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Studies on the Constituents of a Brazilian Folk Infusion. Isolation and Structure Elucidation of New Triterpene Saponins from *Ilex amara* Leaves

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The isolation of three new triterpene saponins 3β-*O*-β-D-glucopyranosyl-(1→3)-α-L-2-*O*-acetylarabinopyranosylolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**2**), 3β-*O*-β-D-glucopyranosyl-(1→2)-α-L-*O*-arabinopyranosylurs-12-en-28-oic acid (**3**), and 3β-*O*-β-D-glucopyranosyl-(1→2)-β-D-*O*-galactopyranosylurs-12-en-28-oic acid (**4**) together with five known saponins and one flavonoid glycoside from the aqueous infusion of *Ilex amara* (Vellozo) Loes. leaves is reported. All structures were elucidated by spectroscopic methods, including the concerted application of one-dimensional (^{1}H, TOCSY, ^{13}C, and ^{13}C DEPT NMR) and two-dimensional NMR techniques (DQF-COSY, HSQC, and HMBC).

KEYWORDS: ***Ilex amara*** **(Vellozo) Loes.; Aquifoliaceae; mate; infusion; triterpene saponins; 1D and 2D NMR**

INTRODUCTION

Ilex amara (Vellozo) Loes. (Aquifoliaceae) is a Brazilian plant species the leaves of which are widely used in the form of an aqueous infusion. Because the leaves have a morphology similar to that of *Ilex paraguariensis* and the infusion has a similar taste and flavor, *I. amara* is also popularly known as "mate". In fact, some adulterations have been found in the local market. Many reports indicate that saponins are the major constituents of *Ilex* species (*1*−*5*). The occurrence of flavonoids (*6*, *7*), xanthines (*8*, *9*), aldehydes (*10*), hemiterpene glycosides (*11*), triterpenes and alkanes (*12*), anthocyanins (*13*), pentyl esters, hexyl esters, and other lipophilic compounds (*14*) has also been reported. Several biological activities were related to the compounds isolated from *Ilex* species, including hypocholesterolemic (*15*) and antioxidant (*16*−*21*) activities and inhibition of acyl CoA cholesteryl acyl transferase (ACAT) (*22*). Some authors have also reported an association between the consumption of mate (*I. paraguariensis*) or tea (*Camellia sinensis*) with esophageal and renal cancer (*23*, *24*). In a continuation of our studies of Brazilian food and/or medicinal plants (*25*, *26*) we have investigated the chemical composition of the aqueous infusion of the leaves of *I. amara*. The chemical information obtained could be important not only for understanding folk utilization but also for the future validation of compounds as markers for the assessment of Brazilian mate infusion (*1*).

MATERIALS AND METHODS

Biological Material. The leaves of *I. amara* (Vellozo) Loes. were collected in July 2000 at Fazenda Três Irmãos, Guaratuba Beach, Bertioga, São Paulo, Brazil. Samples were identified by Dr. Milton Groppo from the Instituto de Biociências da Universidade de São Paulo. A voucher sample is deposited at the Herbario of the Universidade de São Paulo, USP - col. M. Groppo Jr. 434, fr. (SPF). The leaves of *I. paraguariensis* were obtained in the local market and identified by Dr. Milton Groppo Jr.

Apparatus. The ES-MS spectra were obtained with a Fisons Platform spectrometer (Altrinchan, U.K.) in both the positive (90 V) and negative (100 V) modes. The sample was dissolved in MeOH and injected directly.

A Bruker DRX-600 spectrometer (Silberstreifen, Germany), operating at 599.19 MHz for ^{1}H and at 150.858 for ^{13}C, using the UXNMR software package was used for NMR experiments in CD_3OD. Distortionless enhancement by polarization transfer (DEPT) experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative ones for CH_2. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. ^{1}H−^{1}H double quantum filtered (DQF) COSY) (*27*, *28*), ^{1}H−^{13}C HSQC, and HMBC (*29*) experiments were obtained using the conventional pulse sequences as described in the literature, and 1D TOCSY spectra (*30*) were acquired using a waveform generator-based GAUSS shaped pulse, a mixing time ranging from 80 to 100 ms, and an MLEV-17 spin−lock field of 10 kHz preceded by a 2 ms trim pulse.

Preparative HPLC separations were performed on a Waters 590 series system (Milford, MA) equipped with a Waters R401 refractive index

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detector and with a Waters μ-Bondapak C-18 column (30 cm × 7.8 mm i.d.) and a U6K injector. Analytical HPLC analyses were performed on a Varian ProStar 330 chromatograph (Sugar Land, TX) managed by an Varian workstation equipped with a Varian ProStar 220 diode array detector (DAD) operating from 200 to 600 nm and a Rheodyne injector with a 20 μL loop. The column used was a Varian C_{18} 250 × 4.6 mm i.d., at 30 °C. Isocratic elution was performed using MeOH/H_2O, 75:25, as mobile phase at a flow rate of 1.0 mL/min. Monitoring wavelength was 205 nm. The infusion was filtered on a 0.45 μm Millex filter and directly injected. Standard solutions of compounds **1**−**8** were dissolved in the mobile phase and injected into the HPLC for qualitative purposes.

