PROTECTIVE EFFECTS OF ETHANOLIC FRACTIONS OF SIDA SPINOSA AND CISSUS QUADRANGULARIS IN STZ-INDUCED DIABETIC NEPHROPATHY RATS

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Abstract: *The current study examined the protective benefits of Sida spinosa&Cissus Quadrangularis ethanolic fractions (SSEF & CQEF) in streptozotocin-induced diabetic nephropathy with the goal of developing complementary and alternative medicine for the treatment of diabetic nephropathy. Globally, diabetic nephropathy is now the most common cause of end-stage renal failure. Sida spinosa &Cissus Quadrangularis may halt the advancement of diabetic nephropathy because of their anti-inflammatory, anti-diabetic, and antioxidant properties. In this work, rats were given a single intraperitoneal injection of 45 mg/kg of streptozotocin (STZ) to cause diabetic nephropathy (DN). After 4 weeks, urine albumin was determined. Urine albumin more than 300 mg/24 hr confirmed the development of DN. For next eight weeks, oral dosages of SSEF & CQEF (200& 400 mg/kg) were administered to STZ-induced DN rats. Blood glucose levels, body & kidney weight were measured at the conclusion of the study after eight weeks. Parameters from serum like creatine, BUN, albumin, HbA1c and urine parameters like creatinine, 24 h urine output, albumin and creatinine clearance were examined. The kidney's antioxidant enzymes vs CAT, SOD & GSH were assessed and the kidney's histopathology was also examined. In the STZ-induced DN rats, SSEF & CQEFsignificantly increased body weight, decreased blood glucose levels, and lowered kidney weight at 400 mg/kg dose. Additionally, the fractions reduced the urine concentrations of albumin, blood urea nitrogen, and HbA1c. In the kidney, SSEF & CQEF markedly raised the antioxidant indices. A histological examination showed that the STZ-induced DN rats treated with SSEF & CQEFhad decreased tubule vacuolar degeneration& basement membranes thickening. Current research shows that SSEF & CQEF may have antioxidant, antihyperglycemic, and renoprotective properties that could be useful in reducing the progression of diabetic nephropathy.*

Keywords: Diabetic nephropathy, *Sida spinosa, Cissus Quadrangularis,* streptozotocin

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1. INTRODUCTION

Hyperglycemia, which arises from deficiencies in either insulin secretion, insulin action, or both, is a hallmark of diabetes mellitus. Chronic hyperglycemia is also linked to long-term harm, malfunction and organ failure. It also plays a significant role in the emergence of numerous problems in diabetic patients. Furthermore, the most frequent cause of progressive kidney failure that requires dialysis or kidney transplantation is diabetes. According to reports, nephropathy develops in 30–40% of diabetic people and is now the primary cause of end-stage renal failure globally. Aberrations in both structure and function are hallmarks of diabetic nephropathy. Advanced glycation end products (AGEs) buildup and inadequate glucose management are major factors in the development of diabetic nephropathy. Moreover, tissue damage linked to diabetic nephropathy has been linked to advanced glycation end products. Urinary albumin excretion, extracellular matrix accumulation, thickening of basement membranes, mesangial growth, hypertrophy, and loss of glomerular epithelial cells (podocytes) within the glomeruli are among the pathological and clinical features of diabetic nephropathy. Glomerular function gradually deteriorates in diabetic nephropathy patients. As of right now, antihypertensive medications—especially those that target the renin-angiotensin system—such as angiotensin receptor-I antagonists and angiotensin

converting enzyme inhibitors are said to be the most successful treatments for progressive diabetic nephropathy. Nevertheless, the onset of diabetic nephropathy cannot be stopped by these treatments [1-7]. For thousands of years, plants have been essential to preserving human health and enhancing quality of life. The majority of primary healthcare needs for around 75% of the world's population are met by traditional medicine, which uses plant extracts or their active ingredients. Since ancient times, herbal medicines have been used to cure diabetes. Several authors have reported using a variety of Indian herbs to treat diabetes conventionally [8-10].

