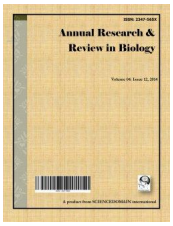

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The Protective Effect of Pomegranate (*Punica granatum*) against Oxidative Stress and Nephropathy Induced by Diabetes in Male Rats: A Biochemical, Molecular and Histopathological Study

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Authors' contributions

This work was carried out in collaboration between all authors. Author GMGS designed the study, performed the biochemical and statistical analysis, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Author AMA participated in the biochemical and statistical analysis of the manuscript, participated in the literature searches and proof read the manuscript. Author AAY performed the histopathological analysis, participated in the literature searches and proof read the manuscript. Author MAMEA performed the molecular analysis, participated in the literature searches, wrote the molecular part and proof read the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Diabetes mellitus is a significant health problem worldwide and type II diabetes is one of the main health problems facing Saudi society, which is often caused by obesity and increased cholesterol in the blood. Due to the seriousness of diabetes and its complications, the present study

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was designed to examine the renoprotective effect of pomegranate in diabetes induced oxidative stress and kidney injury.

Study Design: Adult male Albino rats (200–250 g) were used in this study. Diabetes was induced by streptozotocin (45 mg/kg) followed by treatment for 8 weeks. The animals were randomly divided into four groups (each group, n = 10): normal control (NC); non-diabetic animals fed on commercial diet, diabetic group (DG); diabetic animals fed on commercial diet, pomegranate treated group (PTG); diabetic animals fed on experimental diet contains 20% dried pomegranate, and drug treated group (MTG); diabetic animals fed on commercial diet and treated with metformin (500 mg/kg).

Methodology: At the end of the experimental study (8 weeks) blood glucose levels, lipid peroxidation, biochemical analysis of oxidative stress parameters and biomarkers of kidney damage were determined. The mRNA expression level of oxidative stress defense genes (SOD, CAT ,GR and GPx), as well as the NADPH oxidase (subunits p22phox and p47phox) and the inflammatory factors regulator gene, NF-κB were also evaluated in kidney homogenates using semi-quantitative RT-PCR analysis. Furthermore, histopathological evaluation of kidney was also studied.

Results: Treatment with pomegranate significantly ameliorated the elevated oxidative stress levels in STZ induced diabetic rats resulting in decreased lipid peroxidation and NO concentration, and increased endogenous antioxidant enzymes levels (SOD and GSH). Biomarkers of kidney damage (urea and creatinine) and blood glucose levels were significantly normalized in pomegranate treated group compared to the diabetic group. At the molecular level, a significant enhancement of gene expression of the antioxidant enzyme (SOD, CAT, GR and GPx) was observed in pomegranate treated group compared to the diabetic group. In contrast, significant down-regulation of the NADPH oxidase subunits (p22phox and p47phox) as well as the inflammatory factors regulator gene, NF-κB was recorded. Moreover, the histopathological examinations confirmed the protective effects of pomegranate by normalizing the kidney damage.

Conclusion: This study validates pomegranate as a promising candidate in preventing diabetes associated complications such as nephropathy through its antioxidant activity and its effects on the activity and regulation of oxidative stress defense gene expression.

Keywords: Diabetes; gene expression; lipid peroxidation; nephropathy; oxidative stress; pomegranate.

ABBREVIATIONS

CAT : Catalase,
GSH : Reduced glutathione,
GPx *: Glutathione peroxidase,*
GR : Glutathione reductase,
NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B cells,
NO : Nitric oxide,
ROS : Reactive oxygen species,
SOD : Superoxide dismutase,

1. INTRODUCTION

Diabetes Mellitus is a metabolic as well as vascular disease which causes important complications. The prevalence of diabetes among adults has been increased significantly worldwide. Currently, more than 425 million people are suffering from diabetes worldwide and it is expected to reach 629 million by 2045, with type 2 diabetes being the most expressing form of the disease [1,2]. In diabetes, since the entry of glucose in the tissues is not regulated by insulin, as a result of impaired insulin secretion, impaired insulin action, or both, significant elevation of blood glucose occurs. Chronic exposure to high blood glucose is a leading cause of renal failure, visual loss and a range of other types of tissue damage. Diabetes induced renal damage is one of the severe microvascular complications occurring due to increased flux of glucose into kidney via insulin-independent mechanisms [3]. This is the primary cause of end stage renal failure caused by glomerular injury and neuropathy [4,5] affects more than one third of type I diabetics and an ever increasing amount of type II diabetic patients [6]. Related changes in kidney function, have been reported in streptozotocin induced diabetic rats such as increased levels of blood urea nitrogen and distinct proteinuria [7] as well as histopathological changes such as increase in the glomerular mesangial matrix [8].

