water

MDPI

Article

Assessment of Liver Toxicity in Wistar Rats after Chronic Exposure to Phosphate-Processing Wastewaters from Gafsa-Metlaoui Laundry in Tunisia

Bouthaina Brahmi [1], Emna Nasri [1], Amel Moula [1], Anouar Feriani [1], Nizar Tlili [2], Demetrio Raldua [3], Eduardo Alberto López-Maldonado [4,*] and Mohamed Ali Borgi [1,*]

[1] Laboratory of Biotechnology and Biomonitoring of the Environment and Oasis Ecosystems (LBBEEO), Faculty of Sciences of Gafsa, University of Gafsa, Zarroug, Gafsa 2112, Tunisia; brahmi01@yahoo.com (B.B.); emmna.nasri@gmail.com (E.N.); amelmoula1992@gmail.com (A.M.); ferianianwer@yahoo.fr (A.F.)
[2] Higher Institute of Environmental Science and Technology of Borj Cedria, University of Carthage, Tunis 1073, Tunisia; nizar_fst@yahoo.fr
[3] Institute for Environmental Assessment and Water Research (IDAEA), Consejo Superior de Investigaciones Cientificas (CSIC), Jordi Girona, 18, 08034 Barcelona, Spain; demetrio.raldua@idaea.csic.es
[4] Faculty of Chemical Sciences and Engineering, Autonomous University of Baja, Tijuana 22390, CP, Mexico
* Correspondence: elopez92@uabc.edu.mx (E.A.L.-M.); borgima@fsgf.ugaf.tn (M.A.B.)

Abstract: In the mining basin of the Gafsa region in southwestern Tunisia, environmental exposure to randomly discharged phosphate-processing wastewaters (PPWW) presents a serious threat to health and the surrounding ecosystems. Thus, the contaminated areas are in continuous deterioration over time. There is a paucity of information on the deleterious effects of this kind of effluent. In the current work, the PPWW characterization showed the presence of high contents of Pb (0.90 ± 0.02 mg/L), Cd (0.35 ± 0.27 mg/L), Cr (0.43 ± 0.1 mg/L) and Fe (215.1 ± 2.41 mg/L), exceeding the permissible limits. To assess the chronic toxicity of the effluent in mammalians, two doses of PPWW (50% and 100%) were administered by gavage to Wistar rats for 28 consecutive days. The results revealed that the two PPWW concentrations significantly increased the plasma biochemical markers (bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH)), compared to untreated animals. Moreover, PPWW treatment severely altered the lipid profile by increasing the contents of triglycerides, total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-cholesterol) by 143%, 114%, and 91%, respectively, and significantly reduced the high-density lipoprotein cholesterol (HDL-cholesterol) level by 46%, compared to the control animals. In addition to the significant decrease in activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the liver of intoxicated rats, the malondialdehyde (MDA) level was remarkably altered. All of these were associated with deep histopathological damages, materialized by dilatation of sinusoids, congestion of the centrilobular vein, and inflammatory cell infiltration. These disturbances were accompanied by metal detection in the liver and blood. Additionally, DNA fragmentation detected in hepatic tissues highlighted the genotoxic effects of PPWW. All of the aforementioned effects occurred in a PPWW dose-dependent manner. These findings evidenced, for the first time, the in vivo-deleterious impacts of this type of effluent on mammalians inhabiting the mining basin area and therefore showed the real threats to which humans, as consumers, could be exposed. Accordingly, there is a dire need to pay special attention to PPWW before being discharged into environmental ecosystems without any prior treatments.

Keywords: phosphate-processing wastewaters; heavy metals; oxidative stress; DNA fragmentation; hepatotoxicity; metal accumulation

Citation: Brahmi, B.; Nasri, E.; Moula, A.; Feriani, A.; Tlili, N.; Raldua, D.; López-Maldonado, E.A.; Borgi, M.A. Assessment of Liver Toxicity in Wistar Rats after Chronic Exposure to Phosphate-Processing Wastewaters from Gafsa-Metlaoui Laundry in Tunisia. *Water* **2024**, *16*, 214. https://doi.org/10.3390/w16020214

Academic Editor: Antonio Zuorro

Received: 9 December 2023
Revised: 28 December 2023
Accepted: 4 January 2024
Published: 7 January 2024

1. Introduction

Currently, water pollution is becoming a global challenge that increasingly threatens human and environmental health [1,2]. In fact, rapid industrialization, domestic effluents

from human settlements, and modern agriculture practices (use of fertilizers, herbicides, pesticides, etc.) are the major sources of degradation of inland and coastal waters. It has been documented that around three to four hundred million tons of toxic sludge, heavy metals, solvents, and other unpleased compounds are released into the environment every year worldwide [3–5]. For example, the large amount of hospital wastewaters released into the environment reaches groundwater and therefore threatens the health of water consumers [6]. This category of wastewater induces cytotoxic effects [7], genotoxicity, and mutagenicity [8–10]. Previous reports showed that textile wastewaters comprise high levels of nonbiodegradable compounds, which disturb the aquatic environment [11,12] and consequently human health [13]. Plastic contaminations were also studied for their adverse effects on the human food production chain [14,15]. Considering the undeniable link between the environment and food production, the persistent pollutants discharged into wastewater can end up in food, both of vegetable and animal origins, increasing their levels throughout the food chain [16,17]. It can be noted here that to better understand all these dangerous effects of wastewater and at the same time achieve effective treatment procedures, several reports recommended to look for possible alternatives to limit or identify such spills and create a wastewater quality monitoring system [18].

In Tunisia, the Company of Phosphates of Gafsa (CPG) is ranked among the highest phosphate producers worldwide [19,20]. The company's marketable phosphate production reached 8 million tons per year with water consumption of up to 10.5×10^6 m^3 [20], and the resulting phosphate processing wastewaters (PPWW) were discharged randomly into the environment.

In the literature, few data are available about the harmful consequences of PPWW on the receiving ecosystem. In fact, Mekki et al. [21] and Mekki and Syedi [22] assessed the phytotoxicity of soil irrigated by phosphate ore processing wastewater and its microtoxicity. According to the same authors, PPWW toxicity is correlated with its high contents of heavy metals. Such potentially toxic elements present a real threat, not only on the surrounding ecosystem but also on groundwater of the region, and thus reach humans by drinking water. Herein, it is worth noting that according to the World Health Organization (WHO), the permissible limits of some potentially toxic elements in drinking water have been set at low levels. These levels should not exceed 0.005 mg/L for Cd, 0.05 mg/L for Pb, 0.001 mg/L for Hg, 0.1 mg/L for Ni, 0.01 mg/L for As, 0.01 mg/L for Co, and 0.05 mg/L for Cr [23]. As part of attempts to bio-detoxify PPWW, Moula et al. [24–26] highlighted indigenous bacteria's effectiveness in performing bioremediation assays of toxic PPWW. In a bioremediation context, it should be noted that bio-inspired remediation technologies of heavy metal-contaminated sites increasingly replaced traditional methods, criticized for their high cost and environmental concerns [27]. Hence, Xue et al. investigated the potential of applying the microbially induced carbonate precipitation (MICP) approach to remediate Pb-rich water bodies and Pb-contaminated loess soil [28]. In another study, the authors ensured the immobilization of Pb to prevent its migration, in a harsh environment, from Pb-contaminated water bodies and soils, by means of self-healing microbial-induced calcium carbonate (MICC) materials [29]. So far, studies relating to this new bio-inspired technology have been deepened by the application of the first microcapsule-based self-healing microbial-induced calcium carbonate materials to avoid the migration of Pb ions [30].

Meanwhile, PPWWs are a potential risk for some mammalian species, such as goats and camels, that inhabit this area. These animals live with other wild rodents; right near the areas permanently invaded by PPWW and occasionally drink this wastewater and graze the affected growing indigenous plants. This happening certainly resulted in the passage of heavy metals to humans, as consumers, of these intoxicated animals. As far as we know, the toxic effects of PPWW have never been studied in a mammalian model. There is therefore an urgent need to investigate, for the first time, the in vivo harmful effects of this kind of effluent by using Wistar rats as a model. In mammalians, the liver is known to be a target organ for chemically induced toxicity and for its fundamental role in clearing xenobiotics from the blood [31]. This vital tissue also harbors several markers of

toxicity and has been the subject of extensive toxicological studies [32–34]. Hence, in the present study, we targeted the liver tissue of Wistar rats to explore the impacts of chronic exposure to PPWW (at 50 and 100% concentrations) for 28 consecutive days. To achieve this goal, a set of liver damage markers, including bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), triglycerides, (TG) total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were determined in the plasma. Oxidative stress markers (malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH)), heavy metal (Cd and Pb) determination in the blood and liver, and DNA damage and histopathological changes were explored in treated and untreated rats.

2. Materials and Methods

2.1. Wastewater Sampling and Characterization

The PPWW samples were collected from the effluent stream of the fifth phosphate-washing unit of Gafsa-Metlaoui (Tunisia). The geographical coordinates are latitude 34.193288 and longitude 8.201846. Three samples, at intervals of 15 days, were collected in sterile amber glass bottles, pooled, and stored at −20 °C until use (Figure 1). In the laboratory, samples were filtrated through a 0.45 µm pore nylon membrane filter and a 0.2 µm pore glass fiber filter to remove all suspended matter. After filtration, we only obtained the dissolved fraction, which was used for analytical study and for animal treatments. The effluent pH was measured by a STARTER 2100 pH meter. The electrical conductivity (EC) and the turbidity were measured, respectively, by a Conductimeter type Cond 1970i and a turbidimeter type VTV. After adequate dilution of samples using an acid mixture of HNO_3 and HCl, heavy metal ions (Fe, Cr, Cu, Zn, Cd, and Pb), phosphorus (P), chloride (Cl), potassium (K), calcium (Ca), and sodium (Na) were quantified by an Analtik Jena Nova 400 (Japan) model atomic absorption spectrophotometer (AAS) operating with an air–acetylene flame.

