JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 952–959

Received November 1, 2010
Accepted April 11, 2011
DOI
10.1111/j.2042-7158.2011.01299.x
ISSN 0022-3573

Research Paper

Hepato-/reno-protective activity of Chinese prescription Kangen-karyu through inhibition of AGE formation and fibrosis-related protein expression in type 2 diabetes

Takuya Okamoto[a,b,c], Chan Hum Park[a], Jeong Sook Noh[a], Kazuo Toriizuka[c], Yasuo Sei[b], Jong Cheol Park[d] and Takako Yokozawa[a]

[a]Institute of Natural Medicine, University of Toyama, Sugitani, Toyama, [b]Iskra Industry Co., Ltd, Nihonbashi, Chuo-ku, [c]School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo, Japan, and [d]Department of Oriental Medicine Resources and Research Institute of Korean Oriental Medicines, Sunchon National University, Suncheon, Jeonnam, Korea

Abstract

Objectives This study was conducted to examine whether Kangen-karyu, a Chinese prescription, has an ameliorative effect on diabetes-induced alterations such as advanced glycation endproduct (AGE) formation or the fibrotic response in liver and kidney of type 2 diabetic *db/db* mice.

Methods Kangen-karyu (100 or 200 mg/kg body weight/day, p.o.) was administered every day for 18 weeks to *db/db* mice, and its effect was compared with vehicle-treated *db/db* and *m/m* mice.

Key findings The administration of Kangen-karyu decreased the elevated serum glucose concentration in *db/db* mice. The increased serum creatinine and urea nitrogen levels, which reflect renal dysfunction in *db/db* mice, were significantly lowered by Kangen-karyu administration. The *db/db* mice exhibited the up-regulation of AGEs and its receptor expression in liver and kidney; however, Kangen-karyu treatment significantly reduced expression except for the receptor. Moreover, the augmented expressions of fibrosis-related proteins, transforming growth factor (TGF)-β1, fibronectin and collagen IV were down-regulated by Kangen-karyu administration.

Conclusions These results provide important evidence that Kangen-karyu exhibits a pleiotropic effect on AGE formation and fibrosis-related parameters, representing hepatoprotective and renoprotective effects against the development of diabetic complications in type 2 diabetic *db/db* mice.

Keywords AGE; *db/db* mice; fibrosis; Kangen-karyu; type 2 diabetes

Introduction

In diabetes, chronic hyperglycaemia promotes the formation and accumulation of advanced glycation endproducts (AGEs), which are heterogeneous products formed by non-enzymatic reactions between reducing sugars and free amino groups of proteins, lipids and nucleic acids. AGEs accumulate at sites of microvascular injury in diabetes, including the kidney, retina, vasculature and even liver, and interfere with protein function.[1,2] Especially, AGEs have been shown to exhibit a wide range of chemical, cellular and tissue effects implicated in the development and progression of diabetic nephropathy. Therefore, the design and discovery of inhibitors that either prevent the formation of AGEs or promote the degradation of existing AGEs offer a promising therapeutic approach for diabetes-related complications.[3–5]

AGEs are associated with the expression and activation of a number of pathogenic mediators implicated in the development of diabetic hepatic and renal disease including nonalcoholic steatohepatitis (NASH) and nephropathy. A recent study revealed that AGEs in patients with NASH augmented the proliferation and activation of hepatic stellate cells, a major contributor to liver fibrosis.[6,7] In addition, AGEs are thought to enhance the transcriptional up-regulation of transforming growth factor (TGF)-β1; this appears to be the key intermediate step for diabetic nephropathy.[8,9] Taken together, the formation of AGEs and TGF-β-induced fibrosis may markedly contribute to the functional and morphological alterations that result in diabetic complications in the liver and kidney.

Correspondence: Takako Yokozawa, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.
E-mail: yokozawa@inm.u-toyama.ac.jp

Traditional Chinese prescriptions have attracted much attention due to their beneficial effects observed during clinical experience accumulated over a long time. Among them, Kangen-karyu (Guan-Yuan-Ke-Li), a crude drug developed from a traditional Chinese prescription consisting of six herbs (Paeoniae Radix, Cnidii Rhizoma, Carthami Flos, Cyperi Rhizoma, Saussureae Radix and Salviae Miltiorrhizae Radix), has been clinically used as a treatment for cardiovascular diseases such as angina pectoris and for cerebrovascular disorders. Several studies demonstrated that Kangen-karyu exhibited biological activity such as platelet aggregation inhibition, hypertension suppression, the recovery of learning and memory impairment induced by senescence, neuroprotection and an anti-dementia effect in animal experiments.[10–14] In our previous studies, Kangen-karyu showed favorable ameliorative effects on symptoms of fructose-induced metabolic syndrome, such as hyperglycaemia, hyperlipidaemia and hypertension, through the reduction of triglyceride and cholesterol levels with sterol regulatory element-binding protein-1 expression, and also exhibited protective effects against diet-induced hypercholesterolaemia in rats.[15,16] In addition, we reported the beneficial effect of Kangen-karyu on hyperlipidaemia in streptozotocin-induced type 1 diabetic rats and type 2 diabetic *db/db* mice.[17,18] However, little is known regarding the protective effects of Kangen-karyu on damage to the liver and kidney caused by AGEs or the TGF-β pathway in type 2 diabetes. Therefore, we investigated the effects of Kangen-karyu on AGE formation and fibrosis-related parameters in type 2 diabetic tissue damage, especially in liver and kidney.

