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Pro-apoptotic activity of acylated triterpenoid saponins from the stem bark of *Albizia chevalieri* harms

Olivier Placide Noté[a,b,*], Lin Marcellin Messi[b,c], Joséphine Ngo Mbing[b], Sarah Ali Azouaou[a], Mamadou Sarr[d], Dominique Guillaume[e], Christian Dominique Muller[f], Dieudonné Emmanuel Pegnyemb[b], Annelise Lobstein[a]

[a] *Pharmacognosie et Molécules Naturelles Bioactives, Laboratoire d'Innovation Thérapeutique, UMR 7200, CNRS-Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, F-67401 Illkirch Cedex, France*
[b] *Laboratoire de Pharmacochimie des Substances Naturelles, Département de Chimie Organique, Faculté de Sciences, Université de Yaoundé I, BP 812 Yaoundé, Cameroon*
[c] *Institut de Recherche Agricole pour le Développement (IRAD), BP 2067 Yaoundé, Cameroon*
[d] *UMI 3189, FMPO, University Anta Diop of Dakar, Senegal*
[e] *UFR Médecine-Pharmacie, CNRS-UMR7312, 51 rue Cognacq Jay, 51100 Reims, France*
[f] *Institut Pluridisciplinaire Hubert Curien, UMR 7178 CNRS Université de Strasbourg, 74 route du Rhin, F-67401 Illkirch Cedex, France 67401Illkirch, France*

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ABSTRACT

As a continuation of our interest in apoptosis-inducing triterpenoid saponins from *Albizia* genus, phytochemical investigation of the stem bark of *Albizia chevalieri* led to the isolation of three new oleanane-type saponins, named chevalierosides A–C (**1–3**). Their structures were established on the basis of extensive analysis of 1D and 2D NMR (^{1}H-, ^{13}C NMR, DEPT, COSY, TOCSY, ROESY, HSQC and HMBC) experiments, HRESIMS studies, and by chemical evidence. The pro-apoptotic effect of the three saponins was evaluated on two human cell lines (pancreatic carcinoma AsPC-1 and hematopoietic monocytic THP-1). Cytometric analyses showed that saponins **1–3** induced apoptosis of both human cell lines (AsPC-1 and THP-1) in a dose-dependent manner.

1. Introduction

The genus *Albizia* comprises about 150 species widely distributed in the tropics, with the greatest diversity in Africa and South America (Abdel-Kader et al., 2001). Phytochemical investigations of *Albizia* genus revealed the presence of triterpenoid saponins, consisting mainly of glycosides of oleanolic acid, echinocystic acid, and acacic acid. These glycosides have demonstrated inhibitory activity of the growth of many cancer cells lines *in vitro* (Abdel-Kader et al., 2001; Haddad et al., 2003; Liu et al., 2009; Krief et al., 2005; Zheng et al., 2006; Melek et al., 2007; Cao et al., 2007; Noté et al., 2009, 2015; Miyase et al., 2010a,b). But few reports revealed the proapoptotic function of triterpenoid saponins isolated from plants of *Albizia* genus. In our knowledge, only three studies carried out on *Albizia adianthifolia*, *Albizia zygia*, and *Albizia glaberrima*, respectively, have shown the pro-apoptotic effect of adianthifoliosides A and D (Haddad et al., 2004), zygiaosides A–B (Noté et al., 2016a), and of glaberrimosides A–C (Noté et al., 2016b) on cancer cells lines. One of our recent contribution revealed the first pro-apoptotic activity of glycosides of oleanolic acid, so far isolated from *Albizia* genus with an interesting apoptosis-inducing selectivity degree effect on AsPC-1 cell line (Noté et al., 2016b), which was earlier described as a model of cancer stem cells (CSCs) (Wei et al., 2011). As a continuation of our interest in apoptosis-inducing triterpenoid saponins from *Albizia* genus (et al., 2016a,b; et al., 2016a,b), we screened the saponins content of *Albizia chevalieri.*

A. chevalieri harms (Mimosaceae) is a tree that grows up to 12 m high or a shrub growing under harsher conditions of the dry savannah from Senegal, Niger, Nigeria, and Cameroon (Saidu et al., 2007). Its leaves extract is used for the management of diabetes mellitus in Niger and in Eastern Nigeria, and its stem bark has been reported as a remedy for coughs (Saidu et al., 2007). The decoction of its leaves is used in Northern Nigeria to treat dysentery (Arbonnier, 2004). Its aqueous roots and leaves extracts have been reported to possess significant hypoglycaemic and hypolipidemic effects (Burkill 1995; Saidu et al., 2010). Previous phytochemical studies of *A. chevalieri* revealed the presence of triterpenoids (Mathias et al., 2016) from its stem bark and of glucosides of flavanol from its roots (Tchoukoua et al., 2016).

In the present paper, we report the isolation and structure

* Corresponding author at: Laboratoire de Pharmacochimie des Substances Naturelles, Département de Chimie Organique, Faculté de Sciences, Université de Yaoundé I, BP 812, Yaoundé, Cameroun
E-mail address: oliviernote1@yahoo.fr (O.P. Noté).

