Protective effect of methanolic Peel extract of *Punica granatum* on cadmium-induced hepatotoxicity in mice: Histological and ultrastructural investigation

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ABSTRACT

Cadmium (Cd) is an environmental cumulative pollutant affects many organs specially liver. Recently, plants possessing medicinal activities are investigated in mitigating hepatotoxicity. Pomegranate fruit has been intensively utilized as natural remedy in many cultures. The effect of methanolic extract of pomegranate peels (PEE) against cadmium hepatotoxicity in mice is evaluated. Forty mice were used in this study. Animals were divided into four groups, the 1st control group was given saline solution, the 2nd group was orally treated with 50 mg/kg/b.w. of methanolic pomegranate peels extract (PPE), the 3rd group was i.p injected with 2 mg/kg/bw of $CdCl_2$, and the 4th group was injected with 2 mg/kg/bw of $CdCl_2$ followed by oral administration with 50 mg/kg/b.w. PPE. Histopathological changes of liver were examined by light and transmission electron microscopes. Serum ALT and AST was determined and oxidative stress markers MDA, total antioxidant capacity (TAC) were measured in liver homogenate. The results showed that Cd induced several histological alterations in the liver including congestion of blood vessels, leucocytic infiltration, cytoplasmic vacuolation of the hepatocytes and fatty infiltrations. The ultrastructural changes include mitochondrial degeneration, swelling of rER and pyknosis of nuclei with increase in fat droplets and lysosomes. Biochemically, ALT and AST activities and MDA were increased levels while TAC was decreased. Treating mice with PEE with Cd improved the histological as well as ultrastructural structures. ALT, AST and MDA were reduced with increase in TAC. The results suggests that the ameliorative effect of PPE may be due to its antioxidant properties in combating free radical-induced oxidative stress and tissue injury resulting from cadmium chloride exposure.

Key words: Cadmium, Pomegranate peels, Hepatotoxicity, Histology, Oxidative stress, Mice

INTRODUCTION

Heavy metals occur naturally in the earth's crust. They are environmental contaminants gain entry to body system through food, air and water and bio-accumulate over a period of time [1]. Cadmium is extremely toxic heavy metals causing risks for living organisms and man [2] because little amount of cadmium that enters the body excreted slowly in urine and feces [3]. People are exposed to cadmium from food crops obtained from cadmium containing soils, cigarette

smoke, pigments, plastics, alloys, electronic compounds and rechargeable batteries. Cadmium was classified as human carcinogen by the International Agency for Research on Cancer and is considered major concern for public health by the World Health Organization [4]. Bioaccumulation of cadmium is of intensive global concern. It is accumulated due to increased industrial usage and its inclusion in agricultural fertilizers [5, 6]. Cadmium toxicity induced renal dysfunction, bone diseases in addition to hepatic renal and toxicity [7, 8]. Cadmium is tumorgenic to lung and kidney and is responsible for acute and chronic toxicity in humans [9, 10]. Moreover, cadmium carcinogenicity was explained by enhancing DNA mutation rates and stimulation of mitogenic signaling pathways and expression of oncoproteins that control cellular proliferation [11]. In addition, $CdCl_2$ was found to induce oxidative stress and histological abnormalities in liver and many organs. It elevated the activities of liver enzymes and the bilirubin level as well as levels of uric acid, urea and creatinine were increased in the serum [12].

The liver is the principle target for systemic Cd exposure. Soon after systemic absorption, about half of Cd is accumulated in the liver, resulting in reduced availability of Cd to other organs as the kidneys and testes. Production of reactive oxygen species and the oxidative damage of tissue are associated with Cd hepatotoxicity.

As cadmium initially accumulates in liver, hence acute exposure to toxic doses induces hepatic necrosis and apotosis [13, 14]. Overproduction of reactive oxygen species is the primary mechanism for Cd toxicity [15, 16] .

Cadmium induced oxidative damage to cellular organelles by inducing the generation of ROS which react with cellular biomolecules leading to lipid peroxidation, membrane protein damage, changes the antioxidant system, DNA damage, altered gene expression and apoptosis [17, 18].

Ingredients of dietary origin represent new therapeutic remedy. Plants are used in many countries for treating various ailments. Recently, traditional medicine use is in increase gaining popularity in developing alongside with developed countries [19]. The therapeutic efficiency of many plants was demonstrated and utilized for the management of many diseases. Pomegranate peel represents a source of natural antioxidant. A number of phytochemicals occurs in pomegranate peel extract were evaluated. It was documented that the peel is rich in phenolics, flavonoids, ellagitannins and proanthocyanidin compounds, complex polysaccharides and many minerals including potassium, nitrogen, calcium, magnesium, phosphorus, and sodium [20, 21]. Moreover, the tannins presents in pomegranates possesses free radical-scavenging activities which is highly susceptible to both enzymatic and non-enzymatic hydrolysis [22, 23].

Costantini [24] attributed the antioxidant activity of phenolics mainly to their reducing redox properties, hydrogen donors and metal chelators. Accordingly, it removes free radicals, inhibits the lipid oxidation and microbial growth [25, 26]. Pomegranate has therapeutic capability including anti-inflammatory, antihypertensive, and anti-diabetic properties [27- 30]. Dried pomegranate peel is used for treatment of stomach ache, effective lipoperoxidation preventer [31], has

anti-microbial and anti-carcinogenic properties [32]. Pomegranate efficiently ameliorates fatty liver and total hepatic triglyceride contents [33, 34]. The present work studied the effect of pomegranate peel extract on hepatotoxicity induced by cadmium in male albino mice.