Materials and Methods

Materials

Protease inhibitor cocktail (Cat. #160–19501) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The Bio-Rad protein assay kit (Cat. #500–0006) and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). β-Actin, *o*-phthalaldehyde, phenylmethylsulfonyl fluoride (PMSF) and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. (St Louis, USA). Rabbit polyclonal antibodies against TGF-β1 (sc-146), fibronectin (sc-9068), receptor for AGEs (RAGE) (sc-5563) and collagen IV (ab6586) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and Abcam, Ltd (Abcam, Cambridge, UK). Monoclonal anti-N^{ε}-(carboxyethyl)lysine (CEL) antibody and anti-glycolaldehyde (GA)-pyridine antibody, and polyclonal anti-N^{ε}-(carboxymethyl)lysine (CML) antibody were kindly provided by Dr R. Nagai (Kumamoto University, Japan). Goat anti-rabbit and goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. ECL Western Blotting Detection Reagents were purchased from GE Healthcare (Piscataway, USA).

Preparation of Kangen-karyu extract

Kangen-karyu in the form of a dried powder extract was supplied by Iskra Co., Ltd (Tokyo, Japan). The composition of Kangen-karyu used in this study was: 2.25 g of Paeoniae Radix (*Paeonia lactiflora* Pallas root, Paeoniaceae), 2.25 g of Cnidii Rhizoma (*Cnidium officinale* Makino rhizome, Umbelliferae), 2.25 g of Carthami Flos (*Carthamus tinctrius* L. petal, Compositae), 1.125 g of Cyperi Rhizoma (*Cyperus rotundus* L. rhizome, Cyperaceae), 1.125 g of Aucklandiae Radix (*Aucklandia lappa* Dcne. root, Compositae) and 4.5 g of Salviae Miltiorrhizae Radix (*Salvia miltiorrhiza* Bunge root, Labiatae). This prescription was extracted with 25 volumes of water at 100°C for 1 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 44%, by weight, of the starting materials. Each sample was dissolved in 50% aqueous ethanol with sonication, and filtered through a Cosmonice filter (PVDF, 0.45 μm; Nacalai Tesque, Inc., Kyoto, Japan). Reverse-phase high-performance liquid chromatography was performed using a Cosmosil $5C_{18}$-AR II column (250 × 4.6 mm i.d.; Nacalai Tesque, Inc.) with elution gradients of 4–30% (39 min) and 30–75% (15 min) CH_3CN in 50 mM H_3PO_4 at a flow rate of 0.8 ml/min. The UV absorbance from 200 to 400 nm was monitored with a JASCO MD-910 photodiode array detector (Jasco, Tokyo, Japan). All assigned peaks were identified by carrying out co-injection tests with authentic samples and comparing with UV spectral data. Figure 1 shows the chromatogram obtained for Kangen-karyu. The major compounds detected were paeoniflorin, pentagalloyl glucose, rosmarinic acid, lithospermic acid and lithospermic acid B. A voucher specimen was deposited in the herbarium of the University of Toyama.

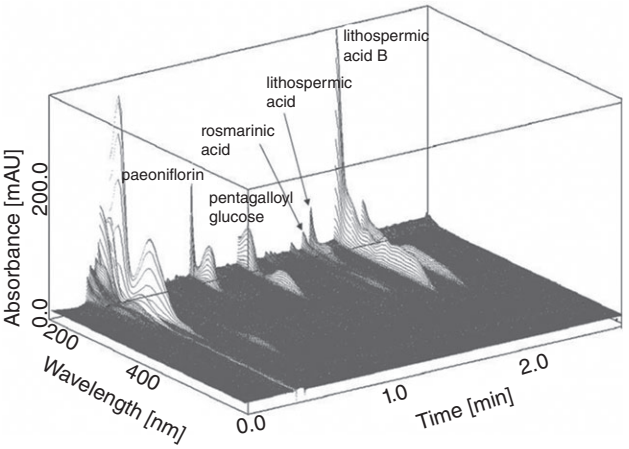

Figure 1 Three-dimensional HPLC analysis of Kangen-karyu showing its major compounds.

Experimental animals and treatment

Animal experiments were performed according to the 'Guidelines for Animal Experimentation' approved by the Ethics Committee of the University of Toyama (Registration No. S-2006 INM-22). Six-week-old male C57BLKS/J *db/db* and age-matched non-diabetic *m/m* mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Mice were maintained under a 12-h light–dark cycle and housed at a controlled temperature (23 ± 3°C) and humidity (about 60%). The mice were allowed free access to laboratory pellet chow (comprising 24.0% protein, 3.5% lipids and 60.5% carbohydrate;

CLEA Japan Inc., Tokyo, Japan) and water. After adaptation (at 6 weeks of age), the glucose level of blood taken from the tail vein was measured, and then *db/db* mice were divided into three groups ($n = 8$/group). Kangen-karyu was orally administered every day at a dose of 100 or 200 mg/kg body weight, respectively, by gavage, while vehicle-treated *db/db* mice were orally given water. The dose of Kangen-karyu used in this study was chosen based on the data obtained in our previous studies.[15–18] The non-diabetic *m/m* mice ($n = 6$) as a normal control group were used for comparisons with diabetic groups. The body weight, food intake and water intake were measured every day during the administration period. After 18 weeks of administration, blood samples were collected by cardiac puncture from anaesthetized mice. The serum was immediately separated from blood samples by centrifugation. Subsequently, mice were perfused with ice-cold physiological saline after cardiac puncture, and the liver and kidney were harvested, snap-frozen in liquid nitrogen and stored at −80°C until analyses.

Measurement of serum parameters

Serum glucose was measured using a commercial kit (Glucose CII-Test, Cat. #439–90901; Wako Pure Chemical Industries, Ltd, Osaka, Japan). Serum leptin (Cat. #200726; Morinaga Institute of Biological Science, Yokohama, Japan) levels were measured based on enzyme-linked immunosorbent assays.

