

Pomegranate peel extract protects against carbon tetrachloride-induced nephrotoxicity in mice through increasing antioxidants status

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Received October 21, 2019; Accepted April 1, 2020

DOI: 10.3892/br.2020.1320

Abstract. Carbon tetrachloride (CCl₄) is a notorious environmental pollutant known for its toxicity. The aim of the present study was to evaluate the possible protective effects of aqueous pomegranate peel extract (PPE) against CCl₄ induced nephrotoxicity in mice. Adult male mice were divided into four groups: Group one was used as the control; Group two was treated with a daily oral dose of PPE (400 mg/kg) for 15 days; the third group was intraperitoneally injected with a dose (1 ml/kg) of CCl₄ twice a week for two weeks; and the final group was injected with the same dose of CCl₄ twice a week concomitantly with a daily oral dose of PPE (400 mg/kg). Biochemical and histopathological data were analyzed along with the gene expression levels of the antioxidant enzymes and immunohistochemistry of the kidney tissue. CCl₄ resulted in a significant increase in the serum urea and creatinine levels with detectable degenerative changes in the Bowman's capsule and glomerulus, with cells exhibiting vacuolization and evidence of necrosis. Co-administration of animals with CCl₄ and PPE resulted in improved biochemical and histopathological conditions. Similarly, increased production of the Caspase-3 and collagen fibers were reduced in mice treated

with PPE. Quantitative analysis of superoxide dismutase, catalase and glutathione peroxidase further accentuated the effects of PPE treatment significantly improving the conditions of the CCl₄-administered group. The results of the present study demonstrate that the phenolic derivative rich PPE is a potent nephroprotective agent and suppresses CCl₄-induced nephrotoxicity in mice.

Introduction

Carbon tetrachloride (CCl₄) is an organic solvent extensively used in cleaning reagents. It has been used in animal models to investigate synthetic poison-induced inner organ damage. When this lethal compound is introduced in to the body by ingestion, inward breath or skin assimilation, it is disseminated throughout the body, often accumulating in the liver, cerebrum, kidney, muscle, fat and blood (1). There are several studies demonstrating CCl₄-induced kidney (2), liver and testicular damage (3), as well as blood disorders (4). CCl₄ intoxication in animals is used experimentally to induce oxidative stress under various physiological conditions (5). Prolonged exposure to CCl₄ induces histopathological features such as inflammatory leukocyte infiltration, necrosis, fibrosis, cirrhosis, and may also result in the development of cancer (6).

Nephrotoxicity is a widely recognized health issue and is often the result of exposure to medication or toxins (7). Renal failure results in a severe decline in the excretory capacity of the kidney, resulting in buildup of nitrogenous waste in the blood (8). Renal failure is a common pathophysiological disturbance caused by CCl₄ that leads to death, and is categorized into acute and chronic renal failure (9). One of the principal causes of acute kidney injury is oxidative stress that subsequently results in the formation of reactive oxygen species (ROS). Direct nephrotoxic effects of toxins such as CCl₄, including phospholipid damage, mitochondrial dysfunction, increased intracellular calcium concentration and lysosomal hydrolase inhibition result in the formation of ROS and thus increases oxidative stress, causing proximal tubular toxicity (10). ROS

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Abbreviations: CCl₄, carbon tetrachloride; PPE, pomegranate peel extract; IP, intraperitoneally; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OD, optical density; RPM, revolutions per minute; ROS, reactive oxygen species; GC-MS, gas chromatography-mass spectrometry

Key words: pomegranate, GC-MS analysis, CCl₄ toxicity, kidney function, histopathology, antioxidant enzymes

contributes to the progression of fibrosis either directly or indirectly by promoting inflammation. Fibrosis and inflammation together may further augment ROS formation or stimulate the production of cytokines and growth factors (11). Reactive oxygen metabolites are postulated to underlie the pathogenesis of CCl₄ nephrotoxicity (12). Thus, the buildup of free radicals in cells can induce lipid peroxidation and the oxidative breakdown of membrane polyunsaturated fats results in readjustments to cell membrane permeability and viscosity (13). *In vivo* and *in vitro* reports have shown that CCl₄ increases lipid peroxidation, reduces oxidized glutathione levels in the kidney cortex and causes a reduction in the activity of enzymes which would result in decreased lipid peroxidation (14). CCl₄ can sub-lethally induce proximal tubular damage in the kidney and cause changes to the granular pneumocytes (15).

Several medicinal plants are known for their remedial properties when used to treat renal disorders, due to the presence of various multifaceted therapeutic chemical compounds (16). When medicinal plants with nephroprotective properties are administered alongside various nephrotoxic agents, they may attenuate toxicity (8). *Punicagranatum* L. (*Punicaceae*), commonly known as pomegranate, is often used in folk lore medicine for the management of various diseases (17). Pomegranate peel extract (PPE) exhibits marked antioxidant properties (18). It has been shown to reduce oxidative stress mediators indicating its antioxidant capacity, which is attributed to the presence of diverse phenolic compounds, such as gallic acids, ellagic acids, ellagitannins, catechins, gallotannins, anthocyanins, quercetins and ferulic acids (19,20). These polyphenols possess antioxidant properties in scavenging free radicals and inhibiting lipid oxidation (21). Furthermore, studies on animals have shown PPE does not exhibit any toxic effects (22). Additionally, the anti-inflammatory (23) as well as the anticancer properties of PPE have also been established (24), and PPE may be a potent nephroprotective agent (25,26).

