Antihyperglycemic Effect of *Cephalotaxus sinensis* Leaves and GLUT-4 Translocation Facilitating Activity of Its Flavonoid Constituents

Wei LI,[a,b] Rong-Ji DAI,[a,b] Yu-Hong YU,*[,a,b] Liang LI,[a,b] Chong-Ming WU,[a] Wei-Wei LUAN,[a] Wei-Wei MENG,[a,b] Xin-Sheng ZHANG,[b] and Yu-Lin DENG*[,a]

[a] *School of Life Science and Technology, Beijing Institute of Technology; Beijing 100081, P. R. China: and* [b] *Beijing BIT & GY Pharmaceutical R&D; Beijing 100081, P. R. China.* Received November 3, 2006; accepted February 16, 2007

The objectives of this study were to investigate the antihyperglycemic effect of *Cephalotaxus sinensis* leaves and to identify the active components. The antihyperglycemic effect of various fractions (FA, FB, FC, FD) of the 80% ethanol extract of the leaves was evaluated in streptozotocin (STZ)-induced diabetic rats. Among the tested fractions, FC was the most active. FC (0.48 g/kg) given orally for 10 d reduced significantly ($p<0.001$) the blood glucose of STZ-induced diabetic rats. The food and water intakes of FC (0.48 g/kg)-treated diabetic rats were reduced significantly ($p<0.001$) when compared to the 0.5% carboxymethyl cellulose (CMC)-treated diabetic rats. The activity-guided fractionation of the ethanol extract of *C. sinensis* leaves furnished three flavonoid compounds, apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (1), apigenin (2), and apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-glucopyranoside] (3). The elevation of GLUT-4 protein level in membrane preparations from mice adipocytes was detected by Western blot analysis after adipocytes were preincubated with FC (0.1, 1, 10 mg/ml), apigenin (0.1, 2 mg/ml) and apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (0.1, 2 mg/ml), respectively. Phytochemical investigation and HPLC-DAD analysis of FC indicated that the flavonoids were the major constituents in this fraction. These results suggest that the fraction from *C. sinensis* leaves is a promising drug for the treatment of diabetes, and that the flavonoids from this plant are the active constituents.

Key words *Cephalotaxus sinensis*; antihyperglycemic effect; GLUT-4; translocation; apigenin; HPLC-DAD

Type 1 diabetes and type 2 diabetes (T2D) rank highly among the top 10 causes of mortality throughout the world. Diabetes patients, particularly those with T2D are at considerable risk of excessive morbidity and mortality from cardiovascular, cerebrovascular, and peripheral vascular diseases leading to myocardial infarction, strokes, and amputations.[1)]

With the rapid advancements in medicine, novel treatments with fewer side effects have become feasible for the long-term control of this disorder. Recent scientific investigation has confirmed the efficacy of many of plant medicines on diabetes mellitus (DM), some of which are remarkably effective.[2)] The recommendation of the WHO committee on DM encouraged research on hypoglycemic agents of plant origin used in Chinese traditional medicine and it has greatly motivated research in this area.[3)]

Insulin-stimulated glucose utilization is the major site for regulation of plasma glucose concentrations.[4)] Glucose transport is the rate-limiting step in carbohydrate metabolism.[5)] A family of glucose transporters (GLUT) mediates glucose transport across the cell membrane, and the subtype 4 form (GLUT-4) is the insulin sensitive glucose transporter. Reduction in insulin-mediated glucose uptake caused by decreasing GLUT-4 protein in diabetes, especially T2D, was observed.[6,7)] Compounds that facilitate GLUT-4 translocation can be potentially beneficial for the treatment of diabetes, especially for T2D.

Cephalotaxus sinensis (REHD *et* WILE) LI, a medicinal plant and widely distributed in southern China, has been used as a Chinese traditional medicine for treatment of dyspepsia, ascariasis, inflammation, and cough.[8)] Alkaloids, flavonoids, biflavonoids, and diterpenes were reported to be constituents of *Cephalotaxus* species.[9—11)] It was reported that the ester type alkaloids possessed antitumour activity.[8)] Recently, osteoblast differentiation stimulating activity of biflavonoids from *Cephalotaxus koreana* NAKAI (Cephalotaxaceae) have been reported.[12)]

To the best of our knowledge, there are no reports on the antihyperglycemic activity of *C. sinensis* in the literature. As part of a series of experiments to obtain natural products with antidiabetic efficacy from plants, the antihyperglycemic activity of various fractions of the ethanol extract of *C. sinensis* leaves were evaluated in chronic STZ-induced diabetic rats. The activity of the isolated compounds from the fraction was estimated at the cellular level *in vitro*. The study elucidated for the first time the biologic properties, namely the activity of facilitating GLUT-4 translocation, of the fraction and its flavonoid constituents from *C. sinensis*. Furthermore, we characterized the main components of the fraction by using phytochemical and HPLC-DAD methods.

