Thieme

Polyphenols from *Euphorbia pekinensis* Inhibit AGEs Formation *In Vitro* and Vessel Dilation in Larval Zebrafish *In Vivo*

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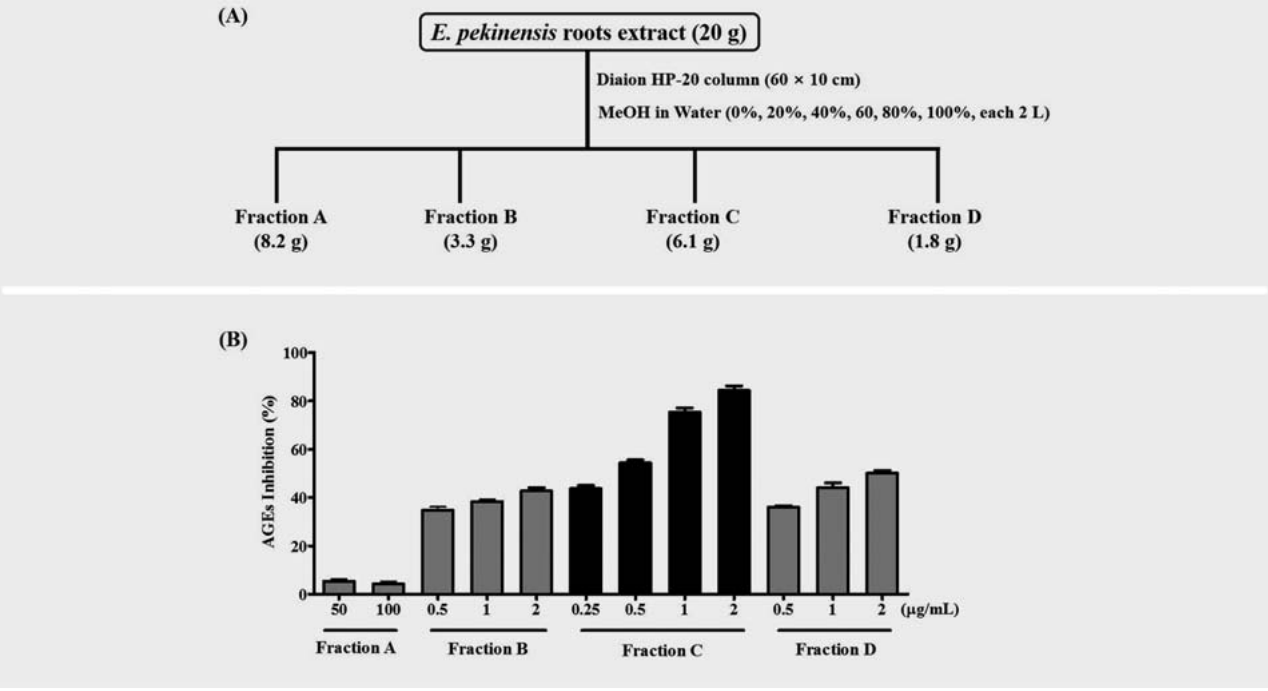
ABSTRACT

To identify active compounds in the roots of *Euphorbia pekinensis* for treatment of diabetic complications, an active column fraction from a 70% EtOH extract of *E. pekinensis* root was purified by preparative reversed-phase high-performance liquid chromatography, leading to the isolation of a new ellagic acid derivative, 3,3′-di-*O*-methylellagic acid 4-*O*-(6′′-*O*-galloyl)-*β*-D-galactopyranoside (**1**), along with three known compounds, geraniin (**2**), 3,3′-di-*O*-methylellagic acid 4-*O*-*β*-D-xylopyranoside (**3**), and ellagic acid 3,3′-dimethyl ether (**4**). The structure of the new compound was established by extensive spectroscopic studies and chemical evidence. The inhibitory effects of isolated compounds **1**–**4** on advanced glycation end-products (AGEs) formation were examined. All compounds exhibited considerable inhibition of AGEs formation and IC_{50} values of 0.41–12.33 µM, compared with those of the positive controls aminoguanidine (IC_{50} = 1122.34 µM) and quercetin (IC_{50} = 27.80 µM). In addition, the effects of **2** and **4** on the dilation of hyaloid-retinal vessels induced by high glucose (HG) in larval zebrafish were investigated; both compounds significantly reduced the HG-induced dilation of hyaloid-retinal vessels relative to the HG-treated control group.

