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Nephroprotective role of alcoholic extract of *Pterocarpus marsupium* heartwood against experimentally induced diabetic nephropathy

[Papel nefroprotector del extracto alcohólico del duramen de *Pterocarpus marsupium* contra la nefropatía diabética inducida experimentalmente]

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Abstract

Context: Heartwood of *Pterocarpus marsupium* has been widely reported for its effect on diabetes clinical or preclinically. However, role in diabetic complications is yet to be revealed.

Aims: To investigate the effect of alcoholic extract of the heartwood of *Pterocarpus marsupium*in experimentally induced diabetic nephropathy in rats.

Methods: The streptozotocin (STZ) 55 mg/kg, i.p., once daily induced diabetes in Sprague-Dawley rats. These animals were treated orally with alcoholic extract of *P. marsupium* (100, 200 and 400 mg/kg) or glimepiride (10 mg/kg) for 60 days. Body weight, blood glucose, glycosylated hemoglobin (HBA_{1c}), biochemical markers of renal function were estimated on day 30 and at the end of study (day 60). Kidney weight measurement, oxidative stress markers such as lipid peroxidation (TBARS), catalase, superoxide dismutase, reduced glutathione were estimated in kidney tissues, and histopathological evaluation was carried out at the end of study period.

Results: The administration of an alcoholic extract of *P. marsupium* showed a decrease in blood glucose, HBA_{1c}, kidney weight, serum creatinine, blood urea nitrogen, serum uric acid, urea, urine volume, urine albumin and the level of TBARS. While the increase in urine creatinine, the activity of superoxide dismutase and glutathione were observed when compared to the diabetic control group. This effect was observed significantly at the highest dose of the plant extract. The histopathological study also confirmed that alcoholic extract prevented structural kidney damage.

Conclusions: These results suggest that the alcoholic extract of *P. marsupium* has renoprotective effects against STZ induced diabetic nephropathy.

Keywords: Antioxidant; biochemical markers; diabetic nephropathy; oxidative stress markers; *Pterocarpus marsupium*; renoprotective.

Resumen

Contexto: El duramen de *Pterocarpus marsupium* ha sido reportado ampliamente por su efecto sobre la diabetes, tanto clínica o preclínicamente. Sin embargo, el papel de las complicaciones diabéticas aún no se ha revelado.

Objetivos: Investigar el efecto del extracto alcohólico del duramen de *Pterocarpus marsupium*in sobre la nefropatía diabética inducida experimentalmente en ratas.

Métodos: La estreptozotocina (STZ) 55 mg/kg, i.p., indujo diabetes en ratas Sprague-Dawley. Los animales se trataron oralmente con extracto alcohólico de *P. marsupium* (100, 200 y 400 mg/kg) o glimepirida (10 mg/kg) durante 60 días. El peso corporal, la glucosa en sangre, la hemoglobina glicosilada (HbA1c), los marcadores bioquímicos de la función renal se determinaron en el día 30 y al final del estudio (día 60). El peso del riñón, los marcadores de estrés oxidativo, tales como peroxidación lipídica (TBARS), catalasa, superóxido dismutasa y glutatión reducido se estimaron en los tejidos renales. La evaluación histopatológica se llevó a cabo al final del período de estudio.

Resultados: El extracto alcohólico de *P. marsupium* mostró una disminución en la glucosa en sangre, la HbA1c, el peso del riñón, creatinina sérica, nitrógeno ureico en sangre, ácido úrico en suero, urea, el volumen de orina, la albúmina de la orina y la concentración de TBARS. Se observó el aumento de la creatinina en orina, la actividad de la superóxido dismutasa y el glutatión en comparación con el grupo de control diabético. Este efecto se observó significativamente en la dosis más alta del extracto vegetal. El estudio histopatológico confirmó también que el extracto alcohólico impidió el daño renal estructural.

Conclusiones: El extracto alcohólico de *P. marsupium* tiene efectos nefroprotectores contra la nefropatía diabética inducida por STZ.

Palabras Clave: Antioxidante; marcadores bioquímicos; marcadores de estrés oxidativo; nefropatía diabética; *Pterocarpus marsupium*; renoprotectivo.

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INTRODUCTION

Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes and one of the leading causes of end-stage renal failure and death in diabetic patients (Evans et al., 2002; Yorek, 2003). Approximately, 30% of all diabetic patients converts into diabetic nephropathy after 10-20 years of diabetes (Kikkawa et al., 2003). One of the primary target of hyperglycemia is tubular cells of kidney and chronic exposure of high blood glucose level contributes early renal pathological alterations in the form of tubulointerstitial changes, increase in tubular basement membrane thickening that further characterized by glomerular and tubular hypertrophy, accumulation of protein matrix and development of renal hypertrophy (Nath, 1998; Gilbert and Cooper, 1999; Young et al., 2010; Habib, 2013).

Studies have reported that the defining metabolic derangements (i.e. hyperglycemia, hyperlipidemia, and hyperinsulinemia), systemic and glomerular hypertension, oxidative stress, and advanced glycation end products (AGEs) increase the risk for diabetic renal disease (Ha and Kim, 1995). It has been proved by many researchers that overproduction of free radicals in persistent hyperglycemic conditions is the main triggering factor for activation of all pathways involved in the pathogenesis of complications of diabetes (Evans et al., 2002; Yorek, 2003; Johansen et al., 2005). Hyperglycemia not only stimulates the production of reactive oxygen species (ROS) but also attenuates antioxidative mechanisms through glycosylation of antioxidative enzymes as described also in previous studies (Paolisso et al., 1993; Ruiz et al., 1999). Many *in vivo* and *in vitro* studies reported the role of oxidative stress as one of the major pathophysiological mechanisms involved in the development of DN and support the theory that antioxidants may play a major role in the improvement of diabetes and its related complications (Paolisso et al., 1993; Ha and Kim, 1995; Ruiz et al., 1999; Johansen et al., 2005). Accumulating biochemical and clinical evidences support the effect of phenolic antioxidant against oxidative stress-mediated disorders. Decreased diabetic nephropathy has been observed in experimental animals after the administration of certain naturally occurring polyphenols (Anjaneyulu and Chopra, 2004; Hazem et al., 2011). Evidence has been established that proper glycemic control and treatment with antioxidant provides significant beneficial role in slowing the progression of nephropathy in diabetes, however, reversal of nephropathy to normal condition is not easy once the duration of diabetes is prolonged (Kowluru et al., 2004; Renu et al., 2004).

