Effect of *Adhatoda zeylanica* Ethanolic Extract on Attenuated Kidney in Streptozotocin-Induced Diabetic Rats

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Abstract

Objective The present study was aimed to evaluate the effect of ethanolic extract of *Adhatoda zeylanica* (EAZ) leaves on streptozotocin (STZ)-induced diabetes mellitus (DM) and its renal complications in male Wistar albino rats.

Materials and Methods Adult male Wistar albino rats were randomly selected from a colony, divided into four groups, namely, A, B, C, and D, with each having six rats (n = 6) and each weighing between 200 and 250 g. Group A served as control and received only water per oral (p.o.). Group B, C, and D animals received a single dose of STZ at 45 mg/kg body weight (kbw) intraperitoneal administration (i.p.) on day 1 and observed for fasting blood glucose (FBG) to induce DM for next 72 hours. After the DM was induced, group B served as DM control, group C received the standard drug glibenclamide (GL) at 5 mg/kbw p.o. once daily, and group D received EAZ of 500 mg/kbw p.o. once daily for 35 days. After the observation period, the animals were euthanized, serum creatinine and blood urea, antioxidants in the kidney tissue homogenate, and histopathological studies were assessed to know the ameliorative effect of the test drugs.

Results Renal parameters, such as serum creatinine, blood urea, antioxidants activities, in group D were nearer to the control when compared with groups B and C. Histopathological studies revealed that there was minimal renal damage in group D when compared with groups B and C.

Conclusion Administration of ethanolic EAZ showed significant ameliorative effects on the FBG, biochemical, oxidative, and histopathological parameters on kidney tissues treated with STZ to induce DM.

Keywords
- diabetic mellitus
- streptozotocin
- *Adhatoda zeylanica*
- glibenclamide nephroprotective

Introduction

Diabetes mellitus is a long-standing disorder manifested by hyperglycemia. Etiology of DM is related to defects in insulin secretion and/or insulin action on cell membranes leading to hyperglycemia.[1] DM has been implicated in several serious health problems including glomerulonephritis, nephropathy, retinopathy, hepatopathy, and cardiomyopathy, all of which can increase the risk of death.[2]

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There is a significant increase in DM-positive population worldwide, and calculations based on a study suggest that there will be 210 million people who will be DM positive during 2013 to 2035 (22 years).[3] Unfortunately, DM in the younger age group has been on the rise, and there is an urgent need to combat this disease. Hyperglycemia can lead to high production of reactive oxygen species (ROS) and a simultaneous reduction of the antioxidant defense mechanisms which can cause oxidative stress. It is proposed that DM makes alterations in the activity of antioxidant enzymes such as catalase, glutathione (GSH-Px) peroxidase, and superoxide dismutase (SOD) that leading to imbalance in oxidative status and damage of tissues.[4] Therefore, hyperglycemia-induced oxidative stress due to the disruption of cellular function and cellular damage has a crucial role in the development and progression of diabetic complications.[5,6]

DM is the leading cause of the end-stage renal disease (ESRD) worldwide, and approximately 40% of ESRD patients were attributed to diabetic nephropathy (DN).[7] The high concentration of blood glucose damages the kidney tissue, leading to altered kidney function in the patients, causing DN and develop an ESRD.[8] The kidney damage in DN is manifested histologically by the thickening of the glomerular basement membrane, mesangial matrix expansion, macrophage infiltration, podocyte loss, and tubular epithelial degeneration.[9] Existing therapy for DM is known to support glycemic control but is believed to do little in regard to the complications to various organs. Besides, these antidiabetic drugs are associated with mild-to-moderate side effects.[10]

Although numerous oral hypoglycemic drugs exist alongside insulin, still there is no promising therapy to cure diabetes. Over the last few decades, the reputation of herbal remedies has increased globally due to its therapeutic efficacy and safety.

