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The Antioxidant and Tyrosinase-inhibiting Activities of 8-*O*-4′ Neolignans from *Crataegus pinnatifida* Seeds

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Abstract: Many tyrosinase inhibitors have been alleged to have serious side effects. To search for relatively mild and safe tyrosinase inhibitors, two new 8-*O*-4′ neolignans, named huangnin A (**1**) and B (**2**), and four known analogs (**3**-**6**) were isolated from the seeds of *Crataegus pinnatifida*. Their structures were elucidated by spectroscopic analyses (1D, 2D NMR, HRESIMS, CD and $Rh_2(OCOCF_3)_4$-induced CD). In addition, the *in vitro* antioxidant and anti-tyrosinase activities of all isolates were evaluated. The results showed that compound **5** has good antioxidant and promising tyrosinase-inhibiting activities.

Keywords: *Crataegus pinnatifida*; Rosaceae; 8-*O*-4′ neolignans; antioxidant activity; tyrosinase-inhibiting activity.

1. Introduction

Melanin production, which is principally responsible for skin color, is a major defense mechanism against the harmful ultraviolet rays in sunlight. However, excess production of melanin after long periods of exposure to the sun can cause dermatological disorders such as melasma, freckles, postinflammatory melanoderma, and solar lentigines [1]. Tyrosinase catalyzes the first two reactions in melanin synthesis, the hydroxylation of L-tyrosine to L-DOPA and the subsequent oxidation of L-DOPA to dopaquinone. This *o*-quinone is a highly reactive compound that can spontaneously polymerizes to form melanin [2]. In addition, the uncontrolled production of reactive oxygen species (ROS) is one of the major factors involved in melanin synthesis and skin damage [3]. Hence, the use of tyrosinase inhibitors and/or antioxidants is becoming increasingly important in the cosmetic and medicinal industries due to their preventive effects in pigmentation disorders and skin aging [4, 5].

Hawthorn is the common name of the plants in the genus *Crataegus* of the Rosaceae family, which are distributed in the Northern Hemisphere mostly in China, Europe and North America. More than 1000 species of the genus have been described [6]. In Europe, the fruits, leaves and flowers of hawthorn are used for astringent, diuretic and the alleviation of cardiovascular disease. In China, hawthorn is consumed as fresh fruit or processed juice and jam to improve digestion and relieve food stasis. The fruits are also used to improve circulation, remove blood stasis and treat hyperlipidemia

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and hypertension [7]. *Crataegus pinnatifida* and *Crataegus scabrifolia* are the major hawthorn species in China, and *Crataegus pinnatifida* is the most commercially important due to its large and delicious fruits [8]. Most of the previous phytochemical investigations of hawthorn have almost exclusively focused on the leaves, fruits and flowers [7]. In this study, two new 8-*O*-4′ neolignans together with four known analogs have been isolated and identified from the seeds of *Crataegus pinnatifida* (Figure 1). The purpose of this study was to determine whether 8-*O*-4′ neolignans from *Crataegus pinnatifida* seeds act as natural antioxidants and/or tyrosinase inhibitors. Thus, the *in vitro* antioxidative and anti-tyrosinase activities of compounds **1**-**6** were evaluated.

