

Ameliorative effects of pomegranate on carbon tetrachloride hepatotoxicity in rats: A molecular and histopathological study

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Abstract. The present study aimed to investigate the molecular mechanism underlying the hepatoprotective effects of pomegranate (POM) against oxidative stress in a rat model of carbon tetrachloride (CCl₄)-induced liver damage. Injection of rats with CCl₄ resulted in hepatic inflammation and lipid accumulation via the upregulation of interleukin (IL)-6 and sterol regulatory element-binding protein 1c (SREBP-1c) mRNA expression. CCl₄ induced downregulation of the anti-inflammatory factors alpha 2-macroglobulin (α -2M) and IL-10 in comparison with the POM treated group. In addition, CCl₄ induced downregulation of superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) expression. Conversely, prior administration of POM counteracted the deleterious alterations induced by CCl₄. POM downregulated CCl₄-induced IL-6 upregulation, normalized the increase in SREBP-1c expression, and prevented CCl₄-induced α -2M downregulation. POM counteracted CCl₄-induced alterations via immunosuppressive, anti-inflammatory and regenerative effects by upregulating transforming growth factor- β 1, HSP70 and IL-10 mRNA expression. In addition, POM increased reactive oxygen species scavenging activity

by augmenting the antioxidant defense mechanism against CCl₄ hepatotoxicity, as demonstrated by detecting SOD, CAT and GST expression. These results confirm that, at the molecular level, POM exerts hepatoprotective effects against CCl₄-induced oxidative stress and liver tissue damage.

Introduction

Oxidative stress occurs due to the inability of various antioxidant mechanisms to scavenge excessive levels of reactive oxygen species (ROS) and/or a reduction in antioxidant defense mechanisms. Consequently, degenerative diseases, including hepatopathies (1) and nephropathies (2) may occur. The liver and kidneys are the first tissues to be affected by oxidative stress produced by infectious agents, alcohol, drugs, toxic industrial chemicals, food additives, and pollutants in the air and water. Furthermore, free radicals and ROS have a crucial role in the initiation and progression of liver disease and cancer (3).

Carbon tetrachloride (CCl₄) is an industrial solvent, which is extensively used as a xenobiotic to induce chemical liver injury. CCl₄-induced oxidative stress is commonly used in rodent models to determine the protective effects of synthetic or natural products against drug-associated hepatotoxicity and nephrotoxicity (4,5). CCl₄ is metabolized by hepatic microsomal cytochrome P450 into trichloromethyl free radicals. Trichloromethyl can react with sulfhydryl groups (glutathione and protein thiols) and antioxidant enzymes, including catalase (CAT) and superoxide dismutase (SOD) (4). Overproduction of trichloromethyl free radicals may initiate membrane lipid peroxidation, eventually leading to various pathological alterations (6). ROS have an important role in the development and progression of human disease, including liver disorders, lung and kidney damage, diabetes mellitus, atherosclerosis and aging (7), via free radical-induced lipid peroxidation and cell membrane damage (8). Furthermore, CCl₄ causes tissue damage during inflammation, cancer and aging (9). Parallel to oxidative stress, CCl₄ may induce alterations in various pathways, which affect the metabolic and healthy state of subjects, including changes in the gene expression of acute phase proteins, cytokines (inflammatory and anti-inflammatory), and genes associated with lipid metabolism.

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Abbreviations: α -2M, alpha 2-macroglobulin; CAT, catalase; GST, glutathione S-transferase; HSP70, heat shock protein 70; i.p., intraperitoneal injection; IL-6, interleukin-6; IL-10, interleukin-10; SOD, superoxide dismutase; ROS, reactive oxygen species; POM, pomegranate; SREBP-1c, sterol regulatory element-binding protein 1c; TGF- β 1, transforming growth factor- β 1; RT-PCR, reverse transcription-polymerase chain reaction

Key words: pomegranate, carbon tetrachloride, protection, gene expression, hepatic toxicity

It has previously been demonstrated that various natural products can protect organs against CCl₄-induced oxidative stress by enhancing the activities of antioxidant enzymes, including CAT, glutathione S-transferase (GST) and SOD (10). Pomegranate (*Punica granatum*; POM) is widely renowned in the Middle East due to its health benefits (11). POM fruit, juice and peel possess a marked antioxidant capacity, exert anti-obesity and antihypertensive effects, and may be used to treat prostate cancer (12-14). POM contains high levels of polyphenols, particularly ellagitannins, condensed tannins and anthocyanins (15). POM juice consumption has been reported to significantly increase sperm quality, spermatogenic cell density, antioxidant activity and testosterone levels in male rats (16). In addition, POM juice has been proposed to exert chemopreventive, chemotherapeutic, antiatherosclerotic and anti-inflammatory effects (14,17); therefore, its consumption has markedly increased (18,19). It is well known that the liver is the main organ responsible for detoxification and drug metabolism; therefore, the present study aimed to investigate the effects of POM on CCl₄-induced hepatotoxicity. In addition, the molecular mechanisms underlying the effects of POM on CCl₄-induced alterations in the expression of antioxidants, cytokines, inflammatory markers and genes associated with lipid metabolism, as well as hepatic histopathology, were examined.

