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SAPONINS FROM *PISONIA UMBELLIFERA*

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Key Word Index—*Pisonia umbellifera*; Nyctaginaceae; oleanolic acid saponins; *seco*-glycosides.

Abstract—Six saponins were isolated from the leaves of *Pisonia umbellifera*. Three are new oleanolic acid saponins, and two of them contain an unusual *seco*-glycopyranosyl moiety. Their structures were determined using a combination of ^{1}H and ^{13}C NMR, and mass spectrometry as 3-*O*-{β-D-glucopyranosyl(1 → 2)[β-D-glucopyranosyl(1 → 2)-β-D-xylopyranosyl(1 → 3)]-β-D-glucuronopyranosyl} 28-*O*-β-D-glucopyranosyl olean-12-en-3β-ol-28-oic acid, 3-*O*-{2′-(2″-*O*-glycolyl)-glyoxylyl-β-D-glucuronopyranosyl} 28-*O*-β-D-glucopyranosyl olean-12-en-3β-ol-28-oic acid and 3-*O*-{2′-*O*-glycolyl)-glyoxylyl-β-D-glucuronopyranosyl} olean-12-en-3β-ol-28-oic acid.

INTRODUCTION

Pisonia umbellifera Seem. is a large tree distributed through the tropical regions of the South Sea Islands, occurring wild in the coast of Vanuatu and New Caledonia. Its young leaves have a different shape from the mature leaves. The tree is valued locally since it is fast growing and its leaves and bark provide food for pigs. The leaves of closely related species, *P. grandis* (the brown cabbage tree) and *P. alba* (the lettuce tree) are used for human consumption. In Vanuatu, the leaves of *P. umbellifera* are also used as a treatment for ciguatera poisoning and as a 'green manure'. Its leaves are buried in the soil by Melanesian market-gardeners in the banana-tree and yam plantations, probably as a means of fighting phytophage nematodes [1]. Apart from scattered reports on polyphenolic constituents, saponins and alkaloids from some species of *Pisonia*, the chemistry of this genus appears to have been neglected [2]. We herein report the isolation and structural elucidation of six saponins. One of these is a new pentasaccharidic bidesmoside of oleanolic acid, two others are new *seco*-glycosides of oleanolic acid.

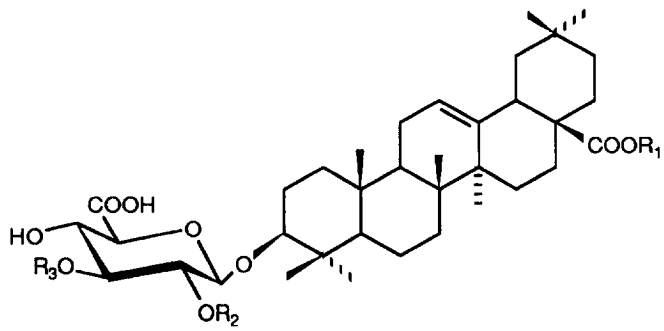
RESULTS AND DISCUSSION

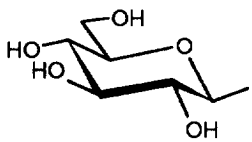
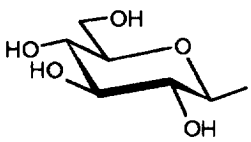
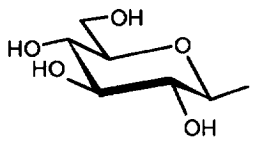
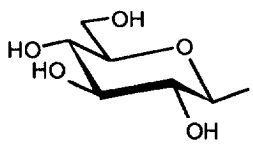
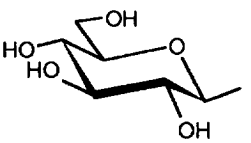
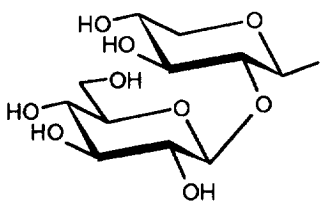
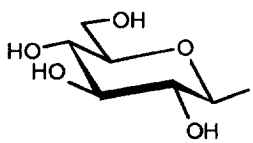
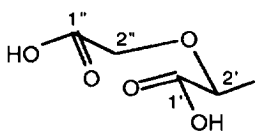
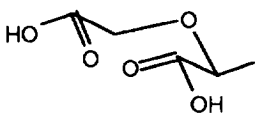
The mature leaves of *P. umbellifera* were successively defatted with petrol and chloroform, then extracted with methanol and aqueous methanol. After concentration, residues were partioned between water and butanol; the butanolic extracts were subjected to chromatographic purification on silica gel column. Saponins **1–6** were separated by repeated chromatography.

