http://dx.doi.org/10.21577/0103-5053.20190209

Article

J. Braz. Chem. Soc., Vol. 00, No. 00, 1-7, 2019
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Minutifloroside, a New Bis-Iridoid Glucoside with Antifungal and Antioxidant Activities and Other Constituents from *Palicourea minutiflora*

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A new bis-iridoid glucoside minutifloroside (**1**) was isolated from *Palicourea minutiflora*, together with asperuloside, (−)-epicatechin, catechin, quercetin, rutin, ursolic acid, oleanolic acid, daucosterol and two monoterpenic indole alkaloids strictosidinic acid and vincosamine. Structural characterization of the compounds was established on their spectral data basis, mainly mass spectrometry (MS) and 1D and 2D nuclear magnetic resonance (NMR). The bis-iridoid showed high activity against *Candida albicans* strain and antioxidant activity.

Keywords: *Palicourea minutiflora*, Rubiaceae, bis-iridoid, alkaloids, antifungal activity

Introduction

Iridoids are monoterpenoids found in plenty of plants families and are present in a number of folk medicinal species.[1,2] These bioactive metabolites were considered chemotaxonomic markers in the Rubiaceae family and exhibit remarkable biological and pharmacological properties such as neuroprotective, antitumor, anti-inflammatory, antiviral, antibacterial, antifungal, antioxidant, antiprotozoal and antiallergic.[3,4]

The presence of dimer iridoids was found in species of Rubiaceae, mainly those belonging to the genus *Saprosma*, *Paederia*, *Mussaenda*, *Lasianthus*, *Randia* and *Asperula*.[5]

The genus *Palicourea* (Rubiaceae) is taxonomically complex and previous phytochemical studies demonstrate the remarkable presence of quinolinic and monoterpenoid indole alkaloids,[6-8] flavonoids,[9] coumarins[10] and terpenoids.[11]

Therefore, as part of the investigative efforts to find compounds from *Rubiaceae* of the Northeastern Brazil flora, this work reports the first chemical study of aerial parts from *Palicourea minutiflora*, endemic species of Atlantic Forest from Brazil. The MeOH extract was investigated resulting in the isolation and structural elucidation of a new bis-iridoid glucoside, minutifloroside (**1**), and the known iridoid asperuloside (**2**), the two monoterpenic indole alkaloids strictosidinic acid (**3**) and vincosamine (**4**), the flavonoids (−)-epicatechin, catechin, quercetin, and rutin, along with ursolic acid, oleanolic acid and daucosterol. The isolation of the vincosamine (**4**) and (−)-epicatechin has been reported for the first time in genus *Palicourea*. This paper deals with the isolation and structure elucidation of the new compound and the assessment of antifungal and antioxidant activities.

Experimental

General experimental procedures

Column chromatography (CC) was performed either over silica gel 60 (Merck, 70-230 mesh) or Sephadex LH-20 (Sigma-Aldrich). Analytical thin layer chromatography (TLC) was performed on precoated silica gel plates (TLC Silica gel $60F_{254}$ from Merck) and the compounds were

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detected by UV light (250 and 366 nm) and by spraying with chromogenic agents including *p*-anisaldehyde-H_2SO_4 and H_2SO_4:MeOH solution, followed by heating at 150 °C or by spraying with Dragendorff solution.

Optical rotations were measured in H_2O in a PerkinElmer 343 digital polarimeter at 20 °C and 589 nm, with an optical cell path of 10 mm. High-resolution electrospray ionization Fourier transform mass spectrometry (ESI-FT-MS) data were performed using a Q-Exactive system (Thermo Fischer Scientific) constituted of a heated electrospray ionization (HESI-II) probe source and a hybrid mass analyzer, quadrupole-Orbitrap. The samples mass spectra were acquired in negative mode. The extract solution was injected by direct infusion with a flow rate of 10 μL min^{-1}. The experimental parameters to ESI(−)-FT-MS analysis were as follows: spray voltage of 4.5 kV, capillary temperature of 300 °C and s-lens of 70 V. For collision, it was applied high collision dissociation (HCD) of 10 and 20 eV. The Xcalibur 3.0.63 software (Thermo Fisher Scientific) was used to acquire and process the data. The exact mass from the *m/z* of each ion was compared with theoretical *m/z* to assign the molecular formula with an error less than 5 ppm. ESI(+)-MS data was acquired using a Premier XE triple quadrupole mass spectrometer (Waters Co.) running in the positive and negative ion mode. Major ESI(+)- and ESI(−)- S source parameters were as follow: capillary voltage of 2.0-3.5 kV, cone voltage of 10-45 V and the quadrupole was set to unitary resolution.

