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Dereplication by HPLC-ESI-MS and antioxidant activity of phenolic compounds from Banisteriopsis laevifolia (Malpighiaceae)

GERSO P. ALEXANDRE, JORGE LUIZ S. SIMÃO, MARIA OLIVIA A. TAVARES, IZABELLA MARIANA S. ZUFFO, STÉPHANIE V. PRADO, JOSEILSON A. DE PAIVA, ABUBAKAR N. MUSTAPHA, ANSELMO E. DE OLIVEIRA, LUCILIA KATO & VANESSA GISELE P. SEVERINO

Abstract: The genus Banisteriopsis (Malpighiaceae) comprises 77 genera and 1,300 species. Despite efforts to provide detailed information about the chemical wealth of B. laevifolia, this article provides the identification and characterization of compounds from the ethanolic extracts of the leaves and flowers using advanced methodologies which include high-performance liquid chromatography coupled to tandem mass spectrometry, which contribute to the knowledge about compounds present in the genus Banisteriopsis. The dereplication aided by Global Natural Products Social Molecular Networking afforded the identification of seven compounds reported for this species for the first time. A mixture of two known flavonoids and a diterpenoid, 18-hydroxy-ent-halima-1(10),13-(E)-dien-15-oic acid, were isolated by conventional separation methods. The elevated 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity observed in some samples was attributed to either the type of extract, the presence of 3,4,5-trihydroxybenzoic acid, or the influence of the substitution pattern and the synergistic effect of the compounds present. Principal Component Analysis was applied to correlate mass spectrometry data with the antioxidant activity of the samples. The high diversity of metabolites found in this study and those which have been reported for Banisteriopsis strongly recommends further investigation into the chemical and biological properties of these species.

Key words: Banisteriopsis laevifolia, phenolic compounds, antioxidant activity, molecular networking, dereplication.

INTRODUCTION

With six biomes, the biodiversity of Brazilian flora is considered the richest in the world. This flora has more than 55 thousand plant species out of which 10 thousand are considered medicinal, aromatic, and useful plants (Batalha & Ming 2004). The Brazilian Cerrado savanna is endowed with ecosystems that are marked by rich vegetation and plant diversity. It is said to represent the second largest biome in Brazil, especially when considering woody species (Guarim-Neto & Morais 2003). Families such as Mimosaceae, Fabaceae, Caesalpiniaceae, Poaceae, Asteraceae, Malpighiaceae, and others have been identified in this domain (Klink & Machado 2005).

The genus Banisteriopsis belongs to the Malpighiaceae family, which is comprised of 77 genera and 1,300 species (92 of these species are mainly distributed in Brazil, Bolivia, Colombia, Ecuador, and Peru), presenting a pantropical
distribution with the greatest biodiversity in the South American continent (Anderson & Davis 2010, Wang et al. 2010).

Several research studies related to the biological potential of the genus *Banisteriopsis* have been reported (Rodrigues & Carvalho 2001, Frias et al. 2011). Classes of compounds with medicinal indications/therapeutics have been identified in this genus. Research describing dihydrophenanthrenes in *B. anisandra* and phytohormones and the psychoactive indole alkaloids with β-carboline structure in *B. caapi* have also been reported. These active hallucinogenic compounds are served in drinks during rituals by the Ayahuasca in occidental countries (Freitas et al. 2015, Schwarz et al. 2003).

The extracts from different species of *Banisteriopsis* have been acclaimed for a number of therapeutic indications. For instance, *B. argyrophylla* has been described with anti-inflammatory action; *B. campestris* is used as diuretic; and *B. megaphylla* is used for antipyretic activity and for the treatment of pulmonary diseases; while the ethanolic extracts of *B. anisandra* showed an *in vitro* antimicrobial activity (Pádua et al. 2013, Frias et al. 2011). The chemical compounds from the essential oils extracted from the leaves of *B. laevifolia* showed antimicrobial activity against yeasts of the genus *Candida* (Nunes et al. 2016). In addition to the established bioactivity and presence of alkaloids and phenolic compounds in *B. laevifolia*, there are folkloric reports on the uses of the roots in the treatment of ovarian haemorrhage (Rodrigues & Carvalho 2001). Due to the activity reported for *B. laevifolia*, the analysis of the antioxidant potential helps to confirm the presence of phenolic compounds in metabolism of this species, as well as contributes to evaluation and selection of substances that have potential to be used as medicinal drugs (Nunes et al. 2016, Alves et al. 2010).

