3,5-Di-O-caffeoyl-epi-quinic Acid from the Leaves and Stems of Erigeron annuus Inhibits Protein Glycation, Aldose Reductase, and Cataractogenesis

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In our ongoing project directed toward the discovery of novel treatments for diabetic complications from herbal medicines, sixteen compounds including three caffeoylquinic acids and four flavonoids were isolated from an EtOAc-soluble extract of the stems and leaves of Erigeron annuus. All the isolates were evaluated in vitro for inhibitory activity on the formation of advanced glycation end products (AGEs) and rat lens aldose reductase (RLAR). Of these, 3,5-di-O-caffeoyl-epi-quinic acid (3) exhibited the most potent inhibitory activity in both the AGEs and aldose reductase (AR) assays. Compound 3 markedly reduced AGEs-bovin serum albumin (BSA) cross-linking in a dose-dependent manner. Furthermore, opacity of lenses was significantly prevented when they were treated with 3 in an ex vivo experiment.

Key words Erigeron annuus; caffeoylquinic acid; advanced glycation end product; aldose reductase; diabetic complication

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. Of these mechanisms, 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. The harmful effects of AGEs (both endogenous and exogenous) 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-κB and release of pro-inflammatory cytokines, growth factors, and adhesion molecules. The polyol pathway focuses on the enzyme aldose reductase (AR; alditol/NADP+ oxidoreductase, E.C.1.1.1.21), which reduces excess d-glucose into d-sorbitol using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Increased accumulation of sorbitol has been linked to cellular damage. AR also has been demonstrated to play important roles in the pathogenesis of diabetic complications such as cataract, neuropathy, nephropathy, and retinopathy. Thus AGEs and AR inhibitors can offer a potential strategy as therapeutics for the prevention of diabetic and other pathogenic complications.

In our ongoing project directed toward the discovery of novel treatments for diabetic complications from herbal medicines, the stems and leaves of Erigeron annuus were chosen for detailed investigation since the EtOAc-soluble fraction led to the isolation of sixteen compounds. All isolates obtained in the present study were evaluated for their potential to inhibit AGEs formation and RLAR. Of these, 3,5-di-O-caffeoyl-epi-quinic acid (3) was subjected to an in vitro AGEs-bovin serum albumin (BSA) cross-linking inhibition assay and an ex vivo experiment. The biological evaluation of the isolates is described herein.

MATERIALS AND METHODS

General Experimental Procedures Melting points were measured by IA9100 melting point apparatus (Barnstead International, U.S.A.) and are quoted uncorrected. Optical rotations were obtained by P-2000 digital polarimeter (Jasco, Japan). UV spectra were recorded by Jasco V-530 spectrometer. IR spectra were recorded by Jasco FTIR-4100 spectrophotometer. EI-MS were recorded by 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 were performed on Kieselgel 60 F254 (Merck) plates (silica gel; 0.25 mm layer thickness); com-
pounds were visualized by dipping plates into 20% (v/v) H$_2$SO$_4$ 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), and reversed-phase silica gel (YMC Co.; ODS-A 12 nm S-150 μm) were used for column chromatography (CC). All solvents used for the chromatographic separations were distilled before use.

**Plant Material** The leaves and stems of *E. annuus* (L.) Pers. (Compositae) were collected in Jeonmin-dong, Yuseong-gu, Daejeon, Korea, in June 2007 and were identified by one of our authors, Prof. J.-H. Kim. A voucher specimen (KIOM-ErAn3E) has been deposited at the Herbarium of the Diabetic Complications Research Center, Korea Institute of Oriental Medicine, Korea.