One of the such plant *Pterocarpus marsupium* Roxb., from the family Leguminosae, is a rich source of phenolics and flavonoids compounds viz. pterostilbene, marsupiun, pterosupin, and liquiritegenin, which are reported to have antioxidant, antihyperglycemic and antihyperlipidemic activities (Jahromi and Ray, 1993; Manickam et al., 1997; Patil and Dattatraya, 2011). Its bark and heartwood are known to be useful medicinally in the management of diabetes since long (Patil and Dattatraya, 2011). In one of the study, the aqueous extract of bark was found to reduce the blood glucose level in alloxan-induced diabetic rats (Vats et al., 2002). So many compounds isolated from the different parts of the *P. marsupium* extracts may serve as a potential source of natural antioxidant as well as for the treatment of diabetes (Jahromi and Ray, 1993; Manickam et al., 1997; Vats et al., 2002; Karanjit et al., 2008; Dilip and Veeresham, 2015). Therefore, in the present study, it was planned to evaluate the effect of an alcoholic extract of heartwood of *P. marsupiumon* the renal function and oxidative stress in streptozotocin (STZ)-induced rat model of DN.

MATERIAL AND METHODS

Plant material

Heartwood of *P. marsupium* was procured from Shashi Phytochemical Industries, Alwar, India. This material was authenticated by the botanist of the Central Research Institute of Homeopathy, Noida. The *P. marsupium* heartwood was shade dried at room temperature, and the dried wood was powdered and stored in an airtight container until further use. A hundred grams of powdered *P. marsupium* heartwood were packed in a Soxhlet apparatus and extracted with 500 mL of absolute ethanol. The ethanol extracts were concentrated in a rotary evaporator (Hei-VAP-G1 Diagonal Heidolph,

Germany). This crude extract was used for further animal studies.

Animals

Healthy male Sprague Dawley rats (weight, 150 g to 200 g) were procured from Chaudhary Charan Singh University. They were housed in polypropylene cages and maintained under standard conditions (12 h light and dark cycles, at 25 ± 2°C and 30 to 70% relative humidity). They were fed with standard pellet diet, and tap water was provided *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee, and the experiments were conducted in accordance with guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Registration no. 574/02/ab/CPCSEA) in India.

Drugs and chemicals

All the chemicals including enzymes were procured from SISCO Research Laboratory, Mumbai, India. STZ were purchased from Himedia (Mumbai, India). Glimepiride was obtained as gift sample from Ind-Swift Laboratories Ltd., Chandigarh, India. Biochemical kits for estimation of serum and urine parameters were procured from AGAPPE Diagnostics Ltd., Delhi, India. Glycosylated hemoglobin (HBA_{1c}) kit procured from was procured from Diasys Diagnostic System, Mumbai, India. All other chemicals and reagents used in the study were of analytical grade.

Induction and assessment of diabetic

Diabetes was induced in the overnight-fasted rats by intraperitoneal administration of STZ at 55 mg/kg dose as a solution in 0.1 mol/L citrate buffer (pH 4.4). STZ was freshly dissolved in citrate buffer (0.1 M; pH, 4.5) and maintained on ice before use. All the animals were administered 5% glucose solution orally (1 mL/100g body weight), 24 h after the STZ challenge (Tesch and Allen, 2007). Three days after STZ injection, blood samples were collected, and serum glucose levels were estimated using the glucometer (Accu-Check, Roche Diagnostics Pvt. Ltd., New Delhi, India). Animals with fasting glucose levels greater than 250 mg/dL were served as diabetic rats and selected for the further study. Selected diabetic rats were divided into six groups, each containing six animals. Group 1: was the normal control administered with vehicle; Group 2: DM control (STZ and vehicle); and Groups 3: DM and positive control (glimepiride 10 mg/kg); Groups 4, 5 and 6: DM and alcoholic extract of *P. marsupium* (100, 200 and 400 mg/kg, respectively).

Treatment group animals received a single dose of the alcoholic extract (100, 200 and 400 mg/kg) daily in a constant volume (10 mL/kg) for 60 days while an equal volume of vehicle (0.5% carboxymethyl cellulose) was administered orally to normal and diabetic control group rats. The day of administration of the first dose was considered the zero-day of treatment. The change in body weight recorded on days 0, 30^{th} and 60^{th} while food intake and water intake were recorded on days 30^{th} and 60^{th} of the study.

Sample collection and biochemical assays

During the experiment, animals were kept for 24 h in metabolic cages individually for urine collection on days 29 and 59 of the study for the measurement of urine parameters viz. urine volume, creatinine, urea, uric acid, total protein and albumin levels by biochemical kits (AGAPPE Diagnostics Ltd., Delhi, India).

Biochemical parameters

The animals were deprived of food overnight at the end of the experimental period and then sacrificed by cervical decapitation. Fasting blood glucose (FBG) concentration was determined in blood samples on 0, 15^{th}, 30^{th}, 45^{th} and 60^{th} days of the study period using strip-operated glucometer (Accu-Check, Roche Diagnostics Pvt. Ltd., New Delhi, India). %HbA1c was determined in EDTA-blood samples by QDxA1c Analyzer (Ceragem Medisys Inc., Korea) on 30^{th} and 60^{th} day of the study. Serum was separated from the blood samples collected on days 30 and 60 and used for the determination of biochemical parameters, such as blood urea nitrogen (BUN), creatinine, urea, uric acid and total proteins. The urine and blood parameters were evaluated using a semi-auto-analyzer (ARTOS ELITA, Swemed Biomedical Pvt Ltd., Bangalore, India). Creatinine clearance was determined to estimate

glomerular filtration rate (GFR). Creatinine clearance was measured using the following equation:

Ccr (mL/min/kg) = [urinary Cr (mg/dL) x urinary volume (mL) / serum Cr (mg/dL)] x [1000/body weight (g)] x [1/1440 (min)].