Materials and methods

Materials. CCl₄ was purchased from Sigma-Aldrich (St. Louis, MO, USA). The DNA ladder (100 bp) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). QIAzol for RNA extraction and oligo-dT primers were purchased from Qiagen, Inc. (Valencia, CA, USA). Rabbit anti-rat heat shock protein 70 (HSP70) primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Experimental animals. Male Wistar rats, weighing 200-250 g, were purchased from the Animal House, King Abdulaziz University (Jeddah, Saudi Arabia). The adult male Wistar rats were divided into four equal groups (n=6/group) in the present study. The rats were kept under observation for ~1 week, in order to acclimate to the environment prior to onset of the experiment. The rats were maintained in stainless steel cages at normal atmospheric temperature (27±5°C), under good ventilation and a 12 h/12 h light/dark cycle. The rats had access to food and water *ad libitum*. All animal procedures were approved by the Ethics Committee of the Dean of Scientific Affairs of Taif University (Taif, Saudi Arabia).

Experimental design and treatments. Group 1 served as a control group, in which the rats were injected intraperitoneally (i.p.) with corn oil (0.5 ml/kg) once daily. Group 2 were administered water daily for 25 days and on days 18 and 20 were injected with 1 ml/kg CCl₄ (50% in corn oil; i.p.). Group 3 were administered POM juice daily [30 ml/kg body weight (BW)], and were injected with corn oil (i.p.) on days 18 and 20. Group 4 were administered POM juice (30 ml/kg BW) daily for 25 days, and were injected with 1 ml/kg CCl₄ (50% in corn oil; i.p.) on days 18 and 20. POM juice was administered to the rats in the morning, 2 h after water deprivation, and the rats consumed

the given dose within 2 h, in order to assure no changes due to environmental conditions. The dose of POM juice was chosen based on a previous study (20).

Sampling. At the end of the experiment, the rats were sacrificed by cervical dislocation following anesthetization by diethyl ether (Sigma-Aldrich) inhalation. The liver tissues of all groups were harvested, homogenized and maintained in either formalin or QIAzol reagent for histopathology and RNA extraction, respectively.

Analysis of gene expression

RNA extraction and cDNA synthesis. Total RNA was extracted from 100 mg tissue samples using QIAzol reagent, according to the manufacturer's instructions. The integrity of the prepared RNA was determined by electrophoresis. RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm (Bio-Rad SmartSpec Spectrophotometer; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The 260/280 optical density ratio of all RNA samples was 1.7-1.9. A total of 2 µg RNA was reverse transcribed using oligo-dT primers and Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (SibEnzyme Ltd., Novosibirsk, Russia). For cDNA synthesis, a mixture of 2 µg total RNA and 0.5 ng oligo dT primer in a total volume of 11 µl sterilized diethylpyrocarbonate (DEPC) water was incubated in a PeX 0.5 thermal Cycler (Bio-Rad Laboratories, Inc.) at 65°C for 10 min for denaturation. Then, 4 µl 5X RT-buffer, 2 µl 10 mM dNTPs and 100 units M-MuLV Reverse Transcriptase (all purchased from SibEnzyme Ltd., Novosibirsk, Russia) was added in a total volume of 20 µl by DEPC water. The mixture was re-incubated in the thermal cycler at 37°C for 1 h, then at 90°C for 10 min in order to inactivate the enzyme.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). RNA from hepatic tissues was analyzed by semi-quantitative RT-PCR using corresponding specific primers for the indicated genes (Table I). The primers were designed using Oligo-4 computer program (version 7.0; Molecular Biology Insights, Cascade, CO, USA) according to the nucleotide sequences published in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>; Table I), and were synthesized by Macrogen Korea (Seoul, South Korea). PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl (10 picomoles) of each primer (forward and reverse), and 12.5 µl PCR Master Mix (Promega Corporation, Madison, WI, USA). The final volume was brought to 25 µl using sterilized, nuclease-free deionized water. PCR was carried out using a PeX 0.5 Thermal Cycler, and the following cycling conditions were used: Denaturation at 94°C for 5 min for one cycle, followed by 27 cycles of denaturation at 94°C for 1 min, annealing at the specific temperature corresponding to each primer (Table I) for 1 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. As an internal reference, glyceraldehyde 3-phosphate dehydrogenase mRNA expression was detected using specific primers (Table I). PCR products subsequently underwent 1.5% agarose gel electrophoresis (Bio Basic Inc., Markham, ON, Canada) in Tris-Borate-ethylenediaminetetraacetic acid buffer at 100 V for 30 min with ethidium bromide staining (Sigma-Aldrich). PCR products were visualized under ultraviolet (UV) light and images

Table I. Sequences and conditions of polymerase chain reaction primers.

