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Antioxidative Caffeoylquinic Acids and Flavonoids from *Hemerocallis fulva* Flowers

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ABSTRACT: Tumor necrosis factor-α (TNF-α)-induced reactive oxygen species (ROS) production in HepG2 was used to screen hepatocyte protective compounds from the flowers of *Hemerocallis fulva*. Three new polyphenols, *n*-butyl 4-*trans*-*O*-caffeoylquinate (**1**), kaempferol 3-*O*-{α-L-rhamnopyranosyl(1→6)[α-L-rhamnopyranosyl(1→2)]}-β-D-galactopyranoside (**2**), and chrysoeriol 7-*O*-[β-D-glucuronopyranosyl(1→2)(2-*O*-*trans*-feruloyl)-β-D-glucuronopyranoside (**3**), together with four caffeoylquinic acid derivatives (**4**−**7**), eight known flavones (**8**−**15**), one naphthalene glycoside, stelladerol (**16**), one tryptophan derivative (**17**), adenosine (**18**), and guanosine (**19**) were isolated from the bioactive fractions of the aqueous ethanol extract of *H. fulva* flowers. The structures of isolated compounds were characterized by means of spectroscopic data. Compounds **1**−**3** were described as first isolated natural products. Among the above-mentioned compounds, the caffeoylquinic acid derivatives are the major components with potent free radical scavenging activity in HepG2 cells and are for the first time isolated from *H. fulva* flowers. A convenient ultraperformance liquid chromatography (UPLC) method was also developed to simultaneously separate and identify caffeoylquinic acids and flavonoids promptly.

KEYWORDS: *Hemerocallis fulva*, flowers, caffeoylquinic acids, flavonoids, TNF-α, free radical scavenger, UPLC

INTRODUCTION

Excess production of reactive oxygen species (ROS) and/or defective cellular antioxidant systems have been implicated in many pathological conditions including chronic liver injury and fibrogenesis.[1−3] Many inflammatory cytokines were expressed through the production of ROS during chronic liver damage.[3] Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, plays a central role in hepatocyte cell death during liver injury.[4] Multiple studies have shown that TNF-α signaling is associated with enhanced generation of ROS, which significantly contributes to TNF-α-induced cell death.[5,6] Phenols such as lignans and flavonoids derived from fruits and vegetables have been regarded as hepatoprotective agents.[7−9]

Hemerocallis fulva L. (daylily) belongs to the Liliaceae family.[10] Both fresh and dried flowers of the plant have been commonly used as a vegetable in eastern Asia. Different kinds of compounds including lactams,[11,12] carotenoids,[13] steroidal saponins,[14] anthraquinones,[15,16] and flavones[17] have been isolated from the aerial parts or roots or flowers of *Hemerocallis* spp. As a part of our interest in liver protective constituents in edible flowers, further investigation on the chemical and biological studies of *H. fulva* flowers was conducted. This paper reports on the isolation and structural elucidation of three new compounds from *H. fulva* flowers and the free radical scavenging activity of the isolates in HepG2 cells. Meanwhile, a convenient ultraperformace liquid chromatography (UPLC) method was developed to simultaneously separate and identify caffeoylquinic acids and flavonoids.

MATERIALS AND METHODS

General Experimental Procedures. Infrared (IR) spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer (Thermo Electron, Akron, OH). Optical rotation was measured on a Jasco P-2000 polarimeter (Hachioji, Tokyo, Japan). Ultraviolet (UV) spectra were measured on a Hitachi U-3310 spectrophotometer (Hitachi, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were run in CD_3OD or DMSO-d_6 on a Varian Unity INOVA-500 or VNMRS 600 (Varian, Palo Alto, CA). ESIMS and HRESIMS mass spectra were recorded on a Finnigan MAT LCQ and Finnigan Mat 95S, respectively. UPLC was performed on a Waters AcQuity Ultra Performance LC (Milford, MA). Silica gel (230−400 mesh) and a semipreparative Si column (LiChrosorb Si-60) (Merck, Darmstadt, Germany) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Solvents (analytical grade) were purchased from Merck. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco BRL (Grand Isand, NY). Dimethyl sulfoxide (DMSO) and analytical grade solvents were from Merck. Trypan blue, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), and TNF-α were purchased from Sigma (St. Louis, MO). CM-H_2DCF-DA was from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade and purchased from commercial suppliers.

Determination of Total Phenolic Content. The total phenolic content was determined according to the Folin−Ciocalteu method.[18] Briefly, 200 μL of each sample (2000 μg/mL) was separately mixed with 2 mL of distilled water, 1 mL of Folin−Ciocalteu reagent, and 5 mL of 20% sodium carbonate. After incubation in the dark for 20 min at room temperature, the absorbance was measured at 735 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The amount of Folin−Ciocalteu reagent was substituted by the same amount of distilled water in the blank. Gallic acid (0, 200, 400, 600, 800, and 1000 μg/mL) was used to make a calibration curve. The total phenolic content was expressed as milligram gallic acid equivalents (GAE) per gram of extract.

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Determination of Total Flavonoid Content. The $AlCl_3$ method was used to determine the total flavonoid content.[18] Briefly, 0.5 mL of each sample (2000 μg/mL) was separately mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm with a microplate reader (Molecular Devices). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in the blank. Catechin (0, 100, 200, 400, 600, and 800 μg/mL) was used to make a calibration curve. The total flavonoid content was expressed as milligram catechin equivalents (CE) per gram of extract.

