4. Materials and Methods

4.1. Drugs, chemicals and antibodies

The drugs, chemicals and antibodies used in this study were represented in **Table 4.1**. All other remaining drugs/chemicals/reagents used were of analytical grade.

Table 4.1: Drugs, chemicals and antibodies

S.No.	Drugs/Chemicals/Antibodies	Source
1	Tetramethylpyrazine	Sigma-Aldrich (St. Louis, MO, USA)
2	Wortmannin	Sigma-Aldrich (St. Louis, MO, USA)
3	Streptozotocin	Sigma-Aldrich (St. Louis, MO, USA)
4	Nicotinamide	Sigma-Aldrich (St. Louis, MO, USA)
5	Rat insulin ELISA kit	Sigma-Aldrich (St. Louis, MO, USA)
6	Rat IL-6 ELISA kit	Sigma-Aldrich (St. Louis, MO, USA)
7	Rat CRP ELISA kit	Sigma-Aldrich (St. Louis, MO, USA)
8	Huminsulin R 100 IU	Eli Lilly and Company (India)
9	SOD assay kit	Sigma-Aldrich (St. Louis, MO, USA)
10	MDA assay kit	Sigma-Aldrich (St. Louis, MO, USA)
11	GSH-Px assay kit	Sigma-Aldrich (St. Louis, MO, USA)
12	BUN assay kit	Sigma-Aldrich (St. Louis, MO, USA)
13	SCR assay kit	Sigma-Aldrich (St. Louis, MO, USA)
14	24 h urinary protein assay kit	Sigma-Aldrich (St. Louis, MO, USA)
15	Tris Buffer	SDFCL (Mumbai, India)
16	Bradford reagent	Sigma-Aldrich (St. Louis, MO, USA)
17	Bovine Serum Albumin (BSA)	SRL (Mumbai, India)
18	3, 3'-diaminobenzidine (DAB)	SRL (Mumbai, India)

19	H_2O_2	SDFCL (Mumbai, India)
20	Phospho-PI3 Kinase-p85 (Tyr458)/p55 (Tyr199) antibody	Cell Signaling Technology (Danvers, USA)
21	Phospho-Akt (Ser473) antibody	Cell Signaling Technology (Danvers, USA)
22	Glut-4 (1F8) Mouse mAb antibody	Cell Signaling Technology (Danvers, USA)
23	PI3 Kinase-p85 antibody	Cell Signaling Technology (Danvers, USA)
24	Akt Antibody	Cell Signaling Technology (Danvers, USA)
25	β-actin antibody	Cell Signaling Technology (Danvers, USA)
26	Phospho-GSK-3β antibody	Cell Signaling Technology (Danvers, USA)
27	GSK-3β antibody	Cell Signaling Technology (Danvers, USA)
28	Bcl-2 antibody	Cell Signaling Technology (Danvers, USA)
29	Bax antibody	Cell Signaling Technology (Danvers, USA)
30	Cleaved Caspase-3 antibody	Cell Signaling Technology (Danvers, USA)
31	HRP-linked, IgG anti-rabbit Antibody	Cell Signaling Technology (Danvers, USA)
32	HRP-linked, IgG anti-mouse Antibody	Cell Signaling Technology (Danvers, USA)
33	QIAzol lysis reagent	Qiagen Science (Maryland, USA)
34	DNase I, RNase-free	Thermo Scientific (EU, Lithuania)
35	RevertAid first strand cDNA synthesis kit	Thermo Scientific (EU, Lithuania)
36	SYBR Green PCR Kit	Qiagen Sciences (Hilden, Germany)
37	Hematoxylin & Eosin	Sigma-Aldrich (St. Louis, MO, USA)

4.2. Equipment and software

The equipment and software used in this study were enlisted in **Table 4.2**.

