *R. anceps* and *U. jamaicensis* showed good to moderate activity for the 4 strains tested. None of the extracts were active on *E. coli*.

The literature provides some recent studies showing the antibacterial activity of lichen extracts (Shrestha et al. 2014, 2016, Jha et al. 2017, Moura et al. 2017, Brakni et al. 2018, Maurya et al. 2018). The work from Moura et al. describes the good activity of *Cladonia substelatta* extract on 136 *Staphylococcus* spp. strains isolated from cats and dogs, with different resistance profiles, showing that lichens can be an interesting source of antimicrobials either for veterinary purpose (Moura et al. 2017). They report meaningful results for a strategic

Table II. MIC values (µg/mL) for lichen extracts or isolated compounds against five bacterial strains.

Extract/ Isolated Compound	MIC (µg/mL)				
	S. aureus **(NEWP 0023)**	***S. aureus*** **(clinic)**	***E. faecalis*** **(NEWP 0012)**	***E. faecium*** **(clinic)**	***E. coli*** **(NEWP 0022)**
C. borealis	7.8	7.8	7.8	7.8	≥ 250
C. confusa	15.6	7.8	7.8	7.8	≥ 250
C. crispatula	250	125	≥ 250	≥ 250	≥ 250
C. furcata	250	250	≥ 250	≥ 250	≥ 250
P. canaliculata	62.5	62.5	125	125	≥ 250
P. lichexanthonicum	250	≥ 500	≥ 250	≥ 250	≥ 250
P. sphaerospora	250	250	≥ 250	125	≥ 250
R. anceps	125	125	62.5	125	≥ 250
S. ramulosum	7.8	7.8	7.8	7.8	≥ 250
U. jamaicensis	62.5	62.5	15.6	62.5	≥ 250
C. cryptochlorophaea	31.25	7.8	7.8	7.8	≥ 250
C. pachyderma	250	250	≥ 250	≥ 250	125
Protocetraric acid	≥ 250	nt	≥ 250	nt	nt
Perlatolic acid	3.9	7.8	3.9	15.6	nt
Usnic acid	3.9	3.9	1.95	7.8	nt
Psoromic acid	≥ 250	≥ 250	≥ 250	≥ 250	nt
Secalonic acid	≥ 250	≥ 250	≥ 250	≥ 250	nt
Barbatic acid	31.3	31.3	7.8	31.3	nt
Salazinic acid	≥ 250	≥ 250	31.25	125	nt
Hypostictic acid	125	≥ 250	62.5	≥ 250	nt
Norstictic acid	≥ 250	125	62.5	125	nt
Atranorin	≥ 250	≥250	≥ 250	≥ 250	nt
Gentamicin	0.5	0.5	7.5	60	1.9

nt: not tested.

area, as zoonotic antibiotic resistance can be transmitted to human pathogens through direct contact between animal and humans (Wegener 2012).

Shrestha et al. draws attention to a study of 34 lichen species from North America, and highlight that extracts with MICs <16 µg/mL over methicillin-resistant *S. aureus* (MRSA) were considered very active (Shrestha et al. 2014). Based on this parameter, extracts from *C. borealis*, *C. confusa*, *S. ramulosum* and *C. cryptochlorophaea* have a great antibacterial potential, since they presented MIC values <16 µg/mL for the two multi-resistant clinical strains evaluated. In addition, in a further work the author selected one of these 34 extracts (*Letharia vulpina*) and studied its mode of antimicrobial action, proven it to interfere in membrane stability and to disrupt cell division processes (Shrestha et al. 2016).

The active extracts were selected for the antibiotic activity assay using the bioautography technique (Honda et al. 2016a), in order to have qualitative indications of which would be the active components of each extract. The assignment of the constituents, which are likely to be responsible for inhibiting microbial growth, was based on chromatograms of the same extracts which were chemically revealed (Table III, Figures S6 to S9). It´s interesting to highlight that cryptochlorophaeic and caperatic acids were found to be the active compounds on *C. cryptochlorophaea* extract. This is the first report of antimicrobial activity for these substances.

Figure 1 shows the structures of the compounds identified as active in the evaluated extracts.

Some isolated compounds were also evaluated for their antimicrobial potential by broth microdilution method. Usnic, perlatolic and barbatic acids and atranorin were selected for assessment because they are present in active extracts and have been identified as responsible for antimicrobial activity, according to the bioautography assay. The other active compounds, according to bioautography, could not be isolated due to small amount of lichen available. Psoromic acid as well as hypostictic, secalonic, norstictic, salazinic, and protocetraric acids, were present in extracts with moderate or no activity, but were also selected to be evaluated separately.

Table III. Active components in bioautography assay of selected extracts.

