JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b03465 • Publication Date (Web): 08 Sep 2016

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Flavonoids and a Limonoid from the Fruits of *Citrus unshiu* and their Biological Activity

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ABSTRACT: The fruits of *Citrus unshiu* are one of the most popular and mostly enjoyed fruits in Korea. As we continue to seek for bioactive metabolites from Korean natural resources, our study on chemical constituents of the fruits of *C. unshiu* resulted in the isolation of a new flavonoid glycoside, limocitrunshin **1**, along with seven other flavonoids **2**–**8** and a limonoid **9**. All structures were identified by spectroscopic methods, namely 1D and 2D NMR, including HSQC, HMBC and ^{1}H-^{1}H COSY experiments, HRMS, and chemical methods. Compounds **3**, **5**, and **9** are reported to be isolated from this fruit for the first time. The isolated compounds were applied to activity tests to verify their inhibitory effects on inflammation and nephrotoxicity. Compounds **5** and **9** showed the most potent inhibitory activity on renal cell damage and nitric oxide production, respectively. Thus, the fruits of *C. unshiu* could serve as a valuable natural source of bioactive components with health benefits for potential application in functional foods.

KEYWORDS: *Citrus unshiu*; Rutaceae; flavonoid; nitric oxide; nephrotoxicity

■INTRODUCTION

Citrus unshiu Markovich (Rutaceae), also known as the Satsuma mandarin or Satsuma tangerine, is cultivated in subtropical countries with moderate climate, such as Korea, Japan, China and Russia. As the fruits of *C. unshiu* are seedless and are easily peeled, they are one of the mostly enjoyed fruits in Korea. Citrus fruit is a major product of Jeju Island in Korea, and many varieties are cultivated there. Peels of mature fruits of *C. unshiu* have been used as a Chinese traditional medicine, as well as in Korea.[1-3] The peels of citrus fruits contain phenolic compounds, such as flavanones and hydroxyl cinnamic acids; hesperidin and narirutin are two main constituents of the flavanones in *C. unshiu* fruit.[4-6] The peels of *C. unshiu* are known to possess a wide variety of biological and pharmacological activity by *in vitro* and *in vivo* studies. For example, the peels showed antioxidant activity[7] and inhibition of the growth of tumor in murine renal cell carcinoma in mice through immune-mediated pathways.[2] The peels also exhibited the inhibitory activities of hydroperoxide production,[8] certain virus such as hepatitis C,[9] as well as growth of certain bacteria.[10] From a recent study, the peels of *C. unshiu* were reported to have the inhibitory effects on the pro-inflammatory cytokines in lipopolysaccharide (LPS)-activated RAW 264.7 cells.[1]

In spite of intensive research on the pharmacological features of *C. unshiu* peels, the flesh of the whole fruit has not drawn much attention in biological research although it is the portion that people actually consume. There are few reports dealing with chemical constituents of the whole fruit.[11] The whole fruit has also been used in natural Korean medicine to improve skin elasticity, relieve cough and fatigue, and prevent bronchitis, flu, and cancers.[12] In our continuation to seek for bioactive metabolites from Korean natural resources, the fresh whole

parts of *C. unshiu* fruit were investigated to explore the anti-inflammatory properties of *C. unshiu* fruits. From our recent study, we reported the isolation of a novel cyclic peptide, citrusin XI, and its anti-inflammatory effects in LPS-stimulated RAW 264.7 cells.[11] As part of our study to seek for novel bioactive constituents from *C. unshiu* fruit, phytochemical investigation of the whole fruit was carried out and nine compounds including a new flavonoid glycoside, seven flavonoids and a limonoid, were isolated. The structure of the new compound was identified using spectroscopic techniques including 1D and 2D NMR, HRMS, and other chemical methods. The isolates were further assessed for the inhibition on nitric oxide (NO) production and renal cell damage.

■MATERIALS AND METHODS

General Experimental Procedures. Infrared (IR) spectra were measured on an IFS-66/s FT-IR spectrometer (Bruker, Karlsruhe, Germany). Ultraviolet (UV) spectra were acquired on an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA). Optical rotations were recorded on a Jasco P-1020 polarimeter (Jasco, Easton, MD). High-resolution (HR)-electrospray ionization (ESI) mass spectra were obtained on an UPLC-QTOF Xevo G2-S mass spectrometer (Waters Corporation, Milford, CT). Nuclear magnetic resonance (NMR) spectra were obtained from a Bruker AVANCE III 700 NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C) (Bruker). Agilent 1200 Series HPLC system (Agilent Technologies) equipped with a photo diode array (PDA) detector was used for preparative high-performance liquid chromatography (HPLC) using a 250 mm × 20 mm i.d., 10 μm, YMC-Pack ODS-AM C18(2) column (YMC America, Inc., Allentown, PA). Agilent

1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer was used for LC-MS analysis using an analytical Kinetex C18 100 Å column (100 mm × 2.1 mm i.d., 5 μm) (Phenomenex, Torrance, CA). Column chromatography used silica gel 60, 230-400 mesh, and RP-C18 silica gel, 230-400 mesh (Merck, Darmstadt, Germany). Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was used for molecular sieve column chromatography. Thin-layer chromatography (TLC) analysis was conducted by using precoated silica gel F_{254} plates and reverse-phase (RP)-18 F_{254s} plates (Merck). Spots on TLC were detected using UV and by heating after dipping in solvent of anisaldehyde-sulfuric acid.

Plant Material. Fully ripe fruits of the 'Miyagawa-wase' variety of Satsuma mandarin (*C. unshiu* Marc.) were cultivated from the National Institutes of Horticultural and Herbal Science, Jeju, Korea, in August 2012. The materials were identified by one of the authors (J. W. Hyun). A voucher specimen (SKKU-CU 2012-8) has been stored in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. The whole parts of fresh *C. unshiu* fruits (1.0 kg) were partially chopped, then extracted with 100% EtOH for 2 d twice at room temperature, accompanied with slight shaking or swirling. The resultant extracts were then filtered and the filtrate was concentrated under vacuum pressure. After suspension of the crude extract (89.0 g) in distilled water (10 L), it was subjected to solvent partition using hexane, EtOAc, and *n*-BuOH, which yielded residues of 230 mg, 3.0 g, and 15.0 g, respectively.

