Ameliorative Effects of Hemp (*Cannabis sativa*) Against Copper-induced Toxicity in *Labeo rohita*

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Abstract

This study investigates the therapeutic effect of dietary hemp seed oil (HSO) and hemp seed (HS) against copper-induced toxicity in Labeo rohita. For this purpose, Fingerlings of Labeo rohita (Rohu) are exposed to sub-lethal levels of copper(Cu) for thirty days, and subsequently, all groups except control are fed on two types of hemp-supplemented diets i-e three of hemp seed oil (HSO); 1 %, 2%, 3%) and three of hempseed (HS); 5%, 10%, 15%), for 50 days. After thirty days of copper toxicity various hematological parameters, immune response, serum biochemistry, and antioxidant enzymes in five fish of each group are checked which include elevated levels of white blood cells, mean corpuscular volume, mean corpuscular hemoglobin concentration, hematocrit, neutrophils, lymphocytes, lysozyme, and immunoglobulin M, along with reduced plasma protein levels. Furthermore, copper exposure led to notable changes in liver function tests (LFTs), including alkaline phosphatase, bilirubin, serum glutamic-pyruvic transaminase, and aspartate transaminase. Additionally, changes in antioxidant enzyme activities, such as catalase, superoxide dismutase, glutathione reductase, and peroxidase, are observed in the brain, gills, liver, and muscle tissues. However, interestingly, supplementation with hemp seed oil and hemp seed effectively reversed the altered parameter to a normal level and reduced the adverse effects of copper intoxication, restoring the fish to normal physiological levels. Overall, the findings suggest that hemp product supplementation can mitigate copper toxicity in fish, highlighting its potential therapeutic role as a feed ingredient in aquaculture practices.

Keywords: Copper Toxicity; Hemp Seed Oil; Hemp Seed; Hematology; Serum; Antioxidant Enzymes; Rohu.

Introduction

With the increasing population of the world, there is an intense need for good quality food to alleviate hunger and malnutrition. In this regard, aquaculture plays an essential role in alleviating these problems, by providing freshwater and marine products, which are important

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components of human nutrition, and also rich sources of protein, vitamins, essential fatty acids, and minerals (FAO, 2010), and rich in omega-3 fatty acids, which lowers the risk of heart diseases thus increases life expectancy (Barlas, 1986; Abbas et al., 2010).

The aquatic environment of the world has faced a major threat due to increasing aquatic pollution by toxic substances (Xia et al., 2011; Akan et al., 2012). Rapid expansion in industrialization has resulted in a generation of solid and liquid iste, and their discharge of istes into natural water bodies poses a threat to aquatic fauna (Babalola et al., 2010). Heavy metal pollution in aquatics environments arises from natural processes such as atmospheric deposition and geological weathering and anthropogenic activities such as agricultural runoff and the disposal of municipal, residential, industrial iste products and toxic substances are added in aquatic environment (Dhanakumar et al., 2015; Maier et al., 2015). These factors affect water quality and cause raised concentrations of heavy metals, and at last the metals and their metallic ions climax in water and accumulate within various organs of fish, particularly the liver and kidneys. The metallic ions in water bodies are emerging as a primary cause of physiological abnormalities in aquatic organisms (Shukla et al., 2007).

Fishes are susceptible to various water-dissolved pollutants and their hematological parameters are susceptible indicators, while exposed to stressors (Rehulka et al., 2004; Tavares-Dias and Barcellos, 2005). The health of fish is assessed on specific parameters, by measuring the count of white blood cells and red blood cells, along with levels of hemoglobin and hematocrit (Shah and Altindag, 2004). The harmful effects of waterborne copper on freshwater fish and its influence on specific organs have been extensively reported by (Grosell et al., 2007; Mustafa et al., 2012). For example, exposure to sub-lethal doses of copper has been linked to a reduction in the count of red blood cells, levels of hemoglobin, mean corpuscular hemoglobin, and packed cell volume, as well as increases in the count of white blood cells, mean corpuscular volume, mean corpuscular hemoglobin concentration, and hematocrit (Mazon et al., 2002; Singh et al., 2008). Metals toxicity either decrease or increase serum parameters. The activity of certain enzymes changes when exposed to copper such as aspartate transaminase (AST), alkaline phosphatase (ALP), and alanine transaminase (ALT) (Oner et al., 2008).

Reactive oxygen species (ROS) formation at a cellular level is a normal activity. Still, metal-induced toxicity leads to the production of ROS and hydroxyl radical (OH^-), which causes oxidative stress. Hydroxyl radicals are involved in lipid peroxidation, DNA damage, and oxidation of bases (Singh et al., 2010; Wu et al., 2014). The anti-oxidative system (AOS)

activates against oxidative stress and is responsible for eliminating excess free radicals. Anti-oxidative systems consist of enzymatic and non-enzymatic pathways (Patlevic et al., 2016; Winzer et al., 2000). The enzymatic pathway consists of enzymes such as glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Rahman, 2007). The non-enzymatic antioxidants obtained from diet source such as Vitamin C and E. Some micronutrients are additional sources of non-enzymatic antioxidants such as hypotaurine, glutathione, taurine, carotene, beta carotene, selenium, glutathione, and zinc (Shahidi and Zhong, 2010). The antioxidant enzymes are the best bio-indicators of environmental pollution in the early stages, and their stimulation reflects on specific pollutants (Borkovic et al., 2005)