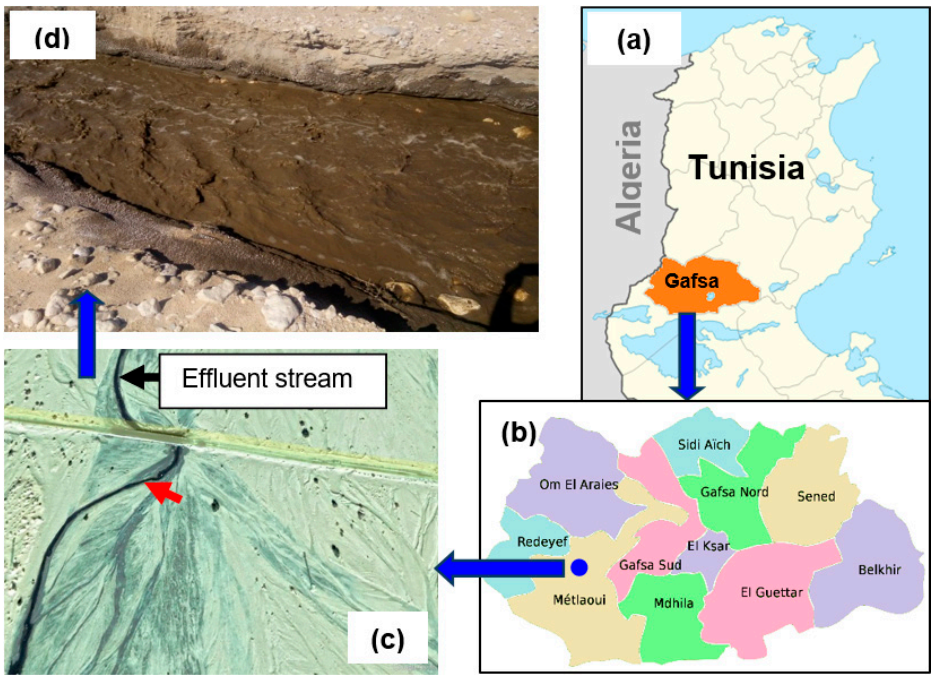

Figure 1. (**a**,**b**) Geographical location of the Gafsa-Metlaoui mining basin in the southwestern dry area of Tunisia (blue point). (**c**) Google Earth Program-generated photo showing the effluent stream and the sampling point (red arrow): latitude 34.193288; longitude 8.201846. (**d**) Image of the phosphate-processing wastewater stream after being discharged from the fifth phosphate washing plant in Metlaoui city.

2.2. Evaluation of Hepatotoxicity

2.2.1. Animal Preparation

All animal experimental procedures were planned and approved by the Ethical Committee for the Care and Use of Laboratory Animals at the University of Gafsa (Reference No: FSG-02-2022). Eighteen male Wistar rats weighing 180–200 g were purchased from Pasteur Institute (Tunisia) and placed under controlled conditions (22 ± 1 °C, humidity of 50%, and alternation between 12 h light and 12 h dark). A standard pellet diet obtained from the Animal Nutrition Society (SNA, Sfax, Tunisia) was used as nutriment for the animals. The rats had free access to water. After a period of adaptation of two weeks, the rats were arbitrarily allocated into three experimental groups (n = 6): group I (control) rats received normal drinking water; Group II (PPWW-50%) and Group III (PPWW-100%) were intragastrically administered 50% and 100% concentrations of PPWW, respectively, for 28 consecutive days. After the treatment period, the rats were anesthetized by an intraperitoneal ketamine hydrochloride injection (30 mg/kg bw) and then euthanized to obtain blood and liver samples. After collecting the blood in EDTA tubes, the samples were centrifuged at 3500 rpm at 4 °C for 15 min, and plasma obtained was stored at −20 °C for further analysis. The rats were dissected, and the liver was excised, weighed, and washed in ice-cold saline buffer. Then, a portion of the liver was kept at −70 °C for biochemical analysis and DNA extraction. For the histopathology study, a part of fresh liver was fixed in neutral buffered formalin (10%).

2.2.2. Assessment of Hepatotoxicity Markers

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activities were determined spectrophotometrically according to standard methods by using commercially available diagnostic kits (Biomaghreb, Ariana, Tunisia), and the values were presented as units per liter. The liver index was estimated using the following formula:

$$\text{Liver index (\%)} = \text{liver weight/body weight} \times 100$$

2.3. Assessment of Oxidative Stress in the Liver Tissue of Experimental Rats

2.3.1. Tissue Extract Preparation

A one-gram portion of the liver was added to 2 mL of ice-cold lysis buffer (pH = 7.4) and homogenized using a Homogenizator Ultraaturex grinder. The resulting homogenate was centrifuged at 4000× *g* for 15 min, and the supernatant was collected and stored at −70 °C until use.

2.3.2. Evaluation of Lipid Peroxidation

The lipid peroxidation in the liver of the control and other treated groups was measured by quantifying thiobarbituric acid reactive substances (TBARS). It was expressed in terms of malondialdehyde (MDA) content (the end product of lipid peroxidation). The MDA level was measured spectrophotometrically according to Buege and Aust's method [35]. The results were expressed in nanomoles of MDA per gram of tissue.

2.3.3. Determination of Liver Enzymatic Antioxidant Activities

The activity of superoxide dismutase (SOD) was determined by measuring the enzyme capacity to inhibit the reduction of nitro-blue tetrazolium (NBT) by the anion superoxide product [36]. The absorbance was measured at 560 nm, and the activity was expressed in units per milligram of protein. The evaluation of catalase activity (CAT) was carried out according to the protocol reported by Aebi [37]. The catalase activity was expressed in micromoles of H_2O_2 decomposed per minute per milligram of protein. Glutathione peroxidase activity (GPx) was determined in the liver homogenate according to the method reported by Flohé and Gunzler [38]. The absorbance was read at 340 nm, and the enzyme activity was expressed in nanomoles of NADPH (Nicotinamide Adenine Dinucleotide

Phosphate) oxidized per minute per milligram of protein. All steps of the evaluation of oxidative stress in rat liver tissue are summarized in Figure 2.

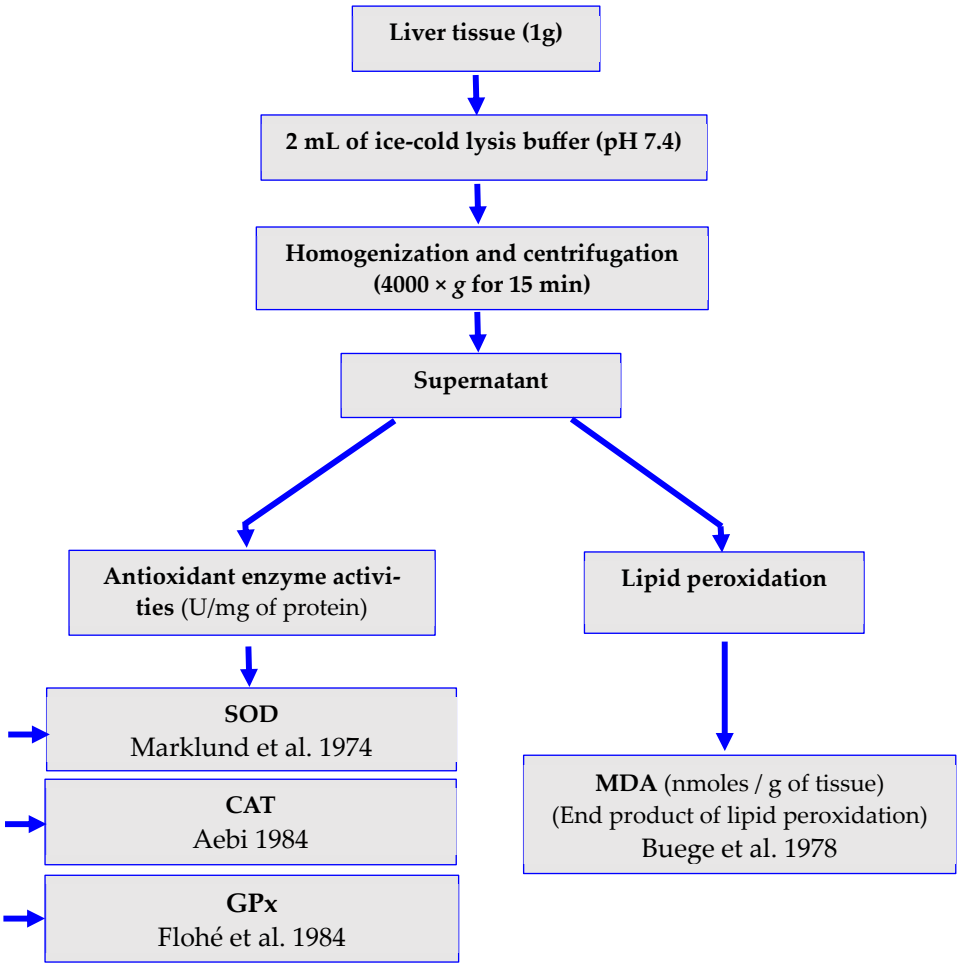

Figure 2. Steps illustrating the experimental evaluation of oxidative stress in rat liver tissue [35–38].

2.4. Protein Quantification

The protein concentration in liver homogenates was determined using the Bradford method [39], and bovine serum albumin (BSA) was used as the standard.

2.5. Histopathological Examinations

Liver samples fixed in phosphate-buffered formalin (10%) were randomly selected for histological analysis. The samples were embedded in paraffin following a sequential dehydration process. Subsequently, 5 µm sections were stained with hematoxylin and eosin. Finally, histopathological analysis of the sections was performed to identify potential changes in liver histology.

2.6. Assessment of DNA Fragmentation

For qualitative evaluation of DNA extracted from the livers of treated and untreated rats, the protocol described by Feriani et al. was used [40]. The DNA fragmentation assay was carried out by electrophoresing genomic DNA samples on agarose/EtBr gel following the procedure reported by Chtourou et al. [41].

2.7. Cadmium and Lead Analysis in Blood and Liver

In the present study, we assessed the contents of cadmium (Cd) and lead (Pb) in the blood and liver of treated and untreated rats. To do so, we used 1 mL of heparin blood

and 1 g of liver tissue. The amount of Cd and Pb was determined by atomic absorption spectrophotometry, following the method described by Andjelkovic et al. [42].

2.8. Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). One-way ANOVA (analysis of variance) with Tukey's post hoc test was used to compare the mean obtained for each treatment group (n = 6 rats) applying Graph-Prism 7.01 (GraphPad, San Diego, CA, USA), with $p < 0.05$ indicating statistical significance.

