Alfalfa (*Medicago sativa* L.) Flavonoids. 1. Apigenin and Luteolin Glycosides from Aerial Parts

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Nine flavones and adenosine have been identified in aerial parts of alfalfa, and their structures were established by spectral (FABMS and NMR) techniques. Five of the identified compounds, including apigenin 7-*O*-[*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranosyl]-4′-*O*-*β*-D-glucuronopyranoside, apigenin 7-*O*-[2-*O*-feruloyl-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranosyl]-4′-*O*-*β*-D-glucuronopyranoside, apigenin 7-*O*-{2-*O*-feruloyl-[*β*-D-glucuronopyranosyl(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}, apigenin 7-*O*-{2-*O*-*p*-coumaroyl-[*β*-D-glucuronopyranosyl(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}, and luteolin 7-*O*-[2-*O*-feruloyl-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranosyl]-4′-*O*-*β*-D-glucuronopyranoside, have not been reported before in the plant kingdom. Additionally, five known compounds, including apigenin 7-*O*-*β*-D-glucuronopyranoside, apigenin 4′-*O*-*β*-D-glucuronopyranoside, apigenin 7-*O*-[*β*-D- glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside], luteolin 7-*O*-*β*-D-glucuronopyranoside, and adenosine, were identified.

Keywords: *Medicago sativa; flavone glucuronides; acylated flavones; adenosine*

INTRODUCTION

The literal translation of the word alfalfa is “father of all food”, and for centuries it has been grown and used as feed for livestock in the form of green feed, hay, or pellets. Alfalfa sprouts are widely consumed by humans as a garnish, and leaf protein concentrates and the dehydrated plant are components of many nutritional supplement products. In addition to the nutritional components (protein and carbohydrates) that are important to its use as an animal feed or food supplements (*1*), alfalfa also contains numerous secondary metabolites that are of interest in human nutrition. These include saponins (*2* and *3*), flavonoids (*4* and *5*), tannins (*6*), coumestrol (*7*), carotenoids, and tocols (*8* and *9*). Characterizing the phytochemical composition of alfalfa can help to improve our understanding of its nutritional value.

A number of flavonoids from different, most often unspecified, parts of the alfalfa plant have been identified (*5*). However, in most cases, only the aglycons after hydrolysis were reported and in only a few instances were the full glycosidic forms structurally determined. Preliminary analyses using liquid chromatography (HPLC with diode array detection) of 47 varieties of alfalfa, selected from USDA stocks and grown in research plots in Southern California, showed that all varieties had similar profiles of flavonoids. Their total concentration ranged from 0.24 to 0.78% in dry matter (*10*). On the basis of the absorption spectra of individual peaks in the chromatograms, these flavonoids were preliminarily classified as glycosides of apigenin, luteolin, and tricin. Because flavonoids have been recently recognized as active principles, showing structure-dependent physiological activity (antioxidant, cancer-preventing, and antimicrobial) (*11*), and their occurrence in food is highly desirable, we deemed it worthy to isolate and establish the structures of individual compounds occurring in the green aerial parts of alfalfa.

MATERIALS AND METHODS

Spectral Analysis. Melting points were uncorrected. The CD spectra were recorded in MeOH at 20 °C on a Jasco P-1020 spectropolarimeter. FABMS spectra were recorded on a MAT 95 (Finnigan) spectrometer with glycerol as a matrix and the ESI−HRMS were recorded on a Mariner Biospectrometry Workstation (Per Septive Biosystems). ^{1}H and ^{13}C NMR spectra were measured on a Bruker DRX-600 spectrometer, and the UXNMR software package was used for NMR measurements in CD_3OD solutions. 2D experiments ^{1}H−^{1}H double quantum filtered−direct chemical shift correlation spectroscopy (DQF−COSY) (*12*), inverse detected ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) (*13*), and heteronuclear multiple bond connectivity (HMBC) (*14*) were obtained using UX−NMR software. 1D-TOCSY (*15*) data were acquired using waveform generator-based GAUSS shaped pulses, with mixing times 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. Alfalfa (*Medicago sativa* L. var. Boja) was grown at the experimental farm of the Institute of Soil Science and Plant Cultivation in Pulawy in 1997. Plants were collected at the beginning of flowering, freeze-dried, and finely powdered.

