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Oleanane-type triterpene saponins with collagen synthesis-promoting activity from the flowers of *Bellis perennis*

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ABSTRACT

The methanol extract from *Bellis perennis* (Asteraceae) flowers was found to promote collagen synthesis in normal human dermal fibroblasts (NHDFs). Seven oleanane-type triterpene saponins, perennisosides XIII–XIX, and two known saponins, bellissaponins BS5 and BS9, were isolated from the methanol extract. The structures were determined based on chemical and physicochemical data, and confirmed using previously isolated related compounds as references. Among the isolates, including 19 previously reported saponins, perennisosides XVIII, I, II, VII, IX, and XI, asterbatanoside D, bernardioside B_2, and bellissaponins BS5 and BS9 significantly promoted collagen synthesis at 3–30 μM without cytotoxicity.

1. Introduction

Bellis perennis is an Asteraceae plant species commonly known as the daisy, which is widely distributed in Europe and North Africa. The whole flowering plant of *B. perennis* has been used for the treatment of bruises, bleeding, muscular pain, purulent skin diseases, and rheumatism in European folk medicine (Gruenwald et al., 2007). During studies exploring the bioactive constituents of *B. perennis* flowers, isolation and structural elucidation of triterpene saponins, flavonoids, and aromatic and acyclic alcohol glycosides were reported (Karakas et al., 2014; Morikawa et al., 2008, 2010, 2011; Yoshikawa et al., 2008). It was also found that the methanol extract and several saponin constituents inhibited elevation of triglyceride levels in plasma (Yoshikawa et al., 2008) and pancreatic lipase activity (Morikawa et al., 2010), and induced gastric emptying in olive oil-loaded mice (Morikawa et al., 2011). Evaluation of the methanol extract established that it can promote collagen synthesis in normal human dermal fibroblasts (NHDFs). Separation of the active constituents in the extract resulted in isolation of seven new oleanane-type triterpene saponins, called perennisosides XIII–XIX (**1**–**7**). This study deals with the isolation and structural elucidation of these new saponins (**1**–**7**), as well as collagen synthesis activity of its saponin constituents.

2. Results and discussion

2.1. Effects of the methanol extract on collagen synthesis in NHDFs

One of the main causes of skin aging is the reduction of type-I collagen, a primary component of the dermal layer of skin (Meigel et al., 1977). Thus, compounds that maintain type-I collagen levels may be useful to prevent skin aging. (Lee et al., 2007). Collagen is a fibrous extracellular matrix protein. In addition, it is a major component of connective tissue in the human body. Approximately 3–6% of the total tissue protein in the body is collagen, and the functional properties of skin depend on the integrity of collagen in the dermis. Collagen deposition is finely controlled, and is dependent on the physiological status of the body. Changes in the rate of collagen deposition occur during wound healing, development of new bone, and aging. Thus, the control of collagen metabolism may be useful for various therapeutic

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and cosmetic applications (Koya-Miyata et al., 2004). Medicinal foods could be potential resources for these applications because of their low toxicity.

Dried flowers of *B. perennis* were extracted with methanol under conditions of reflux to obtain the methanol extract (25.8% from the dried material). The methanol extract was partitioned using ethyl acetate (EtOAc)–H_2O (1:1, v/v) to yield an EtOAc-soluble fraction (6.7%) and an aqueous phase. The latter was subjected to Diaion HP-20 column chromatography (H_2O → MeOH) to yield H_2O- and MeOH-eluted fractions (12.5% and 6.4%, respectively). The methanol extract (1–10 μg/mL) was found to promote collagen synthesis in normal human dermal fibroblasts (NHDFs) (Table 1). Using bioassay-guided fractionation, the MeOH-eluted fraction was found to be the active fraction (% control of collagen content at 10 μg/mL: 147.3 ± 1.9, $p < 0.05$) without cytotoxicity [cell viability (%) in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: 98.8 ± 1.1]. In contrast, the EtOAc-soluble fraction (% control of collagen content at 10 μg/mL: 100.0 ± 4.2; cell viability (%): 102.9 ± 0.8) and the H_2O-eluted fraction (87.8 ± 9.1 and 105.9 ± 0.8, respectively) showed no activity.

