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RESEARCH ARTICLE

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Protective, immunologic, and histopathologic effects of garlic extract (*Allium sativum*) on rainbow trout (*Oncorhynchus mykiss*) exposed to acute toxicity with copper (Cu^{2+})

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ABSTRACT

The present study is an attempt to assess the protective and immunity effects of *Allium sativum* in *Oncorhynchus* mykiss to acute exposure to copper .55 rainbow trout fish with an average weight of 51.20 ± 3.73 g were subjected to various densities of copper (0.02, 0.1, 0.3, and 0.4 mg/l). Under stable conditions, the lethal concentration of copper was detected to be 0.40 mg/l. The treatments included a control with no Cu or garlic treatment (T1), feeding with garlic additive and Cu exposure (T2), and exposure to a lethal dose of Cu with no garlic additive (T3). The blood sample was used to designate hematological indices such as white blood cell (WBC) and red blood cell (RBC) count, hematocrit (HCT), hemoglobin (HB) mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), neutrophils, lymphocytes, monocytes, eosinophils percent and Immunological indices (glucose, total protein, lysozyme, IgM). The results indicated significant differences among the treatments when the concentration of copper was increased ($p < 0.05$). According to the results obtained, there were noteworthy differences in MCV, MCHC, and HCT amongst the treatments ($p < 0.05$). The histopathological results indicated that the main lesions were hyperplasia and necrosis of epithelial cells (in gill), enlargement of Bowman's capsule and tubular degeneration (in kidney), hepatocytes necrosis (in liver) in all the fish. Pathologic severity signs in sampled tissues were increased by increasing in concentration and exposure times of copper sulphate However, the results revealed that the use of garlic in dietary can be beneficial to increasing fish resistance to copper.

Keywords

Garlic extract, Histopathology, Immunity, Protective, Rainbow Trout (Oncorhynchus mykiss)

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Abbreviations

MCV: Mean Corpuscular Volume,
MCHC: Mean Corpuscular Hemoglobin Concentration
Hct: Hematocrit
WBC: white blood cell
RBC: red blood cell (RBC)
HB: hemoglobin
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
Ph: Phosphorus

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Introduction

Rainbow trout (*Oncorhynchus mykiss*) belongs to the family Salmonidae and is the main cultured freshwater fish species worldwide [38]. Fish require copper (Cu) as a micronutrient and obtain it from either water or diet [1]. Copper sulfate is an effective algaecide, but is toxic to many fish species at or near the concentration necessary for algal control [2]. Toxicity experiments show the sensitivity of an organism to a specific toxin. These experiments are useful for determining the acceptable amount of poison in an environment [3]. Toxicity studies are very necessary to predict the harmless amount of compounds in the environment [4].

Garlic (Allium sativum) is a small underground bulb crop from the family Alliaceae or Liliaceae [5, 6]. Some research has also reported the beneficial effects of garlic and its components on the prevention and treatment of fish diseases [7] and toxicosis [8]. However, to the best knowledge of the authors, there are no reports about the resistance rate of rainbow trout (Oncorhynchus mykiss) fed with Allium sativum. Therefore, this research aimed to evaluate the resistance of rainbow trout fed with garlic to acute exposure to copper.

Result

Toxicity Test

This study aimed to examine the poisonous influences of copper sulfate on rainbow trout (O. mykiss) nourished with A. sativum at various period intervals of 24, 48, 72, and 96 h. Table 1 shows the relationship between the mortality rate and the copper sulfate density of O. mykiss. The results presented that mortality reached 100% at the concentration of 0.4 mg/l. The acute poisonousness of copper sulfate indicated that mortality was directly compared to the level of copper sulfate although mortality was not found in the control group (Table 1).

The results attained from the acute static 96-h poisonousness tests of copper sulfate for rainbow trout and the estimated LC50 values with confidence limits are listed in Table 2. The mean LC50 of the effect of copper sulfate on rainbow trout was found to be 0.186 and 0.207 mg/l based on Finney's Probit analysis technique for T3 and T2, respectively. The blood factors of the treatments are shown in Table 2. No mortality was detected in T1 during the experiment.

Hematological and Immunological Changes

The analysis of hematological indices after 96 h revealed numerous significant variations in blood factors in the treatments ($p < 0.05$) (Table 3). Our results indicated that the highest WBC count was detected in T3. There were no significant differences in the Hct percentage of T2 and T3 compared to T1 ($p > 0.05$). Increases were detected in RBC and WBC counts, and Hct levels. Nonetheless, Hct and Hb gradually augmented after toxicity (Table 3).

The IgM and serum lysozyme of the rainbow trout in T2 were significantly ($p < 0.05$) higher than in T3 (Table 4). A lower glucose level was found in fish that received garlic at a lethal dose. The glucose level of the fish significantly rose in the fish exposed to copper ($p < 0.05$). However, the glucose level did not significantly change in T2 and T3 ($p > 0.05$).

