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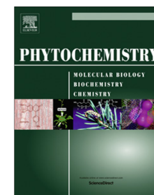

Phytotoxic steroidal saponins from *Agave offoyana* leaves

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ABSTRACT

A bioassay-guided fractionation of *Agave offoyana* leaves led to the isolation of five steroidal saponins (**1**–**5**) along with six known saponins (**6**–**11**). The compounds were identified as (25*R*)-spirost-5-en-2α,3β-diol-12-one 3-*O*-{α-L-rhamnopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside} (**1**), (25*R*)-spirost-5-en-3β-ol-12-one 3-*O*-{α-L-rhamnopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside} (**2**), (25*R*)-spirost-5-en-3β-ol-12-one 3-*O*-{β-D-xylopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside} (**3**), (25*R*)-26-*O*-β-D-glucopyranosylfurost-5-en-3β,22α,26-triol-12-one 3-*O*-{α-L-rhamnopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside} (**4**) and (25*R*)-26-*O*-β-D-glucopyranosylfurost-5-en-3β,22α,26-triol-12-one 3-*O*-{β-D-xylopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside} (**5**) by comprehensive spectroscopic analysis, including one- and two-dimensional NMR techniques, mass spectrometry and chemical methods. The phytotoxicity of the isolated compounds on the standard target species *Lactuca sativa* was evaluated.

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1. Introduction

As part of our ongoing search for phytotoxic natural products, the results of further studies into *Agave offoyana* (Agavaceae) are presented here. The bioassay-guided isolation of phytotoxins from *A. offoyana* flowers was reported in our previous article, in which eleven steroidal saponins, including five new examples (Magueyosides A–E), were described (Pérez et al., 2013). The phytotoxicity results for the isolated compounds against lettuce (*Lactuca sativa* L.) were promising.

Despite the positive results obtained in the aforementioned studies and the fact that the saponins content in the flowers was 3.1% of dry weight, the availability of flowers from *A. offoyana* as a raw material for phytotoxins is limited. In the genus *Agave* the flowering stage takes place after several years of growth (6–8 years). The flowering stage happens only once in the lifetime of the plants and they subsequently die (Bousios et al., 2007). The aim of the current investigation was to isolate the phytotoxic constituents from *A. offoyana* leaves (1.9% of dry weight) in a similar bioassay-guided procedure and to carry out a complete structural characterization of the isolated compounds.

2. Results and discussion

2.1. Characterization of compounds

Dried leaves of *A. offoyana* were extracted exhaustively with EtOH/H_2O (7:3). The extract was partitioned in *n*-BuOH/water and the organic phase was subjected to a bioassay-guided fractionation by VLC on RP-18 to give seven fractions. Eleven steroidal saponins **1**–**11** (Fig. 1) were obtained after multiple separation procedures on the active fractions (see Section 2.2). Six of these compounds were previously reported as Agabrittonoside E (**6**) (Macías et al., 2010), Magueyosides A (**7**) and B (**8**) (Pérez et al., 2013), Agabrittonosides D (**9**) and A (**10**) (Macías et al., 2007) and Cantalasaponin-1 (**11**) (Sati and Pant, 1985). The structures

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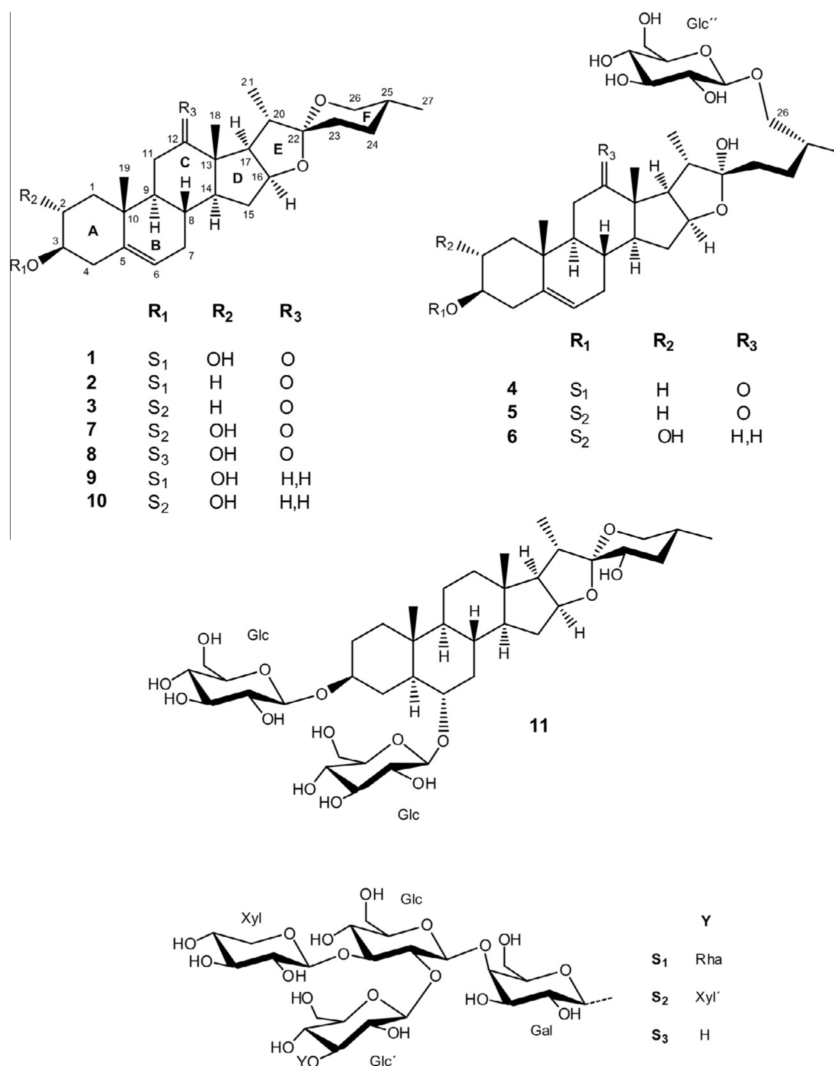

Fig. 1. Chemical structures of saponins **1**–**11**, isolated from *A. offoyana* leaves.

of the compounds were elucidated on the basis of spectroscopic data obtained by ^{1}H, ^{13}C, 2D (HSQC, HMBC, DQF-COSY, TOCSY, ROESY, HSQC-TOCSY), 1D-ROESY (250 ms) and 1D-TOCSY (30, 60, 120 ms) NMR experiments, HRESIMS, ESI-MS/MS and acid hydrolysis. Compounds **6** and **9** were previously characterized as a mixture from *Agave brittoniana* leaves. In the work described here these compounds were isolated in pure form and their HRESI-TOFMS spectra, absolute configuration of sugars and optical rotations were determined.