The nuclear magnetic resonance (NMR) spectra were recorded on a Varian-Mercury plus spectrometer operating at 300.06 (^{1}H) and at 75.45 MHz (^{13}C), respectively, and D_2O as solvent.

Plant material

The aerial parts (leaves and branches) of *P. minutiflora* were collected in January 2014, at the Reserva Particular do Patrimônio Natural Serra Bonita (Camacan, Bahia State, Northeast of Brazil, geographical coordinates 15°23’30”S; 39°33’55”W). Voucher specimens (No. 141.214) were deposited at the Centro de Pesquisa do Cacau (CEPEC) and identified by Dr A. M. Amorim (Universidade Estadual de Santa Cruz) and authenticated by C. M. Taylor (Missouri Botanical Garden, MO).

Extraction and isolation

Dried powdered aerial parts (600.0 g) were extracted by maceration with MeOH at room temperature and concentrated under vacuum to yield 85.3 g of MeOH crude extract. Part of MeOH crude extract (10.0 g) was dissolved in a mixture of MeOH:H_2O (1:1 v/v) and then successively partitioned with different solvents to give *n*-hexane (HF, 0.96 g), $CHCl_3$ (CF, 0.12 g), EtOAc (AcF, 3.95 g) and the remaining hydromethanolic (HMF, 4.97 g) fractions.

The HMF fraction (3.0 g) was subjected to column chromatography on Sephadex LH-20, using MeOH as eluent to obtain 201 fractions, which after TLC analysis were pooled into eight subfractions (HMF_1-HMF_8). HMF_7 was also submitted to successive Sephadex LH-20 columns, by elution with MeOH leading to the isolation of compound **3** (16.1 mg). The subfraction HMF_8 (521.5 mg) was applied to a Sephadex LH-20 column eluted with MeOH yielding 7 subfractions ($HMF_{8.1}$-$HMF_{8.7}$). From fraction $HMF_{8.2}$, it was obtained a precipitate, compound **1** (2.0 mg) as amorphous brown solid.

The AcF fraction (3.05 g) was chromatographed on a Sephadex LH-20 column eluted with MeOH to give 255 fractions, which were grouped into 12 fractions after TLC analysis (AcF_1-AcF_{12}). AcF_1 (310.5 mg) was submitted to CC over silica gel 60 (70-230 mesh) and eluted with a gradient of $CHCl_3$:MeOH yielding 7 subfractions ($AcF_{1.1}$-$AcF_{1.7}$). From $AcF_{1.6}$ it was obtained the quercetin (5.6 mg). The fraction AcF_3 (219.2 mg) was submitted to purification on preparative TLC ($CHCl_3$:MeOH:H_2O, 7:2:1) to afford compound **2** (6.2 mg). The AcF_5 and AcF_6 fractions (496.9 and 691.6 mg) were subjected on Sephadex LH-20 column eluted with MeOH to give 99 and 98 fractions each. The fractions were combined based on their TLC profile into 7 subfractions, respectively ($AcF_{5.1-7}$ and $AcF_{6.1-7}$). Subfractions $AcF_{5.3}$ and $AcF_{6.5}$ showed precipitates, which were washed with $CHCl_3$ and provided the flavonoids (−)-epicatechin (5.5 mg), catechin (2.0 mg) and rutin (12.0 mg). Subfraction $AcF_{6.7}$ (114.3 mg) was analyzed by preparative TLC ($CHCl_3$:MeOH:H_2O, 6:3:1) and this procedure isolated the compound **4** (6.3 mg).

The HF fraction (0.96 g) was fractionated by CC over silica gel eluted with *n*-hexane and mixtures of *n*-hexane/EtOAc in order of increasing polarity (Hex, Hex:EtOAc 10-90%) providing the steroid daucosterol (15.2 mg).

