JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

New Triterpenic Saponins from the Aerial Parts of *Medicago arabica* (L.) Huds

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The reinvestigation of saponin composition from *Medicago arabica* from Italy allowed the detection of nineteen (**1**−**19**) saponins. All of them were purified by reverse-phase chromatography and their structures elucidated by spectroscopic and spectrometric (1D and 2D NMR; ESI-MS/MS) and chemical methods. Fourteen were known saponins, previously found in other plants including other *Medicago* species. They have been identified as glycosides of oleanolic acid, 2β,3β-dihydroxyolean-12-en-28-oic acid, hederagenin, bayogenin and soyasapogenol B. Five saponins, identified as 3-*O*-[-α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-30-*O*-β-D-glucopyranosyl 2β,3β,30-trihydroxyolean-12-en-28-oic acid (**1**), 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-30-*O*-[β-D-glucopyranosyl] 3β,30-dihydroxyolean-12-en-28-oic acid (**2**), 3-*O*-[β-D-glucuronopyranosyl]-30-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl] 2β,3β,30-trihydroxyolean-12-en-28-oic acid (**3**), 3-*O*-[β-D-glucuronopyranosyl]-30-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl] 3β,30-dihydroxyolean-12-en-28-oic acid (**4**) and 3-*O*-[β-D-glucuronopyranosyl]-30-*O*-[β-D-glucopyranosyl] 2β,3β,30-trihydroxyolean-12-en-28-oic acid (**5**), are reported here as new natural compounds. These new saponins, possessing a hydroxy group at the 30-methyl position of the triterpenic skeleton, have never been previously found in the genus *Medicago*.

KEYWORDS: ***Medicago arabica*** **L. Huds; saponins; chemical structure; triterpene glycosides; queretaroic acid; ESI-MS/MS; NMR**

INTRODUCTION

Medicago arabica (L.) Huds, commonly known as "spotted medic", is a wild annual legume species. Plants are 40−65 cm tall, profusely branched from the base, and each leaflet of the trifoliate leaves usually shows an anthocyanin-colored patch in the middle. This medic originates from, and is widespread in, the Mediterranean basin (*1*), and it has become naturalized in Australia (*2*).

Apart from its potential interest as a fodder crop (*2*), *M. arabica* also has interest for its content of saponins, which seems to contribute to the higher biological activity of this species compared to other *Medicago* species. In the genus *Medicago*, saponins are a complex mixture of triterpenic glycosides derived from medicagenic acid, zanhic acid, bayogenin, hederagenin and soyasapogenols. Saponins have been shown to possess a broad spectrum of biological properties, such as antinutritional, antifungal, insecticidal, phytotoxic, and hemolytic activity (*3*).

According to Jurzysta and Waller (*4*), *M. arabica* saponins have 50 times higher fungistatic activity than alfalfa (*M. sativa* L.) saponins. They are also strongly active against plant pathogenic fungi (*5*, *6*) and against dermatophytes (*7*, *8*). Moreover, *M. arabica* saponins were also very effective against Gram positive bacteria (*9*).

As first reported by Bialy et al. (*10*), investigations on the chemical structure of saponins from *M. arabica* led to the identification of hederagenin, bayogenin, oleanolic acid, 2β-hydroxyoleanolic acid and soyasapogenol B glycosides as the main compounds. Further studies on this species allowed the identification of a few additional saponins, with a new triterpene moiety not previously reported in the genus *Medicago* (*3*). The aim of the present work was to acquire further evidence of the previously reported compounds in *M. arabica*, and to elucidate the chemical structure of these newly identified saponins.

MATERIALS AND METHODS

Plant Material. A *M. arabica* population was collected in Siena, mid-Italy (43° 16′ N, 11° 21′ E, 275 m elevation) during spring 2006,

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10.1021/jf8036984 CCC: $40.75
Published on Web 03/03/2009

at the edge of a cereal field, on a clay soil. Fresh aerial vegetation was collected from many different plants in a sampling area of about 20 m^2. Plants were in an early blossom stage. The plant material was dried at 40 °C, ground and used for successive extractions of saponins.

Extraction and Purification. Powdered leaves (500 g) were defatted with $CHCl_3$ in a Soxhlet apparatus (fats 4.3% DM). Defatted material (200 g) was then extracted with 80% MeOH under reflux for 24 h. The solvent was removed under reduced pressure, and the residue resuspended in water. 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 35% MeOH (1 L) to remove sugars and some phenolics; crude saponins (7.2 g; 3.6% yield) were then eluted with 90% MeOH (500 mL) and dried under vacuum.

Fractionation. Total crude saponins (5 g) 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: 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 (1.7 g) and III (2.4 g) contained saponins and were therefore used for the subsequent analytical study. Saponins previously purified from *M. arabica* leaves were used as reference compounds (*10*).

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. 100 μL of MeOH:H_2O (9:1) solutions (30 mg/mL) of each fraction were injected. Saponins were eluted at 2.5 mL/min and detected by UV monitoring at 215 nm. The following saponins were obtained from fraction II under isocratic conditions of 34% A: **1** (37 mg), **2** (22 mg), **3** (31 mg), **4** (18 mg), **5** (50 mg), **6** (11 mg), **7** (8 mg), **8** (7 mg), **9** (15 mg) and **10** (5 mg). Fraction III under isocratic conditions of 49% A afforded saponins **11** (51 mg), **12** (215 mg), **13** (86 mg), **14** (341 mg), **15** (6 mg), **16**, (28 mg), **17** (61 mg), **18** (42 mg) and **19** (28 mg).

HPLC Analyses. The crude mixture of saponins, fractions I−III and all the purified saponins were analyzed by HPLC using a Perkin-Elmer chromatograph equipped with a LC250 binary pump and 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 90% of solvent A in 50 min. Twenty microliters of methanolic solutions (1 mg/mL) of all samples were injected. Saponins were eluted at 1.0 mL/min and detected by UV monitoring at 215 nm.

Hydrolysis of Saponins. Saponin crude mixtures (5 mg) and each individual pure saponin (1 mg) were treated with 1 mL of 2 N HCl in 50% aqueous methanol in a stoppered test tube and stirred at 80 °C for 3 h. After cooling, methanol was eliminated with a stream of N_2 and aglycons were extracted with ethyl acetate (2 × 0.5 mL). Both the organic solutions, containing the aglycons, and the aqueous solution, containing sugars, were dried under N_2 and used for the successive analyses.

Analysis of Sapogenins. Aglycons were identified by TLC, GC/FID and GC/MS methods. Sapogenins were compared to previously identified sapogenins from *Medicago* spp. (*10*−*13*) 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 methyl−peracetyl and methyl−silyl derivatives. Aglycons 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 stirring overnight 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 (Norwalk, CT) 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, carrier gas He at 1.2 mL/min. Retention times and MS spectra were compared to those of previously identified sapogenins, except for the aglycon of saponins **1**−**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 (Fluka, Milano, Italy) spray 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 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 200 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 (0.2 μL) were injected in the "splitless" mode. Authentic reference compounds, treated in the same way as above, were used for identification of sugars. Coinjection of each hydrolysate with the standards gave single peaks. Sugar identification was also carried out by GC/MS as described in Tava et al. (*14*).

NMR Analyses. ^1H and ^{13}C NMR were measured on a Bruker 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 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 ^1H and ^{13}C signals were obtained.

