

Phytochemical Screening and Evaluation of Nephroprotective Activity of *Ficus benghalensis* Leaves on the Kidney of Alloxan Induced Diabetic Rats

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ABSTRACT

The study was carried out to investigate renal protective activity of methanolic extract of leaves of *Ficus Benghalensis* on the kidney of Alloxan induced early diabetic nephropathy in rats and to focus on its possible mode of action. MEFB was also analysed for its phytochemical composition using various qualitative and quantitative methods. Experimental diabetes was induced in wistar albino rats by single intraperitoneal injection of Alloxan monohydrate (150 mg/kg). The methanolic extract of leaves of *Ficus benghalensis* at a dose of 200 and 400 mg/kg body weight was administered at single dose per day to diabetes induced rats for a period of 28 days. At the end of 4 weeks, blood urea nitrogen (BUN), serum creatinine, serum total proteins, serum albumin, glycosylated haemoglobin, was determined. Antioxidant enzymes of kidney were evaluated. Urine was analyzed for albumin, total proteins and creatinine clearance. Kidney of experimental animals was examined to determine structural changes. Our results showed a significant decrease in creatinine, albumin, BUN, total protein, urinary total protein. Whereas significant improvement in glycosylated haemoglobin, oxidative stress parameters of kidney has been observed in MEFB treated diabetic rats. Histopathology of kidney tissue showed structural improvement.

Keywords: Alloxan, Nephroprotective, oxidation stress markers, *Ficus benghalensis*.

INTRODUCTION

Diabetes Mellitus (DM) is clinical condition in which metabolic activity of the carbohydrate, lipid and protein metabolism that contributes to several kinds of complications including diabetic nephropathy. Diabetic nephropathy is one of the major complications of diabetes mellitus. Diabetic nephropathy is the most important cause of death in diabetics, of whom, 30-40% eventually develop end-stage renal failure¹.

It has been reported that diabetic complications are associated with overproduction of Reactive Oxygen Species (ROS) and accumulation of lipid peroxidation by-products². These complications are considered the leading causes for death among these patients.

Oxidative stress is generally considered as an imbalance between pro-oxidant and antioxidant³. Oxidation plays a major role in diabetes. The increase in free radical release accompanied by decrease in antioxidants is a major cause of diabetes⁴.

In diabetes mellitus, there are usually alterations in the endogenous free radical scavenging defenses which leads to ineffective scavenging of reactive oxygen species resulting to oxidative damage⁵. Experimental diabetes induced by alloxan, selectively destroys the β -cells of pancreas by generating excess reactive oxygen species and produces kidney lesions that are similar to human diabetic nephropathy⁶.

Thus, an early control of DM is recommended as one of main strategy to prevent these complications and increase the life span of these patients. The use of herbal medicine for the treatment of DM has gained prominence because of the undesirable side effects of oral anti-diabetic drugs, coupled with more recurrent failure of beta cells to respond to treatment⁷.

The herbal approach received a boost following the WHO recommendation for research on the beneficial uses of medicinal plants in the treatment of DM.⁸ They were considered more potent over most western medicines that are often made of single

chemical compounds effective for direct relief of the symptoms⁹. It is believed that some *Ficus* species can be used as a remedy for visceral obstructive disorders, diabetes, leprosy, respiratory disorders and certain skin diseases¹⁰ and as an absorbent for inflammatory swellings and burns¹¹. *Ficus benghalensis* belongs to the family Moraceae, which is commonly known as Banyan tree. Earlier glucoside, 20- tetratriacontene-2-one, 6-heptatriacontene-10-one, pentatriacontan-5-one, beta sitostirirolalpha- D-glucose and meso-inositol have been isolated from the bark of *Ficus benghalensis*¹². Leaves contain crude protein 9.63%, crude fibres-26.84%, CaO-2.53%, and Phosphorus-0.4 %. It yields latex containing Caoytchoue (2.4%), Resin, Albumin, Cerin, Sugar and Malic acid. It is used in Ayurveda for the treatment of diarrhoea, dysentery and piles^{13,14}, teeth disorders¹⁵ Rheumatism, skin disorders like sores¹⁶ to boost immune system¹⁷ as a hypoglycemic^{18,19}. The extracts of *Ficus benghalensis* were also reported to inhibit insulinase activity from liver and kidney²⁰. However, despite of widespread use of *Ficus benghalensis* as folk medicine to manage DM and other ailments, its protective effects on the renal system has not been established. Therefore, the present study was designed to evaluate the nephroprotective effects of Methanolic leaf extract of *Ficus Benghalensis* on the Kidney of Alloxan Induced Diabetic Rats.

