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Flemingins G–O, Cytotoxic and Antioxidant Constituents of the Leaves of *Flemingia grahamiana*

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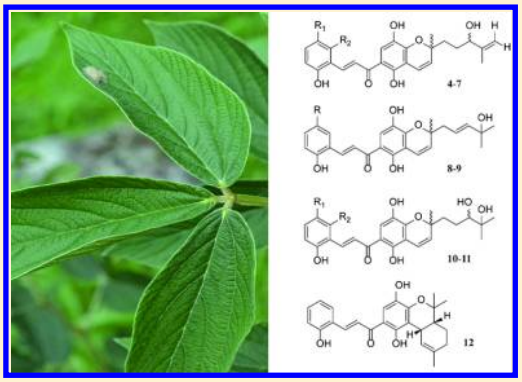
Supporting Information

ABSTRACT: The known flemingins A–C (**1**–**3**) and nine new chalcones, named flemingins G–O (**4**–**12**), along with deoxyhomoflemingin (**13**) and emodin (**14**) were isolated from a leaf extract of *Flemingia grahamiana*. The isolated chalcones were found to have a geranyl substituent modified into a chromene ring possessing a residual chain, as shown by spectroscopic methods. The leaf extract showed an IC_{50} value of 5.9 μg/mL in a DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The chalcones flemingins A, B, C, G, and H were active in the DPPH radical scavenging assay (ED_{50} 4.4–8.9 μM), while flemingins A and C showed cytotoxicity against MCF-7 human breast cancer cells (IC_{50} 8.9 and 7.6 μM, respectively).



Flemingia grahamiana Wight & Arn. (Leguminosae) is a shrub or semishrub reaching a height of up to 1.8 m. It is native to tropical Africa, occurring in open and wooded savannas, sometimes near water in riverine vegetation, on hillsides, in termite mounds, and along roadsides.[1,2] Its fruits and inflorescence are the sources of *warrus* (*warus*), a common cosmetic dye in East Africa, Saudi Arabia, and India.[2,3] This dye has also been obtained from other *Flemingia* species.[3] The roots of *F. grahamiana* are used in Eastern and Southern Africa for the treatment of diarrhea and dysentery, whereas in India the leaves of the plant are used as a purgative and for the treatment of skin diseases.[2] Cardillo et al.[3,4] have analyzed its constituents, from a sample collected in Eritrea, leading to the identification of flemingins A–F, homoflemingin, and deoxyhomoflemingin. Despite its wide use, the biological activities of *F. grahamiana* extracts and constituents have not yet been studied. As part of an ongoing search for novel bioactive agents, the isolation, identification, and the cytotoxic and antioxidant activities of chalcones from the leaves of *F. grahamiana* are reported herein.

RESULTS AND DISCUSSION

Column chromatographic fractionation of a CH_2Cl_2–CH_3OH (1:1) extract of the leaves of *F. grahamiana* with subsequent purification by gel filtration over Sephadex LH-20 and by preparative HPLC led to the isolation of 14 secondary metabolites, including the known flemingins A (**1**), B (**2**), and C (**3**)[3,4] and nine new chalcones (**4**–**12**), together with deoxyhomoflemingin[4,5] (**13**) and emodin[6] (**14**). Compounds **1**–**14** were identified using spectroscopic data interpretation. The identities of the known compounds, **1**–**3**, **13**, and **14**, were confirmed by comparison of their spectroscopic data to those previously published (Tables S1 and S2 and spectra S1–S19 and S99–S07, Supporting Information).[3−6]

Compound **4**, a yellow solid, was obtained as a diastereomeric mixture. Analysis of compound **4** by HRESIMS gave a pseudomolecular ion peak at m/z 439.1771 $[M + H]^+$, corresponding to the molecular formula $C_{25}H_{27}O_7$ ($[M + H]^+$, calcd 439.1757). Its NMR spectra (Tables 1 and 2) resembled those of **1**–**3** (Tables S1 and S2) and were thus indicative of a chalcone substituted with a geranyl group modified into a chromene ring possessing a side chain, which is a common feature of the secondary metabolites of the genus *Flemingia*. Its ^1H NMR spectrum exhibited a pair of doublets at δ_H 8.06 (1H, H-β) and 7.72 (1H, H-α) with the vicinal coupling constant $^3J_{HH}$ = 15.2 Hz indicative of their *trans* relationship. The corresponding carbons resonating at δ_C 141.7 (C-β) and 121.3 (C-α) were identified from the HSQC spectrum (Table 2). Both H-α (δ_H 7.72) and H-β (δ_H 8.06) showed HMBC cross-peaks to the carbonyl carbon at δ_C 194.0, suggesting that they are part of an α,β-unsaturated ketone moiety of a chalcone. The ^1H NMR signals of the A-ring protons of **4** gave a two-proton multiplet at δ_H 6.74 arising from the strongly coupled H-3 and

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Table 1. ^{1}H NMR Spectroscopic Data for Flemingins G–J (4–7) (δ_H, multiplicity (J in Hz))[a]

position	4	5	6	7
3	6.74, m	6.37, d (8.2)	6.93, d (8.0)	6.95, d (8.0)
4	6.74, m	7.02, dd (8.2, 8.2)	7.27, dd (8.0, 7.2)	7.28, dd (8.0, 7.2)
5		6.37, d (8.2)	6.87, dd (8.0, 7.2)	6.88, dd (8.0, 7.2)
6	7.03, dd (1.8, 1.8)		7.83, d (8.0)	7.84, d (8.0)
β	8.06, d (15.2)	8.37, d (15.6)	8.06, d (15.2)	8.07, d (15.2)
α	7.72, d (15.2)	8.14, d (15.6)	7.79, d (15.2)	7.80, d (15.2)
6′	7.33, s	7.28, s	7.43, s	7.49, s
3″	5.63/5.63, d (10.0/10.4)	5.62/5.62,d (10.0/10.0)	5.68, d (9.6)	5.70, d (9.6)
4″	6.76/6.76,d (10.4/10.0)	6.76/6.76,d (10.0/10.0)	6.63, d (9.6)	6.65, d (9.6)
5″	1.46, s	1.46, s	1.38, s	1.39, s
6″a	1.63/1.78, m	1.63/1.77, m	1.63, m	1.53, m
6″b	1.78/1.89, m	1.78/1.89, m	1.68, m	1.76, m
7″a	1.66/1.71, m	1.65/1.67, m	1.22, m	1.24, m
7″b	1.71/1.78, m	1.72/1.74, m	1.50, m	1.53, m
8″	3.99/4.01, m	3.99/4.01, m	3.85, m	3.85, m
10″a	4.80, dd (5.4, 1.0)	4.80, ddd (5.3, 1.8,1.8)	4.70, m	4.72, m
10″b	4.91, dd (5.4, 1.4)	4.91, m	4.83, m	4.85, m
11″	4.68, dd (1.4, 1.0)	1.69, m	1.59, m	1.60, m
OH-2	n.o.[b]	n.o.[b]	10.34, s	10.35, s
OH-2′	n.o.[b]	n.o.[b]	13.54, s	13.55, s
OH-5′	n.o.[b]	n.o.[b]	8.70, s	8.72, s
OH-8″	n.o.[b]	n.o.[b]	10.19, s	10.2, s

