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Research Article

RENOPROTECTIVE EFFECT OF HYDROALCOHOLIC EXTRACT OF AERIAL PARTS OF *CASSIA AURICULATA* LINN. (CEASALPINIACEAE) IN STREPTOZOTOCIN INDUCED EARLY DIABETIC NEPHROPATHY IN RATS

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ABSRACT

Objective: Present study was carried out to investigate renal protective activity of hydroalcoholic extract of aerial parts of *Cassia auriculata L.* (HACA) in streptozotocin induced early diabetic nephropathy in rats, to focus on its possible mode of action and identification of possible phytoconstituents responsible for the proposed activity.

Material and methods: Experimental diabetes was induced in wistar rats by single intraperitonial injection of streptozotocin (65 mg/kg). Animals were divided in six groups (n=6) and treated with variable doses of HACA for 4 weeks. Fasting blood glucose was measured at 0, 7th, 14th, 21st, 28th day of the study. At the end of 4 weeks, oral glucose tolerance test (OGTT), blood urea nitrogen (BUN), serum creatinine, serum total proteins, serum albumin, lipid profile, glycosylated haemoglobin, was determined. Antioxidant enzymes of kidney were evaluated. Urine was analyzed for albumin, total proteins and creatinine clearance. Kidney of experimental animals was examined to determine structural changes. Further, HACA was also analysed for its phytochmeical composition using various qualitative and quantitative methods.

Results: Daily oral administration of HACA for 28 days to diabetic rats produced significant decrease in fasting blood glucose, lipid profile, creatinine, albumin, BUN, total protein, urinary total protein. Whereas significant improvement in glycosylated haemoglobin, oxidative stress parameters of kidney has been observed in HACA treated diabetic rats. Histopathology of kidney tissue showed structural improvement.

Conclusion: The results of our study demonstrate renal protective potential of aerial parts of *Cassia auriculata L.* justifying its use in the indigenous system of medicine.

Keywords: Streptozotocin, Cassia auriculata, Diabetic nephropathy, Quarcetin, Gallic acid.

INTRODUCTION

Diabetic nephropathy (DN) is the most common cause contributing to end stage renal disease. It leads to destruction of function and structure in the kidney, leading to albuminuria which in turn further damage renal tubular structure [1]. Glomerular hyperfiltration, enlargement, mesangial expansion, intratubular fibrosis can be found in diabetic kidney in association with an increase in extracellular matrix [2]. Two hypothesis have been proposed to explain the changes in early DN, the vascular hypothesis and tubular hypothesis. Both mechanisms show that hyperfiltration occurs in early stage of DN and causes increase in glomerular pressure, which further contributes to sclerotic process of the glomeruli [3]. Hyperglycemia induced biochemical abnormalities, viz. formation of advanced glycation end products, over activity of polyol pathway and protein kinase C activation also play a role in the pathogenesis of DN [4].

Current research is focused on the development of newer drug leads from phytoconstituents of medicinal plants which have been used in traditional practices, so as to get more potential and effective agents with lesser side effects than existing hypoglycemic agents [5].

Cassia auriculata Linn. (Family:Caesalpiniaceae) is a tall, branched, bushy shrub growing wild throughout forest along roadside and in waterlands. [6]. Traditionally plant has been used in ayurvedic medicine as 'Avarai Panchanga Chooram' and as constituents of 'kalpa herbal tea' an Indian herbal formulation used in treatment of diabetes to control blood sugar level [7, 8]. The plant is used in the traditional system of medicine for urinary disorders, female antifertility, leprosy, worm infestation, diarrhoea, disease of pittam. Bark is used in skin conditions and as astringent; leaves, flowers and fruits as anthelmintic; seeds for eye troubles and in diabetes [9]. Ethanol extract of roots of Cassia auriculata L. has been reported to have renal protective activity against the cisplatin and gentamycin induced renal injury [10].

The qualitative phytochemical analysis of different parts extract of *Cassia auriculata L.* showed the presence of alkaloids, anthraquinone glycosides, flavonoids, phenolic compounds, saponins, steroids and tannins [11, 12]. Previous studies have proved that the chemical constituents such as flavonoids, bioflavonoids, alkaloids, tannins, saponins are promising agents for treatment of diabetes and its complications [13, 14].

Different components of aerial parts of *Cassia auriculata* L. especially leaves [15], flowers [16] and stem [17] have been reported to possess antihyperglycemic activity which is attributed to presence of phytoconstituents such as alkaloids, anthraquinone glycosides, flavonoids, phenolic compounds, saponins, steroids and tannin [18]. However, till date no studies have so far been reported for diabetic complications. In the absence of any scientific evidence, we have attempted the present study for exploring nephroprotective potential of aerial parts of *Cassia auriculata L.* and to focus on its possible mode of action.

MATERIALS AND METHODS

Collection and authentication of plant material

The aerial parts of *Cassia auriculata* Linn. were collected from the Vita region of Maharashtra state, India in the month of August 2012. The plant was identified and authenticated by Botanical Survey of India, Pune and a voucher specimen (V. No.CAAAAM 5) was deposited in the herbarium for future reference. The aerial parts were dried in shade and subjected to size reduction to a coarse powder by using dry grinder.

Preparation of hydroalcoholic extract

Air dried aerial parts of *C. auriculata* were ground to coarse powder and 100 gm of powder was extracted in methanol: water (70:30) for 72 h at room temperature with intermittent shaking. The extracts were filtered using absorbent cotton wool and filter paper (Whatman No A-1). Filtrates were collected and evaporated on rota evaporator at 40° C to obtain a yield of 21% w/w. The obtained hydroalcoholic extract was preserved in refrigerator till further use.

Experimental animals

Wister rats of either sex (180-220 g) were procured from National Institute of Bioscience, Chaturshrungi, Pune. Animals were housed in

standard polypropylene cages (32.5×21×14 cm) lined with raw husk (renewed after 48h). The animal house was maintained on 12h light/dark cycle at approximately 22±2^{0}C, relative humidity 60–70% and the animals were provided with standard laboratory diet (Nutrivet life sciences, Pune) and water *ad libitum*. The animals were randomly assigned to different groups and a minimum period of 7 days was allowed for adaptation on each experiment. The animals described as fasting were deprived of food for 24 h before experimentation but allowed free access to water throughout. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Modern College of Pharmacy in accordance with the regulations of CPCSEA (884/ac/05/CPCSEA).