Sida spinosa Linn. (Malvaceae) is a tiny perennial herb or shrub with upright branches that grows widely in India's farmed fields, waste areas, roadside ditches, and open clearings. The root is rich in alkaloids, including methyl ester, betaine, α-amyrin, starch, ecdysterone, choline, hypaphorine, ephedrine, si-ephedrine, vasicinol, vasicinone, and vasicine. Roots are employed in fevers and debility as a diaphoretic and nervine tonic. A demulcent used to ease bladder and genitourinary tract irritation. Leaves are used to scald urine and as a demulcent and refrigerant. According to C.P. Khare's ethnobotanical survey, the herb *Sida spinosa* Linn. has hypoglycemic action when extracted ethanolically. Hence study is undertaken to assess *Sidaspinosas'* usefulness in the treatment of DN [11-12].

Cissus quadrangularis Linn. (CQ) is a native of Asia and Africa and is a member of the Vitaceae family. CQ stem is often used in folklore to aid in the healing process after bone fractures. Rat experiments using evidence-based methodology demonstrated the antiosteoporetic properties of CQ stem. Indigenous people and traditional groups have been using many species of *Cissus* to cure diabetes mellitus. Numerous scientific investigations indicate that CQ extracts, either by themselves or in combination with other components, may help assist weight loss and help return blood glucose, cholesterol, and triglyceride levels to normal. According to the subchronic toxicity and mutagenesis investigation, CQ is safe for long-term use and has no side effects at therapeutic levels. In fact, the *Cissus quadrangularis* stem's ethyl acetate fraction is abundant in phenolic chemicals, which have been shown to have strong antioxidant properties. In view of above properties of CQ; it is investigated to assess the beneficial effects in treatment of DN[13-14].

2. MATERIALS AND METHODS

2.1 Plant material

The aerial parts of *Sidaspinosa* were collected from the Pune district of Maharashtra in the month of February. The plant specimen was identifiedand authenticated by Dr. D.N. Mokat, Department of Botany,SavitribaiPhulePune University, Pune and a voucher specimen (SPG-05) has been preserved in our research laboratory for future reference.The stems of *Cissus quadrangularis* Linn *were* collected from Pune District, Maharashtra, India in the month of January. It was authenticated by Dr. J. Jayanthi, Scientist C, Botanical Survey of India, Pune, India. The plant material was then cleaned to remove adhering dust and soil. Then it was dried under shade.

2.2 Chemicals

Streptozotocin (STZ) was purchased from Enzo Life Sciences, USA forinduction of diabetes in the rats.Diagnostic kits for the estimation of biochemical parameters were obtained from Coral Clinical Systems Pvt. Ltd., Mumbai. All other chemicals, reagents and solvents used were of analytical grade.

2.3 Animals

Healthy male Wistar rats weighing between 200 and 250 g were procured from the animal house of the institute and maintained in polypropylene cages at ambient temperature of 22 ± 1 °C and relative humidity of 50-60% with a 12 h light/dark cycle in registered animal house at Institute. The animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the Institutional Animal Ethics Committee (IAEC) (VIVO/PR/2022/P0248). Throughout the experimental period, the animals were fed with standard pellet diet and water *ad libitum*.