MATERIALS AND METHODS

Plant Material The leaves of *C. sinensis* were collected from Liangtian, Dongzhi, Anhui province, P. R. China, in May 2003. The plant was identified by Dr. Sheng-Yuan Xiao (School of Life Science and Technology, Beijing Institute of Technology, Beijing, P. R. China) and Associate Researcher Bin Wen (Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, P. R. China), and a voucher specimen (No. S200305) has been deposited in the lab of the School of Life Science and Technology, Beijing Institute of Technology.

HPLC-DAD Conditions An Elite P230 series HPLC-DAD system consisting of a binary pump and a DAD detector (Elite DAD230, P. R. China) was used for acquiring chromatograms and UV spectra. The HPLC condition was as follows: column, Diamonsil™ C_{18} column (5 μm, 250×4.6 mm, Dikama, P. R. China); column temperature, 25 °C; mobile

* To whom correspondence should be addressed. e-mail: Deng@bit.edu.cn; yyh72@bit.edu.cn

phases: linear gradient system of solvent A (H_2O+0.1% acetic acid) and solvent B (acetonitrile), B/A 10/90 (0 min), 15/85 (10 min), 30/70 (20 min), 70/30 (30 min), 100/0 (35 min); flow-rate, 1 ml/min; detecting wavelength, 200—400 nm; injection volume: 20 μl.

Animals Male rats (Wistar strain, weighing 190—210 g) bred and maintained in our animal house were used for these studies.

Antibody Rabbit polyclonal anti-GLUT-4 antibody was raised against the C-terminal 12-amino acid sequence of rat GLUT-4. This antiserum has been successfully used for immunoblotting at 1 : 100 dilution. Goat anti Rabbit IgG was conjugated to HRP.

Activity-Guided Fractionation and Isolation The dried leaves of *C. sinensis* (600 g) were blended and extracted with 80% ethanol (6 l×3) until exhaustion. The liquid extracts were removed of solvent *in vacuo* to afford a residue (149 g), which was re-suspended in H_2O (6 l). The H_2O phase was filtered and the filtrate was divided into two portions (3 l each). One portion was evaporated to dryness under reduced pressure to afford a residue (FA, 66 g). The other portion was loaded onto a column containing AB-8 resin (1.2 kg), and then eluted sequentially with H_2O, 70% and 95% aqueous solution of EtOH (3 l each). The fractions eluted from H_2O (FB), 70% (FC), 95% (FD) aqueous solution of EtOH were evaporated to dryness under reduced pressure using a rotary evaporator, affording 36, 24, and 3 g, respectively. FC was found to be the most active fraction. One portion of FC (12 g) was further separated on a silica gel column chromatography (200—300 mesh, 1.0 kg) with a gradient system of $CHCl_3$/MeOH/H_2O (80 : 20 : 0→65 : 35 : 1) as elute to afford compound **1** (550 mg), compound **2** (950 mg) and compound **3** (1.1 g).

Induction of Experimental Diabetes Mellitus The overnight fasted rats were made diabetic with STZ (Sigma, St. Louis, MO, U.S.A., 48 mg/kg, i.p). The STZ was freshly dissolved in citrate buffer (0.1 M, pH 3.9) and maintained on ice prior to use; the injection volume was 1 ml/kg. Diabetes was confirmed in the STZ-treated rats by measuring the fasting blood glucose concentration 4 d after injecting STZ. The rats with blood glucose level above 18 mmol/l were considered to be diabetic and were used in the experiment. Animals had free access to food and water after the STZ injection.

Experimental Groups The diabetic animals were classified into six groups (1—6) each consisting of 6 rats. Group 1 as the diabetic control received 1.5 ml of 0.5% CMC-solution (vehicle), the rats of group 2 were given the standard oral hypoglycemic agent Metformin hydrochloride (MH) (1 g/kg bodyweight (bw)) in the same vehicle, while groups 3, 4, 5, 6 received FA (1.26 g/kg bw), FB (0.72 g/kg bw), FC (0.48 g/kg bw), FD (0.06 g/kg bw), respectively. The doses of FA, FB, FC, FD were determined by the yield, and they were from identical amounts of plant. The extracts were redissolved in 1.5 ml of 0.5% CMC-solution and administered orally by a cannula. Besides the above groups, there was one group as normal control including 6 normal rats.

Collection of Blood and Determination of Blood Glucose Blood samples for glucose determination were taken from the tail vein of 12 h fasted rats on days 10 and 17 of the experiments. The samples were allowed to clot for 30 min and the serum was separated by centrifugation.

Serum glucose levels were estimated using the glucose–oxidase–peroxidase method with optical density measured by visible spectrophotometer at 515 nm and calculated with respect to the standard calibration curve.

Intakes of Food and Water Food and water intakes were recorded daily during the experimental period (17 d). Average daily intake was calculated for each animal in different groups.

