

Rat Intestinal Sucrase and α -Glucosidase Inhibitory Activities of Isocoumarin and Flavonoids from the *Zanthoxylum schinifolium* Stems

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Hyperglycemia, an abnormal postprandial increase of blood glucose level, has been linked to the onset of type 2 insulin-dependent diabetes mellitus (NIDDM, Type 2 diabetes) and associated cardiovascular complications including hypertension.^{1,2} The main challenge involved in managing diabetes is maintaining blood glucose levels as close to normal as possible with few major fluctuations.³ α -Amylase and α -glucosidase, such as sucrase, maltase, and isomaltase, are key enzymes involved in starch breakdown and intestinal glucose absorption, respectively. The inhibition of these enzymes can slow down the passage of carbohydrates into the bloodstream significantly decreasing the postprandial increase of blood glucose level after a mixed carbohydrate diet and therefore can be an important strategy in the management of type 2 diabetes.⁴ However previous reports have revealed that excessive inhibition of α -amylase could result in the abnormal bacterial fermentation of undigested starch in the colon and therefore low α -amylase inhibitory activity is useful.^{5,6} A main negative aspect of currently used therapeutic α -glucosidase inhibitors such as the drug acarbose which has strong α -amylase inhibitory activity is digestive tract-disorder such as abdominal distention, flatulence, meteorism, and possibly diarrhea.⁷ Therefore, natural inhibitors from dietary plants are useful as they have lower inhibitory activity against α -amylase and a stronger inhibitory activity against α -glucosidase and can be used as effective therapy for postprandial hyperglycemia with minimal side effects.⁸

Zanthoxylum schinifolium is a dioecious shrub with hooked prickly branchlets belonging to the Rutaceae family. *Zanthoxylum* peel commonly used in traditional oriental to treat the common cold, stomach aches, diarrhoea, and jaundice in Korea, Japan and China.⁹ The majority of phytochemical research on *Z. schinifolium* has focused on essential oils, coumarins, and alkaloids from fruits and leaves.¹⁰⁻¹³ However, components from the stems of *Z. schinifolium* have not been study depth.^{14,15} In this study, we report the isolation and characterization of a novel isocoumarin, schinifolisatin (**1**), along with three known flavonoid glycosides (**2-4**) from the MeOH extract of *Z. schinifolium* stems and their sucrase and α -glucosidase inhibitory activities.

Compound **1** was obtained as a yellowish oil, with the molecular formula C₁₂H₁₂O₅ based on the quasimolecular-ion peak [M+H]⁺ at *m/z* 237.0765 (calcd. 237.0763) in the

HR-ESI-MS spectrum. Its IR spectrum revealed a hydroxy group (3458 cm⁻¹), ester carbonyl group (1761 cm⁻¹), and conjugated carbonyl group (1705 cm⁻¹). The ¹H NMR spectrum of **1** showed tri-substituted resonance signals at δ_{H} 6.79 (d, *J* = 8.2 Hz, H-5), 7.49 (d, *J* = 1.8 Hz, H-8), and 7.51 (dd, *J* = 8.2, 1.8 Hz, H-6) of an aromatic ring; an oxymethine proton signal at δ_{H} 5.51 (dd, *J* = 8.0, 4.0 Hz, H-3); a methylene group at δ_{H} 2.98 (m, H-4); and two methoxy groups at δ_{H} 3.65 (s, 11-OMe) and 3.83 (s, 7-OMe). The above units were confirmed by ¹³C NMR and HSQC spectra, which contained six aromatic carbons at δ_{C} 113.7 (C-8), 115.9 (C-5), 121.7 (C-9), 125.4 (C-6), 148.7 (C-7), and 153.2 (C-10); an oxymethine group at δ_{C} 70.3 (C-3); a methylene group at δ_{C} 37.1 (C-4); and two methoxy groups at δ_{C} 52.5 (11-OMe) and 56.4 (7-OMe). Two carbonyl groups at δ_{C} 167.0 (C-11) and 171.8 (C-1) were also revealed in the ¹³C NMR spectrum. The above signals were indicative of the 3,4-dihydroisocoumarin methyl ester skeleton of **1**.¹⁶ In the HMBC spectrum, the correlations at δ_{H} 5.51 (H-3)/ δ_{C} 167.0 (C-11) and δ_{H} 3.65 (11-OMe)/ δ_{C} 167.0 (C-11) demonstrated that the methyl ester group was attached to C-3 of isocoumarin. The correlation at δ_{H} 3.83 (7-OMe)/ δ_{C} 148.7 (C-7) indicated that the methoxy group was linked to C-7 of the skeleton. The absolute configuration of **1** was determined based on CD analysis. The Cotton effect sign at approximately 240 nm was associated with the configuration of the chiral carbon C-3 in the CD spectra of α,β -unsaturated- δ -lactone.¹⁷ A negative Cotton effect at 285 nm is assignable to the $n \rightarrow \pi^*$ transition of the carbonyl group of the dihydroisocoumarin chromophore.^{18,19} A negative Cotton effect at 265 nm ($\Delta\epsilon$ -0.21) in the CD spectrum of **1** was indicative of a 3*S* configuration.²⁰ Based on these data, compound **1** was characterized as (3*S*)-3,4-dihydro-7-methoxyisocoumarin-3-carboxylic methyl ether, named schinifolisatin.

