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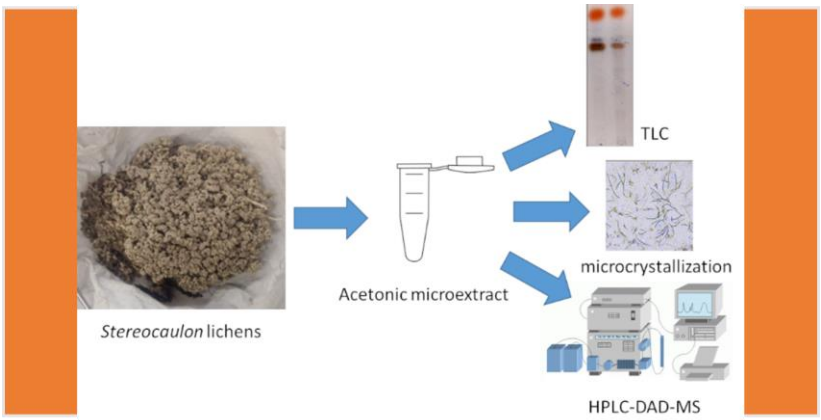
Chemotaxonomic Studies on Some *Stereocaulon* (Stereocaulaceae, Lichenized Ascomycota) from Southern South America and Antarctica

Aline Siqueira Gianini [a], Jean Marc Torres [b], Neli K. Honda [a], Aline P. Lorenz [b], Adriano A. Spielmann [b], and Ana Camila Micheletti* [a]

Lichenized fungi (lichens) produce many compounds especially those derived from the polyketide pathway. These are predominantly phenolic compounds, most of which are unique to lichens, and long-chain fatty acids. These compounds play an important role in the chemotaxonomy of lichens, as they serve as chemical markers for identification at different taxonomic levels. In this work, 100 exsiccates of lichens of the genus *Stereocaulon* collected in Antarctica, Chile and Argentina were analyzed using thin layer-chromatography, microcrystallization and high-performance liquid chromatography coupled with mass spectrometry. A total of 21 compounds were annotated: rangiformic, norrangiformic, pseudonorrangiformic, vinapraesorediosic B and bourgeanic acids (fatty acids); lobaric, norstictic, menegazziaic, hypoconstictic, colensoic, stictic acids (depsidones); atranorin, gyrophoric acid, -ene"-ene"' glomelliferic acid (depsides); strepsilin (dibenzofuran); lobarin (diphenylether), oxysiphulin (chromone) and four new depsidones: 2"-ene lobaric, 3"-ene lobaric and 4"-ene lobaric and – ene"' lobaric acids. The results of the chemical analysis corroborated the identification obtained through morphological analysis and, in some cases, also allowed the species to be separated into chemical groups. The following species were identified: *Stereocaulon corticatulum*, *S. alpinum* (2 chemical groups), *S. melanopotamicum*, *S. implexum*, *S. tomentosum* (4 chemical groups), *S. tomentosum* var. *capitatum* (2 chemical groups), and *Stereocaulon argus* (3 chemical groups).

Graphical abstract



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1. Introduction

[a] Institute of Chemistry, Federal University of Mato Grosso do Sul (UFMS). Av. Costa e Silva - Pioneiros, zip code 79070-900, Campo Grande, Mato Grosso do Sul, Brazil. [b] Institute of Biosciences, Federal University of Mato Grosso do Sul (UFMS). Av. Costa e Silva - Pioneiros, zip code 79070-900, Campo Grande, Mato Grosso do Sul, Brazil. *Corresponding author. E-mail: **ana.micheletti@ufms.br**

Lichens from Maritime Antarctica and southern South America have long been the focus of studies by many researchers, including: Lamb [1-3]; Redon [4]; Øvstedal & Smith [5]; Huneck et al. [6]; George et al. [7]; Adler & Calvelo [8]; Schroeter et al. [9]; Sancho et al. [10], among others. Interest in studying lichens from these regions ranges from species identification to ecology, physiology, and the search for new compounds produced by lichens that can serve as structural models for the study of pharmacological activity or other applications.

The genus *Stereocaulon* (class Ascomycetes, subclass Ascomycetidae, order Lecanorales, and family Stereocaulaceae) is widely distributed throughout the world, being found in different geographic latitudes. This genus has about 150 reported species, many of which have not yet been extensively studied regarding their chemical composition [11]. Compounds from the classes of depsides, depsidones, diphenyl ethers, dibenzofurans, and fatty acids are reported in the genus *Stereocaulon* [3, 11]. In the class of depsidones, lobaric acid and lactonized depsidones from the group of stictic and norstictic acids occur in a number of species of *Stereocaulon*. Therefore, several species of this genus may have similar chemical composition, especially with regard to the major compounds. Recently, Torres et al. [12] studied several specimens of *Stereocaulon alpinum* collected in Argentina (Tierra del Fuego National Park), the South Shetland Islands and the Antarctic Peninsula and concluded that *S. alpinum* has considerable phenotypic variation not related to phylogenetic or geographic patterns.