Table 1.
The relationship between the copper sulfate concentration and the mortality rate of *Oncorhynchus mykiss*

Treatment	Copper concentration (mg/l)		Mortality No.					Cumulative Mortality % (96h)
			NO.	24 h	48 h	72 h	96 h	
Without garlic	0	0	11	0	0	0	0	0
	1	0.02	11	0	0	0	1	9.09
	2	0.1	11	2	1	1	0	36.36
	3	0.3	11	4	3	0	1	72.73
	4	0.4	11	6	5	0	0	100
With garlic	0	0	11	0	0	0	0	0
	1	0.02	11	0	0	0	0	0
	2	0.1	11	1	1	1	0	27.27
	3	0.3	11	3	3	1	1	72.73
	4	0.4	11	5	5	1	0	100

Table 2.
LC1–99 of Cu (mean ± standard error) of *Oncorhynchus mykiss*

LC	Estimate							
	24 h	**24 h**	48 h	48 h	72 h	72 h	**96 h**	**96 h**
	Without garlic	With garlic	Without garlic	With garlic	Without garlic	With garlic	Without garlic	With garlic
LC1	-.082	-.019	-.036	-.022	-.062	-.036	-.094	-.032
LC10	.119	.175	.079	.104	.060	.079	.032	.075
LC20	.204	.257	.127	.158	.111	.127	.085	.120
LC30	.265	.316	.162	.196	.148	.162	.123	.153
LC40	.317	.367	.192	.229	.180	.192	.155	.181
LC50	.366	.414	.220	.260	.209	.220	.186	.207
LC60	.414	.461	.248	.291	.239	.248	.216	.233
LC70	.466	.511	.277	.323	.270	.277	.248	.260
LC80	.527	.570	.312	.362	.307	.312	.287	.293
LC90	.612	.652	.361	.415	.359	.361	.339	.338
LC95	.682	.720	.401	.459	.401	.401	.383	.375
LC99	.813	.847	.476	.542	.480	.476	.465	.445

Table 3.
Hematological changes in Oncorhynchus mykiss acute exposed to copper after 30 minutes. Different lowercase letters within a column show significant effects of the treatments ($p > 0.05$)

Hematological parameters	Treatment 1 (Without garlic and copper)	Treatment 2 (lethal dose, garlic)	Treatment 3 (lethal dose, without garlic)
RBC (10^6 /µl)	0.41 ± 0.18^c	0.58 ± 0.02^b	0.69 ± 0.08^a
WBC (10^3 /µl)	113.35 ± 3.85^b	127.17 ± 2.11^a	131.53 ± 2.7^a
Hb (g /dl)	8.15 ± 0.45^b	8.99 ± 0.21^a	9.19 ± 0.45^a
Hct %	8.35 ± 0.65^b	18.70 ± 0.65^a	19.13 ± 0.20^a
MCV %	201.00 ± 14.90^a	182.63 ± 5.74^b	179.13 ± 6.27^C
MCH (Pg)	246.35 ± 7.15^a	221.17 ± 6.01^b	207.35 ± 5.45^c
MCHC (g /dl)	194.50 ± 3.80^a	171.23 ± 2.61^b	169.35 ± 5.45^b
Neutrophils %	8.20 ± 0.50^b	9.01 ± 0.22^a	9.12 ± 0.31^a
Lymphocytes %	79.01 ± 1.12^c	86.66 ± 2.51^b	96.11 ± 3.04^a
Monocytes	1.19 ± 0.01	1.11 ± 0.31	1.13 ± 0.57
Eosinophils %	1.05 ± 0.51^b	1.38 ± 0.57^a	1.41 ± 0.16^a

Behavioral Changes

The behavioral reaction of rainbow trout fingerlings was assessed every 12 h throughout the acute poisonousness experiments. The control group indicated usual behavior during the experiment time. The behavioral alterations in *O. mykiss* exposed to different concentrations of copper sulfate (ppm level) are as follows:

Control group: There were no behavioral alterations and losses detected during the test. The hypothetical spontaneous reaction was zero.

Test groups:There were downward and vertical swimming forms and unexpected activities. The motion of the fish became enormously relaxed and they showed behavioral irregularities, such as the loss of balance and water capsizing. Finally, the fish sank to

Table 4.
Immunological indices in Oncorhynchus mykiss acute exposed to copper after 30 minutes. Different lowercase letters in a column show significant effects of the treatments ($p > 0.05$)

