Identification of Antidiabetic Effect of Iridoid Glycosides and Low Molecular Weight Polyphenol Fractions of Corni Fructus, a Constituent of Hachimi-jio-gan, in Streptozotocin-Induced Diabetic Rats

Noriko Yamabe,[a] Ki Sung Kang,[a] Yosuke Matsuo,[b] Takashi Tanaka,[b] and Takako Yokozawa*,[a]

[a] *Institute of Natural Medicine, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan: and* [b] *Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyou-cho, Nagasaki 852–8521, Japan.*

In our previous study, Corni Fructus (*Cornus officinalis* Sieb. *et* Zucc.), a component crude drug of the Chinese prescription Hachimi-jio-gan, was reported to reduce glucotoxicities, up-regulate renal function, and consequently ameliorate glycation-associated renal damage as well as Hachimi-jio-gan. Based upon these facts, we prepared Corni Fructus fractions and evaluated which fraction contained the effective components against diabetes, using one iridoid glycoside and three polyphenol fractions, which were expected to possess stronger activities than Corni Fructus, administered orally at a dose of 20 mg/kg body weight/d for 10 d, respectively. As a result, iridoid glycosides and low molecular weight polyphenol fractions could reduce the pathogenesis of diabetic renal damage, each having different mechanisms, *i.e.*, iridoid glycosides successfully decreased the hyperglycemic state and affected renal advanced glycation end-product (AGE) accumulation, such as N^{ε}-(carboxyethyl)lysine and N^{ε}-(carboxymethyl)lysine, while low molecular weight polyphenol fractions could reduce renal lipid peroxidation, the receptor for AGE, and inducible nitric oxide synthase. Overall, these data suggest that iridoid glycosides and low molecular weight polyphenols purified from Corni Fructus improve metabolic parameters associated with the development of diabetic renal damage. The main active components of these fractions are discussed.

Key words   Corni Fructus; active component; streptozotocin; advanced glycation end-product; lipid peroxidation

Diabetes mellitus is a leading cause of many complications, such as atherosclerosis, cardiac dysfunction, retinopathy, neuropathy, and nephropathy. In longitudinal hyperglycemia, advanced glycation end-product (AGE) formation, resulting from the non-enzymatic reactions of carbohydrates and oxidized lipids with proteins, is increased and is also stimulated by oxidative stress which is closely linked with the pathogenesis of diabetic complications as well as the polyol pathway and protein kinase C pathway.[1,2] Therefore, interference with AGE formation has the therapeutic potential to prevent the progression of diabetic complications.

Traditional medicines are recently being focused on to treat diabetes and its complications because of their absence of toxic- and/or side-effects. In our previous study, we reported that Hachimi-jio-gan ameliorated functional and structural features associated with experimental type 1 and 2 diabetic nephropathy rat models,[3,4] and according to the three-dimensional HPLC profile of Hachimi-jio-gan, morroniside and loganin obtained from Corni Fructus (*Cornus officinalis* Sieb. *et* Zucc.) are the major compounds. It was previously discovered that Corni Fructus could ameliorate glucose-associated metabolic disorders, and its mechanisms were intimately related to the formation of AGEs, as well as those of Hachimi-jio-gan.[5] To add to these findings, we evaluated the major active components in Corni Fructus. As the first step, we prepared five fractions from Corni Fructus extract, that is, one sugar with organic acid fraction (S1D1), one iridoid glycoside fraction (S1D2), and three polyphenol fractions (from low to high molecular weights: S2, S3, and S4) as shown in Fig. 1A. Next, we examined the effect of Corni Fructus and its five fractions on hydroxyl radical scavenging activity associated with the glycation reaction, because this reaction is, in part, mediated by hydrogen peroxide

and transition metal ions, called the Fenton reaction.[6] As a result, only the fraction of S1D1 was weaker than Corni Fructus and S1D2 was similar to Corni Fructus; that is, the relation of these activities was as follows, "S1D1<Corni Fructus≦S1D2<S2<S3<S4."

In this study, we evaluated the active fractions of Corni Fructus, that is, one iridoid glycoside and three polyphenol fractions of S1D2, S2, S3, and S4, possessing greater activities compared with Corni Fructus, in streptozotocin (STZ)-induced diabetic rats. This is the first report to clarify the active components not only in Corni Fructus but also in Hachimi-jio-gan against diabetes and associated renal damage.

MATERIALS AND METHODS

Materials   The following reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan): 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid (TBA)), 5-hydroxymethyl-2-furfural (5-HMF), oxalic acid, bovine serum albumin (BSA), 2-amino-2-hydroxymethyl-1,3-propadiol (Tris (hydroxymethyl) aminomethane), Tween 20, phenylmethyl sulfonyl fluoride (PMSF), protease inhibitor mixture DMSO solution, and skim milk powder. Dithiothreitol (DTT) was purchased from BioVision Inc. (Mountain View, CA, U.S.A.). The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Tokyo, Japan). Polyclonal anti receptor for AGE (RAGE) antibody (sc-5563), monoclonal anti NOS2 antibody (sc-7271) (a primary antibody against inducible nitric oxide synthase (iNOS)), polyclonal anti nuclear factor-κB (NF-κB) p65 antibody (sc-109), goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody (sc-2004), and goat anti-mouse IgG HRP conjugated
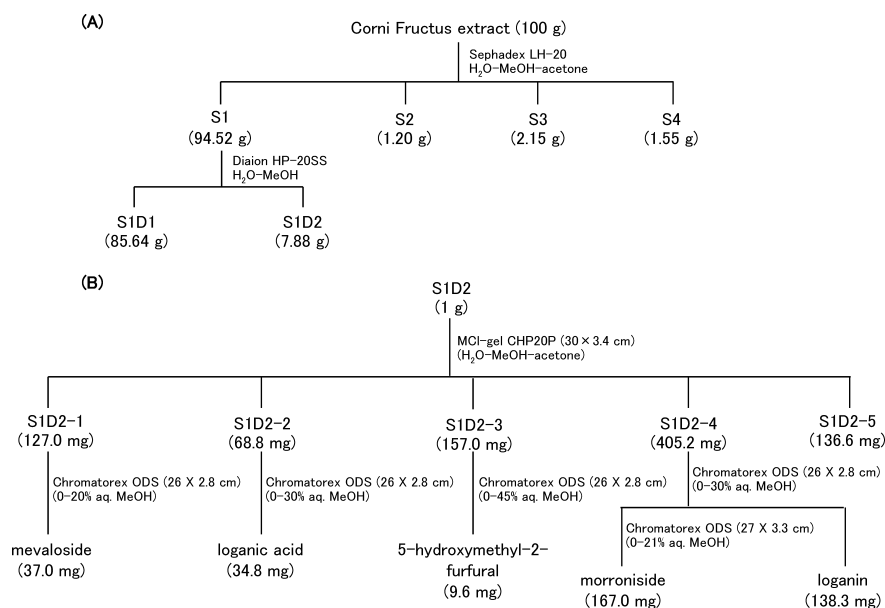
Fig. 1. Fractionation and Isolation of Compounds from Corni Fructus