Acid hydrolysis of the crude saponin extract yielded a single aglycone identified as oleanolic acid according to NMR and mass spectral data, and by comparison with an authentic sample. D-glucose, D-xylose and D-glucuronic acid were also identified and their absolute configurations were determined after separation by prep. TLC. Confirmation of oleanolic acid as the genin in each purified saponin was achieved by analysis of HMBC and HMQC spectra; assignments of the ^{13}C NMR spectra were in accordance with previous publications [3, 4].

Saponins **1**, **2** and **3** are known compounds and their structures were established by analysis of 2D NMR experiments (COSY, HOHAHA, ROESY, HMQC and HMBC) [5] as 3-*O*-β-D-glucuronopyranosyl olean-12-en-3β-ol-28-oic acid, 3-*O*-{β-D-glucuronopyranosyl} 28-*O*-β-D-glucopyranosyl olean-12-en-3β-ol-28-oic acid and 3-*O*-{β-D-glucopyranosyl(1 → 2) [β-D-xylopyranosyl(1 → 3)]-β-D-glucuronopyranosyl} 28-*O*-β-D-glucopyranosyl olean-12-en-3β-ol-28-oic acid respectively. Saponins **1** and **3** were compared by TLC with authentic samples isolated from *Beta vulgaris* [4]. Comparison of the ^{1}H and ^{13}C NMR spectra of saponins **1–3** allowed identification of saponin **2** with chikusetsaponin IVa [6–8].

Saponin **4** displayed in the positive-ion FAB-mass spectrum two molecular adduct ions at *m/z* 1273 $[M + Na]^+$ and 1295.2 $[M + 2Na - H]^+$ suggesting a $C_{59}H_{94}O_{28}$ molecular formula; the intense fragment ion at *m/z* 1087.8 was attributed to the loss of a terminal hexose. The presence of five sugar residues was deduced from the observation of five anomeric carbons at δ 95.7, 101.9, 102.3, 105.8 and 106.3 attached to vicinal protons at δ 5.37 (*d*, $J = 8$ Hz), 5.06 (*d*, $J = 7$ Hz), 5.09 (*d*, $J = 7.5$ Hz), 4.43 (*d*, $J = 8$ Hz) and 4.60 (*m*), respectively, in the HMQC spectrum (Table 1). COSY and HOHAHA experiments allowed immedi-



	R_1	R_2	R_3
1	H	H	H
2		H	H
3			
4			
5		H	
6	H	H	

ate identification of three sugars as glucose, xylose and glucuronic acid. Vicinal couplings constants in these sugars was larger than 7 Hz indicating axial positions for protons in the β-D-configurations. Confirmation of glucuronic acid came from the observation of two carbonyl signals at δ 176.4 and 178.1 (C-28).

