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Beneficial effects of *Terminalia phillyreifolia* (Van Heurck & Müll.Arg.) Gere & Boatwr. bark extract in streptozotocin induced hyperglycaemia and diabetic nephropathy in rats

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ABSTRACT

Introduction: Renal damage is a common clinical manifestation in diabetic patients. Therefore, nephroprotective effect is a desirable property for an antidiabetic agent. Natural compounds belonging to *Terminalia phillyreifolia* (TP) owing to their potent antioxidant and anti-inflammatory activity may prove to be such agents.

Objectives: The objectives of the present study were to evaluate methanolic extract of TP bark for its antihyperglycemic, antioxidant and nephroprotective effect in streptozotocin (STZ) induced diabetes mellitus and nephropathy in rats.

Materials and Methods: Diabetic male Wistar rats were divided into five groups; namely, normal control, disease control, standard (NPH insulin, subcutaneously), BE100 (bark extract 100 mg/kg, p.o.) and BE300 (bark extract 300 mg/kg, orally). Treatment was continued for 8 weeks. Plasma glucose levels, oxidative stress parameters, serum creatinine levels, blood urea nitrogen (BUN) levels, per day urine output, urinary protein excretion (UPE) and kidney hypertrophy index were determined at appropriate time points.

Results: Untreated animals developed severe hyperglycemia and major disturbance in renal function. Rats in standard and BE treated groups had significantly lower plasma glucose levels and oxidative stress markers as compared to disease control animals. BE rats also exhibited nearly normal urine volumes indicating better glomerular filtration rate. They had lower urinary protein, serum creatinine, BUN levels and lower renal hypertrophy index as compared to untreated animals.

Conclusion: TP Bark extract corrected the hyperglycaemia and exerted protective effect against diabetes induced renal damage in rats, which may be partly due to its anti-oxidant effect. Therefore, TP extract can be further evaluated as potential antidiabetic therapy.

Implication for health policy/practice/research/medical education:

This study provides a scientific basis for the age-old use of the plant *Terminalia phillyreifolia*. In this study we evaluated its effect on animal model of diabetic nephropathy. Untreated diabetic animals in this study developed renal damage associated with diabetes mellitus, while animals which were treated with *T. phillyreifolia* extract, demonstrated lower blood glucose levels and preserved kidney function. Thus, our study demonstrated the antidiabetic and nephroprotective effect of *T. phillyreifolia*. The plant extract can be further studied for identification and isolation of active constituent that is responsible for its beneficial effect. It can be the next successful anti-diabetic agent from plant origin subject to scientific findings.

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Introduction

Diabetes mellitus is a chronic disorder of carbohydrate metabolism. Poorly controlled blood glucose levels damage vital organs like the kidney (1). However, factors other than hyperglycaemia also play a significant role in inflicting tissue damage leading to diabetic nephropathy (2). Therefore, a drug that exerts supplementary effects like antioxidant or anti-inflammatory over and above antihyperglycemic action, may give better therapeutic outcomes. The bark extract of *Terminalia phillyreifolia* [synonym; *Anogeissus acuminata* (Roxb. ex Candolle)] has shown hypoglycaemic effect in alloxan induced diabetes mellitus (3). It has shown potent anti-oxidant effects in several studies (4, 5). It has rich flavonoid and tannin content (6), some of which compounds have demonstrated beneficial pharmacological actions. Thus, if used for treatment of diabetes mellitus, it may have renal protective effect and may prevent development of diabetic nephropathy in long-term. Therefore, in the present study, we evaluated the effect of TP bark extract on streptozotocin (STZ) induced diabetes mellitus and its renal complications.

Objectives

The aim of this study is to evaluate the hypoglycaemic and renoprotective effect of TP bark extract against STZ induced diabetic nephropathy in rats.

Materials and Methods

Experimental animals

Adult male Wistar rats were housed in polypropylene cages in standard conditions of light, temperature and humidity. They were allowed standard lab diet and water ad libitum. CPCSEA guidelines were followed for care and use of experimental animals.

Plant material

Terminalia phillyreifolia bark was collected from outskirts of Khedbrahma, Gujarat. The herbarium of collected material was authenticated at NISCAIR, Delhi (Ref No. NISCAIR/RHMD/consult/2013/2290/70). The material was dried in shade and ground to coarse powder. Fifty grams of bark powder was extracted with methanol (200 mL × 3) in a Soxhlet extractor. The filtered extract was dried in vacuum drier and stored at 4°C till use. A suspension was prepared using acacia as suspending agent for oral administration. It was evaluated at two dose levels, selected on the basis of previous studies (3, 7).