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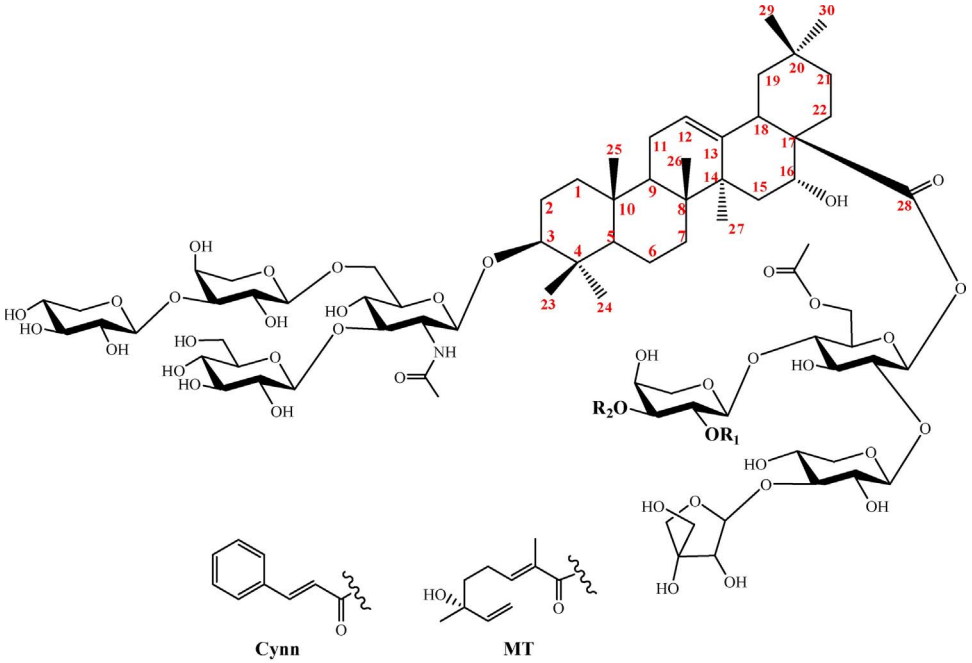

	R_1	R_2
1	Cynn	H
2	MT	H
3	Cynn	MT

Fig. 1. Structures of isolated compounds **1**- **3**.

characterization of three new triterpenoid saponins, named chevalierosides A–C (**1–3**), along with the evaluation for their pro-apoptotic effect on two human tumor cells, a human pancreatic cancer cell line AsPC-1 and a hematopoietic monocytic cancer cell line THP-1, results are reported herein.

2. Results and discussion

The air-dried powdered roots of *A. chevalieri* (150 g) were extracted with 70% EtOH in soxhlet and after evaporation of the solvent, the hydroalcoholic extract was partitioned against *n*-BuOH saturated with water. The *n*-BuOH fraction was then submitted to vacuum-liquid chromatography (VLC) on reversed phase silica gel yielding a methanol fraction that was subjected to a VLC on silica gel. Purification of the eluted fractions by semipreparative high pressure liquid chromatography (HPLC) afforded three new echinocystic acid glycosides, named chevalierosides A–C (**1–3**) (Fig. 1).

Chevalieroside A (**1**), obtained as a white amorphous powder, was assigned a $C_{86}H_{129}NO_{41}$ molecular formula, as deduced from the $[M+NH_4]^+$ ion at *m/z* 1849.8384 in the positive ion mode HRESIMS. Upon acid hydrolysis with 2.0 M HCl, **1** afforded echinocystic acid unit, which was identified with an authentic sample, and the sugar components 2-amino-2-deoxy-glucose (GlcNHAc), glucose (Glc), apiose (Api), xylose (Xyl) and arabinose (Ara), which were identified by co-TLC with authentic samples (Section 3). The absolute configuration of these sugar residues was determined to be D for GlcNHAc, Glc, Api, Xyl, and L for Ara based on GC analysis of their trimethylsilylated derivatives (Section 3) (Chaabi et al., 2010).

Extensive analysis of 1D and 2D NMR spectra (^{1}H NMR, ^{13}C NMR, DEPT, COSY, ROESY, HSQC and HMBC) indicated the presence of seven tertiary methyl groups at δ_H 0.75, 0.93, 0.95, 0.97, 1.06, 1.22, and 1.83, an olefinic broad triplet proton at δ_H 5.55 (brt, J = 3.5 Hz, H-12) attached to a carbon at δ_C 122.8 (C-12), a quaternary carbon at δ_C 144.5 (C-13), one oxymethine proton at δ_H 3.26 (brd, J = 7.6 Hz, H-3), and sugars signals at δ_H 4.83–6.30, evidencing that **1** was an oleanane type triterpenoid saponin. The unambiguous assignment of all ^{1}H and ^{13}C NMR signals of the aglycone of **1** (Table 1), identified as echinocystic, using correlations observed in COSY, ROESY, HSQC, and HMBC spectra, was in full agreement with literature data (Melek et al., 2014; Miyase et al., 2010a, 2010b). Substitutions at C-3 and C-28 of echinocystic acid were evidenced by the observed glycosylation-induced shifts of C-3 at δ_C 89.6 and of C-28 at δ_C 176.0 (Table 1) establishing that **1** was a 3,28-bidesmosidic echinocystic acid derivative with sugar chains linked to C-3 and C-28 of the aglycone through an ether and ester bond, respectively (Woldemichael and Wink, 2001; Sahu and Achari, 2001).

