

Asparagus racemosus Willd (Liliaceae) ameliorates early diabetic nephropathy in STZ induced diabetic rats

Rahul Somani^{a*}, Abhay Kumar Singhai^b, Prashant Shivgunde^c & Dilpesh Jain^c

^aDepartment of Pharmacology, Smt. Kashibai Navale College of Pharmacy, Pune 411 048, India

^bDepartment of Pharmaceutical Sciences, Dr. H S Gour University, Sagar 470 003, India

^cDepartment of Pharmacology, Sinhgad College of Pharmacy, Pune 411 041, India

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Diabetic nephropathy is a major “microvascular” complication of diabetes, differs from other causes of chronic kidney diseases in its predictability, with well-defined functional progression from hyperfiltration to micro- to macroalbuminuria to renal failure. The present study was undertaken to investigate the effect of *Asparagus racemosus* Willd (Liliaceae) on streptozotocin -induced early diabetic nephropathy. Single i.p injection of streptozotocin (55 mg/kg) was administered to induce early diabetic nephropathy in Wistar rats and thereafter treated orally with ethanolic extract of *Asparagus racemosus* (EEAR) at a dose level of 100 and 250 mg/kg daily for 4 weeks. The efficacy of extract was compared with diabetic control rats. *A. racemosus* treatment significantly decreased plasma glucose, creatinine, urea nitrogen, total cholesterol and triglyceride levels. Renal hypertrophy, polyuria, hyperfiltration, microalbuminuria and abnormal changes in the renal tissue as well as oxidative stress were effectively attenuated by EEAR treatment. Basement membrane thickening and mesangial proliferation formation without nodules were seen in diabetic rats, whereas these structural changes were reduced in EEAR treated groups. Results of this study suggested that *A. racemosus* has beneficial effect in the treatment of diabetic nephropathy.

Keywords: *Asparagus racemosus*, Diabetic nephropathy, Microalbuminuria, Streptozotocin

Diabetic nephropathy (DN), a major long-term microvascular complication of diabetes mellitus, is the most common cause of end stage renal disease (ESRD) requiring dialysis¹. Further, DN has been acknowledged as an independent risk factor for cardiovascular disease. So, prevention or retardation of DN has become a major goal in biomedical research². The injurious effects of hyperglycemia are characteristically observed in tissues which are not dependent on insulin for glucose entry into the cell, hence, are not capable of down-regulating glucose transport along with elevation of extracellular glucose levels³. Different biochemical pathways found to be involved in the pathogenesis and reactive oxygen species (ROS) seem to be the common denominator in various pathways⁴.

Metabolic derangements, systemic and glomerular hypertension, oxidative stress and advanced glycation end products (AGEs) found to occur in the progression of DN. In addition, kidney hypertrophy

and hyperfiltration along with metabolic derangements adversely compounds hyperglycemia-induced injury⁵ leading to the development of long-term diabetic renal damage and increased urinary albumin excretion rate (AER)^{6,7}. Thickening of glomerular basement membrane (GBM), glomerular enlargement, mesangial expansion, intertubular fibrosis and albuminuria can be found in diabetic kidney in association with an increase in extracellular matrix (ECM)⁸.

DN can be controlled by multi-targeted therapies, which includes, intensive control of blood glucose and blood pressure by using antihypertensive agents with anti-proteinuric action, lipid lowering strategies and correction of insulin resistance. Although angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) are effective in DN, but not sufficient to completely prevent disease progression, hence development of novel and effective therapeutic strategies are therefore high priorities⁹.

Ayurvedic system has given knowledge about very safe and effective medicinal plants. Ayurvedic literature listed use of the roots of the *Asparagus racemosus* in treatment of nephropathy¹⁰. It has also

*Correspondent author
Telephone: +91 9011093415
E-mail: rahulsomani2001@yahoo.com

been reported to possess hypoglycemic, antidiabetic¹¹, antioxidant¹², insulin secretory¹³ and antilithiatic activity¹⁴. The present investigation was undertaken to study effect *A. racemosus* on streptozotocin (STZ)-induced early DN in experimental animals.

