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Flavonoids from Barrel Medic (*Medicago truncatula*) Aerial Parts

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Twenty-three flavonoids have been identified in the aerial parts of barrel medic, and their structures were established by spectrometric and spectroscopic (ESI-MS/MS and NMR) techniques. Eight of the identified compounds, including apigenin 7-*O*-*β*-D-glucuronopyranosyl-(1→3)-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside, apigenin 7-*O*-[2′-*O*-sinapoyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside], apigenin 7-*O*-{2-*O*-feruloyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucopyranoside}, chrysoeriol 7-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside, chrysoeriol 7-*O*-{2′-*O*-*p*-coumaroyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}, tricin 7-*O*-*β*-D-glucuronopyranosyl-4′-*O*-glucopyranoside, tricin 7-*O*-[2′-*O*-feruloyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucopyranoside], and tricin 7-*O*-{2′-*O*-*p*-coumaroyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}, have not been reported before in the plant kingdom. Additionally, the presence of two luteolin, three apigenin, one chrysoeriol, and six tricin glycosides, previously identified in alfalfa (*Medicago sativa*), was confirmed in *M. truncatula*. Moreover, besides the above flavones, the aerial parts of this species contained three flavonols including rutin, laricitrin 3,7,5′-triglucoside, and laricitrin 3,5′-diglucoside.

KEYWORDS: ***Medicago truncatula*; flavone glucuronides; acylated flavones; flavonols**

INTRODUCTION

Medicago truncatula Geartn. (barrel medic) belongs to the Fabaceae family (*1*). Molecular and phylogenetic studies revealed that *M. truncatula* is very close to the legumes belonging to the genera that comprise economically important crops for human nutrition or animal feed (*2*). This species is closely related to an important forage legume, alfalfa, and has been chosen as a model species for genomic studies in view of its small, diploid genome, short generation time, self-fertility, and high transformation efficiency (*3*−*5*). Plants of *M. truncatula* have higher levels of expression in transgenic than previously reported in alfalfa (*6*). Thus, this is a rapidly developing model for the study of legume biology and an excellent species for fundamental studies on the unique secondary metabolism of legumes (*7*). The plant has also been extensively used for studies on legume−rhizobial and legume−mycorrhizal symbioses (*8*, *9*).

Not much has been known on the phytochemical composition of *M. truncatula*. The saponins were a class of secondary metabolites of this species that have been documented recently (*10*). However, little has been known on the flavonoid composition and their profile. Our previous work on alfalfa provided 22 flavone glycosides showing as aglycones tricin, 3′-*O*-methyltricetin, chrysoeriol, apigenin, and luteolin. Their relatively high concentration in alfalfa (about 3% of dry matter) and acylation with ferulic, coumaric, and synapic acids, which influence UV-B absorption capacity, indicated the importance of these compounds in the adaptation of plants to environmental stresses (*11*−*13*). The flavonols and flavones are of particular importance also in the human and animal diet as there is evidence that they act as antioxidants (*14*, *15*). Epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer (*14*). Thus, the aim of the present work was to isolate and characterize flavonoid glycosides from *M. truncatula* aerial parts and to develop an ultraperfomance liquid chromatography (UPLC) method for their profiling.

MATERIALS AND METHODS

Spectroscopic Analysis. ESI-MS was performed on a Thermo Finnigan LCQ Advantage Max ion-trap mass spectrometer with an electrospray ion source (Thermo Electron Corp., Bellefonte, PA). Compounds were analyzed by direct injection by a syringe pump at a flow rate of 5 *μ*L/min. The spray voltage was set to 4.2 kV and a capillary offset voltage of −60 V. All spectra were acquired at a

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capillary temperature of 220 °C. The calibration of the mass range (400−2000 Da) was performed in negative ion mode. Nitrogen was used as sheath gas, and the flow rate was 0.9 L/min. The maximum ion injection time was set to 200 ms.

NMR spectra in CD_3OD were obtained using a Bruker DRX-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at 599.19 MHz for ^1H and at 150.86 MHz for ^{13}C. Two-dimensional (2D) experiments, $^1H-^1H$ double-filtered direct chemical shift correlation spectroscopy (DQF-COSY), inverse detected $^1H-^{13}C$ heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond connectivity (HMBC), were obtained using UXNMR software. Selective excitation spectra, 1D-TOCSY, were acquired using waveform generator-based GAUSS-shaped pulses, with a mixing time ranging from 100 to 120 ms and an MLEV-17 spin−lock field of 10 kHz preceded by a 2.5 ms trim pulse.

Plant Material. Seeds of *M. truncatula* (A17 Jemalong) were obtained from Dr. XianZhi He, The Samuel Roberts Noble Foundation, Ardmore, OK, where a voucher specimen is deposited, and from Dr. J. M. Prosperi, INRA-SGAP Montpellier, France. Plants were cultivated in an experimental field of the Institute of Soil Science and Plant Cultivation in Pulawy, Poland. They were harvested at the beginning of flowering, lyophilized, finely powdered, and used for the successive extraction.

Extraction. The isolation of flavonoids was performed according to previously developed procedures for alfalfa (*11*). In short, the powdered aerial parts (300 g) were extracted with 80% MeOH at room temperature. After 72 h, the extract was filtered and the residues were extracted two times with 80% MeOH by boiling for 2 h. The extracts were combined, and the solvent was removed under reduced pressure.

Purification. The crude extract (20 g) was suspended in water, and the solution was applied to a 10 cm × 6 cm, 40−63 μm LiChroprep RP-18 (Merck, Warsaw, Poland) preparative column previously preconditioned with water. The column was washed first with water to remove sugars and then with 40% MeOH to elute phenolics. Saponins were eluted with 70% MeOH (*16*).

Fractionation and Separation. The 40% MeOH fraction was condensed nearly to dryness in vacuo, redissolved in distilled water, and loaded onto a 50 cm × 4 cm, 40−63 μm LiChroprep RP-18 column. The column was washed with water and then with increasing concentrations of MeOH in water (5% increments from 0 to 100% MeOH). Ten milliliter fractions were collected, checked by cellulose TLC (Merck) developed in 15% acetic acid, and observed under UV (366 nm). Fractions showing similar TLC patterns (55 fractions) were further analyzed by UPLC. Fractions possessing one compound were combined and evaporated to dryness. Fractions containing more than one compound were further purified on a 50 cm × 2 cm, 40−63 μm RP-18 glass column using an isocratic system (MeCN−1% H_3PO_4) optimized for each fraction on the basis of the analytical separation. This yielded several individual compounds.

1 (10.8 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 251, 351; ESI-MS, *m/z* 1635 [2M − H]$^-$, 817 [M − H]$^-$, 655 [M − 162 − H]$^-$, 493 [M − (2 × 162) − H]$^-$, 331 [M − (3 × 162) − H]$^-$.

2 (0.63 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 254, 347; ESI-MS, *m/z* 637 [M − H]$^-$, 461 [M − 176 − H]$^-$, 251 [2 × 176]$^-$, 285 [M − (2 × 176) − H]$^-$.

