A New Prenylated Isoflavone and a New Flavonol Glycoside from *Flemingia philippinensis*

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A new prenylated isoflavonoid, flemiphilippinin G (**1**), and a new flavonol glycoside, flemiphilippininside (**2**), along with eleven known isoflavonoids were obtained from the roots of *Flemingia philippinensis* (MERR. et ROLFE) LI. Their structures were elucidated on the basis of spectroscopic data. The *in vitro* cytotoxicities of compounds **1**–**13** against MCF-7, A549, and Hep-G2 cell lines were determined by using the MTT (= 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) colorimetric assay, and their antioxidant activities were evaluated by ferric-reducing antioxidant power (FRAP) method. Compound **1** exhibited significant cytotoxicity against all the tested cell lines with IC_{50} values of 4.8–7.3 μM, and compound **2** was found to be inactive. Both compounds **1** and **2** showed weak antioxidant activities with FRAP values of 110 ± 15 and 124 ± 16 μmol/g, respectively.

Introduction. – *Flemingia philippinensis* (MERR. et ROLFE) LI is a shrubby herb mainly growing in tropical and subtropical areas. Its roots have been used in folk medicine for the treatment of rheumatism, arthropathy, leucorrhea, menalgia, menopausal syndrome, and chronic nephritis, and for improving bone mineral density. It was reported that the root extract of *F. philippinensis* exhibited antioxidant, anti-inflammatory, estrogenic, and anti-estrogenic activities [1]. In recent years, the phytochemical composition of *F. philippinensis* has drawn an increasing attention due to the structural complexity and diverse bioactivities, *e.g.*, estrogenic/anti-estrogenic isoflavonoids [1–4], which encouraged us to initiate an extensive study aimed at the identification of isoflavonoids and evaluation of their bioactivities for a better understanding of the active ingredients and action mechanism underlying its traditional medical use. In our study of bioactive compounds from the roots of *F. philippinensis*, one new prenylated isoflavonoid, named flemiphilippinin G (**1**), and one new flavonol glycoside, named flemiphilippininside (**2**), together with eleven known isoflavonoids, **3**–**13**, were obtained. Here, we report the isolation, structure elucidation, and anticancer and antioxidant activities of all the isolated compounds.

Results and Discussion. – The AcOEt-soluble fraction was subjected to repeated column chromatography over silica gel, polyamide, *Sephadex LH-20*, and *ODS* to

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afford two new compounds, **1** and **2**, along with eleven known isoflavonoids (*Fig. 1*), 5,7,3′-trihydroxy-2′-(3-methylbut-2-enyl)-4′,5′-(3,3-dimethylpyrano)isoflavone (**3**) [5], auriculasin (**4**) [5], flemiphilippinin A (**5**) [3], 5,7,3′,4′-tetrahydroxy-2′,5′-diprenyliso-flavone (**6**) [5], 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone (**7**) [4], genistein (**8**) [6], 3′-*O*-methylorobol (**9**) [6], piscigenin (**10**) [7], 8-(1,1-dimethylallyl)genistein (**11**) [5], genistin (**12**) [6], and pallidiflorin (**13**) [8]. Compound **10** was isolated from *F. philippinensis* for the first time. Their structures were identified by means of spectroscopic methods and comparison with the reported data.