3. Results and Discussion

3.1. Physicochemical Characterization of PPWW

To be marketable, rock phosphate treatment in washing units involves several steps, including mechanical separation and flotation, to increase the P_2O_5 content [43]. Hence, phosphate-washing plants dump huge amounts of highly toxic wastewaters, so-called phosphate-processing wastewaters (PPWWs). The physicochemical parameters of the PPWW samples are shown in Table 1. The results indicated that the effluent had a pH of 7.3, with a conductivity (EC) of 9.46 ± 0.03 mS cm^{-1}, associated with a high concentration of chlorides (1660 ± 081 mg/L). The effluent contained high BOD_5 and TOC values of 500 ± 1.03 mg/L and 10.5 ± 0.32 mg/L, respectively. The elevated values of COD and BOD_5 reflected the high degree of water pollution, and the BOD_5/COD ratio, called the "biodegradability ratio" of 1.4 > 0.5, revealed that the effluent is potentially biodegradable and therefore treatable by biological means [44]. The measurements showed the richness of PPWW with calcium (Ca = 835 ± 0.01 mg/L), iron (Fe = 215.1 ± 1.41 mg/L), lead (Pb = 0.9 ± 0.002 mg/L), chromium (Cr = 0.434 ± 0.01 mg/L), and cadmium (Cd = 0.353 ± 0.27 mg/L). These heavy metals are currently considered as being in the top 10 hazardous materials [45]. Their contents were above the limits fixed by international standards for wastewater discharges [46,47] and constitute a real risk, notably after their accumulation in the soil of the receiving medium [22]. In addition to the elevated total suspended solids, the results showed a high amount of phosphorus in the effluent (P = 58.14 ± 1.36 mg/L), which led to waterway eutrophication [48].

Table 1. Physicochemical characterization of phosphate-processing wastewater (PPWW) samples compared to Tunisian national and international standards. Values are the mean of triplicate measurements ± standard deviation (SD). BOD_5: biochemical oxygen demand; COD: chemical oxygen demand; TS: total solids; TOC: total organic carbon; EC: electrical conductivity; COD/BOD_5: ratio of the biodegradability; NI, not identified.

Parameters	PPWW (SD)	OPORT (2018)	APHA (2005)
pH	7.31 (0.04)	8.5	9.00
EC (mS/cm)	9.46 (0.03)	5.00	6.00
TOC (g/L)	10.5 (0.32)	NI	NI
TS (g/L)	54.03 (1.23)	NI	NI
COD (mg/L)	710.8 (9)	200.00	≤70.00
BOD_5 (mg/L)	500 (5)	50.00	≤50.00
COD/BOD_5	1.4 (0.13)	3.00	≤0.71
P (mg/L)	58.14 (1.36)	10.00	≤10.00
Chlorides (mg/L)	1660 (5)	700	NI

Table 1. *Cont.*

Parameters	PPWW (SD)	OPORT (2018)	APHA (2005)
Pb (mg/L)	0.9 (0.02)	0.1	≤0.10
Ca (mg/L)	835 (10)	500	≤200
Na (mg/L)	23 (0.15)	0.50	NI
K (mg/L)	53 (0.12)	50	≤30
Cr (mg/L)	0.434 (0.01)	0.1	NI
Cu (mg/L)	0.342 (0.02)	0.50	≤0.25
Zn (mg/L)	0.173 (0.07)	0.50	≤1.00
Cd (mg/L)	0.353 (0.27)	0.10	≤0.10
Fe (mg/L)	215.1 (2.41)	5.00	≤5.00

3.2. Effect of PPWW on Body and Liver Weights and Blood Glucose Levels

To our knowledge, the in vivo effects of PPWW discharged into the environment have not been previously investigated. The works in this field were restricted to their characterizations in terms of richness in heavy metals, such as Cd, Pb, Cu, Zn, and Fe [22], and other rare earth elements, including lanthanides with Y and Sc [49,50]. Additionally, the potential phytotoxic and microtoxic effects of PPWW were studied by Mekki and Sayadi [22] and Moula et al. [24,25]. Therefore, the in vivo effects of this kind of effluent are worth further investigation.

Changes in body and liver weights and glucose levels in all experimental groups are shown in Figure 3. During the entire experimental period, rats in all treated groups showed no differences in body weights compared to the control. Similarly, the dose PPWW-50% did not affect the liver weight index. However, a significant difference in this parameter was encountered between the PPWW-100 group and the control group ($p < 0.01$) after 4 weeks of effluent exposure. According to Hall et al., the increase in liver weight, in rodents due to exposure to chemicals, can be achieved through histological appearances, some of which clearly showed cytotoxicity and cell death [51]. Additionally, Zhao et al. reported that the wastewater caused the proliferation of liver cells, which led to an increase in liver weight [52]. In another study, it has been documented that the exposure of rats to a Cd and Pb mixture caused liver enlargement and a remarkably increased liver weight index, compared to the untreated animals [53]. The data revealed that PPWW induced a significant increase ($p < 0.0001$) in the blood glucose content (119.6 ± 2.302 mg/dL and 179.6 ± 2.074 mg/dL for PPWW-50% and PPWW-100%, respectively) when compared to the control group (76.40 ± 2.881 mg/dL). This severe hyperglycemia detected in experimental animals might be associated with the alteration of pancreatic cells. Hence, many reports have suggested that the observed hyperglycemia due to toxic effects of pollutant resulted in pancreatic β-cell dysfunction, leading to insufficient insulin secretion [54]. In the same context, Sarmiento-Ortega et al. reported that Cd accumulation in the liver led to mitogenic signals that develop insulin resistance and thus to glucose level elevation in Cd-treated rats [55]. It was also remarkable that the effect of PPWW on blood glucose occurred in a PPWW dose-dependent manner.

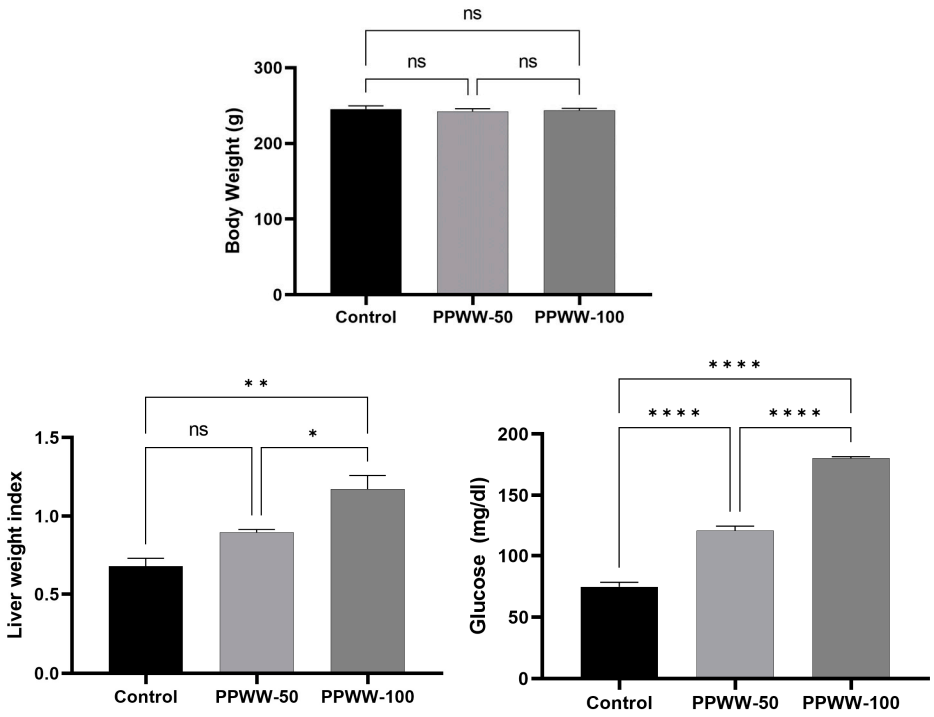

Figure 3. Variation in body weight, liver weight index, and blood glucose level in the control and experimental groups of rats. Values represent the mean ± SD (six animals were treated per group, n = 6). PPWW-50% or PPWW-100% group versus control group. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; ns, not significant.

3.3. Effect on Hepatic Injury Marker Enzymes

The results of the biochemical marker analyses related to hepatic injury in all treated rats are illustrated in Table 2. The PPWW-50%-treated animals showed noticeable increases in the activities of bilirubin, AST, ALT, ALP, and LDH by 95%, 39%, 39%, 149%, and 24%, respectively, compared to the untreated group. For the PPWW-100 dose, these activities were significantly augmented by 153%, 52%, 52%, 189%, and 57%, respectively, compared to the control group. These observations are in line with the work of Chen et al., who attributed the significant elevation of bilirubin, AST, ALT, and LDH activities in mice to the combined effects of Cd and Pb ions [53]. Vineeth Daniel et al. also concluded that the exposure of mice to Pb resulted in a functionally impaired liver, with a dramatic increase in ALT and AST activities [56].

Table 2. Bilirubin, transaminases (AST, ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) activities in plasma of control and treated rats with two doses of phosphate-processing wastewaters (PPWW-50% and PPWW-100%). Values represent the mean ± SD (six animals were treated per group, n = 6). PPWW-50% or PPWW-100% group versus control group. ** $p < 0.01$; **** $p < 0.0001$.

Parameters Treatments	Bilirubin (U/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)
Control	1.333 ± 0.2086	154.4 ± 3.551	102.3 ± 9.609	143.0 ± 5.867	161.1 ± 6.162
PPWW-50%	2.602 ± 0.8175 **	215.0 ± 8.406 ****	142.4 ± 4.118 ****	356.2 ± 6.907 ****	199.9 ± 7.613 ****
PPWW-100%	3.382 ± 0.1479 ****	235.1 ± 5.657 ****	155.8 ± 5.836 ****	414.5 ± 14.92 ****	253.5 ± 9.181 ****

3.4. Effects on Oxidative Stress Markers

The results illustrated in Figure 4 show that chronic exposure of animals to PPWW for four weeks induced oxidative stress in the liver. Lipid peroxidation levels in both the

PPWW-100% group (7.361 ± 0.6598 nmol MDA/g of the liver) and PPWW-50% group (5.951 ± 0.9655 nmol MDA/g of the liver) were significantly higher ($p < 0.0001$) than those of the corresponding controls (2.689 ± 0.7567 nmol MDA/g of the liver). Moreover, the observed effect on oxidative stress was concentration-dependent, with rats from the PPWW-100% group showing lipid peroxidation levels significantly higher than those from the PPWW-50% group ($p < 0.05$). The toxicological effects of heavy metal-rich PPWW were primarily attributed to the presence of heavy metals and other harmful constituents, known for their ability to generate free radicals inside biological systems [57], together with the poor antioxidant defense in intoxicated rats. These observations are in tandem with previous studies showing that oxidative stress is induced by exposure to heavy metals in fish [58], water birds [59], and rats [60,61]. In fact, the incessant generation of reactive oxygen species (ROS) following chronic PPWW administration weakens antioxidant protection and increased lipid peroxidation, as the first step that induces cellular membrane injury [62]. The significant dose-dependent increase in MDA amounts (a biomarker of lipid peroxidation) in the tissue of PPWW-treated animals, indicated the severity of heavy metal toxicity and the high amount of ROS that could be generated. In addition, the activity of several enzymes from the antioxidant defense system (SOD, CAT, and GPx) was significantly inhibited by PPWW (50% and 100%) compared to the control group (Figure 4). However, we did not find a significant difference in the activities of these enzymes between the PPWW-100 and PPWW-50 groups after 28 days of PPWW treatment.

