Natural Product Sciences
19(3) : 206-214 (2013)

Inhibition of Melanin Production and Tyrosinase Expression of Crocetin Derivatives from processed *Gardenia jasminoides*

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Abstract – The crocetin derivatives, crocin (**1**), gentiobiosyl glucosyl crocetin (**3**), and mono-gentiobiosyl crocetin (**4**) were isolated from the fruit of *Gardenia jasminoides* (Gj) and crocetin (**2**) from the processed fruit of Gj (PGj) by column chromatography. Their structures were determined on the basis of spectroscopic methods including IR, MS, and NMR (1D and 2D). These compounds were evaluated for their inhibition activity on melanin production in α-MSH (melanocyte stimulating hormone) activated B16F10 cells. Compounds **1 - 4** reduced melanin content in a dose-dependent manner at concentrations of 20 - 60 uM. They also suppressed tyrosinase protein and m-RNA expressions dose dependently, assayed by western blot analysis, and RT-PCR experiment in B16F10 murine melanoma cells.

Key words – *Gardenia jasminoides,* Crocetin derivatives, B16F10 cells, Melanin content, Tyrosinase

Introduction

Gardeniae Fructus is the dried ripe fruit of *Gardenia jasminoides* Ellis (Rubiaceae) and has been used in the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders, and hypertension (Aburada *et al*., 1976; Miyasita, 1976; Tseng *et al*., 1995). The iridoid components, genipin and genipioside, of this fruit have anti-inflammatory (Lim *et al*., 2008; Nam *et al*., 2010; Zheng *et al*., 2010; Koo *et al*., 2006) activities. Its yellow pigments, crocin has been shown to be a potent antioxidant (Papandreou *et al*., 2006), anticancer (Escribano *et al*., 1996), antidepressant (Akhondzadeh *et al*., 2004) activity. Crocetin, the aglycon form of crocin, exerts antioxidant and anticancer activities (Hsu *et al*., 1999; Chang, *et al*., 1996). Although these various activities were reported, and melanogenesis inhibitory activity of monoterpene glycosides from *Gardeniae Fructus* was published recently (Akihisa *et al*., 2012), but the whitening effect of pigments in *Gardenia jasminoides* has not been studied. To evaluate the potential whitening activity of colorants from *Gardenia jasminoides*, we isolated active compounds and estimated the inhibitory activity on melanin synthesis in α-MSH (melanocyte stimulating hormone) activated B16F10 cells and evaluated protein and m-RNA tyrosinase expressions.

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Experimental

General experimental procedures – IR spectra were obtained using a Jasco FT-IR 430 spectrophotometer. High resolution mass spectra were measured with a JEOL JMS-700. Both ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 500 NMR spectrometer. HPLC was used Agilent 1100 series with DAD. UV-VIS spectra were measured with Jasco UV-975. Muffle furnace was used Thermolyne F-48000. Silica gel (70 - 230 mesh, Merck Co.) and Sephadex LH-20 (Sigma) were used for column chromatography and silica gel 60F254 (Merck) for TLC.

Plant materials – The fruit of *Gardenia jasminoides* was collected at Jangsu, in the Chunbook province, provided by Korea Medicine Herbal Association. The fruit of *G. jasminoides* was ground and heated for 30 min at 180 ºC for the processing procedure. A voucher specimen was deposited at the herbarium in the College of Pharmacy, Sookmyung Women's University.

Extraction and isolation – The fruit (2 kg) of *Gardenia jasminoides* was ground and extracted with methanol for 4 h, yielding, after removal of the solvent under vacuum, 216 g of dried extract. This methanol extract was partitioned with hexane, dichloromethane, ethyl acetate, butanol and water. The butanol fraction (66 g) was applied to silica gel column chromatography and eluted with CH_2Cl_2 : MeOH (10 : 1 → 4 : 1). Fraction 5 (720 mg) and 7 (113 mg) were repeatedly chromatographed

over LH-20 column using MeOH : H_2O (3 : 7 → 8 : 2) as the eluent and we isolated compound **1** (232 mg) from fraction 5 and compound **3** (23 mg) and compound **4** (25 mg) from fraction **7**. The processed fruit (2 kg) of *Gardenia jasminoides* was extracted with methanol and yielded 162 g of dried extract. The EtOAc fraction (14 g) was applied on to a Sephadex LH-20 column with gradient elution (MeOH : H_2O, 3 : 7 → 7 : 3) and fractions were collected. Fraction 3 (102 mg) were repeatedly chromatographed over LH-20 column MeOH:H_2O (4 : 6 → 9 : 1) as the eluent and we isolated compound **2** (20 mg).

***all-trans*-Crocetin di(β-D-gentiobiosyl) ester (1) (crocin)** – Red amorphous powder; $C_{44}H_{64}O_{24}$; IR ν_{max} (DMSO, cm^{-1}): 3450, 2890, 1700, 1570, 1225, 1085; FAB-MS (*m/z*): 977.9 $[M+H]^+$; ^{1}H NMR (500 MHz, DMSO-d_6): 7.36 (2H, br d, J = 10.5 Hz, H-10, 10'), 6.87 (2H, dd, , J = 16.0 Hz, H-15, 15'), 6.82 (2H, d, J = 14.5 Hz, H-12, 12'), 6.67 (2H, dd, , J = 15.0 Hz, H-11, 11'), 6.54 (2H, br dd, J = 10.5 Hz, H-14, 14'), 5.42 (2H, d, J = 8.0 Hz, H-1), 4.17 (2H, d, J = 7.5 Hz, H-1'), 3.99 (2H, dd, J = 10.0 Hz, H-6e), 3.65 (2H, m, H-6'e), 3.59 (2H, dd, H-6a), 3.43 (2H, m, H-6'a), 3.27 (4H, m, H-2, 3, 4, 5), 3.13 (2H, m, H-3'), 3.04 (2H, m, H-4'), 2.96 (2H, m, H-2'), 2.50 (2H, ddd, H-5'), 2.00 (6H, s, H-20, 20'), 1.98 (6H, s, H-19, 19') ; ^{13}C NMR (125 MHz, DMSO-d_6): 166.6 (C-8, 8'), 145.1 (C-12, 12'), 140.4 (C-10, 10'), 137.4 (C-13, 13'), 136.4 (C-14,14'), 132.5 (C-15, 15'), 125.7 (C-9, 9'), 124.4 (C-11, 11'), 103.5 (C-1'), 95.0 (C-1), 77.3 (C-5'), 77.2 (C-3'), 76.7 (C-3, 5), 73.9 (C-2'), 72.9 (C-2), 70.4 (C-4'), 69.7 (C-4), 68.4 (C-6), 61.4 (C-6'), 13.1 (C-19, 19'), 13.0 (C-20, 20').

