



Effect of *Eugenia Jambolana* on Streptozotocin-Nicotinamide induced type-2 Diabetic Nephropathy in Rats

Godwin Selvaraj Esther^{1*}

Alvin Jose Manonmani²

¹ Research Scholar, PRIST University, Vallam, Thanjavur-613403, Tamil Nadu, India./ Department of Pharmacology, Noorul Islam College of Dental Science, NIMS Medicity, Aralummoodu-695123, Trivandrum, Kerala, India.

² Department of Pharmacology, Swamy Vivekanandha College of Pharmacy, Elayampalayam, Tiruchengode-637205, Tamil Nadu, India.

Corresponding Authors:

Godwin Selvaraj Esther
Department of Pharmacology,
Noorul Islam College of Dental
Science, NIMS Medicity,
Aralummoodu-695123,
Trivandrum, Kerala, India.
E-mail :
godwinpharm79@gmail.com

Abstract:

The chronic type-2 diabetes mellitus leads to diabetic nephropathy, which is one of the major microvascular complication of end stage renal disease worldwide and causes premature death in diabetic patients. The objective of the present investigation was to evaluate the antidiabetic activity and protective effect of diabetic induced nephropathy of ethanolic extract of seeds of *Eugenia jambolana* (SEEJ) by using *in-vitro* and *in-vivo* models. The *in-vitro* antidiabetic effect was studied by glucose uptake assay in lymphocyte culture preparation. The *in-vivo* antidiabetic activity and the effect on diabetic nephropathy was evaluated using streptozotocin-nicotinamide induced type-2 diabetes mellitus in male albino *Wistar* rats. The results of *in-vitro* study revealed that SEEJ increased the percentage glucose uptake when calculated in comparison with control group. The *in-vivo* study showed that blood glucose level was significantly reduced in dose dependent manner when compared to the diabetic control group. In addition, it significantly restored the body weight loss, increased kidney weight, glycosylated haemoglobin, blood urea, blood uric acid, blood urea nitrogen, blood creatinine, urine volume and urine microalbumin levels when compared to diabetic control groups. The report of histopathological study of rat kidney tissues strongly supported the protective effect of SEEJ in diabetic nephropathy. The findings of this investigation concluded that SEEJ has significant antidiabetic activity and potential protective effect in diabetic nephropathy.

Keywords: Diabetic nephropathy, streptozotocin, nicotinamide, *Eugenia jambolana*, glucose uptake.

INTRODUCTION

The world is facing an explosive increase in the incidence of type-2 diabetes mellitus (T2DM), which is a chronic metabolic disease with highest rates of prevalence and mortality affects more than 100 million people worldwide. It is caused by an absolute or relative lack of insulin and/or reduced insulin activity (insulin resistance) ⁽¹⁾. The prevalence rate of diabetes is estimated to be 1-5% in India. The number of people with diabetes in India is currently around 50.8 million and expected to rise to 87 million by 2030 unless urgent preventive steps are taken ^(2,3). Hence India leads the world with largest number of diabetic patients

earning the dubious distinction of being the "diabetic capital of the world" ⁽⁴⁾. Diabetes is now considered to be a vascular disease. Diabetic nephropathy is one of the major microvascular complication of type-2 diabetes mellitus and is the major cause of end-stage renal disease (ESRD). The early changes in diabetic nephropathy are characterized by an increase in kidney size, glomerular volume and kidney function followed by the accumulation of glomerular extracellular matrix, increased urinary microalbumin excretion, glomerular sclerosis and tubular fibrosis. Last stage overt diabetic nephropathy is clinically characterized by proteinuria, hypertension and progressive renal insufficiency ⁽⁵⁾. Diabetic

nephropathy has been a growing threat in the world and Eastern countries are not an exception. In Australia type-2 diabetes mellitus patients starting dialysis increased 5-fold between 1993 and 2007 and in India diabetic nephropathy, is expected to rise to 6.6 million of the more than 100 million patients suffering diabetes. So it is a major cause of morbidity in diabetic patients (5, 6). All of the pharmacological modalities show limited efficacy and certain adverse effects such as hepatotoxicity, lactic acidosis, diarrhoea, obesity or weight loss and attenuation of response after prolonged use, dry cough and are expensive particularly for developing countries like India and China. Comparatively very less side effects and low cost of phytopharmaceuticals from natural resources open new avenues for the treatment of various diseases including diabetes. Therefore there is a need for phytochemicals that have antidiabetic potential, which are cost effective, potent and also safe without long-term side effects (1).

The ethanolic extracts of seeds of *Eugenia jambolana* contain saponins which have reported to show the antidiabetic effect in previous studies, appear to be involved in stimulation of pancreatic β -cells and subsequent secretion of insulin (7, 8). Despite the availability of many antidiabetic medicines in the market, diabetes and its microvascular and macrovascular complications continues to be a major medical problem. Plant derivatives with purported antidiabetic activity are used in folk medicine and traditional healing systems around the world (9). Herbal drugs are prescribed widely even when their biologically active ingredients are unknown (10). Substantial efforts have been made in recent years to identify new natural and synthetic antidiabetic drugs. There is flood of scientific data about medicinal

plants including those with antidiabetic potential (11).

