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ORIGINAL ARTICLE

Evaluation of Ethanolic Extract of *Millingtonia hortensis* leaves by Streptozotocin Induced Diabetic Nephropathy and Diuretic Activity in Wistar Rats

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ABSTRACT
Diabetes mellitus is characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic complication of Diabetic nephropathy may be a result of both type 1 and type 2 diabetes mellitus. STZ is a sensitive inducer of the organ lesions in rodents. It destroys pancreatic b-cells inducing hyperglycemia and renal injury. Streptozotocin used for inducing diabetics, metformin used as standard for anti-diabetic effect and furosemide used as standard for diuretic activity. To prevent side effects by therapeutic drugs herbal medicines are used. Ethanolic extract of Millingtonia hortensis used at dose of 200mg/kg and 400 mg/kg and compared with standard control and screened for its diabetic nephropathy activity by using streptozotocin induced diabetic rat model. Activated glycated end products are found in almost all tissues examined from streptozotocin induced diabetic rats. It was capable of decreasing proteinuria, AGEs, blood glucose levels, normalizing the cholesterol and triglycerides levels, ameliorated the serum parameters. Diuretic activity by using furosemide inducing diuresis in rats and increases the urinary volume and electrolyte (sodium and potassium) excretion in urine. The present study revealed that ethanolic extract of Millingtonia hortensis possesses significant diabetic nephropathy and diuretic activity.
KEYWORDS: Hyperglycemia, AGEs, sensitive inducer, proteinuria and ameliorated

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INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1]. Animal models for type 1 diabetes range from animals with spontaneously developing autoimmune diabetes to chemical ablation of the pancreatic beta cells. Type 2 diabetes is modelled in both obese and non-obese animal models with varying degrees of insulin resistance and beta cell failure [2]. Diabetes Mellitus is probably one of the oldest diseases known to man [3]. The synergistic effect of diuretics and drugs that block the renin-angiotensin system makes them an integral part of most modern antihypertensive regimens in diabetic nephropathy.

The chronic complication of Diabetic nephropathy may be a result of both type 1 and type 2 diabetes mellitus [4]. DN is characterized by structural and functional changes like Mesangial expansion, thickening of the basement membrane, glomerulosclerosis (Kimmelstiel–Wilson nodules) and functionally, there's early glomerular hyperfiltration, increased albumin excretion and with advancing nephropathy, increasing proteinuria and declining GFR [5] [6]. The synergistic effect of diuretics and drugs that block the renin-angiotensin system makes them an integral part of most modern antihypertensive regimens in diabetic nephropathy [7]. In spite of the availability of therapeutic agents which retard the progression of diabetic nephropathy, there has been renewed interest in the use of herbal medicines in order to prevent the genesis of this complication [8].*Millingtonia hortensis* Linn a large ornamental tree of Southern Asia is cultivated in various part of India belong to family Bignoniaceae.

Millingtonia hortensis is used as antifungal, antimicrobial, Larvicidal, hepatoprotective and antioxidant [9-12]. The leaves of *M. hortensis* are used as antipyretic, sinusitis, cholagogue and tonic in folklore medicine [13].The aim of the present study is to investigate Diabetic nephropathy and diuretic activity.

MATERIAL AND METHODS

Plant collection and drying

The leaves of *Millingtonia hortensis* was collected from Hyderabad district, telangana in the month of October and was authenticated by botanist harikrishna from Osmania University. The leaves were dried under shade at room temperature for about 20 days and coarsely powdered in a mixer grinder. The powdered material was stored or taken up for extraction process.

Preparation of *Millingtonia hortensis* leaves extract

Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extractor for several times. This process involves extraction followed by evaporation of the solvent. The vapors of the solvent are taken to a condenser and condensed liquid is returned to the drug for continuous extraction.

Preliminary phytochemicals screening

The extract was subjected to preliminary phytochemical screening to identify various phytoconstituents present in *Millingtonia hortensis.*

Acute toxicity testing

Acute toxicity study was carried out in order to check the toxic effects for ethanolic extract of *Millingtonia hortensis* leaves.The study was performed as per Organization for Economic Cooperation and Development (OECD), Up and down procedure (OECD guideline-425) acute toxicity studies. The Limit test is a sequential test that uses a maximum of 5 animals. A test dose of 2000, or exceptionally 5000 mg/kg, may be used. The selection of a sequential test plan increases the statistical power and also has been made to intentionally bias the procedures. Female mice were used for this purpose. The animals were fasted for 3-4 hours, providing only water, after which the extract was administered to the respective groups orally at the dose level of 2000 mg/kg body weight by gastric intubation and the groups were observed continuously for 24 h for behavioral, neurological and autonomic profiles, and then at 24 h and 72 h for any lethality. If mortality is not observed at all, the EEMH is considered as non-toxic.