The EtOAc-soluble fraction (3.0 g) was fractionated on silica gel column chromatography (300 g, 3 × 55 cm) with a gradient solvent system of *n*-hexane-EtOAc [1:1 (0.3 L)], $CHCl_3$-MeOH [50:1 (0.2 L), 10:1 (0.2 L), 5:1 (0.3 L), 2:1 (0.3 L), and 1:1 (0.3 L)], and 100% MeOH (0.5 L) to give thirty fractions (E1 – E30), based on TLC analysis. Fraction E7 (260 mg) was

subjected to an RP-C_{18} silica gel column chromatography (20 g, 1 × 10 cm) using MeOH-H_2O [2:1 (0.3 L)] as a solvent to yield 10 sub-fractions (E7a – E7j). Fraction E7b (52 mg) was further purified by using semi-preparative reverse-phase HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate; 1.5 mL/min) with MeOH-H_2O (2:1) to afford compounds **2** (12.0 mg), **4** (6.4 mg), and **9** (8.0 mg).

The *n*-BuOH-soluble fraction (15.0 g) was fractionated on HP-20 column chromatography (500 g, 5 × 55 cm) with the use of a gradient solvent system of MeOH-H_2O (from 0:1 to 1:0) to give six fractions (B1 – B6) according to TLC analysis. Fraction B2 (4.5 g) was subjected to a column chromatography using silica gel (300 g, 3 × 55 cm) with $CHCl_3$-MeOH-H_2O [7:3:1 (2.0 L)] as a solvent system to obtain 32 sub-fractions (B2(1) – B2(32)). Fraction B2(11) (122 mg) was further purified with preparative reverse phase HPLC (250 mm × 20 mm i.d., 10 μm, YMC-Pack ODS-AM C18(2) column, flow rate; 8.0 mL/min) by using 40% MeOH to obtain compound **8** (38.0 mg). Fraction B2(31) (277 mg) was also separated by preparative HPLC using the same column (flow rate; 10.0 mL/min) with 40% MeOH to yield compound **5** (9.0 mg), along with subfraction B2(31)A (87.0 mg), which was then purified by semi-preparative reverse phase HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate; 2.0 mL/min) with 30% MeOH to furnish compound **7** (30.0 mg). Fraction B4 (2.8 g) was applied to a silica gel column (300 g, 3 × 55 cm) using $CHCl_3$-MeOH-H_2O [8:3:1 (0.8 L), 6:3:1 (0.8 L), and 6:4:1 (0.5 L)] and 100% MeOH (1.0 L) as a gradient solvent system to give 29 sub-fractions (B4(1) – B4(29)). Fraction B4(9) (44 mg) was further purified using semi-preparative RP HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate; 2.0 mL/min) with 40% MeOH to afford compound **6** (5.2 mg). Fraction B4(15) (168 mg) was also separated by semi-preparative HPLC using the same column system with 40% MeOH to yield compound **1** (21.5 mg).

Finally, fraction B6 (3.2 g) was applied to column chromatography with silica gel (300 g, 3 × 55 cm) using a gradient solvent system of $CHCl_3$-MeOH-H_2O [8:3:1 (1.0 L)] and 100% MeOH (1.0 L) to give 20 sub-fractions (B6(1) – B6(20)). Fraction B6(10) (383 mg) was further purified using semi-preparative HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex Luna C18(2) column, flow rate; 3.0 mL/min) with 40% MeOH to obtain compound **3** (6.0 mg).

Limocitrunshin (1). Amorphous yellow powder. $[\alpha]_D^{25}$ +13.57 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.5), 260 (3.2), 272 (3.1) 358 (2.3) nm; IR (KBr) ν_{max}: 3326, 2942, 2826, 1714, 1672, 1590, 1455, 1352, 1110, 1030 cm^{-1}; ^1H NMR (CD_3OD, 700 MHz): δ 7.97 (1H, br s, H-2'), 7.73 (1H, br d, *J* = 8.0 Hz, H-6'), 6.94 (1H, d, *J* = 8.0 Hz, H-5'), 6.29 (1H, br s, H-6), 5.36 (1H, d, *J* = 7.5 Hz, H-1''), 4.25 (1H, dd, *J* = 11.0, 1.0 Hz, H-6''a), 4.14 (1H, dd, *J* = 11.0, 3.0 Hz, H-6''b), 3.98 (3H, s, 3'-OCH_3), 3.94 (3H, s, 8-OCH_3), 3.53 (1H, m, H-2''), 3.50 (1H, m, H-5''), 3.39 (1H, m, H-3''), 3.33 (1H, m, H-4''), 2.52 (4H, m, H-2''', H-4'''), 1.23 (3H, s, H-6'''); ^{13}C NMR (CD_3OD, 700 MHz): δ 178.5 (C-4), 171.5 (C-5'''), 171.3 (C-1'''), 157.9 (C-7), 157.6 (C-2), 157.1 (C-5), 150.1 (C-4'), 149.4 (C-9), 147.5 (C-3'), 134.4 (C-3), 128.2 (C-8), 123.1 (C-6'), 122.1 (C-1'), 115.2 (C-5'), 113.3 (C-2'), 104.7 (C-10), 103.0 (C-1''), 99.3 (C-6), 77.0 (C-3''), 75.0 (C-5''), 74.9 (C-2''), 70.8 (C-4''), 69.7 (C-3'''), 63.6 (C-6''), 61.2 (8-OCH_3), 55.8 (3'-OCH_3), 45.2 (C-2'''), 45.1 (C-4'''), 26.9 (C-6'''); High-resolution (HR)-ESIMS (negative-ion mode) *m/z*: 651.1547 $[M - H]^-$ (calcd for $C_{29}H_{31}O_{17}$, 651.1561).

