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Antioxidant flavonoids from the rhizomes of *Helminthostachys zeylanica*

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Abstract

Eight flavonoids, ugonins E-L (**1**–**8**), were isolated from the rhizomes of *Helminthostachys zeylanica*, and their structures were established on the basis of spectral evidence. Compounds **3**–**8** were evaluated for their antioxidative activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Compounds **6**, **7** and **8** were more active than Trolox, with IC_{20} values of 5.29±0.32, 7.23±0.22, and 7.93±0.31, respectively.

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Keywords: *Helminthostachys zeylanica*; Ophioglossaceae; Flavonoid; Ugonins E–L; Antioxidants; DPPH

1. Introduction

Helminthostachys zeylanica (L.) Hook. (Ophioglossaceae) is a rare plant in lightly shaded regions, and it is the only species of the genus *Helminthostachys* in Taiwan. The rhizomes of *H. zeylanica*, named "Daodi-Ugon", is a Chinese herbal medicine used as an antipyretic and antiphlogistic agent (Chiu and Chang, 1992). It was previously reported that four flavonoids, ugonins A–D, were isolated from the rhizomes of *H. zeylanica* (Murakami et al., 1973a,b). In the course of our investigation on the same material, eight new flavonoids, ugonins E–L (**1**–**8**), including six cyclized geranyl flavonoids (**3**–**8**) were obtained, and their structures were established on the basis of spectral evidence. Among these compounds, **3**–**8** were evaluated for their antioxidative activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Chen et al., 2002).

2. Results and discussion

Chromatographic fractionation of the ethanolic extract from the dried rhizomes of *H. zeylanica* afforded eight flavonoids (**1**–**8**), and their structures were established on the basis of the analyses of spectral data. Compounds **1**–**8** exhibited IR absorptions at ca. 3410 (O-H), 1650 (conj. C=O), 1610 (C=C), and 1570 (C=C) cm^{-1} as well as UV absorptions at 260–295 and 336–373 nm, which indicated their flavonoid character.

Compound **1**, $C_{20}H_{20}O_5$ $[M]^+$ 340, was obtained as a yellow powder. Its ^1H NMR spectrum displayed a pair of double doublets at δ_H 2.67 (1H, $J=2.5$, 17.0 Hz) and 3.09 (1H, $J=13.0$, 17.0 Hz) with a related carbon signal at δ_C 43.9 in the HMQC spectrum, a double doublet at δ_H 5.34 (1H, $J=2.5$, 13.0 Hz) with a related carbon signal at δ_C 80.0, an aromatic singlet at δ_H 6.00 (1H), a pair of broad doublets at δ_H 6.88 (2H, $J=8.5$ Hz) and 7.39 (2H, $J=8.5$ Hz) attributed to the protons of an AA′BB′-type benzene ring, and a singlet at δ_H 12.36 attributed to a hydrogen-bonded hydroxyl group (5-OH). These ^1H NMR spectral features suggested compound 1 to be a flavanone (Meragelman et al., 2001). The ^{13}C NMR spectrum showed five carbons, except for the flavonoid part, and the remaining proton signals included two methyl singlets at δ_H 1.46 and 1.47, a pair of double doublets at δ_H 4.69 (1H, $J=1.0$, 11.0 Hz) and 4.78 (1H, $J=1.0$, 17.5 Hz) attributed to an olefinic methylene, and a double doublet at δ_H 6.18 (1H, $J=11.0$, 17.5 Hz) attributed to an olefinic methine, which is consistent with the presence of a 1,1-dimethylallyl group in **1** (Seo et al., 1997). In the HMBC spectrum of **1** (Fig. 1), the 5-OH proton signal (δ_H 12.36) correlated with signals at δ_C 163.0 (C-5), 104.0 (C-4a), and 98.0 (C-6). The latter

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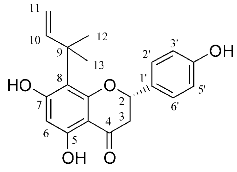

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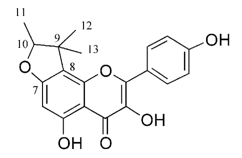

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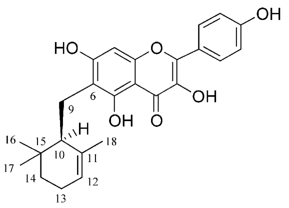

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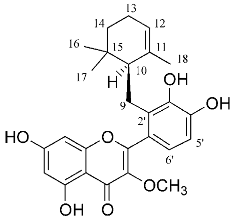

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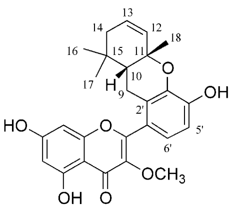

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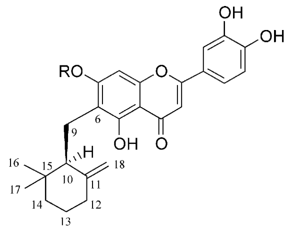

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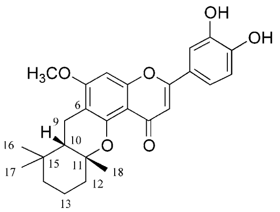
7: R = CH_3



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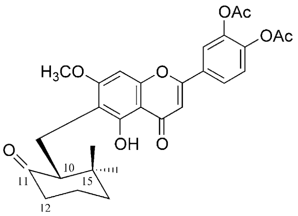