GC separations were run using a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a mass selective detector MSD 5970 MS and an HP-5 fused-silica column (25 m × 0.2 mm i.d., 0.33 μm film).

Combustion analyses were performed on an EA 1110 CHNS-O CE Instruments (Rodano, Italy).

Preparation of Infusions for Qualitative HPLC Analysis. One gram of the air-dried leaves of *I. amara* and *I. paraguariensis* were separately milled and put into 50 mL Erlenmeyer flasks. Ten milliliters of boiled water was added to each sample. The infusion was centrifuged, and the supernatant was filtered through a 0.45 μm Millex filter. Twenty microliters of the filtered solution was directly injected into the analytical HPLC system.

Extraction and Isolation of *I. amara* Constituents. Leaves of *I. amara* were air-dried and milled. The powdered plant (200 g) was boiled for 8−9 min with water (2 L). The mixture was allowed to cool, filtered through filter paper, and evaporated to dryness, affording 3 g of crude extract of each plant. An aliquot (2.0 g) of the extract was dissolved in 10 mL of MeOH and fractionated by column chromatography on a Sephadex LH-20 (1 m × 3 cm i.d.) with a flow rate of 0.5 mL/min. Seventy-five fractions of 8 mL were collected. After TLC analysis (silica gel; *n*-BuOH/AcOH/H_2O, 65:15:25; $CHCl_3$/MeOH/H_2O, 70:30:3), fractions with similar R_f values were combined and further purified by HPLC (μ-Bondpak column, 30 cm × 7.8 mm i.d., flow rate = 2.0 mL/min). Fractions containing saponins were purified using MeOH/H_2O, 75:25, as eluent to yield compounds **1** (18 mg, t_R = 33 min), **2** (14 mg, t_R = 30 min), **3** (12 mg, t_R = 57 min), **4** (22 mg, t_R = 24 min), **5** (33 mg, t_R = 27 min), **6** (19 mg, t_R = 51 min), **7** (17 mg, t_R = 9 min), and **8** (35 mg, t_R = 26 min), and the flavonoid was purified using MeOH/H_2O as eluent to afford compound **9** (10 mg, t_R = 24 min).

TLC Analyses for Hemolytic Activity. Approximately 15 μg of the crude extract of the infusion of *I. amara* and of the isolated compounds **1**−**8** was spotted on TLC silica gel plates (Aldrich, 10 × 20 cm) and developed with CH_3Cl/MeOH/*n*-PrOH/H_2O, 5:6:5:1:4 (upper phase). The plates were dried and sprayed with blood reagent (*31*).

Acid Hydrolysis of Compounds 1—8. A solution of each compound (3 mg) in 6% HCl (3.5 mL) was refluxed for 2 h. The reaction mixture was diluted with H_2O and then extracted with EtOAc. The resulting products were identified by TLC comparison of their R_f values and also by their ^{1}H NMR spectra.

Methanolysis of Compounds 1—8. Each compound (1.0 mg) was heated in a vial for 24 h at 80 °C in MeOH/2% HCl (2 mL). After MeOH and HCl evaporation in an N_2 stream, Ag_2CO_3 and MeOH were added until CO_2 production stopped. The mixture was centrifuged and the centrifugate was dried over P_2O_5. The resulting monosaccharides were treated with Trisil-Z (Pierce) and analyzed by GC-MS. Retention times were identical to those of authentic TMS sugars.

*Compound **1**:* $C_{49}H_{78}O_{18}$; ES-MS, *m*/*z* (rel int.) (negative mode) 994 (20), 953 (2), 791 (55), 749 (100), 587 (15), 455 (absent); (positive mode) 977 (30), 815 (70), 773 (10), 439 (50); ^{1}H and ^{13}C NMR data, see Tables 1 and 2. Combustion analysis: C, 61.53%; H, 8.33%; O, 30.14% (calcd: C, 61.61%; H, 8.23%; O, 30.16%).

*Compound **2**:* $C_{49}H_{78}O_{18}$; ES-MS, *m*/*z* (rel int.) (negative mode) 994 (33), 953 $[M - H]^-$ (5), 791 (51), 749 (100), 587 (29), 455 (absent); (positive mode) 977 (25), 815 (60), 773 (13), 439 (45); ^{1}H and ^{13}C NMR data, see Tables 1 and 2. Combustion analysis: C, 61.49%; H, 8.33%; O, 30.18% (calcd: C, 61.60%; H, 8.24%; O, 30.16%).

