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Evaluation of the renoprotective effect of *Embelia tserjiam-cottam* fruits in a rat model of diabetic kidney disease

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ABSTRACT: The objective of the present study was to determine the renoprotective activity of *Embelia tserjiam-cottam* fruit extract in a streptozotocin-induced model of diabetic nephropathy in rats. The renoprotective effect of the ethanolic extract *of Embelia tserjiam-cottam* (EET) fruit (200mg/kg BW and 400 mg/kg BW) was studied by measuring blood glucose levels, body weight, kidney hypertrophy, serum and urine creatinine, urine microalbumin, blood urea nitrogen (BUN) and glycosylated hemoglobin (HbA1C) levels. Levels of enzymes like malondialdehyde (MDA), superoxide dismutase (SOD), glutathione reductase (GSH), and catalase was also measured. Renal histopathology studies were carried out. Treatment with the high dose of EET 400mg/kg BW caused a significant decrease ($p<0.05$) in blood glucose level, a decrease in renal hypertrophy, a decrease in serum creatinine, an increase in urine creatinine, a decrease in urine microalbumin, decrease in BUN and HbA1C. Oxidative stress decreased significantly on treatment with the high dose of the extract as confirmed by the levels of various antioxidant enzymes like MDA, SOD, GSH, and catalase. Treatment with Ramipril 5mg/kg BW also decreased the progression of renal damage. Histopathology studies of the kidneys showed less glomerular damage in the Ramipril and EET 400mg/kg BW groups. A combination of EET 400mg/kg with Ramipril showed better results than treatment with Ramipril alone. The present study has shown the renoprotective activity of the ethanolic extract of *Embelia tserjiam-cottam* fruit. The combination of the extract with Ramipril further attenuated the renal damage, indicating that EET can be used as an adjuvant along with other established agents to prevent the progression of renal damage in diabetic patients

KEYWORDS: Diabetic nephropathy; urine albumin; embeliatserjiam-cottam; renal hypertrophy; oxidative stress.

1. INTRODUCTION

The incidence of diabetes is on an exponential rise worldwide. Approximately 300 million people will be affected by the disease by the year 2025 as per the estimate by WHO. Unrestrained and prolonged hyperglycemia in diabetic patients ultimately leads to kidney damage known as diabetic kidney disease (DKD) or diabetic nephropathy. Among the many causes of end-stage renal disease, diabetic nephropathy encompasses a major part [1,2]. Retinopathy and neuropathy are among the other microvascular complications of diabetes while cardiovascular problems comprise the macrovascular complications. Kidney dysfunction which arises due to persistently elevated blood glucose levels has more than one causative factor. Metabolic (mesangial expansion and mesangial cell matrix production) and hemodynamic (hyperperfusion and hyperfiltration) alterations lead to progressive damage to the kidneys. The involvement of factors like genetic variations, hypertension, dyslipidemia, etc. in the development of diabetic nephropathy has been well documented [3-5]. Prolonged hyperglycemia generates reactive oxygen species (ROS) and free radicals which activate various signaling pathways like hexosamine pathway, protein kinase C, and polyol pathway and lead to the formation of advanced glycation end products. Increased oxidative stress amounts to kidney damage, extracellular matrix production, and basement membrane thickening. Agents which decrease oxidative stress, ameliorate the pathological damages in the kidney which arise due to hyperglycemia [6,7]. Studies have reported that plant constituents like flavonoids, sylimarin, zinc, curcumin, ellagic acid, ginsenosides, rutin, tangeretin, thymoquinone, and many more decrease hyperglycemia, oxidative stress, hyperlipidemia, and improve glycaemic status thereby preventing

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renal damage. Researchers have also reported that several plant extracts act as angiotensin- converting enzyme inhibitors. Some of these plants and plant constituents were found to have beneficial effects on podocyte biology. The extracts successfully prevented the proliferation of extracellular matrix and the thickening of the glomerular basement membrane. Some of these plant extracts also inhibited cytokines like fibronectin, and NF-κβ involved in diabetic nephropathy [8,9,10,11]. Owing to the presence of various active constituents which can affect multiple targets, medicinal plants can be important as treatment or adjuvant in a disease like diabetic nephropathy where the etiology is multifactorial.

Embelia tserjiam-cottam (synonym - *Embelia basaal*) is a shrub that belongs to the family *Myrsinaceae* with many medicinal properties. The common name of *Embelia tserjiam-cottam* is Vidanga. The different parts of the plant are used for various ailments like dental problems, sore throat, pleuritis, worm infections, insanity, and heart diseases [12]. Researchers have reported that the plant has numerous pharmacological properties like antioxidant, anticaries, antimicrobial, hepatoprotective, antidiabetic, and anti-inflammatory [13-16]. The ethanolic and acetone extract from the fruit also caused 100% inhibition of the angiotensin-converting enzyme [17]. However, no study has been carried out so far to check the activity of *Embelia tserjiam-cottam* extract in the treatment of diabetic nephropathy. This study intends to evaluate the renoprotective activity of *Embelia tserjiam-cottam* fruits in a diabetic kidney disease model in rats.