Recently, generation of the free radicals and connotation between oxidative stress and diabetes complications has extended importance in the field of diabetes. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily

detoxify the reactive intermediates or to repair the resulting damage [9]. Oxidative stress together with inflammation is the major causative factor leading to diabetic complications. An increase in the blood glucose levels together with increased lipid content elicits excessive formation of reactive oxygen species (ROS) and inflammatory cytokines. This alongside depletion of antioxidant defense capacity elevates oxidative stress. The increased ROS leads to epigenetic changes and endothelial dysfunction which sequentially stimulates more ROS production, leading to lipid peroxidation which cause cell membrane instability finally leading to necrosis and damage to the affected tissues or organs [10,11].

In recent years, many researches gave considerable attention to naturally occurring anti-inflammatory and antioxidant compounds to prevent diabetes complications. Most of the naturally occurring anti-inflammatory and antioxidants compounds have shown positive effects in counteracting diabetes complications. Many natural compounds such as salicylate, curcumin and ursolic acid have been proved to alleviate the development of diabetic complications without indicating any effect on hyperglycemia [12-14].

Pomegranate (*Punica granatum*), belongs to the *Punicaceae* family [15], is a traditional small tree in Taif region. Its fruit is a rich source of polyphenolic compounds such as hydrolyzable tannins and anthocyanins, which account for 92% of the antioxidant activity of the whole fruit [16]. As well as, punicalagin, punicalin, gallic acid and ellagic acid as demonstrated by Seeram *et al.*, [17]. It has been revealed that pomegranate has the capability to prevent prostate cancer, inflammation, reductions of blood pressure, anemia, and arthritis [17-19]. Pomegranate also has been proved to serve as an anti-allergic and anti-diabetic [20-23]. Therefore, current study included biochemical, molecular and histopathological examinations in order to validate the protective effects of pomegranate supplementation on kidney damage as a result of diabetes induced oxidative stress and kidney injury.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals and reagents used in this study were of analytical grade. All the chemicals and kits were obtained from local or international companies according to the availability.

2.2 Commercial Diet

A commercial diet was used as basal diet. The commercial diet consists mainly of not more than 64% carbohydrates, not less than 17% protein, not less than 2.67 % fat, not less than 10.33% fiber, and not less than 6% of vitamins and minerals mixture [24].

2.2.1 Preparation of pomegranate and the experimental diet

Fresh fruit of pomegranate were purchased from a local supermarket and were gently washed in cold water just before drying to remove dirt, bacteria, and insects. Pomegranate was peeled, and its edible portion (seed coat) was dried in an electric dehydrator. The dried seed coats were homogenized fine powder and added to the commercial diet (20% W/W) to produce the experimental diet as described by Kamel and his colleagues [25].

2.3 Animals

Adult male albino rats (200–250g) were used in the current study. They were housed in the Institutional animal house. They were fed *ad libitum* and were maintained for acclimatization for seven days before the initiation of the study. The animals were maintained on a 12 h light/dark cycle with temperature 25 ± 2^{o}C and relative humidity 50–70%. The principles of laboratory animal care were followed. The experimental protocol have been examined and approved by the college Ethical Committee before the start of the experiment.

2.3.1 Induction of diabetes

Diabetes was induced in rats with a single intraperitoneal (ip) injection of streptozotocin (45 mg/kg) in citrate buffer (pH 4.6). Five days after induction, fasting blood glucose were estimated. Animals with blood glucose level higher than 250 mg/dl were termed diabetic and were included in the study.

2.3.2 Experimental design and procedures

The animals were randomly divided into four groups (each group, n = 10).

Group 1: normal control group (NC); non-diabetic animals fed on commercial diet.

Group 2: diabetic group (DG); diabetic animals fed on commercial diet.
Group 3: pomegranate treated group (PTG); diabetic animals fed on experimental diet contains 20% dried pomegranate.
Group 4: metformin treated group (MTG); diabetic animals fed on commercial diet and treated with metformin (500 mg/kg) [26].

2.3.3 Samples

At the end of the experimental study (8 weeks). Animals were anaesthetized and blood was withdrawn. Serum and plasma were separated and stored at -20°C till further biochemical analysis. Rats were perfused with normal saline solution and both kidneys were excised. Kidneys were rinsed in ice cold isotonic solution, blot dried and cut into three parts. One part was homogenized in could phosphate buffer (pH 7.4) for biochemical estimations, the other part was kept in TRI reagent for RNA isolation for studying the gene expression and the third part was fixed in 10% formalin solution for histopathological sectioning and examination.