Extract	Active compounds			
	***S. aureus* (NEWP0023)**	***S. aureus* (clinic)**	***E. faecalis* (NEWP0012)**	***E. faecium* (clinic)**
C. borealis	usnic, barbatic and 4-*O*-demethylbarbatic acids	nt	usnic, barbatic and 4-*O*-demethylbarbatic acids	usnic, barbatic and 4-*O*-demethylbarbatic acids
C. confusa	usnic and perlatolic acids	usnic and perlatolic acids	usnic and perlatolic acids	usnic and perlatolic acids
S. ramulosum	atranorin, perlatolic and anziaic acids	atranorin, perlatolic and anziaic acids	atranorin, perlatolic and anziaic acids	perlatolic and anziaic acids
C. cryptochlorophaea	cryptochlorophaeic and caperatic acids	cryptochlorophaeic acid	cryptochlorophaeic acid	cryptochlorophaeic and caperatic acids

nt: not tested.

Table II presents the MIC data for these compounds and shows the great antibacterial potential of usnic and perlatolic acids, which presented low MIC values for the 4 strains tested. Barbatic acid showed good to moderate activity, especially against *E. faecalis* strain for which it was more active. *E. faecalis* was also more susceptible to depsidones norstictic acid, hypostictic acid and salazinic acid, with moderate MIC values.

There have been reports for promising antibiotic activity of usnic and perlatolic acids, the most active compounds found in this work. The recent review published by Galanty et al. presented antimicrobial activity of usnic acid for a large set of bacterial strains, showing MIC values from 2 to 16 µg/mL for *E. faecalis*, *E. faecium*, MSSA (methicillin-susceptible *S. aureus*) and MRSA (methicillin-resistant *S. aureus*) (Galanty et al. 2019). Antonenko et al. (2019) discussed the mechanism of action of this compound, pointing out that it is related to its ability to cause dissipation of membrane potential in bacterial cells (Antonenko et al. 2019). For perlatolic acid, Bellio et al. (2015) showed their relevant antimicrobial activity against 20 strains of MRSA, with MICs ranging from 4 to 64 µg/mL. In addition, they revealed a synergistic relationship between perlatolic acid and some commercial antibiotics, such as clindamycin and gentamicin (Bellio et al. 2015), which has been described as an interesting approach to overcome antimicrobial resistance.

There are also data for anziaic acid (Lin et al. 2013), barbatic acid (Martins et al. 2010) and atranorin (Studzińska-Sroka et al. 2017) against various bacterial strains. The MIC values found throughout this work are comparable to those found in the literature, but for some compounds, such as perlatolic and barbatic acids and atranorin, the activity on *Enterococcus* is being described for the first time.

