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# EUPAFOLIN RHAMNOSIDES FROM *KALANCHOE GRACILIS*

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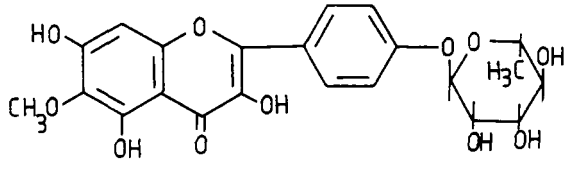
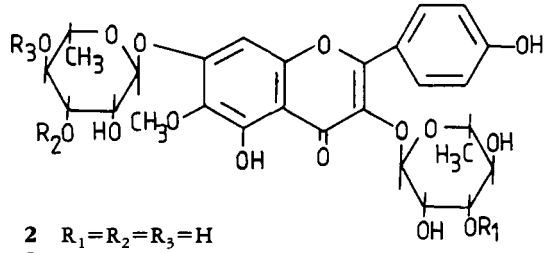
ABSTRACT.—Phytochemical studies on aerial parts of *Kalanchoe gracilis* resulted in the isolation of nine flavonoids and one coumarin. Among the nine flavonoids, four new flavonoids were identified as eupafolin-4′-*O*-rhamnoside [**1**], eupafolin-3,7-di-*O*-rhamnoside [**2**], eupafolin-3-*O*-rhamnosyl-7-*O*-(4-*O*-acetylrhamnoside) [**3**], and eupafolin-3-*O*-(3-*O*-acetylrhamnosyl)-7-*O*-(3-*O*-acetylrhamnoside) [**4**]. The five known compounds were determined as luteolin, quercetin, quercitrin, kaempferol, and eupafolin, based on spectroscopic analysis. The coumarin was identified as 7-hydroxycoumarin.

*Kalanchoe gracilis* Hance (Crassulaceae) is used as a traditional medicine in the treatment of tissue injuries (1,2) in Taiwan. In previous phytochemical studies of the aerial parts of *K. gracilis* (3), the major isolatable constituents proved to be flavonoids. Flavonoids are known to be anti-inflammatory and to have effects on blood circulation (4,5) and therefore the flavonoid constituents of this herb may be considered to be important in its medicinal uses. The presence of flavonoids in the genus *Kalanchoe* has been reported in the literature. Quercetin, kaempferol (6), quercetin 3-diarabinoside, and kaempferol 3-glucoside (7) were isolated from *Kalanchoe pinnata*. Cyanidin 3,5-diglucoside, cyanidin 3-monoglucoside, pelargonidin 3,5-diglucoside, and leucocynidin were isolated from *Kalanchoe blossfeldiana* (8), and kaempferol-coumaroyl arabinoside was found in *Kalanchoe daigremontiana* (9). Recently, patuletin 3,7-di-*O*-rhamnoside was isolated from *Kalanchoe spathulata* (10).

The flavonoids isolated previously from *K. gracilis* in our study (3) were patuletin, its rhamnosides, and novel acetylrhamnosides. They are patuletin, patuletin-3-*O*-rhamnoside, patuletin-3-*O*-rhamnosyl-7-*O*-(3-*O*-acetylrhamnoside), patuletin-3-*O*-rhamnosyl-7-*O*-(4-*O*-acetylrhamnoside), patuletin-3-*O*-rhamnosyl-7-*O*-(3,4-*O*-diacetyl-



**1**



**2** $R_1=R_2=R_3=H$
**3** $R_1=R_2=H$, $R_3=Ac$
**4** $R_1=R_2=Ac$, $R_3=H$

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rhamnoside), patuletin-3-*O*-(4-*O*-acetylrhamnosyl)-7-*O*-(3-*O*-acetylrhamnoside), patuletin-3-*O*-(3-*O*-acetylrhamnosyl)-7-*O*-(3-*O*-acetylrhamnoside), patuletin-3-*O*-(4-*O*-acetylrhamnosyl)-7-*O*-(3,4-diacetylrhamnoside), and patuletin-3-*O*-(4-*O*-acetylrhamnosyl)-7-*O*-(2,4-*O*-diacetylrhamnoside). In continuation of the investigation of the EtOAc extract of *K. gracilis*, one coumarin and nine additional flavonoids were isolated, including novel mono-acetylrhamnosides and diacetylrhamnosides of eupafolin. The structural elucidations were based on uv, $^1$H-nmr, and fabms analysis.

