

Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Ellagic acid inhibits non-enzymatic glycation and prevents proteinuria in diabetic rats

Authors: G. Raghu¹, Sneha Jakhota¹, P. Yadagiri Reddy¹, P. Anil Kumar², G. Bhanuprakash Reddy^{1*}

Author's affiliations: ¹National Institute of Nutrition, Hyderabad, India. ²Department of Biochemistry, University of Hyderabad, Hyderabad, India.

***Corresponding author:** G. Bhanuprakash Reddy
Address: National Institute of Nutrition, Hyderabad-500007, India.
Tel.: +91 40 27197252; fax: +91 40 27019074. E-mail address: geereddy@yahoo.com

Abstract:

The formation of advanced glycation end products (AGEs) is a characteristic feature of diabetic tissues and accumulation of these products has been implicated in the pathogenesis of micro- and macrovascular complications of diabetes including diabetic nephropathy (DN). Compelling evidence suggests that AGEs mediate progressive alteration in the renal architecture and loss of renal function whereas inhibitors of AGEs prevent the progression of experimental DN. We have investigated the potential of ellagic acid (EA), a polyphenol present abundantly in fruits and vegetables, to prevent *in vivo* accumulation of AGE and to ameliorate renal changes in diabetic rats. Streptozotocin-induced diabetic rats were fed with either 0.2% or 2% of EA in the diet for 12 weeks. Dietary supplementation of EA to diabetic rats prevented the glycation mediated RBC-IgG-cross-links and HbA1c accumulation. EA inhibited the accumulation of N-carboxymethyl lysine (CML), a predominant AGE in the diabetic kidney. Further, EA also prevented the AGE-mediated loss of expression of podocyte slit diaphragm proteins: nephrin and podocin. By inhibiting CML formation, EA improved renal function in rats as evidenced by urinary albumin and creatinine levels. In conclusion, EA inhibited AGE accumulation in the diabetic rat kidney and ameliorated AGE-mediated pathogenesis of DN.

Introduction:

Diabetic nephropathy (DN) remains the most common cause of end-stage renal disease (ESRD), accounting for more than 40% of patients on dialysis. Histologically, DN is characterized by expansion of extracellular matrix (ECM) in the mesangial area, thickening of the glomerular basement membrane and loss of the glomerular podocytes[1]. These histological features of DN manifest in the obliteration of the glomerular capillary lumen, loss of glomerular function and proteinuria[2]. Although, the molecular pathogenesis of DN is not fully elucidated, the events of renal injury in DN are mainly caused by complex pathophysiological alterations that are resultant of altered glucose homeostasis[3]. Altered glucose homeostasis resulting in non-enzymatic glycation is implicated in the pathogenesis of multiple diabetic complications [4]. A variety of mechanisms have been studied as contributing to the pathogenesis of diabetic complications under hyperglycemia. These include increased metabolism of glucose through the polyol pathway, increased formation of advanced glycation end products (AGE), and increased oxidative stress[5]. The phenomenon of non-enzymatic glycation involves the reaction between carbonyl (aldehyde or keto) group of reducing sugars and free amino group of proteins resulting a Schiff's base, which spontaneously rearranges into an Amadori's product. Rearrangement, dehydration and condensation of Amadori product lead to irreversible sugar-derived heterogeneous, fluorescent adducts and cross-links, which are collectively known as "advanced glycation end products" (AGEs) [6]. Carboxymethyllysine (CML), argpyrimidine and pentosidine are some of well-characterized AGEs.

Tissue accumulation of AGEs occurs with aging; however, it ensues more rapidly during diabetes owing to hyperglycemia [7-9]. Tissue toxicity of AGEs has emerged as one of the most coherent explanations for the development of many pathological complications associated with aging and diabetes such as arthritis, atherosclerosis, Alzheimer's disease, neuropathy, cataract and diabetic kidney disease[10]. More direct evidence for the role of AGEs in the pathology of DN has come from studies in which treating control rats with exogenous AGEs induced glomerular hypertrophy, mesangial sclerosis and expression of growth factors such as TGF- β and PDGF[11]. The mechanisms by which AGEs contribute to diabetic complications include (i) formation of cross-links between molecules that constitute extracellular matrix (ECM) and (ii) interaction of AGEs with receptors of AGEs (RAGE)[12]. AGE-cross linking on type1 collagen and elastin causes an increase in the area of ECM, resulting in enhanced stiffness of vasculature [12]. Further, non-enzymatic glycation results in increased synthesis of various components of ECM including collagen, laminin and fibronectin via upregulation of TGF- β [13]. On the other hand, interaction of AGEs with RAGE leads to activation of multiple signaling cascades such as MAPK and NF- κ B that in turn transcribe their target genes including vascular cell-adhesion molecule (VCAM), intracellular adhesion molecule-1 (ICAM-1) and vascular endothelial growth factor (VEGF)[12]. Administration of RAGE neutralizing antibodies [14], or genetic deficiency of RAGE [15] proved to be protective against renal injury in rodents. These studies suggest that non-

enzymatic glycation and the resultant accumulation of AGEs are associated with the manifestation of proteinuria in diabetic animals and humans. Hence, inhibition of AGEs formation seems to be an attractive therapeutic option that may alter the pathogenesis and delay the progression of DN and ESRD. Thus, considerable efforts have been focused on suppression of AGE accumulation as a target for intervention. Inhibitors of protein glycation have been shown to block AGEs formation by interacting with intermediates of Maillard reaction and retard the development of micro- and macrovascular complications in experimental diabetic animals [16]. Several agents such as aspirin, aminoguanidine, carnosine, pyridoxamine, OPB-9195, thiamine and thiazolidinediones have been tested *in vitro* and *in vivo* for their anti-AGE properties [17]. Aminoguanidine attenuates albuminuria and prevents mesangial expansion [17]. Although, majority of these agents have been found to be effective in ameliorating both circulating and tissue AGEs, but the fact that many of these elicit side-effects suggests that their use in clinical setup may be confounding. Hence, the identification and testing of novel and natural anti-glycating agents with selective efficacy and safety in humans is very much warranted.