***all-trans*-Crocetin (2)** – Red amorphous powder; $C_{20}H_{24}O_4$; IR ν_{max} (DMSO, cm^{-1}): 3450, 2890, 1700, 1570, 1225, 1085; FAB-MS (*m/z*): 329.3 $[M+H]^+$; ^{1}H NMR (500 MHz, DMSO-d_6): 7.20 (2H, br d, J = 11.5 Hz, H-10, 10'), 6.80 (2H, dd, J = 14.5 Hz, 11.5 Hz, H-15, 15'), 6.72 (2H, d, J = 15.0 Hz, H-12, 12'), 6.64 (2H, dd, J = 15.0 Hz, 11.6 Hz, H-11, 11'), 6.49 (2H, m, H-14, 14'), 1.97 (6H, br s, H-20, 20'), 1.91 (6H, br s, H-19, 19') ; ^{13}C NMR (125 MHz, DMSO-d_6): 143.7 (C-12, 12'), 138.4 (C-10, 10'), 137.1 (C-13, 13'), 135.7 (C-14, 14'), 132.1 (C-15,15'), 132.0 (C-9, 9'), 129.1 (C-11, 11'), 14.0 (C-19, 19'), 9.5 (C-20,20').

***all-trans*-Crocetin-β-D-gentiobiosyl- β-D-glucosyl ester (3)** – Red amorphous powder; $C_{38}H_{54}O_{19}$; IR ν_{max} (DMSO, cm^{-1}): 3450, 2890, 1700, 1570, 1225, 1085; FAB-MS (*m/z*): 837.4 $[M+Na]^+$; ^{1}H NMR (500 MHz, DMSO-d_6): 7.35 (2H, br d, J = 11.5 Hz, H-10,10'), 6.86 (2H, m, H-15, 15'), 6.82 (2H, m, H-12, 12'), 6.65 (2H, dd, J = 14.9 Hz, 11.7 Hz, H-11, 11'), 6.57 (2H, m, H-14, 14'), 5.43 (1H, d, J = 7.5 Hz,H-1''), 5.41 (1H, d, J = 7.5 Hz, H-1), 4.17 (1H, d, J = 8.1 Hz, H-1'), 3.98 (1H, br d, J = 14.5 Hz, H-6e), 3.76 (1H, m, H-6''e), 3.74 (1H, m, H-6'e), 3.68 (1H, m, H-6a), 3.61 (1H, m, H-6''a), 3.57 (1H, m, H-6'a), 3.3 ~3.5 (8H, m, H-2, 3, 4, 5, 2'', 3'', 4'', 5''), 3.28 (1H, m, H-3'), 3.23 (1H, m, H-4'), 3.16 (1H, m, H-2'), 3.13 (1H, m, H-5'), 2.00 (6H, br s, H-19, 19'), 1.97 (6H, br s, H-20, 20') ; ^{13}C NMR (125 MHz, DMSO-d_6): 166.7 (C-8, 8'), 145.1 (C-12), 145.0 (C-12'), 137.4 (C-10), 137.4 (C-10'), 136.4 (C-13,13'), 136.4 (C-14, 14'), 132.5 (C-15, 15'), 125.8 (C-9), 125.7 (C-9'), 124.4 (C-11, 11'), 103.5 (C-1'), 95.1 (C-1), 95.0 (C-1''), 78.3 (C-3'), 77.3 (C-3''), 76.9 (C-3, 5), 76.7 (C-5', 5''), 73.9 (C-2'), 73.0 (C-2, 2''), 70.0 (C-4'), 69.7 (C-4, 4''), 68.0 (C-6), 61.4 (C-6'), 61.4 (C-6''), 13.1 (C-19, 19'), 13.0 (C-20, 20').

***all-trans*-Crocetin mono(β-D-gentiobiosyl) ester (4)** – Red amorphous powder; $C_{32}H_{44}O_{14}$; IR ν_{max} (DMSO, cm^{-1}): 3450, 2890, 1700, 1570, 1225, 1085; FAB-MS (*m/z*): 675.3 $[M+Na]^+$, 653.3 $[M+H]^+$; ^{1}H NMR (500 MHz, DMSO-d_6): 7.35 (1H, br d, J = 11.0 Hz, H-10), 7.09 (1H, br d, J = 9.5 Hz, H-10'), 6.85 (2H,m, H-15, 15'), 6.66 (2H, m, H-12, 12'), 6.61 (2H, m, H-11, 11'), 6.55 (1H, br d, J = 14.0 Hz, H-14'), 6.48 (1H, br d, J = 18.5 Hz, H-14), 5.42 (1H, d, J = 7.0 Hz, H-1), 4.17 (1H, d, J = 8.0 Hz, H-1'), 3.97 (1H, br d, J = 11.0 Hz, H-6e), 3.65 (1H, br d, J = 11.0 Hz, H-6'e), 3.59 (1H,dd, J = 11.5 Hz, J = 5.8 Hz, H-6a), 3.45 (1H, m, H-6'a), 3.41 (2H, m, H-5, 5'), 3.39 (1H, m, H-4), 3.35 (1H, m, H-2), 3.29 (2H, m, H-3, 3'), 3.16 (1H, m, H-4'), 2.98 (1H,m, H-2'), 1.98 (9H, s, H-19, 19'), 1.93 (3H, s, H-20, 20'); ^{13}C NMR (125 MHz, DMSO-d_6): 166.7 (C-8, 8'), 145.2 (C-12, 12'), 140.4 (C-10, 10'), 137.7 (C-13'), 136.6 (C-13), 136.6 (C-14), 132.7 (C-14'), 132.7 (C-15), 130.8 (C-15'), 125.4 (C-9, 9'), 124.0 (C-11, 11'), 103.6 (C-1'), 95.0 (C-1), 77.4 (C-5'), 77.2 (C-3'), 76.8 (C-5), 76.7 (C-3), 73.9 (C-2'), 72.9 (C-2), 70.4 (C-4'), 69.7 (C-4), 68.4 (C-6), 61.4 (C-6'), 13.1 (C-19, 19'), 13.0 (C-20, 20').

