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Anti-diabetic Agents from Medicinal Plants Inhibitory Activity of *Schizonepeta tenuifolia* Spikes on the Diabetogenesis by Streptozotocin in Mice

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The *Schizonepeta tenuifolia* spikes (STS) have been used as a traditional folk medicine for anti-inflammatory, analgesic, anti-pyretic and anti-spasmodic purpose in Korea. Phytosterols (mixture of campesterol 3.68%, stigmasterol 2.30% and β-sitosterol 94.02%) and hesperidin were isolated by chromatography from ether and n-BuOH fractions of STS respectively. These compounds significantly reduced the blood glucose level and lessened the loss of body weight and water consumption dose-dependently when administered at a i.p. doses of 10 and 20 mg/kg for 4 days after the i.v. injection of streptozotocin (180 mg/kg). In the morphologic study, these compounds showed protective activity on the pancreatic islets, especially β-cells, from the degenerative changes by streptozotocin.

Key Words : *Schizonepeta tenuifolia*, Phytosterols. Hesperidin, Anti-diabetogenic activity

INTRODUCTION

The spikes of *Schizonepeta tenuifolia* (Labiatae), which is an annual plant widely cultivated throughout southern Korea, have been used as a traditional folk medicine for anti-inflammatory, analgesic, anti-febrile and anti-spasmodic purpose in Korea (Hahn *et al.*, 1994). In the studies on its chemical constituents, Sasaki (1981) and Kubo (1986) isolated monoterpene glycosides designated schizonepetosides A, B and C, and flavonoid glycosides named apigenin-7-O-β-glucoside and luteolin-7-O-β-glucoside while Oshima (1989) have reported the isolation of another monoterpenoids such as schizonodiol, schizonol and schizonepetosides D and E. As essential oil of this plant, (+)-menthone and (-)-pulegone was reported (Fujita *et al.*, 1973), and the biological activities of these essential oils were identified that (+)-menthone has an anlagesic activity and (-)-pulegone has an analgesic and anti-inflammatory activities in mice model (Yamahara *et al.*, 1980). In addition, we have previously reported the hypoglycemic activity of water extract of *Schizonepeta tenuifolia* in the streptozotocin-induced hyperglycemic mice although its active principle is unknown (Kim *et al.*, 1990).

As a part of our ongoing search for plant-derived anti-diabetic agents, we isolated two biologically active compounds, phytosterols and hesperidin from ether and n-BuOH fractions of *Schizonepeta tenuifolia* spikes respectively. Hesperidin is a flavanone glycoside widely distributed in plants which was reported to have many different biological activities including anti-oxidants (Fraga *et al.*, 1987), anti-allergic (Matsuda *et al.*, 1991), anti-inflammatory (Emim *et al.*, 1994) and anti-cancer activities (Tanaka *et al.*, 1994). Phytosterols were also reported to have hypocholesterolemic (Tabata *et al.*, 1980; Sugano *et al.*, 1976) and anti-tumor activities (Janezic and Rao, 1992; Lee *et al.*, 1980). However, it has not been examined whether these two compounds have any effects on diabetes.

In this study, we examined their effects on the blood glucose level, body weight change, water consumption and pancreatic islets in order to evaluate their anti-diabetogenic activity using streptozotocin as a diabetogenic agent.

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MATERIALS AND METHODS

Instruments and reagents

IR spectra were obtained using a Nicolet MX-S spectrometer. NMR spectra were measured with a Bruker AC-200P spectrometer with TMS as the internal standards. UV was taken on Varian Cary-3 spectrophotometer and GC-MS data were obtained with Hewlett Packard 5890 HP 5970 MSD spectrometer e-

quipped with a 25 m×0.32 mm I.D. capillary column crosslinked with methylsilicone gum (HP 1). The column temperature was 280°C and N_2 (0.8 kg/min.) was used as a carrier gas. FAB-MS was determined on a VG70-VSEQ. TLC was carried out on silicagel 60 F_{254} (Merck). All reagents used in this study were analytical grade.