ESI-MS/MS. Analyses were performed on a 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 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 of 4.0 *m*/*z*, 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).

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

Chemical Data of Saponins 1−19. Saponin **1**: mp 229−231 °C (dec), $[\alpha]_D^{25}$+37.5 (MeOH, *c* 0.32). Anal. Calcd for $C_{47}H_{74}O_{20}$: C, 58.86; H, 7.78. Found: C, 58.92; H, 7.80. ESI-MS (negative ion mode), *m*/*z* (relative intensity): 957.3 (100%) [M($C_{47}H_{74}O_{20}$) − H]$^-$, which fragmented in the MS/MS giving 939.4 (11%) [M − H_2O − H]$^-$; 913.4 (38%) [M − 44(−CO_2) − H]$^-$; 895.3 (58%) [M − 62(−CO_2−H_2O) − H]$^-$; 807.2 (86%) [M − 132(Ara) − H_2O − H]$^-$; 781.3(38%) [M − 176(GluA) − H]$^-$; 763.3 (86%) [M − 132 − (−CO_2−H_2O) − H/M − 176(GluA) − H_2O − H]$^-$; 649.2 (100%) [M − 176(GluA) − 132(Ara) − H]$^-$; 487.2 (14%) [2β-OHQuerAc − H]$^-$/[M − 176(GluA) − 132(Ara) − 162(Glc) − H]$^-$. Saponin **2**: mp 232−233 °C (dec), $[\alpha]_D^{25}$ +11.6 (MeOH, *c* 0.26). Anal. Calcd for $C_{47}H_{74}O_{19}$: C, 59.86; H, 7.91 Found: C, 59.84; H, 7.89. ESI-MS (negative ion mode), *m*/*z* (relative intensity): 941.4 (100%) [M($C_{47}H_{74}O_{19}$) − H]$^-$, which fragmented in the MS/MS giving 923.0 (2%) [M − H_2O − H]$^-$; 897.2 (6%) [M − 44(−CO_2) − H]$^-$; 879.2 (24%) [M − 62(−CO_2−H_2O) − H]$^-$; 791.2 (100%) [M − 132(Ara) − H_2O − H]$^-$; 747.1(19%) [M − 132(Ara) − 62(−CO_2−H_2O) − H/M − 176(GluA) − H_2O − H]$^-$; 633.2 (27%) [M − 176(GluA) +132(Ara) − H]$^-$; 471.1 (2%) [QuerAc − H]$^-$/[M − 176(GluA) − 132(Ara) − 162(Glc) − H]$^-$. Saponin **3**: mp 228−229 °C (dec), $[\alpha]_D^{25}$ +33.3 (MeOH, *c* 0.15). Anal. Calcd for $C_{47}H_{74}O_{20}$: C, 58.86; H, 7.78. Found: C, 58.85; H, 7.83. ESI-MS (negative ion mode), *m*/*z* (relative intensity): 957.4 (100%) [M($C_{47}H_{74}O_{20}$) − H]$^-$, which fragmented in the MS/MS giving 939.1 (62%) [M − H_2O − H]$^-$; 913.1 (23%) [M − 44(−CO_2) − H]$^-$; 895.2 (66%) [M − 62(−CO_2−H_2O) − H]$^-$; 825.2 (29%) [M − 132(Ara) − H]$^-$; 781.2 (100%) [M − 176(GluA) − H]$^-$; 763.2 (92%) [M − 132(Ara) – 62(−CO_2−H_2O) − H/M − 176(GluA) − H_2O − H]$^-$; 649.2(74%) [M − 176(GluA) + 132(Ara) − H]$^-$; 487.2 (15%) [2β-OHQuerAc − H]$^-$/[M − 176(GluA) − 132(Ara) − 162(Glc) − H]$^-$. Saponin **4**: mp 231−233 °C (dec), $[\alpha]_D^{25}$ +10.5 (MeOH, *c* 0.26). Anal. Calcd for $C_{47}H_{74}O_{19}$: C, 59.86; H, 7.91 Found: C, 59.80; H, 7.97. ESI-MS (negative ion mode), *m*/*z* (relative intensity) 941.4 (100%) [M($C_{47}H_{74}O_{19}$) − H]$^-$, which fragmented in the MS/MS giving 923.2 (100%) [M − H_2O − H]$^-$; 897.2 (11%) [M − 44(−CO_2) − H]$^-$; 879.1 (30%) [M − 62(−CO_2−H_2O) − H]$^-$; 765.2 (83%) [M − 176(GluA) − H]$^-$; 747.2 (74%) [M − 132(Ara) − 62(CO_2 − H_2O) − H/M − 176(GluA) − H_2O − H]$^-$; 633.2 (50%) [M − 176(GluA) + 132(Ara) − H]$^-$; 471.1 (11%) [QuerAc − H]$^-$/[M − 176(GluA) − 132(Ara) − 162(Glc) − H]$^-$. Saponin **5**: mp 214−215 °C (dec), $[\alpha]_D^{25}$ +34.5 (MeOH, *c* 0.32). Anal. Calcd for $C_{42}H_{66}O_{16}$: C, 61.00; H, 8.04. Found: C, 59.98; H, 8.01. ESI-MS (negative ion mode), *m*/*z* (relative intensity). 825.3 (100%) [M($C_{42}H_{66}O_{16}$) − H]$^-$, which fragmented in the MS/MS giving 807.0 (9%) [M − H_2O − H]$^-$; 781.1 (31%) [M − 44(−CO_2) − H]$^-$; 763.2 (90%) [M − 62(−CO_2−H_2O) − H]$^-$; 649.2 (100%) [M − 176(GluA) − H]$^-$; 601.1(7%) [M − 62(−CO_2−H_2O) − 162(Glc) − H]$^-$; 487.2 (7%) [2β-OHQuerAc − H]$^-$/[M − 176(GluA) − 162(Glc) − H]$^-$. Saponin **6**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 1059.2 (100%) [M($C_{52}H_{84}O_{22}$) − H]$^-$, which fragmented in the MS/MS giving 897.5 (100%) [M − 162(Glc) − H]$^-$; 765.2 (12%) [M − 162(Glc) − 132(Ara) − H]$^-$; 747.2 (10%) [M − 162(Glc) − 132(Ara) − H_2O − H]$^-$; 603.1 (5%) [M − 162(Glc) − 132(Ara) − 162(Ara) − H]$^-$; 585.2 (3%) [M − 162(Glc) − 132(Ara) − 162(Ara) − H_2O − H]$^-$; 471.3 (2%) [Hed − H]$^-$/[M − 162(Glc) − 132(Ara) − 162(Glc) − H]$^-$. Saponin **7**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 781.0 (100%) [M($C_{41}H_{66}O_{14}$) − H]$^-$, which fragmented in the MS/MS giving 619.5 (100%) [M − 162(Glc) − H]$^-$; 488.2 (10%) [Bayo − H]$^-$/[M − 162(Glc) − 132(Ara) − H]$^-$. Saponin **8**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 941.3 (100%) [M($C_{47}H_{74}O_{19}$) − H]$^-$, which fragmented in the MS/MS giving 779.2 (100%) [M − 162(Glc) − H]$^-$; 629.2 (52%) [M − 162(Glc) − 132(Ara) − H_2O − H]$^-$; 471.2 (21%) [2β-OHOlea − H]$^-$/[M − 162(Glc) − 132(Ara) − 176(GluA) − H]$^-$. Saponin **9**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 927.2 (100%) [M($C_{41}H_{76}O_{18}$) − H]$^-$, which fragmented in the MS/MS giving 765.2 (100%) [M − 162(Glc) − H]$^-$; 603.2 (2%) [M − 162(Glc) − 162(Glc) − H]$^-$; 471.1 (17%) [Hed − H]$^-$/[M − 162(Glc) − 162(Glc) − H]$^-$. Saponin **10**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 765.1 (100%) [M($C_{41}H_{66}O_{13}$) − H]$^-$, which fragmented in the MS/MS giving 603.0 (100%) [M − 162(Glc) − H]$^-$; 471.2 (45%) [Hed − H]$^-$/[M − 162(Glc) − 132(Ara) − H]$^-$. Saponin **11**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 941.7 (100%) [M($C_{48}H_{78}O_{18}$) − H]$^-$, which fragmented in the MS/MS giving 923.6 (100%) [M − H_2O − H]$^-$; 795.5 (13%) [M − 146(Rha) − H]$^-$; 733.5 (34%) [M − H_2O − 146(Rha) − CO_2 − H]$^-$; 632.6 (5%) [M − 146(Rha) − 162(Gal) − H]$^-$; 615.4 (36%) [M − 146(Rha) − 162(Gal) − H_2O − H]$^-$; 457.4 (30%) [SoyaB − H]$^-$/[M − 146(Rha) − 162(Gal) − 176(GluA) − H]$^-$. Saponin **12**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 897.3 (100%) [M($C_{46}H_{74}O_{17}$) − H]$^-$, which fragmented in the MS/MS giving 765.1 (100%) [M − 132(Ara) − H]$^-$; 747.2 (96%) [M − 132(Ara) − H_2O − H]$^-$; 603.2 (50%) [M − 132(Ara) − 162(Ara) − H]$^-$; 585.2 (25%) [M − 132(Ara) − 162(Ara) − H_2O − H]$^-$; 471.1 (10%) [Hed − H]$^-$/[M − 132(Ara) − 162(Glc) − 132(Ara) − H]$^-$. Saponin **13**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 765.4 (100%) [M($C_{41}H_{66}O_{13}$) − H]$^-$, which fragmented in the MS/MS giving 603.1 (100%) [M − 162(Glc) − H]$^-$; 471.1 (16%) [Hed − H]$^-$/[M − 162(Glc) − 132(Ara) − H]$^-$. Saponin **14**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 619.2 (100%) [M($C_{35}H_{56}O_9$) − H]$^-$, which fragmented in the MS/MS giving 487.0 (100%) [Bayo − H]$^-$/[M − 132(Ara) − H]$^-$. Saponin **15**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 779.3 (100%) [M($C_{41}H_{64}O_{14}$) − H]$^-$, which fragmented in the MS/MS giving 717.1(5%) [M − CO_2 − H_2O − H]$^-$; 629.1 (100%) [M − 132(Ara) − H_2O − H]$^-$; 603.1 (5%) [M − 176(GluA) − H]$^-$; 585.1 (27%) [M − 132(Ara) − CO_2 − H_2O − H]$^-$/[M − 176(GluA) − H_2O − H]$^-$; 471.2 (79%) [Hed − H]$^-$/[M − 132(Ara) − 176(GluA) − H]$^-$. Saponin **16**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 763.3 (100%) [M($C_{41}H_{64}O_{13}$) − H]$^-$, which fragmented in the MS/MS giving 701.2 (7%) [M − CO_2 − H_2O − H]$^-$; 613.2 (100%) [M − 132(Ara) − H_2O − H]$^-$; 587.1 (4%) [M − 176(GluA) − H]$^-$/[M − 132(Ara) − CO_2 − H]$^-$; 569.2 (11%) [M − 132(Ara) − CO_2 − H_2O − H]$^-$/[M − 176(GluA) − H_2O − H]$^-$; 455.2 (14%) [Olea − H]$^-$/[M − 132(Ara) − 1762(GluA) − H]. Saponin **17**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 603.2 (100%) [M($C_{35}H_{56}O_8$) − H]$^-$, which fragmented in the MS/MS giving 471.1 (100%) [Hed − H]$^-$/[M − 132(Ara) − H]$^-$. Saponin **18**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 647.3 (100%) [M($C_{36}H_{56}O_{10}$) − H]$^-$, which fragmented in the MS/MS giving 629.0 (3%) [M − H_2O − H]$^-$; 571.1 (12%) [M − Me − CO_2 − H_2O − H]$^-$; 471.1 (100%) [2β-OHOlea − H]$^-$/[M − 176(GluA) − H]$^-$. Saponin **19**: ESI-MS (negative ion mode), *m*/*z* (relative intensity): 631.3 (100%) [M($C_{36}H_{56}O_9$) − H]$^-$, which fragmented in the MS/MS giving 613.1 (33%) [M − H_2O − H]$^-$; 555.1 (60%) [M − Me − CO_2 − H_2O − H]$^-$; 455.2 (100%) [Olea − H]$^-$/[M − 176(GluA) − H]$^-$.

