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ORIGINAL ARTICLE

Punica granatum L. peel extract protects diabetic nephropathy by activating the Nrf-2/HO-1 pathway

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Abstract
Diabetes raises cardiovascular morbidity and mortality worldwide and causes retinopathy, neuropathy, and nephropathy. *Punica granatum* L. (Pomegranate) is a fruit that has been used for its medicinal properties in various cultures. This article aims to investigate the antioxidant, anti-inflammatory, anti-apoptotic activity of *Punica granatum* L. peel ethanol extract (PGE) and its efficacy on NF-κB and Nrf-2/HO-1 signaling pathways in kidney tissue of rats with streptozotocin-induced diabetes. Single dose STZ 60 mg/kg/i.p. rats were given to induce diabetes and blood glucose measurements were taken 7 days later. PGE 10 mg/kg/p.o. administered to the treatment groups for 20 days. Blood, kidney, and pancreas samples taken from anesthetized rats were analyzed biochemically and histopathologically. It was observed that STZ increased the levels of urea, uric acid and creatine in the blood, while PGE significantly decreased these parameters. The diabetic group had higher MDA and lower renal tissue GSH level, CAT, GPx, and SOD activity than the control group. STZ also enhanced inflammation, apoptosis, Bax, Caspase-3, and NF-κB expression, and decreased Bcl-2, HO-1, and Nrf-2 expression. Experimental results showed that PGE has the potential to alleviate the harmful effects on the kidney and pancreas by altering the mentioned parameters in diabetic rats.

Keywords Diabetes · *Punica granatum L* · Oxidative stress · Inflammation · Apoptosis · Kidney

Introduction

The pomegranate (*Punica granatum L.*), a member of the Punicaceae family, is a fruit with an extraordinary structure, known for its mystical properties since ancient times. Throughout history, different cultures have relied heavily on the use of pomegranate, an herb known for being safe, tasty, and free of significant side effects [1]. The pomegranate tree, particularly its fruit, serves as a valuable source of phytochemicals, offering immense potential in folk medicine and urging further exploration of its medicinal properties. The pomegranate tree and its fruit are composed of anatomical parts with various interesting pharmacological effects; seeds, pomegranate juice, peel, leaves, flowers and roots [2]. The antiatherogenic, antioxidant, anticarcinogenic and anti-inflammatory effects of pomegranate and its peel have been demonstrated in various experimental studies [3, 4]. Studies have indicated that pomegranate extract possesses anti-inflammatory characteristics and may potentially mitigate the likelihood of specific chronic ailments, such as cardiovascular disease, diabetes, and certain forms of cancer. It may also have benefits for brain function, including improving memory and cognitive function [5, 6]. Some of the significant components of pomegranate, like ellagic acid, ellagitannins, punicic acid, flavonoids, anthocyanidins, anthocyanins, flavonols, flavone glycosides, and flavones, are thought to protect against diseases [7]. Also, pomegranate contains biomolecules like oleanolic, ursolic, and gallic acids, which are natural products that may have

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hypoglycemic action and are linked to antidiabetic effects [8].

Diabetes mellitus (DM) is a serious illness that has a global prevalence, impacting a significant number of individuals across various age groups. The etiology of DM can be attributed to a range of variables, including disturbances in insulin production, genetic predisposition, environmental influences, and lifestyle choices. A metabolic disorder of carbohydrates, proteins, and lipids is linked to diabetes mellitus, which is characterized by an increase in blood glucose levels [9, 10]. Hyperglycemia, dyslipidemia and inflammation are factors that lead to oxidative stress, and all of these have a significant impact on the progression of diabetes [11]. Hyperglycemia can induce the creation of reactive oxygen species (ROS) by various pathways, including initiation of the polyol pathway, synthesis of advanced glycation end products, and mitochondrial dysfunction. Dyslipidemia can potentially contribute to oxidative stress by raising lipid peroxidation levels and lowering antioxidant concentrations in the body. Also, inflammation can induce the generation of ROS by immune cells, which can further exacerbate the effects of oxidative stress. Finally, oxidative stress and ROS play a critical role in the pathogenesis of diabetes. ROS can contribute to the development of type 1 and type 2 diabetes by harming insulin-producing cells and disrupting insulin signaling pathways [6, 10]. Furthermore, chronic hyperglycemia and hyperlipidemia lower an individual's quality of life by boosting the generation of ROS, which harm several organs, including the testicles and kidneys. [12, 13].

Diabetic nephropathy can be caused by both diabetes and the high blood pressure (hypertension) that often accompanies diabetes. Both of these conditions can harm the kidneys in the body. If diabetic nephropathy is not properly monitored and treated, it can result in kidney failure and, ultimately, death. According to statistics, the majority of diabetic people acquire diabetic nephropathy throughout their lives [14]. Diabetic nephropathy occurs by the production of extracellular matrix, breakdown of the glomerular basement membrane and glomerular atrophy, and degradation in the functional and structural features of the kidney [6, 15]. Streptozotocin (STZ), which is especially used in diabetes research, is one of the most commonly used diabetogenic agents in laboratory settings to trigger experimental diabetes in animal models. STZ, when administered to rats, enters the cell via a glucose transporter and disrupts DNA and dephosphorylates ATP. This results in a decrease in insulin secretion and the development of DM. STZ-induced diabetes in rats is an accepted approach for researching diabetes and evaluating potential treatments for the disease. Additionally, this method lends support to the investigation of the underlying processes of diabetes, as well as oxidative stress and inflammation, among the variables that play a role in the development of the disease [9, 16].