Comparison of the 1D- and 2D-NMR spectral data with reported values led to the identification of structures of the three known compounds (**2-4**) as hesperidin (**2**),²¹ homoesperetin-7-*O*-rutinoside (**3**),²² and hyperin (**4**).²³ Among them, compounds **3** and **4** were isolated from *Zanthoxylum* genus for the first time.

Compounds **1-4** were then evaluated for sucrase and α -glucosidase inhibitory activities at a concentration of 1 mM. Acarbose and quercetin were used as positive control. The

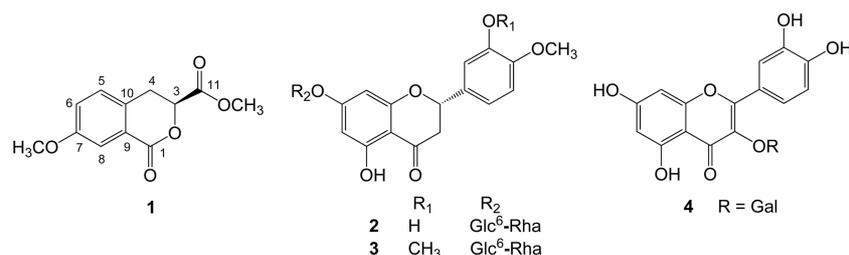


Figure 1. The chemical structures of compounds **1-4** from the stems of *Z. schinifolium*.

results showed that compounds **1-4** exhibited strong sucrose inhibitory activities of 71.6-90.6% at the same concentration, compound **4** also showed significant α -glucosidase inhibitory activity of 58.2% compared with positive control. These results suggested that *Z. schinifolium* may be useful for the treatment of diabetes mellitus.

Experimental

General Experimental Procedures. Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. CD spectra were recorded with a Jasco J-810 spectropolarimeter. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer; The NMR spectra were recorded using a Jeol ECA 600 spectrometer (^1H , 600 MHz; ^{13}C , 150 MHz), High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Column chromatography was performed using a silica gel (Kieselgel 60, 70-230, and 230-400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F₂₅₄ and RP-18 F_{254S} plates (both 0.25 mm, Merck, Darmstadt, Germany).

Plant Material. Dried stems of *Z. schinifolium* were collected from Daejeon, Korea in September 2012 and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 12102) was deposited at the Herbarium of College of Pharmacy, Chungnam National University.

