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Triterpenoid saponins from a cytotoxic root extract of *Sideroxylon foetidissimum* subsp. *gaumeri*

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ABSTRACT

Evaluation of the cytotoxicity of an ethanolic root extract of *Sideroxylon foetidissimum* subsp. *gaumeri* (Sapotaceae) revealed activity against the murine macrophage-like cell line RAW 264.7. Systematic bioassay-guided fractionation of this extract gave an active saponin-containing fraction from which four saponins were isolated. Use of 1D (^{1}H, ^{13}C, DEPT135) and 2D (COSY, TOCSY, HSQC, and HMBC) NMR, mass spectrometry and sugar analysis gave their structures as 3-*O*-(β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid, 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid, 3-*O*-(β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)[β-D-apiofuranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid, and the known compound, 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-protobassic acid. Two further saponins were obtained from the same fraction, but as a 5:4 mixture comprising 3-*O*-(β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)[β-D-apiofuranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid and 3-*O*-(β-D-apiofuranosyl-(1 → 3)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid, respectively. This showed greater cytotoxicity (IC_{50} = 11.9 ± 1.5 μg/ml) towards RAW 264.7 cells than the original extract (IC_{50} = 39.5 ± 4.1 μg/ml), and the saponin-containing fraction derived from it (IC_{50} = 33.7 ± 6.2 μg/ml).

1. Introduction

The Yucatan peninsula is considered a distinct biome, and as such includes the Mexican states of Campeche, Quintana Roo, and Yucatan, as well as parts of Chiapas and Tabasco, the north of Belize, and the Peten region of Guatemala (Durán et al., 2000). Included in its flora are 182 plant families comprising 992 genera and approximately 3000 species, 7.3% of which are endemic (Durán et al., 2000; Espadas-Manrique et al., 2003). Knowledge about the use of plants for therapeutic purposes has been preserved by some indigenous groups, and it is reported that more than 800 species, including 50 endemics, are used in Yucatecan traditional medicine (Durán et al., 1998). These include *Sideroxylon foetidissimum* Jacq. subsp. *gaumeri* Pittier (T.D.Penn), an endemic tree of the Sapotaceae which is used mainly for timber and firewood. The fruits of this species are edible, and local people are said to use the plant for medicinal purposes, although no details on its preparation or application are available (Durán et al., 2000).

Phytochemical and pharmacological studies of *Sideroxylon* are limited, and only a few species have been investigated. MeOH extracts of the root of *Sideroxylon cubense*, a Caribbean species used externally to treat fractures, yielded the triterpenoid saponins sideroxyloside A and 3-*O*-β-D-glucopyranosyl-protobassic acid (Jiang et al., 1994). In addition, two further triterpenoid saponins, sideroxyloside B and sideroxyloside C, were reported from *S.*

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foetidissimum, although the subspecies was not specified, and no bioassay data were given (Nicolas et al., 1995). Of the two known subspecies, *S. foetidissimum* subsp. *foetidissimum* and *S. foetidissimum* subsp. *gaumeri*, only the latter is endemic to the Yucatan peninsula (Durán et al., 2000). Extracts of *S. foetidissimum* subsp. *gaumeri* have been subjected to a range of bioassays, including inhibition of β-glucosidase, DNA interaction, antimycobacterial, antioxidant and cytostatic activity, but no significant activity was noted (Fuentes-García, 2001; Vera-Ku, 2004). Here we report on the detection of cytotoxic activity in the root extract of *S. foetidissimum* subsp. *gaumeri*, and the bioassay-guided isolation and characterisation of saponins **1**–**6**, of which **1**–**3**, **5** and **6** are new examples.

2. Results and discussion

An EtOH extract of the roots of *S. foetidissimum* subsp. *gaumeri* that showed cytotoxic activity (IC_{50} = 39.5 ± 4.1 μg/ml) against the murine macrophage-like cell line RAW 264.7 was subject to bioassay-guided fractionation. This yielded an active fraction from which four saponins **(1**–**4)** and a 5:4 mixture of two further saponins **(5**, **6)** were isolated. The structures of **1**–**6** were determined using MS, NMR and sugar analysis (Section 3.4). Of these, **4** was identified as 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-protobassic acid (Section 3.8), a known compound described by Eskander et al. (2005) as a constituent of the leaves of *Mimusops laurifolia* (Sapotaceae).

Saponin **1** was obtained as a white amorphous powder for which ESI-MS (positive mode) gave a sodiated molecule at *m*/*z* 1555 $[M + Na]^+$, consistent with a molecular formula of $C_{69}H_{112}O_{37}$. MS/MS (MS2) of $[M + Na]^+$ gave fragments at *m*/*z* 867 $[(M + Na) - (3 \times 132) - (2 \times 146)]^+$, attributed to the loss of a pentaglycosidic chain comprising three pentoses and two deoxyhexoses; *m*/*z* 711 $[(M + Na) - (2 \times 162) - 520]^+$, corresponding to the loss of two hexoses and a triterpene moiety, and *m*/*z* 579 $[(M + Na) - (2 \times 162) - 520 - 132]^+$, corresponding to the loss of two hexoses, a triterpene moiety, and a pentose. Complete assignment of all proton and carbon resonances in the ^1H and ^{13}C NMR spectra of **1** was achieved using COSY, TOCSY, HSQC and HMBC data (Tables 1 and 2). Among the characteristic resonances in the ^1H NMR spectrum were those for six quaternary methyl groups at δ 0.89, 0.98, 1.06, 1.30 1.34 and 1.60 (all 3H, *s*), an olefinic proton at δ 5.42, and seven anomeric protons at δ 5.61, 5.16, 5.06, 4.56, 4.45, 4.30 and 4.25. Taken together with the MS data, these suggested that **1** was a triterpenoid saponin with seven sugars. The structure of the triterpenoid moiety was obtained principally from correlations observed in the HMBC spectrum, and confirmed to be that of 16α-hydroxyprotobassic acid (2β,3β,6β,16α,23-pentahydroxy-12-oleanen-28-oic acid) by comparison of NMR data (Table 1) with literature values acquired in the same solvent (Eskander et al., 2005).