RESULTS AND DISCUSSION

GC−FID and GC−MS analyses of derivatized sapogenins, obtained by acid hydrolyses of saponin fractions, confirmed that bayogenin and hederagenin are the major aglycons of *M. arabica* saponins, representing 35.1% and 25.8%, respectively, together with 2β,3β-dihydroxyolean-12-en-28-oic acid (5.1%), oleanolic acid (3.4%), and soyasapogenols, altogether amounting to 1.8%. Two new sapogenins, further identified as 3β,30-dihydroxyolean-12-en-28-oic acid, named queretaroic acid (11.9%), and 2β,3β,30-trihydroxyolean-12-en-28-oic acid (3.6%), were also found. In agreement with Bialy et al. (*10*), neither medicagenic nor zanhic acids were found in the hydrolyzed *M. arabica* saponins.

Crude saponins obtained from the defatted leaves of *M. arabica* (3.6%) were analyzed by HPLC with a good peak resolution as shown in **Figure 1**. Crude saponins were further fractionated by reverse-phase (RP-18) chromatography (open column and preparative HPLC) yielding nineteen saponins **(1−19)** in a pure form, which were structurally elucidated by means of spectroscopic and chemical techniques. Aglycon 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 and to literature data (*10*−*14*). The presence of hederagenin in saponins **6**, **9**, **10**, **12**, **13**, **15** and **17**, bayogenin in saponins **7** and **14**, oleanolic acid in saponins **16** and **19**, 2β-hydroxy oleanolic acid in saponins **8** and **18**,

