

Aqueous extract of *Allium sativum* L bulbs offer nephroprotection by attenuating vascular endothelial growth factor and extracellular signal-regulated kinase-1 expression in diabetic rats

Shiju TM^a, Rajkumar R^a, Rajesh NG^b & Pragasam Viswanathan^{a,*}

^aRenal Research Lab, Biomedical Research Center, School of Bio Sciences and Technology,
VIT University, Vellore 632 014, India.

^bDepartment of Pathology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER),
Dhanvantri Nagar, Puducherry 605006, India

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To investigate the nephroprotective effect of garlic and elucidate the mechanism by which it prevents the progression of diabetic nephropathy in diabetic rats, diabetes was induced by a single ip injection of streptozotocin (45 mg/kg body weight). Garlic extract (500 mg/kg body weight) and aminoguanidine (1 g/L) were supplemented in the treatment groups. Histopathological examination using H&E, PAS staining and the immunohistochemical analysis of vascular endothelial growth factor (VEGF) and extracellular signal-regulated kinase-1 (ERK-1) expression were performed on kidney sections at the end of 12 weeks. Significant change in both, the urine and serum biochemistry confirmed kidney damage in diabetic animals which was further confirmed by the histological changes such as mesangial expansion, glomerular basement membrane thickening, glycosuria and proteinuria. However, the diabetic animals treated with garlic extract showed a significant change in urine and serum biochemical parameters such as albumin, urea nitrogen and creatinine compared to that of diabetic rats. Further, the garlic supplemented diabetic rats showed a significant decrease in the expression of VEGF and ERK-1 compared to diabetic rats, attenuating mesangial expansion and glomerulosclerosis. Thus, garlic extract rendered nephroprotection in diabetic rats.

Keywords: Diabetes, Diabetic nephropathy, Garlic extract, Hypolipidemic, Nephroprotectant

Diabetic nephropathy (DNP) is a chronic kidney disease caused by diabetes mellitus that leads to End Stage Renal Diseases (ESRD). About 25-40% of the patients with diabetes, eventually progress to DNP within 15-20 years after diagnosis¹. The disease has been diagnosed clinically by an increase in the level of albumin in the urine, which is termed as microalbuminuria², a classical biomarker for DNP. This is an indication of kidney damage, which is manifested histologically by the thickening of the glomerular basement membrane, mesangial matrix expansion, macrophage infiltration, podocyte loss and tubular epithelial degeneration³. Although various pathophysiological mechanisms have been predicted till date on DNP, the formation of advanced glycation end products induced by hyperglycemia and dyslipidemia are considered to play the lead role in setting the stage for kidney damage⁴. Therefore, any treatment that targets hyperglycemia and dyslipidemia could possibly prevent the progression of DNP.

The treatment of diabetes and its complications have a long history in which various plant medicines have been utilized⁵. The World Health Organisation recommends the exploration of traditional plant treatments for various diseases, as they are more effective, less toxic and good candidates for oral therapy with fewer side effects⁶. Nature has gifted us with a number of foods that add spice to our life. Most of the spices we consume have medicinal value and garlic is no exception. The beneficial effects of garlic include its antioxidant⁷, anti-glycation⁸, hypolipidemic, hypotensive⁹, and hypoglycemic properties¹⁰. However, its effect on DNP is still unexplored. Hence, the rationale of this study is to examine the role of garlic in preventing the progression of DNP in the experimental albino Wistar rats.

In diabetic patients, the presence of glomerular effluents such as advanced glycation end (AGE) product and large amount of proteins in the tubules, activate various cytokines and growth factors including TGF- β and vascular endothelial growth factor (VEGF)¹¹. The up-regulation of these growth factors enhances extracellular signal-regulated kinase-1 (ERK-1) activation, thus accelerating the progression of DNP¹². Apart from enhancing TGF- β

*Correspondent author

Telephone: +91-416-220 2583

E-mail: pragasam.v@vit.ac.in; pvishvanath@yahoo.com

induced extracellular matrix protein synthesis, VEGF has the ability by itself to stimulate collagen and fibronectin expression, causing mesangial expansion. Further, VEGF which has vascular permeability property has been predicted to be the major cause for proteinuria¹³. Therefore, the present study has been focused in analysing the expression of VEGF and ERK-1, as the possible mechanism in inhibiting the progression of DNP.

Materials and Methods

Chemicals and reagents—Aminoguanidine and streptozotocin (STZ) were purchased from Sigma Aldrich, India. The kits for albumin, urea, triglycerides, total cholesterol and HDL-cholesterol were procured from Span Diagnostics Ltd., Gujarat, India. The glycated haemoglobin kit was purchased from Euro Diagnostics Ltd., Chennai. All the other reagents and chemicals were obtained from Sisco Research Laboratories Ltd., India. Antibodies for VEGF (sc-7269) and ERK-1 (sc-94) were purchased from Santa Cruz Biotechnology, Inc. USA.