Evaluation of Reactive Oxygen Species in HepG2 Cells. ROS in HepG2 cells were measured by the conversion of nonfluorescent 2′,7′-dichlorofluorescein diacetate (DCF-DA) into 2′,7′dichlorofluorescein (DCF). PBS-rinsed cells were treated with TNF-α (200 ng/mL) with or without extract or isolated compound for 2 h and then incubated with 8 μM CM-H_2DCFDA for 30 min at 37 °C. After PBS washing, fluorometric analysis was performed at the indicated time points with the excitation and emission wavelengths at 485 and 530 nm, respectively, in a FLEX Station (Molecular Devices Corp.).[19]

Cell Viability Assay. HepG2 cells were cultured in DMEM medium (containing 10% FBS, pH 7.2−7.4). During 24 h of incubation, cells in serum-free medium were exposed to TNF-α in the absence or presence of extract or isolated pure compounds at the indicated concentrations for 6 h by MTT assay. The cell viability of various test groups was determined by the following equation: (absorbance of the test group/absorbance of the control) × 100%. All test samples mentioned above were dissolved in DMSO. The final concentration of DMSO was <0.1% and the group of 0.1% DMSO was used as the control group.[20]

Isolation and Purification Process. The air-dried flowers of *H. fulva* (10 kg) were purchased from Taitung, Taiwan, in 2009. The dried flowers (10 kg) were soaked with aqueous ethanol (80% EtOH) at 60 °C overnight (3 × 80 L). The combined extracts were evaporated under reduced pressure in a rotary vacuum evaporator. The dry extract (HF, 952 g) was sucessively partitioned with ethyl acetate (EtOAc) and *n*-butanol (BuOH) to give EtOAc- (HF-EtOAc, 353 g), BuOH- (HF-BuOH, 136 g), and water-soluble fractions (HF-H_2O, 450 g). The EtOAc-soluble fraction was then subjected to silica gel (230−400 mesh) column chromatography with successive elution by a Hex/EtOA/MeOH gradient solvent system to obtain six fractions: A (57 g, Hex), B (112 g, 25% EtOAc/Hex), C (79 g, 50% EtOAc/Hex), D (27 g, 75% EtOAc/Hex), E (24 g, EtOAc), and F (48 g, MeOH). Fractions D and E were separated on a Sephadex LH-20 column (MeOH) repeatedly to give **1** (38 mg), **4** (0.95 g), **8** (32 mg), and **9** (35 mg). The H_2O-soluble fraction was chromatogramed over a Diaion HP-20 column and eluted with a gradient of MeOH in H_2O. Fraction 75% MeOH/H_2O eluate was further purified on a Sephadex LH-20 column (80% MeOH/H_2O) to yield **18** (252 mg) and **19** (123 mg). The MeOH eluates of the H_2O-soluble fraction (18 g) and HF-EtOAc-F (52 g) were combined with the BuOH-soluble fraction and further separated on Sephadex LH-20 (MeOH) and RP-18 (preparative and semipreparative) columns repeatedly to give **2** (127 mg), **3** (36 mg), **5** (1.79 g), **6** (64 mg), **7** (85 mg), **10** (78 mg), **11** (141 mg), **12** (108 mg), **13** (43 mg), **14** (34 mg), **15** (18 mg), **16** (25 mg), and **17** (34 mg).

*n-Butyl 4-trans-O-caffeoylquinate (**1**):* colorless amorphous powder; $[\alpha]^{25}_D = -8.08°$ (MeOH, *c* 0.15); IR (KBr) ν_{max} (cm^{-1}), 3406, 1718, 1686, 1627, 1508, 1441, 1069, 1049, 934; UV (MeOH) λ_{max}, nm (log ε), 220 (3.82), 233 (3.78), 243 (3.78), 295 (3.89), 329 (3.98); ^{1}H NMR (500 MHz, CD_3OD), δ 0.93 (3H, t, *J* = 7.2 Hz, H-4′), 1.41 (2H, hepta, *J* = 7.2 Hz, H-3′), 1.64 (2H, hepta, *J* = 7.2 Hz, H-2′), 2.01 (1H, dd, *J* = 13.2, 10.2 Hz, H-6a), 2.06 (1H, m, H-2a), 2.16−2.20 (2H, m, H-2b, -6b), 4.13 (2H, hepta, *J* = 7.2 Hz, H-1′), 4.28 (1H, ddd, *J* = 10.2, 9.6, 4.8 Hz, $w_{1/2}$ = 20.0 Hz, H-3), 4.31 (1H, m, $J_{w1/2}$ = 7.6 Hz, H-5), 4.83 (1H, dd, *J* = 9.6, 2.4 Hz, H-4), 6.35 and 7.62 (1H each, d, *J* = 16.0 Hz, H-α, H-β), 6.78 (1H, d, *J* = 7.8 Hz, H-5″), 6.94 (1H, dd, *J* = 7.8, 2.0 Hz, H-6″), 7.06 (1H, d, *J* = 2.0 Hz, H-2″); ^{13}C NMR (125 MHz, CD_3OD), δ 14.0 (q, C-4′), 20.0 (t, C-3′), 31.6 (t, C-2′), 38.3 (t, C-6), 42.3 (t, C-2), 65.6 (d, C-3), 66.4 (t, C-1′), 69.2 (d, C-5), 76.5 (s, C-1), 78.8 (d, C-4), 115.2 (d, C-α), 115.2 (d, C-2″), 116.5 (d, C-5″), 123.0 (d, C-6″), 127.8 (s, C-1″), 146.7 (s, C-3″), 147.1 (d, C-β), 149.4 (d, C-4″), 169.0 (s, C-9″), 175.3 (s, C-7) Key HMBC correlations: H-1′, H-2, and H-6/C-7; H-4, H-α, and H-β/C-9″. ESIMS, *m*/*z* 409 $[M - H]^-$. HRESIMS, 409.1536 $[M + Na]^+$(calcd 409.1499 for $C_{20}H_{25}O_9$).

