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Protective Role of Fisetin in STZ Induced Diabetic Nephropathy in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: Chronic diabetes mellitus associated with devastating complication the diabetic nephropathy, that further progress to ESRD,a major cause of morbidity and premature mortality in many countries worldwide. Accumulated evidences demonstrated that long standing hyperglycemia induced oxidative stress, inflammatory cytokines, and fibrosis plays a significant role in DN. Fisetin, a bioflavonoid, exhibited variety of promising pharmacological properties such as, anti-diabetic, antioxidant, anti-inflammatory, anti-hyperlipidemic ,and anti-carcinogenics. Hence, the present study was hypothesized to investigate, the effect of fisetin on streptozotocin-induced diabetic nephropathy in rats.
Materials and Methods: Sprague Dawley rats were divided into 6 groups (n=6) as normal control, diabetic control (vehicle), Glimepiride (0.5 mg/kg, orally) and Fisetin treatment (2.5, 5 and 10 mg/kg, orally) groups. After the confirmation of diabetes, vehicle/drug treatments were started and

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continued for 6 weeks. Serum glucose, body weight, were measured on weekly basis.Thereafter, on the last day of treatment protocol, ie 42 day, serum insulin, HbA1c in blood, lipid parameters, creatinine, albumin and urea in serum and in urine creatinine excretion, albumin were measured along with urine volume and creatinine clearance. In addition, weight of kidney and histopathological studies were carried out.

Results: Fisetin treatment significantly attenuated reduction in body weight. Also, it significantly decreased the blood glucose level, ameliorate lipid profile and HbA1c ($p<0.05$) value, but serum insulin level were not much influenced. It also increased albumin in serum, decreased serum urea and creatinine and in urine, it reduced the urine volume, albumin with marked improvement in creatinine excretion and creatinine clearance. Further, the fisetin (10mg/kg) treatment attenuated oxidative stress and cytokines TNF-α ($p<0.01$), IL-1β ($p<0.01$), and IL-6 ($p<0.05$) level in kidney tissue along with amelioration of histopathological alterations compared to diabetic control rats. The standard drug, glimepiride also exhibited similar antidiabetic effect without much influence on oxidative stress, albumin in urine, and cytokine levels.

Conclusions: The results indicated that fisetin ameliorated diabetic nephropathy through its antidiabetic and antioxidant effect which may be attributed to inhibition of downward pathway of glycemia induced oxidative stress, inflammation and necroptosis of renal tissue.

Keywords: Fisetin; nephropathy; insulin; oxidative stress; streptozotocin; inflammation.

1. INTRODUCTION

Diabetes mellitus (DM) is the most common chronic endocrine disorder characterized by persistently high blood glucose levels associated with disrupted metabolism of glucose, fats, and protein, due to inherited or acquired deficiency of insulin production in the pancreas. The prevalence of DM increases worldwide and the International Diabetic Federation (IDF) estimated that globally 700 million people will suffer from diabetes mellitus (DM) by the year 2045. [1]. Long-standing hyperglycemia results in the development of micro and macrovascular complications of DM viz. nephropathy, neuropathy, retinopathy, and cardiovascular disorders [2]. Among these, diabetic nephropathy is the serious pathological complication of DM. Approximately, 30% of the Type-I and 40% of Type-II diabetic patients with uncontrolled, chronic hyperglycemia, developed diabetic nephropathy [3] and currently, it became the most common cause of dialysis, kidney transplantation and the leading cause of end-stage renal disease (ESRD) worldwide [4]. DN is a condition of progressive renal damage, causes morphological and functional changes in the kidney, that characterized by the persistent rise in albuminuria, progressive decline in the glomerular filtration rate (GFR), recruitment of excessive extracellular matrix (ECM) component, mesangial matrix expansion, thickening of glomerular and tubular basement membranes along with glomerulosclerosis and tubulointerstitial fibrosis [3,5].

The current understanding of the pathological process of diabetic nephropathy recognizes the involvement of various hemodynamic, metabolic, and inflammatory factors such as hyperglycemia, activation of the renin-angiotensin system, oxidative stress and fibrosis [6] Among these, the metabolic factor like chronic hyperglycemia plays a crucial role in the glomerular damage via activation of the polyol, hexosamine pathway, protein kinase C (PKC), increased advanced glycation end products (AGEs) formation [7,8] promotes production of reactive oxygen species (ROS) and raise the oxidative stress [9].

The increased ROS production can trigger transcription factor nuclear factor- kappa B (NFκB), that further stimulates various pro-inflammatory cytokines viz. tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-1beta (IL-1β) and interleukin-6 (IL-6) that contributes pathological process of diabetic nephropathy [10,11]. In addition to this, pro-fibrotic cytokine TGF-β1 also involved in renal damage by promoting fibrosis of kidney [12].

All these factors leads to typical morphological and functional alterations in diabetic kidney. Initial structural alterations includes glomerular hypertrophy, mesangial expansion, followed by ECM recruitment, thickening of glomerular and tubular basement membrane that subsequently results in glomerulosclerosis, tubulointerstitial inflammation and fibrosis of the kidney. Functionally, the pre-mature alterations of diabetic kidney includes, glomerular hyperfiltration, followed by progressive

albuminuria, and in later stages it causes declined GFR, and that eventually results in ESRD [5,3].

From a therapeutic standpoint, currently, there is no particular treatment option for DN [13]. However, during early stages, its management can be done by control of hyperglycemia using insulin, oral antidiabetic drugs, and angiotensin-converting enzyme (ACE) inhibitors individually or in combination. [14] Nevertheless, these treatments offer imperfect protection against outset of DN. Further, dialysis or sometimes kidney transplant is needed in the advanced stage of DN [15,16]. Moreover, high cost and due to certain stern side effects of conventional drugs, their dosage may need to be adjusted in the case of kidney failure [17]. Therefore, the research is focused on safe and effective phytoconstituents, as an alternative or an adjunctive therapy in the management of kidney dysfunction in diabetics.