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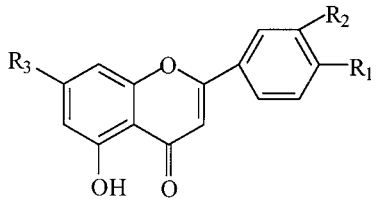

	R_1	R_2	R_3
1	–OGluA	–H	–OGluA(2→1) GluA
2	–OGluA	–H	–OH
3	–OH	–H	–OGluA(2→1) GluA
4	–OH	–OH	–OGluA
6	–OGluA	–H	–OGluA(2→1) GluA–2–*O*–Feruloyl
7	–OH	–H	–OGluA(2→1) GluA–[GluA(1→3)]–2–*O*–Feruloyl
8	–OH	–H	–OGluA(2→1) GluA–[GluA(1→3)]–2–*O*–*p*-Coumaroyl
9	–OH	–H	–OGluA
10	–OGluA	–OH	–OGluA(2→1) GluA–2–*O*–Feruloyl

Figure 1. Chemical formulas of the identified compounds (GluA = glucuronic acid).

Isolation of Flavonoids. *Extraction*. Powdered material (1 kg) was depigmented and defatted in a Soxhlet extractor with chloroform. For extraction, 200 g of defatted material was refluxed for 1.5 h with 2 L of 70% aqueous MeOH. The extract was centrifuged and the supernatant was evaporated under reduced pressure to dryness (51.7 g).

Purification. The extract, suspended in water, was passed through a short column (3 cm × 5 cm, LiChroprep RP-18, 40−63 μm, Merck), preconditioned with water. The column was washed successively with water, 40% aqueous MeOH (5.1 g), and 70% MeOH (3.5 g). Using analytical HPLC as a check, the fraction washed out with 40% MeOH was used for flavonoid separation.

Flavonoid Separation. The 40% MeOH fraction was condensed nearly to dryness in vacuo, redissolved in distilled water, and loaded onto a preparative column (3 × 40 cm, LiChroprep RP-18, 25−40 μm, Merck). The column was washed with water and then with increasing concentrations of MeOH in water (5% increments from 0 to 100% MeOH). Fractions of 10 mL were collected with a fraction collector. Fractions were analyzed with TLC (DC−Alufolien Cellulose, Merck) developed in 15% acetic acid (OHAc) and observed under long wavelength UV (366 nm) illumination. Fractions showing similar TLC patterns were further analyzed by HPLC (Waters with 996 PAD detector, 616 Pump and Millenium software) using an isocratic solvent system (AcN-1%H_3PO_4) with concentration of acetonitrile (AcN) dependent on the specific fraction. Fractions possessing one compound were combined and evaporated to dryness. Fractions containing more than one compound were further separated/purified on a stainless Vertex column (0.8 × 25 cm, Eurospher 100, RP-18, 10μm, Säulentechnik) using an isocratic system (AcN-1%H_3PO_4) optimized for each fraction based on the analytical separation. Separation was monitored with a UV detector. This yielded several individual compounds as shown in Figure 1.

1 (42.4 mg); Amorphous yellow powder; Mp 182−183 °C (with browning); $[\alpha]_D^{20}$ −50.8° (50% MeOH, *c* 0.05). HRMS *m/z*: 797.1387. Calcd for $C_{33}H_{33}O_{23}$ $(M)^-$, 797.1407. FABMS (negative ion mode) *m/z*: 797 $[M-H]^-$, 621 [M-GlcA-H]$^-$, 269 [M-3GlcA-H]$^-$, (positive ion mode) 799 $[M+H]^+$, 623 [M-GlcA+H]$^+$. UV λ_{max}(nm) (MeOH) 266, 323. ^{1}H NMR δ: 7.81 (2H, d, J = 8.0 Hz, H-2′, H-6′), 7.15 (2H, d, J = 8.0 Hz, H-3′, H-5′), 6.77 (1H, d, J = 1.2 Hz, H-8), 6.62 (1H, s, H-3), 6.45 (1H, d, J = 1.2 Hz, H-6), 5.39 (1H, d, J = 7.5 Hz, H-1GluA$_1$), 5.26 (1H, d, J = 7.5 Hz, H-1GluA$_3$) 4.74 (1H, d, J = 7.5 Hz, H-1GluA$_2$). For ^{13}C NMR data see Table 1.