Chemicals

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Preliminary phytochemical study

The HACA was screened for the presence of various phytoconstituents like alkaloids, glycosides, flavonoids, tannins, carbohydrates, amino acids and proteins [19].

Determination of total phenolic content

Total phenolic content was determined using $KMnO_4$ colorimetric assay. An aliquot (1 ml) of hydroalcoholic extract (10-50 µg/ml) or standard quercetin (10-50 µg/ml) was added to 10 ml volumetric flask containing 9 ml of 60 µg/ml solution of $KMnO_4$. The solution was well mixed and optical density was measured against prepared reagent blank colorimetrically at 520 nm. The total phenolic content was determined from the calibration curve and expressed as mg quercetin equivalents. All the determinations were carried out in triplicate and mean values were calculated.

Determination of total tannin content

Total tannin content was determined by hide powder test according to the WHO procedure. Weight difference between tanned and untanned hide powder was used for quantitative determination of tannins [20].

Determination of total flavonoids content

Total flavonoids content was measured by means of an aluminium chloride assay [21] with slight modification. An aliquot (1 ml) of the fraction (1 mg/ml) or standard solutions of quercetin (10-50 µg/ml) was added to a 10 ml volumetric flask containing 4 ml of 50% solution of methanol. To the flask, 0.3 ml of 5 % $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ was added. At the sixth minute, 2 ml of NaOH (1 M) solution was added, the total volume was made upto 10 ml with distilled water. The solution was well mixed and absorbance was measured against reagent blank at 510 nm. The total flavonoid content (mg/g) was determined from the calibration curve and expressed as mg quercetin equivalents. All the determinations were carried out in triplicate, and the mean values were calculated.

TLC analysis of hydroalcoholic extract

TLC chromatography was performed on 20×10 cm aluminum Lichrosphere TLC plates precoated with 200 µm layers of silica gel 60F254 (E. Merck, Germany). Mobile phase used was Ethyl acetate (100): Formic acid (11): Glacial acetic acid (11): Water (2.6) and Chloroform (7.5): Ethyl acetate (6): Formic acid (0.5) for flavonoids and tannins respectively. The plates were observed under UV light (240 and 300 nm) and R_f values of fluorescent substances were recorded [22].

HPTLC analysis of hydroalcoholic extract

HPTLC chromatography was performed on 20×10 cm aluminum Lichrosphere HPTLC plates precoated with 200 µm layers of silica gel 60F254 (E. Merck, Germany). HACA (10 µl) and standard quercetin (600 ng/band) and gallic acid (800ng/band) were applied as bands 6 mm wide and 10 mm apart by means of Camag Linomat V sample applicator (Muttenz, Switzerland) equipped with a 100-µl syringe. The constant application rate was 160 nl/s. Linear ascending development with toluene: ethyl acetate: formic acid (5:4:1, v/v/v) as mobile phase was performed in a twin-trough glass chamber 20×10 cm (Camag) previously saturated with mobile phase for 15 min at room temperature (25±2^o C) and relative humidity 60%±5%. The development distance was 8 cm (development time 10 min) and amount of mobile phase used was 20 ml. HPTLC analysis was performed at 270 nm in reflectance mode with a Camag TLC scanner III operated by WinCATS software (Version 1.2.0). The slit dimensions were 5 mm×0.45 mm and the scanning speed was 20 mm/s [23].

Acute toxicity study

Acute toxicity study was performed according to OECD guidelines no. 423 [24]. Female Wistar rats selected by random sampling technique were employed in this study. The animals were fasted overnight with free access to water. Ethyl acetate fraction was administered orally to different groups at increasing dose levels of 50, 100, 300, 2000 and 5000 mg/kg body weight. After dosing, the animals were observed for 2 hours and then intermittently for further 4 hours for changes in behavioural (alertness, restlessness, irritability, fearfulness), neurological (spontaneous activity, convulsion, gait, bleeding, touch and pain response), autonomic (defecation, micturition) profiles and finally recording mortality up to 24 hours till 14 days [11].

Experimental induction of diabetes

Rats were fasted overnight before being injected intraperitoneally with a single dose of freshly prepared solution of streptozotocin (STZ, 65 mg/kg) in ice cold citrophosphate buffer (pH 4.3). STZ was first weighed individually for each animal according to their weight and administered within 5 min to prevent its degradation. Since STZ is capable of producing fatal hypoglycemia as a result of massive insulin release, rats were treated with 5% glucose solution for 24 h. Normal control rats received an equivalent volume of citrophosphate buffer. After 3 days of STZ administration, fasting blood glucose levels of each rat were determined. Rats showing fasting blood glucose more than 200 mg/dl were considered diabetic and used for the study [25].Treatment commenced on 7th day of STZ administration [26] was considered as the first day of study.

Experimental groups

A total of 36 rats (6 normal; 30 diabetic) were used to accommodate the designated study. They were divided into six different groups consisting of six animals in each group as follows. Study was carried for 28 days.

Group 1: Normal control rats administered cold citrophosphate buffer (pH 4.3).

Group 2: Diabetic control rats i.e. rats treated with STZ (65 mg/kg i.p).

Group 3: Diabetic rats treated with 100 mg/kg of HACA

Group 4: Diabetic rats treated with 200 mg/kg of HACA

Group 5: Diabetic rats treated with 400 mg/kg of HACA

Group 6: Diabetic rats treated with 5 mg/kg of Glibenclamide (Standard oral hypoglycemic drug)

Fasting blood glucose

Fasting blood glucose was determined on 0, 7th, 14th, 21st and 28th day of study period using glucometer (Accu check, Germany).

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test was performed in overnight fasted (18 h) diabetic rats at the end of 28th day of study. Glucose (3g/kg) was fed 30 min after the administration of extracts of standard drug. Blood glucose was determined at 0, 30, 60, 90 and 120 min of glucose administration [27].

Body weight, food and fluid intake

During 28 days study period body weight, food and fluid intake of animals were recorded.

Glycosylated hemoglobin (HbA1c%)

HbA1c % was determined in EDTA-blood samples obtained at the end of the 28th day study using commercial assay kit (Crest biosystems, Goa, India).