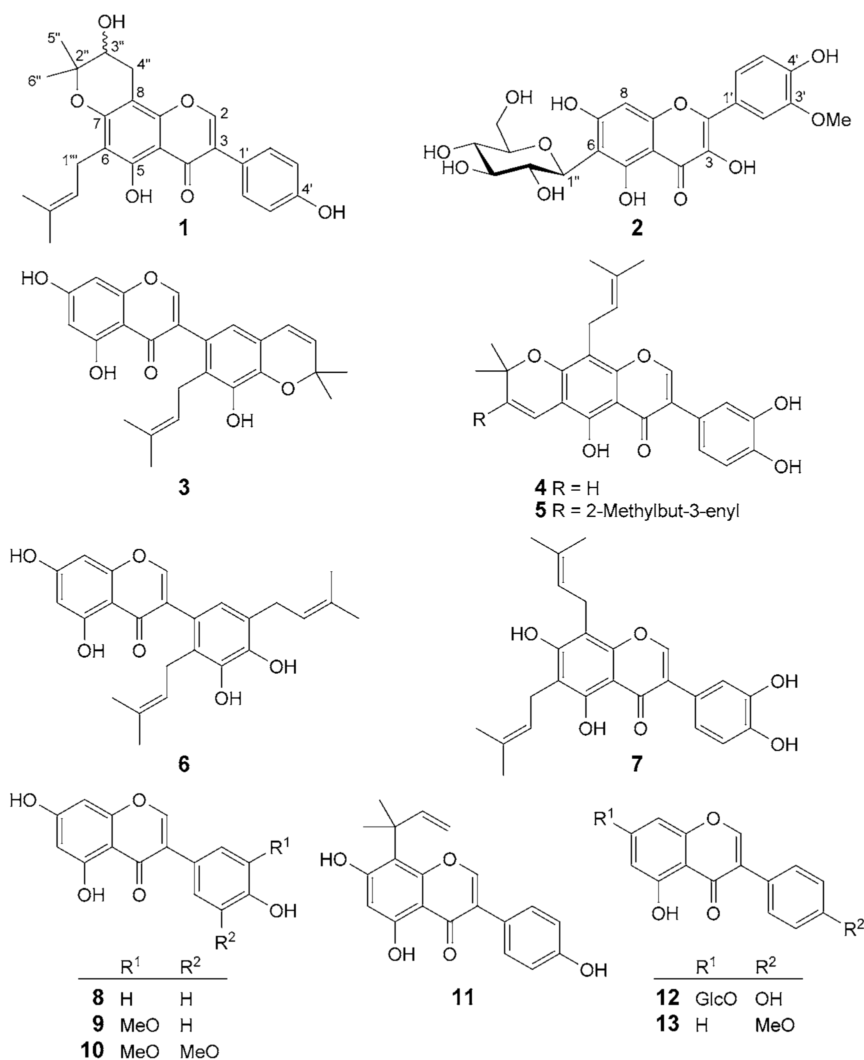

Fig. 1. *The structures of compounds* **1** – **13**

Compound **1** was obtained as a pale yellow amorphous powder. The molecular formula was established as $C_{25}H_{26}O_6$ by HR-EI-MS (*m/z* 422.1718 ($C_{25}H_{26}O_6^+$; calc. 422.1724)) and NMR data. The UV absorption (λ_{max} 272 nm in MeOH) and IR spectrum (3425, 1643, and 1435 cm^{-1}) suggested the presence of either an isoflavonoid or flavonoid skeleton. The ^{1}H-NMR spectrum showed a H-atom *singlet* at δ(H) 8.45 (*s*, H–C(2)), which is characteristic of an isoflavone (*Table 1*). An *AB* system (δ(H) 6.83 (*d*, *J* = 8.4, H–C(3′,5′)), 7.38 (*d*, *J* = 8.4, H–C(2′,6′))), indicating a 4′-monosubstituted *B* ring, and one OH signal at δ(H) 13.06 (*s*, 5-OH) were observed. The signals at δ(H) 1.62 (*s*, Me(4‴)), 1.74 (*s*, Me(5‴)), 3.23 (*d*, *J* = 7.2, CH_2(1‴)), and 5.17 (*t*, *J* = 7.2, H–C(2‴)) were consistent with the presence of a prenyl group [9]. Generally, the geminal dimethyl H-atom signals (δ(H) 1.24 (*s*, Me(5″)) and 1.33 (*s*, Me(6″))), one O-bearing CH group signal (δ(H) 3.72 – 3.75 (*m*, H–C(3″))), and two diastereotopic H-atom signals (δ(H) 2.63 (*dd*, *J* = 7.0, 16.5, H_a–C(4″)), 2.93 (*dd*, *J* = 5.2, 16.5, H_b–C(4″))) indicated the presence of a 3-hydroxy-2,2-dimethyldihydropyran moiety [10]. All these groups, including the prenyl chain, the dihydropyran unit, and the OH group, were attached at the *A* ring of the isoflavonoid. To further assign the position, the HMBC spectrum was applied. In the HMBC spectrum (*Fig. 2*), long-range correlations from H–C(1‴) (δ(H) 3.23) to C(5) (δ(C) 156.1) and C(7) (δ(C) 156.5), and from HO–C(5) (δ(H) 13.06) to C(5), C(6) (δ(C) 111.1), and C(10) (δ(C) 104.8), indicated that the prenyl and the OH groups are attached at C(6) and C(5), respectively. Moreover, the long-range couplings from H–C(4″) (δ(H) 2.63, 2.93) to C(7) (δ(C) 156.5), C(8) (δ(C) 98.6), and C(9) (δ(C) 152.8) supported that the dihydropyran moiety is fused along the C(7) – C(8) bond (*Fig. 1*). Thus, from all the informations above, compound **1** was determined to be a new isoflavone, named flemiphilippinin G (**1**)[1]).

