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Extract of the *Punica granatum* Flowers Attenuates Progression of Hepatoxicity against Cadmium Chloride-Induced Liver Injury in Rats

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ABSTRACT

Background: *In the present study, we investigated the role of cadmium in acute liver injury, as well as the underlying mechanisms.* ***Methods:*** *On the other hand, we investigated whether Punica granatum flower extract (PG) has a healing effect on CD-induced liver damage. Control groups (G1-G2) received water and 200 mg/kg PG, respectively. Toxicity groups (G3-G6) received 2 different doses of Cd (15-30 mg/kg b.w) orally in a single administration. To evaluate liver function, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) were detected in the serum, superoxide dismutase (SOD), glutathione peroxidase (GPx), thiobarbituric acid reactive substances (TBARS) were established in the liver and 8-hydroxy-20-deoxyguanosine (8-OHdG) levels were determined for the antioxidative and anti - inflammatory effects of PG. Tissue sections were also evaluated histopathologically.* ***Results:*** *We found that Cd exposure decreased levels of SOD, GPx and, increased expression of caspase-3 and levels of TBARS and 8-OHdG. Biochemical and histopathological analysis revealed the toxic effects of cadmium on the liver for the rats in oral acute toxicity study. Functional parameters were significantly improved in PG-treated groups and the severity of the liver injury and apoptosis were significantly decreased in this group.* ***Conclusion:*** *As a result, PG can be consumed as a protective agent against acute liver injuries.*

Keywords: Cadmium, Caspase-3, Liver, *Punica granatum* flowers

INTRODUCTION

Cadmium (Cd) is a highly toxic environmental pollutant which is accumulating in the agricultural soils mainly due to anthropogenic sources such as sewage sludge, mining, industrial effluents and the application of phosphorus fertilizers [1]. It accumulates in different body organs [2]. Increased oxidative stress during Cd toxicity can affect the vascular wall and myocardial tissues, thereby leading to hypertension and cardiotoxicity [3]. The World Health Organization (WHO) has published a list of 10 chemicals or groups of chemicals of concern for human health, which includes Cd. Although cadmium is not a redox reactive metal, its toxicity is predominantly based on the induction of oxidative stress [4,5]. The main source of heavy metal exposure is through anthropogenic sources such as transport, agriculture, mining, and other related operations [6]. Therefore, there is a need for a substance to eliminate the accumulation of Cd or to reduce its toxicity in the body organs. Hepatic injury is usually caused by toxic chemicals, drugs, or pathogen infections [7,8]. Liver damage caused by animal metabolism disorders, bile formation, and excretion disorders and a series of pathological changes and the corresponding syndrome greatly reduces the detoxification ability, seriously affects the animal's performance, and endangers the livestock and poultry industry [9]. To gain a better understanding of the effects of Cd on liver metabolic parameters, the present project was aimed at characterizing the prooxidant reactions that occur in subcellular fractions by measuring the levels of thiobarbituric acid reactive substances (TBARs).

Polyphenols are naturally occurring antioxidants and represent one of the most numerous and widely distributed groups of substances in the plant kingdom; as much as 8000 phenolic structures are currently categorized into 4 groups (flavonoids, stilbenes, lignans, and phenolic acids) [10]. According to recent reports, pomegranate is a polyphenol-rich fruit and showed potential as anti-inflammatory and antioxidative medicine in several experimental models [11].

The pomegranate flower extract also contains a large number of polyphenols and possesses potent antioxidant and hepatoprotective property [12]. In Ayurvedic medicine, the pomegranate is considered "a pharmacy unto itself," and its different parts have been used as an anthelmintic, vermifuge, antidiarrheal, blood tonic, antiabortifacient, and antidiabetic [13]. Furthermore, it is believed that in Turkey, the pomegranate flower has a lowering effect of blood pressure. Therefore pomegranate flower leaves are brewed and drunk as a tea. This study was designed to evaluate the effect of the extract of *P. granatum* flowers on Cd-induced liver injury in the rat.

MATERIALS AND METHODS

Drugs and Chemicals

Anhydrous cadmium chloride ($CdCl_2$) was supplied from Sigma Chemicals Company and dissolved in bi-distilled water before administration.