3 (2.3 mg): amorphous yellow powder; UV, λ_{max}(nm) (MeOH) 265, 336; ESI-MS, *m/z* 1243 [2M − H]$^-$, 621 [M − H]$^-$, 351 [2 × 176 − H]$^-$, 269 [M − (2 × 176) − H]$^-$.

4 (1.45 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 251, 346; HRMS 651.1253 [calcd for $C_{28}H_{27}O_{18}$ (M)$^-$: 651.1199]; ESI-MS, *m/z* 1303 [2M − H]$^-$, 651 [M − H]$^-$, 351 [2 × 176 − H]$^-$, 299 [M − (2 × 176) − H]$^-$; ^1H NMR δ 7.60 (1H, d, J = 1.2 and 8.5 Hz, H-6′), 7.59 (1H, d, J = 1.2 and 8.5 Hz, H-6′), 6.99 (1H, d, J = 8.5 Hz, H-5′), 6.88 (1H, d, J = 1.2 Hz, H-8), 6.71 (1H, s, H-3), 6.54 (1H, d, J = 1.2 Hz, H-6), 5.40 (1H, d, J = 7.5 Hz, H-1GluA$_1$), 4.72 (1H 2d, J = 7.5 Hz, H-1GluA$_2$), 4.01 (s, OCH$_3$), 4.00 (1H, d, J = 9.0 Hz, H-5GluA$_1$), 3.80 (1H, dd, J = 9.0, and 9.0 Hz, H-3GluA$_1$), 3.80 (1H, d, J = 9.0 Hz, H-5GluA$_2$), 3.79 (1H, dd, J = 7.5 and 9.0 Hz, H-2GluA$_1$), 3.67 (1H, dd, J = 9.0 and 9.0 Hz, H-4GluA$_1$), 3.47 (1H, dd, J = 9.0 and 9.0 Hz, H-3GluA$_2$), 3.47 (1H, dd, J = 9.0 and 9.0 Hz, H-4GluA$_2$), 3.32 (1H, dd, J = 7.5 and 9.0 Hz, H-2GluA$_2$). For ^{13}C NMR see **Tables 1** and **2**.

Table 1. ^{13}C NMR Data of Flavonoid Aglycones in CD_3OD[a]

C	4	11	13	14	15	20	22	23
2	166.9	166.5	166.0	166.4	166.4	166.6	166.5	165.7
3	104.3	103.6	103.8	103.9	103.9	104.0	103.5	105.6
4	183.3	184.0	183.4	184.3	184.3	184.2	184.0	183.9
5	162.7	162.7	162.6	163.0	162.6	163.2	162.6	162.1
6	100.3	100.4	100.2	100.3	100.1	100.3	100.7	100.9
7	164.6	164.2	163.6	164.4	163.9	164.6	164.2	164.8
8	95.9	95.6	95.9	95.6	95.9	95.9	95.8	96.1
9	158.6	158.5	158.4	158.7	158.5	158.8	158.7	158.4
10	106.8	106.7	106.7	106.7	106.5	106.8	106.7	106.7
1′	123.6	122.6	123.2	122.8	122.6	122.6	122.6	128.1
2′	111.0	129.3	110.4	105.0	129.2	105.0	129.4	105.5
3′	149.8	116.6	149.3	149.3	116.9	149.6	116.6	154.3
4′	153.0	162.5	151.6	141.0	162.7	141.1	162.7	138.8
5′	116.4	116.6	116.3	149.3	116.9	149.6	116.6	154.3
6′	121.7	129.3	121.2	105.0	129.2	105.0	129.4	105.5
OMe	56.5		56.5			56.7 (×2)		56.9 (×2)

[a] Assignments were confirmed by HSQC and HMBC experiments.

Table 2. ^{13}C NMR Data of Sugar and Acylating Phenolic Acid in CD_3OD[a]

C	4	11	13	14	15	20	22	23
sugar								
1″	99.5	99.3	99.2	99.2	98.4	98.0	99.7	100.7
2″	83.5	83.4	82.0	82.4	82.4	82.0	83.6	73.6
3″	76.4	75.8	76.0	76.0	76.1	75.3	76.2	77.0
4″	72.1	72.9	72.6	72.4	70.9	70.6	72.3	72.9
5″	76.0	76.0	76.0	75.9	77.6	77.4	76.0	76.7
6″	172.0	172.0	172.0	172.0	61.9	61.9	172.2	172.1
1‴	105.2	103.0	101.7	102.3	101.2	100.1	105.0	103.9
2‴	75.0	75.3	73.4	73.5	73.6	74.6	74.7	75.0
3‴	77.0	75.8	83.9	83.8	83.5	75.5	86.0	77.2
4‴	72.8	73.4	71.7	71.5	71.6	72.9	71.5	70.7
5‴	76.6	76.0	76.2	76.0	76.1	75.4	76.3	77.9
6‴	172.0	172.1	172.0	172.0	172.2	172.1	172.0	61.7
1⁗			104.4	104.8	104.3		104.9	
2⁗			74.3	73.9	74.0		74.9	
3⁗			76.8	77.0	76.9		77.0	
4⁗			72.9	72.7	72.6		72.8	
5⁗			76.0	75.8	75.8		75.8	
6⁗			172.2	172.5	172.2		172.2	
phenolic acid								
1		126.0	126.6	127.1	122.9	127.7		
2		107.2	130.6	131.2	111.2	110.7		
3		149.5	116.3	117.1	148.7	149.1		
4		139.2	161.0	161.0	150.1	150.3		
5		149.5	116.3	117.1	115.6	116.2		
6		107.2	130.6	131.2	124.0	123.6		
α		116.7	114.7	115.4	114.4	115.0		
β		146.5	146.6	146.6	146.7	146.7		
C═O		167.3	168.1	168.8	168.1	168.9		
OMe		57.0 (x2)			56.4	55.8		

[a] Assignments were confirmed by HSQC and HMBC experiments.

5 (3.06 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 253, 347; ESI-MS, *m/z* 923 [2M − H]$^-$, 461 [M − H]$^-$, 285 [M − 176 − H]$^-$.

6 (80.8 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 351; ESI-MS, *m/z* 1363 [2M − H]$^-$, 681 [M − H]$^-$, 351 [2 × 176 − H]$^-$, 299 [M − (2 × 176) − H]$^-$.

7 (11.2 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 268, 331; ESI-MS, *m/z* 1947 [2M − H]$^-$, 973 [M − H]$^-$, 797 [M − 176 − H]$^-$, 703 [4 × 176 − H]$^-$, 269 [M (4 × 176) − H]$^-$.

8 (0.93 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 255, 353; ESI-MS, *m/z* 1219 [2M − H]$^-$, 609 [M − H]$^-$, 463 [M − 146 − H]$^-$, 301 [M − 146 − 162 − H]$^-$.