Table 1. ^{13}C NMR Assignments (δ) of the Aglycon Moiety of Compounds **1**–**4** in CD_3OD

position	1	2	3	4
1	39.5	39.7	39.6	39.2
2	26.6	26.8	26.7	26.5
3	90.5	90.0	90.3	90.1
4	40.0	40.2	40.0	40.0
5	56.8	56.9	56.7	56.9
6	18.7	19.3	18.9	18.9
7	32.2	32.6	32.1	32.0
8	39.3	40.7	39.0	39.4
9	48.8	48.0	48.9	48.9
10	38.8	37.8	38.5	38.9
11	24.0	24.5	24.2	23.9
12	127.5	123.8	127.5	127.2
13	138.6	144.7	138.7	138.3
14	42.1	42.8	42.0	42.2
15	28.0	28.9	28.1	28.2
16	24.0	24.2	24.6	24.7
17	48.1	47.1	48.1	48.4
18	54.0	42.6	53.4	53.4
19	39.1	47.2	39.3	39.4
20	39.5	31.5	39.2	39.1
21	30.1	34.9	30.7	30.9
22	37.2	33.2	36.8	36.9
23	27.9	28.0	27.9	27.8
24	16.0	15.6	16.3	15.9
25	14.9	16.4	15.0	14.5
26	17.1	17.1	17.0	17.0
27	23.6	25.2	23.5	23.8
28	176.9	178.1	182.0	182.0
29	17.1	33.3	16.8	16.8
30	20.9	23.8	21.0	20.7

*Compound **3**:* $C_{41}H_{66}O_{12}$; ES-MS, *m*/*z* (rel int.) (100 V, negative mode) 790 (22), 749 (3), 587 (45), 455 (absent); (100 V, positive mode) 773 (27), 611 (63), 439 (45); ^{1}H and ^{13}C NMR data, see Tables 1 and 2. Combustion analysis: C, 65.69%; H, 8.73%; O, 30.58% (calcd: C, 65.56%; H, 8.86%; O, 25.57%).

*Compound **4**:* $C_{42}H_{68}O_{13}$; ES-MS, *m*/*z* (rel int.) (negative mode) 820 (19), 779 (3), 617 (40), 455 (2); (positive mode) 803 (23), 641 (68), 439 (40); ^{1}H and ^{13}C NMR data, see Tables 1 and 2. Combustion analysis: C, 64.66%; H, 8.42%; O, 26.92% (calcd: C, 64.68%; H, 8.78%; O, 26.64%).

*Compound **5**:* ES-MS, *m*/*z* (rel int.) (negative mode) 952 (21), 911 (5), 749 (51), 587 (29), 455 (5); (positive mode) 935 (25), 773 (60), 611 (13), 479 (10), 457 (3), 439 (35); ^{1}H NMR (CD_3OD) δ 0.87 (s, Me), 0.92 (d, J = 8 Hz, Me), 0.93 (d, J = 10 Hz, Me), 0.94, 0.98, 1.08, 1.14 (s, 4 × Me), 2.87 (dd, J = 13 and 5 Hz, H-18), 5.27 (m, H-12), 4.31 (d, J = 7 Hz, H-1 glucose), 4.58 (d, J = 7 Hz, H-1 arabinose), 5.37 (d, J = 7 Hz, H-1 glucose linked at C-28).

*Compound **6**:* ES-MS, *m*/*z* (rel int.) (negative mode) 936 (17), 895 (3), 733 (55), 749 (33), 587 (57), 455 (2); (100 V, positive mode) 919 (32), 757 (56), 611 (11), 479 (8), 457 (2), 439 (35); ^{1}H NMR (CD_3-OD) δ 0.88, 0.89, 0.91, 0.97, 0.97, 1.05, 1.18 (s, 7 × Me), 2.93 (dd, J = 13 and 5 Hz, H-18), 5.24 (m, H-12 and H-1 rhamnose), 1.25 (d, J = 6 Hz, Me-6-Rha), 4.53 (2H, m, H-1 glucose and H-1 arabinose).

*Compound **7**:* ES-MS, *m*/*z* (rel int.) (negative mode) 968 (19), 927 (5), 765 (51), 603 (29), 471 (2); (positive mode) 951 (25), 789 (60), 627 (13), 495 (9), 473 (3), 455 (38); ^{1}H NMR (CD_3OD) δ 0.80, 0.87, 0.95, 0.96, 1.04, 1.22, 1.35 (s, 7 × Me), 2.54 (s, H-18), 5.32 (m, H-12), 4.43 (d, J = 8 Hz, H-1 arabinose), 4.51 (d, J = 7 Hz, H-1 galactose), 5.35 (d, J = 7 Hz, H-1 glucose linked at C-28).

*Compound **8**:* ES-MS, *m*/*z* (rel int.) (negative mode) 806 (21), 765 (5), 603 (53), 471 (3); (positive mode) 789 (25), 627 (60), 495 (11), 473 (4), 455 (40); ^{1}H NMR (CD_3OD) δ 0.86, 0.88, 0.95, 0.97, 1.00, 1.22, 1.34 (s, 7 × Me), 2.59 (s, H-18), 5.30 (m, H-12), 4.43 (d, J = 7.2 Hz, H-1 galactose), 4.51 (d, J = 6.5 Hz, H-1 arabinose).

*Compound **9**:* ES-MS, *m*/*z* (rel int.) (negative mode) 593 (55), 447 (25), 285 (88); (positive mode) 617 (45), 595 (60), 449 (23), 287 (90); ^{1}H NMR (CD_3OD) δ 6.07 (d, J = 2.0 Hz, H-6), 6.22 (d, J = 2.0 Hz, H-8), 6.89 (d, J = 8.4 Hz, H3′/5′), 8.09 (d, J = 8.4 Hz, H2′/ H6′), 4.55 (br s, H-1 rhamnose), 5.00 (d, J = 7.2 Hz, H-1 glucose), 1.22 (d, J = 6.0 Hz, H-6 rhamnose).

