JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Triterpenoid Glycosides from the Leaves of Two Cultivars of *Medicago polymorpha* L.

Aldo Tava,*[,†] Luciano Pecetti,[†] Massimo Romani,[†] Mariella Mella,[‡] and Pinarosa Avato[§]

[†]CRA-FLC Centro di Ricerca per le Produzioni Foraggere e Lattiero Casearie, Viale Piacenza 29, 26900 Lodi, Italy

[‡]Dipartimento di Chimica Organica, Università di Pavia, Viale Taramelli 10, 27100 Pavia, Italy

[§]Dipartimento Farmaco-Chimico, Università di Bari Aldo Moro, Via Orabona 4, 70125 Bari, Italy

ABSTRACT: The saponin composition of leaves from the *Medicago polymorpha* cultivars 'Santiago' and 'Anglona' belonging to the botanical varieties *brevispina* and *vulgaris*, respectively, was investigated by a combination of chromatographic, spectroscopic, and spectrometric techniques. Several compounds were detected and quantitated by HPLC analysis using the external standard method. Twelve triterpene saponins (**1**−**12**) were purified by reverse-phase chromatography and their structures elucidated by spectroscopic (1D and 2D NMR, ESI-MS/MS) and chemical methods. They were identified as glycosides of echinocystic acid, hederagenin, caulophyllogenin, bayogenin, and soyasapogenol B. Two of them (**2**, **10**) were previously reported in *M. polymorpha*; five of them (**4**, **6**, **7**, **9**, **12**) were already identified in other *Medicago* species; and three of them (**1**, **8**, **11**) were found in other plant genera. The two saponins identified as 3-*O*-α-L-arabinopyranosyl-28-*O*-[β-D-glucopyranosyl(1→6)β-D-glucopyranoside] echinocystic acid (**3**) and 3-*O*-α-L-arabinopyranosyl-28-*O*-β-D-glucopyranoside echinocystic acid (**5**) are newly identified natural compounds. The presence of echinocystic acid is reported here for the first time in the genus *Medicago*. Saponins from the cultivar 'Anglona' were characterized by a higher amount of echinocystic acid glycosydes, whereas saponins from the cultivar 'Santiago' were characterized by a higher amount of hederagenin glycosydes.

KEYWORDS: *Medicago polymorpha* L., saponins, chemical structure, triterpene glycosides, echinocystic acid, ESI-MS/MS, NMR, HPLC quantification

INTRODUCTION

Burr medic (*Medicago polymorpha* L.) is one of the agronomically most important medic species, mainly used for pastures.[1] Originating from the Mediterranean basin, where it occurs naturally, it spread worldwide and became a cultivated species in Australia.[2] In recent decades, an agronomic interest also arose in the Mediterranean region,[3,4] where this species shows a wide range of adaptation to pedoclimatic conditions.[5] Morphologically, it is a very variable species.[2] Among the several recognized botanical varieties, var. *brevispina* (Benth.) Heyn and var. *vulgaris* (Benth.) Shinners differ in the presence or absence of spines on the pod, the number of coils per pod, or the spine length. The breeding work in Australia privileged the spineless *brevispina* variety, because of the lower risk of pods hooking to sheep's wool with subsequent depreciation of the wool itself.[6] Incomplete success of Australian selections can be experienced in the Mediterranean region, owing to different climatic constraints and exploitation methods.[5,7] A specific breeding, relying on a wider genetic base than just the *brevispina* form, was pursued in Mediterranean countries, which led to the selection of possibly more adapted germplasm from local ecotypes.[3,4]

Saponins so far isolated in the genus *Medicago* consist of a complex mixture of triterpenic pentacyclic glycosides. Although many species have already been analyzed, studies are still in progress to elucidate the chemical structure, biosynthesis, and biological activities of saponins in several other species within the *Medicago* genus.[8,9] Quantitative evaluation of saponins in plant material[10−12] and their use as natural bioactive compounds in agriculture are other interesting aspects investigated by us in recent years.[8,13]

Literature data on saponins of *M. polymorpha* report the presence of short sugar chain bidesmosides of hederagenin and caulophyllogenin, with a unique feature in the genus, that is, the presence of the 1→6 interglycosidic linkage between two glucose units at the C-28 position of the aglycones.[14]

The aim of the current investigation was to assess the saponin concentration and composition in two cultivars of *M. polymorpha* belonging to two botanical varieties, namely, cv. 'Santiago' (var. *brevispina*) selected in Australia and cv. 'Anglona' (var. *vulgaris*) selected in Italy.

MATERIALS AND METHODS

Plant Material. Cultivars 'Santiago' and 'Anglona' belonging to the variety *brevispina* (Benth.) Heyn and *vulgaris* (Benth.) Shinners of *M. polymorpha*, respectively, were field-grown in Lodi, northern Italy (45° 19′ N, 9° 30′ E, 81 m elevation), a site characterized by rather favorable, subcontinental climatic conditions (802 mm long-term average annual rainfall; 320 mm average March−July rainfall). Sowing took place in early spring (April 13) in unreplicated 4.5 m^2 plots. Leaf sampling was carried out at the beginning of flowering of each cultivar, namely, mid-June for 'Santiago' and late June for 'Anglona'. Samples were immediately oven-dried at 40 °C for 2 days. Ground leaves were used for subsequent extractions of saponins.

Received: February 11, 2011
Revised: April 26, 2011
Accepted: April 28, 2011
Published: April 28, 2011

ACS Publications © 2011 American Chemical Society

dx.doi.org/10.1021/jf2005854 | *J. Agric. Food Chem.* 2011, 59, 6142–6149

Extraction and Purification. Powdered leaves (250 g of each cultivar) were defatted with $CHCl_3$ in a Soxhlet apparatus (fats were 8.9% of dry matter in cv. 'Anglona' and 8.2% in cv. 'Santiago'). Defatted material (200 g of each cultivar) was extracted with 80% MeOH under reflux for 24 h. The solvent was removed under reduced pressure, and the residue was suspended in 30% MeOH. The solution was applied onto a 100 × 60 mm, 40−63 μm LiChroprep RP-18 column (Merck, Darmstadt, Germany), preconditioned with 30% MeOH. Elution was carried out with 30% MeOH (500 mL) and successively with 40% MeOH (500 mL) to remove sugars and some phenolics. Total saponins were then eluted with 90% MeOH (700 mL) and dried under vacuum. From 200 g of defatted plant material, 4.1 g (2.1% yield) and 3.4 g (1.7% yield) of crude saponins were obtained from 'Anglona' and 'Santiago' cultivars, respectively.

