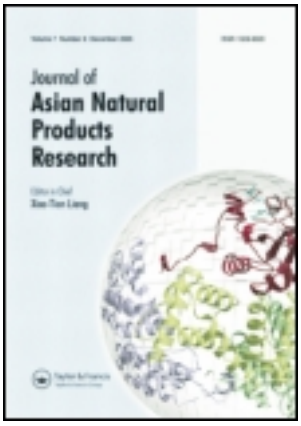

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Lu-Jun Li [a b], Yue Zhang [a], Peng Zhang [a], Hui-Fang Pi [a], Han-Li Ruan [a] & Ji-Zhou Wu [a]

[a] Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, Tongji School of Pharmaceutical Sciences, Huazhong University of Science and Technology, Wuhan, 430030, China

[b] Hubei Key Laboratory of Bio-Technology of Traditional Chinese Medicine, Hubei University, Wuhan, 430062, China

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Two new 5,8-quinoflavans from the leaf of *Ilex centrochinensis*

Lu-Jun Li[ab], Yue Zhang[a], Peng Zhang[a]*, Hui-Fang Pi[a], Han-Li Ruan[a] and Ji-Zhou Wu[a]

[a]*Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, Tongji School of Pharmaceutical Sciences, Huazhong University of Science and Technology, Wuhan 430030, China;* [b]*Hubei Key Laboratory of Bio-Technology of Traditional Chinese Medicine, Hubei University, Wuhan 430062, China*

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Two new 5,8-quinoflavans were isolated from the leaf of *Ilex centrochinensis*, and their structures were elucidated as (2*R*)-7,3′,4′-trimethoxy-5,8-quinoflavan and (2*S*)-7-methoxy-4′-hydroxy-5,8-quinoflavan on the basis of spectroscopic methods, especially 1D and 2D NMR, CD, and mass spectral analyses. Both of them exhibited weak cytotoxic activity against HuH7 cell lines and no cytotoxic activity against CaCO-2 cell lines.

Keywords: *Ilex centrochinensis*; (2*R*)-7,3′,4′-trimethoxy-5,8-quinoflavan; (2*S*)-7-methoxy-4′-hydroxy-5,8-quinoflavan; cytotoxic activity

1. Introduction

In the course of our searching for active compounds of *Ilex* species used commonly in Chinese folk medicine, the constituents of the leaf of *Ilex centrochinensis*, which has been used to treat rheumatoid arthritis for the property of expelling wind and removing dampness [1], were investigated. Previous phytochemical investigations of this species have resulted in the isolation of many flavonoids, triterpenoids, sterols, and cyano compounds [2–4]. We previously reported the isolation of four flavans from this species [5]. As a continuation of our research, two new compounds were obtained and their structures were elucidated as (2*R*)-7,3′,4′-trimethoxy-5,8-quinoflavan (**1**) and (2*S*)-7-methoxy-4′-hydroxy-5,8-quinoflavan (**2**) (Figure 1) on the basis of NMR and MS technologies. The present paper describes the isolation and structural elucidation of these two new compounds. In addition, their cytotoxic activities against human liver cancer cell lines HuH7 and human colon cancer cell lines CaCO-2 were also tested.

2. Results and discussion

Compound **1** was isolated as a yellowish green amorphous solid. The positive mode HR-ESI-MS of **1** gave a quasi-molecular ion at *m/z* 353.0975 $[M + Na]^+$, indicating the molecular formula $C_{18}H_{18}O_6$. The IR spectrum showed absorption bands for aromatic ring (1622, 1594, and 1523 cm^{-1}) and carbonyl (1689 and 1639 cm^{-1}) functional groups. Eighteen proton signals were observed in the ^1H NMR spectrum and 18 carbon signals in the ^{13}C NMR spectrum, which were sorted by the DEPT experiment as three methyl, two methylene, five methine, and eight quaternary carbons. The ^1H and ^{13}C NMR spectra showed the existence of a flavan skeleton. In the ^1H NMR spectrum, two

*Corresponding author. Email: zpeng023@gmail.com

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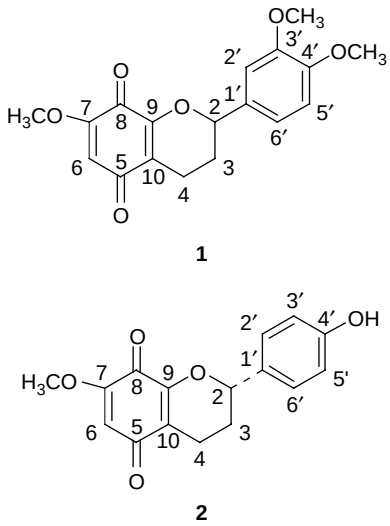