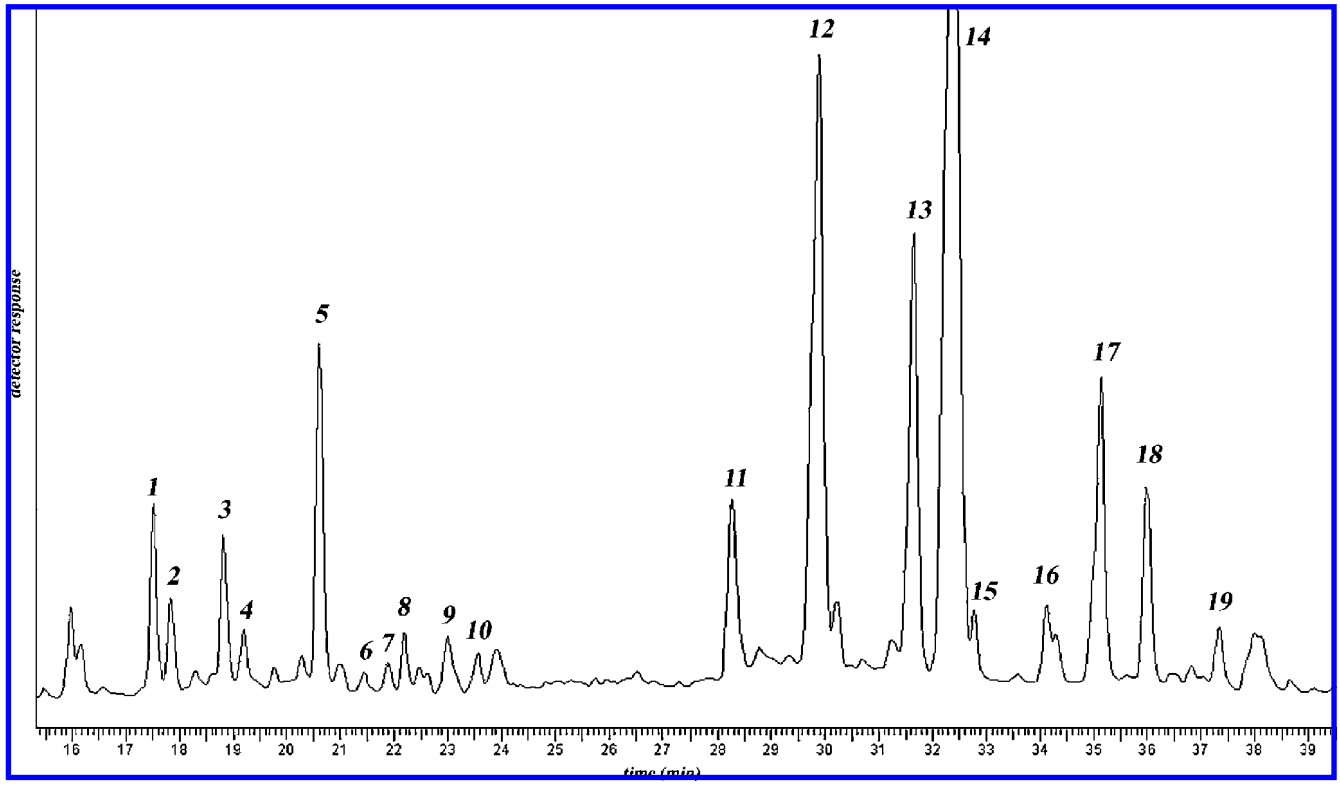


Figure 1. HPLC chromatogram of *Medicago arabica* saponins. The structures of compounds **1**−**19** are reported in **Figure 2**; for chromatographic conditions see Materials and Methods.

and soyasapogenol B in saponin **11** could be established. Two new aglycons, both containing a hydroxylated methyl group at C-30, were detected in saponins **1**, **3**, and **5**, and saponins **2** and **4**, respectively.

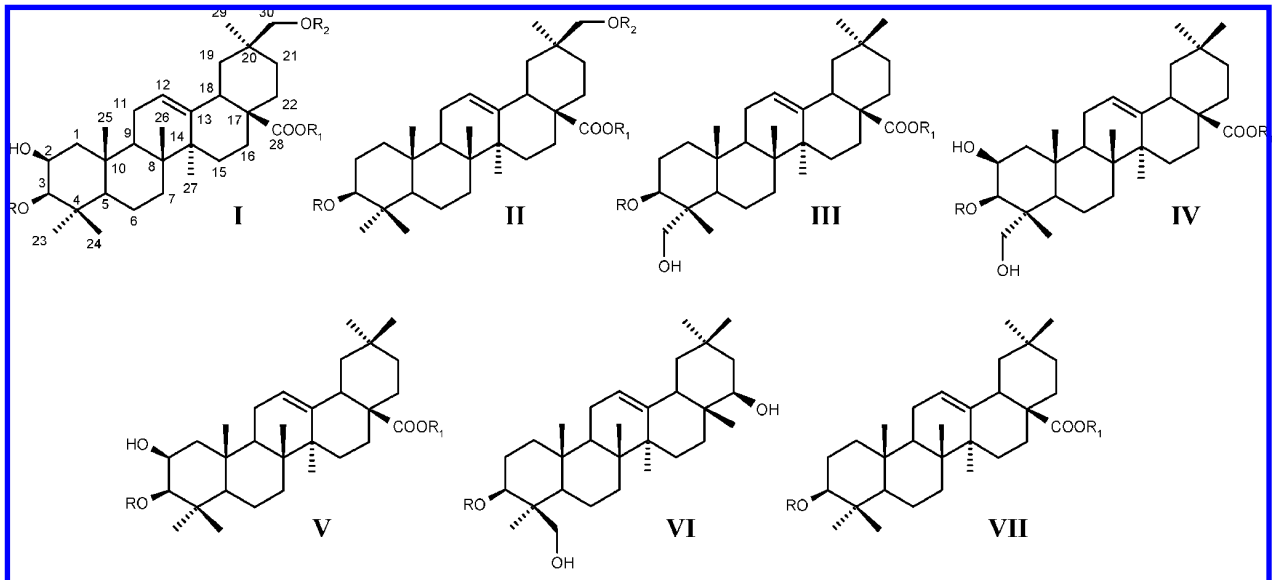
Sugar moieties were identified by means of TLC, comparing the R_f values with those of reference compounds. The absolute configuration of the sugar residues was obtained from GC analysis on a chiral column.

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**−**19** are reported in **Figure 2**, ^{13}C NMR chemical shifts of saponins **1**−**5** are reported in **Table 1**, and ^{1}H NMR data are reported in **Tables 2** and **3**. ESI-MS/MS data for all the isolated saponins **1**−**19** are reported in the text. All the monosaccharides were determined to be in the pyranose form from their ^{13}C NMR data, and by comparison with literature values (*10*−*13*, *15*).

Saponin **1**, 37 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{47}H_{74}O_{20}$ (MW 958). The acid hydrolysis of this saponin gave sapogenin I and α-L-arabinose, β-D-glucose and β-D-glucuronic acid in the ratio 1:1:1.