MATERIALS AND METHODS

Collection and Authentication of Plant

The leaves of the tree of *Ficus benghalensis* were collected from the local areas of Nellore district and authenticated by Dr. Madhava Chetty, Assistant Professor, Dept. of Botany S.V. University - Tirupathi. A portion of the sample was kept in the department museum for future reference.

Chemicals

All the chemicals used was of analytical grade and procured from Sigma chemicals Co, USA and Qualigens fine chemicals, Mumbai, India.

Animals

Mice of either sex weighing 25-30g and male Wistar rats weighing 150-220g were obtained from the animal house of Aravind remedies Pvt Ltd. Animals were fed on conventional diets and water *ad libitum* and they were maintained under standard conditions of humidity, temperature (20-24°C) and light (12-h light: 12-h dark cycle).

The rats were randomly assigned to control and different treatment groups, six animals per group. The Institutional Animal Ethics Committee approved the experimental protocol and the conditions in the animal house approved by Committee for Supervision on Experiments on Animals. The study was conducted in accordance with IAEC guidelines (Registration no- 1696/PO/a/13/CPCSEA/IAEC/22.06.2013). The animals were acclimatized for one week under laboratory conditions.

Extraction of Leaves by Hot Percolation Method

500 g of powder was weighed and extracted with 2 litres of methanol by successive extraction in a soxhlet apparatus for 72 h. Then the solvent is subjected to distillation and concentrated the extracts. The extract is concentrated with rotary evaporator under reduced pressure then the dried extract was weighed and the percentage yield calculated. The percentage yield of extracts was 3.38%w/v.

Preliminary phytochemical study

The MEFB was screened for the presence of various phytoconstituents like alkaloids, glycosides, flavonoids, tannins, carbohydrates, amino acids and proteins.

Acute toxicity studies

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD-423) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. All observations are systematically recorded.

Induction of Diabetes

Hyperglycemia was induced by a single i.p. injection of 150 mg/kg of alloxan monohydrate (Sigma-Aldrich, U.S.A.) in sterile saline solution. Following injecting rats with alloxan by one hour, rats were allowed to be fed standard pellets and water *ad libitum*. After that, the experimental rats were administered 20% glucose solution for 24 hours to prevent hypoglycemia. After 72hr of Injection, fasting blood glucose level (estimated by glucometer)

was measured. Hyperglycemia was confirmed after 5 days of alloxan injection, and hyperglycemic rats (glucose level > 150mg/dl) were separated and selected for the study.

Experimental Design

Total of 24 diabetic surviving and 6 nondiabetic rats were divided in to 5 groups (n=6) as follows-**Group I**-Normal rats received only saline (0.5 mL/kg body weight) and served as positive control,**Group II**-Diabetes induced control rats [treated with alloxan monohydrate in sterile saline (150 mg/kg-bw by i.p. injection, in a single dose),**Group III**-rats received standard drug Glibenclamide at a dose of 0.5 mg/kg orally for 28 days + a single dose of Alloxan.**Group IV**-Received lower dose of *MEFB* 200 mg/kg/d suspended in the vehicle (10 ml/kg) for 28 days+ a single dose of Alloxan.**Group V**-Received higher dose of *MEFB* (400 mg/kg/d suspended in the vehicle (10 ml/kg) for 28 days+ a single dose of Alloxan.

During 28 days study period body weight, food and fluid intake of animals were recorded. HbA1c % was determined in EDTA-blood samples obtained at the end of the 28th day study using commercial assay kit (Crest biosystems, Goa, India).

Parameters of nephroprotective activity

Serum total protein, urinary total protein, serum albumin and urinary albumin were estimated by using kit (Autozyme, India). Blood urea nitrogen, serum creatinine and urinary creatinine clearance was estimated using commercial kit (Crest biosystems, India). Urine volume was measured at 0, 7, 14, 21 and 28th day of treatment period using metabolic cages. Kidney weight was taken of all animals at the end of study Ratio of kidney weight to body weight was determined for all experimental groups under study.

