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Article

Antidiabetic and Renoprotective Effects of Acankoreagenin from the Leaves of *Acanthopanax Gracilistylus* in Streptozotocin-Induced Type I Diabetic Rats

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Abstract: This present study was designed to find out whether the acankoreagenin showed the antidiabetic and renoprotective effects in streptozotocin (STZ)-induced diabetic nephropathy (DN) rats. Type I diabetes was induced by a single intraperitoneal injection of STZ (70 mg/kg). At the end of the experiment, rats were euthanized and serum/plasma was separated for the determination of glucose, insulin, glycated hemoglobin A1c (HbA1c), C-peptide, biochemical parameters, and kidney function. One kidney was used for determining glutathione, superoxide dismutas, malondialdehyde, and tumor necrosis factor-alpha levels. The other kidney and pancreas were used for histopathological studies and immunohistochemical measurement of transforming growth factor beta (TGF-β) or NF-κB. Acankoreagenin (2 mg/kg) treatments led to a significant reduction in blood glucose assessed via oral glucose tolerance test (OGTT) in diabetic rats at 2 h. The treatment also resulted in improved body weight, decreased HbA1c, restored lipid profile, and renal oxidative stress. By inhibiting NF-κB, the release of proinflammatory cytokines was suppressed and by inhibiting TGF-β, the renal fibrosis was suppressed in STZ-induced diabetic rat model. Histopathological injury was also observed in pancreatic and renal tissues. These findings support the beneficial effect of acankoreagenin treatment in DN, which could be attributed to its antidiabetic and renoprotective effects.

Keywords: diabetes mellitus; blood glucose; kidney; diabetic neuropathy; pancreas

1. Introduction

Diabetes mellitus is a fatal metabolic disorder with a high global prevalence and affecting multiple organ systems. It is characterized by insulin-secreting β-cells failure which leading to relative or absolute insulin deficiency. Pancreatic β-cells function plays

an important role in maintaining glucose homeostasis and in preventing the onset of diabetes. Therefore, more and more researches focus on how to maintain or restore the function of beta β-cells. The details of the mechanism underlying causes of increased prevalence of diabetes remain unclear. Type I diabetes is an autoimmune disease, thought to be induced by selective destruction of insulin producing β-cells of the Langerhans islets [1].

DN is caused by diabetes-related metabolic abnormalities of glomerulosclerosis, accompanied by proteinuria, is one of the most common serious complications in diabetic patients, affecting the capillaries, and which may be the cause of disability. Since the 1950s, kidney disease has been recognized as a common complication of diabetes. DN affecting diabetic patients with 20-40%, and there is currently no satisfactory method or treatment target for DN [2]. Therefore, how to prevent the progression of DN has become a research hotspot.

According to the overseas studies, renal fibrosis is one of the ultimate main pathways of DN, which can lead to renal failure and subsequently cause sudden death [3]. The major morphological changes associated with renal dysfunction in diabetic patients include deposition, contraction of extracellular matrix (ECM) material and overproduction, primarily collagen and fibronectin, in the glomeruli [4]. In DN, The increased expression of transforming growth factor-beta 1 (TGF-β1) could promoted the accumulation of ECMs proteins, such as epithelial–mesenchymal transition of proximal tubules, apoptosis, collagens and fibronectin, and dedifferentiation of podocytes, all of which are considered to facilitate renal hypertrophy and dysfunction [5].

The whole plant or plant-derived compounds, such as *Terminalia chebula* [6], *Cuscuta reflexa* [7], *Sanguis draconis* [8], aliskire [9], *Trichilia catigua* [10], *Morinda lucida* [11], Korean red ginseng [12], and *Inonotus obliquus* [13], have been shown to exert hypoglycemic effects or DN in response to STZ *in vivo*.

Acanthopanax gracilistylus W. W. Smith is widely distributed in China, and the root bark, which has been listed in the Chinese pharmacopoeia, is used as medicine for the treatment for paralysis, bone pains, arthritis, rheumatism and sinew, and as a tonic in

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traditional Chinese medicine [14]. Moreover, the leaves of *Acanthopanax gracilistylus* (LAG) contain phenylpropanoids, triterpenoids, lignans, diterpenoids, polyacetylenes, and flavonoids [15-17]. One previous pharmacological studies of this plant have reported anti-inflammatory actions [18] and suppressive effects on human lymphocytes [19]. Recently, the constituents of LAG have been reported that include lupane-triterpenoids, which have anti-diabetic activities [20]. In our very recent study showed that acankoreagenin, isolatef from LAG, has an anti-diabetic effect by STZ-induced type I diabetes in vitro study [21].

In the present study, we investigate the anti-diabetic effect of acankoreagenin from the LAG and whether this preparation can attenuate the development of DN in STZ-induced type I diabetic rats.

2. Results

2.1. Body Weight, Food Intake and Water Intake of Acankoreagenin in Normal and Diabetic Rats

As shown in Table 1, significantly decreases ($P < 0.05$) of body weight (BW) and BW gains were detected in all diabetes groups than non-diabetes group. Type I diabetes control group showed the most decreased of BW and BW gains and showed the lowest of food intakes. At the concentration of 2 mg/kg in acankoreagenin group showed the highest FER than other diabetes groups ($P < 0.05$).

On the first week, water intake was no significant change between each group (Table 2). At the end of the experiment, the concentration of 2 mg/kg in acankoreagenin group showed the lowest water intake than other diabetes groups. These results suggest that acankoreagenin may reduce the blood glucose and thus the rats showed the lower water intake.

2.2. Effect of Acankoreagenin on Blood Glucose and OGTT Levels in Normal and Diabetic Rats

After injection with STZ, there was no significant change between diabetes groups on the fasting blood glucose levels (Figure 1). After 3 weeks of administration, the blood glucose levels of the positive control significantly lower ($P < 0.05$) than the control group. At the concentration of 2 mg/kg in acankoreagenin group showed much decreased of blood

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glucose levels than the positive control group, but it was higher than normal group ($P < 0.05$). These suggest that acankoreagenin could decrease the fasting blood glucose and showed the same effect than positive control.