Experimental protocol Animal procurement

Wistar albino mice (Approx. 20 to 25 gms) & albino rats (Approx 200-250 gms) were procured from Albino research, Hyderabad. Present study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India. (Reg. No. 1175/Po/Re/S/08/CPCSEA).

Evaluation of Diabetes Nephropathy activity

Streptozotocin induced diabetes model

Streptozotocin is a glucosamine-nitrosourea compound. As with other alkylating agents in the nitrosourea class, it is toxic to cells by causing damage to the DNA. DNA damage induces activation of PARP which is likely more important for diabetes induction than the DNA damage itself. Streptozotocin is similar enough to glucose to be transported into cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporter. This explains its relative toxicity to beta cells, since these cells have relatively high levels of GLUT2.

Method

The Male albino rats weighing 200-250 gm will be selected. Diabetes will be induced by streptozotocin (45 mg/kg *i.p*). Blood glucose level will be measured 48hrs after induction. Rats with blood glucose level above 200 mg/dL were considered as diabetic and were used for further study. Study design for Streptozotocin induced diabetes model Group –I serves as Control (Normal saline) Group –II receive Streptozotocin (45 mg/kg, *i.p*), Group –III and IV receives Streptozotocin (45mg/kg.. *i.p)* with ethanolic extract of *Millingtonia hortensis i*n 200mg/kg, *p.o* and 400mg/kg, *p.o*for 8 weeks and Group –V will receive Streptozotocin (45mg/kg. *i.p*) with standard Metformin (100mg/kg, *p.o*).

Blood glucose levels were measured before treatment and on 1st, 2nd, 4th, 6th & 8th weeks. Body weight of each animal will be determined at the initiation and end of the study. On the completion of 8weeks, blood is withdrawn via retro orbital plexus and biochemical parameters will be estimated [14].

Evaluation of Diuretic activity

Lipschitz model

Furosemide, like other loop diuretics, acts by inhibiting the luminal Na-K-Cl cotransporter in the thick ascending limb of the loop of Henle, by binding to the chloride transport channel, thus causing sodium, chloride, and potassium loss in urine. The action on the distal tubules is independent of any inhibitory

effect on carbonic anhydrase or aldosterone; it also abolishes the corticomedullary osmotic gradient and blocks negative, as well as positive, free water clearance.

Method

Male Albino rats will be used for the study. The animals placed in metabolic cages (1per cage), especially designed to separate urine and feces, and kept at a controlled temperature of 22-25°C [15]. At the end of 6h, the volume of urine collected and measured using pH meter. During this period, no food and water is available to the animals. During the 7days of experimental period, body weight, total urine volume, and concentrations of Na and K concentrations are determined by flame flourimeter.

Study design of Furosemide induced diuretic model is Group –I serves as Control (Normal saline) were as Group –II receives Furesomide (20 mg/kg., *i.p*) for 7 days, Group –III and IV receives ethanolic extract of *Millingtonia hortensis* (200mg/kg and 400 mg/kg, *p.o*) for 7 days.

RESULTS

Ethanolic extract of *Millingtonia hortensis* leaves was explored for its diabetic nephropathy and diuretic activities using suitable animal models was screened. All the results obtained in the studied were included below.

Extractive yield of *Millingtonia hortensis* obtained by soxhlation

The ethanolic extract of *Millingtonia hortensis* leaves was prepared by Soxhlation technique. The percentage yield of the extract was calculated by using the following formula.

% Yield of Extract =Amount of extract obtained /Amount of powder used x 100

% Yield of extract = 120/550 X 100 = 21.8 % w/w

Preliminary Phytochemical analysis

The preliminary phytochemical investigation of ethanolic extract of *Millingtonia hortensis* leaves showed the presence of phenolic compounds, glycosides, flavonoids, terpenoids, tannins, steroids *etc*.

Table 1: Preliminary Phytochemical analysis

Phytochemical Constituents	Results
Flavonoids	++
Phenols	++
Terpinoids	++
Tannins	++
Glycosides	++
Steroids	++

Note: ++ indicates present; - indicates absent.