2.4 Extraction and fractionation procedure

Fresh aerial parts and stems of *Sida spinosa*&*Cissus Quadrangularis*plants were carefully washed free from debris with clean water and allowed to dry in an open space away from sun reach until a constant mass was attained. The resultant dried leaves were pulverized using an electric mixer grinder. A known weight (2000 g) of powdered aerial parts and stems of *Sida spinosa*&*Cissus Quadrangularis* was macerated in 6.0 L of absolute ethanol (98 %) in a flat-bottomed sterile vessel. Filtration and concentration of the resultant solution were performed with Whatman No. 1 filter paper and a rotary evaporator set at 45 °C and reduced pressure, respectively. The ethanolic extract thus obtained was partitioned (3 times each) in a fractionating column using different solvents (n-hexane, ethyl acetate, and ethanol) in order of their polarity. The combined leaf fraction in each instance was concentrated using a rotary evaporator (at a similar condition). The concentrated leaf fractions were stored in sterile and well-labeled screw-capped vessels at 3–5 °C until needed for the study. The ethanolic fraction of *Sidaspinosa* (SSEF) &*Cissus Quadrangularis (CQEF)*was used in the study[15-16].

2.5 Phytochemical Analysis

The fractions of *Sidaspinosa*&*Cissus Quadrangularis* were subjected to the preliminary phytochemical investigation to reveal the presence or absence of carbohydrates (Molisch' test),reducing sugar (Fehling'test), saponins (Frothing test), tannins (FeCl3 test), flavonoids (Schinoda'stest), steroids (Salkowski test), glycosides (Keller Killiani test), alkaloid (Wagner's reagent test) andterpenoids

2.6 Acute Toxicity Studies

The fractions of *Sidaspinosa*&*Cissus Quadrangularis* were tested for the acute toxicity in Wistar rats according to the OECD guideline number 423[17]. The three groups containing five rats in each group, group I served as vehicle control and received only distilled water, while group II&III received single oral dose of 2000 mg/kg of ethanolic fractions SSEF & CQEF respectively. Mortality and general behavior of the animals were observed continuously for the initial four hrs and intermittently for the next six hrs and then again at 24 hrs and 48hrs following dose administration. The parameters observed were grooming, hyperactivity, sedation, loss of righting reflex, respiratory rate and convulsions.

2.7 Induction of Diabetic Nephropathy

Diabetes was induced by a single intraperitoneal injection of 45 mg/kg body weight ofstreptozotocin dissolved in freshly prepared 0.1 M Citrate buffer (PH of 4.5) in overnight fasted rats.Streptozotocin-injected animals were given 5% glucose solution for 24 h following streptozotocin injection to prevent initial drug-induced hypoglycemic mortality. After 5 days of STZ injection, the animals having a stable plasma blood glucose level of over 250 mg/dl were considered diabetic. After 4 weeks, the urine was collected using metabolic cages. The urine albumin was checked and animals showing microalbuminuria more than 300mg/24 hr were included in the study.

2.8 Experimental Design

The rats were divided into six groups; each group containing six animals and received following treatment:

Group I: Normal control rats treated with vehicle only (Distilled water)
Group II: Diabetic nephropathycontrol rats treated with vehicle only (Distilled water)
Group III: DN rats treated with 200 mg/kg of SSEF
Group IV: DN rats treated with 400 mg/kg of SSEF
Group V: DN rats treated with 200 mg/kg of CQEF
Group VI: DN rats treated with 400 mg/kg of CQEF

Freshly prepared solutions of the extract& distilled water were orally administered using an intragastric tube once daily for 8 weeks. At the end of the experimental period, the animals were fasted overnight and blood was collected from retro orbital plexus for various biochemical estimations.

2.9 Serum and urine parameters

At the end three weeks of experimentation, blood was obtained by retro-orbital puncture and used for analysis of different biochemical parameters. Blood glucose analysis was done by using glucometer (Accu Check, Roche, Germany) by glucose oxidase-peroxidase method. Glycosylated hemoglobin (HbA1C%) was determined in EDTA-blood samples using commercial assay kit. Body weight of each animal was determined at the initiation and end of the study. Urine (24hr) is collected by using metabolic cages. Urine is analyzed for creatinine and albumin. Blood samples were centrifuged at 8000 rpm for 15 min at 4°C to separate the serum. Serum albumin, creatinine, BUN and HbA1c were measured using commercial kits. At the end, animals were sacrificed & kidneys were removed for histopathological studies.

