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Original Article

Nephroprotective effect of *Paeonia emodi* via inhibition of advanced glycation end products and oxidative stress in streptozotocin–nicotinamide induced diabetic nephropathy

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ABSTRACT

The present study aimed to evaluate the effect of alcohol (PA) and hydroalcohol (PHA) extract of *Paeonia emodi* Royale roots in treatment of streptozotocin–nicotinamide induced diabetic nephropathy. Diabetes mellitus was induced in male Wistar rats by streptozotocin (65 mg/kg intraperitoneally) 15 minutes after nicotinamide (230 mg/kg, intraperitoneally) administration and diabetic nephropathy was assessed by measuring serum glucose, renal parameters (urea, uric acid, creatinine, and blood urea nitrogen level) and lipid profile. The rats were treated with different doses of extracts (100 mg/kg, 200 mg/kg, and 400 mg/kg) for 45 days. Oxidative stress was assessed by measuring tissue antioxidant enzymes level along with the formation of advanced glycation end-products (AGEs) in kidney. PA and PHA (400 mg/kg) produced significant attenuation in the serum glucose level (165.08 ± 3.353 mg/dL and 154.27 ± 2.209 mg/dL, respectively) as compared to control. Elevated renal parameters, lipid levels, tissue antioxidant enzymes and AGE formation were also restored in a dose-dependent manner. These findings suggest that by amelioration of oxidative stress and formation of AGEs, PA and PHA significantly inhibited the progression diabetic nephropathy in rats.

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1. Introduction

Diabetic nephropathy (DN) is one of the major complications of diabetes mellitus, and the morbidity and mortality due to this disease are constantly progressing in industrialized nations. If left untreated, 20–40% of diabetic patients with microalbuminuria will progress to overt nephropathy and 20% of them will develop end-stage renal failure within 20 years. DN is characterized by initial oxidative stress, inflammatory response, thickening of basement membranes, expansion of mesangial matrix and interstitial fibrosis, podocyte and renal cell death, increased albuminuria, and renal dysfunction [1]. Chronic hyperglycemia eventually leads to the irreversible formation of long-lived nonenzymatic glycated proteins, known as advanced glycation end-products (AGEs), in serum and tissues. These play an important role in the development of DN [2]. Moreover, hyperglycemia induces oxidative stress in

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diabetic nephrons and results in activation of multiple biochemical pathways such as glucose flux through the polyol pathway, the hexosamine pathway, excess/inappropriate activation of protein kinase C isoforms and accumulation of AGEs. While activation of each pathway may be injurious alone, collectively they cause an imbalance in the mitochondrial redox state of the cell and lead to excess formation of reactive oxygen species (ROS). These activated pathways are a major source of damage and are potential therapeutic targets in DN [3]. Herbs are a mine of medicinal agents and many medicinal herbs are found to be efficacious, cheap, and safe in preventing diabetes and diabetic complications. Moreover, use of herbal medicines for the treatment of diabetic complications is very important in developing countries where the cost of conventional medicines is a burden to the population. More than 30% of all plant species, at one time or another, were used for medicinal purposes [4].

Paeonia emodi Royle (Paeoniaceae) is an erect glabrous, leafy perennial herb indigenous to north-west Himalayas from Kashmir to Kumaon commonly known as *Ood-saleeb* [5,6]. Several phytochemical studies have revealed that the root contains monoterpene glycosides [7], monoterpene galactosides (paeonin A, B, and C) [8], triterpene (emodinol) [9], 1,5-dihydroxy-3-methyl-anthraquinone, ethyl gallate, and methyl grevillate [10]. Various component of plant root showed β-glucouronidase inhibitory activity [10], lipoxygenase inhibiting activity, hydroxyl radical scavenging activity, neuroprotection [11], and anticonvulsant activities [12,13]. Paeoninol from the methanolic extract of the fruit of *P. emodi* [14] and a novel β-glucuronidase inhibiting triterpene [1β, 3β, 5α, 23, 24-pentahydroxy-30-norolean-12, 20(29)-dien-29-oic acid] have also been isolated from the plant [10]. Root powder of the plant is used with *Selinum vaginatum* in folk medicine recipe for the treatment of diabetes [15] So the present study was undertaken to assess the protective effects of alcohol (PA) and hydroalcohol (40%: PHA) extracts of roots of *P. emodi* Royle in DN rats via inhibition of oxidative stress and AGEs. To the best of our knowledge, this is first report on nephroprotective effect of *Paeonia emodi* in streptozotocin (STZ)-induced diabetic nephropathy.

2. Methods

2.1. Animals

Adult male Wistar rats weighing 250–300 g were housed in standard environmental conditions maintained at 23 ± 2°C with 12 hour light–dark cycle. Animals were fed standard rodent diet and water. Experimental protocol was approved by Institutional Animal Ethical Committee (MMCP/IAEC/13/09) and the experiments were performed according to the Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA) guidelines.

2.2. Collection of plant material

Roots of *P. emodi* were procured from Green Earth Pvt. Ltd. (New Delhi, India) in May, 2013 and identified by Dr Sunita Garg (NISCAIR, New Delhi, India). A voucher specimen (NISCAIR/RHMD/Consult/2013/2296/76) was deposited in the herbarium of NISCAIR for future reference. The botanical name of the plant was verified from published literature and database [16,17].

2.3. Preparation of extract

The roots were dried in the shade, powdered, and then used for the extraction of potential antidiabetic constituents into different solvents (petroleum ether, chloroform, ethanol, and hydroalcohol). Roots were sequentially extracted with solvents in order of increasing polarity, i.e., petroleum ether 60–80°C, chloroform, ethanol, and hydroalcohol (40%) by Soxhlet extraction method. The extraction procedure was continued until the extract gave no coloration. The extracts were distilled and concentrated under reduced pressure and finally freeze dried. PA and PHA extracts were used for further studies.

2.4. Chemicals

STZ was obtained from Sigma–Aldrich (Milwaukee, WI, USA) and nicotinamide (NAD) from Finar India Ltd. Diagnostic kits for the biochemical estimations were obtained from Reckon Diagnostics Pvt. Ltd. (Vadodara, Gujarat, India). All the other chemicals used were of analytical grade.

2.5. Phytochemical screening

The PA and PHA were used to test the chemical compounds such as alkaloids, carbohydrates, fixed oils and fats, terpenoids, phenols, tannins, glycosides, saponins, proteins, amino-acids, and flavonoids in accordance with the methods of Trease and Evans [18], Harbourne [19] with slight modifications. Phytochemical profile of PA and PHA was also determined using high-performance thin layer chromatography (HPTLC) fingerprinting techniques on an automated HPTLC system (CAMAG, Muttenz, Switzerland) according to the instructions of the manufacturer. The developed plates were air dried and viewed in ultraviolet radiations and then scanned by Densitometer (CAMAG TLC Scanner) at 254 nm and 366 nm. The R_f values and fingerprint data were obtained using WIN CATS software (CAMAG, Muttenz, Switzerland).

2.6. Quantitative analysis

2.6.1. Determination of total flavonoids

A 10-g sample of root powder was extracted repeatedly with 100 mL of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42. The filtrate was later transferred into a crucible and evaporated into dryness and weighed to a constant weight [20].

2.6.2. Terpenoid extraction

A 50-g sample of the powdered roots was extracted with solvent combination of methanol and water (4:1) at room temperature for 24 hours. The solution was filtered using Whatman filter paper No. 1 and the filtrate was then evaporated to 1/10 volume at 40°C. The remaining filtrate was acidified with 2M sulfuric

acid (pH 0.89) followed by chloroform extraction (3 times the volume), stirred and allowed to stand in a separating funnel. Out of the two layers formed, the nonaqueous layer was taken and evaporated until dryness. The dried extract contained terpenoids [19].

2.7. Experimental design and animals

Adult male Wistar rats, procured from NIPER (Mohali, India) with initial weights of 280–300 g, were used in this study. Rats were housed under normal conditions with a 12-hour light/dark cycle at 23 ± 1°C and 40% humidity. Animals were fed standard rodent chow and water.

