JOURNAL OF NATURAL PRODUCTS

Volume 62, Number 3 March 1999

Full Papers

Immunomodulatory Principles of *Dichrocephala bicolor*

Lie-Chwen Lin, Yuh-Chi Kuo, and Cheng-Jen Chou*

National Research Institute of Chinese Medicine, Shih-Pai, Taipei 11221, Taiwan, Republic of China

Received April 17, 1998

From the ethanolic extract of *Dichrocephala bicolor* eight compounds—4,5-dicaffeoyl quinic acid (**1**); 3,4-dicaffeoyl quinic acid (**2**); 3,5-dicaffeoyl quinic acid (**3**); ethyl 4,5-dicaffeoyl quinate (**4**); methyl 3,5-dicaffeoyl quinate (**5**); 5-caffeoyl quinic acid (**6**); caffeic acid (**7**); and quercetin-3-*O*-rutinoside (**8**)—were isolated and identified. All of them were selected for immunopharmacological activity tesing. Human mononuclear cells (HMNC) were used as target cells. Cell proliferation was determined by ^{3}H-thymidine uptake. Compounds **2** and **6** potently enhanced HMNC proliferation and interferon-γ production. Enhancement mechanisms may involve the increase of cytokines production.

Dichrocephala bicolor (Roth) Schltdl. (Asteraceae), an annual herbaceous plant of the Compositae family, is widely distributed at altitudes ranging from 500 to 3000 m in Taiwan.[1] It is used as a folk medicine in Taiwan for pneumonia, hypertension, fever, and urotoxia.[2] Although *D. bicolor* has been commonly used in traditional Chinese medicine for a long time, there is relative scarcity of definitive evidence to prove its pharmacological activity.

In the inflammatory process, early responses provide protection by restricting the tissue damage to the site of infection or tissue injury.[3] Several immune cells, including lymphocytes, neutrophils, monocytes, eosinophils and basophils, are involved in this response.[4] They secrete cytokines at the site of inflammation, which then participate in the clearance of the antigen and healing of the tissue.[4] Interferon-γ (IFN-γ) is one of the most important inflammatory factors for the attraction of immune cells.[5] Both leukocytes and cytokines play important roles in inflammatory response regulation. Thus, in our studies, the human mononuclear cells (HMNC) were used as target cells. The effects of those compounds isolated from *D. bicolor* on HMNC proliferation and IFN-γ production were determined.

In this study, we report the isolation of a series of caffeoylquinic acids from *D. bicolor*. Furthermore, the results of immunopharmacological assays of individual caffeoylquinic acids are presented.

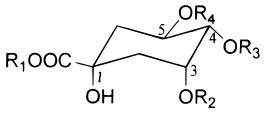
Results and Discussion

The ethanolic extract of *D. bicolor* was partitioned between water and organic solvents. Column chromatography of the EtOAc and *n*-BuOH extracts yielded seven caffeoyl compounds and one flavonol glycoside. They are 4,5-di-*O*-caffeoylquinic acid (**1**); 3,4-di-*O*-caffeoylquinic acid (**2**); 3,5-di-*O*-caffeoylquinic acid (**3**); ethyl 4,5-di-*O*-caffeoyl quinate (**4**); methyl 3,5-di-*O*-caffeoyl quinate (**5**); 5-*O*-caffeoylquinic acid (**6**); caffeic acid (**7**); and quercetin-3-*O*-rutinoside (**8**). The structures of these compounds were elucidated from their ^{1}H and ^{13}C NMR spectra with the aid of ^{1}H−^{1}H COSY, ^{1}H−^{13}C COSY, ^{1}H−^{13}C COLOC, and spin−spin decoupling experiments and by comparison with existing data from the literature.[6−10] Compounds **4** and **5** are probably artifacts formed during the isolation.

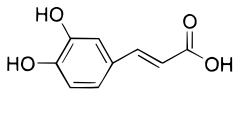
All of these compounds were evaluated for their effects on HMNC proliferation. With the exception of compounds **2** and **6**, no enhancement activity on HMNC proliferation

* To whom correspondence should be addressed. National Research Institute of Chinese Medicine, No. 155-1, Sec. 2, Li-Nung St., Shih-Pai, Taipei (11221), Taiwan, Republic of China. Tel.: 886-2-28201999, ext. 7101. Fax: 886-2-28250743. E-mail: choucj@cma23.nricm.edu.tw.

