

## ***Parmotrema screminiae* (Parmeliaceae), a Novel Lichen Species from Brazil with Potent Antimicrobial Activity**

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**Abstract:** *Parmotrema* is a genus of major interest in the lichen flora of Mato Grosso do Sul state, Brazil, since many of its species are sources of important bioactive compounds. *Parmotrema screminiae* Spielmann & Canêz, a novel species, is a noteworthy source of norlobaridone (a depsidone), protolichesterinic acid and atranorin (a depside). Extract composition was determined by TLC and NMR techniques (<sup>1</sup>H, <sup>13</sup>C, and DEPT-135). The acetone extract was evaluated for antibiotic activity against Gram-negative (*Escherichia coli*, ATCC 25922) and Gram-positive bacteria (*Staphylococcus aureus*, ATCC 25923 and clinical strains), *Enterococcus faecalis* (ATCC 51299), and *E. faecium* (vancomycin-resistant clinical strain). Highly promising results were obtained, since the extract proved active against Gram-positive bacteria alone (MIC = 31.25 µg/mL for *E. faecalis*, 15.6 µg/mL for both *E. faecium* and clinical-strain *S. aureus*, and 7.8 µg/mL for standard *S. aureus*). Bioautography showed norlobaridone and protolichesterinic acid to be responsible for the antibiotic activity.

**Keywords:** antibiotic activity; bioautography; lichen; *Parmotrema*; norlobaridone; protolichesterinic acid

### 1. INTRODUCTION

A highly diverse flora is a remarkable feature among the natural resources of Mato Grosso do Sul state, in Midwest Brazil, which comprises a variety of lichenized fungi. The earliest study of local lichens dates back to 1897, when specimens were collected by Swedish botanist Gustav O. A. Malme [1, 2]. In 1956, Argentinian researcher J. E. Montes amassed a collection of lichens from Ponta Porã county, later acquired by Héctor Osorio, who subsequently published data based on this material [3-5]. In 1979-1980, German investigator Klaus Kalb journeyed Aquidauana, Campo Grande, Rio Verde de Mato Grosso, and Coxim counties collecting material, later partially reported in monographs on tropical lichens [6, 7]. Brazilian lichenologist Marcelo Pinto Marcelli accompanied Kalb in sampling expeditions in 1980, subsequently collecting additional material from Corumbá county in 1990 and from Aquidauana,

Palmeiras, Piraputanga, Terenos, and Campo Grande in 1992, 1993, and 1997, by invitation of the Lichen Study Group, created at the Universidade Federal de Mato Grosso do Sul (UFMS) in 1987. Vital to the chemical investigation of lichens has been the indefatigable collection and identification effort made by Mariana Fleig in 1989-1993, part of which was published in 1991 [8].

In Mato Grosso do Sul, the genus *Parmotrema* is represented by numerous species that are widely distributed. A number of species collected from the localities of Palmeiras (in Dois Irmãos do Buriti county) and Piraputanga (in Aquidauana county) have been investigated for chemical composition, biological activity of isolated compounds [9-13], and chemotaxonomic traits. Taxonomic investigations in the state have revealed one novel species. This article reports the morphological description and chemical analysis of this species, in addition to the study of the antibiotic activity of its extract.

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## 2. MATERIAL AND METHODS

### General procedures

Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker DPX-300 spectrometer operating at 300.13 MHz for  $^1\text{H}$  and 75.48 MHz for  $^{13}\text{C}$ . The dry extract was dissolved in  $\text{DMSO-}d_6$  and the solvent's signal was employed as an internal reference. Thin-layer chromatography (TLC) was performed on plates pre-coated with silica gel 60 F<sub>254</sub> (Merck) using toluene : acetic acid (85:15, v:v) as the eluent. The spots were visualized by spraying the plates sequentially with (a) water, (b) 10%  $\text{H}_2\text{SO}_4$  methanol solution followed by heating until complete appearance of spots, and (c) *p*-anisaldehyde:sulfuric acid followed by reheating. Substance migration was expressed as retention factor ( $R_f$ ). The solvents were purified by distillation. Substance characterization was based on NMR data and on comparisons between  $R_f$  values obtained using different eluents and published values [14].

