Galloyl Glucoses from the Seeds of *Cornus officinalis* with Inhibitory Activity against Protein Glycation, Aldose Reductase, and Cataractogenesis *ex Vivo*

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Received October 19, 2010; accepted November 15, 2010; published online December 8, 2010

In an ongoing project directed toward the discovery of novel treatments for diabetic complications from traditional herbal medicines, six galloyl glucoses, 1,2,3-tri-*O*-galloyl- β -D-glucose (1), 1,2,6-tri-*O*-galloyl- β -D-glucose (2), 1,2,3,6-tetra-*O*-galloyl- β -D-glucose (3), 1,2,4,6-tetra-*O*-galloyl- β -D-glucose (4), 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (5), and tellimagrandin II (6), and two phenolic acids, gallic acid 4-*O*- β -D-glucoside (7) and gallic acid 4-*O*- β -D-(6'-*O*-galloyl)-glucoside (8), were isolated from an EtOAc-soluble fraction of the seeds of *Cornus officinalis* (Cornaceae). The structures of the compounds were identified using physical and spectroscopic methods, as well as by comparison of their data with values reported in the literature. All the isolates were evaluated *in vitro* for inhibitory activity against the formation of advanced glycation end-products (AGEs) and rat lens aldose reductase (RLAR). Compounds 1–6 were subjected to further bioassay to examine their inhibitory effects on AGE cross-linking. The opacity of lenses was significantly prevented when treated with 3 in an *ex vivo* experiment.

Key words *Cornus officinalis*; galloyl glucose; advanced glycation end-product; advanced glycation end-product-bovine serum albumin cross-linking; cataractogenesis; diabetic complication

The formation of advanced glycation end-products (AGEs), increased polyol pathway flux, activation of protein kinase C isoforms, and increased hexosamine pathway flux have been recognized as major factors in the pathogenesis of complications associated with diabetes.¹⁾ Enhanced formation and accumulation of AGEs, which are generally formed through Amadori-type compounds from proteins and reducing sugars, have been implicated as a major pathogenic process leading to diabetic complications, normal aging, atherosclerosis, and Alzheimer's disease.^{2,3)} The harmful effects of AGEs result from structural and functional alterations in plasma and extracellular matrix (ECM) proteins, in particular from cross-linking of proteins and interaction of AGEs with their receptors and/or binding proteins, which leads to enhanced formation of reactive oxygen species with subsequent activation of nuclear factor- κ B and release of proinflammatory cytokines, growth factors, and adhesion molecules.⁴⁾ The polyol pathway focuses on the enzyme aldose reductase (AR; alditol/nicotinamide adenine dinucleotide phosphate (NADP⁺) oxidoreductase, E.C.1.1.1.21), which reduces excess D-glucose to D-sorbitol using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. The increased accumulation of sorbitol has been linked to cellular damage.⁵⁾ AR has also been demonstrated to play important roles in the pathogenesis of diabetic complications such as cataract, neuropathy, nephropathy, and retinopathy.^{6,7)} Thus AGEs and AR inhibitors can offer a potential therapeutic strategy for the prevention of diabetic and other pathogenic complications.^{8,9)}

In our ongoing project directed toward the discovery of preventive agents against diabetic complications from herbal medicines, the seed of *Cornus officinalis* was chosen for detailed investigation, since its EtOAc-soluble fraction showed significant *in vitro* inhibitory effects against both AGE formation (IC₅₀ value of 1.52 μ g/ml) and rat lens AR (IC₅₀ value of 3.28 μ g/ml). The pericarp of *C. officinalis* SIEB. *et* ZUCC.

(Corni Fructus, Cornaceae) is a well-known traditional medicine for its tonic, analgesic, and diuretic properties in Korea, Japan, and China.¹⁰⁾ Corni Fructus has been shown to exert antidiabetic, antioxidative, and AGE-mediated renal injury-protective effects in streptozotocin-induced diabetic rats.^{11,12)} The seeds of *C. officinalis* also exert antihyperglycemic effect *in vivo*.¹³⁾ Recently, we have found that the seeds of *C. officinalis* exhibit much stronger inhibitory activity against AGE formation *in vitro* than its pericaps.¹⁴⁾

In the present study, repeated chromatography of the EtOAc-soluble fraction of the seeds of *C. officinalis*, which exhibited excellent inhibitory activity in our preliminary study, led to the purification of six galloyl glucoses (1–6) as well as two phenolic acids (7, 8), and their structures were determined by spectroscopic data interpretation. All the isolates were evaluated for their potential to inhibit AGE formation and rat lens aldose reductase (RLAR). Compounds 1–6 were subjected to further bioassay to examine their inhibitory effects on AGEs-bovine serum albumin (BSA) cross-linking. 1,2,3,6-Tetra-*O*-galloyl- β -D-glucose (3) was subjected to an *ex vivo* experiment. The biological evaluation of the isolates is described in this paper.