[a] ^{1}H NMR spectra for compounds **4** and **5** were acquired in CD_3OD, while those for compounds **6** and **7** were obtained in DMSO-d_6 at 25 °C. [b] n.o., not observed.

H-4, and a doublet of doublets ($^4J_{HH}$; $^5J_{HH}$ =1.8, 1.8 Hz) integrating for one proton at δ_H 7.03 (H-6), the spin system of which was confirmed using the COSY spectrum. The assignment was supported by the HMBC cross-peaks of H-6 to C-β ($^3J_{CH}$ to δ_C 141.7), C-2 ($^3J_{CH}$ to δ_C 152.3), and C-4 ($^3J_{CH}$ to δ_C 120.5). The HMBC cross-peaks H-β to C-2 ($^3J_{CH}$ to δ_C 152.3) and C-6 ($^3J_{CH}$ to δ_C 115.6) provided confirmation of the nature of the A-ring. A NOE cross-peak between H-α (δ_H 7.72) and a singlet at δ_H 7.33 (Figure 1) allowed the assignment of this signal to H-6′, which is the only proton in the B-ring.

The substitution pattern of the B-ring of compound **4**, with a chromene at C-3′ and C-4′ formed by cyclization of a geranyl group, and two hydroxy groups at C-2′ and C-5′, was found to be similar to those of compounds **1**–**3**,[3] the only exception being the nature of the C-2″ substituent. The assignment of the singlet at δ_H 7.33, 1H, to H-6′ was confirmed by its HMBC correlations to three quaternary aromatic carbons, C-2′ ($^3J_{CH}$ to δ_C 155.7), C-4′ ($^3J_{CH}$ to δ_C 150.1), and C-5′ ($^2J_{CH}$ to δ_C 138.9), and the carbonyl carbon, C=O ($^3J_{CH}$ to δ_C 194.0). The low chemical shift of C-5′ could be attributed to the shielding effect of oxygenation at C-2′ and C-4′. Duplicate signals of two *ortho* olefinic protons at δ_H 5.63/5.63 (1H, d, ^3J = 10.1/10.4 Hz) and 6.76/6.76 (1H, d, ^3J = 10.1/10.4 Hz) were assigned to H-3″ and H-4″, respectively, of the chromene ring, for which the placement at C-3′/C-4′ was defined by the HMBC cross-peaks of H-3″ to C-3′ ($^3J_{CH}$ to δ_C 110.7) and of H-4″ to C-2′ ($^2J_{CH}$ to δ_C 155.7) and C-4′ ($^3J_{CH}$ to δ_C 150.1). The deduction that the side chain of compound **4** has a terminal double bond at C-9″ (δ_C 148.6) and a hydroxy group at C-8″ (δ_C 76.5/76.6) was based on the signal at δ_H 3.99–4.01 (1H, m) attributed to H-8″, the multiplets at δ_H 4.80 (1H) and 4.91 (1H) due to the H-10″a (1H, dd) and 10″b (1H, dd) methylene protons of the terminal double bond, and the allylic methyl singlet at δ_H 1.68 (3H, H-11″). The latter proton signal showed HMBC correlations to C-8″ ($^3J_{CH}$ to δ_C 76.6/76.5), C-10″ ($^3J_{CH}$ to δ_C 111.4/111.7), and C-9″ ($^2J_{CH}$ to δ_C 148.6). Moreover, H-6″a/b (δ_H 1.63/1.78, 1H and 1.78/1.89, 1H) exhibited $^3J_{CH}$ correlations to C-3″ (δ_C 128.2) and C-5″ (δ_C 27.4/27.6), indicating that C-2″ is the position of attachment of the substituent on the chromene ring. Assignment of the 10″a and 10″b protons (δ_H 4.80, 1H and 4.91, 1H) was made based on the observation of a NOE correlation between H-10″a and CH_3-11″ (δ_H 1.68, 3H), as well as one between H-10″b (δ_H 4.91, 1H) and H-8″ (δ_H 3.99/4.01, 1H) (Figure 1). The signal duplications, observed in the ^{1}H and ^{13}C NMR spectra, for positions 3″, 4″, 6″, and 8″ indicated that compound **4** was isolated as a diastereomeric mixture, with its stereocenters being at C-2″ and C-8″. This new compound was therefore characterized as 2,5,2′,5′-tetrahydroxy-2″-(3‴-hydroxy-4‴-methylpent-4‴-enyl)-2-methylpyrano-[5″,6″:3′,4′]-chalcone and was assigned the trivial name flemingin G.

Compound **5** was identified as a diastereomeric mixture of a chalcone. Its NMR spectra (Tables 1 and 2) showed close similarities to those of compound **4**, except for the C-2 and C-6 oxygenation of its A-ring, providing an AX_2 spin system with δ_H 7.02 (1H, dd, ^3J = 8.4, 8.2 Hz, H-4) and 6.37 (2H, d, ^3J = 8.2 Hz, H-3/5). Its HRESIMS peak at m/z 439.1809 $[M + H]^+$ was consistent with the molecular formula $C_{25}H_{27}O_7$ ($[M + H]^+$, calcd 439.1757). On the basis of the above data this new compound, flemingin H, was characterized as 2,6,2′,5′-tetrahydroxy-2″-(3‴-hydroxy-4‴-methylpent-4‴-enyl)-2-methylpyrano-[5″,6″:3′,4′]-chalcone.