## RESULTS AND DISCUSSION

The concentrate from the EtOH extract of the dried plant material was successively partitioned between petroleum ether and $H_2O$, $CHCl_3$ and $H_2O$, and EtOAc and $H_2O$. The residue obtained from the EtOAc portion was fractionated on a polyamide column. Each flavonoid-containing fraction, which was monitored on tlc, was further separated by a combination of Si gel and Sephadex LH-20 cc. Nine other flavonoids and one coumarin were isolated. The coumarin compound was found to be 7-hydroxycoumarin. Five flavonoids were identified as luteolin, quercetin, quercitrin, kaempferol (11), and eupafolin (12) based on comparisons of their spectra with published data. The other four flavonoid glycosides are compounds **1–4**, new derivatives of eupafolin.

Compound **1** was observed as a yellow spot on tlc under $uv_{366}$, indicating a flavonol. In the $^1$H-nmr spectrum (Table 1), the presence of a double doublet signal for H-2′, H-6′ at δ 8.17 (2H, dd, $J = 1, J = 9$) and a double doublet signal for H-3′, H-5′ at δ 6.93 (2H, dd, $J = 1, J = 9$) was evidence for a 4′-oxygenated substituent on the B ring. The

TABLE 1. $^1$H-nmr Spectral Data of Compounds **1**, **2**, **3**, and **4**.[a]

| Proton | Compound | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| eupafolin aglycone | | | | |
| 6-OMe | 3.93 s | 3.87 s | 3.90 s | 3.93 s |
| H-8 | 6.65 s | 6.65 s | 6.67 s | 6.65 s |
| H-2′ | 8.17 dd (1,9) | 7.83 dd (1,9) | 7.85 dd (1,9) | 7.9 dd (1,9) |
| H-3′ | 6.93 dd (1,9) | 6.95 dd (1,9) | 6.96 dd (1,9) | 6.99 dd (1,9) |
| H-5′ | 6.93 dd (1,9) | 6.95 dd (1,9) | 6.96 dd (1,9) | 6.99 dd (1,9) |
| H-6′ | 8.17 dd (1,9) | 7.83 dd (1,9) | 7.85 dd (1,9) | 7.9 dd (1,9) |
| C-3-*O* rhamnose[b] | | | | |
| H-1 | 5.57 d (1) | 5.40 d (1) | 5.41 d (1) | 5.48 d (1) |
| H-2 | 4.10 dd (2,5) | 4.11 dd (2,5) | 4.13 dd (2,5) | 4.27 dd (2,5) |
| H-3 | 3.91 dd (2,10) | 3.74 dd (2,10) | 3.73 dd (2,10) | 5.02 dd (2,10) |
| H-4 | 3.50 t (10) | 3.34 t (10) | 3.1–4.0 m | 3.56 t (10) |
| H-5 | 3.5–3.8 m | 3.5–3.8 m | 3.1–4.0 m | 3.3–3.9 m |
| 6-Me | 1.28 d (7) | 0.94 d (7) | 0.95 d (7) | 0.90 d (7) |
| 3-OAc | | | | 2.15 s |
| C-7-*O* rhamnose | | | | |
| H-1 | | 5.57 d (1) | 5.62 d (1) | 5.60 d (1) |
| H-2 | | 4.25 dd (2,5) | 4.23 dd (2,5) | 4.41 dd (2,5) |
| H-3 | | 3.9 dd (2,10) | 4.05 dd (2,10) | 5.22 dd (2,10) |
| H-4 | | 3.51 t (10) | 5.05 t (10) | 3.71 t (10) |
| H-5 | | 3.5–3.8 m | 3.1–4.0 m | 3.3–3.9 m |
| 6-Me | | 1.27 d (7) | 1.15 d (7) | 0.99 d (7) |
| 3-OAc | | — | — | 2.17 s |
| 4-OAc | | — | 2.09 s | — |

[a]The spectra were recorded at 250 MHz in $CD_3OD$. Chemical shifts are in ppm from TMS, and coupling constants are in parentheses in Hz.

