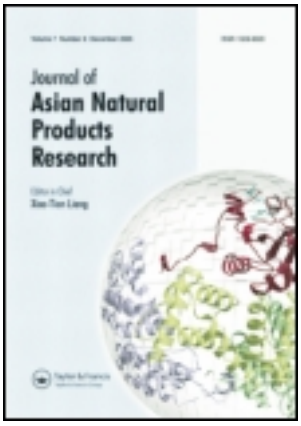

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Naphthoquinones from the root barks of *Juglans cathayensis* Dode

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Naphthoquinones from the root barks of *Juglans cathayensis* Dode

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Phytochemical investigation of *Juglans cathayensis* Dode root barks led to the isolation of two novel nitrogen-containing naphthoquinones named juglonbutine (**1**) and binaphthine (**2**). Their structures were elucidated by the extensive analysis of the spectroscopic data. In addition, six known naphthoquinones (**3**–**8**) were also isolated from the same material for the first time.

Keywords: *Juglans cathayensis*; nitrogen-containing naphthoquinones; juglonbutine; binaphthine

1. Introduction

Juglans cathayensis Dode (Juglandaceae), an ancient tertiary relict plant like *Ginkgo biloba* and *Taxus chinensis*, also called Chinese Walnut, is widely distributed in Mainland China and Taiwan Province. The root barks of the tree have been used in the folk medicine for the treatment of liver and lung cancer as well as esophageal carcinoma [1]. In our previous prescreening study for the anti-tumor activity, the EtOAc soluble fraction partitioned from the EtOH extract of *J. cathayensis* root barks showed significant anti-tumor activity *in vitro*, with the inhibitory rates of 71, 72, 55, and 59% against S180, Caco-2, MCF-7, and HepG-2 cell lines at a concentration of 1 mg/ml, respectively. These results prompted us to initiate a chemical investigation on the EtOAc soluble fraction.

In this study, we describe the isolation and structural elucidation of two new nitrogen-containing naphthoquinones (**1**–**2**) together with six known naphthoquinones identified as juglone (**3**) [2], 5,8-dihydroxy-1,4-naphthoquinone (**4**) [3], 2-ethoxy juglone (**5**) [4], 2-methoxy juglone (**6**) [5], 3-methoxy juglone (**7**), [5] and engelharquinone (**8**) [6] by comparing with the corresponding literature data (Figure 1). The six known compounds were detected in this plant for the first time.

2. Results and discussion

Repeated chromatography of the EtOAc fraction of *J. cathayensis* root barks led to the isolation of **1**–**8**.

Compound **1** was obtained as dark red crystals with $[\alpha]_D^{20}$ and MP 228–230°C. The molecular formula of **1** was determined as $C_{14}H_{13}NO_5$ according to the $[M - H]^-$ ion peak at *m/z* 274.0715 in the HR-ESI-MS with 9 degrees of unsaturation. The IR spectrum of **1** indicated the presence of hydroxyl (3435 cm^{-1}), α,β-unsaturated carbonyl (2923 cm^{-1}), and carbonyl (1697 cm^{-1}) groups [7]. The UV spectrum exhibited absorption maxima at 231, 260, 286, and 472 nm. The ^1H NMR

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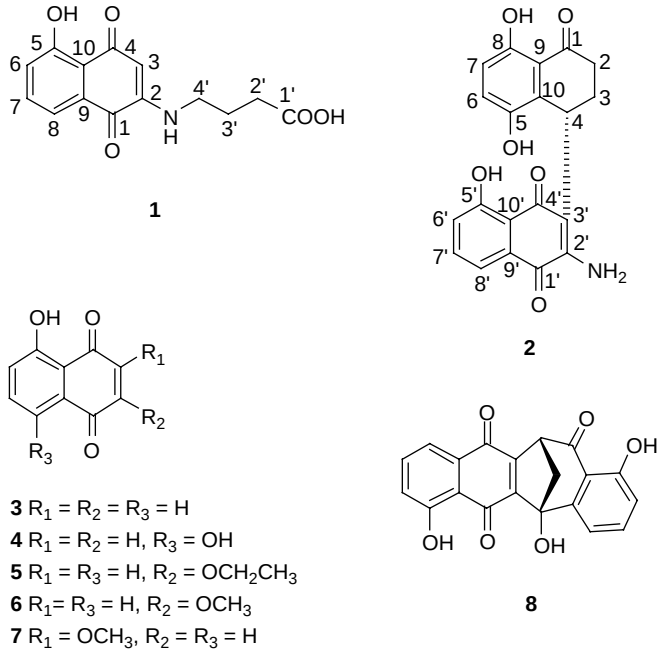

