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ORIGINAL ARTICLE

Stereological study of kidney in streptozotocin-induced diabetic mice treated with ethanolic extract of *Stevia rebaudiana* (bitter fraction)

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Abstract Ethanolic extract of bitter fraction of *Stevia rebaudiana* (*Srbf*) was extracted to investigate its antihyperglycemic and protective effects on renal structural changes in STZ-induced diabetes. Thirty-five male mice were divided into five groups randomly; the first group as non-diabetic control, the second group as untreated diabetic, the third group treated with glibenclamide 0.5 mg/kg, and the fourth and fifth groups treated with *Srbf* by 200 and 400 μg/kg bw through gavage, respectively, for 15 days. Diabetes was induced in the second to fifth groups by administration of 60 mg/kg bw of streptozotocin intraperitoneally. Serum glucose level was monitored every day. At the 16th day, the subjects were sacrificed and their left kidneys were removed. Tissue sections were stained by periodic acid Schiff and used for stereological analysis. The means were compared by one-way ANOVA and Tukey's post hoc test at the significance level of $p \leq 0.05$. The results showed that *Srbf* significantly restored the blood glucose level toward normal level faster than glibenclamide. High dose of *Srbf* could significantly decrease the length and volume of proximal and distal tubules and vessels and the volume of the interstitial tissue in the diabetic treated group. Both doses of *Srbf* could significantly prevent the glomerular hypertrophy and reduction of glomerular number in comparison with the untreated diabetic group. It can be concluded that the antihyperglycemic properties of a bitter fraction of *S. rebaudiana* are better than glibenclamide, and at high dose, it can ameliorate structural nephropathy in diabetic mice.

Keywords *Stevia rebaudiana* · Diabetes · Mice · Stereology · Kidney

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Introduction

Renal hypertrophy as well as glomerular hyperfiltration are two known complications that occur in the initial stages of diabetes mellitus (Hostetter et al. 1981). Further, renal disorders following diabetes mellitus may have a specific pattern compared to the other renal diseases (McCrary et al. 1981). Some studies had revealed that in early diabetes, glomerular hyperfiltration and renal hypertrophy could be reversed by insulin treatment (Mogensen and Anderson 1975; Christiansen et al. 1982). Whereas, in chronic diabetes, glomerular hyperfiltration could be improved by severe control of blood glucose level, but renal hypertrophy is irreversible (Wiseman et al. 1985). Although renal hypertrophy and glomerular hyperfiltration play a pivotal role in the development of diabetic nephropathy, the relationship between them is still unclear (Hostetter 2003).

Nowadays, therapeutic features of the medicinal plants in both modern and traditional medicine are accepted. On the other hand, the suspected efficiency and side effects of chemical antidiabetic drugs are worrying for people (El-Demerdash et al. 2005). Therefore, the usage of natural and herbal antidiabetic products has been widely welcomed.

Stevia rebaudiana (Bertoni) commonly known as "sweet leaf of Paraguay" is one of these plants that belong to the Compositae (Asteraceae) family with 950 genera. Natural components such as stevioside; rebaudiosides A, B, C, D, and E; dulcoside A; and steviolbioside found in the leaves of the plant are responsible for its sweetness. Steviosides are intensely sweet (150–300 times sweeter than sugar) compounds and constituted up to 4–15% of these components (Curi et al. 1986).

The effects of *S. rebaudiana* on the blood pressure (Chan et al. 1998; Lee et al. 2001), rotavirus (Takahashi et al. 2011), fungi (Silva et al. 2008), and tumors (Satishkumar et al. 2008) were approved.

For the first time, we have extracted *S. rebaudiana* into two fractions—bitter and sweet—and have attempted to investigate the antihyperglycemic effect of the bitter fraction and its probable protective effect on renal structural changes in streptozotocin (STZ)-induced diabetic mice quantitatively.

Materials and methods

Plant collection and extraction

Stevia rebaudiana was cultured for the first time in the Faculty of Agriculture, Razi University, Kermanshah, Iran. Five hundred grams of the plant was obtained and shade dried for 1 week. Then, it was ground and 250 g of the obtained powder was extracted with 750 mL of 100% ethanol for 2 h at 40 °C with continuous shaking. The extract was left for 24 h at room temperature, then it was filtered through Whatman paper No. 2. To obtain the bitter fraction of *S. rebaudiana*, at first, the extract was sediment with n-hexane solvent. Then, it was concentrated under reduced pressure using a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) for an hour at 80 °C until a semi-solid sticky mass was obtained. The components of the obtained extract were analyzed with gas chromatography/mass spectrometry (GC/MS) in the Academic Center of Education, Culture and Research of Razi University. The result of GC/MS analyses is presented in Table 1.

Table 1 The components of bitter fraction of *S. rebaudiana* resulted from GC/MS

No.	Compound	Area (%)
1	Nerolidol	1.79
2	Octadecanol	3.92
3	Khusinol	1.01
4	Manoyl oxide	1.92
5	Epi-a-cadinol	0.46
6	3-Keto-manoyl oxide	0.68
7	Sphatulenol	2.89
8	Austroinulin	32.93
9	*n*-Tetracosane	14.08
10	Jhanol	6.58
11	*n*-Pentacosane	9.39
12	b-Sitosterol	0.3
13	3-a-Acetoxy-manol	0.75
14	a-Amyrine	0.3
15	b-Amyrine	0.3

Animals

Thirty-five healthy male Balb/c mice weighing 36 ± 3 g were provided by the Center of Laboratory Animal Breeding, Kermanshah University of Medical Sciences. The animals were housed under standard environmental conditions (at 25 ± 3 °C temperature and 12:12 h light and dark). All animals were fed with standard pellet diet and water ad libitum during the experiment. This study was approved by the ethics committee of Kermanshah University of Medical Sciences.