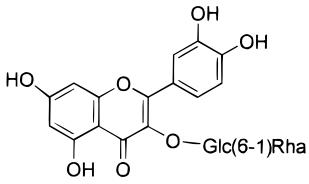
10.1021/np980153o CCC: $18.00
Published on Web 03/04/1999



1: $R_1=R_2=H$ $R_3=R_4=$caffeoyl
2: $R_1=R_4=H$ $R_2=R_3=$caffeoyl
3: $R_1=R_3=H$ $R_2=R_4=$caffeoyl
4: $R_1=$ethyl $R_2=H$ $R_3=R_4=$caffeoyl
5: R1=methyl $R_2=R_4=$caffeoyl $R_3=H$
6: $R_1=R_2=R_3=H$ $R_4=$caffeoyl



7



8

was detected. As shown in Figure 1, the ^{3}H-thymidine uptake in neither the resting nor stimulated states was affected by DMSO (0.1%) treatment. The interleukin-2 (IL-2) was used as a positive control, and it enhanced HMNC proliferation. Compound **7** had little effect on proliferation in either resting or phytochemagglutinin (PHA)-activated cells. By contrast, compounds **2** and **6** enhanced proliferation in either resting or PHA-activated cells. At 10 μg/mL, compound **2** and compound **6** enhanced PHA-treated HMNC to the extent of 78.4 ± 7.6% and 97.9 ± 1.1%, respectively. Similarly, the ^{3}H-thymidine uptake of resting HMNC treated with either compound **2** or **6** was significantly higher than that of nontreated resting cells (725.5 ± 38.9 cpm vs 203.3 ± 3.1 cpm; 909.7 ± 96.4 cpm vs 203.3 ± 3.1 cpm; $p < 0.001$). To study whether the increase in HMNC proliferation was related to cytokine production, the cell supernatants were collected, and IFN-γ concentration was determined by EIA. The results are shown in Figure 2. The IFN-γ production of HMNC was enhanced by IL-2 treatment. Although compound **7** had no effect, IFN-γ production in activated HMNC was significantly enhanced by compounds **2** and **6**. The IFN-γ production in resting HMNC was also increased in compound **2**- or **6**-treated cells (30.5 ± 1.1 pg/mL vs 22.5 ± 1.2 pg/mL; 38.5 ± 1.2 pg/mL vs 22.5 ± 1.2 pg/mL; $p<0.001$). The results demonstrated that enhancement activities of compounds **2** and **6** were comparable to that of IL-2. As shown in Figures 3 and 4, compounds **2** and **6** enhanced lymphoproliferation and IFN-γ production of HMNC in a dose-dependent manner. The EC_{50} values *o*f compounds **2** and **6** on proliferation of resting HMNC were 4.4 ± 1.0 μg/mL and 1.54 ± 0.8 μg/mL, respectively. On the other hand, the EC_{50} values *o*f compounds **2** and **6** on PHA-activated HMNC proliferation were 8.1 ± 1.5 μg/mL and 4.9 ± 0.6 μg/mL, individually. The EC_{50} values of compounds **2** and **6** on IFN-γ production in resting HMNC were 1.25 ± 0.5 μg/mL and 3.9 ± 1.6 μg/mL, respectively. However, the EC_{50} values of compounds **2** and **6** on IFN-γ production in PHA-activated HMNC were 5.0 ± 1.7 μg/mL and 2.8 ± 1.0 μg/mL, individually. This indicated that compounds **2** and **6** had mitogenic activity for HMNC and that they were immunomodulatory agents. The enhancement mechanisms of compounds **2** and **6** may involve increased cytokine production. Plans are underway for the elucidation of their mechanisms of action.