Experimental Animals

Water-soluble Cadmium chloride ($CdCl_2$) was used as a source of Cadmium (Cd). In this study, 35 male Wistar Albino rats weighing 230-250 g were used. The rats were obtained from Ataturk University Experimental Research and Application Center. Animals were housed in standard cages under well-regulated conditions (relative humidity range: 45 ± 5%, temperature: 24 ± 1°C and a 12-h light/12-h dark cycle). During the experiment, rats were fed with standard rat diet and water ad libitum. Standard rat pellets were purchased from Bayramoglu Yem (Erzurum, Turkey, 3.5% fat, 7.5% carbohydrates, 23% protein, 1-2% vitamins and minerals; 3% trace elements, iron, selenium, manganese, zinc, cobalt, iodide, 270 kcal 100 g^{-1}). The experiments were designed and conducted according to ethical norms approved by the Local Animal Care Committee of Ataturk University, Erzurum, Turkey (12.05.2014/54826478-217).

Study Plan

Group classification: Total 35 male Wistar rats were allocated into 6 groups (n=7, G-1 to G-6), G-1: Received water, G-2: Received 200 mg/kg PG, G-3: Received 15 mg/kg cadmium, G-4: Received 15 mg/kg cadmium , G-5: Received 200 mg/kp PG flower extract +15 mg/kg cadmium and G6: Received 200 mg/kp PG flower extract +30 mg/kg cadmium [14-16]. The treatment of all animals was performed by oral gavage in a volume of 1 mL/kg b.w. Animals were sacrificed 24 h after treatment under anesthesia.

Plant material collection, handling, and extraction process: The leaves of *P granatum L.* flowers were harvested from Antalya during April-May. The plant material was shade dried, powdered, and extracted at 60°C for 48 hr, with 70% methanol using Soxhlet apparatus [17].

Blood Sampling

At the end of the experiment, rats were anesthetized, then blood samples were directly collected by cardiac puncture and serum was separated and stored frozen until the biochemical assays.

Serum Parameters

Serum aminotransferases ALT, AST and GGT activities were determined according to the method of [18].

Tissue Preparation and homogenization

Before biochemical assay of 8-OHdG, liver tissues were weighed, broken down into very small pieces, and placed in empty glass tubes. About 1 ml of 140 mM KCl solution per gram of tissue was added to each tube, and then all tissues were homogenized in a motor-driven homogenizer. The homogenate was centrifuged at 2800 g for 10 minutes at 4°C. The resulting supernatant was used for the determination of 8-OHdG levels.

Determination of Antioxidant Enzyme Activities and Oxidative Stress Biomarkers

Glutathione peroxidase (GPx) activity was measured according to the kit manufacturer's instructions (703102,

Batch: 0541320, Cayman Chemical Co., Ann Arbor, MI) using an ELISA microplate reader (Bio-Tek PowerWave XS, Potton, UK). Briefly, 50 µl assay buffer, 50 µl co-substrate mixture, 50 µl NADPH and 20 µl diluted GPx for positive control or 20 µl homogenized sample were added to the microplate wells. About 20 µl microliters cumene hydroperoxide (Sigma-Aldrich, St. Louis, MO) was added quickly to all of the wells, the plate was shaken for a few seconds to mix, and then the absorbance was read at 340 nm once each minute.

Superoxide dismutase (SOD) activity was measured in the supernatant according to the manufacturer's assay kit instructions (706002, Batch: 0543624, Cayman Chemical Co.) using an ELISA Microplate reader (Bio-Tek PowerWave XS). Briefly, 200 µl of the diluted radical detector and 10 µl of the standard were added per well; 200 µl of the diluted radical detector and 10 µl of sample than were added to the wells. Then, 20 µl diluted xanthine oxidase was added to all the wells. The plate was shaken, incubated for 30 min at room temperature and the absorbance was read at 440-460 nm, evaluating the antioxidant enzymes activity.

Lipid peroxidation was estimated by the thiobarbiturate assay [3,6]. Briefly, a 0.5 ml aliquot of each sample was mixed by a 1:1 ratio to trichloroacetic acid 20% (Merck, Darmstadt, Germany) and centrifuged at 12,000 g and 40°C for 10 minutes. The supernatant was then removed and incubated with 1 ml of PBS (pH: 7.0, Merck, Darmstadt, Germany) and 1 ml of thiobarbituric acid 0.6% (Merck, Darmstadt, Germany) for 20 minutes at 900°C. Optical density was read at 535 nm (ELISA microplate reader-Bio-Tek PowerWave XS). Malondialdehyde was then determined at mM by a standard curve created with 1, 1, 3, 3-tetramethoxy-propane (Merck, Darmstadt, Germany). A 0.5 ml water sample treated in the same way was applied as a blank. All determinations were performed in duplicate and their mean was applied. The intra-day coefficient of variation of the assay was 5.45% and the inter-day coefficient of variation of the assay 13.89%. 8-OHdG kit (Elabscience Biotechnology Co., Ltd., Hubei, China) was used for biochemical assay.