Lipid profile

Serum triglycerides (TG), total cholesterol (TC) and high density lipoprotein (HDL) levels were estimated using standard kits (Autozyme Diagnostics, India). Very low density lipoproteins (VLDL) and low density lipoproteins (LDL) levels were calculated using Friedewald formula [28].

VLDL= TG/5

LDL= TC – (HDL+ VLDL)

Enzymatic and non-enzymatic biomarkers of oxidative stress in kidney

Estimation of reduced glutathione (GSH)

GSH activity was studied by method described by Kaur et al [29]. Tissue homogenate (1.0 ml) was precipitated with 1.0 ml of sulfosalicylic acid (4.0%). The samples were kept at 4°C for 1 h and then subjected to centrifugation at 1200 rpm for 15 min at 4ºC. The assay mixture contained 0.5 ml of filtered aliquot, 2.3 ml of sodium phosphate buffer (0.1M, pH 7.4) and 0.2 ml of dithiobisnitrobenzoate (DTNB) in a total volume of 3 ml. The optical density of yellow colour thus developed was read immediately at 412. The unknown GSH concentration was obtained by extrapolation from the standard curve.

Estimation of MDA lipid peroxidation

MDA activity was studied by method described by Kumar et al [30]. Briefly, the homogenate was incubated with 15% TCA, 0.38% TBA and 5N HCl at 95°C for 15 min. The mixture was cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm against appropriate blank.

Estimation of catalase

Catalase activity was studied by method described by Sahreen et al [31].The sample readings were taken by placing 1 ml of phosphate buffer and 5 µl of tissue homogenate in the reference cuvette and test cuvette. Hydrogen peroxide (10 µl) was then added in the test cuvette in the spectrophotometer. Reading was taken at 240 nm, 1 min after placing the cuvettes in the spectrophotometer.

Estimation of superoxide dismutase (SOD)

SOD activity was measured according to method of Marklund [32]. Assay mixture consisted of 2.95 ml Tris-HCl buffer, 25 µl of pyrogallol and 0.05 ml of tissue homogenate in total volume of 3 ml. The difference between the optical densities obtained at 1.30 and 3.30 minutes was determined and expressed as U/mg protein.

Parameters of nephroprotective activity

Serum total protein, urinary total protein, serum albumin and urinary albumin were estimated by using kit (Autozyme, India). Blood urea nitrogen, serum creatinine and urinary creatinine clearance was estimated using commercial kit (Crest biosystems, India). Urine volume was measured at 0, 7, 14, 21 and 28th day of treatment period using metabolic cages. Kidney weight was taken of all animals at the end of study Ratio of kidney weight to body weight was determined for all experimental groups under study.

Histopathalogical studies of kidney

At the end of study, animals were sacrificed, kidney were isolated for histopathalogical estimation [33].

Statistical analysis of data

All the data are presented as mean ± SEM of measurements made on six animals in each group. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnet's multiple test for comparison using Graph Pad Instat (version-3) software. A value of $p<0.05$ was considered to be statistically significant compared with the respective control.

RESULTS

Qualitative phytochemical screening

During preliminary phytochemical investigation HACA showed presence of flavonoids, phenolic compounds, tannins and alkaloids.

Quantitative phytochemical anlalysis

The total phenolic content of HACA was found to be 235.60 mg quercetin equivalent/ g of fraction. Total tannin content of HACA was found to be 17% w/w. Assay of total flavonoid content of HACA was found to be 112.32 mg quercetin equivalent/ g of fraction.

TLC analysis of hydroalcoholic extract

TLC analysis confirmed the presence of flavonoids (0.90 R_f value) and tannins (0.88 R_f value) by comparison with R_f values of standards quarcetin and gallic acid respectively.

HPTLC analysis of hydroalcoholic extract

The overlay spectras of HACA with gallic acid and HACA with quercetin are shown in Fig. 1 and 2 respectively. Gallic acid and quercetin were resolved at R_f 0.93 and R_f 0.91 respectively. The spectra clearly reveal that HACA contains both quercetin and gallic acid.

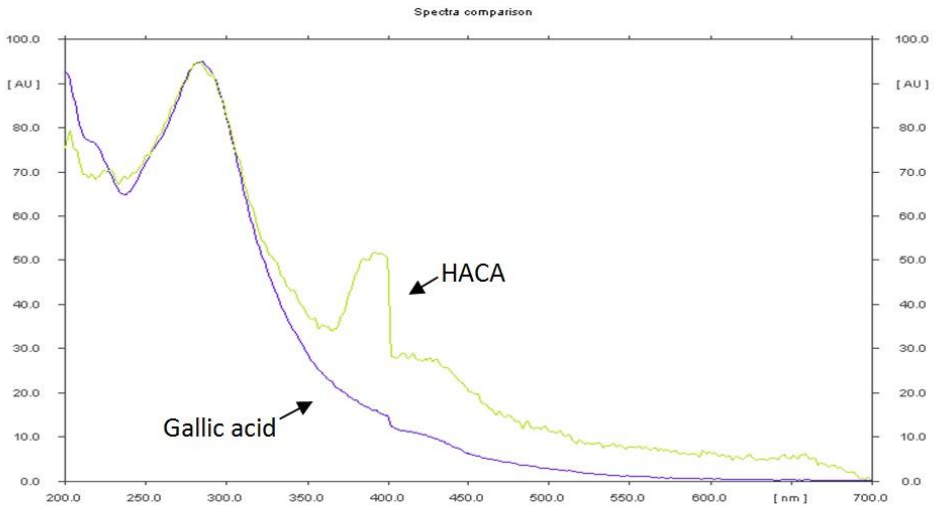

Fig. 1: HPTLC chromatographic overlay spectra of HACA with standard biomarker gallic acid.

