

Original Research Article

Mechanistic Insight of Transient Receptor Potential Melastatin 8 Role in Streptozotocin Induced Diabetic Nephropathy

Running Title: TRPM8 role in diabetic nephropathy

ABSTRACT

Background: The transient receptor potential melastatin 8 (TRPM8) channel is a cold-sensing non-selective cation channel involved in cellular proliferation and signaling, yet its role in diabetic nephropathy (DN) remains poorly characterized. The TRPM8 agonist, geraniol (GE) is a dietary acyclic monoterpene alcohol known for its anti-oxidant, hypoglycemic and renal chemoprotective potentials. This study aims at defining the role of TRPM8 via the use of its agonist GE in an animal model of diabetic nephropathy.

Methods: A total of 80 male Wistar rats were equally divided into 4 equal groups: control, GE sham, diabetic rats received a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg) and STZ+GE diabetic rats received GE orally at a dose of (100 mg/kg/day). Indices of DNA damage, 8-Hydroxyguanosine (8-OHdG), nephrotoxicity and metabolic derangements parameters were measured eight weeks after diabetes induction. Real-time PCR was performed to detect mRNA expression levels of renal TRPM8 and podocyte marker. Renal histopathological and ultrastructural changes were recorded. Western blotting and immunohistochemistry were performed to determine TRPM8 protein expression.

Results: Diabetic rats displayed downregulation of TRPM8 mRNA and protein expression levels in renal tissues. Upon administration of GE, biochemical, ultrastructural and oxidative stress findings were significantly improved in treated diabetic animals compared to control groups and coincided with upregulation of renal TRPM8 expression as well as enhancement of podocytopathy.

26 **Conclusion:** The present study reveals that the ameliorative effect of GE in DN is TRPM8
27 mediated and highlights a mechanistic role of TRPM8 and its agonists in management of
28 diabetic renal complications.

29

30 **Key words:** Geraniol; Streptozotocin; TRPM8; Oxidative stress; Diabetic nephropathy

31 1. INTRODUCTION

32 Diabetic nephropathy (DN) is considered one of the most common and serious complication
33 of diabetes and a major cause of end-stage renal disease (ESRD) [1]. DN is recognized as one
34 of the major podocyte-associated diseases [2]. Its hallmarks comprise glomerular hypertrophy,
35 glomerular basement membrane (GBM) thickening and eventually proteinuria [3].
36 Intracellular calcium signaling plays a robust pathogenic mechanisms in podocytopathy and
37 DN [4]. The transient receptor potential melastatin 8 (TRPM8) is a cold-sensing
38 non-selective calcium permeable cation channel that plays a pivotal role in regulating the cell
39 cycle and Ca^{2+} signalling [5]. TRPM8 is expressed in a subset of normal tissues, including
40 urinary bladder, urogenital tract and temperature-sensing neurons [6, 7]. TRPM8 knockout
41 mice have increased rates of insulin clearance and degradation compared to wild type ones,
42 revealing a novel role of TRPM8 as a regulator of serum insulin [8]. Additionally, TRPM8 is
43 expressed in brown adipose tissue and its activation can prevent obesity [9]. Different
44 plant-derived natural products with variable potencies have been identified as TRPM8
45 agonists, including menthol, eugenol, neferine, eucalyptol and geraniol [10]. Geraniol (GE)
46 (trans-3,7-Dimethyl-2,6-octadien-1-ol) is an acyclic monoterpene alcohol normally isolated
47 from *Cymbopogon flexuosus* and also presents in essential oils of various herbs and food
48 additives [11]. Geraniol is as a TRPM8 agonist with structural similarity to menthol in a dose
49 relevant and cell type specific manner [12, 13]. Geraniol has been reported to have a plethora

of therapeutic potentials as an antioxidant, hypoglycemic, hypolipidemic, anti-inflammatory, antiproliferative and antiangiogenic [14, 15]. Geraniol has been implicated to control multiple signaling pathways that are involved in diverse biological processes, such as cellular proliferation, apoptosis, autophagy and metabolism [16]. However, its role in alleviating diabetic nephropathy has been reported in terms of hypoglycemic and antioxidant potentials [17]. TRPM8 expression has been well characterized in the context of diabetic neuropathy, [18]. Nonetheless, a role for TRPM8 in DN has yet to be elucidated. Therefore, it is intriguing to investigate the potential role of TRPM8 in an animal model of diabetic nephropathy before and after administration of its agonist geraniol.

2. MATERIALS AND METHODS

2.1 Chemicals

Streptozotocin (STZ), Geraniol (GE) and all other chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of high analytical grade.

2.2 Animals

Adult male Wistar rats weighing approximately 160- 180 g were obtained by the Faculty of Medicine Tanta University and acclimatized under laboratory conditions for 1 week prior to experiments. Rats were housed in polyplastic cages with steel wire tops and maintained under standard conditions of temperature ($23\pm 2^{\circ}\text{C}$) and humidity ($50\pm 10\%$) with (12/12 h light/dark cycle) and allowed access to commercial rodent chow and tap water *ad-libitum*.

All animal protocols were approved by the Ethical Committee of Faculty of Medicine, Tanta University, Egypt with the number 31210/11/16.

2.3 Experimental Design

Animals were allocated into 4 equal groups ($n= 20$); group I (normal control group) rats

received neither STZ nor GE, only vehicles; group II (GE treated sham group) rats received only oral GE at a dose of (100 mg/kg body weight/day) in vegetable oil; group III (STZ-induced diabetic Group) overnight fasted rats received a single dose of streptozotocin (STZ), 65 mg/kg body weight, intraperitoneally) in 0.1 M cold citrate buffer (pH 4.5); group 4 (GE treated diabetic group abbreviated as STZ+GE) rats received GE orally at a dose of (100 mg/kg body weight/day) in vegetable oil for 8 weeks starting from the 4th day after STZ injection. After 3 days of induction, rats with marked hyperglycemia (fasting blood glucose >250 mg/dl) were selected as diabetic group that was left for 8 weeks untreated to induce early diabetic nephropathy. Other groups were injected with equivalent amounts of citrate buffer as a vehicle. Blood glucose levels were monitored every other day from the tail vein. At the end of the experimental period, animals were placed in individual metabolic cages for 24h to collect urine samples that were purged of air and stored at -80°C. After an overnight fast, all animals were weighted, and sacrificed. Plasma/serum samples were obtained by centrifugation at 3000 g for 10 min, aliquoted and stored at -80°C for further assays. Tissues were weighted, snap frozen in liquid nitrogen and stored at -80°C till analysis. For microscopy, tissues were fixed in 10% neutral-buffered formalin, 2.5% gluteraldehyde and/or 4% paraformaldehyde (PFA).