Histological Analysis

Liver tissues were fixed in 10% neutral formalin and routinely processed in paraffin. Liver tissues were also trimmed into cassettes, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin wax. Sections of 5 µm for hematoxylin and eosin (H and E) staining were prepared prior to microscopic analysis.

Immunohistochemical Analysis

Immunohistochemical staining was conducted with Caspase-3 antibody (Orb382909 biorbyt, USA). Caspase-3 immunoreaction was examined semi-quantitatively with Nikon Eclipse 80i light microscope. To determine the staining intensity, 10 areas in each section were examined with X100 magnification, and they were scored as (-) no staining, (+) poor staining, (++) moderate staining, and (+++) severe staining. Photographs were taken with a Nikon image analysis system.

Statistical Analysis

The differences in variance were analyzed statistically using a one-way analysis of variance (ANOVA) test by Graph pad prism 5.0 statistics software (GraphPad, La Jolla, CA, USA). Tukey's test was used as a post-hoc.

RESULTS

Hepatic Biochemical Variables

Two different doses of Cd produced a significant increase in the serum levels of AST, ALT, and GGT compared to the control group ($p<0.001$). There was a slight decrease in AST, ALT and GGT levels in low dose Cd+PG when compared to the other Cd treatments groups ($p<0.01$). Investigated enzyme activities in rat serum are shown in Figure 1.

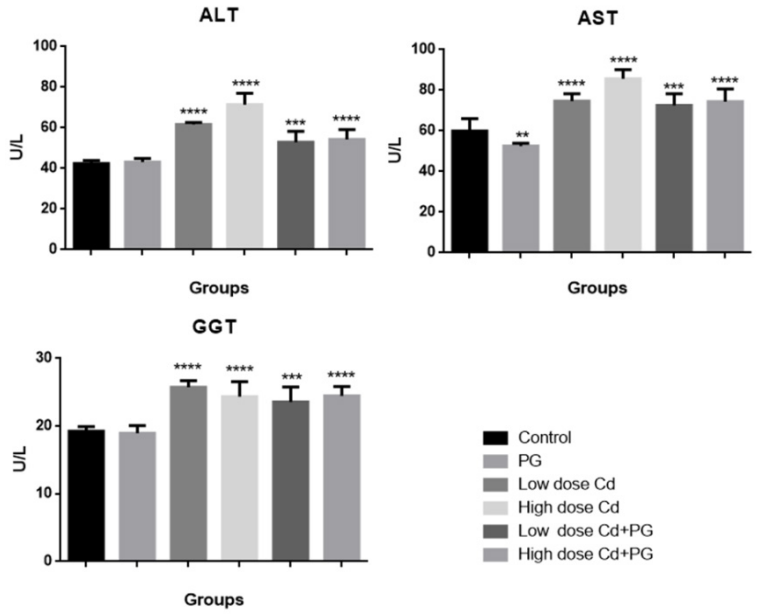

Figure 1 Comparison of liver biochemistry of six experimental groups. Serum ALT, AST and GGT levels of the CD treatment groups (G3, G4, G5, G6) were significantly higher than that in the control groups (G1 and G2) p<0.001. Data are presented as mean ± Standard Error of Mean (SEM) (n=7). * denotes significant differences between other studied groups and control (*: p < 0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001),) by Tukey's multiple range tests

Antioxidant Enzymes in Liver

To comprehensively discuss the antioxidant activity of PG on liver injury, the SOD, GPx activity and MDA level in liver tissue of rats were determined. SOD, GPx activity and MDA levels detected in the liver are summarized in Figure 2. In all experimental groups, except PG group we noticed a decreasing trend in SOD and GPx levels ($p<0.001$) whereas MDA level was increased. Changes in TBARS were noticed in both Cd-treated groups when compared to the controls ($p<0.001$). Regarding the liver, PG did not significantly alter the SOD concentration in the high-dose Cd group. No significant changes were detected in liver GPx activity, due to PG administration especially after treatment of high dose Cd. Additionally, low dose Cd+PG treatment caused a lower MDA level ($p<0.01$) in contrast to the high dose Cd+PG.

