Hepatoprotective Role of the Pomegranate (*Punica Granatum*) Juice on Carbon Tetrachloride-Induced Oxidative Stress in Rats

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Abstract: In the present study, the possible protective role of pomegranate (*Punica granatum*) on the free radical damage of liver caused by carbon tetrachloride in rats was investigated. Treatment of rats with carbon tetrachloride (2 ml/kg, intraperitoneally) produced severe liver injury, as demonstrated by dramatic elevation of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT) and Total bilirubin (TB) levels and typical histopathological changes including hepatocyte necrosis and apoptosis. In addition, carbon tetrachloride (CCl_4) administration caused oxidative stress in rats, as evidenced by increased malondialdehyde (MDA) and Nitrite/nitrate (NO) concentrations in the serum and liver of rats, along with a remarkable reduction in hepatic catalase (CAT), superoxide dismutase (SOD), glutathione transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) activities and GSH content. However, simultaneous oral treatment with *P. granatum* significantly attenuated carbon tetrachloride-induced hepatotoxicity. It ameliorated most biochemical markers tested as well as histopathological, apoptosis and necrosis features. It is therefore suggested that *P. granatum* can provide a definite protective effect against chronic hepatic injury caused by CCl_4 in rats, which may mainly be associated with its antioxidative effect.
[Hany M. Yehia, Ebtesam M. Al- Olayan, Manal F. Elkhadragy. **Hepatoprotective Role of the Pomegranate (*Punica Granatum*) Juice on Carbon Tetrachloride-Induced Oxidative Stress in Rats.** *Life Sci J* 2013; 10(4): 1534-1544]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 202

Keywords: Carbon tetrachloride; *Punica granatum*; oxidative stress; liver.

1.Introduction

The liver is a vital organ that plays a key role in many toxication cases (Karakus *et al.,* 2010), and various pathological factors such as fatty livers, hepatic virus and chemical hepatotoxins induced liver injury (Xie *et al.,* 2010). Carbon tetrachloride (CCl_4) is a well established hepatotoxin. Previous studies showed that both liver and kidneys are the target organs of CCl_4. Extensive evidence demonstrates that CCl_4 is activated in the liver to highly reactive trichloromethyl radical which initiates free radical-mediated lipid peroxidation of the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane, which leading to accumulation of lipid-derived oxidants causing liver injury (Singh, 2008). CCl_4-induced damage is also able to alter the antioxidant status of the tissues, which is manifested by abnormal histopathological changes (Rajesh and Latha, 2004). Which trichloromethyl radical can react with sulfhydryl groups of glutathione (GSH) and protein thiols. In addition, CCl4 also alters the antioxidant profile of the liver including the antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione transferase (GST) (Srivastava, Shivanandapp, 2006). Histopathologically, exposure to CCl_4 can results in hepatic steatosis, centrilobular necrosis, and cirrhosis in the liver (Xu *et al.,* 2010).

There is an intimate relationship between nutrition and the antioxidant defense system, as some exogenous low molecular weight antioxidants may be supplied by the diet. These two main systems of the antioxidant defense act in coordination, their levels being regulated by each other, to avoid oxidative stress events (Masella *et al.,* 2005). In the past few years, a considerably large group of molecules widespread in plants has come into focus.

Pomegranate (*Punica granatum*) is one of the oldest edible fruit and belongs to the Punicacea family (Fadavi *et al.,* 2006). Pomegranate is extensively cultivated in the Mediterranean area and most Near- and Far East countries. This botanic isolation is coincident with a unique biochemistry, the seeds contain an oil of which about 80% is a rare trans 18 carbon fatty acid (punicic acid) (Schubert *et al.,* 1999), and possess the highest botanical concentration of a sex steroid, estrone, at 17mg/kg dried seed (Kim *et al.,* 2002). Pharmacological properties of pomegranate extracts have been scrutinized, with anti-microbial, anti-parasitic, anti-viral, and anti-cancer effects noted (Kim *et al.,* 2002). The fermented juice is potently

antioxidant and oil polyphenols inhibit eicosanoid enzymes cyclooxygenase and lipoxygenase (Schubert *et al.,* 1999). An extract of the flowers lowers blood sugar in rodents (Jafri *et al.,* 2000), and the fresh juice inhibits LDL oxidation and atheromatous plaque formation in rodents and humans (Aviram *et al.,* 2000) . The juice contains flavonoids including anthocyanins and phenolic acids, and the pericarps tannins and ellagitannins (Ben Nasr *et al.,* 1996). Flavonoids (Caltagirone *et al.,* 2000) and tannins (Wang *et al.,* 1999) inhibit cancer cell growth *in vitro* and *in vivo*, while the class of 18C trans fatty acids known as conjugated linoleic acid, structurally related to punicic acid, possess cancer arrestive properties (Igarashi and Miyazawa, 2000). Thus, the present study aims to investigate the hepatoprotective role of *P. granatum* aqueous juice against CCl_4-induced liver toxicity in rats.

