Studies on the Constituents of *Anaxagorea luzonensis* A. Gray

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Five new xanthones, 1,3,6-trihydroxy-5-methoxy-4-prenylxanthone (1), 1,3,5-trihydroxy-6-methoxy-2-prenylxanthone (2), 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl) xanthone (3), 1,3,6-trihydroxy-4-prenylxanthone (4), 3,6-dihydroxy-1,5-dimethoxyxanthone (5) and one new flavonoid, 3,5,7,4′-tetrahydroxy-2′-methoxyflavone (6) along with seven known xanthones and seven known flavonoids were isolated from the bark of *Anaxagorea luzonensis* A. Gray and their chemical structures were determined by means of chemical and spectral studies. Almost all flavonoids and one xanthone (13) showed antioxidant activity.

Key words *Anaxagorea luzonensis*; Annonaceae; xanthone; flavonoid; antioxidative activity

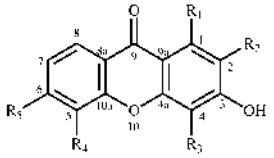
The plant *Anaxagorea luzonensis* A. Gray (Annonaceae) is a tree indigenous to Thailand and is used as a traditional medicinal plant as a blood tonic, stomachic, antipyretic and for treatment of muscular pain.[1)] Recently Akiyama and his colleagues[2,3)] reported that prenylflavonoids obtained from this plant had an estrogenic activity. The prenylflavonoids are reported to possess a wide range of biological activities.[4)] This paper deals with the isolation and structural determination of several xanthones and flavonoids having antioxidant activity from the heartwood of *Anaxagorea luzonensis*.

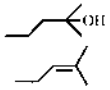
The dried and chopped heartwood (5 kg) of this plant was extracted with methanol under reflux. The methanol extract was partitioned between 90% aqueous methanol and *n*-hexane. Evaporation of the 90% aqueous methanol layer yielded a reddish brown syrup which was further partitioned between ethylacetate and water and the ethylacetate layer was evaporated. Then, the residue was partitioned with chloroform–methanol–water (13 : 7 : 6) and the lower layer was evaporated and was chromatographed on silica gel column using chloroform–methanol (20 : 1, 10 : 1, 5 : 1) as an eluent to give eight fractions (fr. A—H).

Compound **1** was isolated as a yellow powder from fr. C, and the molecular formula was determined as $C_{19}H_{18}O_6$ ($[M]^+$; *m*/*z* 342.1104) by high-resolution mass spectrometry (HR-MS). The presence of a prenyl moiety in the molecule was indicated by the ^{1}H-NMR signals at δ 3.33 (2H, br s), 5.29 (1H, t, J=7.0 Hz), 1.79 (3H, s) and 1.61 (3H, s). Further, the ^{1}H-NMR spectrum revealed a chelated hydroxyl group at δ 13.77, a 1H singlet at δ 5.86, two doublet 1H signals at δ 7.39 (d, J=8.6 Hz) and 6.39 and a methoxyl signal at δ 3.76 (3H, s). On irradiation of the methoxyl group at δ 3.76, there was no NOE enhancement at any proton signal. In the heteronuclear multiple bond correlation (HMBC) spectrum of **1**, the presence of a significant C–H three-bond correlation between a hydrogen-bonded proton at δ 13.77 and a carbon at δ 98.3 bearing a proton at δ 5.86 (H-2) suggested the location of the prenyl moiety at C-4 (not at C-2). Another signal at δ 3.33 was correlated with carbon signals at 154.0 (C-4a), 106.1 (C-4) and 160.6 (C-3). On the basis of these results, the structure of **1** is proposed to be 1,3,6-trihydroxy-5-methoxy-4-prenylxanthone.

Compound **2** was isolated as a yellow powder. The molecular formula was determined as $C_{19}H_{18}O_6$ ($[M]^+$; *m*/*z* 342.1103) by HR-MS, and the UV spectrum indicated of 1,3,5,6-tetrasubstituted xanthone. The presence of a prenyl moiety in the molecule was indicated by the ^{1}H-NMR signals at δ 3.13 (d, J=7.0 Hz), δ 1.72 and 1.61 (each 3H, s) together with a signal at 5.20 (t). Further, the ^{1}H-NMR spectrum showed a chelated hydroxyl group (δ 13.97) and a methoxyl signal (δ 3.73), a 1H singlet at δ 6.04, and two doublet 1H signals at δ 7.39 and 6.37. In differential NOE experiments, irradiation of the methoxyl group at δ 3.73 enhanced the signal at δ 6.37. In the HMBC spectrum of **2**, methylene proton signal of the prenyl moiety at δ 3.13 showed long-range correlations with the carbon signals at 159.3 (C-1), 109.8 (C-2) and 159.3 (C-3). This result suggested the location of the prenyl moiety at C-2. From these spectral data, we proposed the structure **2** for 1,3,5-trihydroxy-6-methoxy-2-prenylxanthone.