Induction of diabetes

Diabetes was induced by a single intraperitoneal (IP) administration of streptozotocin (60 mg/kg bw). Fasting blood glucose (FBG) level was measured everyday by glucometer strips. A blood glucose level higher than 250 mg/dL was considered diabetic.

Experimental design

Three days after diabetes induction, the mice were divided into five following groups ($n = 7$):

1. Control group (C) which received 200 μL normal saline orally
2. Untreated diabetic group (UTD)
3. Treated diabetic mice which received 0.5 mg/kg glibenclamide for 15 days (TDG)
4. Treated diabetic mice which received 200 μg/kg of the ethanolic extract of *Srbf* (TD 200) for 15 days.
5. Treated diabetic mice which received 400 μg/kg of the ethanolic extract of *Srbf* (TD 400) for 15 days

Blood sampling

Blood samples were obtained daily from tail vein in routine tubes to assess the blood glucose level by glucometer strips. At the end of the experiment, all animals were weighed and euthanized with deep chloroform inhalation. Immediately, blood samples were drawn from animals' heart. The collected samples were centrifuged at 10,000 rpm for 15 min and serum separated.

Stereological study

Volume density

After dissection, the left kidney was removed and then weighed. The kidney was fixed in 10% neutral buffered formalin solution for 7 days. An immersion method (Silva and Merzel 2001) was then used to determine the primary volume of the kidney. For estimation of the final volume of the organs, the amount of tissue shrinkage must be specified (Gundersen et al. 1988; Braendgaard and Gundersen 1986). Isotropic, uniform random (IUR) sections must be obtained for estimating tissue shrinkage and tubular length (Gundersen et al. 1988; Nyengaard 1990). These sections were achieved using an orientator method. A description of this method is presented in Fig. 1. Totally, 7–10 slabs were obtained from each kidney through the orientator method. A circular piece was sampled from a kidney slab, and the area of this piece was calculated. The slabs and circular piece were processed, sectioned (5 μm thickness), and stained by a periodic acid-Schiff (PAS) method. The area of the circular piece was calculated again, and tissue shrinkage was estimated as follows (Nyengaard 1990):

$$\text{Volume shrinkage} = 1-\left(\frac{AA}{AB}\right)^{1.5}$$

where AA and AB are the areas of the circular piece after and before tissue processing. The total volume of the organ was then estimated using

$$V_{\text{final}} = V_{\text{primary}} \times (1-\text{volume shrinkage})$$

Tissue sections were examined using a videomicroscopy system composed of a microscope (Olympus CX2, Japan) connected to a video camera (30.5 mm, DinoCapture ver.5, dino-lit.com) and a P4 PC, and the stereological parameters were estimated. The fractional volume of the renal structures was estimated using a point probe (with an area of 100 cm^2 and containing 25 points) and the following formula (Fig. 2):

$$V_v = \frac{P_{\text{structure}}}{P_{\text{reference}}}$$

$P_{\text{structure}}$sum of points hitting to the interested structures
$P_{\text{reference}}$sum of points hitting to the reference space

