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Triterpenic saponins from *Medicago marina* L

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ABSTRACT

The saponin composition of leaves and roots from *Medicago marina* L., sea medic, was investigated by a combination of chromatographic, spectroscopic and spectrometric (GC, LC, ESI-MS/MS, NMR) methods. Several compounds were detected and quantified by HPLC using the external standard method. Saponins from this plant species consist of a mixture of high molecular weight bidesmosidic derivatives of medicagenic and zanhic acid, containing up to six sugars in the molecules. Six of the detected saponins were previously isolated and reported as constituents of other *Medicago* spp.; one saponin was previously described in other plant species; four saponins are undescribed compounds in *Medicago* and never reported before in other plant species. These are: 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*β*-D-apiofuranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester; 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester; 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylmedicagenic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*α*-L-arabinopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester and 3-*O*-*β*-D-glucopyranosylmedicagenic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*α*-L-arabinopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester. The specific saponins synthesized by *M. marina* may have a role in its tolerance to environment, representing a reservoir of osmolytic sugars.

1. Introduction

As a continuation of our previous studies on the phytochemical profile of different species of *Medicago* (Fabaceae), we have characterized the saponins from sea medic (*Medicago marina* L.). Sea medic is a wild perennial species endemic to the Mediterranean and Black Seas regions. It grows exclusively on seashores, usually in loose sand, on the primary and secondary dunes of sea coast, also characterized by the presence of *Ammophyla* species, where it has an important role in soil consolidation (Acosta and Ercole, 2015). It is the only *Medicago* spp. that grows with such habitat requirements. Sea medic is a creeping and rampant plant, with deep roots and vegetative organs densely covered with simple, whitish hairs giving the plant a greyish appearance and helping to avoid excessive loss of water (Lesins and Lesins, 1979; Pignatti, 1982).

Saponins from *Medicago* species consist of a complex mixture of triterpenic pentacyclic glycosides with medicagenic acid, zanhic acid and soyasapogenols as the most frequent aglycones (Tava and Avato, 2006). Although many species within this genus have already been analysed, studies are still in progress to elucidate the chemical structure, biosynthetic pathways and biological activities of saponins from several other *Medicago* species, such as annual medics, which have poorly or never been investigated previously (Tava et al., 2011a; Biazzi et al., 2015; Carelli et al., 2011; Tava and Pecetti, 2012). Quantitative evaluation of saponins in plant material (Tava and Avato, 2006; Tava et al., 2009, 2011b; Tava and Pecetti, 2012) and their use as natural bioactive compounds in pharmaceutical applications and in agroindustry are other interesting aspects investigated by us in the recent years (D'Addabbo et al., 2011; Abbruscato et al., 2014; Paparella et al., 2015; Montanari et al., 2016; Avato et al., 2017; Maestrini et al., 2019).

Due to the increasing economic interest in these natural molecules as high-added value compounds, the present study reports on the structural characterization of saponins from *M. marina* L. leaves and roots. Saponins from this species have never been investigated before. This study proved to be of interest both from a phytochemical, ecological and taxonomical point of view.

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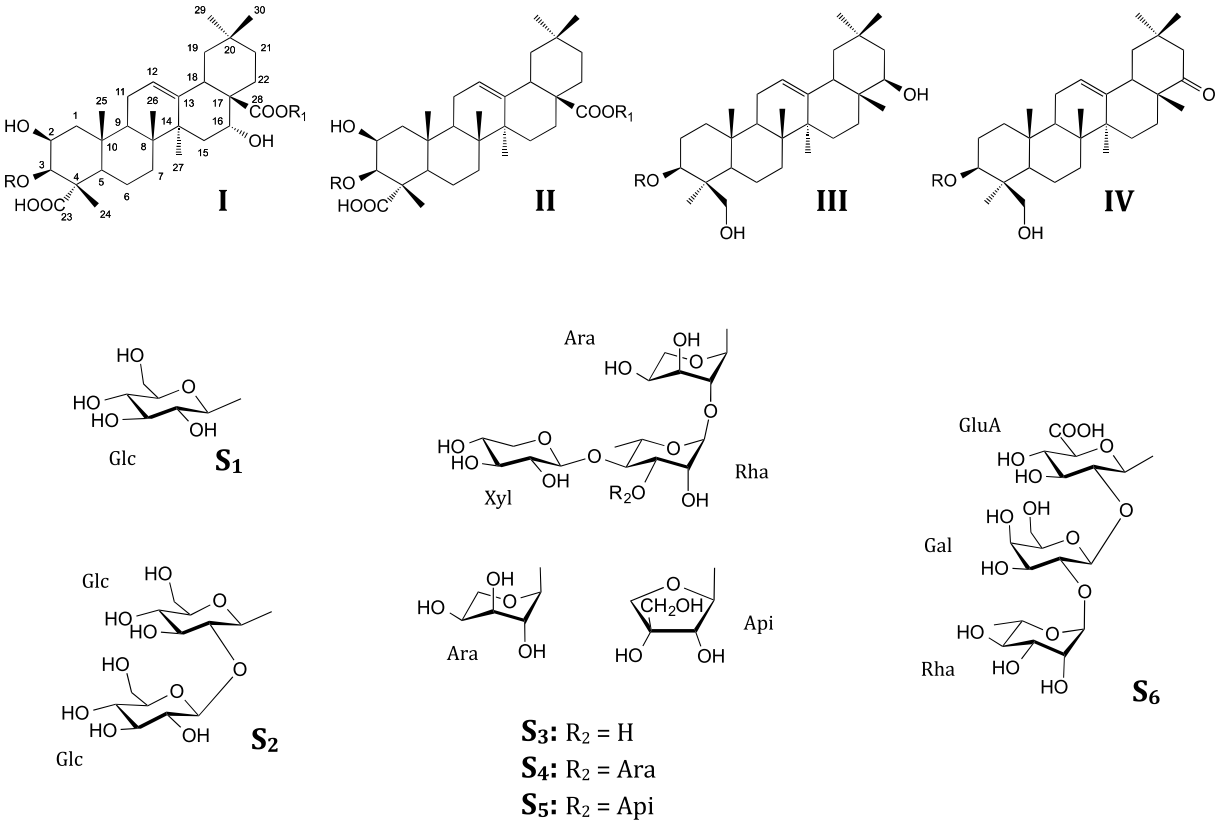

Compound	Aglycone	R	R_1
1	I	S_2	S_4
2	I	S_2	S_5
3	I	S_1	S_4
4	I	S_2	S_3
5	I	S_2	S_3
6	II	S_2	S_4
7	II	S_2	S_5
8	II	S_1	S_4
9	II	S_1	S_3
10	III	S_6	-
11	IV	S_6	-

Fig. 1. Structure of *M. marina* saponins **1–11**. I, zanhic acid; II, medicagenic acid; III, soyasapogenol B; IV, soyasapogenol E; Api: *β*-D-apiofuranosyl; Ara: *α*-L-arabinopyranosl; Glc:*β*-D-glucopyranosyl; GluA: *β*-D-glucuronopyranosyl; Rha: *α*-L-rhamnopyranosyl; Xyl: *β*-D-xylopyranosyl.

