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Pomegranate ameliorates the inflammatory status and oxidative stress in carbon tetrachloride-induced hepatotoxicity in rats.

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ABSTRACT

In Egypt, the highest mortality rates are among patients with hepatic diseases that alter the metabolism of the liver. In recent years, there is great potential for the use of bioactive-rich pomegranate extracts as ingredients in functional foods and nutraceuticals. The present study was focused on the protective effect of pomegranate on the CCl_4-induced dysfunction of rat liver. Rats were divided into five groups. Rats received 1ml/kg distilled water by gavage as a control group. Olive oil and 30% CCl_4 (1ml/kg, i.p) were given every 72hrs for 10 days to rats in the second and third groups, respectively. Rats in the fourth and fifth groups were injected with CCl_4 and received 1ml/kg peels extract or pomegranate juice, respectively for 10 days. CCl_4 causes hepatocyte injury that is characterized by the elevation of serum ALT and AST levels, in addition to activation of the proinflammatory cytokines. CCl_4 administration down-regulates the expression of catalase and glutathione reductase. However, hepatic hemoxygenase-1 (HO-1) was up-regulated at both gene and protein expression levels. The protective effects of pomegranate against hepatotoxicity may, at least in part, be due to its phytochemicals which have demonstrated antioxidant properties and inhibit inflammation and other deleterious processes involved.

Keywords: carbon tetrachloride; hepatotoxicity; oxidative stress; inflammation.

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INTRODUCTION

The liver is the main organ responsible for a multitude of essential functions and plays an essential role in the metabolism of foreign compounds entering the body. In Egypt, liver diseases are one of the most prominent killers specifically hepatitis C virus (HCV) and cirrhosis that alter the metabolism of the liver [1].

Administration of carbon tetrachloride (CCl_4) to rodents is a widely used model to study mechanisms of hepatic injury. CCl_4 hepatotoxicity depends on the reductive dehalogenation of CCl_4 catalyzed by cytochrome P450 in the hepatic endoplasmic reticulum producing unstable trichloromethyl free radical ($^{\bullet}CCl_3$) which is reported as a highly reactive species [2]. In the presence of oxygen/hydrogen, $^{\bullet}CCl_3$ may convert to $^{\bullet}CCl_3OO$, one of highly reactive oxygen species (ROS) [3]. These free radicals bind covalently to unsaturated membrane lipids resulting in increased lipid peroxides followed by pathological changes and finally hepatocyte damage.

In the Holy Qur'an, about nineteen medicinal plants have been identified. Therefore, these plants have been attracted by the botanists, biochemists and pharmacognosists for research purposes [4]. Among these documented plants, pomegranate (*Punica granatum*) has shown profuse pharmaceutical and pharmacological activities against diseases. It also used as foods due to containing high nutritional values. Pomegranate is used in the traditional medicine of different Asian and Ancient Egyptian cultures [5]. The biological activity of pomegranate has been widely investigated, including *in vitro*, *in vivo,* and clinical studies. The beneficial effects are mostly the cardiovascular protective role, neuroprotective activity, hypoglycemic effect, and anticancer properties [6-8].

Realizing the fact, this work was carried out to study the mechanism of hepatoprotective activity of pomegranate peels extract and juice against CCl_4-induced liver damage in rats.

MATERIALS AND METHODS

Carbon tetrachloride (CCl_4), other chemicals and solvents were of analytical grade and were purchased from Sigma Chemical Company. Fresh pomegranates were purchased from local commercial sources (Cairo, Egypt).

Fresh pomegranate fruits were washed and peeled manually where the edible pulp (arils) was blended, while peels (20 g) were left to dry in the shade, extracted and macerated in 200 ml boiled distilled water for 24 hrs. Juice and peels extract were filtrated through Whatman No. 1 filter paper and stored at −20°C for no longer than 1 month [9].

Preliminary Phytochemical Screening

Filtrates were subjected to preliminary phytochemical screening, to identify the chemical constituents. The methods of analysis employed were those described by [10-16].

The total phenolic content in filtrates was determined by the Folin-Ciocalteu method [17] and was expressed as gallic acid equivalents using a gallic acid (10–50 µg/ml) standard curve. The flavonoids content was calculated and expressed as rutin equivalents using a rutin (10–50 µg/ml) standard curve [18,19].