*Kaempferol 3-O-{α-L-rhamnopyranosyl(1→6)-[α-L-rhamnopyranosyl-(1→2)]}-β-D-galactopyranoside (**2**):* yellow amorphous; IR (KBr) ν_{max} (cm^{-1}), 3410, 1667, 1608, 1505, 1069; UV (MeOH) λ_{max} nm (log ε), 265 (3.96), 348 (3.89); ^{1}H NMR (500 MHz, DMSO-d_6), δ 0.79 and 0.94 (3H each, d, *J* = 6.0 Hz, rhamnosyl H-6′′′ and 6′′′), 4.30 and 5.04 (1H each, br s, anomeric H-1′′′ and -1′′′), 5.47 (1H, d, *J* = 7.5 Hz, anomeric H-1″), 6.18 and 6.38 (1H each, d, *J* = 2.0 Hz, H-6 and -8), 6.86 and 7.93 (2H each, d, *J* = 8.5 Hz, H-(3′(5′)) and -2′(6′)), 10.10, 10.80, and 12.63 (1H each, br s, O*H*-4′, O*H*-7, O*H*-5); ^{13}C NMR (125 MHz, DMSO-d_6), δ 17.3 (q, C-6′′′′), 17.7 (q, C-6′′′), 66.9 (t, galactopyranosyl C-6″), 68.2 (d, rhamnopyranosyl, C-5′′′), 68.3 (d, rhamnopyranosyl, C-5′′′′), 70.3 (d, rhamnopyranosyl C-2′′′(2′′′′)), 70.5 (d, rhamnopyranosyl C-3′′′′), 70.6 (d, rhamnopyranosyl, C-3′′′), 70.6 (d, galactopyranosyl C-4″), 71.8 (d, rhamnopyranosy C-4′′′ (4′′′′)), 75.7 (d, galactopyranosyl C-3″), 77.1 (d, galactopyranosyl C-2″), 77.2 (d, galactopyranosyl C-5″), 93.5 (d, C-8), 98.6 (d, C-6), 98.7 (d, galactopyranosyl C-1″), 100.6 (d, rhamnosyl, C-1′′′), 100.8 (d, rhamnosyl, C-1′′′′), 104 (s, C-10), 115.0 (d, C-3′(5′)), 120.9 (s, C-1′), 130.7 (d, C-2′(6′)), 132.6 (s, C-3), 156.4 (s, C-9), 156.9 (s, C-2), 159.8 (C-4′), 161.2 (s, C-5), 164.0 (s, C-7), 177.2 (s, C-4). Key HMBC correlations: H-6, H-8, 7-O*H* (δ 10.80)/C-7; 5-O*H* (δ 12.63)/C-5, C-6, C-10; 4′-O*H* (δ 10.10), H-2′(6′), H-3′(5′)/C-4′; H-1″/C-3, C-2″, C-3″; H-1′′′/C-2″, C-2′′′, C-3′′′, C-5′′′; H-1′′′′/C-6″, C-2′′′′, C-3′′′′, C-5′′′′; H-6′′′/C-4′′′, C-5′′′; H-6′′′′/C-4′′′′, C-5′′′′. ESIMS: *m*/*z* 739 $[M - H]^-$.

*Chrysoeriol 7-O-[(2-O-trans-feruloylglucuronopyranosyl) (1→2)]-O-β-D-glucuronopyranoside (**3**):* yellow amorphous powder; IR (KBr) ν_{max} (cm^{-1}), 3358, 1716, 1657, 1605, 1593, 1514, 1262, 1187, 1159, 1088, 1056; UV (MeOH) λ_{max} nm (log ε), 248 (3.95), 270 (3.88), 334 (4.00); (+NaOAc) λ_{max} nm (log ε), 249 (3.95), 270 (3.88), 335 (4.00); (+$AlCl_3$) λ_{max} nm (log ε), 277 (3.95), 296 (3.88), 338 (3.97), 387 (3.85); (+$AlCl_3$+HCl) λ_{max} nm (log ε), 278 (3.95), 296 (3.88), 337 (3.98), 387 (3.85); ^{1}H NMR (500 MHz, DMSO-d_6), δ 3.54 (1H, m, H-2″), 4.63 (1H, t, *J* = 8.5 Hz, H-2′′′), 4.90 (1H, d, *J* = 8.5 Hz, anomeric H-1′′′), 5.37 (1H, d, *J* = 7.5 Hz, anomeric H-1″), 6.39 and 7.49 (1H each, d, *J* = 15.5 Hz, feruloyl H-α and H-β), 6.41 (1H, d, *J* = 2.0 Hz, H-6), 6.76 (1H, d, *J* = 8.0 Hz, feruloyl H-5), 6.78 (1H, d, *J* = 2.0 Hz, H-8), 6.93 (1H, d, *J* = 8.5 Hz, H-5′), 6.94 (1H, s, H-3), 7.06 (1H, dd, *J* = 8.0, 2.0 Hz, feruloyl H-6), 7.24 (1H, d, *J* = 2.0 Hz, feruloyl H-2), 7.56 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), 7.57 (1H, d, *J* = 2.0 Hz, H-2′), 9.55 (1H, br s, feruloyl 4-O*H*), 9.99 (1H, br s, 4′-O*H*), 12.96 (1H, br s, 5-O*H*); ^{13}C NMR (125 MHz, DMSO-d_6), δ 55.7 (q, OCH_3), 56.0 (q, OCH_3), 71.4 (d, glucuronopyranosyl-C-4″), 71.9 (d, glucuronopyranosyl-C-4′′′), 73.4 (d, glucuronopyranosyl-C-2″), 73.9 (d, glucuronopyranosyl-C-3″), 74.9 (d, glucuronopyranosyl-C-5″), 75.0 (d, glucuronopyranosyl-C-3′′′), 75.6 (d, glucuronopyranosyl-C-5′′′), 79.9 (d, glucuronopyranosyl-C-2′′′), 94.9 (d, C-8), 97.2 (glucuronopyranosyl-C-1″), 99.2 (d, C-6), 100.7 (d, glucuronopyranosyl-C-1′′′), 103.5 (s, C-3), 105.5 (s, C-10), 110.4 (d, C-2′). 111.2 (d, feruloyl-C-2), 115.0 (d, feruloyl-C-α), 115.5 (d, feruloyl-C-5), 115.8 (d, C-5′), 120.5 (d, C-6′), 121.5 (s, feruloyl C-1), 122.9 (d, feruloyl C-6), 125.8 (s, C-1′), 144.6 (d, feruloyl C-β), 147.9 (s, C-3′), 148.1 (s, feruloyl C-3′), 149.2 (s, feruloyl C-4), 150.9 (s, C-4′), 156.9 (s, C-9), 161.2 (s, C-5), 162.2 (s, C-7), 164.2 (s, C-2), 165.8 (s, feruloyl C═O), 169.9 (s, glucuronopyranosyl C-6″), 170.1 (s, glucuronopyranosyl C-6′′′), 182.1 (s, C-4). Key HMBC correlations:

Table 1. Phenol and Flavonoid Contents of the Aqueous Ethanol Extract and the Subtractions of *H. fulva*

extract of *H. fulva* flowers	total phenols (mg gallic acid equiv/g extracts)	total flavonoids (mg catechin equiv/g extracts)
HF	30.9 ± 0.7	15.1 ± 0.9
HF-EA	25.3 ± 2.7	10.4 ± 2.3
HF-BuOH	34.6 ± 2.7	19.5 ± 1.3
HF-H_2O	28.8 ± 0.7	14.6 ± 0.5

H-3/C-2, C-4; 5-OH/C-5, C-6 and C-10; H-8/C-6, C-7, C-9 and C-10; H-2‴/feruloyl C═O and C-1‴; H-2″/C-1″, C-1‴, C-3″, C-4″; H-1″/C-7; H-1‴/C-2‴. Key NOE correlations: OCH_3 (δ_H 3.73)/ feruloyl H-2; OCH_3 (δ_H 3.79)/H-2′. ESIMS: *m/z* 827 $[M - H]^-$, 651 $[M - H - 175]^-$, 527 $[M - H - 300]^-$, 299. HRESIMS: 827.1831$[M - H]^+$(calcd 827.1670 for $C_{38}H_{35}O_{21}$).

Hydrolysis of ***2*** *and* ***3****:* Compound **2** (10 mg) was dissolved in 5% HCl/aqueous ethanol (2 mL) at 80 °C for 30 min. After neutralization with $NaHCO_3$, the mixture was evaporated. The residue was resuspended in H_2O and then filtered to yield kaempferol (3 mg). The sugar components in the filtrate were identified by TLC (on Si gel, developed with ethyl acetate/butanone/formic acid/H_2O = 5:3:1:1) as D-galactose (R_f 0.20) and L- rhamnose (R_f 0.55) in comparison with authentic samples. Compound **3** (5 mg) was conducted by basic hydrolysis (5% NaOH in 50% aqueous ethanol at 80 °C) for 1 h to give compound **15** and ferulic acid.

UPLC Analysis of Isolated Caffeoylquinic Acids and Flavonoids. UPLC analyses were carried out on a Waters AcQuity Ultra Performance LC, which was equipped with a UPLC column (ACQUITY BEH C18, 1.7 μm, 2.1 × 100 mm) and a guard column (VanGuard BEH C18, 1.7 μm, 2.1 × 5 mm) at a constant flow rate of 0.4 mL/min. Mobile phases A (acetonitrile) and B (0.1% formic acid) were run on a programmed protocol: 0−3 min, 2−18% A; 3−5 min, 18−18% A; 5−7 min, 18−40% A; 7−9 min, 40−70% A. The DAD was set at a wavelength between 200 and 400 nm. The chromatographs were plotted at an absorbance of 254 nm. An aliquot of sample (1 μL) was directly introduced into the column through the sample manager.

Statistical Analysis. Data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used. The statistical significance between control and compound-treated group was evaluated by Student's *t* test. A $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Phenol and Flavonoid Compositions. Phenolic compounds are ubiquitously distributed in the plant kingdom. Phenolic compounds such as caffeoylquinic acid derivatives and flavonoids are the most abundant polyphenols in human diet and have been shown to possess different kinds of bioactivities.[21−24] Levels of phenolic contents and flavonoids were shown to exhibit antioxidant capacity and redox properties, such as reducing agents, hydrogen donors, and reactive oxidative species scavengers.[25] As Table 1 shows, the total phenolic contents of HF, HF-EA, HF-BuOH, and H_2O were 30.9 ± 0.7, 25.3 ± 2.7, 34.6 ± 2.7, and 28.8 ± 0.7 mg gallic acid equiv (GAE)/g of extract, respectively. The flavonoid contents of three subfractions were 10.4 ± 2.3, 19.5 ± 1.3, and 14.6 ± 0.5 mg catechin equiv (CE)/g of extracts), respectively. Only the HF-BuOH subfraction has a flavnoid content higher than that in crude extract (15.1 ± 0.9 mg CE/g of extracts). Total flavonoid/total phenolic contents in the crude extract and three subfractions were 0.49 (HF), 0.41 (HF-EtOAc), 0.56 (HF-BuOH) and 0.51 (HF-H_2O), respectively. The results suggest that both flavonoids and phenols play major roles in ROS scavenging in the active fractions.

