

Piper sarmentosum Water Extract Attenuates Diabetic Complications in Streptozotocin induced Sprague-Dawley Rats

(Ekstrak Air *Piper sarmentosum* Mengurangkan Komplikasi Diabetes dalam Tikus Sprague-Dawley Teraruh Streptozotocin)

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ABSTRACT

Piper sarmentosum has been shown to possess antihyperglycemic effect. The effect of water extract of PS leaves was determined on the diabetic complications in streptozotocin induced rats. Eighteen male Sprague Dawley rats (n=18) were randomly divided into three groups with six rats each, namely, control, diabetic untreated and PS treated diabetic groups. Diabetes was induced with intramuscular injection of STZ (50 mg/kg). Ten days following the induction, the diabetes was confirmed with fasting blood sugar level more than 8 mmol/L and PS extract was administered orally (0.125 g/kg) for 28 days. The left kidneys were collected to analyze. The body weight and kidney weight index showed significant differences between control and diabetic groups ($p < 0.05$). However, the lesser extent of body weight gain was observed in diabetic group compared with the control groups. The fasting blood sugar level was reduced in PS treated group. The percent area occupied by the glomerulus over a renal corpuscle was found to be 74.5% in DPS, 72% in DNT and 75% in C group; however it was statistically insignificant. Histological study revealed marked inflammatory cells infiltration and glomeruli contraction with widened urinary spaces revealed in DNT group following 28 days of hyperglycemic state whereas the DPS group showed features of improvement. The water extract of PS leaves has the potential preventive effect on the diabetic nephropathy by reducing hyperglycemia.

Keywords: Diabetic nephropathies; *Piper sarmentosum*; streptozotocin

ABSTRAK

Piper sarmentosum telah terbukti mempunyai kesan antihiperglisemik. Kesan ekstrak air daun PS telah ditentukan ke atas komplikasi diabetes dalam tikus teraruh streptozotocin. Lapan belas tikus Sprague Dawley jantan (n = 18) telah dibahagikan secara rawak kepada tiga kumpulan dengan enam tikus masing-masing, iaitu kumpulan kawalan, kencing manis tidak dirawat dan kencing manis dirawat PS. Diabetes diaruh dengan suntikan STZ (50 mg/kg) secara intramuskular. Sepuluh hari selepas induksi, diabetes telah disahkan dengan aras gula darah ketika berpuasa lebih daripada 8 mmol/L dan ekstrak PS telah diberi secara oral (0.125 g/kg) selama 28 hari. Buah pinggang kiri dikumpulkan untuk dianalisis. Berat badan dan indeks berat buah pinggang menunjukkan perbezaan yang signifikan antara kumpulan kawalan dan diabetes ($p < 0.05$). Walau bagaimanapun, sedikit peningkatan berat badan diperhatikan dalam kumpulan diabetes berbanding dengan kumpulan kawalan. Aras gula darah ketika berpuasa menurun dalam kumpulan dirawat PS. Peratus kawasan yang diduduki oleh glomerulus ke atas kopusel buah pinggang adalah 74.5% dalam DPS, 72% dalam DNT dan 75% dalam kumpulan C, namun ia tidak signifikan secara saintifik. Kajian histologi mendedahkan infiltrasi sel inflamatori dan penguncupan glomerulus dengan ruang urinari yang lebih lebar dalam kumpulan DNT selepas 28 hari berada dalam keadaan hiperglisemik manakala kumpulan DPS menunjukkan ciri-ciri penambahbaikan. Ekstrak air daun PS mempunyai potensi kesan pencegahan pada nefropati diabetes dengan mengurangkan hiperglisemia.

Kata kunci: Nefropati diabetes; *Piper sarmentosum*; streptozotocin

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disorder characterised by hyperglycemia due to deficiency of insulin or insulin insensitivity to the tissue or both (WHO 1999). Kidney is one of the target organs affected by chronic uncontrolled DM. The prevalence of diabetic nephropathy among diabetics in Asian population is alarming which is 58.6% and might lead to economic consequences (Wu et al. 2005).

In early stage of diabetic nephropathy, there is a renal hyperperfusion and consequently increased in glomerular filtration rate (GFR) due to the poor hyperglycemic control (Raptis & Viberti 2001; van Dijk & Berl 2004). This attributes to the renal hypertrophy (García et al. 1981). Mesangial hypertrophy and focal glomerulosclerosis occurred in later stage due to increase in glomerular pressure (Raptis & Viberti 2001; van Dijk & Berl 2004).