MATERIALS AND METHODS

General Experimental Procedures Melting points were measured on an IA9100 melting point apparatus (Barnstead International, U.S.A.) and are quoted uncorrected. Optical rotations were obtained using a P-2000 digital polarimeter (Jasco, Japan). UV spectra were recorded with a Jasco V-530 spectrometer. IR spectra were recorded using a Jasco FTIR-4100 spectrophotometer. Electron ionization-mass spectra (EI-MS) were recorded on an Autospec (Micromass, U.K.). NMR experiments were conducted on a DRX-300 or Avance 500 FT-NMR (Bruker, Germany), and the chemical shifts were referenced to the residual solvent signals. TLC analyses

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were performed on Kieselgel 60 F₂₅₄ and 60 RP-18 F₂₅₄S plates (Merck, 0.25-mm layer thickness); compounds were visualized by dipping plates into 20% (v/v) H₂SO₄ reagent (Aldrich) and then heated at 110 °C for 5–10 min. Silica gel (Merck 60A, 70–230 or 230–400 mesh ASTM), Sephadex LH-20 (Amersham Pharmacia Biotech), Diaion HP-20 (Supelco, U.S.A.), and reverse-phase silica gel (YMC Co., ODS-A 12 nm S-150 μ m) were used for column chromatography (CC).

Plant Material The seeds of *C. officinalis* SIEB. et ZUCC. (Cornaceae) were collected in Gurye-gun, JeollaNam-do, Republic of Korea, in November 2006 and identified by Prof. J.-H. Kim, Department of Life Science, Kyungwon University, Republic of Korea. A voucher specimen (no. KIOM-COOF1) has been deposited at the Herbarium of the Diabetic Complications Research Center, Division of Traditional Korean Medicine (TKM) Integrated Research, Korea Institute of Oriental Medicine, Republic of Korea.

Extraction and Isolation The dried and ground plant material (5.0 kg) was extracted with 20 l of MeOH three times by maceration. The extracts were combined and concentrated *in vacuo* at 40 °C to give an MeOH extract (951 g). The MeOH extract (936 g) was suspended in H₂O (2 l) and successively extracted with *n*-hexane (3 $\times$ 2 l), EtOAc (3 $\times$ 2 l), and *n*-BuOH (3 $\times$ 2 l) to give the *n*-hexane- (11 g), EtOAc- (283 g), *n*-BuOH- (384 g), and water-soluble extracts (258 g), respectively. The EtOAc-soluble fraction (277 g) was chromatographed over silica gel (ϕ 11 $\times$ 50 cm, 70–230 mesh) as the stationary phase using a CHCl₃/MeOH/water gradient (4:1:0.1 $\rightarrow$ 5:4:1 $\rightarrow$ 0:1:0 v/v) to afford 10 fractions (F01–F10). Fraction F06 (8.8 g) was subjected to Sephadex CC (ϕ 5.0 $\times$ 65 cm) with MeOH to produce nine subfractions (F0601–F0609). Gallic acid 4-*O*- β -D-(6'-*O*-galloyl)-glucoside (**8**, 23.6 mg), gallic acid 4-*O*- β -D-glucoside (**7**, 28.7 mg), and 1,2,3-tri-*O*-galloyl- β -D-glucose (**1**, 35.0 mg) were purified from subfractions F0604, F0605, and F0607, respectively, by repeated CC. Fraction F07 (71.3 g) was chromatographed over a Diaion HP-20 column (ϕ 6.5 $\times$ 63 cm), with an MeOH/water gradient (1:4 $\rightarrow$ 1:0) as a solvent system, yielding five subfractions (F0701–F0705). Subfraction F701 was further fractionated using Sephadex CC (ϕ 5.0 $\times$ 65 cm) with an MeOH/water gradient (0:1 $\rightarrow$ 1:0) as a solvent system to give 1,2,6-tri-*O*-galloyl- β -D-glucose (**2**, 54 mg), 1,2,3,6-tetra-*O*-galloyl- β -D-glucose (**3**, 132 mg), and 1,2,4,6-tetra-*O*-galloyl- β -D-glucose (**4**, 250 mg). 1,2,3,4,6-Penta-*O*-galloyl- β -D-glucose (**5**, 160 mg) and tellimagrandin II (**6**, 84 mg) were obtained from subfraction F0705 by a further Sephadex LH-20 CC (ϕ 4.6 $\times$ 48 cm) with an MeOH/water gradient (7:3 $\rightarrow$ 1:0) as a solvent system.