### Collection and morphological study

The lichen was collected from Dois Irmãos do Buriti county (Mato Grosso do Sul, Brazil) near the locality of Palmeiras, from a small elevation at the Estrada Parque (MS-450) roadside (20°29'51.5"S, 55°26'11.1"W, 230 m altitude, 30.VI.2011, leg. A.A. Spielmann & L.S. Canêz 9243; holotype: CGMS 53551).

The material was collected following the usual methodology described in Lichenology [15, 16]. Description followed the protocol proposed by Spielmann [17]. Thalli were color tested with potassium hydroxide solution (K) and sodium hypochlorite solution (C) using liquid household bleach, and also with *p*-phenylenediamine (1,4-phenylenediamine) (P) [18, 19].

### Extract preparation

Thallus portions (635.0 mg) were cleaned, fragmented, and exhaustively treated with acetone at room temperature. After solvent evaporation in a rotary evaporator, the extract was kept in a desiccator. Yield was 8.0%. TLC and NMR were employed for extract analysis. [Supplementary Material](#) available: NMR data ( $^1\text{H}$ ,  $^{13}\text{C}$ , and DEPT-135) of extract.

### Antibiotic activity

### Microorganisms and media

The test organisms used in this study were *S. aureus* ATCC 25923, *E. faecalis* ATCC 51299, *E. coli* ATCC 25922, and clinical strains isolated from the UFMS Hospital Universitário (clindamycin-, erythromycin-, and penicillin G-resistant *S. aureus* and vancomycin-resistant *E. faecium*). All media were purchased from Sigma-Aldrich.

### Determination of minimum inhibitory concentrations

Plates with 96 wells were prepared by dispensing 100  $\mu\text{L}$  of Mueller–Hinton broth into each well. A 2 mg/mL stock solution of the extract was prepared and serial dilutions were performed to attain final concentrations in the 1-1000  $\mu\text{g/mL}$  range, with a 100  $\mu\text{L}$  final volume in each well. For gentamicin, final concentrations ranged from 60 to 0.5  $\mu\text{g/mL}$ . For each bacterial species, the inoculum was an overnight culture grown in Mueller–Hinton agar diluted in sterile saline solution (0.45%) to approximately  $10^8$  CFU/mL. This solution was diluted 1:10 in saline solution (0.45%), and a 5  $\mu\text{L}$  aliquot ( $10^4$  CFU/mL) was added to each well containing a test sample. All experiments were performed in triplicate and the microdilution trays were incubated at 36 °C for 18 h. A 20  $\mu\text{L}$  volume of an aqueous solution (0.5 %) of triphenyl tetrazolium chloride (TTC) was subsequently added to each well and the trays were again incubated at 36 °C for 2 h. In the wells exhibiting bacterial growth, TTC shifted from colorless to red. MIC (expressed in  $\mu\text{g/mL}$ ) was defined as the lowest concentration of each substance at which no color shift occurred.

### Bioautography assays

Volumes of the extract were spotted on silica gel TLC plates (Merck, Silica gel 60 F254), subsequently developed in toluene : acetic acid (85:15, v:v), placed in petri dishes containing thin Mueller–Hinton agar, and covered with 2 mm–deep soft agar. Finally, inocula ( $10^8$  CFU/mL) were spread over the solidified medium and the dishes were incubated at 37 °C for 24 h. An aqueous solution (0.5 %) of TTC was sprayed over the plates containing *S. aureus*. Absence of color change indicated spots containing active substances.

## 3. RESULTS AND DISCUSSION

***Parmotrema screminiae* Spielmann & Canêz, sp. nova** (MycoBank No.: MB 820069)

Type: Brazil, Mato Grosso do Sul, Dois Irmãos do Buriti county, near the locality of Palmeiras, small elevation at the Estrada Parque (MS-450) roadside, alt. 230 m, 30.VI.2011, leg. A.A. Spielmann & L.S. Canêz 9243 (holotype: CGMS 53551).

