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CONSTITUENTS FROM THE RHIZOMES OF *CURCUMA COMOSA* AND THEIR WNT SIGNAL INHIBITORY ACTIVITIES

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Dedicated to Professor Victor Snieckus on the occasion of his 77th birthday

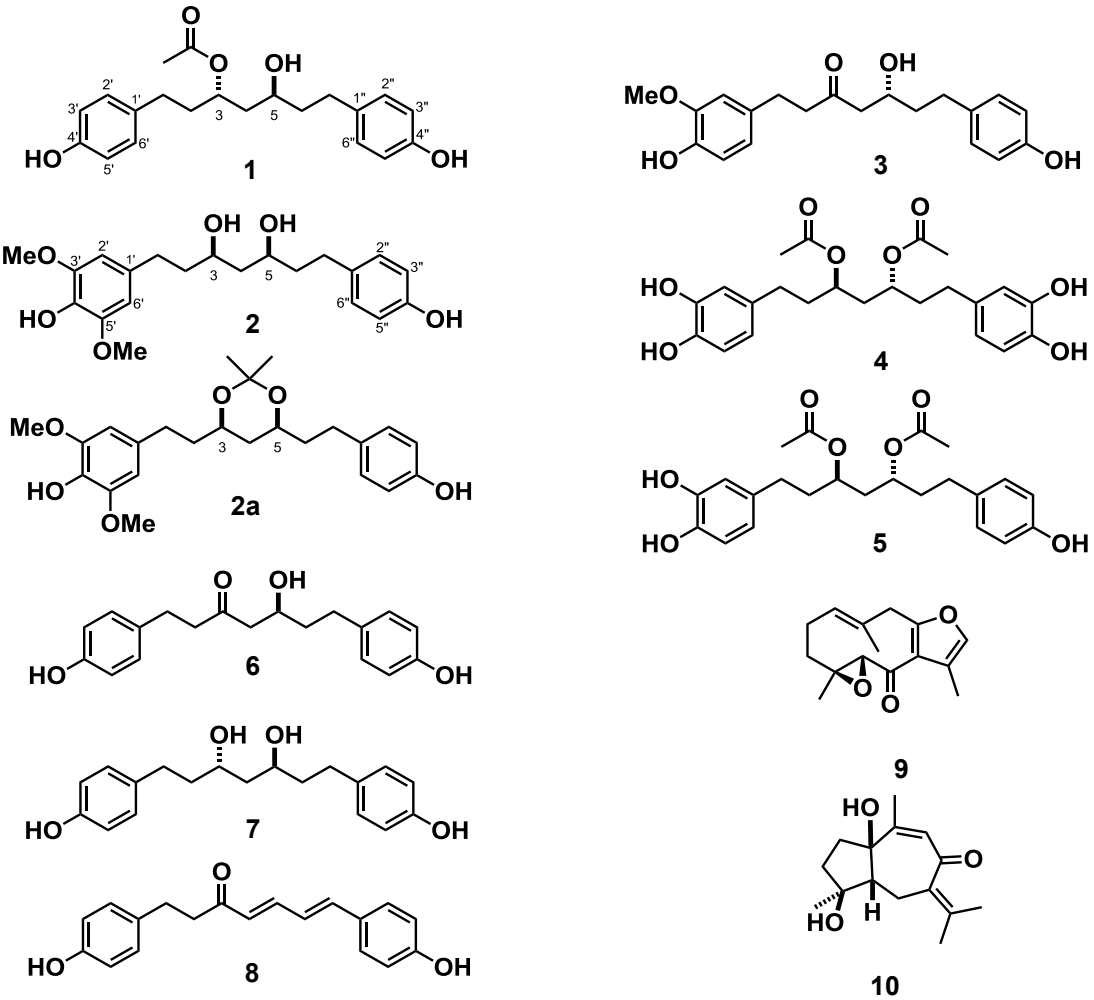
Abstract – Activity-guided fractionation of the *Curcuma comosa* rhizome MeOH extract that inhibited Wnt signal activity led to the characterization of two new diarylheptanoids (**1** and **2**) together with six known diarylheptanoids (**3**-**8**) and two known sesquiterpenoids (**9** and **10**). Their structures were identified by 1D and 2D NMR, HRESIMS, optical rotation, and chemical evidences. Compounds **1** and **8** exhibited dose-dependent inhibitory activity against β-catenin/TCF transcriptional activity.