Evidence has been established that proper glycemic control and replenishment of antioxidant plays a significant beneficial role in slowing the progression of DN; however, reversal of nephropathy to normal condition is not easy once the duration of DM is prolonged.[11]

In view of this, the present study has investigated the effect of a plant extract in management of DM in streptozotocin (STZ)-induced Wistar rats. STZ is frequently used to induce DM in experimental animals through its toxic effects on pancreatic β-cells and as a potential inducer of oxidative stress. Various studies have been done to establish models of DN in rat using STZ.[12]

The medicinal plant used for this study was *Adhatoda zeylanica* which belongs to family fabaceae and subfamily acanthaceae.

It is popularly and frequently used in Ayurveda and Unani medicines and thus, included in the World Health Organization (WHO) manual, entitled The Use of Traditional Medicine in Primary Health Care.[13]

Adhatoda zeylanica, also known as the Malabar nut tree, is a part of the acanthaceae plant family. It is a common small evergreen, subherbaceous bush distributed throughout India, especially in the lower Himalayas (up to 1,300 m above sea level), India, Sri Lanka, Burma, and Malaysia. In Ayurveda, in the ancient system of Indian medicine, there is a well-known plant in the indigenous system of medicine, commonly known as vasaca.[14] It grows to about a height of 1.5 to 2.0 m with leaves approximately 10- to 15-cm long, 5.0-cm wide, with white or purple flowers, four-seeded fruits. The leaf extract of *A. zeylanica* (EAZ) contains a class of alkaloids, namely, vasicine and vasicinone,[15] and phytochemical, like phenols, tannins, alkaloids, anthraquinones, saponins, flavonoids, amino acids, and it has also been reported earlier that it helps in reducing sugar levels.[16] EAZ has been shown to possess multifarious medicinal properties such as antioxidant,[17] antidiabetic,[18] antibacterial,[19] antimicrobial,[20] anti-inflammatory,[21] and hepatoprotective[22] activities.

However, systemic and scientific reports on the investigation of *A. zeylanica* leaves for its effect on renal function are scarce. In our study, EAZ was evaluated for its nephroprotective effect in STZ-induced DM rats.

Materials and Methods

Plant Material

The leaves of *A. zeylanica* were identified, collected, and authenticated by a botanist. The leaves were dried in the shade and powdered in our research laboratory with the help of a pulverizer. The powder was then subjected to extraction in a Soxhlet apparatus for 72 hours using 90% ethanol at a temperature of 70 to 80°C. The extract was concentrated using a rotary evaporator. The percentage yield of the extract was (9.2% w/w) from dry leaves.[23]

Chemicals

All the chemicals and reagents were purchased from commercial sources and were of analytical grade.

Experimental Animals

The animals were procured and housed in the animal house attached to the Department of Pharmacology, K.S. Hegde Medical Academy, Mangalore. Individual rats were housed in separate polypropylene cages with paddy husk bedding. All animals were maintained under standard laboratory conditions, with a constant 12-hour light/dark cycle and controlled temperature (25 ± 2°C) with access to drinking water and pellet diet ad libitum.

Methodology

Acute Oral Toxicity Study

Acute toxicity for EAZ was performed to know the median lethal dose (LD50) in female adult Wistar albino rats and also for the dose fixation for conducting the further study. Each group consists of six rats. For an overnight fasting rats, a single dose of EAZ was administered per oral (p.o.). Groups 1, 2, and 3 had received 175, 550, and 2,000 mg/kg body weight (kbw) of EAZ, respectively. The rats were monitored continuously for first 4 hours, then hourly on day 1, followed by once daily for next 14 days for the body weight, toxicity signs, and mortality.[24]

Induction of Diabetes

To the overnight fasted rats but with free access to water a single dose of STZ intraperitoneal administration (i.p.) at a dose of 45 mg/kbw, dissolved in 0.1 M Citrate buffer (pH = 4.5).[25] Then 5% sucrose was supplemented for 24 hours to prevent the animals from fatal hypoglycemia. After 72 hours of STZ administration, fasting blood glucose (FBG) level was checked using the (Accu-Chek Active, Roche Diabetes Care, Sandhofer Strasse, Mennheimt, Germany) from the tail vein. The rats with a FBG more than 300 mg/dL were considered diabetic and included in the study.[26,27]