Animals

A total of 50 adult female Swiss albino rats weighing 150–200g were used throughout this study. The animals were purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Helwan, Egypt). Rats were housed in steel mesh cages (5/cage) under a controlled condition of a 12h light/12h dark cycle, temperature (25°C±5) and humidity (50%±10). The animals were maintained for a week acclimatization period on a commercial pellet diet. Food and water were provided *ad libitum*.

Experimental design:

Rats were randomly divided into 5 groups (10 rats each) as follows: **Group I (Con):** Rats served as normal control and received distilled water (1ml/kg body weight) orally for 10 days. **Group II (Olive):** Animals received olive oil (1ml/kg body weight i.p) after every 72hrs. **Group III (CCl_4):** Rats received 30% CCl_4 in olive oil (1 ml/kg body weight i.p) after every 72hrs (the hepatotoxic group) [20]. **Groups IV and V:** 1 ml/kg body weight of peels extract **(Peels)** or pomegranate juice **(Juice)**, respectively, was administered orally for 10 days to CCl_4 injected rats. The animals were maintained in their respective groups for 10 days.

Twenty-four hours after the last administration period, animals were anesthetized by urethane (1 g/kg body weight i.p). Blood samples were taken from the retro-orbital venous plexus using a glass capillary tube after a fast of 12hrs, centrifuged at 4000 rpm for 10 min and stored at -20°C. The liver was excised, rinsed from blood in isotonic sterile saline and immediately frozen in liquid nitrogen for studying the expression of TNF-α, catalase, glutathione reductase (GSH-R), cytochrome P450-2E1 (cytP2E1) and hemeoxygenase-1(HO-1) genes as well as HO-1 activity.

Biochemical Assays

Liver marker enzymes, Alanine aminotransaminase (ALT) and Aspartate aminotransaminase (AST) were determined in serum using the commercial assay kits (Spectrum, Egypt). Serum was examined for proinflammatory cytokines TNF-α and IL-6 using ELISA kit (Quantikine R&D system, USA). Serum level of catalase was determined by the colorimetric method using commercial assay kit (Biodiagnostic, Egypt). Hepatic Heme-oxygenase1activity was determined according to the method of Xia et al., [21]. Briefly, hepatic tissue was incubated with heme (50 mmol), rat liver cytosol (5mg/mL), $MgCl_2$ (2mM), glucose-6-phosphate dehydrogenase (1U), glucose-6-phosphate (2mM), NADPH (0.8mM) in 0.5mL of 0.1M phosphate buffer saline (pH 7.4) for 60 minutes at 37°C. The reaction was stopped by cooling the tubes on crushed ice and the reaction solution was extracted with chloroform. The rate of bilirubin formation was monitored at 464nm and 520nm by a spectrophotometer and then calculated using an extinction coefficient of 40 mM^{-1} cm^{-1}.

Real time - polymerase chain reaction (RT-PCR)

***RNA extraction*:**

Total RNA was isolated from liver tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturer's instruction. The RNA concentration was determined spectrophotometrically at 260nm using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm.

***cDNA synthesis*:**

Exactly 1 µg of RNA was used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Promega, Madison, WI, USA). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with $MgCl_2$ (25mM), RTase buffer (10X), dNTP mixture (10mM), oligo d(t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/µl). This mixture was incubated at 42°C for 1h.

***Quantitative real time PCR (qRT-PCR)*:** was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions (10 min at 95°C followed by 40 cycles of 15s at 95°C and 60s at 60°C). The reaction contained SYBR Green Master Mix (Applied Biosystems), 10 µM gene-specific forward and reverse primers (table 1), cDNA and nuclease-free water. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the β-actin which was used as the control housekeeping gene. Primer sequences are shown in table 1.

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Table 1. Primer sequences applied for gene expression analyses in liver.

Target gene	Primer sequence
TNF-α	Forward: 5' GTAGCCCACGTCGTAGCAAAC 3' Reverse: 5' AGTTGGTTGTCTTTGAGATCCATG 3'
Catalase	Forward: 5' GAATGGCTATGGCTCACACA 3' Reverse: 5' CAAGTTTTTGATGCCCTGGT 3'
GSH-R	Forward: 5' ATCAAGGAGAAGCGGGATG 3' Reverse: 5' GCGTAGCCGTGGATGACTT 3'
cytP2E1	Forward: 5' ACTTCTACCTGCTGAGCAC 3' Reverse: 5' TTCAGGTCTCATGAACGGG 3'
HO-1	Forward: 5' ATCGCATGAAAACAGTCCAG 3' Reverse: 5' CAAATAAGCCCACGGCGAC 3'
β-actin	Forward: 5' TCTGGCACCACACCTTCTACAATG 3' Reverse: 5' AGCACAGCCTGGATAGCAACG 3'

