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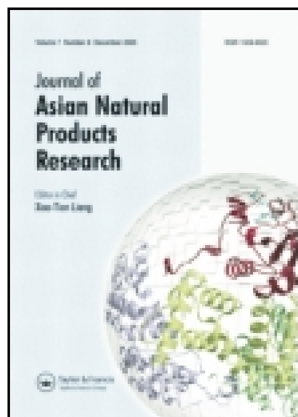

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Flavonoids from silkworm droppings and their promotional activities on heme oxygenase-1

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Flavonoids from silkworm droppings and their promotional activities on heme oxygenase-1

Ji-Hae Park[a], Dae-Young Lee[a], Pyeong Yun[b], Seung-Woo Yeon[c], Jong Hee Ko[c], Yong-Soon Kim[d] and Nam-In Baek[a]*

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A new flavane glucoside, 7,2′-dihydroxy-8-hydroxyethyl-4′-methoxyflavane-2′-*O*-β-D-glucopyranoside (**3**), along with three known flavonoids, 7,2′-dihydroxy-8-prenyl-4′-methoxyflavane (**1**), euchrenone a_7 (**2**), and 7,2′-dihydroxy-8-prenyl-4′-methoxy-2′-*O*-β-D-glucopyranosylflavane (**4**), was isolated from silkworm droppings. The structures of the compounds were elucidated on the basis of 1D and 2D NMR spectroscopic analyses and optical rotational characteristics. The compounds isolated from silkworm droppings were evaluated for their effects on heme oxygenase-1 (HO-1) activity. Compounds **1** and **3** increased the expression of HO-1 in HepG2 cells. HO-1 is an antioxidant enzyme that catabolizes heme to carbon monoxide, free iron, and biliverdin, all of which are involved in the suppression of inflammatory mediators.

Keywords: *Bombyx mori*; 7,2′-dihydroxy-8-hydroxyethyl-4′-methoxyflavane-2′-*O*-β-D-glucopyranoside; HO-1; silkworm droppings

1. Introduction

Silkworm droppings, also known in Korea as Jambun, Jamsa, Wonjamsa, and Wonjamsi, refer to the excrement of the silkworm (*Bombyx mori* L.). When the silkworm ingests the leaves of the mulberry tree, more than half of the leaves are excreted without being digested [1]. Therefore, silkworm droppings are likely composed of both mulberry leaf and various constituents biotransformed by microbes or enzymes in the intestine of the silkworm. Studies have demonstrated that silkworm droppings are nontoxic and can be used to strengthen the internal organs of humans, treat diabetes, and protect against diplegia [2]. Additionally, silkworm droppings were shown to lower cholesterol levels and blood pressure, and to exhibit anti-diabetic characteristics and anticancer activity [3,4].

We have also found that alcohol extracts of silkworm droppings exhibited higher immunosuppressive activities than those of cyclosporine, a well-known immune inhibitor, against IL-6, MCP-1, and MIP-1a cytokines. We searched for immune inhibition compounds in silkworm droppings using SiO_2 and octadecyl silica gel (ODS) column chromatography (CC) and isolated a new flavane, 7,2′-dihydroxy-8-hydroxyethyl-4′-methoxyflavane-2′-*O*-β-D-glucopyranoside (**3**), together with three known flavonoids **1**, **2**, and **4** (Figure 1).

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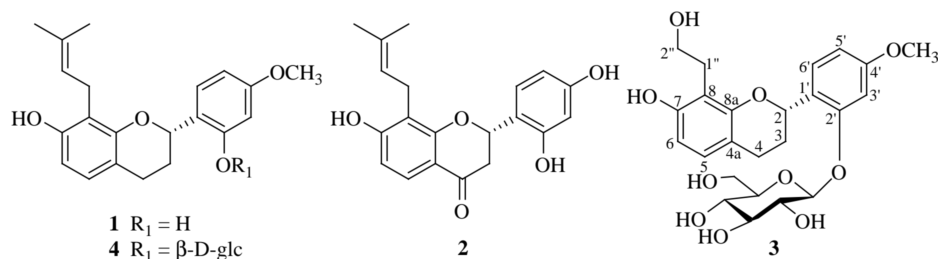

Figure 1. Chemical structures of flavonoids isolated from silkworm droppings. β-D-glc is β-D-glucopyranoside.