2. Results and discussion

Preliminary TLC investigation of saponin extracts from *M. marina* L. (Fig. S1 in Supplementary material), showed several spots with a low R_f (0.12–0.35) and this feature, if compared with saponin extracts from other *Medicago* species previously studied (Bialy et al., 2006; Tava et al., 2005, 2009; 2011a, 2011b), suggested that constituents of leaves and root saponin extracts of *M. marina* L. are more polar and might contain several sugars in the molecules.

GC/FID and GC/MS analysis of derivatized sapogenins obtained after acid hydrolysis of their related saponins, confirmed that medicagenic acid was the major aglycone, representing 73.8% and 79.4% of the total aglycones from leaves and roots, respectively, followed by zanhic acid (16.8% leaves and < 0.1% roots), soyasapogenol B (6.2% leaves and 12.5% roots), and soyasapogenol E (0.3% leaves and 2.3% roots). Identification and quantitation of all sapogenins was achieved on the basis of all the peaks, including derived artefact compounds obtained after acid hydrolyses of the corresponding saponins. As reported in our previous study (Tava et al., 2017), the presence of a hydroxyl group in the γ position of the double bond of the triterpenic pentacyclic structure, such as in soyasapogenol B and zanhic acid (see Fig. 1), promote the double bond transposition and other rearrangements, giving artefact compounds. The knowledge of the nature of these compounds obtained from acid hydrolysis of a particular saponin is

fundamental for the exact quantification of that saponin. This helps to obtain an appropriate quantitative determination of saponins/sapogenins in the plant materials (Tava et al., 2017).

Crude saponins obtained from the defatted leaves of *M. marina* L. (4.7%) were analysed by HPLC (Fig. S2) and fractionated by a combination of RP-18 open column chromatography and semi-preparative HPLC to afford eleven saponins (**1–11**) in a pure form (see Experimental).

To elucidate their chemical structure, spectroscopic and chemical techniques were employed. Aglycone moieties obtained from each of the pure saponins, were identified by TLC R_f values, GC/FID, GC/MS and NMR data compared to those of reference compounds and to literature data (Tava et al., 2005, 2009; 2011b). Sugar moieties were identified by means of TLC, comparing the R_f values with those of reference compounds and their absolute configuration was obtained from GC analysis on a chiral column. The structure elucidation of all saponins was performed by analyzing NMR (^{1}H, ^{13}C and 2D experiments: COSY, HSQC, HMBC, TOCSY) and ESI-MS/MS data (see Supplementary Material Figs. S3–S34).

The chemical structure of the identified saponins **1–11** is reported in Fig. 1. ^{13}C NMR chemical shifts of undescribed compounds, **2**, **4**, **6** and **8**, are reported in Tables 1 and 2, while their ^{1}H NMR data are reported in Tables 3 and 4. All the monosaccharides but apiose were determined to be in the pyranose form from their ^{13}C NMR data. Sugar configurations were also established by comparison of their spectroscopic data with literature values (Bialy et al., 1999, 2006; Kapusta et al., 2005; Tava et al., 2005, 2009; 2011b).

Saponin **1**, 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*α*-L-arabinopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester, and saponin **3**, 3-*O*-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*α*-L-arabinopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester, were previously identified in *M. arborea* L. (Tava et al., 2005). Compound **5**, 3-*O*-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester, was peviously identified in *M. truncatula* (Kapusta et al., 2005), while compound **7**, 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylmedicagenic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*β*-D-apiofuranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester, was previously reported in *Muraltia ononidifolia* E. Mey (Elbandy et al., 2002). Saponin **9**, 3-*O*-*β*-D-glucopyranosylmedicagenic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester, saponin **10**, also known as soyasaponin I and saponin **11**, known as dehydrosoyasaponin I, were already discovered in other *Medicago* spp. (Tava and Avato, 2006).

Saponin **2**, 13 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{63}H_{100}O_{33}$ (MW 1384) by elemental analysis (calcd for C, 54.62; H, 7.28; found C, 54.56; H, 7.20). The acid hydrolysis of this saponin followed by GC analyses of functionalized hydrolysis products, allowed identification of zanhic acid and L-arabinose, D-apiose, L-rhamnose, D-xylose and D-glucose, in the ratio 2:1:1:1:1. The ^{13}C NMR spectrum shows the presence of six anomeric signals at δ_C 111.7 (C-1$_{Api\ V}$), 105.5 (C-1$_{Glc\ II}$), 105.0 (C-1$_{Xyl\ VI}$), 104.9 (C-1$_{Glc\ I}$), 101.1 (C-1$_{Rha\ IV}$), 93.8 (C-1$_{Ara\ III}$), confirmed in the ^{1}H spectrum in which six anomeric protons [δ_H 5.72 (d, J = 3.2 Hz, H-

Table 1
^{13}C NMR spectral data (δ_C, CD_3OD-10%D_2O) of the aglycone moieties of saponins **2**, **4**, **6** and **8**.

No.	2	4	6	8
1	44.7	44.7	44.8	44.8
2	70.4	70.4	71.4	71.2
3	86.4	86.4	86.6	86.5
4	53.4	53.4	53.8	53.8
5	53.3	53.3	53.2	53.2
6	21.4	21.3	22.0	22.0
7	31.5	31.5	33.5	33.5
8	41.0	41.1	41.2	41.2
9	48.6	48.6	49.7	49.7
10	37.3	37.4	37.5	37.5
11	24.5	24.5	23.9	23.9
12	123.7	124.1	124.0	124.0
13	144.4	144.9	145.1	145.1
14	42.7	42.8	43.3	43.3
15	36.2	36.2	29.1	29.1
16	72.6	72.6	24.8	24.8
17	50.3	50.3	48.5	48.5
18	42.0	42.0	42.8	42.8
19	47.5	47.5	47.3	47.3
20	31.2	31.2	31.8	31.7
21	36.1	36.2	35.0	35.0
22	33.8	33.8	33.9	33.9
23	182.2	182.2	183.5	183.5
24	14.0	14.0	14.4	14.3
25	17.3	17.3	17.5	17.5
26	17.8	18.8	18.1	18.1
27	27.4	27.4	26.7	26.7
28	177.1	177.2	178.4	178.4
29	33.3	33.3	33.8	33.8
30	25.2	25.2	24.3	24.3

Table 2
^{13}C NMR spectral data (δ_C, CD_3OD-10%D_2O) of the saccharide moieties of saponins **2**, **4**, **6** and **8**.