We have evaluated a number of traditional and common dietary sources and reported that some spice principles, fruits and vegetables have the potential to inhibit AGE formation under *in vitro* conditions and prevent AGEs mediated complications in animal models, particularly by preventing the formation of CML, a predominant AGE accumulated in diabetic tissues [18, 19], [20]. Flavonoids are abundantly found in many of these dietary sources, and ellagic acid (EA; 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione) is one of the commonly found dietary polyphenols, found in numerous fruits, vegetables and many other plant foods including those dietary sources that we reported to have antiglycating activity (18). Using a range of *in vitro* protein glycation systems and lens organ culture model, we recently reported on the antiglycating activity of EA [21]. EA is already known to have antioxidant, anti-inflammatory and anticarcinogenic properties [22-25]. In the current study, we investigated the antiglycating effect of EA in diabetic animal model. Our studies showed that dietary supplementation of EA prevented CML accumulation and RAGE expression in kidney, prevented histological changes and improved proteinuria in diabetic rats.

Materials and Methods:

Chemicals and reagents:

Streptozotocin, Freund's complete and incomplete adjuvants, glyoxalic acid, sodium cyanoborohydride, BSA, Tri-reagent, Triton X-100, acrylamide, bis-acrylamide, ammonium persulfate, β -mercaptoethanol, SDS, TEMED, PMSF, aprotinin, HRP-conjugated anti-rabbit (A6154), and anti-mouse (A9044) secondary antibodies were purchased from Sigma Chemicals (St. Louis, MO). Research grade quality free ellagic acid (ASB # 00005070-101) was obtained from ChromaDex (Irvine, CA, USA). ECL-

detection kit was obtained from GE Health Care (Buckinghamshire,UK).All the primary antibodies used in this study were purchased from Santa Cruz Biotechnology(Texas, USA). RNA isolation kit and QuantiTect SYBR Green RT-PCR kit were obtained from QIAGEN. All primers used for RT-PCR analysis were procured from Integrated DNA Technologies (Hyderabad, India).Complete mini protease inhibitor tablets were obtained from Roche (Mannheim, Germany).

Experimental design and dietary regimen:

Two-month-old male Wistar-NIN rats with an average bodyweight of 232 ± 16 g were used in the study. All the animals were fed with AIN-93 diet ad libitum. The control (Group I) rats received 0.1 M citrate buffer, pH 4.5 as vehicle while the experimental rats received a single i.p injection of STZ (35 mg/kg) in citrate buffer. 72 h post-injection with STZ, fasting blood glucose levels were monitored. Animals with blood glucose levels <150 mg/dl were excluded from the experiment and the rest were distributed into three groups (Groups II-IV). Animals in group II received AIN-93 diet alone; group III animals received the AIN-93 diet supplemented with 0.2% EA (EA1) whereas group IV animals received AIN-93 diet enriched with 2% EA (EA2). Each group comprised of 6 animals. The dose of EA was decided based on a pilot study. Animals were housed in individual cages in a temperature (22°C) and humidity-controlled room with a 12 h light/dark cycle. All the animals had free access to water. All experiments dealing live animal were performed in compliance with the relevant laws and institutional guidelines, and the animal care and protocols were approved by the Institutional Animal Ethics Committee of the National Institute of Nutrition (Hyderabad, India).

Blood, kidney collection, and processing:

Blood was collected once a week from the retro-orbital plexus for estimation of glucose. For urinary albumin excretion, prior to sacrifice animals were placed in metabolic cages for collection of urine over 24 h. At the end of 12 weeks duration of diabetes, the animals were sacrificed by CO_2 asphyxiation. Kidneys were perfused in vivo via the abdominal aorta with 100 mL of normal saline at 4°C . The left renal vein was punctured to permit the perfusate to drain, and the kidney was removed immediately and placed in 4% paraformaldehyde for subsequent histologic studies. The other kidney was snap frozen in liquid nitrogen and stored at -80°C for further analysis.

Biochemical estimations:

Serum glucose by the glucose oxidase-peroxidase (GOD-POD) method, HbA1c (RBC), albumin, creatinine, urea in urine and plasma were estimated using commercially available kits (BioSystems, Spain).

Analysis of RBC-IgG cross-linking:

Blood was collected from the rats in heparinized tubes and plasma was carefully removed. Then, RBC was washed 4-5 times with ice-cold PBS, and 0.2 ml of packed RBC was collected from the bottom of the tube and diluted in PBS to desirable dilution and used for further analysis. The amount of IgG bound to RBC was quantified using ELISA as described by vasan et al[26]. The bound IgG was calculated $(100 \times (\text{OD}_{410} \text{ control} - \text{OD}_{410} \text{ treated}) / \text{OD}_{410} \text{ control})$.

Quantitative Real-Time PCR:

Total RNA was extracted using tri-reagent (Invitrogen) according to manufacturer instructions. Isolated RNA was further purified by RNeasy Mini Kit (Qiagen) and quantified by measuring the absorbance at 260 nm on NanoDrop (ND 1000). Four μg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit. Reverse transcription reaction was carried out using a thermocycler (ABI-9700) and the reaction conditions were as follows: initial RT for 10 min at 25°C, followed by 37°C for 120 min and inactivation of reverse transcriptase at 84°C for 5 min. Real-time PCR (using ABI-7500) was performed in triplicates with 25 ng cDNA templates using SYBR green RT-PCR kit with gene specific primers (Table 1). Normalization and validation of data were carried using β -actin as an internal control and data were compared between control and diabetic samples according to comparative CT ($2^{-\Delta\Delta\text{ct}}$) method. The reaction conditions were as follows: 40 cycles of initial denaturation temperature at 95°C for 30 s followed by annealing at 52°C for 40 s and extension at 72°C for 1 min and product specificity was analyzed by melt curve analysis.