Compound **3** was isolated as a yellow powder. Its molecular formula was determined as $C_{18}H_{18}O_6$ ($[M]^+$; *m*/*z* 330.1103) by HR-MS. The UV spectrum with absorptions at



	R_1	R_2	R_3	R_4	R_5
1	OH	H		OMe	OH
2	OH		H	OH	OMe
3	OH	H		OH	H
4	OH	H		H	OH
5	OMe	H	H	OMe	OH

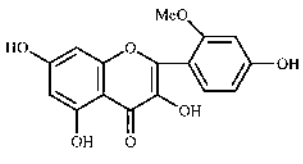
6 

Chart 1

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244 and 319 nm was indicative of a 1,3,5-trioxygenated xanthone moiety, similar to that reported for 1,3,5-trihydroxy-4-prenylxanthone.[5] The ^{1}H-NMR spectrum of **3** showed the presence of signals at δ 2.79 (2H, dd, J=8.6, 4.9 Hz), 1.61 (2H, dd, J=8.6, 4.9 Hz), 1.21 (6H, s) and four aromatic proton signals at δ 7.53, 7.28, 7.24 and 6.27, and a chelated hydroxyl group (δ 12.83). Three of the four aromatic signals are coupled and appear at a lower field than the fourth, the singlet at δ 6.27. Consideration of the hydrogen-bonded hydroxyl group led us to postulate that the aromatic proton at δ 6.27 should be located at C-2 or C-4. Comparison of suitable reference compounds indicates that the other hydroxyl group is in the 5-position. Thus the signal of the lowest field aromatic proton at C-8, the A part of an ABC system, appears as a quartet at δ 7.53, owing to *ortho*- and *meta*-splitting (J=7.3, 1.8 Hz, respectively) by the BC protons at C-6 and C-7, which give rise to the complex signal at δ 7.28 and 7.24. In the HMBC spectrum, the proton (δ 7.53) gave cross peaks with the carbonyl carbon (δ 180.0), one CH aromatic carbon (δ 120.3, C-6) and one aromatic carbon (δ 144.9, C-10a) to be assigned to H-8. In the CH-COSY experiment the two equivalent methyl protons at δ 1.21 correlated with the carbon signal at δ 28.9; two methylene proton absorptions at δ 1.61 are connected to the carbon at δ 42.6 while the other methylene protons (δ 2.79) are linked to the carbon at δ 17.2. The complete ^{13}C-NMR chemical shift assignment of **3** is given in Table 1. The presence of a significant C–H three bond correlation between a hydrogen-bonded proton at δ 12.83 and a carbon at δ 97.6 bearing a proton at δ 6.27 (H-2) suggested the location of the 3-hydroxy-3-methylbutyl moiety at C-4 (not at C-2). In addition, the 3-hydroxy-3-methylbutyl moiety was attached to the C-4 position due to the correlation between the proton signal of C-11 methylene proton (δ 2.79) and C-4 signal at δ 107.6ppm, C-4a (δ 154.2) and C-3 (δ 164.2) observed in the HMBC spectrum. So **3** was 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone.

Compound **4** was determined as $C_{18}H_{16}O_5$ ([M]$^+$; m/z 312.1009) by HR-MS. The presence of a prenyl moiety in the molecule was indicated by the ^{1}H-NMR signals at δ 3.24 (2H, d), 5.18 (1H, t), 1.80 (3H, s) and 1.60 (3H, s). Further, the appearance of an ABC system at δ 7.31 (d, J=2.8 Hz), 7.24 (d, J=8.9 Hz) and 7.10 (dd, J=8.9, 2.8 Hz) ppm indicated the presence of hydroxyl group substituted at position C-6. In the HMBC spectrum, methylene proton signal of the prenyl moiety at δ 3.24 was correlated at δ 154.1 (C-4a), 108.1 (C-4) and 160.7 (C-3). These results suggested that **4** was 1,3,6-trihydroxy-4-prenylxanthone.