The seeds of *Eugenia jambolana*, belonging to the family of Myrtaceae has been indicated in Ayurveda for its use in diabetes mellitus (12). It is reported to have hypoglycemic (2, 11, 13, 14), hypolipidemic (13), antiulcer (14), antibacterial (15), anti-inflammatory (16), neuropsychopharmacological (17), anti HIV (18) and antidiarrhoeal activity (19).

Although the seeds of *Eugenia jambolana* has been used in traditional medicine (13) yet scientific validation of its use in type-2 diabetes mellitus and its effect on diabetic nephropathy needs to be studied. Hence this investigation was undertaken to evaluate the antidiabetic activity and protective effect of diabetic induced nephropathy of ethanolic extract of seeds of *Eugenia jambolana* (SEEJ) by using *in-vitro* and *in-vivo* models. The *in-vitro* antidiabetic effect was studied by glucose uptake assay in lymphocyte tissue culture preparation. The *in-vivo* antidiabetic activity and the effect on diabetic nephropathy was evaluated using streptozotocin-nicotinamide induced type-2 diabetes mellitus in male albino *Wistar* rats.

MATERIALS AND METHODS

Plant material and extract preparation

The fruits of *Eugenia jambolana* was collected during January 2012, from Kaliakkavilai, Tamil Nadu. It was identified and authenticated by botanist Dr. K. Paul Raj and voucher specimen was deposited in the Herbarium, department of botany, Nesamony Memorial Christian College, Marthandam (NMCC/69/2012). Seed was separated and dried in shade and coarse powdered (2000 gm) in a mixer grinder. It was

extracted with soxhlet using 95% ethanol for 72 hours, concentrated on water bath (70°C), kept in oven (30°C) for drying and stored in desiccator. The yield of ethanolic extract of SEEJ was 36.8 gm (1.84%).

***In-vitro* antidiabetic effect by glucose uptake assay**

Lymphocyte culture preparation

Human peripheral lymphocytes (HPLs) were cultured in Rosewell Park memorial institute (RPMI) 1640 low glucose (Himedia, Mumbai, India) media, supplemented with 10% heat inactivated fetal bovine serum (FBS) (Himedia, Mumbai, India), antibiotics (Penicillin and Streptomycin), Phytohaem agglutinin (PHA) (Himedia, Mumbai, India) was used as the stimulant for cell proliferation. The culture was filtered using 0.2 µm pore sized cellulose acetate filter (Sartorius, Japan) in completely aseptic conditions. Lymphocytes were separated from the blood using Hisep (Lymphocyte separation medium, Himedia, Mumbai, India). 3 ml of lymphocytes were transferred to centrifuge tube with 3 ml diluted blood. It was then centrifuged at 2600 rpm at room temperature for 30 minutes. Centrifugation should sediment erythrocytes and polynuclear leukocytes and band mononuclear lymphocytes above HiSep. The pellets were washed with phosphate buffered saline and diluted to 10⁵ cells/ ml and used for studies⁽²⁰⁾.

Individual cultures were exposed to 300 mM glucose followed by plant extracts in increasing concentrations (50, 100, 200 µg/ml) and incubated for 2 hours. Pioglitazone (PG) was used as the standard drug and a positive control with glucose alone was maintained. The glucose consumption was measured in cell free media using glucose assay kit (Sigma Aldrich, USA) as per

manufacturer's instructions. All experiments were repeated in triplicates and mean average was used for calculations. Briefly cells were collected and spun at 7500 rpm and clear supernatant was collected and used for the assay. Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with O-dianisidine in the presence of peroxidase to form a colored product. Oxidized O-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink color is measured at 540 nm and % glucose uptake was measured using the following formula⁽²¹⁾.

$$\% \text{ Glucose uptake} = \frac{\text{Optical density of control} - \text{Optical density of test}}{\text{Optical density of control}} \times 100$$

***In-vivo* antidiabetic effect of SEEJ against streptozotocin-nicotinamide induced type 2 diabetes mellitus and their effect on diabetic nephropathy**

Animals

Male albino *Wistar* strain rats weight about 180-220 gm were procured from the central animal house of Swamy Vivekanandha College of Pharmacy were used for the study. They were maintained in temperature 21± 2°C, standard laboratory conditions and the relative humidity of 55-60 % with a 12 hour light: 12-hour dark cycle. They were allowed access to food with standard pellet diet and water ad libitum. The study protocol was approved by the institutional animal ethical committee of Swamy Vivekanandha College of Pharmacy, Elayampalayam, Tiruchengode, Tamil Nadu. (Protocol no: SVCP/IAEC/Ph.D/019/Feb/2012) and studies were carried out in accordance with the guidelines of Committee for the Purpose of control and supervision of experiments on animals (CPCSEA), India.

