Antioxidant and Tyrosinase Inhibitory Effects of Neolignan Glycosides from *Crataegus pinnatifida* Seeds

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Key words
- *Crataegus pinnatifida*
- Rosaceae
- hawthorn
- neolignan glycosides
- tyrosinase-inhibiting activity
- antioxidant activity

Abstract

In our efforts to find an inhibitor of melanin formation and develop potential depigmenting agents for skin-protecting cosmetics and medicinal products from natural resources, we focused on the seeds of *Crataegus pinnatifida* which showed antioxidant and tyrosinase-inhibiting activities. By activity-guided fractionation of an extract of *C. pinnatifida* seeds, four new neolignan glycosides, pinnatifidaninsides A–D (**1**–**4**), along with two known compounds (**5**–**6**), were isolated. Their structures were elucidated by spectroscopic analyses, especially 1D, 2D NMR and CD spectra. The antioxidant and tyrosinase-inhibiting activities of all isolates were assayed. Compound **6** showed good activity against 2,2-diphenyl-1-pikrylhydrazyl, while compounds **1, 2, 5**, and **6** exhibited strong 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity, being as effective as, or even more effective than the positive control Trolox. Moreover, compounds **5** and **6** displayed a moderate mushroom tyrosinase inhibitory activity.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Drug discovery based on natural products has become a major strategy in modern pharmaceutical research and development, and roughly half of the currently used drugs are directly or indirectly derived from natural products [1]. To search for novel constituents with antioxidant and/or tyrosinase-inhibiting activities as lead structures for inhibitors of melanin formation, many medicinal plants have been screened using an *in vitro* antioxidant and tyrosinase inhibition assay. Among these plants and their extracts, the 70% ethanol extract of the seeds of *Crataegus pinnatifida* was found to exhibit a potent antioxidant and tyrosinase-inhibiting activity. *C. pinnatifida* Bge. (Rosaceae), also referred to as "hawthorn", is associated with about 280 wood plant species which are distributed in the Northern Hemisphere, mainly in China, Europe, and North America [2]. *C. pinnatifida* is one of the most popular medicinal and food sources in the world, and its fruits, leaves, bark, and flowers contain significant amounts of phenolic compounds which exhibit potent antioxidant and antityrosinase activity [3]. Using activity-guided fractionation of an extract of *C. pinnatifida* seeds, four new neolignan glycosides, pinnatifidaninsides A–D (**1**–**4**), along with two known compounds (**5**–**6**), have been isolated. In the present study, we evaluated the antioxidant and anti-tyrosinase activities of all isolates in order to obtain information about the structural features associated with the inhibitory activity of melanin formation and develop potential depigmenting agents for skin-protecting cosmetics and medicinal products.

Results and Discussion

Pinnatifidaninside A (**1**) was obtained as a yellow oil. The molecular formula of **1** was established as $C_{27}H_{38}O_{12}$ based on positive HRESIMS [*m/z* 577.2251 $(M + Na)^+$, calcd. for $C_{27}H_{38}O_{12}Na$, 577.2255]. In its ^1H NMR spectrum (**Table 1**), two sets of ABX proton signals at δ 6.92 (1H, d, J = 1.3 Hz), 6.74 (1H, d, J = 8.0 Hz), 6.80 (1H, dd, J = 8.0, 1.3 Hz) and 6.78 (1H, d, J = 1.7 Hz), 6.72 (1H, d, J = 8.2 Hz), 6.65 (1H, dd, J = 8.2, 1.7 Hz)

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Table 1 ^{1}H NMR data of compounds **1**–**4** (in CD_3OD).