Hematological parameters	Treatment 1 (Without garlic and copper)	Treatment 2 (lethal dose, garlic)	Treatment 3 (lethal dose, without garlic)
Glucose (mg/dl)	109.21 ± 6.18^{b}	129.21 ± 4.18^{a}	134.21± 6.18^{a}
Total protein (g/dl)	3.35 ± 0.05^{a}	1.18 ± 0.05^{b}	1.21 ± 0.01^{b}
Lysozyme (u/ml)	32.60 ± 2.38^{a}	26.20 ± 2.38^{b}	21.60 ± 3.38^{c}
IgM (mg/ml)	16.71 ± 1.74^{a}	12.31 ± 2.07^{b}	10.01 ± 1.14^{c}

the bottom and became immobile. Variations in behavioral reactions were initiated 45 min after dosing. In contradiction of the control group, slowing motion, losing balance, and spending more time at the bottom were detected. After 1 h, the fish were continually swimming indirectly with increased operculum motion and opening mouth for oxygen. Afterwards, the dead fish were observed while their mouth and operculum were open. Nevertheless, the fish exposed to copper indicated two patterns of behavior throughout the first 24 h. Those exposed to higher concentrations showed tension followed by unpredictable swimming and arranging on the water surface instantly after the addition of the material. However, those exposed to lower concentrations remained standing, made small movements, and rest at downward on the containers. Although there were similar signs in all treatments at high concentrations of copper, the fish fed with garlic showed high resistance to toxicity at low concentrations.

Histopathology

The histological samples indicated that the lesions of the fish gill, including edema, hyperemia, hyperplasia, hemorrhage, inflammation, epithelial cells necrosis of gill, and expansion of secondary lamella, were observed in diverse cases exposed to the fatal density of copper (Figures 1 and 2). The main lesions were found on the kidney of the fish, such as the expansion of Bowman's capsule, degenerated tubules of the kidney, epithelial cells necrosis of the kidney, hyperemia, hemorrhage, and migration of inflammatory cells. No lesions were detected in the kidney of the control fish samples (Figure 3). The liver lesions in the samples comprised inflammatory cell infiltration, hepatocyte necrosis, hemorrhage, and hyperemia (Figures 4 and 5). However, the control samples showed no lesions. Nevertheless, the impacts of poisonousness were diverse amongst the studied fish.

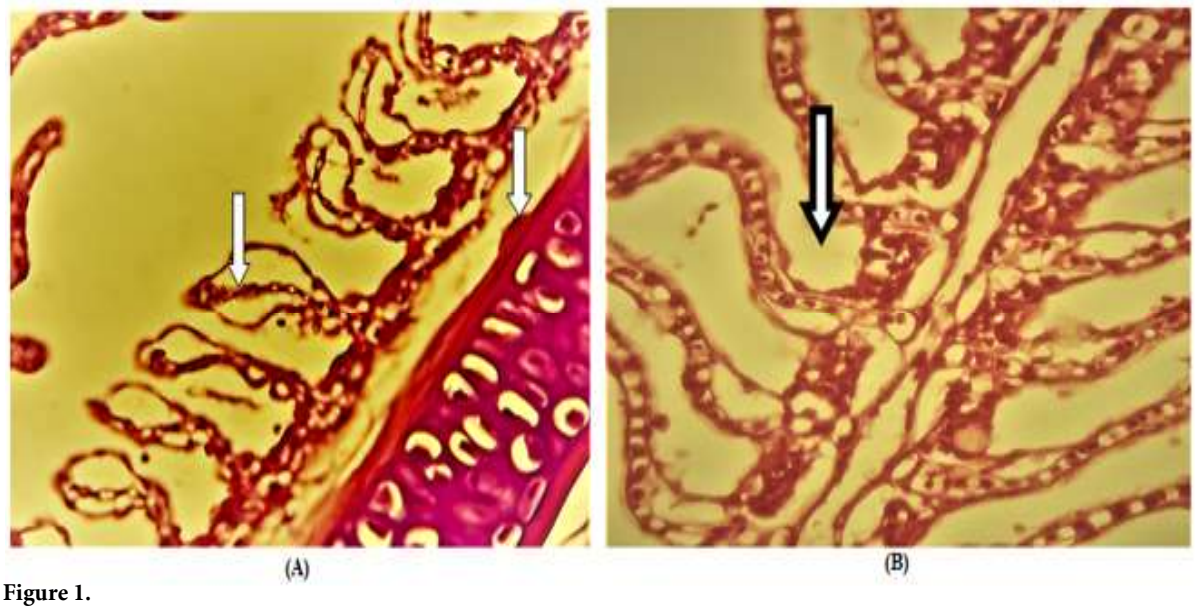


Figure 1.
Photomicrographs of gill in the fish exposed to lethal copper toxicity. Arrows show edema; A: with garlic, b: without garlic, (H & E, ×400)