The blood glucose level in the normal group rose to a peak value 1 h after glucose load and decreased to near normal levels at 2 h (Figure 2). In the diabetes control group, the peak increase in blood glucose concentration was observed after 1 h and remained high over the next 1 h. Even though the blood glucose level in the positive control group decreased after 1 h, it was not to near normal levels at 2 h. Only at the concentration of 2 mg/kg in acankoreagenin group, no significant decrease ($P < 0.05$) in blood glucose concentration at 2 h compared with 0 h.

2.3. Effect of Acankoreagenin on Plasma Insulin, HbA1c and C-peptide Levels in Normal and Diabetic Rats

There was a significantly elevation ($P < 0.05$) the level of HbA1c while the levels of plasma insulin and C-peptide decreased during type I diabetes groups when compared to normal group (Table 3). At the concentration of 0.4 mg/kg in acankoreagenin group showed the same plasma insulin level when compared to the positive control, and in 2 mg/kg group showed higher level than the positive control. At the concentration of 2 mg/kg in acankoreagenin group also showed lower level than the positive control. Only at the concentration of 2 mg/kg group showed the same C-peptide level as the positive control. These suggest that acankoreagenin may have the antidiabetic effect.

2.4. Effect of Acankoreagenin on Biochemical Parameters in Normal and Diabetic Rats

There showed a significantly elevation ($P < 0.05$) the level of AST, ALT, TB, TG, and T-C while the level of H-C decreased during type I diabetes groups when compared to normal group (Table 4). At the concentration of 2 mg/kg in acankoreagenin group, AST showed lower level than the positive control. While it also showed the lower ALT, T-Bil, TG and T-Chol levels than the positive control. And it showed higher H-Chol level than the positive control. These suggest that relatively to normal rats, diabetic rats expressed significant elevation in plasma T-Chol and TG as well as reduction in H-Chol. Acankoreagenin may improve the lipid profiles in type I diabetes rats.

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2.5. Effect of Acankoreagenin on Kidney Function Tests in Normal and Diabetic Rats

The levels of BUN, creatinine, and albumin in normal and experimental groups in type I diabetes rats were shown in Table 5. There was a significantly elevation ($P < 0.05$) the level of BUN, creatinine, and albumin during type I diabetes groups when compared to normal group. At the concentration of 2 mg/kg in acankoreagenin group had the same BUN level when compared to the positive control. At the concentration of 0.4 mg/kg in acankoreagenin group had the same creatinine level when compared to the positive control, while in 2 mg/kg acankoreagenin group had lower level than positive control. At the concentration of 0.4 mg/kg in acankoreagenin group had the same albumin level when compared to the positive control, while in 2 mg/kg acankoreagenin group was had lower level than positive control. These suggest that acankoreagenin can improve the relieving kidney damage in type I diabetes rats.

2.6. Effect of Acankoreagenin on TNF-α Level in Normal and Diabetic Rats

The level of TNF-α in normal and experimental groups in type I diabetes rats were shown in Figure 3. There was a significantly elevation ($P < 0.05$) the level of TNF-α during type I diabetes groups when compared to normal group. The positive control had much decreased the TNF-α level, but it was significantly higher ($P < 0.05$) than compared to the normal group. At the concentration of 2 mg/kg in acankoreagenin group was significantly decreased ($P < 0.01$) when compared to the normal group.

2.7. Effect of Acankoreagenin on the Oxidative Stress Biomarkers in Normal and Diabetic Rats

The levels of GSH, MDA, and SOD in normal and experimental groups in type I diabetes rats were shown in Table 6. There was a significantly elevation ($P < 0.05$) the level of MDA while the level of GSH, and SOD decreased during type I diabetes groups when compared to normal group. At the concentration of 0.4 mg/kg in acankoreagenin group had the same GSH level when compared to the positive control, while in 2 mg/kg in acankoreagenin group had higher level than positive control. At the concentration of 0.4 mg/kg in acankoreagenin group had the same MDA level when compared to the positive control, while in 2 mg/kg acankoreagenin group was had lower level than positive control.

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At the concentration of 2 mg/kg in acankoreagenin group was had higher SOD level than positive control while it was significantly lower ($P < 0.05$) than compared to the normal group. These suggest that acankoreagenin can improve in kidney oxidative stress in type I diabetes rats.

2.8. Effect of Acankoreagenin on NF-kB Expressions in Normal and Diabetic Rats Pancreas

NF-κB and I-κBα expression in the pancreas tissues from each group were shown in Figure 4. NF-κB expression in the control group showed much higher than those in the normal group, while I-κBα expression showed much lower than those in the normal group. At the concentration of 2 mg/kg in acankoreagenin group showed a higher I-κBα expression and lower NF-κB expression than positive control group.

2.9. Effect of Acankoreagenin on TGF-β1 Expressions in Normal and Diabetic Rats Kidney

NF-κB, p-p38 and TGF-β1 expression in the kidney tissues from each group were shown in Figure 5. NF-κB, p-p38 and TGF-β1 expression in control group showed much higher than those in the normal group. At the concentration of 2 mg/kg in acankoreagenin group showed a low expression in NF-κB, p-p38 and TGF-β1.

2.10. Effect of Acankoreagenin on Histopathological Findings in Normal and Diabetic Rats Pancreas and Kidney

The effect of acankoreagenin ameliorated the pancreas histopathology as shown is Figure 6. Three weeks after the establishment of the diabetes model, the diabetic rats showed severely damaged Langerhans islets were visible compared to normal rats. In diabetic rats with acankoreagenin treatment, high-dose of concentration was reduced Langerhans islets damaged, necrosis or inflammation when compared to the normal rats.