Introduction

Euphorbia pekinensis Rupr. (Euphorbiaceae), a species in the genus *Euphorbia,* which comprises approximately 2000 species, is a perennial herb that is distributed widely, particularly in tropical and subtropical regions of southwestern and eastern America and in Africa, Asia, and certain parts of Europe. It is considered one of the 50 most important medicinal herbs used in traditional Chinese medicine [1]. The root of this plant has been used in China for thousands of years for the treatment of edema, fullness of the chest, viscous sputum, epilepsy, dyspnea, ascites, and tubercular disease [2] and has been shown to possess various pharmacological activities, including those attributable to cytotoxic, antiviral, and anti-inflammatory properties [3–5]. Numerous bioactive substances, such as phenolics, hydrolysable tannins, flavonoids, diterpenes and triterpenes, have been isolated from *E. pekinensis*, and their pharmacological activities have been verified *in vitro* [6–13].

In our ongoing efforts to identify effective, naturally sourced therapeutic agents for the treatment of diabetic complications, we found a 70% EtOH extract of *E. pekinensis* root exhibited considerable inhibition of advanced glycation end-products (AGEs) formation. This prompted us to investigate active compounds in the roots of *E. pekinensis*. This report describes the isolation and structural elucidation of active compounds (**1**–**4**) from the roots of *E. pekinensis*, as well as their inhibitory effects on AGEs formation. We further investigated the effects of two isolates (**2** and **4**) on the high glucose (HG)-induced dilation of hyaloid-retinal vessels in larval zebrafish.



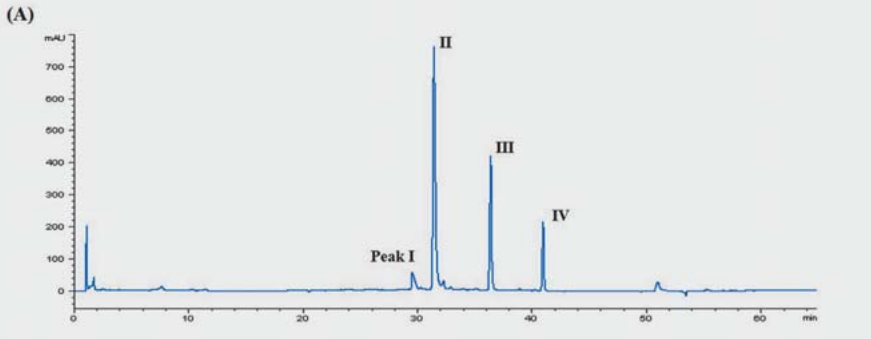
▶ **Fig. 1** Gel chromatography of a 70% EtOH extract of *E. pekinensis* root, performed with a Diaion HP-20 column (**A**), and AGEs formation inhibitory activity of column fractions A–D (**B**).