Measurement of hepatic and renal functional parameters

Hepatic functional parameters (alanine aminotransferase (ALT), aspartate aminotransferase (AST)) were measured using a Wako kit (Transaminase CII-Test, Cat. #431–30901). Renal functional parameters (creatinine and urea nitrogen) were measured using CRE-EN Kainos (Cat. #000215) and BUN Kainos (Cat. #000048), obtained from Kainos Laboratory Inc. (Tokyo, Japan).

Measurement of hepatic and renal glucose content

The hepatic and renal glucose levels were determined by employing the method of Momose *et al*.,[19] with minor modifications. Hepatic and renal tissue were homogenized with ice-cold 0.9% NaCl buffer, and then the homogenate was deproteinized with 0.15 M $Ba(OH)_2$ and 5% $ZnSO_4$. The supernatant was obtained by centrifugation at 1670*g* for 15 min, and then the glucose level was evaluated using a Wako kit (Glucose CII-Test; Cat. #439–90901), as described above.

Preparation of post-nuclear fraction

The post-nuclear fraction was extracted from the liver and kidney of each mouse. In brief, the hepatic and renal tissue were homogenized with ice-cold lysis buffer (pH 7.4) containing 137 mM NaCl, 20 mM Tris-HCl, 1% Tween 20, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail. The homogenate was then centrifuged at 2000*g* for 10 min at 4°C. The protein concentration of post-nuclear fraction was determined using a Bio-Rad protein kit (Cat. #500–0006; Bio-Rad Laboratories, Hercules, USA).

Western blot analyses

For the determination of RAGE, CEL, CML, GA-pyridine, TGF-β1, fibronectin and collagen IV, 30 μg of protein from each post-nuclear fraction was electrophoresed through an 8–15% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane, blocked with 5% (w/v) skimmed milk solution for 1 h, and then incubated with respective primary antibodies overnight at 4°C. After the blots were washed, they were incubated with IgG HRP-conjugated secondary antibody for 1.5 h at room temperature. Each antigen–antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-4000 (Fujifilm, Tokyo, Japan). Band densities were determined using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to β-actin. The protein expression levels are expressed relative to those of *m/m* mice (represented as 1).

Statistical analysis

Data are expressed as means ± SEM. Significance was assessed by one-way analysis of variance followed by Dunnett's multiple comparison test (SPSS 11.5.1 for Windows, 2002; SPSS Inc., USA). $P < 0.05$ was considered significant.

Results

General characteristics

After the 18-week treatment, *db/db* mice showed a significant increase in body weight gain and liver and kidney weights compared with *m/m* mice (Table 1). However, there were no significant weight changes among the experimental *db/db* mice groups (Veh, K-100 and K-200). Also, *db/db* mice exhibited type 2 diabetic characteristics, such as hyperglycaemia and hyperleptinaemia compared with *m/m* mice, but Kangen-karyu administration significantly reduced serum glucose and leptin concentrations at a dose of 200 mg/kg. Typical hepatic functional parameters, serum ALT and AST levels, among *db/db* groups showed no difference (Table 2). The serum creatinine and urea nitrogen levels, renal functional parameters, in *db/db* vehicle mice were elevated compared with *m/m* mice (1.57 fold and 1.47 fold, respectively), whereas these augmented levels were significantly decreased in Kangen-karyu 200 mg/kg-administered *db/db* mice (83.3% ($P < 0.05$) and 78.2% ($P < 0.01$), respectively) (Table 2).

Hepatic and renal glucose levels

The levels of glucose in both liver and kidney of the vehicle-treated *db/db* mice were significantly elevated compared with those of *m/m* mice (Figure 2). Kangen-karyu administration led to marked reduction of the hepatic and renal glucose levels of *db/db* mice from the lower dose.

AGE-related protein expression in liver and kidney

Since Kangen-karyu treatment attenuated hyperglycaemia and decreased glucose levels in the liver and kidney, we further investigated expression of AGE-related proteins,

Table 1 Biochemical analyses

Item	*m/m*	*db/db*		
		Veh	K-100	K-200
Body weight gain (g/18 weeks)	4.7 ± 0.2***	18.9 ± 1.1	17.9 ± 1.5	18.7 ± 1.4
Tissue weight (g)				
Liver	1.47 ± 0.10***	4.08 ± 0.20	3.90 ± 0.36	3.62 ± 0.59
Kidney	0.31 ± 0.01**	0.39 ± 0.01	0.42 ± 0.01	0.43 ± 0.02
% of tissue weight (g/100 g body weight)				
Liver	5.75 ± 0.37**	7.75 ± 0.41	7.41 ± 0.61	7.02 ± 1.24
Kidney	1.23 ± 0.08***	0.75 ± 0.03	0.80 ± 0.05	0.82 ± 0.05
Serum glucose (mg/dl)	136.6 ± 4.1***	483.3 ± 10.3	442.8 ± 24.6	436.6 ± 13.4*
Serum leptin (ng/ml)	1.95 ± 0.31***	19.87 ± 0.91	19.27 ± 1.11	15.43 ± 0.33**

m/m, Misty; Veh, vehicle-treated *db/db* mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± SEM ($n = 6$ or 8). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs *db/db* vehicle-treated mouse values.

Table 2 Hepatic and renal function parameters in serum

Parameter	*m/m*	*db/db*		
		Veh	K-100	K-200
ALT (IU/l)	36.80 ± 2.37*	92.92 ± 15.07	83.81 ± 8.24	80.85 ± 12.95
AST (IU/l)	11.17 ± 0.46***	56.74 ± 3.97	50.96 ± 6.61	42.19 ± 7.34
Creatinine (mg/dl)	0.23 ± 0.01***	0.36 ± 0.02	0.30 ± 0.02*	0.30 ± 0.02*
Urea nitrogen (mg/dl)	15.24 ± 0.51**	22.44 ± 2.03	18.46 ± 1.33	17.54 ± 0.75**

m/m, Misty; Veh, vehicle-treated *db/db* mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± SEM ($n = 6$ or 8). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs *db/db* vehicle-treated mouse values.