Each of the two last sugars, hexoses according to molecular weight, displayed an ABX system at δ 3.75 (A, *dd*, $J = 11.5$, 5 Hz), 3.88 (B, *dd*, $J = 11.5$, 2 Hz), 3.30 (X, *m*) and at δ 3.67 (A, *dd*, $J = 11.5$, 5 Hz), 3.80 (B, *dd*, $J = 11.5$, 2 Hz), 3.35 (X, *m*). One sugar esterified C-28 of the genin (δ_H 5.37 and δ_C 95.7). The assignment of osidic carbons was obtained with HMQC experiments; the chemical shifts for C-3 → C-6 of the hexoses were very similar and suggested the presence of three terminal β-D-glucose units in saponin **4**. The nature and sequence of the sugar units was secured by the preparation of the peracetylated derivative **4a**. The COSY spectrum of **4a** allowed identification of three terminal glucoses amongst which were the sugar ester, of one xylose substituted in position C-2 (δ_H 3.70) and of one glucuronic acid disubstituted at positions C-2 and C-3 (multiplet at δ_H 3.93) (Table 1).

Sequencing of the sugar chain in saponin **4** was

Table 1. ^{1}H and ^{13}C NMR chemical shifts for osidic part of saponins **4**, **5**, **6** (in CD_3OD) and derivative **4a** (in $CDCl_3$)

	4			**4a**		**5**			**6**		
	δ_C	δ_H	*J*	δ_H	*J*	δ_C	δ_H	*J*	δ_C	δ_H	*J*
β-D-Glucuronic acid											
1	105.8	4.43 *d*	8	4.51 *m*	$W_{1/2} = 12$	106.3	4.39 *d*	7.8	106.3	4.40 *d*	7.9
2	78.5	3.92 *t*	8	3.93 *m*		75.5	3.42 *t*	7.7	75.5	3.40 *t*	7.7
3	84	3.80 *t*	9	3.93 *m*		87.1	3.56 *m*		87.3	3.56 *m*	
4	71.6	3.58 *m*		5.10 *t*	8.5	72.7	3.56 *m*		72.7	3.56 *m*	
5	77.4	3.58 *m*		3.98 *d*	9	76.4	3.56 *m*		76.5	3.56 *m*	
6	176.4					176.8			176.9		
β-D-Glucose (ester)											
1	95.7	5.37 *d*	8	5.59 *d*	8	95.7	5.37 *d*	8			
2	73.9	3.31 *t*	8	5.19 *t*	8	74	3.31 *t*	8			
3	78.3	3.38 *m*		5.26 *t*	9	78.3	3.41 *t*	9			
4	71.7	3.35 *m*		5.13 *t*	9.5	71.2	3.34 *m*				
5	78.2	3.35 *m*		3.80 *ddd*	10; 4.5; 2	78.7	3.34 *m*				
6	62.5	3.67 *dd*	11.5; 5	4.06 *dd*	12; 2	62.5	3.67 *dd*	12; 4			
		3.80 *brd*	11.5; 2	4.29 *dd*	12; 4.5		3.81 *dd*	12; 5			
β-D-Xylose											
1	101.9	5.06 *d*	7	4.91 *d*	5						
2	84.3	3.50 *brt*	8; 7	3.70 *dd*	8; 5						
3	77.2	3.63 *t*	8	5.07 *t*	8						
4	71	3.58 *m*		4.85 *td*	8; 5						
5	66.6	3.25 *dd*	11.5; 9	3.38 *dd*	12; 7						
		3.94 *d*	11.5; 5	4.01 *dd*	12; 5						
β-D-Glucose′ (at C-2 of Glu.A)											
1	102.3	5.09 *d*	7.5	4.98 *d*	8						
2	76.1	3.20 *dd*	9; 7.5	5.03 *m*							
3	78.5	3.43 *t*	9	5.10 *m*							
4	72.7	3.09 *t*	9	5.13 *m*							
5	78	3.37 *m*		3.86 *m*							
6	63.7	3.53 *dd*	12; 4	4.18 *dd*	12; 3						
		3.80 *brd*	12	4.25 *dd*	12; 2.5						
β-D-Glucose″ (at C-2 of xyl.)											
1	106.3	4.60 *m*		4.86 *d*	8						
2	75.9	3.33 *m*		4.99 *dd*	9; 8						
3	78.7	3.33 *m*		5.20 *t*	9						
4	71.8	3.33 *m*		5.10 *t*	9						
5	77.7	3.30 *m*		3.84 *m*							
6	62.9	3.75 *dd*	11.5; 5	4.12 *dd*	12; 3						
		3.88 *dd*	11.5; 2	4.35 *dd*	12; 4						
Substituent (at C-3 of Glu.A)											
2′						103.1	5.01 *s*		103	5.02 *s*	
1′						175.5			175.6		
1″						177.4			177.6		
2″						68	4.02 *d*	15	68.4	4.02 *d*	14.7
							4.34 *d*	15		4.40 *d*	14.7