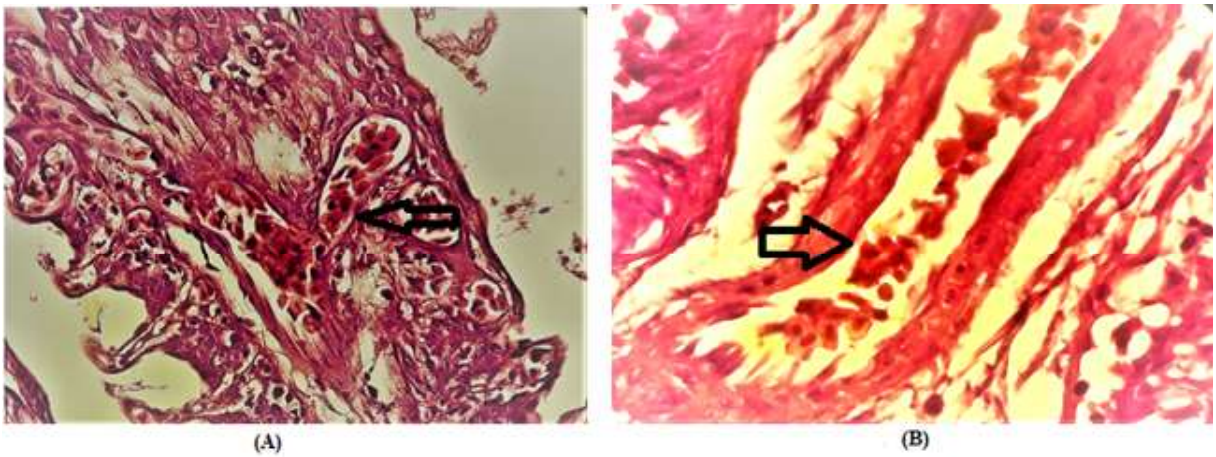


Figure 2.
Photomicrographs of gill in the fish exposed to lethal copper toxicity. Arrows show hyperemia; A: with garlic, B: without garlic, (H & E, ×400)

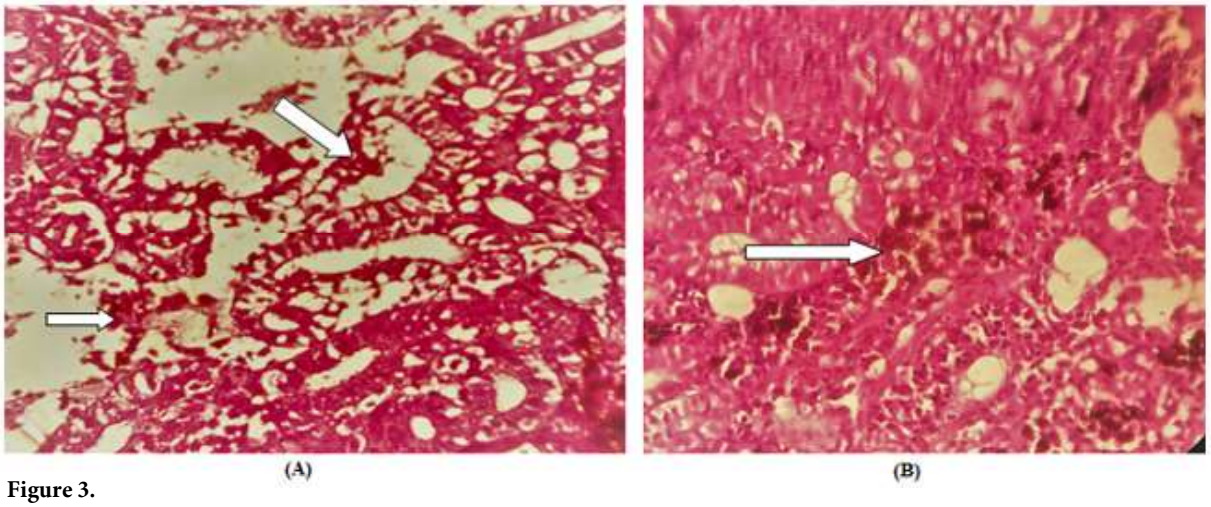


Figure 3.
Photomicrographs of the kidney in the fish exposed to lethal copper toxicity. Arrows show hemorrhage and degenerated tubules of the kidney; A: with garlic, B: without garlic, (H & E, ×400)

