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Formononetin attenuates kidney damage in type 2 diabetic rats

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ABSTRACT

Aim: Diabetic nephropathy is the commonly developed complication of vasculature in type 2 diabetic patients. Chronic hyperglycemia leads to nephropathy in diabetics because of the formation of excessive reactive oxygen species and advanced glycation end products which is reflected in the form of glomerulosclerosis, tubular atrophy and interstitial fibrosis. As per the various reports reduction in SIRT1 expression in kidney tissue is key factor in the development of nephropathy in diabetes because its reduction in tissue is linked with excessive formation of ROS. Formononetin is a polyphenolic compound reported for its effect on SIRT1 and ROS.

Main methods: Type 2 diabetes was induced in rats by diet modification using high fat diet for fifteen days prior to streptozotocin regimen (35 mg/kg, *i.p.*). Treatment of formononetin was started after confirmation of diabetes and continued for 16 weeks. Formononetin was administered orally to the diabetic animals at the dose of 10. 20 and 40 mg/kg.

Key findings: Formononetin treatment for 16 week was able to control hyperglycemia and insulin resistance in diabetic animals. It has also been reduced triglyceride and cholesterol in blood. Formononetin treatment reduced blood concentration of creatinine, blood urea nitrogen and increased albumin concentration. Formononetin treatment also enhanced creatinine clearance in diabetic animals. Oxidative stress burden was also reduced significantly after formononetin treatment along with increased SIRT1 expression in kidney tissues of diabetic animals.

Significance: Formononetin is a potential molecule which increases the expression of SIRT1 in kidney tissue of diabetic. Thus formononetin is an effective molecule to control nephropathy in type 2 diabetes mellitus.

1. Introduction

Diabetic nephropathy (DN) is the most frequently observed vascular complication in approximately 40% of patients suffering from type 2 diabetes [1]. Long lasting kidney diseases and end stage renal failure are the two foremost infirmity observed in the last stage of untreated nephropathy [2]. Diabetic nephropathy represented by glomerulosclerosis, tubular atrophy and interstitial fibrosis, which results into constant decline in glomerular filtration rate [3]. DN is also increases risk of hypertension and cardiovascular complications in diabetics [4]. Mutigenetic factors are involved in development of diabetic nephropathy. However hyperglycemia and associated oxidative stress plays pivotal role to produce vascular damage and alteration in hemodynamic changes such as microalbuminuria, glomerular hyperfiltration and shear stress [5,6]. Hyperglycemia, dyslipidemia, systemic and glomerular hypertension are the key factors of oxidative stress in DN. Oxidative stress generated owing to hyperglycemia and hyperlipidemia affects entire glomerular filtration barrier. Initially, it affects the interaction of endothelial cells with podocytes and glycocalyx layer followed by extracellular matrix deposition and alters the cellular function [7]. Reactive oxidative species also disturb cellular function in kidney by alternating function of various transcription factors [6]. Evidence shows that excessive generation of reactive oxidative species control the activity of SIRT1 in kidney tissue (silent information regulator 2) [8]. In diabetic nephropathy increased oxidative stress causes SIRT1 down regulation in proximal tubule and leads to epigenetic changes in podocytes which is reflected in the form of albuminuria in diabetic patients [9].

Formononetin is an isoflavonoids type of phytoestrogen found in soy and other legume plants. Formononetin is known for its cardioprotective and anti-hyperlipidemic effect [10]. It has also been reported to reduced generation of reactive oxygen species and have significant antioxidant potential [11]. Formononetin has also been used as one of the ingredient of polyhedral formulation for management nephropathy [12]. Recent report shows that formononetin provides anti-inflammatory effect via inhibiting HMGB1 release. In the same study, it

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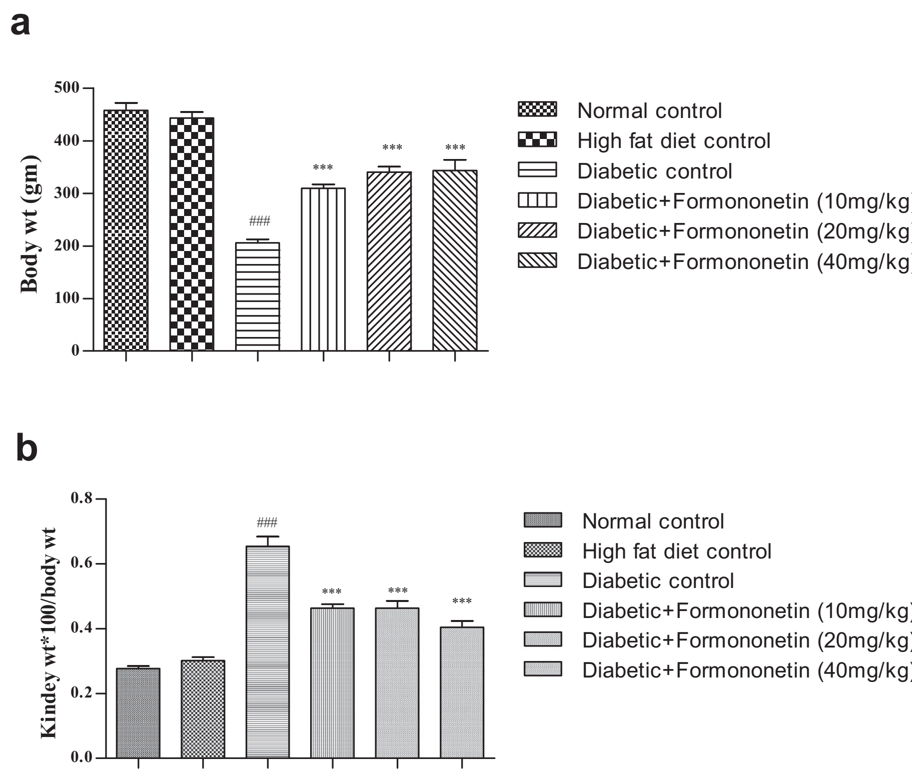

Fig. 1. Effect of formononetin on body weight and kidney hypertrophy in type 2 diabetic rats. **a**. body weight, **b**. ratio of kidney weight to body weight. All values are expresses as Mean ± SEM. $^{###}p < 0.001$, when compared with normal control. $^{***}p < 0.001$, when compared with diabetic control.

has also been reported that inhibition of HMGB1 release is mainly because of up regulation of SIRT1 which leads to reduction in acetylation of HMGB1 [13]. Furthermore, we have reported that formononetin reduces hyperglycemia, improves insulin sensitivity in type 2 diabetic rat along with increased expression of SIRT1 in pancreas [14]. However, there are no systematic study reports available for its effect in diabetic nephropathy. Thus, based on the outcome of our previous study of formononetin treatment in type 2 diabetes mellitus, we designed present study to evaluate the effect on formononetin regimen in type 2 diabetic nephropathy.

2. Materials and methods

2.1. Ethics statement

The animals experiment was carried out with the approval of Institutional Animal Ethics Committee.

2.2. Experimental animals

Sprague-Dawely rats (Male) were purchased from National Institute of Biosciences, Pune, India. The animals were housed at a temperature (22 ± 2 °C) and humidity (40–60%) controlled area with 12/12 h light-dark cycle. Acclimatization of experimental animals was done for one week before starting the experiment.

2.3. Induction of type 2 diabetes mellitus

Type 2 diabetes mellitus was induced in the rats using high fat diet + low dose of streptozotocin method reported previously [15]. Briefly, all animals were randomly assigned to normal pellet diet (Group-1 Normal control) or high fat diet. Rats feed with high fat diet were further divided as vehicle control (Group-2 high fat diet) or receiving streptozotocin (35 mg/kg) intraperitonially along with high fat diet. After one week of streptozotocin injection plasma glucose were measured. The experimental rats with blood glucose > 300 mg/dl considered diabetic. The animals with established type 2 diabetic condition were randomly separated into four groups: Group-3 diabetic control, Group-4 diabetic + Formononetin (10 mg/kg), Group-5 diabetic + Formononetin (20 mg/kg), Group-6 diabetic + Formononetin (40 mg/kg). Formononetin was given orally in the form of suspension (0.5% CMC) for 16 weeks after confirmation of type 2 diabetes mellitus.

2.4. Body weight

The change in body weight was measured at the end of sixteenth week of treatment duration.

2.5. Sample collections

Urine, blood and kidney tissue were collected from each rats after 16 weeks of treatment with formononetin. For the collection of urine samples each animal was kept in metabolic cage (BIK industries, India) for 24 h. Blood samples were collected and plasma was separated by centrifugation of sample at 8000 RPM and stored at −80 °C temperature. The animals were anaesthetized with urethane (1.2 g/kg) and kidney tissue were removed, washed with normal saline and weighed. The left kidney was fastened in 10% neutral buffered formalin solution. The right kidney was stored at −80 °C temperature for future use.

2.6. Estimation of biochemical parameters

Clinical biochemical parameters were measured in plasma samples using biochemical analyzer (Erba Chem 7, Germany) and available diagnostic kits (Transasia Biomedicals Ltd., India). Following parameters were measured: blood glucose, creatinine, total protein, albumin, blood urea nitrogen (BUN), total cholesterol (TC) and triglyceride (TG).

