Constituents of Leaves of *Phellodendron japonicum* MAXIM. and Their Antioxidant Activity

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Three new flavonoid derivatives, 6”-O-acetyl amurensin (1), 6”-O-acetyl phellamurin (3) and (2R)-phellodensin-F (5), together with thirty known compounds have been isolated from the leaves of *Phellodendron japonicum* MAXIM. Their structures were established by means of spectroscopic analysis, including extensive 2D NMR and Mass spectra. The known compounds were identified by comparison with published physical and spectral data. The isolated compounds were screened for their *in vitro* antioxidant activity through DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay. Compounds quercetin and phellodenin-A demonstrated significant radical scavenging activity.

Key words *Phellodendron japonicum*; flavonoid; antioxidant activity; DPPH assay

*Phellodendron* is a small genus of aromatic deciduous trees of East Asia often having thick corky bark. This bark has found application in Chinese traditional medicine for various diseases like meningitis, bacillary dysentery, pneumonia, tuberculosis, and liver cirrhosis.1–3 Previous phytochemical work on the members of *Phellodendron* has reported the isolation of berberine and aporphine type alkaloids, flavonoids, coumarins, lignans and limonoids.3,4) During the course of our investigation on the bioactive chemical components of *Phellodendron* species, we have focused on *Phellodendron japonicum* MAXIM, which is a deciduous tree found widely in Honshu, Japan.4a Few reports encountered on the isolation of flavonoids as major constituents from this plant.5 The current study describes the isolation and structure determination of three new flavone derivatives as well as thirty known compounds from the methanol extract of the leaves of title plant and their DPPH radical scavenging activity.

Results and Discussion

6”-O-Acetyl amurensin (1) was isolated as yellow powder with elemental composition C_{29}H_{30}O_{12} from its HR-FAB-MS ([M+H] m/z 559.1819). The IR absorption bands at 3361, 1719 and 1646 cm<sup>-1</sup> indicated the presence of hydroxyl, ester and conjugated hydrocarbon groups, respectively. The UV absorptions at 271, 327 and 373 nm were typical of a flavonol skeleton.6) The 1H-NMR of 1 revealed an A<sub>2</sub>B<sub>2</sub> system of proton signals at δ 8.18 (2H, d, J=8.8 Hz, H-2’, 6’) and 7.03 (2H, d, J=8.8 Hz, H-3’, 5’), a D<sub>2</sub>O exchangeable hydroxyl signal at δ 12.14 (1H, s, 5-OH). A singlet at δ 6.67 was ascribed to H-6 of A-ring as it has HMQC with a carbon at δ<sub>c</sub> 159.3 (C-5) and 109.0 (C-8), respectively. Also, characteristic prenyl proton signals were observed at δ 3.65 (2H, m, H-1’), 5.28 (1H, brt, J=6.8 Hz, H-2’), 1.65 (3H, s, CH<sub>3</sub>-4’), and 1.82 (3H, s, CH<sub>3</sub>-5’). The position of the prenyl group was determined to C-8 based on the HMBC correlations between H-1’ and C-7 (δ<sub>c</sub> 160.8), C-8. The presence of an anomeric proton at δ 5.14 (d, J=7.6 Hz) and the carbon signals at δ 101.0, 77.3, 74.5, 74.0, 70.7, and 63.6 suggested the presence of one glucosyl moiety with β-configuration. From NOESY studies, glucose residue in 1 was found to be linked to C-7 as NOE of anomeric proton with H-6 were observed. There were very similar to those amurensin (2).7) An acetyl methyl singlet at δ 2.04 (3H, s) in 1H-NMR spectrum together with the 13C-NMR signals at δ 170.3 and 20.0 inferred that 1 was an acetyl derivative of amurensin. The location of the acetyl group was determined to C-6” due to the 3J correlation between H-6” (δ<sub>H</sub> 4.21) and acetyl-carbonyl (δ<sub>C</sub> 170.3) in the HMBC experiment. This was also supported by the down field shifts of C-6” to δ<sub>c</sub> 63.6 and up field shift of C-5” to δ<sub>c</sub> 74.5. Thus, the structure of 1 was elucidated as 6”-O-acetyl amurensin.