Materials and Methods

Drugs and chemicals—Aminoguanidine hydrogen carbonate (Spectrochem Pvt. Ltd., Mumbai), streptozotocin, malondialdehyde (MDA), tetrabutyl ammonium and superoxide dismutase (Sigma-Aldrich, St. Louis), Catalase (Hi Media Laboratories Pvt. Ltd., Mumbai) and commercial diagnostic kits (Biolab, Mumbai) were purchased from local supplier.

Preparation of extract—Roots of *A. racemosus* were purchased from local market of Pune, India. The plant material was authenticated by Agharkar Research Institute; Pune and voucher specimen (Auth 08-132) was deposited. Roots were dried in an oven at 40 °C and grinded into a fine powder. The powder material was macerated with 95% ethanol, filtered and concentrated under reduced pressure using rotary evaporator and yield was found 6.42% w/w.

Experimental animals—Wistar rats of either sex, weighing 150-200 g were purchased (Haffkine Bio-Pharma Corporation Ltd., Mumbai) and maintained under standard laboratory conditions at temperature of 23 ± 2 °C with relative humidity 55°C ± 10 % in 12 h light and dark cycle throughout the experiments. Animals had free access to water and standard laboratory feed *ad libitum*. All the experimental procedures and protocols used in this study were reviewed and approved (SCOP/IAEC/Approval/2008-09/10) by the Institutional Animal Ethics Committee of Sinhgad College of Pharmacy, Pune, constituted under Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Induction of early diabetic nephropathy—Single intraperitoneal injection of STZ (55 mg/kg), prepared in 0.1 M cold citrate buffer (pH 4.5) was administered to induce diabetes in rats whereas, normal control rats were received 0.1 M cold citrate buffer (pH 4.5) only. Hyperglycemia was confirmed 48 h after STZ injection by GOD/POD method²¹. After 4 weeks of diabetes induction plasma glucose level was estimated and those with a plasma glucose level >300 mg/dl were selected as diabetic rats and used for further study.

Experimental design—Normal and diabetic rats were divided into 5 groups (n=5) and were treated daily for 4 weeks.

Group I – Normal Control (NC) received 2% gum acacia (1 ml/kg/day, *p.o*)

Group II – Diabetic control (DC) received 2% gum acacia (1 ml/kg/day, *p.o*)

Group III – Diabetic rats treated with EEAR (100 mg/kg/day, *p.o*)

Group IV – Diabetic rats treated with EEAR (250 mg/kg/day, *p.o*)

Group V – Diabetic rats treated with aminoguanidine hydrogen carbonate (1 g/L) in drinking water with daily fresh preparation.

After 4 weeks treatment, rats were fasted overnight and blood sample were collected and analyzed for the estimation of various biochemical parameters in plasma. Rats were individually placed in metabolic cage, 24 h total urine volume was measured and the same was used for estimation of renal function. Rats were sacrificed and kidneys were collected to study oxidative stress as well as histopathological observations.

Estimations of plasma biochemical parameters—Plasma glucose²¹, creatinine²², urea nitrogen²³, total cholesterol²⁴ and triglyceride²⁵ were determined using commercial diagnostic kits.

Body and kidney weight and relative kidney weight—At the end of treatment, body and kidney weight was measured by gravimetric method using electronic weighing balance and relative kidney weight was calculated.

Estimations of biochemical parameters in urine—Creatinine and albumin level were estimated using commercial diagnostic kit and glomerular filtration rate (GFR)²⁶ and AER²⁷ were calculated.