MATERIALS AND METHODS

Preparation of methanol extract of Pomegranate:

Pomegranate was purchased from local markets in Zagazig City, Egypt. The peels were prepared according to the method described by El-Toumy and Rauwald [35]. Pomegranate fruits were washed in tap water then twice in distilled water. Peels were removed, cut into small pieces and mixed in a mechanical blender with 80%methanol and left at room temperature for 72 hours. The extract was filtered and concentrated to dryness under reduced pressure in a rotary evaporator at 40–50 ^{O}C in order to reduce the MeOH. The obtained pomegranate peel extract (PPE) was stored at −20 ^{O}C until used. PPE was dissolved in saline and orally given to mice at a dose level of 50 mg/kg b.wt. [34].

Experimental design:

Forty adult male mice aged 6–8 weeks (25± 5 g) were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. Animals were kept in plastic cages with temperature (25 ± 2°C), humidity (55±5%) with 12:12 hour dark/light cycles. Animals were fed standard rodent diet and water *ad libitum*. They were adapted for one week prior the experiment to acclimatize them to laboratory conditions. Mice were treated in accordance with the Institutional Guidelines for the Care and Use of Animals approved by Menoufia University (Approval No. MNSH142), Egypt. Animals were randomly divided into four groups; each group contained 10 animals as follows:

***Group1*:** Control mice received only saline solution.

Group 2: *P. granatum peels* extract (PPE) group mice administered orally by PPE solution (50 mg / kg b.w) daily for 14 days.

Group 3: Mice received i.p. injection of cadmium chloride (Cd) at a dose of 2 mg/kg b. w. daily for 14 days according to Ali [36]. Cadmium chloride obtained from Sigma Chemical Company was dissolved in normal mammalian saline.

***Group 4*:** PPE+ Cd group. Mice received $CdCl_2$ at a dose of 2 mg/kg followed by aqueous extract of *P. granatum* peels (50 mg/kg b w) daily for 14 days.

Light and Electron microscopic study

At the end of the predetermined period, 5 mice from each group were decapitated and their livers were removed. For light microscopic examination, liver pieces were fixed in 10% neutral formaline and processed for routine paraffin embedding. 5 micron thick sections were stained with hematoxylin and eosin. For electron microscopy, small liver pieces were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. Specimens were washed in 0.1 M phosphate buffer at 4^{O}C, then post fixed in 1% osmium tetroxide for 2 h at 4^{O}C. Specimens were dehydrated in ascending grades of ethyl alcohol then put in propylene oxide and embedded in Epon resin. Semithin sections (1µm) were stained with toluidine blue in borax and examined with light microscope.

Ultrathin sections (40 nm) were cut, mounted on copper grids and stained with uranyl acetate and lead citrate (Bancroft et al. [37]), examined and photographed with Jeol 1200 EX transmission electron microscope, College of Medicine, Tanta University, Egypt.

Biochemical study

Blood samples were collected from the retroorbital venous plexus in centrifuge tubes and the serum was conserved at −80°C. Hepatic markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities were measured in serum using the method of Reitman and Frankel [38].

For estimation of oxidative stress parameters as well as the antioxidant capacity, pieces from liver tissue (0.25 g) were ice cooled, homogenized in 2.5 ml phosphate buffer (pH 7.4) then centrifuged at 3000xg for 15 min at 4^{O}C. Total antioxidant capacity (TAC) was determined in liver tissue using the method of Koracevic et al. [39]. Malondialdehyde (MDA) assay was detected by TBARS analysis according to Ohkawa et al. [40].

Statistical analysis

The data were expressed as mean ± SD and analyzed using Student's *t*-test and homogeneity of variances (Levene test) using statistical program of social science (SPSS) software for windows. $P < 0.05$ value.

RESULTS

i. Histological observations

Liver of control mice showed the typical histologic structures presenting distinctive hepatic lobules, hepatocytes and the sinusoids appeared around the central vein radiating towards the periphery (Fig.1a). The liver of mice treated with PPE showed no histopathological alterations and the hepatocytes appeared like those in the control (Fig.1b). Liver sections of mice treated with $CdCl_2$ revealed congestion of blood vessels (Fig.1c), inflammatory infiltrate (Fig.1d), hyperactive kupffer cells and bile duct proliferation (Fig.2a), cloudy swelling and cytoplasmic vacuolization of the hepatocytes (Fig.2b) and fatty infiltration. There is focal necrosis aggregation of hepatocytes and leucocytic infiltration around central vein and accumulation of lymphocytes traped the parenchymal cells with scattered apoptotic bodies (Fig. 2c). Animals given $CdCl_2$ and PPE demonstrated less cytotoxicity. More normal hepatocytes, few leucocytic infiltrations and dilatation of interhepatic veins were seen (Fig.2d).

ii. Ultrastructure results:

Ultrastructurally, hepatocytes of control mice revealed normal nucleus, with obvious nucleolus. The chromatin is allocated into heterochromatin and euchromatin. The cytoplasm contains numerous various sized- oval and elongated mitochondria, numerous cisternae of rough endoplasmic reticulum with ribosomes on their cytoplasmic surface (Fig.3a). The bile canaliculi located throughout the hepatocytes with normal microvilli. Hepatocytes of animals treated with

pomegranate extract showed normal structure (Fig.3b). Various deteriorations were observed in mice liver treated with $CdCl_2$. Mitochondria appeared swollen with destructed cristae and rER was dilated (Fig.3c). The sinusoid contains debris bodies with enlarged Kupfer cell, erythrocyte and numerous variable size autophagosomes (Fig.4a).The bile canaliculi appeared with destructed microvilli (Fig.4b).Some nuclei were degenerated (Fig.4c) others were pyknotic with clumped chromatin (Fig.5a). Fat droplets of different sizes were also encountered (Fig.5b). After treatment with pomegranate extract and CdCl2, most liver cells appeared with ordinary nuclei while some with irregular contour, most mitochondria had normal cristea and bile canaliculi with well-organized microvilli were evident (Fig. 5c).