Antifungal assay

For susceptibility testing, we used the broth microdilution method according to the standards of the Clinical and Laboratory Standards Institute (M27-A3),[12] with some modifications for natural products.[13] The experiment was performed with standard strains: *Candida albicans* (ATCC 90028) and *C. glabrata* (ATCC 90030). The final cellular density of the yeast was adjusted to 0.5-5 × 10^3 colony-forming units (CFU) mL^{-1} in RPMI (Roswell Park Memorial Institute, Gibco) with L-glutamine

(without sodium bicarbonate) and 0.165 M 3-(*N*-morpholino) propanesulfonic acid (pH 7.2). The test was performed in flat-bottom 96-well microtiter plates (Techno Plastic Products, Switzerland). For the assay with compound **1**, we tested concentrations of 1250 to 9.765 μg mL^{-1} and fluconazole (Sigma) was used as reference antifungal drug. The plates were incubated at 35 °C for 48 h. The minimum inhibitory concentration (MIC) of compound **1** was considered the lowest concentration at which no fungal growth was evident, by visual reading.

Antioxidant assay

The radical scavenging capacity of the bis-iridoid (**1**) was investigated from their ability to reduce the radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) by TLC bioautography analysis.[14] The experiment was performed with Macherey-Nagel precoated silica gel 60 F254 plates (Düren, Germany) as the stationary phase and $CHCl_3$:MeOH:H_2O (6:3:1) as the mobile phase. After application of the compound **1** (1.0 mg mL^{-1}) and development, the plate was immersed for 2 s in 0.25% (m/v) $DPPH^{\bullet}$ methanolic solution. The antiradical activity results appeared as yellow spots against the purple-blue background. The flavonoid rutin was used as positive control.[15]

Minutifloroside (1)

HRESIMS *m/z*, calcd. for $C_{32}H_{14}O_{21}$ $[M-H]^-$: 761.2140, found: 761.2147; $[\alpha]_D^{20}$ = –65.0° (*c* 0.002, H_2O); ^1H NMR (300.06 MHz, D_2O) and ^{13}C NMR (75.45 MHz, D_2O), see Table 1. Precursor ion of *m/z* 761.21472 fragmented to the product ions of *m/z* 717.2242, 419.1190, 389.1084, 227.0556, 209.0450, and 183.0657 (see Figures S8 and S9, Supplementary Information (SI) section).

Results and Discussion

Iridoids glucosides minutifloroside (**1**) and asperuloside (**2**),[16] indole alkaloids strictosidinic acid (**3**)[17] and vincosamine (**4**)[18] (Figure 1), along with (−)-epicatechin,[19] catechin,[20] quercetin,[21] rutin,[22] ursolic acid,[23] oleanolic acid,[24] and daucosterol,[25] were isolated from the aerial parts extract of *Palicourea minutiflora*. The structures of known compounds were identified and elucidated using a combination of spectroscopic techniques (^1H, ^{13}C NMR and 2D NMR) and by comparisons with literature data.[16-25]

The structure of the new compound **1** was elucidated by spectrometric methods, including 1D and 2D NMR experiments and HRESIMS.

Compound **1** was isolated as a brown amorphous powder, $[\alpha]_D^{20}$ = –65.0° (H_2O), and the molecular formula was assigned as $C_{32}H_{14}O_{21}$, based on its negative ion HRESIMS, through the precursor ion peak of *m/z* 761.21472 $[M-H]^-$.

The MS/MS spectra (Supplementary Information Figures S8 and S9) for *m/z* 761.21472 presented four main pathways. The first two pathways proposed (Scheme 1) the formation of the ions of *m/z* 717.2242 ($[M-H-(CO_2)]^-$) (CO_2, 43.9898) and of *m/z* 419.1190 ($[M-H-(C_{15}H_{18}O_9)]^-$) ($C_{15}H_{18}O_9$, 342.0951), that were formed from the decarboxylation of the deprotonated molecule, and a hydroxyl group rearrangement, respectively. The hydroxyl group rearrangement was proposed as an analogous mechanism to dehydration reactions by eliminating a water molecule and forming double bond.[26] The other two pathways (Scheme 2), that formed the ions of *m/z* 389.1084 ($[M-H-(C_{16}H_{20}O_{10})]^-$) ($C_{16}H_{20}O_{10}$, 372.1056) and of *m/z* 227.0556 ($[M-H-(C_{22}H_{30}O_{15})]^-$) ($C_{22}H_{30}O_{15}$, 534.1585), were proposed toward neutral loss of the molecule $C_{16}H_{20}O_{10}$ by typical remote hydrogen rearrangement,[26-27] and the formation of the ion *m/z* 227.0556 ($[C_{10}H_{11}O_6]^-$) corresponding to regular profile observed in study of iridoid glycosides fragmentation forming epoxide moiety of the neutral molecule $C_{22}H_{30}O_{15}$ (534.1585) (Scheme 2).[28] It was also observed that the ion of *m/z* 389.1084 ($[C_{16}H_{21}O_{11}]^-$) fragmented by water loss to the ion of *m/z* 371.0978.[26,28] The fragment of *m/z* 227.0556 may be formed from the precursor ion and the product ion of *m/z* 389.1084.[28,29] This ion presents several fragments, providing more information of the structure resulting in two possible ions pathways, one by its decarboxylation resulting in the ions of *m/z* 183.0657 ($[C_9H_{11}O_4]^-$) and the other fragment of *m/z* 209.0450 ($[C_{10}H_9O_5]^-$) was formed by elimination of a water.[28]