Extraction and Isolation. Dried stems (2.3 kg) were extracted with MeOH (5 L \times 3) under reflux. The MeOH extract (102.0 g) was suspended in water and partitioned with *n*-hexane, EtOAc and *n*-BuOH. The EtOAc fraction (12.0 g) was subjected to silica gel (5 \times 30 cm) column chromatography with a gradient of hexane-EtOAc-MeOH (10:1:0, 6:1:0, 3:1:0, 1.5:1:0.1, 1:1:0.2; 1.5 L for each step) to give 6 fractions (Fr. 1A-1F). The fraction 1C was separated using a silica gel (1.5 \times 80 cm) column chromatography with a gradient of hexane-acetone-MeOH (7:1:0.1, 6:1:0.1, 5:1:0.1; 1.0 L for each step) elution solvent to give compound **1** (11.0 mg). The *n*-BuOH fraction (28.0 g) was subjected to silica gel (3.0 \times 30 cm) column chromatography with a gradient of CH_2Cl_2 -MeOH- H_2O (16:1:0, 10:1:0, 7.5:1:0.1, 3:1:0.15, 1.5:1:0.2; 2.5 L for each step) to give 6 fractions (Fr. 2A-2F). The fraction 2C was subjected to silica gel (1.0 \times 70 cm) column chromatography with a

gradient of CH_2Cl_2 -MeOH- H_2O (12:1:0, 10:1:0, 8.5:1:0.1, 6.5:1:0.1, 4:1:0.1; 1.5 L for each step) to give four sub-fractions (Fr. 2C-1-2C-4). The fraction 2C-3 was separated using an YMC (1.0 \times 80 cm) column chromatography with a MeOH- H_2O (0.2:1, 0.3:1, 0.6:1, 1:1; 750 mL for each step) elution solvent to give compounds **2** (43.0 mg) and **3** (21.0 mg). The fraction 2C-4 was separated using an YMC (1.0 \times 80 cm) column chromatography with a MeOH- H_2O (0.2:1, 0.67:1; 1.0 L for each step) elution solvent to give compound **4** (12.0 mg).

Schinifolisatin (1): Yellowish oil; $\text{C}_{12}\text{H}_{12}\text{O}_5$; $[\alpha]_{\text{D}}^{25}$: +56.8 (*c* 0.3, MeOH); CD (MeOH): $\Delta\epsilon$ 240+0.16, $\Delta\epsilon$ 265+0.21, $\Delta\epsilon$ 285-0.18 (*c* 0.71×10^7 M); IR (KBr): ν_{max} 3458, 1761, 1705, 1610, 1471 cm^{-1} ; ^1H NMR (methanol-*d*₄, 600 MHz) and ^{13}C NMR data (methanol-*d*₄, 150 MHz), see Table 1; HR-ESI-MS: m/z 237.0765 [M+H]⁺ (calcd. 237.0763).

Hesperidin (2): Amorphous powder, mp 268-271 °C. $[\alpha]_{\text{D}}^{25}$: -36.7 (*c* 0.3, pyridine); IR (KBr): ν_{max} 3474, 2918, 1649, 1609, 1096, 1069 cm^{-1} ; FAB-MS m/z : 611 [M+H]⁺. This compound exhibited comparable spectroscopic data (^1H - and ^{13}C -NMR) to published values.¹⁷

Homoesperetin-7-O-rutinoside (3): Amorphous powder; $[\alpha]_{\text{D}}^{25}$: -42.7 (*c* 0.3, pyridine); IR (KBr): ν_{max} 3470, 2921, 1655, 1612, 1073 cm^{-1} ; FAB-MS m/z : 625 [M+H]⁺. This compound exhibited comparable spectroscopic data (^1H - and ^{13}C -NMR) to published values.¹⁸

Hyperin (4): Yellow needles. IR ν_{max} (KBr) cm^{-1} : 3316, 2900, 1655, 1607, 1060. FAB-MS m/z : 465 [M+H]⁺. This compound exhibited comparable spectroscopic data (^1H -

Table 1. ^1H (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data of compound **1** (methanol-*d*₄, δ , ppm, *J*/Hz)

