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Proanthocyanidins from *Spenceria ramalana* and Their Effects on AGE Formation in Vitro and Hyaloid-Retinal Vessel Dilation in Larval Zebrafish in Vivo

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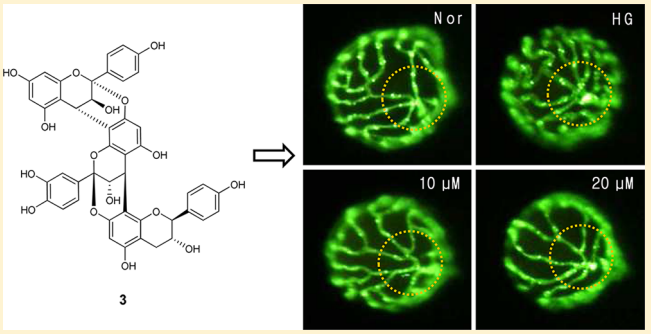
Supporting Information

ABSTRACT: Three new A-type proanthocyanidins (**1**−**3**), *ent*-epiafzelechin-(2α→*O*→7,4α→8)-*ent*-afzelechin 3′-*O*-β-D-glycopyranoside (**1**), *ent*-epiafzelechin-(2α→*O*→7,4α→8)-*ent*-epiafzelechin-(2α→*O*→7,4α→8)-*ent*-afzelechin (**2**), and *ent*-epiafzelechin-(2α→*O*→7,4α→8)-*ent*-epicatechin-(2α→*O*→7,4α→8)-*ent*-afzelechin (**3**), and three known compounds (**4**−**6**) were isolated from the whole plant of *Spenceria ramalana*. The structures of the new proanthocyanidins were established by spectroscopic and chemical studies. The inhibitory effects of compounds **1**−**6** on the formation of advanced glycation end products were examined in vitro. Compounds **3** and **6** showed the strongest inhibition, with IC_{50} values of 17.4 ± 0.5 and 14.1 ± 1.6 μM, respectively. The effects of these isolates on the dilation of hyaloid-retinal vessels induced by high glucose (HG) in larval zebrafish were also investigated. Compound **3** reduced the dilation of HG-induced hyaloid-retinal vessels most effectively. This compound reduced the diameters of HG-induced hyaloid-retinal vessels by about 157.7% and 164.1% at 10 and 20 μM, respectively, versus the HG-treated control group.



Diabetic vascular complications are leading causes of end-stage renal failure, acquired blindness, various neuropathies, and accelerated atherosclerosis. Chronic hyperglycemia plays a major role in the initiation of diabetic vascular complications through various hyperglycemia-induced metabolic and hemodynamic derangements, including increased advanced glycation end-product (AGE) formation, increases in the polyol pathway, activation of protein kinase C isomers, and increases in the hexosamine pathway.[1−4] Of these factors implicated in the pathogenesis of diabetic vascular complications, enhanced formation and accumulation of AGE, heterogeneous molecules derived from nonenzymatic glycation between amino acid residues and oxidative derivatives of glucose or pentose, is irreversible, causing structural and functional changes in proteins, such as collagen, elastin, and albumin, leading to the development of diabetic vascular complications, including diabetic retinopathy, neuropathy, and nephropathy.[5] Because of the potential role of AGE in diabetic vascular complications, the development of pharmacological compounds that inhibit the formation of these glycated products may provide a therapeutic approach for delaying and preventing diabetic vascular complications.

Spenceria ramalana Trimen, a species in the genus *Spenceria*, belonging to the family Rosaceae, is a perennial herb, native to China, distributed in a large cross-section of Shanyu Tibet, Yunnan, and Sichuan. It has been used in traditional folk medicine in China for the treatment of various ailments, including lumbago, stomachache, and dysentery.[6] To date, there has been no study of the chemical constituents or biological activities of this plant. In searching for new AGE inhibitors from natural sources, we found that the EtOAc-soluble fraction of the 80% EtOH extract of the whole plant of *S. ramalana* had a considerable inhibitory effect on AGE formation (IC_{50} = 19.92 ± 0.13 μg/mL). Further phytochemical study of this fraction resulted in the isolation of three new (**1**−**3**) and three known dimeric A-type proanthocyanidins (**4**−**6**). In this report, we describe the isolation and structural elucidation of these compounds, as well as the characterization of their inhibitory effects on AGE formation. The effects of these isolates on the dilation of high-glucose (HG)-induced hyaloid-retinal vessels in larval zebrafish were also investigated.

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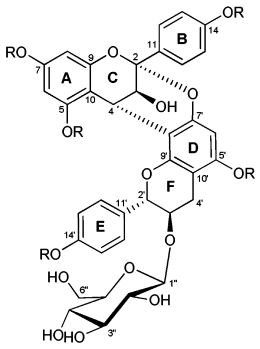

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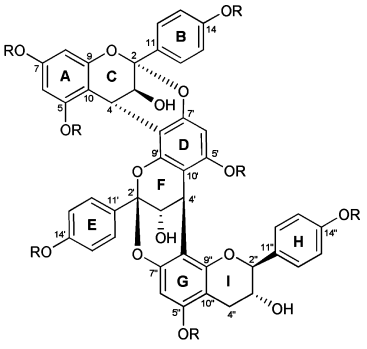

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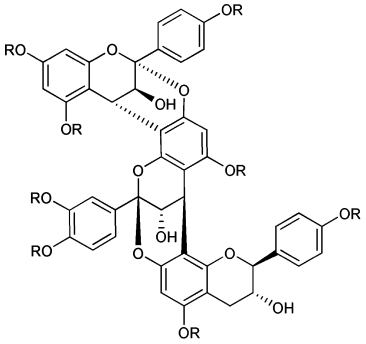

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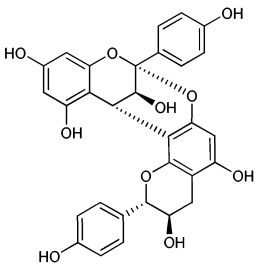

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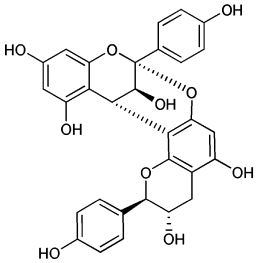

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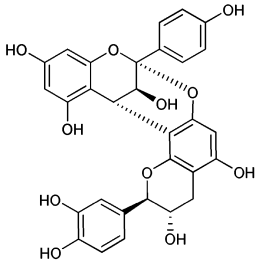

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RESULTS AND DISCUSSION

An 80% (v/v) EtOH extract of the whole *S. ramalana* plant was suspended in H_2O and successively partitioned using *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc-soluble fraction was subjected to a series of chromatographic steps, resulting in the isolation of six proanthocyanidins (**1**–**6**). Of these, three were identified as *ent*-epiafzelechin-(2*α*→*O*→7,4*α*→8)-*ent*-afzelechin (**4**),[7] *ent*-epiafzelechin-(2*α*→*O*→7,4*α*→8)-afzelechin (**5**),[8] and *ent*-epiafzelechin-(2*α*→*O*→7,4*α*→8)-catechin (**6**),[9] by comparison of observed and published physicochemical data.