The ^{1}H NMR spectrum of the sugar portion of compound **1** showed eight anomeric signals at δ_H 5.08 [d, J = 7.3 Hz, 2-amino-2-deoxy-glucose (GlcNHAc)], 5.28 [d, J = 6.6 Hz, arabinose (Ara I)], 4.83 [d, J = 2.2 Hz, xylose (Xyl I)], 5.55 [d, J = 7.6 Hz, glucose (Glc I)], 6.06 [d, J = 8.2 Hz, glucose (Glc II)], 5.00 [d, J = 8.0 Hz, arabinose (Ara II)], 5.40 [d, J = 7.6 Hz, xylose (Xyl II)], and 6.30 [d, J = 2.2 Hz, apiose (Api)], which correlated with eight anomeric carbon atom resonances at δ_C 104.5, 103.5, 108.0, 104.5, 92.9, 103.1, 105.2, and 111.6, respectively in the HSQC spectrum (Table 2). The ^{1}H and ^{13}C NMR data (Table 2) of the monosaccharide residues were assigned starting from the readily identifiable anomeric proton of each hexosyl or pentosyl unit, by means of COSY, TOCSY, ROESY, and HMBC spectra (Table 2). The ^{13}C NMR data indicated that the sugar residues Glc, GlcNHAc, Xyl, and Ara were in their pyranose form, whereas the Api residue was in furanose form. The anomeric proton of Xyl I was determined to have the α-orientation based on its relatively small $^3J_{H\text{-}1,H\text{-}2}$ value of 2.2 Hz, whereas Xyl II was *β*-orientated based on its relatively large $^3J_{H\text{-}1,H\text{-}2}$ value of 7.6 Hz. The anomeric protons of Api was *β*-orientated based on its relatively small $^3J_{H\text{-}1,H\text{-}2}$ value of 2.2 Hz. The remaining anomeric protons of Glc, GlcNHAc, and Ara, were determined to have *β*, and α anomeric configuration, respectively, based on their relative large $^3J_{H\text{-}1,H\text{-}2}$ values (6.6–8.2 Hz) in their pyranose form. The anomeric signal at δ_H 5.08 (d, J = 7.3 Hz) was assigned to 2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl moiety (GlcNHAc) based on the observation its characteristic signals at δ_H 9.08 (d, J = 8.0 Hz,

Table 1
NMR spectroscopic data (500 MHz for ^{1}H and 125 MHz for ^{13}C) for the aglycone moieties of compounds **1-3** (δ in ppm and J in Hz).

No	**1**		**2**		**3**	
	δ_C	δ_H (mult.)	δ_C	δ_H (mult.)	δ_C	δ_H (mult.)
1a	38.7	0.92	39.4	0.94	38.8	0.92
1b		1.45		1.54		1.48
2a	26.4	1.70	26.1	1.79	26.7	1.77
2b		2.13		2.20		2.16
3	89.6	3.26	89.7	3.25	89.8	3.25
4	39.3	–	39.8	–	39.4	–
5	56.0	0.78	56.8	0.78	56.2	0.82
6a	18.6	1.27	18.3	1.32	18.9	nd
6b		1.57		1.59		nd
7a	33.6	1.52	33.9	1.51	33.8	1.52
7b		1.58		1.62		1.62
8	40.1	–	40.9	–	40.2	–
9	47.2	1.76	46.9	1.79	47.2	1.79
10	37.0	–	36.7	–	37.1	–
11a	23.8	1.93	23.9	1.99	24.3	1.98
11b		2.00		2.13		2.16
12	122.8	5.55	122.5	5.64	123.1	5.56
13	144.5	–	144.2	–	144.4	–
14	42.0	–	41.7	–	42.1	–
15a	36.4	1.76	36.5	1.85	36.2	1.78
15b		2.36		2.39		2.40
16	75.3	5.65	75.2	5.69	75.0	5.65
17	49.1	–	49.5	–	49.2	–
18	41.2	3.44	41.0	3.51	41.4	3.48
19a	47.1	1.32	47.8	1.37	47.4	1.33
19b		2.79		2.83		2.80
20	30.9	–	30.6	–	31.0	–
21a	35.9	1.20	35.6	1.28	36.2	1.22
21b		2.42		2.47		2.40
22a	32.3	2.17	32.7	2.20	32.5	2.16
22b		2.29		2.37		2.29
23	28.2	1.22 (s)	28.9	1.28 (s)	28.4	1.22 (s)
24	17.1	0.93 (s)	17.7	0.98 (s)	17.4	0.92 (s)
25	15.6	0.75 (s)	16.8	0.83 (s)	15.9	0.78 (s)
26	17.4	0.95 (s)	18.1	1.01 (s)	17.7	0.94 (s)
27	27.2	1.83 (s)	27.9	1.85 (s)	27.6	1.83 (s)
28	176.0	–	176.0	–	176.1	–
29	33.3	0.97 (s)	33.9	1.02 (s)	33.6	0.98 (s)
30	24.6	1.06 (s)	24.2	1.12 (s)	24.0	1.07 (s)

Assignments were based on the HMBC, HSQC, COSY and DEPT experiments.

Table 2
NMR spectroscopic data (500 MHz for ^{1}H and 125 MHz for ^{13}C) for the sugar moieties of compounds **1-3** (δ in ppm and J in Hz).