9 (1 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 266, 337; ESI-MS, *m/z* 891 [2M − H]$^-$, 445 [M − H]$^-$, 269 [M − H − GluA]$^-$.

10 (74.5 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 249, 337; ESI-MS, *m/z* 1003 [M − H]$^-$, 827 [M − 176 − H]$^-$, 651 [M − (2 × 176) − H]$^-$, 475 [M − (3 × 176) − H]$^-$, 299 [M − (4 × 176) − H]$^-$.

11 (0.76 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 267, 334; HRMS 827.2435 [calcd for $C_{38}H_{35}O_{21}$ (M)$^-$: 827.1672]; ESI-MS, *m/z* 1655 [2M − H]$^-$, 827 [M − H]$^-$, 621 [M − 206 − H]$^-$, 557 [(2 × 176) + 206 − H]$^-$, 351 [(2 × 176) − H]$^-$, 269 [M − (2 × 176) − 206 − H]$^-$; ^{1}H NMR δ 7.83 (2H, d, J = 8.5 Hz, H-2′, H-6′), 7.40 (1H, d, J = 16 Hz, H-β sinapic acid), 6.98 (2H, d, J = 8.5 Hz, H-3′, H-5′), 6.70 (1H, d, J = 1.2 Hz, H-8), 6.60 (2H, s, H-2, H-6, sinapic acid), 6.49 (1H, d, J = 1.2 Hz, H-6), 6.43 (1H, s, H-3), 6.13 (1H, d, J = 16 Hz, H-α sinapic acid), 5.50 (1H, d, J = 7.5 Hz, H-1GluA$_1$), 5.18 (1H, d, J = 7.5 Hz, H-1GluA$_2$), 4.95 (1H, dd, J = 7.5 and 9.0 Hz, H-2GluA$_2$), 4.00 (1H, d, J = 9.0 Hz, H-5GluA$_1$), 3.90 (1H, dd, J = 9.0 and 9.0 Hz, H-3GluA2), 3.89 (1H, d, J = 9 Hz, H-5GluA$_2$), 3.80 (1H, dd, J = 7.5 and 9.0 Hz, H-2GluA$_1$), 3.74 (1H, dd, J = 9.0 and 9.0 Hz, H-4GluA$_2$), 3.73 (s, 2 × OCH$_3$, sinapic acid), 3.73 (1H, dd, J = 9.0 and 9.0 Hz, H-3GluA$_1$), 3.65 (1H, dd, J = 9.0 and 9.0 Hz, H-4GluA$_1$).

12 (1.4 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 338; ESI-MS, *m/z* 1033 [M − H]$^-$, 857 [M − 176 − H]$^-$, 703 [(4 × 176) − H]$^-$, 329 [M − (4 × 176) − H]$^-$.

13 (6.6 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 269, 324; HRMS *m/z* 973.1851 [calcd for $C_{43}H_{41}O_{26}$ (M)$^-$: 973.1887]; ESI-MS, *m/z* 973 [M − H]$^-$, 673 [(3 × 176) + 145 − H]$^-$, 299 [M − 673 − H]$^-$; ^{1}H NMR δ 7.61 (1H, d, J = 16 Hz, H-β *p*-coumaric acid), 7.58 (1H, dd, J = 1.2 and 8.5 Hz, H-6′), 7.57 (1H, d, J = 1.2 Hz, H-2′), 7.43 (2H, d, J = 8.5 Hz, H-2, H-6, *p*-coumaric acid), 6.97 (1H, d, J = 8.5 Hz, H-5′), 6.81 (1H, d, J = 1.2 Hz, H-8), 6.78 (2H, d, J = 8.5 Hz, H-3, H-5 *p*-coumaric acid), 6.67 (1H, s, H-3), 6.51 (1H, d, J = 1.2 Hz, H-6), 6.35 (1H, d, J = 16 Hz, H-α *p*-coumaric acid), 5.43 (1H, d, J = 7.5 Hz, H-1 GluA$_1$), 5.12 (1H, d, J = 7.5 Hz, H-1 GluA$_2$), 5.10 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_2$), 4.50 (1H, d, J = 7.5 Hz, H-1GluA$_3$), 4.08 (1H, d, J = 9.0 Hz, H-5 GluA$_1$), 4.01 (s, OCH$_3$), 4.00 (2H, m, H-3 GluA$_2$, H-5 GluA$_2$), 3.83 (1H, d, J = 9.0 Hz, H-5 GluA$_3$), 3.76 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_1$), 3.74 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_2$), 3.69 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_1$), 3.67 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_1$), 3.49 (1H, dd, J = 9.0 and 9.0 Hz, H-4GluA$_3$), 3.32 (1H, dd, J = 9.0 and 9.0 Hz, H-3GluA$_3$), 3.24 (1H, dd, J = 7.5 and 9.0 Hz, H-2GluA$_3$). For ^{13}C NMR see **Tables 1** and **2**.

14 (2.1 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 270, 322; HRMS, *m/z* 1003.2071 [calcd for $C_{44}H_{43}O_{27}$ (M)$^-$: 1003.1993]; ESI-MS, *m/z* 1003 [M − H]$^-$, 857 [M − 146 − H]$^-$, 673 [(3 × 176) + 146 − H]$^-$, 329 [M − (3 × 176) − 146 − H]$^-$; ^{1}H NMR δ 7.61 (1H, d, J = 16 Hz, H-β *p*-coumaric acid), 7.26 (2H, s, H-2′, H-6′), 7.42 (2H, d, J = 8.5 Hz, H-2, H-6 *p*-coumaric acid), 6.74 (1H, d, J = 1.2 Hz, H-8), 6.78 (2H, d, J = 8.5 Hz, H-3, H-5 *p*-coumaric acid), 6.58 (1H, s, H-3), 6.50 (1H, d, J = 1.2 Hz, H-6), 6.35 (1H, d, J = 16 Hz, H-α *p*-coumaric acid), 5.42 (1H, d, J = 7.5 Hz, H-1 GluA$_1$), 5.11 (1H, d, J = 7.5 Hz, H-1 GluA$_2$), 5.10 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_2$), 4.50 (1H, d, J = 7.5 Hz, H-1 GluA$_3$), 4.06 (1H, d, J = 9.0 Hz, H-5 GluA$_1$), 4.02 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_2$), 4.00 (s, 2 × OCH$_3$), 4.00 (1H, d, J = 9.0 Hz, H-5 GluA$_2$), 3.83 (1H, d, J = 9.0 Hz, H-5 GluA$_3$), 3.75 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_1$), 3.74 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_2$), 3.70 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_1$), 3.67 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_1$), 3.49 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_3$), 3.32 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_3$), 3.24 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_3$). For ^{13}C NMR see **Tables 1** and **2**.