Compound **1** was obtained as a white powder, with the molecular formula $C_{36}H_{34}O_{15}$, as established by HRESIMS, based on the $[M + H]^+$ peak (*m*/*z* 707.1970). The IR spectrum exhibited absorption bands for hydroxy (3450 cm^{-1}) and aromatic (1610 and 1520 cm^{-1}) functional groups. A positive vanillin–sulfuric acid test and a UV absorption maxima observed at 272 nm suggested the presence of a flavan structure in **1**.[10] The ^1H NMR spectrum (Table 1) of **1** exhibited characteristic peaks for two *meta*-coupled doublets [δ_H 6.07 and 5.96 (each d, J = 2.2 Hz)], an aromatic singlet (δ_H 6.09), and two pairs of A_2B_2 aromatic system protons [δ_H 7.51 and 6.83 (each d, J = 9.0 Hz) and δ_H 7.34 and 6.84 (each d, J = 8.4 Hz)]. Additionally, an isolated AB system [δ_H 4.15 and 4.31 (each d, J = 3.6 Hz)] in the heterocyclic proton region was observed, characteristic of the C-ring protons of A-type proanthocyanidins, consisting of two flavanyl units.[11] Such a structural type was supported by the typical acetal carbon resonance at δ_C 100.7 in the ^{13}C NMR spectrum (Table 1). The interflavonoid linkage between the two flavanyl units was established by methylation of **1** with diazomethane, yielding pentamethyl ether (**1a**). The ^1H NMR spectrum of **1a** exhibited, together with four methoxy signals at δ_H 3.72–3.82, one upfield methoxy signal at δ_H 3.31 assignable to MeO-5 due to the anisotropic shielding of the E-ring in the lower flavanyl unit.[12,13] This suggests that the two flavanyl units were linked through the C-4 and C-8′ positions. The existence of a (2→O→7′) rather than a (2→O→5′) ether linkage was deduced from the NOE correlation between a methoxy signal at δ_H 3.75 (3H, s, MeO-5′) and H-4′ methylene signals (δ_H 2.72–2.92, F-ring) in **1a**. The (4→8′, 2→O→7′) interflavonoid linkage of **1** was further supported by observation of the HMBC cross-peaks of H-3 with C-8′ and of H-4 with C-7′, C-8′, and C-9′ (Figure 1). The absolute configuration at C-4 on the heterocyclic C-ring was determined from the sign of the Cotton effect in the region 220–240 nm.[14] A negative Cotton effect around 230 nm ($[\Theta]_{232}$ −9230) in the electronic circular dichroism (ECD) spectrum of **1** leads to assignment of a 4*S*-configuration of the C-ring, thus confirming the (2*α*,4*α*)-configuration of the C-ring because the linkage of the two units of A-type proanthocyanidins must be *cis*.[15] The NOESY cross-peak between H-3 (δ_H 4.15, C-ring) and H-6′ (δ_H 5.96, D-ring) indicated the 3,4-*trans* configuration of the C-ring (Figure 2). On the basis of these data, the absolute configuration of the C-ring was deduced to be 2*R*,3*S*,4*S*, and thus the upper flavanyl unit of **1** was identified as *ent*-epiafzelechin. The absolute configuration at C-2′ of the F-ring was established from the ECD spectrum. Flavan-3-ols with 2*R* and 2*S* absolute configuration give rise to negative and positive Cotton effects, respectively, in the 260–280 nm region of their ECD spectra.[16] Thus, the weak positive Cotton effect around 270 nm ($[\Theta]_{271}$ +580) observed in **1** indicated that the absolute configuration at C-2 of the F-ring differed from that of the C-ring, indicating a 2*S*-configuration of the F-ring.[7,15] The large coupling constant (J = 8.0 Hz) between H-2′ and H-3′ of the F-ring reflected a relative *trans*-configuration.[17] Consequently, the absolute configurations at C-2 and C-3 of the F-ring were deduced to be *S* and *R*, respectively, and the lower flavanyl unit of **1** was thus identified as *ent*-afzelechin. The carbon signals at δ_C 103.9, 78.2, 77.8, 75.4, 71.7, and 62.9 and an anomeric proton signal at δ_H 4.12 in **1** were typical of a glucopyranosyl unit. Acid hydrolysis of **1** yielded **4** as the aglycone and a monosaccharide unit identified as D-glucose upon GC analysis. When the ^{13}C NMR spectrum of **1** was compared with that of **4**, a downfield shift was observed for C-3′ of the F-ring by about 7 ppm, indicating the linkage position of a glucopyranosyl moiety at C-3′ of the lower flavanyl unit, further supported by the HMBC cross-peak between the anomeric proton (δ_H 4.12) and the aglycone carbon (δ_C 75.3) for C-3 of the F-ring (Figure 1). Moreover, the large coupling constant (J = 7.5 Hz) of the anomeric proton indicated that the glucopyranosyl unit was linked in a *β*-configuration. Thus, the structure of **1** was determined as *ent*-epiafzelechin-(2*α*→*O*→7,4*α*→8)-*ent*-afzelechin 3′-*O*-*β*-D-glycopyranoside.

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Table 1. NMR Data for Compounds 1−3 in Methanol-d_4 (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR)