2. Materials and Methods

2.1. Chemicals

Carbon tetrachloride (CCl_4), Tris-HCl buffer and 50, 50-dithiobis-2-nitrobenzoicacid (DTNB), were purchased from Sigma (St. Louis, MO, USA). Perchloric acid, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Merck. All other chemicals and reagents used in this study were of analytical grade. Double-distilled water was used as the solvent. In addition, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), total bilirubin (TB), alkaline phosphatase (ALP), nitric oxide (NO), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) commercial kits (Biodiagnostic, Egypt) were used.

2.2. Animals

Adult male Wistar albino rats weighing 120–150g (7-9 weeks) were obtained from The Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). Animals were kept in wire bottomed cages in a room under standard condition of illumination with a 12-hours light-dark cycle, 55+5% relative humidity and at 25±2^{o}C room temperature. They were provided with balanced standard pellet (VACSERA) as a diet and sterile water *ad libitum*. The animals were kept in clean and a low stress environment and an enclosed door provided a substantial amount of sound proofing. Stressful cage motion, frequent cage handling and other uncontrolled stress-induced practices were avoided. The experiments were approved by the state authorities and followed Egyptian rules on animal protection.

2.3. Pomegranate juice preparation

Ten kg of pomegranates (*Punica granatum*) were washed and manually peeled, without separating the seeds. Juice was obtained using a commercial blender (Braun, Germany), filtrated with a buchner funnel and immediately diluted with distal water to volume of 1:3 and stored at 20 ºC for no longer than 2 months (Faria *et al.,* 2007).

2.4. Experimental protocol

To study the protective effect of pomegranate on carbon tetrachloride mediated reproductive toxicity, twenty four adult male Wistar albino rats were randomly allocated to four groups of six rats each. Group I (Con) served as control and received 300 µl of saline by intraperitoneal (i.p.) injection route each week. Group II (CCl_4) received weekly i.p. injection of 2 ml CCl_4/kg body wt. for 10 weeks as described by Sohn *et al.* (Sohn *et al.,*1991). Group III (pomegranates; *P. granatum*) received juice supplied on dark water bottles and renewed every 2-3 days (Faria *et al.,* 2007). Their average daily intake of fluid was 5.2 to 6.5 ml and the animals of group IV (CCl_4+ *P. granatum*) received *P. granatum* juice as group III for 2 weeks before CCl_4 treatment. The group IV was then interperitoneally injected with 2 ml CCl_4/kg for 10 weeks. After one week of the last i.p. injection of CCl_4, the animals of all groups were cervically dislocated and blood samples were collected from retro-orbital plexus of all rats. Followed by standing for half an hour and then centrifuged at 500 g for 15 min at 4^{0}C to separate serum and stored at -70^{0}C. Part of livers were weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl and 300 mM sucrose (Tsakiris *et al.,* 2004). The homogenate was centrifuged at 500 g for 10 min at 4^{0}C. The supernatant (10%) was used for the various biochemical determinations.

2.5. Biochemical estimations

2.5.1. Liver function test

Serum and liver alanine aminotransferase (ALT), aspartate aminotransferase: (AST). Colorimetric determination of ALT or AST was estimated by measuring the amount of pyruvate or oxaloacetate produced by forming 2, 4-dinitrophenylhydrazine. The color of which was measured at 546 nm according to (Reitman and Franke, 1957). γ-glutamyl transpeptidase (γGT) and alkaline phosphatase were assayed in homogenate of liver using kits provided from Biodiagnostic Co. (Giza, Egypt). Also, Total bilirubin (TB) of serum was assayed according to the method of Schmidt and Eisenburg (Schmidt and Eisenburg, 1975).

2.5.2. Determination of nitrite/nitrate and malondialdehyde levels

Nitrite/nitrate (NO) and malondialdehyde (MDA) were assayed colorimetrically in liver homogenate according to the method of Berkels *et al.,* (2004) and Ohkawa *et al.,* (1979), respectively, where MDA determined by using 1 ml of trichloroacetic acid 10%

and 1 ml of thiobarbituric acid 0.67% and were then heated in a boiling water bath for 30 min. TBARS were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) formed. Nitric oxide determined where in acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide is coupled with N-(1–naphthyl) ethylenediamine. The resulting azo dye has a bright reddish – purple color which can be measured at 540 nm.

2.5.3. Estimation of reduced glutathione and anti-oxidant enzymes

The hepatic and serum reduced glutathione (GSH) were determined by the methods of Ellman, (1984). The method based on the reduction of Elman's reagent (5,5` dithiobis (2-nitrobenzoic acid) "DTNB") with GSH to produce a yellow compound . The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm. In addition, the levels of hepatic antioxidant enzymes as catalase (CAT) was assayed by the method of Aebi (1984). As a result, catalase reacts with a known quantity of H_2O_2. The reaction is stopped exactly after one minute with catalase inhibitor. In the presence of peroxidase (HRP), the remaining H_2O_2 reacts with 3,5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity that is inversely proportional to the amount of catalase in the original sample.