2 (4.9 mg); ($C_{21}H_{18}O_{11}$). FABMS (negative ion mode) *m/z*: 445 $[M-H]^-$, 269 [M-H-GlcA]$^-$. UV, λ_{max}(nm) (MeOH) 266, 318. ^{1}H NMR δ: 7.96 (2H, d, J = 8.3 Hz, H-2′, H-6′), 7.24 (2H, d, J = 8.3 Hz, H-3′, H-5′), 6.82 (1H, d, J = 1.5 Hz, H-8), 6.72 (1H, s, H-3), 6.49 (1H, d, J = 1.5 Hz, H-6), 5.18 (1H, d, J = 7.0 Hz, H-1GluA), 4.14 (1H, d, J = 9.5 Hz, H-5 GluA), 3.67 (1H, dd, J = 9.5 and 9.5 Hz, H-4 GluA), 3.58 (1H, dd, J = 7.5 and 9.5 Hz, H-2 GluA) 3.58 (1H, dd, J = 9.5 and 9.5 Hz, H-3 GluA). For ^{13}C NMR data see Table 1.

3 (5 mg); ($C_{27}H_{26}O_{17}$). FABMS (negative ion mode) *m/z*: 621 $[M-H]^-$, 445 [M-H-GluA]$^-$, 269 [M-H-2GluA]. UV, λ_{max}(nm) (MeOH) 266, 337. ^{1}H NMR δ 7.92 (2H, J = 8.3 Hz, H-2′, H-6′), 6.96 (2H, d, J = 8.3 Hz, H-3′, H-5′), 6.82 (1H, d, J = 1.2 Hz, H-8), 6.65 (1H, s, H-3), 6.50 (1H, d, J = 1.2 Hz, H-6), 5.44 (1H, d, J = 6.5 Hz, H-1 GluA$_1$), 4.71 (1H, d, J = 7.5 Hz, H-1 GluA$_2$). For ^{13}C NMR data see Table 1.

4 (31.5 mg); ($C_{21}H_{18}O_{12}$). FABMS (negative ion mode) *m/z*: 461 $[M-H]^-$, 285 [M-H-GluA]$^-$. UV, λ_{max}(nm) (MeOH) 252, 347. ^{1}H NMR δ 7.44 (1H, dd, J = 1.4 and 8.3 Hz, H-6′), 7.43 (1H, d, J = 1.4 Hz, H-2′), 6.93 (1H, d, J = 8.3 Hz, H-5′), 6.81 (1H, d, J = 1.2 Hz, H-8), 6.67 (1H, s, H-3), 6.52 (1H, d, J = 1.2 Hz, H-6), 5.21 (1H, d, J = 7.0 Hz, H-1 GluA), 4.13 (1H, d, J = 9.5 Hz, H-5 GluA), 3.66 (1H, J = 9.5 and 9.5 Hz, H-4 GluA), 3.58 (1H, dd, J = 7.0 and 9.5 Hz, H-2 GluA), 3.58 (1H, J = 9.5 and 9.5 Hz, H-3 GluA). For ^{13}C NMR data see Table 1.

5 (5.9 mg); FABMS (positive ion mode) *m/z*: 133 $[C_5O_4H_9]^+$, 136 $[C_5N_5H_6]^+$. UV, λ_{max}(nm) (MeOH) 248, 271, 328. For ^{13}C NMR data see Table 1.

6 (12.2 mg); Amorphous yellow powder; Mp 205−206 °C; $[\alpha]_D^{20}$ −13.8° (MeOH, *c* 0.1). HRMS *m/z*: 973.1935 [Calcd for $C_{43}H_{41}O_{26}$ $(M)^-$, 973.1881]. FABMS (negative ion mode) *m/z*: 973 $[M-H]^-$, 797 [M-H-ferulic acid]$^-$, 445 [M-H-ferulic acid-2GluA]$^-$, 269 [M-H-ferulic acid-3GluA]$^-$. UV, λ_{max}(nm) (MeOH) 271, 323. ^{1}H NMR δ 7.74 (1H, d, J = 15.9 Hz, H-β ferulic acid), 7.74 (2H, d, J = 8.0 Hz, H-2′, H-6′), 7.14 (1H, d, J = 1.7 Hz, H-2 ferulic acid), 7.10 (2H, d, J = 8.0 Hz, H-3′, H-5′), 7.04 (1H, dd, J = 1.7 and 8.3 Hz, H-6 ferulic acid), 6.78 (1H, d, J = 8.3 Hz, H-5 ferulic acid), 6.72 (1H, d, J = 1.2 Hz, H-8), 6.56 (1H, s, H-3), 6.40 (1H, d, J = 1.2 Hz, H-6), 6.38 (1H, d, J = 15.9 Hz, H-α ferulic acid), 5.35 (1H, d, J = 7.5 Hz, H-1GluA$_1$), 5.23 (1H, d, J = 7.5 Hz, H-1GluA$_3$) 5.05 (1H, d, J = 7.5 Hz, H-1GluA$_2$), 4.88 (1H, dd, J = 7.5 and 9.5 Hz, H-2GluA$_2$), 3.83 (OCH_3). For ^{13}C NMR data see Table 1.