2. Materials and Methods

2.1. General

Optical rotations were measured on a JASCO P-1020 polarimeter (Jasco Co., Tokyo, Japan). UV spectra were determined on a Shimadzu double-beam 210A spectrometer (Shimadzu Corporation, Shimadzu, Japan). IR spectra were obtained on a Shimadzu ftir-8400s spectrophotometer (Shimadzu Corporation, Kyoto, Japan). CD spectra were obtained using Jasco CD-MDS 450 spectrophotometer (Jasco Co., Tokyo, Japan). NMR spectra were recorded on Bruker ARX-600 instruments (Bruker Co., Billerica, MA, USA). HRESIMS experiments were performed on an Agilent G6520 Q-TOF spectrometer (Agilent technologies Inc., Santa Clara, CA, USA). Semipreparative RP-HPLC isolation was achieved with an Agilent 1100 instrument using YMC 5 μm C18 column (250 mm×10 mm). Peak detection was made with a refractive index detector (RID) (Agilent technologies Inc., Santa Clara, CA, USA). Silica gel (200-300 mesh, Qingdao Marine Chemistry Ltd., Qingdao, China), and Cosmosil ODS (40-80 μm, Nacalai Tosoh Inc., Uetikon, Switzerland) were used for column chromatography. The absorbances in bioassays were measured and recorded on Varioskan Flash Multimode Reader (Thermo scientific). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), diphenylpicrylhydrazyl (DPPH), mushroom tyrosinase, Trolox, and arbutin standard were all purchased from Sigma-Aldrich (Steinheim, Germany) for screening of antioxidant activities on compounds. Solvents were of industrial purity and distilled prior to use**.**

*2.2. Absolute configuration of C-7 in **1** and **2***

Compound **1** or **2** (0.5 mg) was dissolved in dried solution of the dirhodium trifluoroacetate [$Rh_2(OCOCF_3)_4$] complex (1.0 mg) in CH_2Cl_2 (600μL). After mixing, the first CD spectrum was recorded immediately, and the time evolution was monitored until stationary (about 10 min). The inherent CD spectrum was subtracted. The sign of the E band at around 350 nm in the induced CD data was correlated to the absolute configuration of the secondary alcohol [9].

2.3. ABTS assay and DPPH assay

ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol, to an absorbance of 0.7±0.02 at 734 nm. An ethanolic solution (50 μL) of the samples at various concentrations was mixed with 150 μL diluted $ABTS^{\bullet+}$ solution. After reaction at room temperature for 20 min, the absorbance at 734 nm was measured. Lower absorbance of the reaction mixture indicates higher $ABTS^{\bullet+}$ scavenging activity [10].

The 0.1 mM solution of DPPH radical in ethanol was prepared and 100 μL of this solution was mixed with 100 μL of sample solution. The mixture was incubated for 30 min in a dark room at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm [10].

The capability to scavenge the DPPH or $ABTS^{\bullet+}$ was calculated using the formula given below: DPPH/$ABTS^{\bullet+}$ scavenging activity (%) = [1-(S-SB)/(C-CB)]×100% where S, SB, C and CB are the absorbencies of the sample, the blank sample, the control, and the blank control, respectively.

2.4. Assay of inhibitory activity to tyrosinase

40 μL of mushroom tyrosinase solution (100 units/mL), 40 μL of 0.1 mg/mL L-tyrosine solution in phosphate-buffered saline (PBS) solution (25 mM, pH 6.8), 80 μL of PBS solution (25 mM, pH 6.8), and 40 μL of sample (compounds **1**-**6** and arbutin) in 20% MeOH solution (500 μg/mL) were added to a 96-well microplate. The assay mixture was incubated at 37°C for 30 min. A 20% MeOH solution was added to a blank solution. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 492 nm in the microplate reader. The percentage of the inhibition of tyrosinase activity was calculated by the following equation: inhibition (%) = [(A-B)-(C-D)]/(A-B)×100, where A is absorbance of blank solution after incubation, B is absorbance of blank solution before incubation, C is absorbance of sample solution after incubation, and D is absorbance of sample solution before incubation [4].

2.5. Plant material

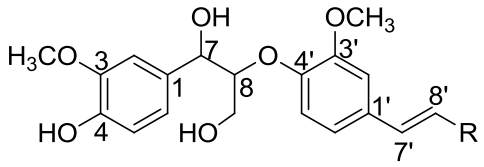
The hawthorn seeds were collected from Shijiazhuang, Hebei province, P. R. China, in June 2011, and were identified by professor Jin-Cai Lu (Department of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, P. R. China.). A voucher specimen (No. 20110701) has been deposited in the Herbarium of Shenyang Pharmaceutical University, Liaoning, P.R. China.