Compound **5** was determined as $C_{15}H_{12}O_6$ ([M]$^+$; m/z 288.0623) by HR-MS. Its ^{1}H-NMR spectrum exhibited two pairs of doublets at δ 7.64, 6.88 (each 1H, d, J=8.6 Hz) and at δ 6.45, 6.37 (each 1H, d, J=2.1 Hz), together with two methoxyl signals [δ 3.89 and 3.83 (each 3H, s)]. In differential NOE experiments, irradiation of the methoxyl group at δ 3.83 enhanced the signal at δ 6.37 (H-2), but on irradiation of another methoxyl signal at δ 3.89, there was no NOE enhancement at any proton signal. In the HMBC spectrum of **5**, the presence of a significant correlation between the methoxyl proton at δ 3.83 and a carbon at δ 161.8 (C-1) and also the methoxyl proton (δ 3.89) correlated to δ 134.0 at C-5. From these spectral data, we proposed the structure for **5** to be 3,6-dihydroxy-1,5-dimethoxyxanthone.

Table 1. ^{13}C-NMR Data of **1**—**6** (100 MHz, DMSO-d_6/TMS, δ ppm)

No.	**1**	**2**	**3**	**4**	**5**	No.	**6**
1	160.6	159.3	160.1	160.7	161.8	2	148.1
2	98.3	109.8	97.6	99.7	95.8	3	136.6
3	160.6	159.3	164.2	160.7	163.7	4	176.0
4	106.1	94.2	107.6	108.1	95.2	5	160.8
4a	154.0	155.5	154.2	154.1	158.9	6	98.2
5	135.2	133.1	146.3	108.1	134.0	7	164.5
6	154.0	135.1	120.1	152.1	149.1	8	93.4
7	120.4	117.9	127.7	121.9	113.3	9	156.8
8	120.4	120.5	114.3	117.8	121.1	10	103.1
8a	125.8	105.8	120.6	120.7	115.9	1′	110.3
9	176.6	176.5	180.0	180.4	172.9	2′	158.5
9a	102.5	98.0	101.7	106.5	104.7	3′	99.3
10a	150.6	150.4	144.9	148.4	155.1	4′	160.7
11	21.6	21.3	17.2	21.4		5′	107.0
12	124.2	124.3	42.6	124.1		6′	131.8
13	129.1	128.9	69.2	130.0		OMe	55.5
14	25.6	25.6	28.9	25.5			
15	17.8	17.8	28.9	17.7			
OMe	59.2	59.2			55.9 (1), 60.7 (5)		

Table 2. Radical Scavenging Effect of *Anaxagorea luzonensis* Extracts and Related Compounds on DPPH Radical

Sample	50% reduction (μg)	Sample	50% reduction (μg)
MeOH ext.	2.69	**6**	3.31
Upper layer	2.19	**16**	56.23
Lower layer	3.24	**17**	2.09
1	776.25	**18**	1.91
2	269.15	**19**	2.40
3	1047.13	**20**	1.12
4	269.15	α-Tocopherol	3.55
5	630.96	Ascorbic acid	1.70
7	1659.59		
8	676.08		
10	1421.55		
11	307.82		
12	147.91		
13	2.19		

1,3,5-trihydroxy-4-prenylxanthone (**7**), 1,3,5-trihydroxy-2-prenylxanthone (**8**), 1,3,6-trihydroxy-5-methoxyxanthone (**9**), gentisein (1,3,7-trihydroxyxanthone) (**10**), 1,3,5-trihydroxyxanthone (**11**), 3,5-dihydroxy-1-methoxyxanthone (**12**), and 1,3,5,6-tetrahydroxy xanthone (**13**) were identified by comparison of spectral data (^{1}H-NMR, ^{13}C-NMR) with literature values.[7—11]