Compound **1** was obtained as a white amorphous solid and its molecular formula was assigned as $C_{56}H_{88}O_{28}$ based on data from HRESI-TOFMS (positive ion mode; *m*/*z* 1231.5345 $[M+Na]^{+}$, calcd. 1231.5360). ^{1}H and ^{13}C NMR assignments for the aglycone moiety of **1** (Table 1) were in good agreement with those of the aglycone moiety of **7** (Pérez et al., 2013), which suggested that Kammogenin (Marker et al., 1943) was the aglycone of **1**. However, significant differences between compounds **1** and **7** were observed concerning the sugar portion. The ^{1}H NMR spectrum of **1** showed five anomeric signals at δ 4.92, 5.14, 5.24, 5.51 and 6.12 (Table 2) and these showed correlations in the HSQC spectrum with carbons at δ 103.7, 105.3, 104.6, 104.6 and 103.1, respectively. This result indicated a glycosidic chain of five sugar units. Individual sugar units were identified by a combination of ^{1}H–^{1}H COSY, 1D-TOCSY and 1D-ROESY experiments. The latter two spectra were acquired from the selective excitation of each anomeric proton. In this way, signals at δ 5.24 and δ 5.51 revealed typical spin systems of β-glucopyranosyl units (Glc and Glc′) and the signal at δ 5.14 was assigned to a β-xylopyranosyl unit (Xyl). Likewise, a typical spin system for a β-galactopyranosyl unit was detected for the anomeric signal at δ 4.92, which was confirmed by correlations with H-5_{Gal} (δ 4.02, dd, J = 7.7, 6.4 Hz) and H-3_{Gal} (δ 4.14, m) in the 1D ROESY experiment. The 1D TOCSY (120 ms) experiment on the anomeric signal at δ 6.12 showed that magnetization was not properly transferred beyond H-2_{Rha} (δ 4.68, dd, J = 1.7, 3.5 Hz). This finding, together with the presence of a methyl doublet at δ 1.64 (J = 6.2 Hz), is characteristic of a rhamnopyranosyl unit. A further selective TOCSY experiment on this methyl doublet (H-6_{Rha}) allowed a sequential assignment of the signals from H-6_{Rha} to H-2_{Rha} and the presence of a rhamnopyranosyl unit was confirmed. The correlations observed in the 1D ROESY experiment between H-1_{Rha} and H-2_{Rha}, and the absence of a cross peak with H-3_{Rha} and H-5_{Rha} suggested the α-anomer. Finally, the ^{13}C signal assignments for each sugar unit were made through an exhaustive analysis of the correlations in HSQC and HSQC-TOCSY experiments.

The sequence of sugars chain and the connection with the aglycone were established by means of interglycosidic HMBC/ROESY correlations, which were observed between H-1_{Rha} (δ 6.12) and C-$3_{Glc'}$ (δ 83.9)/H-$3_{Glc'}$ (δ 4.23), H-$1_{Glc'}$ (δ 5.51) and C-2_{Glc} (δ 81.1)/H-2_{Glc} (δ 4.27), H-1_{Xyl} (δ 5.14) and C-3_{Glc} (δ 87.6)/H-3_{Glc} (δ 4.07), H-1_{Glc} (δ 5.24) and C-4_{Gal} (δ 79.2)/H-4_{Gal} (δ 4.60), H-1_{Gal} (δ 4.92)

Table 1
^{13}C and ^{1}H NMR data (*J* in Hz) of the aglycone moieties of compounds **1**–**5** (pyridine-d_5, 500 MHz).[a]

