

IN VITRO RADICAL-SCAVENGING ACTIVITY, TOXICITY AGAINST *A. salina*, AND NMR PROFILES OF EXTRACTS OF LICHENS COLLECTED FROM BRAZIL AND ANTARCTICALuciana M. Ravaglia^a, Kerolayne Gonçalves^a, Nathália Mitsuko Oyama^a, Roberta Gomes Coelho^a, Adriano A. Spielmann^b and Neli K. Honda^{a,*}^aInstituto de Química, Universidade Federal de Mato Grosso do Sul, 79070-900 Campo Grande – MS, Brasil^bDepartamento de Biologia, Universidade Federal de Mato Grosso do Sul, 79070-900 Campo Grande – MS, Brasil

Recebido em 10/12/2013; aceito em 17/03/2014; publicado na web em 17/06/2014

Extracts of six lichen species collected from Brazil and Antarctica were investigated for their potential toxicity and radical-scavenging properties. The composition of the extracts was investigated using TLC and NMR, leading to identification of atranorin (**1**), along with salazinic (**2**), barbatic (**3**), α -alectoronic (**4**), α -collatolic (**5**), cryptochlorophaeic (**6**), caperatic (**7**), lobaric (**8**), and protolichesterinic (**9**) acids. All acetone extracts were evaluated for their 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and subjected to *Artemia salina* bioassay. The free-radical-scavenging activities of each extract (100 μ g) ranged from $8.9 \pm 0.1\%$ to $38.7 \pm 2.5\%$ and the EC_{50} values ranged from 0.24 ± 2.10 to 3.54 ± 0.28 mg mL⁻¹, while the toxicity of the extracts against *A. salina* were low (151.0 to >600 μ g mL⁻¹).

Keywords: lichens; phenolic compounds; NMR; radical-scavenging potential; *Artemia salina*.

INTRODUCTION

Plants, fungi, mosses, algae, and lichens are responsible for the production of numerous compounds with enormous structural diversity, primarily classified according to their biosynthetic origins. Among these, phenolic compounds, such as flavonoids, tannins, xanthenes, caffeic acid and its analogues, and resveratrol, are potent antioxidants and free-radical scavengers, which exert a potent inhibitory effect against oxidative damage to human cells—impairment that can lead to diseases such as cancer, osteoporosis, and degenerative conditions.¹ Phenolic compounds are important antiviral, anti-inflammatory, antiatherosclerotic, and anticancer agents, and are also known for their cardioprotective effect, among other properties.² Among natural phenolic compounds, those generated by lichens have been widely studied for their potential activity. The biosynthesis of these compounds in lichens occurs via two major routes: acetate-polymalonate and shikimic acid pathways. The acetate-polymalonate pathway accounts for production of the majority of typical lichenic substances, such as depsides, depsidones, dibenzofurans, usnic acids, anthraquinones, xanthenes, and higher aliphatic acids. The shikimic acid pathway is the source for pulvinic acid derivatives. Non-phenolic substances, including terpenes, steroids, and carotenoids, are synthesized through the mevalonate route.³ Because lichens are potential sources of pharmacologically active compounds, investigations to evaluate the biological activities (antimicrobial, antiviral, antitumor, antioxidant, allelopathic, etc.),⁴ including free-radical-scavenging activity⁵ of their extracts or the isolated compounds (and their structurally modified analogues) have been conducted.

Despite the potential radical-scavenging activity of many phenolic compounds, these compounds can be toxic and pose health risks. Therefore, extract toxicity is a crucial feature; however, it can be easily evaluated by assaying the extracts against the brine shrimp *Artemia salina*. This assay has proven to be excellent for preliminary screening of the toxicity of compounds; the indicated toxicity is subsequently evaluated by additional tests. Nonetheless, despite its advantages, the *A. salina* assay has seldom been used for lichen extracts or for compounds isolated from lichens.⁶

To identify new sources of anti-oxidizing agents and free-radical-scavenging compounds potentially useful as food additives or in cosmetics and other products of interest, several studies have addressed the antioxidant activity of plant and lichen extracts, however, only few have identified the compounds responsible for these properties.⁷

With a view to expand the available knowledge on free-radical scavengers, the present study evaluates the extracts of six lichens in a TLC-based 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Free-radical-scavenging activity was quantified spectrophotometrically and toxicity was evaluated on *A. salina* larvae. TLC and NMR analyses were used to elucidate the composition of each extract.

RESULTS AND DISCUSSION

Table 1 shows the results obtained from free-radical-scavenging activity assay, EC_{50} values, and that from the toxicity assay. In terms of free-radical-scavenging activity (FRSA) and EC_{50} values, the most promising extracts were those of *P. wainioi* and *C. cryptochlorophaea* (FRSA% = $28.14 \pm 0.41\%$ and $38.7 \pm 2.5\%$; EC_{50} = 0.56 ± 1.34 and 0.24 ± 2.1 mg mL⁻¹, respectively). Extracts of *P. cetratum*, *C. aggregata*, and *S. alpinum* exhibited inhibitory potentials ranging from 12.53% to 13.85% and EC_{50} values of 1.88 ± 0.76 mg mL⁻¹ (for *P. cetratum*), 3.54 ± 0.28 mg mL⁻¹ (for *C. aggregata*), and 1.55 ± 0.9 mg mL⁻¹ (for *S. alpinum*), while *P. mesotropum* extract showed the lowest activity ($8.9 \pm 0.1\%$ and EC_{50} of 3.13 ± 0.56 mg mL⁻¹). However, extracts of *P. wainioi*, *C. cryptochlorophaea*, and *C. aggregata* were less toxic to *A. salina* than those of *P. mesotropum*, *P. cetratum*, and *S. alpinum*.