15 (2 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 268, 331; HRMS 959.2065 [calcd for $C_{43}H_{43}O_{25}$ (M)$^-$: 959.2095]; ESI-MS, *m/z* 1919 [2M − H]$^-$, 959 [M − H]$^-$, 689 [(3 × 176) + 162 − H]$^-$, 269 [M − (3 × 176) − 162 − H]$^-$; ^{1}H NMR δ 7.90 (2H, d, J = 8.5 Hz, H-2′, H-6′), 7.56 (1H, d, J = 15.9 Hz, H-β ferulic acid), 7.09 (1H, d, J = 1.7 Hz, H-2 ferulic acid), 6.98 (1H, dd, J = 1.7 and 8.3 Hz, H-6 ferulic acid), 6.96 (2H, d, J = 8.5 Hz, H-3′, H-5′), 6.74 (1H, d, J = 1.2 Hz, H-8), 6.72 (1H, d, J = 8.3 Hz, H-5 ferulic acid), 6.58 (1H, s, H-3), 6.50 (1H, d, J = 1.2 Hz, H-6), 6.30 (1H, d, J = 15.9 Hz, H-α ferulic acid), 5.37 (1H, d, J = 7.5 Hz, H-1Glc), 5.16 (1H, d, J = 7.5 Hz, H-1 GluA$_1$), 5.10 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_1$), 4.48 (1H, d, J = 7.5 Hz, H-1 GluA$_2$), 4.00 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_1$), 3.99 (1H, d, J = 9.0 Hz, H-5 GluA$_1$), 3.90 (1H, dd, J = 2.5 and 12.0 Hz, H-6 Glc), 3.85 (s, OCH$_3$, ferulic acid), 3.78 (1H, d, J = 9.0 Hz, H-5 GluA$_2$), 3.74 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_1$), 3.71 (1H, dd, J = 7.5 and 9.0 Hz, H-2 Glc), 3.70 (1H, dd, J = 9.0 and 9.0 Hz, H-3 Glc), 3.70 (1H, dd, J = 5.0 and 12.0 Hz, H-6 Glc), 3.54 (m, H-5 Glc), 3.48 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_2$), 3.44 (1H, dd, J = 9.0 and 9.0 Hz, H-4 Glc), 3.30 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_2$), 3.23 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_2$). For ^{13}C NMR see **Tables 1** and **2**.

16 (2 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 341; ESI-MS, *m/z* 1775 [2M − H]$^-$, 887 [M − H]$^-$, 557 [(2 × 176) + 206 − H]$^-$, 329 [M − (2 × 176) − 206 − H]$^-$.

17 (2 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 351; ESI-MS, *m/z* 1011 [2M − H]$^-$, 505 [M − H]$^-$, 329 [M − 162 − H]$^-$.

18 (25 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 269, 331; ESI-MS, *m/z* 1715 [2M − H]$^-$, 857 [M − H]$^-$, 527 [(3 × 176) − H]$^-$, 329 [(3 × 176) − H]$^-$.

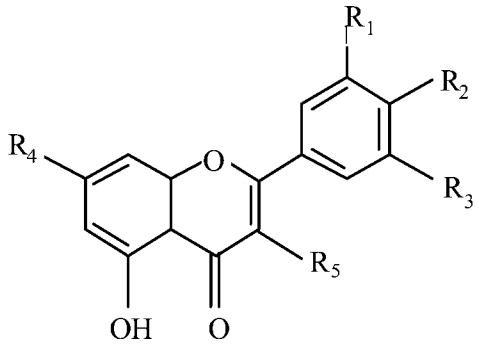
19 (29 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 270, 322; ESI-MS, *m/z* 1655 [2M − H]$^-$, 827 [M − H]$^-$, 497 [(2 × 176) + 146 − H]$^-$, 329 [M − (2 × 176) − 146 − H]$^-$.

20 (3 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 338; HRMS, *m/z* 843.1999 [calcd for $C_{39}H_{40}O_{21}$ (M)$^-$: 843.1985]; ESI-MS, *m/z* 1687 [2M − H]$^-$, 843 [M − H]$^-$, 513 [(2 × 176) + 162 − H]$^-$, 329 [M − (2 × 176) − 162 − H]$^-$; ^{1}H NMR δ 7.49 (1H, d, J = 16.0 Hz, H-β ferulic acid), 7.27 (2H, s, H-2′, H-6′), 6.97 (1H, d, J = 1.2 Hz, H-2 ferulic acid), 6.89 (1H, dd, J = 1.2 and 8.5 Hz, H-6 ferulic acid), 6.76 (1H, d, J = 1.2 Hz, H-8), 6.66 (1H, d, J = 8.5 Hz, H-5 ferulic acid), 6.59 (1H, s, H-3), 6.51 (1H, d, J = 1.2 Hz, H-6), 6.20 (1H, d, J = 16.0 Hz, H-α ferulic acid), 5.44 (1H, d, J = 7.5 Hz, H-1 Glc), 5.16 (1H, d, J = 7.5 Hz, H-1 GluA), 4.94 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA), 4.0 (OCH$_3$ × 2), 3.96 (1H, d, J = 9.0 Hz, H-5 GluA), 3.92 (1H, dd, J = 2.5 and 12.0 Hz, H-6 Glc), 3.79 (1H, dd, J = 9.0 and 9.0 Hz, H-3 Glc), 3.79 (OCH$_3$, ferulic acid), 3.74 (1H, dd, J = 7.5 and 9.0 Hz, H-2 Glc), 3.72 (1H, dd, J = 5.0 and 12.0 Hz, H-6 Glc), 3.71 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA), 3.64 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_2$), 3.60 (m, H-5 Glc), 3.48 (1H, dd, J = 9.0 and 9.0 Hz, H-4 Glc). For ^{13}C NMR see **Tables 1** and **2**.

21 (1.5 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 252, 351; ESI-MS, *m/z* 1311 [2M − H]$^-$, 655 [M − H]$^-$, 493 [M − 162 − M]$^-$, 331 [M − (2 × 162) − H]$^-$.

22 (1 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 265, 336; HRMS 797.1492 [calcd for $C_{33}H_{33}O_{23}$ (M)$^-$: 797.1414]; ESI-MS, *m/z* 1595 [2M − H]$^-$, 797 [M − H]$^-$ 527 [3 × GluA − H]$^-$ 351 [2 × GluA − H]$^-$, 269 [M − 3GluA − H]$^-$; ^{1}H NMR δ 7.95 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.96 (2H, d, J = 8.5 Hz, H-3′, H-5′), 6.82 (1H, d, J = 1.2 Hz, H-8), 6.67 (1H, s, H-3), 6.51 (1H, d, J = 1.2 Hz, H-6), 5.38 (1H, d, J = 7.5 Hz, H-1 GluA$_1$), 4.79 (1H, d, J = 7.5 Hz, H-1 GluA$_2$), 4.70 (1H, d, J = 7.5 Hz, H-1 GluA$_3$), 4.05 (1H, d, J = 9.0 Hz, H-5 GluA$_1$), 3.88 (1H, d, J = 9.0 Hz, H-5 GluA$_2$), 3.78 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_1$), 3.78 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_1$), 3.76 (1H, d, J = 9.0 Hz, H-5 GluA$_3$), 3.74 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_2$), 3.70 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_1$), 3.62 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_2$), 3.52 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_2$), 3.48 (1H, dd, J = 9.0 and 9.0 Hz, H-4 GluA$_3$), 3.47 (1H, dd, J = 9.0 and 9.0 Hz, H-3 GluA$_3$), 3.34 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA$_3$). For ^{13}C NMR see **Tables 1** and **2**.

