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Triterpenoid saponins from the aerial parts of *Trifolium argutum* Sol. and their phytotoxic evaluation

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ABSTRACT

Four triterpenoid saponins (**1**–**4**) were isolated from the aerial parts of *Trifolium argutum* Sol. (sharp-tooth clover) and their structures were elucidated by comprehensive spectroscopic analysis, including 1D and 2D NMR techniques, mass spectrometry and chemical methods. Two of them are new compounds, characterized as 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-ene-22-oxo-29-oic acid (**1**) and 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β,24-dihydroxyolean-12-ene-22-oxo-29-oic acid (**2**). The occurrence of 3β,24-dihydroxyolean-12-ene-22-oxo-29-oic acid (melilotigenin) in its natural form is reported for the first time as a triterpenoid aglycone within *Trifolium* species. The phytotoxicity of compounds was evaluated on four STS at concentration 1 μM to 333 μM. Compound **1** was the most active, showing more than 60% inhibition on the root growth of *L. sativa* at the higher dose, with IC_{50} (254.1 μM) lower than that of Logran® (492.6 μM), a commercial herbicide used as positive control. The structure–activity relationships indicated that both aglycones and glycosidic parts may influence the phytotoxicity of saponins.

1. Introduction

Clover genus (*Trifolium* L.) is one of the largest genera in the family of Leguminosae (Fabaceae) with approximately 250 species. They are widely grown as livestock forage and green manure crops and at least 16 species are actively cultivated (Ellison et al., 2006). One of them is sharp-tooth clover, *Trifolium argutum* Sol. an annual herb plant distributed in Europe, Middle East, Australia and Egypt (Bisby et al., 1994), from which scarce phytochemical information is available in the literature. The occurrence of triterpenoid saponins among *Trifolium* species have extensively been documented (Jurzysta et al., 1989; Sakamoto et al., 1992; Mohamed et al., 1995; Simonet et al., 1999; Oleszek and Stochmal, 2002; Pawelec et al., 2013; Pérez et al., 2013b). Saponins in these plants are a mixture of triterpenoid pentacyclic glycosides which are often derivatives of soyasapogenol A, B, C and E., having hydroxymethyl group at C-24 and double bond between C-12 and C-13 as a common feature. The sugar portion consists of one or three units, where β-D-glucuronic acid is linked at C-3 position of the aglycone moiety. Despite the wide range of pharmacological properties attributed to triterpenoid saponins, they have also been described for their detrimental effects over the plant growth when releasing into the soil (Marchaim et al., 1974; Oleszek et al., 1992; Oleszek, 1993). Triterpenoid saponins have recently been investigated for their phytotoxic activity (Hernández et al., 2011; Scognamiglio et al., 2012). This paper reports the isolation and structure elucidation of two new triterpenoid saponins (**1**, **2**), along with two known analogs (**3**, **4**) from *T. argutum* Sol. aerial parts, and their phytotoxic evaluation against the germination and development of four standard target species (STS).

2. Results and discussion

2.1. Characterization of the compounds

Dried aerial parts of *T. argutum* were extracted exhaustively with 70% EtOH. The hydroalcoholic extract was partitioned in *n*-BuOH/H_2O and the organic phase was subjected to chromatographic fractionation using reverse-phase RP-18 on a gradient

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of Me_2CO/H_2O and other multiple separation procedures to give two new triterpenoid saponins (**1** and **2**) and two known analogs: 3-*O*-β-D-glucuronopyranosyl melilotigenin (caraganin B) (**3**) (Zheng et al., 2013), 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl] soyasapogenol B (soyasaponin-I) (**4**) (Kitagawa et al., 1988) (Fig. 1). The structures of the compounds were elucidated on the basis of spectroscopic data obtained from ^1H, ^{13}C, 2D (HSQC, HMBC, DQF-COSY, TOCSY, ROESY, HSQC-TOCSY), 1D-ROESY (250 ms) and 1D-TOCSY (30, 60, 120 ms) NMR experiments, HRESI-TOFMS, ESI-MS/MS and acid hydrolysis.