The effect of acankoreagenin ameliorated the renal histopathology as shown is Fig. 7. Three weeks after the establishment of the diabetes model, the diabetic rats showed severely damaged glomerulus was visible compared to normal rats. In diabetic rats with acankoreagenin treatment, high-dose of acankoreagenin was reduced glomerulus damaged. Especially, it showed a better renoprotective effect when compared to positive control rats.

3. Discussion

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STZ-induced diabetes is characterized by pancreatic β-cell damage and insufficient insulin synthesis [22], which are all related to oxyradicals. In this study, sharply reduced body weights and increased fasting blood glucose levels were observed in rats with STZ-induced type I diabetes, and significantly reversed by administration of acankoreagenin. We were encouraged to find that acankoreagenin enhanced the serum levels of insulin, indicating their amelioration of the metabolic disturbance of glucose enzymes and had renoprotective effect in diabetic rats.

The changes of body weight, food consumption and water intake have previously been reported as important parameters in pathophysiology of STZ-induced diabetes [23]. In the present study, STZ administration inhibited the percentage weight gain, induced an increase in water and food consumption as compared to the normal control animals. The OGTT is a more sensitive measure of early abnormalities in glucose regulation than fasting plasma glucose or HbA1c level testing [24]. The OGTT showed that blood glucose levels peaked and then returned to fasting values after 2 h in both normal and acankoreagenin-treated diabetic rats. In untreated diabetic rats, blood glucose levels remained greater than 450 mg/dl, even after 2 h. Thus acankoreagenin treatment effectively prevented the increase in blood glucose.

In the insulin synthesis pathway, preproinsulin, consisting of an A-chain, a B-chain, a C-peptide, and a signal sequence, is first translocated into the endoplasmic reticulum of the pancreas beta cells. The signal peptide is cleaved from the N- terminal of the peptide by peptide peptidase, producing proinsulin. In the Golgi apparatus (beta-granules), when proinsulin is packaged into vesicles, the C-peptide is removed, leaving the A and B chains bound together by disulfide bonds that make up the insulin molecule [25]. *In vivo* studies in type I diabetes animal models have established that C-peptide administration significantly improves neurological and renal function. Therefore, in animals with early symptoms of DN, replacement doses of C-peptide therapy can improve peripheral nerve function, as evidenced by increased nerve conduction velocity, increased nerve Na +, K + ATPase activity, and significantly improved nerve structural changes [26]. ALT and AST are important hepatic markers, high plasma concentrations of these markers are indicators

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of liver pathology, as evidenced hepatic. The increase of ALT and AST activity in STZ intoxication has been reported previously, and was confirmed in the present study [27]. However, further investigations are needed to clarify the detailed roles of the oxidative system in acankoreagenin-mediated antidiabetic activity.

DN is a major cause of end-stage renal disease, can be defined as progressive reduction in the glomerular filtration rate, increased proteinuria, and tubulointerstitial fibrosis due to renal pathological changes. Renal fibrosis is a common consequence of DN and other chronic kidney diseases, and may eventually lead to serious clinical complications and dysfunction [28,29]. Compared with the diabetic control group, the renoprotective effect of acankoreagenin was manifested by normalization of BUN, creatinine, and albumin levels. Major antioxidant enzymes, such as SOD and GSH, are thought to be the first line of defense against ROS produced by peroxide decomposition during oxidative stress, while blocking lipid peroxidation [30]. The content of MDA is an important indicator of oxidative stress, and its formation is promoted in the kidney by reactive oxy gen species (ROS). Antioxidants can effectively alleviate the symptoms of diabetes and DN [31]. In the present study, the levels of SOD and GSH showed a significantly increased and a strong reduction in MDA concentration after a 3-week treatment with acankoreagenin.

NF-κB, such as TNF-α and IL-1β, is a transcription factor involved in cellular response and regulates the expression of inflammatory cytokines. Activation of NF-κB contributes to the gradual decrease of β-cells during diabetes, whereas blocking this process protects β-cells against cytokine-induced apoptosis [32]. In diabetes patients, increased NF-κB levels in many tissues have been observed. Via inhibition of NF-κB activities transferrin effectively suppressed the intracellular signaling essential for I-κB gene expression [33]. Our study found that by inhibiting NF-κB, the release of proinflammatory cytokines was suppressed in STZ-induced diabetic rats model. An increased influx of glucose through the hexosamine pathway leads to the increased of TGF-β formation. In the development of glomerulosclerosis TGF-β plays an important role, through stimulating the ECM collagen types I, III, and IV and fibronectin to lead to the tubulointerstitial fibrosis [34]. TGF-β1 signal transduction involves the activation of complex intracellular networks, thereby

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regulating the biological functions of multipotency. Our study found that by inhibiting TGF-β, the renal fibrosis was suppressed in STZ-induced diabetic rat model.

TGF-β1 signaling involves activation of complex intracellular networks to regulate pleiotropic biological functions. As shown in Figure 8, the active form of TGF-β1 is transmitted signals by type I and II receptors, TβRI and TβRII. These cell surface receptors contain threonine kinases, which phosphorylate the transcription factor proteins called Smads to initiate the typical TGF-β signaling pathway [35]. TGF-β1 also activates Smad-independent signaling, like the MAPK, mediated via the Ras-Raf-MEK-ERK pathway, and TGF-β-activated kinase 1 (TAK1), mediated via the TAK1-TAK1-binding protein 1 (TAB1) pathway. TGF-β1-induced activation of TAK1 transmits signals via MKK4-JNK and MKK3-p38 pathways, activates transcription factors activator protein-1 (AP-1) and activator transcription factor-2 (ATF-2), respectively, and activates NF-κB-mediated pro fibrotic responses [36].

