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Flavonoid Glycosides and Cannabinoids from the Pollen of *Cannabis sativa* L.

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Chemical investigation of the pollen grain collected from male plants of *Cannabis sativa* L. resulted in the isolation for the first time of two flavonol glycosides from the methanol extract, and the identification of 16 cannabinoids in the hexane extract. The two glycosides were identified as kaempferol 3-*O*-sophoroside and quercetin 3-*O*-sophoroside by spectroscopic methods including high-field two-dimensional NMR experiments. The characterisation of each cannabinoid was performed by GC-FID and GC-MS analyses and by comparison with both available reference cannabinoids and reported data. The identified cannabinoids were Δ^9-tetrahydrocannabiorcol, cannabidivarin, cannabicitran, Δ^9-tetrahydrocannabivarin, cannabicyclol, cannabidiol, cannabichromene, Δ^9-tetrahydrocannabinol, cannabigerol, cannabinol, dihydrocannabinol, cannabielsoin, 6a, 7, 10a-trihydroxytetrahydrocannabinol, 9, 10-epoxycannabitriol, 10-*O*-ethylcannabitriol, and 7, 8-dehydro-10-*O*-ethylcannabitriol.

Keywords: NMR; pollen; flavonoids; cannabinoids; *Cannabis sativa* L.

INTRODUCTION

Cannabis sativa L. is one of the oldest plants known in medicine and one that has been most studied with respect to its phytochemistry (Turner *et al*., 1980). Currently, a total of 483 constituents have been identified in cannabis of which 66 are cannabinoids (Ross *et al*., 1995). About 20 flavonoids have been reported to be present in the cannabis plant as aglycones or as conjugated *O*-glycosides or *C*-glycosides (Paris *et al*., 1973, 1975; Gellert *et al*., 1974; Turner *et al*., 1980; Ross and ElSohly, 1995). The exact structure of most of the glycosides has not been not determined because of the uncertainty of the exact linkage and/or the number of sugar moieties present. Paris *et al.* (1975) reported the detection of two flavonoids in the extracts of pollen grain from *C. sativa*, which provided, upon acid hydrolysis, two genins identified as apigenin and luteolin, indicating the presence of the respective *O*-glycosides in the pollen. Flavonoid glycosides are claimed to be of benefit in treating conditions characterised by capillary bleeding and are also known as powerful antioxidants (Dewick, 2002). Flavonol glycosides are known to have inhibitory activities against HIV-1 reverse transcriptase and HIV-1 integrase (Tewtrakul *et al.*, 2002).

The purpose of the present investigation was to study the composition of the flavonoids and cannabinoids present in the pollen grains of cannabis. Pollen grains collected from male plants of *C. sativa* were successively extracted with *n*-hexane and methanol. The hexane extract was analysed by GC-FID and GC-MS using 4-androstene-3, 17-dione as the internal standard (Ross *et al.*, 1995); 16 cannabinoids could be identified and quantified. Repeated CC over silica gel of the methanol extract, followed by reversed-phase HPLC and separation over Sephadex LH-20 afforded the 3-*O*-sophorosides of kaempferol and of quercetin.

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EXPERIMENTAL

Instrumental analysis. The ^{1}H- and ^{13}C-NMR spectra as well as 2D spectra (COSY, ROESY, HMQC and HMBC) of kaempferol 3-*O*-sophoroside (**11**) and quercetin 3-*O*-sophoroside (**12**) were recorded in DMSO-d_6 and CD_3OD on a Bruker (Billerica, MA, USA) DRX 400 spectrometer operating at 400 MHz for ^{1}H and 125 MHz for ^{13}C. Mass spectra were measured on a Finnegan (Thermo Electric Corp, Waltham, MA, USA) AQLC-MS system. HRESIMS data were acquired on Bruker BioAPEX 30es. Optical rotations were determined using a Perkin-Elmer (Wellesley, MA, USA) model 343 polarimeter, and UV spectra were measured on a Hewlett-Packard (Palo Alto, CA, USA) model 8453 spectrophotometer.