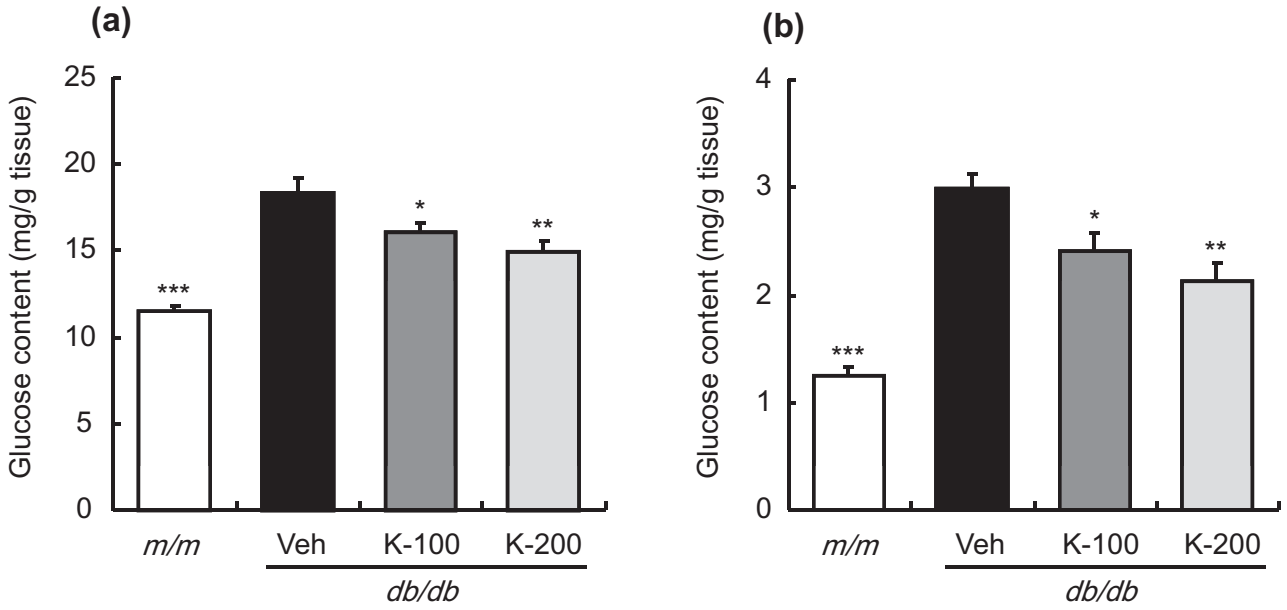

Figure 2 Effect of Kangen-karyu on mouse hepatic (a) and renal (b) glucose content. *m/m*, misty; Veh, vehicle-treated *db/db* mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± SEM ($n = 6$ or 8). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs vehicle-treated *db/db* mouse values.