secondary antibody (sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Monoclonal anti N^ε-(carboxyethyl)lysine (CEL) antibody and polyclonal anti N^ε-(carboxymethyl)lysine (CML) antibody were kindly provided by Dr. R. Nagai (Kumamoto University, Japan). STZ, glycerol, Nonidet P 40 (NP-40), and anti-mouse β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). ECL Western blotting detection reagents were purchased from GE Healthcare (Piscataway, NJ, U.S.A.).

General Experimental Procedures for the Identification of Corni Fructus Components Optical rotations were measured with a Jasco DIP-370 digital polarimeter. ^{1}H-, ^{13}C-NMR, ^{1}H–^{1}H COSY, NOESY, HSQC, and HMBC spectra were recorded at 27 °C with a Varian Unity plus 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Mass spectra (MS) were recorded on a JEOL JMS-700N spectrometer, and glycerol was used as the matrix for FAB-MS measurements. Column chromatography was performed with Diaion HP20SS, MCI-gel CHP 20P (75—150 μm) (Mitsubishi Chemical Co., Japan), Sephadex LH-20 (25—100 μm) (GE Healthcare, Piscataway, NJ, U.S.A.), and Chromatorex ODS (Fuji Silysia Chemical Ltd., Aichi, Japan). TLC was performed on 0.2 mm precoated Kieselgel 60 F_{254} plates (Merck & Co., Inc., Whitehouse Station, NJ, U.S.A.) with benzene–ethyl formate–formic acid (1 : 7 : 1, v/v) or chloroform–methanol–water (14 : 6 : 1, v/v). Spots were detected *via* UV illumination and by spraying with 2% ethanolic FeCl$_3$ or 10% sulfuric acid reagent followed by heating. Analytical HPLC was performed on a 250×4.6 mm i.d. Cosmosil 5C$_{18}$-AR II column (Nacalai Tesque Inc., Kyoto, Japan) with gradient elutions of CH$_3$CN in 50 mM H$_3$PO$_4$ from 10—30% in 30 min and 30—75% in 15 min at a flow rate of 0.8 ml/min and detection with a Jasco MD-910 photodiode array detector.

Preparation of Corni Fructus Fractions The Corni Fructus extract (100 g), which was produced by Tsumura & Co. (Tokyo, Japan) was fractionated by Sephadex LH-20 column chromatography (32 cm×5 cm) with water containing increasing proportions of methanol (0—100%, 10% stepwise gradient elution) and finally 60% acetone to give four fractions, S1 (94.52 g), S2 (1.20 g), S3 (2.15 g), and S4 (1.55 g). The fraction S1 was further separated by Diaion HP-20SS column chromatography (28 cm×5 cm) with water–methanol (0—100%, 10% stepwise gradient elution) to give S1D1 (85.64 g) and S1D2 (7.88 g). TLC and HPLC analysis which were performed as mentioned above showed that S1D1 and S1D2 mainly contained sugars and iridoid glycosides, and S2, S3, and S4 contained phenolic substances (Fig. 1A). A portion of S2 (150 mg) was further purified by MCI-gel CHP20P column chromatography (28 cm×2 cm) with 0—10% MeOH to give 7-O-galloyl sedoheptulose.[7] Further separation of S1D2 (1 g) by MCI-gel CHP20P (30 cm×3.4 cm) with water–MeOH (0—100%, 10% stepwise gradient elution) gave five fractions, S1D2-1 (127.0 mg), S1D2-2 (68.8 mg), S1D2-3 (157.0 mg), S1D2-4 (405.2 mg), and S1D2-5 (136.6 mg). Purification of S1D2-1, S1D2-2, and S1D2-3 by Chromatorex ODS column chromatography (26 cm×2.8 cm) using 0—20% MeOH, 0—30% MeOH, and 0—45% MeOH as an elution solvent yielded mevaloside (37.0 mg),[8] loganic acid (34.8 mg),[9] and 5-HMF (9.6 mg),[10] respectively. A similar separation of S1D2-4 afforded morroniside (167.0 mg)[11,12] and loganin (138.3 mg).[13] The structure of mevaloside was determined on the basis of COSY, HSQC, and HMBC spectral analysis, and other products were identified by ^{1}H- and ^{13}C-NMR comparison (Fig. 1B). The chemical structures purified from S1D2 and S2 are shown in Fig. 2.

