Flavans from the leaf of Ilex centrochinensis

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Available online: 30 Mar 2011

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Phytochemical investigation of the leaf of *Ilex centrochinensis* led to the isolation and characterization of four flavans, one flavanone (**5**), and one flavone (**6**), including a new compound whose structure was elucidated as (2S)-5,3′,4′-trihydroxy-7-methoxyflavan (**1**) and a new natural product whose structure was elucidated as (2S)-5-hydroxy-7,3′,4′-trimethoxyflavan (**4**) on the basis of spectroscopic methods especially 1D and 2D NMR, CD, and mass spectral analyses. Compounds **1** and **4** exhibited weak cytotoxic activity against Huh7 cell line and no cytotoxic activity against Caco-2 cell line.

Keywords: *Ilex centrochinensis*; (2S)-5,3′,4′-trihydroxy-7-methoxyflavan; (2S)-5-hydroxy-7,3′,4′-trimethoxyflavan; cytotoxic activity

1. Introduction

Ilex centrochinensis is a commonly used *Ilex* species in Chinese folk medicine. The leaves are used as a traditional beverage, known as Ku-Ding-Cha in some areas of China [1]. They are also used for the treatment of rheumatoid arthritis for the property of expelling wind and removing dampness [2]. In previous reports, many flavonoids, triterpenoids, sterols, and cyano-compounds have been isolated from the leaves [3–5]. As part of our systematic studies on the chemical constituents of the commonly used medicinal *Ilex* species in China, we carried out the chemical study of *I. centrochinensis*. From this source, a new compound, (2S)-5,3′,4′-trihydroxy-7-methoxyflavan (**1**), and a new natural product, (2S)-5-hydroxy-7,3′,4′-trimethoxyflavan (**4**) [6] (Figure 1), were isolated together with

four known compounds 5,4′-dihydroxy-7,3′-dimethoxyflavan (**2**) [7], 5,4′-dihydroxy-7-methoxyflavan (**3**) [8], naringenin (**5**) [9], and quercetin (**6**) [10]. The structures of the known compounds were identified by comparison of their NMR spectral data with those reported in literatures. In this paper, we report the isolation and structural elucidation of compounds **1** and **4**. In addition, we test their cytotoxic activities against human liver cancer cell lines Huh7 and human colon cancer cell lines Caco-2.

2. Results and discussion

Compound **1** was isolated as a pale yellow crystal. The molecular formula was determined as $C_{16}H_{16}O_5$ by observation of a quasi-molecular ion peak at *m/z* 287.0922 $[M - H]^-$ in the HR-ESI-MS. The IR

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1 R$_1$=OH, R$_2$=H
2 R$_1$=OCH$_3$, R$_2$=H
3 R$_1$=H, R$_2$=H
4 R$_1$=OCH$_3$, R$_2$=CH$_3$
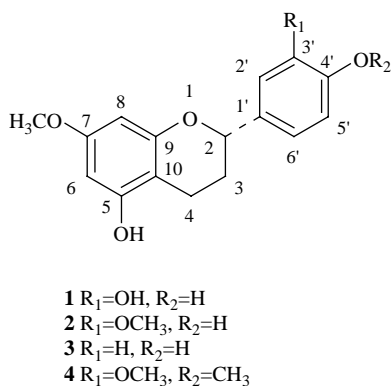
Figure 1.   Structures of compounds **1–4**.

spectrum indicated the presence of hydroxy (3364 cm^{-1}) and aromatic ring (1626, 1611, 1519 cm^{-1}) functionalities. Sixteen proton signals were observed in the ^{1}H NMR spectrum and 16 carbon signals in the ^{13}C NMR spectrum (Table 1), which were sorted by the DEPT experiment as

one methyl, two methylene, six methine, and seven quaternary carbons. The ^{1}H and ^{13}C NMR spectra showed the existence of a flavan skeleton [7,11]. Indeed, in the ^{1}H NMR spectrum, two sets of signals characteristic for aromatic protons at δ 5.94–6.04 and 6.76–6.94 could be assigned to rings A and B, respectively. The ring C protons of the flavan skeleton were located at δ 4.86 (1H, dd, J = 10.4, 2.0 Hz, H-2), 2.13 and 1.92 (m, each 1H, H-3$_{eq}$, and H-3$_{ax}$), and 2.66 (2H, m, H-4). The flavan skeleton of compound **1** was further confirmed by the ^{13}C NMR spectrum, which displayed the ring C carbon signals at δ 77.2 (C-2), 29.4 (C-3), and 19.1 (C-4). The above data were in good agreement with the data reported for this type of compounds [7,11]. In addition, ^{1}H NMR spectrum recorded displayed resonances of one aromatic methoxyl (δ 3.68) and three hydroxy (δ 8.21, 7.84, 7.83) moieties.

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** and **4**.