The aim of the present study was to evaluate the protective properties of pomegranate peel aqueous extract against CCl₄ induced kidney damage in mice. Three major endogenous antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) expression levels were assessed, alongside biochemical, histopathological and immunohistochemical changes, to evaluate the antioxidant capacity of the PPE.

Materials and methods

Preparation of the plant extract. The dried peel of pomegranates was obtained from local markets. To prepare a water extract of the pomegranate peel, the peels were cleaned with distilled water, desiccated and crushed to a fine powder. A total of 1 kg peel powder was added to L boiling water, after which the mixture was kept in a bolted vessel for cooling. The solvent was filtered and concentrated in a water bath until the extract was reduced to a volume of 100 ml. The herb/extract ratio was 10/1. PPE was finally diluted to 10% as described previously (27).

Gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS analysis of the aqueous peel extract of *P. granatum* was performed using a GC-MS examination system (Trace

GC Ultra and ISQ Single Quadruple MS; Thermo Fisher Scientific, Inc.) at a flow rate of 1.5 ml/min. as described previously (28). The database of the National Institute Standard and Technology (NIST, chemdata.nist.gov/) was consulted for the identification of mass spectrum GC-MS.

Experimental animals. A total of 40 adult male CD1 albino mice weighing 20-30 g were acquired from the Animal House of VACSERA, Co. The mice were maintained under normal environmental conditions of temperature and humidity and were given adequate food and water. The mice were allowed to acclimatize for 1 week prior to beginning the experiments. The present study was performed in accordance with published guidelines (29) and approved by the Internal Research Regulation and the Animal Ethics Committee of the Department of Zoology, Faculty of Science, Helwan University (Helwan, Egypt).

Experimental design. To study the effects of PPE on CCl₄ mediated nephrotoxicity, CCl₄ was mixed with olive oil as a vehicle in a 1:1 proportion. The adult male mice were divided into four groups of 10 mice each. The first group was the control group. The second group was treated with a daily oral dose of PPE (400 mg/kg) for two weeks. Group three was injected with 1 ml/kg CCl₄ dissolved in olive oil twice a week for two weeks. The fourth group was injected intraperitoneally (IP) with CCl₄ and treated with PPE, both as above. The dose of CCl₄ and treatment period were based on previous studies (30-32). An equal quantity of olive oil was given IP to the control group. A blank fifth group, not administered olive oil, did not exhibit any differences compared with control group, and therefore the data are not presented.

Biochemical analysis. Animals were anesthetized with inhalant isoflurane (3%) and blood samples were collected and stored in vacuum tubes with clot activator. These samples were centrifuged at 3,000 x g for 10 min at room temperature to separate the serum, and the serum was stored at -20°C. The quantity of serum urea and creatinine was assessed using commercial kits from Reflotron; Liquicolor analysis according to the manufacturer's protocol. Serum urea (33) and creatinine concentrations (34) were measured as described previously.

Histological examination. Mice were euthanized using isoflurane (6%) to avoid stressing the mice (35). The 2 mm thick mouse kidney tissues were fixed in 10% formalin for 48 h at room temperature, and tissues were processed for microscopic examination. The sections were dyed with Harris's hematoxylin and eosin (36) and Mallory's trichrome stain for collagen fibers as described previously (37). The kidney sections of the control and experimental groups were observed using light microscope, and images were captured for analysis.

Immunohistochemistry analysis. Immunohistochemical detection of Caspase-3 was performed using an anti-Caspase3 primary antibody (Labvision; Thermo Fisher Scientific, Inc.) as described previously (38), using a streptavidin-biotin system. Positive reactions for Caspase 3 were observed as brown coloration of the cytoplasm in treated cells. The mean optical pixel density of the kidney tissue was analyzed by