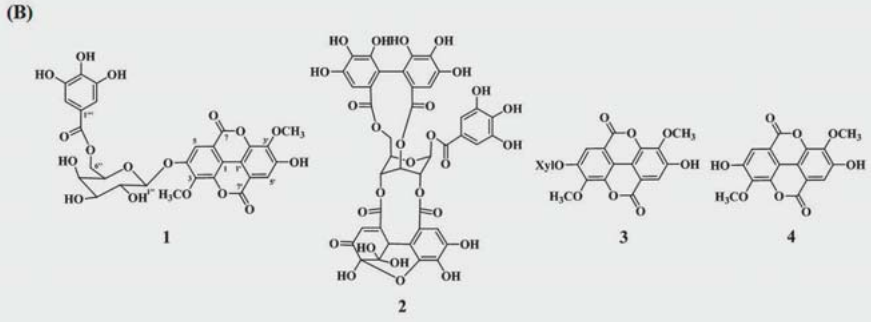
Results and Discussion

The 70% EtOH extract of *E. pekinensis* root that significantly inhibited AGEs formation [IC_{50} = 3.00 µg/mL] was subjected to Diaion HP-20 column chromatography and divided into four fractions (A–D) based on TLC results (▶ **Fig. 1 A**). The AGEs formation inhibitory activity of these fractions was evaluated; fraction C exhibited the strongest activity, with an IC_{50} value of 0.44 µg/mL (▶ **Fig. 1 B**). HPLC analysis of this fraction at 254 nm produced a chromatogram with one small peak (I) and three large peaks (II–IV; ▶ **Fig. 2 A**). Fraction C was further separated by preparative reversed-phase HPLC, leading to the isolation of four compounds (**1**–**4**). Based on the comparison of physicochemical and spectral data on these compounds with those in the literature, three known compounds were identified: geraniin (**2**), 3,3′-di-*O*-methylellagic acid 4-*O*-*β*-D-xylopyranoside (**3**), and ellagic acid 3,3′-dimethyl ether (**4**; ▶ **Fig. 2 B**).

Compound **1** was obtained as an amorphous white powder with the molecular formula $C_{29}H_{24}O_{17}$, as established by HRESIMS, based a molecular ion peak at *m*/*z* 667.0902 $[M+Na]^+$. Characteristic UV absorption maxima (at 250, 280, and 355 nm) suggested the presence of an ellagic acid moiety [14, 15]. ^{1}H- and ^{13}C-NMR data suggested that compound **1** was di-*O*-methylellagic acid monoglycoside with a galloyl group (▶ **Table 1**). The ^{1}H-NMR spectrum exhibited characteristic peaks for two aromatic proton singlets [δH 7.85 and 7.54 (each 1H, s)] of an ellagic acid moiety, a galloyl group [δH 6.86 (2H, s)], two methoxy groups [δH 4.05 and 4.06 (each 3H, s)], and sugar proton signals. The ^{13}C-NMR data, combined with DEPT (distortionless enhancement of polarization transfer) data, exhibited 27 carbon signals, three carbonyl carbons due to two *α*,*β*-unsaturated lactones (δC 158.4 and 158.3) and an ester carbonyl group (δC 165.7), 18 aromatic carbons, two methoxy carbons (δC 61.6 and 61.0), and six carbons for a 6-deoxysugar. The six carbon signals at δC 101.2, 76.1, 73.9, 73.3, 69.6, and 63.1 and an anomeric proton signal at δH 5.28 of **1** were typical of a galactose unit, which was identified as D-galactose by GC analysis of the acid hydrolysate. Moreover, the large coupling constant (*J* = 6.8 Hz) of the anomeric proton indicated that the galactose unit was linked in a *β*-configuration [16]. The complete assignment of the chemical shifts of **1** and its substitution pattern were made by various 2D NMR techniques, such as ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY. The location of two methoxy groups at C-3 and C-3′ on the ellagic acid structure was deduced from the HMBC correlations of δH 4.05 with C-3 (δC 141.7) and δH 4.06 with C-3′ (δC 140.2; ▶ **Fig. 3**). The HMBC correlation between the anomeric proton at δH 5.28 (H-1′′) and C-4 (δC 151.2) clearly indicated that the galactose unit was linked to C-4 of the di-*O*-methylellagic acid structure. This linkage was further confirmed by the observation of an NOE correlation between the anomeric proton (δH 5.28) and H-5 (δH 7.85; ▶ **Fig. 3**). A galloyl group was determined to be adjacent to the 6-position of the galactose moiety by the downfield-shifted H-6′′ protons (δH 4.45 and 4.27). HMBC correlations from the proton H-6′′ of the galactose moiety to C-7′′′ (δC 165.7, carbonyl group) and C-1′′ (δC 119.2) were in accordance with this conclusion. Hence, the structure of **1** was established as 3,3′-di-*O*-methylellagic acid 4-*O*-(6′′-*O*-galloyl)-*β*-D-galactopyranoside.

To assess the effects of the isolated compounds (**1**–**4**) on diabetic complications, the inhibitory effects of these compounds on AGEs formation were examined *in vitro* using commercially avail-





▶ **Fig. 2** HPLC profile (at 254 nm) of the column fraction C from an extract of *E. pekinensis* root (**A**) and chemical structures of isolated compounds (**1**–**4**) from column fraction C (**B**).