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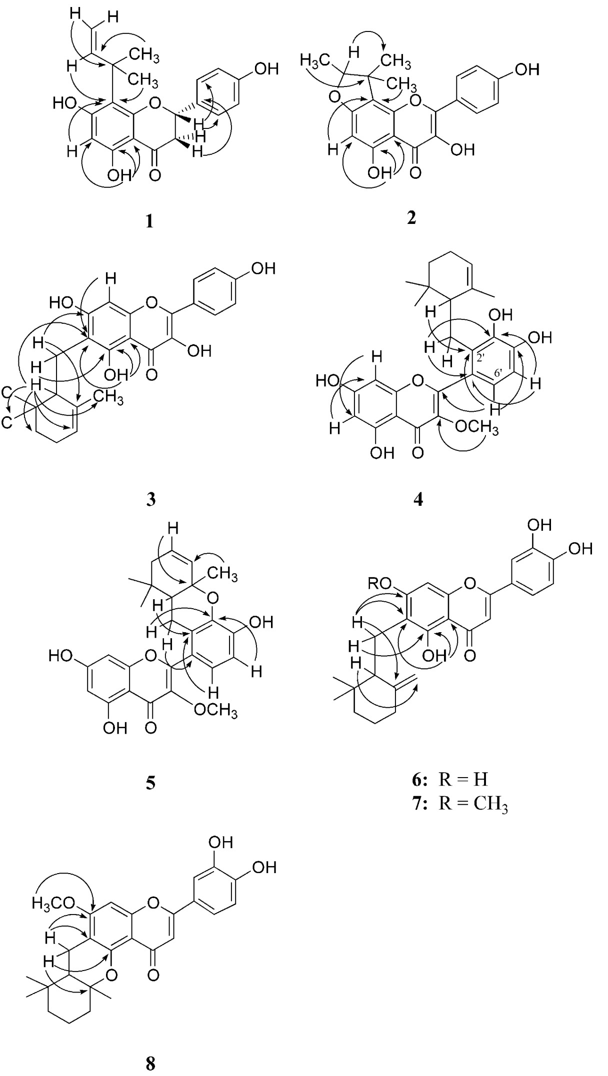

Fig. 1. HMBC correlations of **1**–**8**.

signal exhibited a HMQC correlation with the proton signal at δ_H 6.00, which, in turn, could be assigned to H-6. Moreover, the H-6 signal showed a key correlation with δ_C 113.7 (C-8) which also had a correlation with two methyl proton signals at δ_H 1.46 and 1.47 (H-12 and H-13); the 1,1-dimethylallyl group was thus located at C-8. The stereochemistry at C-2 was determined to be *S* due to the presence of a positive Cotton effect at 350 nm and a negative Cotton effect at 292 nm in the CD spectrum of **1** (Meragelman et al., 2001). Therefore, compound **1** was established as 5,7,4′-trihydroxy-8-(1,1-dimethylallyl)flavanone and named ugonin E.

The ^{1}H NMR spectrum compound **2**, $C_{20}H_{18}O_6$ $[M]^+$354, displayed an aromatic singlet at δ_H 6.18 (1H), a pair of broad doublets at δ_H 7.04 (2H, *J* = 8.5 Hz) and 8.12 (2H, *J* = 8.5 Hz) attributed to the protons of the AA′BB′-type ring B, and a low field singlet at δ_H 12.44 attributed to 5-OH. The presence of a 2,3-dihydro-2,3,3-trimethylfuran ring was indicated by the signals at δ_H 1.33 (3H, *s*), 1.40 (3H, *d*, *J* = 7.0 Hz), 1.60 (3H, *s*), and 4.56 (1H, *q*, *J* = 7.0 Hz) (Seo et al., 1997). The HMBC spectrum of **2** (Fig. 1) exhibited similar correlations as **1**, and the correlation of the H-12 and H-13 signals to C-8 signal (δ_C 113.5) suggested that the 2,3-dihydro-2,3,3-trimethylfuran ring was linked to C-7 and C-8 . Therefore, compound 2 was determined to be 4″,5″-dihydro-3,5,4′-trihydroxy-4″,4″,5″-trimethylfurano[2″,3″:7,8]flavone and named ugonin F.

Compounds **3**–**8** were obtained as yellow powders and ^{1}H and ^{13}C NMR spectra revealed the presence of a monoterpene unit in their structures. The ^{1}H NMR spectrum of **3**, $C_{25}H_{26}O_6$ $[M]^+$422, showed a monoterpene unit of a cyclized geranyl group. The proton signals appeared at δ_H 0.88, 0.98, and 1.51 (each 3H, *s*), whereas resonances at δ_H 1.16 (1H, *m*), 1.70 (1H, *m*), and 2.01 (2H, *m*) were attributed to a pair of aliphatic methylenes, and the signal of additional resonances δ_H 2.28 (1H, *t*, *J* = 7.0 Hz) corresponded to an allylic methine, δ_H 2.66 (1H, *dd*, *J* = 7.0, 13.5 Hz) and 2.87 (1H, *dd*, *J* = 6.5, 13.5 Hz) resulted from a benzylic methylene, and the signal of δ_H 5.23 (1H, *br s*) was for an olefinic methine (Thoison et al., 1992). An HMBC experiment (Fig. 1) showed the correlation between the 5-OH signal (δ_H 12.47) and carbon signals at δ_C 159.3 (C-5), 103.9 (C-4a), as well as δ_C 113.1 (C-6) which also correlated with an aromatic proton signal at δ_H 6.58 (H-8). Moreover, the benzylic methylene proton signals (δ_H 2.66 and 2.87, H_2-9) exhibited a correlation with C-5 and C-7 (δ_C 163.2) signals, which deduced the linkage of the cyclized geranyl group to C-6. Based on the above evidence, compound **3** was determined to be 3,5,7,4′-tetrahydroxy-6-(2,6,6-trimethyl-2-cyclohexenylmethyl)flavone and named ugonin G.