Evaluation of oxidative stress—Right kidney of individual rat was isolated, washed in cold saline and prepared 10% w/v homogenate using 0.15 M KCl by centrifuging at 10500 g for 10 min at 4 °C. The supernatant obtained was used for the estimation of lipid peroxidation (MDA)²⁸ and catalase (CAT)²⁹. Homogenate was further centrifuged at 1000 g for 20 min at 4 °C and the supernatant was used for estimation of superoxide dismutase (SOD)³⁰ and glutathione (GSH)³¹. Protein concentrations of homogenates were determined according to Lowry *et al.*³².

Histopathological studies—Left kidney of individual rat stored in 10% formalin solution was embedded with paraffin and stained with Haematoxylin-Eosin (H & E). Stained samples were observed under light microscope.

Statistical analysis—All the data were expressed as the mean ± S.E.M. Data were subjected to one-way analysis of variance (ANOVA) followed by the Dunnett's test, where *P* < 0.05 was considered as statistically significant.

Results

Biochemical parameters in plasma—STZ induced diabetic rats exhibited significant increase in plasma glucose levels compared with normal control rats ($P < 0.001$). The treatment of EEAR (100 and 250 mg/kg) ameliorated plasma glucose level when compared to diabetic control rats (Table 1). Diabetic rats also exhibit marked increase in creatinine as well as urea nitrogen levels as compare to normal rats ($P < 0.001$). EEAR (250 mg/kg) and aminoguanidine treatment significantly decreased plasma creatinine and urea nitrogen level as compared to the diabetic control rats (Table 1). Elevated level of total cholesterol and triglyceride in the diabetic rats have also been significantly ($P < 0.001$) reduced after four weeks daily treatment with EEAR (100 and 250 mg/kg) and aminoguanidine (Table 1).

Body weight, kidney weight and relative kidney weight—Significant ($P < 0.001$) decrease in body weight and increase in kidney weight (KW) as well as relative kidney weight of diabetic rats were observed when compared to normal control rats. The treatment

of EEAR (250 mg/kg) and aminoguanidine in diabetic rats significantly ($P < 0.01$; $P < 0.001$, respectively) restored the body weight and decreased kidney weight and relative kidney weight when compared to the diabetic control rats (Table 2).

Biochemical parameters in urine—After 8 weeks, significant ($P < 0.001$) increase in 24 h total urine volume, creatinine, GFR and AER were observed in the diabetic rats. EEAR (250 mg/kg) and aminoguanidine treatment for four weeks significantly ($P < 0.001$) prevented the rise in 24 h total urine volume as well as creatinine level. Whereas elevated level of GFR and AER were also been significantly reduced in all the treated groups (Table 3).

Oxidative stress—Diabetic condition produced significant ($P < 0.001$) increase in renal MDA, SOD, GSH and decrease in CAT levels. The treatment of EEAR for four weeks (100 and 250 mg/kg) and aminoguanidine showed dose dependant decrease in MDA, SOD, GSH and increase in CAT level when compared to diabetic control rats (Table 4).

Table 1—Effect of four weeks treatment of EEAR on biochemical parameters in plasma
[Values are mean \pm SEM of 5 animals]

Groups	Glucose (mg/dl)	Creatinine (mg/dl)	Urea nitrogen (mg/dl)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
NC	79.20 \pm 5.19	0.77 \pm 0.07	22.05 \pm 1.15	67.41 \pm 3.07	61.02 \pm 5.69
DC	362.80 \pm 5.83 ^S	1.57 \pm 0.04 ^S	38.67 \pm 1.03 ^S	118.10 \pm 2.91 ^S	140.80 \pm 4.07 ^S
EEAR (100)	243.60 \pm 7.00 [#]	1.34 \pm 0.11	36.61 \pm 0.50	94.65 \pm 2.62 [#]	118.60 \pm 4.43 [*]
EEAR (250)	165.80 \pm 6.92 [#]	0.78 \pm 0.07 [#]	32.19 \pm 1.31 [@]	68.27 \pm 3.03 [#]	89.32 \pm 4.44 [#]
AMG (1)	358.5 \pm 7.72	0.97 \pm 0.03 [#]	30.30 \pm 1.73 [#]	83.46 \pm 4.66 [#]	94.57 \pm 5.26 [#]

