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Multivariate Analysis of UPLC-MS/MS Metabolomic Profiles in Four *Hiraea* Species (Malpighiaceae)

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Abstract: The presence of bioactive compounds is reported in several Malpighiaceae species. However, little metabolomic information is documented in the genus *Hiraea* (Malpighiaceae). Thus, the objective was to identify secondary metabolites in the leaves of *Hiraea cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) and to compare the profiles by VIP score (partial least squares discriminant analysis, PLS-DA). Leaves were extracted with ethanol–water (4:1 *v*/*v*) and subjected to UPLC-MS/MS. The UPLC-MS/MS chromatographic profiles (in both positive and negative ionization modes) were separately processed and compared using the VIP score (PLS-DA). Fifty compounds were annotated, forty-five for the first time in the genus Hiraea, including flavonoids and phenolic acids, such as chlorogenic acid. The VIP score analysis revealed differences in the intensities of the compounds identified in *Hiraea* leaves (95% confidence), with rutin and myricitrin as the key metabolites for distinguishing among the four *Hiraea* species. These findings contributed to an understanding of the chemical diversity within *Hiraea*, suggesting possible ecological adaptations and potential pharmacological applications.

Keywords: *H. cuiabensis*; *H. hatschbachii*; *H. reclinata*; *H. restingae*

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1. Introduction

Hiraea is a genus of neotropical lianas in the botanical family Malpighiaceae, with approximately 80 species of woody vines or shrubs found from Mexico to Argentina [1]. Botanical studies confirm a significant variation in the leaf shape of the *Hiraea* species. The differences in size, indumentum, and glands of the leaf blades of *Hiraea* are well described in the literature and are recurrently updated with the analysis of new materials [2,3]. Despite valuable botanical information, the identification of metabolites in this genus is scarce.

The presence of flavonoids, terpenoids, alkaloids, steroids, phenolics, tannins, and other metabolites is well-documented in several genera of Malpighiaceae, such as *Banisteriopsis*, *Peixotoa*, and *Heteropterys* [4–7]. However, in the genus *Hiraea*, we only found the identification of flavonoids and phenolic compounds in the leaves of *Hiraea reclinata*, a species mainly found in rainforests of Central America [6]. Other species from different neotropical biomes, such as *H. cuiabensis* from the Cerrado savannas and *H. hatschbachii* and *H. restingae* from the Atlantic rainforests, lack metabolomic annotations and chemometric analyses.

Metabolomic analyses in leaves of Malpighiaceae species using liquid chromatography have contributed to separating and identifying bioactive compounds with antioxidant, anti-

depressant, and soothing activities [8–11]. In addition, high-resolution mass spectrometry analyses have contributed to unprecedented identifications of the secondary metabolites in Malpighiaceae, such as flavonoids, alkaloids, phenylpropanoids, and phenolic acids in the genus *Banisteriopsis*, *Diplopterys*, *Heteropterys*, and *Tetrapterys*. Therefore, from the metabolomic profile and phytochemical activities of the compounds present in Malpighiaceae species, new phylogenetic classifications have been reported for this family [4–6].

The combination of chromatographic profiles, high-resolution mass analysis, and chemometric analyses contributes to assessing the similarity and dissimilarity of metabolites between species of the same genus [4]. Differences in compounds present between species, drying methods, extraction protocols, and phytochemical activities have been discussed in the literature using chemometric tools, such as Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) [4,10].

The objectives of this study were to identify secondary metabolites in leaves of *H. cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) and compare these chromatographic profiles by VIP score (PLS-DA). Secondary metabolites were chosen as the focus due to their ecological and pharmacological significance, as well as their role in species differentiation [12].

2. Materials and Methods

2.1. Plant

The leaves of four *Hiraea* species (*Hiraea cuiabensis*, *H. hatschbachii*, *H. reclinate*, and *H. restingae*) were collected from adult plants between 2012 and 2014 (Table 1). The species were identified by botanist R. F. de Almeida (School of Animal, Plant, and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa). For each species (Table 1), three batches of leaves were sampled.

Table 1. Plant samples analyzed.

***Hiraea* Leaves**	**Collection Herbarium (Voucher Number)**	**Local**	**Date (dd/mm/yyyy)**
H. cuiabensis	Francener (1218-SP)	Araguainha/MT-Brazil	29 December 2012
H. hatschbachii	Almeida (HUEFS)	Foz do Iguaçu/PR-Brazil	8 June 2013
H. reclinata	Pace (518-SPF)	Iquitos/Loreto-Peru	18 September 2014
H. restingae	Almeida (SP-518)	Soretano/ES-Brazil	20 January 2012

After collection, the leaves were dried at 40 °C for three days. The material was then stored in the HUEFS herbarium at 18 °C under constant air conditioning (24 h per day) until the extract was prepared.

2.2. Preparation of Extracts

The *H. cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* leaves were separately extracted with ethanol (Sigma-Aldrich, St. Louis, MO, USA) and water (Fair Lawn, NJ, USA) in a proportion of 4:1 (*v*/*v*), 20 mg of leaves to 1 mL of hydroethanolic solvent. Ethanol–water mixtures are well-established solvents for Malpighiaceae metabolomics [6,11]. The plant material and hydroethanolic solvent were homogenized (Qiagen TissueLyzer II, Qiagen, Hilden, Germany) at 25 MHz for 5 min and extracted again for 30 min. The extracts were centrifuged for 15 min, and 300 µL of the supernatant was collected. The solvent was dried in a CentriVap (Labconco®, Kansas City, MO, USA) and stored at −80 °C before UPLC-MS/MS analyses.