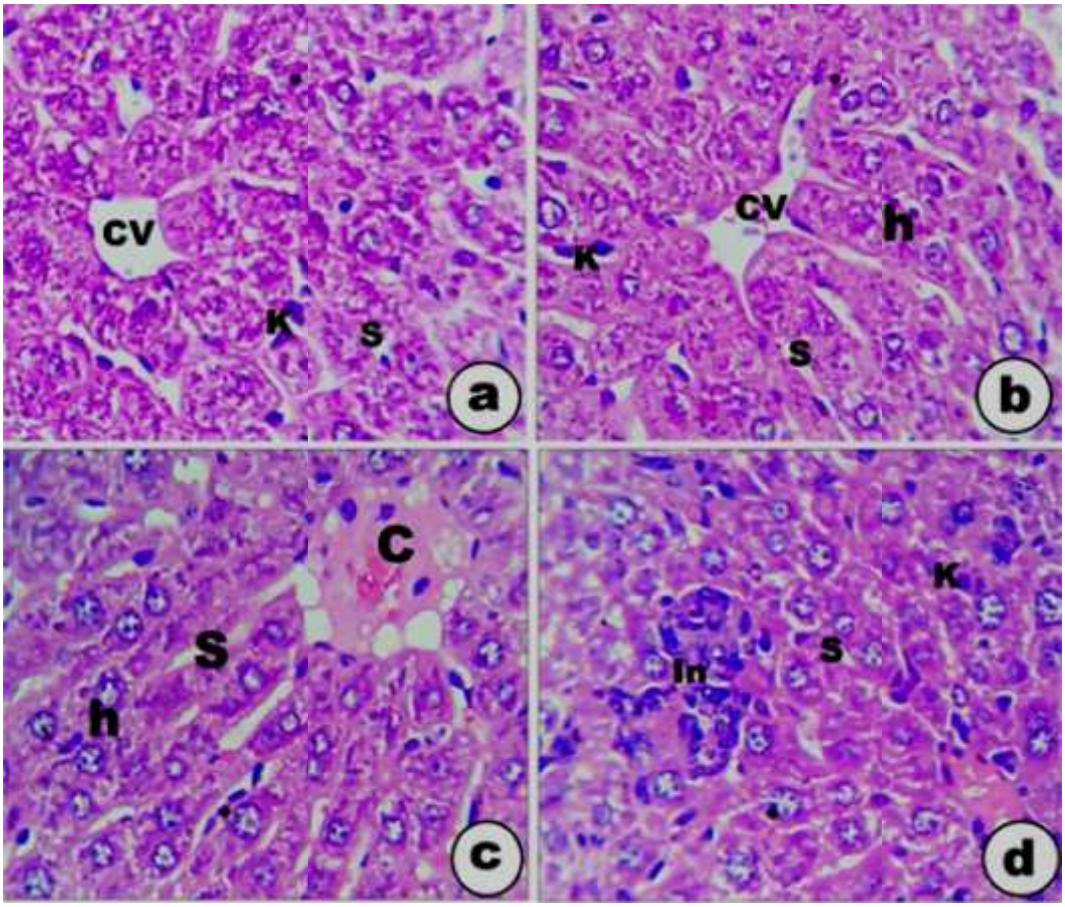

Fig 1: Sections in liver of (a, b): control mice showing hepatocytes (h) with polyhedral contour and basophilic bodies, sinusoids (S), central vein (CV) and Kupffer cells (K); (c): a mouse treated with $CdCl_2$ revealed congestion of central vein (C). (d): inflammatory infiltrate (In) was observed. (X400)