Estimation of effective dose of EAZ in STZ-induced DM in male adult Wistar albino rats was done. Adult male Wistar albino rats were randomly selected from a colony divided into five groups with each having six rats per group. DM was induced in them by administration of single dose of STZ at 45 mg/kbw i.p. to induce DM on day 1. After 72 hours, FBG level was checked for analyzing the DM by using glucometer. After the DM phenotype developed, the rats have received the single dose of test drug orally on day 4, and FBG was repeated on the day 5 to know the effectiveness of EAZ in STZ-induced DM rats. The study chart is mentioned below[27]:

- Group 1: control
- Group 2: STZ-induced DM rats
- Group 3: STZ + EAZ one-fourth of its LD50
- Group 4: STZ + EAZ one-eighth of its LD50
- Group 5: STZ + EAZ one-twelfth of its LD50

Estimation of EAZ Effect on Attenuated Kidney in STZ-Induced DM Rats

Adult male Wistar albino rats were selected randomly from a colony, and they were divided into four groups, namely, A, B, C, and D, with each having six rats (n = 6) and each weighing between 200 and 250 g. They were kept fasting for overnight (but with free access to water). Group A served as control and received only water p.o. Group B, C, and D animals received a single dose of STZ at 45 mg/kbw i.p. to induce DM on day 1. After 72 hours, FBG level was checked for analyzing the DM by using a glucometer. After the DM is induced from day 3, group B animals served as DM control, group C animals received the standard drug glibenclamide (GL) at 5 mg/kbw p.o., once daily for 35 days. Group D received EAZ of 500 mg/kbw p.o. once daily for 35 days. FBS levels were measured on before the experiment began and also on days 4, 19, and 39 of the study.

Sample Collection

At the end of the observation period, the animals were deeply anesthetized with ether. All the animals were observed for any gross/macroscopic pathological changes. Whole blood collected by cardiac puncture, and the serum was separated by centrifugation at 2,000 rpm for 20 minutes for analysis.

Kidneys from the representative groups of animals were removed and weight was noted. One side's kidney tissue was homogenized for the evaluation of the oxidation status and other side's tissue was stored in the formaldehyde for fixation to further histopathological study.

Preparation of Kidney Homogenate

Kidneys were excised and cleaned with ice cold saline and stored at–20°C in freezer. Tissues were thawed and homogenized in phosphate buffered saline, pH of 7.4, centrifuged at 10,000 rpm for 15 minutes using refrigerated centrifuge and supernatant was stored at–20°C. The supernatant was subjected to determination of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) using the method of Mohun and Cook,[28] SOD assay by the methods of Beauchamp and Fridovich,[29] and GSH-Px by Ellman's method.[30]

Renal Function Test

Blood urea (urease, Berthelot's method) and serum creatinine (Jaffe's method) levels were measured using the respective assay kits (Agapee Diagnostic, Bangalore, Karnataka, India) using a semiautomatic biochemical analyzer.

Histopathological Examination

Kidneys were kept in 10% formalin for 24 hours (postfixation). Kidney tissue paraffin blocks and tissue sections were prepared and stained with hematoxylin and eosin by using standard histological procedures. Tubular dilation, tubular degeneration, expansion of Bowman's capsule, interstitial inflammation, and congestion were observed under light microscope.[31]

Ethical Permission

This study was performed at the K.S. Hegde Medical Academy of Mangalore in a CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals)-approved laboratory under registration number 115/1999/CPCSEA, following all ethical practices as laid down in the guidelines for animal care. This study has been approved by the Institutional Animal Ethics Committee (IAEC), KSHEMA/IAEC/31/2011.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) by SPSS, Version 22.0 software where ever it is applicable.