In the HSQC spectrum of **1**, anomeric proton resonances at δ_H 5.61, 5.16, 5.06, 4.56, 4.45, 4.30 and 4.25 correlated with δ_C 94.2, 102.2, 101.4, 106.4, 104.3, 104.5 and 103.4, respectively, indicating the presence of seven *O*-linked sugars. A full set of ^1H and ^{13}C NMR assignments for each sugar residue was derived from a combination of COSY, TOCSY, HSQC and HMBC data (Table 2). The strategy for sequential assignment included the acquisition of TOCSY spectra at two different mixing times, 60 and 100 ms. The shorter mixing time gave correlations from H-1 to H-2 and H-3 of the sugars, whereas at 100 ms, total correlation from H-1 to 5-CH_2 (Ara, Xyl), 6-CH_2 (Glc) and 6-CH_3 (Rha) was observed. Taken together with coupling constant data for the anomeric protons and other key

Table 1
^1H and ^{13}C NMR spectral data for the aglycone moieties of saponins **1**–**3** (CD_3OD, 30 °C).

Atom	**1**		**2**		**3**	
	δ ^1H (*J* in Hz)	δ ^{13}C	δ ^1H (*J* in Hz)	δ ^{13}C	δ ^1H (*J* in Hz)	δ ^{13}C
1	2.07 *m*, 1.22 *m*	46.5	2.06 *m*, 1.19 *m*	46.7	2.08 *m*, 1.21 *m*	46.7
2	4.34 *br m*	70.7	4.34 *br m*	71.4	4.34 *br m*	70.7
3	3.57 *m*	83.9	3.58 *m*	83.8	3.57 *m*	83.9
4	–	44.0	–	44.1	–	44.0
5	1.33 *m*	48.9	1.33 *m*	49.0	1.33 *m*	48.9
6	4.47 *br m*	68.8	4.47 *br m*	68.8	4.45 *br m*	68.7
7	1.82 *m*	41.5	1.83 *m*	41.5	1.81 *m*	41.5
	1.55 *br d* (14.7)		1.55 *dd* (14.8, 3.5)		1.57 *br d* (14.6)	
8	–	40.1	–	40.1	–	40.1
9	1.66 *dd* (11.4, 6.2)	48.8	1.66 *dd* (11.5, 6.2)	48.8	1.65 *dd* (11.4, 6.0)	48.8
10	–	37.2	–	37.2	–	37.2
11	2.11 *m*, 1.99 *m*	24.7	2.13 *m*, 1.97 *m*	24.7	2.11 *m*, 2.01 *m*	24.7
12	5.42 *br m*	124.2	5.41 *br m*	124.2	5.42 *br m*	124.2
13	–	144.0	–	144.0	–	144.0
14	–	43.5	–	43.5	–	43.5
15	1.83 *m*	36.5	1.83 *m*	36.4	1.82 *m*	36.4
	1.42 *dd* (14.9, 3.5)		1.42 *dd* (14.8, 3.5)		1.42 *dd* (14.9, 3.5)	
16	4.49 *br m*	74.7	4.49 *br m*	74.6	4.48 *br m*	74.7
17	–	50.5	–	50.5	–	50.5
18	3.08 *br dd* (14.5, 4.1)	42.3	3.08 *br dd* (14.5, 4.1)	42.3	3.08 *br dd* (14.5, 4.1)	42.5
19	2.28 *m*, 1.06 *m*	47.7	2.28 *m*, 1.05 *m*	47.7	2.27 *m*, 1.05 *m*	48.0
20	–	31.4	–	31.4	–	31.4
21	1.86 *m*, 1.16 *m*	36.5	1.89 *m*, 1.17 *m*	36.5	1.87 *m*, 1.17 *m*	36.6
22	1.90 *m*, 1.77 *m*	31.8	1.91 *m*, 1.77 *m*	31.9	1.89 *m*, 1.80 *m*	31.7
23	3.72 *m*, 3.41 *m*	65.6	3.73 *m*, 3.42 *m*	65.6	3.72 *m*, 3.42 *m*	65.6
24	1.30 *s*	16.5	1.32 *s*	16.4	1.30 *s*	16.5
25	1.60 *s*	19.2	1.62 *s*	19.3	1.60 *s*	19.3
26	1.06 *s*	19.1	1.05 *s*	19.1	1.06 *s*	19.2
27	1.34 *s*	27.4	1.33 *s*	27.4	1.34 *s*	27.5
28	–	177.1	–	177.1	–	177.1
29	0.89 *s*	33.4	0.89 *s*	33.4	0.89 *s*	33.5
30	0.98 *s*	25.2	0.98 *s*	25.2	0.98 *s*	25.2

Table 2
^{1}H and ^{13}C NMR spectral data for the glycosidic moieties of saponins **1**–**3** (CD_3OD, 30 °C).