Table 4.2: List of equipment and software

S.No.	Equipment/Software	Source
1	One Touch Select Simple glucometer	LifeScan (Scotland, UK)
2	Microplate reader	BioTek Instruments (USA)
3	Biomaster touch screen biochemistry analyser (Auto-analyser)	Fisher Scientific (USA)
4	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE; Mini-PROTEAN® Tetra Cell)	Bio-Rad (CA, USA)
5	Polyvinylidene difluoride (PVDF) membrane	Millipore Corporation (MA, USA)
6	Mini Trans-Blot® Electrophoretic Transfer Cell	Bio-Rad (CA, USA)
7	Gel Doc EZ Imager	Bio-Rad (CA, USA)
8	Nanodrop instrument	Thermo Scientific (USA)
9	Rotor-Gene Q 2plex HRM real-time PCR System	Qiagen (Germany)
10	Ax200 Electronic balance	Shimadzu (Japan)
11	pH meter	Eutech Instruments (Germany)
12	UV/Visible spectrophotometer	Shimadzu (Japan)
13	HPLC grade water system	Sartorius (Germany)
14	Cooling centrifuge	Remi (India)

15	Micropipettes	Eppendorf (UK)
16	Refrigerator	Kelvinator (India)
17	Deep freezer	Haier (China)
18	Tissue homogenizer	IKA (Germany)
19	Digital microscope	Dewinter (India)
20	Image Lab software	Bio-Rad (CA, USA)
21	Rotor-Gene Q Series software	Qiagen (Germany)
22	GraphPad Prism 5.0	GraphPad Software, Inc. (USA)
23	ImageJ software	Downloaded from NIH website

4.3. Experimental animals

The present study was performed in compliance with the standards of the *Guide for the Care and Use of Laboratory Animals*, published by the National Institute of Health (NIH publication no. 86–23, revised 1996). All the investigations on animals were carried out with the permission of Institutional Animal Ethical Committee (Reference No. Dean/2016/CAEC/1656) of Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. Adult male Wistar rats about five-weeks-old weighing 180–200 g were procured from the Central Animal House (Registration Number: 542/02/ab/CPCSEA) of IMS, BHU, Varanasi. All the rats used in the experiment were housed in polypropylene cages of dimension 40 × 33 × 17 cm (two animals per cage) under the regulated environmental conditions (temperature: 25 ± 1°C; relative humidity: 45–55%; 12-h light/12-h dark cycle). Animals were supported with free access to commercial pellet diet available for rats and water ad libitum. All the essential measures were taken to decrease the animal pain and to use the minimum number of animals necessary to generate trustworthy results.

4.4. Induction of T2D

4.4.1. Induction of T2D in rats through the HFD-STZ model (for experiments related to the objective I)

After one week of acclimatisation, except the normal control rats, the rats in the experiment were fed on standardised HFD containing 70% normal diet, 18% sugar and 12% lard oil for four weeks to build insulin resistance. Later, the freshly prepared solution of STZ in citrate buffer (0.1M, pH 4.5) was administered to the rats at the 40 mg/kg (i.p.) dose to create moderate destruction of β-cells excluding normal control group [6]. After 5 days of STZ induction, blood samples were obtained from the

tail vein under mild anaesthesia from rats fasted for overnight, and blood glucose was determined with a One Touch Select Simple glucometer (LifeScan, Scotland, UK). The rats having FBG level over 250 mg/dl were considered as successfully induced with diabetes and included in the experiment. The experiment on the diabetic rats was initiated on the fourth day (day 0) after three days of pacification. HFD was carried on for the overall period of the experiment.