Despite efforts to provide detailed information about the chemical wealth of *B. laevifolia* species, it has been observed that little is known about the metabolic fingerprinting of the extracts of some parts of this species that have been reported for their marked folkloric indications. In view of this, this article presents the compounds identified and characterized from the ethanolic extracts of the leaves and flowers of *B. laevifolia*. Advanced and adopted/modified methodologies were used which include high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). In addition, the antioxidant activity of the compounds isolated from the flowers is also reported.

**MATERIALS AND METHODS**

**Plant Material**

Access to the genetic heritage was registered at the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) under code No. A11AE20. Flowers and leaves of *B. laevifolia* were collected in March 2016 at the Serrinha Reserve (16°43’25”S 49°15’50”W) located in Goiânia city, state of Goiâs, Brazil. The materials were identified by Dr. Aristônio Magalhães Teles and a voucher (UFG-60052) was deposited in the herbarium of the Federal University of Goiâs (UFG), Brazil.

**Equipment**

$^1$H (500 MHz) and $^{13}$C (125 MHz) nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III 500 (11,75T). Compound (1) was dissolved in CDCl$_3$ (Sigma-Aldrich, St. Louis, MO, USA) and a mixture of (2) and (3) was dissolved in CD$_3$OD (Sigma-Aldrich, St. Louis, MO, USA). HPLC was performed on a Shimadzu apparatus with an LC-18A pump, an SPD-20A detector, a CBM-20A-communications module (Shimadzu),
and a Rheodyne injector with a loop of 500 µL. The liquid chromatography coupled to the high-resolution tandem mass spectrometry (HPLC-ESI-HRMS/MS) system consisted of an Ultimate 3000 HPLC (Thermo Scientific) coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Scientific) controlled by software Xcalibur™, version 4.2. An H-ESI (heated electrospray ionization) source in negative mode was used for monitoring the compounds by HRFS (high resolution full scan) and in the parallel reaction monitoring (PRM) experiments. An UV-VIS spectrophotometer, model Sp 22 Biospectro, was used with glass cells of 1 cm path length.

**Extraction**

The fresh flowers (618 g) were extracted by maceration with ethanol (Merck, Darmstadt, Germany) (3 × 1 L, every 3 days) at room temperature. The material was filtered and then concentrated in a rotary evaporator to obtain the ethanolic extract of flowers (EEF) (49.7 g, 8.0%). The air-dried leaves (719 g) were pulverized in a knife mill, extracted at room temperature with ethanol (EtOH) (3 × 3 L, 3 days each) and filtered. The filtrate was concentrated to yield the ethanolic extract of leaves (EEL) (79.2 g, 11.0%).

Liquid-liquid extraction was used to fractionate a portion of the EEF (15 g) into the following fractions: hexane (Merck, Darmstadt, Germany) (1.1 g), dichloromethane (Merck, Darmstadt, Germany) (2.8 g), ethyl acetate (Merck, Darmstadt, Germany) (6.4 g), and n-butanol (Merck, Darmstadt, Germany) (3.8 g). Thereafter, a portion of the dichloromethane fraction (480 mg) was subjected to a Sephadex LH-20 (Amersham, Pharmacia Biotech, Little Chalfout, UK) column and eluted with CH₂Cl₂:CH₃OH (Merck, Darmstadt, Germany) (2:8) to give six sub-fractions (D1 to D6). Sub-fraction D2 (66.8 mg) was again chromatographed on a Sephadex LH-20 column eluted with CH₃OH:CH₂Cl₂ (8:2) to give five sub-fractions (D2.1 to D2.5). Sub-fraction D2.5 availed compound (11) (5 mg). Sub-fraction D3 (72.0 mg) was subjected to silica gel (Merck, Darmstadt, Germany) column chromatography eluted with solvents of increasing polarity [hexane:ethyl acetate (1:0 to 0:1) and ethyl acetate:CH₃OH (1:0 to 0:1)] to give nine sub-fractions (D3.1 to D3.9). Sub-fraction D3.9 (111 mg) was purified by HPLC using CH₃OH:CH₂Cl₂ (8:2), isocratic mode with a flow rate of 5.0 mL min⁻¹, to yield a mixture of compounds (1) and (2) (5.2 mg). The ethyl acetate fraction (3.9 g) was subjected to a Sephadex LH-20 column and eluted with CH₃OH:CH₂Cl₂ (8:2) to yield fourteen sub-fractions (EA1 to EA14). Sub-fraction EA7 (520 mg) was further purified by HPLC using CH₃OH:CH₂Cl₂ (8:2), isocratic mode with a flow rate of 5.0 mL min⁻¹, to yield seven sub-fractions (EA7.1 to EA7.13). Sub-fraction EA7.11 (95.8 mg) was purified by HPLC using CH₃OH:CH₂Cl₂ (8:2), isocratic mode with a flow rate of 5.0 mL min⁻¹, to yield six sub-fractions (EA7.11.1 to EA7.11.6).