2.10 Preparation of kidney homogenate

Immediately after sacrifice, both the kidneys were dissected; rinsed with isotonic saline and weighed. After weighing, each kidney was cut into two halves. One half was used for histopathological evaluation. Other half was minced and a homogenate was prepared with 10% (w/v) phosphate-buffered (0.1 M, pH 7.4) using a homogenizer. The kidney homogenate was centrifuged and the supernatant was estimated for kidney antioxidant parameters.

2.11 Kidney antioxidant parameters

5-5-dithiobis (2-nitrobenzoic acid) (DTNB) reagent was used to estimate reduced glutathione (GSH) level in tissue homogenates and the absorbance was read at 412 nm. The amount of GSH in the sample was calculated in microgram per ml from a standard curve obtained and represented in GSH per total tissue protein. Evaluation of kidney homogenate for superoxide dismutase (SOD) and catalase (CAT) activities was carried out following the method published by Nishi et al., and Halliwell and Chirico [18-19].

2.12 Statistical analysis

The results were expressed as Mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple test of comparison using Graph Pad Prism software version 10. A value of $p < 0.0001$ was considered statistically significant.

3 RESULTS

3.1 Preliminary phytochemical screening

The percentage yield of SSEF & CQEF was found to be 3.4and 7% w/w respectively. The ethanolic fractions contained phenolic compounds, flavonoids, alkaloids and tannins.

3.2 Acute oral toxicity study

Ethanolic fractions treated animals did not show any changes in their behavioral patterns and all rats survived during the whole experimental period. There was no significant difference in the body weights and food consumption when compared to vehicle treated rats.Hence, it is concluded that the SSEF & CQEF were safe at the dose of 2000 mg/kg. From this 200 mg/kg and 400 mg/kg dose was selected for further studies.

3.3 Blood glucose level

Administration of STZ lead to significant increase in fasting blood glucose levels in the DN group as compared to normal control group. Furthermore, SSEF & CQEF demonstrated significant decrease in fasting glucose level at 400 mg/kg dose after 8 weeks of treatment (Table 1).

Table 1: Effect of ethanolic fractions of *Sida spinosa* and *Cissus Quadrangularis* on blood glucose levels in STZ-induced DN rats

Group No.	Treatment (dose)	Blood glucose (mg/dl)	
		Before Treatment	After 8 Weeks of Treatment
I	Vehicle Control (DW 10 ml/kg)	99.57±7.90	108.53±13.59
II	DN Control (STZ 45 mg/kg) (DW 10 ml/kg)	297.5±6.80	312.80±4.26
III	DN+SSEF(200 mg/kg)	291.07±8.55	263.83±8.18
IV	DN+SSEF(400 mg/kg)	299.2±8.73	208.66±9.87
V	DN+CQEF (200 mg/kg)	302.26±6.16	260.16±10.38
VI	DN+CQEF (400 mg/kg)	147.00±5.40	199.55±11.88

3.4 Effect on body weight& kidney weight

During 8-week experiment, STZ-diabetic rats exhibited significant weight loss when compared with normal rats. At the end of 8 weeks treatment, the body weight of rats treated with SSEF & CQEF at the dose of 200 mg/kg and 400 mg/kg was significantly increasedcompared with the diabetic control group (Table 2)**.**The kidney weight was significantly increased as compared to those in the normal control group. Treatment with SSEF & CQEF at the dose of 200 mg/kg and 400 mg/kg decreased the kidney weight significantly.