Table 2. ^{1}H and ^{13}C NMR Assignments (δ) of the Sugar Moiety of Compounds **1**–**4** in CD_3OD

	1		**2**		**3**		**4**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
3-Ara								
1	104.9	4.45 d (7.8)	104.9	4.45 d (7.9)	105.5	4.31 d (7.6)		
2	72.5	5.20 dd (7.8; 8.4)	72.5	5.20 dd (7.9; 8.4)	78.0	3.73 dd (7.6, 8.4)		
3	81.2	3.84 dd (3.6; 8.4)	81.2	3.65 dd (3.6; 8.4)	72.6	3.65 dd (3.6, 8.4)		
4	69.7	4.08 m	69.7	4.08 m	68.5	4.04 m		
5	66.5	3.62 dd (12.0; 3.0)	66.5	3.62 dd (12.0; 3.0)	65.3	3.60 dd (12.5, 3.0)		
		3.90 dd (12.0; 2.4)		3.90 dd (12.0; 2.4)		3.81 dd (12.5, 2.0)		
CH_3CO	21.3	2.09 s	21.0	2.07 s				
$\overline{CH_3}CO$	171.0		171.1					
Glc		**(3→1) Glc**		**(3→1) Glc**		**(2→1) Glc**		**(2→1) Glc**
1	106.0	4.41 d (7.8)	106.0	4.41 d (7.8)	104.3	4.59 d (7.8)	104.0	4.69 d (7.8)
2	74.5	3.22 dd (9.6; 7.8)	74.5	3.22 dd (9.6; 7.8)	75.4	3.21 dd (7.8, 9.0)	75.5	3.25 dd (7.8, 9.0)
3	77.9	3.42 t (9.6)	77.9	3.42 t (9.6)	77.9	3.42 t (9.0)	77.8	3.40 t (9.0)
4	71.7	3.38 t (9.6)	71.7	3.38 t (9.6)	71.1	3.37 t (9.0)	71.0	3.37 t (9.0)
5	77.9	3.34 m	77.9	3.34 m	77.2	3.27 m	77.2	3.29 m
6	61.8	3.67 dd (11.5; 6.0)	61.8	3.67 dd (11.5; 6.0)	62.3	3.70 dd (4.5, 11.5)	62.0	3.70 dd (4.5, 11.5)
		3.80 dd (11.5; 2.0)		3.80 dd (11.5; 2.0)		3.85 dd (2.5, 11.5)		3.85 dd (2.5, 11.5)
3-Gal								
1							103.5	4.42 d (7.6)
2							78.2	3.89 dd (7.6, 9.0)
3							75.2	3.70 dd (4.0, 9.6)
4							71.4	3.85 dd (2.5, 4.0)
5							76.2	3.54 m
6							62.2	3.75 dd (12.0, 4.5)
								3.79 dd (12.0, 2.5)
28-Glc								
1	95.5	5.37 d (8.0)	95.5	5.41 d (8.0)				
2	73.6	3.34 dd (9.5; 8.0)	73.6	3.34 dd (9.5; 8.0)				
3	78.3	3.38 t (9.5)	78.2	3.36 t (9.5)				
4	71.9	3.32 t (9.5)	71.9	3.32 t (9.5)				
5	78.2	3.35 m	77.5	3.29 m				
6	61.8	3.67 dd (12.6; 5.9)	61.8	3.71 dd (12.6; 5.9)				
		3.80 dd (12.6; 2.0)		3.85 dd (12.6; 2.0)				

[a] Chemical shift values are in parts per million, and *J* values in hertz are presented in parentheses. All signals were assigned by 1D-TOCSY, DQF-COSY, HSQC, and HMBC studies. Ara = α-L-arabinopyranosyl. Gal = β-D-galactopyranosyl. Glc = β-D-glucopyranosyl.

RESULTS AND DISCUSSION

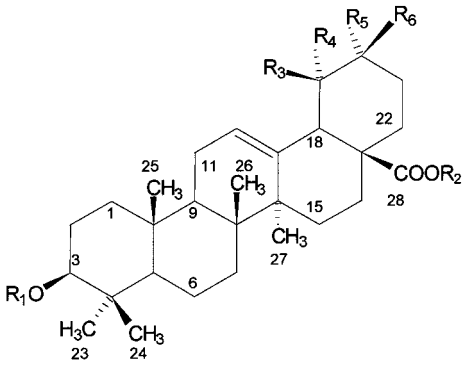
The saponins from the leaves of *I. amara* were readily extracted by the addition of hot water. The infusion was fractionated by chromatograph to separate its chemical constituents, and fractions were further purified by reversed-phase HPLC to yield pure triterpene saponins (**1**−**8**) and flavonoid (**9**) as the major constituents of this infusion (Figure 1). The structures of the compounds were determined by one- and two-dimensional NMR experiments as well as by mass spectrometry.

Acid hydrolysis of **1**, **3**, and **4** released ursolic acid, whereas **2** released oleanolic acid. These aglycons were identified by their ^{1}H and ^{13}C NMR spectra and also by TLC with authentic standards. The gas chromatographic analysis of the methanolysis products showed the presence of glucose and arabinose, in the ratios 2 Glc/1 Ara for both **1** and **2**, 1 Glc/1 Ara for **3**, and 1 Glc/1 Gal for **4**.

