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Protective effects of pomegranate peel extract on the gill, liver, and kidney in experimental cadmium poisoning in common carp (*Cyprinus carpio*)

Hossein Jafarzadeh[a], Soodeh Alidadi[a], Davar Shahsavani[b]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

This study aimed to investigate the protective effects of pomegranate peel (PoP) extract on the gill, liver, and kidney tissues of common carp exposed to cadmium (Cd). For this purpose, 150 common carp weighing 65± 0.85 g were randomly divided into five groups with three triplicates for each group (30 fish per group). The control group received a standard diet without cadmium chloride ($CdCl_2$), the Cd group was exposed to 0.5 mg/L $CdCl_2$, and the extract groups received PoP extract with concentrations of 1%, 2%, and 4% (percentage of food weight) along with 0.5 mg/L $CdCl_2$ in the water. After four weeks, tissue samples were collected from the gill, liver, and kidney and stained with hematoxylin and eosin for histopathological examination. In the gills of the Cd group, lesions included congestion, hemorrhage, clubbing or fusion of the secondary lamellae, and telangiectasia of the lamellae. The liver tissue of the Cd group exhibited severe degeneration and necrosis of hepatocytes, while hemorrhage, congestion, cellular degeneration or necrosis, and hyaline cast were visible in the kidney tissue of this group. The severity of the mentioned lesions was significantly reduced in the PoP extract groups, particularly at concentrations of 2% and 4% ($p < 0.05$). Based on the results, it can be concluded that PoP extract has significant protective effects on the gill, liver, and kidney tissues of common carp exposed to $CdCl_2$.

Keywords

Cadmium; Pomegranate peel extract; Liver; Kidney; Gill; Histopathology

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Abbreviations

Cd: Cadmium
$CdCl_2$: Cadmium chloride
PoP: Pomegranate peel
ROS: Reactive oxygen species
AgNPs: Silver nanoparticles
NO: Nitric oxide.

Corresponding author: Soodeh Alidadi and Davar Shahsavani
Email:salidadi@um.ac.ir
shahsavani@um.ac.ir
Tel: +98(51)3880-5644

Introduction

Heavy metals are significant pollutants in the aquaculture industry worldwide due to domestic and industrial activities [1,2]. Cadmium (Cd), along with other heavy metals like lead and mercury, poses a public health hazard. The United States Agency for Toxic Substances and Disease Registry ranks Cd as the seventh most dangerous agent [3]. Additionally, the International Organization for Cancer Research classified Cd as a human carcinogen in 1993 [4]. Human exposure to Cd occurs through food and inhalation, with cigarette smoke being a predominant source, containing approximately 1.5 to 2 µg of Cd per cigarette [5]. It has been shown that Cd can adversely affect various systems, including the respiratory, reproductive, nervous, immune, endocrine, cardiovascular systems, and the liver, and it is a potent carcinogenic agent [5-7].

In cases of fish poisoning with heavy metals, such as Cd, some organs like the gill, liver, and kidneys are known to be the main organs exposed to $CdCl_2$ in water through respiration and ingestion [2]. These organs are the primary. Numerous studies have demonstrated that Cd can accumulate in various tissues of fish, including muscles, which can have negative implications for human health [7-11]. Cd has been shown to disrupt iron metabolism, leading to anemia and alteration of blood parameters [13,13]. In addition, it suppresses antioxidant mechanisms, leading to lipid peroxidation and oxidative stress [14,15]. There is, therefore, a critical need to minimize or prevent the deleterious effects of Cd exposure.

The use of herbal medicines as supplements or alternatives is growing worldwide. Pomegranate (*Punica granatum L.*), widely cultivated in the Middle East, particularly in Iran, has a long history of use in Iranian herbal medicine [16]. During agricultural production and processing, wastes from pomegranates are generated. These by-products, such as pomegranate peel, offer economic potential as they are a rich source of bioactive substances, including phenolic acids and tannins [17]. One study has shown that the peels of fruits like pomegranates, oranges, apples, and peaches contain higher phenolic content compared to their edible fleshy parts [18]. Pomegranate peel (PoP) is particularly rich in flavonoids and phenolic compounds like tannins or tannic acids, making it a valuable source of bioactive substances [18-20]. Furthermore, it has been shown that PoP extracts have potent wound healing, antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, and anticancer properties, and they can decrease blood lipid levels [19,21-23].