Gene	Product size (bp)	Annealing temp. (°C)	Number of PCR cycles	Direction	Sequence (5'-3')
GST	575	55	29	Sense	GCTGGAGTGGAGTTTGAAGAA
				Antisense	GTCCTGACCACGTCAACATAG
SOD	410	55	28	Sense	AGGATTAAGTGAAGGCGAGCAT
				Antisense	TCTACAGTTAGCAGGCCAGCAG
Catalase	652	55.5	30	Sense	GCGAATGGAGAGGCAGTGTAC
				Antisense	GAGTGACGTTGTCTTCATTAGCACTG
α -2M	325	56	30	Sense	GCTCCTGTCTGTTTCCTTAGTT
				Antisense	ATTGGCCTTTTCGTGGTTTAG
IL-6	485	57	32	Sense	AGTTGCCTTCTTGGGACTGA
				Antisense	GAGCATTGGAAGTTGGGGTA
IL-10	259	57	33	Sense	ACCAGCTGGACAACATACTGC
				Antisense	TCATTCTTCACCTGCTCCACT
TGF- β 1	456	58	32	Sense	TGAGTGGCTGTCTTTTGACG
				Antisense	TGGTTGTAGAGGGCAAGGAC
SREBP-1c	191	58	33	Sense	GGAGCCATGGATTGCACATT
				Antisense	AGGAAGGCTTCCAGAGAGGA
G3PDH	309	52	25	Sense	AGATCCACAACGGATACATT
				Antisense	TCCCTCAAGATTGTCAGCAA

GST, glutathione S-transferase; SOD, superoxide dismutase; AGP, ; α -2M, alpha 2-macroglobulin; IL, interleukin; TGF- β 1, transforming growth factor- β 1; FAS, ; SREBP-1c, sterol regulatory element-binding protein 1c; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

were captured using an InGenius 3.0 gel documentation system (Syngene, Frederick, MD, USA). Band intensities from the various rats from each group were quantified densitometrically using ImageJ software version 1.47 (<http://imagej.en.softonic.com/>).

Immunohistochemical staining of HSP70. Tissue sections were deparaffinized and were then treated with 3% H₂O₂ for 10 min, in order to inactivate peroxidases. Subsequently, the sections were heated in 10 mM citrate buffer at 121°C for 30 min for antigen retrieval, blocked in 5% normal serum for 20 min, and were incubated with primary polyclonal anti-HSP70 [1:100 in phosphate-buffered saline (PBS); sc-33575; Santa Cruz Biotechnology, Inc.] overnight at 4°C. After three extensive washes with PBS, sections were incubated with a biotin-conjugated goat anti-rabbit secondary antibody (1:2,000 in PBS; sc-2040; Santa Cruz Biotechnology, Inc.) for 20 min at 32°C. Following a further incubation with horseradish peroxidase-labeled streptavidin (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions, antibody binding was visualized with diaminobenzidine (Sigma-Aldrich) and sections were counterstained with hematoxylin (Sigma-Aldrich). Tissue slides were visualized using a Wolfe S9-0982 microscope (Carolina Biological Supply Co., Burlington, NC, USA) and photos were captured using a Canon Power Shot SX500 IS digital camera (Canon, Tokyo, Japan).

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Statistical analysis for the obtained results was

conducted using one-way analysis of variance followed by the least significant difference test for multiple comparisons among groups. The analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of POM on the hepatic mRNA expression levels of interleukin (IL)-6, sterol regulatory element-binding protein 1c (SREBP-1c) and alpha 2-macroglobulin (α -2M). To examine whether POM was able to modulate cytokine expression in order to exert its hepatoprotective effects, the mRNA expression levels of IL-6 were detected (Fig. 1A). The mRNA expression levels of IL6 were upregulated following CCl₄ injection. Furthermore, when administered alongside CCl₄, POM normalized the expression of IL6 mRNA. In addition, SREBP-1c, a lipogenic transcription factor and key regulator of hepatic lipid metabolism, was detected. The mRNA expression levels of SREBP-1 were significantly upregulated following CCl₄ injection ($P < 0.05$). Furthermore, when administered alongside CCl₄, POM completely normalized SREBP-1c mRNA expression (Fig. 1B). α -2M is known to stabilize target protein and enhance its regeneration. In the present study, α -2M expression was significantly downregulated in the CCl₄ group compared with the control ($P < 0.05$). However, treatment with POM increased α -2M mRNA expression. Concurrent POM administration in the CCl₄-injected group resulted in a further upregulation in α -2M expression (Fig. 1C).