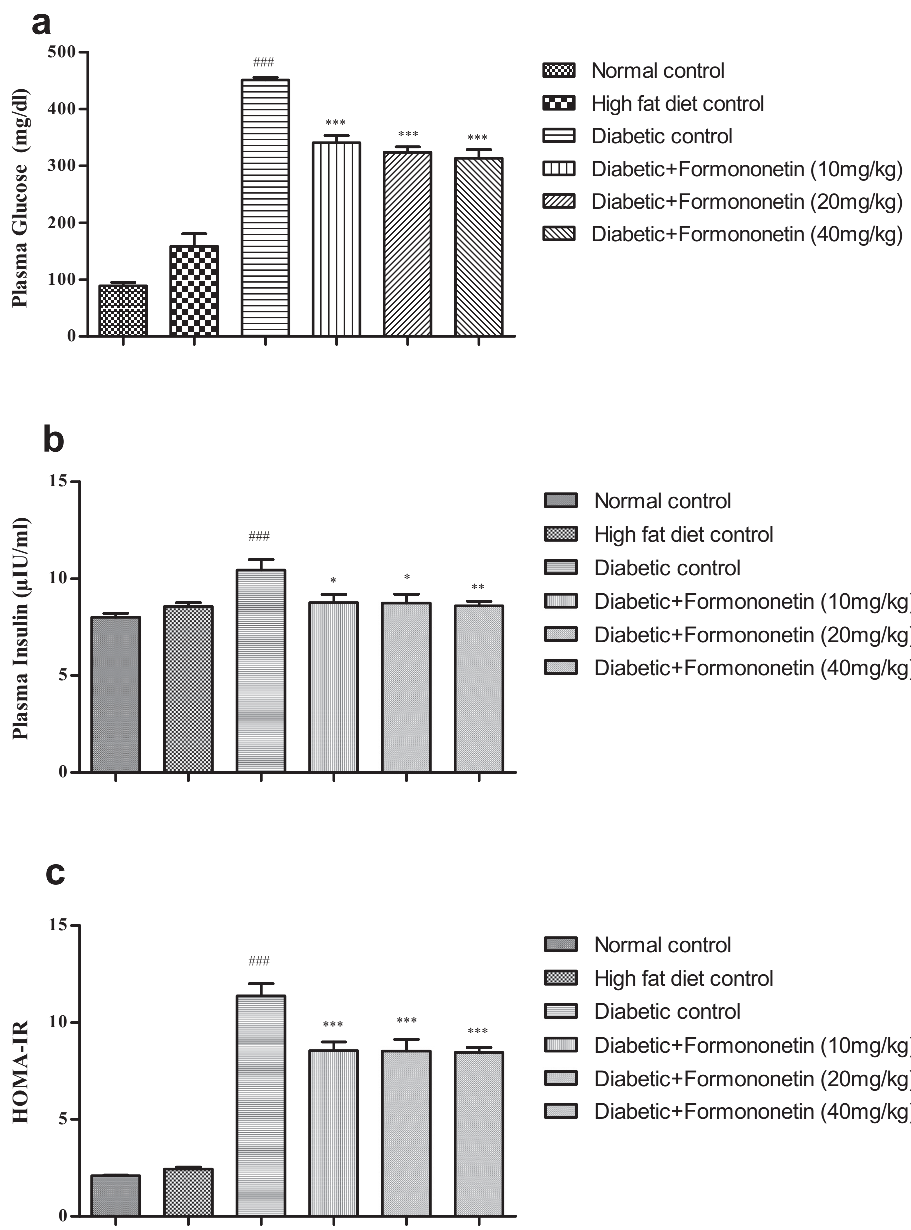

Fig. 2. Effect of formononetin on plasma glucose, insulin and HOMA-IR. **a**. plasma glucose, **b**. plasma insulin and **c**. HOMA-IR. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.

Plasma insulin was determined using rat insulin enzyme linked immunosorbent assay (ELISA) kits (RayBiotech Inc., Norcross, GA, USA).

2.7. Estimation of urine parameters

The following parameters were assessed in urine samples collected for 24 h: Urine volume, creatinine, albumin, total protein and urea. Creatinine clearance was calculated using the formula C_{cr} = urinary creatinine (mg/dl) × urine volume (ml/min)/creatinine in plasma (mg/dl) [16].

2.8. Estimation of oxidative stress markers in kidney tissue

The total homogenate of kidney tissue was prepared in 5% (w/v) phosphate buffer (pH 7.4) using polytron homogenizer. The fraction of total homogenate was centrifuged at 2500 *g* for 10 min at 4 °C to separate Post-Nuclear Supernatant (PNS). The Post-mitochondrial Supernatant (PMS) was prepared from total homogenate by centrifugation at 10,000*g* for 20 min at 4 °C. Malondialdehyde level in kidney tissue was measured by thiobarbituric acid reactive substances (TBARS) assay described by Ohkawa et al. [17]. In the TBARS assay, total homogenate was used to determine thiobarbituric acid reactive substances. Reduced glutathione level in kidney tissue was estimated as per the method given by Ellman [18]. PNS was used to estimate catalase activity in kidney tissue as per the method described by Luck [19]. Sodium oxide dismutase activity was measured in post mitochondrial supernant as per the method described by Paoletti and Mocali [20].

2.9. Kidney hypertrophy

Kidney hypertrophy was determined by calculating the ratio of kidney weight to body weight.

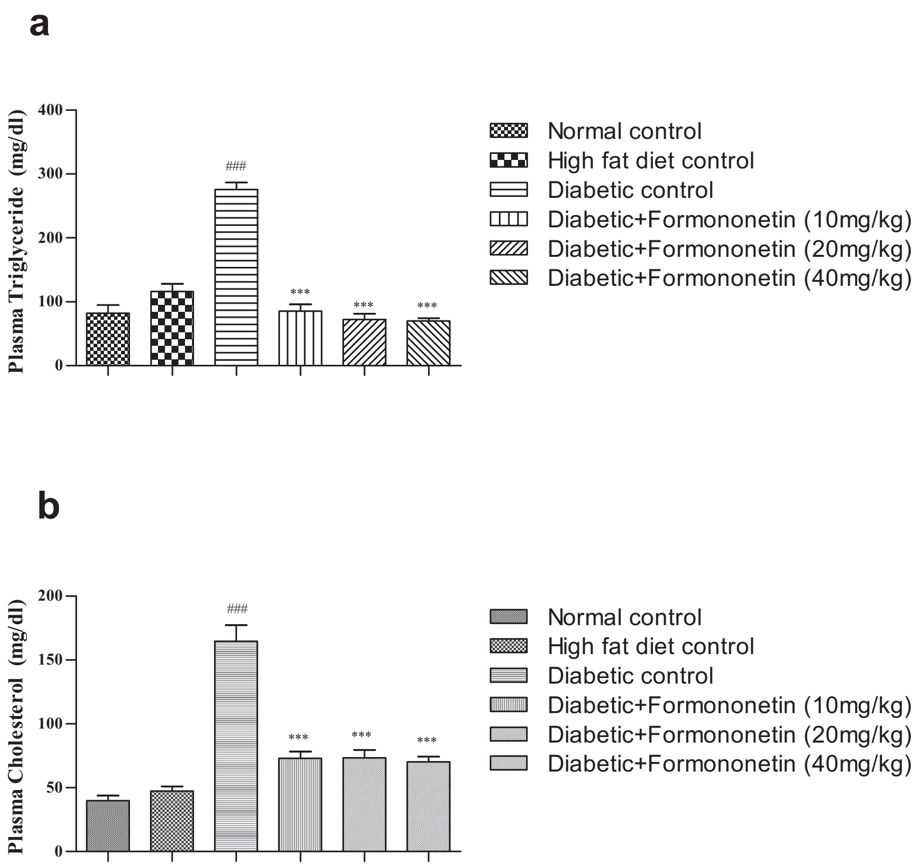

Fig. 3. Effect of formononetin on plasma triglyceride and total cholesterol. **a**. plasma triglyceride, **b**. plasma cholesterol. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. ***p < 0.001, when compared with diabetic control.

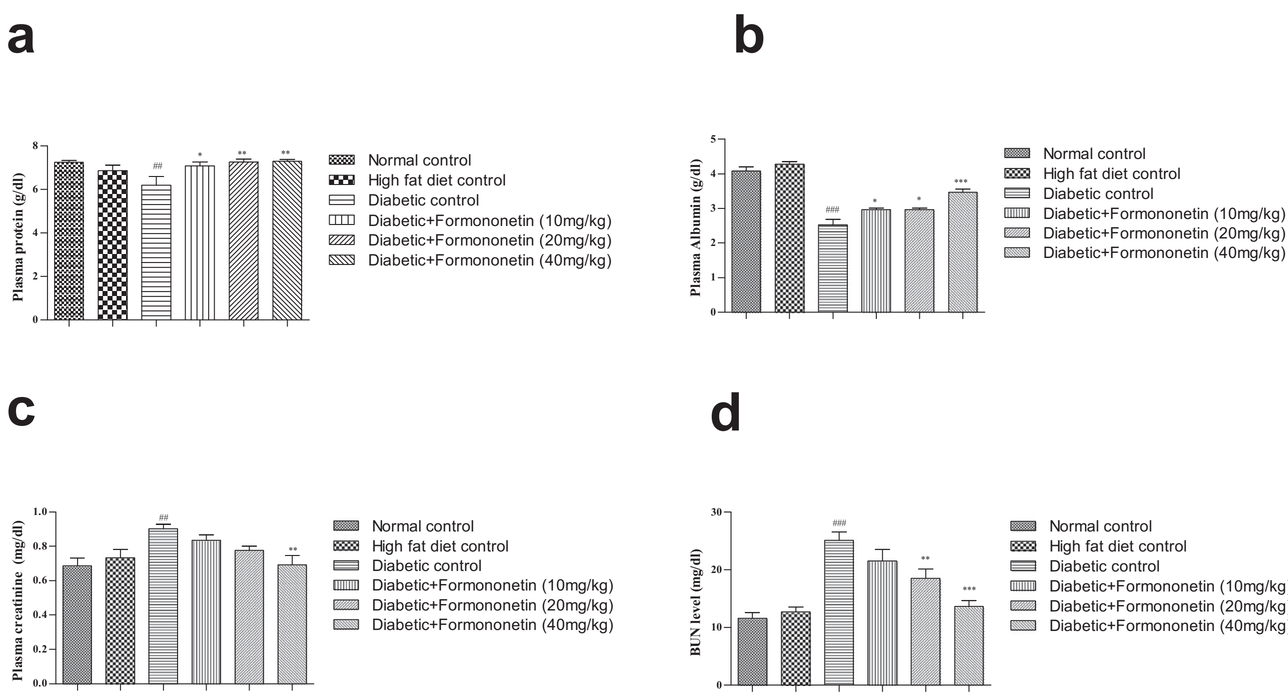

Fig. 4. Effect of formononetin on plasma protein, albumin, creatinine and blood urea nitrogen. **a**. plasma protein, **b**. plasma albumin, **c**. plasma creatinine and **d.** blood urea nitrogen. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.