Enzymatic and non-enzymatic biomarkers of oxidative stress in kidney

Isolated kidney was minced and homogenized

(10 % w/ v) in ice-cold 0.1 M sodium phosphate buffer (pH-7.4). The homogenate was centrifuged at 10,000 rpm for 15-20 min at 4 °C twice to getenzyme fraction. The resultant supernatant was used for various biochemical assays. LPO was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a productformed due to the peroxidation of membrane lipids²¹. SOD activity was assessed according to the method of Marklund and Marklund²². CAT activity was assayed following the method of Aebi²³. GSH content was determined according to the method of Ellman²⁴.

Physical Parameters

Body Weight

The weight of the animals before starting and at the end of the treatment should be measured and the change in body weight should be noted.

Histopathology

The kidneys were quickly removed after sacrifice of rats and were fixed in 10% neutral buffered formalin solution for histopathological processing. Sections were stained with haematoxylin and eosin before being observed under an Olympus microscope at 40X magnification.

Statistics

Data obtained in the experiment were expressed in terms of mean \pm SEM. Statistical significance of data was assessed by analysis of variance (one-way ANOVA) followed by a comparison between different groups using "Dunnet" test. The significance level was set at $P < 0.05$.

RESULTS

PHYTOCHEMICAL SCREENING

Preliminary Phytochemical Investigation of methanol extracts of *Ficus benghalensis leaf extract* showed the presence of triterpenoids, alkaloids, tannins and flavonoids. (Table: 1)

Table 1: Phytochemical investigation of *Ficus benghalensis* leaves extract

S. No.	Phytochemical tests	Present (+) /absent (-)
1	Test for Carbohydrates	-
2	Test for Proteins and Amino acids	-
3	Test for Triterpenoids	+
4	Test for Alkaloids	+
5	Test for Cardiac glycosides	-
6	Test for Anthraquinone glycosides	-
7	Test for Saponins	-
8	Test for Flavonoids	+
9	Test for tannins and phenolic compounds	+
10	Test for fixed oil and fats	-

ACUTE TOXICITY STUDY

Acute toxicity study revealed the non-toxic nature of MEFB. It produced no mortality at 2000 mg/kg. Therefore, one-tenth of the maximum no mortality dose of extract was selected as therapeutic lowest dose (200 mg/kg) and just double of it as highest dose (400 mg/kg) respectively in this study.

Parameters of nephroprotective activity

Treatment with MEFB for 28 days significantly ($p < 0.01$) decreased glycosylated haemoglobin level in treatment group as compared to diabetic control group. Total protein and albumin was significantly decreased in the serum and increased in urine of diabetic control group as compared to normal control group. Treatment with MEFB significantly ($p < 0.01$) increased serum total protein and albumin as compared to diabetic control group. (Table: 2) On the other hand, total protein and albumin in urine was significantly ($p < 0.01$) reduced by MEFB treatment. Blood urea nitrogen and serum creatinine increased significantly ($p < 0.01$) whereas creatinine clearance decreased steeply in diabetic control rats as compared to normal control group indicating a decreased glomerular filtration rate. Treatment with MEFB (400 mg/kg) significantly ($p < 0.01$) decreased the alteration in glomerular filtration rate by decreasing serum creatinine and increasing creatinine clearance compared to diabetic control group. Kidney weight/body weight ratio (KW: BW) of diabetic control rats was significantly higher as compared to other groups which was

normalized significantly ($p < 0.01$) by MEFB treatment. Urine volume of Alloxan induced diabetic control rats was found to be significantly ($p < 0.01$) more compared to normal control rats. (Table: 3) After 4 weeks of treatment with MEFB, urine volume significantly ($p < 0.01$) decreased compared to diabetic control groups.

Enzymatic and non-enzymatic biomarkers of oxidative stress in kidney

Diabetes resulted in significant decrease in antioxidant enzymes like GSH, catalase and SOD. Moreover the levels of MDA were significantly increased. MEFB exhibited improvements in antioxidant enzymatic activity compared to diabetic control group and nearly normalized the levels of SOD, MDA, catalase and GSH. (Table: 4).