	1		2		3	
C(ring)	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2(C)		100.7		101.5		101.5
3(C)	4.15 d (3.6)	67.8	4.28 d (3.5)	67.3	4.28 d (3.5)	68.2
4(C)	4.31 d (3.6)	29.4	4.37 d (3.5)	29.8	4.38 d (3.5)	29.8
5(A)		156.8		157.0		156.9
6(A)	5.96 d (2.2)	98.3	5.92 d (2.2)	98.6	5.93 d (2.2)	98.6
7(A)		158.4		158.5		158.5
8(A)	6.07 d (2.2)	96.8	6.04 d (2.2)	96.8	6.04 d (2.2)	96.8
9(A)		154.3		154.2		154.2
10(A)		104.2		103.6		103.6
11(B)		131.8		131.0		131.0
12(B)	7.51 d (9.0)	129.7	7.67 d (9.0)	129.7	7.67 d (9.0)	129.7
13(B)	6.83 d (9.0)	115.7	6.90 d (9.0)	116.0	6.91 d (9.0)	116.0
14(B)		159.1		159.5		159.5
15(B)	6.83 d (9.0)	115.7	6.90 d (9.0)	116.0	6.91 d (9.0)	116.0
16(B)	7.51 d (9.0)	129.7	7.67 d (9.0)	129.7	7.67 d (9.0)	129.7
2′(F)	5.15 d (8.0)	81.4		100.8		100.8
3′(F)	4.38 ddd (5.6, 8.0, 8.8)	75.3	4.18 d (3.5)	67.6	4.18 d (3.5)	67.6
4′(F)	ax:[a] 2.78 dd (8.8, 16.4) eq:[b] 2.87 dd (5.6, 16.4)	26.9	4.36 d (3.5)	29.6	4.36 d (3.5)	29.6
5′(D)		156.2		158.5		158.4
6′(D)	6.09 s	96.9	6.11 s	96.9	6.11 s	96.9
7′(D)		152.5		154.0		154.0
8′(D)		106.8		108.7		108.6
9′(D)		150.6		147.1		147.1
10′(D)		102.9		103.4		103.5
11′(E)		130.4		131.6		131.6
12′(E)	7.34 d (8.5)	129.5	7.52 d (9.0)	129.7	6.95 d (2.1)	115.5
13′(E)	6.84 d (8.5)	116.6	6.84 d (9.0)	115.7		146.9
14′(E)		159.0		159.2		146.6
15′(E)	6.84 d (8.5)	116.6	6.84 d (9.0)	115.7	6.83 d (6.8)	116.5
16′(E)	7.34 d (8.5)	129.5	7.52 d (9.0)	129.7	6.84 dd (2.1, 6.8)	120.3
2″(I)			4.78 d (8.0)	84.0	4.78 d (8.0)	83.9
3″(I)			4.12 ddd (5.5, 8.0, 9.0)	68.3	4.12 ddd (5.5, 8.0, 9.0)	67.3
4″(I)			eq: 2.60 dd (9.0, 16.5) ax: 3.06 dd (5.5, 16.5)	29.5	eq: 2.60 dd (9.0, 16.5) ax: 3.06 dd (5.5, 16.5)	29.5
5″(G)				157.1		157.0
6″(G)			5.91 s	98.6	5.91 s	98.6
7″(G)				150.2		150.2
8″(G)				108.5		108.5
9″(G)				149.8		149.6
10″(G)				103.9		103.7
11″(H)				130.3		131.0
12″(H)			7.35 d (8.5)	130.1	7.52 d (8.5)	129.7
13″(H)			6.86 d (8.5)	116.5	6.84 d (8.5)	115.7
14″(H)				159.1		159.2
15″(H)			6.86 d (8.5)	116.5	6.84 d (8.5)	115.7
16″(H)			7.35 d (8.5)	130.1	7.52 d (8.5)	129.7
Glc-1	4.12 d (7.5)	103.9				
Glc-2	3.10	75.4				
Glc-3	3.18	78.2				
Glc-4	3.22	71.7				
Glc-5	3.23	77.8				
Glc-6	3.86, 3.64	62.9				

[a]Axial proton. [b]Equatorial proton.

Compound **2** was obtained as a white powder and gave a positive result in the vanillin−sulfuric acid test. Its molecular formula, $C_{45}H_{34}O_{15}$, was determined on the basis of its $[M-H]^-$ peak (m/z 813.1826) in the HRESIMS and NMR data, suggesting the presence of a triflavonoid structure. The ^{1}H NMR spectrum (Table 1) was similar to those of **4** and **5**, except that **2** had additional signals consistent with the presence of a flavanyl unit [δ_H 7.35 and 6.86 (each d, J = 8.5

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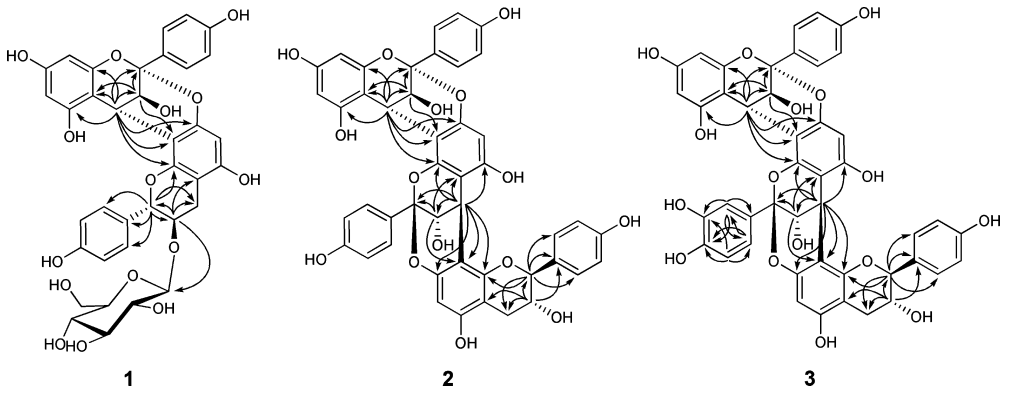

Figure 1. Key HMBC (H→C) correlations of compounds **1**–**3**.

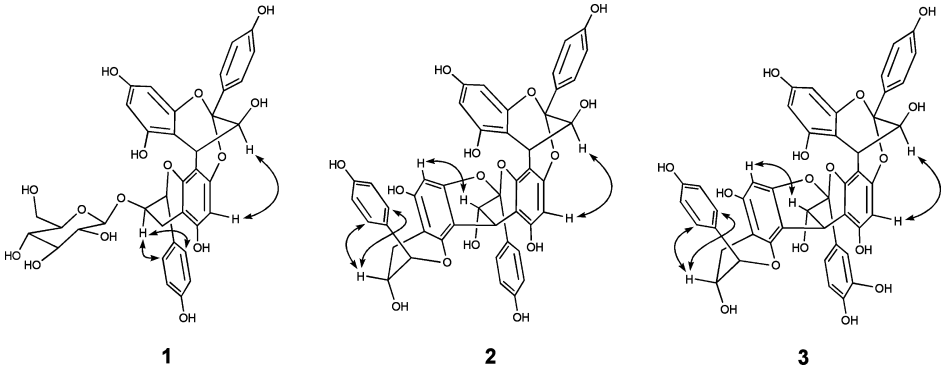

Figure 2. Key NOE (H↔H) correlations of compounds **1**–**3**.