Statistical analysis

The data are presented as mean ± SE. One way analysis of variance (ANOVA) followed by post hoc – least significant difference analysis (LSD) was performed using the statistical package for social science (SPSS) version 16 to compare all the treated groups .The value of $p \leq 0.05$ was considered statistically significant**.**

RESULTS

Preliminary phytochemical studies showed the presence of phenols, tannins and flavonoids in both the juice and peels extract of pomegranate (Tables 2 and 3).

Table 2. Phytochemical screening of pomegranate peel extract and juice

Chemical constituent	Peel	Juice
Alkaloids	+ve	+ve
Glycosides	+ve	+ve
Cardiac glycosides	-ve	-ve
Saponines	+ve	+ve
Phenols	+ve	+ve
Sterols	-ve	-ve
Tannins	+ve	+ve
Flavonoids	+ve	+ve
Diterpenes	+ve	+ve

Table 3. Total phenols and flavonoids concentrations in pomegranate peel extract and juice expressed as Mean±SE.

Chemical constituent	Peel	Juice
Total phenols (mg/g Gallic acid)	200.30±0.50	297.60±0.40
Flavonoids (mg/g Ruttin)	106.60±0.10	88.70±0.08

Data in Table 4 illustrate that administration of CCl_4 to rats caused a significant elevation ($p< 0.001$, 0.0001) in serum ALT and AST levels, respectively, compared to the control group. Parallel elevations in the serum levels of IL-6 and TNF-α were noticed in CCl_4 group ($p< 0.0001$, 0.002, respectively), compared to the control group. Pomegranate peels extract and juice gave rise to a comparable reduction in the serum levels of ALT and AST ($p< 0.001$), as well as serum TNF-α and IL-6 ($p< 0.006$ & 0.007 and 0.008 & 0.01, respectively), compared to the CCl_4 group. Although the expression of TNF-α was significantly up-regulated in all the studied groups (compared to the control group), pomegranate peels extract and juice down-regulated TNF-α expression significantly, compared to the CCl_4 group (Figure 1).

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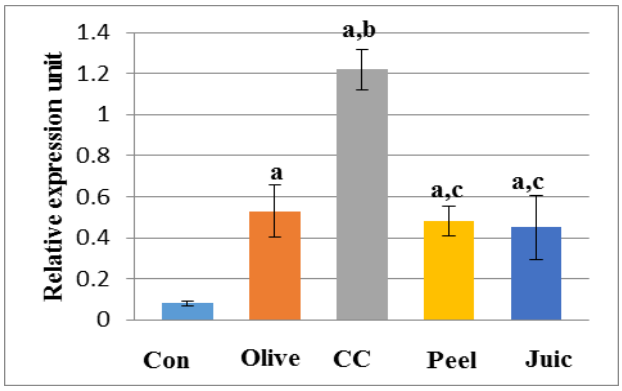

Figure 1. Effects of pomegranate peel extract and juice on the expression levels of hepatic TNF-α. These effects were determined using RT-PCR taking β-actin as an internal control. Data are presented as Mean ± SEM. The effects of peel extract and juice were analyzed using ANOVA followed by least significant difference analysis (LSD) for multiple comparisons. Similar characters denote insignificance between groups. The mean difference is significant at p<0.05. Each group contained 10 rats.

CCl_4 administration induced down-regulation in the expression of both catalase and glutathione reductase as compared to the untreated rats (Figures 2A, B). Moreover, the protein expression of catalase was significantly reduced in serum ($p< 0.01$), compared to the control group (Table 4). Pomegranate peels extract and juice improved both the expression of the previous genes and the serum level of catalase significantly ($p< 0.01$ and 0.03, respectively) as compared to the CCl_4 group.