No.	2	4	6	8
	Glc (I)	Glc (I)	Glc (I)	Glc (I)
1	104.9	104.9	104.7	104.9
2	**84.4**	**84.4**	**86.8**	74.5
3	78.1	78.1	78.1	77.6
4	71.6	71.6	71.6	71.4
5	78.7	78.6	78.6	77.7
6	62.2	62.3	62.3	62.3
	Glc (II)	Glc (II)	Glc (II)	
1	105.5	105.4	105.2	
2	75.6	75.6	75.6	
3	77.7	77.7	77.7	
4	69.8	69.8	69.8	
5	77.7	77.7	77.7	
6	62.3	62.6	62.7	
	Ara (III)	Ara (III)	Ara (III)	Ara (II)
1	93.8	93.8	94.2	94.2
2	**75.8**	**75.7**	**75.8**	**75.8**
3	71.4	71.4	71.4	71.3
4	67.2	67.2	67.3	67.2
5	63.1	63.2	63.9	63.9
	Rha (IV)	Rha (IV)	Rha (IV)	Rha (III)
1	101.1	101.1	101.1	101.1
2	72.1	72.0	72.2	72.2
3	**81.4**	71.8	**82.3**	**82.3**
4	**79.2**	**82.3**	**79.3**	**79.2**
5	69.3	69.3	69.2	69.2
6	18.2	18.3	18.6	18.5
	Api (V)		Ara (V)	Ara (IV)
1	111.7		106.2	106.2
2	78.6		72.9	72.9
3	80.1		75.5	75.2
4	74.9		70.2	70.1
5	65.0		67.7	67.7
	Xyl (VI)	Xyl (V)	Xyl (VI)	Xyl (V)
1	105.0	105.0	105.0	105.2
2	74.5	74.6	74.5	75.5
3	77.1	77.3	77.3	78.6
4	71.0	71.0	71.1	71.0
5	66.9	67.0	67.0	67.0

Table 3
^{1}H NMR spectral data (δ_H, CD_3OD-10%D_2O) of the aglycone moieties of saponins **2**, **4**, **6** and **8**.

No.	2	4	6	8
1	1.28 and 2.12, 2H, dd (14.0, 4.0)	1.26 and 2.14, 2H, dd (14.0, 4.0)	1.27 and 2.12, 2H, dd (14.0, 4.0)	1.25 and 2.13, 2H, dd (14.0, 4.0)
2	4.36, 1H, bq (4.0)	4.32, 1H, bq (4.0)	4.34, 1H, bq (4.0)	4.34, 1H, bq (4.0)
3	4.13, 1H, d (4.0)	4.11, 1H, d (4.0)	4.11, 1H, d (4.0)	4.12, 1H, d (4.0)
4	–	–	–	–
5	1.60, 1H, dd (12.0, 4.0)	1.60, 1H, dd (12.0, 4.0)	1.62, 1H, dd (12.0, 4.0)	1.61, 1H, dd (12.0, 4.0)
6	1.32 and 1.58, 2H[a]	1.28 and 1.58, 2H[a]	1.19 and 1.62, 2H[a]	1.20 and 1.62, 2H[a]
7	1.21 and 1.65, 2H[a]	1.22 and 1.64, 2H[a]	1.21 and 1.73, 2H[a]	1.23 and 1.75, 2H[a]
8	–	–	–	–
9	1.69, 1H[a]	1.65, 1H[a]	1.63, 1H[a]	1.62, 1H[a]
10	–	–	–	–
11	1.95–2.10, 2H[a]	1.96–2.08, 2H[a]	1.95–2.10, 2H[a]	1.95–2.10, 2H[a]
12	5.32, 1H, t (3.0)	5.33, 1H, t (3.0)	5.32, 1H, t (3.0)	5.32, 1H, t (3.0)
13	–	–	–	–
14	–	–	–	–
15	1.38 and 1.78, 2H[a]	1.38 and 1.77, 2H[a]	1.10 and 1.76, 2H[a]	1.08 and 1.77, 2H[a]
16	4.48, 1H[a]	4.49, 1H[a]	2.05–2.20, 2H[a]	2.05–2.20, 2H[a]
17	–	–	–	–
18	3.08, 1H, dd (14.0, 4.0)	3.02, 1H, dd (14.0, 4.0)	2.88, 1H, dd (14.0, 4.0)	2.89, 1H, dd (14.0, 4.0)
19	1.05 and 2.20, 2H[a]	1.05 and 2.23, 2H[a]	1.18 and 1.71, 2H[a]	1.15 and 1.70, 2H[a]
20	–	–	–	–
21	1.21 and 1.40, 2H[a]	1.21 and 1.40, 2H[a]	1.18 and 1.42, 2H[a]	1.18 and 1.43, 2H[a]
22	1.32 and 1.59, 2H[a]	1.32 and 1.58, 2H[a]	1.32 and 1.62, 2H[a]	1.34 and 1.61, 2H[a]
23	–	–	–	–
24	1.40, 3H, s	1.41, 3H, s	1.37, 3H, s	1.37, 3H, s
25	1.30, 3H, s	1.31, 3H, s	1.27, 3H, s	1.28, 3H, s
26	0.81, 3H, s	0.78, 3H, s	0.78, 3H, s	0.78, 3H, s
27	1.35, 3H, s	1.35, 3H, s	1.16, 3H, s	1.17, 3H, s
28	–	–	–	–
29	0.93, 3H, s	0.92, 3H, s	0.91, 3H, s	0.92, 3H, s
30	0.98, 3H, s	0.98, 3H, s	0.94, 3H, s	0.95, 3H, s

Assignments were established by HSQC, DQF-COSY and TOCSY spectra. *J* values (in hertz) are given in parentheses. [a] Multiplicities not assigned due to overlapped signals.

Table 4
^{1}H NMR data (δ_H, CD_3OD-10%D_2O) of the saccharide moieties of saponins **2**, **4**, **6** and **8**.

No.	2	4	6	8
	Glc (I)	Glc (I)	Glc (I)	Glc (I)
1	4.55, d (7.5)	4.54, d (7.5)	4.46, d (7.5)	4.41, d (7.6)
2	3.67	3.64	3.65	3.25
3	3.53	3.52	3.53	3.38
4	3.31	3.32	3.32	3.37
5	3.30	3.31	3.30	3.32
6	3.72, 3.85	3.72, 3.83	3.71, 3.85	3.70, 3.82
	Glc (II)	Glc (II)	Glc (II)	
1	4.65, d (7.5)	4.62, d (7.5)	4.63, d (7.6)	
2	3.23	3.25	3.24	
3	3.30	3.32	3.31	
4	3.38	3.36	3.35	
5	3.33	3.34	3.33	
6	3.65, 3.88	3.67, 3.90	3.68, 3.90	
	Ara (III)	Ara (III)	Ara (III)	Ara (II)
1	5.72, d (3.2)	5.66, d (3.5)	5.64, d (3.5)	5.64, d (3.4)
2	3.82	3.83	3.84	3.83
3	3.95	3.92	3.99	3.95
4	3.91	3.87	3.87	3.88
5	3.52, 3.90	3.54, 3.92	3.55, 3.91	3.55, 3.92
	Rha (IV)	Rha (IV)	Rha (IV)	Rha (III)
1	5.01, br s	5.06, br s	5.09, br s	5.05, br s
2	4.04	3.88	4.11	4.09
3	3.92	3.85	3.92	3.91
4	3.72	3.62	3.71	3.70
5	3.77	3.73	3.75	3.75
6	1.28, d (6.0)	1.29, d (6.0)	1.28, d (6.0)	1.28, d (6.0)
	Api (V)		Ara (V)	Ara (IV)
1	5.32, d (3.8)		4.53, d (7.3)	4.53, d (7.3)
2	4.02		3.62	3.61
3	–		3.55	3.52
4	3.81, 4.15		3.85	3.86
5	3.65		3.58, 3.89	3.61, 3.92
	Xyl (VI)	Xyl (V)	Xyl (VI)	Xyl (V)
1	4.71, d (7.8)	4.71, d (7.9)	4.71, d (7.9)	4.71, d (7.9)
2	3.15	3.19	3.15	3.11
3	3.31	3.30	3.27	3.30
4	3.52	3.54	3.55	3.53
5	3.18, 3.87	3.20, 3.88	3.19, 3.89	3.20, 3.88