Therefore, this research aimed to investigate the potential benefits of PoP extract as a natural remedy for alleviating Cd-induced toxicity in the gill, liver, and kidney tissues of common carp (*Cyprinus carpio*) through histopathological examination.

Results

Histopathological findings

Gill

Histopathological examination revealed various lesions in the Cd group, including congestion, telangiectasia of lamellae, hemorrhage, disruption of the gill structure, and hypertrophy and hyperplasia of the lamellar epithelium, leading to distal clubbing or fusion of the secondary lamellae (Figure 1). Treatment with different concentrations of PoP extract ameliorated the lesions caused by Cd administration (Figure 1).

Statistical analysis showed that the Cd group exhibited severe lesions, while all concentrations of the PoP extract reduced the severity of the lesions. This improvement was significant for all the mentioned lesions in the PoP extract groups ($P < 0.05$) (Figure 2). However, no significant difference was observed among the three PoP extract groups ($P > 0.05$). Although the PoP extract significantly improved the Cd-induced lesions, the normal and healthy gills in

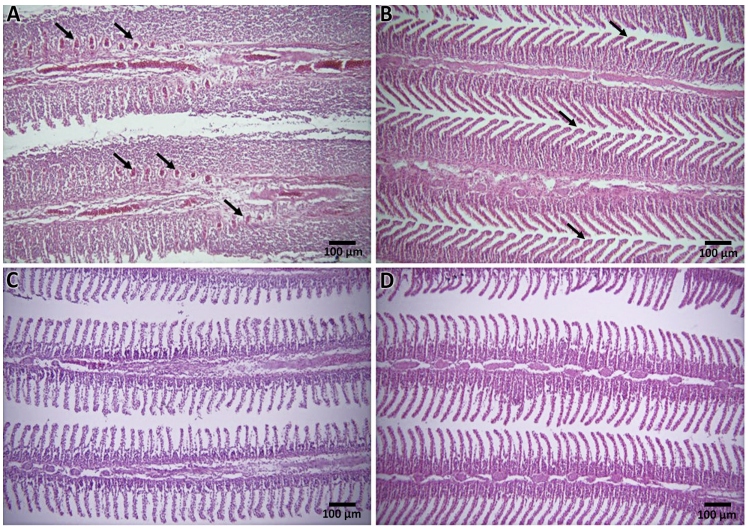

Figure 1.
Micrographs of the gills in common carp that were exposed to CdCl2 and subsequently treated with pomegranate peel extract (n = 9 for each group). In the Cd group (A), congestion, the severe fusion of secondary lamellae, and lamellar telangiectasia or aneurysm can be (arrows). There is club formation of secondary lamellae in the 1% PoP extract group (B). The 2% (C) and 4% (D) PoP extract groups exhibited slight distal clubbing of the lamellae. Hematoxylin and eosin (H & E) staining, scale bars = 100 µm for all.

the control group had lower scores compared to the PoP extract groups ($p < 0.05$).

Liver

In the tissue sections from the liver of the Cd group, severe degeneration and necrosis of hepatocytes were observed. The cytoplasm of the hepatocytes appeared hypertrophied, almost transparent, and clear, with only the cell membrane visible, along with a vesicular nucleus typically located centrally within the cell (Figure 3). Cell nuclei were lost in some hepatocytes, and they showed necrotic changes. In some cases, which were limited to the Cd group, infiltrations of inflammatory cells, predominantly lymphocytes (lymphocytic hepatitis and pancreatitis), were found (Figure 3).

In the groups that received $CdCl_2$ along with different amounts of PoP extract, particularly 2% and 4%, significant improvement in the lesions was observed ($p < 0.05$). However, the liver tissues in