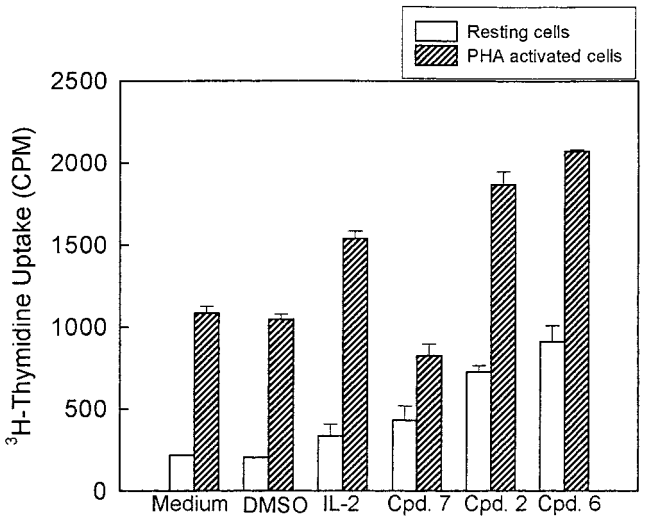


Figure 1. The effects of compounds **7**, **2**, and **6** on human mononuclear cells proliferation; 2×10^5 HMNC were either in resting state or activated by PHA (5 μg/mL). The cells were treated with or without 10 u/mL of IL-2 or 10 μg/mL of compounds **7**, **2**, or **6** for 3 days. Then ^{3}H-thymidine was pulsed for 16 h before harvest. Radioactivity was determined by a scintillation counter. Each bar represents the mean of three independent experiments.

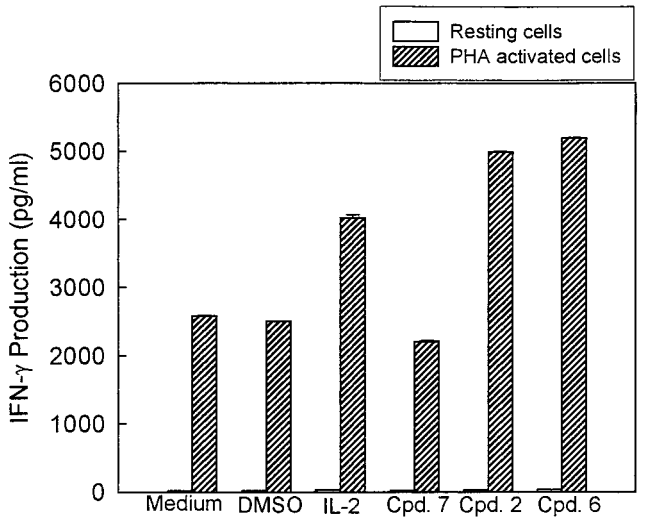


Figure 2. The IFN-γ production in compounds **7**, **2**, and **6** treated human mononuclear cells. The resting or PHA (5 μg/mL)-activated HMNC were cultured with or without 10 u/mL of IL-2 or 10 μg/mL of compounds **7**, **2**, or **6** for 3 days. IFN-γ productions in the cell supernatants were assayed by EIA. Each bar represents the mean of three independent experiments.

Experimental Section

General Experimental Procedures. FABMS spectra were recorded in a glycerol matrix in the negative ion mode on a JEOL JMS−SX 102A mass spectrometer. ^{1}H,^{13}C, and 2D NMR spectra were measured on a Bruker ACP-300 spectrometer with deuterated solvent as internal standard. Column chromatography was performed on Sephadex LH-20 (Pharmacia) or Si gel 60 (70−230 or 230−400 mesh, E. Merck). Si gel 60F_{254} (E. Merck) was used for normal TLC (0.25 mm) with solvent A [EtOAc−butanone−HCO_2H−H_2O (5:3:1:1)] or B (EtOAC). The solvent system I is A−B = 1:3, and II is A−B = 1:12. Spots were visualized by $FeCl_3$ reagent and by spraying 10% H_2SO_4 followed by heating.

Plant Material. The whole plant of *Dichrocephala bicolor* was collected at Wulai, Taipei Hsien, Taiwan, in May 1993. A voucher specimen has been deposited in the herbarium of the Department of Botany of the National Taiwan University.