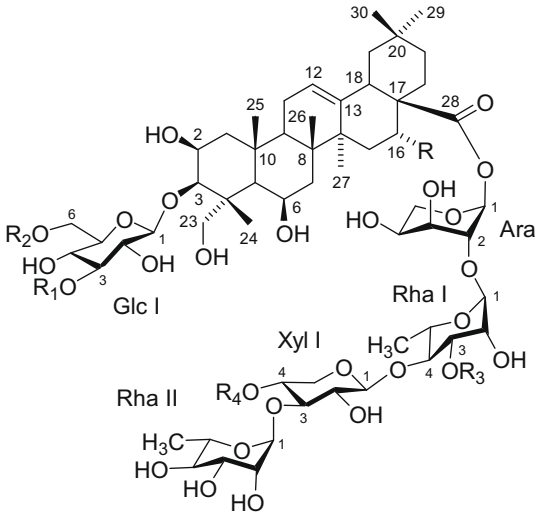
Atom		**1**		**2**		**3**	
		δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
At C-3							
3-*O*-β-Glc I	1	4.45 *d* (7.6)	104.3	4.44 *d* (7.6)	105.4	4.44 *d* (7.6)	104.3
	2	3.31 *m*	75.4	3.29 *m*	75.5	3.30 *m*	75.4
	3	3.37 *m*	78.3	3.36 *m*	78.3	3.37 *m*	78.1
	4	3.48 *m*	71.6	3.37 *m*	71.2	3.36 *m*	71.6
	5	3.48 *m*	76.5	3.28 *m*	77.8	3.48 *m*	76.5
	6	4.15 *m*	69.9	3.81 *m*	62.4	4.15 *dd* (10.9, 2.3)	70.0
		3.72 *m*		3.70 *m*		3.71 *m*	
$6^{Glc\ I}$-*O*-β-Glc II	1	4.30 *d* (7.7)	104.5			4.30 *d* (7.7)	104.5
	2	3.22 *dd* (9.1, 7.7)	75.3			3.22 *dd* (9.1, 7.7)	75.3
	3	3.37 *m*	78.0			3.37 *m*	78.1
	4	3.27 *m*	71.8			3.28 *m*	71.8
	5	3.28 *m*	78.1			3.28 *m*	78.0
	6	3.87 *m*	62.9			3.88 *m*	62.9
		3.65 *m*				3.65 *m*	
At C-28							
28-*O*-α-Ara	1	5.61 *d* (3.9)	94.2	5.61 *d* (3.8)	94.2	5.61 *d* (3.9)	94.2
	2	3.80 *dd* (5.4, 3.9)	75.7	3.80 *dd* (5.4, 3.9)	75.7	3.80 *dd* (5.4, 3.9)	75.7
	3	3.86 *m*	71.5	3.86 *m*	71.5	3.88 *m*	71.2
	4	3.82 *m*	67.2	3.83 *m*	67.3	3.82 *m*	67.0
	5	3.91 *m*	64.0	3.91 *m*	64.0	3.91 *m*	63.7
		3.52 *m*		3.52 *m*		3.50 *m*	
2^{Ara}-*O*-α-Rha I	1	5.06 *br s*	101.4	5.06 *br s*	101.4	5.02 *d* (1.8)	101.3
	2	3.85 *m*	72.4	3.85 *m*	72.4	4.00 *dd* (3.2, 1.8)	72.2
	3	3.85 *m*	72.5	3.85 *m*	72.5	3.85 *dd* (9.4, 3.2)	81.9
	4	3.58 *m*	83.1	3.58 *m*	83.1	3.72 *m*	78.3
	5	3.71 *m*	69.1	3.71 *m*	69.1	3.77 *m*	69.1
	6	1.30 *d* (6.2)	18.2	1.29 *d* (6.1)	18.2	1.27 *d* (6.2)	18.3
$3^{Rha\ I}$-*O*-β-Api	1					5.25 *d* (3.9)	112.0
	2					4.04 *d* (3.9)	78.3
	3					–	80.3
	4					4.12 *d* (9.7)	74.9
						3.76 *d* (9.7)	
	5					3.58 *m*	64.9
$4^{Rha\ I}$-*O*-β-Xyl I	1	4.56 *d* (7.6)	106.4	4.56 *d* (7.6)	106.4	4.65 *d* (7.7)	105.1
	2	3.38 *m*	76.8	3.38 *m*	76.8	3.29 *m*	76.0
	3	3.63 '*t*' (8.8)	80.2	3.64 *m*	80.2	3.44 *m*	84.2
	4	3.74 *m*	75.0	3.74 *m*	75.0	3.55	70.3
	5	4.06 *m*	64.6	4.06 *m*	64.6	3.88 *m*	67.1
		3.30 *m*		3.30 *m*		3.18 *m*	
$3^{Xyl\ I}$-*O*-α-Rha II	1	5.16 *d* (1.7)	102.2	5.16 *d* (1.5)	102.2	5.11 *d* (1.8)	102.8
	2	3.93 *dd* (3.4, 1.7)	72.5	3.93 *dd* (3.4, 1.7)	72.5	3.95 *dd* (3.4, 1.8)	72.4
	3	3.75 *m*	72.2	3.75 *m*	72.2	3.72 *m*	72.3
	4	3.36 *m*	74.2	3.37 *m*	74.2	3.39 *m*	74.1
	5	4.23 *dd* (9.5. 6.2)	69.6	4.22 *dd* (9.5. 6.2)	69.6	4.00 *m*	70.1
	6	1.22 *d* (6.2)	18.1	1.22 *d* (6.2)	18.1	1.25 *d* (6.2)	18.0
$4^{Xyl\ I}$-*O*-β-Xyl II	1	4.25 *d* (7.6)	103.4	4.25 *d* (7.5)	103.4		
	2	3.16 *m*	75.1	3.16 *m*	75.1		
	3	3.28 *m*	78.0	3.28 *m*	78.1		
	4	3.51 *m*	71.2	3.51 *m*	71.2		
	5	3.88 *m*	67.1	3.88 *m*	67.1		
		3.16 *m*		3.16 *m*			

resonances (Table 2), the sugars present were identified as α-Ara*p* (H-1 at δ_H 5.61, *d*, $J_{1,2}$ = 3.9 Hz, $J_{2,3}$ = 5.4 Hz), α-Rha*p* (H-1 of Rha I at δ_H 5.06, *br s*, $J_{5,6}$ = 6.2 Hz; H-1 of Rha II at δ_H 5.16, *d*, $J_{1,2}$ = 1.7 Hz, $J_{5,6}$ = 6.2 Hz), β-Xyl*p* (H-1 of Xyl I at δ_H 4.56, *d*, $J_{1,2}$ = 7.6 Hz; H-1 of Xyl II at δ_H 4.25, *d*, $J_{1,2}$ = 7.6 Hz) and β-Glc*p* (H-1 of Glc I at δ_H 4.45, *d*, $J_{1,2}$ = 7.6 Hz; H-1 of Glc II at δ_H 4.30, *d*, $J_{1,2}$ = 7.7 Hz) (Duus et al., 2000). The coupling constant data for α-Ara*p* indicated that it was present in the $^1C_4$ conformation, as observed in similar triterpenoid saponins (Eskander et al., 2006; Li et al., 1994). This glycosidic profile was in agreement with the results of sugar analysis (Section 3.4), by which the absolute configurations of the constituent monosaccharides of **1** released on acid hydrolysis were determined as L-Ara, D-Glc, L-Rha and D-Xyl.