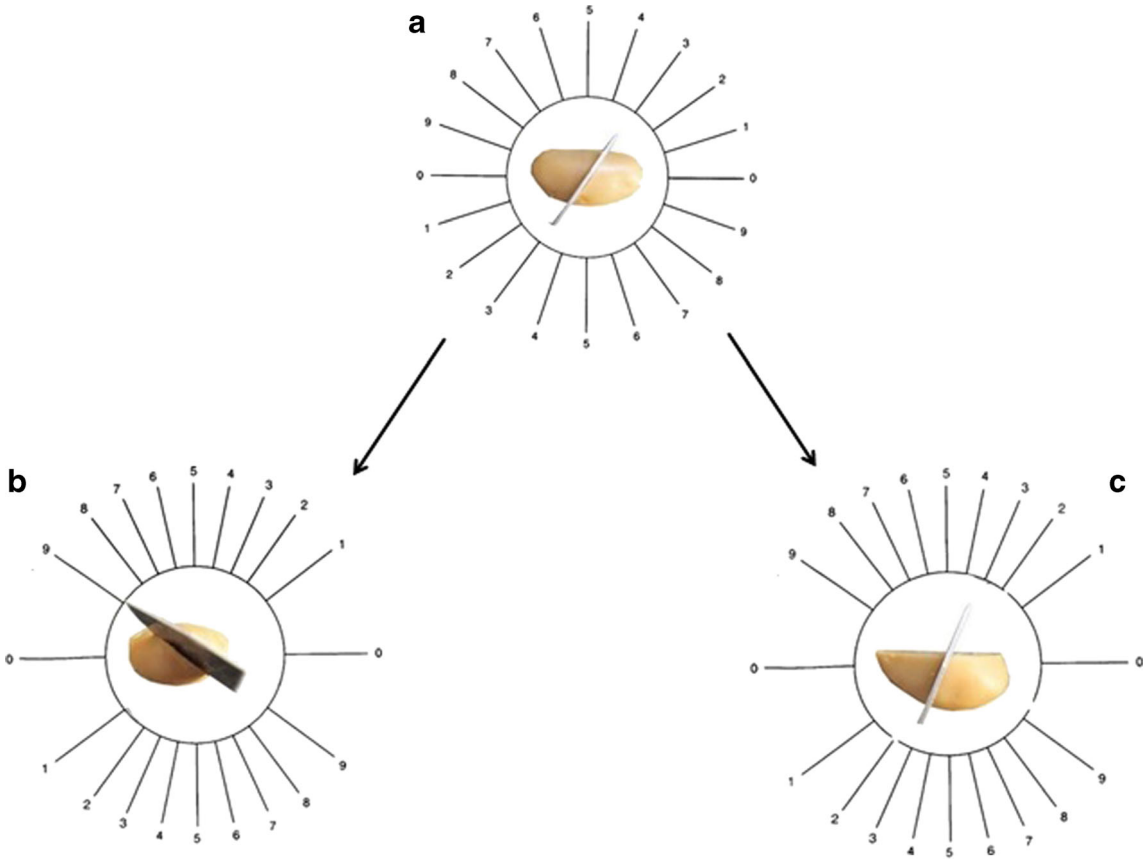

Fig. 1 Orientator method to obtain isotropic uniform random (IUR) sections. **a** The kidney is placed on a circle divided into 10 equal distances. The kidney is cut into two halves in the direction of a random selected number between 0 and 10 (here 3). **b**, **c** The cut surface of each half of the kidney is placed on the 0–0 direction of the second circle with 10 unequal sinus-weighted divisions and the second cuts done in the directions of the tow random selected numbers between 0 and 10 (here 9 and 3). The entire kidney was then sectioned into slabs in the direction of the second cuts with a 1-mm interval, and 7–10 slabs from each kidney were collected

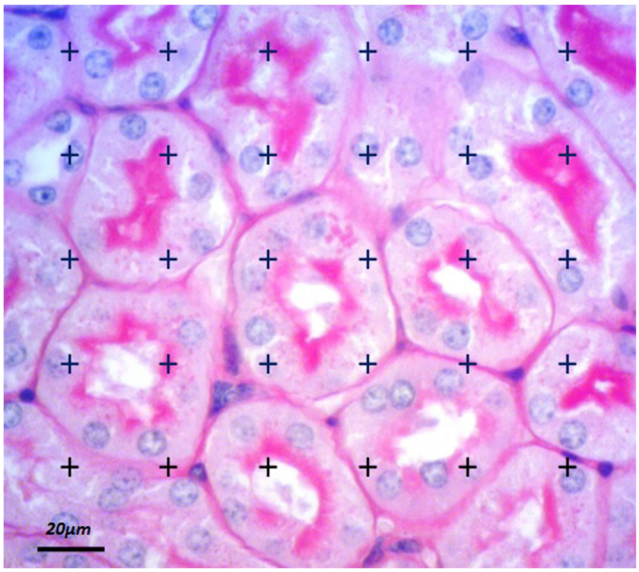

Fig. 2 Estimation of volume density using a point probe. The sum of points hitting each component in 15 microscopic fields was divided by the sum of points hitting the reference space (PAS, ×400)

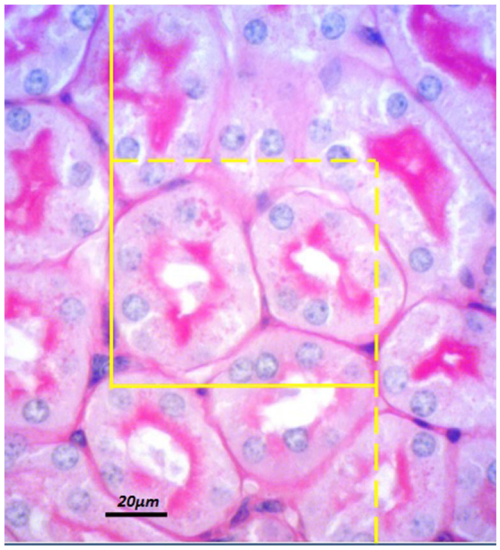

Fig. 3 Estimation of length density of the renal tubules using a counting probe. The tubule structures completely or partly inside the counting frame but only touching the top and right lines are considered (here four proximal convoluted tubules) (PAS, ×400)

Length density

The length density of the tubules and vessels was estimated using an unbiased counting probe (740 × 740 μm). The tubule structures were considered if they were lying completely or partly inside the counting probe and did not touch the down and left lines. Otherwise, they were not considered. The length density was estimated as

$$L_v = 2 \times \frac{\sum Q}{a(\text{frame}) \times \sum \text{frame}}$$

∑Q is the sum of the tubules counted, a(frame) is the probe area, 547,600 μm, and ∑frame is the total number of the counted frames (Fig. 3).

Numerical density

A physical disector procedure (Sterio 1984) was applied for estimating the numerical density of glomeruli. Two parallel sections with 20 μm distance (1th and 5th sections) were prepared: the 1st section as the reference plane (Fig. 4a) and the 5th section as the look-up plane (Fig. 4b). Two counting probes with an area of 547,600 μm^2 were attached on the monitor at the final magnification ×135. The counting rules of the physical disector were applied. Thus, a glomerulus was considered if it was found in the reference plane but not in the look-up plane and did not hit to the down and left lines of the probe. The numerical density of glomeruli was estimated using

$$N_V = \frac{\sum Q^-}{a(\text{frame}) \times h \times \sum P}$$

$\sum Q^-$ is the sum of the counted glomeruli, a(frame) is the probe area, ∑P is the total number of the examined fields, and h is the disector height. The absolute value of each parameter was calculated by multiplying its density by the reference space (Mandarim-de-Lacerda 2003).