Streptozotocin (STZ) is a commonly used agent to develop diabetic animal model. A single dose of STZ induction produced hyperglycemia on day 2 (Gross et al. 2004). The frequent dose range for intravenous injection is 40-60 mg/kg body weight and the same or higher dose was used in intraperitoneal injection (Szkudelski 2001). The intramuscular injection of STZ with the dosage of 50 mg/kg body weight has been reported to cause the hyperglycemia in rats at day 3 (Teoh et al. 2010). The previous study was carried out to determine the duration for the development of diabetic nephropathy. Almost 90% of Sprague Dawley rats developed diabetic nephropathy within one week following the injection of streptozotocin at 55 mg/kg and microscopic findings revealed nodular and diffuse glomerulosclerosis (Greg & Terri 2007).

Intense researches have been done to develop new drugs and adjunct herbal products. Many natural herbs possess medicinal efficacy for different diseases. The natural herb such as bitter melon possesses antihyperglycemic effect (Shetty et al. 2005). The previous study showed that *Piper sarmentosum*, known as Daun kaduk in Malaysia, possesses hypoglycemic effect (Peungvicha et al. 1998). It is a creeping terrestrial herb of Piperaceae family and can be found at the relatively moist area. The leaves are simple, alternate cordate, soft oval, thin, dark green, shiny and round with nerve. Vascular bundle scattered in transverse section in monocotyledon like manner. The spike consists of a single flower spike with very small flower glows opposite a leaf. The fruits are small, ovoid berry and sweet tasting (Mathew et al. 2004). PS is a traditional medicine in Malaysia because of its anti-amoebic, anti-bacterial, anti-tuberculosis, anti-neoplastic, neuromuscular blocking, anti-malarial, anti-oxidant and anti-angiogenic properties (Khalid et al. 2011).

To date, there is no report against the effect of PS on the diabetic complications such as nephropathy. Knowing the beneficial medicinal properties of PS, the current study is aimed to determine whether water extract of PS leaves would be able to prevent the disease progression in the kidney of STZ-induced diabetic rats and discuss the roles of its antioxidant, anti-hyperglycemia and anti-inflammatory properties in prevention of diabetic complications.

MATERIALS AND METHODS

STUDY PROTOCOL

Eighteen male Sprague-Dawley rats with 150 ± 50 g body weight were obtained from the institutional animal house and reared accordance with the ethical guideline. The ethical approval was obtained from the Institutional Animal Ethics Committee prior to the experiment. The rats were kept in one animal per cage with 12 h light and dark cycle in temperature controlled room (22-24°C) and fed with tap water and standard rat chow (Gold Coin, Malaysia) *ad libitum*. One week after acclimatization, the rats were randomly allocated into three groups namely non-diabetic or control (C, $n=6$), untreated diabetic (DNT,

$n=6$) and diabetic treated with PS (DPS, $n=6$). Body weight was recorded daily.

INDUCTION OF DIABETES AND TREATMENT

The diabetic induction was done with intramuscular injection of a single dose of STZ (50 mg/kg) (Sigma Chemical Co. USA), being freshly prepared in 20 mM citrate buffer (pH4.5) after overnight fasting (Teoh et al. 2010). Fasting blood glucose levels were estimated 72 h after the STZ injection. The rats with fasting blood glucose level more than 8 mmol/L were considered as diabetes. The PS treatment was started on day 11 and the dose of 0.125 g/kg was given daily for 28 days (Peungvicha et al. 1998). Fasting blood sugar level was monitored weekly throughout the experiment by using Accu-Chek Advantage glucometre (USA) from the tail vein.

PREPARATION OF PLANT EXTRACT

The PS leaves was purchased from the local supplier and botanical identification was done in Herbarium Unit, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The extract was freeze-dried in the Forest Research Institute of Malaysia (FRIM). Briefly, the extraction procedure was as follows. Five kilograms of fresh leaves of PS were cleaned with tap water and dried at room temperature before chopped into small pieces. Then, the dried leaves pieces were boiled with distilled water at 80°C for 3 h. The water extract was then concentrated and freeze-dried to produce powder form. The powdered extract then stored at 4°C until further use. The powdered extract (0.125 g/kg) was prepared freshly in 5 mL 0.9% normal saline (Rahman et al. 2011).

