The experimental protocol used in the present study was approved by the Institutional Animal Ethical Committee. Age matched young wistar rats weighing about 200-240 g were employed in the present study. Rats were fed on standard chow diet and water *ad libitum*. They were acclimatized in institutional animal house and were exposed to normal cycles of day and night.

**Assessment of diabetes and lipid profile**

The experimental diabetes mellitus was induced in rats by single injection of streptozotocin (STZ) (55 mg/kg *i.p.*,.) dissolved in freshly prepared ice cold citrate buffer of pH 4.5. The blood sugar level was monitored once daily for first week after administration of STZ. Then, at the end of the experimental protocol (8 weeks after administration of STZ), the blood samples were collected and serum was separated. The serum samples were frozen until analyzing the biochemical parameters. The serum glucose concentration was estimated by glucose oxidase peroxidase (GOD-POD) method (Trinder *et al.*, 1969) using the commercially available kit (Transasia Bio-Medical Ltd., Solan, India). The serum total cholesterol was estimated by cholesterol oxidase peroxidase (CHOD/PAP) method (Allain *et al.*, 1974) using the commercially available kit (Transasia Bio-Medical Ltd., Solan, India). The serum triglyceride was estimated by glycerol phosphate oxidase (GPO/PAP) method (Bucolo *et al.*, 1973) using the commercially available kit (Transasia Bio-Medical Ltd., Solan, India). The serum high density lipoprotein (HDL) was estimated by polyethylene glycol (PEG) precipitation method (Allain *et al.*, 1974) using the commercially available kit (Crest Biosystems, Goa, India).
Assessment of diabetic nephropathy

The diabetes mellitus-induced nephropathy was assessed biochemically by estimating serum creatinine, blood urea nitrogen and proteinuria.

*Estimation of serum creatinine*

The serum creatinine concentration was estimated by alkaline picrate method (Bonsnesand Taussky, 1945) using the commercially available kit (Crescent biosystems, Goa, India). Briefly, 2.0 ml of picric acid reagent was added to 0.2 ml of serum for deproteinization of specimen, which was mixed well and centrifuged at 3000 rpm to obtain a clear supernatant. 100 µl of buffer reagent was added to 1.1 ml of supernatant, 0.1 ml of standard creatinine and 0.1 ml of distilled water to prepare test, standard and blank, respectively. 1.0 ml of picric acid reagent was added to blank and standard. The test tubes were mixed well and kept at room temperature for 20 minutes. The alkaline picrate reacts with creatinine to form the orange coloured complex, which was read at 520 nm spectrophotometrically. The serum creatinine concentration was calculated using the following formula:

\[
\text{The serum creatinine concentration (mg/dl) = } \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 2
\]

*Estimation of blood urea nitrogen*

The blood urea was estimated by Berthelot method (Fawcett and Scott, 1960) using the commercially available kit (Kamineni Life Sciences Pvt. Ltd. Hyderabad, India). 1000 µl of working reagent-I containing urease reagent, and a mixture of salicylate, hypochlorite and nitroprusside was added to 10 µl of serum, 10 µl of
standard urea (40 mg/dl) and 10 µl of purified water to prepare test, standard and blank, respectively. All the test tubes were mixed well and incubated at 37 °C for 5 min. Then 1000 µl of reagent-II containing alkaline buffer, was added to all the test tubes, which were incubated at 37 °C for 5 min. Urease catalyses the conversion of urea to ammonia and carbon dioxide. The ammonia thus released reacts with a mixture of salicylate, hypochlorite and nitroprusside to yield indophenol, a blue-green coloured compound. The intensity of the colour produced is directly proportional to the concentration of urea in the sample and is measured spectrophotometrically at 578 nm. The blood urea was calculated using the following formula:

\[
\text{Blood urea (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 40
\]

Blood urea nitrogen (mg/dl) = Serum urea \times 0.467

**Estimation of protein in urine**

The proteinuria was assessed by pyrogallol red method (Watanabe et al., 1986) using the commercially available kit (Transasia Bio-Medical Ltd., Solan, India). 1000 µl of reagent (pyragallol dye) was added to 10 µl of urine sample, 10 µl of standard protein and 10 µl of purified water to prepare test, standard and blank, respectively. All the test tubes were mixed and incubated at 37 °C for 10 min. The absorbances of test and standard samples were noted against blank at 600 nm spectrophotometrically. When the pyrogallol red-molybdate complex binds to basic amino groups of protein molecules, there is a shift in reagent absorbance. The absorbance is directly proportional to protein concentration present in the sample.
The urinary protein was calculated using the following formula:

\[
\text{Uriney protein concentration (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 100
\]

Total microprotein excreted (mg/24 hours) = \text{Urinary protein concentration} \times \text{Urinary protein concentration (mg/dl)} \times 10 \times \text{total volume of urine (liters) excreted for 24 hours}

**Histopathological study**

The early diabetic changes in glomeruli were assessed histologically as previously described (Cha et al., 2004; Tomohiro et al., 2007). The kidney was excised and immediately immersed in 10% formalin. The sections from kidney were dehydrated in graded concentrations of alcohol, immersed in xylene and then embedded in paraffin. From the paraffin blocks, sections of 3-5 μm thickness were made and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) using standard histologic procedures to assess the pathological changes occur in kidney using light microscopy.
Assessment of renal oxidative stress

The development of oxidative stress in the kidney was assessed by estimating renal thiobarbituric acid reactive substances (TBARS) and reduced form glutathione (GSH).