Position	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
3-*O*-Sugars						
GlcNHAc						
1	104.5	5.08 (d, 7.3)	104.2	5.10 (d, 7.3)	104.8	5.09 (d, 7.3)
2	57.7	4.47	56.8	4.55	57.9	4.50
3	81.0	4.86	81.3	4.88	81.2	4.89
4	73.4	4.50	73.2	4.59	73.4	4.51
5	75.0	4.00	74.9	4.05	75.0	3.97
6	68.2	4.69; 4.78	67.8	4.67; 4.80	68.4	4.69; 4.77
$NHCOCH_3$	23.8	2.16	23.4	2.16	23.9	2.16
Ara-I						
1	103.5	5.28 (d, 6.6)	103.0	5.30 (d, 6.6)	103.8	5.28 (d, 6.6)
2	73.3	4.51	73.2	4.53	73.4	4.51
3	83.5	4.53	82.5	4.59	83.7	4.54
4	69.7	4.14	69.4	4.15	69.7	4.13
5	66.5	3.65; 4.22	66.3	3.74; 4.29	66.8	3.64; 4.23
Xyl-I						
1	108.0	4.83 (d, 2.2)	107.1	4.85 (d, 2.2)	108.2	4.84 (d, 2.2)
2	75.4	4.05	74.5	4.07	75.3	4.07
3	78.3	3.97	77.6	4.01	78.4	3.97
4	71.1	4.27	71.1	4.26	71.2	4.25
5	67.5	3.49; 4.36	66.3	3.53; 4.38	67.7	3.47; 4.35
Glc-I						
1	104.5	5.55 (d, 7.6)	104.0	5.51 (d, 7.6)	104.7	5.56 (d, 7.6)
2	75.2	4.05	74.6	4.06	75.4	4.05
3	78.2	4.30	77.7	4.31	78.4	4.30
4	71.1	4.28	71.1	4.26	71.2	4.28
5	78.4	3.95	77.9	4.01	78.5	3.98
6	62.3	4.33; 4.49	62.5	4.35; 4.54	62.5	4.35; 4.52
28-*O*-Sugars						
Glc-II						
1	92.9	6.06 (d, 8.2)	92.7	6.06 (d, 8.2)	93.1	6.06 (d, 8.2)
2	78.7	4.29	77.8	4.31	78.3	4.28
3	76.0	4.14	76.0	4.15	76.0	4.14
4	80.2	4.03	80.2	4.06	80.0	4.05
5	73.9	3.89	73.3	3.91	73.3	3.87
6	63.5	4.51; 4.60	63.5	4.54; 4.65	63.2	4.52; 4.61
Ara-II						
1	103.1	5.00 (d, 8.0)	101.8	5.17 (d, 8.0)	103.1	4.98 (d, 8.0)
2	75.2	5.65	75.0	5.67	75.2	5.65
3	74.0	4.13	74.4	4.16	74.0	4.14
4	68.9	4.26	71.1	4.26	69.1	4.27
5	67.5	3.76; 4.37	66.3	3.57; 4.39	67.6	3.73; 4.77
Xyl-II						
1	105.2	5.40 (d, 7.6)	104.4	5.39 (d, 7.6)	105.1	5.39 (d, 7.6)
2	75.3	4.04	74.8	4.03	75.2	4.04
3	84.8	4.15	84.9	4.16	84.9	4.14
4	71.1	4.27	71.0	4.26	71.0	4.28
5	66.6	3.65; 4.26	66.2	3.67; 4.28	66.7	3.65; 4.26
Api						
1	111.6	6.30 (d, 2.2)	110.9	6.23 (d, 2.2)	111.9	6.30 (d, 2.2)
2	78.1	4.82	77.7	4.85	78.1	4.83
3	80.6	–	80.3	–	80.7	–
4	75.3	4.37; 4.79	74.8	4.41; 4.79	75.5	4.38; 4.79
5	65.8	4.21; 4.25	64.8	4.23; 4.25	65.8	4.21; 4.26

Assignments were based on the HMBC, HSQC, COSY and DEPT experiments.

$NHCOCH_3$) and δ_H 2.16 (s, $NHCOCH_3$) (Abdel-Kader et al., 2001). This assignment was supported by the correlation observed in the HMBC spectrum between the proton signal at δ_H 4.47 (H-2 of GlcNHAc) and the carbonyl signal at δ_C 170.2 ($NHCOCH_3$). In addition, the ^{1}H NMR spectrum of compound **1** exhibited two proton signals of a *trans*-olefinic group at δ_H 6.85 (d, J = 16.2 Hz) and δ_H 8.02 (d, J = 16.2 Hz), and five aromatic proton signals for a monosubstituted benzene group for a cinnamoyl moiety. It was also observed signals characteristic for acetyl group (δ_H/δ_C 1.86/170.8). The sequencing of the glycoside chains was achieved by analysis of HMBC and ROESY experiments.

For the sugar chain attached at C-3 of the aglycone, the cross peak correlations observed in the HMBC spectrum between H-1 (δ_H 5.08) of GlcNHAc and C-3 (δ_C 89.6) of the aglycone, and in the ROESY spectrum between H-1 (δ_H 5.08) of GlcNHAc and H-3 (δ_H 3.26) of echinocystic acid, suggested that GlcNHAc was directly attached to C-3 of the aglycone. Moreover, the HMBC correlation observed between H-1 (δ_H 5.55) of Glc I and C-3 (δ_C 81.0) of GlcNHAc established the connectivity between the two sugar units, which was confirmed by the ROESY correlation observed between H-3 (δ_H 4.86) of GlcNHAc and H-1 (δ_H 5.55) of Glc I. On the other hand, the HMBC correlation observed between H-1 (δ_H 5.28) of Ara I and C-6 (δ_C 68.2) of GlcNHAc allowed us to locate Ara I at C-6 of GlcNHAc. This was supported by the ROESY correlation observed between H-1 (δ_H 5.28) of Ara I and H-6a (δ_H 4.69) of GlcNHAc. This Ara I was substituted at its C-3 by Xyl I, as evidenced by the direct and reverse correlations observed in the HMBC spectrum between H-1 (δ_H 4.83) of Xyl I and C-3 (δ_C 83.5) of Ara I and between H-3 (δ_H 4.53) of Ara I and C-1 (δ_C 108.0) of Xyl I. This was supported by the ROESY correlation observed between H-1 (δ_H 4.83) of Xyl I and H-3 (δ_H 4.53) of Ara I. The terminal position of Xyl I was evidenced by the absence of any ^{13}C NMR glycosylation shifts for this sugar moiety. Thus, the tetrasaccharide α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[β-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-β-D-glucopyranosyl moiety was established to be linked at C-3 of the aglycone (Fig. 2).