As an outcome of this information, the purpose of this research is to investigate the antioxidant, anti-inflammatory, and anti-apoptotic properties of pomegranate peel extract in kidneys, as well as its influence on the NF-κB and Nrf-2/HO-1 signaling pathways. Furthermore, it demonstrates that pomegranate peel extract has the potential to have an antidiabetic impact in rats; this finding is supported by histological evidence.

Materials and methods

Materials

The documentation of commercially available *Punica granatum* L. was undertaken by Ataturk University Faculty of Agriculture, specifically the Department of Plant Protection. For the research, Streptozotocin (STZ) was procured from the Sigma Aldrich.

Preparation of *Punica granatum* L. peel ethanol extract

Pomegranate peel extraction was carried out using a modified version of the specified method [17]. Pomegranates were selected, and their peels were carefully separated from the seeds and subsequently dried in a shaded environment before being crushed. A total of 100 g of pomegranate peel was placed within a 1-liter flask, to which 500 mL of ethanol was added, and left undisturbed for a period of 48 h. Following this incubation period, the resultant mixture underwent filtration to eliminate coarse particles. The organic solvent portion was then separated from the mixture using an evaporator, thereby yielding the desired extract. The extract was stored at a temperature of + 4ºC until employed in the experimental stage of the study.

Animals

In this investigation, 28 Sprague-Dawley rats were employed, with four groups divided evenly ($n = 7$). The rats in question were of an age range between 5 and 6 weeks and had a weight ranging from 200 to 250 g. The rats were given sustenance in the form of pellets and tap water. At 21 ºC (±2), they were subjected to a light/dark cycle of 12 h each. Animals were followed for one week for adaptation to the environment before administering the chemical

components. Animal trials were conducted at the Ataturk University Medical Experimental Research Center. The Animal Experiments Local Ethics Committee at Ataturk University granted consent for the experiment (Protocol No: 2014-45-203).

Experimental design

- *Control group (C)*: Rats were administered physiological saline orally for 20 days.
- *Punica granatum L.* extract group (PGE): Rats were administered 10 mg/kg/p.o. PGE for 20 days.
- STZ-DM *group* (DM): The rats received a single intraperitoneal dose of STZ at a concentration of 60 mg/kg.
- STZ-DM *& Punica granatum L.* extract *group* (DM + PGE): The rats were orally supplied 10 mg/kg of PGE daily for a period of 20 days, whereas a single intraperitoneal dose of 60 mg/kg of STZ was given.

In order to induce diabetes, STZ was dispersed in citrate buffer (0.1 M, pH = 4.5) and then given intraperitoneally following an overnight fast (free access to water) of 18 h [18, 19]. At the end of the twentieth day, the rats were euthanized under sevoflurane anesthesia, and blood and tissue samples were taken. (Sevorane liquid 100%, Abbott Laboratories, Istanbul, Turkiye). Some of the tissues were separated for histopathological examination, some were stored in the freezer at -80 °C until used in Western blot and other biochemical analyzes.

Determination of the blood glucose levels

Rats with a blood glucose concentration equal to or exceeding 250 mg/dl, as determined by glucometer readings taken 7 days after STZ administration, were classified as diabetic and extracts were administered [20]. Blood glucose levels (mg/100 mL) were measured utilizing a Glucometer-elite (Bayer) industrial test that relies on the glucose oxidase technique.

Tissue samples

Kidney tissues taken for biochemical analysis were ground with liquid nitrogen and then homogenized with 0.15 M potassium chloride (KCl) in a homogenizer at 16,000 rpm for 3 min, and lipid peroxidation level and antioxidant enzyme activities were measured. Measurements were made from the supernatant after centrifugation of the homogenate at 5000 g for 1 h (+4 °C).

Renal function analysis

The levels of urea, uric acid, and creatine (Cre) were assessed using a renal function analysis meter (Hangzhou Medasia Trading, China) obtained from Medasia.store. [17].

Lipid peroxidation level and antioxidant enzyme activities analysis in kidney

CAT activity [21], MDA level [22], SOD activity [23], GPx level [24] in kidney tissue was determined by methods. Tissue extraction and GSH analysis were measured according to the methods specified by [25, 26]. All methods given above were measured with Biotech Epocha UV-Visible EIA Spectrophotometer.

Western blot analysis of kidney tissues

50 mg of weighed kidney tissue was subjected to RIPA lysis and homogenized in order to prepare the samples. The samples were homogenized and then centrifuged for 20 min at 16,000 g. Smith developed the Thermo PierceTM BCA measurement kit, which was used to measure proteins. After that, the supernatants were mixed with Laemmli buffer and vortexed. The 10% SDS-PAGE Gel wells were filled with the prepared samples. Proteins were transferred to a PVDF membrane following electrophoresis. Subsequently, 5% BSA was used to block the membrane for 1.5 h. Following blocking, three 5-minute TBS-T washes were performed on the membrane. Primary antibodies were added, and the mixture was incubated overnight at + 4 °C for β-tubulin (sc-47778), Bax (sc-20067), Bcl-2 (sc-7382), Caspase-3 (sc-56053), Nrf-2 (sc-365949), HO-1 (sc-390991), and NF-κB (sc-8008). Following incubation, membranes were cleaned five times for five minutes each using TBS-T after the primary antibodies were removed. Goat anti-mouse IgG secondary antibodies (sc-2005, Santa Cruz Biotechnology, Inc., Texas, USA) were then applied to the membrane and left for 1.5 h. After the membrane was incubated with secondary antibodies for 5 min each, it was rinsed three times with TBS-T. Protein bands were recorded with the Biorad Gel Doc XR + Imaging System (Bio-Rad, Hercules, USA) and visualized with the Trident femto Western HRP Substrate (Catalog Number: GTX14698). The bands were subjected to densitometric analysis using the ImageLab program from Bio-Rad. [27, 28].