Cannabis sativa L. (Famous as marijuana or Indian hemp) is the oldest crop, used for multiple purposes and widely cultivated throughout the tropical and temperate regions of the world (Small, 2017; Chandra et al., 2017). This plant has been used for thousands of years for Medicinal, and nutritional purposes. (Callaway, 2004). Photochemical analysis of hemp leaves showing presence of chemicals such as flavonoids, alkaloids, terpins, cardiac glycosides, resins, and steroids (Audu et al., 2014). Pharmaceutically hemp leaves possess properties that induce abortion, alleviate pain, constrict tissues (especially skin cells), produce intoxication, invigorate, aid digestion, and enhance sexual desire (Whiting et al., 2015). Current research favors the application of hemp seed oil and hemp seed in the food, pharmaceutical, and cosmetic industries (Montserrat-de la Paz et al., 2014). Hemp seed oil contains 80 percent of polyunsaturated fatty acids (PUFAs). Furthermore, it contains vitamin E (tocopherols), linoleic acid and α-linolenic acid that act as antioxidants; these are considered not only the growth metabolites but therapeutically these reduce the production of free radicals such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and superoxide anion O^{2-} (Kriese et al., 2004). Hemp feeding in diets in percentage inclusion enhanced the ratio of omega-3 to omega-6 fatty acids, and reduced the ratio of saturated fatty acids to unsaturated fatty acids to fish for fifty days (Afridi et al., 2019).

The present study aimed to explore the potential applications of hemp seed oil and hemp seed in mitigating metal toxicity, while considering the previous studies of hemp's nutritional importance (Afridi et al., 2019) and pharmacological activity (Callaway, 2004; Whiting et al., 2015). We hypothesized that copper-induced toxicity and its oxidative stress would alter Rohu blood parameters, blood serum, and antioxidant levels, and feeding hemp product in diets will revert and stabilize the altered parameters to their normal level.

Materials and Methods

Preparation of Hemp (C. sativa) Supplemented Feed

For hemp diet preparation, hemp seed oil (Organic extra virgin Canada) is purchased from a registered supplier company. The hemp seeds are purchased in Tirah Maidan and transported to Aquaculture and fisheries at Laboratory Quaid-i-Azam University. Other ingredients of feed mentioned are purchased from a registered company. Following Afridi et al, (2019), seven diets of 35 % crude protein (CP) are prepared, six diets having hemp products, in which three of hemp seed oil (HSO; 1%, 2%, and 3%), and three of hemp seed (HS; 5%, 10%, 15%), while a control or basal diet lacking any hemp supplementations.

Preparation of Standard Solution

Using the Molecular formula, taking 5.44 g analytical grade of copper sulfate pentahydrate ($CuSO_4.5H_2O$), equivalent to one gram copper is shifted to the one-liter volumetric flask, making the exact volume of one liter some deionized water is added in it up to the mark of 1000 ppm (1000mg/l). The solution is shaken well and kept at room temperature for making further dilution. Further dilution (from 0.2 to 4.00ppm) are made for determination of lethal concentration / median tolerance limit (LC_{50}) for 96 h at fingerlings of Rohu under semi-static condition, protocols reported by (Afridi et al., 2019).

Experimental Animals

To gather Rohu fingerlings, induced breeding experiments are carried out at Quaid-i-Azam University's Fisheries and Aquaculture Research Station, having fish breeding facilities. After the yolk sac absorption, fertilized eggs are raised up to swim up fry and then moved to an earthen pond to be raised to the fingerling stage. Fingerling of Rohu with an average weight of 42.60 ± 7.95 g and a length of 16.32 ± 1.28 cm are selected as test animals. Rohu fingerlings are first acclimatized in 5000 liter circular fiberglass tanks in laboratory conditions for a week with good ventilation.

Experimental Design

After acclimatization, a total of 315 Rohu fingerlings are stocked into 21 glass aquaria (120 × 60 × 60 cm) in triplicates, well equipped with water heaters to maintain temperature and air stones connected to aerators. The water temperature is set at 26.5°C during the feeding trial. During experimental period, they are fed a control diet twice a day (at 9:00 and 16:00). After every 48 hours, uneaten feed and fecal matter from each

aquarium are removed through siphoning. A stocking density of 2 g/L (15 fingerlings/aquarium) is used to stock them. To ensure water quality, its parameters are regularly checked before and during the bioassay tests**.**

Determination of LC_{50} for 96h (Acute Toxicity Test)

Under semi-static conditions, the median tolerance limit (LC_{50}) of the Rohu fingerlings is determined over 96 hours. Based on available literature on various carp species, and pre-pretrial experiments, Rohu fingerlings are exposed to 19 different concentrations of copper, ranging (0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0 ppm) to determine the median lethal concentration (LC_{50}/96 h) of copper that causes 50% mortality. No copper treatment is given to control group. Fish mortality is recorded every 24 hours up to 96 hours to calculate the median lethal concentration (LC_{50}/96 h), and dead fish are removed to prevent contamination of water. Using the SPPS probit approach, the LC_{50} for copper is determined for 96 hours with a 95% confidence level (Finney, 1971). The results showed that the median lethal concentration (LC_{50}/96h) of *Labeo rohita* is 1.34 ppm.

Sub-lethal Concentration /Chronic Toxicity Test

Subsequently, fingerlings of *Labeo rohita* from all aquaria (except control group) are exposed for 30 days to a sub-lethal concentration of Cu, or 1/5th or (20%) of (LC50/96h). The value of LC_{50} is 1.34 ppm, and its $1/5^{th}$ is equivalent to the 0.268 ppm, and fingerlings of Rohu are exposed to $1/5^{th}$ value of LC_{50} as chronic toxicity of copper for thirty days, previously reported by (Afridi et al., 2019). Each aquarium's uneaten food and faeces are emptied by siphoning, and a fresh metal concentration is maintained every 24 hours.

