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FLAVONOIDS FROM *EPHEDRA APHYLLA*

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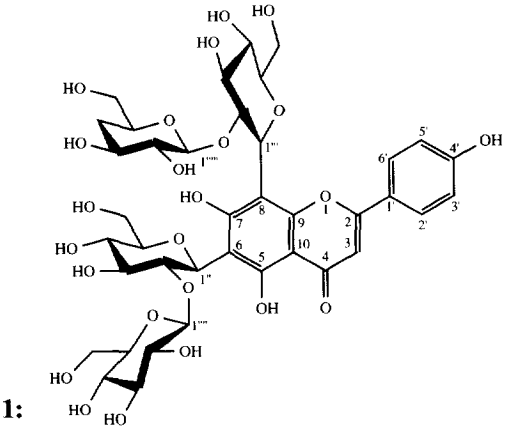
Key Word Index—*Ephedra aphylla*; Ephedraceae; *C*-glycosylflavones; 6,8-di-*C*-sophorosylapigenin; flavonols; herbacetin 3-*O*-rhamnoside-8-*O*-glucoside; herbacetin 7-methyl ether; ESI-mass spectrometry; NMR.

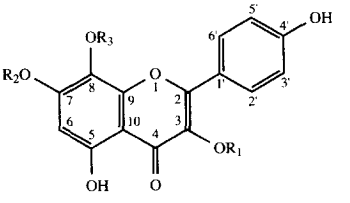
Abstract—A new di-*C*-glucosylflavone, 2″,2‴-di-*O*-β-glucopyranosyl-vicenin II and a new flavonol di-*O*-glycoside, herbacetin 3-*O*-α-rhamnopyranoside-8-*O*-β-glucopyranoside, were isolated from the aerial parts of *Ephedra aphylla*. Vicenin II, the 7-methoxy-4-quinolone 2-carboxylic acid, ephedralone, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic acids and herbacetin 7-methyl ether were also isolated. The ^{13}C NMR spectrum of the latter compound has been assigned for the first time.

INTRODUCTION

As a result of our continuing searches among Egyptian *Ephedra* plants for novel phenolics which might possess biological activity, we were previously able to identify 11 phenolic compounds including herbacetin 8-methyl ether 3-*O*-glucoside 7-*O*-rutinoside, herbacetin 7-*O*-(6″-quinylglucoside) [1] and the quinolone alkaloid, ephedralone from extracts of *Ephedra alata* [2]. We now report the isolation and structure elucidation of the new natural products, 2″,2‴-di-*O*-β-glucopyranosylvicerin II (**1**), and herbacetin 3-*O*-α-rhamnopyranoside 8-*O*-β-glucopyranoside (**2**) from the aqueous ethanolic aerial part extract of *Ephedra aphylla* which grows wild in the sandy dunes of the Sainai peninsula. In addition, the known compounds vicenin II (**3**), ephedralone (**4**), *p*-hydroxybenzoic (**5**), *p*-coumaric (**6**), protocatechuic (**7**) and herbacetin 7-methyl ether (**8**) were also isolated and characterized. it should be noted that only a few ^{13}C NMR spectra have been recorded for 8-oxygenated flavonols [3, 4], and none for herbacetin 7-methyl ether.



1:



2: R_1 = Rha, R_2= H and R_3 = Glc

3: $R_1 = R_3$ = H and R_2 = Me

RESULTS AND DISCUSSION

The phenolics of the aqueous ethanolic aerial part extract of *Ephedra aphylla* were isolated by standard procedures (CC and prep. PC). Two of the separated compounds (**1** and **2**) are new. The remaining compounds (**3**–**8**) are known and exhibited chromatographic, UV spectral, hydrolytic, ESI-mass spectrometry, ^1H and ^{13}C NMR analytical data identical with those of vicenin II [1], ephedralone [2], *p*-hydroxybenzoic, *p*-coumaric, protocatechuic acids [5] and herbacetin 7-methyl ether [6], respectively. The ^{13}C NMR data of **8** were recorded and assigned for the