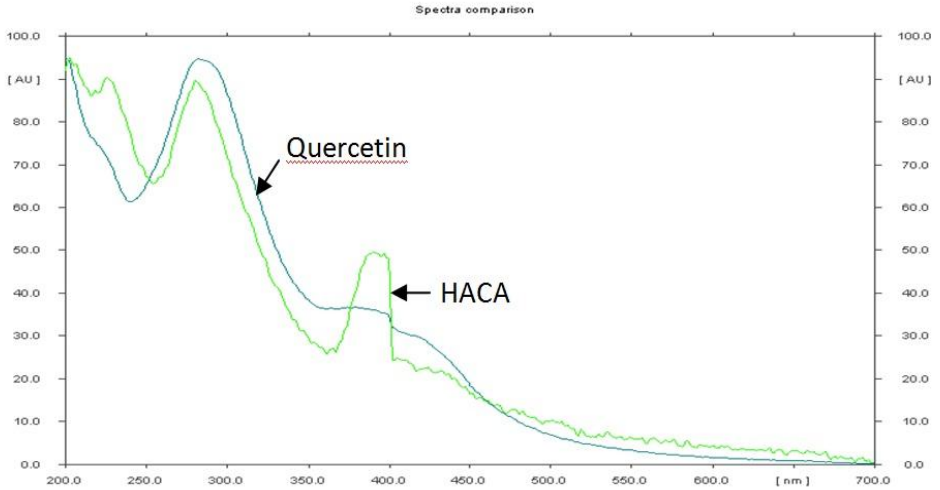

Fig. 2: HPTLC chromatographic overlay spectra of HACA with standard biomarker quercetin.

Acute toxicity study

Acute toxicity study revealed the non-toxic nature of HACA. There was no lethality or any toxic reaction in animals at a single large dose of 5000 mg/kg. No mortality was recorded within the 14 days of observation.

Fasting blood glucose

Treatment with HACA for 4 weeks exhibited a significant ($p<0.01$) decrease in fasting blood glucose in STZ diabetic rats as compared to diabetic control (Fig.3). In diabetic rat's blood glucose level was reduced by 56.85.49%, 68.21% and 75.68% at 100, 200 and 400 mg/kg doses of the extract respectively. The standard oral hypoglycemic drug glibenclamide showed 69.41 % reduction in blood glucose level as compared to diabetic control group.

Oral glucose tolerance test (OGTT) in diabetic rats

The effect of HACA on oral glucose tolerance test (OGTT) is shown in Fig. 4. Blood glucose levels of normal and diabetic rats increased significantly ($p< 0.01$) at 30 min after glucose administration. HACA at all dose levels significantly ($p<0.01$) reduced the increase in blood glucose at 90 min in glucose loaded rats compared with diabetic control rats which show elevation in blood glucose throughout the total measurement period (120 min).

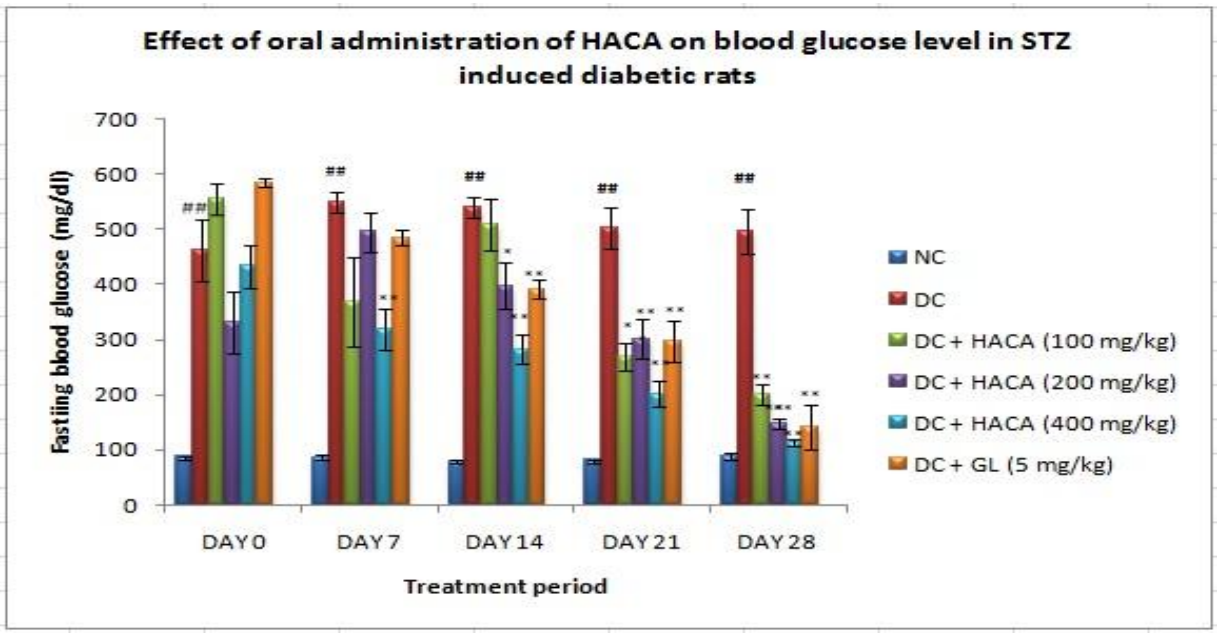

Fig. 3: Effect of HACA on fasting blood glucose in STZ diabetic rats.

NC: Normal control; DC: Diabetic control; HACA: hydroalcoholic extract of *Cassia auriculata* aerial parts, GL: Glibenclamide; n=6, Values are mean ± S.E.M., $^{\#}p<0.05$, $^{\#\#}p<0.01$ as compared to normal control group; $^{*}p<0.05$, $^{**}p<0.01$ as compared to diabetic control group Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison.

Lipid profile

The protective effect of HACA on lipid profile has been shown in Fig. 5. There was a significant ($p<0.01$) decrease in T-CH, TG, LDL-CH, VLDL-CH and significant ($p<0.01$) elevation in serum HDL-CH in diabetic rats when compared to normal rats. HACA (400 mg/kg) treated diabetic rats showed decreased levels of T-CH by 26.02 %, TG by 61.52 %, LDL-CH by 90.61 % and VLDL-CH by 61.56 %. Whereas HACA (400 mg/kg) treated group showed a significant ($p<0.01$) increase in HDL-CH as compared to diabetic control group.