The ^{1}H NMR spectrum of compound **4**, $C_{26}H_{28}O_7$ [M] 452, showed a methoxyl singlet at δ_H 3.57, a pair of *m*-coupled doublets at δ_H 6.19 (1H, $J=2.0$ Hz, H-6) and 6.26 (1H, $J=2.0$ Hz, H-8), a pair of *o*-coupled doublets at δ_H 6.76 (1H, $J=8.5$ Hz, H-5′) and 6.79 (1H, $J=8.5$ Hz, H-6′) for the protons of AA′-type ring B, and a set of signals similar to those of the cyclized geranyl group in compound **3** (see Experimental). The structure of **4** was established by the following HMBC correlations (Fig. 1). The H-6′ signal (δ_H 6.79) correlated with δ_C 130.4 (C-2′), 161.5 (C-2), and 148.0 (C-4′); H-5′ signal (δ_H 6.76) showed correlations with δ_C 124.0 (C-1′) and 145.3 (C-3′). The benzylic methylene signals at δ_H 2.50 and 3.10 (H_2-9) correlated with C-1′ and C-3′ signals. Therefore, the position of the cyclized geranyl group was determined to be at C-2′. A NOE enhancement was observed at the H-6′ signal (δ_H 6.79) on irradiation of the methoxyl signal at δ_H 3.57, suggesting that the methoxyl group was located at C-3 (δ_C 140.1). From the above data, compound 4 was determined to be 5,7,3′,4′-tetrahydroxy-3-methoxy-2′-(2,6,6-trimethyl-2-cyclohexenylmethyl)flavone and named ugonin H.

Compound **5**, $C_{26}H_{26}O_7$ $[M]^+$450, was also a 3-methoxyflavone. Its ^{1}H NMR spectrum differed in part of the cyclized geranyl group from 4. The DEPT spectrum of 5 showed one more olefinic methine carbon instead of one aliphatic methylene carbon in **4**. In the ^{1}H NMR spectrum of **5**, a pair of olefinic protons was observed at δ_H 5.76 (1H, *d*, $J=10.0$ Hz) and 5.81 (1H, *m*), which were assigned to H-12 and H-13, respectively, based on HMBC spectral analysis (Fig. 1). The benzylic methylene proton signals at δ_H 2.93 and 3.05 (H_2-9) of the cyclized geranyl group exhibited HMBC correlations with the C-1′ (δ_C 120.9) and C-3′ (δ_C 141.6) resonances. Thus, the cyclized geranyl group was linked to C-2′ and C-3′. In a NOE experiment, irradiation of the methyl signal at δ_H 1.36 (H_3-18) caused an enhancement of the signal at δ_H 1.85 (*d*, $J=8.0$ Hz, H-10), indicating a *cis* configuration between H_3-18 and H-10. Accordingly, structure 5 was determined to be 4″a,5″,6″,8″a-tetrahydro-5,7,4′-trihydroxy-3-methoxy-5″,5″,8″a-trimethyl-4*H*-chromeno[2″,3″: 3′,2′]flavone and named ugonin I.

Compounds **6**–**8** were assigned as 5,6,7,3′,4′-pentasubstituted flavones. The ^{1}H NMR spectrum of **6**, $C_{25}H_{26}O_6$ $[M]^+$422, showed a pair of singlets at δ_H 6.52 and 6.53 attributed to H-8 and H-3, respectively, three signals for the protons of ABX-type ring B at δ_H 6.97 (1H, *d*, $J=8.0$ Hz, H-5′), 7.40 (1H, *dd*, $J=2.0$, 8.0 Hz, H-6′), and 7.45 (1H, *d*, $J=2.0$ Hz, H-2′), as well as a set of resonances attributable to a cyclized geranyl group (see Experimental). The DEPT spectrum of **6** indicated that the cyclized geranyl part contained two methyls, one benzylic methylene, three aliphatic methylenes, and one olefinic methylene, respectively. The linkage site of this group was determined to be at C-6 since the benzylic methylene proton signals at δ_H 2.74 and 2.96 (H_2-9) correlated with C-5 (δ_C 160.3) and C-7 (δ_C 162.7) in the HMBC spectrum of **6** (Fig. 1). Thus, structure **6** was established as 5,7,3′,4′-tetrahydroxy-6-(6,6-dimethyl-2-methylene-cyclohexylmethyl)flavone and named ugonin J.