The ^1H and ^{13}C NMR spectra indicated the presence of the signals of two distinct moieties of an iridoid glucoside structure and suggested this compound is a bis-iridoid glucoside which is hereafter referred to as units A and B (Table 1). The ^1H NMR spectrum showed signals at δ 7.66 (brs, H-3), 4.99 (d, *J* 8.8 Hz, H-1), 4.88 (m, H-6), 6.03 (brs, H-7), 4.47 (d, *J* 15.9 Hz, H-10a), 4.27 (d, *J* 15.9 Hz, H-10b) of unit A, based on analysis of the heteronuclear multiple quantum correlation (HMQC), ^1H-^1H correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) spectra. Signals at δ 4.86 in the ^1H NMR and at δ 101.9 in the ^{13}C NMR spectra stand for the terminal group of the sugar. In the nuclear Overhauser effect spectrum (NOESY), a signal at δ 2.66 (H-9) correlated with 4.99 (H-1), 3.11 (H-5) and 4.88 (H-6) indicating that these bonds have the same *cis*-configuration. The moiety (unit A) was established by

Table 1. NMR spectroscopic data (300.06 and 75.45 MHz, D_2O) for minutifloroside (**1**)

Position	δ_H (mult., *J* in Hz)	δ_C	COSY	HMBC
Unit A				
1	4.99 (d, 8.8)	103.7	2.66	–
3	7.66 (brs)	157.6	3.11	43.6, 103.7, 110.5, 175.9
4	–	110.5	–	–
5	3.11 (dd, 6.5, 6.5)	43.6	2.66, 7.66	103.7, 110.5, 131.8
6	4.88 (m)	77.0	–	131.8, 152.2
7	6.03 (brs)	131.8	–	43.6, 47.3, 77.0
8	–	152.2	–	47.3, 63.1
9	2.66 (dd, 8.1, 8.1)	47.3	3.11, 4.99	77.0, 103.7, 131.8, 152.2
10	4.47 (d, 15.9) 4.27 (d, 15.9)	63.1	– –	131.8, 152.2 –
11	–	175.9	–	–
1’	4.86[a]	101.9	3.38	103.7
2’	3.38[a] (m)	75.8	4.86	78.6
3’	3.51[a] (m)	78.6	–	72.4
4’	3.41[a] (m)	72.4	–	79.0
5’	3.42[a] (m)	79.0	–	–
6’	3.77[a] (m)	63.8	–	66.4
Unit B				
1	5.26 (d, 5.9)	99.9	3.14	48.7, 101.6, 154.1
3	7.40 (brs)	154.1	3.04	46.5, 99.9, 114.5
4	–	114.5	–	–
5	3.04 (m)	46.5	3.14, 7.40	48.7, 99.9, 131.5, 114.5, 154.1
6	4.61 (m)	83.4	–	83.4, 48.7, 62.4, 83.4
7	5.84 (brs)	131.5	–	62.4
8	–	148.5	–	99.9
9	3.14 (m)	48.7	3.04, 5.26	–
10	4.31-4.25 (d, 15.0)	62.4	–	–
11	–	175.9	–	75.6, 99.9
1”	4.79[a]	101.6	3.33	78.7
2”	3.38 (d, 9.0 and 8.0)	75.6	4.79	72.4
3”	3.51[a] (m)	78.7	–	79.1
4”	3.41[a] (m)	72.4	–	–
5”	3.42[a] (m)	79.1	–	72.4
6”	4.02-3.70[a] (m)	66.8	–	–