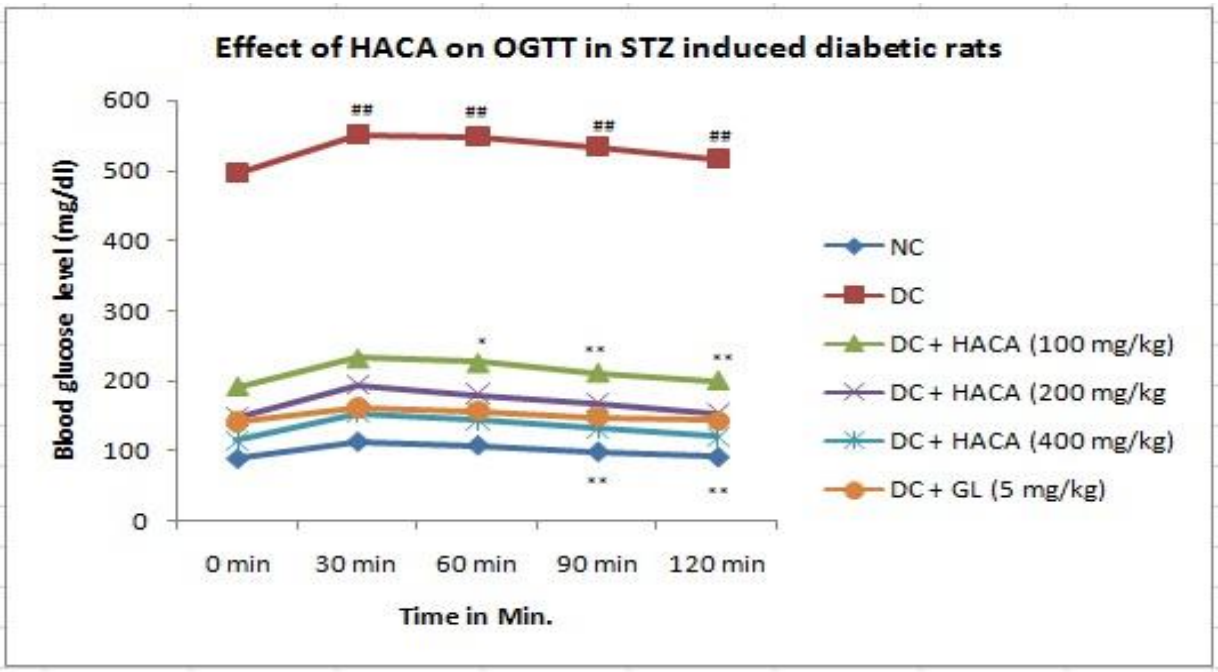

Fig. 4: Effect of HACA on oral glucose tolarance test in STZ diabetic rats.

NC: Normal control; DC: Diabetic control; HACA: hydroalcoholic extract of *Cassia auriculata* aerial parts, GL: Glibenclamide; n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to normal control group *p<0.05, **p<0.01 as compared to diabetic control group Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison

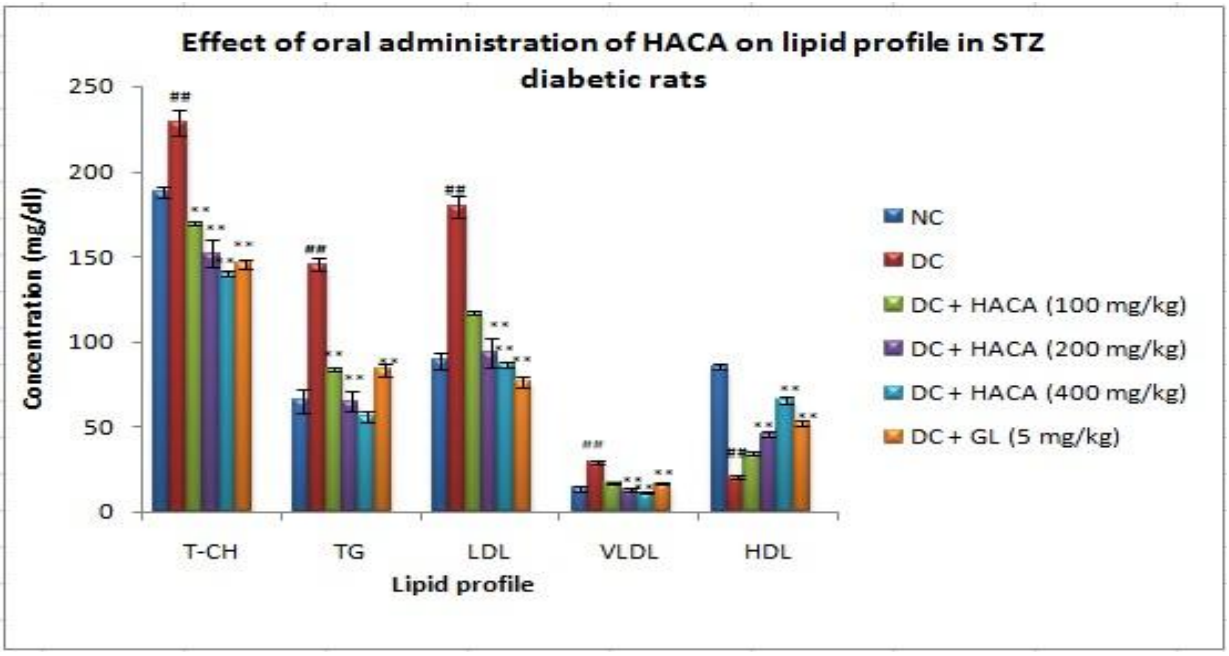

Fig.5: Effect of HACA on lipid profile in STZ diabetic rats.

NC: Normal control; DC: Diabetic control; HACA: hydroalcoholic extract of *Cassia auriculata* aerial parts, GL: Glibenclamide; n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to normal control group *p<0.05, **p<0.01 as compared to diabetic control group. Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Parameters of nephroprotective activity

Treatment with HACA for 28 days significantly (p<0.01) decreased glycosylated haemoglobin level in treatment group as compared to diabetic control group.

Total protein and albumin was significantly decreased in the serum and increased in urine of diabetic control group as compared to normal control group. Treatment with HACA significantly (p<0.01) increased serum total protein and albumin as compared to diabetic control group. On the other hand, total protein and albumin in urine was significantly (p<0.01) reduced by HACA treatment. Blood urea nitrogen and serum creatinine increased significantly (p<0.01) whereas creatinine clearance decreased steeply in diabetic control rats as compared to normal control group indicating a decreased glomerular filtration rate. Treatment with HACA (400 mg/kg) significantly (p<0.01)

decreased the alteration in glomerular filtration rate by decreasing serum creatinine and increasing creatinine clearance compared to diabetic control group. Kidney weight/body weight ratio (KW: BW) of diabetic control rats was significantly higher as compared to other groups which was normalized significantly ($p<0.01$) by HACA treatment. Urine volume of streptozotocin induced diabetic control rats was found to be significantly ($p<0.01$) more compared to normal control rats. After 4 weeks of treatment with HACA, urine volume significantly ($p<0.01$) decreased compared to diabetic control groups.