The Wnt pathway is one of the signaling pathways that plays a significant role in the regulation of several embryonic development and cellular processes.[1] When the Wnt signal is active, β-catenin accumulates, translocates to the nucleus, and activates T-cell factor/lymphoid enhancing factor (TCF/LEF) for the transcription of target genes.[2] However, abnormal activation of this pathway may lead to the development of various diseases and abnormalities. Most cancer cells, particularly colon and melanoma cancer cells, harbor mutations upregulate the Wnt signaling pathway.[3,4] Furthermore, deregulated activation of the Wnt pathway has been linked to the incidence of type II diabetes, and contributes to abnormalities including high bone mass and oligodontia.[1,4] Thus, inhibition of the Wnt signal may present promising therapeutic strategies to treat or manage these diseases.

Several small molecules have been reported to inhibit the Wnt signal. Our group previously reported natural products with potent inhibitory activity against the β-catenin transcriptional pathway.[5-7] In our

continuing search for bioactive natural products exhibiting effects on Wnt signaling, we recently identified *Curcuma comosa* as a hit plant in our screening program. *C. comosa* is widely used in Thailand as a medicinal plant to treat inflammation. Previous chemical studies showed that the rhizome of this plant contained diarylheptanoids and sesquiterpenes.[8,9] Pharmacological studies also demonstrated that it had estrogenic[10] and anti-inflammatory activities,[11] which were attributed to the presence of diarylheptanoids. In this study, we isolated and characterized the constituents of *C. comosa* with potent inhibitory activity of the Wnt signal. Activity-guided isolation with a luciferase cell-based assay system to evaluate the inhibition of β-catenin/TCF transcriptional activity yielded two new diarylheptanoids (compounds **1** and **2**).

Based from our screening using the SuperTOPFlash assay, the MeOH rhizome extract of *C. comosa* showed 82% inhibition of TOP activity at 50 μg/mL. The viability of STF/293 reporter cells at the same concentration was 84%. Through activity-guided isolation, the active compounds were purified using silica gel, ODS column chromatography, and HPLC to give two new (**1** and **2**) together with six known (**3**-**8**) diarylheptanoids and two known sesquiterpenoids, zederone (**9**) and aerugidiol (**10**).



Compound **1** was isolated as a colorless solid and gave a molecular formula of $C_{21}H_{26}O_5$ as determined by HRESIMS (*m/z* 381.1634 $C_{21}H_{26}O_5Na$, Δ -4.4 mmu). Both the ^1H and ^{13}C NMR data (Table 1) of **1** are consistent with published data of its reported enantiomer (**ent-1**).[12] **1** gave a negative optical rotation, which suggests a 3*S*,5*S* configuration, while its reported 3*R*,5*R* enantiomer (**ent-1**) had a positive value. Deacetylation of **1** gave a product whose NMR data were identical with those of hannokinol.[13,14] Optical rotation of the deacetylated product was determined using reversed phase HPLC equipped with an optical rotation detector (ORD) and its response was compared with the isolated (-)-hannokinol (**7**). The 3*R*,5*R* form (**ent-7**) was previously reported to be $[\alpha]^{26}_D$ +4.0 (*c* 0.10 MeOH),[14] while the 3*S*,5*S* form (**7**) was found to be $[\alpha]^{25}_D$ -19.0.[13] Compound **7** and the deacetylated product of **1** both gave a negative response in the ORD. These findings suggest that the deacetylated product of **1** had a negative optical rotation. Therefore, the structure of compound **1** was determined to be (-)-(3*S*,5*S*)-3-acetoxy-5-hydroxy-1,7-bis(4-hydroxyphenyl)heptane.