2.4 RNA extraction, cDNA synthesis and real time PCR

Total RNA was extracted from renal tissues using Isogene (Nippon Gene, Toyama, Japan) according to manufacturer's instructions. RNA was stored at -80 °C until use. 5 µg of total RNA was subjected to first strand cDNA synthesis in using the random hexamers and M-MuLV reverse transcriptase of Viva 2-Steps RT-PCR kit (#RTPL12, Vivantis, USA) according to manufacturer's instructions. The resulting cDNA was amplified using Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and sequence

specific primers for TRPM8 and VEGF-A. PCR condition was set as follows: a single cycle of DNA polymerase activation for a 10 min hold at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min for annealing and 60°C for 1 min for extension, followed by a melting curve analysis to confirm the amplicon specificity. Real-time PCR assays were run on a Rotor-Gene Q 6plex machine (Qiagen, Valencia, CA, USA). Relative gene expression was calculated by the Rotor-Gene Q series software 2.0.3 (Qiagen, Valencia, CA, USA) using the comparative threshold (Ct) method [19] normalized against the housekeeping gene GAPDH that was used as endogenous control in all experiments.

Sequences of primers used for Real time-PCR are detailed in (Table 1). Sequence specific primers were designed by Primer3 software [20].

Table (1) Sequences of primers used in the study

Gene	Accession Number	Nucleotide sequence	Amplicon (base pairs)
Rat TRPM8	NM_134371.2	F 5'-GAAGCCCATTGACAAGCACAAG-3' R 5'-ACGAAGACCAGGGCATAGAG-3'	189
Rat VEGF-A	NM_031836.3	F 5'-AGGAAAGGGAAAGGGTCA-3' R 5'-ACAAATGCTTTCTCCGCT-3'	98
Rat GAPDH	NM_017008.4	F 5'-TCAACTACATGGTCTACATGTTCCAG-3' R 5'-TCCCATTCTCAGCCTTGACTG-3'	113

2.5 Biochemical assays

Enzyme linked immunosorbent assay (ELISA) was used to detect plasma Insulin levels by RayBio® Rat Insulin ELISA Kit (Ray RayBiotech, Inc., Norcross, GA). Fasting Blood Sugar (FBS) was measured by the oxidase method (Biodiagnostic, Egypt), total lipid profile including total cholesterol (TC), triglycerides (TG) and HDL-cholesterol (HDL-Ch) were measured by enzymatic-colorimetric methods (Biodiagnostic, Egypt). LDL cholesterol (LDL-Ch) concentration was calculated according to Friedewald equation [21]. Urinary

albumin, plasma protein, blood urea nitrogen (BUN), uric acid and creatinine were estimated using commercial kits (HUMAN Diagnostics, Wiesbaden, Germany) according to supplier's instructions.

2.6 Pro-oxidant/antioxidant status assessment

For determination of 8-OHdG (8-Hydroxyguanosine), urine samples were centrifuged at 2,000g for 20 min, and the supernatant was used for the by a standard sandwich ELISA kit (Chongqing Biospes Co., Ltd, Chongqing, China) according to supplier's instructions. Levels of Malondialdehyde (MDA) and superoxide dismutase (SOD) enzyme activity in renal tissues were assayed by available commercial kits (Biodiagnostic, Egypt).

2.7 Antibodies, homogenization and western blotting

Antibodies used in this study were as follow: anti-TRPM8 (#KM060, TransGenic Inc., Kobe, Japan), anti-TRPM8 (#ab109308, Abcam, Cambridge, UK), and anti- β actin (#8226; Abcam). Total protein extracts were prepared by homogenization of tissues into a lysis buffer containing (50mM Tris-HCl pH7.6, 250mM NaCl, 1% Triton X-100, 0.5% Triton X100, 3mM EDTA, 3mM EGTA, 10% glycerol, 2mM DTT, 1mM PMSF and 1mM sodium orthovanadate), supplemented with mini complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by Bradford method [22] with bovine serum albumin as a standard. Total proteins of 25 μ g were used after boiling it with Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris [pH 6.8]) at 95°C for 5 min. Proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA). Blots were blocked with 5% skim milk in 0.1% TBST buffer (20 mM Tris HCl [pH 7.5]), 48 mM NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature prior to incubation with anti-TRPM8 and anti- β -actin primary antibodies, at a 1:1000; 1:5000

dilutions in blocking buffer; respectively at 4°C for an overnight. Following three washes in TBST, blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (MBL, Nagoya, Japan; diluted in blocking buffer), as appropriate, for 1h at room temperature. Protein bands were developed using chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Western blot bands were scanned and band intensities were identified by densitometric analysis using the image analysis software package; ImageJ version 1.50i. (NIH, Bethesda, MD, USA).

2.8 Immunohistochemistry and electron microscopy

Hematoxylin and Eosin (H&E) and electron microscopy (EM) preparation was performed as previously described [23]. For TRPM8 staining 4 µm thick sections were incubated with anti-TRPM8 antibody at (1:100 dilution). Peroxidase anti-peroxidase was used as a secondary reagent (DakoCytomation, Cambridge, UK) and 3,3-diaminobenzidine/ H₂O₂ as a substrate. Slides were viewed by (Leica Imaging System LTD., Cambridge, UK). Immunoreactivity of TRPM8 appeared as brown staining of varying degrees of intensity. EM was performed by a transmission electron microscope (JEOL, Tokyo, Japan). Morphometric analysis was carried out to evaluate the foot process effacement on 80kV electron micrographs by using ImageJ version 1.50i. The width of the foot process was calculated according to [24].

Five measurements were taken for each ultrathin section of each group.

2.9 Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6.0 software (GraphPad Inc, San Diego, California, USA). Results are presented as means ± s.e.m (standard error of the mean). Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test. Nonparametric data were analyzed using Kruskal-Wallis test followed by Dunn's post-hoc test. Statistical significance was considered when the *P* value

165 was ≤ 0.05 .

166 **3. RESULTS**

167 **3.1 Effects of Geraniol on body and kidney weight and glycemic indices**

168 Rats injected with STZ developed type 1 diabetes characterized by the significant increase in
169 plasma glucose level and decrease in plasma insulin level (Fig. 1 A, B). Diabetic rats
170 experienced a significant decrease in body weight as well as a significant gain in kidney
171 weight (as a marker of diabetic nephropathy) (Fig.1 C, D) when compared to normal rats.
172 However, GE supplementation significantly decreased plasma glucose levels, improved
173 plasma insulin and decreased renal hypertrophy (Fig. 1 A-D) strengthening the described
174 hypoglycemic effect GE.