4. Material and Methods

4.1. Plant Materials and Isolation of Acankoreagenin from LAG

The LAG were collected at Changsha, Hunan province, China, in October 2015 and confirmed by Professor Liu xiangqian at the Hunan University of Chinese Medicine. A voucher specimen (no.20151006) was deposited in the authorized laboratory. The following isolation method of acankoreagenin from LAG was described by Liu xiangqian [37]. The dried leaves of the plant (500 g) were extracted repeatedly with hot MeOH to give an extract (71.36 g), which was chromatographed on Diaion HP-20P column by using gradient elution with H_2O, 30 % MeOH, 50 % MeOH, 80 % MeOH and MeOH. A saponin mixture eluted with 80 % MeOH was evap-orated to dryness in vacuo, and was subsequently chromatographed on a silica gel by using gradi-ent elution with V ($CHCl_3$) : V (MeOH) : V (H_2O) =9 : 1 : 0.1→7 : 3 : 0.5 to give seven fractions. Fr.2 was chromatographed again on silica gel column using V ($CHCl_3$): V (MeOH): V (H_2O) = 9: 1: 0.1→8: 2: 0.2 to obtain compound 1 (3.59 g). The chemical structure of compound 1 was characterized on the basis of ^{1}H- and ^{13}C-NMR spectral analysis, and comparisons with published spectral data. The purity of the compounds was over 98%. The chemical structure of acankoreagenin was shown in Figure 9.

4.2. Animals and Experimental Group Design

Adult male Sprague-Dawly (SD) rats, 6 weeks (190-210 g) were purchased from Samtako (Osan, Korea), 3 animals per cage, rats were housed in a laminar flow cabinet with a 12-h light/dark cycle (06:00-18:00 h) and temperature (23.0 ± 1 °C). All animals were

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maintained on standard laboratory chow ad libitum. Food and water intake were evaluated daily, and measured body weight weekly. After one week of adaptation, the animals were divided into six groups (n = 6 animals/group); **(N)** normal, non-diabetic group; **(C)** control, type I diabetes group; **(Gua)** guava, 500 mg/kg, positive group; **(Acan 0.08)** acankoreagenin 0.08 mg/kg; **(Acan 0.4)** acankoreagenin 0.4 mg/kg; **(Acan 2)** acankoreagenin 2 mg/kg. The current study protocol was also approved by the Institutional Animal Care and Use Committee of Wonkwang University (Permit No. WKU 17-92).

4.3. Induction of Type I Diabetes

To induce type I diabetes, rats were overnight and then treated via intraperitoneal injection with STZ (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M sodium citrate buffer (pH 4.0), by a single dose of 70 mg/kg. Three days after STZ administration, blood was taken from the lateral veins of the tail and blood glucose was measured by a glucometer (ACCU-CHEK® Advantage, Roche Diagnostics, Mannheim, Germany), and the animals that showed fasting blood glucose > 250 mg/dl were considered diabetes.

4.4. Sample Treatment

All the groups were treated daily with orally administered by via gavage for 3 weeks. The samples were diluted in 0.2% carboxymethyl cellulose (CMC) and treated daily with orally administered. The dilution was performed immediately prior to the intragastric administration. Rats from non-treated groups received vehicle solution (0.2% CMC) without sample. The treatment occurred between 10:00 and 11:00 a.m.

4.5. OGTT

An overnight fast, oral glucose load was administered via gavage 2 g/kg of body weight. Blood glucose levels were measured from tail bleeds. To assess the fasting blood glucose levels, blood samples were collected from each subject at 0, 30, 60 and 120 min after administration of glucose.

4.6. Determination of Plasma Insulin, HbA1c and C-peptide

Plasma insulin level was assayed using with rat insulin ELISA kit (ALPCO Co). Plasma HbA1c level was assayed using with Rat Glycated hemoglobin A1c ELISA Kit (Cusabio Biotech Co., Ltd., WH, HB, China). Serum C-peptide level was assayed using

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with rat C-peptide ELISA kit (ALPCO Co).

4.7. Blood Biochemical Parameters

Blood samples were used for measurement of aspartate transaminase (AST), alanine transaminase (ALT), Total Bilirubin (T-Bil), Total Cholesterol (T-Chol), HDL-Cholesterol (H-Chol) and Triglyceride (TG) were using with clinical chemistry test instrument (Spotchem Ez, koka, konan, Japan).

4.8. KidneyFfunction Tests

Plasma creatinine was assayed using with creatinine detection kit (Arbor assay). Serum albumin was assayed using with rat albumin ELISA kit (Shibayagi, Shibukawa, Gunma, Japan). Blood urea nitrogen (BUN) was assayed using clinical chemistry test instrument.

4.9. Kidney Tumor Necrosis Factor-Alpha (TNF-α) level

TNF-α level in kidney was assessed using with rat TNF-α ELISA kit (BD Biosciences, San Diego, CA, USA).

4.10. Oxidative Stress Biomarkers

Kidney reduced glutathione (GSH) content was assayed using with GSH detection kit (Enzo, Farmingdale, NY, USA). Kidney malondialdehyde (MDA) was assayed using with lipid peroxidation (MDA) assay kit (Sigma-Aldrich). Serum superoxide dismutase (SOD) activity was assayed using with SOD (Sigma-Aldrich) assay kit.

4.11. Western Blotting Analysis

Take each group the frozen kidney and pancreas tissue were homogenized in lysis buffer (iNtRON Biotech) on ice and the centrifuged 17,000 rpm for 10 min at 4℃. The resultant protein extracts were denatured and separated by SDS-PAGE and then transferred to PVDF membranes for 2 h. Membranes were blocked in 5% skim milk and probed with primary antibodies (1 μg/ml) against I-κBα, iNOS, NF-κB, β-actin (Cell Signaling, Beverly, MA, USA) p-38, TGF-β1 (Santa Cruz, CA, USA) were incubated overnight at 4℃. Then the membranes were incubated with anti-rabbit or anti-mouse IgG antibodies for 2 h at room temperature. Reactive bands were visualized using ECL reagent and protein expressions were analyzed by the signals captured on the PVDF membranes using a Fluor Cheme E image analysis.