Preparation of aqueous garlic extract—Several investigators confirmed that the medicinal properties of garlic pertain to the water-soluble compounds such as Allin, s-allylcysteine, s-methylcysteine, and γ -glutamylcysteine¹⁴. Therefore, the aqueous extract of garlic was prepared and utilized in this study. Aqueous garlic extract was prepared based on the method followed by Lilia and Francois¹⁵, with slight modifications. Garlic (*Allium sativum*) bulbs were purchased from a local market in Vellore, India. The bulbs were separated into cloves without any damage, and the cloves were frozen at -20 °C in plastic bags overnight. The cloves were heated to 90 °C for one hour and then cooled to room temperature. The cloves were ground with distilled water (1:1, w/v) and the water-soluble compounds were extracted and filtered using muslin cloth. Based on the investigation done by Omotoso *et al.*¹⁶ 500 mg/kg/day was considered as the optimum dosage, which was prepared from the filtrate. The characterization of the garlic extract was done by the method followed by Bernhard *et al.*¹⁷ and it was found that the extract contained 0.8% alliin by comparing with that of the standard (commercial alliin).

Experimental design—Eight weeks old male albino Wistar rats weighing 200-250 g were used. They were maintained under standard laboratory conditions and

supplied with regular pellets and water *ad libitum* in VIT Animal house, Vellore. The animals were cared for as per the principles of the 'Guide for the care and use of experimental animals' and the Institutional Animal Ethical Committee approved this entire study (Approval number: VIT/IAEC/III/18/2010).

Induction of diabetes—Diabetes was induced by a single intraperitoneal injection of STZ, at a dose of 45 mg/kg body weight dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5)¹⁸. The animals were fasted for 16 h before the STZ injection, and after the injection 5% sucrose was supplemented for 24 h in order to prevent from fatal hypoglycemia. One week after STZ injection, blood drawn from the tail vein was analysed for the blood glucose level using the glucometer. The animals with a blood glucose level of more than 300 mg/dL were considered diabetic and included in the study¹⁹.

Grouping of animals—The animals were segregated into following 5 groups of 6 animals each: Gr. 1: control rats (Con), Gr 2: rats supplemented orally with 500 mg/kg body weight of garlic extract (Con+GE), Gr. 3: diabetic rats (Dia), Gr. 4: diabetic rats treated with 1 g/L of aminoguanidine dissolved in drinking water (Dia+AMG), and Gr. 5: diabetic rats supplemented with 500 mg/kg body weight of garlic extract (Dia+GE). Since aminoguanidine is a potential anti-glycation agent and is proven to ameliorate nephropathy, it was considered as a positive control in the present study. The treatment was started after two weeks of STZ injection when the animals recovered from mild nephrotoxic effects of STZ²⁰.

Measurement of serum and urine albumin—Albumin content was quantified by Bromocresol Green method using the commercially available kit. To 10 μ L of the sample, 1 mL of reagent was added and incubated for 1 min and the absorbance was measured at 630 nm. The albumin present in the sample binds to the anionic dye bromocresol green forming a green colored complex. The absorbance measured was compared with the standard to find the albumin content in the sample.

Quantification of serum and urinary creatinine—Creatinine content was measured by Jaffe method followed by Farrell and Bailey²¹. Briefly, 2 μ L of sample was added to 240 μ L of working reagent, which is a mixture of picric acid, sodium lauryl sulphate and sodium tetra borate in a multi-well plate. This mixture was incubated at room temperature for 30 min and the absorbance was measured at 505 nm. 20 μ L of 30% acetic acid was added to the wells and

further incubated for 10 min. The absorbance was again read at 505 nm and the difference in absorbance was observed. It was compared with the standard graph and the concentration of creatinine was calculated. Creatinine clearance was computed using the following formula:

Creatinine clearance ($\mu\text{L}/\text{min}$) = [Urinary creatinine (mg/dL) / Serum creatinine (mg/dL)] \times Urine volume ($\mu\text{L}/\text{min}$)

Measurement of blood and urine urea nitrogen—Urea nitrogen content was quantified using the commercially available kit. 10 μL of diluted urine (1:20 v/v) or serum was added to the reagents and kept in boiling water bath for 10 min. Urea present in the sample reacted with diacetylmonoxime in presence of thiosemicarbazide to form a purple coloured complex which was measured at 525 nm. The absorbance was compared with that of the standard and the urea nitrogen content was calculated.

Determination of glycated haemoglobin—Glycated haemoglobin level was measured by ion-exchange resin method²². Briefly, 50 μL blood was added to lysing reagent and incubated at room temperature for haemolysate preparation. Haemolysate (100 μL) was added to ion exchange resin tube and the resin separator was inserted into it so that the rubber sleeve is 1 cm above the resin suspension. The tubes were vortexed for 5 min and the resin separator was pushed inside until the resin was completely packed. The supernatant was aspirated and the absorbance was read at 415 nm against distilled water and was noted as ΔGHb . Haemolysate (20 μL) was added to 5 mL of distilled water and the absorbance read at 415 nm was noted as $\Delta\text{TotalHb}$. The percentage of glycated haemoglobin content was calculated using the formula, $\text{GHb}(\%) = (\Delta\text{GHb} / \Delta\text{TotalHb}) \times 4.61$ (assay factor)

Quantification of serum lipid profiles—The plasma lipid parameters such as triglycerides, total cholesterol, and high density lipoprotein-cholesterol (HDL-C) were estimated by the enzymatic GPO-PAP method, CHOD-PAP method, and PEG-CHOD-PAP methods respectively using the commercial kits. The low density lipoprotein-cholesterol (LDL-C) and the atherogenic index (AI) were estimated based on the Friedewald equation²³,

$\text{LDL-cholesterol} = \text{Total cholesterol} - (\text{Triglycerides}/5 - \text{HDL cholesterol})$