2. RESULTS

Intraperitoneal injection of STZ at 55 mg/kg rendered the animals diabetic and after 72 hours those animals with a blood glucose level greater than 200 mg/dl were made a part of the study. Nephropathy develops after 8 weeks so all analyses were done at the end of 8 weeks.

2.1. Phytochemical test

The phytochemical screening of the ethanolic extract of *Embelia tserjiam-cottam*(EET) was performed as per the given method. The presence of alkaloids, tannins, terpenoids, and phenolic compounds was detected.

2.2. IR spectra

The FTIR of EET was done using an FTIR spectrometer. The FTIR spectrum is shown in Fig.1. and the results for the interpretation of the bands are tabulated in Table. 1

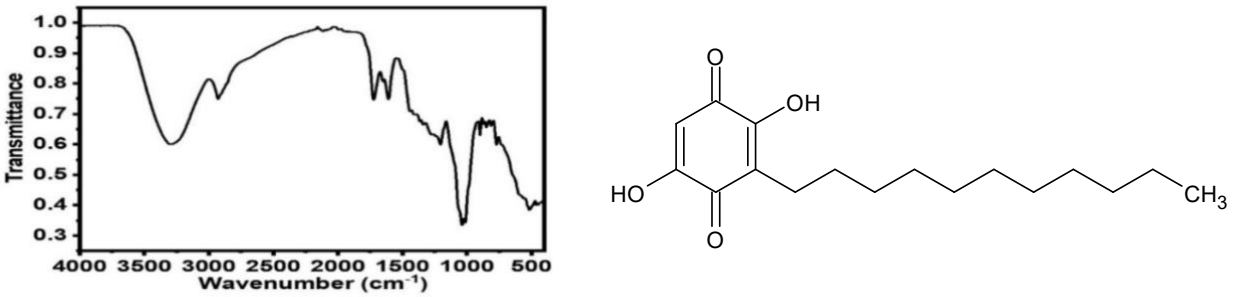

Figure 1. IR spectra of EET and Structure of Embelin (Structure is based on https://en.wikipedia.org/wiki/Embelin)

Table 1. IR bands interpretation.

Functional group	Reported value(cm^{-1})	Observed value(cm^{-1})
-OH	3400-3200	3250
-C=O	1700-1675	1730
	1644-1617	1625
-CH	3000-2840	2900
-CH(bending vibration)	1300-1000	1020,1200
-CH(out-of-plane)	900-690	900,750

2.3. Body weight, Urine volume, and Kidney hypertrophy

In the study, there was a reduction in the body weight of diabetic animals. Ramipril 5 mg/kg treated rats did not produce any change in body weight when compared to the diabetic rats. Treatment with 200mg/kg EET also showed no significant difference in body weight. However, the animals given 400 mg/kg EET had a considerable increase in body weight. Combination of Ramipril with the high dose of the extract produced a further increase in body weight as compared to Ramipril treatment alone (Table 2).

The urine output was higher in the diabetic rats in comparison to normal control showing signs of polyuria. Rats treated with both 200mg/kg and 400 mg/kg of EET showed a significant decrease in urine output in 8th week with the decrease being more in the high dose treated group. Combination treatment of the high dose of the extract with Ramipril caused a further reduction in polyuria.

There was also a considerable decrease in kidney hypertrophy in standard treatment, high-dose EET, and combined standard and high-dose groups.

Table 2. Effect of EET on the weight of animals (g), urine volume (ml), and kidney hypertrophy.

Groups	Body weight	Urine Volume	Kidney hypertrophy
Control	196.67 ± 6.15	10.83±0.48	0.45±0.03
STZ	135 ± 6.19a***	25.67±0.49 a***	1.05±0.04 a***
STZ+STD	148.33 ± 6.01	14.67±0.21 b***	0.8±0.05 b***
STZ+LD	150 ± 3.65	18.83±0.54 b***	0.94±0.04
STZ+HD	173.33 ± 4.22***	15±1.37 b***	0.72±0.04 b***
STZ+STD+LD	152.5 ± 1.12	14.17±0.54	0.6±0.03 c*
STZ+STD+HD	170 ± 1.29a***	11.5±0.62 c*	0.52±0.03 c***

Values indicate Mean±SEM, n=6, a***: p<0.001 compared to normal control, b***: p<0.001, compared to STZ, c***: p<0.001, compared to STZ+STD, c*: p<0.05, compared to STZ+STD

2.4. Results for biochemical parameters

2.4.1. Blood glucose levels

The blood glucose levels were found to be significantly higher in the diabetic rats in the week following induction and throughout the duration of the study (Table 3). Treatment with Ramipril did not produce any decrease in blood sugar levels. Treatment with both high and low dose of EET decreased the blood glucose level with the decrease being more prominent in the high dose group. The combination of the high dose and low dose of EET with Ramipril produced a significant lowering of blood glucose level as compared to Ramipril alone.