HMBC connectivities detected between aglycone and sugar resonances indicated that **1** was a bisdesmoside. At C-3, correlations were observed between H-3 of the aglycone (δ_H 3.57) and C-1 of Glc I (δ_C 104.3), and from H-1 of Glc I (δ_H 4.45) to C-3 (δ_C 83.9). The downfield shifted resonances of C-6 (δ_C 69.9) and 6-CH_2 (δ_H 4.15, 3.72) of Glc I indicated further substitution at this position, as confirmed by HMBC correlations from 6-CH_2 of Glc I to C-1 of Glc II (δ_C 104.5), and H-1 of Glc II (δ_H 4.30) to C-6 of Glc I. Thus a β-D-Glc*p*-(1 → 6)-β-D-Glc*p* moiety was *O*-linked at C-3. The remaining five sugars were present as a pentasaccharide *O*-linked at C-28. The primary sugar was α-Ara*p*, as confirmed by a correlation in the HMBC spectrum between its anomeric proton at δ_H 5.61 and C-28 (δ_C 177.1). HMBC data were also used to define the inter-

glycosidic linkages of the pentasaccharide chain, as illustrated in Fig. 2. Saponin **1** was therefore determined to be 3-*O*-(β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4) α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid (Fig. 1).

Saponin **2** was isolated as a white amorphous powder for which ESI-MS experiments gave a deprotonated molecule at *m*/*z* 1369 $[M - H]^-$ and a sodiated molecule at *m*/*z* 1393 $[M + Na]^+$. These results were consistent with a molecular formula of $C_{63}H_{102}O_{32}$. The ^1H NMR spectrum was similar to that of **1**, except that only six anomeric proton resonances were observed (δ 5.61, 5.16, 5.06, 4.56, 4.44 and 4.25), indicating that **2** was a triterpenoid saponin with six sugars. The difference between the *m*/*z* values for the deprotonated molecules (likewise the sodiated molecules) of **1** and **2** was equivalent to 162 a.m.u., which suggested that the latter had only one Glc residue, rather than two. Full assignment of the ^1H and ^{13}C NMR spectra of **2** using 1D and 2D datasets equivalent to those acquired for **1** confirmed that both saponins were based on 16α-hydroxyprotobassic acid (Table 1), and had the same *O*-linked pentasaccharide at C-28 (Table 2). However, **2** had only a single Glc residue *O*-linked at C-3, rather than the diglucoside of **1**. As expected, MS/MS of $[M + Na]^+$ gave a fragment at *m*/*z* 711 $[(M + Na) - 162 - 520]^+$, corresponding to the loss of a triterpene moiety and one hexose. The absolute configurations of the constituent monosaccharides of **2** released on acid hydrolysis were confirmed to be L-Ara, D-Glc, L-Rha and D-Xyl (Section 3.4). Thus saponin **2** was 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid.



	R	R_1	R_2	R_3	R_4
1	OH	H	β-D-Glc II	H	β-D-Xyl II
2	OH	H	H	H	β-D-Xyl II
3	OH	H	β-D-Glc II	β-D-Api	H
4	H	H	H	H	β-D-Xyl II
5	OH	H	H	β-D-Api	H
6	OH	β-D-Api	H	H	β-D-Xyl II

Fig. 1. Saponins of *Sideroxylon foetidissimum* subsp. *gaumeri*.

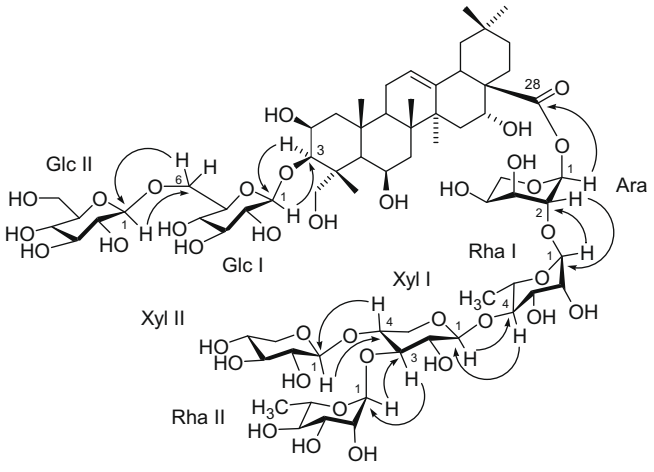


Fig. 2. Key HMBC connectivities of saponin **1**.