Polyphenols viz flavonoids and phenolic acid are the secondary metabolites commonly present in grains, fruits, coffee and tea [18]. Fisetin (3,3',4',7 tetrahydroxyflavone) is an important bioactive flavonol molecule present in various fruits (strawberries, persimmon and apples), vegetables (onion and tomatoes), wine and nuts [19]. Fisetin exhibited multiple pharmacological activities such as antihyperglycemic [20], antihyperlipidemic [21], anti-inflammatory [22], anti-oxidant [23], attenuated ischemia/reperfusion (I/R)-induced cardiac injury [24]. In addition, Fisetin showed potential to attenuate various diabetic complications such as diabetic neuropathy [25] and diabetic cardiomyopathy [26]. Furthermore, Fisetin reported to produce reno-protective effect against cisplatin-induced nephrotoxicity in rats [27]. Despite the potential of fisetin to attenuate DM, its co-morbid conditions and oxidative stress, no attempts were exercised to identify its influence on streptozotocin (STZ) induced diabetic nephropathy in rats. Therefore, the current study has been designed to evaluate the effect of fisetin in amelioration and prevention of DN progression in STZ-induced diabetic nephropathy in rats.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Streptozotocin (STZ), Thiobarbituric acid were obtained from Sigma Aldrich, USA. Fisetin was purchased from Pro Lab. Marketing Pvt. Ltd., New Delhi, India. Glimepiride was procured as a gift sample from Sun Pharma Advance Research Centre, Vadodara, India. Biochemistry reagent kits for evaluation of glucose, kidney function tests, lipids profile etc. were purchased from Transasia Bio-Medicals, Mumbai. ELISA kits used to measure the amount of insulin in serum and cytokines (TNF-α, IL-1β, and IL-6) in kidney tissue homogenate, were purchased from Cusabio and Crystalchem. Kits were used as per the instructions given by the manufacturer. All other chemicals used in the experiment were of analytical grade.

2.2 Experimental Animals

Male Sprague Dawley rats weighing 200-250g of were purchased from National institute of Pharmaceutical education and research NIPER, Mohali Animals were housed in propylene cages and were maintained in a controlled environmental conditions of 12 12 hr light/dark cycle temperature 30±2°C and relative humidity 50±55% They were fed with commercial pellet diet and water *ad libitum* .

2.3 Induction of Diabetes

Diabetes was induced in overnight fasted rats by single dose of STZ 55mg/kg i.p., [28]. prepared in chilled citrate buffer solution of 0.1M concentration and pH 4.4. After 6 hrs of STZ administration, rats were supplied with 10% glucose solution for next 24hrs to prevent mortality in animals due to hypoglycemic shock that caused enormous insulin release from degenerating pancrease. Thereafter 3 days of STZ injection diabetes was confirmed by estimation of blood glucose by glucometer. Diabetic rats having >250mg/dl fasting blood glucose level were selected to conduct experiments.

2.4 Grouping and Treatments

Animals were divided into 6 groups, (n=6) as follows:

Group I: Rats of normal control group, received vehicle DMSO 10% orally for 42 days
Group II: Rats of diabetic control group, received vehicle DMSO 10% orally for 42 days
Group III: Diabetic animals received glimepiride (0.5 mg/kg, p.o.) orally for 42 days
Group IV: Diabetic animals received Fisetin (2.5 mg/kg/day) dissolved in 10% DMSO, orally for 42 days

Group V: Diabetic animals received Fisetin (5 mg/kg/day) dissolved in 10% DMSO, orally for 42 days

Group VI: Diabetic animals received Fisetin (10 mg/Kg/day) dissolve in 10% DMSO, orally for 42 days

After the induction of hyperglycemia, the vehicle or drug treatments were given to rats by oral gavage, once daily for 6 weeks. The doses of fisetin were chosen based on those used in previous studies [20]. The change in blood glucose and physiological parameters such as relative body weight, were monitored at regular time interval. After the completion of 42 days of protocol, rats were kept for overnight fasting, and then were deeply anaesthetized and sacrificed by method of cervical dislocation, for the collection of blood samples and kidneys to carry out biochemical and histopathological studies, respectively.

2.5 Physiological Parameters

Body weights of all the rats were recorded individually before the treatment and at weekly intervals thereafter.

2.6 Serum and Urine Parameters

Blood sample was obtained from animals, fasted for 10 h by puncturing retro orbital sinus [29] on 3rd day, after STZ injection (considered as day 1) of the experiment and afterwards on 7, 14, 21, 28, 35, and 42 day. The blood sample was allowed to centrifuged at 3000 rpm for 10 min to get a clear serum for carrying out different biochemical estimations. Levels of serum glucose were determined weekly using Glucose Oxidase-Peroxidase (GOD-POD) method to confirm the persistent hyperglycemia while lipids viz. total cholesterol, triglycerides, high density lipoprotein cholesterol, kidney function parameters such as albumin, urea, and creatinine were determined in serum on day 42 i.e. the last day of present study. Glycated haemoglobin (HbA1c) level were estimated in blood sample by turbidimetric analysis using spectrophotometer on last day of study.

The 24 hr urine samples of animals were collected using metabolic cages (Orchid Scientific and Scientific Innovations Pvt. Ltd, Nasik, India). Thereafter, urine total volume was measured, filtered, albumin and creatinine were estimated. Creatinine clearance (CCr) was calculated by the following formula and expressed as ml/min/kg body weight [30].

$$\text{CCr} = \frac{\text{Urinary Creatinine (mg/dl) x Urine volume (ml)}}{\text{Serum Creatinine (mg/dl)}} \times \frac{[1000]}{[\text{Body weight (g)}]} \times \frac{[1]}{[1440\ (\text{min})]}$$

All biochemical test were carried on ERBA Chem Touch Analyzer using biochemical kits (Transasia Bio-medicalsPvt. Ltd., Mumbai). The serum insulin levels were evaluated using ELISA kit following manufacturer's instructions.