Hz) and 5.91 (s) in the aromatic region; δ_H 4.78 (d, J = 8.0 Hz), 4.12 (ddd, J = 5.5, 8.0, 9.0 Hz), 3.06 (dd, J = 5.5, 16.5 Hz), and 2.60 (dd, J = 9.0, 16.5 Hz) in the heterocyclic region], suggesting that **2** is a trimeric A-type proanthocyanidin.[18] The two doubly linked units were also demonstrated by the typical acetal carbon resonances at δ_C 101.5 and 100.8 in the ^{13}C NMR spectrum (Table 1). The methylation of **2** with diazomethane yielded heptamethyl ether (**2a**). The ^{1}H NMR spectrum of **2a** exhibited two upfield methoxy signals (δ_H 3.33 and 3.40), assignable to the methoxy groups at C-5 (A-ring) and C-5′ (D-ring), together with five methoxy signals at δ_H 3.70–3.83. The unusual upfield shifts of the two methoxy groups indicated the presence of a through-space interaction with the E- and H-rings,[12,13] suggesting that the two interflavonoid C–C linkages were (4→8′) and (4′→8″). The NOESY cross-peaks between a methoxy signal at δ_H 3.40 (3H, s, MeO-5′) and H-4′ (δ_H 4.30, F-ring) and between a methoxy signal at δ_H 3.70 (3H, s, MeO-5″) and H-4″ (δ_H 2.58–3.10, I-ring) of **2a** indicated the presence of (2→O→7′) and (2′→O→7″) ether linkages. The unsubstituted phloroglucinol A-ring carbons C-6 (δ_C 98.6) and C-8 (δ_C 96.8), C-6 (δ_C 96.9) of the D-ring, and C-6 (δ_C 98.6) of the G-ring in **2**, being similar to those of geranin D isolated from *Geranium niveum*,[19] further supported that the interflavonoid linkage between the three flavanyl units was (4→8, 2→O→7′) and (4′→8″, 2′→O→7″). Although the ^{1}H and ^{13}C NMR spectra of **2** were found to be identical to those of geranin D, **2** showed a Cotton effect with opposite sign around 240 nm ($[\Theta]_{240}$ −5820) relative to that of geranin D ($[\Theta]_{236}$ +7760), indicating that the C- and F-rings had 4*S*-configurations in **2**. The absolute configurations at C-2 of the C-ring and C-2′ of the F-ring were thus automatically assigned as *R* and *S*, respectively. Furthermore, the NOESY cross-peaks between H-3 (δ_H 4.15, C-ring) and H-6′ (δ_H 6.11, D-ring) and between H-3′ (δ_H 4.15, F-ring) and H-6″ (δ_H 5.91, G-ring) indicated the 3,4-*trans* configurations of the C- and F-rings (Figure 2). Thus, both the upper and middle flavanyl units of **2** were identified as *ent*-epiafzelechin. The absolute configuration at C-2‴ of the I-ring was determined from the sign of the Cotton effect observed in the 260–280 nm region of the ECD spectrum of **2**. Compared to **1**, the sign of the Cotton effect around 270 nm was identical, but the amplitude in **2** ($[\Theta]_{271}$ +820) was larger than that in **1**, $[\Theta]_{271}$ +580. This indicates a 2*S*-configuration of the I-ring of **2** because the absolute configurations at C-2 of the C-ring and C-2′ of the F-ring of **2** are identical to those of **1**. Because H-2″ and H-3″ of the I-ring were *trans*-oriented ($J_{2,3}$ = 8.0 Hz), the lower flavanyl unit was characterized as *ent*-afzelechin. Thus, the structure of **2** was determined to be *ent*-epiafzelechin-(2α→*O*→7,4α→8)-*ent*-epiafzelechin-(2α→*O*→7,4α→8)]-*ent*-afzelechin.

Compound **3** gave a positive result in the vanillin–sulfuric acid test and showed a molecular formula of $C_{45}H_{34}O_{16}$, from its $[M - H]^-$ peak (m/z 829.1771) in the HRESIMS, which was assumed to be a trimeric flavonoid.[17] The ^{1}H NMR spectrum (Table 1) displayed two isolated AB systems [δ_H 4.28, 4.38 and 4.18, 4.36 (each d, J = 3.5 Hz)] in the heterocyclic proton region, ascribed as a diagnostic feature of the C- and F-ring protons of A-type proanthocyanidins,[15] indicating the presence of two doubly linked flavanyl units. The location of the interflavanyl linkage of **3** was established by analysis of the ^{1}H NMR spectrum and NOE correlations of the corresponding octamethyl ether (**3a**). The two upfield methoxy signals (δ_H 3.31 and 3.38), assignable to the methoxy groups at C-5 (A-ring) and C-5′ (D-ring), and the NOE correlations of a methoxy signal at δ_H 3.38 (3H, s, MeO-5′) with H-4′ (δ_H 4.30,

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F-ring) and a methoxy signal at δ_H 3.78 (3H, s, MeO-5″) with H-4″ (δ_H 2.60−3.11, I-ring) indicated, as for **2**, the presence of (4→8, 2→O→7′) and (4′→8″, 2′→O→7″) interflavanyl linkages in **3**. This was confirmed by the unsubstituted phloroglucinol A-ring carbons C-6 (δ_C 98.6) and C-8 (δ_C 96.8), C-6 (δ_C 96.9) of the D-ring, and C-6 (δ_C 98.6) of the G-ring in **3**. The ^{1}H NMR features of **3** were similar to those of **2**, but there were marked differences between **3** and **2** in the chemical shifts and splitting patterns of the aromatic protons of the E-ring; an AMX system [δ_H 6.83 (d, J = 6.8 Hz), 6.84 (dd, J = 2.1, 6.8 Hz), and 6.95 (dd, J = 2.1 Hz)] was observed in **3**, instead of an A_2B_2 system in **2**. Thus, **3** differed from **2** in the substitution pattern of the E-ring, substantiated by the ^{13}C NMR chemical shifts of C-4′ (δ_C 146.6) and C-3′ (δ_C 146.9) of the E-ring, as well as analysis of the HMBC spectrum (Figure 1). The absolute configuration of **3** was assigned as being identical to that of **2** on the basis of the Cotton effects and NOE correlations (Figure 2). Thus, the upper and middle flavanyl units of **3** were identified as *ent*-epiafzelechin and *ent*-epicatechin, respectively, and the lower flavanyl unit was characterized as *ent*-afzelechin, due to a positive Cotton effect around 272 nm ($[\Theta]_{272}$ +780) in the ECD spectrum and the *trans* relationship ($J_{2,3}$ = 8.0 Hz) between H-2 and H-3 of the I-ring. Consequently, the structure of **3** was assigned as *ent*-epiafzelechin-(2α→O→7,4α→8)-*ent*-epicatechin-(2α→O→7,4α→8)-*ent*-afzelechin.