Determination of AGE Formation Following a well-established method,¹⁵⁾ the reaction mixture [BSA (10 mg/ml, Sigma, U.S.A.; 700 μ l) in phosphate buffer 50 mM (pH 7.4) with 0.02% sodium azide] was added to fructose 0.2 M and glucose (100 μ l). In screw-cap tubes (1.5 ml), the reaction mixture was then mixed with 200 μ l of serially diluted compounds or aminoguanidine (Sigma). After incubating at 37 °C for 14 d, the fluorescent reaction products (200 μ l) were transferred to 96-well plates and assayed on a spectrofluorometric detector (Bio-Tek, Synergy HT, U.S.A.; excitation wavelength, 350 nm; emission wavelength, 450 nm). AGE assay was performed in triplicate. The concentration of

each test sample giving 50% inhibition of the activities (IC₅₀) was estimated based on the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

AGE Cross-Linking Inhibition Assay The ability of compounds to inhibit AGE cross-links was measured using a previously reported method with modifications.¹⁶⁾ Briefly, a mixture of AGE-BSA 1.0 μ g and either test concentrations of compounds or aminoguanidine were added to each well of collagen-coated microtiter plates, and then the plates were incubated for 4 h at 37 °C. Formation of the collagen-AGE-BSA complex was measured using anti-AGE-BSA monoclonal antibody (Wako Pure Chemical Industries, Japan), horseradish peroxidase-linked goat anti mouse immunoglobulin G (IgG) antibody, and H₂O₂ substrate containing ABTS chromogen. The optical density (OD) was measured on an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek, Synergy HT, U.S.A.) with a sample wavelength of 410 nm. Inhibition of cross-linking was expressed as the percentage decrease in OD when AGE-BSA was incubated with collagen in the presence of compounds.

RLAR Inhibition Assay All experiments were performed in accordance with the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The study was approved by the authors' institute's Committee on Animal Care. Crude RLAR was prepared from lenses that were removed from the eyes of 7–8-week-old male Sprague-Dawley rats (Orient Co., Korea).^{17,18)} The protein content of the enzyme preparation was determined in the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) with a BSA (Sigma) standard. The activity of RLAR was determined by measuring the amount of NADP converted from NADPH per unit time at 37 °C and pH 7.0. To determine their effects on RLAR activity, the isolates and the positive control (epalrestat) were assayed according to the method described previously with slight modifications.¹⁹⁾ Briefly, the incubation mixture contained Na,K-phosphate buffer 135 mM (pH 7.0), lithium sulfate 100 mM, NADPH 0.03 mM, DL-glyceraldehyde 0.04 mM, and 100 μ l of an enzyme preparation, with or without 50 μ l of isolates or positive inhibitor, in a total volume of 1.0 ml. The reaction was initiated by adding NADPH at 37 °C and stopped by adding 0.3 ml of 0.5 N hydrochloric acid. Then, 1 ml of 6 N NaOH containing imidazole 10 mM was added, and the mixture was incubated at 60 °C for 10 min to convert NADP to a fluorescent product. The fluorescence was measured at room temperature with a spectrofluorophotometer (excitation/emission wavelength, 360 nm/460 nm; Synergy HT, Bio-Tek, Winooski, VT, U.S.A.). All measurements were performed in triplicate. The concentration of compounds that resulted in an IC₅₀ value was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

Rat Lens Organ Culture and Analysis of Lens Opacity This study was also approved by the authors' institute's Committee on Animal Care. For *ex vivo* examination of lens opacity, lenses were dissected from 6-week-old male Sprague-Dawley rats, as described previously.^{20,21)} Briefly, each isolated lens was incubated in 2 ml of modified TC-199 medium with antibiotics in 24-well plates and incubated under conditions of 95% air and 5% CO₂ at 37 °C with xy-

lose 20 mM for a period of 2 d. Compound **3** was prepared in DMSO and filtered before being added to the medium. The medium was changed every day and supplemented with compound **3** (5, 10 $\mu\text{g/ml}$) or epalrestat (3 $\mu\text{g/ml}$) as a positive inhibitor, along with xylose 20 mM. All the reagents used in lens culture were filtered through a 0.2- μm filter. Lenses were examined for the development of generalized opacity under an optical microscope with a CCD camera. The opaque area of the lens was then analyzed (Image Analysis 42D 3D measuring software, TDI Scope Eyemedia 3.0, Olysia, Japan).