For the sugar chain attached at C-28 of the aglycone, the cross peak observed in the HSQC spectrum at δ_H/δ_C 6.06/δ 92.9 (Glc II H-1/C-1)

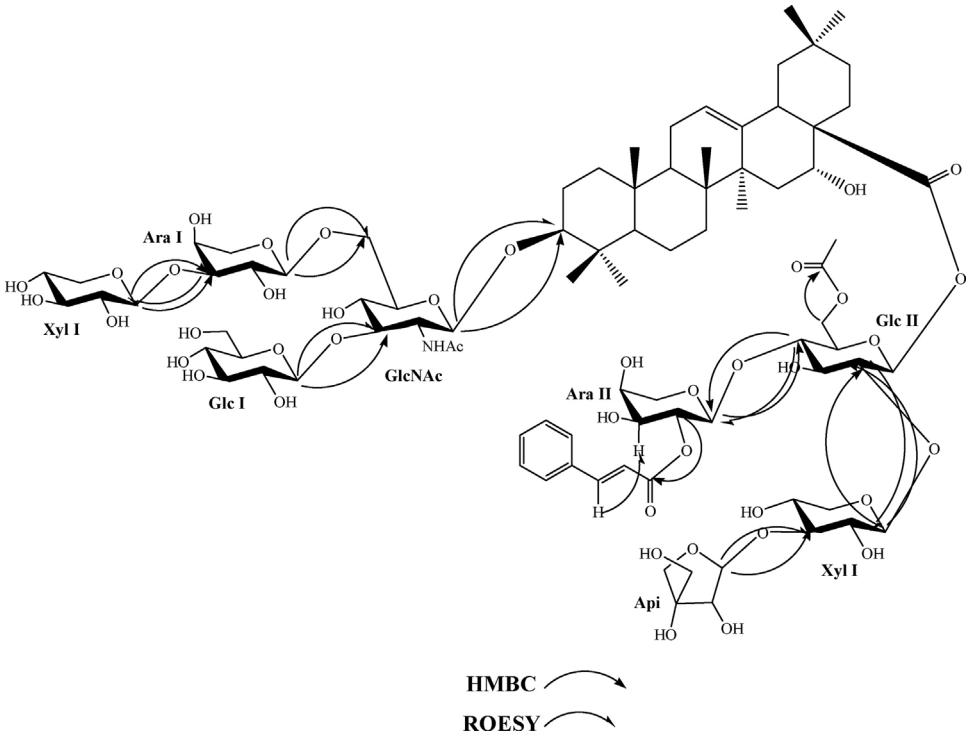

Fig. 2. Key HMBC and ROESY correlations for Compound **1**.

suggested that this sugar should be directly attached to C-28 of the aglycone through an ester bond. The direct and reverse correlations observed in the HMBC spectrum between H-1 (δ_H 5.40) of Xyl II and C-2 (δ_C 78.7) of Glc II and between H-2 (δ_H 4.29) of Glc II and C-1 (δ_C 105.2) of Xyl II, and the ROESY correlation observed between H-1 (δ_H 5.40) of Xyl II and H-2 (δ_H 4.29) of Glc II allowed us to locate Xyl II at C-2 of Glc II. Moreover, the HMBC correlation observed between H-1 (δ_H 6.30) of Api and C-3 (δ_C 84.8) of Xyl II permitted us to attach Api at C-3 of Xyl II. This was supported by the ROESY correlation observed between H-1 (δ_H 6.30) of Api and H-3 (δ_H 4.15) of Xyl II. On the other hand, the direct and reverse correlations observed in the HMBC spectrum between H-1 (δ_H 5.00) of Ara II and C-4 (δ_C 80.2) of Glc II and between H-4 (δ_H 4.03) of Glc II and C-1 (δ_C 103.1) of Ara II, and the ROESY correlation observed between H-1 (δ_H 5.00) of Ara II and H-4 (δ_H 4.03) of Glc II allowed us to locate Ara II at C-4 of Glc II. In addition, the location of the acetyl group at C-6 of Glc II was also evidenced by the observation of the signals of C-6 of Glc II at δ_C 63.5 (deshielded, +1.2 ppm) and of C-5 of Glc II at δ_C 73.9 (shielded, −4.9 ppm) in comparison to Glc I (δ_C 78.4). Furthermore, the observation of the signal of H-2 of Ara II at δ_H 5.65 (deshielded, +1.15 ppm) and of C-2 of Ara II at δ_C 75.2 (deshielded, + 1.8 ppm) in comparison to Ara I (δ_H 4.51 and 73.3) indicated that C-2 of Ara II was esterified. Accordingly, the cinnamoyl moiety was placed at C-2 of Ara II. This was supported by the HMBC and ROESY correlations, observed between H-2 (δ_H 5.65) of Ara II and the carbonyl group (δ_C 166.2) of the *trans*-cinnamoyl moiety, and between H-3 (δ_H 4.13) of Ara II and the olefinic proton (δ_H 8.02) of the *trans*-cinnamoyl moiety, respectively (Fig. 2). Thus, the tetrasaccharide *β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-[(2-*O*-cinnamoyl)-α-D-arabinopyranosyl-(1 → 4)]-(6-*O*-acetyl)-*β*-D-glucopyranosyl moiety was established to be attached at C-28 of the aglycone (Fig. 2). Based on the above evidences, the structure of compound **1** was established as 3-*O*-*β*-{α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[*β*-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl}-echinocystic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-{2-*O*-cinnamoyl-α-D-arabinopyranosyl-(1 → 4)}- (6-*O*-acetyl- *β*-D-glucopyranosyl) ester.