The inhibitory effects of the isolated compounds (**1**−**6**) on AGE formation were examined in vitro according to a previously described procedure[20] using commercially available aminoguanidine, the first AGE inhibitor for the treatment of diabetic nephropathy,[21] as a positive control. As shown in Table 2, all of the tested compounds exhibited considerable inhibition of AGE formation, with IC_{50} values ranging from 14.1 to 39.8 μM, compared with that of aminoguanidine (IC_{50} = 965.9 ± 15.5 μM). Of the tested compounds, compounds **3** and **6**, both of which have a catechol unit in the molecule, were the strongest inhibitors of AGE, with IC_{50} values of 17.4 ± 0.5 and 14.1 ± 1.6 μM, respectively, and their activities were approximately twice those of the other isolates (**1**, **2**, **4**, and **5**) that do not contain a catechol unit, suggesting that the presence of catechol unit(s) in these A-type proanthocyanidins increases their AGE-inhibitory properties. We additionally found that epicatechin and epicatechin-3-*O*-gallate, commercially available compounds containing catechol units, notably inhibited AGE formation at IC_{50} values of 13.6 ± 0.3 and 15.1 ± 0.2 μM, respectively. Because the presence of catechol unit(s) in flavonoids is important to show substantial antioxidant activity, our result is consistent with previous reports that the AGE formation inhibitory activities of several flavonoids were closely associated with their antioxidant capacity.[22,23]

Table 2. Inhibitory Effects of Compounds 1−6 from *S. ramalana* against AGE Formation[a]

compound	IC_{50}[b] (μM)
1	32.1 ± 0.5
2	39.8 ± 3.6
3	17.4 ± 0.5
4	34.7 ± 2.8
5	33.5 ± 2.0
6	14.1 ± 1.6
aminoguanidine[c]	965.9 ± 15.5

[a]Results are expressed as means ± SEM (n = 3). [b]IC_{50} indicates the concentration (μM) at which the inhibition percentage of the AGE formation was 50%, and the values were determined by regression analysis. [c]Positive control.

Accumulation of AGE in retinal blood vessels plays an important role in the onset and development of diabetic retinopathy.[24] Experimental studies have shown that specific inhibitors of AGE formation effectively reduce retinal microvascular lesions in diabetic animal models.[25,26] Recently, it has been reported that the retinal vessels of the hyperglycemic adult zebrafish increase in diameter (compared to normal animals).[27] Thus, to evaluate the effects of compounds **1**−**6** on retinopathy, an in vivo diabetic zebrafish model was used. The change in the extent of hyaloid-retinal vessel dilation was assessed using zebrafish *flk*:EGFP transgenic embryos held under high-glucose (30 mM) conditions. Dilation was reduced by treatment with compounds **1**−**6**; compound **3**, which contains a catechol unit, was most effective in this regard. Vessels treated with **3** were significantly thinner than were those of the control group (Figure 3B−D), and no cytotoxicity was evident at the concentrations tested. Quantitative analysis revealed that **3** reduced the diameters of HG-induced hyaloid-retinal vessels by 157.7% and 164.1% when used at 10 and 20 μM, respectively, compared to the HG-treated control group (Figure 3F). Compound **6** also reduced HG-induced dilation of hyaloid-retinal vessels, albeit to a lesser extent (104.0% inhibition at 20 μM) than did **3** (see Supporting Information). Our results suggest that compound **3** inhibits development of experimental retinopathy at early larval stages.

Proanthocyanidins represent a unique class of oligomeric and polymeric end-products of the flavonoid biosynthetic pathway. The materials are widespread throughout the plant kingdom and exhibit a number of biological properties, including antioxidant, antibacterial, antiviral, anticarcinogenic, anti-inflammatory, and antiallergic activities.[28−30] Our current study of novel AGE inhibitors from *S. ramalana* resulted in isolation of three new (**1**−**3**) and three known dimeric A-type proanthocyanidins (**4**−**6**). This is the first report of the occurrence of A-type proanthocyanidins in the genus *Spenceria*. Compounds **1**−**6** notably inhibited AGE formation with IC_{50} values ranging from 14.1 to 39.8 μM. Compound **3** also significantly reduced dilation of HG-induced hyaloid retinal vessels in a diabetic zebrafish model. Our results suggest that *S. ramalana* and active components thereof may be beneficial to treat and prevent diabetic vascular complications and other related diseases.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-2000 digital polarimeter. ECD spectra were measured on a JASCO J-715 spectrometer. IR spectra were recorded on a JASCO 100 IR spectrometer. All 1D (^{1}H and ^{13}C) and 2D (HMQC, HMBC, and NOESY) NMR spectra were obtained using a Bruker Avance 500 NMR spectrometer with TMS as an internal standard. HRESIMS were recorded on a Shimadzu LCMS-IT-TOF spectrometer. Column chromatography was performed using silica gel (70−230 mesh and 230−400 mesh, Merck) and Sephadex LH-20 (Amersham Pharmacia Biotech). Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 F_{254s} plates (0.25 mm, Merck). Spots were detected by UV light (254 nm) and spraying with 10% H_2SO_4 followed by heating.

Plant Material. The whole plant of *S. ramalana* was collected from an alpine meadow in Lijiang, China, in September 2008, and identified

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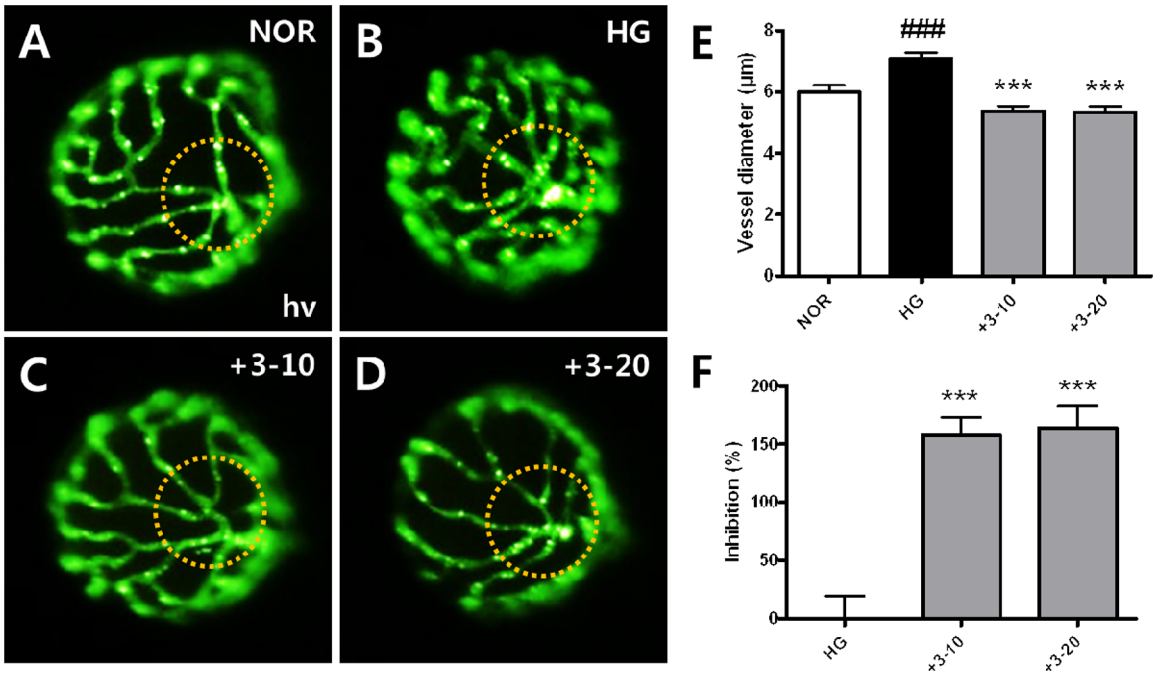