Assignments were established by HSQC, DQF-COSY and TOCSY spectra. J values (in hertz) are given in parentheses.

$1_{Ara\ III}$), 5.32 (d, J = 3.8 Hz, H-$1_{Api\ V}$), 5.01 (br s, H-$1_{Rha\ IV}$), 4.71 (d, J = 7.8 Hz, H-$1_{Xyl\ VI}$), 4.65 (d, J = 7.5 Hz, H-$1_{Glc\ II}$), 4.55 (d, J = 7.5 Hz, H-$1_{Glc\ I}$)] were observed. The HSQC spectra allowed correlation between H and C signals. The structure of the saccharide units was determined by 2D-NMR spectroscopy. A DQF-COSY experiment allowed the sequential assignment of most resonances for each sugar unit, starting from the well isolated proton signals. Complete assignments of all the proton resonances in each sugar unit were achieved by a combination of DQF-COSY and TOCSY results. Furthermore, extensive 2D NMR analysis of the native saponins allowed the identification of *α*-L-arabinopyranosyl (Ara), *α*-L-rhamnopyranosyl (Rha), *β*-D-glucopyranosyl (Glc), *β*-D-xylopyranosyl (Xyl), *β*-D-apiofuranosyl (Api). In the HMBC experiment the anomeric signal at δ_H 4.55 (H-$1_{Glc\ I}$) showed a long-range correlation with the signal at δ_C 86.4 (C-3), indicating that glucose was directly linked to the triterpenic structure at C-3. The position of a second saccharide unit was indicated in the HMBC experiment in which the two anomeric protons at δ_H 4.55 (H-$1_{Glc\ I}$) and δ_H 4.65 (H-$1_{Glc\ II}$) gave a correlation with the same carbon (C-$2_{Glc\ I}$) δ_C 84.4. The 2-D NMR experiments revealed the presence of a sugar chain at the C-28 position. The resonance of the C-28 carboxylic group at δ_C 177.1 indicated the presence of a sugar moiety linked at this position. This carbon gave a clear correlation in HMBC experiment with the anomeric proton at δ_H 5.72 (H-$1_{Ara\ III}$) indicating that arabinose was esterified at the C-28 position as the first sugar in the chain. The sugar linked to arabinose was determined to be a rhamnose. The position of this sugar was deduced from the HMBC experiments in which the two anomeric protons at δ_H 5.72 (H-$1_{Ara\ III}$) and δ_H 5.01 (H-$1_{Rha\ IV}$) gave a correlation with the same carbon (C-$2_{Ara\ III}$) at δ_C 75.8. The third sugar of the chain was determined as apiose and its anomeric proton at δ_H 5.32 (H-$1_{Api\ V}$) gave a correlation with C-$3_{Rha\ IV}$ at δ_C 81.4. The fourth sugar of the chain should then be xylose and consistently its anomeric proton at δ_H 4.71 (H-$1_{Xyl\ VI}$) gave a correlation with C-$4_{Rha\ IV}$ at δ_C 79.2. These findings were also deduced from MS spectra. Based on these spectral data, saponin **2** was elucidated as 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*β*-D-apiofuranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester.

Compound **4**, 9 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{58}H_{92}O_{29}$ (MW 1252) by elemental analysis (calcd for C, 55.58; H, 7.40; found C, 55.52; H, 7.32). After acid hydrolysis it released zanhic acid and L-arabinose, L-rhamnose, D-xylose and D-glucose, in the ratio 1:1:1:2. The ^{13}C NMR

Table 5
Quantitative evaluation (% and mg/g DM) of identified saponins in leaves and roots of *M. marina*. For compound identification see Fig. 1.

compound	MW	leaves		roots	
		%	mg/g DM	%	mg/g DM
1	1384	4.55 ± 0.02	1.56 ± 0.01	–	–
2	1384	2.45 ± 0.13	0.84 ± 0.04	–	–
3	1222	2.95 ± 0.06	0.90 ± 0.02	–	–
4	1252	3.17 ± 0.03	0.99 ± 0.01	–	–
5	1090	0.62 ± 0.03	0.17 ± 0.01	–	–
6	1368	11.65 ± 0.26	3.96 ± 0.08	0.54 ± 0.04	0.04 ± 0.01
7	1368	19.92 ± 1.66	6.77 ± 0.55	1.69 ± 0.67	0.12 ± 0.07
8	1206	40.25 ± 1.54	12.05 ± 0.49	63.17 ± 1.02	3.88 ± 0.64
9	1074	4.03 ± 0.17	1.07 ± 0.05	16.31 ± 3.34	0.91 ± 0.34
10	942	3.96 ± 0.03	0.93 ± 0.01	9.85 ± 2.01	0.46 ± 0.01
11	940	0.08 ± 0.01	0.01 ± 0.00	6.25 ± 0.94	0.31 ± 0.03

spectrum of saponin **4** shows the presence of five anomeric signals at δ_C 105.4 (C-$1_{Glc\ II}$), 105.0 (C-$1_{Xyl\ V}$), 104.9 (C-$1_{Glc\ I}$), 101.1 (C-$1_{Rha\ IV}$), 93.8 (C-$1_{Ara\ III}$), confirmed in the ^{1}H spectrum in which the corresponding five anomeric protons were detected at δ_H 5.66 (d, J = 3.5 Hz, H-$1_{Ara\ III}$), 5.06 (br s, H-$1_{Rha\ IV}$), 4.62 (d, J = 7.5 Hz, H-$1_{Glc\ II}$), 4.71 (d, J = 7.9 Hz, H-$1_{Xyl\ V}$), 4.54 (d, J = 7.5 Hz, H-$1_{Glc\ I}$). The HSQC spectra allowed correlation between H and C signals. The sugar directly linked at the C-3 position of the sapogenin was determined to be glucose: its anomeric signal at δ_H 4.54 showed a long-range correlation with C-3 at δ_C 86.4 in the HMBC experiments. The position of the second saccharide unit was deduced from the HMBC experiment in which the two anomeric protons at δ_H 4.54 (H-$1_{Glc\ I}$) and δ_H 4.62 (H-$1_{Glc\ II}$) gave a correlation with the same carbon (C-$2_{Glc\ I}$) at δ_C 84.4. The sequence of the saccharide chain at C-28 was also defined by HMBC experiment. A cross-peak between C-28 (δ_C 177.2) and H-$1_{Ara\ III}$ (δ_H 5.66) was revealed. Additionally, cross-peaks between C-$2_{Ara\ III}$ (δ_C 75.7) and H-$1_{Rha\ IV}$ (δ_H 5.06) and C-$4_{Rha\ IV}$ (δ_C 82.3) and H-$1_{Xyl\ V}$ (δ_H 4.71), respectively, were observed, indicating that the terminal xylose was linked at C-4 of rhamnose, which in turn was attached at C-2 of arabinose, directly linked to the sapogenin at 28 COOH. On the basis of these spectral data compound **4** was elucidated as 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylzanhic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester.