Extraction and isolation

The fresh spikes of *Schizonepeta tenuifolia* (3 kg) purchased from the market with botanical identification, were extracted three times with 70% MeOH by reflux for 6 h. The MeOH extract (143 g) was evaporated *in vacuo* to dryness, and was partitioned between H_2O and ether. The ether fraction (15 g) was loaded on a silica gel column which was eluted with $CHCl_3$-MeOH (50:1). The fractions which contains one compound with Rf value 0.88 on TLC (developing solvent; $CHCl_3$:MeOH:H_2O=6:4:1, spray reagent; 10% H_2SO_4) was collected and evaporated *in vacuo* which gave compound 1 (350 mg) as white powder. The residual H_2O layer was extracted three times with n-BuOH. The n-BuOH fraction (23 g) was chromatographed on a silica gel column and eluted with $CHCl_3$-MeOH-H_2O (65:30:8). Based on TLC (developing solvent; $CHCl_3$:MeOH:H^2O=70:30:4, spray reagent; 10% H^2SO_4, 5% $FeCl^3$), the fractions which contains one compound with Rf value 0.36 was pooled and evaporated to yield compound 2 (400 mg) as yellow powder.

Compound 1: mp 134-135°C; IR ν_{max} (KBr) cm^{-1} 3420, 2980, 1636; GC-MS m/e ① 400 (M^+), 382 ② 412 (M^+), 394, 369, 301 ③ 414 (M^+), 396, 381, 303, 273, 231, 213

Compound 2: mp 260-261°C; IR ν_{max} (KBr) cm^{-1} 3390, 1650, 1610, 1525, 1435, 1089; UV λ_{max} (MeOH) nm 283, 327, λ_{max} (MeOH+NaOMe) nm 287, 360, λ_{max} (MeOH+$AlCl_3$) nm 312, 383, λ_{max} (MeOH+$AlCl_3$+HCl) nm 312, 383, λ_{max} (MeOH+NaOAc) nm 283, 327, λ_{max} (MeOH+NaOAc+H_3BO_3) nm 283, 327; FAB-MS m/e 611 $(M-H)^+$, 303; ^{1}H-NMR (80 MHz, DMSO-d_6) δ 12.0 (1H, s, 5-OH), 6.92 (3H, d, *J*=7.4 Hz, H-2', 5', 6'), 6.12 (2H, d, *J*=2.5 Hz, H-6, 8), 5.37 (1H, d, *J*=5.11 Hz, H-2), 4.96 (1H, d, *J*=9.8 Hz, glucose anomeric H), 4.51 (1H, s, rhamnose anomeric H), 3.76 (3H, s, OCH3-4'), 2.76 (1H, q, *J*=20, 3 Hz, H-3), 1.08 (3H, d, *J*=6.2 Hz, rhamnose-CH_3); ^{13}C-NMR (50 MHz, DMSO-d6) δ 78.3 (C-2), 40.7 (C-3), 196.9 (C-5), 162.9 (C-5), 96.3 (C-6), 165.3 (C-7), 95.5 (C-8), 162.4 (C-9), 103.3 (C-10), 130.9 (C-1'), 114.1 (C-2'), 147.9 (C-3'), 146.4 (C-4'), 112.0 (C-5'), 117.8 (C-6'), 99.4 (glc-1), 72.9 (glc-2), 76.2 (glc-3), 70.6 (glc-4), 75.4 (glc-5), 66.0 (glc-6), 100.5 (rham-1), 70.2 (rham-2), 69.5 (rham-3), 72.0 (rham-4), 68.2 (rham-5), 17.7 (rham-6).

Hydrolysis of compound 2

Compound 2 (20 mg) was dissolved in 0.5N-H_2SO_4 20 ml and refluxed for 4 h. The reaction mixture was neutralized with $Ba(OH)_2$, and filtered. The filtrate was detected on silica TLC ($CHCl_3$-MeOH-H_2O=70:30:4) in comparison with authentic sugar samples.

Animals

Male ICR mice (16-20g) were purchased from Joongang animal Co., (Seoul). They were housed in a storage room under conditions of 25±1°C, humidity (55±5%) and 12-h light-dark cycle (from 8 a.m. to 8 p.m.). They were allowed to access commercial diet (Samyang food Co., Ltd. Seoul) and water *ad libitum* except for the specified periods.