Figure 3. Effect of compound **3** on dilation of hyaloid-retinal vessels from a high-glucose (HG)-induced diabetic retinopathy model. (A–D) Hyaloid-retinal vessels of *flk*:EGFP transgenic zebrafish. (A) Untreated normal group (Nor). (B) HG-treated control group (HG). (C) 10 μM and (D) 20 μM compound **3** in the HG-treated group. (E) Data are displayed as International System of Units (SI) for vessel diameters. (F) Percentage inhibition (10 μM = 157.7%, 20 μM = 164.1%) of hyaloid-retinal vessel dilation of compound **3** in HG-induced embryos. The diameters of hyaloid-retinal vessels were measured at locations proximal to the optic disc (yellow dotted circle). The hyaloid vessel diameter of each lens was measured three times, and the experiment was performed in triplicate. $^{###}p < 0.001$ vs Nor, $^{***}p < 0.001$ vs HG.

by one of the authors (J.-H.K.). A voucher specimen (No. DiAB-2008-063) has been deposited in the Herbarium of the Diabetic Complications Research Team, Korea Institute of Oriental Medicine, Republic of Korea.

Extraction and Isolation. The dried whole plant of *S. ramalana* (4.0 kg) was extracted with EtOH (3 × 60 L) at room temperature for 7 days with maceration and filtering and then concentrated to give an EtOH extract (330 g). The EtOH extract (320 g) was suspended in H_2O (4 L) and partitioned successively with *n*-hexane (3 × 4.0 L), EtOAc (3 × 4.0 L), and *n*-BuOH (3 × 4.0 L) to afford *n*-hexane- (13.8 g), EtOAc- (198.1 g), and *n*-BuOH-soluble fractions (100.5 g), respectively.

The EtOAc-soluble fraction (180.0 g), which significantly inhibited AGE formation, was subjected to silica gel column chromatography (70–230 mesh, 40 × 9.5 cm), eluting with a gradient solvent system consisting of $CHCl_3$–MeOH (100:1 → 0:1) to afford nine fractions (A–I). Fraction B (18.8 g) was chromatographed on a silica gel column (70–230 mesh, 50 × 10 cm) using a stepwise gradient of $CHCl_3$–MeOH (50:1 → 5:1) to yield nine fractions (B1–B9), one of which, B7 (2.1 g), was further chromatographed on a silica gel column (230–400 mesh, 40 × 7.5 cm), eluted with a $CHCl_3$–MeOH gradient (50:1 → 5:1), yielding seven subfractions (B2.1–B2.7). Subfractions B2.2 (700 mg) and B2.4 (600 mg) were separately chromatographed on a Sephadex LH-20 column (60 × 2.5 cm); elution of B2.2 with a MeOH–H_2O gradient (6:4 → 7:3) yielded compounds **4** (110 mg) and **5** (7 mg), and elution of B.2.4 with MeOH–H_2O (7:3) yielded compound **6** (145 mg). Chromatography of fraction D (21.1 g) on a silica gel column (70–230 mesh, 50 × 10 cm), eluting with a stepwise gradient of $CHCl_3$–MeOH (50:1 → 5:1), afforded six fractions (D1–D6). Fraction D2 (600 mg) was further purified on a Sephadex LH-20 column (60 × 2.5 cm) using a MeOH–H_2O gradient (65:35 → 70:30) to obtain compound **1** (25 mg). Fraction D3 (2.6 g) was applied to a silica gel column (230–400 mesh, 40 × 7.5 cm) and eluted using a $CHCl_3$–MeOH gradient (50:1 → 10:1) to yield eight subfractions (D3.1–D3.8). Compounds **2** (21 mg) and **3** (16 mg) were isolated from fraction D3.8 (350 mg) using a Sephadex LH-20 column (60 × 2.5 cm) eluting with a MeOH–H_2O gradient (60:40 → 70:30).

ent-Epiafzelechin-(2α→O→7,4α→8)-ent-afzelechin 3′-O-β-D-glycopyranoside ***(1):*** white powder; $[\alpha]_D^{25}$ −101 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 224, 272 nm; ECD (*c* 0.0002 M, MeOH) [Θ] (nm) −9230 (232), +580 (271); IR (KBr) ν_{max} 3450, 1610, 1520, 1140, 830 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 707.1970 $[M + H]^+$ (calcd for $C_{36}H_{35}O_{15}^+$, 707.1970).

ent-Epiafzelechin-(2α→O→7,4α→8)-ent-epiafzelechin-(2α→O→7,4α→8)]-ent-afzelechin ***(2):*** white powder; $[\alpha]_D^{25}$ −101 (*c* 0.13, MeOH); UV (MeOH) λ_{max} 226, 272 nm; ECD (*c* 0.0002 M, MeOH) [Θ] (nm) −5820 (240), +447 (254), +820 (271); IR (KBr) ν_{max} 3300 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 813.1826 $[M - H]^-$ (calcd for $C_{45}H_{33}O_{15}^-$, 813.1825).

ent-Epiafzelechin-(2α→O→7,4α→8)-ent-epicatechin-(2α→O→7,4α→8)-ent-afzelechin ***(3):*** white powder; $[\alpha]_D^{25}$ −90 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 224, 274 nm; ECD (*c* 0.0002 M, MeOH) [Θ] (nm) −5635 (240), +371 (254), +780 (272); ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 829.1771 $[M - H]^-$ (calcd for $C_{45}H_{33}O_{16}^-$, 829.1774).