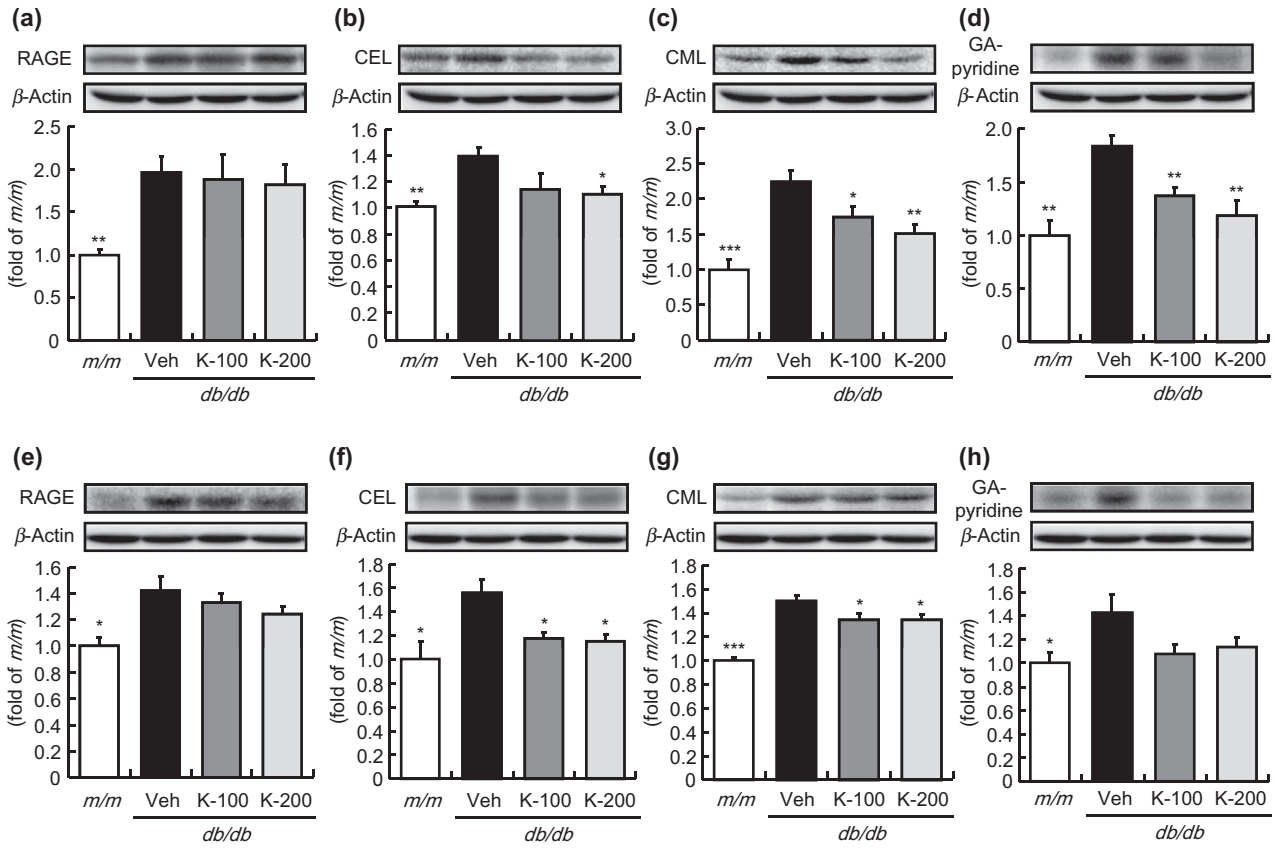

Figure 3 Effect of Kangen-karyu on mouse hepatic and renal AGE-related protein expression. Representative Western blotting analysis of hepatic tissue ((a) RAGE, (b) CEL, (c) CML and (d) GA-pyridine) and renal tissue ((e) RAGE, (f) CEL, (g) CML and (h) GA-pyridine). *m/m*, misty; Veh, vehicle-treated *db/db* mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± SEM ($n = 6$ or 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle-treated *db/db* mouse values.

such as RAGE, CEL, CML and GA-pyridine by employing Western blotting analyses. Expression levels of AGE-related proteins were enhanced in the *db/db* mice liver and kidney compared with those of *m/m* mice (Figure 3). In Kangen-karyu-treated *db/db* mice, up-regulated RAGE protein expression exhibited no changes in the liver and kidney (Figure 3a and 3e); however, the increased expression of CEL, CML and GA-pyridine were significantly attenuated in the liver and kidney (Figure 3b–3d, 3f and 3g), except for renal GA-pyridine (Figure 3h).

Fibrosis-related protein expression

Next, we quantified TGF-β1, fibronectin and collagen IV protein expression (Figure 4). The fibrosis-related protein expression in vehicle-treated *db/db* mice was significantly augmented in both liver and kidney compared with *m/m* mice. However, treatment with Kangen-karyu suppressed these proteins in the liver and kidney of *db/db* mice; especially, collagen IV was attenuated nearly to normal levels in both 100 and 200 mg Kangen-karyu-treated groups.

Discussion

Diabetes is a metabolic disorder that generates unfavorable alterations in various tissues and causes complications triggered by hyperglycaemia, dyslipidaemia, oxidative stress, inflammation and advanced glycation.[20] Among these pathogenic factors in diabetes, hyperglycaemia-induced oxidative and carbonyl stress (so-called glucotoxicity) plays a central role in the initiation and progression of diseases related to diabetes.[21] In this study, C57BLKS/J *db/db* mice developed obesity-induced diabetes, showing hyperglycaemia and hyperleptinaemia, as a result of leptin resistance as well as uncontrolled food intake and systemic fuel metabolism.[22,23] However, Kangen-karyu administration moderated hyperglycaemia and hyperleptinaemia. Also, hepatic and renal functional parameters, such as ALT, AST, creatinine and urea nitrogen levels, were all increased in the serum of type 2 diabetic *db/db* mice; however, ALT and AST were slightly reduced and creatinine and urea nitrogen were significantly lowered by Kangen-karyu treatment. These results show that Kangen-karyu treatment may ameliorate diabetic pathological conditions induced by hyperglycaemia and hyperleptinaemia, and may lead to improvement in the impaired hepatic and renal function of *db/db* mice.

Nonenzymatic glycation is caused by the reaction of sugar and amino moiety, and in the last stage, stable glycation products, AGEs, are formed. Protein glycation and AGE formation are accompanied by increased free radical activity that contributes to the molecular damage in diabetes. The