COLLECTION OF SAMPLES AND HISTOMORPHOLOGICAL ANALYSIS

Following 28 days of treatment, the rats were sacrificed and the left kidneys were collected and recorded the weight. The collected tissues were fixed in 10% formalin, dehydrated through graded alcohol series (50-100%), cleared in xylene and lastly embedded in paraffin wax. Then, the tissue was sectioned to 5 μm thickness and stained with Hematoxylin and Eosin (H&E). Pictures of the stained slides were taken using light microscope (Leica, Germany) attached to a digital camera (Pixelink, Canada). The body weights of the rats were analyzed by comparing the weight at the day of STZ induction (D0) with that of the 28th day (D28). The kidney weight index was expressed as the percentage of kidney weight to the body weight at day 28. The glomerular size in term of area (μm^2) and the area of renal corpuscle (μm^2) were measured by using VideoTesT- Master Morphology, version 5.2. The percent area occupied by the glomerulus over the renal corpuscle was calculated with the formula (glomerular area/ renal corpuscle area $\times 100$). The analysis was done on the three histological sections per specimen with the interval of 10 serial sections. The average readings of 15 glomeruli per

specimen over three microscopic views were recorded with 100× magnification by two independent observers. The overall histological changes were also observed.

STATISTICAL ANALYSIS

The data were analyzed with paired sample T test and one-way ANOVA test by using SPSS version 17. The significant level of the data was considered if p value was less than 0.05.

RESULTS

BODY WEIGHT CHANGES IN DIABETES

The changes in the body weight were recorded (Table 1). The rats in control group showed significant weight gain compared with the diabetic groups ($p<0.05$). The diabetic rats showed lesser extent of weight gain at the end of experiment as expected. While comparing the treated and untreated diabetic rats, there were no significant difference in body weight changes at the end of experiment ($p>0.05$) as shown in Figure 1.

FASTING BLOOD GLUCOSE LEVEL

The mean fasting blood sugar (FBS) level was significantly higher in the diabetic groups induced by STZ compared with

the control group ($p<0.05$). The mean FBS level of untreated diabetic rats steadily maintained from 22.18 ± 1.87 before treatment to 23.75 ± 1.54 at the end of experiment. The PS treated group reduced the FBS level from 26.58 ± 2.74 to 23.33 ± 3.24 mmol/L; however, it was not statistically significant ($p>0.05$) as shown in Table 1 and Figure 2.

CHANGES IN THE KIDNEY OF DIABETIC ANIMAL

Histomorphometric Changes The kidney weight index of diabetic rats at day 28 was significantly higher than the control group ($p<0.05$) as shown in Table 2 and Figure 3. The mean glomerular area (GloA) in diabetic groups became smaller proportionately with the renal corpuscle area (RCA). When compared with the control group, it was found statistically insignificant ($p>0.05$). However, the results of the percent area occupied by the glomerulus over a renal corpuscle was found as 75% in control, 72.3% in untreated diabetic and 74.5% in PS treated groups as shown in Table 2 and Figure 4. This indicated that the glomeruli in untreated diabetic group became contracted; however, these changes were corrected by treating with PS extract although the finding is statistically insignificant.

Histological Changes The untreated diabetic group revealed the contracted glomeruli with widened urinary spaces (Figure 5). It also revealed the marked inflammatory

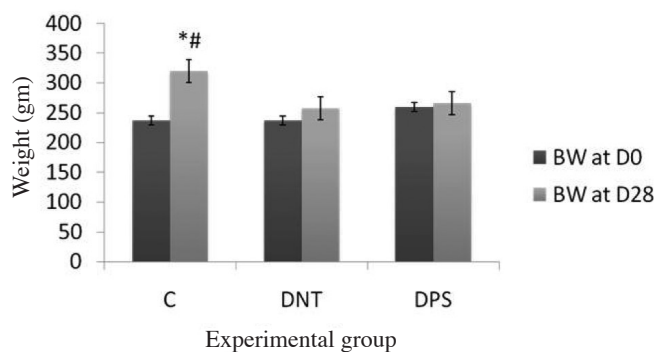
TABLE 1. Effect of PS extract on the body weight and fasting blood sugar level changes in STZ-induced diabetic rats