Compounds **6** and **7** eluted as distinct fractions by preparative HPLC and were identified by NMR as being diastereomers. Their NMR spectra indicated a chalcone core with identical B-ring substitution and the same C-2″ substituent as those of compounds **4** and **5**, and a C-2 monohydroxyated A-ring analogous to flemingin A **1**.[3] Hence, the A-ring of compound **6** gave rise to a doublet of doublets at δ_H 6.87 (1H, ^3J = 8.0, 7.2 Hz, H-5), a doublet at δ_H 6.93 (1H, ^3J = 8.0 Hz, H-3), a doublet of doublets at δ_H 7.27 (1H, ^3J = 8.0, 7.2 Hz, H-4), and a doublet at δ_H 7.83 (1H, ^3J = 8.0, Hz), attributable to H-6.

The C-2 oxygenation of this ring was supported by HMBC correlations of H-4 (δ_H 7.27, 1H) to the quaternary carbon C-2 ($^3J_{CH}$ to δ_C 157.4) and to the aromatic methine carbon C-6 ($^3J_{CH}$ to δ_C 129.3). Moreover, H-3 (δ_H 6.93, 1H), H-6 (δ_H 7.83, 1H), and H-α gave $^3J_{CH}$ correlations to the quaternary carbon C-1 (δ_C 121.3), and H-β to C-2 (δ_C 157.4) and C-6 (δ_C 129.3).

Compound **7** showed virtually identical NMR spectra to **6**, with very minor differences in chemical shifts. The largest difference in chemical shift between the ^{1}H NMR signals of compounds **6** and **7** was observed for the H-6′ of their B-ring, resonating at δ_H 7.43 and 7.49, respectively. The assignment of these signals was confirmed by the $^3J_{CH}$ HMBC correlation of H-6′ to the C=O moiety (δ_C 192.0), for both compounds **6** and **7**. Furthermore, H-6″a and H-6″b resonated at δ_H 1.63 and 1.68, respectively, for compound **6**, but at δ_H 1.53 and 1.76 for compound **7**. The very similar chemical shifts and the identical

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Table 2. ^{13}C NMR Spectroscopic Data for Compounds 4−7[a]

position	4	5	6	7
1	123.5, C	111.5, C	121.3, C	121.3, C
2	152.3, C	160.6, C	157.4, C	157.4, C
3	117.7, CH	107.8, CH	116.3, CH	116.3, CH
4	120.5, CH	132.9, CH	132.2, CH	132.2, CH
5	151.3, C	107.8, CH	119.5, CH	119.5, CH
6	115.6, CH	160.6, C	129.3, CH	129.3, CH
β	141.7, CH	138.0, CH	139.6, CH	139.6, CH
α	121.3, CH	122.4, CH	119.8, CH	119.8, CH
C═O	194.0, C	195.2, C	192.0, C	192.0, C
1′	113.8, C	114.1, C	112.1, C	112.1, C
2′	155.7, C	155.6, C	153.8, C	153.8, C
3′	110.7, C	110.7, C	108.9, C	109.0, C
4′	150.1, C	149.7, C	149.0, C	148.9, C
5′	138.9, C	138.8, C	137.9, C	137.9, C
6′	116.0, CH	116.0, CH	115.4, CH	115.4, CH
2″	81.7/81.8, C	81.5/81.7, C	80.2, C	80.1, C
3″	128.3 CH	128.2, CH	127.8, CH	127.9, CH
4″	118.0, CH	117.8, CH	115.9, CH	115.9, CH
5″	27.4/27.6, CH_3	27.4/27.5, CH_3	26.7, CH_3	26.6, CH_3
6″	38.5/38.5, CH_2	38.5/38.5, CH_2	36.9, CH_2	36.9, CH_2
7″	30.2/30.3, CH_2	30.2/30.4, CH_2	29.0, CH_2	29.0, CH_2
8″	76.5/76.6, CH	76.5/76.6, CH	73.7, CH	73.8, CH
9″	148.6, C	148.6, C	147.9, C	147.8, C
10″	111.4/111.7, CH_2	111.7, CH_2	110.1, CH_2	110.2, CH_2
11″	17.5/17.7, CH_3	17.5/17.7, CH_3	17.6, CH_3	17.5, CH_3

[a] ^{13}C NMR spectra for compounds **4** and **5** were acquired in CD_3OD, and for compounds **6** and **7** in DMSO-d_6 (25 °C).

Figure 1. Key NOE correlations (blue arrows) observed for compound **4** (mixing time 700 ms, CD_3CN, 25 °C, 800 MHz, Spectrum S23, Supporting Information).

coupling pattern of the NMR signals of **6** and **7** (Tables 1 and 2) suggested that these compounds are diastereomers. The HRESIMS pseudomolecular ion at m/z 423.1821 and 423.1843 $[M + H]^+$, observed for **6** and **7**, respectively, were consistent with the same molecular formula $C_{25}H_{27}O_6$ ($[M + H]^+$, calcd 423.1808). The CD spectra of these compounds, acquired in methanol, did not show significant Cotton effects, indicating both substances to be racemic. On the basis of these data, compounds **6** and **7** were characterized as 2,2′,5′-trihydroxy-2″-(3‴-hydroxy-4‴-methylpent-4‴-enyl)-2″-methylpyrano-[5″,6″:3′,4′]-chalcone diastereomers. These new compounds, for which the diastereomers were not distinguished, were given the trivial names flemingins I and J, respectively.