6”-O-Acetyl phellamurin (3) was obtained with HPLC as white powder. The 1H- and 13C-NMR spectra of 3 showed signals due to H-2 [δ 5.09 (d, J=12.0 Hz), H-3 [δ 4.64 (dd, J=12.0, 4.4 Hz)] and 3-OH [δ 4.71 (d, J=4.4 Hz, D<sub>2</sub>O exchangeable)] of a flavanone suggested that 3 was a 2,3-trans-dihydroflavonol derivative.6) A broad singlet at δ 11.64 exchangeable with D<sub>2</sub>O was assigned to a chelated hydroxyl group at C-5. A set of A<sub>2</sub>B<sub>2</sub> doublets at δ 7.43 (2H, J=8.8 Hz) and 6.91 (2H, J=8.8 Hz) was assigned to 2’, 6’- and 3’, 5’-protons of B-ring. A singlet at δ 6.36 was ascribed to H-6 of A-ring as it correlated to carbon at δ 96.4 in HMOC spectrum. Also, characteristic prenyl proton signals were observed at δ 3.13 (1H, dd, J=14.4, 7.6 Hz, H-1”), 3.31 (1H, m, H-1”), 5.16 (1H, m, H-2”), 1.58 (3H, s, CH<sub>3</sub>-4”), and 1.61 (3H, s, CH<sub>3</sub>-5”). The position of the prenyl group was determined at C-8, since H-1” showed HMBC correlation with C-8. The presence of an anomeric proton at δ 5.08 (d, J=7.6 Hz) and the carbon signals at δ 101.2, 77.9, 75.1, 74.5, 71.2, and 64.2 suggested the presence of one glucosyl moiety with β-configuration. From ROESY studies, glucose residue in 3 was found to be linked to C-7 as of anomeric proton had NOE with H-6. An acetyl methyl singlet at δ 1.93

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(3H, s) in 1H-NMR spectrum together with the 13C-NMR signals at δ 170.9 and 20.6 were also observed. These data and coupling patterns were very close to those of phellamurin (4)7 except for the acetyl signals. Thus, compound 2 was postulated as being an acetyl derivative of phellamurin. The location of the acetyl group was determined on C-6° due to the J correlation between H-6° (δC 4.21) and an acetyl-carbonyl carbon (δC 170.9) in the HMBC experiment. The attachment of an acetyl group to C-6° was also evidenced by the down field shifts of C-6° to δC 64.2 and up field shift of neighbouring carbon C-5° to δC 75.1 in 3, in comparison to those of 4. Thus the structure of 3 was assigned to 6-O-acetyl phellamurin.

(2R)-Phellodensin-F (5) was isolated as white platelets with elemental composition C26H30O10 from its HR-FAB-MS (M+1)+ m/z 503.1919. The IR absorption bands at 3380, 2928, 1635 cm−1 were also observed. Thus the structure of 5 was assigned to 6-O-acetyl phellamurin.

The nine compounds of the isolates 2, 4, 6, 16, 17, 18, 20, 22, and 26 were examined for their antioxidant properties using the α,α-diphenyl-β-pirclyhydrazyl free radical (DPPH) scavenging assay. The results were compared with α-tocopherol, which was commonly used in the food industry as antioxidant (IC50 27.0 μM). Among them, compounds 6 and 26 showed strong DPPH radical-scavenging activity with an IC50 values of 17.5 and 21.5 μM, respectively. Compounds 2, 18 and 20 showed moderate scavenging activity with an IC50 values of 94.0, 87.6, 59.4 μM, respectively. These results implied that P. japonicum 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 recorded on a Jasco DIP-370 polarimeter. The 1H- and 13C-NMR spectra were recorded on a Hitachi 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). Optical rotations were recorded on a Jasco DIP-370 polarimeter. Spectral rotations were recorded on a Jasco DIP-370 polarimeter. CD spectra were recorded on a Jasco J-720 spectropolarimeter.