**Extraction and Isolation** The dried plant material (3.3 kg) was extracted with 201 of MeOH three times by maceration. The extracts were combined and concentrated in vacuo at 40°C to give a MeOH extract (330 g). The extract was suspended in H$_2$O (2 l) and successively extracted with n-hexane (3 x 2 l), EtOAc (3 x 2 l), and BuOH (3 x 2 l) to give n-hexane- (51 g), EtOAc- (29 g), BuOH- (56 g), and water-soluble extract (174 g), respectively. The EtOAc-soluble fraction (27 g) was chromatographed over silica gel (φ 6.5 x 47 cm; 70—230 mesh) as the stationary phase using a CHCl$_3$—MeOH gradient (from 20:1 to 0:1 v/v) to afford twelve fractions (F01—F12). 3-Hydroxyprop-4-ene (80 mg), apigenin (4, 18 mg), and daucosterol (235 mg) were purified from fractions F03 (2.81 g), F04 (0.81 g), and F06 (1.18 g), respectively, by recrystallization (in MeOH). Fraction F07 (1.31 g) was purified further over a Sephadex column (φ 5.0 x 65 cm) with MeOH, yielding 6′-O-(4-hydroxy-3,5-dimethoxybenzoyl)-erigeroside (10, 15 mg), eugenyl O-β-D-glucopyranoside (64 mg), and 1-O-β-D-glucopyranosyl-6α-hydroxyeudesman-4(15)-ene (39 mg). 3,5-Di-O-cafeoylquinic acid methyl ester (2, 80 mg) was obtained from fraction F08 (1.7 g) through Sephadex CC (φ 5.0 x 65 cm, MeOH). Fraction 9 (3.0 g) was further fractionated using Sephadex CC (φ 5.0 x 65 cm, MeOH) to give astragalin (6, 10 mg), 3′-O-cafeoylgeraniol (8, 53 mg), 6′-O-cafeoylgeraniol (9, 55 mg), and 6′-O-coumaroyl-3′-O-feruloylquinic acid methyl ester (11, 34 mg). Fraction F10 (2.67 g) was subjected to Sephadex CC (φ 5.0 x 65 cm, MeOH) to afford 3,5-di-O-cafeoyl-epi-quinic acid (3, 40 mg), isosculetarraine 4′-methyl ether 8′-O-β-D-glucuronide (7, 22 mg), and erigeronide (34 mg). Finally, 3-cafeoylquinic acid (1, 57 mg) and apigenin 7- O-β-D-glucuronide (5, 75 mg) were isolated from fraction F12 (3.0 g) by repeated CC.

**Formation of AGEs** According to a well-established method, the reaction mixture (bovine serum albumin [10 mg/ml; Sigma, St. Louis, MO, U.S.A.; 700 μl] in 50 mM phosphate buffer [pH 7.4] with 0.02% sodium azide) was added to 0.2 mM fructose and glucose (100 μM). In screw cap tubes (1.5 ml), the reaction mixture was then mixed with 200 μl of serial diluted compounds or aminoguanidine (Sigma). After incubating at 37°C for 14 d, the fluorescence reaction products (200 μl) were transferred to 96 well plates and assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350, Em: 450 nm). AGEs assay was performed in triplicate. The concentration of each compound giving 50% inhibition of the activities (IC$_{50}$) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

**AGEs Cross-Linking Inhibition and Breaking Assay** The ability of compounds to inhibit or break AGEs cross-links was measured by a previously reported method with modifications. Briefly, for breaking assay, 1.0 μg AGEs-bovin serum albumin (BSA) (MBL, Nagoya, Japan) was added to each well of collagen-coated, micrortite plates (Sigma). AGEs-BSA was allowed to react with collagen at 37°C for 4 h, then test concentrations of compounds or ALT-711 [4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride; Alteon Inc., Ramsey, NJ, U.S.A.] were added to wells and incubated at 37°C. For inhibition assay, the mixture of 1.0 μg AGEs-BSA and either test concentrations of compounds or aminoguanidine were added to each well of collagen-coated, micrortite plates, then the plate was incubated at 37°C for 4 h. Formation of the collagen-AGEs-BSA complex was measured using anti-AGEs-BSA monoclonal antibody (Wako Chemicals, Japan), horseradish peroxidase-linked goat anti-mouse immunoglobulin G (IgG) antibody and H$_2$O$_2$ substrate containing ABTS chromogen. The optical density (OD) was measured by ELISA reader (BIO-TEK, Synergy HT, U.S.A.) with the sample wavelength of 410 nm. Inhibition or breaking 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 National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. The study was approved by our 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). The protein content of the enzyme preparation was determined by 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 by Drufner et al. with slight modifications. Briefly, the incubation mixture contained 135 mM Na$_2$-phosphate buffer (pH 7.0), 100 mM lithium sulfate, 0.03 mM NADPH, 0.04 mM dl-glyceraldehyde, and 100 μl of an enzyme preparation, with or without 50 μl 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 10 mM imidazole 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 spectrofluorometer (Ex/Em=360 nm/460 nm; Synergy HT, Bio-Tek, Winooski, VT, U.S.A.). All measurements were performed in triplicate. The concentration of compounds that led to IC$_{50}$ value was estimated from the least square 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 our 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 previously described.\textsuperscript{25,26} 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% CO\textsubscript{2} at 37 °C with 20 mm/l xylose for a period of 2 d. Compound 3 was prepared in dimethyl sulfoxide (DMSO) and filtered before being added to the medium. Medium was changed every day and supplemented with (DMSO) and filtered before being added to the medium.