	Magueyoside F (**1**)		Magueyoside G (**2**)		Magueyoside H (**3**)		Magueyoside I (**4**)		Magueyoside J (**5**)	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1_{ax}	45.6	1.26 dd (12.5, 11.4)	37.3	0.88 ddd (13.8, 13.7, 4.1)	37.3	0.87 ddd (13.7, 13.7, 4.1)	37.3	0.88 ddd (13.1, 12.9, 3.3)	37.3	0.87 ddd (13.3, 13.2, 3.4)
1_{eq}		2.20 dd (12.5, 4.5)		1.49 ddd (13.8, 3.5, 3.5)		1.47 ddd (13.7, 3.6, 3.6)		1.49[b]		1.47 ddd (13.3, 4.1, 4.1)
2_{ax}	70.1	4.06 ddd (11.4, 8.8, 4.5)	30.2	1.67[b]	30.2	1.65[b]	30.2	1.68[b]	30.2	1.66[b]
2_{eq}				2.08[b]		2.06[b]		2.08[b]		2.06[b]
3	84.5	3.82 ddd (11.5, 8.8, 5.1)	78.3	3.84 dddd (11.4, 11.4, 4.3, 4.3)	78.3	3.84 dddd (11.4, 11.4, 4.7, 4.7)	78.3	3.84 dddd (11.2, 11.2, 4.3, 4.3)	78.3	3.83 dddd (11.4, 11.4, 4.2, 4.2)
4_{ax}	37.9	2.56[b]	39.4	2.41 m	39.4	2.39 m	39.4	2.41 m	39.4	2.39 m
4_{eq}		2.75 dd (13.9, 5.1)		2.68 ddd (13.4, 4.3, 2.2)		2.68 ddd (13.5, 4.7, 2.2)		2.67 ddd (13.3, 4.3, 2.0)		2.68 ddd (13.5, 4.2, 2.1)
5	140.3	–	141.2	–	141.2	–	141.2	–	141.2	–
6	122.1	5.29 m	121.8	5.28 m	121.8	5.27 m	121.8	5.27 m	121.8	5.26 m
7_{ax}	32.1	1.46[b]	32.1	1.47[b]	32.1	1.46[b]	32.1	1.46[b]	32.1	1.45[b]
7_{eq}		1.86[b]		1.87[b]		1.86[b]		1.87[b]		1.86[b]
8	30.7	1.84[b]	31.2	1.84 dddd (11.2, 11.2, 10.9, 5.2)	31.2	1.83 dddd (11.0, 11.0, 10.9, 5.1)	31.2	1.84[b]	31.2	1.84[b]
9	52.4	1.41 ddd (11.8, 12.5, 5.7)	52.6	1.31 ddd (11.2, 13.1, 5.8)	52.6	1.30 ddd (11.0, 12.7, 5.8)	52.7	1.31 ddd (11.9, 13.1, 5.6)	52.7	1.30 ddd (12.2, 12.9, 5.7)
10	38.8	–	37.9	–	37.9	–	37.9	–	37.9	–
11_{ax}	37.9	2.56 dd (14.9, 12.5)	37.9	2.53 dd (14.6, 13.1)	37.9	2.52 dd (14.8, 12.7)	37.9	2.55 dd (14.5, 13.1)	37.9	2.54 dd (14.6, 12.9)
11_{eq}		2.42 dd (14.9, 5.7)		2.30 dd (14.6, 5.8)		2.29 dd (14.8, 5.8)		2.31 dd (14.5, 5.6)		2.30 dd (14.6, 5.7)
12	212.8	–	213.0	–	213.0	–	213.2	–	213.2	–
13	55.3	–	55.3	–	55.3	–	55.7	–	55.7	–
14	56.1	1.43 ddd (13.8, 10.9, 5.5)	56.3	1.43 ddd (13.6, 11.2, 5.1)	56.3	1.43 ddd (13.4, 10.9, 5.3)	56.3	1.43 ddd (12.9, 11.9, 5.4)	56.3	1.43 ddd (13.6, 10.8, 5.3)
15_{ax}	31.9	1.61 ddd (13.8, 12.3, 6.6)	31.9	1.61 ddd (13.6, 12.0, 6.5)	31.9	1.62 ddd (13.4, 12.0, 6.6)	32.2	1.63 ddd (12.9, 12.8, 6.4)	32.2	1.63 ddd (13.6, 12.1, 6.5)
15_{eq}		2.11 ddd (12.3, 7.1, 5.2)		2.11 ddd (12.0, 7.2, 5.1)		2.10 ddd (12.0, 7.1, 5.3)		2.09 ddd (12.8, 6.8, 5.4)		2.08 ddd (12.1, 7.2, 5.3)
16	80.1	4.49 ddd (8.6, 7.1, 6.6)	80.1	4.49 ddd (8.6, 7.2, 6.5)	80.1	4.49 ddd (8.6, 7.1, 6.6)	80.1	4.88 ddd (8.5, 6.8, 6.4)	80.1	4.88 ddd (8.5, 7.2, 6.5)
17	54.4	2.81 dd (8.6, 6.8)	54.4	2.81 dd (8.6, 6.9)	54.4	2.81 dd (8.6, 6.8)	55.2	2.96 dd (8.5, 6.8)	55.2	2.95 dd (8.5, 6.8)
18	16.3	1.10 s	16.3	1.10 s	16.2	1.10 s	16.4	1.16 s	16.4	1.16 s
19	20.3	1.01 s	19.2	0.93 s	19.1	0.91 s	19.2	0.94 s	19.1	0.91 s
20	43.0	1.92 dq (6.8, 6.9)	43.0	1.93 dq (6.9, 6.9)	43.0	1.92 dq (6.9, 6.8)	41.6	2.22 dq (6.8, 6.8)	41.7	2.22 dq (6.8, 6.8)
21	14.3	1.35 d (6.9)	14.3	1.36 d (6.9)	14.3	1.35 d (6.9)	15.6	1.56 d (6.8)	15.6	1.56 d (6.8)
22	109.7	–	109.7	–	109.7	–	111.2	–	111.2	–
23_{ax}	32.2	1.63[b]	32.2	1.64[b]	32.2	1.63[b]	37.5	2.05[b] (2H)	37.5	2.05[b] (2H)
23_{eq}		1.71[b]		1.70[b]		1.70[b]				
24	29.6	1.57[b] (2H)	29.6	1.57[b] (2H)	29.6	1.57[b] (2H)	28.7	1.68[b]; 2.05 o	28.7	1.68[b]; 2.04 o
25	30.9	1.58[b]	30.9	1.58[b]	30.9	1.58[b]	34.6	1.94 m	34.6	1.94 m
26_{ax}	67.3	3.49 dd (10.8, 10.8)	67.3	3.49 dd (10.5. 10.5)	67.3	3.49 dd (10.7, 10.7)	75.6	3.62 dd (9.4, 6.2); 3.96 dd (9.4, 7.2)	75.7	3.62 dd (9.4, 6.3); 3.96 dd (9.4, 7.2)
26_{eq}		3.59 dd (10.8, 3.6)		3.59 dd (10.5, 3.6)		3.59 dd (10.7, 3.7)				
27	17.7	0.70 d (5.8)	17.7	0.70 d (5.8)	17.7	0.70 d (5.8)	17.8	0.99 d (6.7)	17.8	0.99 d (6.7)

[a] Assignments were confirmed by DQF-COSY, 2D-TOCSY, HSQC, HSQC-TOCSY and HMBC experiments.
[b] Overlapped.

Table 2

^{13}C and ^{1}H NMR data (*J* in Hz) of the sugar portions of compounds **1–5** (pyridine-d_5, 500 MHz).[a]

