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CHEMICAL CONSTITUENTS WITH ANTIOXIDANT ACTIVITY FROM THE PERICARPS OF *Juglans sigillata*

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Juglans sigillata (Juglandaceae), a fast growing hardwood species, is indigenous in valleys and on mountain slopes of Guizhou, Sichuan, Tibet, and Yunnan provinces of southwest China [1]. Its fresh pericarps have long been used in traditional medicines for the treatment of various diseases, such as esophageal, gastric, cardiac, and lung cancer [2]. However, the chemical constituents and biological activity of this species has not been reported to date. In this phytochemical investigation of *J. sigillata*, five chemical constituents were isolated from its fresh pericarps, and their structures were elucidated as gallic acid (**1**) [3], ellagic acid (**2**) [4], (+)-catechin(4$\alpha\rightarrow$8)-(+)-catechin (**3**) [5], 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (**4**) [6], and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**5**) [7]. Compounds **1–5** were identified from *J. sigillata* for the first time. By means of physiochemical techniques and spectroscopic methods (NMR, MS, 2D NMR), the first complete assignments of the ^{1}H and ^{13}C NMR chemical shifts for 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**5**) were achieved here.

Plant Material. The fresh pericarps of *J. sigillata* were collected in August 2009 from Santai of Sichuan province, China. Taxonomic identification was done by Professor Dan Wang, Institute of Chemical Industry of Forest Products, CAF, China. A voucher specimen (No. 200908001) has been deposited at the Herbarium of Tianjin Key Laboratory of Pulp & Paper, College of Material Science and Chemical Engineering, Tianjin University of Science and Technology, China.

Equipment. NMR spectra were recorded in MeOH-d_4 with TMS as an internal standard using a Bruker Avance DPX 400 spectrometer at the operating frequency of 400 MHz (^{1}H) and 100 MHz (^{13}C) in Central Laboratory of Kangwon National University, Korea. EI- and FAB-MS spectroscopy was performed with a micromass autospec M363 spectrometer, and MALDI-TOF-MS spectroscopy was done on a Model Voyager-DE STR spectrometer. Eluents were collected with a fraction collector (Gilson FC 204). TLC analyses were performed on DC-Plastikfolien Cellulose F (Merck) plates and developed with *t*-BuOH–AcOH–H_2O (3:1:1, v/v, solvent A) and AcOH–H_2O (3:47, v/v, solvent B). Visualization was by UV light (254 and 365 nm) or by spraying with vanillin–AcOH–EtOH (60:0.15:6, w/v/v) or 1% ethanolic $FeCl_3$ solution followed by heating.

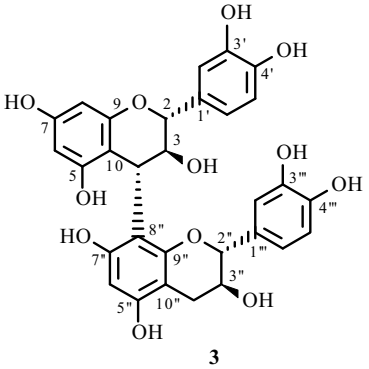

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TABLE 1. NMR Data of Compounds **4** and **5** (MeOH-d_4, δ, J/Hz)

C atom	**4**		**5**	
	δ_H	δ_C	δ_H	δ_C
		Glucose		
1	6.10 (1H, d, J = 8.20)	94.33	6.25 (1H, d, J = 8.37)	94.24
2	5.57 (1H, t, J = 8.99)	72.79	5.91 (1H, t, J = 9.65)	72.61
3	5.51 (1H, t, J = 8.28)	76.88	5.61 (1H, t, J = 9.65)	74.53
4	3.98 (1H, m)	70.05	4.16 (1H, m)	70.21
5	4.40 (1H, m)	77.02	4.41 (1H, m)	74.83
6	4.58 (1H, m)/4.70 (1H, m)	64.42	4.70 (1H, m)/4.72 (1H, m)	63.54
		Galloyl-A		
1′	–	120.25	–	120.13
2′, 6′	6.93 (2H, s)	110.67	6.91 (2H, s)	110.75
3′, 5′	–	146.77	–	146.70
4′	–	140.40	–	140.55
7′	–	166.77	–	166.65
		Galloyl-B		
1"	–	120.80	–	120.65
2″, 6"	7.02 (2H, s)	110.75	6.99 (2H, s)	110.83
3″, 5"	–	146.82	–	146.85
4"	–	140.43	–	140.78
7″	–	167.65	–	167.45
		Galloyl-C		
1‴	–	121.38	–	120.77
2‴, 6‴	7.03 (2H, s)	110.82	7.06 (2H, s)	110.89
3‴, 5‴	–	146.91	–	146.88
4‴	–	140.65	–	141.19
7‴	–	168.18	–	167.73
		Galloyl-D		
1⁗	–	121.63	–	121.45
2⁗, 6⁗	7.12 (2H, s)	110.85	7.12 (2H, s)	111.04
3⁗, 5⁗	–	146.94	–	146.97
4⁗	–	141.12	–	141.11
7⁗	–	168.64	–	168.36
		Galloyl-E		
1′′′′′			–	120.61
2′′′′′, 6′′′′′			6.96 (2H, s)	110.80
3′′′′′, 5′′′′′			–	146.80
4′′′′′			–	140.73
7′′′′′			–	167.35