2.7 Renal Hypertrophy (Kidney Weight) and Organ Collection

On the last day of 6 weeks study, i.e. on day 42, rats from each group were sacrificed employing carbon dioxide, both the kidneys were removed and rinsed using phosphate-buffered saline (PBS). Afterward, kidneys were weighed and then kidney to body weight ratio was calculated as a measure of renal hypertrophy [31]. One kidney of each animal was fixed immediately in normal 10% neutral buffered formalin (NBF) for histopathological study and another kidney was kept to prepare its homogenate, for various biochemical estimations.

2.8 Measurement of Antioxidant and Other Biochemical Parameters in Kidney Tissues

To prepare kidney homogenate, samples of kidney were homogenized with 10% isotonic phosphate-buffered saline (PBS) solution (0.1 M, pH 7.4). Thereafter, homogenate was centrifuged at 16000xg to obtain clear supernatant for evaluating biomarkers markers of oxidative burden and other inflammatory parameters. Oxidative stress markers such as lipid peroxidation (LPO) [32] superoxide dismutase (SOD) [33] catalase (CAT) [34] and reduced glutathione (GSH) [35] were determined as per previously published methods. The content of protein in kidney tissue was estimated by the Biuret method [36].

The amount of inflammatory cytokines in tissue such as TNF-α, IL-1β and IL-6 were estimated employing highly sensitive ELISA kits following manufacturer's instructions.

2.9 Histopathology of kidney

Kidney samples stored in formalin were dehydrated with series of graded alcohol and

impregnated into paraffin blocks then they were subjected to section cutting of around 5 µm in thickness using a rotary microtome. The renal sections were stained with tissue stain hematoxylin and eosin and periodic acid schiff. Thereafter, slides were examined under the microscope at magnification of 400x to note any pathological abnormalities.

2.10 Statistical Analysis

One-way analysis of variance (ANOVA) was used to statistically analyze the data by using the software Graph Pad Prism ver. 5.0 followed by Bonferroni post hoc test. All values were expressed in mean ± standard error mean; where n=6. Data having the value of $p<0.05$ were considered significant statistically in all cases.

3. RESULTS

3.1 Effect of Fisetin Treatment on Body Weight

Results analyzed with a one-way ANOVA suggested that Fisetin treatment notably influenced the body weight in diabetic control rats (Table 1). Further, the Bonferroni test indicated that single-dose administration of STZ i.p. results in a significant ($p<0.001$) decline in the bodyweight of diabetic animals as compared to animals of the normal control group and Fisetin treatment at a dose of 10 mg/kg notably attenuated the reduction ($p<0.01$) in the bodyweight of animals. Fisetin at moderate 5mg/kg and lower dose 2.5 mg/kg, did not cause any notable ($p>0.05$) influence on the decline in the body weight of animals as compared to the diabetic animals of the control group. The standard drug Glimepiride also showed a comparable effect as that of Fisetin 10 mg/kg.

3.2 Effect of Fisetin Treatment on Hyperglycemia

One-way ANOVA revealed, Fisetin treatment significant affect the hyperglycemia in STZ-induced diabetic rats (Table 2). The Bonferroni post hoc test showed that STZ i.p. administration produce significant hyperglycemia on day 3 which remain consistently elevated throughout 6 weeks experiment duration in rats of diabetic control group than rats of a normal control group. Administration of fisetin at 10mg/kg and 5 mg/kg to diabetic rats from day 21 to day 42 significantly $p<0.001$ and $p<0.01$ respectively, decrease the glucose level in blood when compared to the group of diabetic control rats. However, Fisetin at low dose (2.5 mg/kg), did not show any significant ($p>0.05$) effect on blood glucose levels of diabetic rats. Treatment with standard drug Glimepiride, to diabetic animals from day 14 to 42 of present study, also notably ($p<0.001$) decreases the level of blood glucose (Table2).

3.3 Effect of Fisetin Treatment on Lipid Parameters

One-way ANOVA followed by Bonferroni test shown that Fisetin treatment significantly affect the lipid levels in serum (Table 3). Diabetic control rats exhibited increased total cholesterol (TC) ($p<0.001$), triglycerides (TG) ($p<0.001$) and decined HDL-c ($p<0.001$) level in serum as compared to animals of normal control group. Fisetin treatment at (10mg/kg) dose cause significant decline in TC($p<0.001$), TG ($p<0.01$) and increased HDL-c ($p<0.05$) levels. Fisetin at (5mg/kg) decrease the TC ($p<0.01$), TG ($p<0.05$) but not affect the HDL-c ($p>0.05$) levels as compared to untreated diabetic rats. However, Fisetin at (2.5mg/kg) did not show any significant on lipid parameters. Glimepiride, the standard drug, shown more marked effect than fisetin.

3.4 Effect of Fisetin Treatment on HbA1c and Serum Insulin

One-way ANOVA depicts the significant effect of Fisetin treatment on glycosylated hemoglobin (HbA1c) and serum insulin.(Table 3). Untreated diabetic rats showed significantly ($p<0.001$) elevated in HbA1c value and decline in levels of serum insulin on last day of study (42^{nd} day) when compared to rats of normal group. Fisetin(10mg/kg) oral administration for 42 days to diabetic rats exhibited significant ($p<0.05$) decrease in HbA1c value but did not show any notable ($p>0.05$) effect on serum insulin when compared to untreated diabetic animals. Standard antidiabetic drug - glimepiride also caused ($p<0.01$) decrease in HbA1c value without any significant ($p>0.05$) effect on the insulin levels as compared to diabetic control animals. Fisetin in lower dose (5mg/kg and 2.5mg/kg) did not show any significant change in HbA1c value and serum insulin as compared to diabetic control rats.