Five fish are taken from each tank after 30 days of copper exposure to evaluate the chronic effect of Cu exposure on the hematological parameters, liver function test (LFT), serum, and antioxidant level of Rohu.

To investigate the therapeutic effects of hemp remaining fish are kept on experimental trail of fifty days on sis diets of hemp, three diets: of hempseed oil (HSO) at concentrations of (1, 2, and 3%). and three of hempseed (HS) at concentrations of (5, 10, and 15%) at a body weight of 5%. The control diet did not contain any hemp ingredients. Before end of experiments, fish are starved for twenty-four hours.

Complete Blood Profile

Five fish are randomly selected from each aquarium and immediately sedated with newly made MS-222 (0.10 gL-1 buffered with

sodium bicarbonate). Besides the lateral line and caudal peduncle, majority of fish blood is collected directly from the heart with great expertise, using a tiny 3-ml heparinized syringe (24G, ShifaR Changzhou Tangda Medical App. Co., LTD) in lavender top K2 VACUETTER EDTA tubes. Fish erythrocytes are delicate, and they begin to degrade after six hours. Thus, as reported by (Ullah et al., 2018), the samples are processed in time for the estimation of various blood parameters such as RBCs (103 µl-1), WBCs (103 µl-1), Hb (g dl-1), HCT (%), MCV (fL), MCH (pg), and MCHC (g dl-1).

Serum Chemistry

The collected blood sample in red top (EDTA VACUETTER tubes) is immediately, transported to the laboratory for serum collection, blood samples is centrifuged for five minutes at 3000 rpm, the obtained serum is stored at 4°C. The levels of immunoglobulin M (IgM), total plasma proteins, and lysozyme activity in the plasma are assessed using the corresponding standard procedures that had previously been published by (Ullah et al., 2018). Blood remained from blood profile are not discarded but used for liver function test (LFT), with help of chemistry analyzer. The AST/GOT kit (AMEDA Laboro diagnostik GmbH, Graz, Austria) is utilized to determine the AST values.

Analysis of Antioxidants

Four tissues such as brain, muscle, gill, and liver tissues (90 mg each) are collected from five fish in each group and homogenized in 100 mmol KH_2PO_4 buffer with the help of a handheld electrical homogenizer (Model AHS 200). To collect supernatant, the homogenate is centrifuged at 12000X g for 30 min at 4˚C. With slight modification, the level of antioxidants enzymes such as Catalase (CAT) Peroxidase (POD) Superoxide Dismutase (SOD), and Glutathione Reductase (GR) is determined in targeted tissues of Rohu by following standard methods of (Britton and Mehley, 1955; Kakkar et al., 1984). Total Protein levels in all tissues are also estimated by Lowry's method (1951) and used for antioxidant calculation.

Statistical Analysis

All data is represented as mean ± SEM. One-way ANOVA is used to find significant differences between the controls, copper-induced, and hemp-supplemented groups in terms of hematological, immunity, liver function test, and antioxidant levels. After significant differences are found, the Tukey's HSD post hoc test is used to analyze the mean comparison

using SPSS. P-values that fell below 0.05 are considered statistically significant.

Results

The finding of this study demonstrated the toxic effects of copper and the ameliorative effects of different levels of hempseed oil (HSO), and hempseed (HS) in the diet on complete blood profile (Table 1), immunological parameters in (Table 2), LFT in (Table, 3), and antioxidant enzymes in (Tables 4 to 7) are shown in fingerling of Rohu.

Table1: Showing chronic copper toxicity and post effect of hemp seed oil and hemp seed on complete blood profile (CBP) of Rohu (Labeo rohita).