The ^{1}H NMR spectrum of the aglycon moiety of saponin 1 (**Table 2**) showed signals for six tertiary methyl groups at δ 0.83, 0.97, 1.09, 1.11, 1.18 and 1.26, that correlate in the HSQC experiments with the carbon signals at δ 18.09, 28.95, 18.59, 30.06, 26.74 and 17.14, respectively. A further feature was the signal at δ 5.33 (1H, t, J = 3.3) typical of H-12 of Δ^{12} oleanene skeleton, which was confirmed by the presence of the signals at δ 124.29 and δ 145.28 attributable to C-12 and C-13 in the ^{13}C NMR spectrum (**Table 1**). A signal at δ 182.15 and the carbon resonances of ring D in the ^{13}C NMR spectrum similar to those of oleanolic acid suggested the occurrence of a free carboxylic group at the C-28 position. The presence of two secondary alcoholic functions was also deduced from the signals at δ 3.21 (1H, t, J = 4.0) and δ 4.24 (1H, bq, J = 4.0), which correlated in the HSQC spectrum with the carbon resonances at δ 91.16 and δ 71.66, respectively. These signals are typical of ring A of a 2β-hydroxy substituted oleanolic acid (*10*−*13*, *15*, *16*). The stereochemistry of C-2 and C-3 in the triterpene structure was confirmed by 2D-NOESY experiments, in which a cross peak between H-2 (δ 4.24) and H-3 (δ 3.21) was observed, confirming the presence of a 2β,3β-dihydroxyoleanane skeleton. The NMR spectra of compound **1** contained one methyl group less than oleanolic acid and two coupled signals at δ 3.48 and 3.91 (2H, m) in the ^{1}H NMR spectrum that correlate to δ 74.63 in the ^{13}C NMR spectrum, suggesting that one of the methyl groups of the oleanolic acid was replaced by a hydroxymethyl group. This group was located at C-30 on the basis of the downfield shift (+4.0 ppm) exhibited by C-20 (δ 35.91) and the highfield shifts (−4.4, −4.2, −4.8) experienced, respectively, by C-19 (δ 43.17), C-21 (δ 30.60) and C-29 (δ 28.95) in comparison with the same carbon resonances in an oleanene skeleton bearing a Me-30 (*14*). More indications were from the ROESY spectrum, in which a correlation between the signal at δ 2.93 (H-18) and the double signal at δ 3.48 and 3.91 (CH_2-30) was clearly observed.

The ^{1}H and ^{13}C NMR spectra of saponin **1** (**Tables 1** and **3**) showed the presence of three anomeric protons at δ 4.58, 4.57 and 4.24 and carbons at δ 106.74, 105.35 and 105.18. 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 4.58 ($H\text{-}1_{GluAI}$) showed a long-range correlation with the signal at δ 91.16 (C-3), indicating that glucuronic acid is directly linked to the triterpenic



Compound	Aglycone	R	R_1	R_2
1	I	α-L-Ara(1→2)-β-D-GluA	-	β-D-Glc
2	II	α-L-Ara(1→2)-β-D-GluA	-	β-D-Glc
3	I	β-D-GluA	-	α-L-Ara(1→2)-β-D-Glc
4	II	β-D-GluA	-	α-L-Ara(1→2)-β-D-Glc
5	I	β-D-GluA	-	β-D-Glc
6	III	α-L-Ara(1→2)-β-D-Glc-(1→2)-α-L-Ara	β-D-Glc	-
7	IV	α-L-Ara	β-D-Glc	-
8	V	α-L-Ara(1→2)-β-D-GluA	β-D-Glc	-
9	III	β-D-Glc-(1→2)-α-L-Ara	β-D-Glc	-
10	III	α-L-Ara	β-D-Glc	-
11	VI	L-Rha-(1→2)-β-D-Gal-(1→2)-β-D-GluA	-	-
12	III	α-L-Ara(1→2)-β-D-Glc-(1→2)-α-L-Ara	-	-
13	III	β-D-Glc-(1→2)-α-L-Ara	-	-
14	IV	α-L-Ara	-	-
15	III	α-L-Ara(1→2)-β-D-GluA	-	-
16	VII	α-L-Ara(1→2)-β-D-GluA	-	-
17	III	α-L-Ara	-	-
18	V	β-D-GluA	-	-
19	VII	β-D-GluA	-	-

Figure 2. Structures of saponins **1**−**19** (I, 2β,3β,30-trihydroxyolean-12-en-28-oic acid; II, queretaroic acid; III, hederagenin; IV, bayogenin; V, 2β,3β-dihydroxyolean-12-en-28-oic acid; VI, soyasapogenol B; VII, oleanolic acid).

structure at C-3. The sugar linked to glucuronic acid is arabinose. The position of this sugar was indicated in the HMBC spectra in which the two anomeric protons at δ 4.58 ($H\text{-}1_{GluAI}$) and δ 4.57 ($H\text{-}1_{AraII}$) correlate with the same carbon ($C\text{-}2_{GluAI}$) at δ 83.28. A clear correlation between the signal at δ 4.24 ($H\text{-}1_{GlcIII}$) and the signal at δ 74.63 (C-30) was observed in the HMBC experiments, showing that glucose was instead linked to the triterpene skeleton at the C-30 position.

The GC/MS analyses of the methylated−silylated aglycon from saponin **1** showed more evidence of its triterpenic structure. The molecular ion $[M]^+$ was detected at *m*/*z* 718 (2%), which corresponds to $C_{40}H_{74}O_5Si_3$, from which the loss of an acetic acid unit gave *m*/*z* 658 (4%), and the loss of a trimethylsilanol gave *m*/*z* 628 (7%). The typical retro Diels−Alder fragmentation of the cyclic triterpene skeleton gave rise to the ions at *m*/*z* 367 (4%) and *m*/*z* 350 (7%). From the ion at *m*/*z* 367 the loss of trimethylsilanol gave *m*/*z* 278 (43%). The loss of a trimethylsilanol from the ion *m*/*z* 350 gave *m*/*z* 260 (31%), from which the loss of an acetic acid unit gave *m*/*z* 201 (72%). This finding is a characteristic diagnostic tool for the presence of a 12−13 double bond in triterpenes of the β-amyrin class (*17*, *18*). Additional ions originating by successive loss of a trimethylsilanol and an acetic acid

Table 1. ^{13}C NMR Data (δ, CD_3OD) of the Aglycon and Monosaccharide Moieties of Saponins **1**−**5** from *Medicago arabica*

aglycon						monosaccharide					
C	1	2	3	4	5	C	1	2	3	4	5
1	44.89	40.04	44.84	40.06	44.81		Glu Ac (I)	Glu Ac (I)	Glu Ac (I)	Glu Ac (I)	Glu Ac (I)
2	71.66	27.26	71.40	27.28	71.45	1	105.35	103.67	106.81	107.23	106.78
3	91.16	91.27	91.41	91.26	91.42	2	**83.28**	**84.42**	75.37	75.66	75.34
4	39.80	40.83	39.75	40.82	39.75	3	77.76	78.00	77.99	77.98	77.91
5	57.41	57.28	57.29	57.28	57.28	4	73.40	73.95	73.65	73.95	73.57
6	19.39	19.62	19.41	19.61	19.41	5	76.78	76.63	76.63	76.62	76.61
7	34.33	34.30	34.30	34.28	34.30	6	173.95	173.55	173.83	173.51	173.33
8	40.93	40.47	40.93	40.46	40.94						
9	49.63	49.62	49.63	49.59	49.62		Ara (II)	Ara (II)			
10	37.98	38.18	38.01	38.17	38.01	1	106.74	106.85			
11	24.71	24.79	24.71	24.43	24.70	2	74.04	73.75			
12	124.29	124.11	124.21	124.09	124.27	3	74.44	74.25			
13	145.28	145.36	145.39	145.36	145.29	4	69.97	69.73			
14	43.32	43.15	43.33	43.15	43.32	5	67.80	67.60			
15	29.08	29.15	29.09	29.17	29.08						
16	24.89	24.93	24.90	24.69	24.89		Glc (III)	Glc (III)	Glc (II)	Glc (II)	Glc (II)
17	47.66	47.94	47.66	47.93	47.66	1	105.18	107.22	103.66	103.67	105.18
18	42.19	42.11	42.13	42.10	42.19	2	75.58	75.67	**84.40**	**84.42**	75.58
19	43.17	43.02	43.00	43.00	43.18	3	78.21	77.99	77.98	78.01	78.21
20	35.91	35.87	35.87	35.87	35.91	4	72.10	71.97	72.00	71.95	72.10
21	30.60	30.44	30.45	30.42	30.60	5	78.46	78.06	78.08	78.06	78.46
22	33.89	33.81	33.81	33.80	33.89	6	63.23	63.12	63.15	63.11	63.23
23	30.06	29.05	30.21	29.06	30.21						
24	18.59	17.26	19.41	17.26	19.41				Ara (III)	Ara (III)	
25	17.14	16.24	17.14	16.24	17.14	1			106.74	106.84	
26	18.09	18.02	18.08	18.01	18.08	2			73.95	73.76	
27	26.74	26.67	26.73	26.67	26.74	3			74.25	74.24	
28	182.15	182.11	182.11	182.12	182.15	4			69.72	69.73	
29	28.95	28.76	29.06	28.76	28.95	5			67.58	67.61	
30	74.63	74.62	73.88	73.84	74.62						