Induction of diabetes

Rats were fasted for 6 hours prior to administration of STZ (Himedia Labs, Mumbai). STZ was prepared in citrate buffer (pH: 4.5) and administered at a dose of

50 mg/kg intraperitoneally. Post injection animals were supplied with 10% glucose solution overnight in place of drinking water. Blood glucose levels of the animals were tested with a glucometer (One Touch Glucometer, Johnson and Johnson Ltd). Rats with blood glucose level > 250 mg/dL were selected for the study.

Experimental design

Hyperglycemic animals were divided into 4 groups of six animals each. Normal animals of the similar age and weight were kept in the normal control group. Treatment was continued as follows for 8 weeks.

- Group I: Normal Control (NC): 1mL Blank acacia suspension, orally
- Group II: Disease Control (DC): 1 mL Blank acacia suspension, orally
- Group III: Standard (Std): Human NPH Insulin (4 IU/kg/d), subcutaneously
- Group IV: (BE100): Bark extract suspension, 100 mg/kg bw, orally
- Group V: (BE300): Bark extract suspension, 300 mg/kg bw, orally.

Biochemical parameters

Plasma glucose levels (PGLs) were determined from blood collected from retro-orbital plexus of the rat at 0, 1, 2, 4 and 8 weeks after starting the treatment. PGL was determined by glucose oxidase method using Selectra ELITech Clinical Systems fully automated biochemistry analyser.

Collection of urine and determination of urinary protein concentration

At 8 weeks animals were placed in metabolic cages for collection of urine. Solid particles from collected urine were removed by centrifugation. Volume of collected sample was recorded as 24 hr urinary volume. Urinary protein excretion (UPE) was measured using sulfosalicylic acid method (8). Standard plot was prepared by taking absorbance (at 500 nm) of reaction mixtures of albumin at different concentrations as mentioned in Table 1. From test tube number 1-5, 1.25 mL was transferred in test tubes labelled a-e respectively. To each of these test tubes a-e, 3.75 mL of 3% sulfosalicylic acid solution was added. Absorbance was recorded after 5 minutes at 500 nm. A graph of concentration versus absorbance was plotted. R² value and formula for line was determined using Microsoft Excel, 2010.

For test samples, 1.25 mL of filtered urine was mixed with 3.75 mL of 3% sulfosalicylic acid solution in the test tube. Blank solution was prepared by mixing 3.75 mL of 0.9% NaCl solution instead of sulfosalicylic acid. Absorbance was measured after 5 minutes in a

Table 1. Preparation of standard curve for sulfosalicylic acid

Test tube no.	Volume of albumin 10 mg/mL solution added (mL)	0.9% NaCl (mL)	Final Protein Concentration (g/L)
1	0.05	9.95	0.05
2	0.1	9.9	0.1
3	0.2	9.8	0.2
4	0.5	9.5	0.5
5	1.0	9.0	1.0

spectrophotometer at 500 nm. Concentration of protein in urine sample was calculated using formula derived from standard curve.

Determination of BUN and serum creatinine levels

Blood samples were collected at 8 weeks from overnight fasted rats. Blood urea nitrogen (BUN) and serum creatinine levels were determined using Selectra ELITech Clinical Systems fully automated biochemistry analyser.

Kidney weight to body weight ratio

At the end of 8 weeks, animals were sacrificed, kidneys were isolated and weighed. Kidney weight to body weight ratio was calculated using the following formula:

$$\text{Kidney weight/100 g of body weight} = \frac{\text{Kidney weight (g)} \times 100}{\text{Body weight (g)}}$$

Determination of oxidative stress parameters

Serum catalase (9), Serum malondialdehyde (MDA) (10) and reduced glutathione (11) levels were measured at 2 and 8 weeks using suitable methods.

Ethical issues

The project was approved by Institutional Animal Ethics Committee of Parul Institute of Pharmacy. Prior to the experiment, the protocols were confirmed to be in accordance with the guidelines of Animal Ethics Committee of Parul Institute of Pharmacy (921/PO/ReBi/S/05/CPCSEA) and also in accordance with The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. This study is a part of a PhD thesis of Gujarat Technological University

(<http://hdl.handle.net/10603/192049>).

