Recibido el 24-07-2012
Aprobado el 04-09-2012

BIOLOGICAL ACTIVITIES OF 13, 28-EPOXYOLEANANE TRITERPENE SAPONINS FROM TWO PERUVIAN *MYRSINACEAE*

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ABSTRACT

Two known 13,28-epoxy-oleanane triterpene saponins (**1**) and (**2**), were isolated from the 95% ethanolic extract of the roots of *Myrsine coriaceae* and *Myrsine andina*. Their structures were deduced by combined spectral analysis and chemical evidences based on data reported in the literature. Compounds **1** and **2** were evaluated *in vitro* against different cellular models such as *Mycobacterium tuberculosis*, *Leishmania amazonensis* axenic amastigotes, six human cancer cell lines (Hs683, T98G, U251, HT29, MCF7, SKMEL28) and two murine cell lines (CT26 and B16F10). Compound **1** was found to exhibit antileishmanial activity (IC_{50} = 16 µg/mL) whereas compound **2** was inactive (IC_{50} > 50 µg/mL). Furthermore, compound **1** exhibited stronger inhibition activity on human cancer cells (IC_{50} = 15 µg/mL) and on murine cell lines (IC_{50} = 10 µg/mL) than compound **2** (IC_{50} > 82 and 42 µg/mL, respectively**)**. As the only difference between **1** and **2** is due to a substitution of an aldehyde group by a hydroxymethyl moiety, these results showed the crucial role of the aldehyde function at C-30 for the cytotoxicity. In contrast, none of the tested compounds revealed activity against *M. tuberculosis*.

Key words: Saponins, Myrsinaceae, leishmaniasis, tuberculosis

RESUMEN

Dos saponinas triterpénicas de tipo 13,28-epoxi-oleanano (**1**) y (**2**), han sido aisladas a partir del extracto etanólico al 95% de las raíces de *Myrsine coriaceae* y *Myrsine andina*. Las estructuras fueron establecidas mediante la combinación de técnicas espectrales y evidencias químicas con datos reportados en la literatura. Los compuestos **1** y **2** fueron evaluados con ensayos biológicos *in vitro* frente a diferentes modelos celulares, tales como *Mycobacterium tuberculosis***,** amastigotes axénicos de *Leishmania amazonensis* , macrófagos extraídos del peritoneo de ratón, seis líneas celulares de cáncer humano (Hs683, T98G, U251, HT29, MCF7, SKMEL28) y dos líneas de células murinas (CT26 y B16F10). El compuesto **1** mostró una buena actividad antileishmania (IC_{50} = 16 µg/mL) mientras que el compuesto **2** no mostró actividad alguna (IC_{50}> 50 µg/mL). Por otra parte, el compuesto **1** mostró una mayor actividad de inhibición de las células cancerosas humanas (IC_{50} = 15 µg/mL) y en líneas celulares de ratón (IC_{50} = 10 µg/mL) que el compuesto **2** (IC_{50}> 82 y 42 µg/mL, respectivamente). Como la única diferencia entre **1** y **2** se debe a una sustitución de un grupo aldehído por un grupo hidroximetilo, estos resultados demuestran el papel esencial de la función aldehído en C-30

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para la citotoxicidad. En contraste, ninguno de los compuestos ensayados reveló actividad frente a *M. tuberculosis*.

Palabras clave: Saponinas, Myrsinaceae, leishmaniasis, tuberculosis

INTRODUCTION

Infectious diseases such as leishmaniasis and tuberculosis (TB), commonly being considered as neglected diseases, are prevalent in Third World countries. Because most of the affected population live in developing countries and cannot afford existing drugs, both diseases have been ignored by pharmaceutical industry. Leishmaniasis is a parasitic protozoal disease caused by parasites belonging to the genus *Leishmania*, occurring in subtropical and tropical regions. An estimated 12 million people are infected worldwide in 88 countries with an annual incidence of about 2–3 million.[1,2] Clinically, leishmaniasis occurs in visceral, cutaneous and mucocutaneous forms, 90% of the latter occurring in Bolivia, Brazil and Peru. It is a major public health problem in tropical and subtropical regions for which development of drug resistance by the parasites has worsened this problem. In the absence of effective vaccines, chemotherapy still plays a critical role in treating this disease.[3]

As far as TB is concerned, this is a common and in some cases deadly infectious disease, caused by an important intracellular pathogen *Mycobacterium tuberculosis. This disease* causes 2 million deaths per year worldwide, with 98% occurring in developing countries. One of the highest incidence rates in the Americas occurs in Peru.[4,5] Current standard treatments for tuberculosis include antibacterial drugs such as Rifampin and Isoniazid, which require between six to twelve month-therapies to fully eliminate *Mycobacterium* from the body. As very few treatments are available, there is an urge to discover new treatments with less toxicity and low manufacturing costs.

