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Isolation, Identification, and Quantification of Tyrosinase and α-Glucosidase Inhibitors from UVC-Irradiated Mulberry (*Morus alba* L.) Leaves

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ABSTRACT: Methanol extracts from ultraviolet (UV) C-irradiated mulberry leaves (UVC-IML) exhibit stronger tyrosinase and α-glucosidase inhibitory activities than those from unirradiated mulberry leaves. Through a bioassay-guided fractionation and purification process, two oxyresveratrol derivatives, oxyresveratrol (ORT) and 4'-prenyloxyresveratrol (PORT), and six 2-arylbenzofuran derivatives [moracin B (MCB), moracin C (MCC), moracin M (MCM), moracin N (MCN), 6,5'-dimethoxymoracin M (DMMCM), and chalcomoracin (CMC)] were isolated from the methanol extract from UVC-IML. Their chemical structures were determined by UV, mass, and nuclear magnetic resonance spectrometry. ORT and PORT showed potent tyrosinase inhibitory activities with the half maximal inhibitory concentration (IC_{50}) values of 0.57 and 0.90 μM, respectively, and CMC exhibited significant tyrosinase and α-glucosidase inhibitory activity with IC_{50} values of 5.61 and 6.00 μM, respectively. Levels of these eight compounds were increased significantly following irradiation compared with untreated mulberry leaves; ORTs increased approximately 4 fold and moracins increased 2∼16 fold. These data suggest that UVC-IML may represent a promising source of nutraceuticals and cosmeceuticals for prevention of diabetes and skin aging.

Keywords: mulberry (*Morus alba*) leaf, UVC irradiation, oxyresveratrol, moracin, enzyme inhibition

INTRODUCTION

Mulberry (*Morus alba* L.) leaves have widely been used in Korean traditional medicine for treatment of diabetes, hypertension, obesity, fever, and liver damage (1). Mulberry leaves are rich in various functional components, such as 1-deoxynojirimycin (1-DNJ), γ-amino-butyric acid (GABA), flavonoids, stilbenes, and 2-arylbenzofurans (moracins, MCs), which have anti-hyperglycemic, anti-hypertensive, anti-hyperlipidemic, anti-aging, and antioxidant activities (2,3). The mulberry leaf has recently been suggested as an anti-diabetic ingredient in functional food in Korea (4), where the various food-grade mulberry products include drink powders, herbal teas, pills, and tablets (3,5).

Mulberry leaves contain major phytochemicals, such as chlorogenic acid, 1-DNJ, GABA, and flavonoids, whereas mulberry twigs and root barks contain major bioactive constituents, including prenylflavonoids, stilbenes, and MCs (2). Of these phytochemicals, oxyresveratrol (ORT) and MC derivatives, which are found in mulberry twig and root barks, are as great interest as skin-whitening and anti-diabetic agents (6-9). Recently, mulberry leaves are receiving great interest as functional sources since ORT and MC derivatives with anti-hyperglycemic and anti-hyperpigmentation activities have also been found in mulberry leaves (10-13). Despite the presence of these important phytochemicals in mulberry leaves, screening and isolation of functional ORTs and MCs from mulberry leaves of Korea remains limited. Mulberry leaves containing high amounts of ORTs and MCs could be widely used as rich sources of nutraceuticals and cosmeceuticals.

Ultraviolet (UV) irradiation is well-known to improve the quality and biological capacity of foods through inducing the formation of bioactive secondary metabolites in plants (14,15). In particular, UVB and UVC irradiations have been found to increase phytochemical polyphenols, including flavonoids, anthocyanins, and resveratrols in plant leaves and fruits (16-18). A recent study reported that UVB irradiation induces and increases two MC derivatives, chalcomoracin (CMC) and moracin N (MCN), in mulberry leaves (19). However, the effects of

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UV irradiation on phytochemicals and the biological activity of mulberry leaves grown in Korea have not yet been reported. Moreover, production of high quality mulberry leaves by UV irradiation is necessary prior to preparation of value-added mulberry leaf products in Korea.

The objective of this study was to isolate and identify major components with strong tyrosinase and α-glucosidase activities from the methanol extract of UVC-irradiated mulberry leaves (UVC-IML), and to determine their constituents in UVC treated- and untreated-mulberry leaves using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Mulberry leaves and UV irradiation

The leaves of mulberry tree 'Iksuppong' were harvested in September 2017 at Yeongcheon mulberry farmer, Gyeongbuk, Korea. Leaves were transported to the UV-irradiation room, where they were treated by pilot UV apparatus within 2 h of harvest. Mulberry leaves were placed on a convey belt with the back of the leaves facing upwards, and irradiated with UVC light for 5 min at room temperature. UVC irradiation was performed using a UVC light (UV_{254nm}) equipped with ten lamps (upper layer: 6 lamps, with a maximum intensity 10,320 mW/cm^2; lower layer: 4 lamps, with a maximum intensity of 2,755 mW/cm^2; 60 cm distance between the layers) of 155W T-40 M (Philips Co., Amsterdam, Netherlands). The UVC treated mulberry leaves were put into fabric bags at room temperature for one day and then dried in a dry oven at 40±5°C. The plant material was identified by Dr. Sung KB, Sericulture and Entomology Experimental Station, National Institute of Agricultural Science, Wanju, Jeonbuk, Korea, for identifying mulberry leaves. α-Glucosidase (EC 3.2.1.20), mushroom tyrosinase (EC 1.14.18.1), and reagents used for enzyme assays were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All solvents for HPLC analysis were of Merck HPLC grade. All other reagents used in this study were of analytical grade.