TLC and NMR were used to investigate the nature of compounds present in the extracts. All the extracts contained at least one compound exhibiting DPPH radical scavenging activity as determined by TLC bioautography. When visualized with methanol:sulfuric acid and *p*-anisaldehyde or FeCl₃ solutions, constituent phenolic compounds separated by TLC in each extract could be identified. NMR spectra of all species showed chemical shift signals that led to structural characterization of the principal compounds, while signals of lower intensity were attributed to functional groups present in minor constituents.

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Table 1. Composition and activity of lichen extracts

Extracts	% dry wt.	DPPH (FRSA %) ^a EC ₅₀ (mg mL ⁻¹) ^b	<i>A. salina</i> (LC ₅₀ in µg mL ⁻¹ , 95% CI) ^c	Extract composition
<i>C. cryptochlorophaea</i>	33.3	38.7±2.5 0.24±2.10	>600.0 µg mL ⁻¹	Atranorin Cryptochlorophaic acid Caperatic acid
<i>P. wainioi</i>	25.6	28.14±0.41 0.56±1.34	>600.0 µg mL ⁻¹	Atranorin α-Alectoronic acid α-Collatolic acid
<i>S. alpinum</i>	15.3	13.85±0.90 1.55±0.90	151.25 µg mL ⁻¹ (99.0 – 231.1)	Atranorin Lobaric acid
<i>C. aggregata</i>	7.1	13.25±0.10 3.54±0.28	690.6 µg mL ⁻¹ (370.5 – 1287.0)	Atranorin Barbatic acid
<i>P. cetratum</i>	21.0	12.5±0.4 1.88±0.76	383.5 µg mL ⁻¹ (110.9 – 1325.7)	Atranorin Salazinic acid
<i>P. mesotropum</i>	5.8	8.9±0.1 3.13±0.56	207.3 µg mL ⁻¹ (95.0 – 452.0)	Atranorin Protolichesterinic acid
Orcinol	—	22.60±0.58 1.48±0.65	<50.00 µg mL ⁻¹	—
Quinine sulfate	—	NT	65.70 µg mL ⁻¹ (23.5 – 183.4)	—

^aFRSA%: free-radical-scavenging activity of extract (100 µg); ^bEC₅₀: concentration of extract in the reaction mixture needed to decrease the initial DPPH concentration by 50%; ^cLC₅₀: concentration that causes 50% death; 95% CI: 95% confidence interval. NT: not tested.

The phenolic compounds in the extracts are derived from orsellinic or β-methyl orsellinic acids. Depsides and depsidones derived from orsellinic acid have alkyl groups (one, three, five, or seven carbons) linked to C-6 and C-6'. Phenolic hydroxyls (either free or as a methoxy group) occur at C-2, C-4, C-2', and C-4'. In the depsidone structures, in addition to the ester linkage between aromatic rings, an ether linkage between C-2 and C-5' is present. In addition to -CH₃, -CH₂OH, -CHO, or -COOH groups linked to C-3 and C-3', depsides and depsidones derived from β-methyl orsellinic acid have the same substituent groups as the depsides and depsidones derived from orsellinic acid.⁸

Analysis of the NMR spectra and chromatographic behavior led to identification of the compounds in each extract (Figure 1) and indicated those with DPPH-scavenging properties.

Atranorin (**1**), a depside, was present in all the extracts investigated. TLC and NMR spectra provided evidence of its presence. Evidence from ¹H-NMR include low-intensity signals at 3.80-3.93 ppm corresponding to a methyl ester, four signals at 10.20-12.55 ppm corresponding to the hydrogen of the CHO and three hydroxyls, and two singlets at 6.40 and 6.70 ppm corresponding to hydrogens on C-5 and C-5'. Evidence from ¹³C- and DEPT-135 NMR spectra include signals at 192.0-193.9 ppm (for CHO), at 109.0-109.8 and 115.6-116.0 ppm (C-5 and C-5' methine carbons), at 52.0-53.0 ppm (methyl ester), and those at 24.0-25.0, 20.0-22.0, and 9.0-9.8 ppm (three methyls attached to aromatic rings).⁹ Chromatographic migration of atranorin was compared against an authentic sample (not shown).