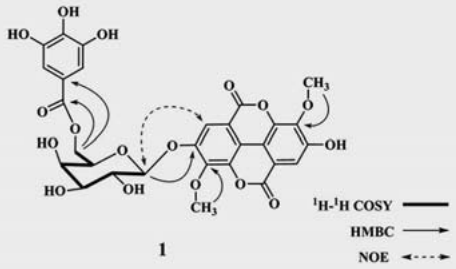
able aminoguanidine, the first AGEs inhibitor used for the treatment of diabetic nephropathy [17], and quercetin, a natural compound, as positive controls. As shown in ▶ **Table 2**, all tested compounds exhibited considerable inhibition of AGEs formation, with IC_{50} values ranging from 0.41 to 12.33 µM, compared with those of aminoguanidine (IC_{50} = 1122.34 µM) and quercetin (IC_{50} = 27.80 µM). Of the compounds tested, geraniin (**2**), a hydrolyzable tannin, was the strongest inhibitor of AGE formation, with an IC_{50} value of 0.41 µM. Its activity was 20 times more effective than that of the other isolates (**1**, **3**, and **4**), which contained ellagic acid moieties, suggesting that garaniin contributes to a major portion of the AGEs formation inhibitory effect of the active column fraction A3 from an extract of *E. pekinensis* root.

Retinal vessel dilatation is a clinically and pathologically noted retinal change associated with diabetic retinopathy [18]. Thus, compounds **2** and **4**, which potently inhibited AGEs formation *in vitro*, were examined to determine their potential effects on retinopathy *in vivo* in a diabetic zebrafish model. In this study, the change in hyaloid-retinal vessel dilation was assessed in transgenic zebrafish embryos expressing enhanced green fluorescent protein (EGFP) in the vasculature under HG conditions. The toxic effects on zebrafish were examined initially, and the results indicated that compounds **2** and **4** were not toxic at concentrations up to 20 µM (data not shown). Thus, to evaluate the effects of **2** and **4** on the HG-induced dilation of hyaloid-retinal vessels, *flk:*EGFP transgenic zebrafish embryos were treated with these compounds at concentrations of 5, 10, and 20 µM. Both compounds significantly reduced the diameters of hyaloid-retinal vessels with HG-induced dilation in a concentration-dependent manner compared with the HG-treated control group (▶ **Fig. 4**). At the highest concentration used (20 µM), these compounds reduced the diameters of hyaloid-retinal vessels with HG-induced dilation by about 82% and 84%, respectively, relative to the HG-treated control group. Treatment with a vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor, VEGFR-2/Flt3/c-Kit inhibitor, at a concentration of 1 µM, used as a positive control, reduced HG-induced dilation of hyaloid-retinal vessels by 77%.

Chronic hyperglycemia plays a crucial role in the initiation of diabetic complications through various hyperglycemia-induced metabolic and hemodynamic derangements [19–21]. Of various metabolic derangements implicated in the pathogenesis of diabetic complications, the accumulation of AGEs, heterogeneous molecules derived from non-enzymatic glycation between amino acid residues and oxidative derivatives of glucose or pentose, are among the most important. AGEs induce irreversible structural and functional changes in proteins, such as collagen, elastin, and albumin. These changes are believed to contribute, in part, to the development and progression of diabetic complications [22]. Due to these potential roles of AGEs in diabetic complications, the development and investigation of AGE inhibitors, particularly natural anti-AGE agents without side effects, may provide insight leading to the development of a therapeutic approach to delay or prevent diabetic complications [23]. Several studies have been conducted to identify medical herbs and dietary plants that inhibit protein glycation. These plants are rich in flavonoids or polyphenolic substances, which are reported to reduce AGEs formation [24–26]. In the current study, which sought to identify effective agents for diabetic complications derived from *E. pekinensis* root, several poly-

▶ **Table 1** ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) and HMBC data for **1** (in DMSO-d_6).