NC: normal control; DC: diabetic control, EEAR: ethanolic extract of *A. racemosus*; AMG: aminoguanidine hydrochloride

One way ANOVA followed by Dunnett's test; where ^S $P < 0.001$ when compared to normal control ^{*} $P < 0.05$, [@] $P < 0.01$, [#] $P < 0.001$ when compared to diabetic control

Table 2—Effect of 4 weeks treatment of EEAR on body weight, kidney weight and relative kidney weight
[Values are mean \pm SEM of 5 animals]

Groups	Body wt (g)	Kidney wt (g)	Relative kidney wt
NC	225.00 \pm 8.51	0.70 \pm 0.03	3.14 \pm 0.19
DC	158.40 \pm 1.88 ^S	1.25 \pm 0.03 ^S	7.94 \pm 0.17 ^S
EEAR (100)	178.80 \pm 2.47 [*]	1.19 \pm 0.05	6.69 \pm 0.22 [@]
EEAR (250)	188.00 \pm 2.21 [#]	1.07 \pm 0.03 [@]	5.72 \pm 0.11 [#]
AMG (1)	185.40 \pm 4.37 [@]	1.05 \pm 0.04 [@]	5.73 \pm 0.30 [#]

Details as in Table 1

Table 3—Effect of four weeks treatment of EEAR on biochemical parameters in urine

Groups	24 h total urine volume (mL)	Creatinine (mg/dl)	GFR (ml/min)	AER (μ g/24 h)
NC	10.20 \pm 1.16	50.06 \pm 7.00	0.47 \pm 0.08	241.8 \pm 71.15
DC	36.00 \pm 3.34 ^S	140.90 \pm 2.29 ^S	2.22 \pm 0.19 ^S	1561 \pm 37.12 ^S
EEAR (100)	28.80 \pm 4.24 [@]	118.00 \pm 8.00	1.70 \pm 0.12 [#]	1239 \pm 58.75 [@]
EEAR (250)	18.40 \pm 1.03 [#]	55.90 \pm 8.42 [#]	0.90 \pm 0.08 [#]	638.2 \pm 66.28 [#]
AMG (1)	18.94 \pm 1.61 [#]	58.37 \pm 5.01 [#]	0.76 \pm 0.10 [#]	723 \pm 51.18 [#]

Details as in Table 1

Histopathological studies—After 8 weeks of study, diabetic animals showed the presence of GBM thickening and mesangial proliferation without nodules, while normal control animals revealed no abnormalities. Treatment with EEAR (250 mg/kg) and aminoguanidine significantly attenuated these progressions (Fig. 1).

Discussion

Hyperglycemia is the principle factor responsible for structural alterations at the renal level. Diabetes Control and Complications Trial Research Group (DCCTRG) has elucidated that hyperglycemia is directly linked to diabetic microvascular

Table 4—Effect of 4 weeks treatment of EEAR on oxidative stress

Groups	MDA (nmol/mg)	SOD (U/mg)	GSH (ng/mg)	CAT (μ g/mg)
NC	15.63 \pm 0.84	4.48 \pm 0.15	9.86 \pm 0.43	113.1 \pm 4.24
DC	42.36 \pm 1.74 ^{\$}	7.04 \pm 0.38 ^{\$}	35.97 \pm 1.08 ^{\$}	37.46 \pm 2.34 ^{\$}
EEAR (100)	34.32 \pm 1.54 [@]	6.03 \pm 0.12 [*]	27.67 \pm 1.57 [#]	74.73 \pm 4.21 [#]
EEAR (250)	24.11 \pm 1.52 [#]	4.99 \pm 0.11 [#]	14.49 \pm 1.55 [#]	101.9 \pm 3.70 [#]
AMG (1)	24.2 \pm 1.74 [#]	5.17 \pm 0.26 [#]	11.91 \pm 1.32 [#]	107.7 \pm 3.52 [#]