**Holotype description**

*Thallus* greyish green in nature, becoming buff in herbarium, lobate, more or less adnate, at some points with rhizines strongly attach to the rock, saxicolous, about 10 cm broad. *Lobes* irregularly branched, laterally overlapped to somewhat crowded in the central parts, 6-12 mm wide, *surface* continuous to irregular, usually smooth in the apices, becoming rugose towards the center, sublustrous; apical zone rounded; margin smooth to slightly crenate, rarely with sublacinules, undulated. *Maculae* absent. *Cilia* absent. *Lobules*, *Phyllidia*, *dactyls* or *lacinules* absent. *Pustules* absent. *Soralia* grayish, linearly interrupted when marginal or capitate, especially when laminal; *soredia* subfarinose. *Isidia* absent. *Medulla* white. *Undersurface* black, lustrous, smooth to rugose or papillate only at some points; *marginal zone* brown, usually light brown or beige under the sorediate lobes, lustrous, 1-6 mm wide, naked, with sharp to more often attenuated limit, smooth, rugose, or papillate; *rhizines* black, simple, sometimes joining adjacent rhizines when touching the substrate, 0.2-0.5 × 0.05-0.10 mm, few, distributed in groups.

*Apothecia* cupuliform to concave, 2-6 mm in diameter, stipitate, laminal, margin crenate or commonly incised when mature, sometimes the incisions reaching the center of the disc, smooth to sorediate, amphithecium sorediate, disc brown, epruinose, imperforate, smooth or wrinkled; *ascospores* ellipsoid, sometimes long-ellipsoid, 12-15 × 6-8 μm, epispodium ca. 1 μm. *Pycnidia* submarginal, inconspicuous, without prominent margin, rare, ostiole black; *conidia* sublageniform, 5-6 × ca. 1 μm.

*Color tests*: cortex K+ yellow, UV–; medulla K+ yellow, C–, KC+ very faint rose, P– UV–.

*Chemistry*: cortex with atranorin (major); medulla with atranorin (major), norlobaridone (major), protolichesterinic acid (major), and loxodin (trace).

*Distribution*: Known only from type location, where

it was collected twice.

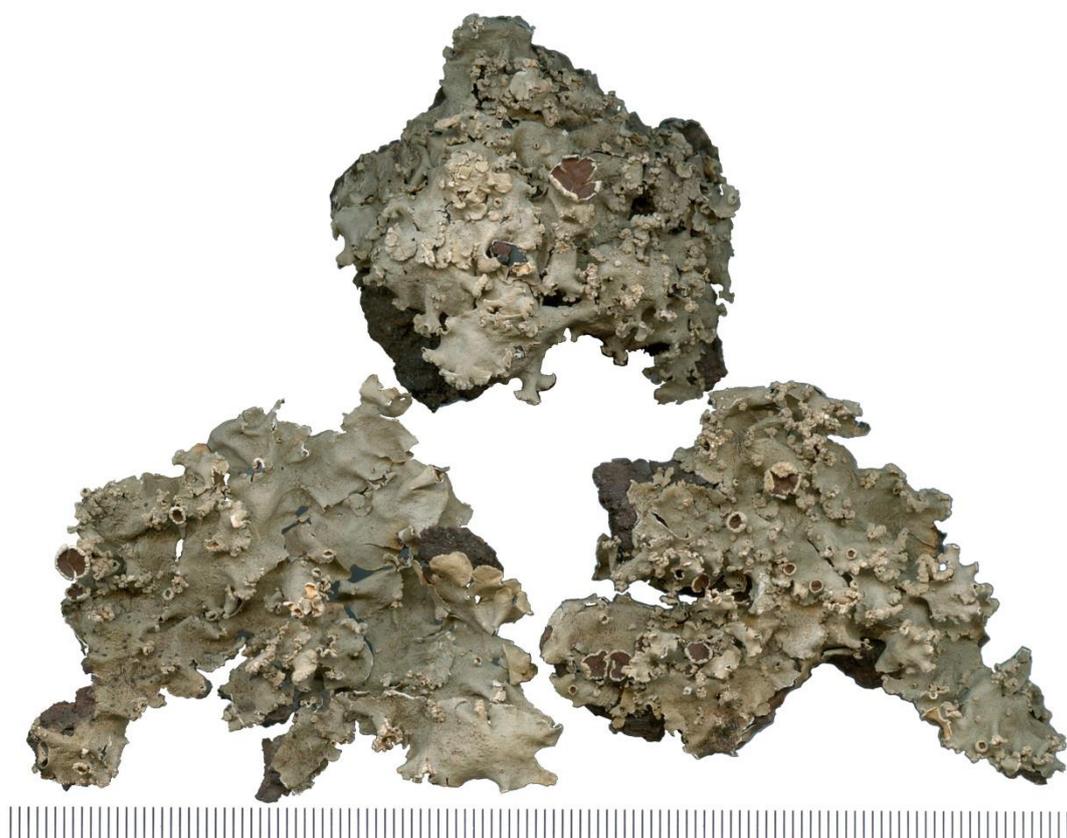
*Additional specimens examined*: Same as the type, 28.XI.2014, leg.: A.A. Spielmann, A. Pott, V.J. Pott & F.M. Alves 12151 (CGMS 53552), saxicolous, in open forest.