The taxonomic identification of lichens can be carried out through morphological and chemical analyses and the study of chemical composition for taxonomic purposes involves, in addition to spot tests, Microcrystallization (MC) and chromatoghaphic techniques, like TLC (Thin Layer Chromatography) and HPTLC (high performance TLC) are also frequently used. NMR analysis has also been applied and, more recently, Mass Spectrometry (MS) has been successfully implemented for lichen chemotaxonomy [13-18]. In this work we present the results of the chemical analysis and the identification of species of the genus *Stereocaulon* collected in Argentina, Chile and the Antarctic Peninsula

2. Material and Methods

2.1 General Procedures

Thin-layer chromatography (TLC) was performed on plates precoated with silica gel 60 GF_{254} (Macherey-Nagel). High-performance liquid chromatography coupled to a diode array detector and mass spectrometry (HPLC-DAD-MS) analyses were performed on equipment with the following specifications: UFCL LC-20AD Shimadzu Prominence coupled to a high-resolution mass spectrometer with an electrospray ionization source (MicrOTOF-Q III- Bruker Daltonics, Billerica, MA, USA). UV/VIS was monitored between 240-800 nm and the Kinetex C18 column (2.6µ, 100 A, 150 x 2.1 mm, Phenomenex) was used.

2.2 Lichens

Specimens of the genus *Stereocaulon* (100 exsiccates) were collected from several points in the Antarctic Continent, in Tierra del Fuego National Park, Argentina, and in Tierra del Fuego, Chile (**Table 1S**). The collections were carried out from 2015 to 2017 by the Lichenology research group of the Institute of Biosciences of UFMS, Brazil, under the coordination of Prof. Dr. Adriano A. Spielmann. The species identification was performed by Prof. Dr. Adriano A. Spielmann and Jean Marc Torres Pineda. All exsiccates are deposited in the CGMS herbarium (Campo Grande, MS, Brazil).

2.3 Extract preparation

Small fragments of lichen thalli were removed from each exsiccata and cleaned using a small brush and, when necessary, distilled water was used to help remove residues adhered to the thalli and after cleaning they were dried at room temperature. For preliminary chemical analysis, approximately 50-100 mg of thalli were fragmented and then treated with distilled acetone. The extraction with acetone (3-10 mL) was repeated four times, the extraction time at each stage being approximately 30 minutes at room temperature. The extracts were pooled and, after evaporation of the solvent, stored in a desiccator.

2.4 TLC

For chromatographic analysis, the extracts were solubilized in acetone and applied to silica gel plates, and elution was performed in toluene:dioxane:acetic acid (90:25:4 v/v/v) and in toluene:acetic acid (85:15 v/v) [19, 20]. The spots were visualized in the chromatograms under UV light at 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. Fatty acids were revealed with water. The chromatographic migration of the constituents present in the extracts (expressed as Rf x 100) was compared with data from the literature [21, 22] with the chromatographic behavior of pure substances and those present in extracts of known composition.

2.5 Microcrystallization

Microcrystallization (MC) of the extracts was performed using the following solutions: GE 1:3 - Glycerol: acetic acid (1:3 v/v), GE 3:1- Glycerol: acetic acid (3:1 v/v), GAW - Glycerol: ethanol: water (1:1:1 v/v/v) and GAoT - Glycerol: ethanol: *o*-toluidine (2:2:1 v/v/v) [21, 23]. The crystal structures of the substances present in the extracts were observed under a microscope Nikkon Eclipse E 220, with 10x magnification and compared with photographs from the literature [21] or with those produced by crystallization of pure substances or extracts of known composition.

2.6 Mass spectra

Mass spectra were obtained as described in Torres et al. [12]. The identification of the compounds was carried out through the analysis of the generated ions $[M-H]^-$ and MS/MS fragments and compared with data available in the literature. [24-37]. All sample chromatograms were adjusted to the same intensity. Information on the putatively annotated compounds present in the acetone extract of *Stereocaulon* is summarized in the **Tables 2S – 4S**. Substances compatible with those of the secondary metabolism of lichens were identified.

3. Results and Discussion

The TLC analysis of the extracts from the 100 exsiccates allowed, according to the chromatographic profile, to preliminarily separate them into three groups, one of them containing atranorin and fatty acids (12 exsiccates), another

containing atranorin and lobaric acid (40 exsiccates) and the third containing atranorin and lactonized depsidones (48 exsiccates) (**Fig. 1 a, b, c**). Based on these results and those obtained from the microcrystallization and HPLC-MS analyses, it was possible to identify 21 compounds from the classes of fatty acids, depsides, depsidones, dibenzofurans, cromones and diphenylethers (**Fig. 1Sa, 1Sb**).