$\text{AI} = (\text{Total cholesterol} - \text{HDL cholesterol}) / \text{HDL cholesterol}$

Histopathological examination—At the end of 12 weeks, animals from all the five groups were

ethanized by cervical decapitation under mild anaesthesia to avoid pain and stress. Kidneys were removed carefully without any damage, washed with phosphate buffer saline (PBS), weighed and fixed in 10% neutral buffered formalin. The kidneys were then processed and embedded in paraffin. Sections (4 μm thick) were cut on a Leica RM 2126 microtome and stained with Haematoxylin & Eosin (H&E) and Periodic acid schiff (PAS) stains for histopathological analysis. The sections were then photographed under a photomicroscope (Olympus BX51; Olympus optical, Tokyo, Japan) at a magnification of $\times 400$. The sections stained with H&E were used to evaluate glomerulosclerosis and the mesangial matrix expansion was determined in terms of PAS positive signals present in the mesangial region excluding cellular elements.

Immunohistochemical observation—Paraffin sections (4 μm thick) were cut, mounted on silanised slides, dewaxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 15 min. After washing with phosphate buffered saline containing 0.1% Tween 20, the slides were incubated overnight with the primary antibody for ERK-1 (1:200 dilutions) and VEGF (1:200 dilutions) at 4 °C. The immunoreactivity was performed by incubation with horseradish peroxidase conjugated goat-anti-rabbit IgG antibody for 30 min at room temperature. The detection step was performed by treatment with 3,3'-diaminobenzidine (Dako) as chromogen. Slides were counterstained with hematoxylin, rinsed in tap water, dehydrated, placed in xylene, and mounted. The sections were then photographed under a photomicroscope at a magnification of $\times 400$. At least 30 glomeruli per section were observed and analyzed for the percentage distribution and intensity of the expression of VEGF and ERK-1.

Statistical analysis—The data were analysed on Graph Pad Prism 5.01 software and expressed as means \pm S.D (n=6). Statistical analysis was performed by One-way ANOVA followed by Dunnet's test to compare the diseased and the treated groups. At the same time, the statistical difference between the normal and diseased was analysed by Un-paired *t*-test. The results were considered statistically significant, if $P < 0.05$.

Results

Effect of aqueous garlic extract on blood glucose and glycated haemoglobin content—The diabetic

animals showed increased blood glucose level and glycated haemoglobin content throughout the study period. No change in both, the blood glucose level and glycated haemoglobin content was observed in diabetic animals supplemented with garlic extract even at the end of the study. Though various studies have proven the anti-hyperglycemic potential of garlic extract^{10,24-26}, it was not observed in the present study.

Effect of treatment on urine volume—The hyperglycemic state maintained in diabetics leads to an osmotic imbalance between the body fluids and the cellular contents. This results in more water consumption by the experimental animals, which subsequently leads to an increase in the urine volume. Significant increase ($P < 0.001$) in the urine volume was observed in the diabetic animals with the progression of the disease (Table 1) and the garlic supplementation was able to decrease ($P < 0.001$) the urine volume very close to normal at the end of 12 weeks.

Effect of garlic extract on the biomarker of DNP—Microalbuminuria is considered as the biomarker for DNP. In diabetic patients, hyperglycemia decreases the level of sulphation of proteoglycans thus decreasing the anionic nature of the membrane². This allows the albumin to pass from blood into urine thus increasing the level of albumin in the urine of diabetics. Significant increase ($P < 0.001$) in the urinary albumin concentration (Fig. 1) and a decrease ($P < 0.05$) in the serum albumin (Table 1) were observed in the diabetic animals, which lead to infer that the animals were progressing into diabetic nephropathy. However, in the animals treated with garlic extract, the urinary albumin level was brought back to normal ($P < 0.001$) with a small increase in the serum albumin content, which proves

that garlic is highly effective in preventing the progression of DNP.

Improvement of urea nitrogen content after treatment—In diabetic patients, the lack of glucose metabolism induces the catabolism of proteins thus increasing the level of urea in the blood. The urea thus formed is usually excreted when the kidney is normal, and a decrease in the level of urine urea nitrogen indicates kidney damage. Figure 2 shows the decrease in the urine urea nitrogen content in the diabetic animals ($P < 0.001$) with an increase in the blood urea nitrogen content ($P < 0.05$) (Table 1). This proves that the animals that were induced with diabetes have progressed to DNP. At the same time, the urea nitrogen content of the diabetic animals that were treated with garlic extract and aminoguanidine was very close to normal ($P < 0.01$).