**RESULTS AND DISCUSSION**

Sixteen compounds including three quinic acid derivatives (1—3) and four flavonoids (4—7) were isolated from an EtOAc-soluble extract of the leaves and stems of *E. annuus* (Fig. 1). Their structure was identified as 3-cafeoylquinic acid (1),\textsuperscript{25,27} 3,5-di-O-cafeoylquinic acid methyl ester (2),\textsuperscript{28} 3,5-di-O-cafeoyl-epi-quinic acid (3),\textsuperscript{29} apigenin (4),\textsuperscript{30} apigenin 7-O-β-d-glucuronide (5),\textsuperscript{30} astragal (6),\textsuperscript{31} isoscutellarein 4’-methyl ether 8-O-β-d-glucuronide (7),\textsuperscript{32} 3’-O-cafeoylerigeroside (8),\textsuperscript{33} 6’-O-cafeoylerigeroside (9),\textsuperscript{34} 6’-O-(4-hydroxy-3,5-dimethoxybenzoyl)-erigeroside (10),\textsuperscript{35} 6’-O-coumaroyl-3-O-feruloyl sucrose (11),\textsuperscript{36} eugenyl O-β-d-glucopyranoside,\textsuperscript{37} 1-O-β-d-glucopyranosyl-6′-O-hydroxydeuesman-4(15)-ene,\textsuperscript{38} daucosterol,\textsuperscript{31} erigeroside,\textsuperscript{39} and 3-hydroxypryan-4-one\textsuperscript{40} by spectroscopic data measurement and by comparison with published values. To the best of our knowledge, this is the first report on the isolation of compounds 1, 3, 7, 9, and 11 from this plant.

All isolates obtained in the present study were evaluated for their potential to inhibit AGEs formation and RLAR (Table 1). Of these, dicaffeoylquinic acids, 3,5-di-O-cafeoyl-epi-quinic acid (3) and 3,5-di-O-cafeoylquinic acid methyl ester (2), exhibited the most potent inhibitory activity against AGEs formation with observed IC\textsubscript{50} values of 6.06 and 12.8 µM, respectively, while a monocafeoylquinic acid, 3-cafeoylquinic acid (1), was not effective. Two caffeyl erigerosides (8, 9) and a sucrose ester (11) also showed much stronger inhibitory activity against AGEs formation (IC\textsubscript{50} values ranging from 15.9 to 24.2 µM) than aminoguanidine (IC\textsubscript{50} value of 961 µM). Aminoguanidine, a hydrazine-like molecule, was 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.\textsuperscript{41}