Cell Preparation Adipocytes were isolated from epididymal fat pads from Kun-Ming mice (30±5 g) by the modified method of Rodbell,[13] Gliemann[14] and Marshall *et al.*[15] Following dissection the fat pads were minced and incubated in KRBH-buffer containing 25 mM HEPES, 200 nM adenosine, 1% (w/v) BSA; 1.5 mg/ml collagenase was added and incubation continued for 1 h at 37 °C. The cells were filtered through nylon screen and centrifuged (1200×***g***, 1 min). The pellet was discarded and the supernatant was saved. The cell suspension was incubated at 37 °C for 40 min with addition of vehicle control (DMSO) or various samples of insulin (1, 10, 100, 200 nM), Glipizide (1, 10 nM), Methylamine (10, 100 nM), FC (0.1, 1, 10 mg/ml), apigenin (0.01, 0.1, 2 mg/ml), and apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (0.01, 0.1, 2 mg/ml).

Membrane Preparation The adipocyte membranes were prepared as described previously.[16,17] Briefly, the cell suspensions fell into pieces in ultrasonic at 4 °C, and were centrifuged at 3000×***g*** for 15 min. Fat cakes were discarded, and the supernatant, a fat-free extract, was centrifuged at 12000×***g*** for 25 min. The pellet was resuspended as membrane fraction in Medium I (10 mM Tris–HCl, 1 mM EDTA, 250 mM sucrose, pH 7.4).

Determination of Translocation of the Insulin-Regulated Glucose Transporter GLUT-4 GLUT-4 content was assessed in Western blots. Briefly, the membrane samples were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (10% acrylamide gel) (80 and 120 V during the stacking and separation gels, respectively). Protein was transferred to nitrocellulose (NC) membranes using a Trans-Blot system (4 h at 15 V in 25 mmol/l Tris, 190 mmol/l glycine, and 20% MeOH). Following transfer, the membrane was washed with phosphate-buffered saline (PBS) and blocked overnight (12 h) at 4 °C with 5% (w/v) skim milk powder in PBS/Tween 20 (0.1% by vol.). Blots were then incubated for 2 h at 37 °C with an immunoglobulin G (IgG) monocolonal rabbit anti-rat antibody (1 : 100) in 5% (w/v) skim milk powder dissolved in PBS/Tween 20 to bind GLUT-4. After the removal of primary antibody, the blots were extensively washed with PBS/Tween 20 thrice, each 10 min. Blots were then incubated for 1 h at 37 °C with the appropriate peroxidase conjugated secondary antibody dilution in 5% (w/v) skim milk powder dissolved in PBS/Tween 20. Following removal of the secondary antibody, the blot was washed with PBS/Tween 20 thrice, each 10 min and the blot was developed with the NBT/BCIP chromogenic agent and photographed.

Statistical Analysis The data from the experiments are presented as mean±S.D. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. p values less than 0.05 ($p<0.05$) were considered as indicative of significance.

RESULTS

Effects on Blood Glucose STZ (48 mg/kg) administration resulted in a significant elevation of glucose level and the MH (1 g/kg), FA (1.26 g/kg), and FC fraction (0.48 g/kg) were able to correct this metabolic aberration significantly ($p<0.001$). The results of all the fractions tested are presented in Fig. 1. MH (1 g/kg), FA (1.26 g/kg), and FC (0.48 g/kg) showed 53.0%, 36.0% and 39.2% reduction in blood glucose, respectively, in comparison to the diabetic control after once daily administration for 17 d. The FC fraction showed 48.9% antidiabetic tendency at 0.48 g/kg given orally for 10 d as compared to the basal blood glucose. Figure 1 also showed that there was a tendency for FC to be more effective than FA, although FC was derived from FA.

Intakes of Food and Water The food and water intakes in control and experimental animals are shown in Table 1; the induction of STZ-diabetes resulted in elevated intake of both. The food and water intakes of MH (1 g/kg), FA (1.26 g/kg), and FC (0.48 g/kg)-treated diabetic rats were reduced significantly ($p<0.001$) when compared to that of the 0.5% CMC-treated diabetic rats.

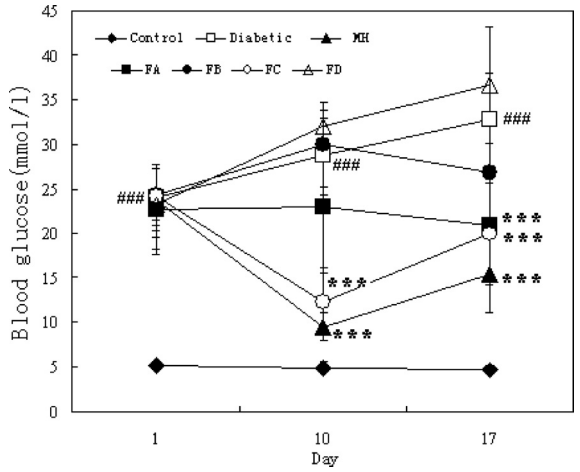

Fig. 1. Effect of Various Fractions of Ethanol Extract from *C. sinensis* Leaves on Blood Glucose Level in STZ-Induced Diabetic Rats

MH (1.00 g/kg), FA (1.26 g/kg), FB (0.72 g/kg), FC (0.48 g/kg), FD (0.06 g/kg) were administered orally to the rats for 17 d. The diabetic control rats received the same volume of 0.5% CMC-solution. Blood samples were taken for glucose determination on days 1, 10 and 17. Values are mean±S.D. from 6 rats in each group; diabetic control is compared with normal control; experimental groups are compared with diabetic control; values are statistically significant at ### $p<0.001$ as compared with normal control, *** $p<0.001$ as compared with the diabetic control.

