JOURNAL OF NATURAL PRODUCTS

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Volume 71, Number 6 June 2008

Full Papers

Antioxidant 2-Phenylbenzofurans and a Coumestan from *Lespedeza virgata*

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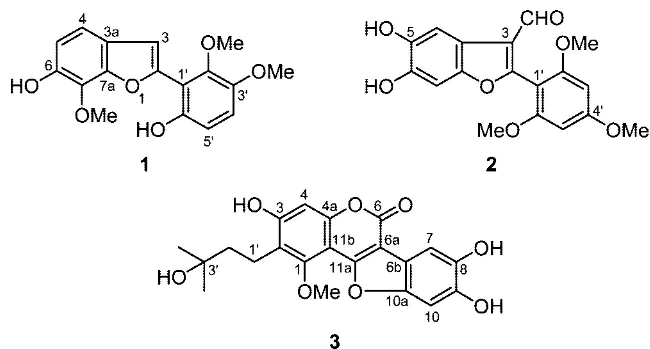
Received January 8, 2008

Two 2-phenylbenzofurans, lespedezavirgatol (**1**) and lespedezavirgatal (**2**), and a coumestan, lespedezacoumestan (**3**), were isolated from the aerial parts of *Lespedeza virgata*, together with nine known flavonoids. Their structures were determined on the basis of spectroscopic data. The three new compounds showed strong oxygen radical absorbance capacity and potent inhibition toward lipid peroxidation in both plasma and kidney homogenate of renal failure rats.

Oxidative stress or reactive oxygen species (ROS) are presumably involved in the pathogenesis of nephritis.[1] In particular, lipid peroxidation can cause cellular injuries by inactivation of membrane enzymes and receptors,[2] which results in the initiation of nephropathies. Antioxidants may also delay the onset of lipid peroxidation, stall the further production of free radicals, and inhibit the damaging effects by enzymes in kidney.[2,3]

Lespedeza species (Leguminosae) are distributed in East Asia and North America and have been used as folk medicines for the treatment of urinary system diseases.[4] Several classes of compounds with antioxidant activity have been identified, including flavonoids, stilbenoids, and other phenolic compounds.[5–9] *L. virgata* (Thunb.) DC. is a Chinese herb used for the treatment of various nephritides in central China. Our previous investigation showed that the *n*-BuOH-soluble fraction of the EtOH extract of this plant could improve the pathological change of glomeruli on an experimental rat model for minimal change nephropathy (MCN) and suggested that this herb effectively counteracted the oxidative damage on kidney possibly by its antioxidant effect.[10] This finding prompted us to investigate its active principles, and herein, we describe the isolation, structural elucidation, and bioactivities of three new phenolics from this herb.



Results and Discussion

Repeated column chromatography and HPLC of the *n*-BuOH-soluble fraction of the EtOH extract of *L. virgata* yielded two new 2-arylbenzofurans, lespedezavirgatol (**1**) and lespedezavirgatal (**2**), and a new coumestan, lespedezacoumestan (**3**), along with nine known flavonoids, tricin,[11] kaempferol,[12] apigenin,[13] kaempferitrin,[14] luteolin,[15] quercetin-3-*O*-α-L-rhamnoside,[16] kaempferol-7-*O*-L-rhamnopyranoside,[17] 7-*O*-α-L-rhamnopyransylkaempferol-3-*O*-β-D-glucopyranoside,[18] and quercetin.[16] The structures of the known compounds were determined by interpretation of their spectroscopic data as well as by comparison with reported data.