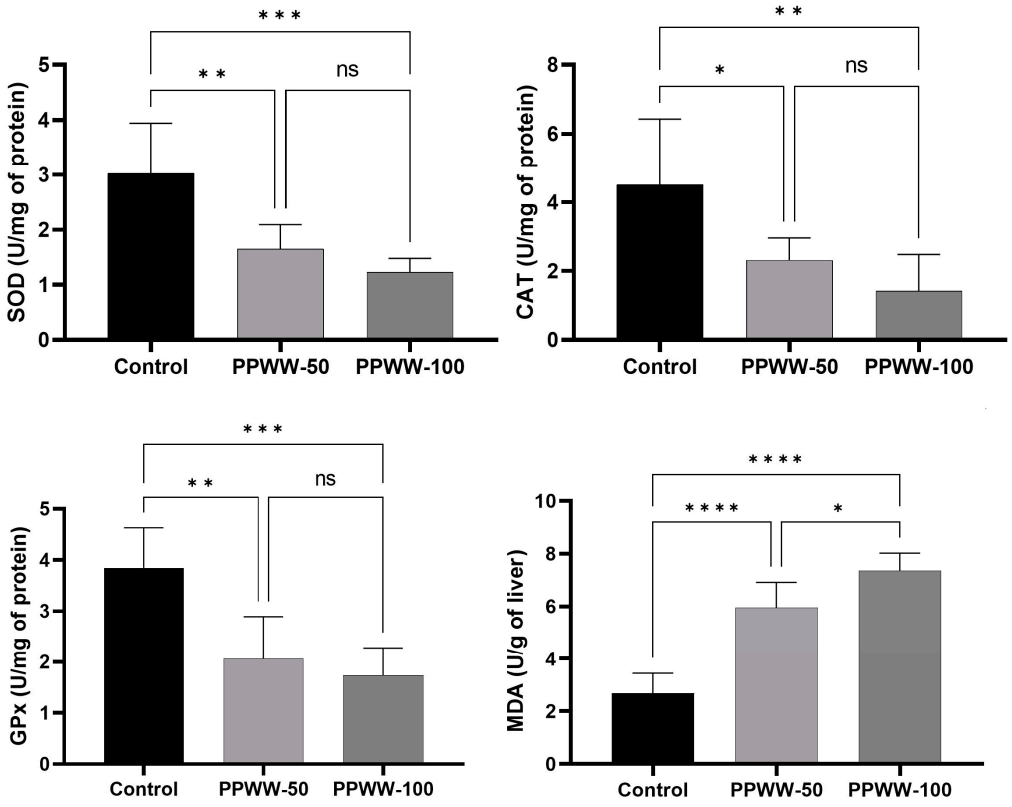

Figure 4. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities and MDA levels in the liver tissues of control and treated rats after 28 days of exposure to 2 doses of phosphate-processing wastewaters (PPWW-50% and PPWW-100%). Values represent the mean ± SD (six animals were treated per group, n = 6). PPWW-50% or PPWW-100% group versus control group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

It is well documented that the antioxidant system, namely, SOD, CAT, and GPx, plays a substantial role in the protection of hepatocytes against lipid peroxidation or inflammation, preventing the occurrence of hepatic damage. In the PPWW-exposed animals, the

significant reduction in antioxidant enzyme activities (SOD, CAT, and GPx) was probably related to the large amount of accumulated Pb and Cd. It has been established that these two metals markedly disrupt the antioxidant system by binding to sulfhydryl groups inside antioxidant enzymes and replacing zinc and copper, which are important cofactors for these enzymes [63–65]. Similar observations were also encountered by Andjelkovic et al. who reported that only a mixture of heavy metals (Cd and Pb) could generate AOPP (advanced oxidation protein products) [42]. In addition to the effects above, the enormous PPWW iron concentration could have a deleterious effect on the antioxidant enzymes. It has been reported that iron overload in rats induces lipid peroxidation and antioxidant depletion caused by a decrease in SOD, GPx, and CAT activities, which causes liver cell injury and apoptosis in animals [66,67]. Other studies also highlighted the impacts of iron overload on the generation of hydroxyl radicals, which are responsible for lipid peroxidation [68–70], oxidative stress, and hepatic fibrogenesis [66,71], as well as an alteration of stress protein expression in rat liver [72]. Bloomer and Brown [73] also reported other liver diseases caused by excessive iron administration.

3.5. Effects on Lipid Profile

Figure 5 illustrates the TG, TC, LDL-C, and HDL-C concentrations in the plasma of control and experimental rats. The administration of PPWW significantly increased the TG (112.47% and 138.78% for PPWW-50% and PPWW-100%, respectively), TC (93.90% and 117.13% for PPWW-50% and PPWW-100%, respectively), and LDL-C concentrations (70.91% and 109.71% for PPWW-50% and PPWW-100%, respectively) compared to control animals. In contrast, PPWW-50% and PPWW-100% doses induced a remarkable decrease in HDL-C levels in plasma by 30.99% and 40.55%, respectively, compared to untreated animals. Nevertheless, the reduction in HDL-C between animals treated with the two doses was insignificant.

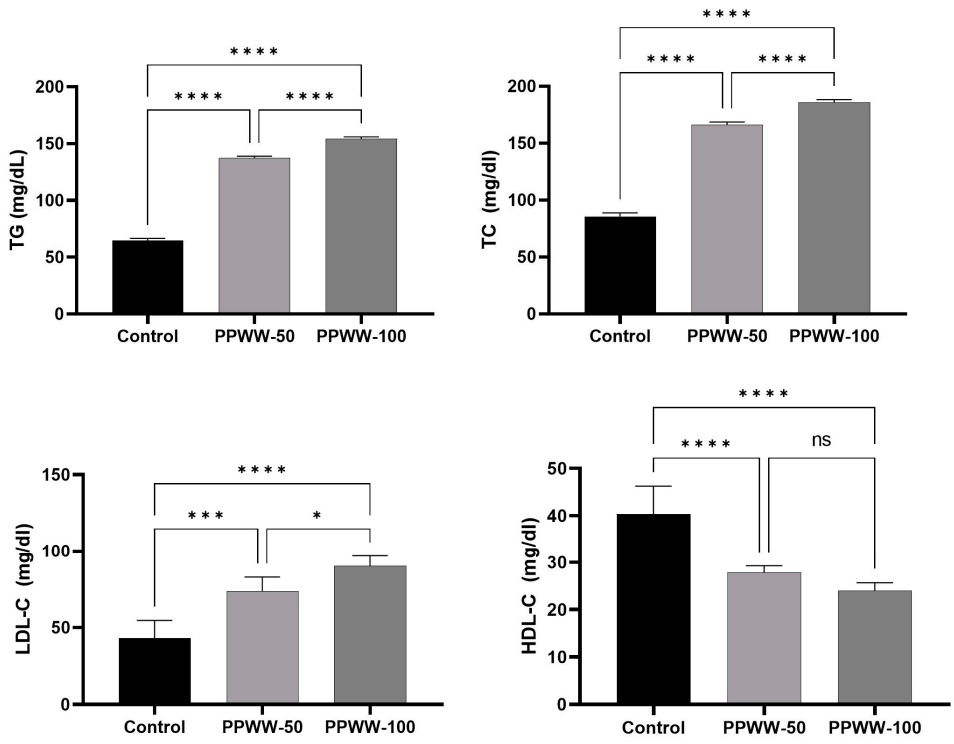

Figure 5. Effect of phosphate-processing wastewaters applied at two doses (50%: PPWW-50%; 100%: PPWW-100%) to rats for 28 days on plasma triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-cholesterol) and high-density lipoprotein cholesterol (HDL-cholesterol). Values represent the mean ± SD (six animals were treated per group, n = 6). PPWW-50% or PPWW-100% group versus control group. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

These harmful effects of PPWW on lipid parameter contents are complex and multivariate. It is possible that this hyperlipidemic effect was the result of the activation of pancreatic lipase activity, leading to an increase in lipid absorption [74]. Moreover, the upregulation of HMG-CoA reductase gene expression, a key enzyme in cholesterol biosynthesis, could also arise. In addition, it has been previously suggested that heavy metals can cause hepatic dyslipidemia and enhance acetyl-CoA carboxylase activity, an important enzyme that regulates de novo lipogenesis [56].

3.6. Histopathological Study

The fixed hepatic portions from animals using H-E (hematoxylin and eosin) were examined to assess the histopathological alterations. As shown in Figure 6, typical structures were observed in the livers of control rats. Compared to control rats, histopathological analysis of the liver of rats in the intoxicated group showed that while the liver of control rats exhibited a normal structure, rats from the treated group (PPWW-50% and PPWW-100%) presented dilatation of sinusoids, congestion of the centrilobular vein, and inflammatory cell infiltration.