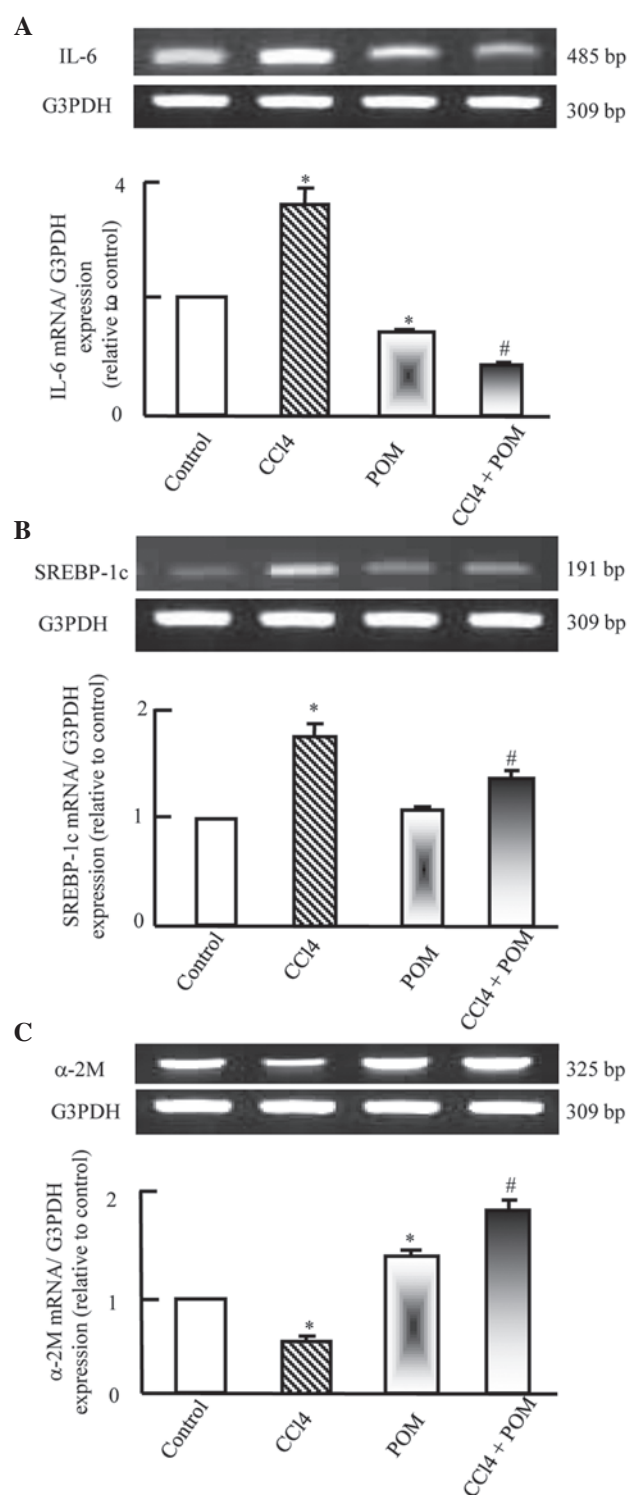


Figure 1. Semi-quantitative reverse transcription-polymerase chain reaction analysis of (A) interleukin (IL)-6, (B) sterol regulatory element-binding protein 1c (SREBP-1c) and (C) alpha 2-macroglobulin (α -2M) mRNA expression in the liver. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal reference. Experimental groups were administered corn oil as a control, carbon tetrachloride (CCl₄), pomegranate (POM), or CCl₄ plus POM. Data are presented as the mean \pm standard error of the mean obtained from three independent experiments. * P <0.05 vs. the control group; # P <0.05 vs. the CCl₄ group.