4.4.2. Induction of T2D in rats through the STZ-NCT model (for experiments related to the objective II)

After an adaptation period of 1 week, T2D was induced in the experimental rats as proposed earlier by Masiello et al. with some modification by using a standardised dose of STZ except in normal control rats [84]. The freshly prepared STZ solution in 0.1 M citrate buffer (pH 4.5) was injected at the dose of 55 mg/kg (i.p.) in a volume of 1 ml/kg to overnight-fasted rats, 15 min after i.p administration of NCT (210 mg/kg). In the induction of T2D, the administration of NCT prior to the introduction of STZ can partially protect β-cells by scavenging NO and inhibiting STZ-induced apoptosis [84]. The hyperglycemic condition was confirmed by the high blood glucose levels, measured after 96 h of STZ-NCT administration. Rats with FBG levels above 250 mg/dl (determined through a One Touch Select Simple glucometer, LifeScan, Scotland, UK) were considered diabetic and subsequently included in the experiment.

4.5. Experimental design

4.5.1. Study design for objective I

In this study, total 36 rats (6 normal and 30 diabetic rats) were used. In five groups (Group II to VI), diabetic rats were randomly allocated with n = 6 in each group according to the following design of an experiment:

Group I	NC	Normal control rats were fed with chow diet and received normal saline (1 ml/100 g body weight)
Group II	DC	Diabetic control rats were fed with a high-fat diet and received normal saline (1 ml/100 g body weight)
Group III	D+T-1	Diabetic rats were fed with a high-fat diet and treated with TMP (100 mg/kg body weight) in normal saline
Group IV	D+T-2	Diabetic rats were fed with a high-fat diet and treated with TMP (150 mg/kg body weight) in normal saline
Group V	D+T-3	Diabetic rats were fed with a high-fat diet and treated with TMP (200 mg/kg body weight) in normal saline
Group VI	D+T-3+W	Diabetic rats were fed with a high-fat diet and treated with TMP (200 mg/kg body weight) in normal saline and PI3K inhibitor wortmannin (W) (15 µg/kg body weight/day [85], i.v.)- 10 min before TMP administration

The test drugs or vehicle were administered orally once daily in the afternoon, for 28 days using an oral gavage.

4.5.2. Study design for objective II

In the whole experiment, thirty rats (twenty-four diabetic and six normal) were employed. Diabetic rats were randomly divided into four groups, each of six animals in accordance with the following experimental design:

Group I	NC	Normal control rats were fed with chow diet and received normal saline (1 ml/100 g b wt.)
Group II	DC	Diabetic control rats were fed with chow diet and received normal saline (1 ml/100 g b wt.)
Group III	D+T-1	Diabetic rats were fed with chow diet and treated with TMP (100 mg/kg b wt.) in normal saline
Group IV	D+T-2	Diabetic rats were fed with chow diet and treated with TMP (150 mg/kg b wt.) in normal saline
Group V	D+T-3	Diabetic rats were fed with chow diet and treated with TMP (200 mg/kg b wt.) in normal saline

The vehicle or test drugs were administered orally once daily in the morning, using an intragastric gavage for eight weeks.

The oral dose range of 100, 150 and 200 mg/kg body weight/day of TMP were chosen in both the studies based on a previous study evaluating anti-hyperglycemic potential and diabetic nephropathy amelioration effect of TMP in rats [33].

4.6. Experimental procedure

4.6.1. Study procedure for objective I

The body weights and FBG were measured on days 0 (before treatment), 14, 21 and 28 during the study. The insulin level in rats was measured in the serum on 0 and 28th day employing ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) as per instructions of the

manufacturer. The insulin resistance (IR), and the function of β-cell (B%) were calculated with the help of fasting glucose and fasting insulin levels through a homeostatic model assessment (HOMA). The following two equations were used for the calculation: HOMA-IR = (Glucose x Insulin) / 405 and HOMA-B = (360 x Insulin) / (Glucose-63) % [86]. On the 14^{th} day, the oral glucose tolerance test (OGTT) was carried out. In this test, 6 h fasting was given to each rat in the study and 2 g/kg body weight glucose solution was orally given to the rats through intra-gastric gavage exactly 30 min after the administration of TMP and vehicle. At time 0 (before glucose infusion) and 30, 60 and 120 min after glucose intake the blood glucose level in each rat was estimated. After treatment of 21 days, the rats were subjected to an insulin tolerance test (ITT). Here, after 6 h of fasting, animals were treated with insulin (1.2 U/kg body weight) in normal saline intraperitoneally, and blood glucose level was evaluated as in OGTT. At the end of the study, the animals were given fasting for overnight, and blood was withdrawn from the orbital vein for the evaluation of serum biochemical parameters and inflammatory cytokines. Then, the rats were euthanised by cervical dislocation under mild anaesthesia following animal ethical guidelines. The skeletal muscle, heart and adipose tissue were excised and frozen rapidly in liquid nitrogen for successive western blot and gene expression analysis.

