Constituents from the Leaves of *Phellodendron amurense* and Their Antioxidant Activity

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Three new coumarins, phellodenols F—H (1—3) and a new glutaric acid derivative, phellodendric acid-A (4) were isolated from the leaves of *Phellodendron amurense* together with twenty-nine known compounds. Extensive 1D and 2D NMR experiments and other spectroscopic studies were employed to determine the structures of 1—4. The isolated compounds were screened for their antioxidant activity through DPPH (α,α-diphenyl-β-picrylhydrazyl) radical scavenging assay. Compounds quercetin, quercetin-3-*O*-β-D-glucoside, quercetin-3-*O*-β-D-galactoside and kaempferol-3-*O*-β-D-glucoside demonstrated significant radical scavenging activity comparable to vitamin E.

Key words *Phellodendron amurense*; coumarin; glutaric acid; antioxidant activity; DPPH assay

There are about 10 species in the genus *Phellodendron*, distributed widely over the tropical and subtropical areas of Asia. The thick corky bark of *Phellodendron amurense* has a long history of ethnobotanical use among native peoples in East Asia. Diabetes mellitus, meningitis, pneumonia, antistomachic, intestinal function control, anti-inflammatory, anti-psychic, hest relief, bacillary dysentery, diarrhea, tuberculosis and liver cirrhosis treatments are among the indications listed for Huangbai, as the baric is commonly known.[1—3] Plants of the genus *Phellodendron* are known to be rich sources of berberine and aphorphine alkaloids, flavonoids, various coumarins, lignans and limonoids.[3—9] In our ongoing investigation of biologically active compounds form the titled plant, we have isolated three new coumarins and a new glutaric acid derivative along with twenty-nine known compounds from the $CHCl_3$ soluble portion of the MeOH extract of the leaves of the *P. amurense*. In this paper, we describe the isolation, structure elucidation of four new compounds and DPPH radical scavenging activity of the compounds.

Results and Discussion

The methanolic extract of the leaves of *P. amurense* was partitioned with $CHCl_3$ and H_2O. The $CHCl_3$ soluble portion were purified by various column chromatographies and HPLC method using different solvent combinations to afford compounds **1**—**4** (Fig. 1) and 29 known compounds. The known compounds were identified as amurensin (**5**),[6] phellamurin (**6**),[6] kaempferol (**7**),[10] kaempferol-3-*O*-β-D-glucoside (**8**),[10] kaempferol-3-*O*-β-D-galactoside (**9**),[11] quercetin (**10**),[12] quercetin-3-*O*-β-D-glucoside (**11**),[10] quercetin-3-*O*-β-D-galactoside (**12**),[13] flavaprenin 7,4′-diglucoside (**13**),[14] hexandraside E (**14**),[15] umbelliferon (**15**),[16] esculetin (**16**),[17] phellodenol A (**17**),[7] scopoletin (**18**),[16] demethylsuberosin (**19**),[18] 7-hydroxy-6-(2-hydroxy-3-methyl-3-butenyl) coumarin (**20**),[19] scoparone (**21**),[20] xanthyletin (**22**),[21] skimmin (**23**),[22] *p*-hydroxybenzaldehyde (**24**),[23] methylparaben (**25**),[24] *p*-hydroxybenzoic acid (**26**),[23] anisaldehyde (**27**),[25] methyl *p*-anisate (**28**),[26] sodium (2*R*) 3-phenyllactate (**29**),[7] methyl caffeate (**30**),[6] ferulic acid (**31**),[27] lupenone (**32**),[28] and 2-acetyl-5-methoxyfuran (**33**)[29] by comparison of their spectra with literature data.