Figure 1. Structures of compounds **1** and **2**.

sets of aromatic protons at δ 6.95–7.05 and 5.89 could be assigned to rings B and A, respectively. The ^{1}H NMR signals at δ 5.08 (1H, dd, $J = 10.2$, 2.4 Hz, H-2), 2.20–2.26 (1H, m, H-3), 1.95–2.02 (1H, m, H-3), and 2.42–2.58 (2H, m, H-4) were assigned to the protons of ring C. The flavan skeleton of **1** was further confirmed by the ^{13}C NMR spectrum, which displayed the ring C carbon signals at δ 79.9 (C-2), 28.8 (C-3), and 19.4 (C-4). The above data were in agreement with those reported for this type of compounds [6,7]. In addition, ^{1}H NMR spectrum displayed resonances of three aromatic methoxyls (δ 3.83, 3.81, 3.81). ESI-MS presented one principal ion fragment at *m/z* 189, resulting from typical Retro Diels–Alder (RDA) cleavage for the loss of fragment at *m/z* 164. As a result, ring B bears two methoxyl substituents and ring A bears the other methoxyl. The substitution pattern of ring B was easy to determine from the ^{1}H NMR spectrum as an ABX system with three proton signals at δ 7.05 (1H, d, $J = 1.6$ Hz, H-2′), 6.96 (1H, d, $J = 8.4$ Hz, H-5′), and 6.98 (1H, dd, $J = 8.4$, 1.6 Hz, H-6′). Thus, the C-3′ and C-4′ of ring B were substituted by methoxyls. The appearance of a singlet at δ 5.89 in the ^{1}H NMR spectrum and two carbonyl carbon signals at δ 176.7 (C-5) and 187.2 (C-8) in the ^{13}C NMR spectrum confirmed the presence of a 5,8-benzoquinone moiety in the A ring, and similar spectroscopic data were obtained for the *p*-benzoquinone [8]. This was further verified by the HMBC correlations of H-6 (δ 5.89) with C-10 (δ 119.5), C-5 (δ 176.7), C-8 (δ 187.2), and C-7 (δ 158.2), as shown in Figure 2. The location of the methoxyl group of ring A was determined to be at C-7, based on HMBC correlation between the methoxyl protons at δ 3.83 and C-7 at δ 158.2.

As compound **1** showed a weak positive optical rotation ($[\alpha]_D^{20}$ +5.58), the absolute configuration at C-2 has been determined by CD experiment. The positive Cotton effect at λ_{max} 292 nm in the CD spectrum was contrary to that of the known compound (2*S*)-4′-hydroxy-5,7,3′-trimethoxyflavan [6], which confirmed that the absolute configuration at C-2 was *R*. Based on the above description, the structure of **1** was unambiguously established as (2*R*)-7,3′,4′-trimethoxy-5,8-quinoflavan.

Compound **2** was isolated as a yellow amorphous solid. Its ^{1}H and ^{13}C NMR

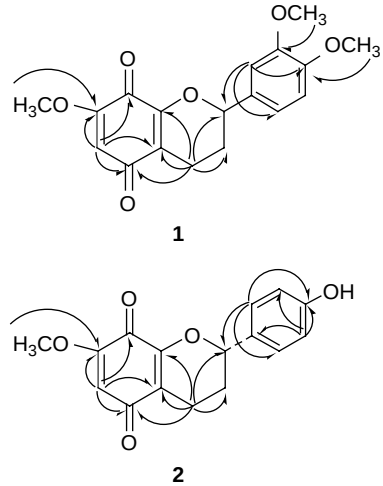

Figure 2. Key HMBC correlations of **1** and **2**.