No.	1[a]	2[a]	3[c]	4[b]
2	6.92 (d, 1.3)	6.97 (d, 1.7)	7.02 (d, 1.3)	7.01 (d, 1.8)
5	6.74 (d, 8.0)	6.89 (d, 8.0)	6.75 (d, 8.1)	6.75 (d, 8.1)
6	6.80 (dd, 8.0, 1.3)	6.83 (dd, 8.0, 1.7)	6.85 (dd, 8.1, 1.3)	6.83 (dd, 8.1, 1.8)
7	4.37 (d, 6.4)	4.44 (d, 5.9)	4.86 (d, 6.1)	4.84 (d, 5.7)
8	4.31 (m)	4.26 (m)	4.11 (m)	4.21 (m)
9	3.60 (m)	3.63 (m)	3.69 (dd, 11.3, 5.3)	3.87 (dd, 11.9, 4.5)
	3.21 (m)	3.25 (m)	3.43 (m)	3.42 (m)
2′	6.78 (d, 1.7)	6.85 (d, 1.9)	6.83 (d, 2.6)	6.81 (d, 2.7)
5′	6.72 (d, 8.2)	6.78 (d, 8.2)	6.97 (d, 8.8)	6.84 (d, 8.1)
6′	6.65 (dd, 8.2, 1.7)	6.70 (dd, 8.2, 1.9)	6.62 (dd, 8.8, 2.6)	6.61 (dd, 8.1, 2.7)
7′	2.64 (2H, t, 7.4)	2.66 (2H, t, 7.5)		
8′	1.89 (2H, m)	1.90 (2H, m)		
9′	3.89 (m)	3.87 (m)		
	3.55 (m)	3.54 (m)		
1′′	4.26 (d, 7.8)	4.25 (d, 7.7)	4.79 (d, 7.2)	4.80 (d, 6.6)
2′′	3.21 (m)	3.22 (m)	3.43 (m)	3.45 (m)
3′′	3.27 (m)	3.26 (m)	3.43 (m)	3.45 (m)
4′′	3.30 (m)	3.28 (m)	3.35 (m)	3.38 (m)
5′′	3.36 (m)	3.35 (m)	3.43 (m)	3.45 (m)
6′′	3.68 (m)	3.65 (m)	3.89 (dd, 10.5, 2.0)	3.92 (dd, 11.9, 1.8)
	3.86 (m)	3.85 (m)	3.68 (m)	3.70 (dd, 11.9, 5.9)
3-OCH_3	3.76 (3H, s)	3.82 (3H, s)	3.82 (3H, s)	3.80 (3H, s)
7-OCH_3	3.24 (3H, s)	3.25 (3H, s)		
3′-OCH_3	3.80 (3H, s)	3.83 (3H, s)	3.83 (3H, s)	3.83 (3H, s)

Coupling constants (*J*) in Hz are given in parentheses; chemical shift values are expressed in ppm. [a] for 300 MHz; [b] for 400 MHz; [c] for 600 MHz

were attributed to two 1,3,4-trisubstituted benzene rings, three methoxyl groups protons at δ 3.80 (3H, s), 3.76 (3H, s), and 3.24 (3H, s), and a glucopyranosyl anomeric proton at δ 4.26 (1H, d, J = 7.8 Hz). In addition, the ^{13}C NMR data (**Table 2**) exhibited 27 carbon resonances, which were classified by a DEPT experiment into three tertiary methyl, five methylene, 13 methine, and six quaternary carbons.

The ^{1}H-^{1}H COSY correlations in combination with HSQC spectroscopic data also revealed the presence of 1,2,3-propanetriol [δ_H 4.37 (H-7), 4.31 (H-8), 3.60 (H-9a), 3.21 (H-9b); δ_c 83.8 (C-7), 85.7 (C-8), 62.2 (C-9)] and 1-propanol [δ_H 2.64 (H-7′), 1.89 (H-8′), 3.89 (H-9′a), 3.55 (H-9′b); δ_c 32.7 (C-7′), 32.6 (C-8′), 69.9 (C-9′)]. The above evidence suggested the presence of two C6-C3 units arising both from a neolignan and a glucose moiety, which was supported by analysis of the ^{13}C NMR, COSY, and HMBC spectra. One and two-dimensional NMR techniques (DEPT, COSY, HMQC, and HMBC) permitted assignments of all the ^{1}H and ^{13}C NMR signals for **1** (**Table 1** and **2**). The HMBC correlation peaks of H-8 and C-4′ and the anomeric proton resonance of glucose H-1′′ to C-9′ indicated that compound **1** was an 8-*O*-4′-neolignan-9′-glucopyranoside. The cross peaks observed in NOESY spectrum between H-2 and 3-OCH_3, between H-2′ and 3′-OCH_3, and between H-7 and 7-OCH_3 placed the methoxy groups at C-3, C-3′, and C-7.