P. cetratum – According to Hale and Fletcher,¹⁰ this species contains atranorin, along with salazinic and consalazinic acids; all substances of taxonomic relevance. TLC of its extract revealed atranorin (**1**, R_f = 0.73) and salazinic acid (**2**, R_f = 0.33). Consalazinic acid was not detected in the extract, probably occurring at low concentration in this lichen. ¹H-NMR spectral analysis identified intense signals at 4.69, 6.91, and 10.49 ppm, which are assigned to two hydrogens of a methylene group (-CH₂OH), one methine hydrogen (C-5), and an aldehyde proton, respectively. In addition, a large signal at 8.37 ppm indicates a hydroxyl linked to a lactol ring. These signals can be

assigned to the depsidone, salazinic acid. In the ¹³C-NMR (DMSO-*d*₆) spectrum, the signals at 52.7, 192.8, and 94.7 ppm confirm methylene, aldehyde, and lactol carbons, respectively. These and additional signals confirm the presence of salazinic acid.¹¹ Low-intensity signals in ¹H- and ¹³C-NMR spectra could be due to atranorin. The NMR spectrum of the acetone extract of *P. cetratum* did not exhibit signals identifying consalazinic acid. *P. cetratum* therefore contains atranorin and salazinic acid, both of which proved to exhibit DPPH scavenging ability. However, FRSA of the extract (100 µg) was low (12.5 ± 0.4%). The EC₅₀ was 1.88 ± 0.76 mg mL⁻¹ and the toxicity to *A. salina* (LC₅₀) was 383.5 µg mL⁻¹.

Cladia aggregata – According to Ahti et al.,¹² *C. aggregata* contains stictic, constictic, norstictic, and cryptostictic acids. Elsewhere in Brazil and through most of the range of *C. aggregata*, a barbatic acid chemotype is dominant. TLC of the acetone extract revealed one spot with R_f = 0.46. ¹H-NMR spectrum of the compound contained four sets of signals which are attributed to methyl groups, one methyl ether (3.84 ppm), two aromatic hydrogens (6.60 and 6.68 ppm), and one -OH group at 10.73 ppm. Comparison of NMR data of the predominant compound with that of an authentic sample of purified barbatic acid (**3**) (depside) confirmed the structure of the compound. TLC revealed the DPPH scavenging activity of **3**. Toxicity, however, was low (LC₅₀ = 690.6 µg mL⁻¹), and 100 µg of the extract inhibited 13.25 ± 0.10% of DPPH activity while the EC₅₀ value was 3.54 ± 0.28 mg mL⁻¹.

Parmotrema wainioi – The ¹H- and ¹³C-NMR spectra contained less intense chemical shifts. In the ¹H-NMR spectrum, a large singlet at 10.94 ppm suggests a hydroxyl in a lactol ring. A multiplet (resembling a triplet) at 2.58 ppm indicates the presence of a deshielded -CH₂- group, adjacent to a single -CH₂- group, hence the possibility that the -CH₂- group is linked to a carbonyl group. Such hydrogens are present in α-alectoronic (**4**) and α-collatolic (**5**) acids. The ¹H-NMR spectrum of the extract exhibits an agglomeration of signals in the region below 4 ppm, making it difficult to assign the chemical shifts accurately, which is not surprising given the structural similarity of these compounds. The ¹³C-NMR spectrum also shows