Statistical analysis

The results are presented as mean ± standard deviation. One-way ANOVA followed by Tukey's post hoc test at the significance level of $p \leq 0.05$ was used to compare the mean groups statistically.

Results

Effects of Srbf on blood glucose level

The values of blood glucose level of treated and untreated diabetic mice are presented in Table 2. The blood glucose levels of untreated diabetic mice increased to approximately 170% ($p \leq 0.05$) of the control mice in a time-dependent manner. However, treatment of STZ diabetic mice with the ethanolic extract of *Srbf* at low and high doses (200 and 400 μg/kg) could significantly ($p \leq 0.05$) reduce the blood glucose levels compared to the glibenclamide-treated group during days 2–5 and 10–13 of the experiment. But the difference between low and high doses of *Srbf* was not significant ($p \geq 0.05$). Moreover, there was no significant ($p \geq 0.05$) difference between the two doses of *Srbf and* glibenclamide at days 14–16 of the experiment. The reference drug glibenclamide produced its maximum response on days 14–

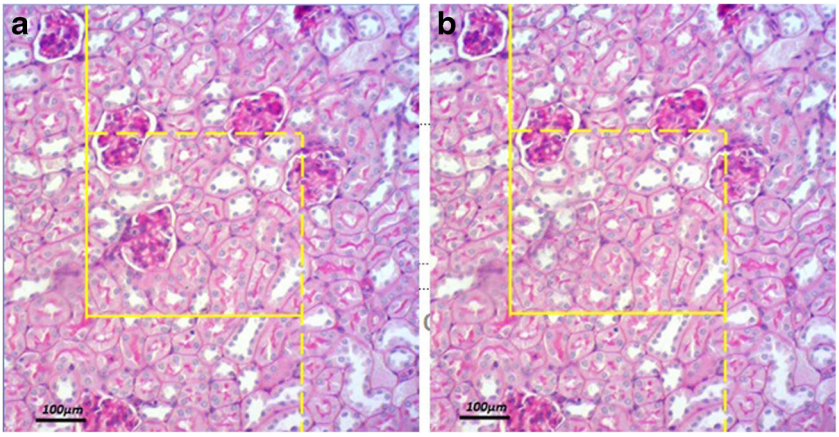

Fig. 4 Physical disector method and estimation of numerical density of glomeruli. **a** First section as reference plane and **b** fifth section as look-up plane with 20 µm distance. A glomerolus was counted if it was located completely or partly in the frame and was not hit by exclusion (continuous) lines in the reference plane as well as if it was not seen in the look-up plane (here 1) (PAS, ×100)

16, which was similar to the response that was observed for 200 and 400 µg/kg of *Srbf.*

Effects of Srbf on body weight

A significant ($p \leq 0.05$) decrease in body weight of diabetic mice was observed when compared to the control ones. Consumption of *Srbf* at high dose significantly ($p \leq 0.05$) restored the body weight in comparison with the untreated diabetic mice (Table 3).

Effects of Srbf on volume

The data of the weight, mean absolute volume, and subcomponents of the kidney in treated and untreated diabetic groups are presented in Tables 3 and 4. The results demonstrated that the kidney weight and volume were increased by 42 and 56% ($p \leq 0.05$), respectively, in the untreated diabetic mice when compared to the control ones. Cortical volume increased by 80% ($p \leq 0.05$) in this group, but the medullary volume increased by 37.5% which was not significant ($p \geq 0.05$) compared to the control group. Treatment of diabetic mice with high dose (400 µg/kg) of *Srbf* could significantly ($p \leq 0.005$) improve the kidney weight and consequently renal volume in comparison with low dose (200 µg/kg) and glibenclamide. Moreover, the effect of glibenclamide was better than the low dose. The changes in medullary volume were not significant ($p > 0.05$) (Table 3).

The volume of PrT (proximal tubule), DiT (distal tubule), vessels, and interstitial tissue was increased by 62, 62.5, 75, and 52%, ($p \leq 0.05$), respectively, in untreated diabetic mice compared to the controls (Table 4). Administration of *Srbf* at high dose to the diabetic mice could significantly ($p \leq 0.05$) decrease the volume of the above structures compared with the untreated diabetic group. Low dose of *Srbf* has no significant ($p > 0.05$) effect on DiT, CoD, and interstitial tissue volume compared to the untreated diabetic and TDG groups.

The glomerular volume was increased significantly (40%) in the untreated diabetic mice compared to the control ones. Treatment with high dose of *Srbf* could significantly inhibit glomerular hypertrophy as compared to the untreated ones. In this respect, there was no significant ($p > 0.05$) difference between glibenclamide and low dose of *Srbf* (Table 5).