Table 2: Effect of ethanolic fractions of *Sida spinosa* and *Cissus Quadrangularis* on body weight and kidney weight levels in STZ-induced DN rats

Group No.	Treatment (dose)	Body Weight (g)		Kidney Weight (g)
		Before Treatment	After 8 Weeks of Treatment	
I	Vehicle Control (DW 10 ml/kg)	224.16±15.85	239.50±15.39	0.755±0.06
II	DN Control (STZ 45 mg/kg) (DW 10 ml/kg)	230.23±11.86	129.16±11.75#	1.44±0.28#
III	DN+SSEF(200 mg/kg)	227.12±15.19	152.83±12.60	1.05±0.14
IV	DN+SSEF(400 mg/kg)	222.83±6.24	180.16±16.17*	0.88±0.21*
V	DN+CQEF (200 mg/kg)	234.50±9.39	154.16±10.55	1.04±0.13
VI	DN+CQEF (400 mg/kg)	128.16±14.86	193.32±15.02*	0.99±0.10

3.4. Serum & urine parameters

The STZ-induced DN rats showed a significant decrease in the levelof creatinine in urine with a significant increase in serum ascompared to the normal control group. After treatment with SSEF & CQEF,the creatinine content increased significantly in urine with a significantdecrease in serum. The decreased creatinine clearance was also significantly restored by the treatment with SSEF & CQEF. The STZ-induced DN rats showed a significantincrease in the albumin content in urine, with a significantdecrease in the level of albumin in serum as compared to thenormal group. The albumin level of the STZ-induced DN rats treatedwith SSEF & CQEF was brought back to normal. ThSTZ-induced DN ratsexhibited significant increase in the blood urea nitrogen contentwhereas treatment with SSEF & CQEF significantly decreased this level.There is increase in HbA1c observed in STZ-induced DN rate which is significantly lowered by 8 weeks treatment with SSEF & CQEF. Diabetes induced polyuria in STZ-induced DN rats was significantly lowered by the treatment with SSEF & CQEF (Table 3 & 4).

Table 3: Effect of ethanolic fractions of *Sida spinosa* and *Cissus Quadrangularis* on serum parameters in STZ-induced diabetic nephropathy rats.

Group No.	Groups (dose)	Serum Albumin (mg/dl)	Serum Creatinine (mg/dl)	Serum BUN (mg/dl)	HbA_1c %
I	Vehicle Control (DW 10 ml/kg)	3.55±0.02	0.44±0.04	19.37±1.58	6.07±1.36
II	DN Control (STZ 45 mg/kg)	2.19±0.03#	1.93±0.05#	43.26±3.30#	12.43±1.73#
III	DN+SSEF(200 mg/kg)	2.24±0.02	1.22±0.03*	29.66±0.56*	9.27±1.36
IV	DN+SSEF(400 mg/kg)	2.98±0.18*	0.71±0.13*	24.96±0.81*	7.26±1.54*
V	DN+CQEF (200 mg/kg)	2.37±0.25	1.19±0.04*	32.25±1.33*	9.63±1.04
VI	DN+CQEF (400 mg/kg)	3.06±0.18*	0.69±0.09*	28.61±1.34*	7.01±1.87*

Table 4: Effect of ethanolic fractions of *Sida spinosa* and *Cissus Quadrangularis* on urine parameters in STZ-induced diabetic nephropathy rats.

Group No.	Groups (dose)	Urine Albumin (mg/dl)	Urine Creatinine (mg/dl)	24 hr urine output (ml/24 hr)	Creatinine clearance (ml/min)
I	Vehicle Control (DW 10 ml/kg)	0.08±0.01	22.53±0.43	13.06±2.96	0.46±0.04
II	DN Control (STZ 45 mg/kg)	0.42±0.03#	10.81±0.90#	40.16±6.11#	0.15±0.01#
III	DN+SSEF(200 mg/kg)	0.25±0.06*	15.61±0.56*	22.78±5.82*	0.20±0.04
IV	DN+SSEF(400 mg/kg)	0.22±0.02*	18.6±0.62*	20.37±3.74*	0.36±0.02*
V	DN+CQEF (200 mg/kg)	0.26±0.04*	14.35±0.44*	24.43±4.40*	0.20±0.03
VI	DN+CQEF (200 mg/kg)	0.24±0.05*	17.38±0.25*	19.24±2.27*	0.33±0.02*

3.5 Kidney antioxidant parameters

SOD, CAT and GSH activities were significantly decreased in the kidney of STZ-induced DN rats as compared to the normal control rats. Treatment with SSEF & CQEF significantly increased activities of SOD and GSH at 400 mg/kg dose levels.