Table 1. Effects of Various Fractions of Ethanol Extract from *C. sinensis* Leaves on Food and Water Intakes in Chronic STZ-Induced Diabetic Rats

Group	Dose (g/kg)	Food intake (g/rat/d)	Water intake (ml/rat/d)
Control		28.13±1.93	32.03±2.77
Diabetic	—	38.53±4.48##	199.89±21.93##
MH	1.00	24.23±7.67***	90.12±30.32***
FA	1.26	30.99±4.92***	132.89±12.24***
FB	0.72	38.09±6.05	221.40±34.78
FC	0.48	25.09±4.93***	106.00±23.89***
FD	0.06	37.20±5.01	194.08±30.56

Values are mean±S.D. from 6 rats in each group; diabetic control is compared with normal control; experimental groups are compared with diabetic control; values are statistically significant at ## $p<0.01$ as compared with normal control, *** $p<0.001$ as compared with the diabetic control.

Structural Elucidation of Compounds The isolated compounds were identified as apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (**1**), apigenin (**2**), apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-glucopyranoside] (**3**) by analysis of NMR and MS data, together with comparison of their spectral data with those in the literature.[18—20]

Compound **1**: White crystal (MeOH). ESI-MS: *m/z* 620.9 $[M+H]^+$, 618.9 $[M-H]^-$. UV λ_{max} (MeOH) nm: 262.7, 332.1. IR (KBr) cm^{-1}: 3400, 1720, 1600, 1490. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.76 (1H, s, 7-OH), 10.22 (1H, s, 4′-OH), 7.86 (2H, d, J=8.8, H-2′, 6′), 6.91 (2H, d, J=8.8, H-3′, 5′), 6.60 (1H, s, H-8), 6.53 (1H, s, H-3), 6.49 (1H, s, H-6), 5.17 (1H, s, H-1‴), 5.44 (1H, d, J=5.7, H-1″), 3.10—4.65 (10H, m), 1.87 (3H, s, 6″-AcCH_3), 1.06 (3H, m, 6—H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 162.2 (C-2), 105.8 (C-3), 175.6 (C-4), 157.1 (C-5), 99.0 (C-6), 160.2 (C-7), 96.3 (C-8), 158.7(C-9), 107.3 (C-10), 121.3 (C-1′), 127.9 (C-2′, 6′), 115.8 (C-3′, 5′), 160.5 (C-4′), 99.6 (C-1″), 72.2 (C-2″), 76.5 (C-3″), 76.7 (C-4″), 73.3 (C-5″), 62.9 (C-6″), 97.0 (C-1‴), 69.8 (C-2‴), 70.5 (C-3‴), 70.3 (C-4‴), 68.6 (C-5‴), 17.9 (C-6‴), 170.1 (O<u>C</u>OCH_3), 20.4 (OCO<u>C</u>H_3).

Compound **2**: Yellow powder. ESI-MS: *m/z* 271.4 $[M+H]^+$. UV λ_{max} (MeOH) nm: 268.5, 336.2. IR (KBr) cm^{-1}: 3400, 1620, 1490. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.95 (1H, s, 5-OH), 10.80 (1H, s, 7-OH), 10.37 (1H, s, 4′-OH), 7.91 (2H, d, J=8.7, H-2′, 6′), 6.91 (2H, d, J=8.7, H-3′, 5′), 6.77 (1H, s, H-3), 6.48 (1H, s, H-8), 6.18 (1H, s, H-6). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 181.7 (C-4), 165.2 (C-2), 163.8 (C-7), 161.6 (C-9), 161.5 (C-4′), 157.5 (C-5), 128.6 (2C, C-2′, 6′), 121.3 (C-1′), 116.2 (2C, C-3′, 5′), 103.5 (C-10), 102.9 (C-3), 99.2 (C-6), 94.3 (C-8).