Diabetes was induced by STZ intraperitoneally at a dose of 65 mg/kg (dissolved in pH 4.5 citrate buffer immediately before injection) 15 min after NAD (230 mg/kg, intraperitoneally). Induction of the diabetes was confirmed by measuring fasting blood glucose levels 72 hours after STZ-NAD injection. The rats with fasting blood glucose level $\geq$ 250 mg/dL were included in the study. Different doses of the extracts (100 mg/kg, 200 mg/kg, and 400 mg/kg) were selected on the basis of oral acute toxicity studies reported in the literature [21]. As DN symptoms typically develop after 30–45 days, the levels of serum urea, uric acid, creatinine, and blood urea nitrogen (BUN) was estimated on the 30th day. Elevated levels of serum urea, uric acid, creatinine, and BUN suggest development of DN. Then treatment with extract and standard was continued for a further 45 days.

Animals were divided into nine groups consisting of six rats each. Group 1 was normal control; Group 2 was DN control; in Groups 3–5, DN rats were treated with 100 mg/kg, 200 mg/kg, or 400 mg/kg PA, respectively; in Groups 6–8 DN rats received 100 mg/kg, 200 mg/kg, or 400 mg/kg PHA, respectively; and Group 9 consisted DN rats receiving 10 mg/kg glimepride.

Blood glucose level, body weight, glycated hemoglobin (Hb1Ac), lipid profile, renal function tests were estimated on the 30th day, 45th day, 60th day, and 75th day after STZ induction. Biochemical estimation was carried out using commercially available kits of Reckon Diagnostics. Liver, kidney and pancreas were obtained and stored at −70°C until use for histopathological examination.

2.8. Body weight and blood glucose estimation

The body weight of each animal was measured before administration of STZ-NAD and animals with similar weight were grouped together. Body weight of each group was measured periodically till end of study. Fasting blood glucose level was estimated after 72 hours of STD administration to confirm diabetes and then at interval of 15 days using commercial enzymatic kits purchased from Reckon Diagnostics Pvt. Ltd. INDIA throughout the study.

2.9. Lipid profiles assay

Serum total cholesterol (TC), triglycerides (TG), low density lipoproteins (LDL), very low density lipoproteins (VLDL), and high density lipoproteins (HDL) were measured on the 30th day, 45th day, 60th day, and 75th day using commercial enzymatic kits purchased from Reckon Diagnostics.

2.10. Renal function tests

Blood samples were collected on the 30th day, 45th day, 60th day, and 75th day from DN control and treated rats for the estimation of serum creatinine, urea, uric acid and BUN using commercial enzymatic kits (Reckon Diagnostics). The animals were placed in individual metabolic cages for 12 hours to collect urine samples before the experimental rats were killed. Urinary creatinine and albumin excretion (UAE) were assessed using kits from Reckon Diagnostics. Creatinine clearance was calculated according to the standard formula:

Ccr (mL/min/100 g body weight) = [urinary creatinine (mg/mL) × urine volume (mL)/serum creatinine (mg/mL)] × [100/body weight (g)] × [1/720 (min)]. (1)

2.11. Estimation of antioxidant enzyme levels

Tissue (kidney, pancreas, and liver) homogenate was used to estimate thiobarbituric acid reactive substances (TBARS) [22] and level of antioxidant enzymes superoxide dismutase (SOD) and reduced glutathione (GSH).

2.12. AGE estimation in kidneys

AGE levels in the kidneys were determined by a method as previously described by Sensi et al [23]. Briefly, perfused kidneys were homogenized in 2 mL of 0.25M sucrose followed by centrifugation at 900*g* at 5°C and the supernatant was separated. The pellet was re-suspended in 2 mL sucrose solution and centrifuged and the supernatant obtained was mixed with the previous one. The proteins present were precipitated by adding equal volume of trichloroacetic acid. Following centrifugation at 4°C at 900*g*, the protein pellet obtained was mixed with 1 mL methanol twice to remove the lipid fraction. The insoluble protein, after washing with 10% cooled trichloroacetic acid was centrifuged and the residue was solubilized in 1 mL of 1N NaOH and the protein concentration was

Table 1 – High-performance thin layer chromatography fingerprinting profile of *Paeonia emodi*.

Sample No.	Rf values			
	Alcohol extract of *P. emodi*		Hydro-alcohol extract of *P. emodi*	
	254 nm	366 nm	254 nm	366 nm
1.	0.21	0.29	—	0.09
2.	0.40	0.51	—	0.32
3.	0.69	0.94	—	0.67
4.	0.76	1.27	—	0.87
5.	1.03	1.38	—	0.94
6.	1.18	—	—	—
7.	1.28	—	—	—
8.	1.37	—	—	—

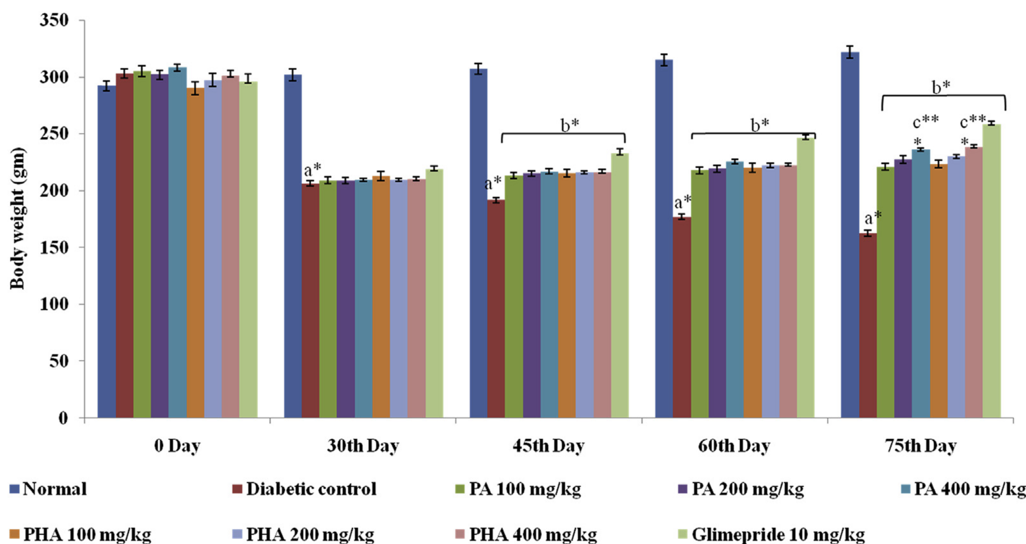

Figure 1 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on body weight. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test. Values are represented as mean ± standard error of the mean ($n = 6$). * $p < 0.001$. ** $p < 0.05$. a = versus control, b = versus diabetic control, c = versus *P. emodi* extract 100 mg/kg.

estimated by measuring the absorbance at 280 nm against BSA standard curve. The AGE content was then measured fluorometrically with an emission at 440 nm and excitation at 370 nm, and the results were expressed as relative fluorescence units (RFU)/mg protein.

2.13. *Histopathology*

Liver, kidney, and pancreatic tissues were obtained from the sacrificed animals and fixed in 10% neutral buffered formalin solution, dehydrated in ethanol and embedded in paraffin. Sections of 5 μm were prepared using a rotary microtome and stained with hematoxylin and eosin for microscopic observations.

2.14. *Statistical analysis*

Statistical analysis was performed using Graphpad Prism 6 (GraphPad Software, Inc., California Corporation). Values were expressed as mean ± standard error of the mean, and one-way analysis of variance (ANOVA) was used for statistical analysis. ANOVA was followed by Tukey's as *post hoc* multiple comparison test. The results were considered significant if $p \leq 0.05$.