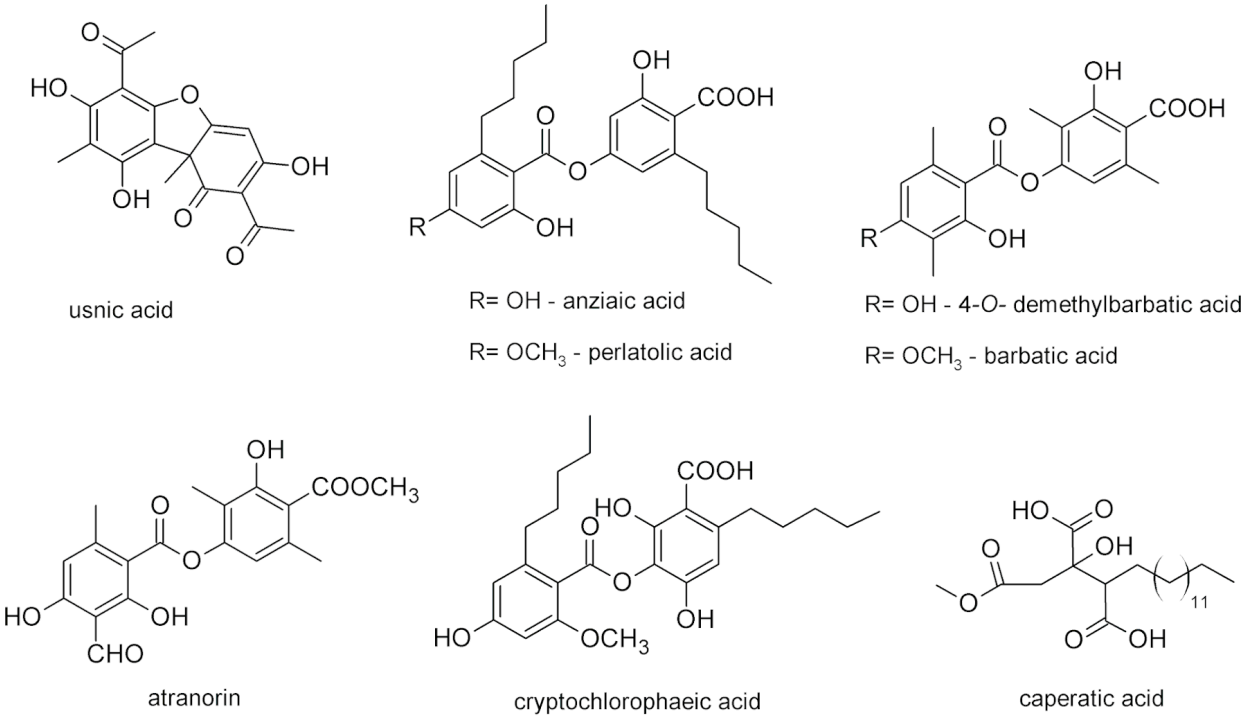

Figure 1. Chemical structures of compounds with antimicrobial activity according to bioautography test.

The extracts of *C. confusa, U. jamaicensis, C. borealis* and *R. anceps* present usnic acid as one of their chemical components, and this substance was strongly active against the evaluated bacteria, however, the antimicrobial profile of the extracts did not show the same behavior. For this reason, ^{1}H NMR spectra were obtained from the extracts to compare the relative proportion of the major metabolites enabling to understand how the variation of the concentration of the substances may be influencing the evaluated biological activity (Table IV, Figures S2 to S5).

With this analysis, it was possible to observe that activity was directly linked to proportion of active compounds. *R. anceps* and *U. jamaicensis* extracts contain no or few usnic acid, and it correlates with their poor antibiotic activity. For the first one, it can be observed that the MIC values of the extract and isolated norstictic acid are the same for 3 of the 4 strains evaluated. The behavior observed for the *U. jamaicensis* extract follows the same trend, as psoromic acid, the major component, was not active when evaluated alone and the low activity of the extract reflects the low proportion of usnic acid in the mixture.

The relative proportion observed between usnic acid and barbatic acid was approximately 1:1 in *C. borealis* extract. It was not found in the ^{1}H NMR spectrum of the extract signals that could be assigned only to 4-*O*-demethylbarbatic acid, so it was not possible to establish its relative proportion in the mixture. The antibacterial activity of the extract generally seems to assume a median behavior between the activity of the two compounds alone, being more active than barbatic acid and less active than usnic acid.

Table IV. Porportion of main compounds found in selected lichen extracts by NMR analisys.

Extract	Main Compounds and Proportion
R. anceps	norstictic acid
U. jamaicensis	usnic acid and psoromic acid (1:5)
C. borealis	usnic acid and barbatic acid (1:1)
C. confusa	usnic acid and perlatolic acid (1:1.5)

The *C. confusa* extract presented a relative proportion between usnic and perlatolic acids of approximately 1:1.5, being the perlatolic acid the major component. Both compounds have outstanding antibacterial activity (Table II); however, the extract shows a higher MIC than the isolated components for the two standard strains. Nevertheless, the reduction in activity does not appear to be significant enough to state that the effect between the compounds is antagonistic (Owen & Laird 2018).

CONCLUSION

Extracts from 12 lichen species had their chemical profile evaluated, in order to attribute their major chemical components. These extracts presented a wide variety of compounds, from the classes of usnic acids, depsides, depsidones, xanthones and fatty acids. The extracts were also tested for their antibacterial potential by broth microdilution assay against 5 bacterial strains, and among the active ones, there was selectivity for Gram-positive bacteria, which included 2 standard strains and 2 multi-resistant clinical strains. *C. borealis*, *C. confusa*, *S. ramulosum* and *C. cryptochlorophaea* extracts were the most active, with MIC values between 7.8 and 31.25 µg/ mL. It was possible to assign the active components of each extract through the bioautography technique: usnic acid, barbatic and 4-*O*-demethylbarbatic acids, perlatolic and anziaic acids, atranorin, cryptochlorophaeic and caperatic acids. For these last two acids,

it is the first report of antibiotic activity. Other compounds found in the extracts were also evaluated by microdilution and proved active, like usnic, perlatolic, barbatic, salazinic, hypostictic and norstictic acids.

As usnic acid was strongly active and some extracts containing this component were inactive, an NMR analysis was performed to assign the relative proportion between the major components of 4 extracts containing this substance (*C. borealis, C. confusa, R. anceps*. and *U. jamaicensis*) making it clear that the proportion in which this active component is found in the extract is directly related to the potency of the activity. When correlating the MIC values found for the isolated compounds and extracts, no evidence of synergistic or antagonistic effect was found between the components.

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SUPPLEMENTARY MATERIAL

Figures S1-S9

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ACM and NKH conceived the study. AAS and NKH were responsible for the collection and identification of lichens. NKH, TM and LMR worked on chemical composition by TLC and NMR. ACM and TM realized the biological assays. ACM, NKH, TM and LMR analysed and interpreted the data. ACM drafted the manuscript. All authors commented on drafts on the paper. All authors have approved the final draft of the manuscript.