Acid Hydrolysis of Compound 1. Compound **1** (1.0 mg) was hydrolyzed with 1 N HCl (1.0 mL) for 6 h at 100 °C. Then the hydrolysate was cooled and filtered, and a yellowish precipitate was obtained, which was identified by NMR to be the aglycone portion, limocitrin.[13] The filtrate was then neutralized by passage through an Amberlite IRA-67 ion-

exchange resin column (Rohm and Haas, Philadelphia, PA). The H_2O eluent was repeatedly evaporated until the liquid was completely removed, then was analyzed using TLC over silica gel ($CHCl_3$/MeOH/H_2O, 8:5:1), loaded with authentic sugar [TLC R_f (glucose) = 0.30] for the comparison. The sugar residue and L-cysteine methyl ester hydrochloride (1.0 mg) were dissolved in 0.1 mL of anhydrous pyridine, and the resultant mixture was gently stirred at 60 °C for 2 h. The mixture was then evaporated under vacuum, and the reaction mixture was trimethylsilylated using 0.3 mL of hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO)/trimethychlorosilane (TMCS, Sigma-Aldrich)/pyridine (3:1:9) at 60 °C for another 1.5 h. It was then concentrated and was solvent-partitioned using *n*-hexane, to yield *n*-hexane-soluble and H_2O-soluble layers. The *n*-hexane layer was examined using gas chromatography (GC).[14,15] The t_R value of the standard D-glucose (D-Glc) derivative prepared in the same way was 18.58 min. D-Glc was detected from compound **1** by co-injection of hydrolysate with standard silylated sample, giving a single peak at 18.59 min.

Determination of the Absolute Configuration for Compound 1. (*S*)-1-Phenylethylamine (1.9 μL, 15.0 μmol), Et_3N (3.2 μL, 22.5 μmol), (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, Sigma-Aldrich) (5.8 mg, 11.5 μmol), and hydroxybenzotriazole (HOBt, Sigma-Aldrich) (2.0 mg, 15.0 μmol) were added to the solution containing compound **1** (4.9 mg, 7.5 μmol) and 0.3 mL of DMF under ice-cooling, and the resultant mixture was stirred at 25 °C for 9 h.[16] The reaction was quenched with dilute aqueous HCl, and a yellowish residue was obtained after drying under vacuum. The residue was separated on Sephadex LH-20 column chromatography using 50% MeOH to furnish amide **1a** (4.2 mg). Compound **1a** was identified by LC-MS analysis, where a molecular ion peak at *m*/*z* 756.2 $[M + H]^+$ was observed. $LiBH_4$ (2.6 mg, 79.5 μmol) was added to the solution containing **1a** (4.0 mg, 5.3 μmol) and THF (0.3 mL) under ice-cooling.

The solution was stirred for 24 h at 25 °C, then the reaction was quenched with dilute aqueous HCl and the resultant mixture was extracted with EtOAc. The resulting extract was separated using a silica gel Waters Sep-Pak Vac 6 cc ($CHCl_3$-MeOH, 3:1) and a colorless oil was obtained, which was then acetylated with Ac_2O (2.5 μL, 26.5 μmol) in pyridine (30 μL). The reaction mixture was stirred for 24 h at 25 °C, diluted with H_2O, extracted with EtOAc, and concentrated to yield **1b** (4.5 mg) as a colorless oil. The ^{1}H NMR spectrum of **1b** was found to be consistent with that of (3*R*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]-mevalonamide when compared, rather than the (3*S*) isomer previously reported.[17,18]

(3*R*)-5-*O*-Acetyl-1-[(*S*)-phenylethyl]-mevalonamide (1b). Colorless oil. ^{1}H NMR ($CDCl_3$, 700 MHz): δ 7.27–7.37 (5H, m, Ph), 6.11 (1H, br s, NH), 5.14 (1H, m, H-1'), 4.23 (2H, t, J = 6.5 Hz, H-5), 2.41, 2.28 (each 1H, d, J = 14.5 Hz, H-2), 2.04, (3H, s, Ac), 1.85-1.83 (2H, m, H-4), 1.50 (3H, d, J = 6.5 Hz, H-2'), 1.23 (3H, s, H-6); ESIMS m/z 294.1 $[M + H]^+$.

Chemicals and Reagents. Cisplatin and LPS were obtained from Sigma-Aldrich (Seoul, South Korea). The cell viability assay kit (Ez-Cytox) was obtained from Dail Lab Service Co. (Seoul, Korea). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen Co. (Grand Island, NY).

Inhibitory Activity towards NO Production by LPS-induced Macrophages. RAW 264.7 cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in DMEM (Cellgro, Manassas, VA) supplemented with 10% FBS, 1% penicillin and streptomycin (Invitrogen Co.) and 4 mM L-glutamine in an atmosphere of 5% CO_2 at 37 °C. When the cells were approximately 80% confluent, they were seeded in 96-well culture plates at 1×10^5 cells per well and incubated for 24 h for adhesion. The cells were then treated with

control (0.5% DMSO) or with the indicated concentrations of the isolates **1-9** or 1 μg/mL of LPS. After incubation for 24 h, 80 μL of cell culture medium was mixed with 80 μL of Griess reagent and the mixture was incubated for 10 min. The absorbance was measured at 540 nm using a microplate reader. The quantity of nitrite was determined from a sodium nitrite standard curve. After the nitric oxide assay, cell viability was determined. When the cells were approximately 80% confluent, they were seeded in 96-well culture plates at 5×10^5 cells per well and incubated for 24 h for adhesion. The cells were treated with control (0.5% DMSO), with the indicated concentrations of isolates **1-9**, or with 1 μg/mL of LPS. After incubation for 24 h, 10 μL of Ez-Cytox reagent was added to each well, and the cells were incubated for 2 h. Quercetin was used as a positive control. Cell viability was measured by absorbance at 450 nm using a microplate reader.

Protective Effect against Cisplatin-induced Damage in LLC-PK1 Renal Cells. Pig kidney epithelium LLC-PK1 cells were purchased from the American Type Culture Collection (Rockville) and cultured in DMEM (Cellgro), supplemented with 10% FBS, 1% penicillin and streptomycin (Invitrogen Co.), and 4 mM L-glutamine in an atmosphere of 5% CO_2 at 37 °C. Cell viability was determined using the Ez-Cytox cell viability detection kit. Pig kidney epithelium LLC-PK1 cells were used to evaluate renoprotective activity against cisplatin-induced cytotoxicity. When the cells were approximately 80% confluent, they were seeded in 96-well culture plates at 1×10^4 cells per well and incubated for 24 h for adhesion. Then cells were treated with control (0.5% DMSO) or the indicated concentrations of isolates **1-9**. After incubation for 2 h, 30 μM of cisplatin was added to each well, and incubated for another 24 h. After incubation, 10 μL of Ez-Cytox reagent was added to each well, and the cells were incubated for 2 h. *N*-acetyl cysteine (NAC) was used as a positive control. Cell viability was measured by absorbance at 450 nm using a microplate reader.