Blood Parameter	Control	Cu Exposed	Hemp seed Oil			Hemp Seed		
			HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
WBC (10^3 µL)	226 ±59^{b}	263 ±75^{a}	260 ±56^{a}	260 ±49^{b}	224 ±96^{b}	251 ±37^{a}	234 ±43^{b}	225 ±20^{b}
RBC (10^6 µL)	2.27 ±0.08^{b}	1.35 ±0.05^{d}	1.39 ±0.15^{d}	1.54 ±0.11^{d}	2.08 ±0.911^{bc}	1.42 ±0.11^{d}	1.75 ±0.18^{cd}	2.78 ±0.33^{a}
Hb (g dL^{-1})	10.21 ±0.17^{a}	7.68 ±0.26^{d}	7.94 ±0.20^{cd}	8.57 ±0.38^{bc}	8.94 ±0.25^{b}	8.17 ±0.34^{bcd}	8.86 ±0.21^{b}	10.08 ±0.24^{a}
HCT (%)	21.2 ±0.51^{d}	28.5 ±1.12^{a}	27.1 ±0.78^{ab}	24.9 ±1.05^{bc}	23.8 ±0.88^{cd}	27.4 ±1.18^{ab}	26.1 ±0.91^{abc}	23.7 ±1.25^{cd}
MCV (10^{-15} L)	111 ±4.42^{e}	147 ±3.44^{ab}	148 ±3.19^{a}	128 ±2.85^{d}	115 ±1.58^{e}	138 ±4.65^{bc}	135 ±0.88c^{d}	112 ±3.39^{e}
MCH (pg)	43.8 ±2.43^{a}	40.3 ±2.53^{ab}	38.9 ±0.77^{b}	40.8 ±0.85^{ab}	40.7 ±1.32^{ab}	38.2 ±1.26^{b}	41.1 ±0.83^{ab}	41.2a ±0.99^{b}
MCHV (g dL^{-1})	32.8 ±0.78^{cd}	37.5 ±0.56^{a}	36.2 ±0.91^{ab}	36.3 ±1.13^{ab}	33.1 ±0.37^{bcd}	35.5 ±2.14^{abc}	32.1 ±0.95^{d}	32.5 ±1.12^{cd}
Platelets (10^3 µL)	19 ±3.6^{c}	13 ±13.0^{a}	72 ±8.9^{b}	36 ±6.1^{c}	22 ±2.6^{c}	41 ±8.1^{bc}	24 ±4.5^{c}	18 ±2.4^{c}
Neutrophil	4.00 ±1.00^{b}	6.00 ±0.57^{ab}	6.33 ±0.66^{ab}	7.33 ±0.66^{a}	5.66 ±1.20^{ab}	5.66 ±1.20^{ab}	5.33 ±0.88^{ab}	4.33 ±0.88^{b}
Lymphocytes	82.67 ±1.20^{d}	90.67 ±0.66^{a}	87.33 ±2.33^{abc}	86.33 ±1.20^{bcd}	83.33 ±1.20^{cd}	90.00 ±0.57^{ab}	84.00 ±1.73^{cd}	82.33 ±1.20^{d}
Monocytes	2.33 ±0.33^{bc}	1.67 ±0.33^{c}	3.00 ±1.00^{abc}	3.33 ±0.33^{ab}	2.66 ±0.33^{abc}	4.00 ±0.57^{a}	2.67 ±0.57^{abc}	3.00 ±0.57^{abc}
Eosinophil	1.67 ±0.33^{a}	2.00 ±0.05^{a}	2.00 ±0.57^{a}	2.33 ±0.33^{a}	1.67 ±0.33^{a}	2.00 ±0.57^{a}	1.33 ±0.33^{a}	1.67 ±0.33^{a}

The data are presented as Means ± SE, with a sample size of n=5. Different letters following the means within the same row indicate significant differences ($P < 0.05$), as determined by ANOVA followed by Tukey's HSD test. Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil; HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp seed.

Complete Blood Profile

The graded concentrations of hemp supplementation in the feed, including hemp seed oil (HSO: 1%, 2%, 3%) and hemp seed (HS: 5%,

10%, 15%), exhibited positive effects on various haematological parameters in *Labeo rohita* fingerlings after a 30-day chronic copper toxicity are shown in (Table 1).

Copper exposure significantly ($P < 0.05$) increased WBCs, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHV), Platelets, neutrophil, and lymphocytes, while the values of RBCs, Hb, and mean corpuscular hemoglobin (MCH) contents are decreased significantly ($P < 0.05$) in comparison to the control group. After applying one-way ANOVA followed by Tukey's HSD post hoc test, it is confirmed that all of the hematological parameter values significantly ($P < 0.05$) returned to normal in the hemp-supplemented feed groups compared to the copper exposure group. The effects of the results are dose-dependent / diets-dependent in percentage inclusion of hemp following copper toxicity and demonstrated significant and positive effect in the fish's blood characteristics

Immunological Parameters

Toxic effects of copper and the ameliorative effects of hemp product in diets on the levels of lysozymes, plasma proteins, and immunoglobulin M (IgM) in fingerlings of Rohu are shown in (Table 2). Copper exposure significantly ($P < 0.05$), increased lysozymes and IgM in the fingerling of Rohu. Analysis of variance (ANOVA) revealed significant changes in IgM, lysozyme, and plasma protein levels of hemp-fed groups after exposure to copper toxicity in fingerling Rohu. Results showing that significantly altered parameters lysozymes, plasma proteins, and immunoglobulin M (IgM) are reverted and stabilized to normal level by feeding hemp for fifty days to fingerling of Rohu.

Table 2: Showing chronic copper toxicity and post effect of hemp seed oil and hemp seed on immunological parameters of Rohu (Labeo rohita).

Serum Parameter	Groups							
	Control	Cu Exposed	HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
Lysozymes ($\mu g ml^{-1}$)	3.59 $\pm 0.55^{b}$	7.45 $\pm 0.53^{a}$	5.86 $\pm 0.48^{ab}$	3.78 $\pm 0.87^{b}$	4.30 $\pm 0.65^{b}$	5.56 $\pm 0.45^{ab}$	3.38 $\pm 0.71^{b}$	3.00 $\pm 0.26^{b}$
Plasma protein ($g\ dL^{-1}$)	5.53 $\pm 0.48^{b}$	4.06 $\pm 1.14^{c}$	8.53 $\pm 0.33^{ab}$	6.66 $\pm 1.42^{ab}$	5.35 $\pm 0.62^{b}$	9.46 $\pm 1.52^{a}$	5.75 $\pm 0.50^{b}$	7.06 $\pm 1.095^{a}$
IgM ($mg\ ml^{-1}$)	9.09 $\pm 1.15^{b}$	16.54 $\pm 0.72^{a}$	11.24 $\pm 1.60^{ab}$	12.41 $\pm 1.64^{ab}$	8.92 $\pm 0.89^{b}$	8.94 $\pm 2.00^{b}$	8.95 $\pm 1.42^{b}$	9.43 $\pm 0.54^{b}$

The data are presented as Means ± SE, with a sample size indicated by n=5. Different letters following the means within the same row signify statistically significant differences ($P < 0.05$), as determined by ANOVA followed by Tukey's HSD test. Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil: HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp seed.