Cancer, responsible for 7.6 million deaths worldwide in 2008 with approximately 70% occurring in low- and middle-income countries, represents another major health issue.[6] In such countries.[7a-d] including Peru,[8] some plant extracts are used to combat various types of cancers and provide evidence that plants represent a major reservoir within which we may identify novel anticancer drugs.[9a-c]

In the course of our investigations on compounds with pharmacological properties and since it has been shown that the 13,28-epoxy bridge in the saponin skeleton was putatively assigned as crucial for antileishmania activity,[10] we carried out a literature search in order to find saponins showing the same structural features as the Maesabalides. These compounds, possessing a 13,28-epoxy-oleanane bridge and isolated previously from *Maesa balansae* (Myrsinaceae),[11] have been shown to possess potent and highly specific *in vitro*[12] and *in vivo*[13] antileishmania activity. Vermeersch *et al.*, (2009)[14] reviewed all those saponins from Myrsinaceae, Primulaceae, Aceraceae and Icacinaceae families possessing a completely saturated pentacyclic triterpene skeleton and a 13,28-epoxy bridge, in which C(28) was a methylene or a hydroxymethylene group, excluding the C(28) carbonyl derivatives. For all the studied plants, a clear correlation was found between the presence of close analogue 13,28-epoxy-oleanane triterpene saponins and a potent and selective antileishmania activity. Furthermore, Aegicerin, an oleanane-type pentacyclic triterpene skeleton with this 13,28-epoxy bridge, has shown a consistently high level of activity against a large panel of both sensitive and resistant strains of *M. tuberculosis*[15].

In this article, we report the isolation and structure elucidation of two known triterpenoid saponins, Ardisiacrispin B (**1**) and Ardisicrenoside A (**2**), previously isolated from several Myrsinaceae plants.[16,17] Both compounds were found to possess the 13,28-epoxy bridge previously demonstrated as crucial for antileishmanial activity *in vitro*[12] and *in vivo*.[13] We

evaluated their activity *in vitro* against *Leishmania amazonensis* axenic amastigotes alongside other pathogenic cellular model, e.g. *Mycobacterium tuberculosis*, as well as two mouse and six human cancer cell lines.

To the best of our knowledge, this is the first report of the isolation of saponins from the *Myrsine andina* (Mez.) Pipoly and *Myrsine coriacea* (Sw.) R. Br. ex Roem. & Schult

EXPERIMENTAL

General

Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a sodium lamp (k = 589 nm) in a 1 cm microcell. The structures of the isolated compounds were identified by nuclear magnetic resonance (NMR; Bruker Avance 500 equipped with a TBI z-gradient 5 mm probe). ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and 2D-NMR analysis in C_5D_5N; NMR experiments were performed at 298 K using standard pulse sequences. Chemical shifts () are given in ppm relative to TMS with coupling constant (*J*) reported in Hz. ESI-MS (positive and negative-ion mode) spectra were recorded on an ion trap LCQ Finnigan spectrometer. Column chromatography and medium-pressure column chromatography were performed on silica gel 60 SDS 70–200 m and 6–35 m, respectively. TLC was carried out on precoated silica gel 60 F254 aluminium plates (Merck). Spots were detected under UV (254 and 366 nm) before spraying with diphenylborate reagent or a vanillin sulfuric solution followed by heating the plate at 110 °C. All solvents were spectral grade or distilled from glass prior to use.

Plant material

The roots of *Myrsine andina* (Mez.) Pipoly and *Myrsine coriacea* (Sw.) R. Br. ex Roem. & Schult were collected near Oxapampa, Chacos, Peru by M. Haddad, C. Amasifuen, J. Mateo, J. Perea in July 2009, and identified by the botanists J. Mateo and C. Amasifuen. A voucher specimen of each (3472 and 1207, respectively) were deposited at the National Herbarium of the San Marcos University in Lima, Peru (UNMSM).