Results

Acute Toxicity Study of EAZ in STZ-Induced DM in Male Adult Wistar Albino Rats

No significant changes in the body weight and acute toxicity signs were noticed at the observation period. It is also noticed that no mortality was observed in animal of all the groups, indicating that toxicity is more than 2,000 mg of EAZ in female adult Wistar albino rats.

Estimation of the Effective Dose of EAZ in STZ Induced DM in Male Adult Wistar Albino Rats

The study found that EAZ at dose one-fourth of its LD50 had shown a significant decrease in the FBG levels in the STZ-induced DM in male adult Wistar albino rats (►**Table 1**; $p < 0.05$).

Effect of EAZ on Attenuated Kidney in STZ-Induced DM Rats

The study showed a significant decrease in the FBG levels in the groups C (80.47%) and D (78.81%) on day 39 in STZ-induced DM rats when the same is compared with group B (►**Table 2**; $p < 0.05$).

The STZ-induced DM rats when administered with the EAZ for consecutive 35 days have shown the significant increase in the concentration of GSH in the kidney tissues when compared with the groups B and C rats (►**Table 3**).

The activities of SOD, GOT, and GPT were significantly decreased ($p < 0.05$) in the group D rats when compared with groups B and C (►**Table 3**).

STZ-induced rats in the groups B and C had shown an increase in serum creatinine and blood urea levels when compared with the control, and significant restoration of these parameters to near normal was noticed in group D (►**Table 4**).

Histological studies in group D have shown minimal damage to kidney tissues'' cytoarchitecture compared with groups B and C (►**Fig. 1**). But the groups B and C had demonstrated moderate-to-severe degenerative features, like dilated tubules, degenerated tubules, glomerular congestion, interstitial inflammatory infiltration, and atrophy of glomerulus, seen with enlarged glomerular space. The degenerated tubule cells with pyknotic nuclei and vacuolated cytoplasm were observed. Sloughing of epithelium was noticed in tubular lumens.

Discussion

STZ is well known for its selective pancreatic islet β-cell cytotoxicity and has been extensively used to induce DM in animals and interfere with cellular metabolic oxidative mechanisms.[32] STZ-induced diabetic rodents are seen to develop kidney disorders similar to the early stage of human diabetic-associated disorders of the kidney.[33] In our study, 45 mg/kbw of STZ through i.p. route effectively induced DM in physiologically normal rats. Pancreatic β-cell degeneration,

Table 1 Fasting blood glucose levels in the streptozotocin-induced diabetes mellitus in male adult Wistar albino rats

Groups	Fasting blood glucose (mg/dL)
1	84.03 ± 14.03
2	372.52 ± 50.53[a]
3	103.43 ± 10.45
4	180.62 ± 30.63[a]
5	270.43 ± 25.72[a]

Note: Fasting blood glucose levels (mg/dL) was assessed in streptozotocin-induced diabetes mellitus rats ($n = 6$) on day 1. Values are expressed as mean ± standard deviation.
[a]Indicates significant difference from the control group ($p < 0.05$).

Table 4 The effect of extract of Adhatoda zeylanica (EAZ) leaves on renal functions

Groups	Urea (mg/dL)	Creatinine (mg/dL)
Normal	27.435 ± 6.017	0.76 ± 0.1
Diabetic control	103.87± 2.39[a]	1.8± 0.16[a]
Diabetic + glibenclamide	61.326± 5.39[a]	1.18± 0.04
Diabetic + streptozotocin	38.58± 1.03	0.82 ± 0.6

Note: The effect of EAZ leaves on renal functions ($n = 6$). Values are expressed as mean ± standard deviation.
[a]Indicates significantly difference from the control group ($p < 0.05$).