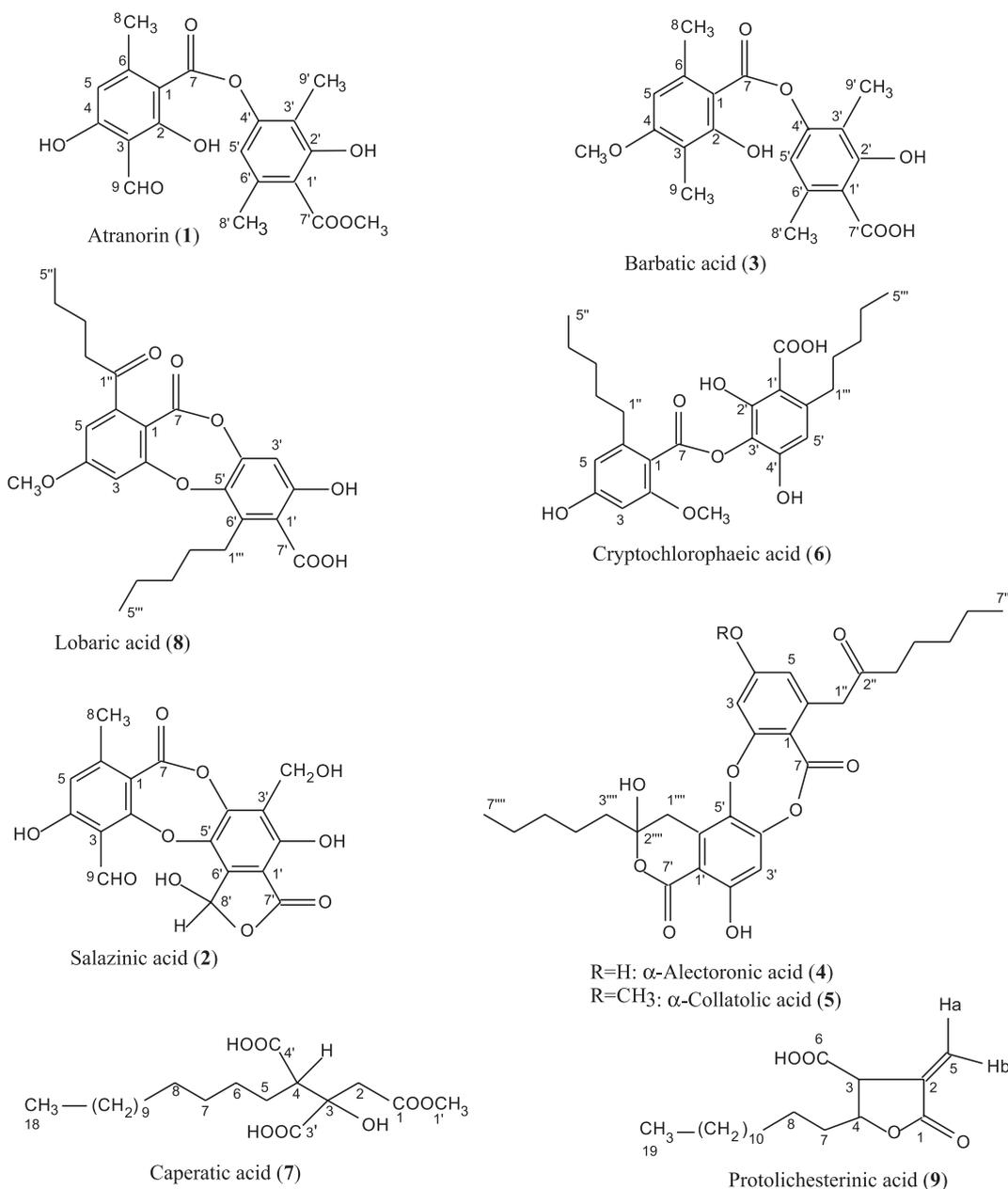


Figure 1. Structures of compounds 1-9 identified in the lichen extracts

a number of signals in the region of aliphatic carbons. The intense signals in this spectrum include one at 13.90 ppm, characteristic of a alkyl terminal $-\text{CH}_3$, and several others in the 22–40 ppm range, attributed to $-\text{CH}_2-$ groups. Signals at 207.2 and 209.4 ppm correspond to ketone carbonyls, while two deshielded signals at 42.8 and 48.0 ppm can be attributed to methylenes located in the vicinity of the ketone groups. Many signals appear to be duplicated in the ^{13}C -NMR spectrum, owing to the structural similarity of the two depsidones, which only differ by the methoxyl substituent at C-4 of α -collatolic acid. These and other signals in the ^{13}C -NMR spectrum indicate the presence of both α -alectoronic (4) and α -collatolic (5) acids in the extract.¹³ These data were confirmed by TLC analysis; α -alectoronic ($R_f = 0.15$) and α -collatolic ($R_f = 0.36$) acids were well visualized with methanol: FeCl_3 . Compounds 4 and 5 proved to be active against DPPH free radical activity, 100 μg of the extract inhibited $28.14 \pm 0.41\%$ of the activity and had an $\text{EC}_{50} = 0.56 \pm 1.34 \text{ mg mL}^{-1}$. Toxicity was low ($\text{LC}_{50} > 600 \mu\text{g mL}^{-1}$).

Canoparmelia cryptochlorophaea – TLC showed three spots,

one of them corresponded to atranorin ($R_f = 0.71$). The NMR spectrum showed signals attributable to atranorin, in addition to signals corresponding to $-\text{CH}_2-$ groups. These groups can be indicative of alkyl chains linked to aromatic rings. Analysis of ^1H -, ^{13}C -, and DEPT-135 NMR signals, coupled with the chromatographic profile of the extract, suggests the presence of cryptochlorophaeic acid (6) in the extract.¹⁴ However, the ^{13}C -NMR spectrum contains signals indicating a compound with a long alkyl chain and signals at 174.4, 170.8, and 168.2 that can be attributed to carboxyl groups, in addition to others indicative of caperatic acid (7), which was not visualized with ferric chloride on TLC. According to Elix *et al.*,¹⁵ cryptochlorophaeic (6) and caperatic (7) acids, along with atranorin (1), are produced by *C. cryptochlorophaea*. Cryptochlorophaeic acid ($R_f = 0.29$) inhibited DPPH activity. This extract (100 μg) was the most active, producing $38.7 \pm 2.5\%$ inhibition and an EC_{50} of $0.24 \pm 2.1 \text{ mg mL}^{-1}$, but exhibited low toxicity ($\text{LC}_{50} > 600.0 \mu\text{g mL}^{-1}$).