Saponin **3** was isolated as a white amorphous powder which in ESI-MS gave a deprotonated molecule at *m*/*z* 1531 $[M - H]^-$ and a sodiated molecule at *m*/*z* 1555 $[M + Na]^+$. These values were consistent with a molecular formula of $C_{69}H_{112}O_{37}$. In common with **1** and **2**, the aglycone of **3** was readily determined as 16α-hydroxyprotobassic acid, as the comparative NMR data in Table 1 confirm. Seven resonances corresponding to anomeric protons could be distinguished in the ^1H NMR spectrum of **3**, at δ 5.63, 5.25, 5.11, 5.02, 4.65, 4.44, and 4.30. Thus like **1**, saponin **3** contained seven sugar residues. Their glycosylation profiles at C-3 were found to be identical, with both characterised by an *O*-linked β-D-Glc*p*-(1 → 6)-β-D-Glc*p* moiety (Table 2). The difference between **1** and **3** (which have the same M_r) therefore resided in the *O*-linked pentasaccharide at C-28, the structure of which was established using correlations in HMBC spectra. In both saponins the primary sugar *O*-linked at C-28 was α-Ara, to which α-Rha I was linked at 2-OH. In comparison to **1**, however, the resonance of C-3 of α-Rha I (δ_C 81.9) was downfield shifted by +9.4 ppm, suggesting additional glycosylation at this position. This was confirmed by a correlation detected in the HMBC spectrum of **3** between δ_H 5.25 and C-3 of α-Rha I (δ_C 81.9). Full assignment of the ^1H and ^{13}C resonances of the sugar moiety with the anomeric proton at δ_H 5.25 (d, $J_{1,2}$ = 3.9 Hz), which was absent from **1**, revealed it to be a β-apiofuranosyl residue. Characteristic spectral features included the doublet resonance of H-2 at δ_H 4.04 ($J_{1,2}$ = 3.9 Hz), quaternary C-3 at δ_C 80.3, and two coupled doublets at δ_H 4.12 and 3.76 with J = 9.7 Hz, corresponding to 4-CH_2 (Table 2). The remaining sugar chain comprised β-Xyl I linked to 4-OH of α-Rha I, and a terminal residue, α-Rha II, linked to 3-OH of β-Xyl I. Thus **3** lacked the terminal residue, β-Xyl II, found in **1** (Table 2). The results of sugar analysis indicated that the absolute configurations of the constituent monosaccharides of **3** released on acid hydrolysis were D-Api, L-Ara, D-Glc, L-Rha and D-Xyl (Section 3.4). The structure of saponin **3** was therefore 3-*O*-(β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)[β-D-apiofuranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid.

Despite additional attempts at purification, the saponin mixture containing **5** and **6** could not be further separated. Use of ESI-MS (negative mode) indicated deprotonated molecules $[M - H]^-$ at *m*/*z* 1369 (**5**) and 1501 (**6**). Likewise, in positive mode, sodiated molecules $[M + Na]^+$ were detected at *m*/*z* 1393 (**5**) and 1525 (**6**). Analysis of the ^1H NMR spectrum of the mixture revealed that **5** and **6** were present in the ratio 5:4, based on integrated intensities of common resonances. Thus two sets of anomeric proton resonances were readily distinguished, the first, corresponding to **5**, at δ 5.63, 5.25, 5.11, 5.03, 4.65 and 4.44, and the second (**6**), at δ

5.61, 5.27, 5.16, 5.07, 4.56, 4.47 and 4.25. The assignment strategy for each individual sugar moiety was as described for **1**–**3**, except that NMR datasets acquired at both 600 and 400 MHz were utilised. Interglycosidic linkages and sites of glycosylation were defined from correlations in HMBC spectra, as before. The aglycone of both **5** and **6** was determined to be 16α-hydroxyprotobassic acid, for which a full set of NMR spectral assignments similar to those of **1**–**3** was obtained (Table 2 and Section 3.9).

Saponin **5**, in common with **1**–**4**, was a bisdesmoside. In the HMBC spectrum, the anomeric proton at δ 4.44, corresponding to a β-Glc residue, correlated with C-3 of the aglycone (δ_C 83.8), and only one sugar was present at this glycosylation site (Table 3). The structure of the pentasaccharide *O*-linked at C-28 was identical to that of **3**, an excellent match being obtained between the resonance assignment sets (^{1}H and ^{13}C) for these glycosyl moieties (Tables 2 and 3). Saponin **6** was also a bisdesmoside. In this case, the pentasaccharide *O*-linked at C-28 was found to be identical to that of **1**, **2** and **4** (Tables 2 and 3). The primary sugar residue *O*-linked at C-3 was β-Glc, as confirmed by a correlation in the HMBC spectrum between its anomeric proton resonance at δ 4.47, and C-3 (δ_C 83.8). In addition, the downfield shift of C-3 of β-Glc, which appeared at δ_C 86.0 (compared to δ_C 78.3 in **5**), indicated further substitution at this position. The remaining sugar moiety, corresponding to the anomeric proton at δ_H 5.27 (*d*, $J_{1,2}$ = 2.8 Hz), was a β-apiofuranosyl residue. This exhibited the characteristic features of a doublet resonance for H-2 at δ_H 3.99 ($J_{1,2}$ = 2.9 Hz), quaternary C-3 at δ_C 80.5, and two coupled doublets at δ_H 4.12 and 3.79 with J = 9.6 Hz, corresponding to 4-CH_2. Confirmation of the interglycosidic linkage followed from a connectivity in the HMBC spectrum between H-3 of β-Glc (δ_H 3.45) and C-1 of β-Api (δ_C 111.5). Similarly, H-1 of β-Api (δ_H 5.27) correlated with C-3 of β-Glc (δ_C 86.0). This defined an *O*-linked β-Api*f*-(1 → 3)-β-Glc*p* moiety at C-3 of **6**. Sugar analysis showed that the absolute configurations of the constituent monosaccharides of the mixture of **5** and **6** released on acid hydrolysis were D-Api, L-Ara, D-Glc, L-Rha and D-Xyl (Section 3.4). Thus the structures of these saponins were confirmed to be 3-*O*-(β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)[β-D-apiofuranosyl-

Table 3
^{1}H and ^{13}C NMR spectral data for the glycosidic moieties of saponins **5** and **6** (CD_3OD, 30 °C).