Biomarkers of oxidative stress

A 10% w/v of kidney homogenate was prepared in 0.15 M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 2000 ×*g* for 20 min at 4°C to remove the cell debris and then the supernatant was centrifuged (Remi-C24bL, Remi Laboratories Instruments, Mumbai, India) at 12000 ×*g* for 1 h at 4°C. The supernatant obtained were used for the determination of lipid peroxidation (Niehius and Samuelsson, 1968), reduced glutathione content (Morgon et al., 1979), superoxide dismutase (SOD) (Kono, 1978) and catalase (CAT) (Aebi, 1974). All assays are performed in triplicate.

Estimation of lipid peroxidation

The procedure described by Niehius and Samuelsson (1968) was adopted in the present study with slight modification for the estimation of lipid peroxide in kidney colorimetrically by measuring thiobarbituric acid reactive substances (TBARS). Briefly 0.1 mL of tissue homogenate (PMS; 0.1 M phosphate buffer, pH 7.4) mixed with 2 mL of (1:1:1 ratio) TBA: TCA: HCl reagent (0.37 % TBA, 0.25 N HCl and 15% TCA). The mixture was placed on boiling water bath for 15 minutes, cooled and centrifuged (remi-C24bL, Remi laboratories instruments, Mumbai, India) at room temperature for 10 minutes at 1000×*g*. The absorbance of the clear supernatant was measured against reference blank at 535 nm using UV–VIS spectrophotometer (UV 3000+, Labindia, Mumbai, India). The TBARS content was calculated and expressed as nmol TBARS formed/min/mg protein (Nishi et al., 2013).

Estimation of reduced glutathione (GSH)

Reduced glutathione in the tissue was determined according to the method suggested by Morgon et al. (1979). Tissue homogenate (1.0 mL) was precipitated with 1.0 mL of sulfosalicylic acid (4.0%). The samples were kept at 4°C for 1 h and then subjected to centrifugation at 1200×*g* for 15 min at 4°C. The assay mixture contained 0.5 mL of filtered aliquot, 2.3 mL of sodium phosphate buffer (0.1 M, pH 7.4) and 0.2 mL of dithiobisnitrobenzoate (DTNB) in a total volume of 3 mL. The optical density of yellow color thus developed was read immediately at 412 nm in spectrophotometer (UV 3000+, Labindia, Mumbai, India). The unknown GSH concentration was obtained by extrapolation from the standard curve (10 - 100 µg/mL).

Estimation of superoxide dismutase (SOD)

The reduction of nitroblue tetrazolium (NBT), which was initiated by the superoxide dismutase, is measured at 560 nm in spectrophotometer as described by Kono (1978). Briefly, the reaction was started by the addition of 0.05 mL of 20 mM hydroxylamine hydrochloride to the reaction mixture containing 96 mM of NBT and kidney homogenate. The SOD activity was expressed as units/mg protein.

Estimation of catalase

The procedure described by the Aebi (1974) was adopted to analyze the catalase activity. Briefly, 0.05 mL of the supernatant of the tissue homogenate was mixed with 1 mL of phosphate buffer (pH 7.0) and 0.01 mL H_2O_2. CAT activity was estimated spectrophotometrically (UV 3000+, Labindia, Mumbai, India) at 240 nm and expressed in terms of units/mg protein.

Histopathological studies

The animals were sacrificed by cervical dislocation, and both kidneys were dissected out. The right kidney was then removed, weighed, and kidney/body weight ratio calculated. Data were expressed as relative organ weight of kidney to 100 g of total body weight. The right kidney was used for the biochemical estimation. The left kidney was stored in 10% formalin solution and stained with periodic acid–Schiff's (PAS) reagent to identify kidney structure and with hematoxylin to distinguish cell nuclei. Histopathological changes in the section of the kidney was observed under the microscope (Olympus-CX21i, Olympus Medical Systems India Private Limited, India) at 400X magnification (Kiran et al., 2012).

Statistical analysis

The values of continuous variables were expressed as mean ± standard error. The one-way analysis of variance, followed by the Tukey multiple comparison tests, was used to analyze the effect of different doses of alcoholic extract of *P. marsupium* when compared to control, with the help of Graph Pad Prism (version 6.0, Graph Pad Inc., San Diego, CA, USA) software. P<0.05 was considered statistically significant.

RESULTS

Effect of *P. marsupium* alcoholic extract on body weight, feed and water intake

Changes in the body weight, feed and water intake in control and experimental groups of rats were represented in the Figs. 1, 2 and 3. A gradual increase in body weight gain was observed in the control group of animals. The STZ treated diabetic nephropathic animals showed significant (p<0.05) decrease in body weight gain along with high intake of feed and water compared to control group of animals. Administration of alcoholic extract of *P. marsupium* at the administered doses (100, 200 and 400 mg/kg) showed significantly (p<0.05) gain in body weight with inhibition of polyphagia and polydipsia when compared to diabetic control group animals.

Effect of *P. marsupium* alcoholic extract on kidney weight

Table 1 represents the data of absolute and relative kidney weight of the treatment as well as control groups. In the DM control group, the kidney weights significantly (p<0.05) increased (1.741 ± 0.21 g) when compared to the normal control group. In the diabetic rats treated with the alcoholic extract of *P. marsupium* showed the reduction in absolute as well as relative kidney weight when compared to the DM control group. However, it was significant (p<0.05) at the highest dose (400 mg/kg) of *P. marsupium*. Glimepiride at 10 mg/kg also showed a significant (p<0.05) reduction in kidney weight when compared to diabetic control group.