2.2. Isolation

In previous studies, 34 saponin constituents were isolated from the active MeOH-eluted fraction including perennisosides I (**8**, 0.0122%), II (**9**, 0.0110%), III (**10**, 0.0026%), VII (**11**, 0.0089%), VIII (**12**, 0.0124%), IX (**13**, 0.0076%), XI (**14**, 0.0132%), and XII (**15**, 0.0111%), perennisaponins B (**16**, 0.0048%), F (**17**, 0.0586%), and K (**18**, 0.0268%), asterbatanoside D (**19**, 0.0063%), bernardioside B_2 (**20**, 0.0128%), bellidioside A (**21**, 0.0575%), bellissaponins BS1 (**22**, 0.0035%) and BS6 (**23**, 0.0233%), and bellisosides D (**24**, 0.0262%), E (**25**, 0.1906%), and F (**26**, 0.0262%) (Morikawa et al., 2008, 2010, 2011; Yoshikawa et al., 2008).

In the present study, several additional constituents in the MeOH-eluted fraction were identified using normal-phase silica gel and reversed-phase ODS column chromatography, followed by HPLC. Seven new oleanane-type triterpene saponins, called perennisosides XIII (**1**, 0.0022%), XIV (**2**, 0.0130%), XV (**3**, 0.0007%), XVI (**4**, 0.0143%), XVII (**5**, 0.0130%), XVIII (**6**, 0.0117%), and XIX (**7**, 0.0005%), and two known saponins, bellissaponins BS5 (**27**, 0.0059%) (Glensk et al., 2001; Wray et al., 1992) and BS9 (**28**, 0.0029%) (Glensk et al., 2001) were isolated as shown in Fig. 1.