Table 3. Effect of EET on blood glucose level (mg/dl).

Groups	1st week	2nd week	4thweek	6th week	8th week
Control	74±1.13	75.33±1.2	73.67±1.28	75.33±1.26	74.83±1.38
STZ	253.33±2.11a***	255.67±0.33 a***	255±1.11 a***	252.67±1.2a***	255.67 ±0.33a***
STZ+STD	254.5±7.11	256.33±0.33	254.67±0.88	255±0.63	253.5±1.12
STZ+LD	242.5±2.81	246.17±0.48 b*	246.5±0.56 b***	237.83±0.95 b***	238±1.00 b***
STZ+HD	235.33±0.21 b**	210±2.58 b***	198.33±1.67 b***	152.83±1.82 b***	145.33±0.21b***
STZ+STD+LD	245.83±2.39	246.83±0.6 c*	246.5±1.09 c ***	238.33±0.95c ***	237.50±1.67c ***
STZ+STD+HD	235.5±0.43 c**	208.33±4.01 c***	198.5±1.5 c***	153.00±1.10 c***	145.17±0.31c***

Values indicate Mean ±SEM, n=6, a***: p<0.001 compared to normal control, b***: p<0.001, compared to STZ, c***: p<0.001, compared to STZ+STD.

2.4.2. Serum and Urine Creatinine

The level of serum creatinine was increased considerably in diabetic rats. In the Ramipril treatment group, the serum creatinine levels decreased when compared to the diabetic rats (Table 4). Treatment with the high dose of EET also resulted in a decrease in serum creatinine levels (p<0.001, compared to diabetic rats). The combination of Ramipril with the high dose of EET led to further lowering of serum creatinine when compared to the Ramipril treatment alone.

The urine creatinine values were significantly lowered in the STZ group compared to the normal control animals. Ramipril and 400 mg/kg of EET resulted in higher urine creatinine levels compared to diabetic animals. When the high dose of the extract was given along with Ramipril it was seen that the urine creatinine levels were increased further.

Table 4. Effect of EET on serum creatinine (mg/dl) and urine creatine (mg/dl).

Groups	Serum Creatinine	Urine creatinine
Control	0.77±0.04	20.02±0.53
STZ	3± 0.08a***	8.37±0.16a***
STZ+STD	1.85±0.08b***	12.3±0.29b***
STZ+LD	2.67±0.08b#	9.48±0.12b#
STZ+HD	2.07±0.07b***	10.75±0.07b***
STZ+STD+LD	1.6±0.11c#	13.53±0.13c*
STZ+STD+HD	1.2±0.06c***	16.38±0.16c***

Values indicate Mean ± SEM, n=6, a***: p<0.001 compared to control, b***: p<0.001, compared to STZ, c***: p<0.001, compared to STZ+STD, c*: p<0.05, compared to STZ+STD

2.4.3. Urine albumin

The level of urine albumin in the STZ group of rats was found to be higher in comparison with the normal control animals (Table.5). Treatment with the standard drug Ramipril and the high dose of EET decreased the urine albumin level compared to the diabetic animals. Combined administration of Ramipril with the high dose of EET led to a further decrease in urine albumin level in comparison to Ramipril treatment alone.

2.4.4. Blood urea nitrogen

The blood urea nitrogen levels in diabetic rats increased considerably in comparison with the normal control rats. Treatment with the standard drug Ramipril and the high dose of EET led to a decrease in BUN levels significantly in comparison to the diabetic rats. Low-dose EET did not cause any change in BUN levels as compared to diabetic rats. The combination of high dose EET and Ramipril produced a further decrease in BUN level when compared to Ramipril treatment alone (Table. 5).

2.4.5. Glycosylated hemoglobin

Glycosylated hemoglobin levels in the diabetic animals were found to be increased (Table. 5). Treatment with Ramipril did not alter glycosylated hemoglobin levels when compared with the diabetic animals. The high dose of EET decreased glycosylated hemoglobin levels significantly. Combination treatment with Ramipril and the high dose of EET decreased glycosylated hemoglobin significantly as compared to treatment with Ramipril alone.

Table 5. Effect of EET on urine albumin(mg/dl), blood urea nitrogen(mg/dl), and glycosylated hemoglobin (%).