Extraction and Isolation. Whole specimens of the air-dried plant (3.7 kg) was extracted with 95% EtOH (20 L) three times at 60 °C for 24 h. The EtOH extracts were combined and concentrated in vacuo at ca. 50 °C to give 315 g of residue. The crude extract was partitioned successively between H_2O and EtOAc, followed by *n*-BuOH (each 1 L × 3), yielding 157, 77, and 70 g, respectively. The *n*-BuOH extract was subjected to Diaion HP-20 column (10 × 120 cm) chromatography with H_2O, 50% MeOH−H_2O, and MeOH as eluents (4 L each). Three fractions (1−3) were collected. Fraction 2 was separated by

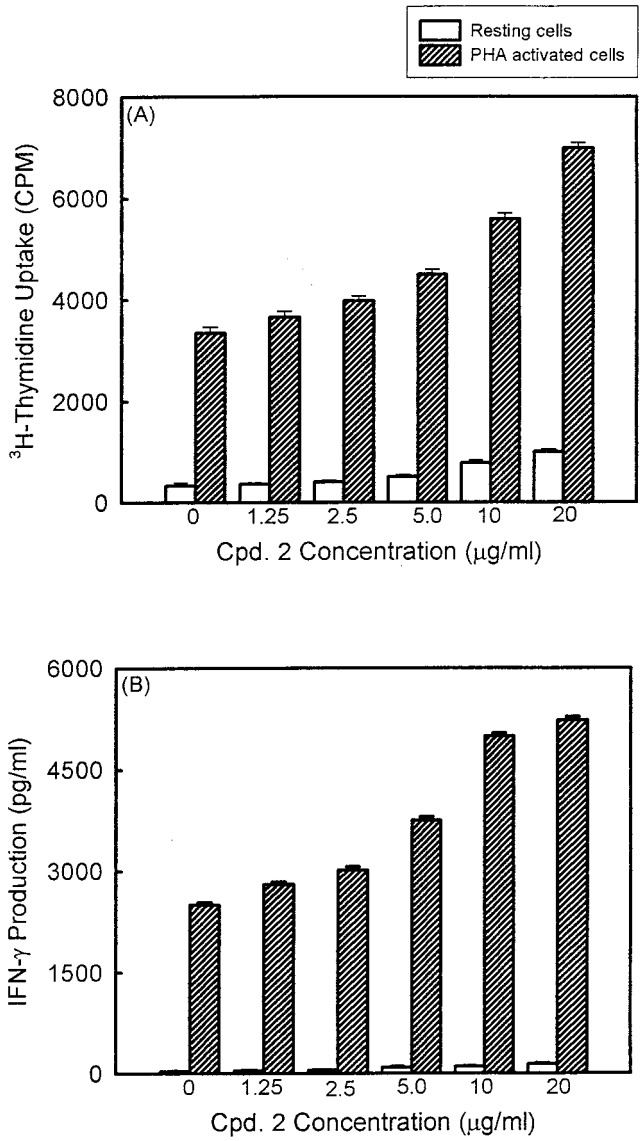


Figure 3. The dosage responses of compound **2** in lymphoproliferation and IFN-γ production of HMNC; 2×10^5 HMNC were either in resting state or activated by PHA (5 μg/mL). The cells were treated with or without various concentrations of compound **2** for 3 days. (**A**) The cell proliferation was determined by the ^{3}H-thymidine uptake. Radioactivity was determined by a scintillation counter. (**B**) IFN-γ productions in the cell supernatants were assayed by EIA. Each bar represents the mean of three independent experiments.