Compound **3**: White crystal (MeOH). ESI-MS: *m/z* 579.0

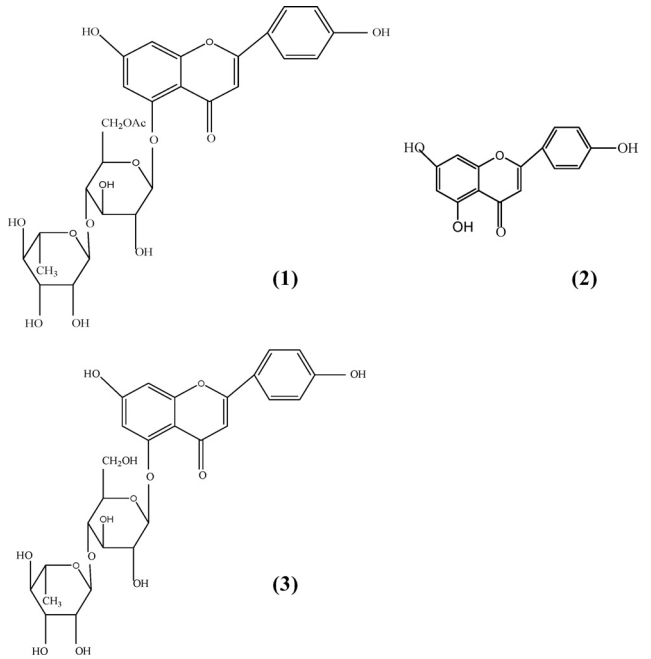

Fig. 2. Structure of Compounds **1**, **2**, **3** Isolated from *C. sinensis*

Apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (**1**), apigenin (**2**), apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-glucopyranoside] (**3**).

$[M+H]^+$, 577.0 $[M-H]^-$. UV λ_{max} (MeOH) nm: 262.7, 331.2. IR (KBr) cm^{-1}: 3400, 1610, 1490. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 10.76 (1H, s, 7-OH), 10.21 (1H, s, 4′-OH), 7.86 (2H, d, J=8.7, H-2′, 6′), 6.91 (2H, d, J=8.7, H-3′, 5′), 6.60 (1H, s, H-8), 6.52 (1H, s, H-3), 6.50 (1H, s, H-6), 5.19 (1H, s, H-1‴), 5.24 (1H, d, J=5.9, H-1″), 3.10—4.96 (10H, m), 1.17 (3H, d, J=6, 6‴-H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 161.9 (C-2), 105.8 (C-3), 175.2 (C-4), 157.1 (C-5), 99.2 (C-6), 161.2 (C-7), 96.3 (C-8), 158.7 (C-9), 107.3 (C-10), 121.4 (C-1′), 127.9 (C-2′, 6′), 115.8 (C-3′, 5′), 160.5 (C-4′), 99.4 (C-1″), 72.2 (C-2″), 76.5 (C-3″), 76.1 (C-4″), 73.7 (C-5″), 61.8 (C-6″), 97.0 (C-1‴), 69.8 (C-2‴), 70.4 (C-3‴), 70.3 (C-4‴), 68.5 (C-5‴), 18.4 (C-6‴).

Effects on the GLUT-4 Translocation The effects of insulin (1, 10, 100, 200 nM), Glipizide (1, 10 nM), Methylamine (10, 100 nM), FC (0.1, 1, 10 mg/ml), apigenin (0.01, 0.1, 2 mg/ml), and apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (0.01, 0.1, 2 mg/ml) on GLUT-4 translocation were estimated by measuring the relative content of GLUT-4 of mice adipocyte membrane after adipocytes were pre-incubated with the different samples above (Fig. 3). The significant increase in GLUT-4 protein level in adipose tissue treated with insulin (10, 100, 200 nM) or Glipizide (1, 10 nM) was observed as compared to control. The GLUT-4 level reached the maximum when pretreated with 100 nM insulin, and 200 nM insulin could not increase the GLUT-4 content than 100 nM insulin. In contrast, Methylamine (10, 100 nM), Semicarbazide-Sensitive Amine Oxidase (SSAO) substrate, dose-dependently decreased the GLUT-4 protein level in adipose tissue. FC (0.1, 1, 10 mg/ml), apigenin (0.1, 2 mg/ml) potently increased GLUT-4 protein level in comparison to that of control in a dose-dependent manner. In the tested dose range of 0.1—2 mg/ml, apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] weakly increased GLUT-4 protein level. Densitometric scanning revealed increasing in GLUT-4 translocation by *ca.* 2.7- and 1.4-fold by FC (10 mg/ml) and apigenin (2 mg/ml), respectively, as compared with control.