2.3. UPLC-MS/MS Analyses

The chromatographic analyses were carried out on an UltiMate 3000 UPLC system (Thermo Scientific®, Waltham, MA, USA) using a Kinetex (Phenomenex®, Torrance, CA, USA) 1.7 mm C_{18} reversed phase UPLC column (50 × 2.1 mm) and a Maxis Impact Q-TOF mass spectrometer Bruker Daltonics®, (Billerica, MA, USA). The mass spectrometer, equipped with an electrospray source and Q-TOF mass analyzer, was operated under the following conditions: source temperature of 200 °C, nitrogen gas used as the nebulizer at a pressure of 2 bar, and capillary voltage of 4200 V.

For UPLC-MS/MS analysis, the extracts were resuspended in 200 µL of methanol–water (4:1, *v*/*v*) and 2 µM sulfachloropyridazine (internal standard). Sulfachloropyridazine was used to monitor the injections, such as retention time [5]. The pump system used water (A) and acetonitrile (B), both acidified with 0.1% (*v*/*v*) formic acid, at a flow rate of 0.5 mL/min. Metabolite separation was performed starting with 5% solvent B for 1 min, followed by a linear gradient from 5% to 100% over 5 min. The column was then washed with 100% solvent B for 2 min, returned to the initial 5% over 1 min, and equilibrated at 5% solvent B for an additional 1 min. The spectra were acquired using data-dependent acquisition (DDA), with the most intense ions subjected to fragmentation MS/MS.

The UPLC-MS/MS profiles, such as the chromatogram shown in Figure 1, were processed using MZmine 2.53 with the following parameters: block mass correction using hexakis 1H,1H,2H-difluoroethoxyphosphazene (Synquest Laboratories, Alachua, FL, USA); mass detection (MS1 = 1.0×10^3 and MS2 = 1.0×10^1—centroid mode); peak deconvolution; minimum extension time 0.01 min; baseline level 1.0×10^3 and median *m*/*z* centering. The baseline and signal-to-noise ratio were adjusted for each ionization mode; therefore, each ionization mode was analyzed individually by VIP score (PLS-DA).

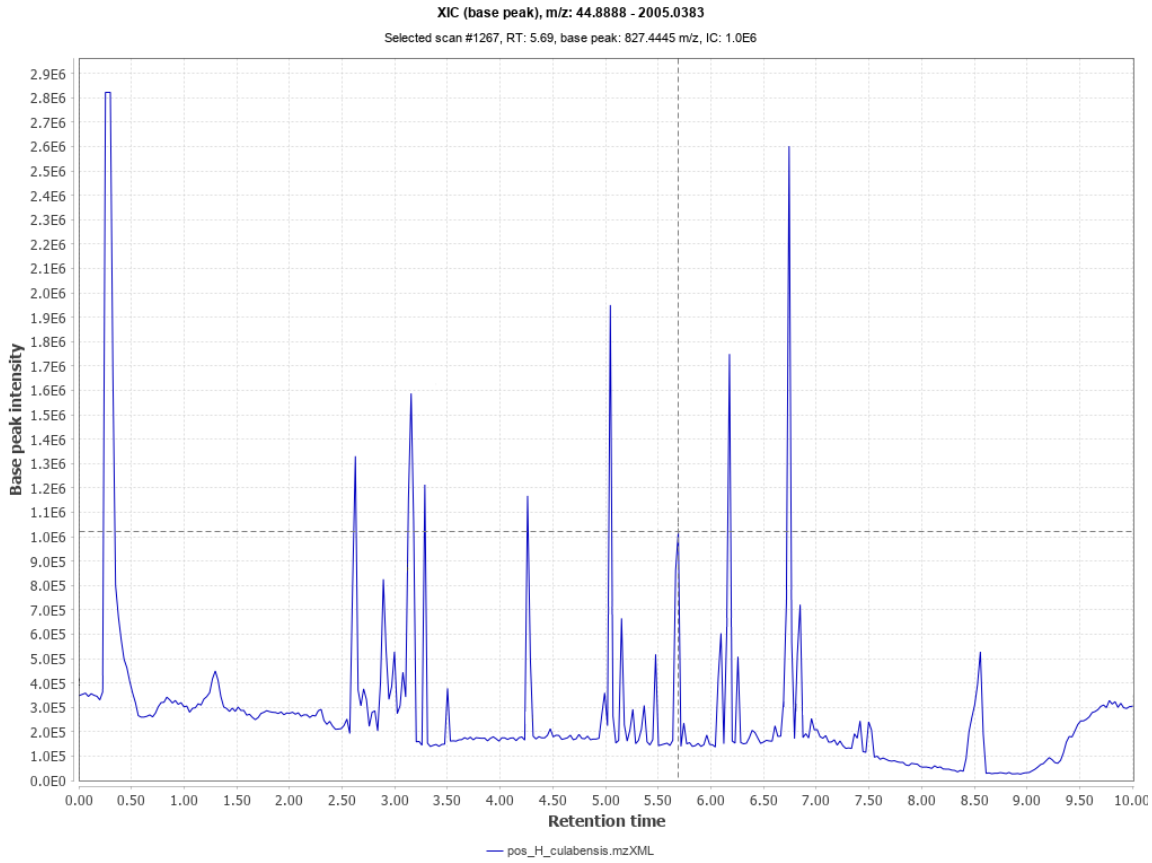

Figure 1. Original chromatogram of the hydroethanolic extract of *Hiraea cuiabensis* leaves obtained by UPLC-MS/MS (positive ionization mode).