Drugs and chemicals

A gift sample of Pioglitazone was obtained from Sun Pharmaceuticals Industries Ltd., Mumbai, India. The following drugs and chemicals were purchased from various companies: Streptozotocin (Sisco Research Laboratories Pvt. Ltd., Mumbai, India), Nicotinamide (Ranbaxy Chemicals Ltd., Mumbai, India), Carboxyl methyl cellulose (Loba Chemicals Pvt. Ltd., Mumbai, India), Formaldehyde (Nine chemicals Pvt. Ltd., Mumbai, India), Sodium citrate (Loba chemicals Pvt. Ltd., Mumbai, India), Citric acid (Loba chemicals Pvt. Ltd., Mumbai, India), Sodium phosphate monobasic (Loba chemicals Pvt. Ltd., Mumbai, India), Sodium phosphate dibasic (Loba chemicals Pvt. Ltd., Mumbai, India) and used for the study.

Induction of type-2 diabetes mellitus

Streptozotocin was freshly dissolved in (0.1 M, PH 4.5) citrate buffer and nicotinamide was dissolved in normal physiological saline and maintained on ice prior to use. Non-insulin dependent diabetes mellitus (T 2 DM) was induced in overnight fasted rats by a single intraperitoneal (i.p) injection of streptozotocin (4.5 mg/kg, b.w), 15 min after the intraperitoneal administration of nicotinamide (110 mg/kg, b.w). The elevated plasma glucose level was determined on 3rd day of streptozotocin and nicotinamide administration and those rats with fasting glucose levels greater than 250 mg/dl were served as diabetic rats and used in the study. Treatment with SEEJ was started on the third day after streptozotocin and nicotinamide induction and continued for 90 days. Nephropathy was noted in diabetic rats between 4-8 weeks after the administration of streptozotocin and nicotinamide ⁽¹⁾.

Treatment protocol

The animals were separated into 6 groups, containing 6 animals each (n=6); a total of 36 rats (30 diabetic surviving rats, 6 normal control rats) were used. SEEJ was suspended in 1% w/v carboxy methyl cellulose (CMC) in water and administered orally using an intra gastric tube.

Group I : Normal control (1% W/V CMC in water, p.o, 1 ml/100 gm b.w.)

Group II : Diabetic rats treated with 1% W/V CMC in water, p.o, 1 ml/100 gm b.w.

Group III : Diabetic+ Pioglitazone (4.05 mg/kg/day in 1% W/V CMC in water, p.o, 1 ml/100 gm b.w.)

Group IV : Diabetic+ SEEJ (100 mg/kg/day in 1% W/V CMC in water, p.o, 1 ml/100 gm b.w.)

Group V : Diabetic+ SEEJ (200 mg/kg/day in 1% W/V CMC in water, p.o, 1 ml/100 gm b.w.)

Group VI : Diabetic+ SEEJ (400 mg/kg/day in 1% W/V CMC in water, p.o, 1 ml/100 gm b.w.)

The initial and final body weight of various groups were recorded. At the end of 90 days treatment, the 24 hours urine was collected in metabolic cages (Instruments & Chemicals Pvt. Ltd, Ambalacity, India) and the volume of urine was noted in all the groups. Then, it was used for the estimation of urine microalbumin. After that, in overnight fasted animals, blood samples were collected in tubes containing EDTA by cardiac puncture after anaesthetizing them using ketamine hydrochloride (24 mg/kg, b.w, i.m injection) and used for the estimation of blood glucose, glycosylated haemoglobin

(HbA_{1c}), blood urea, blood uric acid, blood urea nitrogen (BUN) and blood creatinine levels. Blood samples were collected from retro orbital sinus and blood sugar levels were estimated in every 15 days throughout the 90 days study. The urine and blood parameters were evaluated using semi auto analyzer - Mind Rays Ba-88a., (Mind Rays

Medical India Pvt. Ltd., Mumbai, India) following suitable methods.

Histopathological study of kidneys

After 90 days of treatment, the anaesthetized rats were sacrificed by cervical decapitation and kidneys were excised quickly and stored in 10% buffered formalin solution (formaldehyde, 100 ml; sodium phosphate monobasic, 4 gm; sodium phosphate dibasic, 6.5 gm; and water, 900 ml) and subjected to further processing for histopathological studies.

Statistics

All the data were expressed as mean \pm SEM. The One-way analysis of Variance (ANOVA) followed by Tukey's multiple comparison test was used to analyze the statistical significance for the effect of different doses of SEEJ when compared to control with the help of Graph pad InStat software, version 3.01; values are considered statistically significant when $P < 0.05$.

RESULTS

In-Vitro antidiabetic effect by glucose uptake assay method in lymphocyte culture preparation

The percentage glucose uptake of SEEJ 50 $\mu\text{g/ml}$ was 22.46 %, 100 $\mu\text{g/ml}$ was 40.18 % and 200 $\mu\text{g/ml}$ was 42.08 %. Pioglitazone 6 $\mu\text{g/ml}$ showed 74.3 % glucose uptake (Figure 1).