23 (3 mg): amorphous yellow powder; UV, λ_{max} (nm) (MeOH) 269, 326; HRMS 667.1590 [calcd for $C_{29}H_{31}O_{18}$ (M)$^-$: 667.1511]; ESI-MS, *m/z* 1335 [2M − H]$^-$, 667 [M − H]$^-$, 505 [M − 162 − H]$^-$, 491 [M − 176 − H]$^-$, 329 [M − 162 − 176 − H]$^-$; ^{1}H NMR δ 7.37 (2H, s, H-2′, H-6′), 6.98 (1H, d, J = 1.2 Hz, H-8), 6.86 (1H, s, H-3), 6.58 (1H, d, J = 1.2 Hz, H-6), 5.19 (1H, d, J = 7.5 Hz, H-1 GluA), 5.13 (1H, d, J = 7.5 Hz, H-1 Glc), 4.01 (2 × OCH$_3$), 3.94 (1H, d, J = 9.0 Hz, H-5 GluA), 3.79 (1H, dd, J = 2.0 and 12.0 Hz, H-6 Glc), 3.71 (1H, dd, J = 4.5 and 12.0 Hz, H-6 Glc), 3.62 (2H, dd, J = 9.0 and 9.0



Comp.	R_1	R_2	R_3	R_4	R_5
Flavones					
2	OH	OH	H	–OGluA(2→1) GluA	H
3	H	OH	H	–OGluA(2→1) GluA	H
4	OCH_3	OH	H	–OGluA(2→1) GluA	H
5	OH	OH	H	–OGluA	H
6	OCH_3	OH	OCH_3	–OGluA(2→1) GluA	H
7	H	OH	H	–OGluA(2→1) GluA–[GluA(1→3)]–2–O–Feruloyl	H
9	H	OH	H	–OGluA	H
10	OCH_3	OH	H	–OGluA(2→1) GluA–[GluA(1→3)]–2–O–Feruloyl	H
11	H	OH	H	–OGluA(2→1) GluA-2-*O*-Sinapoyl	H
12	OCH_3	OH	OCH_3	–OGluA(2→1) GluA–[GluA(1→3)]–2–O–Feruloyl	H
13	OCH_3	OH	H	–OGluA(2→1) GluA–[GluA(1→3)]–2–O–*p*-Coumaroyl	H
14	OCH_3	OH	OCH_3	–OGluA(2→1) GluA–[GluA(1→3)]–2–O–*p*-Coumaroyl	H
15	H	OH	H	–OGlc(2→1) GluA–[GluA(1→3)]–2–O–Feruloyl	H
16	OCH_3	OH	OCH_3	–OGluA(2→1) GluA-2-*O*-Sinapoyl	H
17	OCH_3	OH	OCH_3	–OGluA	H
18	OCH_3	OH	OCH_3	–OGluA(2→1) GluA-2-*O*-Feruloyl	H
19	OCH_3	OH	OCH_3	–OGluA(2→1) GluA-2-*O*-*p*-Coumaroyl	H
20	OCH_3	OH	OCH_3	–OGlc(2→1) GluA-2-*O*-Feruloyl	H
22	H	OH	H	–OGluA(2→1) GluA(3→1)GluA	H
23	OCH_3	OGlc	OCH_3	–OGluA	H
Flavonols					
1	OCH_3	OH	-OGlc	-OGlc	-OGlc
8	OH	OH	H	OH	-ORutinoside
21	OCH3	OH	-OGlc	H	-OGlc

Figure 1. Chemical structures of isolated flavonoids.

Hz, H-3, H-4 GluA), 3.61 (1H, dd, J = 7.5 and 9.0 Hz, H-2 GluA), 3.57 (1H, dd, J = 7.5 and 9.0 Hz, H-2 Glc), 3.52 (1H, dd, J = 9.0 and 9.0 Hz, H-3 Glc), 3.50 (1H, dd, J = 9.0 and 9.0 Hz, H-4 Glc), 3.29 (m, H-5 Glc). For ^{13}C NMR see **Tables 1** and **2**.

UPLC Analysis. The Acquinity ultraperformance liquid chromatograph (Waters) consisting of a binary solvent manager, sample manager, PDA detector, and Empower Pro 2.0 software was used. Profiling was performed on a 50 mm × 2.1 mm i.d., 1.7 μm, UPLC BEH C_{18} column (Waters) utilizing a gradient elution profile and a mobile phase consisting of 0.1% acetic acid in water and 40% MeCN. The column was maintained at 50 °C, and the flow rate was kept constant at 0.35 mL/min. One gram of dried and finely powdered barrel medic tops (leaves and stems) was extracted overnight with 50 mL of 80% MeOH at room temperature. The extract was filtered, and the residues were additionally extracted twice by refluxing with 50 mL of 80% MeOH for 1 h. The extracts were combined, and the solvent was removed under reduced pressure. The crude extract was suspended in water (10 mL), and a 2 mL portion was passed through a C_{18} Sep-Pak cartridge (Waters Associates) preconditioned with water. The cartridge was washed first with water to remove sugars and then with 40% MeOH to elute phenolics. This fraction was evaporated and redissolved in MeOH (1 mL) for analyses. The location of individual compounds in the profile was performed by spiking the extract with a purified standard.

RESULTS AND DISCUSSION

Analysis of the flavonoid profile obtained from analytical liquid chromatography of the methanol extract of the aerial green parts of barrel medic using photodiode array detection showed the presence of a number of flavonoid compounds. Twenty of these compounds showed absorption spectra characteristic for apigenin (six glycosides), luteolin (two glycosides), chrysoeriol (three glycosides), and tricin (nine glycosides) derivatives (*11*, *12*). An additional three compounds showed absorption spectra indicating the presence of quercetin, laricitrin, and myricetin derivatives. Separation of the extracts by low-pressure liquid chromatography followed by purification of fractions on a semipreparative C_{18} column afforded 23 single compounds for which structures (**Figure 1**) have been determined by spectrometric and spectroscopic analyses (ESI/MS, NMR).

ESIMS of compounds **5** and **2** gave molecular ions at *m*/*z* 461 and 637, respectively. They both fragmented under an MS/MS experiment to the ion *m*/*z* 285, characteristic for luteolin (*11*). In the fragmentation pattern of compound **2** was found a strong ion at *m*/*z* 351, which indicated the presence of two

linearly connected glucuronic acid units (2 × 176 − H). These MS data together with NMR analysis suggested that compounds **2** and **5** were luteolin 7-*O*-[*β*-D-glucuronopyranosyl-(1→2)]-*O*-*β*-D-glucuronopyranoside and luteolin 7-*O*-*β*-D-glucuronopyranoside, respectively (*17*).