Hepatic superoxide dismutase (SOD) activity was assayed by the method of Nishikimi *et al.* (1979). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. Glutathione-S-transferase (GST) activity in the liver was assayed by the method of Habig *et al.* (1974). The total GST activity was done by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample.

Hepatic glutathione peroxidase (GPx) activity was measured by the method of (Paglia and Valentine, 1967) and the assay was an indirect measurement of the activity of GPx. Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by GPx, was recycled to its reduced state by the enzyme glutathione reductase (GR). The reaction was initiated by the addition of hydrogen peroxide, and the oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. Glutathione reductase activity of liver was assayed by the method of Factor *et al.* (1998). Glutathione reductase catalyzes the reduction of glutathione in the presence of NADPH, which is oxidized to NADPH+. As a result, the decrease in absorbance at 340 nm was measured.

2.6. Flow cytometry

Tissue samples were prepared by manual disaggregation procedure. Briefly, a few drops of RPMI were added to tissue and then minced until complete tissue disaggregation was achieved. Suspended cells were filtered using a 50 µm pore size mesh and then centrifuged at 1000 rpm for 10 min. Cells were resuspended in PBS, counted and washed by calcium buffer then centrifuged at 1500 rpm for 5 min. The pellet was resuspended and then cells were counted. Annexin-PI apoptotic assay was carried out using IQP-120F Kit (IQ Products, Groningen, Netherlands). FAC scan Becton-Dickinson (BD) flow-cytometer was used and data were analyzed using cell Quest software.

2.7. Histopathological examination

Conventional techniques of paraffin-wax sectioning and haematoxylin–eosin staining were used for histological studies (Drury and Wallington, 1981). Slices of fresh liver tissues were cut and fixed in buffered neutral formalin fixative for 24 h. Following fixation, the tissues were washed and processed through an ascending series of alcohol (30%, 50%, 70%, 90% and 100%), cleared in methyl salicylate and infiltrated with wax at 57 °C. The tissues thus cleared were embedded in paraffin. Sections of 5 µm thickness were cut, stained by aqueous haematoxylin and alcoholic-eosin and examined by Nikon microscopy at a magnification of 400×.

2.8. Statistical analysis

The obtained data were presented as means ± standard error. One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Duncan's test using a statistical package program (SPSS version 17.0). All *P*-values are two-tailed and $P<0.05$ was considered as significant for all statistical analysis in this study.

3. Results

Levels of the serum marker enzymes of hepatic damage, ALT, AST, γ-GT, ALP and total bilirubin increased significantly in CCl_4 treated rats (120.65%, 119.48%, 25.87%, 65.38% and 58.89%, respectively) compared to the control group. In contrast, *P. granatum* at 2 ml/rat prevented the liver damage as judged by the decreased enzyme levels (Figures 1 & 2) as compared to CCl4-induced liver damage (ALT; 71.63%, AST; 35.96%, γ-GT; 10.41%, ALP; 19.23% and TB; 16.09%, respectively). In addition, pomegranate administration alone caused a non-significant change in all liver function enzymes except for ALP where it was diminished significantly (-34.6%, $p<0.05$).

Moreover, treatment of pomegranate alone caused a significant decrease in nitric oxide

concentration in both serum and liver homogenate, this decreased was -28.6% for serum and -21.1% for hepatic tissue. The effect of *P. granatum* on CCl_4-induced lipid peroxidation and nitric oxide elevation in the liver is shown in Figure 3. CCl_4 increased the serum and hepatic MDA and NO concentration significantly (serum MDA was increased significantly by 93.42% and hepatic MDA by 54.37%; serum and liver nitric oxide elevated significantly by 113.68% and 51.39%, respectively), this increased which was inhibited by *P. granatum* treatment to 37.87% and 29.27% for serum and hepatic MDA and to 66.01% and 20.22% for serum and liver nitric oxide, respectively.

The hepatic antioxidant enzyme activities were decreased in the liver of rats administered with CCl_4 (CAT; -57.0%, SOD; -54.1%, GPx; -80.2% GR; -26.2%, and GST; -80.4%). Activities of SOD, CAT, GPx, GR and GST were changed positively when rats treated with pomegranate juice where the activity of GR restored by Pom juice treatment. Also, SOD and GPx activities and GSH contents in both liver homogenate and serum were increased significantly when compared with CCl_4 group. However, pomegranate treatment filled to reduce the toxic effect of CCl_4 in both CAT and GST. Further, Pom juice treatment, by itself, significantly boosted the antioxidant enzyme activities as CAT and SOD in the liver, as well as, GSH contents in both serum and liver homogenate (Table 1 and Figure 4).