**Mass Spectrometry Data Acquisition**

The both extracts and sub-fractions D3.5, D3.7, EA7.3, EA7.6, EA7.11.1 and EA7.11.4 were subjected to liquid chromatography coupled to the high-resolution tandem mass spectrometer (HPLC-ESI-HRMS/MS) for identification of phenolic compounds, and each sample (1.0 mg) was dissolved in 1.0 mL of CH₃OH and filtered through a cellulose acetate filter (0.45 µm). The chromatographic separation was carried out using an NST 18 column (4.6 mm × 100 mm, 5.0 µm) at 20 °C with mobile phases of deionized water (A) and acetonitrile (Sigma-Aldrich) (B), both acidified with 0.1% formic acid (Sigma-Aldrich). The applied gradient was 50 to 100% B over 40 minutes, then 100% B was maintained for 5 minutes at a flow rate of 1.0 mL min⁻¹ and a 10 µL injection volume. The MS parameters used were as follows: spray voltage 4 kV, sheath gas flow rate 30 arbitrary units, auxiliary gas flow.
rate 10 arbitrary units, capillary temperature 350 °C, auxiliary gas heater temperature 300 °C, S-lens 55 and collision energy offset of 20 eV. The samples were analysed by a Data Dependent Acquisition (DDA) method using the HR full-scan experiment set up in the m/z range of 150 to 700 Da. The precursor ions were selected and subsequently performed by parallel reaction monitoring (PRM) to obtain MS/MS spectra.

**Total Phenolic Content**

Total Phenols Content (TPC) was assayed according to the methodology described by Sousa et al. (2007) with modifications by Nunes et al. (2016). 3,4,5-trihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO, USA) standard solutions (10.0, 20.0, 40.0, 60.0 and 80.0 μg mL⁻¹) were prepared in CH₃OH and used to construct a calibration curve. The phenolic content was expressed as milligrams of 3,4,5-trihydroxybenzoic acid equivalents per gram of dry weight (mg GAE g⁻¹). Both samples were analysed in quintuplicate.

**Total Flavonoid Content**

Total Flavonoids Content (TFC) was measured according to the methodology described by Woisky & Salatino (1998) with modifications by Nunes et al. (2016). 3,3',4',5,7-pentahydroxyflavone (Sigma-Aldrich, St. Louis, MO, USA) standard solutions (5.0, 10.0, 20.0, 30.0, and 40.0 μg mL⁻¹) were prepared in CH₃OH and used to construct a calibration curve. Total flavonoid content was expressed as quercetin equivalents per gram of dry extract (mg QE g⁻¹). Both samples were analysed in quintuplicate.

**DPPH Radical-Scavenging Activity**

The samples EEF and EEL extracts and the sub-fractions D3.5, D3.7, EA7.3, EA7.6, EA7.11.1, and EA7.11.4 were solubilized in CH₃OH (1 mg mL⁻¹) and diluted to different concentrations ranging from 500 to 7.81 µg mL⁻¹, and the assays were conducted by adding 2800 μL of methanolic solution of DPPH (Sigma-Aldrich, St. Louis, MO, USA) (100 μM) to 200 μL of sample. The mixture was incubated for 40 min in the dark, and the absorbance of unreacted DPPH was used as the control. DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was determined spectrophotometrically at 517 nm. 3,4,5-trihydroxybenzoic acid was used as the standard, and the percent DPPH scavenging effect was calculated by using the following equation:

\[
\text{% DPPH scavenging} = \frac{(\text{Abs}_{517\text{nm}} \text{ (control)} - \text{Abs}_{517\text{nm}} \text{ (sample)})}{\text{Abs}_{517\text{nm}} \text{ (control)}} \times 100
\]

