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Cinnamaldehyde ameliorates diabetes-induced biochemical impairments and AGEs macromolecules in a pre-clinical model of diabetic nephropathy

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Abstract

Purpose Cinnamaldehyde, has various therapeutic potentials including glucose-lowering effect, and insulinotropic effect; however, its glycation inhibitory mechanism is not known yet. In this study, we explored the effects of cinnamaldehyde for its AGEs inhibitory mechanism in a streptozotocin-complete Freund's adjuvant (STZ-CFA) induced diabetic nephropathy (DN) rat model.

Methods Pre-clinical DN model was developed by the administration of multiple low doses of STZ-CFA in rats, mainly characterized by abnormal blood parameters and nephrotic damages. Diabetes-related systemic profile and histopathological hallmarks were evaluated using biochemical assays, microscopic imaging, immunoblot, and real-time PCR analyses, supported by cinnamaldehyde-albumin interaction assessed using STD-NMR and in silico site-directed interactions in the presence of glucose.

Results Cinnamaldehyde-treatment significantly reversed DN hallmarks, fasting blood glucose (FBG), serum insulin, glycated hemoglobin (HbA1c), urinary microalbumin, and creatinine contrasted to non-treated DN rats and aminoguanidine, a positive reference advanced glycation end products (AGEs) inhibitor. The pathological depositions of AGEs, receptor for advanced glycation end products (RAGE), and carboxymethyl lysine (CML), and transcriptional levels of AGE-RAGE targeted immunomodulatory factors (IL1β, TNF-α, NF-κB, TGF-β) were significantly improved in cinnamaldehyde treated rats as compared to aminoguanidine. Cinnamaldehyde post-treatment improved pancreatic pathology and systemic glycemic index (0.539 ± 0.01 vs. 0.040 ± 0.001, $P < 0.001$) in DN rats. Subsequently, in silico profiling of cinnamaldehyde defined the competitive binding inhibition with glucose in AGE and RAGE receptors that was further confirmed by in vitro STD-NMR analysis.

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Conclusion These findings suggest potential role of cinnamaldehyde in reversing STZ-induced diabetic nephropathic impairments; therefore, appears promising candidate for further pharmacological explorations towards diabetes-associated complications.

Keywords Cinnamaldehyde, Diabetic complications, AGEs, CML, Diabetes nephropathy model

Introduction

Diabetic nephropathy, one of the inevitable complications, is defined by ultrastructural glomerular basement membrane thickening, mesangial expansion, and tubular injury collectively resulting in end-stage renal disease [1, 2]. The underlying mechanisms of diabetic nephropathy include advanced glycation end-products (AGEs) deposition and entangled renal microvasculature [2]. The elevated levels of glucose trigger glycation of proteins and/or lipids, forming AGEs, the major macromolecule that binds to the receptor of AGEs (RAGE), and accelerates oxidative stress [3, 4], as well as inflammation-related pathologies via nuclear factor kappa B (NF-κB) signaling pathway [5]. These initiated cascades exert deleterious changes in the body, which eventually develop diabetic co-morbid conditions [5–9]. Moreover, recent studies suggest that AGEs have been linked to various degenerative diseases [10–12] including diabetes [13], Alzheimer's disease [14, 15], chronic kidney disease [16], atherosclerosis [17, 18], and cancer via receptor-mediated signaling cascade [19]. Clinical investigations revealed that carboxymethyl lysine (CML) is the most prevalent AGEs in vasculature lesions and glomerular tissues [20]. Albuminuria and creatinine retention serves as a clinical marker for chronic kidney dysfunction (CKD) [21, 22]. Persistent glycemic insults leading to increased inflammatory responses that exacerbate complications in diabetic nephropathy, resulting in proteinuria and end-stage renal disease. NF-κB controls cytokine production and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized low-density lipoprotein (LDL), etc [23, 24]. The interaction between AGEs-RAGE activates the NF-κB signaling pathway while Tumor necrosis factor α (TNF-α), the pro-inflammatory cytokine, further amplifies the cell signaling involved in systemic inflammation. Moreover, TNF-α and IL-6, two significant pro-inflammatory cytokines have been correlated with micro- and macro-vascular complications of diabetes [25]. Raised degrees of TNF-α and IL-6 have been exhibited in advanced diabetic patients than in healthy subjects [26–28]. Also, IL-6 has been connected to glomerular basement membrane thickening [29]. Thus, breakage of AGEs interaction may restore structural changes towards normal physiology; however, the mechanisms underlying such reversal with therapeutic intervention are yet to be further understood.

Since accumulating evidences have demystified the involvement of AGEs in the progression of diabetic complications, it is considered that prevention of AGEs formation may inhibit the disease progression and related complications. However, development of drug candidates owing anti-AGE activity is a challenge due to complex reactions carried out during AGE formation. Aminoguanidine, a nucleophilic hydrazine compound, was identified as promising candidates in vitro and in animal models exhibited AGE inhibitory effects and entered into phase three clinical trials [30–32]. However, the trial was terminated due to its lower efficacy and various safety concerns including flu-like symptoms, gastrointestinal disturbances and anemia [30, 32]. Numerous other agents such as pyridoxamine, taurine, carnosine, phenyl thiazolium bromide, and 2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) have been investigated in several pre-clinical studies and have shown promising results [30, 33, 34]. However, except pyridoxamine, none has progressed, as yet, to the stage of clinical trials. Therefore, there is a need for developing new antiglycation agents combining higher levels of efficacy, selectivity and biocompatibility in humans.

Phytotherapies contain bioactive entities that via molecular associations have remedial and disease-preventing trends to delay the onset of chronic diseases, thereby conferring a beneficial physiological effect. Previous literature identified many phytochemicals from traditional medicinal plants that can target diabetogenic targets [35]. Cinnamon, as proven anti-diabetic potentials in folk medicine, have long been used in various investigations with increased discoveries against diabetes [36–38]. Recently few potent compounds from cinnamon such as cinnamic acid, and cinnamaldehyde were identified with blood glucose lowering effects, insulinotropic properties, and pancreatic β-cell protection [37, 39]. However, one discipline of diabetes research, AGEs inhibition, has still an unanswered area to elucidate how cinnamaldehyde modulate AGE expression for controlling pathological sequalae, and systemic impairments, particularly diabetes nephropathy and kidney fibrosis, although some data point out the potential of cinnamon against AGE-mediated diabetic pathogenesis in vivo [37]. This study focuses first on the establishment of a rodent model of diabetes nephropathy with typical features of AGEs formation and secondly on the elucidation of cinnamaldehyde towards interventions against diabetes-induced

nephropathy and future prospects of nutritional therapy for diabetic complications.