Details as in Table 1

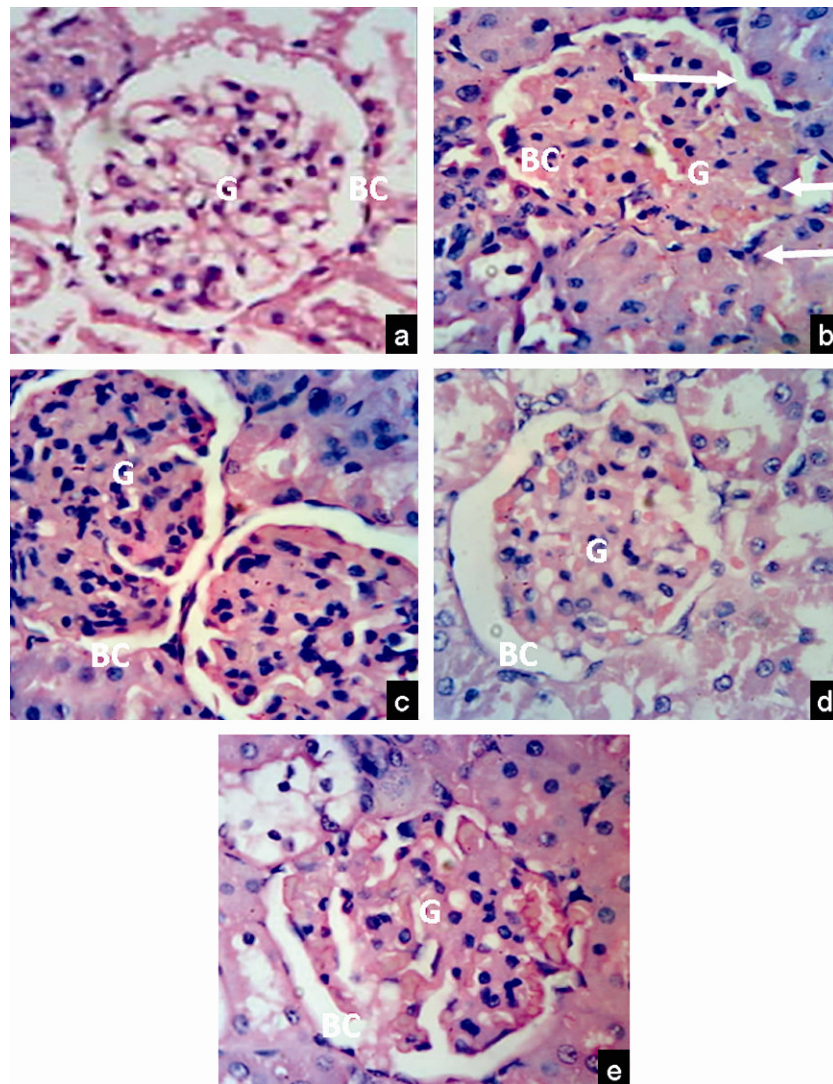


Fig. 1— Histological section of kidneys stained with H & E- 1000 \times (a)-normal control (NC) showing no abnormalities; (b)-diabetic control (DC) showing a necrotic area in glomerulus shown by white arrows. Thickenings in basement membrane are well observed; (c)-DC+EEAR (100 mg/kg); (d)-DC+EEAR (250 mg/kg) and (e)-DC+AMG showing features of healing like normal basement membrane and absence of necrotic cells in glomerulus.[BC:Bowman's capsule; G:glomerulus; P:proximal convoluted tubule]

complications³³, particularly in the kidney. In the present study, STZ induced persistent hyperglycemia for 8 weeks and produced an early DN, characterized by increased urinary albumin excretion and loss of renal function. *Asparagus racemosus* has been traditionally used for the treatment of diabetes and several reports showed good results in control of glycaemic condition.