Extraction and isolation of oxyresveratrol and moracin derivatives

The dried UVC-irradiated mulberry leaves (10 kg) were extracted twice with 80 L of 70% aqueous (*aq*.) methanol under an ultrasonicator (Power Sonic 420, Hwashin Instrument Co., Ltd., Seoul, Korea) for 12 h, followed by filteration and concentration *in vacuo*. The crude extract (1.8 kg) was solubilized in 70% *aq*. Methanol (MeOH) and defatted with *n*-hexane by fractionation. The defatted crude extract (1.66 kg) was suspended in distilled water and partitioned successively with ether (Et_2O), ethyl-acetate (EtOAc), and *n*-butanol (*n*-BuOH). Three solvent fractions were evaporated and obtained: Et_2O (203.4 g), EtOAc (122.8 g), and *n*-BuOH (535.1 g). Among these fractions, the Et_2O fraction exhibited the strongest inhibitory activity against both tyrosinase and α-glucosidase, with the half maximal inhibitory concentration (IC_{50}) of 20.73 and 30.58 μg/mL, respectively. The Et_2O fraction was then subjected to chromatography over a silica gel (70～230 mesh, 12 kg, Merck, Darmstadt, Germany) column (8.5×70 cm), subjected to gradient elution using $CHCl_3$-MeOH (15:1, 10:1, 7:1, and 5:1, v/v, each volume 18 L) as the eluent, to yield seven fractions (Fr. 1～7). Fr. 2 and 4 were successively chromatographed on a octadecyl-silica (ODS)-A (YMC Inc., Milford, MA, USA) column (4.5×60 cm) with 80% *aq*. EtOH and a Sephadex LH-20 column (2.5×80 cm) with 90% *aq*. MeOH; this yielded Comp. 1 (14.2 mg) from Fr. 2, and Comp. 2 (14.5 mg) from Fr. 4, respectively. Fr. 5 and 6 were also subjected to the same purification procedure using ODS-A (60% *aq*. EtOH) and Sephadex LH-20 (90% *aq*. MeOH) columns to yield Comp. 3 (10.1 mg) and Comp. 4 (94.5 mg) from Fr. 5, and Comp. 5 (126.1 mg) from Fr. 6, respectively. Finally, Fr. 7 was separated by ODS-A (eluted gradiently with 40%, 60%, and 80% *aq*. EtOH, each volume 50 mL) to generate four fractions (Fr. 7A～7D). Fr. 7A, 7B, and 7D were purified on a Sephadex LH-20 column (2.5×80 cm) with 90% *aq*. MeOH and yielded Comp. 6 (74.2 mg) from Fr. 7A, Comp. 7 (8.7 mg) from Fr. 7B, and Comp. 8 (39.6 mg) from Fr. 7D, respectively. A schematic for the isolation and purification of ORT and MC derivatives from UVC-IML is presented in Fig. 1.

Identification of isolated compounds

The UV absorption spectra of the eight isolated compounds (in MeOH) were obtained with a photodiode array UV-vis spectrophotometer (S-1100, UNICO, Dayton, NJ, USA). The ^{1}H-nuclear magnetic resonance (NMR) (600 MHz) and ^{13}C-NMR (150 MHz) spectra of the isolated compounds were measured in methanol-d4 (CD_3OD) on a spectrometer (VNS-600, Varian Inc., Palo Alto, CA, USA); chemical shifts are given as δ value using tetramethylsilane (TMS) as an internal standard. The fast atom bombast mass spectrometry (FABMS) spectra were obtained using a JMS HX-100 mass spectrometer (JEOL, Ltd., Tokyo, Japan).

Assay of tyrosinase

The inhibitory effect on tyrosinase were measured using the spectrophotometric method previously described, using L-tyrosine in place of L-dihydroxyphenylalanine as a substrate (20). Sample solutions (25 μL) of varying concentrations were mixed with 25 μL of tyrosinase (2,000 U/mL) in 0.1 M sodium phosphate buffer (pH 6.8), 250 μL of the same buffer and 225 μL of ultrapure water. Af-

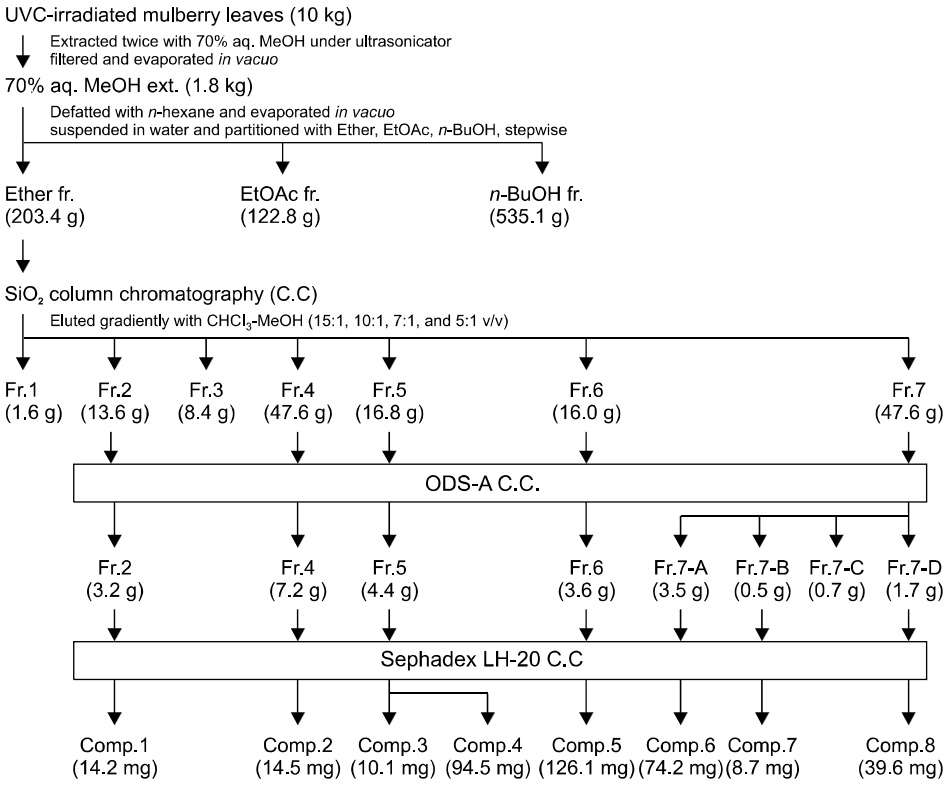

Fig. 1. Schematic procedure for isolation and purification of oxyresveratrol and moracin derivatives from ultraviolet C-irradiated mulberry leaves.

ter preincubation at 37°C for 10 min, 250 μL of L-tyrosine (0.03%) in an ultrapure water was added. After incubation at 37°C for 10 min, the amount of dopachrome in the reaction mixture was determined at 475 nm using a microplate reader. The percentage inhibition of tyrosinase activity was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - \frac{A-B}{C-D} \times 100$$

where A is absorbance at 475 nm of the test sample with enzyme, B is the absorbance at 475 nm of the test sample without enzyme, C is the absorbance at 475 nm with enzyme but without test sample, and D is the absorbance at 475 nm without test sample and enzyme. The inhibitory activity of the sample was expressed as the concentration which inhibits 50% of the enzyme activity. Arbutin was used as the positive control.