Body weight, food intake and water intake

Body weight of Alloxan induced diabetic control rats was found to be significantly ($p < 0.05$) less compared to normal control rats. After 4 weeks of treatment with MEFB body weight significantly ($p < 0.05$) increased compared to diabetic control groups. Food intake was significantly high in diabetic control rats as compared to normal control. At the end of 28 days of treatment food intake of treated groups significantly ($p < 0.01$) decreased as compared to diabetic control. Significant decrease ($p < 0.01$) in water intake was observed in treated groups as compared to diabetic control at the end of study period. (Table: 5).

Table 2: Effect of MEFB on serum parameters in ALLOXAN diabetic rats

Parameters	Normal control	Diabetic control	DC + MEFB (200 mg/kg)	DC+ MEFB (400 mg/kg)	DC+ GL (5 mg/kg)
HbA1c (%)	5.31±0.28	7.38± 0.04##	6.28± 0.07**	5.76± 0.10***	5.22± 0.25***
Serum total protein (g/dl)	7.51± 0.49	3.39±0.617##	4.80±0.25**	6.67±0.47**	7.66±0.45***
Serum albumin (g/dl)	5.46± 0.36	3.12± 0.52##	4.81± 0.21**	5.29± 0.21***	5.96± 0.12***
Serum creatinine (mg/dl)	1.41± 0.69	2.67± .84##	2.02± 0.66**	1.51± 0.25 ***	1.321±0.54 ***
BUN (mg/dl)	20.06±0.98	50.54±3.32##	43.60±0.94 **	39.26±0.42 **	29.65±0.94***

All the values are mean± SEM, n=6, ns=not significant one way ANOVA followed by multiple compression.

Dunnett's test *** P<0.001, ** P<0.01, * P<0.05 as compared to control. ## P <0.001 when compared with normal.

Table 3: Effect of MEFB on Urine parameters in ALLOXAN diabetic rats

Parameters	Normal control	Diabetic control	DC + MEFB (200 mg/kg)	DC+ MEFB (400 mg/kg)	DC+ GL (5 mg/kg)
Urinary total protein (g/dl)	8.14±0.46	12.87±0.44##	9.35±0.56**	7.94±0.49**	7.86±0.38***
Urinary albumin(g/L)	0.69±0.03	3.55±0.11##	1.41±0.07 **	1.16 ±0.12 ***	2.00±0.09 ***
Urinary creatinine clearance(g/L)	0.96±0.40	4.67±0.87##	3.27±0.13**	4.31±0.05***	2.51±0.17***
Urine volume (ml/24hrs)	13.83±0.42	29.16± 2.49##	21.78±0.65***	17.44±0.65***	14.83± 1.22 ***

All the values are mean± SEM, n=6, ns=not significant one way ANOVA followed by multiple compression.

Dunnett's test *** P<0.001, ** P<0.01, * P<0.05 as compared to control. ## P <0.001 when compared with normal.

Table 4: Effect of MEFB on renal oxidative parameters on Alloxan diabetic rats

Parameters	Normal control	Diabetic control	DC+ MEFB (200 mg/kg)	DC + MEFB (400 mg/kg)	DC + GL (5 mg/kg)
Kidney SOD (U/mg protein)	5.571±0.16	3.35±0.16##	5.043±0.053**	5.451±0.086***	5.961±0.18***
Kidney Catalase(U/mg protein)	77.56±1.68	35.64±1.54##	48.97±0.60**	51.05±0.42**	61.09±1.20***
Kidney GSH(nmol/mg protein)	4.35±1.42	2.73±0.06##	4.173±0.05**	4.43±0.06**	4.15±0.05***
Kidney MDA(nmol/mg protein)	107.92±2.509	186.87±6.58##	158.00±3.39**	132.95±3.011***	121.86±9.51***

All the values are mean± SEM, n=6, ns=not significant one way ANOVA followed by multiple compression.

Dunnett's test *** P<0.001, ** P<0.01, * P<0.05 as compared to control. ## P <0.001 when compared with normal.

Histopathology study of kidney

The histological changes in the renal specimen of normal and experimental animals are shown in figure. Diabetic glomeruli showed some areas of mesangial matrix expansion,

thickening of glomerular basement membrane, dilation of tubule and cell infiltrations. Treatment with MEFB lead to regeneration of tissues that were earlier affected with Alloxan.