The ^{1}H NMR spectral data of compound **7**, $C_{26}H_{28}O_6$ $[M]^+$436, closely resembled those of compound **6** except for an additional *O*-methyl group. The presence of a HMBC correlation (Fig. 1) between the methoxyl proton at δ_H 3.88 and the C-7 signal at δ_C 163.3 resulted in the assignment of this methoxyl group at C-7. Therefore, structure **7** was proposed for ugonin K. Compound **8** had the same molecular formula as **7**, but their structures differed by the nature of the cyclized geranyl group. The ^{13}C NMR and DEPT spectra of **8** showed one more methyl carbon (δ_C 19.9, H-18) instead of the olefinic methylene carbon, and a C-11 signal shifted to δ_C 78.8 compared to **7**. The HMBC spectrum of **8** (Fig. 1) exhibited similar correlations as for **7**, in which the benzylic methylene proton signals at δ_H 2.29 and 2.70 (H_2-9) correlated with two *O*-bearing carbon signals at δ_C 154.5 and 162.3 of ring A. In NOE experiment, irradiation of the methoxyl signal at δ_H 3.95 caused enhancement of the signal at δ_H 6.69. The above data thus indicated the proper position of the methoxyl group was at C-7 (δ_C 162.3). In turn, the signal at δ_H 6.69 was attributed to H-8, and the cyclized geranyl substituent could be linked to C-5 and C-6. Additionally, a NOE effect was observed at the H-10 signal (δ_H 1.58) on irradiation of the methyl signal at δ_H 1.22 (H_3-18), indicating a *cis* configuration between H_3-18 and H-10. Therefore, structure **8** was determined to be 4″a,5″,6″,7″,8″,8″a-hexahydro-3′,4′-dihydroxy-7-methoxy-5″,5″,8″a-trimethyl-4*H*-chromeno[2″,3″:5,6]flavone and named ugonin L. The absolute stereochemistry of ugonins F, I, and L (**2**, **5**, and **8**) has not been defined. A *S*-configuration at C-10 of ugonin K (**7**) was established from the presence of a positive Cotton effect at 300 nm (Δε0.6) in the CD spectrum of its carbonyl derivative (**9**) (Nakanishi et al., 1994). The CD spectra of ugonins G, H, and J (**3**, **4**, and **6**) showed similar characteristics as that of **7** (see Experimental), which suggested that these three compounds also have the *S*-configuration at their C-10 positions.

With the exception of **1** and **2**, which were insufficient in quantity for additional analysis, the other six compounds (**3**–**8**) were tested for their antioxidative activity using the DPPH test, and the results are shown in Table 2. All compounds, with the exception of **5**, showed potent antioxidative activity. Compounds **6**, **7** and **8** were more potent than Trolox, with IC_{20} values of 5.29±0.32, 7.23±0.22, and 7.93±0.31, respectively.

3. Experimental

3.1. General

Melting points were determined on a Yanaco MP-I3 micro melting point apparatus and are uncorrected. IR spectra were taken on a Nicolet Avatar 320 FT-IR spectrometer, whereas UV spectra were recorded on a JASCO model 7800 UV/vis spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter, and CD spectra were obtained on a JASCO J-715 spectrometer. EIMS spectra were acquired using either Finnigan MAT 95S or Finnigan MAT GCQ spectrometers. ^{1}H NMR and ^{13}C NMR spectra were measured with a Varian Unity Inova-500 spectrometer.

3.2. Plant material

The rhizomes of *H. zeylanica* (L.) Hook. were collected from Kontum, Vietnam, in July 1999. A voucher specimen (NRICM-99-003) is deposited in the Herbarium of the National Research Institute of Chinese Medicine, Republic of China. The plant was also identified by comparison with a voucher specimen already deposited at the Herbarium of the Department of Botany, National Taiwan University, Republic of China.

3.3. Extraction and isolation

The roots of *H. zeylanica* (12 kg) were extracted with EtOH (20 l×3) at 50 °C for 24 h. The concentrated EtOH extract (460 g) was partitioned between EtOAc and H_2O, and the EtOAc extract (153 g) was applied to a silica gel column eluted with gradient solvent systems of *n*-hexane–EtOAc (20:1–1:10) and EtOAc–MeOH (10:1–1:1) to yield 16 fractions (Fr-1–Fr-16). Fraction Fr-5, the eluate of *n*-hexane–EtOAc = 2:1, was further subjected to silica gel CC eluting with *n*-hexane–Me_2CO (7:1 to 4:1) to give 10 fractions (Fr-5-1–Fr-5-10). Fraction Fr-5-4 was further repeatedly separated over Sephadex LH-20 columns eluting with MeOH and MeOH–H_2O (5:1) to afford ugonin F (**2**, 5.3 mg). The same treatment for Fr-5-5 as Fr 5-4 gave ugonin E (**1**, 4.2 mg) and ugonin I (**5**, 11.7 mg), respectively. Fraction Fr-5-6 was also applied to a Sephadex LH-20 column (MeOH) to afford ugonin G (**3**, 8.5 mg). Fraction Fr-6, eluate of *n*-hexane–EtOAc = 1:2, was further repeatedly separated over silica gel (*n*-hexane–Me_2CO = 7:1 to 1:1) and Sephadex LH-20 columns (MeOH–H_2O = 5:1) to obtain ugonin H (**4**, 8.7 mg). Fraction Fr-7, the eluate of *n*-hexane–EtOAc = 1:2, was further subjected to a silica gel CC (CH_2Cl_2–MeOH = 30:1) and Sephadex LH-20 (MeOH–H_2O = 5:1) to give ugonin J (**6**, 26.3 mg) and ugonin K (**7**, 18.6 mg), respectively. Finally, fraction Fr-12, the eluate of *n*-hexane–EtOA = 1:10, was further purified over silica gel (CH_2Cl_2–MeOH = 30:1–20:1) and Sephadex LH-20 (MeOH–H_2O = 3:1) columns to afford ugonin L (**8**, 12.3 mg).