Fractionation. The crude saponins were dissolved in 30% MeOH and submitted to a chromatographic separation with a 200 × 60 mm, 40−63 μm LiChroprep RP-18 column. Three fractions were eluted from both saponin extracts: fraction I with 50% MeOH (500 mL), fraction II with 70% MeOH (500 mL), and fraction III with 90% MeOH (500 mL). Solvent was reduced under vacuum, and fractions were checked by 60H silica gel TLC plates (Merck), developed with ethyl acetate/acetic acid/water (7:2:2). Spots were visualized by spraying with methanol/acetic anhydride/sulfuric acid (10:1:1 v/v) followed by heating at 120 °C. Only fractions II (2.3 and 0.9 g for cv. 'Anglona' and 'Santiago', respectively) and III (1.1 and 1.9 g for cv. 'Anglona' and 'Santiago', respectively) contained saponins and were used, therefore, for the subsequent analytical study.

Separation. Pure saponins were obtained from fractions II and III by means of preparative HPLC using a 250 mm × 20 mm i.d., 5 μm, Discovery 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. One hundred microliters of MeOH/H_2O (9:1) solutions (30 mg/mL) of each fraction was injected. Saponins were eluted at 2.5 mL/min and detected by UV monitoring at 215 nm. The following pure saponins were obtained from cv. 'Anglona': from fraction II under isocratic conditions of 34% A, saponins **1** (24 mg), **2** (22 mg), **3** (122 mg), **4** (56 mg), **5** (10 mg), **6** (11 mg), and **7** (16 mg); from fraction III under isocratic conditions of 49% A, saponins **8** (10 mg), **9** (8 mg), **10** (12 mg), **11** (37 mg), and **12** (12 mg). From the cv. 'Santiago' saponin mixture, the following pure compounds were recovered: from fraction II under isocratic conditions of 34% A, saponins **2** (56 mg), **3** (18 mg), and **7** (12 mg); from fraction III under isocratic conditions of 49% A, saponins **8** (4 mg), **9** (5 mg), **10** (88 mg), **11** (9 mg), and **12** (5 mg).

HPLC Analyses. The crude mixture of saponins, fractions I−III, and all of the purified saponins were analyzed by HPLC using a Perkin-Elmer (Norwalk, CT) chromatograph equipped with am LC 250 binary pump and a DAD 235 detector. Separation was performed on a 250 mm × 4.6 mm i.d., 5 μm, Discovery C18 column (Supelco) using the same mobile phase as above. Chromatographic runs were carried out under gradient elution from 25% (5 min isocratic condition) to 50% of solvent A in 50 min and then to 90% of solvent A in 30 min. Saponin solutions were filtered by syringe filter with a nylon membrane (0.2 μm, Nalgene, Rochester, NY). Twenty microliters of methanolic solutions (1 mg/mL) of all samples was injected. Saponins were eluted at 1.0 mL/min and detected by UV monitoring at 215 nm.

Saponin Quantification. Determination of saponin content was performed on each sample by three independent extractions. All samples were redissolved in a defined amount of MeOH to obtain approximately a 1 mg/mL saponin solution and filtered by syringe filter with a nylon membrane (0.2 μm, Nalgene). Twenty microliters of methanolic solutions of all samples was injected. The quantification of all the identified saponins was performed by an external standard method using the purified and identified saponins from *M. polymorpha* as reference compounds. Standard solutions of pure saponins were prepared and injected under the same HPLC conditions as described above for the sample mixtures. A series of calibration graphs were obtained for each pure compound between 0.10 and 5.20 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 of saponin per gram of dried plant material.

Hydrolysis of Saponins. Saponin crude mixtures (5 mg) and each individual pure saponin (1−2 mg) were treated with 1 mL of 2 N HCl in 50% aqueous methanol in a stoppered test tube under stirring at 80 °C for 3 h. After cooling, methanol was eliminated with a stream of N_2 and aglycones were extracted with ethyl acetate (2 × 1 mL). For each sample, both the organic solution, containing the aglycones, and the aqueous solution, containing the sugars, were dried under N_2 and stored for the subsequent analyses.

Analysis of Sapogenins. Aglycones were identified by TLC, GC-FID and GC-MS methods. Sapogenins were compared to previously identified sapogenins from *Medicago* spp.[15−18] by TLC (Merck silica gel 60H) elution with petroleum ether/$CHCl_3$/AcOH (7:2:1) or benzene/MeOH (9:1) and were visualized by spraying the developed TLC with MeOH/acetic anhydride/sulfuric acid (10:1:1 v/v) followed by heating at 120 °C. Sapogenins were also analyzed by GC-FID and GC-MS as their methylperacetyl and methylsilyl derivatives. Aglycones were dissolved in 0.5 mL of MeOH and treated with CH_2N_2. This solution was divided into two subsamples and the solvent eliminated under a stream of N_2. Acetylation was performed by using 0.2 mL of pyridine/acetic anhydride/4-dimethylaminopyridine (1:1:0.1) and, after overnight stirring at room temperature, water was added and methylated−peracetylated compounds were extracted with ethyl acetate (3 × 1 mL). Samples were dried over anhydrous Na_2SO_4, concentrated under a stream of N_2, and used for GC-FID and GC-MS analyses. Silylation was performed on the methylated sapogenin subsamples using 0.2 mL of a mixture of pyridine/hexamethyldisilazane/chlorotrimethylsilane (2:1:1) at 70 °C for 10 min. Reacted samples were diluted with isooctane and analyzed by GC-FID and GC-MS. GC-FID analyses of both methylated−peracetylated and methylated−silylated sapogenins were carried out using a Perkin-Elmer model 8500 GC equipped with a 30 m × 0.32 mm i.d., 0.25 μm, DB-5 capillary column. Injector and detector temperatures were set at 350 °C, and 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. Helium was the carrier gas with a head pressure of 12.2 psi. GC-MS analyses were carried out using a Perkin-Elmer Clarus 500 GC equipped with a MS detector and a 30 m × 0.25 mm i.d., 0.25 μm, Elite-5MS capillary column using the same chromatographic conditions as for GC-FID. Mass spectra were acquired over a 50−850 amu range at 1 scan/s with ionizing electron energy of 70 eV; transfer line, 300 °C; and carrier gas He, 1.2 mL/min. Retention times and MS spectra were compared to those of previously identified sapogenins,[15−18] except for the aglycones of saponins **1**, **3**, and **5** further characterized by NMR experiments.