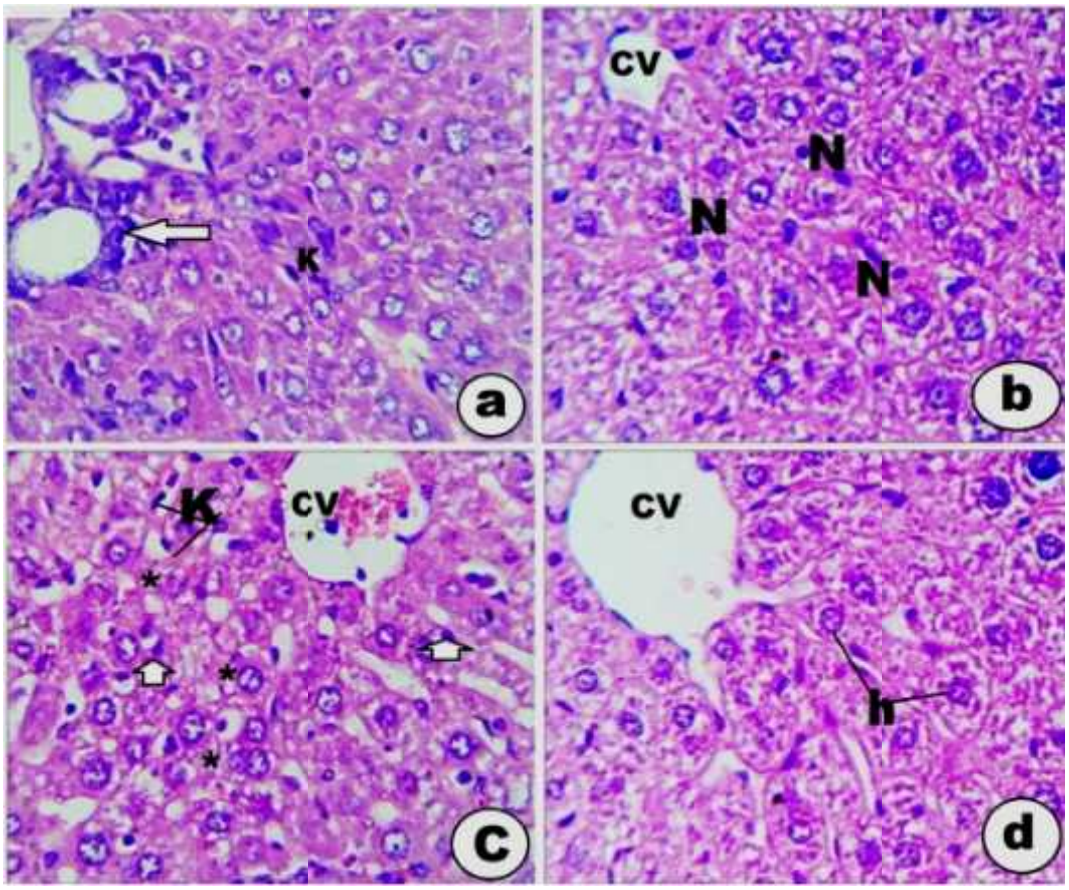

Fig 2: Section in liver of (a): a mouse treated with $CdCl_2$ for 14 days revealed hyperactive kupffer cells (K) and bile duct proliferation (arrow); (b): Cloudy swelling and pyknotic nuclei (N), (c): vacuolated cytoplasm (*) and fatty infiltration (Arrow head), activated kupffer cells (K), congested vein (CV) , (d): a mouse treated with $CdCl_2$ and PPE demonstrated normal hepatocytes (h) and central vein (CV). (X 400)

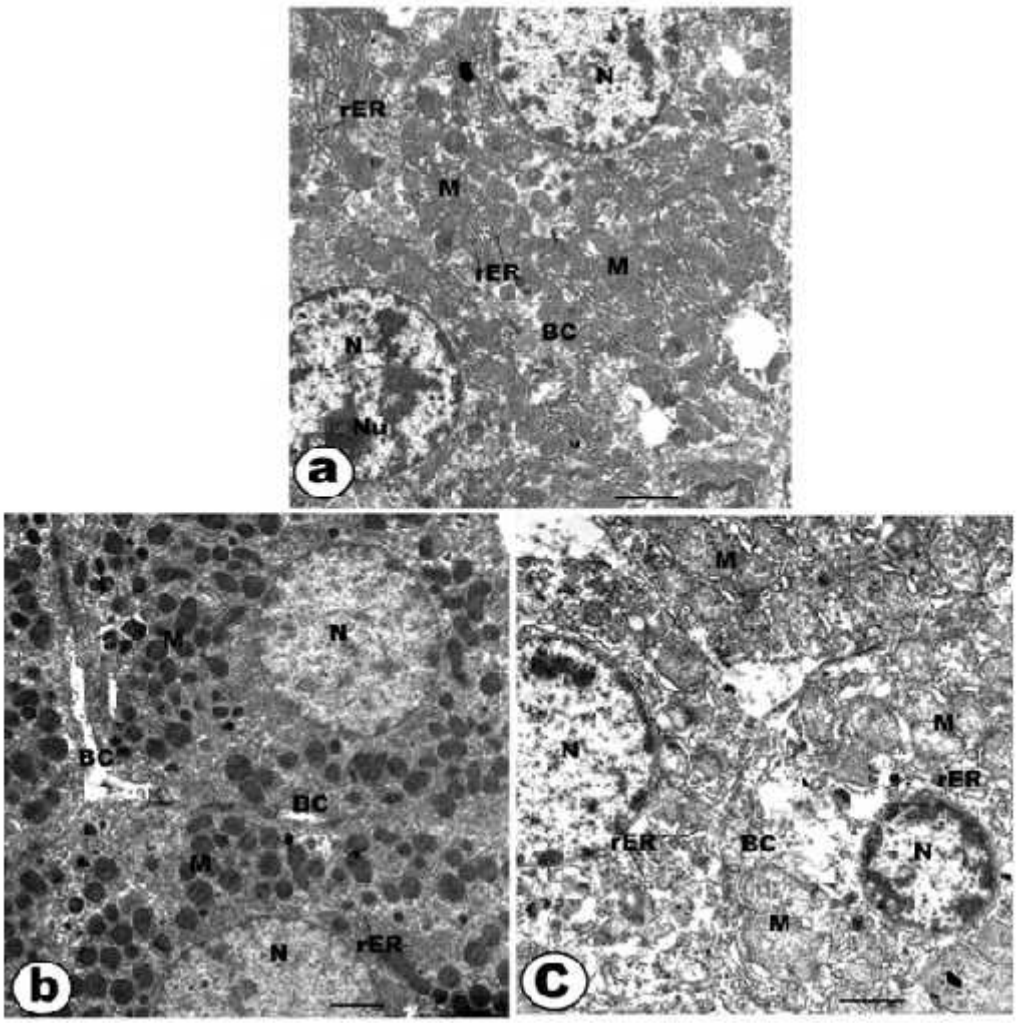

Fig.3: TEM micrograph showing (a): control mouse with regular mitochondria (M), rough endoplasmic reticulum (rER) and bile canaliculi (BC) between two hepatocytes with normal microvilli; (b): liver from mouse treated with pomegranate extract showing normal structure; (c): displaying liver treated with $CdCl_2$ having swollen mitochondria (M) with destructed cristae and expanded rER. (Scale bar =2µm).