The ES-MS (negative mode) mass spectrum of **1** gave the adduct $[M - H + MeCN]^-$ at *m/z* 994 and the pseudomolecular ion $[M - H]^-$ at *m/z* 953. Fragment ions occurred at *m/z* 791 $[(M - H) - 162]^-$, at *m/z* 749 $[(M - H) - 162 - 42]^-$, and at *m/z* 587 $[(M - H) - 162 - 42 - 162]^-$, which were attributed to independent losses of one terminal hexose, one acetyl group, and a second terminal hexose moiety, respectively. In the ES-MS in the positive ion mode we observed the adduct with sodium $[M + Na]^+$ at *m/z* 977. The fragment at *m/z* 815 $[M + Na - 162]^+$ corresponds to the loss of one terminal hexose. Sequential loss of an acetyl unit would yield the fragment $[M + Na - 162 - 42]^+$ at *m/z* 773. The fragment at *m/z* 439 $[M - 162 - 42 - 162 - 132 - 18]^+ = [A + H - 18]^+$ corresponds to the losses of two hexoses, an acetyl group, one pentose, and one water molecule, leading to the dehydrated protonated aglycon fragment.

The structure of **1** was fully elucidated by 1D and 2D NMR experiments at 600 MHz. The ^{1}H NMR spectrum of **1** (Tables 1 and 2) displayed signals for methyl groups at δ 0.77, 0.86, 0.92 (d, $J = 6.6$ Hz), 0.96 (6H), 1.00 (d, $J = 6.6$ Hz), and 1.14. A signal due to H-3 was evident at δ 3.14 (dd, $J = 11.0$; 4.0 Hz), and the olefinic proton appeared at δ 5.27 (br s, H-12). These values were compatible with the ursolic acid aglycon (*32*).

The ^{13}C NMR shifts of the aglycon part of **1** (Table 1) also corresponded well with the shifts for ursolic acid, the most significant differences being those corresponding to C-3 and C-28 (*33*). These shifts were analogous to those reported when both 3-OH and 28-COOH groups are glycosylated in a triterpene saponin (*34*). Three anomeric protons were easily identified in the spectra of **1**. They resonated at δ 5.37 (d, $J = 8.0$ Hz), 4.45 (d, $J = 7.8$ Hz), and 4.41 (d, $J = 7.8$ Hz) and correlated to carbons at δ 95.5, 104.9, and 106.0, respectively. From the assigned aglycon and sugar values (Tables 1 and 2), it appared that **1** was a bisdesmosidic saponin. The structure of saccharide chain was assigned by a combination of 1D TOCSY, 2D DFQ-COSY, HSQC, and HMBC experiments. The isolated anomeric signals resonating in uncrowded regions of the spectrum, between δ 5.37 and 4.41, were the starting points for the 1D TOCSY experiments. Because of the selectivity of multistep coherence transfer, the 1D TOCSY subspectra of the single monosaccharide units could be extracted from the overlapping region of the spectrum (between δ 3.0 and 4.0). Each subspectrum could be attributed to one set of coupled protons such as



	R_1	R_2	R_3	R_4	R_5	R_6
1	Glc(1→3)-(2-OAc)-Ara	Glc	CH_3	H	CH_3	H
2	Glc(1→3)-(2-OAc)-Ara	Glc	H	H	CH_3	CH_3
3	Glc(1→2)-Ara	H	CH_3	H	CH_3	H
4	Gal(1→2)-Ara	H	CH_3	H	CH_3	H
5	Glc(1→3)-Ara	Glc	CH_3	H	CH_3	H
6	Glc(1→3)-[Rha(1→2)]-Ara	H	H	H	CH_3	CH_3
7	Gal(1→2)-Ara	Glc	CH_3	OH	CH_3	H
8	Gal(1→2)-Ara	H	CH_3	OH	CH_3	H

Figure 1. Saponins isolated from *I. amara* leaves: (**1**) 3*β*-*O*-*β*-D-glucopyranosyl-(1→3)-α-L-2-*O*-acetylarabinopyranosylurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester; (**2**) 3*β*-*O*-*β*-D-glucopyranosyl-(1→3)-α-L-2-*O*-acetylarabinopyranosylolean-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester; (**3**) 3*β*-*O*-*β*-D-glucopyranosyl-(1→2)-α-L-arabinopyranosylurs-12-en-28-oic acid; (**4**) 3*β*-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-galactopyranosylurs-12-en-28-oic acid; (**5**) 3*β*-*O*-*β*-D-glucopyranosyl-(1→3)-α-L-arabinopyranosylurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester; (**6**) 3*β*-*O*-[*β*-D-glucopyranosyl-(1→3)]-[α-L-rhamnopyranosyl(1→2]-α-L-arabinopyranosylolean-12-en-28-oic acid; (**7**) 3*β*-*O*-*β*-D-galactopyranosyl-(1→2)-α-L-arabinopyranosyl-19-hydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester; (**8**) 3*β*-*O*-*β*-D-galactopyranosyl-(1→2)-α-L-arabinopyranosyl-19-hydroxyurs-12-en-28-oic acid.

