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Saponins from *Ilex pseudobuxus*

Alexandre T. C. TAKETA and Eloir P. SCHENKEL *

Faculdade de Farmácia, UFRGS, Av. Ipiranga 2752, 90610-000 Porto Alegre, RS. Brazil.

SUMMARY. ***Ilex pseudobuxus*** **Reissek is one of the species refered as an adulterant of Mate (*Ilex paraguariensis* St. Hil.). Two main saponins have been isolated from its leaves and their structures were established as pomolic acid 28-O-α-L-rhamnopyranosyl-(1-2)-β-D-glucopyranoside and rotungenic acid 28-0-α-L-rhamnopyranosyl-(1-2)-β-D-glucopyranoside.**

RESUMEN. "Saponinas de *Ilex pseudobuxus*". *Ilex pseudobuxus* Reissek es una de las especies que ha sido mencionada como adulterante de la yerba mate *(Ilex paraguariensis* St. Hil.). El análisis de la fracción glucosídica de las hojas mostró la presencia predominante de dos saponinas, el 28-O-α-L-ramnopiranosil-(1-2)-β-D-glucopiranósido del ácido pomólico y el 28-0-α-L-ramnopiranosil-(1-2)-β-D-glucopiranósido del ácido rotungénico.

INTRODUCTION

Genus *Ilex,* with some 600 species, is numerically the most important of the family *Aquifoliaceae.* From an economical and sociocultural point of view, the main species is *Ilex paraguariensis* St. Hil., widely used in South Brazil, Argentina, Paraguay and Uruguay as a beverage (Mate) and also as a traditional medicinal plant [1]. The historical and also the current utilization of other plants in the mate preparation are well known, being the congeneric adulteration the more documented one [2,3].

Following our work on the saponins from *Ilex paraguariensis* [4], other *Ilex* species used to adulterate mate have been studied for their saponin content. In the present study we report the isolation and structural elucidation of two saponins from the leaves of *Ilex pseudobuxus* Reiss., one of the species refered as an adulterant of mate and also as a medicinal plant with febrifuge activity [2,3].

KEY WORDS: *Aquifoliaceae, Ilex pseudobuxus,* saponins.
PALABRAS CLAVE: Aquifoliaceae, Ilex pseudobuxus, saponinas.

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

General experimental procedures

Mp's were determined with Kofler's Apparatus and are uncorrected. FABMS spectra were performed on Kratos MS 80 RFA. NMR spectra of compound 2 were recorded with Varian XL 300 (1H-NMR: 300 MHz; 13C-NMR: 75 MHz); compound 1 with Bruker AM 400 (400/100 Hz) and Bruker AMX 500(500/125 MHz).

TLC were performed on silica gel Merck GF, using the following solvent systems: chloroform: ethanol: water, 140:50: 8 (system 1); petrolether: ethyl acetate, 50:50 (system 2); chloroform: ethanol: water, 65:50:8 (system 3); and ethyl acetate: methanol: acetic acid: water, 60:15:15:10 (system 4). Spray reagents: anisaldehyde-H2SO4/UV 360 nm for aglycones and saponins, and aniline-phthalate/UV 360 nm for sugars.

Plant material

The leaves of *I. pseudobuxus* were collected in Campo Bom, State of Rio Grande do Sul, Brazil, on November 1989. A herbarium specimen is deposited in the Botany Department Herbarium of Rio Grande do Sul Federal University (ICN-83281), Porto Alegre.

Isolation of the saponins

The dried leaves (180 g) were crushed, extracted through maceration with ethanol (1 l) and the extract was subsequently evaporated to dryness (36 g). A portion of the extract (6,6 g) was redissolved in n-butanol (250 ml) and successively extracted with Na_2CO_3 5% and NaOH 1% solutions, until phenolic compounds were eliminated (TLC follow up). The resulting butanolic fraction (1,6 g) with the saponin mixture was chromatographed on a column with silica gel (170 g) by gradient elution (chloroform: ethanol: water 140:50:8 and then 130:50:8). 80 fractions of 10 ml each were collected. TLC showed that fractions 11-32 contained mainly compound 1 (187 mg), fractions 33-50 contained a mixture of compounds 1 and 2 (74 mg) and fractions 51-76 contained mainly compound 2 (315 mg). Fractions with compound 1 were rechromatographed over silica gel (20 g) by gradient elution (chloroform: ethanol: water 200:50:8 to 120:50:8), resulting in 23 mg chromatographically pure compound 1. Because the same procedure was not successful for compound 2, a purification by preparative TLC (silica gel 60, chloroform: ethanol: water 70:50:8) was carried out. The subsequent extraction of the silica with methanol, resulted in 34 mg pure compound 2.