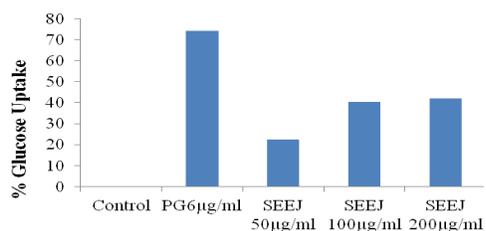


Figure 1: Effect of SEEJ on % glucose uptake, PG: pioglitazone

In-vivo antidiabetic effect of SEEJ and its effect on diabetic nephropathy

The effect of SEEJ on body weight and kidney weight

Body weight: After 90 days of treatment the final body weight of diabetic control rats were significantly ($P < 0.001$) decreased when compared to the final body weight of normal control rats. In animals treated with pioglitazone 4.05 mg/kg, the final body weight was significantly ($P < 0.001$) increased when compared to the final body weight of diabetic control group. In diabetic animals treated with SEEJ 100 mg/kg ($P < 0.001$) SEEJ 200 mg/kg ($P < 0.001$) and SEEJ 400 mg/kg ($P < 0.001$), the final body weights were significantly increased when compared to the final body weight of diabetic control animals (Table 1).

Kidney weight: The kidney weight of diabetic control group increased significantly ($P < 0.001$) when compared to normal control group. Diabetic rats treated with SEEJ 200 mg/kg ($P < 0.001$) and SEEJ 400 mg/kg ($P < 0.001$) significantly decreased the kidney weights when compared to diabetic control rats (Table 1).

Table 1: The effect of SEEJ on body weight and kidney weight

Groups	Treatment	Mean body weight (gms)		Mean kidney weight (gms/100gm body weight)
		Initial	Final (After 90 days of treatment)	
I	Normal control	199.17 \pm 5.54	250.83 \pm 4.36	0.700 \pm 0.03
II	Diabetic control	200.00 \pm 6.19	175.83 \pm 5.69 ***	1.303 \pm 0.06***
III	Diabetic+ P.G. 4.05mg/kg	203.33 \pm 4.41	260.00 \pm 4.28###	1.280 \pm 0.06
IV	Diabetic+ SEEJ (100mg/kg)	201.67 \pm 5.58	214.17 \pm 5.07 ###	1.356 \pm 0.08
V	Diabetic+ SEEJ (200mg/kg)	198.33 \pm 6.15	255.00 \pm 5.77 ###	0.745 \pm 0.03###
VI	Diabetic+ SEEJ (400mg/kg)	207.5 \pm 5.12	252.67 \pm 5.43 ###	0.735 \pm 0.02###

$n=6$, The values are expressed as mean \pm SEM; *** $P < 0.001$ when compared to normal control group ### $P < 0.001$ when compared to diabetic control group.

The effect of SEEJ on blood parameters

Blood glucose levels: In diabetic control rats, the fasting blood glucose level was significantly (P<0.001) increased on the 3rd day after streptozotocin-nicotinamide administration and was maintained same in every fifteen days of blood glucose analysis till the 90th day of treatment schedule when compared to normal control rats. When compared to diabetic control rats, pioglitazone 4.05 mg/kg significantly (P<0.001) reduced the fasting blood glucose level

from the 15th day onwards, until the completion of 90 days treatment. In the diabetic rats treated with SEEJ 100 mg/kg the fasting blood sugar level was significantly decreased (P<0.05) on 60 days treatment, (P<0.01) on 75 days treatment and (P<0.001) on 90 days treatment. Diabetic rats treated with SEEJ 200 mg/kg and SEEJ 400 mg/kg the fasting blood glucose level was significantly decreased (P<0.001) from 30 days to 90 days treatment when compared to diabetic control rats (Table 2, Figure 2).

Table 2: The effect of SEEJ on blood glucose levels (mg/kg)

S. No.	Treatment	Mean blood glucose levels (mg/dl)							
		0 day	3 rd day	15 th day	30 th day	45 th day	60 th day	75 th day	90 th day
I	Normal control	91.63 ± 0 5.73	91.76 ± 03.97	89.48 ± 04.66	93.05 ± 06.25	86.35± 04.42	91.63± 05.62	90.50 ± 04.26	90.77 ± 05.61
II	Diabetic control	99.15 ± 06.57	351.10 ± 27.31 ^{***}	390.37 ± 19.47 ^{***}	395.27 ± 15.36 ^{***}	401.82 ± 17.93 ^{***}	416.9 ± 16.41 ^{***}	421.82 ± 18.17 ^{***}	422.17 ± 21.27 ^{***}
III	Diabetic + P.G 4.05 mg/kg	95.02 ± 04.76	363.43 ± 19.09	301.38 ± 05.04 ^{###}	256.27 ± 08.84 ^{###}	215.45 ± 11.49 ^{###}	122.80 ± 05.80 ^{###}	120.42 ± 03.59 ^{###}	98.17 ± 01.82 ^{###}
IV	Diabetic + SEEJ (100mg/kg)	98.02 ± 05.93	397.83 ± 15.17	382.52 ± 14.55	367.43 ± 14.43	346.13 ± 10.87 [#]	339.58 ± 10.68 ^{###}	332.77 ± 11.42 ^{###}	327.12 ± 11.35 ^{###}
V	Diabetic + SEEJ (200mg/kg)	102.62 ± 04.30	405.33 ± 020.81	349.88 ± 016.36	276.72 ± 015.09 ^{###}	191.97 ± 05.71 ^{###}	134.02 ± 02.60 ^{###}	122.25 ± 03.07 ^{###}	104.7 ± 04.56 ^{###}
VI	Diabetic + SEEJ (400mg/kg)	99.22 ± 06.33	418.57 ± 17.01	348.48 ± 15.05	288.22 ± 14.72 ^{###}	217.78 ± 15.62 ^{###}	150.83 ± 07.19 ^{###}	117.88 ± 04.38 ^{###}	102.58 ± 04.58 ^{###}