Statistical analysis

The observations were expressed as mean \pm SEM (Standard Error of Mean). Further, statistical analysis was performed by either One-way ANOVA or two-way ANOVA, which was followed by appropriate post hoc test, using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA). *P* values <0.05 were considered as significant.

Results

Effect on blood glucose levels of diabetic rats

Diabetic rats had significantly elevated PGL as compared to untreated diabetic rats (DC). Insulin as well as BE treatment resulted in consistently lower PGL (Table 2).

Urinary volume and proteinuria

DC resulted in severe polyuria at two weeks, while there was a significant fall in urinary volume at 8 weeks (4.92 \pm 0.60 mL) indicating loss of glomerular filtration rate (GFR) owing to nephropathy. Urinary volume in BE treated animals at 8 weeks was significantly higher (8.30 \pm 0.86 and 8.78 \pm 0.44 mL for BE100 and BE300 respectively) as compared to DC rats. There was also higher UPE in DC rats (6.51 \pm 0.21 mg/d) indicating significant tubular damage at 8 weeks. BE treatment resulted in significantly lower levels of proteinuria (3.60 \pm 0.19 and 1.76 \pm 0.22 mg/d for BE100 and BE300 respectively) (Figure 1).

Serum creatinine and BUN levels

BE treatment resulted in lower serum creatinine

Table 2. Effect of BE treatment on plasma glucose levels of diabetic rats

	NC	DC	Insulin	BE100	BE300
0 week	83.67 \pm 4.0	405.5 \pm 35.4 ⁵	440.0 \pm 11.0	344.0 \pm 34.79	369.83 \pm 20.9
1 week	84.83 \pm 3.1	368.0 \pm 9.4 ⁵	159.0 \pm 2.2***	154.67 \pm 18.48***	137 \pm 17.9***
2 weeks	88.0 \pm 2.0	363.0 \pm 15.3 ⁵	161.83 \pm 5.9***	158.17 \pm 14.16***	138.67 \pm 18.8***
4 weeks	85.67 \pm 2.5	367.5 \pm 14.3 ⁵	172.67 \pm 8.9***	165.5 \pm 20.73***	142.17 \pm 19.1***
8 weeks	83.33 \pm 1.5	357.5 \pm 8.6 ⁵	161.5 \pm 7.0***	166.83 \pm 9.06***	146 \pm 12.9***

Values are expressed as Mean \pm SEM, N=6.

⁵ Value differs significantly from corresponding normal control group (*P* < 0.01).

* Significantly different from diabetic control (*P* < 0.05), ** (*P* < 0.01), *** (*P* < 0.001).

(0.89 ± 0.10 and 0.68 ± 0.10 mg/dL for BE100 and BE300) and BUN (128.5 ± 12.9 , 107.6 ± 11.7 mg/dL for BE100 and BE300) levels as compared to those in disease control (DC) animals (1.09 ± 0.03 mg/dL and 189.3 ± 13.4 mg/dL for Creatinine and BUN respectively) (Figure 1).

Effect on kidney weight to body weight ratio

Persistently elevated blood glucose levels resulted in kidney hypertrophy and fluid accumulation indicated by increased kidney weight to body weight ratio (0.94 ± 0.04). While this ratio was significantly lower in BE treated groups (0.65 ± 0.06 and 0.62 ± 0.08 for BE100 and BE300) (Figure 2).

Effect of oxidative stress parameters in diabetic rats

Oxidative stress was highly increased in diabetic rats at both time points. At 8 weeks, BE treatment could significantly reduce MDA level and increase catalase and GSH levels, while insulin treatment could also reverse changes except in catalase (Table 3).

Discussion

Diabetes mellitus results in major disturbance in metabolism and energy homeostasis. This stimulates the hunger drive mediated via hypothalamus resulting in hyperphagia in animals. Such increased energy intake however, fails to have metabolic benefits on the body due to lack of insulin action resulting in weight loss or lack of normal weight gain. Diabetic animals in our study demonstrated similar effect of weight loss in spite of increased food intake. However, animals treated with TP bark extract lacked any notable derangements in food intake and body weight.

Hyperglycemia also results in polydipsia. Accordingly, disease control rats in our study had increased water intake parallel to their elevated blood glucose levels, while, BE treatment largely prevented such disturbance in water intake.