The IC₅₀ value, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated from the regression equation for the concentration of sample and percentage inhibition. The data were evaluated statistically using Minitab 18.0 software. The Ryan-Joiner test was used for analysis of normality distribution; this was followed by one-way analysis of variance (ANOVA) and the Tukey test post hoc. The level of statistical significance was defined as \( p = 0.05 \).
Data Processing for Chemometrics Analysis
All calculations were run using R software version 3.6.3 (TEAM 2020). MS data was analysed using R packages Msnbase version 2.12.0 (GATTO & LILLEY 2012) and MALDIquant version 1.19.3 (GIBB & STRIMMER 2012). Pre-treatment (peak alignment by the correlation optimized warping technique) and pre-processing (profile data) of the data matrices were applied. Peak data was binned using 0.0015 Da (5 ppm) bins and the resulting data matrix was Pareto scaled (peak intensities) using the R package MetabolAnalyze (GIFT et al. 2010). Hierarchical clustering analyses (HCA) and Principal component analyses (PCA) were run on the MS data to discriminate the metabolite contents in each extract. HCA was run using square Euclidean distance and Ward’s method to link the clusters.

Classical Molecular Networking Workflow Description
A molecular network was created using the online workflow on the GNPS website (http://gnps.ucsd.edu). Initially mass data obtained in .RAW format were converted to .mzXML files using the Msconvert, version 3.0, from ProteoWizard software (ProteoWizard, Palo Alto, CA, USA). The MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least 6 matched peaks (Wang et al. 2016).

In the analysis, network annotation propagation (NAP) was utilized where the cosine value is to sub select inside a cluster of 0.65, N first candidates for consensus score of 10, accuracy for exact mass candidate search (ppm) of 15, acquisition mode was negative, adduct ion type was [M-H], and structure databases were GNPS and SUPNAT (da Silva et al. 2018). The non-annotated nodes in the classical analysis were submitted to NAP and later manually checked using patterns and mass data from the MoNa library (www.mona.fiehnlab.ucdavis.edu) in the respective fragmentation energy.

RESULTS
Total Phenolic, Flavonoid and Tannin Contents
The measured values of TPC, TFC, and TTC to EEL and EEF of B. laevifolia are presented in Table I. The ANOVA test identified statistical differences (p = 0.05) in phenolic content between the extracts of B. laevifolia, which observed larger quantities of phenolic compounds, flavonoids, and tannins for EEF.

DPPH Radical-Scavenging Activity
The Ryan-Joiner test showed that the data have a normal distribution, which described the continuous behaviour of the data, and therefore parametric tests were used to assess the equality of the IC_{50} means of the samples. The ANOVA test showed different levels (p = 0.05) for radical scavenging capacity, which varied between IC_{50} values of 24.38 ± 0.07 μg mL^{-1} and 679.18 ± 0.06 μg mL^{-1} (Table II). The ANOVA analysis was supplemented by the Tukey test which allows for the evaluation and comparison between different IC_{50} means as well as showing the similarity between the antioxidant capacity of EEF (24.38 ± 0.07 μg mL^{-1}) and the 3,4,5-trihydroxybenzoic acid standard (21.28 ± 0.03 μg mL^{-1}).

Dereplication of phenolic compounds of B. laevifolia by GNPS
The GNPS molecular networking and NAP analysis allowed annotation of ten phenolic compounds (Figure 1), organized in the molecular
Table I. Total phenolic content (expressed in mean ± SD mg 3,4,5-trihydroxybenzoic acid equivalent/g dry weight), flavonoids content (expressed in mean ± SD mg 3,3',4',5,7-pentahydroxyflavone equivalent/g dry weight), and tannins content (expressed in mean ± SD mg 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol equivalent/g dry weight) in Banisteriopsis laevifolia extracts.

| Total Content       | Ethanolic Extract of Flowers (EEF) | Ethanolic Extract of Leaves (EEL) |
|---------------------|-----------------------------------|-----------------------------------|
| Phenolic compounds  | 288.67 ± 0.04a                    | 225.67 ± 0.07b                    |
| Flavonoids          | 199.87 ± 0.13c                    | 173.30 ± 0.08d                    |
| Tannins             | 90.13 ± 0.17e                     | 30.61 ± 0.22f                     |

Values (means of five replicates) followed by different letters are significantly different at p = 0.05.