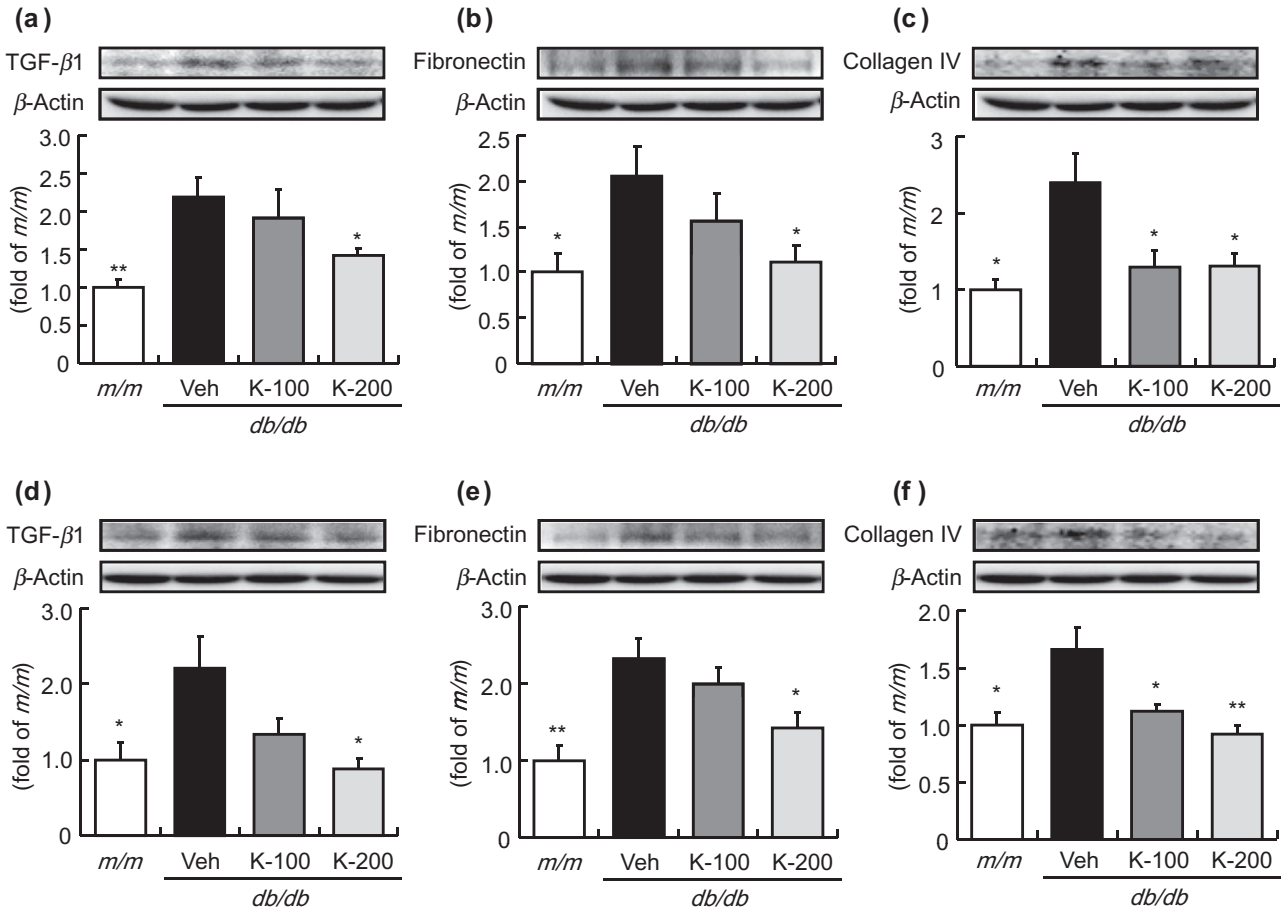

Figure 4 Effect of Kangen-karyu on mouse hepatic and renal fibrosis-related protein expression. Representative Western blotting analysis of hepatic tissue ((a) TGF-β1, (b) fibronectin and (c) collagen IV) and renal tissue ((d) TGF-β1, (e) fibronectin and (f) collagen IV). *m/m*, misty; Veh, vehicle-treated *db/db* mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± SEM ($n = 6$ or 8). *$P < 0.05$, **$P < 0.01$ vs vehicle-treated *db/db* mouse values.

formation and accumulation of AGEs in various tissues are known to irreversibly progress during normal aging and at an extremely accelerated rate in diabetes due to hyperglycaemia. Therefore, we investigated whether Kangen-karyu treatment effectively suppressed the formation of AGEs, such as CEL, CML and GA-pyridine, which are thought to be major AGE products.[24,25] One of the major AGEs, CEL, is reported to form during the reaction of methylglyoxal with lysine residues and it accumulates in human lens proteins with age.[26] Also, CML accumulates with lipid peroxidation in glomerular lesions, resulting in structural and functional alterations in extracellular matrix (ECM) proteins.[27] In addition, GA is a Maillard-reaction intermediate and can be formed by the reaction of L-serine with the myeloperoxidase system. GA reacts with proteins to form AGEs, such as GA-pyridine, which is specific for protein modification by GA. It was reported that GA-pyridine accumulates in atherosclerotic lesions and in the mesangium in renal disease.[25,28] Moreover, RAGE is activated by AGEs, and AGE–RAGE interaction increases reactive oxygen species (ROS) generation with the subsequent activation of nuclear factor (NF)-κB and release of pro-inflammatory cytokines.[29] In this study, type 2 diabetic *db/db* mice showed an augmented hepatic and renal glucose content; however, Kangen-karyu treatment significantly reduced those levels. The glucose-lowering activity of Kangen-karyu, at least in part, influenced the down-regulated expression of hepatic and renal AGEs in *db/db* mice. However, enhanced RAGE expression in *db/db* vehicle mice was not changed by Kangen-karyu treatment. These results suggested that Kangen-karyu could modulate hepatic and renal AGE formation via lowering the tissue glucose content, which was not associated with RAGE expression.

Tissue fibrosis is characterized by the excessive production, deposition and contraction of the ECM, which is associated with diabetic nephropathy, liver cirrhosis and arteriosclerosis.[30,31] The TGF-β pathway plays a critical role in renal pathophysiology by promoting ECM deposition and fibrosis; TGF-β promotes fibroblast proliferation and increases the synthesis of a number of ECM proteins, including collagens and fibronectin, via binding to serine/threonine kinase receptors on the plasma membrane and subsequently activating Smad molecules and additional signalling proteins that coordinately regulate gene expression or cytoplasmic processes.[32] In fact, TGF-β is reported to express in excess in areas of progressive ECM accumulation, and TGF-β blockade successfully inhibited renal sclerotic and fibrotic changes.[33,34]

In this study, experimental type 2 diabetes resulted in the increased protein expression of TGF-β1, fibronectin and collagen IV, whereas these elevated expressions were markedly reduced by Kangen-karyu administration in both the liver and kidney. Of note, fibronectin (liver), TGF-β1 (kidney) and collagen IV (liver and kidney) expressions were fully recovered, to the levels of *m/m* mice, by Kangen-karyu treatment at a dose of 200 mg/kg. These results demonstrated that the anti-fibrotic effects of Kangen-karyu are associated with the down-regulation of fibrotic cytokines, TGF-β1 and the ECM components fibronectin and collagen IV in the liver and kidney of type 2 diabetic mice.