spectral data were similar to those of **1**, suggesting that **2** also had a 5,8-quinoflavan skeleton. The molecular formula was determined as $C_{16}H_{14}O_5$ by a quasi-molecular ion peak at *m/z* 309.0707 $[M + Na]^+$ in the HR-ESI-MS, 44 mass units less than that of **1**. ESI-MS presented the same typical RDA fragment ion at *m/z* 189 as **1**, suggesting that both of them shared the identical ring A. The ^{1}H NMR spectral data indicated the presence of one hydroxy (δ 8.49) and one methoxyl (δ 3.82) groups. Thus, the hydroxy group was located at ring B. The exact position of the hydroxy group was easy to determine from the ^{1}H NMR spectrum as an AA′BB′ system with four proton signals at δ 7.27 (2H, d, $J = 8.4$ Hz, H-2′, 6′) and 6.86 (2H, d, $J = 8.4$ Hz, H-3′, 5′). Thus, the hydroxy group was located at the C-4′ of ring B. The absolute configuration at C-2 of **2** was determined as *S* by its negative Cotton effect at λ_{max} 291 nm in the CD spectrum, which was similar to that of the known compound (2*S*)-4′-hydroxy-5,7,3′-trimethoxyflavan [6]. The structure of **2** was thus established as (2*S*)-7-methoxy-4′-hydroxy-5,8-quinoflavan.

Their cytotoxic activities against human liver cancer cell lines HuH7 and human colon cancer cell lines CaCO-2 were evaluated *in vitro* using the methyl thiazolyl tetrazolium (MTT) method [9]. They both exhibited relative weak cytotoxic activity against HuH7 cell lines with IC_{50} values of 20.20 and 22.48 μg/ml, respectively. However, neither of them displayed cytotoxic activity against CaCO-2 cell lines.

3. Experimental

3.1 General experimental procedures

Melting points were measured using an XT4–100X micro-melting point apparatus and are uncorrected. Optical rotations were recorded on a JASCO *p*-1010 polarimeter. UV spectra were obtained on UV-2550 spectrophotometer. IR spectra were obtained on Spectrum One IR spectrometer with KBr disks. CD spectra were measured on a JASCO J-810 spectropolarimeter. 1D and 2D NMR spectra were recorded on a Bruker ultrashield™ 400 plus spectrometer at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) in acetone-d_6 with TMS (^{1}H NMR) as the internal reference. HR-ESI-MS were recorded on a Bruker Daltonik micrOTOF-Q using a direct inlet system. Silica gel (200–300 mesh; Qingdao Marine Chemical Group Co., Qingdao, China) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

3.2 Plant material

The leaves of *I. centrochinensis* were collected in Enshi, Hubei Province, China, in September 2007, and identified by Prof. J.Q. Li (Wuhan Botanical Garden, the Chinese Academy of Science). Voucher specimens (No. ICC-20070915) have 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 fresh collected leaves of *I. centrochinensis* were shade dried and coarsely powdered. Dried powdered plant materials (9 kg) were macerated in 95% ethanol (40 liters) for 3 days at room temperature for three times. All extracts were pooled together and concentrated by rotary evaporator under reduced pressure at 45°C to give a crude extract. The crude extract was suspended in water (5 liters) and successively partitioned with petroleum ether, ethyl acetate (EtOAc), and *n*-butanol. The EtOAc extract (1.07 kg) was chromatographed

over diatomite column (3.5 kg, 10 × 120 cm), eluted with petroleum ether–EtOAc (100:0, 20:1, 5:1, 2:1, 1:1, 0:100 each 8 liters), and divided into six subfractions A–F. Subfraction C (200 g) was separated by column chromatography on silica gel (1.0 kg, 5 × 120 cm), eluted with gradient solvent systems of petroleum ether–EtOAc (5:1–2:1) to afford four fractions (1–4). Fraction 1 (200 g) was chromatographed by silica gel column chromatography ($CHCl_3$–MeOH, 150:1–100:1, v/v) and Sephadex LH-20 ($CHCl_3$–MeOH, 1:1, v/v) to give compound **1** (7 mg). Fraction 3 (82 g) was further purified by MPLC ($CHCl_3$–MeOH, 100:1–80:1, v/v) to afford five subfractions (1–5), and subfraction 3 (6 g) was further purified by Sephadex LH-20 (MeOH 100%) and crystallized in $CHCl_3$–MeOH (1:1) to give compound **2** (4 mg).