Extraction and isolation

The air-dried and powdered roots of *Myrsine andina* and *Myrsine coriacea* (500 g and 300 g, respectively) were exhaustively macerated with 95% EtOH (3 x 3 L) at room temperature to yield 29.1 g and 52.8 g of crude extract after evaporation of the solvent in vacuo, respectively. Both residues were dissolved in the least amount of MeOH and the solution was diluted with tenfold amount of diethyl-ether to precipitate 2 g and 2.3 g of crude saponin mixture, respectively, and then partitioned with *n*-BuOH saturated with H_2O (3 x 500 mL). The combined *n*-BuOH extract was evaporated under vacuum to dryness to afford the n-BuOH layer residue (1.1 g and 1 g, respectively). The saponin rich precipitates (1.1 and 1 g, respectively) were collected by filtration and fractionated by successive MPLC (silica gel 60, (15–40 μm), $CHCl_3$–MeOH–H_2O 7.5:2.5:0.25 and $CHCl_3$ – MeOH – H_2O 8:2:0.25) to yield **1** and **2**.

Compound 1: Ardisiacrispin B

White amorphous powder. ^{1}H-NMR (C_5D_5N, 500 MHz) and ^{13}C-NMR (C_5D_5N, 125 MHz), (tables 1); ESI-MS (positive-ion mode) *m/z* 1097 $[M + Na]^+$, ESI-MS (negative-ion mode) *m/z* 1073 $[M – H]^-$, 927 $[(M – H) – 146]^-$, 765 $[(M – H) – 146 – 162]^-$, 603 $[(M – H) – 146 – 162 – 162]^-$, and 471 $[(M – H) – 146 – 162 – 162 – 132]^-$.

Compound 2: Ardisicrenoside A

White amorphous powder. ^{1}H-NMR (C_5D_5N, 500 MHz) and ^{13}C-NMR (C_5D_5N, 125 MHz), (tables 1); ESI-MS (positive-ion mode) *m/z* 1099 $[M + Na]^+$. ESI-MS (negative-ion mode) *m/z* 1075 $[M – H]^-$, 929 $[(M – H) – 146]^-$, 767 $[(M – H) – 146 – 162]^-$ and 605 $[(M – H) – 146 – 162 – 162]^-$.

Table 1. NMR Spectroscopic data of aglycon and sugar moieties of compounds **1** and **2.**

Aglycone	**1**		**2**		Sugar	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
1	0.81, 1.61	38.9	0.79, 1.61	38.9	Ara 1	4.95	104.2	4.94	104.2
2	1.82, 1.99	26.3	1.81, 1.98	26.4	2	4.59	80.6	4.58	80.5
3	3.13 br d (9.0)	88.8	3.11 br d (9.2)	88.8	3	4.49	72.2	4.49	72.0
4	-	39.5	-	39.5	4	4.58	74.4	4.58	74.4
5	0.62	55.4	0.62	55.4	5	3.79, 4.39	63.4	3.79, 4.40	63.4
6	1.37, [b]	17.7	1.38, [b]	17.7	Glc-1	5.37 d (7.5)	105.2	5.37 d (7.7)	105.2
7	1.49, [b]	34.3	1.50, [b]	34.1	2	4.07	76.2	4.07	76.2
8	-	44.0	-	42.4	3	4.29	77.9	4.23	77.9
9	1.22	50.1	1.23	50.3	4	4.22	71.5	4.13	71.6
10	-	36.7	-	36.7	5	4.06	77.8	4.06	77.9
11	1.70, nd	18.8	1.39, 1.72	18.9	6	4.38, [b]	62.6	4.38, 4.49	62.4
12	1.52, 1.94	32.1	1.46, 2.10	32.7	Glc-1'	5.23 d (7.5)	102.9	5.24 d (7.4)	102.9
13	-	86.1	-	86.3	2'	4.28	77.7	4.28	77.0
14	-	42.3	-	44.4	3'	4.19	79.4	4.20	79.4
15	1.24, 2.15	36.6	1.50, 2.24	36.8	4'	4.13	71.6	4.14	71.6
16	4.20	77.7	4.20	76.9	5'	3.79	78.2	3.60	77.6
17	-	43.9	-	44.4	6'	4.29, 4.45	62.4	4.30, 4.46	62.3
18	2.06	50.9	1.85	50.9	Rha-1	6.41 br. *s*	101.5	6.42 br. *s*	101.4
19	2.82, nd	33.1	2.03, 2.76	33.2	2	4.50	72.5	4.73	72.2
20	-	48.2	-	36.6	3	4.68	72.4	4.69	72.6
21	2.52, 2.66	30.3	1.68, 2.59	32.4	4	4.25	79.0	4.28	74.6
22	1.55, nd	32.1	1.67, 1.87	31.5	5	5.05	69.2	5.04	69.2
23	1.15	27.5	1.14	27.5	6	1.79	18.2	1.80	18.7
24	1.10	16.3	1.00	16.3					
25	0.80	16.3	0.80	16.2					
26	1.26	18.3	1.31	18.3					
27	1.52	19.6	1.58	19.5					
28	3.15, 3.57	77.5	3.30, 3.60	77.7					
29	1.00	23.9	1.33	28.8					
30	9.60	207.3	3.74, 3.99	65.7					