2.6. Extraction and isolation

The air-dried seeds of hawthorn (30 kg) were crushed to pieces and refluxed with 70% EtOH for 3 times×30 L×4 h, and the ethanol extract was concentrated under reduced pressure. The residue (1500 g) was suspended in water (20 L), and then partitioned with EtOAc and BuOH successively. The ethyl acetate extract (420 g) was suspended in H_2O (5 L) and then chromatographed over D101 macroporous resin CC (12×60 cm) using H_2O-EtOH (from 100:0 to 5:95) as eluents. The H_2O-EtOH (70:30) fraction (128.0 g) was subjected to silica gel CC (12×60 cm) eluted with CH_2Cl_2-MeOH (from 100:0 to 50:50) to afford eight fractions (1-8). Fraction 3 (15.1 g) was further purified over an ODS CC (10×60 cm) using MeOH-H_2O as the mobile phase with a gradient (from 95:5 to 50:50) to afford five fractions ($F_{3\text{-}1}$-$F_{3\text{-}5}$) based on HPLC analysis. $F_{3\text{-}2}$ (3.1 g) was subjected to another silica gel CC (2×30 cm) and eluted with CH_2Cl_2:MeOH (from 95:5 to 85:15) to afford eleven fractions ($F_{3\text{-}2\text{-}1}$-$F_{3\text{-}2\text{-}11}$) based on TLC analysis. $F_{3\text{-}2\text{-}1}$ was subjected to semipreparative PR-HPLC eluted with CH_3OH-H_2O (45:55) at 3 mL/min to yield **1** (3 mg, t_R 39 min) and **2** (2 mg, t_R 35 min). $F_{3\text{-}2\text{-}4}$ was subjected to semipreparative PR-HPLC eluted with MeOH-H_2O (40:60) at 3 mL/min to yield **3** (16 mg, 40 min) and **4** (15 mg, 36 min). $F_{3\text{-}2\text{-}2}$ was subjected to semipreparative PR-HPLC eluted with MeOH-H_2O (32:68) at 3 mL/min to yield **5** (36 mg, t_R 54 min) and **6** (33mg, t_R 44 min).

Huangnin A (**1**) yellow oil; $[\alpha]_D^{20}$ +12.2 (*c*=0.02, MeOH); CD (MeOH) nm: 229 (-3.05); $Rh_2(OCOCF_3)_4$-induced CD (CH_2Cl_2) nm: 350 (-0.28); UV (MeOH) λ max (logε): 231 (3.44) nm, 280 (1.30); IR (KBr) ν_{max}: 3430, 2920, 1608, 1464, 1140, 1033, 848, 778, 618 cm^{-1}; HRESIMS *m/z* 427.1493 $[M+Na]^+$ (calcd for $C_{22}H_{28}O_7Na$, 427.1473); ^1H and ^{13}C NMR date (in CD_3OD), see Table 1.

Huangnin B (**2**) yellow oil; $[\alpha]_D^{20}$ +7.2 (*c*=0.02, MeOH); CD (MeOH) nm: 231 (-4.40); $Rh_2(OCOCF_3)_4$-induced CD (CH_2Cl_2) nm: 352 (0.58); UV (MeOH) λ max (logε): 231 (4.53), 279 (1.24); IR (KBr) ν_{max}: 3396, 2920, 1605, 1464, 1133, 1030, 858, 776, 618 cm^{-1}; HRESIMS *m/z* 427.1473 $[M+Na]^+$ (calcd for $C_{22}H_{28}O_7Na$, 427.1473); ^1H and ^{13}C NMR date (in CD_3OD), see Table 1.