4.12. Histopathology

Pancreas and kidney specimens were immediately fixed in 10 % formalin solution, dehydrated, embedded in histological paraffin and sectioned (5 μm) in non-serial cuts. Specimens were stained with hematoxylin & eosin (H&E) to identify morphological changes. The tissue sections were deparaffinized, rehydrated and blocked against endogenous peroxidase, washed in 0.01 M phosphate (PBS, pH 7.4) buffer asline and then the sections were incubated with Protein Block Serum-Free (DAKO, Carpinteria, CA, USA) to block non-specific staining. Digital images from the pancreas and kidney using 40 digital images (100 magnification) from each group.

4.13. Statistical Analysis

All experiments were performed in triplicate. Dates were analyzed using the SPSS (Statistical Package for the Social Science, Ver. 18.0) program. The data are expressed as the mean ± SEM values. The differences between the means of the experimental and control groups were performed using Student's *t*-test. And comparisons between multiple groups were made by ANOVA and Duncan's tests. Differences with a p value <0.05 were considered statistically significant.

5. Conclusions

In conclusion, the ability of acankoreagenin to decrease blood glucose levels in STZ-induced type I diabetic rats towards normal levels confirms its antidiabetic activity. It also inhibited diabetic complications by preventing changes in plasma concentration of TG and cholesterol, as well as AST and ALT activities. These findings suggested a mechanism by which acankoreagenin improves renal tissue fibrosis. Furthermore, this study demonstrated that treatment with acankoreagenin could attenuate the progression of renal dysfunction in STZ-induced type I diabetic rats. The protective effect of acankoreagenin may be correlated with TGF-β1 signaling through NF-κB, p-p38 pathway. These data indicate that acankoreagenin may have a beneficial effect in preventing the progress of type I diabetes. However, the proposed mechanism and its clinical prospect in protection against DN require further investigation.

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Author Contributions: Xiang-Qian Liu and Eun-Hee Hwang designed the experiments and revised the paper. Yang Yang performed the experiments, analyzed the data, and wrote the paper. Man-Xia Lu and Qin-Peng Zou helped analyze experimental data. All authors read and approved the final manuscript.

Conflict of interest: The authors declare that there are no conflicts of interest in this paper.

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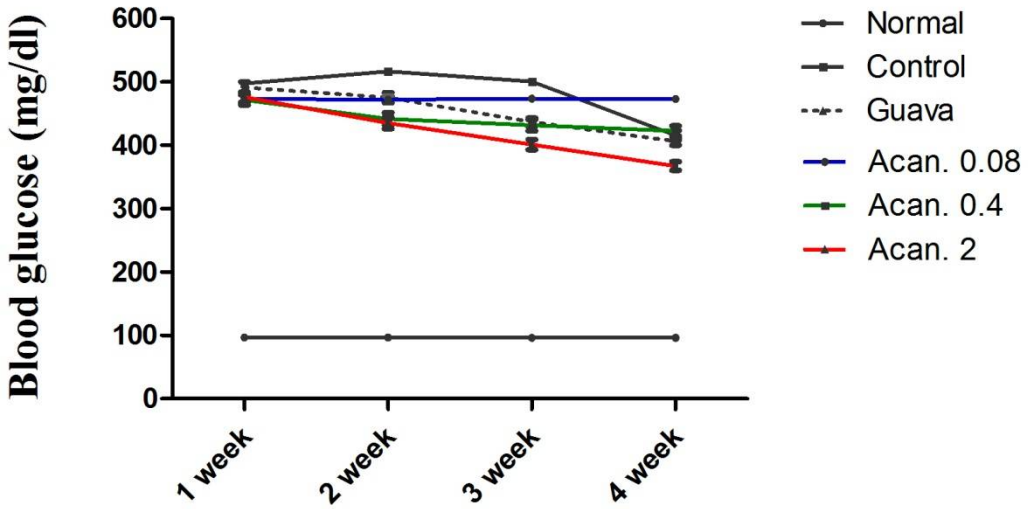

Figure 1. Blood glucose of normal and diabetic rats per one day during 3 weeks. **Acan. 0.08**: acankoreagenin 0.08 mg/kg; **Acan. 0.4**: acankoreagenin 0.4 mg/kg; **Acan. 2**: acankoreagenin 2 mg/kg.

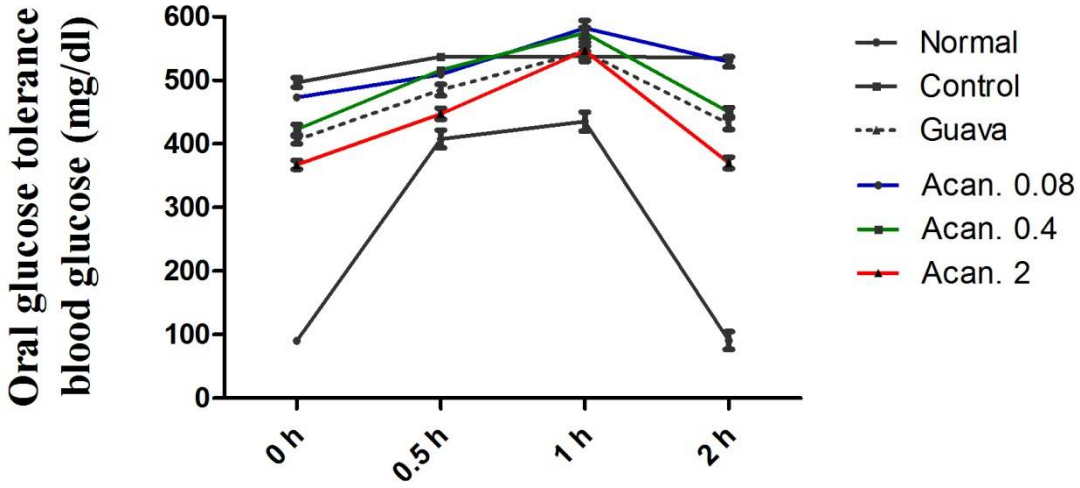

Figure 2. OGTT levels of normal and diabetic rats. The groups are the same as described in Figure 1.