Compound Isolation and Structural Identification. Nineteen compounds including three new compounds (**1**−**3**), together with four known caffeoylquinic acid derivatives, chlorogenic acid methyl ester (**4**),[26,27] chlorogenic acid (**5**),[26] 4-caffeoylquinic acid (**6**),[28] and 5-caffeoylquinic acid (**7**),[28] eight known flavones, including kaempferol (**8**),[26] quercetin (**9**),[26] astragalin (**10**),[26] isoquercitrin (**11**),[26] kaempferol 3-*O*-rutinoside (**12**),[29] rutin (**13**),[29,30] quercetin 3-*O*-{α-L-rhamnopyranosyl-(1→6)[α-L-rhamnopyranosyl(1→2)]}-β-D-galactopyranoside (**14**),[31] chrysoeriol 7-*O*-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside (**15**),[28] a naphthalene glycoside, stelladerol (**16**),[16] a tryptophan derivative, lycoperodine-1 (**17**),[32] adenosine (**18**),[26] and guanosine (**19**) were isolated from high phenolic subfractions of the aqueous ethanol extract of *H. fulva* flowers (Figure 1). The structures of known compounds were elucidated by spectral methods and their spectroscopic data compared with those of an authentic sample or with published data. Compound **4** is probably an artifact during the isolation.

Compound **1** was obtained as a colorless amorphous powder, $[\alpha]^{25}_D = -8.08°$ (MeOH, *c* 0.15), and gave a molecular ion peak at *m/z* 409 $[M - H]^-$ in agreement with a molecular formula of $C_{20}H_{25}O_9$ by HRESIMS and DEPT NMR. The IR spectrum showed absorption bands for hydroxyl (3406 cm^{-1}), ester (1718 cm^{-1}), conjugated carbonyl (1686 cm^{-1}), and an aromatic group (1627 and 1508 cm^{-1}). ^1H and ^{13}C NMR indicated signals for a butyroyl [δ_H 0.93 (3H, t, *J* = 7.2 Hz), 1.41 and 1.64 and 4.13 (2H each, hepta, *J* = 7.2 Hz); δ_C 14.0 (q), 20.0 (t), 31.6 (t), and 66.4 (t)], two methylenes [δ_H 2.01 (1H, dd, *J* = 13.2, 10.2 Hz), 2.06 (1H, m), and 2.16−2.20 (2H, m); δ_C 38.3 (t, C-6) and 42.3 (t, C-2)], three oxymethines [δ_H 4.28 (1H, ddd, *J* = 10.2, 9.6, 4.8 Hz), 4.31 (1H, m), 4.83 (1H, dd, *J* = 9.6, 2.4 Hz); δ_C 65.6 (d, C-3), 69.2 (d, C-5), and 78.8 (d, C-4)], one *trans*-caffeoyl [δ_H 6.35 and 7.62 (1H each, d, *J* = 16.0 Hz,), 6.78 (1H, d, *J* = 7.8 Hz), 6.94 (1H, dd, *J* = 7.8, 2.0 Hz), and 7.06 (1H, d, *J* = 2.0 Hz); δ_C 115.2 (d), 115.2 (d), 116.5 (d), 123.0 (d), 127.8 (d), 146.7 (s), 147.1 (d), 149.4 (s), and 169.0 (s)], a quaternary oxygenated carbon [δ_C 76.5 (s)], and a carbonyl [δ_C 175.3 (s)]. From the data mentioned above, compound **1** was suggested as the *n*-butyl ester of 4-caffeoylquinic acid. The coupling constants of H_3 and H_4 (*J* = 9.6 Hz) with a large half-width value ($J_{w1/2}$ = 20.0 Hz), and a small one between H_4 and H_5 (*J* = 2.4 Hz) in the quinate moiety indicated they had *trans*-diaxial protons and axial−equatorial gauche configuration, respectively.[33] The acylation of hydroxyl groups produced the downfield shifts of H-1′ [δ_H 4.13 (2H, hepta, *J* = 7.2 Hz)] and H-4 [δ_H 4.83 (1H, dd, *J* = 9.6, 2.4 Hz)], which displayed the presence of acyloxy groups at both positions. This assignment was further confirmed by HMBC correlations from H-1′, H-2, and H-6 to C-7 and from H-4, H-α, and H-β to C-9″.

Compound **2** showed the *quasi* molecular ion peak at *m/z* 739 $[M - H]^-$. ^1H and ^{13}C NMR signals were assigned by 1D-TOCSY, $^1H-^1H$-COSY, HMQC, and HMBC NMR experiments and suggested kaempferol, a galactopyranosyl, and two rhamnopranosyl units were included in the skeleton. The upfield shift (about 1.8 ppm) of C-3 (δ_C 132.6) and a lower field shift (about 9.7 ppm) of C-2 (δ_C 156.9) in the kaempferol moiety (compared to kaempferol (**8**)) indicated *O*-glycosidation at C-3.[21] The coupling constants of the anomeric proton signals at δ_H 5.47 (1H, d, *J* = 7.5 Hz), 4.30, and 5.04 (1H each, br s), and