H−C(1) to H−C(4) for arabinose or H−C(1) to H−C(6) for glucose.

1D TOCSY subspectra obtained by irradiation of the signals at δ 5.37 and at δ 4.41 showed two sets of coupled resonances in a sugar, ascribable from H-1 to H-6 of two glucose units. The 1D TOCSY subspectrum obtained by irradiating the signal at δ 4.45 showed connectivities to three methine groups (δ 5.20, 4.08, and 3.84). The coherence transfer to H-5 was not obtained because of the small J_{H4-H5} value of this arabinose unit. The double doublet at δ 5.20 (J = 5.0 and 8.4 Hz) clearly indicated that this was an acetylated sugar. The DFQ-COSY spectrum showed the connectivity between the anomeric signal at δ 4.45 and the signal at δ 5.20, thus establishing that the acetyl group was located at position 2 of the arabinose moiety. The sequential assignments of all these sugar protons as shown in Table 2 derived from their distinctive DQF-COSY patterns. The assignments of all proton resonances for the sugar moieties, immediately permitted assignment of the resonances of the linked carbon atoms by HSQC (Table 2).

Information about the sequence of the saccharide chain was deduced from an HMBC experiment. Key correlation peaks were observed between the anomeric proton of the glucose (δ 5.37) and C-28 of the ursolic acid aglycon (δ 176.9), the anomeric proton signal of the arabinose (δ 4.45) and C-3 of the aglycon (δ 90.5), and the anomeric proton of glycose (δ 4.41) and C-3 of the arabinose (δ 81.2). Further correlation was also observed between H-3 (δ 3.14) and C-1′ of the arabinose unit (δ 104.9). The β-configuration at the anomeric position for the glucopyranosyl units (J_{1-2} = 7.8 Hz) was easily deduced from their relatively large $^3J_{1-2}$ coupling constants (7−8 Hz). The large coupling also allowed the α-configuration for the arabinopyranosyl unit (J_{1-2} = 7.8 Hz) to be established (*35*).

These data suggested that the structure of **1** is 3*β*-*O*-*β*-D-glucopyranosyl-(1→3)-α-L-2-*O*-acetylarabinopyranosylurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester. This compound was reported from *I. paraguariensis* (*1*), but no spectroscopic data were found in the literature.

The ES-MS (negative mode) mass spectrum of **2** gave the adduct $[M - H + MeCN]^-$ at *m*/*z* 994 and the quasimolecular ion $[M - H]^-$ at *m*/*z* 953. Fragment ions occurred at *m*/*z* 791 $[(M - H) - 162]^-$, *m*/*z* 749 $[(M - H) - 162 - 42]^-$, and *m*/*z* 587 $[(M - H) - 162 - 42 - 162]^-$ and were interpreted as independent losses of one terminal hexose, one acetyl group, and a second terminal hexose moiety, respectively. The ES-MS in the positive ion mode presented a fragmentation pattern similar to that of compound **1** with ions at *m*/*z* 977 $[M + Na]^+$, *m*/*z* 815 $[M + Na - 162]^+$, *m*/*z* 773 $[M + Na - 162 - 42]^+$, and *m*/*z* 439 $[A + H - 18]^+$.

Compound **2** showed ^{1}H and ^{13}C NMR spectra similar to those of compound **1**. The main differences related to the aglycon. The ^{1}H NMR spectrum of **2** displayed signals for methyl groups at δ 0.76, 0.82, 0.94, 0.96, 0.97, 0.98, and 1.18. The H-3 absorbs at δ 3.15 (d, J = 11.5 and 4.5), and the olefinic proton appears at δ 5.27 (br, H-12). These values were in good agreement with oleanolic acid as aglycon (*33*). The ^{13}C NMR shifts of the aglycon part of **2** (Table 1) also corresponded well with the shifts for oleanolic acid. The most significant differences were the downfield shift caused by glycosylation at C-3 and the upfield shift caused by substitution at C-28 (*36*, *34*).

The sugar region of compound **2** was almost superimposable on that of compound **1** (Table 2). The only difference was the chemical shift of the anomeric proton of the glucose moiety bonded to C-28, which absorbed at δ 5.41 (d, J = 8.0 Hz). From these considerations the structure of the new saponin (**2**) was established as 3*β*-*O*-*β*-D-glucopyranosyl-(1→3)-α-L-2-*O*-acetylarabinopyranosylolean-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester.

The ES-MS (negative mode) mass spectrum of **3** gave the adduct $[M - H + MeCN]^-$ at *m*/*z* 790, the pseudomolecular ion $[M - H]^-$ at *m*/*z* 749, and an ion at *m*/*z* 587 $[(M - H) - 162]^-$, the latter one due to the loss of one terminal hexose. In the ES-MS in the positive ion mode we observed the adduct with sodium $[M + Na]^+$ at *m*/*z* 773. The fragment at *m*/*z* 611 $[M + Na - 162]^+$ corresponded to the loss of the terminal hexose. The fragment at *m*/*z* 439 $[M - 162 - 132 - 18]^+ = [A + H - 18]^+$ corresponded to the loss of one hexose, one pentose, and one water molecule, leading to the dehydrated protonated aglycon fragment.