Analyses of Sugars and Determination of Absolute Configuration. Sugars were separated on Merck cellulose plates with benzene/butanol/pyridine/water (1:5:3:3), made visible with a silver nitrate spray (Fluka, Milano, Italy), and identified by comparison with authentic reference compounds (Sigma-Aldrich, Milano, Italy). The determination of sugar absolute configurations was carried out by GC-FID using a 30 m × 0.32 mm i.d., 0.25 μm, Chirasil-Val column (Alltech, Deerfield, IL). Sugar samples were suspended in MeOH (0.5 mL) and treated with CH_2N_2. Solvent was eliminated under a stream of N_2, the residue dissolved in 1-(trimethylsilyl)imidazole (Tris-Z) (VWR International, Milano, Italy) and pyridine (1:1, 0.3 mL), and the solution stirred at 60 °C for 5 min. After drying of the solution under N_2, the residue was separated by water and CH_2Cl_2 1:1 (1 mL). The organic layer was used for GC analyses as follows: 60 °C for 3 min, raised to

dx.doi.org/10.1021/jf2005854 |*J. Agric. Food Chem.* 2011, 59, 6142–6149

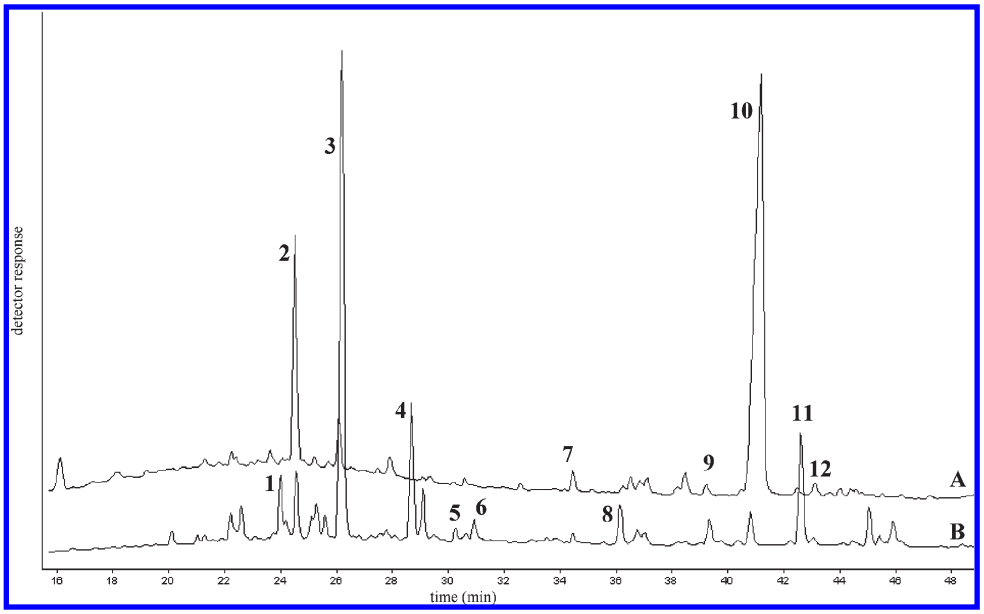


Figure 1. HPLC chromatogram of *M. polymorpha* saponins from (A) cv. 'Santiago' and (B) cv. 'Anglona'. The structures of compounds **1**−**12** are reported in Figure 2.

200 °C at 5 °C/min; injector and detector temperatures were set at 200 and 250 °C, respectively. Helium was the carrier gas with a head pressure of 12 psi; samples (0.5 μL) were injected in the splitless mode. Authentic reference compounds, treated in the same way as above, were used for the identification of sugars. Co-injection of each hydrolysate with the standards gave individual peaks. Sugar identification was also carried out by GC-MS as described previously.[15]

NMR Analyses. ^{1}H and ^{13}C NMR were measured on a Bruker (Bremen, Germany) AV-300 spectrometer at the operating frequencies of 300.13 and 75.13 MHz, respectively. Saponins were examined as solutions in CD_3OD (5−10 mg/0.5 mL) in 5 mm diameter tubes at 25 °C. TMS was used as internal reference. 2D NMR experiments (H,H DQF-COSY; H,H TOCSY; H,H NOESY; H,H ROESY; H,C HSQC; H,C HMBC) were carried out on all compounds using the phase-sensitive method. Based on 2D NMR analyses, assignments of ^{1}H and ^{13}C signals were obtained.

ESI-MS/MS. Analyses were performed on an 1100 series Agilent LC/MSD Trap-System VL. An Agilent Chemstation (LC-MSD Trap-Software 4.1) was used for acquisition and processing of the data. All of the analyses were carried out using an ESI ion source type in the negative mode with the following settings: capillary voltage, 4000 V; nebulizer gas (N_2), 15 psi; drying gas (N_2), heated at 350 °C and introduced at a flow rate of 5 L/min. Full scan spectra were acquired over the range of m/z 100−2200 with a scan time of 13000 m/z/s. Automated MS/MS was performed by isolating the base peaks (molecular ions) using an isolation width ofm/z 4.0 , fragmentation amplitude of 1.0 V, threshold set at 100, and ion charge control on, with maximum acquiring time set at 300 ms. Samples were dissolved in MeOH/H_2O (9:1) at the concentration of 20−30 ppm and injected by direct infusion at a flow rate of 10 μL/min with a syringe pump (KD Scientific, Holliston, MA).

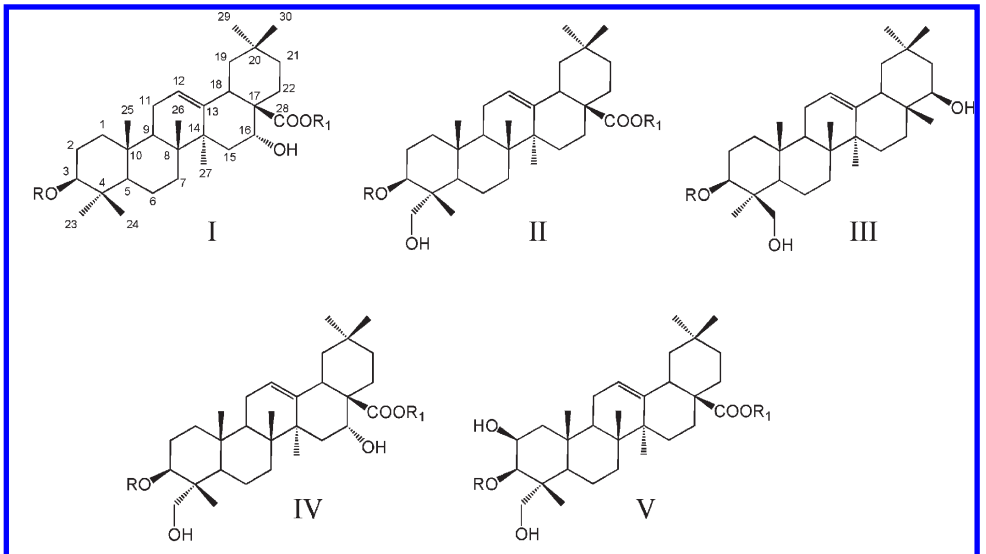
Melting points were determined using a Büchi (Uster, Switzerland) apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Elemental analyses were carried out on a Carlo Erba (Milano, Italy) instrument.