Figure 1. The structures of compounds **1**–**8**.

spectrum (DMSO-d_6) revealed the presence of an AMX system of 1,2,3-trisubstituted benzene at δ_H 7.26 (dd, $J = 7.9$, 1.2 Hz), 7.57 (t, $J = 7.9$ Hz), 7.50 (dd, $J = 7.9$, 1.2 Hz), and an ethylene proton at δ_H 5.69 (1H, s). Three aliphatic methylene signals [δ_H (2.30 (2H, t, $J = 7.2$ Hz), 1.78 (2H, m), 3.23 (2H, m)], an NH (δ_H 8.08, 1H, t), a chelated hydroxyl proton (δ_H 13.44, 1H, s), and a carboxyl proton (δ_H 12.13, 1H, s) were also observed in the ^{1}H NMR spectrum. A structural unit of —NH—CH_2(C-4′)—CH_2(C-3′)—CH_2(C-2′)— was deduced from the ^{1}H–^{1}H COSY correlations of NH/H-4′/H-3′/H-2′. The ^{13}C NMR and DEPT spectra (DMSO-d_6) showed signals for three methylene at δ_C 41.4, 30.8, and 22.7; four aromatic methine at δ_C 134.1, 125.1, 118.4, and 98.0; and seven quaternary carbons, including four aromatic tertiary carbons and three carbonyl carbons at δ_C 180.7, 187.9, and 174.1 (Table 1). The above spectral characteristics of **1** resembled that of a typical naphthoquinone skeleton [3] with a —NH—CH_2—CH_2—CH_2— side chain. The nitrogen atom was located at C-2 by the ^3J HMBC correlations from NH to C-1 and C-3 and from H-4′ to C-2.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of compound **1** in DMSO-d_6.

Position	δ_C, mult.	δ_H, mult. (J in Hz)
1	180.7, C	
2	149.8, C	
3	98.0, CH	5.69, s
4	187.9, C	
5	160.2, C	
5-OH		13.44, s
6	125.1, CH	7.26, dd (7.9, 1.2)
7	134.1, CH	7.57, t (7.9)
8	118.4, CH	7.50, dd (7.9, 1.2)
9	130.6, C	
10	114.5, C	
1′	174.1, C	
1′-COOH		12.13, brs
2′	30.8, CH_2	2.30, t (7.2)
3′	22.7, CH_2	1.78, m
4′	41.4, CH_2	3.23, m
NH		8.08, t (6.1)

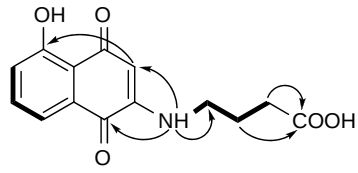

Figure 2. The key COSY and HMBC correlations of compound **1**.

The connection between the methylene group and the carboxyl group was confirmed by the HMBC correlations from H-3′ to C-1′ and from H-2′ to C-1′ as shown in Figure 2. From the above evidence, the structure of **1** was determined to be 2-(3-carboxypropylamino)-5-hydroxy-naphthalen-1,4-dione, named juglonbutine.

Compound **2** was obtained also as dark red crystals with $[\alpha]_D^{20} + 27.1$ and MP 263–265°C. The molecular formula of **2** was determined as $C_{20}H_{15}NO_6$ according to the $[M - H]^-$ ion peak at *m/z* 364.0825 in the HR-ESI-MS with 14 degrees of unsaturation. The IR spectrum of **2** showed absorptions at 3477, 3361 (amino and hydroxyl group, overlapped), 2927 (α,β-unsaturated carbonyl group), and 1680 (carbonyl group) cm^{-1} [7]. The UV spectrum of **2** exhibited absorption maxima at 218, 229, 263, and 378 nm. The ^1H NMR spectrum (DMSO-d_6) indicated the presence of a 1,2,3-trisubstituted phenolic AMX system at δ_H 7.46 (dd, $J = 7.7$, 1.3 Hz), 7.53 (t, $J = 7.7$ Hz), 7.27 (dd, $J = 7.7$, 1.3 Hz), and two *ortho*-coupled aromatic protons at δ_H 6.92 (d, $J = 8.8$ Hz) and 6.63 (d, $J = 8.8$ Hz). Two aliphatic methylene, one aliphatic methine (Table 2), one NH_2 (δ_H 7.40, 2H, br s), and two chelated hydroxyl [δ_H 12.02 (1H, s) and