Methylation of 1–3. The methylation of **1** (10 mg) was done with an excess of CH_2N_2 in MeOH–Et_2O at room temperature for 48 h. After a routine workup, the product was purified by HPLC [Agilent 1200 system; YMC-pack Pro C_{18} column (250 × 10 mm, i.d.); MeOH–H_2O (30:70, v/v); UV detection, 270 nm; flow rate, 3.0 mL/min] to give pentamethyl ether (**1a**, 6 mg). The methylation of **2** (7 mg) and **3** (10 mg) was similarly performed to afford heptamethyl ether (**2a**, 3 mg) and octamethyl ether (**3a**, 2.5 mg).

1a: white powder; ^1H NMR (300 MHz, $CDCl_3$) δ_H 2.72–2.92 (2H, m, H-4′), 3.31 (3H, s, MeO-5), 3.72 (3H, s, MeO-7), 3.75 (3H, s, MeO-5′), 3.80 (3H, s, MeO-14), 3.82 (3H, s, MeO-14′), 4.14 (1H, d, *J* = 3.5 Hz, H-3), 4.25 (1H, d, *J* = 3.5 Hz, H-4), 5.02 (1H, d, *J* = 8.0 Hz, H-2′), 6.10 (1H, d, *J* = 2.2 Hz, H-6), 6.20 (1H, s, H-6′), 6.24 (1H, d, *J* = 2.2 Hz, H-8), 6.94–7.60 (8H, aromatic protons in rings B and E), 4.13 (1H, d, *J* = 7.5 Hz, glc-1), 3.12–3.85 (6H, m, glc-2, 3, 4, 5, 6); ESIMS *m*/*z* 777.2 $[M + H]^+$.

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2a: white powder; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 2.58−3.10 (2H, m, H-4″), 3.33 (3H, s, MeO-5), 3.40 (3H, s, MeO-5′), 3.70 (3H, s, MeO-5″), 3.73 (3H, s, MeO-7), 3.78 (3H, s, MeO-14″), 3.81 (3H, s, MeO-14′), 3.83 (3H, s, MeO-14), 4.04 (1H, m, H-3″), 4.12 (1H, d, J = 3.5 Hz, H-3′), 4.25 (1H, d, J = 3.5 Hz, H-3), 4.30 (1H, d, J = 3.5 Hz, H-4 or H-4′), 4.32 (1H, d, J = 3.5 Hz, H-4 or H-4′), 4.70 (1H, d, J = 8.0 Hz, H-2″), 5.98 (1H, d, J = 2.2 Hz, H-6″), 6.08 (1H, d, J = 2.2 Hz, H-6), 6.18 (1H, d, J = 2.2 Hz, H-6′), 6.20 (1H, d, J = 2.2 Hz, H-8), 6.92−7.70 (12H, aromatic protons in rings B, E, and H); ESIMS m/z 911.2 $[M - H]^-$.

3a: white powder; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 2.60−3.11 (2H, m, H-4″), 3.31 (3H, s, MeO-5), 3.38 (3H, s, MeO-5′), 3.68 (3H, s, MeO-13′), 3.74 (3H, s, MeO-14′), 3.78 (3H, s, MeO-5″), 3.83 (3H, s, MeO-7), 3.87 (3H, s, MeO-14″), 3.90 (3H, s, MeO-14), 4.03 (1H, m, H-3″), 4.14 (1H, d, J = 3.5 Hz, H-3′), 4.26 (1H, d, J = 3.5 Hz, H-3), 4.30 (1H, d, J = 3.5 Hz, H-4′), 4.34 (1H, d, J = 3.5 Hz, H-4), 4.70 (1H, d, J = 8.0 Hz, H-2″), 5.96 (1H, d, J = 2.2 Hz, H-6″), 6.07 (1H, d, J = 2.2 Hz, H-6), 6.19 (1H, d, J = 2.2 Hz, H-6′), 6.20 (1H, d, J = 2.2 Hz, H-8), 6.90−7.72 (11H, aromatic protons in rings B, E, and H); ESIMS m/z 941.3 $[M - H]^-$.

Acid Hydrolysis of 1. Compound **1** (5 mg) in 10% HCl−dioxane (1:1, 1 mL) was heated at 80 °C for 3 h in a water bath. The mixture was neutralized with Ag_2CO_3, filtered, and extracted with EtOAc (20 mL). The EtOAc layer was evaporated, and the residue subjected to reversed-phase HPLC [Agilent 1200 system; YMC-pack Pro C_{18} column (250 × 10 mm, i.d.); MeOH−H_2O (30:70, v/v); UV detection, 270 nm; flow rate, 3.0 mL/min] to give *ent*-epiafzelechin-(2α→O→7,4α→8)-*ent*-afzelechin (**4**, 2 mg), identified by comparing spectroscopic data with literature data.[7] 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 completed, the solution was treated with Ac_2O (3 mL) at 60 °C for 1 h. Authentic samples were prepared by the same procedure. The acetate derivatives were subjected to gas chromatography (GC) analysis. GC conditions: GC-2010 (Shimadzu) instrument; detector, FID; column, TC-1 capillary column (0.25 mm × 30 m; GL Science, Inc.); column temperature, 230 °C; programmed increase, 38 °C/min; carrier gas, N_2 (1 mL/min); injection and detector temperature, 270 °C. The sugar derivative thus obtained showed a retention time of 21.30 min, identical to that of authentic D-glucose.

Determination of AGE Formation. According to a well-established method, the reaction mixture [bovine serum albumin (10 mg/mL, Sigma, St. Louis, MO, USA; 700 μL) in 50 mM phosphate buffer (pH 7.4) with 0.02% sodium azide] was added to 0.2 M fructose and glucose (100 μL). In screw cap tubes (1.5 mL), the reaction mixture was mixed with 200 μL of serially diluted compounds and aminoguanidine (Sigma). After incubating at 37 °C for 7 days, the fluorescent reaction products (200 μL) were transferred to 96-well plates and assayed on a spectrofluorometric detector (Bio-Tek, Synergy HT, USA; excitation wavelength, 350 nm; emission wavelength, 450 nm). The AGE assay was performed in triplicate. The concentration of each test sample giving 50% inhibition of the activity (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against remaining activity.

Measurement of Vessel Dilation in Larval Zebrafish. 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 Tg (transgenic) fish and raised in egg water (sea salt, 0.06 g/L). One-day *flk*:EGFP Tg embryos were placed into 24-well plates (five embryos per well) and maintained in 2 mL volumes of egg water with 30 mM glucose. HG-induced embryos were treated with 10 or 20 μM of compounds from 1 day postfertilization (dpf) to 6 dpf. At 6 dpf, HG-induced embryos were fixed with 4% (v/v) paraformaldehyde, and each lens containing hyaloid retinal vessels was isolated and aligned so that the optic disc (OD) was facing upward. Fluorescence images were obtained using an Olympus SZX16 stereomicroscope. The diameters of hyaloid vessels were measured in 3−4 main branches of the OD, to the first sub-branch, using the Image J software. All experiments were performed in triplicate.