Histopathology and immunohistochemistry of kidney and pancreas tissue

Pancreas and kidney tissue samples were immersed in a 10% formalin solution following the euthanasia. They

were washed in flowing tap water for 10 h after approximately 48 h of fixation. Paraffin blocks were employed for embedding subsequent to conventional tissue examination. Slides were subsequently generated by removing 4 µm thick sections from each block. In the analysis of the preparations for histopathological examination, they were examined with Hematoxylin-Eosin (HE) staining and light microscopy (Olympus Bx51 with DP72 camera system). 10 areas were randomly selected in the kidney tissues and x20 size images were taken under a high light microscope. Scoring is; Grade 0= - (none); Grade 1 = + 1 (mild); Grade 2 = + 2 (intermediate); Grade 3 = + 3 (severe) was reported.

For immunoperoxidase examination, all sections mounted on adhesive (poly-L-lysine) slides were deparaffinized and dehydrated by passing through xylene and alcohol series. Endogenous peroxidase was inactivated by washing with phosphate buffer solution (PBS, pH 7.2) for 5 min and keeping the sections in 3% H_2O_2 for 10 min. To prevent nonspecific background staining, the sections were incubated with protein block compatible with all primary and secondary antibodies for 5 min. After the excess block solution remaining in the tissue sections at the end of incubation was poured off, primary antibodies (8 OHdG Cat no: sc-66036, dilution ratio: 1/100, US; Insulin cat no: ab181547, dilution ratio: 1/200, UK) were added without washing. After washing, AEC (3-amino-9-ethyl carbazole) chromogen was applied to the pancreatic sections and kept for 5–10 min depending on the retention of the chromogen. For background staining, the sections were kept in Mayer hematoxylin for 1–2 min and then washed in tap water. Coverslips were closed using water-based adhesive. Kidney tissues were kept in 3–3′ diaminobenzidine (DAB) chromogen for 5–10 min. After keeping in Mayer hematoxylin for 1–2 min, they were passed through a series of alcohols and kept in xylene for 5 min, then coverslips were closed using Entellan and examined with a light microscope (Leica DM 1000). In other stages, the incubation time with primary antibodies, whether antigen retrieval or enzyme treatment will be applied, and the dilution ratios of primary antibodies may vary depending on the commercial kit used. Sections were evaluated as absent (-), mild (+), moderate (++) and severe (+++) according to their immune positivity.

Statistical analysis

The statistical evaluation in groups was conducted using the SPSS software package version 22.0, which employed one-way analysis of variance (ANOVA). In addition, Post-hoc Tukey's test was used to perform multiple comparisons. The values were reported as the mean ± standard error of means (SEM), and a p-value of less than 0.05 was considered statistically significant. In the histopathological examination, the Kruskal-Wallis test, one of the nonparametric tests, was used to analyze the differences between the groups in the semiquantitative data obtained, and the Mann Whitney U test was used to compare the paired groups. SPSS 20.0 package program was used for these statistical analyses.

Results

Levels of blood glucose

The diabetic control rats had much higher levels of glucose in their blood. When treated with PGE, diabetic rats exhibited a statistically significant ($p < 0.001$) drop in blood glucose levels to values that were very close to normal (Table 1).

Blood urea, uric acid, and creatine levels

Diabetic rats showed an increase in the level of urea in blood compared to the control group. After treatment with PGE, the urea level significantly decreased. Also, uric acid and creatine levels were significantly increased in STZ-diabetic rats, whereas treatment with PGE significantly decreased this level (Table 2).

Analysis of oxidative stress

Malondialdehyde (MDA), a marker of lipid peroxidation, has been observed at significantly elevated ($p < 0.001$) levels in the kidneys of STZ-diabetic rats. SOD, CAT ($p < 0.001$), and GPx activities, as well as GSH levels ($p < 0.01$), were markedly reduced in the kidneys of STZ-diabetic rats compared to normal control rats. Treatment with PGE (10 mg/kg/bw) significantly decreased the increased level of MDA and significantly increased the decreased activities of

Table 1 Effect of PGE on glucose in the blood of the control and experimental groups

C	PGE	DM	DM + PGE	*p*
122.50 ± 0.92^{a}	123.00 ± 0.89^{b}	340.00 ± 20.7^{b}	128.33 ± 0.82^{b}	***

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ significant from the control rats; ns – non-significant. The values were reported as the mean ± standard error (SEM), and a p-value of less than 0.05 was considered statistically significant. Significant variations in means within the same column are represented by distinct letters, a and b. Abbreviations: C, control rats; PGE, *Punica granatum L.* extract rats; DM, diabetes rats; DM + PGE, *Punica granatum L.* extract-treated rat.

Table 2 Effect of PGE on blood urea, uric acid, and creatine levels in STZ-induced diabetes

Parameter	C	PGE	DM	DM + PGE	*p*
Urea (mg/dL)	5.46 ± 0.01^{c}	4.92 ± 0.22^{c}	28.05 ± 0.31^{a}	12.66 ± 0.56^{b}	***
Uric Acid (mg/dL)	0.09 ± 0.0^{b}	0.8 ± 0.0^{c}	0.10 ± 0.0^{a}	0.9 ± 0.0^{b}	***
Crea (mg/dL)	0.40 ± 0.0^{c}	0.40 ± 0.0^{c}	0.92 ± 0.0^{a}	0.51 ± 0.0^{b}	***

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ significant from the control rats; ns – non-significant. The values were reported as the mean ± standard error (SEM), and a p-value of less than 0.05 was considered statistically significant.