	Magueyoside F (**1**)		Magueyoside G (**2**)		Magueyoside H (**3**)		Magueyoside I (**4**)		Magueyoside J (**5**)	
		β-D-Gal		β-D-Gal		β-D-Gal		β-D-Gal		β-D-Gal
1	103.7	4.92 d (7.5)	103.2	4.86 d (7.6)	103.2	4.87 d (7.7)	103.2	4.86 d (7.7)	103.2	4.88 d (7.9)
2	73.0	4.52 dd (7.9, 9.7)	73.5	4.43 dd (7.6, 9.6)	73.5	4.40 dd (7.7, 9.7)	73.5	4.43 dd (7.7, 9.7)	73.5	4.40 dd (7.9, 9.4)
3	75.9	4.14 m	75.9	4.11 m	75.8	4.11 dd (9.7, 3.1)	75.9	4.11 dd (9.7, 3.5)	75.8	4.12 dd (9.4, 3.7)
4	79.2	4.60 d (2.5)	80.0	4.59 d (2.8)	80.0	4.60 d (3.1)	80.0	4.59 brs	80.0	4.60 brs
5	76.1	4.02 dd (7.7, 6.4)	75.7	3.97 dd (8.6, 5.5)	75.7	3.97 dd (8.5, 5.7)	75.7	3.97 dd (8.8, 5.4)	75.6	3.98 dd (8.9, 5.4)
6	61.1	4.18 dd (10.5, 6.4)	61.0	4.18 dd (10.6, 5.5)	61.0	4.18 dd (10.5, 5.7)	61.0	4.18 dd (10.6, 5.4)	61.0	4.18 dd (10.0, 5.4)
		4.57 dd (10.5, 7.7)		4.65 dd (10.6, 8.6)		4.67 dd (10.5, 8.5)		4.65 dd (10.6, 8.8)		4.67 dd (10.0, 8.9)
		β-D-Glc		β-D-Glc		β-D-Glc		β-D-Glc		β-D-Glc
1	104.6	5.24 d (7.9)	105.3	5.18 d (7.9)	105.3	5.19 d (8.0)	105.1	5.18 d (7.8)	105.3	5.19 d (7.9)
2	81.1	4.27 dd (7.9, 8.5)	81.3	4.33 dd (7.9, 8.7)	81.1	4.40 dd (8.0, 8.5)	81.3	4.33 dd (7.8, 8.5)	81.1	4.40 dd (7.9, 8.5)
3	87.6	4.07 dd (8.5, 8.5)	87.5	4.10 dd (8.7, 8.7)	87.0	4.14 dd (8.5, 8.9)	87.5	4.10 dd (8.5, 8.7)	87.0	4.14 dd (8.5, 8.8)
4	70.7	3.82[b]	70.7	3.81 dd (8.7, 9.1)	70.8	3.81 dd (8.9, 9.2)	70.7	3.81 dd (8.7, 8.7)	70.8	3.81 dd (8.8, 9.1)
5	78.0	3.83[b]	78.0	3.84 ddd (9.1, 6.8, 3.0)	78.0	3.88 ddd (9.2, 6.8, 2.4)	78.0	3.84 ddd (8.7, 6.3, 3.1)	78.0	3.88 ddd (9.1, 6.2, 2.3)
6	63.3	4.07[b]	63.3	4.06 dd (11.9, 6.8)	63.3	4.06 dd (12.0, 6.8)	63.3	4.06 dd (11.9, 6.3)	63.3	4.06 dd (12.6, 6.2)
		4.50 dd (9.8, 2.2)		4.51 dd (11.9, 3.0)		4.52 dd (12.0, 2.4)		4.51 dd (11.9, 3.1)		4.52 dd (12.6, 2.3)
		β-D-Glc′		β-D-Glc′		β-D-Glc′		β-D-Glc′		β-D-Glc′
1	104.6	5.51 d (8.0)	104.7	5.51 d (8.0)	104.4	5.59 d (7.5)	104.7	5.51 d (7.9)	104.4	5.59 d (7.4)
2	76.8	3.97 dd (8.0, 9.3)	76.8	4.00 dd (8.0, 9.3)	75.5	4.09[b]	76.8	4.00 dd (7.9, 9.2)	75.5	4.10[b]
3	83.9	4.23 dd (9.2, 9.2)	83.5	4.23 dd (9.3, 9.3)	87.1	4.06[b]	83.5	4.23 dd (9.2, 9.2)	87.1	4.06[b]
4	69.1	4.01 m	69.6	4.13 dd (9.3, 7.4)	69.5	4.06[b]	69.6	4.12 dd (9.2, 8.9)	69.5	4.06[b]
5	78.4	3.75 ddd (9.3, 4.9, 2.3)	78.8	3.79 m	78.7	3.90 m	78.8	3.79 m	78.7	3.90 m
6	62.8	4.38 dd (11.9, 4.9)	62.6	4.37 dd (11.9, 3.4)	62.5	4.30 dd (11.8, 4.8)	62.6	4.36 dd (11.8, 3.0)	62.5	4.30 dd (12.3, 4.8)
		4.49 dd (11.9, 2.3)		4.52 dd (11.9, 2.3)		4.54 dd (11.8, 2.6)		4.52 dd (11.8, 1.7)		4.54 dd (12.3, 2.3)
		β-D-Xyl		β-D-Xyl		β-D-Xyl		β-D-Xyl		β-D-Xyl
1	105.3	5.14 d (7.6)	105.3	5.14 d (7.6)	105.3	5.16 d (7.7)	105.3	5.14 d (7.6)	105.3	5.16 d (7.8)
2	75.7	3.95 dd (7.6, 8.8)	75.6	3.95 dd (7.6, 8.8)	75.5	3.96 dd (7.7, 8.3)	75.6	3.95 dd (7.6, 8.9)	75.6	3.96 dd (7.8, 8.3)
3	78.9	4.01 dd (8.8, 8.8)	78.9	4.01 dd (8.8, 8.8)	78.7	4.07 dd (8.3, 8.7)	78.9	4.01 dd (8.9, 8.9)	78.7	4.07 dd (8.3, 8.9)
4	71.0	4.12 m	71.0	4.11 m	71.1	4.12 m	71.0	4.11 m	71.1	4.12 m
5_{ax}	67.6	3.64 dd (10.8, 10.9)	67.6	3.65 dd (10.8, 11.2)	67.7	3.66 dd (9.7, 11.4)	67.6	3.65 dd (10.7, 11.0)	67.7	3.66 dd (10.1, 11.0)
5_{eq}		4.22 dd (10.9, 5.3)		4.22 dd (11.2, 5.1)		4.22 dd (11.4, 4.4)		4.22 dd (11.0, 5.0)		4.23 dd (11.0, 4.4)
		α-L-Rha		α-L-Rha		β-D-Xyl′		α-L-Rha		β-D-Xyl′
1	103.1	6.12 d (1.7)	103.1	6.11 d (1.3)	106.6	5.10 d (7.5)	103.1	6.11 brs	106.6	5.11 d (7.4)
2	72.7	4.68 dd (1.7, 3.5)	72.7	4.68 dd (1.3, 3.7)	75.8	3.94 dd (7.5, 8.0)	72.7	4.68 d (3.4)	75.8	3.94 dd (7.4, 8.0)
3	73.0	4.49 dd (3.5, 8.9)	72.9	4.48 dd (3.7, 8.9)	78.2	4.07 dd (8.0, 8.7)	72.9	4.48 dd (3.4, 8.8)	78.2	4.08 dd (8.0, 8.7)
4	74.5	4.31 dd (8.9, 9.3)	74.5	4.29 dd (8.9, 9.2)	71.2	4.12 m	74.5	4.29 dd (8.8, 9.2)	71.2	4.13[b]
5_{ax}/5	70.1	4.91 dq (9.3, 6.2)	70.1	4.93 dq (9.2, 6.1)	67.5	3.57 dd (11.2, 9.5)	70.1	4.93 dq (9.2, 6.2)	67.6	3.57 dd (11.2, 9.5)
5_{eq}/6	19.0	1.64 d (6.2)	19.0	1.65 d (6.1)		4.23 dd (11.2, 4.4)	19.0	1.65 d (6.2)		4.23 dd (11.2, 4.3)
								26-*O*-β-D-Glc″		26-*O*-β-D-Glc″
1							105.4	4.83 d (7.8)	105.4	4.83 d (7.8)
2							75.6	4.04 dd (7.8, 8.2)	75.6	4.04 dd (7.8, 8.3)
3							79.0	4.25 [b]	79.0	4.25 [b]
4							72.1	4.24[b]	72.1	4.24[b]
5							78.9	3.96 m	78.9	3.96 ddd (8.5, 4.5, 2.5)
6							63.2	4.40 dd (12.0, 4.3)	63.2	4.40 dd (12.0, 4.5)
								4.56 dd (12.0, 2.2)		4.57 dd (12.0, 2.5)

[a] Assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, HSQC-TOCSY and HMBC experiments.
[b] Overlapped.

and C-3 (δ 84.5)/H-3 (δ 3.82) of the aglycone moiety. These results revealed that the sugar chain was the same as in Agabrittonoside D (**9**) (Macías et al., 2007).

After acid hydrolysis of **1**, the absolute configurations of the sugars were determined by a slight modification of the method reported by Tanaka et al. (2007). Sugars were converted into the thiazolizine derivatives and then into the arylthiocarbamate using L-cysteine methyl ester and *o*-tolylisothiocyanate. The reaction mixture was then directly analyzed by UPLC–UV–SRM/MS and the retention times (*Rt*) were compared with values obtained for derivatives of authentic samples of each D- or L-sugar. In this way, D-galactose, D-glucose, D-xylose and L-rhamnose were identified.