Assay of α-glucosidase

The inhibitory effect on α-glucosidase was measured using the spectrophotometric method described previously (20). Sample solutions (10 μL) of varying concentrations were mixed with 50 μL of α-glucosidase (2 U/mL) in 0.1 M sodium phosphate buffer (pH 6.8). After preincubation at 37°C for 15 min, 50 μL of 4-nitrophenyl-α-D-glucopyranoside (5 mM) was added. Following 5 min incubation at 37°C, the amount of 4-nitrophenol in the reaction mixture was measured spectrophotometrically at 405 nm using a microplate reader. The percentage inhibition of α-glucosidase activity and IC_{50} value were calculated by the same method of above tyrosinase assay. Acarbose was used as the positive control.

Quantification of ORT and MC derivatives by HPLC

Dried UVC-irradiated mulberry leaves (2 g) were homogenized with 20 mL of 70% *aq*. MeOH in tissue homogenizer (T 25 digital ULTRA-TURRAX®, IKA, Freiburg im Breisgau, Germany) for 3 min and centrifuged at 3,000 rpm for 30 min. The upper layer was taken and filled up 20 mL with the same solvent. The aliquot was passed through a 0.45 μm membrane filter (polyvinylidene difluoride syringe filter, Finetech Research and Innovation Corp., Taichung, Taiwan) and injected into an analytical HPLC. HPLC was performed using a Waters e2690/5 HPLC system (Waters, Milford, MN, USA) equipped with a 2998 photodiode array detector at 320 nm and an autosampler. HPLC analysis was carried out using an YMC-Pack Pro C_{18} column (46 mm i.d×250 mm, YMC Inc.) with a Guard-Pak C_{18} precolumn insert. The separation was conducted using a linear gradient of two solvent systems (solvent A, 0.05% H_3PO_4 in H_2O, and solvent B, CH_3CN) at a flow rate of 0.8 mL/min. Eight polyphenols, including ORT and MC derivatives, were identified by comparisons of their retention times with those of the eight standards isolated previously. Linear correlation coefficients were superior to 0.999 for each polyphenol. Levels of polyphenols were determined by calibration curves of eight standard polyphenols [ORT, y=5.7681x −4.3606; 4'-prenyloxyresveratrol (PORT), y=3.1425x−

1.3712; moracin B (MCB), y=5.1445x+2.6872; moracin C (MCC), y=5.9803x+3.3987; moracin M (MCM), y=4.7938x−1.0534; MCN, y=6.4804x+1.3008; 6,5'-dimethoxymoracin M (DMMCM), y=5.4493x+1.2256; CMC, y=3.6986x+4.4093] and expressed in mg per 100 g of the dried weight of mulberry leaves. Recovery rates for the eight polyphenols were above 93%.

Statistical analysis

All data were expressed as mean±standard deviation (SD) of three determinations. Statistical analyses were performed using IBM SPSS Statistics 19.0 software (IBM Corp., Armonk, NY, USA). Statistical comparisons were carried out using the Student's *t*-test for independent samples, with results where $P<0.05$ considered to be statistically significant.