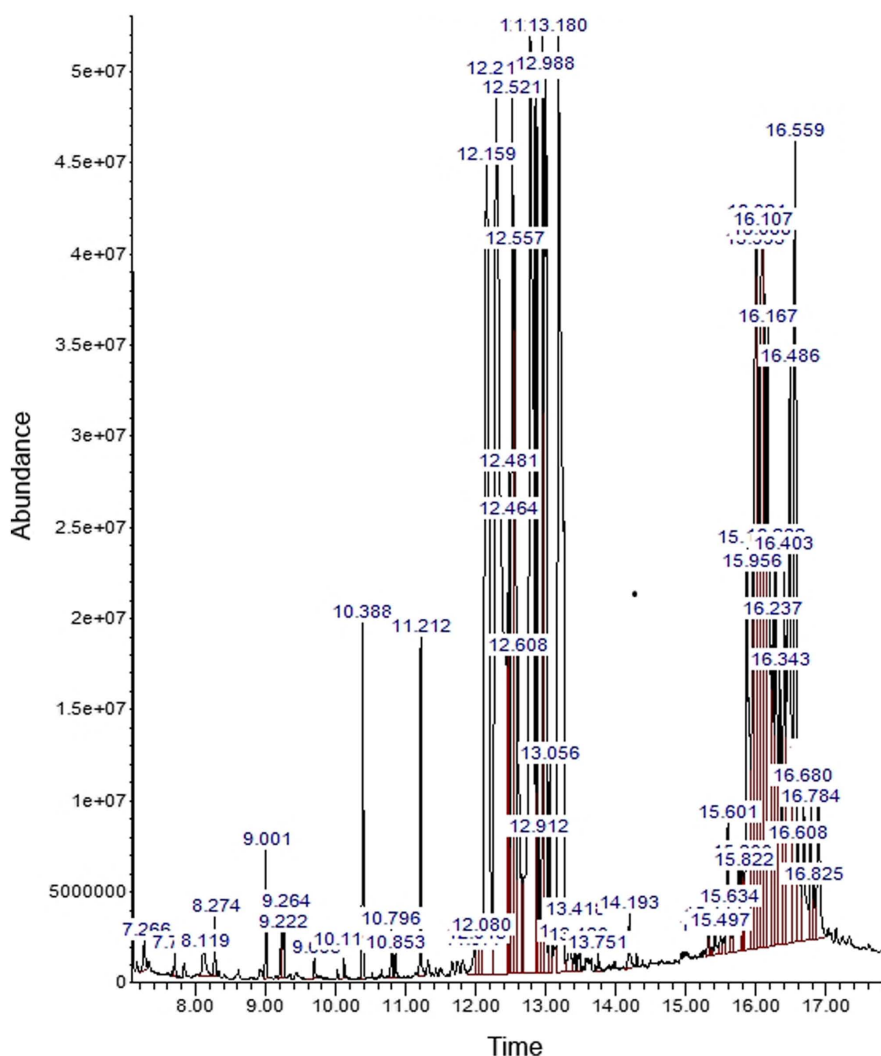


Figure 1. Gas chromatography-mass spectrometry chromatogram of aqueous extract of *Punica granatum* peel.

using Image Pro Plus version 6.0 (Media Cybernetics, Inc.) and is expressed as the mean optical density (MOD). For each sample, 10 random fields of view were averaged to determine the mean. MOD values were only determined for Caspase-3 staining and not for the collagen staining.

Antioxidant enzyme expression. Quantitative analysis of endogenous antioxidant enzymes, such as SOD, CAT and GPx was performed. A PureLink FFPE RNA Isolation kit, (Thermo Fisher Scientific, Inc.) was used for RNA isolation. A total of 6 slices of 10 μm thick kidney tissue sections were fixed and preserved in formalin, embedded in paraffin, placed into a sterile microcentrifuge tube and RNA was extracted according to the manufacturer's protocol. Briefly, the tissue was separated from the melted paraffin by centrifugation at 3,000 \times g for 5 min at 4°C and digested with Proteinase K. The tissue lysate was then processed by selective binding of RNA to a silica-based membrane in the Spin Cartridge. Wash Buffer was used to remove impurities by thorough washing. The final product was eluted as total RNA in RNase-Free water. Quantity and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Eluted RNAs with an absorbance ratio OD

260/280 >2.0 were used for further analysis. RNA quality was determined using agarose gel electrophoresis on 1.5% gel, and the RNA was frozen at -20°C for further use.

The primers were designed using the nucleotide sequence alignment NCBI BLAST tool (blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the primers were: CAT forward, 5'-TCC GGGATCTTTTAAACGCCATTG-3' and reverse, 5'-TCG AGCACGGTAGGGACAGTTCAC-3'; SOD forward, 5'-AGC TGCACCAGCAAGCAC-3' and reverse 5'-TCCACCACC CTTAGGGCTGA-3'; GPx forward 5'-GGCAAGGTACTA CTTATCGAG-3' and reverse, 5'-GTTACCTCGCACTT CTCGAAG-3'; and GAPDH forward 5'-GGATTTGGTCGT ATTGGG-3' and reverse 5'-CGACATACTCAGCACCGG-3'. GAPDH was used as the house keeping gene. Primers were purchased from MacroGen, Inc.