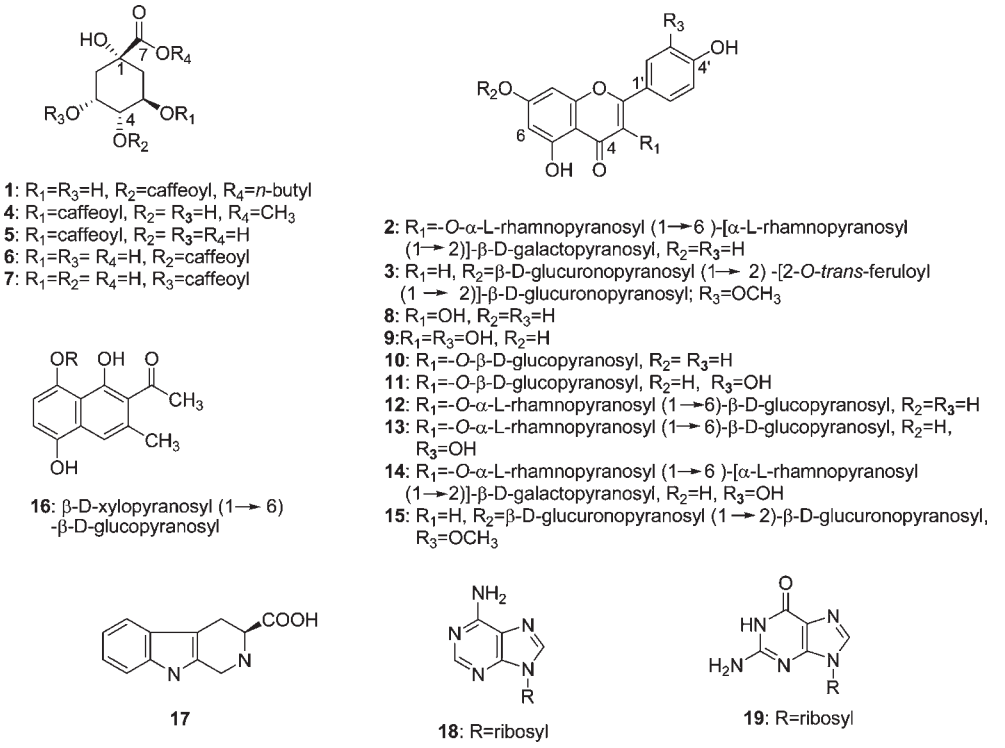

Figure 1. Structures of the isolated compounds.

two methyl group at δ_H 0.79 and 0.94 (3H each, d, J = 6.0 Hz) revealed **2** with one β-D-galactosyl and two α-L-rhamnosyl units. Acidic hydrolysis of **2** yielded kaempferol, D-galactose, and L-rhamnose. The downfield shift of C-2 (δ_C 77.1) and C-6 (δ_C 66.9) of the galactopyranosyl indicated the interglycosidic linkage at both positions. The assignment was further supported by J_2 and J_3 correlations between H-1″ (δ_H 5.47) and C-3 (δ_C 132.6), between H-1‴ (δ_H 5.04) and C-2″ (δ_C 77.1), and between H-1⁗ (δ_H 4.30) and C-6″(δ_C 66.9). The ^{1}H and ^{13}C NMR spectral data of **2** were similar to those of **14** except for signals assignable to the aglycone moiety in place of kaempferol in **2**. The structure was therefore assigned as kaempferol 3-*O*-{α-L-rhamnopyranosyl(1→6)-[α-L-rhamnopyranosyl(1→2)]}-β-D-galactopyranoside.

The molecular formula of compound **3** was determined to be $C_{38}H_{36}O_{21}$ on the basis of ^{13}C and DEPT spectra and the HRESIMS molecular ion at m/z 827.1831 $[M - H]^+$. The UV characteristic absorption bands at 232, 270, and 333 nm indicated compound **2** to be a flavone derivative.[34] The presence of a signal at δ_H 12.96 and a bathochromic shift of 30 nm of band II (248 nm shifts to 278 nm) in the presence of $AlCl_3$/HCl indicated that C-5 was hydroxylated.[34] Meanwhile, the absence of a 7-hydroxyl group was elucidated by no bathochromic shift on adding shift reagent NaOAc.[34] The ^{1}H, ^{13}C, and DEPT spectra revealed the presence of chrysoeriol, two glucuronopyranosyl, and *trans*-feruloyl moieties. Alkali hydrolysis gave compound **15** and ferulic acid. Long-range correlations between H-2‴ [δ_H 4.63 (t, J = 8.5 Hz)] and carbonyl of feruloyl (δ_C 165.8), between H-1‴ [δ_H 4.90 (d, J = 8.5 Hz)] and C-2″ (δ_C 79.9), and between H-1″ [δ_H 5.37 (d, J = 7.5 Hz) and C-7 (δ_C162.2) elucidated their connectivities. The structure was further confirmed by the NOE correlations between methoxyl at δ_H 3.73 and feruloyl H-2 at δ_H 7.24 and between methoxyl at δ_H 3.79 and H-2′ at δ_H 7.57 (d, J = 2.0 Hz).

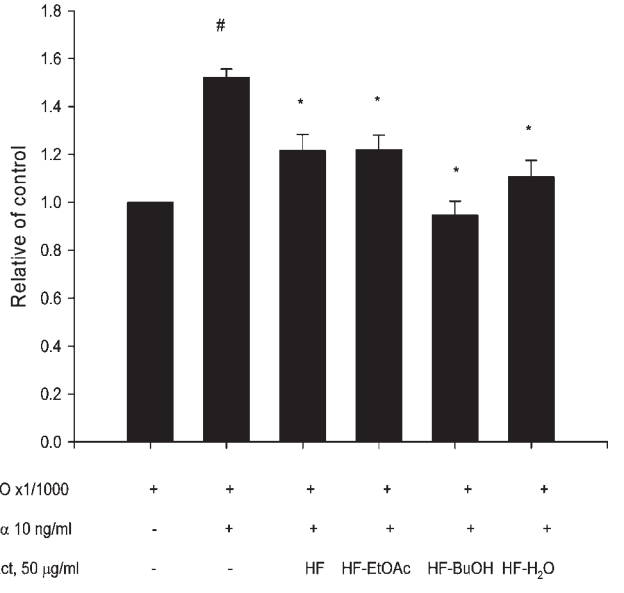

Figure 2. Effects of the ethanol extract of *H. fulva* on TNF-α-induced HepG2 cell ROS production. HepG2 cells were seeded at 8 $\times 10^4$ cells/well in 96 wells overnight. PBS-rinsed cells were treated with TNF-α (10 ng/mL) with or without HF extract for 2 h and then incubated with 8 μM of CM-H_2DCFDA for 30 min at 37 °C. The fluorometric analysis was measured with the excitation and emission wavelengths at 485 and 530 nm. Each column represents the mean ± SEM of three experiments. Resveratrol was used as a positive control. (#) $P < 0.05$, compared with control; (∗) $P < 0.05$, compared with TNF-α-induced group.