[a]Overlapped signals. COSY: correlation spectroscopy; HMBC: heteronuclear multiple bond correlation.

comparing their spectroscopic data with those reported for deacetylasperulosidic acid.[30]

The remaining spectral data revealed a second iridoid unit (part B) of the new bis-iridoid. Signals at δ 7.40 (brs, H-3), 5.26 (d, *J* 5.9 Hz, H-1), 4.61 (m, H-6), 5.84 (brs, H-7), 4.31 and 4.25 (d, *J* 15.0, H-10a,b). The terminal group of the sugar was revealed by signals at δ 4.79 in the ^{1}H NMR and at δ 101.6 in the ^{13}C NMR spectra. In the NOESY spectrum, a signal at δ 3.14 (H-9) correlated with 5.29 (H-1) and 3.04 (H-5) indicating the *cis*-junction between the two rings and the *O*-glycosyl residue C-1” with a β configuration. The ^{1}H and ^{13}C NMR data of the moiety indicated signals similar to those of scandoside,[30] and further confirmed by the detailed analyses of ^{1}H-^{1}H COSY and HMBC spectra. Further proof of the linkage was obtained from the HMBC correlations between H-6’ at δ 3.77 (m) of the unit A and C-6” δ 66.8 of precoated unit B, and by extensive analysis in mass (MS/MS) experiment.

The compound **1** was assayed for their antifungal activities against the yeasts *C. albicans* and *C. glabrata* by broth microdilution method. This compound exhibited the highest antifungal activity in *C. albicans* than *C. glabrata* species. The MIC was 9.765 µg mL^{-1} for *C. albicans* and killed at *C. glabrata* at 1250 µg mL^{-1}.