n=6, The values are expressed as mean ±SEM; ^{***}P<0.001 when compared to normal control group, [#]P<0.05, ^{##}P<0.01, ^{###}P<0.001 when compared to diabetic control group.

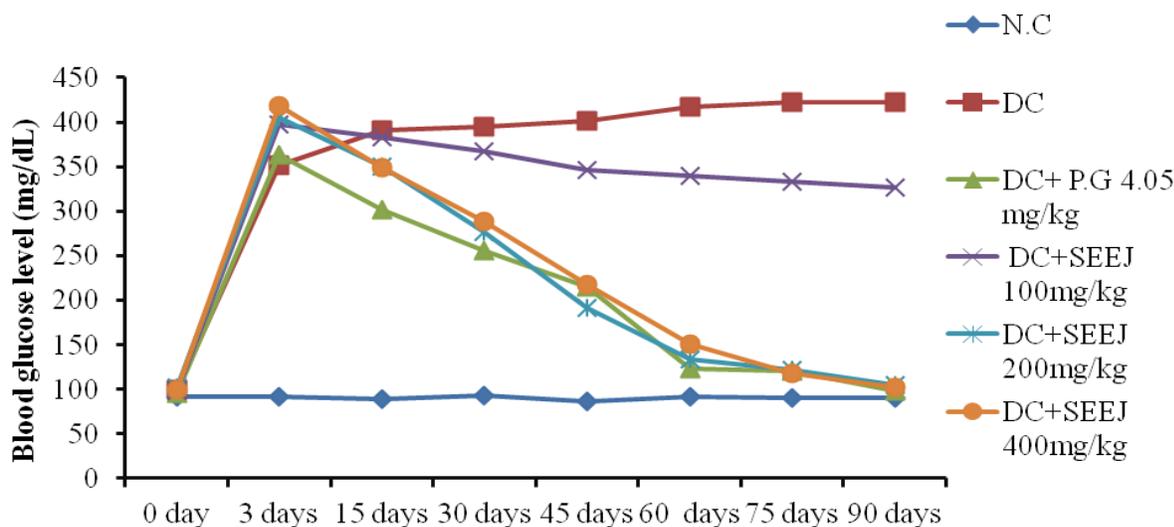


Figure 2: The effect of SEEJ on blood glucose level (mg/dL), NC: Normal control, DC: Diabetic control, PG: Pioglitazone

Glycosylated haemoglobin level: The levels of glycosylated haemoglobin (HbA_{1c}) were significantly ($P<0.001$) increased in diabetic control rats, when compared to normal control rats. The pioglitazone 4.05 mg/kg treated group showed significant ($P<0.001$) decrease in HbA_{1c} when compared to diabetic control rats. In SEEJ 100 mg/kg ($P<0.05$), SEEJ 200 mg/kg ($P<0.001$) and SEEJ 400 mg/kg ($P<0.001$) treated diabetic rats showed significant decrease in HbA_{1c} when compared to diabetic control rats (Table 3).

Blood urea level: The blood urea level in diabetic control group was significantly ($P<0.001$)

increased when compared to normal control group. The diabetic group treated with SEEJ 400 mg/kg significantly ($P<0.05$) decreased the blood urea level when compared to diabetic control group (Table 3).

Blood uric acid level: The blood uric acid level of diabetic control group was significantly ($P<0.001$) increased when compared to normal control group. In diabetic rats treated with SEEJ 200 mg/kg ($P<0.001$) and SEEJ 400 mg/kg ($P<0.001$) the blood uric acid level was significantly decreased when compared to diabetic control group (Table 3).