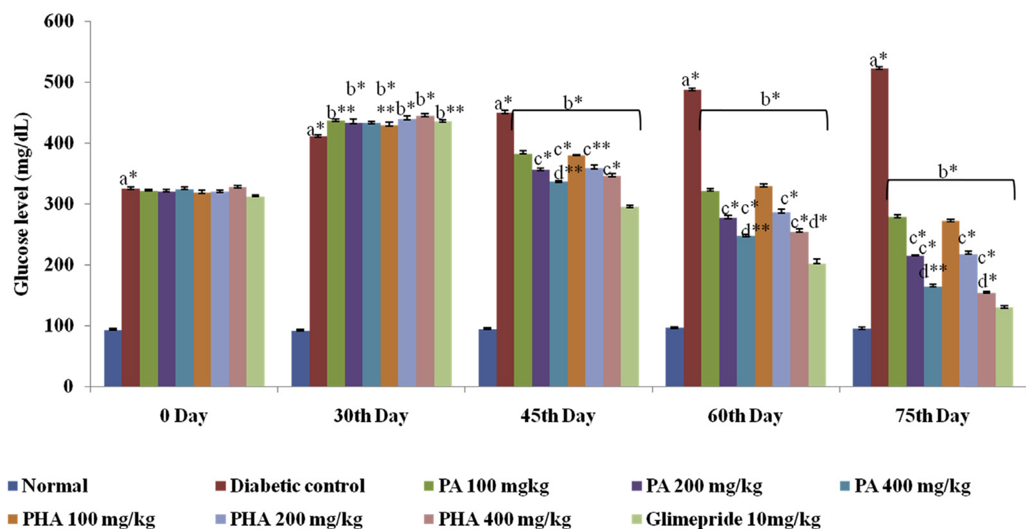

Figure 2 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on blood glucose level. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test. Values are represented as mean ± standard error of the mean ($n = 6$). * $p < 0.001$. ** $p < 0.01$. *** $p < 0.05$. a = versus control, b = versus diabetic control, c = versus *P. emodi* extract 100 mg/kg, d = versus *P. emodi* extract 200 mg/kg.

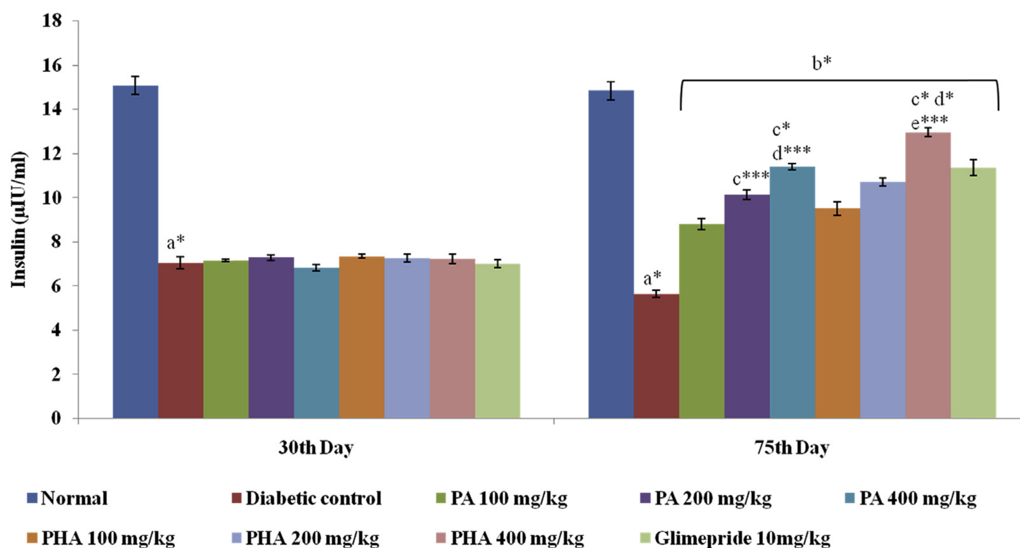

Figure 3 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on insulin level. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test. Values are represented as mean ± standard error of the mean ($n = 6$). * $p < 0.001$. ** $p < 0.01$. *** $p < 0.05$. a = versus control, b = versus diabetic control, c = versus *P. emodi* extract 100 mg/kg, d = versus *P. emodi* extract 200 mg/kg.

3. Results

3.1. *Phytochemical screening*

Preliminary phytochemical screening of PA showed the presence terpenoids and phenolic compounds whereas polyphenolic compounds, flavonoids, tannins, and terpenoids were present in PHA. HPTLC fingerprinting of the plant extracts is presented in Table 1, which confirms the presence of different types of phytochemical compounds in the PA and PHA. The presence of bands of different Rf values indicated the occurrence of several phytochemicals in the extract of *P. emodi*.

3.2. *Quantitative analysis*

The quantity of flavonoids and terpenoids was found in the following order 12.70% > 4.12%, respectively.

3.3. *Effect of PA and PHA on body weight*

DN control rats showed significant attenuation in body weight during the study. Administration of PA at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg increased the body weight significantly by 5.74%, 9.13%, and 12.91%, respectively. PHA increased the body weight by 5.18%, 10.04%, and 13.87%, respectively, at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg.

Table 2 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on renal function estimation in diabetic-nephropathy Wistar rats.[a]

Parameters	Urea (mg/dL)		Uric acid (mg/dL)		Creatinine (mg/dL)	
Groups	30th day	75th day	30th day	75th day	30th day	75th day
Normal	33.15 ± 0.789	33.28 ± 0.629	5.22 ± 0.216	5.27 ± 0.198	0.72 ± 0.009	0.81 ± 0.009
Diabetic control	$82.13 \pm 1.64^{a*}$	$97.96 \pm 0.518^{a*}$	$12.59 \pm 0.854^{a*}$	$16.04 \pm 0.612^{a*}$	$2.83 \pm 0.046^{a*}$	4.22 ± 0.062
PA 100 mg/kg	$88.60 \pm 1.616^{b***}$	$60.97 \pm 0.447^{b*d**}$	11.85 ± 0.340	$8.20 \pm 0.142^{b*}$	$3.48 \pm 4.106^{b***}$	$2.05 \pm 0.050^{b*}$
PA 200 mg/kg	89.04 ± 1.097	$55.87 \pm 1.774^{b*c**}$	12.16 ± 0.336	$7.04 \pm 0.120^{b*}$	$3.63 \pm 2.05^{b**}$	$1.79 \pm 0.064^{b*}$
PA 400 mg/kg	$81.48 \pm 0.673^{c**d**}$	$43.08 \pm 0.803^{b*c*d*}$	13.49 ± 0.273	$6.11 \pm 0.124^{b*c*}$	3.18 ± 1.795	$1.05 \pm 0.013^{b*c*d*}$
PHA 100 mg/kg	85.17 ± 1.546	$61.56 \pm 0.588^{b*d*}$	11.72 ± 0.303	8.05 ± 0.052	$4.32 \pm 1.058^{b*}$	$2.32 \pm 0.058^{b*}$
PHA 200 mg/kg	80.95 ± 1.469	$51.96 \pm 0.801^{b*c*}$	12.99 ± 0.330	$7.10 \pm 0.101^{b*}$	$3.88 \pm 2.321^{b*}$	$1.97 \pm 0.066^{b*}$
PHA 400 mg/kg	84.96 ± 1.636	$40.92 \pm 0.759^{b*c*d*}$	12.81 ± 0.370	$6.34 \pm 0.139^{b*c**}$	$3.20 \pm 1.978^{c*}$	$1.10 \pm 0.054^{b*c*d*}$
Glimipride 10 mg/kg	83.87 ± 1.098	$34.35 \pm 0.933^{b*}$	12.29 ± 0.634	$6.01 \pm 0.167^{b*}$	3.00 ± 0.223	$0.96 \pm 0.052^{b*}$

* $p < 0.001$.
** $p < 0.01$.
*** $p < 0.05$.
a = versus control, b = versus diabetic control, c = versus *P. emodi* extract 100 mg/kg, d = versus *P. emodi* extract 200 mg/kg.
a Each group ($n = 6$) represents mean ± standard error of the mean. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test.

Glimepride also increased the body weight by 18.36% in DN rats (Figure 1). The effect on body weight was found to be dose dependent in treatment groups. Food and water intake in diabetic rats significantly increased compared with the normal rats. Chronic treatment with PA and PHA significantly ameliorated these physiological parameters.