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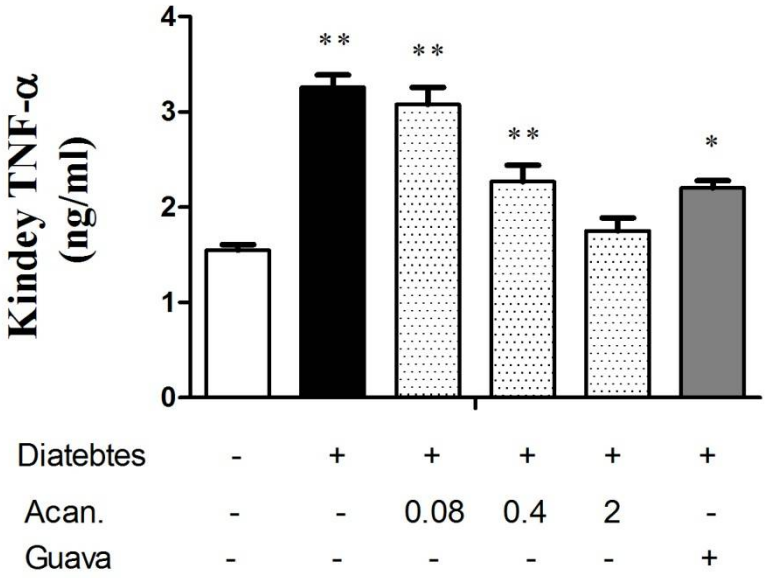

491 **Figure 3.** The changes of the TNF-α level of acankoreagenin in rat kidney. The groups are
492 the same as described in Figure 1. Bars indicate the mean ± SEM (n=6). *, $P < 0.05$ and **,
493 $P < 0.01$ versus normal group.

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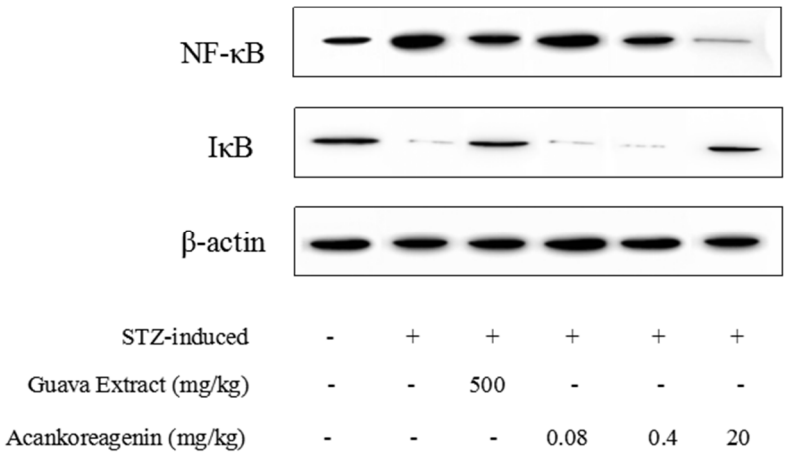

497 **Figure 4.** NF-κB and I-κBα expression in the pancreas tissues from each group. Normalized
498 by β-actin.

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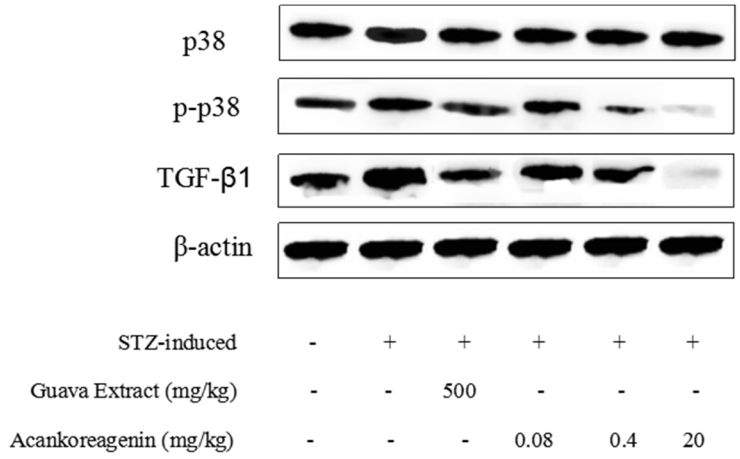

506 **Figure 5.** NF-κB, p-p38 and TGF-β1 expression in the kidney tissues from each group.
507 Normalized by β-actin.

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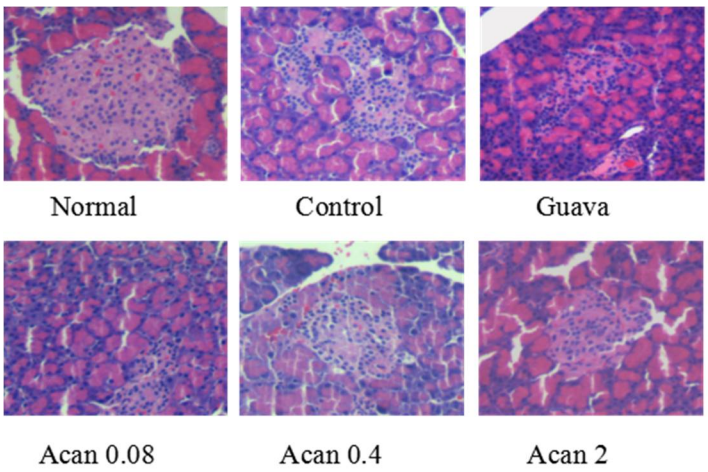

515 **Figure 6.** Effects on the histological change in diabetic rat pancreas. The cellular
516 morphologies of islets were examined after counterstaining with H&E. **(**Magnification
517 100×). The groups are the same as described in Figure 1.