Acid hydrolysis

The saponins were hydrolysed as described by Kartning and Wegschaider [5] by two dimensional TLC and analysed for aglycone (system 2) and sugar components (systems 3 and 4).

Alkaline hydrolysis

The isolated compounds 1 and 2 (each 5 mg) were refluxed in KOH 10 % solutions in ethanol. After neutralization with HCl (18%) and extraction with ethyl

acetate, the aqueous phase was concentrated and extracted with pyridine. The organic phase was analysed by TLC for the aglycone (system 2) and the pyridine extract for sugar components (systems 3 and 4).

Acetylation of compound 1

Compound 1 (13.8 mg) was acetylated with acetic anhydride and pyridine at room temperature for 18 h, followed by usual work up, to yield 15 mg acetylated compound 1 as a colorless powder.

Saponin T1

White powder, mp 189-190 °C. TLC: Rf 0.30 (system 1). FAB/MS *m/z:* 803 $[M+Na]^+$, 413, 149. For ^1H and ^{13}C NMR data, see Tables 1 and 2.

Acetylated compound 1

mp 119 °C. ^1H NMR δ($CDCl_3$): 5.37 (1H, br t, 12-H), 3.43 (s, 18-H), 4.84 (dd, 3-H); methylprotons: 0.73 (s, 3H), 0.85 (s, 6H), 0.92 (s, 3H), 0.94 (d, J = 6 Hz), 1.22 (s, 3H), 1.25 (s, 3H); sugar protons (assigned by HH-Cosy): 5.65 (d, 8 Hz, 1'-H), 3.90 (t, 8 Hz, 2'-H), 5.19 (t, 9.4 Hz, 3'-H), 5.14 (t, 9.4 Hz, 4'-H), 3.84 (m, 5'-H), 4.78 (dd, 12.0/ 4.0 Hz, 6'-H), 4.00 (dd, 12.0/ 2.5 Hz, 6'-H) for the glucopyranose and 5.08 (s, 1''-H), 5.11 (partial overlap, 2''-H), 5.21 (partial overlap, 3''-H), 5.065 (t, 10Hz, 4''-H), 3.96 (dq, 10.0/ 6.0 Hz, 5''-H), 1.19 (d, 6.0 Hz,6''-H) for the rhamnopyranose.

Saponin T2 (2)

White powder, mp 193 °C. TLC: Rf 0.17 (system 1). FABMS *m/z (positive mode):* 819 $[M+ Na]^+$, 835 $[M+K]^+$, 550, 453, 391. (Negative mode) 795 $[M-H]^-$, 612, 487, 459. NMR data for the sugar chain: see Table 2; ^1H NMR for the aglycone: see Table 3; ^{13}C NMR for the aglycone (carbon 1 to 30) δ (C_5D_5N): 39.0, 28.0, 80.3, 43.2, 56.6, 19.3, 34.1, 40.6, 48.0, 37.2, 24.4, 128.4, 139.3, 42.3, 29.6, 26.2, 48.8, 54.8, 72.9, 42.0, 26.8, 37.6, 23.7, 64.6, 17.5, 16.3, 24.5, 176.5, 27.1, 16.9.

RESULTS AND DISCUSSION

The ethanol extract of the leaves of *I. pseudobuxus* yielded, after elimination of phenolic compounds, a butanolic fraction with two glycosides (pseudobuxus saponins T1 and T2), which were purified by repeated column chromatography and preparative TLC. Acid hydrolysis on TLC allowed the characterization of glucose and rhamnose as sugar components for both compounds. The aglycones could not be clearly detected as many spots appeared on the TLC. This suggested a degradation of these compounds by the acidic hydrolysis procedure. Alkaline hydrolysis resulted in the same sugars, suggesting ester glycoside structures.

Compound 1 exhibited an intense molecular ion peak $[M+Na]^+$ at *m/z* 803 on FAB-MS. The ^1H-broadband-decoupled ^{13}C-NMR spectrum contains 42 signals; the DEPT subspectra revealed eight methyl, ten methylene, sixteen methine and eight quaternary C atoms. Considering the presence of glucose, rhamnose and the mass spectrum, the empirical formula $C_{42}H_{68}O_{13}$ can be derived. The ^1H and 13C spectra confirmed the presence of one α-rhamnopyranosyl unit (H1' δ = 6.64, s; C1' δ = 101.3) and one ester bounded β-glucopyranosyl unit (H1' δ = 6.15, d, J = 8 Hz; C1' δ = 94.9). The ^1H-NMR displayed a ^1H singlet signal at δ = 2.88, that considered together with the ^{13}C-NMR signal at δ = 72.6 for a quaternary oxygenated carbon indicated a 19-hydroxylated ursane derivative.