The annotations were performed by comparing the obtained spectra with spectral libraries NIST and Massbank. The fragmentation patterns and m/z ratios of each detected ion were matched against these libraries. The confidence level of the annotated compounds was determined solely based on MS/MS data [13]. For each annotation, the error (ppm) was calculated using the exact mass obtained from Exact Mass Online. Only annotations with an error (ppm) below 7 were considered.

The original chromatographic profiles obtained by UPLC-MS/MS (positive and negative ionization mode) are available on the MassIVE platform (MSV000085119).

2.4. VIP Score (PLS-DA)

VIP score analysis was performed to differentiate the intensity secondary metabolites in the UPLC-MS/MS chromatographic profiles of hydroethanolic extracts of *H. cuiabensis*, *H. hatschbachii*, *H. reclinate*, and *H. restingae* leaves (Malpighiaceae), with 95% confidence. The chromatographic profiles were compiled into matrices, in which the columns correspond to the sample and the lines correspond to the chromatographic data (retention time and m/z value) for the fragments. A matrix was compiled for each ionization mode. The intensities of the compounds annotated in the UPLC-MS/MS profiles were added to the rows, and the samples were inserted into each column (three columns for each sample). The VIP score was performed in Metaboanalyst 6.0 software. The chromatographic profiles dataset was normalized by sum, transformed (log10), and auto-scaled.

To validate the VIP score model (PLS-DA), the Q2 and R2 parameters from cross-validation were provided by Metaboanalyst for each ionization mode obtained through UPLC-MS/MS analysis. Q2 is an estimate of the predictive ability of the model and is calculated via cross-validation (CV). In each CV, the predicted data are compared with the original data, and the sum of squared errors is calculated. The prediction error is then summed over all samples (Predicted Residual Sum of Squares or PRESS). The PRESS is divided by the initial sum of squares and subtracted from 1 to resemble the scale of the R2. Good predictions will have low PRESS or high Q2 (close to 1.0).

3. Results

Hydroethanolic extracts of *H. cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* were analyzed by UPLC-MS/MS for the annotation of secondary metabolites. Fifty compounds (in both positive and negative ionization modes) were annotated (Tables 2 and 3), including 45 metabolites not previously reported in these species. Eighteen compounds in positive ionization mode and twenty-seven in negative ionization mode, previously unreported in *H. cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae*, were annotated. The compounds annotated for the first time in *Hiraea* species are listed in Tables 2 and 3, indicated by an asterisk (*).

A total of eleven compounds (adenosine, 1-hexadecanoyl-2-glycero-3-phosphate, isoquercitrin, petunidin 3-galactoside, flavonol, rutin, pyrocatechuic acid, aeculetin, catechin, quercetin-3-*O*-beta-D-xylopyranoside, and hyperoside) were annotated in all *Hiraea* species, suggesting common phytochemical activities between species. However, some annotations were peculiar to each species. Therefore, VIP scores (PLS-DA) were applied separately for each ionization mode to identify the metabolites responsible for discriminating among the analyzed *Hiraea* leaves.

Table 2. Annotated compounds in the chromatographic profiles UPLC-MS/MS (positive ionization mode) of the *Hiraea cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* leaves. The base peak of each metabolite is highlighted in bold.

N°	Ion (*m*/*z*)	Exact Mass	Adduct	Error (ppm)	Fragment	Metabolites (Class)	Molecular Formula	R_t ** (min)	Leaves	VIP Score Values
1	265.1452	265.1439	$[M + H]^+$	4.90	155.1085, **142.1008**	Abscisic acid * (hormone)	$C_{15}H_{20}O_4$	2.34	*H. cuiabensis*; *H. hatschbachii*; *H. reclinata*	1.2780
2	268.1055	268.1045	$[M + H]^+$	3.72	**136.0627**, 119.0358, 108.0425	Adenosine (nitrogenous)	$C_{10}H_{13}N_5O_4$	0.38	All	0.4441
3	433.2312	433.2331	$[M + Na]^+$	−4.38	**158.0090**	1-Hexadecanoyl-2-glycero-3-phosphate * (glycerol)	$C_{19}H_{39}O_7P$	5.26	All	1.2552
4	435.0916	435.0927	$[M + H]^+$	−2.52	**303.0491**	Quercetin-3-*O*-pentoside (flavonoid)	$C_{20}H_{18}O_{11}$	3.12	*H. cuiabensis*; *H. hatschbachii*	1.2161
5	463.1221	463.1240	$[M]^+$	−4.10	**317.0641**	Peonidin-3-*O*-glucoside * (anthocyanin)	$C_{22}H_{23}O_{11}$	3.04	*H. hatschbachii*; *H. reclinata*; *H. restingae*	1.0280
6	465.1041	465.1033	$[M + H]^+$	1.72	**303.0521**	Isoquercitrin * (flavonoid)	$C_{21}H_{20}O_{12}$	2.82	All	0.2580
7	477.1366	477.1396	$[M + H]^+$	−6.28	**315.0838**	Cirsimarin * (flavonoid)	$C_{23}H_{24}O_{11}$	3.42	*H. restingae*	1.2780
8	480.1259	480.1267	$[M + H]^+$	−2.85	**318.0731**, 301.0704	Petunidin-3-galactoside * (anthocyanin)	$C_{22}H_{23}O_{12}$	3.02	All	- ***
9	480.1268	480.1267	$[M + H]^+$	0.35	318.0740, **317.0661**	Petunidin-3-*O*-glucoside * (anthocyanin)	$C_{22}H_{23}O_{12}$	3.31	*H. reclinata*	1.1070
10	491.1156	491.1189	$[M + H]^+$	−6.71	**295.0574**	Kaempferol-*O*-acetylhexoside * (flavonoid)	$C_{23}H_{22}O_{12}$	3.30	*H. cuiabensis*	0.2621