Saponin **6**, 8 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{63}H_{100}O_{32}$ (MW 1368) by elemental analysis (calcd for C, 55.25; H, 7.36; found C, 55.18; H, 7.33). After acid hydrolysis it released medicagenic acid and L-arabinose, L-rhamnose, D-xylose and D-glucose, in the ratio 2:1:1:2. Six anomeric signals related to the sugars were observed in the ^{13}C NMR spectrum at δ_C 106.2 (C-$1_{Ara\ V}$), 105.2 (C-$1_{Glc\ II}$), 105.0 (C-$1_{Xyl\ VI}$), 104.7 (C-$1_{Glc\ I}$), 101.1 (C-$1_{Rha\ IV}$), 94.2 (C-$1_{Ara\ III}$), and at δ_H 5.64 (d, J = 3.5 Hz, H-$1_{Ara\ III}$), 5.09 (br s, H-$1_{Rha\ IV}$), 4.71 (d, J = 7.9 Hz, H-$1_{Xyl\ VI}$), 4.63 (d, J = 7.6 Hz, H-$1_{Glc\ II}$), 4.53 (d, J = 7.3 Hz, H-$1_{Ara\ V}$), 4.46 (d, J = 7.5 Hz, H-$1_{Glc\ I}$) in the ^{1}H NMR spectrum. The HSQC spectra allowed correlation between H and C signals. The 2D NMR analysis of the sugar portion of saponin **6** revealed the presence of the same monosaccharide at C-3 position as in saponins **2** and **4** and the same sugar chain at C-28 position of the sapogenin as in compound **3**. In the HMBC experiment H-$1_{Glc\ I}$ (δ_H 4.46) gave a correlation with C-3 at δ_C 86.6, and the two anomeric protons at δ_H 4.46 (H-$1_{Glc\ I}$) and δ_H 4.63 (H-$1_{Glc\ II}$) correlated with the same carbon (C-$2_{Glc\ I}$) at δ_C 86.8. The sequence of the sugar chain at C-28 was derived by the following HMBC correlations: H-$1_{Rha\ IV}$ (δ_H 5.09) with C-$2_{Ara\ III}$ (δ_C 75.8), H-$1_{Ara\ V}$ (δ_H 4.53) with C-$3_{Rha\ IV}$ (δ_C 82.3) and H-$1_{Xyl\ VI}$ (δ_H 4.71) with C-$4_{Rha\ IV}$ (δ_C 79.3). The attachment of the tetrasaccharide moiety to C-28 of the aglycone was based on a HMBC correlation of H-$1_{Ara\ III}$ (δ_H 5.64) with C-28 (δ_C 178.4) of the aglycone. On the basis of these spectral data compound **6** was elucidated as 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosylmedicagenic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*α*-L-arabinopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester.

Compound **8**, 43 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{57}H_{90}O_{27}$ (MW 1206) by elemental analysis (calcd for C, 56.71; H, 7.51; found C, 55.70; H, 7.48). After acid hydrolysis it released medicagenic acid and L-arabinose, L-rhamnose, D-xylose and D-glucose, in the ratio 2:1:1:1. The ^{1}H and ^{13}C NMR spectra of **8** exhibited five anomeric protons at δ_H 5.64 (d, J = 3.4 Hz, H-$1_{Ara\ II}$), 5.05 (br s, H-$1_{Rha\ III}$), 4.71 (d, J = 7.9 Hz, H-1_{XylIV}), 4.53 (d, J = 7.3 Hz, H-$1_{Ara\ IV}$), 4.41 (d, J = 7.6 Hz, H-$1_{Glc\ I}$) and carbons at δ_C 106.2 (C-$1_{Ara\ IV}$), 105.2 (C-$1_{Xyl\ V}$), 104.9 (C-$1_{Glc\ I}$), 101.1 (C-$1_{Rha\ III}$), 94.2 (C-$1_{Ara\ II}$). The HSQC spectra allowed correlation between H and C signals. The sugar directly linked at the C-3 position of the sapogenin was determined to be glucose: its anomeric signal at δ_H 4.41 showed a long-range correlation with C-3 at δ_C 86.5 in the HMBC experiments. The sequence of the sugar chains at C-28 was determined by the following HMBC correlations: H-$1_{Rha\ III}$ (δ_H 5.09) with C-$2_{Ara\ II}$ (δ_C 75.8), H-$1_{Ara\ IV}$ (δ_H 4.53) with C-$3_{Rha\ III}$ (δ_C 82.3) and H-$1_{Xyl\ V}$ (δ_H 4.71) with C-$4_{Rha\ III}$ (δ_C 79.2). A cross-peak between C-28 (δ_C 178.4) and H-$1_{Ara\ II}$ (δ_H 5.64) was also observed. On the basis of these spectral data compound **8** was elucidated as 3-*O*-*β*-D-glucopyranosylmedicagenic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-[*α*-L-arabinopyranosyl-(1 → 3)]-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl ester.

Data reported here indicate that almost all of the purified compounds from *M. marina* L. were glycosides of only zanhic acid (**1**–**5**), and medicagenic acid (**6**–**9**). No other aglycone commonly found in other *Medicago* spp., excluding soyasapogenol B and E, was found (Tava and Avato, 2006). Saponins **2**, **4**, **6** and **8** are undescribed compounds, while saponins **1**, **3**, **5** and **9** were previously characterized and reported as constituents of *M. arborea* L. and *M. truncatula* Gaertn. (Kapusta et al., 2005; Tava et al., 2005). Saponin **7** was previously isolated from other plant species containing saponins (Elbandy et al., 2002).

Quantitative analyses of all the detected saponins was performed by HPLC (Fig. S2 in Supplementary material) by an external standard method (Tava et al., 2011b), and results are shown in Table 5, in which their percentage composition and their amount as mg/g dry matter (DM) are reported. No saponins of zanhic acid were isolated from the roots of *M. marina* L. Saponin **8** was the most abundant compound in both the examined plant organs, representing the 40.25 ± 1.54% (12.05 ± 0.49 mg/g DM) and 63.17 ± 1.02% (3.88 ± 0.64 mg/g DM) of the total saponins from leaves and roots, respectively. Compound **7** (19.92 ± 1.66%, 6.77 ± 0.55 mg/g DM) and compound **6** (11.65 ± 0.26%, 3.96 ± 0.08 mg/g DM) were the second most abundant saponins in leaves, while saponin **9** (16.31 ± 3.34%, 0.91 ± 0.34 mg/g DM) was the most abundant in roots.