Table 1. Effect of Fisetin on body weight of STZ -induced hyperglycaemic rats

Treatments	Body Weight (g)						
	Day1	Day7	Day 14	Day 21	Day 28	Day 35	Day 42
Normal control	226.8±2.15	231.3±3.20	237.8±3.33	244.5±3.86	251.1±3.91	256.3±3.37	260.4±3.83
Diabetic Control	234.4±3.06	227.4±3.99	215.5±4.95#	202.1±4.73*	191.1±4.35*	179.7±4.09*	171.4±4.96*
Glimepiride 0.5	233.3±3.63	228.6±3.75	222.4±4.26	215.5±3.65	209.5±3.42†	202.5±3.55	197.2±3.94
Fisetin 2.5	227.6±4.26	220.0±5.64	211.4 ±4.36	203.7±4.66	196.0±3.78	187.3±3.73	176.8±3.79
Fisetin 5	229.3±2.63	223.7±3.90	215.8±4.27	208.2±3.28	202.3±2.92	194.2±3.77	187.5±3.90
Fisetin 10	231.7±4.39	225.8±3.81	218.1±4.08	213.4±4.09	206.9±3.90	199.6±4.21†	193.9±4.29

*Values are mean±SEM (n=6), Doses are expressed in mg/kg, #P<0.05 and *P<0.001 when compared to normal control, †P<0.05, P<0.01 when compared to diabetic control*

Table 2. Anti-hyperglycemic effect of Fisetin in STZ-induced diabetic rats

Treatments	Fasting Serum Glucose (mg/dl)						
	Day1	Day7	Day 14	Day 21	Day 28	Day 35	Day 42
Normal control	95.18±7.55	107.5±8.73	111.6±10.91	109.8±9.99	113.4±10.37	106.6±10.12	97.45±9.83
Diabetic Control	334.9±11.20*	338.1±10.77*	341.3±12.61*	346.7±14.89*	348.9±13.10*	347.8±13.46*	349.3±14.57*
Glimepiride 0.5	352.4±8.49	330.8±9.78	288.4±10.58†	252.2±10.01§	218.3±11.28§	188.1±12.58§	153.8±12.69§
Fisetin 2.5	359.2±10.80	354.2±10.41	343.5 ±9.91	331.4±10.29	322.6±9.61	316.7±11.55	307.5±9.67
Fisetin 5	355.4±9.67	343.6±11.12	327.4±8.99	309.9±12.66	294.2±12.74†	282.8±14.14†	267.8±13.82
Fisetin 10	349.6±11.44	331.4±12.74	297.4±11.43	266.9±14.50	224.6±10.90§	195.7±11.88§	162.1±13.34§

*Values are mean±SEM (n=6), Doses are expressed in mg/kg, *P<0.001 when compared to normal control; †P<0.05, P<0.01, §P<0.001 when compared to diabetic control*

Table 3. Effect of Fisetin on altered HbA1c, insulin and lipid profile in STZ- induced diabetic rats

Groups	Lipid Profile			HbA1c (%)	Insulin (ng/ml)
	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-c (mg/dl)		
Normal control	69.04±3.30	79.73±3.73	35.21±1.96	4.35±0.25	2.86±0.17
Diabetic control	127.8±4.48*	122.4±4.15*	20.74±2.55*	9.68±0.52*	1.05±0.23*
Glimepiride 0.5	90.52±3.99§	91.85±2.80§	31.84±2.45†	6.25±0.63	1.93±0.19
Fisetin 2.5	119.5±4.20	115.5±3.08	24.53±2.29	8.83±0.51	1.35±0.21
Fisetin 5	106.0±2.44	106.2±3.79†	27.94±1.81	7.45±0.54	1.72±0.19
Fisetin 10	98.37±3.53§	99.47±3.43	30.98±1.63†	6.78±0.73†	1.89±0.14

*Values are mean ± SEM (n=6), Doses are expressed in mg/kg, *P<0.001 when compared to normal control, †P<0.05, P<0.01, §P<0.001 when compared to diabetic control*

3.5 Effect of Fisetin Treatment on kidney Function Parameters

One-way ANOVA indicated that treatment with fisetin significantly affect the various parameters of kidney function (Table 4). In untreated diabetic rats, there is significant ($p<0.001$) rise in serum creatinine, urea, urine volume and urinary albumin and decrease in serum albumin alongwith urinary creatinine excretion and creatinine clearance. Treatment of diabetic rats with Fisetin (10 and 5 mg/kg, p.o.) significantly decline the level of serum creatinine, urea, urine volume, urinary albumin, raised level of albumin in serum, urinary creatinine excretion and creatinine clearance when compared to untreated diabetic rats. However, Fisetin at low dose (2.5 mg/kg), did not show any significant ($p>0.05$) effect on kidney function parameters. Further, standard drug Glimepiride cause decline in serum creatinine, urea and volume of urine but did not show much effect on albumin in serum and urine, creatinine clearance and excretion of creatinine in urine when compared to untreated diabetic rats.

3.6 Effect of Fisetin Treatment on Renal Weight and Renal Hypertrophy

One-way ANOVA indicated that treatment with fisetin significantly influenced the renal weight of animals (Table 5). Diabetic control rats showed significant increase in renal weight to body weight ratio indicating increased renal hypertrophy index. Fisetin (10mg/kg) treatment, orally for 6 weeks notably attenuated increase in renal weight and renal hypertrophy index as compared to untreated diabetic rats. However, Fisetin at (5, 2.5 mg/kg) dose and glimepiride, did not cause similar attenuation.