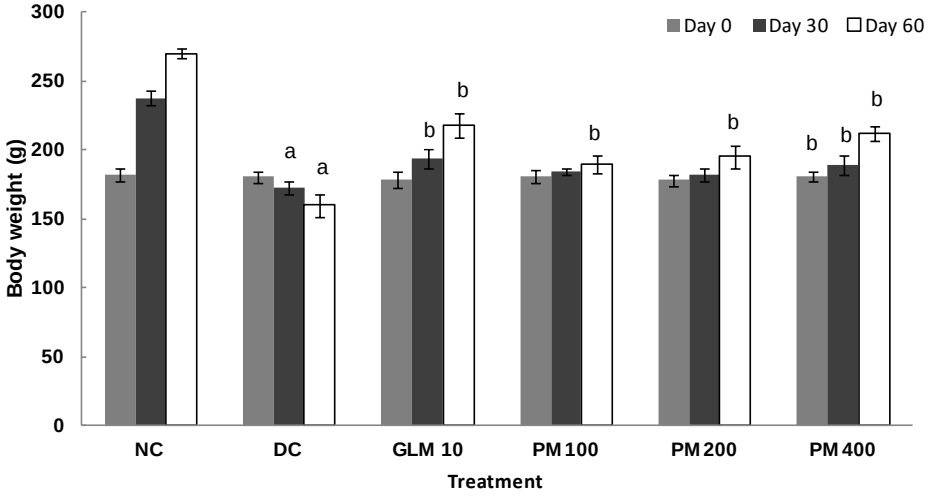

Figure 1. Effect of the treatment of alcohol extract of *P. marsupium* on the body weight.

NC: Normal control treated with vehicle (0.5% carboxymethyl cellulose); DC: diabetic control treated with streptozotocin (55 mg/kg); GLM: Glimepiride (10 mg/kg); PM: Alcoholic extract of *Pterocarpus marsupium* (100 - 400 mg/kg).

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean ± SEM.(n=6). [a]Significant difference (p<0.05) when compared to vehicle control; [b]Significant difference (p<0.05) when compared to diabetic control.

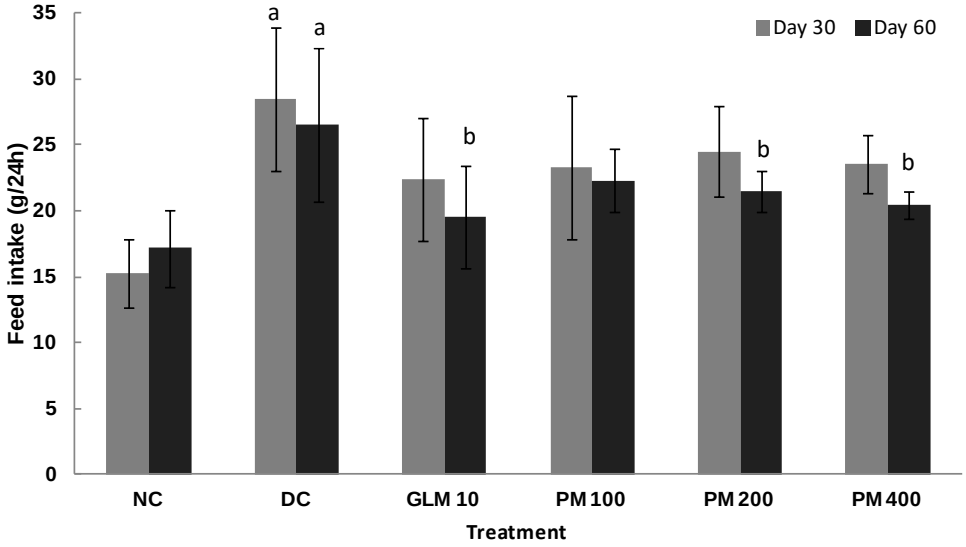

Figure 2. Effect of the treatment of alcohol extract of *P. marsupium* on the food intake.

NC: Normal control treated with vehicle (0.5% carboxymethyl cellulose); DC: diabetic control treated with streptozotocin (55 mg/kg); GLM: Glimepiride (10 mg/kg); PM: Alcoholic extract of *Pterocarpus marsupium* (100 - 400 mg/kg).

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean ± SEM. (n=6). [a]Significant difference ($p<0.05$) when compared to vehicle control; [b]Significant difference ($p<0.05$) when compared to diabetic control.

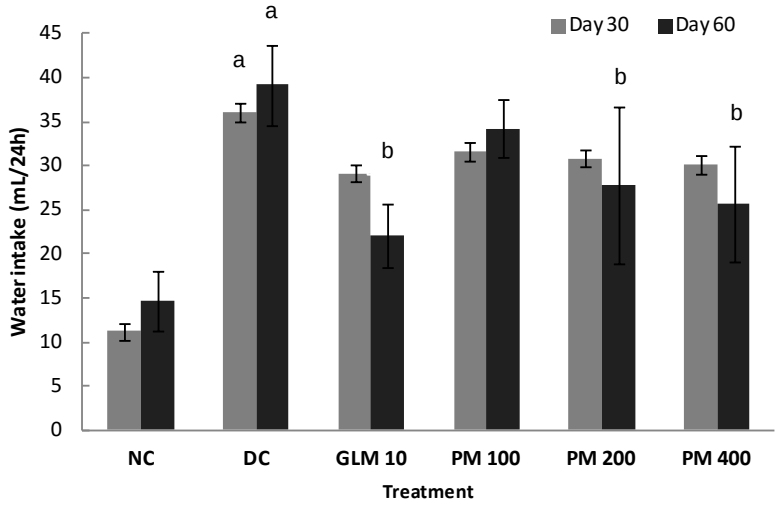

Figure 3. Effect of the treatment of alcohol extract of *P. marsupium* on the water intake.