From the anomeric carbon (δ_C 104.5) and the anomeric proton [δ_H 4.26 (1H, d, J = 7.8 Hz)] and other characteristic NMR resonances, the sugar unit was identified as β-glucopyranose, which was further confirmed by strong NOESY signals between H-1′′ and H-3′′, H-5′′ [4]. Acid hydrolysis of **1** [5] with 2 M HCl afforded D-glucose by GC analysis following conversion to the trimethylsilyl thiazolidine derivatives. Regarding possible staggered conformers with intramolecular hydrogen bonding of the benzylic hydroxyl and aryloxyl groups, the large and small J values for H-7 and H-8 of 8-*O*-4′ neolignan diastereoisomers correspond to the *threo* form and *erythro* form, respectively [6]. So **1** was hydrolyzed with β-glucosidase to prepare its aglycone. After hydrolysis, the aglycone **1a** was obtained. In the ^{1}H NMR spectra of **1a** in $CDCl_3$, a large coupling constant $J_{7,8}$ = 8.0 Hz was observed, thus the relative configuration of C-7 and C-8 of **1a** and **1** was determined to be in the *threo* form. The absolute configuration at C-8 of **1** was established on the basis of CD spectroscopic evidence. The fact that the CD spectrum of **1** showed positive Cotton effects at 237 nm indicated that compound **1** had a 7*S*,8*S*-configuration according to a study of a related system [7, 8]. Thus, the structure of **1** was definitely that of (7*S*,8*S*)-*threo*-3,7,3′-trimethoxy-8-*O*-4′-neolignan-9′-*O*-β-D glucopyranoside.

The molecular formula of **2** was identified as $C_{27}H_{38}O_{12}$ by HRESIMS. Its ^{13}C NMR spectroscopic data were similar to those of **1** except for a small difference in the chemical shifts of C-7 and C-8 ($\Delta\delta$ 0.5–0.9). Based on analysis of the 2D NMR spectra, assignments of all the proton and carbon signals were possible (**Table 1** and **2**), and the planar structure of **2** was established as the same as that of **1**. Accordingly, compound **2** was identified as a diastereisomer of **1**. β-Stereochemistry of the anomeric carbon was determined by the coupling constant (J = 7.7 Hz) of the anomeric proton [4]. The D-configuration of glucose was determined by GC analysis following conversion to the trimethylsilyl thiazolidine derivatives [5]. In the ^{1}H NMR spectrum of the aglycone **2a**, obtained by enzymatic hydrolysis of **2**, a small coupling constant of H-7 (J = 4.4 Hz) was observed, which was different from that of **1a** and indicated that the relative configuration of C-7 and C-8 of **2** was in the *erythro*-form. The positive CD effect at 233 nm of **2** supported a 7*R*,8*S*-configuration as shown in **Fig. 1**. Therefore, **2** was characterized as (7*R*,8*S*)-*erythro*-3,7,3′-trimethoxy-8-*O*-4′-neolignan-9′-*O*-β-D glucopyranoside and named pinnatifidaninside B.

Compound **3** exhibited a quasimolecular ion peak at *m/z* 521.1626 $[M + Na]^+$ in positive HRESIMS analysis, corresponding

Table 2 ^{13}C NMR data of compounds **1–4** (at 75 MHz, in CD_3OD).

No.	1	2	3	4
1	131.0	131.0	133.8	134.2
2	112.4	112.2	111.8	111.8
3	148.9	149.1	148.8	148.7
4	147.4	147.9	147.2	147.0
5	115.7	116.0	115.9	115.7
6	122.0	122.0	120.8	120.9
7	83.8	84.3	74.2	74.1
8	85.7	86.2	88.4	87.3
9	62.2	62.1	61.8	62.1
1′	137.8	137.8	155.0	155.0
2′	114.2	114.2	104.0	104.0
3′	151.7	151.7	152.5	152.7
4′	147.3	147.5	144.8	144.4
5′	119.3	119.1	120.7	120.7
6′	121.8	121.6	109.8	109.6
7′	32.7	32.7		
8′	32.6	32.7		
9′	69.9	69.9		
1″	104.5	104.5	103.2	103.2
2″	75.2	75.2	74.9	74.9
3″	77.9	77.9	78.0	78.0
4″	71.7	71.7	71.6	71.5
5″	78.1	78.1	78.2	78.2
6″	62.8	62.8	62.6	62.6
3-OCH_3	56.4	56.4	56.5	56.4
7-OCH_3	57.0	57.2		
3′-OCH_3	56.5	56.6	56.4	56.4

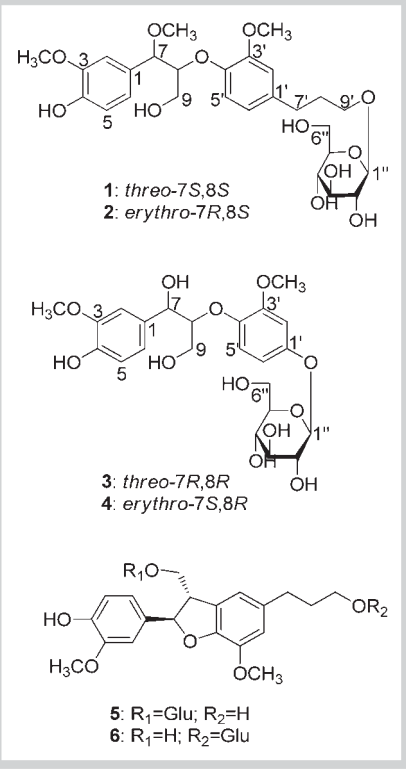

Fig. 1 Structures of compounds **1–6**.