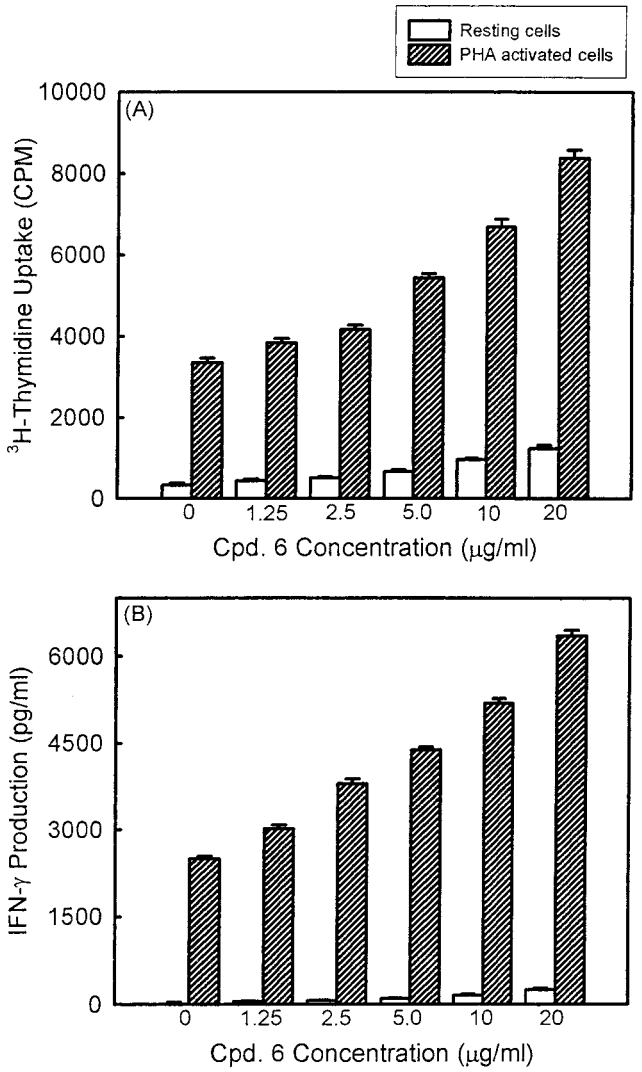


Figure 4. The dosage responses of compound **6** in lymphoproliferation and IFN-γ production of HMNC; 2×10^5 HMNC were either in resting state or activated by PHA (5 μg/mL). The cells were treated with or without various concentrations of compound **6** for 3 days. (**A**) The cell proliferation was determined by ^{3}H-thymidine uptake. Radioactivity was determined by a scintillation counter. (**B**) IFN-γ productions in the cell supernatants were assayed by EIA. Each bar represents the mean of three independent experiments.

Sephadex LH-20 cm^3 with MeOH to yield compound **1** (110.2 mg). Fraction 3 was chromatographed over Sephadex LH-20 with a mixture of MeOH−H_2O (2:8−7:3) into six fractions (fractions 3-1−3-6). Fractions were collected in 100-mL portions and pooled according to their TLC profile (solvent system I). Subsequent separation of fraction 3-4 on Sephadex LH-20 with MeOH afforded compounds **6** (40.6 mg) and **8** (1.02 g). Compounds **2** (36.8 mg) and **3** (87.1 mg) were obtained from fraction 3-5 in the same way as described above. The EtOAc extract was subjected to Si gel column (10 × 120 cm) with a mixture of EtOAc−*n*-hexane (1:2−1:0) to afford a further four fractions A−D (solvent system II). Fraction C was rechromatographed repeatedly over Sephadex LH-20 with MeOH as eluent to yield **4** (10.4 mg), **5** (3.8 mg), and **7** (18.3 mg).

Biological assays: Lymphoproliferation test. Heparinized human peripheral blood (20 mL) was obtained from a healthy donor. HMNC was isolated by the Ficoll−Hypaque method as described previously.[11] The blood was centrifuged at 2500 rpm, 4 °C for 10 min to remove plasma. Blood cells were then diluted with PBS buffer and centrifuged in a Ficoll−Hypaque discontinuous gradient at 1500 rpm in room temperature for 30 min. The HMNC layers were collected and washed with cold distilled H_2O and 10× Hank's buffer saline solution to remove red blood cells. The density of HMNC was adjusted to 2×10^6 cells/mL before use. Cell suspension solution (100 μL) was introduced into each well of a 96-well flat-bottomed (Nunc 167008, Nunclon, Roskilde, Denmark) with or without 10 μg/mL PHA (Gibco, Grand Island, NY). IL-2 (10 u/mL) or various compounds (10 μg/mL) obtained from *D. biocolor* were co-cultured with the cells. The plates were put aside in 5% CO_2-air humidified atmosphere at 37 °C for 3 days. Subsquently, ^{3}H-thymidine (6.70 Ci/mmol, 1 μCi/well, NEN) was added into each well. After a further 16-h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dentate, MultiMate 2000, Billingshurst, UK). Radioactivity in the filters was measured by a scintillation counter. The enhancement activity of each compound on HMNC proliferation was calculated by the following equation:

$$\text{enhancement activity (\%)} = \frac{\text{experimental group (cpm)} - \text{control group (cpm)}}{\text{control group (cpm)}} \times 100$$