1: R=$CH_2OCH_2CH_3$; *threo*-7*R*,8*R*; 7'*E*
2: R=$CH_2OCH_2CH_3$; *erythro*-7*S*,8*R*; 7'*E*
3: R=CH_2OH; *threo*-7*R*,8*R*; 7'*E*
4: R=CH_2OH; *erythro*-7*S*,8*R*; 7'*E*
5: R=CHO; *threo*-7*R*,8*R*; 7'*E*
6: R=CHO; *erythro*-7*S*,8*R*; 7'*E*

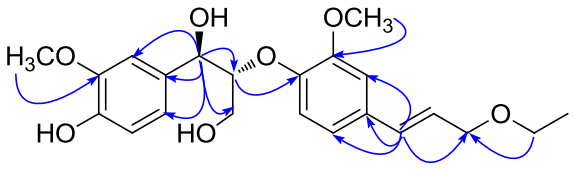


Figure 1. The structures of compounds **1**-**6** and the key HMBC correlations of **1**

3. Results and Discussion

Compound **1** gave the molecular formula $C_{22}H_{28}O_7$ from an HRESIMS ion at *m/z* 427.1493 $[M+Na]^+$ (calcd for $C_{22}H_{28}O_7Na$, 427.1473). The ^1H NMR spectrum of **1** (Table 1) showed six aromatic proton signals at δ 7.09 (1H, d, *J*=1.8 Hz, H-2), 6.77 (1H, d, *J*=8.1 Hz, H-5), 6.88 (1H, dd, *J*=8.1, 1.8 Hz, H-6), 7.05 (1H, d, *J*=1.8 Hz, H-2′), 7.02 (1H, d, *J*=8.3 Hz, H-5′) and 6.94 (1H, dd, *J*=8.3, 1.8 Hz, H-6′), revealing the presence of two ABX system aromatic rings. The proton signals at δ 4.90 (1H, d, *J* = 5.9 Hz, H-7), 4.32 (1H, m, H-8), 3.75 (1H, dd, *J* = 11.9, 4.0 Hz, H-9_a), 3.49 (1H, dd, *J* = 11.9, 5.4 Hz, H-9_b) and 6.58 (1H, d, *J* = 15.9 Hz, H-7′), 6.24 (1H, dt, *J* = 15.9, 6.2 Hz, H-8′), 4.14 (2H, d, *J* = 6.2 Hz, H-9′) established the occurrence of an 1,2,3-propanetriol moiety and a (*E*)-3-phenylprop-2-en-1-ol moiety. Additionally, two methoxyl groups protons attached to the aromatic ring at δ 3.84 (3H, s, 3-OCH_3), 3.90 (3H, s, 3′-OCH_3) and an ethoxyl group protons at δ 3.57 (2H, q, *J* = 7.0 Hz, H-1″) and 1.24 (3H, t, *J* = 7.0 Hz, H-2″) were observed. Analysis of the ^{13}C NMR (Table 1) of **1** revealed the presence of 22 carbon resonances, ascribed to two methoxy groups, an ethoxy group, twelve aromatic, four oxygenated carbons and two olefinic carbons signals. The HMBC correlations of H-7 at δ 4.90 with C-1, C-2, C-6, C-8 and C-9 and of H-7′ at δ 6.58 with C-1′, C-2′, C-6′ and C-9′ confirmed the presence of two phenyl propanoid units. In the HMBC spectrum, the correlation of H-8 at δ 4.32 with C-4′ at δ 149.4 suggested that **1** was an 8-*O*-4′ system neolignan. In addition, the HMBC correlations (Fig. 1) of the methoxyls and ethoxyl also confirmed their position. Analysis of HMBC spectra of **1** permitted the assignment of all proton and carbon resonances.