Plant Material The leaves of P. japonicum MAXIM. were collected in August 2000 from Japan, and authenticated by Prof. C. S. Kuoh (Department of Life Science, National Cheng Kung University, Tainan, Taiwan). A voucher specimen of the plant (NCKU Wu 20000809) has been deposited at the herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Separation The air-dried and powdered leaves of P. japonicum (2.3 kg) were extracted with hot methanol (51×6) and concentrated to give dark brown syrup and colorless crystals 2 (23.6 g). The syrup was allowed to recrystallization in acetone to get 4 (15.7 g). Then the remaining syrup was partitioned between water and chloroform and the resulted chloroform solubles (90 g) were chromatographed over silica gel using a gradient of chloroform and methanol to afford 12 fractions. Fraction...
3 was rechromatographed over silica gel using mixture of n-hexane and ethyl acetate as eluents, and purified by preparative TLC to yield 27 (2.7 mg) and 29 (4.5 mg). Fraction 4 on column chromatography with n-hexane and ethyl acetate (9:1) yielded 28 (3.1 mg) and 30 (1.2 mg). Fraction 5 was chromatographed over silica gel using a gradient of chloroform and diisopropyl ether to afford 9 (1.3 mg), 17 (4.3 mg), and 28 (6.7 mg). Fraction 6 was subjected to column chromatography over silica gel with chloroform–diisopropyl ether (12:1) to give 9 (3.1 mg), 14 (10.0 mg), 16 (7.2 mg), 17 (2.5 mg), 21 (1.8 mg), and 15 (0.2 mg). Similarly, fraction 8 was also chromatographed over silica gel with chloroform–acetonitrile (9:1) to give 10 (3.1 mg), 13 (6.0 mg), and 18 (13.2 mg). Silica gel column chromatography of fraction 9 by chloroform–diisopropyl ether (6:1) resulted in 32 (2.7 mg), 20 (8.4 mg), 31 (4.1 mg). The last fraction of chloroform layer was separated with silica gel column chromatography using diisopropyl ether and methanol in 9:1 ratio to afford 2 (5.5 mg), 4 (7.2 mg), and 18 (8.8 mg). The water-soluble fraction (32.0 mg) was chromatographed over Diaion HP-20 using water–methanol gradients, which yielded 10 fractions. Fraction 3 was column chromatographed with Diaion HP-20 gel using gradients of water and methanol to afford 20 (6.7 mg) and 22 (28.0 mg). Fraction 4 on column chromatography over Diaion HP-20 with water–methanol gradients afforded 11 (5.6 mg), 13 (3.9 mg), 25 (4.2 mg), 33 (6.7 mg), and 26 (205.2 mg). Fraction 8 was chromatographed over silica gel using mixture of ethyl acetate, methanol and water as eluents (95:4:1) and successively purified by HPLC [Cosmosil 5C-18-AR-II waters (5 μm)] with methanol–water (35:65) to afford 6 (2.6 mg), 2 (1.6 g), 7 (0.7 mg), 8 (0.7 mg), 4 (3.2 mg), 16 (16.7 mg), 19 (6.4 mg), 21 (3.8 mg), 3 (0.9 mg), 1 (1.8 mg), 5 (0.2 mg), 23 (3.4 mg), and 24 (2.5 mg).