3.3. *Effect of PA and PHA on blood glucose*

STZ-NAD administration resulted in significant increase in fasting blood glucose of diabetic rats with time, when compared to nondiabetic rats. Treatment of diabetic rats with the extract reduced the blood glucose level in a dose-dependent manner. A significant reduction in fasting blood glucose level was observed in DN rats treated with PA and PHA (400 mg/kg) from initial level of 324.49 mg/dL to 165.08 mg/dL and of 327.75 mg/dL to 154.27 mg/dL, respectively (Figure 2). The results of PHA at a dose of 400 mg/kg were comparable to that of glimepride (10 mg/kg) that reduced blood glucose level up to 130.41 mg/dL at the end of the study.

3.4. *Effect of PA and PHA on HbA1c*

Hb1Ac level was measured at the start of treatment and end of the study. Substantial elevation in Hb1Ac level was observed in untreated DN control group (8.20 ± 0.06%) in comparison to normal control (4.02 ± 0.029%) on the 30th day and the level of Hb1Ac was further increased in DN rats at the end of study (75th day) to 11.01 ± 0.03%. After treatment with PA and PHA (100 mg/kg, 200 mg/kg, and 400 mg/kg) for 45 days produced significant decrease in Hb1Ac level in comparison to DN control group, i.e., Hb1Ac level 6.56 ± 0.21%, 5.46 ± 0.07%, 4.77 ± 0.16% (PA), respectively, and 6.54 ± 0.18%, 5.38 ± 0.085%, 4.98 ± 0.07% (PHA), respectively.

3.5. *Effect of PA and PHA on fasting insulin level*

A significant decrease in fasting insulin level (5.64 ± 0.17 μU/mL) was observed in STZ- treated diabetic nephropathy rats in comparison to normal control rats (14.84 ± 0.41 μU/mL). Administration of 100 mg/kg, 200 mg/kg, and 400 mg/kg of PA and PHA for 45 days produced significant increase in serum insulin level [PA (8.80 ± 0.24 μU/mL, 10.13 ± 0.23 μU/mL, and 11.38 ± 0.14 μU/mL, respectively); PHA (9.51 ± 0.30 μU/mL, 10.71 ± 0.18 μU/mL, and 12.94 ± 0.20 μU/mL, respectively; Figure 3].

3.6. *Effect of PA and PHA on renal function*

UAE was found to be increased in DN control rats to a significant extent (1640.25 ± 0.93 μg/24 hours) in comparison to normal control (219.66 ± 0.32 μg/24 hours). Administration of PA and PHA at 100 mg/kg, 200 mg/kg, and 400 mg/kg significantly reduced UAE to 496.52 ± 5.38 μg/24 hours, 483.81 ± 5.84 μg/24 hours, and 475.05 ± 9.17 μg/24 hours (PA); 503.9 ± 10.45 μg/24 hours, 476.02 ± 10.13 μg/24 hours, and 462.60 ± 5.18 μg/24 hours (PHA) respectively at 75th day. In DN control rats a significant increase in urea (97.96 ± 0.52 mg/dL), uric acid (15.54 ± 0.66 mg/dL), creatinine (4.10 ± 0.12 mg/dL), and BUN (45.74 ± 0.24 mg/dL) levels was observed at 75th day of study in comparison to normal control. Administration of PA and PHA (100 mg/kg, 200 mg/kg, and 400 mg/kg) attenuated the increased renal parameters in a dose-dependent manner (Table 2). Increased kidney weight/body weight ratio was significantly ameliorated by the treatment with PA and PHA. Twelve-hour urine volume and urinary creatinine increased significantly increased in diabetic control rats in comparison to normal control. Treatment with PA and PHA (100 mg/kg, 200 mg/kg, and 400 mg/kg) significantly reduced the level of urine volume and urine creatinine level. In addition, treatment with PA and PHA significantly improved creatinine clearance (Table 2).

3.7. *Effect of PA and PHA on lipid profile*

Serum TC, TG, LDL, and VLDL were found to be significantly increased in DN rats. Administration of PA, PHA and glimepride significantly reduced these levels. At the 75th day of study PA, PHA (400 mg/kg), and glimepride showed similar results in ameliorating TC level by 104.85 mg/dL, 105.42 mg/dL, and 102.54 mg/dL, respectively. TG levels were reduced by 35.06%, 41.17%, and 52.98% with administration of PA at

BUN (mg/dL)		Urine output (mL)		Urine creatinine (mg/dL)		Creatinine clearance (mL/min/kg)	
30th day	75th day	30th day	75th day	30th day	75th day	30th day	75th day
15.48 ± 0.368	15.54 ± 0.294	14.77 ± 0.763	15.44 ± 0.575	0.07 ± 0.016	0.08 ± 0.012	3.28 ± 0.238	3.05 ± 0.065
38.35 ± 0.769^{a*}	45.74 ± 0.242^{a*}	85.40 ± 1.614^{a*}	86.90 ± 0.941^{a*}	0.04 ± 0.018^{a*}	0.04 ± 0.007^{a*}	4.79 ± 0.381^{a**}	3.75 ± 0.179
41.38 ± 0.751^{b***}	28.47 ± 0.207^{b*d**}	83.29 ± 1.710	54.62 ± 1.440^{b*}	0.04 ± 0.012	0.05 ± 0.009^{b*}	3.50 ± 0.228	3.63 ± 0.147
41.58 ± 0.510^{b***}	26.09 ± 0.825^{b*c**}	84.25 ± 1.058	52.75 ± 0.796^{b*}	0.04 ± 0.007	0.06 ± 0.011^{b*}	3.56 ± 0.167	4.03 ± 0.244
38.05 ± 0.313	20.12 ± 0.373^{b*c*d*}	85.19 ± 1.263	45.19 ± 1.263^{b*c*}	0.04 ± 0.017	0.06 ± 0.008^{b*c*}	3.96 ± 0.090	6.75 ± 0.530^{b*c*}
39.77 ± 0.719	28.74 ± 0.273^{b*d*}	85.79 ± 1.520	44.62 ± 1.440^{b*}	0.04 ± 0.024	0.05 ± 0.008^{b*}	3.07 ± 0.261	4.78 ± 0.151
37.80 ± 0.683	24.26 ± 0.372^{b*c*}	85.58 ± 0.862	41.08 ± 1.486^{b*e***}	0.04 ± 0.017	0.06 ± 0.011^{b*c**}	3.39 ± 0.137	5.40 ± 0.281^{b***}
39.69 ± 0.760	19.11 ± 0.353^{b*c*d*}	85.19 ± 1.211	35.19 ± 1.263^{b*c**e*}	0.05 ± 0.002	0.07 ± 0.001^{b*c*}	4.33 ± 0.336	8.49 ± 0.473^{b*c*}
39.01 ± 0.512	16.04 ± 0.477^{b*}	85.20 ± 1.633	50.53 ± 1.070^{b*}	0.04 ± 0.016	0.06 ± 0.009^{b*}	4.19 ± 0.324	8.93 ± 0.386^{b*}

100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively. PHA at same doses reduced TG levels by 31.32%, 40.12%, and 49.68%, respectively. Similarly, levels of LDL and VLDL were also reduced significantly by administration of PA, PHA, and glimepride in DN rats. HDL-cholesterol significantly decreased in DN rats and the treatment with PA, PHA, and glimepride significantly increased the level of HDL-cholesterol (Table 3).

3.8. Effect of PA and PHA on antioxidant enzymes and lipid peroxidation

GSH level was significantly decreased in kidney, liver, and pancreas of DN rats. Treatment with PA and PHA for 45 days increased level of antioxidant enzyme in comparison to DN control group. Similar level of SOD was also reduced significantly in DN rats. Intervention with PA and PHA increased these levels significantly in kidney, liver, and pancreas. Oxidative stress increases in DN due to increased lipid peroxidation and decreased level of antioxidant enzymes. Lipid peroxidation in term of TBARS was found to be high in case of DN control rats in comparison to normal control group (Table 4).

3.9. Effect of PA and PHA on AGEs in kidneys

Induction of DN in rats led to a significant increase in kidney AGE levels when compared to normal animals. Administration of PA and PHA (100 mg/kg, 200 mg/kg, and 400 mg/kg) significantly reduced ($p < 0.001$) the AGE levels in kidneys when compared to control group. However, administration of glimepride (10 mg/kg) also produced significant change ($p < 0.05$) in AGE levels when compared to the control group (Figure 4).