Comparative Analysis of the Components of Various Fractions of the Ethanol Extract and Standardization of FC Fraction The components of various fractions of the ethanol extract were analyzed by the HPLC-DAD. The HPLC

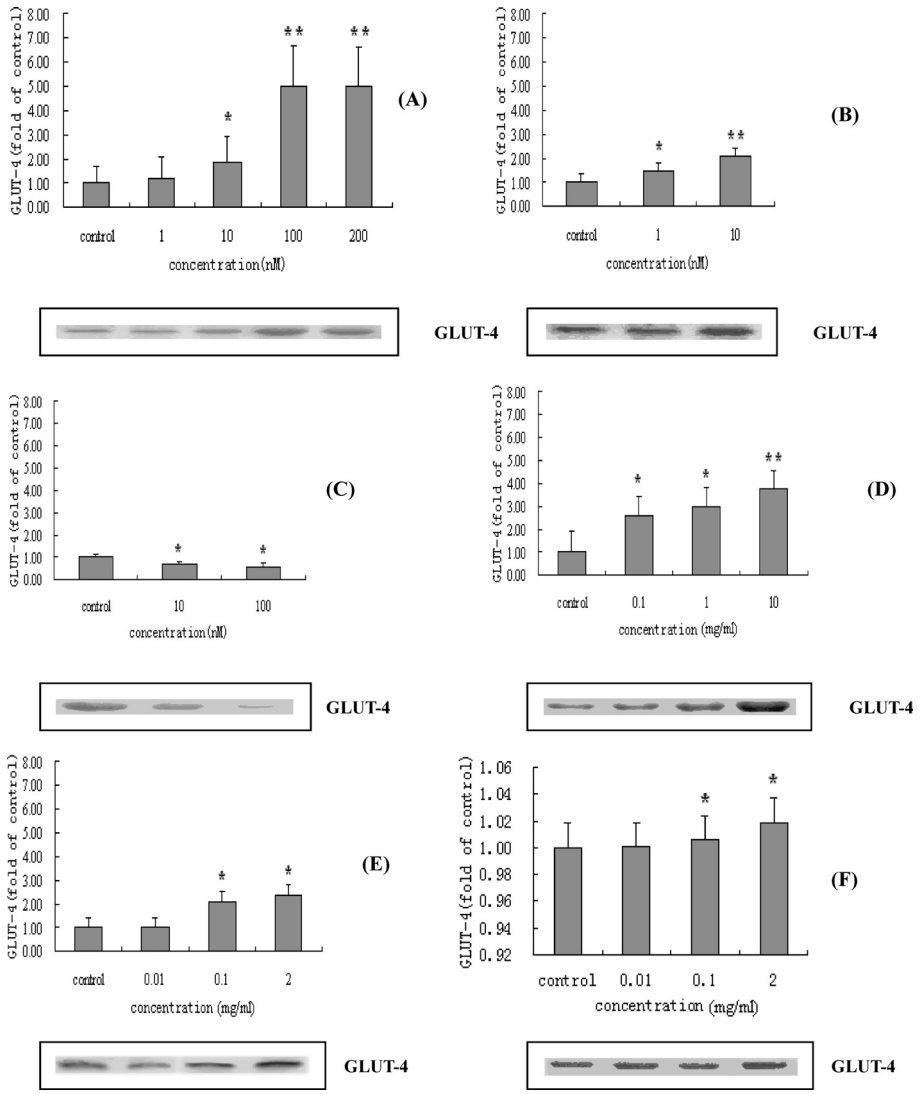

Fig. 3. Effects of Insulin (A), Glipizide (B), Methylamine (C), FC (D), Apigenin (E), Apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (F) on GLUT-4 Translocation (n=3).

Densitometric quantifications and representative Western blots are shown. Value represents mean±S.D. $*p<0.05$ *vs.* control, $**p<0.01$ *vs.* control.

chromatograms are shown in Fig. 4. The results showed that the FA was well divided into three fractions, namely FB, FC, FD, respectively.

The active components in the FC fraction were investigated. This fraction was selected because it showed the strongest antihyperglycemic tendency. The major peaks (1—11) in the HPLC chromatogram of FC exhibited characteristic UV maximum absorbance of flavonoids, namely, two maximum absorbance bands: band I (300—400 nm), band II (220—280 nm) (Fig. 5A). The three flavonoid constituents (compounds **1**, **2**, **3**) were detected. Their retention times were 23.7, 29.2 and 10.7 min, respectively, detected by UV monitoring at 254 nm (Fig. 5A). In the HPLC chromatogram, Peaks 1, 9, 11 corresponded to apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-glucopyranoside] (compound **3**), apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] (compound **1**) and apigenin (compound **2**), respectively (Fig. 5). Phytochemical investigation and HPLC-DAD analysis indicated that the major principles contained in the fraction FC were flavonoids.