The structure of **1** was established as Kammogenin-3-*O*-{α-L-rhamnopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside}. This compound has not been described previously and we propose to name it Magueyoside F.

Compound **2** was isolated as a white amorphous solid and has the molecular formula $C_{56}H_{88}O_{27}$. The ^{1}H NMR spectrum of **2** showed five anomeric signals at δ 4.86, 5.14, 5.18, 5.51 and 6.11 (Table 2) and these were correlated in the HSQC spectrum with carbons at δ 103.2, 105.3, 105.3, 104.7 and 103.1, which were identified as being due to β-D-galactopyranosyl, β-D-xylopyranosyl, β-D-glucopyranosyl, β-D-glucopyranosyl and α-L-rhamnopyranosyl units, respectively. A rigorous study of the HMBC and ROESY correlations allowed us to establish the sequence of the sugars chain in compound **2** as being the same as in **1**. Despite the fact that ^{1}H and ^{13}C NMR data for rings C–F of the aglycone moieties of **1** and **2** were almost superimposable (Table 1), significant differences for ring A were observed. Analysis of the 2D TOCSY, ^{1}H–^{1}H COSY and HSQC-TOCSY spectra allowed the spin system for ring A to be identified as [–CH_2–CH_2–CHOH–CH_2–] with only one oxygenated sp^3 carbon (δ 78.3, C-3). The lack of a hydroxyl group at C-2 of the aglycone moiety of **2** is consistent with the molecular formula. Therefore, the aglycone moiety of **2** was elucidated as (25*R*)-spirost-5-en-3β-ol-12-one, which is known as gentrogenin (Wall et al., 1957; Huang et al., 1997; Xie et al., 2009). Based on the evidence outlined above, the structure of **2** was finally determined as gentrogenin-3-*O*-{α-L-rhamnopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside}, which has not been reported previously and we propose to name this compound Magueyoside G.

The spectroscopic data for compound **3** ($C_{55}H_{86}O_{27}$) showed that it was a spirostane pentaglycoside that is closely related to **2**, except for one terminal monosaccharide constituent. The ^{1}H NMR spectrum of **3** showed, instead of the anomeric resonance signal of the α-rhamnopyranosyl unit at δ 6.11, an anomeric doublet at δ 5.10 (*J* = 7.5 Hz), which was identified as being due to a β-D-xylopyranosyl unit. HMBC and ROESY correlations for **3** suggested the same sugars chain as in Agabrittonoside A (**10**) (Macías et al., 2007) and Magueyoside A (**7**) (Pérez et al., 2013). Consequently, the structure of compound **3** was elucidated as gentrogenin-3-*O*-{β-D-xylopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside}, which has not been described previously and we propose to name this compound Magueyoside H.

Compound **4** has the molecular formula $C_{62}H_{100}O_{33}$ and this suggests an extra hexosyl unit plus a hydroxyl group in comparison with **2**. The ^{1}H NMR signal assignments for the aglycone moiety of **4** were in good agreement with those of **2**, with the exception of the signals for rings E and F. The ^{13}C NMR data for the aglycone moiety of **4** (Table 1) suggest a furostane skeleton with the usual hemiketalic function at C-22 (δ 111.2). A clear diagnostic cross peak was observed in the ROESY spectrum between the H-20 (δ 2.22) and H_2-23 protons (δ 2.05, 2H) and this provided evidence of an α-orientation of the hydroxyl group at C-22 and a 22*R* configuration (Macías et al., 2010). The 25*R* configuration was deduced according to Agrawal's rule, which establishes a 25*R* configuration when the difference in the chemical shifts between geminal protons of the glycosyloxy methylene H_2-26 ($\Delta_{ab} = \delta_a - \delta_b$) is less than 0.48 (Agrawal, 2004, 2005). The ^{1}H NMR spectrum of **4** showed an additional anomeric doublet at δ 4.83 (*J* = 7.8 Hz) (Table 2), which was identified as being due to a β-D-glucopyranosyl unit (Glc″). The HMBC cross peak between H-$1_{Glc''}$ and the carbon signal at δ 75.6 (C-26 of the aglycone), as well as ROESY correlations between this anomeric proton and the signals for H_2-26 (δ 3.96 and δ 3.62), confirmed the presence of a 26-*O*-glucosylated furostanol saponin. Thus, the structure of compound **4** was elucidated as (25*R*)-26-*O*-β-D-glucopyranosylfurost-5-en-3β,22α,26-triol-12-one 3-*O*-{α-L-rhamnopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside}, which is a 26-*O*-glucosylated furostanol derivative of **2**. This compound has not been reported previously and we propose to name it Magueyoside I.

Compound **5** ($C_{61}H_{98}O_{33}$) showed spectroscopic features similar to those of **4**. The ^{1}H and ^{13}C NMR assignments for the aglycone moiety of **5** (Table 1) were consistent with those of **4**, while the NMR data for the glycosidic portion (Table 2) were consistent with those of **3**. Accordingly, the structure of **5** was identified as a 26-*O*-glucosylated furostanol derivative of **3**. Thus, compound **5** was identified as (25*R*)-26-*O*-β-D-glucopyranosylfurost-5-en-3β,22α,26-triol-12-one 3-*O*-{β-D-xylopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-galactopyranoside}. This saponin has not been described previously and we propose to name it Magueyoside J.

It is interesting to note that only three saponins isolated from *A. offoyana* flowers (Pérez et al., 2013) were found in the leaves. Regarding sugar moieties, rhamnose derivatives were not found in the flowers. Only one saponin with a short sugar chain was isolated per studied organ. The compound present in the leaves was the 23-hydroxylated derivative of saponin found in the flowers. This fact suggests a possible role of saponins in the flowering stage or lifespan of *Agave* species and this possibility warrants further investigation.

2.2. Bioassay of extracts, fractions and saponins

In order to carry out the initial bioactivity evaluation, extracts and fractions were assayed on etiolated wheat coleoptiles at 800, 400 and 200 ppm (Fig. 2). This is a rapid preliminary test that shows a good correlation with the phytotoxicity of saponins (Pérez et al., 2013). Fractions F3 to F6 showed the best inhibition profiles and they were therefore selected for the phytotoxicity evaluation.

The assay was performed using *L. sativa* L. (lettuce), *Lycopersicum esculentum* Will. (tomato), *Lepidium sativum* L. (cress), and *Allium cepa* L. (onion) as standard target species (STS) (Macías et al., 2000). Significant effects were observed only on the root growth of STS, especially in lettuce (Fig. 3). The inhibition values for fractions F4–F6 on the root growth of lettuce at 800 ppm were higher than those of Logran® (Fig. 2A). Strong inhibition profiles were also obtained for all tested fractions, including F3 on onion (Fig. 2D).