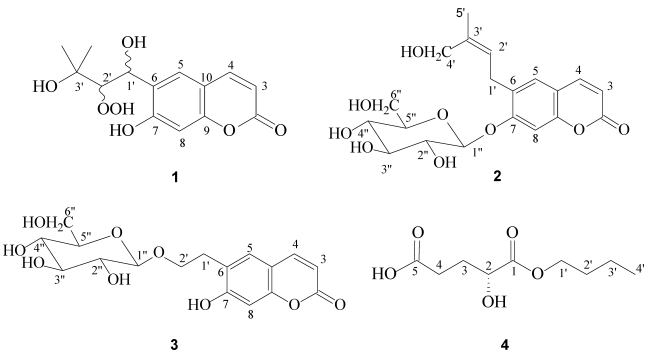

Fig. 1. Structures of New Compounds **1**, **2, 3** and **4**

Phellodenol-F (**1**) was obtained as yellow syrup with elemental composition of $C_{14}H_{16}O_7$ determined from its HR-EI-MS ($[M]^+$ *m/z* 296.0897). The UV absorption maxima at 225, 305 (sh), and 327 nm and the IR bands at 3400 and 1719 cm^{-1} consistent with the presence of hydroxyl and conjugated ester carbonyl groups. The ^{1}H-NMR spectrum revealed AB-type protons at δ 7.90 and 6.17 (J=9.4 Hz) signals indicative of the H-3 and H-4 protons of the coumarin nucleus. Two aromatic protons singlets at δ 7.61 and 6.70 were assignable for H-5 and H-8, respectively. The up-field shift of H-8 suggested that a free hydroxyl group substituted at C-7. This was supported by the bathochromic shift of 47 nm observed in the UV absorption maxima after addition of NaOAc.[7] The ^{1}H-NMR spectrum also revealed signals for two oxygenated methine signals (δ 5.44, 1H, br, 4.40, 1H, d, J=4.4 Hz) and two methyl groups (δ 1.28 and 1.27, each 3H, s). The ^{13}C-NMR spectrum also revealed corresponding signals at δ 99.0, 71.5, 70.3, 25.2 and 25.1. Since molecular formula contained 7 oxygen atoms and the above spectral analysis accounted for 6 oxygen atoms, the presence of peroxy group was inferred. The downfield chemical shifts of oxygenated methine proton H-2′ and the corresponding carbon at δ 4.40 and 99.0 suggested a hydroperoxide group at C-2′. The location of peroxyl group at C-2′ was also indirectly evidenced by ^3J correlations between δ_C 25.1, 25.2 (4′,5′-Me) and a very down field shifted oxymethine proton δ_H 4.40 (H-2′) in HMBC experiment (Fig. 2). These data confirmed the

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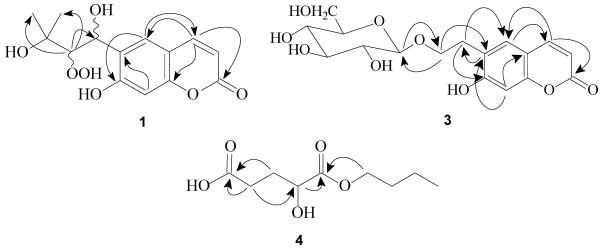

Fig. 2. HMBC Correlations of **1**, **3** and **4**

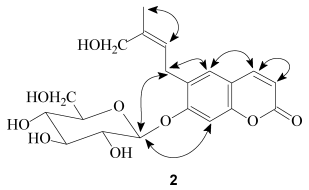

Fig. 3. NOESY Correlations of **2**

presence of 1,3-dihydroxy-2-hydroperoxy-3-methylbutyl side chain on the coumarin skeleton. Thus, the structure of phellodenol-F was elucidated as **1**.