Table 2. ^1H NMR Data (δ, CD_3OD) of the Aglycon Moieties of Saponins **1**−**5**[a]

H	1	2	3	4	5
1	1.20 and 2.05, 2H[b]	1.12 and 1.68, 2H[b]	1.21 and 2.06, 2H[b]	1.12 and 1.68, 2H[b]	1.21 and 2.07, 2H[b]
2	4.24, 1H, bq (4.0)	1.71 and 1.98, 2H[b]	4.23, 1H, bq (4.0)	1.72 and 1.98, 2H[b]	4.22, 1H, bq (4.0)
3	3.21, 1H, d (4.0)	3.27, 1H, dd (14.0, 4.0)	3.21, 1H, d (4.0)	3.28, 1H, dd (14.0, 4.0)	3.20, 1H, d (4.0)
4	–	–	–	–	–
5	0.86, 1H, dd (12.0, 4.0)	0.90, 1H, dd (12.0, 4.0)	0.92, 1H, dd (12.0, 4.0)	0.89, 1H, dd (12.0, 4.0)	0.91, 1H, dd (12.0, 4.0)
6	1.49 and 1.62, 2H[b]	1.51 and 1.64, 2H[b]	1.49 - 1.60, 2H[b]	1.50 and 1.64, 2H[b]	1.49 and 1.62, 2H[b]
7	1.26−1.33, 2H[b]	1.25 and 1.36, 2H[b]	1.25 and 1.37, 2H[b]	1.25 and 1.38, 2H[b]	1.23 and 1.37, 2H[b]
8	–	–	–	–	–
9	1.56, 1H[b]	1.56, 1H[b]	1.55, 1H[b]	1.57, 1H[b]	1.57, 1H[b]
10	–	–	–	–	–
11	1.61 and 2.03, 2H[b]	1.60 and 2.02, 2H[b]	1.59 and 2.04, 2H[b]	1.60 and 2.03, 2H[b]	1.59 and 2.05, 2H[b]
12	5.33, 1H, t (3.3)	5.31, 1H, t (3.4)	5.33, 1H, t (3.4)	5.32, 1H, t (3.4)	5.33, 1H, t (3.4)
13	–	–	–	–	–
14	–	–	–	–	–
15	1.09 and 1.78, 2H[b]	1.11 and 1.81, 2H[b]	1.08 and 1.76, 2H[b]	1.10 and 1.79, 2H[b]	1.08 and 1.78, 2H[b]
16	1.88−2.01, 2H[b]	1.90−2.00, 2H[b]	1.89−2.01, 2H[b]	1.90−2.00, 2H[b]	1.88−2.00, 2H[b]
17	–	–	–	–	–
18	2.93, 1H, dd (14.0, 4.0)	2.94, 1H, dd (14.0, 4.0)	2.91, 1H, dd (14.0, 4.0)	2.90, 1H, dd (14.0, 4.0)	2.94, 1H, dd (14.0, 4.0)
19	1.41 and 1.62, 2H[b]	1.42 and 1.62, 2H[b]	1.43 and 1.62, 2H[b]	1.42 and 1.61, 2H[b]	1.42 and 1.60, 2H[b]
20	–	–	–	–	–
21	1.32 and 1.60, 2H[b]	1.33 and 1.63, 2H[b]	1.33 and 1.61, 2H[b]	1.32 and 1.63, 2H[b]	1.32 and 1.61, 2H[b]
22	1.40−1.55, 2H[b]	1.38−1.55, 2H[b]	1.41−1.53, 2H[b]	1.38−1.52, 2H[b]	1.40−1.54, 2H[b]
23	1.11, 3H, s	0.96, 3H, s	1.09, 3H, s	0.98, 3H, s	1.09, 3H, s
24	1.09, 3H, s	0.97, 3H, s	1.09, 3H, s	0.98, 3H, s	1.08, 3H, s
25	1.26, 3H, s	1.18, 3H, s	1.28, 3H, s	1.18, 3H, s	1.26, 3H, s
26	0.83, 3H, s	0.82, 3H, s	0.84, 3H, s	0.82, 3H, s	0.84, 3H, s
27	1.18, 3H, s	1.08, 3H, s	1.18, 3H, s	1.09, 3H, s	1.18, 3H, s
28	–	–	–	–	–
29	0.97, 3H, s	0.83, 3H, s	0.97, 3H, s	0.87, 3H, s	0.97, 3H, s
30	3.48, 1H, d (11.5) and 3.91, 1H, d (11.5)	3.51, 1H, d (11.5) and 3.93, 1H, d (11.5)	3.52, 1H, d (11.5) and 3.90, 1H, d (11.5)	3.49, 1H, d (11.5) and 3.89, 1H, d (11.5)	3.46, 1H, d (11.5) and 3.81, 1H, d (11.5)

[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.

unit from the ion at *m*/*z* 337 (not detected) from the cleavage of the central ring of the β-amyrin structure were detected at *m*/*z* 247 (95%) and *m*/*z* 187 (34%). The GC/MS spectrum of the methylated−peracetylated derivative gave similar results.

This saponin was identified as 3-*O*-[-α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-30-*O*-β-D-glucopyranosyl 2β,3β,30-trihydroxyolean-12-en-28-oic acid (**1**) and represents a new identified compound in this plant material.

Table 3. ^{1}H NMR Data (δ, CD_3OD) of the Saccharide Moieties of Saponins **1**−**5**[a]

H	1	2	3	4	5
	GluA (I)	GluA (I)	GluA (I)	GluA (I)	GluA (I)
1	4.58, d (7.2)	4.50, d (7.2)	4.43, d (7.1)	4.38, d (7.1)	4.45, d (7.2)
2	3.65[b]	3.64[b]	3.38[b]	3.39[b]	3.39[b]
3	3.64[b]	3.62[b]	3.41[b]	3.41[b]	3.42[b]
4	3.63[b]	3.61[b]	3.52[b]	3.54[b]	3.53, t (9.1)
5	3.81[b]	3.83[b]	3.80[b]	3.80[b]	3.81, d (9.1)
6	−	−	−	−	−
	Ara (II)	Ara (II)			
1	4.57, d (2.1)	4.48, d (2.1)			
2	3.68[b]	3.66[b]			
3	3.61[b]	3.60[b]			
4	3.81[b]	3.79[b]			
5	3.55, 3.89[b]	3.52, 3,90[b]			
	Glc (III)	Glc (III)	Glc (II)	Glc (II)	Glc (II)
1	4.24, d (7.5)	4.23, d (7.5)	4.38, d (7.4)	4.37, d (7.5)	4.24, d (7.5)
2	3.24[b]	3.23[b]	3.42[b]	3.41[b]	3.20[b]
3	3.37[b]	3.38[b]	3.58[b]	3.57[b]	3.38[b]
4	3.30[b]	3.31[b]	3.31[b]	3.33[b]	3.30[b]
5	3.32[b]	3.34[b]	3.30[b]	3.31[b]	3.32[b]
6	3.72, dd, (11.0, 5.0),3.91[b]	3.74, dd, (11.0, 5.0), 3.95[b]	3.73, dd, (11.0, 5.0), 3.92[b]	3.73, dd, (11.0, 5.0), 3.90[b]	3.72, dd, (11.0, 5.0), 3.91, dd (11.0, 5.0)
			Ara (III)	Ara (III)	
1			4.46, d (2.0)	4.46, d (2.1)	
2			3.67[b]	3.65[b]	
3			3.60[b]	3.58[b]	
4			3.82[b]	3.81[b]	
5			3.56, 3.92[b]	3.55, 3.91[b]	

[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.