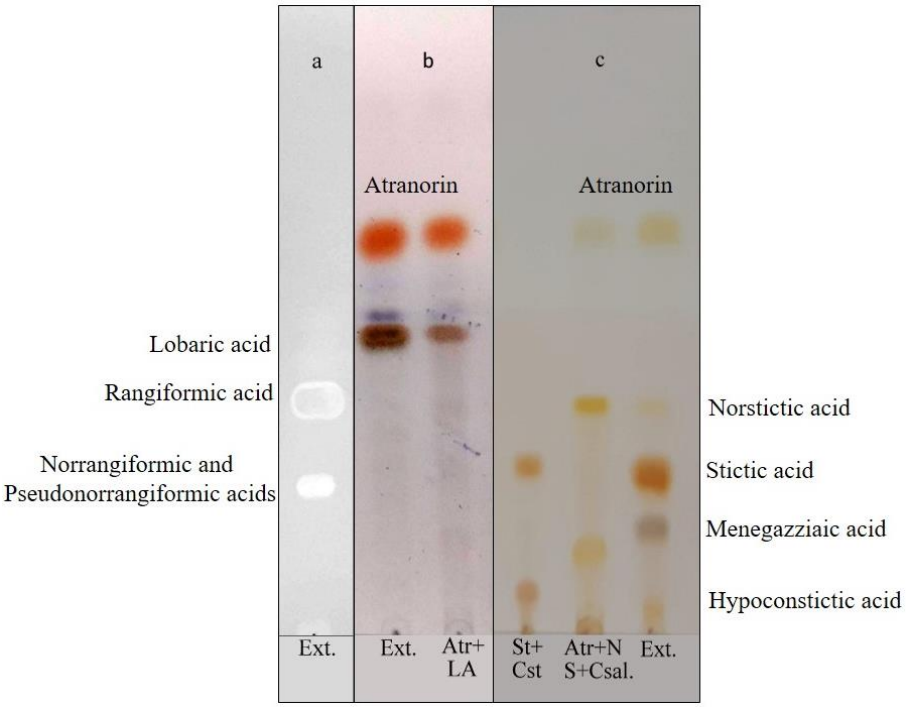

Fig. 1. TLC of acetone extracts representing each group of compounds. **a** – Fatty acids; **b** – Atranorin, lobaric acid and other compounds; **c** – Atranorin, stictic, menegaziaic and hypoconstictic acids. Eluents: (**a** and **b**) - Toluene: acetic acid 85:15 v/v; (**c**) - Toluene: Dioxane: Acetic Acid 90:25:4 v/v/v. Visualization with water (**a**), sulfuric acid and heating followed by *p*-anisaldehyde and reheating (**b** and **c**). Ext. - acetone extract; Atr+LA – Atranorin + lobaric acid; St+Cst – stictic + constictic acids; Atr+N+S+Csal – Atranorin + norstictic + salazinic + consalazinic acids.

The fatty acids presented Rf values of 24 and 38, respectively, in the eluent Toluene: Acetic Acid 85:15 v/v (**Fig. 1a**). The microcrystallization of these extracts in GE 3:1 showed structures that, when compared to photos in the literature [21], suggested the presence of rangiformic, norrangiformic and pseudonorrangiformic acids (**Fig. 2)**

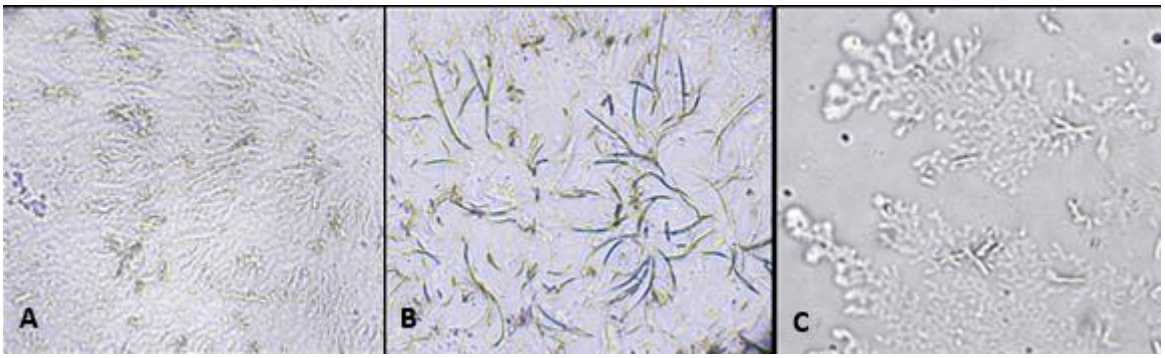

Fig. 2. Photos of microcrystallization in 3:1 GE of rangiformic (A), norrangiformic (B) and pseudonorrangiformic (C) acids.

Rangiformic acid presents one of the carboxyls esterified and therefore should migrate in the TLC a little more than the norrangiformic acid. The pseudonorrangiformic acid is a isomer of norrangiformic acid and should present the same chromatographic migration.

The extracts containing atranorin and lobaric acid showed the presence of other substances in the TLC, some migrating very close to this acid, and the microcrystallization analysis confirmed the presence of atranorin and lobaric acid. Although the TLC (**Fig. 1b**) showed the presence of other substances, not all of them formed crystalline structures, either because they were present in low concentration or because they do not crystallize under the test conditions. In the TLC of the extracts containing stictic acid and atranorin, it was also possible to identify menegaziaic and hypoconstictic acids. Norstictic acid is also present in some specimens of this group, however, in low concentration (**Fig. 1c**).

To corroborate the identity of several constituents, present in the extracts of Stereocaulon specimens and to investigate the nature of others that could not be identified through TLC and MC analysis, representative extracts from each group were selected for analysis by high-performance liquid

chromatography coupled to a diode array detector and mass spectrometry (HPLC-DAD-MS), in negative mode. The base peak chromatograms of the extracts from the three groups analyzed are shown in **Fig. 3**, **4** and **5**, respectively.