achieved by a ROESY experiment carried out on the native compound **4**. Observation of an intense Overhauser effect between H-3 of oleanolic acid and H-1 of glucuronic acid confirmed the attachment of a four-sugar chain at this position. ROEs were observed between H-1 of glucose (δ 5.09) and H-2 of glucuronic acid (δ 3.92), between H-1 of xylose (δ 5.06) and H-3 of glucuronic acid (δ 3.80), and between H-1 of glucose (δ 4.60) and H-2 of xylose (δ 3.50).

On the basis of the above findings, the structure of the new saponin **4** is concluded to be 3-*O*-{β-D-glucopyranosyl($1\rightarrow2$) [β-D-glucopyranosyl($1\rightarrow2$)-β-D-xylopyranosyl($1\rightarrow3$)]-β-D-glucuronopyranosyl} 28-*O*-β-D-glucopyranosyl olean-12-en-3β-ol-28-oic acid.

The high polarity of saponins **5** and **6**, and the difficulty of their purification, stemmed from the presence of several acid functions in these saponins. Saponin **5** showed in the positive FAB-mass spectrum, two molecular adduct ions at *m/z* 949.9 $[M+Na+H]^+$ and 971.1 $[M+2Na-H]^+$ corresponding to $C_{46}H_{70}O_{19}$. Its negative FAB-mass spectrum exhibited a quasi-molecular ion at *m/z* 925.3 $[M-H]^-$, and two molecular adduct ions at *m/z* 947.2 $[M+Na-2H]^-$ and 969.8 $[M+2Na-3H]^-$ suggesting the presence of two carboxylic acid functions in compound **5**. Negative fragments at *m/z* 763.3, 785.3 and 807.2 were attributed to the loss of a terminal hexose from these pseudo-molecular ions. The nature of this hexose was deduced to be a β-D-glucopyranose from analysis of COSY and HOHAHA spectra showing seven protons in correlation, with five of them possessing large vicinal *trans*-diaxial couplings ($J>8$ Hz) (Table 1). The anomeric signals were at δ_H 5.37 and δ_C 95.7, indicating that the sugar esterified an acid function. The observation of a HMBC cross-peak between the anomeric proton and the C-28 of oleanolic acid confirmed the link of the glucose to the genin.

The presence of a second sugar in saponin **5** was deduced from the observation of a proton doublet at δ 4.39 directly bound to an anomeric carbon at δ 106.3 (HMQC). The 2D H–H experiments allowed the identification of one uronic acid. Measurement of the coupling constants of H-1, H-2 and H-3 showed that these three protons were axial. Comparison of proton and carbon chemical shifts of saponin **5** with those of saponin **2**, and with those related in the literature [4–9], indicated the presence of a β-D-glucuronic acid substituted in position C-3. This glucuronic acid was attached to C-3 of oleanolic acid as demonstrated by observation of $^3J_{H\text{-}C}$ HMBC correlations between these two elements.