Groups	Urine albumin	Blood urea nitrogen	Glycosylated hemoglobin
Control	0.07±0.014	10.73±0.4	4.6±0.29
STZ	0.68±0.03a***	45.87±0.58a***	10.22±0.16a***
STZ+STD	0.41±0.04b***	21.83±0.91b***	9.63±0.18
STZ+LD	0.62±0.01	42.7±0.63	9.4±0.17b*
STZ+HD	0.54±0.02b**	31.9±0.6b***	7.7±0.15b***
STZ+STD+LD	0.36±0.03	22.08±0.84	9.42±0.2
STZ+STD+HD	0.28±0.01c*	15.86±1.01c***	7.62±0.17c***

Values indicate Mean ± SEM, n=6, a***: p<0.001 compared to normal control, b***: p<0.001, compared to STZ, b**: p<0.01, compared to STZ, b*: p<0.01, compared to STZ, c***: p<0.001, compared to STZ+STD, c*: p<0.05, compared to STZ+STD

2.5. Assessment of oxidative stress

The diabetic rats showed an elevation in the TBARS value and reduction in the values of the superoxide dismutase, catalase, and glutathione reductase enzymes (Fig. 2, 3, 4, 5). On treatment with Ramipril and high dose of EET, the level of TBARS decreased whereas levels of GSH, SOD and Catalase showed an increase. Combination of Ramipril with the high dose of EET produced a further decrease in the oxidative stress as compared to Ramipril given alone. Treatment with low dose of EET caused a slight increase in the levels of SOD and catalase when compared to the diabetic rats. Combination treatment with Ramipril and low dose EET caused an increase in the SOD and catalase levels somewhat when compared to Ramipril treatment alone.

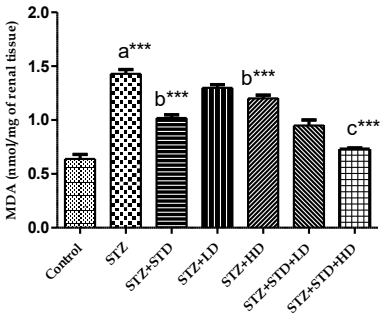

Figure 2. Effect of EET on MDA levels. Values are compared to normal control and indicate: Mean±SEM, n=6, a***: p<0.001, b***: p<0.001, compared to STZ, c***: p<0.001, compared to STZ+STD.

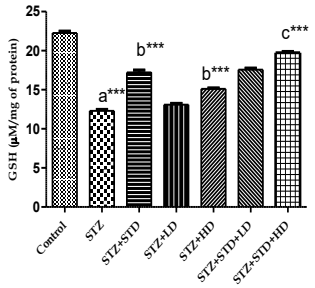

Figure 3. Effect of EET on GSH levels. Values are compared to normal control and indicate;: Mean±SEM, n=6, a***: p<0.001, b***: p<0.001, compared to STZ, c***: p<0.001, compared to STZ+STD.

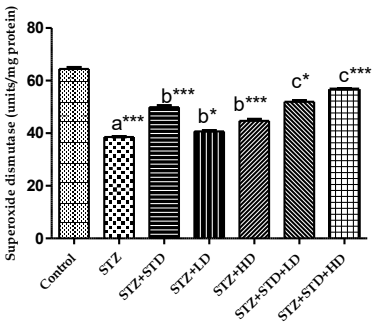

Figure 4. Effect of EET on SOD levels. Values indicate Mean±SEM, n=6, a***: p<0.001 compared to normal control, b***: p<0.001, compared to STZ, b*: p<0.05 compared to STZ, c*: p<0.05, compared to STZ+STD c***: p<0.001, compared to STZ+STD.

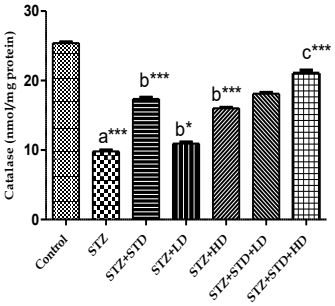

Figure 5. Effect of EET on Catalase levels. Values indicate Mean±SEM, n=6, a***: p<0.001 compared to normal control, b***: p<0.001, compared to STZ, b*: p<0.05 compared to STZ,c*: p<0.05, compared to STZ+STD c***: p<0.001, compared to STZ+STD.

2.6. Renal histopathology

The histopathology picture of the vehicle control group showed normal size of glomeruli, no dilation of subcapsular space, and no vacuolar degeneration (A). The renal histopathology studies in the diabetic group showed enlarged glomeruli with dilated subcapsular space and vacuolar degeneration (B). Treatment with Ramipril prevented the glomerular enlargement but there was some dilation of subcapsular space (C). The high dose of EET also prevented the structural change in the kidney (E) to some extent while the low dose of EET did not show any effect (D). The combination of Ramipril with 400 mg/kg EET was the most effective in preventing glomerular enlargement and preserving the normal architecture of the kidneys (G).

3. DISCUSSION

Long term hyperglycemia related kidney complications are a major health risk for diabetic patients and in the long run, it could culminate in total kidney failure. In diabetic nephropathy, there is an interplay of numerous factors like abnormal glucose metabolism, hypertension, disturbances in lipid metabolism, oxidative stress; involvement of vasoactive substances, chemokines, growth factors, etc. Diabetic nephropathy is marked by abnormalities in hemodynamic mechanisms and pathological changes like glomerulosclerosis. Hyperfiltration and microalbuminuria are seen during the initial stages of diabetic nephropathy. Early treatment can prevent the progression of the disease and hence effective intervention at this stage can be lifesaving [33].