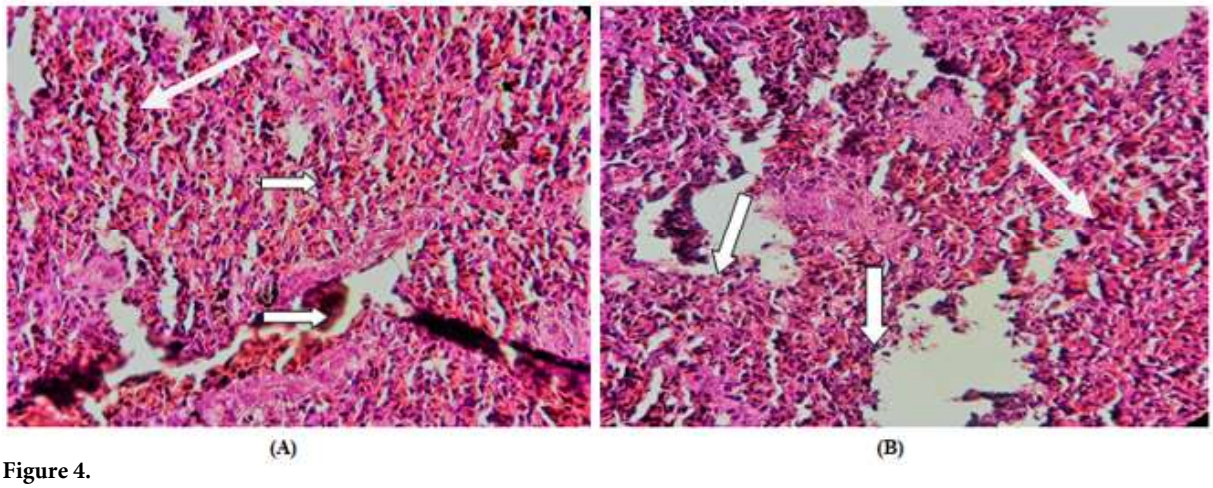
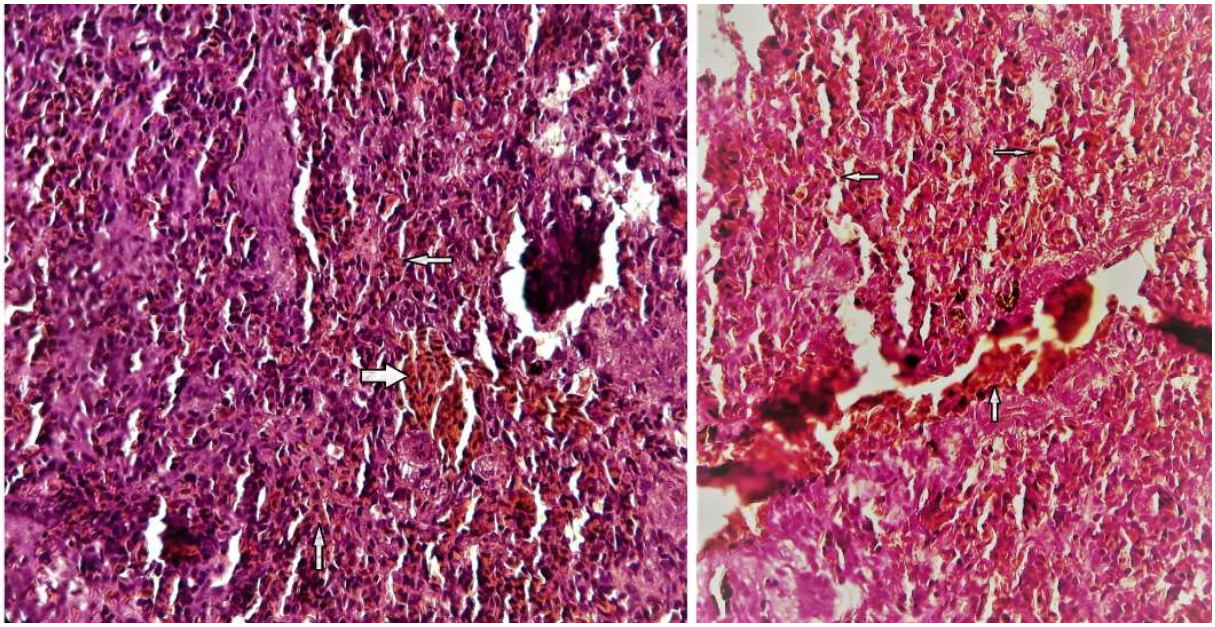


Figure 4.
Photomicrographs of the liver in the fish exposed to lethal copper toxicity. Arrows show hemorrhage, inflammatory cells infiltration, and hepatocytes necrosis; a: with garlic, b: without garlic, (H & E, ×400)



(A) (B)

Figure 5.
Photomicrographs of the liver in the fish exposed to lethal copper toxicity. Arrows show hemorrhage and hyperemia; a: with garlic, b: without garlic, (H & E, ×400)

Discussion

The current study aimed to investigate the poisonous impacts of copper on rainbow trout fish nourished with garlic at diverse period intervals of 24, 48, 72, and 96 h. Each species responds differently to material, so the significance of leading poisonousness examinations with diverse animals [9]. Nevertheless, behavioral variations in fish are a suitable biomarker for screening contamination and managing the marine environment [10]. In this research, the fish exposed to copper were detected to be highly irritable and showed demoniac swimming when handled. Their bodies were protected with thick mucus and finally deceased with open mouths. At the beginning of the bioassays and especially at higher concentrations, the fish would move to the water surface.

After 6-8 h of confusion at the upper concentrations, the behavior was more irregular amongst the rainbow trout. Fish became stiff, keeping a situation perpendicular to the downward of the tank and gulping air. However, the control fish were kept under the same conditions as the test fish in all respects except for the addition of copper toxicant. The fish acted regularly, showing none of the indications detected in the fish exposed to copper. Several authors have described the same variations in the gills of the fish exposed to diverse types of poisons.