Position	**1** δ_H	δ_C[a]	**4** δ_H	δ_C[a]
2	4.86 (1H, dd, J = 10.4, 2.0)	77.2	4.93 (1H, dd, J = 10.4, 2.0)	78.3
3	2.13 (1H, m)	29.4	2.17 (1H, m)	30.4
	1.92 (1H, m)		1.97 (1H, m)	
4	2.66 (2H, m)	19.1	2.67 (2H, m)	20.2
5		156.9		157.9
6	5.94 (1H, d, J = 2.4)	93.1	5.97 (1H, d, J = 2.4)	94.1
7		159.3		160.3
8	6.04 (1H, d, J = 2.4)	94.0	6.05 (1H, d, J = 2.4)	95.2
9		156.1		157.1
10		102.2		103.1
1′		133.9		135.8
2′	6.93 (1H, d, J = 2.0)	113.4	7.06 (1H, d, J = 2.0)	111.3
3′		144.9		150.5
4′		144.6		150.1
5′	6.82 (1H, d, J = 8.0)	114.9	6.94 (1H, d, J = 8.0)	112.8
6′	6.77 (1H, d, J = 8.0, 2.0)	117.6	6.98 (1H, d, J = 8.0, 2.0)	119.4
7-OCH$_3$	3.68 (3H, s)	54.3	3.68 (3H, s)	55.4
3′-OCH$_3$			3.83 (3H, s)	56.3
4′-OCH$_3$			3.81 (3H, s)	56.3
5-OH	8.21 (1H, s)		8.22 (1H, s)	
3′-OH	7.84 (1H, s)			
4′-OH	7.83 (1H, s)			

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

Thus, the flavan bears one methoxyl and three hydroxy substituents. ESI-MS presented two principal ion fragments at *m/z* 135 and 151, resulting from typical Retro-Diels–Alder cleavage. Based on these data, we concluded that the former fragment ion corresponds to the B-ring with two hydroxy substituents, and the latter corresponds to the A-ring with one methoxyl and one hydroxy groups. The substitution pattern of ring B was easy to determine from the ^{1}H NMR spectrum, and an ABX system was visible with three signals attributable to one *meta*-coupling proton (δ 6.93, d, $J = 2.0$ Hz, H-2'), one *ortho*-coupling proton (δ 6.82, d, $J = 8.0$ Hz, H-5'), and one *ortho–meta*-coupling proton (δ 6.77, dd, $J = 8.0$, 2.0 Hz, H-6'). In addition, the chemical shifts of the oxygenated carbons observed in the ^{13}C NMR spectrum at δ 144.9 and 144.6 confirmed their *ortho* position. Thus, ring B was 3',4'-dihydroxy substitution. The substitution pattern of ring A was also determined based on the NMR considerations. Indeed, a pair of *meta*-coupled doublets at δ 5.94 (d, $J = 2.4$ Hz, H-6) and 6.04 (d, $J = 2.4$ Hz, H-8) were observed in the ^{1}H NMR spectrum, while resonances of the oxygenated carbons at δ 156.9, 159.3, and 156.1 (C-5, C-7, C-9) confirmed that their relative positions were in *meta* position. Thus, 5,7-disubstitution was clearly established. Lastly, the exact position of methoxyl was further elucidated by 2D NMR spectra. In the HMBC spectrum (Figure 2), the long-range correlation between the methoxyl protons at δ 3.68 and C-7 at δ 159.3 suggested that the methoxyl was linked at C-7. The result was further confirmed by the NOESY experiment. Presaturation of the methoxyl resonance at δ 3.68 gave the enhancement of signals H-8 (δ 6.04) and H-6 (δ 5.94).

As compound **1** showed a weak negative optical rotation ($[\alpha]_D^{20} - 6.96$), the absolute configuration at C-2 was determined by the CD experiment. The negative Cotton effect at λ_{max} 277 nm
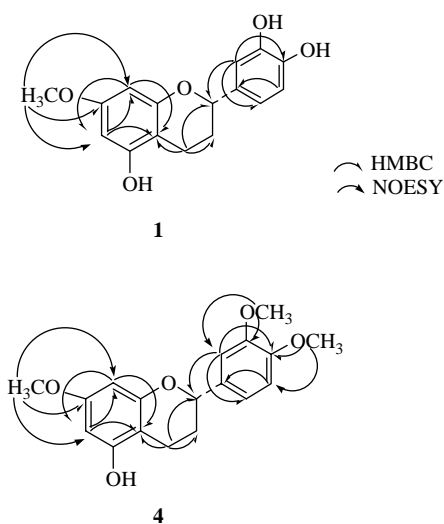

Figure 2. Key HMBC and NOESY correlations of **1** and **4**.

in the CD spectrum was similar to that of the known compound (2S)-4'-hydroxy-5,7,3'-trimethoxyflavan [6]. Therefore, the absolute configuration at C-2 of compound **1** was assigned to be *S*. Thus, the structure of compound **1** was unambiguously established as (2S)-5,3',4'-trihydroxy-7-methoxyflavan, a new compound.