The chromatogram in **Fig. 3** shows three peaks that indicate the elution of the compounds present in the analyzed extract.

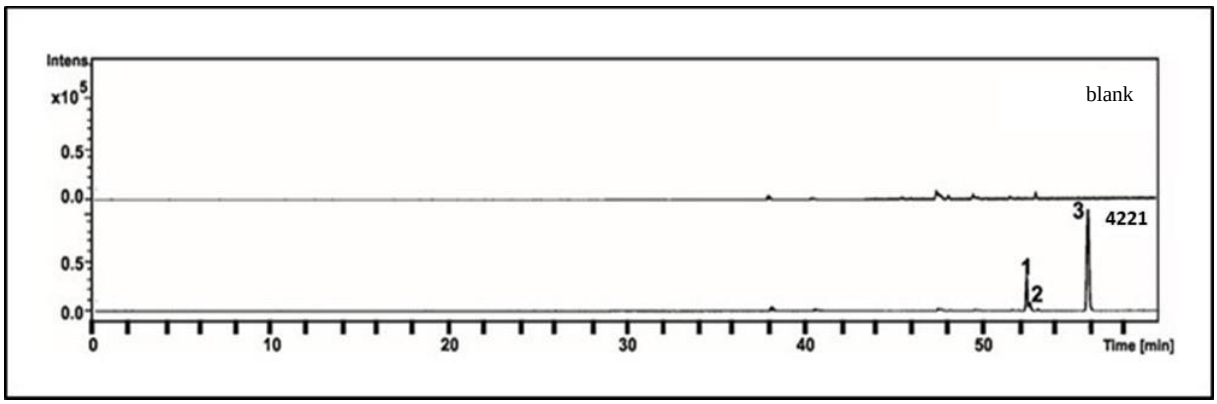

Fig. 3. Chromatogram of the base peak obtained by HPLC-DAD-MS of the acetone extract of *Stereocaulon* representative of the species containing fatty acids (**Fig. 1a**), in negative mode. Peaks 1-3 represent compounds present in these extracts.

The analysis of the ions generated from the fragmentation of the compound eluted in peak 1 (RT 52.5 min), the $[M-H]^-$ of m/z 371.2461 ($C_{20}H_{35}O_6$) and the MS/MS fragments of m/z 353 $[M-H_2O-H]^-$ and m/z 265 $[M-H_2O-2CO_2H]^-$, led to the identification of norrangiformic acid (**Fig. 2S**). According to the MC results, extracts from this group of *Stereocaulon* may also contain pseudonorrangiformic acid, which has no chemical structure described in the literature, only its molecular formula is known [21]. It is an isomer of norrangiformic acid and the difference lies in the arrangement of the carboxyl groups present in the structure of this acid. No other fragments were eluted in this peak, only those mentioned in the identification of norrangiformic acid, however, other structural arrangements are possible for the same m/z and **Fig. 3S** brings a proposed fragmentation for pseudonorrangiformic acid.

The compound eluted in peak 2 (RT 52.7 min) was identified through the $[M-H]^-$ ion of m/z 373.0941 ($C_{19}H_{17}O_8$) and the second-order fragments of m/z 195 $[M-C_9H_7O_4-H]^-$, m/z 177 $[M-C_{10}H_{11}O_4-H]^-$ resulting from the breaking of the ester bond of depsides, and the m/z 163 $[M-C_9H_7O_4-CH_3OH-H]^-$ resulting from the breakdown of the methyl ester. These fragments allowed us to confirm atranorin (**Fig. 4S**), already identified in the TLC analysis.

Analysis of the spectral data generated from the fragmentation of the compound eluted in peak 3 (RT 56.1 min), the $[M-H]^-$ ion of m/z 385.2628 ($C_{21}H_{37}O_6$) and the MS/MS fragment of m/z 265 $[M-CH_3OH-2CO_2-H]^-$ led to the identification of rangiformic acid (**Fig. 5S**). **Table 2S** summarizes the information about this group.

Therefore, the 12 specimens of this group of *Stereocaulon* presented atranorin and the rangiformic, norrangiformic and pseudonorrangiformic acids. Two species of *Stereocaulon* containing atranorin, rangiformic and norrangiformic acids are reported in the literature [3,20], *S. corticatulum* and *S. delisei*. Then, evaluation of the morphological characteristics indicated that the 12 specimens analyzed are *S.corticatulum*. Lamb [3] cites for this species, atranorin, the rangiformic and norrangiformic acids and an unidentified fatty acid. This should possibly be the pseudonorrangiformic acid that we identified by microcrystallization.

The chromatographic profile of extracts from 40 specimens containing atranorin and lobaric acid showed, in addition to these compounds, others in low concentrations; one of the specimens (APL 767) also showed a spot suggesting the presence of the dibenzofuran strepsilin. For HPLC-DAD-MS analysis in negative mode, a representative specimen of the group (AAS 3419) and specimen APL 767 were selected (**Fig. 4**, **Table 3S**). The base peak chromatograms of these analyzed extracts showed 13 peaks, of which 2-10, 12 and 13 are common in all extracts. Fourteen compounds were identified in these extracts and are from the classes of dibenzofurans, diphenylethers, fatty acids, chromones, depsides and depsidones.