*Parmotrema screminiae* (Figure 1) has a sorediate, saxicolous thallus and eciliate lobes. Atranorin, norlobaridone, and protolichesterinic acid are major constituents. Compared with *Parmotrema* species known to synthesize norlobaridone (see key in Supplementary Material), the novel species resembles eciliate *P. applanatum* Marcelli & Ribeiro, which, however, is a small lichen with a very adnate thallus and exclusively linear, marginal soralia. Color test results (cortex K+ yellow, medulla K+ yellow, C–, KC+ very faint rose, P–) together with chromatographic profiles (Figure 2) indicated the presence of atranorin (major constituent) in the cortex and atranorin (major), norlobaridone (major), protolichesterinic acid (major), and loxodin (trace) in the medulla. The medulla reacts K+ yellow, indicating the presence of atranorin—a feature also observed in *P. mordenii* (Hale) Hale, a species that also produces protolichesterinic acid. However, norlobaridone is absent from *P. mordenii* and likewise from *P. praesorediosum* (Nyl.) Hale [20]. These three species are similar in overall morphology, and studies using molecular markers are expected to further clarify the specific delimitation of saxicolous, sorediate *Parmotrema* lichens. Currently, differences in chemistry are among the most reliable features allowing these species to be distinguished. With regard to distribution, the new species is known only from the type location, where it was collected twice. The epithet *screminiae* is named after Professor [Edna Scremin Dias](#), of the UFMS Department of Biology, for her outstanding contribution to the implementation of research lines involving lichen species native to Mato Grosso do Sul.

The acetone extract chromatogram also showed several spots possibly corresponding to trace components, or even to degradation products or pigments. <sup>1</sup>H, <sup>13</sup>C, and DEPT-135 NMR spectra were recorded to further elucidate extract composition (Figures 3S-19S). The <sup>1</sup>H spectrum shows a cluster of signals indicative of aliphatic chains; aromatic CH<sub>3</sub>, methoxyl, or methyl ester groups; hydrogens pertaining to olefinic systems; and aromatic, aldehyde carbonyl, and phenolic hydroxyl hydrogens (Figures 3S, 6S, 8S, 9S). Signals at 10.33, 3.93, 6.51, and 6.76 ppm in the <sup>1</sup>H spectrum are indicative of aldehyde

carbonyl and methyl ester groups, as well as hydrogens at C-5 and C-5', respectively, in the structure of the depside atranorin. Signals are also seen in the region from 0.91 to 2.82 ppm, indicating an alkyl chain, while signals at  $\delta$  6.60, 6.62, 6.82, and 6.85 ppm can be assigned to hydrogens attached to carbons C-3, C-5, C-3', and C-1' of the norlobaridone structure. In addition to the chemical shift signals

assigned to atranorin and norlobaridone, other signals in the  $^1\text{H}$  spectrum are indicative of extra compounds. In addition to one singlet at 3.92 ppm, that suggests the loxodin depsidone, chromatographically shown to be present at low concentration in the extract (Figure 2), the doublets at 5.97 ( $J$ , 2.7 Hz) and 6.26 ppm ( $J$ , 2.7 Hz) suggest hydrogens in an olefinic system.