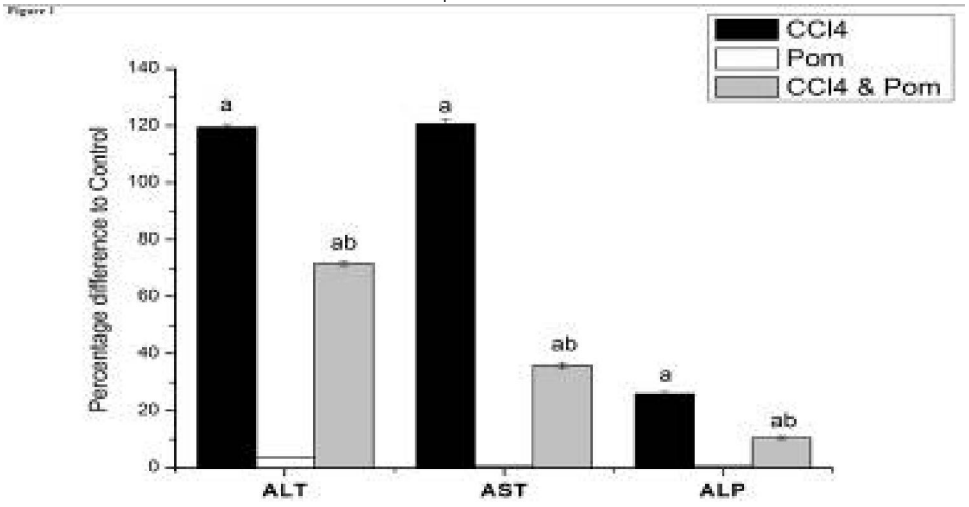


Figure 1. Effects of pomegranate juice on serum ALT, AST levels and hepatic ALP activity in rats treated with CCl_4 (2 ml CCl_4/kg body wt. for 10 weeks)

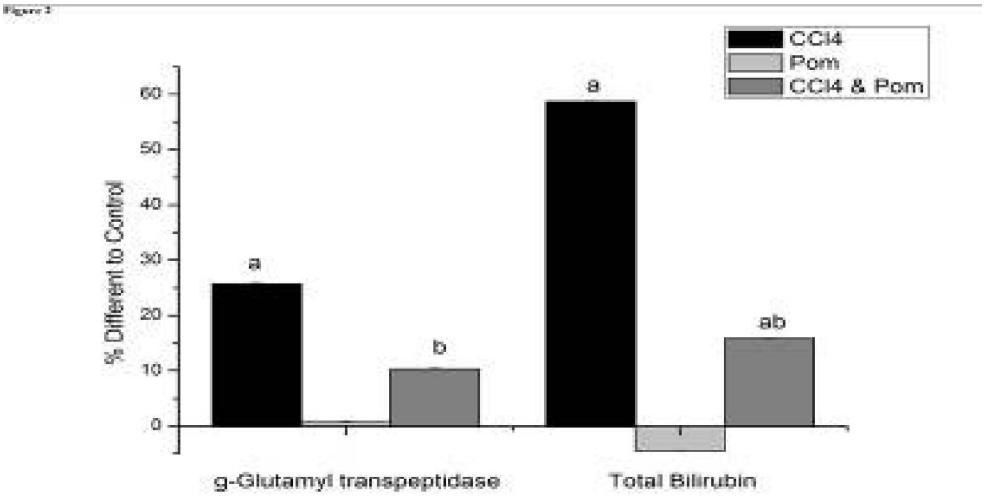


Figure 2. Protective role of pomegranate juice on hepatic γ-GT activity and serum TB level in rats treated with CCl_4 (2 ml CCl_4/kg body wt. for 10 weeks)

