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Triterpenoids from the leaves of *Ilex theezans* Martius ex Reiss.*

Margareth L. ATHAYDE [1], Eloir P. SCHENKEL [1] **,
Grace GOSMANN [1] and Dominique GUILLAUME [2]

[1] *Faculdade de Farmácia, UFRGS, Av. Ipiranga, 2752, 90610-000 Porto Alegre, RS, Brazil.*
[2] *Laboratoire de Chimie Thérapeutique, URA 1310 du CNRS, Faculté des Sciences Pharmaceutiques et Biologiques, Paris V, France.*

SUMMARY. From *Ilex theezans* leaves, a species reported as adulterant of the genuine erva-maté *(Ilex paraguariensis* St. Hil.), two saponins and one triterpene have been isolated. Their structures were elucidated by mean of spectroscopic methods. The saponins were identified as the 28-O-β-D-glucopyranosylester of rotundic acid (pedunculoside, 1) and the 28-O-β-D-glucopyranosylester of rotundioic acid (2). The triterpene was identified as rotundic acid (3).

RESUMEN. "Triterpenoides de las hojas de *Ilex theezans* Martius". *Ilex theezans* Martius ex Reiss. es una de las especies que ha sido mencionada como adulterante de la yerba mate verdadera *(Ilex paraguariensis* St. Hil.). Dos saponinas y un triterpeno de las hojas fueron aislados y sus estructuras químicas elucidadas a través de métodos espectroscópicos como el éster 28-O-β-D-glucopiranósido del ácido rotúndico (pedunculósido, **1**) y el éster 28-O-β-D-glucopiranósido del ácido rotundioico (**2**). El triterpeno fue identificado como el ácido rotúndico (**3**).

INTRODUCTION

During the last years we have systematically studied the saponins of *Ilex paraguariensis* (genuine erva-maté) and other species reported as substitute or adulterant of erva-maté [1]. The utilization of other *Ilex* species in this traditional beverage has to be seen within the historical context of maté production considering that, until the middle of this century, some of these species have been proposed as maté substitutes. After then, the sanitary legislation of several South American countries clearly established maté as a product exclusively obtained from *Ilex paraguariensis,* forbidding the alimentary use of its near relatives. These latter are generally known as "caúna" or "congonha" followed by a second name related to other characteristics, at the most case to the geographical occurrence, for example "caúna-da-serra" (caúna of the mountain) or "caúna-da-praia" (caúna of the beach). Although the production of erva-maté is becoming more industrialized and controlled, its adulteration by variable quantities of leaves of other *Ilex* species is still a problem in some regions where the wild collected raw material play an important role for the maté production.

After having established the accumulation of saponins in the leaves of *Ilex paraguariensis* [2-5], we have investigated the saponins of *Ilex dumosa* [6,7], *Ilex taubertiana* [8], *Ilex pseudobuxus* [9] and *Ilex argentina* [10]. Continuing this work, we report herein our results concerning the isolation and structural elucidation of two saponins and an acid triterpene from the leaves of *Ilex theezans* Mart. ex Reiss., popularly called as "caúna amargosa" (bitter caúna). This species is known to impart a bitter taste to the maté beverage [11] and, notwithstanding this property, it had been proposed as a substitute for *Ilex paraguariensis.*

MATERIALS AND METHODS

General experimental procedures

EIMS, CIMS and FABMS spectra were per-

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PALABRAS CLAVE: ácido rotúndico, ácido rotundioico, Aquifoliaceae, *Ilex theezans,* pedunculósido, saponinas

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** Author to whom correspondence should be addressed. *E-mail:* Schenkel@farmacia.ufrgs.br

formed on a Kratos MS-80 RF spectrometer. Optical rotation was determined on a Perkin-Elmer 141 polarimeter or on a Perkin-Elmer 341 polarimeter. 1H- and 13C-NMR spectra were obtained on a Bruker AC 300 spectrometer. Analytical TLC was carried out on silica gel Merck GF 254 nm plates, using chloroform: ethanol (7:3, v/v) as eluant for compounds **1** and **2** and chloroform:ethanol (9:1, v/v) for compound **3**; detection was performed with anisaldehyde-H_2SO_4/100 °C. For TLC different authentic samples of triterpenes and sugars were used.