**Figure 1.** *Parmotrema screminiae*: holotype.

The  $^{13}\text{C}$  spectrum also shows a cluster of signals for  $\text{CH}_3$ ,  $\text{CH}_2$ , and  $\text{CH}$ , in addition to  $\text{CH}_3\text{O}$ - or  $\text{CH}_3\text{OCO}$ -, aromatic  $\text{CH}$ , aromatic  $\text{C}$ ,  $\text{COOH}$ ,  $\text{CHO}$ , and  $\text{C=O}$  groups. In the  $^{13}\text{C}$  and DEPT-135 spectra (Figures 11S and 18S, respectively), signals at 193.7 ( $\text{CHO}$ ), 52.0 (methyl ester), 110.7 and 116.4 (methine C-5 and C-5', respectively), and 24.5, 23.7, and 8.5 ppm (methyls attached to aromatic rings) confirm the structure of atranorin [21]. Evidence of atranorin presence in the extract is provided by NMR and TLC data.

$^{13}\text{C}$  and DEPT-135 spectra show signals of chemical shift corresponding to alkyl groups ( $\delta$  13.3-42.8 ppm) and methine carbons at  $\delta$  105.4, 107.4, 111.8, and 113.2 ppm, indicative of a structure

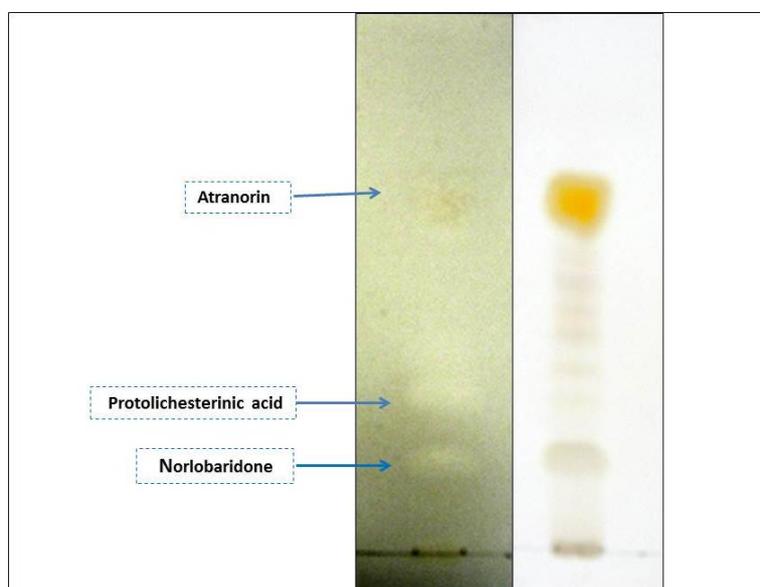
derived from orsellinic acid. In the  $^{13}\text{C}$  spectrum, a signal at 202.80 ppm, indicating a  $\text{C=O}$  group in an alkyl chain (Figure 11S), can be assigned to a keto group at C-1'' or C-1''''. Assignment of other signals (Figure 1S) suggests the structure of norlobaridone. The  $^{13}\text{C}$  and DEPT-135 spectra reveal a number of low-intensity signals with chemical shifts closely resembling those of norlobaridone signals and another at 53.5 ppm in the  $^{13}\text{C}$  spectrum, suggesting the structure of loxodin. Comparisons with lobaric acid allowed chemical shift signals to be attributed to norlobaridone [22]. Signals at 79.1, 49.50, and 174.0 ppm in the  $^{13}\text{C}$  spectrum are indicative of a lactone ring and signals at 123.3 and 134.5 ppm, corroborate the presence of an olefinic system. Figure 2S shows the signals assigned to the structure of



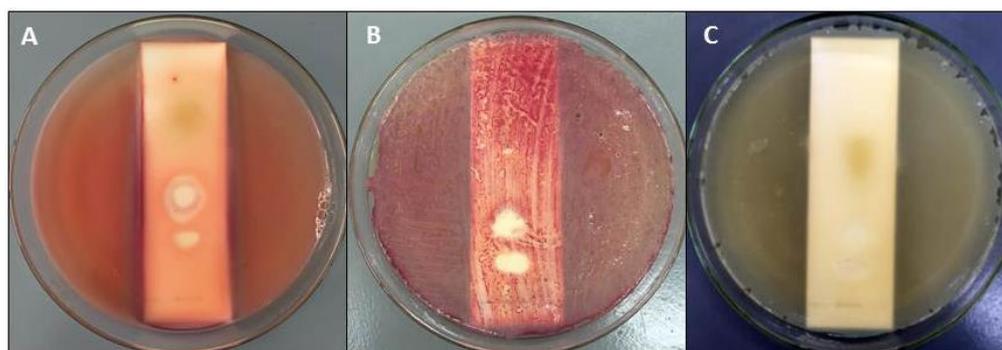
The relative ratios between compounds, based on NMR signals and the chromatographic spots, reveal atranorin, norlobaridone, and protolichesterinic acid to be the principal substances present in the extract, which also contains loxodin.