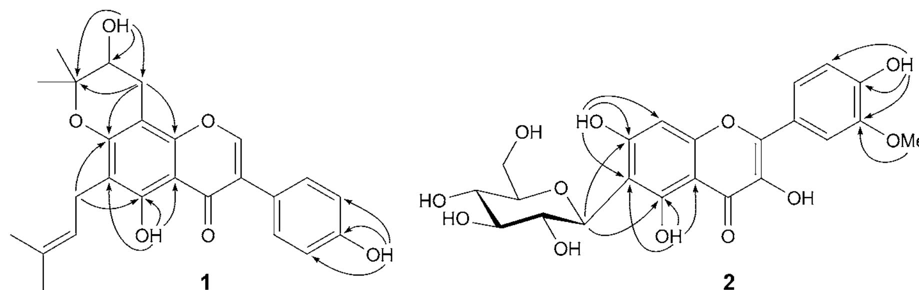

Fig. 2. *Key HMBCs* (H → C) *of* **1** *and* **2**

Compound **2** was obtained as a yellow amorphous powder. The molecular formula was established as $C_{22}H_{22}O_{12}$ by HR-EI-MS (*m/z* 478.1111 ($C_{22}H_{22}O_{12}^+$; calc. 478.1106)) and NMR data. The UV absorptions (λ_{max} 256 and 376 nm in MeOH) and IR spectrum (3418, 1624, and 1468 cm^{-1}) indicated a free OH group at C(3) of the flavonol skeleton [11]. The ^{1}H-NMR spectrum (*Table 1*) displayed a MeO signal (δ(H) 3.84 (*s*, MeO–C(3′))), three aromatic H-atom signals (δ(H) 6.94 (*d*, *J* = 8.0, H–C(5′)), 7.69

[1]) For systematic name, see the *Exper. Part*.

Table 1. *^{1}H- and ^{13}C-NMR Data of Compounds* **1** *and* **2** *in (D$_6$)DMSO* (δ in ppm, J in Hz). For atom numbering, see *Fig. 1*.