[b]C-4′-*O*-rhamnose for compound **1**.

signals for H-8 at δ 6.65 (1H, s) and for one methoxyl proton at δ 3.93 (3H, s) were observed, which supported a flavonoid with substitutions at C-5, C-6, and C-7 on ring A. The uv spectrum of **1** in NaOMe exhibited a bathochromic shift of 66 nm in the band I region with a decrease in intensity as compared with the spectrum in MeOH, indicating the presence of a 4′-hydroxyl group which was substituted.

The fabms spectrum of **1** showed a molecular ion at *m/z* 463 $[M+H]^+$. The fragment at *m/z* 317 $[AH]^+$ suggested the presence of one methoxyl and four hydroxyl groups in the aglycone. Furthermore, the fragment at *m/z* 301 $[A-15]^+$ with lower intensity than that of the *m/z* 317 ion indicated that the methoxyl group was at the C-6 position (13). The presence of a 4′-oxygenated substituent and a C-3 with a free hydroxyl group, determined from uv analysis, meant that the other two hydroxyl groups had to be at the C-5 and C-7 positions. The substitution of C-4′ was revealed in the $^1$H-nmr spectrum (Table 1); the downfield signal for an anomeric proton of a sugar appeared at δ 5.57 (1H, d, $J=1$), together with signals in the upfield region for sugar protons H-2 at δ 4.10 (1H, dd, $J=2, J=5$), H-3 at δ 3.91 (1H, dd, $J=2, J=10$), H-4 at δ 3.50 (1H, t, $J=10$), and H-5 at δ 3.5–3.8 (1H, m), indicating a glycoside. The characteristic doublet signal at δ 1.28 (3H, d, $J=7$) for methyl protons confirmed that the glycoside was a rhamnoside. Therefore, compound **1** was determined as eupafolin 4′-*O*-rhamnoside.

The $^1$H-nmr spectrum for compound **2** was similar in the aromatic region to that for compound **1**, suggesting another substituted eupafolin (Table 1). However, the uv spectrum of **2** exhibited a bathochromic shift of 40 nm of band I with an increase of intensity after addition of NaOMe, demonstrating a free 4′-hydroxyl group. The $^1$H-nmr also exhibited signals suggesting substitution with rhamnose. The two doublet signals at δ 1.27 (3H, d, $J=7$) and 0.94 (3H, d, $J=7$), typical for rhamnose methyl protons, indicated the substitution of the aglycone with two rhamnose moieties. The signals for protons on two sugar moieties were clearly observed. The two downfield signals for the anomeric protons appeared at δ 5.57 (1H, d, $J=1$) and δ 5.40 (1H, d, $J=1$); the signals for two H-2 were located at δ 4.25 (1H, dd, $J=2, J=5$) and δ 4.11 (1H, dd, $J=2, J=5$); the signals for two H-3 were located at δ 3.90 (1H, dd, $J=2, J=10$) and δ 3.74 (1H, dd, $J=2, J=10$), and signals for two H-4 appeared at δ 3.51 (1H, t, $J=10$) and δ 3.34 (1H, t, $J=10$). In the fabms spectrum, the $[M+Na]^+$ ion appeared at *m/z* 631, and the fragmentation peaks at *m/z* 463 $[M+H-\text{rhamnose}]^+$ and 317 $[M+H-2\times\text{rhamnose}]^+$ confirmed the presence of two rhamnose moieties. In addition, the fragment at *m/z* 301 [A − 15] also supported the assignment of a methoxyl substitution at C-6. The appearance of dark purple color under $uv_{366}$ on tlc and the absence of bathochromic shift after the addition of NaOAc in the uv spectrum indicated that both C-3 and C-7 were substituted. It was concluded that the rhamnoses were attached separately on the C-3 and C-7 positions of the aglycone. On the basis of these data, compound **2** was assigned the structure of eupafolin 3,7-di-*O*-rhamnoside.