Effects of Srbf on glomerular number

The glomerular number of the untreated diabetic mice was reduced significantly (19.6%) in comparison with the control

Table 2 Blood glucose level on different days in the control and experimental groups treated with *Srbf* (mean ± SD)

Groups ($n = 7$)	Blood glucose level (mg/dL)				
	Day 1	2–5 days	6–9 days	10–13 days	14–16 days
C	97.2 ± 3.3	69 ± 2.5	75.8 ± 5.7	81.5 ± 4.4	62.6 ± 3.7
UTD	233.8 ± 13.3*	214 ± 20.8*	221.8 ± 12.2*	211.2 ± 14.9*	236.2 ± 8.8*
TDG	210.2 ± 40.1*	194.6 ± 20.3*	185.2 ± 11.9*	186.4 ± 27.4*	130.2 ± 7.8*·***
TD 200	226.4 ± 11.2*	145.8.1 ± 17.9*·**·***	162 ± 10.5*·***	148.2 ± 10.7*·**·***	129.4 ± 12.3*·***
TD 400	225.8 ± 6*	141.4 ± 9.6*·**·***	157.8 ± 7.5*·***	147.8 ± 14.3*·**·***	117.2 ± 15.6*·***

C control, *UTD* untreated diabetic, *TDG* glibenclamide treated, *TD200* treated diabetics with 200 µg/kg of *Srbf*, *TD400* treated diabetics with 200 µg/kg of *Srbf*

*$p \leq 0.05$ vs control; **$p \leq 0.05$ vs glibenclamide-treated group; ***$p \leq 0.05$ vs untreated diabetic group

Table 3 Animal weight (g), kidney weight (mg), and final volume (mm^3) of the kidney and its subcomponents in the control and experimental groups treated with *Srbf* (mean ± SD)

Groups (n = 7)	Parameters				
	Body weight	Kidney weight	Kidney volume	Cortex volume	Medulla volume
C	36 ± 3	135.2 ± 1.4	110.5 ± 2.9	77.77 ± 4.3	25.3 ± 3.2
CE				(% 2)	(% 4.8)
UTD	29.2 ± 2.8*	192.7 ± 15.6*	171 ± 14.7*	135 ± 11.8*	34.9 ± 3.7
CE				(% 3.3)	(% 4)
TDG	33.5 ± 2.45	160.5 ± 10.9*·***	143.2 ± 10.2*·***	114.8 ± 10.3*·***	30.2 ± 5.1
CE				(% 3.4)	(% 6.4)
TD200	31 ± 4.37*	176.5 ± 8.7*·***	160.7 ± 7.18*	125 ± 8*	28.1 ± 6
CE				(% 2.46)	(% 8.2)
TD400	35.2 ± 3.5***	136.1 ± 11.2**·***	121.5 ± 10.18**·***	91 ± 14**·***	29.3 ± 5.8
CE				(% 5.9)	(% 7.6)

C control, *UTD* untreated diabetic, *TDG* glibenclamide treated, *TD200* treated diabetics with 200 μg/kg of *Srbf*, *TD400* treated diabetics with 200 μg/kg of *Srbf*, *CE* coefficient error

*$p \leq 0.05$ vs control; **$p \leq 0.05$ vs glibenclamide-treated group; ***$p \leq 0.05$ vs untreated diabetic group

group. Whereas, glomerular number loss was significantly prevented by the ethanolic extract of *Srbf* at doses of 200 and 400 μg/kg compared to the untreated ones. The difference between the treated groups and the control group was not significant ($p \geq 0.05$) (Table 5).

Effects of Srbf on length

The length of the PrT, DiT, vessels, and interstitial tissue was significantly ($p \leq 0.05$) increased (68, 82, 75, and 30%, respectively) in untreated diabetic mice compared to the control ones (Table 6). Low and high doses of *Srbf* could significantly ($p \leq 0.001$) decrease the length of the PrT and DiT compared to the untreated diabetic group. However, the difference between the two doses was not significant ($p > 0.05$). The length of vessels was decreased significantly ($p \leq 0.05$) following high dose of *Srbf* compared to the untreated diabetic group.

Discussion

Hypoglycemic and antihyperglycemic activities of *S. rebaudiana* have been reported previously (Chen et al. 2005; Ferreira et al. 2006; Abudula et al. 2004). As far as we know, this is the first investigation on the effects of bitter

Table 4 Final volume (mm^3) of proximal and distal tubules (PrT, DiT), collecting ducts (CoD), loop of Henle (LoH), interstitial tissues (InT), and vessels (V) in the control and experimental groups treated with *Srbf* (mean ± SD)

Groups (n = 7)	Parameters					
	PiT	DiT	CoD	LoH	V	InT
C	68.5 ± 4	16.5 ± 3	20.9 ± 4	1.3 ± 0.38	9.1 ± 1.2	11.4 ± 2
CE	(% 2.3)	(% 6.9)	(% 7.2)	(% 11)	(% 5)	(% 6.7)
UTD	111 ± 9.9*	26.7 ± 8.1*	25.4 ± 6	1.76 ± 0.38	15.9 ± 2.8*	17.3 ± 3*
CE	(% 3.3)	(%11)	(% 8.9)	(% 8.1)	(% 6.6)	(% 6.5)
TDG	87.4 ± 11*·***	21.5 ± 4.8	23.8 ± 5.7	1.2 ± 0.21	11.6 ± 1.6*·***	13.4 ± 3***
CE	(% 4.7)	(% 8.4)	(% 9)	(% 6.6)	(% 5.2)	(% 8.4)
TD 200	95.4 ± 7.2*	18.6 ± 4.6***	24.1 ± 4.6	1.1 ± 0.16	13.4 ± 1.2*	10.9 ± 2.7***
CE	(% 2.8)	(% 11.2)	(% 7.4)	(% 5.5)	(% 3.4)	(% 9.3)
TD 400	48.3 ± 11**·***	15.2 ± 4**·***	22.9 ± 3.6	1.1 ± 0.08	8.1 ± 1.3***	10.6 ± 3.2***
CE	(% 8.6)	(% 9.9)	(% 5.5)	(% 2.7)	(% 6)	(% 11.4)