	1		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
C(2)	8.45 (*s*)	153.9		146.4
C(3)		122.3		135.7
C(4)		180.5		176.1
C(5)		156.1		159.8
C(6)		111.1		108.2
C(7)		156.5		163.1
C(8)		98.6		93.2
H–C(8)			6.50 (*s*)	
C(9)		152.8		155.0
C(10)		104.8		102.7
C(1′)		121.3		121.9
H–C(2′)	7.38 (*d*, J = 8.4)	130.2	7.76 (*d*, J = 2.0)	111.7
H–C(3′)	6.83 (*d*, J = 8.4)	115.1		147.3
C(4′)		157.4		148.8
H–C(5′)	6.83 (*d*, J = 8.4)	115.1	6.94 (*d*, J = 8.0)	115.6
H–C(6′)	7.38 (*d*, J = 8.4)	130.2	7.69 (*dd*, J = 2.0, 8.0)	121.6
H–C(1″)			4.60 (*d*, J = 8.8)	73.1
C(2″)		78.7		70.6
H–C(3″)	3.72–3.75 (*m*)	66.7		78.9
H–C(4″)	2.63 (*dd*, J = 7.0, 16.5), 2.93 (*dd*, J = 5.2, 16.5)	24.9		70.2
H–C(5″)	1.24 (*s*)	20.9		81.6
H–C(6″)	1.33 (*s*)	25.3		61.4
H–C(1‴)	3.23 (*d*, J = 7.2)	21.1		
H–C(2‴)	5.17 (*t*, J = 7.2)	121.9		
C(3‴)		130.6		
H–C(4‴)	1.62 (*s*)	17.7		
H–C(5‴)	1.74 (*s*)	25.5		
HO–C(3)			9.47 (*s*)	
HO–C(5)	13.06 (*s*)		13.06 (*s*)	
HO–C(7)			10.55 (*s*)	
*Me*O–C(3′)			3.84 (*s*)	55.7
HO–C(4′)	9.61 (*s*)		9.76 (*s*)	
HO–C(3″)	5.32 (*d*, J = 4.4)			

(*dd*, J = 8.0, 2.0, H–C(6′)), and 7.76 (*d*, J = 2.0, H–C(2′))), indicating a 3′,4′-disubstitution of ring *B*. Four phenolic-OH signals (δ(H) 9.47 (*s*, HO–C(3)), 9.76 (*s*, HO–C(4′)), 10.55 (*s*, HO–C(7)), 13.06 (*s*, HO–C(5))), and an aromatic H-atom *singlet* at δ(H) 6.50 (*s*, H–C(8)) were also observed. The ^{13}C-NMR spectrum (*Table 1*) revealed the presence of 15 flavonol C-atoms (δ(C) 146.4 (C(2)), 135.7 (C(3)), 176.1 (C(4)), 159.8 (C(5)), 108.2 (C(6)), 163.1 (C(7)), 93.2 (C(8)), 155.0 (C(9)), 102.7 (C(10)), 121.9 (C(1′)), 111.7 (C(2′)), 147.3 (C(3′)), 148.8 (C(4′)), 115.6 (C(5′)), 121.6 (C(6′))), and a MeO C-atom (δ(C) 55.7 (MeO–C(3′))). The rest of the C-atom signals suggested the presence of a sugar residue (six glucosyl C-atom signals at δ(C) 61.4 (C(6″)), 70.2 (C(4″)), 70.6 (C(2″)), 73.1 (C(1″)), 78.9 (C(3″)), and 81.6 (C(5″))), and

the ^{1}H-NMR signal at δ(H) 4.60 (d, $J = 8.8$, H–C(1″)) evidenced the β-configuration of the glucosyl residue. In the HMBC spectrum, the long range correlations from HO–C(5) (δ(H) 13.06) to C(5) (δ(C) 159.8), C(6)), and C(10) (δ(C) 102.7), from HO–C(7) (δ(H) 10.55) to C(6), C(7) (δ(C) 163.1), and C(8) (δ(C) 93.2), from HO–C(4′) (δ(H) 9.76) to C(3′) (δ(C) 147.3), C(4′) (δ(C) 148.8), and C(5′) (δ(C) 115.6), and from MeO–C(3′) (δ(H) 3.84) to C(3′) indicated that three OH groups and one MeO group were attached at C(5), C(7), and C(4′), and C(3′), respectively (*Fig. 2*). The correlation from H–C(1″) (δ(H) 4.60) to C(5) (δ(C) 159.8), C(6) (δ(C) 108.2), and C(7) (δ(C) 163.1), indicated that the glucosyl moiety was attached at C(6), which was further confirmed by the significant downfield shift of C(6) (from δ(C) 99.0 to 108.2), compared to quercetin [12], and that the flavone is a *C*-glycoside flavonol [11]. Therefore, the structure of compound **2**, named flemiphilippininside[1]), was deduced as depicted in *Fig. 1* (**2**).