Phellodenol-G (**2**) was isolated as white powder and exhibited a molecular ion peak in its HR-FAB-MS at *m/z* 408.1417 associated with the molecular formula $C_{20}H_{24}O_9$. The UV and IR spectral data and the ^{1}H-NMR signals at δ 7.86 (1H, d, J=9.6 Hz, H-4), 6.26 (1H, d, J=9.6 Hz, H-3), 7.39 (1H, s, H-5) and 7.11 (1H, s, H-8) were similar to those of **1**, indicating the presence of a 6,7-disubstituted coumarin basic skeleton in **2**. Additionally, characteristic signals for a 4-hydroxy-3-methyl-2-butenyl group at δ 5.42 (1H, t, J=7.4 Hz), 4.28 (1H, d, J=12.2 Hz), 4.13 (1H, d, J=12.2 Hz), 3.50 (2H, m) and 1.81 (3H, s) were also observed. The *Z* geometry for double bond was inferred by the NOE cross-peaks between H-2′ and H-5′ (Fig. 3). The presence of an anomeric proton at δ 5.05 (d, J=7.6 Hz) and the carbon signals at δ 100.7, 77.2, 76.9, 73.5, 70.0, and 61.2 suggested the presence of one glucosyl moiety with β-configuration. Unambiguous location of the glucose residue at C-7 and hence of the 4-hydroxy-3-methyl-2-butenyl side chain at C-6 was inferred by using NOESY experiment. The NOESY spectrum showed strong cross-peaks between H-1′, H-2′ and H-5, and also between H-1′ and H-1″, H-1″ and H-8. These spectroscopic data defined the structure of phellodenol-G as **2**.

Phellodenol-H (**3**), obtained as white powder, was determined to have molecular formula $C_{17}H_{20}O_9$ from its HR-FAB-MS. The UV absorptions at 309 (sh) and 331 nm, IR bands at 3395, 1714 and 1627 cm^{-1} were indicative of 7-hydroxy coumarin skeleton in **3**. The typical AB-type signals at δ 7.84 and 6.17 (each 1H, J=9.6 Hz) for H-4 and H-3, respectively and two singlet aromatic protons at δ 7.47 and 6.71 in the ^{1}H-NMR spectrum, indicated the basic coumarin skeleton as 7-hydroxy-6-substituted coumarin. In the high-field region, four mutually coupled signals at δ 2.98 (2H, t, J=7.1 Hz), 3.82 (1H, dt, J=9.6, 7.1 Hz) and 4.11 (1H, dt, J=9.6, 7.1 Hz) were indicative of a 2-oxygenate ethyl group in **3**. An anomeric proton doublet at δ 4.32 with coupling constant of J=8.0 Hz and the carbon signals at δ 104.5, 78.1, 78.0, 75.1, 71.6, and 62.7 suggested the presence of β-glucopyranosyl unit. From the HMBC studies (Fig. 2), glucosyl residue in **3** was found to be linked to C-2′ as anomeric proton had ^3J correlation with C-2′. Besides, the ^2J, ^3J correlations of H-1′ with C-6, C-5, and C-7 and ^3J correlation of H-2′ with C-6 in the HMBC spectrum inferred that the 2-glucosyloxyethyl group was connected to C-6 of coumarin nucleus. Thus the structure of phellodenol-H was established as **3**.

Phellodendric acid-A (**4**) was isolated as an optically active white powder and exhibited a molecular ion peak in its HR-EI-MS at *m/z* 204.0997 corresponding to the molecular formula $C_9H_{16}O_5$. The IR absorptions at 3389 and 1725 cm^{-1} accounted for the hydroxyl and carboxyl carbonyl groups, respectively. Presence of the mutually coupled oxygenated methine proton at δ_H 4.25 and two pairs of methylene protons at δ 2.10 (1H, m), 2.20 (2H, m), and 2.46 (1H, m) in the ^{1}H-NMR spectrum together with two carbonyls at δ_C 177.4 and 172.9, an oxygenated methine at δ_C 64.8, and two methylene carbons at δ_C 28.6 and 25.0 in ^{13}C-NMR established the 2-hydroxyglutaric acid unit in **4**. The remaining signals at δ_H 4.12 (2H, m), 1.62 (2H, m), 1.39 (2H, m) and 0.91 (3H, t, J=7.4 Hz) in ^{1}H-NMR and the signals at δ_C 55.4, 30.7, 19.0 and 13.3 in ^{13}C-NMR indicated an oxybutyl side chain. The attachment of this side chain was confirmed at C-1 due to the ^3J correlation between C-1 (δ_C 172.9) and H-1′ (δ_H 4.12) in the HMBC experiment (Fig. 2). The absolute configuration at C-2 was determined to be *R* on the basis of its positive optical rotation, which was compared with a series of *R*-(+)-dialkyl 2-hydroxyglutarate.[30]