[a] ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of **1** and **2** in C_5D_5N. The assignments were based on DEPT, ^{1}H , ^{1}H-COSY, TOCSY, NOESY, HMQC and HMBC experiments.

[b] Not determined.

Bioassays

Compounds **1** and **2** were evaluated against *L. amazonensis* axenic amastigotes *in vitro*[18] and against *M. tuberculosis in vitro*[15]. Two independent experiments were carried out to determine the *in vitro* growth inhibitory activity of compounds **1** and **2** in cancer cell lines. This growth inhibitory activity was determined by means of the sulforhodamine B (SRB) assay method and the MTT colorimetric assay, respectively, as detailed previously.[19a-b; 20a-d]

RESULTS

Extraction of the roots of *Myrsine andina* (Mez.) Pipoly and *Myrsine coriacea* (Sw.) R. Br. ex Roem. & Schult and purification of the extract as described in the experimental section yielded compounds **1** and **2**. The elucidation of their structures was performed mainly by 500 MHz NMR analysis, including 1D- and 2D-NMR (COSY, TOCSY, NOESY, HSQC, HMBC), and mass spectrometry.

Compound **1** was obtained as a white crystalline powder with molecular formula $C_{53}H_{86}O_{22}$, which was determined by analysis of the MS data. Its positive ESI-MS showed the pseudomolecular ion peak at *m/z* 1097 $[M + Na]^+$. This was confirmed from its negative ion ESI-MS that showed the quasimolecular ion peak at *m/z* 1073 $[M - H]^-$. Other fragment ion peaks were observed at *m/z* 927 $[(M - H) - 146]^-$, 765 $[(M - H) - 146 - 162]^-$, 603 $[(M - H) - 146 - 162 - 162]^-$, and 471 $[(M - H) - 146 - 162 - 162 - 132]^-$ corresponding to the successive loss of one deoxyhexosyl, two hexosyl and one pentosyl moieties, respectively.

Of the 53 carbons in the ^{13}C-NMR spectrum (pyridine-d_5), 30 were assigned to the triterpenoid skeleton and 23 to the oligosaccharide moiety. Among the 30 carbons of the triterpene skeleton in the ^{13}C-NMR spectrum, 6 were assigned to the methyl carbons at δ_C 16.3, 16.3, 18.3, 19.6, 23.9 and 27.5 ppm, and the methyl protons were identified by the HSQC experiments at δ_H 1.10, 0.80, 1.26, 1.52, 1.00 and 1.15 ppm, respectively. Two methine carbons bearing oxygen were found at δ_C 88.8 and 79.4 ppm. The structural assignment was initiated from the long-range coupling networks observed between the methyl protons and the adjacent carbons from the HMBC experiments. Extensive NMR analysis (*table 1*) showed that the aglycone was an oleanane skeleton with an oxygen bridge between Agly- 13 (δ_C 86.1) and Agly-28 (δ_C 77.5). This was confirmed by comparison of the NMR data with known spectral data for structurally related compounds.[11,21,22]