The extensive analysis of 1D and 2D NMR experiments showed that the oligosaccharide chains at C-3 and C-28 of the echinocystic acid were common in the three saponins. They will not be further discussed in the structural elucidation of compounds **2** and **3**.

Chevalieroside B (**2**), obtained as a white amorphous powder, was assigned a $C_{87}H_{137}NO_{42}$ molecular formula, as deduced from the $[M+NH_4]^+$ ion at *m/z* 1885.8960 in the positive ion mode HRESIMS. Detailed comparison of NMR data of **2** (1D and 2D NMR analysis) with those of **1**, indicated identical acyl moiety at C-6 of Glc-II and the disappearance of the *trans*-cinnamoyl moiety signals observed for **1**. In addition, the ^{1}H NMR spectrum of compound **2** exhibited one tri-substituted olefinic proton signal at δ_H 7.24 (d, J = 7.2 Hz) and one group of one-substituted olefin proton signals at δ_H 6.23 (dd, J = 10.9; 17.6 Hz), 5.25 (d, J = 10.9 Hz) and 5.47 (d, J = 17.6 Hz), indicating that compound **1** had one unit of monoterpenoid moiety (MT) (Liu et al., 2010). All proton and carbon signals of this MT moiety were fully assigned by extensive analysis of HSQC and further confirmed by HMBC spectrum. The stereochemistry of the tri-substituted double bond was determined as *E* from the chemical shift of H-3 at δ_H 7.24 since the *Z* isomer would appear at higher field (Zhang et al., 1999). The configuration of C-6 of the MT moiety was determined as *S* based on the chemical shift values of C-5 (δ_C 41.0), C-7 (δ_C 144.1), C-8 (δ_C 112.7) and C-10 (δ_C 23.6) which were very close to the corresponding values in the ^{13}C NMR data of (6*S*)-menthiafolic acid 6-*O*-*β*-D-quinovoside (Liang et al., 2005). The esterification of the hydroxyl group at C-2 of Ara II by MT was suggested from the deshielded position of H-2 of Ara II and C-2 of Ara II resonances at δ_H 5.67 and δ_C 75.0, respectively. This was supported by the HMBC correlation observed between H-2 (δ_H 5.67) of Ara II and C-1 (δ_C 167.3) of MT moiety. Accordingly, the structure of compound **2** was established as 3-*O*-*β*-{α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[*β*-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl}-echinocystic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl]-2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl]- α-L-arabinopyranosyl-(1 → 4)}-(6-*O*-acetyl- β-D-glucopyranosyl) ester.

Chevalieroside C (**3**), obtained as a white amorphous powder, was assigned a $C_{96}H_{143}NO_{43}$ molecular formula, as deduced from the $[M+NH_4]^+$ ion at *m/z* 2016.9416 in the positive ion mode HRESIMS. Detailed comparison of NMR data of **3** (1D and 2D NMR analysis) with those of **2**, indicated also identical acyl moiety at C-6 of Glc-II, and the

presence of a cinnamoyl moiety (Cynn) and monoterpenoid moiety (MT), identified in **1** and **2**, respectively. In addition, the NMR spectra of compound **3** showed acylation shifts at H-6 (δ_H 4.52, 4.61) and C-6 (δ_C 63.2) of Glc-II and at H-2 (δ_H 5.65) and C-2 (δ_C 75.2), and H-3 (δ_H 4.14) and C-3 (δ_C 74.0) of Ara-II. The observation of the HMBC correlations between H-2 (δ_H 5.65) of Ara II and C-1 (δ_C 166.4) of the Cynn moiety, and between H-3 (δ_H 4.14) of Ara II and C-1 (δ_C 167.4) of the MT moiety, confirmed the positions of the Cynn and the MT moieties at C-2 and C-3 of Ara II, respectively. Consequently, the structure of **3** was established as 3-*O*-*β*-{α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[*β*-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl}-echinocystic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl-3-*O*-cinnamoyl]-2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl-3-*O*-cinnamoyl]-α-L-arabinopyranosyl-(1 → 4)}-(6-*O*-acetyl- *β*-D-glucopyranosyl) ester.

The pro-apoptotic activity of compounds **1–3** was evaluated using Annexin V-FITC binding assay on two human cell lines: the pancreatic carcinoma AsPC-1 and the cancerous monocytic THP-1 cell lines. In result, percentage of apoptotic cells following 24 h of treatment was increased in a concentration-dependent manner for AsPC-1 and THP-1 cells, respectively (Fig. 3). Computed concentrations of each compound to induced half-maximal effects (EC_{50}) on cell apoptosis were determined as being 17.91 and 8.11 µM, 13.72 and 5.69 µM, and 3.10 and 8.32 µM on AsPC-1 and THP-1 cells for chevalierosides A–C, respectively. The strong pro-apoptotic effect of chevalieroside C (**3**) on the pancreatic carcinoma AsPC-1 cells observed at lowest concentration may be probably due to the number and nature of acyl moieties in compound **3** compared to compounds **1** and **2**. Someone can also wonder if the pro-apoptotic effect of saponins **1–3** on THP-1 cells is not due to the presence of acyl moieties in their sugar chains, since the increase of the percentage of apoptotic cells was not observed for the THP-1 cancerous cells when the non-acylated glaberrimosides A–C recently isolated from *A. glaberrima* (Noté et al., 2016a) were tested. Nevertheless, further studies are necessary to support this conclusion. Furthermore, the next steps will be to further explain the apoptotic route induced by chevalieroside C (**3**) specifically to this highly resistant cancer cells. In addition, this study contributes to complete the knowledge of the chemical composition of *Albizia* species, which are natural sources of bioactive triterpenoid saponins.

