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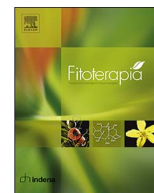

Phenylpropanoids from the fruit of *Crataegus pinnatifida* exhibit cytotoxicity on hepatic carcinoma cells through apoptosis induction

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ABSTRACT

Eight new phenylpropanoids (**1a/1b**, **2**–**4**, **5a/5b** and **6**) including two pairs of enantiomers (**1a/1b** and **5a/5b**), along with a known analogue (**7**) were isolated from the fruit of *Crataegus pinnatifida*. Their structures were elucidated using comprehensive spectroscopic methods. Compounds **1a/1b** and **5a/5b** were separated successfully by chiral chromatographic column. The absolute configurations of enantiomers were determined by comparison between the experimental and calculated electronic circular dichroism (ECD) spectra. The *in vitro* antitumor activities of the isolates were evaluated against two human hepatocellular carcinoma HepG2 and Hep3B cells. Five compounds (**1a/1b**, **2**–**4**) exhibited more potent cytotoxicity and their structure-activity relationships were also discussed. Annexin V-FITC/PI staining using flow cytometry was carried out to examine cell apoptosis, and the results showed that compounds **3**–**4** with the presence of two methoxy groups substituted at C-3′ significantly induced apoptosis in HepG2 cells.

1. Introduction

Hepatocellular carcinoma (HCC), one of the most lethal malignancies, is considered as the second major cause of cancer-related death [1,2]. Though the treatment strategies have been greatly improved over the past decade, the survival rate of HCC patients is still unsatisfactory with a dismal prognosis [3]. Therefore, much attention has been focused on developing novel, highly efficacious and well-tolerated therapies for HCC treatment. Natural products are rich sources of lead compounds for drug discovery due to their unique structures and bioactivities [4,5]. However, racemates sometimes exist in natural products, and they are difficult to separate by conventional methods because of their similar physical and chemical properties [6,7]. It has been long established that different absolute configurations may lead to marked differences in biological activity [4,8]. Therefore, it is of utmost importance to obtain optically pure compounds and evaluate their pharmacological effects respectively.

Crataegus pinnatifida (Chinese hawthorn), belonging to the family of Rosaceae, is widely distributed in China, Europe and North America [9]. It has been used as a well-known traditional medicine for the prevention and treatment of heart disease [10,11]. *C. pinnatifida* has attracted increasing attention in the fields of food, nutraceuticals, and phytomedicine because of its wide health benefits [12]. Our previous phytochemical studies on the seeds of *C. pinnatifida* have resulted in the isolation of a series of lignans, some of which showed promising anti-tumor activities [13,14].

As the continuation of our previous research, we have undertaken a further phytochemical investigation on the fruit of *C. pinnatifida* in order to search for more potential anti-tumor agents. It led to the isolation of eight new phenylpropanoids (**1a/1b**, **2**–**4**, **5a/5b** and **6**) including two pairs of enantiomers (**1a/1b** and **5a/5b**) along with a known analogue (**7**). The two pairs of new enantiomers were separated successfully using chiral chromatographic column. All compounds were tested for their cytotoxic activities on two hepatocellular carcinoma cell lines (Hep3B and HepG2 cells). In addition, the apoptosis induced by **1a/1b** and **2**–**4** in HepG2 cells were further evaluated using flow cytometry.

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Table 1
^{1}H NMR and ^{13}C NMR spectroscopic data for compounds **1–4** (in $CDCl_3$).[a]