The HMBC correlations between two angular methyl groups at δ_H 1.10 (3H, *s*, Me(24)) and δ_H 1.15 (3H, *s*, H–C(23)) with δ_C 88.8 (C(3)) confirmed the location of the first secondary alcoholic function at Agly-3. The absolute configuration of Agly-3 and Agly-16 was determined from a NOESY experiment: cross-peaks at δ_H 3.13 (1H, *d*, *J* = 9.0, Agly-3)/ δ_H 0.62 (1H, br. *d*, *J* = 12.0, Agly-5), and δ_H 4.20 (1H, br. *s*, Agly-16)/ δ_H 1.26 (3H, *s*, Me(26)), confirmed the absolute configuration of the aglycone. Based on these findings, the aglycone was identified as 3β,16α dihydroxy-13β,28-epoxyoleanane.

The ^{1}H-NMR spectrum of **1** displayed signals for four anomeric protons at δ_H 4,94 (d, *J* = 4,27 Hz), 5,24 (d, *J* = 7,91 Hz), 5,37 (d, *J* = 7,63 Hz), 6.41 (H, br. s), which correlated with the carbon signals at δ_C 104.2, 102.9, 105.2, and 101.5, respectively, in the HSQC spectrum.

The ring protons of the monosaccharide residues were assigned starting from the anomeric protons by means of the COSY, TOCSY, HSQC, and HMBC NMR plots (table 1), and the sequence of the oligosaccharide chains was obtained from the HMBC and NOESY experiments. All the protons within each spin system were delineated using COSY with the aid of TOCSY and NOESY spectra. After assignments of the protons, the ^{13}C-NMR resonances of each sugar unit were identified by HSQC and further confirmed by HMBC. HPTLC of the acid hydrolysate of **1** in comparison with reference sugars, NMR data of related structures[11,21] lead to the identification of the four monosaccharide units as β-D-glucose (Glc x2), one α-L-arabinose (Ara), and one α-L-rhamnose (Rha). The COSY and TOCSY spectra confirmed the presence of the one α-rhamnopyranosyl (Rha) from their typical pattern in the COSY spectrum[23,24] and the α-configuration of this latter was also confirmed by observation of NOESY correlations between δ_H (Rha-1), δ_H (Rha-3), and δ_H (Rha-5). This information was confirmed by the δ_C (Rha-1) non-splitting pattern of rhamnose unit and the three-bond HMBC correlations from δ_H (Rha-1) to δ_C (Rha-3) and δ_C (Rha-5) indicated that its anomeric H-atom was equatorial, thus possessing an α-configuration in the $^1C_4$ form.[25]

The relatively large $^3J_{H\text{-}1,H\text{-}2}$ values of the Glc (7.5–7.8), indicated a β anomeric orientation for the Glc.[26]

A cross peak observed in the NOESY spectrum at δ_H 4.94 (1H, d, *J* = 4.27, Ara-1)/ $_H$ 3.13 (1H, br. *d*, *J* = 9.0, Agly-3), revealed a substitution at C(3) of the aglycon by an arabinosyl moiety. This was confirmed by a correlation observed in the HMBC spectrum between an anomeric signal at δ_H 4.94 (1H, d, *J* = 4.27, Ara-1) and δ_C 88.8 (Agly-3) and by the reverse correlation at δ_H 3.13 (1H, br. *d*, *J* = 9.0, Agly-3) and δ_C 104.20 (Ara-1). The HMBC correlation between