Liver Function Tests

The incremental levels of hemp supplementation in the feed exhibited concentration-dependent positive effects on the LFT enzymes, including alkaline phosphatase, bilirubin, SGPT, and AST, following 30-day copper-induced chronic toxicity in Rohu are shown in (Table 3).

Table 3: Showing chronic copper toxicity and post effect of hemp seed oil and hemp seed on LFTs of Rohu (Labeo rohita).

LFT	Groups							
	Control	Cu Exposed	HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
SGPT	18.33 ±1.76^{c}	58.66 ±4.17^{a}	37.66 ±6.56^{abc}	24.00 ±4.04b^{c}	17.667 ±2.40^{c}	41.333 ±4.05^{ab}	29.667 ±2.60^{bc}	34.33 ±7.05^{bc}
AST	4.68 ±0.44^{b}	14.45 ±0.70^{a}	9.72 ±1.41^{ab}	11.68 ±1.53^{ab}	4.79 ±0.33^{b}	10.59 ±1.68^{ab}	9.32 ±2.88^{ab}	5.67 ±0.47^{b}
Bilirubin	0.93 ±0.24^{c}	3.60 ±0.11^{a}	2.96 ±0.18^{ab}	2.08 ±0.37^{bc}	1.79 ±0.51^{bc}	2.88 ±0.22^{ab}	0.84 ±0.27^{c}	0.80 ±0.11^{c}
Alkaline Phosphatase	63.66 ±2.96^{ab}	95.00 ±1.15^{a}	72.66 ±2.90^{ab}	69.33 ±8.83^{ab}	74.33± 9.82^{ab}	74.33 ±12.54^{ab}	64.66 ±2.18^{ab}	60.66 ±0.88^{b}

The data are presented as Means ± SE, with a sample size denoted by n=5. Different letters following the means within the same row indicate statistically significant differences ($P < 0.05$), determined by ANOVA followed by Tukey's HSD test. Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil; HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp seed.

Copper exposure alone increased significantly ($P < 0.05$), alkaline phosphatase, bilirubin, SGPT, and AST in fingerling of Rohu. The altered parameters of serum are reverted to their normal level as revealed by analysis of one-way ANOVA followed by Tukey's HSD post hoc test, in fingerling of Rohu. Results showing positive effects of graded levels of hemp-supplemented feed on alkaline phosphatase, bilirubin, SGPT, and AST, in fingerlings of Rohu.

Antioxidants Enzymes

The inclusion percentage of hemp supplement feed showed positive effects on the antioxidant levels of Peroxidase, Catalase, Superoxide dismutase, and Glutathione reductase in selected tissues of fingerlings of Rohu following 30-day copper induced toxicity are shown in (Tables 4 to 7).

Catalase (CAT)

The Catalase activities in copper-induced toxicity group and hemp feeding in diets group are shown in (Table 4). Results showing that activities of Catalase are significantly ($P < 0.05$) increased in all tissues

due to chronic toxicity of copper exposure for thirty days. However interestingly, all altered values of catalase in selected tissues of Rohu fingerlings are reverted to normal level after feeding hemp in diets for fifty days. Results showing ameliorative effect of hemp against copper induced toxicity in fingerling of Rohu.

Table 4: Examine the subsequent impact of hemp seed (HS) and hemp seed oil (HSO) on Catalase (CAT) activity (µmolmin^{-1}mg^{-1} protein) in various tissues of Rohu (Labeo rohita) after exposure to chronic copper toxicity.

Tissues	Groups							
	Control	Cu Exposed	HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
Brain	22.26 ±0.82^{c}	55.67 ±2.16^{a}	42.73 ±3.21^{ab}	35.85 ±2.84^{bc}	29.81 ±1.26^{bc}	42.54 ±3.08^{ab}	34.96 ±4.28^{bc}	20.22 ±8.26^{c}
Gills	28.67 ±1.72^{f}	61.98 ±1.82^{a}	51.50 ±3.56^{bc}	47.64 ±1.71^{bcd}	39.68 ±1.61d^{e}	54.73 ±1.64^{ab}	42.30 ±2.67^{cde}	35.17 ±1.40^{ef}
Liver	49.27 ±3.00^{d}	118.95 ±2.31^{a}	93.31 ±9.67^{b}	75.29 ±4.87^{bc}	57.97 ±1.40^{cd}	85.27 ±2.25^{b}	76.61 ±1.81^{bc}	58.35 ±5.27^{cd}
Muscle	18.08± 0.83^{c}	43.65 ±3.02^{a}	33.89 ±2.42^{b}	26.85 ±1.24^{bc}	22.82 ±1.04^{c}	33.27 ±1.72^{b}	26.75 ±1.69^{bc}	21.83 ±1.85^{c}

The data are presented as Means ± SE, with the sample size denoted by n=5. Different letters following the means within the same row indicate statistically significant differences ($P < 0.05$), as determined by ANOVA followed by Tukey's HSD test. Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil; HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp seed.

Superoxide dismutase (SOD)

The Superoxide dismutase activities in selected tissues of Rohu fingerlings are shown in copper induced toxicity group and hemp feeding group are shown in (Table 5). Results showing that activities of Superoxide dismutase are significantly ($P < 0.05$) increased in all tissues due to chronic toxicity of copper exposure for thirty days. However remarkably, all altered values of superoxide dismutase in selected tissues of Rohu fingerlings are reverted to normal level after feeding hemp in diets for fifty days. Results demonstrating hemp's ability to protect Rohu fingerlings against copper-induced toxicity.