3. Experimental

3.1. General

Optical rotations were measured on a Jasco P-2000 polarimeter. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature in pyridine-d_5 using a Bruker AVANCE III 500 spectrometer. Chemical shifts are given in δ (ppm) value relative to TMS as internal standard. HRESIMS spectra were recorded on a microTOF ESI-TOF mass spectrometer (Agilent) operating in positive mode. Analytical HPLC was performed on Varian 920-LC apparatus equipped with an autosampler, a pump, a diode array detector (DAD), and Galaxie software. Semipreparative HPLC was performed on a Gilson apparatus equipped with Trilution LC software using a Nucleodur 100-5 C18ec (21 × 250 mm, 5 µm) column purchased from Machery-Nagel (Germany). Thin layer chromatography (TLC) was performed on precoated silica gel plates (60 F_{254}, Merck) using the system solvent *n*-BuOH-AcOH-H_2O, 65:15:25 as eluent. The spots were observed after spray with Komarowsky reagent, which is a mixture (5:1) of *p*hydroxybenzaldehyde (2% in MeOH) and Ethanolic H_2SO_4 (50%). Vacuum-liquid chromatography (VLC) was carried out using RP-18 silica gel 60 (25–40 µm) and silica gel 60 (15–40 µm and 40–63 µm).

3.2. Plant material

The stem bark of *A. chevalieri* were harvested at Nkolbisson, Yaoundé peripheral quarter, in Cameroon in October 2011 under the guidance of Mr. Paul Nana, botanist of the National Herbarium of Cameroon (NHC), where one specimen (47196/HNC) was deposited.

3.3. Extraction and isolation

The air-dried, powdered stem bark of *A.* chevalieri (150 g) were

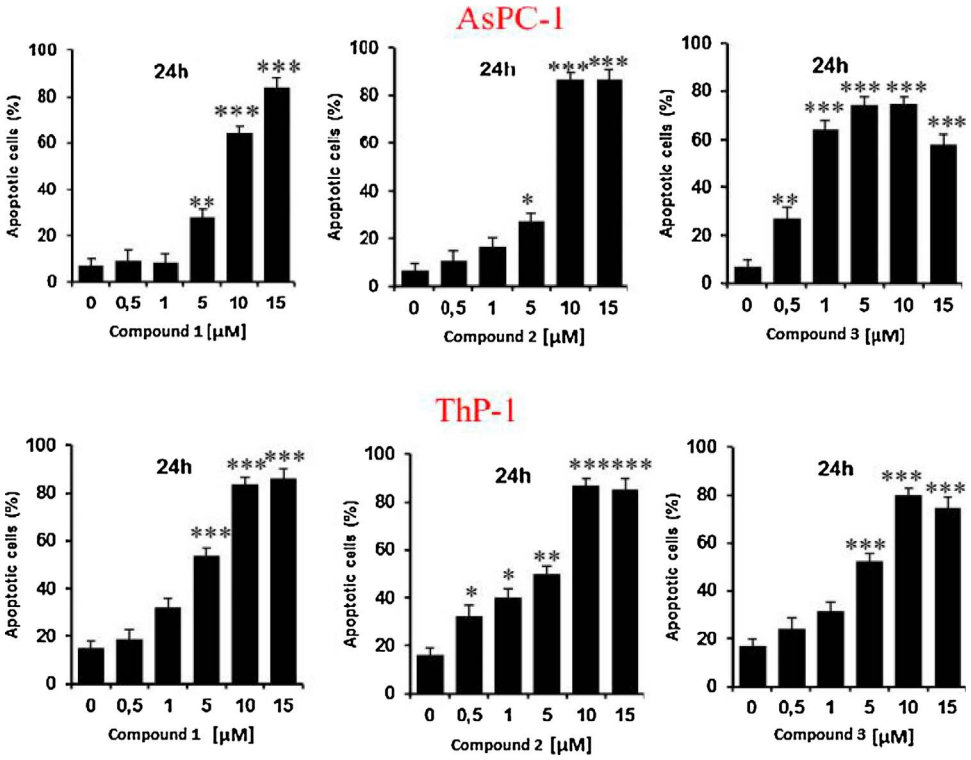

Fig. 3. The apoptotic effect of the three purified saponins was investigated on 2 human cell lines (AsPC-1 and THP-1) by capillary cytometry using the Annexin-V FITC/PI staining assay (n = 3).
*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

extracted with 70% EtOH in soxhlet apparatus. The resulting hydro-alcoholic solution was then evaporated to dryness under reduce pressure to yield a brown residue (4.83 g). This residue was suspended in 200 ml of H_2O and partitioned with *n*-BuOH sat. H_2O (3 × 300 ml). The *n*-BuOH soluble phase was evaporated to dryness affording 6.14 g of brown gum residue which was taken in a minimum of water (10 ml) and then submitted to vacuum-liquid chromatography (VLC) using RP-18 silica gel 60 (25–40 µm) eluted with a gradient of MeOH-H_2O (100% H_2O → 100%MeOH). The 70–85% MeOH extract (600.5 mg) was subjected to VLC using silica gel 60 (15–40 µm) eluted with $CHCl_3$-MeOH-H_2O (80:20:2, 70:30:5, 60:33:7, and 60:40:10) to give four main subfractions (ACE1- ACE4). Subfraction ACE4 (126.3 mg) was purified by semipreparative HPLC using gradient system of CH_3CN-H_2O (20 ml/min) to yield compounds **1** (t_R, 13.20 min, 7.9 mg), **2** (t_R, 13.68 min, 8.5 mg), and **3** (t_R, 16.16 min, 7.2 mg).