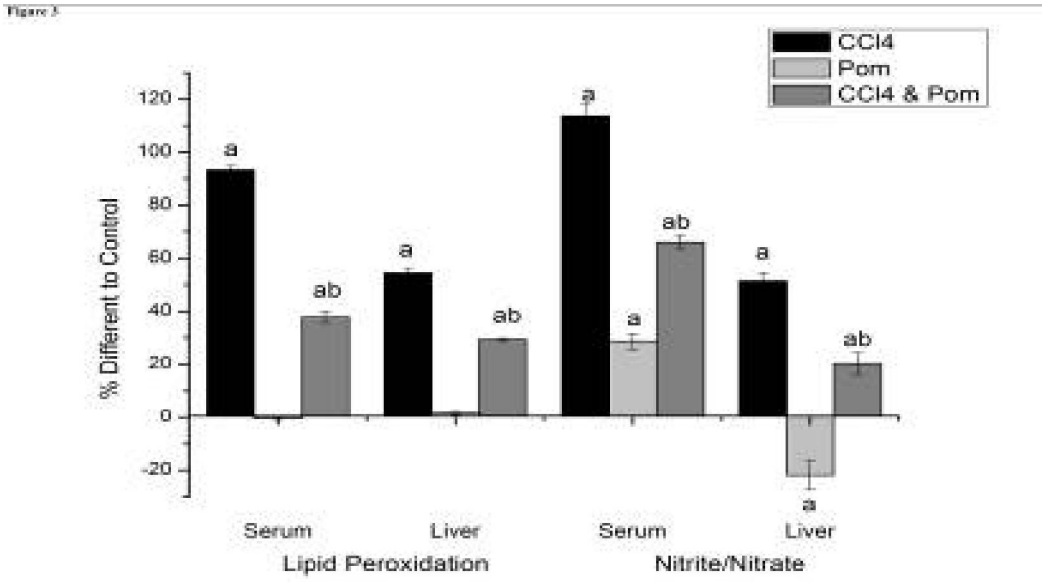


Figure 3. Ameliorative effects of pomegranate juice on serum and hepatic MDA and NO levels in rats treated with CCl_4 (2 ml CCl_4/kg body wt. for 10 weeks)

Table 1. Changes in the levels of GST, GPx and GR in liver homogenate of male rats treated with carbon tetrachloride (CCl_4), Pomegranate (Pom) and CCl_4+Pom.

Groups	**GST** (µmol/h/ g tissue)	**GPx** (U/g tissue)	**GR** (µmol/ g tissue)
Con	0.64±0.02	1553.6±89.03	10.25±0.90
CCl_4	0.12±0.01[a]	302.6±16.36[a]	7.57±0.41[a]
Pom	0.64±0.02	1459.0±71.94	11.79±0.49
CCl_4+Pom	0.22±0.01[a]	798.1±59.92[ab]	8.54±0.49

Values are means ±SE (n=6). a: Significant change at $p < 0.05$ with respect to Con, b:significant change at $p < 0.05$ with respect to CCl_4.

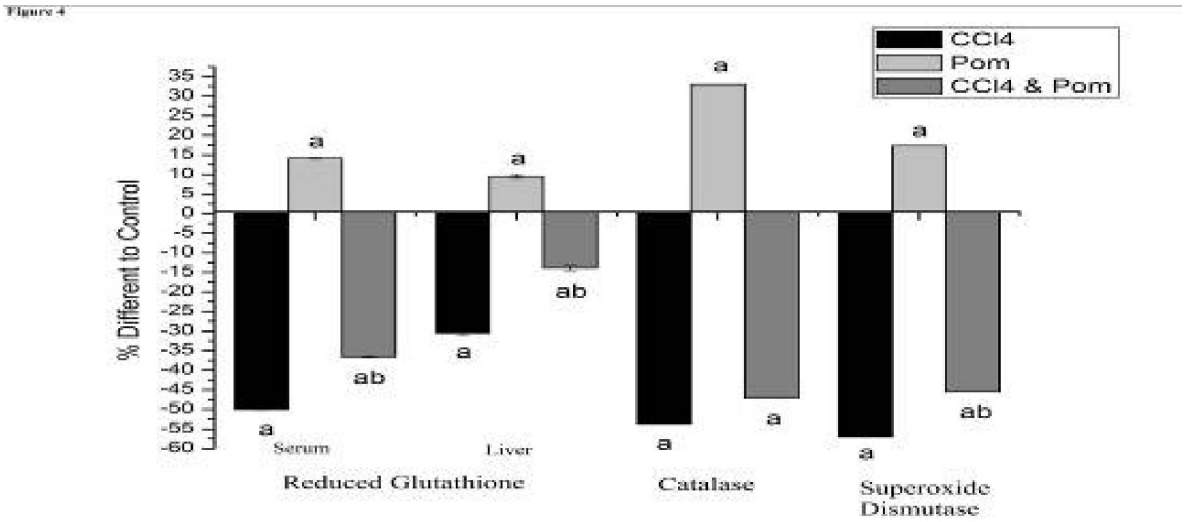


Figure 4. The protective effects of pomegranate juice on serum and liver GSH levels and hepatic CAT and SOD activity in rats treated with CCl_4 (2 ml CCl_4/kg body wt. for 10 weeks)