Position	**1a/1b**		**2**		**3**		**4**	
	δ_H (multi, *J* in Hz)	δ_C	δ_H (multi, *J* in Hz)	δ_C	δ_H (multi, *J* in Hz)	δ_C	δ_H (multi, *J* in Hz)	δ_C
1	–	131.6	–	131.5	–	131.5	–	131.6
2	7.14 (d, 1.8)	112.0	7.14 (d, 1.8)	112.0	6.82 (br.s)	105.7	7.14 (d, 1.8)	111.8
3	–	152.0	–	152.0	–	153.5	–	151.9
4	–	146.4	–	146.6	–	134.3	–	146.1
5	7.12 (d, 8.1)	123.0	7.12 (d, 8.1)	122.9	–	153.5	7.12 (d, 8.1)	123.0
6	7.16 (dd, 8.1, 1.8)	122.5	7.16 (dd, 8.1, 1.8)	122.5	6.82 (br.s)	105.7	7.16 (dd, 8.1, 1.8)	122.4
7	7.44 (d, 15.9)	152.3	7.44 (d, 15.9)	152.3	7.42 (d, 15.9)	152.6	7.44 (d, 15.9)	152.1
8	6.66 (dd, 15.9, 7.7)	128.4	6.66 (dd, 15.9, 7.7)	128.3	6.67 (dd, 15.9, 7.7)	128.7	6.66 (dd, 15.9, 7.7)	128.3
9	9.70 (d, 7.7)	193.6	9.69 (d, 7.7)	193.6	9.70 (d, 7.7)	193.5	9.70 (d, 7.7)	193.5
1′	–	157.4	–	157.9	–	156.0	–	156.8
2′	4.68 (d, 2.1)	90.6	4.71 (d, 2.1)	90.6	4.50 (d, 2.2)	87.4	4.65 (d, 2.1)	90.5
	4.14 (d, 2.1)		4.14 (d, 2.1)		3.96 (d, 2.2)		4.13 (d, 2.1)	
3′	5.02 (s)	99.5	5.06 (s)	98.7	5.04 (s)	100.1	4.96 (s)	100.3
2-OCH_3	3.87 (s)	56.1	3.87 (s)	56.1	3.86 (s)	56.4	3.87 (s)	56.1
6-OCH_3	–	–	–	–	3.86 (s)	56.4	–	–
3′-OCH_2CH_3	3.78 (m)	61.9	3.77 (m)	61.7	–	–	–	–
	3.63 (m)		3.63 (m)		–		–	
	1.28 (t, 7.1)	15.3	1.27 (t, 7.1)	15.3	–	–	–	–
3′-OCH_3	3.44 (s)	53.1	–	–	3.45 (s)	52.8	3.45 (s)	53.1

[a] ^{1}H NMR spectra recorded at 400 MHz and ^{13}C NMR spectra recorded at 100 MHz for compounds **1–4**.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter. The UV spectra were obtained on a Shimadzu UV-1700 spectrometer. CD spectra were taken on a MOS 450 detector (Bio-Logic Science Instruments, Seyssinet-Pariset, France). HRESIMS experiments were measured by a MicroTOF spectrometer (Bruker Co., Karlsruhe, Germany). The 1D and 2D NMR spectra were recorded on Bruker ARX-400 and AV-600 spectrometers (Bruker Corporation, Bremen, Germany), respectively, with trimethylsilane (TMS) as internal standard. Semipreparative RP-HPLC isolation was achieved with an instrument equipped with a LC-6AD pump and a SPD-20A ultraviolet-visible light absorbance detector using an YMC C_{18} column (250 mm × 10 mm, 5 µm, Shimadzu, Tokyo, Japan). The Chiralpak IC column (4.6 × 250 mm, 5 µm, Daicel Polymer Ltd., Tokyo, Japan) was used in the HPLC system. The organic solvents were distilled prior to the separation process. Column chromatography was carried out on silica gel (100–200 or 200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Dianion HP-20 macroporous resin (Mitsubishi Chemical Co., Japan), ODS gel (60–80 µm, Merck, Germany). MTT assays were performed on a Varioskan Flash Multimode Reader (Thermo Scientific Co. Ltd, Massachusetts, USA).

2.2. Plant material

The fruit of *C. pinnatifida* was collected from Hebei province, China, in October 2016 and authenticated by Professor Jin-Cai Lu (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). A voucher specimen (no. 20170402) has been deposited in the Herbarium of Shenyang Pharmaceutical University, Liaoning, China.