TLC separations were performed on Merck (Darmstadt, Germany) TLC plates pre-coated with $Si_{60}F_{254}$. Visualisation was achieved by spraying with 5% sulphuric acid in ethanol followed by heating. HPLC analyses were carried out on Waters (Milford, MA, USA) 486 system interfaced to a UV detector or a Waters 2487 Dual λ Absorbance Detector: a normal phase NP-PREP column (250 × 21.2 mm i.d.; 5 µm/300 × 57 mm i.d.; 55 µm. Plenomenex, Torrance, CA, USA) was employed. For GC-FID analyses, a Hewlett-Packard 5880A gas chromatograph equipped with a capillary injector (with Merlin Microseal) and a dual flame ionisation detector was employed. The same GC interfaced with an HP model 5970A mass selection detector (MSD)

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was used for GC-MS analyses. Both GC-FID and GC/MS systems were fitted with a J&W Scientific (Folsom, CA, USA) DB-1 column (15 m × 0.25 mm i.d.; 0.25 μm film thickness). The operating conditions were: GC splitless mode; the initial temperature was 170°C held for 1 min, then raised to 250°C at 10°C/min, and then held for 10 min; the detector temperature was 260°C; the carrier gas was helium at a flow rate of ca. 1 mL/min; the flow rate of hydrogen in the FID was 30 mL/min; the make-up gas was helium at a flow rate of 20–30 mL/min.

Standard cannabinoids. Ten cannabinoid standards (Δ^9-tetrahydrocannabinol, Δ^8-tetrahydrocannabinol, Δ^9-tetrahydrocannabivarin, cannabidiol, cannabichromene, cannabigerol, cannabinol, cannabicyclol, cannabielsoin and cannabitriol) were obtained from the National Institute on Drug Abuse (NIDA) or isolated in-house from plant material. Solutions of standard cannabinoids were prepared in 100 μg/mL concentrations in methanol.

Plant material and extraction. A Mexican variety of *Cannabis sativa* L. was cultivated in the experimental garden of the School of Pharmacy, University of Mississippi. The pollen was collected in the summer of 1995 and kept under refrigeration until required for use. Pollen grains (200 g) were extracted three times by maceration overnight at room temperature with hexane (500 mL each extraction). Defatted pollen was extracted with methanol (500 mL × 2) and the combined methanol extract concentrated under vacuum to afford 52.2 g of residue (26.1% yield).

Isolation of flavonoid glycosides. The methanol extract (40 g) was chromatographed on a silica gel column (800 g silica gel) using a chloroform: methanol step gradient. Four fractions were collected: fraction C_2F_1 (9.24 g) eluted with 80:20 (chloroform: methanol), C_2F_2 (2.45 g), with 70:30, C_2F_3 (1.78 g) with 50:50, and C_2F_4 (30.04 g) with 30:70. The whole fraction C_2F_2 was rechromatographed using a chloroform: methanol step gradient (from 100:0 to 0:100) on a silica gel column (140 g silica gel) and six fractions were collected, namely C_3F_1 (0.3 g) eluted with 80:20 (chloroform: methanol), C_3F_2 (0.75 g) with 80:20, C_3F_3 (0.55 g) with 75:25, C_3F_4 (0.3 g) with 75:25, C_3F_5 (0.3 g) with 70:30, and C_3F_6 (0.1 g) with 60:40. Fraction C_3F_2 was then separated by normal phase preparative HPLC using chloroform: methanol (85:15) as mobile phase at a flow rate of 20 mL/min to yield three major fractions, i.e. C_3F_2A (35.0 mg), C_3F_2B (12.0 mg) and C_3F_2C (98.0 mg). Repeated separation of C_3F_2C (98.0 mg) on a Sephadex LH-20 column (115 × 3 cm i.d.) using methanol as the eluent yielded two pure compounds **1** (27.8 mg) and **2** (19.0 mg). These compounds (10 mg each) were separately hydrolysed according to a previously published procedure (El-Sayed *et al*., 2002). The acid hydrolysis of **1** yielded kaempferol as the aglycone, whilst **2** produced quercetin as the aglycone. Both compounds produced glucose as the sugar moieties.