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Table 1. ^{13}C NMR chemical shifts (ppm) of the isolated *Ephedra aphylla* flavonoids

2″,2‴-Di-*O*-glucosyl vicenin II		Herbacetin 3-*O*-rhamnoside-8-*O*-glucoside		Herbacetin 7-methyl ether	
Aglycone moiety		Algycone moiety			
2	164.31	2	157.42	2	147.50
3	102.82	3	133.59	3	135.57
4	182.49	4	175.92	4	176.57
5	158.75	5	153.84	5	153.72
6	107.60	6	97.88	6	96.80
7	161.37	7	157.42	7	159.39
8	105.43	8	127.91	8	126.15
9	155.26	9	148.67	9	152.40
10	104.09	10	102.70	10	103.52
1′	121.67	1′	121.37	1′	122.04
2′	129.17	2′	130.71	2′	129.85
3′	116.09	3′	115.49	3′	115.62
4′	160.96	4′	159.80	4′	159.39
5′	116.09	5′	115.49	5′	115.62
6′	129.17	6′	130.71	6′	129.85
				OMe	56.03
C-Glucosyl moiety		3-*O*-Rhamnoside moiety			
1″ & 1‴	71.15 & 72.11	1″	102.48		
2″ & 2‴	82.00 & 82.10	2″	70.67		
3″ & 3‴	79.00 & 79.10	3″	70.55		
4″ & 4‴	70.59 & 70.75	4″	71.58		
5″ & 5‴	81.04 & 81.90	5″	70.48		
6″ & 6‴	61.49 & 61.40	Me	17.74		
O-Glucosyl moiety		8-*O*-Glucoside moiety			
1⁗ & 1⁗′	103.10 & 103.20	1‴	102.00		
2⁗ & 2⁗′	73.47 & 74.21	2‴	74.09		
3⁗ & 3⁗′	75.04 & 75.00	3‴	77.80		
4⁗ & 4⁗′	69.28 & 69.40	4‴	69.41		
5⁗ & 5⁗′	77.90 & 77.94	5‴	77.34		
6⁗ & 6⁗′	60.00 & 60.05	6‴	61.08		

first time. The spectrum revealed distinct 13 carbon resonances (Table 1), among which the most upfield one at δ 56.03 was assigned to a methoxyl carbon whose α-aromatic carbon is attached to at least one none oxygenated vicinal (β) carbon. Oxygenation of the vicinal carbons on both side of an aromatic one bearing a methoxyl group would bring the resonance of the methoxyl carbon characteristically downfield to $\simeq$60 ppm. That compound **8** is a flavonol in which the B-ring is hydroxylated at its 4′ position is proved by the carbon resonances at δ 147.5, 135.57 and 176.57 ppm, assignable to C-2, C-3 and C-4, respectively, and by the present pattern of carbon resonances typical for 4′-hydroxylated B ring (Table 1) as well. In the spectrum, the presence of only one carbon resonance in the region from δ 90 to 103, at 95.08 ppm, as well as the presence of carbon resonances at δ 126.15 and at 159.39 could be interpreted only in terms of hydroxylation at position 8 and a methyl ether at position 7 of **8**; an interpretation which agrees well with the chemical shift value of the above described methoxyl carbon resonance. These data confirm the structure of **8** as a herbacetin 7-methyl ether.

The new compound **1**, obtained as a light yellow amorphous powder possessed chromatographic properties and colour reactions similar to those of polyglycosylated apigenin derivatives (Table 2). It showed an $[M+Na-2H]^-$ ion at *m/z* 939.4 in the negative ESI-mass spectrum, corresponding to a M_r of 918. Fragments at *m/z* 755.1 and 593 were also observed. These results, together with the UV spectral data (Table 2) suggested **1** to be a tetrahexoside apigenin whose 5, 7 and 4′ hydroxyl groups are free of substitutents. Consequently, **1** is most probably of *C*-glycosidic nature. Complete acid hydrolysis (methanolic 2 M HCl, for 7 hr) of **1** gave 6,8-di-*C*-glucosylapigenin, or vicenin II (CoPC, UV and ^1H NMR spectral analysis) and glucose (CoPC). The former was also obtained on enzymic hydrolysis by β-glucosidase. Compound **1** is therefore di-*O*-β-glucosylvicenin II. The ^1H NMR spectrum revealed a vicenin II pattern of resonances at δ 6.72 (*s*, H-3); 6.9 (*d*, J = 8 Hz, H-