Table 2. *Cont.*

N°	Ion (*m*/*z*)	Exact Mass	Adduct	Error (ppm)	Fragment	Metabolites (Class)	Molecular Formula	R_t ** (min)	Leaves	VIP Score Values
11	493.1312	493.1346	$[M]^+$	−6.89	**329.0628**	Malvidin-3-*O*-galactoside * (anthocyanin)	$C_{23}H_{25}O_{12}$	3.19	*H. hatschbachii*	1.2161
12	551.1017	551.1037	$[M + H]^+$	−3.62	**303.0485**	Flavonol (flavonoid)	$C_{24}H_{22}O_{15}$	3.05	All	1.0077
13	565.1550	565.1557	$[M + H]^+$	1.23	325.1134, **287.0558**	Isoschaftoside * (flavonoid)	$C_{26}H_{28}O_{14}$	2.78	*H. reclinata*; *H. restingae*	0.4825
14	573.0847	573.0856	$[M + Na]^+$	−1.57	**308.1107**	Quercetin-3-*O*-malonylglucoside * (flavonoid)	$C_{24}H_{22}O_{15}$	3.04	*H. hatschbachii*	1.2161
15	595.1654	595.1663	$[M + H]^+$	−1.51	**287.0557**	Kaempferol-3-*O*-rutinoside (flavonoid) *	$C_{27}H_{30}O_{15}$	3.14	*H. reclinata*	1.0281
16	595.1672	595.1663	$[M]^+$	1.51	**287.0564**	Cyanidin-3-rutinoside * (anthocyanin)	$C_{27}H_{31}O_{15}$	3.26	*H. hatschbachii*	- ***
17	597.1461	597.1455	$[M]^+$	1.00	**301.0354**	Delphinidin-3-*O*-sambubioside * (anthocyanin)	$C_{26}H_{29}O_{16}$	3.92	*H. cuiabensis*	0.2621
18	609.1822	609.1819	$[M + H]^+$	0.49	303.0793, **301.0715**	Diosmetin-7-*O*-rutinoside * (flavonoid)	$C_{28}H_{32}O_{15}$	3.11	*H. cuiabensis*	- ***
19	611.1606	611.1612	$[M + H]^+$	0.98	**303.0509**	Rutin (flavonoid)	$C_{27}H_{30}O_{16}$	2.93	All	1.2944
20	627.1570	627.1561	$[M + H]^+$	1.43	**301.0352**	Myricetin-3-rutinoside * (flavonoid)	$C_{27}H_{30}O_{17}$	2.69	*H. cuiabensis*; *H. restingae*	- ***
21	649.2180	649.2191	$[M + H–H_2O]^+$	−1.69	149.0450, **148.0371**	Maltotetraose * (carbohydrate)	$C_{24}H_{42}O_{21}$	0.38	*H. cuiabensis*	1.0281
22	675.6782	675.6766	$[2M + H]^+$	2.36	**343.4278**, 329.4122	Docosenamide * (amide)	$C_{22}H_{43}NO$	6.82	*H. cuiabensis*; *H. hatschbachii*; *H. restingae*	- ***

* First reported in the analyzed *Hiraea* species; ** Retention time; *** Not significant for the discriminant analysis. Therefore, they have no value.

Table 3. Annotated compounds in the chromatographic profiles UPLC-MS/MS (negative ionization mode) of the *Hiraea cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* leaves. The base peak of each metabolite is highlighted in bold.

N°	Ion (*m*/*z*) [M–H]$^-$	Exact Mass	Error (ppm)	Fragment	Metabolites (Class)	Molecular Formula	R_t *** (min)	Leaves	VIP Score Values
1	153.0193	153.0187	3.92	**109.0284**, 108.0206	Pyrocatechuic acid (phenolic * compound)	$C_7H_6O_4$	1.24	All	- ****
2	177.0195	177.0187	4.51	176.0109, **106.0054**	Aesculetin * (flavonoid)	$C_9H_6O_4$	2.69	All	- ****
3	201.1138	201.1126	5.92	184.1111, **139.0759,** 137.0602	Decanedioic acid * (acids)	$C_{10}H_{18}O_4$	3.43	*H. hatschbachii*; *H. reclinata*	1.0431
4	204.0309	204.0296	6.37	161.0476, **160.0398**, 159.0320	Xanthurenic acid * (acids)	$C_{10}H_7NO_4$	1.26	*H. hatschbachii*	1.0431
5	210.0777	210.0766	5.23	148.0524, **124.0524**, 118.0054	Enicoflavine * (flavonoid)	$C_{10}H_{13}NO_4$	2.98	*H. hatschbachii*	1.0431
6	269.0463	269.0450	4.83	**245.1595**	Apigenin * (flavonoid)	$C_{15}H_{10}O_5$	4.19	*H. cuiabensis*; *H. reclinata; H. restingae*	1.0431
7	282.0852	282.0838	4.96	**150.0430**	Guanosine * (flavonoid)	$C_{10}H_{13}N_5O_5$	0.74	*H. reclinata*	0.9781
8	285.0410	285.0399	3.85	**284.0332**	Luteolin (flavonoid)	$C_{15}H_{10}O_6$	3.49	*H. reclinata*	0.6566
9	289.0726	289.0712	4.84	**125.0226**	Catechin * (flavonoid)	$C_{15}H_{14}O_6$	3.92	All	1.0431
10	325.0937	325.0923	4.30	**146.0316**	Coumaroyl hexoside * (phenolic glycoside)	$C_{15}H_{18}O_8$	2.59	*H. reclinata*; *H. restingae*	1.0431
11	351.0880	351.0868	3.41	**191.0539**	Pinastric acid * (acids)	$C_{20}H_{16}O_6$	1.61	*H. cuiabensis*; *H. hatschbachii*	1.0431
12	353.0883	353.0872	3.11	**191.0518**, 135.0418	Caffeoyl quinic acid * (phenolic compound)	$C_{16}H_{18}O_9$	1.46	*H. hatschbachii*; *H. reclinata*; *H. restingae*	1.0431
13	353.0886	353.0872	3.96	**191.0530**, 179.1326, 135.0426	Chlorogenic acid * (phenolic compound)	$C_{16}H_{18}O_9$	3.08	*H. cuiabensis*; *H. reclinata*; *H. restingae*	1.0436