Based on the available quantities of the saponins, lettuce was chosen as a model plant to test their phytotoxicities; at 333, 100, 33, 10, 3.3 and 1 μM (Fig. 4). Magueyoside A (**7**), Magueyoside B (**8**) and Agabrittonoside A (**10**) were excluded from this test because they had been assayed previously under identical conditions (Pérez et al., 2013).

All of the saponins tested showed a significant effect on root growth as fractions with lower IC_{50} values than Logran®(IC_{50}

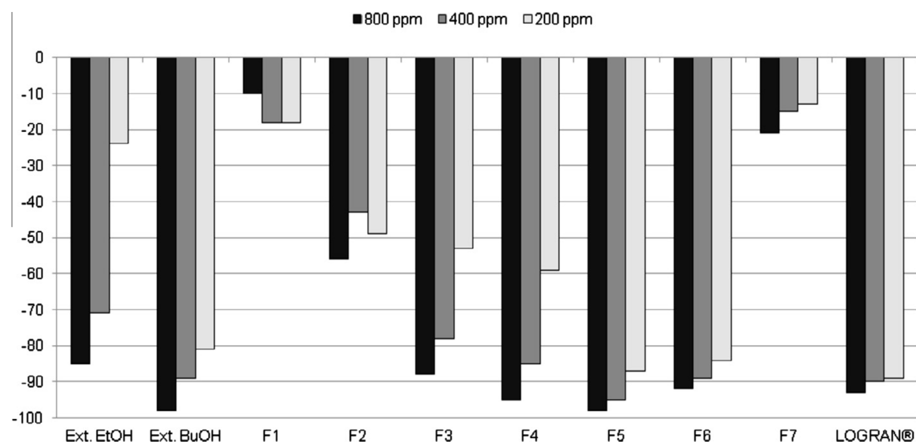

Fig. 2. Effect of extracts and fractions of *A. offoyana* leaves on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage of the control.

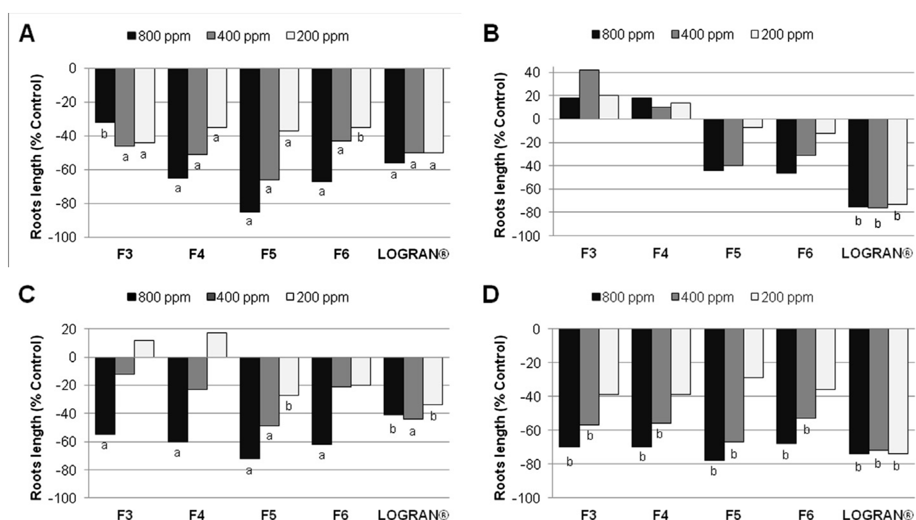

Fig. 3. Effect of fractions F3–F6 on root growth of STS: (A) *Lactuca sativa*; (B) *Lycopersicum esculentum*; (C) *Lepidium sativum*; (D) *Allium cepa*. Values are expressed as percentage of the control and are not significantly different with $P > 0.05$ for Welch's test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

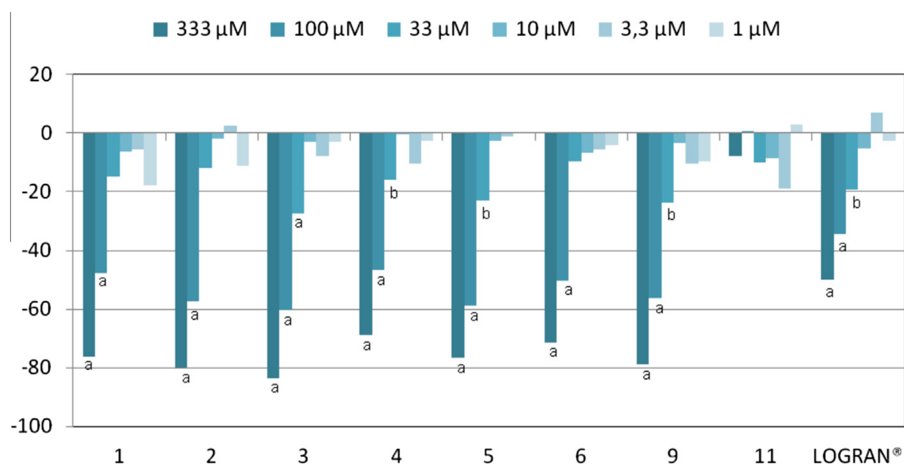

Fig. 4. Effect of compounds **1**–**6**, **9** and **11** on root growth of *Lactuca sativa* L. Values are expressed as percentage of the control and are not significantly different with $P > 0.05$ for Welch's test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

251.1 μM), except for Cantalasaponin-1 (**11**) (Table 3). As a result, a long glycosidic chain linked at C-3 of the aglycone moiety was suggested as a key factor for phytotoxicity. Although the bioactivities of compounds **1**–**6** and **9** were not significantly different (IC_{50} in the range 85–160 μM) some points should be highlighted. A β-D-xylopyranosyl unit instead of an α-L-rhamnopyranosyl unit at the

Table 3
Phytotoxicity of compounds **1–6**, **9** and **11** on roots of *Lactuca sativa* L.

Compounds	IC_{50} (μM)	r^2
1	159.6	0.9865
2	101.4	0.9771
3	90.4	0.9949
4	137.7	0.9632
5	89.5	0.9855
6	135.5*	0.9832
9	109.6	0.9844
11	n.d	n.d
LOGRAN®	251.1*	0.9778

* The data were not adjusted to the dose–response curve; n.d, not determined.

end of glycosidic chains may slightly enhance the phytotoxicity, as shown by comparison of compounds **3** (IC_{50} 90.4 μM), **2** (IC_{50} 101.4 μM), **5** (IC_{50} 89.5 μM) and **4** (IC_{50} 137.7 μM). In contrast, the relationship between aglycone structure and phytotoxicity remains unclear. Comparison of the activity of spirostane and furostane saponins also highlighted an interesting result as the difference between them was negligible.