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) spectral data for compounds **1** and **2**

Position	**1**[a]		**2**[b]	
	^1H (δ, ppm; *J*, Hz)	^{13}C (δ, ppm)	^1H (δ, ppm; *J*, Hz)	^{13}C (δ, ppm)
1	2.48 (2H, m)	32.0	2.56 (2H, m)	30.7
2	1.87 (2H, m)	38.0	1.74 (2H, m)	40.0
3	5.06 (1H, m)	73.0	3.85 (1H, m)	72.4
4	1.72 (1H, m)	43.0	1.56 (2H, m)	42.8
	1.60 (1H, m)			
5	3.51 (1H, m)	68.1	3.85 (1H, m)	72.5
6	1.63 (2H, m)	41.0	1.74 (2H, m)	39.9
7	2.63 (1H, m)	31.8	2.66 (2H, m)	31.9
	2.54 (1H, m)			
1’		133.7		132.9
2’	6.96 (1H, d, 8.7)	130.3	6.39 (1H, s)	104.9
3’	6.67 (1H, d, 8.7)	116.1		146.9
4’		156.4		132.8
5’	6.67 (1H, d, 8.7)	116.1		146.9
6’	6.96 (1H, d, 8.7)	130.3	6.39 (1H, s)	104.9
1”		134.2		133.3
2”	6.98 (1H, d, 8.7)	130.3	6.99 (1H, d, 8.3)	129.4
3”	6.68 (1H, d, 8.7)	116.1	6.73 (1H, d, 8.3)	115.3
4”		156.4		154.2
5”	6.68 (1H, d, 8.7)	116.1	6.73 (1H, d, 8.3)	115.2
6”	6.98 (1H, d, 8.7)	130.3	6.99 (1H, d, 8.3)	129.4
3-OAc	1.98 (3H, s)	21.1		
		173.1		
3’,5’-OMe			3.82 (6H, s)	56.2
3,5,4’,4”-OH			4.40 (4H, br s)	

[a] measured in CD_3OD
[b] measured in $CDCl_3$

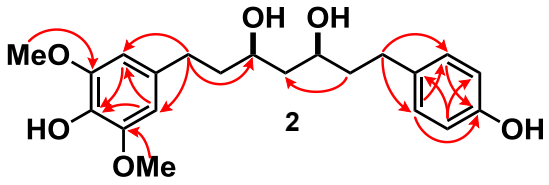


Figure 1. Selected HMBC of **2**

Compound **2** was obtained as a mixture with a minor unknown compound. An intense peak at *m/z* 399.1762 ($[M+Na]^+$, $C_{21}H_{28}O_6Na$ Δ -2.1 mmu) was observed in HRESIMS, which is consistent with the molecular formula of $C_{21}H_{28}O_6$. The ^1H NMR spectra of **2** and the unknown compound exhibited some overlapping peaks, which indicated that they are structurally-related compounds. A singlet peak at δ 3.82 (6H) was due to the two symmetric methoxy groups and were assigned at C-3' and C-5'. The presence of two symmetric benzene protons was indicated by the correlations observed between a singlet proton peak at δ 6.39 and carbon peak at δ 104.9 in both HMBC (Figure 1) and HMQC. They were assigned at H-2' and H-6'. The two doublets at δ 6.99 (J = 8.3 Hz) and 6.73 (J = 8.3 Hz) suggested the presence of 1,4-disubstituted aromatic protons and were assigned to four aromatic protons, H-2"/H-6" and H-3"/H-5", respectively. From these results, **2** was assigned as 3,5-dihydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxyphenyl)heptane.

Acetonidation of compound **2** using Rychnovsky's method yielded its acetonide derivative **2a**. The HMQC data of **2a** showed correlations between the two methyl peaks at δ 1.32 and δ 1.62, and ^{13}C peaks at δ 21.9 and δ 32.5, respectively. These results suggested that **2a** was produced from a *syn*-1,3-diol.[15] An intense peak at *m/z* 439.2070 ($[M+Na]^+$, $C_{24}H_{32}O_6Na$, Δ -2.7 mmu) was observed based on HRESIMS and corresponded to **2a**. The absolute stereochemistry of **2** remains undefined.

The structure of the six known diarylheptanoids (5*R*)-5-hydroxy-7-(4-hydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-3-heptanone (**3**),[12,16] (3*R*,5*R*)-3,5-diacetoxy-1,7-bis(3,4-di- hydroxyphenyl)heptane (**4**),[17] (3*R*,5*R*)-3,5-diacetoxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)hep-tane (**5**),[12] (5*S*)-5-hydroxy-1,7-bis(4-hydroxyphenyl)heptan-3-one (**6**),[18,19] (-)-hannokinol (**7**),[13] 1,7-bis(4-hydroxyphenyl)hepta-4*E*,6*E*-dien-3-one (**8**),[20] and two known sesquiterpenoids, zederone (**9**)[21] and aerugidiol (**10**)[22,23] were identified by comparing their spectral values and optical rotations with those in the literature.