Groups	BW_D0± SEM (g)	BW_28±SEM (g)	FBS_D10 ± SEM (mmol/L)	FBS_D28±SEM (mmol/L)
Control (C)	237.25±4.85	320.08±10.65*#	5.68±0.19#	5.81±0.24#
DNT	236.91±6.04	257.83±9.43	22.18±1.87	23.75±1.54
DPS	259.91±11.8	266.33±16.79	26.58±2.74	23.33±3.24

* significant difference between day 28 compared with day 0 ($p<0.05$)

significant difference between control and diabetic groups (DNT&DPS) ($p<0.05$);

D0: at the day of STZ induction; D10: day 10 of STZ induction; D28: at the day of sacrificed; BW: body weight; FBS: fasting blood sugar



* significant difference between day 28 compared with day 0 ($p<0.05$)

significant difference between control and diabetic groups (DNT&DPS) ($p<0.05$); D0: at the day of STZ induction; D28: at the day of sacrificed

FIGURE 1. Effect of PS extract on the body weight changes in STZ-induced diabetic rats

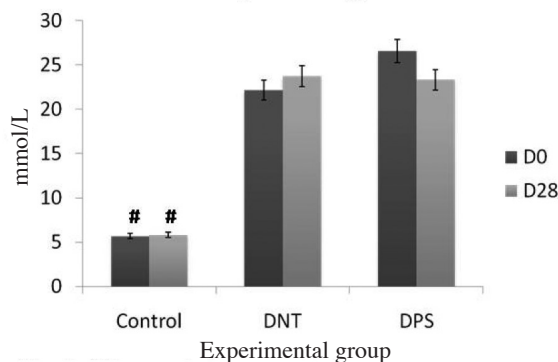
TABLE 2. Effect of PS extract on the histomorphometric changes in kidney of STZ-induced diabetic rats

	Control (C) (g)	DNT (g)	DPS (g)
Kidney weight index	0.34±0.03 [#]	0.44±0.06	0.43±0.03
Percent glomerular area in a renal corpuscle	75.05±1.97	72.33±6.38	74.53±1.85

significant difference between control and diabetic groups (DNT&DPS) ($p < 0.05$)

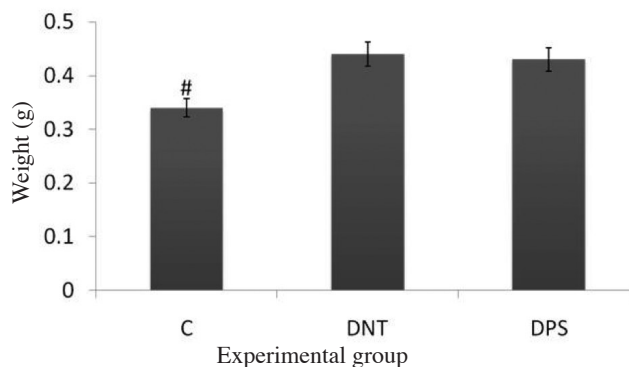
Values are expressed as mean \pm SD

Kidney weight index (KWI)=(kidney weight in gram/body weight at day 28) \times 100; Percent glomerular area in a renal corpuscle = (glomerulus area/renal corpuscle area) \times 100



significant difference between control and diabetic groups (DNT&DPS) ($p < 0.05$); D0: at the day of STZ induction; D28: at the day of sacrificed

FIGURE 2. Effect of PS extract on the FBS level of STZ-induced diabetic rats



significant difference between control and diabetic groups (DNT&DPS) ($p < 0.05$)

FIGURE 3. Effect of PS extract on the kidney weight index of STZ-induced diabetic rats

cells infiltration in both the renal cortex and medulla and the glomerular membrane thickening which could also be due to the inflammatory cell infiltration. However, the PS treatment attenuated the histology changes in the kidney following STZ induction (Figure 5). The histology finding supported the histomorphometric results. The kidneys of PS treated diabetic rats showed mild inflammatory cells infiltration and reduced the size of urinary space. The glomerular membrane thickening was not found in the PS treated kidneys.