2.3.3.1 Kidney antioxidant parameters

The supernatants of kidney homogenates were used for the assessment of endogenous antioxidant parameters (Superoxide dismutase (SOD), Reduced glutathione (GSH), malondialdehyde (MDA)) and nitric oxide using commercially available enzymatic kits (Bio-diagnostic company, 29 Tahreer St., Dokki, Giza, Egypt. www.bio-diagnostic.com) according to the references listed in the instruction manual [27-30].

2.3.3.2 Biochemical assessment of kidney function, glucose, Nitric oxide and antioxidant parameters in blood

Kidney function tests [31,32], glucose, nitric oxide [30] and antioxidant parameters (Superoxide dismutase (SOD), Reduced glutathione (GSH), malondialdehyde (MDA)) [27-29] were measured by commercially available enzymatic kits according to the instruction manual (Bio-diagnostic company, 29 Tahreer St., Dokki, Giza, Egypt. www.bio-diagnostic.com).

2.3.3.3 Semi quantitative gene expression analysis

Total RNA was extracted from tissue samples of kidney using TRIZOL® reagent (Invitrogen), according to the manufacturer's instructions and as previously described [33]. A total RNA of 3µg was denaturized first by incubation at 65˚C for 10 min after adding 1.0ng Oligo dT primer and used for cDNA synthesis with a reverse transcription system using reverse transcriptase (Promega, Madison, WI, USA) [34]. The specific primers for semi quantitative gene expression analysis of antioxidant genes and NF-κB gene Table 1. were designed based on the published sequence [34,35] and synthesized by (Macrogen genes Co., Seoul, South Korea). Semi quantitative polymerase chain reactions (PCR) were conducted in a Bio Rad T100™ Thermal Cycle machine in a total volume of 25 µl consisting of 12.5µl Promega master mix (2x), 2µl of each of the forward and reverse primers (1pM), 1µl of template cDNA and 7.5µl RNAs free water. The following PCR cycling conditions were used: 95˚C for 5 min for initial denaturation, followed by 29 cycles of denaturation at 95˚C for 60 sec,

Table 1. PCR conditions for examined genes in the kidney

Name	Sense	Anti-sense	Annealing Temperature	Size (bp)
Cat	ACGAGATGGCACACTTTGACAG	TGGGTTTCTCTTCTGGCTATGG	55°C	341
GPx	AAGGTGCTGCTCATTGAG AATG	CGTCTGGACCTACCAGGAACTT	57°C	406
SOD	AGGATTAACTGAAGGCGAGCAT	TCTACAGTTAGCAGGCCAGCAG	55°C	410
GR	CCATGTGGTTACTGCACTTC	CTGAAGCATCTCATCGCAG	58°C	545
P22phox	GGACGCTTCACGCAGTGGTA	GGACAGCAGTAAGTGGAGGACA	59°C	209
P47phox	ATGGGACTGCCCGTGAAGAT	GGATGATGGGACCCGTGATG	60°C	189
NF-κB	ACTGCCGGGATGGCTTCTAT	CTGGATGCGCTGGCTAATGG	61°C	238
GAPDH	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA	52°C	309

Cat,catalase; GPx, Glutathione peroxidase; SOD, superoxide dismutase; GR, glutathione reductase; P22phoxand P47phox, NADPH oxidase subunits ; NF-κB, nuclear factor-κB; GAPDH, glyceradehyde-3-phosphate dehydrogenase.

annealing as presented in Table 1. for 60 sec and extension at 72˚C for 60 sec, with an additional final extension at 72˚C for 10 min. Expression of Glyceraldehyde 3 phosphate dehydrogenase was served as a reference and as an internal standard. The PCR products were electrophoresed in 2% agarose gel at 100 V for 30 min and stained with ethidium bromide in Tris Borate EDTA buffer. The PCR products were visualized using the InGenius 3.0 gel documentation system (Syngene, Frederick, MD, USA) and under ultraviolet light. The densitometric analysis for PCR bands was performed using ImageJ software version 1.47 (http://imagej.en.softonic.com/).

2.3.3.4 Histopathological examinations

The fixed kidney samples in 10% formalin were dehydrated through graded alcohol series and were embedded in paraffin. The paraffin blocks were subsequently cut into 4 mm sections and stained with hematoxylin & eosin (H&E). The slides were examined under light microscopy at 200 magnifications.