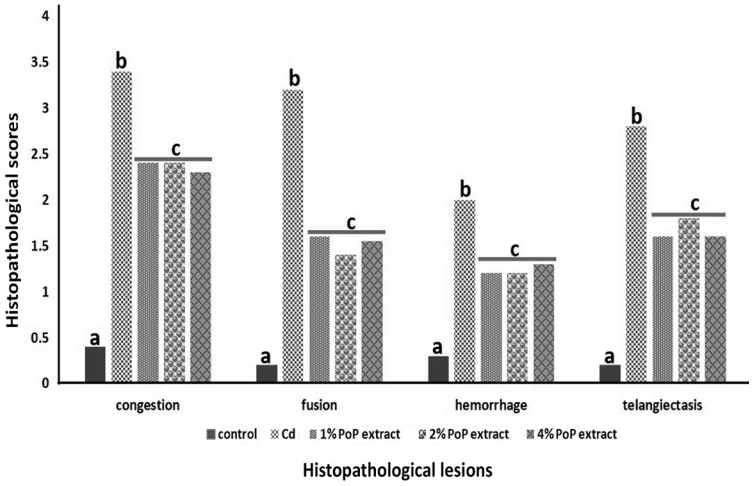

Figure 2.
Statistical analysis of the scores of various histopathological lesions, including congestion, fusion of the secondary lamellae, hemorrhage, and telangiectasis, in the gill tissue related to different groups after four weeks of exposure. The scores are reported as Mean ± SEM and analyzed using Kruskal-Wallis and Mann-Whitney tests. Different letters indicate a significant difference ($p < 0.05$)