Experimental methods

Materials

Cinnamaldehyde (purity > 98%) was purchased from Alfa Aesar (MA, USA). Streptozotocin was bought from Calbiochem (Merck, USA). The glucose estimation kit was bought from Randox (Crumlin, UK), rat insulin ELISA kit, rat hemoglobin A1c (HbA1c), rat creatinine, and serum AGEs ELISA kits were from Crystal Chemicals (Downers Grove, IL, USA), and rat microalbumin ELISA kit purchased from Bethyl Laboratories (Montgomery, USA). Primary antibodies used in immunofluorescence and immunoblot analysis; rabbit polyclonal anti-AGEs, mouse monoclonal anti-CML, rabbit polyclonal anti-RAGE; were purchased from Abcam (Cambridge, UK), while rabbit polyclonal anti-actin, mouse monoclonal anti-glucagon, and guinea pig monoclonal anti-insulin were bought from Sigma (Missouri, USA). Secondary antibodies, namely, Alexa fluor® 594 goat anti-mouse from Invitrogen (California, USA), Alexa fluor® 594 goat anti-rabbit, Alexa fluor® 594 donkey anti-guinea pig, Cy2 donkey anti-mouse, peroxidase-conjugated goat anti-rabbit, and peroxidase-conjugated goat anti-rabbit, were purchased from Jackson ImmunoResearch (Baltimore, USA).

Animals and experimental design

Age-matched male Wistar rats (180–220 g body weight) were procured from the animal resource facility of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. The animals were handled following international guidelines and in compliance with institutional ethical committee protocols (ASP number 2016-0031).

The animals were housed in conventional animal cages, with a maximum occupancy of 3 rats per cage on standard conditions i.e., 23–25 °C, 12-h light-dark cycle, and open access to food and water as per the international guidelines and standard animal study protocol. The animals were allowed to be acclimatized to the environment before experimentation.

In the current study, a total of 40 animals were randomly allocated to four experimental groups (Fig. S9). For in vivo diabetic nephropathic model, streptozotocin (30 mg/kg body weight) was injected intravenously with complete Freund' adjuvant 100 µL (intra-peritoneal) following overnight fasting [40]. This approach was chosen due to its higher induction rate, little to no chance of relapse, and lower mortality (<25%) compared to acute high doses of STZ. The animal was given a 5% glucose solution for the next 24 h to prevent acute hypoglycemic effects or mortality due to β-cell destruction. Three doses of STZ were administered weekly, and the animals were housed in standard cages for 8 weeks ad libitum access to food and water. The animals with fasting glucose levels >270 mg/dl and significant albuminuria were included for further experimentation (Fig. S1). Then, diabetic animals were either treated with cinnamaldehyde (20 mg/kg) or aminoguanidine (350 mg/kg) during the second half of the 4-months study period. Cinnamaldehyde (20 mg/kg) was selected on the basis of plasma glucose lowering effect in glucose challenge diabetic rats (Fig. S2). While, aminoguanidine, the standard AGEs inhibitor, was selected based on the documentation [32]. Following treatment, a comprehensive evaluation encompassing systemic glycemic profile, plasma insulin, renal functional and structural parameters over 16 weeks of experimental diabetes treated with or without cinnamaldehyde/aminoguanidine have been evaluated.

Group 1: Control (Con; non-diabetic, non-treated rats, $n = 12$)
Group 2: Diabetic nephropathic (DN; STZ-induced rats, $n = 12$)
Group 3: Cinnamaldehyde-treated diabetic nephropathic rats (DN+CD, $n = 8$)
Group 4: Aminoguanidine-treated diabetic nephropathic rats (DN+AG; positive control, $n = 8$)

Bio-sampling and handling

By the end of 16th week, all animals were anesthetized through intraperitoneal injection using sodium thiopental (60 mg/kg, *i.p*) followed by cervical dislocation with minimal distress then, surgical dissection was performed and their organs (pancreas, kidney) and blood plasma were collected and stored at −80 °C for further experimental analysis. 24-hours urine samples were collected a day before dissection by segregating individual animal in metabolic cages. The collected urine was centrifuged at 3000 rpm for 15 min and assessed for microalbumin and creatinine according to their respective protocols. For histopathological analysis, the harvested pancreas and kidney were processed for histological and biochemical analysis as described below.

Biochemical examinations

Biochemical parameters such as fasting glucose, insulin, AGEs, and creatinine were measured from serum collected in tubes without EDTA. The HbA1c was estimated from the whole blood collected in the EDTA tubes. 24-hour urine, microalbumin, and creatinine were measured in urine samples to evaluate kidney function in each group. All these biochemical parameters were assessed using commercially available kits following the manufacturer's protocol. Values were calculated against

their respective standards using Curve Expert Software 1.4® (Roche, Switzerland).

Histological and immunohistochemical analysis

For general histological analysis, 5 µm thick formalin fixed paraffin embedded pancreatic or kidney sections were stained with hematoxylin and eosin according to previously described protocol [41]. Briefly, pancreatic sections were deparaffinized using xylene, rehydrated by serial dilutions of isopropanol and distilled water. The rehydrated sections were first stained with Hematoxylin for 2 min, then wash the excess stain and immerse into the Eosin for 30 s. The stained sections were again dehydrated and mounted, then examined under microscope (Nikon Eclipse 90i, Japan).

For the identification of glycation markers (AGEs and CML), tissue sections were rehydrated and subjected to antigen retrieval by immersion in citrate buffer (pH 4.5) for 90 min to unmask the targeted antigens. To prevent non-specific binding, off-targeted antigens were blocked using 2% animal serum, the sections were first incubated with primary antibody, insulin-glucagon/AGEs/CML for 2 h, then incubated with their respected secondary antibody. The nuclei were counter stained with DAPI for 2 min, then the slides were mounted by Fluoromount (Sigma). The immune-stained slides were observed under Nikon Eclipse 90i fluorescent microscope (Osaka, Japan) with NIS-Elements image analysis software AR 3.0 (Nikon, Osaka, Japan). Minimal image processing was done by Adobe Photoshop CS3.

Immunoblotting

Protein expression analysis among experimental groups was done according to previously reported protocol [42]. Briefly, isolated tissues were homogenized into ice-cold RIPA Lysis Buffer and total protein was estimated by Bradford assay. From each experimental group, a protein sample of 15 µg was resolved in 10% SDS-PAGE, then transferred on to the 0.22 µm nitrocellulose membrane. Following off targeted protein blocking using 5% skimmed milk, the membrane was first incubated with primary antibody (AGEs/RAGE/CML/actin) for 2-hours then incubated with their respective HRP-labeled secondary antibody for 1-hour. The unbounded antibody was washed, then the blot was subjected to ECL treatment and visualized by gel documentation system (Alpha Ease FC imaging system, Fluor chem, Alpha Innotech, USA). Band intensities of proteins were measured using Image Studio Lite and normalized by expression of actin in each sample.

Real-time PCR studies

Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. The concentration and purity of isolated RNA were measured at 260 nm and 260/280, respectively using a Nanodrop 2000 spectrophotometer (Thermoscientific, MA, USA). The extracted RNA was stored at −80 °C till further processing. From total extracted RNA, 1 µg was used for cDNA synthesis using a cDNA revert-first aid synthesis kit (Thermofisher) following the manufacturer's guidelines. The expression of specified genes was analyzed using Thermocycler-480 Real-time PCR instrument (Roche) using SYBR Green I Master mix (Roche, Basel, Switzerland) according to the protocol describe previously [43]. The forward and reverse primers for targeted genes were designed by primer3 software and NCBI primer-BLAST (Table S2). GAPDH is used as an endogenous control for quantitative RT-PCR analysis.