Cell culture – B16F10 murine melanoma cells were purchased from ATCC. The cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 ug/mL streptomycin and 0.25 ug/mL amphotericin. Cells were maintained at 37 ºC under 5% CO_2 in a fully humidified atmosphere.

MTT assay for viability – B16F10 cells were seeded at 1.5 × 10^4 cells/mL in 48-well plates and incubated for 24 h, then treated with varying concentrations of compounds **1 - 4**. The cells were incubated for 48 h and then moved to fresh medium containing 0.5 mg/mL of

MTT. The incubation continued for an additional 4 h at 37 ºC. The medium was removed and the MTT-formazan was dissolved in 400 uL of DMSO. The extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance of the DMSO solution at 570 nm using an ELISA reader (Mosmann, 1983). Cytotoxicity was calculated as the reduction in cell viability.

Measurement of melanin content – B16F10 cells were plated at a density of 1×10^5 cells/mL in 6-well cell culture plates. Cells were incubated for 24 h then treated with various concentrations of test samples in the presence and absence of 0.4 uM α-MSH for 48 h. Cells were harvested and washed twice with PBS, and then incubated in 1 N NaOH with 10% DMSO for 1 h at 70 ºC and centrifuged at 12000 rpm for 30 min. Optical density of the supernatant was measured at 405 nm using ELISA plate reader.

Assay of tyrosinase activity – B16F10 cells (1×10^5 cells/mL) were incubated for 24 h then treated with various concentrations of test samples in the presence and absence of 0.4 uM α-MSH for 48 h. Cells were harvested and washed twice with PBS, and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40%, 0.5% sodium deoxychlolate, 0.1 SDS) and centrifuged at 12000 rpm for 30 min. After quantifying protein levels, the supernatants were mixed with 100 uL of freshly prepared enzyme solution [(0.1% L-DOPA in 0.1 M sodium phosphate buffer (pH 6.8)], then incubated at 37 ºC for 2 h. The absorbance was measured by using a microplate reader at 490 nm.

Tyrosinase zymography – Cell lysates were prepared from B16F10 cells treated with test samples after 2 days of treatment, as described above. The lysates containing 50 ug of protein were mixed with loading buffer without β-mercaptoethanol and separated in SDS-polyacrylamide gel without a boiling step. After electrophoresis, the gels were washed with 0.1 M sodium phosphate buffer (pH 6.8) for 30 min 4 times at room temperature and then incubated in 10 mM L-DOPA solution for 2 h at room temperature.

Western blot analysis – B16F10 cells were seeded at 1×10^5 cells/mL in 60 mm cell culture plates containing 4 mL of culture medium. Cells were incubated for 24 h. and then treated with 0.4 uM α-MSH with various concentrations of test samples for 48 h. The cells were rinsed with PBS and lysed with iced-lysis buffer (Complete Lysis-B(2X), Roche) for 1 h and then centrifuged at 12000 rpm for 20 min. The lysates were analyzed protein contents quantified using the Bradford method, and boiled for 5 min at 95 ºC. Total cell lysates (20 ug) were applied on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-rabbit tyrosinase (Santacruz, USA) for 1 h and incubated with a horseradish peroxidase conjugated anti-IgG in blocking buffer for 1 h. The blots were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Bio-science, England) according to the manufacturer's instruction.

Table 1. Sequences of tyrosinase and β-actin primers

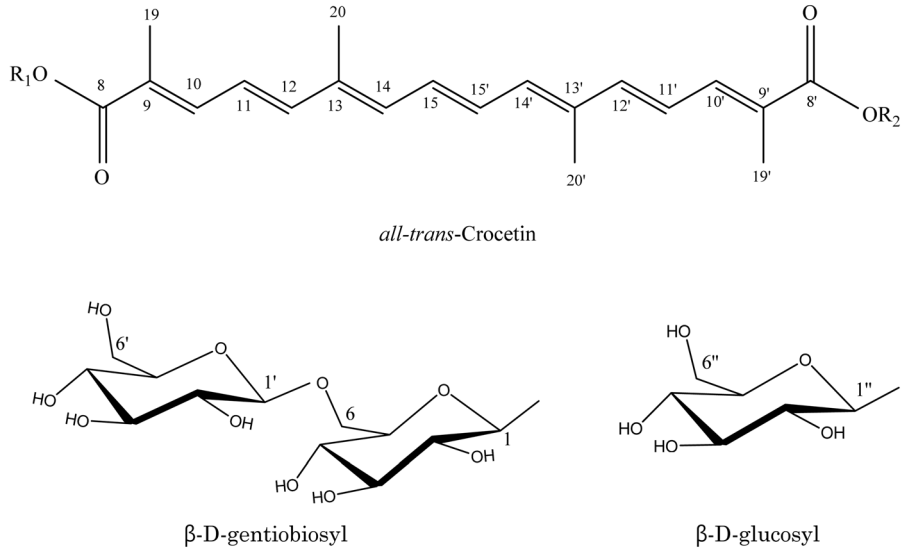
Gene	Primer sequence
Tyrosinase	
Forward	CGC CAG CTT TCA GGC AGA GGT
Reverse	TGG TGC TTC ATG GGC AAA ATC
β-actin	
Forward	ACC GTG AAA AGA TGA CCC AG
Reverse	TAC GGA TGT CAA CGT CAC AC

Reverse transcriptase and polymerase chain reaction (RT-PCR) – B16F10 cells were seeded (1×10^6 cells/mL) in 100 mm cell culture plates containing 10 mL of culture medium. Cells were treated with test samples together with 0.4 uM α-MSH. The RNA was isolated with RNA isoplus™ (Takara Biotechnology, Japan) and each RNA extract (2 ug) was reverse transcribed into cDNA using superscript II reverse transcriptase (Gendepot, USA). PCR was performed in 20 uL of a solution containing Top-Taq™ PreMix (CoreBio System, Korea). The primer sequences are listed in Table 1. PCR was performed for 25 amplification cycles in a DNA thermal cycler (TaKaRa PCR Thermal Cycler, Japan). PCR products were separated by electrophoresis on 1.2% agarose gels and visualized using ethidinium bromide staining.