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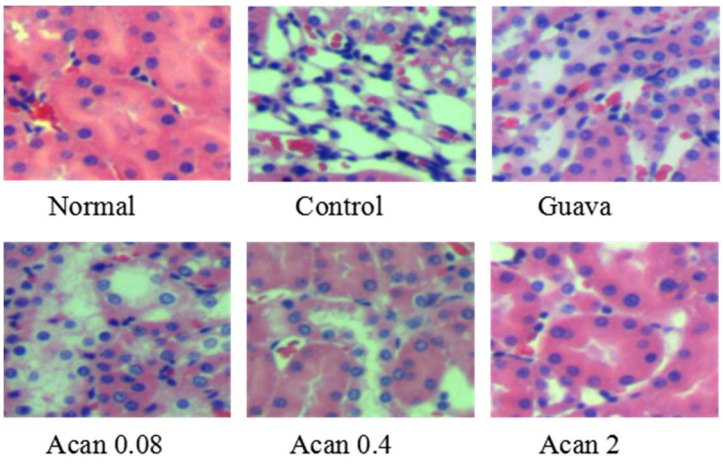

524 **Figure 7.** Effects on the histological change in diabetic rat kidney. The cellular
525 morphologies of kidney were examined after counterstaining with H&E. **(**Magnification
526 100×). The groups are the same as described in Figure 1.

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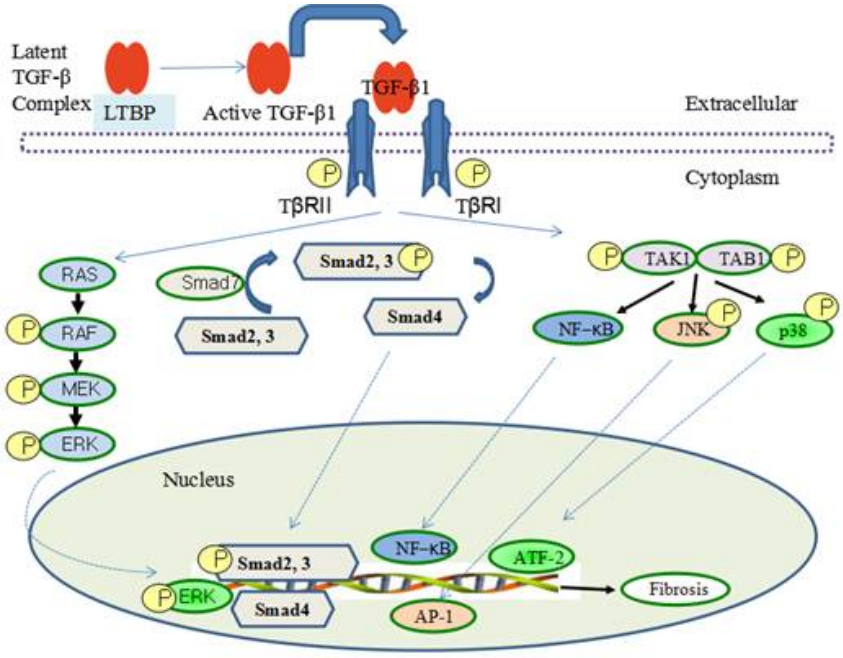

530 **Figure 8.** Transforming growth factor-β1 (TGF-β1) signaling pathways**.**

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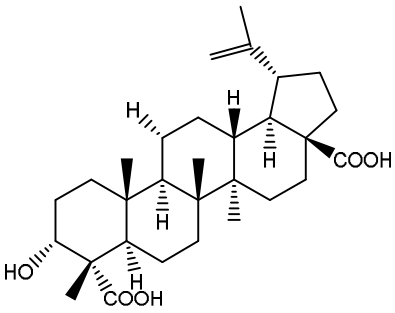

Figure 9. The chemical structure of acankoreagenin.

Table 1. The changes of body weight, food intakes and food efficiency ratio (FER) of rats during 4 weeks.

Group	Initial BW (g)	Final BW (g)	BW gains (g/d)	Food intakes (g/d)	FER [1]
Normal	163.41 ± 3.21	311.67 ± 4.01^{a}	5.49 ± 3.82^{a}	91.66 ± 1.20^{b}	0.059 ± 0.82^{a}
Control	162.72 ± 3.52	173.33 ± 2.11^{e}	0.39 ± 3.14^{f}	66.00 ± 2.51^{c}	0.005 ± 0.41^{ef}
Guava	163.97 ± 3.62	181.67 ± 3.07^{de}	0.65 ± 2.92^{e}	91.00 ± 4.0 ^{b}	0.007 ± 0.67^{e}
Acan 0.08	162.46 ± 4.37	185.00 ± 8.46^{d}	0.83 ± 7.15^{d}	92.66 ± 3.38^{b}	0.009 ± 0.17^{de}
Acan 0.4	162.06 ± 2.72	240.00 ± 7.05^{c}	2.88 ± 9.74^{c}	100.64 ± 4.20^{ab}	0.028 ± 4.33^{c}
Acan 2	162.27 ± 6.30	260.00 ± 6.32^{b}	3.62 ± 5.68^{b}	103.52 ± 2.45^{a}	0.035 ± 0.68^{b}

The data were expressed as the mean ± SEM (n=6). $P < 0.05$ versus $^{a-f}$ normal, non-diabetic group. [1] Weight gain/Food intake.

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Table 2. The changes of water intake of acankoreagenin in rats during 4 weeks.

Group	Water intake (ml/d)			
	1 week	2 week	3 week	4 week
Normal	41.66 ± 0.33	71.33 ± 0.33^{f}	85.33 ± 0.33^{f}	85.35 ± 1.45^{f}
Control	41.00 ± 0.57	546.66 ± 3.33^{a}	556.66 ± 3.33^{a}	560.00 ± 7.54^{a}
Guava	41.66 ± 0.33	530.00 ± 5.77^{bc}	453.33 ± 3.36^{de}	400.00 ± 7.52^{d}
Acan 0.08	42.00 ± 0.57	521.00 ± 1.00^{c}	516.66 ± 3.36^{b}	513.37 ± 3.35^{b}
Acan 0.4	42.00 ± 0.57	513.33 ± 6.66^{cd}	476.66 ± 3.33^{c}	453.33 ± 8.01^{c}
Acan 2	41.33 ± 0.66	503.33 ± 3.33^{e}	446.66 ± 3.38^{e}	363.33 ± 6.64^{e}

The data were expressed as the mean ± SEM (n=6). $P < 0.05$ versus $^{a-f}$ normal, non-diabetic group.