C control, *UTD* untreated diabetic, *TDG* glibenclamide treated, *TD200* treated diabetics with 200 μg/kg of *Srbf*, *TD400* treated diabetics with 200 μg/kg of *Srbf*

*$p \leq 0.05$ vs control; **$p \leq 0.05$ vs glibenclamide-treated group; ***$p \leq 0.05$ vs untreated diabetic group

Table 5 Final volume (mm^3) and number of the glomeruli in the control and experimental groups treated with *Srbf* (mean ± SD)

Groups (n = 7)	Parameters	
	Volume	Number
C	0.002 ± 0.0001	27,790.4 ± 1325.2
CE	(%1.9)	(%1.8)
UTD	0.003 ± 0.0003*	22,534.8 ± 574.8*
CE	(%3.8)	(%0.9)
TDG	0.0025 ± 0.0003	25,011 ± 1214***
CE	(%4.5)	(%1.8)
TD200	0.0024 ± 0.0002	25,881.9 ± 1385.7***
CE	(%3.2)	(%2)
TD400	0.002 ± 0.0001****	26,454 ± 1275.4***
CE	(%1.9)	(%1.8)

C control, *UTD* untreated diabetic, *TDG* glibenclamide treated, *TD200* treated diabetics with 200 μg/kg of *Srbf*, *TD400* treated diabetics with 200 μg/kg of *Srbf*

*$p \leq 0.05$ vs control; **$p \leq 0.05$ vs glibenclamide-treated group; ***$p \leq 0.05$ vs untreated diabetic group

fraction of *S. rebaudiana* on hyperglycemia and renal structural changes in diabetes using stereological methods. The present study shows that the administration of the *Srbf* extract could reduce toward normal levels of fasting blood glucose and ameliorate the renal morphological changes due to STZ-induced diabetes in mice.

It is well known that renal hypertrophy occurs in the initial stage of diabetes mellitus (Mogensen and Andersen 1973; Kroustrup et al. 1977; Osterby and Gundersen 1980) and is not completely reversible even with prolonged treatment (Tuttle et al. 1991; Wiseman et al. 1985). However, this event can be improved by insulin treatment at the onset of the diabetes (Tuttle et al, 1991), but belated treatment is unsuccessful (Stackhouse et al. 1990). In this experiment, 15 days of STZ-induced diabetes resulted in 56% increase of kidney volume. This renal enlargement was due to a further increase in cortical volume (80%) and its subcomponents, namely, proximal and distal convoluted tubules, glomeruli, vessels, and interstitial tissue rather than medullary volume (37.5%). Treatment of diabetic mice with high dose of *Srbf* could completely preserve the kidney weight and volume.

Increase in the total length, surface area, and number of capillaries of glomeruli in the initial stages of diabetes leads to glomerular dysfunction (Osterby and Gundersen 1980). It has been revealed that in glomerular hypertrophy associated with type 1 diabetes, increase in the number of capillaries is more pronounced than increase in the length of existing capillaries. The present study showed a 40% increase in glomerular volume which is normalized with high dose of *Srbf*. Based on previous reports, some plant extracts including garlic, ginger (Qattan et al. 2008), and *Ginkgo biloba* (Welt et al. 2007) can also prevent glomerular hypertrophy in experimental diabetes. The glomerular number reduction was significantly prevented by *Srbf* compared to the untreated diabetic mice, and the difference between the mice treated and the control ones was not significant. Obviously, nephrogenesis is restricted to the embryonic life and cannot be seen during the postnatal life. Therefore, glomerular number reduction is irreversible and can be interpreted as a one-way road. Maintenance of glomerular number in the diabetic mice indicates that *Srbf* could act as a nephroprotective agent.

During renal hypertrophy, both volume and length of renal tubules are increased. There are two mechanisms for these enlargements: increase in tubular cell volume and/or increase

Table 6 Final length (m) of the proximal and distal tubules (PrT, DiT), collecting ducts (CoD), loop of Henle (LoH), and vessels (V) in the control and experimental groups treated with *Srbf* (mean ± SD)

Groups (n = 7)	Parameters				
	PrT	DiT	CoD	LoH	V
C	33.8 ± 5.7	23 ± 3.3	42.3 ± 5.7	19.5 ± 4.4	56.6 ± 11.7
CE	(%6.3)	(%5.4)	(%5)	(%8.5)	(%7.8)
UTD	56.8 ± 7.3*	42 ± 6.8*	52.1 ± 8.9	28.3 ± 6.3*	73.5 ± 6.8*
CE	(%4.8)	(%6)	(%6.4)	(%8.4)	(%3.5)
TDG	46.4 ± 8.3*	37.4 ± 9.8*	54.8 ± 9.7	27.1 ± 5.6	65.6 ± 11
CE	(%6.7)	(%9.9)	(%6.7)	(%7.8%)	(%6.3)
TD 200	37.4 ± 7.8******	27.1 ± 4.9********	53.8 ± 11.9	24.6 ± 5.6	62.6 ± 12.3***
CE	(%7)	(%6.8)	(%8.3)	(%8.6)	(%7.4)
TD 400	33.6 ± 7.7********	22.6 ± 6********	50.7 ± 8.2	20.6 ± 4.3********	55.5 ± 10.7********
CE	(%8.6)	(%10)	(%6)	(%7)	(%7.3)

C control, *UTD* untreated diabetic, *TDG* glibenclamide treated, *TD200* treated diabetics with 200 μg/kg of *Srbf*, *TD400* treated diabetics with 200 μg/kg of *Srbf*

*$p \leq 0.05$ vs control; **$p \leq 0.05$ vs glibenclamide-treated group; ***$p \leq 0.05$ vs untreated diabetic group

in tubular cell number. However, studies showed that tubular cells tend to increase the number relatively more than they tend to increase the volume in response to hyperglycemia. Hyperplasia, therefore, seems to be the initial response to hyperglycemia (Nyengaard et al. 1993).