Significant variations in means within the same column are represented by distinct letters, a, b, and c. Abbreviations: C, control rats; PGE, *Punica granatum L.* extract rats; DM, diabetes rats; DM + PGE, *Punica granatum L.* extract-treated rat.

Table 3 Effect of PGE on kidney tissue MDA, SOD, CAT, GSH, and GPx levels/activities on STZ-induced diabetes

Parameter	C	PGE	DM	DM + PGE	*p*
MDA (nmol/g)	64.13 ± 3.55^{b}	61.95 ± 2.43^{b}	92.68 ± 1.47^{a}	63.45 ± 4.61^{b}	***
SOD (EU/mg)	18.35 ± 0.44^{a}	19.15 ± 0.25^{a}	15.16 ± 0.22^{c}	16.81 ± 0.31^{b}	***
CAT (kU/g)	161.14 ± 3.85^{a}	170.73 ± 5.03^{a}	74.28 ± 2.82^{b}	159.52 ± 3.02^{a}	***
GSH (nmol/g)	1.35 ± 0.06^{ab}	1.49 ± 0.02^{a}	1.17 ± 0.09^{b}	1.31 ± 0.03^{ab}	**
GPx (U/mg)	3.51 ± 0.12^{ab}	3.69 ± 0.09^{a}	2.93 ± 0.25^{b}	3.04 ± 0.07^{b}	**

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ significant from the control rats; ns – non-significant. The values were reported as the mean ± standard error (SEM), and a p-value of less than 0.05 was considered statistically significant.

Significant variations in means within the same column are represented by distinct letters, a, b, and c. Abbreviations: C, control rats; PGE, *Punica granatum L.* extract rats; DM, diabetes rats; DM + PGE, *Punica granatum L.* extract-treated rat.

Table 4 Histopathological statistical evaluation of the effect of PGE on kidney tissue against STZ-DM

Parameter	C	PGE	DM	DM + PGE
Interstitial nephritis	0.00 ± 0	0.00 ± 0	2.00 ± 0	0.00 ± 0
Degeneration of tubular epithelium	0.00 ± 0	0.00 ± 0	3.00 ± 0	1.00 ± 0
Necrosis of tubular epithelium	0.00 ± 0	0.00 ± 0	2.00 ± 0	0.00 ± 0
Dilatation of Bowman's capsule	0.00 ± 0	0.00 ± 0	2.00 ± 0	0.00 ± 0

Abbreviations: C, control rats; PGE, *Punica granatum L.* extract rats; DM, diabetes rats; DM + PGE, *Punica granatum L.* extract-treated rat

antioxidant enzymes (SOD, CAT, GPx) and reduced glutathione (Table 3).

Evaluation of western blot analysis

Western blot findings are designed by bands and groups in Figs. 1 and 2. When the expression levels of Bax and Caspase-3 proteins were examined, it was determined that the highest level was in the DM group. A decrease was observed in the PGE treatment group (DM + PGE) compared to the DM group. Bcl-2 level was found to be lowest in the DM group. It was determined that PGE treatment groups increased Bcl-2 levels compared to the damage group (DM). According to this result, it was observed that PGE significantly reduced the damage in diabetes-related kidney damage by activating the Bcl-2 pathway (Fig. 1). In the experimental groups, the highest NF-κB protein expression was produced in the DM group. It was determined that PGE decreased NF-κB protein expression in the treatment group (DM + PGE). Nrf-2 protein expression level was found to be highest in PGE and lowest in the DM group. It was determined that the PGE dose administered together with DM increased Nrf-2 protein expression.

According to our findings in HO-1 protein expression, it was determined that there was a decrease in the DM group compared to the control group, and HO-1 protein increased in DM + PGE compared to the damage group (Fig. 2).

Histopathological evaluation

The glomerulus, cortex, and medullary tubules of C and PGE groups rats' kidney tissues were normal histologically (Fig. 3). Examining the kidney tissues of rats with diabetes-induced interstitial nephritis revealed mild tubular dilatation, hydropic degeneration of tubular epithelium, coagulation necrosis of tubular epithelium, hyperemia of intertubular arterioles, dilatation of glomerulus, Bowman's capsule, and hyperemia of glomerular capillaries. In the kidney tissues of the rats in the diabetic rat group treated with PGE, the glomerulus was normal, but hydropic degeneration was detected in the tubular epithelium of some tubules (Table 4). However, no necrotic cells were found in the tubular epithelium (Fig. 3).