The cytotoxicities of all the isolated compounds **1**–**13** were evaluated against human breast carcinoma cell line (MCF-7), human lung cancer cell line (A549), and human hepatoma cell line (Hep-G2), using the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) colorimetric assay. The IC_{50} values of compounds **1**–**13** on the viability of cancer cells after 72 h of incubation are compiled in *Table 2*. Compared to tamoxifen and doxorubicin, which were used as positive control in this study, compound **1** exhibited strong cytotoxicities against all the tested cell lines with IC_{50} values of 5.1 ± 0.1, 7.3 ± 0.2 and 4.8 ± 0.1 μM, respectively. Compounds **3** and **9** exhibited significant cytotoxicities against MCF-7 cells with IC_{50} values of 7.5 ± 0.2 and 11.3 ± 0.1 μM, respectively. Compounds **3**, **10**, and **11** showed remarkable cytotoxicities against Hep-G2 cells with IC_{50} values of 11.9 ± 0.2, 6.8 ± 0.1, and 12.2 ± 0.1 μM, respectively. Furthermore, all the isolated compounds except **2** and **12** exhibited moderate or weak inhibitory activities against A549 cells with IC_{50} values from $14.5 \pm$

Table 2. *Cytotoxicities of Compounds* **1**–**13** *by the MTT Assay* (IC_{50}, [μM])

	MCF-7	A549	Hep-G2
1	5.1 ± 0.1	7.3 ± 0.2	4.8 ± 0.1
2	> 100	> 100	> 100
3	7.5 ± 0.2	24.8 ± 0.3	11.9 ± 0.2
4	34.0 ± 0.3	15.8 ± 0.2	34.7 ± 0.3
5	24.5 ± 0.2	14.5 ± 0.1	36.0 ± 0.3
6	18.8 ± 0.1	20.5 ± 0.2	29.6 ± 0.3
7	16.0 ± 0.1	15.0 ± 0.2	28.8 ± 0.2
8	20.0 ± 0.2	60.1 ± 0.4	24.2 ± 0.2
9	11.3 ± 0.1	90.9 ± 0.5	44.5 ± 0.3
10	25.3 ± 0.2	> 100	6.8 ± 0.1
11	17.2 ± 0.2	24.8 ± 0.2	12.2 ± 0.1
12	> 100	> 100	> 100
13	65.8 ± 0.4	79.2 ± 0.4	27.4 ± 0.2
Tamoxifen[a])	5.5 ± 0.1	12.0 ± 0.1	6.3 ± 0.1
Doxorubicin[a])	3.4 ± 0.1	3.8 ± 0.1	9.0 ± 0.1

[a]) Positive controls.

0.1 to 90.9 ± 0.5 μM. While **2** and **12** were found to be inactive against all the tested cell lines (IC_{50} > 100 μM), other compounds showed moderate inhibitory effects against MCF-7 and Hep-G2 with IC_{50} values ranging from 16.0 ± 0.1 to 65.8 ± 0.4 μM. The remarkable cancer cell anti-proliferative activities of **1** and other isoflavonoids has shed some light on the active ingredients and action mechanism supportive to the beneficial properties and rationale for the use of title plant as folk medicine.

The antioxidant activities of compounds **1**–**13** were evaluated by the FRAP assay. As shown in *Table 3*, the FRAP values of the compounds ranged from 110 ± 15 to 9252 ± 401 μmol/g. Compounds **1** and **2** exhibited weaker antioxidant activities compared to the reference compound, L-ascorbic acid, with the values of 110 ± 15 and 124 ± 16 μmol/g, respectively. Compounds **7**, **10**, and **11** showed potent antioxidant activities with the values of 4338 ± 481, 9252 ± 401, and 7962 ± 253 μmol/g, respectively.