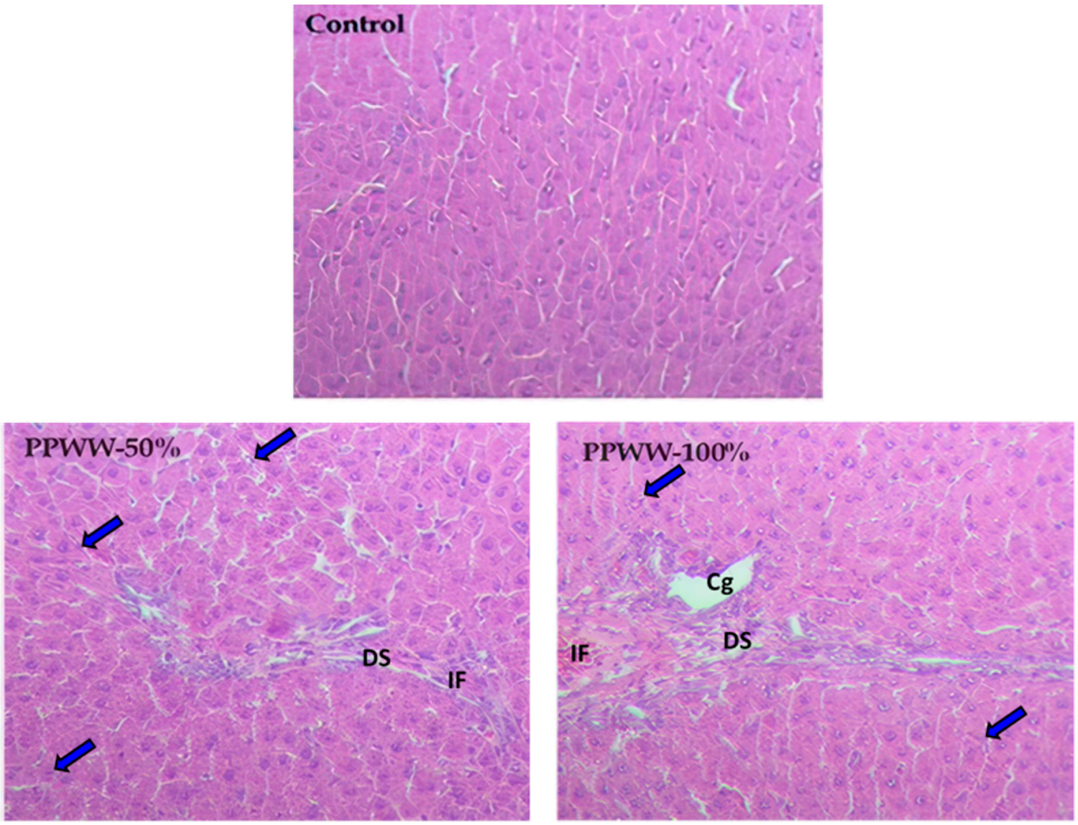

Figure 6. Photomicrographs of the liver in control and experimentally treated rats (PPWW-50% and PPWW-100%). Hepatic tissue sections were stained with hematoxylin and eosin (G×400). IF: inflammatory cell infiltration; Cg: congestion of the centrilobular vein. Arrows: proliferation of Kupffer cells; DS: dilatation of sinusoids.

The liver is one of the most sensitive predictors of chemical toxicity and correlates well with serum biochemistry parameters, DNA structure, and histopathology with modest interanimal variations [75]. This organ is a target for many heavy metals. For instance, it has been reported that following the oral treatment of rats with river water contaminated by heavy metals, the liver exhibited more significant metal accumulation than the kidney and brain, which occurred in a concentration-dependent manner [76].

The significant dose-dependent increase in bilirubin levels and activities of AST, ALT, LDH, and ALP in plasma is consistent with the histopathological findings. In fact, we detected a lobular disordered structure, congestion of the centrilobular vein, appearance of necrotic cells, and activation of Kupffer cells, in the liver of the treated rats. These deleterious effects support the accumulation of heavy metals derived from PPWW in the liver. Similar liver damages were also documented in rats during their exposure to heavy-metal mixtures [77,78]. Compared to control animals, the observed damage structure of the liver could be a result of the generation of free radicals caused by continuous accumulation of heavy metals in the hepatic tissue. Many studies have signaled that hepatocyte cell death induced by oxidative stress plays a significant role in the liver tissue damage [79,80].

3.7. Effect of PPWW on DNA of Liver Tissue

As can be seen in the gel electrophoresis (Figure 7), the DNA extracted from the liver cells of the control group presented an approximately intact band. Significant qualitative alterations in the genomic DNA profile were observed for the DNA extracted from 50% (PPWW-50%) and 100% (PPWW-100%) effluent-treated animals. The treatment of rats with PPWW-100% markedly affected the DNA quality by generating a "ladder"-like pattern, revealing liver DNA fragmentation and apoptosis. There was a significant change in DNA fragmentation among the treated groups with PPWW-50% and those treated with PPWW-100%.

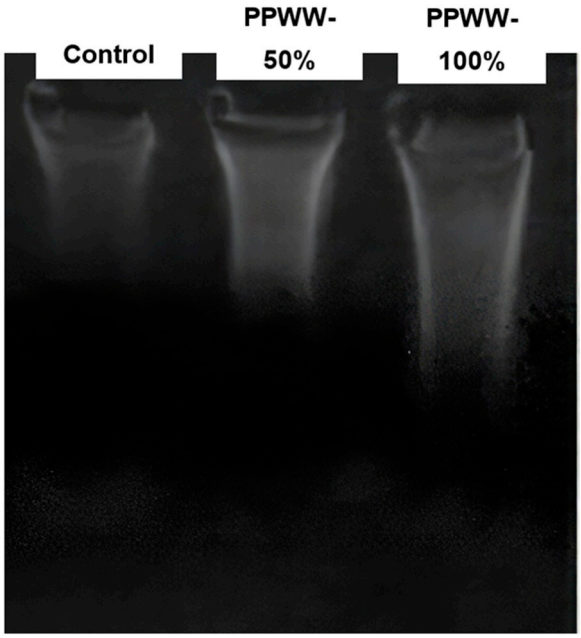

Figure 7. Photo of agarose gel electrophoresis showing qualitative analyses of the DNA extracted from liver tissues of control and experimental rats. Control: DNA isolated from liver samples of untreated control rats. PPWW-50%: DNA isolated from the livers of animals treated with 50% concentrations of phosphate-processing wastewater. PPWW-100%: DNA isolated from animals treated with 100% concentrations of phosphate-processing wastewater.

It has been reported that experimental rats exposed to toxic compounds suffer from severe genotoxicity, materialized by DNA fragmentation. This phenomenon may be related to repair mechanism-induced DNA lesions and conformational alterations, leading to carcinogenesis, cell-cycle modulation, or apoptosis of nuclear proteins and DNA [81]. In this context, previous studies have documented the mechanism of heavy metal-induced hepatotoxicity, particularly DNA damage, when they go beyond permissible limits [78,82]. In these recent works, the authors emphasized the essential role of the generated reactive oxygen species (ROS), which cause numerous injuries and undesirable changes in hepatic

tissues. Additionally, Beltifa et al. [9] demonstrated that DNA damages occurred in the hepatic cells of rats exposed to hospital wastewater, previously characterized by their rich ness in heavy metals [7].

In this work, the animals were exposed to a mixture of potentially toxic elements collected in situ but not a mixture of standards under laboratory well-controlled exposure assays. The damage and alterations in the cellular function of liver tissue observed in our work could be partially associated with the presence of elevated amounts of iron in PPWW samples, as has been reported by Al-Basher [66]. Previous research has reported that oxidative damage to DNA resulting from high metal levels may be related to the development of hepatocellular carcinoma (HCC). Furthermore, the increased DNA damage seems to be associated with the overexpression of the p53 gene, inducing apoptotic cell death [80]. In addition, the ability of heavy metals to generate oxidative stress by the overproduction of ROS (reactive oxygen species), which induces DNA injury, has been demonstrated previously [83,84].

3.8. Heavy Metal Concentration in Blood and Liver Tissue

The experimental group of rats treated with the two doses of PPWW had significantly higher blood and liver concentrations of Cd ($p < 0.001$ in blood and $p < 0.0001$ in liver) and Pb ($p < 0.0001$ in blood and liver) than the control group. In addition, exposure to PPWW-100% significantly increased the Cd concentration by 33% and 35% in the blood and liver, respectively, compared to the PPWW-50% group. However, the measured levels of Pb in the blood and liver in the experimental group receiving the PPWW-100% concentration exhibited no significant difference when compared to the group exposed to PPWW-50% (Figure 8).

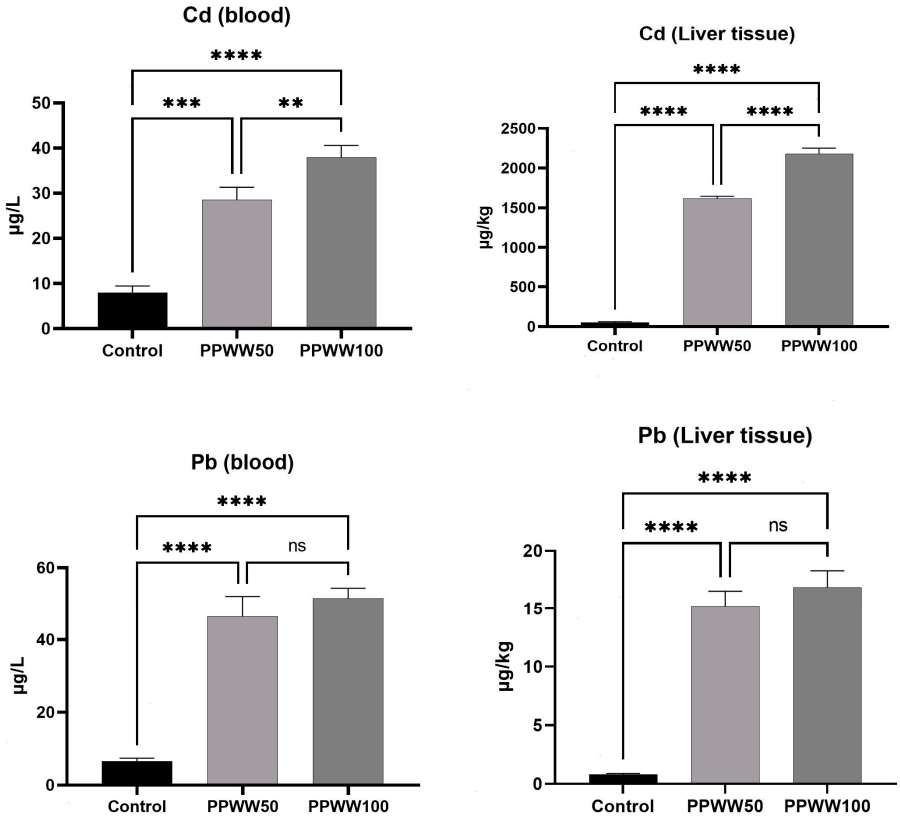

Figure 8. Cadmium and lead concentrations in rat blood (µg/L) and liver (µg/kg of tissue) of the control and experimental groups of rats. Values represent the mean ± SD (six animals were treated per group, n = 6). PPWW-50% or PPWW-100% group versus control group. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant.