Table 2. ^1H (400 MHz) and ^{13}C (100 MHz) NMR and HMBC spectral data of compound **2** in DMSO-d_6.

Position	δ_C, mult.	δ_H, mult. (*J* in Hz)	HMBC
1	206.6, C		
2	36.6, CH_2	2.58, dt (17.6, 5.6)	1, 4
		2.76, ddd (17.6,10.8, 4.8)	1, 4
3	26.9, CH_2	2.08, dt (18.8, 5.6)	1
		2.22, br ddd (18.8, 10.8, 4.8)	1
4	30.9, CH	4.33, t (4.8)	3, 10, 2′, 4′
5	146.6, C		
5-OH		8.92, s	5, 6, 10
6	124.6, CH	6.92, d (8.8)	5, 8, 10
7	115.1, CH	6.63, d (8.8)	5, 8, 9
8	155.2, C		
8-OH		12.02, s	6, 7, 8, 9
9	117.9, C		
10	131.4, C		
1′	181.6, C		
2′	148.2, C		
2′-NH_2		7.40, brs	
3′	114.8, C		
4′	187.7, C		
5′	160.5, C		
5′-OH		13.14, s	
6′	125.6, CH	7.27, dd (7.7, 1.3)	5′, 8′, 10′
7′	134.5, CH	7.53, t (7.7)	5′, 9′
8′	118.4, CH	7.46, dd (7.7, 1.3)	1′, 6′,10′
9′	130.5, C		
10′	114.9, C		

13.14 (1H, s)] protons were also observed from the ^{1}H NMR spectrum. The ^{13}C NMR, DEPT, and HSQC spectra indicated two methylene, six methine (five sp^2 carbon), and 12 quaternary sp^2 (including three carbonyl carbon at δ_C 181.6, 187.7, and 206.6) carbons. The spin system of —CH(C-4)—CH$_2$(C-3)—CH$_2$(C-2)— was determined by the ^{1}H–^{1}H COSY correlations of H-2/H-3/H-4 as shown in Figure 2. The above spectral evidences suggested the presence of one 1,4-napthoquinone unit and one α-tetralone unit [6]. The linkage between the two units was established at C-4 and C-3′ by the HMBC correlations from H-4 to C-4′, C-3′, and C-2′ (Figure 2). Two hydroxyl groups [δ_H 8.92 (1H, s) and 12.02 (1H, s)] were located at C-5 and C-8, respectively, which was supported by the HMBC correlations from 5-OH to C-5, C-6, and C-10 and from 8-OH to C-7, C-8, and C-9. The chelated hydroxyl proton at δ_H 13.14 (1H, s) was located at C-5′ by the NOESY correlation between the OH and H-6′ [5]. The amino group was located at C-2′ by the NOESY correlation between H-4 and NH$_2$ (Figure 3). From the above evidences, the planar structure of **2** was established as 2′-amino-5,8,5′-trihydroxy-2,3,4-pentahydro-[4,3′]-binaphthalene-1, 1′,4′-trione, named binaphthine.

Chiral HPLC analysis (see Experimental section) of **2** showed a single peak and the optical rotation revealed a positive value of +27.1 ($c = 0.33$, CH$_3$OH). According to the literature, all of the known similar naphthoquinones with 4S configuration exhibited a positive value of the optical rotation; on the other hand, all of the naphthoquinones with 4R configuration exhibited a negative value of the optical rotation [8–12]. Thus, the absolute configuration of C-4 was very likely to be S. The small coupling constant (4.8 Hz) between H-4 and H-3 in ^{1}H NMR spectrum suggested that H-4 was equatorial in cyclohexenone ring, which was in good agreement with the observed NOESY correlations, as shown in Figure 4. The naphthalene group on C-4 of **2** had α-quasi-axial orientation, which is different from the naphthoquinones given in the literature [8]. And the conformation of compound **2** was supposed to be caused by the steric hindrance between the 1,4-napthoquinone and the α-tetralone units.