Hyperglycemia is the main pathological factor causing target tissue damage in diabetic patients causing long-term complications. Elevated blood glucose levels promote non-enzymatic glycation of vital molecules including proteins resulting in AGE (advanced glycation end-product) formation. Such AGEs play a key role in disturbance of tissue homeostasis. Hyperglycemia also increases formation of reactive oxygen species (ROS) due to derangement of electron transfer mechanism of mitochondria (12). These ROS cause damage leading to inflammation and cell death. STZ injection resulted in hyperglycemia in all our experimental animals. However, BE treatment could significantly lower blood glucose levels in a dose dependent manner. In previous

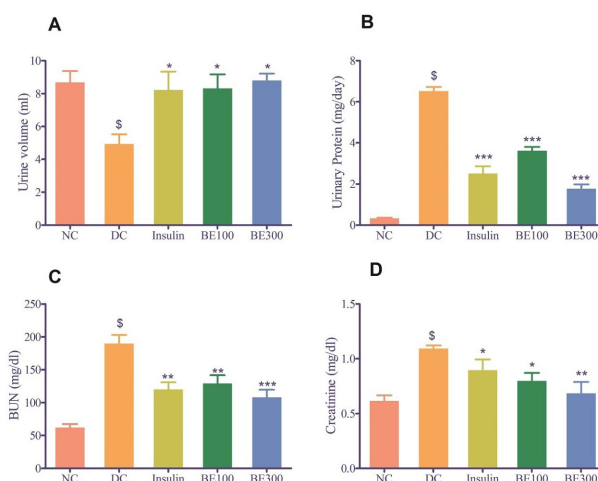


Figure 1. Effect of *Terminalia phillyreifolia* bark extract on (A) urinary volume (B) urinary protein (C) BUN and (D) serum creatinine level in diabetic rats at 8 weeks. Data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. *** Values differ significantly from diabetic control (DC) group ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$). [§] Value differs significantly from normal control (NC) group ($P < 0.001$). Each point represents mean \pm SEM of 6 experiments. NC: normal control animals, DC: diabetic control animals, BE100: diabetic animals treated with bark extract (100 mg/kg, p.o./day), BE300: diabetic animals treated with bark extract (300 mg/kg, p.o./day).

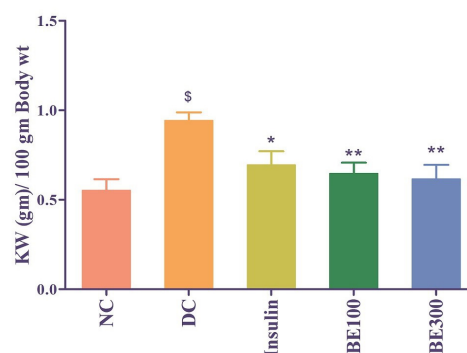


Figure 2. Effect of *Terminalia phillyreifolia* bark extract on relative kidney weight in diabetic rats at 8 weeks. Data were analysed by one way ANOVA followed by Tukey's multiple comparison test. ** Values differ significantly from diabetic control (DC) group ($P < 0.01$), * ($P < 0.05$). [§] Value differs significantly from normal control (NC) group ($P < 0.001$). Each point represents mean \pm SEM of 6 experiments. NC: normal control animals, DC: diabetic control animals, BE100: diabetic animals treated with bark extract (100 mg/kg, p.o./day), BE300: diabetic animals treated with bark extract (300 mg/kg, p.o./day).

study by Manosroi et al, TP bark extract demonstrated glucose lowering effect that was comparable to insulin (1.1 fold) (3). In a study by other investigators also TP extracts demonstrated a potent hypoglycemic effect (13). However, ours is the first study to demonstrate the consistent hypoglycemic effect of TP bark extract on STZ induced DM over long treatment period.

Diabetes induced by STZ in rats is known to develop

Table 3. Effect of BE treatment on oxidative stress parameters of diabetic rats

Parameter	Time	NC	DC	Insulin	BE100	BE300
MDA levels (nmol/mL)	2 week	1.60 ± 0.49	4.50 ± 0.56 [§]	2.27 ± 0.62*	1.93 ± 0.37*	2.13 ± 0.52*
	8 week	1.97 ± 0.79	5.27 ± 0.62 [§]	2.9 ± 0.59*	2.73 ± 0.92*	2.37 ± 0.54**
Catalase (U/mL)	2 week	0.773 ± 0.07	0.336 ± 0.03 [§]	0.603 ± 0.09**	0.651 ± 0.05**	0.693 ± 0.07***
	8 week	0.81 ± 0.04	0.466 ± 0.03 [§]	0.57 ± 0.06	0.68 ± 0.06*	0.738 ± 0.06**
GSH levels (µg/mL)	2 week	11.81 ± 1.79	5.81 ± 0.76 [§]	9.25 ± 2.20	9.90 ± 0.86	10.82 ± 0.62*
	8 week	11.39 ± 0.80	4.45 ± 0.74 [§]	9.97 ± 1.36**	9.57 ± 1.40*	9.82 ± 1.38*

Values are expressed as mean ± SEM, n = 6.