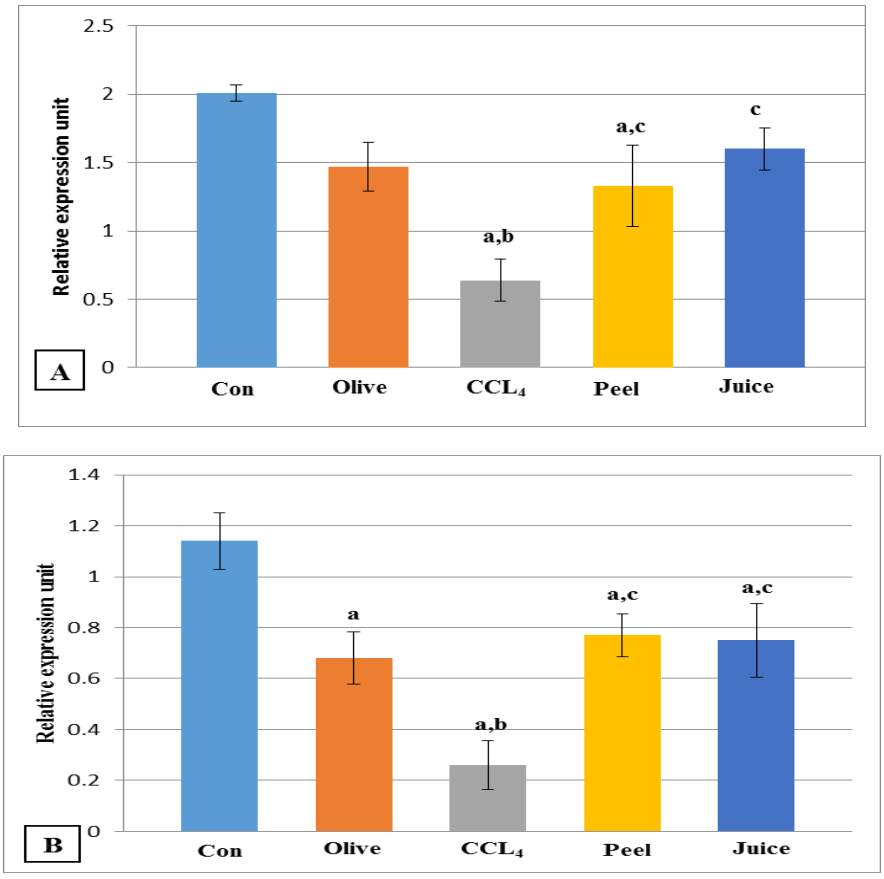

Figure 2. Expression levels of hepatic catalase (A) and GSHR (B) in the different studied groups. RT-PCR was used for determination of the effects of pomegranate on gene expression taking β-actin as an internal control. Data are presented as Mean ± SEM. The effects of peel extract and juice were analyzed using ANOVA followed by least significant difference analysis (LSD) for multiple comparisons. Similar characters denote insignificance between groups. The mean difference is significant at p<0.05. Each group contained 10 rats.

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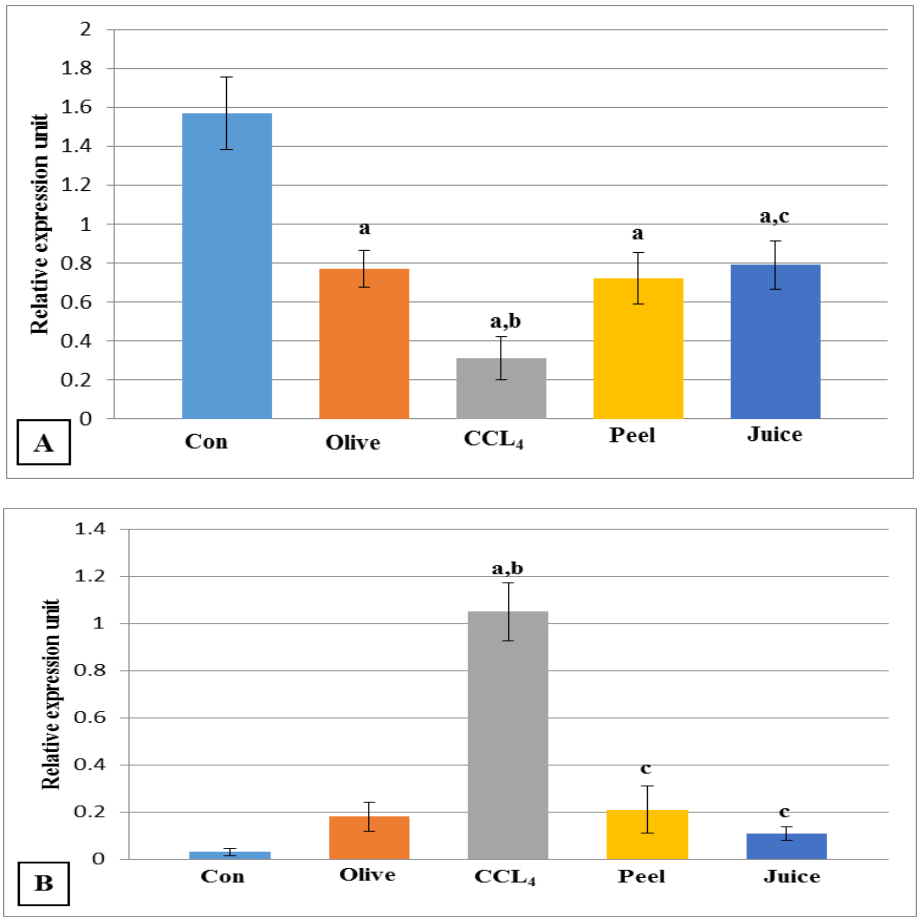