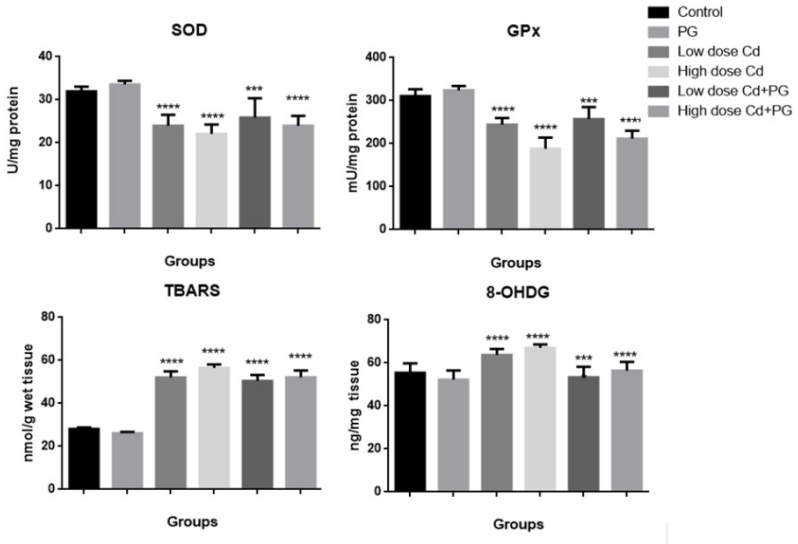

Figure 2 The effects of PG on liver SOD, GPx, TBARS and 8-OHDG levels after liver injury. Data are presented as mean ± Standard Error of Mean (SEM) (n=7). *denotes significant differences between other studied groups and control (*: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001), by Tukey's multiple range tests.

Histological Examination

Histopathological examination in the liver of control rats showed the normal architecture, the cytoplasmic staining was homogeneous, and the cell nuclei were distinct. The liver morphology was also normal in the PG group (Figure 3).

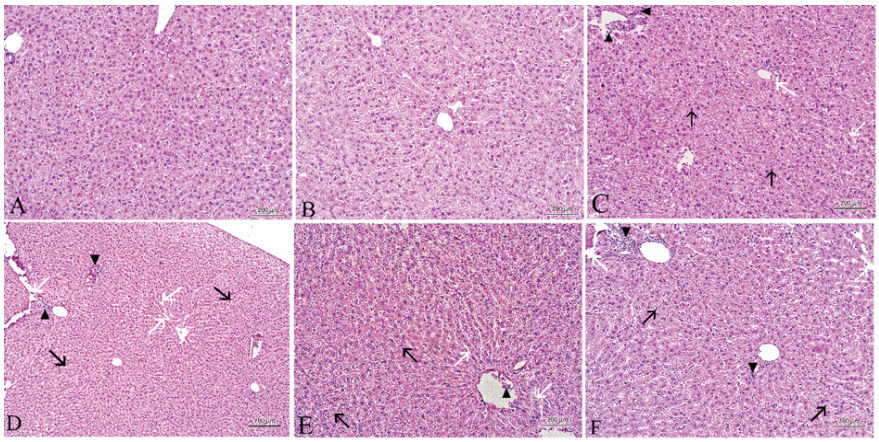

Figure 3 (A) Normal rat liver (100x); (B)Liver section from rat administered 200 mg/kg PG flower extract showing normal architecture; (C-D) Liver section from rat administered 15-30 mg/kg body weight of CD showing; loss of the cellular architecture. The cells exhibits pyknotic nuclei (black arrows), cellular infiltration (arrow heads) and intracellular vacuolation (white arrows) (100X); (D-E) Photomicrograph of 15-30 CD mg/kg administered with PG flower extract showing; mild injury compare treatment with only CD. Cells exhibit pyknotic nuclei (black arrows), cellular infiltration (arrow heads) and intracellular vacuolation (white arrows). vacuolar degeneration, microvesicular fatty change, focal collection of lymphocytes and vascular congestion (100X)

Liver Caspase-3 Immunoexpression

Livers of high dose Cd-treated rats exhibited strong Caspase-3 immunopositivity compared to control and PG treated groups (Figure 4). In the low dose, Cd+PG group showed a mild Caspase-3 immunopositivity compared to low dose Cd-treated rats. In addition, comparing to the control treated rats, the livers of high dose treated group exhibited a significant increase in the area Caspase-3 immunoreactivity compared to the control group. Meanwhile, Caspase-3 area was declined, not reached the level of control, after PG co-treatment. The administration of PG alone had no significant effect on the area of Caspase-3 immunoreactivity compared to the control group. Hepatocytes with morphological characteristics of apoptotic activity were also detected, supporting the finding of elevated Caspase-3 activity in the liver.