Thus, saponin **5** contained saponin **2** as a subunit, substituted at position C-3 of glucuronic acid by a $C_4H_5O_5$ fragment. The negative fragment ion at *m/z* 631.2 $[M-H-\text{hexose}-132]^-$ confirmed the terminal position of this supplementary residue. The absence of accompanying ions at *m/z* 653 and 675 indicated that this unit contained the other two acid functions. Subtraction of two carboxylic acids $-C_2H_2O_4-$ from the $C_4H_5O_5$ fragment left a C_2H_3O composition for the undetermined part of saponin **5**. The three proton resonances were found as one pair of doublets at δ 4.02 and 4.34 ($J=15$ Hz) and one singlet at δ 5.01. The HMQC experiment revealed that they were directly attached to one methylene at δ 68 and one methine at δ 103.1, respectively (Table 1). The proton and carbon chemical shifts of this methine group were analogous to those of an anomeric position. The carbonyl signals for the carboxylic acids were observed at δ 175.5 and 177.4. Analysis of the HMBC spectrum showed that the geminal protons correlated with one of the carbonyls (δ 177.4) and with the pseudo-anomeric carbon. The proton singlet exhibited a correlation with the methylene (δ 68) and with the C-3 of glucuronic acid. Even though no $^2J_{H\text{-}C}$ correlation was observed between this singlet and the other carbonyl carbon (δ 175.5), the only possible structure fitting these elements corresponds to *seco*-glycoside **5**.

Saponin **6** was related to **5**; the major difference was the absence of signals for the β-D-glucose in **6** (Table 1). Its positive FAB-mass spectrum showed two molecular adduct ions at *m/z* 787.6 $[M+Na]^+$ and 809.6 $[M+2Na-H]^+$ which confirmed a formula at $C_{40}H_{60}O_{14}$. Three quasi-molecular negative ions at *m/z* 763.3 $[M-H]^-$, 785.2 $[M+Na-2H]^-$ and 807.2 $[M+2Na-3H]^-$ were detected, and only one negative fragment was observed, at *m/z* 631.2 $[M-H-132]^-$.

The new saponins **5** and **6** may originate from normal pentose glycosides through a series of oxidative cleavages. Analogous *seco*-glycoside compounds were recently isolated from two other sources; *Achyranthes fauriei* (Amaranthaceae) [10, 11] and *Beta vulgaris* (Chenopodiaceae) [9, 12] which also belong to the order of Centrospermales. It is not impossible that the presence of *seco*-glycosides is a chemotaxonomic marker of this botanical order. The nature of the asymmetric center on the *seco*-glycosides remains to be determined.

EXPERIMENTAL

General. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz. 2D experiments were performed using standard Bruker microprograms. Hardware modifications of the AC-300 spectrometer allowed acquisition of the C-H correlations in the reverse mode. FAB-MS were recorded on an AutoSpec or a ZAB2-SEQ spectrometer (Fisons-VG); samples were dissolved in MeOH and 1–2 μl of the soln was mixed to 1–2 μl of glycerol or thioglycerol, and of thioglycerol with 1% of TFA for saponins **5** and **6**.

Plant material. Mature leaves of *P. umbellifera* were collected in Vaté island in Vanuatu. The herbarium sample (GB 1422) is deposited in the National Herbarium of Port-Vila (Vanuatu), the herbarium of ORSTOM centre of Nouméa (New Caledonia), the Museum of Natural History (France) and Kew gardens (England).