Effects of Subfractions and Isolated Polyphenols on ROS scavenging in HepG2 Cells. Flavonoids, phenethyl glycosides,

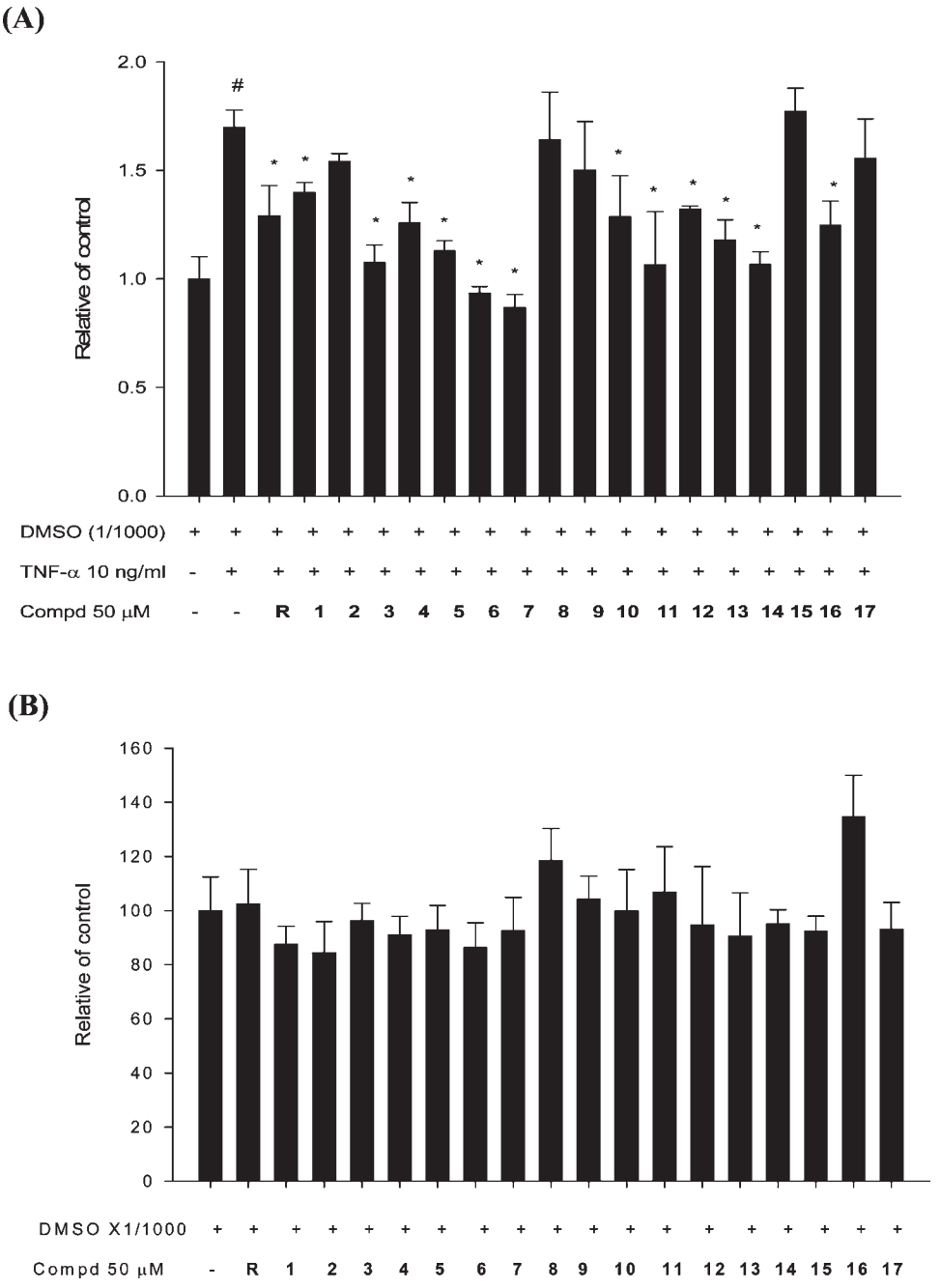

Figure 3. Effects of the isolated polyphenols and flavonoids from *H. fulva* on (A) ROS production and (B) cell viability in TNF-α-induced Hep G2 cells. (A) PBS-rinsed cells were treated with TNF-α (10 ng/mL) with or without test compound for 2 h AND then incubated with 8 μM CM-H_2DCFDA for 30 min at 37 °C. The fluorometric analysis was measured with the excitation and emission wavelengths at 485 and 530 nm. (B) cell viability in TNF-α-induced HepG2 cells was tested by MTT assay. Each column represents the mean ± SEM of three experiments. Resveratrol was used as a positive control. (#) $P < 0.05$, compared with control group; (∗) $P < 0.05$, compared with TNF-α-induced group.