Based on the presence of medicagenic and zanhic acid as common aglycones found in saponins from other *Medicago* spp. and considering the sugars of saponins isolated from *M. marina* L., some considerations can be drawn. Saponins isolated until now from *Medicago* species (Tava

and Avato, 2006) typically contain several sugars or sugar chains generally linked by an *O*-heterosidic linkage at C-3 position of the aglycone in monodesmosidic types. Bidesmosidic saponins have additional sugars at C-28 position linked by an ester linkage to the triterpene structure. Tridesmosidic saponins with an extra sugar at C-23 position seems to be very rare in *Medicago* species. Until now only one tridesmosidic saponin has been isolated from *M. sativa* L. and *M. truncatula* Gaertn. (Tava and Avato, 2006). Number of sugars characterizing the structure of saponins from *Medicago* has been found to vary among the species and the plant organs. In some species such as for example in *M. arborea* L., saponins isolated from the aerial parts consist of up to seven units of sugars in the molecule (Tava et al., 2005). As regards sugar linkage in the molecule, in all the *Medicago* species studied until now (Tava et al., 2011a) medicagenic and zanhic acid containing saponins are always glycosylated with glucose or glucuronic acid as the first sugar at C-3, while the second sugar, often represented by glucose, is predominantly linked at the C-2 position. Only in *M. truncatula* the second glucose unit is linked at the C-3 position (Kapusta et al., 2005). In *M. arborea* L., *M. sativa* L. and *M. truncatula* Gaernt. the C-28 glycosilated saponins of medicagenic and zanhic acid are often characterized by the presence of the same linear sugar chain: Xyl(1 → 4)Rha (1 → 2)Ara. Moreover, these species also synthetize saponins with a branched sugar chain at the C-28 position (Tava et al., 2011a).

Accordingly, all the detected saponins in *M. marina* L. are bidesmosidic compounds with the C-3 position characterized by the presence of the same sugar, glucose (compounds **3, 5, 8** and **9**), or by the disaccharide chain Glc(1 → 2)Glc (saponins **1, 2, 4, 6, 7**). Additionally, the same sugar chain Xyl(1 → 4)Rha(1 → 2)Ara at C-28 was detected in all the saponins, with branching points formed by Ara or Api linked (1 → 3) at Rha as in saponins **1–3** and **6–8**.

Medicago genus includes 83 species which are grouped based on their morphological facets into four different subgenera; in addition each subgenus is divided up into some sections (Lesins and Lesins, 1979). Thus the subgenus *Medicago* includes four sections, among which sect. *Marinae* Grossheim. *M. marina* L. is the only representative *Medicago* species in this section indicating that it does not share strict morphological, environmental and genetic relationships with the other *Medicago* species (Lesins and Lesins, 1979).

Chemical composition of saponins from its leaves and roots also suggests a separation of *M. marina* from other *Medicago* species in that 60–63% of the total saponin mixture resulted made up by new structural types, never isolated before from the other investigated *Medicago* species. Quite interesting, the three dominant saponins in both tissues seems to represent marker compounds for this species (Table 5).

On the other hand, chemical results obtained in this work also indicate that saponins of *M. marina* L. have general structural features similar to those isolated from other *Medicago* species. Thus in *M. marina* L., glucose is often present at C-3 position as already described for *M. arborea* L. (Tava et al., 2005) and *M. sativa* L. (Bialy et al., 1999) saponins, with a linkage (1 → 2) in this side chain. In addition, glucose is often present as the first sugar linked at C-3 position as found in *M. truncatula* Gaertn. (Kapusta et al., 2005). Typical of *M. marina* saponins is the presence of a higher amount of C-28 branched sugar chains saponins, that represents the 81.8% and 64.4% of total saponins from leaves and roots, respectively (Table 5). This kind of compounds were also detected but in lower amount in *M. arborea* (Tava et al., 2005), *M. sativa* L. and *M. truncatula* Gaernt. (Tava and Avato, 2006).

Although *M. arborea* L. and *M. sativa* L. belong to different sections (sect. Arboreae and Falcago, respectively) they are included in the same subgenus *Medicago* as *M. marina*; *M. truncatula* belongs instead to the separate subgenus Spirocarpus.

M. marina L. is a salt tolerant legume plant growing along coastal zones where high salinity of soil occurs and the chemical peculiarity of this species of *Medicago* might reasonably be related with its environmental adaptation.

It is known that general metabolites such as sugars, polyols, amino acids, etc. act as osmolytes mediating osmotic stress (Slama et al., 2015). Role of specialized metabolites to the salt tolerance of plants is instead poor determined. A specific investigation on soybean (Wu et al., 2008) revealed that composition of isoflavonones and saponins could discriminate between salt-sensitive and salt-tolerance soybean varieties. In addition, due to their structural features, saponins act as surfactants being able to enhance plant nutrients and water uptake. An accumulation of bioactive saponins was also detected in *Quillaja brasiliensis* leaves when plants were subjected to biotic and abiotic stresses (de Costa et al., 2013).

Thus, we can suggest that specific saponins synthesized by *M. marina* may have a role in its tolerance to environment. Moreover, since they are highly glycosylated, we may also envisage that saponins in *M. marina* represents a reservoir of osmolytic sugars utilized by the plant for oxidative stress protection.