Saponin **2**, 22 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{47}H_{74}O_{19}$ (MW 942). The acid hydrolysis of this saponin gave sapogenin II and α-L-arabinose, β-D-glucose and β-D-glucuronic acid in the ratio 1:1:1.

The NMR spectra of the aglycon moiety of this saponin showed some similarities to that of saponin **1**. Six tertiary methyl groups at δ 0.82, 0.83, 0.96, 0.97, 1.08 and 1.18, that correlate in the HSQC experiments with the carbon signals at δ 18.02, 28.76, 29.05, 17.26, 26.67 and 16.24, respectively, were detected. The presence of a 12−13 double bond, showing a typical signal at δ 5.31 (1H, t, $J = 3.4$) in the ^{1}H NMR spectrum and at δ 124.11 and δ 145.36 in the ^{13}C NMR spectrum and the free carboxylic group at the C-28 position at δ 182.11, were also detected. The same downfield shift (+4.0 ppm) exhibited by C-20 (δ 35.87) and the highfield shifts (−4.3, −4.0, −4.6) of C-19 (δ 43.02), C-21 (δ 30.44) and C-29 (δ 28.76), respectively, in comparison with the same carbon resonances in an oleanene skeleton bearing a Me-30 were observed, suggesting the presence of the hydroxyl function at C-30. In the ROESY spectrum, a correlation between the signal at δ 2.94 (H-18) and the two signals at δ 3.51 and 3.93 (CH_2-30) was clearly observed. Only one secondary alcoholic function at 3.27 in the ^{1}H NMR spectra, correlating with the signal at δ 91.27 in the ^{13}C NMR spectra, was observed instead, indicating the presence of the OH group at the C-3 position. These findings are in good agreement with data reported for 3β,30-dihydroxyolean-12-en-28-oic acid, named queretaroic acid (*19*−*21*).

The 2D NMR analysis of the sugar portion of saponin **2** revealed the presence of the same sugar chain at the C-3 position and the same monosaccharide at the C-30 position of the sapogenin as in compound **1**. In the HMBC experiment, H-1_{GluAI} (δ 4.50) gave a correlation with the C-3 at δ 91.27, and a cross peak between H-1_{AraII} (δ 4.48) and C-2_{GluAI} (δ 84.42) was revealed. A cross peak between H-1_{GlcIII} (δ 4.23) and C-30 (δ 74.62) was also observed.

The GC/MS spectrum of methylated−silylated aglycon from saponin **2** showed the molecular ion $[M]^+$ at *m*/*z* 630 (3%), which corresponds to $C_{37}H_{66}O_4Si_2$, from which the loss of an acetic acid unit gave *m*/*z* 570 (5%), and the loss of a trimethylsilanol gave *m*/*z* 540 (4%). The retro Diels−Alder fragmentation of the cyclic triterpene skeleton gave rise to the ions at *m*/*z* 279 (9%) and *m*/*z* 350 (5%). From the ion at *m*/*z* 279 the loss of trimethylsilanol gave *m*/*z* 190 (29%). The loss of a trimethylsilanol from the ion at *m*/*z* 350 gave *m*/*z* 260 (22%), which, by loss of an acetic acid unit, gave the ion at *m*/*z* 201 (80%). Additional ions from the cleavage of the central ring of the β-amyrin structure were at *m*/*z* 247 (93%) and *m*/*z* 187 (28%), originating from the ion at *m*/*z* 337 (not detected) by successive loss of a trimethylsilanol and an acetic acid unit (*17*,*18*). The GC/MS spectrum of the methylated−peracetylated derivative gave similar results.

This saponin was identified as a new saponin of queretaroic acid, named 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-30-*O*-[β-D-glucopyranosyl] 3β,30-dihydroxyolean-12-en-28-oic acid (**2**).

Saponin **3**, 31 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{47}H_{74}O_{20}$ (MW 958). The acid hydrolysis of this saponin gave sapogenin I and α-L-arabinose, β-D-glucose and β-D-glucuronic acid in the ratio 1:1:1.

This compound has the same aglycon moiety as saponin **1**, as shown by NMR spectra and GC/MS analyses of the hydrolysis products. Three anomeric signals were observed in the ^{13}C NMR spectrum at δ 103.66, 106.74 and 106.81 and at δ 4.38, 4.43 and 4.46 in the^{1}H NMR spectrum. The HMBC experiments showed the presence of a monosaccharide unit at the C-3 position and a sugar chain at the C-30 position. The

C-3 position of the triterpene was glycosylated with a glucuronic acid; its anomeric signal at δ 4.43 correlated indeed with C-3 at δ 91.41. The sequence of saccharide chains at the C-30 was also defined by HMBC experiments. A cross peak between C-30 (δ 73.88) and H-1_{GlcII} (δ 4.38) and a cross peak between C-2_{GlcII} (δ 84.40) and H-1_{AraIII} (δ 4.46) were revealed. This saponin was identified as 3-*O*-[β-D-glucuronopyranosyl]-30-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl] 2β,3β,30-trihydroxyolean-12-en-28-oic acid (**3**).

Saponin **4**, 18 mg, was isolated as an amorphous solid. The molecular formula was determined to be $C_{47}H_{74}O_{19}$ (MW 942). The acid hydrolysis of this saponin gave sapogenin II and α-L-arabinose, β-D-glucose and β-D-glucuronic acid in the ratio 1:1:1.

Spectroscopic data of the aglycon moiety of saponin **4** indicated the presence of the same genin as in saponin **2**, identified as queretaroic acid. The 2D NMR analysis of the sugar portion of this saponin revealed the presence of the same sugar chain at the C-3 position and the same monosaccharide unit at the C-30 position of the sapogenin as in compound **3**. Three anomeric signals were observed in the ^{13}C NMR spectrum at δ 103.67, 106.84 and 107.23 and at δ 4.37, 4.38 and 4.46 in the ^{1}H NMR spectrum. In the HMBC experiment H-1_{GluAI} (δ 4.38) gave a correlation with C-3 at δ 91.26. A cross peak between C-30 (δ 73.84) and H-1_{GlcII} (δ 4.37) and a cross peak between C-2_{GlcII} (δ 84.42) and H-1_{AraIII} (δ 4.46) were revealed. This saponin was identified as 3-*O*-[β-D-glucuronopyranosyl]-30-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl] queretaroic acid (**4**).