To the best of our knowledge, nitrogen-containing naphthoquinones are rarely isolated from natural sources and have never been reported from *Juglans* plants. Compounds **1** and **2** were supposed to be derived from acetyl coenzyme A through the intermediates A–C by a series of biochemical reactions such as amination

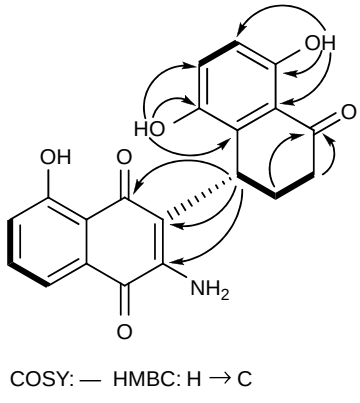

Figure 3. The key COSY, HMBC, and NOESY correlations of compound **2**.

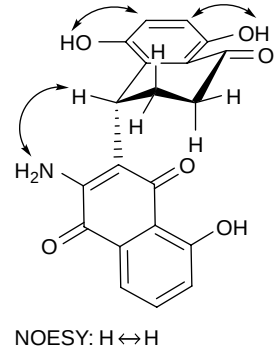

Figure 4. The probable conformation of the cyclohexenone ring and key NOESY correlations of compound **2**.

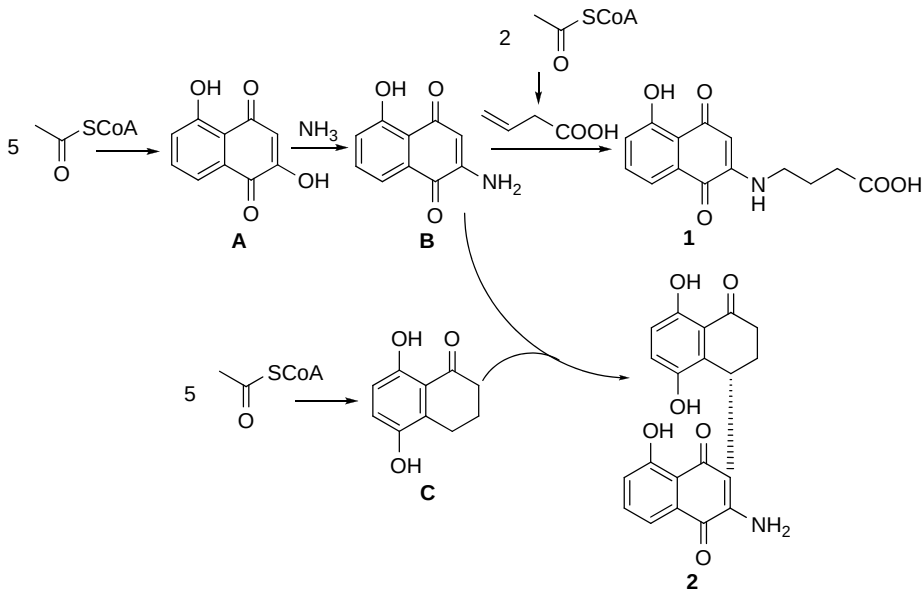

Figure 5. The plausible biosynthetic pathway for **1** and **2**.

and carbon–carbon coupling. The plausible biosynthetic pathways for **1** and **2** were postulated as shown in Figure 5.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on an automatic polarimeter (PE-341LC, Perkin-Elmer Co, Waltham, MA, USA). UV spectra were carried out on a Shanghai Spectrum 756PC spectrophotometer. IR spectra were recorded on a Bruker VERTEX 70 spectrometer with KBr pellets. 1D and 2D NMR spectra were measured on a Bruker AM-400 spectrometer with TMS as internal standard. High-resolution ESI-MS spectra were obtained on a Thermo Scientific LTQ–Orbitrap XL mass spectrometer. Column chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical, Inc., Qingdao, China) and Sephadex LH-20 gel (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Thin-layer chromatography was carried out on silica gel 60 F254 over glass plates (Qingdao Marine Chemical, Inc.) using various solvent systems.