Table II. Scavenging activity (mean ± SD), expressed as inhibitory concentration (μg mL-1), in the DPPH test with extracts and sub-fractions of Banisteriopsis laevifolia.

| Sample                     | DPPH (IC50) |
|----------------------------|-------------|
| 3,4,5-trihydroxybenzoic acid | 21.28 ± 0.03a |
| EEF                        | 24.38 ± 0.07a |
| EEL                        | 107.21 ± 0.09b |
| D3.5                       | 120.57 ± 0.12bc |
| D3.7                       | 154.52 ± 0.24c |
| EA7.3                      | > 1000d     |
| EA7.6                      | 573.08 ± 0.13ef |
| EA7.11.1                   | 679.18 ± 0.06e |
| EA7.11.4                   | 401.42 ± 0.02f |

Values (means of five replicates) followed by different letters are significantly different at p = 0.05. EEF: ethanolic extract of flowers; EEL: ethanolic extract of leaves; D3.5, D3.7, EA7.3, EA7.6, EA7.11.1, and EA7.11.4: sub-fractions.

network in six main spectral families (Figure 2). In other words, family (2a) was formed by derivatives of 3',4',5,7-tetrahydroxyflavone (m/z 285.041) (1), 3',4',5,7-pentahydroxyflavanone (m/z 301.036) (2), 4',5,7-trihydroxyflavanone (m/z 271.062) (3), and 3,4,5-trihydroxybenzoic acid (m/z 169.014) (4); family (2b) by derivatives of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (m/z 301.036) (5), family (2c) by derivatives of 3-[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-Benzopyran-4-one (m/z 609.147) (6) and 2-(3,4-dihydroxyphenyl)-3-(β-D-glucopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one (m/z 463.095) (7); family (2d) by derivatives of 2,5-dihydroxybenzoic acid (m/z 153.019) (8); family (2e) by derivatives of 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol (m/z 289.073) (9); and family (2f) by derivatives of 3,4-dihydroxybenzoic acid (m/z 153.019) (10). The annotated compounds in B. laevifolia were confirmed to results in level 2 (MS/MS match and accurate mass – Sumner et al. 2007) and are shown in table III. Further information about MS/MS spectrum are provided in supplementary material (Figures S1 - S9).
Figure 1. Structures of phenolic compounds identified by GNPS and NAP.

Figure 2. Full classical molecular network realized using MS/MS data and visualized in Cytoscape from the ethanolic extracts of flowers (EEF), leaves (EEL), and sub-fractions obtained from EEF of B. laevifolia.
### Table III. Compounds annotated at flowers and leaves extracts of *B. laevifolia*.

| #  | tR (min) | Molecular Formula | [M-H]- Error (ppm) | MS2 | Compound | Checking | Sample                |
|----|----------|-------------------|--------------------|-----|----------|----------|-----------------------|
| 1  | 28.22    | C_{15}H_{10}O_{6} | 285.04089 3.42     | 285.04089, 133.02847 | 3',4',5,7-tetrahydroxyflavone | Standard  | Flower and Leave       |
| 2  | 23.77    | C_{15}H_{10}O_{7} | 301.03573 3.60     | 301.03546, 257.04559, 215.03444, 151.00269, 149.02335 | 3',4',5,5,7-pentahydroxyflavone | NMR and HRMS | Flower and Leave       |
| 3  | 28.03    | C_{15}H_{12}O_{7} | 271.06165 3.69     | 271.06165, 177.01872, 151.03049, 119.04926, 93.03345 | 4',5,7-trihydroxyflavanone | Standard  | Flower and Leave       |
| 4  | 17.31    | C_{7}H_{6}O_{3}   | 169.01353 1.01     | 169.01353, 168.96231, 154.94655, 151.00284, 125.02343, | 3,4,5-trihydroxybenzoic acid | Standard  | Flower and Leave       |
| 5  | 27.68    | C_{15}H_{10}O_{7} | 301.03571 2.92     | 301.03571, 178.99376, 151.00285, 121.02850, 107.01277, 83.01266, 65.00207 | 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one | Standard  | Flower and Leave       |
| 6  | 25.50    | C_{27}H_{30}O_{16} | 609.14703 2.41     | 301.03574, 300.02795, 271.02524, 255.03014, 151.00288 | 3-{[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy}-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-Benzopyran-4-one | Standard  | Flower and Leave       |
| 7  | 25.63    | C_{27}H_{30}O_{12} | 463.09548 -        | 301.03571, 300.02789, 271.02524, 255.03009, 151.00285 | 2-(3,4-dihydroxyphenyl)-3-{β-D-glucopyranosyl}oxy)-5,7-dihydroxy-4H-1-benzopyran-4-one | Standard  | Leave                 |
| 8  | 19.23    | C_{15}H_{14}O_{6} | 153.01848 1.99     | 153.01848, 109.02840 | 2,5-dihydroxybenzoic acid | Standard  | Flower and Leave       |
| 9  | 20.10    | C_{15}H_{14}O_{6} | 289.07217 3.30     | 289.07217, 245.08203, 205.05028, 203.07100, 179.03438, 125.02345, 109.02844 | 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol | Standard  | Flower and Leave       |
| 10 | 24.47    | C_{15}H_{14}O_{6} | 153.01854 1.60     | 153.01854, 109.02843 | 3,4-dihydroxybenzoic acid | MoNa      | Flower and Leave       |
Isolated Compounds
A mixture of flavonoids (1) and (2) and the diterpenoid 18-hydroxy-ent-halima-1(10),13-(E)-dien-15-oic acid (11) were isolated and identified by NMR (1D & 2D) spectra and HRMS. The analysis in negative mode very clearly shows the ion peak at 285.04047 [M-H]⁻ (error 3.8 ppm) indicating the molecular formula C₁₅H₉O₆ which suggested the identification of flavonoid (1); flavonoid (2) was identified according to the another set of ¹H NMR aromatic hydrogen signals, by comparison from the literature, and the ion [M-H]⁻ 301.03573 (error 3.6 ppm), indicating the molecular formula C₁₅H₉O₇ (Guzel 2020).