Stereocaulon alpinum – According to Øvstedal and Smith,¹⁶ this species contains atranorin and lobaric acid. TLC of the acetone extract

revealed the presence of atranorin ($R_f = 0.63$) and another compound ($R_f = 0.40$). The extract (100 μg) inhibited DPPH activity (13.85 \pm 0.90%). The EC_{50} value was 1.55 \pm 0.90 mg mL^{-1} and this extract was the most toxic against *A. salina* ($\text{LC}_{50} = 151.25 \mu\text{g mL}^{-1}$). The $^1\text{H-NMR}$ spectrum of the extract contains no signals identifying atranorin, but contains several other signals indicating two alkyl chains, a methoxy group, and four aromatic hydrogens. The DEPT-135 NMR spectrum confirms the presence of alkyl groups (δ 13.8–41.0 ppm), a methoxy group (δ 56.5 ppm), and methine carbons at 105.7, 106.1, and 111.5 ppm. These three methine carbons indicate a depsidone structure derived from orsellinic acid. In the $^{13}\text{C-NMR}$ spectrum, the signal at 203.1 ppm can be attributed to a keto group at C-1'' or C-1''' of the alkyl chain linked to an aromatic ring. The location of the other signals suggests that the structure of the compound is that of lobaric acid (**8**). These data are in agreement with the results reported by Sundholm and Huneck.¹⁵

Parmotrema mesotropum – This extract showed the lowest inhibition of DPPH activity (8.9 \pm 0.1%; $\text{EC}_{50} = 3.13 \pm 0.56 \text{ mg mL}^{-1}$) and proved to be toxic to *A. salina* ($\text{LC}_{50} = 207.3 \mu\text{g mL}^{-1}$). Signals in the $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra confirmed the presence of atranorin. In addition, the two singlets at 6.2 and 5.9 ppm can be attributed to geminal hydrogens in an olefinic system. In the $^{13}\text{C-NMR}$ spectrum, signals at 124.1 and 133.9 ppm confirm the presence of an olefinic system. Signals at 4.67 ppm, two singlets at 1.22 and 0.84 ppm, a doublet at 1.63, and a signal at 3.54 ppm in the $^1\text{H-NMR}$ spectrum suggest an aliphatic chain structure. In the $^{13}\text{C-}$ and DEPT-135 NMR spectra, two $-\text{CH}-$ groups at 79.1 and 49.2 ppm suggest a lactone ring. Signals at 10.3 ppm ($\delta^{13}\text{C}$) and 0.84 ppm ($\delta^1\text{H}$) correspond to a $-\text{CH}_3$ group at the end of the alkyl chain, while others at 22 to 40 ppm reveal $-\text{CH}_2-$ groups. These data are compatible with the structure of protolichesterinic acid (**9**),¹⁷ a compound that occurs as (+) and (–) isomers, which was not visualized on TLC after spraying with methanol:sulfuric acid and heating. Atranorin and protolichesterinic acid (**9**) were thus identified in the extract. The low activity of this extract against DPPH probably results from the scavenging activity of atranorin alone.

The phenolic structures identified in the extracts contain (in addition to phenolic hydroxyls) substituents including $-\text{COOH}$, $-\text{CHO}$, $-\text{CH}_2\text{OH}$, $-\text{CH}_3$, or an alkyl chain. The phenolic hydroxyls can be methylated. Phenols are capable of donating a hydrogen atom to free radicals, and its antioxidant activity depends on the number and position of hydroxyl groups and other substituents in the molecule.¹⁸

Free radicals, such as hydroxyls, reactive oxygen species, and hydrogen peroxide, are crucial to many cellular processes. Physiological mechanisms involving these radicals are regulated by enzymes, including tyrosinase, catalase, protein kinase C, and superoxide dismutase, or compounds such as ascorbic acid and tocopherol.¹⁹ However, when the mechanism of antioxidant protection becomes unbalanced, side effects occur due to the attack of free radicals on unsaturated fatty acids, proteins, and nucleic acids in cell membranes, potentially resulting in diseases, among them cancer, atherosclerosis, emphysema, arthritis, and cirrhosis.²⁰ These processes of oxidative deterioration of biomolecules involve enzymatic and non-enzymatic oxidation. Antioxidants can protect against non-enzymatic oxidation, whereas enzymatic oxidation can be prevented by typical inhibitors.²¹ Supplements or foods containing antioxidants can be used to reduce oxidative damage.²² Synthetic additives have been widely employed, although their safety and toxicity have been the subject of debate.²³ The *A. salina* assay is currently used to screen extracts or compounds for toxicity. Assay results can be correlated with toxicity against tumor cells, a correlation akin to that established by Solis *et al.*²⁴ for this assay and the toxicity of compounds against KB (human nasopharyngeal carcinoma) cells.

Cytotoxicity of phenols is related to lipophilicity, as expressed by the base-10 logarithm of the octanol-water partition coefficient ($\log P$), a parameter of importance in chemical toxicology as it can indicate metabolic fate, biological transport properties, and intrinsic biological activity. Electronic character has also been considered in QSAR studies.²⁵ The degree of ionization ($\text{p}K_a$) of phenols is another parameter considered in toxicity investigations.²⁶