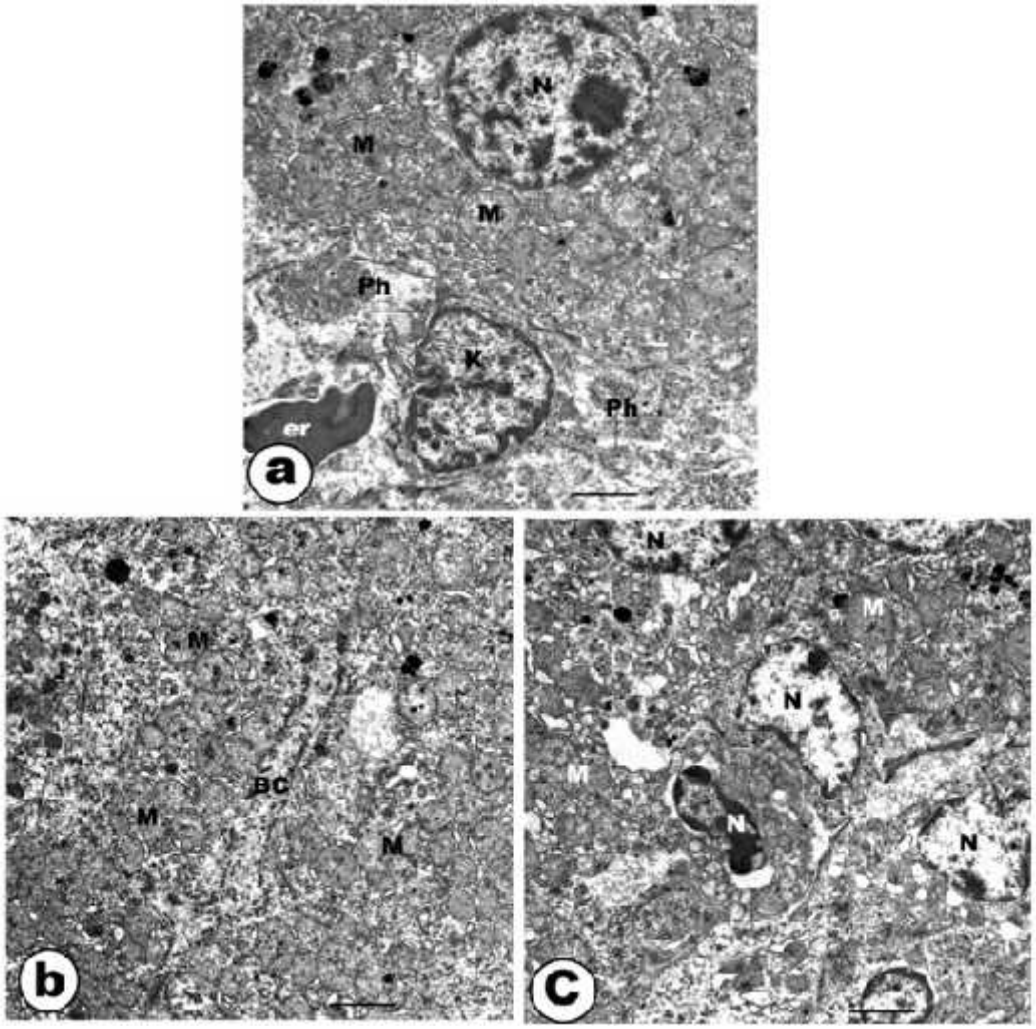

Fig.4: TEM micrograph of mice treated with $CdCl_2$, (a): the sinusoid contains debris bodies, dilated Kupfer cell (K), erythrocyte (er) and numerous autophagosomes (ph) with variable size (b): the bile canaliculi with destructed microvilli, (c): degenerated and irregular shape nuclei (N) with few chromatin materials. (Scale bar =2µm).