Lespedezavirgatol (**1**) was isolated as a pale yellow powder. Its molecular formula was determined as $C_{17}H_{16}O_6$ from the HRESIMS ion at *m/z* 339.0839 $[M + Na]^+$. Its UV spectrum showed absorption maxima at 234, 280, 317, and 330 nm. The ^1H NMR spectrum

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10.1021/np800016e CCC: $40.75 © 2008 American Chemical Society and American Society of Pharmacognosy
Published on Web 05/17/2008

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data for **1** and **2** in DMSO-d_6

	1		2	
position	δ_H	δ_C	δ_H	δ_C
2		150.8		159.8
3	7.05 s	103.3		118.8
3a		123.3		115.2
4	7.07 d (8.4)	114.5	7.36 s	105.5
5	6.76 d (8.4)	113.4		143.9[a]
6		146.4		145.0[a]
7		132.3	6.98 s	98.1
7a		145.6		148.5[a]
1′		115.1		97.9
2′		150.6		160.0
3′		141.3	6.38 s	91.1
4′	7.43 d (8.4)	120.8		163.5
5′	6.75 d (8.4)	112.3	6.38 s	91.1
6′		151.6		160.0
2′-OMe	3.86 s	60.0	3.73 s	56.0
3′-OMe	3.79 s	60.2		
7-OMe	4.01 s	60.2		
5-OH				
6-OH	9.20 br s			
6′-OH	9.76 br s			
3-CHO			9.60 s	186.8
4′-OMe			3.87 s	55.6
6′-OMe			3.73 s	56.0

[a] Assignments are interchangeable in vertical column.

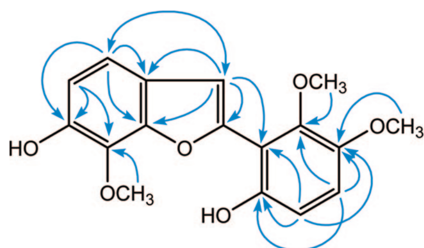

Figure 1. Key HMBC correlations of **1**.

(Table 1) showed four doublets (1H each, J = 8.4 Hz) at δ 7.07 (H-4), 6.76 (H-5), 7.43 (H-4′), and 6.75 (H-5′) for two sets of *ortho*-coupled aromatic protons, indicating the presence of two *ortho*-tetrasubstituted benzene rings. The spectrum also exhibited a singlet at δ 7.05 (1H, H-3) for a separate aromatic proton, three singlets at δ 4.01, 3.86, and 3.79 (3H each) corresponding to three aromatic methoxy groups, and two broad singlets at δ 9.76 and 9.20 (1H each) assignable to two phenolic hydroxy groups. These findings suggested the 2-arylbenzofuran skeleton for **1**.[19] The ^{13}C NMR spectrum (Table 1) and the DEPT experiments indicated the presence of three methoxy groups and fourteen sp^2 carbons comprising five methine carbons and nine quaternary carbons including seven oxygenated [δ 151.6 (C-2), 150.8 (C-2), 150.6 (C-2′), 146.4 (C-2), 145.6 (C-7a), 141.3 (C-2), and 132.3 (C-7)]. In the HMBC spectrum (Figure 1), long-range correlations were observed from H-3 to C-2 (150.9), C-3a (123.4), C-4 (114.5), C-7a, and C-1′ (115.1), from H-4 to C-3a, C-3, and C-7a, from H-5 to C-3a, and from H-5′ to C-1′, indicating the skeleton of 2-phenylbenzofuran. The positions of the three methoxy groups were established by the HMBC correlations (Figure 1) from the protons at δ 4.01, 3.86, and 3.79 to C-7, C-3′, and C-2′, respectively. Therefore, the structure of **1** was determined as 2-(6′-hydroxy-2′,3′-dimethoxyphenyl)-6-hydroxy-7-methoxybenzofuran.