Acute toxicity studies

Ethanolic extract of *Millingtonia hortensis* leaves was tested on albino Swiss mice up to a dose of 2000 mg/kg bd. wt. The animal did not exhibit any signs of toxicity or mortality up to 2000 mg/kg, bd. wt. various morphological and behavioral characters were observed during the study. The other parameters like food and water consumption were also observed. All the animals were found to be safe even after 14 days of observation. Hence the extract was found to be safe up to 2000 mg/kg, bd. wt. Dose selection: From the above toxicity studies 2000 mg/kg, bd. wt. was found to be safe and the working dose was considered as 1/5th&1/10th *i.e.,* 200 mg/kg, bd. wt. and 400 mg/kg. bd. wt. was selected for the study.

***In vivo* Evaluation of Diabetes Nephropathy activity**

Ethanolic extract of *Millingtonia hortensis* was explored for its diabetic nephropathy activity in Streptozotocin induced rat model. All the results obtained in this study were included below.

Effect of EEMH on Streptozotocin induced diabeticrat

Diabetic nephropathy activity for ethanolic extract of *Millingtonia hortensis* on streptozotocin induced rat model.

Table 2: Effect of EEMH on blood glucose levels in Streptozotocin induced diabetic rats

Groups	Blood Glucose Levels (mg/dL)					
	After induction	1st week	2nd week	4th week	6th week	8th week
Normal control	95.6±0.80	97.6±0.94	98.8±0.94	10±0.966	102.3±0.88	102.5±0.763
Disease control	260±0.96** A	325±0.99 ** Aa	324±0.96** Ab	340±0.99*A	345±0.94**Aa	352±0.97 ** aB
EEMH (200 mg/kg)	257.6±0.8** Aa	208.5±0.76**Aa	203.6±0.88** Ab	190.8±0.945** Aa	184.5±0.763** AB	179.6±0.88**AB
EEMH (400 mg/kg)	259.5±0.7**a	214.8±0.945** AB	189.5±0.763**AB	173.6±0.88** AB	148.8±0.945*Bb	144.6±0.88*Aa
Streptozotocin	263.8±0.7* B	209±0.966**AB	192.8±0.94*B	17±0.966* b	159±0.966**Aa	141.1±0.94**Bb

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared control group (** = p < 0.01, * = p < 0.05), negative control (A = p< 0.01, B= p < 0.05) and standard (a == p< 0.01, b= p < 0.05).

In diabetic group the fasting blood glucose levels was increased after the administration of STZ and it was found to be significant compared to the normal control group throughout 8 weeks. In STZ-diabetic treated group, both the doses of EEMH i.e. 200 mg/kg and 400 mg/kg produced significant reduction of fasting blood glucose levels at the end of 8 weeks. In STZ-diabetic standard group, the fasting blood glucose level was decreased at the end of 8 weeks.

Effect of EEMH on Serum Parameters in STZ diabetic rats

In our experiments, elevated level of biomarkers i.e., serum albumin, serum creatinine and Blood Urea Nitrogen indicated the development of Diabetic nephropathy. Serum triglycerides, serum cholesterol were found to be significantly elevated in diabetic rats as shown in the table 3.

Table 3: Effect of EEMH on Serum Parameters in STZ diabetic rats 3.5 *In vivo* diuretic activity.

GROUPS	SERUM ALBUMIN (MG/DL)	SERUM CREATININE (MG/DL)	BUN (MG/DL)	CHOLESTEROL (MG/DL)	TRIGLYCERIDES (MG/DL)
NORMAL CONTROL	5.36±0.08	1.75±0.09	15.6±0.918	58±0.966	79±0.966
DISEASE CONTROL	2.45±0.07**AA	2.11±0.09** AA	28.1±0.945**AA	80.3±0.88*BB	153.1±0.945** AA
EEMH (200 MG/KG)	3.58±0.09**A	0.88±0.094** BB	22.6±0.988** AA	71.5±0.763**AA	122.3±0.881** BB
EEMH (400 MG/KG)	4.55±0.07* AA	0.85±0.09** A	27±0.966*AB	62.8±0.945* AB	95.8±0.945**A
STREPTOZOTOCIN	5±0.096*B	1±0.096*B	21.8±0.94** A	66±0.966*BB	83±0.966**AA

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared with control group (** = p < 0.01, * = p < 0.05), negative control (A = p< 0.0, B= p < 0.05) and standard (a == p< 0.01, b= p < 0.05).