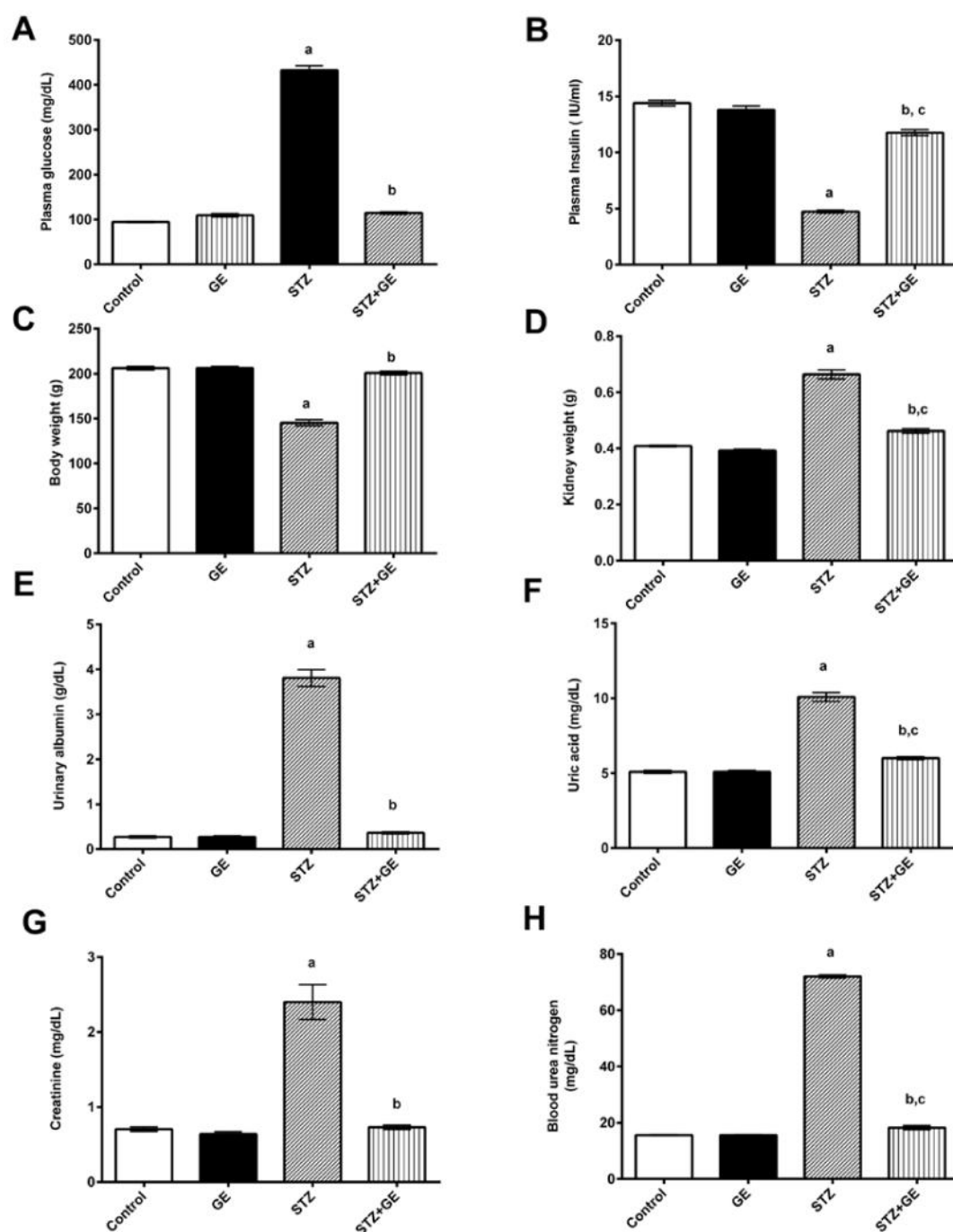
175 **3.2 Effects of GE on STZ-induced nephrotoxicity**

176 STZ-induced diabetic rats exhibited significant alterations in nephrotoxicity markers where,
177 urinary albumin, plasma uric acid, creatinine and plasma BUN showed significant elevation
178 (Fig. 1 E-H). However, GE treatment efficiently reduced those alterations and appeared to act
179 as a renoprotective agent in DN.

180 **3.3 Effects of GE on lipid profiling and DNA damage indices**

181 STZ- induced diabetic rats exhibited significant dyslipidemia, 8 weeks GE treatment weeks
182 significantly restored lipid profiling to near control levels (Fig. 2 A-D). In our present study,
183 nephrotoxicity was associated with imbalance between kidney antioxidant/pro-oxidant status
184 with heightened oxidative stress that was evident by significant increase in lipid peroxidation
185 product MDA, urinary oxidative stress marker 8-OHdG excretion levels and decrease the
186 antioxidant activity of SOD (Fig. 2 E-G). Treatment with GE effectively decreased the
187 alterations in these oxidative stress related parameters revealing GE as a good antioxidant
188 tool that protects rat kidney from diabetes-induced oxidative damage.

189 **Fig. 1**



190 **Fig.1. Effects of geraniol on glycemic and nephrotoxic indices of STZ-induced diabetic rats.**

191 Control: normal control; GE: treated with geraniol; STZ: STZ-induced (diabetic); STZ+GE: GE treated
 192 post to STZ induction (A) plasma glucose, (B) Plasma Insulin (C) body weight, (D) kidney weight, (E)
 193 urinary albumin, (F) uric acid, (G) creatinine, (H) blood urea nitrogen. Data present mean $\pm$ s.e.m., n=
 194 20. “a” indicates significant difference between the normal control and STZ-induced groups, “b”
 195 indicates the significant difference between STZ-induced and GE treated groups and “c” indicates the
 196 significant difference between the STZ+GE group and normal control group using ANOVA followed
 197

by Tukey's post-hoc test. ($P^a < 0.0001$, $P^b < 0.0001$, $P^c < 0.0001$).
Fig.2