Lespedezavirgatal (**2**) was obtained as a yellowish powder. Its molecular formula was determined as $C_{18}H_{16}O_7$ by the HRESIMS in combination with the NMR data. The UV spectrum showed absorption maxima at 287, 318, 356, and 384 nm, resembling those of arylbenzofuran derivatives.[20] The ^{1}H NMR spectrum (Table 1) showed a singlet at δ 9.60 corresponding to an aldehyde group, two singlets at δ 3.73 (6H) and 3.87 (3H) for three aromatic methoxy groups, and three singlets at δ 7.36 (1H, H-4), 6.98 (1H, H-7), and 6.38 (2H, H-2′ and 6′) for four aromatic protons. The ^{13}C NMR (Table 1) and DEPT spectra indicated the presence of three methoxy groups, an aldehyde group, four aromatic methines, and 10 aromatic quaternary carbons, of which seven were oxygenated. In the HMBC spectrum (Figure 2), cross-peaks were observed between the proton at δ 3.73 and the quaternary carbon at δ 160.5 (C-2′ and C-6′), between the proton at δ 3.87 and carbon at δ 163.5 (C-4′), and between the proton at δ 6.38 and the carbons at δ 163.5, 160.5, and 97.9 (C-1′), indicating the presence of a 2,4,6-trimethoxyphenyl structural moiety. The HMBC correlations (Figure 2) from both H-4 and H-7 to C-3a, C-5, C-6, and C-7, from H-4 to C-3, and from the aldehydic proton to C-2, C-3, and C-3a were also observed, indicating the presence of a 5,6-dihydroxybenzofuran-3-carbaldehyde moiety. The connection between two moieties was thus exclusively assigned from C-2 to C-1′, and the structure of **2** was established as shown.

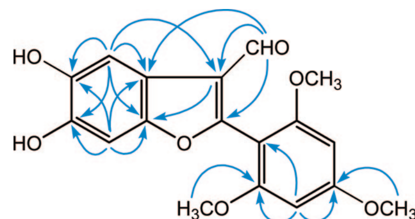

Figure 2. Key HMBC correlations of **2**.

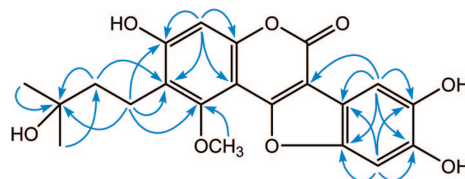

Figure 3. Key HMBC correlations of **3**.

Lespedezacoumestan (**3**) was obtained as a yellow powder. The molecular formula was determined as $C_{21}H_{19}O_8$ by the HRESIMS, which gave a pseudomolecular ion at m/z 399.1074 [M − H]$^-$. The UV absorption of three maxima at 214, 253, and 347 nm is characteristic for a coumestan derivative.[21] The ^{1}H NMR spectrum displayed three sharp singlets at δ 6.73 (1H, H-4), 7.16 (1H, H-7), and 7.25 (1H, H-10), assignable to a pentasubstituted and a 1,2,4,5-tetrasubstituted aromatic ring. The ^{13}C NMR and DEPT spectra exhibited signals for 15 sp^2 carbons, including three methine and 12 quaternary carbons. These findings supported that **3** had a coumestan skeleton. Furthermore, the ^{1}H, ^{13}C, and DEPT NMR spectra showed the presence of an aromatic methoxy group [δ_H 3.90 (3H, s); δ_C 62.4 (q)] and a 3-hydroxylated isoprenyl group [δ_H 1.17 (6H, s, H$_3$-4′ and H$_3$-5′), 1.57 (2H, m, H$_2$-2′), and 2.65 (2H, m, H$_2$-1′); δ_C 29.1 (q, C-4′ and C-5′), 69.0 (s, C-3′), 43.3 (t, C-2′), and 18.3 (t, C-1′)]. In the HMBC spectrum (Figure 3), the correlations from the methoxy protons at δ 3.90 to C-1 (δ 153.7), from H$_2$-1′ to C-2 (δ 121.0), C-1, and C-3 (δ 159.3), and from H-4 to C-2, C-3, C-4a, and C-11b, and the absence of correlation between H-4 and C-11a (δ 157.7) indicated that the methoxy group and the 3-hydroxylated isoprenyl group were attached to C-1 and C-2, respectively, and further a hydroxy group substituted at C-3. HMBC correlations were also observed from both H-7 and H-10 to C-8 (δ 144.4), C-9 (δ 145.6), C-6b (δ 113.8), and C-10a (δ 149.0) and from H-7 to C-6a (δ 102.3), indicating that C-8 and C-9 were each substituted by a hydroxy group. Thus, the structure of **3** was established as depicted.