3. Experimental

3.1. General experimental procedures

Melting points were determined using a Buchi (Uster, Switzerland) apparatus. Optical rotations were measured on a PerkinElmer 241 polarimeter. Elemental analyses were carried out on a Carlo Erba (Milano, Italy) instrument. Molecular formulas obtained by elemental analysis of saponins were in agreement with data obtained by MS analysis. ^{1}H and ^{13}C NMR were measured on a Bruker AV-300 spectrometer at the operating frequencies of 300.13 and 75.13 MHz, respectively. Purified saponins were examined as solutions in CD_3OD-10% D_2O (5 mg/mL) in 5 mm tubes at 25 °C. ^{1}H and ^{13}C chemical shifts were expressed in ppm relative to TMS as standard. 2D NMR experiments (COSY; TOCSY; HSQC; HMBC) were carried out on all compounds using the phase sensitive method. For ESI/MS-MS a Jasco UPLC system equipped with a binary pump system, photo diode array detector (Jasco Chemstation ChromNAV) and coupled to a Thermo LTQ (linear ion trap mass detector) with an electrospray ionization (ESI) source was used. All data were acquired and processed using Thermo Xcalibur Qual Browser software. Chromatographic runs were carried out with an Acquity UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particles, 13 nm pore size) (Waters) under a linear gradient of solvent A (H_2O/0.1% HCOOH) and solvent B (CH_3CN/0.1% HCOOH) as follows: 0.0–5.0 min (20% B), 30.0 min (30% B), 20.0 min (80% B), 30.0 min (100% B). The flow rate was 0.3 mL/min, and the column temperature was 30 °C. Samples were dissolved in MeOH:H_2O 9:1 (3 mg/mL) and used (5.0 μL) for analysis. The eluates were spectrophotometrically checked at 9 different wavelengths (from 210 nm to 290 nm). For MS detection, negative ESI was used as ionization mode. Capillary voltage, 3100 V; sheath gas (He), aux gas (He), sweep gas (He) heated at 275 °C and introduced with a source heater temperature of 80 °C. Full scan spectra were acquired over the range of 100–2000 m/z. Automated MS/MS was performed by isolating the base peaks (molecular ions) using an isolation width of 2.0 m/z, normalized collision energy of 25 V, threshold set at 500 and ion charge control on, with max acquire time set at 300 ms. HPLC analyses were performed using a PerkinElmer (Norwalk, CT, USA) chromatograph equipped with a LC250 binary pump and DAD 235 detector. GC/FID analyses were carried out using a PerkinElmer model 8500 GC. GC/MS analyses were carried out using a PerkinElmer Clarus 500 GC equipped with a MS detector. LiChroprep RP-18 silica gel (40–63 μm Merck, Milano, Italy) was used for column chromatography (CC). 60H silica gel F_{254} plate and cellulose plate from Merck, were used for thin layer chromatography (TLC). All solvents (analytical grade) were from Sigma Aldrich (Milano, Italy).

3.2. Plant material

The *M. marina* samples were collected by R.A. at the beginning of flowering at Ugento (Lecce, Italy), 39°53′10″ N 18°07′13″E. Leaves

were separated from stems, and dries at 40 °C to a constant weight. Roots were washed with water and dried at 40 °C. All samples were ground and used for the successive extractions. Specimens of *M. marina* (MM1018) are deposited at Orto Botanico-DISTEBA, Università del Salento, Lecce, Italy.

3.3. Extraction and purification of saponins

Powdered plant material (70 g leaves and 15 g roots) were defatted with $CHCl_3$ in a Soxhlet apparatus (fats 3.9% DM leaves and 1.6% DM roots). Defatted material (50 g leaves and 10 g roots) was separately extracted with 80% MeOH under reflux for 24 h. The solvent was removed under reduced pressure, and the residue was re-suspended in 30% MeOH. The solution was applied onto a 100 × 60 mm RP-18 (40–63 μm) column, preconditioned with 30% MeOH. Elution was carried out with 30% MeOH (500 mL) to remove sugars and some phenolics. Total saponins were then eluted with 90% MeOH (400 mL) and dried under vacuum. 2.37 g of crude saponins were obtained from defatted leaves (4.7% yield), while 0.12 g of crude saponins were obtained from defatted roots (1.2% yield).

3.3.1. Fractionation

Crude saponins from leaves (2.0 g) were dissolved in 30% MeOH and submitted to a 200 × 60 mm RP-18 (40–63 μm) column. Three fractions were eluted: fraction I with 50% MeOH (350 mL), fraction II with 70% MeOH (350 mL) and fraction III with 90% MeOH (350 mL). Separation allowed to obtain 0.38 g of fraction I, 1.28 g of fraction II and 0.15 g of fraction III. After removing the solvent under vacuum, purity of each fraction was checked by silica gel TLC plates, developed with ethyl acetate/acetic acid/water (7:2:2). Spots were visualized by spraying the TLC plates with MeOH/acetic anhydride/H_2SO_4 (10:1:1 v/v) followed by heating at 120 °C.

3.3.2. Separation

Pure saponins were obtained from fractions I-III by means of semi-preparative HPLC using a 250 mm × 21.2 mm i.d., 5 μm, Discovery® HS C18 column (Supelco, Milano, Italy) with a mobile phase consisting of solvent A, CH_3CN/0.05% CF_3COOH, and solvent B H_2O/1% MeOH/0.05% CF_3COOH. 100 μL of MeOH/H_2O (9:1) solutions (25 mg/mL) of each fraction were injected. Saponins were eluted at 2.5 mL/min and detected by UV monitoring at 215 nm. Several sub-fractions were obtained, checked by silica gel TLC as described above and those containing compounds with the same eluting time were combined. From fraction I under linear elution gradient from 20% A to 40% A in 45 min the following pure saponins were obtained: saponin **1** (17 mg), **2** (13 mg), **3** (10 mg), **4** (9 mg) and **5** (6 mg). From fraction II under isocratic condition of 63% A, a mixture of **6** and **7** (35 mg), and saponins **8** (43 mg) and **9** (3 mg) were obtained. Saponin **6** (8 mg) and **7** (12 mg) were separated under linear gradient condition from 40% A to 65% A in 40 min. From fraction III under isocratic elution condition of 75% A, compounds **10** (10 mg) and **11** (3 mg) were obtained.

3.3.3. 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosylzanhic acid 28-O-β-D-xylopyranosyl-(1 → 4)-[β-D-apiofuranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (2)

Amorphous, white powder; mp: 285–287 °C, $[\alpha]_D^{25}$ -12.1 (MeOH *c* 0.42); for ^1H and ^{13}C NMR data see Tables 1–4; ESI-MS (negative ion mode), *m/z* 1383.6 [M ($C_{63}H_{100}O_{33}$)–H]$^-$, which fragmented in the MS/MS giving *m/z* 997.8 [M-H-162(Glc)-162(Glc)–CO_2–H_2O]$^-$, *m/z* 841.6 [M-H-132(Ara)-146(Rha)-132(Xyl)-132(Api)]$^-$, *m/z* 679.5 [M-H-132(Ara)-146(Rha)-132(Xyl)-132(Api)-162(Glc)]$^-$, *m/z* 455.3 [517(Zanh)–CO_2–H_2O]$^-$.

3.3.4. 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosylzanhic acid 28-O-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (4)

Amorphous, white powder; mp: 262–264 °C, $[\alpha]_D^{25}$ -9.3 (MeOH *c* 0.51); for ^1H and ^{13}C NMR data see Tables 1–4; ESI-MS (negative ion mode), *m/z* 1251.6 [M ($C_{58}H_{92}O_{29}$)–H]$^-$, which fragmented in the MS/MS giving *m/z* 927.7 [M-H-162(Glc)-162(Glc)]$^-$, *m/z* 865.3 [M-H-162(Glc)-162(Glc)–CO_2–H_2O]$^-$, *m/z* 841.4 [M-H-132(Ara)-146(Rha)-132(Xyl)]$^-$, *m/z* 455.3 [517(Zanh)–CO_2–H_2O]$^-$.