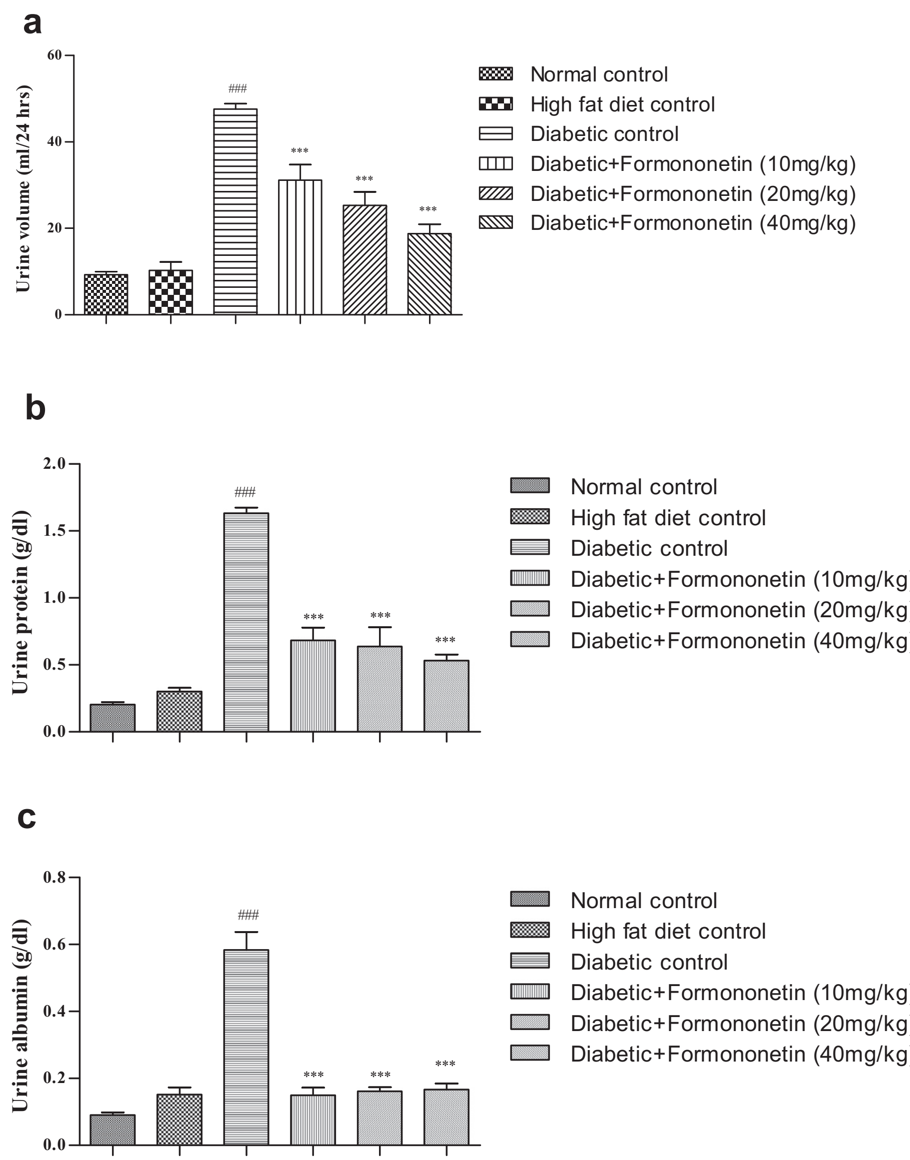

Fig. 5. Effect of formononetin on urine volume, urine protein and urine albumin **a**. Urine volume, **b**. Urine protein and **c**. Urine albumin All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. ***p < 0.001, when compared with diabetic control.

2.10. Histological assessment of renal injury

The kidney tissue embedded in paraffin blocks were sliced into sections with thickness of 3 mm. The sections were stained with hematoxylin-Eosin (HE), Masson Trichome and Periodic Acid Schiff (PAS). Morphometric analysis was performed to evaluate the damage occurred in kidney tissue [21]. Glomerular damage was measured by semi quantitative scoring method in HE stain kidney tissue. The sections were observed under the magnification of 400× and scoring was done as: 0 for no lesion, 1 for < 25% damage, 2 for 25 to 49% damage, 3 for 50–74% damage and 4 for 75–100% damage [22].

Thickening of basement membrane capillary plexus of glomeruli and stromal hyperplasia developed in mesangial area of glomeruli was observed in PAS stain sections under 400× magnification and mesangial matrix index was measured using ImageJ 1.51a software (NIH, USA) as per the method described by Garud and Kulkarni [21].

The severity of tubulointerstitial fibrosis was evaluated in Masson's trichome stained sections. The sections were observed at 100× magnification and semi quantitative scoring was done to find out tubulointerstitial fibrosis index (TFI). The following parameters were considered during the scoring: deposition of extracellular matrix, presence of inflammatory cells, tubular atrophy and interstitial cell proliferation. The degree of interstitial fibrosis was graded as 0 for absence of fibrosis and cell infiltration, 1 for single focus of lesion, 2 for more than two isolated foci, 3 for greater than five isolated loci, 4 for > 10 isolated foci or fibrosis and infiltration of cells [21]. The percentage of collagen content was determined in kidney tissue stained with trichome stain using ImageJ1.51a software.

2.11. Western blot analysis of kidney tissue

Western blot analysis was performed to find out the expression of SIRT1 in kidney tissue [23,24]. Briefly, the tissue homogenate was prepared using radio-immuno-precipitation assay buffer and proteins were isolated. The concentration of protein in tissue was determined as per Lowry method. The separation of proteins was performed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride membrane (PVDF membrane) by electrophoresis (Bio-Rad, California, USA). Immunoblots analysis was carried out using anti-SIRT1 antibody (mouse;

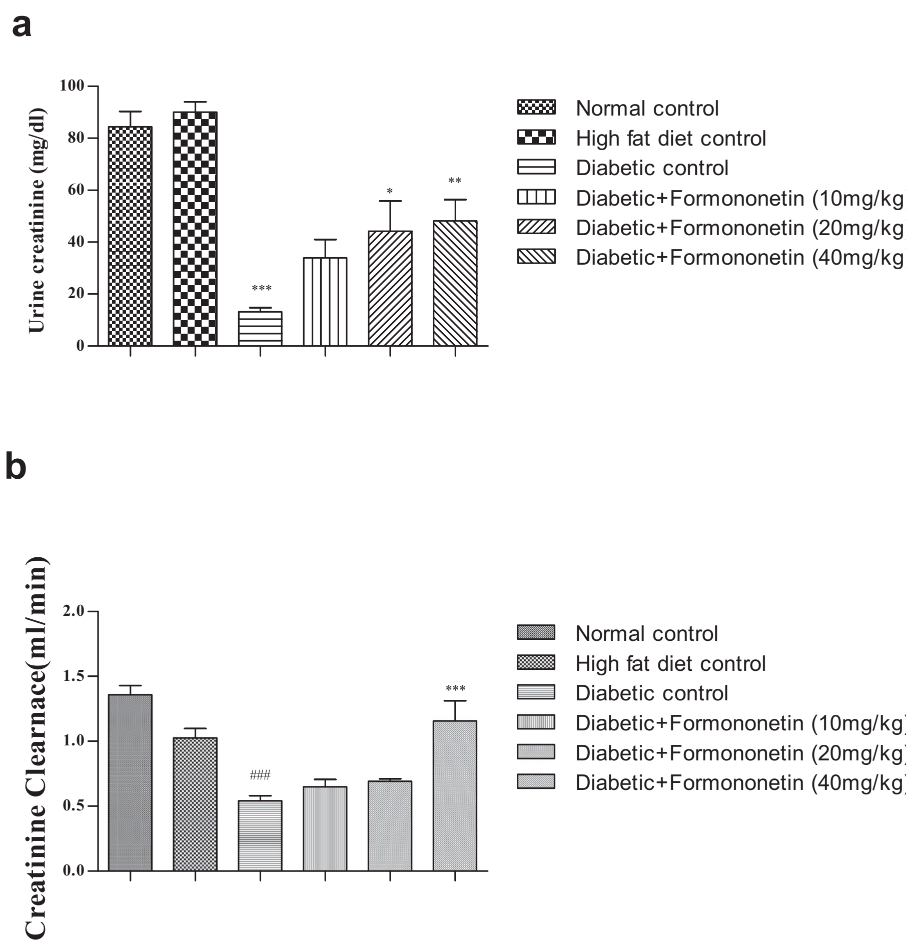

Fig. 6. Effect of formononetin on urine creatinine and creatinine clearance. **a**. Urine creatinine **b**. Creatinine clearance. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.

1:1000), β-actin (goat; 1:2000) and HRP-conjugated secondary antibodies (anti-rabbit: 1:2000). Immunoblots were detected using enhanced chemiluminescence (ECL) method and results were relatively expressed (SIRT1/β-actin).

2.12. *Immunohistochemical analysis of kidney tissue*

Immunochemical analysis for the expression of SIRT1 in tissue samples of kidney was performed as per previously reported method [25]. The semi quantitative analysis was performed to determine for the expression of SIRT1 in kidney tissue using ImageJ1.51a software.

2.13. *Statistical analysis*

All data are expressed as mean ± SEM. Results were statistically evaluated by using GraphPad Prism ver. 5.00 for Windows. Significant differences between the experimental groups were assessed by ANOVA (analysis of variance) test followed by Dunnett's Multiple Comparison. *p* value < 0.05 were considered to be significant.

3. Results

3.1. *Effect of formononetin on body weight and renal hypertrophy*

The effect of formononetin treatment on body weight and renal hypertrophy was measured after sixteen week treatment. As shown in Fig. 1a the average body weight (205.7 ± 6.922) of diabetic animals decreased significantly as compare to the average weight of normal (457.5 ± 14.40) and high fat diet (443.0 ± 11.99) fed animals. Treatment with all doses of formononetin showed increase in body weight when compared with diabetic animals at significant level.

The effect of formononetin treatment on renal hypertrophy was assessed by measuring the ratio of kidney weight to body weight of animals in each group. As shown in Fig. 1b diabetic animals showed significant increase in renal hypertrophy as compare to normal control animals($p < 0.001$). While formononetin treatment showed reduction in renal hypertrophy in diabetic animals. All the three doses were found effective to reduced renal hypertrophy significantly ($p < 0.001$). However, 40 mg/kg dose of formononetin showed better effect than lower doses of formononetin.