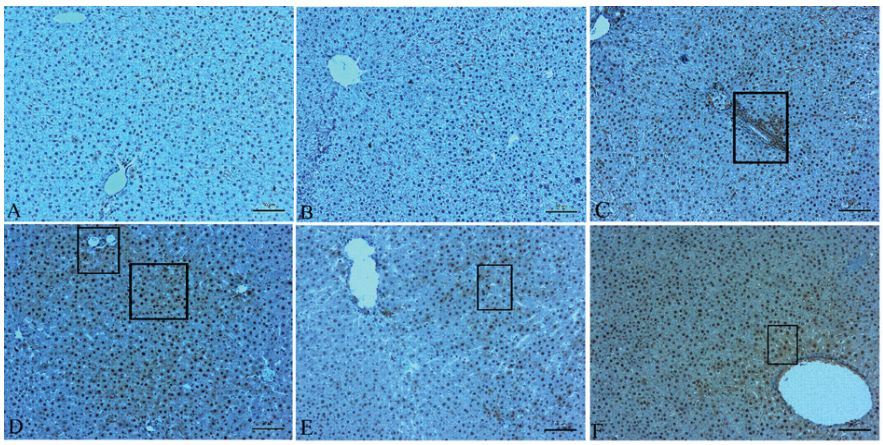

Figure 4 Caspase-3 IHC staining for apoptosis in the liver tissue. Localization of caspase-3 appears as brown staining. (A) and (B) control and PG groups, respectively showed negative immunoreactivity for Caspase-3. Formation of caspase-3, was significantly higher in rats found in low and high dose Cd groups C and D, respectively, than control rats (A) (inside square). The low dose Cd+PG (E) and High dose Cd+ PG (F) (inside square) groups showed moderate positive immunoreactivity for Caspase-3 (100X)

DISCUSSION

Cd is a common pollutant of the natural environment and food in developing and industrialized countries and it is forecasted that exposure to this toxic heavy metal will grow [19]. Humans are generally exposed to Cadmium (Cd) by 2 main routes, inhalation, and ingestion of Cd containing products such as iron coating, copper alloys, stabilizers in rubber and plastics, cigarette papers and fungicides [20]. The liver and kidneys are exposed to Cd toxicity as they are the organs responsible for its excretion [21].

According to our literature survey, combined therapy with PG in Cd-induced liver injury has not been identified so far. Thus, this study provides, for the first time, evidence of the use of PG against liver injury. Liver damage is the basis of acute liver failure, and severe or persistent liver damage ultimately leads to liver failure and is frequently induced by viruses, alcohol, and chemicals [22].

Increases in the levels of ALT, AST, ALP, and LDH are valid indicators of liver injury [23]. Acute exposure to the investigated Cd administered alone or with PG extract resulted in the altered profile of some biochemical parameters. In the current study, PG extract reduced the high levels of ALT and AST elevated by low dose Cd. As Cd causes structural and functional damage to the cell membrane of the hepatocytes and increases the membrane permeability, leading to the leakage of cytoplasm enzymes into the blood [24]. As a result of oxidative stress, Cd toxicity results in tissue damage [24]. These results show that PG extract can effectively remove free radicals in liver cells and enhance the body's antioxidant capacity, thereby improving the anti-damage ability of the liver. To the best of our knowledge, this is the first report suggesting the protective effects of PG in Cd-induced hepatotoxicity in rat. Both the administration of PG with low dose Cd and administration of PG with high dose Cd reduced the histopathological damage, indicating that PG is therapeutic and preventive. The histopathological findings of the present study were consistent with the findings in the literature. For example, in a study conducted by Wei, et al., on diabetes combining non-alcoholic fat liver disease, the protective effect of 75 mg/kg, 150 mg/kg and 300 mg/kg PG was reported [25]. There have been reports suggesting that ethanolic extract of flowers of *P. granatum* modulate different functions through no signaling pathway [26].

Caspase-3 is a mediator of the mitochondrial apoptotic pathway and is known to be an indicator of oxidative stress-induced necrosis [27]. It was observed that caspase-3 immunostaining in the Cd groups apoptotic cells was quite significant, while the staining was very weak in Cd+PG-treated groups apoptotic cells. In the present investigation, treatment with methanolic extract of flowers of *P. granatum* (200 mg/kg i.p.) attenuated Cd-induced liver dysfunction.

The current study also revealed that Cd-induced the oxidative stress as evidenced by increased levels of MDA and inhibit the activities of SOD and GPx in the tissue of rats compared to those of the control group [28,29]. These results correlate well with the previous studies [30].

CONCLUSION

In conclusion, our data revealed that PG flower extract has a protective effect on acute liver injury and could modulate oxidative stress, apoptosis, and DNA damage, lipid peroxidation.

DECLARATIONS

Acknowledgments

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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