NC: Normal control treated with vehicle (0.5% carboxymethyl cellulose); DC: diabetic control treated with streptozotocin (55 mg/kg); GLM: Glimepiride (10 mg/kg); PM: Alcoholic extract of *Pterocarpus marsupium* (100 - 400 mg/kg).

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests.

Values are expressed as mean ± SEM (n=6).

[a] Significant difference ($p<0.05$) when compared to vehicle control; [b]Significant difference ($p<0.05$) when compared to diabetic control.

Table 1: Effect of the alcoholic extract of *P. marsupium* on kidney weight.

Parameter	Normal control	Diabetic control	Glimepiride (10 mg/kg)	Alcoholic extract of *P. marsupium* (mg/kg)		
				100	200	400
Absolute kidney weights (g)	1.356 ± 0.11	1.741 ± 0.21[a]	1.426 ± 0.09[b]	1.624 ± 0.15	1.576 ± 0.11	1.499 ± 0.13[b]
Kidney weights (g/100 g b.w.)	0.502 ± 0.09	1.089 ± 0.17[a]	0.65 ± 0.08[b]	0.86 ± 0.11	0.81 ± 0.08	0.71 ± 0.10[b]

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean ± SEM (n=6).

Effect of *P. marsupium* alcoholic extract on fasting blood glucose and glycosylated hemoglobin

The effect of repeated oral administration of alcohol extract of *P. marsupium* on FBG concentrations in STZ-diabetic rats is presented in Table 2. STZ-induced diabetic rats showed approximately four to five-fold increases in the blood glucose levels throughout the study. Administration of alcoholic extract at different doses of 100, 200 and 400 mg/kg to STZ-treated diabetic rats caused reduction of blood glucose level, which was significant ($p<0.05$) at the highest dose from day 15 and at all the doses from day 30 onwards. This reduction in blood glucose was related to dose and duration of treatment. Glimepiride at 10 mg/kg exhibited significant ($p<0.05$) reduction in blood glucose level when compared to diabetic control.

The value of HbA1c was significantly ($p<0.05$) increased in STZ-diabetic rats when compared with normal rats (Table 3). Administration of the alcoholic extract at different doses (100, 200 and 400 mg/kg) for 60 days reduced the level of %HbA1c in diabetic treated rats as compared to untreated diabetic rats. Although, all the dose of the alcoholic extract ameliorated the concentration of FBG and %HbA1c, however, the dose level of 400 mg/kg b.w. was found to be significantly ($p<0.05$) more effective.

Effect of *P. marsupium* alcoholic extract on renal function parameters

Table 4 represents the level of serum creatinine, BUN, uric acid and total protein of control and experimental groups of rats. There was significantly ($p<0.05$) increased in the levels of serum creatinine, BUN, uric acid and total protein levels in STZ induced diabetic rat from fourth week onwards when compared with non-diabetic animals. Treatment with the alcoholic extract at the doses of 100, 200 and 400 mg/kg per oral resulted in significant ($p<0.05$) reduction in serum creatinine level, BUN, uric acid and total protein level compared to diabetic rats. Also, the effect of the alcoholic extract on the renal function was found to be dose-dependent.

The changes in the level of urine volume, urine creatinine, total protein and albumin in non-diabetic control, STZ-induced diabetic control and alcoholic extract treated rats were illustrated in Table 4. During the experiment period, STZ-diabetic rats showed marked polyuria by significant ($p<0.05$) increase in 24 h urine volume, accompanied by an increased in urinary albumin and total proteins excretion. On the other side, an important renal marker urinary creatinine and creatinine clearance showed significant ($p<0.05$) decreased compared to non-diabetic rat group. After 60 days treatment of alcoholic extract and glimepiride, 24 h urine volume and 24 h urine protein excretion of STZ-diabetic rats were markedly decreased along with a remarkable increase in urinary creatinine and creatinine clearance than their vehicle-treated counterparts. This effect was more prominent and significant ($p<0.05$) at the highest dose level (400 mg/kg) of alcoholic extract.

Renal antioxidant status parameters after treatment of *P. marsupium* alcoholic extract

TBARS content was elevated, and SOD, GSH and CAT activities were decreased significantly ($p<0.05$) in the kidney of diabetic rats as compared to normal rats (Table 5). Treatment with the alcoholic extract of *P. marsupium* at dose levels of 100,200 and 400 mg/kg b.w. significantly ($p<0.05$) de-

creased the increased level of TBARS and significantly ($p<0.001$) increased the decreased activities of SOD, GSH and catalase (Table 6). Although all the dose levels of alcoholic extract of *P. marsupium* restored the antioxidant status close to the normal values; however, 400 mg/kg b.w. dose level was found to be more effective and significant ($p<0.05$). These results indicate that the alcoholic extract of *P. marsupium* ameliorates oxidative stress in the diabetic rats in a dose-dependent manner.

Table 2. Change in blood glucose levels (mg/dL) in each experimental group of animals.

Day	Normal control	Diabetic control	Glimepiride (10 mg/kg)	Alcohol extract *P. marsupium* (mg/kg)		
				100	200	400
0	91.04 ± 2.30	313.24 ± 1.49[a]	321.82 ± 2.73[a]	328.61 ± 2.00[a]	320.21 ± 1.34[a]	317.42 ± 2.98[a]
15	89.52 ± 3.21	343.45 ± 4.62	274.88 ± 5.33	322.41 ± 2.64	311.55 ± 1.81	285.42 ± 4.44[b]
30	84.35 ± 2.10	435.03 ± 2.55	210.31 ± 3.71[b]	255.72 ± 3.30[b]	228.69 ± 4.11[b]	224.40 ± 6.10[b]
45	82.53 ± 1.97	450.41 ± 2.65	172.23 ± 2.74[b]	207.48 ± 1.80[b]	214.16 ± 2.72[b]	184.82 ± 5.06[b]
60	84.58 ± 2.16	415.31 ± 2.58	149.75 ± 4.02[b]	201.09 ± 2.28[b]	189.22 ± 2.59[b]	175.01 ± 6.19[b]

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean ± SEM (n=6).
[a]Significant difference ($p<0.05$) when compared to vehicle control; [b] Significant difference ($p<0.05$) when compared to diabetic control.