RESULTS AND DISCUSSION

Pollen grains collected from male plants of *Cannabis sativa* L were successively extracted with *n*-hexane and methanol. The hexane extract was analysed by GC-FID and GC-MS using 4-androstene-3, 17-dione as the internal standard (Ross *et al.*, 1995) and 19 peaks were detected. The characterisation of each cannabinoid was performed by comparison against reference cannabinoids and/or reported data. Out of the detected 19 peaks, 16 cannabinoids were identified and quantified (Table 1), namely, Δ^9-tetrahydrocannabinol, cannabichromene, cannabicitran, cannabinol, cannabigerol, cannabidiol, cannabicyclol, Δ^9-tetrahydrocannabivarin, cannabidivarin, dihydrocannabinol, cannabielsoin, 9,10-epoxycannabitriol, Δ^9-tetrahydrocannabiorcol, 10-*O*-ethylcannabitriol, 6a,7,10a-trihydroxytetrahydrocannabinol and 7,8-dehydro-10-*O*-ethylcannabitriol. From Table 1, it is clear that Δ^9-tetrahydrocannabinol was the predominant cannabinoid present in the pollen and constituted 81% of the total cannabinoids, followed by cannabichromene (8.3%), cannabinol (3.6%) and cannabigerol (3.4%).

Compound 1

Yellow solid (27.8 mg); $[\alpha]_D^{26.6}$ –22.3° (MeOH, *c* 0.67); UV (MeOH) λ_{max} (nm) 214, 266, 344; ESIMS *m/z* 609 $[M\text{-}H]^+$. The ^{1}H- and ^{13}C-NMR in (CD_3OD) were comparable with data published for kaempferol-3-*O*-sophoroside (Markham *et al.*, 1978).

Compound 2

Yellow solid (19.0 mg); $[\alpha]_D^{26.6}$ –16.7° (MeOH, *c* 0.34); UV (MeOH) λ_{max} (nm) 216, 256, 354. ESIMS *m/z* 625 $[M\text{-}H]^+$. The ^{1}H- and ^{13}C-NMR in (CD_3OD) were comparable with data published for quercetin-3-*O*-sophoroside (Gluchoff-Fiasson *et al.*, 1997).

Compound **1** was obtained as a yellow amorphous solid. In the ESIMS, **1** showed an ion $[M\text{-}H]^+$ at *m/z* 609 in the negative mode suggesting a molecular formula of $C_{27}H_{30}O_{16}$, which is 16 mass units less than that of **2**. The UV spectra of **1** also showed characteristic flavone absorption maxima at 216, 256 and 354 nm. Analysis of the ^{1}H-NMR together with ^{1}H-^{1}H-COSY spectra of **1** showed the presence of two AM type spin systems at δ 6.18 (br s) and 6.43 (br s), assigned to a meta substituted A ring, and the other at δ 6.88 (2H, d, J = 8.2 Hz) and 8.00 (2H, d, J = 8.2 Hz) assigned to a 4′ substituted C ring of a flavone nucleus. Similarly, analysis of the DEPT, HMQC and HMBC spectra suggested that the aglycone portion of **1** was a substituted kaempferol. In H-NMR spectra, **1** also showed the two characteristic anomeric proton peaks at δ 5.56 (d, J = 6.4 Hz.) and 4.59 (d, J = 6.4 Hz) and 12 proton multiplet peaks at δ 3.03 ≈ 3.46. Comparing the NMR data of **1** with that of **2** and analysing the DEPT, HMQC and HMBC spectra, **1** was determined to be kaempferol-3-*O*-sophoroside (El-Sayed *et al*., 2002).

Compound **2** was obtained as yellow amorphous solid. In the ESIMS, **2** exhibited an ion $[M\text{-}H]^+$ at *m/z* 625 in the negative mode suggesting a molecular formula of $C_{27}H_{30}O_{17}$ with 13 degree of unsaturation (Gluchoff-Fiassson *et al.*, 1997). The UV spectra of **2** showed characteristic flavonol absorption maxima at 216, 256 and 354 nm. Analysis of the ^{1}H-NMR together with the ^{1}H-^{1}H-COSY spectra showed the presence of a two-spin system around the aromatic region. The first spin system of

Table 1. GC-UV and GC-MS data from the analysis of the hexane extract of pollen grains from *Cannabis sativa* L.