DISCUSSION

The development and disease progression in diabetes is highly associated with the extent of oxidative stress (Baynes & Thorpe 1999; Ceriello 2000). The increase in free radical production and the changes in the activity of innate antioxidants such as glutathione and superoxide dismutase (SOD) were exhibited in chemically induced diabetic models (Maritim et al. 2003). There was an alteration in malondialdehyde (MDA) level, an oxidative cellular damage marker, in streptozotocin (STZ) induced

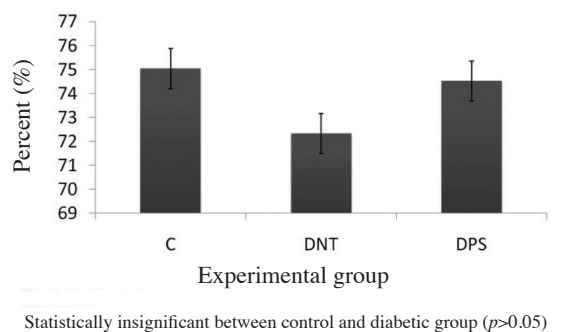


FIGURE 4. Effect of PS extract on the percentage of glomerular area occupied in a renal corpuscle

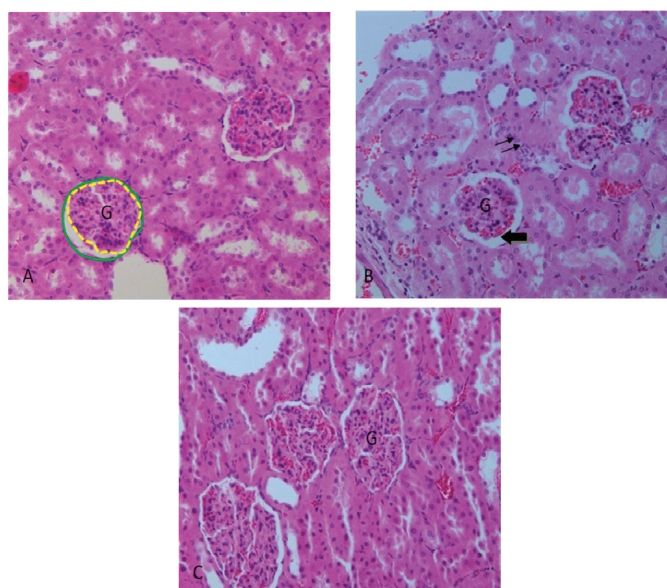


FIGURE 5. Effect of PS extract on the histological changes in kidney of STZ-induced diabetic rats (H&E X200) (a) Control (b) Untreated diabetic kidney (DNT) (c) PS treated diabetic kidney (DPS): Glomerulus (G); urinary space (bold arrow); Inflammatory cells (thin arrows); glomerular area (yellow-dotted line), renal corpuscle area (green line)

diabetic rats (Azzat et al. 2010). The inflammatory marker, $\text{TNF-}\alpha$, is involved in insulin resistant diabetic conditions (Lee et al. 2005). A linkage has been reported between the oxidative stress and inflammatory reaction (Mariappan et al. 2007). All of these might attribute to the development of diabetic complications such as nephropathy, retinopathy and neuropathy.

The natural herb, PS, possesses antioxidant activity (Khalid et al. 2011; Subramaniam et al. 2003). An earlier study done by Rahman et al. (2011) showed that the water extract of PS leaves increased the erythrocyte SOD level and reduced the MDA level in STZ-induced diabetic rats. Hence, its antioxidant property plays a role in prevention of the disease progression in STZ-induced diabetic kidney. Moreover, the water extract of the whole PS plant showed hypoglycemic effect and the LD_{50} was more than 10 g/kg per oral administration (Peungvicha et al. 1998). The dose

(0.125 g/kg) in the present study was much lower than the LD_{50} . The insignificant reduction of FBS level found in PS treated diabetic rats in the present study may be explained that the leaves of PS possess less potent hypoglycemic effect than that of the whole plant. However, the detail analysis is required to explain the underlying mechanism. According to an earlier study done by Rukachaisirikul et al. (2004), they identified the various chemical constituents from fruit of PS such as eight amides: pellitorine, guineensine, brachystamide B, sarmentine, brachyamide B, 1-piperetyl pyrrolidine, 3',4',5'-trimethoxycinnamoyl pyrrolidine and sarmentosine; and two lignans: (+)-asarinin and sesamin, and four other compounds, 1-(3,4-methylenedioxyphenyl)-1E-tetradecene, methyl piperate, a mixture of β -sitosterol and stigmasterol. However, there is a paucity of reports on the PS constituents that possess dominant hypoglycemic effect.