3.2 Plant material

The root barks of *J. cathayensis* were collected in Shengnongjia Mountainous Area of Hubei Province of China in September 2008 and identified by Mr Shigui Shi from Shennongjia Institute for Drug Control. A voucher specimen (P20080910) has been deposited at the Faculty of Pharmaceutical Sciences, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China.

3.3 Extraction and isolation

The dried root barks of *J. cathayensis* (20 kg) were extracted three times (12 h each time) with 95% ethanol at room temperature and concentrated *in vacuo* to give a crude extract (2500 g). Then the extract was suspended in H_2O and partitioned sequentially with petroleum ether, EtOAc, and *n*-BuOH to yield petroleum

ether fraction (190 g, yield 0.95%), EtOAc fraction (360 g, yield 1.8%), and *n*-BuOH fraction (480 g, yield 2.4%).

A portion of the EtOAc fraction (320 g) was subjected to silica gel column chromatography with a gradient of petroleum ether–EtOAc (99:1 → 1:2, v/v) as the mobile phase to provide 100 fractions. Fractions 5–10 (1.0 g) were subjected to silica gel column chromatography by eluting petroleum ether–EtOAc (10:1, v/v) to give compound **3** (100 mg). Fractions 20–25 (1.0 g) were subjected to silica gel column chromatography by eluting petroleum ether–EtOAc (8:1, v/v) to give 30 subfractions. From the subfractions 5–10 (200 mg), compound **4** (15 mg) was obtained after repeated purification by column chromatography over Sephadex LH-20 using acetone as the mobile phase. Subfractions 15–20 were further chromatographed over a silica gel column eluted with petroleum ether–EtOAc (6:1, v/v) to give compound **5** (200 mg). Fractions 25–30 were subjected to silica gel column chromatography by eluting petroleum ether–EtOAc (5:1) to give 20 subfractions. Subfractions 5–15 were further subjected to silica gel column chromatography eluted with cyclohexane–CH_2Cl_2 (1:3, v/v) to give compounds **6** (400 mg) and **7** (100 mg). Fractions 55–60 (2.0 g) were subjected to silica gel column chromatography by eluting petroleum ether–EtOAc (5:1, v/v) to afford subfractions 1–10. From the subfractions 1–5 (500 mg), compound **8** (20 mg) was obtained over a further silica gel column eluted with petroleum ether–EtOAc (6:1, v/v). Compounds **1** (20 mg) and **2** (15 mg) were obtained by repeated column chromatography over Sephadex LH-20 using acetone as the mobile phase.

*3.3.1 2-(3-Carboxypropylamino)-5-hydroxy-naphthalene-1,4-dione (**1**)*

Dark red crystals; $[\alpha]_D^{20}$; UV (MeOH) λ_{max} (log ε): 231 (1.95), 260 (1.56), 286 (0.62), and 472 (0.34) nm; IR (KBr) ν_{max}: 3435, 2923, and 1697 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) spectral data (see Table 1); and HR-ESI-MS *m/z* 274.0715 $[M - H]^-$ (calcd for $C_{14}H_{12}NO_5$, 274.0715).

*3.3.2 2′-Amino-5,8,5′-trihydroxy-2,3,4-pentahydro-(4,3′(-binaphthalene-1,1′,4′-trione (**2**)*

Dark red crystals; $[\alpha]_D^{20} + 27.1$; UV (MeOH) λ_{max} (log ε) 218 (2.63), 229 (2.34), 263 (1.55), and 378 (0.43) nm; IR (KBr) ν_{max}: 3477, 3361, 2927, and 1680 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) spectral data (see Table 2); and HR-ESI-MS *m/z* 364.0825 $[M - H]^-$ (calcd for $C_{20}H_{14}NO_6$, 364.0821).

3.4 Chiral HPLC analysis of compound 2

Compound **2** was analyzed by chiral HPLC [column, Chiralcel OD-H (4.6 mm i.d. × 25 cm, Daicel Chemical Industries, Ltd, Tokyo, Japan); mobile phase, *n*-hexane-iso PrOH–diethylamine (90:10:0.1); UV detector, 254 nm; flow rate, 0.5 ml/min; column temperature, 20°C]. Compound **2** exhibited one peak at 26.5 min.

Acknowledgements

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