δ_H 5,37 (d, J = 7,63 Hz, Glc-1), and δ_C 80.6 (Ara-2) and the reverse correlation at δ_H 4.59 (1H, m, Ara-2) and (C) 105.2 (Glc-1) indicated that Glc was linked to C(2) of Ara. This was also confirmed by a NOESY cross peak between δ_H 5,37 (d, J = 7,63 Hz, Glc-1) and δ_H 4.59 (1H, m, Ara-2). Furthermore, the HMBC correlation between δ_H 5.23 (1H, *d*, J = 7.91, Glc-1')) and δ_C 74.4 (Ara-4), together with the reverse correlation at δ_H 4.58 (1H, *m*, Ara-4) and δ_C 102.9 (Glc-1')) and the NOESY correlation between δ_H 5.23 (1H, *d*, J = 7.5, Glc-1') and δ_H 4.58 (1H, *m*, Ara-4) established the (14) linkage between Glc' and Ara. The substitution of the glucopyranosyl moiety at the δ_H Glc-2') position by a rhamnopyranosyl residue was deduced according to the reverse correlations in the HMBC spectra at δ_H 6.41 (1H, br. *s*, Rha-1)/ δ_C 77.7 (Glc-2') and δ_H 4.28 (1H, *m*, Glc-2')/ δ_C 101.5 (Rha-1)), together with the additional NOESY cross-peaks at δ_H 6.41 (1H, br. *s*, Rha-1)/ δ_H 4.28 (1H, *m*, Glc-2').

On the basis of the above results, the structure of **1** was elucidated as 3-β-Oα-l-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→3)-[β-D-glucopyranosyl (1→2)]-β-d-arabinopyranosyl}-16α-hydroxy-13β,28-epoxy-oleanane.

Compound **2** was obtained as a white crystalline powder with molecular formula $C_{53}H_{88}O_{22}$ that was determined by analysis of the MS data. Its positive ESI-MS showed the pseudomolecular ion peak at *m/z* 1099 $[M + Na]^+$. This was confirmed from its negative ion ESI-MS that showed the quasimolecular ion peak at *m/z* 1075 $[M - H]^-$. Other fragment ion peaks were observed at *m/z* 929 $[(M - H) - 146]^-$, 767 $[(M - H) - 146 - 162]^-$ and 605 $[(M - H) - 146 - 162 - 162]^-$ corresponding to the successive loss of one deoxyhexosyl and two hexosyl, respectively.

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Among the 30 carbons of the triterpene skeleton in the ^{13}C-NMR spectrum, 6 were assigned to the methyl carbons at δ_C 16.2, 16.3, 18.3, 19.5, 27.5 and 28.8 ppm, and the methyl protons were identified by the HSQC experiments at δ_H 0.80, 1.00, 1.31, 1.58, 1.14 and 1.33 ppm, respectively. Two methine carbons bearing oxygen were found at δ_C 88.8 and 76.9 ppm. The structural assignment was initiated from the long-range coupling networks observed between the methyl protons and the adjacent carbons from the HMBC experiments. Extensive NMR analysis (*table 1*) showed that the aglycone was an oleanane skeleton with an oxygen bridge between Agly-13 (δ_C 86.3) and Agly-28 (δ_C 77.7). This was confirmed by comparison of the NMR data with known spectral data for structurally related compounds.[11,21,22]

The HMBC correlations between two angular methyl groups at δ_H 1.00 (3H, *s*, Me-24) and δ_H 1.14 (3H, *s*, Me-23) with δ_C 88.8 (Agly- 3) confirmed the location of the first secondary alcoholic function at C(3). The absolute configuration of Agly-3 and Agly- 16 was determined from a NOESY experiment: cross-peaks at δ_H 3.11 (1H, *d*, J = 9.2, Agly-3)/ δ_H 0.62 (1H, br. *d*, J = 12.0, Agly-5), and δ_H 4.20 (1H, br. *s*, Agly- 16)/ δ_H 1.31 (3H, *s*, Me(26)), confirmed the absolute configuration of the aglycone. Based on these findings, the aglycone was identified as 3β,16α dihydroxy-13β,28-epoxyoleanane.

Table 2. Antimycobacterial (MIC μg/mL) and antiprotozoal (IC_{50} μM) activity.

Compounds	*M. tuberculosis*		*L. amazonensis amastigotes*
	$H_{37}Rv$	MDR	
1	> 25	> 25	16.0
2	> 25	> 25	> 50
Anf.[a]	-	-	0.2
Inh.[b]	0.125	32.0	-

[a]Amphotericin B. [b]Isoniazid.