3.7 Effect of Fisetin Treatment on Oxidative Stress Parameters in Renal Tissue

One-way ANOVA indicated that treatment with Fisetin significantly influenced the parameters of oxidative in renal tissue (Table 6). Untreated diabetic rats showed significantly increased levels of malondialdehyde (MDA), decline in superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) content in renal tissue. Treatment of diabetic rats with Fisetin (10 and 5 mg/kg), orally for 6 weeks notably attenuated the rise in malondialdehyde levels and raised the activities of CAT, SOD and GSH content when compared to untreated diabetic rats. Fisetin at dose of 2.5 mg/kg and glimepiride, fail to show comparable attenuation in parameters of oxidative stress.

3.8 Effect of Fisetin Treatment on Inflammatory Cytokines in Renal Tissue

One-way ANOVA showed Fisetin treatment significantly influenced inflammatory cytokines in renal tissues (Table 7). Untreated diabetic rats exhibited notably rise in the levels of inflammatory cytokines such as IL-6, IL-1β and TNF-α, in renal tissue when compared to normal rats. Treatment with Fisetin (5 and 2.5 mg/kg, orally) for 6 weeks results in notable decline in level of IL-1β and TNF-α and Fisetin treatment (10mg/kg) orally for 6 weeks results in more notable reduction in every inflammatory cytokines parameters when compared to untreated diabetic rats. However, glimepiride treated group did not show similar significant reduction when compared to untreated diabetic animals, except in TNF-α .

3.9 Effect of Fisetin Treatment on Renal Histopatholgical Changes

The characteristic histopathological images are represented in Figs 1 and 2. H&E and PAS staining employed to assess the morphological changes of renal tissues at 400X magnification. The renal microscopic sections of rats of the normal control group exhibited normal architecture of glomerulus, Bowman's capsule, mesangium, tubules, and mesangial cellularity without any pathological changes noticed (Fig 1a). Conversely, H and E stained renal sections from animals of the diabetic control group revealed glomerulus shrinkage, severe glomerulosclerosis, and expanded Bowman's space (Fig 1b). Also, Fistein (2.5 mg/kg) treated rats shown almost similar pathology as that of the diabetic control group viz. glomerulosclerosis and increased glomerular space (Fig 1d). Besides, the kidney sections of glimepiride and Fisetin (5 mg/kg) treated rats showed mild improvement with restored glomerular space, an increase in mesangial matrix and mild degeneration of capillaries network in the glomerulus (Fig 1c, 1e) whereas Fisetin 10mg/kg treated renal sections improved greatly and showed decreased mesangial expansion and glomeruli hypertrophy (Fig 1f).

Table 4. Effects of Fisetin on kidney functions parameters

Groups	Serum Creatinine (mg/dl)	Serum Urea (mg/dl)	Serum Albumin (mg/dl)	Urine			Creatinine clearance
				Urine output (ml/24 hr)	Creatinine (mg/dl)	Albumin (gm/24hr)	(ml/min/kg bw)
Normal control	0.60±0.09	24.43±1.75	4.58±0.18	11.02±1.67	56.72±2.94	0.05±0.02	2.76±0.08
Diabetic control	1.65±0.16*	48.29±1.88*	3.12±0.22*	38.08±4.10*	19.32±1.7*	0.33±0.03*	1.79±0.15*
Glimepiride 0.5	0.97±0.11†	40.31±1.61†	3.52±0.19	24.33±2.14†	25.63±2.98	0.20±0.04	2.24±0.11
Fisetin 2.5	1.13±0.14	42.98±1.48	3.46±0.14	29.7±2.98	21.5±2.23	0.22±0.03	2.20±0.10
Fisetin 5	0.93±0.16†	39.75±1.54†	3.84±0.20	21.78±4.12¶	28.17±3.52	0.17±0.02†	2.42±0.14†
Fisetin 10	0.78±0.08¶	36.98±1.58§	4.12±0.17†	17.07±2.12§	33.05±2.85†	0.15±0.02¶	2.57±0.11¶

*Values are mean±SEM (n=6), Doses are expressed in mg/kg, *P<0.001 when compared to normal control; †P<0.05, P<0.01, §P<0.001 when compared to diabetic control*

Table 5. Effects of Fisetin on altered kidney weight and kidney hypertrophy in STZ induced diabetic rats

Groups	Body weight on 42nd day (g) (A)	Kidney weight (B) (g)	Kidney hypertrophy Index (%) [(B÷A)x100]
Normal control	260.4±3.83	1.78±0.06	0.68±0.03
Diabetic control	171.4±4.96*	2.35±0.12‡	1.37±0.04*
Glimepiride 0.5	197.2±3.94¶	2.12±0.08	1.07±0.04†
Fisetin 2.5	176.8±3.79	2.20±0.06	1.25±0.09
Fisetin 5	187.5±3.90	2.14±0.09	1.14±0.05
Fisetin 10	193.9±4.29¶	1.93±0.07†	0.99±0.06¶

*Values are mean±SEM (n=6), Doses are expressed in mg/kg, ‡P<0.01, *P<0.001 when compared to normal control; †P<0.05 and P<0.01, when compared to diabetic control*