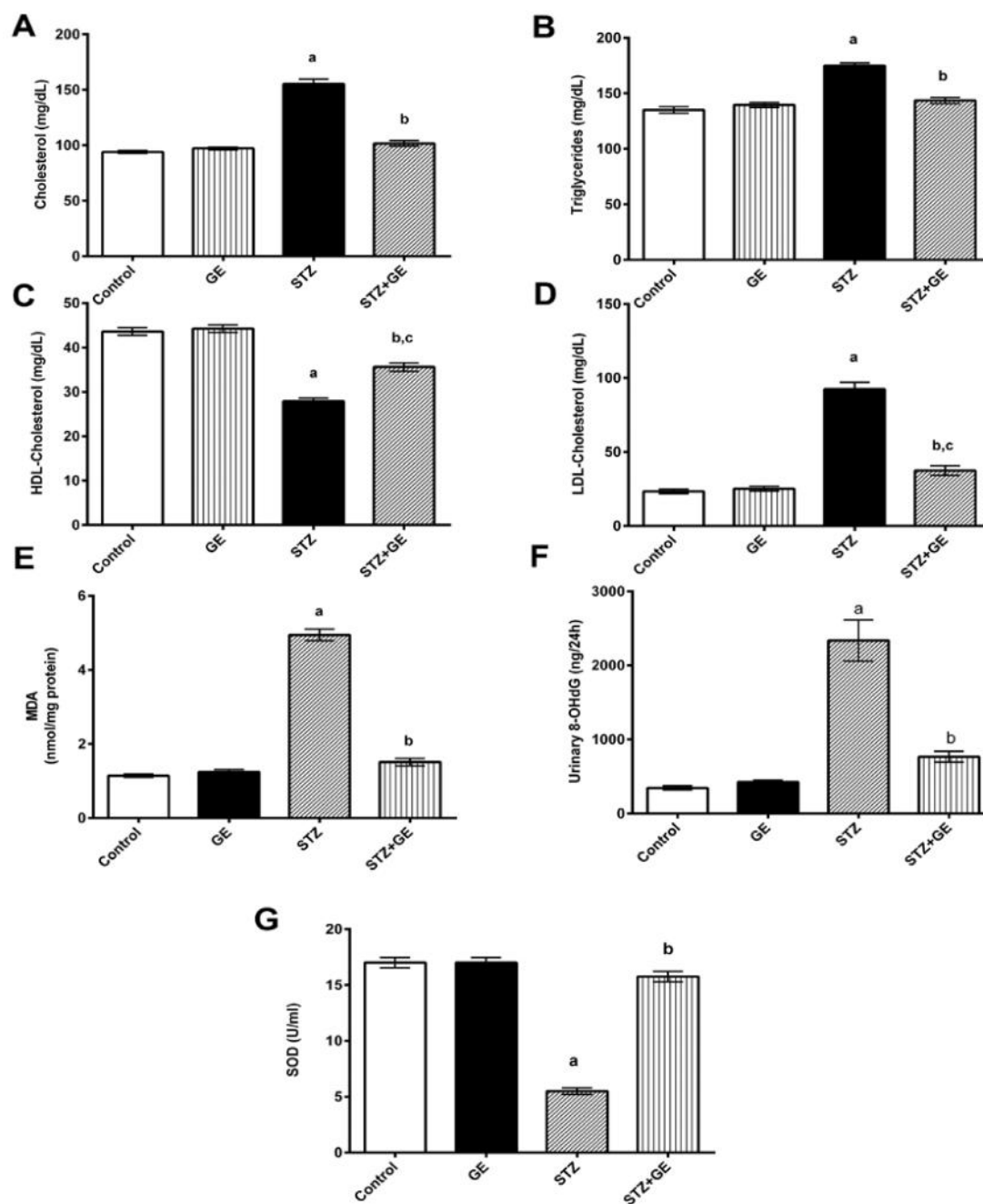


Fig. 2. Effects of geraniol on lipid profiling and DNA damage indices of STZ-induced diabetic rats. Control: normal control; GE: treated with geraniol; STZ: STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction (A) Cholesterol, (B) Triglycerides, (C) HDL-Cholesterol, (D) LDL-Cholesterol, (E) MDA, (F) urinary 8-OHdG, (G) SOD. Data present mean $\pm$ s.e.m., $n = 20$. "a" indicates significant difference between the normal control and STZ-induced groups, "b" indicates the significant difference between STZ-induced and GE treated groups and "c" indicates the significant difference between the STZ+GE group and normal control group

using ANOVA followed by Tukey's post-hoc test. ($P^a < 0.0001$, $P^b < 0.0001$, $P^c < 0.05$). HDL: high density lipoproteins; LDL: low density lipoproteins; MDA: malondialdehyde; 8-OHdG: 8- Hydroxyguanosine; SOD: superoxide dismutase.

3.4 Geraniol protects from STZ-induced renal injury

Histological examination of H&E stained sections from diabetic kidney showed characteristic diabetic nephropathy in the form of increased glomerular size, lobulation, degenerative changes in the proximal tubules, cellular infiltration and areas of hemorrhage, (Fig. 3 c) relative to the preserved renal structures from the non-diabetic groups (Fig. 3 a, b). These alterations were effectively decreased post treatment with GE (Fig. 3 d). These results confer a protective action of GE in diabetic renal injury.

3.5 Analysis of TRPM8 expression in STZ-induced diabetic rats

In order to explore that GE observed effects were TRPM8 mediated we investigated the expression of TRPM8 mRNA in kidney of diabetic rats at 2 and 8 weeks intervals. Following 2 weeks of STZ- injection, renal TRPM8 mRNA expression was downregulated as compared to other groups. However, that reduction was not of statistical significance (Fig. 4 A).

At 8 weeks after diabetes induction there was a significant decrease of TRPM8 mRNA expression of the kidney relative to the control group (Fig. 4 B). Meanwhile, GE treatment for 8 weeks significantly increased the altered renal TRPM8 mRNA expression, when compared to diabetic rats (Fig. 4 B). We furtherly confirmed renal TRPM8 protein expression levels by western blotting followed by densitometry, that showed significant reduction of TRPM8 levels in renal tissues from diabetic rats relative to control ones. TRPM8 protein expression levels were increased 8 weeks following GE treatment to reach about 90% of controls (Fig. 5 A, B). Immunohistochemistry of kidney sections demonstrated decreased immunoreactivity of TRPM8 in sections from diabetic rats relative to control ones (Fig. 5 C,a). GE administration for 8 weeks efficiently increased protein expression of TRPM8 with

intense immunoreactivity noted at both distal and proximal renal tubules (Fig. 5 C, b).