Table 2. Chromatographic and UV data of flavonoids isolated from *Ephedra aphylla*

Compound	Chromatographic properties R_f-values (× 100)			UV spectral data, λ_{max}(nm)				
	H_2O	HOAc-H_2O	BAW	MeOH	NaOAc	NaOAc–H_3BO_3	$AlCl_3$	NaOMe
2″,2‴-Di-*O*-glucosyl vicenin II	44	59	30	272, 332	280, 390	283, 390	280, 302, 345, 385	274, 360, 405
Vicenin II	23	55	33	272, 333	282, 393	283, 390	280, 305, 345, 383	275, 361, 402
Herbacetin-3-*O*-rhamnoside 8-*O*-glucoside	72	75	26	273, 320, 358	283, 312, 391	276, 318, 364	282, 314, 355, 415	282, 335, 412
Herbacetin-8-*O*-glucoside	10	22	43	276, 330, 378	278, 330sh, 374	282, 323sh, 376	278, 364, 445	287, 336, 425, decomp
Herbacetin-7-methyl ether	00	02	67	278, 332, 382	278, 330, 380	278, 330, 380	280, 370, 444	255sh, 295sh, 370, decomp
Herbacetin	00	04	62	276, 335sh, 380	278, 330sh, 375	285, 320sh, 375	265sh, 280, 370, 448	decomp.

3′ & H-5′) and 8.0 (*d*, $J = 8$ Hz, H-2′ & H-6′). In addition, a complicated multiplet, integrated to four protons revealed itself in the region from δ 4.65 to 4.83 ppm, was assigned to the four anomeric β-glucosyl protons. Other glucose protons (24 protons) together with the hydroxyl and H_2O protons showed resonances as a complex pattern localized in the region from δ 3.12 to 3.92 ppm. ^{13}C NMR spectral analysis exhibited, in addition to the characteristic 6,8-di-*C*-substituted apigenin pattern of carbon resonances (Table 1), a group of 24 glucose carbon resonances, each possessing a distinct chemical shift value. Di-*O*-glucosylation followed from the anomeric carbon signals at δ 103.1 and 103.22, while di-*C*-glucosylation was proved by the C-1 resonances at δ 71.15 and 72.11 ppm. The four carbon resonances localized in this spectrum at δ 60.00, 60.05, 61.49 and 61.40 were assigned to the C-6 carbons in both the *O*-glucosyl and the *C*-glucosyl moieties, respectively, and reflect no substitution of the geminal hydroxyl groups of the C-6 in the *C*-glucosyl moieties. That *O*-glucosylation in **1** is present at each of the C-2 carbons of the *C*-glucosyl moieties was then confirmed by the downfield location of the C-2 carbon resonances in both the 6- and 8-*C*-glucosyl moieties at δ 73.47 and 74.21 ppm (α-effect) as well as by the upfield shift of the signals of the vicinal C-1 and C-3 carbons of the same moieties (β-effect) to δ 71.15, 72.11, 79.10 and 79.00 ppm, respectively (all in comparison with the resonances of the corresponding carbons in 6- and 8-*C*-glucosylapigenin [7]). Other glucosyl carbon resonances, in this spectrum possessed chemical shift values which were in close agreement with the structure of **1** as 2″,2‴-di-*O*-β-glucopyranosyl-6,8-di-*C*-glucopyranosylapigenin, which represents, to the best of our knowledge, a new natural product.