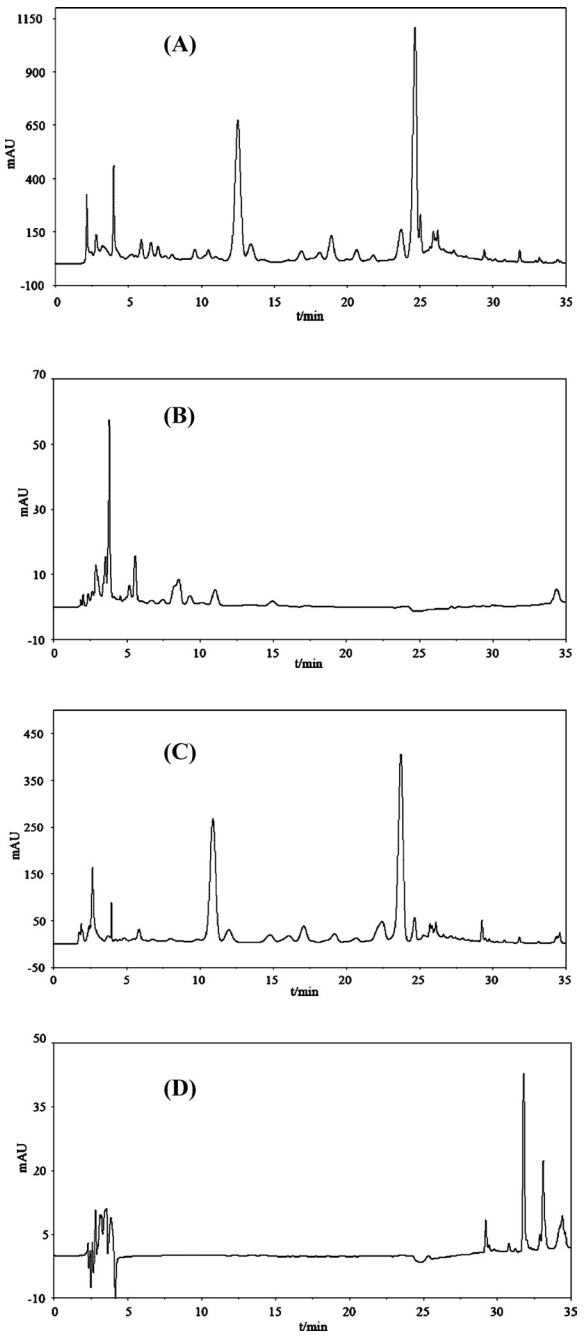

Fig. 4. HPLC Analysis of Various Fractions FA (A), FB (B), FC (C), FD (D) of the Ethanol Extract at 254 nm

DISCUSSION

STZ is a diabetogenic agent[21] and β cell cytotoxic, and it can be used to induce experimental diabetes in rodents.[22] The results of the present study indicated that *C. sinensis* leaves were an effective herb against chronic STZ-induced diabetes and also in preventing the gain of food and drink intake induced as a consequence of diabetes. The possible mechanism by which FC brings about its antihyperglycemic effect may be potentiation of the insulin effect of plasma.

T2D, which may be caused by the metabolic syndrome, is characterized by a reduced sensitivity to insulin signaling and a reduced efficiency of glucose transport, primarily in adipocytes and muscle cells, leading to hyperglycemia and hyperinsulinemia.[23] Glucose transport, which is the first step in glucose utilization, has been found to be a rate-limiting step in glucose metabolism in ruminants as in non-ruminants, and to be regulated by insulin.[24,25] Glucose transport across the plasma membrane is carried out by five facilitative glucose transporter proteins in both monogastrics and ruminants: the insulin-sensitive (GLUT-4), which are mainly expressed in adipose tissue and skeletal muscles, and the non-insulin sensitive glucose transporters (GLUT-1, 2, 3, 5).[26] Insulin resistance in T2D is manifested by decreased insulin stimulated glucose transport and metabolism in adipocytes and skeletal muscle resulting in down-regulation of the major insulin-responsive glucose transporter, GLUT-4.[27] GLUT-4 is translocated to the cell membrane to form glucose transport channels after being activated.[28,29] This increase in GLUT-4 translocation is compatible with the increased glucose uptake. GLUT-4 in rat adipocytes resides mostly in intracellular sites in the basal state, while cellular insulin treatment caused GLUT-4 redistribution, leading to a drastic increase in the plasma membrane GLUT-4 level.[30,31] GLUT-4 is also shown to be expressed in human skeletal muscle by cloning and characterization of insulin responsive glucose transporters.[32] Compounds that activate the insulin-mediated glucose transport signaling pathway can be potentially beneficial for the treatment of metabolic syndrome and diabetes,[33] especially for T2D.

A simple and efficient experimental method by simulation of the above study in adipocytic cell following the modified previous method was developed in our lab, which could help us to better screen the active constituents. Our observations revealed activated GLUT-4 translocation by insulin. Glipizide, an anti-T2D drug, also showed the positive result. In contrast, Methylamine (10, 100 nM), SSAO substrate, which can inhibit the GLUT-4 translocation, dose-dependently decreased the GLUT-4 protein level in adipose tissue. Moreover, in our study we found that the FC (0.1, 1, 10 mg/ml), apigenin (0.1, 2 mg/ml) could significantly facilitate the GLUT-4 translocation. In the tested dose range of 0.1—2 mg/ml, apigenin-5-*O*-[α-L-rhamnopyranosyl-(1→4)-6-*O*-β-D-acetylglucopyranoside] weakly facilitated the GLUT-4