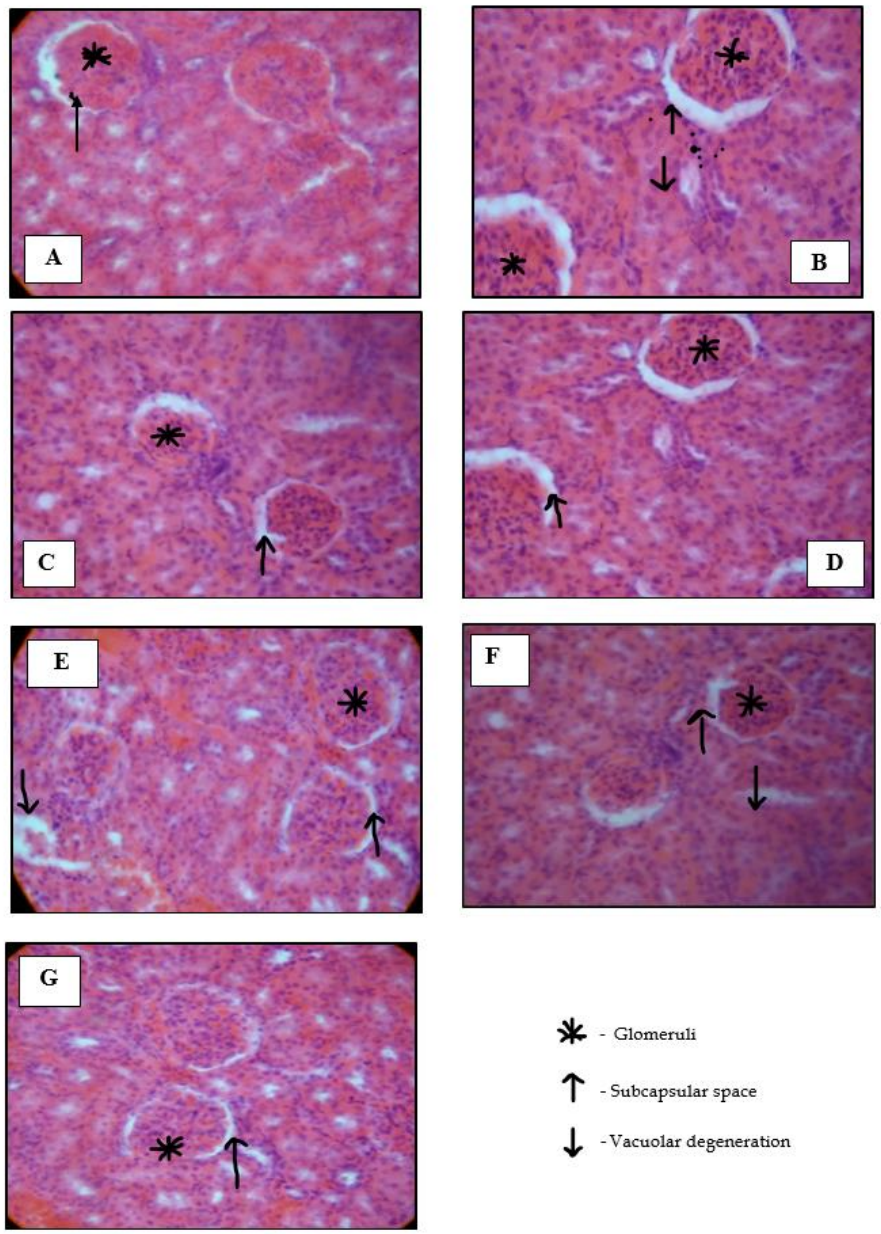

Figure 6. Histopathology picture of rat kidney (H&E stained renal tissue-magnification 40X). **A.** Normal control- Normal architecture of glomeruli with normal subcapsular space. **B.** Glomeruli enlarged with dilated subcapsular space and vacuolar degeneration. **C.** Normal architecture of glomeruli with slight dilation of subcapsular space. **D**. Enlarged glomeruli with vacuolar degeneration. **E.** Slightly enlarged glomeruli with some vacuolar degeneration but normal subcapsular space. **F.** Glomerular architecture is mostly normal with some dilation of subcapsular space and slight vacuolar degeneration is seen . **G.** Glomerular architecture and subcapsular space normal.

Although therapeutic agents have been studied for diabetic nephropathy treatment none of them have been completely successful. Also, most of these drugs may cause serious toxicities. Owing to the presence of various phytochemicals which possess multiple therapeutic properties medicinal plants may be promising agents for the treatment of diabetic kidney disease [34].

Streptozotocin-induced diabetic nephropathy is a well-established rodent model which mimics the changes seen in the initial stages of the disorder. Streptozotocin destroys the pancreatic beta cells selectively with very little effect on other organs. Nephropathy develops in 4-8 weeks and can be measured by various biochemical parameters like urine albumin, serum and urine creatinine, blood urea nitrogen, body weight, kidney hypertrophy, and kidney histopathology to name a few [35].