Sixteen compounds of the isolates **4**, **5**, **6**, **8**, **9**, **10**, **11**, **12**, **15**, **19**, **20**, **23**, **26**, **31**, **32**, and **33** were examined for their antioxidant properties using the α,α-diphenyl-β-picrylhydrazyl free radical (DPPH) scavenging assay. The results were compared with α-tocopherol, which was commonly used in the food industry as antioxidant (IC_{50}, 25.2 μM). Among them, compounds **5**, **8**, **10**, **11**, **12**, **26** and **31** showed strong DPPH radical-scavenging activity with IC_{50} values of 91.0, 20.5, 15.5, 22.6, 19.0, 89.3 and 43.0 μM, respectively. These results implied that *P. amurense* might be able to afford protection against oxidative damage.

Experimental

General Procedures Melting points were recorded on Yanaco MP-S3 melting point apparatus without correction. The UV spectra were recorded on a Hitachi UV-3210 spectrophotometer. The IR spectra were measured on a Jasco IR Report-100 spectrophotometer as KBr disks. ^{1}H, ^{13}C, HMQC, HMBC, and NOESY NMR spectra were recorded on Bruker AC-200, AMX-400 and Varian-400 Unity Plus spectrometers, using tetramethylsilane (TMS) as internal standard; all chemical shifts are reported in parts per million (ppm, δ). Mass spectra (EI or FAB) were performed on a VG 70-250 S spectrometer. Optical rotations were recorded on a Jasco DIP-370 polarmeter.

Plant Material The leaves of *P. amurense* were collected in October 2001 from Shenyang, China, and authenticated by Prof. C. S. Kuoh. A voucher specimen of the plant (NCKU Wu 20011005) has been deposited at the herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Separation The air-dried and powdered leaves of *P. amurense* (3.0 kg) were extracted with hot methanol (51×6) and concentrated to give dark brown syrup. The syrup was partitioned between water and chloroform, and the resulted $CHCl_3$ soluble portion (120 g) was chromatographed over silica gel using a gradient elution of chloroform and methanol to afford 10 fractions. Fraction 4 was rechromatographed over silica gel using mixture of *n*-hexane and chloroform (5 : 1) as eluents, and purified by preparative TLC to yield **32** (275.3 mg). Fraction 6 on column chromatography with *n*-hexane and acetone (6 : 1) yielded **15** (32.6 mg). Fraction

7 was chromatographed over silica gel using a gradient elution of *n*-hexane and chloroform to afford **15** (78.6 mg) and **32** (55.3 mg). Fraction 8 was subjected to column chromatography over silica gel with *n*-hexane–diisopropyl ether (1 : 1) to give **22** (1.6 mg), **24** (3.4 mg), and **25** (2.2 mg). Similarly, fraction 9 was also chromatographed over silica gel with chloroform–diisopropyl ether (50 : 1) to give **15** (21.8 mg), **19** (74.1 mg), **20** (9.6 mg), and **25** (1.2 mg). The last fraction of chloroform layer was separated with silica gel column chromatography using chloroform and acetone in 29 : 1 ratio to afford **15** (14.6 mg). The water-soluble fraction (500 g) was chromatographed over Diaion HP-20 using water–methanol gradients, which yielded 7 fractions. Fraction 3 was chromatographed over Diaion HP-20 gel using gradient elution of water and methanol to afford **23** (18.3 mg). Silica gel column chromatography of fraction 4 by diisopropyl ether–methanol–water (3 : 1 : 0.1) resulted in **3** (3.6 mg), **10** (4.6 mg), and **12** (5.5 mg). Compound **5** (17.5 g) was recrystallized from fraction 5 in methanol. The mother liquid was allowed to recrystallization in acetone to get **6** (26.2 g). Then the remaining syrup was chromatographed over silica gel using mixture of chloroform, methanol and water as eluents (3 : 1 : 0.1) and successively purified by HPLC [Cosmosil 5C-18-AR-II waters (5 μm)] with methanol–water (35 : 65) to afford **1** (0.8 mg), **2** (7.4 mg), **4** (3.5 mg), **7** (5.2 mg), **8** (26.5 mg), **9** (253.2 mg), **10** (3.3 mg), **11** (1.5 g), **13** (8.6 mg), **14** (312.6 mg), **16** (4.1 mg), **17** (2.3 mg), **18** (3.3 mg), **21** (3.1 mg), **24** (2.8 mg), **26** (2.5 mg), **27** (1.7 mg), **28** (14.3 mg), **29** (2.6 mg), **30** (4.8 mg), **31** (23.5 mg), and **33** (1.5 mg). Fraction 6 was separated with silica gel column chromatography using diisopropyl ether–methanol–water in 3 : 1 : 0.1 ratio to afford **5** (23.6 mg) and **10** (15.9 mg).