Similar to the results obtained in this study, condensing of the lamellar epithelium (fusion) was described following the exposure of Nile tilapia (*Oreochromis niloticus L.*) to deltamethrin and glyphosate [11]. In the current study, Finney's Probit analysis presented 96-h LC50 value for *O. mykiss* exposed to copper sulfate densities of 0.186 and 0.207 mg/l for T2 and T3, respectively. Gul et al. [12] stated that 96-h LC50 for *P. reticulata*, Pallas, 1859 in hard water (235 mg CaCO3 / l, pH: 7.88, DO = 5.8 mg/l) was 30.8 and 30.9 mg/l based on Finney's Probit and Behrens-Karber's methods, respectively. Their reported values are slightly higher than our results. The dissimilarity can be attributed to changes in the water temperature, pH, and hardness. Bagdonas and Vosylienė [13] investigated Cu toxicity for rainbow trout. The 96-hr LC50 value for Cu was 0.2 mg/l. Hosseini et al. [14] reported the LC50 value of copper sulfate for rainbow trout fish in 24, 48, 72, and 96 h as to be 0.96, 4.79, 8.38, and 10 mg/l, respectively. Finlayson and Verrue [15] evaluated the toxicity of zinc, cadmium, and copper mixture for juvenile Chinook salmon. Bello-Olusoji and Adebola [16] investigated the toxicity of copper for freshwater prawn, *Caridina Africana*. The 96-h median fatal concentration for copper salts was 0.15 mg/l.

The variations in toxicity stated by different researchers may result from the changes in species, life stage, organism dimension, examination method, and water quality [9]. The fatal level varies with fish depending on species, age, and environmental factors. Inyang et al. [17] proved that hardness and water pH had a noteworthy direct relationship with the 96-h LC50 of copper in the examined fish. The highest death rate occurred throughout the early hours of exposure. However, all these remarks were more noticeable with the increasing densities of the poisons. A fish extremely vulnerable to the toxicity of a metal can be less or not vulnerable to the toxicity of another

metal at a similar concentration [18].

Salmonids are usually more susceptible to toxic metals in comparison with other fish species. Different experiments have revealed that the lethal concentration of copper is 0.1-10 mg/l for fish. We observed that the fatal level of copper was 0.4 mg/l for all the treatments. As CWQC [19] recommended, there was a safe level of LC50 96-h concentration (0.01 mg/l) for all aquatic animals. In the present study, NOEC was 0.02 mg/l in fish fed with *A. sativum*, whereas at the 0.02 mg/l concentration of copper, the mortality rate was 9.09% after 96 h in fish not fed *A. sativum*. This reveals that fish fed *A. sativum* are more resistant than others. However, given the classification of Louis et al. [20] for the degree of metal toxicity and the lethal concentration obtained in this research (0.4 mg/l), copper sulfate is very toxic (at the rate of 0.1-10 mg/l) to rainbow trout.

Hematological factors, such as complete blood cell count, are usually used for checking the health condition of farmed fish to several infections [21]. According to our results, a significant increase was observed in some hematologic indices, namely RBC, WBC, and HCT. The WBC and RBC count gradually rose after exposure. We observed that garlic could decrease WBC, MCV, and HCT values after 96 h exposure (Table 1). A decreasing effect was noted in the MCHC value in T2 and T3 after 96 h compared to the control value. Such damage to the cell organelles has been reported in various studies [22, 23]. According to KO et al. [22], hematological parameters, such as RBC count, Hct, and Hb value are sensitive indicators in the evaluation of fish metabolism under metal stress. In the present study, copper exposure induced an important rise in RBC, WBC, and Hct values in rainbow trout, which may be attributed to the augmentation in oxygen supply. The increased level of stress hormones (corticosteroids) caused a fall in leucocytes and erythrocytes in studied fish [7]. Kumar and Banerjee [24] reported that metals directly affected hematopoietic cells in the kidney and spleen and induced anemia by reducing the oxygen supply due to RBC concentration and decreased Hb. First, leucocytes initially increase to keep the phagocytosis mechanism and create antibacterial or antiviral compounds to halt the extent of the agent [25]. The attained results indicated a fascinating pattern of reaction in the hematological factors to garlic. Therefore, copper exposure induced a significant increase in WBC and RBC count in the treatments. Studies have shown that RBC and WBC counts were lower in fish fed garlic (T2). This reveals that the use of garlic could improve fish resistance. Similar results have been observed in erythrocytes and leucocytes in fish exposed to various toxicants and pathogens [7, 25]. On the other hand, the reduction in MCV detected after copper exposure can cause the shrinkage of erythrocytes due to hypoxia or microcytic anemia [25]. However, the quality and quantity of leukocytes are generally used to determine immune reactions, disease, and toxicants. Leukocytes are normally lower in healthy fish than in infected fish. As a result, they can be used as an indicator of infectious diseases similar to our study [26]. However, diminished Hb or RBC count can be an indicator of anemia [27]. Alterations in differential leukocyte count are identified as susceptible indices of poisons or dysfunction in hematological tissues or some infected illnesses [28]. Lymphocyte percentage was lower than usual lymphopenia and can be an appropriate marker of immune system shortage and xenobiotic material treatments that can also reduce the body's source of lymphocytes [16]. Furthermore, increases in monocyte and eosinophil and the reduction of lymphocytes were detected in WBCs (Table 1). According to Banaee et al. [29], utmost infections cause a type of neutrophilia. Analysis after 96 h exposure indicated that copper treatment (T3) was most efficient in RBC and WBC. The decrease in RBC and HCT levels in toxicant-treated fish can be attributed to the disturbance in erythropoiesis and the development of RBC [30]. Several researchers have described that the reduction in RBC count and HCT levels could be associated with the pressure after little exposure to metal [28, 30]. According to the results, garlic can rise antibody production and inhibit the undesirable effects of copper. During the experiment, RBC and hemoglobin rose in the garlic treatments in fatal doses in comparison with the other investigated treatments.