Statistical analysis – All data are presented as the mean value ± standard deviation (S.D.) from three independent experiments. Significant differences between the control and the experimental groups were assessed by the Student's t-test. Results were considered significant at $p < 0.01$ and $p < 0.001$.

Results and Discussion

Compound **1** was obtained as a red amorphous powder and the mass spectrum was in accordance with the formula $C_{44}H_{64}O_{24}$. The presence of hydroxyl groups (3450 cm^{-1}), ester (1700 cm^{-1}), and double bond (1570 cm^{-1}) were indicated by the IR spectrum. The ^1H NMR spectrum showed the presence of methyl peaks at δ 2.00 and 1.98. The presence of a double bond was further indicated by its signals at δ 7.36, 6.87, 6.82, 6.67, and



Compound **1** : R1 = R2 = β-D-gentiobiosyl

Compound **2** : R1 = R2 = H

Compound **3** : R1, R2 = β-D-gentiobiosyl, β-D-glucosyl

Compound **4** : R1, R2 = β-D-gentiobiosyl, H

Fig. 1. Structures of the crocetin derivatives from *Gardenia jasminoides*.

6.54. The anomeric proton signal of the first glucose was presented at δ 5.42, and the anomeric proton signal of the second glucose was presented at δ 4.17. The equitorial proton of position 6 was presented at δ 3.99, and position 6' was presented at δ 3.65. The axial proton of position 6 was presented at δ 3.59, and position 6' was presented at 3.43. A total of 44 carbon signals were observed in the ^{13}C NMR spectrum, including methyl carbon signals at δ 13.1 and 13.0, double bonded carbons at δ 166.6, 145.1, 140.4, 137.4, 136.4, 132.5, 125.7, and 124.4. The gentiobiosyl carbons were presented at δ 103.5, 95.0, 77.3, 77.2, 76.7, 73.9, 72.9, 70.4, 69.7, 68.4, and 61.4. Accordingly, the structure of compound **1** was elucidated as all-trans-crocetin di (β-D-gentiobiosyl) ester. All assignments in the ^{1}H and ^{13}C NMR were based on measurements of HSQC, HMBC, DEPT, and ^{1}H-^{1}H COSY. The final structure of compound **1** was confirmed by a comparison of spectroscopic values to those reported in the literature (Pfister *et al*., 1996; Calsteren *et al*., 1997; Choi *et al*., 2001; Hong *et al*., 2004) (Fig. 1).

Compound **2** was purified a red amorphous powder and the molecular ion peak was found at *m/z* 329 in the MS spectrum, suggesting that the molecular formula of compound **2** was $C_{20}H_{24}O_4$. The IR spectrum showed an absorption band at 3393 cm^{-1} due to hydroxyl group and 1715 cm^{-1} of double bond. The ^{1}H NMR spectrum showed the presence of methyl peaks at δ 1.97 and 1.91. The presence of a double bond was further indicated by its signals at δ 7.20, 6.80, 6.72, 6.64, and 6.49. Total of 20 carbon signals were observed in the ^{13}C NMR spectrum, including methyl carbon signals at δ 14.0 and 9.5, double bonded carbons at δ 143.7, 138.4, 137.1, 135.7, 132.1, 132.0, and 129.1. With all measurements of HSQC, HMBC, DEPT and ^{1}H-^{1}H COSY, and on a comparison of our data with published spectral data (Calsteren *et al*., 1997), the final structure of compound **2** was confirmed as crocetin (Fig. 1).

Compound **3** was obtained as red amorphous powder and the mass spectrum of compound **3** was in accordance with the formula $C_{38}H_{54}O_{19}$. Absorption band at 3394 cm^{-1} in the IR spectrum confirmed the presence of hydroxyl group. The ^{1}H NMR spectrum showed the presence of

methyl peaks at δ 2.00 and 1.97. The presences of double bonds were indicated by its signals at δ 7.35, 6.86, 6.82, 6.65, and 6.57. Because of the non-symmetric sugars on each side of aglycon moiety, the chemical shift of sugar moieties varied from one to another. The anomeric proton signal of the glucose was presented at δ 5.43, the anomeric proton signal of the first glucose unit of gentiobiose was presented at δ 5.41, and the anomeric proton signal of the second glucose unit of gentiobiose was presented at δ 4.17. The equitorial proton of position 6 was presented at δ 3.98, position 6" was presented at δ 3.76, and position 6' was indicated at δ 3.74. The axial proton of position 6 was presented at δ 3.68, position 6" was presented at 3.61, and position 6' was indicated at δ 3.57. A total of 38 carbon signals were observed in the ^{13}C NMR spectrum, including methyl carbon signals at δ 13.1 and 13.0. Double bonded carbon signals were indicated at δ 166.7, 145.1, 145.0, 137.4, 137.4, 136.4, 136.4, 132.5, 125.8, 125.7, and 124.4. Based on this observation, and on a comparison of our data with published spectral data (Calsteren *et al*., 1997; Hong *et al*., 2004), compound **3** was identified as all-trans-crocetin mono (β-D-gentiobiosyl) mono (β-D-glucosyl) ester (Fig. 1).