Fig.3

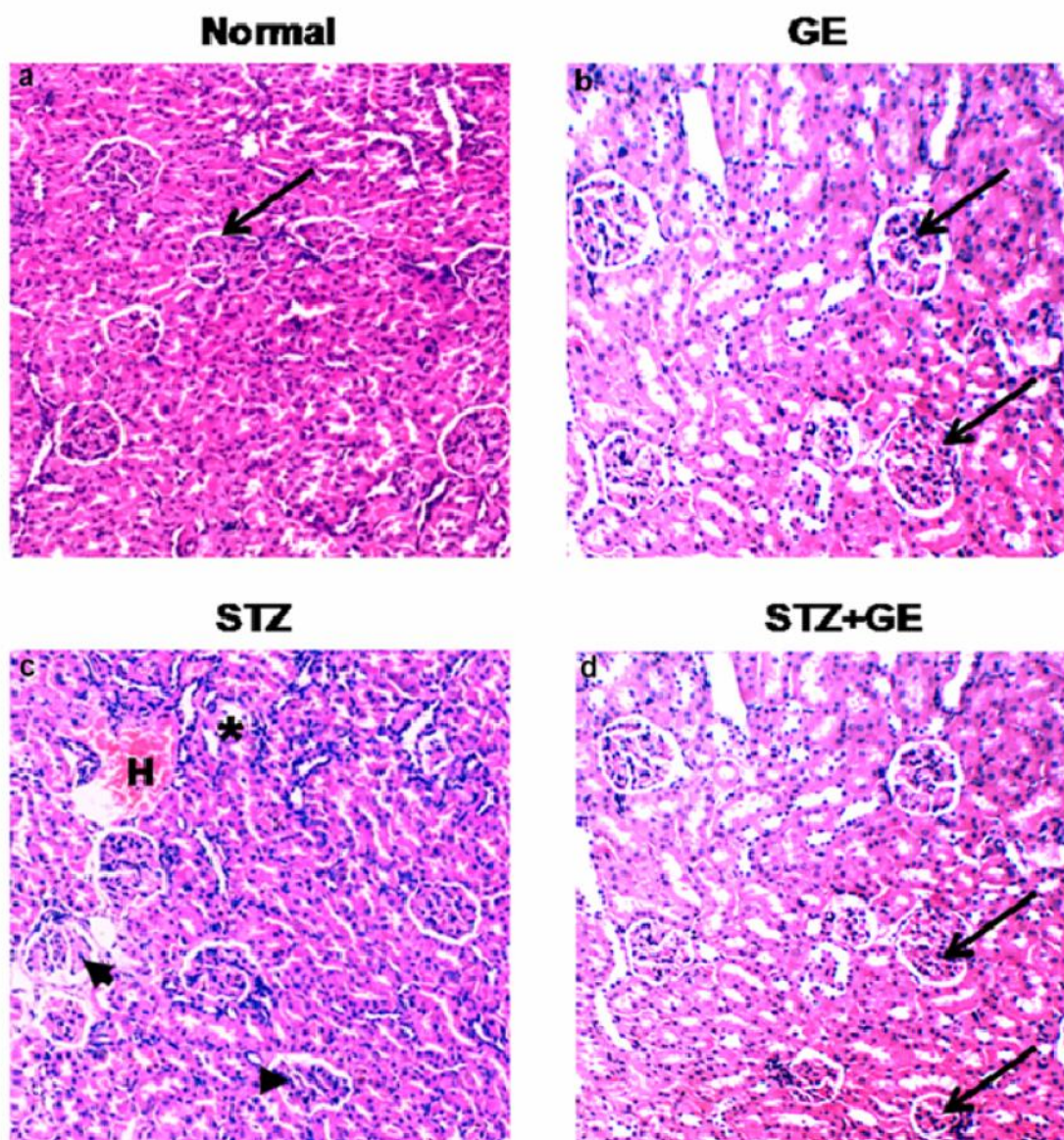
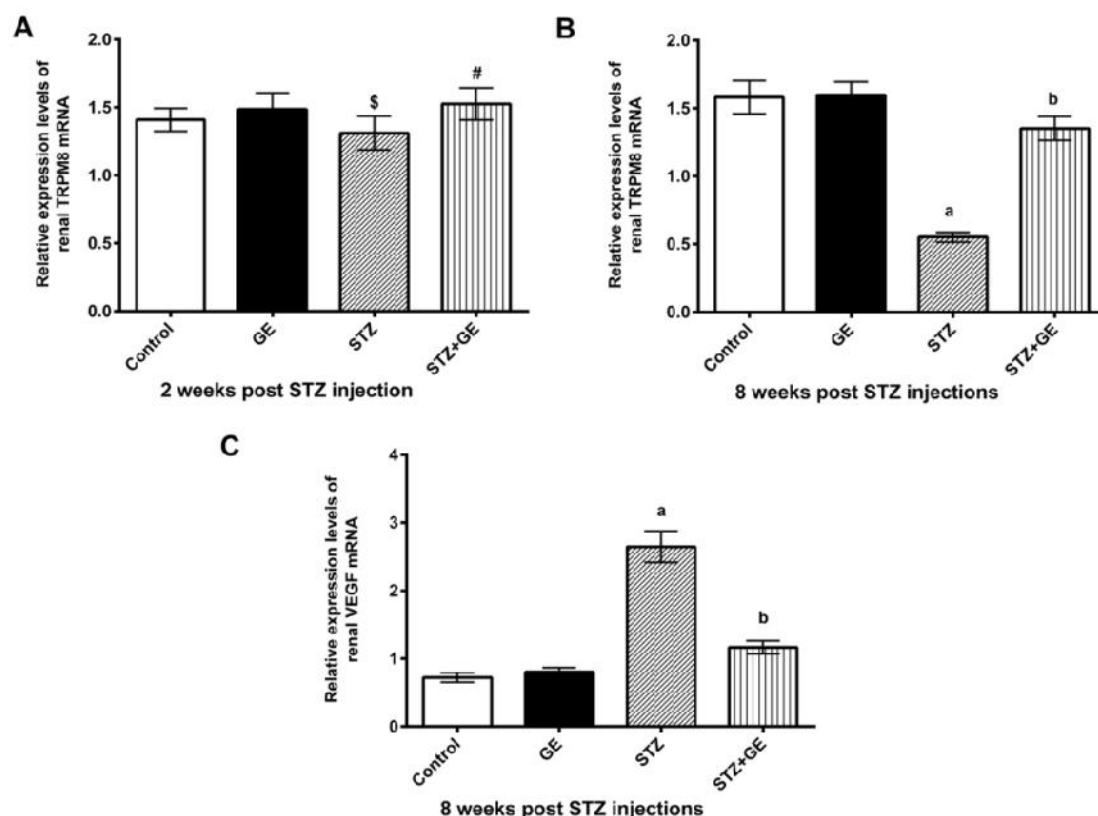


Fig. 3. Effects of geraniol on STZ-induced diabetic nephropathy in rats. Photomicrographs of rat kidney stained with H&E (X100) showing (a) normal control group with normal renal architecture (b) GE only treated group with preserved renal architecture, (c) STZ-induced diabetic group showing massive cellular infiltration (*), areas of interstitial hemorrhage (H) and lobulated glomeruli (double arrows) (d) GE treated diabetic group with improved renal architecture. Black arrows represent normal sized glomeruli.

246 **Fig. 4**



247 **Fig. 4. Effect of geraniol on TRPM8 and VEGF mRNA expression in STZ-induced diabetic rats.**

248 Total RNA was extracted from kidney tissues from; control: normal control; GE: treated with geraniol; STZ:
 249 STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction, followed by real time PCR. Histogram
 250 showing (A) TRPM8 relative mRNA expression at 2 weeks, \$ denotes STZ *VS* control, $P=0.928$ and # denotes
 251 STZ *VS* STZ+GE, $P=0.544$ (non-significant). (B) TRPM8 relative mRNA expression at 8 weeks (C) VEGF
 252 relative mRNA expression at 8 weeks. GAPDH was used as internal control. Data present mean $\pm$ s.e.m., $n=20$.
 253 “a” indicates significant difference between the normal control and STZ-induced groups, “b” indicates the
 254 significant difference between STZ-induced and GE treated groups using ANOVA followed by Tukey’s post-hoc
 255 test. ($P^a < 0.0001$, $P^b < 0.0001$).
 256

257 3.6 Effects of geraniol treatment on angiogenesis and podocytes in diabetic rats

258 Next we assessed, whether GE could mitigate the diabetic podocytopathy. Renal vascular
 259 endothelial growth factor (VEGF), an angiogenic and podocyte marker, mRNA expression
 260 was significantly increased in diabetic rats compared to control ones, (Fig. 4 C).
 261 Ultrastructural studies by transmission electron microscopic examination of kidney from
 262 diabetic animals displayed podocytopathy, effacement, swelling and/or fusion of foot
 263 processes with narrowing of the subpodocytic space (Fig. 6, A) as well as significant increase
 264

of podocyte foot processes width (FPW) in nm (Fig. 6, B) confirming that decreased renal TRPM8 expression was coupled with cytoskeletal derangement and podocytopathy during DN. Eight weeks of GE administration significantly reduced VEGF mRNA expression levels to control levels. Meanwhile, at the ultrastructural level GE+ STZ treated animals revealed restoring of normal structure with preserved podocyte morphology and significant lowering of foot processes width (FPW) in nm (Fig. 6 A,B). Collectively these findings convey an effective role of GE in alleviating diabetic nephropathy via modifying TRPM8 gene expression and remodeling podocytopathy.