Chemical Data of Saponins 1−3, 5, 8, 10, 11. Saponin **1**: ESI-MS (negative ion mode), m/z (relative intensity) 1089.0 (100%) $[M(C_{53}H_{86}O_{23}) - H]^-$, which fragmented in the MS/MS giving 765.2 (100%) [M − H − 162(Glc) − 162(Glc)]$^-$; 603.3 (7%) [M − H − 162(Glc) − 162(Glc) − 162(Glc)]$^-$; 471.1 (10%) [M − H − 162(Glc) − 162(Glc) − 162(Glc) − 132(Ara)]$^-$. Saponin **2**: ESI-MS (negative ion mode), m/z (relative intensity) 1073.0 (100%) $[M(C_{53}H_{86}O_{22}) - H]^-$, which fragmented in the MS/MS giving 749.2 (100%) [M − H − 162(Glc) − 162(Glc)]$^-$; 603.0 (5%) [M − H − 162(Glc) − 162(Glc) − 146(Rha)]$^-$; 471.1 (11%) [M − H − 162(Glc) − 162(Glc) − 146(Glc) − 132(Ara)]$^-$. Saponin **3**: mp 226−227 °C (dec), $[\alpha]_D^{25}$ −9.2 (MeOH, *c* 1.7). Anal. Calcd for $C_{47}H_{76}O_{18}$: C, 60.76; H, 8.28. Found: C, 60.98; H, 8.61. ESI-MS (negative ion mode), m/z (relative intensity) 927.9 (100%) $[M(C_{47}H_{76}O_{18}) - H]^-$, which fragmented in the MS/MS giving 603.3 (100%) [M − H − 162(Glc) − 162(Glc)]$^-$; 471.2 (3%) [M − H − 162(Glc) − 162(Glc) − 132(Ara)]$^-$. Saponin **5**: mp 183 °C (dec), $[\alpha]_D^{25}$ +5.8 (MeOH, *c* 0.29). Anal. Calcd for $C_{41}H_{66}O_{13}$: C, 64.12; H, 8.67. Found: C, 64.29; H, 8.84. ESI-MS (negative ion mode), m/z (relative intensity) 765.0 (100%) $[M(C_{41}H_{66}O_{13}) - H]^-$, which fragmented in the MS/MS giving 603.1 (100%) [M − H − 162(Glc)]$^-$; 471.2 (6%) [M − H − 162(Glc) − 132(Ara)]$^-$. Saponin **8**: ESI-MS (negative ion mode), m/z (relative intensity) 619.0 (100%) $[M(C_{35}H_{56}O_9) - H]^-$, which fragmented in the MS/MS giving 487.1 (6%) [M − H − 132(Ara)]$^-$. Saponin **10**: ESI-MS (negative ion mode), m/z (relative intensity) 749.1 (100%) $[M(C_{41}H_{66}O_{12}) - H]^-$, which fragmented in the MS/MS giving 603.0 (87%) [M − H − 146(Rha)]$^-$; 471.1 (100%) [M − H − 146(Rha) − 132(Ara)]$^-$. Saponin **11**: ESI-MS (negative ion mode), m/z (relative intensity) 603.0 (100%) $[M(C_{35}H_{56}O_8) - H]^-$, which fragmented in the MS/MS giving 471 (23%) [M − H − 132(Ara)]$^-$.

RESULTS AND DISCUSSION

The HPLC analysis gave a good peak resolution of the crude saponins obtained from the defatted leaves of both cv. 'Anglona' and 'Santiago' (Figure 1). Crude saponins were further fractionated by reverse-phase (RP-18) chromatography (open column and preparative HPLC), allowing the separation of 12 saponins (**1**−**12**) in a pure form. Aglycone moieties obtained from each of them were identified by TLC R_f values, GC-FID, GC-MS, and NMR data compared to those of reference compounds already available from *Medicago* ssp. and to literature data.[14,16−24] The presence of echinocystic acid in saponins **1**, **3**, **5**, and **11**, hederagenin in saponins **2**, **4**, **6**, **10**, and **12**, soyasapogenol B

dx.doi.org/10.1021/jf2005854 |*J. Agric. Food Chem.* 2011, 59, 6142–6149



compound	aglycone	R	R_1
1	I	β-D-Glc(1→2)-α-L-Ara	β-D-Glc(1→6)-β-D-Glc
2	II	α-L-Rha-(1→2)-α-L-Ara	β-D-Glc(1→6)-β-D-Glc
3	I	α-L-Ara	β-D-Glc(1→6)-β-D-Glc
4	II	β-D-Glc(1→2)-α-L-Ara	β-D-Glc
5	I	α-L-Ara	β-D-Glc
6	II	α-L-Ara	β-D-Glc
7	III	α-L-Rha-(1→2)-β-D-Gal-(1→2)-β-D-GluA	-
8	IV	α-L-Ara	-
9	V	α-L-Ara	-
10	II	α-L-Rha-(1→2)-α-L-Ara	-
11	I	α-L-Ara	-
12	II	α-L-Ara	-

Figure 2. Structure of saponins **1**−**12** (**I**, echinocystic acid; **II**, hederagenin; **III**, soyasapogenol B; **IV**, caulophyllogenin; **V**, bayogenin).

in saponin **7**, caulophyllogenin in saponin **8**, and bayogenin in saponin **9** was thus established.

Sugar moieties were identified by means of TLC, comparing the R_f values with those of reference compounds. The absolute configuration of the sugar residues was obtained from GC analysis on a chiral column. The assignment of all ^{1}H and ^{13}C signals for each pure saponin was obtained on the basis of 2D NMR experiments. The structure elucidation of all saponins was performed by combining NMR, GC-MS, and ESI-MS/MS data. The molecular weights were obtained from elemental analyses and MS spectra and evaluated from NMR signals in which all carbons were revealed. MS fragmentation ions were also used to establish sugar chains in the molecule. The chemical structures of the identified saponins **1**−**12** are reported in Figure 2, and ^{1}H and ^{13}C NMR chemical shifts of saponins **3** and **5** are reported in Tables 1 and 2, respectively. All of the monosaccharides were determined to be in the pyranose form as deduced by their ^{13}C NMR data and by comparison with literature values.[14,16−23]

Compound **1**, 24 mg, MW 1090, released after acid hydrolysis echinocystic acid and α-L-arabinose and β-D-glucose in the ratio 1:3. This saponin had spectroscopic characteristics identical to those of 3-*O*-[β-D-glucopyranosyl(1→2)α-L-arabinopyranosyl]-28-*O*-[β-D-glucopyranosyl(1→6)β-D-glucopyranoside] echinocystic acid. This compound was previously identified in *Polyscias guilfoyley* [22] but is reported here for the first time as a new saponin in the genus *Medicago*.