Streptozotocin treatment leads to the development of severe hyperglycemia. Hyperglycaemia stimulates many pathways. Activation of the polyol pathway causes a rise in sorbitol levels, contributing to the microvascular complications of diabetes [36]. The Diabetic Control and Complications Trial (DCCT) concluded that tight control of blood glucose is important to prevent microvascular complications [37]. Treatment with the high dose of EET showed a decrease in glucose levels. The low dose of EET also produced some reduction in blood glucose levels but the decrease was not as significant as the high dose of EET. Treatment with Ramipril however did cause any reduction in glucose level indicating that Ramipril does not possess any antihyperglycemic action. Combination of Ramipril with EET did not produce any additional lowering of blood levels of glucose.

Impaired creatinine levels are one of the predictive markers for progressive decline in kidney function. Many studies have pointed towards a correlation between microalbuminuria and impaired creatinine levels with deteriorating filtration properties of diabetic kidneys [38]. In the present study high dose of EET and a combination treatment of a high dose of EET with Ramipril successfully reversed the impaired creatinine profile.

In diabetes, there is also loss of body weight due to the catabolic effects causing a decrease in muscle mass [39]. In this work, there was a reduction in the body weight of the diabetic group. Treatment with high dose of the plant extract prevented the decrease in body weight which can be attributed to its antihyperglycemic and hence anticatabolic effects. However, neither Ramipril treatment nor treatment with low dose of the extract affected the body weight. The lack of effect of Ramipril on body weight reduction maybe because it does not possess any antihyperglycemic effect.

Kidney hypertrophy is also a common feature seen in diabetic kidneys. Hyperfiltration and hypertrophy are the forerunners of further structural damage in the kidney. Hypertrophy may occur as a result of activation of growth factors, increase in protein synthesis, or decline in degradation of extracellular components [40]. The present study revealed a significant increase in renal hypertrophy in diabetic animals. In the treatment groups, there was a considerable reduction in hypertrophy. However, low dose of EET extract did not produce any significant decrease in renal hypertrophy. Thus, treatment with high dose EET and Ramipril successfully halted the initial structural changes. Combination treatment of Ramipril with high dose EET yielded better results. Polyuria was seen in the diabetic rats indicating hyperfiltration. Treatment with high dose EET reversed the polyuria. Combination of Ramipril with high dose EET showed better prevention of polyuria. It has been well documented that maintaining good glycaemic control averts the increase in glomerular filtration rate and shields against kidney impairment.

Albuminuria is a harbinger of progressive nephropathy. High blood glucose levels damage the tiny filters of the glomerulus and albumin leaks out into the urine. The presence of albuminuria can be very harmful as it indicates worsening renal function. If metabolic control is initiated early progression to renal failure can be prevented. In this study, there was a considerable increase in urine albumin levels in the diabetic group. Treatment with high dose EET reversed the albuminuria to a considerable extent. Combination treatment of Ramipril with high dose EET showed further improvement. Blood urea nitrogen is also elevated during diabetic nephropathy. Diabetic rats showed an increase in blood urea nitrogen levels [41]. There was a reversal of albuminuria and increased blood urea nitrogen levels in the Ramipril treated, high dose and combination treatment groups indicating a renoprotective role.

Glycosylated hemoglobin is an advanced glycosylation end product (AGE). Hemoglobin A1c (Hb A1c) is useful as an indicator of average glycemia. Lower values of Hb A 1c, reduce the risk of complications [42]. In the present study, glycosylated hemoglobin level was elevated in the diabetic control group compared to the normal control. Treatment with high dose of the extract caused a reduction in the levels of glycosylated hemoglobin. Treatment with Ramipril did not lower the glycosylated hemoglobin level as Ramipril does not have any glucose-lowering effect. This shows that Ramipril exerts a renoprotective effect that is independent of hypoglycaemic action.

Hyperglycaemia causes increased levels of reactive oxygen species (ROS), which impairs the antioxidant mechanisms. ROS activates various signaling pathways culminating in extracellular matrix proteins accumulation in the diabetic kidney [43]. Treatment with Ramipril and high dose of the plant extract prevented the decline in antioxidant defense mechanisms. The combination treatment of ramipril with the plant extracts lowered the oxidative stress further. It has been reported that renin angiotensin aldosterone system (RAS) when activated causes worsening of oxidative damage. Coupling of antioxidant mechanism with inhibition of RAS, thus may give better reno-protection.

The hematoxylin and eosin-stained sections of the kidney showed that treatment with Ramipril and high dose of the plant extract had a significant protective action and prevented the pathological changes in the glomerulus and kidney tubules. The glomerular size was reduced and subcapsular space was less dilated. Combination treatment with Ramipril and plant extract led to better preservation of glomerular architecture as compared to the treatments given alone.