Table 3. *Cont.*

N°	Ion (*m*/*z*) [M–H]$^-$	Exact Mass	Error (ppm)	Fragment	Metabolites (Class)	Molecular Formula	R_t *** (min)	Leaves	VIP Score Values
14	359.0787	359.0766	5.84	198.0892, 177.0187, **164.0109**	Rosmarinic acid * (phenolic compound)	$C_{18}H_{16}O_8$	3.53	*H. hatschbachii*; *H. reclinata*	1.2542
15	367.1045	367.1029	4.33	**196.0735**, 179.0708	3-*O*-feruloylquinic * acid (phenolic compound)	$C_{17}H_{20}O_9$	2.77	*H. hatschbachii*	1.0431
16	431.0996	431.0978	4.17	**283.0187**	Isovitexin (flavonoid)	$C_{21}H_{20}O_{10}$	3.74	*H. cuiabensis*	1.1601
17	433.0788	433.0770	4.15	303.0234302.0156, **301.0079**	Avicularin * (flavonoid)	$C_{20}H_{18}O_{11}$	4.02	*H. cuiabensis*	1.5502
18	433.0792	433.0770	5.07	**301.0080**	Quercetin-3-*O*-beta-D-xylopyranoside * (flavonoid)	$C_{20}H_{18}O_{11}$	3.55	All	1.0431
19	459.0944	459.0927	3.70	**177.0400**	Oroxindin * (flavonoid)	$C_{22}H_{20}O_{11}$	3.53	*H. hatschbachii*	0.1471
20	463.0889	463.0876	2.80	**301.0264**, 178.9916	Myricitrin * (flavonoid)	$C_{21}H_{20}O_{12}$	2.98	*H. cuiabensis*; *H. reclinata*	1.6217
21	463.0893	463.0876	3.67	**175.0256**	Hyperoside * (flavonoid)	$C_{21}H_{20}O_{12}$	3.69	All	0.6566
22	477.1048	477.1033	3.14	**313.0364**, 301.0364	Isorhamnetin-3-*O*-glucoside * (flavonoid)	$C_{22}H_{22}O_{12}$	3.60	*H. cuiabensis*; *H. reclinata*; *H. restingae*	0.7729
23	479.0826	479.0825	0.20	**478.0747**	Myricetin-3-*O*-beta-D-galactopyranoside * (flavonoid)	$C_{21}H_{20}O_{13}$	6.42	*H. restingae*	1.0431
24	515.1213	515.1189	4.65	**190.0472**, 174.0522	Dicaffeoyl quinic acid * (acids)	$C_{25}H_{24}O_{12}$	3.18	*H. hatschbachii*	0.1248
25	579.1463 **	579.1502	−6.73	**124.0312**	Epicatechin (flavonoid)	$C_{15}H_{14}O_6$	2.21	*H. reclinata*	- ****
26	623.1586	623.1612	−4.17	**317.0293**, 301.0348, 300.0270	Isorhamnetin-3-galactoside-6″-rhamnoside * (flavonoid)	$C_{28}H_{32}O_{16}$	3.46	*H. cuiabensis*; *H. hatschbachii*	0.5496
27	739.2062	739.2085	−3.11	597.9844, **596.5766**	Kaempferol-3-glucoside-2″-rhamnoside-7-rhamnoside * (flavonoid)	$C_{33}H_{40}O_{19}$	3.14	*H. cuiabensis*	0.1179
28	895.1954 **	895.1932	2.45	**447.0928**	Quercetin-3-*O*-rhamnoside * (flavonoid)	$C_{21}H_{20}O_{11}$	3.51	*H. cuiabensis*	1.0692

* First reported in the analyzed *Hiraea* species; ** [2M–H]$^-$; *** Retention time; **** Are not significant for the discriminant analysis. Therefore, they have no value.

The comparison of the profiles obtained by UPLC-MS/MS from the VIP score analysis (PLS-DA) is presented in Figure 2 (positive ionization mode) and Figure 3 (negative ionization mode).