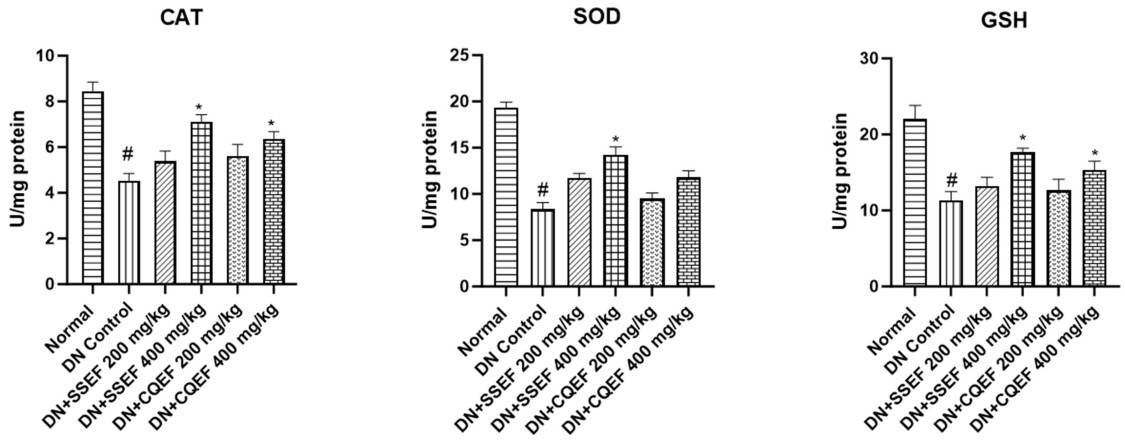
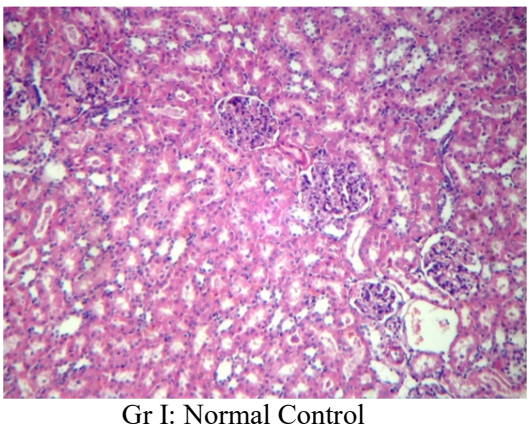


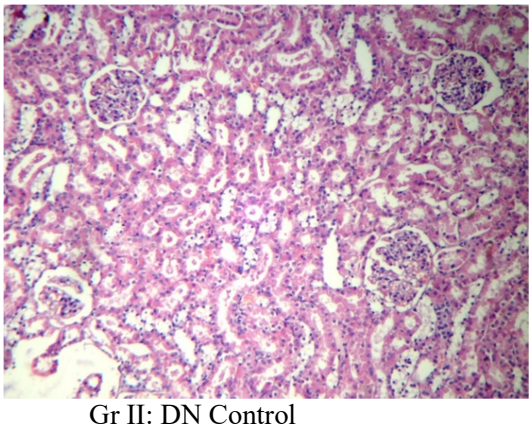
Fig. 2. Effect of SSEF & CQEF on antioxidant parameters ofkidney tissue homogenate in STZ-DN rats. * Treatment group was compared with DN control, p < 0.0001; ### DN control group was compared with normal control, p <0.0001; using one-way ANOVA with Dunnett's test.