In previous study, 4 weeks treatment of EEAR in diabetic rats significantly decreased plasma glucose level which might be attributed to its antidiabetic¹² and insulin secretory activity¹³. Functional alterations such as elevated plasma creatinine and urea nitrogen level were significantly reversed after 4 weeks repeated dose treatment of EEAR. Hypercholesterolemia and hypertriglyceridemia have been reported to occur in diabetic rats³⁴. Furthermore, kidneys are involved in catabolism of lipoprotein[a]; therefore, clearance of lipoprotein[a] in patients with nephropathy is decreased. Increased levels of plasma triglycerides and total cholesterol were significantly reduced by EEAR and aminoguanidine treatment. DN is characterized by increased AER and 24 h total urine volume, hyperfiltration occurred in the setting of normoalbuminuria as well as microalbuminuria and that loss of renal function began in the context of proteinuria³⁵. Urinary albumin level is a selective marker of glomerular injury and elevated AER represents progressive nephropathy, results into a loss of auto-regulation and inflammation²⁷. Several studies have shown that reduction of microalbuminuria indicates a better prognosis³⁶. EEAR and aminoguanidine treatment effectively reversed microalbuminuria and attenuated hyperfiltration, suggests EEAR attenuates the progression of early diabetic nephropathy.

Diabetes caused excessive break down of tissue protein, results into loss of body weight³⁷. DN has also been known to produce hypertrophy and increase in kidney weight³⁸. Therefore, the relative kidney weight in diabetic rats was significantly increased than normal rats. EEAR (250) and aminoguanidine treatment significantly restored body weight and decreased kidney weight, suggest EEAR has preventive effect on kidney hypertrophy. This is in agreement with previous findings³⁹.

Increased formation of reactive oxygen species is a major cause for development and progression of diabetic microvascular complications such as nephropathy and it has been demonstrated that modulation of oxidative stress through treatment with antioxidants effectively reduced diabetic snag⁴⁰.

Preventive antioxidants like superoxide dismutase (SOD), catalyses the dismutation of superoxide to H₂O₂ and catalase (CAT) breaks it into water. Lipid peroxidation (LP) is a free radical mediated process, initiates chain of reaction that gives rise to many products of toxicological interest like malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and various 2-alkenals⁴¹. Oxidative stress was found to be inhibited dose dependently by EEAR as assessed by decrease in renal MDA levels. Diabetic rats exhibited elevated levels of enzymic antioxidants SOD, non-enzymic antioxidant GSH and reduced CAT. EEAR treatment reversed these changes; whereas effect of EEAR (250) was comparable to aminoguanidine. Elevations in GSH and SOD activity may be compensatory mechanisms for the chronic overproduction of free radicals and oxidative stress⁴⁰.

Pathogenesis of DN results in expansion of mesangial matrix and thickening of GBM, due to accumulation of extracellular matrix (ECM) components⁴². Young *et al.*⁴³ reported that mesangial cell proliferation occurs in experimentally induced DN exhibit detectable changes in mesangial ECM deposition. In the present study, structural abnormalities in glomerulus such as basement membrane thickening and mesangial proliferation without nodules were observed in the diabetic kidney as compared to normal control rats. However nodular lesions, capsular drops, mesangiolysis, fibrin cap and mesangial sclerosis was not observed in the present study. Glomerulus is a principle site for the action of reactive oxygen species, leading to glomerulonephritis⁴⁴. EEAR treatment significantly prevented the GBM thickening and mesangial proliferation.

In conclusion, ethanolic extract of *A. racemosus* reduced hyperglycemia, creatinine, urea nitrogen level, AER, GFR and oxidative stress in diabetic rats, which are important factors relating to the progression of DN. Therefore, these findings showed that *A. racemosus* has beneficial effect in the treatment of early diabetic nephropathy.

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