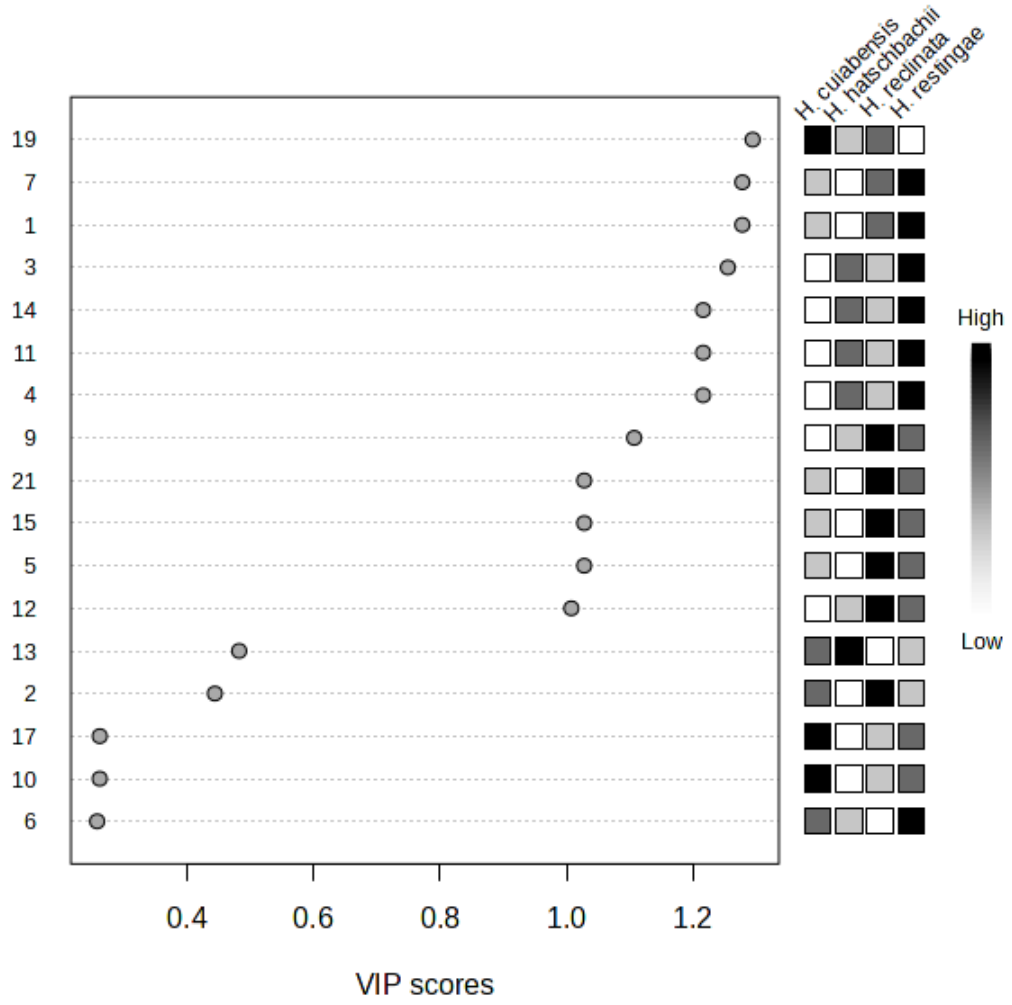

Figure 2. VIP score from the comparison between UPLC-MS/MS chromatographic profiles (positive ionization mode) of the leaves of *Hiraea cuiabensis*, *H. hatschbachii*, *H. reclinate*, and *H. restingae*. The numbers on the left correspond to the metabolites listed in Table 2, while the colored boxes on the right represent the relative intensities of each metabolite across the *Hiraea* samples.

Table 4 shows the Q2 and R2 values of the VIP score (PLS-DA), comparing the profiles generated by UPLC-MS/MS under both positive and negative ionization modes.

Table 4. Q2 and R2 values of the VIP score (PLS-DA) comparing the profiles UPLC-MS/MS (positive and negative ionization mode) of *Hiraea* species.

Measure	Positive Ionization Mode	Negative Ionization Mode
Q2	0.9987	0.9998
R2	0.9991	0.9997