Compound (11) was identified by the ¹H NMR spectrum from the diagnostic olefinic hydrogen at δ 5.35 (s), two tertiary methyl groups at δ 0.96 (s) and δ 0.94 (s), and one secondary methyl group at δ 0.84 (d, 7.5 Hz), suggesting a typical halimane type diterpenoid, with presence of hydroxy methylene group at δ 3.34 (d, 10 Hz, 1H) and δ 3.50 (d, 10 Hz, 1H) was attributed to C₄ as in an ent-halimane diterpene found and isolated by Monteiro et al. (2015). The HMBC analysis showed the α, β unsaturated carboxy group side chain (Supplementary Material – Table SI). The MS analysis in the negative mode presents molecular ion peaks m/z 319.22742 [M-H]⁻ and positive mode m/z 321.24228 [M+H]⁺ which suggested the molecular formula C₂₀H₃₂O₃ (error negative mode = 0.6 ppm and positive mode = 2.1 ppm).

DISCUSSION
The Malpighiaceae family and species of the genus Banisteriopsis have a large number of phenolic compounds (Frias et al. 2012). These compounds form a versatile class of secondary metabolites widely present in higher plants. They are varied in terms of structures and are classified according to the number of aromatic rings and the structure that connects the rings (Heleno et al. 2015, Dias et al. 2015, Khan et al. 2020). Flavonoids and tannins are important groups of polyphenols that show important functions in vegetables, and the content of these metabolites in a plant results from a sum of physiological and environmental factors (Jin 2019, Khan et al. 2020).

Our results (Table I) have demonstrated a higher content of phenolic compounds, flavonoids, and tannins in the flowers (EEF) of B. laevifolia. A comparison of the minimum statistically significant difference, Tukey test, between EEF and EEL measured contents confirmed the preferential accumulation of these compounds in flower tissues. According to Medini et al. (2014), this accumulation process may occur during the plant development stage. Phenolic compounds might be found in all parts of a plant during the flowering stage; however, they are preferentially accumulated in the epidermal tissues of flowers and contribute to the mechanisms of defence and attraction of pollinating agents (Pietta 2000, Medini et al. 2014, Jiang et al. 2016).

The phenolic compounds represent an important class of metabolites to combat and prevent oxidative stress caused by free radicals, and inactivation mechanisms occur through reduction reactions, by means of electron transfer, which yield stable molecules (Alves et al. 2010, Braham et al. 2020, Khan et al. 2020). The evaluation of potential sequestration of the DPPH radical for B. laevifolia samples was performed, followed by analysis of variance and the Tukey test for all possible pairs of IC₅₀ for B. laevifolia present in Table II. The results pointed out a statistical similarity between the standard (3,4,5-trihydroxybenzoic acid standard) and the EEF sample, demonstrating the high antioxidant activity of the sample. The antioxidant capacity
for the other samples were classified, according to Mensor et al. (2001), as moderate (EEL, D3.5, and D3.7) and weak (EA7.3, EA7.6, EA7.11.1, and EA7.11.4).