Atom		**5**		**6**	
		δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
At C-3					
3-*O*-β-Glc	1	4.44 *d* (7.6)	105.4	4.47 *d* (7.6)	105.2
	2	3.29 *m*	75.5	3.42 *m*	75.1
	3	3.36 *m*	78.3	3.45 *m*	86.0
	4	3.36 *m*	71.2	3.43 *m*	69.8
	5	3.28 *m*	77.8	3.30 *m*	77.5
	6	3.81 *m*, 3.70 *m*	62.4	3.80 *m*, 3.70 *m*	62.3
3^{Glc}-*O*-β-Api	1			5.27 *d* (2.8)	111.5
	2			3.99 *d* (2.9)	78.0
	3			–	80.5
	4			4.12 *d* (9.6), 3.79 *d* (9.6)	75.0
	5			3.60 *m*	65.2
At C-28					
28-*O*-α-Ara	1	5.63 *d* (3.6)	94.1	5.61 *d* (3.9)	94.2
	2	3.79 *m*	75.9	3.80 *m*	75.7
	3	3.88 *m*	71.3	3.86 *m*	71.7
	4	3.83 *m*	67.0	3.82 *m*	67.3
	5	3.91 *m*, 3.51 *m*	63.8	3.91 *m*, 3.52 *m*	63.8
2^{Ara}-*O*-α-Rha I	1	5.03 *d* (1.8)	101.4	5.07 *br d* (1.4)	101.4
	2	4.00 *m*	72.2	3.85 *m*	72.3
	3	3.84 *m*	81.9	3.85 *m*	72.4
	4	3.71 *m*	78.3	3.59 *m*	83.2
	5	3.76 *m*	69.1	3.72 *m*	69.1
	6	1.27 *d* (6.1)	18.3	1.30 *d* (6.1)	18.2
$3^{Rha\ I}$-*O*-β-Api	1	5.25 *d* (4.0)	112.0		
	2	4.05 *d* (3.9)	78.3		
	3	–	80.1		
	4	4.10 *d* (9.6), 3.76 *d* (9.7)	74.9		
	5	3.58 *m*	64.9		
$4^{Rha\ I}$-*O*-β-Xyl I	1	4.65 *d* (7.8)	105.0	4.56 *d* (7.6)	106.5
	2	3.29 *m*	76.0	3.38 *m*	76.8
	3	3.44 *m*	84.2	3.64 *m*	80.2
	4	3.53 *m*	70.2	3.74 *m*	75.0
	5	3.87 *m*, 3.17 *m*	67.1	4.06 *m*, 3.31 *m*	64.6
$3^{Xyl\ I}$-*O*-α-Rha II	1	5.11 *d* (1.8)	102.8	5.16 *d* (1.8)	102.2
	2	3.94 *dd* (3.3, 1.7)	72.5	3.93 *dd* (3.4, 1.7)	72.5
	3	3.71 *m*	72.4	3.75 *m*	72.3
	4	3.39 *m*	74.1	3.36 *m*	74.2
	5	4.00 *m*	70.1	4.22 *dd* (9.6, 6.3)	69.6
	6	1.24 *d* (6.2)	17.9	1.22 *d* (6.3)	18.1
$4^{Xyl\ I}$-*O*-β-Xyl II	1			4.25 *d* (7.5)	103.4
	2			3.16 *m*	75.0
	3			3.28 *m*	78.1
	4			3.51 *m*	71.2
	5			3.88 *m*, 3.16 *m*	67.1

(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid **(5)**, and 3-*O*-(β-D-apiofuranosyl-(1 → 3)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)[β-D-xylopyranosyl-(1 → 4)]-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid **(6)**.

Saponins **1**–**3**, **5** and **6** have not been reported previously. Together with **4** they possess the 'core' tetrasaccharide α-L-Rha*p*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-α-L-Rha*p*-(1 → 2)-α-L-Ara*p* *O*-linked at C-28, a relatively common feature in the saponins of Sapotaceae and some other plant families (Eskander et al., 2005). In contrast, sideroxyloside A, isolated previously from the roots of *S. cubense* (Jiang et al., 1994) had the tetrasaccharide α-L-Rha*p*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-α-L-Rha*p*-(1 → 2)-β-D-Xyl*p* *O*-linked at C-28 (i.e., with a different primary sugar to **1**–**6**). Sideroxylosides B and C, from the roots of *S. foetidissimum*, have the pentasaccharide β-D-Api*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-α-L-Rha*p*-(1 → 4)-α-L-Rha*p*-(1 → 2)-α-L-Ara*p* as a common unit at C-28 (Nicolas et al., 1995). Sideroxylosides A–C all possess protobassic acid as aglycone, as was also found for **4** in the present study.

The original fraction containing saponins **1–6**, purified samples of **1**, **2** and **4**, and the 5:4 mixture of **5** and **6** were tested for cytotoxicity against the murine macrophage-like cell line RAW 264.7 (Section 3.10). Of these, the fraction containing **1**–**6** and the 5:4 mixture of **5** and **6** showed moderate and strong cytotoxicity, respectively. Comparison of their IC_{50} values, at 33.7 ± 6.2 μg/ml and 11.9 ± 1.5 μg/ml, respectively, showed a significant difference (unpaired *t*-test, $n = 3$, $p = 0.0009$, 95% confidence). Saponins **1**, **2** and **4** were not active in this assay. Thus the aglycone moiety common to **1**, **2**, **5** and **6** (16α-hydroxyprotobassic acid) is not a structural determinant of cytotoxicity. Although various types of biological activity have been associated with the sugar moieties of saponins (Kalinowska et al., 2005; Sparg et al., 2004), bisdesmosides are often inactive, and are thought to serve primarily as transport and/or storage forms of monodesmosides (Hostettmann et al., 1991; Hostettmann and Marston, 1995). In this context the cytotoxic activity of the 5:4 mixture of **5** and **6**, which comprised two bisdesmosidic saponins, is noteworthy.

3. Experimental

3.1. General experimental procedures and instrumentation

Silica gel 60 F_{254} (Sigma–Aldrich) plates were used for TLC. After development in $CHCl_3$–MeOH–H_2O (14:7:1), plates were sprayed with 1% vanillin in EtOH, immersed in H_2SO_4 (5% in H_2O), and heated with hot air until spots appeared. HPLC was carried out using a Waters system (600E pump, 996 photodiode array detector) with a Spherisorb ODS2 column (250 mm × 10 mm i.d.; 5 μm particle size). HPLC grade solvents were used (Fisher Scientific and BDH), and all chromatograms monitored at 210 nm.