Saponin **5**, 50 mg, was isolated as an amorphous solid. The molecular formula was $C_{42}H_{66}O_{16}$ (MW 826). The acid hydrolysis of this saponin gave sapogenin I and glucose and glucuronic acid, in the ratio 1:1.

The same aglycon moiety as in saponin **1** was present from the NMR data. Two anomeric signals were observed in the ^{13}C NMR spectrum at δ 105.18 and 106.78 and at δ 4.24 and 4.45 in the^{1}H NMR spectrum. In the HMBC experiment the signal at δ 4.45 (H-1_{GluAI}) gave a correlation with C-3 at δ 91.42, and the signal at δ 4.24 (H-1_{GlcII}) with C-30 at δ 74.62. These findings allowed us to identify this saponin as 3-*O*-[β-D-glucuronopyranosyl]-30-*O*-[β-D-glucopyranosyl] 2β,3β,30-trihydroxyolean-12-en-28-oic acid (**5**).

Compound **6**, 11 mg, MW 1096, released hederagenin and α-L-arabinose and β-D-glucose, in the ratio 1:1 after acid hydrolysis. Spectroscopic characteristics of this saponin were consistent with those of 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]-28-O-[β-D-glucopyranoside] hederagenin which was previously found in *M. arabica* (*10*) and *M. sativa* (*11*).

Compound **7**, 8 mg, MW 782, released bayogenin and α-L-arabinose and β-D-glucose after acid hydrolysis, in the ratio 1:1, and had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl]-28-*O*-[β-D-glucopyranoside] bayogenin. This saponin was previously isolated from *M. arabica* (*10*).

Compound **8**, 7 mg, MW 942, released 2β-hydroxy oleanolic acid and α-L-arabinose, β-D-glucose and β-D-glucuronic acid in the ratio 1:1:1 after acid hydrolysis. This saponin had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranoside]-2β-hydroxy oleanolic acid and already reported in *M. arabica* (*10*).

Compound **9**, 15 mg, MW 928, released hederagenin and α-L-arabinose and β-D-glucose in the ratio 1:2 after acid hydrolysis. This saponin had spectroscopic characteristics matching those of 3-*O*-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]-28-*O*-[β-D-glucopyranoside] hederagenin, and was previously isolated from *M. arabica* (*10*) and *M. sativa* (*11*).

Compound **10**, 5 mg, MW 766, released hederagenin and α-L-arabinose and β-D-glucose after acid hydrolysis in the ratio 1:1. This saponin had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl]-28-*O*-[β-D-glucopyranoside] hederagenin, which was previously found in *M. arabica* (*10*).

Saponin **11**, 51 mg, MW 942, yielded a small amount of soyasapogenol B after acid hydrolysis and soyasapogenols C, D and F as artifacts (*22*). 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. Based on these characteristics and spectroscopic data, this compound 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 from Leguminosae, and it has been previously detected in several *Medicago* spp. (*3*).

Compound **12**, 215 mg, MW 898, released hederagenin and α-L-arabinose and β-D-glucose in the ratio 2:1 after acid hydrolysis, and had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] hederagenin. This saponin was previously characterized in *M. sativa* (*11*).

Compound **13**, 86 mg, MW 766, released hederagenin and α-L-arabinose and β-D-glucose in the ratio 1:1 after acid hydrolysis, and had spectroscopic characteristics identical to those of 3-*O*-[β-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl] hederagenin. This saponin was previously identified in *M. arabica* (*10*), *M. hybrida* (*13*) and *M. sativa* (*11*).

Compound **14**, 341 mg, MW 620, released bayogenin and α-L-arabinose after acid hydrolysis, and had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl] bayogenin. This saponin was previously found in *M. arabica* (*10*).

Saponin **15**, 6 mg, MW 648, released hederagenin, α-L-arabinose and β-D-glucuronic acid after acid hydrolysis, and had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl] hederagenin. This saponin was previously found in *Hedera nepalensis* (*23*) and *Aralia armata* (*24*).

Saponin **16**, 28 mg, MW 764. The acid hydrolysis of this saponin gave oleanolic acid and α-L-arabinose and β-D-glucuronic acid, in the ratio 1:1. This compound was identified as 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl] oleanolic acid, and it was previously found in *Cynara cardunculus* (*25*).

Compound **17**, 61 mg, MW 604, released hederagenin and α-L-arabinose after acid hydrolysis, and had spectroscopic characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl] hederagenin. This saponin is one of the widely occurring hederagenin glycosides in plants, and it was previously found also in *M. arabica* (*10*).

Saponin **18**, 42 mg, MW 648. The acid hydrolysis of this saponin gave 2β-hydroxy oleanolic acid and β-D-glucuronic acid. This compound was identified as 3-*O*-[β-D-glucuronopyranosyl] 2β-hydroxy oleanolic acid, and it was previously identified in fruits of *Caryocar glabrum* (*26*).

Saponin **19**, 28 mg, MW 632. The acid hydrolysis of this saponin gave oleanolic acid and β-D-glucuronic acid. This compound was identified as 3-*O*-[β-D-glucuronopyranosyl] oleanolic acid, and has been isolated from several plant species (*26*−*29*).

A very interesting feature of saponins from *M. arabica* described in this study is the identification of queretaroic acid and its 2β-hydroxy derivative, 2β,3β,30-trihydroxyolean-12-en-28-oic acid, as new aglycons for saponins of *Medicago* species. The most common aglycons isolated so far within the *Medicago* genus are medicagenic acid, zhanic acid, bayogenin, hederagenin, caulophyllogenin, oleanolic acid, 2β-hydroxy oleanolic acid and soyasapogenols (*3*). Recently the 2β,3β-dihydroxy-23-oxo-12-en-28-oic acid has been identified as a novel aglycon in *M. arborea* (*12*) and *M. hybrida* (*13*).

Distribution of queretaroic acid is rather rare in plants. This triterpenoid was first isolated from the Mexican cactus *Lemaireocereus queretaroensis* (*30*) and subsequently found in several other cacti (*31*−*33*), suggesting a systematic relevance of its presence in such plants. Queretaroic acid has been found sporadically as the aglycon component of saponins from plants belonging to the Convolvulaceae (*Pharbitis nil)* (*34*), Verbenaceae (*Clerodendron serratum*) (*35*) or Chenopodiaceae (*Chenopodium quinoa*) (*36*) families.

Queretaroic acid has the olean-12-ene skeleton and, together with glycyrrhetic acid, is one of the few naturally occurring triterpenes which is oxygenated at C-30. Isolation of this compound in *M. arabica* should therefore be considered of importance. As it described above and shown in **Figure 2**, the olean-12-ene skeleton characterizes all the saponins extracted from the aerial parts of *M. arabica*, and in general all the saponins isolated from *Medicago* species (*3*). Queretaroic acid is supposed to be synthesized in vivo by a CYP P450 dependent hydroxylation of oleanolic acid (*37*). In this context, if a biosynthetic pathway leads from oleanolic acid (VII) to queretaroic acid (II), 2β,3β,30-trihydroxyolean-12-en-28-oic acid (I) should also be considered as likely to originate from a CYP P450 dependent hydroxylation of 2β-hydroxy oleanolic acid (V). To our knowledge, this last new sapogenin has never been found in plants.

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Received for review November 27, 2008. Revised manuscript received January 29, 2009. Accepted February 2, 2009.

JF8036984