Fig.5

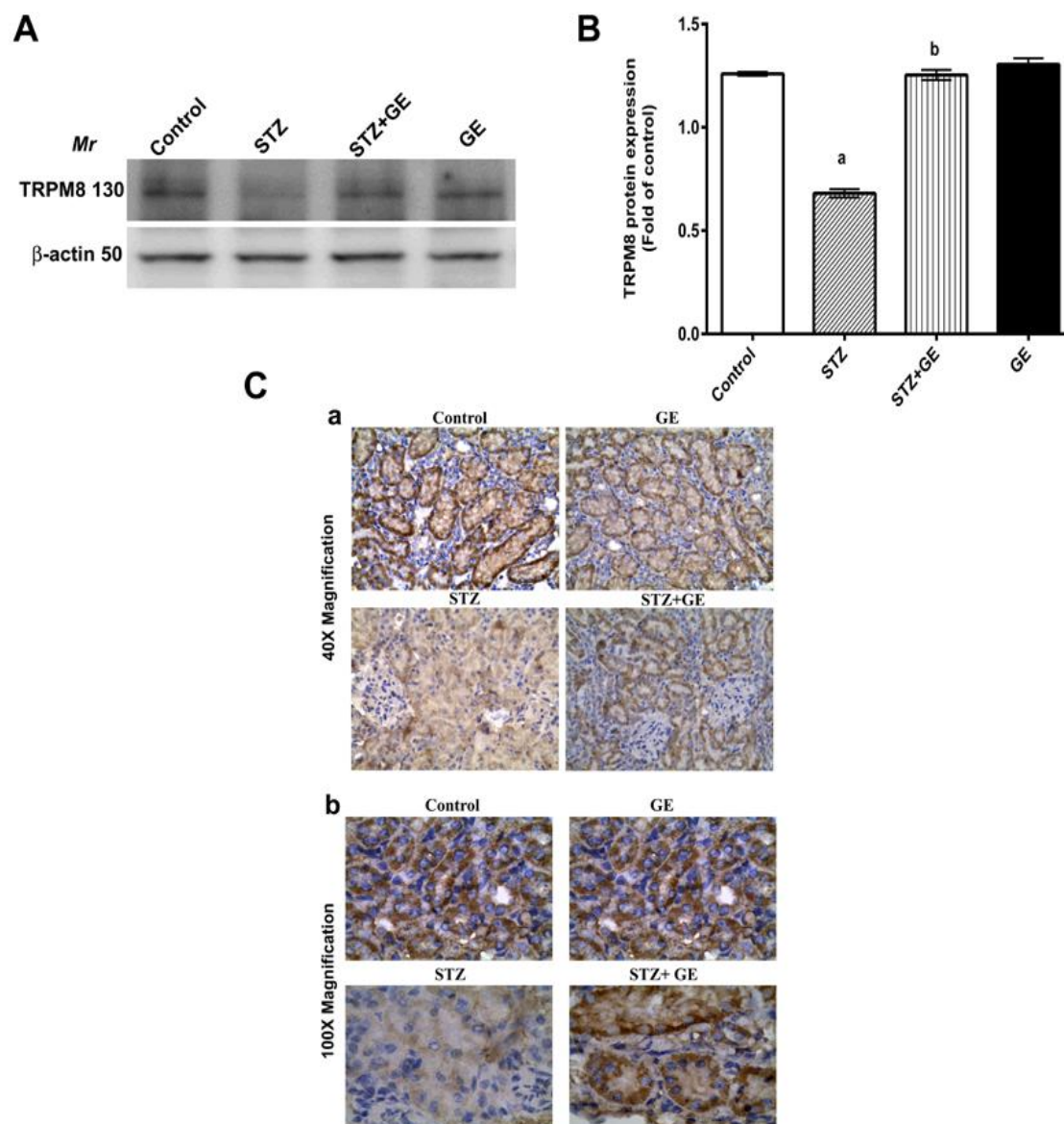


Fig. 5. Decreased TRPM8 protein expression in STZ-induced diabetic rats.

(A) Lysates from kidney tissues from control: normal control; STZ: STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction; GE: treated with geraniol, were analyzed by western blotting for TRPM8 protein levels; β-actin was used as a loading control. (B) Bands from (A) were quantified by densitometry, and expressed as a value relative to control (β- actin). *Mr*; Molecular weight marker in kDa (kilodaltons). Values are presented as means± s.e.m., n= 6, “a” indicates significant difference between the normal control and STZ-induced groups, “b” indicates the significant difference between STZ-induced and GE treated groups using Kruskal-Wallis test followed by Dunn's multiple comparisons test.

($P^a < 0.0001$, $P^b < 0.0001$). (C) Photomicrographs showing immunohistochemistry of rat kidney cortical sections of groups as in (A), stained with anti-TRPM8 (brown), counterstained with Hematoxylin (blue), (a) 40X magnification (b) High power 100x magnification, showing decreased TRPM8 immunoreactivity at renal tubules of diabetic rats, GE treated tubules showing intense TRPM8 immunostaining.