Peak number	GC-UV Retention time (min)	Relative Retention time (min)[a]	GC-MS Retention time (min)	Amount (g %)	$[M]^+$	Base peak	Other significant ions or fragments	Identification
1	6.28	0.602	3.996	0.004	258	243	215, 201, 175	Δ^9-Tetrahydrocannabiorcol
2	7.03	0.674	4.311	<0.003	286	203	271, 174	Cannabidivarin
3	7.45	0.714	4.475	0.007	314	231	299, 271, 258	Cannabicitran
4	7.70	0.738	4.557	0.051	286	271	243, 203	Δ^9-Tetrahydrocannabivarin
5	7.72	0.740	4.628	0.005	314	231	299, 271, 260, 245, 207,174	Unknown
6	8.19	0.785	4.768	0.017	314	231	299, 267, 174	Cannabicyclol
7	8.59	0.824	4.944	0.044	314	231	299, 246, 192, 174	Cannabidiol
8	8.69	0.833	5.004	0.324	314	231	299, 174	Cannabichromene
9	8.88	0.851	5.098	0.005	328	231	313, 285, 258, 213, 187, 174	Unknown
10	9.00	0.863	5.169	0.026	314	299	271, 258, 243, 231	Unknown
11	9.46	0.907	5.430	3.123	314	299	271, 258, 243, 231	Δ^9-Tetrahydrocannabinol
12	9.91	0.950	5.680	0.131	316	193	297, 273, 247, 231, 219	Cannabigerol
13	10.01	0.960	5.751	0.135	310	295	250, 238, 230, 193	Cannabinol
14	10.10	0.970	5.811	<0.003	312	312	297, 269, 256, 231, 213, 193	Dihydrocannabinol
15	10.93	1.048	6.167	<0.003	330	247	315, 287, 231	Cannabielsoin
16	11.21	1.075	6.266	<0.003	362	207	344, 327, 304, 286, 273	6a,7,10a -Trihydroxy-tetrahydrocannabinol
17	11.47	1.100	6.533	0.005	328	328	313, 285, 271, 231, 193	9,10-Epoxycannabitriol
18	11.68	1.112	6.663	0.003	374	332	348, 315, 297, 259, 247, 231, 209, 193, 175	10-*O*-Ethylcannabitriol
19	13.59	1.303	7.747	<0.003	372	342	315, 285, 235, 205	7,8-Dehydro-10-*O*-ethylcannabitriol

[a] Relative to the internal standard.

the AM type at δ 6.19 (d, J = 1.9 Hz) and 6.38 (d, J = 1.9 Hz) was assigned to a meta substituted A ring, and the other AMX type spin system at δ 6.89 (d, J = 8.4 Hz.), 7.67 (d, J = 1.9 Hz.) and 7.54 (dd, J = 1.9, 8.4 Hz) was assigned to a 4′, 5′ di-substituted C ring of a flavone nucleus. Analysis of the DEPT, HMQC and HMBC spectra suggested that the aglycone part of **2** was a substituted quercetin. In the ^{1}H-NMR spectra, two peaks characteristic for anomeric protons at δ 5.28 (d, J = 6.4 Hz) and 4.76 (d, J = 6.4 Hz) and 12 proton multiplet peaks at δ 3.30 ≈ 3.43 indicated the presence of two sugar moieties in this flavonoid. The ^{13}C-NMR shift of the sugar unit was very similar to that of sophoroside in quercetin-3-*O*-sophoroside. The proton at δ 5.28 (H-1″) showed a cross peak to the carbon at C-3 (d 134.14) whilst the proton at 4.76 (H-1‴) showed a cross peak to the carbon at C-2″ (d 81.99). Therefore, **2** was assigned to be quercetin-3-*O*-sophoroside (Markham *et al.*, 1978).

Compounds **1** and **2** were tested for anti-malarial, anti-microbial and anti-inflammatory activities, but no significant activities were demonstrated even though flavonoid glycosides are known to have potential anti-oxidant (Plumb *et al.*, 1997), anti-bronchospastic and anti-inflammatory activities (Circosta *et al.*, 1990, 1992).

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