Production of transforming growth factor-β (TGF-β) by mesangial components, overproduction of oxygen-free radicals following hyperglycemia, and inducible nitric oxide synthase (iNOS) expressed in response to cytokines are involved in the pathogenesis of renal hypertrophy and diabetic nephropathy (Chaiyasut et al. 2011; Sharma and Ziyadeh 1995; DeRubertis and Craven 1994). Lipophilic antioxidants such as vitamin E and probucol could reduce renal hypertrophy and inhibit the progression of diabetic nephropathy (Kim et al. 2000). In our experiment, GC/MS analysis revealed that austroinulin constitutes the main compound (32.93%) of the bitter fraction of *S. rebaudiana*. Based on previous studies, austroinulin has antioxidant and anti-inflammatory activities and inhibits from NO, iNOS, and pro-inflammatory cytokine (TNF-a, IL-6, IL-1b, and MCP-1) production through suppressing the IFN-β/STAT1 pathway by blocking the activation of IRF3 and NF-κB (Woo Byun 2012).

In diabetes, glycogenolysis and gluconeogenesis are increased in liver, and in parallel, utilization of glucose by tissues is decreased. These events lead to hyperglycemia. Stevioside, a main glycoside of *S. rebaudiana*, reinforces insulin secretion and decreases PEPCK gene expression in liver which slows down gluconeogenesis and thereby regulates hyperglycemia (Chen et al. 2005).

In this study, the serum glucose results showed that *Srbf* at low and high doses could restore the blood glucose level toward normal level. It has been revealed that a close correlation between the degree of renal injury and fasting plasma glucose concentrations could exist (Seyer-Hansen 1977). The hypoglycemic action of *Srbf* seems to attenuate the renal injury that occurred in the treated diabetic mice. Although no significant difference was seen between classic antidiabetic drug glibenclamide and experimental doses of *Srbf* at the end of the experiment (day 14–16), *Srbf*-treated mice attained nearby normal serum glucose level on days 2–5 of the experiment, whereas glibenclamide-treated mice attained nearby normal serum glucose level on days 14–16 of the experiment. The results indicate that *Srbf* not only is as effective and potent as glibenclamide but also can act faster than glibenclamide. Misra et al. (2011) investigated the antidiabetic activity of medium-polar extract from the leaves of *S. rebaudiana* on alloxan-induced diabetic rats and reported that medium-polar leaf extract of *S. rebaudiana* at 200 and 400 mg/kg produced a delayed but significant decrease in the blood glucose level in comparison with glibenclamide which is in contrast with our finding. This could be due to the different types of extract we used.

Conclusion

In conclusion, the present data indicate that hyperglycemia and renal structural changes could be improved by high dose of bitter fraction of *S. rebaudiana*. This extract possibly acts as an antioxidant due to its compounds such as austroinulin.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the ethics committee of Kermanshah University of Medical Sciences.

References

Abudula R, Jeppesen PB, Rolfsen SE, Xiao J, Hermansen K (2004) Rebaudioside A potentially stimulates insulin secretion from isolated mouse islets: studies on the dose-, glucose-, and calcium dependency. Metabolism 53:1378–1381

Braendgaard H, Gundersen HJ (1986) The impact of recent stereological advances on quantitative studies of the nervous system. J Neurosci Methods 18(1–2):39–78

Chan P, Xu DY, Liu JC, Chen YJ, Tomlinson B, Huang WP (1998) The effect of stevioside on blood pressure and plasma catecholamines in spontaneously hypertensive rats. Life Sci 63:1679–1684

Chen TH, Chen SC, Chan P, Chu YL, Yung HY, Cheng JT (2005) Mechanism of hypoglycemic effects of Stevesiodes, a glycoside of *Stevia rebaudiana*. Planta Med 71:108–113

Chaiyasut C, Kusirisin W, Lailerd N, Lerttrakarnnon P, Suttajit M, Srichairatanakool S (2011) Effects of phenolic compounds of fermented Thai indigenous plants on oxidative stress in streptozotocin-induced diabetic rats. J Evid Based Complementary Altern Med 2011:1–10

Christiansen JS, Gammelgaard J, Tronier B, Svendsen PA, Parving H-H (1982) Kidney function and size in diabetes before and during initial insulin treatment. Kidney Int 21:683–688

Curi R, Alvarez M, Bazotte RB, Botion LM, Godoy JL, Bracht A (1986) Effect of *Stevia rebaudiana* on glucose tolerance in normal adult humans. Brazilian J Med Biol Res 19:771–774

DeRubertis FR, Craven PA (1994) Activation of protein kinase C in glomerular cells in diabetes: mechanisms and potential links to the pathogenesis of diabetic glomerulopathy. Diabetes 43:1–8