Diabetogenesis by streptozotocin

Diabetes in mice was induced by streptozotocin according to the Yamahara method (Yamahara *et. al.*, 1981). Briefly, ICR mice fasted for 24 h were treated with intravenous injection of freshly prepared streptozotocin (180 mg/kg) in cold citrate buffer (10 mM, pH 4.5). Compounds 1 and 2 dissolved in DMSO followed by dilution with physiological saline to adjust the final DMSO concentration less than 1%, was intraperitoneally administered 1 h after the intravenous injection of streptozotocin (180 mg/kg) on Day 1, and twice a day on Days 2 and 3. On Day 4, compounds 1 and 2 were administered once in the morning. Four hours after the last administration, venous blood was drawn from the retro-orbital sinus from the mice fasted for 24 h with microhematocrit tubes, and the blood glucose level was determined with a glucose autoanalyzer by the glucose oxidase method (Lott *et al.*, 1975). The volume of water consumption and weight change for 3 days were also recorded.

Morphologic examination of pancreatic islets

Pancreatic tissue excised after the blood drawn was fixed in Bouin's solution, and pancreatic islets were examined with light microscopy after staining with hematoxylin-eosin and aldehyde fuchsin (Cook *et al.*, 1974).

Data analysis

The results obtained were evaluated by employing Student's t test.

RESULTS AND DISCUSSION

Compound 1 obtained as white powder, showed positive reaction to Liebermann-Burchard test. Its IR spectrum with strong absorption at 3420, 2980 and

Table I. Effects of compounds 1 and 2 on blood glucose level in streptozotocin-induced diabetic mice

Drugs	Dose (mg/kg, i.p.)	Blood glucose level[a] (mg/dl)
Control (untreated)	-	101.5±7.4
Control(treated)	-	321.4±8.0
Compound 1	5	287.8±9.9
	10	251.1±3.9*
	20	225.0±6.9**
Compound 2	5	307.0±4.2
	10	247.3±5.2*
	20	160.1±9.7**
Glibenclamide	1	197.0±9.5**

Drugs were administered 1 h after the injection of streptozotocin (180 mg/kg, i.v.) on Day 1, and twice a day on Days 2 and 3, and once 4 h before the blood draw. Control group was administered the vehicle only.
[a]Blood glucose level was determined with a glucose autoanalyzer by the glucose oxidase method, and values are expressed as a mean±S.E.M. of 6 mice.
Significantly different from treated control. *p<0.05, **p<0.01

Table II. Effects of compounds 1 and 2 on the body weight change and water consumption in streptozotocin-induced diabetic mice

Drugs	Dose (mg/kg, i.p.)	Body weight change (%/3 days)	Water consumption (ml/3 days)
Control(untreated)	-	8.7±2.6	6.3±1.9
Control(treated)	-	-21.2±3.4	22.1±3.0
Compound 1	5	-10.1±2.8	18.0±2.0
	10	-7.3±2.4*	12.2±2.1*
	20	-6.9±1.4*	10.5±1.9*
Compound 2	5	-9.9±2.7	17.1±2.2
	10	-5.1±2.6*	10.3±1.6*
	20	-4.0±1.4*	8.7±2.0**
Glibenclamide	1	0.4±0.8**	10.0±1.8*

For description of experimental conditions, refer to Table I.
Significantly different from treated control. *p<0.05, **p<0.01

1636 cm^{-1} made possible to assume compound 1 as a phytosterols. In GC-MS data, it was revealed that compound 1 is a mixture of three different compounds with retention time 13.736, 14.016 and 14.629 respectively. Furthermore, direct comparison of molecular ion peak of these three compounds with their authentic sample showed that compound 1 is a mixture of campesterol 3.68%, stigmasterol 2.30% and β-sitosterol 94.02%. to Mg+HCl test, and the strong absorption at 3390, 1650, 1435 and 1089 cm^{-1} in the IR spectrum indicated the presence of hydroxy, conjugated carbonyl, aromatic group. In the ^{1}H-NMR spectrum (80 MHz, DMSO-d6) of compound 2, a doublet and singlet signal of glucose and rhamnose anomeric H at 4.96 and 4.51, doublet and quertet signal at 5.37 and 2.76 by H-2 and H-3, and doublet signal at 1.08 by rhamnose-CH_3 was observed. ^{13}C-NMR spectrum of compound 2 showed 9 peaks due to benzopyrone ring, and 6 peaks due to phenyl ring which is carried at the C-2 position of the benzopyrone ring. The carbon peaks of sugar moieties of compound 2 were assigned by comparison with the chemical shift of authentic sample, and identified on a TLC plate from the acid hydrolysate of compound 2. From these results in addition to FAB-MS data [$(M-H)^+$ m/e 611], compound 2 was identified as hesperidin.