3.4. Ugonin E (1) (= 5,7,4′-trihydroxy-8-(1,1-dimethylallyl)flavanone)

Yellow powder; $[\alpha]_D^{25}$ −45.4° (*c* 0.11, MeOH); UV λ_{max}^{MeOH} nm (log ε): 336 (3.43), 295 (3.94), 217 (4.15); IR ν_{max} (KBr) cm^{-1}: 3406 (OH), 2964, 2922, 2865, 1640, 1614, 1598, 1514, 1467, 1403, 1377, 1267, 1220, 1156, 1067, 826; CD (*c* 6.47×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 31.7 (218), −38.8 (292), 2.9 (350); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ 12.36 (1H, *s*, 5-OH), 7.39 (2H, *br d*, *J* = 8.5 Hz, H-2′, 6′), 6.88 (2H, *br d*, *J* = 8.5 Hz, H-3′, 5′), 6.18 (1H, *dd*, *J* = 11.0, 17.5 Hz, H-10), 6.00 (1H, *s*, H-6), 5.34 (1H, *dd*, *J* = 2.5, 13.0 Hz, H-2), 4.78 (1H, *dd*, *J* = 1.0, 17.5 Hz, H-11), 4.69 (1H, *dd*, *J* = 1.0, 11.0 Hz, H-11), 3.09 (1H, *dd*, *J* = 13.0, 17.0 Hz, H-3), 2.67 (1H, *dd*, *J* = 2.5, 17.0 Hz, H-3), 1.46, 1.47 (each 3H, *s*, H-12, 13); For ^{13}C

Table 1
^{13}C NMR spectral data (δ) for **1–8**

No.	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[a]	**6**[a]	**7**[c]	**8**[a]
2	80.0	146.8	146.6	161.5	158.7	164.7	163.9	161.5
3	43.9	136.5	136.6	140.1	139.6	104.2	103.0	107.5
4	198.3	176.8	176.5	179.9	178.7	183.0	181.8	177.1
4a	104.0	104.8	103.9	105.9	105.3	104.9	104.2	109.5
5	163.0	162.8	159.3	163.1	162.4	160.3	157.8	154.5
6	98.0	94.9	113.1	100.1	99.1	112.4	112.0	108.7
7	166.3	165.9	163.2	166.9	165.5	162.7	163.3	162.3
8	113.7	113.5	93.9	95.2	94.1	93.7	90.1	91.5
8a	162.8	152.9	155.6	159.0	157.8	156.3	155.3	158.9
9	41.4	44.4	25.3	30.8	20.0	21.9	20.7	18.7
10	151.3	91.4	48.2	49.2	43.5	53.0	52.2	47.4
11	108.1	14.4	138.5	138.2	73.9	150.5	149.1	78.8
12	29.6	21.7	120.6	121.4	130.9	32.0	30.7	40.3
13	30.0	26.2	23.9	24.1	128.2	24.1	22.8	20.4
14			31.1	30.4	41.6	35.2	34.4	42.1
15			33.5	33.7	32.5	35.2	34.4	34.0
16			27.5	27.1	20.8	28.1	27.5	20.8
17			27.8	28.8	30.0	28.5	28.0	32.3
18			24.3	25.2	26.9	109.4	108.8	19.9
1′	130.9	123.4	123.4	124.0	120.9	123.9	121.5	124.0
2′	128.9	130.3	130.4	130.4	121.9	113.9	113.4	113.9
3′	116.0	116.3	116.3	145.3	141.6	146.3	145.7	146.5
4′	158.5	160.2	160.1	148.0	147.9	149.8	149.7	149.5
5′	116.0	116.3	116.3	113.3	112.2	116.5	115.9	116.5
6′	128.9	130.3	130.4	122.1	122.1	120.0	118.9	119.2
OCH_3				61.1	60.2		56.0	56.4

[a] Measured in acetone-d_6.
[b] Measured in CD_3OD.
[c] Measured in DMSO-d_6.

Table 2
Free radical scavenging activity of **3–8** and trolox in the DPPH assay

Sample	Free-radical scavenging activity IC_{20} (μM)
3	12.63 ± 0.42^a
4	9.84 ± 0.22^b
5	>100
6	5.29 ± 0.32^e
7	7.23 ± 0.22^d
8	7.93 ± 0.31^c
Trolox	10.39 ± 0.56^b

Data are expressed as mean ±S.D. Averages followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test.

NMR (Me_2CO-d_6, 125 MHz), see Table 1; EIMS *m/z*: 340 $[M]^+$ (76), 325 (100), 305 (30), 206 (88); HREIMS *m/z* 340.1301 (calc. for $C_{20}H_{20}O_5$, 340.1305).