Compound **4** was obtained as a red amorphous powder and the mass spectrum of compound **4** was in accordance with the formula $C_{32}H_{44}O_{14}$. Absorption bands at 1664 and 1587 cm^{-1} in the IR spectrum confirmed the presence of carbonyl and double bonds. The ^{1}H NMR spectrum showed the presence of methyl peaks at δ 1.98 and 1.93. The presences of double bonds were indicated by its signals at δ 7.35, 7.09, 6.85, 6.66, 6.61, 6.55, and 6.48. Because of the non-esterified carboxylic acid functional group, the chemical shift of aglycon moieties varied from one to another. The anomeric proton signal of the first glucose was presented at δ 5.42, and the anomeric proton signal of the second glucose was presented at δ 4.17. The equitorial proton of position 6 was presented at δ 3.97, and position 6' was presented at δ 3.65. The axial proton of position 6 was presented at δ 3.59, and position 6' was presented at 3.45. A total of 32 carbon signals were observed in the ^{13}C NMR spectrum, including methyl carbon signals at δ 13.1 and 13.0, double bonded carbons at δ 166.7, 145.2, 140.4, 137.7, 136.6, 136.6, 132.7, 132.7, 130.8, and 125.4. The gentiobiosyl carbons were presented at δ 103.6, 95.0, 77.4, 77.2, 76.8, 76.7, 73.9, 72.9, 70.4, 69.7, 68.4, and 61.4. Accordingly, the structure of compound **4** was elucidated as all-trans-crocetin mono (β-D-gentiobiosyl) ester. All assignments in the ^{1}H and ^{13}C NMR were based on measurements of HSQC, HMBC, DEPT and ^{1}H-^{1}H COSY. The final structure of compound **4** was confirmed by a comparison of spectroscopic values to those reported in the literature (Pfister *et al*., 1996; Calsteren *et al*., 1997; Hong *et al*., 2004) (Fig. 1).

Melanin is a complex biopolymer synthesized in melanocytes, and provides protection against UV induced damage by absorbing and scattering UV radiation. But, overproduction and accumulation of melanin result in various hyperpigmentation disorders. Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase related protein-1 (TRP-1), and TRP-2 (Olivares and Solano, 2009). Studies have revealed that most skin lightening agents inhibit the conversion of tyrosine to melanin, and act mostly as tyrosinase inhibitors (Draelos, 2007). Several natural sources have been reported that those products inhibit melanin synthesis by regulating these enzymes. Coenzyme Q10 inhibits tyrosinase activity (Zhang *et al*., 2012), and arbutin from *Arctostaphylos uva-ursi* (Maeda *et al*., 1996), oxyresveratrol from *Morus alba* (Shin *et al*., 1998), and flavonoids (Shimizu *et al*., 2000) were reported to inhibit melanogenesis.

Recently, studies have revealed that reactive oxygen species (ROS) play significant roles in the regulation of melanocyte proliferation and melanogenesis, and reported that antioxidants and ROS scavengers inhibit hyperpigmentation and melanogenesis (Sapkota *et al*., 2010). Although plenty of studies on *Gardenia jasminoides* revealed their antioxidant activities, but their inhibition activity on melanogenesis has rarely been studied. Recent study on melanogenesis inhibitory activity of monoterpene glycosides from *Gardeniae Fructus* was published (Akihisa *et al*., 2012), but the whitening effect of pigments in *Gardenia jasminoides* has not been studied.

Thus, we isolated active compounds and estimated the inhibitory activities on melanin synthesis in α-MSH (melanocyte stimulating hormone) activated B16F10 cells and their tyrosinase expressions on protein and mRNA levels. These active compounds were determined on the basis of IR, MS, and NMR analysis, and identified as crocetin derivatives. Compounds **1 - 4** inhibited melanin production in B16F10 cells with the concentration of 20 uM, 40 uM, and 60 uM in a dose-dependent manner (Fig. 2). Compounds **1 - 4** had no significant cytotoxic effect at concentration of 20-60 uM in the presence and absence of α-MSH (Fig. 3). This inhibition of melanin biosynthesis was not due to cytotoxicity, as determined by MTT assay. The treatment of compounds **1 - 4** to the B16F10 cell reduced a significant tyrosinase activity in the presence and absence of α-MSH (Fig. 4). The result of tyrosinase zymography was similar that reduced the tyrosinase

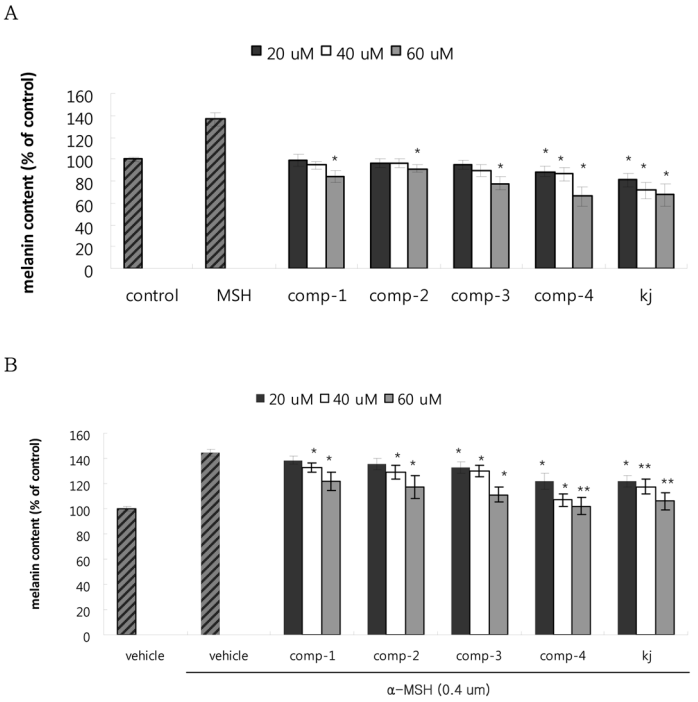


Fig. 2. Inhibitory effects of compounds on melanin production in B16F10 melanoma cells without (A) or with (B) α-MSH (0.4 μM). Cells were seeded at 1×10^5 cells/mL. After 24 hours, cells were treated with various concentrations of compounds **1 - 4** (20 - 60 μM) without (A) or with (B) α-MSH (0.4 μM) and were cultured for 48 hours. Then, melanin contents were measured at 490 nm. Kojic acid (kj) was used as positive control. Each value represents the mean ± S.D of three experiments. *Indicates a significant difference from control, *$p < 0.01$, **$p < 0.001$ (comp-1: crocin, comp-2: crocetin, comp-3: gentiobiosyl glucosyl crocetin, comp-4: mono-gentiobiosyl crocetin, kj: kojic acid).