Compound **1** was obtained as a white amorphous powder and its molecular formula was assigned as $C_{48}H_{74}O_{20}$ based on data from HRESI-TOFMS (*m/z* 969.4696 $[M-H]^-$, calcd. for $C_{48}H_{73}O_{20}$, 969.4695). In the negative ESI-MS/MS spectrum, the deprotonated molecule $[M-H]^-$ at *m/z* 969 suffered the successive neutral losses of H_2O (18 Da) and CO_2 (44 Da) to produce the *m/z* 951 and 907 ions, respectively, indicating the presence of a carboxylic group. Further fragment ions at *m/z* 761 and 599 indicated the neutral losses of a deoxyhexose unit (146 Da) and a hexose unit (162 Da), respectively. The successive neutral loss of 114 Da to generate the ion at *m/z* 485, suggested the presence of a hexuronic acid unit. It is originated from the ion at *m/z* 599, corresponding to a molecular rearrangement derived from hexuronic acid after losses of water and carbon dioxide (Gülcemal et al., 2013). The ^1H NMR spectrum of **1** showed for the aglycone moiety signals of six tertiary methyl groups at δ 0.90, 0.98, 0.99, 1.13, 1.26 and 1.26 (Table 1) and these were correlated in the HSQC spectrum with carbons at δ 16.3, 17.3, 21.0, 21.6, 23.4 and 25.8 respectively. One olefinic proton at δ 5.38 (H-12, t, *J* = 3.4 Hz), together with two olefinic carbons at δ 125.8 (C-12) and δ 142.2 (C-13) suggested the presence of a Δ^{12}-oleanene skeleton. An oxygen-bearing methine proton signal at δ 3.42 (dd, *J* = 11.6, 4.5 Hz) and its long-range correlations observed in the HMBC spectrum with the carbon resonances at δ 23.4 (C-23), 44.7 (C-4) and 64.3 (C-24) suggested the occurrence of an equatorial secondary alcoholic group at C-3. The typical 24-hydroxymethyl group found among *Trifolium* saponins (Pérez et al., 2013b) was deduced from the ^1H NMR signals at δ 3.21 (d, *J* = 11.5 Hz) and δ 4.14 (d, *J* = 11.5 Hz) which correlated in the HSQC spectrum with the carbon resonance signal at δ 64.3, as well as from their ROESY correlations with proton signals at δ 0.90 (H-25, methyl group) and δ 1.83 ($H\text{-}2_{ax}$). The less shielded signals in the ^{13}C NMR spectrum were found at δ 180.4 and 218.5, which were assigned as being due to a carboxylic group at C-29 and a keto group at C-22, respectively (Table 1). Therefore, the structure of the aglycone moiety of **1** was recognized to be 3β,24-dihydroxyolean-12-ene-22-oxo-29-oic acid, which is known as melilotigenin and its NMR assignments were in good agreement with those previously reported (Kang and Woo, 1988; Takeshita et al., 1991; Zheng et al., 2013). Melilotigenin has previously been identified as its methyl ester artifact in *Trifolium repens* (Sakamoto et al., 1992), however to the best of our