Western Blotting Analysis. LLC-PK1 cells cultured in 6-well plates were treated with 250 μM of **5** and **8** for 24 h, and cells were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) immediately before use. Concentration of protein was determined using the Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts (20 μg/lane) of protein (whole-cell extracts) were separated by electrophoresis and transferred onto PVDF transfer membranes. Specific proteins were analyzed using epitope-specific primary antibodies to phospho-JNK, p53, cleaved caspase-3, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP) conjugated anti-rabbit antibodies (Cell Signaling Technology). Bound antibodies were detected using ECL Advance Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and visualized with a FUSION Solo Chemiluminescence System (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Statistical Analysis. One-way analysis of variance (ANOVA) followed by a multiple comparison test with Bonferroni adjustment was used for statistical analysis using SPSS ver. 19.0 (SPSS Inc., Chicago, IL). *P* values of less than 0.05 were considered statistically significant.

■RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Compounds. Whole *C. unshiu* fruits were extracted with 100% EtOH to give crude EtOH extracts (89.0 g) which were solvent-partitioned using hexane, EtOAc, and *n*-BuOH. Chemical investigation of the two fractions,

EtOAc-soluble and *n*-BuOH-soluble fractions, using repeated column chromatography and HPLC purification resulted in isolation and identification of a novel flavonoid glycoside, limocitrunshin **1**, along with seven other flavonoids **2**–**8** and a limonoid **9** (**Figure 1**).

Compound **1** was isolated as an amorphous, yellowish powder; its molecular formula, $C_{29}H_{32}O_{17}$ was established by HR-ESIMS in negative ion mode at *m/z* 651.1547 $[M - H]^-$ (calcd for $C_{29}H_{31}O_{17}$, 651.1561). The absorption bands for OH (3326 cm^{-1}), carbonyl (1714 cm^{-1}), conjugated carbonyl (1672 cm^{-1}), and benzyl groups (1590 and 1455 cm^{-1}) were observed in the IR spectrum. In the UV spectrum, absorption bands that appeared at 260, 272, and 358 nm suggested that compound **1** possesses a flavonol skeleton. The ^1H and ^{13}C NMR data consisted of signals that are similar to that of 5,7,8,3',4'-pentasubstituted flavonol glycoside[19] with two methoxy groups [δ_H 3.98 (s); δ_C 55.8 and δ_H 3.94 (s); δ_C 61.2] and one sugar unit as shown by signals of an anomeric proton at δ_H 5.36 (d, J = 7.5 Hz, H-1") and its corresponding anomeric carbon at δ_C 103.0 (C-1"). The heteronuclear multiple bond correlations (HMBC) between δ_H 3.98 (OCH_3) and δ_C 147.5 (C-3') and between δ_H 3.94 (OCH_3) and δ_C 128.2 (C-8) was observed and thus confirmed the locations of methoxy groups at C-3' and C-8 of the aglycone, respectively (**Figure 2**). Acid hydrolysis of **1** yielded a sugar and limocitrin, 3,4',5,7-tetrahydroxy-3',8-dimethoxyflavone, which was verified through comparison with reported NMR data.[13] The sugar was identified as D-glucose using GC analysis, where the retention time of the derivative of the sugar residue and the standard sugar were compared. The position of glycosidic linkage was determined by the correlation between δ_H 5.36 (H-1") and δ_C 134.4 (C-3) in HMBC experiment, indicating the glucose unit to be located at C-3 of the aglycone. The remaining signals in the ^1H and ^{13}C NMR spectra of **1** were identified as a 3-hydroxy-3-methylglutaryl (HMG)-moiety by the HMBC correlations of H-2'" (δ_H 2.52)/C-1'" (δ_C 171.3), H-4'" (δ_H 2.52)/C-5'" (δ_C 171.5), and H-6'" (δ_H 1.23)/C-2'"

(δ_C 45.2), C-3''' (δ_C 69.7), and C-4''' (δ_C 45.1). On the basis of the HMBC correlations between δ_H 4.25 and 4.14 (H-6'') and δ_C 171.3 (C-1'''), the HMG substituent was established to be located at C-6 of the glucose moiety (**Figure 2**). The absolute configuration of the HMG unit was determined by the refined method using the steps of amination and reduction.[16] Amination with (*S*)-1-phenylethylamine gave compound **1a** (**Figure 3**). Reduction of **1a** with $LiBH_4$ followed by acetylation with Ac_2O yielded 5-*O*-acetyl-1-[(*S*)-phenylethyl]-mevalonamide (**1b**): the ^{1}H NMR data of **1b** were identical to those of (3*R*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]-mevalonamide, instead of (3*S*) isomer.[17,18] Thus, with the above evidences, compound **1** was unambiguously identified as limocitrin-3-*O*-[(*S*)-3-hydroxy-3-methylglutaryl-(1→6)]-β-D-glucopyranoside, and was named limocitrunshin. Previously, the gross structure of **1** was identified from *C. unshiu* without verifying the absolute configuration,[20,21] but the complete structure of **1** including the identification of the (3*S*)-HMG-moiety were established for the first time in this study. The previously reported NMR data in the literature were very similar to those of compound **1**, but they had apparent differences in the ^{13}C NMR data of the HMG unit [particularly, δ_C 69.7 (C-3''') in **1** and δ_C 68.8 (C-3''') in the reported data],[21] which suggested that compound **1** is not identical to the previously reported one. Compound **1** is the first example of the limocitrin 3-β-D-glucopyranoside possessing the (3*S*)-HMG-moiety.