Cinnamaldehyde-based STD-NMR interactions

The saturation transfer difference NMR interaction of compounds with albumin protein was carried out in this study using cinnamaldehyde to albumin at the ratio of 1:1000. All STD NMR experiments were performed on an AVANCE III HD-400 MHz NMR spectrometer (Bruker BioSpin Pte Ltd.), equipped with room temperature probe for water suppression. Data processing was performed using the TopSpin 3.5 pl6 program suite (Bruker, MA, USA).

Cinnamaldehyde-driven docking studies on AGE and RAGE

Induced fit docking methodology was led to consider conceivable binding modes within receptor active sites comprehensively. The unique procedure allows scientists to rapidly anticipate dynamic site calculations with minimal cost, limiting the risk of misleading positive results. MOE software was utilized to complete the in silico studies [44]. All the compounds were outlined by utilizing MOE builder suite and go through for geometry improvement, nuclear charges, topological data (rotatable bonds) with the expansion of polar hydrogens, and energy minimization by utilizing MM94x force field. 3D crystal structure of Human serum albumin (HSA) was accessed from RCSB protein data bank (PDB code: 4IW2) and RAGE (PDB code: 3O3U) to look at the impact of cinnamaldehyde in presence of glycated states for HSA and site coordinated docking for hindering RAGE-AGE. The receptor was likewise pre-arranged utilizing MOE and energy was minimized by Amber10EHT force field. Re-docking of HSA-related ligands was completed to assess the dependability of the software, with the least RMSD of the binding pose concerning the native structure of the crystal pose. Afterward, we docked our compounds at the Sudlow site I. At the same time, the VC1 space in RAGE is related to mediating AGE reactions through RAGE and consequently, this site is decided to figure out the binding pattern of CD.

Statistical analyses

All results in this study were expressed as median ± SEM. For the statistical analysis, one-way ANOVA with Bonferroni post hoc tests were performed and evaluated by GraphPad Prism version 8.0 (GraphPad, La Jolla, CA). $p < 0.05$ was considered statistically significant.

Result and discussion

Cinnamaldehyde administration regulates diabetes-related major biochemistry

Persistent hyperglycemia in diabetes has been associated with increased glycation of macromolecules, leading to primary and secondary complications including nephropathy [45, 46]. In order to assess the therapeutic effect of cinnamaldehyde, first we established an induced STZ-mediated pre-clinical model of diabetic nephropathy in rodents to mimic an impaired biochemical profile characteristic and pathophysiological features associated with diabetic nephropathy (DN).

We categorized the findings of cases into the pre-clinical diabetic nephropathy model, tested cinnamaldehyde, and standard aminoguanidine groups. The mean difference of diabetes-related biochemical parameters such as FBG, serum insulin, and insulin-to-glucose ratio of baseline and STZ-induced DN model was observed with significant increase ($P < 0.0001$) in the DN group; however, potentially reduced in post-treatment groups cinnamaldehyde (20 mg) and aminoguanidine (350 mg) for the average duration of two months (Fig. 1). Diabetes nephropathic rats showed significantly high fasting blood glucose (20.2 ± 1.2 vs. 4.5 ± 0.2 mmol/L, $P < 0.01$), with low serum insulin levels (0.81 ± 0.08 vs. 3.55 ± 0.18 pmol/L, $P < 0.01$) contrasted to vehicle control group. STZ specifically targets and destroy the pancreatic insulin producing cells, leading to a compromised ability to release insulin in response to high blood glucose levels. However, chronic cinnamaldehyde post treatment significantly improve the FBG (10.0 ± 0.7 vs. 20.2 ± 1.2 mmol/L, $P < 0.01$) and serum insulin (3.55 ± 0.18 vs. 5.39 ± 0.04 pmol/L, $P < 0.01$). Furthermore, insulin-to-glucose ratio was increased (0.539 ± 0.01 vs. 0.040 ± 0.001, $P < 0.01$) exclusively in the cinnamaldehyde post-treated DN rats.

Diabetic complication i.e., nephropathy related biochemical parameters, HbA1c (8.46 ± 1.2 vs. 12.47 ± 2.7), serum AGEs (0.34 ± 0.04 vs. 0.81 ± 0.04), urinary microalbumin (0.11 ± 0.04 vs. 5.98 ± 0.04), and CCR (7.82 ± 0.05 vs. 0.48 ± 0.08) were significantly normalized in cinnamaldehyde- and aminoguanidine- (standard/positive control) post-treated DN rats as compared to that of DN group, but there is no significant effect of aminoguanidine observed on FBG, urinary protein, CCR, and kidney to body weight ratio (Fig. 1). Consistently, clinical evidences demonstrated hyperglycemia mediated AGEs generation as a key contributor in the pathogenesis of diabetic nephropathy that manifested as microalbuminuria and renal histopathological features, eventually progress to renal failure [46, 47]. These biochemical assessments suggest therapeutic improvements when correlated with the basic STZ-initiated renal impedances and the recovery with cinnamaldehyde intervention.

Cinnamaldehyde-treated rats exhibit a notably normalized STZ-impaired histopathology

The impaired biochemical parameters of DN might exhibit pathobiology of the diabetic kidney, which goes through a bunch of significant underlying metabolic and functional changes. Persistent diabetes associated hyperglycemia mainly affects small vessels and ultimately progresses to tissue dysfunction. The glycoproteins (or AGEs) adhere to the microvasculature leading these sites exposed to the positive charges. The change in the membrane charge (polarity) allows the negatively charged proteins (collagens and laminins) to get attached to the microvasculature, thus making them thickened and more prone to be leaked or damaged. One of the latest is raised hydrostatic pressure and low oncotic pressure, permitting escape of plasma proteins that gather as periodic acid—Schiff—positive deposits in the vessel walls. In addition, the extracellular matrix expounded by cells near the vessels is raised, due to excessive production and turnover of its constituent proteins and glycosaminoglycans. As a consequence, the thickness of the basement membrane is increased in several tissues, comprising mesangial expansion in the kidney [48]. Therefore, in this study, we have shown the impact of impaired biochemical parameters on structural features of nephrotic sites and their treatments, as shown by H&E, PAS as well as immunohistochemically stained kidney sections of experimental rats (Fig. 2).

H&E-stained kidney sections of DN rats showed significant renal histopathological changes, notably severe distortion in the tubular cells and glomeruli, glomerulosclerosis with some lesions, tubulointerstitial fibrosis and atrophy as well as penetrations and mesangial expansion (Fig. 2). Hyperglycemia activates various mediators including VEGF, TGF-β1, that facilitates the accumulation of collagen within the glomerular basement membrane, contributing to its thickening, which is a critical pathological process in the progression of diabetes associated nephron damage [49]. In DN rats treated with cinnamaldehyde and aminoguanidine, the overall renal structure was protected from advanced stage of diabetic nephropathy, for example, clear glomeruli with Bowman's space and fine meshwork, which seemed normal with no rupturing when contrasted with DN groups. PAS-stained kidney sections of DN group showed glomerular distortions and burst capsular wall (stained purple), thickened basement membranes, and