Plant material

Leaves of *Ilex theezans* Martius ex Reissek were collected in Osório, State of Rio Grande do Sul, Brazil, on september 1991. An herbarium specimen is deposited in the Botany Department Herbarium of Rio Grande do Sul Federal University (ICN-7167), Porto Alegre.

Extraction and isolation

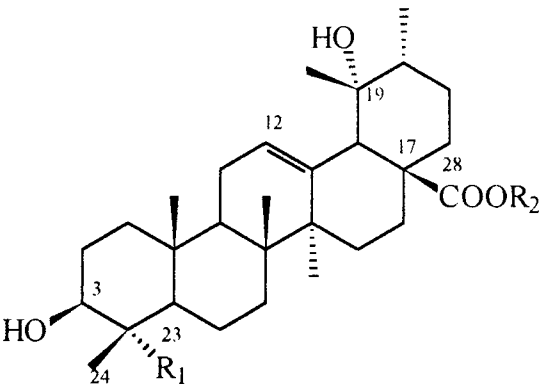
Air-dried leaves (200 g) were crushed and extracted with ethanol-water (6:4, v/v) for 30 minutes at 100 °C. The ethanol was removed under reduced pressure and the aqueous suspension was successively extracted with chloroform and ethyl acetate. The ethyl acetate phase was evaporated to dryness (8.4 g) and a part of it (1 g) was repeatedly chromatographed on a silica gel column using chloroform-ethanol (7:3, v/v) to give compounds **3** (4.8 mg), **1** (60 mg), and an additional fraction that was purified by preparative TLC (silica gel, chloroform:ethanol, 8:2, v/v), affording 13.2 mg of pure compound **2**.

Acid hydrolysis

Compounds **1** and **2** were hydrolyzed as described by Kartnig and Wegschaider [12] using two dimensional silica gel TLC. The first migration was carried out using chloroform-ethanol (7:3, v/v) as eluent. In order to analyze the aglycone, petrolether-ethyl acetate (1:1, v/v) was used. Sugar components were analyzed using ethyl acetate-methanol-acetic acid-water (60:15:15:10, v/v).

Alkaline hydrolysis

Compound **1** (26 mg) was refluxed in 4% NaOH ethanolic solution (20 ml) for 1 h. After evaporation to dryness the residue was suspended in water and then extracted with ethyl acetate. The ethyl acetate phase was analyzed by TLC.



1	R_1=CH_2OH	R_2= glucose
2	R_1= COOH	R_2= glucose
3	R_1= CH_2OH	R_2= H

Figure 1. Compounds **1**, **2** and **3** isolated from *Ilex theezans* leaves.

Acetylation of compound **2**

Compound **2** (10 mg) was acetylated with acetic anhydride and pyridine at room temperature for 12 h furnishing **2a** in quantitative yields.

Compound **1**

$[\alpha]_D$ = + 49,5 ° (c = 0.5, pyridine, 25°C); CIMS *m/z* 668 $(M + NH_3)^+$, 651 $(M + H)^+$, 650 $(M)^+$; EIMS *m/z* 426 [M - hexose - COO]$^+$ (10%) , 264 (8%), 246 (10%), 219 (10%), 201 (35%), 146 (70%). ^{1}H-NMR (C_5D_5N) δ: 1.04, 1.20, 1.31, 1.41, 1.62 (3H each, s, CH_3 x 5), 1.06 (3H, d, *J* = 6.5 Hz, 30-CH_3), 2.95 (1H, s, H-18), 6.25 (1H, d, *J* = 8 Hz, Glc-1). ^{13}C-NMR: see Table 1.