3. RESULTS

3.1 Biochemical results

Table 2 shows the effect of supplementation with 20% pomegranate (PTG) on blood content of malondaldehyde (MDA), nitric oxide (NO), and reduced glutathione (GSH) and the activities of superoxide dismutase (SOD) compared to the control (NC), diabetic (DG) and metformin treated groups (MTG). The results indicated that malondaldehyde significantly increased in the diabetic group (mean±SD=10.37±1.09) compared to the normal control group (mean±SD=1.95±0.52) (the percent increase was 431.8 %). However, the treatment with metformin drug or the supplementation with (20%) pomegranate reduced the percent changes to 96.41% and 114.87%, respectively. The percentage reductions observed in diabetic rats treated with drug or fed on pomegranate compared to diabetic group were -63.07% and -60.95%, respectively. There was no significant deference between the drug and pomegranate treatments.

A dramatic and significant increase in nitric oxide (% change = 1423.9) was observed in the diabetic group compared to the normal control group. Nevertheless, the treatment with metformin or pomegranate reduced the percent changes to 432.58% and 594.26%, respectively. The percentage reductions observed in rats treated with drug or fed on pomegranate supplemented diet compared to diabetic group were -186.10% and -119.51%, respectively (Table 2).

In contrast, a significant reduction in the reduced glutathione (GSH) contents was observed in the diabetic group (Table 2). The percent decrease was -79.43% compared to the normal control group. The decrease in GSH was -9.67% and -40% in drug and pomegranate treated groups, respectively. The results represented that the treatment with metformin or pomegranate ameliorate the diabetic effect and increased the level of GSH compared to the diabetic group (339.19% and 188.27% increase, respectively).

As shown in Table 2 a significant reduction in superoxide dismutase (SOD) activity was observed in the diabetic group compared to the control group (mean±SD were 6.24±4.2 and 16.22±2.46, respectively). The percent decrease was -61.5% compared to the normal control. The decrease in SOD activity in drug and pomegranate treated groups was -5.73% and -17.08%, respectively. The results represented that the treatment with metformin or pomegranate ameliorate the diabetic effect and increased the level of SOD activity compared to the diabetic group (145.03% and 115.54% increase, respectively).

Similar results for the previous parameters were observed in the kidney tissue homogenate (Table 3). An increase of MDA and NO content in diabetic group (mean±SD were 10.62±0.55 and 92.50±24.14, respectively) compared to the control group (mean±SD were 1.77±0.47 and 25.67±15.18, respectively). The percent increase were 500% and 260.3%, respectively. This increase was ameliorated with metformin and pomegranate treatments. The percent changes with metformin and pomegranate supplementation compared to diabetic control were -76.74% and -72.69% for MDA and were -71.57% and -28.14% for NO, respectively.

On the other hand, a decrease in GSH contents and SOD activity was observed in diabetic group (mean±SD were 27.34±13.3 and 3.04±1.52, respectively) compared to control group (mean±SD were 79.57±38.31 and 6.5±3.00, respectively). The percent decrease were

Table 2. The effect of pomegranate supplementation on malondaldehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) and superoxide dismutase (SOD) activities in blood

Groups		MDA nmol/ml	NO µmol/L	GSH mg/dL	SOD U/ml
Control (NC)	Mean±SD	1.95±0.52^{c}	4.88±1.88^{d}	73.81±14.58^{a}	16.22±2.46^{a}
Diabetic (DG)	Mean±SD	10.37±1.09^{a}	74.37±15.15^{a}	15.18 ±3.39^{c}	6.24±4.2^{cb}
	%Change	431.8	1423.9	-79.43	-61.5
Metformin (MTG)	Mean±SD	3.83±0.86^{b}	25.99±3.94^{cb}	66.67±8.76^{a}	15.29±3.18^{a}
	%Change	96.41	432.58	-9.67	-5.73
	%Change*	-63.07	-186.1	339.19	145.03
Pomegranate (PTG)	Mean±SD	4.19±2.41^{b}	33.88±17.67^{b}	43.76±30.01^{b}	13.45±1.17^{b}
	%Change	114.87	594.26	-40.71	-17.08
	%Change*	-60.95	-119.51	188.27	115.54

Values represent the mean ± standard deviation (n = 10) in each group. Values that are followed by different letters within each column are significantly different (p < 0.05).
% change: comparison with control; % change: comparison with diabetic group*

Table 3. The effect of pomegranate supplementation on malondaldehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) and superoxide dismutase (SOD) activities in kidney tissues