PCA was then applied to identify chemical similarities on the MS data that might be related to the observed antioxidant activity. The first two principal components (PC1 and PC2) explained 50% of the total MS data variance and according to the score plot in Figure 3a; PC1 discriminates EEL (right side) from the rest of the samples. According to the plot of the loadings for PC1, Figure 3b, the m/z peaks having the highest loading values (>0.2) are 56.596, 161.408, 260.134, and 125.686, and these are the most important ions responsible for distancing EEL. The ion m/z 125.686 (the farthest point on the right in Figure 3b) comes from the fragmentation of 3,4,5-trihydroxybenzoic acid. This compound is
the most abundant phenolic acid in *B. laevifolia* metabolism, and it has been demonstrated to be an important antioxidant component responsible for the efficient radical scavenging (Badhani et al. 2015).

All sub-fractions but D3.5 are grouped together at the centre of Figure 3a. The sub-fraction D3.5 (top left) presents a different pattern, mainly because of the high positive loading values from m/z peaks 99.857, 270.653, and 126.142 (Figure 3b). The sample EEF (bottom left) is also discriminated from the rest of the samples owing to the m/z peaks 165.460, 59.603, and 202.267.

The dendrogram in Figure 3c presents the clustering beginning from sub-fractions (at the left) and follows until the extracts (at the right), except for the sample D3.5. The grouping in Figure 3c is very similar to the pattern found from the PCA results. Although the four compounds identified in sub-fraction D3.5 are not as many as the eight compounds identified in EEF, it is possible that the antioxidant elevation of this sub-fraction is associated with the presence of 3,4,5-trihydroxybenzoic acid, confirmed by a reference standard, as well as the synergistic effect of the fewer compounds (Heim et al. 2002).

According to Figure 3a, there are four groups: 1) EEL; 2) EEF; 3) D3.5, and 4) remaining sub-fractions. A new PCA (supplementary material, Figure S10) was applied to the group 4 samples and characteristic ions identified. However, since these ions presented small m/z values, they could not be related to any specific chemical compound in database libraries.

Through PCA analysis, the similarity in chemical composition between extracts and fractions of *B. laevifolia* can be observed through the common molecular fragments present in the samples. Thus, in order to elucidate the families of compounds among the extracts and fractions, GNPS dereplication was employed. After processing the MS/MS data on the GNPS platform, the molecular networks obtained were processed and analyzed using Cytoscape software, version 3.8.2, followed by NAP analysis in order to note the possible corresponding structures of phenolic compounds present in *B. laevifolia* (Sumner et al. 2007, Mannocchio-Russo et al. 2020). The families were formed by nodes with the same mass fragmentation profiles. In family 2a, derived structure of (1) with molecular ion [M-H]⁻ m/z 285.041 and ion [M-H-152]⁻ and derived of (2) molecular ion [M-H]⁻ m/z 301.036 were observed from EEF in sub-fractions D3.7 and EA7.6. Also, in this family, derived of (3) were annotated from EEL and EEF in sub-fractions D3.5, D3.7, and EA7.6 with the quasi-molecular ion [M-H]⁻ m/z 271.062 and ions [M-H-94], [M-H-120], and [M-H-152]. From compound (3), it was possible to search compound (4) from EEL and EEF in sub-fractions D3.5, D3.7, and EA7.6 with quasi-molecular ion [M-H]⁻, m/z 169.014 and ions [M-H-18] (annotated through NAP) and [M-H-44] (Boudiar et al. 2019).