Regarding the initiation and progression of diabetic tissue damage in the liver and kidney, a complicated reciprocal relationship with hyperglycaemia, AGEs and fibrosis seems to exist. There is increasing evidence about the relationship between AGE and TGF-β. That is, the diabetic condition activates the TGF-β-dependent Smad signalling pathway to stimulate collagen synthesis,[35] and also AGE-RAGE-mediated ROS generation activates TGF-β-Smad signalling and subsequently induces hypertrophy and fibrosis by autocrine production of angiotensin II.[36] In this study we found that Kangen-karyu administration to *db/db* mice could markedly inhibit AGE formation as well as fibrosis-related protein expression, and thus we successfully confirmed the efficacy of Kangen-karyu on this pathway. However, in addition to the TGF-β-dependent pathway, there is other evidence that AGEs induce connective tissue growth factor and promote ECM accumulation,[37] but also that the mitogen-activated protein kinase (MAPK)-Smad signalling cross-talk pathway is a key mechanism in diabetic scarring, because AGE-induced Smad activation and collagen synthesis are inhibited by ERK/p38 MAPK inhibitors, but not by TGF-β blockade, representing the TGF-β-independent pathway.[38,39] Hence, these mechanistic details of Kangen-karyu need to be identified in future studies.

Although the detailed mechanisms of Kangen-karyu against diabetes were not clarified in this study, Kangen-karyu significantly reduced the serum triglyceride and AGE levels, the lipid peroxide levels in both the serum and hepatic tissue, and the expression volumes of CML, RAGE, NF-κB, inducible nitric oxide synthase and cyclooxygenase-2, that were increased in streptozotocin-induced diabetic rats.[17] These effects of Kangen-karyu may be attributed to its antioxidant activity, resulting from the harmonization of its six components, because all components are commonly known to be rich in antioxidant compounds.[40–45] Similar effects, in which Kangen-karyu may improve oxidative stress via the regulation of dyslipidaemia, were observed in type 2 diabetic *db/db* mice.[18,46] It is well known that antioxidant agents effectively regulate biochemical factors due to oxidative stress.[47–50] Based on our continual studies, along with the present experiment, Kangen-karyu may function as an ameliorator of oxidative stress and show beneficial effects on diabetes and diabetic complications.

We also carried out an investigation of the side effects or toxicity of Kangen-karyu (data not shown). The results showed normal ranges of haematological data, such as ALT, AST, creatinine and urea nitrogen, as well as changes in body and tissue weights, although the maximum concentration for oral administration was of a higher level (1000 mg/kg body weight/day) than the dose used for clinical administration (7.5 g/day, 125 mg/kg body weight/day). Therefore, we suggest that Kangen-karyu is safe and non-toxic.

Conclusions

This study supports the concept that, in hyperglycaemia, AGE formation and TGF-β-related fibrosis are associated with the progression of diabetic complications. Kangen-karyu treatment effectively alleviated these unfavorable conditions in the presence of diabetic damage of the liver and kidney. Therefore, this study suggests that Kangen-karyu exerts its hepatoprotective and renoprotective potential through the inhibition of AGE formation and fibrosis-related diabetic complications in the liver and kidney of type 2 diabetics.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

1. Ramasamy R *et al*. Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* 2005; 15: 16R–28R.
2. Ahmed N, Thornalley PJ. Advanced glycation endproducts: what is their relevance to diabetic complications? *Diabetes Obes Metab* 2007; 9: 233–245.
3. Peyroux J, Sternberg M. Advanced glycation endproducts (AGEs): pharmacological inhibition in diabetes. *Pathol Biol (Paris)* 2006; 54: 405–419.
4. Reddy VP, Beyaz A. Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. *Drug Discov Today* 2006; 11: 646–654.
5. Desai K, Wu L. Methylglyoxal and advanced glycation endproducts: new therapeutic horizons? *Recent Pat Cardiovasc Drug Discov* 2007; 2: 89–99.
6. Hyogo H *et al*. Elevated levels of serum advanced glycation end products in patients with non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2007; 22: 1112–1119.
7. Iwamoto K *et al*. Advanced glycation end products enhance the proliferation and activation of hepatic stellate cells. *J Gastroenterol* 2008; 43: 298–304.
8. Lal MA *et al*. Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. *Kidney Int* 2002; 61: 2006–2014.
9. Yamagishi S *et al*. Advanced glycation end products inhibit de novo protein synthesis and induce TGF-β overexpression in proximal tubular cells. *Kidney Int* 2003; 63: 464–473.
10. Takahashi M *et al*. *Kangenkaryu* prevents the decrease of cholinergic markers following the nucleus basalis magnocellularis lesion. *Jpn J Pharmacol* 1992; 60: 307–310.
11. Gao M *et al*. Studies on preventive effect of 'Kangenkaryu', Chinese herbal medicine, on stroke in SHR-SP. *J Trad Med* 2001; 18: 245–250.