Compound **2**, 78 mg, MW 1074, released after acid hydrolysis hederagenin and α-L-arabinose, α-L-rhamnose, and β-D-glucose, in the ratio 1:1:2, and had spectroscopic characteristics identical to those of 3-*O*-[α-L-rhamnopyranosyl(1→2)α-L- arabinopyranosyl]-28-*O*-[β-D-glucopyranosyl(1→6)β-D-glucopyranoside] hederagenin. This saponin was previously reported from *M. polymorpha*.[14]

Compound **3**, 140 mg, was isolated as an amorphous solid; the molecular formula was estimated as $C_{47}H_{76}O_{18}$ (MW 928). The acid hydrolysis of this saponin gave echinocystic acid and α-L-arabinose and β-D-glucose in the ratio 1:2. The triterpenoid glycosidic structure of this compound was also suggested by NMR spectra (Tables 1 and 2). The ^{1}H NMR spectrum of the aglycone moiety of compound **3** (Table 2) showed signals for seven tertiary methyl groups at δ 0.80, 0.86, 0.90, 0.98, 0.99, 1.06, and 1.38, which correlate in the HSQC experiments with the

dx.doi.org/10.1021/jf2005854 |*J. Agric. Food Chem.* 2011, 59, 6142–6149

Table 1. ^{13}C NMR Data of the Aglycone and Monosaccharide Moieties of Compounds 3 and 5 from *M. polymorpha*

	δ, CD_3OD				
	aglycone			monosaccharide	
C	3	5	C	3	5
1	40.2	40.2		Ara (I)	Ara (I)
2	32.1	32.0	1	107.5	107.5
3	91.0	91.0	2	73.1	73.1
4	42.9	43.0	3	74.6	74.7
5	57.2	57.5	4	69.8	69.8
6	19.7	19.7	5	66.7	66.7
7	36.7	36.8			
8	40.5	40.5		Glc(II)	Glc(II)
9	48.5	48.7	1	96.0	96.0
10	38.2	38.2	2	75.4	75.3
11	24.8	24.8	3	78.2	78.7
12	123.9	123.9	4	71.2	71.4
13	144.9	144.9	5	78.1	79.1
14	41.1	41.2	6	**69.8**	62.7
15	27.4	27.4			
16	74.2	74.3		Glc(III)	
17	50.2	50.4	1	104.9	
18	42.3	42.4	2	75.2	
19	48.1	48.1	3	78.2	
20	31.6	31.6	4	71.8	
21	36.6	36.6	5	78.4	
22	34.5	34.5	6	63.0	
23	28.9	28.9			
24	17.3	17.3			
25	16.6	16.4			
26	18.2	18.1			
27	27.6	27.6			
28	177.5	177.5			
29	33.7	33.7			
30	25.4	25.3			

carbon signals at δ 18.2, 17.3, 33.7, 16.6, 25.4, 28.9, and 27.6, respectively (Table 1). A further feature was the signal at δ 5.32 (1H, t, J = 3.0 Hz) typical of H-12 of the Δ12 oleanene skeleton, which was confirmed by the presence in the ^{13}C NMR spectrum of the signals at δ 123.9 and 144.9 attributable to C-12 and C-13 (Table 1). The signal at δ 177.5 in the ^{13}C NMR spectrum suggested the occurrence of a carboxylic group, whereas the presence of the ^{1}H NMR signals at δ 3.15 (1H, dd, J = 12.0 and 4.0) and δ 4.54 (1H, br s), correlating in the HSQC experiments with the carbon resonances at δ 91.0 and δ 74.2, respectively, suggested the presence of two secondary alcoholic groups. From the ^{1}H and ^{13}C spectroscopic data, the location of the hydroxy groups was determined to be at 3β and 16α, respectively, in agreement with data from the literature.[19−23] The presence of echinocystic acid was also confirmed by GC-MS analyses of the methylated−silylated aglycone, showing the ions at m/z 279 (6%) and m/z 350 (9%) originated from the cleavage of the central ring of the β-amyrin structure through the typical retro Diels−Alder fragmentation of the molecular ion $[M]^+$ at m/z 630 ($C_{37}H_{66}O_4Si_2$). From the ion at m/z 279 the loss of trimethylsilanol gave the peak at m/z 189 (31%). The loss of a trimethylsilanol from the ion m/z 350 gave the ion at m/z 260 (60%), from which the loss of an acetic acid unit gave the base peak at m/z 201. The GC-MS spectrum of the methylated−peracetylated derivative gave concurring results.

The ^{1}H and ^{13}C NMR spectra of saponin **3** (Tables 1 and 2) showed the presence of three anomeric protons at δ 4.29, 4.35, and 5.34 and carbons at δ 96.0, 104.9, and 107.5. Complete assignments of all the proton resonances in each sugar unit were achieved by a combination of DQF-COSY and TOCSY data. In the HMBC experiments the anomeric signal at δ 4.29 ($H\text{-}1_{AraI}$) showed a long-range correlation with the signal at δ 91.0 (C-3), indicating that arabinose is directly linked to the triterpenic structure at C-3. The presence of the chain Glc(1→6)Glc linked to the C-28 position of the aglycone was also evidenced by 2D NMR experiments. In the HMBC experiments, a clear correlation between the signal at δ 5.34 ($H\text{-}1_{GlcIII}$) and the signal at δ 177.5 (C-28) was observed, and a cross peak between $H\text{-}1_{GlcIII}$ (δ 4.35) and $C\text{-}6_{GlcII}$ (δ 69.8) was also revealed. This saponin was identified as 3-*O*-α-L-arabinopyranosyl-28-*O*-[β-D-glucopyranosyl-(1→6)β-D-glucopyranoside] echinocystic acid (**3**) and represents a new identified natural compound.