Among the extracts assayed, *C. cryptochlorophaea* and *P. wainioi* extracts exhibited highest radical-scavenging activity and lowest toxicity to *A. salina*. The extract of *C. aggregata* was the least toxic to *A. salina* and showed intermediate radical-scavenging activity. *S. alpinum*, and *P. cetratum* extracts showed radical-scavenging activity in the same range as *C. aggregata*, despite being more toxic to *A. salina*. *P. mesotropum* extract displayed the lowest radical-scavenging activity and proved toxic. Toxicity of *P. mesotropum*, *P. cetratum*, and *S. alpinum* extracts is possibly related to the presence of atranorin and protolichesterinic, salazinic, and lobaric acids. Proper interpretation of DPPH-scavenging results and toxicity against *A. salina* requires, however, a deeper investigation than merely taking into account the effects of chemical groups present in each molecule of a given extract. The $\log P$ values, for instance, ranged from 5.77 \pm 0.40 to 7.76 \pm 0.57 (except for salazinic acid, $\log P = 1.84 \pm 0.62$), revealing a predominantly lipophilic character among the compounds identified. Regarding the $\text{p}K_a$ values of substituent groups, it appears that in cryptochlorophaeaic acid, the presence of three phenolic hydroxyls (6.14 \pm 0.50 to 7.53 \pm 0.25) and a carboxyl (the latter ionized under the assay conditions) favor radical-scavenging activity, which could explain the higher activity displayed by the *C. cryptochlorophaea* extract. Barbatic acid, present in *C. aggregata*, is also ionized under the same conditions and contains two phenolic hydroxyls, but the extract of *C. aggregata* exhibited lower free-radical scavenging activity than that of *C. cryptochlorophaea*. In *P. wainioi* extract, α -alectoronic and α -collatolic acids, and atranorin lack free carboxyl groups. Although α -alectoronic and α -collatolic acids contain two and one phenolic hydroxyl(s), respectively, scavenging activity of the *P. wainioi* extract was greater than that of *C. aggregata* (containing atranorin and barbatic acid) or *P. cetratum* (atranorin and salazinic acid). Barbatic and salazinic acids each have two phenolic hydroxyls. Furthermore, barbatic acid also contains a carboxyl that becomes ionized under the assay conditions (Table 2). None of the extracts exhibited scavenging activity significantly different from that of orcinol ($p > 0.05$). In all extracts evaluated, DPPH-scavenging activity and toxicity to *A. salina* may result not only from lipophilic and electronic effects, but also from synergistic or antagonistic effects between compounds present in the extracts.

Among the species studied, only *C. aggregata* and *S. alpinum* have been cited in the literature. Barbatic acid, isolated from the former, has been tested against four multi-drug resistant *Staphylococcus aureus* strains and the MIC assays place this compound in the same level of inhibition as other lichen substances.²⁷ The ethanolic extract of *C. aggregata* has been evaluated against four *Malassezia* strains, which are responsible for pityriasis versicolor and seborrheic dermatitis in humans. The results indicate *C. aggregata* as a potential source for cosmaceuticals.²⁸ Lobaric acid isolated from *S. alpinum* has been evaluated for its thermodynamic, photophysical, and photochemical properties, however its SPF (Sun Protection Factor) is very low when compared to homosalate (reference solar filter).²⁹ Lobaric acid has also been evaluated against twelve human cancer cells lines and is active against K-562 (leukemia), T47-D (mammary carcinoma), and Capan-1 cells, and inhibition of tubulin polymerization is observed.^{30,31}

Protolichesterinic acid, the aliphatic γ -methylene lactone, inhibits two lipoxygenases (5-LOX and 12-LOX) *in vitro*.^{30,32} In addition, the

Table 2. Log *P* values of compounds and p*K*_a values of ionizable groups

Extract Composition	log <i>P</i> ^a	p <i>K</i> _a ^a
Atranorin (1)	6.14 ± 0.49	C-2 (OH) 5.64 ± 0.45; C-4 (OH) 5.98 ± 0.30; C-2' (OH) 9.12 ± 0.20
Cryptochlorophaeic acid (6)	7.45 ± 0.43	C-2 (OH) 7.53 ± 0.25; C-2' (OH) 6.14 ± 0.50; C-4' (OH) 6.40 ± 0.50; C-7' (COOH) 2.81 ± 0.43
Caperatic acid (7)	7.54 ± 0.41	C-3' (COOH) 2.90 ± 0.36; C-3 (OH) 11.11 ± 0.29; C-4' (COOH) 3.64 ± 0.36
α-Alectoronic acid (4)	6.30 ± 0.59	C-4 (OH) 6.93 ± 0.40; C-2' (OH) 8.71 ± 0.40; C-2''' (OH) 11.18 ± 0.20
α-Collatolic acid (5)	5.95 ± 0.55	C-2' (OH) 8.73 ± 0.40; C-2''' (OH) 11.18 ± 0.20
Lobaric acid (8)	7.76 ± 0.57	C-2' (OH) 7.12 ± 0.20; C-7' (COOH) 2.60 ± 0.20
Barbatic acid (3)	5.95 ± 0.42	C-2 (OH) 7.63 ± 0.45; C-2'' (OH) 7.51 ± 0.45; C-7' (COOH) 2.68 ± 0.25
Salazinic acid (2)	1.84 ± 0.62	C-2 (OH) 5.39 ± 0.40; C-2' (OH) 8.53 ± 0.40; C-8' (CH ₂ OH) 13.43 ± 0.10; C-9' (OH) 9.86 ± 0.20
Protolichesterinic acid (9)	5.77 ± 0.40	C-6 (COOH) 3.05 ± 0.40