The uv spectra of **3** with diagnostic reagents exhibited the same characteristics as that of **2**, indicating a free 4′-hydroxyl group on the B ring of **3**.

The $^1$H-nmr spectrum of **3** (Table 1) was almost identical to that for compound **2** except that there was a singlet at δ 2.09, typical for methyl protons of an acetyl group. The acetyl group was considered to be on the sugar moiety. In fact, the downfield shift of a triplet signal at δ 5.05 (1H, t, $J=10$) compared with compound **2** was evidence for the H-4 of either the 3-*O*- or 7-*O*-rhamnose moiety (14). In the fabms spectrum, the molecular ion at *m/z* 651 $[M+H]^+$ demonstrated **3** to be a eupafolin glycoside containing one acetyl group and two rhamnose moieties. The significant ion at *m/z* 505 due to the loss of one rhamnose from C-3 and the fragment ion at *m/z* 317 due to a further loss of one acetylrhamnose indicated that the acetylated rhamnose moiety was on the oxygen

of C-7; therefore acetylation was at the 4-*O*-position of this rhamnose moiety. The structure of **3** was thus assigned as eupafolin 3-*O*-rhamnosyl-7-*O*-(4-*O*-acetylrhamnoside).

The $^1$H-nmr spectra of **4** indicated a compound almost identical with **2**. The only difference occurred at the two signals for acetyl groups at δ 2.17 (3H, s) and δ 2.15 (3H, s), which showed the presence of one more acetyl group in **4** than **3**. In the downfield region, two signals for anomeric protons at δ 5.60 and δ 5.48 were observed. Two double doublet signals for H-3 of the 3-*O*- and 7-*O*- rhamnose moieties appeared at δ 5.22 (1H, dd, $J=2, J=10$) and δ 5.02 (1H, dd, $J=2, J=10$). The downfield shifts suggested that the C-3 hydroxyls on both rhamnose units were acetylated (14). The signals for H-2, δ 4.41 (1H, dd, $J=2, J=5$) and δ 4.27 (1H, dd, $J=2, J=5$), and the signals for H-4, δ 3.71 (1H, t, $J=10$) and δ 3.56 (1H, t, $J=10$), of both rhamnose moieties appeared as expected; they were in the same regions as the corresponding rhamnose protons of **2**. Therefore compound **4** was identified as eupafolin-3-*O*-(3-*O*-acetylrhamnosyl)-7-*O*-(3-*O*-acetylrhamnoside).

The additional compounds were identified as luteolin, quercetin, quercitrin, kaempferol, and eupafolin by correspondence of the uv, $^1$H-nmr, and ms spectra with published data (11,12). The final compound was determined as 7-hydroxycoumarin by comparison of the uv spectral data and tlc with an authentic sample (15).

The flavonoids have well documented anti-inflammatory activity (4), with evidence that luteolin, quercetin, and rutin are particularly effective inhibitors of lipoxygenation and prostaglandin synthesis (4); quercetin is also a known inhibitor of platelet aggregation (16). All these factors are important during tissue damage. Furthermore quercitrin is known to have a capillary stabilizing effect (5), and using the method of Tajima *et al.* (17) it has been shown to have antihemorrhagic activity (18). Quercitrin represented 0.35% of the flavonoid content of the EtOAc fraction from 2.5 kg (dry wt) of *K. gracilis*. The premise that the flavonoids of *K. gracilis* are particularly responsible for the plant's use in traditional medicine may therefore be a valid one. Because the acetylated rhamnosides of patuletin and eupafolin represent 6.79% of the EtOAc fraction, their potential biological activities in this area and their bioavailability as compared with the medicinally used ethoxylated derivatives of rutin are of some interest.

## EXPERIMENTAL

PLANT MATERIAL.—The aerial parts of *K. gracilis* were obtained in 1987, from the medicinal plant garden of the School of Pharmacy, National Taiwan University, Taipei, Taiwan. An herbarium specimen, identified by Mr. Y.H. Tsen, was kept in the Pharmacognosy Laboratory of the School.