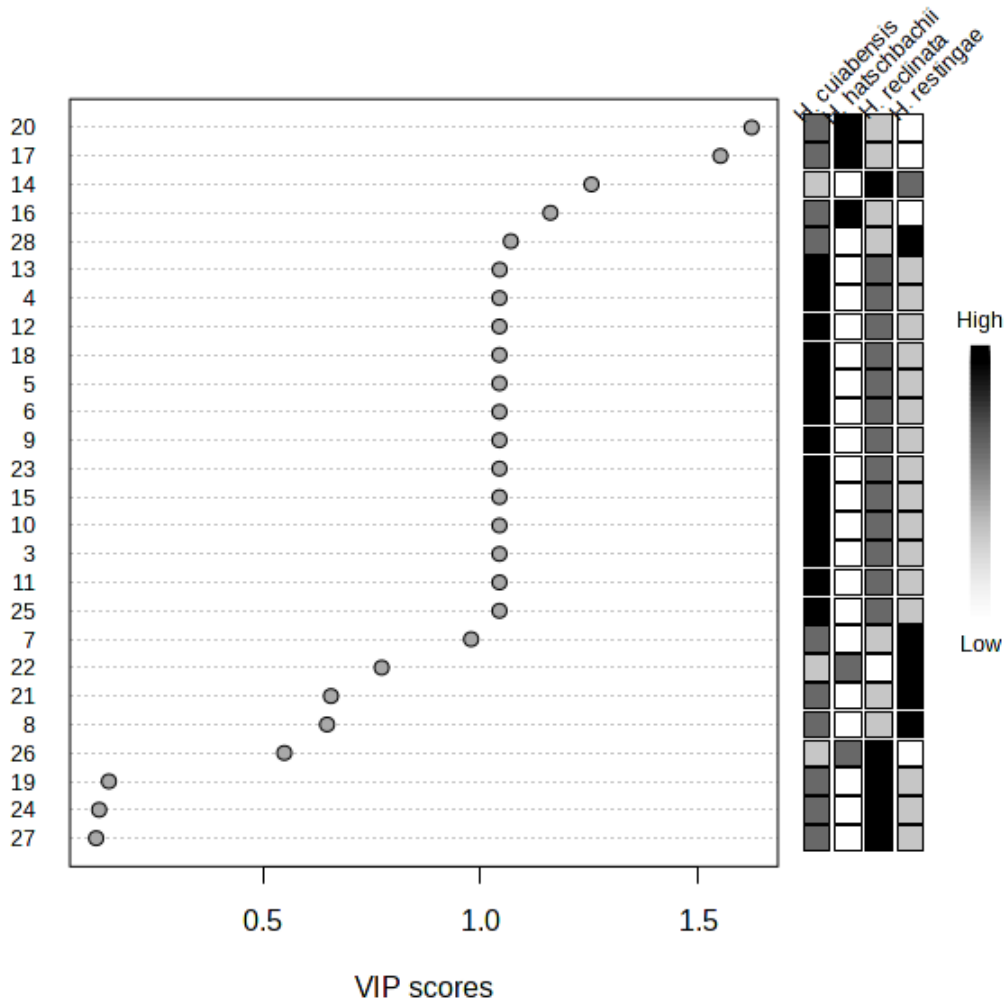

Figure 3. VIP score from the comparison between UPLC-MS/MS chromatographic profiles (negative ionization mode) of the leaves of *Hiraea cuiabensis*, *H. hatschbachii*, *H. reclinate*, and *H. restingae*. The numbers on the left correspond to the metabolites listed in Table 3, while the colored boxes on the right represent the relative intensities of each metabolite across the *Hiraea* samples.

4. Discussion

Twenty-two compounds were annotated in the UPLC-MS/MS profiles (positive ionization mode). Table 2 shows compounds annotated in *Hiraea* leaves. The metabolites annotated in the UPLC-MS/MS profiles suggest biological, medicinal, and ecological activities in *Hiraea* leaves. Despite the absence of phytochemical evaluations and screening in the leaves of *H. cuiabensis*, *H. hatschbachii*, *H. reclinate*, and *H. restingae,* we found in the literature biological and chemical properties related to the annotated compounds (Table 2).

Abscisic acid was annotated in *H. cuiabensis*, *H. hatschbachii*, and *H. reclinata* (compound **1**—Table 2).

Abscisic acid is a plant hormone present in several plants and is one of the foremost antiviral defense compounds in plants [14]. In addition to immunological action, abscisic acid regulates plant responses in dry environments and abiotic stresses, such as salt and temperature stress [14,15]. Thus, this annotation suggests some abiotic stress or a need for viral defense in these leaves.

Cirsimarin was annotated only in the leaves of *H. restingae* (compound **7**–Table 2). Cirsimarin is a flavone found in other plants, such as the leaves of *Banisteriopsis laevifolia* (Malpighiaceae), leaves and stems of *Abrus precatorius* (Fabaceae), and *Teucrium arduini* (Lamiaceae) [4,16,17]. Among the reported activities, antibacterial was attributed to cirsi-

marin, with a potent inhibition against the bacteria *K. pneumonia* and *B. subtilis* [17]. Thus, biological activities that have not yet been reported may be associated with *H. restingae*.

Petunidin-3-*O*-glucoside (compound **9**—Table 2) was annotated only in *H. reclinate*. The presence of anthocyanins in Malpighiaceae has been reported in the literature for different species. Petunidin-3-glucoside, petunidin-3-acetylglucoside, and petunidin-3-p-coumaroylglucoside were annotated in *Byrsonima* (Malpighiaceae) [5]. The presence of anthocyanins in plants enhances their resistance to dry environments, increasing their tolerance to water scarcity [18]. Petunidin 3-galactoside (compound **8**—Table 2) was annotated in all *Hiraea* leaves, suggesting similarities between species of the genus *Hiraea* and other genera.

The annotated flavonoids, such as isoquercetin, flavonol, isoschaftoside, rutin, and myricetin-3-rutinoside are also reported in other genera of Malpighiaceae such as *Banisteriopsis*, *Bunchosia*, *Byrsonima*, *Camarea*, and *Heteropterys* [4,6]. Flavonoids act as sun filters on leaves, also supporting plant growth and development in abiotic environments [19]. Additionally, the antioxidant potential of plants is attributed to flavonoids [20]. Therefore, in addition to ecological activities, the annotation of flavonoids also suggests phytochemical activities.

The UPLC-MS/MS profiles (positive ionization mode) were subjected to VIP score analysis (PLS-DA) to verify the differences in the intensities of the compounds annotated in the *Hiraea* leaves. The objective of the VIP score analysis was to discriminate among *Hiraea* species and to compare the intensities of these metabolites across the four species.

The VIP score (PLS-DA) showed a difference between the intensities of the compounds annotated in *Hiraea* leaves (95% confidence). For each compound (y-axis), a VIP score value (x-axis) was calculated. The highest VIP score value is assigned to the most critical metabolites in the discrimination of *Hiraea* species (Figure 2). The color of the boxes in the VIP score plot (Figure 2) classifies metabolite intensities as high and low. Therefore, we confirmed a discrimination of the annotated compounds based on their intensities and VIP score values > 1.2. Thus, the intensity of the annotated compounds discriminates among *H. cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae* species.