In diabetes, unexplained weight loss is one of the associated complications. It might be due to the enhanced amino acids catabolism which is attributed by lack of energy due to cellular deprivation of glucose (Charlton & Nair 1998). The deficiency of insulin like growth factors contributes to the unintentional weight loss in diabetic animals (Brown et al. 1997). In the present study, there was no weight loss in the diabetic animals. However, the diabetic rats in the present study revealed the lesser extent of weight gain than that of the control group which was similar to the finding of Helal et al. (2003). This could probably be due to the imbalance between protein synthesis and breakdown as a consequence of insulin deficiency. Moreover, it could be influenced by the activity of water extract of PS on the reduction of visceral fat (Azlina et al. 2009).

Other than unexplained weight loss, kidney is one of the target organs being affected by chronic uncontrolled hyperglycemia. The earlier study mentioned that the microscopic changes seen in diabetic nephropathy include glomerular lesions, renal vascular lesions and pyelonephritis, depending on the stage of the disease progression (Kumar et al. 2007). The most prominent glomerular lesions are thickening of capillary basement membrane, diffuse mesangial sclerosis and nodular glomerulosclerosis. However, thickened capillary basement membranes can be seen prominently under electron microscope. Whereas, the feature of diffuse mesangial sclerosis is identified as diffuse mesangial matrix expansion along with mesangial cell proliferation and it is always associated with the basement membrane thickening. Nodular glomerulosclerosis is described as ball-like deposits of laminated matrix found in the periphery of glomerulus which can be seen prominently only with PAS stain. Renal vascular lesion involves renal atherosclerosis and arteriosclerosis and it constitutes as part of the macrovascular changes in diabetics. Lastly, in diabetic kidney, the most common type of pyelonephritis is necrotizing papillitis pattern (Kumar et al. 2007). However, all the above mentioned changes were not seen in the present study. Perhaps the shorter duration of study might be one of the factors that contributed to the less significant changes observed in the kidney of diabetic rats.

Furthermore, the dose and route of administration of STZ, a diabetic induction agent, could also influence on the development of diabetic renal changes. The earlier study mentioned that nodular and diffuse glomerulosclerosis were seen one week after STZ injection with the dose of 55 mg/kg by using intravenous route (Greg & Terri 2007). However, the results of the present study contradicted the finding of Greg and Terri (2007) although the hyperglycemia was static for 28 days. Moreover, the individual body homeostasis would also be considered as one of the factors.

As a consequence of hyperglycemia, the increased kidney weight index (KWI) found in the diabetic animals of the present study could be due to increase of renal plasma flow and glomerular filtration rate (García et al. 1981).

The previous studies showed that the glomerular size in diabetic rats was increased compared with the non-diabetic rats (Butcher et al. 1977; Stefan et al. 2000) which was attributed by an enlargement of glomerular tuft due to the enlargement of individual glomerular cells (Osterby & Gundersen 1975).

Saraheimo et al. (2003) found the inflammatory cells infiltration in renal cortex and medulla of the diabetic rats. The present study also found the similar pattern in the kidneys of diabetic rats. However, the PS treated diabetic kidneys revealed mild inflammation, probably due to anti-inflammatory property of the PS (Amran et al. 2011; Zakaria et al. 2010). According to the diabetic nephropathy classification described by Tervaert et al. (2010), the PS treated diabetic kidney in the present study was considered as class I diabetic nephropathy because it showed only mild changes and the similar features was found in the control group under light microscope. Moreover, the PS treatment prevents the glomerular contraction which was obviously found in DNT group in term of the percentage of glomerular area occupied in a renal corpuscle. Therefore, the medicinal properties of water extract of PS such as antioxidant, anti-hyperglycemic and anti-inflammatory properties attenuate the further progression of diabetic complications.

As a conclusion, this preliminary research showed that anti-hyperglycemic property of PS prevents further progression of diabetic nephropathy. It is promising to reveal the prominent protective effect if the future study being extended to explore the effect of active constituent of this medicinal plant (PS) on diabetic complications like nephropathy. Furthermore, the dose and duration of induction and treatment should be properly adjusted and the molecular basis on the mechanism of action of PS on the full-blown diabetic nephropathy models should be carried out in future research.

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