Compound **2a**

Amorphous powder, $[\alpha]_D$ = +32 ° (c = 0.125, MeOH, 20 °C). FAB-MS *m/z* 687 $(M + Na)^+$. ^{1}H-NMR ($CDCl_3$) δ: 0.74, 0.96, 0.97, 0.98, 1.28 (3H each, s, CH3 x 5), 1.23 (3H, d, *J* = 4.5 Hz, 30-CH_3), 2.54 (1H, s, H-18), 5.41 (1H, br t, H-12), 5.55 (1H, d, *J*= 8 Hz, Glc-1). ^{13}C-NMR: see Table 1.

Compound **3**

Chromatographic behavior of this compound suggested its non glycosidic nature. TLC comparison of **3** with the aglycone of **1** indicated the same compound. Bidimensional and co-chromatography with triterpene authentic samples confirmed compound **3** as rotundic acid (3β,19α,23-trihydroxyurs-12-en-28-oic acid).

RESULTS AND DISCUSSION

Compound **1** afforded glucose on acid hydrolysis. Its ^{1}H-NMR and ^{13}C-NMR data indicated

Carbon	**1** (C_5D_5N)	**2a** ($CDCl_3$)
1	38.9	37.7
2	27.0	25.7 [a)]
3	73.8	77.0
4	42.8	51.6
5	48.7	50.5
6	18.8	22.8
7	33.2	32.4
8	40.6	40.0
9	47.8	47.0
10	37.2	36.5
11	24.1	23.9
12	128.4	128.7
13	139.3	137.3
14	42.1	40.9
15	29.2	28.1
16	26.7	23.5 [a)]
17	48.6	47.7
18	54.4	52.8
19	72.7	72.9
20	42.1	40.9
21	26.1	25.1
22	37.7	36.2
23	68.2	180.2
24	13.0	11.5
25	17.5	15.5
26	16.6	15.9 [b)]
27	24.5	24.0
28	177.1	175.6
29	27.5	27.2
30	15.1	16.6 [b)]
glucose		
1'	95.8	91.5
2'	73.9	69.7
3'	78.8	72.7
4'	71.3	67.8
5'	79.1	72.3
6'	62.4	61.4

Table 1. ^{13}C NMR spectral data for compound **1** and peracetylated compound **2a.**
[a,b)] Assignments may be interchanged in each column

the presence of only one β-glucosyl unit (δ 6.25, 1H, d, J = 8 Hz, H-1'; δ 95.8, C-1'), both chemical shifts evidencing an ester linkage between the sugar portion and the aglycone. The ^1H-NMR spectrum of **1** showed six methyl singlets and one methyl doublet. In the ^{13}C-NMR spectrum, one set of glucose signals was observed and thirty aglycone signals, including three oxygenated carbon signals at δ 73.8 (CH), 72.7 (C) and 68.2 (CH_2). The first signal was attributed to C-3 substituted with a free hydroxyl group and the second was assigned to C-19, bearing a tertiary hydroxyl group. The quaternary nature of C-19 is inferred by the proton singlet signal at δ 2.91 (1H, br s), assignable to H-18 of a 19-hydroxylated ursane skeleton [13]. The third oxygenated carbon was assigned to an hydroxymethylene group at C-23 or C-24, considering the molecular peak at *m/z* 650 (CIMS), and the EIMS, that showed two characteristic peaks at *m/z* 264 (8%) and 246 (10%) denoting the retro-Diels-Alder cleavage fragments commonly found in olean-12-ene or urs-12-ene derivatives possessing two hydroxyl groups in rings A/B and hydroxyl and carboxyl groups in rings D/E. The hydroxymethylene group was assigned to C-23 considering the ^{13}C chemical shifts of the methyl groups. The shielded methyl group (δ 13.0, s) was assignable to the 4β-methyl group considering that an α-methyl group in the same neighborhood was observed at δ 23.7 [13]. This is confirmed by the C-4 and C-5 chemical shifts observed at high field (δ 42.8 and 48.7, respectively). Comparison of the ^{13}C-NMR data of compound **1** with those reported for pedunculoside (28-O-β-D-glucopyranosylester of rotundic acid) [14] established the identity of the two compounds. Pedunculoside has previously been isolated from *Ilex rotunda, I. pedunculosa* and *I. oldhami* [15] and was also isolated as the main saponin from the leaves of *I. taubertiana Loes* [8].