Histopathology of kidney sections of Alloxan diabetic rats treated with MEFB

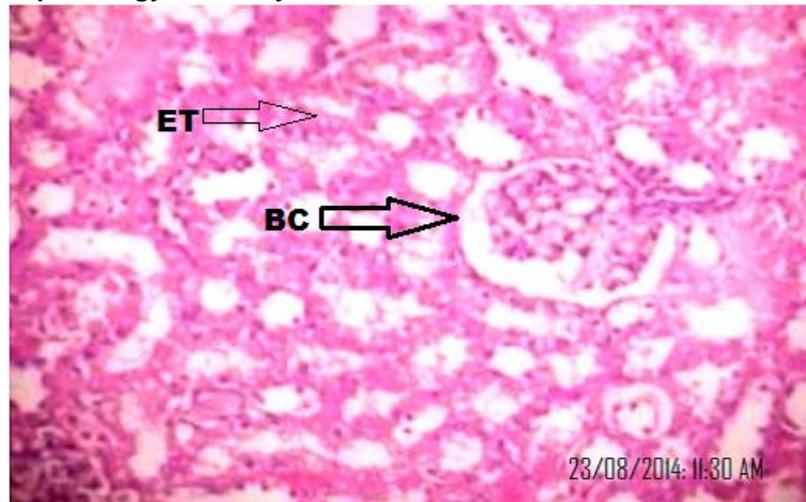


Fig. 1: Normal control

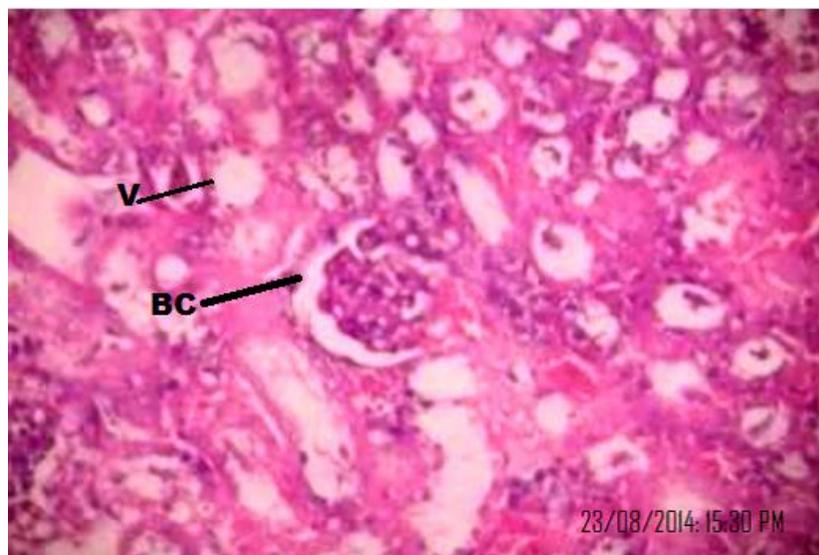


Fig. 2: Alloxan treated group (DC)

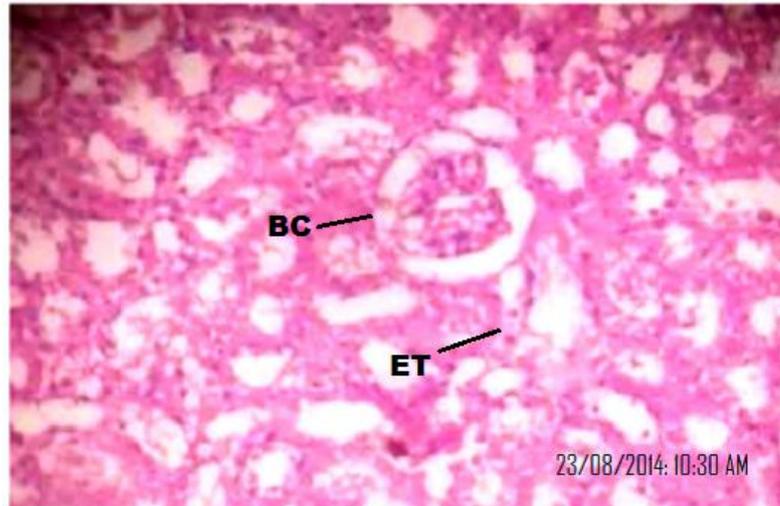


Fig. 3: DC+Glibenclamide(5 mg/kg)