Anti-diabetogenic activity of these compounds were evaluated in the mice treated with streptozotocin, and the results are shown in Tables I and II. When injected intraperitoneally to the mice treated with streptozotocin, compounds 1 and 2 elicited hypoglycemic effect. Blood glucose level of the compound 2 treated group were significantly decreased in a dose-dependent manner; 247.3 and 160.1 mg/dl for 10 and 20 mg/kg of compound 2 treated group respectively, where corresponding blood glucose level for control mice was 321.4 mg/dl (Table I). Also, administration of compound 2 lowered the loss of body weight and water consumption for 3 days dose-dependently which was consistent with the significant decrease in blood glucose level (Table II). These activity of compound 2 at a dosage of 20 mg/kg was comparable to that of glibenclamide at a dosage of 1 mg/kg. In morphologic examination of pancreatic islets (Figs. 1 and 2), the mice received streptozotocin injection uniformly showed marked degenerative changes such as architectural disarray and increased intercellular space with hematoxylin-eosin staining (Fig. 1-2), and β-cells in islets showed remarkable decrease in number and size with aldehyde fuchsin staining (Fig. 2-2). However, in the compound 2 treated group, pancreatic islet cells were much more populated (Fig. 1-3). Moreover, the number of β-cells in islets was increased compared to that of control group (Fig. 2-3). These histologic improvement were consistently compatible with its hypoglycemic activity. In addition, intraperitoneal administration of compound 1 also exhibited significant anti-diabetogenic activity in terms of reduced blood glucose level and lowered loss of body weight and water consupmtion (Tables I and II). The histologic findings of pancreatic islets treated with compound 1 also showed its protective effect on the pancreatic islets from the degenerative changes caused by streptozotocin injection (Fig. 1-4 and 2-4).

Streptozotocin is a specific β-cytotoxic agent and cause necrosis of the majority of pancreatic β-cells. While the mechanism by which streptozotocin exert its diabetogenic action on the pancreatic β-cells are still remained to be elucidated (Srivastava *et al.*, 1982; Wilson *et al.*, 1984; Tanaka *et al.*, 1995), it was suggested that the diabetogenic action of streptozotocin is mediated by noxious oxygen free radicals which is supported by the experiments utilizing free radical