C	δ_C	DEPT	H-C correlations	H-H correlations
1	39.1	CH_2	A: 0.93 B: 1.55	1.55 0.93 / 1.82-1.87
2	28.1	CH_2	1.82-1.87	3.39 / 1.55
3	78.2	CH	3.39 (dd, 11 Hz, 5 Hz)	1.82-1.87
4	39.3	C		COLOC: 0.83 / 0.99 / 1.16
5	55.9	CH	0.83	1.37 / 1.48 COLOC: 0.96 / 1.16 / 1.55 / 1.74
6	19.0	CH_2	1.37 (dl, 14 Hz) 1.48*	0.83
7	33.8	CH_2	1.60 (dl) 1.68	1.48
8	40.5	C		COLOC: 1.18 / 1.71
9	47.8	CH	1.74	
10	7.3	C		COLOC: 0.96 / 1.18 / 1.48
11	24.0	CH_2	2.06 (m)	5.59
12	128.3	CH	5.59 (t, 4 Hz)	2.06 COLOC: 2.88 / 2.06
13	139.3	C		COLOC: 2.88 / 1.71 / 2.06
14	142.2	C		2.16 / 1.71 / 1.18
15	29.6	CH_2	1.65 (m) 2.26 (ddd, 14 Hz, 14 Hz, 4 Hz)	1.65 / 2.17
16	26.1	CH_2	2.17 (m, 12 Hz) 3.20 (ddd, 14 Hz, 12 Hz, 4 Hz)	3.20 / 2.26 1.65 / 2.26
17	48.7	C		COLOC: 1.25 / 1.65 / 2.88
18	54.7	CH	2.88 (s)	COLOC: 1.44
19	72.6	C		COLOC: 1.44 / 1.45 / 1.25
20	41.8	CH	1.45*	
21	26.6	CH_2	1.25 (dl, 11 Hz) 2.02 (m)	2.02 1.25
22		CH_2	1.9 (dd, 14 Hz, 4 Hz) 2.05*	2.05 1.95 / 2.02
23	28.7	CH_3	1.16(s)	COLOC: 0.99
24	16.5	CH_3	0.99 (s)	COLOC: 0.83
25	15.7	CH_3	0.96 (s)	COLOC: 0.83
26	17.5	CH_3	1.18 (s)	
27	24.3	CH_3	1.71 (s)	COLOC: 2.26
28	176.9	C		COLOC: 2.88
29	27.0	CH_3	1.44 (s)	
30	16.6	CH_3	1.08 (d. J = 6 Hz)	1.45

* Multiplicity and/or coupling constants obscure, due to partial overlap.

Table 1. ^1H and ^{13}C NMR spectral data for the aglycone of compound 1 (pyridine-d_5, δ values).

For further structure elucidation, the connectivity was established by the combination of two dimensional NMR methods (HH-Cosy, CH-Cosy and CH-Coloc), according to Table 1 and 2. These data correspond to a pomolic acid glycoside-6, with the sugar chain at C-28.

For the sugar chain, using the HH and HC correlations, it was possible to assign all carbon and proton signals. The low field displacement of the glucose C-3 (δ = 80.1) suggested it to be the attachment point of the terminal rhamnose.

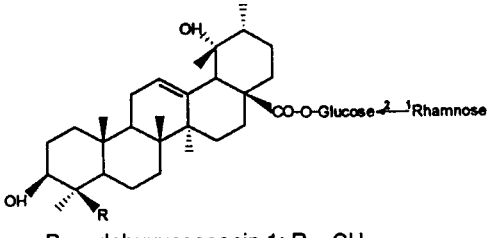
Compound 1			Compound 2
Carbon δ_C	DEPT	δ_H	δ_C δ_H
Glucose			
C1	94.9 CH	H_1' = 6.155 (d = 8 Hz)	94.9 H1' = 6.14 (d = 8 Hz)
C2	75.0 CH	H_2' = 4.525 (t = 8 Hz)	75.1 H2' = 4.525 (t = 8 Hz)
C3	80.1 CH	H_3' = 4.35 (t = 9 Hz)	80.1 H3' = 4.34 (t = 9 Hz)
C4	71.5 CH	H_4' = 4.285 (t = 9 Hz)	71.6 H4' = 4.28 (t = 9 Hz)
C5	79.0 CH	H_5' = 4.02 (ddd; J = 12; 4; 2.5 Hz)	79.0 H5' = 4.01 (ddd; J = 12; 4; 2.5 Hz)
C6	62.3 CH_2	4.465 (dd; J = 12.0; 2.5 Hz) 4.35 (dd; J = 12.0; 4.0 Hz)	62.4 4.45 (dd; J = 12.0; 2.5 Hz) 4.35*
Rhamnose			
C1	101.3 CH	H_1" = 6.57 (br s)	101.3 H1' = 6.67 (d. 1 Hz)
C2	72.3 CH	H_2" = 4.8 (dd; J = 1.5; 3.5 Hz)	72.4 H2' = 4.79 (dd; J = 1.5; 3.5 Hz)
C3	72.5 CH	H_3" = 4.59 (dd; J = 3.5; 10 Hz)	72.6 H3' = 4.57 (dd; J = 3.5; 10 Hz)
C4	73.8 CH	H_4" = 4.32 (t; J = 9.5 Hz)	73.8 H4' = 4.315 (t ; J = 9.5 Hz)
C5	69.7 CH	H_5" = 4.65 (dq; J = 10.0; 6.0 Hz)	69.7 H5' = 4.65 (dq; J = 10.0; 6.0 Hz)
C6	18.7 CH_3	H_6" = 1.75 (d; J = 6.0 Hz)	18.8 H = 1.74 (d; J = 6.0 Hz)