Immunohistochemistry:

Kidneys were collected at the end of the animal experiment and fixed in 4% paraformaldehyde in sodium phosphate buffer (pH 7.2), followed by embedding and sectioning in paraffin using standard protocols. Three kidneys from each group were used for analysis. Immunolocalization of nephrin, podocin, CML and VEGF, Direct immunofluorescence of IgG (Goat anti-rat IgG (Alexa fluor 488, Invitrogen) were carried out on 4 μm thick paraffin sections of Wistar rat kidneys prepared on chrome alum gelatin coated slides. The kidney sections were deparaffinized on a hot plate at 60°C for 45 minutes followed by incubating the slides in xylene for 10 min, and the same was repeated twice. The

sections were then hydrated in decreasing grades of isopropyl alcohol (90%, 70% and 50%). Antigen retrieval was done by heating the slides in 10 mM sodium citrate, pH 6.0 for 10 min at 60°C in a microwave oven. The endogenous peroxidase activity was quenched by incubating the slides in 3% H₂O₂ for 30 min. Two washes of 5 min interval each time were given in phosphate buffer saline (PBS). To prevent non-specific binding of the antibody, blocking was done by incubating the slides in 10% normal goat serum in PBS at room temperature for 1 hour. Later the slides were incubated overnight at 4 °C with primary antibody in 1.5% normal goat serum in PBS. After incubation slides were washed with PBS and incubated with biotinylated secondary antibody solution for 30 min and followed by incubation of slides for 30 min with Vectastain Elite ABC reagent (ABC Elite kit, Vector Laboratories). The protein was localized by brown staining in the kidney sections upon the addition of DAB solution containing H₂O₂. Slides were observed under an epifluorescence microscope (LMD 6000, Leica Microsystems, Germany), and images were captured using appropriate filters.

Western Blot Analysis:

Tissue lysates were prepared in homogenization buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS) containing protease inhibitor cocktail (Complete mini, Roche, Mannheim, Germany), 1 mM sodiumorthovanadate and 50 mM NaF. Lysates were centrifuged at 12,000 rpm and aliquots of the supernatants were separated on 12 % SDS-PAGE and transferred on to the nitrocellulose membrane (Immobilon-P, Millipore). After probing with corresponding primary antibodies, antigen-antibody complexes were detected with horseradish peroxidase-labeled secondary antibodies and visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

Statistical analysis:

Data were expressed as mean \pm SEM unless otherwise stated. A one-way analysis of variance (ANOVA) with pairwise comparisons according to the Tukey method was used in this study. Differences were considered significant if the p-value was less than 0.05.

Results:

EA has no effect on blood glucose in diabetic rats:

Fasting blood glucose levels were elevated in STZ treated diabetic rats and persisted for 12 weeks of the study compared with control animals. Average fasting blood glucose levels of STZ-induced diabetic

rats were 332 ± 35 mg/dl compared with that of control rats' 90 ± 15 mg/dl. Diabetic rats showed decreased body weight and increased food intake compared with control rats (Table 2). Dietary supplementation of EA did not improve hyperglycemia and body weights in diabetic rats (Table 2). Feeding EA to control rats did not elicit any deleterious effects.

EA ameliorates diabetes mediated protein glycation in blood:

The in vivo anti-AGE effect of EA was assessed by estimating the amount of glycated hemoglobin (HbA1c). HbA1c levels in untreated diabetic rats (12%) were significantly higher compared with control animals (5%; Fig.1A). Dietary supplementation of EA ameliorated HbA1c accumulation in experimental diabetic rats (EA1- 9% & EA2-7%; Fig 1A). Further, we have investigated the ability of EA to prevent cross-linking of IgG on red blood cell surface (RBC-IgG). During diabetic conditions, there is a considerable increase in RBC-IgG cross-linking that provide an index of AGE mediated protein cross-linking. We assayed the % decrease in RBC-IgG cross-linking in treated rats compared with untreated diabetic rats. Dietary supplementation with EA significantly decreased RBC-IgG cross-linking (EA1-67% and EA2-72%) compared with untreated diabetic rats (38%) (Fig.1B).

EA ameliorate diabetes mediated protein glycation in glomeruli:

In addition to analysis of the glycation of hemoglobin, we have estimated the extent of protein glycation in kidney in terms of IgG cross-linking to glomerular regions and effect of EA in preventing protein glycation, if any. In glomeruli from diabetic rats, we have noticed an accumulation of significant amount of IgG compared with control rats (Con- 0.4×10^5 vs 3×10^5 ; Fig. 1C&D). Interestingly, EA supplementation to rats (EA1 & EA2) significantly ameliorated IgG accumulation in glomerular regions (Fig.1C &D).

Dietary feeding of EA prevented CML accumulation in diabetic rat kidney:

CML is a predominant AGE that is abundant in renal tissues of diabetic rats [27]. Therefore, we measured CML levels in glomerular extracts of control and experimental rats. Both immunoblotting and immunohistochemical examination revealed the elevated accumulation of CML in the glomerular region of diabetic rats (Fig 2A-D). Our experiments revealed that feeding EA prevented the CML formation in glomeruli of diabetic rats (Fig. 2A-D). The cytotoxicity of AGE (also CML) at the cellular level is mediated via RAGE (Receptor for AGE). Therefore, we measured the expression of RAGE in these experimental animals. Increased expression of RAGE was observed in diabetic rat kidney compared with control rats (Fig. 2A, C). Interestingly, hyperglycemia-induced RAGE expression was prevented by dietary supplementation of EA (Fig. 2A, C).