Compound **2** was found to possess chromatographic properties (dull black colour, unchanged by ammonia vapour on PC under UV light) and UV spectral data (Table 2) resembling those reported for 3,8-*O*-substituted herbacetin [8]. Negative ESI-mass spectrometry spectra exhibited a $[M-H]^-$ molecular ion at m/z 609, corresponding to a M_r of 610, in addition to two fragment ions at m/z 462.9 and 447. On acid hydrolysis **2** yielded herbacetin (coPC, UV and ^1H NMR spectral data) together with glucose and rhamnose (coPC). On controlled acid hydrolysis or α-rhamnosidase [9] of (**2**), an intermediate (**2a**) was released, separated (prep. PC) and identified through its chromatographic behaviour, UV spectral data (Table 2) and the results of negative ESI-mass spectrometry analysis ($[M-H]^-$: m/z 463, corresponding to a M_r of 464) to be herbacetin 8-*O*-β-glucoside. These analytical data showed compound **2** to be herbacetin 3-*O*-α-rhamnoside 8-*O*-β-glucoside. The ^1H NMR spectrum of (**2**) was in accordance with the proposed structure. In this spectrum the rhamnose anomeric proton resonance was at δ 5.65 ppm, proving the attachment of this moiety to C-3 of the aglycone herbacetin. The half-line width of this resonance

(*ca* 4 Hz) confirmed the α-configuration at the rhamnose anomeric carbon. The anomeric β-glucoside proton was found resonating as a doublet (J = 8 Hz) at δ 5.05 ppm. Consequently, **2** is identified as herbacetin 3-*O*-α-rhamnoside-8-*O*-β-glucoside. The ^{13}C NMR spectral analysis (Table 1) of **2** finally confirmed this structure.

EXPERIMENTAL

^1H NMR spectra were measured at 400 MHz, and the chemical shifts were measured relative to TMS. ^{13}C NMR resonances were measured relative to DMSO-d_6 and converted to the TMS scale by adding 39.5. Typical conditions: spectral width = 6000 Hz for ^1H and 22 000 Hz for ^{13}C, 32K data points and a flip angle of 45°. ES-MS (negative mode): The direct flow injection technique was applied, sample in MeOH was introduced (1.25 ml min^{-1}) together with MeOH sheath-liquid (5 ml min^{-1}) by a Harvard infusion pump 9 ml min^{-1} SF_6 sheath gas in to the ESI ion source of a Finnigan MAT 4600 Spectrometer. PC was carried out on Whatman no. 1 paper, using solvent systems: (1) H_2O; (2) HOAc–H_2O (3:17); (3) *n*-BuOH–HOAc–H_2O (4:1:5, upper layer); (4) C_6H_6–*n*-BuOH–H_2O–pyridine (1:5:3:3, upper layer). Solvents 2 and 3 were used for prep. PC on Whatman no. #MM paper and solvents 3 and 4 for sugar analysis.

Plant material. Shrubs of *E. aphylla* were collected from the Suez desert in Egypt during March 1995 and classified by Dr L. Boulos, Professor of Botany, National Research Centre, Cairo.

Isolation and identification. Aerial part material was extracted with EtOH–H_2O (3:1). The concd extract was applied to a polyamide 6S CC (Riedel–De Häen AG, Seelze Hanover, Germany) and eluted with H_2O–EtOH mixts of decreasing polarities. The successive eluates were individually dried *in vacuo* and subjected to 2D-PC, Compounds **1** and **2** were isolated pure from the 80:20 fr. through repeated column fractionation over Sepadex LH-20, using H_2O as solvent. Pure **1** was sepd from the 70:30 fr. by crystallization. Pure (**4**–**8**) were isolated pure by prep. PC from the 90:10 fr., using HOAc–H_2O as solvent.