C	δ_c	δ_H (*J* in Hz)	HMBC (H → C)
1	114.4		
2	141.0		
3	141.7		
4	151.2		
5	112.1	7.85 s	112.8, 114.4, 141.0, 141.7, 151.2, 158.3
6	112.8		
7	158.3		
1′	111.1		
2′	141.9		
3′	140.2		
4′	152.9		
5′	111.6	7.54 s	111.1, 112.1, 140.2, 141.9, 152.9, 158.4
6′	112.1		
7′	158.4		
1′′	101.2	5.28 d (6.8)	73.3, 151.2
2′′	73.3	3.87	73.9, 101.2
3′′	73.9	3.79	69.6, 73.3
4′′	69.6	3.41	73.9, 76.1
5′′	76.1	3.40	63.1, 69.6
6′′	63.1	4.27 dd (5.2, 12.0), 4.45 dd (2.1, 12.0)	76.1, 119.2, 165.7
1′′′	119.2		
2′′′	108.6	6.86 s	119.2, 138.4, 145.4, 165.7
3′′′	145.4		
4′′′	138.4		
5′′′	145.4		
6′′′	108.6	6.86 s	119.2, 138.4, 145.4, 165.7
7′′′	165.7		
3-OMe	61.6	4.06 s	61.6
3′-OMe	61.0	4.05 s	61.0



▶ **Fig. 3** Key ^{1}H-^{1}H COSY, HMBC, and NOE correlations of compound **1**.

▶ **Table 2** Inhibitory effects of compounds **1**–**4** against AGEs formation.

Compounds	IC_{50} (95% CI) μM[a]
1	10.23 (9.64–10.82)
2	0.41 (0.40–0.42)
3	12.33 (12.06–12.60)
4	8.94 (7.98–9.90)
Aminoguanidine[b]	1122.34 (1112.45–1132.21)
Quercetin[b]	27.80 (26.52–29.08)

[a] Results are expressed as IC_{50} values and 95% confidence intervals (95% CI) and IC_{50} indicates the concentration (μM) at which the inhibition percentage of the AGEs formation was 50%. The values were determined by regression analysis. [b] Positive controls.

phenols were isolated from an active column fraction of an extract of this root. Although *E. pekinensis* is classified as a toxic herb in Chinese medicine, reports suggest that this toxicity is associated with the presence of many diterpenoids in this plant [10–13]. Thus, our results suggest that active polyphenols or a polyphenol-rich column fraction of an extract of *E. pekinensis* root would have beneficial uses in the development of therapeutic and/or preventive agents for diabetic vascular complications and other related diseases.

Materials and Methods

General experimental procedures

Optical rotations were measured using the JASCO P-2000 digital polarimeter. UV spectrum was recorded using the JASCO V-550 UV/VIS spectrometer. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained using the Bruker DRX-400 spectrometer with tetramethylsilane as an internal standard. 2D NMR experiments (COSY, HSQC, HMBC, and NOESY) were performed on the Bruker Avance 500 NMR spectrometer. HRESIMS was performed using the Shimadzu LCMS-IT-TOF spectrometer. Column chromatography was performed using Diaion HP-20 (Supelco). TLC was performed on pre-coated silica gel 60 F254 (0.25 mm, Merck) and RP-18 F254s plates (0.25 mm, Merck). Spots were detected by UV light (254 nm) and spraying of 10% H_2SO_4 followed by heating. The HPLC analysis was performed on an Agilent 1200 HPLC system with a binary pump (G1312A), a vacuum degasser (G1322A), a thermostatted column compartment (G1316A), a multiple wavelength detector (G1365B, MWD), and an autosampler (G1329A), using a Luna C18 (250 × 10 mm, 5.0 μm, Phenomenex) column.

Plant material

The roots of *E. pekinensis* were purchased from Namseongdang herbal medicine store (Daegu, Republic of Korea). The origin of the herbal medicine was identified by Prof. J. H. Kim, Gachon University, Republic of Korea, and a voucher specimen (KIOM-EUPE) has been deposited in the Herbarium of Korea Institute of Oriental Medicine, Republic of Korea.