Glutathione reductase (GR)

The Glutathione reductase activities in selected tissues of Rohu fingerlings are shown in copper induced toxicity group and hemp feeding group shown in (Table 6). The chronic toxicity of copper exposure for thirty days resulted in a significant ($P < 0.05$) increase in Superoxide dismutase activity across all organs. Surprisingly, though, after feeding hemp in diets for fifty days, all changed values due to copper toxicity of

Glutathione reductase in certain tissues of Rohu fingerlings reverted to normal levels. Findings of results indicating that hemp can protect Rohu fingerlings from the harmful effects of copper in fifty days hemp feeding in diets in graded level.

Table 5: Examine the subsequent impact of hemp seed (HS) and hemp seed oil (HSO) on superoxide dismutase (SOD) activity (µmolmin^{-1}mg^{-1} protein) in various tissues of Rohu (Labeo rohita) after exposure to chronic copper toxicity.

Tissues	Groups							
	Control	Cu Exposed	HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
Brain	314.3 ±2.5^{f}	670.9 ±4.4^{a}	523.0 ±5.3^{b}	475.7 ±6.1^{c}	417.7 ±7.7^{e}	534.8 ±6.9^{b}	467.7 ±8.7^{cd}	439.7 ±8.6^{de}
Gills	383.9 ±6.9^{f}	853.4 ±5.9^{a}	747.5 ±6.7^{b}	711.8 ±6.5^{cd}	638.3 ±4.8^{e}	718.6 ±2.5^{bc}	677.9 ±14.8^{d}	611.6 ±1.7^{e}
Liver	532.4 ±4.7^{e}	981.4 ±4.6^{a}	762.6 ±14.2^{b}	652.2 ±13.6^{cd}	620.7± 5.6^{d}	698.1 ±17.5^{c}	641.6 ±0.7^{d}	606.6 ±4.3^{d}
Muscle	707.2 ±4.9^{d}	1115.3 ±8.2^{a}	942.8 ±6.4^{b}	865.6 ±7.6^{c}	733.4 ±6.9^{d}	944.3 ±1.8^{b}	718.5 ±3.4^{d}	730.7 ± 9.9^{d}

The data are presented as Means ± SE, with the sample size indicated by n=5. Different letters following the means within the same row indicate statistically significant differences ($P < 0.05$), as determined by ANOVA followed by Tukey's HSD test. Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil: HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp seed.

Table 6: Examine the subsequent impact of hemp seed (HS) and hemp seed oil (HO) on Glutathione reductase (GR) activity (µmolmin-1mg-1 protein) in various tissues of Rohu (Labeo rohita) after exposure to chronic copper toxicity.

Tissues	Groups							
	Control	Cu Exposed	HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
Brain	1198.4 ±39.9^{c}	1676.4 ±29.9^{a}	1557.3 ±22.4^{ab}	1462.8 ±16.5^{b}	1417.9 ±47.1^{b}	1490.3 ±33.2^{b}	1514.3 ±20.9^{b}	1460.0 ±14.6^{b}
Gills	1268.1 ±42.3^{d}	1781.2 ±38.6^{a}	1626.9 ±29.7^{ab}	1519.3 ±57.6^{bc}	1460.2 ±19.2^{c}	1697.6 ±23.9^{a}	1532.4 ±19.2^{bc}	1464.1 ±16.4^{bc}
Liver	1393.0 ±21.8^{c}	1962.6 ±82.3^{a}	1673.7 ±34.5^{b}	1514.4 ±19.4^{bc}	1448.0 ±16.5^{c}	1683.2 ±32.3^{b}	1539.7 ±3.5^{bc}	1480.7 ±23.9^{c}
Muscle	659.4 ±42.5^{cd}	1068.5 ±80.5^{a}	852.8 ±13.7^{bc}	561.9 ±32.8^{d}	604.5 ±30.4^{d}	912.9 ±11.6^{ab}	567.5 ±33.8^{d}	565.9 ±36.4^{d}

The data are presented as Means ± SE, with the sample size denoted by n=5. Different letters following the means within the same row indicate statistically significant differences ($P < 0.05$), as determined by ANOVA followed by Tukey's HSD test. Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil: HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp seed.

Peroxidase (POD)

The Peroxidase activities in copper induced toxicity group and hemp feeding in diets group are shown in (Table 7). Results showing that activities of Peroxidase are significantly ($P < 0.05$) increased in all tissues due to chronic toxicity of copper exposure for thirty days. However interestingly, all altered values of Peroxidase in selected tissues of Rohu fingerlings are reverted to normal level after feeding hemp in diets for fifty days. Results showing ameliorative effect of hemp against copper induced toxicity in fingerling of Rohu.

Table 7: Examine the subsequent impact of hemp seed (HS) and hemp seed oil (HSO) on Peroxidase (POD) activity (µmolmin^{-1}mg^{-1} protein) in various tissues of Rohu (Labeo rohita) after exposure to chronic copper toxicity.