12. Makino T *et al*. Effects of Kangen-karyu on coagulation system and platelet aggregation in mice. *Biol Pharm Bull* 2002; 25: 523–525.
13. Pu F *et al*. Ameliorating effects of Kangen-karyu on neuronal damage in rats subjected to repeated cerebral ischemia. *J Nat Med* 2010; 64: 167–174.
14. Zhao Q *et al*. Kangen-karyu improves memory deficit caused by aging through normalization of neuro-plasticity-related signaling system and VEGF system in the brain. *J Ethnopharmacol* 2010; 131: 377–385.
15. Yokozawa T *et al*. The protective role of Chinese prescription Kangen-karyu extract on diet-induced hypercholesterolemia in rats. *Biol Pharm Bull* 2006; 29: 760–765.
16. Yokozawa T *et al*. The protective role of Kangen-karyu against fructose-induced metabolic syndrome in a rat model. *J Pharm Pharmacol* 2007; 59: 1271–1278.
17. Kim HY *et al*. Beneficial effects of Chinese prescription Kangen-karyu on diabetes associated with hyperlipidemia, advanced glycation endproducts, and oxidative stress in streptozotocin-induced diabetic rats. *J Ethnopharmacol* 2009; 124: 263–269.
18. Yamabe N *et al*. Effect of Chinese prescription Kangen-karyu on lipid metabolism in type 2 diabetic *db/db* mice. *J Ethnopharmacol* 2010; 129: 299–305.
19. Momose T *et al*. Organic analysis. XLIV. A new deproteinizing agent for determination of blood sugar. *Chem Pharm Bull* 1963; 11: 968–972.
20. Stumvoll M *et al*. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 2005; 365: 1333–1346.
21. Poitout V, Robertson RP. Glucolipotoxicity: fuel excess and β-cell dysfunction. *Endocr Rev* 2008; 29: 351–366.
22. Jéquier E. Leptin signaling, adiposity, and energy balance. *Ann NY Acad Sci* 2002; 967: 379–388.
23. Sharma K *et al*. Diabetic kidney disease in the *db/db* mouse. *Am J Physiol Renal Physiol* 2003; 284: F1138–F1144.
24. Bierhaus A *et al*. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. *Cardiovasc Res* 1998; 37: 586–600.
25. Greven WL *et al*. Mesangial accumulation of GA-pyridine, a novel glycolaldehyde-derived AGE, in human renal disease. *Kidney Int* 2005; 68: 595–602.
26. Ahmed MU *et al*. N^{ε}-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 1997; 324: 565–570.
27. Heidland A *et al*. Advanced glycation end products and the progressive course of renal disease. *Am J Kidney Dis* 2001; 38: S100–S106.
28. Nagai R *et al*. Identification in human atherosclerotic lesions of GA-pyridine, a novel structure derived from glycolaldehyde-modified proteins. *J Biol Chem* 2002; 277: 48905–48912.
29. Bierhaus A *et al*. RAGE in inflammation: a new therapeutic target? *Curr Opin Investig Drugs* 2006; 7: 985–991.
30. Bedossa P, Paradis V. Liver extracellular matrix in health and disease. *J Pathol* 2003; 200: 504–515.
31. Kanwar YS *et al*. Diabetic nephropathy: mechanisms of renal disease progression. *Exp Biol Med* 2008; 233: 4–11.
32. Verrecchia F *et al*. Transforming growth factor-β signaling through the Smad proteins: role in systemic sclerosis. *Autoimmun Rev* 2006; 5: 563–569.
33. Ziyadeh FN. Mediators of diabetic renal disease: the case for TGF-β as the major mediator. *J Am Soc Nephrol* 2004; 15: S55–S57.
34. Qi W *et al*. Transcriptional regulation of the TGF-β1 promoter by androgen receptor. *Biochem J* 2008; 416: 453–462.
35. Li JH *et al*. Role of TGF-β signaling in extracellular matrix production under high glucose conditions. *Kidney Int* 2003; 63: 2010–2019.
36. Fukami K *et al*. AGEs activate mesangial TGF-β-Smad signaling via an angiotensin II type I receptor interaction. *Kidney Int* 2004; 66: 2137–2147.
37. Zhou G *et al*. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor β-independent pathway. *Am J Pathol* 2004; 165: 2033–2043.
38. Li JH *et al*. Advanced glycation end products activate Smad signaling via TGF-β-dependent and -independent mechanisms: implications for diabetic renal and vascular disease. *FASEB J* 2004; 18: 176–178.
39. Yang F *et al*. Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-β-dependent and -independent Smad pathways. *Hypertension* 2009; 54: 877–884.
40. Ohta S *et al*. Studies on chemical protectors against radiation. XXX. Radioprotective substances of Cnidii Rhizome. *Yakugaku Zasshi* 1990; 110: 746–754.
41. Lee SC *et al*. Antioxidative constituents from *Paeonia lactiflora*. *Arch Pharm Res* 2005; 28: 775–783.
42. Zhou L *et al*. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* 2005; 45: 1345–1359.
43. Kim EO *et al*. Antioxidant properties and quantification of phenolic compounds from safflower (*Carthamus tinctorius* L.) seeds. *Food Sci Biotechnol* 2007; 16: 71–77.
44. Pandey MM *et al*. *Saussurea costus*: botanical, chemical and pharmacological review of an ayurvedic medicinal plant. *J Ethnopharmacol* 2007; 110: 379–390.
45. Yazdanparast R, Ardestani A. In vitro antioxidant and free radical scavenging activity of *Cyperus rotundus*. *J Med Food* 2007; 10: 667–674.
46. Noh JS *et al*. Chinese prescription Kangen-karyu prevents dyslipidaemia and oxidative stress in mouse model of type 2 diabetes. *J Pharm Pharmacol* 2011; 63: 111–119.
47. Scott JA, King GL. Oxidative stress and antioxidant treatment in diabetes. *Ann NY Acad Sci* 2004; 1031: 2004–2013.
48. Aragno M *et al*. Up-regulation of advanced glycated products receptors in the brain of diabetic rats is prevented by antioxidant treatment. *Endocrinology* 2005; 146: 5561–5567.
49. Lapolla A *et al*. Importance of measuring products of non-enzymatic glycation of proteins. *Clin Sci* 2005; 38: 103–115.
50. Yagmur E *et al*. Elevation of N^{ε}-(carboxymethyl)lysine-modified advanced glycation end products in chronic liver disease is an indicator of liver cirrhosis. *Clin Biochem* 2006; 39: 39–45.