[§] Value differs significantly from corresponding normal control group ($P < 0.01$)

* Significantly different from diabetic control ($P < 0.05$), ** $P < 0.01$, *** $P < 0.001$.

features of nephropathy at approximately 8 weeks; therefore, we continued the treatment with test agents for 8 weeks to observe its effect on development of diabetic nephropathy. Hyperglycemia and other pathological factors like oxidative stress, lipid peroxidation, damage by advanced glycation end products and inflammatory cytokines cause substantial kidney damage resulting in loss of nephrons. This causes reduction in functional capacity of kidney causing decrease in urine formation. The same effect was observed in rats of disease control group, who had highly diminished urine formation. BE treatment could prevent such fall in renal function indicating a protective effect against diabetic renal damage.

Renal tubular damage due to hyperglycemia and other factors also results in loss of barrier function of tubular and glomerular membrane (14). It causes disturbance of ability of glomerular barrier to prevent protein excretion based on charge and size selectivity (15). Such renal damage is clinically reflected as microalbuminuria initially, which further progresses to macroalbuminuria and finally to end-stage renal disease. Thus, UPE is an important marker for diagnosis and staging of renal damage (16). BE treated rats had significantly low UPE as compared to untreated diabetic rats. BE300 treatment was able to reduce UPE even lower than that in insulin treated animals. This also indicates that additional mechanism (e.g. antioxidant action) other than hypoglycemic action may be partly responsible for nephroprotective effect of BE.

Serum creatinine and BUN levels are sensitive markers of kidney function, which were both significantly reduced by BE treatment. This indicates a protective effect of BE on the renal function.

Inflammation triggered by glucose toxicity, ROS and AGE, results in edema and fluid accumulation in the kidney. Chronic inflammatory changes result in glomerulosclerosis and tubulointerstitial fibrosis (17). There is deposition of myofibroblasts and extracellular matrix stimulated by activation of various growth factors such as platelet-derived growth factor and transforming growth factor-beta and interleukin-1 resulting in reduced excretion efficiency of the kidney (18). These changes

are reflected in elevated relative kidney weight. The same pathology was also observed in untreated diabetic animals, which had increased kidney to body weight ratio. Both standards as well as test treatment could reduce this increased kidney weight, however, it was lowest in BE300 treated animals.

A better effect of BE300 on all parameters of nephropathy, in spite of nearly equal hypoglycemic efficiency to insulin, indicates additional mechanism of BE in preventing renal damage. TP extract has demonstrated potent antioxidant effect in several *in vivo* and *in vitro* studies (3-5). In the present study it was able to reduce oxidative stress in diabetic animals. Lipid peroxidation is an important mechanism that leads to cell membrane damage triggering apoptosis or necrosis (19). MDA is the end-product and sensitive marker of lipid peroxidation. BE treated animals had lower levels of MDA indicating less lipid peroxidation. Catalase and GSH are enzymatic antioxidants, the levels of which are depleted in DC animals due to uncontrolled oxidative stress. Such depletion in antioxidant reserve of body was not observed in BE treated animals, indicating a potent antioxidant effect. Thus, antioxidant effect in addition to hypoglycemic effect of the BE may be potentially responsible for its nephroprotective effect. Our previous study using leaf extract of the plant also demonstrated a potent antidiabetic and renoprotective effect (20). Thus, further study is required that investigates the active principle involved in the beneficial effects in diabetic animals and its mechanism of action.

Conclusion

Methanolic extract of TP bark demonstrated blood glucose lowering action comparable to insulin. It also protected against development of diabetic nephropathy in present animal model, which may be at least partially due to its anti-oxidant action. However, it needs to be further evaluated for its other potential mechanisms.

Authors' contribution

AMN was the principal investigator of the study. DJP and ANP were involved in study design and other technical

inputs. All authors were involved in preparation, review, editing of the manuscript. ANP proofread the manuscript. All authors have reviewed and agreed upon the content of the manuscript and confirmed the accuracy and integrity of the work.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication and animal use) have been fully considered by the authors.

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