3.2. *Effect of formononetin on plasma glucose, insulin and HOMA-IR*

Glucose level in plasma samples of all the animals was measured to check the outcome of treatment with formononetin after 16 week treatment. As shown in Fig. 2a diabetic animals showed significantly ($p < 0.001$) augmented blood glucose level as compare to normal animals. While formononetin treated animals showed significant ($p < 0.001$) reduction in blood glucose level as compared to diabetic animals. As shown in Fig. 2b, Insulin level was also increased in diabetic animals potentially ($p < 0.001$) as compare to normal animals. Conversely, formononetin regimen at three different dose demonstrated significant decline in plasma insulin level ($p < 0.05$ for 10 mg/kg, 20 mg/kg dose, $p < 0.01$ for 40 mg/kg). Insulin resistance was also found significantly ($p < 0.001$) high in diabetic animals indicated by increased HOMA-IR value. Formononetin treatment exhibited

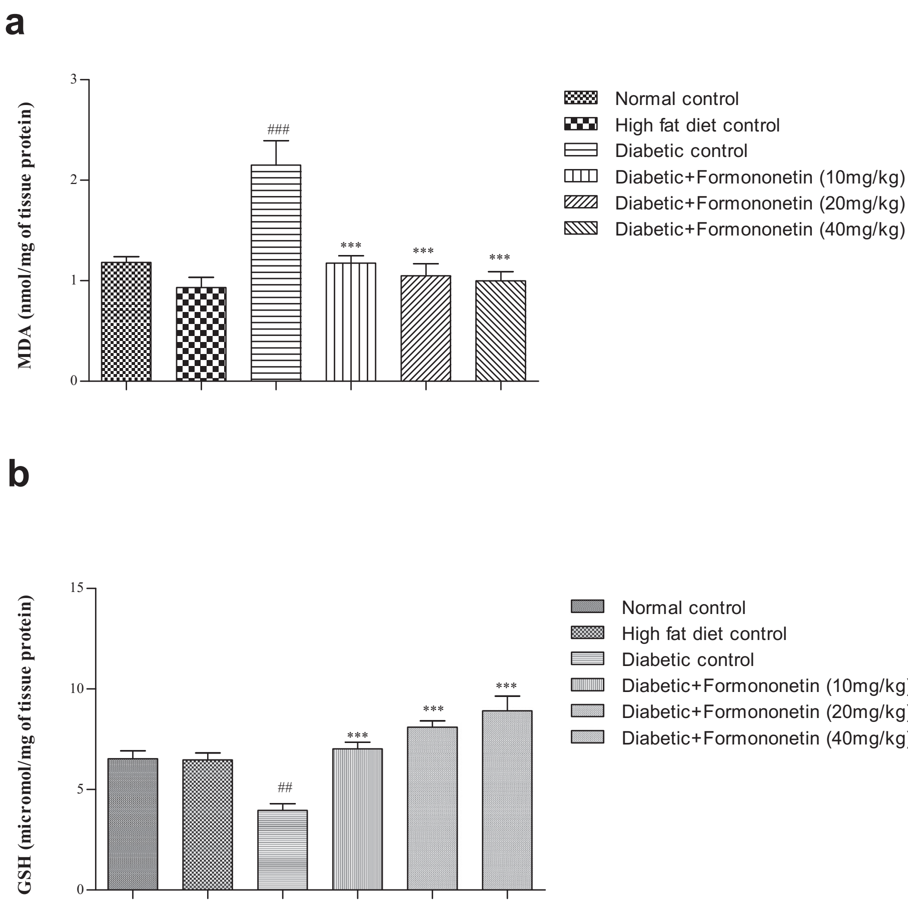

Fig. 7. Effect of formononetin on MDA and GSH. **a**. MDA, **b**. GSH. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.

reduction in insulin resistance at selected dose levels significantly ($p < 0.001$).

3.3. Effect of formononetin on triglyceride and cholesterol level in blood

Fig. 3a and b shows the outcome of formononetin dosing on triglyceride and cholesterol concentration in plasma. Diabetic animals displayed significant escalation of ($p < 0.001$) triglyceride and cholesterol in blood as compared to normal animals. However, formononetin treatment demonstrated significant reduction ($p < 0.001$) of triglyceride at selected dose levels. Formononetin treatment also reduced cholesterol at significant level ($p < 0.001$). Normal animal feeded with high fat diet showed increased amount of triglyceride and cholesterol in blood but not at significant level when compared with normal animals.

3.4. Effect of formononetin on plasma biomarkers

The effect of formononetin regimen on renal biomarkers is represented in Fig. 4. Untreated diabetic animals showed significant reduction of total protein ($p < 0.01$) and albumin ($p < 0.001$) in blood. Formononetin treatment normalized protein level in diabetic animals. Albumin level was also increased significantly ($p < 0.05$ for 10 and 20 mg/kg), ($p < 0.001$ for 40 mg/kg) after formononetin treatment. Plasma creatinine and BUN levels were increased significantly in diabetic animals ($p < 0.01$ and $p < 0.001$ respectively). However formononetin treatment reduced plasma creatinine significantly at 40 mg/kg dose ($p < 0.01$) and BUN at 20 and 40 mg/kg ($p < 0.01$, 0.001).

3.5. Effect of formononetin on urine biomarkers

Figs. 5 and 6 shown effect of formononetin regimen on urine biomarkers. Output of urine was increased drastically in untreated diabetic animals after 16 weeks ($p < 0.001$). Formononetin treatment exhibited reduction in urine output at significant level ($p < 0.001$). Diabetic animals also showed significantly ($p < 0.001$) increased excretion of protein and albumin in urine. While formononetin treatment significantly ($p < 0.001$) reduced protein and albumin excretion in urine. Formononetin treatment also escalated creatinine excretion in urine and significantly improved creatinine clearance in diabetic animals. As compare to other doses the higher dose of formononetin showed remarkable improvement in creatinine clearance ($p < 0.001$).

3.6. Effect of formononetin on oxidative stress marker in kidney tissue

As shown in Figs. 7 & 8, diabetic condition increases oxidative stress indicated by increased concentration of MDA and reduced level of GSH, SOD and Catalase. Formononetin treatment significantly reduced MDA ($p < 0.001$) concentration in kidney tissue. Formononetin treatment also improved GSH and SOD concentration in kidney tissue at significant level ($p < 0.001$). Catalase concentration was also improved significantly ($p < 0.01$) after formononetin treatment in diabetic animals at dose of 40 mg/kg.

3.7. Effect of formononetin treatment on histopathological changes

Histopathological changes in kidney tissue of various groups have been summarized in Table 1.

As shown in Fig. 9 diabetic animals after 16 week of type 2 diabetes

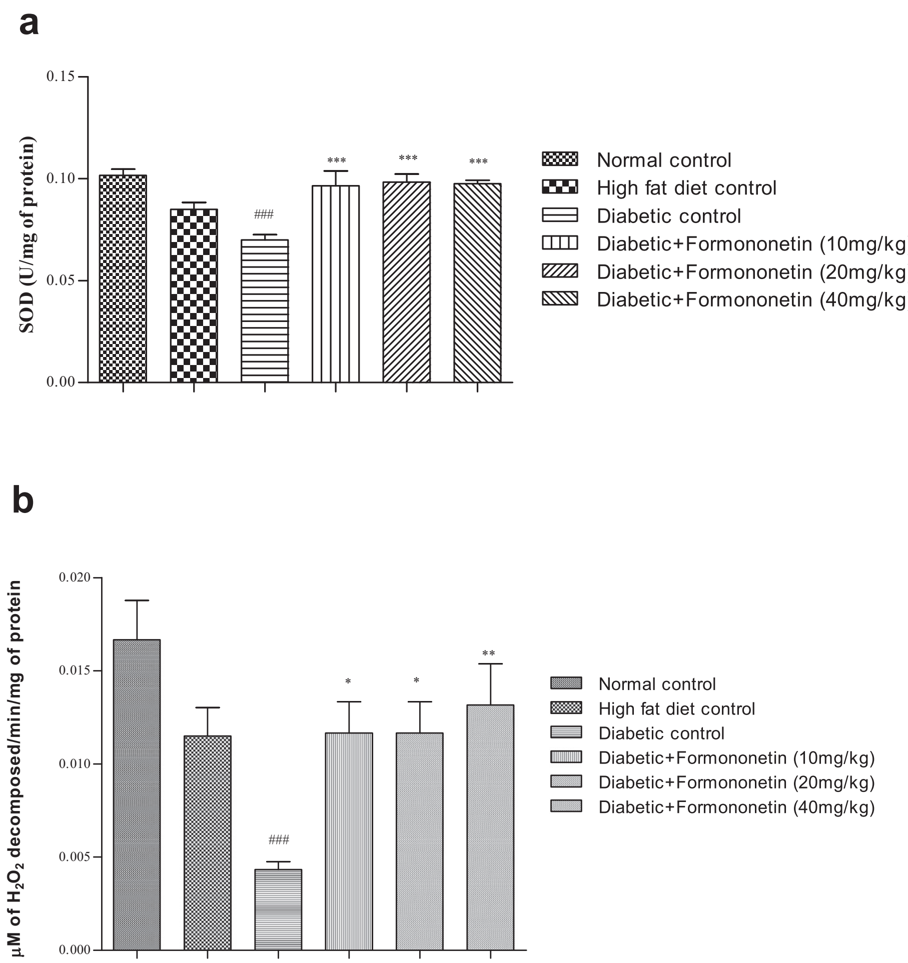

Fig. 8. Effect of formononetin on catalase and SOD. **a**. SOD, **b**. Catalase. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.

Table 1
Effect of formononetin treatment on histopathology and SIRT1 expression in kidney tissues.

Group	Glomerular damage score	Mesangial matrix index	Tubulo-interstitial fibrosis index	Collagen content (%)	SIRT1 expression (OD)
Normal control	0	4.5 ± 0.79	0	2.83 ± 0.68	0.19 ± 0.008
High fat diet control	2.00 ± 0.63	11.12 ± 2.45	2 ± 0.63	3.50 ± 0.92	0.15 ± 0.007
Diabetic control	2.83 ± 0.17 ##	24.43 ± 4.18###	2.33 ± 0.33 ##	12.76 ± 1.91###	0.12 ± 0.012##
Diabetic + formononetin (10 mg/kg)	2.00 ± 0.63	9.67 ± 2.63**	1.83 ± 0.60	6.55 ± 1.55**	0.15 ± 0.008
Diabetic + formononetin (20 mg/kg)	1.33 ± 0.62	8.75 ± 1.5***	1 ± 0.44	5.90 ± 1.17**	0.15 ± 0.013
Diabetic + formononetin (40 mg/kg)	0.67 ± 0.42*	8.75 ± 1.6***	0.66 ± 0.49*	3.21 ± 0.41***	0.18 ± 0.009**

All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.
Mesangial matrix index = (mesangial matrix area ∗ 100/total glomerular area).

induction showed significant (p < 0.01) glomerular damage. Higher dose of formononetin (40 mg/kg) showed significant (p < 0.05) improvement in kidney histology when compared with diabetic control. Animals fed with high fat diet also showed damage in kidney however the damage was not significant when compared with disease control.