Improvement of creatinine content after treatment—Creatinine is a waste product formed in the muscle by creatine phosphate catabolism. When

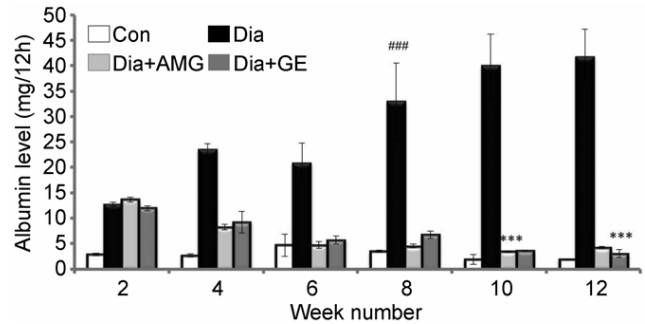


Fig. 1—Effect of aqueous garlic extract on urine albumin. Graph showing the urine albumin level (mg per 12 h) of the animals from the groups; Con, Con+GE, Dia, Dia+AMG, and Dia+GE. Values are given as mean \pm standard deviation (n=6). P values: ###,*** < 0.001; #, ** < 0.01; *, * < 0.05 (#, ###, ###, ###: Con vs Dia groups; ****, ****: Dia vs Dia + AMG and Dia vs Dia + GE groups)

Table 1—Effect of garlic extract (GE) on Metabolic parameters and serum biochemistry at the end of 12 week [Values are mean \pm SD from 6 samples each]

Parameters	Groups				
	I	II	III	IV	VI
Body weight (g)	294 \pm 3.5	279 \pm 4.6	151 \pm 1.7###	186 \pm 3**	205 \pm 3.4***
Urine volume (ml/12h)	3.75 \pm 0.4	4 \pm 1.4	24.5 \pm 0.7###	9 \pm 1.4***	8.3 \pm 0.4***
Blood glucose (mg/dl)	100 \pm 2	120 \pm 3	582 \pm 12###	433 \pm 6***	469 \pm 9***
Glycated haemoglobin (%)	6 \pm 0.7	6.1 \pm 0.5	9.8 \pm 0.2#	7.6 \pm 0.7	9.7 \pm 0.8
Serum albumin (mg/dl)	4.1 \pm 0.1	4.3 \pm 0.2	3.2 \pm 0.2#	3.4 \pm 0.1	3.4 \pm 0.1
Serum creatinine (mg/dl)	0.9 \pm 0.05	0.9 \pm 0.06	1.7 \pm 0.03###	1.5 \pm 0.03**	1.5 \pm 0.12**
Blood urea nitrogen (mg/dl)	15 \pm 0.5	14 \pm 1	59 \pm 3.5##	21 \pm 3.8**	24 \pm 3.7**
Creatinine clearance (μ L/min)	8.16 \pm 0.5	8.8 \pm 0.7	5.9 \pm 0.3###	11.5 \pm 0.7**	9.5 \pm 0.6***

P values: ###,*** < 0.001; #, ** < 0.01; *, * < 0.05 (#, ###, ###, ###: Gr I vs Gr III; IV, V; ****, ****: Gr III vs Gr IV and Gr. III vs Gr. V
Gr. I=Control (Con); Gr. II: Con+GE; Gr. III: Diabetic (Dia); Gr. IV: Dia+Aminoguanide; Gr. V; Dia+GE

there is an abnormality in the kidney, there is an increase in the serum creatinine content and a decrease in the urine creatinine content, which makes it a good biomarker for kidney damage. In this study, a significant decrease ($P<0.001$) in the urine creatinine content (Fig. 3) with a significant increase in the serum ($P<0.01$) was observed (Table 1), which confirms that the diabetic animals have progressed to DNP. Further, the decreased creatinine clearance observed in diabetic animals confirmed that the animals have encountered kidney damage. However, the supplementation of garlic extract improved the creatinine clearance which evinced that garlic is an effective nephroprotectant.

Effect of garlic on serum lipid profile—
The changes in the serum lipid level of the diabetic rats supplemented with garlic extract are shown in Table 2. There was a significant increase in the level of triglycerides ($P<0.001$), total cholesterol ($P<0.001$), LDL-cholesterol ($P<0.001$), and

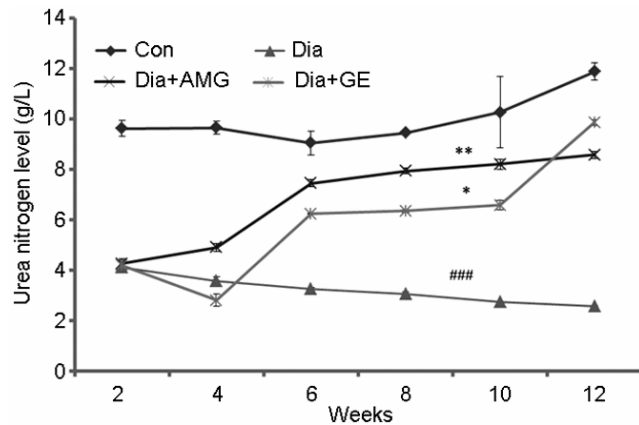


Fig. 2—Effect of aqueous garlic extract on urine urea nitrogen. Graph showing the level of urine urea nitrogen (gram per litre) of the animals from the groups; Con, Con+GE, Dia, Dia+AMG, Dia+GE. Values are given as mean \pm standard deviation (n=6). P values: ###.*** <0.001 ; ##.*** <0.01 ; *.** <0.05 (###.###: Con vs Dia groups; *.***: Dia vs Dia + AMG and Dia vs Dia + GE groups)

atherogenic index ($P<0.05$) with a significant decrease in the HDL-cholesterol level ($P<0.05$) in the diabetic animals. After treatment with garlic extract, there was a significant decrease ($P<0.05$) in triglycerides, total cholesterol, and LDL-cholesterol with very little change in the HDL-cholesterol level. The results obtained in the present study were comparable to the studies conducted by Anwar *et al.*⁷ wherein the rats were supplemented with flax and pumpkin seed mixture after inducing DNP.