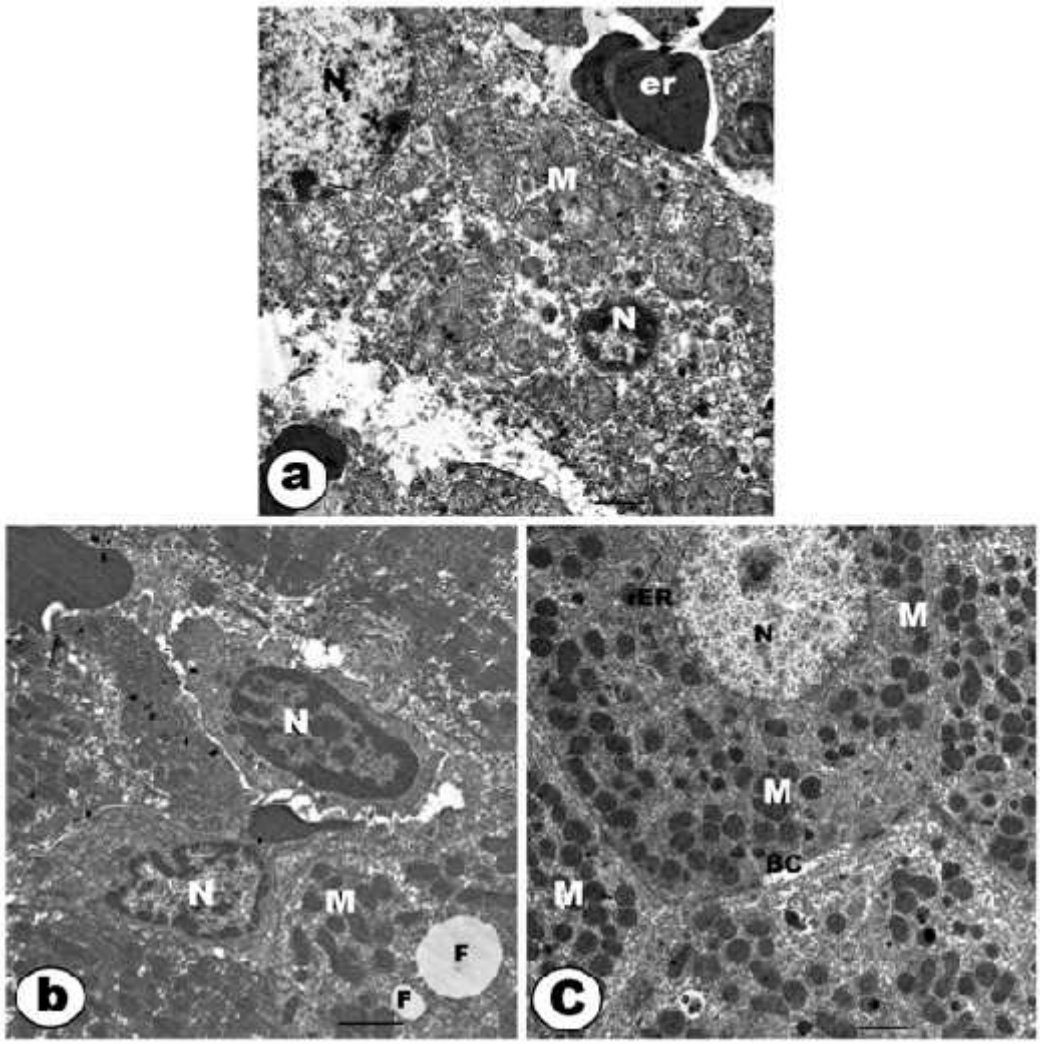

Fig.5: TEM micrograph of mice treated with $CdCl_2$ displaying; (a): hepatocyte with clumped and pyknotic chromatin, degenerated mitochondria and sinusoid; (b): fat droplets (F) of different sizes; (c): liver of a mouse treated with $CdCl_2$ and pomegranate extract with normal nucleus but other with irregular contour, normal mitochondria (M) with normal cristea and bile canaliculi(BC) with an efficient microvilli were observed, (Scale bar =2µm).

iii. Biochemical results

Serum hepatic marker enzyme levels for control and treated mice have been summarized in Figs.6& 7 .The levels of AST and ALT enzymes were significantly increased ($p<0.05$) as a result treatment with $CdCl_2$ for 14days in comparison with that of the control. On the other hand, a decrease in these parameters was recorded in animals treated with $CdCl_2$ and pomegranate in respect with control. There is no significant difference in the activity of ALT and AST in the sera of control group or animals group pomegranate.

The data obtained from mice treated with $CdCl_2$ for 14days showed a significant ($p<0.05$) increase in the hepatic MDA levels compared to control

(Fig.8) but after treatment with $CdCl_2$ and PPE extract, MDA showed an obvious decrease. Animals treated with PPE only showed no difference from control. In respect to the total antioxidant capacity (TAC), mice treated with $CdCl_2$ showed low value compared to the control. On the other hand, insignificant increase in the TAC was recorded in liver homogenate of animals treated with $CdCl_2$ and pomegranate (Fig. 9).

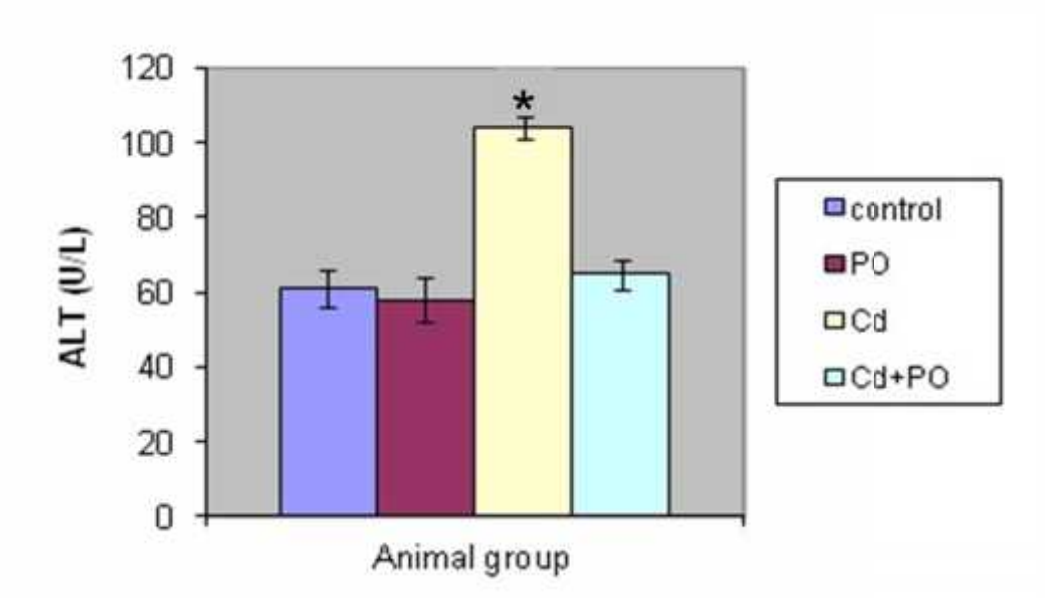

Fig.6: Effects of cadmium chloride and PPE extract on liver (ALT) (U/L), (*) significant at ($p<0.05$) .

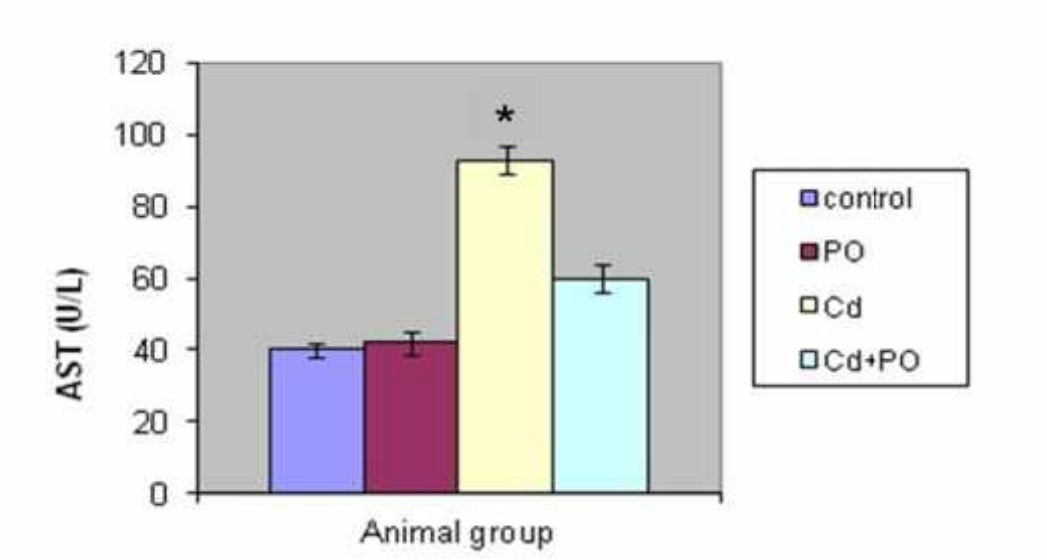

Fig.7: Effects of cadmium chloride and PPE extract on liver (AST) (U/L). (*) significant at ($p<0.05$) .