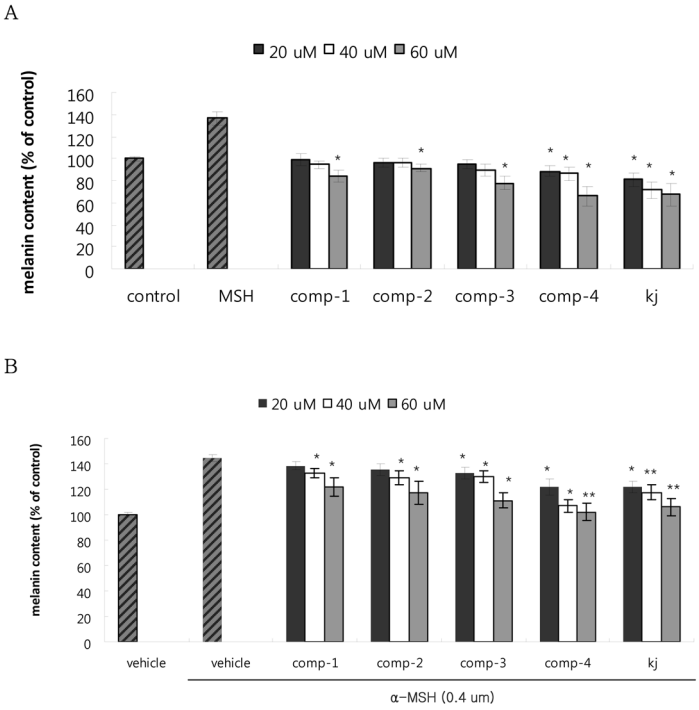


Fig. 3. Effects of compounds from *Gardenia jasminoides* on the viability of B16F10 melanoma cells without (A) or with (B) α-MSH (0.4 μM). B16F10 cells (1.5×10^4 cells/mL) were incubated 24 hours in DMEM containing 10% FBS and were treated for 48 hours. Results are expressed as percentage of control. Values are means ± SD and were obtained from three different experiments (comp-1: crocin, comp-2: crocetin, comp-3: gentiobiosyl glucosyl crocetin, comp-4: mono-gentiobiosyl crocetin, kj: kojic acid).

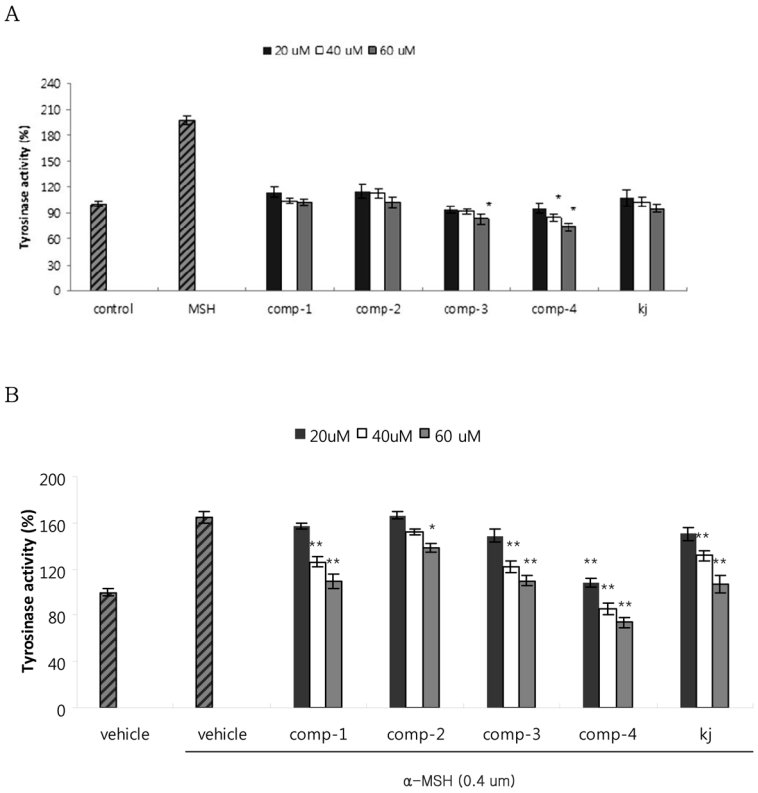


Fig. 4. Inhibitory effects of compounds on tyrosinase activity in B16F10 melanoma cells without (A) or with (B) α-MSH (0.4 μM). Cells were seeded at 1×10^5 cells/mL. After 24 hours, cells were treated with various concentrations of compounds 1 - 4 (20 - 60 μM) without (A) or with (B) α-MSH (0.4 μM) and were cultured for 48 hours. Then, tyrosinase activity was measured at 490 nm. Each value represents the mean ± S.D of three experiments. *Indicates a significant difference from control, $*p < 0.01$, $**p < 0.001$ (comp-1: crocin, comp-2: crocetin, comp-3: gentiobiosyl glucosyl crocetin, comp-4: mono-gentiobiosyl crocetin, kj: kojic acid).

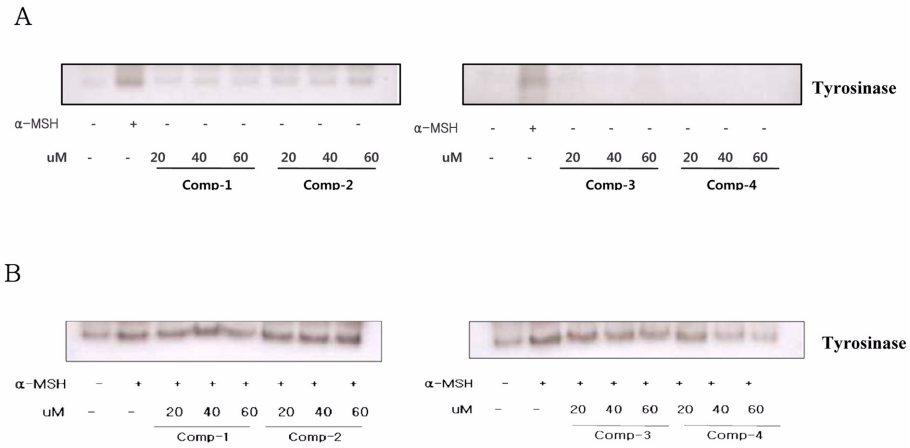


Fig. 5. Gel-dopa staining after treatment with various concentrations of compounds without (A) or with (B) α-MSH (0.4 μM) in B16F10 melanoma cells. Cells were treated with various concentrations of compounds **1 - 4** (20 - 60 μM) in absence (A) and presence (B) of α-MSH (0.4 μM) for 48 hours. After cell lysates were electrophoresed on SDS polyacrylamide gel, the gel was incubated in L-DOPA solution to visualize tyrosinase activity (comp-1: crocin, comp-2: crocetin, comp-3: gentiobiosyl glucosyl crocetin, comp-4: mono-gentiobiosyl crocetin).