3.10. Histopathology

Kidney of normal control animals showed normal renal parenchyma with renal glomeruli as a glomerulus and Bowman's capsule and surrounded by proximal and distal tubules. Kidney of DN rats showed mesangial expansion and thickening of glomerular capillaries. Glomeruli infiltrated by inflammation cells along with infiltration were seen in the cortex and medulla areas. Atrophy of glomeruli was seen in STZ-induced diabetic rats. In the glimepiride-treatment group, necrotic condition was reduced in convoluted tubules with reduced infiltration of inflammation cells in cortex and medulla. *P. emodi* extracts treated group also showed protection, i.e. reduction in mesangial expansion, membrane thickness and atrophy (Figure 5).

Normal central vein with radiating sinusoid cords was present in liver of normal rats. There was no sinusoid congestion, swelling, or necrotic cells. DN rats demonstrated perivenular inflammatory collection and hyperplasia of Kupffer cell with condensed nuclei and fatty infiltration. These pathological changes were reversed by *P. emodi* extracts administered DN rats (Figure 6).

Pancreatic cells of the normal control group showed normal architecture with normal acini and islets cells with no signs of edema or inflammation. In DN rats, inflammation, disorganization of the islets and steatosis were observed. Cell infiltration was seen in the acinar cells along with necrosis and shrinkage of islet cells. Treatment with *P. emodi* extracts and glimepride, showed protective effect on islets of Langerhans and acinar cells as compared to diabetic rats and further reduction in edema, inflammation, and shrinkage of islets (Figure 7).

4. Discussion

STZ-induced diabetes in rats has been described as a useful experimental model to study DN. The selective destruction of pancreatic β-cells by STZ leads to the poor sensitivity of insulin for glucose uptake [24]. NAD is an antioxidant that exerts a protective effect on the cytotoxic action of STZ by scavenging free radicals and causes lesser damage to pancreatic β-cell mass producing type 2 diabetes [25]. Chronic exposure to hyperglycemia leads to activation of multiple biochemical pathways including polyol pathway. Activation of these pathways leads to generation of ROS, causing oxidative stress, defective insulin gene expression, and increased apoptosis of β-cells [26].

Sustained hyperglycemia is an important factor in mediating the development and progression of diabetic kidney disease [27–29]. Kidneys remove metabolic wastes such as urea, uric acid, creatinine, and ions in order to maintain optimum chemical composition of body fluids. The concentrations of the metabolites increase in blood during renal diseases or renal damage associated with uncontrolled diabetes mellitus [30]. DN is characterized by an increase in blood urea, uric acid, creatinine, and BUN level.

The relationship between oxidant generation, oxidative stress, hyperglycemia, and dyslipidemia is well known [31]. Lipids play an important role in the pathogenesis of complications associated with diabetes mellitus. Dyslipidemia could lead to DN via activation of TGF-β pathway, subsequently accelerating the production of ROS leading to glomerular damage [32]. Moreover, increased polyol pathway activity induced by hyperglycemia has been reported to contribute to abnormalities such as increased osmotic and oxidative stress factors that have been cited as promoters of diabetic microvascular diseases, including DN [33]. Although complete treatment of DN is not achievable, attenuation of hyperglycemia and oxidative stress associated with DN can ameliorate the progression of diabetic complications.

Since diabetes has been known as an oxidative stress disorder caused by an imbalance between free radical formation and the ability of the body's natural antioxidants, many studies have suggested that oxidative stress can play a role in systemic inflammation, endothelial dysfunction, impaired secretion of pancreatic β-cells and impaired glucose utilization in peripheral tissues [34], that lead to long-term secondary complications. Numerous epidemiological studies suggest that herbs/diets rich in phytochemicals and antioxidants execute a protective role in health and disease [35]. *P. emodi* Royle is a traditional herb used for the management of diabetes which supported our present study [36]. Phytochemical evaluation of PA and PHA showed the presence of terpenoids, phenolic compounds including flavonoids, and tannins. Out of these, PA was found to be rich in phenolic compounds

Table 3 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on lipid profile in diabetic-nephropathy Wistar rats.[a]

Parameters	TC (mg/dL)		TG (mg/dL)		LDL (mg/dL)		VLDL (mg/dL)		HDL (mg/dL)	
Groups	30th day	75th day	30th day	75th day	30th day	75th day	30th day	75th day	30th day	75th day
Normal	103.5 ± 2.75	103.6 ± 2.02	72.44 ± 2.06	71.38 ± 1.46	33.00 ± 2.20	32.96 ± 2.47	33.07 ± 0.94	32.59 ± 0.66	56.09 ± 0.63	56.44 ± 0.54
Diabetic control	250.4 ± 2.54^{a*}	295.8 ± 2.49^{a*}	161.72 ± 3.27^{a*}	203.26 ± 1.12^{a*}	187.26 ± 2.31^{a*}	229.13 ± 2.72^{a*}	73.84 ± 1.49^{a*}	92.81 ± 0.51^{a*}	30.86 ± 0.424^{a*}	26.07 ± 0.329^{a*}
PA 100 mg/kg	268.6 ± 3.0^{b*d**}	169.7 ± 0.501^{b*d*}	154.64 ± 1.563	100.29 ± 1.057^{b*d*}	210.98 ± 2.72^{b*d*}	113.65 ± 1.61^{b*d*}	70.61 ± 0.711	45.97 ± 0.480^{b*d*}	31.36 ± 0.787^{b*}	26.24 ± 0.411^{b*d*}
PA 200 mg/kg	251.1 ± 3.020^{c**}	134.4 ± 1.055^{b*c*}	153.19 ± 1.362^{b***}	90.09 ± 0.773^{b*c*}	193.27 ± 3.23^{c*}	76.32 ± 1.38^{b*c*}	69.95 ± 0.619	43.13 ± 0.351^{b*c*}	26.75 ± 0.765^{b**}	36.04 ± 0.775^{b*c*}
PA 400 mg/kg	241.5 ± 2.141^{c*}	104.85 ± 0.789^{b*c*d*}	151.68 ± 1.723^{b***}	71.01 ± 2.0153^{b*c*d*}	184.96 ± 2.85^{c*}	47.08 ± 2.26^{b*c*d*}	69.26 ± 0.783^{b***}	32.42 ± 0.916^{b*c*d*}	27.19 ± 0.553^{b*}	40.05 ± 0.747^{b*c*d**}
PHA 100 mg/kg	260.5 ± 3.93	162.29 ± 0.801^{b*d*}	155.61 ± 3.049	108.68 ± 1.363^{b*d*}	198.08 ± 4.85	106.34 ± 2.13^{b*d*}	71.05 ± 1.386	49.62 ± 0.620^{b*d*}	26.29 ± 0.745^{b*}	43.57 ± 0.579^{b*d**}
PHA 200 mg/kg	246.56 ± 2.026	142.39 ± 0.724^{b*c*}	162.04 ± 2.397	97.55 ± 1.177^{b*c*}	201.26 ± 1.68^{b***}	83.46 ± 1.49^{b*c*}	73.99 ± 1.090	44.54 ± 0.535^{b*c*}	25.88 ± 0.918^{b**}	34.21 ± 0.570^{b*c**}
PHA 400 mg/kg	250.36 ± 2.369^{d***}	105.43 ± 0.655^{b*c*d*}	161.59 ± 1.577	81.23 ± 0.825^{b*c*d*}	186.71 ± 2.69^{d***}	44.87 ± 1.56^{b*c*d*}	73.78 ± 0.717	37.09 ± 0.375^{b*c*d*}	26.83 ± 0.784^{b**}	39.41 ± 0.810^{b*c*d*}
Glimepride10 mg/kg	252.2 ± 2.91	102.5 ± 1.33^{b*}	158.89 ± 2.24	71.68 ± 2.02^{b*}	189.74 ± 3.19	34.39 ± 1.57^{b*}	72.55 ± 1.02	32.73 ± 0.92^{b*}	30.68 ± 0.703	53.81 ± 0.295^{b*}

* $p < 0.001$.
** $p < 0.01$.
*** $p < 0.05$.
a = versus control, b = versus diabetic control, c = versus *P. emodi* extract 100 mg/kg, d = versus *P. emodi* extract 200 mg/kg.
[a] Each group ($n = 6$) represents mean ± standard error of the mean. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test.