The second uniform group of compounds showing similar UV absorption spectra characteristic for apigenin glycosides included flavonoids **3**, **7**, **9**, **11**, **15**, and **22**. Under ESI-MS/MS experiments all of these compounds gave a peak at *m*/*z* 269, characteristic for apigenin. On the basis of UV spectra some additional information was obtained. Compounds **7**, **11**, and **15** showed substantially increased absorbance of band I as compared to the flavonoids **3**, **9**, and **22**, which indicated their acylation with hydroxycinnamic acids (*18*). Compound **9** gave a molecular ion peak at *m*/*z* 445 and a second peak at *m*/*z* 269 corresponding to the loss of uronic acid from the parent molecule. Moreover, its retention time (UPLC) and NMR characteristics were identical to those obtained for apigenin 7-*O*-*β*-D-glucuronopyranoside, previously isolated from alfalfa aerial parts (*11*). Compound **3** gave a molecular ion peak at *m*/*z* 621, and two other peaks at *m*/*z* 445 and 269 were ascribed to the loss of a uronic acid unit and an aglycone, respectively. Moreover, a strong ion at *m*/*z* 351 corresponded to two glucuronic acid units, thus suggesting the linkage between the two sugar units. On the basis of the mass spectrum it was evident that compound **3** had two uronic acid units attached to apigenin. Its retention time and NMR characteristics were identical with those of apigenin 7-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-gluronopyranoside] previously identified in alfalfa (*11*).

Compound **22** showed the molecular ion at *m*/*z* 797, and three other peaks at *m*/*z* 527, 351, and 269 were ascribed to three uronic acid units, two uronic acid units, and an aglycone, respectively. On the basis of the mass spectrum it was evident that compound **22** had three uronic acid units attached to apigenin, and the presence of a strong ion at *m*/*z* 527 indicated the linkage among the three sugar units. Analysis of ^{1}H and ^{13}C NMR spectra (**Tables 1** and **2**) of **22** clearly suggested the occurrence of apigenin and three glucuronic acid units. Selected 1D-TOCSY spectra obtained by irradiating each anomeric proton signal yielded the subspectrum of each glucuronic acid unit with high resolution. The results of 1D-TOCSY and DQF-COSY experiments allowed the sequential assignments of all the proton resonances of the three sugar units. HSQC experiments, which correlated all of the proton resonances with those of each corresponding carbon, allowed the identification of the glycosidation sites. Glycosidation shifts were observed for C-2 $GluA_1$ (δ 83.6) and C-3 $GluA_2$ (δ 86.0). On the basis of HMBC correlations between H-1 $GluA_1$ (δ 5.38) and C-7 of the aglycone (δ 164.2), between H-1 $GluA_2$ (4.79) and C-2 $GluA_1$ (δ 83.6), and between H-1 $GluA_3$ (4.70) and C-3 $GluA_2$ (δ 86.0), compound **22** was determined as apigenin 7-*O*-*β*-D-glucuronopyranosyl-(1→3)-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside.

Compound **11** gave a molecular ion at *m*/*z* 827 and four additional peaks at *m*/*z* 621, 557, 351, and 269, two of which corresponded to the loss of 206 units (621) and aglycone (269). Peaks at *m*/*z* 557 and 351 corresponded to the sugar portion with (176 + 176 + 206 − H) and without (176 + 176 − H) the acyl moiety and indicated that sugars were attached as one chain to the aglycone. The loss of 206 units from the parent ion suggested acylation of the sugar chain with sinapic acid. By comparison of the ^{1}H NMR spectra of **3** and **11** it was evident that flavonoid **11** contained additionally a singlet for two protons (δ 6.60 s), indicating three oxygenated positions in the phenolic moiety, together with a signal corresponding to two methoxyl groups (δ 3.73 s) and signals at δ 7.40 (d, J = 16.0) and 6.13 (d, J = 16.0), typical of a trans double bond. This evidence along with ^{13}C NMR data confirmed the occurrence of a sinapoyl moiety, which was deduced to be at C-2 $GluA_2$ on the basis of the HMBC correlation between H-2 $GluA_2$ (δ 4.95, dd, J = 7.5 and 9.0 Hz) and C═O (δ 167.3) of the sinapoyl unit. On the basis of these findings, flavonoid **11** was identified as apigenin 7-*O*-[2′-*O*-sinapoyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside].

Compound **7** showed a molecular ion at *m*/*z* 973, which was 176 mu higher than that found for compound **22**, suggesting another molecule of glucuronic acid or acylation with ferulic acid. Increased absorption of band I in the UV spectrum of **7** as compared to spectrum **22** proved acylation with ferulic acid. A strong ion in the MS at *m*/*z* 703 was indicative of the linear attachment of three glucuronic acids and ferulic acid. On the basis of these data together with NMR analysis flavonoid **7** was identified as apigenin 7-*O*-{2-*O*-feruloyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside}. Its identity was further supported by comparing the retention time with that obtained for original standard previously isolated from alfalfa (*11*).

The molecular ion peak of compound **15** was at *m*/*z* 959, which was 14 mu lower than that obtained for flavonoid **7**. This ion fragmented to two peaks at *m*/*z* 689 and 269. The ion at *m*/*z* 689 corresponded to the sugar chain and was interpreted as (3 × 176 + 162), suggesting that a glucuronic acid unit in **7** was replaced by a hexose unit in **15**. The high ratio of absorption of band I/band II in the UV spectrum indicated acylation of **15**. The structure was supported by ^{13}C NMR analysis of **15**, which showed the presence of two glucuronic acid units, a ferulic acid moiety, and six carbons characteristic of a glucose unit (**Table 2**). On the basis of 1D-TOCSY spectra and DQF-COSY all of the proton resonances of the three sugar units were assigned. In the HSQC spectrum glycosidation shifts were observed for C-2 Glc (δ 82.4) and C-3 $GluA_1$ (δ 83.5). A HMBC experiment, which showed long-range correlations between H-1 Glc (δ 5.37) and C-7 of the aglycone (δ 163.9), between H-1 $GluA_1$ (δ 5.16) and C-2 Glc (δ 82.4), between H-1 $GluA_2$ (δ 4.48) and C-3 $GluA_1$ (δ 83.5), and between H-2 $GluA_1$ (δ 5.10) and C═O of the feruloyl moiety (δ 168.1), allowed us to deduce that compound **15** differed from compound **7** only in the replacement of the glucuronic acid unit linked to the aglycone by a glucose unit. Thus, flavonoid **15** was identified as apigenin 7-*O*-{2-*O*-feruloyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucopyranoside}.

A second group of isolated compounds included flavonoids **6**, **12**, **14**, **16**−**20**, and **23**. Their MS/MS spectra showed the ion at *m*/*z* 329, characteristic for tricin. Also, UV spectra supported an aglycone structure; however, two groups could be distinguished on the basis of the ratio of the UV absorption of band I/band II: nonacylated compounds **6**, **17**, and **23** and acylated with hydroxycinnamic acids compounds **12**, **14**, **16**, and **18**−**20**.