Table 3. Effect of the alcoholic extract of *P. marsupium* on HbA1c, serum creatinine, uric acid, BUN, albumin and total protein.

Parameters	Day	Normal Control	Diabetic control	Glimepiride (10 mg/kg)	Alcohol extract of *P. marsupium* (mg/kg)		
					100	200	400
HbA1c (%)	30	4.12 ± 0.21	13.13 ± 0.45[a]	9.19 ± 0.42[b]	11.25 ± 0.31	10.22 ± 0.53	9.98 ± 0.86[b]
	60	4.75 ± 0.45	11.63 ± 0.34[a]	7.44 ± 0.56[b]	10.95 ± 0.48	9.19 ± 0.48	8.75 ± 0.48[b]
Creatinine (mg/dL)	30	0.69 ± 0.04	3.71 ± 0.15[a]	1.61 ± 0.14[b]	2.70 ± 0.07	2.58 ± 0.10	1.85 ± 0.16[b]
	60	0.71 ± 0.03	3.47 ± 0.24[a]	1.03 ± 0.20[b]	1.68 ± 0.17[b]	1.45 ± 0.11[b]	1.11 ± 0.11[b]
BUN (mg/dL)	30	15.72 ± 0.72	51.38 ± 2.33[a]	35.01 ± 0.51[b]	45.38 ± 0.75[b]	41.59 ± 0.51	38.05 ± 0.31[b]
	60	16.35 ± 0.44	43.05 ± 0.36[a]	21.12 ± 0.61[b]	41.06 ± 0.49	39.38 ± 0.47[b]	29.95 ± 0.49[b]
Uric acid (mg/dL)	30	2.39 ± 0.31	4.43 ± 0.92[a]	3.40 ± 0.52[b]	4.10 ± 0.52	4.08 ± 0.75	3.62 ± 0.22[b]
	60	2.23 ± 0.21	4.99 ± 0.58[a]	2.88 ± 0.29[b]	3.86 ± 0.24	3.63 ± 0.16	3.01 ± 0.13[b]
Albumin (mg/dL)	30	3.15 ± 0.56	2.11 ± 0.21[a]	2.66 ± 0.51	2.19 ± 0.44	2.20 ± 0.45	2.45 ± 0.32
	60	3.47 ± 0.41	2.34 ± 0.72[a]	3.01 ± 0.23[b]	2.23 ± 0.66	2.44 ± 0.21	2.73 ± 0.33
Total protein (g/dL)	30	6.14 ± 0.23	3.12 ± 0.23[a]	4.34 ± 0.41[b]	3.89 ± 0.56	4.23 ± 0.45[b]	4.85 ± 0.69[b]
	60	6.73 ± 0.19	2.59 ± 0.23[a]	5.98 ± 1.25[b]	4.35 ± 0.34[b]	4.89 ± 0.94[b]	5.85 ± 0.66[b]

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean ± SEM (n=6).
[a]Significant difference ($p<0.05$) when compared to vehicle control; [b]Significant difference ($p<0.05$) when compared to diabetic control.

Table 4. Effect of the alcoholic extract of *P. marsupium* on urine albumin,creatinine, urine protein and creatinine clearance.

Parameter	Day	Normal control	Diabetic control	Glimepiride (10 mg/kg)	Alcohol extract *P. marsupium* (mg/kg)		
					100	200	400
24 h Urine output (mL)	30	9.12 ± 3.73	38.64 ± 5.00[a]	26.17 ± 3.04	28.17 ± 4.71	29.77 ± 4.59	24.15 ± 3.9[b]
	60	11.75 ± 2.33	37.67 ± 4.26	18.17 ± 4.44[b]	32.17 ± 5.77	22.83 ± 5.50[b]	19.15 ± 4.90[b]
Albumin (mg/dL)	30	3.43 ±0.46	15.92 ± 0.52[a]	8.21 ± 2.31[b]	13.33 ± 2.11	11.28 ± 0.41	9.41 ± 0.85
	60	3.57± 0.41	17.44 ± 1.59[a]	6.27 ± 0.31[b]	12.53 ± 2.78	9.56 ± 0.39[b]	7.41 ± 0.59[b]
Creatinine (mg/dL)	30	42.25 ± 4.12	28.84 ± 2.81[a]	36.62 ± 3.26[b]	28.01 ± 4.59	31.28 ± 2.36	35.72 ± 5.12[b]
	60	46.55 ± 5.65	18.35 ± 2.36[a]	41.66 ± 3.25[b]	31.23 ± 3.59[b]	35.69 ± 3.31[b]	39.59 ± 4.19[b]
Total protein (mg $24h^{-1}$)	30	13.58 ± 0.81	32.89 ± 1.68[a]	18.24 ± 0.79[b]	29.16 ± 2.09	28.40 ± 0.89	25.46 ± 0.94[b]
	60	14.01 ± 1.23	37.46 ± 5.89[a]	20.48 ± 3.58[b]	26.23 ± 0.56[b]	25.40 ± 0.89[b]	21.42 ± 1.89[b]
Creatinine clearance (mL/min)	30	3.17 ± 1.1 1	1.18 ± 0.82[a]	2.04 ± 0.73[b]	1.09 ± 0.64	1.10 ± 0.86	1.53 ± 0.49
	60	3.61 ± 1.41	0.81 ± 0.64[a]	2.64 ± 0.98[b]	1.92 ± 0.56[b]	1.95 ± 0.81[b]	2.45 ± 0.64[b]

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean ± SEM (n=6).
[a]Significant difference (p<0.05) when compared to vehicle control; [b]Significant difference (p<0.05) when compared to diabetic control.