The Wnt signal inhibitory activities of the characterized compounds were determined using the luciferase reporter assay described previously.[5] Quercetin (IC_{50} = 30.4 μM),[5] which was previously shown to possess β-catenin/TCF transcriptional inhibitory activity,[23] was used as a positive control. Compounds **1**, **7**, and **8** showed the highest inhibitory activity in the SuperTOPflash assay with IC_{50} values of 3.0, 1.0, and 2.9 μM, respectively. However, only **1** and **8** did not affect SuperFOPflash activity up to 5 μM, which

suggests that they have inhibitory activity against β-catenin/TCF transcriptional pathway (Figure 2). The other diarylheptanoids (**2**-**7**) as well as zederone (**9**) also showed dose-dependent inhibitory activity against the SuperTOPflash assay; however, since their IC_{50} values were higher than 10 μM (data not shown), they were not subjected to further assays. Aerugidiol (**10**), on the other hand, was inactive.

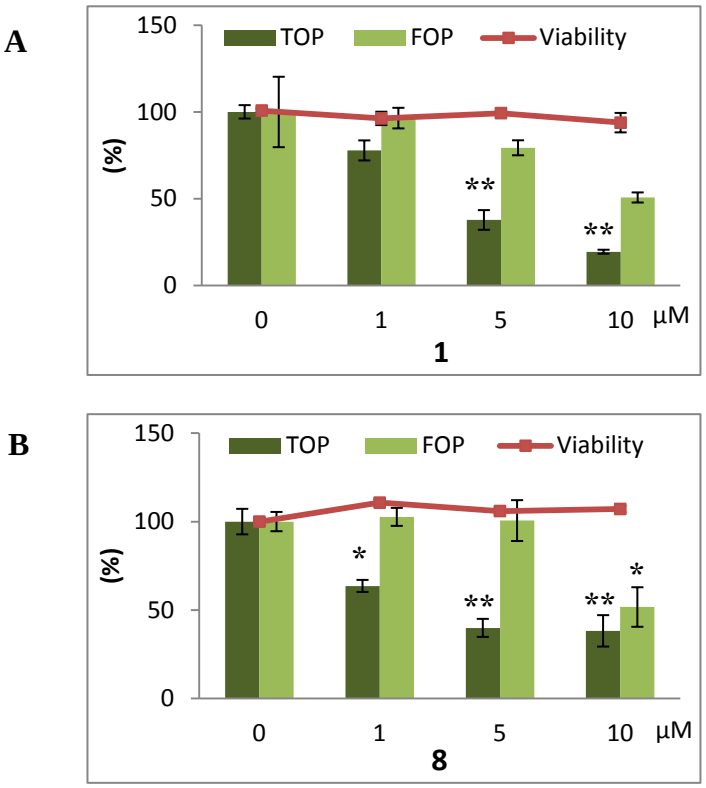


Figure 2. Bar graphs showing the dose-dependent TOPflash and FOPflash activities of **1** (A) and **8** (B). The line graph represents cell viability All data are presented as the mean ± SEM (n=3). *p < 0.05; **p < 0.01, Tukey's test, significantly different from control (0 μM).

Since the assay involved the addition of LiCl, an inhibitor of glycogen synthase kinase-3 (GSK3), the data may suggest that **1** and **8** act downstream of the Wnt signaling pathway or on β-catenin itself. However, western blot analysis using SW480 cells showed that β-catenin levels in both cytoplasmic and nuclear fractions were not affected. Some curcumin derivatives exhibiting Wnt/β-catenin pathway inhibitory activity were also reported to have no effect on β-catenin levels. Instead, they showed to decrease the level of p300, which is a positive regulator of the Wnt signal pathway.[24] The FMCA assay was used to determine the effect of **1** and **8** on the viability of colon cancer cells, SW480, HCT116, and DLD1. Several studies previously reported that inhibiting β-catenin/TCF transcriptional activity decreased the viability of colon cancer cells.[24,25] However, after 24 hr incubation, neither compounds exhibited inhibitory activity on the growth of these three colon cancer cells.