This paper describes the procedures for the isolation and structural determination of the four flavonoids from silkworm droppings in addition to evaluating the flavonoids for their effects on the expression of heme oxygenase-1 (HO-1).

2. Results and discussion

When 80% methanol extracts of silkworm droppings were developed on silica gel thin layer chromatography (TLC), some spots exhibited UV absorption and yellow and dark brown coloration when sprayed with 10% H_2SO_4 solution and heated, indicating the presence of flavonoids in the extracts. The methanol extracts were fractionated into EtOAc, *n*-BuOH, and H_2O layers through solvent fractionation. Repeated silica gel and ODS CC of the EtOAc fractions produced four flavonoids. The chemical structures of the compounds were determined through the analysis of data from ^{1}H, ^{13}C NMR, DEPT, gradient correlation spectroscopy, gradient heteronuclear single-quantum coherence (gHSQC), gradient heteronuclear multiple-bond connectivity (gHMBC), electron ionization mass spectrometry (EI-MS), fast atom bombardment mass spectrometry (FAB-MS), and infrared spectroscopy (IR). The three compounds **1**, **2**, and **4** were identified to be 7,2′-dihydroxy-8-prenyl-4′-methoxyflavane (**1**), euchrenone a_7 (**2**), and 7,2′-dihydroxy-8-prenyl-4′-methoxy-2′-*O*-β-D-glucopyranosylflavane (**4**) on the basis of spectroscopic data and chemical evidence. These findings were confirmed by comparison of the physical and spectroscopic data with those reported in the literature [5,6].

Compound **3** was isolated as a pale yellow amorphous powder and showed IR absorbance bands of hydroxyl (3364 cm^{-1}) and aromatic (1609 cm^{-1}) groups. The molecular weight was determined to be 478 from the molecular ion peak *m/z* 477 $[M-H]^-$ in the negative FAB-MS spectrum, and a molecular formula of $C_{24}H_{30}O_{10}$ was determined by the pseudo-molecular ion ($[M-H]^-$, *m/z* 477.1772) in the HR-FAB-MS. The ^{1}H NMR spectrum exhibited three olefin methine signals of a 1,2,4-trisubstituted benzene ring at δ_H 7.34 (1H, d, $J = 8.4$ Hz), 6.85 (1H, d, $J = 2.4$ Hz), and 6.63 (1H, dd, $J = 8.4$, 2.4 Hz), and two olefin methine signals of a 1,2,3,4-tetrasubstituted benzene ring were seen at δ_H 6.73 (1H, d, $J = 8.0$ Hz) and 6.33 (1H, d, $J = 8.0$ Hz). An oxygenated methine signal at δ_H 5.50 (1H, dd, $J = 9.6$, 2.0 Hz), an oxygenated methylene signal at δ_H 3.65 (2H, m, H-2″), a methoxy signal at δ_H 3.78 (3H, s, 4′-OMe), and three methylene signals at δ_H 2.90 (2H, m, H-1″), 2.85 (1H, m, H-4_a), 2.61 (1H, m, H-4_b), 2.19 (1H, m, H-3_a), and 1.82 (1H, m, H-3_b) were also observed. In addition, a hemiacetal proton signal at δ_H 4.91 (1H, d, $J = 7.2$ Hz), oxygenated methylene signals at δ_H 3.92 (1H, dd, $J = 12.0$, 2.0 Hz) and 3.70 (1H, dd, $J = 12.0$, 6.4 Hz), and methine signals at δ_H 3.45 (1H, m), 3.44 (1H, m), and 3.38 (1H, m) resulted from the hexose moieties.