Effect of garlic extract on the kidney histology—
The control rats that were supplemented with garlic extract did not show any change in the kidney histology (Fig. 4 (a)-(d)) The diabetic rats showed evidence for mesangial expansion and nodular glomerulosclerosis with increase in the thickening of the glomerular capillary membrane. There is evidence of glycosuria and proteinuria in the tubules (Fig. 5 (a) and (b)). Normal kidney histology was observed in diabetic rats supplemented with garlic while aminoguanidine treated rats showed mild mesangial expansion in glomeruli (Fig. 6).

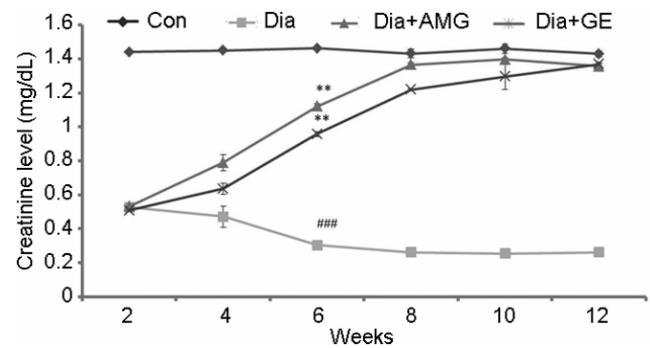


Fig. 3—Effect of aqueous garlic extract on urine creatinine. Graph showing the urinary creatinine level (mg/dl) of the animals from the groups; Con, Con+GE, Dia, Dia+AMG, Dia+GE. Values are given as mean \pm standard deviation (n=6). P values: ###.*** <0.001 ; ##.*** <0.01 ; *.** <0.05 (###.###: Con vs Dia groups; *.***: Dia vs Dia + AMG and Dia vs Dia + GE groups)

Table 2—Effect of garlic extract on Serum lipid profile
[Values are mean \pm SD from 6 samples each]

Serum parameters	Groups				
	I	II	III	IV	VI
Triglycerides (mg/dl)	50 \pm 3.5	49 \pm 0.5	112 \pm 3.5###	92 \pm 0.6*	95 \pm 1.2*
Total cholesterol (mg/dl)	103 \pm 2.6	95 \pm 2.1	196 \pm 9###	123 \pm 3.1*	142 \pm 1.9*
HDL-cholesterol (mg/dl)	50 \pm 0.1	50 \pm 0.5	44 \pm 1.2#	52 \pm 3	49 \pm 4.8
LDL-cholesterol (mg/dl)	42 \pm 3.3	35 \pm 1.6	105 \pm 4.5###	53 \pm 0.1**	70 \pm 1.5*
Atherogenic index	1 \pm 0.05	0.9 \pm 0.02	3.5 \pm 0.3#	1.4 \pm 0.1*	1.82 \pm 0.2*
LDL/HDL ratio	0.8 \pm 0.06	0.7 \pm 0.02	2.5 \pm 0.3#	1 \pm 0.1*	1.5 \pm 0.26

P values: ###.*** <0.001 ; ##.*** <0.01 ; *.** <0.05 (###.###: Gr I vs Gr III; IV, V; *.***: Gr III vs Gr IV and Gr. III vs Gr. V
Gr. I=Control (Con); Gr. II: Con+GE; Gr. III: Diabetic (Dia); Gr. IV: Dia+Aminoguanide; Gr. V; Dia+GE

Effect of garlic on VEGF and ERK-1 expression—
 To investigate the mechanism by which garlic extract prevents the progression of DNP, the expression of ERK-1 and VEGF in kidney was observed. Increased expression of both ERK-1

and VEGF was observed in STZ-induced diabetic rats. However, the diabetic rats that were supplemented with garlic extract showed a marked reduction in the expression of ERK-1 and VEGF (Figs 7 and 8).