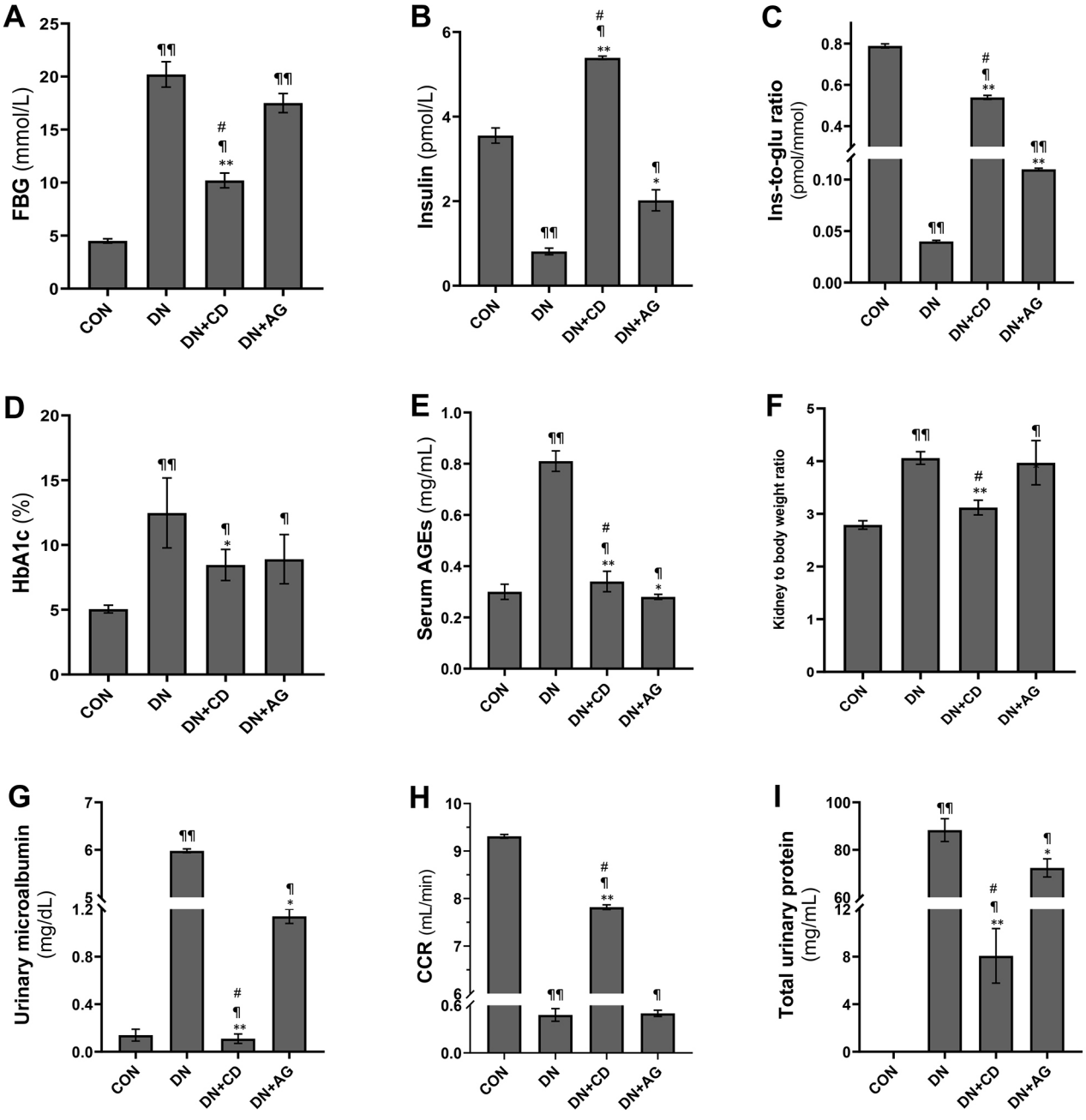

Fig. 1 Comparison of biochemical profile parameters across trial rats. (**A**) FBG, (**B**) Insulin, (**C**) Ins-glu-ratio, (**D**) HbA1c, (**E**) serum AGES, (**F**) Kidney to body weight ratio, (**G**) Urinary microalbumin, (**H**) CCR, (**I**) total urinary protein. In DN rodents treated with cinnamaldehyde (CD), a significant reduction in biochemical indexes, such as FBG, HbA1c, serum AGEs, and urinary microalbumin levels was noticed. In parallel, an overlap change increment was seen in serum insulin level and creatinine clearance. The cinnamaldehyde results are comparable with aminoguanidine (AG), used as a positive control; however, no change in FBG, serum insulin, and creatinine clearance was observed in the AG group. Data expressed as median ± SEM, $n = 8–12$. $^{¶}p < 0.01$, $^{¶¶}p < 0.001$ *vs.* Control, $^{*}P < 0.01$, $^{**}P < 0.001$ vs. DN, $^{\#}P < 0.001$ vs. AG. One way ANOVA was performed with Bonferroni post hoc tests

hypocellularity in the interstitial components. In DN rats treated with cinnamaldehyde and aminoguanidine, the mesangial tissues were delineated and interstitial tissue did not indicate any abnormalities. The glomerular capsular walls were clear as PAS-positive purple, showing a typical glomerular tuft meshwork. No abnormalities with purple-stained (glycogen) membranes of tubules were apparent. Immunohistochemical investigation of DN rats highlighted increased deposition of AGEs and CML all through the kidney areas generally around the glomerulus and Bowman's capsule. Whereas, in treated groups, there was a significant reduction in AGEs/CML depositions in the renal parts and the glomerulus. The cinnamaldehyde treatment in DN rats indicates the best