4.6.2. Study procedure for objective II

Body weight and FBG were measured on 0, 28^{th} and 56^{th} day of treatment. On the 49^{th} day, the OGTT was performed in rats which were fasted for 6 h, and glucose solution (2 g/kg b wt.) was given orally to every animal in the experiment exactly 30 min after the administration of vehicle or TMP. The blood glucose level was measured at 0 min (immediately before glucose infusion) and 30, 60 and 120 min after administration of glucose. After 8 weeks of treatment with TMP, blood was

collected from the retro-orbital plexus of anaesthetised rats and then centrifuged for 15 min at 8000 x g to separate the serum for further analysis of various biochemical parameters. The fasting serum insulin (FSI) level was measured using ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) as per guidelines of the manufacturer. Finally, the rats were euthanised by cervical dislocation under pentobarbital anaesthesia (30 mg/kg, i.p.), and all the animal ethical guidelines were followed. Kidney tissues were immediately cut, and a part of it was rapidly frozen in liquid nitrogen and kept at -80°C for subsequent analysis of oxidative stress parameters and western blot, and another part was fixed in formalin (10 %) and preserved for histopathological observation.

4.7. Biochemical estimations

4.7.1. Estimation of lipid profile and glycosylated haemoglobin content

In the serum, the level of triglyceride (TG), total cholesterol (TC), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol were measured using auto-analyser (Fisher scientific, Biomaster touch screen biochemistry analyser). Nayak and Pattabiraman proposed the method through which the level of glycosylated haemoglobin (HbA1c) was assessed in whole blood [87].

4.7.2. Estimation of pro-inflammatory cytokines in serum

Using the commercially available ELISA kits (Sigma-Aldrich, St. Louis, MO, USA), the pro-inflammatory cytokines IL-6 and CRP level were estimated. Here the concentrations of test samples were obtained through the construction of a standard curve. The whole study was carried out according to the guidelines of the kit manufacturer.

4.7.3. Western blot analysis

Western blotting was performed as explained by the method proposed by Martin et al. with few modifications [88]. Initially, the lysis buffer (20 mM Tris–HCl (pH 7.4), 2 mM EGTA, 2 mM EDTA, 30 mM NaF, 1 mM PMSF, 30 mM sodium pyrophosphate, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail) was used to make the 10% w/v skeletal muscle, heart, kidney and adipose tissue homogenate and the homogenate was solubilised at 4°C for 1 h and centrifuged at 8000 g for 15 min. The supernatants were collected, and the protein content was estimated according to Bradford's method and then stored at −20°C until further analysis [89].

On the individual lanes of polyacrylamide gel, 40 μg of protein samples were loaded and separated by Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE; Bio-Rad, Mini-PROTEAN® Tetra Cell) using Tris-glycine-SDS running buffer (**Table 4.3**). At the beginning, we ran the gel at the voltage of 80 V when the protein sample was running on stacking gel (upper gel; **Table 4.4**), and then, when the protein sample came into the resolving gel (lower gel; **Table 4.5**), we usually adjust the voltage to 100-130 V to speed up the running process. Proteins were relocated to polyvinylidene difluoride (PVDF) (Millipore Corporation, MA, USA) membrane employing Bio-Rad (Mini Trans-Blot® Electrophoretic Transfer Cell) using transfer buffer (**Table 4.6**), at 30 V constant, 90 mA (overnight transfer) or 100 V constant, 350 mA (high-intensity field 1 h transfer).