to a molecular formula of $C_{23}H_{30}O_{12}$. The ^1H NMR of **1** showed signals attributed to two 1,3,4-trisubstituted aromatic rings at δ 7.02 (H-2), 6.75 (H-5), 6.85 (H-6) and 6.83 (H-2′), 6.97 (H-5′), 6.62 (H-6′), together with signals attributed to two aromatic methoxys at δ 3.82 and 3.83. In addition, an arylglyceryloxy unit was indicated by signals of a vicinal coupling system attributed to two oxymethines at δ 4.86 (H-7) and 4.11 (H-8) and an oxymethylene at δ 3.69 (H-9_a) and 3.43 (H-9_b). A doublet, assignable to an anomeric proton at δ 4.79, partially overlapped signals attributed to oxymethylene and oxymethine protons between δ 3.40 and 3.95, suggesting that there was a glycosyl moiety in **3**. Analysis of the ^{13}C NMR and DEPT spectra (**Table 2**) of **3** revealed the presence of 23 carbon resonances, ascribed to two tertiary methyl, two methylene, 13 methine, and six quaternary carbons. Overall, the proton and carbon signals in the ^1H and ^{13}C NMR data of **3** were similar to those of **1** except for reduction of the side chain (C-7′, C-8′, and C-9′ of **1**), indicating a derivative of 8-*O*-4′ neolignan glycoside. The HMBC and NOESY correlations (**Fig. 2**) of the two methoxyls also confirmed their positions. The correlation of HMBC between the signal at δ 155.0 (C-1′) and δ 4.79 (H-1″) indicated that the glucopyranosyl group was located at C-1′.

β-Stereochemistry of the anomeric carbon was determined by the coupling constant (J = 7.2 Hz) of the anomeric proton, which was further confirmed by strong NOESY signals between H-1″ and H-3″, H-5″ [4]. The absolute configuration of glucose was confirmed by acidic hydrolysis of **3**, which produced β-D-glucopyranose as the sole sugar, as identified by GC comparison with an authentic sample [5]. The *threo* configuration of **3** was confirmed by the fact that the $\Delta\delta_{C8\text{-}C7}$ value of **3** was larger than that of **4**. It has been reported that the $\Delta\delta_{C8\text{-}C7}$ values, eliminating the effect of systematic errors [$\Delta\delta_{C8\text{-}C7}$ (*threo*) > $\Delta\delta_{C8\text{-}C7}$ (*erythro*)], could also be used 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 [9, 10]. The absolute configuration at C-8 of **3** was established on the basis of the CD spectroscopic evidence [8, 9]. The fact that the CD spectra of **3** showed negative Cotton effects at 229 nm indicated that compound **3** had a 7*R*,8*R*-

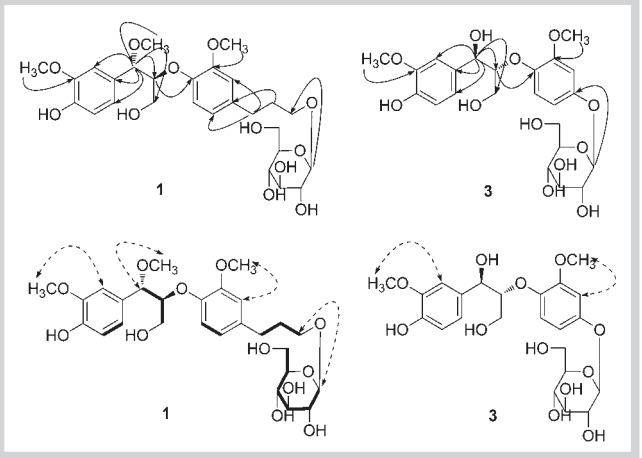

Fig. 2 Key HMBC (→), ^{1}H-^{1}H COSY (—), and NOESY (↔) correlations of compounds **1** and **3**.

configuration according to the study of a related system. Therefore, the structure of **3** was identified, and it was named pinnatifidaninside C.