Effects of POM on the mRNA expression levels of transforming growth factor (TGF)- β 1 and IL-10. The mRNA expression levels of TGF- β 1 were significantly upregulated

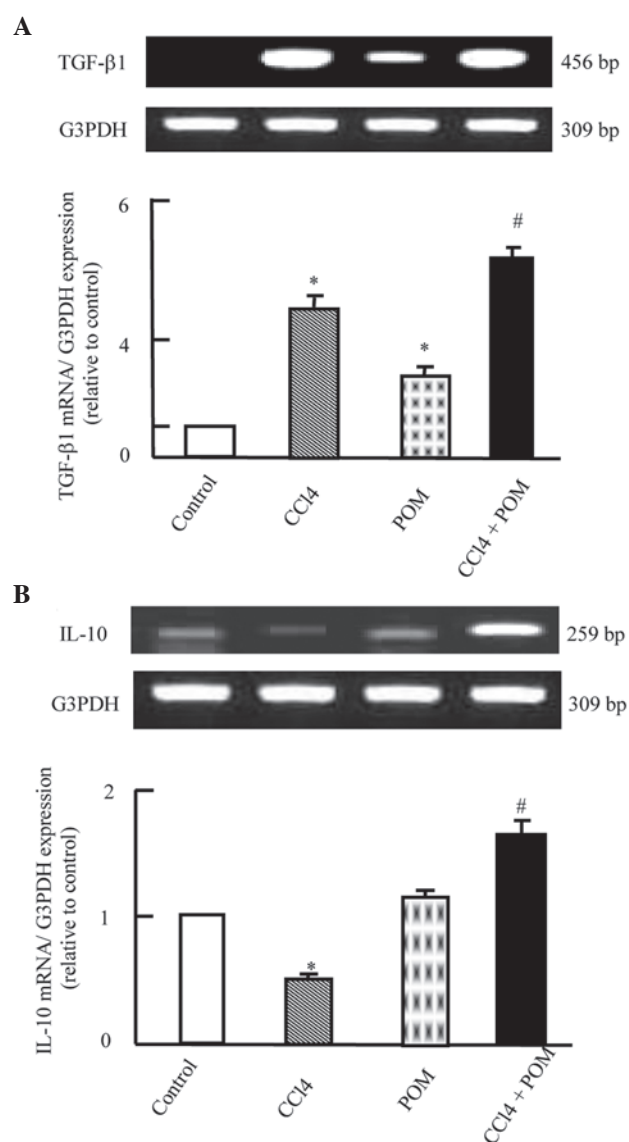


Figure 2. Semi-quantitative reverse transcription-polymerase chain reaction analysis of (A) transforming growth factor (TGF)- β 1 and (B) interleukin (IL)-10 mRNA expression in the liver. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal reference. Experimental groups were administered corn oil as a control, carbon tetrachloride (CCl₄), pomegranate (POM), or CCl₄ plus POM. Data are presented as the mean \pm standard error of the mean obtained from three independent experiments. * P <0.05 vs. the control group; # P <0.05 vs. the CCl₄ group.

in the CCl₄-treated group compared with in the control group (P <0.05). POM treatment alone only slightly upregulated TGF- β 1 mRNA expression, as compared with the control group. However, when the rats were co-treated with CCl₄ and POM the mRNA expression levels of TGF- β 1 were further significantly upregulated compared with in the CCl₄ group (P <0.05; Fig. 2A). Treatment of the rats with CCl₄ significantly suppressed the mRNA expression levels of the anti-inflammatory cytokine IL-10, as compared with the control (P <0.05). However, co-treatment with POM and CCl₄ significantly upregulated interleukin-10 mRNA expression compared with the control and CCl₄-treated groups (P <0.05; Fig. 2B).

Effects of POM on hepatic antioxidant enzyme expression. CCl₄ induced the downregulation of the expression of CAT, GST