Table 3: The effect of SEEJ on glycosylated haemoglobin, blood urea and blood uric acid level

Groups	Treatment	Mean glycosylated haemoglobin level(%)	Mean blood urea level (mg/dl)	Mean blood uric acid levels (mg/dl)
I	Normal control	5.08 ± 0.30	35.6 ± 1.32	0.95 ± 0.12
II	Diabetic control	10.3 ± 0.28***	42.27 ± 0.73***	2.03 ± 0.07***
III	Diabetic + P.G(4.05mg/kg)	6.6 ± 0.14###	43.42 ± 1.04	1.93 ± 0.11
IV	Diabetic + SEEJ (100mg/kg)	8.9 ± 0.22#	42.88 ± 0.63	1.62 ± 0.17
V	Diabetic + SEEJ (200mg/kg)	5.7 ± 0.36###	41.78 ± 0.71	0.97 ± 0.18###
VI	Diabetic + SEEJ (400mg/kg)	6.17 ± 0.37###	37.98 ± 1.26#	1.12 ± 0.16###

n=6, The values are expressed as mean ± SEM; *** $P<0.001$ when compared to normal control group, # $P<0.05$, ### $P<0.001$ when compared to diabetic control group.

Blood urea nitrogen level: In the diabetic control group the blood urea nitrogen (BUN) was significantly ($P<0.001$) increased when compared to normal control group. In diabetic rats treated with SEEJ 100 mg/kg ($P<0.05$), SEEJ 200 mg/kg ($P<0.001$) and SEEJ 400 mg/kg ($P<0.001$) significantly decreased the blood urea nitrogen level when compared to diabetic control group (Table 4).

Blood creatinine level: The blood creatinine level of diabetic control group was increased significantly ($P<0.001$) when compared to normal control group. But diabetic rats treated with SEEJ 200 mg/kg ($P<0.01$) and SEEJ 400 mg/kg ($P<0.001$) showed significant decrease in blood creatinine

level when compared to diabetic control group (Table 4).

Table 4: The effect of SEEJ on BUN and creatinine levels (mg/dL)

Groups	Treatment	Mean BUN levels (mg/dl)	Mean creatinine levels (mg/dl)
I	Normal control	15.48 ± 0.79	0.44 ± 0.04
II	Diabetic control	24.7 ± 0.57***	0.68 ± 0.06***
III	Diabetic + P.G(4.05mg/kg)	23.53 ± 0.53	0.65 ± 0.03
IV	Diabetic + SEEJ (100mg/kg)	21.5 ± 0.33#	0.59 ± 0.01
V	Diabetic + SEEJ (200mg/kg)	16.33 ± 0.69###	0.49 ± 0.01##
VI	Diabetic + SEEJ(400mg/kg)	16.3 ± 1.06###	0.46 ± 0.01###

n=6, The values are expressed as mean ± SEM; *** $P<0.001$ when compared to normal control group, # $P<0.05$, ## $P<0.01$, ### $P<0.001$ when compared to diabetic control group.

The effect of SEEJ on urine parameters

Volume of urine: In the diabetic control group of rats, the volume of urine was increased significantly ($P < 0.001$) when compared to normal control rats. In diabetic rats treated with pioglitazone 4.05 mg/kg ($P < 0.001$), SEEJ 100 mg/kg ($P < 0.001$), SEEJ 200 mg/kg ($P < 0.001$) and SEEJ 400 mg/kg ($P < 0.001$) significantly decreased the volume of urine when compared to diabetic control group (Table 5).

Urine microalbumin level: The urine microalbumin levels in the diabetic control group of rats were significantly ($P < 0.001$) increased when compared to normal control group of rats. In diabetic control rats treated with SEEJ 100 mg/kg ($P < 0.01$), SEEJ 200 mg/kg ($P < 0.001$) and SEEJ 400 mg/kg ($P < 0.001$) showed significant decrease in the urine microalbumin levels when compared to diabetic control rats (Table 5).

Table 5: The effect of SEEJ on volume of urine and urine microalbumin levels

GTO I,II,III,IV,V,VI	Treatment	Mean volume of urine (ml)	Mean urine microalbumin levels (mg/dl)
I	Normal control	2.18±0.30	0.33±0.05
II	Diabetic control	19.6±1.02***	0.77±0.05***
III	Diabetic + P.G(4.05mg/kg)	3.05±0.54###	0.58±0.06
IV	Diabetic + SEEJ (100mg/kg)	13.65±0.88###	0.50±0.04##
V	Diabetic + SEEJ (200mg/kg)	3.41±0.55###	0.23±0.03###
VI	Diabetic +SEEJ(400mg/kg)	3.71±0.59###	0.27±0.04###

n=6, The values are expressed as mean ± SEM; *** $P < 0.001$ when compared to normal control group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ when compared to diabetic control group.

Histopathological studies

The histopathological observations of the rat kidneys revealed that the normal control group rats shows normal glomeruli and kidney tubules with healthy epithelial cells. The kidneys of diabetic control group rats shows thickening of

vesicles disrupted tubules, degeneration and necrosis of epithelial cells and intertubular haemorrhage. But the kidneys of diabetic rats treated with SEEJ 200 mg/kg and SEEJ 400 mg/kg showed regeneration of tubular epithelium depicting normal tubules with intact epithelium and presence of few RBCs in between tubules.