The NMR spectrum of **4** was identical to that of **3**, suggesting that the planar structure of **4** was the same as that of **3**. The *β*-form of glucose was indicated by the coupling constant of the anomeric proton signal at δ_H 4.80 (1H, d, J = 6.6 Hz) in the ^{1}H NMR data, which was further confirmed by NOESY signals [4]. The absolute configuration of glucose was determined by acid hydrolysis and comparison with an authentic sample by GC [5]. The small $\Delta\delta_{C8\text{-}C7}$ value of **4** suggested a relative *erythro* configuration [6, 9, 10]. The negative CD effect at 229 nm of **4** supported a 7*S*,8*R*-configuration [7, 8] as shown in **Fig. 1**, and the compound was named pinnatifidaninside D.

By comparing the physical and spectroscopic data with literature values, the known compounds were readily identified as 7*R*,8*S*-dihydrodehydrodiconiferyl alcohol-9-*O*-*β*-D-glucoside (**5**) [11] and 7*R*,8*S*-dihydrodehydrodiconiferyl alcohol-9′-*O*-*β*-D-glucoside (**6**) [12].

Melanin, the major pigment of skin, plays an important role in protecting it against the harmful effects of UV-irradiation. However, an increase in the levels of epidermal melanin synthesis and its uneven distribution can cause esthetic problems such as freckles, melasma, and aging spots [13]. Melanin synthesis is controlled by a cascade of enzymatic reactions. Tyrosinase, which is the rate-limiting enzyme in melanin synthesis, catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone [14]. In addition, the uncontrolled production of reactive oxygen species (ROS) is one of the major factors involved in melanin synthesis and skin damage. ROS may induce melanocyte proliferation and the release of a-melanocyte-stimulating hormone (a-MSH), associated with an excess of melanogenesis, leading to abnormal pigmentation. Antioxidants can prevent or delay pigmentation by several different mechanisms, e.g., by scavenging ROS or by reducing o-quinones or other intermediates in melanin biosynthesis, thereby delaying oxidative polymerization [15]. Hence, the use of antioxidants and/or tyrosinase inhibitors is becoming increasingly important in the areas of cosmetics and medicinal product development due to their preventive effects in pigmentation disorders and skin aging [16].

Table 3 Free radical scavenging activity of compounds **1**–**6**.

Compound	DPPH (IC_{50}, μg/mL)	ABTS (IC_{50}, μg/mL)
1	64.65 ± 2.82	7.40 ± 0.84
2	79.86 ± 3.24	8.40 ± 0.89
3	65.83 ± 2.23	26.13 ± 1.42
4	66.46 ± 2.00	32.03 ± 2.23
5	64.73 ± 3.83	13.60 ± 2.59
6	24.06 ± 1.47	5.22 ± 1.24
Trolox[a]	9.96 ± 1.01	14.10 ± 2.66

[a] Trolox was used as positive control; values are mean ± SD of triplicate determinations

Several studies have suggested a strong correlation between the phenolic composition of the sample and its free radical scavenging activity [17]. Neolignan glycosides have also been claimed to possess antioxidation activity and could serve as lead compounds for the development of antioxidant agents [18–20]. The 2,2-diphenyl-1-pikrylhydrazyl (DPPH) radical scavenging assay is usually used to evaluate the ability of new compounds or extracts to capture free radicals by producing the reduced form DPPH-H through a hydrogen-donating action. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) cation ($ABTS^+$) is another synthetic radical which is more versatile than DPPH: the $ABTS^+$ model can be used to assess the scavenging activity for both polar and nonpolar samples [21, 22]. The results of the DPPH and $ABTS^+$ radical scavenging activities of all the isolates are summarized in **Table 3**. Among the tested compounds, **6** showed the most potent antioxidant activity in the DPPH assay, although it was slightly weaker than the positive control Trolox, and other compounds also showed moderate antioxidant activity. In the $ABTS^+$ assay, compounds **1, 2, 5**, and **6** showed strong $ABTS^+$ radical scavenging activity with IC_{50} values of 7.40, 8.40, 13.60, and 5.22 μg/mL, respectively, indicating that these compounds are as effective as, or even more effective than the positive control. The structural requirements of the active constituents were examined. The stronger antioxidant activity of **1** and **2** compared to **3** and **4** implies the importance of the side chain in enhancing the radical scavenging capacity of 8-*O*-4′ neolignan glycosides. It is also notable that compound **6** exhibited more significant antioxidant activity than compound **5** suggesting that the antioxidant activity of dihydrobenzofuran neolignan glycosides is probably related to the position of the glucopyranosyl moiety. In consideration of the structures of the pair of structural isomers, we found that the 9′-*O*-*β*-D-glucoside may enhance the antioxidant activity of dihydrobenzofuran neolignan glycosides.