Compound **4**, 56 mg, MW 928, under acid hydrolysis released hederagenin and α-L-arabinose and β-D-glucose, in the ratio 1:2. Spectroscopic characteristics of this saponin were consistent with those of 3-*O*-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]-28-*O*-[β-D-glucopyranoside] hederagenin, which was previously found in *Medicago arabica*[18] and *Medicago sativa*.[23]

Saponin **5**, 10 mg, was isolated as an amorphous solid; the molecular formula was determined as $C_{41}H_{66}O_{13}$ (MW 766). The acid hydrolysis of this saponin produced echinocystic acid and α-L-arabinose and β-D-glucose in the ratio 1:1. Two anomeric protons at δ 4.29 and 5.37 and carbons at δ 96.0 and 107.5 were evidenced in its ^{1}H and ^{13}C NMR spectra (Tables 1 and 2). In the HMBC experiments the anomeric signal at δ 4.29 ($H\text{-}1_{AraI}$) showed a long-range correlation with the signal at δ 91.0 (C-3), whereas a cross peak between the signal at δ 5.37 ($H\text{-}1_{GlcII}$) and the signal at δ 177.5 (C-28) was observed. This saponin was identified as 3-*O*-α-L-arabinopyranosyl-28-*O*-β-D-glucopyranosyl echinocystic acid (**5**) and represents a new identified compound in plants.

Compound **6**, 11 mg, MW 766, after acid hydrolysis released hederagenin and α-L-arabinose and β-D-glucose in the ratio of 1:1. Its spectroscopic characteristics were identical to those of 3-*O*-α-L-arabinopyranosyl-28-*O*-β-D-glucopyranoside hederagenin and already reported for *M. arabica*.[18]

Compound **7**, 28 mg, MW 942, after acid hydrolysis yielded a small amount of soyasapogenol B and soyasapogenols C, D, and F as byproducts.[24] α-L-Rhamnose, β-D-galactose, and β-D-glucuronic acid in the ratio 1:1:1 were identified as the sugar constituents after acid hydrolysis. On the basis of these characteristics and spectroscopic data, saponin **7** was identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl (1→2)-β-D-glucuronopyranoside] soyasapogenol B, also known as soyasaponin I. It is often found as a constituent of saponins in the Leguminosae family, and it was previously detected in several other *Medicago* spp.[8]

Compound **8**, 14 mg, MW 620: the acid hydrolysis of this saponin gave caulophyllogenin and α-L-arabinose. This compound was identified as 3-*O*-α-L-arabinopyranosyl caulophyllogenin, and it was previously described in *Caulophyllum thalictroides* (L.) Michx.[19] It has never been reported in the genus *Medicago* before this investigation.

dx.doi.org/10.1021/jf2005854 |*J. Agric. Food Chem.* 2011, 59, 6142–6149

Table 2. [1]H NMR Data of the Aglycone and Monosaccharide Moieties of Saponins 3 and 5 from *M. polymorpha*[a]

	δ, CD_3OD				
	aglycone			monosaccharide	
H	3	5	H	3	5
1	1.07 and 1.65, 2H[b]	1.05 and 1.61, 2H[b]		Ara(I)	Ara(I)
2	1.82 and 1.95, 2H[b]	1.82 and 1.94, 2H[b]	1	4.29, d (6.5)	4.29, d (6.4)
3	3.15, 1H, dd (12.0, 4.0)	3.10, 1H, dd (12.0, 4.0)	2	3.57, dd (6.5, 7.0)	3.56, dd (6.4, 7.0)
4	–	–	3	3.33[b]	3.32[b]
5	0.75, 1H[b]	0.75, 1H[b]	4	3.72 br s	3.72 br s
6	1.53 and 1.40, 2H[b]	1.52 and 1.39, 2H[b]	5a	3.56, d (10.7)	3.55, d (10.8)
7	1.19 and 1.37, 2H[b]	1.20 and 1.37, 2H[b]	5b	3.84[b]	3.82[b]
8	–	–			
9	1.71, 1H[b]	1.71, 1H[b]		Glc(II)	Glc(II)
10	–	–	1	5.34, d (7.9)	5.37, d (7.9)
11	1.68 and 1.91, 2H[b]	1.69 and 1.91, 2H[b]	2	3.21, dd (7.9, 8.1)	3.50, dd (7.9, 8.0)
12	5.32, 1H, t (3.0)	5.34, 1H, t (3.0)	3	3.38, t (8.1)	3.42, t (8.0)
13	–	–	4	3.41, t (8.1)	3.29, t (8.0)
14	–	–	5	3.50, m	3.26, m
15	1.71 and 1.88, 2H[b]	1.71 and 1.89, 2H[b]	6a	3.78, dd (11.8, 5.0)	3.67, dd (11.9, 5.0)
16	4.54, 1H, br s	4.55, 1H, br s	6b	4.15, dd (11.8, 3.0)	3.89, dd (11.9, 3.0)
17	–	–			
18	3.03, 1H, dd (14.0, 4.0)	3.01, 1H, dd (14.0, 4.0)		Glc(III)	
19	1.08 and 1.55, 2H[b]	1.07 and 1.56, 2H[b]	1	4.35, d (7.7)	
20	–	–	2	3.52, dd (7.7, 8.0)	
21	1.75−1.95, 2H[b]	1.77−1.95, 2H[b]	3	3.42, t (8.0)	
22	1.38 and 1.53, 2H[b]	1.40 and 1.55, 2H[b]	4	3.28, t (8.0)	
23	1.06, 3H, s	1.06, 3H, s	5	3.25, m	
24	0.86, 3H, s	0.86, 3H, s	6a	3.67, dd (11.9, 5.1)	
25	0.98, 3H, s	0.99, 3H, s	6b	3.89, dd (11.9, 3.0)	
26	0.80, 3H, s	0.81, 3H, s			
27	1.38, 3H, s	1.39, 3H, s			
28	–	–			
29	0.90, 3H, s	0.90, 3H, s			
30	0.99, 3H, s	0.99, 3H, s			

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

Compound **9**, 13 mg, MW 620, after acid hydrolysis released bayogenin and α-L-arabinose. Its spectroscopic features matched those of 3-*O*-α-L-arabinopyranosyl bayogenin. It was already isolated from *M. arabica*.[18]

Compound **10**, 100 mg, MW 750, released after acid hydrolysis hederagenin and α-L-arabinose and α-L-rhamnose in the ratio 1:1. This saponin had spectroscopic characteristics identical to those of 3-*O*-[α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranosyl] hederagenin, which was previously found in *M. polymorpha*.[14]

Compound **11**, 46 mg, MW 604, released after acid hydrolysis echinocystic acid and α-L-arabinose, and its spectroscopic features were identical to those of 3-*O*-α-L-arabinopyranosyl echinocystic acid. This saponin was previously characterized in *Tetrapanax papyriferum* (Hook.) Koch,[20] but it is here reported for the first time in *Medicago*.