In conclusion, we isolated two new diarylheptanoids alongside six known diarylheptanoids and two known sesquiterpenoids. Our results indicate that although compounds **1** and **8** showed inhibitory activity against the Wnt signal, they did not affect the proliferation of various colon cancer cells. Another mechanism also involving β-catenin may compensate for the loss of β-catenin/TCF transcriptional activity. Or, cell permeability of the compounds may provide an explanation why level of β-catenin and proliferation of the cancer cells were not affected.

EXPERIMENTAL

General Experimental Procedure. ^{1}H and ^{13}C NMR spectra were recorded on JEOL JNM-ECA600 spectrometers with deuterated solvent. HRESIMS were measured on a JEOL JMS-T100LP spectrometer. Optical rotation was determined with a JASCO P-1020 polarimeter. UV spectra were obtained in a Shimadzu UV mini-1240 spectrometer. IR spectra were measured on ATR in a JASCO FTIR 230 spectrophotometer.

Plant Materials. The rhizome of *C. comosa* was collected from Khon Kaen, Thailand and identified by T. Kowithyakorn. A voucher specimen (7-196) was deposited in our laboratory.

Cell culture. The STF/293 cell line was a generous gift from Prof. Jeremy Nathans (John Hopkins Medical School); HEK293, HCT116, and DLD1 cells were purchased from ATCC; SW480 cells were derived from the Institute of Development, Aging, and Cancer, Tohoku University. STF/293, HEK293, SW480, DLD1, and HCT116 were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FBS. Cultures were maintained in a humidified incubator at 37 ^{o}C in 5% CO_2/95% air.

Luciferase assay. TCF/β-catenin transcriptional activity was determined using the method previously described.[5] STF/293 cells (3×10^4), which were stably transfected with SuperTOPflash, were seeded into 96-well plates and, after 24 h incubation, treated with compounds combined with 15 mM LiCl. After 24 h, cells were lysed and luciferase activity was measured. HEK293 cells (1×10^5) were briefly split into 24-well plates for the control reporter assay (FOPFlash). After 24 h, cells were transfected with 1 μg of the luciferase reporter construct (SuperFOPflash) and 2 ng of pRL-CMV (Promega, USA) for normalization. At 3 h post-transfection, the compounds combined with 15 mM LiCl were added. Cells were lysed after 24 h incubation, and luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

Cell viability assay. Cell viability was measured using the FMCA assay.[26] Cells were inoculated into 96-well plates for 24 h. Compounds were then added and incubated for another 24 h. After incubation, they were treated with fluorescein diacetate (Wako) in PBS buffer. Fluorescence was detected after 1 h incubation.

Isolation of the constituents. The dried rhizome of *C. comosa* (226 g) was extracted using MeOH. The

MeOH crude extract (6.6 g) of *C. comosa* was directly subjected to silica gel column chromatography (φ50 x 270 mm) eluting with the hexane-EtOAc gradient system (1:0 to 0:1) to give fractions 1A-1L. Fraction 1E (76.5 mg) was recrystallized to give compound **9** (28.4 mg). Fraction 1J (548 mg), which showed high activity on the TOP assay, was then subjected to ODS flash column chromatography (φ25 x 180 mm) to yield fractions 3A-3H. Active fraction 3D (59 mg) was further purified using silica gel column chromatography (φ25 x 170 mm; $CHCl_3$:MeOH gradient system (1:0 - 0:1)) to give compound **4** (11.2 mg). Another fraction from 3D was further purified by preparative HPLC [Cosmosil 5C_{18}-AR-II; φ10 x 250 mm; 60% MeOH] to give compounds **1** (5.7 mg) and **5** (4.9 mg). Fraction 3B (58.2 mg) was subjected to flash silica column chromatography (φ25 x 230 mm) using $CHCl_3$: MeOH (1:0 to 0:1) to give fractions 6A-6H. Fraction 6B was identified as compound **10** (6.2 mg). Fraction 6F was further purified by preparative HPLC [Cosmosil 5C_{18}-AR-II; φ10 x 250 mm; 53% MeOH] to afford compound **6** (2.9 mg). Fraction 6C gave compound **3** (1.1 mg) after purification by HPLC [Cosmosil 5C_{18}-AR-II; φ10 x 250 mm; 45% MeOH]. The active fraction 1K was subjected to ODS flash column chromatography (φ45 x 180 mm) and eluted with increasing MeOH (67-100%), followed by silica gel column chromatography (φ30 x 235 mm) using $CHCl_3$:MeOH (1:0 to 0:1) yielded ten fractions (11A-11J). Fraction 11H was identified as compound **7** (14.8 mg). Fraction 11E, which showed weak activity, was further purified using by preparative HPLC [Cosmosil π-NAP; φ10 x 250 mm; 60% MeOH] to give a mixture (6.8 mg) of compound **2** and an unknown compound. Another active fraction, 1I (203.2 mg), was subjected to ODS flash column chromatography (φ40 x 170 mm) and eluted with increasing MeOH (60%-80%) to give 11 fractions (15A-15K). Fraction 15F (18.3 mg), which showed strong activity, was subjected to silica gel column chromatography using $CHCl_3$:MeOH (10:1-0:1) and yielded 8 fractions (16A-16H). Fraction 16D (7.1 mg) was further purified using Sephadex LH-20, followed by silica gel column chromatography [φ25 x 150 mm; $CHCl_3$:MeOH (1:0-0:1)] to afford compound **8** (0.7 mg).