3.5. Ugonin F (2) (=4″,5″-dihydro-3,5,4′-trihydroxy-4″,4″,5″-trimethylfurano[2″,3″:7,8]flavone)

Yellow powder; $[\alpha]_D^{25}$ 3.1° (*c* 0.32, MeOH); UV λ_{max}^{MeOH} nm (log ε): 373 (4.02), 310 (3.84), 271 (4.10), 220 (4.09); IR ν_{max} (KBr) cm^{-1}: 3427 (OH), 2962, 2917, 2863, 1642, 1619, 1596, 1516, 1419, 1388, 1269, 1220, 1135, 1070; CD (*c* 5.65×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 17.2 (203), 1.1 (210), 0.9 (214), 1.0 (218); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ 12.44 (1H, *s*, 5-OH), 8.12 (2H, *br d*, *J*=8.5 Hz, H-2′, 6′), 7.04 (2H, *br d*, *J*=8.5 Hz, H-3′, 5′), 6.18 (1H, *s*, H-6), 4.56 (1H, *q*, *J*=7.0 Hz, H-10), 1.40 (3H, *d*, *J*=7.0 Hz, H-11), 1.60, 1.33 (each 3H, *s*, H-12, 13); For ^{13}C NMR (Me_2CO-d_6, 125 MHz), see Table 1; EIMS *m/z*: 354 $[M]^+$ (74), 340 (100), 242 (14); HREIMS *m/z* 354.1100 (calc. for $C_{20}H_{18}O_6$, 354.1098).

3.6. Ugonin G (3) (=3,5,7,4′-tetrahydroxy-6-(2,6,6-trimethyl-2-cyclohexenylmethyl)flavone)

Yellow powder; $[\alpha]_D^{25}$ 98.9 (*c* 0.94, MeOH); UV λ_{max}^{MeOH} nm (log ε): 368 (4.13), 301 (3.87), 271 (4.15); IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2955, 2912, 2850 1650, 1624, 1560, 1486, 1451, 1361, 1272, 1220, 1177, 1083, 809; CD (*c* 6.63×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 7.1 (212), 3.6 (235), 1.3 (269), 0.9 (331); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ ; 12.47 (1H, *s*, 5-OH), 8.11 (2H, *br d*, *J*=8.5 Hz, H-2′, 6′), 6.99 (2H, *br d*, *J*=8.5 Hz, H-3′, 5′), 6.58 (1H, *s*, H-8), 5.23 (1H, *br s*, H-12), 2.87 (1H, *dd*, *J*=6.5, 13.5 Hz, H-9), 2.66 (1H, *dd*, *J*=7.0, 13.5 Hz, H-9), 2.28 (1H, *t*, *J*=7.0 Hz, H-10), 2.01 (2H, *m*, H-13), 1.70 (1H, *m*, H-14), 1.16 (1H, *m*, H-14), 1.51 (3H, *s*, H-18), 0.98 and 0.88 (each 3H, *s*, H-16, H-17); For ^{13}C NMR (Me_2CO-d_6, 125 MHz), see Table 1; EIMS *m/z*: 422 $[M]^+$ (19), 300 (100); HREIMS *m/z* 422.1723 (calc. for $C_{25}H_{26}O_6$, 422.1724).

3.7. Ugonin H (4) (=5,7,3′,4′-tetrahydroxy-3-methoxy-2′-(2,6,6-trimethyl-2-cyclohexenylmethyl)flavone)

Yellow powder; $[\alpha]_D^{25}$ 130.5° (*c* 0.95, MeOH); UV λ_{max}^{MeOH} nm (log ε): 336 (4.03), 303 (3.99), 260 (4.34), 206 (4.70); IR ν_{max} (KBr) cm^{-1}: 3416 (OH), 2923, 2860 1661, 1609, 1561, 1493, 1450, 1351, 1288, 1220, 1156, 1083, 804; CD (*c* 5.31×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 17.6 (212), 9.5 (228), 2.5 (250), −3.7 (276), 6.8 (303); ^{1}H NMR (CD_3OD, 500 MHz) δ 12.79 (1H, *s*, 5-OH in Me_2CO-d_6), 6.79 (1H, *d*, *J*=8.5 Hz, H-6′), 6.76 (1H, *d*, *J*=8.5 Hz, H-5′), 6.26 (1H, *d*, *J*=2.0 Hz, H-8), 6.19 (1H, *d*, *J*=2.0 Hz, H-6), 4.92 (1H, *br s*, H-12), 3.57 (3H, *s*, 3-OCH_3), 3.10 (1H, *dd*, *J*=5.5, 13.0 Hz, H-9), 2.50 (1H, *dd*, *J*=10.0, 13.0 Hz, H-9), 1.93 (1H, *dd*, *J*=5.5, 10.0 Hz, H-10), 1.75 (1H, *m*, H-13), 1.53 (1H, *m*, H-13), 1.44 (1H, *m*, H-14), 1.05 (3H, *s*, H-18), 0.97 (1H, *m*, H-14), 0.87 and 0.73 (each 3H, *s*, H-16, 17); For ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; EIMS *m/z* 452 $[M]^+$ (75), 436 (19), 422 (27), 330 (81), 316 (100), 300 (34); HREIMS *m/z* 452.1830 (calc. for $C_{26}H_{28}O_7$, 452.1830).