Table 5. The effects of the alcoholic extract of *P. marsupium* on the antioxidant profile in diabetic nephropathic rats.

Experimental group	Dose (mg/kg)	TBARS (nmol/mg protein)	CAT (U/mg protein)	GSH (g of GSH/mg protein)	SOD (U/mg protein)
Normal control	0	0.89 ± 0.01	41.87 ± 0.69	51.60 ± 0.29	19.61 ± 0.132
Diabetic control (Streptozotocin)	55	1.87 ± 0.08[a]	26.89 ± 0.89[a]	24.10 ± 0.67[a]	10.19 ± 0.042[a]
Glimepiride	10	1.25 ± 0.15[b]	38.86 ± 1.39[b]	40.17 ± 1.06[b]	16.83 ± 0.029[b]
P. marsupium	100	1.66 ± 0.17	27.70 ± 0.99	28.95 ± 0.79	12.17 ± 0.019[b]
	200	1.53 ± 0.12[b]	29.01 ± 0.92	30.34 ± 0.89	12.89 ± 0.029[b]
	400	1.37 ± 0.11[b]	34.36 ± 0.32[b]	36.92 ± 0.38[b]	14.21 ± 0.021[b]

Data was analyzed by one-way ANOVA followed by Tukey multiple comparison tests. Values are expressed as mean±SEM (n=6).
[a]Significant difference (p<0.05) when compared to vehicle control; [b]Significant difference (p<0.05) when compared to diabetic control.

Histopathological studies

The histopathological studies, as illustrated in Fig. 4A-C, revealed that the kidney section of control group rats showed normal kidney tubules with healthy epithelial cells, prominent cortex, and medulla with the perfect presence of glomeruli. The kidney specimens of the diabetic control group rats treated with STZ 55 mg/kg i.p showed severely disrupted tubules, tubular vacuolization, glomerular hypertrophy degeneration and necrosis of epithelial cells, interstitial inflammatory cell infiltration, and intertubular hemorrhage. Pharmacological treatment with the alcoholic extract of *P. marsupium* (100, 200 and 400 mg/kg b.w. for 60 days) prevented the diabetes-induced pathological changes in the kidney by improving tubular and glomerular injury in diabetic rats. This improvement was more prominent at highest dose level compared to low and mid doses of the alcoholic extract.

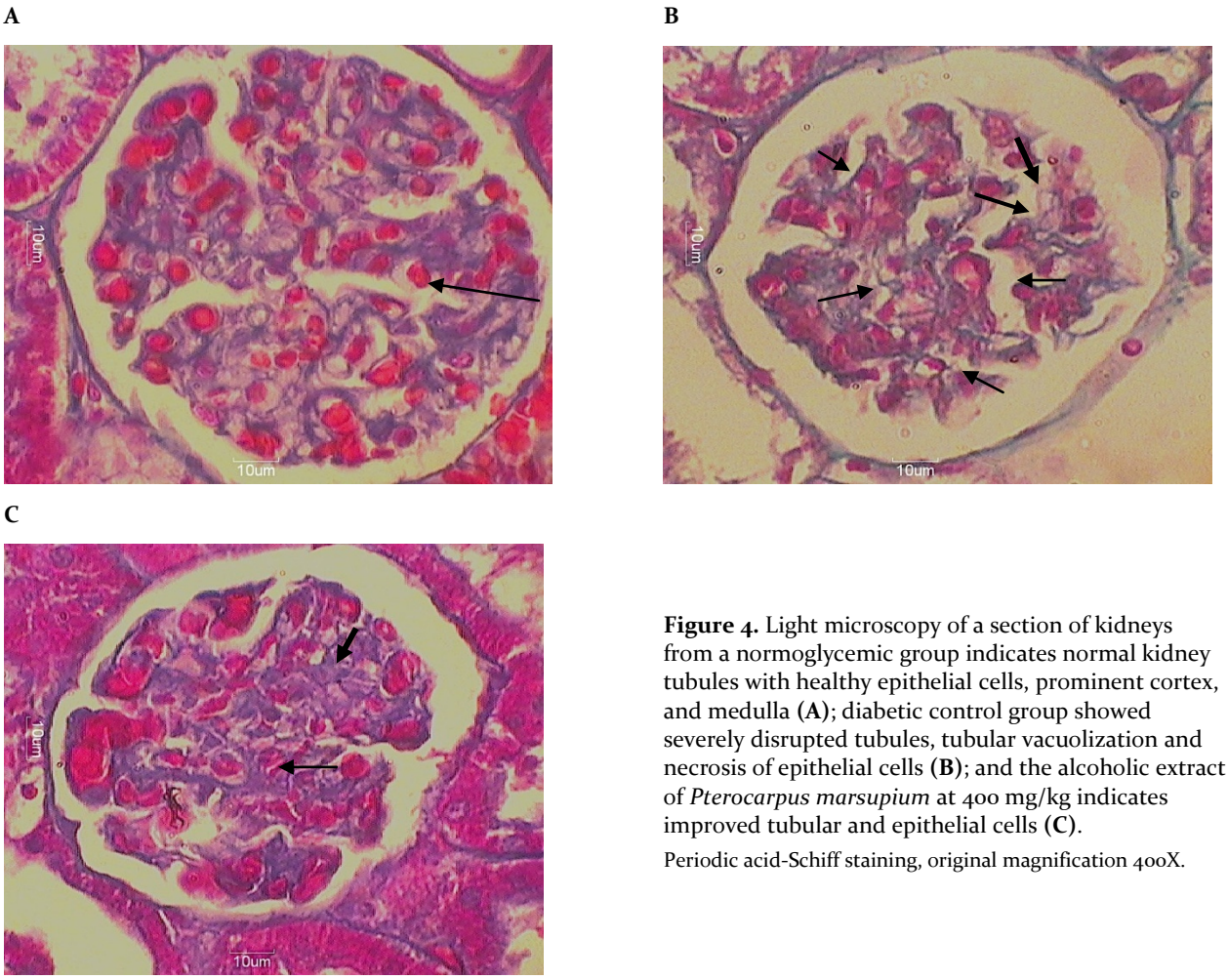

Figure 4. Light microscopy of a section of kidneys from a normoglycemic group indicates normal kidney tubules with healthy epithelial cells, prominent cortex, and medulla **(A)**; diabetic control group showed severely disrupted tubules, tubular vacuolization and necrosis of epithelial cells **(B)**; and the alcoholic extract of *Pterocarpus marsupium* at 400 mg/kg indicates improved tubular and epithelial cells **(C)**.