El-Demerdash FM, Yousef MI, Abou El-Naga NI (2005) Biochemical study on the hypoglycemic effects of onion and garlic in alloxaninduced diabetic rats. Food Chem Toxicol 43:57–63

Ferreira EB, de Assis Rocha Neves F, da Costa MA, do Prado WA, de Araújo Funari Ferri L, Bazotte RB (2006) Comparative effects of *Stevia rebaudiana* leaves and stevioside on glycaemia and hepatic gluconeogenesis. Planta Med 72:691–696

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K (1988) Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 96(5):379–394

Hostetter TH, Troy JL, Brenner BM (1981) Glomerular hemodynamic in experimental diabetes. Kidney Int 19:410–415

Hostetter TH (2003) Hyperfiltration and glomerulosclerosis. Semin Nephrol 23:194–199

Kim SS, Gallaher DD, Csallany AS (2000) Vitamin E and probucol reduce urinary lipophilic aldehydes and renal enlargement in streptozotocin-induced diabetic rats. Lipid 35:1225–1237

Kroustrup JP, Gundersen HJ, Osterby R (1977) Glomerular size and structure in diabetes mellitus. III. Early enlargement of the capillary surface. Diabetologia 13:207–210

Lee CN, Wong KL, Liu JC, Chen YJ, Cheng JT, Chan P (2001) Inhibitory effect of stevioside on calcium influx to produce anti-hypertension. Planta Med 67:796–799

Mandarim-de-Lacerda CA (2003) Stereological tools in biomedical research. An Acad Bras Cien 75(4):469–486

McCrary RF, Pitts TO, Puschett JB (1981) Diabetic nephropathy. Natural course, survivorship, and therapy. Am J Nephrol 1:206–218

Misra H, Soni M, Silawat N, Mehta D, Mehta BK, Jain DC (2011) Antidiabetic activity of medium-polar extract from the leaves of *Stevia rebaudiana* Bert. (Bertoni) on alloxan-induced diabetic rats. J Pharm Bioall Sci 3:242–248

Mogensen CE, Andersen MJ (1973) Increased kidney size and glomerular filtration rate in early juvenile diabetes. Diabetes 22:706–712

Mogensen CE, Anderson MJF (1975) Increased kidney size and glomerular filtration rate in untreated juvenile diabetes: normalization by insulin-treatment. Diabetologia 11:221–224

Nyengaard JR (1990) Stereologic methods and their application in kidney research. J Am Soc Nephrol 10(5):1100–1123

Nyengaard JR, Flyvbjeg A, Rasch R (1993) The impact of renal growth, regression and regrowth in experimental diabetes mellitus on number and size of proximal and distal tubular cells in the rat kidney. Diabetologia 36:1126–1131

Osterby R, Gundersen HJG (1980) Fast accumulation of basement membrane material and the rate of morphological changes in acute experimental diabetic glomerular hypertrophy. Diabetologia 18:493–500

Qattan KA, Thomson M, Muslim A (2008) Garlic (*Allium sativum*) and ginger (*Zingiber officinale*) attenuate structural nephropathy progression in streptozotocin-induced diabetic rats. Eur J Clin Nutr Metab 3:62–71

Satishkumar J, Sarvanan MM, Seethalakshmi I (2008) In-vitro antimicrobial and antitumor activities of *Stevia rebaudiana* (Asteraceae) leaf extracts. Trop J Pharm Res 7:1143–1149

Seyer-Hansen K (1977) Renal hypertrophy in experimental diabetes: relation to severity of diabetes. Diabetologia 13:141–143

Sharma K, Ziyadeh FN (1995) Hyperglycemia and diabetic kidney disease: the case for transforming growth factor-β as a key mediator. Diabetes 44:1139–1146

Silva PA, Oliveira DF, Prado NR, Carvalho DA, Carvalho GA (2008) Evaluation of the antifungal activity by plant extracts against Colletotrichum gloeosporioides PENZ. Ciência e Agrotecnologia 32:420–428

Silva MA, Merzel J (2001) Stereological determination of the volume of the rat hemimandible tissues. Anat Rec 263(3):255–259

Stackhouse S, Miller PL, Park SK, Meyer TW (1990) Reversal of glomerular hyperfiltration and renal hypertrophy by blood glucose normalization in diabetic rats. Diabetes 39:989–995

Sterio DC (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127–136

Takahashi K, Matsuda M, Ohashi K, Taniquchi K, Nakaqomi O, Abe Y (2011) Analysis of anti-rotavirus activity of extract from *Stevia rebaudiana*. Antivir Res 49:15–24

Tuttle KR, Bruton JL, Perusek MC, Lancaster JL, Kopp DT, DeFronzo RA (1991) Effect of strict glycemic control on renal hemodynamic response to amino acids and renal enlargement in insulin-dependent diabetes mellitus. N Engl J Med 324:1626–1632

Welt K, Weis J, Martin R, Hermsdorf T, Drews S, Fitzl G (2007) *Ginkgo biloba* extract protects rat kidney from diabetic and hypoxic damage. Phytomedicine 14(2–3):196–203

Wiseman MJ, Saunders AJ, Keen H, Viberti GC (1985) Effect of blood glucose control on increased glomerular filtration rate and kidney size in insulindependent diabetes. N Engl J Med 312:617–621

Woo Byun M (2012) Anti-inflammatory activity of austroinulin from *Stevia rebaudiana* in LPS-induced RAW264.7 cells. J Korean Soc Food 41(4):456–461