The membranes were blocked with bovine serum albumin (5%) in TBS-T (0.1%Tween20) (**Table 4.7**) for 2 h and overnight incubated at 4°C with primary antibodies: Phospho-PI3 Kinase-p85, PI3 Kinase-p85, Phospho-Akt, Akt, Glut-4, Phospho-GSK-3β, GSK-3β, Bcl-2, Bax, Cleaved Caspase-3 and β-actin (internal control) (Cell Signaling Technology, Danvers, USA). The primary antibody solution

was prepared in 1:1000 dilution in 1% BSA-TBS-T. The membranes were then washed with TBS-T solution gently and subsequently incubated with either HRP-linked, IgG anti-mouse or anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, USA) for 1 h. The secondary antibody solution was prepared in 1:500 dilution in 1% BSA-TBS-T. The desired blots were washed with TBS-T three times (each of 10 min), and were visualised using 3, 3'-diaminobenzidine (DAB) (SRL, Mumbai, India) and H_2O_2 (SDFCL, Mumbai, India) as substrates (**Table 4.8**) employing Bio-Rad Gel Doc™ EZ Imager system. The densitometric analysis was achieved using NIH Image J analysis software.

Table 4.3: Composition of SDS-PAGE Running buffer (1 L, pH 8.3)

Tris base (25 mM)	3.0285 g
Glycine (190 mM)	14.25 g
SDS (0.5%)	5.00 g
Distilled H_2O	Up to 1 L

Table 4.4: Solutions for preparing 5% Stacking gels for Tris-glycine SDS-PAGE

Solution components	**Component volumes (ml) per gel mold volume of 5 ml**
H_2O	3.4
30% acrylamide mix	0.83
1.0 M Tris (pH 6.8)	0.63
10% SDS	0.05
10% APS	0.05
TEMED	0.005

Table 4.5: Solutions for preparing Resolving gels for Tris-glycine SDS-PAGE

Solution components	**Component volumes (ml) per gel mold volume of 10 ml**	
	8 % gel	**10 % gel**
H_2O	4.6	4.0
30% acrylamide mix	2.7	3.3
1.5 M Tris (pH 8.8)	2.5	2.5
10% SDS	0.1	0.1
10% APS	0.1	0.1
TEMED	0.006	0.004

Table 4.6: Composition of Transfer buffer (1 L, pH 8.3)

Tris base (25 mM)	3.0285 g
Glycine (190 mM)	14.25 g
SDS (0.5%)	5.00 g
Methanol	20% of the whole volume (200 ml)
Distilled H_2O	Up to 1 L

Table 4.7: Composition of Tris Buffer Saline (TBS, 1x) (1 L, pH 7.5)

Tris base	6.05 g
NaCl	8.76 g
Distilled H_2O	Up to 1 L

Table 4.8: Composition of DAB development solution (For 1 membrane)

DAB	8 mg
50 mM Tris (pH 7.6)	13 ml
H_2O_2	13 µl

4.7.4. Real-time polymerase chain reaction (RT-PCR)

A. RNA isolation

a) Added 1 ml of QIAzol lysis reagent per 100 mg of skeletal muscle, adipose and cardiac tissue to the samples and homogenized using a homogenizer. Incubated samples for 5 min to permit complete dissociation of the nucleoproteins complex.

b) Added 0.2 ml of chloroform per 1 ml of QIAzol reagent used for lysis, then securely caped the tube and incubated for 2–3 min.

c) Centrifuged the samples for 15 min at 12,000 × *g* at 4°C. The mixture gets separated into a lower red phenol-chloroform, interphase, and a colourless upper aqueous phase.

d) Transferred the aqueous phase containing the RNA to a new tube.

e) Added 0.5 ml of isopropanol to the aqueous phase, per 1 ml of QIAzol reagent used for lysis, then incubated for 10 min and centrifuged for 10 min at 12,000 × *g* at 4°C.