Figure 5

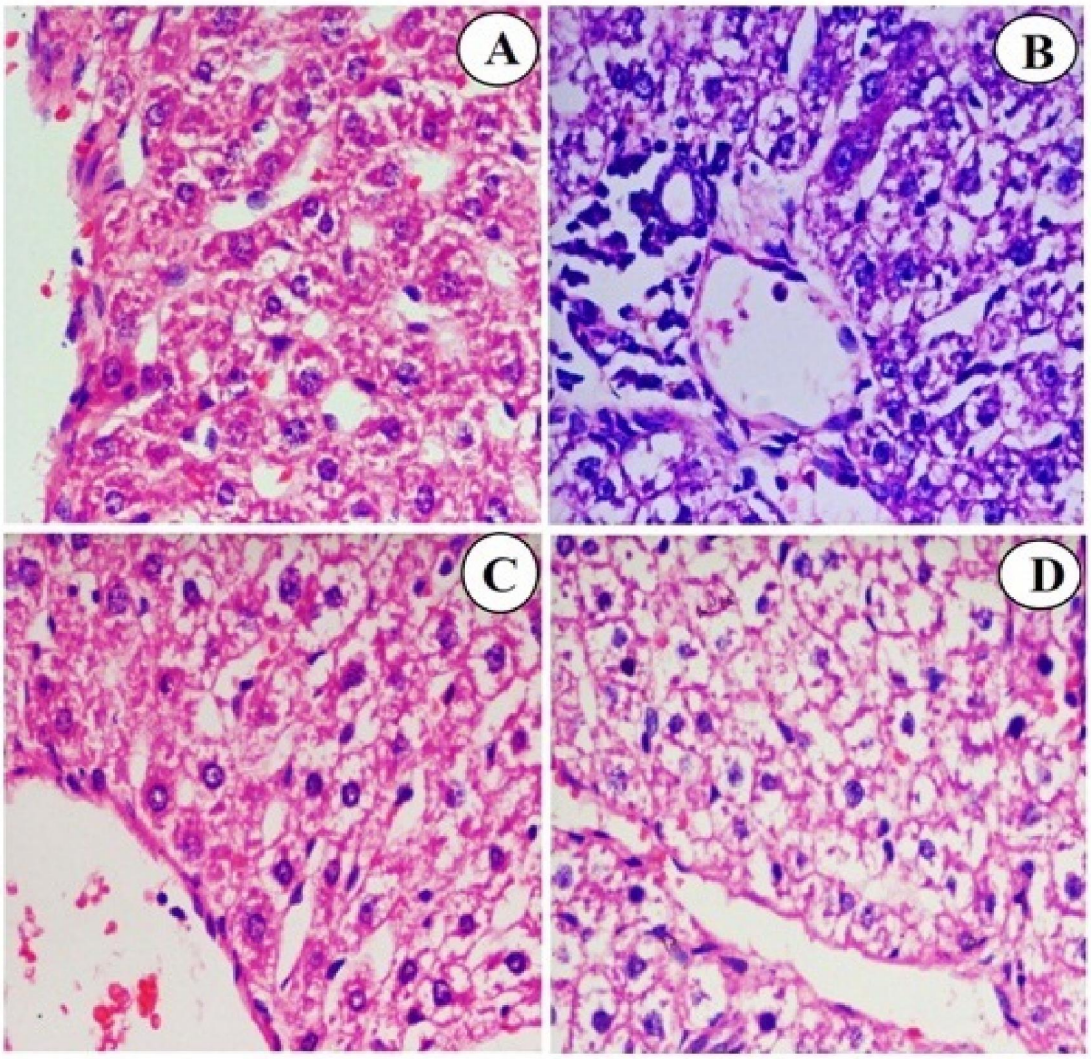
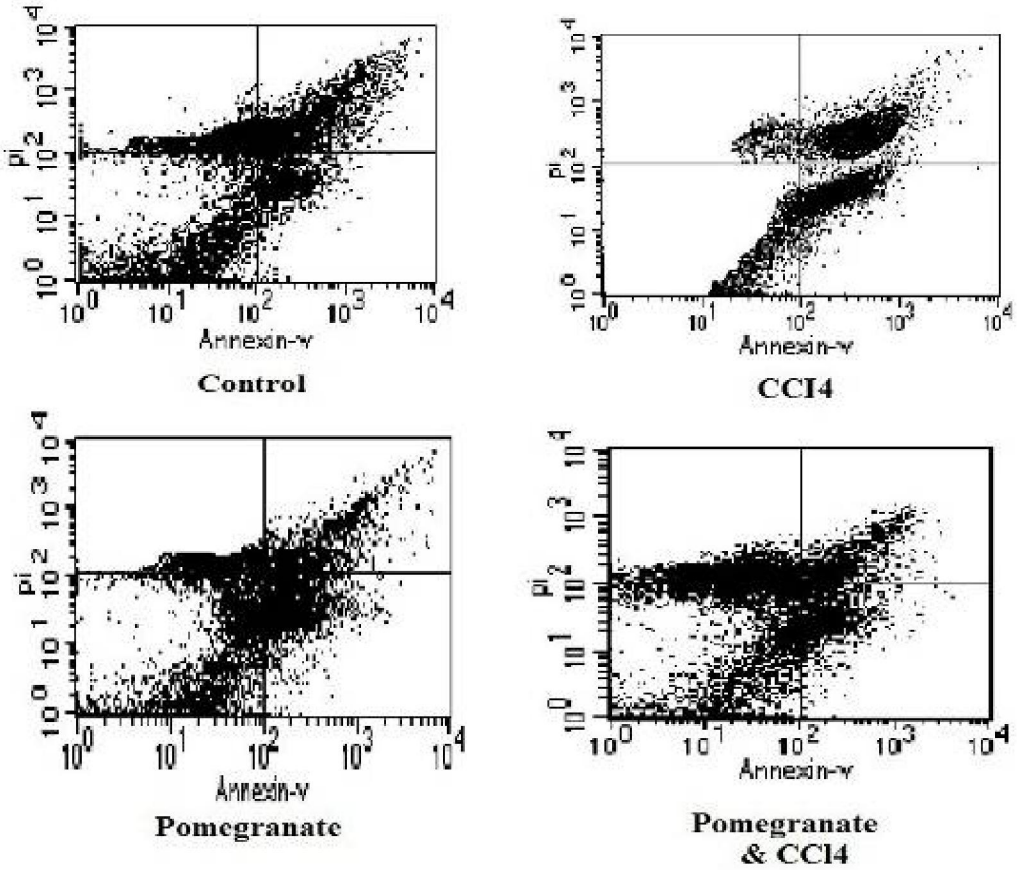