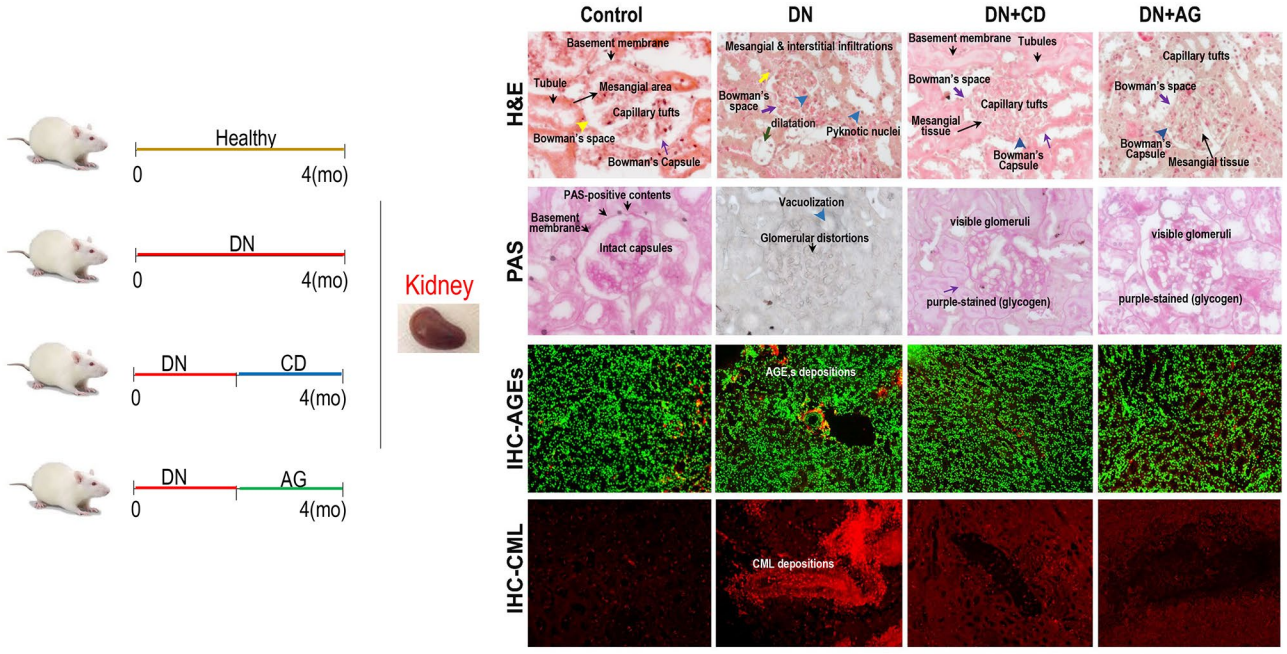

Fig. 2 Comparative histopathology of trial rodents: (A) Representative (H&E and PAS panel) expressed renal lesions and suggestive impairments; whereas, (immunofluorescent-AGEs and -CML depositions appeared as red) were examined in kidney segments of (i) control; (ii) STZ-induced DN, diabetic nephropathy rats; (iii) DN treated with CD, cinnamaldehyde; and (iv) DN treated with AG, aminoguanidine. Treatment groups were compared with diabetic nephropathy. In the AGEs panel, DAPI was shown as pseudo-green. Schematic plan (left panel). Magnification IHC-AGEs: ×100; H&E and IHC-CML: ×200; PAS: ×400

protection against AGEs/CML deposition in the renal sections. Our study is in strong agreement with previous findings where pravastatin has been shown to ameliorate tubular damage in diabetic nephropathy by inhibiting the AGEs-induced apoptosis via suppression of RAGE expression [50].

Cinnamaldehyde-administration alleviates AGEs, CML, and RAGE expression in renal tissues

In several studies, AGEs, especially carboxymethyl-lysine in diabetic kidneys has been shown to relate the upregulation of RAGE [51]. Moreover, accumulation of AGEs and CML have not only shown to be detected in the renal tissue but also their depositions are in direct correlation with the severity of diabetic nephropathy [51, 52]. Hence, we tested whether cinnamaldehyde ameliorates these pathological depositions in DN-induced kidneys (Fig. 3). To analyze this further, we performed immunoblotting of renal tissues from cinnamaldehyde post-treated DN group (DN+CD) to examine AGEs, CML, and expression of RAGE. AGEs bands were observed relating to the molecular weight of ~62-kD on the blot that in DN group, was significantly increased ($P<0.001$) as compared to control group. AGEs were significantly reduced in the DN+CD lane (treated group) and DN+AG (positive control) as compared to DN group only. The data indicate that cinnamaldehyde and aminoguanidine decreased the total AGEs concentration in the kidneys of diabetic rats. Besides, the accumulation of CML in the renal tissues of different trial groups were observed as one heavy CML (~50-kD) and a light CML (~21-kD). In DN group, both heavy (HMW)- and light (LMW)-CML were increased (~1.9-fold; $P<0.001$) compared to the control group. In DN+CD treatment group, the concentration of both CML significantly decreased ($P<0.001$) as compared to DN group only. In DN+AG group (positive control), the concentration of HMW- and LMW-CML were similarly decreased ($P<0.001$) compared to DN group (Fig. 3B). To evaluate ligand and receptor-protein interaction for AGE-RAGE pathway in renal tissues of animal groups, the co-relative expression of AGEs and RAGE was assessed by western blotting (Fig. 3A, C). In DN group, the expression of full-length RAGE receptor was increased by 2-folds ($P<0.001$) than control group, whereas downregulated ($P<0.05$) in DN+CD group, as compared to DN group. The DN+AG group (positive control), the expression of full-length RAGE receptor was decreased by 1.24-folds ($P<0.05$) compared to the DN group. Spliced variants of RAGE can also be seen at ~42-kD, significantly up-regulated ($P<0.001$) in DN compared to the control group while significantly ($P<0.001$) down regulation in DN+CD group compared to DN group. The DN+AG group (positive control) has also shown downregulation of RAGE variant by 13.1-folds ($P<0.001$) than DN group. Another RAGE variant could be seen at ~34 kD, and it was up-regulated in DN group

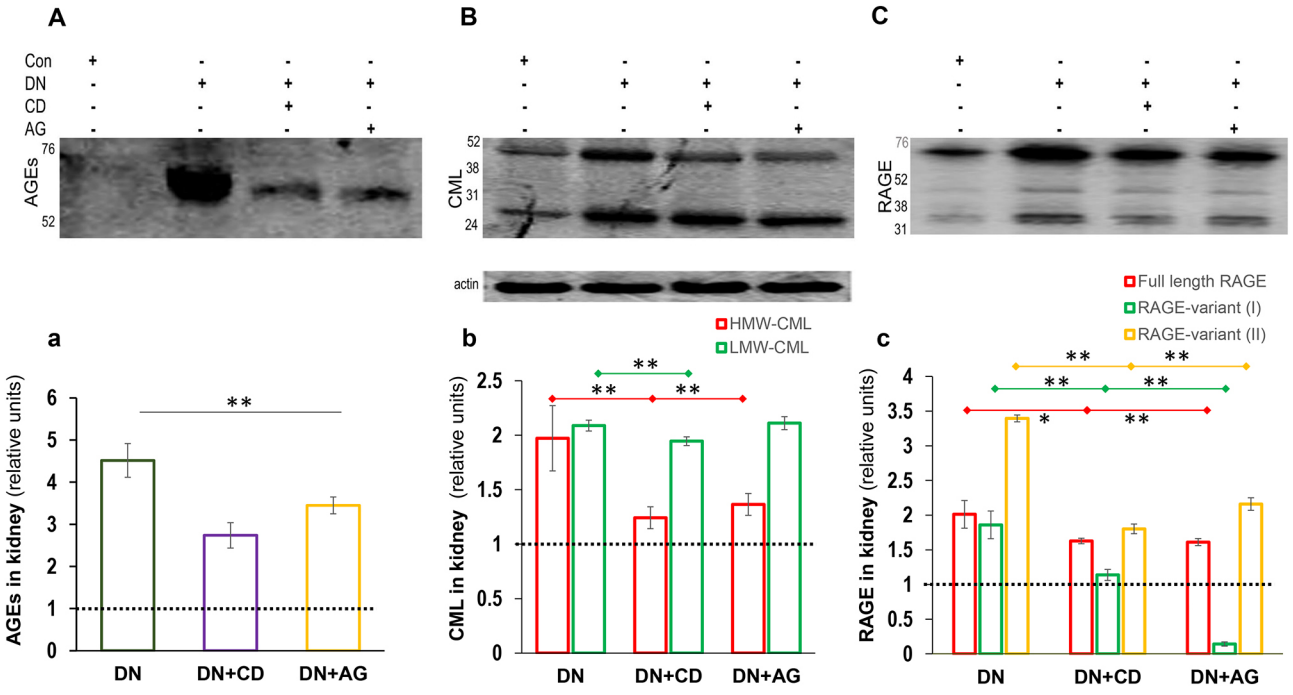

Fig. 3 Comparison of designated protein levels examined by western blot pattern in renal tissue from experimental rats: **Aa** Protein bands and quantification of AGEs levels **Bb** Protein bands and quantification of HMW- and LMW-CML and **Cc** Protein bands and quantification of RAGEs (spliced variants), separately: The increased STZ-induced depositions were significantly diminished in DN-treated cinnamaldehyde, CD (20 mg/kg) and -aminoguanidine, AG groups. Actin served as a loading control. Quantification values were expressed mean ± SD as per column ($n = 8–12$ per group). *$P < 0.01$, **$P < 0.001$ *vs.* DN group, (One-way ANOVA was performed with Bonferroni post hoc test) assorted relatively against control group set as 1 (indicated in black-dashed lines). *HMW-CML* High Molecular Weight-n-Carboxymethyl lysine, *LMW-CML* Low Molecular Weight-n-Carboxymethyl lysine