The naturally occurring products conjugated with HMG group are seldom present in various types of natural products including sesquiterpenoids,[18,22] diterpenoids,[23] triterpenoids,[24-27] steroids,[28] and flavonoids.[29-33] The majority of compounds with the HMG moiety belong to flavonoid glycosides, and the HMG moiety tends to be attached at C-6 of sugar such as glucose or galactose.[29-33] Citrus fruits have been reported to contain the flavonoid glycosides carrying the HMG unit,[20,21,30,31] but this study is the first report of the verification of 3*S*-

297 configuration for the HMG group using the refined method in the flavonoid glycosides of *C.*
298 *unshiu*. An extensive literature survey showed that the HMG group does not seem to have
299 significance in biological activities. Several HMG-conjugated flavonoid glycosides did not
300 have inhibitory effects on NO production in lipopolysaccharide (LPS)-induced RAW 264.7
301 cells[33] nor antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA)
302 and *Helicobacter pylori* (*H. pylori*), although the other related flavonoids without the HMG
303 group showed the antimicrobial activity.[30] In addition, the presence of the HMG group in
304 triterpenoids did not affect cytotoxic activities against several cancer cell lines[25,26] nor
305 inhibitory effects on LPS-induced NO production in murine microglia BV-2 cells.[26] However,
306 there was an interesting report for the HMG group that the linkage of the HMG group at C-3
307 in triterpenoids markedly increased the selective inhibition of COX-1 activity when
308 compared to the related triterpenoids without the HMG group, which were selective
309 inhibitors of COX-2.[27]
310 The known compounds were identified as nobiletin (3',4',5,6,7,8-hexamethoxyflavone) (**2**),[34]
311 kaempferol 3-*O*-rutinoside (**3**),[35] limocitrin 3-glucoside (**4**),[19] kaempferol 3-(2^{G}-
312 rhamnosylrutinoside) (**5**),[35] didymin (4'-methoxyl naringenin 7-*O*-rutinoside) (**6**),[36] (2*S*)-
313 narirutin 4'-*O*-glucoside (4'-β-D-glucosyl naringenin 7-*O*-rutinoside) (**7**),[37] naringenin 7-*O*-
314 rutinoside (**8**),[38] and methyl nomilinate (**9**)[39] respectively, by comparing their spectroscopic
315 and physical data with those in the literature as well as by measurement of their specific
316 rotations. Compounds **3**, **5**, and **9** were reported from *C. unshiu* for the first time.
317 **Evaluation of Biological Activity of Compounds 1–9.** Compounds **1**–**9** were evaluated for
318 inhibitory effects on inflammation and nephrotoxicity to verify their potential health benefits.
319 Murine macrophage RAW 264.7 cells were used for evaluating inhibitory activity towards
320 NO production by LPS-activated macrophages.[40,41] Treatment of RAW 264.7 cells with up to

200 μM of compounds **1-9** did not show any cytotoxic effects (**Figure 4A**). Conversely, all of the compounds significantly inhibited NO production (**Figure 4B**), which is involved in inflammatory processes.[42] Particularly, compound **9** (IC_{50} = 65 μM) was the strongest inhibitor as compared to the positive control used in this study [IC_{50} (quercetin): 150 μM].[43,44] The other compounds also showed inhibitory activity on NO with IC_{50} values in the range 70-110 μM [IC_{50} (**1**): 75 μM, IC_{50} (**2**): 95 μM, IC_{50} (**3**): 110 μM, IC_{50} (**4**): 75 μM, IC_{50} (**5**): 70 μM, IC_{50} (**6**): 85 μM, IC_{50} (**7**): 70 μM, and IC_{50} (**8**): 70 μM]. The most potent inhibitor, methyl nomilinate (**9**) is a class of limonoids which are highly oxygenated nortriterpenoids with a prototypical structure and a β-substituted furan ring. Its occurrence is abundant in citrus fruits and other plants of the families Rutaceae and Meliaceae. There has been only limited work that focused on the anti-inflammatory effects of limonoids, but recently many limonoids were reported to inhibit NO production in RAW 264.7 macrophage cells induced by LPS,[45-48] which suggests the potential of limonoids for the development of anti-inflammatory agents. The other active compounds were all flavonoids and their inhibitory effects on NO production have been extensively studied.[49] In the structure-activity relationships, it has been reported that a C-2,3 double bond in the flavonoid skeleton is essential for the activity and that the efficacy of activity was dependent upon the substitution patterns within the flavonoids.[49]

Next, the kidney protective effects of compounds **1-9** were assessed in LLC-PK1 cells using a WST assay.[50] The kidney protection effects of isolates **1-9** are shown in **Figure 5A**. Pretreatment of LLC-PK1 cells with compounds **1**, **2**, **5**, **8** and **9** at concentrations of 125 and 250 μM significantly abrogated cisplatin-induced nephrotoxicity (**Figure 5A**). Of these, compounds **5** and **8** were selected for further mechanistic studies because of their ameliorating effects on cell viability damage, leading to recovery of more than 90% at the

250 μM concentration (**Figure 5A**).

Western blotting was performed in order to investigate the protective mechanism of compounds **5** and **8** on the expression of proteins involved in the apoptotic response. Results from Western blot analysis are shown in **Figure 5B**. We determined that cisplatin promoted high levels of phosphorylated JNK (phospho-JNK), p53 and cleavage of caspase-3, which triggered apoptosis of LLC-PK1 cells, while pretreatment with compounds **5** and **8** decreased levels of phospho-JNK, p53, and cleaved caspase-3 protein (**Figure 5B**). Therefore, the kidney cell protective effects of compounds **5** and **8** are shown to involve the inhibition of pathways of apoptosis through the JNK-p53-caspase apoptotic cascade.

Recently, it was reported that several flavonoids isolated from peat moss *Sphagnum palustre* showed the protective effects against kidney damage induced by cisplatin[51] and that flavonoids in a multi-herbal decoction, known as Chungsimyeonja-tang, possessed protective effects against cisplatin-induced nephrotoxicity.[52] Unfortunately, it was difficult to find any significant relevance between structure and activity of the flavonoid molecules in this kidney protection assay.

In conclusion, chemical investigation of the EtOH extract of *C. unshiu* fruit led to the isolation and identification of a total of 9 components including a new flavonoid glycoside, which may be at least partially responsible for the health benefits of *C. unshiu* fruit. Among the isolates, compound **9** inhibited potent NO production in LPS-stimulated macrophages, and compounds **5** and **8** showed the most potent inhibition of renal cell damage. Thus, this study revealed the possible application of the fruits of *C. unshiu* as a beneficial natural source of bioactive metabolites with health benefits in functional foods.