EA prevented AGE induced TGF- β expression:

It has been known that AGEs activate transforming growth factor- β (TGF- β) and its downstream signaling pathway in glomerular cells [28]. Our study also demonstrated increased expression of TGF- β in glomeruli from diabetic rats (Fig 3A). TGF- β is a fibrogenic cytokine and a key mediator in advanced diabetic renal disease[29]. Therefore, we have investigated for the morphological changes in kidney sections employing PAS staining for glycated proteins and Masontrichome staining for collagen accumulation (Fig. 3B&C). We noticed increased PAS staining in untreated diabetic rats (Fig.3B). However, in diabetic animals fed with EA (both 0.2 and 2%), the intensity of PAS staining was decreased compared to untreated diabetic rats. Nevertheless, in EA fed diabetic rats, there was a significant reduction of Masontrichome staining in both interstitial and glomerular regions suggesting that EA prevented collagen deposition possibly by attenuating TGF- β expression in diabetic rat kidneys.

EA prevented depletion of podocyte slit diaphragm proteins in diabetic rats:

We analyzed the steady state abundance of major slitdiaphragm proteins: nephrin and podocin (Fig. 4A-F).Quantification of mRNA by RT-PCR revealed that expression of both nephrin and podocin were decreased by 35-40% in diabetic rat glomerulus compared to that of control rat glomeruli (Fig. 4A,B). Dietary supplementation of EA attenuated the hyperglycemia mediated loss of glomerular nephrin and podocin expression. Inconcurrency with RT-PCR analysis, both immunohistochemical and immunoblotting studies revealed diminished expression of nephrin and podocin in untreateddiabetic rats whereas supplementation of EA mitigatedthe loss of glomerular nephrin and podocin expression (Fig. 4C-F).

EA ameliorates proteinuria in diabetic rats:

Since EA prevented depletion of slit diaphragm proteins in glomerular lysates, we assessed glomerular function by quantifying protein content in urine. Diabetic rats excreted significantly elevated levels of albumin compared to control rats (136.2 vs. 6.01 mg/24 h). Urinary albumin content is significantly decreased in rats fed EA in a dose-dependent manner (45.03 & 29.61 mg/24 h). Diabetic rats excreted greater amounts of creatinine compared with non-diabetic control rats (23.15 vs. 7.67 mg/24 h) whereas feeding diabetic rats with EA manifested in the diminished excretion of creatinine (8.94 & 7.19 mg/24 h) which is also reflected in albumin to creatinine ratio (Fig. 5A).Furthermore, we estimated urea content in the plasma and urine. Diabetic animals displayed 65.87 ± 6.21 mg/dl urea in plasma against 29.27 ± 2.71 mg/dl in control rats (Table 2). Plasma urea content was restricted to 53.55 ± 7.34 and 44.16 ± 7.01 mg/dl in EA 0.2% and 2% fed rats, respectively. Simultaneously we have also measured urea content in the urine. Diabetic rats excreted increased quantity of urea in the urine when compared with

control rats (Fig. 5B) and in rats fed with EA the amount of urea excreted in urine decreased significantly (Fig. 5B).

Discussion:

The accumulation of AGEs, consequent activation of RAGE and resultant aberrant cellular events are implicated in the pathogenesis of diabetic complications including nephropathy. AGEs manifest in altered signaling events and injury to various glomerular cells including podocytes that eventually result in proteinuria. Therefore, it is reasonable to presume that blocking AGEs formation and/or preventing RAGE activation would be a therapeutic target to combat AGE mediated renal pathology and improve proteinuria [30]. In the present study our experiments reveal that EA prevented non-enzymatic glycation as evidenced by HbA1c, IgG and RBC cross-linking and importantly, CML accumulation in renal tissues. EA prevented AGE mediated activation of TGF- β and resultant fibrosis and mesangial expansion in diabetic rats. This study highlights the fact that loss of podocyte slit diaphragm proteins, podocin and nephrin is ameliorated in diabetic rats fed with EA, thus improved proteinuria in diabetic rats.

The bioavailability of dietary EA, however, has been under the scanner owing to its insolubility in aqueous solutions and stability. Ellagic acid undergoes extensive metabolism in the gut by the microbiota producing urolithins which are better absorbed and circulate as glucuronide conjugates [31]. However, studies have also shown some amount of free EA in plasma [32]. Though, the number of in vivo studies is still limited, the biological effects of EA could be attributed to both free EA and its metabolites, particularly urolithins. However, we did not measure urolithins in this study. Nevertheless, urolithins have been proved to prevent protein glycation and neurodegeneration [33] and also shown to exhibit carbonyl scavenger activity in vitro [34]. Therefore, it is possible that free EA and/or urolithins might contribute to inhibition of AGE formation in vivo. However, further investigations are needed to delineate the contribution of various metabolites of EA including free EA in preventing AGE formation and amelioration of DN.

DN has become the most frequent cause of terminal renal failure. The clinical course of DN includes the development of histological and functional degenerative changes in the kidney including, thickening of GBM, mesangial matrix expansion, arteriolar hyalinosis and podocytopathy that are characteristic aspects of DN [35]. Hyperglycemia is implicated in the development of degenerative changes via formation of AGEs [36]. Immunochemical studies have detected AGEs in the mesangium, endothelium, tubular and glomerular basement membranes. CML is the major AGE that is detected in these compartments of the kidney. In this study, we have focused on the protective effect of EA on podocytes under diabetic conditions.

Podocytes are terminally differentiated visceral epithelial cells located at the interface of blood-urine barrier adhered to GBM. Podocytes have a specialized structure with a cell body, primary and secondary foot processes. A slit diaphragm connects secondary foot processes of neighboring podocytes, which offers a major size-selective filtration barrier that allow only water and small molecular size solutes to pass through and retains high molecular weight protein in plasma. Podocyte foot processes, GBM, and the fenestrated endothelial cells together form the glomerular filtration barrier. Thus, podocytes regulate the composition of primary glomerular filtrate and proteinuria ensues when the structure of podocytes is destroyed by disruption of the slit diaphragm[37]. AGEs essentially via upregulation and activation of RAGE are implicated in several degenerative changes in podocytes[38]. AGE-rich culture media and glycated collagen IV have been found to induce podocyte apoptosis by inducing the expression of FOXO4 and Bim [39]. Podocyte apoptosis has been reported in patients with diabetes. However, diabetic mice that are devoid of RAGE expression are protected from podocyte injury, suggesting a preponderant role of AGEs in the pathology of podocytopathy [15]. AGE-RAGE axis has been shown to reduce the expression of slit diaphragm proteins such as zonulaoccludins, and α -actinin 4, an actin filament cross-linking protein required for normal podocyte adhesion[40]. Reduced expression of silt-diaphragm proteins results in increased albumin permeability through the podocytes. Thus, AGEs trigger development of proteinuria. Further, loss of integrity of actin cytoskeleton may result in detachment of podocytes from GBM. AGEs, particularly CML, induced inflammatory molecules such as MCP-1 in podocytes [41].