Kidney tissue stained with PAS stain showed significantly high level of stromal hyperplasia in mesangial cells and glomerular basement membrane thickening in diabetic animals. (Fig. 10) Measurement of mesangial matrix index demonstrated increase in disposition of mesangial matrix in renal tissue of diabetic animals. Conversely, Formononetin treatment at selected three dose levels showed significant reduction (p < 0.01 for 10 mg/kg, p < 0.001for 20 and 40 mg/kg) in kidney damage indicated by reduction in mesangial matrix index.

Masson trichomes stain was used to measure fibrosis level and collagen content in the diabetic animals. (Fig. 11) The tubulointerstitial fibrosis was significantly (p < 0.01) higher in the kidney tissue of diabetic animals. While formononetin regimen demonstrated considerable reduction (p < 0.05) in fibrosis at higher dose.

Collagen deposition was significantly (p < 0.001) high in the kidney tissue of diabetic animals. While treatment with formononetin demonstrated significant reduction (p < 0.05, 0.01 and 0.001 for 10, 20 and 40 mg/kg respectively) in collagen deposition in kidney tissue at all the three selected doses.

3.8. Effect of formononetin treatment on SIRT1 expression

Fig. 13(a) shows results of SIRT1 expression in kidney by western blot method. Diabetic animals exhibited significant decrease

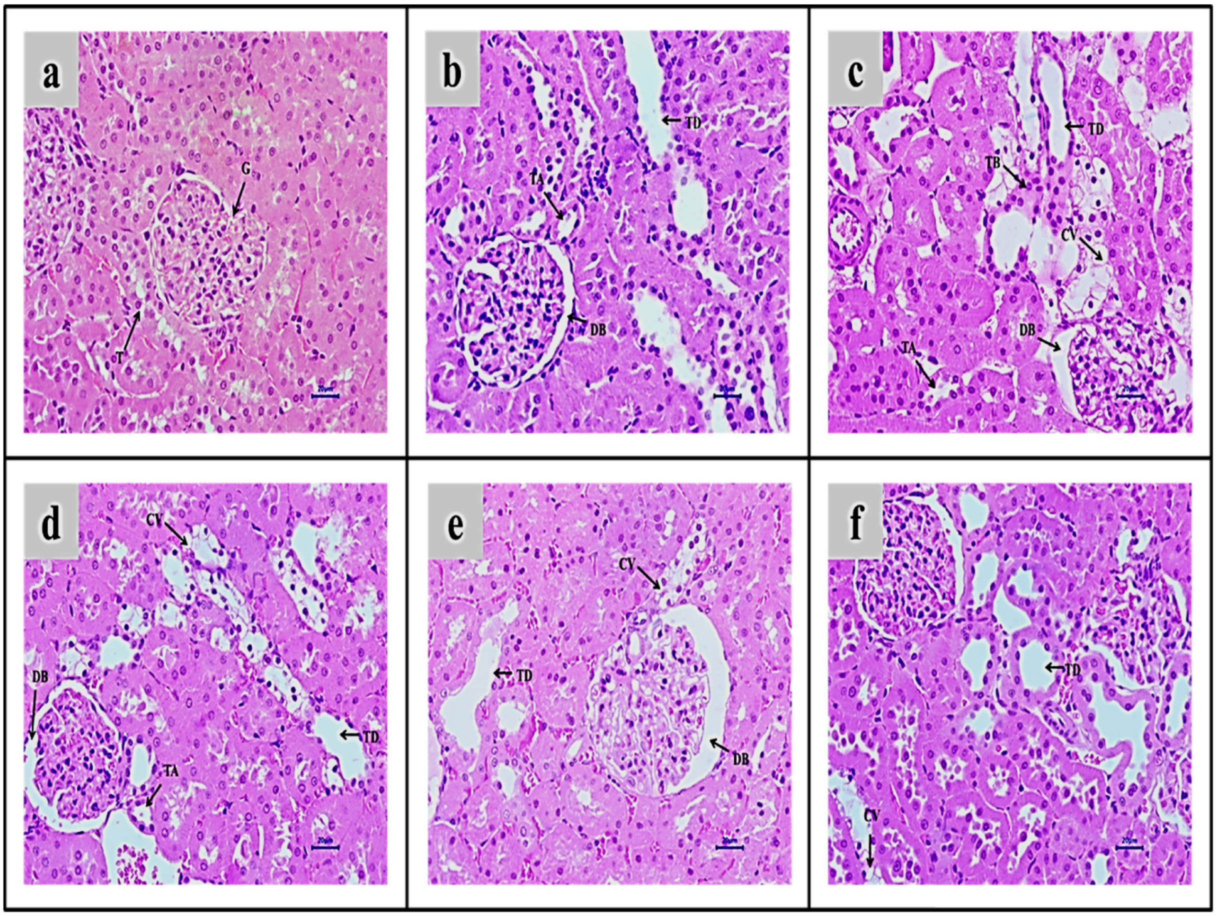

Fig. 9. Formononetin treatment protects kidney tissue from hyperglycemia induced damage in type 2 diabetes. (**a**) Normal control: histology of Renal Tubule (T) and Glomeruli (G). (**b**) High fat diet control: Tubular Dilation (TD), Tubular atrophy (TA), Dilated bowman's space (DB). (**c**) Diabetic control: Tubular Dilation (TD), Tubular atrophy (TA), Cytoplasmic vacuolation (CV), Thickening of tubular basement membrane (TB), Dilated bowman's space (DB). (**d**) Diabetic + Formononetin (10 mg/kg): Tubular Dilation (TD), Tubular atrophy (TA), Cytoplasmic vacuolation (CV), Dilated bowman's space (DB) (**e**) Diabetic + Formononetin (20 mg/kg): Tubular Dilation (TD), Cytoplasmic vacuolation (CV), Dilated bowman's space (DB) (f) Diabetic + Formononetin (40 mg/kg): Tubular Dilation (TD), Cytoplasmic vacuolation (CV). All sections were stained with H&E stain and observed at 400× magnification.

($p < 0.001$) in SIRT1 expression in kidney tissue as mentioned in Fig. 13(a). Higher dose of formononetin showed significant increase ($p < 0.05$) in SIRT1 expression in kidney tissue.

Diabetic animals showed significant reduction in SIRT1 expression demonstrated by decrease in optical density in Fig. 13(b). While formononetin regimen showed increase in SIRT1 expression in kidney section stained immunohistochemically. (Fig. 12) Furthermore, higher dose of formononetin demonstrated significant ($p < 0.01$) escalation in SIRT1 expression in kidney tissues.

4. Discussion

The current study was centered on hypothesis that increase in SIRT1 expression can reduce insulin resistance, oxidative stress and maintain normal lipid profile which is considered as major factor for the development of nephropathy in type 2 diabetic conditions.

The development of nephropathy in type 2 diabetic rats was confirmed by hyperglycemia, insulin resistance, hypertriglyceridemia, hypercholesterolemia, increased concentration of plasma creatinine and BUN, increased renal hypertrophy, oxidative stress, proteinuria and impaired creatinine clearance.

Uncontrolled hyperglycemia in type 2 diabetes invokes kidney damage due to hemodynamic dyregulation and functional changes in nephron. This results into increased permeability of plasma proteins especially albumin through glomerular filtration barrier and leads to increased excretion of albumin in urine. Chronic hyperglycemia is also responsible for the formation of reactive oxygen species (ROS) and advanced glycation end product in kidney tissue of diabetics, which causes depletion of podocytes and results in diabetic nephropathy [26]. Extracellular matrix proteins present in kidney tissue are susceptible to advanced glycation end product formation through matrix-matrix and cell-matrix interaction, which is responsible for glomerulosclerosis in diabetic nephropathy [27]. These reports indicate that chronic hyperglycemia is the major culprit for development of vascular complication in diabetics including nephropathy [28]. Hyperglycemia results into proteinuria, hyperalbuminuria, glomerular hypertrophy and extracellular matrix accretion in diabetic nephropathy [29]. In our study also we have observed similar results in untreated diabetic animals. Diabetic animals showed high amount of glucose in their blood along with hypercholesterolemia and triglycerdemia might be due to continuous feeding of high fat diet and diabetic condition. Chronic treatment with formononetin for 16 week not totally normalize blood glucose like normal animals, but able to decrease hyperglycemia significantly in diabetic animals by reducing insulin resistance. Hypertriglyceridemia and hypercholesterolemia are the independent factors associated with diabetic nephropathy [30]. In our study also untreated diabetic animals showed severe hypertriglyceridemia and hypercholesterolemia. Number of reports showed that formononetin has lipid lowering effect [31,32]. In present study also formononetin showed tremendous effect in decreasing triglyceride and cholesterol