It has been reported that ethanolic and acetone extracts of the fruits of *Embelia tserjiam-cottam* inhibit angiotensin converting enzyme (ACE) to the extent of 100% [17]. The inhibition of ACE is reported to halt the progression of renal damage in diabetic patients [44]. Embelin which is an important constituent of the plant *Embelia tserjiam-cottam,* causes the downregulation of inflammatory mediators like TNF-α, IL-1β, and IL-6. Administration of embelin also increases insulin sensitivity, and activates expression of GLUT 4 in adipose tissue. The constituent also exerts a protective effect on pancreatic β cell by decreasing the generation of ROS [45,46]. *Embelia tserjiam-cottam* affects many molecular targets implicated in diabetic nephropathy. The IR spectra of EET showed functional groups similar to that present in the structure of Embelin. Therefore, there is a high possibility that the activity of EET is owing to the presence of Embelin in it.

In this work, the high dose of the plant extract showed good antihyperglycemic action. There was a significant decrease in oxidative stress in rats that received Ramipril and high dose of the plant extract. Ramipril and high dose of the extract also prevented the impairment of various important biochemical markers like urine albumin, serum, urine creatinine, glycosylated hemoglobin, etc. Less pathological abnormalities were seen in the glomeruli of the rats which received Ramipril and high dose of EET. Moreover, combination of Ramipril with high dose of EET resulted in better renoprotective action indicating a synergistic action. Therefore, we can conclude that the ethanolic extract of *Embelia tserjiam-cottam* has good renoprotective action and can be used as an adjuvant to the conventional therapies used presently for diabetic nephropathy. It can also be used as a combination treatment with antidiabetic drugs.

4. CONCLUSION

The results obtained from the study indicate that as hypothesized the ethanolic extract of *Embelia tserjiam-cottam* fruits has good activity in preventing kidney damage due to diabetes. There is a convincing possibility that this renoprotective action is due to the antihyperglycemic, antioxidant, and ACE inhibitory properties of the plant extract. This activity can be attributed to the presence of Embelin in the plant extract as predicted from the IR data.

5. MATERIALS AND METHODS

5.1. Plant material

The fruits of *Embelia tserjiam-cottam* were obtained from a local supplier in Patiala, Punjab. The fruits were authenticated by Dr. Sunita Garg, Head, Raw materials herbarium and Museum, CSIR-NISCAIR, Delhi.

5.2. Extraction

The fruits were washed properly with water and dried in shade before being subjected to size reduction. The coarsely powdered plant material was extracted with ethanol using the Soxhlet apparatus. The extraction was carried out for a duration of 48 hours. The extract obtained was filtered and the filtrate was dried using a Rotary evaporator. The calculated yield was 8.2%.

5.3. Recording of IR spectra

The IR spectra of EET was recorded using an FTIR spectrometer(Model RZX, Perkin Elmer, USA). Pellets of the extract were prepared using potassium bromide and scanned over a range of 400-4000 cm^{-1}.

5.4. Animals

To conduct the study both male and female Wistar rats weighing 180-220g were selected. Animals were acquired from the animal house (1201/PO/Re/S/08/CPCSEA). The animals were kept under standard housing conditions. Temperature and humidity were maintained as per CPCSEA and IAEC guidelines. The experimental protocol was designed to adhere to the guidelines set by CPCSEA. A light: dark cycle of 12:12 was followed. Acclimatisation period for the animals was 7 days. The animals were given standard rat chow and water on an as and when required basis.

5.5. Phytochemical test

The extract was tested to detect the various phytoconstituents like saponins, carbohydrates, alkaloids, steroids, flavonoids, and phenolic compounds as per the standard methods reported in literatüre [18,19].

5.6. Selection of doses

From literature review it was found that no toxicity was detected with the extract up to a dose of 2000mg/kg body weight (bw). As per OECD guidelines, one-tenth (200mg/kg bw) and one-fifth (400 mg/kg bw) of 2000mg/kg were chosen as the treatment doses.

5.7. Treatment protocol

After acclimatization, the animals were weighed and numbered. The rats were administered 55mg/kg bw of Streptozotocin (prepared in 0.1M citrate buffer at a pH of 4.5) by intraperitoneal injection. Mortality may occur following streptozotocin injection as the pancreas is damaged and it leads to sudden high levels of insulin which may cause severe hypoglycaemia in the animals. To prevent hypoglycaemic mortality the rats were administered 5% w/v solution of glucose in drinking water for 5-6 hours after streptozotocin injection. Fasting blood glucose was then estimated 72 hours after injection of streptozotocin. The estimation of blood glucose was done using a calibrated glucometer. The criteria for selecting the rats for the study was a blood glucose level of 200mg/dl or more. The weight of the animals was then recorded, the animals were numbered and distributed into 7 groups of 8 animals each (Table.6). The experimental procedures were carried out for 8 weeks at the end of which all estimations were done [20,21].

Table 6. Experimental protocol.