Conclusion:

There is compelling evidence to suggest that the formation and accumulation of AGEs mediate the progressive alteration in renal architecture and loss of renal function. It is evidenced from current and earlier studies that inhibitors of advanced glycation ameliorate the progression of proteinuria in experimental diabetic rats. AGEs are also implicated in the pathology of several diabetic complications such as cataract, retinopathy, and cardiomyopathy. Considering the magnitude of diabetic complications and their impact on human health and economy, inhibitors of glycation like EA from dietary sources are of major importance and immense use.

Acknowledgements: GBR received grants from the Department of Biotechnology, India under 7th FP of Indo-EU collaborative grant on functional foods (Grant agreement #245030). GR was supported by a postdoctoral fellowship from the grant; SJ received a research fellowship from University Grants Commission, India; PAK was supported by INSPIRE-Faculty fellowship from Department of Science & Technology, India. The authors thank Ms. G. Sailaja from Department of

Pathology, National Institute of Nutrition, Hyderabad, for the help in processing and preparing the tissues sections.

Conflicts of interest statement: The authors declare no conflict of interests

Abbreviations: **AGE**, advanced glycation end product; **CML**, carboxy methyl lysine; **DAB**, 3,3'-diaminobenzidine; **DN**, diabetic nephropathy; **EA**, ellagic acid; **ESRD**, end stage renal disease; **ECM**, extracellular matrix, **ICAM**, intracellular adhesion molecule; **PBS**, phosphate buffer saline; **PMSF**, phenyl methane sulfonyl fluoride; **SDS**, sodium dodecyl sulphate; **STZ**, streptozotocin; **TEMED**, N,N,N',N'- tetra methyl ethylene diamine; **VCAM**, vascular cell adhesion molecule.

Keywords: Advanced glycation end-products, carboxymethyllysine, diabetic nephropathy, ellagic acid, proteinuria

Table 1: List of Primers used for RT-PCR analysis

Gene	Sequence
<i>β-Actin</i>	5' CGA CAA CGG CTC CGG CAT GT 3' 5' GGG GCC ACA CGC AGC TCA TT 3'
<i>VCAM</i>	5' TCT TCG GAG CCT CAA CGG TAC T 3' 5' TGG TGC TGC AAG TCA GGA GCA T 3'
<i>TGF-β</i>	5' AGC TGG TGA AAC GGA AGC GCA T3' 5' TTG TAC AAA GCG AGC ACC GCC T 3'
<i>Nephrin</i>	5' ACA GCG TGC TGG TGA TGA CTG T3' 5' TGG TAA TGG CGC TTG GGG GAA A3'
<i>Podocin</i>	5' AGC CAT CCA GTT CCT GGT GCA A3' 5' TGC CCC AAA CAC AGG TCA CTG A3'

Table 2: Biochemical estimation in urine, serum and, plasma of control and experimental rats.

Parameters	Control	Diabetic (D)	D+EA1	D+EA2
Average food intake (g/day)	16±0.55	32±2.24*	30±2.07*	29±2.39*
Body Weights(g)	309±4	199.6±23.85*	199.7±22.36*	195.5±12.67*
Blood glucose (mg/dL)	90.31±15.41	332.2±35.52*	338.1±47.50*	328.4±23.46*
HbA1C	6.03±0.98	11.61±2.03*	9.17±1.70	7.58±0.52
Plasma urea (mg/dL)	29.27±2.71	65.87±6.21*	53.55±7.34	45.16±7.09
Serum creatinine (mg/dL)	0.84±0.02	0.79±0.05	0.83±0.07	0.84±0.04
Urinary albumin (mg/24h)	6.01±0.57	136.2±30.4*	45.03±27.06*	29.61±11.73*
Urinary creatinine (mg/24h)	7.67±0.45	23.15±5.08*	8.94 ±1.03	7.19±2.11
Urinary urea (mg/24h)	411.8±32.66	1104±277.6*	797.5±154.8*	666.5±190*
Creatinine clearance (mL/min)	0.65±0.02	3.14±0.16*	1.07±0.14	0.87±0.11

Data are presented as mean ± SE, n=6 (*p<0.05)

References:

1. Kolset SO, Reinholt FP, Jenssen T. Diabetic nephropathy and extracellular matrix. *J Histochem Cytochem* 60: 976-986.
2. Lopez-Novoa JM, Rodriguez-Pena AB, Ortiz A et al. Etiopathology of chronic tubular, glomerular and renovascular nephropathies: clinical implications. *J Transl Med* 9: 13.
3. Gnudi L, Karalliedde J. Beat it early: putative renoprotective haemodynamic effects of oral hypoglycaemic agents. *Nephrol Dial Transplant*.
4. Brownlee M. Glycation products and the pathogenesis of diabetic complications. *Diabetes Care* 1992; 15: 1835-1843.
5. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; 414: 813-820.
6. Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. *Circ Res* 2004; 95: 233-238.
7. Brownlee M, Vlassara H, Cerami A. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 1984; 101: 527-537.
8. Dyer DG, Dunn JA, Thorpe SR et al. Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 1993; 91: 2463-2469.
9. Price DL, Rhett PM, Thorpe SR, Baynes JW. Chelating activity of advanced glycation end-product inhibitors. *J Biol Chem* 2001; 276: 48967-48972.
10. Reddy VP, Beyaz A. Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. *Drug Discov Today* 2006; 11: 646-654.
11. Tsuchida K, Makita Z, Yamagishi S et al. Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195. *Diabetologia* 1999; 42: 579-588.
12. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* 2006; 114: 597-605.
13. Pugliese G, Pricci F, Romeo G et al. Upregulation of mesangial growth factor and extracellular matrix synthesis by advanced glycation end products via a receptor-mediated mechanism. *Diabetes* 1997; 46: 1881-1887.
14. Flyvbjerg A, Denner L, Schrijvers BF et al. Long-term renal effects of a neutralizing RAGE antibody in obese type 2 diabetic mice. *Diabetes* 2004; 53: 166-172.
15. Myint KM, Yamamoto Y, Doi T et al. RAGE control of diabetic nephropathy in a mouse model: effects of RAGE gene disruption and administration of low-molecular weight heparin. *Diabetes* 2006; 55: 2510-2522.
16. Booth AA, Khalifah RG, Todd P, Hudson BG. In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways. *J Biol Chem* 1997; 272: 5430-5437.
17. Forbes JM, Cooper ME. Mechanisms of diabetic complications. *Physiol Rev* 93: 137-188.
18. Mrudula T, Suryanarayana P, Srinivas PN, Reddy GB. Effect of curcumin on hyperglycemia-induced vascular endothelial growth factor expression in streptozotocin-induced diabetic rat retina. *Biochem Biophys Res Commun* 2007; 361: 528-532.
19. Saraswat M, Reddy PY, Muthenna P, Reddy GB. Prevention of non-enzymic glycation of proteins by dietary agents: prospects for alleviating diabetic complications. *Br J Nutr* 2009; 101: 1714-1721.
20. Muthenna P, Raghu G, Kumar PA et al. Effect of cinnamon and its procyanidin-B2 enriched fraction on diabetic nephropathy in rats. *Chem Biol Interact* 222C: 68-76.
21. Muthenna P, Akileshwari C, Reddy GB. Ellagic acid, a new antiglycating agent: its inhibition of N-(carboxymethyl)lysine. *Biochem J* 2012; 442: 221-230.

22. Jurenka JS. Therapeutic applications of pomegranate (*Punica granatum* L.): a review. *Altern Med Rev* 2008; 13: 128-144.
23. Corbett S, Daniel J, Drayton R et al. Evaluation of the anti-inflammatory effects of ellagic acid. *J Perianesth Nurs* 25: 214-220.
24. Lee WJ, Ou HC, Hsu WC et al. Ellagic acid inhibits oxidized LDL-mediated LOX-1 expression, ROS generation, and inflammation in human endothelial cells. *J Vasc Surg* 2010; 52: 1290-1300.
25. Heber D. Multitargeted therapy of cancer by ellagitannins. *Cancer Lett* 2008; 269: 262-268.
26. Vasani S, Zhang X, Kapurniotu A et al. An agent cleaving glucose-derived protein crosslinks in vitro and in vivo. *Nature* 1996; 382: 275-278.
27. Luevano-Contreras C, Chapman-Novakofski K. Dietary advanced glycation end products and aging. *Nutrients* 2: 1247-1265.
28. Fukami K, Ueda S, Yamagishi S et al. AGEs activate mesangial TGF-beta-Smad signaling via an angiotensin II type I receptor interaction. *Kidney Int* 2004; 66: 2137-2147.
29. Ziyadeh FN. Mediators of diabetic renal disease: the case for TGF-beta as the major mediator. *J Am Soc Nephrol* 2004; 15 Suppl 1: S55-57.
30. Yamagishi S, Nakamura K, Matsui T et al. Agents that block advanced glycation end product (AGE)-RAGE (receptor for AGEs)-oxidative stress system: a novel therapeutic strategy for diabetic vascular complications. *Expert Opin Investig Drugs* 2008; 17: 983-996.
31. Espin JC, Larrosa M, Garcia-Conesa MT, Tomas-Barberan F. Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. *Evid Based Complement Alternat Med* 2013; 2013: 270418.
32. Yang J, Lee R, Henning SM et al. Soy protein isolate does not affect ellagitannin bioavailability and urolithin formation when mixed with pomegranate juice in humans. *Food Chem* 2016; 194: 1300-1303.
33. Verzelloni E, Pellacani C, Tagliacozzi D et al. Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites. *Mol Nutr Food Res* 2011; 55 Suppl 1: S35-43.
34. Liu W, Ma H, Frost L et al. Pomegranate phenolics inhibit formation of advanced glycation endproducts by scavenging reactive carbonyl species. *Food Funct* 2014; 5: 2996-3004.
35. Amann K, Benz K. Structural renal changes in obesity and diabetes. *Semin Nephrol* 33: 23-33.
36. Yamagishi S, Nakamura K, Imaizumi T. Advanced glycation end products (AGEs) and diabetic vascular complications. *Curr Diabetes Rev* 2005; 1: 93-106.
37. Benzing T. Signaling at the slit diaphragm. *J Am Soc Nephrol* 2004; 15: 1382-1391.
38. Ramasamy R, Vannucci SJ, Yan SS et al. Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* 2005; 15: 16R-28R.
39. Anil Kumar P, Welsh GI, Saleem MA, Menon RK. Molecular and cellular events mediating glomerular podocyte dysfunction and depletion in diabetes mellitus. *Front Endocrinol (Lausanne)* 5: 151.
40. Zhang A, Huang S. Progress in pathogenesis of proteinuria. *Int J Nephrol* 2012: 314251.
41. Gu L, Ni Z, Qian J, Tomino Y. Pravastatin inhibits carboxymethyllysine-induced monocyte chemoattractant protein 1 expression in podocytes via prevention of signalling events. *Nephron Exp Nephrol* 2007; 106: e1-10.