Compound **6** was determined as $C_{16}H_{12}O_7$ ([M]$^+$; m/z 316.0572) by HR-MS. Its UV spectrum was characteristic of a flavone (249, 306, 347 nm). The ^{1}H-NMR spectrum exhibited a methoxyl group at δ 3.74 (s), ABX type signals at δ 7.25 (d, J=8.5 Hz), 6.53 (d, J=2.1 Hz) and 6.47 (dd, J=8.5, 2.1 Hz) due to the 2′,4′-disubstituted ring B, along with a signal due to ring A at δ 6.25 (d, J=1.8 Hz) and 6.16 (d, J=1.8 Hz). The chemical shift values of the relevant protons and carbons were similar to those of morin (3,5,7,2′,4′-pentahydroxyflavone)[12] except for the presence of signals due to a methoxyl group. The methoxyl group was deduced to be attached to the C-2′ based on the correlation between the proton signal of the methoxyl group (δ 3.74) and the C-2′

signal at δ 158.5 ppm in the HMBC spectrum. Further, in the NOE spectrum, irradiation of the methoxyl group enhanced the signal at δ 6.53 (H-3′). These results suggested that 6 was 3,5,7,4′-tetrahydroxy-2′-methoxyflavone.

Known flavonoids, biochanin A (**14**), chrysin (**15**), 3′-methylorobol (**16**), orobol (**17**), taxifolin (**18**), kaempferol (**19**) and quercetin (**20**) were identified by comparison of their spectral data with literature values.[6,12—14] These compounds were isolated for the first time from this plant.

The radical scavenging activity was determined using the stable α,α-diphenyl-β-picrylhydrazyl (DPPH) radical. As shown in Table 2, the methanol extract and the ethylacetate extracts (upper and lower layer) scavenged the DPPH radical.[15,16] Compound **6** scavenged the DPPH radical to the same degree as α-tocopherol, but the five new xanthones (**1**—**5**) had little antioxidative activity.

Experimental

General Procedures ^{1}H- and ^{13}C-NMR spectra, with tetramethylsilane (TMS) as internal standard were recorded on a JEOL FT-NMR A 500. HR-MS and DI-EI-MS were measured on a JEOL JMX-GX 303 mass spectrometer. UV spectra were measured on a Shimadzu UV-160A. TLC was conducted on precoated silica gel plates (Merck 60F-254) and RP-18. Column chromatography was carried out on silica gel (Merck Kieselgel 60F) and Sephadex LH-20 (Pharmacia). HPLC and MPLC were carried out on a column of Asahipak ODP-50 (4.6 mm×250 mm, 10 mm×250 mm) and Lober RP-18 (LiChroprep RP-18).

Materials The traditional Thai medicine ("Kam-lang-wua-talerng") was purchased in Chiang-Mai (Thailand) from a commercial supplier in 1985 and the voucher sample (EAR-0028) was placed in Sankyo Co., Ltd. in Tokyo, Japan. Kam-lang-wua-talerng has the botanical name *Anaxagorea luzonensis* A. Gray, Annonaceae, and is a tree indigenous to Thailand. Its heartwood is available in chips of varying size, mostly 5—10 cm in length, consisting of reddish-brown heartwood to which a little of the whitish bark still adheres. The wood is hard, but easily split.

Extraction and Isolation The dried and chopped heartwood (5 kg) of *A. luzonensis* was extracted three times with methanol under reflux. After filtration, the methanol extract was concentrated under reduced pressure and the residue was then partitioned between 90% aqueous methanol and *n*-hexane. Evaporation of the 90% aqueous MeOH layer yielded a reddish-brown syrup which was further partitioned between EtOAc and water. The EtOAc fraction, after removal of the solvent, afforded EtOAc extract (756 g). The water layer was then extracted with *n*-butanol, and the evaporation of the solvent afforded 55 g of *n*-butanol extract.

A part of the EtOAc extract (260 g) was partitioned with $CHCl_3$–MeOH–H_2O (13 : 7 : 6) (2600 ml) and the lower layer was concentrated *in vacuo* to give a residue (86 g). A portion of the residue (10 g) was dissolved in MeOH and then was chromatographed on a silica gel column. The absorbed substances were eluted with $CHCl_3$–MeOH (20 : 1, 10 : 1, 5 : 1) to give eight fractions (A—H). Fraction B (907 mg) was further rechromatographed on silica gel with $CHCl_3$–MeOH (50 : 1) to give four fractions. Fraction 2 (221.6 mg) was further rechromatographed on a silica gel column with hexane–EtOAc (2 : 1) and yielded **7** (115.8 mg) and **14** (25.8 mg).