Groups		MDA (nmol/g tissue)	NO (µmol/g tissue)	GSH (mg/g tissue)	SOD (U/mg tissue)
Control (NC)	Mean±SD	1.77±0.47^{b}	25.67±15.18^{b}	79.57±38.31 ^{a}	6.5±3.00^{a}
Diabetic (DG)	Mean±SD	10.62±0.55^{a}	92.50±24.14^{a}	27.34±13.3 ^{b}	3.04±1.52^{b}
	%Change	500.0	260.3	-65.64	-53.32
Metformin (MTG)	Mean±SD	2.47±2.08^{b}	26.30±14.77^{b}	63.56±41.18^{a}	4.1±0.46^{a}
	%Change	39.54	2.45	-20.12	-36.92
	%Change*	-76.74	-71.57	132.48	34.87
Pomegranate (PTG)	Mean±SD	2.90±0.52^{b}	66.47±39.8^{b}	67.83±35.04^{a}	5.43±2.67^{a}
	%Change	63.84	158.9	-14.75	-16.46
	%Change*	-72.69	-28.14	148.1	78.62

Values represent the mean ± standard deviation (n = 10) in each group. Values that are followed by different letters within each column are significantly different (p < 0.05).
% change: comparison with control; % change: comparison with diabetic group*

65.64% and 53.32%, respectively. The treatment with metformin and pomegranate supplementation significantly recovered the GSH contents and SOD activity. The percent increase in the contents and activity with metformin and pomegranate compared to diabetic control were 132.48% and 148.1% for GSH, and 34.87% and 78.62% for SOD, respectively (Table 3).

Table 4 shows the effect of pomegranate on the serum concentration of creatinine and urea compared to the control, diabetic and metformin treated groups. The results indicated that both creatinine and urea were significantly increased in the diabetic group (mean±SD=1.80±0.29 and 78.53±11.61, respectively) compared to the normal control group (mean±SD=0.68±1.48 and 17.94±4.85, respectively). The percent increase was 164.71 % and 337.74%, respectively. Both metformin and pomegranate treatments ameliorated the diabetic effect and reduce the increase of creatinine and urea concentration. The percent changes with metformin and pomegranate compared to diabetic control were -40.0% and -63.89% for creatinine and were -63.66% and -69.27% for urea, respectively. Regarding creatinine, the recovery was segnificant in the pomegranate treated group compared to metformin treated group. While, there was no significant difference between the two treatments regarding urea. The blood glucose levels at the beginning and at the end of the experiment are shown in Table 4. The results indicated that pomegranate treatment significantly normalized the blood glucose concentration compared to the diabetic group and there was no significant difference between pomegranate and metformin treatments.

Table 4. The effect of pomegranate supplementation on Kidney function tests and blood glucose levels

Groups		Creatinine mg/dL	Urea mg/dL	Glucose* mg/dl	Glucose** mg/dl
Control (NC)	Mean±SD	0.68±1.48[c]	17.94±4.85[c]	75.87±17.26[b]	77.37±16.25[a]
Diabetic (DG)	Mean±SD	1.80±0.29[a]	78.53±11.61[a]	267.19±33.45[a]	245.26±40.89[b]
	%Change	164.71	337.74	252.17	217.0
Metformin (MTG)	Mean±SD	1.08±0.51[b]	28.54±20.62[b]	245.84±77.69[a]	80.03±11.85[a]
	%Change	58.80	59.09	224.0 -7.99	3.44
	%Change*	-40.0	-63.66		-67.37
Pomegranate (PTG)	Mean±SD	0.65±0.12[c]	24.13±3.22[b]	257.9±35.19 [a]	86.54±20.32[a]
	%Change	-4.41	34.41	239.9	11.85
	%Change*	-63.89	-69.27	-3.48	-64.72

Values represent the mean ± standard deviation (n = 10) in each group. Values that are followed by different letters within each column are significantly different (p < 0.05). % change: comparison with control; % change: comparison with diabetic group. *The blood glucose levels at the beginning and **at the end of the experiment*

3.2. Gene Expression Results

Semi-quantitative PCR experiments were performed on kidney samples to determine possible gene expression alternation of some antioxidant genes and some kidney-functions biomarker genes in the four animal groups. (Fig. 1) showed down-regulation of all the antioxidant genes (GR, GPx, Cat and SOD) in the diabetic animals in comparison with that of control group animals. While, the diabetic animals administrated with pomegranate retained higher expression levels of SOD and GPx genes, they retained the same expression levels of GR, and Cat genes, comparing with those of control animals. The diabetic animals administrated with