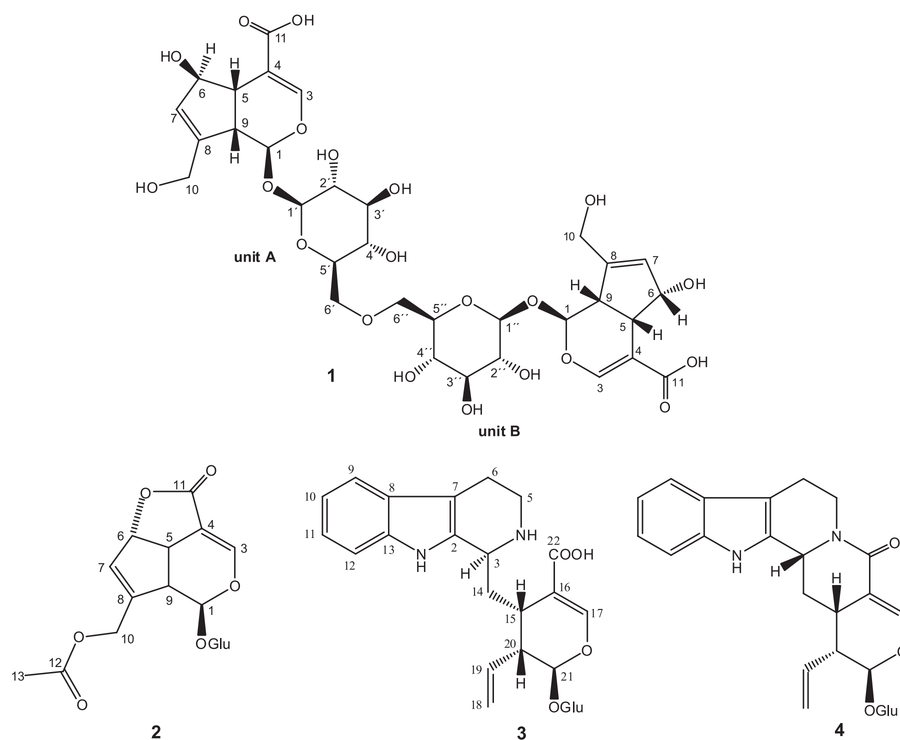
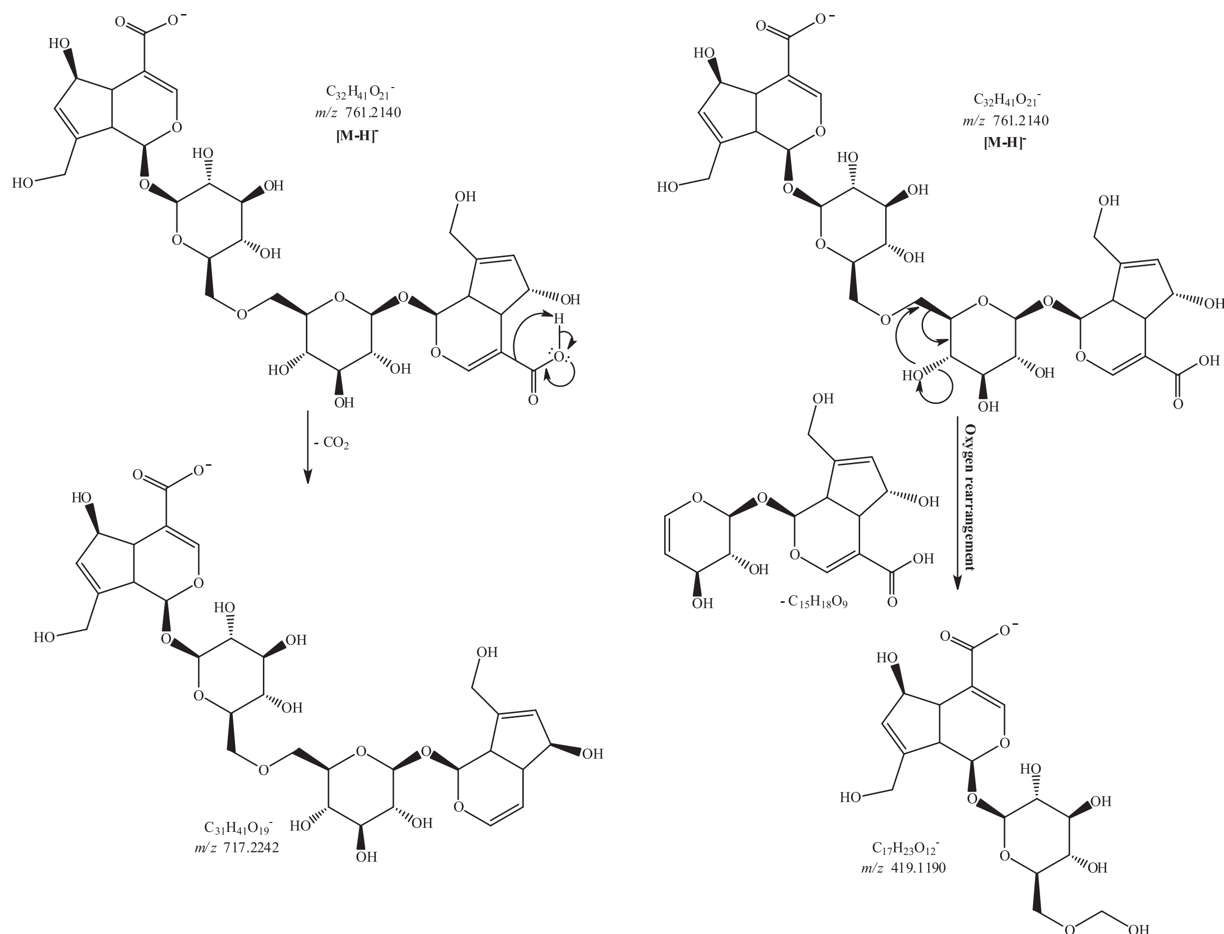
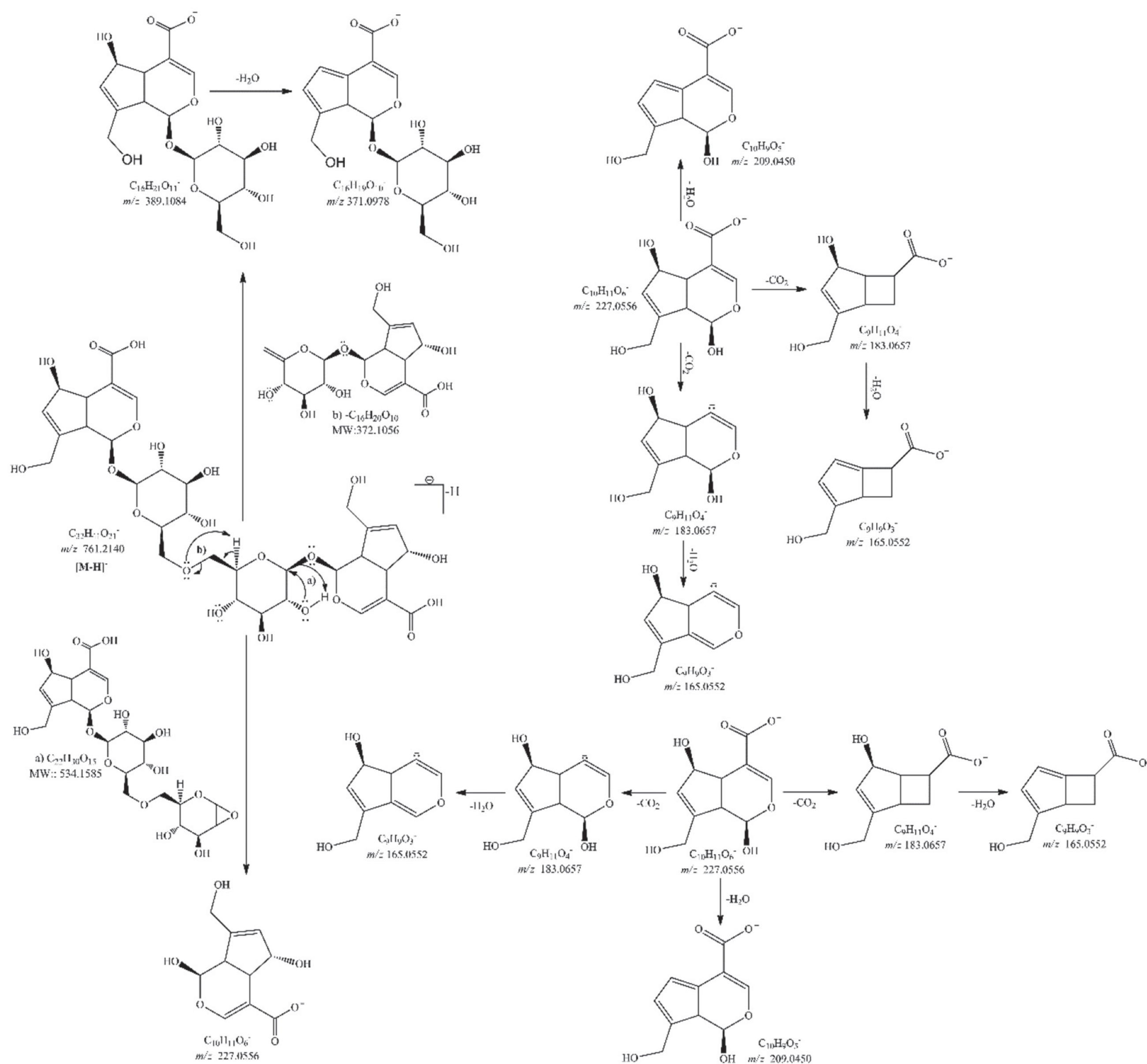


Figure 1. Chemical structure of compounds **1-4** isolated from aerial parts of *Palicourea minutiflora*.



Scheme 1. Proposed fragmentation mechanism for precursor ion and two fragments for compound **1**.



Scheme 2. Proposed fragmentation mechanism for precursor ion and two fragments for compound **1**.

The capacity to scavenge DPPH$^•$ radical using TLC bioautography method was also carried out with compound **1**.[14] The antioxidant activity was positive for compound **1** at 1 mg mL^{-1} by visualization of a yellow spot against the purple background.

Conclusions

In summary, in this study two monoterpenic indole alkaloids, four flavonoids and three terpenoids were isolated from the methanolic extract from *P. minutiflora* Müll. Arg., including asperuloside and a novel bis-iridoid, minutifloroside. This bis-iridoid exhibited high antifungal activity against *C. albicans* and showed antioxidant capacity.

Supplementary Information

1D and 2D NMR spectra for compound **1** are available online free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

We acknowledge the Coordenação de Aperfeiçoamento de Nível Superior (CAPES) for financial support.

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Submitted: February 25, 2019
Published online: September 2, 2019

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