Compound **4** was obtained as a colorless crystal and assigned the molecular formula of $C_{18}H_{20}O_5$, as established from the quasi-molecular ion peak at *m/z* 315.1239 $[M - H]^-$ in the HR-ESI-MS, 28 mass units greater than that of compound **1**. Twenty proton signals were observed in the ^{1}H NMR spectrum and 18 carbon signals in the ^{13}C NMR spectrum (Table 1), which were sorted by the DEPT experiment as three methyl, two methylene, six methine, and seven quaternary carbons. The IR and ^{1}H NMR spectra of **4** were similar to those of compound **1**, suggesting that compound **4** also has a flavan skeleton. Comparison of the ^{1}H NMR spectral data of **4** with those of **1** indicated the presence of three methoxyl (δ 3.68, 3.81, 3.83) and one hydroxy (δ 8.22) moieties. ESI-MS presented a same

principal ion fragment at *m/z* 151 which corresponds to the A-ring with one methoxyl and one hydroxy groups as compound **1**. As a result, the B-ring has two methoxyl substituents. In a similar manner, the ^{1}H NMR, HMBC, and NOESY experiments indicated that the substitution patterns of rings A and B were the same as those of compound **1**. The absolute configuration at C-2 of **4** was also determined as *S* by its negative Cotton effect at λ_{max} 276.5 nm in the CD spectrum [6]. Thus, the structure of compound **4** was established as (2*S*)-5-hydroxy-7,3′,4′-trimethoxyflavan, a new natural product.

The cytotoxicities of compounds **1**–**6** were evaluated *in vitro* using the MTT method [11]. They all displayed no cytotoxic activity against Caco-2 cell lines. Compounds **2** and **3** showed strong cytotoxic activity against Huh7 cell lines with IC_{50} values of 8.98 and 13.04 μg/ml, respectively. Compounds **1** and **4** exhibited relative weak cytotoxic activity with IC_{50} values of 25.77 and 26.43 μg/ml, respectively.

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. IR spectra were obtained on Spectrum one IR spectrometer (Perkin Elmer, CA, USA) 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 was recorded on a Bruker Daltonics micrO-TOF-Q mass spectrometer 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 from 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, 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) and eluted with gradient solvent systems of petroleum ether–ethyl acetate (5:1–2:1) to afford four fractions (1–4). Fraction 4 (48 g) was chromatographed by MPLC silica gel column chromatography (CH_3Cl–MeOH, 20:1) and Sephadex LH-20 (MeOH 100%) to give compound **1** (150 mg). Fraction 3 (82 g) was further purified by MPLC

(CH$_3$Cl–MeOH, 100:1–80:1, V/V), followed by Sephadex LH-20 (MeOH 100%), and crystallized in CHCl$_3$–MeOH (1:1) at 20°C to give compounds **2** (25 mg), **3** (20 mg), and **5** (8 mg). Compound **4** (15 mg) was isolated from fraction 2 (32 g) 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). Subfraction D (180 g) was separated by column chromatography on silica gel (1.0 kg, 5×120 cm) and eluted with gradient solvent systems of petroleum ether–ethyl acetate (3:1–1:1) to afford three fractions (1′–3′). Fraction 2′ (60 g) was further purified by MPLC (CHCl$_3$–MeOH, 11:1 V/V), followed by Sephadex LH-20 (MeOH 100%) to give compound **6** (80 mg).

3.3.1   Compound **1**

Pale yellow crystal; mp 174–176°C; $[\alpha]_D^{20}$ − 6.96 (*c* = 0.70, acetone); CD (MeOH) $\Delta\varepsilon_{277.0\,nm}$ − 0.98; IR (KBr) ν_{max} (cm^{-1}): 3365 (OH), 2962, 2937, 2844, 1626, 1611, 1519, 1445, 1283, 1119, 1142, 1072, 1050, 810; ^{1}H and ^{13}C NMR spectral data, see Table 1; ESI-MS *m/z* (rel. int.): 287 [M − H]$^-$, 151 (32), 135 (23). HR-ESI-MS (negative ion): *m/z* 287.0922 [M − H]$^-$ (calcd for C$_{16}$H$_{15}$O$_5$, 287.0925).

3.3.2   Compound **4**

Colorless crystal; mp 183–185°C; $[\alpha]_D^{20}$ = −13.69 (*c* = 0.16, acetone); CD (MeOH) $\Delta\varepsilon_{276.5\,nm}$ − 1.12; IR (KBr) ν_{max} (cm^{-1}): 3448 (OH), 2922, 2873, 2840, 1630, 1595, 1513, 1257, 1150, 1138, 1077, 1026, 811; ^{1}H and^{13}C NMR spectral data, see Table 1; ESI-MS *m/z* (rel. int.): 315 [M − H]$^-$, 151 (22); HR-ESI-MS (negative ion): *m/z* 315.1239 [M − H]$^-$ (calcd for C$_{18}$H$_{19}$O$_5$, 315.1238).

3.4   Cytotoxic bioassay

Cytotoxicity was determined by the MTT method [12] using human liver cancer cell lines Huh7 grown in Dulbecco's modified Eagle medium (DMEM) supplied with 10% FBS and human colon cancer cell lines Caco-2 grown in DMEM 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 IC$_{50}$. 5-FU was used as a positive control.

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