7 (10 mg); Amorphous yellow powder; Mp 210−211 °C; $[\alpha]_D^{20}$ −50.8° (MeOH, *c* 0.1). HRMS *m/z*: 973.1940 [Calcd for $C_{43}H_{41}O_{26}$ $(M)^-$, 973.1881]. FABMS (negative ion mode) *m/z*: 973 $[M-H]^-$, 797 [M-H-ferulic acid]$^-$, 445 [M-H-ferulic acid-2GluA]$^-$, 269 [M-H-ferulic acid-3GluA]$^-$.; UV, λ_{max}(nm) (MeOH) 271, 323. ^{1}H NMR δ 7.88 (2H, d, J = 8.0 Hz, H-2′, H-6′), 7.57 (1H, d, J = 16 Hz, H-β ferulic acid), 7.11 (1H, d, J = 1.2 Hz, H-2 ferulic acid), 7.00 (1H, d, J = 8.0 and 1.2 Hz, H-6 ferulic acid), 6.96 (2H, d, J = 8.0 Hz, H-3′, H-5′), 6.75 (1H, d, J = 8.0 Hz, H-5 ferulic acid), 6.74 (1H, d, J = 1.2 Hz, H-8), 6.60 (1H, s, H-3), 6.48 (1H, d, J = 1.2 Hz, H-6), 6.33 (1H, d, J = 16 Hz,

Table 1. ^{13}C NMR Data of Compounds (1−10) in CD_3OD[a]

	δ									
C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
					Aglycons					
2	166.1	166.0	166.9	166.9	153.7	165.8	166.6	166.7	166.8	166.7
3	104.5	105.2	105.5	104.2		104.8	103.8	103.9	104.2	105.7
4	183.9	184.1	184.2	184.1	152.4	183.9	184.3	184.3	184.1	184.0
5	162.7	162.9	162.9	162.9	118.4	162.7	162.0	163.3	162.9	162.8
6	101.8	101.6	100.9	101.1	155.3	101.3	100.4	100.8	101.2	101.4
7	164.3	164.5	164.1	164.5		164.3	163.8	164.0	164.5	164.3
8	95.3	96.1	96.2	96.0	138.6	95.8	96.2	95.5	96.1	96.1
9	158.7	158.9	159.0	158.9		158.7	158.7	158.8	158.9	158.9
10	107.1	107.0	107.2	107.2		107.1	106.9	106.9	107.3	107.4
1′	125.8	126.2	123.2	123.5	90.3	125.6	123.0	123.0	123.1	126.7
2′	129.2	129.4	129.7	114.3	72.3	129.2	129.7	129.7	129.7	115.1
3′	117.2	118.1	117.1	147.1	75.6	117.7	117.0	116.7	117.1	148.0
4′	161.2	161.8	162.9	151.2	87.4	161.2	162.7	162.8	162.9	149.7
5′	117.2	118.1	117.1	116.8	63.2	117.7	117.0	116.7	117.1	117.7
6′	129.2	129.4	129.7	120.5		129.2	129.7	129.7	129.7	120.8
					Sugars					
1″	99.3		100.0	101.5		99.3	98.9	99.2	101.4	99.2
2″	83.7		84.1	74.4		83.4	82.2	82.4	74.5	82.9
3″	75.7		76.3	77.2		75.8	75.8	76.0	77.2	75.4
4″	72.4		72.4	72.8		72.9	72.6	72.4	72.9	72.6
5″	76.1		76.7	76.8		76.0	76.0	75.9	76.7	75.9
6″	172.0		172.3	172.0		172.0	171.9	172.0	172.3	172.1
1‴	104.3		104.1			103.0	101.9	102.1		103.1
2‴	74.5		75.5			75.3	73.3	73.5		75.2
3‴	77.6		77.2			75.8	83.6	83.8		75.8
4‴	72.0		73.3			73.4	71.6	71.6		73.8
5‴	76.0		76.8			76.0	75.8	76.0		76.3
6‴	172.0		172.3			172.0	171.8	172.0		172.0
1⁗	101.0	101.3				101.0	105.0	104.8		101.2
2⁗	74.5	74.5				74.4	73.8	73.9		74.3
3⁗	77.0	77.3				77.1	76.9	77.0		77.3
4⁗	72.6	72.9				72.9	72.9	72.7		72.6
5⁗	76.2	76.6				76.5	75.7	75.8		76.4
6⁗	172.0	172.1				172.0	172.3	172.7		172.0
					Phenolic acid					
1						127.8	127.6	127.1		127.0
2						111.7	111.3	131.2		111.8
3						149.5	149.4	117.1		149.1
4						150.5	150.4	161.1		150.4
5						116.4	116.6	131.2		116.6
6						124.1	124.3	117.1		124.2
α						115.5	115.3	115.6		115.5
β						147.0	147.0	146.8		146.9
C═O						168.8	168.5	168.8		168.6
OMe						56.4	56.3			56.4

[a] Assignments confirmed by HSQC and HMBC experiments.