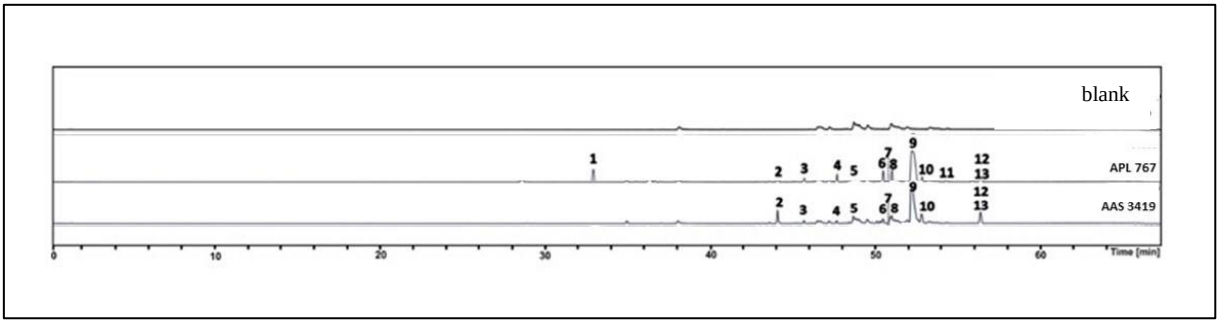

Fig. 4. Chromatograms of the base peak obtained by HPLC-DAD-MS of the acetone extracts of *Stereocaulon* representative of the species containing lobaric acid (AAS 3419 and APL 767) (**Fig. 1b**), in negative mode. Peaks 1-13 represent compounds present in these extracts.

Peak 1 eluted a compound with $[M-H]^-$ m/z 269.0453 ($C_{15}H_9O_4$) and a single second-order fragment with m/z 197. The anthraquinones emodin and islandicin, as well as strepsilin – a dibenzofuran, have the same molecular formula of $C_{15}H_{10}O_4$. It was not possible to propose, through the analysis of the fragment with m/z 197 $[M-C_2O_3-H]^-$ which of these substances could generate it. However, it was possible to note in the TLC analysis, after development with sulfuric acid and heating, the appearance of a grayish spot with an Rf compatible with strepsilin, which allowed this peak to be attributed to this dibenzofuran. This information is further reinforced by the fact that the other possible isomers, emodin and islandicin, under the same conditions, would be revealed with an orange coloration. Additionally, strepsilin has already been reported in the genus *Stereocaulon* [38,39].

Peak 2 eluted a compound with $[M-H]^-$ m/z 473.1821 and molecular formula $C_{25}H_{29}O_9$. There are two possibilities: loxidinol and lobarin – they are substances in the diphenyl ether class with structural similarity. Loxodinol presents its hydroxyl groups free and the carboxyl esterified, while lobarin presents a methylated hydroxyl group and the carboxyl group in free form. Mass spectra of compounds in the diphenyl ether class are expected to present fragments originating from the cleavage of the ether bond, in addition to those generated by dehydration and/or decarboxylation, among others [24,28]. Analysis of the generated fragments indicates that the compound eluted in peak 2 is lobarin, since only lobarin can generate, through the breaking of the ether bond, the fragments of m/z 251 and m/z 239. Furthermore, lobarin can generate the fragment of m/z 383 formed by the loss of two CO_2 molecules, one from the lactol ring and the carboxyl group, in addition to one H_2 molecule (**Fig. 6S**).

Gyrophoric acid was eluted in peak 3 (RT 45.5 min) and identified through the $[M-H]^-$ ion of m/z 467.0974 ($C_{24}H_{19}O_{10}$) and its fragments of m/z 317 $[M-C_8H_6O_6-H]^-$, m/z 167 $[M-2C_8H_6O_6-H]^-$ and m/z 149 $[M-2C_8H_6O_6-H_2O-H]^-$ (**Fig. 7S**). The chromone oxysifulin eluted in peak 4 (RT 47.5 min) was identified through the $[M-H]^-$ ion of m/z 411.1551 ($C_{24}H_{25}O_8$) and the MS/MS fragments of m/z 397 $[M-CO_2-H]^-$ and m/z 369 $[M-CO_2-CO-H]^-$ (**Fig. 8S**). In peak 10 (52.7 min) atranorin was eluted and was identified as previously described. At peak 11 (RT 54.1 min) was eluted vinapraesorediosic acid B, identified by the ion $[M-H]^-$ of m/z 395.2801 ($C_{23}H_{39}O_5$) and its second-order fragments m/z 351 $[M-CO_2-H]^-$ and m/z 323 $[M-C_4H_8O-H]^-$ (**Fig. 9S**). Bourgeanic acid was the compound eluted at peak 12 (RT 56.2 min) and presented the ion $[M-H]^-$ of m/z 385.2972 ($C_{22}H_{41}O_5$) and the second-order fragment of m/z 202 $[M-C_{11}H_{21}O_3-H]^-$ (**Fig. 10S**). Peak 13 eluted a compound that presented a deprotonated molecular ion of m/z 441.1910 ($C_{25}H_{29}O_7$). This formula corresponds to two substances, colensoic acid and picroliquenic acid. However, observing the fragments (peak 13 - **Table 2S**), it is likely that the fragments of m/z 397 $[M-CO_2-H]^-$, m/z 370 $[M-C_5H_{12}-H]^-$, m/z 353 $[M-2CO_2-H]^-$ and m/z 338 $[M-2CO_2-CH_3-H]^-$ are formed from colensoic acid, as shown in **Fig. 11S**. Picroliquenic acid does not generate the fragments of m/z 353 and 338, as they involve the loss of two CO_2 molecules. Thus, the substance eluted in peak 13 is colensoic acid. The compounds eluted in peaks 5,6,7,8 have structures related to lobaric acid. Lobaric acid was identified as peak 9 (RT 52.0 min) based on analysis of the $[M-H]^-$ ion of m/z 455.1661 ($C_{25}H_{27}O_8$) and its fragments: m/z 411 $[M-CO_2-H]^-$, m/z 393 $[M-CO_2-H_2O-H]^-$, m/z 384 $[M-C_5H_{11}-H]^-$, m/z 367 $[M-2CO_2-H]^-$, m/z 352 $[M-2CO_2-CH_3-H]^-$, m/z 296 $[M-2CO_2-C_5H_{11}-H]^-$, and m/z 281 $[M-2CO_2-CH_3-C_5H_{11}-H]^-$ (**Fig. 12S**).