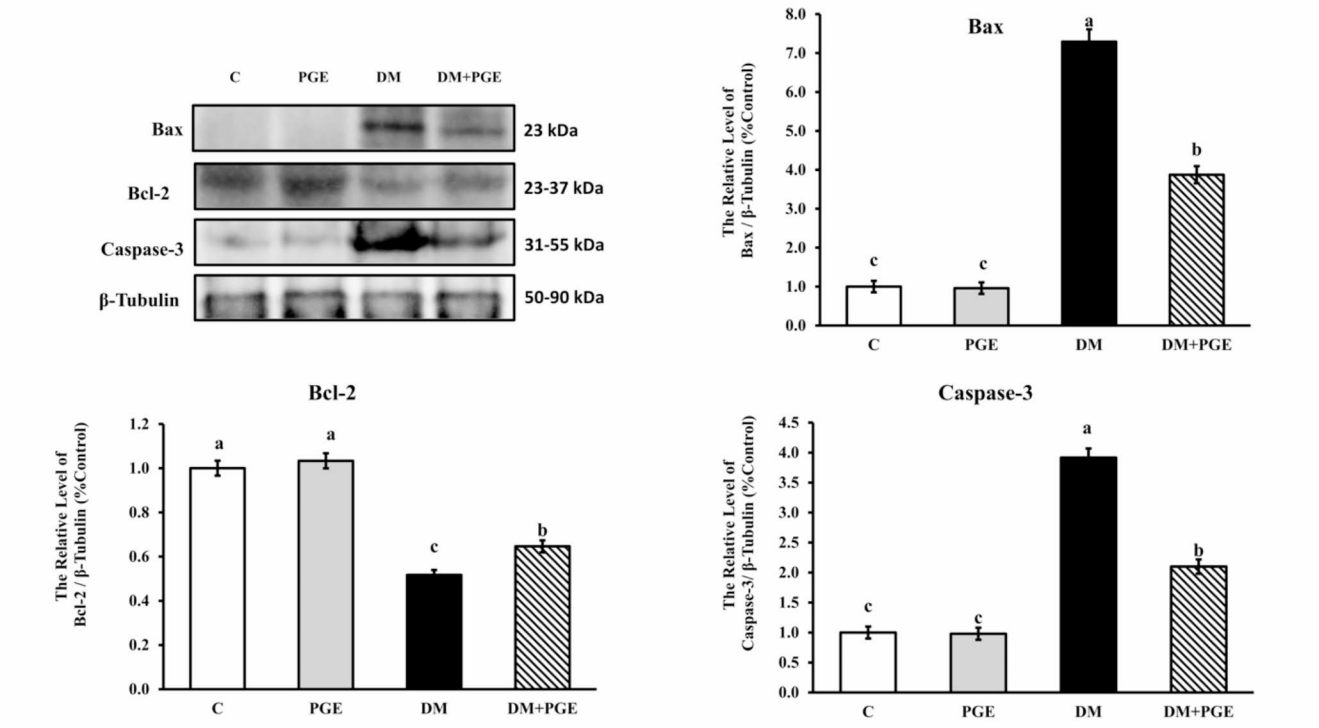

Fig. 1 Effects of PGE on STZ-induced diabetes proteins levels of Bax, Bcl-2, and Caspase-3 genes in the kidney. The bar graph is shown as mean ± standard error (SEM) ($n = 7$) (One-way ANOVA and Tukey's test) ($p < 0.001$). Abbreviations: C, control rats; PGE, *Punica granatum* L. extract rats; DM, diabetes rats; DM + PGE, Punica granatum L. extract-treated rat. Significant variations in means within the same column are represented by distinct letters, a, b, and c

Immunohistochemistry evaluation

It was noted that the expression of 8-hydroxy-2-deoxyguanosine (8-OHdG) was low in the renal tissues of rats in both the control group and the group treated with pomegranate (Fig. 3). Severe cytoplasmic positive cells were found in both tubular epithelium and glomerular endothelium in kidney tissues of DM group rats. Mild cytoplasmic positive cells were detected in the renal tubular epithelium in the tissues of the group treated with pomegranate against diabetes. When the DM and DM + PGE groups were compared in terms of 8-OHdG positive cell numbers, there was a statistically significant difference ($p < 0.05$).

In addition, tissues stained with insulin were examined immunohistochemically to determine the level of insulin secretion in the pancreas. Although severe insulin expression was observed in the islets of langerhans of the pancreatic tissues of the rats in the control and pomegranate groups, very little insulin expression was detected in the islets of langerhans of the pancreatic tissues of the rats in the diabetes group (Fig. 3). Moderately positive cells were determined in the islets of langerhans of the pancreatic tissues of DM + PGE group rats (Fig. 3). When the DM and DM + PGE groups were compared in terms of 8-OHdG positive cell numbers, there was a statistically significant difference ($p < 0.05$) (Table 5).

Discussion

Diabetes mellitus is a global health concern associated with various complications, including kidney damage. Diabetic nephropathy, or kidney damage, is a common and severe complication of diabetes mellitus. It is characterized by the progressive deterioration of renal function, resulting in increased proteinuria, glomerular hypertrophy, and eventually, end-stage renal disease [29, 30]. Traditional pharmacological interventions have limitations, prompting researchers to explore alternative treatments such as plant-based extracts. *Punica granatum* L., commonly known as pomegranate, has gained attention due to its diverse pharmacological properties. The peel of *Punica granatum* L. contains bioactive compounds, including polyphenols, flavonoids, and ellagitannins, which have demonstrated potential therapeutic effects in various diseases, including diabetes mellitus and its associated complications [7].