■ ASSOCIATED CONTENT

*Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at

■AUTHOR INFORMATION

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Author Contributions

H.J.E., D.L., S.L., and H.J.N. performed most of the experimental work. H.J.N. conceived the project and designed the experiments. J.W.H. and P.H.Y. provided the needed materials. H.J.E. and K.H.K. designed and implemented the separation and purification protocols. D.L. and K.S.K. designed and implemented the biological test protocols. K.S.K., S.L., and K.H.K. drafted and revised the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2015R1C1A1A02037383).

Note

The authors declare there are no conflicts of interest.

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Figure captions

Figure 1. Chemical structures of compounds **1-9**.

Figure 2. Key ^{1}H-^{1}H COSY (—) and HMBC (→) correlations of **1**.

Figure 3. Determination of the absolute configuration of HMG group of compound **1**.

Figure 4. (A) Comparison of the cell viability of compounds **1-9** in RAW 264.7 cells. (B) Nitric oxide inhibition of compounds **1-9** in LPS-activated macrophage RAW 264.7 cells. *p<0.05 means compared to the LPS-treated value.

Figure 5. (A) Comparison of the protective effects of compounds **1-9** against cisplatin-induced nephrotoxicity in LLC-PK1 cells. (B) Effects of compounds **5** and **8** on phospho-JNK, JNK, phospho-p53, p53, and cleaved caspase-3 expression on cisplatin-induced nephrotoxicity in LLC-PK1 cells. *p<0.05 means compared to the cisplatin-treated value.

577

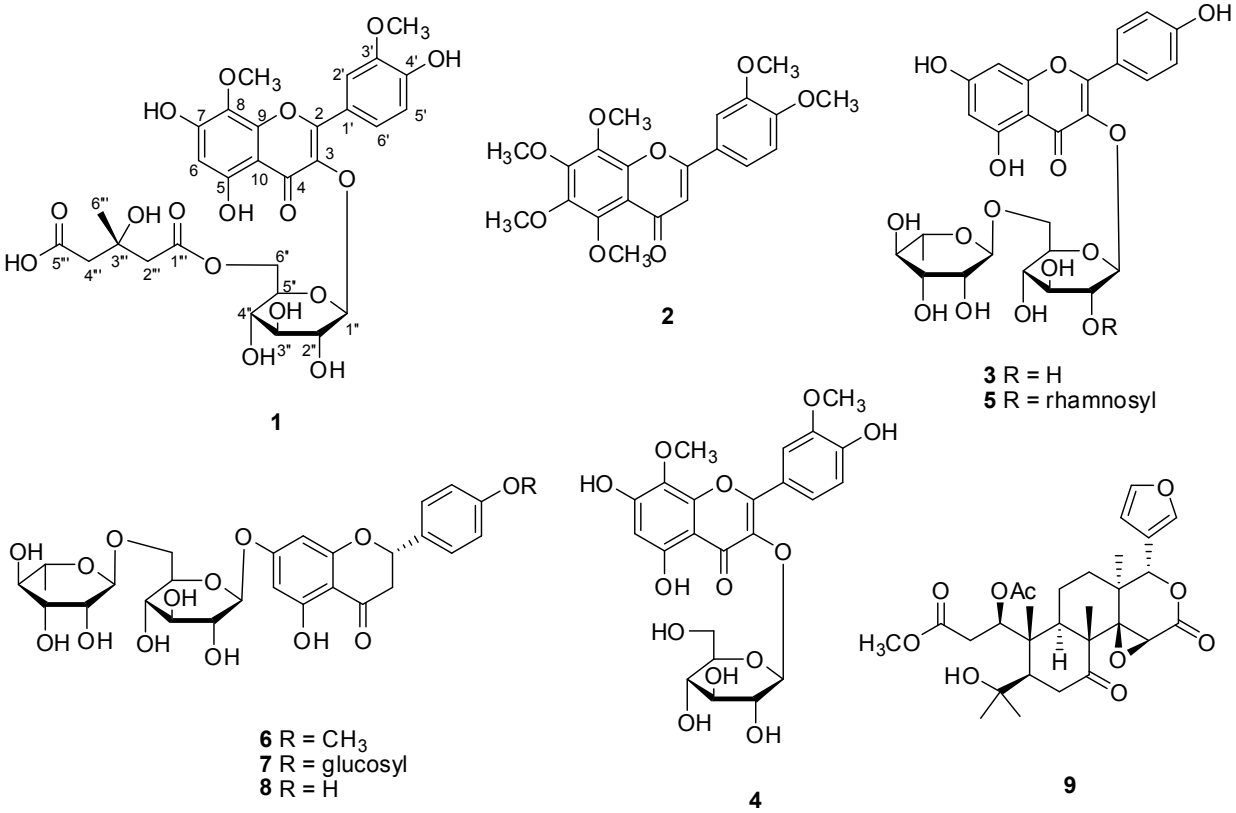


Figure 1.