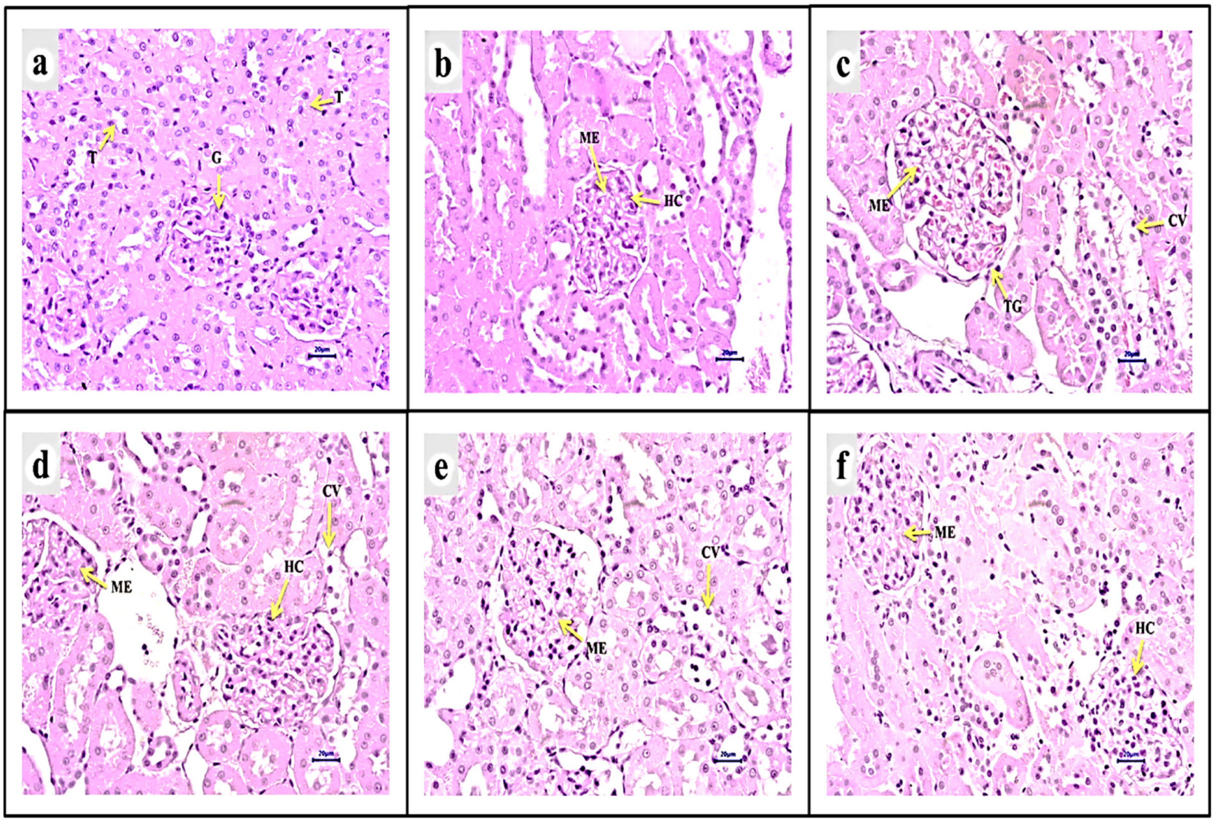

Fig. 10. Formononetin treatment reduced expansion of mesangial matrix in kidney tissue of type 2 diabetic rats. (a) Normal control, (b) High fat diet control, (c) Diabetic control, (d) Diabetic + Formononetin (10 mg/kg), (e) Diabetic + Formononetin (20 mg/kg), (f) Diabetic + Formononetin (40 mg/kg). T; Tubules,G: glomeruli, ME; mesangial matrix expansion, CH; cell hypercellularity, CV; cytoplasmic vacuolation, TG; thickened glomerular basement membrane. All sections were stained with Periodic Acid-Schiff (PAS) stain and observed at 400× magnification.

level in diabetic animals and it was found comparable with normal animals.

The most commonly observed sign of diabetic nephropathy in its early or late phase include increase in glomerular filtration rate, glomerulosclerosis and proteinuria. (Xue et al. 2011) Total protein, creatinine, albumin and blood urea nitrogen (BUN) are the functional parameters in the diabetic nephropathy. In the present study total protein and albumin were decreased in blood while creatinine and blood urea nitrogen was found in higher concentration in blood samples. Total protein and albumin concentration was found high in urine sample while creatinine level was very low in urine samples along with reduction in creatinine clearance in diabetic animals. This result indicates that development of nephropathy in diabetic animals was successfully achieved. Formononetin treatment showed improvement in creatinine clearance and reduction in blood urea nitrogen in diabetic animals along with improving plasma concentration of protein, albumin and creatinine. Formononetin treatment also decreased urine volume and albumin concentration and increased creatinine excretion in urine. This suggested that formononetin treatment provide beneficial effect in reducing the risk of development of diabetic nephropathy along with reducing hyperglycemia in type 2 diabetics.

Hyperglycemia induced reactive oxygen species damage kidney tissue in type 2 diabetes mainly by enzymatic mechanisms (NADPH oxidase) and non-enzymatic (electron transport chain in mitochondrial) pathways [33]. Activation of these pathways generates excess amount of superoxide species which subsequently generate secondary reactive oxygen species [34]. This situation causes increased production of superoxide and reduction in antioxidant activity mainly superoxide dismutase. This is reflected in the form of reduced level of SOD in kidney tissues of diabetics suffering from nephropathy [35]. Catalase is another important enzyme responsible for the protection of kidney tissue against oxidative stress. Catalase present in peroxisome found in kidney cell converts H_2O_2 generated during lipid metabolism into water and oxygen. However hyperglycemia and associated increased level of free fatty acids in type 2 diabetics cause peroxisomal dysfunction which results into reduction in catalase level in kidney tissue [36].

In our study chronic hyperglycemia condition in diabetic animals showed excessive production of ROS, this was reflected in form of reduction in SOD and catalase activity. While formononetin treatment showed improvement in SOD concentration and catalase activity. Increased oxidative stress accelerates lipid peroxidation results into increase in formation of malondialdehyde (MDA) and further damage the kidney tissue [21]. The level of glutathione, an important non-protein thiol responsible for maintaining redox balance at cellular level is decreased in diabetic nephropathy [37]. In the present study MDA level was found high and GSH level was decreased in diabetic animals due to increased lipid peroxidation in kidney tissue of untreated diabetic animals. However, formononetin treatment reduced MDA concentration and improved GSH concentration in kidney tissue of diabetic animals. This shows that formononetin treatment for chronic period of time could reduce the formation of ROS.

Renin angiotensin system is associated with further damage in diabetic nephropathy. Increased level of Ang II, a major hormone of RAS results into hypertrophy in tubular epithelial cells and mesangial cells. (Chawla et al., 2010) Histochemical observation using different types of staining reagent such as H&E, PAS and Masson trichomes stained indicated that there was a severe damage to the kidney tissues of untreated diabetic animals. Degenerative changes such as lymphocytic infiltration, hyperplastic lesions, fibrosis in kidney tissue along with increased mesangial matrix expansion and increased collagen deposition in kidney tissue in diabetic animals. The outcome of the study showed that treatment with formononetin protected kidney

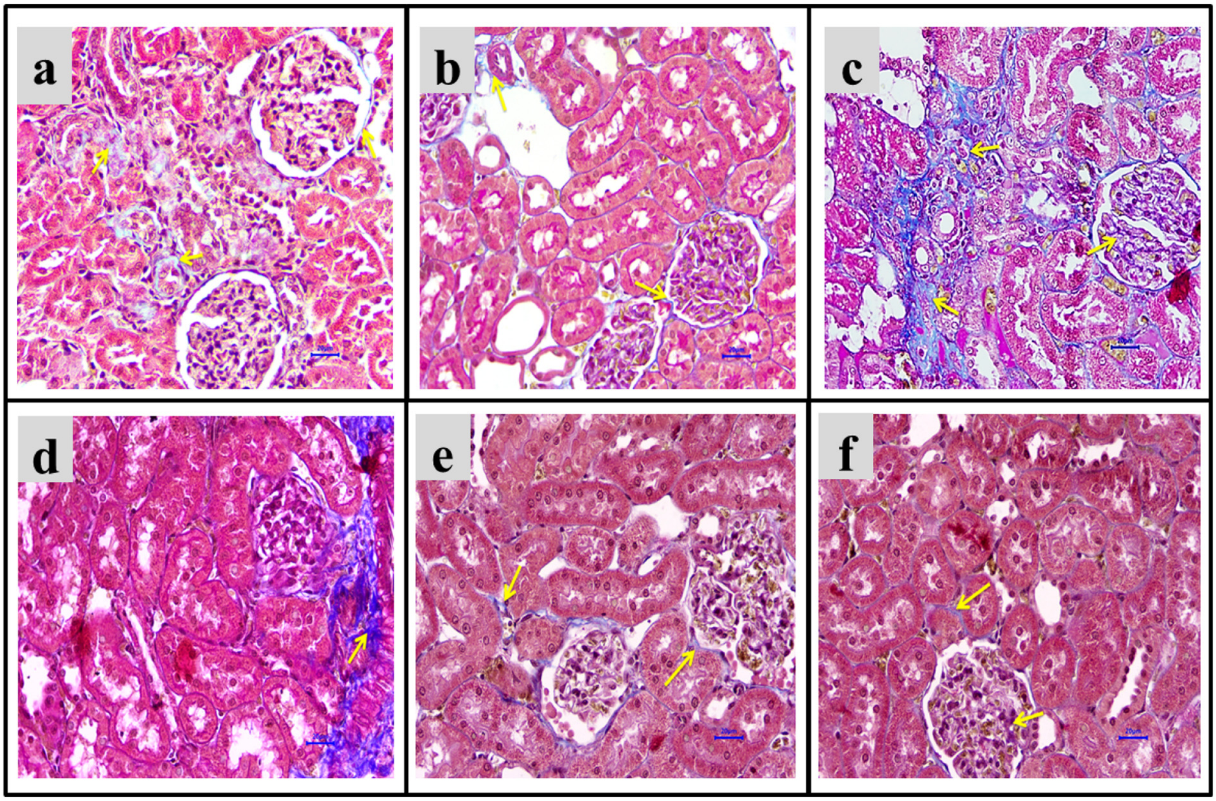

Fig. 11. Formononetin treatment decreased fibrosis in kidney tissues of type 2 diabetic rats. (a) Normal control, (b) High fat diet control, (c) Diabetic control, (d) Diabetic + Formononetin (10 mg/kg), (e) Diabetic + Formononetin (20 mg/kg), (f) Diabetic + Formononetin (40 mg/kg). (Arrow indicate collagen stained region.) All sections were stained with Masson's trichome stain and observed at 400× magnification.