Compound **8** was obtained as a yellow solid and was assigned a molecular formula of $C_{25}H_{26}O_7$ based on analysis of its HRESIMS ($C_{25}H_{27}O_7$, $[M + H]^+$ m/z 439.1733, calcd 439.1757). This substance was identified as being a chalcone derivative with NMR spectroscopic features very similar to those of compound **4**, but with an aliphatic chain attached to C-2″, isomeric to that of **4**, and thus carrying a hydroxy group at C-9″ and a double bond between C-7″ and C-8″. This double bond was identified by the COSY correlations of the diastereotopic protons δ_H 2.42 (1H, m, H-6″a) and 2.49 (1H, m, H-6″b) with the olefinic protons resonating at δ_H 5.68 (2H, m, H-7″ and H-8″, Table 4). The assignment was supported by the HMBC correlation of H-6″ (δ_H 2.42 and 2.49) and the olefinic carbon at δ_C 121.8 (C-7″) and of the two methyl groups CH_3-10″ and CH_3-11″ (δ_H 1.16, 3H and δ_H 1.17, 3H) to the sp^2 methine carbon at δ_C 143.4 (C-8″, $^3J_{CH}$) as well as to an sp^3 oxygenated quaternary carbon at δ_C 71.2 (C-9″, $^2J_{CH}$). As the $^3J_{HH}$ was not readable due to the identical chemical shift of H-7″ and H-8″, the proposed *E*-configuration of the C-7″−C-8″ double bond was based on the chemical shift of its carbons (predicted values for *E*: $\delta_{C\text{-}8''}$ 142 and $\delta_{C\text{-}7''}$ 124, and for *Z*: $\delta_{C\text{-}8''}$ 137 and $\delta_{C\text{-}7''}$ 120; observed $\delta_{C\text{-}8''}$ 143.4 and $\delta_{C\text{-}7''}$ 121.8).[7] While this assignment is less reliable than that based on the magnitude of the $^3J_{HH}$ of the olefinic protons, it is in good agreement with conclusions made by biogenetic considerations.[8] This compound, flemingin K, was therefore characterized as 2,5,2′,5′-tetrahydroxy-2″-((*E*)-4‴-hydroxy-4‴-methylpent-2‴-enyl)-2″-methylpyrano-[5″,6″:3′,4′]-chalcone (**8**).

Compound **9** was isolated as a yellow solid. The HRESIMS at m/z 423.1832 $[M + H]^+$ was compatible with the molecular formula, $C_{25}H_{27}O_6$ ($[M + H]^+$, calcd 423.1808). On the basis of spectroscopic similarities to **4**-**8**, compound **9** was also identified as a chalcone. The spectroscopic features of the B- and C-rings, as well as for the C-2″ substituent, were virtually identical to those of compounds **8** and **9** (Tables 3 and 4). However, compound **9** was found to possess a 2-hydroxyated A-ring, as revealed by its coupling pattern, exhibiting a multiplet at δ_H 6.88 (1H, H-3), a doublet of doublets of doublets at δ_H 7.25 (1H, J = 7.8, 7.8, 2.0 Hz, H-4), a multiplet at δ_H 6.90 (1H, H-5), and a doublet of doublets centered at δ_H 7.63 (1H, J = 8.0, 2.0 Hz, H-6). Although the scalar coupling of H-7″ and H-8″ was not measurable due to their similar shifts in CD_3OD, its

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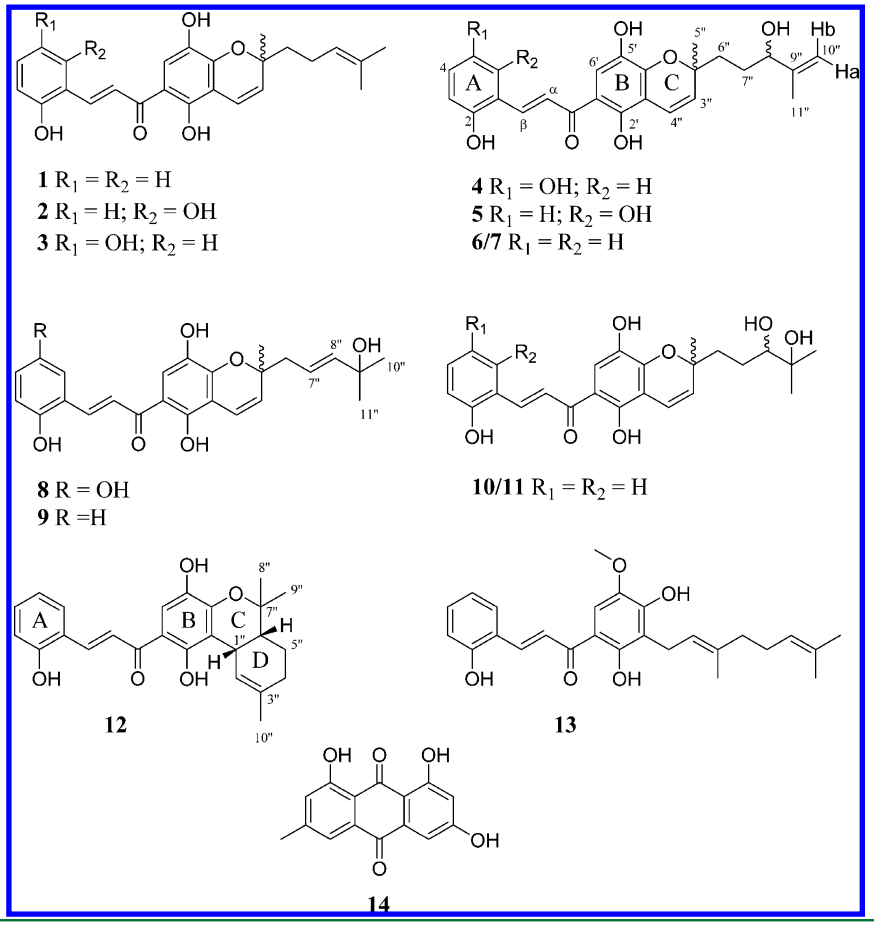