ESI-MS and MS/MS mass spectrometry analyses were performed using a Micromass (Manchester, UK) Quattro 11 tandem mass spectrometer with an electrospray, Z-spray ion source (+/− ve). Flow injection analysis was employed with a solvent mixture of CH_3OH and H_2O each with 0.1% HCOOH 50:50 v/v and a flow rate of 10 μl/min. The analyte concentration was 10 μg/ml. For tandem mass spectrometry, a collision cell with 2×10^{-5} torr argon, and product ion scans were employed.

NMR spectra were acquired in CD_3OD at 30 °C on either Bruker Avance 400 MHz or Varian 600 MHz instruments. Standard pulse sequences and parameters were used to obtain 1D ^1H, 1D ^{13}C, DEPT135, COSY, TOCSY, HSQC and HMBC spectra. For the TOCSY experiments, mixing times of both 60 and 100 ms were used. Chemical shift referencing was carried out with respect to TMS at 0.00 ppm.

3.2. Plant material

S. foetidissimum subsp. *gaumeri* was collected on July 20th, 2001, from the town Othón P. Blanco, Quintana Roo, Mexico (approximately 191005N and 883530W). The plant was identified by Mr. Filogonio May-Pat and a voucher specimen (FMay 1945) deposited at the herbarium of the Centro de Investigación Científica de Yucatán in Mexico.

3.3. Extraction and isolation

Roots of *S. foetidissimum* subsp. *gaumeri* were dried, first for a week at room temperature, and then for 4 days in an oven at 55 °C. The dried roots were ground using a Pagani mill (model 1520, 5 HPI), and 1242 g of material extracted (48 h, ×3) using EtOH (1200 ml/250 g, batchwise) at room temperature. The solvent was removed under reduced pressure by rotary evaporation to give a crude extract (69.2 g), a portion of which (12.1 g) was subjected to open Si gel column chromatography (4 cm × 8 cm i.d.) and eluted successively with $CHCl_3$–MeOH–H_2O (80:30:2, 3500 ml), (70:30:3, 750 ml), (60:40:4, 1750 ml) and (50:50:5, 750 ml). Twenty-seven 250 ml fractions were collected and combined into 10 fractions (A–J) after TLC analysis. Fraction J (410 mg) was redissolved in 2 ml H_2O, and part of this solution (J.1) subjected to semi-preparative HPLC with isocratic elution (65% MeOH for 15 min at 1.5 ml/min), yielding four main subfractions (J.1a–J.1d). Of these, J.1d (5.7 mg) comprised saponin **4** (confirmed as pure by ^1H NMR). A further portion of the same solution (J.2) was subjected to semi-preparative HPLC (repetitive 50 μl injections), but with gradient elution. Three solvents MeOH (A), MeCN (B), and H_2O (C) were employed, using the program, $t = 0$ min, A = 33%, B = 12%, C = 55%, flow = 2.0 ml/min (initial conditions); $t = 45$ min, A = 33%, B = 12%, C = 55%, flow = 2.0 ml/min; $t = 47$ min, A = 40%, B = 30%, C = 30%, flow = 2.5 ml/min; $t = 60$ min, A = 40%, B = 30%, C = 30%, flow = 2.5 ml/min; $t = 62$ min, A = 33%, B = 12%, C = 55%, flow = 3.0 ml/min; $t = 70$ min, A = 33%, B = 12%, C = 55%, flow = 3.0 ml/min; $t = 71$ min, A = 33%, B = 12%, C = 55%, flow = 2.0 ml/min (return to initial conditions). Six fractions, J.2a–J.2f, were collected at t_R 4.4–6.3, 17.8, 19.9, 30.6, 33.4 and 51.4 min, respectively. ^1H NMR analysis established that J.2b and J.2d comprised saponins **1** (30.2 mg) and **2** (29.5 mg), respectively, in pure form.

Fraction J.2f (35.6 mg) was redissolved in 1 ml of H_2O and subjected to semi-preparative HPLC (repetitive 50 μl injections) using isocratic elution with 65% MeOH over 25 min at 1.5 ml/min. A subfraction collected at t_R 15.8 min comprised an additional amount of saponin **4** (19.9 mg). Similarly, fraction J.2f (11 mg) was redissolved in H_2O and subjected to semi-preparative HPLC using isocratic elution with MeOH–MeCN–H_2O (33:12:55) for 35 min at 1.5 ml/min. A subfraction collected at t_R 22.8 min afforded saponin **3** (5.1 mg). Finally, fraction J.2e (21.9 mg) was redissolved in H_2O and subjected to semi-preparative HPLC using gradient elution with MeOH–MeCN–H_2O (33:12:55) for 45 min at 1.5 ml/min. Collection of a subfraction at t_R 35.0 min gave a 5:4 mixture of saponins **5** and **6** (10.4 mg).

3.4. Sugar analysis

Acid hydrolysis of **1**–**4** and the 5:4 mixture of **5** and **6** was carried out by dissolving approximately 0.7 mg of each in 2 ml dioxan:2 M HCl (1:1) and heating at 100 °C for 1 h. The hydrolysate was transferred to a 7 ml vial and dried under a stream of N_2 on a heating block at 40 °C. The absolute configurations of the constituent monosaccharides of **1**–**6** released by acid hydrolysis were determined by GC–MS analysis of their trimethylsilylated thiazolidine derivatives, which were prepared using the method of Ito et al. (2004). Conditions for GC were: capillary column, DB5-MS

(30 m × 0.25 mm × 0.25 μm), oven temperature program, 180–300 °C at 6 °C/min; injection temperature 350 °C; carrier gas, He at 1 ml/min. Compounds **1**–**4** and the 5:4 mixture of **5** and **6** gave D-xylose, L-arabinose, L-rhamnose and D-glucose at t_R 9.48, 9.52, 10.19 and 12.20 min, respectively. In addition, **3** and the 5:4 mixture of **5** and **6** gave D-apiose at t_R 9.27 min (identical to authentic standards).