Animals and Treatment The Guidelines for Animal Experimentation approved by the University of Toyama were followed in all experimental studies. Five-week-old male Wistar rats (120—130 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan), kept in wire-bottomed cages, and exposed to a 12-h light/dark cycle. The room temperature and humidity were maintained automatically at about 25 °C and 60%, respectively. They were allowed free access to laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid, and 60.5% carbohydrate) and

(A)

mevaloside

5-hydroxymethyl-2-furfural

morroniside

loganic acid

loganin
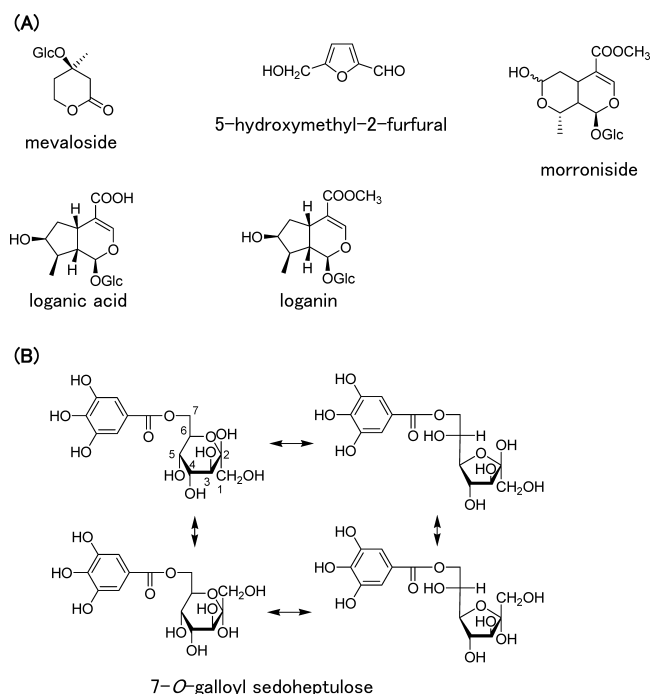
(B)

7-*O*-galloyl sedoheptulose

Fig. 2. Chemical Structures of Isolated Compounds in S1D2 (A) and S2 (B) Fractions

water. After several days of adaptation, the rats were injected intraperitoneally with STZ (50 mg/kg body weight) in 10 mM citrate buffer (pH 4.5). Ten days after the injection, the body weights were measured and blood samples were obtained between 10 a.m. and 11 a.m., avoiding the influence of food consumption, to determine glucose levels, and the rats with glucose levels >400 mg/dl were randomly divided into five experimental groups containing eight rats each. The diabetic control group was given water, while the other diabetic groups were orally given the Corni Fructus fractions (S1D2, S2, S3, and S4) dissolved in water at dose of 20 mg/kg body weight/d *via* gavage. The dose was determined by the activity and the content of Corni Fructus and its fractions, that is, 200 mg of Corni Fructus could effectively ameliorate the diabetic disorders in our previous study[5] and the content of S1D2 (7.88%) was considered as the standard, because its activity was less than the other polyphenol fractions on hydroxyl radical scavenging capability. The rats which underwent a sham injection of citrate buffer without STZ were also used as normal controls containing five rats. After 10 d of the administration period, 24-h urine samples were collected using metabolic cages and blood samples were obtained between 10 a.m. and 11 a.m. from the abdominal aorta under pentobarbital anesthesia (50 mg/kg body weight, intraperitoneally), and then the serum was immediately separated from the blood samples by centrifugation. After renal perfusion through the renal artery with ice-cold physiological saline, the kidneys were removed from each rat and frozen at −80 °C until analysis.

Clinical Characteristics of Animals Serum levels of glucose, total protein, total cholesterol, urea nitrogen (urea-N), and creatinine (Cr) were examined using commercial reagents (Glucose CII-Test Wako, A/G B-Test Wako, and Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); BUN Kainos and CRE-EN

Kainos obtained from Kainos Laboratories, Inc. (Tokyo, Japan)). Serum glycosylated protein was measured by the TBA assay of McFarland *et al.*[14] in which non-enzymatically bound glucose is released as 5-HMF and quantitated colorimetrically. TBA-reactive substance levels were determined using the methods of Naito and Yamanaka.[15] Urine component levels were determined as follows: protein by the sulfosalicylic acid method[16] and Cr using a commercial reagent (CRE-EN Kainos). Cr clearance (Ccr) was calculated on the basis of urinary Cr, serum Cr, urine volume, and body weight using the following equation: Ccr (ml/min/kg body weight)= [urinary Cr (mg/dl)×urinary volume (ml)/serum Cr (mg/dl)]× [1000/body weight (g)]×[1/1440 (min)].

AGE Level in Kidney The renal AGE level was determined by the method of Nakayama *et al.*[17] In brief, minced kidney tissue was delipidated with chloroform and methanol (2 : 1, v/v) overnight. After washing, the tissue was homogenized in 0.1 N NaOH, followed by centrifugation at 8000×*g* for 15 min at 4 °C. The amounts of AGEs in these alkali-soluble samples were determined by measuring the fluorescence at an emission wavelength of 440 nm and an excitation wavelength of 370 nm. A native BSA preparation (1 mg/ml of 0.1 N NaOH) was used as a standard, and its fluorescence intensity was defined as one unit of fluorescence. The fluorescence values of samples were measured at a protein concentration of 1 mg/ml and expressed in arbitrary units (AU) compared with a native BSA preparation.

Mitochondrial TBA-Reactive Substance Level in Kidney Mitochondria were prepared from kidney homogenate by differential centrifugation (800×*g* and 12000×*g*, respectively) at 4 °C according to the methods of Johnson and Lardy[18] and Jung and Pergande,[19] respectively, with minor modifications. Each pellet was resuspended in preparation medium and the concentration of TBA-reactive substance was determined by the method of Uchiyama and Mihara.[20] The protein level was examined by the method of Itzhaki and Gill[21] with BSA as the standard.