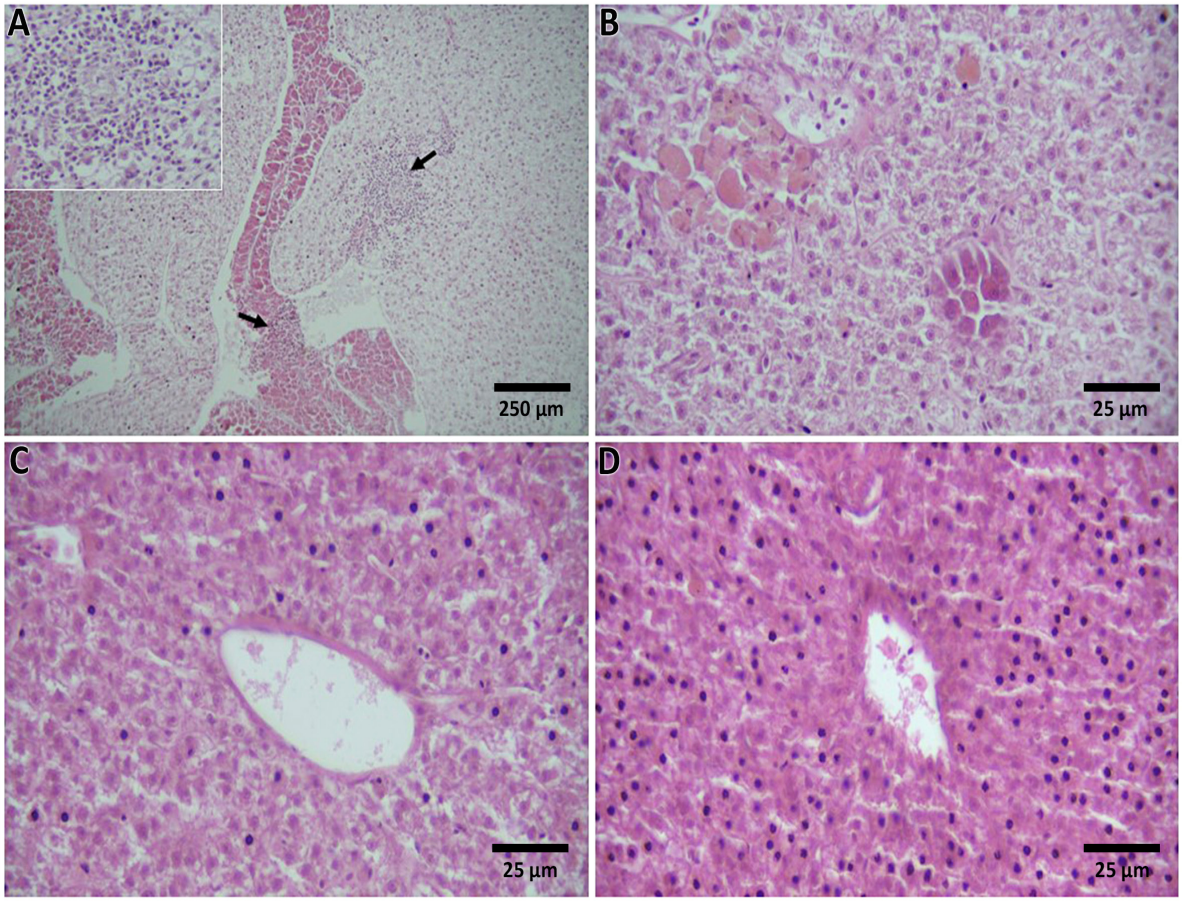

Figure 3.
Histopathological examination of the liver of common carp following CdCl2 poisoning and subsequent treatment with PoP extract (n = 9 for each group). In the Cd group (A), severe degeneration and necrosis of hepatocytes, as well as infiltration of mononuclear inflammatory cells with the predominance of lymphocytes can be observed (arrows) (H & E staining, scale bar = 250 µm). In the upper corner: infiltration of lymphocytes is shown at a higher magnification. In the 1% (B), 2% (C), and 4% (D) PoP extract groups, there is an improvement in cell degeneration and necrosis of hepatocytes. No evidence of inflammation is observed in the PoP extract groups. H & E staining, scale bars = 25 µm for B, C, and D.

these groups remained different from normal and healthy tissues in the control group. The 1% PoP extract group showed some recovery in the lesions, but it was not statistically significant (p = 0.095). Moreover, there was no significant difference between the treatment groups receiving PoP extract (Figure 4).

Kidney

The control group exhibited the normal structure of the kidney tissue, while the Cd group showed various lesions such as congestion, hemorrhage, degeneration of the tubular epithelium characterized by hydropic degeneration or cell swelling with narrowed tubular lumen, and cell necrosis (Figure 5). Eosinophilic hyaline casts were also observed in the tubular lumens in this group. These lesions were more severe in the Cd group, but PoP extracts reduced the CdCl2-induced lesions (Figure 5).

Compared to the Cd group, the PoP extract groups, especially 2% and 4%, showed significant improvements in the lesions, including hemorrhage, hyaline casts, and cell degeneration and necrosis (p < 0.05). However, there was no significant difference in the scores among the three PoP extract groups (p > 0.05) (Figure 6).

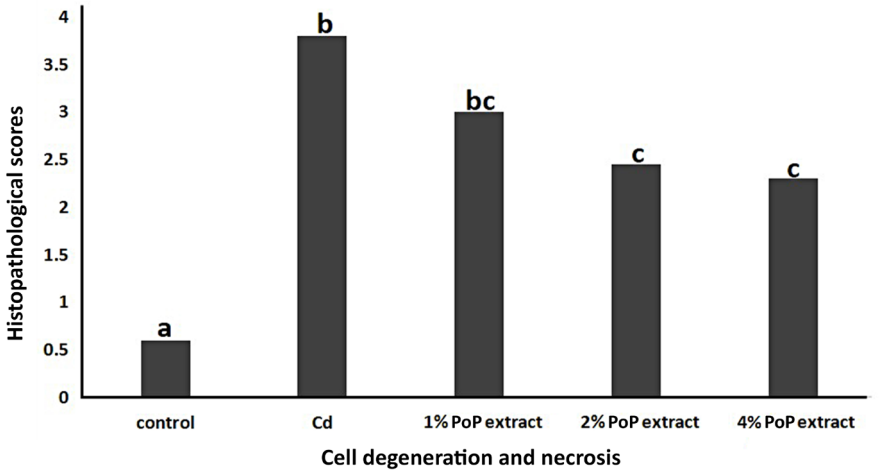

Figure 4.
Statistical analysis of the scores related to cell degeneration and necrosis in the liver tissue of the different groups after four weeks of exposure. The scores are reported as Mean ± SEM and analyzed using Kruskal-Wallis and Mann-Whitney tests. Different letters are significantly different (p < 0.05).