3.5. Saponin *1*

Amorphous white powder. ^{1}H and ^{13}C NMR: see Tables 1 and 2; ESI-MS (negative mode) *m*/*z*: 1531 $[M-H]^-$; ESI-MS (positive mode) *m*/*z*: 1555 $[M+Na]^+$; MS/MS of *m*/*z* 1555 $[M+Na]^+$ *m*/*z*: 867 $[(M+Na)-(3\times132)-(2\times146)]^+$, 711 $[(M+Na)-(2\times162)-520]^+$, 579 $[(M+Na)-(2\times162)-520-132]^+$, 563 $[(M+Na)-(2\times162)-520-146]^+$.

3.6. Saponin *2*

Amorphous white powder. ^{1}H and ^{13}C NMR: see Tables 1 and 2; ESI-MS (negative mode) *m*/*z*: 1369 $[M-H]^-$; ESI-MS (positive mode) *m*/*z*: 1393 $[M+Na]^+$; MS/MS of *m*/*z* 1393 $[M+Na]^+$ *m*/*z*: 711 $[(M+Na)-162-520]^+$, 579 $[(M+Na)-162-520-132]^+$, 563 $[(M+Na)-162-520-146]^+$, 433 $[(M+Na)-162-520-132-146]^+$, 287 $[(M+Na)-162-520-132-(2\times146)]^+$, 155 $[(M+Na)-162-520-(2\times132)-(2\times146)]^+$.

3.7. Saponin *3*

Amorphous white powder. ^{1}H and ^{13}C NMR: see Tables 1 and 2; ESI-MS (negative mode) *m*/*z*: 1531 $[M-H]^-$; ESI-MS (positive mode) *m*/*z*: 1555 $[M+Na]^+$.

3.8. Saponin *4*

Amorphous white powder. ^{1}H and ^{13}C NMR: identical to lit. (Eskander et al., 2005; compound **2**); ESI-MS (negative mode) *m*/*z*: 1353 $[M-H]^-$; ESI-MS (positive mode) *m*/*z*: 1377 $[M+Na]^+$.

3.9. Saponins *5* and *6*

Amorphous white powder comprising 5:4 mixture of **5** and **6**. ^{1}H and ^{13}C NMR of glycosidic resonances: see Table 3; ^{1}H NMR of aglycone (CD_3OD, assignments identical for **5** and **6**): δ 2.06, 1.18 (2 × *m*, H-1a,b), 4.34 (*br m*, H-2), 3.58 (*m*, H-3), 1.33 (*m*, H-5), 4.47 (*br m*, H-6), 1.82, 1.56 (2 × *m*, H-7a,b), 1.65 (m, H-9), 2.12, 1.98 (2 × *m*, H-11a,b), 5.42 (*br m*, H-12), 1.84, 1.41 (2 × *m*, H-15a,b), 4.49 (*br m*, H-16), 3.08 (*dd*, *J* = 14.4, 4.1 Hz, H-18), 2.27, 1.06 (2 × *m*, H-19a,b), 1.90, 1.16 (2 × *m*, H-21a,b), 1.90, 1.79 (2 × *m*, H-22a,b), 3.72, 3.43 (2 × *m*, H-23a,b), 1.31 (s, 24-CH_3), 1.63 (s, 25-CH_3), 1.05 (s, 26-CH_3), 1.34 (s, 27-CH_3), 0.89 (s, 29-CH_3), 0.98 (s, 30-CH_3); ^{13}C NMR of aglycone (CD_3OD, assignments identical for **5** and **6**): δ 46.8 (C-1), 71.4 (C-2), 83.8 (C-3), 44.1 (C-4), 49.0 (C-5), 68.7 (C-6), 41.5 (C-7), 40.0 (C-8), 48.8 (C-9), 37.3 (C-10), 24.6 (C-11), 124.2 (C-12), 144.0 (C-13), 43.5 (C-14), 36.4 (C-15), 74.6 (C-16), 50.5 (C-17), 42.3 (C-18), 47.7 (C-19), 31.4 (C-20), 36.5 (C-21), 31.8 (C-22), 65.6 (C-23), 16.4 (C-24), 19.3 (C-25), 19.1 (C-26), 27.4 (C-27), 177.1 (C-28), 33.4 (C-29), 25.2 (C-30); ESI-MS (negative mode) *m*/*z*: 1369 $[M-H]^-$ (**5**), 1501 $[M-H]^-$ (**6**); ESI-MS (positive mode) *m*/*z*: 1393 $[M+Na]^+$ (**5**), 1525 $[M+Na]^+$ (**6**).

3.10. Cytotoxicity bioassay by MTT

The murine macrophage-like cell line RAW 264.7 was used for the cell viability assays. The cell line was purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). The medium used for the maintenance of the cell line and for the *in vitro* bioassay was Dulbecco's modified eagle's medium (Sigma–Aldrich), supplemented with 10% of heat inactivated foetal bovine serum (Sigma–Aldrich), 2.5 μg/ml amphotericin B, 30 μg/ml streptomycin and 30 IU/ml penicillin. The modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich) assay was used to detect the cellular viability (Habtemariam, 1995). Briefly, cells were harvested (5×10^3 cells/well) in 100 μl in 96-well plates. After 24 h incubation at 37 °C and 5% CO_2 to allow cell establishment, different concentrations of compounds or extracts made with 1:3 or 1:2 serial dilutions were added. The starting concentration was 100 μg/ml for pure compounds and 500 μg/ml for crude extracts or fractions. Four replicates were set up for each experiment and the experiment was repeated four times ($n = 4$). Samples were left in contact with cells for 24 h and cell viability was assessed by adding 10 μl of MTT solution (5 mg/ml) during the last 4 h of incubation. The absorbance associated with formazan-blue formation was then measured using a Thermo Labsystems-Multiskan EX ELISA reader at 570 nm. Calculation of IC_{50} values (concentration required to kill 50% of cells) was carried out using the corresponding Microsoft Excel formula for correlation and regression. Significant differences (95% confidence) between fractions as the fractionation steps advanced were obtained using the GraphPad Prism 5.01 software. The positive control, podophyllotoxin, gave an IC_{50} value of 29 ± 5 nM against RAW 264.7 cells under the same experimental conditions.

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