In an oxygen radical absorbance capacity (ORAC) fluorescein assay,[22] the three new compounds exhibited dose-dependent oxygen radical scavenging activity as shown in Figure 4. At a concentration of 1.5 μM, the average ORAC values of compounds **1**−**3** for Trolox

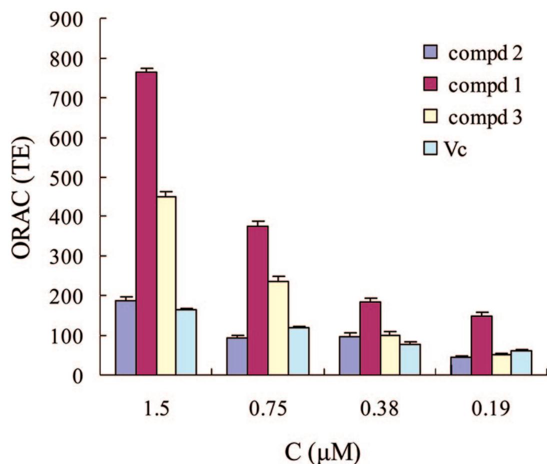

Figure 4. Dose-dependent ORAC of compounds **1**−**3**. Vitamin C (Vc) was used as a positive control. The ORAC value was calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. The results represent the mean ± SD for three independent experiments.

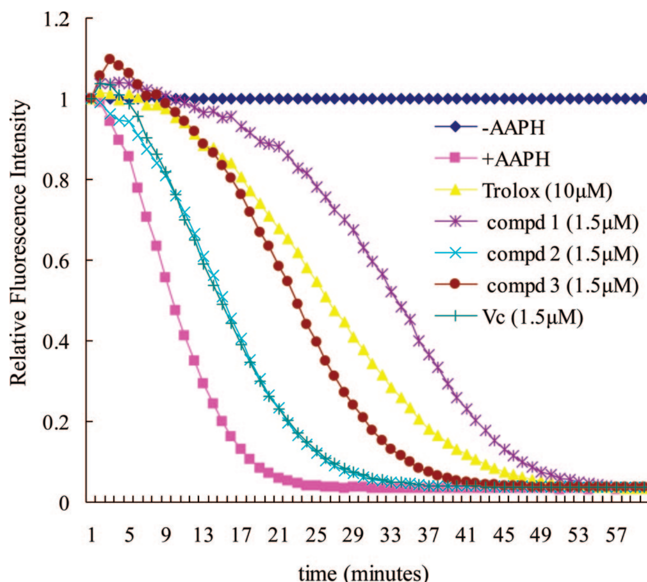

Figure 5. Inhibition of AAPH-induced fluorescence decay by compounds **1**−**3**. Curves of fluorescence decay induced by AAPH as peroxy radical generator in the presence of compounds **1**−**3** and vitamin C (positive control) at the same concentration (1.5 μM) are shown. Trolox was used as a control standard. The antioxidative activity of samples is expressed as net area under the curve.

equivalents (TE) were determined to be 762.96, 188.99, and 449.62, respectively. All were higher than that of vitamin C (TE = 164.56) ($p < 0.01$) (Figure 5

Compounds **1**−**3** were further evaluated for the inhibitory activity against lipid peroxidation in rat kidney homogenate and plasma using a thiobarbituric acid reactive substances (TBARS) assay.[23] The IC_{50} values of **1**−**3** and vitamin C toward malondialdehyde (MDA) levels in plasma and kidney homogenates of kidney failure rats are shown in Table 2. Compounds **1**−**3** were potent inhibitors of lipid peroxidation *in vitro*. Thus, the three new compounds could potently counteract oxidative damage on kidney.

Experimental Section

General Experimental Procedures. UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 25 UV−vis spectrophotometer. ^{1}H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were recorded on a Bruker DRX-400 in DMSO-d_6 with the residual solvent peak (δ_H 2.49 and δ_C 39.51) as reference. ESIMS were collected on an MDS SCIE API 2000 LC/MS/MS instrument. For column chromatography, macroporous resin D101 (Tianjin Ou-Rui Bio-Tech Co. Ltd., China) and Si gel 60 (100−200 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Develosil ODS (10 μm, Nomura Chemical Co. Ltd., Japan), polyamide (Shanghai Hushi Chemical Co. Ltd., China), and Sephadex LH-20 were used. Vitamin C, disodium fluorescein, 6-hydro-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Table 2. Inhibitory Activity of **1**−**3** against MDA in Plasma and Kidney Homogenate of Kidney Failure Rats (IC_{50}, mM)[a]

compound	plasma	kidney homogenate
1	0.18 ± 0.006	0.16 ± 0.002
2	0.75 ± 0.032	0.64 ± 0.016
3	0.19 ± 0.002	0.45 ± 0.453
vitamin C	5.54 ± 0.182	3.05 ± 0.113

[a] Data represent mean ± SD for three independent experiments.