In our study, we only assessed the concentrations of Pb and Cd in the blood and liver of treated and untreated animals as well-known elements for their toxicity [42,82]. Until today, these two elements have been the subject of many studies, in which scientists are increasingly discovering their innumerable harmful effects as well as innovative bio-inspired technologies for their remediation [30,53,55]. Obviously, our choice does not exclude the involvement of other metals in the effluent.

The mechanistic aspects by which potentially toxic elements exert their harmful effects are not fully understood. In addition, several factors act on the accumulation of metals in the organs, including the characteristics of the metal, the dose, and the duration of the exposure, as well as their capacity for binding to ligands [82]. In comparison to unexposed animals, both treated groups displayed significantly higher Pb and Cd contents in the blood along with the liver, where only a Cd accumulation occurred in a dose-dependent manner. For the Pb level, we did not find a significant difference between the two applied doses of the effluent. This finding could be explained by the difference in gastrointestinal absorption of heavy metals and saturation of their binding sites. In addition, metals' toxicity, bioavailability, and antagonistic competitive effects could be involved in their levels inside the different compartments and tissues of exposed organisms [85–87]. Hence, it can be concluded that the disturbances in liver structure and function resulted from the cumulative effects of potentially toxic elements.

4. Conclusions

The obtained results provide evidence, for the first time, that hepatic tissue might be altered following chronic exposure to PPWW. Given the richness of PPWW in heavy metals, it was remarkable that the detrimental effects obtained in the current work could result from synergistic, antagonistic, or additive impacts of heavy metals. Hence, a specific focus on the effects of combined heavy metals on the liver is essential in risk assessments during contact with heavy metal-containing effluents. In light of these results, it appears necessary to complete the research by (1) investigating the interactions among the different metals present in wastewater and the potential resulting cocktail effect and (2) estimating deeply the potential bioaccumulation and biomagnification of PPWW residues induced hepatotoxicity and apoptosis in rat livers. In the light of the present study, and as future guidelines for these bioassays, we plan to extend our work by analyzing other organs that may be affected following exposure to the effluent and reinforce our findings using toxicokinetic studies. Investigating the impact of chronic exposure to PPWW on other animal and plant species, particularly those related to human food, is one of our short-term prospects. Finally, this work constitutes a strong message, regarding the alarming situation caused by this wastewater, freely discharged into the environment. Hence, the use of existing or innovative biological and physicochemical approaches to detoxify PPWW has become a priority in the mining basin of the Gafsa region in Tunisia.

Author Contributions: Conceptualization, B.B., E.N., A.F., D.R. and M.A.B.; Methodology, A.F., D.R., A.M., E.N. and M.A.B.; Validation, B.B., E.N., A.F., D.R. and M.A.B.; Formal analysis, A.F., D.R. and M.A.B.; Investigation, B.B., A.F., D.R. and M.A.B.; Resources, A.F., E.N. and M.A.B.; Data curation, A.F., E.N. and M.A.B.; Writing—original draft preparation, A.F., E.N., N.T., A.M., E.A.L.-M. and M.A.B.; Writing—review and editing, A.F., E.N., N.T., E.A.L.-M. and M.A.B.; Visualization, A.F., N.T., D.R., E.A.L.-M. and M.A.B.; Supervision, A.F., D.R., E.A.L.-M. and M.A.B.; Project administration, A.F., D.R. and M.A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Higher Education and Scientific Research of Tunisia (Fund allocated to LR21ES26).

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors thank all the institutions that contributed to the development of this work.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Yeh, G.; Hoang, H.-G.; Lin, C.; Bui, X.-T.; Tran, H.-T.; Shern, C.-C.; Vu, C.-T. Assessment of Heavy Metal Contamination and Adverse Biological Effects of an Industrially Affected River. *Environ. Sci. Pollut. Res.* **2020**, *27*, 34770–34780. [CrossRef] [PubMed]
2. Alibi, S.; Beltifa, A.; Hassen, W.; Jaziri, A.; Soussia, L.; Zbidi, F.; Ben Mansour, H. Coastal Surveillance and Water Quality Monitoring in the Rejiche Sea—Tunisia. *Water Environ. Res.* **2021**, *93*, 2025–2033. [CrossRef] [PubMed]
3. Alam, M.M.; Alothman, Z.A.; Naushad, M.; Aouak, T. Evaluation of Heavy Metal Kinetics through Pyridine Based Th(IV) Phosphate Composite Cation Exchanger Using Particle Diffusion Controlled Ion Exchange Phenomenon. *J. Ind. Eng. Chem.* **2014**, *20*, 705–709. [CrossRef]
4. Xiao, J.; Xie, Y.; Cao, H. Organic Pollutants Removal in Wastewater by Heterogeneous Photocatalytic Ozonation. *Chemosphere* **2015**, *121*, 1–17. [CrossRef] [PubMed]
5. Xiao, Y.; De Araujo, C.; Sze, C.C.; Stuckey, D.C. Toxicity Measurement in Biological Wastewater Treatment Processes: A Review. *J. Hazard. Mater.* **2015**, *286*, 15–29. [CrossRef] [PubMed]
6. Emmanuel, E.; Pierre, M.G.; Perrodin, Y. Groundwater Contamination by Microbiological and Chemical Substances Released from Hospital Wastewater: Health Risk Assessment for Drinking Water Consumers. *Environ. Int.* **2009**, *35*, 718–726. [CrossRef]
7. Nasri, E.; Machreki, M.; Beltifa, A.; Aroui, S.; Ghorbel, A.; Saad, A.; Feriani, A.; Borgi, M.A.; Ghazouani, L.; Sire, O.; et al. Cytotoxic Effects of Seven Tunisian Hospital Wastewaters on the Proliferation of Human Breast Cancer Cell Line MDA-231: Correlation with Their Chemical Characterization. *Environ. Sci. Pollut. Res.* **2017**, *24*, 20422–20428. [CrossRef]
8. Sharma, P.; Mathur, N.; Singh, A.; Sogani, M.; Bhatnagar, P.; Atri, R.; Pareek, S. Monitoring Hospital Wastewaters for Their Probable Genotoxicity and Mutagenicity. *Environ. Monit. Assess.* **2015**, *187*, 4180. [CrossRef]
9. Beltifa, A.; Verschaeve, L.; Mansour, H. Genotoxicity Assessment of the Hospital Wastewaters in Tunisia. *J. Environ. Toxicol. Anal. Res.* **2019**, *1*, 104.
10. Afsa, S.; Hamden, K.; Lara Martin, P.A.; Mansour, H.B. Occurrence of 40 Pharmaceutically Active Compounds in Hospital and Urban Wastewaters and Their Contribution to Mahdia Coastal Seawater Contamination. *Environ. Sci. Pollut. Res.* **2020**, *27*, 1941–1955. [CrossRef]
11. Manu, B.; Chaudhari, S. Anaerobic Decolorisation of Simulated Textile Wastewater Containing Azo Dyes. *Bioresour. Technol.* **2002**, *82*, 225–231. [CrossRef] [PubMed]
12. Garcia, V.S.G.; De Freitas Tallarico, L.; Rosa, J.M.; Suzuki, C.F.; Roubicek, D.A.; Nakano, E.; Borrely, S.I. Multiple Adverse Effects of Textile Effluents and Reactive Red 239 Dye to Aquatic Organisms. *Environ. Sci. Pollut. Res.* **2021**, *28*, 63202–63214. [CrossRef] [PubMed]
13. Rocha, P.S.; Luvizotto, G.L.; Kosmehl, T.; Böttcher, M.; Storch, V.; Braunbeck, T.; Hollert, H. Sediment Genotoxicity in the Tietê River (São Paulo, Brazil): In Vitro Comet Assay versus in Situ Micronucleus Assay Studies. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 1842–1848. [CrossRef] [PubMed]
14. Bouwmeester, H.; Hollman, P.C.H.; Peters, R.J.B. Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology. *Environ. Sci. Technol.* **2015**, *49*, 8932–8947. [CrossRef] [PubMed]
15. Waring, R.H.; Harris, R.M.; Mitchell, S.C. Plastic Contamination of the Food Chain: A Threat to Human Health? *Maturitas* **2018**, *115*, 64–68. [CrossRef]
16. Mancini, F.R.; Busani, L.; Tait, S.; La Rocca, C. The Relevance of the Food Production Chain with Regard to the Population Exposure to Chemical Substances and Its Role in Contaminated Sites. *Ann. dell'Istituto Super. Sanità* **2016**, *52*, 505–510.
17. Jiang, F.; Ren, B.; Hursthouse, A.; Deng, R. Evaluating Health Risk Indicators for PTE Exposure in the Food Chain: Evidence from a Thallium Mine Area. *Environ. Sci. Pollut. Res.* **2020**, *27*, 23686–23694. [CrossRef]
18. Mariacrocetta, S.; Piazza, S.; Freni, G. Bayesian Identification of Brackish Water Infiltration by Lagrangian Sensors. In Proceedings of the 39th IAHR World Congress (Granada, 2022), Granada, Spain, 19–24 June 2022. [CrossRef]
19. Narasiah, S.; Shoiry, J.; Morasse, C. Effets des Variations des Températures Saisonnières sur les Modifications des Phosphates Dans des Eaux Usées. *Rev. Sci. l'Eau* **1988**, *1*, 305–320. [CrossRef]
20. Tijani, A.; Fakhfakh, H. Les Projets Sociaux Sont-Ils des Leviers de Création de la Valeur? *J. Bus. Econ.* **2013**, *1*, 94–105.
21. Mekki, A.; Awali, A.; Aloui, F.; Loukil, S.; Sayadi, S. Characterization and Toxicity Assessment of Wastewater from Rock Phosphate Processing in Tunisia. *Mine Water Environ.* **2017**, *36*, 502–507. [CrossRef]
22. Mekki, A.; Sayadi, S. Study of Heavy Metal Accumulation and Residual Toxicity in Soil Saturated with Phosphate Processing Wastewater. *Water Air Soil Pollut.* **2017**, *228*, 215. [CrossRef] [PubMed]
23. World Health Organization. *Guidelines for Drinking-Water Quality*, Incorporating the First Addendum, 4th ed.; World Health Organization: Geneva, Switzerland, 2017; ISBN 978-92-4-154995-0.
24. Moula, A.; Borgi, M.A.; Loukil, S.; Chaieb, M.; Mekki, A. Assessment of Phosphate Laundries Wastewater Phytotoxicity and Biotreatment Assays. *Clean–Soil Air Water* **2020**, *48*, 2000077. [CrossRef]
25. Moula, A.; Ellafi, A.; Borgi, M.A.; Rhimi, S.; Akermi, N.; Namsi, A.; Chaieb, M.; Mekki, A. Enhanced Bioremediation of Heavy Metals from Phosphate Processing Wastewater Using the Indigenous Bacterium *Serratia rubidaea* NCTC12971. *Geomicrobiol. J.* **2021**, *38*, 914–923. [CrossRef]
26. Moula, A.; Borgi, M.A.; Ellafi, A.; Chaieb, M.; Mekki, A. Biosorption of Heavy Metals from Phosphate-Processing Effluent by *Serratia rubidaea* NCTC12971 Immobilized in Ca-Alginate Beads. *Int. Microbiol.* **2022**, *26*, 149–160. [CrossRef] [PubMed]