3-*O*-*β*-{α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[*β*-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl}-echinocystic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-{2-*O*-cinnamoyl-α-D-arabinopyranosyl-(1 → 4)}-(6-*O*-acetyl-*β*-D-glucopyranosyl) ester (**1**), white amorphous powder; $[\alpha]_D^{25}$ −28.8 (c 0.01, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) data, see Tables 1 and 2. Positive HRESIMS: *m/z* 1849.8384 $[M+NH_4]^+$.

3-*O*-*β*-{α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[*β*-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl}-echinocystic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl]-2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl]-α-L-arabinopyranosyl-(1 → 4)}-(6-*O*-acetyl-*β*-D-glucopyranosyl) ester (**2**), white amorphous powder; $[\alpha]_D^{25}$ −45.7 (c 0.01, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) data, see Tables 1 and 2. Positive HRESIMS: *m/z* 1885.8960 $[M+NH_4]^+$.

3-*O*-*β*-{α-D-xylopyranosyl-(1 → 3)-α-L-arabinopyranosyl-(1 → 6)-[*β*-D-glucopyranosyl-(1 → 3)]-2-(acetamido)-2-deoxy-*β*-D-glucopyranosyl}-echinocystic acid 28-*O*-*β*-D-apiofuranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl-3-*O*-cinnamoyl]-2)-{2-*O*-[(*6S,2E*)-2,6-dimethyl-2,7-octadienoyl-3-*O*-cinnamoyl]-α-L-arabinopyranosyl-(1 → 4)}- (6-*O*-acetyl- *β*-D-glucopyranosyl) ester (**3**), white amorphous powder; $[\alpha]_D^{25}$ −50.5 (c 0.01, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) data, see Tables 1 and 2. Positive HRESIMS: *m/z* 2016.9416 $[M+NH_4]^+$.

3.4. Acidic hydrolysis of isolated saponins

Each saponin (2 mg) was hydrolyzed with 2 ml of 2 M HCl at 85 °C during 2 h. After cooling, the solvent was removed under reduced pressure. The sugar mixture was extracted from the aqueous phase (10 ml) and washed with CH_2Cl_2 (3 × 5 ml). The combined CH_2Cl_2 extracts were washed with water to give after evaporation the aglycone moiety, which was compared with an authentic standard sample by Co-TLC. The sugars were first analyzed by TLC over silica gel ($CHCl_3$-MeOH-H_2O, 8:5:1) by comparison with standard samples. The absolute configuration of each monosaccharide was determined from GC–MS analysis of their trimethylsilylated derivatives by comparison with authentic samples using the method previously described (Chaabi et al., 2010). The following sugars were detected: D-glucose, 2-(acetamido)-2-deoxy-D-glucose, D-xylose, L-arabinose, and D-apiose.

3.5. Pro-apoptotic evaluation

3.5.1. Cell lines and culture conditions

The human pancreatic carcinoma cell line ASPC-1 was a gift from Dr. Timo Kehl (Deutsches Krebsforschungszentrum Heidelberg, Heidelberg, Germany), and THP-1 (TIB-202) cell lines, were purchased from ATCC (LGC Standards,Molsheim, France). ASPC-1 cells were cultivated in DMEM-based media (Sigma-Aldrich, Saint-Quentin-Fallavier, France), supplemented with 10% (v/v) fetal bovine serum (BioWhittaker, Verviers, Belgium), 2 mM ultraglutamine, 50 µM non-essential amino acids, 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich). THP-1 cells were grown in RPMI 1640 medium with 2 mM L-alanyl-L-glutamine additionally supplemented with 10% (v/v) fetal bovine serum and 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich). Cultures were kept at 37 °C in a humidified incubator equilibrated with 5% CO_2. Before confluency adherent cells were trypsinized and subcultured twice a week.

3.5.2. Pro-apoptotic evaluation

Cells were treated with the three different compounds of chevalieriosides A–C (**1–3**) in ranging concentrations (1–15 µM) then collected for apoptosis induction estimation. A minimum of 5000 cells was acquired per sample and analyzed on the InCyte software (Guava/Millipore/Merck, CA, USA). Apoptosis rates were assessed by capillary cytometry (Guava EasyCyte Plus, Millipore Merck) using Annexin V-FITC (ImmunoTools, Germany) and PI (MiltenyiBiotec Inc., USA) according to the manufacturer's recommendations. Gates were drawn around the appropriate cell populations using a forward scatter (FSC) versus side scatter (SSC) acquisition dot plot to exclude debris. Final concentration of DMSO applied to cells during incubation with tested drugs was always 0.5%. In the tested setup that concentration had no adverse effects on cell viability, nor cell morphology. To discriminate between negative and positive events in the analysis, a non-stained control sample from each culture condition always accompanied acquisition of the stained cells to define their cut off. Negative control, i.e. sample with cells without compounds but with the same amount of DMSO as for diluted compounds, as well as positive control with 15 µM Celastrol, a natural pentacyclic triterpenoid (Enzo Life Sciences, Farmingdale, US), were included in each experiment. Cytometers performances were checked weekly using the Guava Easy Check Kit 4500-0025 (Merck/Millipore/Guava Hayward, CA, USA).

3.5.3. Statistical analysis

Data presented as bar graphs, were expressed as means ± S.E.M. of at least three independent experiments. Statistical evaluation was performed with the one-way ANOVA test followed by the post-hoc Bonferroni test using GraphPad Prism software (Prism version 5.04 for Windows, GraphPad Software, CA, USA); a *p*-value less than 0.05 was considered as significant (*), less than 0.01 very significant (**) and less than 0.001 highly significant (***).

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