In family 2b, all nodes have the same mass as the precursor ion. Thus, compound (5) was identified in EEL and EEF with the quasi-molecular ion [M-H]⁻ m/z 301.036 and other fragment ions with m/z 178.994, 151.003, 121.028, 107.013, 83.013, and 65.002 (Oliveira et al. 2018). The family 2c presents nodes with differences of m/z -146.052. In this family, derived of (6), with quasi-molecular ion [M-H]⁻ m/z 609.147 and ions m/z 301.036 and m/z 300.028, was annotated from EEL and EEF in sub-fractions D3.5 and EA7.6 (Boudiar et al. 2019, Cuyckens & Claey 2004). It is evident that the neighbouring node is derived from the loss of a rhamnosyl fragment (Okonkwo et al. 2016). This suggests the presence of derived of (7) from EEL and EEF in sub-fractions D3.5, D3.7, EA7.3, and EA7.6, with ions m/z 301.036, 300.028, 271.025, 255.030, and 151.003 (Cifuentes et al. 2020).
In the family 2d and 2f, the clusters formed by ions from EEF and EEL samples, showed the presence of compounds (8) and (10). The quasi-molecular ion $m/z$ [M-H]$^-$: 153.019 availed compound (8), while the base peak ion $m/z$ 109.028 (Zhang et al. 2018) suggested compound (10). The existence and fragmentation pattern of compound (10) agrees with the data reported in the literature (Bhagya & Chandrashekar 2020, Mallmann et al. 2020). However, precise information on retention time would be necessary, as these dihydroxy acids have at least six isomers that present the $m/z$ [M-H]$^-$ and [M-CO$_2$$]$^-$ (Belaya 2020). The family 2e presents a node identified as compound (9), which was obtained from EEL and EEF, with a quasi-molecular ion $m/z$ 289.073 and ions 245.082, 205.050, 203.071, 179.034, 125.023, and 109.028.

Compounds (5) and (9) fragmentation profiles were also identified and reported for B. argyrophylla (Oliveira et al. 2018). In addition, compounds (6) and (9) were also reported in B. laevifolia species (Nunes et al. 2016). To the best of our knowledge, this is the first time that compounds (1 – 4), (7), (8) and (10) are reported in B. laevifolia. This finding may contribute to an understanding of the antioxidant activity of the leaves and flower extracts of B. laevifolia, and the identification of these nine phenolic compounds brings added importance from the chemical, biological, and species preservation point of view and to the benefits that these phenolic compounds can bring to human health (Durazzo et al. 2019, Imran et al. 2019).

In spite of the inadequate studies and scarce information in the literature about the B. laevifolia species, this work on dereplication by HPLC-ESI-MS and antioxidant activity of phenolic compounds from B. laevifolia (Malpighiaceae) contributes to knowledge about the genus Banisteriopsis in general and opens avenues for continuing research on the B. laevifolia species in particular. From the findings reported herein, it is possible to understand the differences in the antioxidant activities between extracts and sub-fractions through data processing with PCA. The antioxidant activity is related to the chemical composition of the extracts or sub-fractions with particular reference to the HO$^-$ groups as well as to the substitution pattern on the flavonoid structures (Heim et al. 2002). Phenolic compounds that have two HO$^-$ substitutions in ring B exhibited greater antioxidant activity than those with an HO$^-$ substitution. Hence, compound (5) and its derivatives have greater antioxidant activity than 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one (Cao et al. 1997).

Although ent-halimane diterpene (11) was previously isolated from Hymenaea stigonocarpa (Monteiro et al. 2015), this study pioneered its presence for the first time in B. laevifolia species. Further phytochemical studies with other species of Banisteriopsis are needed to fully understand the chemosystematic significance of these compounds in this genus. The high diversity of metabolites found in this study and those which have reported for the Banisteriopsis genus strongly recommend further investigation into the chemical and biological properties of its species.

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SUPPLEMENTARY MATERIAL

Figure S1. MS/MS spectral pattern of 3’,4’,5,7-tetrahydroxyflavone.

Figure S2. MS/MS spectral pattern of 4’,5,7-trihydroxyflavanone.

Figure S3. MS/MS spectral pattern of 3,4,5-trihydroxybenzoic acid.

Figure S4. MS/MS spectral pattern of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one.

Figure S5. MS/MS spectral pattern of 3-[(6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one.

Figure S6. MS/MS spectral pattern of 2-(3,4-dihydroxyphenyl)-3-(β-D-glucopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one.

Figure S7. MS/MS spectral pattern of 2,5-dihydroxybenzoic acid.

Figure S8. MS/MS spectral pattern of 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol.

Figure S9. MS/MS spectral pattern of 3,4-dihydroxybenzoic acid.

Figure S10. Principal component plots for MS data of the sub-fractions D3.7, EA7.3, EA7.6, EA7.11.1, and EA7.11.3: a) score plot for PC1 x PC2; b) loadings for PC1 x PC2.

Table SI. 1H and 13C NMR spectroscopic data for compound (11).