27. Pagliaccia, B.; Carretti, E.; Severi, M.; Berti, D.; Lubello, C.; Lotti, T. Heavy metal biosorption by Extracellular Polymeric Substances (EPS) recovered from anammox granular sludge. *J. Hazard. Mater.* **2021**, *424*, 126661. [CrossRef]
28. Xue, Z.-F.; Cheng, W.-C.; Xie, Y.-X.; Wang, L.; Hu, W.; Zhang, B. Investigating immobilization efficiency of Pb in solution and loess soil using bio-inspired carbonate precipitation. *Environ. Pollut.* **2023**, *322*, 121218. [CrossRef]
29. Xue, Z.-F.; Cheng, W.-C.; Wang, L.; Xie, Y.-X.; Qin, P. Effect of a harsh circular environment on self-healing microbial-induced calcium carbonate materials for preventing Pb^{2+} migration. *Environ. Technol. Innov.* **2023**, *32*, 103380. [CrossRef]
30. Xue, Z.-F.; Cheng, W.-C.; Wang, L.; Qin, P.; Xie, Y.-X.; Hu, W. Applying the first microcapsule-based self-healing microbial-induced calcium carbonate materials to prevent the migration of Pb ions. *Environ. Res.* **2023**, *239*, 117423. [CrossRef]
31. Sauer, J.-M.; Stine, E.R.; Gunawardhana, L.; Hill, D.A.; Sipes, I.G. The Liver as a Target for Chemical-Chemical Interactions. *Adv. Pharmacol.* **1997**, *43*, 37–63.
32. McBurney, R.N.; Hines, W.M.; VonTungeln, L.S.; Schnackenberg, L.K.; Beger, R.D.; Moland, C.L.; Han, T.; Fuscoe, J.C.; Chang, C.-W.; Chen, J.J.; et al. The liver toxicity biomarker study phase I: Markers for the effects of tolcapone or entacapone. *Toxicol Pathol.* **2012**, *40*, 951–964. [CrossRef]
33. Soldatow, V.Y.; Lecluyse, E.L.; Griffith, L.G.; Rusyn, I. In Vitro Models for Liver Toxicity Testing. *Toxicol. Res.* **2013**, *2*, 23–39. [CrossRef] [PubMed]
34. Thakkar, S.; Chen, M.; Fang, H.; Liu, Z.; Roberts, R.; Tong, W. The Liver Toxicity Knowledge Base (LKTB) and drug-induced liver injury (DILI) classification for assessment of human liver injury. *Expert Rev. Gastroenterol. Hepatol.* **2018**, *12*, 31–38. [CrossRef] [PubMed]
35. Buege, J.A.; Aust, S.D. Microsomal Lipid Peroxidation. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1978; Volume 52, pp. 302–310. ISBN 978-0-12-181952-1.
36. Marklund, S.; Marklund, G. Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *Eur. J. Biochem.* **1974**, *47*, 469–474. [CrossRef] [PubMed]
37. Aebi, H. Catalase in Vitro. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1984; Volume 105, pp. 121–126. ISBN 978-0-12-182005-3.
38. Flohé, L.; Günzler, W.A. Assays of Glutathione Peroxidase. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1984; Volume 105, pp. 114–120. ISBN 978-0-12-182005-3.
39. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
40. Feriani, A.; Tir, M.; Aldahmash, W.; Mnafgui, K.; Hichem, A.; Gómez-Caravaca, A.M.; Del Mar Contreras, M.; Taamalli, A.; Alwasel, S.; Segura-Carretero, A.; et al. In Vivo Evaluation and Molecular Docking Studies of *Schinus molle* L. Fruit Extract Protective Effect against Isoproterenol-Induced Infarction in Rats. *Environ. Sci. Pollut. Res.* **2022**, *29*, 80910–80925. [CrossRef]
41. Chtourou, Y.; Slima, A.B.; Makni, M.; Gdoura, R.; Fetoui, H. Naringenin Protects Cardiac Hypercholesterolemia-Induced Oxidative Stress and Subsequent Necroptosis in Rats. *Pharmacol. Rep.* **2015**, *67*, 1090–1097. [CrossRef]
42. Andjelkovic, M.; Djordjevic, A.B.; Antonijevic, E.; Antonijevic, B.; Stanic, M.; Kotur-Stevuljevic, J.; Spasojevic-Kalimanovska, V.; Jovanovic, M.; Boricic, N.; Wallace, D.; et al. Toxic Effect of Acute Cadmium and Lead Exposure in Rat Blood, Liver, and Kidney. *Int. J. Environ. Res. Public Health* **2019**, *16*, 274. [CrossRef]
43. ÖZbelge, H.Ö.; Al-Fariss, T.F.; Abdulrazik, A.M. Use of Sea Water in the Flotation of Carbonate Rich Sedimentary Phosphate Rock. *Fertil. Res.* **1993**, *34*, 217–222. [CrossRef]
44. Andrio, D.; Asmura, J.; Yenie, E.; Putri, K. Enhancing BOD_5/COD Ratio Co-Substrate Tofu Wastewater and Cow Dung during Ozone Pretreatment. *MATEC Web Conf.* **2019**, *276*, 06027. [CrossRef]
45. ATSDR. The Agency for Toxic Substances and Disease Registry. A Priority List of Hazardous Substances U.S. Available online: https://www.atsdr.cdc.gov/spl/#2017spl (accessed on 10 August 2017).
46. OPORT. Ministère des Affaires Locales et de l'Environnement, Official Printing Office of the Republic of Tunisia, Décret Gouvernemental n° 2018315 du 26 Mars 2018, Annexe 1, Rejet Dans le Domaine Public Maritime, Hydraulique et Réseau Public d'Assainissement. *J. Off. R. Tunis.* **2018**, *26*, 824. (In French). Available online: https://documents1.worldbank.org/curated/pt/254711575642731435/pdf/Annexes.pdf (accessed on 30 March 2018).
47. APHA. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association/American Water Works Association/Water Environment Federation: Washington, DC, USA, 2005.
48. Sharifi, Z.; Sinegani, A.A.S. Arsenic and Other Irrigation Water Quality Indicators of Groundwater in an Agricultural Area of Qorveh Plain, Kurdistan, Iran. *Am.-Eurasian J. Agric. Environ. Sci.* **2012**, *12*, 548–555.
49. Khelifi, F.; Besser, H.; Ayadi, Y.; Liu, G.; Yousaf, B.; Harabi, S.; Bedoui, S.; Zighmi, K.; Hamed, Y. Evaluation of Potentially Toxic Elements' (PTEs) Vertical Distribution in Sediments of Gafsa–Metlaoui Mining Basin (Southwestern Tunisia) Using Geochemical and Multivariate Statistical Analysis Approaches. *Environ. Earth Sci.* **2019**, *78*, 53. [CrossRef]
50. Yang, X.; Makkonen, H.T.; Pakkanen, L. Rare Earth Occurrences in Streams of Processing a Phosphate Ore. *Minerals* **2019**, *9*, 262. [CrossRef]
51. Hall, A.P.; Elcombe, C.R.; Foster, J.R.; Harada, T.; Kaufmann, W.; Knippel, A.; Küttler, K.; Malarkey, D.E.; Maronpot, R.R.; Nishikawa, A.; et al. Liver hypertrophy: A review of adaptive (adverse and non-adverse) changes—Conclusions from the 3rd International ESTP Expert Workshop. *Toxicol. Pathol.* **2012**, *40*, 971–994. [CrossRef] [PubMed]