Periodic acid-Schiff staining, original magnification 400X.

DISCUSSION

Streptozotocin-induced diabetes mellitus causes the destruction of beta cells of islets of Langerhans (Kavalali et al., 2003) due to this reduction in insulin release takes place, which further leads to diabetes and its related complications. In the present study, STZ-injected rats demonstrated typical characteristics of diabetes mellitus such as hyperglycemia, polyuria, loss of body weight, hyperphagia, polydipsia along with the elevation of the kidney weight. The loss of body weight throughout the study could be due to dehydration and catabolism of fats and proteins (Hoftizer and Carpenter, 2000). Kidney weight in diabetic rats also significantly increased as compared to normal rats. Despite a significant reduction in food consumption, the oral administration of the alcoholic extract of *P. marsupium* showed a remarkable increase in body weight probably due to the protective effect in controlling muscle wasting, i.e. reversal of gluconeogenesis (Young et al., 2010) and decreased kidney weight. These findings suggest that alcoholic extract of *P. marsupium* may prevent kidney hypertrophy.

The fundamental mechanism underlying hyperglycemia involves overproduction of glucose by excessive hepatic glycogenolysis and gluconeogenesis and decreased utilization by the tissues (Sugimoto et al., 2001) and controlling the glucose can prevent the occurrence and development of DN (Sameer et al., 2006). A high glucose present in the blood reacts with hemoglobin to form glycosylated hemoglobin (Al-Yassin and Ibrahim, 1981). The observed high levels of glycated hemoglobin in STZ induced rats at the

end of the study reveals poor glycemic control. Since the glycosylation of protein is an oxidation reaction, flavonoids should be able to prevent this reaction as they are considered as effective antioxidants. Data of current study revealed that administration of an alcoholic extract of *P. marsupium* to STZ induced rats reduced blood glucose level and the formation of glycosylated hemoglobin by its normoglycemic activity (Premalatha and Parameswari, 2012).

The abnormalities in kidney function progress by an alteration in renal hemodynamics, which leads to proteinuria, glomerulosclerosis, and renal dysfunction. It has also been observed that impaired balance of nitrogen coupled with lowered protein synthesis leads to the formation of non-protein nitrogenous compounds such as BUN and creatinine in diabetic nephropathy conditions. This increased concentration of BUN, uric acid and creatinine in serum of diabetic rats indicates progressive renal damage, which is taken as an index of altered GFR in diabetic nephropathy (Sameer et al., 2006; Hemant and Bodhankar, 2013). Functional changes in the kidney of STZ treated animals was further proved with the elevated level of urine markers viz., urine volume, albumin and protein. These alterations in functional changes were further testified with the histopathological findings of the kidney of STZ treated rats. Creatinine clearance is calculated by creatinine levels in serum and urine, which is an indicator of alteration in the renal function. High creatinine level in blood and low level in urine, as observed in the diabetic rats, indicate an alteration in the kidney function. The oral administration of the alcoholic extract improved renal function by lowering serum creatinine, BUN, uric acid, creatinine clearance, urine volume level and increased creatinine level in urine, which was prominently significant at the highest dose (400 mg/kg).

Chronic hyperglycemia, a well-recognized pathogenetic factor of long-term complications in diabetes mellitus, is reported to generate not only more ROS but also attenuates antioxidative mechanism through glycation of the scavenging enzymes (Krishan and Chakkarwar, 2011; Elmarakby and Sullivan, 2012). Oxygen free radicals exert their cytotoxic effects on membrane phospholipids resulting in the formation of lipid peroxidation marker TBARS (Cheng et al., 2011). Furthermore, enhanced oxidative stress due to diabetes may also cause dysfunction in the defense system against free radicals, such as reduction in glutathione or inactivation of SOD and CAT. Reduced activities of SOD and catalase can have deleterious effects on kidney as a result of superoxide anion and hydrogen peroxide accumulation and damage (Yu et al., 2011; Matough et al., 2012). The present study showed that the renal concentration of TBARS was elevated and that of GSH, SOD, and CAT decreased in diabetic animals indicates increased oxidative stress in diabetic kidneys. A marked improvement in renal function by the alcoholic extract of *P. marsupium* in diabetic rats by decreased malondialdehyde content and increased SOD, GSH, and CAT concentration suggest its inhibitory effect on lipid peroxidation, ROS and protective effect against diabetic kidney cellular damage.

CONCLUSIONS

In this investigation, elevation of blood glucose, glycosylated hemoglobin, BUN, serum creatinine, serum uric acid, and urine albumin levels in the diabetic control group were significantly reversed by an alcoholic extract of *P. marsupium* heartwood in streptozotocin-induced type 2 diabetic nephropathy along with antioxidant property. Therefore, this study suggested that oxidative stress plays a prime role in the pathophysiology of diabetic nephropathy, and alcoholic extract of *P. marsupium* showed the ability to attenuate the renal damage in diabetes through its antioxidant action.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Concepts	x			x	x		x	
Design	x			x	x		x	
Definition of intellectual content	x							
Literature search	x							
Clinical studies								
Experimental studies	x	x	x	x	x	x		
Data acquisition	x	x	x	x		x		
Data analysis	x			x				x
Statistical analysis	x							
Manuscript preparation	x					x		
Manuscript editing	x					x	x	x
Manuscript review				x	x		x	x

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