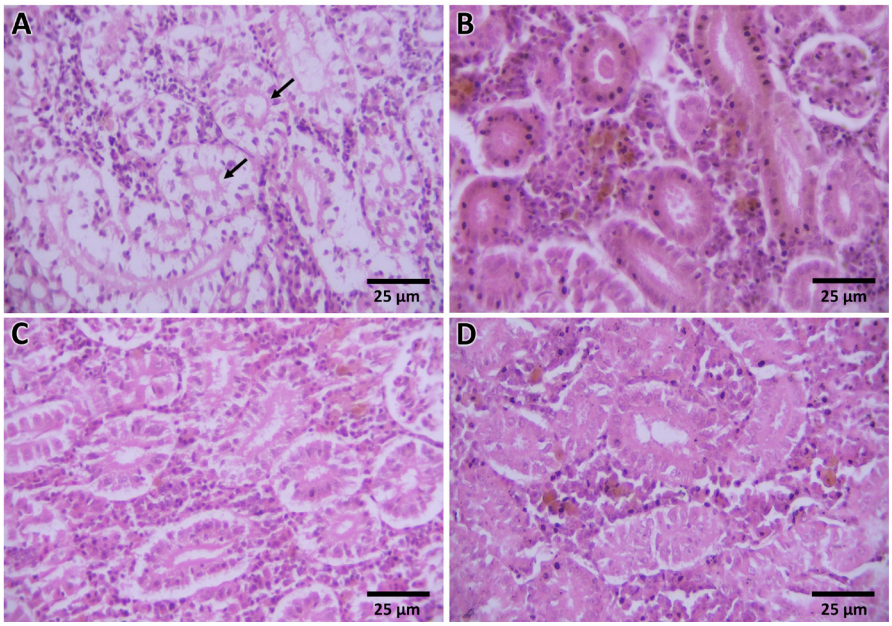

Figure 5.
Photomicrographs of the kidney tissues exposed to CdCl2 and treated with PoP extract (n = 9 for each group). In the Cd group (A), there are severe hydropic degenerations in the epithelial cells of the tubules, as well as narrowing of the lumen (arrows). In the 1% (B), 2% (C), and 4% (D) PoP extract groups, there is a recovery and reduction of hydropic degeneration in the tubular epithelial cells. H & E staining, scale bars = 25 µm for all.

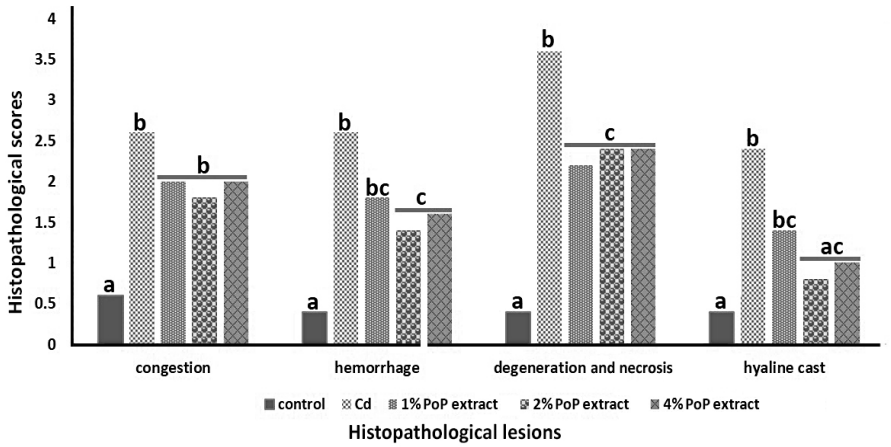

Figure 6.
Statistical analysis of the scores related to histopathological lesions, including congestion, hemorrhage, degeneration and necrosis, and hyaline cast, in the kidney tissue of different groups four weeks after the CdCl2 exposure. The scores are reported as Mean ± SEM and analyzed using Kruskal-Wallis and Mann-Whitney tests. Dissimilar letters are regarded as significantly different (p < 0.05).

Discussion

In the present study, CdCl2 caused various histopathological lesions in the gill, liver, and kidney tissues of common carp. Previous studies have also reported similar tissue damage induced by Cd exposure. For instance, Ahmed et al. [24] investigated the toxic effects of Cd in climbing perch (Anabas testudineus) and observed epithelial cell necrosis, separation of the epithelial layer, and fusion of secondary lamellae in the gill tissue. They also reported congestion, cell degeneration, and necrosis in the liver, as well as vacuolation in the kidney [25]. They showed that heavy metals like Cd can cause significant histopathological changes in various tissues of fish. Likewise, Peykanheraty et al. [24] found hyperplasia, clubbing, and fusion of the lamellae in the gill of Chondrostoma regium exposed to CdCl2, along with congestion and focal necrosis in the liver. They indicated that the gill and liver tissues can be regarded as the main organs exposed to the harmful effects of Cd.