^aLog *P* and p*K*_a values were calculated using ACD 6.0 software.

acid significantly inhibits [³H]-thymine uptake in Capan-1, PANC-1 (both pancreatic cancer), PC-3 (prostatic adenocarcinoma), T47-D (mammary carcinoma), NIH:OVCAR-3 (ovarian adenocarcinoma), NCI-H1417 (small-cell lung cancer), and JURKAT (T-cell leukemia) cell lines, with EC₅₀ values ranging from 2.4 ± 0.9 μg mL⁻¹ to 4.3 ± 3.3 μg mL⁻¹. It exhibits moderate activity against AGS (gastric adenocarcinoma), HL-60 (acute promyelocytic leukemia), Capan-2 (pancreatic cancer), and K-562 (erythroleukemia) cell lines, with EC₅₀ values = 7.0 ± 0.9–10.7 ± 0.1 μg mL⁻¹.³⁰ Protolichesterinic and lobaric acids show a strong cytotoxic effect on HCT-116 and HeLa cell lines in a concentration-dependent manner.⁷ The antiproliferative activity of protolichesterinic acid against HeLa cells at 40 μM has been related to induction of programmed cell death through a caspase-dependent pathway.⁷ Atranorin induces apoptosis and inhibits cell proliferation in nine human cancer cell lines.³³ Salazinic acid shows strong activity toward FemX (human melanoma) and LS174 (human colon carcinoma) cell lines,⁷ but low cytotoxicity against MM98 (malignant mesothelioma) and A431 (vulvar carcinoma) cells, as well as against HaCaT keratinocytes.³⁴ α-Alectoronic acid shows a two-fold higher IC₅₀ value than that observed for cisplatin against B16 murine melanoma cells.¹³ Information on the toxicity of cryptochlorophaeic, α-alectoronic, α-collatolic, and barbatic acids remains scarce. To the best of our knowledge, an evaluation of the chemical composition and radical-scavenging and toxic activities of *P. wainioi*, *P. cetratum*, *P. mesotropum* and *C. cryptochlorophaea* extracts has not been reported previously.

CONCLUSION

In conclusion, *C. cryptochlorophaea* and *P. wainioi* extracts were most active against DPPH free-radical activity and proved to be the least toxic against *A. salina*, while *P. mesotropum*, *P. cetratum*, and

S. alpinum extracts were the least active against DPPH activity and most toxic against *A. salina*. Extracts of *C. aggregata* exhibited DPPH-scavenging activity similar to that of the latter three lichens, albeit proving to be less toxic. DPPH-based TLC bioautography and NMR analysis revealed that atranorin (1), along with salazinic (2), barbatic (3), α-alectoronic (4), α-collatolic (5), cryptochlorophaeic (6), and lobaric (8) acids, contributes to the radical-scavenging activity of the extracts investigated. These compounds, as well as caperatic (7) and protolichesterinic (9) acids, probably accounted for the toxicity against *A. salina*. All the lichen species investigated are promising sources for compounds 1–9, which possess important biological activities.

EXPERIMENTAL SECTION

Species selection and preparation

Parmotrema cetratum (Ach.) Hale, *Parmotrema wainioi* (A. L. Smith) Hale and *Canoparmelia cryptochlorophaea* (Hale) Elix and Hale were collected near Piraputanga village in Aquidauana country, Mato Grosso do Sul state, Brazil (20°27'21.2"S, 55°29'00.9"W; alt. ~200 m; *P. cetratum* on rock, *P. wainioi* and *C. cryptochlorophaea* on corticolous substrate in open forests). These species were identified by Dr. Mariana Fleig and the vouchers were deposited at the Campo Grande Herbarium of the Universidade Federal de Mato Grosso do Sul (CGMS 37950, for *P. cetratum*; CGMS 38012, for *P. wainioi*; and CGMS 37949, for *C. cryptochlorophaea*). *Parmotrema mesotropum* (Müll. Arg.) Hale was collected from Praça Bolívia, Santa Fé neighborhood, Campo Grande country, Mato Grosso do Sul state, Brazil (20°26'57.05"S, 54°35'8.72"W, on corticolous substrate). This species was identified by Dr. Luciana Canêz and a voucher was deposited at the Campo Grande Herbarium (CGMS 37545). *Cladia aggregata* (Sw.) Nyl. was collected from Fortaleza canyon at the Parque Nacional da Serra Geral, Cambará do Sul country, Rio Grande do Sul state, Brazil (29°04'04.3"S, 49°57'50.8"W, alt. ~1030 m, on terricolous substrate). *Stereocaulon alpinum* Laurer was collected from Keller Peninsula, King George Island, South Shetland Islands, Antarctica (62°04'40.50"S, 58°25'50"W, alt. ~10 m, on mosses). These two species were collected and identified by A. A. Spielmann and were deposited at the Campo Grande Herbarium (CGMS 37946, for *C. aggregata*, and CGMS 37544, for *S. alpinum*). Species were selected based on availability and botanical identification. Fragments of each lichen (200–500 mg) were cleaned (removing the substrates) and triturated in a mortar and the resulting powder exhaustively treated with acetone at room temperature. After solvent evaporation, the extracts were stored in a desiccator. TLC and ¹H-, ¹³C-, and DEPT-135 NMR methods were employed for analysis of the extracts.