DISCUSSION

Chronic diabetes mellitus causes multiple complications like diabetic nephropathy and premature mortality, accounting for atleast 10 % of total health care expenditure in many countries ⁽¹⁾. The results of *in-vitro* antidiabetic effect of SEEJ by glucose uptake assay revealed the increase in percentage glucose uptake in lymphocyte culture preparation. When compared to pioglitazone on increasing the dose of SEEJ, the % glucose uptake also increased proportionally. Pioglitazone increase the % glucose uptake by skeletal muscle via glucose transporters and the report of the SEEJ also showed the increase in percentage glucose uptake. So the current observations confirms the role of % glucose uptake and are indeed may be due to the activation of PPAR γ by PPAR γ agonist (insulin sensitisers) which are currently being used in the treatment of insulin resistance associated with type-2 diabetes mellitus and thus influenced the peripheral glucose uptake. Drugs like thiazolidinediones and Insulin cause differentiation of pre-adipocytes into adipocytes. The adipocytes then stimulate glucose uptake and this aid in reducing the blood glucose levels. Therefore, drugs which exhibit glucose uptake activity would be desirable for patients with T2DM. The drug SEEJ exhibited increase in % glucose uptake and thus can be explored as

glucose lowering agent to treat T2DM ^(22, 23). The study supports this hypothesis and given a lead to explore the role of SEEJ in glucose uptake.

The results of *in-vivo* antidiabetic effect of SEEJ by streptozotocin-nicotinamide induced type-2 diabetes mellitus revealed that the reduction of final body weight of diabetic control group is due to increased muscle wasting in diabetes ⁽²⁴⁾. But diabetic rats treated with SEEJ showed an increase in body weight as compared to the diabetic control which may be due to its protective effect in controlling muscle wasting i.e reversal of gluconeogenesis. This observation is consistent with the results of previous researchers, as they reported that any drug possessing antidiabetic activity protects the muscle wasting in diabetic animals ⁽²⁵⁾.

Diabetic rats treated with SEEJ significantly decreased the kidney weights when compared to diabetic control rats. The increase in kidney weight was due to renal enlargement, which is one of the key features occurring during nephropathy, a hypertrophy and hyperfunction of the kidneys with typical increase in kidney size and glomerular filtration rate can be observed. This is due to the factors such as glomerular hypertrophy and nephromegaly (whole kidney enlargement), an early feature of both experimental and human diabetes occurs due to combination of tubular hypertrophy hyperplasia and interstitial expansion ⁽²⁶⁾.

In the diabetic rats treated with SEEJ the fasting blood sugar level was significantly decreased when compared to diabetic control rats. When compared to pioglitazone, the SEEJ was also equally effective at 200 mg/kg and 400 mg/kg doses in controlling fasting blood glucose level in diabetic rats. Liver is mainly responsible for maintaining normal concentrations of blood

glucose by its ability to store glucose as glycogen and to produce glucose from glycogen breakdown or from gluconeogenic precursors. Selective destruction of pancreatic β -cells by streptozotocin using experimental diabetes results in the decreased plasma insulin levels. This in turn leads to the defective glucose oxidation and causes hyperglycemia in diabetes involves over-production (excessive hepatic glycogenolysis and gluconeogenesis) and decreased utilization of glucose by the tissues ⁽¹⁾. The activation of PPAR γ by PPAR γ agonists (insulin sensitizers) which are currently being used in the treatment of insulin resistance associated with type-2 diabetes mellitus and thus influenced the peripheral glucose uptake. PPAR γ , a transcription factor belonging to the nuclear receptor family. Drugs like thiazolidinediones and Insulin cause differentiation of pre-adipocytes into adipocytes. The adipocytes then directly enhances insulin signaling and stimulate glucose uptake in muscle on binding with PPAR γ agonists and thus aid in reducing the blood glucose levels. Therefore, drugs which exhibit glucose uptake activity would be desirable for patients with T2DM. The drug SEEJ exhibited significant reduction in blood glucose level and thus can be explored as glucose lowering agent to treat T2DM ^(22, 23). The reports of the present study was consistent with the results of previous researchers who reported the SEEJ showed significant decrease in fasting blood glucose level in drug treated group when compared to diabetic control group⁽¹³⁾. This indicates that the SEEJ has possessing significant antidiabetic effect at 200 mg/kg and 400 mg/kg dose levels.