H-α ferulic acid), 5.42 (1H, d, J = 7 0.5 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 $GluA_2$), 4.49 (1H, d, J = 7.5 Hz, H-1 $GluA_3$), 4.10 (1H, d, J = 9.5 Hz, H-5 $GluA_1$), 4.00 (1H, d, J = 9.5 Hz, H-5 $GluA_2$), 3.99 (dd, J = 9.5 and 9.5 Hz, H-3 $GluA_2$), 3.86 (d, J = 9.5 Hz, H-5 $GluA_3$), 3.83 (OC*H*$_3$), 3.75 (1H, dd, J = 7.5 and 9.5 Hz, H-2 $GluA_1$), 3.75 (1H, dd, J = 9.5 and 9.5 Hz, H-4 $GluA_2$), 3.70 (1H, dd, J = 9.5 and 9.5 Hz, H-3 $GluA_1$), 3.65 (1H, dd, J = 9.5 and 9.5 Hz, H-4 $GluA_1$), 3.50 (1H, dd, J = 9.5 and 9.5 Hz, H-4 $GluA_3$), 3.33 (1H, dd, J = 9.5 and 9.5 Hz, H-3 $GluA_3$), 3.26 (1H, dd, J = 7.5 and 9.0 Hz, H-2 $GluA_3$). For ^{13}C NMR data see Table 1.

8 (36 mg); Amorphous yellow powder; Mp 203−204 °C; $[\alpha]_D^{20}$ −61.0° (MeOH, *c* 0.1). HRMS *m*/*z*: 943.1755 [Calcd for $C_{42}H_{39}O_{25}$ $(M)^-$, 943.1775]. FABMS (negative ion mode) *m*/*z*: 943 $[M-H]^-$, 797 [M-H-coumaric acid]$^-$, 445 [M-H- coumaric acid-2GluA]$^-$, 269 [M-H-coumaric acid-3GluA]$^-$. UV, λ_{max}(nm) (MeOH) 271, 323. ^1H NMR δ 7.89 (2H, d, J = 8.0 Hz, H-2′, H-6′), 7.60 (1H, d, J = 16 Hz, H-*β* *p*-coumaric acid), 7.42 (2H, d, J = 8.0 Hz, H-2, H-6, *p*-coumaric acid), 6.95 (2H, d, J = 7.5 Hz, H-3′, H-5′), 6.76 (2H, d, J = 8.0 Hz, H-3, H-5 *p*-coumaric acid), 6.76 (1H, d, J = 1.2 Hz, H-8), 6.62 (1H, s, H-3), 6.47 (1H, d, J = 1.2 Hz, H-6), 6.34 (1H, d, J = 16 Hz, H-α *p*-coumaric acid), 5.41 (1H, d, J = 7.5 Hz, H-1 $GluA_1$), 5.11 (1H, d, J = 7.5 Hz, H-1 $GluA_2$), 5.09 (1H, dd, J = 7.5 and 9.5 Hz, H-2 $GluA_2$), 4.50 (1H, d, J = 7.5 Hz, H-1 $GluA_1$) 4.08 (1H, d, J = 9.0 Hz, H-5 $GluA_1$), 3.99 (1H, d, J = 9.0 Hz, H-5 $GluA_2$), 3.99 (1H, dd, J = 9.5 and 9.5 Hz, H-3 $GluA_2$), 3.85 (1H, d, J = 9.5 Hz, H-5 $GluA_3$), 3.75 (1H, dd, J = 7.5 and 9.5 Hz, H-2 $GluA_1$), 3.75 (1H, dd, J = 9.5 and 9.5 Hz, H-4 $GluA_2$), 3.68 (1H, dd, J = 9.5 and 9.5 Hz, H-3 $GluA_1$), 3.64 (1H, dd, J = 9.5 and 9.5 Hz, H-4 $GluA_1$), 3.50 (1H, dd, J = 9.5 and 9.5 Hz, H-4 $GluA_3$), 3.33 (1H, dd, J = 9.5 and 9.5 Hz, H-3 $GluA_3$) 3.24 (1H, dd, J = 7.5 and 9.5 Hz, H-2 $GluA_3$). For ^{13}C NMR data see Table 1.