Table 4 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on level of antioxidant enzymes and lipid peroxidation (TBARS) in diabetic-nephropathy Wistar rats.[a]

Parameters	SOD (U/mg protein)			GSH (μM/mg protein)			TBARS (nmol/mg protein)		
Groups	Kidney	Pancreas	Liver	Kidney	Pancreas	Liver	Kidney	Pancreas	Liver
Normal	4.87 ± 0.083	4.17 ± 0.298	3.67 ± 0.096	74.93 ± 0.621	69.45 ± 0.289	66.21 ± 0.476	0.55 ± 0.01	0.46 ± 0.011	0.41 ± 0.005
Diabetic control	1.23 ± 0.049^{a*}	1.15 ± 0.058^{a*}	1.06 ± 0.045^{a*}	38.60 ± 0.481^{a*}	41.90 ± 0.347^{a*}	45.53 ± 0.482^{a*}	2.98 ± 0.017^{a*}	2.71 ± 0.068^{a*}	2.48 ± 0.066^{a*}
PA 100 mg/kg	2.048 ± 0.022^{b*d*}	1.32 ± 0.017^{d**}	1.15 ± 0.020^{d*}	43.34 ± 0.548^{b*d*}	43.21 ± 0.297^{d*}	48.55 ± 0.336^{b*d*}	2.46 ± 0.033^{b*d*}	1.84 ± 0.019^{b*d*}	1.47 ± 0.015^{b*d***}
PA 200 mg/kg	2.81 ± 0.036^{b*c*}	2.20 ± 0.023^{b*c**}	2.06 ± 0.023^{b*c*}	53.23 ± 0.761^{b*c*}	50.96 ± 0.625^{b*c*}	53.61 ± 0.494^{b*c*}	1.93 ± 0.011^{b*c*}	1.33 ± 0.008^{b*c*}	1.31 ± 0.022^{b*c***}
PA 400 mg/kg	3.76 ± 0.026^{b*c*d*}	3.08 ± 0.007^{b*c*d**}	3.08 ± 0.012^{b*c*d*}	64.71 ± 0.407^{b*c*d*}	57.80 ± 0.316^{b*c*d*}	57.33 ± 0.237^{b*c*d*}	1.27 ± 0.021^{b*c*d*}	1.10 ± 0.023^{c*d*}	1.12 ± 0.007^{b**c*d**}
PHA 100 mg/kg	2.17 ± 0.015^{b*d*}	1.49 ± 0.025^{d*}	1.28 ± 0.013^{d*}	46.69 ± 0.378^{b*d*}	45.15 ± 0.359^{b*d*}	48.11 ± 0.160^{b*d*}	2.19 ± 0.011^{b*d*}	1.74 ± 0.013^{b*d*}	1.42 ± 0.009^{b*d*}
PHA 200 mg/kg	3.01 ± 0.019^{b*c*}	2.44 ± 0.010^{b*c*}	2.24 ± 0.008^{b*c*}	56.61 ± 0.286^{b*c*}	53.59 ± 0.347^{b*c*}	51.86 ± 0.221^{b*c*}	1.83 ± 0.020^{b*c*}	1.21 ± 0.005^{b*c*}	1.22 ± 0.005^{b*c*}
PHA 400 mg/kg	3.98 ± 0.017^{b*c*d*}	3.21 ± 0.019^{b*c*d**}	3.15 ± 0.010^{b*c*d*}	65.38 ± 0.282^{b*c*d*}	56.88 ± 0.253^{b*c*d*}	59.04 ± 0.086^{b*c*d*}	1.84 ± 0.020^{c*d*}	1.01 ± 0.02^{c*d*}	0.97 ± 0.012^{c*d*}
Glimepride10 mg/kg	3.86 ± 0.018^{b*}	4.03 ± 0.148^{b*}	3.63 ± 0.110^{b*}	67.83 ± 0.535^{b*}	60.77 ± 0.375^{b*}	61.26 ± 0.350^{b*}	1.19 ± 0.03^{b*}	0.98 ± 0.020^{b*}	0.92 ± 0.031^{b*}

* $p < 0.001$.
** $p < 0.01$.
*** $p < 0.05$.
a = versus control, b = versus diabetic control, c = versus *P. emodi* extract 100 mg/kg, d = versus *P. emodi* extract 200 mg/kg; GSH = glutathione; SOD = superoxide dismutase, TBARS = thiobarbituric acid reactive substances.
[a] Each group ($n = 6$) represents mean ± standard error of the mean. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test.

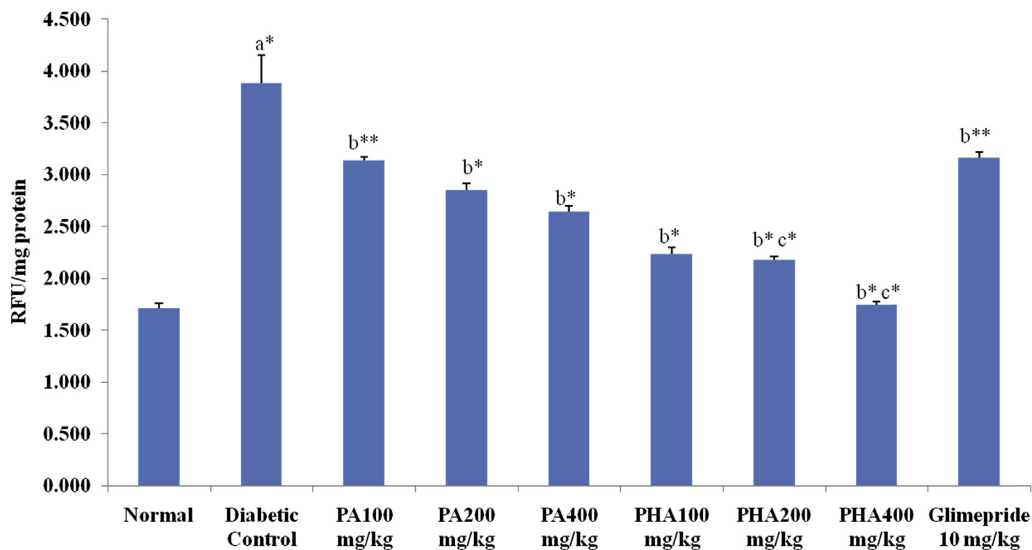

Figure 4 – Effect of alcohol (PA) and hydroalcohol (PHA) extracts of *Paeonia emodi* Royale roots on level of advanced glycation end-products. Data were analyzed by using one-way analysis of variance followed by Tukey's multiple test. Values are represented as mean ± standard error of the mean ($n = 6$). * $p < 0.001$. ** $p < 0.01$. *** $p < 0.05$. a = versus control, b = versus diabetic control, c = versus standard.