Reverse transcription-quantitative (RT-q)PCR. First-strand cDNA was synthesized from 2 μg total RNA with primers for each gene. Briefly, 20 μl reverse transcription reaction mix was prepared using M-MLV Reverse Transcriptase system (Thermo Fisher Scientific, Inc.). cDNA was synthesized by incubating the reaction mix at 42°C for 1 h and stored at -20°C or used immediately. qPCR was performed using a 7500

Table I. Phytochemicals identified in the aqueous extract peel of *P. granatum* by gas chromatography-mass spectrometry.

| Number of peaks | Retention time | Area percentage | Compound name |
|-----------------|----------------|-----------------|--|
| | | | Trisiloxane, octamethyl- |
| | | | Pyrazol-5(4H)-one, 3-(4-methoxyphenyl)- |
| | | | 3-Methoxyphenol, TMS derivative |
| | | | 4-Pentamethyldisilyloxyhexadecane |
| | | | Silanol, trimethyl-, phosphate (3:1) |
| | | | Butanedioic acid, bis(trimethylsilyl) ester |
| | | | 2-Propenoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester |
| 1 | 10.391 | 0.7193 | Malic acid |
| | | | Isoindole-1,3(1H,3H)-dione, 2-[2-(4-methylphenylhydrazono)propyl]- |
| 2 | 11.2118 | 0.8076 | D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 1) ^a |
| | | | Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, γ -lactone, d- |
| | | | L-(-)-Arabitol, 5TMS derivative |
| 3 | 12.1597 | 7.1061 | D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 2) |
| 4 | 12.2921 | 12.0624 | D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 2) |
| 5 | 12.4615 | 1.1313 | Citric acid, 4TMS derivative |
| 6 | 12.5198 | 3.9131 | D-Allofuranose, pentakis(trimethylsilyl) ether |
| 7 | 12.5568 | 2.5652 | β -D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)- |
| 8 | 12.6098 | 1.2826 | Erythritol, 4TMS derivative |
| 9 | 12.7792 | 9.0392 | 1,2,3-Tri-O-galloyl- -glucopyranose |
| 10 | 12.8692 | 1.9619 | D-(+)-Xylose, 4TMS derivative |
| | | | 2-Hydroxybenzimidazole, 2TBDMS derivative |
| 11 | 12.9593 | 2.7779 | 2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)- |
| 12 | 12.9857 | 3.6912 | 2- α -Mannobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 1) |
| 13 | 13.0546 | 5.8949 | Gallic acid, 4TMS derivative (6.4499) |
| 14 | 13.1817 | 8.3094 | Mannopyranoside, trimethylsilyl 2,3,4,6-tetrakis-O-(trimethylsilyl)- |
| | | | 1,5-Anhydrohexitol, 4TMS derivative |
| | | | Palmitic Acid, TMS derivative |
| | | | 2,4-Hexadienedioic acid, (E, E)-, 2TMS derivative |
| | | | 11-Octadecenoic acid, (E)-, TMS derivative |
| | | | Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, γ -lactone, d- |
| | | | Sucrose, 8TMS derivative |
| | | | Lactulose, octakis(trimethylsilyl) ether (isomer 2) |
| 15 | 15.8717 | 2.5257 | [4-Bromo-2-(hydrazono-phenyl-methyl)-phenyl]-carbamic acid, ethyl ester |
| 16 | 15.9564 | 1.4316 | Ethyl 2-[4-chlorophenyl]-7,8-benzocinchoninate |
| 17 | 15.9935 | 3.1568 | D-Fructose, 5TMS derivative |
| 18 | 16.0888 | 3.3861 | D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 2) |
| 19 | 16.1047 | 3.2436 | Sucrose, 8TMS derivative |
| 20 | 16.1682 | 4.3471 | D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 2) |
| 21 | 16.2371 | 1.2581 | D-Fructose, 5TMS derivative |
| 22 | 16.4859 | 4.0291 | Acrylonitrile, 2-chloro-3,3-bis-(4-nitrophenoxy)- |
| 23 | 16.5601 | 3.7202 | Molybdenum, tricarbonyl[(1,2,3,4,5,6-eta.)-1,4-dimethylbenzene]- |
| | | | 2-(2-Bromo-4-methylphenoxy)-N'-([1-(4-nitrophenyl)-2-pyrrolidinyl] |
| | | | methylene)acetylhydrazide |
| | | | D-Psicopyranose, 5TMS derivative (isomer 2) |

Applied Biosystems RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc. For each sample, a 20 μ l reaction mixture consisting of 1 μ l diluted cDNA (1:20), 5 pmol each of the forward and reverse primers, and 10 μ l 2x SYBR Premix Ex Taq II (Takara Bio, Inc.). Expression in each sample was

assessed in triplicate. Relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method (39).

Statistical analysis. Data are presented as the mean \pm standard deviation and were analyzed using GraphPad Prism version 7

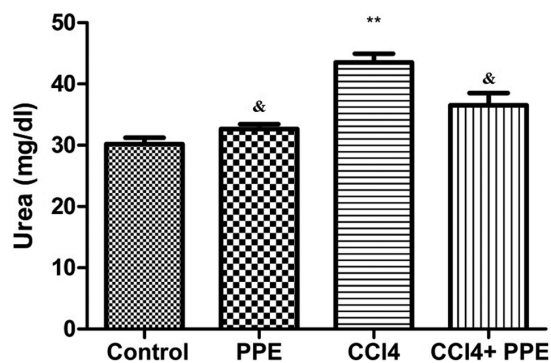


Figure 2. Effects of PPE and/or CCl₄ administration on serum urea levels in mice. **P<0.001 represents CCl₄ vs. PPE and PPE + CCl₄ group; &P>0.05 represents the comparison between the control vs. PPE and CCl₄ + PPE group; PPE, pomegranate peel extract; CCl₄, carbon tetrachloride.