9 (25 mg); ($C_{21}H_{18}O_{11}$). FABMS (negative ion mode) *m*/*z*: 445 $[M-H]^-$, 269 [M-H-GluA]$^-$, (positive ion mode) 447 $[M+H]^+$, 271 $[M+H-GluA]^+$. UV, λ_{max}(nm) (MeOH) 266, 337. ^1H NMR δ 7.88 (2H, J = 8.3 Hz, H-2′, H-6′), 6.96 (2H, d, J = 8.3 Hz, H-3′, H-5′), 6.82 (1H, d, J = 1.2 Hz, H-8), 6.66 (1H, s, H-3), 6.50 (1H, d, J = 1.2 Hz, H-6), 5.23 (1H, d, J = 7.0 Hz, H-1 GluA), 4.15 (1H, J = 9.5 Hz, H-5 GluA), 3.67 (1H, J = 9.5 and 9.5 Hz, H-4 GluA), 3.59 (1H, dd, J = 7.0 and 9.5 Hz, H-2 GluA), 3.59 (1H, dd, J = 9.5 and 9.5 Hz, H-3 GluA). For ^{13}C NMR data see Table 1.

10 (2 mg); Amorphous yellow powder; Mp 214−215 °C (with browning); $[\alpha]_D^{20}$ −38.4° (MeOH, *c* 0.1). HRMS *m*/*z*: 989.1860 [Calcd for $C_{43}H_{41}O_{27}$ $(M)^-$, 989.1830]. FABMS (negative ion

mode) *m/z*: 989 [M-H]$^-$, 813 [M-H-ferulic acid]$^-$, 461 [M-H-ferulic acid- 2GluA]$^-$, 285 [M-H-ferulic acid-3GluA]$^-$. UV, λ_{max}(nm) (MeOH) 247, 328. ^{1}H NMR δ 7.64 (1H, d, J = 16.0 Hz, H-6′), 7.46 (1H, d, J = 1.5 Hz, H-2′), 7.25 (1H, d, J = 8.2 Hz, H-5′), 7.18 (1H, d, J = 1.7 Hz, H-2 ferulic acid), 7.08 (1H, dd, J = 1.7 and 8.2 Hz, H-6 ferulic acid), 6.84 (1H, d, J = 1.2 Hz, H-8), 6.82 (1H, d, J = 8.2 Hz, H-5 ferulic acid), 6.69 (1H, s, H-3), 6.54 (1H, d, J = 1.2 Hz, H-6), 6.39 (1H, d, J = 16 Hz, H-α ferulic acid), 5.26 (1H, d, J = 7.5 Hz, H-1 $GluA_1$), 5.23 (1H, d, J = 7.5 Hz, H-1 $GluA_3$), 5.18 (1H, d, J = 7.5 Hz, H-1 $GluA_2$), 4.89 (1H, dd, J = 7.5 and 9.5 Hz, H-2 $GluA_2$), 3.91 (OMe). For ^{13}C NMR data see Table 1.

RESULTS AND DISCUSSION

Analysis of the flavonoid profile obtained from analytical liquid chromatography of the methanol extract of the aerial green parts of alfalfa using photodiode array detection showed the presence of a number of flavonoid compounds. Nine of these compounds showed absorption spectra characteristic for apigenin and luteolin derivatives (*16*). Separation of the extracts by low-pressure liquid chromatography, followed by purification of fractions on a semipreparative C18 column, afforded several single compounds for which structures have been determined by spectral analyses (FABMS, NMR).

Thus, FAB mass spectrometry of **9** gave the molecular ion peak at *m/z* 445 and a second peak at 269 corresponding to the loss of uronic acid from the parent molecule. The ^{13}C NMR spectrum showed the presence of 21 carbons, 15 of which corresponded to an aglycon molecule with chemical shifts consistent with those obtained for apigenin (*16* and *17*). Similarly, in the ^{1}H NMR spectrum, six proton signals in the range of 6.50−7.88 ppm corresponded to the aglycon part of the molecule. The remaining five proton signals in the region of 3.59−5.23 ppm and six carbon signals (Table 1) corresponded to a glucuronic acid molecule (*17*). The position of the sugar was confirmed by HMBC spectra; cross-peaks were observed between H-1 of the sugar (δ 5.23) and C-7 of the aglycon (δ 164.5). Thus, the structure of **9** was established as apigenin 7-*O*-β-D-glucuronopyranoside.