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The side chain was deduced to be an ethyl alcohol moiety based on the signals of an oxygenated methylene proton at δ_H 3.65 and a methylene proton at δ_H 2.90. As a result, compound **3** was assumed to be a flavane monoglycoside combined with an ethyl alcohol group.

The ^{13}C NMR spectrum showed 24 carbon signals, including a hexose carbon signal at δ 102.9 (C-1‴), 78.2 (C-3‴), 78.1 (C-5‴), 74.9 (C-2‴), 71.4 (C-4‴), and 62.5 (C-6‴), which were identified as those of a β-glucopyranose. The multiplicity of each carbon was determined using a DEPT experiment. Two benzene rings showed 12 carbon signals: four oxygenated olefin quaternary signals at δ 161.3 (C-4′), 156.1 (C-2′), 155.5 (C-7), and 155.2 (C-8a), three olefin quaternary signals at δ 125.4 (C-1′), 114.4 (C-4a), and 113.4 (C-8), and five olefin methine signals at δ 128.2 (C-5), 127.9 (C-6′), 108.8 (C-5′), 108.4 (C-6), and 103.1 (C-3′). There were also one oxygenated methine signal at δ 73.5 (C-2), one methoxy signal at δ 55.8, and two methylene signals at δ 30.4 (C-3) and 25.8 (C-4). Consequently, compound **3** was identified as a flavane glucoside. The stereostructure of the anomer carbon was confirmed to be β from the coupling constant of the hemiacetal proton signal ($J = 7.2$ Hz) in the ^{1}H NMR spectral data. The side chain was determined to be an ethyl alcohol group from an oxygenated methylene at δ 62.6 (C-2″) and a methylene at the δ 27.7 (C-1″) carbon signals. To determine the locations of the glucose and the ethyl alcohol group in compound **3**, 2D NMR, such as gHSQC and gHMBC experiments, was conducted. In the gHMBC spectrum, the methylene proton signal at δ_H 2.90 (H-1″) showed cross peaks with two oxygenated olefin quaternary carbon signals at δ 155.5 (C-7) and 155.2 (C-8a), and with one olefin quaternary carbon signal at δ 113.4 (C-8). Also, the anomeric proton signal at δ_H 5.50 (H-1‴) showed a correlation with the oxygenated olefin quaternary carbon signal at δ 156.1 (C-2′). Based on these results, the ethyl alcohol and the β-D-glucose moieties were determined to be located at C-8 and C-2′, respectively. As a result, compound **3** was determined to be a 7,2′-dihydroxy-8-hydroxyethyl-4′-methoxyflavane-2′-*O*-β-D-glucopyranoside. The stereostructure of a chiral carbon, C-2, was determined as *S* by the comparison of not only the chemical shifts (δ_C 73.5, δ_H 5.50) of the carbon and proton signals, but also through the comparison of the specific rotation value of −48.1 with those of similar compounds in the literature [7]. The chemical shifts of C-2 and H-2 in flavane moiety with 2*R* structure were usually observed at δ_C 78.5 and δ_H 4.85, whereas those with 2*S* structure were at δ_C 73.5 and δ_H 5.50 [8]. The sugar of compound **3** was determined to be D-glucopyranose from the specific rotation value ($[\alpha]_D^{25}$ +47.4, $c = 1.2$, H_2O) of the sugar obtained through acid hydrolysis of the glycoside **3**. As a result, compound **3** was determined to be a new compound.

To determine whether the isolated compounds induced the expression of HO-1, we exposed HepG2 cells to 20 μM concentration of compounds **1–4**. The results showed that compounds **1** and **3** increased the expression of HO-1 in a concentration-dependent manner (Figure 2). The inductions of HO-1

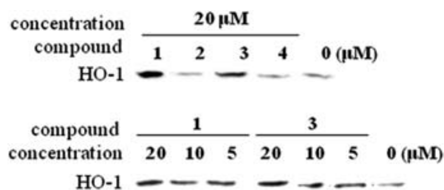

Figure 2. HO-1 expressions in HepG2 cells exposed to compounds **1** and **3** isolated from silkworm droppings. Cells were incubated with 20 μM concentrations of compounds **1–4** for 16 h. HO-1 protein expression was evaluated using antiHO-1 antibodies as described in the experimental methods. One representative Western blot from three independent experiments is shown.