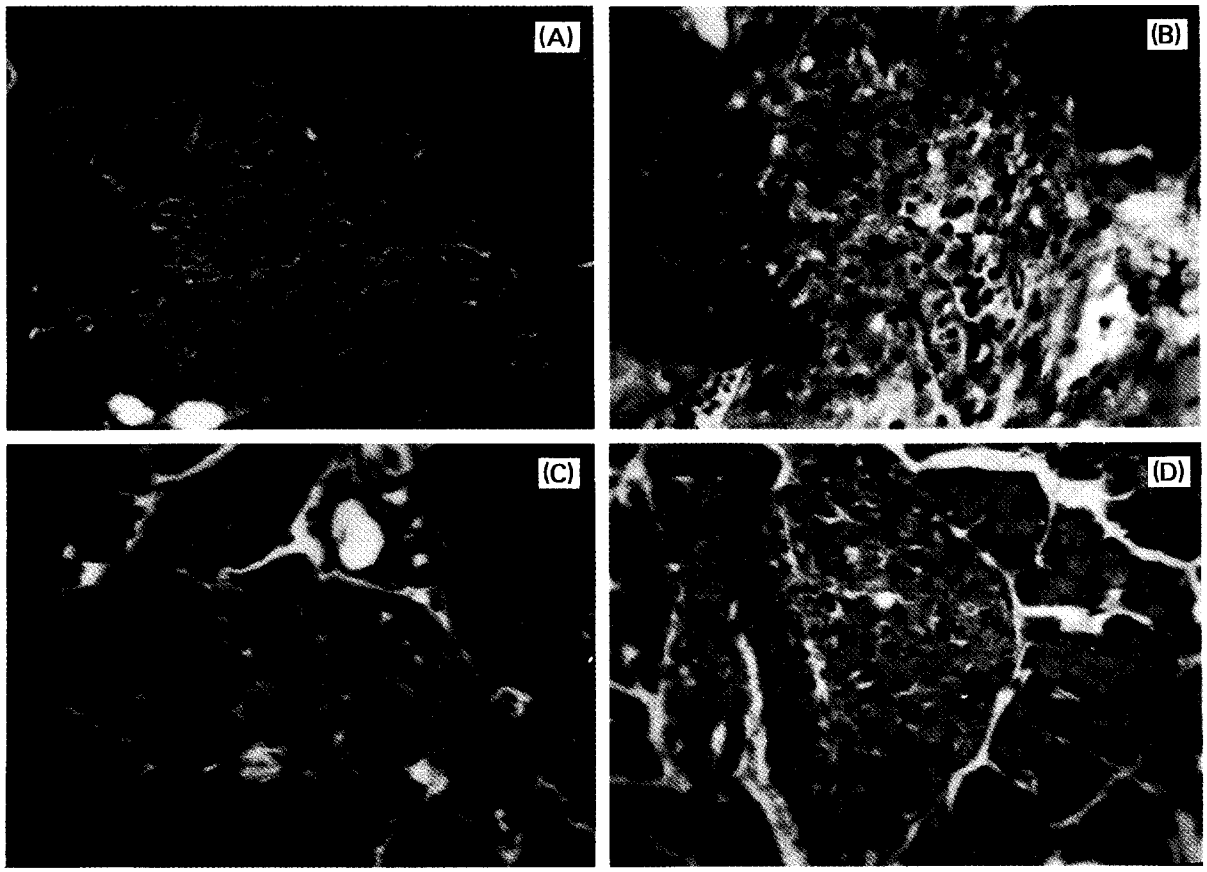

Fig. 1 (A) Pancreas of normal mouse. Acinar and islets components are distinctly separated. Islet cells are compactly arranged. Intercellular spaces are negligible (Hematoxylin & Eosin, ×400). (B) Pancreas of control mouse showing evident architectural disarray and marked atrophy resulted in increased intercellular spaces (Hematoxylin & Eosin, ×400). (C) Pancreas of compound 2 (20 mg/kg) treated mouse. The structure of pancreatic parenchyma is well-maintained with more populated islet cells (Hematoxylin & Eosin, ×400). (D) Pancreas of compound 1 (20 mg/kg) treated mouse. Architectural disarray is improved than control (Hematoxylin & Eosin, ×400).

scavengers such as superoxide dismutase and dimethyl urea (Robbins *et al.*, 1980; Sandler and Andersson, 1982). Other mechanistic investigations have suggested that the depletion of cellular NAD resulting from its catabolism by the nuclear enzyme poly(ADP-ribose)polymerase is critical in streptozotocin-induced toxicity (Yamamoto *et al.*, 1981; Radons *et al.*, 1994; Bellmann *et al.*, 1995).

In this study, we demonstrated that constituents of *Schizonepeta tenuifolia*, compounds 1 and 2 have anti-diabetogenic activity. Because the initial hyperglycemia following i.v. injection of streptozotocin had a characteristic delay in onset of about 45 to 60 mins (Rerup, 1970), the experimental model we employed in this study in which the first administration of tested compounds was given 1 h after streptozotocin injection can be used to evaluate the anti-diabetogenic activity of the compounds. As judged from the lowered blood glucose level and morphologic findings, it is conceivable that amelioration of the diabetic states by these compounds in this model using a single streptozotocin injection is presumably mediated by preventing β-cells from the destruction or apoptosis induced by streptozotocin. Although it is not examined in this study, these preventing activity of the compounds may be due to their free radical scavenging ability (Husain *et al.*, 1987; Robak and Gryglewski, 1988; Chen *et al.*, 1990; Sichel *et al.*, 1991). Another possible mechanism by which compounds 1 and 2 can improve the diabetic states after streptozotocin injection is their effects on the activities of several enzymes involved in glucose metabolism such as glucose-6-phosphatase, hexokinase, and glucose-6-phosphate dehydrogenase (Kiho *et al.*, 1994; Ghosh *et al.*, 1994). Because the administration of compounds 1 and 2 lowered the loss of body weight, these compounds may promote glucose uptake by liver and skeletal muscle resulting from the increase in hexokinase and glucose-6-phosphate dehydrogenase activity and decrease in glucose-6-phosphatase activity.