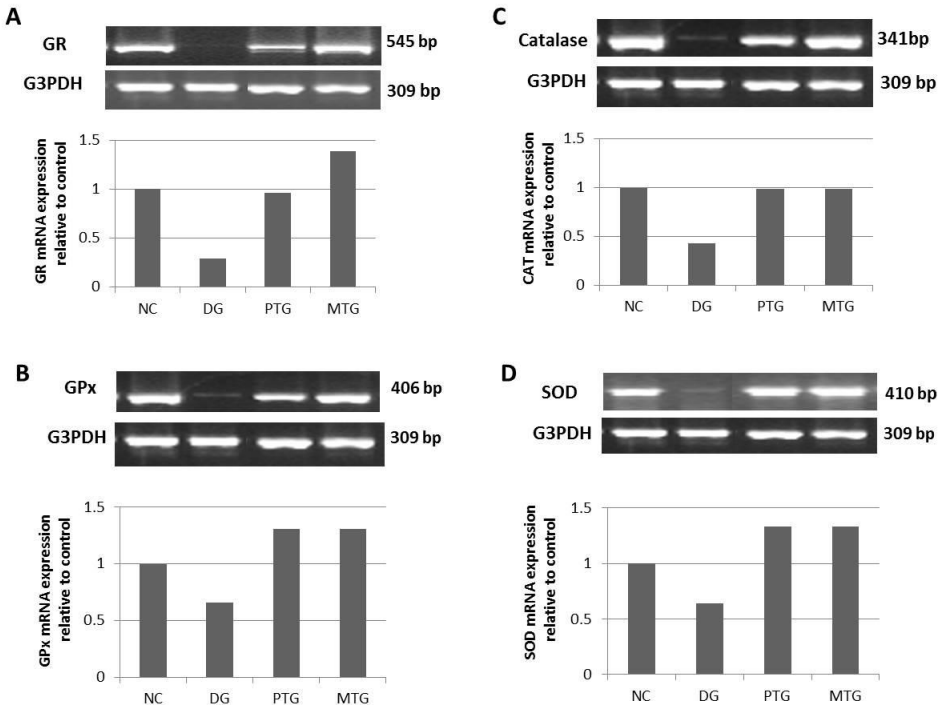

Fig. 1. Semi-quantitative RT-PCR analysis of antioxidant enzymes and its corresponding G3PDH in renal tissues. A: glutathione reductase (GR), B: glutathione peroxidase (GPx), C: catalase and D: superoxide dismutase (SOD). Experimental groups were normal control (NC), diabetic group (DG), pomegranate treated group (PTG), and metformin treated group (MTG) as described in materials and methods. Values are means ± SEM obtained from 5 different rats per group. P*< 0.05 vs. control group

the chemical drug metformin showed higher expression levels of GR, GPx, and SOD and same expression level of Cat gene comparing with those of control animals.

In addition, the mRNA expression of NADPH oxidase subunits P47phox, P22phox and the nuclear factor-κB genes was up-regulated in the diabetic animals in comparison with that of control group animals (Fig., 2). While, comparing with control animals, the diabetic animals administrated with pomegranate showed same or less expression level of P22phox and the nuclear factor-κB genes, respectively and up-regulated expression level of the P47phox gene.

3.3 Histopathological Examination

As shown in (Fig. 3), the kidneys of the control group rats (NC) showed normal histological structure of renal corpuscles and tubules. The diabetic group (DG) kidneys showed minimal degenerative changes in the form of tubular epithelium cloudy swelling and vacuolation, and cystic dilatation of the capsular cavity. The severity and extension of the renal parenchyma degenerative lesions were mild and similar