Table 1: Effect of HACA on nephroprotective parameters in STZ diabetic rats

Parameters	Normal control	Diabetic control	DC+ HACA (100 mg/kg)	DC + HACA (200 mg/kg)	DC+HACA (400 mg/kg)	DC+ GL (5 mg/kg)
HbA_{1c} (%)	5.41± 0.28	7.48± 0.04##	6.78± 0.09	5.48± 0.07**	6.46± 0.10*	5.2± 0.25**
Serum total protein (g/dl)	7.61± 0.49**	3.29± 0.617##	4.20± 0.75**	4.90± 0.25**	6.77± 0.47**	7.86± 0.45**
Serum albumin (g/dl)	8.56± 0.36**	3.02± 0.52##	4.22± 0.18	4.71± 0.21**	5.99± 0.21**	6.36± 0.12*
Serum creatinine (mg/dl)	6.31± 0.69**	15.07± 0.84##	11.62± 0.89**	10.42± 0.66**	8.31± 0.25**	8.91± 0.54**
BUN (mg/dl)	20.06± 0.98**	50.44± 3.32##	46.50± 0.70	43.50± 0.94*	39.96± 0.42**	29.85± 0.94**
Urinary total protein (g/dl)	8.14± 0.46**	12.87± 0.44##	9.95± 0.94**	9.35± 0.56**	7.94± 0.49**	7.86± 0.38**
Urinary albumin (g/L)	0.69± 0.03**	3.55± 0.11##	1.79± 0.05**	1.41± 0.07**	1.16 ± 0.12**	2.00± 0.09**
Urinary creatinine clearance (g/L)	0.96± 0.40**	4.67± 0.87##	2.73± 0.28**	3.27± 0.13**	4.31± 0.05**	2.51± 0.17**
KW:BW	3.02 ± 0.15**	7.15 ±0.62##	5.45 ±0.52**	5.87 ± 0.69*	4.88 ± 0.26**	3.40 ± 0.27**
Urine volume (ml/24hrs)	13.83± 0.42**	52.16± 2.49##	16.32± 1.54#**	21.78± 0.65**	17.44± 0.65**	14.83± 1.22**

NC: Normal control, DC: Diabetic control , HACA: hydroalcoholic extract of *Cassia auriculata* aerial parts, GL: Glibenclamide; n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Table 2: Effect of HACA on renal oxidative parameters in STZ diabetic rats

Parameters	Normal control	Diabetic control	DC+HACA (100 mg/kg)	DC+HACA (200 mg/kg)	DC + HACA (400 mg/kg)	DC + GL (5 mg/kg)
Kidney SOD (U/mg protein)	5.671± 0.16**	3.35± 0.16##	4.186± 0.16**	5.043± 0.053**	6.151± 0.086**	6.11± 0.18**
Kidney Catalase(U/mg protein)	78.66± 1.68**	36.64± 1.54##	45.08± 1.16**	49.97± 0.60**	52.05± 0.42**	62.09± 1.20**
Kidney GSH(nmol/mg protein)	4.55± 1.42**	2.73± 0.06##	3.90± 0.06**	4.173± 0.05**	4.43± 0.06**	4.15± 0.05**
Kidney MDA(nmol/mg protein)	196.92± 2.509*	389.87± 6.58##	282.62± 3.71*	254.00± 3.39**	223.95± 3.011**	271.86± 9.51**

NC: Normal control, DC: Diabetic control, HACA: hydroalcoholic extract of *Cassia auriculata* aerial parts GL: Glibenclamide; .n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Table 3: Effect of HACA on body weight, food and water intake in STZ diabetic rats

Experimental Groups	Body weight (g)		Food intake (g/24 hrs)		Water intake (ml/24hrs)	
	Initial	Final	Initial	Final	Initial	Final
Normal control	189.16± 8.78	197± 8.45	14.5± 0.77	18.08± 1.08	11± 0.38	11.16± 0.35
Diabetic control	183.16± 7.11	139.16± 4.527	21± 1.75	29.75± 1.24	44.33± 3.19	75± 3.84
DC+HACA (100 mg/kg)	175± 8.169	178.33± 6.878	25.91± 0.436	13.33± 0.909	58.83± 4.62	36.5± 1.197
DC+HACA (200 mg/kg)	164.33± 6.76	183.16± 6.405	26.33± 1.815	16.83± 0.97	58.83± 4.62	34.25± 1.59
DC+HACA (400 mg/kg)	195.16± 5.05	201.33± 3.12	31.66± 2.24	18.66± 1.02	52.16± 2.15	19.66± 1.45
DC+GL (5 mg/kg)	164.33± 5.42	168.16± 7.46	18.33± 0.90	16.5± 0.70	48.33± 0.90	16.5± 0.70

NC: Normal control, DC: Diabetic control, HACA: hydroalcoholic extract of *Cassia auriculata* aerial parts GL: Glibenclamide; n=6, Values are mean ± S.E.M., #p<0.05, ##p<0.01 as compared to NC; group *p<0.05, **p<0.01 as compared to DC: Data analysed by one way Analysis of Variance (ANOVA) followed by Dunnet's Multiple Test for comparison.

Enzymatic and non-enzymatic biomarkers of oxidative stress in kidney

Diabetes resulted in significant decrease in antioxidant enzymes like GSH, catalase and SOD. Moreover the levels of MDA were significantly increased. HACA exhibited improvements in antioxidant enzymatic activity compared to diabetic control group and nearly normalized the levels of SOD, MDA, catalase and GSH.