f) Total RNA gets precipitated and formed a white gel-like pellet at the bottom of the tube. Discarded the supernatant with a micro pipettor.

g) Resuspended the pellet in 1 ml of 75% ethanol per 1 ml of QIAzol reagent used for lysis. Then, vortexed the sample briefly, and centrifuged for 5 min at 7500 × *g* at 4°C.

h) Discarded the supernatant with a micropipettor and air-dried the RNA pellet for 10 min.

i) Resuspended the pellets in 50 µl of RNase-free water by pipetting up and down, then incubated on heat block set at 55–60°C for 10 min and stored the RNA at –70°C.

j) Determined the RNA yield through a Nanodrop instrument (Thermo Scientific, USA).

B. Removal of genomic DNA from RNA preparations

a) Firstly, to the RNase-free tube, the following components were added:

Table 4.9: Composition of RNA purification solution

RNA	1 µg
10x Reaction Buffer with $MgCl_2$	1 µl
DNase I, RNase-free	1 µl (1 U)
Water, nuclease-free	Up to 10 µl

b) Incubated at 37°C for 30 min, then 1 µl 50 mM EDTA was added to the tubes and again incubated at 65°C for 10 min.

c) Used the prepared RNA as a template for reverse transcription reaction.

C. First Strand cDNA Synthesis

a) After thawing, mixed and briefly centrifuged the components of the kit and stored on ice.

b) Added the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Table 4.10: Composition of first strand cDNA synthesis solution (contd.)

Template RNA	1 µg
Oligo $(dT)_{18}$ primer	1 µl
Water, nuclease-free	Up to 12 µl
Total volume	12 µl

c) Then, added the following components in the indicated order:

Table 4.11: Composition of first strand cDNA synthesis solution

5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 U/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV RT (200 U/µl)	1 µl
Total volume	20 µl

d) Mixed gently and centrifuged briefly the components in the tube and incubated for 60 min at 42°C.

e) Terminated the reaction by heating at 70°C for 5 min. Then, the reverse transcription reaction product was stored at -70°C.

f) The synthesised cDNA content was quantified using a Nanodrop instrument.

D. Polymerase chain reaction (PCR)

Real-time PCR was carried out on the Rotor-Gene Q 2plex HRM real-time PCR System (Qiagen, Germany) using the QuantiFast SYBR Green PCR Kit, according to manufacturer's protocol (**Table 4.12**). After initial pre-incubation and denaturation for 10 min at 95°C, PCR amplification was executed at 95°C for 10 s (denaturation) and at

60°C for 45 s (combined annealing/extension) for 40 cycles. The relative gene expression levels were normalised using β-actin gene and quantified by the $2^{-\Delta\Delta Ct}$ method. The primer sequences employed in the study were listed in **Table 4.13**.

Table 4.12: Composition of PCR mixture

Components	**Volume**
2x QuantiFast SYBR Green PCR Master Mix	5 µl
Forward primer	1 µl
Reverse primer	1 µl
cDNA Template	1 µl
Nuclease-free water	2 µl
Total reaction volume	10 µl

Table 4.13: Primer sequences used for RT-PCR

Target gene	**Primer sequence**		**Gene bank accession number**
PI3K	Forward	5'-GACAATGCCTCTCCACAGTAA-3'	AB009636.1
	Reverse	5'-GGTTTCTCCTCCACATCTTC-3'	
Akt	Forward	5'-ATGGAGCAGAGATTGTGTCAG-3'	DQ198085.1
	Reverse	5'-GCACAAGCCAAAGTCAGTAATC-3'	
Glut4	Forward	5'-ACTTAGGGCCAGATGAGAATG-3'	BC085757.1
	Reverse	5'-GTAAGGGAAGAGAGGGCTAAAG-3'	
β-actin	Forward	5'-TTACTGCTCTGGCTCCTAGCAC-3'	NM007393.5
	Reverse	5'-ACTCCTGCTTGCTGATCCAC-3'	