Fraction C (425 mg) was further chromatographed on silica gel with $CHCl_3$–MeOH (20 : 1) to give two fractions, and fr. 1 (76.4 mg) was rechromatographed on silica gel eluted with hexane–EtOAc (2 : 1) to give three fractions (fr. 1-1, 2, 3). Repetitive semi-preparative HPLC of fr.1-1 (60.0 mg) on an Asahipak ODP-50 column (10 mm×250 mm) yielded **1** (4.0 mg, 0.002%). **2** (4.6 mg, 0.002%) was obtained from fr. 1-2 (6.7 mg) by using a silica gel column with hexane–EtOAc (2 : 1). Fraction 2 (348.1 mg) was also chromatographed on silica gel with hexane–EtOAc (2 : 1) to give two fractions (fr. 2-1, -2). Fraction 2-1 (136.3 mg) was repetitively chromatographed on a silica gel and further applied on a Sephadex LH-20 column chromatograph with MeOH to give **15** (4.0 mg). Fraction 2-2 (134.2 mg) was chromatographed on silica gel with C_6H_6–CH_3COCH_3 (10 : 1) repeatedly to give **3** (5.4 mg, 0.003%) and **16** (5.8 mg).

Fraction D (2.17 g) was rechromatographed on silica gel with $CHCl_3$, $CHCl_3$–MeOH (50 : 1, 20 : 1) to give five fractions (fr. 1—5). Fraction 4 was separated into a precipitation (Ppt) and solution. Ppt (49.6 mg) was subjected to preparative TLC with hexane–EtOAc (1 : 2) and MeOH as developing solvent to obtain **8** (2.6 mg). Part of the solution (159.0 mg) was further chromatographed on silica gel with hexane–EtOAc (3 : 1) and C_6H_6–EtOAc (10 : 1) to give **4** (1.5 mg, 0.001%). Fraction 5 (1.42 g) was recrystallized with hexane–EtOAc (2 : 1) to give **9** (216.4 mg).

Fraction E (1.66 g) was chromatographed on silica gel with $CHCl_3$–MeOH (50 : 1) to give five fractions (fr. 1—5). Fraction 2 (316.0 mg) was further chromatographed on silica gel with hexane–EtOAc (1 : 2) to give four fractions (2-1—-4). Fraction 2-1 (22.2 mg) was rechromatographed in the same manner to give **10** (2.5 mg) and fr. 2-2 (175.9 mg) was crystallized with C_6H_6–EtOAc (2 : 1) to give **11** (70.3 mg).

Fraction F (1.93 g) was chromatographed on silica gel with hexane–EtOAc (2 : 1, 1 : 1, 1 : 3). From the hexane–EtOAc (2 : 1) elution, **17** (177.4 mg) was obtained. From hexane–EtOAc (1 : 3) eluate **12** (51.9 mg) and **5** (43.5 mg, 0.022%) were obtained, respectively.

Fraction G (1.69 g) was chromatographed in the same manner on fraction F to give **18** (74.3 mg).

Fraction H (1.58 g) was chromatographed on silica gel with C_6H_6–CH_3COCH_3 (20 : 1, 10 : 1, 5 : 1, 2 : 1) and further applied on a Sephadex LH-20 column with MeOH. From each developing solvents was obtained: **19** (3.2 mg), **6** (47.6 mg, 0.024%), **20** (39.6 mg) and **13** (20.4 mg).

Compound **1**; 1,3,6-Trihydroxy-5-methoxy-4-prenylxanthone: High resolution positive-mode DI-MS: Calcd for $C_{19}H_{18}O_6$ $[M]^+$: 342.1109. Found: 342.1104. UV λ_{max}^{MeOH} nm: 242, 322, 362. ^{1}H-NMR (DMSO-d_6) δ: 13.77 (1H, OH), 7.39 (1H, d, J=8.6 Hz, H-8), 6.39 (1H, d, J=8.6 Hz, H-7), 5.86 (1H, s, H-2), 5.29 (1H, t, J=7.0 Hz, H-12), 3.33 (2H, br s, H-11), 1.79 (3H, s, H-14), 1.61 (3H, s, H-15), 3.76 (OMe). ^{13}C-NMR (DMSO-d_6): Table 1.

Compound **2**; 1,3,5-Trihydroxy-6-methoxy-2-prenylxanthone: High resolution positive-mode DI-MS: Calcd for $C_{19}H_{18}O_6$ $[M]^+$: 342.1111. Found: 342.1103. UV λ_{max}^{MeOH} nm: 245, 320. ^{1}H-NMR (DMSO-d_6) δ: 13.97 (1H, OH), 7.39 (1H, d, J=8.9 Hz, H-8), 6.37 (1H, d, J=8.9 Hz, H-7), 6.04 (1H, s, H-4), 5.20 (1H, t, J=7.0 Hz, H-12), 3.13 (2H, d, J=7.0 Hz, H-11), 1.72 (3H, s, H-14), 1.61 (3H, s, H-15), 3.73 (OMe). ^{13}C-NMR (DMSO-d_6): Table 1.