3.6 Histopathological evaluation

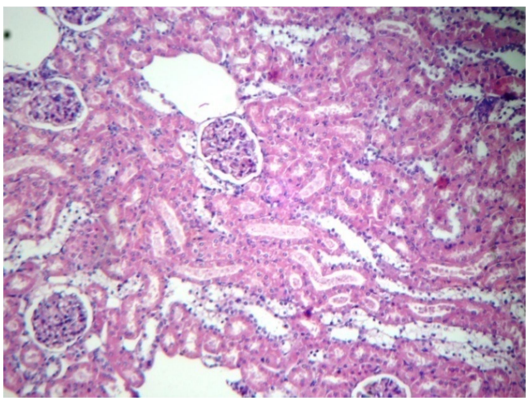
Kidneys stained with PAS exhibited moderate intensity PAS positivity in glomeruli and mild degree basement membrane thickening in the DN control group. Treatment with SSEF & CQEF reduced the PAS positivitystaining intensity in glomeruli at all dose levels and basement membrane thickening in STZ-induced DN rats at 400 mg/kg dose level respectively

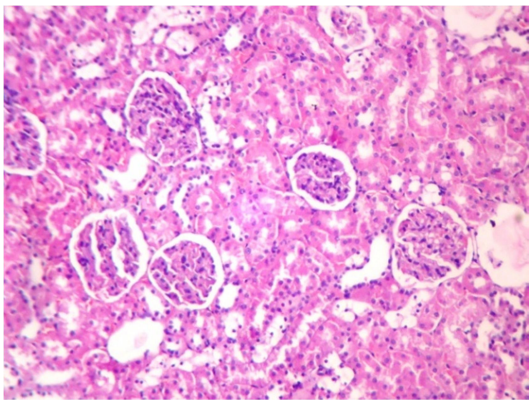


Gr I: Normal Control

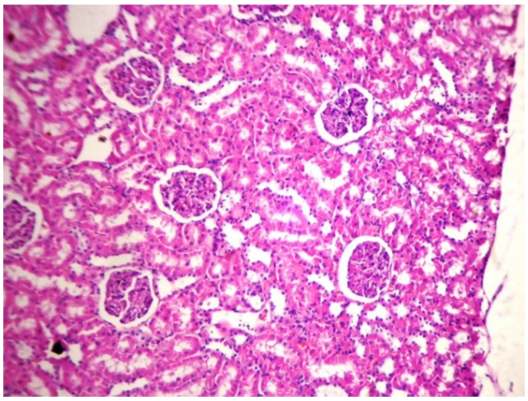


Gr II: DN Control

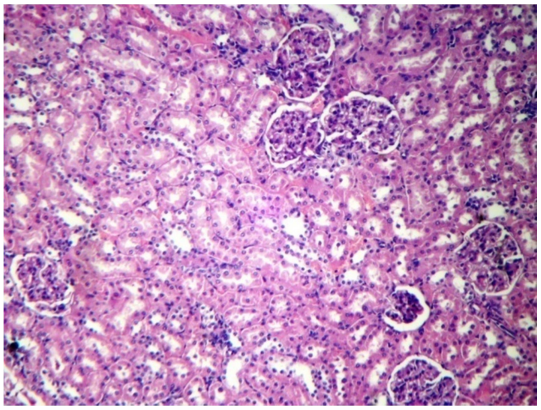




Gr III: DN+SSEF (200 mg/kg)



Gr IV: DN+SSEF (400 mg/kg)



Gr V: DN+CQEF (200 mg/kg)

Gr VI:DN+CQEF (400 mg/kg)

4. DISCUSSION

End stage renal failure is most frequently caused by diabetic nephropathy, a dangerous consequence of diabetes mellitus. Diabetic nephropathy affects 15–25% of people with type 1 diabetes and 30–40% of people with type 2 diabetes. Although there are medications that can slow down the development of diabetic nephropathy, there is still interest in using herbal remedies to stop the condition from starting in the first place [20].