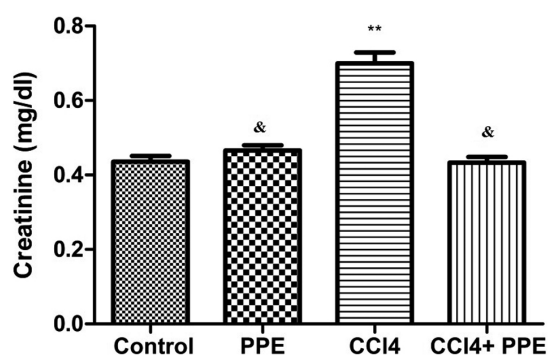


Figure 3. Effects of PPE and/or CCl₄ administration on serum creatinine levels in mice. **P<0.001 represents CCl₄ vs. PPE and PPE + CCl₄ group; &P>0.05 represents the comparison between the control vs. PPE and CCl₄ + PPE group. PPE, pomegranate peel extract; CCl₄, carbon tetrachloride.

(GraphPad Software, Inc.). A two-way ANOVA was used for multiple comparisons with a post-hoc Bonferroni correction. A Student's t-test was used to compare each treated sample with the control. P<0.05 was considered to indicate a statistically significant difference.

Results

GC-MS analysis. Analysis of aqueous extract of pomegranate peel using GC-MS showed the presence of a variety of phenolic compounds, heterocyclic compounds, organic acids, fatty acids and sugars (Fig. 1). The peaks in the chromatogram were assimilated and equated using the NIST Mass Spectral Library database of known compounds stored in the GC-MS library. The phytoconstituents were identified and are presented in Table I. The extract contained a total of 23 peaks characteristic of phenolic components which are carbohydrates; gallotannins and gallotannin derivatives (1,2,3-Tri-O-galloyl-glucopyranose); phenolic acids (predominantly gallic acid); and organic acids such as citric acid and malic acid. In addition, fatty acids (octadecatrienoic acid and palmitic acid) were present in small concentrations.

Biochemical analysis. Changes in the urea and creatinine concentrations in serum of adult male mice in the control

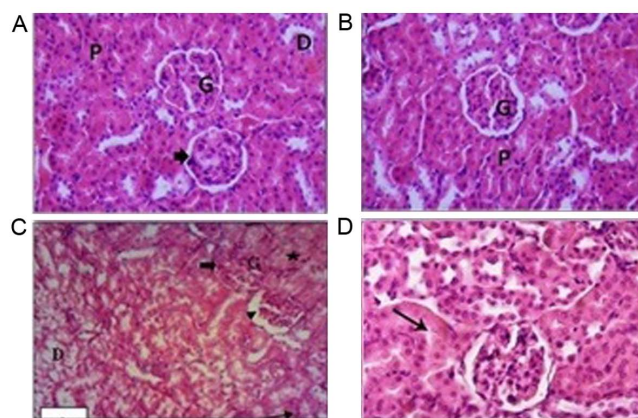


Figure 4. Images of kidney tissues stained with hematoxylin and eosin. (A) Kidney tissues in the control group showed normal histological architecture of the mouse kidney cortex, normal glomeruli, normal Bowman's space (arrowhead), and normal distal and proximal tubules. (B) Kidney tissues of the PPE-treated group showed normal glomeruli, renal tubules and normal proximal tubules. (C) Kidney tissue of the CCl₄-treated group showed hypercellularity of glomeruli (thick arrow) and widening of the Bowman's space (arrowhead). Some tubules exhibited swollen cells in the epithelial lining that narrowed the lumen (star). Some renal tubules exhibited extensive vacuolar degeneration and their lumens were filled with cellular debris (arrow). (D) Kidney tissues in the CCl₄ + PPE treated group. Glomeruli exhibited slight congestion and some of the cells of the renal tubules appeared slightly swollen (arrow). Magnification, x100. G, glomeruli; D, distal tubules; P, proximal tubules; PPE, pomegranate peel extract; CCl₄, carbon tetrachloride.