by 3.3-folds ($P<0.001$) than control. In DN+CD group, there was down regulation of RAGE variant by 1.8-folds ($P<0.001$) than DN group. In DN+AG group (positive control), there was down regulation of RAGE variant by 1.5-folds ($P<0.001$) than DN group (Fig. 3c).

Overall histology and immunoblotting results suggested that AGEs, CML, and RAGE deposition has been increased in DN rats depicting the pathological features of diabetic nephropathy. Elevated AGEs results in continuous stimulation of RAGE receptors via positive feedback mechanism; hence, facilitating the immunopathological cascade resulting in severity of disease [53, 54].

Cinnamaldehyde alleviates AGE-RAGE induced inflammatory expressions

The deposition of glycated proteins not only cause structural damages but also initiates the downstream pathways after binding with their ligand, RAGE. Accumulating evidence showed that the pathogenesis of DN is exaggerated by immunomodulatory mechanisms, rather than the consequence of disease [55, 56]. Individuals have been shown low degree inflammation for years before clinical manifestations of diabetes nephropathy [57]. In this perspective, immunogenic components involved in the process of inflammation and endothelial damage have attracted our attention to be investigated in the current study. Real Time q-PCR of different genes from renal tissues of experimental rats were performed to evaluate the antiglycation effects of cinnamaldehyde on transcriptional levels on AGE-RAGE related inflammatory cascades (Fig. 4). In DN group, the mRNA expression of several genes related to diabetes nephropathy was significantly increased namely, RAGE (~4.6-folds); to that of control group. In chronic CD- and AG- post-treatments started to two months DN rats, significantly reduced mRNA expression, shown as cinnamaldehyde vs AG in RAGE (2.8-folds; $P<0.001$ vs. 3.9-folds; $P<0.01$), to that of control group (Fig. 4). The activation of RAGE pathway leads to the oxidative burst, production and release of proinflammatory mediators and cytokines. Increased inflammatory signaling mediators were observed in STZ induced diabetic rats via activation of immunogenic cells. In DN group, the mRNA expression of related genes was increased namely, IL-1β 3.8-fold ($P<0.001$); TNF-α ~5-fold ($P<0.001$); TGF-β 2.6-fold ($P<0.001$); in comparison to control group. In chronic cinnamaldehyde and aminoguanidine post-treatments started to two months DN rats significantly reduced mRNA expression, shown in fold between cinnamaldehyde vs. aminoguanidine: IL-1β (1.8 $P<0.001$ vs. 3.5-fold; $P<0.05$), TNF-α (2.4; $P<0.001$ *vs.* 3.4; $P<0.001$), TGF-β (1.4; $P<0.001$ vs. 1.9; $P<0.01$), in comparison to that of DN group (Fig. 4). Conclusively,

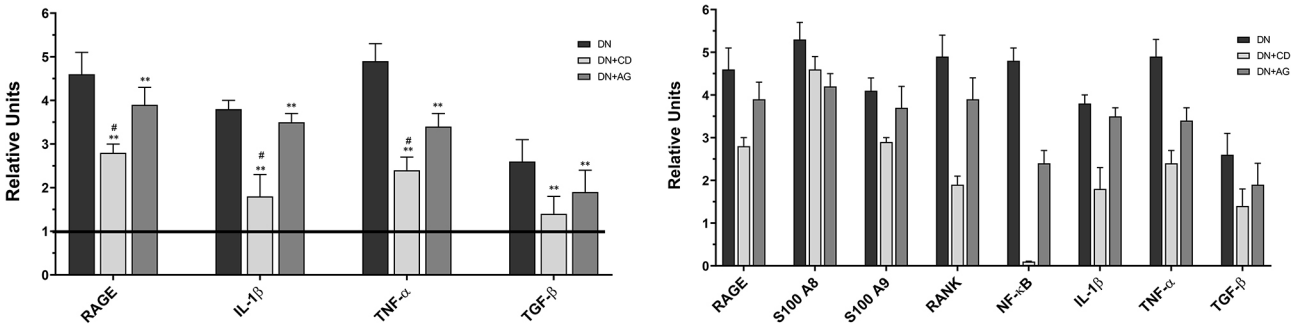

Fig. 4 Comparison of transcription levels of targeted genes in test rats: In DN rats treated with cinnamaldehyde, the mRNA expressions of targeted genes were significantly downregulated. Data expressed as mean ± SD, $n = 8–12$. (One-way ANOVA was performed with the Bonferroni post hoc test)

elevated RAGE receptors on immune cells stimulate inflammatory mediators that initiate the immuno-oxidative cascades depicting diabetic inflammatory conditions in DN rats. While, chronic cinnamaldehyde treatment ameliorates the AGE-RAGE mediated inflammatory signaling cascade, thus tissue remodeling and structural and functional changes were halted in the kidneys of DN+CD. Current findings are in strong agreement with previous studies, where they investigated neuroprotective effects shown by cinnamaldehyde [58], cinnamon [59], against neuroinflammatory changes in Alzheimer's disease and Parkinson's disease animal models. Moreover, cinnamaldehyde also demonstrates a compelling ability to inhibit iNOS, thereby, mitigating inflammatory responses, while simultaneously enhancing eNOS activity to support vascular health thus, combating the inflammatory response effectively and support overall vascular function [60]. The standard AGEs inhibitor, aminoguanidine, has also been reported to have a profound role in modulation of nitric oxide synthase (NOS) enzymes, iNOS and eNOS [61]. Aminoguanidine selectively inhibit the iNOS, the key contributor of inflammation and tissue damage, thus, effectively reduces NO levels, and mitigates the inflammatory responses. However, aminoguanidine affects the eNOS to a lesser extent, that may lead to decreased NO availability, potentially compromising vascular function and contributing to endothelial dysfunction.