In the RLAR assay, compound 3 also showed the strongest inhibitory activity (IC\textsubscript{50} value of 0.44 µM) although it is less active than epalrestat (IC\textsubscript{50} value of 0.067 µM). Epalrestat is a commercial synthetic AR inhibitor that is approved in Japan for the improvement of subjective neuropathy symptoms, abnormality of vibration sense, and abnormal changes in heart beat associated with diabetic peripheral neuropathy.\textsuperscript{52} Compounds 1, 2, 4—9, and 11 also showed significant RLAR inhibitory activity (IC\textsubscript{50} values ranging from 0.79 to 7.18 µM), while compound 10 exhibited weak activity (IC\textsubscript{50} value of 75.8 µM). Although various natural products including flavonoids and phenolics have been reported as AR or protein glycation inhibitors,\textsuperscript{43—48} this is the first report of compounds 3 and 7—11 having inhibitory activity against AR or protein glycation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory effect (IC\textsubscript{50} value; µM)\textsuperscript{a}</th>
<th>RLAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;141</td>
<td>1.67±0.03</td>
</tr>
<tr>
<td>2</td>
<td>12.8±0.20</td>
<td>0.79±0.21</td>
</tr>
<tr>
<td>3</td>
<td>6.06±0.11</td>
<td>0.44±0.09</td>
</tr>
<tr>
<td>4</td>
<td>17.8±0.42</td>
<td>7.18±2.13</td>
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<tr>
<td>5</td>
<td>101.7±0.64</td>
<td>1.28±0.02</td>
</tr>
<tr>
<td>6</td>
<td>101.8±0.39</td>
<td>5.89±1.25</td>
</tr>
<tr>
<td>7</td>
<td>&gt;105</td>
<td>1.77±0.39</td>
</tr>
<tr>
<td>8</td>
<td>15.9±0.35</td>
<td>4.84±0.38</td>
</tr>
<tr>
<td>9</td>
<td>19.0±0.10</td>
<td>2.00±0.20</td>
</tr>
<tr>
<td>10</td>
<td>71.2±11.4</td>
<td>75.8±23.0</td>
</tr>
<tr>
<td>11</td>
<td>24.2±0.84</td>
<td>1.67±0.58</td>
</tr>
<tr>
<td>AG\textsuperscript{b}</td>
<td>961±29</td>
<td>0.067±0.009</td>
</tr>
<tr>
<td>EP\textsuperscript{c}</td>
<td>—</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Inhibitory effect expressed as mean±S.D. of triplicate experiments. IC\textsubscript{50} values were calculated from the dose inhibition curve. \textsuperscript{b} After incubating for 14 d, the fluorescent reaction products were assayed on a spectrofluorometric detector. Eugenyl O-β-d-glucopyranoside, 1-O-β-d-glucopyranosyl-6′-O-hydroxydeuesman-4(15)-ene, daucosterol, erigeroside, and 3-hydroxypryan-4-one were not active (IC\textsubscript{50} value, ≥50 µg/ml) in both RLAR and AGEs assay systems. \textsuperscript{c} Aminoguanidine (AG) and epalrestat (EP) were used as positive control.
AGEs formation.

Compound 3, which exhibited the most potent inhibitory activity in both the AGEs and RLAR assay, was further evaluated for its effect on the inhibition or breaking of AGEs. Compound 3 markedly reduced AGEs-BSA cross-linking in a concentration-dependent manner, and exhibited more potent inhibitory activity than aminoguanidine. However, compound 3 did not show significant breaking activity against preformed AGEs-BSA cross-linking at 1000 μg/ml (data not shown). Thus compound 3 did not act as a breaker of AGEs cross-linking but as an inhibitor of AGEs cross-linking as well as AGEs formation.

In the ex vivo experiment, most lenses were covered with opaque rings after 2 d of incubation with 20 mM xylose (Fig. 3). The effects of compound 3 (5, 10 μM) were measured every 24 h and compared versus untreated xylose lenses. The opacity of the lenses after 2 d of xylose treatment was significantly prevented by compound 3 (10 μM) treatment. Although there are some reports that monocaffeoyl and dicaffeoyl quinic acids including chlorogenic acid inhibit AR and the formation of AGEs, this is the first report on 3,5-dif WWE caffeoyl epigallocatechin as an inhibitor of RLAR, AGEs formation, AGEs-BSA cross-linking, and cataractogenesis.

In summary, sixteen compounds were isolated from the stems and leaves of E. annuus. Compounds 2—6 and 8—11 inhibited both the formation of AGES and RLAR. The most potent compound 3 inhibited AGEs-BSA cross-linking to collagen and prevented opacification of rat lenses. Thus compound 3 seems to be worthy of additional biological tests more fully to evaluate its potential as a therapeutic agent for diabetic complications and related diseases.

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REFERENCES