RESULTS AND DISCUSSION

Eight compounds including six galloyl glucoses (**1**–**6**) and two phenolic acids (**7**, **8**) were isolated from the EtOAc-soluble fraction of the seeds of *C. officinalis*. Their structures were identified as 1,2,3-tri-*O*-galloyl- β -D-glucose (**1**),²² 1,2,6-tri-*O*-galloyl- β -D-glucose (**2**),²³ 1,2,3,6-tetra-*O*-galloyl- β -D-glucose (**3**),^{22,23} 1,2,4,6-tetra-*O*-galloyl- β -D-glucose (**4**),²⁴ 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (**5**),^{23,25} tellimagrandin II (**6**),²⁵ gallic acid 4-*O*- β -D-glucoside (**7**),²⁶ and gallic acid 4-*O*- β -D-(6'-*O*-galloyl)-glucoside (**8**)²⁷ by spectroscopic measurement and by comparison with published values (Fig. 1). To the best of our knowledge, this is the first report on the isolation of compounds **7** and **8** from this plant. All isolates obtained in the present study were evaluated for their potential to inhibit AGE formation and RLAR (Table 1). Compounds **1**–**6** were subjected to further bioassay to examine their inhibitory effects on AGE-BSA cross-linking (Fig. 2). Of these, 1,2,3,6-tetra-*O*-galloyl- β -D-glucose (**3**)

Fig. 1. Structures of Compounds **1**–**8** from the Seeds of *C. officinalis*

Table 1. Inhibitory Effects of Compounds **1**–**8** from the Seeds of *C. officinalis* against AGE Formation and RLAR

Compound	Inhibitory effect (IC_{50} value; μM) ^{a)}	
	AGE formation ^{b)}	RLAR
1	2.81 ± 0.03	2.35 ± 0.27
2	1.89 ± 0.24	4.01 ± 0.12
3	1.99 ± 0.07	0.70 ± 0.12
4	0.80 ± 0.02	0.76 ± 0.07
5	0.87 ± 0.01	1.93 ± 0.01
6	0.90 ± 0.01	0.90 ± 0.08
7	>150.6	82.74 ± 8.49
8	42.16 ± 0.82	11.77 ± 1.23
AG ^{c)}	961.2 ± 29.5	—
EP ^{c)}	—	0.067 ± 0.009

a) Inhibitory effect is expressed as mean $\pm$ S.D. of triplicate experiments. IC_{50} values were calculated from the dose inhibition curve. b) After incubating for 14 d, the fluorescent reaction products were assayed on a spectrofluorometric detector. c) Aminoguanidine (AG) and epalrestat (EP) were used as positive controls.

was subjected to an *ex vivo* experiment (Fig. 3).

In the AGE assay, compound **4** showed the strongest inhibitory activity with an IC_{50} value of $0.80 \mu\text{M}$, followed by compounds **5** ($\text{IC}_{50} = 0.87 \mu\text{M}$) and **6** ($\text{IC}_{50} = 0.90 \mu\text{M}$). Other galloyl glucoses **1**–**3** also exhibited much stronger inhibitory activity (IC_{50} values ranging from 1.89 to $2.81 \mu\text{M}$) than the well-known positive control aminoguanidine ($\text{IC}_{50} =$

Fig. 2. Percentage Inhibition of Compounds **1**–**6** on AGE-BSA Formation Measured Using ELISA with Rabbit Anti-AGE Antibodies (6D12)

Inhibition by different concentrations of compounds **1** (1,2,3-tri-*O*-galloyl- β -D-glucose), **2** (1,2,6-tri-*O*-galloyl- β -D-glucose), **3** (1,2,3,6-tetra-*O*-galloyl- β -D-glucose), **4** (1,2,4,6-tetra-*O*-galloyl- β -D-glucose), **5** (1,2,3,4,6-penta-*O*-galloyl- β -D-glucose), **6** (tellimagrandin II), and aminoguanidine (AG). Data are mean $\pm$ S.D. ($n=3$). * $p < 0.01$ vs. sample incubated with AG.