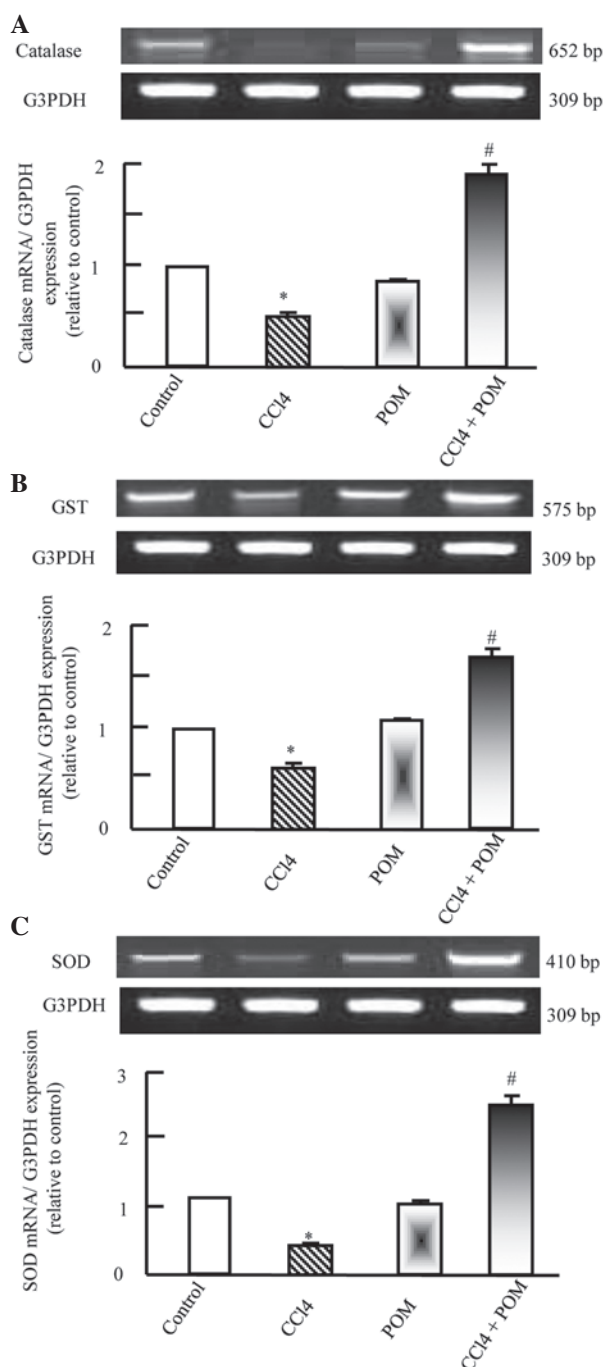


Figure 3. Semi-quantitative reverse transcription-polymerase chain reaction analysis of (A) catalase, (B) glutathione S-transferase (GST) and (C) superoxide dismutase (SOD) mRNA expression in the liver. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal reference. Experimental groups were administered corn oil as a control, carbon tetrachloride (CCl₄), pomegranate (POM), or CCl₄ plus POM. Data are presented as the mean \pm standard error of the mean obtained from three independent experiments. *P<0.05 vs. the control group; #P<0.05 vs. the CCl₄ group.

and SOD, which were normalized following co-administration of POM with CCl₄. Following co-treatment with CCl₄ and POM, the mRNA expression levels of the antioxidant enzymes were completely restored, compared with in the CCl₄ group (Fig. 3A-C).

Effects of POM on hepatic HSP70, as revealed by immunohistochemical staining. Immunostaining of liver

samples from control rats with HSP70 antibodies indicated normal hepatic architecture, with normal hepatic cords, hepatic sinusoids and normal portal area, and an absence of HSP70 expression (Fig. 4A). Liver samples from the CCl₄ group exhibited moderate HSP70 expression in the hepatocytes with increased fat infiltration (Fig. 4B). Liver samples from the POM-supplemented group exhibited a lack of HSP70 expression in hepatocytes, alongside normal hepatic architecture (Fig. 4C). Liver samples from the rats co-treated with CCl₄ and POM exhibited significantly increased HSP70 expression in the hepatocytes (P<0.05), alongside a decrease in the aberrant alterations detected in the CCl₄ group (Fig. 4D).

Discussion

The present study demonstrated that POM exerted a protective effect on the liver against CCl₄-induced oxidative stress. CCl₄ is one of the most commonly used hepatotoxins, and its metabolites trichloromethyl radical (CCl₃) and trichloromethyl peroxy radical (CCl₃COO•) are involved in the pathogenesis of liver and kidney damage (7,21). Both radicals are able to covalently bind macromolecules and induce peroxidative degradation of the cell membrane of liver cells.

IL-6 is produced in the liver by several types of cells, including biliary epithelial cells and cholangiocytes, in response to inflammatory mediators (22,23). IL-6 is a commonly used marker of inflammation, the expression of which has been demonstrated to be markedly increased in the liver of patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (24). In the present study, CCl₄ induced inflammation, as demonstrated by an upregulation in hepatic IL-6 expression. However, downregulation of basal IL-6 mRNA expression levels, and attenuation of the CCl₄-induced upregulation of IL-6 by POM, indicates its anti-inflammatory effects. Parallel with these results, increased levels of the proinflammatory cytokine IL-6 in the ileum of a rat model of necrotizing enterocolitis were normalized following treatment with POM seed oil (25).