Phellodenol-F (**1**): Yellow syrup, HR-EI-MS *m/z*: $[M]^+$ 296.0897 (Calcd for $C_{14}H_{16}O_7$: 296.0896). $[\alpha]_D^{25}$ +2.9° (c=0.027, MeOH). UV $\lambda_{max}^{(MeOH)}$ (log ε) nm: 225 (3.74), 305 (sh) (3.68), 327 (4.00). UV $\lambda_{max}^{(MeOH+NaOAc)}$ nm: 306 (sh), 330, 374. IR (KBr) cm^{-1}: 3400 (OH), 2920, 1719 (C=O), 1623, 1565, 1275, 1037, 998. EI-MS *m/z* (%): 296 (M^+, 27), 268 (15), 245 (23), 226 (40), 135 (22), 105 (23), 57 (100). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 1.27 (3H, s, CH_3), 1.28 (3H, s, CH_3), 4.40 (1H, d, J=4.4 Hz, H-2′), 5.44 (1H, br s, H-1′), 6.17 (1H, d, J=9.4 Hz, H-3), 6.70 (1H, s, H-8), 7.61 (1H, s, H-5), 7.90 (1H, d, J=9.4 Hz, H-4). ^{13}C-NMR (100 MHz, acetone-d_6) δ: 25.1 (3′-CH_3), 25.2 (3′-CH_3), 70.3 (C-3′), 71.5 (C-1′), 97.6 (C-8), 99.0 (C-2′), 112.1 (C-3), 113.1 (C-10), 125.3 (C-5), 128.6 (C-6), 144.4 (C-4), 156.9 (C-9), 160.4 (C-2), 163.9 (C-7).

Phellodenol-G (**2**): White powder, mp 129—131 °C. HR-FAB-MS *m/z*: $[M+1]^+$ 408.1417 (Calcd for $C_{20}H_{24}O_9$: 408.1420). $[\alpha]_D^{25}$ −59.9° (c=0.004, MeOH). UV $\lambda_{max}^{(MeOH)}$ (log ε) nm: 221 (4.03), 294 (3.65), 325 (3.83). IR (KBr) cm^{-1}: 3420 (OH), 2890, 1722 (C=O), 1625, 1560, 1507, 1082, 992. ^{1}H-NMR (400 MHz, acetone-d_6) δ: 1.81 (3H, s, 3′-CH_3), 3.41—3.59 (6H, m, H-1′, 2″, 3″, 4″, 5″), 3.70 (1H, dd, J=12.2, 6.0 Hz, H-6″), 3.91 (1H, dd, J=12.2, 2.4 Hz, H-6″), 4.13 (1H, d, J=12.2 Hz, CH_2OH), 4.28 (1H, d, J=12.2 Hz, CH_2OH), 5.05 (1H, d, J=7.6 Hz, H-1″), 5.42 (1H, t, J=7.4 Hz, H-2′), 6.26 (1H, d, J=9.6 Hz, H-3), 7.11 (1H, s, H-8), 7.39 (1H, s, H-5), 7.86 (1H, d, J=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, acetone-d_6) δ: 20.3 (3′-CH_3), 27.4 (C-1′), 60.2 (C-4′), 61.2 (C-6″), 70.0 (C-4″), 73.5 (C-2″), 76.9 (C-3″), 77.2 (C-5″), 100.7 (C-1″), 102.3 (C-8), 112.9 (C-3), 113.7 (C-10), 124.9 (C-2′), 128.0 (C-6), 128.3 (C-5), 135.9 (C-3′), 144.5 (C-4), 154.1 (C-9), 158.6 (C-7), 162.2 (C-2).