Compound **2** had mass spectral characteristics identical to those of flavonoid **9**. Its UV spectrum, however, showed a shift in band I of the absorption spectra from 337 (in **9**) to 318 nm indicating substitution occurring in ring B of the aglycon. The ^{13}C NMR spectrum of the aglycon portion of **2** was similar to that registered for **9** with some differences for the values of ring B (Table 1). The HMBC correlation between H-1GluA (glucuronic acid) (δ 5.18) and C-4′ of the aglycon (δ 161.8) allowed us to deduce that the sugar moiety was linked to C-4′. Thus, the structure of **2** was established as apigenin 4′-*O*-β-D-glucuronopyranoside.

Compound **3** showed a UV spectrum identical to that of **9**, and three characteristic peaks could be identified in the FABMS spectrum. The molecular ion was registered at *m/z* 621, and two other peaks at *m/z* 445 and 269 were ascribed to the loss of uronic acid molecule and an aglycon, respectively. Based on the mass spectrum it was evident that compound **3** had two uronic acid units attached to apigenin. The ^{13}C NMR values (Table 1) were consistent with the occurrence of two glucuronic acid units with 1→2 linkage. The attachment of the disaccharide moiety to C-7 of the aglycon was deduced from the HMBC correlation between H-1$GluA_1$ (δ 5.44) and C-7 of the aglycon (δ 164.1). Thus, the structure of **3** was established as apigenin 7-*O*-[β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside].

Compound **1** showed a UV spectrum with maxima at 266 and 323 nm, similar to the spectrum of flavonoid **2**. The mass spectrum showed the molecular ion at *m/z* 797 and peaks at *m/z* 623 and 269, corresponding to the loss of uronic acid and to the aglycon, respectively. It was clear that **1** contained apigenin with three uronic acid moieties. Comparing ^{1}H and ^{13}C NMR data of **1** to those of **3** (Table 1) it was evident that **1** differed from **3** only for the occurrence of a further glucuronic acid unit which was located at C-4′ on the basis of the HMBC correlation between H-1$GluA_3$ (δ 5.26) and C-4′ of the aglycon (δ 161.2). Thus the structure of **1** was established as apigenin 7-*O*-[β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranosyl]-4′-*O*-β-D-glucuronopyranoside.

Compound **7** showed a quasi-molecular ion [M-H]$^-$ at *m/z* 973 and further peaks at *m/z* 797, 445, and 269. Analysis of ^{1}H and ^{13}C NMR (Table 1) spectra of **7** clearly suggested the occurrence of apigenin, three glucuronic acid units, and a ferulic acid moiety. 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. In particular the proton signal at δ 5.10, lowfield shifted as reported for acylated kaempferol glycosides (*18*), was assigned to H-2 $GluA_2$. HSQC experiments, which correlated all the proton resonances with those of each corresponding carbon, allowed the identification of the glycosidation sites by comparison of the observed carbon chemical shifts with those of the corresponding methylpyranosides (*19*). Glycosidation shifts were observed for C-2 $GluA_1$ (δ 82.2) and C-3 $GluA_2$ (δ 83.6). The structure of **7** was definitely determined by the HMBC experiment which showed long-range correlations between H-1$GluA_1$ (δ 5.42) and C-7 of the aglycon (δ 163.8), H-1 $GluA_2$ (δ 5.12) and C-2 $GluA_1$ (δ 82.2), H-1 $GluA_3$ (δ 4.49) and C-3 $GluA_2$ (δ 83.6), and H-2 $GluA_2$ (δ 5.10) and *C*═O of the ferulic moiety (δ 168.5). Thus, **7** was determined as apigenin 7-*O*-{2-*O*-feruloyl-[β-D-glucuronopyranosyl(1→3)]-*O*-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside}.

Compound **6** showed in the FABMS spectrum an identical molecular ion at *m/z* 973 and a very similar degradation pattern to compound **7**, indicating a structure closely related to it. Analysis of ^{1}H and ^{13}C NMR data and comparison with those of **7** clearly revealed that the difference between the two compounds should be confined to the position of the third glucuronic acid unit. The chemical shift of H-1 $GluA_3$ (δ 5.23) as well as the absence of any glycosidation shift for the carbons of $GluA_1$ and $GluA_2$ except that exhibited by C-2 $GluA_1$ (δ 83.4) indicated that $GluA_3$ was linked to the aglycon. The HMBC correlation between H-1 $GluA_3$ (δ 5.23) and C-4′ (δ 161.2) suggested that the third glucuronic acid unit was attached at C-4′. Thus, the structure of **6** was established as apigenin 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranosyl]-4′-*O*-β-D-glucuronopyranoside.