*3.8. Ugonin I (5) (=4″a,5″,6″,8″a-tetrahydro-5,7,4′-trihydroxy-3-methoxy-5″,5″,8″a-trimethyl-4*H*-chromeno[2″,3″:3′,2′]flavone)*

Yellow powder; $[\alpha]_D^{25}$ −45.0° (*c* 0.10, MeOH); UV λ_{max}^{MeOH} nm (log ε): 336 (4.66), 302 (4.63), 260 (4.94), 208 (5.25); IR ν_{max} (KBr) cm^{-1}: 3411 (OH), 2969, 2928, 2870 1656, 1609, 1562, 1488, 1451, 1356, 1272, 1220, 1162, 1083, 815; CD (*c* 4.44×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) −11.3 (208), −1.4 (228), −3.4 (252), 0.3 (267), −1.4 (303), −0.1 (367); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ 12.73 (1H, *br s*, 5-OH), 6.95 (1H, *d*, *J*=8.5 Hz, H-6′), 6.76 (1H, *d*, *J*=8.5 Hz, H-5′), 6.36 (1H, *d*, *J*=2.0 Hz, H-8), 6.24 (1H, *d*, *J*=2.0 Hz, H-6), 5.81 (1H, *m*, H-13), 5.76 (1H, *d*, *J*=10 Hz, H-12), 3.66 (3H, *s*, 3-OCH_3), 3.05 (1H, *dd*, *J*=8.0, 18.0 Hz, H-9), 2.93 (1H, *d*, *J*=18.0 Hz, H-9), 1.99 (1H, *d*, *J*=18.0 Hz, H-14), 1.85 (1H, *d*, *J*=8.0 Hz, H-10), 1.85 (1H, *dd*, *J*=6.0, 18.0 Hz, H-14), 1.36 (3H, *s*, H-18), 0.97 and 0.79 (each 3H, *s*, H-16, 17); For ^{13}C NMR (Me_2CO-d_6, 125 MHz), see Table 1; EIMS *m/z* 450 $[M]^+$ (15), 436 (100), 330 (43), 316 (23), 300 (14); HREIMS *m/z* 450.1676 (calc. for $C_{26}H_{26}O_7$, 450.1673).

3.9. Ugonin J (6) (=5,7,3′,4′-tetrahydroxy-6-(6,6-dimethyl-2-methylene-cyclohexylmethyl)flavone)

Yellow powder; $[\alpha]_D^{25}$ 50.0° (*c* 0.30, MeOH); UV λ_{max}^{MeOH} nm (log ε): 347 (4.27), 275 (4.16), 214 (4.51); IR ν_{max} (KBr) cm^{-1}: 3412 (OH), 2927, 2870, 1656, 1614, 1571, 1488, 1356, 1261, 1172, 1077, 815; CD (*c* 4.74×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 12.2 (203), 8.2 (206), 1.7 (224), 2.2 (240), −0.3 (282), 0.6 (331), 0.1 (391); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ 13.30 (1H, *s*, 5-OH), 7.45 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.40 (1H, *dd*, *J* = 2.0, 8.0 Hz, H-6′), 6.97 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.53 (1H, *s*, H-3), 6.52 (1H, *s*, H-8), 4.42 (1H, *br s*, H-18), 4.24 (1H, *br s*, H-18), 2.96 (1H, *br t*, *J* = 13.0 Hz, H-9), 2.74 (1H, *dd*, *J* = 3.5, 13.0 Hz, H-9), 2.51 (1H, *m*, H-12), 2.43 (1H, *dd*, *J* = 3.5, 12.0 Hz, H-10), 1.92 (1H, *m*, H-12), 1.67 (1H, *m*, H-14), 1.56 (1H, *m*, H-13), 1.48 (1H, *m*, H-13), 1.25 (1H, *m*, H-14), 1.05 and 0.93 (each 3H, *s*, H-16, 17); For ^{13}C NMR (Me_2CO-d_6, 125 MHz), see Table 1; EIMS *m/z* 422 $[M]^+$ (100), 300 (40); HREIMS *m/z* 422.1723 (calc. for $C_{25}H_{26}O_6$, 422.1724).

3.10. Ugonin K (7) (= 5,3′,4′-trihydroxy-7-methoxy-6-(6,6-dimethyl-2-methylene-cyclohexylmethyl)flavone)

Yellow powder; $[\alpha]_D^{25}$ −18.7° (*c* 0.16, MeOH); UV λ_{max}^{MeOH} nm (log ε): 346 (4.00), 273 (3.93), 214 (4.23); IR ν_{max} (KBr) cm^{-1}: 3421 (OH), 2923, 2849 1656, 1603, 1563, 1483, 1451, 1382, 1267, 1125, 809; CD (*c* 4.59×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 1.8 (202), 1.9 (206), 0.9 (224), 0.8 (240), 0.4 (331), 0.1 (391); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.13 (1H, *s*, 5-OH), 7.44 (1H, *dd*, *J* = 1.5, 8.0 Hz, H-6′), 7.43 (1H, *d*, *J* = 1.5 Hz, H-2′), 6.89 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.74 (1H, *s*, H-8), 6.69 (1H, *s*, H-3), 4.42 (1H, *br s*, H-18), 4.14 (1H, *br s*, H-18), 3.88 (3H, *s*, 7-OCH_3), 2.84 (1H, *br t*, *J* = 12.5 Hz, H-9), 2.65 (1H, *dd*, *J* = 4.0, 12.5 Hz, H-9), 2.37 (1H, *m*, H-12), 2.22 (1H, *dd*, *J* = 4.0, 12.5 Hz, H-10), 1.93 (1H, *m*, H-12), 1.59 (1H, *m*, H-14), 1.55 (1H, *m*, H-13), 1.44 (1H, *m*, H-13), 1.23 (1H, *m*, H-14), 1.02 and 0.84 (each 3H, *s*, H-16, 17); For ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; EIMS *m/z* 436 $[M]^+$ (12), 313 (100); HREIMS *m/z* 436.1869 (calc. for $C_{26}H_{28}O_6$, 436.1880).