Compound **3**; 1,3,5-Trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone: High resolution positive-mode DI-MS: Calcd for $C_{18}H_{18}O_6$ $[M]^+$: 330.1105. Found: 330.1103. UV λ_{max}^{MeOH} nm: 244, 319. ^{1}H-NMR (DMSO-d_6) δ: 12.83 (1H, OH), 7.53 (1H, dd, J=7.3, 1.8 Hz, H-8), 7.28 (1H, dd, J=7.9, 1.8 Hz, H-6), 7.24 (1H, dd, J=7.3, 7.9 Hz, H-7), 6.27 (1H, s, H-2), 2.79 (2H, dd, J=8.6, 4.9 Hz, H-11), 1.61 (2H, dd, J=8.6, 4.9 Hz, H-12), 1.21 (6H, s, H-14, 15). ^{13}C-NMR (DMSO-d_6): Table 1.

Compound **4**; 1,3,6-Trihydroxy-4-prenylxanthone: High resolution positive-mode DI-MS: Calcd for $C_{18}H_{16}O_5$ $[M]^+$: 312.1057. Found: 312.1009. UV λ_{max}^{MeOH} nm: 233, 267, 317, 380. ^{1}H-NMR (DMSO-d_6) (ppm) δ: 7.31 (1H, d, J=2.8 Hz, H-5), 7.24 (1H, J=8.9 Hz, H-8), 7.10 (1H, dd, J=8.9, 2.8 Hz, H-7), 5.70 (1H, s, H-2), 5.18 (1H, t, J=7.0 Hz, H-12), 3.24 (2H, d, J=7.3 Hz, H-11), 1.80 (3H, s, H-14), 1.60 (3H, s, H-15). ^{13}C-NMR (DMSO-d_6): Table 1.

Compound **5**; 3,6-Dihydroxy-1,5-dimethoxyxanthone: High resolution positive-mode DI-MS: Calcd for $C_{15}H_{12}O_6$ $[M]^+$: 288.0634. Found: 288.0623. UV λ_{max}^{MeOH} nm: 243, 306. ^{1}H-NMR (DMSO-d_6) δ: 7.64 (1H, d, J=8.6 Hz, H-8), 6.88 (1H, d, J=8.6 Hz, H-7), 6.45 (1H, d, J=2.1 Hz, H-4), 6.37 (1H, d, J=2.1 Hz, H-2), 3.89 (3H, s, 5-OMe), 3.83 (3H, s, 1-OMe). ^{13}C-NMR (DMSO-d_6): Table 1.

Compound **6**; 3,5,7,4′-Tetrahydroxy-2′-methoxyflavone: High resolution positive-mode DI-MS: Calcd for $C_{16}H_{12}O_7$ $[M]^+$: 316.0584. Found: 316.0572. UV λ_{max}^{MeOH} nm: 249, 306, 347. ^{1}H-NMR (DMSO-d_6) δ: 7.25 (1H, d, J=8.5 Hz, H-6′), 6.53 (1H, d, J=2.1 Hz, H-3′), 6.47 (1H, dd, J=8.5, 2.1 Hz, H-5′), 6.25 (1H, d, J=1.8 Hz, H-8), 6.16 (1H, J=1.8 Hz, H-6), 3.74 (3H, s, OMe). ^{13}C-NMR (DMSO-d_6): Table 1.

Measurement of DPPH Radical Reducing Activity The sample dissolved in EtOH solution (1.0 ml) and 0.1 M acetic acid buffer solution (pH 5.5, 1.0 ml) was added to DPPH radical EtOH solution (1×10^{-4} M, 0.5 ml) in a 5 ml plastic dispocell (Becton Dickinson, U.S.A.). After 30 min of incubation at 20 °C, the absorbance was measured using a Shimadzu UV-160A spectrophotometer at 517 nm. The difference in absorbance between the test sample and control (ethanol) was taken as the activity. The activity was viewed as the amount required for 50% reduction of DPPH (1×10^{-4} M) solution.

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