RESULTS AND DISCUSSION

Isolation and structural elucidation of ORT and MC derivatives

The MeOH extract of UVC-IML with the strong tyrosinase and α-glucosidase inhibitory activities were partitioned successively with Et_2O, EtOAc, and *n*-BuOH. The Et_2O fraction showing potent enzyme inhibition was further chromatographed onto a silica gel column and was purified using ODS-A and Sephadex LH-20 column chromatographies to generate two ORT and six MC derivatives. The structures of the eight compounds were identified by UV, MS, and NMR spectrometry, and through comparing with published spectrometric data (13,21-23). Comp. 1 showed UV absorption maxima (λ_{max}) at 218, 235 (sh), 290 (sh), 301, and 330 nm, and a positive FABMS $[M+H]^+$ with a molecular peak at *m/z* 245 and a fragment ion peak at *m/z* 135, assignable to a ORT moiety (24). The ^{1}H- and ^{13}C-NMR spectra of Comp. 1 showed 2,4-dihydroxybenzene signals (A ring), two *trans*-olefinic signals, and 3',5'-dihydroxybenzenel signals (B ring). Thus, Comp. 1 was easily identified as an ORT. The FABMS spectrum of Comp. 2 showed a molecular ion at m/z 313 $[M+H]^+$ and fragment ions at m/z 257 $[M^+-C_4H_7]$ and 243 $[M^+-C_5H_9]$, indicating that a prenyl group was attached to Comp. 1. The ^{1}H- and ^{13}C-NMR spectra of Comp. 2 showed a similar spectra to that of Comp. 1 except that 3',5'-dihydroxybenzene was substituted for a 4'-prenyl group (δ 1.65, 1.75, 3.26, and 5.23 ppm) in a B ring. From the above results, Comp. 2 was characterized as a PORT. ORT and PORT have been identified as the major stilbenes in mulberry twigs and root barks (22,24,25), but are rarely detected in mulberry leaves. In addition, six MC derivatives containing a 2-arylbenzofuran skeleton were identified. Comp. 3 showed UV absorption maxima (λ_{max}) at 214, 295 (sh), 320, and 332 (sh) nm, and a positive FABMS $[M+H]^+$ with a molecular peak at *m/z* 243 together with a fragment ion peak at *m/z* 133, which are indicative of a typical 2-arylbenzofuran unit (21). The ^{1}H- and ^{13}C-NMR spectra of Comp. 3 showed one set of ABX type aromatic signals (A ring), a singlet signal (C ring), and A_3 type aromatic signals (B ring), indicating that Comp. 3 was a 6,3',5'-trihydroxy-2-arylbenzofuran compound (22). Comp. 3 was therefore easily characterized as a MCM. Comp. 4 gave a molecular peak at *m/z* 311 $[M+H]^+$, together with fragment peaks at *m/z* 255 $[M^+-C_4H_7]$ and 241 $[M^+-C_5H_9]$ by FABMS spectrometry, indicating that a prenyl group was attached to Comp. 3. The ^{1}H- and ^{13}C-NMR spectrum of Comp. 4 was very similar to those of Comp. 3, except for the existence of one prenyl group at the 4'-position of the benzene B ring, one prenyl signal and two meta-coupled aromatic signals in a B ring. Comp. 4 was therefore elucidated to be 4'-prenylmoracin M, MCC. Comp. 5 had the same molecular ion spectra as Comp. 4 at *m/z* 311 $[M+H]^+$by FABMS spectrometry. The ^{1}H- and ^{13}C-NMR of Comp. 5 showed a typical 2-arylbenzofuran unit with one prenyl group at the 5 position of A ring: two *para*-coupled aromatic signals of one prenyl group (A ring), a downfield singlet signal (C ring), and three *meta*-coupled aromatic signals (B ring). Comp. 5 was therefore identified as a 5-prenylmoracin M, MCN. Comp. 6 gave a molecular peak at *m/z* 271 $[M+H]^+$, together with parent peaks at *m/z* 239 $[M^+-CH_3O]$ and 209 $[M^+-C_2H_6O_2]$. The ^{1}H-NMR of Comp. 6 showed a MCM unit attached with two methoxy group: a ABX type aromatic protons (A ring) at δ 7.43 ppm (1H, d, J=8.4 Hz, H-4), 7.10 ppm (1H, d, J=1.8 Hz, H-7), 6.86 ppm (1H, dd, J=2.4 and 8.4 Hz, H-5): a downfield broad singlet (C ring) at δ 7.01 ppm (1H, br s, H-3); anA_3 type aromatic protons (B ring) at δ 6.88 ppm (2H, d, J=2.4 Hz, H-2'and H-6') and 6.34 ppm (1H, t, J=2.4 Hz, H-4'); and two methoxy protons at δ 3.81 and 3.85 ppm (each 3H, s, H-3', and H-5'). The ^{13}C-NMR spectra of Comp. 6 was very similar to that of Comp. 3 (MCM), attached to each methoxy group at C-6 and C-5' positions: a downfield signal at C-3 (δ 159.80 ppm), C-5' (162.74 ppm), C-6' (105.06 ppm) positions, and a upfield signal at C-4' (δ 102.43 ppm) (21,23). Comp. 6 was therefore identified as 6,5'-dimethoxymoracin M (DMMCM), which has not previously been found in the mulberry tree, even though several methoxymoracin derivatives of Comp. 6 have been identified in mulberry twigs and root barks (21,23,26). Comp. 7 showed a molecular peak at *m/z* 287 $[M+H]^+$ a parent peak at *m/z* 271 $[M^+-OH]$, suggesting that Comp. 7 was a hydroxysubstituted Comp. 6. The structure of Comp. 7 was easily identified by comparing the ^{1}H- and ^{13}C-NMR of Comp. 6 with moracin B (MCB); two *para*-coupled aromatic proton signals at δ 7.14 ppm (1H, s, H-4) and δ 6.92 ppm (1H, s, H-7), and a down-

Table 1. ^{1}H-NMR spectral data of eight compounds isolated from ultraviolet C-irradiated mulberry leaves

Position	Comp. 1 (ORT)	Comp. 2 (PORT)	Comp. 3 (MCM)	Comp. 4 (MCC)	Comp. 5 (MCN)	Comp. 6 (DMMCM)	Comp. 7 (MCB)	Comp. 8 (CMC)
H-3	6.29, 1H, d, J=2.0	6.26, 1H, d, J=2.0	6.90, 1H, s	6.82, 1H, s	6.85, 1H, s	7.01, 1H, br s	6.95, 1H, br s	6.84, 2H, s
H-4			7.34, 1H, d, J=8.4	7.31, 1H, d, J=8.4	7.18, 1H, s	7.43, 1H, d, J=8.4	7.14, 1H, s	7.28, 1H, d, J=9.0
H-5	6.30, 1H, dd, J=2.0, 9.2	6.28, 1H, dd, J=2.0, 9.2	6.73, 1H, dd, J=2.4 and 8.4	6.71, 1H, dd, J=2.4 and 7.8		6.86, 1H, dd, J=2.4 and 8.4		6.83, 1H, m
H-6	7.31, 1H, d, J=9.6	7.29, 1H, d, J=9.0						
H-7			6.88, 1H, d, J=1.8	6.87, 1H, br d, J=1.8	6.88, 1H, s	7.10, 1H, d, J=1.8	6.92, 1H, s	6.84, 2H, s
Hα	7.26, 1H, d, J=16.2	7.21, 1H, d, J=16.2						
Hβ	6.80, 1H, d, J=16.2	6.75, 1H, d, J=16.2						
H-10					3.30, 2H, br d, J=7.0			
H-11					5.37, 1H, br t, J=7.0			
H-13					1.28, 3H, s			
H-14					1.74, 3H, s			
H-2′	6.43, 2H, d, J=1.8	6.45, 2H, d, J=2.0	6.75, 2H, d, J=2.4	6.77, 2H, s	6.74, 2H, d, J=2.4	6.88, 2H, d, J=2.4	6.84, 2H, d, J=2.4	6.80, 2H, br s
H-4′	6.12, 1H, t, J=2.0		6.23, 1H, t, J=2.4		6.22, 1H, t, J=2.1	6.34, 1H, t, J=2.4	6.33, 1H, t, J=2.4	
H-6′	6.43, 2H, d, J=1.8	6.45, 2H, d, J=2.0	6.75, 2H, d, J=2.4	6.77, 2H, s	6.74, 2H, d, J=2.4	6.88, 2H, d, J=2.4	6.84, 2H, d, J=2.4	6.80, 2H, br s
H-7′		3.26, 2H, br d, J=7.0		3.30, 2H, br d, J=7.0				
H-8′		5.23, 1H, br t, J=7.0		5.25, 1H, br t, J=7.0				5.71, 1H, br s
H-9′								4.05, 1H, br s
H-10′		1.75, 3H, s		1.66, 3H, s				4.55, 1H, t, J=4.5
H-11′		1.65, 3H, s		1.77, 3H, s				3.70, 1H, t, J=4.5
H-12′								2.44, 1H, m 2.16, 1H, m
H-13′								1.91, 3H, s
H-19′								6.34, 1H, d, J=9.3
H-20′								8.33, 1H, d, J=9.6
H-23′								6.69, 1H, d, J=2.4
H-25′								6.21, 1H, dd, J=2.4, 8.0
H-26′								6.92, 1H, d, J=8.4
H-27′								3.22, 2H, d, J=7.8
H-28′								5.15, 1H, t, J=7.2
H-30′								1.71, 3H, s
H-31′								1.59, 3H, s
OCH_3						3.85	3.92	
OCH_3						3.81	3.81	