Extraction, fractionation, and isolation

The air-dried roots of *E. pekinensis* (1 kg) were extracted using 70% aqueous EtOH (three times, with 10 L each time) at room temperature for 7 d, filtered, and concentrated to yield a 70% EtOH extract (216 g, 21.6% yield). The extract (20 g) was subjected to Diaion HP-20 column chromatography (60 × 10 cm) and eluted with a gradient solvent system consisting of (A) MeOH and (B) water: 100% B (2 L), 80% B (2 L), 60% B (2 L), 40% B (2 L), 20% B (2 L), and 100% A (2 L). The resulting portions from column chromatographic separation were combined into four fractions (A, 8.2 g; B, 3.3 g; C, 6.1 g; D, 1.8 g) based on TLC results. In this bioassay-guided study, the most active column fraction A3 was further purified by extensive preparative reversed-phase HPLC [Agilent 1200 HPLC system; Luna C18 column (250 × 10 mm, 5.0 µm), Phenomenex; gradient from 20–60% MeOH in water over 50 min; UV detection, 254 nm; flow rate, 2.5 mL/min] to obtain compounds **1** (tR: 29.78 min, 10 mg), **2** (tR: 31.42 min, 180 mg), **3** (tR: 36.36 min, 105 mg), and **4** (tR: 40.94 min, 42 mg). The purity of the isolated compounds ranged from 98.0% to 99.5%, as determined by analytical HPLC [Agilent 1200 HPLC system, Luna C18 column (250 × 4.6 mm, 5.0 µm), Phenomenex; 40% acetonitrile in water; UV detection, 254 nm; flow rate, 1.0 mL/min].

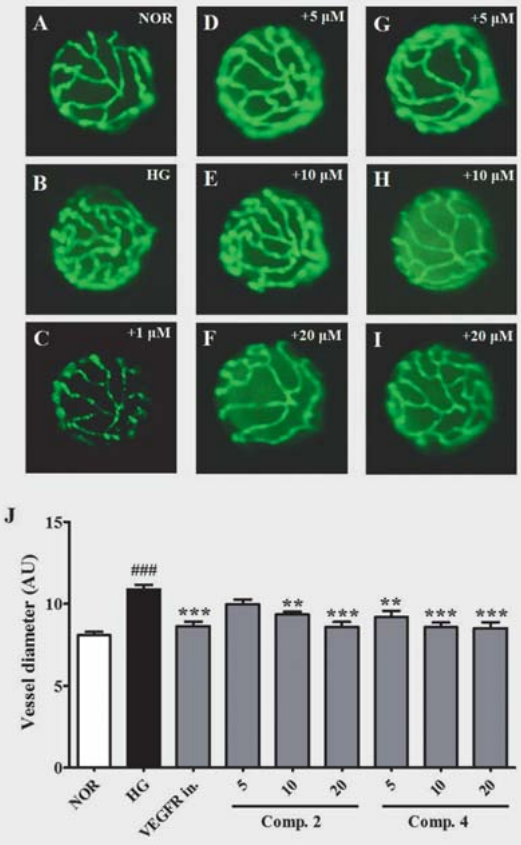
3,3′-Di-O-methylellagic acid 4-O-(6′′-O-galloyl)-β-D-galactopyranoside (**1**): white powder; $[\alpha]_D^{25}$ + 26.0 (*c* 0.1, MeOH); UV (MeOH) λmax 250, 280, and 355 nm; ^{1}H- and ^{13}C-NMR, see ▶ **Table 1**; HRESIMS *m/z* 667.0902 $[M+Na]^+$ (calcd for $C_{29}H_{24}O_{17}Na^+$, 667.0906).

Acid hydrolysis of compound 1

Compound **1** (2 mg) in 10% HCl/dioxane (1 : 1, 1 mL) was heated separately at 80 °C for 3 h in a water bath. The mixture was neutralized with Ag_2CO_3, filtered, and then extracted with ethyl acetate (20 mL). The aqueous layer was evaporated, and the residue was treated with L-cysteine methyl ester hydrochloride (2 mg) in pyridine (0.5 mL) at 60 °C for 1 h. After the reaction was complete, the solution was treated with acetic anhydride (3 mL) at 60 °C for 1 h. Authentic sample was prepared using the same procedure. The acetate derivatives were then subjected to GC analysis using a GC-2010 device (Shimadzu) with a flame ionization detector and TC-1 capillary column (0.25 mm × 30 m; GL Sciences, Inc.) using the following parameters: column temperature, 230 °C; programmed increase, 38 °C/min; carrier gas, N_2 (1 mL/min); and injection and detector temperature, 270 °C. The sugar derivative thus obtained showed a retention time of 17.30 min, identical to that of authentic D-galactose.