The acetone extract was evaluated for antibiotic activity against Gram-positive *S. aureus* (ATCC 25923 and clinical strains), *E. faecalis* (ATCC 51299), and *E. faecium* (vancomycin-resistant clinical strain) and Gram-negative *E. coli* (ATCC 25922). The extract proved active against Gram-positive bacteria alone (MIC = 31.25  $\mu\text{g/mL}$  for *E. faecalis*, 15.6  $\mu\text{g/mL}$  for both *E. faecium* and clinical-strain *S. aureus*, and 7.8  $\mu\text{g/mL}$  for standard *S. aureus*). The results are highly promising, since extracts typically exhibit biological activity only at much higher concentrations.

To identify the compounds responsible for the antimicrobial activity, the extract-sensitive strains were subjected to bioautography, all of which exhibited the same pattern of sensitivity to extract components (Figure 5). Inhibition of bacterial growth was observed in the regions corresponding to norlobaridone and protolichesterinic acid, likely responsible for the extract's activity. Türk et al. [24] reported the antimicrobial activity of protolichesterinic acid isolated from *Cetraria aculeata*. Antiproliferative and pro-apoptotic activities of this compound have been reported elsewhere [25]. No data have been published on the antimicrobial activity of norlobaridone, but this property has been reported for other structurally similar depsidones—such as lobaric and physodic acids—with long side chains containing ketone groups [26-28].



**Figure 4.** Chromatograms of the acetone extract of *Parmotrema screminiae*. **I:** Revelation with water. Image was contrast-enhanced to facilitate visualization of spots. **II:** Revelation with 10% sulfuric acid in methanol, followed by heating.



**Figure 5.** Biochromatography of *Parmotrema screminiae* extract against (A) *Staphylococcus aureus* (ATCC 25923), (B) clinical strain *S. aureus*, and (C) clinical strain *Enterococcus faecalis*. A and B strips were developed with 0.5% triphenyl tetrazolium chloride.

#### 4. CONCLUSION

*Parmotrema screminiae* Spielmann & Canêz, a novel lichen species native to Mato Grosso do Sul, is a noteworthy source of norlobaridone (a depsidone) and protolichesterinic acid (a lactonized fatty acid), which are probably responsible for the potent antibiotic activity against *S. aureus* and *E. faecalis* exhibited by the lichen extract. These compounds can find utility as structural models for the synthesis of new drugs to treat infections caused by these microorganisms.

#### 5. ACKNOWLEDGMENTS

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#### 6. REFERENCES AND NOTES