Urea, uric acid, and creatine concentrations are the most essential markers for assessing renal function. Increased urea and creatinine levels following exposure to a toxic

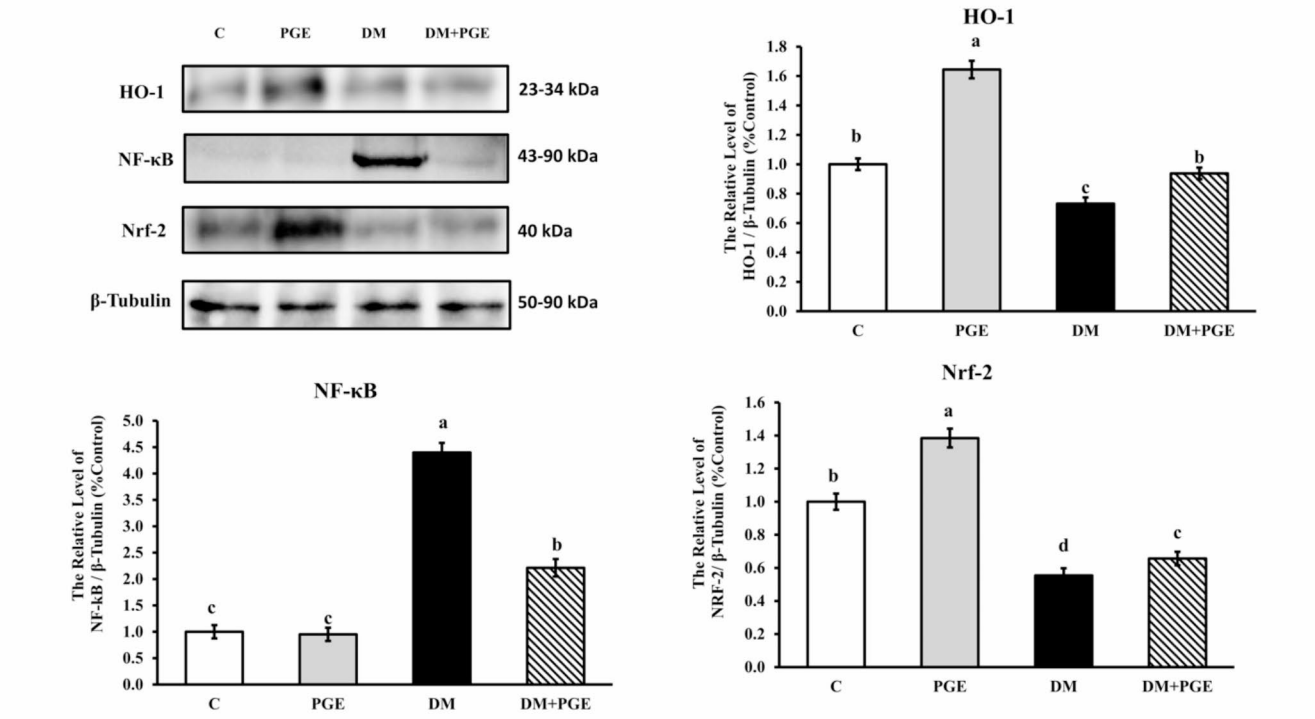

Fig. 2 Effects of PGE on STZ-induced diabetes protein levels of HO-1, NF-κB, and Nrf-2 genes in the kidney. The bar graph is shown as mean ± standard error (SEM) ($n = 7$) (One-way ANOVA and Tukey's test) ($p < 0.001$). Abbreviations: C, control rats; PGE, *Punica granatum L.* extract rats; DM, diabetes rats; DM + PGE, Punica granatum L. extract-treated rat. Significant variations in means within the same column are represented by distinct letters, a, b, c, and d

substance result in kidney injury due to protein catabolism [31, 32]. Various STZ dosages produce renal tubular necrosis and raise blood urea and creatin levels in laboratory animals [6, 33]. This study demonstrated that STZ affects kidney function and increases blood urea, uric acid and creatine levels. It was discovered, however, that pomegranate peel ethanole extract therapy reduced urea, uric acid and creatine levels and reverted renal function. In previous studies using various toxins, treatments using different parts of the pomegranate have been shown to significantly improve kidney function [34, 35]. The positive outcomes in kidney function tests, specifically improvements in urea, uric acid and creatine levels, suggest that *Punica granatum* L. peel ethanol extract can ameliorate renal dysfunction associated with diabetic nephropathy. The findings of this study indicate the potential of the extract as a therapeutic agent to preserve kidney function and prevent progression of kidney damage.

Diabetes is affected by oxidative stress, according to research. Diabetes is a metabolic disorder resulting from elevated blood glucose levels. High blood glucose can increase the production of free radicals and diminish the antioxidant defense. By triggering apoptosis, inflammation, and autophagy, oxidative stress causes tissue injury. It can also reduce insulin production by impairing the function of beta cells in the pancreas [9, 36]. GSH is an essential component in both the removal of harmful free radicals and the upkeep of the antioxidant status of the cell. Superoxide dismutase transforms superoxide into hydrogen peroxide, a less reactive form of oxygen that belongs to the reactive oxygen species category. Subsequently, catalase reduces hydrogen peroxide to water. Thus, catalase helps SOD to completely neutralize ROS [36, 37]. The amount of MDA, which serves as a marker for lipid peroxidation, exhibits an increase with increased oxidative damage. Associated with oxidative stress is a decrease in the concentrations of antioxidants such as SOD, CAT, GSH and GPx, which actively contribute to the body's defense mechanisms [36, 38]. In studies where diabetes was triggered by STZ, it was found that MDA levels in kidney tissue increased and antioxidant activities decreased [39, 40]. In a study using pomegranate leaf extract used against STZ-induced diabetes, it was observed that it increased the concentrations of reduced glutathione, SOD, CAT and decreased the MDA level in the kidney [6]. In the study of Mohan et al., pomegranate juice was administered orally to rats for 4 weeks against STZ-induced diabetes, and it increased GSH, SOD, CAT levels/activities and decreased MDA levels in the kidney tissues [35]. In another research using pomegranate seed oil against STZ-diabetes, it was stated that the MDA level decreased