In STZ-diabetic group the albumin, creatinine, blood urea nitrogen (BUN), cholesterol and triglycerides levels in serum were increased when compared to the control group. The albumin, creatinine, BUN, cholesterol and triglyceride levels in diabetic treated group, at dose of EEMH i.e. 200 mg/kg and 400 mg/kg produced significant decrease compared to control group.

***In vivo* evaluation of diuretic activity**

Ethanolic extract of *Millingtonia hortensis* was explored for its diuretic activity by the Lipschitz Test, has shown a significant diuretic activity by increasing urinary output and increased excretion of sodium, potassium, chloride urinary electrolyte concentrations when compared to control.

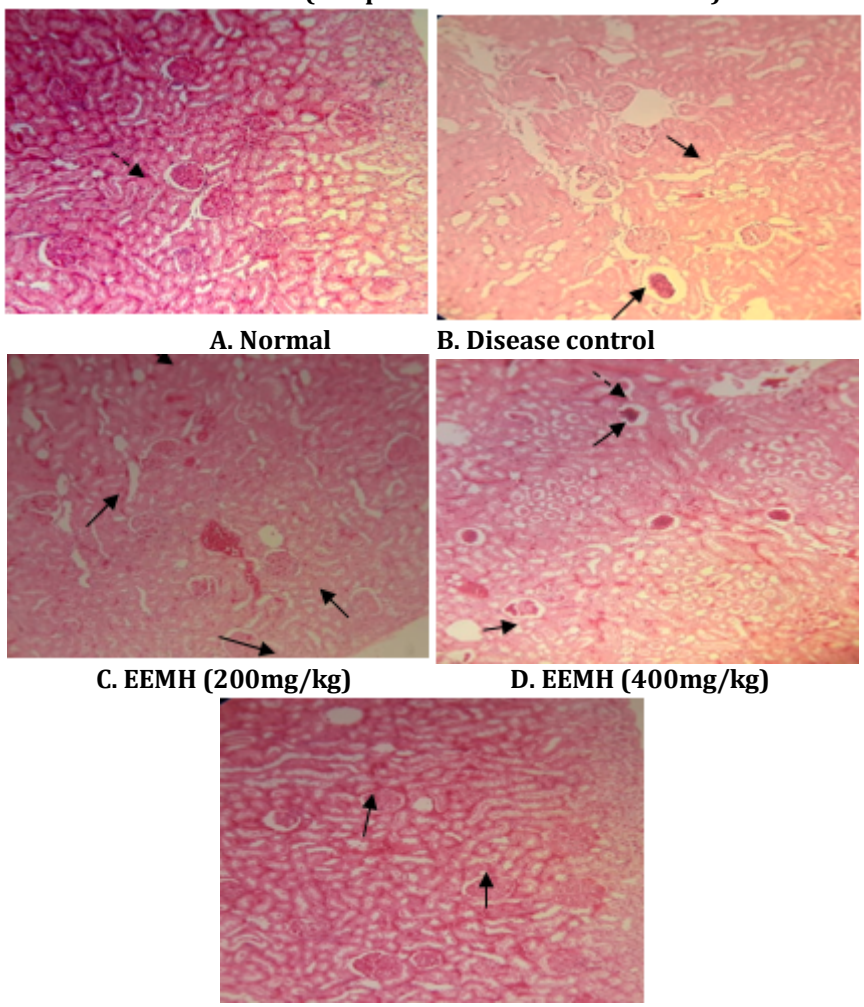
Table 4: Effect of Furosemide and EEMH on Urine Volume

Groups	Urine volume (mL)	Total Na+ (µMoles/kg)	Total K+ (µMoles/kg)
Normal control	9.17±0.10	151.1±13.2	404±4.34
EEMH (200 mg/kg)	13.2±0.19A	202.2±3.21A	106.4±9.1A
EEMH (400 mg/kg)	15.3±0.45A	233.4±0.62*	121.2±13.1A
Furosemide (20 mg/kg)	20.01±0.40*	323.3±4.31*	137.1±8.22*

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared with control group (* = $p < 0.001$, A= $p < 0.001$).

The reference diuretic (furosemide) significantly increased urine output and urinary sodium compared to the normal control group. Administration of the EEMH at dose of 200 mg/kg and 400 mg/kg also resulted in a significant increase in urine volume, urinary sodium concentration and decrease in potassium concentration although less than that found with the furosemide.