Table 6. Effects of Fisetin on oxidative stress parameters in kidney tissue

Groups	MDA (n moles/mg protein)	SOD (U/mg protein)	CAT (µ moles of H_2O_2 consumed/min/ mg protein)	GSH µmol mg protein^{-1}
Normal control	1.56±0.07	20.04±1.35	33.1±2.14	4.94±0.27
Diabetic control	2.93±0.29*	10.43±1.59‡	18.99±2.25‡	1.88±0.32*
Glimepiride 0.5	1.92±0.18†	14.73±1.57	26.65±2.00	3.39±0.32†
Fisetin 2.5	2.11±0.21	13.6±1.38	24.51±2.38	2.89±0.31
Fisetin 5	1.89±0.20†	16.24±1.55	27.32±1.83†	3.47±0.35†
Fisetin 10	1.77±0.17¶	17.14±1.43†	28.91±2.31†	3.82±0.30§

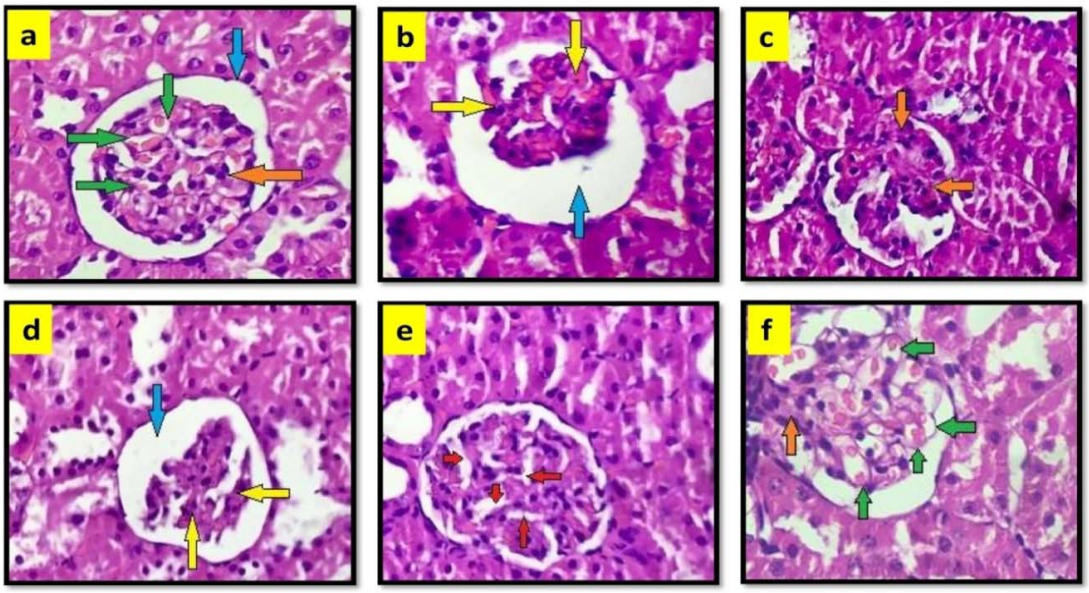
*Values are mean±SEM (n=6), Doses are expressed in mg/kg,*P<0.01,*P<0.001 when compared to normal control; †P<0.05, P<0.01, §P<0.001, when compared to diabetic control.*

Table 7. Effect of Fisetin on inflammatory cytokines in kidney tissue

Groups	TNF-α	IL-6	1L-1β
	pg/mg protein	pg/mg Protein	pg/mg protein
Normal control	97.41±7.58	7.16±0.51	65.5±6.45
Diabetic control	183.1±8.19*	14.64±2.36‡	138.8±8.17*
Glimepiride 0.5	152.7±6.02†	10.47±0.70	108.5±7.76
Fisetin 2.5	164.6±6.35†	12.23±0.81	117.7±5.88
Fisetin 5	151±5.76†	10.16±0.74	104.2±7.29†
Fisetin 10	142.9±6.10¶	9.18±1.06†	94.83±6.56¶

*Values are mean±SEM (n=6), Doses are expressed in mg/kg, ‡P<0.01, *P<0.001 when compared to normal control; †P<0.05, P<0.01 when compared to diabetic control*

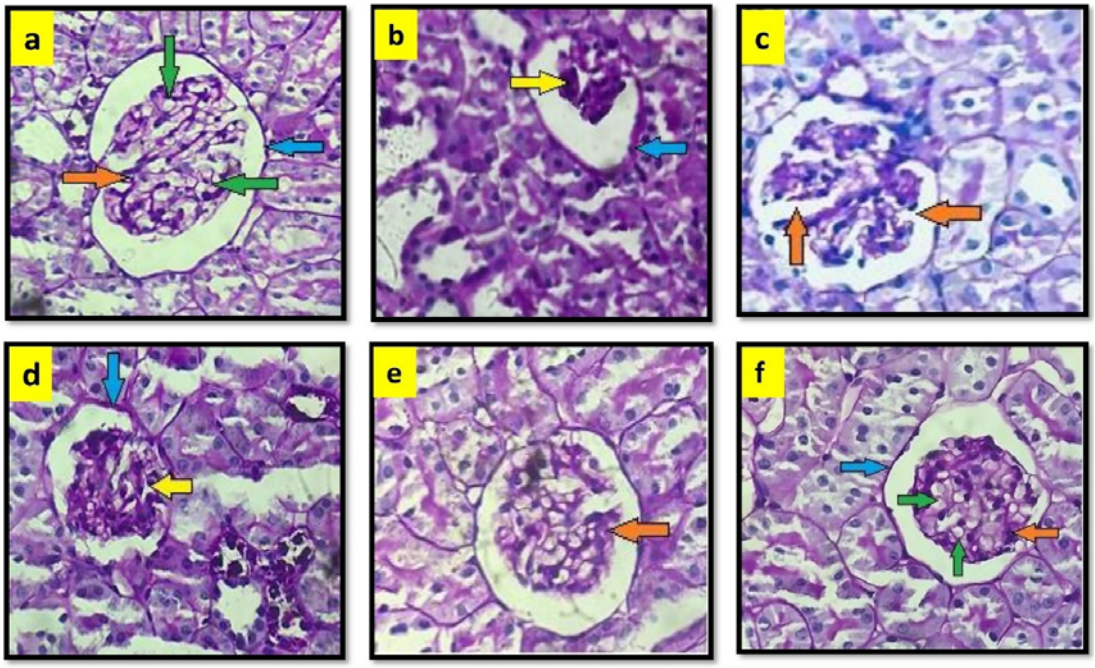
Fig. 1. Effect of Fisetin on histopathological alterations in H &E stained kidney tissue sections(X400)