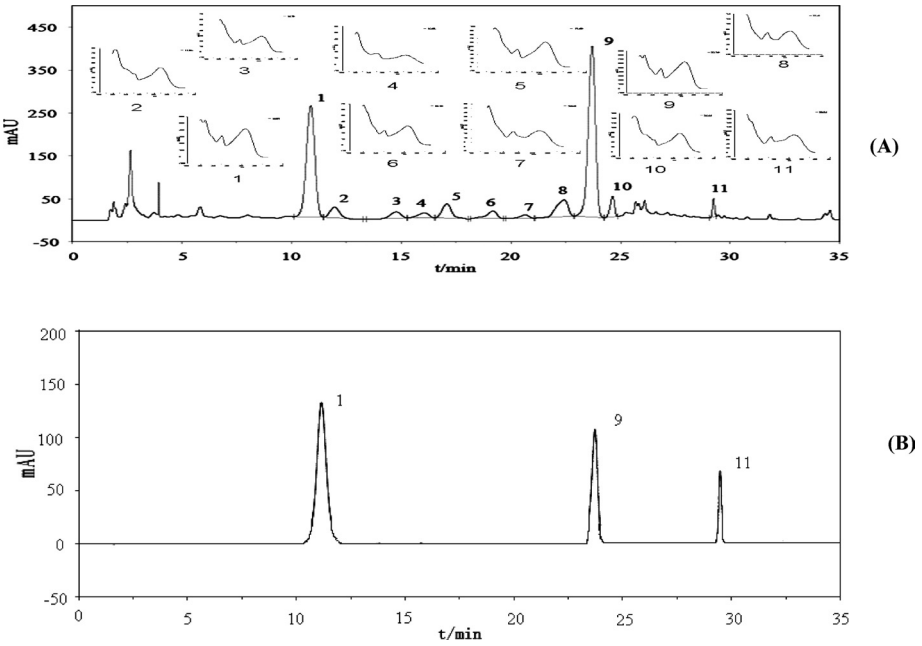

Fig. 5. The Spectrum of Major Peaks (1—11) in HPLC Chromatogram of FC (A) and HPLC Chromatogram of Standard Compounds **1**, **2**, **3** (B)

translocation. It was expected that the extract and its flavonoid constituents possessed the effects like the two positive controls (insulin, Glipizide). These results revealed that the flavonoids from *C. sinensis* were the active constituents.

The macropore absorbance resin affords a good separation method for plant extracts. The AB-8 resin is weak polar. The fractions of FB, FC, FD separated by AB-8 resin represented the polar, medium polar, and less polar components, respectively. The medium polar components corresponding to FC could significantly lower the blood glucose level in STZ-induced rats. Moreover, this fraction had more pronounced antihyperglycemic tendency than the ethanol extract (FA). These showed that the AB-8 resin could effectively concentrate the active components.

Our phytochemical investigation and HPLC-DAD analysis of FC indicated that the flavonoids were the major principles in the fraction.

Flavonoids are naturally occurring phenolic compounds that are widely distributed in plants. Due to the presence of aromatic hydroxyl groups, flavonoids have strong antioxidant properties. They are scavengers of reactive oxygen and nitrogen species and, therefore, inhibit peroxidation reactions. They also protect macrophages from oxidative stress by keeping glutathione in its reduced form.[34,35] Antioxidants can inhibit oxidative glycation (glycoxidation) of tissue proteins with reducing sugars.[36] Increased advanced glycation end-product formation has been implicated as one of main molecular mechanisms of how hyperglycemia causes diabetic complications.[37] The antioxidant properties are thus associated with DM or its complications.

Flavonoids and their derivatives are active inhibitors to aldose reductase, such as quercetin, silymarin, puerarin, baicalim, berberine, apigenin and so on. The aldose reductase inhibitors can effectively prevent and delay diabetic complications, such as diabetic nephropathy, vasculopathy, retinopathy, and peripheral neuropathy.[38,39] Matsuda *et al.* reported that the inhibitory effect of apigenin on aldose reductase, and the IC_{50} (concentration of 50% inhibitory percentage) value was 0.58 mM.[40]

In the FC, many flavonoids were found involving apigenin, apigenin glycoside. It could be concluded that these flavonoids correlated with the antihyperglycemic activity of FC, although we couldn't exclude the effect of other minor substances.

In conclusion, the fraction from *C. sinensis* leaves extract showed a potent antihyperglycemic effect on chronic STZ-induced diabetic rats, and administration of the fraction did not change the blood glucose levels in normal rats (data not shown). There was no case showing acute hypoglycemic conditions during the administration of the fraction. The flavonoids from *C. sinensis* were the active constituents. The activity of facilitating GLUT-4 translocation of the flavonoids was first found. These results show that the fraction from *C. sinensis* leaves extract is a promising drug for the treatment of diabetes, and the flavonoids may be leading compounds for further study as new drugs for the prevention and/or treatment of DM and its complications.

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