Pos.	δ_{H}	δ_{C}	HMBC
1		171.8	H-3, 5, 8
3	5.51 (1H, dd, 8.0, 4.0)	70.3	H-4
4	2.98 (2H, m)	37.1	H-3, 5
5	6.79 (1H, d, 8.2)	115.9	H-4, 6
6	7.51 (1H, dd, 8.2, 1.8)	125.4	H-5, 8
7		148.7	H-5, 6, 8, 7-OMe
8	7.49 (1H, d, 1.8)	113.7	H-6
9		121.7	H-4, 5, 8
10		153.2	H-4, 5
11		167.0	H-3, 11-OMe
7-OMe	3.83 (3H, s)	56.4	
11-OMe	3.65 (3H, s)	52.5	

Table 2. Rat intestinal sucrase inhibitory activities of compounds 1-4

Compounds	% Sucrase inhibitory activity ^a	% α -Glucosidase inhibitory activity ^a
1	87.3 \pm 1.68	10.0 \pm 0.83
2	71.6 \pm 1.19	9.6 \pm 0.36
3	73.1 \pm 2.28	18.9 \pm 2.89
4	90.6 \pm 0.38	100
Positive control	91.1 \pm 0.93 ^b	92.9 \pm 4.88 ^c

^aPercentage of enzyme inhibition at the concentration of 1 mM. ^bAcarbose (0.05 mM) was used as positive control. ^cQuercetin (1 mM) was used as positive control. Data presented is the mean \pm SD of samples runs in triplicate.

and ¹³C-NMR) to published values.¹⁹

Inhibition Assay for Sucrase Inhibitory Activity. The crude enzyme solution prepared from rat intestinal acetone powder Sigma-Aldrich Co. (St. Louis, MO, USA) was used as the small intestinal sucrase, showing specific activities of 0.34 units/mL. Rat-intestinal acetone powder (1.0 g) was suspended in 3 mL of 0.9% saline, and the suspension was sonicated twelve times for 30 sec at 4 °C. After centrifugation (10000 \times g, 30 min, 4 °C), the resulting supernatant was used for the assay. Sucrase inhibitory activities were assayed by modifying a method developed by Kwon *et al.*²⁴ The inhibitory activity was determined by incubating a solution of an enzyme (50 μ L), 0.1 M phosphate buffer (pH 7.0, 100 μ L) containing 0.4 mg/mL sucrose and a solution (50 μ L) with a concentration of sample (1.0 mM) at 37 °C for 30 min. Acarbose (0.05 mM) was used as positive control. The reaction mixture was heated in a boiling water bath to stop the reaction for 10 min, and then the amount of liberated glucose was measured by the glucose oxidase method.²⁵ The inhibitory activity was calculated from the formula as follows. Inhibition (%) = (C-T)/C \times 100, where C is the enzyme activity without inhibitor and T is the enzyme activity with inhibitor.

Inhibition Assay for α -Glucosidase Activity. The α -glucosidase inhibition assay was performed according to (Li *et al.*, 2009) with some modification. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Briefly, a mixture of 50 μ L samples and 50 μ L of 0.1 M phosphate buffer (pH 7.0) containing α -glucosidase solution (0.3 U/mL) and 50 μ L distilled water was incubated in 96 well plates at 37 °C for 15 min. After pre-incubation, 100 μ L of 3 mM p-NPG solution in 0.1 M phosphate buffer (pH 7.0) was added to each well at timed intervals. Quercetin (1 mM) was used as positive control. The reaction mixtures were incubated at 37 °C for 10 min and stopped by adding 750 μ L of 0.1 M Na₂CO₃. The absorbance was recorded at 405 nm by FLUO star Optima (BMG Labtech, Offenburg, Germany). The results were expressed as a percent of μ -glucosidase inhibition and calculated according to the following equation:

$$\% \text{ inhibition} = [(A \text{ control} - A \text{ compound}) / A \text{ control}] \times 100$$

Statistical Analysis. All experiments were performed in triplicate. Data is presented as the means \pm SD. The results were statistically analyzed by ANOVA and Duncan's multiple range tests. Statistical significance was accepted at a level of $p < 0.05$.

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