Determination of IFN-γ Production. The resting or PHA-activated HMNC (2×10^5 cells/well) were co-cultured

with or without IL-2 (10 u/mL) or 10 μg/mL of pure compounds isolated from *D. bicolor* for 3 days. The cell supernatants were then collected and assayed for IFN-γ concentration by the enzyme immunoassay (EIA; Quantikine IFN-γ test kit, R&D systems). A solution (100 μL) of murine monoclonal antibody (3 μg/mL), which was dissolved in PBS buffer and specific for human IFN-γ, was added to each well. The microplate was incubated overnight at room temperature and washed with PBS buffer containing 0.05% Tween 20 three times. To block plate, 300 μL of PBS buffer containing 1% bovine serum albumin (BSA), 5% sucrose, and 0.05% NaN_3 was added to each well. After 2 h, the blocking buffer was aspirated, and the plate was washed three times with PBS buffer containing 0.05% Tween 20. Standards or samples (200 μL) were pipetted into each well and incubated for 2.5 h at room temperature. After washing away any unbound substance, 200 μL of a 200-ng/mL horseradish peroxidase-linked polyclonal antibody-specific IFN-γ was dissolved in pH 7.3, and PBS buffer was added to the wells and incubated at room temperature for 2 h. Following a wash to remove any unbound antibody-enzyme reagent, 200 μL of 100 μg/mL tetramethylbenzidine substrate solution, which was dissolved in pH 6.0, 100 mM sodium citrate buffer, and contained 0.006% H_2O_2, was added to each well, then incubated for 20 min at room temperature. The color development was stopped by adding 50 μL of 2N H_2SO, and the intensity of the color was measured at OD_{450} nm. The color developed in proportion to the amount of IFN-γ bound in the initial step.

Statistical Analysis. Data were presented as mean ± SD. The differences between groups were assessed with Student's *t*-test.

4,5-Di-*O*-caffeoylquinic acid (1): white powder, mp 234−238 °C (dec); R_f 0.55 (solvent system I, Si gel); IR (KBr) ν_{max} 3400 (OH), 1715, 1705 (C=O), 1610 (C=C) cm^{-1}; FABMS *m/z* 515 $[M - H]^-$; ^1H NMR (CD_3OD) δ 2.21−2.03 (4H, m, H_2-2 and H_2-6), 4.13 (1H, dd, J = 5.2, 11.0 Hz, H-3), 5.18(1H, m, H-4), 5.62 (1H, m, H-5), 6.24, 6.30 (each 1H, d, J = 16.0 Hz, H-8′,8″), 6.74, 6.76 (each 1H, d, J = 8.2 Hz, H-5′, 5″), 6.87, 6.93 (each 1H, dd, J = 8.2, 2.0 Hz, H-6′, 6″), 7.01, 7.05 (each 1H, d, J = 2.0 Hz, H-2′, 2″), 7.50, 7.58 (each 1H, d, J = 16.0 Hz, H-7′, 7″); ^{13}C NMR (CD_3OD) δ 38.11, 39.66 (C-2/6), 67.71 (C-3), 69.96 (C-5), 74.57 (C-4), 75.58 (C-1), 114.96, 115.22 (C-8′/8″), 115.09 (C-2′/2″), 116.50 (C-5′/5″), 123.12, 123.22 (C-6′/6″), 127.72, 127.79 (C-1′/1″), 146.73, 146.78 (C-3′/3″), 147.18, 147.39 (C-7′/7″), 149.51, 149.64 (C-4′/4″), 168.45 (C-9′/9″), 181.88 (C-7).

Through our integrated ^1H,^{13}C, $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, and COLOC NMR experiments, the assignment of all carbon signals of **1** was carried out. However, the signals of C-3 and C-5 were reversed in the literatures.[6,7]

3,4-Di-*O*-caffeoylquinic acid (2): amorphous brown solid; R_f 0.56 (solvent system I, Si gel); IR (KBr) ν_{max} 3300 (OH), 1725, 1705 (C=O), 1640, 1610 (C=C) cm^{-1}; FABMS (negative mode) *m/z* 515 $[M - H]^-$; ^1H NMR data (CD_3OD), in good agreement with the published data[7]; ^{13}C NMR data, in good agreement with the published data.[6,7]