Figure 3. Hepatic cytP2E1 (A) and HO-1(B) gene expression in the different studied groups. Gene expression was determined by using the RT-PCR technique taking β-actin as an internal control. Data are presented as Mean ± SEM. The effects of peel extract and juice were analyzed using ANOVA followed by least significant difference analysis (LSD) for multiple comparisons. Similar characters denote insignificance between groups. The mean difference is significant at $p<0.05$. Each group contained 10 rats.

The hepatic hemoxygenase-1 (HO-1) was significantly up-regulated at both gene and protein expression levels in the CCl_4 group as compared to normal group (Figure 3B and Table 4). Administration of pomegranate peels extract and juice improved the expression levels as compared to the CCl_4 group.

The isoform 2E1 of cytochrome P450 (CYP2E1) was down-regulated in all groups. However, only pomegranate juice improved the expression as compared to the CCl_4 group (Figure 3A).

Table 4. Serum liver markers, pro-inflammatory cytokines and catalase levels in addition to hepatic HO-1 activity in the different experimental groups expressed as Mean±SE.

Parameters / Groups	ALT (U/L)	AST (U/L)	TNF-α (pg/ml)	IL-6 (pg/ml)	Catalase (U/L)	HO-1 (pmol bilirubin /mg/min)
Con	15.67±4.33^a	45.33±4.98^a	44.84±8.62^a	47.92±7.10^a	173.00±14.80^a	13.87±1.63^a
Olive	19.00±4.73^a	59±6.66^a	39.10±3.30^a	43.00±11.80^a	158.33±39.41^a	27.77±4.49^{a,c}
CCl_4	43.67±4.33^b	89±8.50^b	82.30±6.96^b	90.83±16.07^b	64.63±11.55^b	52.50±8.17^b
Peel	17.67±3.48^a	63±3.46^a	48.20±4.97^a	60.88±4.98^a	172.67±22.98^a	30.60±6.56^c
Juice	15.67±4.10^a	55±4.16^a	46.80±8.30^a	61.30±4.30^a	155.67±30.78^a	26.23±2.64^{a,c}

- For each parameter similar characters denote insignificance between groups.
- The mean difference is significant at $p<0.05$.
- Each group contained 10 rats.

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DISCUSSION

CCl_4-induced hepatic injuries are commonly used animal models for the screening of hepatoprotective plant extracts. CCl_4-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl_4 by cytochrome P450 to $^{\bullet}CCl_3$ and/or $^{\bullet}CCl_3OO$ which leads to lipid peroxidation and finally necrosis [22,23]. The second phase involves the activation of Kupffer cells, probably by free radicals, accompanied by the production of proinflammatory mediators [24]. As a result of the hepatic injury, the altered permeability of the membrane causes the enzymes from the cells to be released into circulation, as shown by the abnormally high level of serum hepatospecific enzymes, ALT and AST, which were increased markedly after the CCl_4 injection, but these increases were attenuated by pomegranate peels extract and juice. These results indicate that pomegranate preserves the structural integrity of the hepatocellular membrane and protects the liver against CCl_4-induced hepatotoxicity.