Tissues	Groups							
	Control	Cu Exposed	HSO 1%	HSO 2%	HSO 3%	HS 5%	HS 10%	HS 15%
Brain	22.47 ±2.09^{c}	76.47 ±4.00^{a}	57.13 ±2.88^{ab}	70.43 ±4.25^{a}	66.91 ±5.87^{a}	57.63 ±7.09^{ab}	36.08 ±3.42^{bc}	41.67 ±6.77^{bc}
Gills	28.56 ±2.19^{c}	82.67 ±5.25^{a}	64.70 ±4.61^{ab}	44.48 ±3.50^{bc}	45.47 ±4.68^{bc}	68.07 ±6.34^{a}	38.07 ±2.84^{c}	38.67 ±1.42^{c}
Liver	42.77 ±3.73^{c}	161.64 ±9.04^{a}	83.97 ±5.44^{b}	66.67 ±8.37^{bc}	63.27 ±7.74^{bc}	86.70 ±2.68^{b}	66.03 ±4.98^{bc}	64.96 ±6.64^{bc}
Muscle	5.68 ±1.16^{b}	12.47 ±1.68^{a}	9.19 ±1.17^{ab}	9.75 ±1.93^{ab}	6.68 ±0.43^{ab}	9.37 ±0.48^{ab}	7.85 ±0.87^{ab}	7.17 ±0.87^{ab}

Data are represented Means ± SE; n= number of sample (n=5); Means followed by different letter within the row are significantly different ($P < 0.05$). (ANOVA followed by Tukey's HSD test). Control and Copper exposed= basal diet; HSO1%= 1% hemp seed oil, HSO2%= 2% hemp seed oil, HSO3%= 3% hemp seed oil: HS5%= 5% hemp seed, HS10%= 10% hemp seed, HS15%= 15% hemp.

Discussion

Globally, heavy metal addition into aquatic environments has increased due to sewage and industrial iste pollution. It is a fact that heavy metals adversely affect fish's physiological parameters such as blood, serum, and antioxidant defense system (McDonald and Grosell, 2006; Oner et al., 2008; Singh et al., 2008). Fishes are extensively used as bio-indicators to assess the health of aquatic ecosystems. Copper is an essential microelement that is required as a cofactor for more than thirty copper-containing enzymes such as hephaestin, tyrosinase, superoxide dismutase, cytochrome C oxidase, ceruloplasmin, lysyl oxidase, dopamine-β-hydroxylase, that catalyze reactions in fundamental metabolic processes, however its high-level lead to toxic effect on human and animal (Prohaska, 2011).

The findings of the present study regarding the impact of metal intoxication on blood parameters closely align with previous research,

demonstrating notable increases in white blood cell (WBC) and red blood cell (RBC) counts, as well as alterations in hemoglobin concentration and hematocrit levels (Adak; Shalaby, 2001; Vutukuru, 2005). Results indicate that copper exposure significantly ($P < 0.05$) increased WBCs, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHV), Platelets, neutrophil, and lymphocytes, while the values of RBCs, hemoglobin (Hb), and mean corpuscular hemoglobin (MCH) contents are decreased significantly ($P < 0.05$) in comparison to the control group, with agreement to previous studies on conducted on copper toxicity by (Ketpadung and Tangkrock-Olan, 2006; Srivastava and Punia, 2011; Vosylienė and Mikalajūnė, 2006). For example, a decrease in RBC count, hemoglobin percentage, and packed cell volume percentage (PCV) has been shown in *Channa punctatus* when exposed to high levels of Cu. Likewise, exposure to copper resulted in a substantial rise in hematocrit and leukocyte levels with the percentage of monocytes showing no significant change following Cu exposure (Mazon et al., 2002; Vergolyas et al., 2010). Exposure to a sub-lethal concentration of Cu resulted in decreased levels of Hb, and PCV and increased levels of MCV, WBC, MCHC, and MCH (Singh et al., 2008).

In fish toxicology, the evaluation of serum immunological and biochemical analysis are valuable diagnostic methods to determine the overall health status and especially affected target organs (McDonald and Grosell, 2006). In this study, copper exposure significantly ($P < 0.05$), increased lysozymes and IgM in the fingerling of Rohu in comparison to the control. The toxic effect of Cu is evident from elevated serum parameters. In the results of this study, the plasma protein decreased in Cu-exposed in comparison to the control of fingerlings of Rohu. The increased concentration of serum total protein indicates robust immunity (Vasudeva Rao et al., 2006). Upon metal exposure, plasma protein has been decreased, as reported by Srivastava (Srivastava and Punia, 2011). Gopi et al., (2019) reported that sub-lethal concentration of copper affects lysozyme activities in *Oreochromis niloticus*. The elevated level of lysozymes displays a potent natural immune response in blood serum (Ingram, 1980). Lysozyme is a bacteriolytic enzyme that cleaves N-acetylmuranine and glycoside bonds within bacterial cell walls (Wu et al., 2019), by hydrolyzing them. However, the main component of the humoral system is IgM, which is abundant in fish (Wilson and Warr, 1992).

Similarly, copper exposure alone increased significantly ($P < 0.05$), alkaline phosphatase, bilirubin, SGPT, and AST in fingerling of Rohu. The AST is a nonspecific biomarker for environmental pollutants and an important serum marker for the health of animal species (Oner et al., 2008). AST is a biochemical marker and an efficient indicator for the detection

of sub-lethal damage to organs such as kidneys and liver. Kim et al., (2008), reported an increased level of AST is an indicator of the presence of toxic agents in blood serum. Mukhopadhyay reported, that serum transaminases used as a biomarkers for liver health, and an increase in transaminases causes hemolytic anemia and hepatocellular damage (Mukhopadhyay et al., 1982). Several investigations have indicated that certain metals exhibit the capability to elevate or diminish total serum protein, and enzyme levels in serum based on the type of metal, fish species, water quality, and duration of exposure (Munoz et al., 1991). An alternative study reported that metal toxicity changes serum parameters like enzymes and proteins (Garcia-Nino and Pedraza-Chaverrí, 2014; Mutlu et al., 2015).