Flavonoid **17** showed the lowest molecular weight, and its mass spectrum contained three peaks at *m*/*z* 1011, 505, and 329, corresponding to [2M − H], molecular ion, and the aglycone, respectively. The UV spectrum and retention time of this compound were identical to those found for tricin 7-*O*-*β*-D-glucuronopyranoside previously isolated from alfalfa aerial parts (*12*).

The UV spectrum of compound **6** was similar to that of **17**, and its molecular ion was at *m*/*z* 681, which indicated a molecule

176 mu higher than that of **17**. The UV spectrum did not indicate acylation with hydroxycinnamic acid; hence, 176 mu was indicative of the presence of a second glucuronic acid unit. The strong ion present in the MS/MS spectrum at *m*/*z* 351 suggested two glucuronic acid units, which proved a linear connection of these sugars in the flavonoid structure. The retention time of this compound was identical to that of tricin 7-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside previously isolated from alfalfa (*12*).

The MS/MS spectrum of compound **23** contained a molecular ion at *m*/*z* 667 and additional ions at *m*/*z* 505, 491, and 329, corresponding to the loss of hexose and glucuronic acid and to the aglycone, respectively. The lack of an ion at *m*/*z* 337 (hexose + glucuronic acid − H) suggested that the two sugar units were placed in two different positions; thus, compound **23** should be a bidesmosidic glycoside. The ^{13}C NMR spectrum showed, along with signals for tricin, signals attributable to terminal glucose and glucuronic acid units. 1D-TOCSY spectra obtained by selectively irradiating the anomeric proton signal at *δ* 5.19 showed the spin system of the glucuronic acid unit, whereas the 1D-TOCSY spectrum obtained starting from the anomeric proton signal at *δ* 5.13 yielded the spin system of the glucose unit. HMBC correlations between H-1 Glu (*δ* 5.13) and C-4′ (*δ* 138.8) and between H-1 GluA (*δ* 5.19) and C-7 (*δ* 164.8) allowed us to identify compound **23** as tricin 7-*O*-*β*-D-glucuronopyranosyl-4′-*O*-*β*-D-glucopyranoside.

Compounds **16**, **18**, and **19** had molecular ions higher by 206, 176, and 146 mu, respectively, than that of compound **6**, and their UV spectra indicated acylation with hydroxycinnamic acids. The presence of strong ions at *m*/*z* 557 [(2 × 176) + 206 − H]$^-$ in **16**, *m*/*z* 527 [(3 × 176) − H]$^-$ in **18**, and 497 [(2 × 176) + 146 − H]$^-$ in **19** suggested that all three compounds had a linear sugar chain acylated with sinapic, ferulic, and caffeic acid, respectively. Thus, compound **16** was established as tricin 7-*O*-[2′-*O*-sinapoyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside], compound **18** as tricin 7-*O*-[2′-*O*-feruloyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside], and compound **19** as tricin 7-*O*-[2′-*O*-*p*-coumaroyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside]. Structures were further confirmed by comparison of their retention times with those of standards previously isolated from alfalfa aerial parts (*12*).

Flavonoid **20** had a molecular ion at *m*/*z* 843, which was 14 mu lower than that found for **18**. Two additional ions at *m*/*z* 513 and 329 corresponded to the loss of (2 × 176 + 162) sugar units and to the aglycone, respectively. An ion at *m*/*z* 513 suggested a linear structure of the sugar chain, and the UV spectrum indicated acylation of a flavonoid with hydroxycinamic acid. From analysis of the MS/MS spectrum it was clear that the compound had a sugar chain composed of hexose, glucuronic acid, and ferulic acid. The occurrence of a ferulic acid was confirmed by the ^{1}H NMR spectrum, which showed signals at *δ* 6.97 (1H, d, *J* = 1.2 Hz), 6.89 (1H, dd, *J* = 1.2 and 8.5 Hz), and 6.66 (1H, d, *J* = 8.5 Hz), indicative of a 1,3,4-trisubstituted aromatic ring, along with a methoxyl singlet at *δ* 3.85 and signals at *δ* 7.49 (1H, d, *J* = 16.0 Hz) and 6.20 (1H, d, *J* = 16.0 Hz), typical of a trans double bond. Analysis of 1D and 2D NMR data showed the occurrence of three glucuronic acid units. The identity of a hexose unit and its location in the sugar chain were deduced from NMR analysis. In particular, 1D-TOCSY spectra obtained by irradiating the anomeric proton signals at *δ* 5.44 and 5.16 showed the spin systems of a *β*-glucose and a *β*-glucuronic acid, respectively. HSQC, which correlated the proton signals with the corresponding carbons, suggested a glycosidation shift for C-2 Glu (*δ* 82.0). On the basis of the HMBC correlations between H-1 Glu (*δ* 5.44) and C-7 (*δ* 164.6), between H-1 GluA (*δ* 5.16) and C-2 Glu (*δ* 82.0), and between H-2 GluA (*δ* 4.94) and C═O (*δ* 168.9) of the feruloyl moiety, compound **20** was identified as tricin 7-*O*-[2′-*O*-feruloyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucopyranoside].

Compound **12** showed a molecular ion at *m*/*z* 1033, and its UV spectrum indicated acylation with hydroxycinnamic acid. A strong ion at *m*/*z* 703 suggested the presence of a linear sugar chain composed of four units with 176 mu, interpreted as three glucuronic acids and an acylating ferulic acid moiety. The retention time of **12** was identical with that of the standard tricin (7-*O*-{2′-*O*-feruloyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside} previously isolated from alfalfa tops (*12*).

The MS/MS spectrum of **14** contained a molecular ion at *m*/*z* 1003 and three ions at *m*/*z* 857, 673, and 329. The absorption spectrum of this compound suggested acylation with hydroxycinnamic acid, and the ion at *m*/*z* 673 was indicative for a linear structure of the sugar chain. The ion at *m*/*z* 857 showed the loss of 146 mu from the parent molecule, which proved that the terminal molecule was coumaric acid. Its presence in **14** was confirmed by the ^{1}H NMR spectrum, which showed two signals at *δ* 7.42 (2H, d, *J* = 8.5 Hz) and 6.78 (2H, d, *J* = 8.5 Hz) indicative of a 1,4-disubstituted aromatic ring, along with signals at *δ* 7.61 (1H, d, *J* = 16.0 Hz) and 6.35 (1H, d, *J* = 16.0 Hz), typical of a trans double bond. Analysis of 1D and 2D NMR data showed the occurrence of three glucuronic acid units. HMBC correlations between H-1 $GluA_1$ (*δ* 5.42) and C-7 (*δ* 164.4), between H-1 $GluA_2$ (*δ* 5.11) and C-2 $GluA_1$ (*δ* 82.4), between H-1 $GluA_3$ (*δ* 4.50) and C-3 $GluA_2$ (*δ* 83.8), and between H-2 $GluA_2$ (*δ* 5.10) and C═O of the *p*-coumaroyl moiety (*δ* 168.8) allowed us to identif compound **14** as the new tricin 7-*O*-{2′-*O*-*p*-coumaroyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside}.