Chemical shift in δ ppm, coupling constant (J) expressed in Hz in parenthesis and measured in the solvent CD_3OD, taking TMS as an internal standard.
ORT, oxyresveratrol; PORT, 4′-prenyloxyresveratrol; MCM, moracin M; MCC, moracin C; MCN, moracin N; DMMCM, 6,5′-dimethoxymoracin M; MCB, moracin B; CMC, chalcomoracin.

field shift (113.09→144.80 ppm) at C-5 signal (A ring) (21,26). Comp. 7 was also not found in mulberry leaves. Finally, Comp. 8 showed a molecular peak at *m/z* 677 $[M+H]^+$. The ^1H- and ^{13}C-NMR spectra of Comp. 8 showed the presence of a 2-arylbenzofuran unit, a tetra-substituted cyclohexene ring, two 2,4-dihydroxyphenyl

Table 2. ^{13}C-NMR spectral data of eight compounds isolated from ultraviolet C-irradiated mulberry leaves

Position	Comp. 1 (ORT)	Comp. 2 (PORT)	Comp. 3 (MCM)	Comp. 4 (MCC)	Comp. 5 (MCN)	Comp. 6 (DMMCM)	Comp. 7 (MCB)	Comp. 8 (CMC)
C-1	117.84	118.07						
C-2	157.34	157.17	156.15	156.49	154.59	156.44	156.33	155.24
C-3	103.55	103.54	107.18	101.26	102.23	102.38	102.52	100.31
C-4	159.24	157.21	121.98	121.76	121.39	122.09	104.94	121.31
C-5	108.37	108.33	113.24	113.08	126.17	113.09	144.80	111.67
C-6	128.38	128.18	156.87	157.13	155.55	159.80	148.31	155.75
C-7			98.46	98.42	97.85	96.60	95.99	97.00
C-8			157.25	156.59	155.70	157.19	150.62	154.67
C-9			123.04	123.19	122.75	123.81	123.03	121.71
Cα	124.81	125.70						
Cβ	126.50	126.68						
C-10					29.50			
C-11					124.40			
C-12					134.00			
C-13					17.81			
C-14					25.96			
C-1′	142.19	138.50	133.81	130.24	132.80	133.73	133.93	129.96
C-2′	105.64	103.58	103.91	103.83	103.84	105.06	106.20	103.48
C-3′	159.57	159.03	159.97	157.51	159.89	160.08	160.03	155.65
C-4′	102.27	115.39	103.51	116.88	103.31	102.43	102.33	114.74
C-5′	159.57	159.03	159.97	157.51	159.89	162.74	162.70	155.65
C-6′	105.64	105.58	103.91	103.83	103.84	105.06	102.17	103.48
C-7′		23.30		23.31				132.65
C-8′		123.71		124.35				123.54
C-9′		131.01		131.33				32.28
C-10′		25.98		25.98				48.00
C-11′		17.91		17.92				35.68
C-12′								31.48
C-13′								22.39
C-14′								208.76
C-15′								112.30
C-16′								163.18
C-17′								115.69
C-18′								162.44
C-19′								106.61
C-20′								131.11
C-21′								120.39
C-22′								156.39
C-23′								102.05
C-24′								156.55
C-25′								105.78
C-26′								127.53
C-27′								21.01
C-28′								122.20
C-29′								130.38
C-30′								24.46
C-31′								16.45
OCH_3						56.2	56.8	
OCH_3						55.8	55.8	

Chemical shift in δ ppm, coupling constant (J) expressed in Hz in parenthesis and measured in the solvent CD_3OD, taking TMS as an internal standard.
ORT, oxyresveratrol; PORT, 4′-prenyloxyresveratrol; MCM, moracin M; MCC, moracin C; MCN, moracin N; DMMCM, 6,5′-dimethoxy-moracin M; MCB, moracin B; CMC, chalcomoracin.

moieties, and an isoprenyl moiety, indicating that Comp. 8 was identical to a chalcomoracin (CMC). Thus, Comp. 8 was characterized as a chalcomoracin, which has been shown to be a natural Diels-Alder type adduct with anti-microbial and anti-diabetic activities (27-29). The detailed ^{1}H- and ^{13}C-NMR spectral data for the eight ORT and MC derivatives from UVC-IML are given in Table 1 and 2. From the above results, we showed that two ORTs, ORT and PORT, and six MCs, MCB, MCC, MCM, MCN, DMMCM, and CMC, were isolated and identified from UVC-IML for the first time from mulberry leaves; four MC derivatives (MCC, MCM, MCN, and CMC) have previously been isolated and identified from mulberry leaves and UVB-irradiated mulberry leaves (13,19). ORT and MC derivatives are known as phytoalexins from diseased mulberry shoots (30,31). In this study, we speculated that ORT and MC derivatives in mulberry leaves are induced by UVC irradiation. Therefore, the ORT and MC derivatives in UVC-IML could represent phytoalexins with biological activity for preventing several pathological disorders.