Body weight, food intake and water intake

Body weight of streptozotocin induced diabetic control rats was found to be significantly ($p<0.05$) less compared to normal control rats. After 4 weeks of treatment with HACA body weight significantly ($p<0.05$) increased compared to diabetic control groups. Food intake was significantly high in diabetic control rats as compared to normal control. At the end of 28 days of treatment food intake of treated groups significantly ($p<0.01$) decreased as compared to diabetic control. Significant decrease ($p<0.01$) in water intake was observed in treated groups as compared to diabetic control at the end of study period.

Histopathology study of kidney

The histological changes in the renal specimen of normal and experimental animals are shown in figure. Diabetic glomeruli showed some areas of mesangial matrix expansion, thickening of glomerular basement membrane, dilation of tubule and cell infiltrations. Treatment with HACA lead to regeneration of tissues that were earlier affected with STZ.

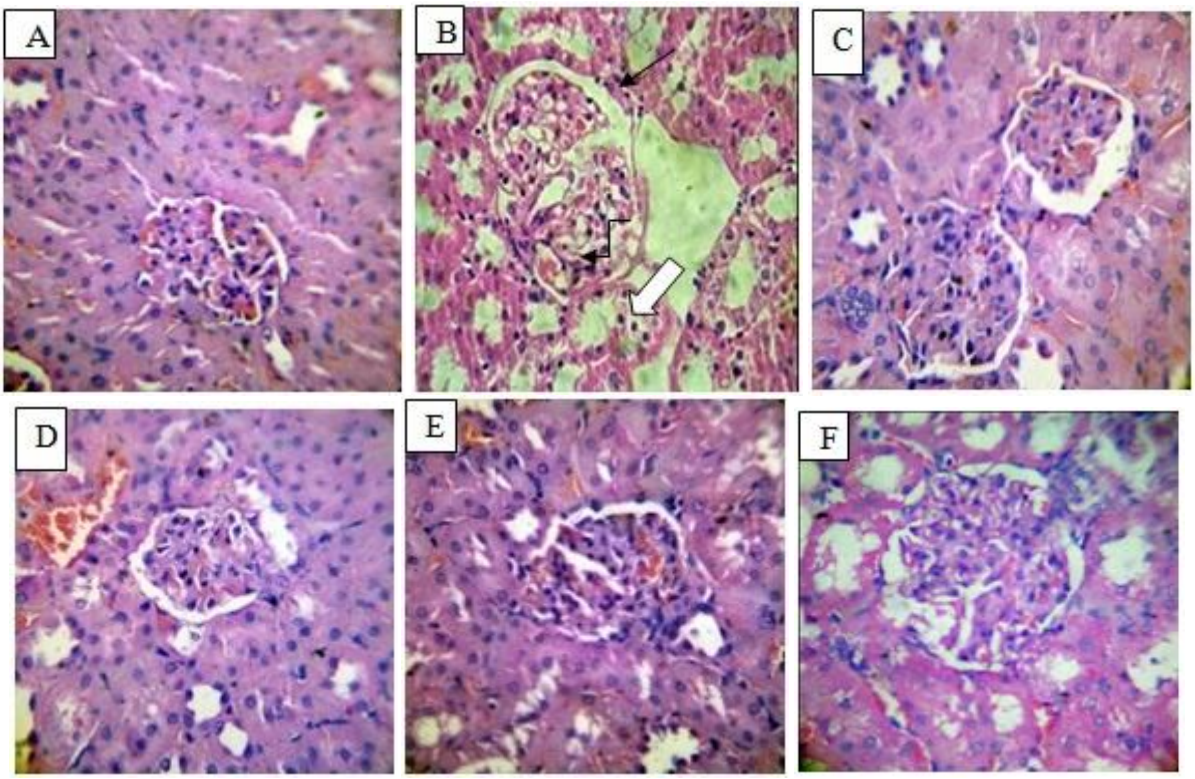

Fig. 6: Histopathology of kidney sections of STZ diabetic rats treated with HACA.

Kidney sections were stained with haematoxylin-eosin and observed under 40X magnification of digital microscope. (A) Normal control with typical histological structure of rat kidney. (B) Diabetic control group showing glomerular basement membrane thickening (➞), tubular dilation (⇨) and cell infiltration (➞). (C) DC + HACA (100 mg/kg). (D) DC+HACA (200 mg/kg). (E) DC+ HACA (400 mg/kg). (F) DC + GL (5 mg/kg). All HACA treated group showed significant improvement in kidney histopathology as compared to DC.

DISCUSSION

Diabetes mellitus is considered as a major health risk worldwide. One of the major morbidity and mortality factors confronted by diabetic patient is an increased risk of developing diabetic nephropathy that often progresses to end stage renal disease. Prevention of initiation of nephropathy or retardation of progression of glomerulosclerosis is an important key for development of therapeutic agent for renal disease. Despite the important progress made in management of diabetes by use of synthetic drugs, during the past few years there has been renewed interest in the use of herbal products [34]. *Cassia auriculata L.* has been reported to possess renal protective activity but its ability to ameliorate diabetic nephropathy has not been established clearly. In the present study, protective role of hydroalcoholic extract of aerial parts of Cassia auriculata L. (HACA) in early diabetic nephropathy is evaluated by using STZ induced diabetic nephropathy in rats as the animal model. The present results suggest that HACA exhibit significant antihyperglycemic, hypolipidemic and nephroprotective effects in STZ induced diabetic rats.

Streptozotocin is widely employed to induce experimental diabetic nephropathy as it selectively destroys the islet of Langerhans [35]. In the present study, induction of diabetic nephropathy by STZ was evidenced by elevated levels of urinary total protein, urinary albumin, serum creatinine, BUN and decreased creatinine clearance, which were taken as direct *in vivo* index for nephropathy in STZ diabetic rats [36].

Hyperglycemia is the principle factor responsible for structural alterations in the renal tissues. Studies have proved that improved glycemic control can significantly decrease the development and progression of diabetic nephropathy [37]. In the present study, STZ induced diabetic rats exhibited significant increase in blood glucose level. Chronic treatment of diabetic rats with HACA reduced blood glucose level in duration dependent manner indicating its potent antihyperglycemic activity which contributes at least in part in delaying the progression of diabetic nephropathy.