Antioxidant enzyme levels serve as the most reliable indicator for early detection of environmental pollution (Borkovic et al., 2005; Velkova-Jordanoska et al., 2008). Moreover, metal-induced toxic states elicit oxidative stress (Halliwell and Gutteridge, 2015), leading to cell death through the oxidation of DNA, proteins, lipids, and various cellular components (Patlevic et al., 2016; Wu et al., 2014). Our study demonstrates copper-induced toxicity significantly changes the levels of catalase, peroxidase, glutathione reductase, and superoxide dismutase in the brain, gills, liver, and muscle of fingerling of Rohu. Previously, Kaminski studies had also reported results in accordance with this study where the activities of antioxidants such as SOD, CAT, in the blood of white sharks (*Ciconia Caronia*) are significantly altered by heavy metals (Cd, Ca, and Mg) (Kamiński et al., 2007). The activities of antioxidants are significantly increased in the copper-exposed group. Sampaio reported, that Cu contamination resulted in increased hepatic superoxide dismutase activity in PA cu (*Piaractus mesopotamicus*) (Sampaio et al., 2012). Radi and Matkovics reported similar findings to our study that Cu-induced toxicity significantly boosted the levels of antioxidant enzymes except glutathione peroxidase and lipid peroxidation in common carp (*Cyprinus carpio*) (Radi and Matkovics, 1988).

Furthermore, the results of the present study clearly show good impacts of 50 days of hemp feeding on the blood parameter of Rohu, and all measured blood parameters returned to their standard levels (Table 1). Copper-induced toxicities significantly changed blood indices (WBC, RBC, Hb, hematocrit, MCV, MCH, MCHV, platelets, neutrophils, and lymphocytes), are restored to almost normal levels in fingerlings of Rohu after hemp-supplemented diet in feed for 50 days. The inclusion of hemp seed has shown better effects on blood parameters in Rohu in comparison to hemp seed oil. Furthermore, hemp seed (HS15%) has demonstrated greater efficacy compared to other hemp inclusions of hemp seed (5% and 10%). Our study is the first documented evidence showing the beneficial

impacts of whole hempseed (powder) and hemp seed oil in counteracting copper-induced toxicity on fish blood parameters.

The incorporation of hemp product in diets in graded level in percentage inclusion, exhibited a favorable impact on the lysozyme levels, plasma proteins, and immunoglobulin M (IgM) contents in fingerlings Rohu. Feeding hemp in the diet for fifty days restored the lysozyme, plasma protein, and immunoglobulin M levels, to normal levels which had been altered by Cu intoxication. The graded supplementation of hemp feed exhibited concentration-specific and significantly positive effects on the LFT enzymes (alkaline phosphatase, bilirubin, SGPT, and AST) of Rohu as reported by other researcher (Callaway, 2004; Afridi et al., 2024).

Results of this study also clearly indicate a good impact of hemp feeding as the levels of different antioxidants in gills, brain, liver, and muscle are reverted in fingerlings of Rohu, especially a diet containing 15% hemp seed (Tables 4 to 7). In Cu intoxicated group, the significantly changed antioxidant are reverted to normal level after hempseed oil and hemp seed feeding for 50 days.

Limited literature exists on therapeutic role of hemp against Cu-induced toxicity in the diets on freshwater fishes. Therapeutically, hemp is rich in essential fatty acids, such as linoleic and α-linolenic acid, as well as tocopherols (Vitamin E), which are good growth metabolites, and renowned for their potent non-enzymatic antioxidant properties and scavenging free radicals (Callaway, 2004; Kriese et al., 2004). Based on the concentration of essential fatty acids such as linoleic acid (18:2n6, LA) constituting approximately 55% and α-linolenic acid (18:3n3, ALA) at about 20%, hemp can be regarded as a super food and a potential alternative to fish oil due to its superior omega ratio (n6/n3) ratio of 2.5 (Callaway, 2004). Given its comprehensive nutritional profile, including an omega-6 to omega-3 (n6/n3) ratio of 2.5, surpassing that of fish oil, hemp oil can used as potential alternative to fish oil.

Compared to other animals, information is scarce regarding the utilization of hempseed products in aquaculture feed. Hemp is recognized as an important source of nutrition but neglected duet o it notorious nature Some researchers have experimented with substituting fish meal with hempseed meal in feed formulations, suggesting its potential in feed for fish farming (Webster et al., 2000). However, literature on the nutritional and therapeutic applications of hempseed products remains limited. Recent studies strongly advocate for the use of hemp seed and hemp seed oil across various industries, including pharmaceuticals, cosmetics, and food (Montserrat-de la Paz et al., 2014).

Remarkably, hemp has been investigated for its pharmacological activities (Callaway, 2004; Whiting et al., 2015), therapeutic role

(Grotenhermen and Russo, 2002), antibacterial (Ali et al., 2012), antifungal, and robust anti-leishmanial activities (Radwan et al., 2009). Based on these facts, the study is designed to use hemp against copper-induced toxicity in a freshwater fish *labeo rohita.* This study reveals the pioneering therapeutic efficacy of hemp (*Cannabis sativa*) in combating copper-induced toxicity in *Labeo rohita* for the first time. In this study, we obtained significant results for hemp, but 15% of hemp seed results are comparatively better than hemp seed oil inclusion.

Conclusions and Recommendations

We propose the inclusion of hemp seed oil and hemp seed in fish diets to enhance both nutritional value and therapeutic benefits. This dietary strategy is expected to not only improve fish health but also positively impact consumer's health.

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