Inhibition of tyrosinase and α-glucosidase activities

Recently, research has focused on the application of naturally occurring crude drugs for the functional ingredients of nutraceuticals and cosmeceuticals. In particular, mulberry extracts are widely used in Korea for treatment of diabetes and skin aging due to the potency of α-glucosidase and tyrosinase inhibitors (2). Tyrosinase catalyzes melanin synthesis in mammals; overproduction of melanin causes skin hyperpigmentations, such as age spots, melasma, and chloasma. Tyrosinase inhibitors could therefore be used as a skin-whitening agent in cosmetics (32). Meanwhile, α-glucosidase is responsible for control of postprandial glucose levels. α-Glucosidase inhibitors have been used clinically for controlling blood glucose levels as potential therapeutic agents for type 2 diabetes mellitus (33). As shown in Table 3, the methanol extract of UVC-IML showed significantly ($P<0.001$) stronger inhibitory activities against tyrosinase (IC_{50}=130.93 μg/mL) and α-glucosidase (IC_{50}=299.41 μg/mL) than those from unirradiated mulberry leaves (IC_{50}=498.86 and 751.75 μg/mL). Furthermore, out of the three solvent

Table 3. Inhibitory effects of methanolic extract and three solvent fractions from the ultraviolet (UV) C-irradiated mulberry leaves on tyrosinase and α-glucosidase activities

Sample	Inhibition (IC_{50}, μg/mL)	
	Tyrosinase	α-Glucosidase
MeOH extract (control)	498.86±7.45	751.75±8.60
MeOH extract (UVC-irradiated)	130.93±1.32*	299.41±11.72*
Et_2O fr.	20.73±0.83*	30.58±0.40*
EtOAc fr.	44.98±1.27*	43.66±0.41*
n-BuOH fr.	114.49±6.89*	62.15±0.54*

Data represent mean±SD of triplicate determinations.
*$P<0.001$ vs control.

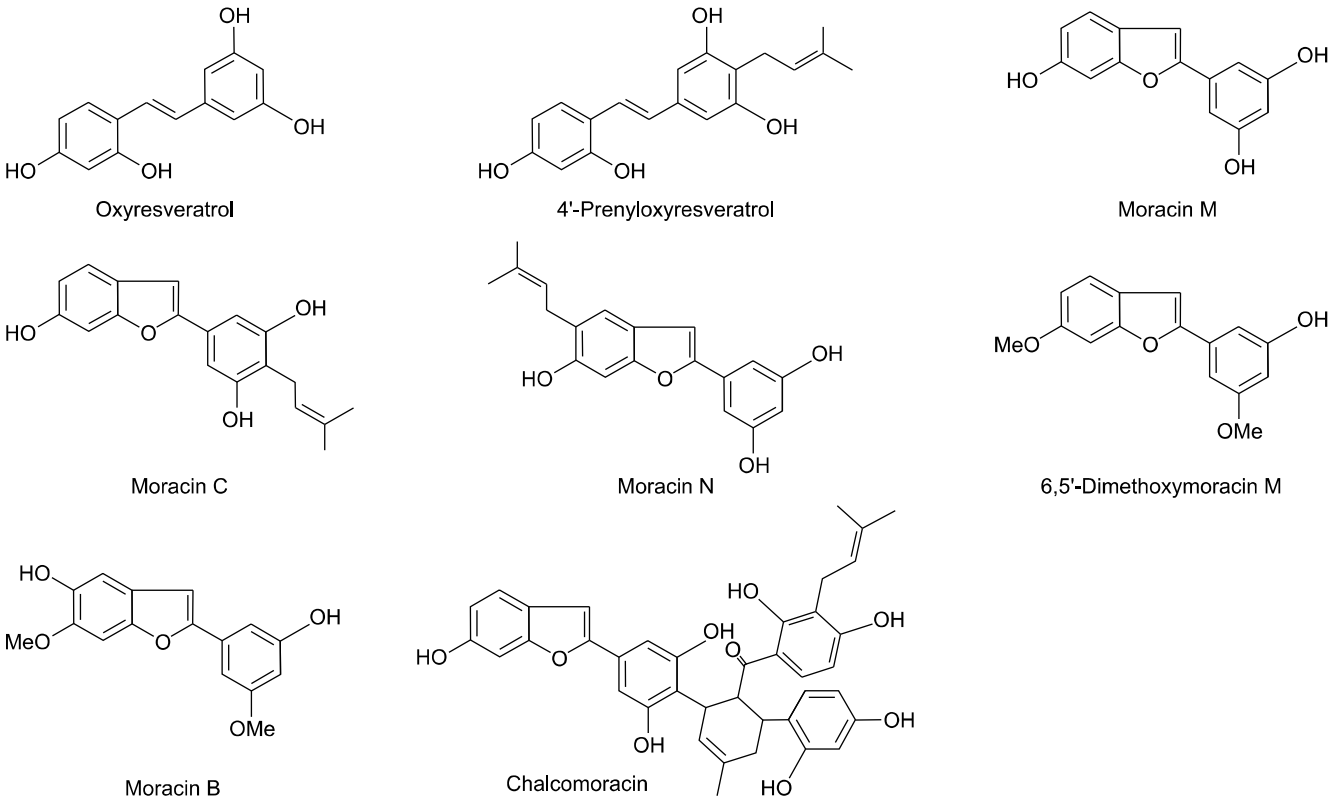

Fig. 2. Chemical structures of oxyresveratrol and moracin derivatives isolated from ultraviolet C-irradiated mulberry mulberry leaves.