activity (Fig. 5). Then we performed western blot analysis to clarify the mechanism for the tyrosinase inhibition by compounds **1 - 4** in α-MSH-activated B16F10 cells. The treatment of compounds **1 - 4** (20 - 60 uM) decreased the expression of tyrosinase activity dose dependently (Fig. 6). The effects of compounds **1 - 4** (20 - 60 uM) on the

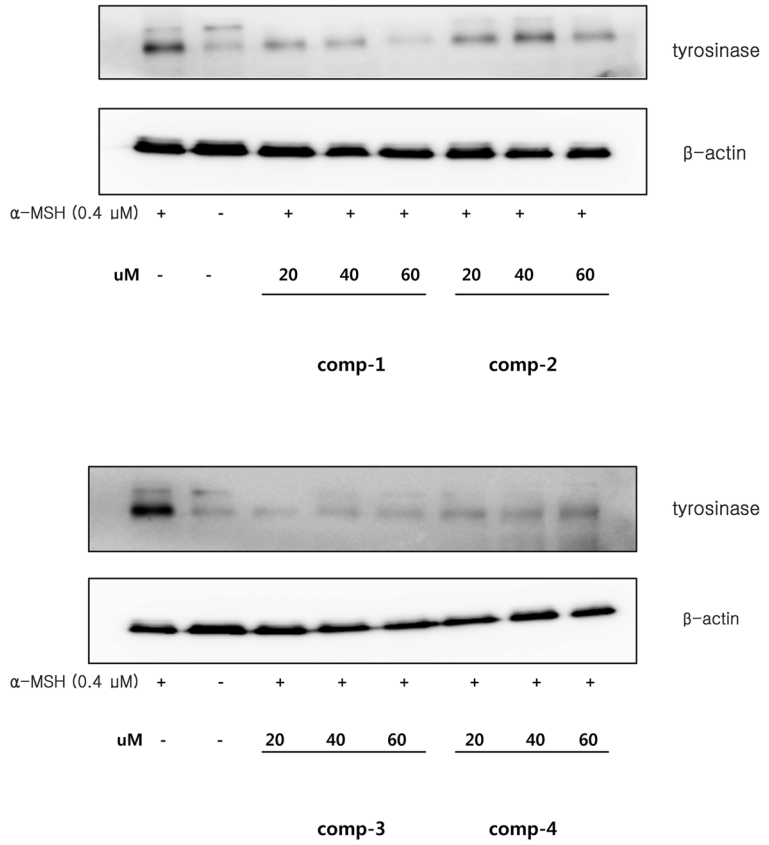


Fig. 6. Effects of compounds on tyrosinase protein expressions in B16F10 melanoma cells. B16F10 cells were treated for 48 hours with α-MSH (0.4 μM) and compounds **1 - 4** (20 - 60 μM). The level of tyrosinase protein in lysates was determined by western blot analysis (comp-1: crocin, comp-2: crocetin, comp-3: gentiobiosyl glucosyl crocetin, comp-4: mono-gentiobiosyl crocetin).

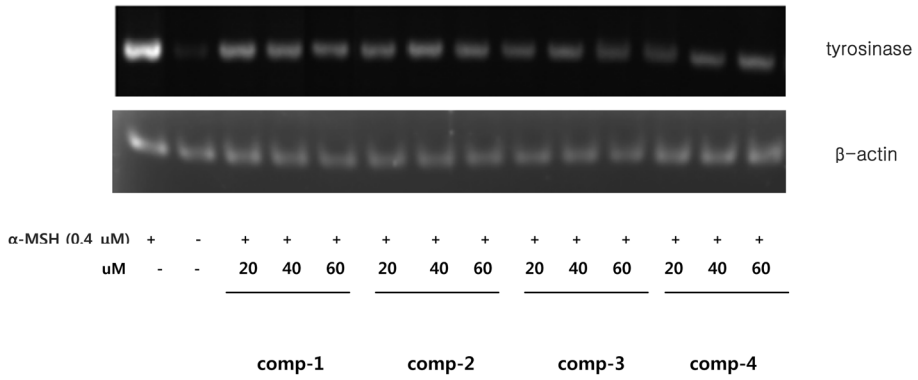


Fig. 7. Effects of compounds on tyrosinase mRNA expressions in B16F10 melanoma cells. B16F10 cells were treated for 48 hours with α-MSH (0.4 μM) and compounds **1 - 4** (20 - 60 μM). The level of tyrosinase mRNA in lysates was determined by RT-PCR analysis (comp-1: crocin, comp-2: crocetin, comp-3: gentiobiosyl glucosyl crocetin, comp-4: mono-gentiobiosyl crocetin).

expression of tyrosinase mRNA were analyzed by RT-PCR. As shown in Fig. 7, compounds **1 - 4** showed significant suppression of mRNA expression in a dose dependent manner.

In conclusion, these results suggested that crocetin derivatives from *Gardenia jasminoides* decrease melanin production in B16F10 melanoma cells, and down-regulate cellular enzymatic activity of tyrosinase as well as decrease mRNA and protein levels, thus might be used as a potential source of skin whitening agents.

References

Aburada, M., Sasaki, H., and Harada, M., Pharmacological studies of

gardenia fruits. II. Contribution of the constituent crude drugs to choleretic activity of 'Inchinko-to' in rats. *Yakugaku Zassh,* **96**, 147-153 (1976).

Akhondzadeh, S., Fallah-Pour, H., Afkham, K., Jamshidi, A.H., and Khalighi-Cigaroudi, F., Comparison of *Crocus sativus L.* and imipramine in the treatment of mild to moderate depression: a pilot doubleblind randomized trial [ISRCTN45683816]. *BMC Complement. Altern. Med.* **4**, 12 (2004).