naphthalene glycosides, and carotenoids have been isolated and claimed as the antioxidative principles of daylily (*Hemerocallis* spp.) flowers[13,16] or leaves.[17] In this study, caffeoylquinate derivatives including *n*-butyl 4-*trans*-*O*-caffeoylquinate (**1**, 38 mg), methyl chloriginate (4, 0.95 g), chlorogenic acid (**5**, 1.79 g), 4-caffeoylquinic acid (**6**, 64 mg), and 5-caffeoylquinic acid (**7**, 85 mg) were isolated as the major constituents in *H. fulva* flowers. Chlorogenic acid and the related caffeoylquinic acids from natural resources have been focused on various bioactivities including antioxidative effect.[22−24] As Figure 2 shows, the crude extract of the flowers of *H. fulva* and three subfractions significantly inhibited TNF-α-induced ROS production in HepG2 cells at the concentration of 50 μg/mL and had no direct cytotoxicity (data not shown). The HF-BuOH subfraction preserved the most potent activity and diminished ROS to a level similar to that of the control group. The isolated caffeoylquinic acids and flavonoids (**1**−**17**) were also tested for ROS scavenging activity (Figure 3A). Among those compounds, caffeoylquinic acid derivatives **1** and **4**−**7**, flavonoids **3** and **10**−**14**, and anthraquinone **16** significantly inhibited

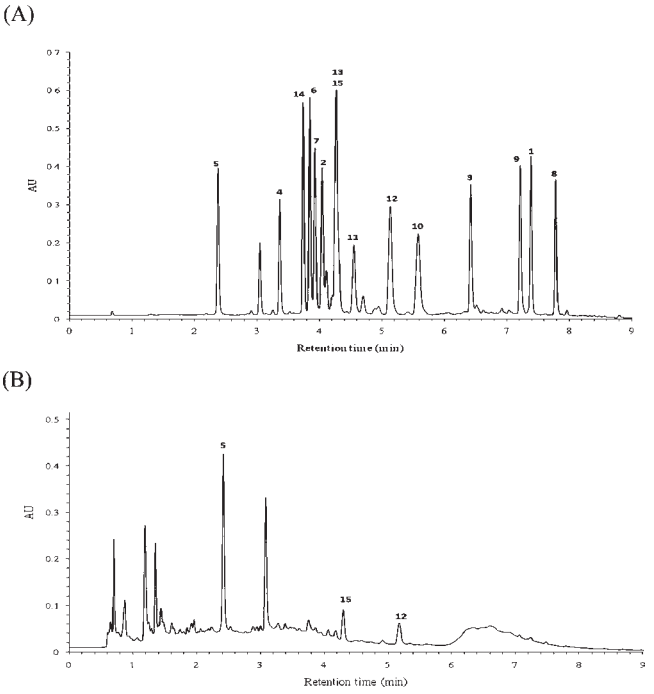

Figure 4. UPLC chromatogram of (A) isolated caffeoylquinic acids and flavonoids and (B) crude aqueous ethanol extract. (**1**) *n*-Butyl 4-caffeoylquinate; (**2**) kaempferol 3-*O*-{α-L-rhamnopyaranosyl(1→6)-[α-L-rhamnopyranosyl(1→2)]}-*β*-D-galactopyranoside; (**3**) chrysoeriol 7-*O*-[(2-*O*-*trans*-feruloylglucuronopyranosyl) (1→2)]-*O*-*β*-D-glucuronopyranoside; (**4**) methyl chlorogenate; (**5**) chlorogenic acid; (**6**) 4-caffeoylquinic acid; (**7**) 5-caffeoylquinic acid; (**8**) kaempferol; (**9**) quercetin; (**10**) astragalin; (**11**) isoquercitrin; (**12**) kaempferol 3-*O*-rutinoside; (**13**) rutin; (**14**) quercetin 3-*O*-{α-L-rhamnopyranosyl(1→6)[α-L-rhamnopyranosyl(1→2)]}-*β*-D-galactopyranoside; (**15**) chrysoeriol 7-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside; (**16**) stelladerol; (**17**) lycoperodine-1.

ROS production in HepG2 cells at 50 μM without direct toxicity under the working concentration (Figure 3B). From our results, both caffeoylquinic acid derivatives and flavonoids are responsible for the ROS scavenging activity in *H. fulva*.

Identification of Caffeoylquinic Acids and Flavonoids by UPLC. The isolated caffeoylquinic acids and flavonoids were analyzed by a UPLC system. A complete UV chromatogram (254 nm) obtained with the standard solution is shown in Figure 4. Peaks were identified by comparison with the retention time and DAD spectra of the authentic samples (Figure 4). The results indicated that caffeoylquinic acids and flavonoids gave good resolutions except rutin (**13**) and chrysoeriol 7-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside (**15**) overlapping. Our results suggest that UPLC analysis can be considered as a convenient analytical method for caffeoylquinic acids and flavonoids simultaneously and promptly.

This is the first report that *H. fulva* flowers contain high levels of caffeoylquinic acids and possess potent ROS scavenging activity. Our results suggest that caffeoylquinic acids, especially chlorogenic acid in *H. fulva* flowers, at least partially contributed to their ROS scavenging effect. Thus, *H. fulva* flowers may be a source of natural ROS scavenger and beneficial to the health of consumers.

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ABBREVIATIONS USED

HF, aqueous ethanolic extract of the flowers of *H. fulva*; EtOAc, ethyl acetate; BuOH, *n*-butanol; HF-EtOAc, ethyl acetate-soluble subfraction of HF; HF-BuOH, *n*-butanol-soluble subfraction of HF; HF-H_2O, H_2O-soluble subfraction of HF; Hex, hexane; MeOH, methanol; GAE, gallic acid equivalents; CE, catechin equivalents; IR, infrared spectra; UV, ultraviolet spectra; NMR, nuclear magnetic resonance; ESIMS, electrospray ionization mass spectra; UPLC, ultraperformance liquid chromatography; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TNF-α, tumor necrosis factor-α; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; ROS, reactive oxygen species; DCF-DA, 2′,7′-dichlorofluorescein diacetate; ANOVA, one-way analysis of variance.

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