On the basis of MS/MS data the third uniform group was formed by compounds **4**, **10**, and **13** as they all had ion *m*/*z* 299, characteristic for chrysoeriol (*12*). From analysis of the ratio of the UV band I/band II absorbance it was evident that compound **4** was not acylated and that compounds **10** and **13** were acylated with hydroxycinnamic acids.

The MS spectrum of **4** contained three dominant peaks at *m*/*z* 651, 351, and 299, corresponding to the molecular ion, sugar portion, and the aglycone. The presence of an ion at *m*/*z* 351 indicated that the sugar chain in **4** consisted of two linearly connected glucuronic acids and was identical to those found in compounds **2**, **3**, and **6**. ^{1}H and ^{13}C NMR data in comparison with those of compounds **2**, **3**, and **6** suggested an identical sugar chain. Thus, compound **4** was identified as chrysoeriol 7-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside.

Compound **10** had a molecular ion at *m*/*z* 1003. The MS/MS fragmentation patterns for this compound were identical with the fragmentation of compound **18**, where two glucuronic acids and ferulic acids were identified in the sugar chain. Thus, the structure of **10** was established as chrysoeriol 7-*O*-{2′-*O*-feruloyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside}. Its identity was further confirmed by cochromatography with the standard previously isolated from alfalfa (*12*).

The molecular ion of flavonoid **13** was at *m*/*z* 973. The strong ion at 673 was assigned to a sugar portion consisting of three

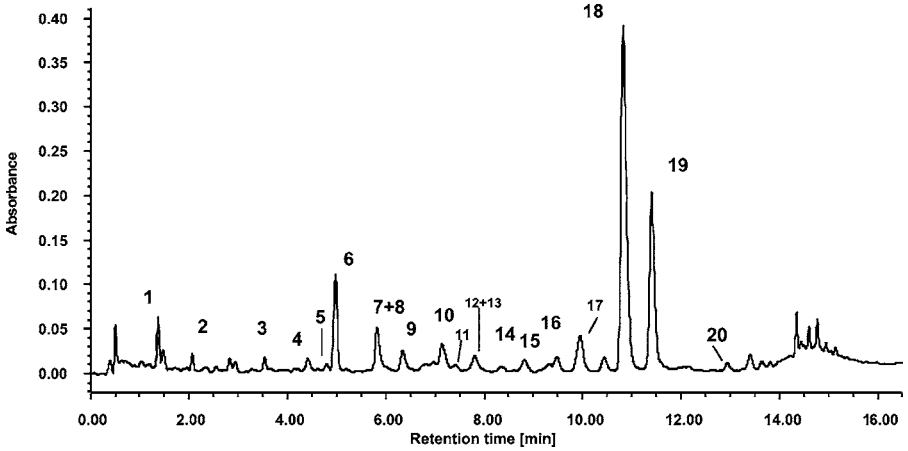


Figure 2. Ultraperformance liquid chromatography profile of *M. truncatula* flavonoids. Peak numbers correspond to the numbering of flavonoids in the text. Flavonoid **21** had $t_R = 3$ min, **22** overlapped with flavonoid **3**, and compound **23** had $t_R = 4.56$ min.

glucuronic acids (3 × 176) and coumaric acid (146), which suggested similarity of sugar chains in **13** and **14**. Analysis of NMR data **(Tables 1** and **2)** in comparison with those of **13** suggested that difference between the two compounds was confined to the aglycone moiety. Thus, flavonoid **13** was identified as chrysoeriol 7-*O*-{2′-*O*-*p*-coumaroyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside}.

Compound **8** had a molecular ion at *m*/*z* 609 and two additional ions at *m*/*z* 463 and 301, indicating the loss of deoxyhexose and hexose from the parent molecule. The ion at *m*/*z* 301 and the UV spectrum of **8** suggested the structure of quercetin 3-*O*-*β*-D-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (rutin). The identity of this compound was further confirmed by cochromatography with authentic rutin standard.

Flavonoids **1** and **21** showed identical UV absorption spectra, and their MS/MS degradation showed the ion representing aglycone moiety at *m*/*z* 331, which is characteristic for laricitrin. The molecular ion of **21** was at *m*/*z* 655, and the MS/MS spectrum contained two additional ions at *m*/*z* 493 and 331 corresponding to the loss of one and two hexoses. The ^{1}H and ^{13}C NMR signals were superimposable on those reported for laricitrin 3,5′-diglucopyranoside (*19*). Flavonoid **1** had a molecular ion at *m*/*z* 817, which was 162 mu higher than that of **21**, suggesting one additional hexose in the molecule. The MS/MS fragmentation patterns of **1** were similar to that found for **21**. Cochromatography with authentic standard confirmed **1** to be laricitrin 3,7,5′-triglucopyranoside.

Some of the flavones identified in the aerial parts of *M. truncatula* (**2**, **3**, **5**−**7**, **9**, **10**, **12**, and **16**−**19**) have been previously identified in alfalfa (*Medicago sativa*) aerial parts. However, to the best of our knowledge, compounds **4**, **11**, **13**−**15**, **20**, **22**, and **23** are novel structures never reported before in the plant kingdom.

In general, flavonoids of *M. truncatula* showed a high level of similarity with alfalfa flavones. The dominant group (nine compounds) included tricin glycosides, followed by apigenin (six compounds), chrysoeriol (three compounds), and luteolin (three compounds). The sugar chains of the majority of flavones were composed exclusively of glucuronic acid, but in contrast to alfalfa, two flavones (**15** and **20**) possessed glucose as the first sugar attached at position C-3. The common feature of *M. truncatula* and *M. sativa* flavones was their acylation with hydoxycinnamic acids. The concentration of particular glycosides in *M. truncatula* was not determined in this study. The UPLC was, however, successfully applied to profile flavonoids in this species (**Figure 2**). From this profile it was evident that dominant flavonoids **6**, **18**, and **19** had tricin as an aglycone. These three peaks overwhelmed other flavonoids, some of which were found in trace amounts, even not observed in the profile. Of these three tricin glycosides, compounds **18** and **19** were acylated with hydroxycinnamic acids. As acylation influences the spectroscopic characteristics of flavones, by increasing their UV-B-absorbing capacity (*18*), their abundance in both species of *Medicago* may underscore the importance of this process in shielding plants against UV-B radiation (*20*).

The presence of flavonols, rutin, and laricitrin glucosides in *M. truncatula* differentiates this species from *M. sativa*. Because aerial parts included leaves, stems, and flowers, there is a possibility that flavonols were constituents of flowers and not necessarily the leaves and stems. The laricitrin and quercetin aglycones were previously identified in hydrolysates of *M. truncatula* flowers (*21*). This presumption needs further clarification.

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