2.3. Extraction and isolation

The air-dried fruit of *C. pinnatifida* (50 kg) was powdered and extracted with 70% EtOH for 3 × 30 L × 4 h. The EtOH extract was concentrated to obtain the crude extract (3800 g). The residue was suspended in H_2O (20*L*) and partitioned continuously with ethyl acetate and *n*-BuOH. The EtOAc extract (600 g) was chromatographed on a silica gel column eluted with CH_2Cl_2:MeOH from 100:0 to 5:1 to yield four fractions (Fr. A-D). Then Fr. A (132 g) was further applied to a HP-20 macroporous resin eluted with H_2O, 50% and 90% EtOH to provide two fractions (Fr. A1-Fr. A2). Fr. A1 (40 g) and Fr. A2 (80 g) were carried on an ODS column using EtOH-H_2O (from 20:80 to 90:10) as the elution system, respectively, and then redistributed in five fractions (Fr. 1–5) on the basis of silica gel TLC analysis. Fr. 5 (10 g) was further chromatographed on silica column with the mobile phase petroleum ether/EtOAc (v/v 50:1 → 0:1) and CH_2Cl_2/MeOH (v/v = 20: 1 → 3:1) to yield Fr. 5.1–5.8. Compound **1** (2.5 mg, t_R = 34.7 min) was separated from fraction 5.3.8 by semipreparative HPLC eluted with CH_3CN-H_2O (45:55). Compound **2** (3 mg, t_R = 39.2 min) was obtained from Fr. 5.2.8 by semi-preparative HPLC eluted with CH_3CN:H_2O (34:56). Compounds **3** (2.4 mg, t_R = 56.5 min), **4** (2.7 mg, t_R = 58.3 min) were separated from fraction 5.3.3 by semipreparative HPLC eluted with MeOH-H_2O (45:55). Fr. 5.3.7 was purified through HPLC chromatography (CH_3CN-H_2O, 45:55) to give compound **5** (4 mg, t_R = 26.8 min). Similarly, Fr. 5.2.3 was applied to HPLC eluted with CH_3CN-H_2O (45,55) to obtain **6** (3.6 mg, t_R = 37.4 min) and **7** (16.1 mg, t_R = 22.8 min). Compound **1** was eluted by Daicel chiralpak IC column (*n*-hexane/isopropanol, *v*/v, 2:1, flow rate 0.6 mL/min) to give **1a** (1.4 mg, t_R = 18.8 min) and **1b** (1.1 mg, t_R = 20.5 min). Compound **5** was performed by Daicel chiralpak IC column (*n*-hexane/isopropanol, v/v, 1:1, flow rate 0.6 mL/min) to obtain **5a** (2.2 mg, t_R = 9.1 min) and **5b** (2.4 mg, t_R = 10.2 min).

Crataegusoid A (**1**): Yellow oil; $[\alpha]_D^{20}$ −0.6 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (logε): 292 nm (3.42); IR (KBr) ν_{max} 3431, 2985, 1629, 1492, 1400, 1369, 1270, 1173, 1124, 1006, 832, 798 cm^{-1}; The ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR data (100 MHz, $CDCl_3$), see Table 1; HRESIMS (*m/z*): 315.1206 $[M + Na]^+$ (calcd for $C_{16}H_{20}O_5Na$, 315.1203).

(−)-Crataegusoid A (**1a**): $[\alpha]_D^{20}$ −23 (*c* 0.10, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 209 (−3.22), 310 (−2.09) nm.

(+)-Crataegusoid A (**1b**): $[\alpha]_D^{20}$ +24 (*c* 0.10, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 214 (+2.88), 307 (+2.09) nm.

Crataegusoid B (**2**): Yellow oil; UV (MeOH) λ_{max} (logε): 292 nm (3.69); IR (KBr) ν_{max} 3438, 2986, 1630, 1492, 1400, 1369, 1173, 1125, 1006, 832, 798 cm^{-1}; The ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR data (100 MHz, $CDCl_3$), see Table 1; HRESIMS (*m/z*): 329.1357 $[M + Na]^+$ (calcd for $C_{17}H_{22}O_5Na$, 329.1359).

Crataegusoid C (**3**): Yellow oil; UV (MeOH) λ_{max} (logε): 310 nm

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