Figure 5. Effect of pomegranate juice on histopathological damages induced by CCl_4 in the liver of rats. Liver sections were stained using the hematoxylin–eosin method. (A) The control rats, without any signs of liver damage. (B) After 10 weeks following CCl_4 treatment (2 ml CCl_4/kg body wt.), liver sections with massive hepatocyte degeneration, apoptosis or necrosis, and inflammatory cells infiltration. (C) Pomegranate juice treatment, liver section with healthy hepatocytes more or less like normal. (D) CCl_4+ pomegranate juice, the liver sections showed reduced signs of liver damage. Original magnifications 400×.

Figure 6



Group	Viable Hepatocytes	Apoptotic Hepatocytes	Dead Hepatocytes	Necrotic Hepatocytes
Control	21.6±1.1	47.6±2.2	18.3±1.2	12.5±0.9
CCl4	10.7±0.8[a]	61.7±2.7[a]	4.3±0.6[a]	26.7±1.2[a]
Pomegranate	28.1±1.4[a]	47.8±2.1	10.3±0.7[a]	13.8±0.8
Pomegranate &CCl4	14.8±0.9[ab]	48.6±1.6[b]	13.4±0.8[b]	23.2±1.5[a]

Values are means ± SE (n=5). a: Significant change at $p < 0.05$ with respect to control group 1, b: significant change at $p < 0.05$ with respect to CCl4 group.

Figure 6. Assessment of apoptosis by annexin-V and PI staining on liver cells from rats treated for 10 weeks with CCl_4 (2 ml CCl_4/kg body wt.) and pomegranate juice.

There was no abnormal appearance or histological changes in the liver of control rats, which injected saline only or in pomegranate juice treated rats, where there are normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus and visible central veins (Figure 5A & C). CCl_4 administration caused classical damage in the rat liver after 10 weeks, as demonstrated by severe hepatocyte necrosis, inflammatory cells infiltration, fatty degeneration, haemorrhage, and hydropic degeneration (Figure 5B). Fibrosis or vacuolar degeneration, and microvesicular steatosis were occasionally observed. Treatment with pomegranate juice was able to ameliorate the CCl_4-induced liver injuries and typical histological changes were markedly absent in the liver sections (Figure 5D).

Flow cytometric analysis was performed to investigate the anti-apoptotic effects of pomegranate juice on liver cells against CCl4-induced apoptosis and necrosis. Cells were dual-stained with Annexin V and propidium iodide (PI) (Figure 6). *In vivo* treated of liver with CCl_4 caused apoptosis (61.7%) and necrosis (26.7%) as compared with large county number of viable cells in control rats. The protective effects of pomegranate juice on CCl4-induced apoptosis in liver cells were abolished by decreasing number of apoptotic cells (48.6%), pomegranate did not alter necrosis (23.2%) that induced by CCl_4.

4. Discussion

Hepatotoxicity induced by CCl_4 is the most commonly used model system for the screening of hepatoprotective activity of plant extracts/ drugs. Administering CCl_4 to rats markedly increases serum AST, ALT, ALP, GGT and TB levels which reflect the severity of liver injury (Srivastava and Shivanandappa, 2006; Abdel-Moneim, 2010) . In this study, significant increase in AST, ALT, GGT, ALP, and TB in the serum were observed after administration of CCl_4, as reported earlier. These marker enzymes are cytoplasmic in origin and are released into the circulation after cellular damage (Lin *et al.,* 2000; Abdel-Moneim, (2010) - . The rise in the enzyme AST is usually accompanied by an elevation in the levels of ALT, which plays a vital role in the conversion of amino acids to keto acids (Sharma and Shukl, 2010). The leakage of large quantities of enzymes into the blood stream was associated with centrilobular necrosis and ballooning degeneration of the liver. However, the increased levels of these enzymes were significantly decreased by treatment with *P. granatum* juice, implying that the juice prevented the liver damage which was further confirmed by the reduced amount of histopathological injuries.

Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by the free radical derivatives of CCl_4 and is responsible for cell membrane damage and consequent release of marker enzymes of heptotoxicity (Danni *et al.,* 1991). In the present study, significantly elevated levels of MDA, products of membrane lipid peroxidation, observed in CCl_4 administered rats indicated hepatic damage. Which CCl_4 metabolites react with polyunsaturated fatty acids and form covalent adducts with lipids and proteins. These events lead to lipid peroxidation and destruction of cell membranes with the consequent liver injury (Szymonik-Lesiu *et al.,* 2003) Treatment of pomegranate juice prevented lipid peroxidation which could be attributed to the radical scavenging antioxidant constituents (Yuan *et al.,* 2008). Antioxidant effect of flavanoids that found in pomegranate enhanced the process of regeneration. This might be due to destruction of free radicals, supplying a competitive substrate for unsaturated lipids in the membrane and/or accelerating the repair mechanism of damaged cell membrane.

The NO radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with $O_2^{\bullet-}$ radicals to form peroxynitrite, that damages biomolecules such as, proteins, lipids and nucleic acids (Gulcin *et al.,* 2002; Gouthamchandra *et al.,* 2010). In our results the pomegranate juice was active and it might be possessing very potent and novel therapeutic agents for scavenging of NO. These juice may also exert their effects on the regulation of pathological conditions caused by excessive generation of NO and its oxidation product—peroxynitrite.

GSH is the main antioxidant found in liver cells and plays a protective role in the metabolism of a large number of toxic agents. It is able to act as a free radical trapping agent and to preserve cytochrome P450 by blocking lipid peroxidation (Levin *et al.,* 1973). In the present study, *P. granatum* juice markedly increased the hepatic GST activity and maintained GSH level even after CCl4 treatment. CCl_4 administration leads to a significant decrease in the glutathione level which can be an important factor in the CCl_4 toxicity. The mechanism of hepatoprotection by *P. granatum* extract against CCl_4 toxicity might be due to restoration of the GSH level.

Among the cellular antioxidants, SOD, CAT, GST, GPx, and GR have received extensive studies. SOD catalyses the dismutation of superoxide anion to H_2O_2 and O_2. Because H_2O_2 is still harmful to cells, catalase and GPx further catalyse the decomposition of H_2O_2 to water. In the reaction catalysed by GPx, GSH is oxidized to GSSG, which can then be reduced back to GSH by GR. GSH is also a cofactor for GST, a phase II enzyme, primarily involved in the

detoxification of electrophilic xenobiotics via catalysing the formation of GSH-electrophile conjugate (Hayes *et al.,* 2005). Thus, the coordinate actions of various cellular antioxidants in mammalian cells are critical for effectively detoxifying free radicals. CCl_4 administration to rats declined antioxidant capacity of the rat liver as evinced in decreased activity of the antioxidant enzymes, which is in agreement with earlier reports (Shahjahan *et al.,* 2004). *P. granatum* treatment prevented the reduction in the Similar studies have shown a positive effect of different classes of polyphenols and flavonoids on antioxidant enzyme activities *in vivo* (Scharf *et al.,* 2003; Shahjahan *et al.,* 2004; Yuan *et al.,* 2008)

Because of the high reactivity and short life, reactive oxygen species (ROS) has been generally analyzed by measuring the changes in antioxidases such as SOD, GPx or the products of lipid peroxidation such as MDA, but there is still an absence of detailed and direct data of ROS changes in studies of acute liver injury (Wu *et al.,* 2007). The results of SOD, GPx and MDA indicated that ROS production was highly elevated in the liver of rats after CCl_4 injection, thus further confirming that free radicals and oxidative damage certainly play a vital role in the pathogenesis of acute liver injury, which also provides strong evidence for natural antioxidants applied in the treatment of toxic hepatopathy.

In conclusion, the hepatoprotective effects of *P. granatum* are presented through multiple ways. *P. granatum* scavenges free radicals that produced by CCl4 and by increases the activity of antioxidant-defense system. These actions of *P. granatum* are of significant clinical importance, as abuse of alcohol or other xenobiotics damages the liver in a manner similar to this CCl4 intoxication model. Further studies with the individual antioxidant compounds isolated from *P. granatum* root extract on hepatocytes are underway which will enable us to understand the exact mechanism of hepatoprotective action by *P. granatum*.

Acknowledgments
This project was supported by King Saud University, Deanship of Scientific Research,
College of Food and Agriculture Sciences, Research Center.

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10/22/2013