Tyrosinase inhibition by the isolates (**1**–**6**) was examined with the expectation that compound **6** would show stronger anti-tyrosinase activity. The inhibitory activities were examined using mushroom tyrosinase as described previously with minor modifications, and arbutin was used as a positive control [23]. The inhibition of mushroom tyrosinase is summarized in **Fig. 3**. Compounds **1**–**4** exhibited weak inhibitory activity against mushroom tyrosinase with 37.58%, 34.54%, 31.15%, and 32.97% inhibition at 500 μg/mL, respectively. However, compounds **5** and **6** exhibited moderate inhibitory activity against mushroom tyrosinase with 46.00% and 58.15% inhibition at the same concentration.

In summary, four new 8-*O*-4′ neolignan glucosides and two known dihydrobenzofuran neolignan glycosides were isolated from *C. pinnatifida* seeds, and their free radical scavenging activities and tyrosinase inhibitory effects were evaluated in the cell-

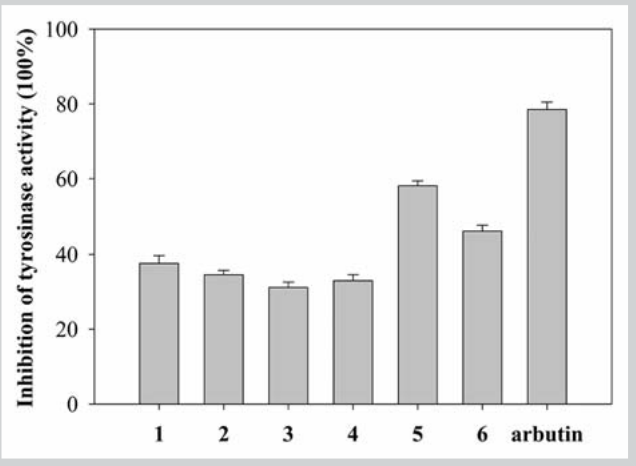

Fig. 3 Tyrosinase inhibition of compounds **1–6** (at 500 µg/mL). Arbutin was used as positive control; each column represents the mean ± SD of triplicate determinations.

free system. Compound **6** showed good activity against DPPH (IC_{50} 24.06 µg/mL), while compounds **1, 2, 5**, and **6** exhibited potent $ABTS^+$ free radical scavenging activity (IC_{50} 5.22–13.60 µg/mL), being as effective as, or even more effective than the positive control. Moreover, **5** and **6** displayed a moderate effect on tyrosinase producing 58.15% and 46.00% inhibition, respectively. However, the ability of these compounds to inhibit melanin cells (such as through inhibition of melanin production in B16-F10 melanoma cells) remains to be determined in future experiments, as well as their toxicity and the safety of their use before practical application.

Materials and Methods

General experimental procedures

Optical rotations were obtained by using a JASCO DIP-370 digital polarimeter. The UV spectra were measured on a Shimadzu UV-1700 spectrometer. CD spectra were obtained using MOS 450 detector from BioLogic in the 200–400 nm wavelength range. The FT-IR spectra were obtained on a Bruker IFS-55 spectrometer. ^{1}H-NMR, ^{13}C-NMR, HMBC, and HSQC spectra were recorded on Bruker ARX-300, Bruker ARX-400, and Bruker AV-600 spectrometers with TMS as an internal standard. HRESIMS experiments were performed on an Agilent G6520 Q-TOF spectrometer. The chromatographic silica gel (200–300 mesh) was produced from Qingdao Ocean Chemical Factory, ODS (50 µm) by YMC Co. Ltd., and Sephadex LH-20 by GE Healthcare. Macroporous adsorption resin D101 was produced by Cangzhou Bon Adsorber Technology Co., Ltd. Semipreparative RP-HPLC isolation was achieved with an Agilent 1100 instrument using a YMC 5 µm C18 column (250 mm × 10 mm) eluting with CH_3CN-H_2O or CH_3OH-H_2O at 4.0 mL/min. Peak detection was made with a refractive index detector (RID). GC analysis was performed on an Agilent 7890A equipped with an H_2 flame ionization detector (FID) and a HP-5 quartz capillary column (30 m × 320 µm × 0.25 µm). The absorbances in bioassays were measured and recorded on Varioskan Flash Multimode Reader (Thermo Scientific). TLC plates were precoated with silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd.) and visualized under a UV lamp at 254 nm or by spraying 5% vanillin-H_2SO_4 (w/v).

Plant material

The seeds of hawthorn were collected from Shijiazhuang, Hebei province, P.R. China, in June 2011, and were identified by Professor J.C. Lu (Department of Natural Products Chemistry, Shenyang Pharmaceutical University). A voucher specimen (No. 20110701) has been deposited in the Herbarium of the Shenyang Pharmaceutical University.