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expression by compounds **1** and **3** were almost the same as those of a chalcone, (*E*)-1-[2-methoxy-4,6-bis(methoxymethoxy)phenyl]-3-(3-nitrophenyl)prop-2-en-1-one [9] and a triterpenoid, 23-hydroxyursolic acid [10], which were reported to significantly increase HO-1 expression. HO-1 is considered to be an antioxidant enzyme that catabolizes heme to carbon monoxide, free iron, and biliverdin, all of which are involved in the suppression of inflammatory mediators or factors that may be used to improve atopy-related symptoms.

3. Experimental

3.1 General experimental procedures

UV spectrum was obtained using UV-vis spectrophotometer (Simadzu UV-1601, Tokyo, Japan). Optical rotation was measured on a JASCO P-1010 digital polarimeter (Jasco, Tokyo, Japan), and IR spectra were obtained using a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). EI-MS was recorded on a JEOL JMS-700 (JEOL, Tokyo, Japan) and FAB-MS was conducted on a JEOL JMSAX-700 (Tokyo, Japan). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR spectrometer (Palo Alto, CA, USA). SiO_2 (Kiesel gel 60, Merck, Darmstadt, Germany) and ODS (LiChroprep RP-18, Merck) resins were used for CC, and TLC analysis was carried out using Kiesel gel 60 F_{254} and RP-18 F_{254S} (Merck) plates. The compounds were detected using a UV lamp Spectroline Model ENF-240 C/F (Spectronics Corporation, New York, NY, USA) and a 10% H_2SO_4 solution. AntiHO-1 antibodies (Stressgen, Victoria, Canada) were used for Western blotting experiments, and proteins from these gels were then electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA, USA).

3.2 Materials

Silkworm droppings were supplied in January 2008 from the Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon, Korea. A voucher specimen (KHU08-0102) has been reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

3.3 Extraction and isolation

Dried silkworm droppings (10 kg) were extracted at room temperature using 80% aqueous MeOH (36 L ×3) for 24 h, followed by filtration. The filtrates were evaporated in vacuum, and the obtained MeOH extracts were suspended in H_2O (3 L) and then extracted successively with ethyl acetate (EtOAc, 3 L ×5) and *n*-butanol (*n*-BuOH, 2.8 L ×3). The organic and aqueous layers were concentrated to produce the residues of the EtOAc fraction (SDE, 61 g), the *n*-BuOH fraction (SDB, 30 g), and the H_2O fraction (SDW, 136 g).

The EtOAc extract (SDE, 60 g) was applied to SiO_2 CC (φ 8.0 × 15.0 cm) and eluted with *n*-hexane–EtOAc (5:1 → 3:1 → 1:1 → 1:3, 30 L of each) and $CHCl_3$–MeOH (10:1 → 7:1 → 5:1 → 3:1, 25 L of each). The eluting solutions were monitored using TLC and ultimately produced 23 fractions (SDE-1 to SDE-23). Fraction SDE-5 [2.9 g, V_e/V_t (elution volume/total volume) 0.07–0.11] was subjected to SiO_2 CC (φ 5.0 × 11.0 cm) and was eluted with *n*-hexane–EtOAc (5:1 → 1:1, 2.5 L of each) and $CHCl_3$–MeOH (10:1 → 7:1, 2.5 L of each), yielding 22 fractions (SDE-5-1 to SDE-5-22). Fraction SDE-5-12 (630 mg, V_e/V_t 0.19–0.24) was purified using ODS CC (φ 3.5 × 6.0 cm) and was eluted with acetonitrile–H_2O (3:2, 2.6 L) to ultimately produce compound **1** [SDE-5-12-6, 7.0 mg, V_e/V_t 0.02–0.03, TLC (ODS F_{254S}) R_f 0.50, acetonitrile–H_2O = 3:1]. Fraction SDE-9 (1.9 g, V_e/V_t 0.35–0.42) was subjected to SiO_2 CC (φ 4.5 × 12.0 cm) and eluted with