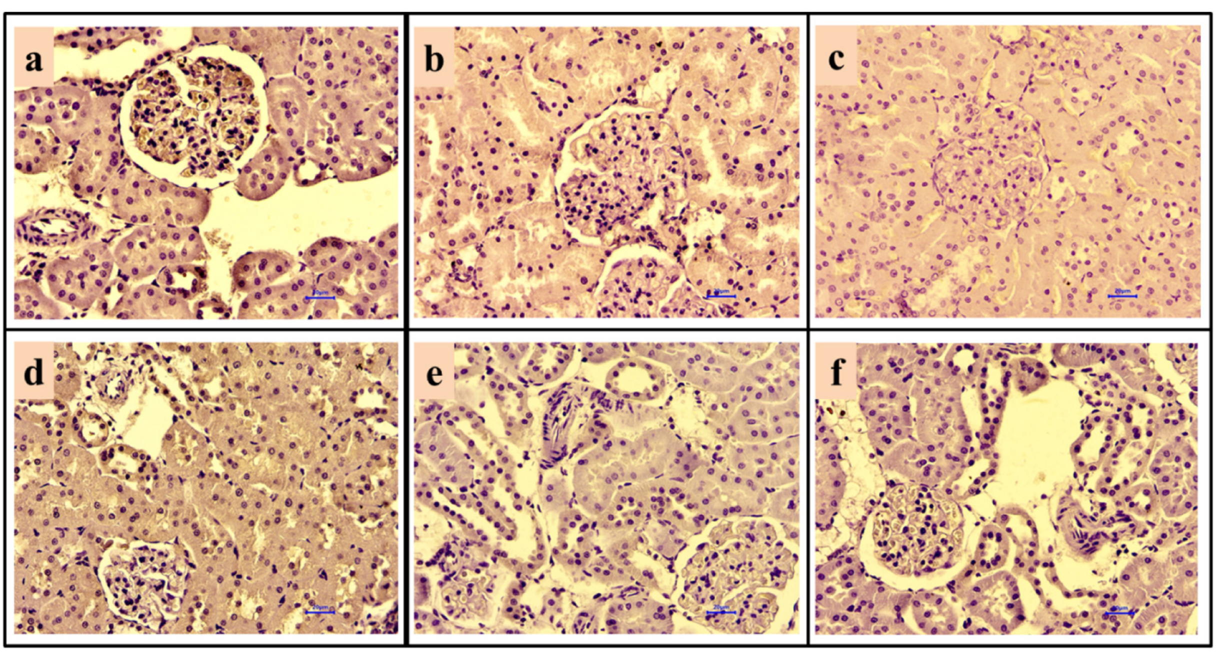

Fig. 12. Formononetin treatment increases the expression of SIRT1 in kidney tissue of type 2 diabetic rats. (**a**) Normal control (**b**) High fat diet control (**c**) Diabetic control (**d**) Diabetic + Formononetin (10 mg/kg) (**e**) Diabetic + Formononetin (20 mg/kg) (**f**) Diabetic + Formononetin (40 mg/kg). All sections were immunohistochemically stained and observed at 400× magnification.

tissues of diabetic animals against degenerative changes generally observed in diabetic nephropathy. This outcome is also supported by other parameters evaluated in the present study.

SIRT1 is known for the maintenance of glucose homeostasis via maintenance of lipid metabolism, hepatic glucose production and reducing insulin resistance [38]. SIRT1 is involved in reduction of oxidative stress via regulating transcriptional activities of various important proteins linked with formation of oxidative stress such as PGC-1α. Recent reports shows that SIRT1 negatively control the expression of $p66^{Shc}$ and reduce oxidative stress in kidney tissue and protects

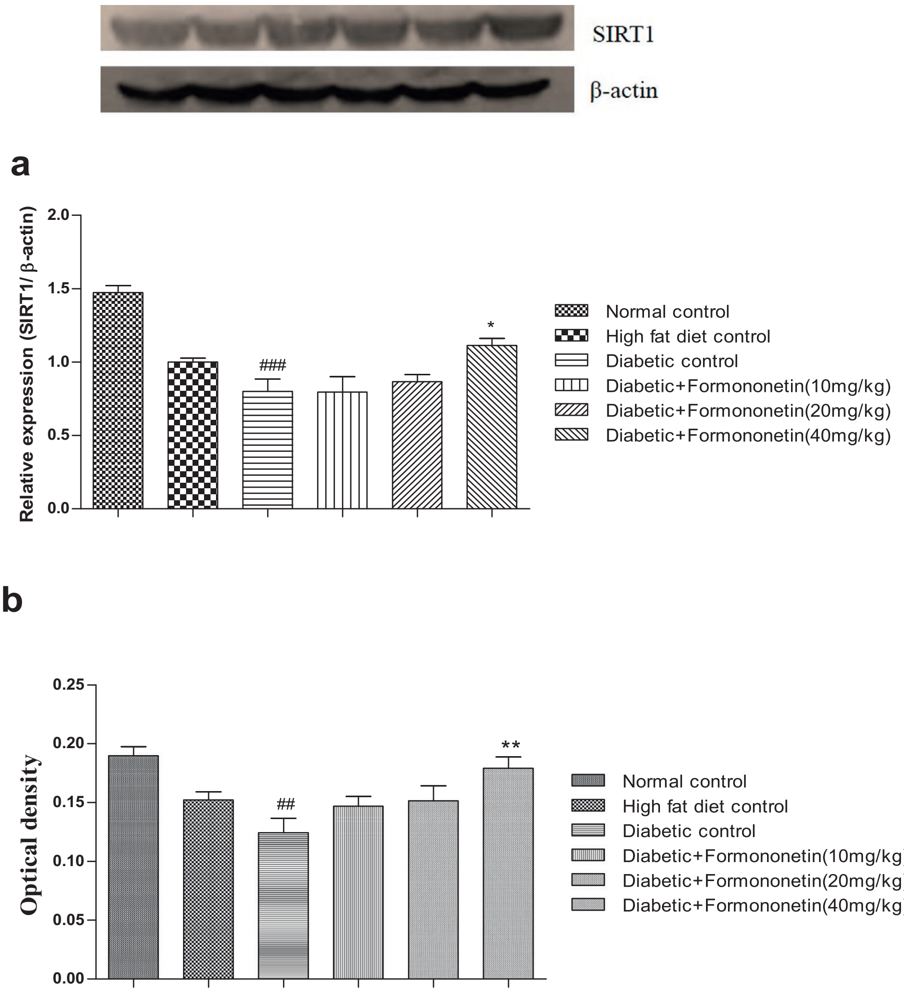

Fig. 13. Expression of SIRT1 in kidney tissue. (**a**) Relative expression of SIRT1 in kidney tissue by western blot method. 1. Normal control 2. High fat diet control 3. Diabetic control 4. Diabetic + Formononetin (10 mg/kg) 5. Diabetic + Formononetin (20 mg/kg) 6. Diabetic + Formononetin (40 mg/kg) (**b**) Expression of SIRT1 in kidney tissue in immunohistochemical staining method. All values are expresses as Mean ± SEM. ###p < 0.001, when compared with normal control. *p < 0.05, **p < 0.01, ***p < 0.001, when compared with diabetic control.

podocytes depletion [39]. SIRT1 also reduce hyperalbuminuria, and fibrosis in proximal tubular cells, also decrease mesangial expansion in diabetic nephropathy [40,41]. SIRT1 regulates RAS system by activating angiotensin-converting enzyme 2 (ACE2) promoters. It also causes vasodilation by decreasing the expression of AT1R (angiotensin II receptor-type I). It also deacetylase eNO and increases nitric oxide production which protects vascular tissue [39,42].

Immunohistochemical analysis is one of the important methods for the quantification of SIRT1 in present study. Formononetin treatment showed increase in expression of SIRT1 in kidney tissue. Immunohistochemical results are also supported by outcome of western blot analysis. There is a strong correlation of SIRT1 expression and generation of ROS in kidney tissues. Thus increased expression of SIRT1 might be the reason for reduction of oxidative stress and kidney tissue damage after treatment with formononetin.

5. Conclusion

The outcome of the present study indicates that increase in expression of SIRT1 by formononetin could reduce the risk of nephropathy in type 2 diabetes. Thus formononetin would be considered as a new therapeutic agent for type 2 diabetic nephropathy in future.

Conflict of interest

None.

References

[1] H.-H. Parving, H. Lehnert, J. Bröchner-Mortensen, R. Gomis, S. Andersen, P. Arner, The effect of Irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes, N. Engl. J. Med. 345 (2001) 870–878, https://doi.org/10.1056/NEJMoa011489.

[2] S. Menini, C. Iacobini, G. Pugliese, C. Pesce, Dietary interventions to contrast the onset and progression of diabetic nephropathy: a critical survey of new data, Crit. Rev. Food Sci. Nutr. 58 (2018) 1671–1680, https://doi.org/10.1080/10408398.2016.1278355.

[3] T. Jourdan, J.K. Park, Z.V. Varga, J. Pálóczi, N.J. Coffey, A.Z. Rosenberg, G. Godlewski, R. Cinar, K. Mackie, P. Pacher, G. Kunos, Cannabinoid-1 receptor deletion in podocytes mitigates both glomerular and tubular dysfunction in a mouse model of diabetic nephropathy, Diabetes. Obes. Metab. 20 (2018) 698–708, https://doi.org/10.1111/dom.13150.

[4] D. de Zeeuw, G. Remuzzi, H.-H. Parving, W.F. Keane, Z. Zhang, S. Shahinfar, S. Snapinn, M.E. Cooper, W.E. Mitch, B.M. Brenner, Proteinuria, a target for renoprotection in patients with type 2 diabetic nephropathy: lessons from RENAAL, Kidney Int. 65 (2004) 2309–2320, https://doi.org/10.1111/J.1523-1755.2004.00653.X.

[5] F.P. Schena, L. Gesualdo, Pathogenetic mechanisms of diabetic nephropathy, J. Am. Soc. Nephrol. 16 (Suppl. 1) (2005) S30–S33, https://doi.org/10.1681/ASN.2004110970.

[6] A.M. Al Hroob, M.H. Abukhalil, R.D. Alghonmeen, A.M. Mahmoud, Ginger alleviates hyperglycemia-induced oxidative stress, inflammation and apoptosis and protects rats against diabetic nephropathy, Biomed. Pharmacother. 106 (2018)

381–389, https://doi.org/10.1016/J.BIOPHA.2018.06.148.

[7] M.K. Sagoo, L. Gnudi, Diabetic nephropathy: is there a role for oxidative stress? Free Radic. Biol. Med. 116 (2018) 50–63, https://doi.org/10.1016/J.FREERADBIOMED.2017.12.040.

[8] A. Salminen, K. Kaarniranta, A. Kauppinen, Crosstalk between oxidative stress and SIRT1: impact on the aging process, Int. J. Mol. Sci. 14 (2013) 3834–3859, https://doi.org/10.3390/ijms14023834.

[9] K. Hasegawa, S. Wakino, P. Simic, Y. Sakamaki, H. Minakuchi, K. Fujimura, K. Hosoya, M. Komatsu, Y. Kaneko, T. Kanda, E. Kubota, H. Tokuyama, K. Hayashi, L. Guarente, H. Itoh, Renal tubular Sirt1 attenuates diabetic albuminuria by epigenetically suppressing Claudin-1 overexpression in podocytes, Nat. Med. 19 (2013) 1496–1504 http://www.ncbi.nlm.nih.gov/pubmed/24141423 (accessed August 2, 2018).