HISTOPATHOLOGY OF KIDNEY TISSUE (Streptozotocin induced diabetes)



A. Normal **B. Disease control**

C. EEMH (200mg/kg) **D. EEMH (400mg/kg)**

E. Standard Metformin (100mg/kg)

Figure 3.1: Histopathological analysis of renal tubule and glomeruli for streptozotocin induced diabetic model

DISCUSSION

Diabetic nephropathy activity

In the present study ethanolic extract of *Millingtonia hortensis* was investigated for its diabetic nephropathy activity by using streptozotocin induced rat model [16] and various biochemical parameters like blood glucose level, serum estimation of albumin, creatinine, blood urea nitrogen, cholesterol, triglyceride levels were also investigated.

DN is one of the major complications of diabetes mellitus and a leading cause of end-stage renal failure in the world today [17]. STZ is a sensitive inducer of the organ lesions in rodents. It destroys pancreatic b-cells inducing hyperglycemia and renal injury [18]. During the course of diabetes, excessive formation of AGEs under hyperglycemic condition plays a major role in the pathogenesis of diabetic nephropathy [19]. AGEs are found in almost all tissues examined from STZ-induced diabetic rats[20].

Phytochemical screening of ethanol extract of *Millingtonia hortensis* showed the presence of various phytoconstituents like phenolic compounds, terpenoids, flavonoids, carbohydrates, steroids, tannins and phytosterol are known to reduce diabetic nephropathy activity by decreasing insulin secretion [21],serum AGEs, proteinuria, systolic blood pressure and decreasingthe receptor of AGEs [22]. Thus EEMH exhibited the potential to protect the kidney by decreasing the formation of AGE in the circulation of the STZ diabetic rats

Administration of EEMH (200 and 400 mg/kg) significantly reduced TC, TG, levels. DN is one of the most serious complications of diabetes and it is characterized by elevated the level of serum creatinine, blood urea nitrogen and creatinine. These changes are attributed to persistent hyperglycemia and increased levels of BUN, creatinine, urea and uric acid. Treatment of diabetic rats with EEMH effectively reduced the levels of urea, uric acid, creatinine and BUN indicating their increased clearance from kidney. Histopathogical studies resulted in degeneration of glomeruli in Disease control and it was noted that EEMH group showed normal glomeruli in significant to Standard metformin group. In our present study, oral administration of EEMH significantly reduced the kidney weight/body weight ratio (kidney index) to near normal value.

Diuretic Activity

In the present study ethanol extract of *Millingtonia hortensis* was investigated for its diuretic activity by using Lipchitz test on urinary volume and electrolyte excretion. Diuresis has two components: an increase in urine volume (water secretion) and a loss of solutes (*i.e*. electrolytes) in the urine. These processes may result from suppression of renal tubular reabsorption of water and electrolytes into the blood stream. Administration of the EEMH showed a significant increase in urine output and electrolyte excretion. The reference drug furosemide, like other loop diuretics acts by inhibiting the luminal Na+ and K+ symporter in the thick ascending limb of the Henle loop. By inhibiting the transporter, the loop diuretics reduce the reabsorption of Na+ in the kidney and also diminish the lumen-positive potential that drives from K+ recycling. However, based upon the sodium/potassium excretion ratio of EEMH at 20 mg/kg dose and furosemide respectively, it appears that the EEMH is more potassium sparing than furosemide. These results demonstrate that the ethanolic extract of the leaves *Millingtonia hortensis* has a moderate diuretic activity. In conclusion, the oral administration of the ethanolic extract of *Millingtonia hortensis* increased significantlyin24 h urine volume after treatment.

CONCLUSION

The EEMH was screened for its diabetic nephropathic activity by using streptozotocin induced diabetic rat model. It was capable of decreasing blood glucose levels, normalizing the cholesterol and triglycerides levels, ameliorated the serum parameters. EEMH was screened for diuretic activity using lipschitz model in rats was capable of increasing the urinary volume and electrolyte (sodium and potassium) excretion. The present study revealed that ethanolic extract of *Millingtonia hortensis* possesses significant diabetic nephropathy and diuretic activity. However further studies are required to perform diabetic nephropathy *in vivo* and to isolate the phytochemical constituents responsible for the diabetic nephropathy and diuretic activities and to confirm their exact mechanism of action which will facilitate the use of this plant as better remedy for diabetic kidney disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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