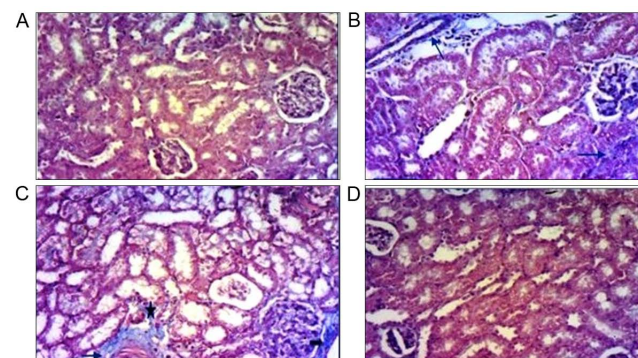


Figure 5. Images of kidney tissue sections stained with Mallory's trichrome stain. (A) Images of renal tissues in the control group showed scant interstitial connective tissue and low collagen content surrounding the glomeruli. (B) Renal tissue of the PPE-treated group exhibited a moderate increase in the connective tissue content in the glomerulus and peritubular areas, and around the blood vessels (arrows). (C) CCl₄ treated mice showed an extensive increase in the collagen content in the peri-glomerular space (thick arrow), surrounding the blood vessels (arrow), and in the intertubular spaces (star). (D) PPE + CCl₄ group exhibited a moderate decrease in the collagen fiber content compared with the CCl₄ group. Magnification, x100. PPE, pomegranate peel extract; CCl₄, carbon tetrachloride.

and experimental groups are presented in Figs. 2 and 3. The data show that administration of CCl₄ for 15 days induced a significant increase in serum urea and creatinine levels compared with the control group (P<0.001). Treatment of the CCl₄-treated group with PPE resulted in a significant reduction in the level of serum urea and creatinine concentrations compared with the CCl₄ treated group (P<0.05), and did not differ significantly compared with the mice in the control group (P>0.05; Figs. 3 and 4). In addition, there were no notable psychological differences observed in any of the

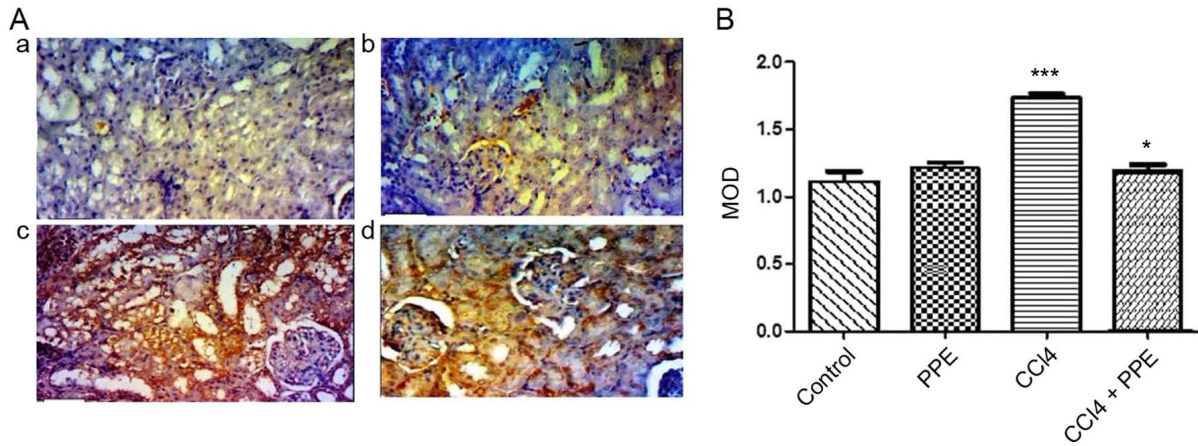


Figure 6. (A) Images of kidney tissue sections stained with Caspase-3 immunostaining. (Aa) Kidney tissue from a control mouse showed low levels of immunoreactivity for Caspase-3 in the renal tubules as shown by the faint brown staining. (Ab) Kidney tissues of the PPE-treated group exhibited weak positive immunoreactivity for Caspase-3. (Ac) Kidney tissues of the CCl₄ treated group showed strong and widespread immunoreactivity for Caspase-3 as shown by the extensive brown coloration in the renal tissues. (Ad) Renal tissues from in the PPE + CCl₄ treated group showed a notable reduction in Caspase-3 immunoreactivity. Magnification, x100. (B) MOD values of Caspase-3 expression in the kidney tissues. Significance: * $P < 0.05$, PPE + CCl₄ vs. PPE and control group; *** $P < 0.001$, CCl₄ vs. PPE + CCl₄ group; PPE, pomegranate peel extract; CCl₄, carbon tetrachloride; MOD, mean optical density.

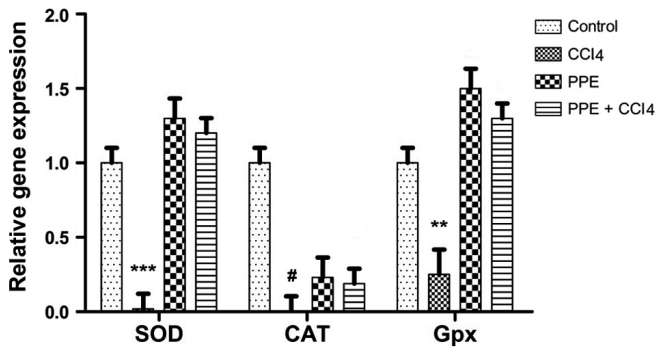


Figure 7. Effects of PPE and/or CCl₄ administration on the mRNA expression levels of SOD, CAT and GPx. SOD expression: *** $P < 0.001$, CCl₄ vs. PPE + CCl₄ and PPE group; GPx expression: ** $P < 0.01$, CCl₄ vs. PPE + CCl₄ and PPE group; CAT expression: # $P < 0.05$ vs. CCl₄ vs. PPE and PPE + CCl₄ group. PPE, pomegranate peel extract; CCl₄, carbon tetrachloride; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

mice. Food and water consumption were regularly monitored and did not deviate noticeably throughout the duration of the experiments (data not shown).