SREBP-1c is an important transcription factor, which regulates the hepatic expression of enzymes associated with the *de novo* synthesis of fatty acids (26). Upregulation of the gene expression levels of SREBP-1c has previously been shown to accompany ethanol-induced fatty liver (27). In the present study, CCl₄ induced the upregulation of SREBP-1c mRNA expression, thus indicating the disturbance of lipid metabolism through which CCl₄ may induce liver degeneration. In addition, treatment with POM normalized SREBP-1c hepatic mRNA expression, thus suggesting that the hepatoprotective effects of POM operate via normalization of lipid metabolism. Consistently, a previous study demonstrated that strawberry seed oil was able to decrease SREBP-1c hepatic content, which was associated with reduced plasma triglycerides and a decreased proportion of liver fat (28).

α -2M is the largest major non-immunoglobulin plasma protein, which is synthesized by numerous cell lineages, including lung fibroblasts, monocytes, macrophages, hepatocytes, astrocytes and adrenocortical cells (29). α -2M is expressed in hepatocytes where it has an important role in the regulation of proteolytic activity in the tissue and pericellular space, and contributes to the clearance of α -2M-proteinase complexes

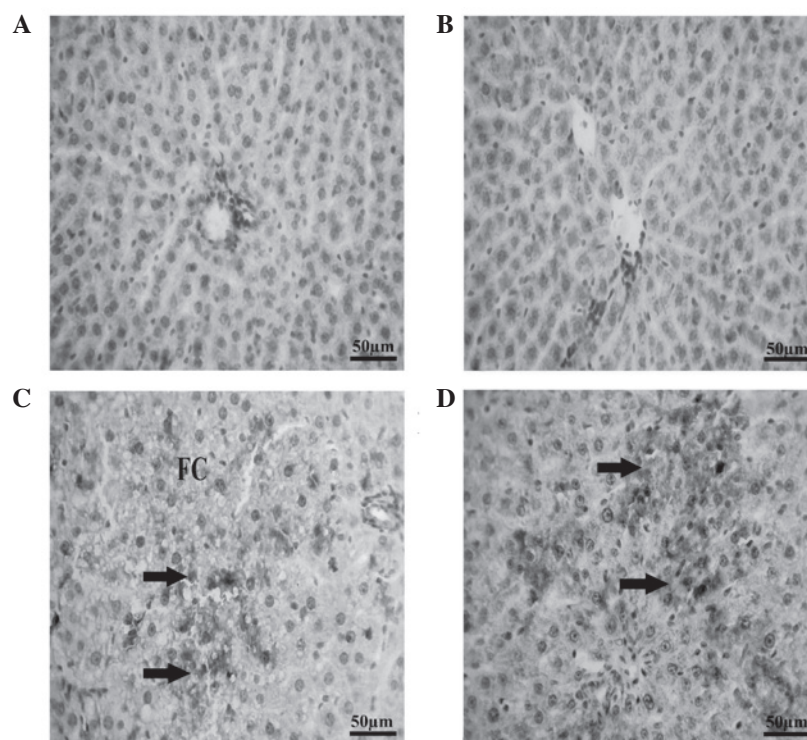


Figure 4. (A) Photomicrograph of a liver sample from a healthy control rat immunostained with heat shock protein 70 (HSP70). HSP70 expression was absent in the hepatocytes, and the sample exhibited normal hepatic cords, hepatic sinusoids (HS) and normal portal area. (B) Photomicrograph of a liver sample from a rat in the carbon tetrachloride (CCl_4) group immunostained with HSP70. Moderate HSP70 expression was detected in the hepatocytes (arrows) with extensive fatty changes (FC). (C) Photomicrograph of a liver sample from a pomegranate-treated rat immunostained with HSP70. HSP70 expression was absent in the hepatocytes, and the sample exhibited normal hepatic cords and HS. (D) Photomicrograph of a liver sample from a rat treated with CCl_4 and pomegranate immunostained with HSP70. Strong HSP70 expression was detected in the hepatocytes alongside regeneration of the degenerative changes. Scale bar=50 μm .

from the circulation (30). In addition, α -2M exerts an inhibitory effect on various types of nonspecific proteases and possesses pronounced immunosuppressive activity (31). The results of the present study demonstrated that treatment with CCl_4 resulted in downregulation of hepatic α -2M mRNA expression compared with the control group. The ability of POM to upregulate basal and CCl_4 -suppressed α -2M mRNA expression indicated that the hepatoprotective effects of POM may operate via upregulation of α -2M expression. Notably, increased α -2M levels have been reported to have an important role in radioprotection via anti-fibrotic, anti-inflammatory, antioxidant, homeostatic, and repair and remodeling mechanisms (32).