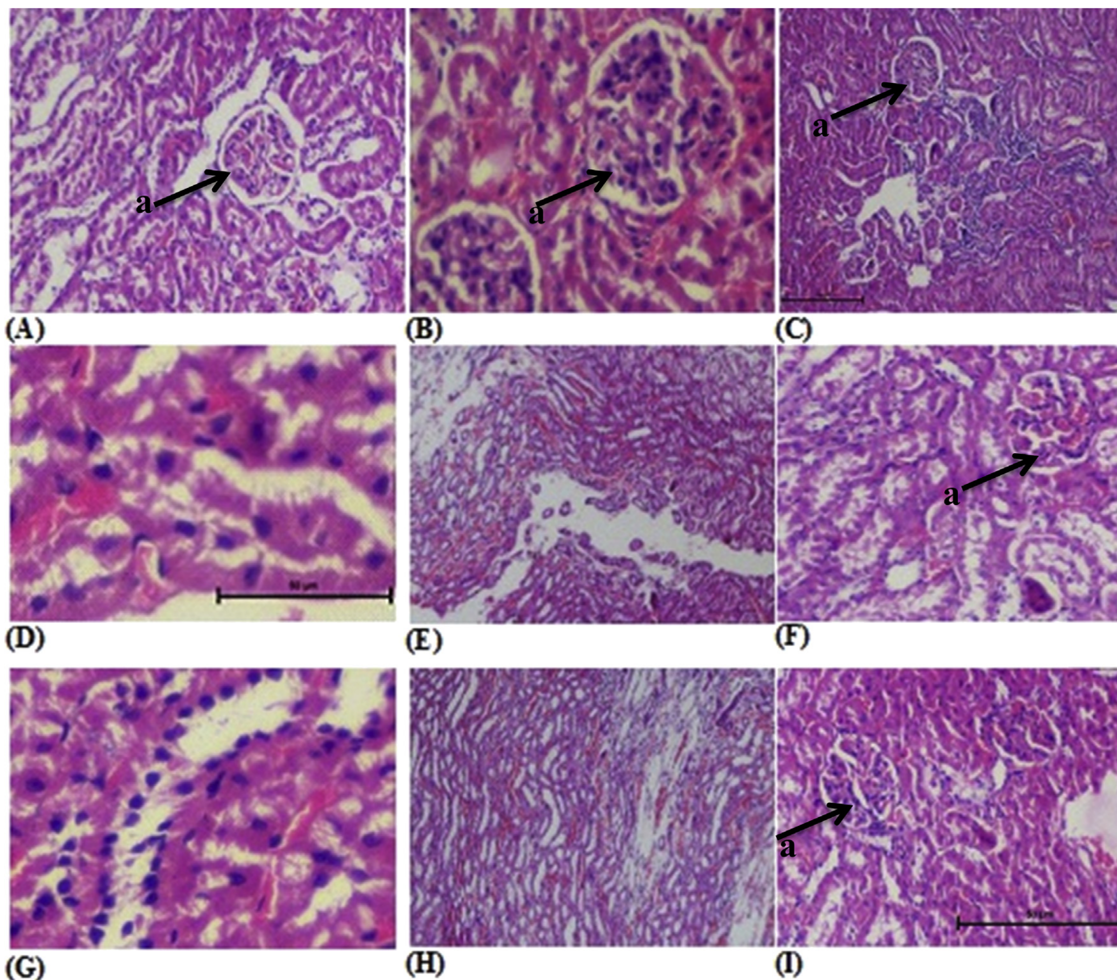

Figure 5 – Histopathological changes in kidney of normal and treated rats (hematoxylin and eosin 10×); “a” shows the glomerulus. (A) Normal; (B) diabetic nephropathy control; (C) standard; (D) treated with 100 mg/kg alcohol extract of *Paeonia emodi* Royale roots (PA); (E) PA 200 mg/kg; (F) PA 400 mg/kg; (G); treated with 100 mg/kg hydroalcohol extract of *Paeonia emodi* Royale roots (PHA); (H) PHA 200 mg/kg; and (I) PHA 400 mg/kg.

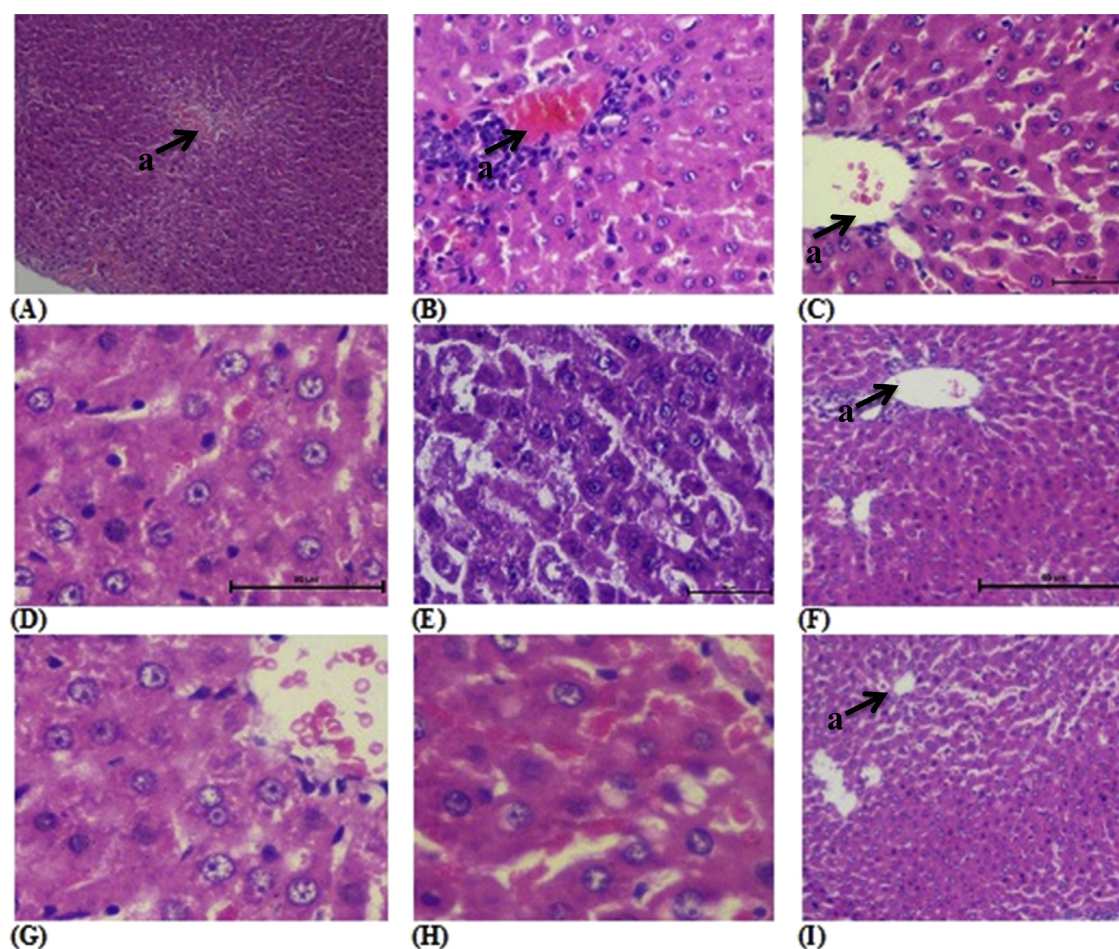

Figure 6 – Histopathological changes in pancreatic islet of normal and treated rats (hematoxylin and eosin; magnification 10×); "a" shows the liver central vein. (A) Normal; (B) diabetic nephropathy control; (C) standard; (D) treated with 100 mg/kg alcohol extract of *Paeonia emodi* Royale roots (PA); (E) PA 200 mg/kg; (F) PA 400 mg/kg; (G); treated with 100 mg/kg hydroalcohol extract of *Paeonia emodi* Royale roots (PHA); (H) PHA 200 mg/kg; and (I) PHA 400 mg/kg.

whereas PHA contained abundant terpenoids. Previous studies showed the presence of triterpenoids in *P. emodi*, which include emodinol [8],1β,3 β,5α,23,24-pentahydroxy-30-nor-olean-12,20(29)-dien-28-oic acid [10], 6α,7α-epoxy-1α,3β,4β,13β- tetrahydroxy-24,30-dinor-olean-20-ene-28,13 β-olide,1α, 3β,4β -trihydroxy-24,30-dinor-olean-6-ene-28-oic acid,11α,12α -epoxy-3β,4β,-dihydroxy-24-nor-olean-28-oic acid, 3β,4β,23,30-tetrahydroxy-24-nor-olean-12-ene-28-oic acid [37], oleanolic acid, betulinic acid [10], β-amyrin, cycloartenol, lupeol, and 24-methylenecycloartanol [38]. Triterpenoids are known to stimulate insulin release from pancreas and reduces oxidative stress thus can be effective in management of diabetes and related complications [39,40].

Phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. Natural antioxidants mainly come from plants in the form of phenolic compounds such as flavonoids and phenolic acids. *In vitro* and *in vivo* experiments have shown that phenolic acids exhibit powerful effects on biological responses by scavenging free radicals and eliciting antioxidant capacity [41]. Phenolic compounds present in *P. emodi* in previous studies include ethyl gallate, methyl grevillate [10], benzoic acid, 3-hydroxy benzoic acid [8], 4-hydroxy benzoic acid, gallic acid, and methyl gallate [14]. Flavonoids and other phenolic compounds are known to reduce oxidative stress, reduce necrosis, and regenerate β-cells [42]. Phenolic compounds are potent free radical scavengers that increase antioxidant enzyme level and reduced lipid peroxidation Thus, the presence of these secondary metabolites may prove beneficial for counteracting the complications associated with diabetes.