Extraction and isolation

The air-dried seeds of hawthorn (30 kg) were crushed to pieces and refluxed with 70% EtOH for 3 × 30 L × 4 h. The solvent was evaporated under vacuum. Then, the extract (1500 g) was suspended in H_2O (20 L) and partitioned with ethyl acetate (3 × 20 L). The ethyl acetate extract (420 g) was suspended in H_2O (5 L) and then chromatographed over D101 macroporous resin column (⌀ 12 × 60 cm) using H_2O-EtOH [100 : 0 (20 L), 70 : 30 (20 L), 40 : 60 (20 L), and 5 : 95 (20 L)] 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-CH_3OH [100 : 0 (5 L), 90 : 10 (5 L), 80 : 20 (5 L), 70 : 30 (5 L), 50 : 50 (5 L)] to afford eight fractions (1–8). Fraction 6 (21.2 g) was further purified over an ODS CC (⌀ 10 × 60 cm) using MeOH-H_2O as the mobile phase with a gradient [95 : 5 (4 L), 90 : 10 (4 L), 80 : 20 (4 L), 70 : 30 (4 L), 50 : 50 (4 L)] to afford five fractions (F_{6-1} – F_{6-5}) based on HPLC analysis. F_{6-2} (4.2 g) was subjected to another silica gel column (⌀ 2 × 30 cm) and eluted with CH_2Cl_2:CH_3OH [95 : 5 (2 L), 90 : 10 (2 L), 85 : 15 (2 L), 80 : 20 (2 L)] to afford eleven fractions (F_{6-2-1} – F_{6-2-11}) based on TLC analysis. F_{6-2-5} was subjected to semipreparative HPLC eluted with CH_3OH-H_2O (40 : 60) at 4 mL/min to yield **1** (17 mg, t_R 47 min) and **2** (18 mg, t_R 53 min). F_{6-2-7} was subjected to semipreparative HPLC eluted with CH_3CN-H_2O (18 : 82) at 4 mL/min to yield **3** (13 mg, t_R 28 min) and **4** (12 mg, t_R 22 min). F_{6-2-6} was subjected to semipreparative HPLC eluted with CH_3CN-H_2O (22 : 78) at 4 mL/min to yield **5** (33 mg, t_R 54 min) and **6** (280 mg, t_R 29 min). The purity of compounds **1**-**6** was greater than 95% as determined by TLC and NMR.

Pinnatifidaninside A (**1**): yellow oil; $[\alpha]_D^{20}$ + 8.2 (*c* = 0.10, CH_3OH); CD (CH_3OH) nm: 237 (3.81); UV (CH_3OH) λ max (log ε): 229 (3.72) nm, 280 (1.39) nm; IR (KBr) ν_{max}: 3445, 2919, 2850, 1641, 1463, 1119, 848, 779, 618 cm^{-1}; HRESIMS *m*/*z* 577.2251 $[M + Na]^+$ (calcd. for $C_{27}H_{38}O_{12}Na$, 577.2255); ^{1}H and ^{13}C NMR, see **Table 1** and **2**.

Pinnatifidaninside B (**2**): yellow oil; $[\alpha]_D^{20}$ − 18.5 (*c* = 0.08, CH_3OH); CD (CH_3OH) nm: 233 (5.25); UV (CH_3OH) λ max (log ε): 231 (3.72) nm, 279 (1.02) nm; IR (KBr) ν_{max}: 3441, 2919, 2850, 1678, 1590, 1463, 1135, 1031, 849, 777, 618 cm^{-1}; HRESIMS *m*/*z* 577.2253 $[M + Na]^+$ (calcd. for $C_{27}H_{38}O_{12}Na$, 577.2255); ^{1}H and ^{13}C NMR, see **Table 1** and **2**.

Pinnatifidaninside C (**3**): yellow oil; $[\alpha]_D^{20}$ − 16.2 (*c* = 0.10, CH_3OH); CD (CH_3OH) nm: 229 (− 7.10); UV (CH_3OH) λ max (log ε): 231 (3.62) nm, 280 (1.31) nm; IR (KBr) ν_{max}: 3405, 2920, 2850, 1606, 1462, 1125, 1031, 848, 618 cm^{-1}; HRESIMS *m*/*z* 521.1626 $[M + Na]^+$ (calcd. for $C_{23}H_{29}O_{12}$, 521.1629); ^{1}H and ^{13}C NMR, see **Table 1** and **2**.