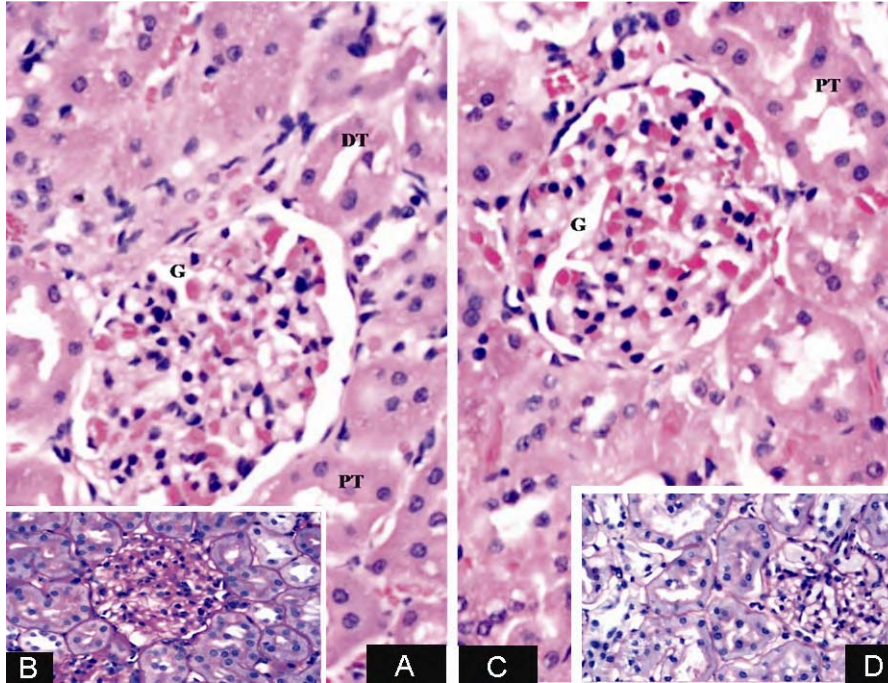


Fig. 4—Figure showing the images of kidney histology from control and rats supplemented with garlic extract. 4(A): control rats showing normal renal parenchyma, H&E, x 400; 4(B) (insert): control rats showing normal renal parenchyma, PAS, x 400; 4(C): rats supplemented with garlic extract showing normal glomerulus, tubules and blood vessels, H&E, x 400; 4(D) (insert): control rats supplemented with garlic extract showing normal glomerulus, tubules and blood vessels, PAS, x 400.

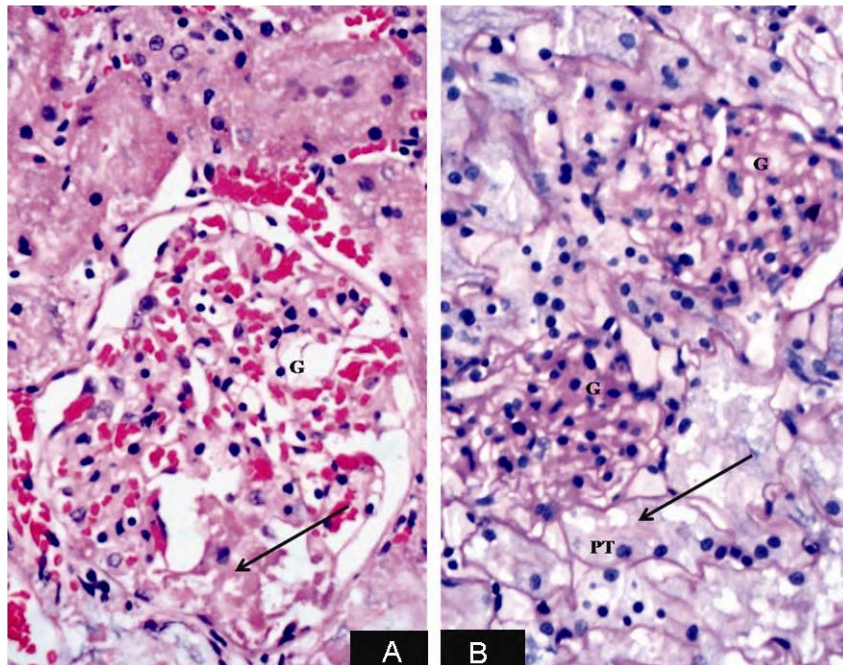


Fig. 5—Figure showing the kidney histological images of diabetic rats. 5(A, B): The section shows mesangial expansion and nodular glomerulosclerosis with increase in glomerular capillary membrane thickening, H&E, PAS respectively, x 400.