Previous studies on the mode of action of phytotoxic saponins mainly concern oleanane-type compounds (Oleszek, 1993; Waller et al., 1996; Hernández Carlos et al., 2011; Scognamiglio et al., 2012). According to Marchaim et al. (1974) the hydrophobic moieties of alfalfa saponin molecules combine with membrane cholesterol in the cell of cotton seeds, causing structural changes in the membranes that result in increased swelling at the fringe of the living cell wall. The ability of saponins to interact with membrane cholesterol, which in turn leads to membrane destabilization, is the classical explanation for their bioactivity. In the case of steroidal saponins, this mechanism takes place in the haemolytic action where furostane-types are generally inactive because the hydrophobic moiety is modified. However, our data do not fully fit the proposed mechanism of saponin membrane activity, since the furostane saponins showed similar phytotoxicities to those of their spirostane analogs. This behavior has also been found for monodesmoside and bisdesmoside oleanane-type saponins (Oleszek, 1993, 2000). Similarly, the structure/cytotoxicity relationship between steroidal saponins was reviewed recently and it was established that there is no clear correlation between haemolytic activity and cytotoxicity (Podolak et al. 2010). In the same way, the SAR data that refer to haemolysis could not be directly extrapolated to relationships for cytotoxic activity.

The discrepancy in phytotoxic activity between furostane and spirostane saponins may be a simple exception or, perhaps, it could indicate a different mode of action. The results presented in this paper show that saponins of *A. offoyana* leaves generally have stronger phytotoxic activity than Logran® on *L. sativa* L. These compounds can therefore be considered as potential herbicides and they warrant further investigation.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined on a Perkin-Elmer 241 polarimeter (589 nm, 20 °C). 1D and 2D NMR spectra were recorded on a Bruker Avance III HD Ascend™-500 spectrometer equipped with 5 mm ^1H {^{109}Ag-^{31}P} broadband inverse (BBI) z-gradient probe. ^1H (500.18 MHz) and ^{13}C (125.77 MHz) NMR spectra were recorded in pyridine-d_5 at 25 °C and chemical shifts are given on the δ scale referenced to residual pyridine, δ_H 8.74, 7.58, 7.22 and δ_C 150.35, 135.91, 123.87. 1D (^{13}C) spectra were obtained using the uniform driven equilibrium Fourier transform sequence (UDEFT) (Piotto et al., 2006). Adiabatic pulse sequences using gradients were applied and all 2D spectra, except for HMBC, were recorded in the phase-sensitive mode. Exact masses were measured on a UPLC-QTOF ESI (Waters Synapt G2, Manchester, UK) high resolution mass spectrometer (HRESI-TOFMS). Mass spectra were recorded in negative or positive ion mode in the m/z range 100–2000, with a mass resolution of 20000 and an acceleration voltage of 0.7 kV. MS/MS fragmentation patterns of saponins were obtained using a Thermo LCQ Advantage Max ion-trap mass spectrometer. Samples were injected by direct infusion. The spectrometer was operated in the negative electrospray mode with the following parameters: spray voltage 3.9 kV, capillary voltage −47 V, tube lens offset −60 V and capillary temperature 240 °C. Semi-preparative HPLC in isocratic mode was performed on a chromatographic system equipped with Gilson 321 pump, a Gilson GX-271 liquid handler with a 2 mL sample loop, a Gilson Prep ELS™ II detector and a semi-preparative reversed phase Atlantis Prep T3, 5 μm, (250 × 10 mm, i.d) column or an Agilent Eclipse Plus C18, 3.5 μm, (150 × 4.6 mm, i.d) analytical column.

3.2. Plant material

Leaves of *A. offoyana* were collected in January 2008 by botanist Dr. Alfredo Noa in Palenque, Remedios City, north of Villa Clara province, Cuba. A voucher specimen was deposited in the Herbarium Dr. Alberto Alonso Triana of the Universidad Central 'Marta Abreu' de Las Villas, Cuba (number HPVC 3017).

3.3. Extraction and isolation

Dried and powdered leaves (1 kg) were extracted three times with ethanol/water (7:3) for 48 h by maceration at room temperature. The solvent was removed under reduced pressure and the syrupy residue (14.7%) was suspended in distilled water, defatted with *n*-hexane, and then extracted with water-saturated *n*-BuOH. After removing the solvent, 15 g of *n*-BuOH extract (12.9% of ethanolic extract) were purified by VLC on LiChrospher RP-18 and eluted with mixtures of $MeOH/H_2O$ to give seven fractions (F1: 1.42 g, F2: 1.19 g, F3: 3.64 g, F4: 2.13 g, F5: 2.14 g, F6: 1.45 g and F7: 1.22 g).

F3 was subjected to MPLC on a Büchi 861 apparatus with a column filled with 40–63 μm LiChrospher RP-18, using Me_2CO/H_2O (3:7) as mobile phase. Six milliliter fractions were collected and checked by TLC on RP-18 F_{254S}, developed with Me_2CO/H_2O (4:6), then sprayed with Oleum reagent and heated at 150 °C. Fractions with similar profiles were combined to give 9 fractions, of which F3–2 and F3–4 contained the major saponins. Further fractionation of F3–2 by HPLC on an analytical C18 column with 15.5% of solvent B (CH_3CN containing 0.2% HCO_2H) in solvent A (H_2O containing 0.2% HCO_2H) as the mobile phase, 1.7 mL/min and 40 °C, yielded compounds **4** (9.2 mg) and **5** (8.7 mg). Under the same conditions, fractionation of F3–4 gave compound **6** (4.2 mg).

Fractionation of F4 by MPLC under the same conditions as described above, but using Me_2CO/H_2O (5:5) as the eluent gave four further fractions. Fraction F4–3 was the major fraction. Subsequent purification of F4–3 by HPLC on a semi-preparative C18 column using 34% of solvent B in solvent A as mobile phase, 5 mL/min and 35 °C, yielded compounds **1** (4.6 mg), **7** (4.8 mg), **8** (4.7 mg) and **11** (5.0 mg).

F5 was subjected to MPLC with a mixture of $Me_2CO/MeOH/H_2O$ (3:3:4) as the mobile phase to give three additional fractions, of which F5–1 was then purified by HPLC on semi-preparative C18 column with 37% of solvent B in solvent A as mobile phase, 5 mL/min and 30 °C, to give compounds **2** (9.6 mg) and **3** (10.1 mg).

Finally, F6 was directly fractionated by HPLC on a semi-preparative C18 column, with 39% of solvent B in solvent A as mobile phase, 5 mL/min and 39 °C, to give compounds **9** (8.1 mg) and **10** (3.5 mg).