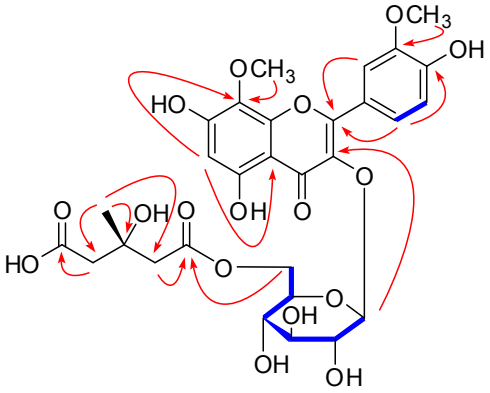
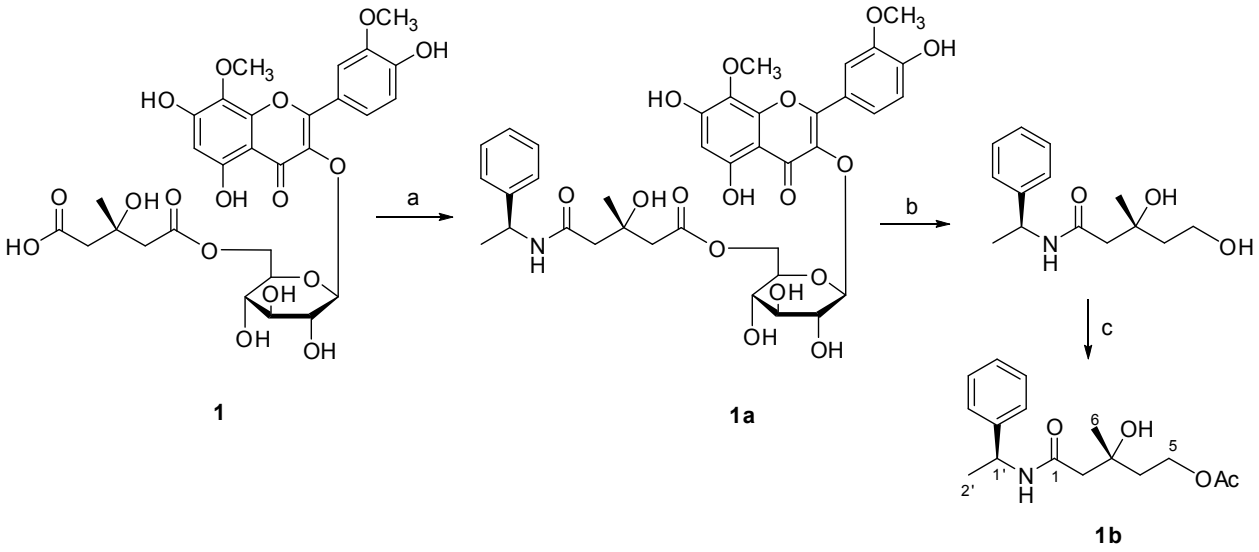


Figure 2.



1 **1a** **1b**

(a) (*S*)-1-phenylethylamine, DMF, Et_3N, PyBOP, HOBt; (b) $LiBH_4$, THF; (c) Ac_2O, pyridine.

Figure 3.

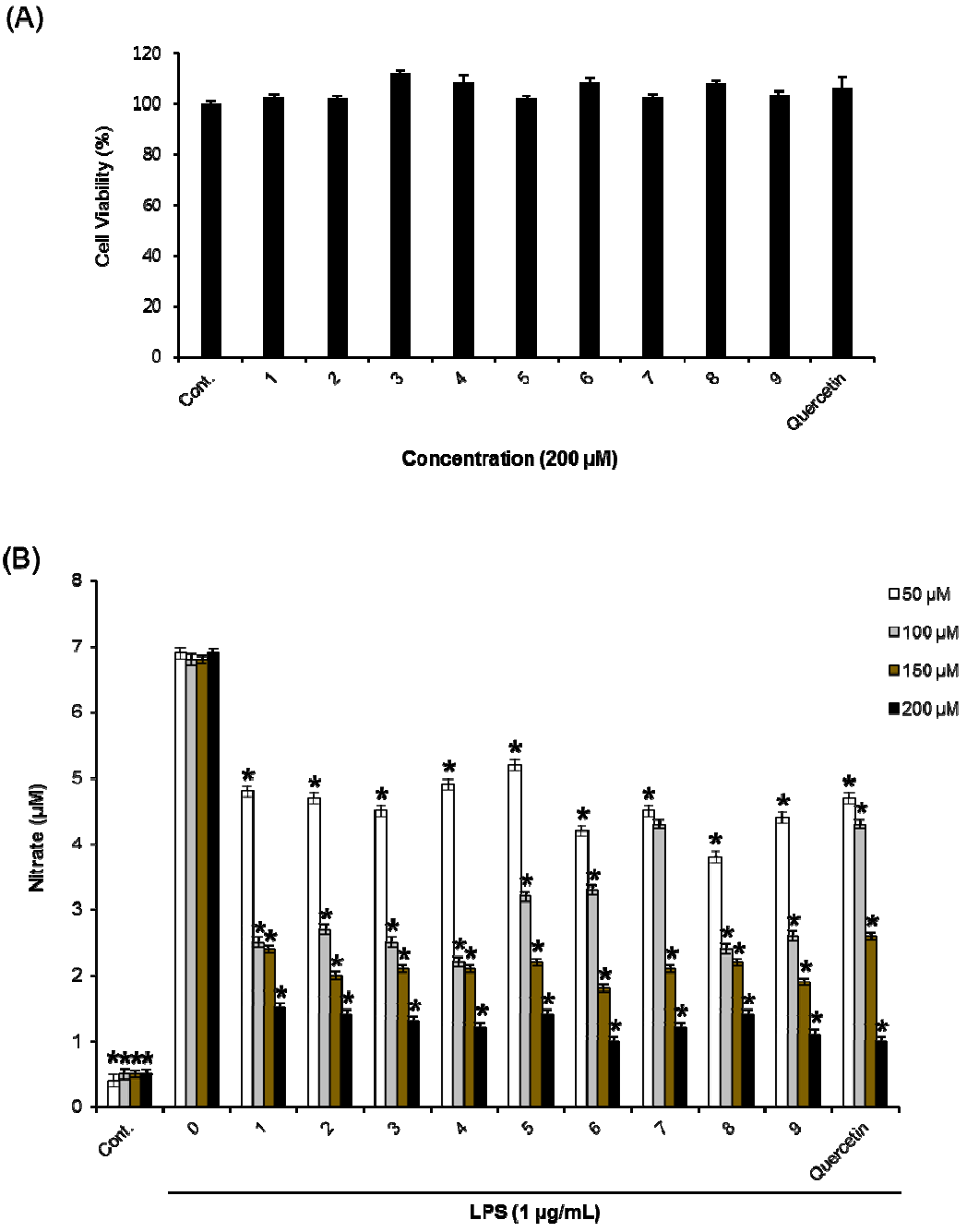

(A)
Cell Viability (%)
120
100
80
60
40
20
0
Cont.
1
2
3
4
5
6
7
8
9
Quercetin
Concentration (200 μM)
(B)
Nitrate (μM)
8
7
6
5
4
3
2
1
0
50 μM
100 μM
150 μM
200 μM
Cont.
0
1
2
3
4
5
6
7
8
9
Quercetin
LPS (1 μg/mL)

Figure 4.