Protein Preparation and Western Blot Analyses Renal cortical sections in all experimental rats were homogenized with ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris–HCl, 1% (v/v) Tween 20, 10% (v/v) glycerol, 1 mM PMSF, and protease inhibitor mixture DMSO solution. After centrifugation (2000×*g* at 4 °C) to ensure equal loading among lanes, the protein concentration of each tissue was determined using a Bio-Rad protein assay kit and BSA as a standard, and then immunoblotting was carried out.

For the determination of RAGE, iNOS, CEL, and CML protein expressions, 30 μg of protein of each sample was electrophoresed through 8 or 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Separated proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 h, and then incubated with primary antibodies to RAGE, iNOS, CEL, CML, and β-actin, respectively, overnight at 4 °C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP conjugated secondary antibody for 90 min at room temperature. Each antigen–antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (Fujifilm, Japan).

Band densities were determined by Scion image software

(Scion Corporation, Frederick, Maryland, U.S.A.) and quantified as the ratio to β-actin. The evaluation of these protein levels at mean values against normal rats is represented as 1 and the corresponding values for the diabetic rats are expressed as the ratios of these values.

Determination of the Expression of NF-κB in Nuclear and Cytosolic Extracts Nuclear and cytoplasmic extracts were obtained from the renal cortex in experimental rats ($n=$ 3 per group) according to the method of Rangan *et al.*[22] with some modifications. In brief, cortical renal tissue (100 mg) was homogenized in 200 μl of ice-cold hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl$_2$, 0.1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor mixture DMSO solution. Then, 65 μl of 2% (v/v) NP-40 were added and the mixture was vortexed for 1 min, incubated on ice for 10 min, and centrifuged at 1000×**g** for 10 min at 4 °C. The resulting supernatant was collected and used as the cytosolic fraction. Crude nuclear pellets were rinsed twice with hypotonic buffer and resuspended in 60 μl of hypertonic buffer containing 50 mM HEPES (pH 7.9), 50 mM KCl, 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor mixture DMSO solution. It was then vortexed twice for 1 min each, and the mixture was centrifuged at 13000×**g** for 10 min at 4 °C to yield a supernatant containing extracted nuclear proteins. The protein concentration was determined as described above and equal amounts of protein (30 μg) were used for each sample in Western blot analyses with anti-NF-κB antibody.

Band densities of NF-κB were determined using image analysis software as described above, and these protein levels were calculated as the ratio to the normal values.

Statistical Analysis The effect of fractions of Corni Fructus on each parameter was examined using one way Analysis of Variance. Individual differences among groups were analyzed by Dunnett's test. $p<0.05$ was considered significant. The results are expressed as means±S.E.M.

RESULTS

Body Weight Change, Kidney Weight, and Food and Water Intake During the 10 d of the experimental period, diabetic control rats showed a significant decrease in the body weight gain and a 1.6-fold heavier kidney weight compared with normal rats; however, oral administration of S1D2 and S2 fractions led to a tendency to increase body weight and significantly decrease the kidney weight (Table 1). In addition, food and water intakes in diabetic control rats were increased 1.4- and 3.2-fold compared to those of normal rats, but the administration of S1D2 and S2 led to a decrease in water intake, while there were no changes in food intake in experimental groups (Table 1).

Serum Constituents Figure 3 shows the serum glucose, glycosylated protein, and lipid levels. At the beginning, STZ-induced diabetic rats showed a serum glucose level of about

Table 1. Body Weight Changes, Kidney Weight, and Food and Water Intake

| Groups	Body weight			Kidney weight (mg/100 g B.W.)	Food intake (g/d)	Water intake (ml/d)
	Initial (g)	Final (g)	Gain (g/10 d)			
Normal rats	246.6±4.3*	297.8±7.1*	51.2±4.4*	0.70±0.02*	21.5±1.3*	42.7±3.8*
Diabetic rats						
Control	199.0±4.7	206.5±6.0	11.4±2.2	1.13±0.02	30.0±1.4	137.6±3.1
S1D2 (20 mg/kg B.W.)	199.3±5.7	224.5±7.3	17.5±3.8	1.02±0.03*	27.0±0.9	120.9±3.8*
S2 (20 mg/kg B.W.)	197.9±6.0	211.1±10.3	14.3±3.9	1.03±0.03*	28.3±1.5	125.7±3.4*
S3 (20 mg/kg B.W.)	197.6±6.3	202.6±9.3	5.0±5.2	1.06±0.02	28.5±0.6	134.8±6.7
S4 (20 mg/kg B.W.)	197.5±3.6	201.1±6.8	3.6±5.1	1.05±0.03	29.6±0.6	128.9±6.3

∗ $p<0.05$ *vs.* diabetic control values.
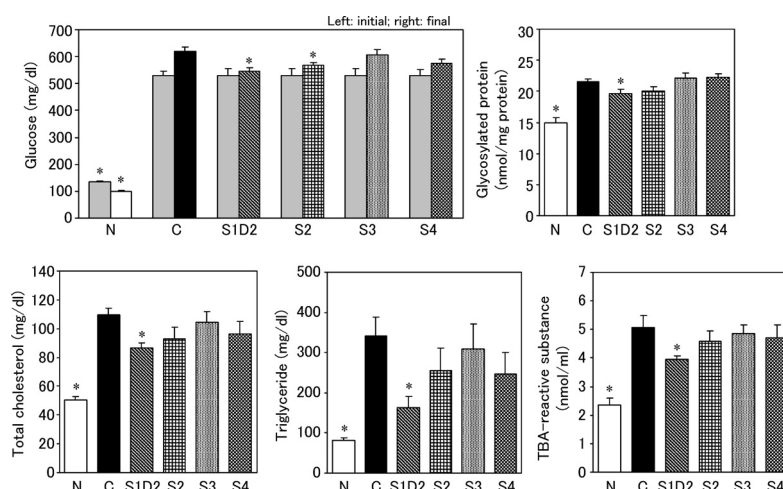
Fig. 3. Effect of Corni Fructus Fractions on Serum Constituents

N, normal rats; C, diabetic control rats; S1D2, S1D2 (20 mg/kg body weight)-treated diabetic rats; S2, S2 (20 mg/kg body weight)-treated diabetic rats; S3, S3 (20 mg/kg body weight)-treated diabetic rats; S4, S4 (20 mg/kg body weight)-treated diabetic rats. Results are shown as the means±S.E.M. ($n=5$ or 8). ∗ $p<0.05$ *vs.* diabetic control values.