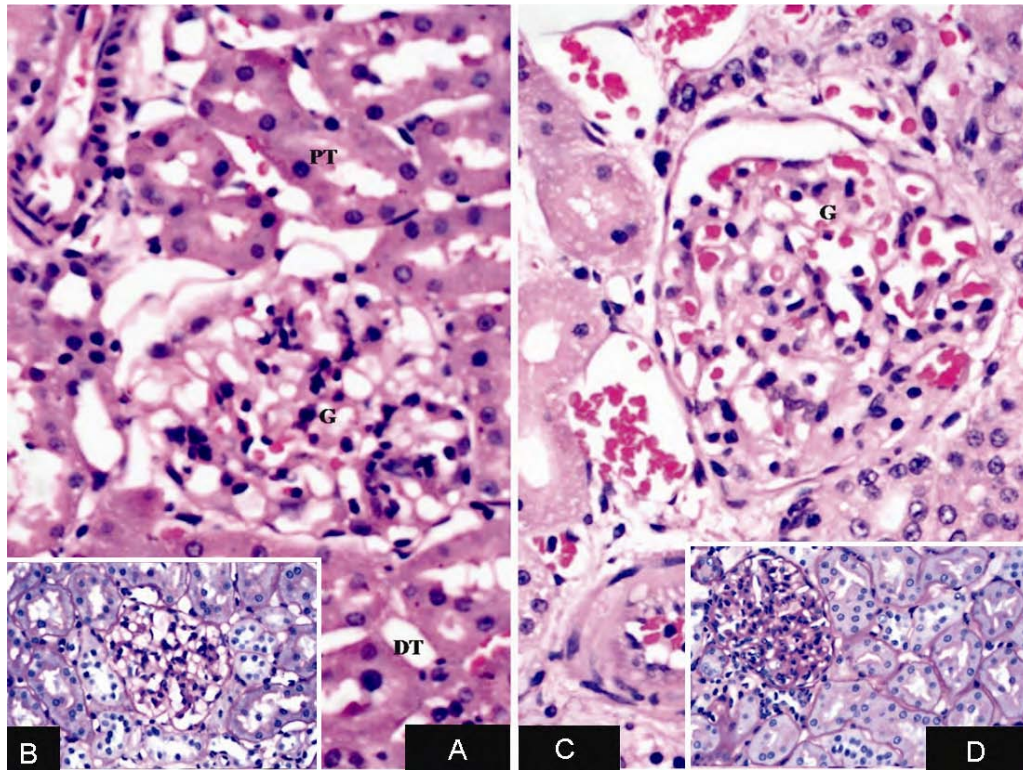


Fig. 6—Histopathological images of kidney from diabetic rats supplemented with aminoguanidine and garlic extract. Figure showing the images of kidney histology from diabetic rats supplemented with aminoguanidine (A, B) and garlic extract (C, D). 6(A): section showing mild mesangial expansion with normal glomerular capillary basement membrane thickening. Tubules and interstitium are within normal limits, H&E, x 400. 6(B) (insert): section showing mild mesangial expansion with no mesangial nodules. Tubules and interstitium are within normal limits, PAS, x 400. 6(C, D (insert)): section showing glomerulus with normal cellularity and membrane thickness. Tubules, interstitium and blood vessels are within normal limits, H&E, PAS respectively, x 400.

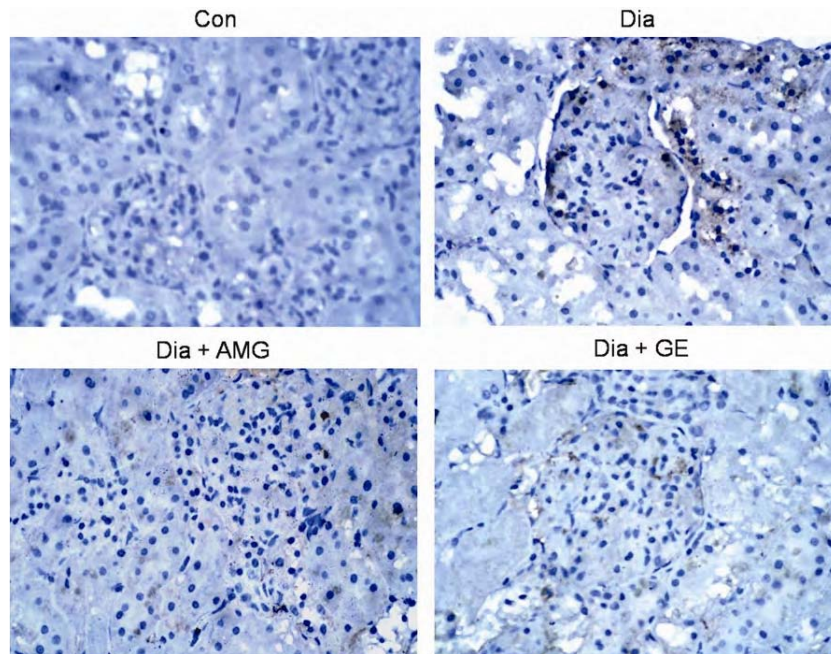


Fig. 7—Immunohistochemical images of VEGF expression. Figure showing the change in expression of VEGF in Con, Dia, Dia + AMG and Dia + GE groups. The diabetic rats showed an increased expression of VEGF. However, marked reduction in the expression of VEGF was observed in diabetic rats administered with garlic extract and aminoguanidine.

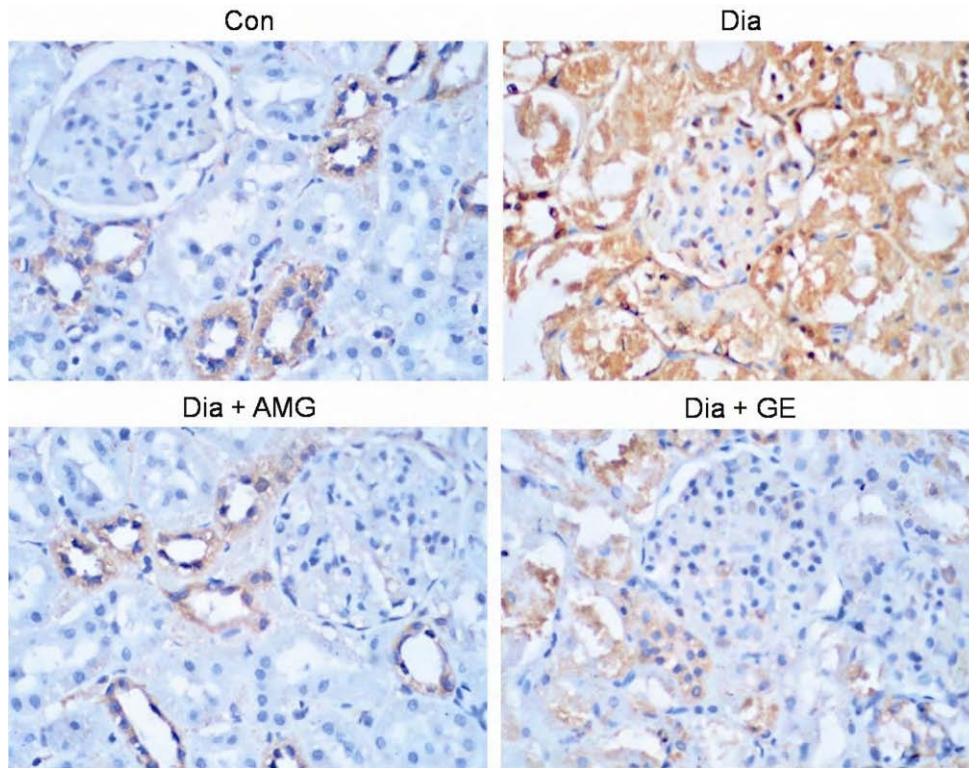


Fig 8—Immunohistochemical images of ERK-1 expression. Figure showing the change in expression of ERK-1 in Con, Dia, Dia + AMG and Dia + GE groups. The diabetic rats showed an increased expression of ERK-1. Supplementation of both garlic extract and aminoguanidine reduced the expression of ERK-1.