Preparation of renal homogenate

The kidney was dissected and washed with ice cold isotonic saline and weighed. The kidney was then minced, and a homogenate (10% w/v) was prepared in chilled 1.15% KCl. The homogenate was used for estimating TBARS, GSH and total protein.

Estimation of TBARS

The renal TBARS, an index of lipid peroxidation, was estimated according to the method described earlier (Ohkawa et al., 1979). The reaction mixture was prepared by mixing 0.2 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA). The reaction mixture was made up to 4.0 ml with distilled water, and then heated in water bath at 95°C for 60 min. After cooling in tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) were added to reaction mixture and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The standard curve using 1,1,3,3-tertramethoxypropane was plotted (Figure 9) to calculate the concentration of TBARS and the results were expressed as nmol/mg of protein.
Estimation of reduced glutathione

The GSH level in the kidney was estimated using the method described by Ellman (Ellman, 1959). Briefly, the renal homogenate was mixed with 10% w/v trichloroacetic acid in ratio of 1:1 and centrifuged at 4 °C for 10 min at 5000 rpm. The supernatant obtained (0.5 ml) was mixed with 2 ml of 0.3 M disodium hydrogen phosphate buffer (pH 8.4) and 0.4 ml of distilled water. Then 0.25 ml of 0.001 M freshly prepared DTNB [5, 5'-dithiobis (2-nitrobenzoic acid) dissolved in 1% w/v sodium citrate] was added. The reaction mixture was incubated for 10 min and absorbance of yellow coloured complex was noted spectrophotometrically at 412 nm. A standard curve was plotted (Figure 10) using reduced form of glutathione and the results were expressed as nmol/mg of protein.

Estimation of total protein

The renal total protein content was estimated by Lowry’s method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. Briefly, 0.3 ml of tissue homogenate was diluted to 1 ml. The 100 μl of diluted supernatant was made up to 1 ml using distilled water. To this, 5 ml of Lowry’s reagent was added. The contents were mixed thoroughly and the mixture was allowed to stand for 15 min at room temperature. Then 0.5 ml of Folin-ciocalteu reagent was added and the contents were vortexed vigorously and incubated at room temperature for 30 min. The standard curve was plotted (Figure 8) using BSA. The protein content was determined spectrophotometrically at 750 nm.
Experimental protocol

Eight groups were employed in the present study and each group comprised 6 rats. The fenofibrate and saxagliptin were suspended in 0.5% w/v of carboxy methyl cellulose (CMC). Group I (Normal Control), rats were maintained on standard food and water and no treatment was given. Group II (Diabetic Control), rats were administered STZ (55 mg/kg, i.p., once) dissolved in citrate buffer (pH 4.5). Group III (Fenofibrate per se), the normal rats were administered fenofibrate (30 mg/kg p.o.) suspended in 0.5% w/v of CMC for 7 weeks. Group IV (Saxagliptin per se), the normal rats were administered saxagliptin (3 mg/kg p.o.) suspended in 0.5% w/v of CMC for 7 weeks. Group V (Fenofibrate Treated), the diabetic rats, after 1 week of STZ administration, were treated with low dose of fenofibrate (30 mg/kg p.o.) for 7 weeks. Group VI (Saxagliptin Treated), the diabetic rats, after 1 week of STZ administration, were treated with saxagliptin (3 mg/ kg p.o.) for 7 weeks. Group VII (Fenofibrate + Saxagliptin Treated), the diabetic rats, after 1 week of STZ administration, were treated with the combination of low dose of fenofibrate (30 mg/kg, p.o.) and saxagliptin (3 mg/kg p.o.) for 7 weeks. Group VIII (Lisinopril Treated), the diabetic rats after 1 week of STZ administration, were treated with lisinopril (1 mg/kg p.o.) for 7 weeks.
Statistical analysis

All values were expressed as mean ± SD. The data obtained from various groups were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison test. The p value of less than 0.05 was considered to be statistically significant and the p values were of two tailed.

Drugs and chemicals

Streptozotocin was obtained from Sigma-Aldrich Ltd., St. Louis, USA. 1,1,3,3-tetra methoxypropane and carboxymethyl cellulose were purchased from R. K. Enterprises, Meerut, India. Fenofibrate was obtained from Ranbaxy Laboratory Ltd., Gurgaon, India. Saxagliptin was obtained from Bristol-Myers Squibb, Mumbai, India. Lisinopril was obtained from Dr. Reddy’s Laboratory Ltd., Hyderabad, India. All other chemicals used in the present study were of analytical grade.
Figure 8. Standard curve for estimation of total protein
Figure 9. Standard curve for estimation of TBARS

\[ y = 0.2065x - 0.0026 \]

\[ R^2 = 0.9995 \]
Figure 10. Standard curve for estimation of GSH