Compound **12**, 17 mg, MW 604, released after acid hydrolysis hederagenin and α-L-arabinose and had spectroscopic characteristics identical to those of 3-*O*-α-L-arabinopyranosyl hederagenin. This saponin was previously identified in *M. arabica*.[18]

Quantitative evaluation of saponins and sapogenins from the leaves of the two cultivars of *M. polymorpha* was achieved by GC and HPLC analyses, respectively. GC-FID and GC-MS analyses of derivatized sapogenins, obtained by acid hydrolyses of the purified saponin fractions, showed the presence of hederagenin (6.70 ± 0.43 mg/g dry matter) as the dominant aglycone in cv. 'Santiago' and echinocystic acid (7.48 ± 0.82 mg/g dry matter) as the dominant aglycone in cv. 'Anglona'. Caulophyllogenin, bayogenin, and soyasapogenol B were also detected in both cultivars, but in lower amount (Table 3). Remarkable compositional differences are also evident between the two cultivars of *M. polymorpha* if the pattern of individual saponin concentrations is considered (Table 4). Monodesmosidic compounds (saponins **7**−**12**) were present in higher amount in cv. 'Santiago' than in cv. 'Anglona', totally amounting to 8.19 and 3.09 mg/g dry matter, respectively. The reverse was true for the bidesmosides (saponins **1**−**6**), totally accounting for 11.88 mg/g dry matter in cv. 'Anglona' and 3.27 mg/g dry matter in cv. 'Santiago'.

dx.doi.org/10.1021/jf2005854 |*J. Agric. Food Chem.* 2011, 59, 6142–6149

Table 3. Individual Sapogenin Concentrations in Two *M. polymorpha* Cultivars[a]

	mg/g dry weight	
compound	'Anglona'	'Santiago'
echinocystic acid (**I**)	7.48 ± 0.82	0.02 ± 0.01
hederagenin (**II**)	0.95 ± 0.15	6.70 ± 0.43
soyasapogenol B (**III**)	0.20 ± 0.07	0.18 ± 0.05
caulophyllogenin (**IV**)	0.45 ± 0.09	0.01 ± 0.01
bayogenin (**V**)	0.15 ± 0.02	0.09 ± 0.02

[a] The structures of aglycones **I**−**V** are reported in Figure 2.

Table 4. Individual Saponin Concentrations in Two *M. polymorpha* Cultivars[a]

	mg/g dry weight	
compound	'Anglona'	'Santiago'
1	0.78 ± 0.18	0.05 ± 0.03
2	0.65 ± 0.04	2.98 ± 0.11
3	8.12 ± 0.55	0.23 ± 0.04
4	1.95 ± 0.32	–
5	0.07 ± 0.02	0.01 ± 0.00
6	0.31 ± 0.04	–
7	0.23 ± 0.10	0.35 ± 0.07
8	0.48 ± 0.12	0.07 ± 0.01
9	0.35 ± 0.09	0.04 ± 0.02
10	0.41 ± 0.07	7.52 ± 0.41
11	1.57 ± 0.19	0.12 ± 0.04
12	0.05 ± 0.03	0.09 ± 0.02

[a] The structures of compounds **1**−**12** are reported in Figure 2.

The most abundant saponin detected in 'Santiago' cultivar was the monodesmoside **10** (7.52 ± 0.41 mg/g dry matter) followed by the bidesmoside **2** (2.98 ± 0.11 mg/g dry matter), whereas in 'Anglona' the most abundant saponins were the bidesmosides **3** (8.12 ± 0.55 mg/g dry matter) and **4** (1.95 ± 0.32 mg/g dry matter) followed by the monodesmoside **11** (1.57 ± 0.19 mg/g dry matter).

Saponins **4** and **6** were not present in cv. 'Santiago', whereas the relative abundance of other saponins varied between the two cultivars. The concentration of saponins **10** and **2** was much higher in 'Santiago' than in 'Anglona', but the reverse was true for the concentration of saponins **3** and **11**.

Our results clearly reveal a different phytochemical profile between the two studied cultivars of *M. polymorpha*, indicating a peculiar biosynthetic capacity of cv. 'Anglona' to synthesize echinocystic acid, which has never been reported until now in any *Medicago* species. Moreover, echinocystic acid represents the aglycone of only one saponin isolated from cv. 'Santiago', which, in contrast to cv. 'Anglona' has a more diversified profile of aglycones. Concerning the sugar portion, all of the identified saponins, except soyasaponin **I** (saponin **7**), have α-L-arabinose as the first monosaccharide unit directly linked at the 3-*O* position of the aglycone moiety, with β-D-glucose or α-L-rhamnose eventually linked 1→2 to α-L-arabinose. Bidesmosidic compounds are instead characterized by the presence of β-D-glucose only as the first sugar linked at the 28-*O* position and, when present, the second β-D-glucose unit is linked 1→6 to the first sugar. As previously reported,[9] the presence of the 1→6 interglycosidic linkage, unique in the saponins from the genus *Medicago*, is further supported by the analysis of *M. polymorpha*, confirming the presence of specific glycosyltransferases in this genus. Four saponins (**4**, **6**, **9**, and **12**) identified in *M. polymorpha* were also reported in *M. arabica*.[12,18] A genetic affinity between these medic species is corroborated by their belonging to the same Leptospiræ section of the *Medicago* genus[2] and the close resemblance of their isoenzymatic profiles.[25]

This being the first assessment of saponin content in two different cultivars of *M. polymorpha*, we may speculate that the observed differences between the two analyzed cultivars (in terms of both sapogenin and saponin concentrations) also relate to the different botanical varieties to which 'Anglona' [var. *vulgaris* (Benth.) Shinners] and 'Santiago' [var. *brevispina* (Benth.) Heyn] belong. Other investigations are needed with larger germplasm samples to verify whether *M. polymorpha* varieties *brevispina* and *vulgaris* (and, possibly, *polymorpha* as well) have evolutionarily developed different compositions of triterpenoid glycosides.

Differences in saponin composition can also be involved in the use of these two varieties as a feed. Although there are no specific data on the biological activity of *M. polymorpha* saponins, the presence of a higher amount of monodesmosidic compounds in cv. 'Santiago' could represent a negative trait for animal feeding, as these compounds are reported to be in general more biologically active compared to the corresponding bidesmosides,[8] which are most abundant in the cv. 'Anglona'.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 0371 404740. Fax: +39 0371 31853. E-mail: aldo.tava@entecra.it.

REFERENCES

(1) Piano, E.; Pecetti, L. Minor legume species. In *Fodder Cops and Amenity Grasses. Handbook of Plant Breeding Series*; Boller, B., Posselt, U. K., Veronesi, F., Eds.; Springer: New York, **2010**; Vol. *5*, pp 477−500.