Plant Material. The aerial parts of *L. virgata* were collected from Luo Tian, Hubei Province, China, in June 2005. The plant was identified by Prof. Ma Ji at the School of Traditional Chinese Medicine, Southern Medical University of China. A voucher sample was deposited at the Department of Pharmacology, Southern Medical University, Guangzhou, China.

Animals. Healthy 6-week-old rats (180 g), obtained from the Center for Laboratory Animal Sciences, Southern Medical University, Guangzhou, China, were used. Animals were kept in separate rooms on a 12 h light/dark cycle, at room temperature (23 ± 1 °C), with free access to food and water.

Extraction and Isolation. Air-dried aerial parts of *L. virgata* (5 kg) were extracted three times with 85% EtOH. The resulting EtOH solution was concentrated under vacuum. The residue (600 g) was suspended in H_2O and partitioned successively with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The *n*-BuOH-soluble extract (82 g) was subjected to macroporous resin D101 CC eluted with EtOH−H_2O mixtures of decreasing polarities (6:4, 7:3, and 9:1) to obtain three fractions (I−III). Fraction II (8.0 g), obtained on elution with EtOH−H_2O (7:3), was chromatographed on a polyamide column using EtOH−H_2O (3:7) to obtain four subfractions (II-1−II-4). Subfraction II-1 (0.45 g), on recrystallization from MeOH, afforded apigenin (14 mg). Subfraction II-2 (1.8 g) was further separated by Si gel CC using $CHCl_3$−MeOH mixtures of increasing polarities (9:1 to 7:3) followed by Sephadex LH-20 CC to afford quercetin (21 mg), kaempferol (18 mg), and tricin (15 mg). Fraction III (31.0 g), which was obtained on elution with EtOH−H_2O (9:1), was further separated by silica gel CC using $CHCl_3$−MeOH mixtures of increasing polarities (98:2 to 7:3), yielding three subfraction (III-1−III-3). Subfraction III-1 (2.1 g), obtained on elution with $CHCl_3$−MeOH (98:2), was further chromatographed on a Sephadex LH-20 column using MeOH to afford kaempferitrin (12 mg), luteolin (9 mg), and compound **1** (16 mg). Subfraction III-2, eluted by $CHCl_3$−MeOH (9:1), was further chromatographed on an RP-18 Si gel column using MeOH−H_2O (6:4) followed by purification with Sephadex LH-20 CC using MeOH to afford compounds **2** (11 mg) and **3** (25 mg). Subfraction III-3 (0.7 g), afforded by elution with $CHCl_3$−MeOH (7:3), was subjected to an RP-18 column using MeOH−H_2O (4:1) followed by CC over Sephadex LH-20 with MeOH to yield kaempferol-7-*O*-L-rhamnopyranoside (8 mg) and 7-*O*-α-L-rhamnopyranosylkaempferol-3-*O*-β-D-glucopyranoside (26 mg).

Lespedezavirgatol (1): pale yellow, amorphous powder; UV (MeOH) λ_{max} (log ϵ) 234 (4.21), 280 (3.97), 317 (4.56), 330 (sh) nm; ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6), see Table 1; ESIMS *m/z* 351 [M + Cl]$^-$, 315 [M − H]$^-$; HRESIMS *m/z* 339.0839 [M + Na]$^+$ (calcd for $C_{17}H_{16}O_6Na$, 339.0845).