The compounds eluted in peaks 6, 7 and 8 (RT 50.2, 50.5 and 50.8 min) are isomeric structures, with the same $[M-H]^-$ ion of m/z 453, with formula $C_{25}H_{25}O_8$. This ion presented 2Da less than that of lobaric acid and the same fragmentation pattern as this acid (m/z 409 $[M-CO_2-H]^-$, 382 $[M-C_5H_{11}-H]^-$, 365 $[M-2CO_2-H]^-$, 350 $[M-2CO_2-CH_3-H]^-$ and 294 $[M-2CO_2-C_5H_{11}-H]^-$), indicating that these isomers have one more double bond than lobaric acid. The fragments of m/z 382 and m/z 294 allowed identifying that the double bond is present in the aliphatic chain of ring A. Thus, the acids 2"-ene lobaric, 3"-ene lobaric and 4"-ene lobaric were identified.

Peak 5 (RT 48.5 min) showed the coelution of two compounds, a depsidone and a depside, isomers to those eluted in peaks 6, 7 and 8. The generated second-order fragments of m/z 391 $[M-CO_2-H_2O-H]^-$, m/z 365 $[M-2CO_2-H]^-$, m/z 350 $[M-2CO_2-CH_3-H]^-$ and m/z 333 $[M-2CO_2-HOCH_3-H]^-$ indicated that depsidone has a structure similar to those eluted in peaks 6, 7 and 8, however, with the double bond located in the aliphatic chain attached to ring B (**Fig. 13S**). It was not possible to define the position of this double bond, therefore, there are 4 possible structures: 1´´´-ene lobaric, 2´´´-ene lobaric, 3´´´-ene lobaric or 4´´´-ene lobaric acids. The second-order fragments m/z 409 $[M-CO_2-H]^-$, m/z 377 $[M-CO_2-CH_3OH-H]^-$ and m/z 231 $[M-C_{12}H_{13}O_3-H_2O-H]^-$ allowed us to identify that the depside coeluted in peak 5 presents two double bonds in the structure. The fragment of m/z 231 indicated that double bonds are in different aliphatic chains, and it was not possible to define the location of these unsaturations, just as it was not possible to locate the position of the keto group in the alkyl chain linked to ring A **(Fig. 14S**). Considering that among the known depsides that present a keto group in the alkyl chain, it is located in the 2'' position in most of these compounds, there are 08 possible structures for this depside: –ene''-ene'''-glomeliferic acid.

It was also not possible to define the stereochemistry of the double bonds of the compounds eluted in peaks 5, 6, 7 and 8. The structures of the two compounds eluted in peak 5 have not yet been fully elucidated due to the small amount of botanical material available for chemical analysis. According to the databases consulted, there are still no reports of these substances in lichens.

The chromatograms shown in **Fig. 4** confirm lobaric acid (peak 9) as the major substance in this group of analyzed specimens. The proportion of other substances present in these extracts varies among specimens, as can be seen from the intensity of the peaks in the chromatograms presented. In TLC (**Fig. 1b**) the smaller spots close to that of lobaric acid can be attributed to minor substances from the depsidone class eluted in peaks 5, 6, 7 and 8 shown in the chromatogram in **Fig. 4**. The depside eluted in peak 5 must migrate more than lobaric acid, as well as colensoic acid. Lobarin and oxysiphulin should migrate little on TLC plate, remaining close to the point of application. Although these substances are present in low proportions and are not currently used for specimen identification, they may serve as a future tool for differentiating species as taxonomic identification methods improve. Currently, identification is conducted by analyzing morphological characteristics and chemical composition (major substances), determined primarily through spot tests and TLC.