Table 2 Fasting blood glucose levels assessed in streptozotocin-induced diabetes rats

Groups	Day 1	Day 4	Day 19	Day 39
Normal (mg/dL)	82.5 ± 20.5	80.4 ± 13.56	87 ± 16.97	79 ± 15.55
Diabetic control (mg/dL)	97 ± 9.89	300.16 ± 10.84[a]	340 ± 48.08[a]	425.40 ± 49.49[a]
Diabetic + glibenclamide (mg/dL)	102.5 ± 7.37	138.35 ± 6.43[a]	85.5 ± 7.77[a]	83.22 ± 19.7[a]
Diabetic + streptozotocin (mg/dL)	98.10 ± 4.43	167.12 ± 12.21	98 ± 11.31	90.12 ± 38.18[a]

Note: Fasting blood glucose levels (mg/dL) was assessed in streptozotocin-induced diabetes rats ($n = 6$). Values are expressed as mean ± standard deviation.
[a]Indicates significant difference from the control group ($p < 0.05$).

Table 3 The effect of EAZ leaves on functional enzymes of rat kidney tissues

Groups	SOD (U/g tissue)	GSH (μmoles/g tissue)	GPT (U/g tissue)	GOT (U/g tissue)
Normal (mg/dL)	312.32 ± 10. 13	2.636 ± 0.380	0.522 ± 0.19	1.695 ± 0.440
Diabetic control (mg/dL)	440.33 ± 25.146[a]	0.240 ± 0.04[a]	0.937 ± 0.031[a]	1.99 ± 0.10
Diabetic + glibenclamide (mg/dL)	327.33 ± 39.79[a]	1.368 ± 0.477[a]	0.586 ± 0.09[a]	1.781 ± 0.159[a]
Diabetic + streptozotocin (mg/dL)	320.03 ± 39.82[a]	1.95 ± 0.31[a]	0.704 ± 0.068[a]	1.720 ± 0.06

Abbreviations: EAZ, extract of *Adhatoda zeylanica*; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; GSH, glutathione; SOD, superoxide dismutase.
Note: The effect of EAZ leaves on functional enzymes of rat kidney tissues ($n = 6$). Values are expressed as Mean ± standard deviation.
[a]Indicates significant difference from the control group ($p < 0.05$).

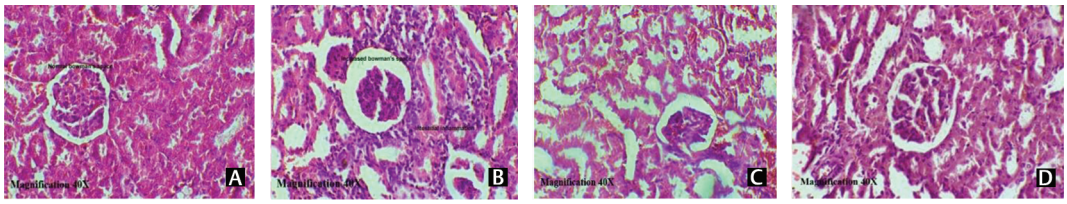

Fig. 1 Histological picture of kidney tissue from the representative animal with hematoxylin and eosin stained; in group B, showing increased Bowman's space, glomerular congestion, atrophy of glomerulus seen with dilated glomerular space and dilated tubules, degenerated tubules, and interstitial inflammatory infiltration. In group C, mildly dilated tubules and degenerative changes of tubular epithelium and slight congestion of glomerular tuft were noticed and in group D, minimal changes in tubule dilation and degeneration with reduced Bowman's space, compared with groups B and C. (magnification × 40).

decreased insulin, and glycogen levels were observed in them. In contrast, *A. zeylanica* leaf extract at the administered dose significantly lowered STZ-induced hyperglycemia, improved insulin, and glycogen contents in extract-treated rats compared with diabetic control animals.