Experiments were conducted on axenic amastigotes of *Leishmania amazonensis* (strain MHOM/BR/76/LTB-012) according to Estevez *et al.*[16] The experiment has been carried twice with 4 replicates per experimental condition

Evaluation of the antimycobacterial activity *in vitro* was performed using the TEMA method[17] against MDR and sensitive strains of *Mycobacterium tuberculosis*. The experiment has been carried once with 6 replicates per experimental condition

The ^{1}H NMR spectrum of **2** displayed signals for four anomeric protons at δ_H 4.94 (d, J = 4.27 Hz), 5.24 (d, J = 7.42 Hz), 5.37 (d, J = 7.71 Hz), 6.42 (1H, br. s), which correlated with the carbon signals at δ_C 104.2, 102.9, 105.2, and 101.4, respectively, in the HSQC spectrum.

The ring protons of the monosaccharide residues were assigned starting from the anomeric protons by means of the COSY, TOCSY, HSQC, and HMBC NMR plots (table 1), and the sequence of the oligosaccharide chains was obtained from the HMBC and NOESY experiments. All the protons within each spin system were delineated using COSY with the aid of TOCSY and NOESY spectra. After assignments of the protons, the ^{13}C-NMR resonances of each sugar unit were identified by HSQC and further confirmed by HMBC. HPTLC of the acid hydrolysate of **2** in comparison with reference sugars, NMR data of related structures[11, 21] lead to the identification of the four monosaccharide units as β-D-glucose (Glc x2), one α-L-arabinose (Ara) and one α-L-rhamnose (Rha). The COSY and TOCSY spectra confirmed the presence of the one α-rhamnopyranosyl (Rha) from their typical pattern in the COSY spectrum[23,24] and the -configuration of this latter was also confirmed by observation of NOESY correlations between Rha-1, Rha-3, and Rha-5'. This information was confirmed by the Rha-1 non-splitting pattern of rhamnose unit and the three-bond HMBC correlations from H– Rha-1 to Rha-3 and Rha-5 indicated that its anomeric H-atom was equatorial, thus possessing an -configuration in the $^1C_4$ form.[25]

The relatively large $^3J_{H\text{-}1,H\text{-}2}$ values of the Glc (7.5–7.8), indicated a β anomeric orientation for the Glc[26].

The ^{1}H- and ^{13}C-NMR signals of **2** assigned from 2D-NMR spectra were almost superimposable on those of **1**, except for the disappearance of the signals corresponding to the aldheyde group (δ_C 207.3, C-30) replaced by a hydroxymethyl function (δ_C 65.7) (table 1).

On the basis of the above results, the structure of **2** was elucidated as 3-β-Oα-l-rhamnopyranosyl (1→2)-β-D-glucopyranosyl (1→3)-[β-D-glucopyranosyl (1→2)]-β-d-arabinopyranosyl}-16α-hydroxy-30-hydroxymethyl-13β,28-epoxy-oleanane.

Compounds **1** and **2** were evaluated *in vitro* against different cellular models such as *Mycobacterium tuberculosis*[15], *Leishmania amazonensis* axenic amastigotes[16], mouse peritoneal macrophages, six human cancer cell lines (Hs683, T98G, U251, HT29, MCF7, SKMEL28) and two murine cell lines (CT26 and B16F10) (table 3).

Table 3. *In vitro* growth inhibition determined in 2 mouse and 6 human cancer cell lines.

Compounds	IC_{50} growth inhibitory concentrations (μM) after 72 h of treatment of human cancer cells with the compounds						
	Hs683	**T98G**	**U251**	**HT-29**	**MCF-7**	**SKMEL-28**	**m**
1	19	22	17	6	22	7	15
2	38	> 100	55	> 100	> 100	> 100	> 82

Compounds	IC_{50} growth inhibitory concentrations (μM) after 72 h of treatment of murine cancer cells with the compounds		
	CT-26.WT	**B16F10**	**m**
1	15	4	10
2	44	40	42

These experiments have been carried out in the Laboratory of Toxicology – Faculty of Pharmacy – Université Libre de Bruxelles (Brussels, Belgium) using the MTT colorimetric assay to determine the IC_{50} growth inhibitory concentrations of compounds **1** and **2** in 2 mouse (CT-26.WT and B16F10) and 6 human cancer cell lines. These cell lines included the Hs683 oligodendroglioma (obtained from the American Type Culture Collection [ATCC], Manassas, VA; code HTB-138), the T98G glioblastoma (ATCC code CRL-1690), the U251 glioblastoma (ECACC code 09063001), the HT-29 colon cancer (ATCC code HTB-38), the MCF-7 breast cancer (Deutsche Sammlung von Mikroorganismen and Zellkulturen [DSMZ], Braunschweig, Germany; code ACC115), the SKMEL-28 melanoma (ATCC code HTB-72), the CT26.WT colon cancer (ATCC code CRL-2638) and the B16F10 melanoma (ATCC code CRL-6475). The experiment has been carried once with 6 replicates per experimental condition.