Phellodenol-H (**3**): White powder, mp 150—152 °C. HR-FAB-MS *m/z*: $[M+1]^+$ 368.1102 (Calcd for $C_{17}H_{20}O_9$: 368.1107). UV $\lambda_{max}^{(MeOH)}$ (log ε) nm: 222 (4.01), 309 (sh) (3.86), 331 (4.08). UV $\lambda_{max}^{(MeOH+NaOAc)}$ nm: 344, 379. IR (KBr) cm^{-1}: 3395 (OH), 2931, 1714 (C=O), 1627, 1398, 1267, 1142, 1087, 1038. ^{1}H-NMR (400 MHz, CD_3OD) δ: 2.98 (2H, t, J=7.1 Hz, H-1′), 3.18 (1H, t, J=8.0 Hz, H-2″), 3.23—3.40 (3H, m, H-3″, 4″, 5″), 3.67 (1H, dd, J=11.6, 4.8 Hz, H-6″), 3.82 (1H, dt, J=9.6, 7.1 Hz, H-2′), 3.87 (1H, dd, J=11.6, 4.8 Hz, H-6′), 4.11 (1H, dt, J=9.6, 7.1 Hz, H-2′), 4.32 (1H, d, J=8.0 Hz, H-1″), 4.32 (1H, s, OH), 6.17 (1H, d, J=9.6 Hz, H-3), 6.71 (1H, s, H-8), 7.47 (1H, s, H-5), 7.84 (1H, d, J=9.6 Hz, H-4). ^{13}C-NMR (100 MHz, CD_3OD) δ: 31.0 (C-1′), 62.7 (C-6″), 70.0 (C-2′), 71.6 (C-4″), 75.1 (C-2″), 78.0 (C-5″), 78.1 (C-3″), 102.7 (C-8), 104.5 (C-1″), 112.2 (C-3), 113.0 (C-10), 125.0 (C-6), 131.3 (C-5), 146.3 (C-4), 155.8 (C-9), 161.1 (C-7), 164.0 (C-2).

Phellodendric Acid-A (**4**): White powder, mp 228—231 °C. HR-EI-MS *m/z*: $[M]^+$ 204.0997 (Calcd for $C_9H_{16}O_5$: 204.0998). $[\alpha]_D^{25}$ +4.5° (c=0.03, MeOH). IR (KBr) cm^{-1}: 3389 (OH), 2964, 1725 (C=O), 1245, 1109. EI-MS *m/z* (%): 204 (M^+, 3), 129 (8), 84 (100), 56 (13). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 0.91 (3H, t, J=7.4 Hz, H-4′), 1.39 (2H, m, H-3′), 1.62 (2H, m, H-2′), 2.10 (1H, m, H-4), 2.20 (2H, m, H-3), 2.46 (1H, m, H-4), 4.12 (2H, m, H-1′), 4.25 (1H, m, H-2), 7.06 (1H, br, OH); ^{13}C-NMR (100 MHz, acetone-d_6) δ: 13.3 (C-4′), 19.0 (C-3′), 25.0 (C-4), 28.6 (C-3), 30.7 (C-2′), 55.4 (C-1′), 64.8 (C-2), 172.9 (C-1), 177.4 (C-5).

Free Radical-Scavenging Activity Assay The effect of isolated compounds on the scavenging of DPPH radical was estimated according to the method of Yamaguchi *et al.*[31] with minor modifications. A sample was dissolved in 0.1 ml DMSO and then added to 0.1 ml of 0.1 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand for 30 min at room temperature in the dark. The absorbance at 517 nm by DPPH was measured by a μ Quant universal microplate spectrophotometer. α-Toc (Sigma Chemical Co.) was used as a standard agent. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{scavenging effect (\%)} = [1 - (\text{absorbance of sample at 517 nm} / \text{absorbance of control at 517 nm})] \times 100$$

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