SEEJ treated diabetic rats showed significant decrease in HbA_{1c} when compared to diabetic control rats. When compared to pioglitazone the

SEEJ was also equally effective at 200 mg and 400 mg doses in controlling HbA_{1c} level in diabetic rats. In an uncontrolled or poorly controlled diabetes, there is an increased glycosylation of a number of proteins, including haemoglobin. HbA_{1c} is 3.4-4.8 % of total haemoglobins in normal human red blood cells and it would found to increase in diabetic patients upto 16 %. The level of HbA_{1c} is the indicator of the degree of control of diabetes in patients and its level reflects the average blood glucose concentration over the past three months ⁽¹⁾. HbA_{1c} is the most abundant glycosylated haemoglobin product, which initiates and participates in multiple organ damage in diabetes patients. The reaction between glucose and haemoglobin forming HbA_{1c} is a type of a nonenzymatic condensation of glucose with the free amino groups of the N-terminals of the b-chain of the haemoglobin molecules. The process is slow, continuous and irreversible. It serves as an indicator of metabolic control in diabetes ⁽²⁷⁾. Each 1% reduction in glycosylated haemoglobin is associated with a 37 % reduction in microvascular complications, 18 % myocardial infarction and 21 % fewer diabetes-related deaths ⁽²⁸⁾. In the present study also, HbA_{1c} level increased in diabetic rats and administration of SEEJ controls the glycation of haemoglobin by an increase in glutathione peroxidase and thus decreases the level of HbA_{1c} in experimental rats ⁽¹⁾.

The diabetic group rats treated with SEEJ significantly decreased the blood urea level when compared to diabetic control group. Elevated levels of urea occurs in diabetes mellitus due to increased protein breakdown and may also be seen in renal disorders like glomerular nephritis and chronic nephritis ⁽²⁾. In the present

investigation elevated level of blood urea in the diabetic group treated with SEEJ was restored to near normal level.

In diabetic rats treated with SEEJ, the blood uric acid level was significantly decreased when compared to diabetic control group. Uric acid is a product of purine metabolism. The increase in uric acid could be due to the fact that filtered uric acid is both reabsorbed and excreted in the proximal tubule through a voltage-sensitive urate channel and a urate-anion exchange mechanism. Hyperuricemia can be a result of either increased production or decreased excretion ⁽²⁹⁾. The SEEJ restored the elevated uric acid level in diabetic rats.

In diabetic rats treated with SEEJ the blood urea nitrogen level was significantly decreased when compared to diabetic control group. Blood urea nitrogen is formed when protein breaks down, which is another marker of kidney function. When blood flows through the body, protein circulates to cells. Cells use the protein and excrete the waste products urea which is filtered out of the blood by kidneys. Urea also contain nitrogen. In diabetic nephropathy urea and nitrogen stay in the blood. The BUN of over 20 mg/dl is an indicator of decreased kidney function. The SEEJ restored the elevated blood urea nitrogen in diabetic rats ⁽³⁰⁾.

But diabetic rats treated with SEEJ showed significant decrease in blood creatinine level when compared to diabetic control group. Creatinine is endogenously produced and released into body fluids and its clearance measured as an indicator of glomerular filtration rate ⁽³¹⁾. If serum creatinine levels increased due to hyperglycemia that causes osmotic diuresis and depletion of extracellular fluid volume ⁽³²⁾.

The SEEJ significantly reversed the elevated blood creatinine in diabetic rats.

In diabetic rats treated with pioglitazone, the volume of urine significantly decreased when compared to diabetic control group of rats. In diabetic control group of rats treated with SEEJ, the volume of urine level was significantly decreased when compared to diabetic control group. Polyuria is the symptom of diabetes, the volume of urine levels increased in diabetic rats, since the renal tubules are unable to absorb all of the glucose filtered in the glomeruli. The renal excretion of glucose requires excretion of water and produces an osmotic diuresis which is called polyuria or excessive urination. It can cause dehydration, resulting in dry skin and blurred vision, which is due to fluctuation in the amount of glucose and water in the lenses of the eye during dehydration. Glucose needs water to flow from the body. Loss of water causes an increase in the serum polarity that stimulates the thirst centre in the hypothalamus ⁽³³⁾. The SEEJ significantly decreases the increased volume of urine output in diabetic rats.

The diabetic control rats treated with SEEJ showed significant decrease in the urine microalbumin levels when compared to diabetic control rats. The increase in urine microalbumin was due to proteins from the kidney, appear in the urine as a consequence of normal process of cell turn over and metabolism. The release of the protein is increased during kidney's functional impairment as happens in diabetes ⁽³⁴⁾. The SEEJ restored the elevated levels of urine microalbumin in the diabetic rats. The report of histopathological studies of rat kidneys strongly supports the outcome of the study by restoring the kidney damage that occurred in the diabetic

rats treated with 200 mg/kg and 400 mg/kg doses of SEEJ.

CONCLUSIONS

In this investigation, SEEJ increased the percentage glucose uptake in lymphocyte culture preparation. The abnormalities in body weight, kidney weight, blood glucose, glycosylated haemoglobin, blood urea, blood uric acid, blood creatinine and urine microalbumin levels in the diabetic control group was significantly reversed by SEEJ in streptozotocin- nicotinamide induced type- 2 diabetic nephropathy in male albino *Wistar* rats. Therefore, this investigation concluded that SEEJ may be used as an antidiabetic agent and renal protective for chronic type-2 diabetes mellitus patients to prevent the nephropathy complications in diabetic populations, after confirming its efficacy and safety in well-controlled clinical trials. If it is confirmed in humans, SEEJ may be a potent, safe and cost effective phytomedicine to prevent nephropathy-induced premature death in diabetic patients.

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