Histological analysis. Kidney sections from control mice stained using hematoxylin and eosin showed normal renal cortex architecture. The proximal convoluted tubules and the distal convoluted tubules appeared normal (Fig. 4A). The renal tissues of PPE treated mice showed several normal glomeruli and renal tubules, and low levels of inflammatory cells in the intertubular spaces (Fig. 4B).

The renal architecture in CCl₄ treated mice was notably affected. The majority of the glomeruli exhibited hypercellularity severe glomerular congestion. Bowman's spaces in the glomeruli were narrowed, whereas in other glomeruli, there was an increase in the Bowman's spaces. The epithelial cells which constituted the lining of tubules were severely swollen with a decreased lumen volume. Some of the renal tubules showed extensive vacuolar degeneration and their lumens were

filled with cellular debris. Cellular infiltration in the renal tissues was visible and notably increased (Fig. 4C).

Administration of PPE to CCl₄ treated mice resulted in marked improvements, and reduced the renal damage caused by CCl₄. The kidney tissues of the CCl₄ + PPE group retained intact histological architectures with reduced damaged areas compared with the CCl₄ group; however some glomeruli appeared to be slightly congested, and a few tubules showed slightly swollen epithelial cells but with normal nuclei (Fig. 4D).

Alteration in collagen fibers. There were notable histopathological changes observed in the collagen fiber content in renal tissues between the control and treated sections. Mallory's triple stain of the renal tissues in the control sections showed that there was less interstitial connective tissue, and where present, it was primarily concentrated around the blood vessels (Fig. 5). The collagen content surrounding the glomeruli was scant (Fig. 5A). The collagen fiber content in renal tissues of the PPE group was similar to that of the control group; a moderate increase in the connective tissue was observed in both glomerular and peritubular areas (Fig. 5B). The renal tissues of CCl₄ treated mice exhibited an extensive increase in the collagen fiber content primarily in the intra-glomerulus, around the renal corpuscles, in the intertubular spaces and around the blood vessels (Fig. 5C). The renal tissue of the CCl₄ and PPE treated mice showed a moderate decrease in the collagen fiber content when compared with the CCl₄ group (Fig. 5D).

Immunohistochemical observations. The results obtained by image analysis for Caspase-3 expression showed that the CCl₄-treated group exhibited strong cytoplasmic immunoreactivity, indicated by the intense brown color in renal tissues of mice and the MOD values were significantly increased compared with the control group ($P < 0.001$; Fig. 6Aa, Ac and B). The PPE treated mice exhibited relatively normal levels of Caspase-3 immunoreactivity (Fig. 6Ab). There was a

significant decrease in the intensity of Caspase-3 expression in the renal tissue, when treated with PPE combined with CCl₄ compared with the CCl₄ group ($P < 0.05$; Fig. 6Ad).

RT-qPCR. RT-qPCR analysis of SOD, CAT and GPx mRNA expression levels are shown in Fig. 7. The results showed a significant increase in the gene expression levels of these genes in the PPE and PPE + CCl₄ treated groups compared with the control group. A significant increase in gene expression levels were observed in all three genes compared with the CCl₄ treated group. The CAT gene expression levels were increased both in PPE and PPE + CCl₄ group compared with the CCl₄ group. However, the expression of CAT in the CCl₄, PPE and PPE + CCl₄ groups were notably lower than the control, unlike the expression of SOD and GPx. The difference between the expression levels of all the genes in the CCl₄ and PPE + CCl₄ groups was significant when compared with the control ($P < 0.001$).

Discussion

Kidneys are responsible for the removal of various chemicals and toxins from the blood stream, and thus are likely to be subjected to consequent damage. Functional impairment of the kidneys is a significant public health problem (8). The present study demonstrated the ameliorative effects of PPE on CCl₄ induced renal toxicity in mice. The results show that treatment with CCl₄ resulted in nephrotoxicity, as indicated by a rise in serum levels of urea and creatinine along with detectable histopathological changes in the kidney tissue which manifested as morphological changes to the glomerulus, renal tubules and vacuolization of the cells. The results of the present study are similar to previous studies examining the histopathological alterations in the kidney tissue induced by administration of CCl₄, and the increase changes in the serum urea and creatinine levels (40-42). These changes are caused by the formation of free radicals, which results in lipid peroxidation and breakdown of the membrane structure, and thus subsequently in the damage of nephron structural integrity (40,43). Additionally, the oxidation of lipids, proteins, carbohydrates, DNA and other biological molecules by noxious ROS can result in DNA mutations, which contribute to impairment of the target cell's function and often results in cell senescence and death (42,44). The capacity of tubular absorption is altered and the nephrons are overloaded, resulting in subsequent renal dysfunction (45,46).