Extraction and purification of saponins. Dried and

powdered leaves (520 g) were extracted with petrol (10 l) and then with $CHCl_3$ (10 l). The residue was boiled under reflux successively in MeOH (10 l) and 20% aq. MeOH (10 l) for 3 hr. After cooling and filtration, MeOH was removed *in vacuo* and the residues were partitioned between H_2O and *n*-BuOH. The organic layers were evapd to provide brown residues of saponin mixtures (9.5 g and 11 g; yield 4%). Part of the mixtures (2 g and 10 g) was purified by CC using a gradient of $CHCl_3$–MeOH–H_2O. Frs eluted with 6:4:0 and 60:40:1 contained saponins **1** and **2**; frs eluted with 60:40:1 and 60:40:2 contained saponins **2**, **3** and **4**; frs eluted with 60:40:5 and 60:40:8, and with pure MeOH contained saponins **5** and **6**. Saponins **1** and **2** were purified by CC followed by prep. TLC in $CHCl_3$–MeOH–H_2O (60:40:7). The mixt. of saponins **3** and **4** was subjected to reversed phase RP-18 CC using a gradient of MeOH–H_2O (from 60:40 to 100:0) and then prep. TLC in $CHCl_3$–MeOH–H_2O (60:40:7 or 60:40:8). Frs containing saponins **5** and **6** were passed through an ion-exchange IRN 77 (H^+) resin column before their purification by CC and prep. TLC in $CHCl_3$–MeOH–H_2O (60:40:8) or by reversed phase CC.

Hydrolysis of saponin mixture. Crude saponin (500 mg) was dissolved in 13 ml of a mixture (1:1) of 6.5% aq. $HClO_4$ and H_2SO_4 0.02N, and heated at 140° in a sealed tube for 2 hr. After cooling, the sapogenin ppt was filtered, rinsed with H_2O and dried *in vacuo* over P_2O_5; 140 mg of oleanolic acid was obtained. The acid aq. layer was neutralized with KOH 0.5 M and freeze-dried. Sugars were identified with authentic samples as xylose, glucose and glucuronic acid by TLC in 2-butanone–*iso*-PrOH–Me_2CO–H_2O (20:10:7:6); after prep. TLC of the sugar mixture, the optical rotation of each purified sugar was measured.

Saponin **4**: $[\alpha]_D$ +2.3° (CD_3OD); *c* 0.7). Positive FAB-MS *m/z*: 1295.2 $[M + 2Na - H]^+$, 1273 $[M + Na]^+$, 1111.8 $[M + Na - glc]^+$, 1088.9 $[M + H - glc]^+$, 1067 $[M + Na - CO_2glc]^+$. ^1H NMR (CD_3OD): δ 0.79 (3H, *s*, H-26), 0.86 (3H, *s*, H-24), 0.9 (3H, *s*, H-29), 0.92 (3H, *s*, H-30), 0.94 (3H, *s*, H-25), 1.06 (3H, *s*, H-23), 1.13 (*m*, H-19), 1.14 (3H, *s*, H-27), 1.6 (*m*, H-2), 1.69 (*m*, H-19), 1.88 (*m*, H-9), 2 (*m*, H-2), 2.85 (*dd*, *J* = 14, 4 Hz, H-18), 3.19 (*dd*, *J* = 12, 4 Hz, H-3), 5.24 (*brt*, *J* = 3 Hz, H-12), see Table 1. ^{13}C NMR (CD_3OD): δ 16 (C-25), 16.9 (C-24), 17.7 (C-26), 19.3 (C-6), 24 (C-30 and C-16), 24.6 (C-11), 26.3 (C-27), 27 (C-2), 28.4 (C-23), 28.9 (C-15), 31.5 (C-20), 33.2 (C-22), 33.5 (C-29), 34 (C-7), 34.9 (C-21), 37.9 (C-10), 39.9 (C-1), 40.5 (C-4), 40.7 (C-8), 42.6 (C-18), 42.9 (C-14), 47.2 (C-19), 48 (C-17), 49.1 (C-9), 57 (C-5), 92.1 (C-3), 123.9 (C-12), 144.8 (C-13), 178.1 (C-28), see Table 1.

Derivative **4a**: ^1H NMR ($CDCl_3$): δ 0.74 (3H, *s*, H-26), 0.83 (3H, *s*, H-24), 0.89 (3H, *s*, H-25), 0.92 (6H, *s*, H-29 and H-30), 1.04 (3H, *s*, H-23), 1.12 (3H, *s*, H-27), 2.82 (*dd*, *J* = 13, 4 Hz, H-18), 3.09 (*dd*, *J* = 11, 4.5 Hz, H-3), 5.32 (*brt*, *J* = 3 Hz, H-12), see Table 1.