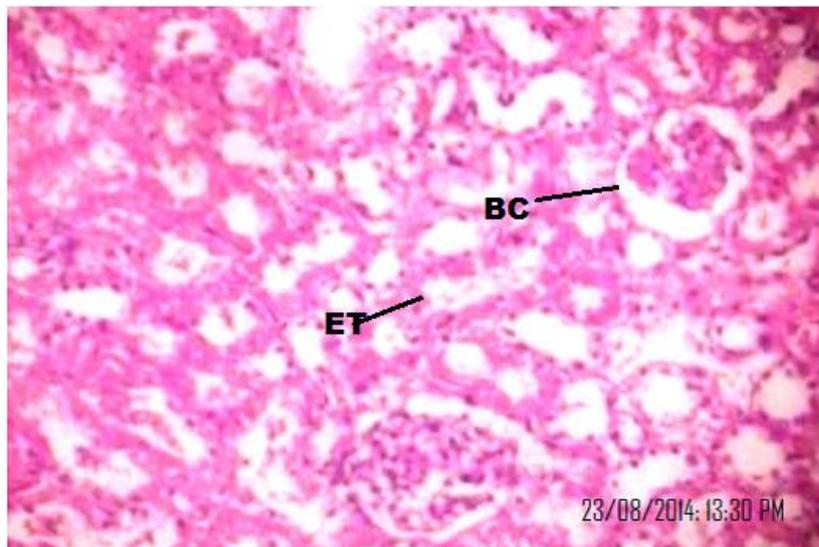


Fig. 4: DC + MEFB (200 mg/kg)

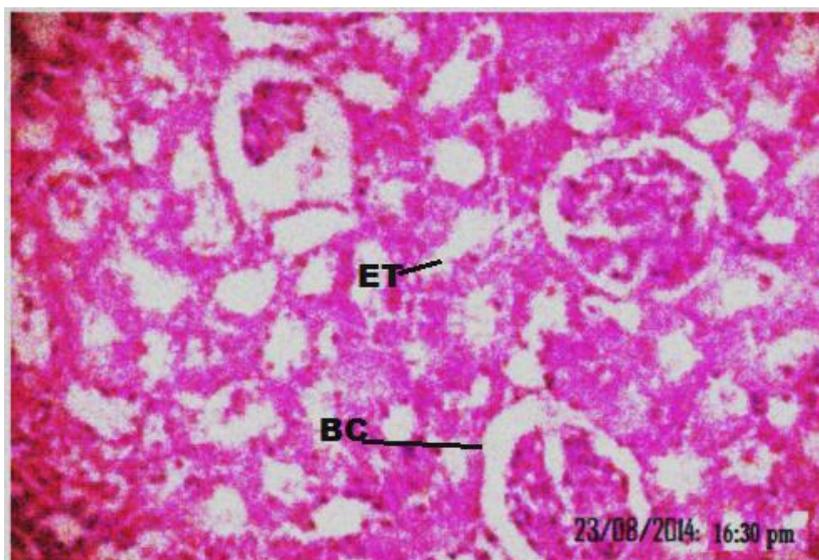


Fig. 5: DC + MEFB (400 mg/kg)
ET: Extended Tubules; BC = Bowmen's capsule ; V = Vacules

DISCUSSION

This study was aimed to evaluate the nephroprotective effects of methanolic leaf extract of *Ficus bengalensis* against alloxan-induced nephrotoxicity in diabetic rats. Diabetic nephropathy is one of the major complications of diabetes that is associated with the excretion of albumin in urine²⁵. Microalbuminuria and proteinuria typically reflect the presence of moderate and severe lesions, respectively, in kidney disease²⁶. However, the development of diabetic nephropathy is characterised by a progressive increase in urinary protein particularly albumin and a decline in glomerular filtration rate, which eventually leading to end-stage renal failure²⁷.

Experimental diabetes induced by alloxan, selectively destroys the β -cells of pancreas by generating excess reactive oxygen species and produces kidney lesions that are similar to human diabetic nephropathy²⁸. In the present study, induction of diabetic nephropathy was evidenced by elevated levels of urinary total protein, urinary albumin, serum creatinine, BUN and decreased creatinine clearance, which were taken as direct *in vivo* index for nephropathy. In the present study, Alloxan induced diabetic rats exhibited significant increase in blood glucose level. Chronic treatment of diabetic rats with MEFB reduced blood glucose level in duration dependent manner indicating its potent anti hyperglycemic activity which contributes at least in part in delaying the progression of diabetic nephropathy.