Compound **1** was found to exhibit antileishmanial activity (IC_{50} = 16 μg/mL) whereas compound **2** was inactive (IC_{50} > 50 μg/mL). Furthermore, compound **1** exhibited stronger inhibition activity on human cancer cells (IC_{50} = 15 μg/mL) and on murine cell lines (IC_{50} = 10 μg/mL) than compound **2** (IC_{50} > 82 and 42 μg/mL, respectively**)**. As the only difference between **1** and **2** is due to a substitution of an aldehyde group by a hydroxymethyl moiety, these results showed the crucial role of the aldehyde function at C-30 for the cytotoxicity. The possibility remains that this growth inhibitory activity is related to compound **1** and **2** mediated cytotoxic effects on the parasites' host cell itself. One possible explanation previously raised to explain the cytotoxicity of such saponins on macrophages is that saponins are amphiphilic and induce the formation of micelles, which can be easily taken up by phagocytic cells.[27]

Although other triterpenes have been shown to exhibit antibacterial activity against *Mycobacterium tuberculosis*, this was not the case for saponins **1** or **2** (IC_{50} > 25 μM). Literature reports that the presence of a carbonyl group at the C-16 position of a triterpene would be crucial to exhibit anti-mycobacterial activity. For example, protoprimulagenin A, bearing a hydroxyl moiety at C-16, displays no antibacterial activity whereas aegicerin, having almost the same structure but with a carbonyl group at C-16, exhibits marked antibacterial activity against a large number of resistant *Mycobacterium* strains.[15]

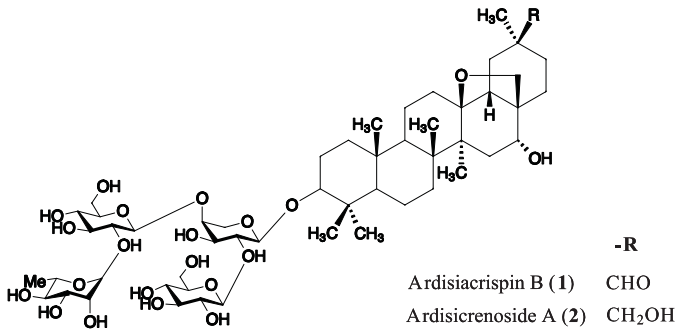

Figure 1. Chemical structures of compounds **1** and **2**.

CONCLUSIONS

- In conclusion, the structures of two known 13,28-epoxy-oleanane triterpene saponins (**1**, **2**) isolated from two Peruvian Myrsinaceae, *Myrsine coriacea* and *Myrsine andina*, were elucidated. This is the first report of saponins isolation from these plants. The present study demonstrated that compound **1** displayed significant anti-tumor activities towards a broad spectrum of human cancer cells. Furthermore, we demonstrated the crucial role of the aldehyde function at C-30 for the cytotoxicity. Further experiments should be carried out in order to investigate their ability to induce apoptosis in different cancer cell lines. These results may be useful in the search for new compounds for cancer prevention and therapy.

ACKNOWLEDGMENTS

The authors are grateful to the FRB (Fondation pour la Recherche sur la Biodiversité, France) for the financial support through a grant to Dr. M. Haddad (Project DIPERSAP). The authors thank Rodolfo Vasquez, Rocío Rojas, José Mateo and Juan Perea from the Missouri Botanical Garden of Oxapampa, Peru and Carlos Amasifuen, for their help to collect the plants for this project. We thank Dr. Nicolas Fabre from the University of Toulouse, France, for assistance in obtaining ^{1}H,^{13}C-NMR and MS spectra. We also thank Laetitia Moreno Y Banuls and Robert Kiss, PhD student and Director of Research, respectively, with the Fonds National de la Recherche Scientifique (FRS-FNRS; Belgium) for performing the biological assays.

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