[10] I. Kaczmarczyk-Sedlak, W. Wojnar, M. Zych, E. Ozimina-Kamińska, J. Taranowicz, A. Siwek, Effect of formononetin on mechanical properties and chemical composition of bones in rats with ovariectomy-induced osteoporosis, Evid. Based Complement. Alternat. Med. 2013 (2013) 457052, , https://doi.org/10.1155/2013/457052.

[11] H. Mu, Y.-H. Bai, S.-T. Wang, Z.-M. Zhu, Y.-W. Zhang, Research on antioxidant effects and estrogenic effect of formononetin from *Trifolium pratense* (red clover), Phytomedicine 16 (2009) 314–319, https://doi.org/10.1016/J.PHYMED.2008.07.005.

[12] S. Yang, M. Liu, Y. Chen, C. Ma, L. Liu, B. Zhao, Y. Wang, X. Li, Y. Zhu, X. Gao, D. Kong, Y. Duan, J. Han, X. Yang, NaoXinTong capsules inhibit the development of diabetic nephropathy in db/db mice, Sci. Rep. 8 (2018) 9158, , https://doi.org/10.1038/s41598-018-26746-1.

[13] J.S. Hwang, E.S. Kang, S.G. Han, D.-S. Lim, K.S. Paek, C.-H. Lee, H.G. Seo, Formononetin inhibits lipopolysaccharide-induced release of high mobility group box 1 by upregulating SIRT1 in a PPARδ-dependent manner, PeerJ 6 (2018) e4208, , https://doi.org/10.7717/peerj.4208.

[14] M.J. Oza, Y.A. Kulkarni, Formononetin treatment in type 2 diabetic rats reduces insulin resistance and hyperglycemia, Front. Pharmacol. 9 (2018) 739, , https://doi.org/10.3389/fphar.2018.00739.

[15] K. Srinivasan, B. Viswanad, L. Asrat, C.L. Kaul, P. Ramarao, Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening, Pharmacol. Res. 52 (2005) 313–320, https://doi.org/10.1016/j.phrs.2005.05.004.

[16] M.S. Garud, Y.A. Kulkarni, Gallic acid attenuates type I diabetic nephropathy in rats, Chem. Biol. Interact. 282 (2018) 69–76, https://doi.org/10.1016/J.CBI.2018.01.010.

[17] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358, https://doi.org/10.1016/0003-2697(79)90738-3.

[18] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77, https://doi.org/10.1016/0003-9861(59)90090-6.

[19] H. Luck, Catalase, Methods of Enzymatic Analysis, Academic Press, New York, 1971, https://books.google.co.in/books?id=joj3tgEACAAJ&dq=methods+of+enzymatic+analysis+1971&hl=en&sa=X&ved=0ahUKEwiyl4SpgdLcAhVEfysKHTLyBx0Q6AEIKDAA , Accessed date: 4 August 2018.

[20] F. Paoletti, A. Mocali, [18] determination of superoxide dismutase activity by purely chemical system based on NAD(P)H oOxidation, Methods Enzymol. 186 (1990) 209–220, https://doi.org/10.1016/0076-6879(90)86110-H.

[21] M.S. Garud, Y.A. Kulkarni, Eugenol ameliorates renal damage in streptozotocin-induced diabetic rats, Flavour Fragr. J. 32 (2017) 54–62, https://doi.org/10.1002/ffj.3357.

[22] M.S. Garud, Y.A. Kulkarni, Attenuation of renal damage in type I diabetic rats by umbelliferone – a coumarin derivative, Pharmacol. Rep. 69 (2017) 1263–1269, https://doi.org/10.1016/J.PHAREP.2017.06.014.

[23] L. Bordone, M.C. Motta, F. Picard, A. Robinson, U.S. Jhala, J. Apfeld, T. McDonagh, M. Lemieux, M. McBurney, A. Szilvasi, E.J. Easlon, S.-J. Lin, L. Guarente, Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic β cells, PLoS Biol. 4 (2005) e31, , https://doi.org/10.1371/journal.pbio.0040031.

[24] S. Ghosh, P. More, A. Derle, R. Kitture, T. Kale, M. Gorain, A. Avasthi, P. Markad, G.C. Kundu, S. Kale, D.D. Dhavale, J. Bellare, B.A. Chopade, Diosgenin functionalized iron oxide nanoparticles as novel nanomaterial against breast cancer, J. Nanosci. Nanotechnol. 15 (2015) 9464–9472 http://www.ncbi.nlm.nih.gov/pubmed/26682367 , Accessed date: 4 September 2018.

[25] M.J. Oza, Y.A. Kulkarni, Biochanin A improves insulin sensitivity and controls hyperglycemia in type 2 diabetes, Biomed. Pharmacother. 107 (2018) 1119–1127, https://doi.org/10.1016/J.BIOPHA.2018.08.073.

[26] S. Fakhruddin, W. Alanazi, K.E. Jackson, Diabetes-induced reactive oxygen species: mechanism of their generation and role in renal injury, J. Diabetes Res. 2017 (2017) 1–30, https://doi.org/10.1155/2017/8379327.

[27] J.M. Forbes, M.E. Cooper, M.D. Oldfield, M.C. Thomas, Role of advanced glycation end products in diabetic nephropathy, J. Am. Soc. Nephrol. 14 (2003) S254–S258, https://doi.org/10.1097/01.ASN.0000077413.41276.17.

[28] D. Fang, H. Guan, J. Liu, G. Wei, W. Ke, B. Yao, H. Xiao, Y. Li, Early intensive insulin therapy attenuates the p38 pathway in the renal cortex and indices of nephropathy in diabetic rats, Endocr. J. 59 (2012) 81–90 http://www.ncbi.nlm.nih.gov/pubmed/22068113 , Accessed date: 24 August 2018.

[29] F.H. Nystrom, M.J. Quon, Insulin signalling: metabolic pathways and mechanisms for specificity, Cell. Signal. 11 (1999) 563–574 http://www.ncbi.nlm.nih.gov/pubmed/10433517 , Accessed date: 24 August 2018.

[30] L. He, L. Hao, X. Fu, M. Huang, R. Li, Severe hypertriglyceridemia and hypercholesterolemia accelerating renal injury: a novel model of type 1 diabetic hamsters induced by short-term high-fat/high-cholesterol diet and low-dose streptozotocin, BMC Nephrol. 16 (2015) 51, , https://doi.org/10.1186/s12882-015-0041-5.

[31] R.D. Sharma, Effect of various isoflavones on lipid levels in triton-treated rats, Atherosclerosis 33 (1979) 371–375, https://doi.org/10.1016/0021-9150(79)90189-8.

[32] P.B. Clifton-Bligh, M.-L. Nery, R.J. Clifton-Bligh, S. Visvalingam, G.R. Fulcher, K. Byth, R. Baber, Red clover isoflavones enriched with formononetin lower serum LDL cholesterol—a randomized, double-blind, placebo-controlled study, Eur. J. Clin. Nutr. 69 (2015) 134–142, https://doi.org/10.1038/ejcn.2014.207.

[33] J.C. Jha, C. Banal, B.S.M. Chow, M.E. Cooper, K. Jandeleit-Dahm, Diabetes and kidney disease: role of oxidative stress, Antioxid. Redox Signal. 25 (2016) 657–684, https://doi.org/10.1089/ars.2016.6664.

[34] H. Fujita, H. Fujishima, S. Chida, K. Takahashi, Z. Qi, Y. Kanetsuna, M.D. Breyer, R.C. Harris, Y. Yamada, T. Takahashi, Reduction of renal superoxide dismutase in progressive diabetic nephropathy, J. Am. Soc. Nephrol. 20 (2009) 1303–1313, https://doi.org/10.1681/ASN.2008080844.

[35] H. Fujita, T. Sakamoto, K. Komatsu, H. Fujishima, T. Morii, T. Narita, T. Takahashi, Y. Yamada, Reduction of circulating superoxide dismutase activity in type 2 diabetic patients with microalbuminuria and its modulation by telmisartan therapy, Hypertens. Res. 34 (2011) 1302–1308, https://doi.org/10.1038/hr.2011.127.

[36] I. Hwang, J. Lee, J.Y. Huh, J. Park, H.B. Lee, Y.-S. Ho, H. Ha, Catalase deficiency accelerates diabetic renal injury through peroxisomal dysfunction, Diabetes 61 (2012) 728–738, https://doi.org/10.2337/db11-0584.

[37] Y. Ueno, M. Kizaki, R. Nakagiri, T. Kamiya, H. Sumi, T. Osawa, Dietary glutathione protects rats from diabetic nephropathy and neuropathy, J. Nutr. 132 (2002) 897–900, https://doi.org/10.1093/jn/132.5.897.

[38] R. Yacoub, K. Lee, J.C. He, The role of SIRT1 in diabetic kidney disease, Front. Endocrinol. 5 (2014) 166, , https://doi.org/10.3389/fendo.2014.00166.

[39] S. Zhou, H.-Z. Chen, Y.-z. Wan, Q.-J. Zhang, Y.-S. Wei, S. Huang, J.-J. Liu, Y.-B. Lu, Z.-Q. Zhang, R.-F. Yang, R. Zhang, H. Cai, D.-P. Liu, C.-C. Liang, Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction, Circ. Res. 109 (2011) 639–648, https://doi.org/10.1161/CIRCRESAHA.111.243592.

[40] S. Kume, T. Uzu, K. Horiike, M. Chin-Kanasaki, K. Isshiki, S. Araki, T. Sugimoto, M. Haneda, A. Kashiwagi, D. Koya, Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney, J. Clin. Invest. 120 (2010) 1043–1055, https://doi.org/10.1172/JCI41376.

[41] L. Zhuo, B. Fu, X. Bai, B. Zhang, L. Wu, J. Cui, S. Cui, R. Wei, X. Chen, G. Cai, NAD blocks high glucose induced mesangial hypertrophy via activation of the Sirtuins-AMPK-mTOR pathway, Cell. Physiol. Biochem. 27 (2011) 681–690, https://doi.org/10.1159/000330077.

[42] R. Miyazaki, T. Ichiki, T. Hashimoto, K. Inanaga, I. Imayama, J. Sadoshima, K. Sunagawa, SIRT1, a longevity gene, downregulates angiotensin II type 1 receptor expression in vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1263–1269, https://doi.org/10.1161/ATVBAHA.108.166991.