*3.3.1 7,3′,4′-Trimethoxy-5,8-quinoflavan (**1**)*

Yellowish green amorphous solid; melting point: 203–205°C; $[\alpha]_D^{20}$ +5.58 ($c = 0.50$, acetone); CD (MeOH) $\Delta\varepsilon_{292.0\,nm}$ + 0.37; UV λ_{max}^{MeOH} nm: 286, 204; IR (KBr) ν_{max} (cm^{-1}): 2914, 2849, 1689, 1639, 1622, 1594, 1523, 1465, 1268, 1242, 1087, and 846; ^1H and ^{13}C NMR spectral data are given in Table 1; ESI-MS *m/z* (relative intensity): 353 $[M + Na]^+$, 189 (23). HR-ESI-MS (positive ion): *m/z* 353.0975 $[M + Na]^+$ (calcd for $C_{18}H_{18}O_6Na$, 353.0996).

*3.3.2 7-Methoxy-4′-hydroxy-5,8-quinoflavan (**2**)*

Yellow amorphous solid; melting point 171–173°C; $[\alpha]_D^{20}$ −2.96 ($c = 0.20$, acetone); CD (MeOH) $\Delta\varepsilon_{291.0\,nm}$ − 0.43; UV λ_{max}^{MeOH} nm: 286; IR (KBr) ν_{max} (cm^{-1}): 3388 (OH), 2914, 2848, 1691, 1636, 1622, 1594, 1242, 1087, and 832; ^1H

Table 1. ^1H and ^{13}C NMR spectral data for compounds **1** and **2**.

	1		**2**	
Position	δ_H	δ_C[a]	δ_H	δ_C[a]
2	5.08 (1H, dd, $J = 10.2, 2.4$)	79.9	5.05 (1H, dd, $J = 10.2, 2.4$)	79.9
3	2.20–2.26 (1H, m)	28.8	2.13–2.23 (1H, m)	28.7
	1.95–2.02 (1H, m)		1.92–2.02 (1H, m)	
4	2.42–2.58 (2H, m)	19.4	2.42–2.58 (2H, m)	19.4
5		176.7		176.8
6	5.89 (1H, d, $J = 2.4$)	107.5	5.88 (1H, d, $J = 2.4$)	107.5
7		158.2		158.5
8		187.2		187.1
9		152.3		153.4
10		119.5		119.4
1′		133.7		132.0
2′	7.05 (1H, d, $J = 1.6$)	111.4	7.27 (1H, d, $J = 8.4$)	128.6
3′		150.6	6.86 (1H, d, $J = 8.4$)	116.2
4′		150.6		158.2
5′	6.96 (1H, d, $J = 8.4$)	112.9	6.86 (1H, d, $J = 8.4$)	116.2
6′	6.98 (1H, dd, $J = 8.4, 1.6$)	119.6	7.27 (1H, d, $J = 8.4$)	128.6
7-OCH_3	3.83 (3H, s)	56.8	3.82 (3H, s)	56.8
3′-OCH_3	3.81 (3H, s)	56.3		
4′-OCH_3	3.81 (3H, s)	56.3		
4′-OH			8.49 (1H, s)	

Note: 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR, acetone-d_6, *J* in Hz, δ in ppm.
[a] Assignments were accomplished using HSQC and HMBC experiments.

and ^{13}C NMR spectral data are given in Table 1; ESI-MS *m/z* (relative intensity): 309 $[M + Na]^+$, 189 (44). HR-ESI-MS (positive ion): *m/z* 309.0707 $[M + Na]^+$ (calcd for $C_{16}H_{15}O_5Na$, 309.0733).

3.4 Cytotoxic bioassay

Cytotoxicity was determined by the MTT method [9] using human liver cancer cell lines HuH7 grown in Dulbecco's Modified Eagle Media (DMEM) supplied with 10% fetal bovine serum (FBS) and human colon cancer cell lines CaCO-2 grown in DMEM medium supplied with 10% FBS and 1% non-essential amino acid (NEAA). Cells in the logarithmic phase were cultured at a density of 10,000 cells/ml per well in a 96-well microtiter plate. Then, different concentrations of the test compounds dissolved in dimethyl sulfoxide (DMSO) were added to each well. Each concentration was tested in quadruplicate. After incubation at 37°C in 5% CO_2 for 24 h, 20 μl of MTT (0.5 mg/ml) was added to each well and incubated for another 4 h, and then the liquid in the wells was removed. DMSO (150 μl) was added to each well. The absorbance was recorded on a microplate reader (Tristar LB 941) at a wavelength of 530 nm. The cytotoxicity was expressed as 50% inhibitory concentration (IC_{50}). 5-FU was used as a positive control.

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