Figure Legends:

Figure 1: Ellagic acid prevented protein glycation and accumulation of AGE in diabetic rats. The anti-AGE effect of EA was assessed by estimation of glycated hemoglobin- HbA1C (A) and RBC-IgG cross-linking (B) in control, diabetic and diabetic rats fed with EA (EA1 and EA2). IgG accumulation in glomerular regions of kidney was assessed using immunofluorescence, images were captured at original magnification 40x (C) and quantified using Image J analysis (D). Data in panel C are representative of three independent experiments. Data in panel A, B and D are presented as mean \pm SE (n=6 for data in panel A and B; n=3 for data in panel D). Significant difference ($p<0.05$) of the mean values among the groups are indicated by a, b, c above the bars.

Figure 2: Ellagic acid treatment prevented CML accumulation in diabetic rat glomeruli. CML and RAGE expression in glomerular preparation from control, diabetic and diabetic rats fed with EA (EA1 and EA2) treated rats were analyzed by immunoblotting (A), and densitometric quantification by normalizing to β -actin (B, C). Data are presented as mean \pm SE (n=3). Significant difference ($p<0.05$) of the mean values among the groups are indicated by a, b above the bars. Accumulation of CML in glomerular regions is assessed using immunohistochemical staining by probing with anti-CML antibodies images were captured at original magnification 40x (D).

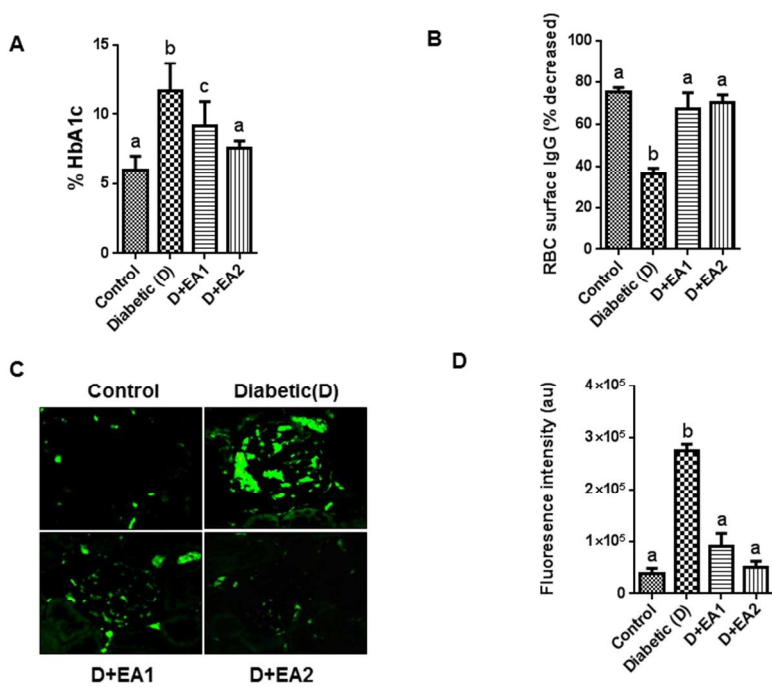
Figure 3: Inhibition of TGF- β and accumulation of ECM in glomeruli by EA. TGF- β expression was measured in control, EA treated and untreated diabetic rats by quantitative real-time PCR (A). Significant difference ($p<0.05$) of the mean values among the groups are indicated by a, b above the bars. Deposition of glycated protein in experimental rats was analyzed using PAS staining procedure (B). Accumulation of ECM in glomerular preparation and interstitium was measured using mason-trichrome staining, images were captured at original magnification 40x (C).

Figure 4: Ellagic acid ameliorated loss of expression of podocyte specific markers. Expression of podocyte specific markers nephrin and podocin was analyzed in glomerular regions from control, diabetic and diabetic rats fed with EA (EA1 and EA2) treated rats by quantitative RT-PCR (A, B), immunohistochemistry (C) and by immunoblotting (D). Data presented in A, B, E and F are presented as mean \pm SE (n=3). Significant difference ($p<0.05$) of the mean values among the groups are indicated by a, b above the bars. Data in panel C and D is representative of three independent analyses.

Figure 5: In vivo administration of EA ameliorated renal function in diabetic rats. Albumin/creatinine ratio (A) and urea (B) was measured in urine collected for 24 h from control, diabetic and EA treated diabetic rats. Data in A and B presented in A and B as mean \pm SE (n=3). Significant difference ($p<0.05$) of the mean values among the groups are indicated by a, b and c above the bars.

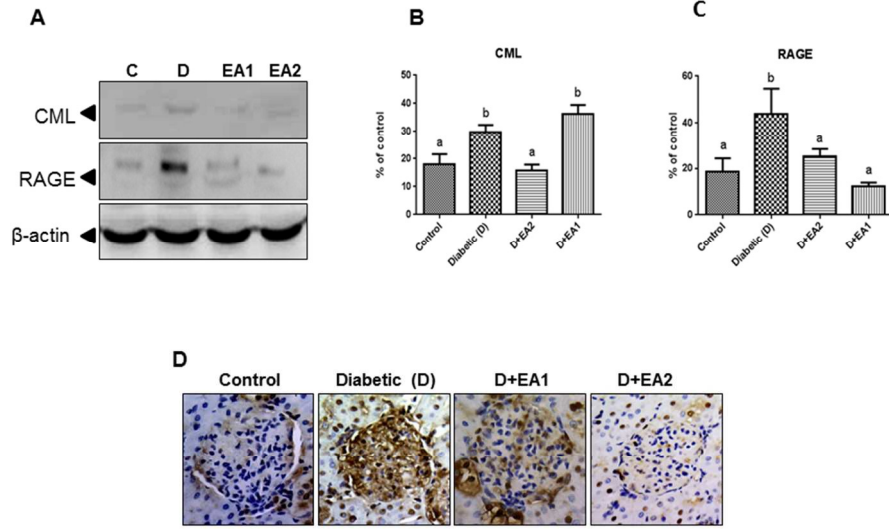
Figure 6: Proposed model for the anti-AGE function of EA and its role in combating proteinuria in diabetes: EA effectively inhibited protein glycation and AGE (CML) formation in diabetic rats, which in turn prevented AGE dependent mesangial expansion, extracellular matrix (ECM) accumulation and glomerulosclerosis. Alternatively, EA prevented AGE dependent activation of VCAM, loss of podocyte slit-diaphragm proteins (nephrin and podocin), thus prevented proteinuria.