Hyperglycemia leads to an increased production of glomerular matrix proteins, the accumulation of which decreases the surface area for filtration leading to decreased glomerular filtration rate (GFR). Decreased GFR is associated with the formation of reactive oxygen species²⁹ that induce oxidative stress which is key pathogenic factor in the development of diabetic complications including diabetic nephropathy³⁰. Hyperglycemia is also responsible for increased oxidative stress in the kidney which induces apoptosis that contribute to the development of diabetic nephropathy³¹. Hyperglycemia induced oxidative stress caused by free radical generation and decrease antioxidant defense system which, has been assessed to estimate the degree of oxidative stress³². Our study showed increased oxidative stress as demonstrated by increase in level of lipid peroxidation products such as MDA and decrease in SOD, GSH and catalase activity in kidneys of diabetic untreated group. MEFB treatment restored the

levels of MDA, SOD, GSH and catalase close to normal control values, which confirms that antioxidant potential of MEFB is responsible for renal protective activity.

Urinary total protein and albumin, which are generally considered as markers of renal function³³, were increased and creatinine clearance was decreased in diabetic rats. Decrease in urinary albumin, serum creatinine and BUN observed in MEFB treated groups with improvement in urinary clearance of creatinine indicates that MEFB ameliorated the loss of renal function and glomerular hyperfiltration in diabetic rats. Magnitude of urinary protein level is further associated with a graded increase in the risk of progression to end stage renal disease and cardiovascular event³⁴. Treatment with MEFB in Alloxan diabetic rats showed significant reduction in urinary protein level which indicate that MEFB may have ability to delay the end stage renal disease and associated cardiovascular complications.

The increase in glycosylated haemoglobin was also observed in alloxan diabetic group. It has been previously reported that the elevation of glycosylated hemoglobin beyond 7% generally leads to diabetic nephropathy³⁵. Treatment with MEFB showed a marked improvement in the glycosylated haemoglobin levels which demonstrates its role in delaying the progression of diabetic nephropathy.

The Decrease in the body weight of Alloxan induced diabetic rat is possibly due to dehydration, increase in muscle wasting³⁶ and catabolism of fats and proteins³⁷. In our study, marked increase in kidney weight was observed which is in agreement with previous studies³⁸. The relative kidney weight in diabetic rats was significantly increased than normal rats. MEFB treatment significantly restored body weight to kidney weight ratio which confirms that MEFB has preventive effect on kidney hypertrophy.

Alloxan induced diabetic rats showed marked increase in food intake and water intake when compared to normal rats, which could be due to poor glycemic control³⁹. Treatment with MEFB in diabetic rats normalized the food and water intake which is because of its ability to improve glycemic control.

The histological features found from the tissue sections of different groups and the Photomicrographs of tissue sections of kidney were presented. The histopathology of tissue sections suggest that the control group had encountered vast histological damages as evidenced by the glomerular and tubular congestion with abnormal Bowman's capsule, blood vessel congestion, epithelial cell

desquamation, and presence of tubular cast. Inflammatory cells were also seen in kidney section from the Alloxan-treated group. In Alloxan group, mononuclear cells infiltrated mainly in the sub-capsular region and interstitial oedema was also noticed. Hyaline changes, vacuolization and necrosis in the proximal tubular epithelial cells were also seen. Concurrent treatment with MEFB (200 mg/kg) was found to reduce such changes in kidney histology induced by Alloxan.

The histological features of the MEFB (400 mg/kg) treated group showed minimal cellular damage in contrast to the control group. The standard drug Glibenclamide showed almost normal glomerular and tubular arrangements with minimal blood vessel congestion, epithelial cell desquamation, and presence of tubular cast with very few inflammatory cells.

CONCLUSION

We concluded that the Methanolic Leaf extract of *Ficus benghalensis* was found to effectively improve the renal function and ameliorate lesions associated with diabetic nephropathy in alloxan-induced nephrotoxicity rats. This was shown by improved activities of metabolic enzymes and recovered renal cells from injuries in diabetic rats. The proposed mechanisms for renal protective activity of MEFB are due to its major components viz. Flavonoids and tannins. These results could further suggest that possible use of Methanolic Leaf extract of *Ficus benghalensis* as a nutraceutical supplement to cope with diabetic-induced detrimental effects and to protect renal cells from damages.

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