3.3.5. 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosylmedicagenic acid 28-O-β-D-xylopyranosyl-(1 → 4)-[α-L-arabinopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (6)

Amorphous, white powder; mp: 282–283 °C, $[\alpha]_D^{25}$ -15.9 (MeOH *c* 0.48); for ^1H and ^{13}C NMR data see Tables 1–4; ESI-MS (negative ion mode), *m/z* 1367.8 [M ($C_{63}H_{100}O_{32}$)–H]$^-$, which fragmented in the MS/MS giving *m/z* 1235.7 [M-H-132(Ara)]$^-$, *m/z* 981.4 [M-H-162(Glc)-162(Glc)–CO_2–H_2O]$^-$, *m/z* 825.3 [M-H-132(Ara)-146(Rha)-132(Xyl)-132(Ara)]$^-$, *m/z* 439.4 [501(Med)–CO_2–H_2O]$^-$.

3.3.6. 3-O-β-D-glucopyranosylmedicagenic acid 28-O-β-D-xylopyranosyl-(1→4)-[α-L-arabinopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (8)

Amorphous, white powder; mp: 275–276 °C, $[\alpha]_D^{25}$ -7.8 (MeOH *c* 0.36); for ^1H and ^{13}C NMR data see Tables 1–4; ESI/MS (negative ion mode), *m/z* 1205.7 [M ($C_{57}H_{90}O_{27}$)–H]$^-$, which fragmented in the MS/MS giving *m/z* 1073.3 [M-H-132(Ara)]$^-$, *m/z* 981.7 [M-H-162(Glc)–CO_2–H_2O]$^-$, *m/z* 663.2 [M-H-132(Ara)-146(Rha)-132(Xyl)-132(Ara)]$^-$, *m/z* 439.4 [501(Med)–CO_2–H_2O]$^-$.

3.4. HPLC analyses

The crude mixture of saponins, fractions I-III and all the purified saponins were analysed by HPLC using a 250 mm × 4.6 mm i.d., 5 μm, Discovery® HS C18 column (Supelco) using the same mobile phase as above. Chromatographic runs were carried out under gradient elution from 20% (5 min isocratic condition) to 30% of solvent A in 30 min, than to 50% solvent A in 20 min and to 90% solvent A in 20 min. Twenty μL of methanolic solutions (1 mg/mL) of each sample were injected. Saponins were eluted at 1.0 mL/min and detected by UV monitoring at 215 nm.

3.5. Saponin quantitation

250 mg of defatted plant material were treated with 5 mL of 30% MeOH in a stoppered tube, heated for 30 min at 50 °C and sonicated for 10 min. The sample was than centrifuged at 3000 × *g*, the supernatant removed and the precipitated re-extracted in the same conditions. The combined solutions were then run through a RP-18 column (400 mg, Merck), preconditioned with 30% MeOH. Elution was carried out with 30% MeOH (5 mL) to remove sugars and some phenolics; crude saponins where then eluted with 90% MeOH (3 mL) and dried under vacuum. Extraction of each sample was performed in triplicate and each obtained extract was run separately in HPLC analyses. All samples were re-dissolved in the proper amount of 90% MeOH to obtain a 1 mg/mL saponin solution and filtered by a syringe filter with a nylon membrane (0.2 μm, Nalgene). Twenty μL of methanolic solutions of each sample were used in HPLC analyses. The quantitation of all the identified saponins was performed by the external standard method using previously purified and identified saponins as reference compounds. Standard solution of pure saponins were prepared and analysed under the same HPLC conditions as described above. A series of calibration graphs were obtained for each pure compound between 0.10 and 5.5 μg injected, and a linear response was observed. These analytical conditions allowed a practical measurable sensitivity of 0.10 μg of saponin per injection, that is, a detection limit of 0.005 mg per gram of dried

plant material.

3.6. Hydrolysis of saponins

Saponin crude mixtures (5 mg) and each individual pure saponin (1–2 mg) were treated with 2 mL of 2 N HCl in 50% aqueous MeOH in a stoppered test-tube under stirring at 80 °C for 8 h. After cooling, MeOH was eliminated with a stream of N_2 and aglycones extracted with AcOEt (3 × 1 mL). Both the organic solution, containing the aglycones, and the aqueous solution, containing the sugars, from each sample, were dried under N_2 and used for the subsequent analyses.

3.6.1. Analysis of sapogenins

Aglycones were identified by TLC, GC/FID and GC/MS methods. Sapogenins were compared to previously purified sapogenins from *Medicago* spp. (Tava et al., 2017) by silica gel TLC elution with petroleum ether/chloroform/acetic acid (7:2:1) and spots were visualized by spraying with MeOH/acetic anhydride/sulphuric acid (10:1:1 v/v) followed by heating at 120 °C. Sapogenins were also analysed by GC/FID and GC/MS as their methyl/silyl derivatives. Aglycones were dissolved in 0.5 mL of MeOH, treated with CH_2N_2 for 15 min and then the solvent eliminated under a stream of N_2. Silylation was performed on the methylated sapogenins using 0.2 mL of a mixture of pyridine-hexamethyldisilazane-chlorotrimethylsilane (Merck) 2:1:1 at 70 °C for 10 min. Samples were diluted with isooctane and used for GC/FID and GC/MS analyses. GC/FID analyses were performed with a 30 m × 0.32 mm, 0.25 μm i.d., DB-5 capillary column. Injector and detector temperatures were set at 350 °C; the oven temperature program was: 90 °C for 5 min, increased at 20 °C/min to 250 °C for 1 min and then increased at 4 °C/min to 350 °C for 15 min. Samples (1 μL) were injected in the "splitless" mode. He was the carrier gas with a head pressure of 12.2 psi. GC/MS analyses were carried out using a 30 m × 0.25 mm, 0.25 μm i.d., Elite-5MS capillary column using the same chromatographic conditions as for GC/FID. Mass spectra were acquired over 50–850 amu range at 1 scan/sec with ionizing electron energy 70 eV. Transfer line 300 °C, carrier gas He at 1.2 mL/min. Retention times and MS spectra were compared to those of previously identified sapogenins.

3.6.2. Analyses of sugars and determination of absolute configuration

Sugars were separated on cellulose plates with benzene/butanol/pyridine/water (1:5:3:3), made visible with a silver nitrate spray and identified by comparison with authentic reference compounds. The determination of sugar absolute configurations was carried out by GC/FID using a 30 m × 0.32 mm, 0.25 μm i.d., Chirasil-Val column (Alltech, Deerfield, IL). Sugar samples were dissolved in 1-(trimethylsilyl)imidazole (Tris-Z) and pyridine (Merck, 1:1, 0.3 mL), and the solution stirred at 60 °C for 5 min. After drying the solution under N_2, the residue was partitioned by water and CH_2Cl_2 (1 mL, 1:1). The organic layer was used for GC analyses. GC temperature gradient was as follows: 60 °C for 3 min, raised to 200 °C at 5 °C/min; injector and detector temperatures were set at 200 °C and 250 °C, respectively. He was the carrier gas with a head pressure of 12 psi; samples (0.2 μL) were injected in the "splitless" mode. Authentic reference compounds from Sigma-Aldrich, treated in the same way as reported above, were used for sugars identification. Co-injection of each hydrolysate with the standards gave single peaks. Sugar identification was also carried out by GC/MS as described previously (Tava et al., 1993).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112333.

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