6,8-*Di*-C-*sophorosylapigenin* (**1**). R_f-values: Table 2. UV data: Table 2. M_r 918, ESI-MS: negative ion: m/z 939.4 $[M+Na-2H]^-$, 755.1 [mono-*O*-glucosylvicenin II $-H]^-$, 593 [vicenin II $-H]^-$. **1** was hydrolysed with 2 N aq. methanolic HCl (1:1) at 100° for 7 hr to give vicenin II and glucose. Vicenin II was precipitated from the cold aq. hydrolysate after evaporating the MeOH. Vicenin II: R_f values and UV data: Table 2; ^1H NMR: aglucone moiety: 6.76 (*s*, H-3), 6.92 (*d*, J = 8 Hz, H-3′ & H-5′), 8.0 (*d*, J = 8 Hz, H-2′ and H-6′); sugar moieties: 4.84 (*br s*, $W_{1/2}$ = 16 Hz, H-1″ and H-1‴), 3.08–3.88 (*m*, 12 sugar protons overlapped by OH protons). Hydrolysis of **1** with β-glucosidase (β-glucosidase, from sweet almond meal, Merck) yielded vicenin II (CoPC). ^1H NMR of **1**: 6.72 (*s*, H-3), 6.9 (*d*, J = 8 Hz, H-3′ and H-5′), 8.0 (*d*, J = 8 Hz, H-2′ and H-6′), 4.65–4.83 (*m*, two *O*-anomeric protons and two *C*-anomeric protons), 3.12–3.92 (*m*, 24 glucosyl protons hidden by OH protons). ^{13}C NMR of **1**: Table 1.

Herbacetin 3-O-α-*rhamnopyranoside*-8-O-β-*glucopyranoside* (**2**). R_f-values: Table 2. UV data: Table 2. M_r 610, ESI-MS: negative ion: m/z 609 $[M-H]^-$, 462.9 [M − rhamnosyl moiety]$^-$, 447 [M − glucosyl moiety]$^-$. Acid hydrolysis (2 N aq. HCl, 1000°, 2 hr) of **1** gave glucose, rhamnose (coPC) and herbacetin. R_f-values and UV data of herbacetin: Table 2; ^1H NMR of herbacetin: δ 6.28 (*s*, H-6), 7.0 (*d*, J = 8 Hz, H-3′ and H-5′), 8.2 (*d*, J = 8 Hz, H-2′ and H-6′). Hydrolysis of **2** with α-rhamnosidase (pectinase, from Koch–Light) yielded herbacetin 8-*O*-glucoside. Controlled acid hydrolysis of **2** (0.5 N aq. HCl, 100°, 1/2 hr) afforded also herbacetin 8-*O*-glucoside: R_f-values and UV data: Table 2; ^1H NMR: δ 6.25 (*s*, H-6), 6.9 (*d*, J = 8 Hz, H-3′ and H-5′), 8.1 (*d*, J = 8 Hz, H-2′ and H-6′); M_r 464, ESI-MS: negative ion: 463.0 $[M-H]^-$. ^1H NMR of **2**: herbacetin moiety: δ 6.25 (*s*, H-6), 6.9 (*d*, J = 7.5 Hz, H-3′ and H-5′), 8.0 (*d*, J = 7.5 Hz, H-2′ and H-6′); sugar moieties: δ 5.65 (*br s*, $W_{1/2}$ = 4 Hz, anomeric rhamnose proton), 5.05 (*d*, J = 8 Hz, anomeric glucose proton), 3.1–3.9 (*m*, 10 sugar protons hidden by OH protons), 0.92 (*d*, J = 6 Hz, methyl rhamnose proton). ^{13}C NMR of **1**: Table 1.

Herbacetin 7-methyl ether (**8**). R_f-values and UV data: Table 2. M_r 316, ESI-MS: negative ion: 314.9 $[M-H]^-$. ^1H NMR: δ 6.45 (*s*, H-6), 7.0 (*d*, J = 7.5 Hz, H-3′ and H-5′), 8.2 (*d*, J = 7.5 Hz, H-2′ and H-6′), 3.9 (*s*, OMe-7). ^{13}C NMR: Table 1.

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