It has been suggested that the hepatic necrosis caused by CCl_4 involves bioactivation by a microsomal cytochrome P450–dependent monooxygenase system, resulting in the formation of trichloromethyl free radicals and ROS, which initiate lipid peroxidation and protein oxidation leading to hepatocellular membrane damage [25]. This process is followed by the release of inflammatory mediators from the activated hepatic macrophages, which are believed to potentiate the CCl_4-induced hepatic injury [26]. In the present study, the serum level of the proinflammatory cytokine TNF-α was increased after CCl_4 administration with a concomitant increase in its gene expression. TNF-α potentiates the apoptotic signaling cascade from ROS-damaged mitochondria [27]. Moreover, elevated expression of TNF-α also stimulates the production of another pro-inflammatory cytokine, IL-6 [28]. Baranova and colleagues [29] reported that hepatic IL-6 expression correlates positively with the plasma IL-6 levels and the degree of hepatic inflammation. On the contrary, Zuinen et al., [30] and Gewiese-Rabsch et al., [31] stated that IL-6 binds to both membrane-bound IL-6 receptor and soluble IL-6 receptor to trigger NF-κB and STAT3-dependent hepatic regeneration.

Evidence for the clinical benefits of pomegranate has been reported in some studies [32-34], but few studies that have assessed its impact on inflammation have been conducted *in vitro* or used pomegranate extract [35,36]. The elevation in the pro-inflammatory cytokines was attenuated by pomegranate, which greatly reduced the expression level of TNF-α induced by CCl_4 exposure at the transcriptional level. This finding is consistent with other reported studies using different antioxidants [37]. In another study, the peels extract demonstrated down-regulation in the expression of inflammatory gene IL-6 in colonic and adipose tissues from obese mice fed a high-fat diet [38].

Many hepatotoxicants including CCl_4 require metabolic activation, especially by liver cytochrome P450 enzymes, to form reactive, toxic metabolites, $^{\bullet}CCl_3$ and $^{\bullet}CCl_3OO$, that in turn cause oxidative stress in the liver in experimental animals and humans [39]. Although several isoforms of P450 may metabolize CCl_4, attention has focused largely on the P450 2El isoform. In the present study expression of cyt P2El isoform was down-regulated in the CCl_4 group as an adaptive mechanism to limit hepatotoxicity [40].

The covalent binding of $^{\bullet}CCl_3$ to cell protein is considered the initial step in a chain event eventually leading to elicits membrane lipid peroxidation [41]. Moreover, $^{\bullet}CCl_3$ reacts with sulfhydryl group in glutathione and protein thiols as well as antioxidant enzymes such as catalase [42], explaining the present results which demonstrate that serum catalase level was reduced in CCl_4 group. In addition, the expression of antioxidant enzymes, catalase and glutathione reductase were also down-regulated in the CCl_4 group.

Phytochemicals of pomegranate such as phenolic acids, flavonoids and tannins have demonstrated antioxidant properties and inhibit inflammation and other deleterious processes involved in degenerative diseases. The pomegranate peels extract and juice were able to prevent CCl_4-induced decay of antioxidant enzyme activities. A similar scavenger role of flavonoids in mice and rats after exposure to CCl_4 had been determined in liver [43,44].

Heme oxygenases (HO) catabolize free heme into equimolar amounts of Fe^{2+}, carbon monoxide (CO), and biliverdin [45]. HO-1 is induced by oxidative stress, cytokines and other mediators produced during inflammatory processes, likely as part of a defense system in cells exposed to stress to provide a negative feedback for cell activation and the production of mediators, which could modulate the inflammatory

response. HO-1 activity results in the inhibition of oxidative damage and apoptosis, with significant reductions in inflammatory events including the production of inflammatory cytokines [46]. Wen et al., [47] and Park et al., [26] reported that the administration of CCl_4 resulted in a time-dependent increase in hepatic HO-1 activity, which reached a maximum at 24h. This increase was accompanied by the rapid and significant induction of HO-1 protein expression, which occurred in the same time-dependent manner following CCl_4 administration. HO-1 is thought to play an important cytoprotective role against oxidative cellular injuries [48]. In this study, pomegranates peels extract or juice, reduced the HO-1 at the molecular and protein levels. These reductions may be due to the improvement in the oxidative state reached by the antioxidants of pomegranate.

In conclusion, these results show that the protective effects of pomegranate against the CCl_4-induced hepatotoxicity may, at least in part, be due to its phytochemicals such as phenolic acids, flavonoids and tannins which have demonstrated antioxidant properties and inhibit inflammation and other deleterious processes involved in degenerative diseases.

Conflict of interest

The author declares that she has no financial/commercial conflicts of interest.
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