Compound **3** showed ^{1}H and ^{13}C NMR spectra similar to those of compound **1** (Tables 1 and 2). The main differences related to the saccharide moiety, as previously revealed by hydrolysis results. The 1D TOCSY subspectrum, obtained by irradiation of the signal at δ 4.59 (d, J = 7.8 Hz), showed a spin system corresponding to a glucose unit with β-configuration, whereas the 1D TOCSY subspectrum obtained by irradiating the signal at δ 4.31 (d, J = 7.6 Hz) showed connectivities to three methine groups (δ 4.04, 3.73, and 3.65), corresponding to the arabinose moiety with an α-configuration. The assignments of all protons and carbons of **3** were established from 1D and 2D NMR experiments (1D TOCSY, DFQ-COSY, DEPT, HSQC, and HMBC) as shown in Tables 1 and 2.

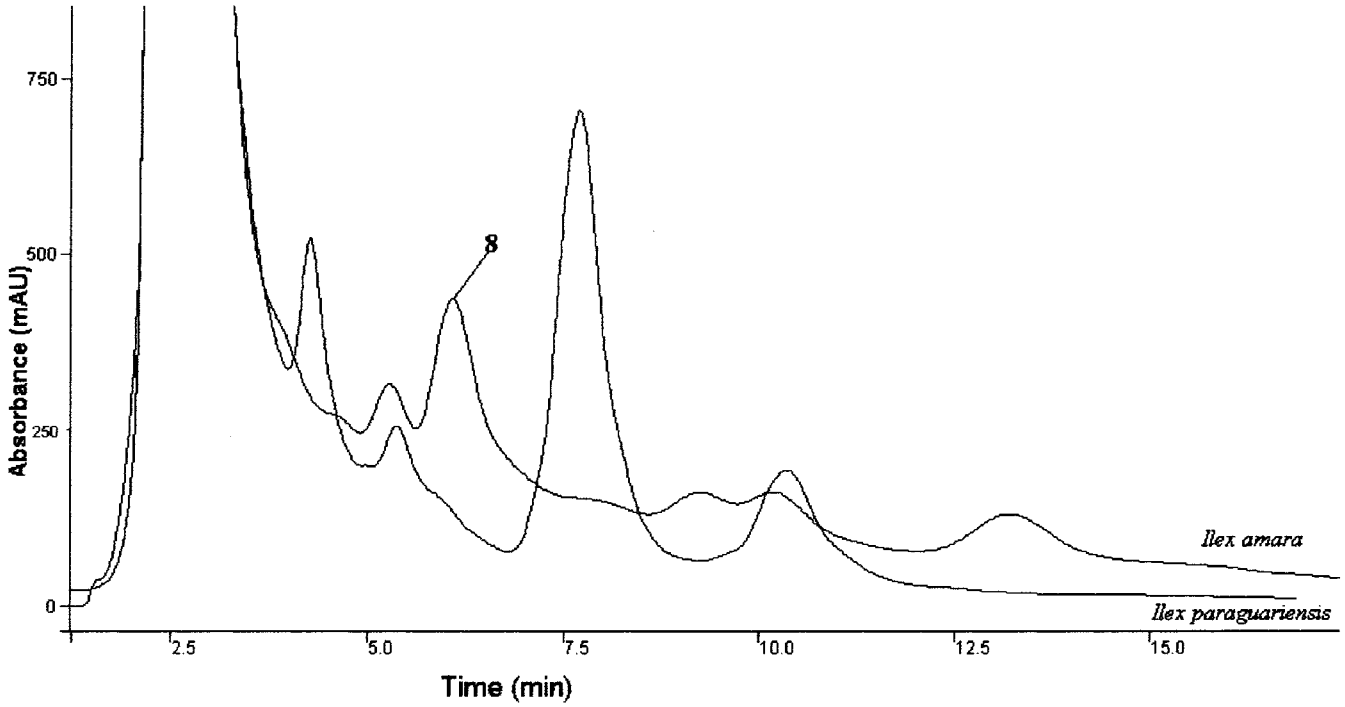


Figure 2. Comparative HPLC analysis of the infusions of *I. amara* and *I. paraguariensis*.

The position of the saccharide on the aglycon and the sequence of the saccharide chain were deduced from an HMBC experiment. Correlation peaks were observed between the anomeric proton of the arabinose (δ 4.31) and C-3 of the aglycon (δ 90.3) and between the anomeric proton of the glucose (δ 4.59) and C-2 of the arabinose (δ 78.0). Further correlation was also observed between H-2 (δ 3.73) of the arabinose and C-1 of the glucose unit (δ 104.3). These data suggested that the structure of **3** is the new 3β-*O*-β-D-glucopyranosyl-(1→2)-α-L-*O*-arabinopyranosylurs-12-en-28-oic acid.