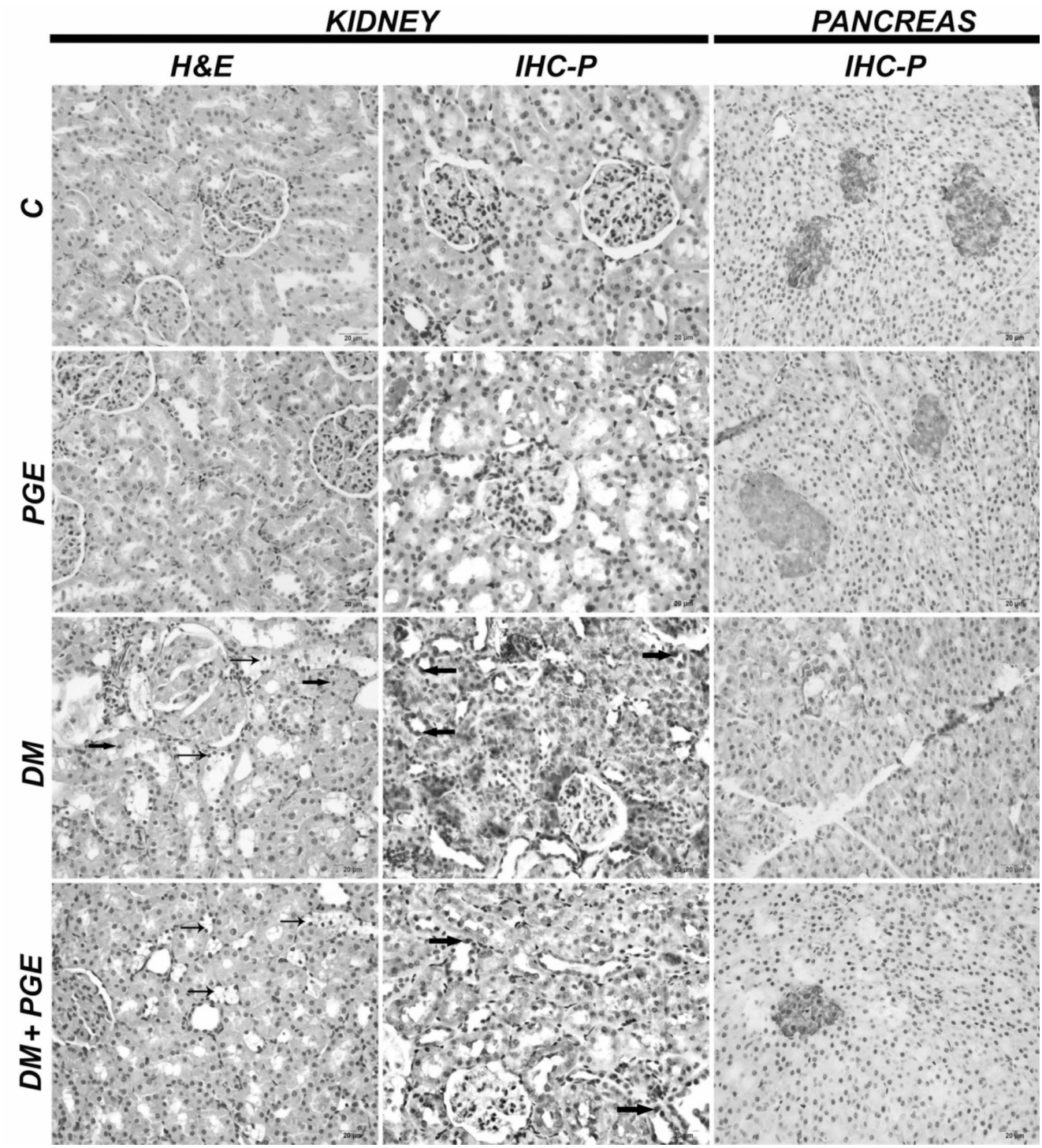

Fig. 3 Representative photomicrographs of H&E-20 µm stained kidney sections of rats in response to PGE treatment versus STZ application are shown. Histological images of kidney tissues in C and PGE rats have normal histological structure. Advanced degeneration (thin arrow) and moderate necrosis (thick arrow) were observed in the DM group. In the DM + PGE group, mild degeneration (thin arrow) was observed. H&E staining, Bar:20 µm. 8-OHdG (IHC-P, DAB chromogen Bar: 20 µm) was stained immunohistochemically to determine. C and PGE groups kidney tissues are 8-OHdG negative. 8-OHdG is positive in severe levels of glomerular endothelium and tubule epithelium in the DM group kidney tissues. DM + PGE group kidney tissues are 8-OHdG positive in very few tubules epithelium. Insulin (IHC-P, AEC chromogen Bar: 20 µm) was stained immunohistochemically to determine. Section of the pancreas of the C and PGE group showed strong immunoreactivity of insulin in beta cells, which occupy most of the islet. A significant decrease in the immunohistochemical expression of insulin was observed in the beta cells of the pancreas in the DM group. Insulin immunoreactivity in pancreatic beta cells of the DM + PGE group also increased significantly compared to the DM group

Table 5 Immunohistochemical evaluation of 8-OHdG positive stained in kidney and insulin positive stained cells in pancreatic tissues

Parameter	C	PGE	DM	DM + PGE
8-OHdG (Kidney)	0.25 ± 0.16	0.25 ± 0.17	0.3 ± 00	0.75 ± 0.16
Insulin (Pancreas)	3.00 ± 0.0	3.00 ± 0.0	0.75 ± 0.16	2.00 ± 0.19

Abbreviations: C, control rats; PGE, Punica granatum L. extract rats; DM, diabetes rats; DM + PGE, Punica granatum L. extract-treated rat

significantly [41]. In parallel with the literature data, while the pomegranate peel ethanol extract, which we applied orally against STZ-diabetes, decreased the MDA level in the kidney tissue, it significantly increased the activity/levels of SOD, CAT, GSH, GPx parameters.