(2) Lesins, K. A. ; Lesins, I. *Genus Medicago (Leguminosae). A Taxogenetic Study*; Dr. W. Junk Publishers: The Hague, The Netherlands, **1979**; pp 196−199.

(3) Porqueddu, C.; Sulas, L.; Rivoira, G. L'impiego di medica polimorfa per migliorare i pascoli mediterranei. *Inform. Agric.* **1996**, *53*, 35–40.

(4) Prosperi, J. M.; Angevain, M.; Bonnin, I.; Chaulet, E.; Genier, G.; Jenczewski, E.; Olivieri, I.; Ronfort, J. Genetic diversity, preservation and use of genetic resources of Mediterranean legumes: alfalfa and medics. In *The Genus Medicago in the Mediterranean Region: Current Situation and Prospects in Research*; Genier, G., Prosperi, J. M., Eds.; Options Méditerranéennes 18; CIHEAM: Zaragoza, Spain, 1996; pp 71−89.

(5) Piano, E.; Francis, C. M. The annual species of *Medicago* in the Mediterranean region. Ecogeography and related aspects of plant introduction and breeding. In *The Future of Lucerne. Biotechnology, Breeding and Variety Constitution*; Rotili, P., Zannone, L., Eds.; ISCF: Lodi, Italy, 1992; pp 373−385.

(6) Cocks, P. S.; Phillips, J. R. Evolution of subterranean clover in South Australia. I. The strains and their distribution. *Aust. J. Agric. Res.* **1979**, *30*, 1035–1052.

(7) Cocks, P. S. Changes in the size and composition of the seed bank of medic pastures grown in rotation with wheat in north Syria. *Aust. J. Agric. Res.* **1992**, *43*, 1571–1581.

(8) Tava, A.; Avato, P. Chemical and biological activity of triterpene saponins from *Medicago* species. *Nat. Prod. Commun.* **2006**, *1*, 1159–1180.

dx.doi.org/10.1021/jf2005854 |*J. Agric. Food Chem.* 2011, 59, 6142–6149

(9) Tava, A.; Scotti, C.; Avato, P. Biosynthesis of saponins in the genus *Medicago*. *Phytochem. Rev.* **2010**, DOI: 10.1007/s11101-010-9169-x.

(10) Huhman, D. V.; Berhow, M. A.; Sumner, L. W. Quantification of saponins in aerial and subterranean tissues of *Medicago truncatula*. *J. Agric. Food Chem.* **2005**, *53*, 1914–1920.

(11) Kapusta, I.; Bogdan, J.; Stochmal, A.; Oleszek, W. Determination of saponins in aerial parts of barrel medic (*Medicago truncatula*) by liquid chromatography−electrospray ionization/mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 7654–7660.

(12) Pecetti, L.; Biazzi, E.; Tava, A. Variation in saponin content during the growing season of spotted medic [*Medicago arabica* L. (Huds.)]. *J. Sci. Food Agric.* **2010**, *90*, 2405–2410.

(13) D'Addabbo, T.; Carbonara, T.; Leonetti, P.; Radicci, V.; Tava, A.; Avato, P. Control of plant parasitic nematodes with active saponins and biomass from *Medicago sativa*. *Phytochem. Rev.* **2010**, DOI: 10.1007/s11101-010-9180-2.

(14) Kinjo, J.; Uemura, H.; Nakamura, M.; Nohara, T. Two new triterpenoidal glycosides from *Medicago polymorpha* L. *Chem. Pharm. Bull.* **1994**, *42*, 1339–1341.

(15) Tava, A.; Oleszek, W.; Jurzysta, M.; Berardo, N.; Odoardi, M. Alfalfa saponins and sapogenins: isolation and quantification in two different cultivars. *Phytochem. Anal.* **1993**, *4*, 269–274.

(16) Tava, A.; Mella, M.; Avato, P.; Argentieri, M. P.; Bialy, Z.; Jurzysta, M. Triterpenoid glycosides from leaves of *Medicago arborea* L. *J. Agric. Food Chem.* **2005**, *53*, 9954–9965.

(17) Bialy, Z.; Jurzysta, M.; Mella, M.; Tava, A. Triterpene saponins from the roots of *Medicago hybrida*. *J. Agric. Food Chem.* **2006**, *54*, 2520–2526.

(18) Tava, A.; Mella, M.; Avato, P.; Biazzi, E.; Pecetti, L.; Bialy, Z.; Jurzysta, M. New triterpenic saponins from the aerial parts of *Medicago arabica* (L.) Huds. *J. Agric. Food Chem.* **2009**, *57*, 2826–2835.

(19) Jhoo, J. W.; Sang, S.; He, K.; Cheng, X.; Zhu, N.; Stark, R. E.; Zheng, Q. Y.; Rosen, R. T.; Ho, C. T. Characterization of the triterpene saponins of the roots and rhizomes of blue cohosh (*Caulophyllum thalictroides*). *J. Agric. Food Chem.* **2001**, *49*, 5969–5974.

(20) Strigunov, V. S.; Grishkovets, V. I.; Shashkov, A. S.; Chirva, V. Y. Triterpene glycosides from *Tetrapanax papyriferum*. III. Minor monodesmoside glycosides from stem bark. *Chem. Nat. Compd.* **2001**, *37*, 462–465.

(21) Nagao, T.; Tanaka, R.; Shimokawa, H.; Okabe, H. Studies on the constituents of *Aster scaber* Thunb. II. Structures of echinocystic acid glycosides isolated from the roots. *Chem. Pharm. Bull.* **1991**, *39*, 1719–1725.

(22) Cioffi, G.; Vassallo, A.; Lepore, L.; Venturella, F.; Dal Piaz, F.; Tommasi, N. Antiproliferative oleanane saponins from *Polyscias guilfoylei*. *Nat. Prod. Commun.* **2008**, *3*, 1667–1670.

(23) Bialy, Z.; Jurzysta, M.; Oleszek, W.; Piacente, S.; Pizza, C. Saponins in alfalfa (*Medicago sativa* L.) root and their structural elucidation. *J. Agric. Food Chem.* **1999**, *47*, 3185–3192.

(24) Mahato, S. B. Triterpenoid saponins from *Medicago hispida*. *Phytochemistry* **1991**, *30*, 3389–3393.

(25) Paredes, M.; Becerra, V.; Correa, P.; Del Pozo, A. Isoenzymatic diversity in accessions of *Medicago polymorpha* collected along an environmental gradient in Chile, and its relationship with other species of *Medicago*. *Rev. Chil. Hist. Nat.* **2000**, *73*, 479–488.