A

B

Fig. 3. Preventive Effects of Compound **3** against Cataractogenesis of Rat Lenses Induced with Xylose 20 mM

(A) Representative image of rat lenses incubated for various times; (B) opacity of each lens. All opacities were analyzed from each lens. Data are expressed as mean $\pm$ S.D. ($n=4$). ### $p < 0.001$ vs. without xylose; *** $p < 0.001$ vs. xylose.

961.2 μM). Aminoguanidine, a hydrazine-like small molecule, is the first AGE inhibitor explored in clinical trials. However, the drug was not ultimately approved for commercial production because side effects were observed in phase III clinical trials in patients with diabetes.²⁸⁾

The galloyl glucoses **1**–**6**, which exhibited potent inhibitory activity in the AGE assay, were further evaluated for their effect on the inhibition of AGE-BSA cross-linking to collagen *in vitro*. All the galloyl glucoses markedly reduced AGE-BSA cross-linking in a concentration-dependent manner, and all exhibited more potent inhibitory activity than aminoguanidine. Thus galloyl glucoses act as inhibitors of AGE cross-linking as well as AGE formation.

In the RLAR assay, the two tetra-*O*-galloyl glucoses **3** and **4** showed the most potent inhibitory activity (IC_{50} values of 0.70, 0.76 μM , respectively) although they were less active than the commercial synthetic AR inhibitor epalrestat (IC_{50} = 0.067 μM). Epalrestat is a commercial synthetic AR inhibitor approved in Japan for the improvement of subjective neuropathy symptoms, abnormality of vibratory sense, and abnormal changes in heartbeat associated with diabetic peripheral neuropathy.²⁹⁾ The other galloyl glucoses **1**, **2**, and **5** also showed significant RLAR inhibitory activity (IC_{50} values ranging from 1.93 to 4.01 μM), while the two gallic acid derivatives **7** and **8** exhibited mild activity.

Compound **3** (1,2,3,6-tetra-*O*-galloyl- β -D-glucose), which exhibited the most potent RLAR inhibitory activity, was further evaluated for its inhibitory effect on cataractogenesis *ex vivo*. In the *ex vivo* experiment, most lenses were covered with opaque rings after 2 d of incubation with xylose 20 mM (Fig. 3). The effects of compound **3** (40, 80 μM) were measured every 24 h and compared with untreated xylose lenses. The opacities of the lenses after 2 d of xylose treatment were significantly improved after compound **3** treatment in a dose-dependent manner.

Various natural products including flavonoids and phenolics have been reported as AR or protein glycation inhibitors.^{30–32)} However, tannins such as galloyl glucoses are not widely known as inhibitors of AR or protein glycation although their fundamental activities, *e.g.*, reduction and antiperoxidation properties and marked biological and pharmacological activities of various potencies have been reported.³³⁾ Two gallotannins, 1,3,4,6-tetra-*O*-galloyl- β -D-glucose and 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (**5**), were previously reported to be potent inhibitors of human placenta AR with IC_{50} values of 0.799 and 0.070 μM , respectively.³⁴⁾ 1,2,3,6-Tetra-*O*-galloyl- β -D-glucose (**3**) was isolated from *Paeoniae Radix* and shown to have potent inhibitory activity against RLAR with an IC_{50} value of 0.63 μM .³⁵⁾ This is in good agreement with our present study (IC_{50} = 0.70 μM for RLAR). To the best of our knowledge, this is the first report of compounds **1**, **2**, **4**, **6**, and **8** as inhibitors of AR and compounds **1**–**8** as inhibitor of AGE formation. Furthermore, the inhibitory activity of compounds **1**–**6** against AGE-BSA cross-linking and the preventive effect of compound **3** against cataractogenesis have not been reported to date.

In summary, galloyl glucoses **1**–**6** were identified as active principles in the seeds of *C. officinalis* which inhibit AGE formation, AGE-BSA cross-linking, and RLAR *in vitro*. In addition, 1,2,3,6-tetra-*O*-galloyl- β -D-glucose (**3**) prevented the opacity of lenses *ex vivo*. Thus these galloyl

glucoses appear to be worthy of additional biological investigation to fully evaluate their potential as therapeutic agents for diabetic complications and other related diseases.

Acknowledgments We thank the Korea Basic Science Institute (KBSI) for performing NMR and MS experiments. This research was supported by grants [K09030 and K10040] from the Korea Institute of Oriental Medicine (KIOM).

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