TLC

The extracts were chromatographed on aluminum plates coated with silica gel GF₂₅₄ (0.20 mm, Macherey-Nagel), using the following eluents: (I) toluene:ethyl acetate:acetic acid, 6:4:1 v/v/v; (II) toluene:acetic acid, 85:15 v/v.³⁵ Spots were visualized under UV (254 nm) and then sprayed with methanol:sulfuric acid (10%) followed by *p*-anisaldehyde:sulfuric acid and finally heated until complete appearance of the spots, or sprayed with a methanolic solution of FeCl₃ (5%). Migration of substances was expressed as rate of flow (*R*_f).

NMR analysis

¹H-, ¹³C-, and DEPT-135 NMR spectra were acquired in DMSO-*d*₆. Chemical shifts were calibrated using the solvent signal

as reference. All NMR experiments were conducted on a Bruker Advance DPX300 instrument (operating at 300.13 MHz for ^1H and 75.48 MHz for ^{13}C).

Evaluation of antioxidant (DPPH-scavenging) activity

The antioxidant activity of extracts, based on their DPPH scavenging activity, was determined as described by Hatano *et al.*³⁶ Solutions of extract in methanol:DMSO (1:1 v/v) were added to freshly prepared solution of DPPH (0.004% w/v) in methanol:DMSO (2.0 mL), which was protected from light. The mixture was incubated in the dark at room temperature for 30 min. Absorbance was recorded at 517 nm using a Biospectro SP220 spectrophotometer. Free-radical-scavenging activity (FRSA%) was calculated as follows:

$$\text{FRSA}\% = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test extract) and A_{sample} is the absorbance of the test extracts. Assays were carried out in triplicate. A test with methanol:DMSO against DPPH showed no variation in absorbance at 517 nm. Orcinol was used as the positive control. Results of FRSA% were expressed for 100 $\mu\text{g mL}^{-1}$ of each extract. EC_{50} values (concentration of extract in the reaction mixture needed to decrease the initial DPPH concentration by 50%) were determined by linear regression analysis of the obtained DPPH-FRSA values (*software* Origin 6.0)

In order to identify the compounds responsible for free-radical-scavenging activity, TLC assays were performed. Extracts (100 μg) solubilized in acetone were spotted on a TLC plate and eluted using appropriate solvents. TLCs of *P. wainioi* and *C. cryptochlorophaea* extracts were conducted using eluent II; eluent I was employed for extracts of *C. aggregata*, *P. cetratum*, *P. mesotropum*, and *S. alpinum*. After drying, the plates were sprayed with a methanolic solution of DPPH (0.5 mg mL^{-1}). The reaction was monitored and after 30 min the compounds exhibiting antioxidation activity appeared as yellow-white spots against a purple background. Another TLC plate, processed under the same conditions, was visualized after heating with methanol:sulfuric acid (10%). Subsequently, *p*-anisaldehyde was applied to the plate, which was then heated. The TLC of *P. wainioi* was visualized with a methanolic solution of FeCl_3 (5%).

For each extract, comparison of chromatographic profiles, made visible by DPPH and by methanol:sulfuric acid or methanol: FeCl_3 , indicated the presence of active substances. Compounds were identified by comparing their chromatographic behavior with that of pure substances or by NMR analysis of the extract.

Statistical analysis

Statistical calculations were performed using Origin 6.0 *software*. One-way analysis of variance (ANOVA) was employed to determine the statistical significance of extract activity.

Toxicity to *Artemia salina*

A. salina assays were conducted as described by Meyer *et al.*³⁷ Dried brine shrimp eggs were bred in saline solution (38 g L^{-1} in distilled water) and the container was illuminated with a 40 W lamp. After 48 h, the nauplii were collected and added (10 per vial) to the solutions of the lichen extracts, which were solubilized in saline solutions with 1% DMSO to give final concentrations of 600, 400, 200, 120, 60, and 30 $\mu\text{g mL}^{-1}$. Quinine sulfate was used as the control. Bioassays were conducted in triplicate, and after 24 h of contact the survivors

were counted and LC_{50} values calculated using Probitos *software*.³⁸ Extracts with LC_{50} lower than 600 $\mu\text{g mL}^{-1}$ were considered active.

SUPPLEMENTARY MATERIAL

Tables 1S, 2S, 3S, and 4S (NMR experimental and literature data), Figures 1S and 2S (TLC of extracts), and Figures 3S–20S (^1H , ^{13}C , and DEPT-135 NMR spectra) are available at <http://quimicanova.sbq.org.br> in PDF format with free access.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Mariana Fleig (Universidade Federal do Rio Grande do Sul) and Dr. Luciana Canêz (Universidade Federal do Rio Grande) for the identification of lichen species and also to FUNDECT-MS for the financial support. R. G. C and L. M. R thank CNPq (Brazil), K. G. thanks PET/UFMS, and N. M. O thanks PIBIC-CNPq for the grant(s) awarded.

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