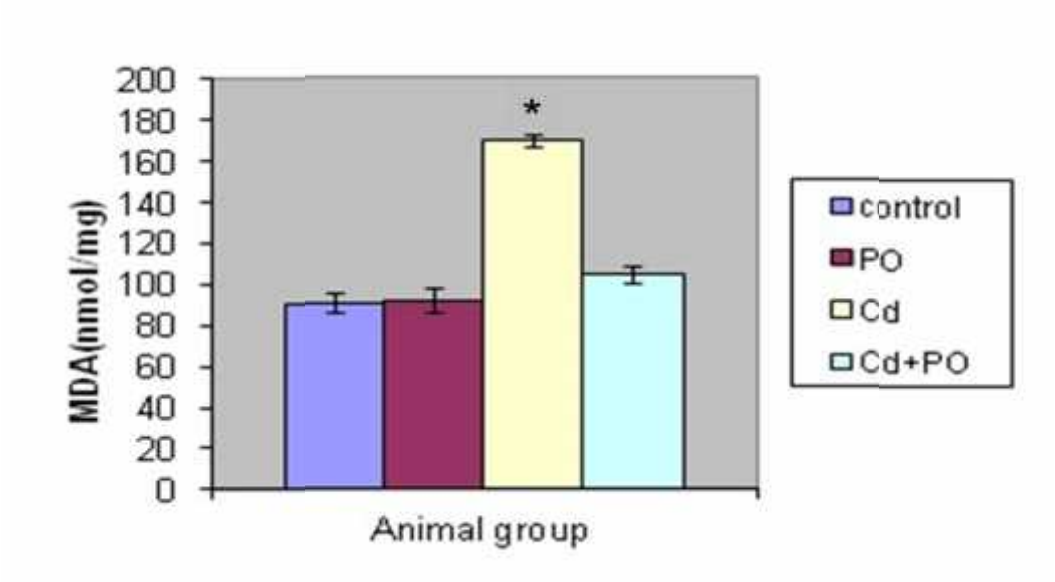

Fig.8: Effects of cadmium chloride and PPE extract on liver (MDA) (nmol/mg tissue). (*) significant at ($p<0.05$) .

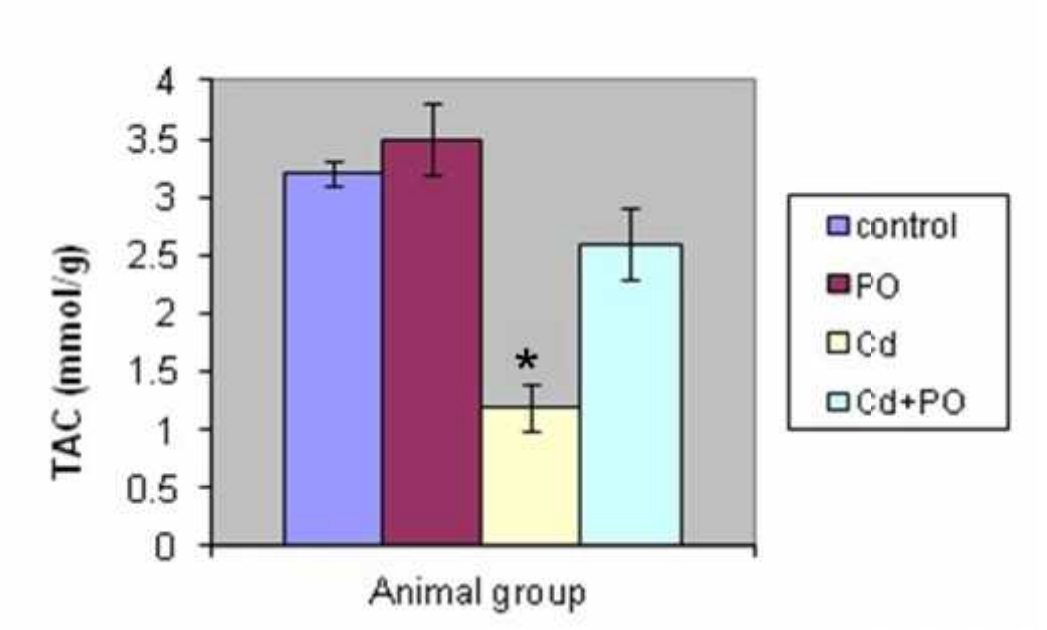

Fig.9 : Effects of cadmium chloride and PPE extract on liver (TAC) (nmol/mg tissue). (*) significant at ($p<0.05$) .

DISCUSSION

By the virtue of its long biological half-life (15–20 years), cadmium accumulates over time within and induces damage to liver, blood, reproductive

organs and kidneys [41]. The present work focused on liver which is a vital organ targeted by cadmium accumulation. The present results showed that Cd caused necrosis, hydropic change and fatty infiltration with inflammatory infiltrate. These histopathologcal alterations coincides with earlier studies in animals exposed to Cd [41- 45]. The histopathological changes were confirmed by the increase in liver function enzymes (ALT, AST) in sera of Cd-treated rats. Similarly, many investigators indicated increase in AST and ALT activities following exposure of rats [46, 47] and mice [48] to $CdCl_2$. The increase in the liver enzyme activities may be due to liver dysfunction and disturbance in the synthesis of these enzymes [49]. Formerly, Williamson et al. [50] indicated that liver injury accompanies Cd administration is explained by increased levels of hepatic marker enzymes in serum pointing to cellular leakage and loss of hepatic membrane architecture functional integrity where elevated ALT and AST levels are followed to detect liver damage.