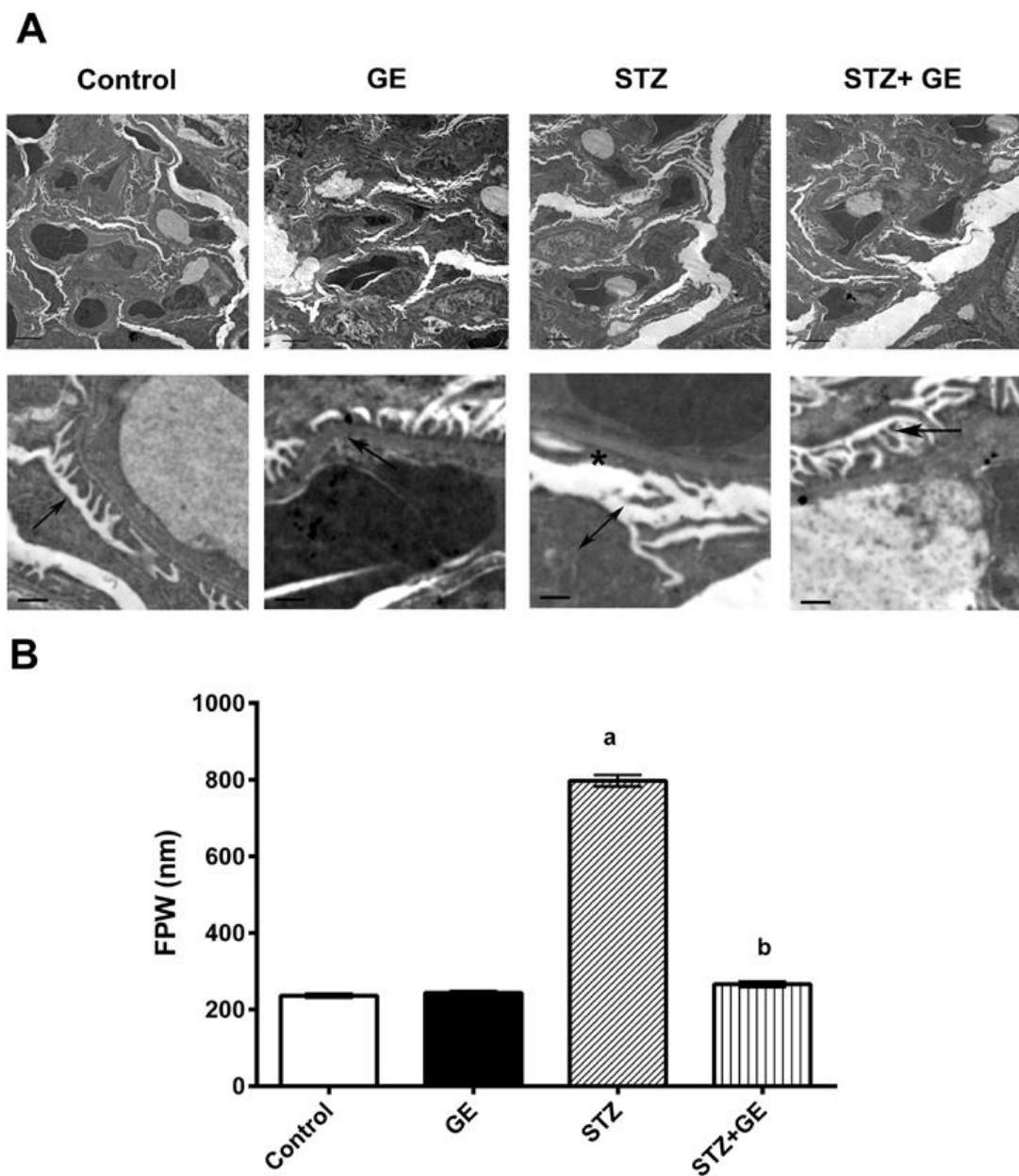


Fig. 6. Effect of geraniol on ultrastructural features of diabetic nephropathy.

(A) Photomicrographs of kidney samples from the four experimental groups were examined under transmission electron microscope as indicated. Upper panel; X1000 magnification (scale bar 2 μ m), lower panel; X6000 magnification (scale bar 5 μ m). STZ group revealed part of Malpighian corpuscle with many blood capillaries (BC) with their covering podocytes. Narrowing and obliteration of subpodocytic (*) space can be seen with focal thickening of the glomerular basement membrane and fusion, effacement and swelling of foot processes (double arrow). Other non-diabetic and treated groups displayed fenestrated blood capillary with its basement membrane of normal thickness covered by regularly spaced foot processes of podocytes (arrow). (B) Quantification of foot process width (FPW) from all indicated groups in (A), data present mean $\pm$ s.e.m., (n= 5). “a” indicates significant difference between the normal control and STZ-induced groups, “b” indicates the significant

difference between STZ-induced and GE treated groups using ANOVA followed by Tukey's post-hoc test. ($P^a < 0.0001$, $P^b < 0.0001$).

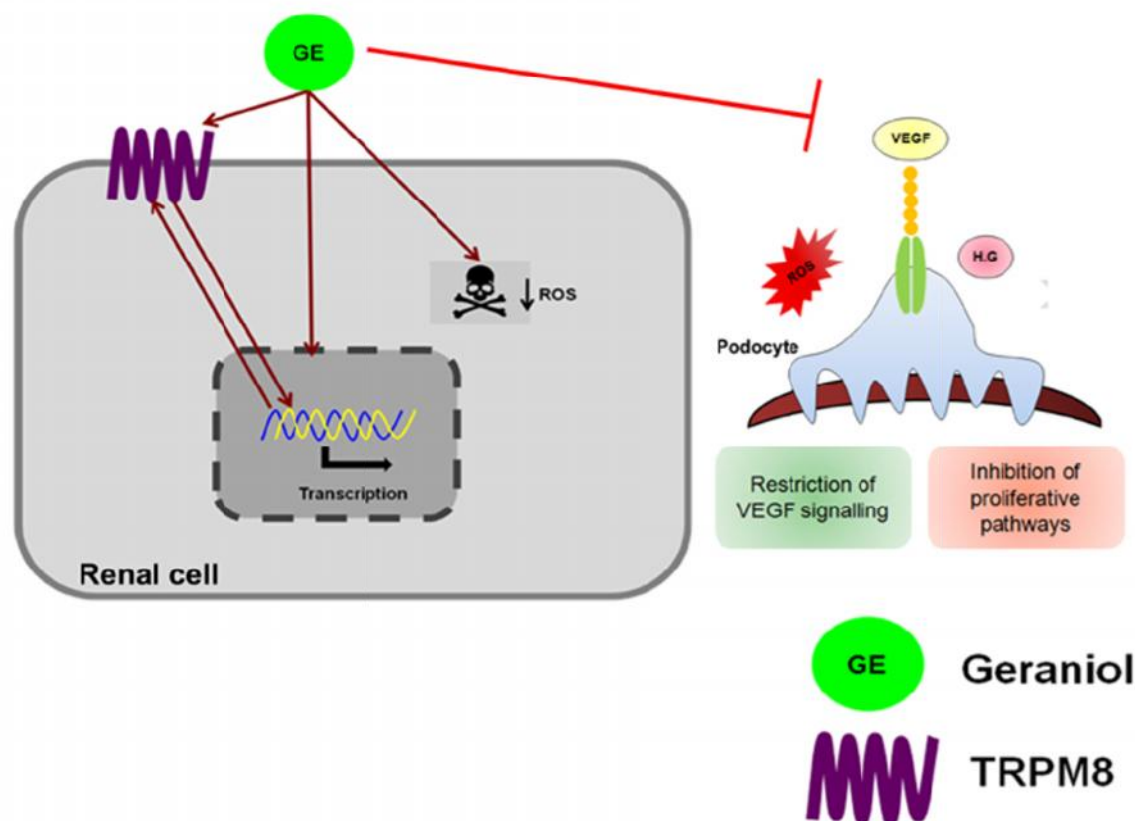


Fig. 7. Proposed model of TRPM8 in diabetic nephropathy.

Under normal condition there is restriction of growth factor signaling. Hyperglycemia, ROS, inflammation, VEGF activation and podocytopathy lead to alteration of TRPM8 expression. GE treatment activated and increased TRPM8 expression that in turn increases its own expression as well as decreased VEGF signalling.

4. DISCUSSION

The molecular mechanisms underlying the development of DN is complex and remains ill defined that accounts for the ineffectiveness of currently available interventions [25]. Although geraniol has been reported to ameliorate hyperglycemia, dyslipidemia and oxidative stress in rodent models of T1DM and metabolic syndrome [17, 26], its effect on DN and the underlying mechanisms remained largely uncharacterized.