Cinnamaldehyde protects the pancreas and improves glycemic index

STZ selectively destroys pancreatic β-cells resulting in decreased insulin production, one of the primary manifestations of diabetes characterized by higher level of blood glucose in diabetic nephropathy model indicating deleterious effects of STZ on the pancreatic β-cells. The insulin therapy in diabetic nephropathy; however, is paradoxical. Decreased serum insulin levels along with increased blood glucose levels were found in diabetic nephropathy model rats indicating deleterious effects of STZ in the pancreas selectively at β-cells. The rats in response to multiple lower doses of STZ, an elevated blood glucose level was observed and no regeneration potential of the pancreas was observed during 4-months period (Fig. 5B). The immunohistochemistry of the pancreas with insulin-glucagon-DAPI reveals the detailed morphology and physiology of α- and β-cells in Langerhans islets (Fig. 5A). In the control rat pancreas, α- and β-cells are arranged in a regular pattern as a large number of β -cells occupy space within islets. A significant number of islets are occupied by β-cells in the center, surrounded by α-cells. This proportion of β-cells to rafts of islets has been altered in the DN group, and the rafts of islets have a small number of β-cells surrounded by α-cells. Upon treatment with CD, a deviation from the DN group has been reserved and the CD-treated DN group showed an increase in the β-cell population within islets. The CD-treated DN group also had a similar number of α- and β-cells as the control rats' group.

STD-NMR and in silico assessments of cinnamaldehyde interactions with albumin protein, AGEs, and RAGE

The STD-NMR of cinnamaldehyde has shown interaction with albumin protein (Fig. 6Aa). The hydrogen atoms from the parent group as well as from the substitution group were participating in the interaction with albumin protein. The hydrogen atoms from the substitution group i.e., H4', H3', and H5' showed maximum interaction (100%), whereas H5', H1, H2', H3, and H2 showed 51, 47, 40, 36, and 18% interaction, respectively. This technique is useful in determining protein-ligand binding and their interactions (Fig. 6A).

To take it further, we co-relate the inhibitory binding mechanism of cinnamaldehyde for AGEs formation. Many exogenous compounds bind to HSA and consequently, glycation is exacerbated in diabetic patients with high levels of blood sugar and induces various complications. That leads to RAGE signal transduction and accumulates as a result of normal aging and inflammatory processes, particularly in diabetes [62]. Several groups are working on finding new analogs for decreasing the glycation level by inhibiting HSA overexpression and competing with glucose moieties in the specific pocket.

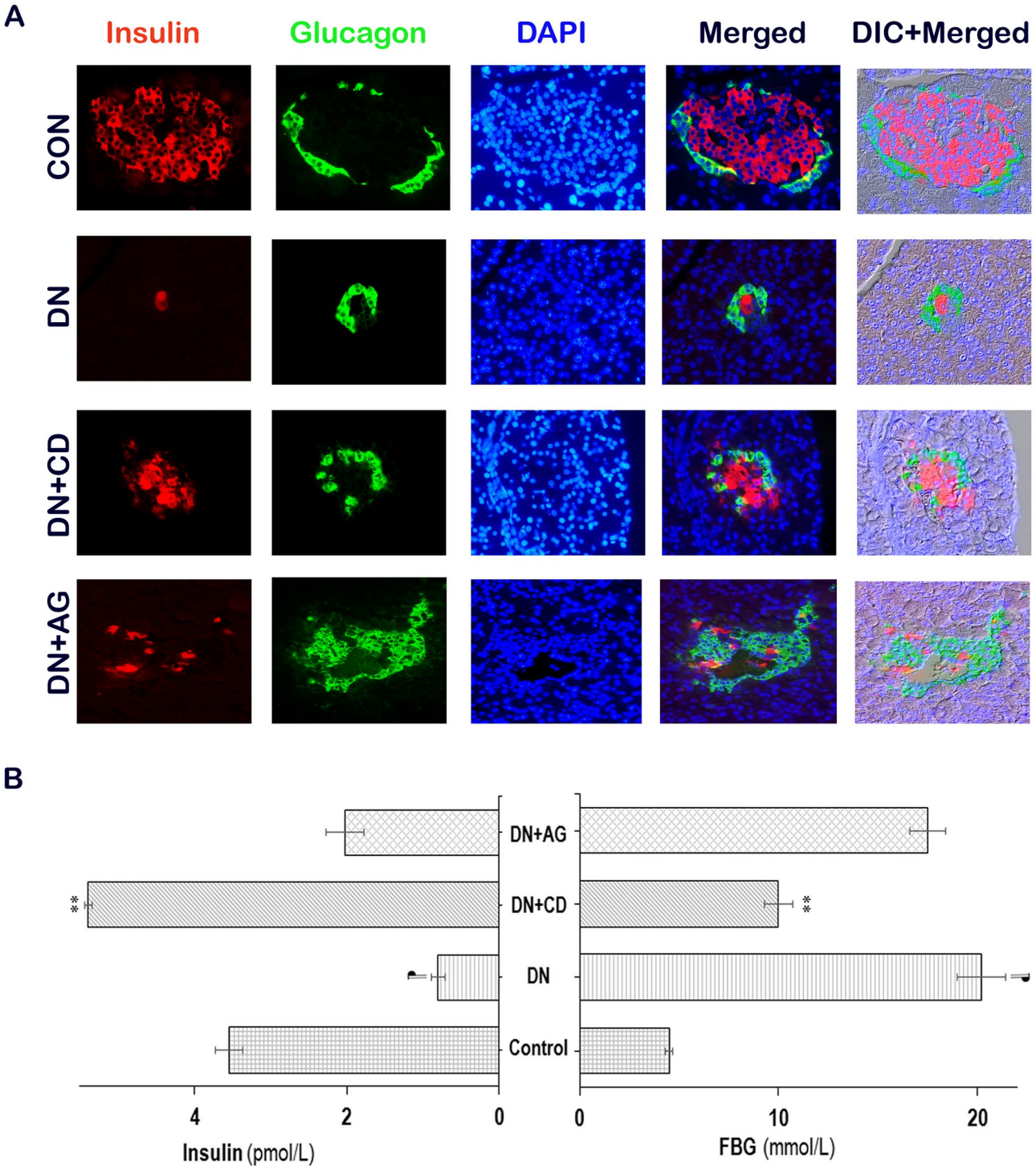

Fig. 5 Comparative immunofluorescence analysis: **A** insulin (red), glucagon (green), DAPI (blue), and merged (black) in pancreas tissue sections of (i) control; (ii) STZ-induced diabetic nephropathy rats; (iii) DN rats treated with CD, cinnamaldehyde; and (iv) DN rats treated with AG, aminoguanidine. Magnification ×200. **B** Fasting blood glucose and serum insulin levels in all experimental rats. Treatment groups were compared with diabetic nephropathy. Data expressed as median ± SEM. $n = 8–12$ (One-way ANOVA was performed with the Bonferroni post hoc test)