Table 3. *Antioxidant Activities of Compounds* **1**–**13** *Determined by FRAP Assays*

Compound No.	FRAP [μmol/g]
1	110 ± 15
2	124 ± 16
3	164 ± 30
4	2977 ± 91
5	588 ± 26
6	1005 ± 25
7	4338 ± 481
8	180 ± 5
9	3591 ± 176
10	9252 ± 401
11	7962 ± 253
12	247 ± 10
13	119 ± 16
L-Ascorbic acid[a])	4930 ± 300

[a]) Positive control.

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Experimental Part

General. 2,4,6-Tripyridyl-*s*-triazine (TPTZ) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from *Sigma Chemical Co.* (St. Louis, MO). L-Ascorbic acid, tamoxifen, and doxorubicin were purchased from *Shanghai Boao Biotech Co., Ltd.* (Shanghai, P. R. China) and *Shenzhen Main Luck Pharmaceuticals Inc.* (Shenzhen, P. R. China), resp. Column chromatography (CC): silica gel (SiO_2; 100–200 and 200–300 mesh, *Qingdao Marine Chemical Ltd.*, Qingdao, P. R. China), *RP-18* silica gel (40 μm, *J. T. Baker*, USA), *Polyamide*, and *Sephadex LH-20.* UV Spectra: *Perkin-Elmer Lambda 35 UV-VIS* spectrophotometer; in MeOH; λ in nm. IR Spectra: *WQF-*

410 FT-IR spectrophotometer; as KBr tablets; in cm^{-1}. ^{1}H (400 MHz), ^{13}C-(100 MHz), and 2D-NMR spectra: *Bruker DRX-400* instrument; δ in ppm, J in Hz; residual solvent peak as reference. HR-EI-MS: *MAT95XP* mass spectrometer. ESI-MS: *MDS SCIEX API 2000 LC/MS/MS* instrument.

Plant Material. The roots of *F. philippinensis* were bought from the *Qingping Herbal Medicine Market* (Guangzhou, P. R. China) in 2008. The plant material was identified by Prof. *Yunfei Deng*, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, P. R. China. A voucher sample (No. 200802) was deposited with the Herbarium of South China Botanical Garden.

Extraction and Isolation. The powdered dry roots of *F. philippinensis* (40 kg) were extracted three times with 95% EtOH (50 l × 3) at r.t., for 3 d each. After evaporation of the solvent *in vacuo*, the combined crude EtOH extract (2 kg) was suspended in H_2O (4 l) to produce an aq. soln., which was then partitioned with AcOEt (4 l) to afford an AcOEt (670 g) extract. The AcOEt-soluble extract was subjected to CC (SiO_2); $CHCl_3$/MeOH 98 : 2 – 10 : 90) to yield six fractions, *Frs. 1 – 6*. *Fr. 2* (87.6 g) was further separated by CC (*Polyamide*, MeOH/H_2O 70 : 30 – 100 : 0) to afford three subfractions, *Subfrs. 2a – 2c*. *Subfr. 2a* (13.8 g) was subjected to CC (SiO_2; petroleum ether (PE)/AcOEt 90 : 10 – 60 : 40) to afford **1** (5 mg) and **5** (20.5 mg). *Subfr. 2b* (5.3 g) was purfied by recrystalization with MeOH to give **3** (150 mg) and **4** (25 mg). *Subfr. 2c* (3.1 g) was further separated by CC (*Sephadex LH-20*; MeOH) to yield **7** (25 mg). *Fr. 3* (65.4 g) was subjected to CC (SiO_2; PE/acetone 80 : 20 – 50 : 50) to afford three subfractions, *Subfrs. 3a – 3c*. *Subfr. 3a* (7.5 g) was subjected to CC (SiO_2; $CHCl_3$/MeOH 90 : 10 – 50 : 50) to yield **6** (100 mg), **8** (20 mg), and **9** (25 mg). *Subfr. 3b* (10.4 g) was subjected to CC (*ODS*) to give **10** (40 mg), **13** (8 mg), and **11** (10 mg). *Subfr. 3c* (2.5 g) was separated by CC (*ODS*; 70% MeOH), followed by CC (*Sephadex LH-20*; MeOH) to give **2** (30 mg) and **12** (50 mg).