Table 3. ^{13}C NMR Spectroscopic Data for Compounds 8−12[a]

position	8	9	10	11	12
1	123.5, C	121.3, C	121.3, C	123.2, C	121.4, C
2	152.3, C	157.0, C	157.4, C	159.0, C	157.3, C
3	118.0, CH	116.1, CH	115.9, CH	117.1, CH	116.3, CH
4	120.5, CH	132.2, CH	132.2, CH	132.9, CH	132.1, CH
5	151.4, C	119.5, CH	119.5, CH	120.9, CH	119.5, CH
6	115.7, CH	129.3, CH	129.3, CH	131.1, CH	129.3, CH
β	141.7, CH	139.6, CH	139.6, CH	141.8, CH	139.2, CH
α	121.3, CH	119.4, CH	119.8, CH	121.5, CH	120.1, CH
C═O	194.0, C	191.2, C	191.9, C	194.2, C	192.0, C
1′	113.9, C	112.1, C	115.3, C	113.9, C	112.7, C
2′	155.7, C	153.8, C	153.8, C	155.8, C	158.6, C
3′	111.0, C	110.0, C	109.0, C	110.8, C	111.3, C
4′	150.2, C	148.9, C	149.0, C	150.1, C	149.8, C
5′	138.9, C	137.9, C	138.0, C	139.0, C	138.8, C
6′	115.9, CH	115.5, CH	112.0, CH	116.0, CH	111.9, CH
1″					31.0, CH
2″	81.5, C	79.9, C	80.5, C	81.8, C	121.6, CH
3″	128.1	127.4, CH	128.0, CH	128.7, CH	133.6, C
4″	117.8, CH	116.3, CH	116.3, CH	117.7, CH	28.9, CH_2
5″	27.2, CH_3	26.3, CH_3	29.0, CH	27.2, CH_3	20.3, CH_2
6″	45.3, CH_2	43.4, CH_2	38.6, CH_2	39.8, CH_2	38.7, CH
7″	121.8, CH	119.8, CH	26.7, CH_2	26.7, CH_2	78.1, C
8″	143.4, CH	143.3, CH	77.6, CH	79.7, CH	24.9, CH_3
9″	71.2, C	68.8, C	71.6, C	73.9, C	25.2, CH_3
10″	29.6, CH_3	29.83, CH_3	24.4, CH_3	24.9, CH_3	23.5, CH_3
11″	29.7, CH_3	29.83, CH_3	25.4, CH_3	25.8, CH_3	

[a]^{13}C NMR spectra for compounds **8**, **9**, and **11** were in CD_3OD, and for compounds **10** and **12** in DMSO-d_6 (25 °C).

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Table 4. ^{1}H NMR Spectroscopic Data for Flemingins K–M (8–10) (δ_H, multiplicity (J in Hz))[a]

position	8	9	10
3	6.74, m	6.88, m	6.94, d (8.3)
4	6.74, m	7.25, ddd (7.8, 7.8, 2.0)	7.28, ddd (8.3, 7.3, 1.6)
5		6.90, dd (7.8, 8.0)	6.89, dd (8.0, 7.3)
6	7.03, dd (1.7, 1.7)	7.63, dd (8.0, 2.0)	7.84, dd (8.0, 1.6)
β	8.05, d (15.2)	8.11, d (15.6)	8.08, d (15.6)
α	7.72, d (15.2)	7.82, d (15.6)	7.81, d (15.6)
6′	7.32, s	7.34, s	7.49, s
3″	5.64, d (9.6)	5.64, d (10.2)	5.69, d (10.1)
4″	6.76, d (9.6)	6.76, d (10.2)	6.65, d (10.1)
5″	1.49, s	1.49, s	1.39, s
6″a,b	2.42, m	2.44, m	1.68, m
	2.49, m	2.47, m	1.95, m
7″a,b	5.68, m	5.68, m	1.23, m
			1.68, m
8″	5.68, m	5.68, m	3.04, dd (10.1, 6.1)
10″	1.16, s	1.16, s	0.96, s
11″	1.17, s	1.17, s	1.03, s

[a] ^{1}H NMR spectra for compounds **8**–**10** were acquired in CD_3OD, and for compound **11** in DMSO-d_6 (25 °C).

Table 5. ^{1}H NMR Spectroscopic Data for Flemingins N and O (11 and 12) (δ_H, multiplicity (J in Hz))[a]

position	11	12
3	6.89, m	6.94, dd (8.2, 1.2)
4	7.25, ddd (8.7, 7.3, 1.6)	7.28, ddd (8.2, 7.5, 1.6)
5	6.90, m	6.88, ddd (7.9, 7.5, 1.6)
6	7.63, dd (8.0, 1.6)	7.84, dd (7.9, 1.6)
β	8.11, d (15.5)	8.06, d (15.6)
α	7.82, d (15.5)	7.81, d (15.6)
6′	7.35, s	7.40, s
1″		3.56, m
2″		6.26, m
3″	5.67, d (10.2)	
4″a	6.76, d (10.2)	1.91, m
4″b		1.98, m
5″a	1.48, s	1.27, m
5″b		1.92, m
6″a	1.73, m	1.83, m
6″b	2.12, m	
7″a	1.45, m	
7″b	1.81, m	
8″	3.25, dd (10.8, 2.0)	1.25, s
9″		1.41, s
10″	1.12, 3H, s	1.63, s
11″	1.15, s	

[a] ^{1}H NMR spectrum for compound **11** was obtained in CD_3OD, and for compound **12** in DMSO-d_6 (25 °C).

magnitude determined in DMSO-d_6 ($^3J_{HH}$ = 10.2 Hz) was indicative of a *trans* configuration. This new compound, flemingin L, was therefore identified as 2,2′,5′-trihydroxy-2″-((*E*)-4‴-hydroxy-4‴-methylpent-2‴-enyl)-2″-methylpyrano-[5″,6″:3′,4′]-chalcone.

Compounds **10** and **11** were obtained by HPLC separation as yellow solids. These compounds were identified by NMR as diastereomers of a chalcone with an identical 2″-substituent to compounds **10** and **11** and possess a C-2-oxygenated A-ring similar to **1**,[3] **6**, and **7**. The assignment of the ABCD spin system of the A-ring was based on COSY and TOCSY spectra and was supported by the NOE interaction of H-β (δ_H 8.08/8.11) and H-6 (δ_H 7.84/7.63). Apart from some small differences in chemical shift for H-6″ and H-7″ (Tables 4 and 5), compounds **10** and **11** showed almost superimposable ^{1}H NMR spectra (Figure 2). Their HRESIMS gave an $[M + H]^+$ peak at m/z 441.1779 and 441.1912 for **10** and **11**, respectively, corresponding to the molecular formula $C_{25}H_{29}O_7$ (calcd 441.1913).