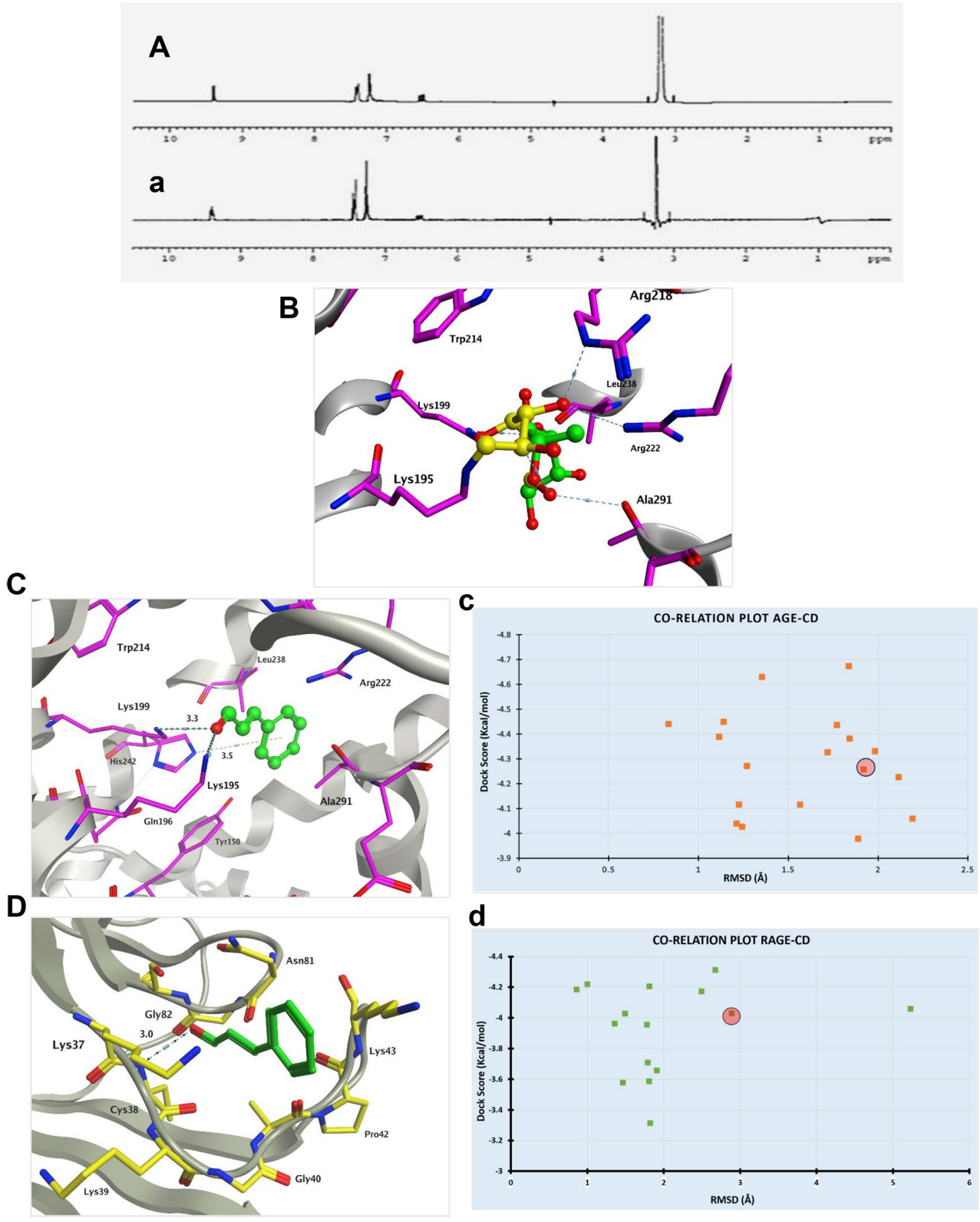

Fig. 6 STD-NMR bindings **A** 1-D NMR of cinnamaldehyde. **a** STD-NMR of cinnamaldehyde (CD) with BSA. Binding patterns **B** Redock pose (green) of glucose in the HSA pocket with a deviation of 2.7 Å from the cognate pose (yellow) **C** Dock pose of CD in HSA Suddlow site I as AGEs followed by **c** Co-relation plot for Dock Score and Rmsd **D** AAGE receptor's binding pose with CD in RAGE VC1 domain followed by its **d** Co-relation plot for Dock Score and Rmsd

Hence, glycated proteins are targeted to control diabetes mellitus. HSA is mostly reported to have Suddlow Site I for AGE binding [63]. Similarly, RAGE has a VC1 domain to mediate the AGE signaling cascade. Here we reported the competitive inhibition and site-directed binding of AGE and RAGE respectively with CD.

In computational docking, re-docking for the open-chain conformations of glucose in HSA-AGE (4iw2) gives a root mean square deviation (RMSD) of 2.7 Å with −4.09 Kcal/mol of binding energy through the Triangle Matcher placement method along with London DG and GBWI scoring functions (Fig. 6B). On the other hand, RAGE binding site was directed to accommodate cinnamaldehyde in the respective binding pocket with the same algorithm used for HSA-AGE. Leads to select the best interacted, top-ranked pose (highlighted in a circle) plus the least deviating pose of each receptor with cinnamaldehyde (Fig. 6c, d). In HSA-AGE (Fig. 6B), HSA showed interactions with the crucial residue of open chain (Lys199, Trp214, Arg222) that are with considerable hydrogen bond (h-bond) distance and hydrophobic interactions plus a covalent interaction has been seen with Lys195. About that cinnamaldehyde (Fig. 6C) with a deviation of 1.9 Å from the cognate pose with a binding affinity of −4.24 kcal/mol has shown to have an h-bond with Lys195 and Lys199 at 3.5 Å and 3.3 Å respectively, hence, cinnamaldehyde presume to have inhibiting activity against glycation end product. In RAGE, the best pose of cinnamaldehyde possesses a binding affinity with −4.02 kcal/mol (Fig. 6c) forms an h-bond through carbonyl's "O", Lys37 at a distance of 3.0 Å while hydrophobic interactions were surrounded by positively charged atoms as depicted in (Fig. 6D) including Cys38, Lys39, Gly40, Pro42, Asn81, and Gly82. HSA is an enormous molecule with multiple drug binding sites, Sudlow site I supposed to be the best for CD to bind since there are many other proteins responsible for glycation thus, we also check the binding affinity of cinnamaldehyde of AGE receptor, RAGE. Results showed that compounds are competitive in the presence of glucose with HSA towards a specific pocket and can inhibit the cascade of glycated proteins thus, plays an important role to decrease the glycation rate targeting complication of diabetes.

Conclusions

In summary, these findings provide new insights into the mechanism of cinnamaldehyde against diabetic nephropathy such as in renal inflammation and demonstrate a novel therapeutic role of this compound in fibrosis, which could be extrapolated beyond kidney tissue. Overall, we suggested cinnamaldehyde as a potent molecule for the diabetic drug research. However, the CD-induced effects are based on preclinical rat models of diabetes nephropathy without going to test against other fibrotic disorders or pleiotropic effects, extrapolations to wider pre-clinical conditions are still open to be investigated in the future.

Abbreviations

IL1β	Interleukin-1 beta
IL6	Interleukin-6
LDL	Low-density lipoprotein
NF-κB	Nuclear factor kappa B
STD-NMR	Saturation Transfer Difference Nuclear Magnetic Resonance
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor alpha
DAPI	4′,6-diamidino-2-phenylindole

Supplementary information

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Supplementary Material 1

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Author contributions

Conceptualization, original draft writing, reviewing, and editing: N.F., H.J., M.I.K. Formal analysis, investigations, funding acquisition, reviewing, and editing: U.Q., Z.U.-H., R.M.H., G.A.S. Resources, data validation, data curation, and supervision: T.A.S., M.D., Y.A.B.J.

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Data availability

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

The ethical committee, of University of Karachi, revised and approved this work under (ASP number 2016-0031). Notably, all animal experimentation was conducted in accordance with applicable laws, regulations, and guidelines, prioritizing animal welfare and minimizing any potential harm.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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