a Normal control: showed normal structure of glomerular capillaries (green arrows), mesangial cells (orange arrow), glomerular capsule (blue arrow). b STZ, Diabetic control: showed glomerulosclerosis (yellow arrows) and wide Bowman's space (blue arrow).c STZ+ Glimepiride (0.5mg/kg): showed moderate mesangial matrix expansion (orange arrows). d. STZ+Fisetin(2.5 mg/kg): showeddamaged glomerular capillary network (yellow arrows),increased capsular space (blue arrow).eSTZ+Fisetin(5 mg/kg): showed mild degeneration of glomerular capillary network. f. STZ+ Fisetin(10mg/kg): showednormal glomerular capillaries (green arrows) and mild mesangial expansion (orange arrow)

Kidney sections of normal control group rats, stained with PAS showed the thin and distinct glomerular capillaries basement membrane and tubules, normal mesangial cellularity, mesangial region and Bowman's space (Fig 2a). However, the renal sections from diabetic control group rats revealed diffused glomerulosclerosis and significantly thickening glomerular capillary basement membrane (Fig 2b). Further, Fistein (2.5 mg/kg) treated rats shown nearly alike pathology as that of the diabetic control group viz. glomerulosclerosis and thickened glomerular capillary basement membrane (Fig 2d). Kidney sections treated with standard drug glimepiride and fisetin (5 mg/kg) showed focal segmental glomerulosclerosis, mesangial cellularity and increased accumulation of mesangial matrix which was less when compared to diabetic control rats (Fig 2c & e), while Fisetin (10 mg/kg) treated rats revealed almost normal architecture of glomerular basement membrane, mild mesangial expansion and lesser accumulation of mesangial matrix when compared to rats of diabetic control group (Fig 2f).

Fig. 2. Effect of Fisetin on histopathological alterations in PAS stained kidney tissue sections (X400)



a Normal control: showed normal glomerular capillaries (green arrows), mesangial region (orange arrow)and glomerular basement membrane (blue arrow). b STZ, Diabetic control: showed glomerulosclerosis (yellow arrow), thick glomerular basement membrane (blue arrow). c STZ+ Glimepiride (0.5mg/kg): showed focal glomerulosclerosis (orange arrows). d STZ+ Fisetin (2.5 mg/kg) : showeddamaged glomerular capillary network (yellow arrow),thick glomerular basement membrane (blue arrow).e STZ+ Fisetin (50 mg/kg) : showed focal glomerulosclerosis (orange arrows). fSTZ+ Fisetin (100mg/kg) : showed normal architecture of glomerular capillaries (green arrows), thin glomerular basement membrane (blue arrow) and slight mesangial expansion (orange arrow)

4. DISCUSSION

The current study results revealed, that daily oral administration of Fisetin prevented the deterioration of renal functioning in STZ induced diabetic nephropathy in rats. This could be due to the effect of Fisetin to ameliorate hyperglycemia, reduced the levels of oxidative burden and inflammation. Experimental rats injected with a single dose of STZ (55 mg/kg) i.p., shown to cause a significant rise in their blood glucose level after 72 hrs. Also, it causes a notably elevated weekly blood glucose, glycated hemoglobin values, and decreased level of insulin when compared to the animals of the normal control group. The increased blood glucose also leads to altered serum lipid levels as observed by the rise in total cholesterol (TC), triglycerides (TG), and decline in HDL-cholesterol level in serum. The results obtained are similar to the previous findings that suggest the progression of diabetes mellitus [37]. The diabetic condition of animals was also manifest from the weekly decline in total body weight, due to increased tissue proteins loss and muscle devitalization [38]. Fisetin treatment ameliorated the diabetic condition of animals as shown by a significant attenuation of high blood glucose and lipid level alterations when compared to the diabetic rats of the STZ control group, however, the decline in total body weight remained unaffected. The treatment with fisetin also attenuated the elevated glycated hemoglobin values in the blood that indicates the blood glucose level is moderately controlled without any notable improvement in the level of serum insulin. This indicated that fisetin does not affect insulin secretion may be due to increased degeneration of insulin-secreting pancreatic beta cells by streptozotocin.

Further, results of present experiments revealed that long-term uncontrolled hyperglycemia leads to impaired kidney function as shown by

increased urea and creatinine level, decline in albumin level in serum along with a notable rise in urinary albumin, urine volume, and decrease in creatinine excretion, and CrCl. Microalbuminuria is an important prognostic marker for nephropathy development due to diabetes. Long-term hyperglycemia in diabetes activates a serious microvascular complication DN [39] that supports the findings.

DN affected the different segments of nephrons in the kidney. In glomeruli, increased extracellular matrix (ECM) deposition promotes basement membrane thickening and mesangial expansion [5]. Renal tubular hypertrophy is the earliest structural change in DN. Further, the kidney's structural changes subsequently result in loss of kidney function. Functionally during the early phase of DN, there is elevated urinary albumin excretion with increased glomerular filtration rate (GFR) and in later stages, there is a rise in proteinuria along with diminished glomerular filtration rate. These conditions are similar to the findings observed in the experimentally controlled diabetic animals.