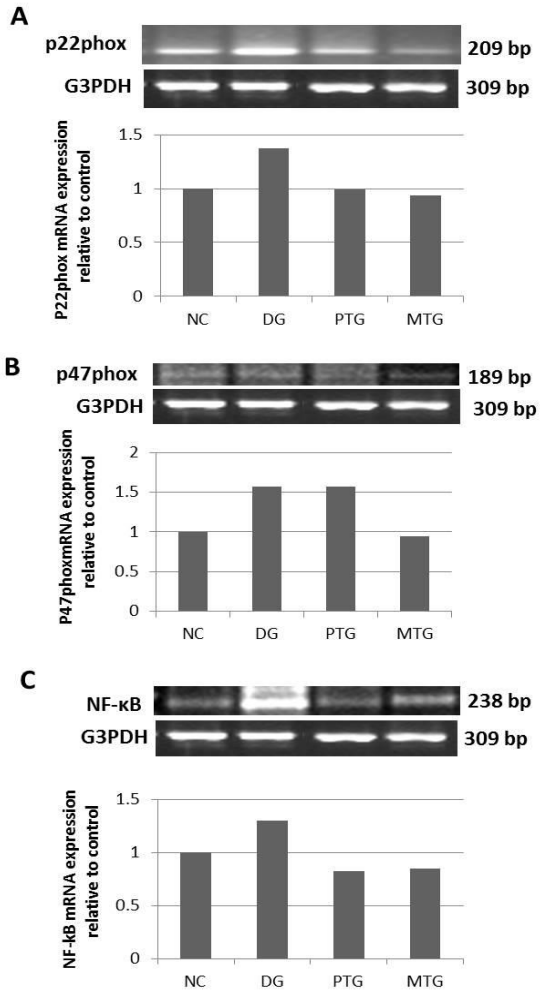

Fig. 2. Semi-quantitative RT-PCR analysis of some kidney functions biomarkers and its corresponding G3PDH in renal tissues. A: NADPH oxidase subunit P22phox; B: NADPH oxidase subunits P47phox and C: NF-κB, nuclear factor-κB. Experimental groups were normal control (NC), diabetic group (DG), pomegranate treated group (PTG), and metformin treated group (MTG) as described in materials and methods. Values are means ± SEM obtained from 5 different rats per group. P*< 0.05 vs. control group

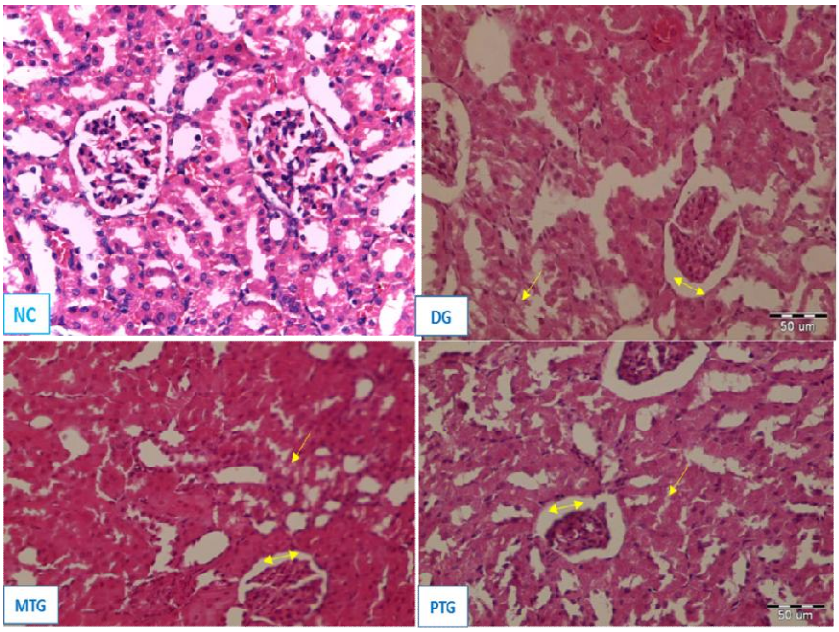

Fig. 3. Histopathological evaluation of metformin and pomegranate treatment on kidneys of diabetic mice. Representative results showing the histopathological picture of kidneys of normal control (NC), diabetic (DG), metformin treated (MTG) and pomegranate treated (PTG) rats in H&E (×200) stained sections. The normal histological picture of renal parenchyma was present in NC rats. DG mice showed minimal degenerative changes in the form of tubular epithelium cloudy swelling and vacuolation (arrow), and cystic dilatation of the capsular cavity (double arrow) ; whereas metformin treated (MTG) and pomegranate treated (PTG) rats revealed similar mild severity and extension of the renal parenchyma degenerative lesions

among rats of both metformin treated (MTG) and pomegranate (PTG) treated groups.

4. DISCUSSION

4.1 Oxidative Strees and the Protictive Effect of Pomogranate

Diabetes is a group of metabolic disorders in which there are high levels of blood glucose over a prolonged period. Hyperglycemia with increased lipid content elicits the formation of free radicals. Excessive formation of reactive oxygen species (ROS) alongside depletion of antioxidant defense capacity elevates oxidative stress. This oxidative stress together with inflammation is the major causative factor leading to diabetic complications. Diabetes is usually associated with lipid peroxidation which causes cell membrane instability finally leading to necrosis and damage to the affected tissues or organs and may also affect the progress of diabetic complications [9-11]. Therefore prevention of lipid peroxidation and maintaining a balanced between the production of ROS and antioxidant defenses may be one mechanism by which natural antioxidants in vegetables, and fruits help preventing diabetes complications. In the current study the level of lipid peroxidation, NO, antioxidant enzymes, kidney function tests were estimated, as well as the expression level of important antioxidant enzymes and inflammatory factors regulator gene were evaluated to examine the renoprotective effect of pomegranate in diabetes induced oxidative stress and kidney injury.