Compound 1. Colorless solid; $[\alpha]^{26}_{D}$ -9.9 (*c* 0.5 MeOH); positive HRESIMS $[M+Na]^+$ *m/z* 381.1634 (calcd. for $C_{21}H_{26}O_5Na$ 381.1678, Δ -4.4 mmu); UV λ_{max} (MeOH) nm (log ε) 278 (3.6) and 223 (4.2) IR ν_{max} 3354, 2924, 1706, 1514, and 1242; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 1.

Deacetylation of 1.[12] A drop of conc. HCl was added to a MeOH (0.5 mL) solution of **1** (1.4 mg, 3.9 μmol) . The mixture was stirred at room temperature overnight, vacuum dried, and purified by silica gel chromatography using $CHCl_3$:MeOH (9:1) to yield **7** (0.5 mg, 1.6 μmol, 42% yield). The optical rotation of **7** was determined through its response (negative) in HPLC with an optical rotation detector (ORD).

Compound 2. Colorless solid; positive HRESIMS $[M+Na]^+$ *m/z* 399.1762 (calcd. for $C_{21}H_{26}O_5Na$ 399.1784, Δ -2.1 mmu; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz), see Table 1.

Acetonidation of 2. The acetonide derivative of **2** was prepared using the procedure presented by

Rychnovsky *et al*.[15] with minor modifications. 2,2-Dimethoxypropane (1 mL) was added to a solution of **2** (1.0 mg, 2.7 μmol) and pyridinium/*p*-toluenesulfonate (2.3 mg, 9.2 μmol) in dehydrated acetone (0.5 mL). The reaction mixture was stirred at room temperature for 1 h and dried in a stream of N_2 vapor. The residue was subjected to silica gel chromatography using $CHCl_3$:MeOH (95:5) as the eluent and afforded **2a** (0.6 mg, 1.4 μmol, 55% yield). ^{1}H NMR (600 MHz, C_6D_6): δ 0.93 (1H, dt, *J* = 1.2, 12.6 Hz, H-4), δ 0.99 (1H, dt, *J* = 1.2, 12.6 Hz, H-4), δ 1.32 (3H, s, CH_3), δ 1.55 (2H, m, H-2 and 6), δ 1.62 (3H, s, CH_3), δ 1.84 (2H, m, H-2 and 6), δ 2.66 (2H, m, H-1 and 7), δ 2.76 (2H, m, H-1 and 7), δ 3.37 (6H, m, 3'-OCH_3 and 5'-OCH_3), δ 6.37 (2H, s, H-2' and 6'), δ 6.52 (2H, d, *J* = 8.7, H-3 and 5), δ 6.95 (2H, d, *J* = 8.7, H-2" and 6"). HRESIMS *m/z* 439.2070 $[M+Na]^+$ (calcd. for $C_{24}H_{32}O_6Na$ 439.2097 Δ -2.7 mmu).

Statistical Analysis. Data for the inhibitory activities and cell viability are presented as the mean ± SEM of the three replicates of one representative experiment from two independent experiments. One way analysis of variance (ANOVA) followed by Tukey's test. Probability (*p*) values less than 0.05 were considered significant.

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