The $\Delta\delta_{C8-C7}$ values eliminating the effect of systematic errors [$\Delta\delta_{C8-C7}$ (*threo*)>$\Delta\delta_{C8-C7}$ (*erythro*)] were applicable to differentiate *threo* and *erythro* aryl glycerols without substituent(s) at C-7 or/and C-8 of the glycerol moiety as well as the *erythro* and *threo* 8-*O*-4′ isomers when the data were obtained in the same solvent [11, 12]. In our study, the $\Delta\delta_{C8-C7}$ value of **1** was larger than **2**, thus the relative configuration at C-7/C-8 of **1** was determined to be in the *threo*-form. The absolute configuration at C-8 was established on the basis of the CD spectroscopic evidence [13]. Since the CD spectra of **1** showed a negative Cotton effect at 229 nm, indicating **1** had the 8*R*-configuration according to the study of related system. On the basis of the bulkiness rule for secondary alcohols [9, 12], a negative Cotton effect at 350 nm (the E band) in the $Rh_2(OCOCF_3)_4$-induced CD spectrum indicated the 7*R* configuration for **1**, which was in agreement with that defined by the 7,8-*threo* and 8*R* configurations assigned above. Therefore, the structure of **1** was elucidated and named huangnin A.

The HRESIMS of **2** showed a quasimolecular ion $[M+Na]^+$ at *m/z* 427.1473 (calcd for $C_{22}H_{28}O_7Na$, 427.1473). The NMR spectra of **2** were identical with those of **1**, suggesting that the planar structure of **2** was the same as that of **1**. The smaller $\Delta\delta_{C8-C7}$ value of **2** suggested a relative-*erythro* configuration [11, 12]. A negative CD effect at 231 nm of **2**, justified a 7*S*,8*R*-configuration [13]. This was also supported by positive Cotton effects in the $Rh_2(OCOCF_3)_4$-induced CD spectra of

2 (352 nm) [9, 12]. Hence, it was concluded that compound **2** was as shown in Fig. 1 and named huangnin B.

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds **1** and **2** (in CD_3OD).

No.	**1**		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
1		133.8		134.1
2	7.09 (d, 1.8)	111.7	7.04 (d, 1.6)	111.9
3		148.8		148.7
4		147.1		147.1
5	6.77 (d, 8.1)	115.8	6.74 (d, 8.1)	115.7
6	6.88 (dd, 8.1, 1.8)	120.9	6.85 (dd, 8.1, 1.6)	121.0
7	4.90 (d, 5.9)	72.3	4.85 (d, 5.7)	74.1
8	4.32 (m)	86.9	4.39 (m)	86.2
9	3.75 (dd, 11.9, 4.0) 3.49 (dd, 11.9, 5.4)	61.9	3.86 (dd, 12.0, 5.9) 3.79 (dd, 12.0, 3.8)	62.2
1′		132.7		132.7
2′	7.05 (d, 1.8)	111.2	7.03 (br.s)	111.4
3′		151.7		151.9
4′		149.4		149.1
5′	7.02 (d, 8.3)	118.6	6.90 (br.s)	118.8
6′	6.94 (dd, 8.3, 1.8)	120.7	6.90 (br.s)	120.8
7′	6.58 (d, 15.9)	133.4	6.55 (d, 15.9)	133.4
8′	6.24 (dt, 15.9, 6.2)	125.6	6.21 (dt, 15.9, 6.2)	125.6
9′	4.14 (2H, d, 6.2)	74.0	4.13 (2H, d, 6.2)	72.3
1″	3.57 (2H, q, 7.0)	66.6	3.57 (2H, q, 7.0)	66.6
2″	1.24 (3H, t, 7.0)	15.4	1.23 (3H, t, 7.0)	15.4
3-OCH_3	3.84 (3H, s)	56.3	3.82 (3H, s)	56.3
3′-OCH_3	3.90 (3H, s)	56.5	3.83 (3H, s)	56.5

By comparing physical and spectroscopic data with literatures, the known compounds were readily identified as *threo*-(7*R*,8*R*)-1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(*E*)-3-hydroxy-1-propenyl]-2-methoxyphe-noxy}-1,3-propanediol (**3**) [14] *erythro*-(7*S*,8*R*)-1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(*E*)-3-hydroxy-1-propenyl]-2-methoxyphe-noxy}-1,3-propanediol (**4**) [14] *threo*-(7*R*,8*R*)-guaiacylglycerol-*β*-coniferyl aldehyde ether (**5**) [15] and *erythro*-(7*S*,8*R*)-guaiacylglycerol-*β*-coniferyl aldehyde ether (**6**) [15] by comparing the physicochemical and spectroscopic data with those reported literatures.