The $^3J_{CH}$ HMBC correlation of H-6′ (δ_H 1.68/1.73 and 1.95/2.12) with C=O (δ_C 191.9/194.2), C-2′ (δ_C 153.8/155.8), and C-4′ (δ_C 149.0/150.1) and its $^2J_{CH}$ correlation to C-5′ (δ_C 138.0/139.0) indicated that these compounds are diastereomers and not regioisomers. Due to the use of DMSO-d_6 as solvent, the chelated hydroxy group OH-2′ could be identified (δ_H 13.6). From the above data, these two new compounds, named flemingins M and N, were identified as diastereomers of 2,2′,5′-trihydroxy-2″-(3‴,4‴-dihydroxy-4‴-methylpentyl)-2″-methylpyrano-[5″,6″:3′,4′]-chalcone. Their CD spectra showed insignificant Cotton effects, thus indicating that both are racemic at C-2″.

Compound **12** was also obtained as a yellow solid and was identified as a chalcone derivative possessing a monooxygenated A-ring, which was revealed by the similar ^{1}H NMR chemical shifts and COSY spectrum to those of compounds **1**, **6**, **7**, **10**, and **11**. The nature of the A-ring was confirmed by the presence of only one carbon, C-2, in this ring with a chemical shift above 150 ppm in the ^{13}C NMR spectrum (Table 3) and also by the NOE correlation of H-β (δ_H 8.06, 1H) and H-6 (δ_H 7.81, 1H) (Figure 3).

Unlike chalcones **1**–**11**, where the C-ring has a residual side chain at C-2″, the side chain of compound **12** was found to be fused with a methylcyclohexene ring. Thus, the ^{13}C NMR spectrum of **12** showed seven aliphatic carbons, two of them tertiary CHs at δ_C 31.0 (C-1″) and 38.7 (C-6″), two methylenes at δ_C 20.3 (C-5″) and 28.9 (C-4″), and three methyl carbons at δ_C 23.5 (C-10″), 24.9 (C-8″), and 25.2 (C-9″). This could be attributed either to a highly saturated geranyl chain or a cyclohexene ring system, with the latter deduced from the COSY correlation of the benzylic/allylic proton δ_H 3.56 (1H, H-1″) to the olefinic proton at δ_H 6.26 (1H, H-2″) and to the aliphatic proton at δ_H 1.83 (1H, H-6″). The presence of a cyclohexene ring in **12** was supported by the HMBC correlations of H-10″ (δ_H 1.63, 3H) to the sp^2 carbons at δ_C 121.6 (C-2″) and 133.6 (C-3″) and to the sp^3 methylene carbon at δ_C 28.9 (C-4″) and the HMBC correlations of CH_3-8″/9″ (δ_H 1.25, 3H and δ_H 1.41, 3H) and C-6″ (δ_C 38.7). The *cis* configuration of the dihydropyran-cyclohexene ring junction was established from the NOE interaction between H-1″ (δ_H 3.56, 1H) and H-6″ (δ_H 1.83, 1H). On the basis of the above data, **12** was identified as (*E*)-1-((6a,10a)-1,4-dihydroxy-6,6,9-trimethyl-6a,7,8,10a-tetrahydro-6*H*-benzo[*c*]chromen-2-yl)-3-(2-hydroxyphenyl)prop-2-en-1-one and was given the trivial name flemingin O.

Motivated by the use of *F. grahamiana* in traditional medicine, the constituents isolated from its leaves were tested against MCF-7 human breast cancer cells. Of the compounds tested (Table 6), flemingins A (**1**) and C (**3**) exhibited cytotoxic activity. Although these compounds may be of interest for the development of novel anticancer agents, this observation indicates a possible risk of using the leaves of *F.*

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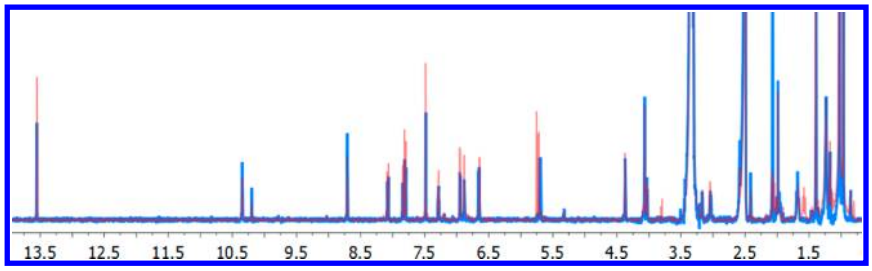

Figure 2. Superimposed ^{1}H NMR spectra (DMSO-d_6, 800.09 MHz, 25 °C) of compounds **10** (red) and **11** (blue).

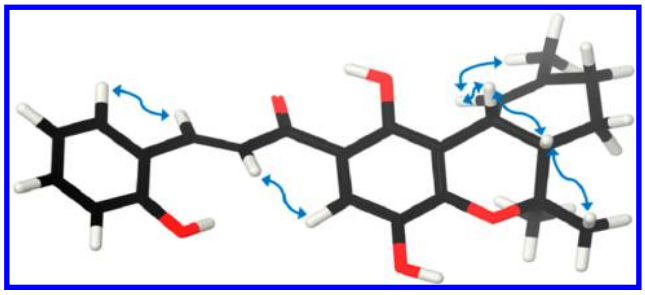

Figure 3. Key NOE correlations (blue arrows) observed for compound **12** (mixing time 700 ms, CD_3CN, 25 °C).