Flemiphilippinin G (*=9,10-Dihydro-5,9-dihydroxy-3-(4-hydroxyphenyl)-8,8-dimethyl-6-(3-methylbut-2-en-1-yl)-4*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-4-one*; **1**). Amorphous, pale-yellow powder. UV (MeOH): 272. IR (KBr): 3425, 1643, 1435. ^{1}H- and ^{13}C-NMR ((D_6)DMSO): see *Table 1*. ESI-MS (pos.): 423.1 ($[M+H]^+$), 444.9 ($[M+Na]^+$). ESI-MS (neg): 421.2 ($[M-H]^-$), 457.4 ($[M+Cl]^-$). HR-EI-MS: 422.1718 ($C_{25}H_{26}O_6^+$; calc. 422.1724).

Flemiphilippininside (*=(1*S*)-1,5-Anhydro-1-*C*-[3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxo-4*H*-1-benzopyran-6-yl]-*D*-glucitol*; **2**). Yellow, amorphous powder. UV (MeOH): 255, 376. IR (KBr): 3418, 1624, 1468. ^{1}H- and ^{13}C-NMR ((D_6)DMSO): see *Table 1*. ESI-MS (pos.): 501.1 ($[M+Na]^+$). ESI-MS (neg.): 477.2 ($[M-H]^-$), 955.6 ($[2M-H]^-$). HR-EI-MS: 478.1111 ($C_{22}H_{22}O_{12}^+$; calc. 478.1106).

Cytotoxicity Assay. Cytotoxicities of compounds **1** – **13** were determined by MTT method [13] using human breast carcinoma (MCF-7), human lung cancer (A549), and human hepatoma (Hep-G2) cell lines. Human cancer cells were plated at 1×10^4 cell/well in 96-well microtiter plates and incubated for 24 h at 37°, 5% CO_2. Then, the cells were treated in triplicate with or without various concentrations of test samples. After 3 d of incubation at 37°, 5% CO_2, MTT reagent (5 mg/ml) was added to each well for 4 h. The resulting crystals were dissolved in DMSO (150 µl) and shaken for another 15 min. The absorbance was then determined with a *CENios* microplate reader (*TECAN*, Austria) at a wavelength of 570 nm. The inhibition percentages were calculated from reduction of absorbance in the control which was treated with 1% DMSO alone. Control wells received only the media without the test samples. The anticancer drugs, tamoxifen and doxorubicin were used as positive control in this study. The half maximal inhibitory concentration (IC_{50}) values were calculated by software SPSS 16.0 from the reduction of absorbance in the control assay. The assay was performed in triplicate, and the data were presented as mean ± S.D.

Anti-Oxidant Assay. This assay was carried out according to a modified protocol of *Griffin* and *Bhagooli* [14]. Briefly, 2.5 ml of a 10 mM TPTZ soln. in 40 mM HCl, and 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 300 mM acetate buffer (pH 3.6) were prepared to give a FRAP soln. A total of 20 µl of test compounds including L-ascorbic acid as a reference compound in DMSO was allowed to react with 180 µl of the freshly prepared FRAP soln. for 20 min at 37° in the dark in quadruplicate. Absorbance of the resulting colored product (ferrous TPTZ complex) was measured with a *Tecan Genios* microplate reader (*Tecan Group Ltd.*, CH-Männedorf) at 595 nm. One ml of various concentrations of $FeSO_4$, and 1 ml of 10 mM TPTZ and 10 ml of 300 mM acetate buffer (pH 3.6) were used for a calibration curve. FRAP Values of test compounds were expressed as means ± standard errors (SE) µM of Fe^{II}/g in quadruplicate.

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