3,5-Di-*O*-caffeoylquinic acid (3): yellow granules, mp 170−172 °C; R_f 0.54 (TLC system I, Si gel); IR (KBr) ν_{max} 3350 (OH), 1725, 1710, 1705 (C=O), 1640, 1610, 1530 (C=C) cm^{-1}; FABMS *m/z* 515 $[M - H]^-$; ^1H NMR data (CD_3OD), in good agreement with the published data;[7,8] ^{13}C NMR data, in good agreement with the published data.[6,7,9]

Ethyl 4,5-di-*O*-caffeoylquinate (4): amorphous white powder; R_f 0.71 (TLC system II, Si gel); FABMS *m/z* 543 $[M - H]^-$, 381 $[M -$ caffeoyl$]^-$; ^1H and ^{13}C NMR data closely resembled those of **1**, except for the ethyl group at δ 1.24 (3H, t, J = 7.0 Hz, $-CH_3$), 4.15 (2H, q, J = 7.0 Hz, $-OCH_2-$), and 14.31 (C-9), 62.71 (C-8).

Methyl 3,5-di-*O*-caffeoylquinate (5): amorphous brown solid; R_f 0.69 (solvent system II, Si gel); FABMS *m/z* 529 $[M - H]^-$; ^1H and ^{13}C NMR data bore close resemblance to those of **3**, except for the methyl group at δ 3.68 (3H, s, $-OCH_3$) and 53.03 (C-8).

5-*O*-Caffeoylquinic acid (6, chlorogenic acid): amorphous brown solid; R_f 0.20 (TLC system II, Si gel); IR (KBr) ν_{max} 3400 (OH), 1705 (C=O), 1610 (C=C) cm^{-1}; FABMS *m/z* 353 $[M - H]^-$, 191 $[M -$ caffeoyl$]^-$, 163 [caffeoyl]$^-$, 135 [caffeoyl $-$ (C=O)]$^-$; ^1H NMR data ($CD_3OD + D_2O$), in good agreement with the published data;[8] ^{13}C NMR ($CD_3OD + D_2O$) δ 38.55, 39.92 (C-2/6), 72.31 (C-3), 72.40 (C-5), 74.29 (C-4), 77.74 (C-1), 115.38 (C-8′), 115.56 (C-2′), 116.82 (C-5′), 123.33 (C-6′), 127.78 (C-1′), 145.84 (C-3′), 147.01 (C-7′), 148.64 (C-4′), 169.72 (C-9′), 181.13 (C-7).

Quercetin-3-*O*-rutinoside (8): yellow needles, mp 186−188 °C; R_f 0.12 (TLC system I, Si gel); ^1H and ^{13}C NMR data (DMSO-d_6), in good agreement with published data.[10]

Acknowledgment. We are grateful to the National Science Council, the Republic of China, for support of this research under grant NSC-86-2113-M-077-002.

References and Notes

(1) Li, H. L. In *Flora of Taiwan*; Editional Committee of the Flora of Taiwan: Taipei, Taiwan, 1978; Vol. IV, pp 845−847.
(2) Kao, M. T. In *Popular Herbal Remedies of Taiwan*; Southern Materials Center: Taipei, 1985; p 166.
(3) Kuby, J. In *Immunology*, W. H. Freeman and Co.: New York, 1994; pp 7−10.
(4) Arai, K.; Lee, F.; Miyajima, A. *Annu. Rev. Biochem*. **1990**, *59*, 783−836.
(5) Dalton, D. K.; Pitts-Meek, S. *Science* **1993**, *259*, 1739−1742.
(6) Nishimura, H.; Nonaka, G. I.; Nishioka, I. *Phytochemistry* **1984,** *23***,** 2621−2623.
(7) Timmermann, B. N.; Hoffmann, J. J. *J. Nat. Prod*. **1983**, *46*, 365−368.
(8) Cheminat, A.; Zawatzky, R.; Becker, H.; Brouillard, R. *Phytochemistry* **1988**, *27*, 2787−2794.
(9) Wald, B.; Wray, V.; Galensa, R.; Herrmann, K. *Phytochemistry* **1989**, *28*, 663−664.
(10) Lin, L. C.; Chou, C. J.; Chen, K. T.; Chen, C. F. *Chin. Pharm. J*. **1993**, *45*, 421−429.
(11) Kuo, Y. C.; Tsai, W. J.; Shiao, M. S.; Chen, C. F.; Lin, C. Y. *Am. J. Chin. Med*. **1996**, *24*, 111−125.

NP980153O