52. Zhao, X.; Zhang, X.; Li, M.; Sun, S.; Yao, L.; Cao, D.; Huang, X.; Guo, H.; Liu, X.; Yu, F. Repeated exposure to the irrigative wastewater in Shijiazhuang induced precancerous lesion associated with cytochrome P450. *Chemosphere* **2019**, *237*, 124467. [CrossRef] [PubMed]
53. Chen, H.; Zhu, C.; Zhou, X. Effects of Lead and Cadmium Combined Heavy Metals on Liver Function and Lipid Metabolism in Mice. *Biol. Trace Elem. Res.* **2023**, *201*, 2864–2876. [CrossRef] [PubMed]
54. Feriani, A.; Hachani, R.; Kaabi, B.; Ncir, M.; El Feki, A.; Allagui, M.S. Protective Effects of *Zygophyllum album* Extract against Deltamethrin-Induced Hyperglycemia and Hepato-Pancreatic Disorders in Rats. *Can. J. Physiol. Pharmacol.* **2016**, *94*, 1202–1210. [CrossRef]
55. Sarmiento-Ortega, V.E.; Moroni-González, D.; Díaz, A.; Eduardo, B.; Samuel, T. Oral Subacute Exposure to Cadmium LOAEL Dose Induces Insulin Resistance and Impairment of the Hormonal and Metabolic Liver-Adipose Axis in Wistar Rats. *Biol. Trace Elem. Res.* **2022**, *200*, 4370–4384. [CrossRef]
56. Daniel, P.V.; Kamthan, M.; Gera, R.; Dogra, S.; Gautam, K.; Ghosh, D.; Mondal, P. Chronic exposure to Pb^{2+} perturbs ChREBP transactivation and coerces hepatic dyslipidemia. *FEBS Lett.* **2019**, *593*, 3084–3097. [CrossRef]
57. Ale, A.; Bacchetta, C.; Cazenave, J. Responses of Multiple Biomarkers in the Fish *Hoplosternum littorale* after Exposure to Chromium and Lead. *Fresenius Environ. Bull.* **2016**, *25*, 4052–4059.
58. Wu, L.; Yu, Q.; Zhang, G.; Wu, F.; Zhang, Y.; Yuan, C.; Zhang, T.; Wang, Z. Single and Combined Exposures of Waterborne Cu and Cd Induced Oxidative Stress Responses and Tissue Injury in Female Rare Minnow (*Gobiocypris rarus*). *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2019**, *222*, 90–99. [CrossRef] [PubMed]
59. Martinez-Haro, M.; Green, A.J.; Mateo, R. Effects of Lead Exposure on Oxidative Stress Biomarkers and Plasma Biochemistry in Waterbirds in the Field. *Environ. Res.* **2011**, *111*, 530–538. [CrossRef] [PubMed]
60. Yildirim, S.; Celikezen, F.C.; Oto, G.; Sengul, E.; Bulduk, M.; Tasdemir, M.; Ali Cinar, D. An Investigation of Protective Effects of Litium Borate on Blood and Histopathological Parameters in Acute Cadmium-Induced Rats. *Biol. Trace Elem. Res.* **2018**, *182*, 287–294. [CrossRef] [PubMed]
61. Saedi, S.; Shirazi, M.R.J.; Totonchi, M.; Zamiri, M.J.; Derakhshanfar, A. Effect of Prepubertal Exposure to $CdCl_2$ on the Liver, Hematological, and Biochemical Parameters in Female Rats; an Experimental Study. *Biol. Trace Elem. Res.* **2020**, *194*, 472–481. [CrossRef] [PubMed]
62. Tabrez, S.; Ahmad, M. Effect of Wastewater Intake on Antioxidant and Marker Enzymes of Tissue Damage in Rat Tissues: Implications for the Use of Biochemical Markers. *Food Chem. Toxicol.* **2009**, *47*, 2465–2478. [CrossRef] [PubMed]
63. Flora, G.; Gupta, D.; Tiwari, A. Toxicity of Lead: A Review with Recent Updates. *Interdiscip. Toxicol.* **2012**, *5*, 47–58. [CrossRef] [PubMed]
64. Casalino, E.; Calzaretti, G.; Sblano, C.; Landriscina, C. Molecular Inhibitory Mechanisms of Antioxidant Enzymes in Rat Liver and Kidney by Cadmium. *Toxicology* **2002**, *179*, 37–50. [CrossRef]
65. Almasmoum, H.; Refaat, B.; Ghaith, M.M.; Almaimani, R.A.; Idris, S.; Ahmad, J.; Abdelghany, A.H.; BaSalamah, M.A.; El-Boshy, M. Protective Effect of Vitamin D3 against Lead Induced Hepatotoxicity, Oxidative Stress, Immunosuppressive and Calcium Homeostasis Disorders in Rat. *Environ. Toxicol. Pharmacol.* **2019**, *72*, 103246. [CrossRef]
66. Al-Basher, G.I. Green Tea Activity and Iron Overload Induced Molecular Fibrogenesis of Rat Liver. *Saudi J. Biol. Sci.* **2019**, *26*, 531–540. [CrossRef]
67. Niu, Q.; He, P.; Xu, S.; Ma, R.; Ding, Y.; Mu, L.; Li, S. Fluoride-Induced Iron Overload Contributes to Hepatic Oxidative Damage in Mouse and the Protective Role of Grape Seed Proanthocyanidin Extract. *J. Toxicol. Sci.* **2018**, *43*, 311–319. [CrossRef]
68. Kadiiska, M.B.; Burkitt, M.J.; Xiang, Q.H.; Mason, R.P. Iron Supplementation Generates Hydroxyl Radical in Vivo. An ESR Spin-Trapping Investigation. *J. Clin. Investig.* **1995**, *96*, 1653–1657. [CrossRef]
69. Bacon, B.R.; Tavill, A.S.; Brittenham, G.M.; Park, C.H.; Recknagel, R.O. Hepatic Lipid Peroxidation in Vivo in Rats with Chronic Iron Overload. *J. Clin. Investig.* **1983**, *71*, 429–439. [CrossRef] [PubMed]
70. Milic, S.; Mikolasevic, I.; Orlic, L.; Devcic, E.; Starcevic-Cizmarevic, N.; Stimac, D.; Kapovic, M.; Ristic, S. The Role of Iron and Iron Overload in Chronic Liver Disease. *Med. Sci. Monit.* **2016**, *22*, 2144–2151. [CrossRef] [PubMed]
71. Brown, K.E.; Dennery, P.A.; Ridnour, L.A.; Fimmel, C.J.; Kladney, R.D.; Brunt, E.M.; Spitz, D.R. Effect of Iron Overload and Dietary Fat on Indices of Oxidative Stress and Hepatic Fibrogenesis in Rats: Dietary Fat and Iron Overload. *Liver Int.* **2003**, *23*, 232–242. [CrossRef] [PubMed]
72. Brown, K.; Broadhurst, K.; Mathahs, M.; Weydert, J. Differential Expression of Stress-Inducible Proteins in Chronic Hepatic Iron Overload. *Toxicol. Appl. Pharmacol.* **2007**, *223*, 180–186. [CrossRef]
73. Bloomer, S.A.; Brown, K.E. Iron-Induced Liver Injury: A Critical Reappraisal. *Int. J. Mol. Sci.* **2019**, *20*, 2132. [CrossRef] [PubMed]
74. Contreras, M.D.M.; Gómez-Cruz, I.; Feriani, A.; Alwasel, S.; Harrath, A.H.; Romero, I.; Castro, E.; Tlili, N. Hepatopreventive Properties of Hydroxytyrosol and Mannitol-Rich Extracts Obtained from Exhausted Olive Pomace Using Green Extraction Methods. *Food Funct.* **2022**, *13*, 11915–11928. [CrossRef]
75. Sturgill, M.G.; Lambert, G.H. Xenobiotic-Induced Hepatotoxicity: Mechanisms of Liver Injury and Methods of Monitoring Hepatic Function. *Clin. Chem.* **1997**, *43*, 1512–1526. [CrossRef]
76. Reddy, U.A.; Prabhakar, P.V.; Rao, G.S.; Rao, P.R.; Sandeep, K.; Rahman, M.F.; Kumari, S.I.; Grover, P.; Khan, H.A.; Mahboob, M. Biomarkers of Oxidative Stress in Rat for Assessing Toxicological Effects of Heavy Metal Pollution in River Water. *Environ. Sci. Pollut. Res.* **2015**, *22*, 13453–13463. [CrossRef]

77. Anyanwu, B.O.; Ezejiofor, A.N.; Nwaogazie, I.L.; Orisakwe, O.E.E. Hepatoprotective Effect of *Costus afer* on Trace Metal Mixture Treated Rats Mediated by Regulation of Oxidative Stress Markers, Inflammatory Cytokines and Bio-Metal Chelation. *Res. Sq.* **2021**. [CrossRef]
78. Renu, K.; Chakraborty, R.; Myakala, H.; Koti, R.; Famurewa, A.C.; Madhyastha, H.; Vellingiri, B.; George, A.; Valsala Gopalakrishnan, A. Molecular Mechanism of Heavy Metals (Lead, Chromium, Arsenic, Mercury, Nickel and Cadmium)–Induced Hepatotoxicity–A Review. *Chemosphere* **2021**, *271*, 129735. [CrossRef] [PubMed]
79. Mekircha, F.; Chebab, S.; Gabbianelli, R.; Leghouchi, E. The possible ameliorative effect of *Olea europaea* L. oil against deltamethrin-induced oxidative stress and alterations of serum concentrations of thyroid and reproductive hormones in adult female rats. *Ecotoxicol. Environ. Saf.* **2018**, *161*, 374–382. [CrossRef]
80. Feriani, A.; Tir, M.; Gómez-Caravaca, A.M.; Del Mar Contreras, M.; Taamalli, A.; Segura-Carretero, A.; Ghazouani, L.; Mufti, A.; Tlili, N.; El Feki, A.; et al. *Zygophyllum album* Leaves Extract Prevented Hepatic Fibrosis in Rats, by Reducing Liver Injury and Suppressing Oxidative Stress, Inflammation, Apoptosis and the TGF-B1/Smads Signaling Pathways. Exploring of Bioactive Compounds Using HPLC–DAD–ESI–QTOF-MS/MS. *Inflammopharmacology* **2020**, *28*, 1735–1750. [CrossRef] [PubMed]
81. Chaouachi, K. Hookah (Shisha, Narghile) Smoking and Environmental Tobacco Smoke (ETS). A Critical Review of the Relevant Literature and the Public Health Consequences. *Int. J. Environ. Res. Public Health* **2009**, *6*, 798–843. [CrossRef] [PubMed]
82. Balali-Mood, M.; Naseri, K.; Tahergorabi, Z.; Khazdair, M.R.; Sadeghi, M. Toxic Mechanisms of Five Heavy Metals: Mercury, Lead, Chromium, Cadmium, and Arsenic. *Front. Pharmacol.* **2021**, *12*, 643972. [CrossRef]
83. Chaudhry, A.S.; Jabeen, F. Assessing Metal, Protein, and DNA Profiles in *Labeo rohita* from the Indus River in Mianwali, Pakistan. *Environ. Monit. Assess.* **2011**, *174*, 665–679. [CrossRef]
84. Ercal, N.; Gurer-Orhan, H.; Aykin-Burns, N. Toxic Metals and Oxidative Stress Part I: Mechanisms Involved in Metal-Induced Oxidative Damage. *Curr. Top. Med. Chem.* **2001**, *1*, 529–539. [CrossRef]
85. Wu, X.; Cobbina, S.J.; Mao, G.; Xu, H.; Zhang, Z.; Yang, L. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. *Environ. Sci. Pollut. Res.* **2016**, *23*, 8244–8259. [CrossRef]
86. Massó, E.L.; Corredor, L.; Antonio, M.T. Oxidative damage in liver after perinatal intoxication with lead and/or cadmium. *J. Trace Elem. Med. Biol.* **2007**, *21*, 210–216. [CrossRef]
87. Witkowska, D.; Słowik, J.; Chilicka, K. Heavy Metals and Human Health: Possible Exposure Pathways and the Competition for Protein Binding Sites. *Molecules* **2021**, *26*, 6060. [CrossRef]