In the current study the level of MDA, which is generated as an end product during the oxidation of lipids has been used as marker for the induction of oxidative stress in kidney cells [36]. SOD, CAT, GST, GPx and GSH are the most common antioxidant enzymes that have been used to investigate the induction of oxidative stress [37]. Therefore, in this study, we aimed to determine the possible effects of the supplementation of pomegranate on GSH contents and SOD enzyme activity as well as the gene expression levels of SOD, CAT, GR and GPx.

Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) to the reduced glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell [38]. GSH known as body's master antioxidant is capable of preventing damage to important cellular components caused by reactive oxygen species (ROS). The thiol group of cysteine in GSH is able to donate a reducing equivalent to other molecules, such as ROS to neutralize them [39]. SOD is an enzyme against the superoxide radical and catalyzes its dismutation into H_2O_2, which is utilized by CAT or GPx [40].

Many studies showed the generation of ROS and stimulation of lipid peroxidation in diabetic trails supporting the function of oxidative stress in diabetic complications [reviewed in 41-43].

In the present study, diabetes increased lipid peroxidation as shown by elevated levels of MDA. Moreover, diabetes induced the generation of NO. This situation suggests the induction of oxidative stress in cells. Furthermore, diabetes caused significant decreases in the antioxidant enzyme activities as compared to the normal control. Similarly with our work, in previous studies, changes in these parameters were observed in diabetic trails [reviewed in 41-43]. Moreover, we observed down-regulation of all the antioxidant genes (GR, GPx, Cat and SOD) in the diabetic animals in comparison with that of control group animals.

The treatment with pomegranate significantly reduced the elevated MDA levels as well as NO. Moreover, pomegranate significantly elevated the antioxidant enzyme activities as well as induced the expression levels of SOD and GPx genes, comparing with those of control animals. While, the diabetic animals administrated with pomegranate retained the same expression levels of GR, and Cat genes.

4.2 Kidney Function and the Protictive Effect of Pomogranate

The renal excretion of the waste compounds can be used to indicate the health status of the kidneys. The biochemical values of urea, uric acid and creatinine, are useful parameters that have been widely used as good indicators for the kidney function. The data of this study revealed that serum urea and creatinine levels increased with diabetes (Table 3). These increases are indicators of kidney damage and renal functional disorders. Our data confirm the data obtained from previous studies which revealed that chemicals and diabetes cause such as abnormal urea, creatinine and uric acid levels [44,45]. The values of these biochemical parameters significantly decreased in the pomegranate treated group which indicate its prevention effect. These investigations are agreed with our light microscopic examinations of the kidneys.

Moreover, the mRNA expression of NADPH oxidase (subunits p22phox and p47phox) and the inflammatory factors regulator gene, NF-κB were also evaluated as an indicator of kidney functions. Oxidative stress induced by chronic exposure to high concentrations of glucose is responsible for the aberrant function of the mesangial cells (MCs), which regulate kidney blood flow, and that is associated with most of diabetes-related kidney disease [46]. p22phox and p47phox, the NADPH oxidase subunits in the cytomembrane and cytoplasm, are primarily expressed in the mesangial cells in kidney. Mesangial cells in a hyperglycemia environment express more NADPH oxidase, which leads to increased ROS in the kidney [47]. The increased ROS resulted in the expression significant increase of NF-κB gene that regulates the expression of inflammatory factors.

In the present study, inhibition in the expression of NADPH oxidase subunit p47phox and NF-κB in the pomegranate treated group was shown (Fig. 2). The amelioration of renal histomorphologic changes and impaired function as a result of the inhibition of NADPH oxidase in kidney was previously reported [41]. Increased expression of NF-κB may activate TGF-β1, which results in inhibition of the degradation of ECM, and accelerates the accumulation of ECM in the glomerular mesangium and expands the mesangial region increased synthesis of matrix proteins such as collagen type IV, fibronectin, and laminin [48-49].

4. CONCLUSION

The biochemical, molecular and histopathological results that reported in the present study validate pomegranate as a promising candidate in preventing diabetes associated complications such as nephropathy through its antioxidant activity and its effects on the activity and regulation of oxidative stress defense gene expression.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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