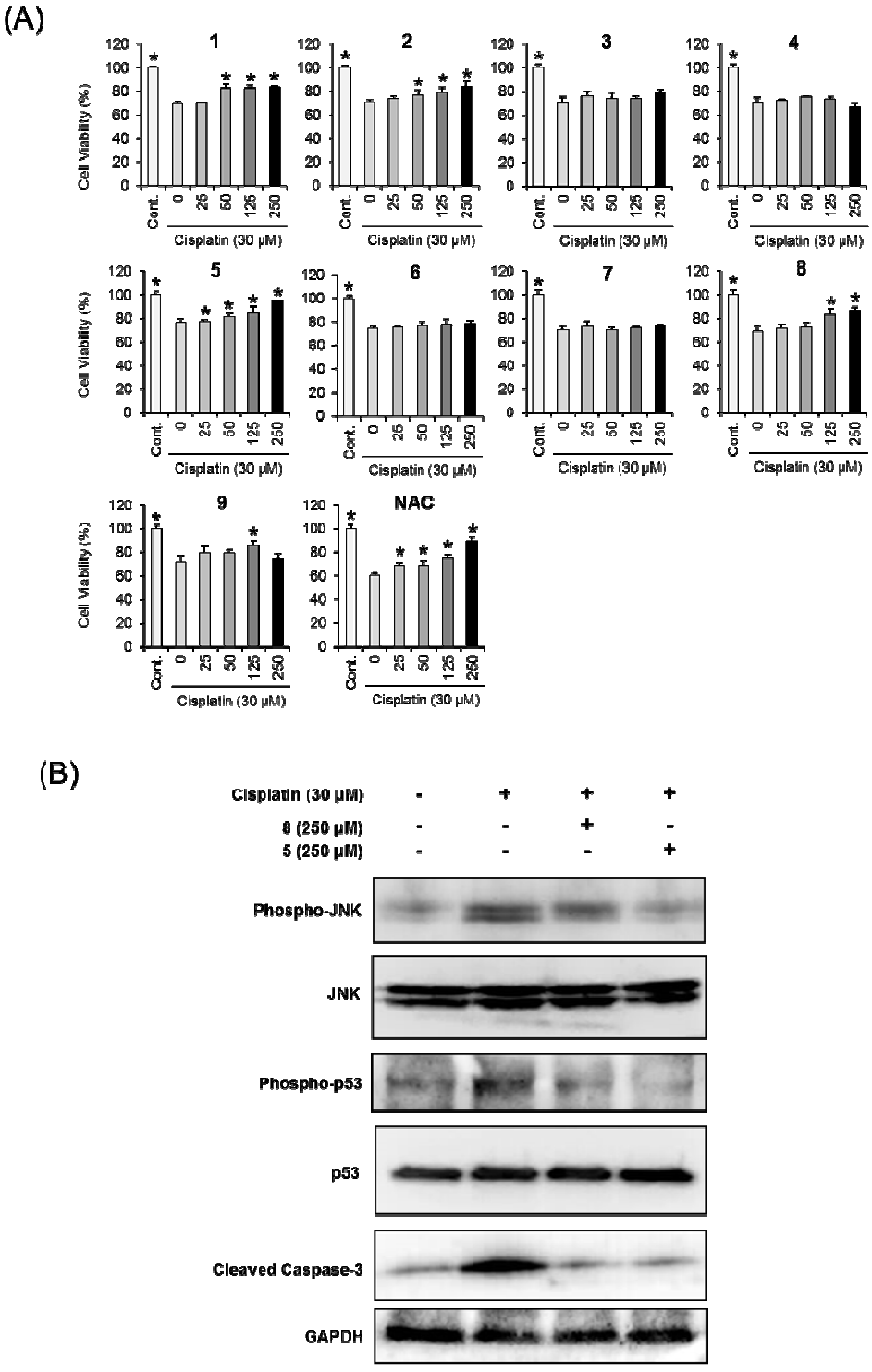
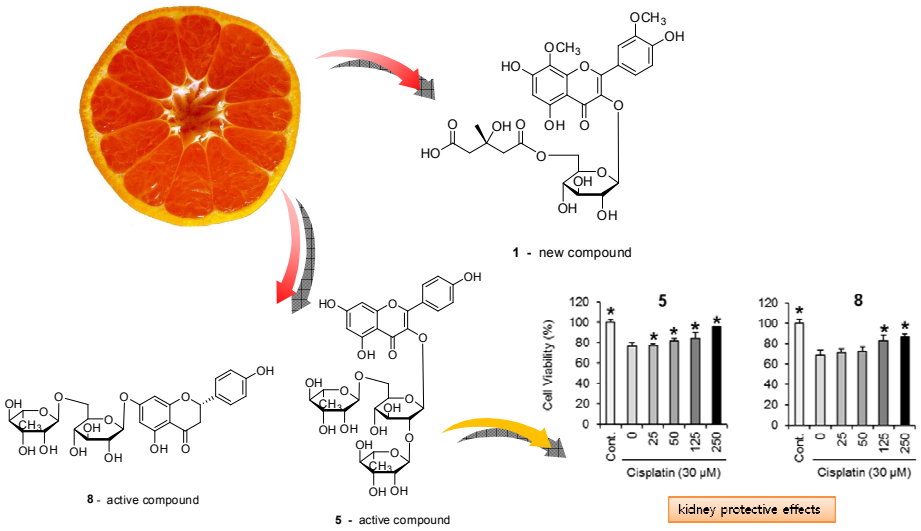

(A)
1
2
3
4
5
6
7
8
9
NAC
Cell Viability (%)
Cont.
0
25
50
125
250
Cisplatin (30 μM)
(B)
Cisplatin (30 μM)
8 (250 μM)
5 (250 μM)
Phospho-JNK
JNK
Phospho-p53
p53
Cleaved Caspase-3
GAPDH

Figure 5.

TOC graphic


1 - new compound
8 - active compound
5 - active compound
5
8
Cell Viability (%)
Cont
0
25
50
125
250
Cisplatin (30 μM)
kidney protective effects