The animals treated with PPE alone for 15 days did not show any toxicity in the histopathological evaluations nor any significant changes in serum urea and creatinine levels when compared with the control. These results are in agreement with a previous study (47), showing that pomegranates exhibit potent antioxidative effects without any noticeable toxic effects. Furthermore, the present study suggested that PPE reduced the CCl₄-induced effects on renal physiology. The reduction in tissue damage was attributed to the high antioxidant and nephroprotective effects of PPE, possibly due to the high levels of phenolic compounds (26,48-50). Administration of the PPE is reported to attenuate the toxicity and oxidative stress induced by chlorpyrifos-ethyl treatment in animals (51). The presence of kidney fibrosis is generally considered as endpoint

organ failure proceeding to loss of function (49). In the present study, in the CCl₄-treated group, there was a substantial increase in collagen content in the intra-glomerular and the intertubular spaces as well as around the renal corpuscles and the blood vessels. CCl₄-induced nephrotoxicity is suggested to impair renal function, increase inflammation and fibrosis, with notably increased collagen deposition as a downstream outcome of increased free radical levels (52). However, the treatment of mice with CCl₄ + PPE resulted in a notable decrease in collagen fiber content; this protective effect may be attributed to the antioxidant and antifibrotic properties of PPE (48,49,53). The histopathological changes described in the present study are limited in their value, due to an unavailability of the high-resolution electron microscopic images. Caspase-3 is the key protein involved in programmed cell death (54) In the present study, immunohistochemical results of kidney tissue from CCl₄-administered mice showed a substantial increase in Caspase-3 production, suggesting an increase in apoptosis compared with the control group. CCl₄ has been shown to induce acute nephrotoxicity with apoptotic renal damage by increasing the renal levels of active Caspases-3. Furthermore, CCl₄ is capable of increasing oxidative stress and apoptosis in other organs such as the liver (55). Co-administration of PPE with CCl₄ resulted in a decrease in Caspase-3 levels, further highlighting the protective effects of PPE against oxidative stress-induced apoptosis, as a result of diethylnitrosamine and phenobarbital mediated apoptosis in the kidney (29). These results suggest that PPE protects against CCl₄-induced renal tissue damage by enhancing the anti-apoptotic activity as well as by reducing the expression of apoptotic proteins. As only total Caspase-3 expression levels were analyzed, it is not possible to concretely say whether apoptosis was increased without assessing cleaved Caspase 3 levels. However, the increase in Caspase 3 levels do suggest a potential increase in apoptosis, and thus, the antiapoptotic effects of PPE.

In the CCl₄ treated mice, the expression levels of SOD, CAT and GPx were significantly decreased, whereas an increase in expression of SOD and GPx was observed in the PPE treated mice group compared with the control group, suggesting an overall improvement in the antioxidative state, even in the absence of injury. In the CCl₄ + PPE treated mice compared with the CCl₄-treated group, there was a significant 1.3 fold increase in SOD and GPx expression. In addition, there was a slight increase in CAT expression. These results highlight the protective effects of PPE against oxidative stress. A previous study showed that phenolic compounds from PPE showed strong antioxidant properties as gauged by the Gallic acid monohydrate, 2,2-diphenyl-1-picrylhydrazyl scavenging activity and ferric reduction tests (56). The results of the present study are in agreement with the previous study, suggesting the protective effects of pomegranate against stress-induced tissue damage. A significant protective effect was observed when Streptozotocin-nicotinamide induced diabetic rats were subjected to 21 days of oral gavage with pomegranate seed-juice (57). Treatment with pomegranate seed-juice significantly increased the activity of SOD and CAT. The qPCR results performed in the present study further support the biochemical and histopathological data, showing a reduction in tissue injury of the Bowman's capsule and glomerular cells.

In conclusion, the results of the present study demonstrate the nephroprotective role of the aqueous PPE against CCl₄-induced nephrotoxicity at the biochemical, histopathological and molecular level. The histopathological improvement may be attributed to the antioxidant properties of PPE. The present study further validates the folk based use of aqueous PPE for the treatment of toxicity-related renal injuries. Pomegranate peel-based tea may protect against diabetes, or against kidney damage in patients who regularly use nephrotoxic medicines. Pomegranate fruit peels are a waste material of the food industry, and the present study highlights its potential clinical value.

Acknowledgements

We would like to thank Dr Shazina Kanwal (Guangzhou Institutes of Biomedicine and Health, China) for reviewing our manuscript and for the valuable suggestions, and Miss Angel Weaver for assistance with revising the language of the manuscript.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MARI and HAO performed the *in vivo* experiments. HAO conducted the biochemical analysis and the plant extract analysis. NME and MARI performed the histopathology and immunohistochemical analysis. SA and TA performed the molecular experiments. All authors contributed equally in experimental design and to writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Internal Research Regulation and the Animal Ethics Committee of the Department of Zoology, Faculty of Science, Helwan University (Helwan, Egypt).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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