Induction of diabetes in the present study caused a significant increase in antioxidant enzyme SOD activity and a decrease in GSH concentration in the kidney tissue; this result is in accordance with a previous study.[34] Normal GSH is needed to preserve the structural and functional integrity of cells. The decrease in the level of GSH in tissues represents increase in the utilization due to oxidative stress induced by STZ.[35] Tissue protein thiols and disulphides are in equilibrium with the GSH–GSSG (oxidized gluthathione disulfide) redox pair.[36] Since GSH is the first-line defense in scavenging free radicals, the acute hyperglycemic condition and free-radical load formed due to β-cell damage by STZ decreased GSH concentration.

In our study, an increase in the SOD enzyme level was noticed in DM-induced group. That shows, may be healthy rats were used to induced with DM, and the study period was concise. Overexpression of these antioxidant might be an adaption response and could be a protective mechanism against oxidative stress and this data are in line with a previous work.[34,37] SOD is a free-radical scavenging enzyme that has a longer life, and an increase in free radicals induces its gene expression. So, its activity is higher in STZ control. In EAZ-treated rats, the possible decrease in free-radical load has decreased SOD activity.

Evaluation of GOT and GPT enzyme activities in kidney tissues showed significantly increased activity in STZ-treated DM rats compared with group A, but the activity of these enzymes significantly reduced in both the groups C and D. GOT and GPT are housekeeping enzymes in liver and kidney tissues involved in protein metabolism. Alterations in the activity of mentioned enzymes in STZ treated DM rats may be due to metabolic abnormalities or cellular damage.[38] It has been reported that the increase in GOT, GPT, alkaline phosphatase (ALP), and pseudocholinesterase (PChE) activities in the kidney tissues of STZ-treated rats is due to the subtle membrane changes that allow passage to intracellular enzymes to the extracellular space.[39] The present study, significantly reduced the enhanced GOT and GPT activities after the administration of *A. zeylanica* to the diabetic rats. The reversal of renal GOT and GPT activity in *A. zeylanica*–treated DM rats toward average level is evidence of preventing cellular and tissue damage under diabetic conditions.

The kidney damage is reflected by an increase in metabolic wastes such as blood urea and creatinine in the blood. In our study, the STZ-induced DM rats showed significant increase in serum creatinine and blood urea. This proved that diabetic rats have progressed to DN. Increased blood urea and serum creatinine indicate glomerular damage, leading to a decrease in these materials' renal excretion.[40] DM seems to increase ROS production, causing damage to oxygenated mediators, altering glomerular filtration and increasing the permeability of the membrane.[41] However, the diabetic rats that were treated with EAZ showed a significant decrease in serum parameters. Thus, showed that the EAZ supplementation had ameliorated kidney damage in diabetic rats, thus attenuating DN.

In accordance with the results obtained in biochemical analysis, morphological changes were also noticed in the kidney tissues. The histological analysis in DM rats treated with GL and EAZ showed that a nonsignificant change was noticed in the histology of kidney tissue (►**Fig. 1**). Thus, the antioxidant property of the plant extract was demonstrated. Similar findings also saw in many previous histopathological studies.[31,42]

EAZ was able to render nephroprotective effect in these models by attenuating oxidative stress. So, it is speculated that the nephroprotective effect of EAZ might be due to its antioxidant properties. The plant extract might contain bioactive components that have the potential to reverse/prevented the undesirable changes in the kidney associated with hyperglycemia-induced oxidative stress. Thus, a corrective measure, even on the histology, of the kidney was noticed.

Conclusion

Our study demonstrated that the ethanolic EAZ possesses antidiabetic potential in STZ-induced DM rats when administered p.o. for 35 days at the dose of 500 mg/kbw.

The biochemical and histological studies have suggested that oxidative stress plays a prime role in the pathophysiology of diabetic nephropathy, and the EAZ showed the ability to attenuate the renal damage in diabetes through its antioxidant action.

Authors' Contributions

P.D. designed and worked on the experiment, R.H. collected the review of literature and also planned for the

experiment, and G.S. helped in planning and statistical analysis of the work. All the three authors have equally contributed for the overall study.

Conflict of Interest
None declared.

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