Many tyrosinase inhibitors, such as kojic acid and arbutin, have been alleged to have serious side effects. For example, kojic acid may cause cytotoxicity, skin cancer, and dermatitis, and it has been banned from use in cosmetics in many countries. Some phenylpropanoids (flavonoids or lignans) have been reported as relatively mild and safe tyrosinase or melanogenesis inhibitors, whereas they exhibit anti-oxidant, anti-inflammatory, and other biological activities [4]. These findings have motivated us to continue our research on natural flavonoids or lignans.

In previous investigations, 8-*O*-4′ neolignans had served as a lead compound for antioxidant agents [16-19]. Oxidative stress is one of the general pathological processes in melanogenesis, thus we assessed the antioxidant properties of the compounds in the seeds of *Crataegus pinnatifida*. Two methods were used, ABTS and DPPH assays. The results showed that compounds **1**-**6** exhibited strong effects against the ABTS radical, with IC_{50} values of 8.75, 13.16, 11.90, 12.40, 3.77 and 11.63 μg/mL, respectively (Table 2), which were stronger than or similar to that of the positive control (Trolox). The stronger activity of **5** compared to compounds **1** and **3** implies the importance of the aldehyde moiety in enhancing the radical-scavenging capacity of 8-*O*-4′ neolignans. In addition, the antioxidant

activities were also found to be related to the relative stereochemistry at C-7/C-8, and the radical-scavenging capacity of compounds **1**, **3** and **5** with the 7,8-*threo*-configuration were considered to be stronger than that of **2**, **4** and **6** with *erythro*-configuration. In the DPPH assay, the isolates that showed better antioxidant capacities were **5** (12.41 μg/mL), and **3** (39.66 μg/mL), in accordance with their antioxidant potencies established in the ABTS assay. Notably, the antioxidant potency of **5** was equivalent to that of Trolox (P>0.05), the positive control used in this study.

Next, we evaluated the tyrosinase inhibition activities of compounds **1**-**6**, abutin was used as positive control. As shown in Table 2, compounds **1**, **2** and **6** showed moderate inhibitory activities against mushroom tyrosinase, with 53.44%, 40.60% and 52.73% inhibition at 500 μg/mL, respectively. Compound **5** exerted the most potent inhibitory activity against mushroom tyrosinase, with 66.67% inhibition at the same concentration.

Our research proved that **5** could be potential inhibitors of melanin formation. However, further investigations are required to determine their abilities to inhibit melanin cells.

Table 2. Free radical scavenging and tyrosinase inhibition activities of compounds **1**-**6.**

Compound	ABTS (IC_{50}, μg/mL)	DPPH (IC_{50}, μg/mL)	Tyrosinase inhibition (%, at 500μg/mL)
1	8.75 ± 1.22	57.07 ± 3.22*	53.44 ± 3.22*
2	13.16 ± 0.97	78.51 ± 3.44*	40.60 ± 2.35*
3	11.90 ± 2.10	39.66 ± 2.12*	36.70 ± 2.44*
4	12.40 ± 1.33	70.11 ± 4.22*	32.50 ± 3.50*
5	3.77 ± 1.43*	12.41 ± 2.10	66.67 ± 2.14
6	11.63 ± 2.10	45.07 ± 2.50*	52.73 ± 3.20*
Trolox [a]	14.10 ± 0.89	9.96 ± 1.15	
Abutin [a]			75.48 ± 3.45

[a] Trolox and abutin were used as positive control; Values are mean±SD of triplicate determinations; * P<0.05 versus positive control group, n=3.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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