6"-O-Acetyl Amurensic (1): Yellow powder, mp: 235—237°C. HR-FAB-MS m/z: 559.1819 [M+H]+ [Caled for C36H38O14: 559.1816]. [α]23D = −91.3° (c=0.05, MeOH). UV λmax (MeOH) (log ε) nm: 271 (4.46), 327 (4.20), 373 (4.36). IR (KBr) cm−1: 3361 (OH), 2923, 1719 (C=O), 1646 (C=O), 1599, 1259, 1081. FAB-MS m/z (%): 559 [M+H]+ (17), 355 (24), 299 (16), 185 (100), 149 (20). 1H-NMR (400 MHz, acetone-d6): δ: 1.65 (3H, s, 4'-CH3), 1.82 (3H, s, 5'-CH3), 2.04 (3H, s, 8'-CH3), 3.45 (1H, m, H-4"'), 3.52—3.74 (4H, m, H-1", 2", 3", 4"'), 3.86 (1H, td, J=8.6, 2.0 Hz, H-5"'), 4.21 (1H, m, H-6"), 4.46 (1H, d, J=9.6 Hz, H-6"'), 4.51 (1H, d, J=4.4 Hz, OH), 4.55 (1H, brs, OH), 4.65 (1H, brs, OH), 5.14 (1H, d, J=7.6 Hz, H-1"), 5.28 (1H, brt, J=6.8 Hz, H-2"'), 6.67 (1H, s, H-6), 7.03 (2H, d, J=8.8 Hz, H-3", 5''), 8.07 (1H, br, 3-OH), 8.18 (2H, d, J=8.8 Hz, H-2", 6''), 9.05 (1H, brs, 4'-OH), 12.14 (1H, s, 5'-OH). 13C-NMR (100 MHz, acetone-d6): δ: 17.5 (C-5'), 20.0 (C-8''), 21.7 (C-1"'), 25.1 (C-4"'), 63.6 (C-6"), 70.7 (C-7"'), 74.0 (C-5"'), 74.5 (C-5"), 77.3 (C-3"'), 98.1 (C-6), 101.0 (C-1"'), 105.1 (C-10), 109.0 (C-8), 115.7 (C-3", 5''), 122.9 (C-2"'), 129.9 (C-2"), 131.4 (C-3"), 147.8 (C-2"), 153.5 (C-9"), 159.6 (C-4"'), 160.8 (C-7"'), 170.3 (C-6"'), 176.4 (C-4').

6"-O-Acetyl Phellamurin (3): White powder. HR-FAB-MS m/z: 561.1969 [M+H]+ [Caled for C36H38O14: 561.1972]. 1H-NMR (400 MHz, acetone-d6): δ: 1.58 (3H, s, 4'-CH3), 1.61 (3H, s, 5'-CH3), 1.93 (3H, s, 8'-CH3), 3.13 (1H, dd), J=14.4, 7.6 Hz, H-1"'), 3.31 (1H, m, H-1"'), 3.43 (1H, m, H-4"'), 3.55—3.58 (2H, m, H-2", 3"'), 3.84 (1H, m, H-5"'), 4.21 (1H, dd, J=11.6, 7.2 Hz, H-6"'), 4.44 (1H, m, H-6"'), 4.51 (1H, m, OH), 4.60 (1H, m, OH), 4.64 (1H, dd, J=12.0, 4.4 Hz, H-3'), 4.71 (1H, d, J=4.4 Hz, 3-OH), 5.08 (1H, d, J=7.6 Hz, H-1"'), 5.09 (1H, d, J=12.0 Hz, H-2'), 5.16 (1H, m, H-2"'),
acetone–methanol solution at room temperature in the dark. The absorbance at 517 nm by DPPH was measured by a method of Yamaguchi et al. (Sigma Chemical Co.) was used as a standard agent. The capability to scavenge the DPPH radical was estimated according to the method of Yamaguchi et al. 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 $\mu$ Quant universal microplate spectrophotometer. $\alpha$-Toc (Sigma Chemical Co.) was used as a standard agent. The capability to scavenge the DPPH radical was calculated using the following equation:

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