Hyperglycemia leads to an increased production of glomerular matrix proteins, the accumulation of which decreases the surface

area for filtration leading to decreased glomerular filtration rate (GFR). Decreased GFR is associated with the formation of reactive oxygen species [38] that induce oxidative stress which is key pathogenic factor in the development of diabetic complications including diabetic nephropathy [39]. Hyperglycemia is also responsible for increased oxidative stress in the kidney which induces apoptosis that contribute to the development of diabetic nephropathy [40]. Hyperglycemia induced oxidative stress caused by free radical generation and decrease antioxidant defense system which, has been assessed to estimate the degree of oxidative stress [41]. Our study showed increased oxidative stress as demonstrated by increase in level of lipid peroxidation products such as MDA and decrease in SOD, GSH and catalase activity in kidneys of diabetic untreated group. HACA treatment restored the levels of MDA, SOD, GSH and catalase close to normal control values, which confirms that antioxidant potential of HACA is responsible for renal protective activity.

Kidney plays important role in removal of metabolic wastes such as urea, uric acid, creatinine and ions to maintain optimal chemical composition of body fluid. The increased concentration of these metabolites in blood is due to metabolic disturbances observed in renal diseases associated with uncontrolled diabetes mellitus [42]. In the present study, urinary total protein and albumin, which are generally considered as markers of renal function [43], were increased and creatinine clearance was decreased in STZ diabetic rats. Decrease in urinary albumin, serum creatinine and BUN observed in HACA treated groups with improvement in urinary clearance of creatinine indicates that HACA ameliorated the loss of renal function and glomerular hyperfiltration in STZ diabetic rats. Magnitude of urinary protein level is further associated with a graded increase in the risk of progression to end stage renal disease and cardiovascular event [44]. Treatment with HACA in STZ diabetic rats showed significant reduction in urinary protein level which indicate that HACA may have ability to delay the end stage renal disease and associated cardiovascular complications.

The HACA plays a key role in controlling coronary heart disease by exerting its effect on altered lipid profile in diabetic rats. In diabetic state, there is inactivation of the lipoprotein lipase by which free fatty acids are converted into phospholipids and cholesterol, which are finally discharged into blood, causing an elevation of serum phospholipid level [45]. HDL helps to scavenge cholesterol from extra hepatic tissues. Decreased HDL can contribute to the increased LDL cholesterol levels as there is a reciprocal relation between the concentration of LDL and HDL [46]. In our study, markedly increased levels of TG, T-CH, VLDL-CH, LDL-CH and decreased level of HDL-CH in STZ diabetic rats contributed to the pathogenesis of diabetic rats as reported in previous studies [47,48,49] This altered levels of TG, T-CH, VLDL-CH, LDL-CH and HDL-CH was reversed towards the normal control level by administration of HACA during treatment period. This implies that HACA may activate lipoprotein lipase which would be helpful to reduce the incidence of lipid born complications such as renal damage [50, 51].

In diabetic state, increased non-enzymatic and auto-oxidative glycosylation of haemoglobin is one of the possible mechanisms that can be linked to major vascular complications [52]. In our study, increase in glycosylated haemoglobin was observed in STZ diabetic rats. It has been previously reported that the elevation of glycosylated hemoglobin beyond 7% generally leads to diabetic nephropathy [53]. Treatment with HACA showed a marked improvement in the glycosylated haemoglobin levels which demonstrates its role in delaying the progression of diabetic nephropathy.

In the present study, decrease in the body weight of STZ diabetic rat is possibly due to dehydration, increase in muscle wasting [54] and catabolism of fats and proteins [55]. In our study, marked increase in kidney weight was observed which is in agreement with previous studies [56]. The relative kidney weight in diabetic rats was significantly increased than normal rats. HACA treatment significantly restored body weight to kidney weight ratio which confirms that HACA has preventive effect on kidney hypertrophy.

STZ diabetic rats in our study showed marked increase in food intake and water intake when compared to normal rats, which could be due to poor glycemic control [57]. Treatment with HACA in diabetic rats normalized the food and water intake which is because of its ability to improve glycemic control. Increase in urine volume in STZ diabetic rats shows early progression to diabetic nephropathy. Urine volume is maintained by Na^+K^+ ATPase pump in the basolateral membrane which hydrolyzes ATP and thus results in transport of Na^+ into the intercellular and interstitial spaces and increases movement of K+ into cells. The increase in Na-K-ATPase activity in diabetic rat results in increased urine flow [58]. Significant decrease in urine volume in HACA treated STZ diabetic rats thus correlate with a significant decrease in Na^+ K^+ ATPase activity.

Morphological changes observed in renal histopathology are an important feature in diabetic nephropathy progression. In our study, kidney sections of STZ diabetic rats showed accumulation of extra cellular matrix (ECM) components, increased mesangial matrix, thickness of glomerular basement membrane, tubular dilation and cell infiltration. Oral administration of HACA revived the structural changes in kidney sections by reducing glomerular basement membrane thicknening, tubular dilation and cell infiltration, which confirms its role as a renal protective agent in STZ induced diabetic nephropathy.

In our study, preliminary phytochemical analysis was carried out to determine phytoconstituents responsible for nephroprotective activity. Thin layer chromatography (TLC) method can be widely used as standard technique for rapid and accurate qualitative determination of phytoconstituents of plant extracts [59]. In the present work, we performed preliminary TLC analysis in which HACA was subjected to mobile phases of different polarity which indicated the presence of flavonoids and tannins compounds. TLC screening of HACA showed the spots with R_f values similar to standard quercetin and gallic acid. Quantitative analysis of HACA by total tannin, total flavonoid and total phenolic assay was carried out which showed higher flavonoid content than tannin content. HPTLC analysis of HACA confirmed the presence of quercetin and gallic acid. Previous studies report that quercetin has a protective effect on kidney [60] and gallic acid adjuvant with quercetin showed better antidiabetic, antihyperglycemic and antioxidant activity [61]. Thus it is clear that the renal protective potential of HACA in the present study may be due to its flavonoid component quercetin along with tannin component gallic acid.

In conclusion, we demonstrated that the administration of HACA effectively ameliorated alterations in early diabetic nephropathy induced by STZ by virtue of its antihyperglycemic, antihyperlipidemic and antioxidant mechanism with improvement in histological alterations in renal tissue. The proposed mechanisms for renal protective activity of HACA are due to its major components viz. flavonoids and tannins. Further studies are required to isolate the major constituents, which will contribute in development of effective therapy for diabetic nephropathy.

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