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$CHCl_3$–MeOH (15:1, 5.4 L), yielding eight fractions (SDE-9-1 to SDE-9-8). Fraction SDE-9-4 (216 mg, V_e/V_t 0.22–0.31) was purified using ODS CC (φ 3.0 × 4.5 cm) and eluted with MeOH– H_2O (3:2, 690 ml) to ultimately produce compound **2** [SDE-9-4-4, 3.5 mg, V_e/V_t 0.22–0.26, TLC (ODS F_{254S}) R_f 0.60, MeOH–H_2O = 3:1]. Fraction SDE-18 (985 mg, V_e/V_t 0.83–0.84) was subjected to SiO_2 CC (4.5 × 14.0 cm) and eluted with $CHCl_3$–MeOH (10:1, 2.6 L) to yield eight fractions (SDE-18-1 to SDE-18-8). Fraction SDE-18-6 (156 mg, V_e/V_t 0.62–0.74) was purified using ODS CC (2.5 × 5.5 cm) and eluted with MeOH–H_2O (3:4, 560 ml) to result in nine fractions (SDE-18-6-1 to SDE-18-6-9), yielding compound **3** [SDE-18-6-2, 11 mg, V_e/V_t 0.12–0.17, TLC (ODS F_{254S}) R_f 0.40, MeOH–H_2O = 1:1]. Fraction SDE-19 (1.2 g, V_e/V_t 0.84–0.87) was subjected to ODS CC and was eluted with MeOH–H_2O (1:1, 2.1 L) to produce six fractions (SDE-19-1 to SDE-19-6) and also yielded compound **4** [SDE-19-2, 16 mg, V_e/V_t 0.11–0.19, TLC (ODS F_{254S}) R_f 0.50, MeOH–H_2O = 2:1].

*3.3.1 7,2′-Dihydroxy-8-prenyl-4′-methoxyflavane (**1**)*

A pale yellow amorphous powder (MeOH); $[\alpha]_D^{20}$ −23.7 (c = 0.1, MeOH); IR (CaF_2 window in $CHCl_3$, cm^{-1}) 3402, 2924, 1715, 1616; ^1H and ^{13}C NMR refer to the literature [5]; EI-MS: *m/z* 340 $[M]^+$.

*3.3.2 Euchrenone a_7 (**2**)*

A pale yellow amorphous powder (MeOH); $[\alpha]_D^{20}$ −34.8 (c = 0.45, MeOH); IR (CaF_2 window in MeOH, cm^{-1}): 3310, 2927, 2853, 1708, 1601; ^1H and ^{13}C NMR refer to the literature [6]; EI-MS: *m/z* 324 $[M]^+$.

*3.3.3 7,2′-Dihydroxy-8-hydroxyethyl-4′-methoxyflavane-2′-O-β-D-glucopyranoside (**3**)*