Histopathological variations in fish tissue can be used to identify the direct toxic impacts of compounds on target tissues because they reveal the loss instigated by the period and severity of exposure to the poisonous component and the tissue's adaptive capability [31, 32]. The gill is the first organ exposed to and influenced by toxins and pollutants. We observed significant deformations in the gill lamellae. The fusion of lamellae and the hyperemia of gill epithelium were apparent and telangiectasia was less common. Gills are in direct contact with water and react to ecological contamination and will be affected by copper. Nevertheless, in gill, at upper levels (> 0.2 mg/l), cell hyperplasia happened and the interlamellar spaces were filled. Several authors have described the same changes in the gills of fish exposed to diverse types of toxicants, such as Deltamethrin on Nile tilapia (*Oreochromis nilotica*) [33] and formalin on *Corydoras melanistius* [9]. The toxicity of copper is the reason for pathological changes in the gills, respiratory imbalance, instigating gill dysfunction, mortality, and osmoregulatory changes correlated with the fact that

the gill epithelium is the main contact surface because of having an enormous contact surface area with the external environments. Therefore, it is a target of the contaminants in the water [34]. Copper can disturb the nervous and cardiovascular systems of the fish when it is aggregated in the gills because it can adjust the transfer of salt (NaCl) into and out of the fish [35]. It can disturb the cellular structure and glucose metabolism of fish [36]. At the high concentrations of copper (> 0.3 mg/l), after 96 h of exposure, observations in the kidneys included the congestion of capillaries, focal necrosis, increases in Bowman's space, and necrosis of renal tubules. Similar results have been reported by others [9]. Various reasons have been put forth for fish mortality, including degenerated renal tubules and glomerulus, disrupted kidney function, disoriented osmoregulation caused by injuries to the gills, brain injuries causing convulsion [37], decreased oxygen transmission by blood, and increased plasma ammonia [35].

Comparing the data and figures demonstrates that at the 0.1 mg/l concentration of copper, the fish fed with garlic showed the lowest symptoms of sickness compared to others. Pathological results in the liver comprised swelling of hepatocytes, cellular deteriorations, and focal necrosis. These histopathological changes approved the toxic impact of copper. The liver is the main organ of several key metabolic pathways. Consequently, the toxic effects of compounds typically become visible in the liver. Numerous carbon-based combinations compel toxicopathic lesions in the liver of fish species. Acute toxic harms generally comprise cloudy swelling or hydropic degenerations along with the pyknosis, karyolysis, and karyorrhexis of nuclei [33].

In the liver, at lower concentrations (0.02 mg/l), as well as in gills, no important alterations were detected throughout the test. Finally, several injuries can result in fish death, including deteriorated renal tubules and glomerulus, and necrosis of hepatocytes. Toxicity disturbs liver and kidney function.

In Conclusion, Fish species were recently suggested as environmental biomarkers. Measuring metals in aquatic organisms may be a bioindicator of their impact on organisms and such information is beneficial in environmental threat evaluation. According to our findings, *A. sativum* is beneficial in the diet for improving fish resistance in fish systems.

Materials and Methods

Experimental Settings

This investigation was conducted in the Fish Research Laboratory of the Fishery Division, Gonbad Kavous University, Golestan, Iran. The rainbow trout fish was prepared from a cold-water fish farm located in Fazel Abad, Golestan province, Iran, and transported to the research laboratory in 2 h in special plastic bags with adequate oxygen. The experimental tanks were of 25-liter capacity. The weight of the fish used was 51.2 ± 3.73 g. The tanks were aerated for 24 h and dissolved oxygen was maintained at a saturation range by aeration. The test temperature (17.1°C ± 0.74°C), pH (8.09 ± 0.15), and dissolved oxygen (8.12 ± 1.02 mg/l) were measured using a portable multi-parameter Hack (Model 2000). The experimental diet was prepared by supplementing a basal formulated diet with 1% of garlic microencapsulation [38]. Fresh garlic (A. sativum) samples were bought from a local grocery store. The fish were fed a phytobiotic-enriched diet at least thrice a day for 40 days. The garlic was peeled and then powdered in the oven (ON-11E). Garlic powder was mixed with ethanol (purity of 70%) in a shaker at room temperature for 48 h. The solution was passed through a Whatman filter paper (42 µ) and placed in Rotary (HS-200S, Korea) at 75°C for 1 h to remove the alcohol. Next, the extract was placed in the oven at 38°C for 30 min. In order to encapsulate garlic extract, 30 g maltodextrin and 10 g Arabic gum were mixed with 60 g distilled water at 70°C-80°C and then homogenized (IKA T 25 digital ULTRA, Germany) for 1 h by homogenizer at 7000 g. The material was stored at 60°C inside the Ben-Marie (Memert. WNB 14, Germany) for 24 h. Coating materials and garlic extract (3:1 ratio) were mixed for 30 min. Microencapsulated garlic extract was preserved in a freeze dryer (Alpha-2 LD plus, Germany) for 24 h [38]. A commercial diet (Bezae Company, Iran) was employed as the experimental diet. The analyzed content was crude protein 44%-45%, crude fat 14%-14.5%, moisture 10%, crude fiber 2%-2.2%, absorbable phosphor 0.8%, and digestible energy 4300 kcal/kg.