The ES (negative mode) mass spectrum of **4** gave the adduct $[M - H + MeCN]^-$ at *m/z* 820 and the pseudomolecular ion $[M - H]^-$ at *m/z* 779. Fragment ions occurred at *m/z* 617 $[(M - H) - 162]^-$ and *m/z* 455 $[(M - H) - 162 - 162]^-$, corresponding to sequential losses of two hexose moieties. In the ES-MS spectrum in the positive ion mode we observed the adduct with sodium $[M + Na]^+$ at *m/z* 803. The fragment at *m/z* 641 $[M + Na - 162]^+$ corresponded to the loss of the terminal hexose. The fragment at *m/z* 439 $[M - 162 - 162 - 18]^+ = [A + H - 18]^+$ corresponded to the loss of two hexose units and one water molecule, leading to the dehydrated protonated aglycon fragment.

With regard to the aglycon moiety, compound **4** showed ^1H and ^{13}C NMR spectra superimposable on those of compound **3** (Table 1). The main differences related to the saccharide chain, and this observation was also supported by hydrolysis experiments. 1D TOCSY experiments were performed to identify the sugars linked to the ursolic acid aglycon. The subspectra obtained by irradiating the signal at δ 4.42 (d, $J = 7.6$ Hz) showed connectivities to three methine groups (δ 3.89, 3.83, and 3.68), corresponding to a galactose unit with β-configuration, and the 1D TOCSY subspectra obtained by irradiation at δ 4.69 (d, $J = 7.8$ Hz) displayed a typical spin system H-1 to H-6 of the glucose moiety with β-configuration.

The sequence of the sugar chain, the position of attachment on the aglycon, and the complete assignments of each proton and carbon of **4** were deduced from DFQ-COSY, DEPT, HSQC, and HMBC experiments (Tables 1 and 2). In the HMBC spectrum, correlation peaks were observed between the anomeric proton of the galactose (δ 4.42) and C-3 of the aglycon (δ 90.1) and between the anomeric proton of the glucose (δ 4.69) and C-2 of the galactose (δ 78.2). Therefore, the structure of **4** was concluded to be the new 3β-*O*-β-D-glucopyranosyl-(1→2)-β-D-*O*-galactopyranosylurs-12-en-28-oic acid.

Compounds **5**−**9** displayed spectroscopic data identical to those reported in the literature: 3β-*O*-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosylurs-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**5**) was previously isolated from *Aralia decaisneana* (*36*); 3β-*O*-[β-D-glucopyranosyl-(1→3)]-[α-L-rhamnopyranosyl(1→2)]-α-L-arabinopyranosylolean-12-en-28-oic acid (**6**) was isolated from *I. paraguariensis* (*37*); 3β-*O*-β-D-galactopyranosyl-(1→2)-α-L-arabinopyranosyl-19-hydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**7**) and 3β-*O*-β-D-galactopyranosyl-(1→2)-α-L-arabinopyranosyl-19-hydroxyurs-12-en-28-oic acid (**8**) were isolated from *I. cornuta* (*38*); and the flavonoid 3-*O*-α-L-rhamnopyranosyl(1→6)-glucopyranosylkaempferol (**9**) was isolated from *Capparis artilogenia* (*39*). All of these structures were confirmed by 1D and 2D NMR experiments and also by ES-MS analyses.

The main objective of this study was to investigate the chemical compounds from *I. amara* because it is a species used in a traditional beverage also called mate. The major compounds of the *I. amara* leaves are saponins. This class of secondary metabolites is among those which provide foods with biological activities and even with antinutritional and toxic properties (*40*).

The structural diversity of saponins depends not only on the triterpene skeleton but also on the variety of sugars attached to the aglycon nucleus. The saponins isolated from *I. amara* are based on ursane, oleanane, and 19-hydroxyursane triterpenes. Although similar saponins were isolated from *I. paraguariensis*, to the best of our knowledge, saponins with 19-hydroxyursane or 19-hydroxyoleanane skeletons were not found in the aqueous infusion of the leaves of *I. paraguariensis*.

To check this hypothesis, we performed a comparative HPLC analysis of the infusions of *I. amara* and *I. paraguariensis* (Figure 2). The chromatographic profiles of the aqueous

infusions of *I. amara* and *I. paraguariensis* could be established by comparing the retention times and UV spectra of the peaks with those of the isolated compounds. Figure 2 shows that the 19-hydroxy saponin (**8**) is present in significant amounts in relation to the other detected compounds only in the infusion of *I. amara* but is almost absent in the infusion of *I. paraguariensis*. Therefore, the presence of the 19-hydroxy saponin (**8**) can be used to establish a chemical differentiation between the aqueous infusions of *I. paraguariensis* and *I. amara*.

Concerning the hemolytic activity, TLC analysis sprayed with blood reagent (*31*) indicated a positive response with 15 μg for compounds **1**−**8** isolated from *I. amara*. Recently, much attention has been paid to the antioxidant activity of the phenolic compounds in the infusion of mate (*I. paraguariensis*) (*20*). However, because saponins are amphiphilic compounds with hemolytic properties, they can be toxic. This toxicity was demonstrated by Takechi (*41*), who reported the high hemolytic activity of di- and triglycosides of methyl ursolate. Moreover, Oda (*42*) suggested that saponins with an acyl residue tended to show increased hemolytic activity. Although there have been no reports that the intake of the aqueous infusion of *I. amara* is harmful, our results demonstrate a need for more information about the biological activities of this tealike beverage.

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