Akihisa, T., Watanabe, K., Yamamoto, A., Zhang, J., Matsumoto, M., and Fukatsu, M., Melanogenesis inhibitory Activity of Monoterpene Glycosides from *Gardeniae Fructus*. *Chemistry & Biodiversity* **9**, 1490-1499 (2012).

Calsteren , M.R., Bissonnette, M.C., Cormier, F., Dufresne, C., Takahito, I., LeBlanc, J.C., Perreault, D., and Roewer, I., Spectroscopic Characterization of Crocetin Derivatives from *Crocus sativus* and *Gardenia jasminoides. J. Agric. Food. Chem*. **45**, 1055-1061 (1997).

Chang, W.C., Lin, Y.L., Lee, M.J., Shiow, S.J., and Wang, C.J., Inhibitory effect of crocetin on benzo(a)pyrene genotoxicity and neoplastic transformation in C3H10T1/2 cells. *Anticancer Res*. **16**, 3603-3608 (1996)

Choi, H.J., Park, Y.S., Kim, M.G., Kim, T.K., Yoon, N.S., and Lim, Y.J., Isolation and characterization of the major colorant in Gardenia fruit. *Dyes and Pigment* **49**, 15-20 (2001).

Draelos, Z.D., Skin lightening preparations and the hydroquinone controversy. *Dermatol. Therapeut.* **20**, 308-313 (2007).

Escribano, J., Alonso, G.L., Coca-Prados, M., and Fernandez, J.A., Crocin, safranal and picrocrocin from saffron (*Crocus sativus L.*) inhibit the growth of human cancer cells *in vitro. Cancer Lett.* **100**, 23-30 (1996).

Hong, Z., Yuhong, Z., Fang, Y., Fang, C., Xinshen, Z., Minhua, L., and Weiguo, L., Semi-Preparative Isolation of Crocins from Saffron (*Crocus sativus L.). Chromatographia* **59**, 691-696 (2004).

Hsu, J.D., Chou, F.P., Lee, M.J., Chiang, H.C., Lin, Y.L., Shiow, S.J., and Wang, C.J., Suppression of the TPA-induced expression of nuclear-protooncogenes in mouse epidermis by crocetin via antioxidant activity. *Anticancer Res*. **19**, 4221-4227 (1999).

Koo, H.J., Lim, K.H., Jung, H.J., and Park, E.H., Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. *J. of Ethnopharmacol*. **103**, 496-500 (2006).

Lim, H., Park, K.R., Lee, D.U., Kim, Y.S., and Kim, H.P., Effects of the constituents of Gardenia Fructus on Prostaglandin and NO production. *Biomol. and Therapeut.* **16**, 82-86 (2008).

Maeda, K. and Fukuda, M., Arbutin: mechanism of its depigmenting action in human melanocyte culture. *J. pharmacol. and Exper. Ther.* **276**, 765-769 (1996).

Miyasita, S., A historical study of Chinese drugs for the treatment of jaundice. *Am. J. of Clin. Med.* **4**, 239-243 (1976).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. **65**, 55-63 (1983).

Nam, K.N., Choi, Y.S., Jung, H.J., Park, G.H., Park, J.M., Moon, S.K., Cho, K.H., Kang, C.H., Kang, I.S., Oh, M.S., and Lee, E.J., Genipin inhibits the inflammatory response of rat brain microglial cells. *Inter. Immunopharmacol*. **10**, 493-499 (2010).

Olivares, C., and Solano, F., New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins. *Pigment. Cell. Melanoma. Res*. **22**, 750-760 (2009).

Papandreou, M.A., Kanakis, C.D., Polissiou, M.G., Efthimiopoulos, S., Cordopatis, P., Margarity, M., and Lamari, F.N., Inhibitory activity on amyloid-beta aggregation and antioxidant properties of *Crocus sativus* stigmas extract and its crocin constituents. *J. Agric. Food. Chem.* **54**, 8762-8768 (2006).

Pfister, S., Meyer, P., Steck, A., and Pfander, H., Isolation and Structure Elucidation of Carotenoid-Glycosyl Esters in Gardenia Fruits (*Gardenia jasminoides Ellis*) and Saffron (*Crocus sativus Linne*). *J. Agric. Food. Chem.* **44**, 2612-2615 (1996).

Sapkota, K., Park, S.E., Kim, J.E., Kim, S., Choi, H.S., Chun, H.S., and Kim, S.J., Antioxidant and Antimelanogenic Properties of Chestnut Flower Extract. *Biosci. Biotechnol. Biochem*. **74**, 1527-1533 (2010).

Shimizu, K., Kondo, R., and Sakai, K., Inhibition of tyrosinase by flvonoids, stilbenes and related 4-substituted resorcinols: structure-activity investigations. *Planta Med*. **66**, 11-15 (2000).

Shin, N.H., Ryu, S.Y., Choi, E.J., Kang, S.H., Chang, I.M., Min, K.R., and Kim, Y., Oxyresveratrol as the potent inhibitor on DOPA oxidase activity of mushroom tyrosinase. *Biochem. Biophys. Res. Commun*. **243**, 801-803 (1998).

Tseng, T.H., Chu, C.Y., Huang, J.M., Shiow, S.J., and Wang, C.J., Crocetin protects against damage in rat primary hepatocytes. *Cancer Lett.* **97**, 61-67 (1995).

Zhang, M., Dang, L., Guo, F., Wang, X., Zhao, W., and Zhao, R., Coenzyme Q_{10} enhances dermal elastin expression, inhibits IL-1α production and melanin synthesis *in vitro. Intern. J. Cosme. Sci.* **34**, 273-279 (2012).

Zheng, X.C., Yang, D., Liu, X., Wang, N., Li, B., Cao, H.W., Lu, Y.L., Wei, G., Zhou, H., and Zheng, J., Identification of a new anti-LPS agent, geniposide, from Gardenia jasminoides Ellis, and its ability of direct binding and neutralization of lipopolysaccharide in vitro and in vivo. *Intern. Immunopharmacol*. **10**, 1209-1219 (2010).

Received March 21, 2013
Revised May 24, 2013
Accepted July 28, 2013