Table 3. The changes of plasma insulin, HbA1c, and C-peptide levels of acankoreagenin.

Group	Plasma insulin (ng/ml)	HbA1c (%)	C-peptide (pM)
Normal	0.94 ± 0.14^{a}	5.73 ± 0.28^{f}	15.32 ± 0.28^{a}
Control	0.32 ± 0.16^{f}	13.05 ± 0.42^{a}	3.31 ± 0.32^{e}
Guava	0.61 ± 0.32^{c}	9.63 ± 0.47^{c}	10.32 ± 0.37^{b}
Acan 0.08	0.37 ± 0.44^{ef}	12.04 ± 0.67^{b}	4.61 ± 0.38^{d}
Acan 0.4	0.55 ± 0.62^{d}	8.98 ± 0.71^{d}	7.02 ± 0.37^{c}
Acan 2	0.72 ± 0.52^{b}	7.01 ± 0.48^{e}	10.38 ± 0.46^{b}

The data were expressed as the mean ± SEM (n=6). $P < 0.05$ versus $^{a-f}$ normal, non-diabetic group.

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Table 4. The changes of the biochemical parameters of acankoreagenin in rats.

Group	AST (IU/l)	ALT (IU/l)	T-Bil (mg/dl)	TG (mg/dl)	T-C (mg/dl)	H-C (mg/dl)
Normal	67.83 ± 1.22^{f}	8.05 ± 0.25^{f}	0.35 ± 0.04^{f}	38.00 ± 0.44^{f}	75.83 ± 1.27^{d}	53.50 ± 1.82^{a}
Control	531.16 ± 7.40^{a}	459.33 ± 4.24^{a}	3.05 ± 0.09^{a}	201.66 ± 7.45^{a}	132.16 ± 3.32^{a}	30.00 ± 1.87^{d}
Guava	360.16 ± 4.02^{c}	229.83 ± 7.85^{d}	1.28 ± 0.10^{d}	59.66 ± 3.16^{e}	93.00 ± 4.34^{c}	40.02 ± 1.73^{bc}
Acan0.08	511.06 ± 5.08^{b}	426.00 ± 6.44^{b}	2.50 ± 0.16^{b}	145.83 ± 5.54^{b}	101.33 ± 4.94^{b}	30.50 ± 1.62^{d}
Acan0.4	347.33 ± 8.02^{d}	280.83 ± 8.52^{c}	1.73 ± 0.14^{c}	125.00 ± 6.19^{c}	91.33 ± 4.80^{c}	38.66 ± 1.42^{c}
Acan2	235.50 ± 8.05^{e}	164.66 ± 8.28^{e}	0.93 ± 0.09^{e}	74.00 ± 3.33^{d}	78.66 ± 2.81^{d}	44.68 ± 1.65^{b}

557 AST: aspartate transaminase; ALT: alanine transaminase; T-Bil: Total Bilirubin; TG: Triglyceride; T-C: Total
558 Cholesterol; H-C: HDL-Cholesterol. The data were expressed as the mean ± SEM (n=6). $P < 0.05$ versus $^{a-f}$normal,
559 non-diabetic group.

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Table 5. The changes of kidney function tests of acankoreagenin in rats.

Group	BUN (mg/dl)	Creatinine (mg/dl)	Albumin (ng/ml)
Normal	10.83 ± 0.74^{e}	0.77 ± 0.04^{e}	787.33 ± 3.62^{a}
Control	61.82 ± 1.16^{a}	2.58 ± 0.13^{a}	219.00 ± 4.21^{f}
Guava	37.00 ± 1.68^{d}	1.40 ± 0.11^{c}	492.33 ± 8.87^{c}
Acan0.08	51.50 ± 1.86^{b}	2.50 ± 0.26^{a}	307.67 ± 4.45^{e}
Acan0.4	44.33 ± 2.48^{c}	1.64 ± 0.27^{b}	453.33 ± 8.53^{d}
Acan2	33.02 ± 1.00^{d}	0.92 ± 0.22^{d}	553.34 ± 7.93^{b}

561 BUN: Blood Urea Nitrogen. The data were expressed as the mean ± SEM (n=6). $P < 0.05$ versus $^{a-f}$normal, non-diabetic
562 group.

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Table 6. The changes of kidney oxidative stress biomarkers of acankoreagenin in rats.

Group	GSH (uM/mg protein)	MDA (nM/g protein)	SOD (U/ml)
Normal	1.86 ± 0.17^{a}	24.43 ± 0.06^{d}	89.06 ± 1.78^{a}
Control	0.26 ± 0.06^{f}	43.40 ± 0.12^{a}	39.96 ± 1.30^{f}
Guava	1.23 ± 0.08^{c}	31.30 ± 0.81^{b}	65.60 ± 0.61^{c}
Acan0.08	0.36 ± 0.15^{e}	40.86 ± 0.27^{a}	46.16 ± 0.40^{e}
Acan0.4	0.99 ± 0.16^{d}	32.30 ± 1.41^{b}	59.06 ± 1.70^{d}
Acan2	1.53 ± 0.17^{b}	27.86 ± 0.74^{c}	73.63 ± 0.63^{b}

563 GSH: reduced glutathione; MDA: malondialdehyde; SOD: Superoxide Dismutase; NAD: Nicotinamide adenine
564 dinucleotide. The data were expressed as the mean ± SEM (n=6). $P < 0.05$ versus $^{a\text{-}f}$ normal, non-diabetic group.
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