Extraction and Isolation. The air-dried and finely powered pericarps (5.0 kg) were extracted with 95% EtOH solution for more than three days at room temperature. After combination, filtration, concentration, and removal of EtOH by a vacuum rotary evaporator, the aqueous residue was successively fractionated with various solvents and subsequently freeze-dried to give fractions soluble in *n*-hexane (14.0 g, yield 0.28%), CH_2Cl_2 (22.4 g, yield 0.45%), EtOAc (48.0 g, yield 0.96%), *n*-BuOH (32.8 g, yield 0.66%), and H_2O (568.0 g, yield 11.36%). Part of the resulting *n*-BuOH soluble fraction powder (25.5 g) was loaded to a Sephadex LH-20 column, eluting with MeOH–H_2O (4:1, v/v) mixtures to furnish five fractions: B_1 (6.2 g), B_2 (2.7 g), B_3 (5.8 g), B_4 (7.8 g), and B_5 (2.9 g), which were guided and monitored by TLC detection. Fraction B_1 was reloaded on a Sephadex LH-20 column with MeOH–H_2O (2:1, v/v) mixtures used as eluents for further purification to yield five subfractions (B_{11}–B_{15}), and the third subfraction (B_{13}, 2.8 g) was eluted stepwise with MeOH–H_2O (1:1 and 1:4, v/v) to give

yellowish compounds **1** (129 mg) and **3** (74 mg). Fraction B_4 was also loaded on a Sephadex LH-20 column, eluting with MeOH–H_2O (2:1, v/v) mixtures for further separation to give four subfractions (B_{41}–B_{44}). Subfraction B_{43} (4.3 g) was reloaded on a Sephadex LH-20 column likewise with MeOH–H_2O (1:4, v/v) and EtOH–hexane (1:2 and 1:4) as eluents to yield compounds **2** (63 mg) and **5** (208 mg). Fraction B_3 was further separated using Sephadex LH-20 column chromatography and preparative TLC with EtOH–hexane (1:1) and MeOH–H_2O (1:1, v/v) as solvents to give compound **4** (112 mg).

Gallic Acid (1). Yellowish amorphous powder; vanillin–HCl–EtOH test: positive (dark red), $FeCl_3$ test: positive (dark brown); R_f 0.54 (solvent A) and 0.42 (solvent B). EI-MS *m/z* $[M]^+$ at 170, suggesting molecular weight 170 and calculated for $C_7H_6O_5$. ^{1}H NMR (400 MHz, MeOH-d_4, δ): 7.09 (2H, s, H-2,6). ^{13}C NMR (100 MHz, MeOH-d_4, δ): 109.85 (C-2,6), 122.36 (C-1), 138.42 (C-4), 145.69 (C-3,5), 170.67 (C-7).

Ellagic Acid (2). Yellowish amorphous powder; vanillin–HCl–EtOH test: positive (dark red), $FeCl_3$ test: positive (dark brown); R_f 0.12 (solvent A) and 0.02 (solvent B). Positive FAB-MS *m/z* $[M + H]^+$ at 303, indicating molecular weight 302 and calculated for $C_{14}H_6O_8$. ^{1}H NMR (400 MHz, MeOH-d_4, δ): 7.47 (2H, s, H-5,5′). ^{13}C NMR (100 MHz, MeOH-d_4, δ): 107.53 (C-1,1′), 110.11 (C-5,5′), 112.22 (C-6,6′), 136.26 (C-2,2′), 139.48 (C-3,3′), 148.00 (C-4,4′), 159.05 (C-7,7′).