Statistical Analysis. Hyaloid vessel diameter was used to calculate the percentage inhibition of compound **3** in HG-treated embryos according to the formula

$$\text{percentage inhibition (\%)} = [1 - (T_n - C_{av})/(HG_{av} - C_{av})] \times 100$$

where C_{av} = average of hyaloid-vessel diameter (SI) in the control embryos, HG_{av} = average of hyaloid-vessel diameter (SI) in the HG-treated embryos, and T_n = hyaloid-vessel diameter (SI) of the HG-treated embryos with compound **3**. The results are expressed as means ± standard error of the mean (SEM) from three independent experiments. Statistical significance was assessed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests with the GraphPad 5.0 Prism software (GraphPad, San Diego, CA, USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR, ^{13}C NMR, NOESY, and mass spectra for the new compounds **1**−**3** and zebrafish data of compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Wei, M.; Gaskill, S. P.; Hanffner, S. M.; Stern, M. P. *Diabetes Care* **1998**, *21*, 1167−1172.

(2) Laakso, M. *Diabetes* **1999**, *48*, 937−942.

(3) Nishikawa, T.; Edelstein, D.; Du, X. L.; Yamagishi, S.; Matsumura, T.; Kaneda, Y.; Yorek, M. A.; Beebe, D.; Oates, P. J.; Hammes, H. P.; Giardino, I.; Brownlee, M. *Nature* **2000**, *404*, 787−790.

(4) Brownlee, M. *Nature* **2001**, *414*, 813−820.

(5) Takenaka, K.; Yamagishi, S.; Matsui, T.; Nakamura, K.; Imaizumi, T. *Curr. Neurovasc. Res.* **2006**, *3*, 73−77.

(6) Li, C.; Ikeda, H.; Ohba, H. In Wu, Z. Y.; Raven, P. H.; Hong, D. Y., Eds. *Flora of China*, Vol. *9*; Science Press: Beijing, and Missouri Botanical Garden Press: St. Louis, 2003; p 384.

(7) Bilia, A. R.; Morelli, I.; Hamburger, M.; Hostettmann, K. *Phytochemistry* **1996**, *43*, 887−892.

(8) Rawat, M. S. M.; Prasad, D.; Joshi, R. K.; Pant, G. *Phytochemistry* **1999**, *50*, 321−324.

(9) Prasad, D. *Fitoterapia* **2000**, *71*, 245−253.

(10) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer: New York, 1970; p 41.

(11) Jacques, D.; Haslam, E.; Bedford, G. R.; Greatbanks, D. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2663−2671.

(12) Lou, H.; Yamazaki, Y.; Sasaki, T.; Uchida, M.; Tanaka, H.; Oka, S. *Phytochemistry* **1999**, *51*, 297−308.

(13) Kolodziej, H.; Sakar, M. K.; Burger, J. F. W.; Engelshowe, R.; Ferreira, D. *Phytochemistry* **1991**, *30*, 2041−2047.
(14) Botha, J. J.; Young, D. A.; Ferreira, D.; Roux, D. G. *J. Chem. Soc., Perkin Trans. 1* **1981**, 1213−1219.
(15) Hikino, H.; Shimoyama, N.; Kasahara, Y.; Takahashi, M.; Konno, C. *Heterocycles* **1982**, *19*, 1381−1384.
(16) Korver, O.; Wilkins, C. K. *Tetrahedron* **1971**, *27*, 5459−5465.
(17) Balde, A. M.; De Bruyne, T.; Pieters, L.; Kolodziej, H.; Berghe, D. V.; Claeys, M.; Vlietinck, A. *Phytochemistry* **1995**, *38*, 719−723.
(18) Morimoto, S.; Nonaka, G. I.; Nishioka, I. *Chem. Pharm. Bull.* **1987**, *35*, 4717−4729.
(19) Calzada, F.; Cedillo-Rivera, R.; Bye, R.; Mata, R. *Planta Med.* **2001**, *67*, 677−680.
(20) Jang, D. S.; Yoo, N. H.; Kim, N. H.; Lee, Y. M.; Kim, C. S.; Kim, J.; Kim, J. H.; Kim, J. S. *Biol. Pharm. Bull.* **2010**, *33*, 329−333.
(21) Brownlee, M.; Vlassara, H.; Kooney, A.; Ulrich, P.; Cerami, A. *Science* **1986**, *232*, 1629−1632.
(22) Morimitsu, Y.; Yoshida, K.; Esaki, S.; Hirota, A. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 2018−2021.
(23) Lou, H.; Yuan, H.; Yamazaki, Y.; Sasaki, T.; Oka, S. *Planta Med.* **2001**, *67*, 345−349.
(24) Stitt, A. W.; Li, Y. M.; Gardiner, T. A.; Bucala, R.; Archer, D. B.; Vlassara, H. *Am. J. Pathol.* **1997**, *150*, 523−531.
(25) Stitt, A.; Gardiner, T. A.; Alderson, N. L.; Canning, P.; Frizzell, N.; Duffy, N.; Boyle, C.; Januszewski, A. S.; Chachich, M.; Baynes, J. W.; Thorpe, S. R. *Diabetes* **2002**, *51*, 2826−2832.
(26) Gardiner, T. A.; Anderson, H. R.; Stitt, A. W. *J. Pathol.* **2003**, *201*, 328−333.
(27) Alvarez, Y.; Chen, K.; Reynolds, A. L.; Waghorne, N.; O'Connor, J. J.; Kennedy, B. N. *Dis. Model. Mech.* **2010**, *3*, 236−245.
(28) Bagchi, D.; Garg, A.; Krohn, R. L.; Bagchi, M.; Tran, M. X.; Stohs, S. J. *Res. Commun. Mol. Pathol. Pharmacol.* **1997**, *95*, 179−189.
(29) Bagchi, M.; Bagchi, D. J.; Balmoori, J.; Stohs, S. J. *Gen. Pharmacol.* **1998**, *30*, 771−776.
(30) Shahat, A. A.; Cos, P.; De Bruyne, T.; Apers, S.; Hammouda, F. M.; Ismail, S. I.; Azzam, S.; Claeys, M.; Goovaerts, E.; Pieters, L.; Vanden Berghe, D.; Vlietinck, A. J. *Planta Med.* **2002**, *68*, 539−541.