GENERAL EXPERIMENTAL PROCEDURES.—Tlc was performed on Si gel 60 $F_{254}$ plates (Merck) using the solvent system $CHCl_3$-MeOH-butanone-$Me_2CO$ (60:20:10:5) and $CHCl_3$-MeOH-butanone-$H_2O$ (40:20:10:1). The spots were visualized under $uv_{366}$ and fumed with $NH_3$ vapor. $^1$H-nmr spectra were recorded at 250 MHz with TMS as internal standard on a Brucker NM 250 instrument. Mass spectra were obtained on a VG Analytical Ltd. ZAB-IF mass spectrometer. Uv spectra were obtained on a Perkin-Elmer 402 spectrophotometer following standard procedures, as previously described (19).

EXTRACTION AND ISOLATION.—The dried plant material (2.5 kg) was extracted with 95% EtOH at room temperature. After removal of the solvent, $H_2O$ was added and the crude extract was partitioned successively between petroleum ether and $H_2O$, $CHCl_3$ and $H_2O$, and EtOAc and $H_2O$. The EtOAc extract (15 g) was chromatographed on a polyamide column using $CHCl_3$ as eluent followed by increasing concentrations of MeOH. The eluates of $CHCl_3$-MeOH (75:25 and 50:50) were further chromatographed on a Si gel (230 mesh) column with a $CHCl_3$/MeOH/butanone mixture of increasing polarity. The fractions containing flavonoids were rechromatographed on Sephadex LH-20. Compounds were isolated and purified through repeated chromatographic separations.

*Eupafolin-4'-O-rhamnoside* [**1**].—Uv λ max (MeOH) 270, 327, 360; (NaOMe) 263, 426; ($AlCl_3$) 266, 350, 430; ($AlCl_3$ + HCl) 265, 350, 430; (NaOAc) 270, 350; (NaOAc/$H_3BO_3$) 270, 350; fabms *m/z* (rel. int.) $[M+H]^+$ 463 (7), $[M+H-146]^+$ $[AH]^+$ 317 (53), $[A-15]^+$ 301 (21), 223 (54), 207 (100).

*Eupafolin-3,7-di-O-rhamnoside* [**2**].—Uv λ max (MeOH) 230 sh, 269, 325, 348 sh; (NaOMe) 252 sh, 269, 388; ($AlCl_3$) 238, 278, 307, 345; ($AlCl_3$ + HCl) 238, 278, 307, 342; (NaOAc) 270, 340; ($NaOAc/H_3BO_3$) 270, 340; fabms *m/z* (rel. int.) $[M+H+Na]^+$ 631 (10), $[M+H-146+Na]^+$ 485 (14), $[M+H-146]^+$ 463 (17), 413 (48), $[M+H-292]^+$ $[AH]^+$ 317 (41), $[A-15]^+$ 301 (34), 223 (100).

*Eupafolin-3-O-rhamnosyl-7-O-(4-O-acetylrhamnoside)* [**3**].—Uv λ max (MeOH) 226 sh, 269, 325, 345; (NaOMe) 253 sh, 267, 388, 396 sh; ($AlCl_3$) 238, 278, 305, 345; ($AlCl_3$ + HCl) 238, 278, 305, 342; (NaOAc) 269, 350; ($NaOAc/H_3BO_3$) 269, 350; fabms *m/z* (rel. int.) $[M+H]^+$ 651 (27), $[M+H-146]^+$ 505 (27), $[M+H-334]^+$ $[AH]^+$ 317 (100), $[A-15]^+$ 301 (25), 147 (23).

*Eupafolin-3-O-(3-O-acetylrhamnosyl)-7-O-(3-O-acetylrhamnoside)* [**4**].—Uv max (MeOH) 270, 328; (NaOMe) 270, 388; ($AlCl_3$) 278, 345; ($AlCl_3$ + HCl) 278, 342; (NaOAc) 270, 340; ($NaOAc + H_3BO_3$) 270, 340; fabms *m/z* (rel. int.) $[M+H-146]^+$ 651 (27), $[M+H-334]^+$ $[AH]^+$ 317 (100), $[A-15]^+$ 301 (25), 147 (23).

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