Saponin **5**: $[\alpha]_D$ +6.8° (CD_3OD); *c* 0.3). Positive FAB-MS *m/z*: (rel. int.): 971.1 $[M + 2Na - H]^+$ (6.5), 945.9 $[M + Na + H]^+$ (6.5). Negative FAB-MS *m/z* (rel. int.): 969.8 $[M + 2Na - 3H]^-$ (1), 947.2 $[M + Na - 2H]^-$ (4), 925.3 $[M - H]^-$ (6.5), 807.2 $[M + 2Na - 3H - glc]^-$ (4), 785.3 $[M + Na - 2H - glc]$, 763.3 $[M - H - glc]^-$ (6), 631.2 $[M - H - glc - 132]^-$ (3). ^1H NMR (CD_3OD): δ 0.79 (3H, *s*, H-26), 0.83 (3H, *s*, H-24), 0.9 (3H, *s*, H-29), 0.92 (3H, *s*, H-30), 0.95 (3H, *s*, H-25), 1.05 (3H, *s*, H-23), 1.15 (3H, *s*, H-27), 2.85 (*dd*, *J* = 14, 4 Hz, H-18), 3.20 (*dd*, *J* = 12, 4 Hz, H-3), 5.24 (*brt*, *J* = 3 Hz, H-12), see Table 1. ^{13}C NMR (CD_3OD): δ 16 (C-25), 17 (C-24), 17.8 (C-26), 19.3 (C-6), 24 (C-16 and C-30), 24.6 (C-11), 26.3 (C-27), 26.9 (C-2), 28.5 (C-23), 28.9 (C-15), 31.5 (C-20), 34 (C-7), 33.5 (C-29), 33.2 (C-22), 35.5 (C-21), 37.9 (C-10 and C-1), 40.2 (C-4), 40.7 (C-8), 42.6 (C-18), 42.9 (C-14), 47.2 (C-19), 48 (C-17), 57.1 (C-5), 90.7 (C-3), 123.9 (C-12), 144.8 (C-13), 178.1 (C-28), see Table 1.

Saponin **6**: $[\alpha]_D$ +10.7° (CD_3OD; *c* 0.2). Positive FAB-MS *m/z* (rel. int.): 809.6 $[M + 2Na - H]^+$ (8.5), 787.6 $[M + Na + H]^+$ (12.5). Negative FAB-MS *m/z* (rel. int.): 807.2 $[M + 2Na - 3H]^-$ (6), 785.2 $[M + Na - 2H]^-$ (6.5), 763.3 $[M - H]^-$ (10), 631.2 $[M - H - 132]^-$ (11), 455.2 $[aglycone - H]^-$ (8). ^1H NMR (CD_3OD): δ 0.83 (3H, *s*, H-26), 0.87 (6H, *s*, H-24 and H-29), 0.94 (6H, *s*, H-25 and H-30), 1.05 (3H, *s*, H-23), 1.12 (3H, *s*, H-27), 2.91 (*dd*, *J* = 14, 4 Hz, H-18), 3.2 (*dd*, *J* = 11.5, 4 Hz, H-3), 5.2 (*brt*, *J* = 3.4 Hz, H-12), see Table 1. ^{13}C NMR (CD_3OD): δ 16 (C-25), 17 (C-24), 18.2 (C-26), 19.4 (C-6), 24.3 (C-30), 24.6 (C-16 and C-11), 26.5 (C-27), 26.8 (C-2), 28.6 (C-23), 29.3 (C-15), 31.8 (C-20), 33.9 (C-29), 34.4 (C-7 and C-22), 35.5 (C-21), 38 (C-10), 39.9 (C-1), 40.6 (C-4), 40.2 (C-8), 43.1 (C-14), 43.5 (C-18), 90.9 (C-3), 122.6 (C-12), 146.6 (C-13), 179 (C-28), see Table 1.

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