- [1] Baptista, L. R. M. *Ciência & Ambiente* **1996**, *13*, 99.
- [2] Marcelli, M. P. *In: Lichenology in Latin America: history, current knowledge and applications*, Marcelli, M.P. & M.R.D. Seaward (Eds). São Paulo, CETESB, 1996, p. 25.
- [3] Osorio, H.S. *Comun. Bot. Mus. Hist. Nat. Montevideo* **1992**, *5*, 1.
- [4] Yoshimura, I.; Osorio, H. *J. Hattori Bot. Lab.* **1975**, *39*, 263.
- [5] Sérusiaux, E. *Nord. J. Bot.* **1983**, *3*, 517. [[CrossRef](#)]
- [6] Kalb, K. *Die Gattung Pyxine*. Bibliotheca Lichenologica 24. Berlin: J. Cramer, 1987. [[CrossRef](#)]
- [7] Staiger, B. *Die Flechten familie Graphidaceae. Studien in Richtung einer natürlicheren Gliederung*. Bibliotheca Lichenologica 85. Berlin: J. Cramer, 2002.
- [8] Fleig, M.; Riquelme, I. *Acta Bot. Bras.* **1991**, *5*, 3. [[CrossRef](#)]
- [9] Micheletti, A. C.; Beatriz, A.; Lima, D. P.; Honda, N. K.; Ó Pessoa, C.; Moraes, M.O.; Lotufo, L.V.; Magalhães, H. I. F.; Carvalho, N. C. P. *Quim. Nova*, **2009**, *2*, 12. [[CrossRef](#)]
- [10] Brandão, L. F. G.; Alcantara, G. B.; Matos, M. F. C.; Bogo, D.; Freitas, D. S.; Oyama, N. M.; Honda, N. K. *Chem. Pharm. Bull.* **2013**, *61*, 176. [[CrossRef](#)]
- [11] Honda, N. K.; Pavan, F. R.; Coelho, R. G.; Leite, S. R. A.; Micheletti, A. C.; Lopes, T. I. B.; Misutsu, M. Y.; Beatriz, A.; Brum, R. L.; Leite, C. Q. F. *Phytomedicine* **2010**, *17* 328. [[CrossRef](#)]
- [12] Honda, N. K.; Lopes, T. I. B.; Costa, R. C. S.; Coelho, R. G.; Yoshida, N. C.; Rivarola, C. R. V.; Marcelli, M. P.; Spielmann, A. A. *Orbital: Electron. J. Chem.* **2015**, *7*, 99. [[CrossRef](#)]
- [13] Peres, M. T. L. P.; Mapeli, A. M.; Faccenda, O.; Honda, N. K. *Orbital: Electron. J. Chem.* **2015**, *7*, 275. [[CrossRef](#)]
- [14] Culberson, C. F.; Kristinsson, H. *J. Chromatogr.* **1970**, *46*, 85. [[CrossRef](#)].
- [15] Fink, B. *The Bryologist* **1905**, *8*, 22. [[CrossRef](#)]
- [16] May, P. F. 2000. Farlow Herbarium, Harvard University, Cambridge, Massachusetts. Available: <http://www.huh.harvard.edu/collections/lichens/collecting.html>.
- [17] Spielmann, A. A. *Estudos taxonômicos em Parmotrema s.l. (Parmeliaceae, Ascomycota liquenizados) com ácido salazínico*. [Doctoral dissertation]. São Paulo Brazil: Instituto de Botânica da Secretaria de Estado do Meio Ambiente. São Paulo, 2009. [[link](#)]
- [18] Hale, Jr. M. E. *The Biology of Lichens*, London, Edward Arnold, 1974.
- [19] Orange, A.; James, P.W.; White, F. J. *Microchemical methods for the identification of lichens*. British Lichen Society, 2001.
- [20] Spielmann, A. A.; Marcelli, M. P. *Hoehnea* **2009**, *36*, 551. [[CrossRef](#)]
- [21] König, G.; Wright, A. D. *Phytochem. Anal.* **1999**, *10*, 279. [[CrossRef](#)]
- [22] Huneck, S.; Yoshimura, I. *Identification of Lichen Substances*, Berlin, Springer, 1996.
- [23] Murta, M.; Azevedo, M. B. M.; Greene, A. E. *J. Org. Chem.* **1993**, *58*, 7537. [[CrossRef](#)]
- [24] Türk, A. O.; Yilmaz, M.; Kivaç, M.; Türk, H. *Zeitsch. für Naturforsch.* **2003**, *58c*, 850. [[CrossRef](#)]
- [25] Bessadottir, M.; Eiríksson, F. F.; Becker, S.; Ögmundsdóttir, M. H.; Ómasdóttir, S.; Thorsteinsdóttir, M.; Ögmundóttir, H. M. *Prostaglandins Leukot. Essent. Fatty Acids* **2015**, *98*, 39. [[CrossRef](#)]
- [26] Türk, H.; Yilmaz, M.; Tay, T.; Türk, A. O.; Kivaç, M. *Zeitsch. für Naturforsch.* **2006**, *61c*, 499. [[CrossRef](#)]
- [27] Thadhani, V. M.; Choudhary, M. I.; Khan, S.; Karunaratne, V. J. *Natn. Sci. Foundation Sri Lanka* **2012**, *40*, 43. [[CrossRef](#)]
- [28] Kosanić, M.; Ranković, B. *J. Med. Food* **2011**, *14*, 1624. [[CrossRef](#)]