Discussion

Various plants such as *Glycine max*²⁷, *Brassica oleracea*²⁸ and *Coscinium fenestratum*²⁹ have been explored for the ameliorative effect of diabetic nephropathy in experimental models. However, garlic with potential anti-oxidant, hypolipidemic, and anti-glycation activity is still unexplored in this regard. In this study, the effect of garlic extract on DNP and the mechanism by which it prevents the progression of DNP in experimental animals have been analysed.

Diabetes is associated with an increase in the level of serum lipids, a risk factor for coronary heart diseases. Drugs that are able to decrease the serum lipid concentration also reduce the risk of diabetic complications³⁰. Increase in the level of serum triglycerides, total cholesterol, and LDL-cholesterol was observed in the diabetic animals that may be due to the breakdown of lipids and mobilization of free fatty acids³¹. After treatment with garlic extract, there was a significant decrease in the serum lipid level, which proves it as a potent hypolipidemic agent.

The classical symptoms of diabetes including polydipsia and polyuria³² were observed throughout

the experimental period in the diabetic rats. Treatment with garlic extract decreased the urine volume significantly normalizing the water intake. Kidney removes the metabolic waste such as urea, uric acid, and creatinine from the body thus maintaining the homeostasis. Any renal damage with uncontrolled diabetes is reflected by the accumulation of these metabolic wastes in the blood³³. In the present study, garlic was able to significantly decrease the level of these metabolites in serum increasing their level in urine, which was evident from the increased creatinine clearance after garlic supplementation. Because of the oncogenic action of STZ in causing liver and kidney tumors³⁴, the progression of diabetic rats to nephropathy cannot be confirmed with only the serum and urine biochemical analysis. So the kidney histology was observed and it was found that the biochemical changes were substantiated by the histological observations. In accordance with the previous reports³⁵, the diabetic rats showed significant changes of diabetic nephropathy including nodular glomerulosclerosis, capillary basement membrane thickening, and mesangial expansion with evidence of glycosuria in tubules at the end of 12 weeks.

However, the diabetic rats that were supplemented with garlic extract showed normal morphology. This proves that garlic could be used as an effective nephroprotectant. Since the supplementation of garlic extract did not have any effect on the blood glucose level, it could be speculated that the nephroprotective effect of garlic extract might be because of its hypolipidemic and anti-glycation property. Though the anti-glycation property of garlic has been proven by several investigators *in vitro*^{8,36}, the effect of garlic in preventing the formation of AGE in diabetic rats has to be elucidated in future.

The hyperglycemic state maintained in the diabetic condition induces advanced glycation end (AGE) product formation³⁷. AGE products interact with the inflammatory cells through cell surface molecules such as macrophage scavenger receptor type-2, RAGE and CD36³⁸, which results in the activation of p21ras, ERK-1, ERK-2 and p38 mitogen activated receptor kinase³⁹. This stimulates NF- κ B, which in turn enhanced the expression of chemokines, cytokines and growth factors such as VEGF and TGF- β ⁴⁰. VEGF, a potent inducer of vasopermeability has been proven to be involved in diabetic albuminuria⁴¹. Further, VEGF has been found to aggravate mesangial matrix expansion and proteinuria by stimulating the expression of matrix proteins such as collagen and fibronectin⁴². The activation of growth factors results in ERK-1 activation, mediating tubular epithelial cell apoptosis⁴³. Further, ERK-1 activation can also enhance cell proliferation and matrix protein synthesis⁴⁴. Since inflammatory pathway mediated by cytokine activation also plays a pivotal role in enhancing mesangial expansion and macrophage infiltration in DNP, the effect of garlic in inhibiting the expression of VEGF, ERK-1 has been analysed in this study.

The treatments inhibiting VEGF and ERK-1 expression have been shown to improve the renal function in diabetic animals^{45,12}. In the present study, the diabetic animals that were supplemented with garlic extract showed a significant decrease in the expression of both VEGF and ERK-1 compared to that of the untreated diabetic animals. This showed that the garlic extract decreases mesangial expansion and early glomerulosclerosis, thus attenuating DNP. Inhibition of VEGF expression also improved albuminuria, which was evident from the results (Fig. 1). The improvement of renal function by inhibition of VEGF and ERK-1 was vivid from the creatinine and urea level, which was brought back

very close to normal after treatment with garlic extract.

From the results, it can be concluded that the garlic extract has a potent nephroprotective effect in STZ induced diabetic animals. In addition, it was also found that the garlic extract has the ability to inhibit VEGF and ERK-1 expression, thus attenuating mesangial expansion and early glomerulosclerosis. This proves that the garlic supplementation is helpful in preventing the progression of diabetic nephropathy. However, further studies are warranted to investigate the active principles responsible for the nephroprotective effect in garlic extract.

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