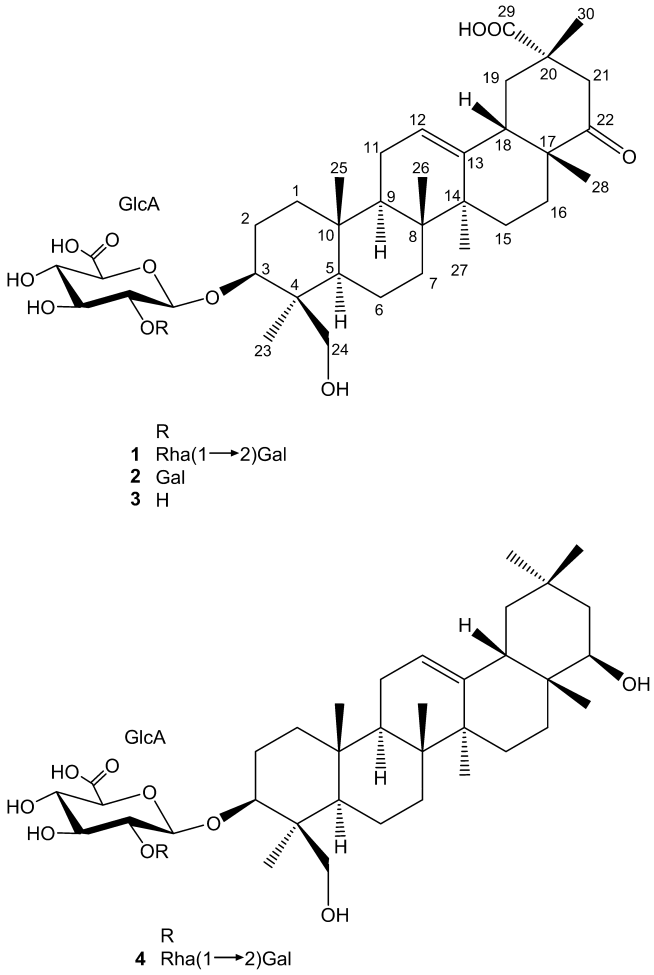

Fig. 1. Structures of saponins (**1–4**) isolated from *T. argutum* aerial parts.

Table 1
^{13}C and ^1H NMR data for the aglycone moiety of compounds **1** and **2** (500 MHz).[a]

	1[b]		**2**[c]	
	δ_C, type	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1_{ax}	39.6, CH_2	1.05[d]	39.6	1.03[d]
1_{eq}		1.65[d]		1.65[d]
2_{ax}	27.1, CH_2	1.83 ddd (13.7, 11.6, 3.6)	27.1	1.83[d]
2_{eq}		2.08[d]		2.00[d]
3	92.4, CH	3.42 dd (11.6, 4.5)	92.2	3.40 dd (11.5, 4.8)
4	44.7, C	–	44.6	–
5	57.2, CH	0.96[d]	57.2	0.97[d]
6_{ax}	19.3, CH_2	1.37[d]	19.4	1.40[d]
6_{eq}		1.65[d]		1.65[d]
7_{ax}	33.9, CH_2	1.57[d]	34.0	1.58[d]
7_{eq}		1.39[d]		1.39[d]
8	40.9, C	–	40.9	–
9	48.7, CH	1.64[d]	48.8	1.64[d]
10	37.4, C	–	37.5	–
11	24.9, CH_2	1.94[d] (2H)	24.9	1.94[d] (2H)
12	125.8, CH	5.38 t (3.4)	125.8	5.38 t (3.2)
13	142.2, C	–	142.2	–
14	42.9, C	–	42.9	–
15	26.1, CH_2	1.13[d]	26.1	1.13[d]
		1.80[d]		1.80[d]
16	28.3, CH_2	1.16[d]	28.2	1.16[d]
		2.15[d]		2.15 m
17	49.2, C	–	49.2	–
18	48.0, CH	2.41 dd (13.9, 4.0)	47.9	2.41 dd (13.8, 3.9)
19_{ax}	42.2, CH_2	2.57 dd (13.8, 13.8)	42.1	2.58 dd (13.8, 13.8)
19_{eq}		1.63[d]		1.62[d]
20	45.4, C	–	45.1	–
21α	46.9, CH_2	3.02 d (14.6)	46.7	3.01 d (14.6)
21β		2.23 dd (14.6, 2.2)		2.23 dd (14.6, 2.1)
22	218.5, C	–	217.6	–
23	23.4, CH_3	1.26 s	23.0	1.24 s
24	64.3, CH_2	3.21 d (11.5)	64.3	3.25 d (11.5)
		4.14 d (11.5)		4.12 d (11.5)
25	16.3, CH_3	0.90 s	16.2	0.91 s
26	17.3, CH_3	0.98 s	17.3	0.98 s
27	25.8, CH_3	1.26 s	25.8	1.26 s
28	21.0, CH_3	0.99 s	21.0	1.00 s
29	180.4, C	–	179.7	–
30	21.6, CH_3	1.13 s	21.5	1.14 s

[a] The assignments were confirmed by DQF-COSY, 2D-TOCSY, HSQC, HSQC-TOCSY and HMBC experiments.
[b] Acquired in methanol-d_4 / D_2O (95:5).
[c] Acquired in methanol-d_4.
[d] Overlapped with other signals.