The mechanism of cadmium-induced hepatotoxicity was explained by different mechanisms. Of significance is interaction with essential organelles as microsomes, mitochondria and peroxisomes that results in increasing free radicals production and lipid peroxidation [51]. In addition, cadmium is a non-redox metal, capable of indirectly causing oxidative damage to the liver by depleting cellular antioxidant levels especially glutathione and protein-bound sulfhydryl groups; that enhances generation of reactive oxygen species (ROS) as superoxide ion, hydroxyl radicals and hydrogen peroxide [52, 53].

In addition, cadmium treatment caused pyknosis of nuclei, mitochondrial degeneration, swelling of ER and fatty degeneration. Similarly, Kim and Yoon [54] and Marcano et al. [55] reported that liver of mice treated with cadmium showed loss of endoplasmic rough reticulum, lysosomes and peroxisomes alteration, condensation of nuclear chromatin and nuclear membrane rupture, dilatation of intracellular space and destruction of mitochondria. Moreover, Mahran et al. [56] and Sakr et al. [44] indicated damage of the nuclear membrane, regression or swelling of mitochondrial cisternae, deterioration of rough endoplasmic reticulum, and proliferation of smooth endoplasmic reticulum with condensation of the nuclear chromatins and appearance of fat droplets in liver of rats treated with $CdCl_2$.

Mitochondria are the most vulnerable cellular organelle to oxidative stress where their degeneration is involved in cell death. Mitochondria are targets in cadmium hepatotoxicity. Increasing body of evidence demonstrated that mitochondrial dysfunction implicated in Cd-induced liver damage [57, 58]. In this work, cadmium was found to cause degeneration of mitochondria. This could be explained on the findings of Thevenod [59]. The intracellular Cd induced damage of mitochondria, and/or cell death. Moreover, cadmium intervenes with oxidative phosphorylation of mitochondria and in higher doses can inhibit basal respiration and affects the regulation of mitochondrial genes as Hsp60 which implicate in cell protection and apoptosis [57]. In addition, Xu et al. [60] indicated that Cd exposure caused mitochondrial fragmentation both in L02 cells and rats' liver. An old study conduct in 1975 by Hoffmann and colleagues [61] indicated degeneration of rough endoplasmic reticulum, proliferation of smooth

endoplasmic reticulum, autophagocytosis, and degeneration of mitochondria after 16 hours of single intravenous injection of cadmium acetate to rats.

Biochemically, there was a marked increase in hepatic MDA and decrease in TAC following administration of $CdCl_2$ to mice. Cd has been demonstrated to stimulate free radical production, resulting in oxidative deterioration of lipids, proteins and DNA and initiating various pathological conditions. In this concern, Bekheet et al. [62] found inhibition in the activities of SOD and CAT and significant increase in MDA level in liver and kidney of rats exposed to cadmium chloride daily for 7 successive days. It has been shown that Cd produces hydroxyl radicals by displacing protein-bound Fenton metals and accordingly it can cause oxidative stress and lipid peroxidation. Over production of ROS normally induces oxidative stress and lipid peroxidation unless it was scavenged with endogenous antioxidant. This over - production attributed to the depletion of antioxidant or to the direct action of cadmium on peroxidation reaction and iron - mediated peroxidation [63]. A single dose of cadmium chloride significantly increased serum aminotransferases, malondialdehyde and significantly decreased catalase, superoxide dismutase, reduced glutathione and glutathione peroxidase in rats while thyme extract provides hepatoprotection against cadmium chloride induced hepatotoxicity in albino rats [64].

The present study proved that *Punica granatum* peel (PPE) plays a role in Cd detoxification. The histological and ultrastructural results revealed an improvement in the liver of mice treated with $CdCl_2$ and PPE together with improvement in level of serum liver function enzymes, ALT and AST. Coincides with this result, Agha et al. [65] indicated amelioration of pentachlorophenol-induced histological alterations in the liver by *P. granatum* peels extract. El-Alfy et al.[66] reported that *Punica granatum* peel methanolic extract effectively ameliorated the significant elevation in serum ALT, AST, ALP activities and bilirubin level in thioacetamide- administered rats. Wei et al. [67] reported protective effect of pomegranate peels extract and seeds in liver fibrosis- induced by carbon tetrachloride in rats. Recently, Çalışkan et al. [68] indicated that pomegranate juice had preventive effect on paracetamol-induced acute liver damage. According to Elwej et al. [69], pomegranate peel mitigated barium-induced oxidative stress in rats liver evidenced by decrease in malondialdehyde, AST and ALT activities and increased CAT and GPx and GSH activities.

In this work, co-treatment with PPE attenuated Cd induced hepatic oxidative damage in mice. These effects were evident from the significant decrease in MDA and increase in TAC. Similarly, Osman et al. [70] reported that ethanolic extract of *Punica granatum* peel decreased MDA levels in rats intoxicated with CCl_4. TAC was increased in animals treated with Cd and PPE. The decrease in MDA and increase in TAC indicate the antioxidant activity of PEE. Similar results were found in mice kidney tissue following treatment with Cd and PPE [71]. Aboonabi et al. [28] indicated that pomegranate can increase antioxidant enzyme, capable of ameliorating oxidative stress and protects liver and kidney in diabetic rats. In this work, pomegranate peel extract is methanolic that gives undeniable ameliorative action against cadmium. In this concern, Middha et al. [72] indicated that pomegranate peel methanolic extract possesses

higher antioxidant activity than the aqueous counterpart. Pomegranate peel and seeds are potent antioxidants due to their active electron donors compounds, that convert free radicals to more stable products and terminate radical chain reaction. It is concluded from this work that Cd- induced hepatotoxicity in mice. Co-administration with PEE ameliorated the toxicity of Cd via its antioxidant activity.

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