3.4. Compound **1**

$[\alpha]_D^{20}$ −47.8 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1231.5345 $[M+Na]^+$ (calcd. for $C_{56}H_{88}O_{28}Na$, 1231.5360). ESI-MS (negative ion mode), *m*/*z* 1207 $[M-H]^-$, which was fragmented in the MS/MS to give *m*/*z* 1075 $[M-H-132]^-$, 929 $[M-H-132-146]^-$, 767 $[M-H-132-146-162]^-$, 605 $[M-H-132-146-162-162]^-$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.5. Compound **2**

$[\alpha]_D^{20}$ −46.4 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1191.5425 $[M-H]^-$ (calcd. for $C_{56}H_{87}O_{27}$, 1191.5435). ESI-MS (negative ion mode), *m*/*z* 1191 $[M-H]^-$, which was fragmented in the MS/MS to give *m*/*z* 1059 $[M-H-132]^-$, 913 $[M-H-132-146]^-$, 751 $[M-H-132-146-162]^-$, 589 $[M-H-132-146-162-162]^-$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.6. Compound **3**

$[\alpha]_D^{20}$ −45.6 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1177.5294 $[M-H]^-$ (calcd. for $C_{55}H_{85}O_{27}$, 1177.5278). ESI-MS (negative ion mode), *m*/*z* 1177 $[M-H]^-$, which was fragmented in the MS/MS to give *m*/*z* 1045 $[M-H-132]^-$, 883 $[M-H-132-162]^-$, 751 $[M-H-132-162-132]^-$, 589 $[M-H-132-162-132-162]^-$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.7. Compound **4**

$[\alpha]_D^{20}$ −37.2 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1371.6042 $[M-H]^-$ (calcd. for $C_{62}H_{99}O_{33}$, 1371.6069). ESI-MS (negative ion mode), *m*/*z* 1371 $[M-H]^-$, which was fragmented in the MS/MS to give *m*/*z* 1239 $[M-H-132]^-$, 1093 $[M-H-132-146]^-$, 931 $[M-H-132-146-162]^-$, 769 $[M-H-132-146-162-162]^-$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.8. Compound **5**

$[\alpha]_D^{20}$ −29.2 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1357.5918 $[M-H]^-$ (calcd. for $C_{61}H_{97}O_{33}$, 1357.5912). ESI-MS (negative ion mode), *m*/*z* 1357 $[M-H]^-$, which was fragmented in the MS/MS to give *m*/*z* 1225 $[M-H-132]^-$, 1063 $[M-H-132-162]^-$, 931 $[M-H-132-162-132]^-$, 769 $[M-H-132-162-132-162]^-$. For ^1H and ^{13}C NMR data see Tables 1 and 2.

3.9. Compound **6**

$[\alpha]_D^{20}$ −43.6 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1359.6084 $[M-H]^-$ (calcd. for $C_{61}H_{99}O_{33}$, 1359.6069).

3.10. Compound **9**

$[\alpha]_D^{20}$ −54.0 (MeOH, *c* 0.1). HRESI-TOFMS, *m*/*z* 1193.5582 $[M-H]^-$ (calcd. for $C_{56}H_{89}O_{27}$, 1193.5591).

3.11. Acid hydrolysis of saponins

Compounds **1**–**6** and **9** (1 mg each) were treated with 2 M HCl in 1,4-dioxane/H_2O (1:1, v/v, 2 mL) at 95 °C for 4 h. After cooling, the solvent was removed with a stream of N_2. The dry residue was suspended in water and aglycones were extracted with ethyl acetate (3 × 2 mL). The aqueous layer containing sugars was neutralized with Amberlite IRA-400 (OH^- form), dried under N_2 and stored prior to analysis.

3.12. Determination of the absolute configuration of sugars

The absolute configurations of monosaccharide constituents of compounds **1**–**6** and **9** were determined according to the method reported by Tanaka et al. (2007) with slight modifications. Sugars from each sample were dissolved in pyridine (0.5 mL) containing L-cysteine methyl ester hydrochloride (1 mg) and heated at 60 °C for 1 h; *o*-tolyl isothiocyanate (2 μL) was then added and the mixture was heated at 60 °C for 1 h. Each reaction mixture was directly analyzed on a Waters UPLC system with a Waters Acquity PDA detector (at 245 nm) and Waters triple quadrupole detector (TQD) operated in selected reaction monitoring (SRM) positive electrospray mode (capillary voltage −3.2 kV, cone voltage −30 V, collision energy −15 V) using the following transitions: *m*/*z* 447 > 298 for hexoses, *m*/*z* 431 > 282 for deoxyhexoses and *m*/*z* 417 > 268 for pentoses. The software used for acquisition and data processing was MassLynx v4.1. An Acquity BEH C18, 1.7 μm (100 × 2.1 mm i.d) (Waters) column was used; 16 min linear gradient from 5% to 50% of solvent B (CH_3CN containing 0.1% HCO_2H) in solvent A (H_2O containing 0.1% HCO_2H), 0.35 mL/min at 30 °C. The derivatives of monosaccharides of D-galactose, D-glucose, D-xylose and L-rhamnose, in the analyzed saponins were identified by comparison of their retention times (*Rt*) with those of authentic samples (Sigma–Aldrich, Steinheim, Germany) treated in the same way as described above. The *Rt* of D-rhamnose was obtained by reaction of its L enantiomer with D-cysteine methyl ester (Tanaka et al., 2007) due to the lack of an authentic sample; (*Rt*: D-galactose 10.10 min, L-galactose 10.20 min, D-glucose 10.40 min, L-glucose 10.18 min, D-xylose 10.69 min, L-xylose 10.54 min, L-rhamnose 11.54 min, D-rhamnose 9.95 min).

3.13. Bioassay

Etiolated wheat coleoptile assays and phytotoxicity bioassays with the monocot *A. cepa* L. (onion) and dicots *L. esculentum* Will. (tomato), *L. sativum* L. (cress) and *L. sativa* L. (lettuce) as standard target species (STS) were conducted under the conditions reported in our previous work (Pérez et al., 2013). Extracts and fractions were assayed at concentrations of 800, 400 and 200 ppm and pure compounds at 333, 100, 33, 10, 3.3, 1 μM. Control samples (buffered aqueous solutions without any test compound) and the commercial herbicide Logran®, a combination of *N*-(1,1-dimethylethyl)-*N*′-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-N-{[(4-methoxy-6-methyl-1,3,5-triazin-2yl)amino]carbonyl}benzene-sulfonamide (Triasulfuron, 0.6%), were used as internal references (Macías et al., 2000) and were tested under the same conditions as the samples.

The evaluated parameters in the phytotoxicity assay (germination rate, root length and shoot length) were recorded using a Fitomed® system (Castellano et al., 2001), which allowed automatic data acquisition and statistical analysis using its associated software. Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition. The concentration that resulted in a 50% inhibition (IC_{50} values) was calculated from the dose–response curve.

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Appendix A. Supplementary data

Supplementary data associated with this article include 1H, 13C (UDEFT), HSQC, HMBC, ROESY, DQF-COSY, TOCSY, HSQC-TOCSY, 1D TOCSY and 1D ROESY NMR spectra, High Resolution Mass Spectra and ESI-MS/MS spectra for the new compounds (**1**–**5**). Etiolated wheat coleoptiles and phytotoxicity bioassay experimental procedures. Figure showing the effects of compounds **1**–**6**, **9** and **11** on germination and shoot growth of Lactuca sativa. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.05.014. These data include MOL files and InChiKeys of the most important compounds described in this article.

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