TGF- β 1 is a powerful pleiotropic cytokine that possesses immune-suppressing and anti-inflammatory properties (33). In the present study, treatment with POM alone elevated TGF- β 1 mRNA expression levels, thus indicating that the modulatory effects of POM on immune status may be caused by TGF- β 1 upregulation. Treatment with CCl_4 induced the upregulation of TGF- β 1 mRNA expression, the expression of which was further upregulated in the rats co-treated with POM and CCl_4 . These results suggested that the anti-inflammatory effects of POM may be due to TGF- β 1 upregulation. In patient-tolerated kidney or liver allografts, type 1 regulatory T cells were demonstrated to exist, and were capable of suppressing naïve T-cell activation and producing high levels of both IL-10 and TGF- β 1 (34).

IL-10 has been reported to exert both pro- and antitumoral effects through the inhibition of nuclear factor- κB signaling; therefore, it is able to downregulate proinflammatory

cytokine expression (35). IL-10 configures development of the immune response and suppresses proinflammatory cytokine expression (36). In addition, IL-10 induces the downregulation of T helper 1 cytokine mRNA expression, and inhibits IL-1 and IL-6 production (37,38). In the present study, CCl_4 -induced downregulation of IL-10 is consistent with the findings of a previous study, which reported that CCl_4 reduced hepatic IL-10 expression, as compared with in the control group (39). In the present study, POM upregulated IL-10 expression, thus suggesting that POM exerts an anti-inflammatory effect, which protects the liver from CCl_4 -induced inflammation. Notably, previous studies have reported that IL-10 may exert anti-fibrotic effects during CCl_4 -induced hepatic fibrosis (40,41). Therefore, POM may protect the liver from CCl_4 -induced inflammation and fibrosis by upregulating the expression of IL-10 (42).

Delaying or inhibiting the oxidation of easily oxidizable macromolecules, such as lipids, is accomplished by antioxidants, including SOD, CAT and GST, which have a major role in protecting these molecules from the actions of free radicals or ROS (43). Conversion of superoxide to less toxic H_2O_2 is catalyzed by SOD, whereas conversion of H_2O_2 into nontoxic H_2O is catalyzed by CAT (44). The present study demonstrated that administration of POM in CCl_4 -administered rats significantly restored mRNA expression of the antioxidants examined, thus suggesting that POM possesses anti-lipid peroxidation and antioxidative properties. Parallel with these results, *Ocimum sanctum* extract has been shown to cause a significant decrease in lipid peroxidation, coupled with a

significant increase in SOD and CAT expression in the liver homogenates of rats exposed to oxidative stress (45). The present study suggested that CCl₄-induced suppression of SOD mRNA expression may be caused by enhancement of lipid peroxidation or inactivation of antioxidant enzymes, which leads to increased accumulation of superoxide radicals and lipid peroxidation acceleration. However, when administered alongside CCl₄, POM prevented CCl₄-induced antioxidant suppression. These results indicated that the ROS scavenging activities of POM juice may protect the liver from CCl₄-induced oxidative stress. A similar protective effect of flavonoids has been reported in a previous study, which detected the ability of flavonoids to scavenge oxidative radicals in the liver of mice and rats following exposure to CCl₄ (46). In accordance with the present results, POM has been reported to exhibit a promising antioxidant capacity, and is an effective scavenger for several ROS, primarily due to its high levels of phenolic acids, flavonoids and other polyphenolic compounds (47), through which POM may protect against free radical-mediated oxidative stress and attenuate CCl₄-induced antioxidant depletion (48).

HSPs are induced in response to various stressors, in order to protect cells from such effects (49). HSP70 has been reported to protect cells from tumor necrosis factor- α , prostaglandin, hydrogen peroxide, ethanol and UV (50). In the present study, HSP70 expression was slightly increased following treatment with CCl₄, which is parallel to the previously reported induction of HSP70 in acute liver damage by CCl₄ (51). POM administration, together with CCl₄, induced strong HSP70 immunoreactivity, thus preventing protein denaturation, which may act as a mechanism to overcome CCl₄-induced hepatic oxidative stress. Parallel with this assumption, CCl₄-induced liver damage has been shown to be attenuated by pre-induction of chaperones by heat treatment (52).

In conclusion, the present study confirmed that POM exerts hepatoprotective effects against CCl₄-induced oxidative stress. POM was demonstrated to possess anti-inflammatory effects by suppressing CCl₄-induced IL-6 expression, and was able to normalize lipid peroxidation by decreasing CCl₄-induced SREBP-1c expression and increasing CCl₄-suppressed α -2M mRNA expression. In addition, POM may exert hepatoprotective activity by its immunosuppressive, anti-inflammatory and regenerative effects via upregulation of TGF- β 1, HSP70 and IL-10, and may increase ROS scavenging activities by augmenting the antioxidant defense mechanism against CCl₄-induced hepatotoxicity and preventing CCl₄-induced SOD, CAT and GST downregulation.

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