Sida spinosa &*Cissus quadrangularis* are rich in polyphenolic compounds including tannins and flavonoids. This has aroused great interest due to their antidiabetic potential which could be considered as a lead to further study the effect on diabetic complications such as nephropathy.Taking this into consideration, in our present investigation, we have evaluated the protective effects of SSEF & CQEF on STZ-induced diabetic nephropathy in rats.

Because streptozotocin can cause selective necrosis of the pancreatic beta cells, which leads to degranulation and a loss of insulin-secreting capacity, it has been the agent of choice for inducing experimental diabetes mellitus. Therefore, STZ was utilized to induce diabetes in rats in the current investigation. The administration of STZ resulted in a notable rise in blood glucose levels, which were subsequently reduced upon treatment with SSEF & CQEF. This validates the extract's antihyperglycemic effect, as previously documented by reputable studies.

A notable decrease in body weight is linked to STZ-induced diabetes because of enhanced muscular atrophy, hypoinsulinemia, hyperglycemia, and tissue protein loss. The STZ-diabetic rats' body weight decreased gradually, while therapy with SSEF & CQEF markedly increased the body weight.

In rats given STZ, there is an increase in kidney weight (hypertrophy). Renal hypertrophy is thought to be caused by local changes in the synthesis of one or more growth factors, such as the overexpression of transforming growth factor-beta 1 in the kidney, particularly in proximal convoluted tubule cells and glomerular mesangial cells. Renal hypertrophy may also result from a rise in the rate of protein synthesis and/or a fall in the breakdown of renal extracellular components [23]. In STZ-diabetic rats, SSEF & CQEF therapy resulted in a decrease in the kidney weight, reversing renal hypertrophy.

In individuals suffering from renal failure, hypoalbuminemia is thought to be the best indicator of mortality. The most prevalent protein in nephrotic urine is by far albumin. Albuminuria was linked to declining kidney function, as evidenced by the considerable drop in serum albumin concentration in STZ-diabetic nephropathy rats and the increase in albumin levels in urine[24]. These levels returned to normal after SSEF & CQEF treatment, demonstrating its protective effects against microalbuminuria.Increased serum creatinine level and BUN along with decreased excretion of creatinine in the urine are indicators of the development of diabetic nephropathy [25]. Reversal of these effects was observed in STZ-diabetic rats treated with SSEF & CQEF.

Oxidative stress plays an important role in the pathogenesis of DN through overproduction of active carbonyl intermediates, reduction of antioxidant enzyme activities, formation of lipid peroxides and reactive oxygen species (ORS). Antioxidant enzymes prevent injuries at cellular and tissue levels. An imbalance between ROS production and antioxidants is the key element in DN. In the present study, induction of Type 1 DM with STZ caused a significant elevation of oxidative stress as indicated decreased activities of the antioxidant enzymes SOD and CAT along with decline in the endogenous antioxidant GSH. This could be attributed to the pathogenesis in the rat kidney and the progression of DN. Treatment of rats with SSEF & CQEFfor 8 weeks post DN confirmation reversed these abnormalities.

Histopathological examination of kidney sections of STZ-induced DN rats showed severe vacuolar degeneration of tubules, increased glomerular space, moderate intensity PAS positivity in glomeruli and basement membrane thickening. Treatment with SSEF & CQEF significantly reduced the aforementioned alterations, thus demonstrating protective role in renal damage.

5. CONCLUSION

In the current study, SSEF & CQEF treatment improved serum and urine parameters, corrected kidney antioxidant status, reduced blood glucose, and normalized the levels of albumin, HbA1c and BUN in STZ-induced DN rats. In conclusion, SSEF & CQEF has antihyperglycemic, antioxidant properties. As a result, it protects against diabetic nephropathy caused by STZ. Furthermore, more research is required to fully understand the cellular and molecular mechanisms of action of SSEF & CQEF.

CONFLICT OF INTEREST

None

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