The fish were not nourished 24 h previous to or throughout Cu exposure. Finally, the fish specimens were considered in three investigational groups (11 fish in separate treatment; replications with 2 fish specimens). The experiment treatments comprised a control with no Cu or garlic treatment (T1), feeding with garlic additive and Cu exposure (T2), and exposure to a lethal dose of Cu with no garlic additive (T3).

Toxicity Test

For the acute bioassay experiments, 11 fish specimens were separated for each concentration. The same number of fish served as control. Stock solutions of copper sulfate were provided by liquidation methodical grade copper sulfate ($CuSO_4.5H_2O$ from Merck) in ddH_2O. The added copper sulfate to each aquarium was considered after the volume of each tank was exactly specified. The control group was preserved in water without adding copper sulfate and garlic. Based on the preliminary tests and previous results (range finding test), the fish were exposed to 0, 0.02, 0.1, 0.3, and 0.4 mg/l of copper to determine LC50 for all the fish. No mortality was observed during this period. However, deceased fish were separated every 12 h and were eliminated from the aquaria immediately. The mortality rate was recorded at 24, 48, 72, and 96 h after the challenge. The behavior variations in all fish and the exposed fish to different doses of copper sulfate were assessed (e.g., breathing and general activity). The tests were performed by a stationary acute investigational technique [12] and the bioassay structure was implemented as defined in identical techniques [39].

Histopathological and Hematological Studies

Nine fish specimens were randomly selected and blood samples were taken from their caudal veins [40] in 2 ml disposable heparinized syringes. The blood samples were kept in test tubes containing EDTA (10 mM Tris–HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6). In blood samples, WBC count, RBC count, Hct, and HB were evaluated [41]. The RBC count, Hct, and HB were analyzed immediately. After diluting with Hen-

dricks's diluting solution, the number of RBCs was counted using an optical microscope equipped with a hemocytometer (Improved Neuberger, Germany). HB concentration was measured by the Cyan-methemoglobin technique (Asan Pharm. Co., Ltd.). Hct value was analyzed by the microhematocrit centrifugation technique using a capillary tube and a microcentrifuge (Hawksley & Sons, Ltd.) [42].

To measure immune factors, blood was obtained from the caudal vein without heparin [43]. The blood samples were centrifuged at 5000 g at 4°C for 10 min and the serum samples were stored at -80oC until analysis [44].

In addition, 12 fish were randomly selected, euthanized, and dissected to collect the whole digestive tract, gill, liver, and kidney tissues [42]. Lysozyme levels were evaluated according to the Ellis method [45]. Immunoglobulin M content was estimated based on the technique explained by Saha et al. [46]. Alternative hemolytic supplement activity (ACH50) was assessed through the method explained by Sunyer and Tort [47] based on the hemolysis of rabbit red blood cells. The volume of the serum yielding 50% hemolysis (ACH50) was found and used to estimate the supplement activity of the samples (ACH50 is in units/ml). Serum glucose and total protein were measured by Pars Azmoon kits (Pars Azmoon Company, Iran) according to the manufacturer protocols. The samples were derived from the kidney, liver, and gill of the fish for histopathological evaluation. The arbitrarily deprived sections for tissue processing were fixed in 10% neutral buffered formalin [48]. Next, the samples were observed under an optical microscope for histological alterations and the histology of the control group was compared with the treated groups.

Statistical Analysis

The acute toxic influence of copper sulfate was assessed on the standard experiment species rainbow trout (O. mykiss) using Finney's [49] Probit Method (LC50 analysis). No observed effect concentration (NOEC) was considered the highest concentration that led to no death, while the lowest observed effect concentration (LOEC) was regarded as the lowest concentration that caused fish death [50]. Statistical analysis was performed by the analysis of variance (ANOVA) followed by Duncan's ($p < 0.05$) [51] Multiple Range Test (DMRT). SPSS version 16 was used for the analyses and the results are presented as mean ± SD.

Authors' Contributions

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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