2.3. Structures of perennisosides XIII–XIX (**1**–**7**)

Perennisoside XIII (**1**) was obtained as an amorphous powder with a positive optical rotation ($[\alpha]_D^{27}$ +7.6 in MeOH). Using positive- and negative-ion FABMS, quasimolecular ion peaks were observed at *m/z* 1229 $[M+Na]^+$ and 1205 $[M-H]^-$, and HRFABMS analysis established that its molecular formula was $C_{58}H_{94}O_{26}$. Treatment of **1** with 0.5% sodium methoxide (NaOMe)–MeOH produced a des-acyl derivative and methyl (*S*)-(+)-3-hydroxybutyrate (Burk et al., 1995), which was identified by HPLC using an optical rotation detector (Morikawa et al., 2010, 2011; Yoshikawa et al., 2008). The des-acyl derivative was treated successively with 5% aqueous sulfuric acid (H_2SO_4)–1,4-dioxane (1:1, v/v) to liberate bayogenin (Kasai et al., 1988), together with L-rhamnose and D-glucose; these were identified by HPLC using an optical rotation detector (Morikawa et al., 2010, 2011; Yoshikawa et al., 2008). The ^1H (Table 2) and ^{13}C NMR (Table 3) spectra (pyridine-d_5) of **1**, which were assigned with the aid of DEPT, TOCSY, ^1H–^1H COSY, HSQC, HMBC, and phase-sensitive ROESY experiments, showed signals assignable to six methyls [δ 0.85, 0.87, 1.23, 1.34, 1.35, 1.67 (3H each, all s, H_3-29, 30, 26, 27, 24, 25)], a methylene, and two methines bearing an oxygen function [δ 3.66, 4.15 (1H each, both d, J = 10.3 Hz, H_2-23), 4.22 (1H, br s, H-3), 4.55 (1H, br s, H-2)], an olefinic proton [δ 5.49 (1H, t-like, J = *ca.* 3 Hz, H-12)], three glucopyranosyl moieties [δ 4.96 (1H, d, J = 7.6 Hz, Glc-H-1‴″), 5.11 (1H, d, J = 7.9 Hz, Glc-H-1‴′), 6.03 (1H, d, J = 7.6 Hz, Glc-H-1′)], and a rhamnopyranosyl moiety [δ 1.60 (3H, d, J = 6.2 Hz, Rha-H_3-6″), 6.51 (1H, br s, Rha-H-1‴)] with a 3-hydroxybutyryl group {δ 1.54 (3H, d, J = 6.2 Hz, 3HB-H_3-4‴″″), [2.80 (1H, dd, J = 4.8, 13.7 Hz), 2.94 (1H, dd, J = 8.2, 13.7 Hz), 3HB-H_2-2‴″″], 4.69 (1H, m, 3HB-H-3‴″″)}. The ^1H and ^{13}C NMR spectra of **1** resembled that of bernardioside B_2 (**20**) (Schöpke et al., 1996), except for the signals due to an additional β-D-glucopyranosyl moiety (Glc-H-1‴″) and a 3-hydroxybutyryl group. As shown in Fig. S1, the TOCSY and ^1H–^1H COSY experiments on **1** indicated the presence of partial structures written in bold lines. In the HMBC experiment of **1**, long-range correlations were observed between the following protons and carbons: Glc-H-1″ and C-28 (δ_C 176.3); Glc-H-2″ [δ 4.32 (m)] and Rha-C-1 (δ_C 101.1); Rha-H-1‴ and C-2″ (δ_C 75.1); Glc-H-1‴″ and C-3″ (δ_C 88.8); Glc-H-1‴″ and C-6″ (δ_C 69.2); and 3HB-H_2-2‴″″, Rha-H-4‴ and 3HB-C-1‴″″ (δ_C 171.9) (Fig. S1). The 28-*O*-sugar moieties in **1** were also characterized by a phase-sensitive ROESY experiment as shown in Fig. 2. The evidence mentioned above led to deduction of the structure of **1** as bayogenin {28-*O*-[4-*O*-(3*S*)-3-hydroxybutyryl]-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 3)]-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl} ester.

Perennisoside XIV (**2**) was obtained as an amorphous powder with a negative optical rotation ($[\alpha]_D^{23}$ −2.3 in MeOH). In the positive-ion FABMS, a quasimolecular ion peak was observed at *m/z* 1305 $[M+Na]^+$, and HRFABMS analysis indicated that the molecular formula was $C_{60}H_{98}O_{29}$. Acid hydrolysis of **2** with 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v) liberated bayogenin together with L-rhamnose and D-glucose, which were again identified by HPLC using an optical rotation detector. The ^1H (Table 2) and ^{13}C NMR (Table 3) spectra (pyridine-d_5) showed signals assignable to the bayogenin portion [δ 0.84, 0.86, 1.19, 1.22, 1.33, 1.62 (3H each,

Table 1
Effects of methanol extract of *B. perennis* flowers and TGF-β1 on collagen synthesis in NHDFs.

		Collagen content (% of control)			
		0 μg/mL	1 μg/mL	3 μg/mL	10 μg/mL
MeOH extract		100.0 ± 1.7 (100.0 ± 3.5)	115.5 ± 2.8[a] (95.4 ± 1.5)	118.9 ± 5.5[b] (95.8 ± 3.0)	140.2 ± 1.4[b] (89.9 ± 0.9)
	0 ng/mL	1 ng/mL	3 ng/mL	10 ng/mL	30 ng/mL
TGF-β1	100.0 ± 1.8 (100.0 ± 2.4)	196.5 ± 5.7[b] (85.2 ± 1.2)	240.0 ± 5.7[b] (74.1 ± 3.1[c])	255.9 ± 10.1[b] (71.0 ± 2.6[c])	261.0 ± 2.0[b] (70.5 ± 2.0[c])

Each value represents the mean ± S.E.M. (N = 4).
Significantly different from the control.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] Cytotoxic effects were observed, and values in parentheses indicate cell viability (%) in MTT assay.

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