Groups	Name	Treatment given	Number of days
I	Vehicle control	Tween 80 (2%v/v) at 10 ml/kg bw, p.o.	8 weeks
II	STZ control	Streptozotocin 55mg/kg bw, i.p.	8 weeks
III	Standard	Streptozotocin 55 mg/kg bw, i.p+5mg/kg bw Ramipril, i.p	8 weeks
IV	STZ+LD	Streptozotocin 55 mg/kg bw, i.p+ Ethanolic extract of *Embelia tserjiam-cottam* (EET) 200mg/kg bw, p.o.	8 weeks
V	STZ+HD	Streptozotocin 55 mg/kg bw, i.p.+ Ethanolic extract of *Embelia tserjiam-cottam* (EET) 400 mg/kg bw, p.o.	8 weeks
VI	STZ+STD+LD	Streptozotocin 55 mg/kg bw, i.p.+ 5mg/kg bw Ramipril, i.p.+ Ethanolic extract of *Embelia tserjiam-cottam* (EET) 200mg/kg bw, p.o.	8 weeks
V	STZ+STD+HD	Streptozotocin 55 mg/kg bw, i.p.+ 5mg/kg bw Ramipril, i.p.+ Ethanolic extract of *Embelia tserjiam-cottam* (EET) 400mg/kg bw, p.o.	8 weeks

5.8. Assessment of weight of animals and volume of urine

The weight of the animals was recorded after the study period. The animals were put inside metabolic cages for a day. The 24-hr urine was collected and the urine volume was estimated [22].

5.9. Assessment of biochemical parameters

5.9.1. Collection of samples for estimation

Twenty-four-hour urine sample was collected by keeping the animals in metabolic cages. Blood was drawn from the animals by cardiac puncture under ether anesthesia for estimation of various parameters.

5.9.2. Biochemical estimations in blood and urine

Blood glucose: The animals were kept on overnight fasting and the blood glucose levels of the animals were estimated on the 10th and 40th day of the study with a standardized glucometer [23].

Serum and Urine creatinine: Estimation of serum and urine creatinine was done by a method of alkaline picrate with the help of a commercially available kit. The procedure involves the reaction of alkaline picrate and creatinine forming a complex that is orange in color. The absorbance of the orange-colored complex was measured at 520 nm using a spectrophotometer (UV-1700 Spectrophotometer, Shimadzu, Japan) [24,25].

Urine Albumin: Urine microalbumin was estimated with the help of a commercially available kit based on the BCG dye binding method [26]. Absorbance of the prepared samples was recorded at a wavelength of 630nm using UV-1700 Spectrophotometer, Shimadzu, Japan.

5.10. Kidney hypertrophy

The extent of kidney hypertrophy was measured after sacrificing the animals at the end of the experiment. To calculate renal hypertrophy the weight of two kidneys was summed. The weight of two kidneys divided by the total weight of animals was taken as an indicator of the degree of hypertrophy [27].

5.11. Measurement of glycated hemoglobin level

Glycosylated hemoglobin levels were estimated with the help of a kit based on the colorimetric assay. The glycosylated hemoglobin with ketoamine bond in hemoglobin is heated in an acidic environment and hexose dewatered partially to form 5-hydroxymethylfurfural. 5-HMF can react further to form a yellow complex [28].

5.12. Renal oxidative stress estimation

The kidneys were removed and the right kidney was used for oxidative stress studies. The kidneys were homogenized in cold potassium phosphate buffer. The homogenates were subjected to cold centrifugation at 5000 rotations per minute for 10 minutes and the superficial layer obtained was utilized for the estimation of:

Thiobarbituric acid reactive substance (TBARS): The lipid peroxidation was measured in terms of concentration of thiobarbituric acid reactive species level [29]. The pink color of the supernatant was measured with a spectrophotometer. Lipid peroxide is expressed as nM of MDA /mg of Renal tissue.

Catalase: The estimation of catalase was done according to a method given by Luck [30]. After centrifugation, the supernatant was added to a reaction mixture. The rate of change of optical density was recorded at a wavelength of 240nm.

Superoxide Dismutase Assay: Assay of superoxide dismutase was carried out as per the procedure given by Kono [31]. The reaction was done by mixing hydroxylamine hydrochloride to nitroblue tetrazolium and the kidney homogenate. The readings were taken at a wavelength of 560 nm.

Reduced glutathione: The method described by Ellman [32] was followed for the estimation of reduced glutathione (GSH). The supernatant obtained after cold centrifugation was mixed with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction yielded a yellow color. The absorbance was read at a wavelength of 412 nm using a spectrophotometer (UV-1700 Spectrophotometer, Shimadzu, Japan).

5.13. Renal histopathology

The left kidney was taken for the purpose of histopathology. The kidney tissue (2-3 mm) was kept in formalin and then dipped in paraffin wax. Thin kidney sections were cut. Kidney pieces were then treated with hematoxylin and eosin (H&E) dye. The thin treated pieces were then studied under a microscope fitted with a camera.

5.14. Statistical Analysis

GraphPad Prism software was used for performing the statistical analysis. The values for mean and standard error of mean (SEM) were computed (n=6). Oneway analysis of variance (ANOVA) was used followed by Tukey multicomparison test to calculate the level of significance.

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