Inflammation is a body response to detrimental stimuli, such as an infection, injury, or damaged tissue. Inflammation is typically a brief process that initiates the healing process and is required to limit injury [42]. The transcription factor NF-kappa B (NF-κB) plays a key function in controlling immune and inflammatory responses [43]. The NF-κB pathway is one of the main inflammatory pathways that plays a central role in the pathogenesis of diabetes. It is usually found within cells in an inactive state. The NF-κB pathway is one of the primary inflammatory pathways that is involved in the development of diabetes and plays an important role in this process. In most cases, it can be discovered inactive within the cells of an organism. upon activation, NF-κB translocates into the nucleus and increases transcriptional activity by stimulating the expression of inflammatory adhesion molecules, chemokines, and inflammatory cytokines, all of which are involved in the induction and progression of DM [44, 45]. In this experimental study, it was determined that the NF-κB activation of the DM group showed a significant increase compared to previous studies, and protein expression decreased with the applied PGE treatment. The result proved that PGE could act by inhibiting the NF-κB pathway to reduce inflammatory responses.

Apoptosis is a normal physiological process, allowing cells to program themselves if they are unnecessary or damaged, triggering their death. Regulation of apoptosis is a complex process and is controlled by a number of signaling pathways [46]. Characteristically, caspases involve cross-interaction between pro-apoptotic members of the Bcl-2 family (e.g. Bax and Bad) and death amplification factors such as cytochrome c released from mitochondria and apoptosis-inducing factor. The apoptotic pathway has a role in the development of several diseases, such as diabetic nephropathy [47, 48]. Bax and Bcl-2 are essential mediators in the regulation of endogenous apoptosis; Bax promotes its activation and Bcl-2 exerts inhibitory effects. In addition, hyperglycemia-associated ROS induce renal cell apoptosis by decreasing Bcl-2 and increasing Bax and Caspase-3 expression [49–51]. The data from our investigation demonstrated that STZ induced oxidative stress, inhibited antioxidant activity, reduced renal Bcl-2 protein expression, and elevated Bax and Caspase-3 activity/levels. The treatment group (DM + PGE) increased Bcl-2 protein expression in kidneys and decreased Bax and Caspase-3 protein expression, demonstrating that PGE is anti-apoptotic.

Various experimental research have confirmed the antioxidant and anti-inflammatory benefits of pomegranate, primarily attributed to its polyphenolic components [52, 53]. Nrf-2/HO-1 has been shown to play an crucial role in defense against oxidative damage and inflammation in diabetic nephropathy [54]. They may also exert their antioxidant potential through activation of the transcription nuclear factor erythroid 2-related factor-2 (Nrf-2) [55]. The anti-inflammatory effects of polyphenols can be linked to many processes, such as the inhibition of nuclear factor kappa light chain-enhancing activator B cells (NF- κB), the mitigation of oxidative stress, and the suppression of proinflammatory cytokines [56, 57]. Experimental findings showed that Nrf-2 and HO-1 protein expression levels were increased and activation of Nrf-2 in the study could lead to heme oxygenase induction. Recently, it has been reported that apoptosis parameters such as Bax, Bcl-2, Caspase-3 affect pathways such as NF-κB and Nrf-2/HO-1 [58, 59]. In the light of the findings, it was determined that DM causes oxidative stress in kidney damage, suppresses antioxidant activity, causes apoptosis, and suppresses renal Nrf-2 and HO-1 expression. It was found that when STZ was given together with PGE treatment, it increased renal Nrf-2 and HO-1 protein levels in the group and showed antioxidant properties.

In histopathological evaluation, severe necrotic-degenerative epithelium, hyperemia and mild interstitial nephritis in the glomerular capillaries were observed in the tubules of the diabetes group, while in the treatment group, the glomeruli had a normal structure and some tubular epithelia tended to heal with mild hydropic degeneration. In our study, as in other diabetes studies, it was concluded that PGE could provide histopathological improvement in parallel with biochemical parameters [6, 34].

Conclusion

In conclusion, our data suggest that PGE effectively mitigates oxidative stress, scavenges free radicals, supports treatment, and strengthens endogenous antioxidant defense mechanisms against STZ-induced diabetic nephropathy. Due to its potent antioxidant, anti-inflammatory, and

anti-apoptotic properties, PGE regulates increased lipid peroxidation and renal function biomarkers, maintaining kidney tissue integrity. Additionally, the downregulation of inflammatory and fibrotic markers indicate that the ethanol extract of Punica granatum L. peel may influence the NF-κB and Nrf2/HO-1 signaling pathways, potentially preventing renal damage and fibrosis in diabetic nephropathy. These findings offer new insights into the mechanisms by which PGE might be beneficial for the prevention and treatment of diabetic nephropathy. Nevertheless, further research is needed to fully elucidate the underlying mechanisms.

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Author contributions BAY, FY and SK designed the research. BAY, EAS, SY and TD conducted experiments and analyzed data. EAS wrote the manuscript. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability On request, information will be made available.

Declarations

Ethical approval Animal trials were conducted at the Atatürk University Medical Experimental Research Center. The Animal Experiments Local Ethics Committee at Atatürk University granted consent for the experiment (Protocol No: 2014-45-203).

Competing interests Authors declare that they have no conflicts to declare.

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