4.7.5. Evaluation of oxidative stress and renal biochemical markers

The parts of kidney tissues stored at -80°C were homogenised, and homogenates were centrifuged at 8000 x g for 15 min, after that the supernatant was separated for kidney oxidative parameters analysis. Serum and kidney SOD, MDA and GSH-Px levels were estimated using commercial specific kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. BUN and SCR levels were estimated using the specific kits (Sigma-Aldrich, St. Louis, MO, USA), and 24 h urine samples were collected utilising the metabolic cages and urinary protein content for a day was calculated using a special kit. All the operational procedures were performed according to the manufacturer's instruction.

4.7.6. Histopathological examination

Kidney tissue samples that were fixed in formalin (10 %) were dehydrated by placing in 70% isopropyl alcohol for 2 h and then in each ascending strength (80%, 90%, 100% isopropyl alcohol) for 1 h each. After that, xylene was added to check for the appearance of milkiness. If milkiness appeared then repeated the dehydration procedure. The dehydrated tissues were impregnated in paraffin wax (melting point: 56ºC) for a period of 1 h at 58–60ºC. Molten paraffin was poured into the L-block along with the tissues and allowed to become hard. The tissues were sectioned into very thin (4 to 6 µm) transverse sections using a microtome and mounted on the slides with Mayer's albumin solution (a mixture of equal parts of egg white and glycerin, beaten and filtered with the addition of 1% sodium salicylate) and incubated in warm oven for 2 h at 60ºC. Slides containing paraffin sections were placed on a slide holder. Then, slides were deparaffinized with Xylene for 30 min and the excess xylene was blotted [90].

Thereafter, the tissues were rehydrated successively with 100%, 90%, 80% isopropyl alcohol for 2–3 min each and put in water for 3 min. The excess water was blotted and the tissues were kept into Hematoxylin stain for 1–2 min and then kept in tap water for 1–2 min. The slides containing tissue sections were dipped into 1N HCl followed by Scott's water (Sodium Bicarbonate 3.5 g, Magnesium sulphate 20 g, distilled water 1 L) for 1 min each. Thereafter, the tissues were immersed in Eosin stain for 30 s, then tissues were dipped into 70% alcohol and then into 90% alcohol and pure alcohol subsequently and left for 2 min in each. Finally, one drop of gum DPX (Dibutylphthalate Polystyrene Xylene) was poured onto the slides and sections were covered with cover slip. The slides were observed under the light microscope with suitable zooming [90].

The glomerular mesangial expansion index (GMEI) and the tubulointerstitial damage index (TDI) were estimated and graded as reported earlier by Border et al. and Matsumoto et al. [91, 92]. The GMEI score was graded as 0, for normal glomerulus; 1, for expansion of matrix in up to 25% area of glomerulus; 2, for expansion of matrix in 25–50% area of glomerulus; 3, for expansion of matrix in 50–75% area of glomerulus; 4, for expansion of matrix in 75-100% glomerular area. The TDI score was graded ranging from 0 to 5 on the ground of percentage of tubular cellularity, cellular infiltration, thickening of basement membrane, atrophy, dilation, interstitial widening or sloughing as mentioned: 0, for no tubulointerstitial injury; 1, for less than 10% tubular injury; 2, for 10 to 25% tubular injury; 3, for 25 to 50% tubular injury; 4, for 50 to 75% tubular injury; and 5, for 75 to 100% injury of tubules.

4.8. Statistical Analysis

All the results of the study were expressed as mean ± standard error of the mean (S.E.M.). The results were analysed using one-way ANOVA followed by Tukey's multiple comparison post hoc tests and two-way ANOVA followed by Bonferroni post-test. Statistical analysis was accomplished by Graph Pad Prism 5.0 software (San Diego, CA, USA). A P value less than 0.05 was considered statistically significant.