The FABMS spectrum of compound **8** showed a molecular ion at *m/z* 943 with additional peaks at *m/z* 797, 445, and 269, corresponding to the successive loss

of 146 amu, two glucuronic acids, and one glucuronic acid, respectively. Inspection of ^{1}H and ^{13}C NMR data of **8** and comparison with those of **7** suggested that the two compounds differed only in the acyl moiety linked to C-2‴, being in **8** *p*-coumaroyl rather than feruloyl as in **7**. The structure of **8** was thus established as apigenin 7-*O*-{2-*O*-*p*-coumaroyl-[*β*-D-glucuronopyranosyl (1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}.

The UV spectrum of compound **4** showed absorption at 252 and 347 nm, characteristic for luteolin (*16*). Compound **4** showed a molecular ion at *m*/*z* 461 and a peak at *m*/*z* 285 corresponding to the aglycon obtained after glucuronic acid loss from parent molecule. The identification of the aglycon as luteolin was further confirmed by its ^{13}C NMR spectrum which was in a good agreement with the data presented by Harborne (*16*). The glucuronic acid was located at C-7 of the aglycon on the basis of the HMBC correlation between H-1 of the sugar (δ 5.21) and C-7 of the aglycon (δ 164.5). Thus, the structure of **4** was established as luteolin 7-*O*-*β*-D-glucuronopyranoside.

Compound **10** showed a molecular ion at *m*/*z* 987 and additional intensive peaks at *m*/*z* 813, 461, and 285 corresponding to the loss of ferulic acid, ferulic acid, and two glucuronic acid molecules and to the aglycon part (luteolin as in **4**), respectively. Analysis of ^{1}H and ^{13}C NMR data of **10** in comparison to these of **6** clearly suggested that the two compounds possessed an identical sugar portion, and differed only for the aglycon, being luteolin in **10** instead of apigenin as in **6**. Thus, the structure of **10** was established as luteolin 7-*O*-[2-feruloyl-*β*-D-glucuronopyranosyl(1→2)-*β*-D-glucuronopyranosyl]-4′-*O*-*β*-D-glucuronopyranoside.

The UV spectra for compound **5** showed three absorption maxima at 248, 271, and 328 nm, indicating dissimilarity of this compound from the remaining flavones. The FABMS spectrum contained two distinct signals at *m*/*z* 133 and 136 corresponding to $[C_5O_4H_9]^-$ and $[C_5N_5H_6]^-$. On the basis of ^{1}H and ^{13}C NMR, compound **5** was identified as adenosine (*19*).

Some of the above apigenin and luteolin structures have been previously reported in alfalfa (**9**) (*5*) or in other plant sources (**3** (*17*) and **9** (*20*)). However, to our knowledge, compounds **1**, **6**, **7**, **8,** and **10** are novel structures and have never before been reported in the plant kingdom. Furthermore, compounds **1**−**8** and **10** have been identified in *Medicago sativa* aerial parts for the first time. Glucuronoflavones are rare in plants, especially those with apigenin and luteolin as aglycons. Diglucuronoflavones have been identified only in a few plant sources, eg. *Clerodendron trichotomum, Medicago radiata, Adenocalymma alliaceum, Elodea canadensis, Secale cereale,* and *Perilla ocimoides* (*17* and *21*). Derivatives of flavonol, flavones, or anthocyanin glycosides acylated with hydroxycinnamic acid (*p*-coumaric, caffeic, ferulic, and sinapic) of flavonol, flavones, or anthocyanin glycosides have been found in a few plant sources including *Matthiola incana* (*22* and 23), *Brassica oleracea* and *Raphanus napus* (*18* and *24*), *Pisum sativum* (*25*), *Salix gilgiana* (*26*), *Asplenium prolongatum* (*27*), and *Brunfelsia grandiflora* (*28*).

Flavonoid glycosides occur ubiquitously in the plant kingdom and are recognized as UV−B radiation protectors. However, great diversity of the glycoside structures within the plant suggest their function in plant−herbivores association (oviposition and feeding stimulants, toxicity and inhibitory effect for insects) (*29*). Their nutritional importance in food formulas has received much attention (*11*). None of the glycosides acylated with hydroxycynnamic derivatives has been examined with respect to such properties. Because alfalfa is an important crop, both as feed for livestock and as a good raw material for food additive preparations, it seems crucial to characterize its unique flavonoid composition.

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