Fig 1:



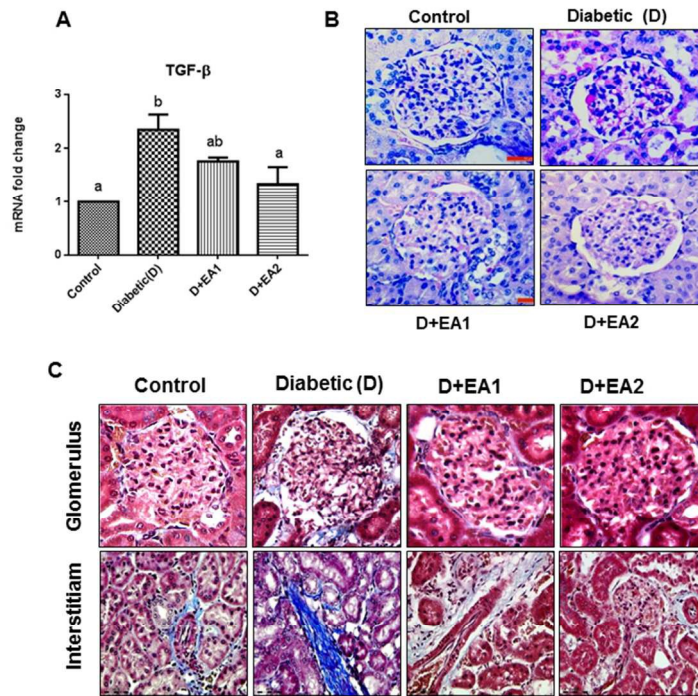
254x190mm (96 x 96 DPI)

Fig 2:



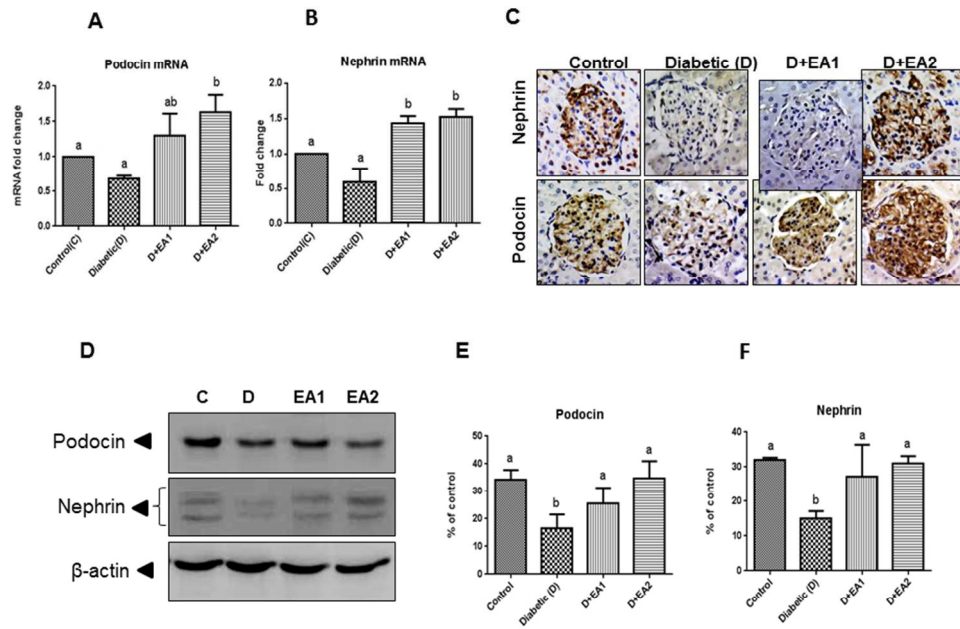
254x190mm (96 x 96 DPI)

Fig 3:



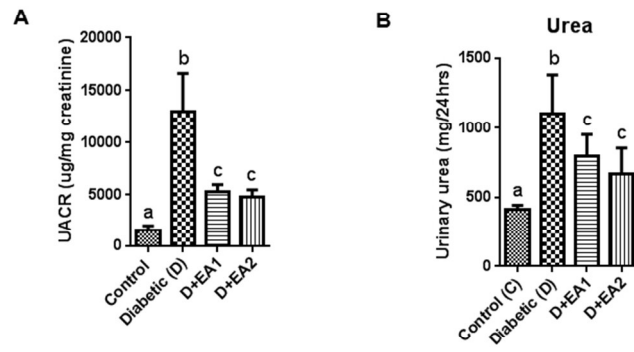
254x190mm (96 x 96 DPI)

Fig 4:



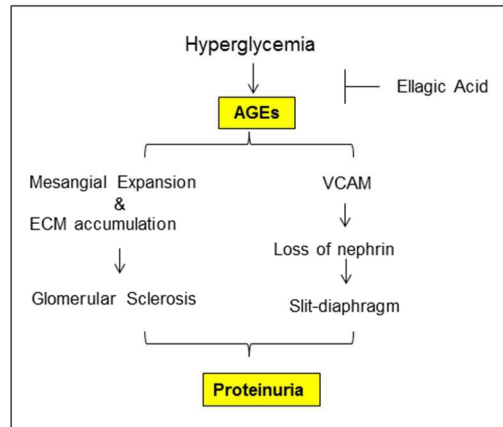
254x190mm (96 x 96 DPI)

Fig 5:



254x190mm (96 x 96 DPI)

Fig 6:



254x190mm (96 x 96 DPI)