Table 6. Cytotoxic (IC_{50} μM) and Radical Scavenging (ED_{50} μM) Activities of Selected Flemingins and of the Crude Leaf Extract of *Flemingia grahamiana*

sample	IC_{50} [b]	ED_{50} [c]	activity index
F. grahamiana (leaf extract)[a]		5.9	
flemingin A (**1**)	8.9	4.4	1.8
flemingin B (**2**)	>236.7	7.8	3.1
flemingin C (**3**)	7.6	5.9	2.4
flemingin G (**4**)	220.8	8.3	3.3
flemingin H (**5**)		8.9	3.5
flemingin I (**6**)	>236.3		
flemingin K (**8**)	>227.7		
flemingin L (**9**)	>236.3		
emodin (**14**)	317.4		
quercetin (standard)		2.5	1.0

[a]The chlorophyll was removed from the leaves by column chromatography on Sephadex LH-20 with CH_2Cl_2–CH_3OH, 1:1, before testing. [b]As positive control 1-isopropyl-3-(pyridin-4-ylethynyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (IC_{50} = 5.0 μM, confidence interval (95%) = 1.4–17.8 nM)[10] was used. [c]ED_{50} is given in μg/mL for crude and in μM for pure compounds. The generally accepted threshold for bioactivity is 10 μM.

grahamiana by indigenous populations as a purgative and against skin diseases.[2]

The crude leaf methanol extract of *F. grahamiana* showed antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.[9] Strong antioxidant activities were observed for flemingins A–C (**1**–**3**), G (**4**), and H (**5**) (Table 6). The 2′,5′-*para*-dihydroxylation of the B-ring is suggested to contribute to the strong antioxidant activity of these flemingins due to the formation of a stable 1,4-quinone upon oxidation. Despite the fact that its A-ring is not *para*-dihydroxylated, unlike **3** and **4**, providing the theoretical ability of radical scavenging by formation of a second quinoid ring, the most potent antioxidant activity among the tested compounds was observed for flemingin A (**1**).

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were obtained on a Hewlett-Packard 8453 spectrophotometer; CD spectra were recorded on a JASCO J-710 CD spectropolarimeter. NMR spectra were acquired on Varian Unity Inova spectrometers operating at 200, 400, 600, and 800 MHz or on a Bruker 300 MHz spectrometer. NMR spectra were processed using MestReNova-9.0. Structural assignment was based on ^{1}H and ^{13}C NMR as well as gCOSY,[11] gNOESY,[12] gHSQC,[13] and gHMBC[14] spectra. The solvent residual peak was used for chemical shift referencing (DMSO-d_6, δ_H 2.50 and δ_C 39.52; CD_3OD, δ_H 3.31 and δ_C 49.00; CD_3CN, δ_H 1.94 and δ_C 1.32). LC-MS (ESI) chromatograms were acquired on a PerkinElmer PE SCIEX API 150EX instrument equipped with a Turbolon spray ion source and a Gemini 5 mm RPC_{18} 110 Å column and applying a H_2O–CH_3CN, 80:20–20:80, gradient solvent system with a separation time of 8 min. HRESIMS were obtained with a Q-TOF-LC/MS spectrometer (Stenhagen Analyslab AB, Gothenburg, Sweden) using a 2.1 × 30 mm, 1.7 μm RPC_{18} column and a H_2O–CH_3CN gradient system (5:95–95:5 gradient and 0.2% formic acid). Column chromatography (CC) was carried out using silica gel 60 (70–230 mesh). Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck) precoated aluminum plates, which after development with an appropriate solvent system were evaluated under UV light (254 and 366 nm). Gel filtration was carried out over Sephadex LH-20 (Pharmacia) suspended in CH_2Cl_2–CH_3OH (1:1). Preparative HPLC was performed on a Waters 600E system using the Chromulan (Pikron Ltd.) software and an RP-C_8 Kromasil column (250 mm × 25 mm) with the solvent system H_2O–CH_3OH or H_2O–CH_3CN (gradient, 90:10 to 5:95, for 30–80 min, flow rate of 8–15 mL/min).

Plant Material. The leaves of *Flemingia grahamiana* were collected from Kitale in Western Kenya in October 2008. The plant material was identified by Mr. Simon Mathenge, a senior botanist at the East African Herbarium, Nairobi, where a voucher specimen (Mathenge 2008/487) is deposited.

Extraction and Isolation. Dried and pulverized leaves (413 g) of *F. grahamiana* were soaked twice in CH_2Cl_2–CH_3OH (1:1) at room temperature, for 24 h in each case, to yield 30 g of crude extract after solvent evaporation. A portion of the extract (20 g) was adsorbed on silica gel 60 (70–230 mesh ASTM) (40 g), loaded onto a column of silica gel (38 cm length and 5 cm diameter, 200 g silica gel), and then eluted with increasing amounts of EtOAc in *n*-hexane to give 20 fractions, of approximately 500 mL each. Fractions 7–20 were purified by gel filtration over Sephadex LH-20 (eluted with CH_2Cl_2–CH_3OH, 1:1). Fractions 7–9, eluted at 5–25% EtOAc in *n*-hexane, were combined and further purified by CC on silica gel deactivated with oxalic acid using an EtOAc in *n*-hexane gradient. This led to the isolation of compounds **1** (46.0 mg) and **14** (2.1 mg). Subfractions 7–9 were combined and subjected to preparative HPLC (RPC_8 250 mm × 25 mm, Kromasil column; H_2O–CH_3CN eluent), yielding compounds **12** (2.5 mg) and **13** (3.0 mg). Fraction 15 (eluted with 50% EtOAc in *n*-hexane) was subjected to preparative HPLC (H_2O–CH_3OH mixtures as eluents) and yielded compounds **4** (15.4 mg) and **5** (6.3 mg). Fractions 16 and 17, eluted with 50–70% EtOAc in *n*-hexane, were mixed and subjected to preparative HPLC (H_2O–CH_3OH as eluent) to give compound **8** (13.2 mg). Fractions 18 and 19, eluted with 70% to 100% EtOAc in *n*-hexane, were combined and separated on preparative HPLC (H_2O–CH_3OH eluent), giving a

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mixed fraction (compounds **10** and **11**). Further purification of the latter by preparative HPLC with a gradient of H_2O–CH_3OH gave compounds **10** (1.5 mg) and **11** (2.3 mg). Fractions 13 and 12, eluted at 40% EtOAc in *n*-hexane, provided a crude mixture of **6**, **7**, and **9** by preparative HPLC (H_2O–CH_3CN eluent), which were then isolated using a gradient of H_2O–CH_3OH to give 3.0, 2.6, and 10.0 mg, respectively. The combined fractions 10–12, eluted with 30% EtOAc in *n*-hexane, were subjected to repeated preparative HPLC (H_2O–CH_3CN eluent) chromatographic purification, yielding compounds **2** (20.6 mg) and **3** (30.7 mg).