Progression of DN stimulates interstitial fibrosis and tubular atrophy of nephrons [40]. This can be observed from the increased relative renal weight of untreated diabetic animals of the control group. Fisetin treatment attenuated the rise in urea, Cr in serum and lowers the urinary albumin excretion and urine volume of rats. Also, fisetin is shown to ameliorate the serum albumin level, urinary creatinine clearance, and creatinine excretion. These results indicates the renoprotective effect of fisetin that was additionally supported by reduced weight of kidney and hypertrophy index owing to fisetin treatment and confirms the anti-hypertrophic effect.

At later stages of diabetes, the long-term hyperglycemia incites the advanced glycosylation end products and reactive oxygen species generation, a major mechanism implicated in the development of DN and other complications of diabetes [41]. In agreement with this, the present study showed a rise in MDA, the decline in SOD, CAT level, and reduced glutathione in the kidney tissues of diabetic control animals, which further confirmed, high blood glucose stimulates renal oxidative stress. Fisetin (10 mg/kg) treatment shown to ameliorate the oxidative burden. Fisetin, exhibited antioxidant activity [42-43] and might have averted the damage due to oxidative stress. However, Fisetin at a dose of (2.5 mg/kg), (5 mg/kg) and standard drug glimepiride did not exhibit any significant effect on the markers of oxidative stress.

Persistent oxidative stress due to long-term hyperglycemia, activates the ubiquitous transcription factor, NF-kB. This further triggers the increased expression of several pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β that leads to inflammation, apoptosis, and immunological response subsequently [44]. Excessive production of these mediators promotes renal inflammatory changes. NF-kB plays a crucial role in the pathophysiology of various inflammatory diseases including DM and its vascular complications such as DN [45-47]. During the earliest stages of DN, TNF-α involved in hyperfiltration and hypertrophy of kidney and in later stages it leads to renal cell apoptosis and necrosis [48]. Furthermore, IL-6 triggers excessive ECM accumulation in podocytes and mesangial cells of the nephron causing mesangial expansion, thickened basement membrane of the glomerulus that further leads to structural abnormality of the glomerulus. Increased expression of IL-6 is also associated with albuminuria [49]. Apart from this, Interleukin-1 (IL-1) is another master cytokine that contributes to local and systemic inflammation. It further comprises two major proinflammatory cytokines namely interleukin-1α (IL-1α) and interleukin-1β (IL-1β) [50]. Hyperglycemia induced intracellular ROS generation incites the inflammasome [51] of immune cells to release IL-1β and IL-1α and these further binds to IL-1 receptor on a renal cell and culminate in the amplified release of chemokine and cytokine [52-53]. Moreover, IL-1β contributes pathogenesis of diabetes [54] and its complications in diabetic nephropathy [55]. Results of present experiments revealed, the notably increased level of pro-inflammatory cytokines in renal tissue such as TNF-α, IL-6 and IL-1β, signifies that long-term high glucose level leads to inflammation-related kidney changes. Fisetin treatment attenuated the rise in inflammatory cytokines level that subsequently alleviate the necroptotic degeneration of the kidney. Inflammatory cell aggregation in kidney cells promotes DN [56] and inhibiting the deposition of these cells shown to have a protective effect in DN, hence supports the results of the present study.

Previously, flavonols such as quercetin and myricetin revealed renoprotective effect by identical mechanisms that further supports the observed renoprotective effect [57-58].

Moreover, the standard oral hypoglycemic drug glimepiride, also presented such amelioration which might be possible due to control of high blood glucose and succeeding damage.

Further, histopathological studies of (H & E) stained kidney sections of controlled diabetic animals revealed severe glomerulosclerosis and increased glomerular space. Also, PAS stained sections displayed thickening of the basement membrane of glomerular capillaries, mesangium expansion, and increased mesangial cellularity, which surely indicated the progression of renal damage. Fisetin treatment to animals significantly attenuated the renal histopathological alterations suggesting its protective effect on high glucose-induced kidney damage.

These findings indicated that fisetin treatment ameliorated the dysfunction of the kidney and attenuated the DN development in animals. It is hard to explicate the precise mechanisms involved in fisetin induced nephroprotective action. It might be possible that fisetin through its antihyperglycemic action or its antioxidant effect attenuates the prolonged hyperglycemia-induced oxidative burden that could potentially inhibit various pathways viz polyol hexosamine and decrease the generation of ROS. This further might arrest NF-κB activation and hinder inflammation through decreasing inflammatory cytokines formation and subsequently decrease the fibrosis that could further protect against renal damage. Thus the renoprotective effect of fisetin may be due to its antihyperglycemic action and antioxidant effect. However to establish the molecular mechanism/exact pathway involved in protective effect in DN further research based on gene and protein expression are essential to accomplish.

Fisetin can be used clinically for renoprotective effect by concurrently prescribing as herbal drugs or dietary supplements, along with standard antidiabetic medication, right from diagnosis of prediabetes or diabetic disorder. This will further halt the progression of DM and the development of DN in patients with diabetes.

5. CONCLUSION

Fisetin showed antihyperglycemic effect, improved dysfunctions of kidney, microalbuminuria and renal oxidative damage in diabetic rats. Hence, it could be concluded that Fisetin presents renoprotective effect in diabetic animals through its antihyperglycemic, antioxidant and anti-inflammatory effect which may be attributed to inhibition of downward pathway of glycemia induced oxidative stress, inflammation and necroptosis of kidney tissue.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the we were not funded by the producing company rather it was funded by personal efforts.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experiments were planned and performed following guidelines of Committee for the purpose of control and supervision of experiments on animals CPCSEA Government of India. The experimental protocol [IAEC-CTIPS/2019/XI/0070 (PCL-D)] was approved by Institutional Animal Ethical Committee IAEC of CT institute of Pharmaceutical Sciences, Punjab, India (Reg No 1704/PO/Re/S/13/CPCSEA).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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