318 The present study provides novel evidence on the renoprotective potential of geraniol via
319 modulation of TRPM8 expression as documented by improvement of DN biochemical
320 markers and restoring of normal renal architecture. Furthermore, GE mitigates diabetic
321 podocytopathy via downregulation of VEGF and alleviation of the observed heightened
322 oxidative stress. Persistent hyperglycemia drives reactive oxygen species (ROS) generation
323 which is considered as one of the dominant mechanisms driving DN development [27].
324 Several studies have illustrated the increased lipid peroxidation in clinical and experimental
325 diabetes [17]. This is in agreement with current results that showed increased levels of lipid
326 peroxidation product (MDA), urinary 8-OHdG, as well as decreased SOD activity at diabetic
327 kidney. GE treatment effectively reduced oxidative stress indices and restored renal SOD
328 antioxidant activity which is attributed to its potent renoprotective and antioxidant effect.
329 These findings are coincident with previous reports [17, 28]. It is of note that GE dose used in
330 the present study (100 mg/kg BW) was efficient to induce antioxidant effects, where a report
331 had implicated a pro-oxidant potential of GE at higher doses [11]. TRPM8 channel is a cation
332 selective one, with special preference for calcium ions [29]. Although the expression of
333 TRPM8 in many tissues has been reported, to the best of our knowledge, this study is the first
334 to report a role of TRPM8 expression in DN. The results of the present study demonstrated
335 TRPM8 mRNA and protein levels to be downregulated in renal tissues of diabetic rats as
336 compared to controls, 8 weeks following T1DM induction. This is in line with a recent study
337 describing that the reactive metabolite methylglyoxal, a byproduct of glycolysis that is
338 increased in the plasma of diabetic patients, inhibits the activity and reduces mRNA
339 expression of TRPM8 in dorsal root ganglion cells [30].

The mRNA and protein expression of renal TRPM8 was up-regulated after GE administration in diabetic treated animals. Interestingly, the observed herein oxidative stress did not elicit increased TRPM8 expression in diabetic animals.

Consequently, cells treated with H_2O_2 failed to increase TRPM8 activity [31]. In disagreement with our current findings, Nocchi et al., [32] stated that H_2O_2 treatment upregulates TRPM8 expression in aged mouse urothelium. This discrepancy may be tissue specific due to different redox potentials in aging animals. Similarly, increased TRPM8 expression was detected at dorsal root ganglion as well as in bladder tissues from diabetic animals [33, 34]. On the other hand Facer et al., [35] did not find any change in TRPM8 expression in skin from diabetic patients; indicating that TRPM8 expression is tissue and cell specific. It is of note that in these contradictory studies TRPM8 expression was not assessed beyond 2 weeks of diabetic induction. In the current study we also could not retrieve a significant downregulation of TRPM8 expression at 2 weeks. Only 8 weeks post-induction and establishment of DN, TRPM8 expression displayed a significant reduction when compared to other studied groups. Therefore, it is seemingly that renal TRPM8 downregulation is linked to the pathogenesis and structural changes of DN.

In addition, our TRPM8 immunostaining results revealed maximum intensity at distal and proximal tubules, inferring a structural and functional role of TRPM8 in normal kidney. The ultrastructural findings of the current study detected characteristic diabetic podocyte dysfunction as previously stated [36]. VEGF has been known to be involved in podocytopathy of DN due to high glucose levels, oxidative stress, dysregulation of podocyte signaling and cytoskeletal remodeling [2, 37]. The present study and other ones have detected upregulation of renal VEGF mRNA expression in diabetic rats as compared to control groups [38, 39]. On the other hand GE treated diabetic rats showed decreased mRNA expression of

364 VEGF, decreased FPW and alleviation of podocytopathy. In agreement with present data,
365 Carnesecchi et al., [40] and others [41] have reported the ability of dietary monoterpenes to
366 inhibit angiogenesis in animal models and cancer cell lines through apoptosis induction and
367 increasing the expression of the pro-apoptotic proteins. Moreover, Zhu et al., [42] has
368 described downregulation of VEGF in response to TRPM8 overexpression. Therefore, GE
369 could modulate TRPM8/VEGF signaling particularly at podocytes. The current proposed
370 mechanism of GE clarifies the role of TRPM8 in normal renal physiology that is
371 downregulated by hyperglycemia in DN. Mechanistically GE observed action herein is
372 multifactorial, first by, activation of TRPM8 receptors that in turn can regulate their function
373 by modulating their own expression at the plasma membrane via rapid vesicular translocation
374 and fusion [10]. Whether TRPM8 itself can modify other genes in renal transcriptome
375 requires further studies. Second, GE acts through direct transcriptional activation and
376 decrease of DNA synthesis, which influences S phase progression of the cell cycle, thus
377 decreasing renal cell proliferation [43]. Meanwhile, GE can produce evident changes in the
378 resting potential and cell membrane polarization that trigger modifications of
379 membrane-bound proteins activities and alter the intracellular signaling transduction
380 pathways in the corticomedullary tubular cells [14, 44, 40]. The observed dyslipidemia in the
381 current work can trigger signaling pathways that are involved in the development of DN [45].
382 GE significantly attenuated the observed hyperlipidaemia in diabetic rats, mostly by
383 modifying the expression of genes involved in lipid metabolism [46]. Intriguingly, an
384 anti-inflammatory role for TRPM8 has been reported, where TRPM8-deficient mice
385 exhibited aggravated inflammatory conditions [47]. Dyslipidemia and inflammatory markers
386 such as tumor necrosis factor- α (TNF- α) associated well with renal impairment in diabetes
387 [48]. Based on the ability of TRPM8 to modulate cytokine expression by increasing the

expression of the anti-inflammatory cytokine; interleukin-10 and decreasing that of TNF- α [29], a TRPM8 mediated role of GE in immunoinflammation can be concluded. TRPM8 knockout mice have increased rates of insulin clearance and degradation compared to wild type ones; revealing a novel role of TRPM8 as a regulator of serum insulin [8]. A role of TRPM8/ insulin signaling can't be omitted, since insulin rapidly and directly signals to podocyte and dynamically remodels its cytoskeleton [49]. Collectively, and as depicted in Fig. 7 diabetic podocytopathy may act on translational or posttranslational pathways to modify renal transcriptome and to determine the progression of nephropathy [50]. Therefore, persistent hyperglycemia, ROS, inflammatory cytokines as well as altered insulin homeostasis would end in activated VEGF signaling, decreased expression of TRPM8, cytoskeletal remodeling and ultimately ESRD.

5. CONCLUSION

The present study demonstrates that GE treatment ameliorated STZ-induced DN in rats via multifaceted molecular mechanisms. Our salient finding is the novel role of TRPM8 in DN. GE increased renal TRPM8 expression, abrogated oxidative stress, decreased VEGF expression and improved podocytopathy. The exact mechanism of TRPM8 at various types of renal cells warrants future studies to open new avenues for developing new therapeutic targets of DN.

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