fractions from the methanol extract of UVC-IML, the Et_2O fraction (IC_{50}=20.73 and 30.58 μg/mL) exhibited the strongest inhibitory activity against the two enzymes (P<0.001). We isolated and purified major components of enzyme inhibition from the Et_2O fraction by column chromatography, and identified two ORTs (ORT and PORT) and six MCs (MCM, MCN, MCC, MCB, DMMCM, and CMC) by NMR and MS spectrometry (Fig. 2). Among them, two ORTs (ORT and PORT) showed potent tyrosinase inhibitory activities with IC_{50} values of 0.57 and 0.90 μM, respectively. These inhibitory activities were higher than that of arbutin (IC_{50}=14.18 μM), a well-known tyrosinase inhibitor (P<0.001) (32). In addition, most of MC derivatives exhibited considerable tyrosinase inhibitory activities, with the exceptions of MCC, MCB, and DMMCM; CMC (IC_{50}=5.61 μM) exerted a significantly higher tyrosinase inhibitory activity than arbutin (P<0.001). ORT is well-known as a potent mushroom tyrosinase inhibitor, which can be isolated from mulberry twigs and roots barks, and is widely used as a whitening agent in the cosmetics industry (24,32). Of the eight compounds examined, PORT (IC_{50}=28.04 μM) and CMC (IC_{50}=6.00 μM) showed significant α-glucosidase inhibitory activities (Table 4), although their activities were lower than that of acarbose (IC_{50}=0.02 μM), a positive α-glucosidase inhibitor (P<0.001) (33). The other compounds exhibited moderate inhibitory activities, with the exceptions of ORT, DMMCM, and MCB. These bioassay results therefore imply that the 3',5'-dihydroxylated benzene B ring of stilbene and the 2-arylbenzofuran skeleton could be crucial for tyrosinase inhibition. However, methylation of a hydroxyl group (DMMCM and MCB) or substitution of an isoprenoid group (MCC) on the benzene B ring may negatively influence the tyrosinase inhibitory effect. In contrast, PORT and MC (MCC and MCN) favorably inhibited the α-glucosidase activity, and methylation of a hydroxyl group (DMMCM and MCB) on the benzene B ring adversely affected the α-glucosidase inhibitory effect. Thus, ORT and MC derivatives could be mainly responsible for the strong tyrosinase and α-glucosidase inhibition of the MeOH extract from UVC-IML. UVC-treated mulberry leaves with functional ORT and MC derivatives could potentially be used as sources of nutraceuticals and cosmeceuticals for prevention of diabetes and skin aging. Yang et al. recently reported that leaves of *Morus* species in China contain MC derivatives (MCC, MCN, and CMC) with strong α-glucosidase and tyrosinase inhibitory activities (21). However, few studies have been reported on the isolation and identification of ORT and MC derivatives with biological activities from mulberry leaves cultivated in Korea; however, functional ORT and MC derivatives have been found in mulberry leaves (10,20). Thus, this study is the first to isolate and identify ORT and MC derivatives with higher tyrosinase and α-glucosidase inhibitory activity from mulberry leaves. Our previous study demonstrated that mulberry leaves exhibit significant anti-diabetic effect in mice fed on a high fat diet (34). In addition, many researchers have reported that mulberry leaves have anti-diabetic activities in diabetic animal models (2). However, no studies are available on the anti-diabetic effects of UVC-irradiated mulberry leaves. Further *in vivo* animal investigations are ongoing to compare the anti-diabetic properties of UVC-treated mulberry leaves with untreated mulberry leaves.

Table 4. Inhibitory effects of oxyresveratrol and moracin derivatives from ultraviolet C-irradiated mulberry leaves on tyrosinase and α-glucosidase activities

Compound	Inhibition (IC_{50}, μM)	
	Tyrosinase	α-Glucosidase
Oxyresveratrol (ORT)	0.57±0.04*	272.91±13.16†
4'-Prenyloxyresveratrol (PORT)	0.90±0.03*	28.04±0.06†
Moracin M (MCM)	17.23±0.37#	167.72±2.44†
Moracin C (MCC)	37.94±0.42*	88.77±1.32†
Moracin N (MCN)	10.16±0.03*	59.35±2.00†
6,5'-Dimethoxymoracin M (DMMCM)	68.15±1.05*	224.27±5.56†
Moracin B (MCB)	45.84±0.73*	469.16±16.61†
Chalcomoracin (CMC)	5.61±0.20*	6.00±0.46†
Arbutin	14.18±0.51	–
Acarbose	–	0.02±0.01

Data represent mean±SD of triplicate determinations.
*P<0.001 and #P<0.002 vs arbutin, and †P<0.001 vs acarbose.

Quantification of ORT and MC derivatives

UV irradiation is known to increase and induce several phytochemicals in plant leaves and fruits (14,18). In our previous study, UVA treatment did not affect polyphenolic compounds in mulberry leaves, while UVB greatly increased the levels of flavonoids and ORTs in mulberry leaves (35). In the present study, we investigated the effect of UVC irradiation on the polyphenolic profiles in mulberry leaves. As shown in Fig. 3, two ORT and six MC derivatives were isolated from UVC-IML, were detected at 320 nm, and were quantitated using HPLC analysis. As presented in Table 5, we showed that UVC treatment significantly increases levels of ORT and MC derivatives about 4.0~4.2 and 2~16 times, respectively, compared with untreated mulberry leaves (P<0.001). Specifically, UVC treatment significantly increased the levels of MCC, MCB, and DMMCM by about 11~16 fold. It is therefore very interesting to note that ORT and MC derivatives were induced and increased by UVC irradiation in the mulberry leaves. These results are not consistent with an earlier report, which showed that UVB irradiation increases MC derivatives, such as CMC and MCN, in mulberry leaves (19). This discrepancy supports earlier reports that show considerable differences in the