Inhibition of AGEs formation assay

In accordance with a well-established method, a reaction mixture of BSA (10 mg/mL, 700 µL; Sigma) in 50 mM phosphate buffer (pH 7.4) with 0.02% sodium azide (Sigma) was added to 0.2 M fructose and glucose (100 µL). In screw-cap tubes (1.5 mL), the reaction mixture was then mixed with 200 µL of serially diluted compounds or aminoguanidine (99% purity; Sigma) or quercetin (99.5% Purity; Sigma). After incubating at 37 °C for 7 d, the fluorescent reaction products (200 µL) were transferred to 96-well plates and assayed on a spectrofluorometric detector (Synergy HT; excitation wavelength, 350 nm, emission wavelength, 450 nm). The AGEs assay was performed in triplicate. The IC_{50} was estimated from the least-squares regression line of the logarithmic concentration plotted against remaining activity using the GraphPad 5.0 Prism software.



▶ **Fig. 4** Effects of compounds **2** and **4** on dilation of hyaloid-retinal vessels in a HG-induced diabetic retinopathy model. Hyaloid-retinal vessels of *flk*:EGFP transgenic zebrafish from the following groups: **A** untreated normal (NOR); **B** HG-treated control (HG); **C** HG-treated specimens receiving 1 µM VEGFR-2/Flt3/c-Kit inhibitor (VEGFR in.); HG-treated specimens receiving (**D**) 5 µM, (**E**) 10 µM, and (**F**) 20 µM compound **2**; and HG-treated specimens receiving (**G**) 5 µM, (**H**) 10 µM, and (**I**) 20 µM compound **4**. **J** Data are displayed as mean artificial units (AU) for vessel diameters. The diameters of hyaloid-retinal vessels were measured at locations proximal to the optic disc (red circle). Scale bar = 50 µm. The hyaloid vessel diameter of each lens was measured three times and the experiment was performed in triplicate. ### $p < 0.001$ vs. NOR, ** $p < 0.01$ vs. HG, *** $p < 0.001$ vs. HG.

Measurement of vessel dilation in larval zebrafish

All experimental protocols for animal care and use were approved by the local ethics board (South Korean Institute of Oriental Medicine Animal Care and Use Committee) Committee (Approval date and number; April 20, 2015 and 15–020, respectively), and animal husbandry and procedures were performed in accordance with institutional guidelines. Adult zebrafish were maintained under standard conditions at 28.5 °C under a 14-h light/10-h dark

cycle. Embryos were obtained from crosses between *flk*:EGFP transgenic fish and raised in egg water (sea salt, 0.06 g/L). One-day-old *flk*:EGFP embryos were placed in a 24-well plate (five embryos per well) and maintained in 2 mL egg water containing 130 mM glucose. HG-induced embryos were treated with **2** and **4** from 1 to 6 d post-fertilization (dpf). At 6 dpf, HG-induced embryos were fixed with 4% paraformaldehyde, and the lens containing the hyaloid-retinal vessels was isolated. Fluorescence was visualized using the Olympus SZX16 stereomicroscope, and diameters were measured using ImageJ software. Experiments were performed in triplicate. VEGFR-2/Flt3/c-Kit inhibitor (99.4% purity; Calbiochem), a VEGFR tyrosine kinase inhibitor, was used as a positive control.

Statistical analysis

Hyaloid vessel diameter was used to calculate the percentage inhibition of sample in HG-treated embryos according to the following formula:

$$\text{Percentage inhibition (\%)} = 100 - [(T - C)/(HG - C)] \times 100$$

where C = hyaloid vessel diameter (AU) of the control embryos; HG = hyaloid vessel diameter (AU) of the HG-treated embryos; and T = hyaloid vessel diameter (AU) of the HG-treated embryos treated with sample. The hyaloid vessel diameter of each lens was measured three times and the experiment was performed in triplicate. Statistical significance was assessed using one-way analysis of variance and Dunnett's multiple comparison tests with the GraphPad 5.0 Prism software.

Acknowledgements

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Conflict of Interest

No conflicts of interest exist.

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