Table 2.   Renal Function Parameters

Groups	s-Urea N (mg/dl)	s-Cr (mg/dl)	Ccr (ml/kg B.W./min)	Urine volume (ml/d)	Urinary protein (mg/d)
Normal rats	22.4±1.0*	0.369±0.004	7.75±0.07*	20.2±2.6*	9.9±1.3
Diabetic rats					
Control	38.5±2.4	0.370±0.007	6.62±0.20	110.9±2.6	11.2±0.3
S1D2 (20 mg/kg B.W.)	33.1±1.7	0.368±0.012	6.78±0.45	93.8±5.9*	9.1±0.9
S2 (20 mg/kg B.W.)	33.9±1.2	0.368±0.008	7.25±0.10*	99.1±4.2*	9.3±0.6
S3 (20 mg/kg B.W.)	36.8±2.1	0.369±0.009	6.68±0.25	106.9±5.2	11.8±0.9
S4 (20 mg/kg B.W.)	34.9±3.8	0.371±0.018	6.68±0.35	102.7±6.4	9.8±0.5

∗$p<0.05$ vs. diabetic control values.

530 mg/dl (normal rats: 133.8 mg/dl), but 10 d after, diabetic control rats showed an increase to 618.2 mg/dl. On the other hand, 20 mg of S1D2 and S2 administered to fraction-treated diabetic rats led to significant decreases (544.8 mg/dl and 567.8 mg/dl, respectively), but no reduction was shown with S3 and S4 treatment.

At the end of this experiment, serum constituents except for glucose levels were measured, as shown in Fig. 3. Diabetic control rats showed an increase in the serum glycosylated protein level compared to normal rats (from 14.9 nmol/mg protein to 21.5 nmol/mg protein), while only the fraction of S1D2 decreased the levels significantly (19.7 nmol/mg protein, $p<0.05$). In addition, serum lipid levels, evaluated with total cholesterol, triglyceride, and TBA-reactive substance levels, were all significantly increased by about 2.18-, 4.23-, and 2.16-fold, respectively, relative to the control levels; however, a notable reduction was shown on treatment with only S1D2, and these effects were similar to those of glucose and glycosylated protein.

Renal Function   Table 2 shows the effect of Corni Fructus fractions on renal function parameters in diabetic rats. The serum urea-N level and urine volume in diabetic control rats were significantly increased, and the Ccr level conversely showed a decrease of 85% compared with the normal level, though there were no remarkable changes between diabetic control and normal rats in terms of serum Cr and urinary protein excretion levels. In contrast, amelioration of the Ccr level was observed in the S2-treated group ($p<0.05$), and both the urine volume and urinary protein excretion level were similarly decreased in S1D2- and S2-treated groups.

AGE and Mitochondrial TBA-Reactive Substance Levels in Kidney   Table 3 shows that the renal AGE and mitochondrial TBA-reactive substance levels were increased significantly by about 1.62- and 1.22-fold, respectively, in STZ-induced diabetic control rats compared to the normal values. After treatment with fractions of Corni Fructus, the AGE level was significantly lowered only by S1D2, but there was a slight decrease with S2 treatment. Conversely, each treated group showed a decrease in the TBA-reactive substance level, being much more effective in the S2-treated group than in the other groups.

Western Blotting of Renal Cortex   To evaluate AGE and oxidative stress-related protein expressions in the renal cortex, we performed Western blot analyses with RAGE, iNOS, CEL, and CML antibodies. As shown in Fig. 4, renal RAGE, iNOS, and CEL and CML, which were estimated to be 65 kDa and a 50 kDa molecules, respectively, and also anti-CEL and CML antibody-reactive proteins, were elevated in STZ-induced diabetic control rats compared with normal

Table 3.   Renal AGE and Mitochondrial TBA-Reactive Substance Levels

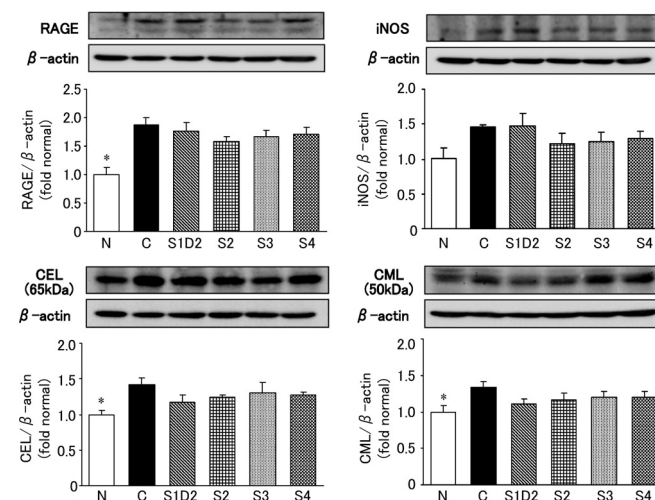| Groups	AGE (AU)	TBA-reactive substance (nmol/mg protein)
Normal rats	2.74±0.14*	1.87±0.09*
Diabetic rats		
Control	4.45±0.18	2.29±0.11
S1D2 (20 mg/kg B.W.)	3.90±0.15*	1.68±0.15*
S2 (20 mg/kg B.W.)	4.05±0.12	1.55±0.16*
S3 (20 mg/kg B.W.)	4.28±0.17	1.72±0.06*
S4 (20 mg/kg B.W.)	4.21±0.12	1.70±0.08*

∗$p<0.05$ vs. diabetic control values.