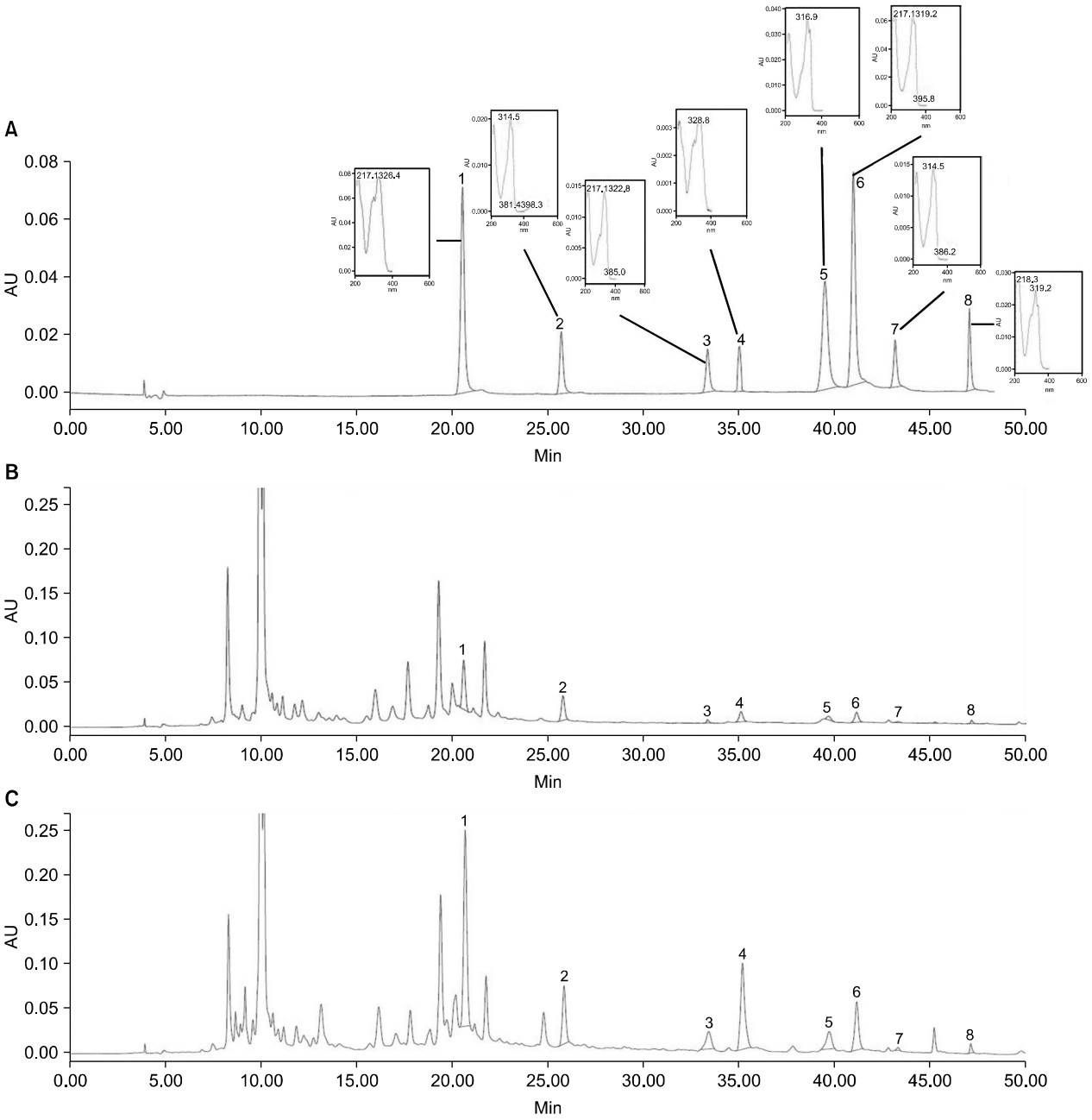

Fig. 3. HPLC chromatograms of eight standard polyphenolic compounds (A) and methanol extracts from untreated (B) and ultraviolet C-irradiated (C) mulberry leaves. 1, oxyresveratrol; 2, moracin M; 3, moracin B; 4, 4′-prenyloxyresveratrol; 5, moracin C; 6, moracin N; 7, 6,5′-dimethoxymoracin M; 8, chalcomoracin.

Table 5. Levels of oxyresevratrol and moracin derivatives of ultraviolet (UV) C-unirradiated and UVC-irradiated mulberry leaves (UVC-UIML and UVC-IML, respectively)

Mulberry Leaf	Contents (mg/100 g, dry weight)							
	ORTs		MCs					
	ORT	PORT	MCB	MCC	MCM	MCN	DMMCM	CMC
UVC-UIML	11.42±0.45	11.34±0.38	0.58±0.02	0.46±0.01	7.00±0.28	2.19±0.07	0.03±0.00	0.34±0.01
UVC-IML	45.40±1.12*	48.30±1.55*	6.78±0.25*	7.28±0.31*	16.61±0.62*	11.83±0.41*	0.33±0.01*	1.16±0.02*

Data present mean±SD of triplicate determinations.
ORT, oxyresveratrol; PORT, 4′-prenyloxyresveratrol; MCB, moracin B; MCC, moracin C; MCM, moracin M; MCN, moracin N; DMMCM, 6,5′-dimethoxymoracin M; CMC, chalcomoracin.
*P<0.001 vs UVC-UIML.

contents of polyphenols, such as phenolic acids, flavonoids, and resveratrols, are present in plants according to type, intensity and duration of UV irradiation (14,16,19). Establishing the optimal conditions for UV irradiation is further required for production of high quality mulberry leaves.

In conclusion, two ORT and six MC derivatives were isolated and identified from UVC-IML grown in Korea. Most of the isolated compounds showed considerable tyrosinase and α-glucosidase inhibitory activities. Irradiation increased the levels of these compounds about 4 fold for ORTs and about 2～16 fold for MCs, compared with unirradiated mulberry leaves. Thus, UVC-irradiated mulberry leaves could represent a potential source of food with anti-diabetic effects, and cosmetics with anti-aging effects. Moreover, UVC-treated mulberry leaves could be utilized as promising materials for production of high-quality mulberry leaf teas and tablets. Further investigation on the anti-diabetic and anti-aging effects of UVC-IML *in vivo* is ongoing.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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