A pale yellow amorphous powder (MeOH); UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 260 (4.02); $[\alpha]_D^{20}$ −54.6 (c = 0.3, MeOH); IR (CaF_2 window in MeOH, cm^{-1}) 3364, 2915, 1609, 1075; ^1H NMR (400 MHz, methanol-d_4, δ_H) 7.34 (1H, d, J = 8.4 Hz, H-6′), 6.85 (1H, d, J = 2.4 Hz, H-3′), 6.73 (1H, d, J = 8.0 Hz, H-5), 6.63 (1H, dd, J = 8.4, 2.4 Hz, H-5′), 6.33 (1H, d, J = 8.0 Hz, H-6), 5.50 (1H, dd, J = 9.6, 2.0 Hz, H-2), 4.91 (1H, d, J = 7.2 Hz, H-1‴), 3.92 (1H, dd, J = 12.0, 2.0 Hz, H-6‴a), 3.78 (3H, s, 4′-OMe), 3.70 (1H, dd, J = 12.0, 6.4 Hz, H-6‴b), 3.65 (2H, m, H-2″), 3.45 (1H, m, H-3‴), 3.44 (1H, m, H-2‴), 3.44 (1H, m, H-4‴), 3.38 (1H, m, H-5‴), 2.90 (2H, m, H-1″), 2.85 (1H, m, H-4_a), 2.61 (1H, m, H-4_b), 2.19 (1H, m, H-3_a), 1.82 (1H, m, H-3_b); ^{13}C NMR (100 MHz, methanol-d_4, δ_C) 161.3 (C-4′), 156.1 (C-2′), 155.5 (C-7), 155.2 (C-8a), 128.2 (C-5), 127.9 (C-6′), 125.4 (C-1′), 114.4 (C-4a), 113.4 (C-8), 108.8 (C-5′), 108.4 (C-6), 103.1 (C-3′), 102.9 (C-1‴), 78.2 (C-3‴), 78.1 (C-5‴), 74.9 (C-2‴), 73.5 (C-2), 71.4 (C-4‴), 62.6 (C-2″), 62.5 (C-6‴), 55.8 (4′-OCH_3), 30.4 (C-3), 27.7 (C-1″), 25.8 (C-4); negative FAB-MS: *m/z* 477 $[M - H]^-$; HR-FAB-MS: *m/z* 477.1772 (calcd for $C_{24}H_{29}O_{10}$, 477.1761).

*3.3.4 7,2′-Dihydroxy-8-prenyl-4′-methoxy-2′-O-β-D-glucopyranosylflavane (**4**)*

A pale yellow amorphous powder (MeOH); $[\alpha]_D^{20}$ −24.2 (c = 0.1, MeOH); IR (CaF_2 window in MeOH, cm^{-1}): 3362, 2927, 1713, 1069; ^1H and ^{13}C NMR refer to the literature [5]; negative FAB-MS *m/z* 501 $[M - H]^-$.

3.4 Hydrolysis of compound, isolation of the sugar, and measurement of specific rotation value

SDE-18-6-1 (21 mg, V_e/V_t 0.00–0.11) and SDE-18-6-3 (32 mg, V_e/V_t 0.18–0.29), which were revealed to contain 95% of compound **3** from the TLC experiment, were combined and dissolved in H_2O (3 ml), followed by addition of 10% hydrochloric acid (0.5 ml). The solution

was hydrolyzed at 120°C for 2 h. The hydrolysate was neutralized by the addition of Ag_2CO_3 in excess and filtered through filter paper. The filtrate was centrifuged at 5000 *g* for 10 min. The supernatant was concentrated *in vacuo* to give a white powder (12 mg). The powder was dissolved in H_2O (1 ml) and was used for the measurement of the specific rotation value.

3.5 HO-1 expression in HepG2 cells

HepG2 cells were plated in 6-well plates at a density of 1×10^6 cells/well and were grown at 37°C. After 24 h, the cells were treated with compounds for 16 h, washed twice with 1 × PBS, and harvested in 1 ml of PBS through centrifugation at 14,000 rpm for 5 min. The cells were then lysed in 100 ml of TEN buffer [50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA], 1% Triton X-100, a protease inhibitor cocktail, and sodium vanadate. The cell lysates were loaded onto a 12% SDS-polyacrylamide gel and separated using electrophoresis. Proteins were electrophoretically transferred to a 0.2-μm PVDF membrane, and the blots were incubated overnight with antiHO-1 primary antibodies (1:1000). Bound antibody was detected using a horseradish peroxidase-linked secondary antibody.

Acknowledgements

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