Fig. 4.   Effect of Corni Fructus Fractions on Protein Expressions in Renal Cortex

N, normal rats; C, diabetic control rats; S1D2, S1D2 (20 mg/kg body weight)-treated diabetic rats; S2, S2 (20 mg/kg body weight)-treated diabetic rats; S3, S3 (20 mg/kg body weight)-treated diabetic rats; S4, S4 (20 mg/kg body weight)-treated diabetic rats. Results are shown as the means±S.E.M. ($n=5$ or 8). ∗$p<0.05$ vs. diabetic control values.

rats (1.88-, 1.45-, 1.42-, and 1.33-fold, respectively). However, the S2-treated group only showed a down-regulation of RAGE and iNOS protein expressions to the value of 1.58- and 1.21-fold compared with those of normal (without significance), while the S1D2-treated group showed slight decreases in the CEL and CML proteins to the value of 1.17- and 1.10-fold of normal.

NF-κB Expression in Nucleus and Cytosolic Extracts of Renal Cortex   Western blot analysis was performed to confirm the presence of NF-κB proteins in nuclear and cytosolic extracts of the renal cortex using experimental rats (Fig. 5). At the end of this experiment, NF-κB protein was expressed at higher levels in both the nucleus and cytosolic
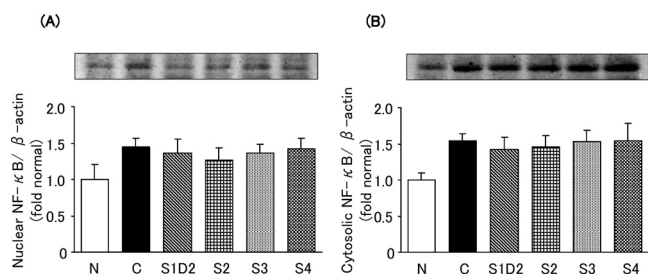
(A)

(B)

Fig. 5.   Effect of Corni Fructus Fractions on NF-κB Protein Expressions in Nuclear (A) and Cytosolic (B) Extracts of Renal Cortex

N, normal rats; C, diabetic control rats; S1D2, S1D2 (20 mg/kg body weight)-treated diabetic rats; S2, S2 (20 mg/kg body weight)-treated diabetic rats; S3, S3 (20 mg/kg body weight)-treated diabetic rats; S4, S4 (20 mg/kg body weight)-treated diabetic rats. Results are shown as the means±S.E.M. ($n=3$). No significant differences *vs.* diabetic control values.

extracts of diabetic control rats than those from normal rats, but these increases were not sufficient to show the significances. In addition, 10 d after administration, there was no significant reduction of either nuclear or cytosolic NF-κB protein levels, but in the nucleus, a slight reduction was observed in the S2 fraction.

DISCUSSION

The genus *Cornus* (dogwood) belongs to the family Cornaceae, which consists of about 55 species, and is widely distributed in the northern hemisphere, eastern Asia, and the eastern and northern part of the United States,[23] and Corni Fructus belongs to the subgroup Cornelian cherries containing iridoid total glycoside such as morroniside and loganin and also a few polyphenols such as cornusiin A, B, and C, monomeric and trimeric hydrolysable tannins, and so on.[24,25] Furthermore, several reports of its use including traditional medicine have been made, *e.g.*, Vareed *et al.*[26] reported that Corni Fructus has been used for improving liver and kidney functions, and iridoid total glycoside has the effect of preventing the overexpression of transforming growth factor-β_1 and matrixes in glomeruli with a diabetic model.[27] Moreover, we have previously reported that not only Hachimi-jio-gan, containing Corni Fructus, could prevent the development of type 1 and 2 diabetic nephropathy,[3,4] and also that Corni Fructus led to a better AGE clearance due to improving the renal function than aminoguanidine, the prototype AGE inhibitor, suggesting that Corni Fructus might become an effective remedy against diabetic AGE accumulation.[5] Therefore, we hypothesized that Corni Fructus would be a major active ingredient in Hachimi-jio-gan, and based upon these facts, this study was designed to evaluate the active fractions of Corni Fructus, in order to clarify the essential compounds in Corni Fructus.

Intensive therapy of the blood glucose level in patients with type 1 diabetes, which was performed by The Diabetes Control and Complication Trial Research Group,[28] has been emphasized to delay the onset and slow the progression of diabetic complications like retinopathy, neuropathy, and nephropathy. Furthermore, AGE formation, one of the metabolic disorders caused by hyperglycemia, has been focused on as a marker of long-term glycemic control in body tissues,[29] and these products have also been strongly implicated in the pathogenesis of diabetic microvascular and

macrovascular diseases.[30]

In this experiment, diabetic rats showed a significant decrease in body weight gain and increase in kidney weight, *i.e.*, renal hypertrophy, despite food and water intake being increased compared with normal rats during the 10 d of the experimental period, representing growth retardation due to the obstruction of glucose uptake caused by the lack of insulin following STZ injection. By the oral administration of fractions, S1D2 and S2 were found to be effective to decrease the kidney weight and water intake, suggesting that these two fractions could ameliorate the renal changes and enlargement. However, body weight gain with S3 and S4 administration decreased compared with that of diabetic control rats, suggesting that they might show toxicity to some extent. In contrast, at the end of the study, the hyperglycemic state had worsened from the initial value in diabetic control rats, but S1D2- and S2-treated rats showed significant decreases. Additionally, diabetic control rats not only showed an increase in serum glycosylated protein, a parameter of Amadori products, *e.g.*, glycosylated hemoglobin (HbA$_{1c}$), produced in the glycation reaction, but also abnormal lipid metabolism, as shown by increased levels of total cholesterol, triglycerides, and TBA-reactive substance, a parameter of lipid peroxidation. On the other hand, only S1D2-treatment affected these parameters significantly, while the S2-treated group showed a decrease next to those of S1D2, but failed to show any significance; hence, we considered that these results seemed to depend on glycemic control.