In conclusion, we have demonstrated that the di-

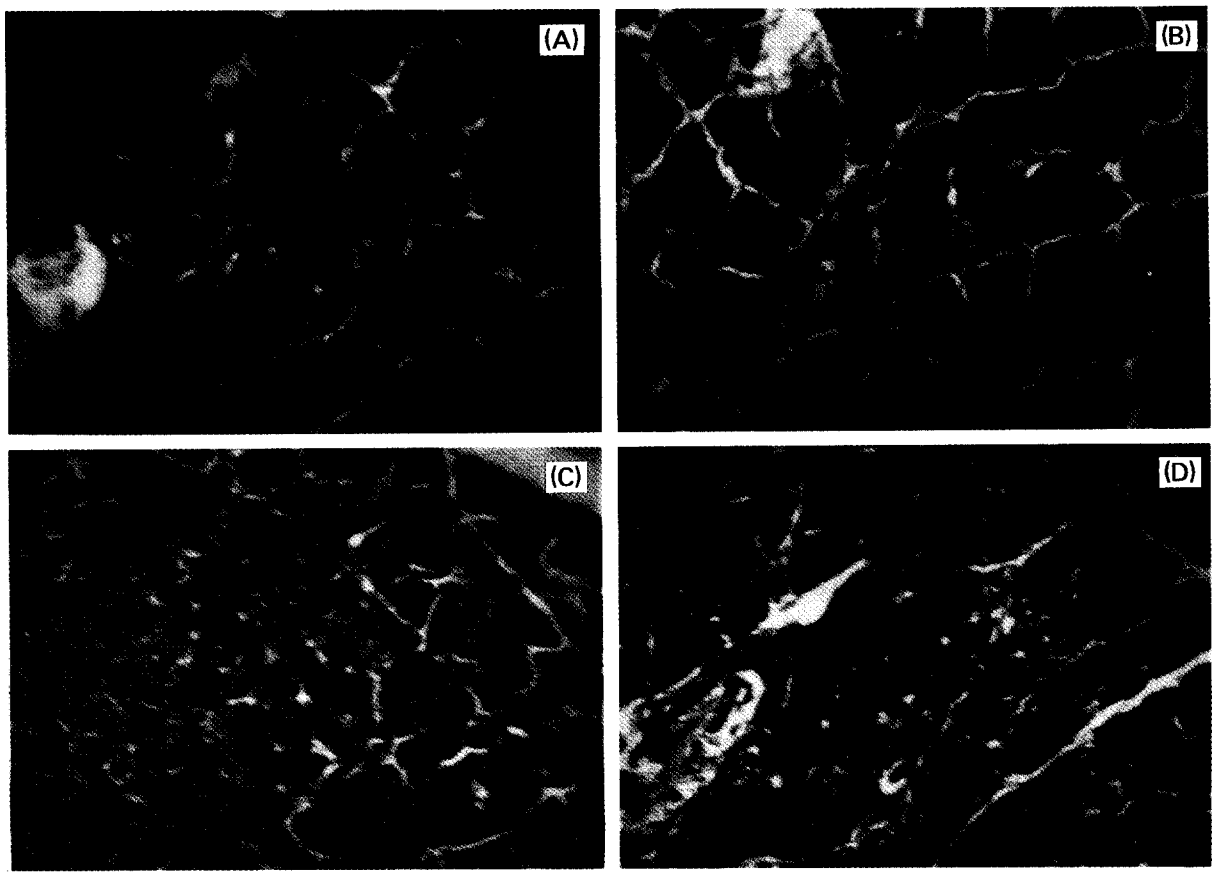

Fig. 2. (A) Pancreatic islets of normal mouse. β-cells in islets are deeply stained with purple (Aldehyde fuchsin, ×400). (B) Pancreatic islets of control mouse. The majority of cells shows degenerative changes and β-cell mass is decreased compared to normal mouse (Aldehyde fuchsin, ×400). (C) Pancreatic islets of compound 2 (20mg/kg) treated mouse. Islet cells are more compactly arranged and β-cells are markedly increased than control (Aldehyde fuchsin, ×400). (D) Pancreatic islets of compound 1 (20 mg/kg) treated mouse. Deeply stained β-cells are markedly increased (Aldehyde fuchsin, ×400).

abetic states such as hyperglycemia, loss of body weight and increased water consumption in streptozotocin treated mice is successfully improved by a administration of compounds 1 (mixture of campesterol 3.68%, stigmasterol 2.30% and β-sitosterol 94.02%) and 2 (hesperidin) which are components of *Schizonepeta tenuifolia* due to their protective activity on the β-cells from the diabetogenic action of streptozotocin.

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