(+)-Catechin(4α→8)-(+)-catechin (3). Yellowish amorphous powder; vanillin–HCl–EtOH test: positive (dark red), $FeCl_3$ test: positive (dark brown); R_f 0.38 (solvent A) and 0.50 (solvent B). FAB-MS: *m/z* $[M + H]^+$ at 579, suggesting molecular weight 578 and calculated for $C_{30}H_{26}O_{12}$. ^{1}H NMR (400 MHz, MeOH-d_4, δ, J/Hz): 2.48 (1H, dd, J = 8.0, 16.3, H-4ax″), 2.77 (1H, dd, J = 5.7, 16.3, H-4eq″), 4.07 (1H, m, H-3″), 4.24 (1H, d, J = 9.4, H-4), 4.34 (1H, t, J = 9.4, 7.8, 8.6, H-3), 4.41 (1H, d, J = 7.8, H-2″), 4.74 (1H, d, J = 7.3, H-2), 5.78 (1H, d, J = 2.3, H-6), 5.89 (1H, d, J = 2.4, H-8), 6.01 (1H, s, H-6″), 6.24–6.99 (6H, br.m, H-2′,2‴,5′,5‴,6′,6‴). ^{13}C NMR (100 MHz, MeOH-d_4, δ): 28.74 (C-4″), 38.85 (C-4), 68.83 (C-3″), 73.97 (C-3), 82.71 (C-2″), 84.21 (C-2), 96.33 (C-8), 97.14 (C-6), 97.83 (C-6″), 100.76 (C-10″), 107.47 (C-10), 108.46 (C-8″), 115.47 (C-2′), 116.21 (C-5′), 116.43 (C-5″), 120.44 (C-6″), 120.90 (C-6′), 132.12 (C-1″), 132.67 (C-1), 145.74 (C-4″), 146.34 (C-3″), 146.34 (C-4′), 146.39 (C-3′), 155.14 (C-5″), 156.12 (C-7″), 157.38 (C-7′), 157.39 (C-9″), 158.90 (C-9′).

1,2,4,6-Tetra-*O*-galloyl-β-D-glucose (4). Yellowish amorphous powder; vanillin–HCl–EtOH test: positive (weak yellow), $FeCl_3$ test: positive (dark brown); R_f 0.48 (solvent A) and 0.51 (solvent B). MALDI-TOF-MS: *m/z* $[M + Na]^+$ at 811 and $[M + K]^+$ at 827, indicating molecular weight 788 and calculated for $C_{34}H_{28}O_{22}$. ^{1}H and ^{13}C NMR data, see Table 1.

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (5). Yellowish amorphous powder; vanillin–HCl–EtOH test: positive (weak yellow), $FeCl_3$ test: positive (dark brown); R_f 0.22 (solvent A) and 0 (solvent B). MALDI-TOF-MS: *m/z* $[M + Na]^+$ at 963 and $[M + K]^+$ at 979, suggesting molecular weight 940 and calculated for $C_{41}H_{32}O_{26}$; ^{1}H and ^{13}C NMR data, see Table 1.

Antioxidant Activity. The antioxidant activity of compounds **1–5** were evaluated by the DPPH free-radical-scavenging assay introduced by Blois, with slight modification [8, 9]. MeOH solutions (4 mL) of samples at different concentrations (2–40 μg/mL) were added to a solution of DPPH (1.5×10^{-4} M, 1 mL) in MeOH. After mixing gently and letting stand at room temperature for 30 min, the optical density was measured at 517 nm with a UV-visible spectrophotometer (Libra S32, Biochrom Ltd.). The results were calculated by taking the mean of all triplicate values. IC_{50} values were obtained through extrapolation from the concentration of sample necessary to scavenge 50% of the DPPH free radicals. Results showed that compounds **1** (IC_{50} 6.47 μM), **2** (IC_{50} 6.71 μM), **3** (IC_{50} 6.60 μM), **4** (IC_{50} 6.57 μM), and **5** (IC_{50} 6.53 μM) exhibited significant antioxidant potential that was comparable with α-tocopherol (IC_{50} 6.70 μM), which was used as positive control. The facts above reveal that fresh pericarps of *J. sigillata* have significant antioxidant potential and can be a promising alternative to more toxic synthetic antioxidants in food, pharmaceutical, and cosmetic products [10, 11].

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