Prolonged exposure to hyperglycemia in diabetes participates in the formation and accumulation of AGEs which correlates with the severity of renal complications in diabetes.[1,31] Additionally, the increased concentration of AGEs is accelerated by the decreased capability to metabolize and/or excrete them. From this experiment, diabetic control rats showed a decrease in Ccr, reflecting a decline in AGE clearance, increase in serum urea-N, and greater urine volume, though there were no changes in serum Cr and the urinary protein excretion level, suggesting that the diabetic control rats were kept in the early stage of diabetes over the experimental period. However, 10 d of S2 administration improved the Ccr level, and the urine volume and urinary protein excretion levels, having a strong correlation with increased water intake and renal hypertrophy, were significantly reduced by the administration of S1D2 and S2. Therefore, we hypothesized that the effect of S1D2 in the reduction of urinary protein level might be independent of renal function but might in part correlate with the marked glycemic control and inhibition of AGE formation in serum, while the reduction of urinary protein level by S2 would be due to the improvement of renal function, which would enhance AGE clearance.

Recently, attention has been focused on the essential roles of AGEs, that is, AGEs alter the structure and function of matrix tissue proteins,[1,2] and AGE-modified proteins stimulate a variety of cellular responses *via* a specific cell surface receptor, resulting in the expression and activation of pathogenic mediators, *e.g.*, extracellular matrix, oxidative stress, cytokines, and growth factors, implicated in the development and stimulation of diabetic renal diseases.[32] For these reasons, we first assessed renal AGE accumulation and mitochondrial lipid peroxidation *via* measuring the fluorescence

and TBA-reactive substance, whereby diabetic control rats showed significant increases in these levels. On the other hand, concerning the level of AGEs, only S1D2 successfully reduced them, while a slight reduction was shown in the S2 group; however, all fractions reduced the TBA-reactive substance level, particularly S2.

For further investigation, we performed Western blot analyses in the renal cortex, evaluating AGE actions with receptors relating intracellular responses and the renal AGE level characterized physicochemically by neither cross-linking nor fluorescence, *e.g.*, CEL and CML, identified in ageing and diabetes-related diseases. That is, these products are not only derived from glucose–metabolic intermediates and metabolites of glycolysis, but also serve as general bio-markers of oxidative stress resulting from carbohydrate and lipid oxidation reactions.[33—35] It is well known that RAGE is the best-characterized receptor for AGE which is composed of multiple members of the immunoglobulin superfamily.[36] Moreover, AGEs trigger the activation of NF-κB *via* interaction with RAGE, leading to its translocation to the nucleus where it induces transcription,[32] and the promoter region of the RAGE gene contains NF-κB binding sites,[37] potentially producing a self-perpetuating pathway, and the iNOS gene also contains the NF-κB binding sites.[38] In this study, we showed increased levels of RAGE and iNOS protein in the diabetic control group; however, these proteins were both down-regulated in the S2-treated group, though there was no effect by the administration of S1D2 on the expression of these proteins. On the contrary, increases of CEL and CML proteins were detected in the diabetic renal cortex, and they were both lower in the S1D2 group, similar to the fluorescent AGE level as described above. These results suggest that S1D2 might reduce these proteins by controlling serum glucose and glycation reactions in comparison with the antioxidative activity. Next, we demonstrated that NF-κB p65 was expressed in the renal cortex of diabetic control rats; however, we observed slight increases in nuclear and cytosolic extracts from diabetic control rats compared with normal rats. Therefore, we suggested that this experiment was not sufficient term to discuss the significant expression level of the NF-κB p65 protein. On the contrary, the nuclear level showed a slight decrease by S2 treatment, and thus, long-term treatment with S2 in advanced diabetic rats might provide evidence that the effect of S2 on RAGE and iNOS proteins is due to a decrease of nuclear NF-κB p65.

Taken together, we estimated that S1D2 had effects on intracellular AGE formation and/or accumulation but weak effects on oxidative stress, and that S2 had effects mainly on oxidative stress caused by the AGE-RAGE signaling pathway *via* ameliorating AGE clearance and renal RAGE expression. However, significant reductions of CEL and CML were not seen in S2, possibly relating to the process of glycolysis, *i.e.*, the administered 7-*O*-galloyl sedoheptulose, a component of S2, would be decomposed by glycosidase and kinase to form sedoheptulose-7-phosphate which can also be produced by a transketolase reaction between xylulose and ribose in the pentose phosphate pathway. In addition, Lal *et al.*[39] have reported that sedoheptulose-7-phosphate was increased in the STZ-induced diabetic rat lens at day 14, as well as other sugars and sugar phosphates, having a cross-linking potential but less than that of fructose-3-phosphate. Therefore, we consid-

ered that S2, *i.e.*, 7-*O*-galloyl sedoheptulose, might have some influence on AGE formation and/or accumulation by its administration. Thus, these mechanical relationships must be clarified in future studies.

In conclusion, we elucidated that S1D2 and S2 were the active fractions of Corni Fructus in STZ-induced diabetic rats, having different mechanisms in the diabetic serum and renal disorders. To add to these findings, elucidation of the major active components in these fractions might clarify the most important contributors to prevent and/or delay the onset of diabetic renal damage. Hence, this evidence may lead to the development of novel therapeutic agents not only from Corni Fructus but also Hachimi-jio-gan.