Cd is known to cause cytotoxicity by binding to thiol groups in mitochondria, leading to mitochondrial dysfunction, cellular degeneration, and necrosis [5]. It also can increase lipid peroxidation, resulting in structural impairment and vacuolization of the liver and other tissues [2,5,24]. Cd may negatively affect the antioxidant system and generate free radicals, including reactive oxygen species (ROS) like superoxide (O2-), hydroxyl (OH-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) in the body [5,7].

The findings achieved from the present study are fully aligned with previous studies, demonstrating that Cd exposure can cause structural disruptions in various tissues. These tissue injuries highlight the importance of implementing effective methods to prevent or mitigate the harmful effects of heavy metals like Cd.

Disposing the agricultural wastes, such as PoP, presents a significant challenge. However, there has been growing interest in extracting valuable nutrients, including phenols, from these agricultural waste materials as safe and affordable sources of natural antioxidants [26-28]. Several studies have reported that pomegranates possess significant antioxidant activity compared to other dietary plants or fruits [27,29,30]. PoP has received considerable attention due to its high content of bioactive substances and antioxidant capacity.

Numerous studies have investigated the effectiveness of PoP extracts in mitigating the cytotoxicity of heavy metals and toxic agents in various animal models [31-34]. For instance, Hamed and Abdel-Tawwab [30] demonstrated that PoP powder inclusion in the diet of Nile tilapia could alleviate the adverse effects induced by silver nanoparticles (AgNPs). In that study, PoP significantly increased antioxidant activity and reduced tissue damage in the liver and kidneys, which are caused due to AgNPs exposure [31]. Likewise, another study showed that PoP extract significantly reduced lipid peroxidation and improved tissue damage and apoptosis in the liver of the Wistar rats exposed to lead (Pb) [32].

Jafari et al. [34] found that PoP extract, particularly at concentrations of 1% and 2% of diet weight (compared to 4%), effectively reversed the decline in liver antioxidant enzyme activity and the increase in lipid peroxidation caused by the $CdCl_2$ exposure over a period of 140 days in fish. They showed that Cd could negatively affect fish activities and physiology, and the PoP extract improved tissue functions. Our study supports these findings, while greater improvements were observed with the 2% and 4% PoP extracts in our study. Jafari and coworkers claimed that the probable bitterness of water due to the higher concentration of 4% may reduce appetite, water consumption, and physiologic activities, leading to reduce the extract intake by the fish and its positive effects [33]. Moreover, the density of 20 fish per aquarium in that study was higher than our study (N=10), and the period of the study was longer (140 vs. 28 days), which could affect the results of the studies.

The antioxidant activity of PoP is mainly attributed to compounds such as vitamin C, flavonoids, quercetin, ellagic acid, gallic acid, tannins, ellagitannins, and gallotannins [18,19,26,27,30]. These compounds can increase antioxidant activities, reduce lipid peroxidation, chelate Cd, and inhibit Cd deposition [5,35,36].

It has been reported that flavonoids and ellagic acid in PoP can function as potent scavengers and chelating agents for O_{2^-} and OH free radicals produced through the metabolism of heavy metals [5,35]. The presence of hydrogen atoms in the structure of gallic acid can delocalize ROS [36,37]. In addition, gallic acid has been found to have anti-inflammatory potentials, and it can reduce the Cd-induced inflammatory markers, including myeloperoxidase, interleukin-6, and NO in the rat brain [36]. Winiarska-Mieczan et al. [38] demonstrated that tannic acid can reduce the Cd accumulation in the rat lung and heart. Moreover, PoP-activated carbon as an adsorbent has been successfully applied to remove $CdCl_2$, the most common form of Cd and highly soluble in water, from aqueous ecosystems [39].

Taken together, the positive effects of PoP extract on the $CdCl_2$-induced lesions in the gill, liver, and kidney tissues suggest its hepatoprotective and nephroprotective potentials and antioxidant properties.

In conclusion, the PoP extracts mitigated the histopathological lesions induced by CdCl2 in the gill,

liver, and kidney tissues of common carp. The extent of improvement was particularly significant at higher concentrations of the extract (2% and 4%), indicating a dose-dependent effect. Although the PoP extract significantly reduced the tissue damage caused by $CdCl_2$, the tissues did not fully recover their normal structure. The presence of active phytocompounds in PoP extract and its protective role against toxic substances like $CdCl_2$ suggest its potential in the field of aquatic toxicology and fish physiology.

Materials and Methods

Ethical statement

The present study was conducted according to the Animal Experimental Guidelines approved by the Institutional Animal Care and Use Committee at Ferdowsi University of Mashhad.