3.10.1. 5-hydroxy-7-methoxy-3′,4′-diacetoxy-6-(6,6-dimethyl-2-oxo-cyclohexylmethyl)flavone (9)

Ugonin K (**7**, 8 mg) in acetic anhydride-pyridine was heated at 50–60 °C for 2 h to yield its 3′,4′-diacetate (8 mg) as a pale yellow powder. Ozone was passed into a stirred solution of the above diacetate (7 mg) in 5 ml CH_2Cl_2 at −70 °C for 2 min, then the solution was purged with nitrogen at −70 °C and the temperature held during addition of dimethyl sulfide (0.3 ml). The solution was stirred and allowed to come to room temperature within 2 h, and stirred further for 1 h. Chromatography of the residue over preparative silica-TLC (CH_2Cl_2-Me_2CO = 18:1) gave compound **9** (5 mg) as a pale yellow powder; CD (*c* 9.96×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 0.5 (212), −0.7 (227), −0.1 (248), −0.9 (271), 1.0 (290), 0.7 (300), −0.1 (360); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ 13.05 (1H, *s*, 5-OH), 8.00 (1H, *dd*, *J* = 2.0, 8.0 Hz, H-6′),7.95 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.47 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.83 (1H, *s*, H-3), 6.77 (1H, *s*, H-8), 3.94 (3H, *s*, 7-OCH_3), 3.09 (1H, *dd*, *J* = 9.5, 13.5 Hz, H-9), 2.80 (1H, *dd*, *J* = 4.5, 13.5 Hz, H-9), 2.66 (1H, *m*, H-12), 2.49 (1H, *dd*, *J* = 4.5, 9.5 Hz, H-10), 2.32 and 2.31 (each 3H, *s*, acetoxyl CH_3), 2.12 (1H, *m*, H-12), 1.83 (2H, *m*, H-13), 1.79 (1H, *m*, H-14), 1.54 (1H, *m*, H-14), 1.03 and 0.93 (each 3H, *s*, H-16, 17); ^{13}C NMR (Me_2CO-d_6, 125 MHz) δ 212.4 (C-11), 183.2, 168.6 and 168.4 (acetoxyl C=O), 164.6, 163.0, 159.3, 157.2, 146.3, 144.0, 130.6, 125.5 (C-6′), 125.3 (C-5′), 122.7 (C-2′), 112.8, 106.8, 106.7 (C-3), 91.2 (C-8), 60.8 (C-10), 56.6 (7-OCH_3), 39.3 (C-12), 39.0, 36.8 (C-14), 28.7 and 25.3 (C-16, 17), 23.2 (C-13), 20.2 and 20.4 (acetoxyl CH_3), 20.1 (C-9); EIMS *m/z* 522 $[M]^+$ (43) for $C_{29}H_{30}O_9$.

3.11. Ugonin L (8) (= 4″a,5″,6″,7″,8″,8″a-hexahydro-3′,4′-dihydroxy-7-methoxy-5″,5″,8″a-trimethyl-4H-chromeno[2″,3″:5,6]flavone)

Yellow powder; $[\alpha]_D^{25}$ 81.8° (*c* 0.22, MeOH); UV λ_{max}^{MeOH} nm (log ε): 337 (4.32), 271 (4.23), 243 (4.22), 214 (4.55); IR ν_{max} (KBr) cm^{-1}: 3406 (OH), 2943, 2870, 1635, 1609, 1571, 1451, 1345, 1272, 1204, 1120, 1088, 815; CD (*c* 4.59×10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 11.7 (212), 2.9 (267), 3.9 (311), −1.7 (350), 0.2 (380); ^{1}H NMR (Me_2CO-d_6, 500 MHz) δ 7.49 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.37 (1H, *dd*, *J* = 2.0, 8.0 Hz, H-6′), 6.96 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.69 (1H, *s*, H-8), 6.40 (1H, *s*, H-3), 3.95 (3H, *s*, 7-OCH_3), 2.70 (1H, *dd*, *J* = 5.5, 17.0 Hz, H-9), 2.29 (1H, *dd*, *J* = 13.5, 17.0 Hz, H-9), 2.06 (1H, *m*, H-12), 1.63 (3H, *m*, H-12, H-13), 1.58 (1H, *dd*, *J* = 5.5, 13.5 Hz, H-10), 1.48 (1H, *m*, H-14), 1.34 (1H, *m*, H-14), 1.22 (3H, *s*, H-18), 1.01 and 0.93 (each 3H, *s*, H-16, 17); ^{13}C NMR (Me_2CO-d_6, 125 MHz), see Table 1; EIMS *m/z* 436 $[M]^+$ (73), 327 (48), 313 (100); HREIMS *m/z* 436.1883 (calc. for $C_{26}H_{28}O_6$, 436.1880).

3.12. Assay for antioxidative activity

The antioxidative activities of the pure compounds were assessed on the basis of the radical scavenging effect of the stable DPPH free radical (Chen et al., 2002). One milliliter of a 100 μM DPPH–ethanol solution was added to 10 μl of sample solutions of different concentrations and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm using a spectrophotometer and converted into the percentage of antioxidative activity (AA) using the following formula:

$$\text{AA\%} = 100 - \left\{\left[\left(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}\right) \times 100\right]/\text{Abs}_{\text{control}}\right\} \tag{1}$$

Ethanol (1.0 ml) plus sample solution (10 µl) was used as a blank and DPPH solution plus ethanol was also used a negative control. The IC_{20} value is the concentration of test sample required to scavenge 20% DPPH free radicals. Trolox was used as a positive control.

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