*Flemingin A (**1**):* yellow solid; ^1H and ^{13}C NMR data, see Tables S1 and S2 in the Supporting Information; ESIMS m/z 407.7 $[M + H^+]$. The NMR and MS data were in agreement with the data given in ref 3.

*Flemingin B (**2**):* yellow solid; UV (MeOH) λ_{max} (log ε) 350 (4.3) nm; ESIMS m/z 423.1 $[M + H^+]$. The ^1H and ^{13}C NMR data, shown in the Supporting Information, were in agreement with the data given in ref 3.

*Flemingin C (**3**):* yellow solid; UV (MeOH) λ_{max} (log ε) 290 (4.2), 375 (4.1), 400 (4.2) nm; ^1H and ^{13}C NMR data, see Supporting Information; ESIMS m/z 423.0 $[M + H^+]$. The NMR and MS data were in agreement with the data given in ref 3.

*Flemingin D (**4**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 295 (3.2) and 405 (3.1) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS m/z 439.1771 $[M + H]^+$ (calcd for $C_{25}H_{26}O_7$, 439.1757).

*Flemingin H (**5**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 170 (3.3) and 365 (3.1) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS m/z 439.1809 $[M + H]^+$ (calcd for $C_{25}H_{26}O_7$, 439.1757).

*Flemingin I (**6**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 275 (3.5), 365 (3.2) nm; CD no Cotton effect; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS m/z 423.1821 $[M + H]^+$ (calcd for $C_{25}H_{26}O_6$, 423.1808).

*Flemingin J (**7**):* yellow, amorphous solid; (CH_3OH) λ_{max} (log ε) 305 (3.5), 340 (3.4), 370 (3.4) nm; CD no Cotton effect; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS m/z 423.1843 $[M + H]^+$ (calcd for $C_{25}H_{26}O_6$, 423.1808).

*Flemingin K (**8**):* yellow, amorphous solid; ^1H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 439.1733 $[M + H]^+$ (calcd for $C_{25}H_{26}O_7$, 439.1757).

*Flemingin L (**9**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 265 (3.2), 285 (3.3), 300 (3.1) nm; ^1H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 423.1832 $[M + H]^+$ (calcd for $C_{25}H_{26}O_6$, 423.1808).

*Flemingin M (**10**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 265 (3.5), 380 (3.2) nm; CD no Cotton effect; ^1H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 441.1779 $[M + H]^+$ (calcd for $C_{25}H_{28}O_7$, 441.1813).

*Flemingin N (**11**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 275 (3.4), 350 (3.4) nm; CD no Cotton effect; ^1H and ^{13}C NMR data, see Tables 3 and 5; HRESIMS m/z 441.1912 $[M + H]^+$ (calcd for $C_{25}H_{28}O_7$ 441.1813).

*Flemingin O (**12**):* yellow, amorphous solid; UV (CH_3OH) λ_{max} (log ε) 300 (3.3), 360 (3.1) nm; CD no Cotton effect; ^1H and ^{13}C NMR data, see Tables 3 and 5; HRESIMS m/z 407.1878 $[M + H]^+$ (calcd for $C_{25}H_{27}O_5$ 407.1858).

*Deoxyhomoflemingin (**13**):* yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 300 (3.2) nm; ^1H and ^{13}C NMR data see Supporting Information; ESIMS m/z 423.4 $[M + H]^+$. The NMR data were in agreement with the data given in ref 4.

Cytotoxicity Assays. Cytotoxicity assays against MCF-7 human breast cancer cells were carried out as described by Endale et al.[15] The cells were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10% v/v), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The medium was preincubated at 37 °C under humid conditions with a mixture of 5% CO_2 and 95% air. Cells were seeded in 96-well plates at optimal cell density to ensure exponential growth for the duration of the assay. The growth medium was replaced with the experimental medium containing an appropriate drug concentration or control (0.1% or 1.0% v/v, dimethyl sulfoxide) after 24 h of preincubation. Cell viability was measured, after 48 h of incubation, using PrestoBlue cell viability reagent (Invitrogen AB Lidingö, Sweden), in accordance with the manufacturer's instructions. The fluorescence was measured using a POLARstar Omega (BMG Labtech, Ortenberg, Germany) plate reader. Results are expressed as means ± SE for six replicates as a percentage of the vehicle control (assumed to be 100%). Experiments were carried out independently at least three times. Statistical analyses were performed using a two-tailed Student's *t*-test. A value of $p < 0.05$ was considered to indicate statistically significant differences.

Antioxidant Activity Assays. The method described by Ohnishi et al.[16] was used with minor modifications. Test sample solutions at concentrations of 320, 160, 80, 40, 20, 10, 5, and 2.5 μg/mL were prepared in double-distilled methanol. From each concentration, 1.0 mL of the sample was added to 2.0 mL of 76 μM DPPH dissolved in methanol. The mixture was allowed to stand at room temperature for 30 min, and the absorbance of the remaining DPPH was then measured at 517 nm. The radical scavenging activity was measured as the decrease in absorbance due to the DPPH radical, expressed as a percentage of the absorbance of the control solution (1.0 mL of methanol and 2.0 mL of 76 μM DPPH solution). Activity was then expressed as EC_{50}, the concentration of the test compound required to give a 50% decrease in the absorbance compared to that of the control solution. Quercetin was used as the positive control. The percentage of scavenged DPPH was calculated as $100 \times (A_{DPPH} - A_{Sample})/A_{DPPH}$, where A_{DPPH} is the absorbance of the solution containing DPPH but without a test sample and A_{Sample} is the absorbance of the mixture of the test sample and DPPH solution. Values of EC_{50} were determined by plotting the percentage of scavenged DPPH versus the initial concentration of each sample. The activity index was calculated by comparing the value of EC_{50} (in μM) for a given test compound with that of the standard.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

1D and 2D NMR, MS, UV, and CD spectra and data for cytotoxicity and antioxidant assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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