The most important metabolites for discrimination showed VIP score values > 1.2. Thus, the discriminating metabolites among the four *Hiraea* species analyzed (positive ionization mode) were as follows: 19 (rutin), 7 (cirsimarin), 1 (abscisic acid), 3 (1-hexadecanoyl-2-glycero-3-phosphate), 14 (quercetin-3-*O*-malonylglucoside), 11 (malvidin-3-*O*-galactoside), and 4 (quercetin-3-*O*-pentoside), respectively (Figure 2).

Rutin, the compound with the highest VIP score, was annotated in all *Hiraea* leaf samples, showing variation in relative intensity among them. Although present in all leaves, rutin exhibited the highest intensity in *H. cuiabensis*, suggesting notable phytochemical activity. Rutin and its derivatives are well known for their antioxidant properties [6].

Compounds **7**, **1**, **3**, **14**, **11**, and **4** exhibit higher relative intensities in *H. restingae*. These secondary metabolites belong to various classes, including acids, glycerol derivatives, anthocyanins, and flavonoids (Table 2). According to the literature, these compounds play multiple ecological and phytochemical roles, such as adaptive responses to dry environments (abscisic acid), as well as antibacterial and antioxidant activities (cirsimarin) [14,17]. The chemical and biological activities of compounds **3**, **14**, **11**, and **4** (Table 2) remain underreported in the current literature. However, the high abundance of these secondary metabolites in *H. restingae* leaves suggests promising bioactivities, highlighting the need for further investigation in natural product research, particularly through comprehensive phytochemical screening.

Additionally, all metabolites annotated by UPLC-MS/MS in positive ionization mode, along with their respective VIP scores, are summarized in Table 2 and visualized in Figure 2.

Twenty-eight compounds (negative ionization mode) were annotated in the UPLC-MS/MS profiles (Table 3). Some acids found in *Hiraea* leaves, such as caffeoyl quinic, rosmarinic, xanthurenic, and chlorogenic, exert physiological, therapeutic, antioxidant and antifungal functions [21,22].

Apigenin, isovitexin, catechin, and epicatechin were also annotated in other leaves of Malpighiaceae, especially in the genus *Banisteriopsis* (*Banisteriopsis laevifolia*, *B. malifolia*, and *B. stellaris* leaves) [4]. Studies associate these compounds with antioxidant activities [6]. Although the secondary metabolites identified in *H. reclinata* leaves are predominantly flavonoids [6], this study reports novel annotations within the genus *Hiraea*, including phenolic compounds, glycosides, and tryptophan derivatives (Table 3).

The VIP score of UPLC-MS/MS chromatographic profiles in negative ionization mode showed three discriminant compounds (VIP score > 1.2) as follows: 20 (myricitrin), 17 (avicularin), and 14 (rosmarinic acid) (Figure 3).

Myricitrin (compound **20**—Table 3) decreases traumatic injury of the spinal cord and exhibits antioxidant and anti-inflammatory activities [23]. Avicularin (compound **17**—Table 3) is a bioactive flavonoid reported to inhibit oxidative stress and the release of pro-inflammatory cytokines. Due to its combined antioxidant and anti-inflammatory properties, avicularin has demonstrated neuroprotective effects against amyloid beta-induced neurotoxicity. Furthermore, it has been shown to mitigate memory impairment associated with amyloid beta-induced Alzheimer's disease [24].

The highest intensities of myricitrin and avicularin were detected in the leaves of *H. hatschbachii*. No chemical or biological activities have been previously reported in the literature for *H. hatschbachii*, nor have the secondary metabolites identified in this study been previously annotated in this species. Therefore, *H. hatschbachii* may possess unexplored medicinal and therapeutic potential.

Rosmarinic acid (compound **14**—Table 3) commonly found in *Boraginaceae* and *Lamiaceae* species, is a bioactive compound with antiviral, antibacterial, anti-inflammatory, and antioxidant properties, contributing to the medicinal and protective functions of plants [25]. The highest relative intensities of this metabolite were detected in the leaves of *H. reclinata* (Figure 3). Although the only phytochemical activity reported for *H. reclinata* to date is anti-HIV activity [6], the presence of this metabolite suggests potential additional roles, including unexplored medicinal and cosmetic applications.

Other metabolites detected by UPLC-MS/MS in negative ionization mode, along with their corresponding VIP scores, are presented in Table 3 and illustrated in Figure 3.

5. Conclusions

This study successfully annotated and compared the metabolomic profiles of four *Hiraea* species (*H. cuiabensis*, *H. hatschbachii*, *H. reclinata*, and *H. restingae*) leading to the annotation of 50 secondary metabolites, 45 for the first time in the genus. VIP score analysis (PLS-DA) revealed significant variations in metabolite intensities among species, with rutin and myricitrin identified as key discriminatory compounds. These findings contributed to an understanding of the chemical diversity within *Hiraea*, suggesting possible ecological adaptations and potential pharmacological applications.

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Abbreviations

The following abbreviations are used in this manuscript:

H.	*Hiraea*
PLS-DA	Discriminant Analysis-Partial Least Squares
UPLC-MS/MS	Ultra-performance Liquid Chromatography coupled to Mass Spectrometry
VIP	Variable Importance in Projection

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