The exsiccates of this group were identified as *Stereocaulon alpinum* Laurer, *Stereocaulon melanopotamicum* I.M. Lamb. and *Stereocaulon implexum* Th.Fr. One of the specimens of *Stereocaulon alpinum* (APL 767) showed a small difference in chemical composition considering the presence of strepsilin and vinapraesorediosic acid B.The species

Stereocaulon melanopotamicum and *Stereocaulon implexum* also do not contain strepsilin and vinapraesorediosic acid B. Lamb [3] cited atranorin and lobaric acid for the species *S. alpinum* and some specimens also contain bourgeanic acid (*S. alpinum* var. *erecta*). These same compounds present in *S. alpinum* are also cited for *Stereocaulon implexum* and *Stereocaulon melanopotamicum*, with the latter also mentioning the occurrence of unidentified trace substances.

Another 48 exsiccates evaluated by TLC showed the presence of atranorin, stictic, menegazziaic and hypoconstictic acids and in some cases norstictic acid (**Fig. 1c**). Two extracts were selected, based on the TLC chromatographic profile, one of them containing norstictic acid and the other in which this acid was not revealed, for analysis by HPLC-DAD-MS (**Fig. 5**).

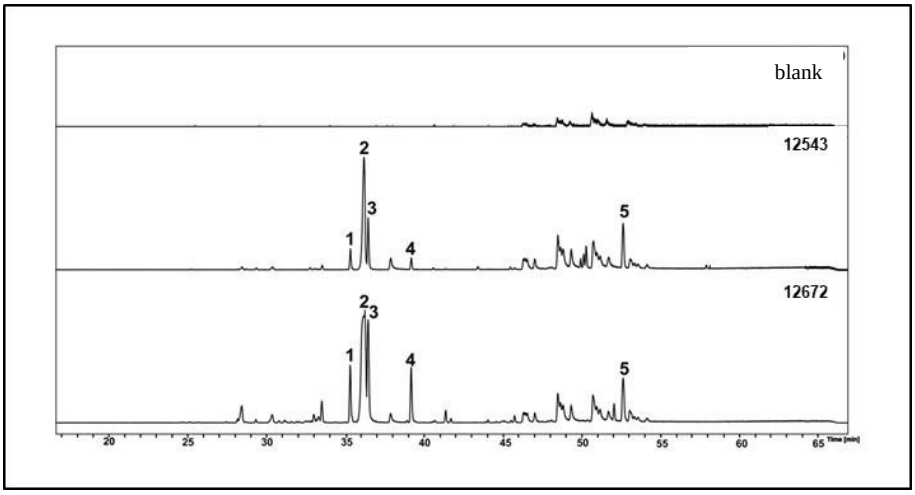

Fig. 5. Chromatogram of the base peak obtained by HPLC-DAD-MS of the acetone extracts of *Stereocaulon* representative of the species containing depsidones (A.A. Spielmann 12543 and A.A. Spielmann 12672) (**Fig. 1c**), in negative mode. Peaks 1-5 represent compounds present in these extracts.

The ions $[M-H]^-$ of m/z 387.0730 ($C_{18}H_{15}O_9$), m/z 343 $[M-CO_2-H]^-$, m/z 299 $[M-2CO_2-H]^-$, m/z 284 $[M-2CO_2-CH_3-H]^-$, m/z 266 $[M-2CO_2-CH_3-H_2O-H]^-$ and m/z 256 $[M-2CO_2-CH_3-CO-H]^-$ obtained from peak 1 (RT 35.3 min) can be attributed to both hypoconstitic acid and cryptoconstitic acid (**Fig. 15S**), however, TLC analysis indicates that the compound found in the analyzed extracts is hypoconstitic acid. Hypoconstictic and cryptostictic acids present very similar chromatographic migration but with a small difference in color when revealed with sulfuric acid. Cryptostictic acid reveals orange and hypoconstictic acid reveals red, thus the substance present in the analyzed extracts was hypoconstictic acid. The compound eluted in peak 2 (RT 36.2 min) was identified as stictic acid through the ion $[M-H]^-$ of m/z 385.0576 ($C_{19}H_{13}O_9$) and the fragments of m/z 297 $[M-2CO_2-H]^-$, m/z 282 $[M-2CO_2-CH_3-H]^-$ and that of m/z 267 $[M-2CO_2-OCH_2-H]^-$ [28,31,34,35].

In peak 3 (RT 36.4 min), menegazziaic acid was eluted and identified through the ion $[M-H]^-$ of m/z 373.0572 ($C_{18}H_{13}O_9$) and the second order fragments of m/z 329 $[M-CO_2-H]^-$, m/z 314 $[M-CO_2-CH_3-H]^-$, m/z 299 $[M-CO_2-OCH_2-H]^-$, m/z 286 $[M-CO_2-CH_3-CO]^-$ and m/z 270 $[M-2CO_2-CH_3-H]^-$ (**Fig. 16S**). In peak 4, norstictic acid (RT 39.2 min) was eluted, identified by the $[M-H]^-$ ion of m/z 371.0424 ($C_{18}H_{11}O_9$) and second-order fragments of m/z 327 $[M-CO_2-H]^-$, m/z 283 $[M-2CO_2-H]^-$ and m/z 255 $[M-2CO_2-CO-H]^-$ (**Fig. 17S**). The presence of atranorin (peak 5 RT 52.6 min) was confirmed as previously described (**Fig. 4S**, **Table 4S**). The HPLC-MS-DAD analysis confirmed the results obtained by the TLC analysis and the chromatogram in **Fig. 5** showed (by the intensity of the peaks) the difference in concentration of the substances present in the two extracts. Although norstictic acid was present in both extracts, the low concentration of this acid in the extract indicated as 12543 justified its non-revealing on the TLC.