Administration of STZ leads to chronic hyperglycemia indicated by elevated fasting blood glucose level in experimental animals due to pancreatic β cell damage, which is also suggested by increase in the levels of HbA1c%, whereas administration of PA and PHA resulted in a significant reduction of the fasting blood glucose level as compared to diabetic rats. Moreover, induction of diabetes with STZ-NAD is associated with the characteristic loss of body weight, which is mainly due to increased muscle wasting [43] and loss of tissue proteins [44]. Oral administration of PA and PHA improved the body weight in diabetic rats by reversing the muscle wasting and protein loss. This may be achieved via the inhibition of hepatic gluconeogenesis and glucose output from the liver, which is accompanied by the suppression of lipolysis in adipose tissue [45].

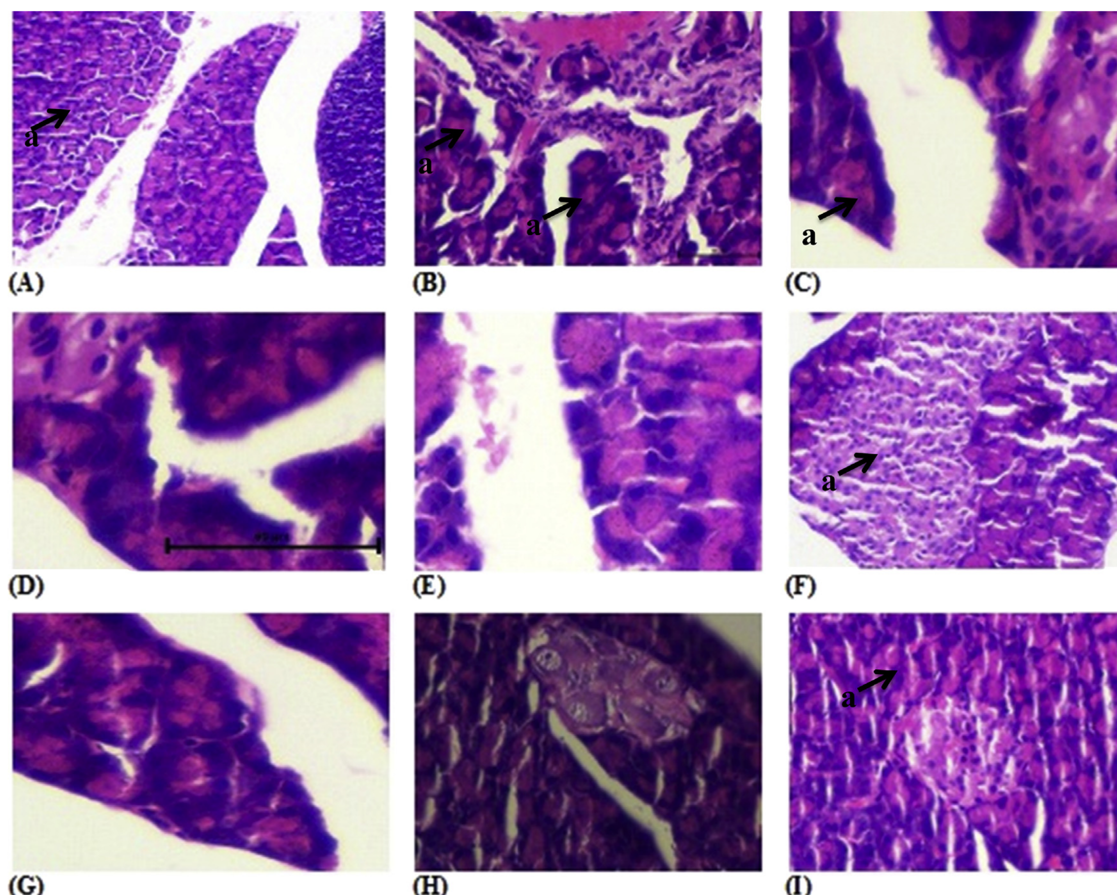

Figure 7 – Histopathological changes in Liver of normal and treated rats (hematoxylin and eosin 10×); "a" shows β-cells. (A) Normal; (B) diabetic nephropathy control; (C) standard; (D) treated with 100 mg/kg alcohol extract of *Paeonia emodi* Royale roots (PA); (E) PA 200 mg/kg; (F) PA 400 mg/kg; (G); treated with 100 mg/kg hydroalcohol extract of *Paeonia emodi* Royale roots (PHA); (H) PHA 200 mg/kg; and (I) PHA 400 mg/kg.

Diabetic rats were found to have significantly increased level of plasma glucose, total cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol, but decreased plasma HDL-cholesterol and insulin levels [46]. Similarly, in our study, significant dyslipidemia was observed in experimental animals and administration of PA and PHA significantly reduced lipid levels. Besides reducing lipid levels, PA and PHA effectively increased HDL-cholesterol level. The antihyperglycaemic effect of PA and PHA and improved diabetic state may lead to a reduction in the concentration of VLDL and consequently the LDL level [47]. A study by Zargar et al [21] on antihyperlipidemic effect of *P. emodi* supported our present study.

Moreover, high glucose-induced renal damage is associated with excessive production of ROS under hyperglycemic conditions [48]. ROS can react with polyunsaturated fatty acids leading to lipid peroxidation and irreversible tissue damage [42]. It is found that high glucose-induced renal damage is associated with excessive production of ROS under hyperglycemic conditions [48]. Typical markers of oxidative stress have been reported in both diabetic humans and animals, including increased ROS, malondialdehyde, and reduced levels of antioxidants [49]. Induction of diabetes in the present study led to significant elevation of malondialdehyde and a reduction of SOD and GSH in the kidneys, pancreas, and liver. Administration of PA and PHA ameliorated oxidative damage in DN rats via increasing level of antioxidant enzymes and reduced LPO. The effectiveness of *P. emodi* against oxidative stress is widely recognized and has been discussed in other studies [21,50,51], which supported our present study.

Renal damage is evident with increase in UAE, serum creatinine, and BUN indicating reduced creatinine and urea clearance. Similar structural changes were found in the present study. Treatment of DN rats with PA and PHA effectively reduced the levels of urea, uric acid, creatinine and BUN indicating their increased clearance from kidney. Moreover, the untreated diabetic rats showed signs of diabetic nephropathy, including the increased urine volume, reduced level of creatinine in urine and creatinine clearance. Administration of PA and PHA for 45 days significantly decreased the level of all these parameters. At the onset of diabetes, the kidney grows large and the glomerular filtration rate becomes disturbed [52].

Formation of AGEs facilitated by oxidative stress is the product of the reaction between carbohydrates and the free amino group of proteins [53]. AGEs have been implicated to play key roles in the pathogenesis of DN by stimulating cytokine and growth factor synthesis leading to extracellular

matrix accumulation in kidneys [54]. Treatment of DN rats with PA and PHA effectively reduced the levels of AGEs in the kidney.

DN is characterized by a series of renal structure abnormalities including basement membrane thickening, mesangial expansion, glomerulosclerosis, and tubulointerstitial fibrosis [55]. In the present study, histopathological observations of kidney showed glomeruli with mesangiocapillary proliferation. Messangial expansion is a crucial structural change leading to loss of renal function in diabetes. Liver showed perivenular inflammatory collection. The ultrastructure of diabetic pancreas showed considerable reduction in the islets of Langerhans and depleted islets [56]. Similar structural changes in pancreas were found in the present study. Oral administration of PA and PHA significantly improved the histological alterations induced by STZ-NAD, which could be attributed to its antioxidant properties of the plant.

5. Conclusion

It can be concluded that PA and PHA showed protective effect against DN, since they exhibited beneficial effects on the blood glucose level, associated biomarkers of DN and AGEs in kidney.

Conflicts of interest

All authors have no conflicts of interest to declare.

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