The ethical approval for this study was issued with the ethical code for grant number 3/58310 from the Committee on Research Ethics of IR.UM.REC.1401.133, based on the Ethical Guidelines of Research from Ferdowsi University of Mashhad. The study was performed in the Aquaculture Laboratory, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Preparation of pomegranate peel extract

To prepare the PoP extract, the PoPs were washed with distilled water, dried in an oven at 40 °C for 10 days, and then ground. In the next step, the PoP powder (10 g) was extracted in a Soxhlet extraction apparatus using an equal mixture of four solvents: water, ethyl acetate, acetone, and ethanol. The extraction process was performed in three repetitions for 6 h each. The obtained extracts were then centrifuged at 4500 rpm for 3 min to remove fine particles and filtered using the Whatman paper (grade No. 41). Finally, the extract was concentrated in a vacuum oven at 40 °C, dried, and ground [40]. The PoP extract was ground, and the powder was stored at -18 °C until further use.

Study procedures

A total of 150 healthy common carp weighing approximately 65 ± 0.85 g with an average body length of 18 ± 1.5 cm and indeterminate sex were used in this study. The fish were randomly distributed among 15 glass aquaria, with a density of 10 fish per aquarium. After seven days of acclimatization period and feeding with a standard commercial fish diet, the fish were divided into five groups, with three replications for each group (30 fish in three aquaria per group).

The five groups (in three replicates) included as follows:

1- Control group: Fish received a standard diet without CdCl2, provided in four meals amounting to 2.5% of their body weight.

2- Cadmium (Cd) group: Fish were fed with the standard diet and exposed to a concentration of 0.5 mg/L of $CdCl_2$ (Merck, Germany) [34,41].

3- 1% PoP extract group: Fish were fed the same diet as the Cd group and received 0.35 g of the powdered PoP extract, which accounted for 1% of food weight [34].

4- 2% PoP extract group: In addition to the diet provided to the Cd group, fish received 0.7 g of PoP extract, representing 2% of the food weight.

5- 4% PoP extract group: Fish received 1.4 g of PoP extract (4% of food weight) along with the standard diet and 0.5 mg/L of $CdCl_2$.

After four weeks from the study, three fish from each aquarium (n = 9 for each group) were randomly selected and caught using an aquarium fish net. These fish (n = 45) were anesthetized with clove powder (0.5 g/L) [16, 34]. On necropsy of the fish, the gill, liver, and kidney tissues were removed, and the samples (with a size of 2 × 2 cm) from these tissues were taken for histopathological examinations.

It should be noted that the rest of the fish were kept for educational purposes in the Department of Aquaculture.

Histopathological examination

The tissue samples from the gill, liver, and kidneys were immediately placed and fixed in a 10% neutral buffered formalin solution. The formalin solution was changed after 24 h with a fresh formalin solution. The tissue samples were then dehydrated with varying degrees of ethanol, cleared with xylene, embedded in paraffin waxes, and cut into 5 µm-thickness sections in the laboratory of the Pathobiology Department. Finally, the sections were stained with hematoxylin and eosin dyes, and the prepared slides were examined under a light microscope equipped with a digital camera (Olympus, Japan) for any histological changes, including congestion, hemorrhage, cellular degeneration or necrosis, and other lesions. Ten fields of view at ×400 magnification (high-power fields) were examined for the histopathological lesions, and each lesion was scored for all groups based on Table 1 [42].

Statistical analysis

The Kruskal-Wallis and Mann-Whitney tests were used to analyze and compare the histopathological scores (Mean ± SEM) between the groups using the statistical package SPSS version 19.0 for Windows. P values lower than 0.05 ($p \leq 0.05$) were considered as significant.

Table 1.
The scoring system used for analysis of each histopathological lesion in the gill, liver and kidney of the fish.

Scoring of lesions	Description
0	Normal structure, with no lesion
1	Lesions in <25% the studied microscopic fields
2	Lesions in 25-50% the studied microscopic fields
3	Lesions in 50-75% the studied microscopic fields
4	Lesions in >75% the studied microscopic fields

Authors' Contributions

H.J. carried out the experiments and contributed to sample preparation. S.A and D.S conceived and planned the experiments, contributed to sample preparation, contributed to the interpretation of the results, and took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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