The analysis of the chemical composition together with the morphological analysis allowed the identification of the specimens of this group as the species *Stereocaulon tomentosum* Fr., *Stereocaulon tomentosum* var. *capitatum* I.M. Lamb. and *Stereocaulon glabrum* (Müll. Arg.) Vain. Four chemical groups were determined for the species *Stereocaulon tomentosum*, one of them containing stictic, menegazziaic, norstictic and hypoconstictic acids; another did not present hypoconstictic acid, the third presented only stictic and norstictic acids and the fourth group containing atranorin, stictic and hypoconstictic acids (**Table 1S**). Lamb [3] cited for this species only atranorin, stictic and norstictic acids and possibly constictic and consalazinic acids, although the latter two were not detected in the specimens analyzed here. Menegazziaic and hypoconstictic acids were also not cited by Lamb [3] for this species. *Stereocaulon tomentosum* var. *capitatum* presented two chemical groups that differed from each other in terms of the presence of hypoconstictic acid. The species *Stereocaulon glabrum* showed the presence of stictic, menegazziaic, norstictic and hypoconstictic acids, another group did not present hypoconstictic acid and a third group presented only stictic and norstictic acids. According to Lamb [3], there are two chemical groups for the species *Stereocaulon glabrum*, one of them containing atranorin, stictic and norstictic acids, the latter in low concentration, and the other, atranorin, norstictic and connorstitic acids. The specimens analyzed here presented atranorin, stictic, norstictic, menegazziaic and hypoconstictic acids. These last two acids were not mentioned by Lamb [3].

4. Conclusions

In the present study, twenty-one substances were identified in extracts from 100 specimens of lichens from the genus *Stereocaulon*. Among the compounds, five are being reported for the first time, although the proposed structures await confirmation. Although TLC led to the identification of

several substances in the analyzed extracts, this was only possible through comparison with the chromatographic behavior of substances used as standards and with literature data. Similarly, microcrystallization, although an important auxiliary tool, allowed the identification of a limited number of substances, and just as TLC, did not permit the identification of trace substances, like for the extracts containing lobaric acid. In this case, the compositional elucidation of the extracts and structural elucidation of the trace components were only possible through the use of mass spectrometry. The results of mass spectrometry analyses reported here corroborate those obtained from the TLC and microcrystallization regarding the major compounds and allowed the identification of new substances present in species of the *Stereocaulon* genus. This more elaborated information on the composition of lichen species may, over time, lead to an updated detail in the taxonomic classification of lichens.

Supporting Information

Table 1S – Specimens of lichens analyzed, collection sites, species and chemical composition. **Table 2S** – Putative compounds annotated for the acetone extract of representative *Stereocaulon* spp. containing fatty acids. **Table 3S** – Putative compounds annotated for the acetone extract of representative *Stereocaulon* spp. containing lobaric acid (AAS 3419 and APL 767). **Table 4S** – Putative compounds annotated for the acetone extract of representative *Stereocaulon* spp. containing depsidones (A.A. Spielmann 12543 and A.A. Spielmann 12672). **Fig. 1Sa** – Chemical structures of the compounds of the classes of fatty acids, dibenzofuran, chromone and depsides putatively identified in the *Stereocaulon* spp. **Fig. 1Sb** – Chemical structures of the compounds of the classes of diphenylether and depsidones putatively identified in the *Stereocaulon* spp. **Fig.2S** – Fragmentation proposal for norrangiformic acid. **Fig. 3S** – Fragmentation proposal for pseudonorrangiformic acid. **Fig. 4S** – Fragmentation of atranorin. **Fig. 5S** - Fragmentation proposal for rangiformic acid. **Fig. 6S** – Fragmentation proposal for lobarin. **Fig. 7S** – Fragmentation of gyrophoric acid. **Fig. 8S** – Fragmentation proposal for oxysiphulin. **Fig. 9S** – Fragmentation proposal for vinapraesorediosic acid B. **Fig. 10S** – Fragmentation proposal for bourgeanic acid. **Fig. 11S** – Fragmentation proposal for colensoic acid. **Fig. 12S** – Fragmentation proposal for lobaric acid. **Fig. 13S** – Fragmentation proposal for depsidone eluted with retention time 48.5 min (peak 5). **Fig. 14S** – Fragmentation proposal for depside ($C_{25}H_{26}O_8$) eluted with retention time 48.5 min (peak 5). **Fig. 15S** – Fragmentation proposal for cryptostictic and hypoconstictic acids. **Fig. 16S** – Fragmentation proposal for menegazziaic acid. **Fig. 17S** – Fragmentation proposal for norstictic acid.

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Author Contributions

NKH, AAS and ACM conceived the study. AAS, APL and JMT were responsible for sample selection and morphological chacacterization. ASG and NKH worked on elucidation of chemical composition (microcrystallization, chromatographic analyses and mass spectrometry). NKH and ACM drafted the manuscript. All authors commented on drafts on the paper. All authors have approved the final draft of the manuscript.

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