Peracetylated compound **2 (2a)** was obtained as amorphous powder and afforded glucose on acid hydrolysis. Its ^1H-NMR indicated the presence of one β-glucosyl unit (H-1: δ 5.5, d, J= 8 Hz) linked to the aglycone by an ester linkage. Its ^{13}C-NMR spectrum revealed thirty six carbon signals and the thirty belonging to the aglycone included characteristic signals due to two carboxyl groups (δ 180.2 and 175.6), a trisubstituted double bond (δ 128.7 and 137.3) and two oxygenated carbons (δ 77.0 and 72.9). A signal at δ 2.54 (1H, br s, H-18) suggested the presence of a 19-O-substituted urs-12-ene skeleton. Comparison of the ^{13}C-NMR data of **2a** with those of **1** showed that the main differences were one additional carboxyl group in **2a** instead of an hydroxymethylene group in **1**. Comparison of the ^{13}C-NMR data of **2a** with those of the 28-O-β-D-glucopyranosyl ester of rotundioic acid, previously isolated from the fresh leaves of *Ilex rotunda* [15] established the identity of the two compounds.

TLC comparison of compound **3** with authentic samples of triterpenoids and with the aglycone obtained by alkaline hydrolysis of

compound **1** revealed that compound **3** is rotundic acid (3β,19α,23-trihydroxyurs-12-en-28-oic acid). This triterpene was previously isolated from the fruits [13,14], seeds [16] and leaves [17] of *Ilex rotunda;* also from *I. pedunculosa* and *I. oldhami* [17] species distributed in Eastern Asia, and more recently from the leaves of *I. taubertiana* Loes., a South American species [8].

From the South American *Ilex* species so far investigated the chemical structure of *I. paraguariensis* saponins is unique, and they belong to the ursane or oleanane series. The saponins isolated from *Ilex theezans* have a 19-α-hydroxy-ursane acid derivative as aglycone (Figure 1), a type of aglycone that has never been isolated from *I. paraguariensis,* nor from *I. dumosa* [6,7] but that could be identified from *I. taubertiana* [8], *I. pseudobuxus* [9] and *I. argentina* [10]. The knowledge of the chemical composition of these species can be helpful in understanding the infrageneric relationships within the genus *Ilex* and furthermore to the development of methodologies for the quality control of maté products based on the saponins content.

The saponin content of *Ilex paraguariensis* and of other *Ilex* species raised the question of the contribution of these compounds to the taste of maté. Currently, we are testing the bitterness of the aqueous extracts, crude saponin fractions and isolated saponins using the filter paper method [18] by which the minimal amount of extracts or isolated saponins perceivable as a bitter taste is detected. The aqueous extracts of *I. theezans* displayed a lower threshold value of 200 μg in comparison with the value obtained for that of *I. paraguariensis* (500 μg) [19]. So far, the pedunculoside isolated from *I. theezans* and *I. taubertiana* displayed the lowest threshold value of 4 μg, so this compound is roughly one hundred times more bitter than matesaponin 1, fifty times more bitter than matesaponin 2 and twenty five times more bitter than matesaponin 4, all isolated from *Ilex paraguariensis* [19].

Finally, and noteworthy, the presence of adulterants like *Ilex theezans* leaves might, beside the unknown physiological and pharmacological consequences, influences the taste of maté, causing a wide variation on the product bitterness, being therefore an important factor that can affect its acceptance by the consumers.

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