Lespedezavirgatal (2): yellowish powder; UV (MeOH) λ_{max} (log ϵ) 287 (4.17), 318 (4.67), 356 (4.05), 384 (sh) nm; ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6), see Table 1; ESIMS *m/z* 379 [M + Cl]$^-$, 343 [M − H]$^-$; HRESIMS *m/z* 367.0788 [M + Na]$^+$ (calcd for $C_{18}H_{16}O_7Na$, 367.0794).

Lespedezacoumestan (3): yellowish powder; UV (MeOH) λ_{max} (log ϵ) 214 (4.82), 253 (2.02), 347 (2.35) nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.25 (1H, s, H-10), 7.16 (1H, s, H-7), 6.73 (1H, s, H-4), 3.90

(3H, s, 1-OMe), 2.65 (2H, m, H_2-1′), 1.57(2H, m, H_2-2′), and 1.17 (6H, s, H-4′and H-5′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.3 (C-3), 157.8 (C-6), 157.7 (C-11a), 153.7 (C-1), 152.5 (C-4a), 149.0 (C-10a), 145.6 (C-9), 144.4 (C-8), 121.0 (C-2), 104.5 (C-7), 100.0 (C-11b), 99.1 (C-4), 98.9 (C-10), 69.0 (C-2′), 62.4 (C-3′), 43.3 (C-2′), 29.1 (C-4′ and C-5′), 18.3 (C-1′); ESIMS *m/z* 435 $[M + Cl]^-$, 399 $[M - H]^-$, 369 $[M - OCH_3]^-$; HRESIMS *m/z* 399.1074 $[M - H]^-$ (calcd for $C_{21}H_{19}O_8$, 399.1080).

Oxygen Radical Absorbance Capacity. The activity was assessed by the ORAC-fluorescein assay.[22] In this assay, Trolox standards (10 μM), fluorescein (63 nM), and AAPH (12.8 mM) in phosphate buffer (PBS, 75 mM, pH 7.4) were prepared prior to use. Serial 2-fold dilutions of compounds **1**−**3** and vitamin C (positive control) were made in PBS. In a Costar 3631 assay plate, 20 μL each of compound dilutions, 140 μL of AAPH, and 20 μL of fluorescein were sequentially added to each well. The ORAC measurements were carried out on a Labsystems Fluoroskan plate reader with fluorescent filters (excitation at 485 nm and emission at 527 nm). The ORAC values, calculated as difference of the areas under the quenching curves of fluorescein between the blank (PBS) and the samples, were expressed as Trolox equivalents per μM samples.

Inhibition of Lipid Peroxidation. Potassium bromate-induced renal oxidative stress and kidney damage of rat were generated using the method of Watanabe et al.[24] with minor modification. Briefly, the rats were anesthetized 6 h after intraperitoneal administration of $KBrO_3$ (1.2 mM). Blood was collected in tubes containing heparin and centrifuged at 1000 rpm for 10 min to obtain the plasma. After collection of the blood, the rats were sacrificed. The kidneys were immediately excised, washed, and homogenized. The resultant homogenate was centrifuged at 1600 rpm for 10 min at 4 °C. Inhibition of lipid peroxidation on rat kidney homogenates and plasma was evaluated by the TBARS assay.[23] The supernatant of kidney homogenate or plasma (0.5 mL) was added to each of the solutions of compounds **1**−**3** and Vc (0.1 mL each). After standing for 10 min, 100 μL of each reaction solution was transferred to a 5 mL vial, and 100 μL of SDS, 1.5 mL of HOAc, and 1.5 mL of TBA were sequentially added. The mixture was incubated at 95 °C for 1 h. After cooling in an ice bath, the mixture was centrifuged at 1600 rpm for 10 min at 4 °C and allowed to stand at room temperature for 30 min. The upper layer (150 μL) was transferred to the plate. The absorbance at 540 nm was read using an EIA multiwell reader. The IC_{50} value, defined as the concentration giving 50% inhibition of malondialdehyde (MDA) level, was determined from a dose−response curve. The results are shown in Table 2.

Acknowledgment. We are grateful to Prof. Hiroshi Kurihara, College of Pharmacy, Jinan University, Guangzhou, China, for the ORAC measurements.

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