REFERENCES

1) Bohlender J., Franke S., Stein G., Wolf G., *Am. J. Physiol. Renal Physiol.*, **289**, F645—F659 (2005).
2) Thomas M. C., Forbes J. M., Cooper M. E., *Am. J. Ther.*, **12**, 562—572 (2005).
3) Yokozawa T., Yamabe N., Cho E. J., Nakagawa T., Oowada S., *Nephron Exp. Nephrol.*, **97**, e38—e48 (2004).
4) Yamabe N., Yokozawa T., *J. Pharm. Pharmacol.*, **58**, 535—545 (2006).
5) Yamabe N., Kang K. S., Goto E., Tanaka T., Yokozawa T., *Biol. Pharm. Bull.*, **30**, 520—526 (2007).
6) Carubelli R., Schneider E., Jr., Pye Q. N., Floyd R. A., *Free Rad. Biol. Med.*, **18**, 265—269 (1995).
7) Zhang Y., Chen Y., Zhao S., *Acta Pharm. Sin.*, **34**, 153—155 (1999).
8) Tschesche R., Struckmeyer K., Wulff G., *Chem. Ber.*, **104**, 3567—3572 (1971).
9) El-Naggar L. J., Beal J. L., *J. Nat. Prod.*, **43**, 649—707 (1980).
10) Liu Z. L., Slininger P. J., Dien B. S., Berhow M. A., Kurtsman C. P., Gorsich S. W., *J. Ind. Microbiol. Biotechnol.*, **31**, 345—352 (2004).
11) Inouye H., Tobita S., Akiyama Y., Ito K., Shingu T., *Chem. Pharm. Bull.*, **21**, 846—854 (1973).
12) Otsuka H., Kijima K., *Chem. Pharm. Bull.*, **49**, 699—702 (2001).
13) Garcia J., Chulia A. J., *Planta Med.*, **4**, 327—329 (1986).
14) McFarland K. F., Catalano E. W., Day J. F., Thorpe S. R., Baynes J. W., *Diabetes*, **28**, 1011—1014 (1979).
15) Naito C., Yamanaka T., *Jpn. J. Geriat.*, **15**, 187—191 (1978).
16) Sakagishi Y., "Rinsho Kagaku Bunseki II," ed. by Saito M., Kitamura M., Niwa M., Tokyo Kagaku Dojin, Tokyo, 1968, pp. 115—142.
17) Nakayama H., Mitsuhashi T., Kuwajima S., Aoki S., Kuroda Y., Itoh T., Nakagawa S., *Diabetes*, **42**, 345—350 (1993).
18) Johnson D., Lardy H., *Methods Enzymol.*, **10**, 94—96 (1967).
19) Jung K., Pergande M., *FEBS Lett.*, **183**, 167—169 (1985).
20) Uchiyama M., Mihara M., *Anal. Biochem.*, **86**, 271—278 (1978).
21) Itzhaki R. F., Gill D. M., *Anal. Biochem.*, **9**, 401—410 (1964).
22) Rangan G. K., Wang Y., Tay Y. C., Harris D. C. H., *Kidney Int.*, **56**, 118—134 (1999).
23) Fan C., Xiang Q. Y., *Am. J. Bot.*, **88**, 1131—1138 (2001).
24) Hatano T., Ogawa N., Kira R., Tasuhara T., Okuda T., *Chem. Pharm. Bull.*, **37**, 2083—2090 (1989).
25) Wang S. F., Chen X. G., Hu Z. D., Ju Y., *Biomed. Chromatogr.*, **17**, 306—311 (2003).
26) Vareed S. K., Reddy M. K., Schutzki R. E., Nair M. G., *Life Sci.*, **78**, 777—784 (2006).
27) Xu H. Q., Hao H. P., *Biol. Pharm. Bull.*, **27**, 1014—1018 (2004).
28) The Diabetes Control and Complications Trial Research Group, *N. Engl. J. Med.*, **329**, 977—986 (1993).
29) Brownlee M., Cerami A., Vlassara H., *N. Engl. J. Med.*, **318**, 1315—1321 (1988).
30) Fukami K., Ueda S., Yamagishi S., Kato S., Inagaki Y., Takeuchi M., Motomiya Y., Bucala R., Iida S., Tamaki K., Imaizumi T., Cooper M. E., Okuda S., *Kidney Int.*, **66**, 2137—2147 (2004).
31) Giardino I., Edelstein D., Brownlee M., *J. Clin. Invest.*, **94**, 110—117 (1994).
32) Yan S. D., Schmidt A. M., Anderson M., Zhang J., Brett J., Zou Y. S., Pinsky D., Stern D., *J. Biol. Chem.*, **269**, 9889—9897 (1994).
33) Koito W., Araki T., Horiuchi S., Nagai R., *J. Biochem.* (Tokyo), **136**,

831—837 (2004).

34) Lieuw-A-Fa M. L. M., van Hinsbergh V. W. M., Teerlink T., Barto R., Twisk J., Stehouwer C. D. A., Schalkwijk C. G., *Nephrol. Dial. Transplant.*, **19**, 631—636 (2004).

35) Fu M. X., Requena J. R., Jenkins A. J., Lyons T. J., Baynes J. W., Thorpe S. R., *J. Biol. Chem.*, **271**, 9982—9986 (1996).

36) Wendt T., Tanji N., Guo J., Hudson B. I., Bierhaus A., Ramasamy R., Arnold B., Nawroth P. P., Yan S. F., D'agati V., Schmidt A. M., *J. Am. Soc. Nephrol.*, **14**, 1383—1395 (2003).

37) Li J., Schmidt A. M., *J. Biol. Chem.*, **272**, 16498—16506 (1997).

38) Xie Q. W., Kashiwabara Y., Nathan C., *J. Biol. Chem.*, **269**, 4705—4708 (1994).

39) Lal S., Szwergold B. S., Taylor A. H., Randall W. C., Kappler F., Wells-Knecht K., Baynes J. W., Brown T. R., *Arch. Biochem. Biophys.*, **318**, 191—199 (1995).