Table 2. NMR spectral data for the sugar chain at compound 1 and compound **2** (pyridine-d_5).

compound 2			rotungenic acid methylester	
H	**δ (ppm)**	**J (Hz)**	**δ (ppm)**	**J (Hz)**
3	3.4 bd	12	3.44 brdd	11.5; 4.5
12	5.4 brt	3.5	5.35 brt	3.5
16	2.96 brt	12		
18	2.68 s		2.6 brs	
23	1.26 s		1.26 s	
24	3.4 brd 4.24 d	 11	3.35 brd 4.2 d	11 11
25	0.86 s		0.86 s	
26	0.68 s		0.66 s	
27	1.26 s		1.25 s	
29	1.3 s		1.25 s	
30	0.95 d	6	0.97 d	7

Table 3. ^{1}H-NMR (300 MHz, pyridine-d_5/D_2O spectral data of 2 and rotungenic acid methylester (360 MHz, $CDCl_3$) *.

* cited from reference 9



Pseudobuxussaponin 1: R = CH_3

Pseudobuxussaponin 2: R = CH_2OH

However, a recent publication [7] for another *Ilex* species, reported the same ^{13}C-values (deviation 0.5 ppm) for a glycoside obtained by enzymatic hydrolysis of Ilexoside IX, elucidated as pomolic acid 28-O-α-L-rhamnopyranosyl-(1-2)-β-D-glucopyranoside. The authors [7] assigned the signals at δ = 79.8 and δ = 75.5 to carbons 2 and 3 of the glucose. According to the correlation spectra of compound 1, the signal at δ = 80.1 corresponds to carbon 3, though this does not represent a proof for linkage at this point. Therefore the correlation spectra of the peracetylated compound 1 was measured. The abnormally high shielding of the glucose H2 (δ = 3.90) is typical for a proton at the sugar linkage[8], showing that this position is not acetylated, therefore corresponding to the branching point. Consequently, compound 1 has the same structure as the glycoside reported for *Ilex crenata* fruits [7]. Thus this work contributes to the correct assignment for this known compound and might be of chemotaxonomical importance, since the same saponin occurs in both *Ilex* species.

Compound 2 exhibited a molecular ion at *m/z* 819 $[M+ Na]^+$ at the positive modus, and at *m/z* 795 $[M-H]^-$ at the negative modus. The DEPT subspectra revealed seven methyl, eleven methylene, sixteen methine and eight quaternary C atoms, suggesting a formula $C_{42}H_{68}O_{13}$ (molecular mass: 796). The analysis of the HH and HC correlation spectra indicated the same features as in compound 1. A 1-H singlet signal (δ = 2.68) assignable to 18-H of a 19-hydroxylated ursane, an oxygenated quaternary carbon (δ = 72.7) and a carbon signal at δ = 94.9 for the glucose moiety linked with 28-COOH, in the β-configuration. The ^{13}C-NMR spectra of the two compounds were very similar, except for the the signals attributable to carbons 3, 4 and 5 and that one of the eight methyl groups in compound 1 was replaced with a hydroxymethyl group (δ = 64.6). It is reported that a hydroxymethyl group at the 4α-position (C23) provokes a high field shielding for the C24-methyl group (to *ca.* δ = 11-13). Considering the absence of signals upfield of δ = 16.0, the most probable position for the hydroxygroup is C24. A comparison with the reported spectral data for hydroxylated triterpenoid acids indicated that the ^{13}C- and 1H-values for the aglycone of compound 2 are coherent with the values for rotungenic acid (Table 3) [9]. As to the sugar chain, compound 2 shows the same values as compound 1. These results led to the proposal of saponin T2 as rotungenic acid 28-O-α-L-rhamnopyranosyl-(1-2)-β-D-glucopyranoside. Rotungenic acid has been first reported for *Ilex rotunda* [9] and the derivative here described is a new natural compound.

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