Pinnatifidaninside D (**4**): yellow oil; $[\alpha]_D^{20}$ − 4.5 (*c* = 0.08, CH_3OH); CD (CH_3OH) nm: 229 (− 8.25); UV (CH_3OH) λ max (log ε): 230 (3.77) nm, 282 (1.41) nm; IR (KBr) ν_{max}: 3404, 2919, 1605, 1462, 1383, 1125, 1031, 848, 618 cm^{-1}; HRESIMS *m*/*z* 521.1600 [M +

Na]$^+$ (calcd. for $C_{23}H_{29}O_{12}$, 521.1629); ^{1}H and ^{13}C NMR, see ◉ **Table 1** and **2**.

Confirmation of glycosyl units

Compounds **1**–**4** (2.0 mg) were hydrolyzed with 2 M TFA (1.5 mL), heated for 3 h at 110 °C and extracted with $CHCl_3$ (3 × 1.5 mL). The aqueous layer was concentrated to dryness under a stream of nitrogen. The aqueous residue was redissolved in anhydrous pyridine (1.0 mL), and L-cysteine methyl ester hydrochloride (3.0 mg) was added to the solution of pyridine. The mixture was heated at 60 °C for 2 h, and 0.5 mL n-trimethylsilyimidazole (TMSI) was added, followed by heating at 60 °C for 1 h. The reaction product was analyzed by GC under the following conditions: GC: Agilent 7890 A equipped with an H_2 FID and a HP-5 quartz capillary column (30 m × 320 µm×0.25 µm); column temperature: 150–250 °C with the rate of 10 °C/min; carrier gas: N_2 (1 mL/min); split ratio: 10 : 1; injection temperature: 280 °C; detector temperature: 280 °C; injection volume: 1.0 µL. The monosaccharides of compounds **1**–**4** were confirmed by comparison of the retention time of monosaccharides derivatives with those of standard sugars (the standard sugars were subjected to the same reaction, D-glucopyranose: 15.2 min; L-glucopyranose: 15.5 min).

Enzymatic preparation of aglycones 1a and 2a from 1 and 2

Compounds **1** and **2** (6.0 mg) were hydrolyzed with 20 mg of *β*-glucosidase (E.C 3.2.1.21, Cat. No. G0395–5 KU, from almonds lyophilized powder, Sigma Inc.) in 1.5 mL of H_2O at 37 °C for 10 h. The reaction mixture was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated *in vacuo* and subjected to semipreparative HPLC using 28% CH_3CN-H_2O (4 mL/min) to give the aglycones **1a** (0.4 mg, t_R 25 min) and **2a** (1.2 mg, t_R 27 min). **1a**: ^{1}H NMR (in $CDCl_3$, at 400 MHz) *δ* 6.50–7.50 (6H, m, H-2, 5, 6, 2′, 5′ and 6′), 4.41 (1H, d, *J* = 8.0 Hz, H-7), 4.25 (1H, m, H-8), 3.91 (3H, s, 3′-OCH_3), 3.89 (3H, s, 3-OCH_3), 3.50–4.20 (2H, m, H-9), 3.66 (2H, t, *J* = 6.4 Hz, H-9′), 2.67 (2H, m, H-7′), 1.86 (2H, m, H-8′). **2a**: ^{1}H NMR (in $CDCl_3$, at 400 MHz) *δ* 6.50–7.00 (6H, m, H-2, 5, 6, 2′, 5′ and 6′), 4.41 (1H, d, *J* = 4.4 Hz, H-7), 4.24 (1H, m, H-8), 3.91 (3H, s, 3′-OCH_3), 3.89 (3H, s, 3-OCH_3), 3.80–4.20 (2H, m, H-9), 3.65 (2H, t, *J* = 6.4 Hz, H-9′), 2.67 (2H, m, H-7′), 1.87 (2H, m, H-8′).

Antioxidant activity assay

In this assay, Trolox (purity > 98%; Sigma) was used as a positive control. A 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. 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.

The capability to scavenge DPPH/$ABTS^{\bullet+}$ was calculated using the formula given below:

$$\text{DPPH/ABTS}^{\bullet+}\text{ scavenging activity (\%)} = [1 - (S - SB)/(C - CB)] \times 100\%$$

where S, SB, C, and CB are the absorbencies of the sample, the blank sample, the control, and the blank control, respectively [22].

Inhibitory activity to tyrosinase

This assay was performed according to the procedure of Dai et al. [23] with slight modifications, using L-tyrosine as a substrate. Arbutin (purity > 98%; Sigma) was used as a positive control.

Supporting information

Biological experimental details as well as ^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC NMR, UV, IR, CD, and HRESIMS spectra of new compounds (**1**–**4**) are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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